AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology

Environmental Microbiology | Full-Length Text

Metagenome meta-analysis reveals an increase in the abundance of some multidrug efflux pumps and mobile genetic elements in chemically polluted environments

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ABSTRACT Many human activities contaminate terrestrial and aquatic environments with numerous chemical pollutants that not only directly alter the environment but also affect microbial communities in ways that are potentially concerning to human health, such as selecting for the spread of antibiotic-resistance genes (ARGs) through horizontal gene transfer. In the present study, metagenomes available in the public domain from polluted (with antibiotics, with petroleum, with metal mining, or with coal-mining effluents) and unpolluted terrestrial and aquatic environments were compared to examine whether pollution has influenced the abundance and composition of ARGs and mobile elements, with specific focus on IS26 and class 1 integrons (*intI*1). When aggregated together, polluted environments had a greater relative abundance of ARGs than unpolluted environments and a greater relative abundance of IS26 and *intI*1. In general, chemical pollution, notably with petroleum, was associated with an increase in the prevalence of ARGs linked to multidrug efflux pumps. Included in the suite of efflux pumps were *mexK*, *mexB*, *mexF*, and *mexW* that are polyspecific and whose substrate ranges include multiple classes of critically important antibiotics. Also, in some instances, β-lactam resistance (TEM181 and OXA-541) genes increased, and genes associated with rifampicin resistance (RNA polymerases subunits *rpoB* and *rpoB2*) decreased in relative abundance. This meta-analysis suggests that different types of chemical pollution can enrich populations that carry efflux pump systems associated with resistance to multiple classes of medically critical antibiotics.

IMPORTANCE The United Nations has identified chemical pollution as being one of the three greatest threats to environmental health, through which the evolution of antimicrobial resistance, a seminally important public health challenge, may be favored. While this is a very plausible outcome of continued chemical pollution, there is little evidence or research evaluating this risk. The objective of the present study was to examine existing metagenomes from chemically polluted environments and evaluate whether there is evidence that pollution increases the relative abundance of genes and mobile genetic elements that are associated with antibiotic resistance. The key finding is that for some types of pollution, particularly in environments exposed to petroleum, efflux pumps are enriched, and these efflux pumps can confer resistance to multiple classes of medically important antibiotics that are typically associated with *Pseudomonas* spp. or other Gram-negative bacteria. This finding makes clear the need for more investigation on the impact of chemical pollution on the environmental reservoir of ARGs and their association with mobile genetic elements that can contribute to horizontal gene transfer events.

KEYWORDS antimicrobial resistance, petroleum pollution, metal pollution, antibiotic pollution, coal mining pollution

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The authors declare no conflict of interest.

See the funding table on p. 13.

Received 23 June 2023
Accepted 17 July 2023
Published 20 September 2023

The United Nations has identified chemical pollution as being one of the three greatest threats to environmental health, along with climate change and the loss of biodiversity (1). It is becoming increasingly clear that environmental degradation is resulting in new emerging infections, and managing this problem must be done through an integrative environment health sciences lens (2). Toxic organic or inorganic chemicals can inhibit the activities of microorganisms that carry out crucially important ecosystem functions, eroding their critical role in shaping the natural environment (3, 4). Some types of chemical pollution may be associated with the evolution of human pathogens in directions that make them a greater health risk. For example, clinical isolates of *Pseudomonas aeruginosa* carry genomic islands and integrative conjugative elements that were likely acquired from co-existing bacteria in the environment (5), such as *P. aeruginosa* clone ST395. This strain has caused important hospital outbreaks in France and the United Kingdom (6) and possesses a genomic island (GI-7) carrying six genes conferring copper resistance that is identical to that carried by non-pathogenic *Pseudomonas* spp. isolated from copper-contaminated environments. Petitjean et al. (6) hypothesized that GI-7 was selected for in copper-polluted environments and acquired by clone ST395 through horizontal gene transfer (HGT), allowing it to survive in copper-based hospital water distribution networks. Another example of the consequences of the environmental application of chemicals to human health is the strong association of the use of triazole fungicides for crop protection and the development of fungicide resistance in clinical isolates of *Aspergillus fumigatus* (7).

Human impacts on the environment may be accelerating the pace of microbial evolution by, for example, increasing the frequency of HGT and decreasing the fidelity of DNA replication (8). Anthropogenic activities that increase the abundance of antibiotic-resistance genes (ARGs) in the environment or that promote their genetic association with mobile genetic elements (MGEs) will increase the likelihood that humans or food animals are exposed to ARGs through contaminated water or foodstuffs and thus increase the probability that ARGs will be acquired by pathogens of human or animal health concern (9, 10). Such activities include environmental contamination with fecal wastes from humans or food-producing animals, antibiotic residues in pharmaceutical manufacturing effluents, and antibiotics used in aquaculture or crop production (11). Furthermore, the global bacterial metapangenome carries an enormous reservoir of ARGs (the "resistome") and MGE (the "mobilome") (12, 13), underscoring the potential for diverse ARGs to move into and among human pathogens.

The rapid pace of antimicrobial resistance (AMR) development, and the consequent erosion of the ability to treat and resolve infections, is a seminal public health challenge (14). Mandated authorities and public health policymakers recognize that any effective strategy to mitigate the pace of AMR development must deploy measures across the One Health continuum, including the environment (15, 16).

Numerous types of MGEs can facilitate the exchange of ARGs between environmental bacteria (17). Insertion sequences (IS) are the simplest type of MGE, consisting of an element that carries only the genes necessary for transposition (17). Notably, the IS IS26 plays a major role in the dissemination of ARGs in a wide range of Gram-negative bacteria including pathogens of human or animal health concern (18). In Gram-negative bacteria, IS26 recruits ARGs into the mobilizable gene pool by forming transposons carrying numerous varied ARGs (19). For example, multidrug resistance plasmids carrying IS26 have been detected in *Escherichia coli* from swine, avian wildlife, retail food vegetables, and agricultural soil, indicating that IS26 is widely disseminated across the One Health continuum (20–23). Integrons are genetic elements capable of capturing a variety genes, including ARGs, as part of gene cassettes (24). Integrons lack the capability to move themselves, however, relying upon other MGEs for mobility (25). Class 1 integrons can carry genes conferring resistance to multiple antibiotics and are widely found in human pathogens but also non-pathogenic environmental bacteria (24, 26). Class 1 integrons have been proposed as an indicator of anthropogenic impacts on the environment as they are more abundant in polluted than unpolluted environments (27).

Fecal pollution entrains enteric microorganisms, antibiotics, and other potentially co-selective chemical residues into the environment (28–31). In fecally polluted environments, the observed increase in ARGs can be adequately explained by the introduction of enteric bacteria, rather than an impact of (co)-selecting chemicals on environmental bacteria (32). However, the potential impact of chemical pollutants on the environmental resistome or mobilome is difficult to discern due to the overlapping presence of genes carried by enteric bacteria.

