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Bioavailability of potentially toxic elements influences antibiotic resistance gene and mobile genetic element abundances in urban and rural soils

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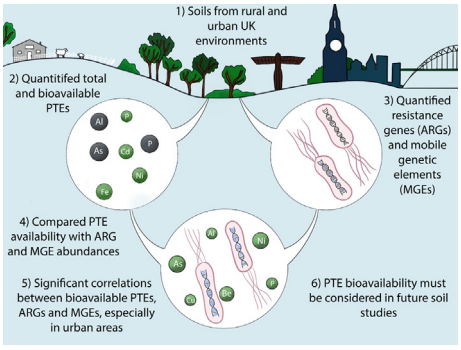
HIGHLIGHTS

- Patterns of antibiotic resistance genes differ between urban and rural landscapes.
- In rural settings, antibiotic resistance is directly associated with mobile genetic elements.
- In urban sites, antibiotic resistance is more directly linked with potentially toxic elements.
- The presence of antibiotic resistance genes and mobile genetic elements correlate better with bioavailable elements (metals).

GRAPHICAL ABSTRACT



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ABSTRACT

Antibiotic resistance genes (ARGs) that can encode resistance traits in bacteria are found across the environment. While it is often difficult to discern their origin, their prevalence and diversity depends on many factors, one of which is their exposure to potentially toxic elements (PTE, i.e., metals and metalloids) in soils. Here, we investigated how ambient ARGs and mobile genetic elements (MGEs) relate to the relative bioavailability of different PTEs (total versus exchangeable and carbonate-bound PTE) in rural and urban soils in northeast England. The average relative abundances of ARGs in rural sites varied over a 3-log range (7.24×10^{-7} to 1.0×10^{-4} genes/16S rRNA), and relative ARG abundances in urban sites varied by four orders of magnitude (1.75×10^{-6} to 2.85×10^{-2} genes/16S rRNA). While beta-lactam and aminoglycoside resistance genes dominated rural and urban sites, respectively, non-specific ARGs, also called multidrug-resistance genes, were significantly more abundant in urban sites ($p < 0.05$). Urban sites also had higher concentrations of total and exchangeable forms of PTE than rural sites, whereas rural sites were higher in carbonate-bound forms. Significant positive Spearman correlations between PTEs, ARGs and MGEs were apparent, especially with bioavailable PTE fractions and at urban sites. This study found significant positive correlations between ARGs and beryllium (Be), which has not previously been reported. Overall, our results show that PTE bioavailability is important in explaining the relative selection of ARGs in soil settings and must be considered in future co-selection and ARG exposure studies.

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1. Introduction

Antibiotics have revolutionised modern healthcare by treating once previously fatal infections. However, the increased use of antibiotics has resulted in many microorganisms becoming resistant to treatment, ultimately contributing to the global antimicrobial resistance (AMR) problem. AMR's health and economic consequences (Howard et al., 2003) have driven the need for further investigations into the connections between human, animal, and environmental sections (Robinson et al., 2016)—representing the One Health paradigm. Antibiotic resistance genes (ARGs) encode bacteria's resistance to antibiotics (White and Hughes, 2019) and are naturally present in soils (Dcosta et al., 2011). However, human activity has been shown to enhance natural resistance in soils, including through agricultural practices such as irrigation and the application of manure fertiliser (Graham et al., 2019). The human impact on resistance in soils has been observed in soil archive data from the Netherlands, which showed a significant increase in the relative abundance of ARGs after 1940, when antibiotics started being widely used (Knapp et al., 2010). The environment allows one to investigate ARGs at their origins and elucidate the drivers of their formation and transfer, which can mobilise to impact clinical and agricultural settings (Graham et al., 2016). Furthermore, the co-presence of mobile genetic elements (MGEs), including transposons and integrases (Gillings et al., 2015; Ma et al., 2017; Stokes and Gillings, 2011), exacerbates the issue by facilitating the spread of acquired resistance.

Potentially toxic elements (PTE; Pourret and Hursthouse, 2019), comprising of metals and metalloids, have been reputed to contribute to the selection of ARGs in many environments (e.g., Wright et al., 2006, Wright et al., 2008; Knapp et al., 2010, 2011, 2017; Seiler and Berendonk, 2012) due to co-

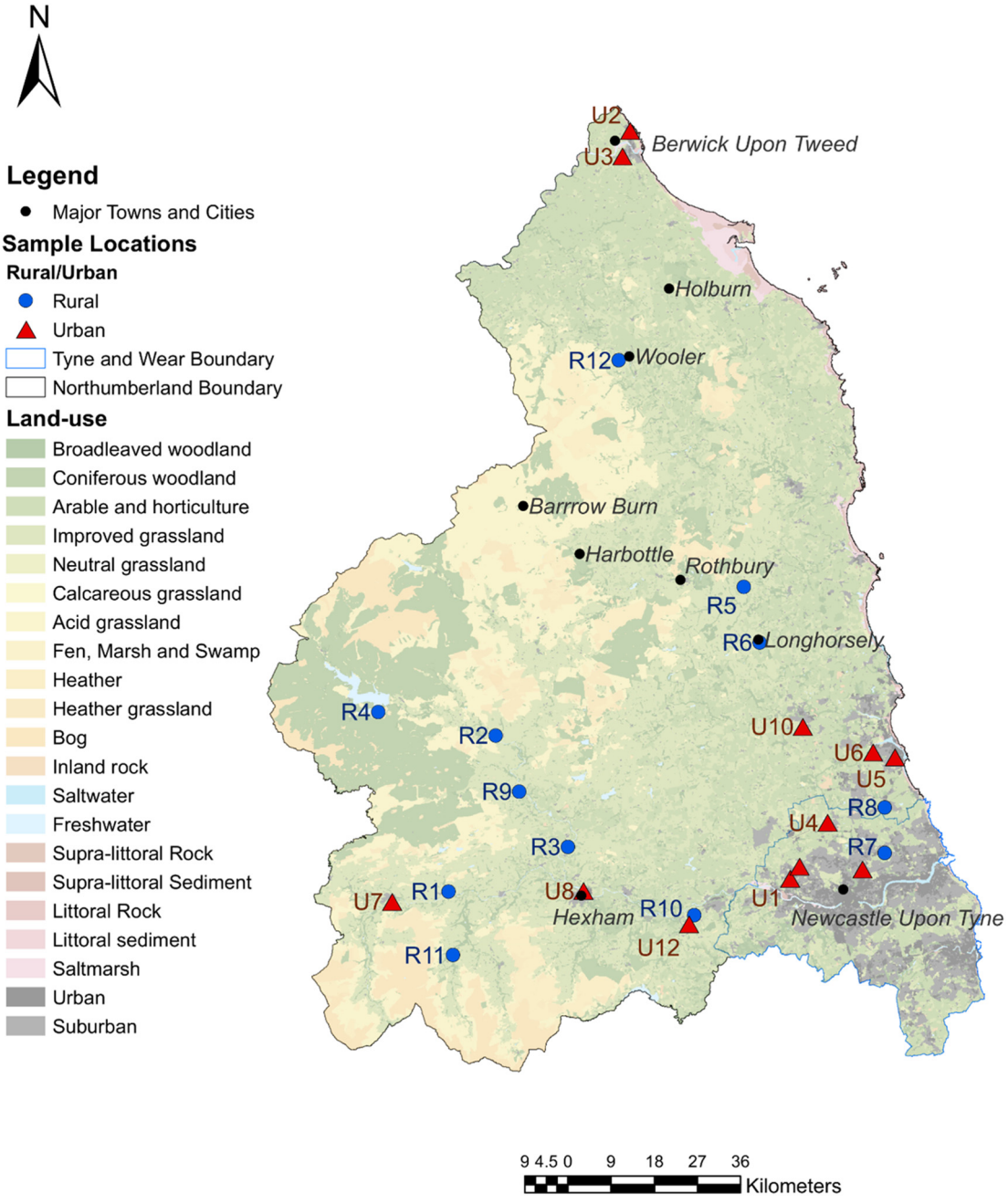

Fig. 1. Antibiotic resistance gene (ARG) abundance in rural and urban sites and land use in Northumberland. The map reveals the sample locations in rural (R1-R12) and urban (U1-U12) sites produced using the ArcGIS software ArcMap version 10.6 (ESRI, 2018). Boundary data were downloaded from the Census boundary data (UK Data Service, 2019); and land-use data was obtained from Digimap® (Rowland et al., 2017).

