



Microbial community structure and antibiotic resistance genes in Dinaric karst groundwater under contrasting hydrological conditions

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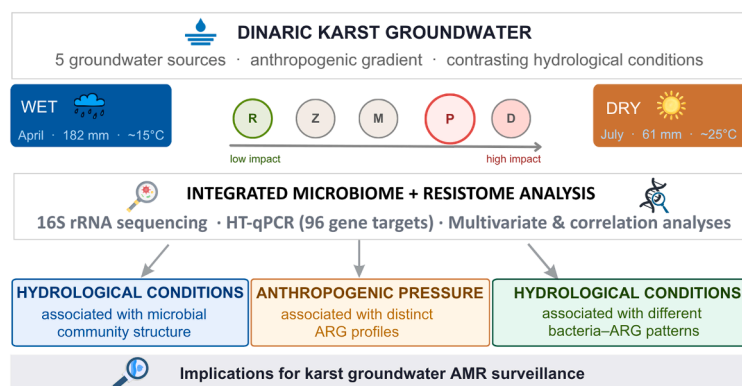
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HIGHLIGHTS

- Hydrological conditions were associated with microbial community differences.
- TOC was the strongest variable associated with microbial community composition.
- Anthropogenic influence increased ARG abundances, especially under wet conditions.
- *Escherichia/Shigella* and *Klebsiella* showed contrasting ARG associations.
- Vancomycin and colistin resistance genes occurred at low relative abundances.

GRAPHICAL ABSTRACT



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ABSTRACT

Karst aquifers are vulnerable drinking water resources, yet microbiome-resistome studies remain scarce. We investigated microbial community composition and antibiotic resistance gene (ARG) profiles in five karst groundwater sources along an anthropogenic gradient in coastal Croatia under contrasting hydrological conditions. 16S rRNA amplicon sequencing showed that hydrological conditions were associated with community composition. Distance-based redundancy analysis identified total organic carbon as the variable associated with community structure, while ammonium and *Escherichia coli* were associated with dry hydrological conditions. High-throughput qPCR (96 gene targets) revealed distinct resistome profiles across sites and conditions. Sites with greater anthropogenic influence showed higher ARG abundances than the reference site, particularly under wet hydrological conditions. Efflux pump genes were prevalent across sites and conditions, while aminoglycoside and trimethoprim ARGs were particularly prominent at the most impacted site under dry conditions. *Escherichia/Shigella* and *Klebsiella* showed the broadest ranges of significant ARG correlations under wet and dry hydrological conditions, respectively, although several associations were sensitive to individual sampling locations. Clinically relevant ARGs, including vancomycin and colistin resistance genes, were detected at low relative abundances.

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These findings demonstrate associations between hydrological conditions, anthropogenic influence, and karst groundwater microbiomes and resistomes, highlighting the potential of molecular AMR surveillance in drinking water sources.

1. Introduction

Groundwater accounts for 97% of accessible freshwater worldwide [1] with Dinaric karst aquifers representing a major drinking water source in the Mediterranean region [2]. Karst systems are characterised by high permeability, rapid infiltration of precipitation through conduit networks, and thin soil cover, which limit natural filtration and make groundwater flow dynamics highly responsive to hydrological conditions [3–5]. These properties facilitate the transport and persistence of agricultural runoff, stormwater, and sewage leakage into groundwater, potentially altering its physicochemical stability and microbial composition [5,6].

Antimicrobial resistance (AMR) is recognized as a major global public health threat, with groundwater increasingly considered as an environmental reservoir and potential transmission pathway [7,8]. Surface runoff and infiltration can introduce antibiotics, antibiotic-resistant bacteria (ARB), and antibiotic resistance genes (ARGs) into groundwater systems [4,9]. Climate change may further exacerbate this issue by altering hydrological regimes through increased precipitation variability, flooding, rising temperatures, and drought, thereby influencing microbial community structure and potentially favouring the persistence of ARGs and pathogenic bacteria [10,11]. This is particularly relevant given that karst groundwater supplies up to 50% of drinking water in some European regions [12].

Despite its importance, AMR monitoring in groundwater remains largely absent from regulatory frameworks. While the European Groundwater Directive 2006/118/EC [13] sets quality standards for nitrates and pesticides, with emerging attention to PFAS and pharmaceutical residues [14], no established framework currently addresses AMR in groundwater. Microbiological parameters are monitored only in groundwater used for drinking water under the European Drinking Water Directive 2020/2184 [15], highlighting a significant regulatory gap from a One Health perspective [16,17].

Advances in molecular tools have improved our ability to characterise microbial communities and resistomes in low-biomass environments such as karst groundwater. 16S rRNA amplicon sequencing enables comprehensive profiling of bacterial community composition and temporal dynamics [18], while high-throughput quantitative PCR (HT-qPCR) provides sensitive and cost-effective quantification of ARGs across large gene panels [19]. Together, these approaches provide an integrated framework for environmental AMR surveillance.

Although AMR has been widely studied in wastewater [20,21], rivers [22], and drinking water systems [23], knowledge of microbial diversity and ARG profiles in karst groundwater remains limited. Recent studies have begun to address this gap using molecular and culture-based approaches in rural karst springs [24], karst rivers [25] and urban karst groundwater [4], yet integrated microbiome-resistome studies across contrasting hydrological conditions in Dinaric karst groundwater are still lacking. To our knowledge, this is the first study to combine 16S rRNA amplicon sequencing with HT-qPCR resistome profiling in Dinaric karst groundwater, providing an assessment of how contrasting hydrological conditions are associated with variation in microbial community composition and resistome profiles in this hydrogeological unique system. Furthermore, this study provides new insights into the occurrence of clinically relevant resistance determinants, including carbapenemase genes and vancomycin and colistin resistance determinants, across karst groundwater sources used for drinking water supply and spanning different levels of anthropogenic influence. We hypothesized that variation in hydrological conditions and anthropogenic pressure contributes to differences in microbial community composition and resistome

profiles in karst groundwater. To address this knowledge gap, we investigated five karst groundwater sources used for drinking water supply with varying anthropogenic influence, sampled under contrasting hydrological conditions, to (i) characterise microbial community composition and resistome profiles; (ii) identify physicochemical predictors associated with community and resistome variation; and (iii) assess site-specific patterns and patterns associated with contrasting hydrological conditions.

2. Materials and methods

2.1. Sample collection

The study area is located in a Dinaric karst aquifer system in the Kvarner area, coastal Croatia. Groundwater samples were collected from five groundwater sources representing karst springs and wells with varying degrees of anthropogenic influence, ranging from forested catchments to urban and peri-urban areas (sites R, Z, M, P and D). The main characteristics of the sampling sites are summarised in Table 1. Sampling campaigns were conducted at two time points selected based on real-time hydrological monitoring of the study area from January to July 2025 to represent contrasting hydrological conditions: April 2025 (182.3 mm cumulative rainfall and mean air temperature ~15°C in the 10 days prior to sampling) and July 2025 (61.3 mm cumulative rainfall and mean air temperature ~25°C in the 10 days prior to sampling). Sampling was conducted under relative stable hydrological conditions in both periods, several days after the preceding rainfall events, to capture the integrated hydrological signal rather than the immediate flush response to individual precipitation events. At each sampling site, triplicate samples were collected, each consisting of 10 L for DNA extraction and 2.5 L for microbiological analyses, transported in a cooler in sterile canisters and processed in the laboratory within 12 h of collection. For molecular analyses, each 10 L sample was filtered through 0.22 µm PES membrane filters (Merck Millipore, Ireland). Filters were stored at –20°C until DNA extraction.

2.2. Physicochemical and microbiological analyses

Physicochemical properties of all groundwater samples were

Table 1
Characteristics of the five karst groundwater sampling sites [26–28].