In the present study, it was hypothesized that various types of chemical pollution may increase the relative abundance of ARGs in microbial communities, either by increasing the frequency of HGT, or by skewing populations to favor individuals that carry ARGs. To explore whether chemical pollution impacts the abundance and composition of MGEs and ARGs, a collection of publicly available metagenome studies were analyzed. The relative abundance of genes related to MGEs and ARGs was examined in metagenomes sequenced from soil, water, or sediment environments polluted with antibiotics, coal- or metal-mining effluents, or petroleum hydrocarbons. These were compared to metagenomes from unpolluted terrestrial or aquatic environments to determine if pollution increased the abundance of ARGs or MGEs and, if so, which ones.

RESULTS

Impact of chemical pollution on the environmental resistome

Overall, the relative abundance of ARGs was significantly higher in environments exposed to chemical pollution compared to unpolluted environments (Wilcoxon rank sum, $P = 0.000104$) (Fig. 1A). Parsing out the metagenomes according to the type of chemical pollution (antibiotic residues, coal-mining effluents, metal-mining effluents, or petroleum) revealed that those exposed to antibiotic residues (Wilcoxon rank sum, $P = 0.00000251$) had significantly higher relative abundances of ARGs compared to unpolluted environments (Fig. 1B). In contrast, environments exposed to metal-mining effluents (Wilcoxon rank sum, $P = 0.158$), coal-mining effluents (Wilcoxon rank sum, $P = 0.0198$), or petroleum (Wilcoxon rank sum, $P = 0.0856$) did not exhibit a significantly enriched relative abundance of ARGs (Fig. 1B).

The principal component analysis (PCA) indicated that the overall composition of ARGs from unpolluted environments differed from those impacted with antibiotic (PERMANOVA $P = 0.045$) and petroleum pollution (PERMANOVA $P = 0.035$), while no significant differences were found between unpolluted and metal or coal mining environments (PERMANOVA $P > 0.05$; Fig. 2).

A total of 94 ± 40 ARGs were detected in the unpolluted environments (Fig. 3), with most of them having mechanisms belonging to antibiotic efflux (46.43%), antibiotic target replacement/antibiotic target protection (35.58%), and antibiotic target protection (8.22%) classes as categorized by CARD (Fig. 4; Table S2). Considering drug resistance, 44% and 39% of the ARGs detected in unpolluted environments confer multidrug resistance and resistance to rifamycin antibiotics, respectively (Fig. 4; Table S2). The richness of ARGs was similar in environments exposed to petroleum residues and in unpolluted environments ($P = 0.72$; Fig. 3). However, the relative abundance of genes associated with efflux pumps (>70% of the total genes) was higher in the petroleum-polluted sites (Fig. 4; Table S2). In petroleum-polluted sites, the most abundant ARGs correspond to genes conferring resistance to multiple drugs (66.6%), β-lactams (13%), and rifamycin (7.2%), but for rifamycin resistance, these genes are in lower relative abundance compared to unpolluted environments (Fig. 4; Table S2). Environments exposed to antibiotic residues presented significantly higher ARGs richness (156 ± 45) than unpolluted environments (Fig. 3), mainly represented by genes conferring resistance to multiple drugs (51%), rifamycin (28.3%), aminoglycosides (7.3%), phenicol (3%), and sulfonamide (2.5%; Fig. 4; Table S2). Notably, aminoglycoside, sulfonamide, and phenicol resistance genes were higher in relative abundance compared to unpolluted environments.

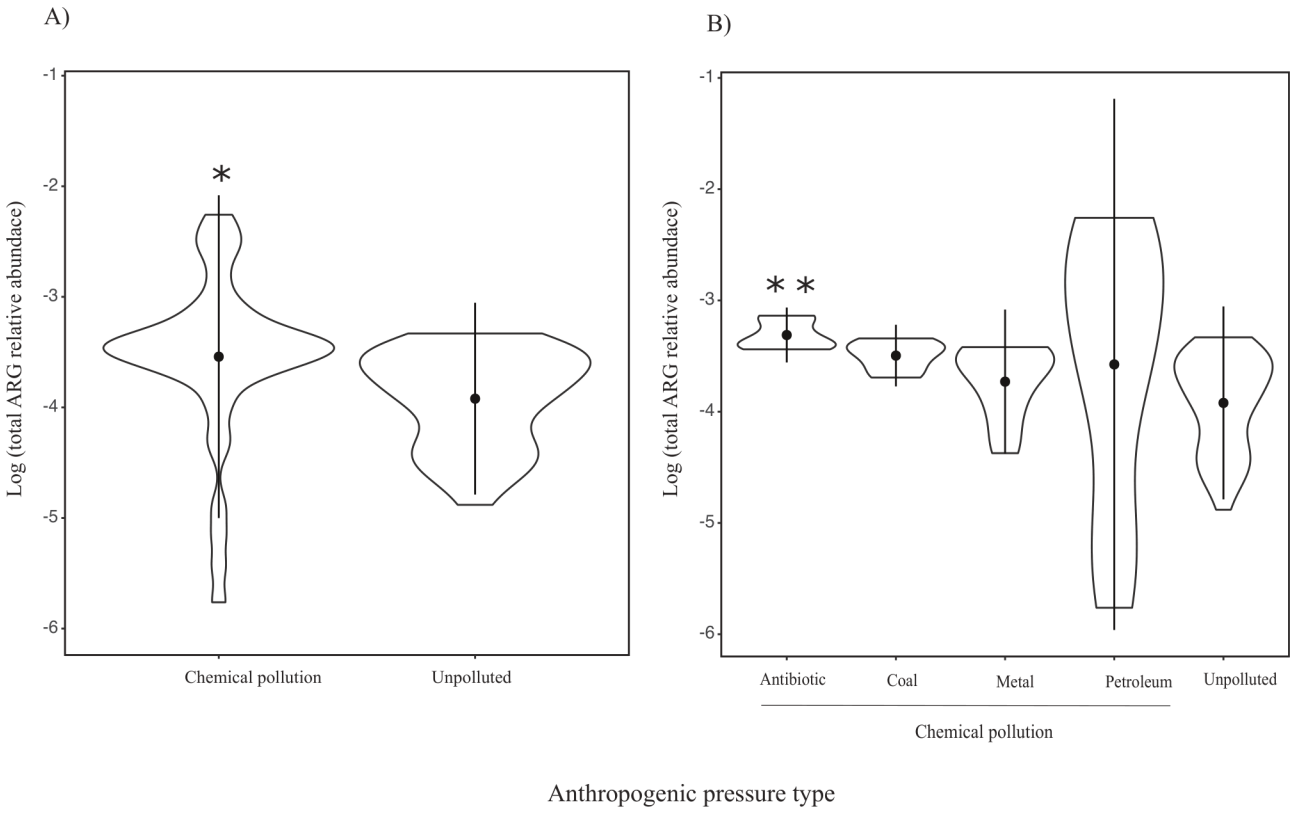

FIG 1 Distribution of the relative abundances of ARG sequences in environments exposed to (A) chemical pollution (samples from all types of pollution grouped together; *n* = 43) and (B) specific pollution types: antibiotic residues (*n* = 11), metal-mining effluent (*n* = 12), coal-mining effluent (*n* = 5), and petroleum (*n* = 15) compared to unpolluted environments (*n* = 39). The circle represents the geometric mean, and the vertical black line represents the SD. An asterisk (*) indicates a significant difference in abundance between unpolluted and polluted environments ($P < 0.05$). Two asterisks (**) indicate a significant difference in abundance between unpolluted and a specific type of chemically polluted environments with the alpha threshold for significance adjusted to $P < 0.0125$ to correct for multiple comparisons.

