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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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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   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

1. Introduction

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

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

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

2

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 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

4

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_{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_{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): $TI_{metals}= \sum(C_i/EC_{50i})$, 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 μl 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 μl qPCR reaction mixture consisted of 10 μl 2 × LightCycle 480

167 SYBR Green I Master, one μg μl^{-1} bovine serum albumin, one μM of each primer, one ng μl^{-1} DNA as

168   template and six μl 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 (1.39×10^{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_{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

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 (6.8×10^2 to

225   1.7×10^8 copies per gram of soil) with an average of 3.4×10^7 (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   229×10^7 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 6.6×10^6 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 - 43×10^7 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 8.7×10^3 to 9.0×10^8 with an average of 2.2×10^8

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 2-38 $\times$ 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

12

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) (λ= 0.187, P < 0.01) and a significant indirect positive effect on number of

296   detected ARGs (λ = 0.251, P < 0.001) (Figure 4b) and normalized abundance of co-occurred ARGs

297   (copies per 16S rRNA gene) (λ = 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 (λ = 0.521, P <

302   0.001) and subsequently influenced the number of detected ARGs and abundance of co-occurring ARGs

303   (λ = 0.483, P < 0.001 and λ = 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

4. Discussion

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

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

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

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

380 **Acknowledgments**

389

References

391   An XL, Chen QL, Zhu D, Zhu YG, Gillings MR, Su JQ, 2018. Impact of Wastewater Treatment on the

392   Prevalence of Integrons and the Genetic Diversity of Integron Gene Cassettes. Appl Environ

393   Microbiol, 84.

394   Ashbolt NJ, Amezquita A, Backhaus T, Borriello P, Brandt KK, Collignon P, et al, 2013. Human Health

395   Risk Assessment (HHRA) for environmental development and transfer of antibiotic resistance.

396   Environ Health Perspect, 121: 993-1001.

397   Azarbad H, Niklinska M, Laskowski R, van Straalen NM, van Gestel CA, Zhou J, et al, 2015. Microbial

398   community composition and functions are resilient to metal pollution along two forest soil

399   gradients. FEMS Microbiol Ecol, 91: 1-11.

400   Baker-Austin C, Wright MS, Stepanauskas R, McArthur JV, 2006. Co-selection of antibiotic and metal

401   resistance. Trends Microbiol, 14: 176-82.

402   Berendonk TU, Manaia CM, Merlin C, Fatta-Kassinos D, Cytryn E, Walsh F, et al, 2015. Tackling

403   antibiotic resistance: the environmental framework. Nat Rev Microbiol, 13: 310-7.

404   Berg J, Brandt KK, Al-Soud WA, Holm PE, Hansen LH, Sørensen SJ, et al, 2012. Selection for Cu-
405   tolerant bacterial communities with altered composition, but unaltered richness, via long-term Cu
406   exposure. Appl Environ Microbiol, 78: 7438-7446.

407   Berg J, Thorsen MK, Holm PE, Jensen J, Nybroe O, Brandt KK, 2010. Cu exposure under field
408   conditions coselects for antibiotic resistance as determined by a novel cultivation-independent
409   bacterial community tolerance assay. Environ Sci Technol, 44(22):8724-8.

410   Brandt KK, Frandsen RJ, Holm PE, Nybroe O, 2010. Development of pollution-induced community
411   tolerance is linked to structural and functional resilience of a soil bacterial community following a
412   five-year field exposure to copper. Soil Biology and Biochemistry, 42(5):748-57.

413   Deng W, Wang Y, Liu Z, Cheng H, Xue Y, 2014. HemI: a toolkit for illustrating heatmaps. PLoS One, 9:
414   e111988.

415   Eisenhauer N, Bowker MA, Grace JB, Powell JR, 2015. From patterns to causal understanding: Structural
416   equation modeling (SEM) in soil ecology. Pedobiologia, 58: 65-72.

417   EMEA, 2006. European Medicines Agency, Guideline on the Environmental Risk Assessment of
418   Medicinal Products for Human Use.

