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Evidence for co-selection of antibiotic resistance genes and mobile genetic elements in metal polluted urban soils

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4 1 **Evidence for co-selection of antibiotic resistance genes and mobile genetic elements in metal polluted urban**
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6 2 **soils**
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Highlights:

1. Fifty archived urban soils from the Belfast area Tellus Survey were analyzed.
2. Antibiotic resistance determinants were profiled by high throughput qPCR chip.
3. Evidence for metal-induced co-selection of antibiotic resistance genes (ARGs).
4. Total ARGs were positively correlated with total mobile genetic elements.
5. Metals may confer persistent selection pressures for ARGs in urban soils.

1 **Abstract**

2 Antibiotic resistance genes (ARGs) constitute emerging environmental pollutants and pose risks to public
3 health. Toxic metals are known to select for metal-resistant bacteria in metal-contaminated soils, but there
4 is growing concern that metal contaminants can also act as co-selective agents thereby causing
5 environmental proliferation of antibiotic resistance. In this study, we quantified ARGs and selected
6 mobile genetic elements (MGEs) known to constitute potential ARG hosts in 50 archived urban and
7 suburban soils from the Belfast metropolitan area using a high-throughput qPCR ARG chip. ARG
8 prevalence was linked to concentrations of individual metals and a soil metal toxicity index calculated
9 based on the relative toxicity of different metals to soil microbial processes. A total of 164 ARGs were
10 detected across the 50 soils analyzed with an average absolute abundance of 3.4×10^7 ARG gene copies
11 per gram of soil. A significant correlation between abundance of ARGs and MGEs was observed,
12 suggesting the importance of horizontal gene transfer for ARG dissemination. Network analysis revealed
13 significant co-occurrence patterns between specific metals (As, Cd, Co, Cr, Cu, Hg, Ni and Zn) and
14 associated ARGs. Path analysis further indicated that the soil metal toxicity index significantly affected
15 the number of detected ARGs ($\lambda = 0.32$, $P < 0.001$) and the abundance of metal co-occurring ARGs ($\lambda =$
16 0.612 , $P < 0.001$) via effects on MGEs. Collectively, our results indicate a role of soil metals in co-
17 selection of ARGs and MGEs in urban and semi-urban soils and suggest a risk for environmental ARG
18 dissemination via horizontal gene transfer.

19

20 **Keywords**

21 Antibiotic resistance genes, mobile genetic elements, co-selection, metal toxicity, qPCR chip, urban soils

22

23

24 **1. Introduction**

25 The rapid emergence of antibiotic-resistant bacteria is occurring worldwide, posing threats to global
26 public health, food security and development (Ventola, 2015). According to the World Health
27 Organization (WHO), a post-antibiotic era is emerging, in which antibiotic resistance threatens the
28 effective prevention and successful treatment of an ever-increasing range of bacterial infections. The
29 critical role of the environment for development and dissemination of antibiotic resistance has now been
30 recognized (Ashbolt et al., 2013). Consequently, antibiotic resistance genes (ARGs) and mobile genetic
31 elements carrying these genes can be regarded as emerging environmental pollutants (Gillings et al., 2008;
32 Pruden et al., 2006).

33

34 The widespread use of antibiotics is generally considered to be the primary cause for elevated levels of
35 ARGs in pathogenic bacteria (Ventola, 2015), but there is growing concern that contaminants such as
36 metals and biocides may also co-select for antibiotic resistance (Baker-Austin et al., 2006; Berendonk et
37 al., 2015; Hoffman et al., 2005; Pal et al., 2017; SCENIHR, 2009). Co-selection of antibiotic and
38 metal(loid) resistance have been associated with arsenic (As), cadmium (Cd), cobalt (Co), chromium (Cr),
39 copper (Cu), mercury (Hg), nickel (Ni), lead (Pb) and zinc (Zn) (Knapp et al., 2011; Pal et al., 2017; Pal
40 et al., 2015; Seiler and Berendonk, 2012; Yazdankhah et al., 2014). Several co-selection mechanisms are
41 known (Baker-Austin et al., 2006). The genes encoding resistance to antibiotics and metals may for
42 instance be found on the same mobile genetic elements (plasmid, integron or transposon), and this
43 physical linkage results in co-resistance. Cross-resistance is another co-selection mechanism which occurs
44 when single genes encode resistance to both antibiotics and metals.

45

46 The relative importance of antibiotics and co-selecting agents for the selection of antibiotic resistance is
47 likely to differ between different environments. Antibiotic residues primarily accumulate to toxic levels

48 able to strongly select bacterial communities in habitats where antibiotics are used by humans (e.g.,
49 human or animal gut), whereas antibiotics only rarely accumulate to toxic levels in soil or water (Brandt
50 et al., 2015). By contrast, metals frequently accumulate to toxic levels in some environmental
51 compartments including both agricultural and urban soils (Brandt et al., 2010; Berg et al., 2012; Imfeld et
52 al., 2011; McLaughlin and Smolders, 2001). Indeed, toxic metals may in some cases provide stronger and
53 more persistent selective pressures for environmental selection of antibiotic resistance as compared to
54 antibiotic residues (Song et al., 2017). Consequently, metal-induced co-selection for ARGs in metal
55 contaminated environments represents a risk factor for the expansion of the soil bacterial resistome and
56 may thus represent a barrier for reversal of antibiotic resistance even if antibiotic residues are prevented
57 from reaching the environment.

58

59 Continuous accumulation of metal(loid) contaminants widely occurs in urban soils (Luo et al., 2012).
60 Over half of the world's population currently lives in urban areas, and the urban population continues to
61 grow (United Nations, 2014) suggesting increasingly important linkages between urban environmental
62 quality and human health (Li et al., 2018). Due to rapid urbanization and intensive anthropogenic activity,
63 a massive volume of potential selective agents (such as heavy metals) and microbes carrying ARGs
64 swarm into urban soils and successfully persist, increasing the level of ARG pollution in urban
65 environments (Wang et al., 2014). These ARGs spread amongst humans and in the environment by
66 horizontal gene transfer (HGT), developing into pathogenic antibiotic-resistant bacteria (pARB) and
67 thereby raising the risk of failure of antibiotic treatments (Zhu et al., 2018). The linkage between soil
68 metals and ARGs from urban and industrially polluted soils has been observed in previous studies (Knapp
69 et al., 2017, Berg et al. 2010). However, only a few metals and a limited number of ARGs were targeted
70 and comprehensive evidence for the ability of metals to co-select antibiotic-resistance in urban soils is
71 still largely lacking (Larsson et al., 2018). Therefore, monitoring ARG distribution in urban environments
72 and its association with metals as co-selective agents is necessary.

73

74 In this study, we profiled ARGs in metal-contaminated soils within an urban area to improve our
75 understanding of the role of metal contaminations in developing ARGs. To this end, 50 archived soils
76 across the urban area of Belfast, Northern Ireland with a gradient of various metal contaminations were
77 selected and examined using a high-throughput qPCR based ARG chip. The ARG profile was
78 subsequently linked to MGEs and metal contamination by multivariable statistical analyses and network
79 analysis. Cluster analysis, permanova test with land use and distance-decay analysis was applied to depict
80 the influence of geographic factors (spatial location) on ARG distribution. The path analysis was further
81 performed to test our hypotheses of metal-induced co-selection on ARGs.