resistance and cross-resistance processes (Baker-Austin et al., 2006; Cantón and Ruiz-Garbajosa, 2011; Seiler and Berendonk, 2012; Wright et al., 2006), including when antibiotics are not present (Song et al., 2017). However, previous work has tended to focus on "total metals" (or "total PTEs") in comparisons (Dickinson et al., 2019; Knapp et al., 2017, 2011; McCann et al., 2019; Seiler and Berendonk, 2012; Zhao et al., 2020, 2019). While correlations sometimes exist, the true impact of PTE contamination in soils also depends on the concentration and chemical-related species, which cannot be determined using only "total concentration" data (Huang et al., 2010). As such, we hypothesise that "bioavailable PTEs" better represent attainable forms for cellular uptake by microorganisms and, therefore, have greater importance in environmental studies (Rodríguez et al., 2009).

Selective chemical extraction techniques are typically used to assess the bioavailability of PTEs and their interactions in soil (Ahnstrom and Parker, 1999; Qiao et al., 2003; Silveira et al., 2006; Tessier et al., 1979). One of the most widely used is the Tessier procedure (Tessier et al., 1979), which classifies PTEs into five fractions: exchangeable, carbonate-bound, Fe—Mn oxide, organic-matter and residual fractions. Exchangeable fractions are bioavailable, whereas carbonate-bound, Fe—Mn oxide and organic matter fractions are potentially bioavailable (Wang et al., 2021; Qasim and Motelica-Heino, 2014; Ahumada et al., 2008). Residual fractions are non-bioavailable (He et al., 2005; Rodríguez et al., 2009).

Besides lacking comparisons between PTE fractions and ARGs in the environment, most studies have not compared the influence of PTEs in urban vs rural sites. Here, we simultaneously compare PTE pollution in rural and urban sites and show that land-use influences PTE concentrations, bioavailability, and as a result, ARG/MGE abundance. Therefore, here we assess the relative influence of "total" and "bioavailable" PTEs and the abundance and diversity of ARGs and MGEs in rural and urban sites to understand better the drivers of resistance selection in different landscapes.

2. Methodology

2.1. Sample collection and locations

The top 20 cm of soil profiles were grab sampled in June 2016 across Northumberland, NE England (Fig. 1; Table S1). Twenty-four sites were selected across landscapes and categorised as either "rural" (R1-R12) or "urban" (U1-U12) according to apparent land use based on a subjective assessment of their geographic setting and landscape character. Soil samples were stored at −20 °C until further analysis.

2.2. Total and bioavailable PTE extractions

Soils were air-dried and sieved to <2 mm. Soil pH and total organic carbon (TOC) were quantified according to ISO 1090:2005 and ISO 10694 with a LECO CS233 carbon analyser (LECO Instrument Ltd., U.K.), respectively (Table S2). The following PTEs were selected for analysis: aluminium (Al), arsenic (As), beryllium (Be), cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), mercury (Hg), manganese (Mn), nickel (Ni), phosphorous (P), lead (Pb) and zinc (Zn).

PTE were characterised on dried, homogenised soils using previously described methods (Gray et al., 2014) on a Perkin Elmer ICP-OES (Inductively Coupled Plasma Optical Emission Spectroscopy, Optima 5300 DV). However,

Table 1
Total and bioavailable PTE concentrations (mg/kg) in Northumberland soils.

Symbol	Name	Speciation	Rural		Urban		Mann Whitney (rural vs urban)
			Mean	Min-Max	Mean	Min-Max	*p*-value (greater value)
Al	Aluminium	Total	8400	600–12,000	10,200	7500–13,000	0.033 (urban)
As	Arsenic	Total	8.9	5.3–14	11.6	4–29	0.378
Be	Beryllium	Total	0.8	0.5–1	1.1	0.5–1.8	0.033 (urban)
Cd	Cadmium	Total	0.5	0.2–1.4	0.9	0.1–2.8	0.551
Cr	Chromium	Total	19	23–28	28	16–63	0.014 (urban)
Cu	Copper	Total	36	16–71	53	25–112	0.050 (urban)
Fe	Iron	Total	27,000	18,000–41,000	27,000	16,000–35,000	1.000
Hg	Mercury	Total	0.1	0.05–0.2	0.2	0.1–0.8	0.374
Mn	Manganese	Total					
Ni	Nickel	Total	22	14–29	25	14–35	0.266
P	Phosphorous	Total	810	430–1400	940	480–1900	0.410
Pb	Lead	Total	120	34–610	250	24–690	0.101 (urban)
Zn	Zinc	Total	160	69–530	280	51–850	0.128
Al	Aluminium	Exchangeable	1.4	0.2–8.6	0.9	0.1–4.7	0.160
As	Arsenic	Exchangeable	0.02	0–0.12	0.03	0–0.1	0.671
Be	Beryllium	Exchangeable	0.0011	0.0006–0.0039	0.0010	0–0.0019	0.514
Cd	Cadmium	Exchangeable	0.03	0.01–0.08	0.07	0–0.35	0.755
Cr	Chromium	Exchangeable	0.015	0–0.063	0.004	0–0.022	0.514
Cu	Copper	Exchangeable	0.010	0–0.031	0.017	0–0.068	0.630
Fe	Iron	Exchangeable	0.28	0.07–1.16	0.37	0.19–0.80	0.068 (urban)
Hg	Mercury	Exchangeable	0	0	0	0	
Mn	Manganese	Exchangeable	6.5	0.3–31	3.5	0.6–9.2	0.843
Ni	Nickel	Exchangeable	0.10	0–0.56	0.06	0–0.22	0.755
P	Phosphorous	Exchangeable	0.64	0–2.48	0.93	0–8.47	0.551
Pb	Lead	Exchangeable	0.08	0–0.58	0.96	0–6.52	0.128
Zn	Zinc	Exchangeable	1.27	0–7.29	5.96	0.0305–39.36	0.347
Al	Aluminium	Carbonate Bound	6.0	0.1–54.5	2.3	0.7–5.8	0.089 (rural)
As	Arsenic	Carbonate Bound	0.05	0–0.3	0.1	0–0.3	0.291
Be	Beryllium	Carbonate Bound	0.0048	0.0009–0.0239	0.0	0	0.478
Cd	Cadmium	Carbonate Bound	0.010	0.003–0.035	0.0	0	0.671
Cr	Chromium	Carbonate Bound	0.014	0–0.045	0.0	0	0.478
Cu	Copper	Carbonate Bound	0.05	0.02–0.16	0.1	0–0.2	0.178
Fe	Iron	Carbonate Bound	2.3	0.4–15.5	1.4	0.7–2.5	0.060 (rural)
Hg	Mercury	Carbonate Bound	0.04	0–0.09	0.0	0–0.1	0.198
Mn	Manganese	Carbonate Bound	5.2	1.7–9.8	3.0	1.2–5	0.002 (rural)
Ni	Nickel	Carbonate Bound	0.06	0–0.15	0.0	0–0.1	0.590
P	Phosphorous	Carbonate Bound	2.1	0.3–5.7	2.9	0.3–18.8	0.887
Pb	Lead	Carbonate Bound	0.9	0–5.5	1.8	0–6.9	0.198
Zn	Zinc	Carbonate Bound	1.6	0.2–6.4	2.8	0.1–11.7	0.799

Table 2
Gene differences and commonalities between urban and rural sites.