Site	Type	Catchment characteristics	Land use	Anthropogenic influence
R	Ascending overflow karst spring	Forested, mountainous catchment	Forest/natural	Low – reference site
Z	Permanent karst spring	Karst catchment, urban fringe, near river	Mixed urban/natural	Low – moderate
M	Drilled well system (8 wells)	Peri-urban catchment, agricultural plain recharge	Mixed urban/agricultural	Moderate – peri-urban, occasional saltwater intrusion ²
P	Karst spring with vertical shaft and horizontal gallery	Coastal catchment, coastal bay	Urban/coastal	High – occasional saltwater intrusion
D	Karst spring with drainage trench	Coastal catchment, coastal bay	Urban/coastal	High – occasional saltwater intrusion

determined using internationally validated ISO methods, as detailed in [Table S1](#). Parameters included indicators of organic matter content (total organic carbon (TOC) and permanganate index), nutrients (nitrates, ammonium, phosphates), major ions (calcium, magnesium, sodium, potassium, chlorides, sulphates, fluorides, and hydrogen carbonate), and trace metals (iron, manganese, zinc, copper, and aluminium). Microbiological analyses included enumeration of *Escherichia coli* by membrane filtration according to ISO 9308-2:2014 (CFU/100 mL).

2.3. DNA extraction

Total DNA was extracted from filters using the DNeasy PowerWater Kit (Qiagen, Germany) with a modified pretreatment protocol [29]. Mechanical lysis was performed using a Bead Ruptor 4 homogenizer (Omni International, USA) at setting 5 for 40 s. DNA purity and quantity were assessed using NanoDrop spectrophotometer (BioSpec Nano, Shimadzu, Japan), and Qubit Fluorometer 3.0 (Thermo Fisher Scientific, USA), respectively. Additionally, procedural blanks, prepared by processing clean, unused filters through the complete DNA extraction protocols, were included as negative extraction controls for both the 16S rRNA amplicon sequencing and HT-qPCR analyses. All samples were stored at -20°C until further analysis.

2.4. Microbial community analysis

Extracted DNA from groundwater samples was used for microbial community analysis by 16S rRNA amplicon sequencing, targeting the V3-V4 hypervariable region. Library preparation and sequencing were performed by Novogene (Munich, Germany) on an Illumina NovaSeq 6000 platform. Raw sequence reads were processed using the QIIME2 software package (release 2024.10). As forward and reverse reads could not be merged due to insufficient overlap, only forward reads were retained for downstream analysis. Quality filtering and denoising were performed using the DADA2 plugin, including declustering and chimera removal. Amplicon sequence variants (ASVs) occurring as singletons or present in only one sample were excluded from further analysis. Taxonomic assignment of remaining ASVs was performed using a naïve Bayes classifier trained on V3-V4 16S rRNA sequences from the SILVA v138 database. An additional filtering step removed chloroplast, mitochondria and unclassified bacterial sequences from the dataset. A phylogenetic tree was constructed using the phylogeny plugin within QIIME2.

Raw sequence reads were deposited in the European Nucleotide Archive (ENA) under accession number PRJEB113026.

2.5. Resistome analysis

The resistome of groundwater samples was characterised by HT-qPCR using the SmartChip qPCR system (Takara Bio, Mountain View, CA, USA), performed by Resistomap Oy (Helsinki, Finland). A total of 96 gene targets were quantified, comprising 80 ARGs selected to cover a broad range of clinically and environmentally relevant resistance determinants as part of a One Health AMR surveillance panel, 8 mobile genetic elements (MGEs) commonly associated with horizontal gene transfer, 5 microbial source tracking (MST) markers, 2 taxonomic markers (*uidA* for *E. coli* and 23S rRNA for *Enterococcus*), and the 16S rRNA gene for normalisation of total bacterial load. The complete list of gene targets is provided in [Table S2](#).

Each DNA sample was diluted to 10 ng/ μL . The SmartChip platform performs 5,184 reactions of 100 nL per run; thermal cycling conditions are provided in [Table S2](#). Each sample was analysed in technical triplicates; a gene was considered detected if a signal was obtained in at least two of three replicates. A Ct value of 28 was used as the cutoff for reliable detection. Amplification efficiency ranged from 1.7 to 2.3. Undetected values (Ct $\geq$ 28 or signals obtained in fewer than 2 of 3 technical replicates) were treated as zero for statistical analyses. This

represents non-detection under the applied analytical criteria rather than confirmed absence of the target. Reactions with amplification efficiency outside the predefined accepted range (2 ± 0.3) were excluded from further analysis. A no template control consisting of molecular-grade water was included in each HT-qPCR run. Relative abundance of each target gene was calculated by normalisation to the 16S rRNA gene using the $2^{-\Delta\text{Ct}}$ method ($\Delta\text{Ct} = \text{Ct target gene} - \text{Ct 16S rRNA gene}$) [30]. Detection frequencies (n/30), median Ct values, and Ct ranges among positive samples for all gene targets are provided in [Supplementary Table S2](#).

2.6. Statistical analysis

All statistical analyses and visualisations were performed in R software. Microbial community analyses were conducted in R v4.5.2, and resistome analyses in R v4.4.2, using tidyverse package [31]. Sequencing depth was assessed by rarefaction curve analysis prior to diversity analyses. Two samples (D4_1 and P7_1) did not meet the minimum read count threshold following DADA2 filtering and were excluded from further analysis. A subsampling threshold of 52,546 reads was applied, retaining 28 samples for diversity analysis. As a result, two groups (site D under wet conditions and site P under dry conditions) were represented by two replicates, while all remaining groups retained three replicates. To assess the potential impact of excluding the two low-quality samples, a sensitivity analysis was performed by repeating the alpha diversity and PERMANOVA analyses with D4_1 and P7_1 retained. Inclusion of these samples resulted in a PERMANOVA R^2 of 0.49 ($p = 0.001$), compared with $R^2 = 0.61$ ($p = 0.001$) for the quality-filtered dataset. Chao1 remained significantly different between hydrological conditions, whereas Shannon diversity remained non-significant.

Alpha diversity indices (Chao1 and Shannon) were calculated using the phyloseq package [32]. Beta diversity was assessed using Bray-Curtis dissimilarity and visualised by Principal Coordinates Analysis (PCoA) using the vegan package [33]. Differences in community composition between groups were tested using PERMANOVA (999 permutations). To assess the potential influence of differences in within-group dispersion on PERMANOVA results, multivariate dispersion was evaluated using the betadisper function in the vegan package, with significance assessed by permutation testing. Distance-based redundancy analysis (db-RDA) was performed using the vegan package to assess the relationship between microbial community composition and physicochemical parameters. Environmental variables included in the db-RDA represented the subset of physicochemical parameters consistently measured across all sampling sites; groundwater temperature and dissolved oxygen were not included because these parameters were not available for all sampling locations. Collinearity among environmental variables was assessed using Pearson correlation analysis ([Table S3](#)); variables with $r > 0.7$ were excluded, retaining the more ecologically relevant variable. Variables with a variance inflation factor (VIF) > 10 were additionally excluded. Forward selection of significant environmental variables was performed using the ordstep() function (999 permutations), retaining only variables with $p \leq 0.05$. Final model fit was assessed by adjusted R^2 and VIF for all retained variables.

Resistome data from HT-qPCR, normalised to the 16S rRNA gene as described in [Section 2.5](#), were used for all resistome statistical analyses. Fold-change analysis compared the relative abundance of selected targets (*E. coli*, universal Bacteroidales marker, *int1*, and total ARGs) at each site against with that at the reference site R for each sampled hydrological condition (R4 for wet season comparisons and R7 for dry season comparisons). Fold changes were calculated as $\log_2(\text{mean relative abundance at site X} / \text{mean relative abundance at site R})$, with positive values indicating higher and negative values indicating lower relative abundance than at the reference site. Spearman rank correlation analysis was performed to assess associations between ARG relative abundances and the relative abundances of nine bacterial genera

previously reported to harbour ARGs, with analyses conducted separately for wet and dry hydrological conditions. Only correlations with adjusted $p \leq 0.05$ (Benjamini-Hochberg correction) were considered significant. The robustness of these correlations to the influence of individual sampling locations was further assessed using a leave-one-site-out sensitivity analysis, performed separately for wet and dry hydrological conditions. In each iteration, one sampling location was excluded and the Spearman correlation analysis was repeated. Full results of the sensitivity analysis are provided in Table S8.

