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Title page

Short-term copper exposure as a selection pressure for antibiotic resistance and metal resistance in an agricultural soil

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16 **Abstract:**

17 Owing to the similar mechanisms of antibiotic and metal resistance, there is a growing
18 concern that metal contamination may select for antibiotic resistance genes (ARGs) in the
19 environment. Here, we constructed short-term laboratory microcosms to investigate the
20 dynamics of a wide range of ARGs and two copper (Cu) resistance genes in an agricultural
21 soil amended with a gradient of Cu concentrations (0~1000 mg kg^{-1}). Mobile genetic elements
22 (MGEs) were also quantified as a proxy for the horizontal gene transfer potential of ARGs.
23 We detected 126 unique ARGs across all the soil samples using the high-capacity quantitative
24 PCR array, and multidrug and β-lactam resistance were the most abundant ARG categories.
25 The copper amendments significantly enhanced the absolute and relative abundances of
26 ARGs and MGEs, which gradually increased along the gradient of increasing Cu
27 concentrations. The two Cu resistance genes (*copA* and *pcoR*) were highly enriched in low-
28 level Cu treatment (50 and 100 mg kg^{-1}), and their abundances decreased with the increasing
29 Cu concentrations. The level of metal and antibiotic resistance gradually declined over time in
30 all Cu-amended treatments but was still considerably higher in contaminated soils than
31 untreated soils after 56 days' incubation. Significant associations among ARGs and MGEs
32 were revealed by the network analysis, suggesting the mobility potential of antibiotic
33 resistance in Cu-amended soils. No significant positive correlations were found between
34 ARGs and copper resistance genes, suggesting that these genes are not located in the same
35 bacterial hosts. Taken together, our results provide empirical evidence that short-term copper
36 stress can cause evolution of high-level antibiotic and metal resistance, and significantly
37 change the diversity, abundance and horizontal transfer potential of soil ARGs.

38

39 **Keywords:** Copper; antibiotic resistance genes; copper resistance genes; co-selection;
40 agriculture soil; public health

41

42 **Introduction**

43 The use of synthetic antibiotics for infection treatments and growth promotion has
44 selected for the massive evolution of antibiotic resistance in both clinical and environmental
45 settings (Pruden et al. 2006). The increasing prevalence and propagation of antibiotic

46 resistance genes (ARGs) in bacteria and pathogens have been considered as a global threat to
47 public health and food security (Berendonk et al. 2015). The persistence of ARGs in the
48 environment provides a likelihood of them being horizontally transferred from the
49 environmental to human-related pathogens and other bacteria mediated by the mobile genetic
50 elements (MGEs) such as transposons, integrons and plasmids (Rizzo et al. 2013; Sentchilo et
51 al. 2013; Gillings et al. 2015); or being migrated into the food chain through growing
52 vegetables in ARGs-enriched soils (Chen et al. 2017). Previous studies have reported the
53 sequence similarity between some ARGs detected in the environment and those detected in
54 human feces (Nesme et al. 2014) and human pathogens (Forsberg et al. 2012), indicating the
55 potential of environmental bacteria to share their ARGs with human-related pathogens or
56 commensal bacteria. Therefore, it is important to understand the evolution of antibiotic
57 resistance and routes of resistance dissemination in the environment (Pruden et al. 2006),
58 before we can include soil resistomes in appropriate mitigation options and risk assessment
59 strategies (Ashbolt et al. 2013).
60 To combat the dissemination of antibiotic resistance, it is imperative to accurately assess
61 the risks associated with environmental sources of ARGs and identify the major pathways by
62 which resistance develops and evolves in natural settings (Hu et al. 2018). Land application of
63 organic wastes containing a high proportion of antibiotic residues has been regarded as an
64 important route of ARGs entering into the soil environment (Zhu et al. 2013; Chen et al. 2016;
65 Zhang et al. 2017; Gou et al. 2018). However, the selection pressure imposed by antibiotics is
66 not long-lasting owing to the rapid degradation of antibiotics in soil (Hu et al. 2016a). There
67 is increasing awareness that a wide array of other factors, in addition to antibiotics, may play
68 an important role in the evolution and environmental spread of antibiotic resistance (Hu et al.
69 2017). In particular, heavy metals, with an estimated half-life ranging for hundreds of years,
70 may function as a strong selection agent for antibiotic resistance and metal resistance among
71 bacteria through the common mechanisms of co-resistance (physical genetic linkage between
72 different genes), cross-resistance (single gene encoding resistance to multiple antibiotics and
73 metals) and co-regulation (shared regulatory system to antibiotic and metal resistance) (Berg
74 et al. 2005; Baker-Austin et al. 2006). A few studies have postulated the co-selection
75 mechanisms as a major pathway for the elevated frequencies of antibiotic resistance in heavy-

76 metal contaminated environmental settings (Ji et al. 2012; Hu et al. 2017; Zhang et al. 2018).
77 Heavy metals such as copper (Cu) are commonly utilized as animal feed to control
78 disease and promote growth and can be introduced to soils following land amendment of
79 metal-containing animal wastes, fertilizers and sewage sludge (Seiler and Berendonk 2012).
80 Although Cu is an essential element for various cellular components and physiological
81 functions of the bacterial cells, a high concentration of Cu can be toxic to soil organisms via
82 causing damage to nucleic acids and cellular membranes (Knapp et al. 2011; Seiler and
83 Berendonk 2012). Previous studies have documented the long-term impact of Cu
84 contamination on the occurrence of antibiotic resistance and reported the linkage between Cu
85 pollution and the increasing occurrence of ARGs in soils (Hu et al. 2016b; Poole 2017). It was
86 found that Cu had stronger impact on the patterns of ARGs compared with other types of
87 heavy metals such as lead, nickel and zinc (Knapp et al. 2011; Zhu et al. 2013). Meanwhile,
88 most of previous studies of the long-term metal-induced development of ARGs were
89 conducted in soils contaminated with multiple antibiotics, metals and other antimicrobial
90 agents, which hinders our ability to infer how Cu exposure directly contributes to the
91 evolution of antibiotic resistance. To date, no studies have explored the temporal dynamics of
92 copper resistance genes (CRGs) and a wide spectrum of ARGs in agricultural soils exposed to
93 short-term Cu contamination, and it remains largely unknown whether the short-term Cu
94 exposure can select for both antibiotic and copper resistance. Such information is of great
95 value for us to understand the behaviors of soil bacterial communities under the selection
96 pressure which enables a better prediction of the evolution of ARGs in the environment.
97 This study aimed to examine the effects of short-term Cu exposure on the abundance,
98 diversity, and horizontal transfer potential of 285 ARGs, 10 MGEs and two CRGs (the *copA*
99 and *pcoR* genes) in laboratory soil microcosms amended with a gradient of Cu concentrations
100 using the high-throughput quantitative PCR (HT-qPCR). We explored the possibility that
101 short-term Cu exposure may lead to the co-selection of antibiotic and metal resistance due to
102 the possible genetic linkage of these resistance genes on MGEs. The following hypotheses
103 were tested: (1) Cu amendment can enhance the abundance and diversity of soil ARGs, which
104 are positively correlated with the Cu concentrations; (2) Cu amendment can increase the
105 abundance of CRGs, which might show a similar temporal pattern to that of ARGs; and (3)

106 The relative abundances of ARGs, MGEs and CRGs can be positively correlated if these
107 resistance genes are located on the same MGEs or in the same bacterial hosts.
108

109 **Materials and Methods**

110 ***Soil sampling***

111 Soil samples used for construction of the laboratory microcosm experiment were
112 collected from a long-term research vegetable farm at Clyde (38°07'S, 145°19'E), Victoria,
113 Australia. Mean annual temperature at this site is 19.4 °C, and mean annual precipitation is
114 819 mm. This vegetable farm has been applied with only inorganic fertilizers during the
115 preceding five years without known history of organic fertilizer application. The soil is
116 characterized as a Loamy Sand with a pH (H_2O) value of 6.7. We collected the surface soils
117 (0-10cm) and transported them to the laboratory on ice, passed through 2 mm mesh, and
118 archived at 4 °C prior to the start of the incubation. Details on the soil properties are given in
119 Table 1.

