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Zhao, Y., Cocerva, T., Cox, S., Tardif, S., Su, J. Q., Zhu, Y. G., & Brandt, K. K. (2019). Evidence for co-selection of antibiotic resistance genes and mobile genetic elements in metal polluted urban soils. *Science of the Total Environment*, 656, 512-520. <https://doi.org/10.1016/j.scitotenv.2018.11.372>

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

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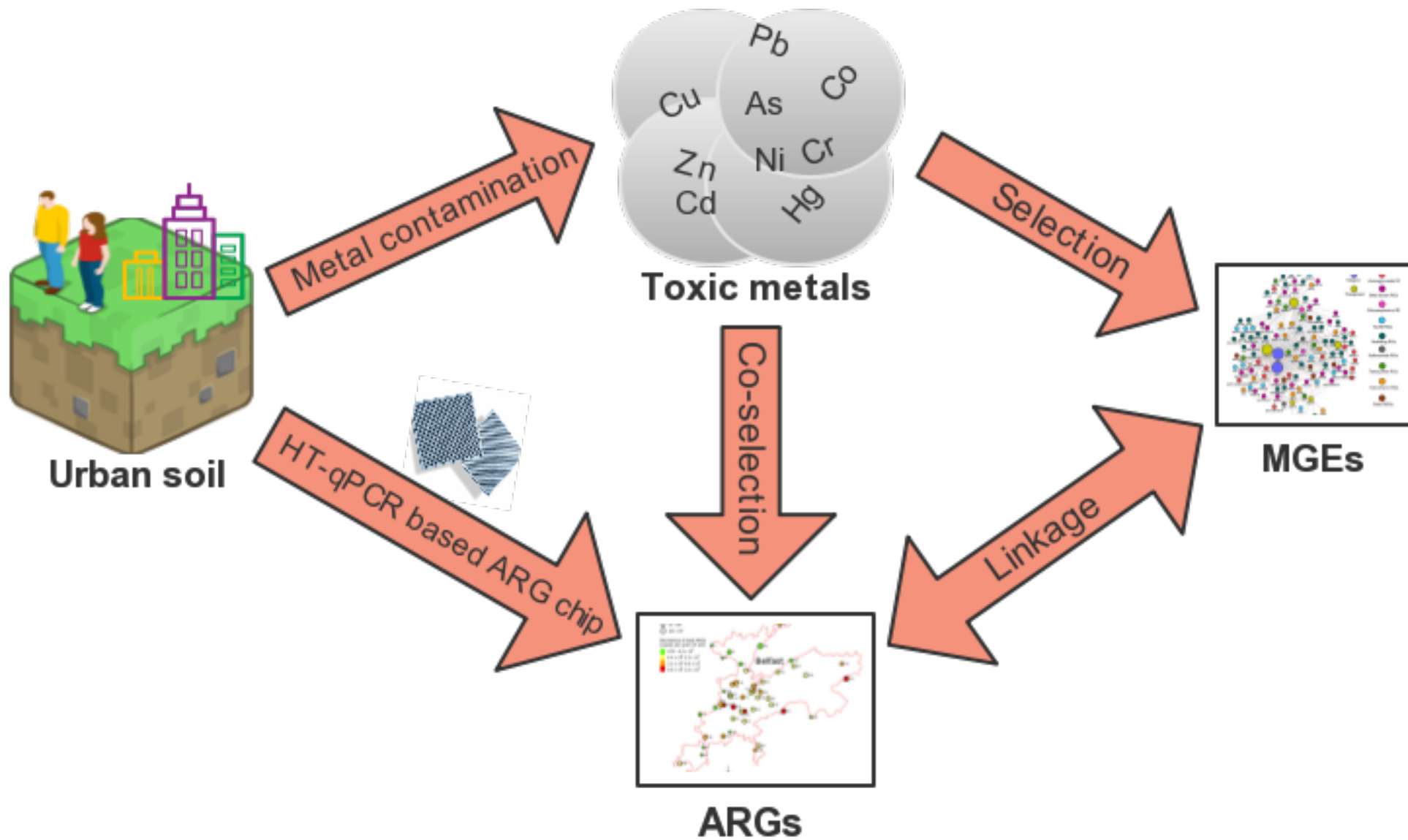
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**Highlights:**