3. Results

3.1. Microbial community composition

A total of 1,970,545 raw reads were recovered from 30 groundwater samples. After quality filtering and denoising with DADA2, 1,686,691 reads were retained, with a mean of $59,786 \pm 3,805$ reads per sample (Table S4). Two samples (D4_1 and P7_1) were excluded due to insufficient read counts, as described in Section 2.6. A total of 1,490 ASVs were identified across the retained samples. Rarefaction curve analysis confirmed sufficient sequencing depth for 28 of the 30 samples, with curves reaching a plateau prior to subsampling (Fig. S1).

3.1.1. Alpha and beta diversity

Estimated species richness (Chao1) was significantly lower under wet than dry hydrological conditions (wet: 67.4 ± 14.8 ; dry: 101.0 ± 28.6 ; $p < 0.05$; Fig. 1A; Table S5). Shannon diversity index did not differ significantly between wet and dry hydrological conditions (dry: 3.94 ± 0.6 ; wet: 3.68 ± 0.5 ; Fig. 1B), although a trend towards higher values under dry hydrological conditions was observed.

PCoA based on Bray-Curtis dissimilarity showed differences in sample distribution between hydrological conditions (PERMANOVA: $R^2 = 0.61$, $p = 0.001$; Fig. 1C). Samples under dry hydrological conditions formed tighter clusters than those under wet hydrological conditions, which showed greater dispersion. PERMDISP confirmed significant differences in within-group dispersion between the sampled hydrological conditions ($F = 5.21$, $p = 0.031$), with greater dispersion under wet hydrological conditions (Fig. 1C). Samples from site P under wet hydrological conditions clustered in proximity to those under dry hydrological conditions.

3.1.2. Environmental factors associated with bacterial community composition

The db-RDA explained 47.8% of total community variation across two constrained axes (dbRDA1 = 30.7%, dbRDA2 = 17.1%; Fig. 2; Table S3). Along dbRDA1, TOC was the variable most strongly associated with microbial community composition, primarily with wet season samples from sites Z and R, where TOC concentrations were highest (0.76 and 0.82 mg/L C, respectively; Table S1). Site P in the wet season was a notable exception, showing the weakest association with TOC and the strongest associations with copper and conductivity among all wet season samples.

Along dbRDA2, two distinct groups of parameters were identified. Ammonium and *E. coli* were co-directional and associated with dry hydrological conditions at sites Z, M and R, while nitrates and the permanganate index were primarily associated with wet hydrological conditions at sites D and M. Copper and conductivity were primarily associated with site P under wet hydrological conditions, whereas site R under dry hydrological conditions showed the weakest associations with organic matter indicators (TOC and permanganate index).

3.2. Bacterial community composition

At the phylum level, *Pseudomonadota* and *Bacillota* were detected across all five sampling sites under both sampled hydrological conditions (Fig. 3A). *Pseudomonadota* was the dominant phylum in all samples, with relative abundances ranging from 52.5% to 99.5%. Under dry hydrological conditions, the relative abundance of *Bacillota* was higher across all sites, ranging from 3.7% to 36.3%, with the highest values observed at sites M, Z and R. *Bacteroidota* was detected at notable relative abundance only under wet hydrological conditions at site P. Additional phyla were present at relative abundances below 0.15%.

At the family level, *Enterobacteriaceae* was the dominant family across all samples, with relative abundances ranging from 36.0% to 70.1% (Fig. 3B). Under wet hydrological conditions, *Flavobacteriaceae* reached relative abundances of 31.9%–34.8% at site P, while remaining below 1.5% at all other sites. Unclassified *Enterobacterales* represented a substantial proportion at sites R and Z (mean relative abundances of 50.5% and 27.2%, respectively). *Aeromonadaceae*, *Bacillaceae*, *Enterococcaceae* and *Morganellaceae* showed higher relative abundances under dry hydrological conditions.

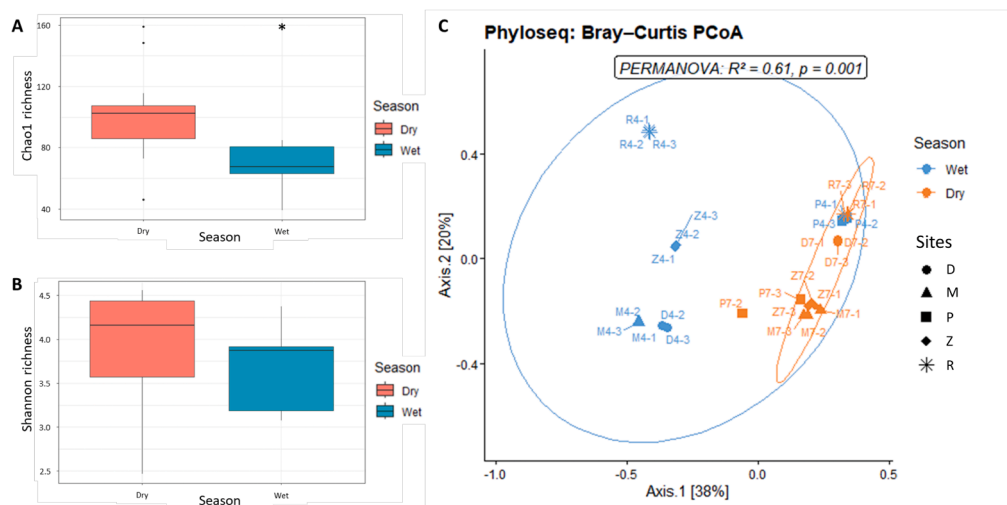


Fig. 1. Alpha and beta diversity of groundwater microbial communities across five sampling sites under contrasting wet and dry season hydrological conditions. (A) Chao1 species richness and (B) Shannon diversity index. Asterisk indicates significant difference between the sampled wet and dry season conditions ($p < 0.05$). (C) PCoA based on Bray-Curtis dissimilarity. Colours indicate sampling season and shapes indicate site.