Rural, exclusive genes (n = 12)	**Urban, exclusive genes** (n = 15)
Aminoglycosie: aadA2	**ß-lactamase**: bla-LI, blaPAO, blaTEM, cepA,
ß-lactamase: ampC-02, blaIMP-01,	mecA, pbp
blaOKP, cfxA	**MLSB**: ereA, mphB, pikR1
MLSB: acrF, qacH, rarD	**Multidrug**: adeA
Sulfonaminde: sul2	**Sulfonamide**: folA
Vancomycin: vanRC4, vanSB, vanYB	**Tetracycline**: tetH
	Vancomycin: vanHD
	f-c-a-phenicol*: catB
	triclosan: fabK

Genes found in both landscapes (n = 83)
Aminoglycoside: aac, aac(6′)l1, aac(6′)-lb(aacA4), aacC, aacC1, aacC4, aadA1, aadA5, aadA9, aadE, aph6ia, aphA1, spcN, strB
ß-lactamase: blaDHA, ampC1, ampC4, ampC5, ampC9, blaAAC1, blaCMY2, blaCTX-M1–2–4-6, blaMOX/CMY, blaOCH, blaOXY, blaROB, blaSFO, blaSHV01, blaVIM, cfiA, sphaA1–2, fox5, ndm1,penA, pbp2x, pbp5
MLSB: carB, erm(34), erm(36), ermK-02, lnuC, matA/mel, mdtA, mefA, mphA-1-2, oleC, pikR2, vatE-1
Multdrug: acrA, acrR,ceoA, emrD, marR, mdtE/yhiU, mepA, mexA, mexE, mexF, mtrC-1, mtrD, oprD, oprJ, qacEΔ1, tolC, ttgA, ttgB, yceE/mdtG, yidY/mdtL,
Tetracycline: tetD, tetG, tetL, tetM, tetPB, tetR
Vancomycin: vanB, vanC, vanHB, vanRB, vanTC, vanXD,vanYD
f-c-a-phenicol*: cmlA1, cmx(A), floA,
Other: bacA, pncA

Genes not found in either landscape
Aminoglycoside: aac6iia, aac6iF, aacA/aphD, aacC2, aadA1, aadA2–03, aadD, aph2, spcN, str, strA
Beta-lactamase: bla1, blaCMY, blaCTX-M-3, blaCTX-M-5, blaGES, blaIMP2, blaXOA10, blaPER, blaPSE, blaSHV2, blaTLA, blaVEB, blaZ
Multidrug: acrB, cmeA, cmr, lmrA-01, lnuA-01, lnuB-01, lnu-B02, mdetl1, mexD, mtrC, mtrD, pmrA, qac, qacA, qacB, qacH, sdeB, yceL/mdtH
MLSB: ereB, erm(35), ermA, ermA/ermTR, ermB, ermC, ermF, ermJ/ermD, ermK-01, ermT-01, mphC, msrA, msrC ermT-02, ermX, ermY, vatB, vatC, vatE, vgaA, vgbB
Sulfonamide: dfrA1, dfrA12, sulA/folP
Tetracycline: tet(32), tet(34), tet(35), tet(36), tet(37), tetA, tetB, tetC, tetD, tetE, tetJ, tetO, tetPA, tetPB, tetQ, tetS, tetT
Vancomycin: vanA, vanC1, vanC2, vanG, vanRA1, vanRA2, vanRC, vanRD, vanSA, vanSC, vanSE, vanTC, vanTE, vanTG, vanWB, vanWG, vanXA, vanXB, vanYD
f-c-a-phenicol*: catA1, catB3, cfr, qnrA
Other: fosB, fosX, nimE, nisB, sat4, speA

Note: f-c-a-phenicol = (fluor-) / (chloro-) / (am-) phenicol resistance.

the extraction methods varied. Total PTE (PTE_{total}) involved digestions with *aqua regia* (3:1 nitric acid: hydrochloric acid). Bioavailable PTEs were characterised according to (Tessier et al., 1979) extractions; however, only the first two Tessier fractions were considered here: exchangeable (PTE_{exch}) and carbonate-bound (PTE_{carb}), which represent the bioavailable fractions in the soil (Wang et al., 2021; Qasim and Motelica-Heino, 2014; Ahumada et al., 2008). Exchangeable fractions were extracted with 1 M sodium acetate (pH 8.2) at room temperature (1 h) and represent PTE susceptible to ionic exchange (Zimmerman and Weindorf, 2010). Whereas the carbonate-bound fractions were extracted from the remaining solids (following exchangeable extractions) with 1 M sodium acetate, but the pH was adjusted to 5.0 with acetic acid; this fraction is susceptible to changes in pH (Zimmerman and Weindorf, 2010). As such, $PTE_{bioavailable}$ represents the sum of PTE_{exch} and PTE_{carb}.

2.3. Quantification of total bacteria, ARGs and MGEs

Following the manufacturer's instructions, the FastDNA Spin Kit for Soil (M.P. Biomedicals, Santa Ana, California, USA) was used for DNA extraction using 0.5 g of soil. DNA concentrations and purity were verified via UV-spectrophotometric analysis (NanoDrop 1000; ThermoFisher Scientific, UK). The extracted DNA was then freeze-dried and transported to the Institute of Urban Environment, Chinese Academy of Science (Xiamen) for high-throughput quantitative PCR (HT-qPCR) using SmartChip Real-Time PCR (WaferGen Biosystems, Takara, Shiga, Japan) system (e.g., Zhu et al., 2017, 2013). The analysis detects 296 ARGs, 16S-rRNA gene (a surrogate measure of "total bacteria"), and seven marker genes for MGEs, including five transposase genes, class-1 integron-integrase gene (*intI*-1), and a clinical-*intI*-1 (*cIntI-1*) as previously described (Looft et al., 2012; McCann et al., 2019).

To facilitate interpretation, individual ARGs were classified by their "ARG family": aminoglycoside, beta-lactamase (beta-lactam), macro-lincosamide-streptogramin B (MLSB), "multidrug-resistance" (i.e., MDR, non-specific mechanisms), sulphonamide, tetracycline, vancomycin, flor-/chlor-/am-phenicols (F/C/A) and "other" genes, which represented multiple antibiotic classes with few gene representatives (e.g., triclosan, pyrazinamide, bacitracin). However, individual genes also were assessed via network analysis (see below). We note to the reader that multidrug-resistance here refers to ARGs that code for general cell functions (e.g., genes for efflux pumps). In contrast, some specific genes (e.g., bla_{NDM-1} or bla_{KPC}) are wide spectrum and cause intense multidrug-resistance, but were not included in our multirug-resistance family.

2.4. Statistical analysis

Either Microsoft *Excel* or *R-Studio* (R Core Team, 2018) were used for statistical analyses. In addition, maps were produced using the ArcGIS software ArcMap version 10.6 (ESRI, 2018); boundary data was downloaded from the Census boundary data (UK Data Service, 2019); and land-use data were obtained from *Digimap*® (Rowland et al., 2017).