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  $3.4 \times 10^7$  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   corresponded ARGs. Path analysis further indicated that the soil metal toxicity index significantly  
15   affected the number of detected ARGs ( $\lambda = 0.32$ ,  $P < 0.001$ ) and the abundance of metal co-occurring  
16   ARGs ( $\lambda = 0.612$ ,  $P < 0.001$ ) via effects on MGEs. Collectively, our results indicate a role of soil metals  
17   in co-selection of ARGs and MGEs in urban and semi-urban soils and suggest a risk for environmental  
18   ARG 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 (Berg et al., 2012; Imfeld et al., 2011;  
52 McLaughlin and Smolders, 2001). Indeed, toxic metals may in some cases provide stronger and more  
53 persistent selective pressures for environmental selection of antibiotic resistance as compared to antibiotic  
54 residues (Song et al., 2017). Consequently, metal-induced co-selection for ARGs in metal contaminated  
55 environments represents a risk factor for the expansion of the soil bacterial resistome and may thus  
56 represent a barrier for reversal of antibiotic resistance even if antibiotic residues are prevented from  
57 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. Therefore, monitoring ARG distribution in urban environments and its association  
72 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 19<sup>th</sup> 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 conclude that the metropolitan area of Belfast is relevant for evaluating the ability  
99 of soil metal(loid)s to co-select antibiotic resistance. The boundaries of the study area have been defined  
100 using the Corine land cover data (European Environment Agency, 2012) satellite images and the spatial  
101 distribution of the available urban soil samples.

102

## 103 2.2. Soil collection

104 The Tellus geochemical and geophysical survey was undertaken across the entire region of Northern  
105 Ireland by the Geological Survey of Northern Ireland (GSNI) between 2004 and 2007. Shallow soil  
106 samples (5-20 cm) from 1166 sampling locations were collected and archived at room temperature (<  
107 25°C). Full details of the sampling strategy are described in a previous study (Smyth, 2007). In this study,  
108 a total of 50 sampling locations were selected ([Figure 1a](#)) and their archived urban soils (Knights, 2006)  
109 were retrieved from the GSNI Tellus survey archive in October 2016. Original sampling coordinates are  
110 listed in [Table S1](#). Sample selection targeted a broad spectrum of toxic elements, with a wide range of  
111 concentrations, and included a variety of different land uses while ensuring samples were spatially  
112 distributed across the city.

113

## 114 2.3. Soil chemical characterization

115 Archived metal concentration data (As, Cd, Co, Cr, Cu, Hg, Ni, Pb and Zn) were retrieved from the  
116 Tellus database for downstream analyses. The spatial distribution of these elements across Belfast has  
117 been reported previously (McIlwaine et al., 2017). Concentrations of As in soils were found to be  
118 controlled by anthropogenic input, while Cu, and Zn were influenced by both anthropogenic input and  
119 geogenic input, and contamination by Ni, Co and Cr were mainly contributed to by geogenic sources  
120 (McIlwaine et al., 2017).

121

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

129

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

133

#### 134 2.4. DNA extraction

135 The 50 selected soil samples were retrieved from the Tellus soil archive and aseptically weighed into  
136 prepared sterile plastic bags. A total of 250 - 400 mg of dry soil was transferred into PowerBead Tubes  
137 (MoBio) and incubated for rehydration at room temperature for 20-30 minutes. DNA was extracted from  
138 rehydrated soil using MoBioPowerSoil® DNA Isolation Kit according to the instruction manual. The  
139 concentration and purity of DNA were checked using ultraviolet absorbance (ND1000, Nanodrop,  
140 Thermo Fisher Scientific). DNA was eluted in 100  $\mu$ l elution buffer and stored at -20 °C for downstream  
141 analysis. Long-term storage has been proven to not cause bias in DNA results (Tzeneva et al., 2009,  
142 Knapp et al., 2010).

143

## 144 2.5. High-throughput qPCR

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

156

157 All HT-qPCR were performed in technical triplicates with negative control. The data from reaction wells  
158 with  $r^2$  smaller 0.99 were discarded. Only data for samples with three technical replicates that all  
159 generated amplification products were regarded as positive detection and used in further data analysis.  
160 Relative copy number was calculated based on previously published method (Looft et al., 2012): relative  
161 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  
162 normalized abundance of a gene (copies per 16S rRNA) was calculated by dividing relative gene copy  
163 number of the gene by relative copy number of reference gene 16S rRNA.

164

165 Absolute 16S rRNA copy numbers (copies per gram of soil) were determined using the standard curve  
166 method on a Roche 480 system. Each 20  $\mu$ l qPCR reaction mixture consisted of 10  $\mu$ l 2  $\times$  LightCycle 480  
167 SYBR Green I Master, one  $\mu$ g  $\mu$ l<sup>-1</sup> bovine serum albumin, one  $\mu$ M of each primer, one ng  $\mu$ l<sup>-1</sup> DNA as

168 template and six  $\mu\text{l}$  nuclease-free PCR-grade water. The thermal cycle consisted of a 10 min enzyme  
169 activation at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s  
170 and extension at 72 °C for 15 s. A plasmid control containing a cloned and sequenced 16S rRNA gene  
171 fragment ( $1.39 \times 10^{10}$  copies per liter) was used to generate calibration curves from a tenfold dilution for  
172 standard calculation. All qPCRs were performed in technical triplicates with negative controls. Absolute  
173 ARG copy numbers were calculated by transforming relative copy numbers by normalization from  
174 absolute 16S rRNA gene copy number.

