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***Title Page**

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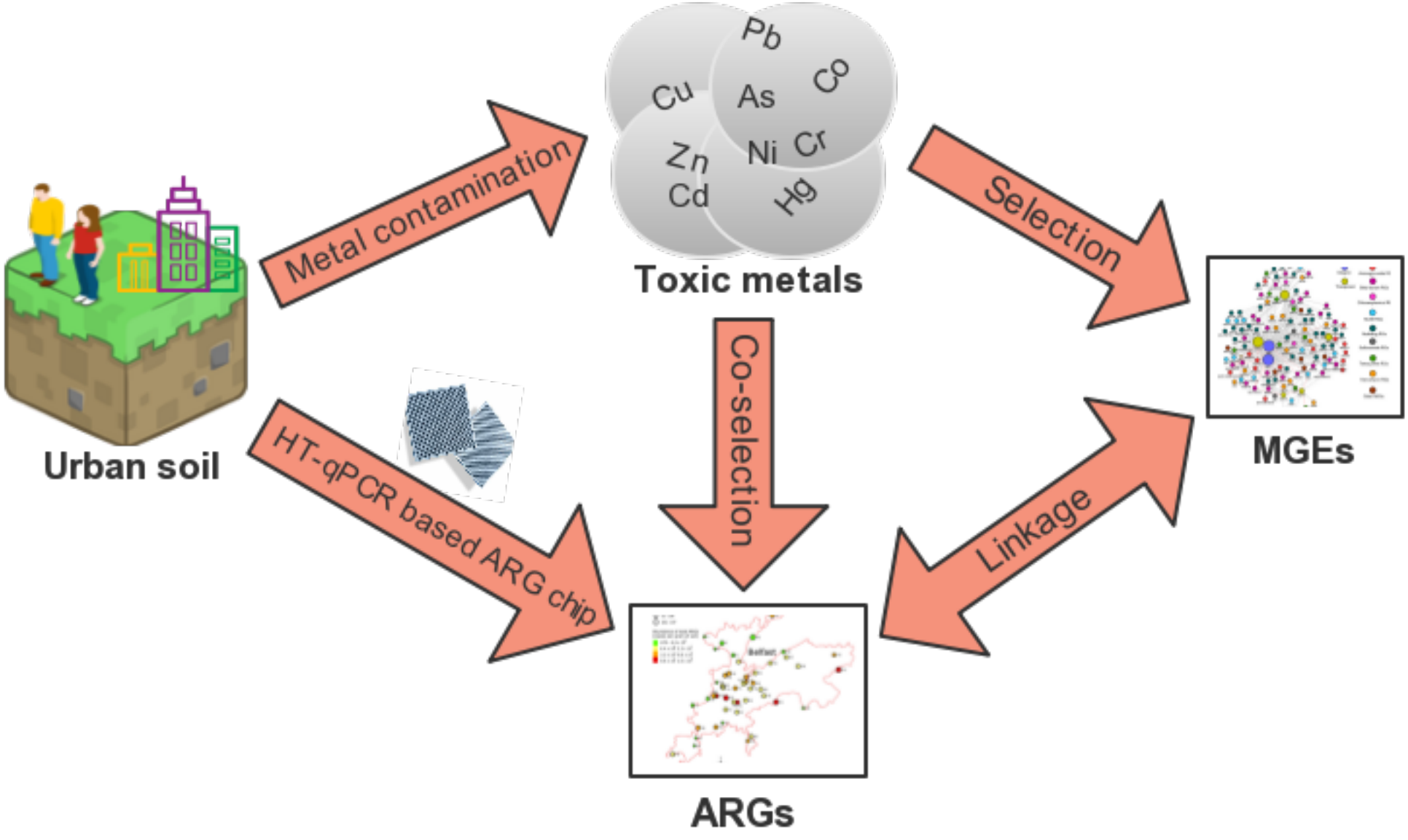
*Graphical Abstract



***Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)**

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.