419   ESRI, 2010. Environmental Systems Resource Institute, ArcMap 10.0, Redlands, California.

420   European Environment Agency, 2012. Corine Land Cover 2012 seamless vector data.

421   Finney JM, 1972. Indirect effects in path analysis. Sociological Methods & Research, 1: 175-186.

422   Forsberg KJ, Reyes A, Wang B, Selleck EM, Sommer MO, Dantas G, 2012. The shared antibiotic
423   resistome of soil bacteria and human pathogens. Science, 337: 1107-1111.

424   Gillings M, Boucher Y, Labbate M, Holmes A, Krishnan S, Holley M, et al, 2008. The evolution of class
425   1 integrons and the rise of antibiotic resistance. Journal of bacteriology, 190: 5095-5100.

426   Gillings MR, Gaze WH, Pruden A, Smalla K, Tiedje JM, Zhu YG, 2015. Using the class 1 integron-
427   integrase gene as a proxy for anthropogenic pollution. ISME J, 9: 1269-79.

428   Gillings MR. DNA as a Pollutant: the Clinical Class 1 Integron, 2018. Current Pollution Reports, 4(1):49-
429   55.

430   Hammer Ø, Harper D, Ryan P, 2001. Paleontological statistics software: package for education and data

431   analysis. Palaeontologia Electronica.

432   Hoffman LR, D'Argenio DA, MacCoss MJ, Zhang Z, Jones RA, Miller SI, 2005. Aminoglycoside

433   antibiotics induce bacterial biofilm formation. Nature, 436: 1171-5.

434   Imfeld G, Bringel F, Vuilleumier S, 2011. Bacterial tolerance in contaminated soils: potential of the PICT

435   approach in microbial ecology. Tolerance to Environmental Contaminants, CRC, Boca Raton,

436   333e364.

437   Knapp CW, Callan AC, Aitken B, Shearn R, Koenders A, Hinwood A, 2017. Relationship between

438   antibiotic resistance genes and metals in residential soil samples from Western Australia. Environ

439   Sci Pollut Res Int, 24: 2484-2494.

440   Knapp CW, McCluskey SM, Singh BK, Campbell CD, Hudson G, Graham DW, 2011. Antibiotic

441   resistance gene abundances correlate with metal and geochemical conditions in archived Scottish

442   soils. PLoS One, 6: e27300.

443   Knapp CW, Zhang W, Sturm BS, Graham DW, 2010. Differential fate of erythromycin and beta-lactam

444   resistance genes from swine lagoon waste under different aquatic conditions. Environ Pollut,

445   158(5):1506-12.

446   Knights KV, 2006. A report on the Tellus urban field campaigns of Belfast Metropolitan areas and

447   Londonderry. Commissioned Report CR/07/006N. British Geological Survey.

448   Li G, Sun GX, Ren Y, Luo XS, Zhu YG, 2018. Urban soil and human health: a review. European Journal

449   of Soil Science, 69: 196-215.

450   Looft T, Johnson TA, Allen HK, Bayles DO, Alt DP, Stedtfeld RD, et al, 2012. In-feed antibiotic effects

451   on the swine intestinal microbiome. Proc Natl Acad Sci USA, 109: 1691-6.

452   Luo XS, Yu S, Zhu YG, Li XD, 2012. Trace metal contamination in urban soils of China. Sci Total

453   Environ, 421-422: 17-30.

454   Martínez JL, Coque TM, Baquero F, 2015. What is a resistance gene? Ranking risk in resistomes. Nat

455   Rev Microbiol, 13(2):116.

456   Mata M, Baquero F, Perez‑Diaz J, 2000. A multidrug efflux transporter in Listeria monocytogenes.

457   FEMS Microbiology Letters, 187: 185-188.

458   McIlwaine R, Doherty R, Cox SF, Cave M, 2017. The relationship between historical development and

459   potentially toxic element concentrations in urban soils. Environ Pollut, 220: 1036-1049.

460   McLaughlin MJ, Smolders E, 2001. Background zinc concentrations in soil affect the zinc sensitivity of

461   soil microbial processes—A rationale for a metalloregion approach to risk assessments.