Simper analysis identified the top 20 genes that contributed the most to the differences between unpolluted environments and to each of the chemically polluted environments (Table 1). When grouped by mechanism, 15 of the top 20 genes were identified as efflux pumps and one as a regulator of efflux pump genes (Table 1). The remaining four genes are genes associated with antibiotic target alteration/antibiotic target replacement [the RNA polymerase (RNAP) genes *rpoB* and *rpoB2* which confer resistance to rifamycin] and with antibiotic inactivation (the TEM-181 and OXA-541 genes which are ß-lactamases). The *rpoB* and *rpoB2* genes were significantly lower, and the TEM-181 and OXA-541 genes were significantly higher in relative abundance in petroleum-impacted environments but not in any of the other polluted environments (Table 1). The 16 efflux pump genes confer resistance to a wide range of antibiotics including macrolide, sulfonamide, fluroquinolone, and aminoglycoside and to clinically relevant antibiotics such as carbapenems, cephalosporins, and tetracyclines (Table 1). Compared to unpolluted environments, the *mexK* gene was significantly enriched in environments contaminated with antibiotic residues and especially with petroleum (Table 1), whereas *mexB*, *mexF*, and *mexW* were enriched only in petroleum-contaminated environments (Table 1). The *muxB* gene that confers resistance to macrolide, monobactam, and tetracycline was enriched in metal- and coal-mining-polluted environments (Table 1).

Impact of chemical pollution on the relative abundance of *intI*1 and IS26

The relative abundances of *intI*1 (Wilcoxon rank sum, $P = 0.00105$) and IS26 (Wilcoxon rank sum, $P = 0.00150$) were significantly higher in chemically polluted environments

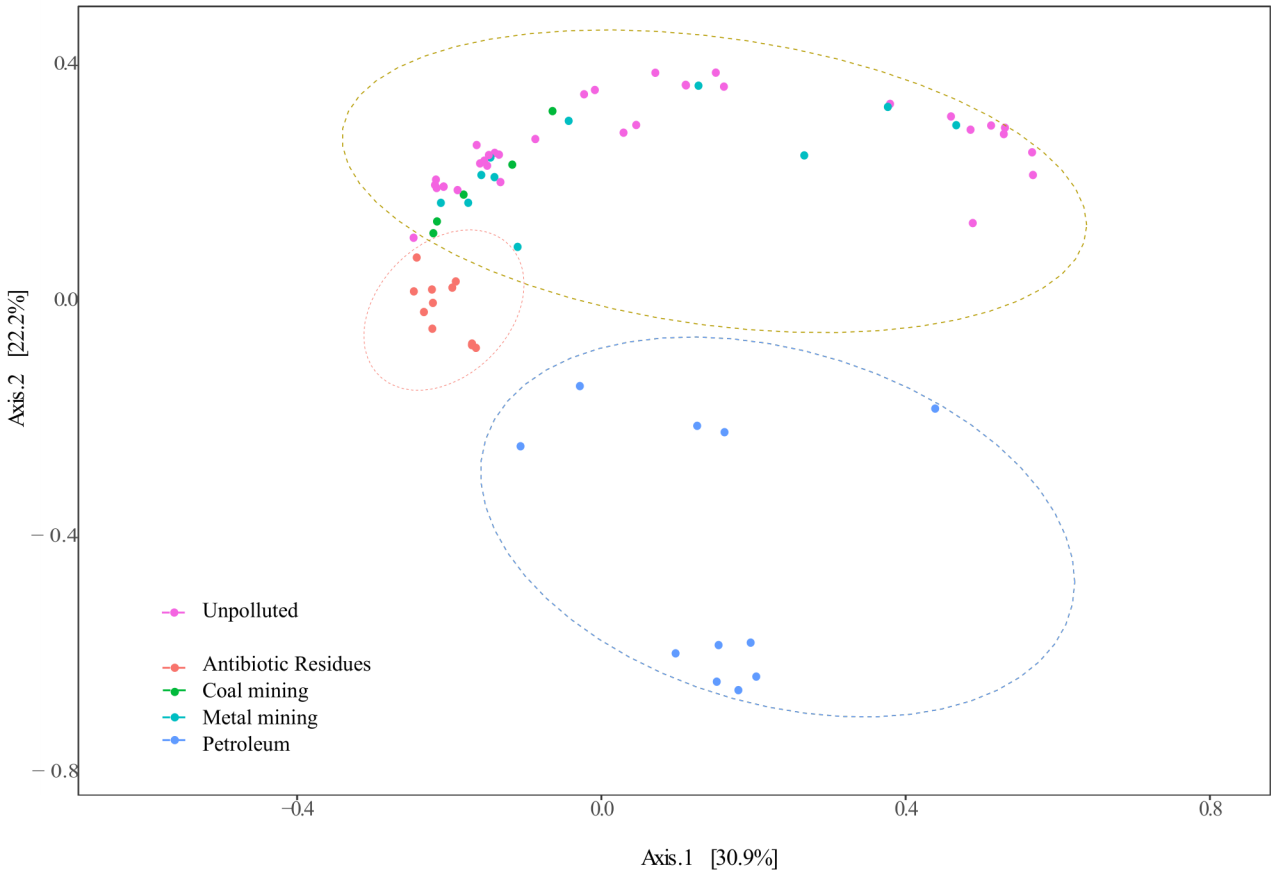

FIG 2 Principal component analysis (PCA) ordination plots to compare the composition of antibiotic resistance genes in unpolluted and chemically polluted environments based on CLR-transformed Aitchison distances. Percentages of total variance explained by each principal component (PC1 and PC2) are displayed in the axis titles. Dotted circles correspond to 95% confidence ellipses for each environmental group.

compared to unpolluted environments (Fig. 5A and B, respectively). Parsing out the metagenomes according to the type of chemical pollution revealed that antibiotic residue exposure significantly increased the relative abundance of both *intI*1 (Wilcoxon rank sum, P = 0.00532; Fig. 5C) and IS26 (Wilcoxon rank sum, P = 0.00342; Fig. 5D). Petroleum pollution significantly enriched the relative abundance of *intI*1 (Wilcoxon rank sum, P = 0.00863; Fig. 5C) but not IS26 (Wilcoxon rank sum, P = 0.0666; Fig. 5D). Exposure to metal-mining effluent or coal-mining effluents did not significantly increase the relative abundance of either *intI*1 (Wilcoxon rank sum, P = 0.186 and P = 0.171, respectively; Fig. 5C) or IS26 (Wilcoxon rank sum, P = 0.165 and P = 0.0291, respectively; Fig. 5D).