Abstract

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

Keywords

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

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

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

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

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

2. Materials and Methods

2.1. Study area

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

soils investigated in this study (5-20 cm depth), and significant top soil contamination is therefore highly unlikely. We therefore conclude that the metropolitan area of Belfast is relevant for evaluating the ability of soil metal(loid)s to co-select antibiotic resistance. The boundaries of the study area have been defined using the Corine land cover data (European Environment Agency, 2012) satellite images and the spatial distribution of the available urban soil samples.

2.2. Soil collection

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

2.3. Soil chemical characterization

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

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

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

2.4. DNA extraction

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

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

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

2.6. Statistical analysis

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

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

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

3. Results

3.1. Diversity and abundance of ARGs in Belfast urban area

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

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

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

To better explore the prevalence of ARGs within the studied soil bacterial communities, the absolute abundance of ARGs and MGEs was normalized relative to the abundance of the 16S rRNA gene. 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 copies per gram. The normalized ARG abundance in 50 soils varied from 0.06 to 0.77 with an average of 0.15 ARG copies per 16S rRNA gene.

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

3.2. MGEs and their associations with ARGs

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

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

3.3. Co-occurrence pattern between metals and ARGs

The soil samples profiled for ARGs and MGEs were contaminated to varying degrees by metals. The metals with the potential of co-selection for ARGs and their concentration ranges in the soil samples were shown in Table 1. The co-occurrence pattern between metals and ARGs was further explored by network analysis based on Pearson's correlation ($r > 0.7$, $P < 0.05$) (Figure 3). The network consists of 25 nodes including eight metals and 17 ARGs. A total of 24 significant positive correlations were found between metals and ARGs. No significant negative correlation was found between metals and ARGs. As, Cd, Co, Cr, Cu, Hg, Ni and Zn all exhibited co-occurrences with specific ARGs. Zinc was found to co-occur with eight resistance genes conferring resistance to aminoglycosides (4), multidrug (3) and beta-lactam antibiotics (1). Copper co-occurred with aminoglycoside resistance genes (*aadA* and *aac*) and MLSB resistance genes (*mefA*). The number of ARGs that co-occurred with Cd, Co, Ni, Hg, Cr and As were 3, 3, 2, 2, 2 and 1, respectively. These metal(loid)s all co-occurred with 17 specific ARGs conferring resistance 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) (λ= 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, $P < 0.001$).

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

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

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

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

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

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

Conflict of interests

The authors declare no conflicts of interests.

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References

An XL, Chen QL, Zhu D, Zhu YG, Gillings MR, Su JQ, 2018. Impact of Wastewater Treatment on the Prevalence of Integrons and the Genetic Diversity of Integron Gene Cassettes. Appl Environ Microbiol, 84.

Ashbolt NJ, Amezquita A, Backhaus T, Borriello P, Brandt KK, Collignon P, et al, 2013. Human Health Risk Assessment (HHRA) for environmental development and transfer of antibiotic resistance. Environ Health Perspect, 121: 993-1001.

Azarbad H, Niklinska M, Laskowski R, van Straalen NM, van Gestel CA, Zhou J, et al, 2015. Microbial community composition and functions are resilient to metal pollution along two forest soil gradients. FEMS Microbiol Ecol, 91: 1-11.

Baker-Austin C, Wright MS, Stepanauskas R, McArthur JV, 2006. Co-selection of antibiotic and metal resistance. Trends Microbiol, 14: 176-82.

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

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

Hoffman LR, D'Argenio DA, MacCoss MJ, Zhang Z, Jones RA, Miller SI, 2005. Aminoglycoside antibiotics induce bacterial biofilm formation. Nature, 436: 1171-5.

Imfeld G, Bringel F, Vuilleumier S, 2011. Bacterial tolerance in contaminated soils: potential of the PICT approach in microbial ecology. Tolerance to Environmental Contaminants, CRC, Boca Raton, 333e364.

Knapp CW, Callan AC, Aitken B, Shearn R, Koenders A, Hinwood A, 2017. Relationship between antibiotic resistance genes and metals in residential soil samples from Western Australia. Environ Sci Pollut Res Int, 24: 2484-2494.