462   Environmental Toxicology and Chemistry, 20: 2639-2643.

463   NISRA, 2016. Population Projections for areas within Northern Ireland: 2016-based. Northern Ireland

464   Statistics and Research Agency.

465   Oksanen J, Kindt R, Legendre P, O'Hara B, Stevens MHH, Oksanen MJ, et al, 2007. The vegan package.

466   Community ecology package, 10: 631-637.

467   Pal C, Asiani K, Arya S, Rensing C, Stekel DJ, Larsson DGJ, et al, 2017. Metal Resistance and Its

468   Association With Antibiotic Resistance. Adv Microb Physiol, 70: 261-313.

469   Pal C, Bengtsson-Palme J, Kristiansson E, Larsson DG, 2015. Co-occurrence of resistance genes to

470   antibiotics, biocides and metals reveals novel insights into their co-selection potential. BMC

471   Genomics, 16: 964.

472   Partridge SR, Tsafnat G, Coiera E, Iredell JR, 2009. Gene cassettes and cassette arrays in mobile

473   resistance integrons. FEMS Microbiol Rev, 33: 757-84.

474   Pruden A, Pei R, Storteboom H, Carlson KH, 2006. Antibiotic resistance genes as emerging contaminants:

475   studies in northern Colorado. Environ Sci & Tech, 40: 7445-7450.

476   Rousk J, Bååth E, Brookes PC, Lauber CL, Lozupone C, Caporaso JG, Knight R, Fierer N, 2010. Soil

477   bacterial and fungal communities across a pH gradient in an arable soil. ISME J. 4(10):1340.

478   Royle SA, Simms A, Clarke HB, Gillespie R, 2007. Belfast, Part II, 1840-1900. Royal Irish Academy.

479   SCENIHR, 2009. SCoEaNIHR. Assessment of the Antibiotic Resistance Effects of Biocides.

480   Seiler C, Berendonk TU, 2012. Heavy metal driven co-selection of antibiotic resistance in soil and water

481   bodies impacted by agriculture and aquaculture. Front Microbiol, 3: 399.

482   Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al, 2003. Cytoscape: a software

483   environment for integrated models of biomolecular interaction networks. Genome research, 13:

484   2498-2504.

485   Smolders E, Oorts K, Van Sprang P, Schoeters I, Janssen CR, McGrath SP, McLaughlin MJ, 2009.

486   Toxicity of trace metals in soil as affected by soil type and aging after contamination: using

487   calibrated bioavailability models to set ecological soil standards. Environ Toxicol Chem,

488   28(8):1633-42.

489   Smyth D, 2007. Methods used in the Tellus geochemical mapping of Northern Ireland.

490   Song J, Rensing C, Holm PE, Virta M, Brandt KK, 2017. Comparison of Metals and Tetracycline as

491   Selective Agents for Development of Tetracycline Resistant Bacterial Communities in

492   Agricultural Soil. Environ Sci Technol, 51: 3040-3047.

493   Tzeneva VA, Salles JF, Naumova N, de Vos WM, Kuikman PJ, Dolfing J, et al, 2009. Effect of soil

494   sample preservation, compared to the effect of other environmental variables, on bacterial and

495   eukaryotic diversity. Res Microbiol, 160: 89-98.

496   United Nations, 2014. Department of Economic and Social Affairs Population Division. World

497   Urbanization Prospects: the 2014 Revision. Highlights (ST/ESA/SER.A/352), United Nations.

498   Ventola CL, 2015. The antibiotic resistance crisis: part 1: causes and threats. Pharmacy and Therapeutics,

499   40: 277.

500   Wang FH, Qiao M, Su JQ, Chen Z, Zhou X, Zhu YG, 2014. High throughput profiling of antibiotic

501   resistance genes in urban park soils with reclaimed water irrigation. Environ Sci Technol, 48:

502   9079-85.

503   Welp G, 1999. Inhibitory effects of the total and water-soluble concentrations of nine different metals on

504   the dehydrogenase activity of a loess soil. Biology and Fertility of Soils, 30(1-2):132-9.

505   Yazdankhah S, Rudi K, Bernhoft A, 2014. Zinc and copper in animal feed - development of resistance

506   and co-resistance to antimicrobial agents in bacteria of animal origin. Microb Ecol Health Dis, 25.

507   Zhao Y, Su JQ, An XL, Huang FY, Rensing C, Brandt KK, et al, 2018. Feed additives shift gut

508   microbiota and enrich antibiotic resistance in swine gut. Sci Total Environ, 621: 1224-1232.