DISCUSSION

Results from the present study indicate that pollution of terrestrial and aquatic environments with various types of chemicals can increase the abundance of some ARGs and MGEs. In general, pollution increased the abundance of ARGs associated with multidrug efflux pumps and ß-lactam resistance (TEM181 and OXA-541) while genes associated with rifampicin resistance decreased (RNAP subunits *rpoB* and *rpoB*2). One possibility for the increase in these ARGs, especially efflux genes, is that these genes confer a fitness advantage in environments polluted with various chemicals, and the relative abundance of bacteria that carry them therefore increases. This is consistent with the composition of microbial communities being profoundly modified in environments impacted by unconventional oil and gas (fracking) wastewater (33), petroleum products

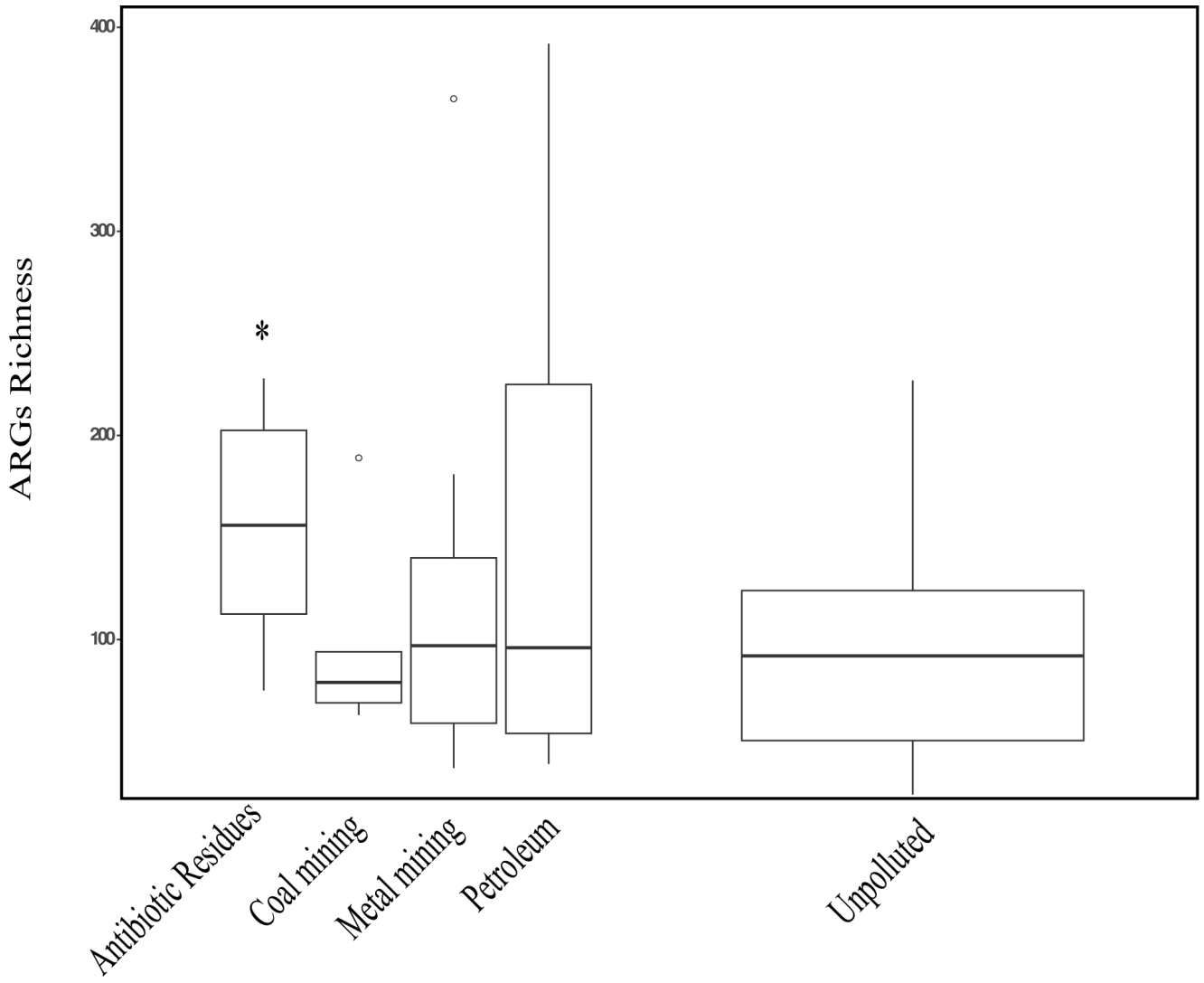

FIG 3 Distribution of the richness of ARG subtypes in unpolluted and chemically polluted metagenomes. Two asterisks (**) indicate a significant difference in abundance between unpolluted and a specific type of chemically polluted environment with the alpha threshold for significance adjusted to $P < 0.0125$ to correct for multiple comparisons.

(34–36), acid mine drainage (37), surface mining (38), and heavy metals such as copper, zinc, and nickel (39).

Exposure to petroleum in particular resulted in an increased number of unique ARGs encoding efflux pumps that confer resistance to various antibiotics (Table 1). Many organic solvents found in petroleum (e.g., toluene, *n*-hexane, xylene, and cyclohexane) are toxic to bacteria as a result of their ability to compromise the integrity of biological membranes (40). The toxicity of organic solvents increases with their lipophilicity (40). Intriguingly, all of the efflux genes whose abundance increased in petroleum-polluted sites encoded for several resistance-nodulation-division (RND) efflux systems found in *Pseudomonas* species, a genus known to be tolerant to organic solvents (40–42). These RND efflux systems included MexAB-OprM, MexEF-OprN, MexJK-OprM, and MexVW-OprM. In *P. aeruginosa*, the MexAB-OprM and MexEF-OprN efflux systems are major contributors to multidrug resistance in both lab and clinical isolates and also provide some level of tolerance to the organic solvents hexane and *p*-xylene (43). MexAB-OprM is a major contributor to intrinsic solvent tolerance in *P. aeruginosa*, while MexEF-OprN and MexCD-OprJ confer low-level tolerance to organic solvents (43). Intriguingly, *in vitro*