Knapp CW, McCluskey SM, Singh BK, Campbell CD, Hudson G, Graham DW, 2011. Antibiotic resistance gene abundances correlate with metal and geochemical conditions in archived Scottish soils. PLoS One, 6: e27300.

Knapp CW, Zhang W, Sturm BS, Graham DW, 2010. Differential fate of erythromycin and beta-lactam resistance genes from swine lagoon waste under different aquatic conditions. Environ Pollut, 158(5):1506-12.

Knights KV, 2006. A report on the Tellus urban field campaigns of Belfast Metropolitan areas and Londonderry. Commissioned Report CR/07/006N. British Geological Survey.

Li G, Sun GX, Ren Y, Luo XS, Zhu YG, 2018. Urban soil and human health: a review. European Journal of Soil Science, 69: 196-215.

Looft T, Johnson TA, Allen HK, Bayles DO, Alt DP, Stedtfeld RD, et al, 2012. In-feed antibiotic effects on the swine intestinal microbiome. Proc Natl Acad Sci USA, 109: 1691-6.

Luo XS, Yu S, Zhu YG, Li XD, 2012. Trace metal contamination in urban soils of China. Sci Total Environ, 421-422: 17-30.

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

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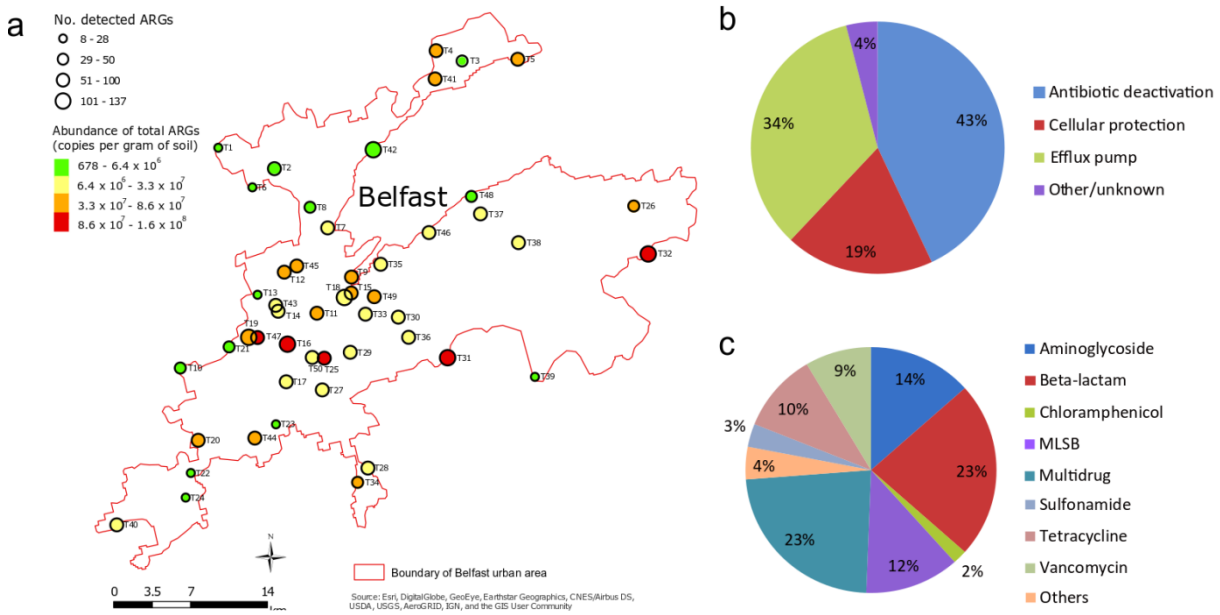

Figure 1 Antibiotic resistance gene (ARG) profile in Belfast urban soils. The map (a) reveals the number of different ARGs detected (dot size scale) and their absolute abundance (copies per gram of soil; color scale) in 50 urban soils from the Belfast metropolitan area. Pie charts depict (b) the percentage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