509   Zhu YG, Gillings M, Simonet P, Stekel D, Banwart S, Penuelas J, 2018. Human dissemination of genes

510   and microorganisms in Earth's Critical Zone. Glob Chang Biol, 24: 1488-1499.

511   Zhu YG, Zhao Y, Li B, Huang CL, Zhang SY, Yu S, et al, 2017. Continental-scale pollution of estuaries

512   with antibiotic resistance genes. Nat Microbiol, 2: 16270.

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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).
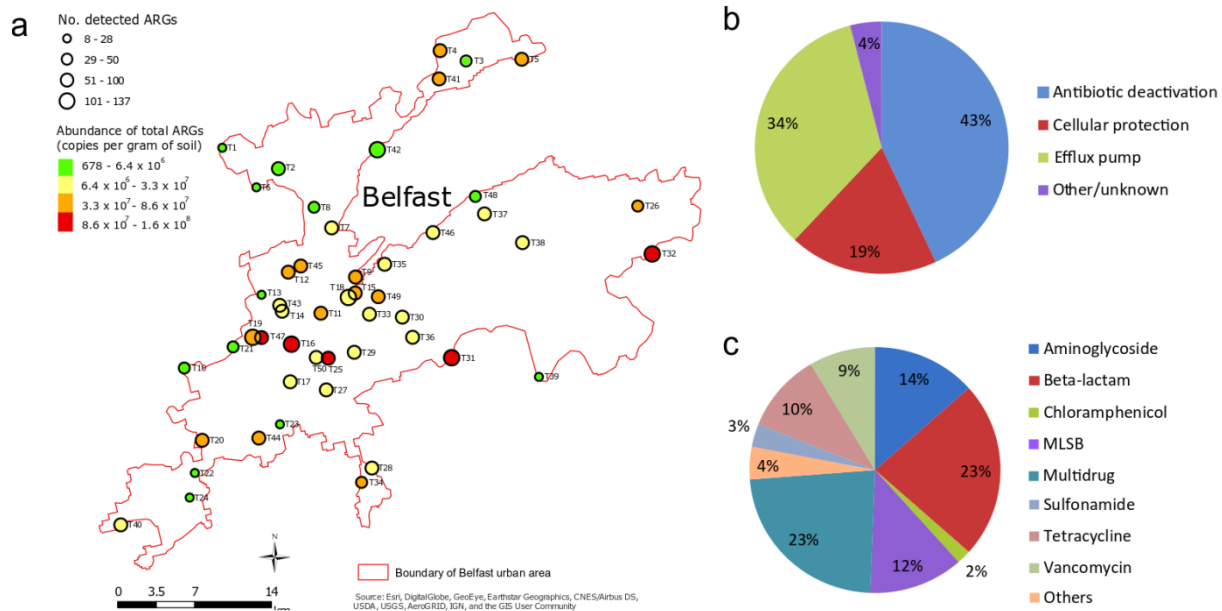
517