175

## 176 2.6. Statistical analysis

177 All HT-qPCR data filtration and calculations were performed using Microsoft Excel 2010. Bar charts and  
178 scatter diagrams were generated using Origin Pro 9.1. Correlation analyses and significance tests were  
179 performed using IBM SPSS Statistics 22. Heatmaps were generated using HemI 1.0 (Deng et al., 2014).  
180 Shannon H index was determined using PAST Statistics Software (Hammer et al., 2001). The distance-  
181 decay analysis and permanova test were conducted using R 3.4.1 with the vegan package (Oksanen et al.,  
182 2007). The co-occurrence patterns between ARGs (normalized abundance) and MGEs (normalized  
183 abundance)/metals (total concentration) were explored using network analysis. To visualize the  
184 correlations in the network interface, a correlation matrix was constructed using all pairwise Pearson's  
185 rank correlations. Only correlations with Pearson's  $r > 0.7$  (or  $< -0.7$ ) and a significance level of  $P < 0.05$   
186 were considered robust and used for forming the co-occurrence networks. Network visualization was  
187 performed in Cytoscape 3.6.0 (Shannon et al., 2003). For spatial data representation, maps were produced  
188 using the ArcGIS software ArcMap version 10 (ESRI, 2010).

189

190 The path analysis, as a special case of structural equation model (SEM), has been proven useful as a  
191 statistical tool to explore the complex networks of causal relationships ecosystems (Eisenhauer et al.,

192 2015). In this study, it was performed to evaluate the overall effect of soil metal contamination  
193 (represented by  $TI_{\text{metals}}$ ) on ARG patterns as represented by both the number of detected ARGs and the  
194 normalized abundance of all co-occurring ARGs using SPSS AMOS. The path model was established  
195 based on the following theoretical assumptions: (i) metal contamination may directly influence ARG  
196 patterns by acting as a direct selective agent; (ii) metal contamination may indirectly affect ARG patterns  
197 through MGE associated co-selection processes, with co-resistance as a mechanism. The data were fitted  
198 to the estimated model using a maximum-likelihood estimation method. The model fit was tested and an  
199 overall goodness-of-fit of the model was indicated by satisfaction of the following criteria: (i) non-  
200 significant Chi-square value ( $P > 0.05$ ); (ii) low root mean square error of approximation as absolute fit  
201 index (RMSEA  $< 0.08$ ); (iii) high increment fit index (CFI, GFI, TLI, NFI  $> 0.95$ ; CFI as comparative fit  
202 index, GFI as goodness of fit, TLI as Tucker-Lewis index and NFI as Normed-fit index). The disturbance  
203 terms (also called the residual error terms) were added into the model to reflect the unexplained variance  
204 and measurement error. The path coefficients (standardized regression weights) and significance were  
205 calculated in an SPSS AMOS, showing the effect of an independent variable on a dependent variable in  
206 the path model. The standardized direct, indirect and total effects were automatically calculated using  
207 SPSS AMOS following the method published previously (Finney, 1972).

208

### 209 **3. Results**

#### 210 3.1. Diversity and abundance of ARGs in Belfast urban area

211 A total of 175 genes (164 ARGs, eight transposase genes, two Class 1 integron-integrase genes and the  
212 16S rRNA gene) were detected by the HT-qPCR chip in the 50 studied urban soils (Figure 1). The  
213 detected ARGs represented most major resistance mechanisms including antibiotic deactivation (43%),  
214 efflux pumps (34%) and cellular protection (19%) (Figure 1b) and confer resistance to most major classes  
215 of antibiotics administered to humans and animals, including aminoglycosides, beta-lactams,

216 chloramphenicols, MLSB, sulfonamides, tetracyclines, vancomycin and multidrug. Almost half of the  
217 detected ARGs confer resistance to beta-lactams (23%) and multidrug (23%) (Figure 1c). The number of  
218 detected ARGs in the Belfast urban area ranged from 8 to 137 with the average at 67 (Figure 1a). The  
219 Shannon diversity H index of ARGs varied from 1.2 to 3.4 in Belfast urban samples (Figure S1). To  
220 compare the spatial similarity of ARG patterns, we examined the presence and absence of ARGs across  
221 all samples. Only two shared core ARGs (*mphA-02* and *cphA-01*) conferring resistance to macrolide and  
222 beta-lactam antibiotics were found in all samples.