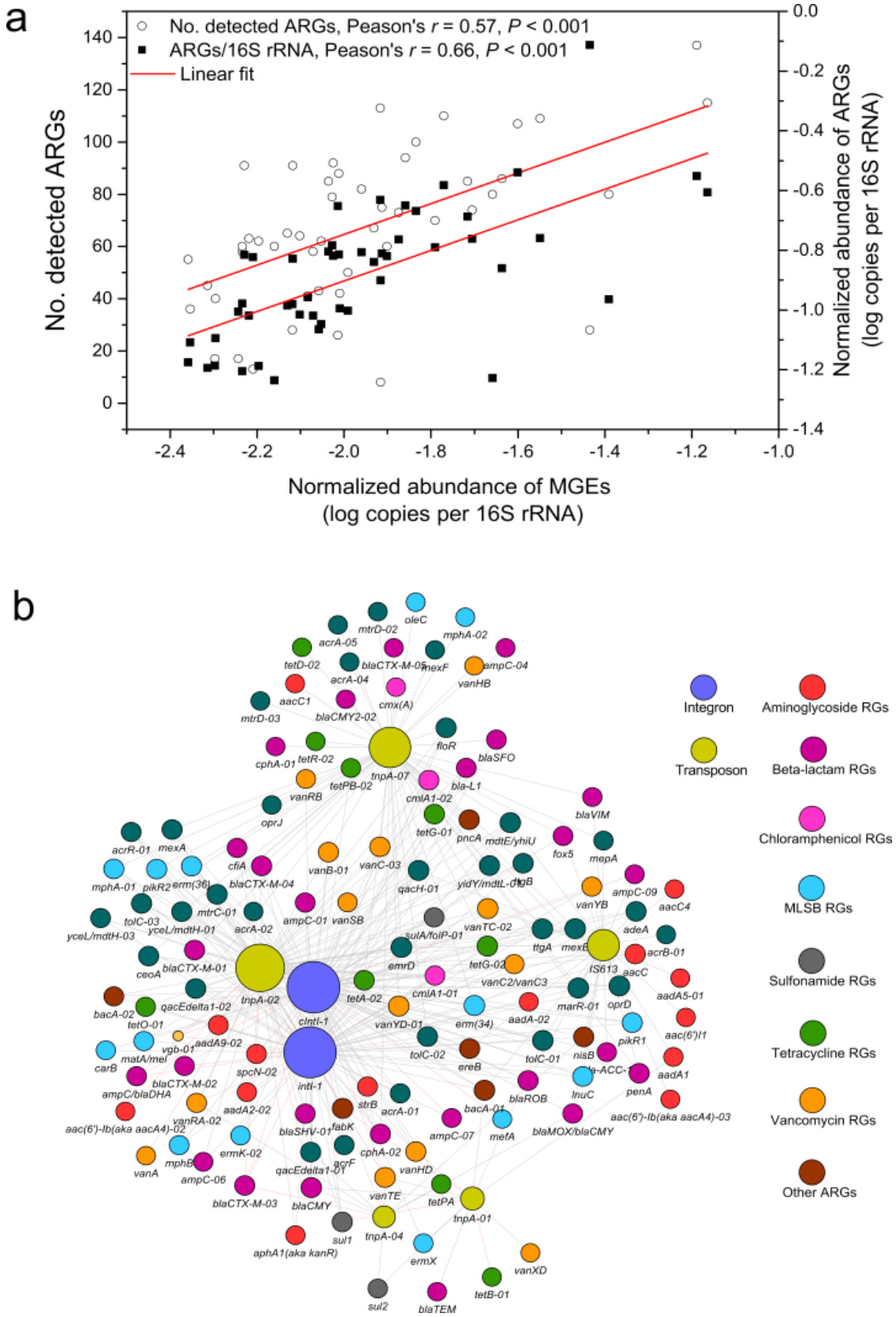
a
No. detected ARGs, Peason's r = 0.57, P < 0.001
ARGs/16S rRNA, Peason's r = 0.66, P < 0.001
Linear fit
No. detected ARGs
Normalized abundance of ARGs (log copies per 16S rRNA)
Normalized abundance of MGEs (log copies per 16S rRNA)
b
Integron
Transposon
Aminoglycoside RGs
Beta-lactam RGs
Chloramphenicol RGs
MLSB RGs
Sulfonamide RGs
Tetracycline RGs
Vancomycin RGs
Other ARGs