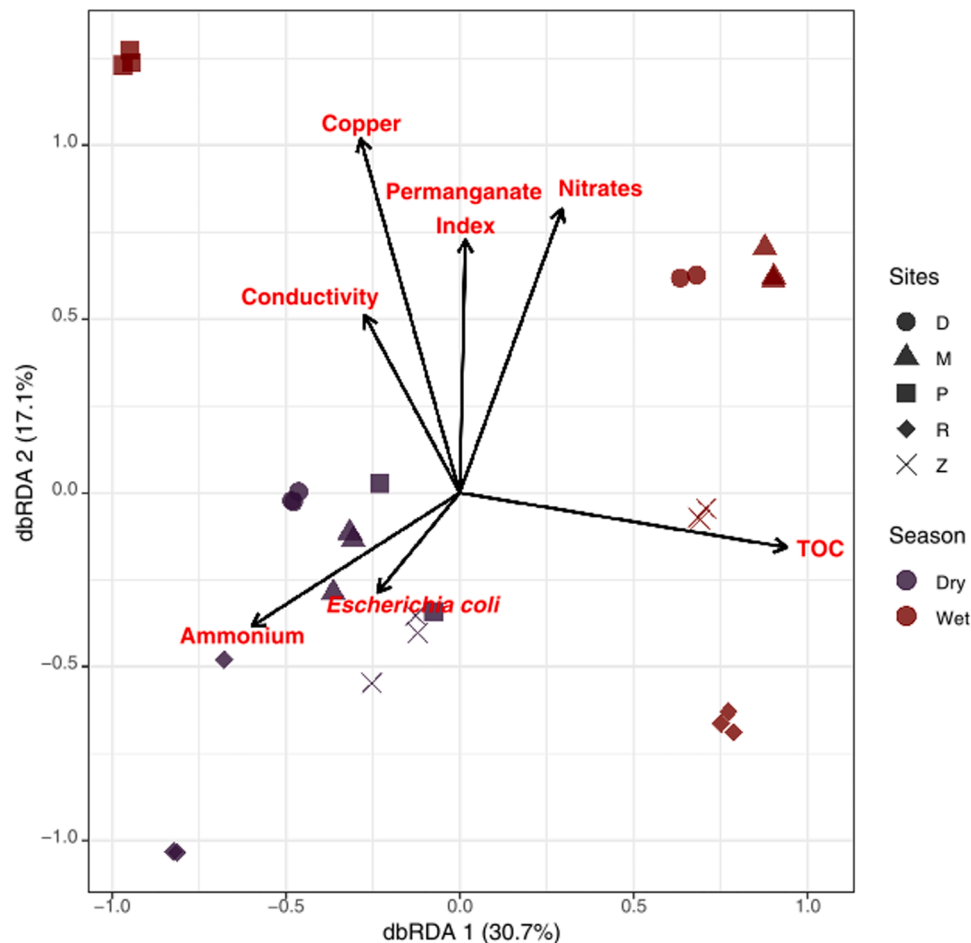


Fig. 2. Distance-based redundancy analysis (db-RDA) of bacterial community composition across five sampling sites and two sampling periods representing contrasting wet and dry season hydrological conditions. Arrows represent significant environmental parameters ($p < 0.05$). Shapes indicate sampling site and colours indicate season.

At the genus level, three bacterial genera were detected in all samples under both sampled hydrological conditions: *Enterococcus*, *Escherichia/Shigella*, and *Morganella* (Fig. 3C; Table S6). *Klebsiella* was predominant under wet hydrological conditions at sites D and M (54.0%–59.0%), while remaining undetected at site R and at site D under dry hydrological conditions. Overall, *Klebsiella* showed lower relative abundances under dry hydrological conditions, except at site P. *Escherichia/Shigella* showed higher relative abundances under dry than wet hydrological conditions at most sites, ranging from 11.9% to 62.8%, except at site P, where mean relative abundance was higher under wet conditions (41.1%) than under dry conditions (18.8%). *Aeromonas* was detected at four of five sites under dry hydrological conditions, with relative abundances ranging from 7.2% to 24.6%. *Myroides* was detected exclusively under wet hydrological conditions at sites D and P, representing the second most abundant genus at site P (31.1%–34.8%). Bacteria outside the ten most abundant genera showed a wider range of relative abundances under dry than wet hydrological conditions (8.1%–30.9% vs. 9.6%–14.8%).

The relative abundance of nine genera previously reported to harbour ARGs was examined under both sampled hydrological conditions (Fig. 3D). Four of these genera were among the ten most abundant taxa, with the remaining five, *Acinetobacter*, *Citrobacter*, *Enterococcus*, *Pseudomonas*, and *Stenotrophomonas*, present at lower abundances. *Escherichia/Shigella* dominated under dry hydrological conditions, while *Klebsiella* co-dominated under wet hydrological conditions, particularly at site D, where it was absent under dry conditions. *Acinetobacter* was the

most abundant of the remaining ARG-associated genera, with relative abundances ranging from undetected to 12.1%, showing higher abundances under wet conditions at sites M, R and Z but under dry conditions at site D. *Pseudomonas*, *Citrobacter*, and *Stenotrophomonas* were detected at mean relative abundances below 1%. *Citrobacter* and *Klebsiella* were not detected at site R under either sampled hydrological condition.

3.3. Correlation between relative ARG abundance and bacterial genera

Spearman correlation matrices were constructed to assess the relationship between relative ARG abundances (HT-qPCR) and the relative abundances of nine bacterial genera previously reported to harbour ARGs (Fig. 4; Fig. S3; Table S7). These genera – *Escherichia/Shigella*, *Klebsiella*, *Enterobacter*, *Acinetobacter*, *Pseudomonas*, *Citrobacter*, *Aeromonas*, *Enterococcus*, and *Stenotrophomonas* – were selected *a priori* based on their established clinical and environmental relevance in aquatic resistome studies.

During wet season sampling, *Escherichia/Shigella* showed the highest number of significant positive correlations, with the most robust associations retained across all five leave-one-out iterations, involving efflux pump genes (*acrR_2*, *mdtA*, *mdtE*, and *tolC_2*), beta-lactam resistance genes (*ampC_1* and *bla_{CMY_2}*), and *tet(X)* (Fig. 4A; Table S8). Associations with carbapenemase genes (*bla_{KPC_2}* and *bla_{VIM}*) and *vanB_1* were retained in 3–4 of 5 iterations. *Klebsiella* showed the most robust positive correlation with *tet(M)* (5/5 iterations), whereas MGE associations (*IS26_1* and *tnpA_2*) were less stable, being retained in 2–3 of 5