82

83 **2. Materials and Methods**

84 2.1. Study area

85 Belfast is the capital and the largest city in Northern Ireland with a population of approximately 700 000
86 (NISRA, 2016). During the 18th and 19th centuries, Belfast grew to be the leading industrial city in
87 Ireland, with thriving linen and shipbuilding industries. Belfast continued to play an important role
88 throughout the industrial revolution in the 19th century and is also historically recognized for tobacco-
89 processing, rope making, glass manufacturing, tobacco production and distilleries (Royle et al., 2007).
90 Concentrations of metal(loid)s in the Belfast area have been shown to act as an ‘urbanization tracer’
91 (McIlwaine et al., 2017) and differential metal(loid) concentrations can, therefore, be largely attributed to
92 anthropogenic contamination. By contrast, anthropogenic contamination of the Belfast area soils with
93 antibiotics or ARGs can generally be considered negligible. Human fecal wastes are almost exclusively
94 released to aquatic recipients through wastewater collection and treatment processes (EMEA, 2006).
95 Leakage of untreated wastewater from the collection network into soil could potentially provide a route
96 for antibiotics and ARGs to enter the soil. However, the collection network is at a greater depth than the

97 soils investigated in this study (5-20 cm depth), and significant top soil contamination is therefore highly
98 unlikely. We therefore assume that ARGs present in the sampled top soils of the Belfast area were
99 primarily derived from the natural, ‘background’ antibiotic resistome (e.g. ARGs in soil-dwelling bacteria
100 producing or degrading antibiotics; (D’Costa et al., 2007; Dantas et al., 2008)) with potential
101 contributions from other diffuse sources (e.g. ARGs in fecal matter from wildlife, rain water, or particles
102 deposited via air). Hence, the metropolitan area of Belfast provides an opportunity to evaluate the ability
103 of soil metal(loid)s to co-select antibiotic resistance. The boundaries of the study area have been defined
104 using the Corine land cover data (European Environment Agency, 2012) satellite images and the spatial
105 distribution of the available urban soil samples.

106

107 2.2. Soil collection

108 The Tellus geochemical and geophysical survey was undertaken across the entire region of Northern
109 Ireland by the Geological Survey of Northern Ireland (GSNI) between 2004 and 2007. Shallow soil
110 samples (5-20 cm) from 1166 sampling locations were collected and archived at room temperature (<
111 25°C). Full details of the sampling strategy are described in a previous study (Smyth, 2007). In this study,
112 a total of 50 sampling locations were selected ([Figure 1a](#)) and their archived urban soils (Knights, 2006)
113 were retrieved from the GSNI Tellus survey archive in October 2016. Original sampling coordinates are
114 listed in [Table S1](#). Sample selection targeted a broad spectrum of toxic elements, with a wide range of
115 concentrations, and included a variety of different land uses while ensuring samples were spatially
116 distributed across the city.

117

118 2.3. Soil chemical characterization

119 Archived metal concentration data (As, Cd, Co, Cr, Cu, Hg, Ni, Pb and Zn) were retrieved from the
120 Tellus database for downstream analyses. The spatial distribution of these elements across Belfast has

121 been reported previously (McIlwaine et al., 2017). Concentrations of As in soils were found to be
122 controlled by anthropogenic inputs. By contrast, concentrations of Cu and Zn were influenced by both
123 anthropogenic and geogenic inputs, while concentrations of Ni, Co and Cr were mainly attributed to
124 geogenic sources (McIlwaine et al., 2017).

125

126 A metal toxicity index (TI_{metals}) was calculated for each soil sample to provide a normalized measure of
127 the bacterial selection pressure posed by the toxic metals present in each sample. TI_{metals} was calculated
128 based on previously recorded effects of individual metal(loid)s (As, Cd, Co, Cr, Cu, Hg, Ni, Pb and Zn)
129 on soil microbial processes (Welp, 1999) following a previously established procedure (Azarbad et al.,
130 2015; Stefanowicz et al., 2008): $TI_{\text{metals}} = \sum(C_i/EC_{50i})$, where C_i equals the total concentration of the
131 element i in the soil and EC_{50i} equals the half-maximal effective concentration for that particular element i
132 (Welp, 1999).

133

134 Archived soil pH and land use data were also retrieved from the Tellus database ([Table S1](#)). Full details
135 of the analytical methods used and quality assurance/quality control procedures adopted in the Tellus
136 geochemical survey can be found in a previous study (Smyth, 2007).

137

138 2.4. DNA extraction

139 The 50 selected soil samples were retrieved from the Tellus soil archive and aseptically weighed into
140 prepared sterile plastic bags. A total of 250 - 400 mg of dry soil was transferred into PowerBead Tubes
141 (MoBio) and incubated for rehydration at room temperature for 20-30 minutes. DNA was extracted from
142 rehydrated soil using MoBioPowerSoil® DNA Isolation Kit according to the instruction manual. The
143 concentration and purity of DNA were checked using ultraviolet absorbance (ND1000, Nanodrop,

144 Thermo Fisher Scientific). DNA was eluted in 100 µl elution buffer and stored at -20 °C for downstream
145 analysis. Long-term storage has been proven to not cause bias in DNA results (Tzeneva et al., 2009,
146 Knapp et al., 2010).

147

148 2.5. High-throughput qPCR

149 A total of 296 primer sets (Table S2) were used to examine Belfast urban soils. These 296 primer sets
150 targeted antibiotic resistance genes (285 primer sets), mobile genetic elements (10 primer sets) and a 16S
151 rRNA gene as a reference gene (Zhao et al., 2018; Zhu et al., 2017). Collectively, the targeted ARGs
152 confer resistance to all major classes of antibiotics including aminoglycoside, beta-lactamase,
153 chloramphenicol, macrolide-lincosamide-streptogramin B (MLSB), multidrug, sulfonamide, tetracycline
154 and vancomycin. Targeted mobile genetic elements included transposase genes (8 primer sets) and Class
155 1 integron-integrase gene (2 primer sets). The HT-qPCR was performed with an HT-qPCR based ARG
156 chip using the WaferGen SmartChip Real-time PCR system. Negative controls were included. The
157 thermal cycle consisted of 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 s and
158 annealing at 60 °C for 30 s. Melting curve analyses were automatically conducted by Wafergen
159 SmartChip qPCR software.

160

161 All HT-qPCR were performed in technical triplicates with negative control. The data from reaction wells
162 with r^2 smaller 0.99 were discarded. Only data for samples with three technical replicates that all
163 generated amplification products were regarded as positive detection and used in further data analysis.

164 Relative copy number was calculated based on previously published method (Looft et al., 2012): relative
165 gene copy number = $10^{(31-C_T)/(10/3)}$, where C_T refers to the qPCR results and 31 refers the cut-off point. The
166 normalized abundance of a gene (copies per 16S rRNA) was calculated by dividing relative gene copy
167 number of the gene by relative copy number of reference gene 16S rRNA.

168

169 Absolute 16S rRNA copy numbers (copies per gram of soil) were determined using the standard curve
170 method on a Roche 480 system. Each 20 μl qPCR reaction mixture consisted of 10 μl $2 \times$ LightCycle 480
171 SYBR Green I Master, one μg μl^{-1} bovine serum albumin, one μM of each primer, one ng μl^{-1} DNA as
172 template and six μl nuclease-free PCR-grade water. The thermal cycle consisted of a 10 min enzyme
173 activation at 95 $^{\circ}\text{C}$, followed by 40 cycles of denaturation at 95 $^{\circ}\text{C}$ for 30 s, annealing at 60 $^{\circ}\text{C}$ for 30 s
174 and extension at 72 $^{\circ}\text{C}$ for 15 s. A plasmid control containing a cloned and sequenced 16S rRNA gene
175 fragment (1.39×10^{10} copies per liter) was used to generate calibration curves from a tenfold dilution for
176 standard calculation. All qPCRs were performed in technical triplicates with negative controls. Absolute
177 ARG copy numbers were calculated by transforming relative copy numbers by normalization from
178 absolute 16S rRNA gene copy number.