120 ***Soil microcosms and DNA extraction***

121 We established six treatments in 250 ml vials with three replicates containing 20 g of
122 fresh soils: untreated soil samples (control), and treatments with 50, 100, 250, 500, 1000 mg
123 Cu kg^{-1} soil (Cu50, Cu100, Cu250, Cu500 and Cu1000, respectively). The $CuCl_2$ solution was
124 added to the soil microcosms to reach the corresponding Cu concentrations. All the soil
125 microcosms were maintained at 60% of the water filled pore space through regular
126 replenishment of sterilized water. The aerobic condition was maintained through refreshing
127 air in the vials twice a week. A total of 126 soil microcosms were incubated in the dark at
128 25°C, and destructively sampled at days 0, 7, 14, 21, 28, 42 and 56. The DNeasy PowerSoil
129 Kit (QIAGEN INC., Germantown, MD, USA) was used to isolate microbial genomic DNA
130 from the soil samples following the manufacturer's instructions.

131 ***High-throughput qPCR (HT- qPCR) analysis of ARGs and MGEs***

132 The Wafergen SmartChip Real-time PCR platform (WaferGen Biosystems, Fremont,
133 CA, USA) was used for the high-throughput qualification of ARGs and MGEs in soils as
134 previously described (Su et al. 2015; Gou et al. 2018). A total of 285 probes targeting all the
135 major categories of antibiotic resistance and 10 probes targeting MGEs were included in the

136 current version of the HT-qPCR array (Looft et al. 2012; Johnson et al. 2016). The 100 nl
137 qPCR reaction mixture contained the SensiMix SYBR No-ROX reagent, primers and
138 genomic DNA. The qPCR reaction was run in triplicate for each sample using the
139 amplification conditions: 10 min at 95°C, followed by 40 cycles of 30 s at 95°C and 30 s at
140 60°C. The HT-qPCR results were quality-filtered according to the criteria as described
141 previously (Su et al. 2015; Hu et al. 2017) and the relative abundances of ARGs and MGEs
142 (as compared to 16S rRNA gene) were calculated using the comparative C_T method
143 (Schmittgen and Livak 2008).

144 ***Quantitative PCR analysis of copper resistant genes (CRGs) and 16S rRNA gene***

145 The Bio-Rad CFX384™ Real-Time PCR Detection System (Bio-Rad, Herculers, CA,
146 USA) was used to qualify the *copA* and *pcoD* genes using the primer pairs
147 copAF2010/copAR2333 (Li et al. 2012) and pcoR1/pcoR2 (Trajanovska et al. 1997),
148 respectively. The 10 µl PCR reaction mixture consisted of 5 µl SensiMix SYBR No-ROX
149 reagent (Bioline), 0.5 µl of each primer (10 µM), 2 µl of 10-fold diluted DNA template, and 2
150 µl of nuclease-free water. The thermal-cycling conditions were: 95°C for 10 min, 40 cycles of
151 95°C for 30 s, 51°C for 30s, and 72°C for 45 s. The bacterial 16S rRNA gene was quantified
152 using the primer pair BACT1369F/PROK1492R (Suzuki et al. 2000). Amplification
153 conditions were: 95°C for 10 s, 35 cycles of 95°C for 15 s and 56°C for 1 min. The CRGs (i.e.
154 the *copA* and *pcoD* genes) and the bacterial 16S rRNA gene were PCR-amplified from the
155 soil DNA, linked to the pGEM®-T Easy Vectors, and transferred to JM109 High Efficiency
156 Competent Cells (Promega, Madison, USA). Plasmid DNA was extracted from positive
157 clones using the PureYield™ Plasmid Miniprep System (Promega) and sequenced to confirm
158 their identities. Standard curves were prepared from 10-fold serial dilutions of plasmids with
159 inserts of the targeted gene sequences. We conducted melting curve analysis after each qPCR
160 run to verify the specificity of PCR amplicons.

161 ***Network analysis and visualization***

162 The CoNet Cytoscape plug-in method was employed to explore the co-occurrence
163 patterns among the detected ARGs, MGEs and CRGs in all the samples as previously
164 described (Soffer et al. 2015; Hu et al. 2017). The pairwise interaction scores were calculated
165 with Pearson and Spearman correlation methods and Kullback-Leibler and Bray-Curtis

166 dissimilarity methods. The potential false-positive correlations were eliminated by the
167 conduction of the ReBoot procedure with 100 permutations and inclusion of ARGs with an
168 occurrence of > 30 across all the samples. We merged the resultant P-values using the Brown
169 method. The significant pairwise correlations (with a correlation coefficient above 0.6 and a
170 significance level below 0.05) were utilized to construct the co-occurrence network and
171 visualized using the Fruchterman Reingold algorithm on the interactive Gephi platform
172 (Bastian et al. 2009).

173 ***Statistical analysis***

174 Significant differences ($P < 0.05$) in the relative and absolute abundance of ARGs,
175 MGEs and CRGs across treatments were detected using the one-way analysis of variance
176 (ANOVA) test after correction for multiple testing. Pearson correlation test was performed to
177 assess the correlations between the abundances of ARGs, MGEs, and CRGs. Non-metric
178 multidimensional scaling (NMDS) analysis was conducted to visualize the differences in the
179 relative abundance of ARGs across different treatments based on the Bray-Curtis dissimilarity
180 matrices. The effects of Cu-amended treatments on the ARG patterns were tested by
181 performing the permutational multivariate analysis of variance (PerMANOVA) using the
182 'vegan' package of the R software with 999 permutations.

183

184 **Results and discussion**

185 ***Copper amendment increased the diversity and abundance of ARGs, MGEs and CRGs***

186 While many studies have examined the impacts of heavy metals on soil ARGs in long-
187 term experimental sites, less is known about the short-term dynamics of ARGs and MGEs
188 following Cu amendment to soils. In this study, the HT-qPCR array was employed to assess
189 the abundance and diversity of ARGs and MGEs in an agricultural soil treated with different
190 Cu concentrations (0~1000 mg kg^{-1}) in laboratory microcosms. A total of 126 unique ARGs
191 and 6 MGEs were detected across all the samples. In line with previous profiling of ARGs in
192 animal farms (Zhu et al. 2013), manure-contaminated soils (Hu et al. 2016a), antibiotics-
193 treated soils (Zhang et al. 2017), and human-impacted estuaries (Zhu et al. 2017), the detected
194 ARGs in this study could encode resistance to all the major categories of common antibiotics,
195 with the most frequent types being multidrug (24.3%), aminoglycoside (19.9%), tetracycline