223  
224 The absolute abundance of ARGs in Belfast urban soils varied over six orders of magnitude ( $6.8 \times 10^2$  to  
225  $1.7 \times 10^8$  copies per gram of soil) with an average of  $3.4 \times 10^7$  (Figure 1a). The predominant ARGs  
226 encoded beta-lactam, multidrug, aminoglycoside or chloramphenicol resistance (Figure S2). The three  
227 most abundant ARGs were *mexF*, *cphA-01* and *cmx(A)*. The *mexF* gene, which encodes a multidrug  
228 resistance efflux pump for chloramphenicol and fluoroquinolone, was detected in 49 soil samples at  $5 -$   
229  $229 \times 10^7$  copies per gram of soil. The *cphA* gene confers resistance to different beta-lactams antibiotics  
230 (penicillin, cephalosporin and carbapenem) via hydrolysis of the beta-lactam ring and was detected in all  
231 50 soils with an average abundance of  $6.6 \times 10^6$  copies per gram of soil. The *cmx(A)* gene encodes a  
232 chloramphenicol exporter and was found in 49 samples, varying in abundance from  $2 - 43 \times 10^7$  copies  
233 per gram of soil.

234  
235 To better explore the prevalence of ARGs within the studied soil bacterial communities, the absolute  
236 abundance of ARGs and MGEs was normalized relative to the abundance of the 16S rRNA gene.  
237 Bacterial abundances in soils were in the range of  $8.7 \times 10^3$  to  $9.0 \times 10^8$  with an average of  $2.2 \times 10^8$   
238 copies per gram. The normalized ARG abundance in 50 soils varied from 0.06 to 0.77 with an average of  
239 0.15 ARG copies per 16S rRNA gene.

240

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

251

### 252 3.2. MGEs and their associations with ARGs

253 A total of two integron genes and eight transposon genes were targeted and detected in this study. The  
254 total absolute abundance of these MGEs ranged from  $2\text{-}38 \times 10^7$  copies per gram of soil with an average  
255 of  $3.4 \times 10^6$ . The normalized abundance of MGEs ranged from 0.004 to 0.069 copies per 16S rRNA gene  
256 with average at 0.014. Class 1 integron, *intI-1*, was found in all 50 soils. Significant correlations were  
257 found between MGEs and ARGs (Figure 2). The normalized abundance of MGEs was positively  
258 correlated with the number of ARGs detected (Pearson's  $r = 0.57$ ,  $P < 0.001$ ), as well as normalized  
259 abundance of ARGs (Pearson's  $r = 0.66$ ,  $P < 0.001$ ) (Figure 2a). Positive correlations were also found  
260 between the absolute abundance of ARGs and the MGEs: Class 1 integrons (Pearson's  $r = 0.97$ ,  $P < 0.001$ )  
261 and transposons (Pearson's  $r = 0.66$ ,  $P < 0.001$ ) (Figure S5).

262



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

273

### 274 3.3. Co-occurrence pattern between metals and ARGs

275 The soil samples profiled for ARGs and MGEs were contaminated to varying degrees by metals. The  
276 metals with the potential of co-selection for ARGs and their concentration ranges in the soil samples were  
277 shown in Table 1. The co-occurrence pattern between metals and ARGs was further explored by network  
278 analysis based on Pearson's correlation ( $r > 0.7$ ,  $P < 0.05$ ) (Figure 3). The network consists of 25 nodes  
279 including eight metals and 17 ARGs. A total of 24 significant positive correlations were found between  
280 metals and ARGs. No significant negative correlation was found between metals and ARGs. As, Cd, Co,  
281 Cr, Cu, Hg, Ni and Zn all exhibited co-occurrences with specific ARGs. Zinc was found to co-occur with  
282 eight resistance genes conferring resistance to aminoglycosides (4), multidrug (3) and beta-lactam  
283 antibiotics (1). Copper co-occurred with aminoglycoside resistance genes (*aadA* and *aac*) and MLSB  
284 resistance genes (*mefA*). The number of ARGs that co-occurred with Cd, Co, Ni, Hg, Cr and As were 3, 3,  
285 2, 2, 2 and 1, respectively. These metal(loid)s all co-occurred with 17 specific ARGs conferring resistance  
286 to aminoglycosides, beta-lactams, MLSBs, multidrug, tetracycline and vancomycin. Among the 24

287 connections, almost half were contributed by a combination of aminoglycoside (25%) and multidrug  
288 resistance genes (21%).