179

180 2.6. Statistical analysis

181 All HT-qPCR data filtration and calculations were performed using Microsoft Excel 2010. Bar charts and
182 scatter diagrams were generated using Origin Pro 9.1. Correlation analyses and significance tests were
183 performed using IBM SPSS Statistics 22. Heatmaps were generated using HemI 1.0 (Deng et al., 2014).
184 Shannon H index was determined using PAST Statistics Software (Hammer et al., 2001). The distance-
185 decay analysis and permanova test were conducted using R 3.4.1 with the vegan package (Oksanen et al.,
186 2007). The co-occurrence patterns between ARGs (normalized abundance) and MGEs (normalized
187 abundance)/metals (total concentration) were explored using network analysis. To visualize the
188 correlations in the network interface, a correlation matrix was constructed using all pairwise Pearson's
189 rank correlations. Only correlations with Pearson's $r > 0.7$ (or < -0.7), a significance level of $P < 0.05$,
190 and p-values lower than the critical value calculated by the Benjamini-Hochberg procedure (false
191 discovery correction) were considered robust and used for forming the co-occurrence networks. The

192 network structure was confirmed by randomization procedure from randomized data. Network
193 visualization was performed in Cytoscape 3.6.0 (Shannon et al., 2003). For spatial data representation,
194 maps were produced using the ArcGIS software ArcMap version 10 (ESRI, 2010).

195

196 The path analysis, as a special case of structural equation model (SEM), has been proven useful as a
197 statistical tool to explore the complex networks of causal relationships ecosystems (Eisenhauer et al.,
198 2015). In this study, it was performed to evaluate the overall effect of soil metal contamination
199 (represented by TI_{metals}) on ARG patterns as represented by both the number of detected ARGs and the
200 normalized abundance of all co-occurring ARGs using SPSS AMOS. The path model was established
201 based on the following theoretical assumptions: (i) metal contamination may directly influence ARG
202 patterns by acting as a direct selective agent; (ii) metal contamination may indirectly affect ARG patterns
203 through MGE associated co-selection processes, with co-resistance as a mechanism. The data were fitted
204 to the estimated model using a maximum-likelihood estimation method. The model fit was tested and an
205 overall goodness-of-fit of the model was indicated by satisfaction of the following criteria: (i) non-
206 significant Chi-square value ($P > 0.05$); (ii) low root mean square error of approximation as absolute fit
207 index (RMSEA < 0.08); (iii) high increment fit index (CFI, GFI, TLI, NFI > 0.95 ; CFI as comparative fit
208 index, GFI as goodness of fit, TLI as Tucker-Lewis index and NFI as Normed-fit index). The disturbance
209 terms (also called the residual error terms) were added into the model to reflect the unexplained variance
210 and measurement error. The path coefficients (standardized regression weights) and significance were
211 calculated in an SPSS AMOS, showing the effect of an independent variable on a dependent variable in
212 the path model. The standardized direct, indirect and total effects were automatically calculated using
213 SPSS AMOS following the method published previously (Finney, 1972).

214

215 **3. Results**

216 3.1. Diversity and abundance of ARGs in Belfast urban area

217 A total of 175 genes (164 ARGs, eight transposase genes, two Class 1 integron-integrase genes and the
218 16S rRNA gene) were detected by the HT-qPCR chip in the 50 studied urban soils (Figure 1). The
219 detected ARGs represented most major resistance mechanisms including antibiotic deactivation (43%),
220 efflux pumps (34%) and cellular protection (19%) (Figure 1b) and confer resistance to most major classes
221 of antibiotics administered to humans and animals, including aminoglycosides, beta-lactams,
222 chloramphenicols, MLSB, sulfonamides, tetracyclines, vancomycin and multidrug. Almost half of the
223 detected ARGs confer resistance to beta-lactams (23%) and multidrug (23%) (Figure 1c). The number of
224 detected ARGs in the Belfast urban area ranged from 8 to 137 with the average at 67 (Figure 1a). The
225 Shannon diversity H index of ARGs varied from 1.2 to 3.4 in Belfast urban samples (Figure S1). To
226 compare the spatial similarity of ARG patterns, we examined the presence and absence of ARGs across
227 all samples. Only two shared core ARGs (*mphA-02* and *cphA-01*) conferring resistance to macrolide and
228 beta-lactam antibiotics were found in all samples.

229

230 The absolute abundance of ARGs in Belfast urban soils varied over six orders of magnitude (6.8×10^2 to
231 1.7×10^8 copies per gram of soil) with an average of 3.4×10^7 (Figure 1a). The predominant ARGs
232 encoded beta-lactam, multidrug, aminoglycoside or chloramphenicol resistance (Figure S2). The three
233 most abundant ARGs were *mexF*, *cphA-01* and *cmx(A)*. The *mexF* gene, which encodes a multidrug
234 resistance efflux pump for chloramphenicol and fluoroquinolone, was detected in 49 soil samples at $5 -$
235 229×10^7 copies per gram of soil. The *cphA* gene confers resistance to different beta-lactams antibiotics
236 (penicillin, cephalosporin and carbapenem) via hydrolysis of the beta-lactam ring and was detected in all
237 50 soils with an average abundance of 6.6×10^6 copies per gram of soil. The *cmx(A)* gene encodes a
238 chloramphenicol exporter and was found in 49 samples, varying in abundance from $2 - 43 \times 10^7$ copies
239 per gram of soil.

240

241 To better explore the prevalence of ARGs within the studied soil bacterial communities, the absolute
242 abundance of ARGs and MGEs was normalized relative to the abundance of the 16S rRNA gene.
243 Bacterial abundances in soils were in the range of 8.7×10^3 to 9.0×10^8 with an average of 2.2×10^8
244 copies per gram. The normalized ARG abundance in 50 soils varied from 0.06 to 0.77 with an average of
245 0.15 ARG copies per 16S rRNA gene.

246

247 We further investigated the spatial distribution of ARGs with distance-decay analysis and cluster analysis.
248 However, our results show that ARG distribution in the Belfast urban area was unlikely to have been
249 driven by geographic factors. The geographic distance did not show any significant correlation with the
250 similarity of ARG communities between samples (Pearson's $r = -0.026$, $P = 0.355$) (Table S3). Likewise,
251 land use at the time of soil sampling could not explain the observed ARG patterns. Combining the cluster
252 analysis with a heatmap to visualize the ARG profiles in different land uses, we thus did not find any
253 significant effect of land use (cluster) on the ARG distribution in Belfast urban soils (Figure S3&4). This
254 conclusion was further confirmed by permanova test ($R^2 < 0.02$, $P > 0.05$; Table S4). Both results
255 indicated the inconsequential role that geographic factors played in determining the fate of ARGs in
256 Belfast urban soils.