196 (14.4%), and β-lactam (10.9%) (Fig. 1A). The detected ARGs were classified into different
197 resistance mechanisms including efflux pump (41.6%), antibiotic deactivation (38.3%) and
198 cellular protection (18.9 %) (Fig. 1B). The average numbers of detected ARGs highly varied
199 across the different Cu-amended treatments, with the highest values recorded in the Cu50
200 treatment (Fig. 1C). The diversity of ARGs in the high-level Cu treatments (Cu250, Cu500
201 and Cu1000) was significantly lower than that in the Cu50 treatment ($P < 0.05$). The diversity
202 of MGEs also significantly changed across the different Cu-treated treatments (Fig. 1D).
203 In contrast to the diversity patterns, the absolute abundance and relative abundance (as
204 compared to the bacterial 16S rRNA gene) of ARGs and MGEs constantly increased with the
205 increasing Cu concentrations (Fig. 2). The highest abundance of ARGs and MGEs was
206 observed in Cu1000 and significantly higher than that in untreated soils and low-level Cu
207 treatments (Cu50 and Cu100) ($P < 0.05$). In particular, the abundances of genes encoding
208 resistance to multidrug, aminoglycoside and sulfonamide significantly increased with the
209 increasing Cu concentrations (Fig. 2A, 2C). The NMDS analysis of the relative abundance of
210 individual ARG subtypes based on the Bray-Curtis dissimilarity matrices revealed that ARG
211 profiles gradually shifted across the gradient of Cu contamination (Fig. 3), which revealed
212 that Cu contamination was a major determinant shaping the ARG patterns (PerMANOVA, $P <$
213 0.05). The absolute and relative abundances of the *copA* and *pcoR* genes exhibited different
214 patterns from that of ARGs and MGEs along the gradient of Cu concentration (Fig. 4). The
215 abundance of the *copA* gene was significantly higher than that of the *pcoR* gene across all the
216 treatments ($P < 0.05$). The absolute abundance of the *copA* gene was found to be highest in
217 the Cu100 treatment, which was significantly higher than that in other treatments ($P < 0.05$)
218 (Fig. 4C). The *pcoR* gene showed the similar variation tendency, but with the highest values
219 recorded in the Cu50 treatment (Fig. 4B, 4D).
220 Different from the previous studies of the long-term impacts of Cu contamination on the
221 distribution patterns ARGs (Berg et al. 2005; Hu et al. 2016b), our results showed that even a
222 short-term Cu exposure can significantly enhance the abundance of antibiotic resistance and
223 influence the compositions of soil ARGs. This finding suggested that soil bacterial
224 communities can quickly evolve under the metal stress and develop resistance to Cu and
225 antibiotics in a short term of two months. In line with our results, previous studies reported

226 that freshwater bacterial communities exposed to heavy metal contamination can lead to
227 increased levels of multi-resistant microorganisms (Stepanauskas et al. 2006), and long-term
228 heavy metal (e.g., copper and nickel) exposure can induce bacterial adaptations that result in
229 increased levels of resistance to multiple antibiotics in soils (Berg et al. 2005; Knapp et al.
230 2011; Hu et al. 2016b, 2017). Heavy metal elements in swine feed had strong correlations
231 with the occurrence and abundances of multidrug-resistant bacteria (Zhu et al. 2013;
232 Medardus et al. 2014). Our study provided further evidence that soil resident bacteria are
233 highly adaptable and can rapidly evolve resistance to the added heavy metals through the
234 development of both antibiotic and metal resistance. However, it was found that the
235 abundances of ARGs and CRGs showed different responses to Cu amendment, for example,
236 ARG abundances increased with increasing Cu concentrations (Fig. 2) while CRG
237 abundances showed a general declining trend along the gradient of Cu concentrations (Fig. 4).
238 This suggests that the targeted ARGs and CRGs might reside in different bacterial hosts, and
239 ARG-bearing bacteria and CRG-bearing bacteria might have different preference for Cu
240 concentrations, with ARG-bearing bacteria favoured at high Cu concentrations while CRG-
241 bearing bacteria favoured at low Cu concentrations. Therefore, given the anthropogenic levels
242 of metal contamination in the environment, it is assumed that metal pollution can exert a
243 strong selection pressure for antibiotic resistance, but antibiotic resistance and metal
244 resistance are not necessarily positively associated under the Cu stress.

245 ***Temporal changes of ARGs, MGEs and CRGs***

246 The temporal changes of ARGs, MGEs and CRGs in the soil microcosms were explored
247 at seven time points (days 0, 7,14, 21, 28, 42 and 56). Owing to the very similar temporal
248 patterns of ARGs, MGEs and CRGs in different Cu-amended treatment, only the treatments
249 with the highest gene abundances were compared to the control treatment (Fig. 5). The
250 absolute abundances of total ARGs (Fig. 5A) and MGEs (Fig. 5B) increased shortly following
251 the Cu addition, reached the highest level at day 14, and then gradually declined over time
252 during the incubation. Similarly, the absolute abundances of the *copA* and *pcoR* genes (Fig.
253 5C, 5D) in Cu-amended soils climbed to the highest level at day 14 and showed the similar
254 declining patterns to that of ARGs and MGEs. By contrast, the absolute abundances of ARGs,
255 MGEs and CRGs in the control treatment remained largely unchanged during the short-term

256 microcosm incubation. Our results suggested that soil resident microorganisms can rapidly
257 develop resistance to the Cu stress in less than two weeks possibly through the evolution of
258 antibiotic resistance and copper resistance. However, exposure of soil resident bacteria to
259 heavy metals for more than two weeks in soil microcosms can be toxic and damage cellular
260 membranes and genomic DNA, hence, sensitive bacterial hosts could be killed, resulting in
261 the reduction of ARGs (Burch et al. 2013) and CRGs (Wang et al. 2016). The reduction of
262 ARGs/CRGs over time following after day14 can be also attributed to the gradual out-
263 competition of ARGs/CRGs-bearing bacteria by the soil resident microbiomes, as the ability
264 of resistance to antibiotics/metals can be an additional energy cost of bacterial hosts (Martinez
265 2009), which can become less competitive when nutrients are becoming limited in a soil
266 microcosm. However, it should be noted that the level of antibiotic resistance is still
267 significantly higher than the background level in the control treatment, suggesting the
268 enrichment of ARGs during to short-term Cu exposure can be long-standing in soils. This
269 finding might help explain the phenomenon that despite tremendous efforts to reduce the
270 global consumption of antibiotics, there is increasing prevalence of ARGs in environmental
271 settings (Li et al. 2017).

272 ***Relationships among ARGs, MGEs and CRGs***

273 Pearson correlation analysis demonstrated that the relative abundances of total ARGs
274 and specific categories of ARGs (including aminoglycoside, FCA and sulfonamide resistance)
275 had significantly positive correlation with that of total transposase and intergrase genes ($P <$
276 0.05, Table 2). Moreover, the MLSB (Macrolide-Lincosamide-Streptogramin B), multidrug
277 and tetracycline resistance genes had also significantly positive relationships with integrase
278 genes ($P < 0.05$, Table 2). This finding was further supported by the co-occurrence network
279 analysis which revealed intensive correlations between ARGs and MGEs (Fig. 6). The
280 resultant network included 23 nodes (ARG or MGE subtypes) and 80 edges which could be
281 separated into three modules. The *tnpA-05* gene (belonging to MGEs) was intensively
282 connected with multiple genes encoding potential resistance to aminoglycoside, multidrug,
283 FCA and sulfonamide, indicating that these co-occurring ARGs have a likelihood of dispersal
284 to other bacteria and pathogens through the mechanism of horizontal gene transfer (HGT). It
285 has been well-documented that some transposons harbor metal-resistance operons and