PTE_{total} concentrations were compared to the average concentrations found in the U.K. soil and herbage pollutant survey (UKSHS) (Environment Agency, 2007)—except for Al, Be, Fe and P, which the UKSHS did not measure.

Dataset normality and scedasticity were assessed using the Shapiro-Wilk and Levene's tests. The relative abundance of ARGs, MGEs and PTE concentrations were log-transformed prior to statistical analysis to improve sample distribution. Significance was defined as $p < 0.05$ unless otherwise stated. A Kruskal-Wallis test was performed to analyse differences between PTE concentrations and ARG/MGE abundances in rural and urban landscapes.

Table 3
Positive Spearman correlations ($p < 0.10$) between mobile genetic elements and antimicrobial-resistance gene families.

	Rural landscapes					Urban landscapes				
	ARG_{total}	# ARG	MGE_{total}	Integrase	Transposase	ARG_{total}	MGE_{total}	# ARG	Integrase	Transposase
Total ARG_{total}	–			0.63*	0.61*	–				
# ARG		–		0.57	0.78**		–		0.71**	0.66*
Total MGE_{total}			–		0.77**			–		0.94**
Aminoglycoside			0.83**	0.83**				0.53		
Beta-lactam	0.86**	0.60*			0.58*	0.73**	0.70*	0.65*	0.70*	0.68*
F/C/A	0.87**	0.89**			0.67*					
MLSB	0.69*	0.72**	0.90**	0.90**	0.80**	0.58*				
Multi-drug	0.83**	0.83**		0.56	0.26*	0.59*	0.66*	0.71**	0.66*	0.58*
Tetracycline	0.60*	0.55	0.92**	0.92**	0.65*					
Vancomycin						0.69*				

* Significance, $p < 0.05$
** Significance, $p < 0.01$

Spearman correlations were used as a non-parametric method (i.e., based on "rankings") to determine bivariate relationships with skewed data distributions. We first examined the entire dataset, but we also divided the dataset to compare "urban" and "rural" landscapes individually. Correlations aimed to 1) provide a better understanding of underlying environmental factors related to ARG development and dissemination; 2) determine the extent to which PTE bioavailability contributes to ARG presence. The *p*-values were adjusted according to Benjamin Hochberg (1995) to reduce false-positive results. Strongly positive correlations ($r_s > 0.7, p < 0.05$) were further visualised using network analysis based on the *igraph R* package and *Gephi* software (Bastian et al., 2009).

3. Results

3.1. PTE concentrations

The study assessed relationships between PTE bioavailability and AMR at rural and urban sites. The urban sites had higher PTE_{total} and PTE_{exch} concentrations than rural sites (Table 1), whereas the rural sites tended to have greater PTE_{carb} levels (Al, Fe, and Mn). Therefore, there were considerable variations in the range of concentrations among sites. For most bioavailable PTEs (i.e., $PTE_{bioavailable}$), the carbonate-bound form was more prevalent than the exchangeable form, except for Cd, Cr, Ni (rural sites) and Zn (urban sites). On a total-element basis, urban sites had higher Al, Be, Cr, Cu, and Pb (Table 1).

PTE presence reflects either their natural occurrence as a mineral or a pollutant. To provide some context of PTE concentrations versus geochemical conditions in UK soils, PTE_{total} concentrations were compared with the UKSHS (Environment Agency, 2007) Table S4), which provides baseline levels with which extraneous pollutant levels can be contrasted. In general, predominant PTEs in rural and urban sites were Al and Fe (not listed in UKSHS), representing natural Northumberland mineralogy comprising Al-containing feldspar in sandstone and Permian coal measures (via pyrite, FeS_2). Regarding the twelve rural sites, all but two sites (R4 and R9) had above-average Cu concentrations; furthermore, rural sites R1, R8, R10, and R11 exceeded average Cd, Cu, Hg, Ni, Pb and Zn levels in the UK survey. No sites had any exceedances for Cr, and only one rural site (R11) had excessive Zn.

Among the urban sites, each sample ($n = 12$) had at least one PTE concentration exceeding average UKSHS urban-soil concentrations, except for

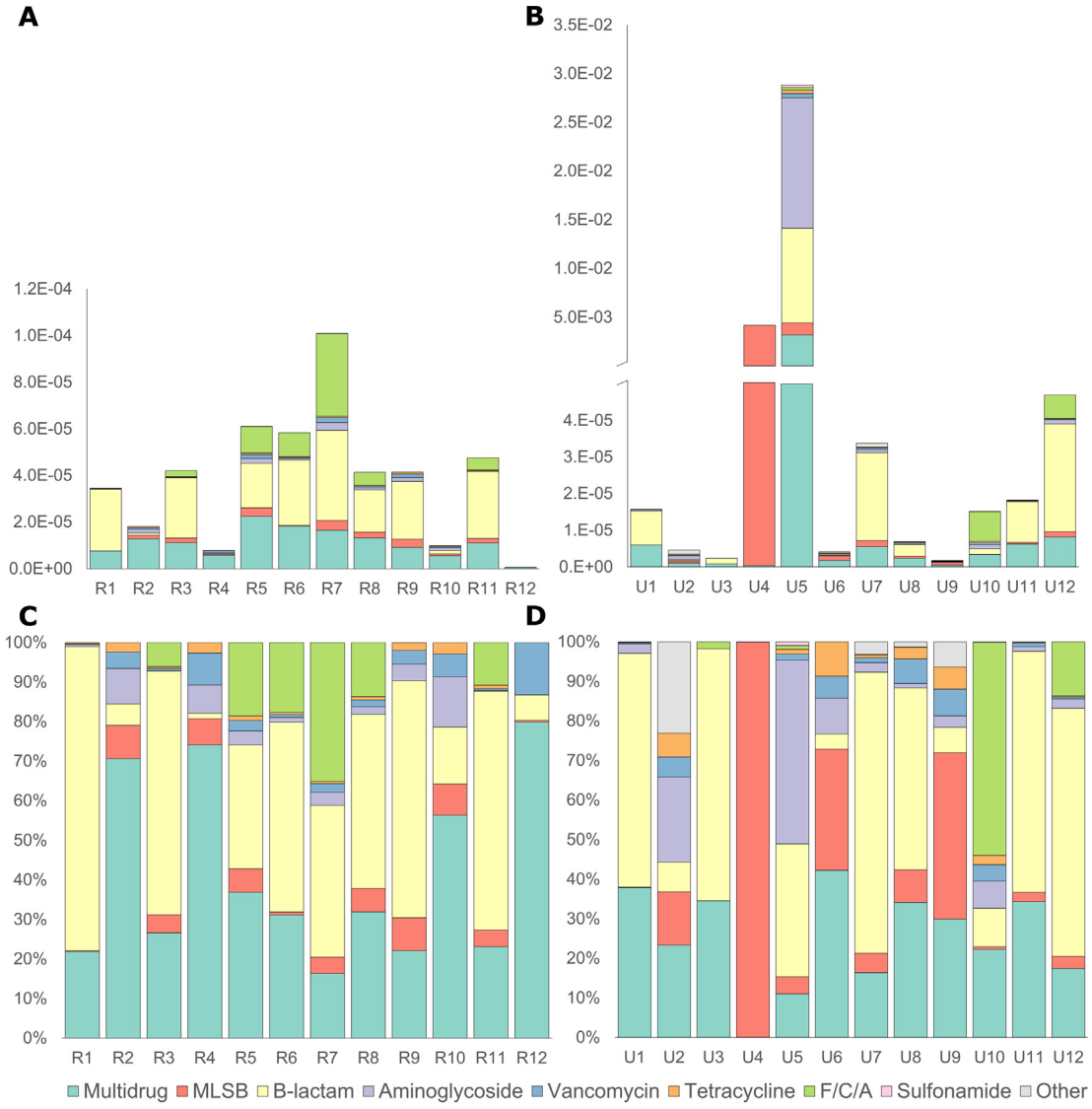

Fig. 2. The relative abundance of ARGs in rural (A) and urban areas (B) with their corresponding proportions of ARG family (based on relative abundance) in rural (C) and urban (D) areas. The relative abundance genes were normalised to 16S-rRNA abundance. MLSB denotes Macrolide-Lincosamide-Streptogramin. 'Other' represents ARGs that do not directly have an antibiotic class (e.g., bacitracin, pyrazinamide and triclosan). F/C/A denotes the flor−/chlor−/am-phenicol antibiotic class.