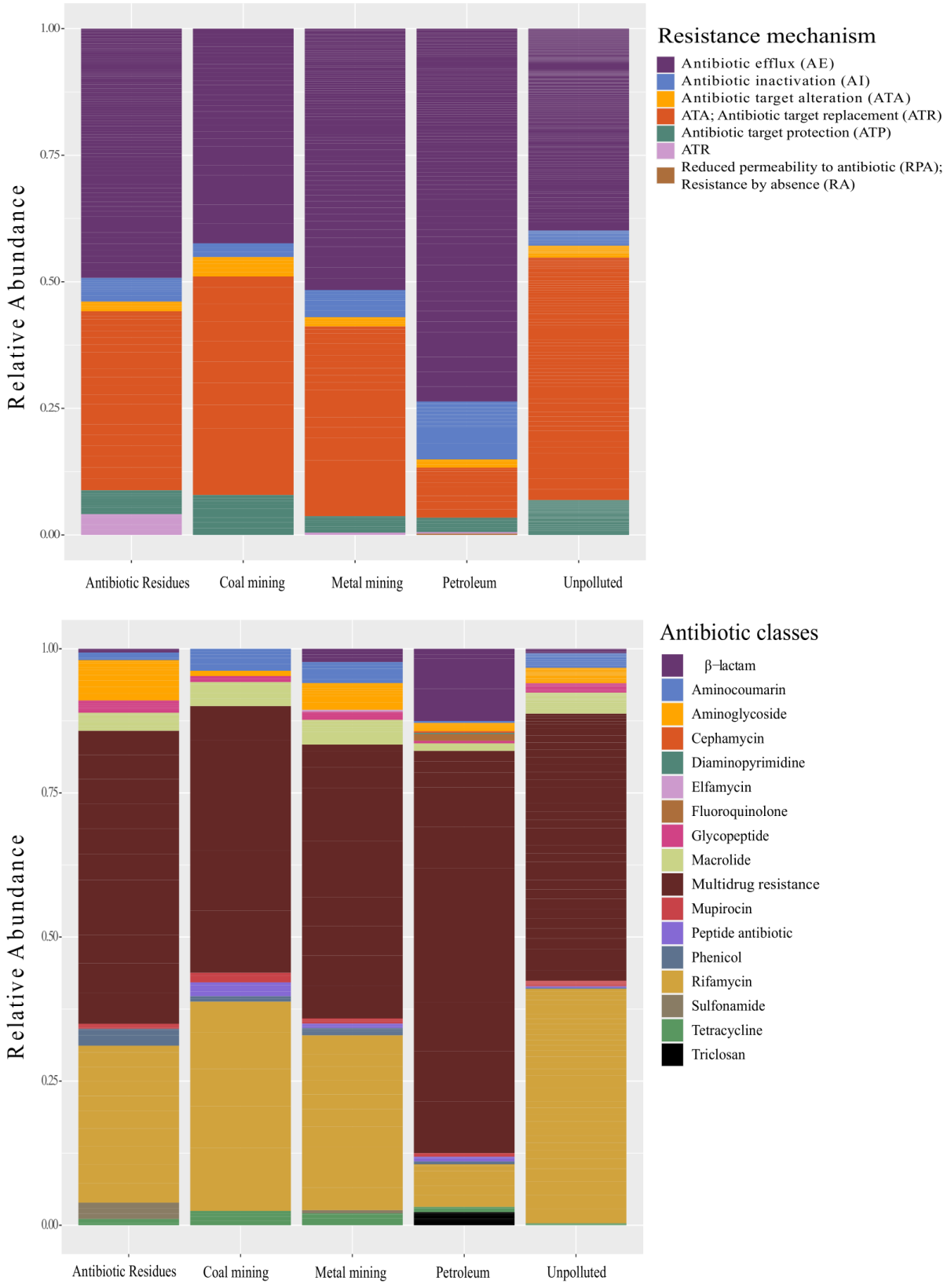

FIG 4 Composition of ARGs in unpolluted and chemically polluted metagenomes grouped by (A) resistance mechanism and (B) antibiotic class. Only categories with a relative abundance >0.02% were retained for the analysis. The lower and upper edges of each boxplot are the first and third quartiles, the midline shows the median, and the whiskers extend from the minimal and maximal values.

TABLE 1 Mean relative abundance (normalized to the number of filtered sequences in each metagenome) of the 20 genes that contributed the most to the differences between unpolluted environments and to each of the chemically polluted environments[a]

	Resistance mechanism	Antibiotic efflux															
	Gene family	Resistance-Nodulation-Division (RND)															
		REG	Efflux-pumps components														
	Gene	mexI	mexB	mexQ	mexF	mexD	mexK	mexW	muxB	muxC	smeE	axyY	smeB	ceoB	acrF	cmeB	axyX
Antibiotic residues	Mean relative abundance	4.00948E-06	6.50E-06	1.10E-05	3.68E-07	5.75E-06	**1.01E-05**	6.68E-06	1.83E-06	1.40E-05	8.15E-06	1.20E-05	5.66E-06	**2.32E-05**	0.00E + 00	0.00E + 00	7.74E-07
	SD	2.99549E-06	5.19E-06	1.37E-06	3.32E-06	4.71E-06	**8.45E-06**	5.04E-06	1.47E-05	1.09E-05	7.13E-06	1.10E-05	4.43E-06	**2.95E-05**	0.00E + 00	0.00E + 00	1.19E-06
Metal mining	Mean relative abundance	2.32889E-06	2.73E-06	3.26E-06	8.59E-07	2.73E-06	2.63E-06	3.38E-06	**2.29E-05**	1.61E-05	2.62E-06	3.76E-06	2.29E-06	4.30E-07	2.94E-07	0.00E + 00	0.00E + 00
	SD	1.71847E-06	3.26E-06	2.91E-06	1.10E-06	2.76E-06	3.89E-06	2.26E-06	**1.88E-05**	1.49E-05	3.43E-06	3.23E-06	2.09E-06	4.23E-07	3.92E-07	0.00E + 00	0.00E + 00
Coal mining	Mean relative abundance	6.5542E-06	4.92E-06	5.49E-06	1.45E-06	2.00E-06	0.00E + 00	5.00E-06	**3.66E-05**	1.80E-05	4.62E-06	1.15E-05	1.66E-06	3.30E-07	4.40E-07	6.13E-07	0.00E + 00
	SD	3.6045E-06	2.12E-06	3.80E-06	7.77E-07	1.64E-06	0.00E + 00	2.74E-06	**1.38E-05**	9.85E-06	2.78E-06	4.35E-06	1.68E-06	4.93E-07	6.43E-07	7.91E-07	0.00E + 00
Petroleum	Mean relative abundance	2.28322E-05	**1.30E-04**	1.38E-05	**1.84E-05**	2.96E-05	**1.30E-04**	**5.52E-05**	8.70E-06	3.28E-05	2.26E-05	1.97E-05	1.26E-05	2.30E-07	0.00E + 00	0.00E + 00	0.00E + 00
	SD	4.83422E-05	**1.92E-04**	4.47E-05	**4.23E-05**	5.74E-05	**1.86E-04**	**1.03E-04**	1.22E-04	5.65E-05	3.89E-05	2.98E-05	2.01E-05	2.93E-07	0.00E + 00	0.00E + 00	0.00E + 00
Unpolluted	Mean relative abundance	1.67519E-06	1.49E-06	2.98E-06	6.21E-07	1.36E-06	1.43E-06	2.29E-06	1.10E-06	7.69E-06	1.28E-06	2.02E-06	1.21E-06	1.04E-06	0.00E + 00	0.00E + 00	0.00E + 00
	SD	1.49882E-06	1.49E-06	2.77E-06	8.65E-07	1.66E-06	2.09E-06	2.16E-06	1.56E-05	1.09E-05	1.32E-06	2.11E-06	1.32E-06	7.28E-07	0.00E + 00	0.00E + 00	0.00E + 00