286 integrons containing multiple ARGs (Baker-Austin et al. 2006) and might play important roles
287 in the horizontal transfer of antibiotic and metal resistance genes in environmental microbial
288 populations (Gillings et al. 2015). Although a range of biotic and abiotic factors (e.g. soil pH,
289 temperature, soil moisture contents, soil types, and vegetation) can affect the frequencies of
290 HGT of ARGs in soils (Aminov 2011), our results indicated the HGT potential of multiple
291 antibiotic resistance in bacterial communities of Cu-contaminated soils. In addition, it should
292 be noted that the network analysis can only provide correlative evidence for the possible links
293 between ARGs and MGEs, which requires further validation experiment to confirm their
294 connections in bacterial hosts.
295 In contrast to the previous reports about the co-occurrence of CRGs and ARGs under
296 the Cu stress, for example the co-occurrence of *pcoA* with *erm*(A) and *intl*1 (Besaury et al.
297 2016), co-transmission of *tcrB*/*cueO* with *erm*(B) in *Enterococcus* and *Enterococci*
298 (Amachawadi et al. 2013; Silveira et al. 2014), no significant correlation was found between
299 the relative abundances of ARGs and the *pcoR* gene, and the *copA* gene had significantly
300 negative relationships with the aminoglycoside, FCA, sulfonamide resistance genes and
301 MGEs (Table 3). These results suggested that the targeted *copA* and *pcoR* genes and the
302 detected ARGs may be not intimately linked in the same gene cassettes or these genes might
303 reside in different bacterial strains. Similarly, previous studies did not find the co-occurrence
304 of the *pcoA* gene with ARGs in *Salmonella* (Yin et al. 2017), and low co-occurrence
305 frequency between ARGs and CRGs (including the *copA* gene) was observed on plasmids
306 under the stress of both antibiotics and metals (Pal et al. 2015). These findings suggest that
307 bacteria resistant to antibiotics and those resistant to Cu might belong to different groups, and
308 no co-selection is actually occurring in this short-term microcosm.
309

310 **Conclusions**

311 To be able to prevent further development of environmental resistome, it is important to
312 understand how bacterial antibiotic resistance evolves in the environment. Our study provided
313 empirical evidence that even short-term exposure to copper can contribute to the development
314 of bacterial antibiotic resistance in agricultural soils. The changes in antibiotic resistance are
315 not significantly and positively associated with that of metal resistance, indicating that co-

316 selection might be not the main mechanism for the evolution of antibiotic and metal resistance
317 in short-term Cu-contaminated soil microcosms. Moreover, the transposase genes had strong
318 and positive relations with ARGs during the incubation, suggesting the HGT potential of
319 ARGs might be enhanced by the Cu exposure. Together, our findings caution that the heavy
320 metals such as Cu widely present in the soil environment can lead to a high degree of
321 antibiotic resistance and may contribute to the dissemination of environmental resistomes.
322
323
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327
328 **Conflict of Interest:** The authors declare that they have no conflict of interest.
329
330 **References**
331 Amachawadi RG, Scott HM, Alvarado CA, Mainini TR, Vinasco J, Drouillard, JS, Nagaraja
332 TG (2013) Occurrence of the transferable copper resistance gene tcrB, among fecal
333 *enterococci* of U.S. feedlot cattle fed copper supplemented diets. Appl Environ Microbiol
334 79: 4369-4375
335 Aminov RI (2011) Horizontal gene exchange in environmental microbiota. Front Microbiol 2:
336 158
337 Ashbolt NHJ, Amezquita A, Backhaus T, Borriello P, Brandt KK, Collignon P (2013) Human
338 health risk assessment (HHRA) for environmental development and transfer of antibiotic
339 resistance. Environ Health Perspect 121: 993-1001
340 Baker-Austin C, Wright MS, Stepanauskas R, McArthur JV (2006) Co-selection of antibiotic
341 and metal resistance. Trends Microbiol 14: 176-182
342 Bastian M, Heymann S, Jacomy M (2009) Gephi: an open source software for exploring and
343 manipulating networks. Icwsm 8:361-362
344 Besaury L, Pawlak B, Quillet L (2016) Expression of copper-resistance genes in microbial
345 communities under copper stress and oxic/anoxic conditions. Environ Sci Pollut Res Int

346 23: 4013-4023
347 Berendonk TU, Manaia CM, Merlin C, Fatta-Kassinos D, Cytryn E, Walsh F et al (2015)
348 Tackling antibiotic resistance: the environmental framework. Nat Rev Microbiol 13: 310-
349 317.
350 Berg J, Tom-Petersen A, Nybroe O (2005) Copper amendment of agricultural soil selects for
351 bacterial antibiotic resistance in the field. Lett Appl Microbiol 40:146-151
352 Burch TR, Sadowsky MJ, Lapara TM (2013) Aerobic digestion reduces the quantity of
353 antibiotic resistance genes in residual municipal wastewater solids. Front Microbiol 4:17
354 Chen Q, An X, Li H, Su J, Ma Y, Zhu YG (2016) Long-term field application of sewage
355 sludge increases the abundance of antibiotic resistance genes in soil. Environ Int 92:1-10
356 Chen Q, An X, Zhu YG, Su JQ, Gillings MR, Ye ZL, Cui L (2017) Application of struvite
357 alters the antibiotic resistome in soil, rhizosphere, and phyllosphere. Environ Sci Technol
358 51: 8149-8157
359 Forsberg KJ, Reyes A, Wang B, Selleck EM, Sommer MOA, Dantas G. (2012) The shared
360 antibiotic resistome of soil bacteria and human pathogen. Science 337: 1107-1111.
361 Gillings MR, Gaze WH, Pruden A, Smalla K, Tiedje JM, Zhu YG (2015) Using the class 1
362 integron-integrase gene as a proxy for anthropogenic pollution. ISME J 9:1269-1279
363 Gou M, Hu HW, Zhang YJ, Wang JT, Hayden H, Tang YQ, He JZ (2018) Aerobic composting
364 reduces antibiotic resistance genes in cattle manure and the resistome dissemination in
365 agricultural soils. Sci Total Environ 612:1300-1310
366 Hu HW, Han XM, Shi XZ, Wang JT, Han LL, Chen D, He JZ (2016a) Temporal changes of
367 antibiotic resistance genes and bacterial communities in two contrasting soils treated with
368 cattle manure. FEMS Microbiol Ecol 92: fiv196
369 Hu HW, Wang JT, Li J, Li JJ, Ma YB, Chen D, He JZ (2016b) Field-based evidence for
370 copper contamination induced changes of antibiotic resistance in agricultural soils. Environ
371 Microbiol 18: 3896-3909
372 Hu HW, Wang JT, Li J, Shi XZ, Ma Y, Chen D, He JZ (2017) Long-term nickel contamination
373 increases the occurrence of antibiotic resistance genes in agricultural soils. Environ Sci
374 Technol 51: 790-800
375 Hu HW, Wang JT, Singh BK, Liu, YR, Chen YL, Zhang YJ, He JZ (2018) Diversity of

376 herbaceous plants and bacterial communities regulates soil resistome across forest biomes.
377 Environ Microbiol. http://doi.org/10.1111/1462-2920.14248.
378 Ji X, Shen Q, Liu F, Ma J, Xu G, Wang Y, Wu M (2012) Antibiotic resistance gene
379 abundances associated with antibiotics and heavy metals in animal manures and
380 agricultural soils adjacent to feedlots in Shanghai; China. J Hazard Mater 235: 178-185
381 Johnson TA, Stedtfeld RD, Wang Q, Cole JR, Hashsham SA, Looft T, Zhu YG, James MT
382 (2016) Clusters of antibiotic resistance genes enriched together stay together in swine
383 agriculture. mBio 7: e02214-e02215
384 Knapp CW, McCluskey SM, Singh BK, Campbell CD, Hudson G, Graham DW (2011)
385 Antibiotic resistance gene abundances correlate with metal and geochemical conditions in
386 archived scottish soils. PloS ONE 6: 1-6
387 Legros S, Doelsch E, Feder F, Moussard G, Sansoulet J, Gaudet JP, Rigaud S, Doelsch IB,
388 Macary HS, Bottero JY (2013) Fate and behaviour of Cu and Zn from pig slurry spreading
389 in a tropical water-soil-plant system. Agric Ecosyst Environ 164: 70-79
390 Li XF, Yin HB, Su JQ (2012) An attempt to quantify Cu-resistant microorganisms in a paddy
391 soil from Jiaxing, China. Pedosphere 22: 201-205
392 Li LG, Xia Y, Zhang T. (2017) Co-occurrence of antibiotic and metal resistance genes
393 revealed in complete genome collection. ISME J 11: 651-662.
394 Looft T, Johnson TA, Allen HK, Bayles DO, Alt DP, Stedtfeld RD, Sul WJ, Stedtfeld TM,
395 Chai B, Cole JR (2012) In-feed antibiotic effects on the swine intestinal microbiome. Proc
396 Natl Acad Sci USA 109: 1691-1696
397 Martinez JL (2009) Environmental pollution by antibiotics and by antibiotic resistance
398 determinants. Environ Pollut 157: 2893-2902
399 Mao D, Luo Y, Mathieu J, Wang Q, Feng L, Mu Q, Feng C, Alvarez PJJ (2014) Persistence of
400 extracellular DNA in river sediment facilitates antibiotic resistance gene propagation.
401 Environ Sci Technol 48: 71-78
402 Medardus JJ, Molla BZ, Nicol M, Morrow WM, Rajala-Schultz PJ, Kazwala R, Gebreyes WA
403 (2014) In-feed use of heavy metal micronutrients in US swine production systems and its
404 role in persistence of multidrug-resistant *salmonellae*. Appl Environ Microbiol 80: 2317-
405 2325