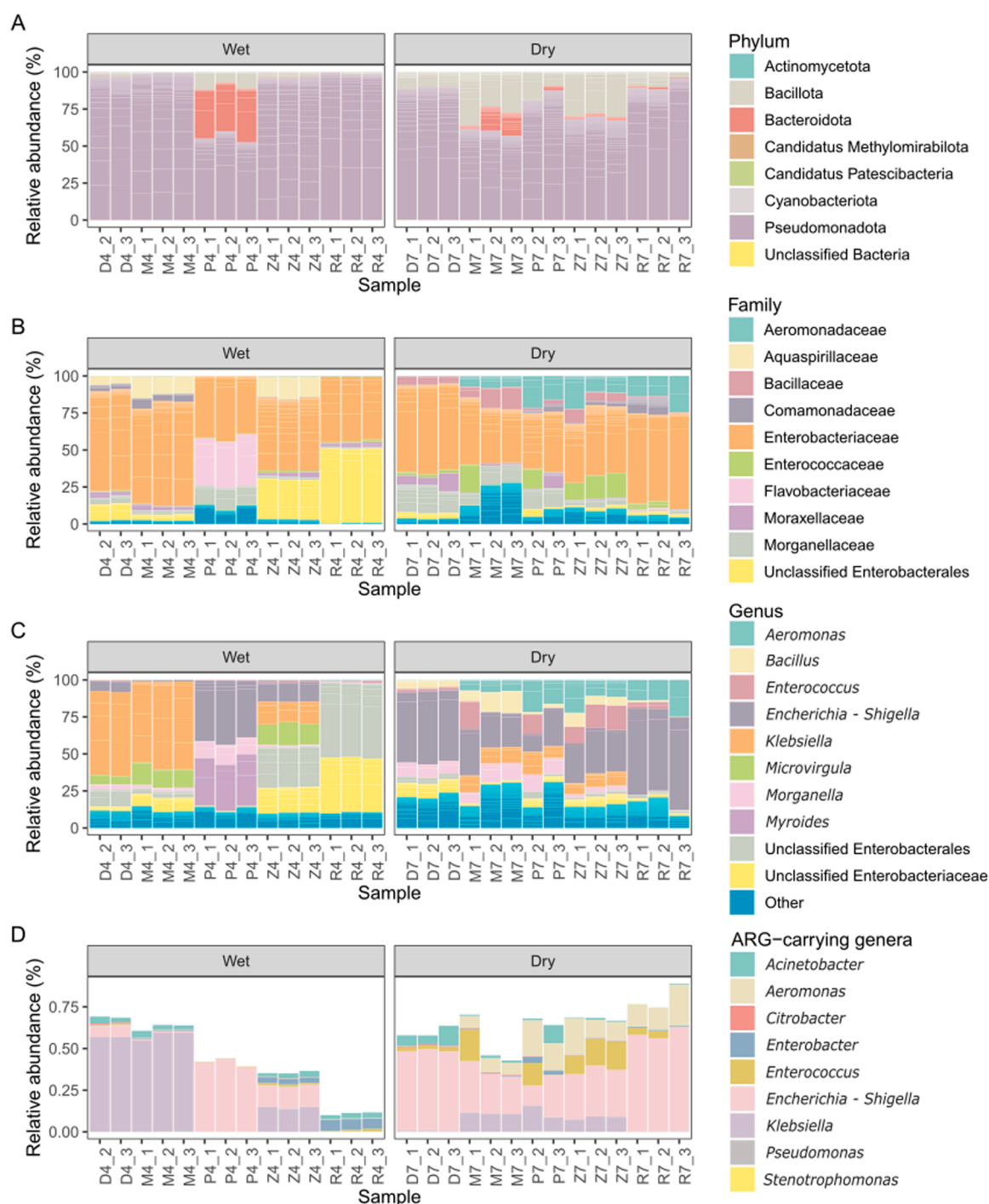


Fig. 3. Relative abundance (%) of the most abundant bacterial taxa across five sampling sites under contrasting wet and dry season hydrological conditions. Panels show composition at the (A) phylum, (B) family and (C) genus level, and (D) relative abundance of nine genera previously reported to harbour ARGs. Top ten taxa shown at each taxonomic level in panels A-C; remaining taxa pooled as 'Other'.

iterations. *Citrobacter* showed robust associations with *APH(6)-Id* and *bla_{TEM}* (5/5 iterations; Fig. 4A; Table S8).

In dry season samples, *Klebsiella* showed the highest number of significant positive correlations, involving multiple resistance- and mobility-related targets, including *int1*, *tnpA*, and ARGs associated with several antibiotic classes (Fig. 4B; Table S8). The most robust *Klebsiella* associations were with *bla_{SHV-1}* and *qnrB* (5/5 leave-one-site-out iterations), followed by *bla_{OXA-1}*, *lnuB*, *qnrS-1*, *sul1-1*, *sul2-2*, and *catB3* (4/5). The remaining associations, spanning multiple additional resistance classes, were less stable (2–3/5), except for *ermF* and *cfxA* (1/

5 each). *Escherichia/Shigella* showed positive correlations primarily with *ampC-1* and efflux pump genes *acrA-1*, *acrR-2*, and *mdtE*, all retained in 3 of 5 leave-one-site-out iterations. *Citrobacter* and *Pseudomonas* showed additional associations with ARGs and MGEs, with several associations showing low retention across leave-one-site-out iterations (Fig. 4; Table S8). The strongest positive correlation across the dataset was observed between *Acinetobacter* and *bla_{OXA-51}* ($p = 0.97$, $p < 0.001$; Table S8), which was retained across all five iterations. Notably, several ARGs positively correlated with *Klebsiella* showed negative correlations with *Escherichia/Shigella* (Table S8).



Fig. 4. Significant positive Spearman correlations between bacterial genera (rows) and antibiotic resistance genes/mobile genetic elements (ARGs/MGEs, columns), grouped by antibiotic class, shown separately for (A) wet season and (B) dry season samples. Cell colour indicates the strength of the Spearman correlation coefficient (ρ scale 0.6–1.0); only statistically significant (adjusted $p < 0.05$) positive correlations with $\rho \geq 0.7$ are shown, and only targets with at least one significant positive association in either panel are displayed. The number within each coloured cell indicates leave-one-site-out (LOSO) retention, i.e., the number of iterations (out of five, corresponding to the five sampling sites) in which the association remained significant after exclusion of one site at a time. Exact ρ and p -values and full per-site LOSO results for all significant correlations, including the negative associations not shown here, are provided in Table S8.

3.4. Distribution and relative abundance of ARGs

Log2 fold-change analysis was used to compare the relative abundances of *E. coli* (*uidA*), universal Bacteroidales, *intI1* and total ARGs at four sampling sites against the reference site R for each sampled hydrological condition (R4 for wet season comparisons and R7 for dry season comparisons) (Fig. 5). In wet season samples, all four sites showed higher relative abundance of *E. coli* than site R4, with fold-changes of 1.95 log2FC (site M), 4.56 log2FC (site D), 5.95 log2FC (site Z) and 7.38 log2FC (site P). Universal Bacteroidales showed higher relative abundance at sites Z and P, while sites M and D showed similar or lower relative abundances compared with R4. *intI1* relative abundance was similar to R4 across all wet season sites. Total ARG relative abundance was highest at site P (2.91 log2FC), followed by site Z (1.25 log2FC), while sites M and D showed similar total ARG relative abundances to R4. In dry season samples, all sites showed lower relative abundance of *E. coli* relative to R7. For universal Bacteroidales, sites Z and M showed higher relative abundances compared with R7, while sites P (−0.66 log2FC) and D (−4.14 log2FC) showed lower relative abundances. *intI1* was not detected at site D in dry season samples, while remaining similar to R7 at all other sites. Total ARG relative abundance was similar to or slightly lower than R7 across all sites, with fold-changes ranging from

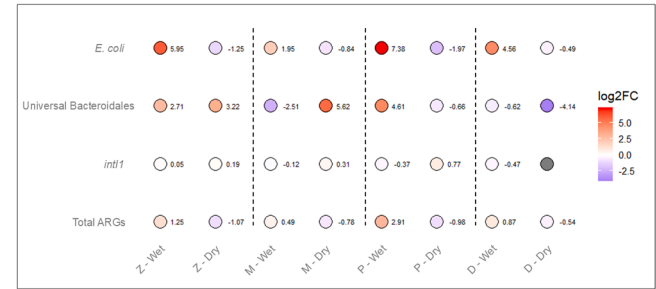


Fig. 5. Log2 fold-change (log2FC) in relative abundance of *E. coli*, universal Bacteroidales, *intI1* and total ARGs at four sampling sites (Z, M, P and D) under wet and dry season hydrological conditions, relative to the reference site (site R); wet season samples compared with R4, dry season samples compared with R7. Red indicates increased relative abundance relative to the reference, and blue indicates decreased relative abundance relative to the reference.

−1.07 log2FC (site Z) to −0.54 log2FC (site D) (Fig. 5). PCoA based on Bray-Curtis dissimilarity was performed to assess the distribution of ARG relative abundances under the sampled hydrological conditions (Fig. 6). Two distinct clusters were identified, separating wet season samples (Z4, M4, and D4) from dry season samples (Z7, M7, D7, and R7), with significant differences in ARG composition between the sampled hydrological conditions (PERMANOVA: $R^2 = 0.26$, $p = 0.0002$). The wet season sample from site P (P4) clustered with the dry season group, while R4 and P7 were identified as outliers. The proportion of ARG variation associated with the sampled hydrological conditions ($R^2 = 0.26$) was lower than that observed for microbial community composition ($R^2 = 0.61$). Detailed examination of the 30 most abundant ARGs (Fig. 7) revealed that site R had consistently lower relative ARG abundances than the other sites under wet hydrological conditions. The beta-lactamase gene *ampC_1* and efflux pump-encoding genes (*mdtA*, *acrR_2*, *acrB_1*, *acrA_1*, *mdtE*, and *tolC_2*) were among the most abundant ARGs across most sites and both sampled hydrological conditions, with the exception of site P under dry hydrological conditions, where aminoglycoside (*aadA_1* and *aadA2_3*) and trimethoprim resistance genes (*dfrA17* and *dfrA1_1*) predominated instead. Additionally, *bla*_{TEM} showed higher relative abundance at site P compared with other sites

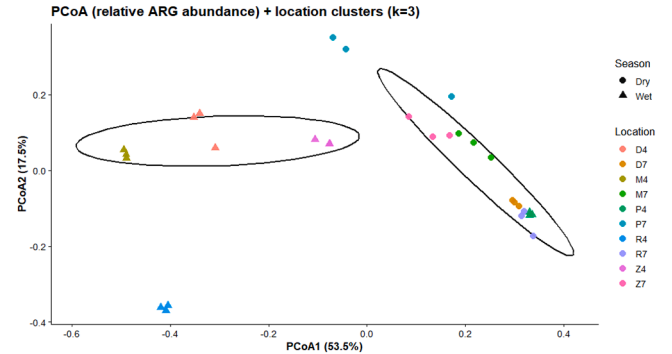


Fig. 6. Principal Coordinates Analysis (PCoA) of ARG relative abundances detected by HT-qPCR across five sampling sites under contrasting wet and dry season hydrological conditions. Shapes indicate season (circle – dry; triangle – wet) and colours indicate sampling site. Ellipses represent 95% confidence intervals.