two sites (U3 and U10). However, there were urban samples with concentrations greater than the maxima in UKSHS: U1 (As, Pb), U7 (Cd, Pb, Zn), U8 (Cd, Pb, Zn) and U12 (Cr). From UKSHS averages and ranges, urban soils tended to have greater PTE values than rural ones. This comparison suggests that the soils had comparatively high concentrations relative to other soils in the UK, particularly in the urban sites, which could be attributed to anthropogenic activities, such as vehicle traffic or street dust (Bolan et al., 2004; Wuana and Okieimen, 2011).

3.2. Diversity of ARGs and MGEs

A total of 110 unique ARGs were detected in urban and rural samples, averaging 37 (ranging from 7 to 50) and 31 (ranging from 7 to 44) positive ARG detections, respectively. Shared vs place-specific genes were examined between rural and urban sites (Table 2; Fig. S1). Eighty-three genes were present in all sites; 12 were specific to rural sites, and 15 were specific to urban environments. Thus, there were similarities across landscapes (>80 %); but some unique signatures existed. Four specific genes were found in the rural sites that code beta-lactam resistance, three genes each from non-specific resistance and vancomycin (respectively), and one gene each for aminoglycoside and sulphonamide. In urban sites, six specific genes were for beta-lactam resistance, two among the MLSB, and one gene (each) for non-specific, sulphonamide, tetracycline, vancomycin, F/C/A and triclosan ARG families.

Here, in rural sites, beta-lactam genes (45.66 % of gene abundance) predominate, followed by non-specific MDR (28.84 %) and F/C/A (15.14 %) resistance genes. In urban sites, the most prevalent class is aminoglycoside (40.94 %), followed by beta-lactam (30.3 %) and MLSB (14.47 %). However, if one excludes U4 and U5, beta-lactams (53.99 %) prevailed, followed by non-specific MDR (24.01 %) and F/C/A (9.81 %) resistance. MDR (i.e., non-specific) genes were found in rural and urban sites. However, they were more significant at urban sites (Kruskal Wallis, $p = 0.012$), which is potentially concerning, as these genes encode resistance potential to multiple antibiotics. Regarding action mechanisms, antibiotic deactivation systems were dominant at all sites, particularly urban sites (rural: 51.78 %, urban: 85.5 %), followed by efflux pump mechanisms (rural: 44 %, urban: 8.5 %).

In terms of MGEs, five transposase genes and two integrases were detected in both landscapes. MGEs were found less frequently in the urban sites, e.g., undetectable in three sites (U2, U4 and U10). Further, transposase or integrase systems appear to associate with the number of genes detected across samples (Table 3). Integrase presence in both landscapes was unsurprisingly ubiquitous (Gillings et al., 2015; Wright et al., 2008). They are common in environmental samples, and their elevated presence is often linked to human impact (Gillings et al., 2015).

3.3. Relative gene abundances

The relative abundance of ARG per gene family are shown in Fig. 2 and Table S6. Rural sites averaged a total relative ARG abundance of 3.87×10^{-5} genes/16S-rRNA ($\pm 2.79 \times 10^{-5}$), with relative ARG abundance across sites averaging over a 3-log range: 7.24×10^{-7} to 1×10^{-4} genes/16S-rRNA. In urban sites, relative abundances of ARGs varied approximately four orders of magnitude, with an average abundance of 2.73×10^{-3} genes/16S-rRNA ($\pm 8.2 \times 10^{-3}$), ranging 1.75×10^{-6} to 2.85×10^{-2} genes/16S-rRNA. The highest relative abundances of ARGs were found at sites U4 and U5. Equivalent high abundances were not seen at other sites in this study, suggesting these sites warrant further investigation, including additional sampling in the areas.

Among rural sites, MGEs were detected in all but one site (R12); Fig. 3 displays their abundances (per family). Transposases and integrases varied by one to two orders of magnitude (5.67×10^{-9} to 2.15×10^{-7} genes/16S-rRNA and 1.7×10^{-6} to 1.77×10^{-5} genes/16S-rRNA, respectively. In urban samples, as mentioned previously, MGEs were less frequent. Furthermore, they had greater variances. Transposases varied by six orders of magnitude (2.67×10^{-9} to 1.63×10^{-3}genes/16S-rRNA), and integrase genes varied by four orders of magnitude (6.95×10^{-7} to 1.46×10^{-3}genes/16S-rRNA).

To further investigate gene and PTE relationships, Spearman correlations were performed. For both landscapes, total ARG abundance correlated with selected individual antibiotic classes (see Tables 3 and S7),

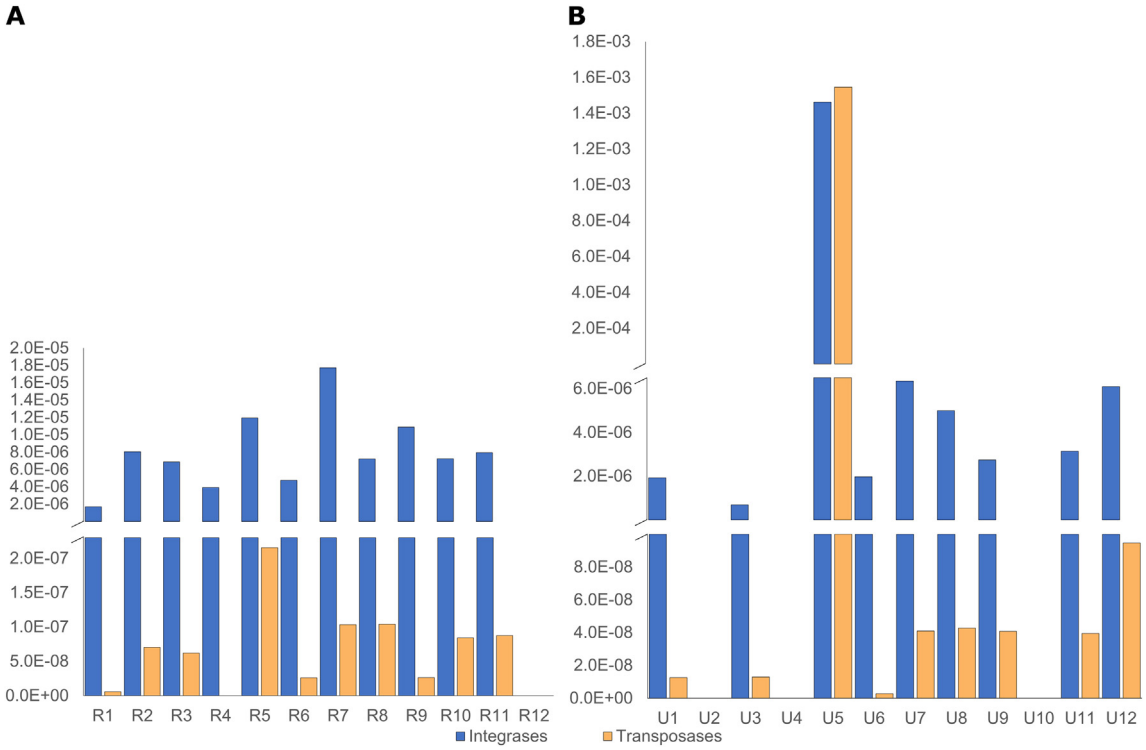

Fig. 3. Relative abundance (genes/16S-rRNA) of mobile genetic elements (MGE) in rural (A) and urban (B) sites.

particularly beta-lactam, MDR and MLSB, which reflect their predominance. Additionally, correlations were found with F/C/A and tetracycline in rural settings and vancomycin in urban sites. More importantly, there were correlation patterns related to the MGEs. In the rural sites, there appears to be a closer relationship between MGEs, including transposons and integrons, and the presence of ARG gene families—both in terms of the number of significantly paired correlations and their strength as correlation coefficients. However, the associations become less evident in urban settings with only integrase and transposase genes correlated with beta-lactams and multi-drug resistance, which were also the predominate gene families (Section 3.2).