406 Nesme J, Cecillon S, Delmont TO, Monier JM, Vogel TM, Simont P (2014) Large-scale
407 metagenomic-based study of antibiotic resistance in the environment. Curr Biol 24: 1096-
408 1100
409 Pal C, Bengtsson-Palme J, Kristiansson E, Larsson DJ (2015) Co-occurrence of resistance
410 genes to antibiotics, biocides and metals reveals novel insights into their co-selection
411 potential. BMC Genomics 16: 964
412 Poole K (2017) At the nexus of antibiotics and metals: the impact of Cu and Zn on antibiotic
413 activity and resistance. Trends Microbiol 25: 820-832
414 Pruden A, Pei R, Storteboom H, Carlson KH (2006) Antibiotic resistance genes as emerging
415 contaminants: studies in Northern Colorado. Environ Sci Technol 40: 7445-7450
416 Rizzo L, Manaia C, Merlin C, Schwartz T, Dagot C, Ploy MC, Michael I, Fatta-Kassinos D
417 (2013) Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and
418 genes spread into the environment: a review. Sci Total Environ 447: 345-360
419 Schmittgen TD, Livak JK (2008) Analyzing real-time PCR data by the comparative C_T
420 method. Nat Protoc 3: 1101-1108
421 Seiler C, Berendonk TU (2012) Heavy metal driven co-selection of antibiotic resistance in
422 soil and water bodies impacted by agriculture and aquaculture. Front Miciobiol 3: 399
423 Sentchilo V, Mayer AP, Guy L, Miyazaki R, Tringe SG, Barry K et al (2013) Community-
424 wide plasmid gene mobilization and selection. ISME J 7: 1173-1186
425 Silveira E, Freitas AR, Antunes P, Barros M, Campos J, Coque TM, Peixe L, Novais C (2014)
426 Co-transfer of resistance to high concentrations of copper and first-line antibiotics among
427 *Enterococcus* from different origins (humans, animals, the environment and foods) and
428 clonal lineages. J Antimicrob Chemother 69: 899-906
429 Soffer N, Zaneveld J, Thurber RV (2015) Phage-bacteria network analysis and its implication
430 for the understanding of coral disease. Environ Microbiol 17: 1203-1218
431 Stepanauskas R, Glenn TC, Jagoe CH, Tuckfield RC, Lindell AH, King CJ et al (2006)
432 Coselection for microbial resistance to metals and antibiotics in freshwater microcosms.
433 Environ Microbiol 8: 1510–1514
434 Su JQ, Wei B, Ou-Yang WY, Huang FY, Zhao Y, Xu HJ, Zhu YG (2015) Antibiotic resistome
435 and its association with bacterial communities during sewage sludge composting. Environ

436 Sci Technol 49: 7356–7363
437 Suzuki MT, Taylor LT, Delong EF. (2000) Quantitative analysis of small-subunit rRNA genes
438 in mixed microbial populations via 50-nuclease assays. Appl Environ Microbiol 66: 4605–
439 4614
440 Trajanovska S, Britz ML, Bhave M (1997) Detection of heavy metal ion resistance in Gram-
441 positive and Gram-negative bacteria isolated from lead-contaminated site. Biodegradation
442 8: 113-124
443 Wang R, Zhang J, Sui Q, Wan H, Tong J, Chen M, Wei Y, Wei D (2016) Effect of red mud
444 addition on tetracycline and copper resistance genes and microbial community during the
445 full scale swine manure composting. Bioresour Technol 216: 1049-1057
446 Yang L, Huang B, Hu W, Chen Y, Mao M, Yao L (2014) The impact of greenhouse vegetable
447 farming duration and soil types on phytoavailability of heavy metals and their health risk in
448 eastern China. Chemosphere 103: 121-130
449 Yin Y, Gu J, Wang X, Song W, Zhang K, Sun W, Zhang X, Zhang Y, Li H (2017) Effects of
450 Copper Addition on Copper Resistance, Antibiotic Resistance Genes, and *intl1* during
451 Swine Manure Composting. Front Microbiol 8: 344
452 Zhang ML, Chen LH, Ye CS, Yu X (2018) Co-selection of antibiotic resistance via copper
453 shock loading on bacteria from a drinking water bio-filter. Environ Pollut 233: 132-141
454 Zhang YJ, Hu HW, Gou M, Wang JT, Chen D, He JZ (2017) Temporal succession of soil
455 antibiotic resistance genes following application of swine, cattle and poultry manures
456 spiked with or without antibiotics. Environ Pollut 231: 1621-1632
457 Zhu YG, Johnson TA, Su JQ, Qiao M, Guo GX, Stedtfeld RD, Hashsham SA, Tiedje JM
458 (2013) Diverse and abundant antibiotic resistance genes in Chinese swine farms. Proc Natl
459 Acad Sci USA 110: 3435-3440
460 Zhu YG, Zhao Y, Li B, Huang CL, Zhang SY, Yu S et al (2017) Continental-scale pollution of
461 estuaries with antibiotic resistance genes. Nat Microbiol 2: 16270
462
463

464 **Table 1** The basic properties of the arable soil used in this study

pH	Color	Texture	Organic matter (%)	Organic carbon (%)	Total carbon (%)	Total nitrogen (%)	Nitrate (mg kg^{-1})	Ammonium (mg kg^{-1})
6.7	grey	Sandy loam	3.8	2.2	2.4	0.27	240	19
C:N ratio	**Sulphur (mg kg^{-1})**	**Phosphorus (mg kg^{-1})**	**Available potassium (mg kg^{-1})**	**Calcium (cmol kg^{-1})**	**Magnesium (cmol kg^{-1})**	**Aluminium (mg kg^{-1})**	**Copper (mg kg^{-1})**	**Zinc (mg kg^{-1})**
8.9	39	620	220	8.4	2.6	<9.0	12	24

465

466

Table 2 Pearson correlations of the relative abundances of ARGs and MGEs across all the soil samples

	Relative abundances of MGEs	
	Transposase	Integrase
Total ARGs	**0.599 (<0.001)**	**0.611 (<0.001)**
Aminoglycoside	**0.820 (<0.001)**	**0.606 (<0.001)**
β-lactam	0.088 (0.380)	0.178 (0.260)
FCA	**0.810 (<0.001)**	**0.446 (0.003)**
MLSB	0.084 (0.397)	**0.323 (0.037)**
Multidrug	0.143 (0.295)	**0.402 (0.008)**
Sulfonamide	**0.853 (<0.001)**	**0.516 (<0.001)**
Tetracycline	0.027 (0.564)	**0.392 (0.010)**
Vancomycin	0.102 (0.319)	0.243 (0.120)
Other	0.051 (0.550)	0.279 (0.073)