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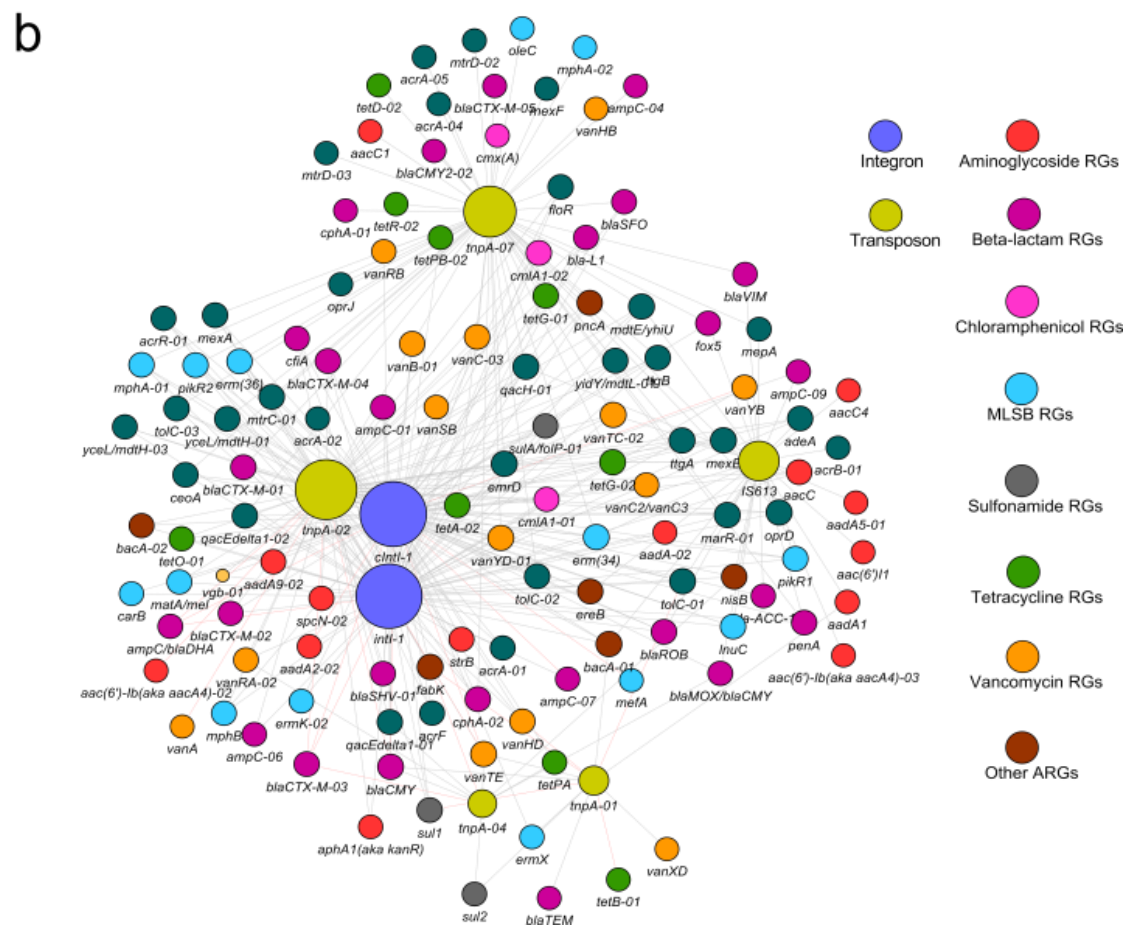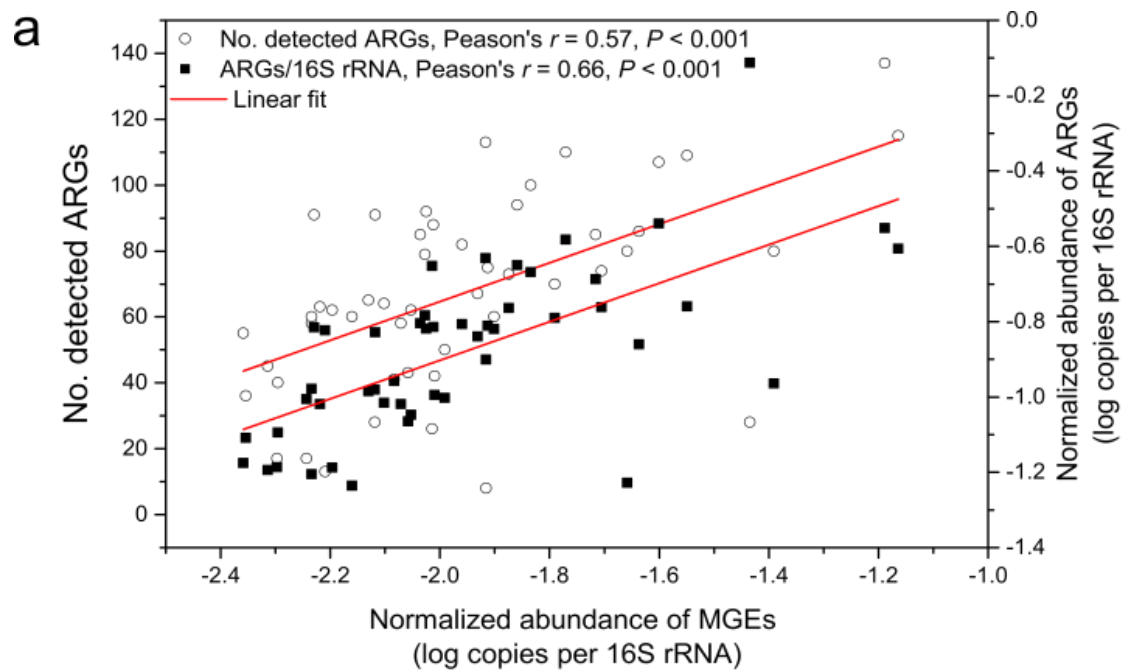
a