289

### 290 3.4. The effects of metal toxicity index and MGEs on ARGs

291 To further assess the effects of metals and MGEs on ARGs, structural equation model (SEM) based path  
292 analysis was performed with a multiple-pathways model based on the theoretical assumptions outlined in  
293 section 2.6 (Figure 4a). The path analysis indicated that the degree of soil metal contamination (metal  
294 toxicity index) had a significant direct positive influence on normalized abundance of co-occurring ARGs  
295 (copies per 16S rRNA gene) ( $\lambda = 0.187$ ,  $P < 0.01$ ) and a significant indirect positive effect on number of  
296 detected ARGs ( $\lambda = 0.251$ ,  $P < 0.001$ ) (Figure 4b) and normalized abundance of co-occurred ARGs  
297 (copies per 16S rRNA gene) ( $\lambda = 0.426$ ,  $P < 0.001$ ) (Figure 4c). The total standardized effects of metal  
298 toxicity index on the number of detected ARGs and normalized abundance of co-occurring ARGs (copies  
299 per 16S rRNA gene) were 0.323 and 0.612, respectively. This suggests that increase of one unit of one  
300 metal toxicity index resulted in 0.323 more numbers of ARGs detected and 0.612 more copies of co-  
301 occurred ARGs per 16S rRNA gene. Metal toxicity changed MGE abundances in soil ( $\lambda = 0.521$ ,  $P <$   
302  $0.001$ ) and subsequently influenced the number of detected ARGs and abundance of co-occurring ARGs  
303 ( $\lambda = 0.483$ ,  $P < 0.001$  and  $\lambda = 0.817$ ,  $P < 0.001$ ) (Figure 4a). Metal toxicity index and MGE abundance  
304 both had a total positive effect on the number of detected ARGs and abundance of co-occurred ARGs.  
305 The path analysis results were further tested and confirmed by correlation analysis. The correlation  
306 analysis showed significant positive correlations among metal toxicity index, the abundance of MGE,  
307 number detected ARGs and abundance of co-occurring ARGs (Figure S6), which further confirmed the  
308 positive effect of metals on ARGs via MGEs. Significant positive correlations were also found between  
309 soil pH and the number of detected ARGs (Pearson's  $r = 0.329$ ,  $P < 0.05$ ), and between the absolute  
310 abundance of the 16S rRNA gene and total ARGs (copies per gram) (Pearson's  $r = 0.905$ ,  $P < 0.001$ ).

311

## 312 **4. Discussion**

### 313 4.1. Evidence for metal-induced co-selection of ARGs in urban soils

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

327

### 328 4.2. Co-selection mechanisms and potential for horizontal gene transfer of ARGs

329 Although our study was not specifically designed to compare the relative importance of different co-  
330 selection mechanisms (Baker-Austin et al., 2006), it clearly demonstrated co-selection of ARGs and  
331 MGEs (Figure 4). The strong linkages between metal toxicity index, MGEs and ARGs indicate a  
332 significant metal impact on both the diversity and abundance of ARGs via MGEs (Figure 4). According  
333 to our results of path analysis, 78% of the total effect of metal toxicity on the number of detected ARGs  
334 and 69% of the total effect of metal toxicity on the abundance of co-occurring ARGs were observed to

335 occur via MGEs. The majority of co-occurring ARGs with metals were found to also co-occur with  
336 MGEs, suggesting an underlying metal-driven co-selection process with co-resistance (i.e. linkage of  
337 genes conferring resistance to metals and antibiotics on the same genetic element) as the major  
338 mechanism for most studied ARGs that do not have any known roles in bacterial metal resistance.  
339 However, the resistance genes *acrF*, *adeA*, *ttgB*, *qacEΔ1*, *rarD*, *tetPA* and *mefA* encode efflux pumps as  
340 their resistance mechanism and cross-resistance with other classes of antimicrobial agents via efflux  
341 cannot be ruled out. For instance, the multi-drug resistance pumps encoded by the genes *acrF*, *adeA*,  
342 *qacEΔ1rarD* and *ttgB* can export both metals and antibiotics for detoxification purposes (Mata et al.,  
343 2000).

344

345 In terms of human health risk assessment, the ability of soil bacteria to transfer ARGs to pathogenic  
346 bacteria of clinical relevance is of considerable concern (Forsberg et al., 2012, Ashbolt et al., 2013).  
347 Importantly, our study indicates that urban soil metal pollution co-selected ARGs that may be prone to  
348 horizontal gene transfer between different species of bacteria. Results of correlation analysis, network  
349 analysis and path analysis all showed that an increase in MGE abundance was strongly correlated with an  
350 increase in ARG diversity and abundance (Figure 2, 4 & S6). The co-occurrence pattern of ARGs and  
351 MGEs revealed by network analysis showed several clusters within the network. Resistance genes such as  
352 *aac* and *aad* genes, known to be carried within integron gene cassettes (An et al., 2018; Partridge et al.,  
353 2009), were strongly correlated with the class 1 integron gene *IntI-1*, clinical class 1 integron gene *cIntI-1*  
354 and transposon genes *tnpA* and *IS613* (Figure 2). The clinical class 1 integron-integrase gene, *cIntI-1*,  
355 exhibits rapid responses to various environmental pressures (including toxic metals) and thus has been  
356 proposed as a marker for anthropogenic pollution and as an emerging pollutant (Gillings et al., 2015;  
357 Gillings, 2018). The significant correlation and co-occurrence pattern between the clinical class 1  
358 integron-integrase gene (*cIntI-1*) and ARGs (Table S5 & Figure 2) may therefore suggest that metal  
359 contamination increases environmental selection of bacteria containing clinical Class 1 integrons

360 conferring resistance to both metals and antibiotics even in the absence of a selection pressure exerted by  
361 antibiotic residues.