Values showed are the R-values obtained from the Pearson correlation test, and values showed in the brackets are the corresponding *P*-values. Significant correlations ($P < 0.05$) are highlighted in bold numbers. (MLSB: Macrolide-Lincosamide-Streptogramin B resistance genes; FCA: fluoroquinolone, quinolone, florfenicol, chloramphenicol and amphenicol resistance genes)

477 **Table 3** Pearson correlations of the relative abundance between CRGs (*copA* and *pcoR*) and
478 ARGs and MGEs across all the samples
479

	Gene copies /16S rRNA copies	
	copA	*pcoR*
Aminoglycoside	**-0.529 (< 0.001)**	-0.179 (0.257)
Beta Lactamase	-0.069 (0.662)	0.123 (0.438)
FCA	**-0.635 (< 0.001)**	-0.151 (0.339)
MLSB	-0.097 (0.541)	-0.262 (0.093)
Multidrug	-0.060 (0.704)	-0.074 (0.643)
Sulfonamide	**-0.591 (< 0.001)**	-0.156 (0.323)
Tetracycline	-0.084 (0.598)	-0.272 (0.081)
Vancomycin	-0.085 (0.592)	0.158 (0.316)
other	0.041 (0.798)	-0.025 (0.877)
Transposase	**-0.545 (< 0.001)**	-0.222 (0.158)
Integrase	**-0.337 (0.029)**	-0.137 (0.386)

480
481 Values showed are the R-values obtained from the Pearson correlation test, and values showed
482 in the brackets are the corresponding P-values. Significant correlations ($P < 0.05$) are
483 highlighted in bold numbers.

484 **Figure legends**

485 **Figure 1** Classification of all the detected ARGs based on the classes of antibiotics (A);
486 Classification of all the detected ARGs based on the resistance mechanisms (B); The number
487 of ARGs (C) and MGEs (D) detected across different Cu-contaminated treatments in the
488 agricultural soil. Different letters above the columns represent a significant difference ($P <$
489 0.05). (MLSB: Macrolide-Lincosamide-Streptogramin B resistance genes; FCA:
490 fluoroquinolone, quinolone, florfenicol, chloramphenicol and amphenicol resistance genes)

491 **Figure 2** The relative and absolute abundance of ARGs (A and C) and MGEs (B and D)
492 detected across all the samples. Different letters above the columns represent a significant
493 difference ($P < 0.05$).

494 **Figure 3** The non-metric multidimensional scaling (NMDS) ordinations showing the Bray-
495 Curtis dissimilarity of the relative abundance of ARGs in all samples along the gradient of Cu
496 concentrations. The 2D stress value is 0.12, which indicates that these data could be well
497 represented by the 2D ordination.

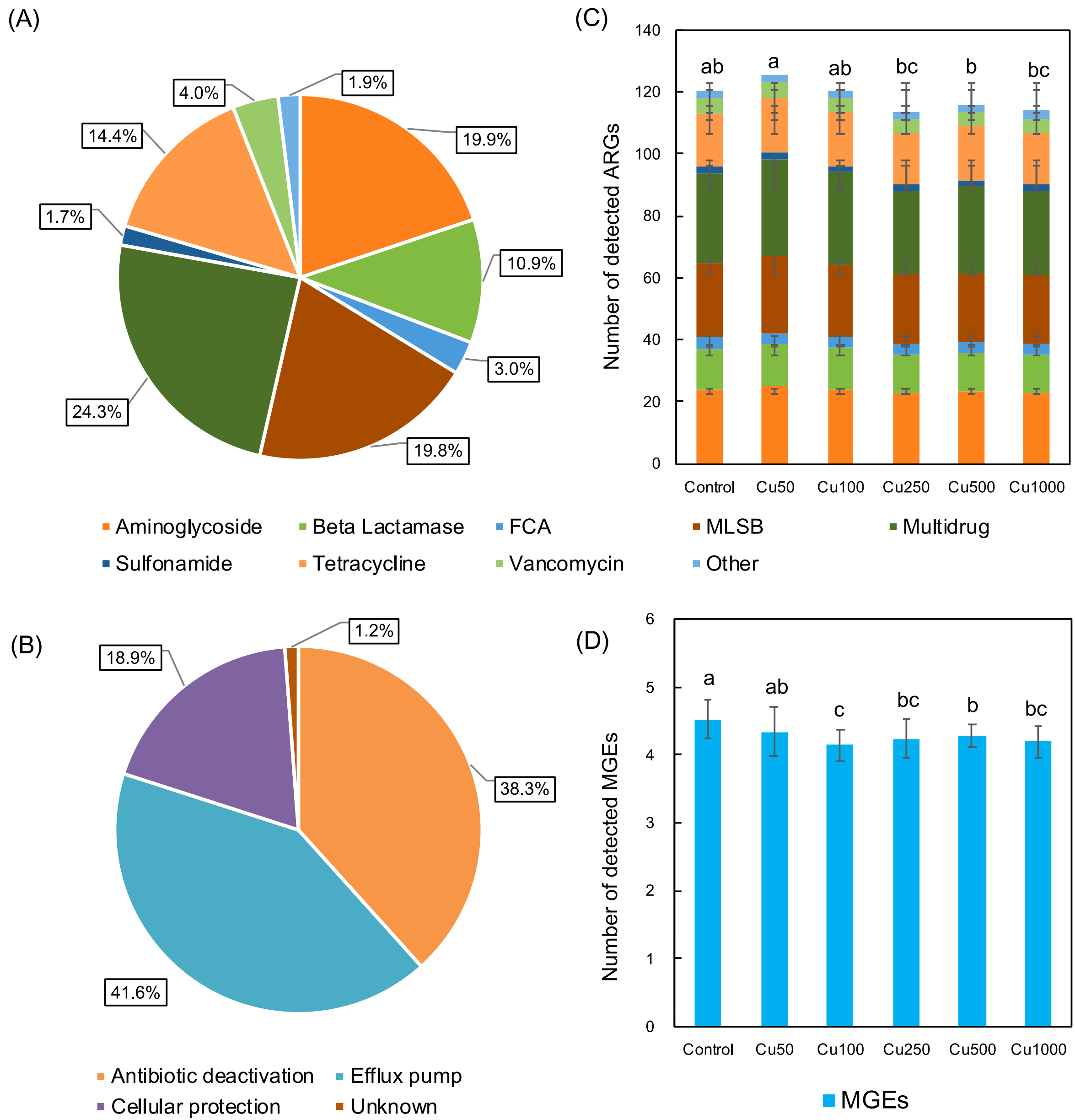
498 **Figure 4** The relative and absolute abundance of copper resistance genes *copA* (A and C) and
499 *pcoR* (B and D). Different letters above the columns represent a significant difference.

500 **Figure 5** The temporal changes of the absolute abundance of ARGs (A) and MGEs (B) as
501 well as the *copA* (C) and *pcoR* (D) genes in the control and Cu-contaminated treatments
502 during the incubation.

503 **Figure 6** The co-occurrence patterns among the detected ARGs and MGEs in all the soils as
504 revealed by the network analysis. Different modularity classes are represented by the nodes
505 with different colors, and strong and significant correlations between nodes are represented by
506 the edges.