257

258 3.2. MGEs and their associations with ARGs

259 A total of two integron genes and eight transposon genes were targeted and detected in this study. The
260 total absolute abundance of these MGEs ranged from $2-38 \times 10^7$ copies per gram of soil with an average
261 of 3.4×10^6 . The normalized abundance of MGEs ranged from 0.004 to 0.069 copies per 16S rRNA gene
262 with average at 0.014. Class 1 integron, *intI-1*, was found in all 50 soils. Significant correlations were
263 found between MGEs and ARGs (Figure 2). The normalized abundance of MGEs was positively

264 correlated with the number of ARGs detected (Pearson's $r = 0.57$, $P < 0.001$), as well as normalized
265 abundance of ARGs (Pearson's $r = 0.66$, $P < 0.001$) (Figure 2a). Positive correlations were also found
266 between the absolute abundance of ARGs and the MGEs: Class 1 integrons (Pearson's $r = 0.97$, $P < 0.001$)
267 and transposons (Pearson's $r = 0.66$, $P < 0.001$) (Figure S5).

268
269 The co-occurrence pattern between specific ARGs and MGEs were revealed by network analysis based
270 on Pearson correlations ($r > 0.7$, $P < 0.05$) (Figure 2b). The network consists of 130 nodes corresponding
271 to 8 MGEs and 122 ARGs. A total of 358 strong correlations between these MGEs and ARGs were found,
272 including 334 positive correlations and 24 negative correlations. MGEs including integrons and
273 transposons both exhibited a co-occurrence pattern with different types of ARGs. A total of 81 ARGs
274 were positively correlated with the class 1 integron, *intI-1*, while 77 ARGs were positively correlated with
275 the clinical class 1 integron, *cIntI-1*. For transposons, 66 ARGs were found positively correlated to *tnpA*
276 and *IS613* genes. Among the 358 correlations between ARGs and MGEs, 31% were contributed by
277 multidrug-resistant genes, while beta-lactam, MLSB, vancomycin, aminoglycoside, tetracycline and
278 chloramphenicol resistant genes accounted for 20%, 12%, 12%, 7%, 6% and 2%, respectively.

279

280 3.3. Co-occurrence pattern between metals and ARGs

281 The soil samples profiled for ARGs and MGEs were contaminated to varying degrees by metals. The
282 metals with the potential of co-selection for ARGs and their concentration ranges in the soil samples were
283 shown in Table 1. The co-occurrence pattern between metals and ARGs was further explored by network
284 analysis based on Pearson's correlation ($r > 0.7$, $P < 0.05$) (Figure 3). The network consists of 25 nodes
285 including eight metals and 17 ARGs. A total of 24 significant positive correlations were found between
286 metals and ARGs. No significant negative correlation was found between metals and ARGs. As, Cd, Co,
287 Cr, Cu, Hg, Ni and Zn all exhibited co-occurrences with specific ARGs. Zinc was found to co-occur with

288 eight resistance genes conferring resistance to aminoglycosides (4), multidrug (3) and beta-lactam
289 antibiotics (1). Copper co-occurred with aminoglycoside resistance genes (*aadA* and *aac*) and MLSB
290 resistance genes (*mefA*). The number of ARGs that co-occurred with Cd, Co, Ni, Hg, Cr and As were 3, 3,
291 2, 2, 2 and 1, respectively. These metal(loid)s all co-occurred with 17 specific ARGs conferring resistance
292 to aminoglycosides, beta-lactams, MLSBs, multidrug, tetracycline and vancomycin. Among the 24
293 connections, almost half were contributed by a combination of aminoglycoside (25%) and multidrug
294 resistance genes (21%).

295

296 3.4. The effects of metal toxicity index and MGEs on ARGs

297 To further assess the effects of metals and MGEs on ARGs, structural equation model (SEM) based path
298 analysis was performed with a multiple-pathways model based on the theoretical assumptions outlined in
299 section 2.6 (Figure 4a). The path analysis indicated that the degree of soil metal contamination (metal
300 toxicity index) had a significant direct positive association on normalized abundance of co-occurring
301 ARGs (copies per 16S rRNA gene) ($\lambda = 0.187$, $P < 0.01$) and a significant indirect positive effect on
302 number of detected ARGs ($\lambda = 0.251$, $P < 0.001$) (Figure 4b) and normalized abundance of co-occurred
303 ARGs (copies per 16S rRNA gene) ($\lambda = 0.426$, $P < 0.001$) (Figure 4c). The total standardized effects of
304 metal toxicity index on the number of detected ARGs and normalized abundance of co-occurring ARGs
305 (copies per 16S rRNA gene) were 0.323 and 0.612, respectively. This suggests that increase of one unit of
306 one metal toxicity index resulted in 0.323 more numbers of ARGs detected and 0.612 more copies of co-
307 occurred ARGs per 16S rRNA gene. Metal toxicity changed MGE abundances in soil ($\lambda = 0.521$, $P <$
308 0.001) and subsequently influenced the number of detected ARGs and abundance of co-occurring ARGs
309 ($\lambda = 0.483$, $P < 0.001$ and $\lambda = 0.817$, $P < 0.001$) (Figure 4a). Metal toxicity index and MGE abundance
310 both had a total positive effect on the number of detected ARGs and abundance of co-occurred ARGs.
311 The path analysis results were further tested and confirmed by correlation analysis. The correlation
312 analysis showed significant positive correlations among metal toxicity index, the abundance of MGE,

313 number detected ARGs and abundance of co-occurring ARGs (Figure S6), which further confirmed the
314 positive association between metals and ARGs via MGEs. Significant positive correlations were also
315 found between soil pH and the number of detected ARGs (Pearson's $r = 0.329$, $P < 0.05$), and between
316 the absolute abundance of the 16S rRNA gene and total ARGs (copies per gram) (Pearson's $r = 0.905$, P
317 < 0.001).

318

319 **4. Discussion**

320 4.1. Evidence for metal-induced co-selection of ARGs in urban soils

321 To the best of our knowledge, this present study provides the most comprehensive investigation of the
322 ability of metal contamination to affect the distribution of ARGs in urban soils to date. The co-
323 occurrence between specific metal contaminants and specific ARGs together with the significant positive
324 effect of overall soil metal loading (metal toxicity index) on soil ARGs are key findings and suggests co-
325 selection of metals and ARGs (Figure 3 & 4). Not only was a diverse range of ARGs detected, many of
326 which displayed significant co-occurrence patterns with both specific metals and overall metal load
327 (metal toxicity index), but we were also able to identify a potential causal link between soil metals and
328 ARGs. Hence, observed ARG patterns could not be explained by current land use or geographic location
329 and with the possible exception of the two included pasture soils (Table S1), the studied soils were
330 generally unlikely to have received significant recent point source inputs of fecal materials from humans
331 or animals treated with antibiotics. Soil pH also affected observed ARG patterns; this could most likely be
332 attributed to the known abilities of pH to modulate bacterial community composition (Rousk et al., 2010)
333 and the bioavailability/toxicity of metals (Smolders et al., 2009).