362

363 4.3. Conclusions and perspectives for the environmental dissemination of ARGs.

364 Our findings provide evidence that metal contamination (As, Cd, Co, Cr, Cu, Hg, Ni and Zn) could  
365 significantly affect the diversity, abundance and mobility potential of a broad spectrum of ARGs in urban  
366 soils. Collectively, our results suggest that urban soil metal contamination increases the potential for  
367 horizontal gene transfer of ARGs via co-selection of ARGs and MGEs thereby generating a pool of high-  
368 risk mobile ARGs (Martínez et al., 2015). The metal-induced co-selection of ARGs in urban soils is thus  
369 of significant public health concern and has implications for controlling the environmental dissemination  
370 of antibiotic resistance. Indeed, it is likely that metals in many soils confer more important selective  
371 agents than antibiotic residues because metals, as opposed to antibiotics, frequently accumulate to  
372 persistent toxic levels in contaminated soils (Song et al., 2017). Hence, we propose that metal-induced co-  
373 selection of ARGs and MGEs needs to be monitored in metal contaminated soils in the interest of both  
374 human and environmental health. Serious consideration is needed to set minimum standards for retarding  
375 ARGs and for mitigating the accumulation of toxic metals in urban soils.

376

#### 377 **Conflict of interests**

378 The authors declare no conflicts of interests.

379

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389

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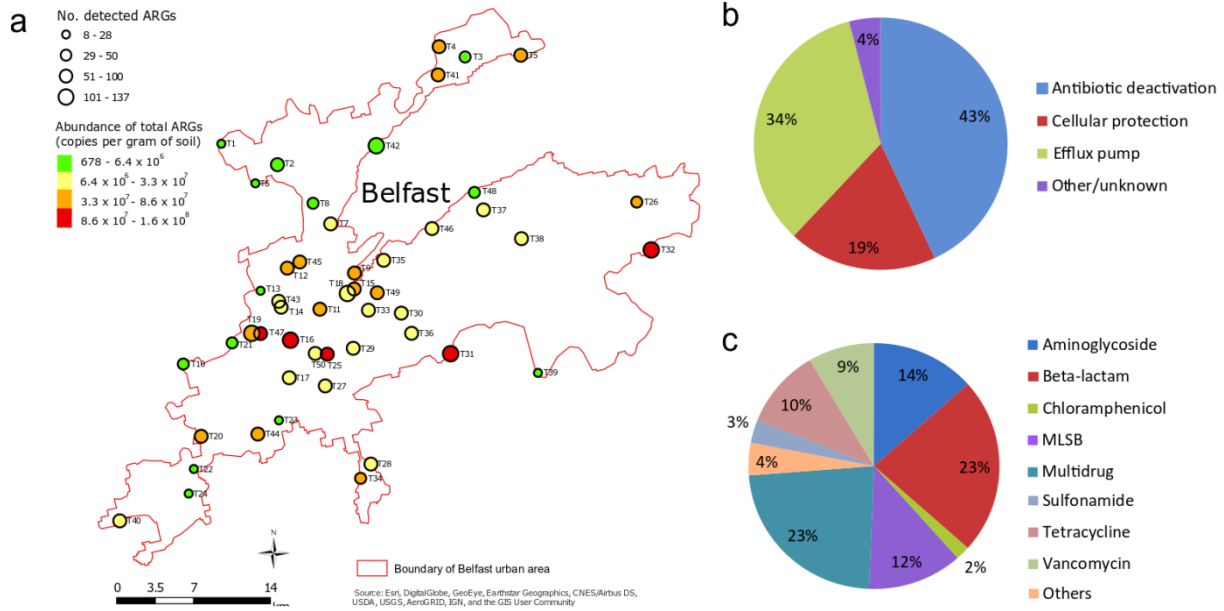
514

515 **Figures and Tables**516 **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