	Resistance mechanism	ATA & ATR		Antibiotic Inactivation	
	Gene family	Rifamycin-resistant*		ß-lactamases	
	Gene	rpoB2*	rpoB2*	blaTEM-181	blaOXA-541
Antibiotic residues	Mean relative abundance	7.37E-06	7.37E-06	2.27E-08	2.18E-06
	SD	1.23E-06	1.23E-06	4.23E-07	2.18E-06
Metal mining	Mean relative abundance	7.37E-06	7.37E-06	4.15E-07	1.37E-06
	SD	2.41E-06	2.41E-06	1.07E-06	3.22E-06
Coal mining	Mean relative abundance	5.32E-05	5.32E-05	3.14E-07	1.85E-06
	SD	1.95E-05	1.95E-05	1.07E-06	1.00E-06
Petroleum	Mean relative abundance	**4.94E-07**	**4.94E-07**	**8.54E-06**	**2.32E-05**
	SD	**2.90E-07**	**2.90E-07**	**1.96E-05**	**2.95E-05**
Unpolluted	Mean relative abundance	2.32E-05	2.90E-07	3.11E-07	4.69E-07
	SD	2.95E-05	2.90E-07	1.63E-06	1.77E-06

[a]Numbers in bold indicate significant differences in abundance between each type of chemically polluted environment and unpolluted environments (Bonferroni-adjusted, $P < 0.003$). REG: regulator of efflux genes; ATA/ATR: antibiotic target alteration/antibiotic target replacement.

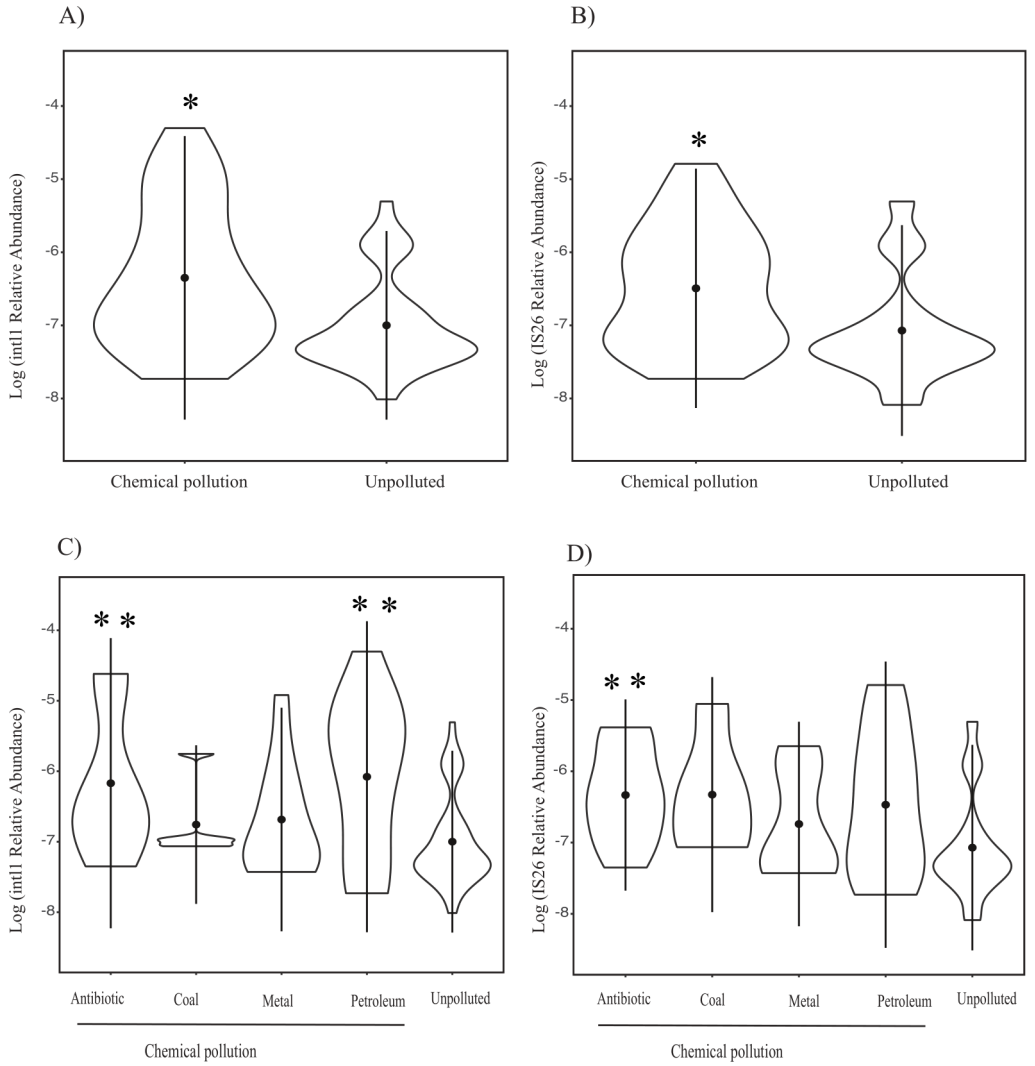

FIG 5 Distribution of the total relative abundances of (A–C) class 1 integrons (*intI1*) and (B–D) IS26 in (A and B) chemically polluted environments (n = 43) or (C and D) environments polluted by antibiotic residues (n = 11), metal-mining effluent (n = 12), coal-mining effluent (n = 5), or oil (n = 15) compared to unpolluted environments (n = 39). The circle represents the geometric mean, and the vertical black line represents the SD. An asterisk (*) indicates the significant difference in abundance between unpolluted and polluted environments ($P < 0.05$). Two asterisks (**) indicate a significant difference in abundance between unpolluted and a specific type of chemically polluted environment with the alpha threshold for significance adjusted to $P < 0.0125$ to correct for multiple comparisons.