334

335 4.2. Co-selection mechanisms and potential for horizontal gene transfer of ARGs

336 Although our study was not specifically designed to compare the relative importance of different co-
337 selection mechanisms (Baker-Austin et al., 2006), it clearly demonstrated co-selection of ARGs and
338 MGEs (Figure 4). The strong linkages between metal toxicity index, MGEs and ARGs indicate a
339 significant metal impact on both the diversity and abundance of ARGs via MGEs (Figure 4). According
340 to our results of path analysis, 78% of the total effect of metal toxicity on the number of detected ARGs
341 and 69% of the total effect of metal toxicity on the abundance of co-occurring ARGs were observed to
342 occur via MGEs. The majority of co-occurring ARGs with metals were found to also co-occur with
343 MGEs, suggesting an underlying metal-driven co-selection process with co-resistance (i.e. linkage of
344 genes conferring resistance to metals and antibiotics on the same genetic element) as the major
345 mechanism for most studied ARGs that do not have any known roles in bacterial metal resistance.
346 However, the resistance genes *acrF*, *adeA*, *ttgB*, *qacEAI*, *rarD*, *tetPA* and *mefA* encode efflux pumps as
347 their resistance mechanism and cross-resistance with other classes of antimicrobial agents via efflux
348 cannot be ruled out. For instance, the multi-drug resistance pumps encoded by the genes *acrF*, *adeA*,
349 *qacEAIrarD* and *ttgB* can export both metals and antibiotics for detoxification purposes (Mata et al.,
350 2000).

351

352 The ability of soil bacteria to transfer ARGs to pathogenic bacteria of clinical relevance is of considerable
353 public health concern (Forsberg et al., 2012, Ashbolt et al., 2013, Graham et al., 2016). Importantly, our
354 study indicates that urban soil metal pollution co-selected ARGs that may be prone to horizontal gene
355 transfer between different species of bacteria. Results of correlation analysis, network analysis and path
356 analysis all showed that an increase in MGE abundance was strongly correlated with an increase in ARG
357 diversity and abundance (Figure 2, 4 & S6). The co-occurrence pattern of ARGs and MGEs revealed by
358 network analysis showed several clusters within the network. Resistance genes such as *aac* and *aad* genes,
359 known to be carried within integron gene cassettes (An et al., 2018; Partridge et al., 2009), were strongly
360 correlated with the class 1 integron gene *IntI-1*, clinical class 1 integron gene *cIntI-1* and transposon

361 genes *tnpA* and *IS613* (Figure 2). The clinical class 1 integron-integrase gene, *cIntI-1*, exhibits rapid
362 responses to various environmental pressures (including toxic metals) and thus has been proposed as a
363 marker for anthropogenic pollution and as an emerging pollutant (Gillings et al., 2015; Gillings, 2018).
364 The significant correlation and co-occurrence pattern between the clinical class 1 integron-integrase gene
365 (*cIntI-1*) and ARGs (Table S5 & Figure 2) may therefore suggest that metal contamination increases
366 environmental selection of bacteria containing clinical Class 1 integrons conferring resistance to both
367 metals and antibiotics even in the absence of a selection pressure exerted by antibiotic residues.

368

369 4.3. Conclusions and perspectives for the environmental dissemination of ARGs.

370 Our findings provide evidence that metal contamination (As, Cd, Co, Cr, Cu, Hg, Ni and Zn) could
371 significantly affect the diversity, abundance and mobility potential of a broad spectrum of ARGs in urban
372 soils. Collectively, our results suggest that urban soil metal contamination increases the potential for
373 horizontal gene transfer of ARGs via co-selection of ARGs and MGEs thereby generating a pool of high-
374 risk mobile ARGs (Martínez et al., 2015). Indeed, it is likely that metals in many soils confer more
375 important selective agents than antibiotic residues because metals, as opposed to antibiotics, frequently
376 accumulate to persistent toxic levels in contaminated soils (Song et al., 2017). Based on the ongoing
377 recruitment of resistance genes by MGEs (Gillings et al., 2015, Gillings, 2018), we further hypothesize
378 that soil metals may become an even more important public health risk factor for the evolution (e.g.
379 selection, mobilization, transfer and persistence) of ARGs in the future. Hence, we call for additional
380 research into metal-induced co-selection of ARGs and MGEs in metal contaminated soils in the interest
381 of public health (Larsson et al., 2018).

382 Urban soils are commonly contaminated with toxic metals (McIlwaine et al., 2017) and our study thus has
383 implications for environmental regulation and management of urban soils worldwide. Hence, urban
384 residents may be exposed to ARGs via ingestion of metal-contaminated urban soils (especially children)

385 or vegetables (urban gardening) unless adequate precautions are taken. Metals and co-selected ARGs may
386 also be leached to groundwater or transported by surface runoff to aquatic receptors implying subsequent
387 human ARG exposure via drinking water (Frey et al., 2015, Ma et al., 2017) or recreational activities
388 (Leonard et al., 2018). Specific metals will probably co-select for different ARGs in different soils and it
389 will therefore not be relevant to monitor only a few ARGs when evaluating risks posed by toxic soil
390 metals. We therefore propose that metal susceptibility in indicator soil bacteria or absence of pollution-
391 induced community tolerance to metals (Lekfeldt et al., 2014) might serve as additional protection goals
392 for environmental risk assessment of toxic metals in soil. In conclusion, serious consideration is needed to
393 set minimum standards for retarding ARGs and for mitigating the accumulation of toxic metals in urban
394 soils.

395

396 **Supporting Information**

397 Identity of the 50 Tellus soil samples selected for this study (Table S1); primers used in this study (Table
398 S2); relationships between geographical distance and distributions of antibiotic resistance genes (ARGs)
399 and metal(loid) contamination as investigated by distance-decay analysis (Table S3); the ability of
400 different land use categories to explain variation in ARG distribution by permanova test (Table S4);
401 correlations between normalized abundance of clinical class 1 integron-integrase gene and total ARGs
402 (Table S5) (in Supporting Information.docx).

403 Shannon H index of 50 soil samples showing ARG diversity (Figure S1); stacked bar chart showed the
404 relative percentage of absolute abundance of ARGs (Figure S2); heatmap showing normalized abundance
405 of ARGs in 50 Tellus soils and their land use (Figure S3); heatmap showing absolute abundance of ARGs
406 in 50 Tellus soils and their land use (Figure S4); correlation between absolute abundances of class 1
407 integron/transposases and ARGs (Figure S5); correlations among metal toxicity index, MGEs and ARGs
408 (Figure S6) (in Supporting Information.docx).

409

410 **Conflict of interests**

411 The authors declare no conflicts of interests.

412

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422

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572

573

574 **Figures Legend**

575 **Figure 1** Antibiotic resistance gene (ARG) profile in Belfast urban soils. The map (a) reveals the number
576 of different ARGs detected (dot size scale) and their absolute abundance (copies per gram of soil; color
577 scale) in 50 urban soils from the Belfast metropolitan area. Pie charts depict (b) the percentage of detected
578 ARGs corresponding to different resistance mechanisms and (c) their classification based on the
579 antibiotics they confer resistance to.

580 **Figure 2** Correlation between mobile genetic elements (MGEs) and antibiotic resistance genes (ARGs)
581 across all 50 Belfast urban soils. (a) The normalized abundance of all targeted MGEs (2 integrons and 8
582 transposons) significantly correlated to the total number of detected ARGs and the normalized abundance of
583 ARGs (copies per 16S rRNA gene) based on Pearson's correlation ($P < 0.001$). (b) Network analysis
584 showing the co-occurrence pattern between individual MGEs and ARGs. A connection represents a
585 strong (Pearson's $r > 0.7$) and significant ($P < 0.05$) correlation. The nodes with different colors represent
586 MGEs and different ARG types. The edges with different colors correspond to positive (grey) and
587 negative (red) correlations between nodes. The size of node is proportional to the number of connections
588 between nodes. The width of edge is proportional to the degree of correlation.