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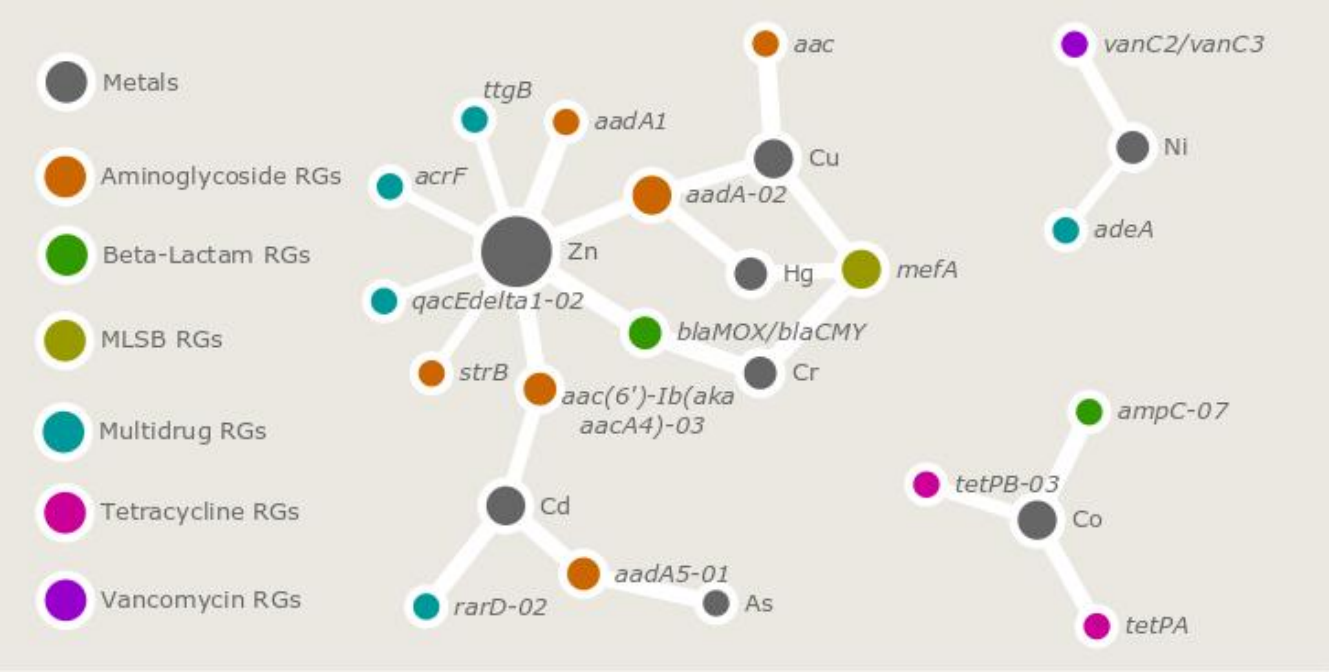
Figure 2 Correlation between mobile genetic elements (MGEs) and antibiotic resistance genes (ARGs) across all 50 Belfast urban soils. (a) The normalized abundance of all targeted MGEs (2 integrons and 8 transpons) significantly correlated to the total number of detected ARGs and the normalized abundance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



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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.