b

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 transpons) 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.
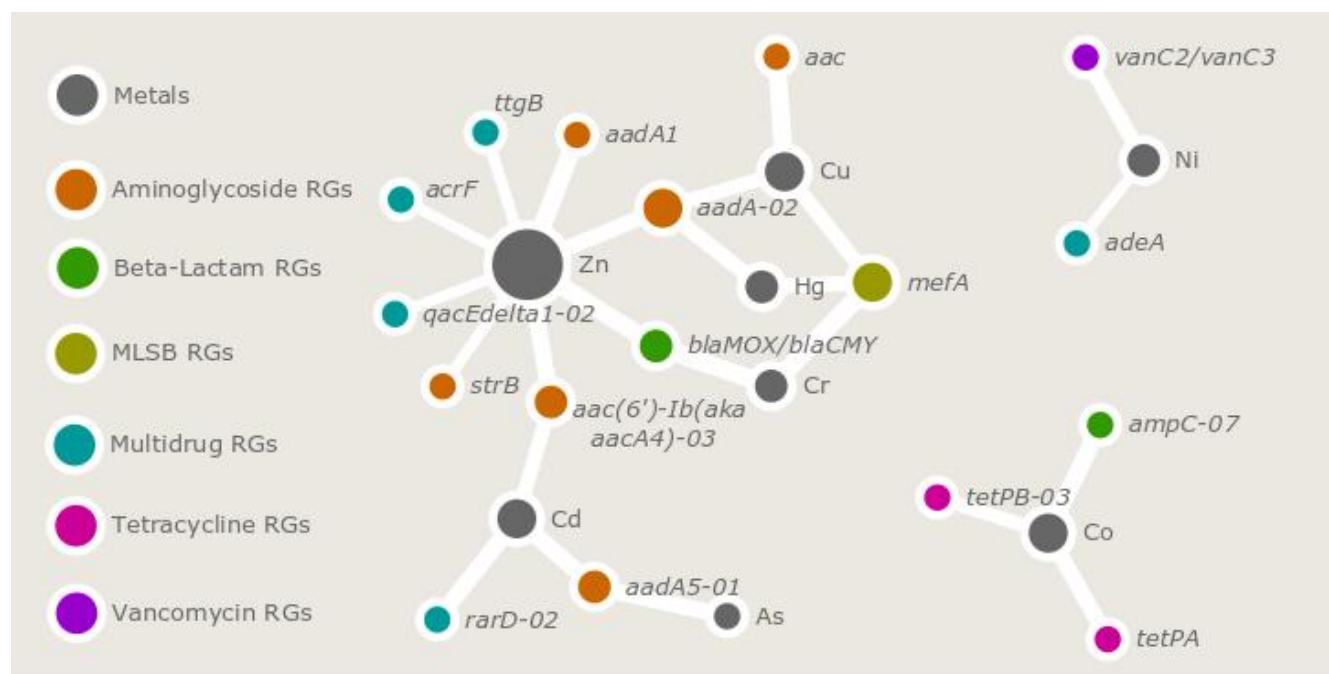
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535

Figure 3 Network analysis showing the co-occurrence pattern between metals (As, Cd, Co, Cr, Cu, Hg,