589 **Figure 3** Network analysis showing the co-occurrence pattern between metals (As, Cd, Co, Cr, Cu, Hg,
590 Ni, Pb, and Zn) and antibiotic resistance genes (ARGs) based on Pearson correlation analysis. A
591 connection represents a strong (Pearson's $r > 0.7$) and significant ($P < 0.05$) correlation. The color of each
592 node represents each metal or the type of ARG. The node size is proportional to the number of the
593 connections. The edge width represents the degree of correlation plotted with Pearson's r . All significant
594 correlations in the data set were positive.

595 **Figure 4** Path analysis showing the modelled effect of soil metal contamination (metal toxicity index) and
596 normalized abundance of mobile genetic elements(MGEs) on (a) the number of detected antibiotic
597 resistance genes (ARGs) and the normalized abundance of co-occurring ARGs (copies per 16S rRNA

598 gene). Path arrows correspond to positive (red) and negative (green) effects with significance level of $P <$
599 0.05 (solid line), and $P > 0.05$ (dotted line) with path directions. Numbers adjacent to the path arrows are
600 path coefficients (standardized regression weights), and the arrow width is proportional to the strength of
601 path coefficients. Bar charts show the standardized direct effect, indirect effect and total effect of metal
602 toxicity index and MGEs on (b) number of detected ARGs and (c) normalized abundance of co-occurring
603 ARGs (copies per 16S rRNA gene) derived from path modelling (a).

Table 1 Total concentrations of metals in Belfast urban soils.

Symbol	Name	Average (mg/kg)	Min - Max (mg/kg)	Category
Pb	Lead	354.5	22.9 - 2910	Post-transition metal
Cd	Cadmium	0.8	0.08 - 3.27	Transition metal
Co	Cobalt	22.3	6.1 - 48	Transition metal
Cr	Chromium	64.1	24 - 345	Transition metal
Cu	Copper	160.0	19 - 954	Transition metal
Hg	Mercury	0.4	0.06 - 1.86	Transition metal
Ni	Nickel	82.3	20.1 - 244	Transition metal
Zn	Zinc	458.4	32 - 5550	Transition metal
As	Arsenic	14.1	2.2 - 51.3	Metalloid

*Analyzed with *aqua regia* digestion followed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (McIlwaine et al., 2017).

Figure
[Click here to download Figure: Figure 1.pdf](#)

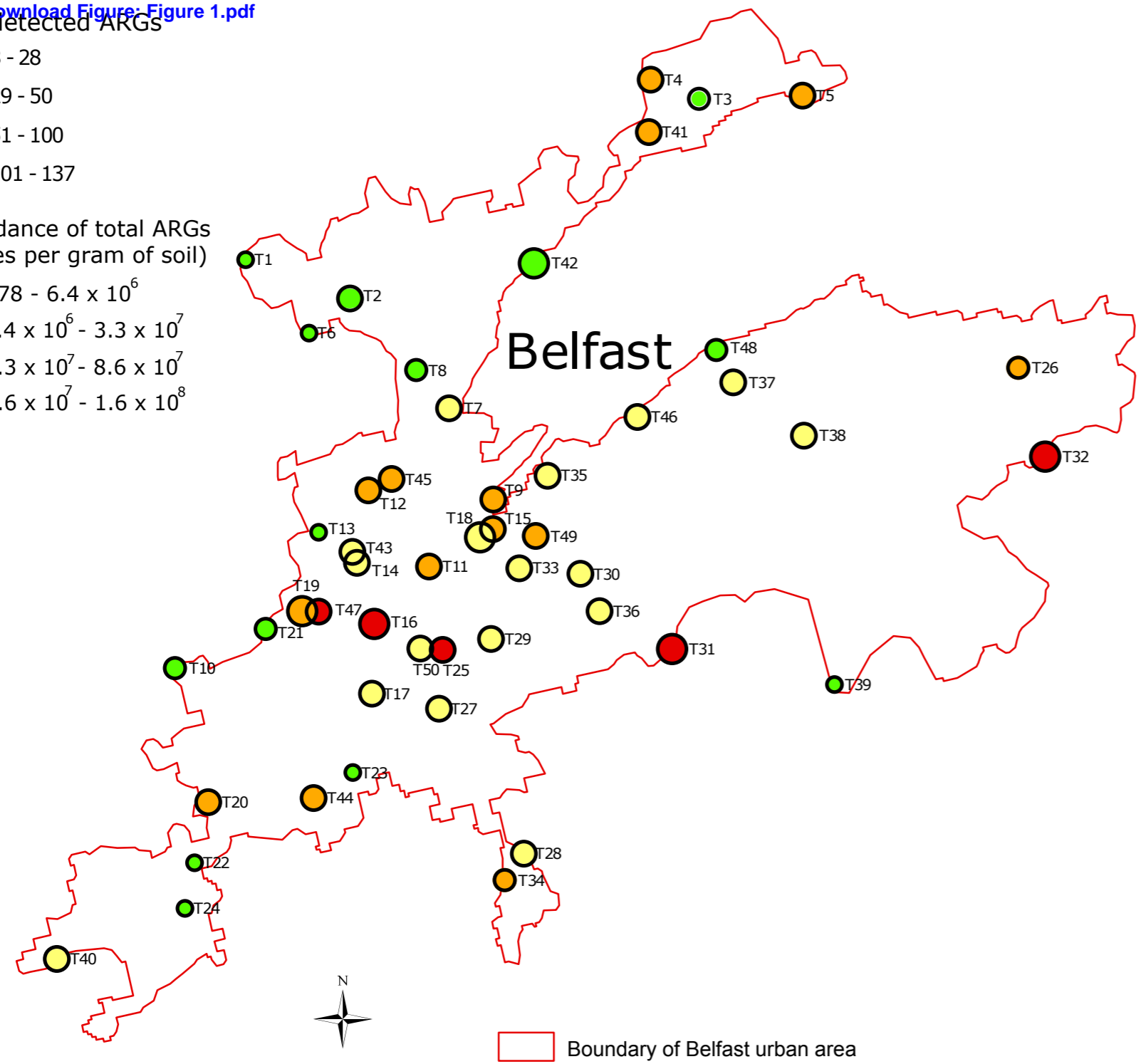
a

No. detected ARGs

- 8 - 28
- 29 - 50
- 51 - 100
- 101 - 137

Abundance of total ARGs
(copies per gram of soil)

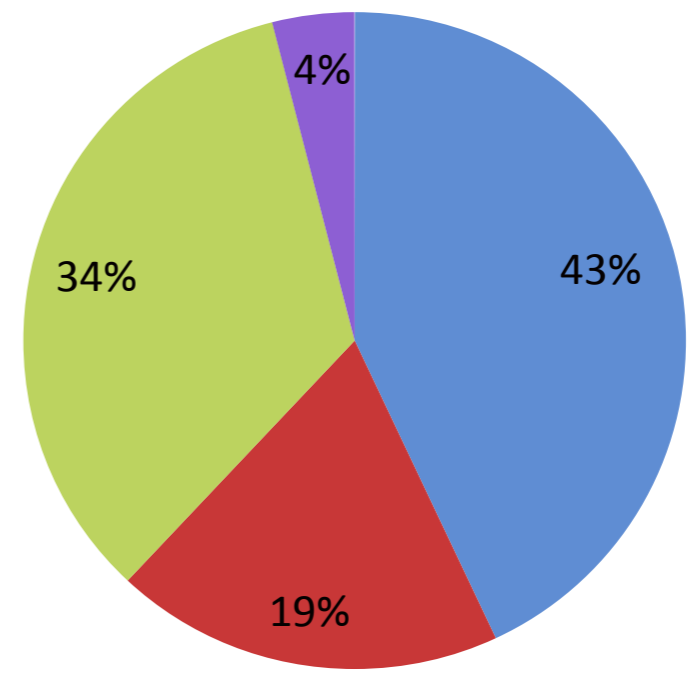
- 678 - 6.4×10^6
- 6.4×10^6 - 3.3×10^7
- 3.3×10^7 - 8.6×10^7
- 8.6×10^7 - 1.6×10^8