A co-occurrence analysis was used to highlight individual gene relationships (Fig. 4) based on significant and strong correlations ($r_s > 0.7$, $p < 0.05$). The rural network shows predominant nodes among the beta-lactams (23.1 %), MDR (20.5 %), MLSB (15.8 %) and aminoglycoside (14.1 %) families. Modularity analysis revealed three different clusters of genes (Fig. S1). Three of the most distinctive clusters in rural sites include (1) the integron gene *IntI,* multidrug-resistance and MLSB genes; (2) *bl1* genes, *aac* and *aad* genes; and (3) the transposase gene *Tn22* and multidrug-resistance genes with genes in beta-lactam and aminoglycoside ARG families. The urban network had similarly predominant nodes in beta-lactam (22.2 %), MLSB (17.3 %), MDR (14.8 %) and aminoglycoside (12.5 %) families. However, three of the most distinctive clusters centred on (1) *yceE/mdtG,* bl_{vim} and *mexA*; (2) *pikR1*, aac_{2ic} and cml_{e3} with beta-lactam genes; and (3) aac_{3ic} and pbp_{2b}/pen_a and the MDRs.

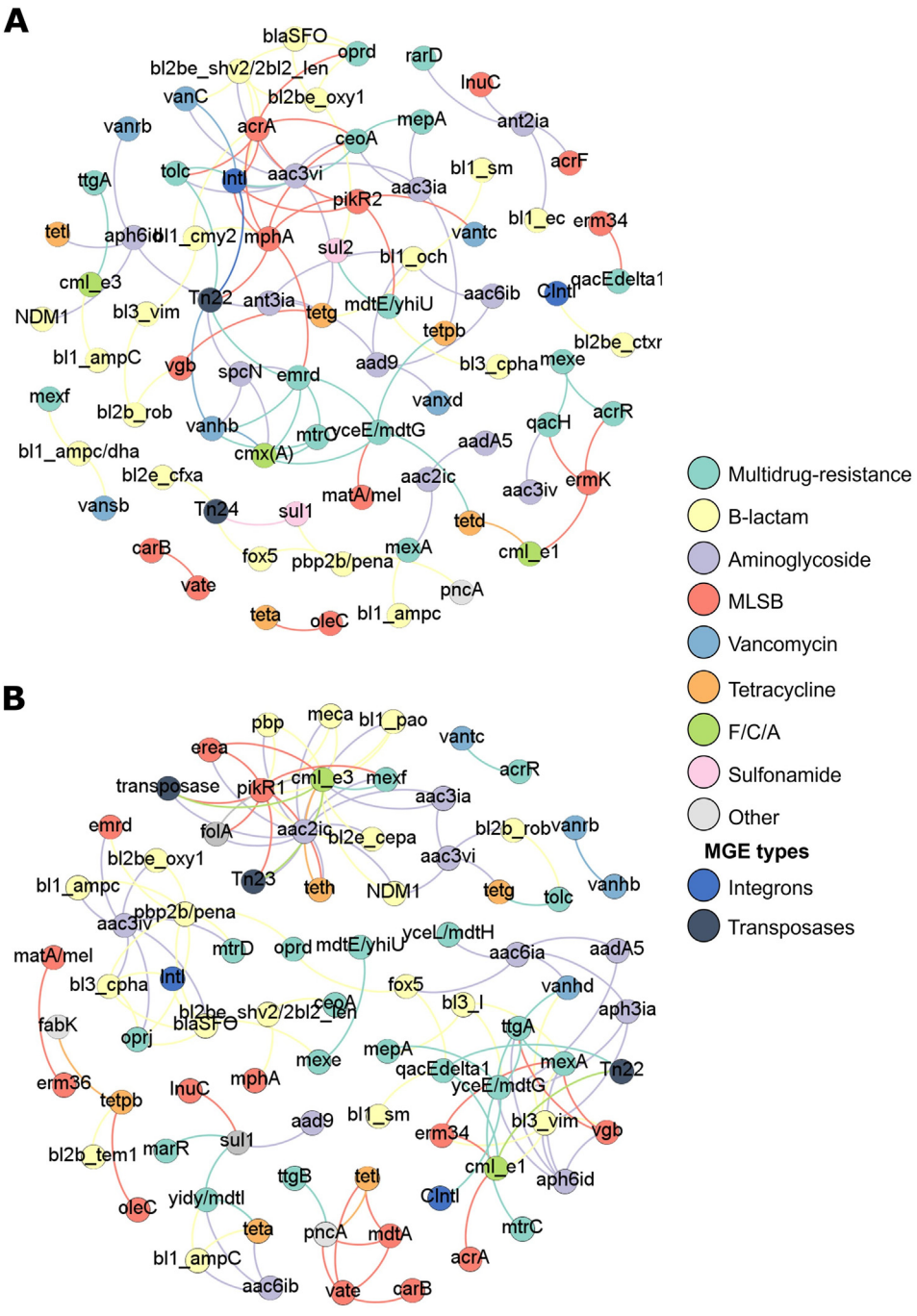

Fig. 4. Network analysis co-occurrence patterns among ARG subtype and MGE subtype in rural sites (A) and urban sites (B). A connection represents a substantial (Spearman correlations $r_s > 0.7$) and a significant correlation ($p < 0.05$).

The network analysis lacked strong correlations between specific ARGs and MGEs in urban sites; however, the elevated abundances of ARGs in sites like U5 coincided with elevated MGE levels. In extreme cases, MGEs possibly played a role in ARG proliferation; however, this did not appear to be the case for most other urban sites in this study.

3.4. Relationship between total and bioavailable PTEs, ARGs and MGEs

Significant associations were previously found between gene presence and MGEs, suggesting integrons and transposons tended to be associated with ARGs, especially among rural sites. Therefore, Spearman correlation analysis was conducted with ARGs (e.g., abundances of "total ARG" and each gene-family and the number of detectable genes) with the various measures of Tessier-extracts PTEs (Table 4, Table S8). When compared with PTE extracts, varied patterns emerged.

In rural settings, significant ($p < 0.05$) correlations among "total" PTE included Cu, Be, Hg, and P, with Cu associated with multiple genes, although the number of specific correlations was comparatively few. However, among the sequential extractions, the correlation coefficients improved in numerous cases (e.g. pyrazinamide and transposons), and additional correlations emerged between genes and PTE, for example, the number of ARG detections, and the F/C/A and MSLB families. This suggests improved ability to observe correlations. Interestingly, there were no correlations between any fraction of PTE and MGE, integrases, and many ARG, including multi-drug resistance.