507

508 **Figure 1**



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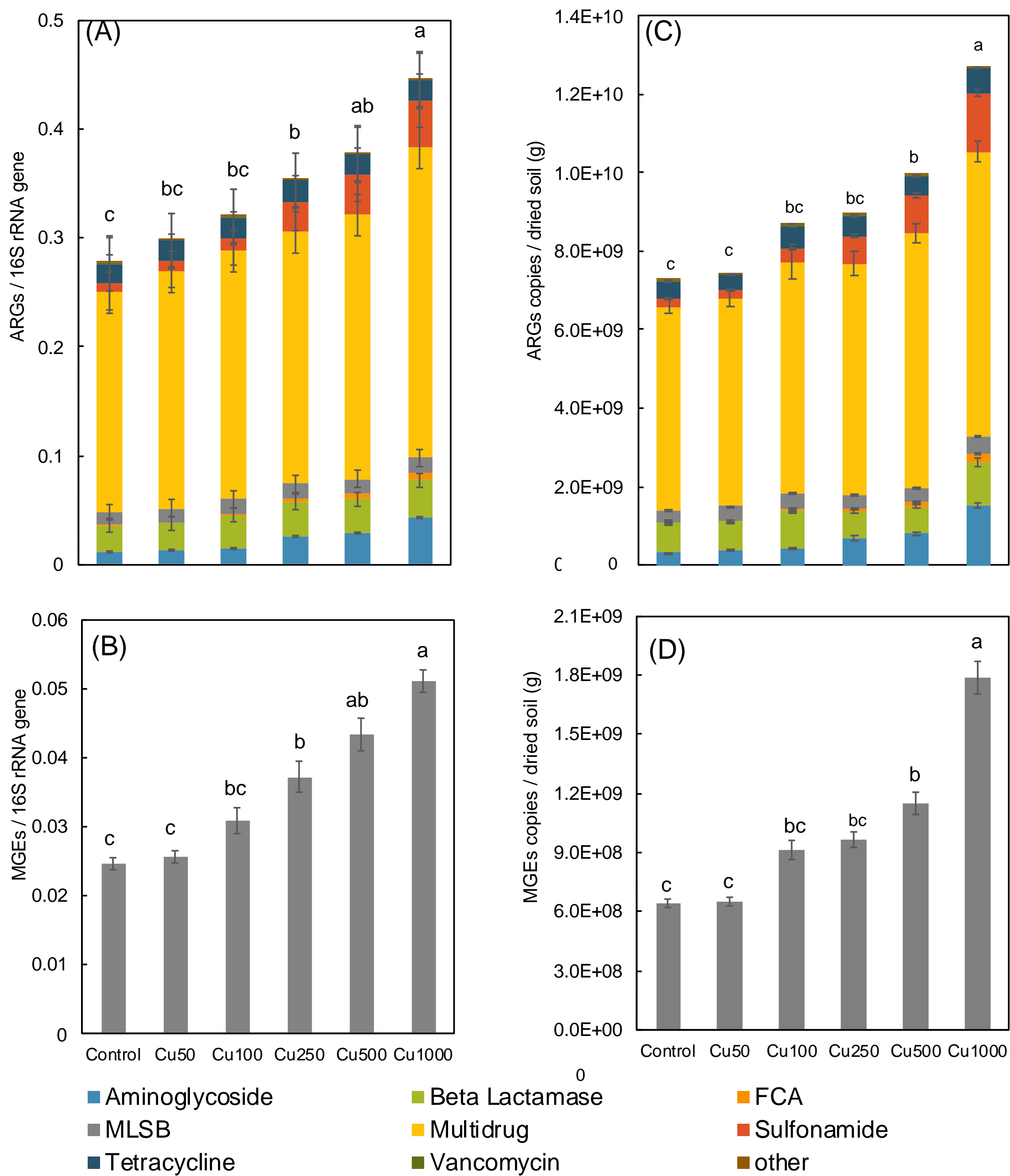
511 **Figure 2**



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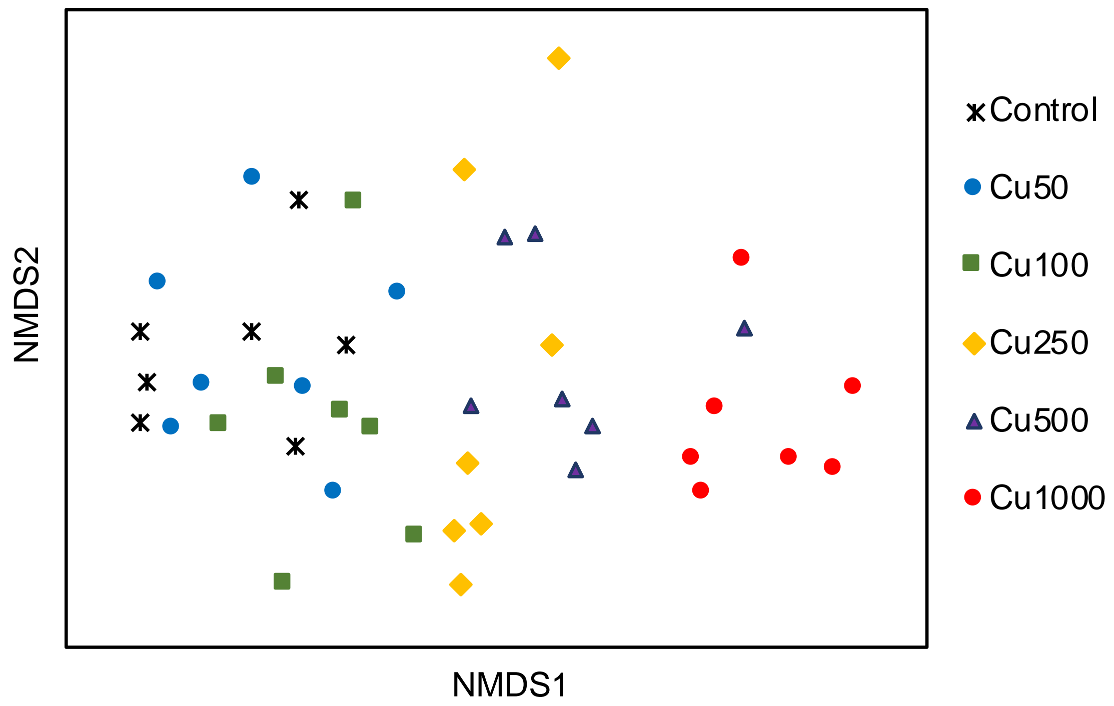
514 **Figure 3**



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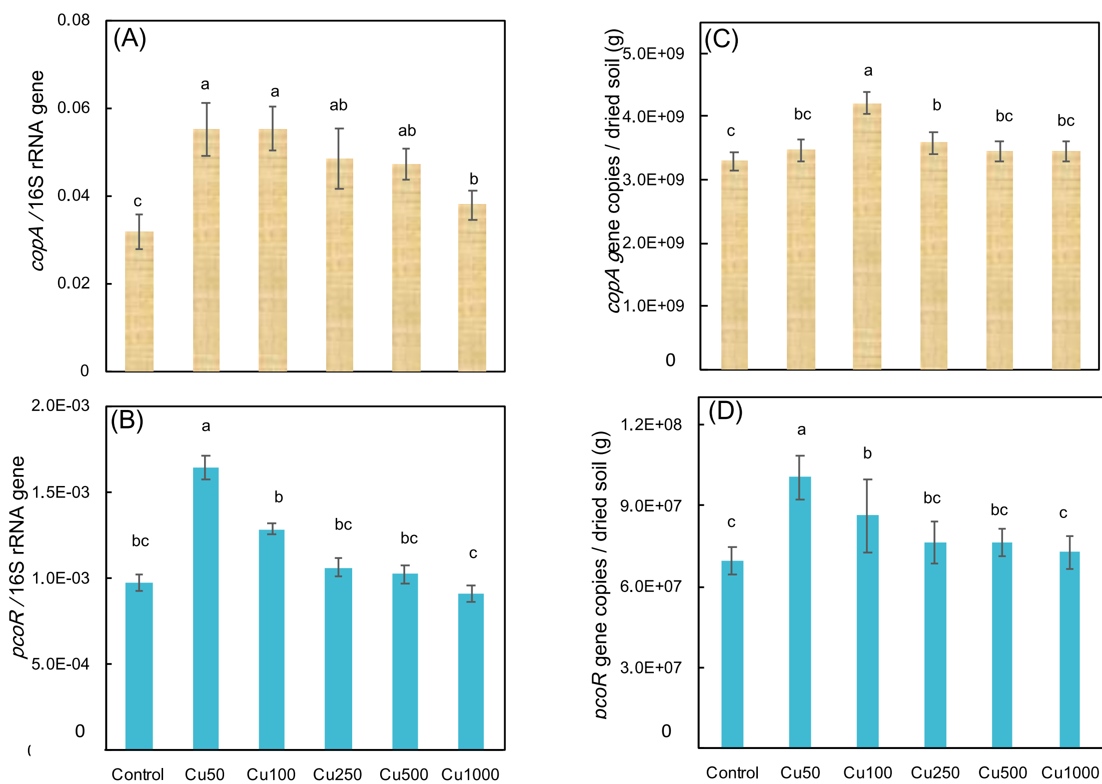
517 **Figure 4**