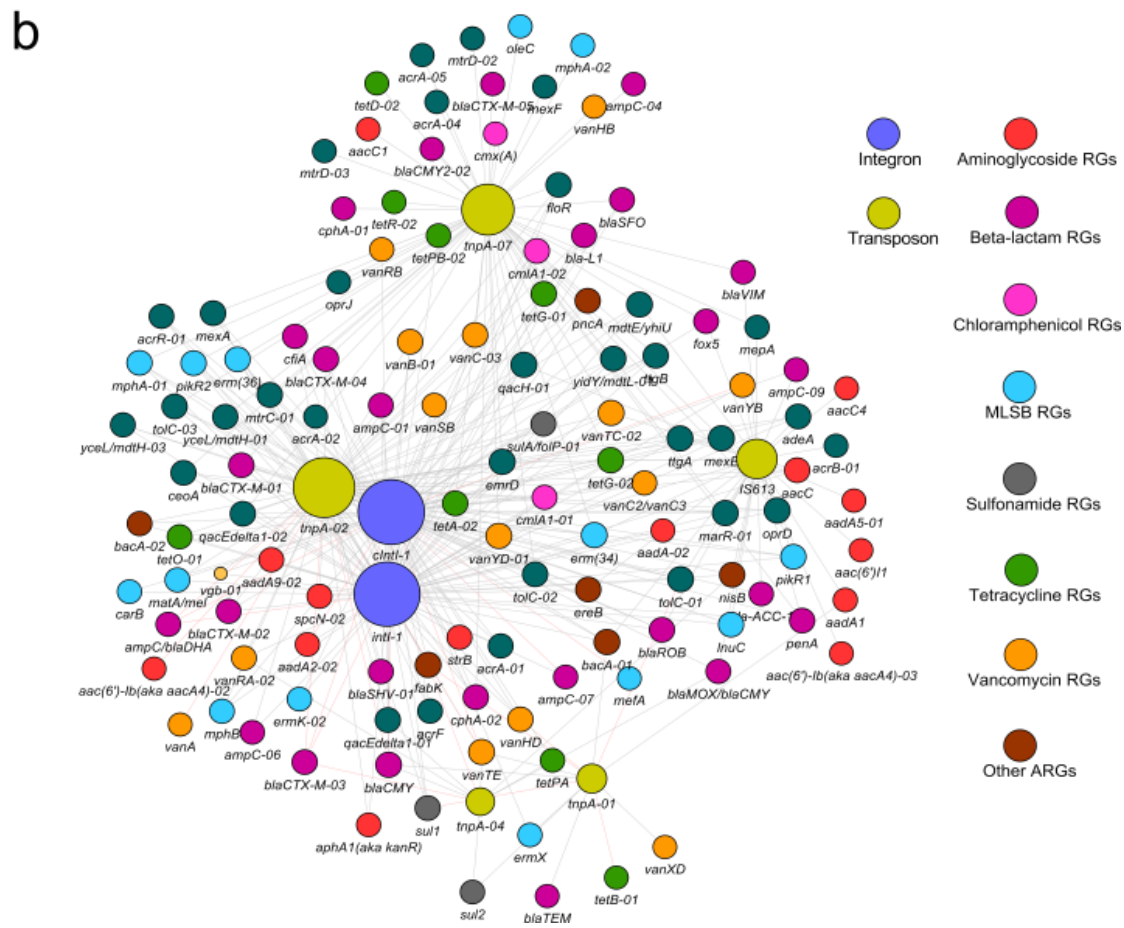
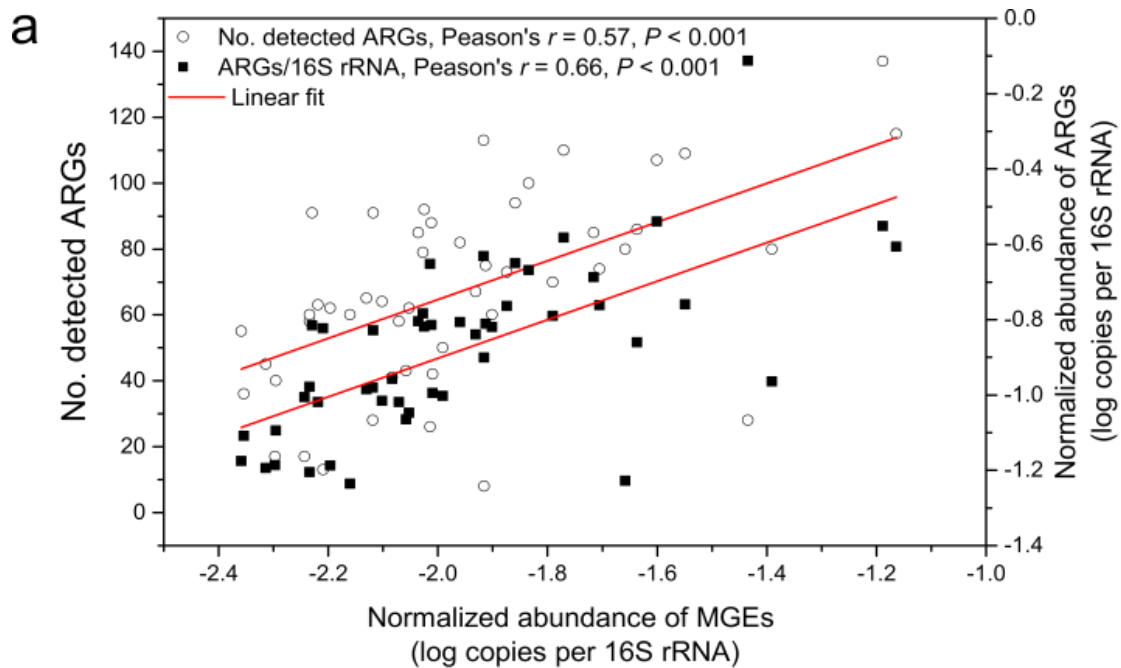
\*Analyzed with *aqua regia* digestion followed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (McIlwaine et al., 2017).