There were more correlations among PTE versus ARGs and MGE in urban landscapes. Cd, Pb, and Zn were common among many ARG relationships, and more importantly, they co-occur with the abundance of integrases and transposons. These relationships exist not only among "total" PTE but often with similar correlation coefficients with bioavailable PTE forms of PTEs. Accordingly, more and stronger correlations emerged when considering bioavailable PTE factions, particularly As, Be, and Ni.

To further facilitate the understanding of the relationship between PTEs and resistance, a network analysis (Fig. 5) was conducted to provide a higher resolution of gene-PTE relationships based on positive and significant Spearman correlations ($r_s > 0.7$, $p < 0.05$) among ARGs, MGEs and total and bioavailable PTE. The number of co-occurrence links among PTEs is summarised in Table 5. Connections in both networks were attributed to bioavailable (50 % in rural and 40.82 % in urban sites) and total PTEs (10.3 % in rural and 12.24 % in urban sites). Further, 12 % were aminoglycoside genes in rural networks, and in urban networks, 12 % to multidrug-resistance and 6.12 % were beta-lactams, the key genes detected in each landscape. Overall, most nodes were either bioavailable or anthropogenic in origin (e.g., As, Cd, Cu, Hg, Ni, Pb, Zn), thus capable of impacting the microorganisms.

Table 4
Positive Spearman correlations ($p < 0.10$) between PTE and antimicrobial-resistance gene families.

	All sites				Rural landscapes				Urban landscapes			
	PTE_{total}	PTE_{exch}	PTE_{carb}	$PTE_{bioavail}$	PTE_{total}	PTE_{exch}	PTE_{carb}	$PTE_{bioavail}$	PTE_{total}	PTE_{exch}	PTE_{carb}	$PTE_{bioavail}$
Total ARG		As 0.46 * Cu 0.35				Cr 0.54 Cu 0.50				Al 0.53 As 0.75** Be 0.68*	Ni 0.52	Ni 0.62*
Total MGE	Cd 0.36	Cd 0.42*	Cd 0.43* Mn 0.42 Ni 0.56*	Cd 0.5*					As 0.51 Cd 0.79** Pb 0.63* Zn 0.74**	Cd 0.79** Zn 0.51	Cd 0.60* Mn 0.51 Pb 0.61* Zn 0.73* Total PTE 0.67**	Cd 0.82** Zn 0.73** Total PTE 0.61*
Number of ARG		Cr 0.35	Mn 0.40 Ni 0.46*	Cd 0.50*		Cu 0.62*		Cu 0.59*	Cd 0.72** Pb 0.62* Zn 0.68*	Cd 0.73** Pb 0.55 Total PTE 0.59*	Pb 0.62* Zn 0.53 Total PTE 0.73**	Cd 0.70* Mn 0.55 Pb 0.65* Zn 0.63* Total PTE 0.68*
Aminoglycoside	Hg 0.35	Ni 0.35	Al 0.35	Al 0.34 Ni 0.43*		Ni 0.53	As 0.53 Fe 0.51			Be 0.60*		Al 0.52 Be 0.54
Beta-lactam		As 0.80 Cr 0.43*			Be 0.58*	Cr 0.82**		Cr 0.54		As 0.06* Cd 0.55		Cd 0.53
F/C/A		Cr 0.35				Cu 0.63*						
MLSB		As 0.41*	Ni 0.52**	Ni 0.35		Cu 0.55*				Be 0.70*	Ni 0.66*	
Multi-drug			Mn 0.40						Cd 0.54 Pb 0.50 Hg 0.52 Zn 0.54	As 0.53 Cd 0.67*		Cd 0.57*
Bactracin									Cr 0.5	Cr 0.7*	Ni 0.5	As 0.6* Cr 0.6*
Pyrazinamide			As 0.59**	As 0.54**	P 0.58*	P 0.76**	P 0.70*	P 0.71*		Ni 0.53	As 0.88**	As 0.83**
Triclosan				As 0.36						Cr 0.52 P 0.50	As 0.51	
Sulfonamide												
Tetracycline											Be 0.77**	Be 0.71*
Vancomycin		Be 0.36 Ni 0.36	Ni 0.53**	Ni 0.49**		Ni 0.56	Fe 0.53 Ni 0.52			Be 0.63*		As 0.64* Fe 0.60* Ni 0.54
Integron	Cd 0.36	Cd 0.46*	Cd 0.43* Ni 0.46*	Cd 0.50*					As 0.51 Cd 0.79** Pb 0.63* Zn 0.74**	Cd 0.79** Zn 0.51	Cd 0.60* Mn 0.51 Pb 0.61* Zn 0.73** Total PTE 0.67*	Cd 0.82** Total PTE 0.61*
Transposase	Cd 0.40* Pb 0.35 Hg 0.35	Cd 0.41*	Cd 0.56** Pb 0.37 Zn 0.46*	Cd 0.47*	Cu 0.58* Hg 0.57* Pb 0.54	Cu 0.73**	Cd 0.55	Cu 0.70** Pb 0.50	Cd 0.66* Pb 0.51 Zn 0.59*	Cd 0.73**	Cd 0.57 Zn 0.68* Total PTE 0.63*	Cd 0.75** Zn 0.66* Total PTE 0.58*