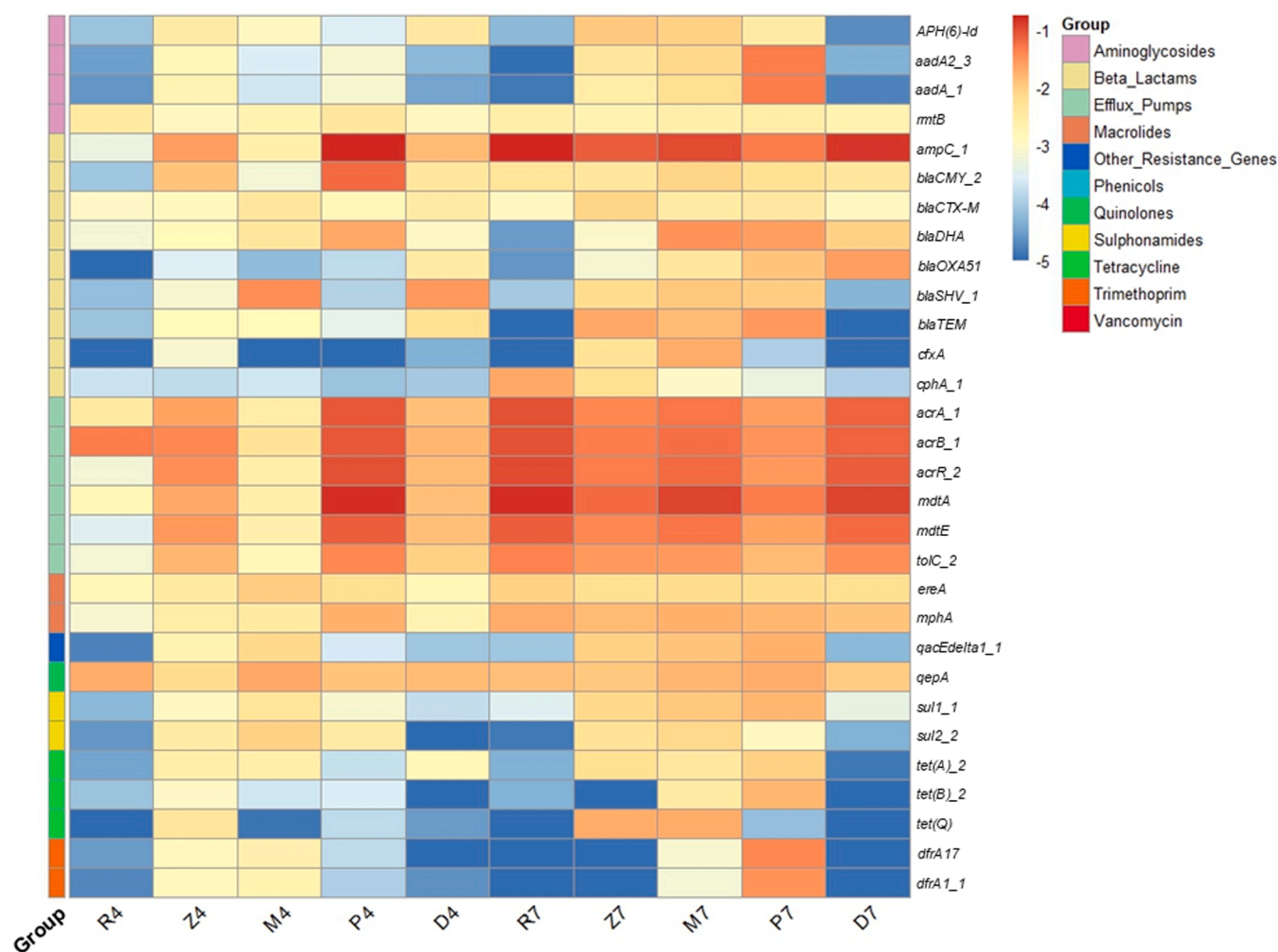


Fig. 7. Heatmap of the 30 most abundant antibiotic resistance genes across all five sampling sites under wet (4) and dry season (7) hydrological conditions. Relative abundance values are log10-transformed. The complete heatmap is provided in Fig. S2.

under dry hydrological conditions.

The complete resistome profile (Fig. S2) revealed additional site- and hydrological-condition-specific patterns. Under wet hydrological conditions, site P showed a distinct ARG profile, with notably higher relative abundances of beta-lactam resistance genes (*ampC_1*, *bla_{DHA}*, and *bla_{CMY_2}*), as well as tetracycline and macrolide resistance genes (*tet(X)* and *mphA*) compared with the other sites.

Sites Z and M showed higher relative abundances of aminoglycoside, beta-lactam, sulphonamide and trimethoprim resistance genes under wet hydrological conditions compared with site R, while site M showed higher relative abundances of aminoglycoside, beta-lactam, sulphonamide and tetracycline resistance genes under dry hydrological conditions. Vancomycin resistance genes (*vanA* and *vanB_1*) and the mobile colistin resistance gene *mcr-1* were detected at low relative abundances across all sites and sampled hydrological conditions, with *vanB_1* showing the lowest relative abundances at sites D and R under wet hydrological conditions.

4. Discussion

4.1. Differences in microbial community structure under contrasting hydrological conditions

Hydrological conditions were significantly associated with microbial community composition (PERMANOVA: $R^2 = 0.61$, $p = 0.001$). PERMDISP analysis, however, indicated significant differences in within-

group dispersion between the sampled hydrological conditions, with greater dispersion among wet season samples, as visible in the PCoA (Fig. 1C). Thus, the observed separation should be interpreted in the context of both differences in community composition between the two sampled hydrological conditions and within-group variability. This greater variability under wet conditions is consistent with the high hydraulic connectivity characteristic of karst systems, where rapid infiltration through conduits and fractures can facilitate transport of surface-derived microorganisms into groundwater following precipitation events [34]. Similar patterns associated with contrasting hydrological conditions have been reported in groundwater microbial communities from karst springs [35], supporting the role of hydrological dynamics as an important structuring factor in karst microbiomes. Estimated species richness (Chao1) was significantly lower under wet hydrological conditions despite the potential influx of surface-derived microorganisms, likely reflecting hydrological dilution, whereby increased infiltration may favour the dominance of a smaller number of rapidly responding taxa while reducing the detectability of rare autochthonous microorganisms [36]. In contrast, Shannon diversity did not differ significantly between the sampled wet and dry hydrological conditions, as it is less sensitive to changes in rare taxa and more strongly reflects the evenness of dominant community members. It should be noted that the statistical analyses employed in this study demonstrate associations rather than causal relationships between environmental factors and variation in microbial community and resistome composition. Furthermore, the two sampling campaigns were designed to capture contrasting hydrological

conditions rather than to characterise the full range of annual hydrological dynamics. Therefore, the observed patterns should be interpreted within the scope of the study design.