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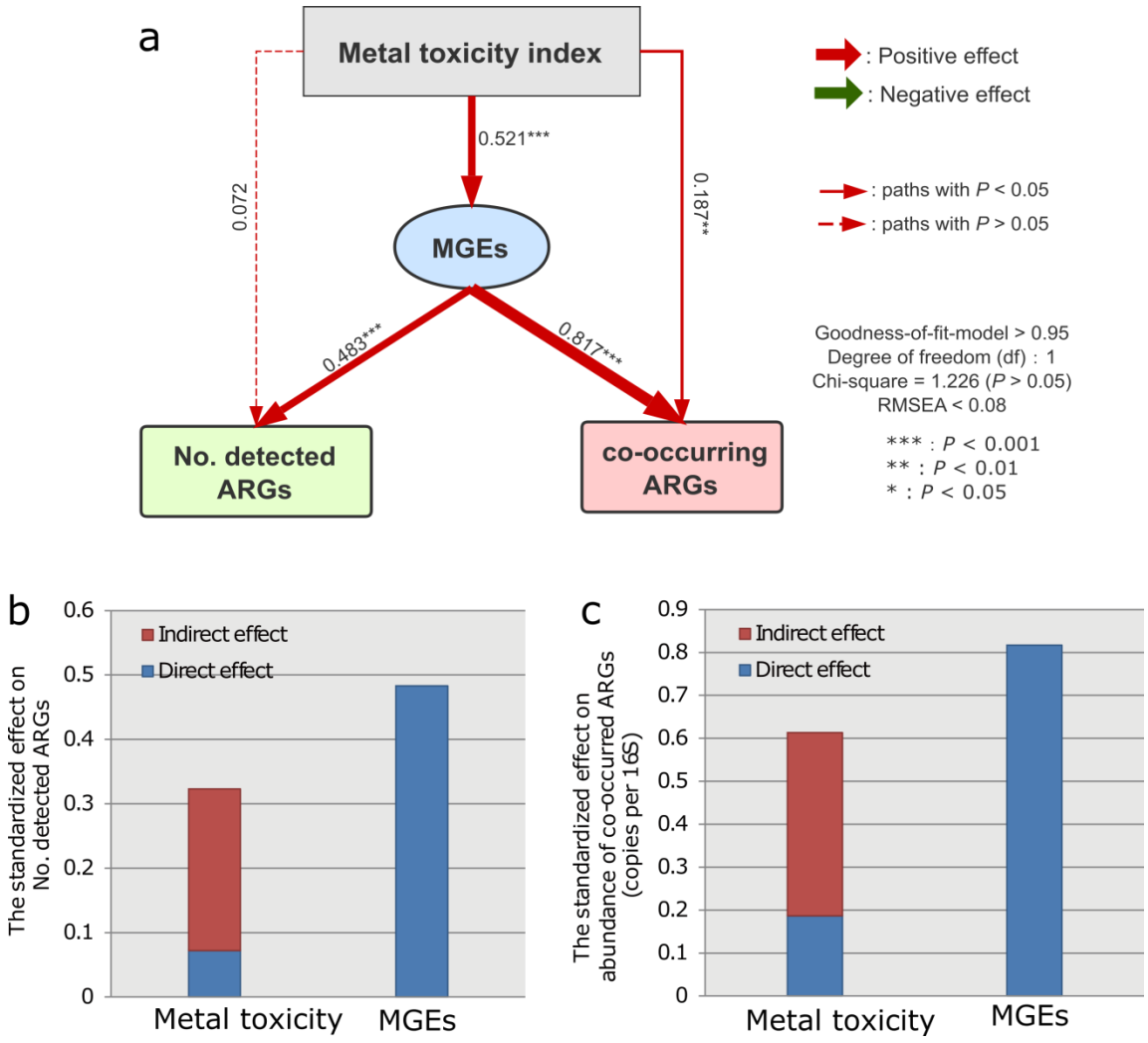

Figure 4 Path analysis showing the modelled effect of soil metal contamination (metal toxicity index) and normalized abundance of mobile genetic elements(MGEs) on (a) the number of detected antibiotic resistance genes (ARGs) and the normalized abundance of co-occurring ARGs (copies per 16S rRNA gene).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