Boundary of Belfast urban area

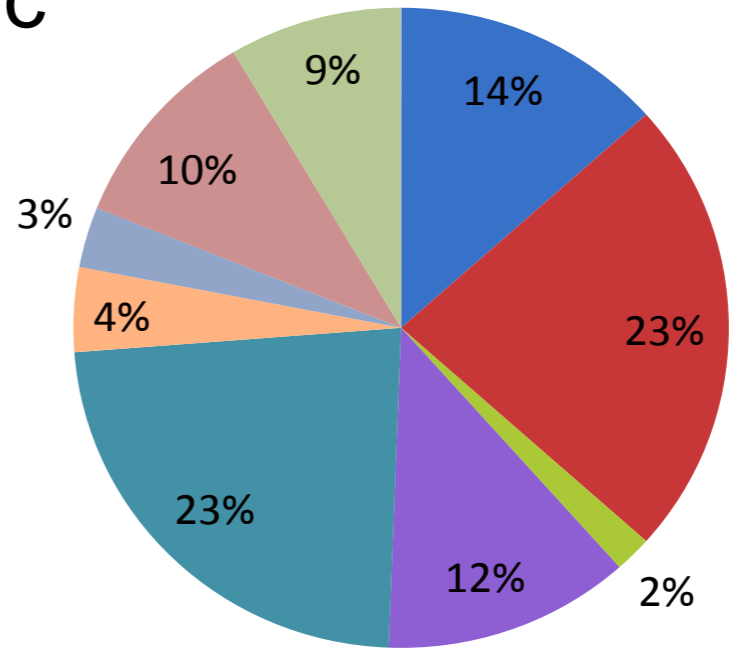
Source: Esri, DigitalGlobe, GeoEye, Earthstar Geographics, CNES/Airbus DS, USDA, USGS, AeroGRID, IGN, and the GIS User Community

b



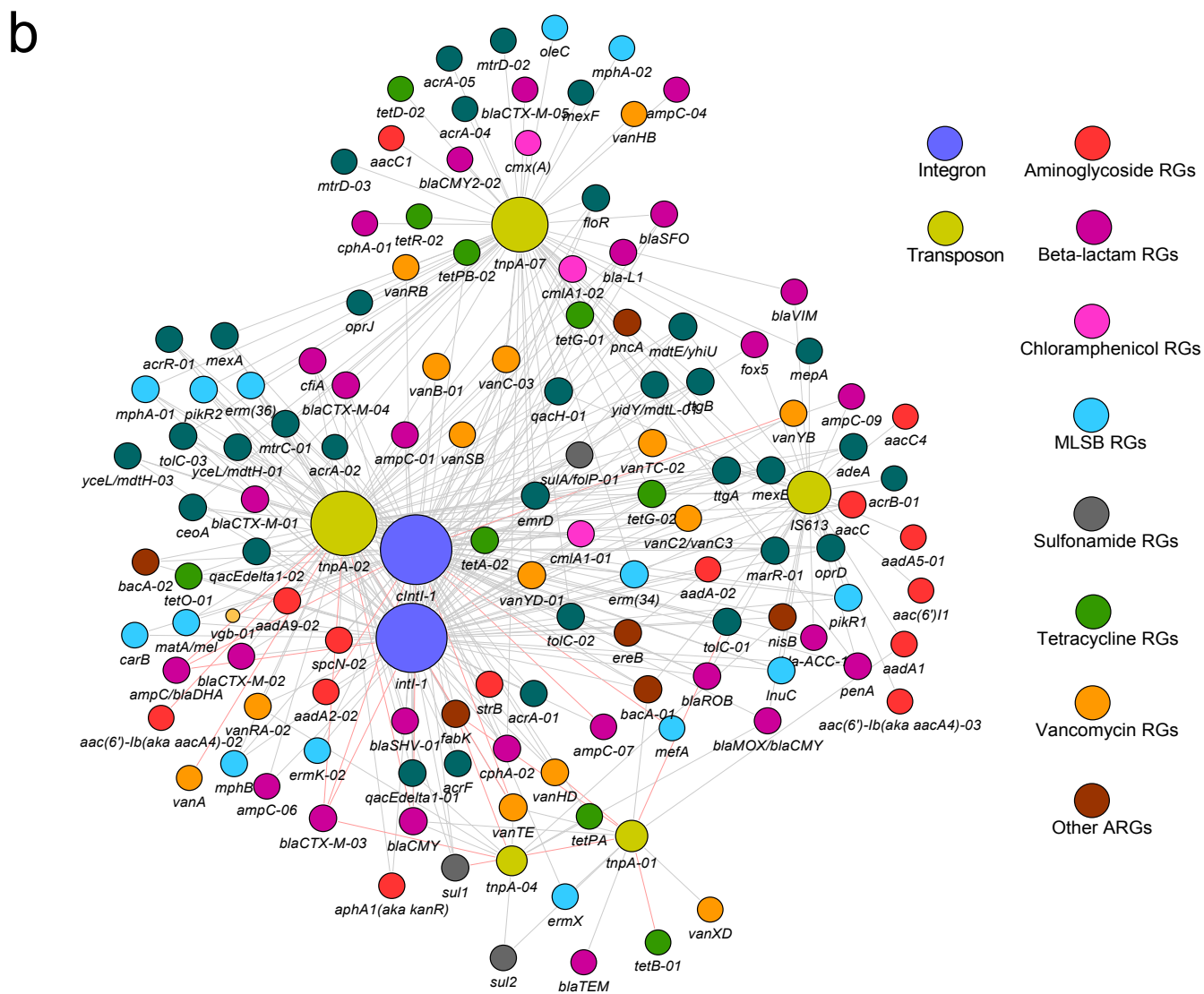
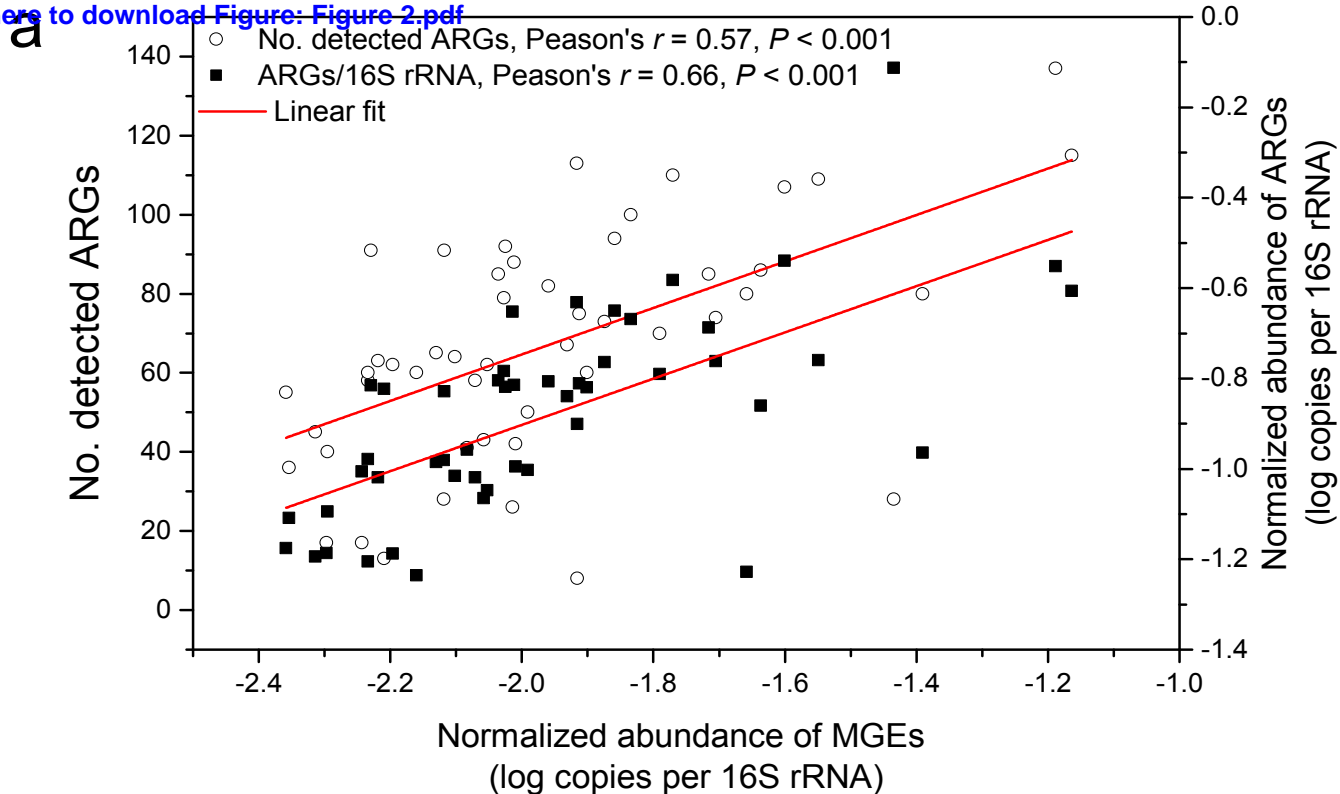
- Antibiotic deactivation
- Cellular protection
- Efflux pump
- Other/unknown