exposure of *P. aeruginosa* to hexane was selected for *nalB*-type *P. aeruginosa* mutants overexpressing MexAB-OprM and thus exhibited a multidrug-resistant phenotype (44). Similarly, in *Pseudomonas putida* strain DOT-T1E, *MexB* (a homolog of the *MexB* transporter in *P. aeruginosa*) also contributes to high-level toluene tolerance (45). The gene *mexB* is essential for the growth of *P. putida* strain DOT-T1E in the presence of toluene (45). The biocidal chemical 2-hydroxybiphenyl is a major product of the bacterial desulfurization of dibenzothiophene, an important component of coal and crude oil (45). *Pseudomonas azelaica* strain HBP-1 is capable of completely mineralizing 2-hydroxybiphenyl (45). This bacterium uses the MexAB-OprM efflux system to adjust the internal concentration of 2-hydroxybiphenyl such that it is available for biodegradation but below the threshold of toxicity. Thus, it may be that enrichment of bacteria capable of metabolizing some of the toxic constituents of petroleum effluent may contribute to the increase in the abundance of genes encoding efflux pumps in the metagenome. While

the MexVW (46) and MexJK (47–49) efflux systems have been implicated in antibiotic resistance in *P. aeruginosa*, their role in organic solvent tolerance is currently unknown and warrants further research.

The *rpoB* and *rpoB2* genes were less abundant in petroleum-contaminated sites than in unpolluted sites. Mutations in *rpoB*, encoding the β subunit of the RNAP core enzyme, can facilitate adaptation to a variety of environmental and antibiotic stresses (50). Therefore, the identification of single nucleotide polymorphisms in the *rpoB* and *rpoB2* genes would be needed to determine if alleles confer an ARG phenotype in the contaminated sites under study. As a core gene that occurs in all genomes, we have no reasonable explanation for why the relative abundance of these genes would be lower in petroleum-polluted sites compared to unpolluted sites, or pollution with antibiotics, coal- or metal-mining effluents.

The *muxB* and *muxC* efflux genes were elevated in metal and coal mining-polluted environments. In *P. aeruginosa*, the gene *muxA* encodes for the membrane fusion protein component and functions with two RND components, MuxB and MuxC, and an outer membrane protein OpmB to form the MuxABC-OpmB efflux system (51). Plasmid-mediated overexpression of this system in *P. aeruginosa* strain YM64 led to elevated minimum inhibitory concentrations (MICs) of aztreonam, macrolides, novobiocin, and tetracycline (51). Mutational inactivation of this efflux system in *P. aeruginosa* strain PAO1 resulted in elevated levels of a ß-lactamase with increased resistance to ß-lactam antibiotics and decreased virulence in plants and insects (52). More recently, mutational inactivation of the MuxABC-OpmB efflux system in mutants deficient in other RND efflux systems was shown to specifically alter the susceptibility to the aminocoumarin antibiotic novobiocin (53). Intriguingly, MuxABC-OpmB was implicated in the secretion of the coumarin-containing siderophore pyoverdine, suggesting that it plays a role in iron acquisition (53). At present, its potential role in providing a fitness advantage in metal- or coal-mining environments is unknown.

With respect to MGEs, pollution with antibiotic residues or petroleum, but not metal or coal-mining effluent, was associated with an increase in the relative abundance of *intI*1. The *intI*1 result was anticipated since it has previously been observed to increase in abundance under anthropogenic pressure (27). IS26, however, was detectably enriched in environments polluted with antibiotic residues, but not other types of chemical pollution. IS26 is associated with plasmids and other MGEs and implicated in the widespread dispersal of ARGs (17, 54). Our results suggest that IS26 is a more important contributor to the dissemination of ARGs that have a role as fitness determinants in antibiotic-polluted environments compared to petroleum, metal, or coal-mining effluent polluted environments. Thus, in these polluted environments, MGEs that carry IS26 are less likely to expand into the bacterial populations. Overall, these results are in agreement with the notion that MGEs are dynamic in response to a diversity of anthropogenic pressures and not only exposure to antibiotics (55). Thus, more research specifically directed at understanding the impacts of various anthropogenic pressures on the dynamics and selective advantages of environmental MGEs, independent of the resistome, is called for.

Except for *bla*OXA-541 and *bla*TEM-181, ARGs that increased in relative abundance in chemically polluted metagenomes were not identified in the CARD database as functionally carrying out either target modification or modification of the antibiotic molecule. These general mechanisms of antibiotic resistance are widely used by pathogens to defeat many classes of critically important antibiotics (55). They are often associated with MGEs and amenable to HGT, likely much more so than efflux pumps that are generally chromosomally encoded (55). Although we did not detect an increase in these types of ARGs, the detection limit for shotgun metagenomics is notoriously poor, relative to qPCR for example, and the approach used in the present study is arguably not sensitive enough to detect variations in relative abundance that might still be significant from a risk point of view. Furthermore, the short-read sequences analyzed here do not reveal the genetic context of ARGs, and thus events of concern such as their accrual into

multidrug resistance plasmids. We emphasize therefore that our conclusions are limited to commenting on the impact of chemical pollution on the relative abundance of efflux pumps, but the dynamics of other ARGs with particular relevance to human or animal health may still be of concern.

A limitation of the retrospective approach used in the present study is that the polluted and unpolluted sites from which the metagenomes were obtained almost certainly vary in multiple aspects that were not captured by the associated metadata. These include, for example, variation in climate, physical and chemical properties of the aquatic or terrestrial environments sampled, vegetation and soil management in the case of terrestrial systems, history and extent of pollution, and so on. The toxicity of metals will vary with factors that determine their speciation. The toxicity of, and therefore selection pressures exerted by, antibiotics or components of petroleum will vary with factors that determine their sorption to organic or mineral components of soil or sediment and thus bioavailability. Likewise, there were variations in the methods for DNA extraction and sequencing and the bioinformatics tools employed in processing the sequences prior to database submission. Thus, the conclusions from this study must be challenged with prospective experiments. Most obviously by sampling chemically polluted environments alongside appropriate proximal unpolluted sites and comparing them using complementary methods that reveal the evolution of ARGs in the microbial communities, including shotgun metagenomics, transcriptomics, qPCR, or culturomics.

MATERIALS AND METHODS

Metagenome data collection

We identified metagenomes deposited in public databases that originated from unpolluted or polluted environments by searching publication databases. A combination of keywords was used to search for published articles in PubMed (conducted between May 2021 and November 2021) that reported metagenomic sequencing of unpolluted and chemically polluted samples from the environment, as well as fecally polluted samples that were used to validate analytical methods (Table S1A). The metagenomes published in these articles were filtered resulting in a total of 117 metagenomes that were selected for the present study. Criteria for inclusion in this study were as follows: sequenced using Illumina HiSeq technologies without PCR amplification and had over 100,000 sequencing reads so that differences in sequencing depth were insignificant in biasing the count of ARGs and MGEs based on sequencing effort.