517



518

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



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

534



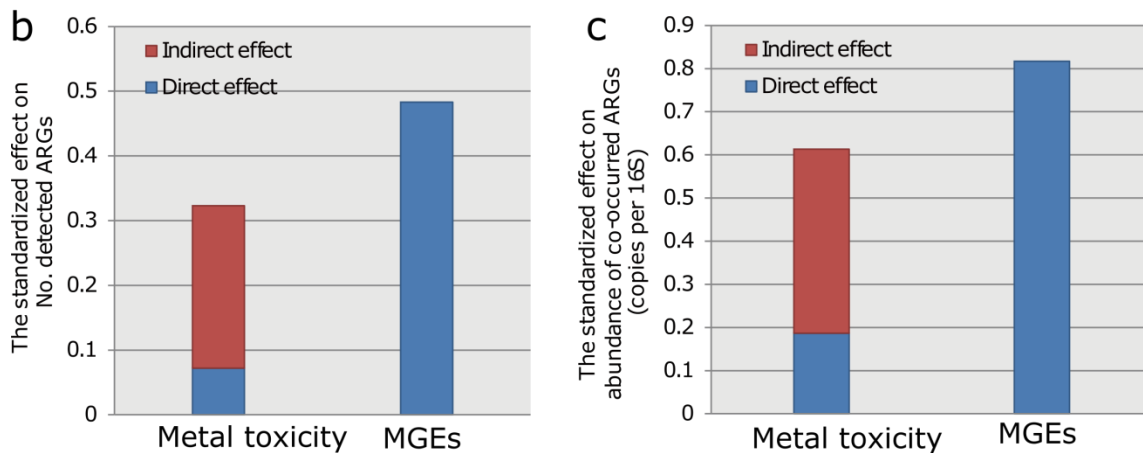
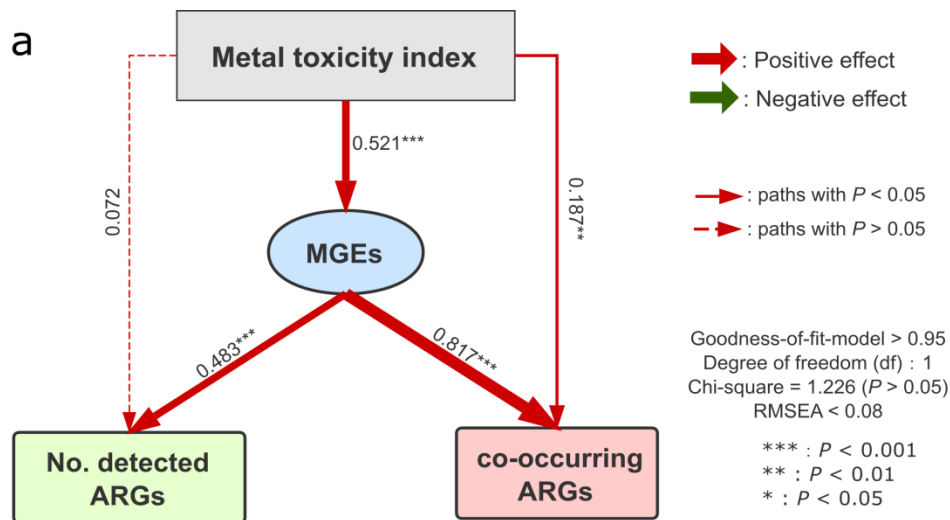
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536 **Figure 3** Network analysis showing the co-occurrence pattern between metals (As, Cd, Co, Cr, Cu, Hg,  
 537 Ni, Pb, and Zn) and antibiotic resistance genes (ARGs) based on Pearson correlation analysis. A  
 538 connection represents a strong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

542

543





544

545 **Figure 4** Path analysis showing the modelled effect of soil metal contamination (metal toxicity index) and

546 normalized abundance of mobile genetic elements(MGEs) on (a) the number of detected antibiotic

547 resistance genes (ARGs) and the normalized abundance of co-occurring ARGs (copies per 16S rRNA

548 gene). Path arrows correspond to positive (red) and negative (green) effects with significance level of  $P <$

549  $0.05$  (solid line), and  $P > 0.05$  (dotted line) with path directions. Numbers adjacent to the path arrows are

550 path coefficients (standardized regression weights), and the arrow width is proportional to the strength of

551 path coefficients. Bar charts show the standardized direct effect, indirect effect and total effect of metal

552 toxicity index and MGEs on (b) number of detected ARGs and (c) normalized abundance of co-occurring

553 ARGs (copies per 16S rRNA gene) derived from path modelling (a).

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