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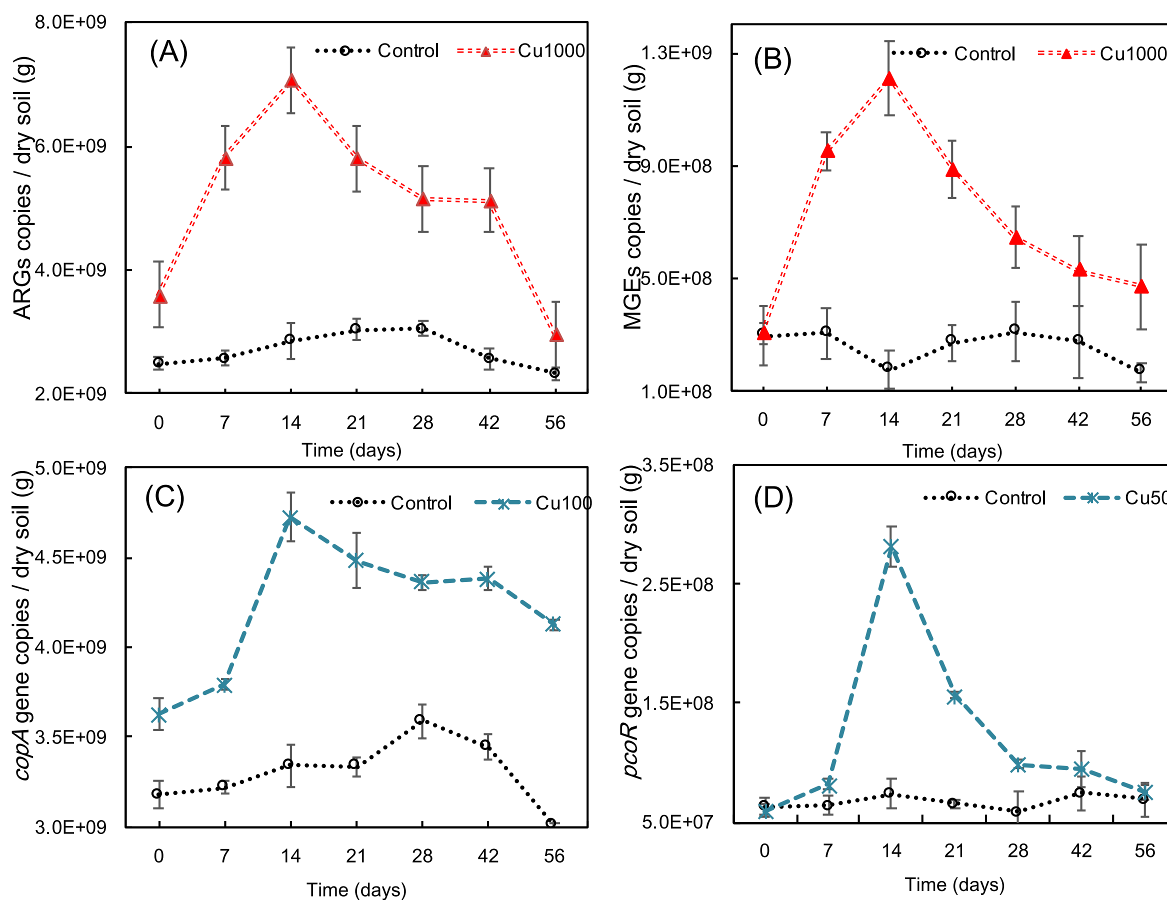
520 **Figure 5**



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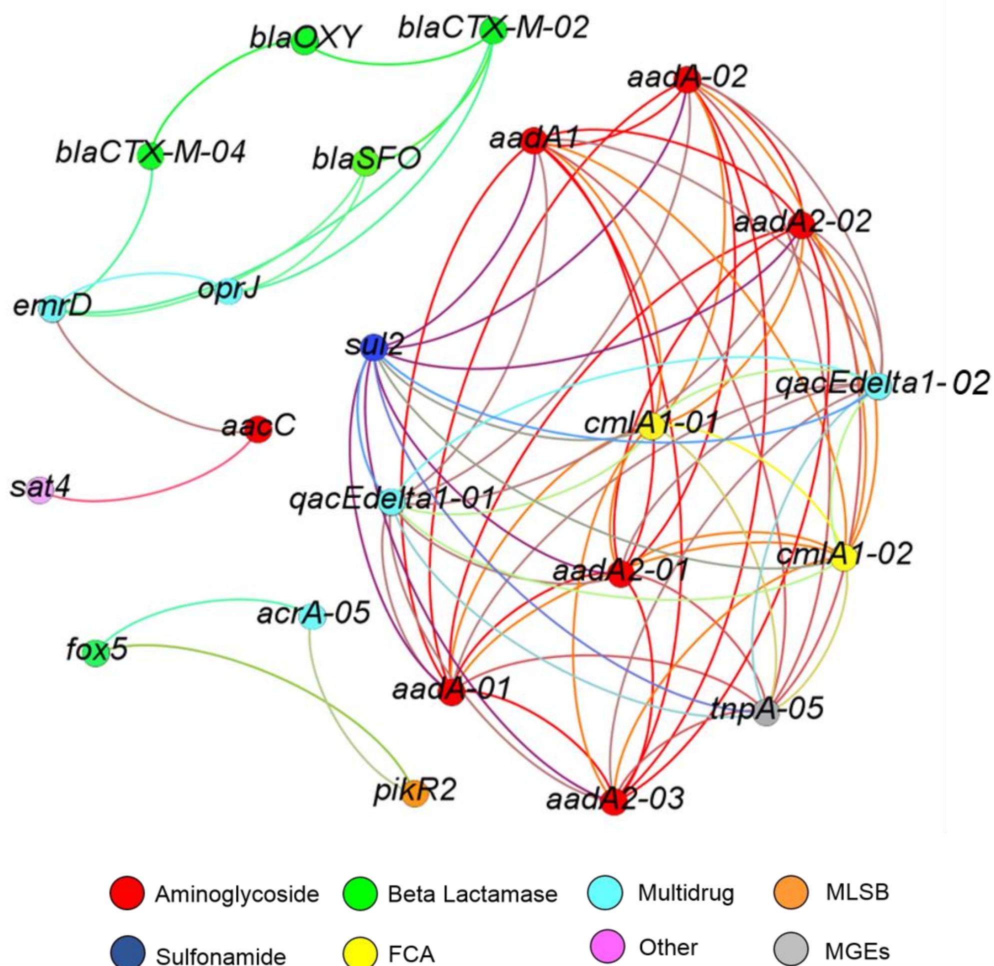
522 **Figure 6**



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