Ni, Pb, and Zn) and antibiotic resistance genes (ARGs) based on Pearson correlation analysis. A

connection represents a strong (Pearson's $r > 0.7$) and significant ($P < 0.05$) correlation. The color of each

node represents each metal or the type of ARG. The node size is proportional to the number of the

connections. The edge width represents the degree of correlation plotted with Pearson's r. All significant
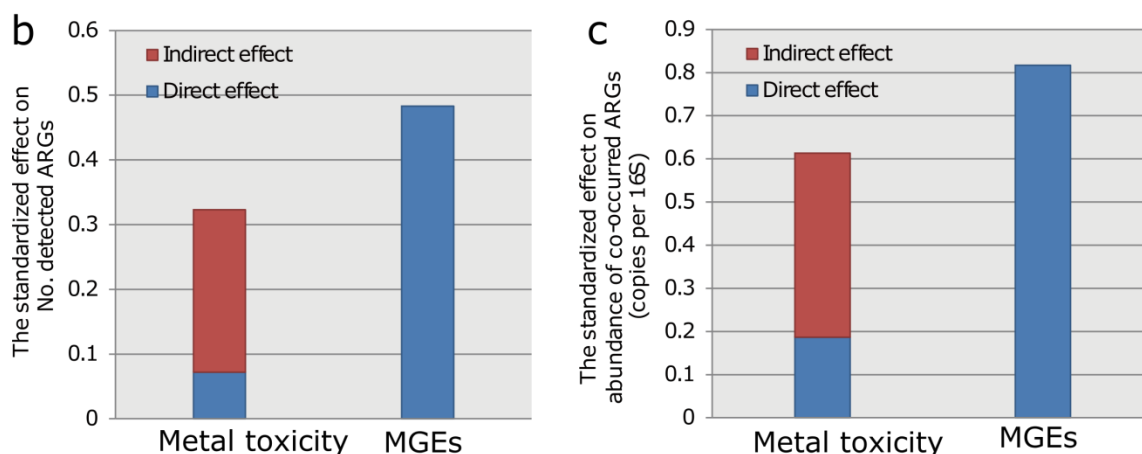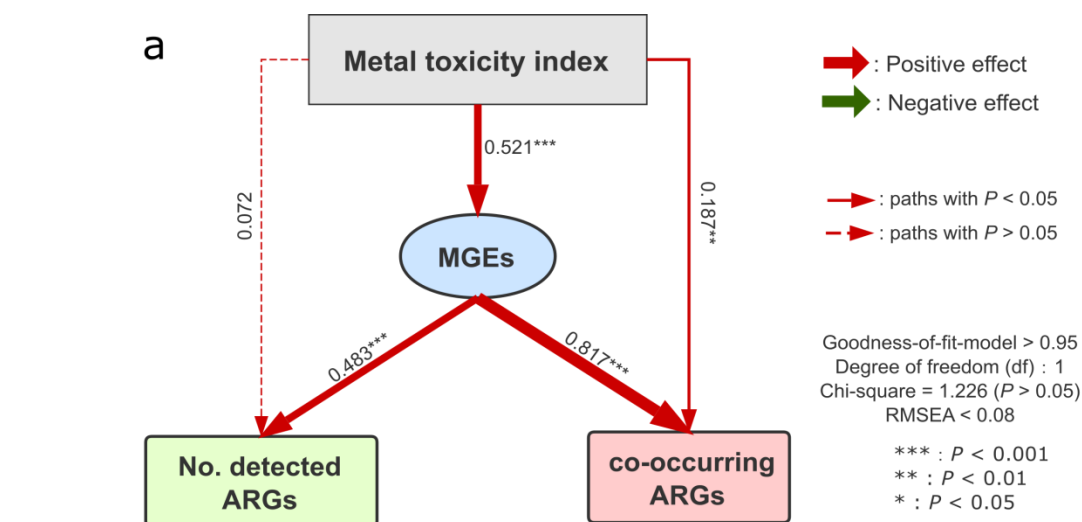
correlations in the data set were positive.

542

543

27

Figure 4 Path analysis showing the modelled effect of soil metal contamination (metal toxicity index) and normalized abundance of mobile genetic elements(MGEs) on (a) the number of detected antibiotic resistance genes (ARGs) and the normalized abundance of co-occurring ARGs (copies per 16S rRNA gene). Path arrows correspond to positive (red) and negative (green) effects with significance level of $P <$ 0.05 (solid line), and $P > 0.05$ (dotted line) with path directions. Numbers adjacent to the path arrows are path coefficients (standardized regression weights), and the arrow width is proportional to the strength of path coefficients. Bar charts show the standardized direct effect, indirect effect and total effect of metal toxicity index and MGEs on (b) number of detected ARGs and (c) normalized abundance of co-occurring ARGs (copies per 16S rRNA gene) derived from path modelling (a).

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