c



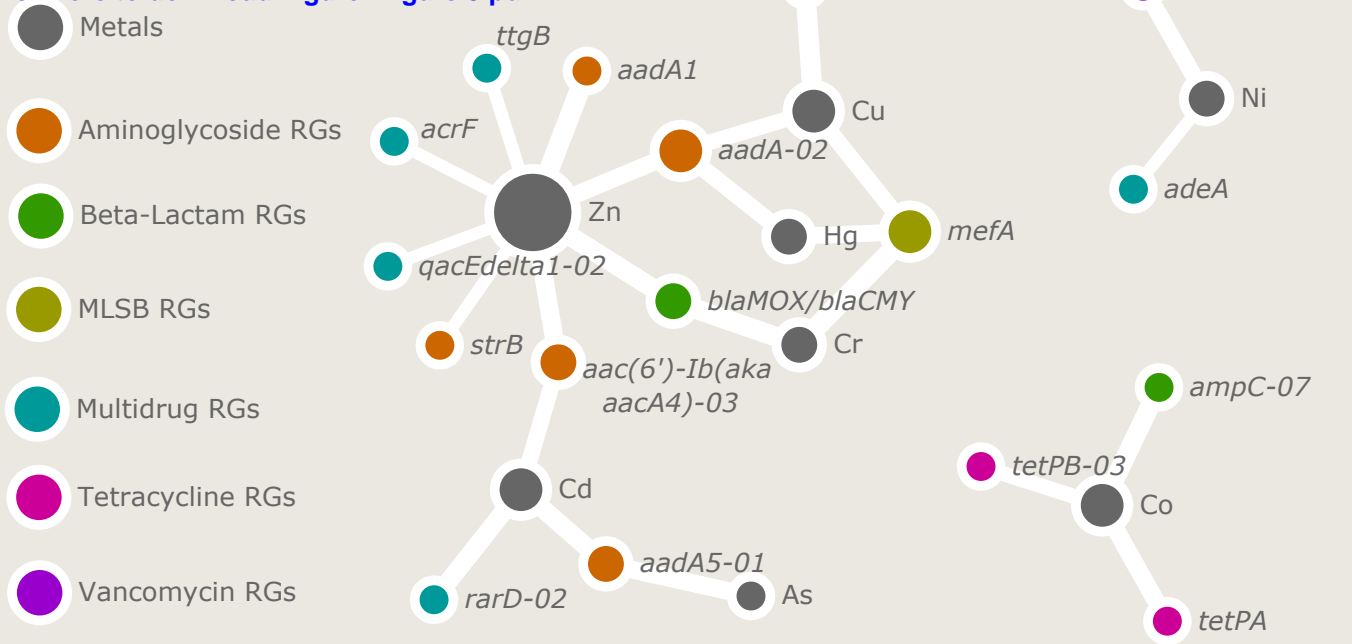
- Aminoglycoside
- Beta-lactam
- Chloramphenicol
- MLSB
- Multidrug
- Sulfonamide
- Tetracycline
- Vancomycin
- Others

Figure
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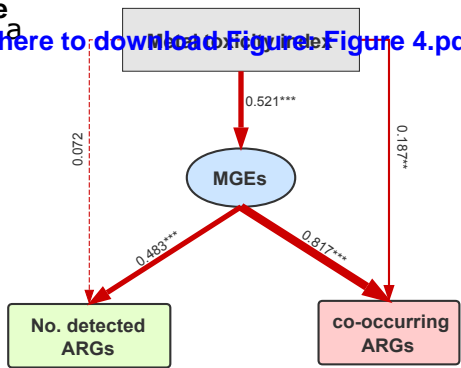
Figure

[Click here to download Figure: Figure 3.pdf](#)



Figure

[Click here to download Figure 4.pdf](#)

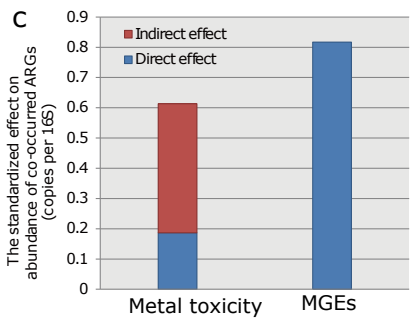
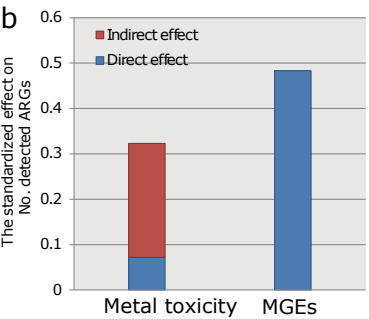


➔ : Positive effect
➔ : Negative effect

➔ : paths with $P < 0.05$
- ➔ : paths with $P > 0.05$

Goodness-of-fit-model > 0.95
Degree of freedom (df) : 1
Chi-square = 1.226 ($P > 0.05$)
RMSEA < 0.08

*** : $P < 0.001$
** : $P < 0.01$
* : $P < 0.05$



Supplementary material for on-line publication only

[Click here to download Supplementary material for on-line publication only: Supporting Information.docx](#)