* Significance, $p < 0.05$
** Significance, $p < 0.01$

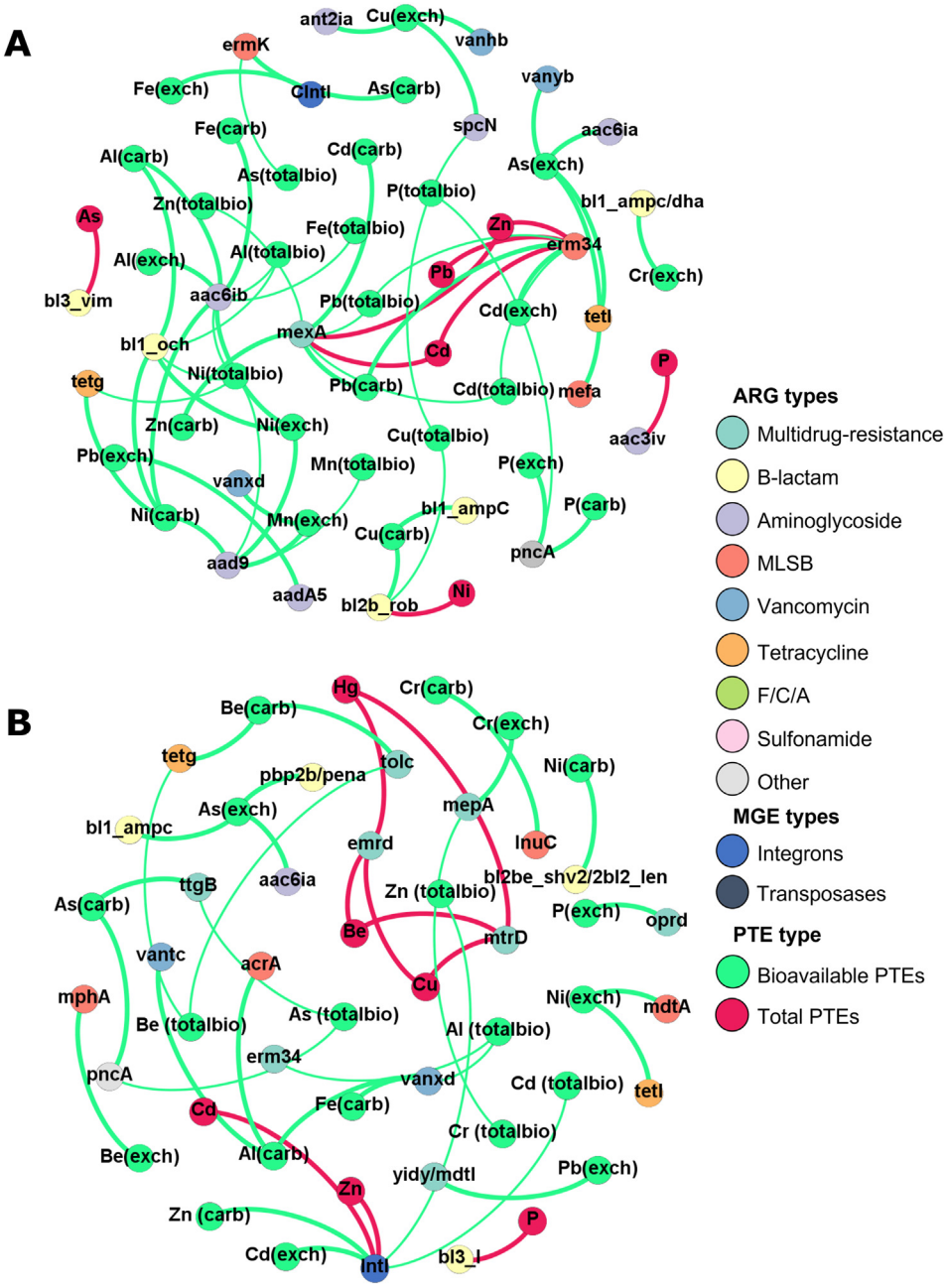

Fig. 5. Network analysis showing co-occurrence patterns between total PTEs, bioavailable PTEs (exchangeable (exch), carbonate-bound (carb) and exchangeable + carbonate-bound (totalbio)), antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) based on Spearman correlation analysis in rural sites (A) and urban sites (B).

Table 5
Frequency of significant ARG gene co-occurrences (links) with PTE (Fig. 5).

Rural			Urban		
PTE fraction	Links	Elements	PTE fraction	Links	Elements
Total	n = 1	As, Pb	Total	*n* = 2	Be, Cu, Hg
				n = 1	P
Bioavailability	*n* = 4	Ni	Bioavaiability	n = 2	Al, As, Be
	n = 2	Al, Cd, Cu, Pb		n = 1	Cd, Cr, Zn
	n = 1	As, Fe, P			
Exchangeable	n = 4	As	Exchangeable	*n* = 3	As
	n = 1	As, Cd, Cr, Fe, P, Zn		n = 1	Be, Cr, P, Pb
Carbonate	n = 1	Al, Cd, P, Pb	Carbonate	n = 3	Al
				n = 2	Al, Be
				n = 1	Fe, Ni

4. Discussion

4.1. PTE availability

The first hypothesis was confirmed in that quantifying the bioavailable fractions of PTE improve our predictions of soil ARG and MGE abundances, which was very apparent. When significant correlations existed for "total" and "bioavailable" fractions, the latter tended to have greater correlation coefficients. Further, the bioavailable forms of PTE identified more significant and positive correlations with ARGs, which total PTE measurements missed. Each bioavailable fraction had nearly twice the significant correlations ($p < 0.10$).

As previously mentioned, many AMR-related investigations have focused on total PTE (metals). Solely examining this measurement

unjustifiably assumes that each fraction's chemical nature and impact on the microbiome are similar (Vilar et al., 2005), which is unreasonable. The physicochemical character of the PTE affects its toxicity, and bioavailability represents the extent of microorganisms' exposure. This depends on its absorption in particles, surface-adsorption dynamics, precipitation and complexation (Arya et al., 2021). Fractions resulting from Tessier sequential extractions better consider these soil-PTE interactions (Tessier et al., 1979). The first two extractions reflect PTE availability in the upper soil horizons (from which these samples were taken), i.e. a) exchangeable fractions, which represent absorption-desorption dynamics depending on the ionic strength of the water, and b) carbonate binding, which is susceptible to changes in pH. The following two extractions can impact bioavailability, either over time or under certain environmental conditions, i.e., c) iron and manganese oxides that effectively bind trace metals but become unstable under anoxic conditions, and d) organically-bound PTE which is released following degradation. The fifth fraction (residual) represents elements structurally incorporated in the mineral matrix and not likely to become bioavailable. An aggressive acid (e.g., *aqua regia*) digestion likely overestimates microorganisms' exposure to PTE.

Knowing this information has implications for future studies investigating pollution impacts on antibiotic resistance, where quantifying bioavailable forms appears to be a better approach for surveillance (Arya et al., 2021). This makes sense because they are more likely to directly impact microorganisms (Amundsen et al., 1997; Bolan et al., 2013).

4.2. Differences between rural and urban landscapes

We also examined whether PTE affected ARGs in different landscapes differently. While there is an 80 % commonality in ARGs, differences emerged in relationships among ARGs, MGEs and bioavailable PTEs. Apparent differences between landscapes in terms of PTE best explained patterns of correlations. P and Cu were commonly associated with resistance in rural environments.

Phosphorus often represents the system's fertility, and previous work has found excessive nutrients as a possible driver of antibiotic resistance selection. While this could be in the form of natural fertilisers (e.g., biosolids; Zhu et al., 2019), the stimulation of microbial activity could also contribute. For example, in previous studies, P has been interpreted as a driver for ARGs in the High Arctic due to faecal contamination (McCann et al., 2019). Copper, although a micro-nutrient, has been commonly found to be associated with ARGs in soil systems at higher concentrations (e.g., Brandt et al., 2010; Knapp et al., 2017, 2011).

On the other hand, Cd, Pb, Zn, As, Be, and Ni were often associated here with ARGs in urban settings. Most of these elements can be linked with anthropogenic pollution, such as industrial or mining activity, and have contributed to MGE enrichment and increased relative ARG abundance (Gupta et al., 2022). Arsenic resistance has been found on the same plasmid as ARGs (Mukhopadhyay and Rosen, 2002; Pal et al., 2015). Significant and positive correlations with genes and PTEs such as Zn, Cr, Pb, Cd and Ni have been observed in previous studies (Knapp et al., 2011; Zhao et al., 2019). Interestingly, no other studies have reported correlations with Be; this warrants further investigation because Be is a toxic metal associated with industrial manufacturing (Taylor et al., 2003).

This study also observes improved correlations with ARGs and MGE when only the bioavailable PTE fraction was considered, although patterns differed slightly between the landscapes. In rural landscapes with lower PTE concentrations, increases in ARG abundances appeared to be primarily associated with the presence of MGEs. In urban landscapes, elevated PTE levels appeared to be more directly associated with ARG prevalence, possibly simply due to greater anthropogenic pollution. In addition, there were fewer correlations between MGEs and ARG abundance in urban sites. The direct toxicological response is observed, especially with PTE commonly associated with pollution (especially those without biochemical relevance).

Integrons, a common MGE platform, are well known for increasing under environmental pressure (Gillings et al., 2015). Along with integrons, transposons facilitate horizontal gene transfer by translocating genes between chromosomes and plasmids. Both contribute to microbial survival, especially to those responding to environmental stress. In cases where high MGE and PTE concentrations co-exist, the consequences could be highly elevated ARG, as seen in R7 and U5, which had the highest MGE and ARG abundance (U5).

Antibiotic resistance remains a severe problem and is frequently exacerbated by PTE contamination. However, investigating the speciation of PTEs and their role in spreading antibiotic resistance has not been previously considered. In addition, considerations in their relationships with MGE must evaluate how they were interconnected. Finally, this work inspires the need for further investigation into the potential for bioavailable metals in maintaining and exacerbating resistance through co-selection.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.157512.

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