The selected metagenomes were downloaded from the European Nucleotide Archive (ENA), which included metagenomes from soil, water, or sediment that were unpolluted (n = 39) or variously exposed to chemical (n = 43) contamination (Table S1B). The chemically polluted environments analyzed were variously exposed to antibiotic residues (n = 11), effluents from coal mining (n = 5), effluents from metal mining (n = 12), or petroleum (n = 15) but had no known exposure to fecal waste. Unpolluted metagenomes were obtained from samples that did not report any known exposure to fecal or chemical pollutants.

In addition, 35 fecal metagenomes were also analyzed as a positive control for the bioinformatic methods used in this study. These fecally polluted metagenomes were obtained from environments exposed to fecal waste from humans or farm animals, reclaimed wastewater, or wastewater treatment plant effluents. Using the analytical pipeline described below, ARGs and MGEs were more abundant in metagenomes from the fecally polluted environments relative to those from the unpolluted environments, as expected (32) (Fig. S1 and S2). On this basis, the methodologies employed in the present study were deemed suitable to investigate metagenomes from chemically polluted environments.

Metagenomic analysis

FastQC (version 0.11.9) was employed to assess the read quality, read length, total number of reads, and overrepresented sequences in each metagenome (56). Sequence reads were trimmed with Trimmomatic (version 0.39) in paired-end mode to remove adaptor sequences, reads below an average window quality of 20, and reads with a length below 90 base pairs (57). After trimming and quality filtering, the metagenomes comprised an average of 24 ± 21 million reads per sample.

Relative abundances of IS26 mobile elements and the class 1 integron-integrase gene (*intI*1)

The relative abundances of IS26 mobile elements and class 1 integrons from the metagenomes were determined by first identifying reads homologous to the IS26 transposase gene (*tnp26*) and the class 1 integron-integrase gene (*intI*1) using BLASTX implemented in the DIAMOND software (58). The *tnp26* gene is an appropriate marker because it is the only gene encoded on IS26 mobile elements, and it must be intact in order to catalyze IS26 movement (19, 59). One sequence of *tnp26* (Genbank ID: *QIQ11582.1*) was used as the database sequence to identify *tnp26* homologs from the metagenome reads. Other known IS26 sequences (three minor variants designated IS15Δ1, IS15Δ2, and IS26**) have >99% nucleotide identity to IS26 and >99% amino acid identity to *tnp26*, thus were not deemed necessary to be included in the BLASTX database (19). The *intI*1 gene sequence used in the BLASTX database was extracted from a custom non-redundant MGE database described in Pärnänen et al. (60) and available on Github (https://github.com/KatariinaParnanen/MobileGeneticElementDatabase).

A read was identified as IS26 or *intI*1 if the sequence similarity search implemented in DIAMOND (BLASTX with an E-value cut-off at 10^{-3}) had ≥90% amino acid identity and coverage over ≥90% of the length of the database sequence (58). This stringent threshold was used in order to minimize the number of false positives (61–63). The relative abundances of IS26 and *intI*1 were calculated by dividing the number of reads identified as IS26 or *intI*1 by the total number of filtered reads in each metagenome.

Relative abundances of ARGs

The filtered metagenome reads were analyzed for the presence of ARGs by comparison with the Comprehensive Antibiotic Resistance Database (CARD) database version 3.1.1 (64). Metagenome reads were mapped to the canonical reference database within CARD using Bowtie2 with default parameters (64, 65). The CARD canonical reference database contains over 1,600 known ARGs that have published experimental evidence of elevated MICs against antibiotics (64).

The relative abundance of ARGs in each metagenome was calculated by dividing the number of reads mapped to all ARGs or mapped to ARGs grouped by resistance mechanism, or mapped to ARGs grouped by antibiotic class, by the total number of filtered reads.

Statistics and data visualization

The Shapiro-Wilk test was used to assess the normality of data sets, which indicated that the distribution of ARGs, IS26, and *intI*1 reads was not normal. Therefore, the non-parametric Wilcoxon rank sum test was used to test whether differences in the log-transformed relative abundances of ARGs, *intI*1, and IS26 were significant between chemically polluted and unpolluted metagenomes, and fecally polluted and unpolluted metagenomes. The level of statistical significance was evaluated at $P < 0.05$. To test for differences in *intI*1, IS26, and ARG relative abundances between unpolluted metagenomes and metagenomes polluted specifically by antibiotic residues, coal-mining effluents, metal-mining effluents, and petroleum, non-parametric Wilcoxon rank

tests were also employed, but the alpha threshold for significance was adjusted to $P < 0.0125$ to correct for multiple comparisons.

To assess ß-diversity, a center log ratio (CLR) transformation of the ARG sequence abundances was used to obtain CLR-transformed relative abundances. Next, a PCA of Aitchison distances, i.e., Euclidean distances calculated using CLR-transformed relative abundances, was performed to investigate differences in the ARG composition between each type of chemically polluted environments (environments polluted with antibiotic residues, effluents from coal mining, and effluents from metal mining or petroleum) and unpolluted environmental metagenomes. A permutational multivariate analysis of variance (ANOVA) (PERMANOVA, using 999 permutations) was used to determine if the distances between metagenomes grouped by pollution type were statistically significant.

The analysis of similarity percentages [SIMPER (66)] was used to identify the top 20 ARGs contributing the most to the difference between unpolluted environments and each of the environments polluted with (i) antibiotic residues, (ii) coal-mining effluents, (iii) metal-mining effluents, or (iv) petroleum. Significant differences in the gene abundances between each group were determined using Dunn's multiple comparison test with Bonferroni correction. All statistics and visualizations used the base stats (v4.1.2) and vegan (v2.6–2) packages in R version 3.5.1 (R Development Core Team, 2015; http://www.R-project.org).

ACKNOWLEDGMENTS

Research funding was obtained competitively through Agriculture and Agri-Food Canada (AAFC) and from the Canadian Federal Genomics Research and Development Initiative on Antimicrobial Resistance (GRDI-AMR). J. Subirats was funded by Juan de la Cierva Formación and Beatriu de Pinós grants.

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FUNDING

Funder	Grant(s)	Author(s)
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ADDITIONAL FILES

The following material is available online.

Supplemental Material

Figure S1 (AEM01047-23-s0001.tif). Figure S1. (A) Distribution of total relative abundances of all ARGs in fecally polluted (n = 40) and unpolluted (n = 39) environments.
Figure S2 (AEM01047-23-s0002.tif). Figure S2. (A) Distribution of total relative abundances of (A) class 1 integrons (intI1), and (B) IS26 in fecally polluted and unpolluted environments.
Table S1 (AEM01047-23-s0003.docx). Table S1. (A) Keywords used to search for and identify metagenomes used in the present study. (B) Metagenomes analyzed in the present study.
Table S2 (AEM01047-23-s0004.xlsx). Relative abundance of resistance classes.

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