4.2. Physicochemical factors associated with microbial community composition

Among the physicochemical parameters assessed by db-RDA, TOC was the variable most strongly associated with microbial community composition. TOC represents an important carbon source supporting microbial growth and metabolism [37], and its association with wet season samples at the low-impacted sites Z and R may reflect increased inputs of dissolved organic carbon during precipitation events [38]. Importantly, the association between TOC and relatively low abundances of genera previously reported to harbour ARGs at sites R and Z (Figs. 2 and 3D) is consistent with a greater contribution of naturally derived organic matter, in agreement with previous observations linking naturally derived TOC to lower ARG richness in drinking water [39]. In contrast, the permanganate index and nitrates were primarily associated with wet season samples from sites under greater urban and agricultural pressure (D and M), consistent with the known vulnerability of karst aquifers to surface-derived nutrient loading [40,41]. While TOC represents total organic matter, including refractory humic substances, the permanganate index provides an estimate of more readily oxidisable organic matter [42]. This divergence may reflect differences in the sources and characteristics of organic matter between low-impacted and more anthropogenically influenced sites. The influx of bioavailable carbon during precipitation events at impacted sites may contribute to the observed differences in microbial community composition [43].

Copper and conductivity were primarily associated with wet season samples from the coastal site P, which may reflect contributions from urban infrastructure and stormwater runoff [44], as well as periodic saltwater intrusion characteristic of coastal karst systems in this area. The highest conductivity values observed at site D (Table S1) may also be influenced by marine water, consistent with its location in the same coastal bay as site P. If periodic saltwater intrusion contributes to the elevated conductivity at these coastal sites, the resulting changes in ionic conditions could also influence microbial community structure and potentially affect resistance-related traits [45,46]. However, disentangling the contributions of saltwater intrusion and anthropogenic contamination to the observed resistome patterns at sites P and D remains challenging, as direct salinity measurements were not available in this study. Copper can contribute to co-selection of AMR under certain conditions [47], and its association with site P, which also exhibited the highest relative ARG abundances, raises the possibility that metal-driven co-selective processes may contribute to the observed resistome profile at this location. The association of contamination indicators (ammonium and *E. coli*) with samples collected under dry hydrological conditions in the db-RDA analysis may reflect reduced dilution under low-flow conditions and potentially enhanced contamination signals. Ammonium in groundwater is commonly associated with wastewater contamination from septic tanks or sewage systems, as well as agricultural fertiliser inputs [48].

Overall, the observed physicochemical associations reflect the joint influence of land-use pressure and site-specific hydrogeological differences on microbial community composition.

4.3. Taxonomic composition and genera associated with ARGs

Differences in bacterial community composition between the two sampled hydrological conditions were reflected across all taxonomic levels. The dominance of *Pseudomonadota* across all sites and under both sampled hydrological conditions is consistent with its reported prevalence in oligotrophic groundwater environments [49]. Although bias associated with the selected V3-V4 primers cannot be fully excluded, similar dominance of *Pseudomonadota* has been reported in previous

studies of groundwater microbial communities [50]. The higher relative abundance of *Bacillota* under dry hydrological conditions, particularly at sites M and Z, may be associated with the lower-low conditions during the dry sampling period. The detection of *Bacteroidota* at notable relative abundance exclusively at the coastal spring (site P) under wet hydrological conditions is consistent with increased influence of surface-derived organic and faecal-associated inputs [51,52].

Genera previously reported to harbour ARGs were detected across most sites and both sampled hydrological conditions, indicating the presence of bacterial taxa previously linked to ARG occurrence under both conditions. It should be noted that patterns of taxonomic dominance under the sampled hydrological conditions do not necessarily correspond to ARG correlation patterns, as the latter reflect co-variation between taxa and ARGs rather than taxon abundance alone. Higher relative abundance of *Escherichia/Shigella* under dry and *Klebsiella* under wet hydrological conditions, particularly at sites D and M, may reflect differences in environmental conditions and potential source contributions. The higher relative abundance of faecal-associated taxa such as *Escherichia/Shigella* under the sampled dry hydrological conditions (61.3 mm rainfall) may be consistent with reduced dilution under lower-flow conditions [53]. Under the sampled wet hydrological conditions (182.3 mm rainfall), greater precipitation may increase the influence of surface runoff and associated microbial inputs, including taxa such as *Klebsiella*, a genus frequently reported in association with agricultural and urban runoff [54]. These differences are consistent with contrasting hydrological conditions represented by the two sampling periods. It should be noted that genus-level classification based on 16S rRNA amplicon sequencing does not allow discrimination between pathogenic and non-pathogenic strains. Therefore, detection of *Escherichia/Shigella* sequences should not be interpreted as confirmation of the presence of pathogenic *E. coli*. *E. coli* counts reported in this study were independently determined by culture-based membrane filtration according to ISO 9308-2:2014. *Aeromonas*, detected at four of the five sites under dry hydrological conditions, represents a widespread environmental ARG-associated taxon due to its common occurrence in aquatic environments and its documented association with intrinsic and acquired resistance determinants [55,56].

4.4. Site-specific anthropogenic influence and ARG profiles

The coastal site P consistently showed the strongest signatures of anthropogenic influence, with higher relative abundance of *E. coli* and total ARGs than the reference site under wet hydrological conditions, together with a distinct resistome profile across both sampling periods. Higher relative abundance of universal *Bacteroidales* at site P under wet hydrological conditions further supports faecal inputs from human or animal sources, consistent with the observed *E. coli* patterns. The water abstraction system at this site, consisting of a vertical shaft and horizontal gallery, may facilitate the rapid transport of surface-derived contaminants, potentially contributing to the transport of ARGs and bacteria carrying resistance determinants from diverse runoff sources, including the nearby port, railway and seasonal tourist activity [28]. Further hydrogeological characterisation of this site is required to fully quantify infiltration pathways and dynamics.

Interestingly, the resistome profile at site P under wet hydrological conditions, characterised by notably higher relative abundances of beta-lactam, tetracycline and macrolide resistance genes than at other sites under the same conditions, is consistent with persistent anthropogenic influence at this location across the two sampled hydrological conditions. Under dry hydrological conditions, site P showed a distinct shift towards aminoglycoside (*aadA1* and *aadA2.3*) and trimethoprim (*dfrA17* and *dfrA1.1*) resistance genes, accompanied by lower efflux pump gene relative abundances than at other sites. This distinct ARG profile (Fig. 7) was consistent with the distinct position of P7 in the ARG-based PCoA (Fig. 6). Aminoglycoside and trimethoprim resistance gene classes have previously been associated with anthropogenic

contamination from urban and clinical settings [4,57,58] and are frequently found in association with MGEs, which may contribute to their potential mobility. Notably, site P under dry hydrological conditions showed a weaker association with ammonium and *E. coli* than the other sites, suggesting that faecal indicator bacteria may not fully explain the distinct resistome pattern at this location. The location of this site within a coastal bay exposed to urban runoff, wastewater-associated inputs, port activities, and seasonal tourism provides plausible sources of diverse human-associated microbial and resistome inputs. While higher ambient temperatures during the dry sampling period (July, $\sim 25^{\circ}\text{C}$) have been proposed as a factor influencing ARG persistence in karst water systems [24,25], the higher ARG relative abundances observed at site P, particularly under wet hydrological conditions (April, $\sim 15^{\circ}\text{C}$), suggest that anthropogenic influence, in addition to variation between the sampled hydrological conditions, may contribute to its distinct resistome profile.

The forested reference site R showed the lowest relative ARG abundances under wet hydrological conditions compared with all other sites (Fig. 7; Fig. S2), supporting its use as a reference location. However, its ARG profile under dry hydrological conditions was comparable to those observed at the other sites, indicating that its reference status varied between the two sampled hydrological conditions. Notably, samples from this site under wet hydrological conditions were identified as outliers in the ARG-based PCoA, with R4 distinguished by its consistently low overall relative ARG abundances, including low relative abundances of efflux pump genes (*acrA_1*, *acrB_1*, *acrR_2*, *mdtA*, *mdtE*, and *tolC_2*) and relatively low relative abundances of other ARG classes (Fig. 7). Nevertheless, the detection of ARGs at this minimally impacted site indicates that even forested karst springs may receive ARG inputs during periods of increased precipitation. This finding has important implications for the definition of reference sites in karst groundwater monitoring programmes, as hydrological dynamics may influence ARG abundances even at sites with no direct anthropogenic pressure. Site R should therefore be considered a hydrological reference rather than an absolute ARG baseline, and fold-change values presented in this study represent comparisons specific to the sampled hydrological conditions rather than fixed baseline estimates.

The lower proportion of ARG variation associated with the sampled hydrological conditions (PERMANOVA: $R^2 = 0.26$) compared to microbial community composition ($R^2 = 0.61$) suggests that, while hydrological conditions were strongly associated with overall bacterial community structure, ARG profiles are additionally associated with site-specific contamination sources and local environmental conditions. Sites Z, M and D exhibited intermediate ARG profiles between the reference site R and the most impacted site P, consistent with their varying degrees of anthropogenic influence. Together, these findings highlight the joint influence of land-use pressure and karst hydrological connectivity on ARG distribution patterns in groundwater systems.

4.5. Associations between bacterial genera and ARGs

Spearman correlation analysis revealed hydrological-condition-specific associations between bacterial genera previously reported to harbour ARGs and resistance determinants. These correlations represent statistical co-variation between relative abundances and do not demonstrate that ARGs are carried by the correlated bacterial taxa. Under wet hydrological conditions, *Escherichia/Shigella* showed the broadest range of significant positive correlations, particularly with efflux pump and beta-lactam resistance genes. Several of these associations were retained across all five leave-one-site-out iterations, indicating that they persisted despite exclusion of each individual sampling location. In contrast, their retention was generally lower under dry hydrological conditions. The association of *Escherichia/Shigella* with efflux pump genes is consistent with the established role of the AcrAB-TolC system in antibiotic resistance in *E. coli* [59]. However, the widespread occurrence of efflux pump genes also reflects their intrinsic

physiological functions, and their relative abundance alone should not be interpreted as evidence of increased AMR risk.

Under wet conditions, *Klebsiella* was positively associated with MGEs and multiple ARGs, including *tet(M)*, which was retained across all five leave-one-site-out iterations. Other associations with MGEs showed lower retention. Similarly, associations of *Citrobacter* with *APH(6)-Id* and *bla_{TEM}* were retained across all five iterations. These findings support the presence of recurring co-occurrence patterns, but co-occurrence between ARGs and MGEs does not demonstrate physical linkage or horizontal gene transfer. Associations involving *Stenotrophomonas* and *Pseudomonas* were generally less robust, with several not retained after exclusion of individual sampling locations.

Under dry conditions, *Klebsiella* showed the broadest range of positive associations, including with *int1*, *tnpA_2*, and multiple ARG classes. The most robust associations were observed for *bla_{SHV_1}* and *qnrB*, which were retained across all five leave-one-site-out iterations, whereas several other associations were retained in four of five iterations. This pattern suggests that some *Klebsiella*-associated relationships were relatively consistent across sampling locations, while others were more sensitive to individual sites. *Escherichia/Shigella* showed a narrower set of associations under dry conditions, including several efflux pump genes that were retained in fewer leave-one-site-out iterations than under wet conditions.

The contrasting association patterns between *Klebsiella* and *Escherichia/Shigella* under wet and dry hydrological conditions may reflect differences in the sources contributing to the groundwater microbial community and in their responses to changing hydrological connectivity. Increased runoff during wet conditions may introduce bacteria and resistance determinants from a wider range of diffuse sources, potentially broadening co-occurrence patterns involving *Escherichia/Shigella*. Under lower-flow conditions, more consistent associations involving *Klebsiella* may reflect a different balance of local and persistent sources. These interpretations are consistent with the broader environmental gradients observed in the db-RDA results, but the correlation analysis itself does not establish causal relationships.

The strongest genus-ARG association was observed between *Acinetobacter* and *bla_{OXA-51}* under dry hydrological conditions ($p = 0.97$, $p < 0.001$), and this association was retained across all five leave-one-site-out iterations. The close relationship between *bla_{OXA-51}* and *Acinetobacter*, particularly *A. baumannii*, is biologically consistent with the established presence of this gene as an intrinsic chromosomal marker in the species [60]. Previous studies have shown that insertion sequences such as *ISAbal* can influence expression of *bla_{OXA-51}*-like genes in *A. baumannii* [61,62], highlighting the importance of distinguishing gene occurrence from evidence of genetic mobility.

Overall, the leave-one-site-out analysis showed that the robustness of genus-ARG associations varied substantially among taxa and resistance determinants. Some associations were consistently retained across sampling locations, whereas others were sensitive to the exclusion of individual sites. Given the limited number of independent sampling locations, these correlations should therefore be interpreted as patterns of co-variation rather than evidence of specific host-gene relationships, physical linkage, or horizontal gene transfer.

4.6. Clinically relevant ARGs and One Health implications

The detection of vancomycin resistance genes (*vanA* and *vanB_1*) and the mobile colistin resistance gene *mcr-1* at low but consistent relative abundances across most sites and under both sampled hydrological conditions is of potential public health concern, as vancomycin and colistin remain critical last-resort treatment options for multidrug-resistant infections [63]. The widespread occurrence of vancomycin and colistin resistance genes across sites with different degrees of anthropogenic influence suggests that clinically relevant resistance determinants are not restricted to heavily contaminated environments. It should be noted that detection of ARG DNA does not confirm the

presence of viable ARB or transmissible resistance determinants, and additional culture-based and viability assays would be required to assess the associated health risk. The absence of antibiotic concentration data limits our ability to directly assess selective pressure as a factor contributing to the observed resistome patterns. Future studies combining resistome profiling with antibiotic quantification would provide a more comprehensive understanding of factors associated with ARG occurrence and persistence in karst groundwater. The absence of an established regulatory framework for AMR surveillance in groundwater represents a major gap in drinking water safety governance. Our findings support the consideration of molecular AMR indicators, including *int1* and clinically relevant resistance gene classes, in future karst groundwater monitoring programmes [64], alongside hydrological and microbiological indicators.

5. Conclusions

This study identified associations between hydrological conditions and differences among groundwater sites in microbial community structure and resistome composition, including patterns consistent with varying degrees of anthropogenic influence in Dinaric karst groundwater. Hydrological conditions were the factor most strongly associated with microbial community composition among the factors examined (PERMANOVA: $R^2 = 0.61$, $p = 0.001$), although significant differences in within-group dispersion between the sampled wet and dry hydrological conditions indicate that this separation should be interpreted with caution. TOC was the physicochemical variable most strongly associated with microbial community composition.

The most impacted coastal site showed higher relative ARG abundances than the reference site under both sampled hydrological conditions, in contrast to other impacted sites where total relative ARG abundances were similar to or slightly lower than the reference site under dry hydrological conditions. Contrasting genus-ARG co-occurrence patterns between the sampled hydrological conditions, together with the detection of MGEs, highlight the co-occurrence of resistance and mobility-related determinants in karst groundwater. The detection of clinically relevant ARGs, including carbapenemase and vancomycin resistance genes, further demonstrates the occurrence of resistance determinants of clinical relevance. However, the sensitivity analysis showed that the robustness of individual associations varied among sampling locations, and these correlations do not demonstrate horizontal gene transfer or physical linkage. Further investigation using approaches capable of resolving host–gene and gene–MGE associations is therefore warranted.

The detection of clinically relevant resistance genes across all sites, including minimally impacted forested springs, highlights the potential value of molecular AMR indicators for monitoring karst groundwater used for drinking water supply. To further investigate temporal ARG dynamics, a longitudinal follow-up study based on a targeted qPCR panel is currently underway. In parallel, potentially pathogenic bacterial isolates from the same sampling sites are being characterised in a separate study to complement the molecular findings presented here.

CRedit authorship contribution statement

Ana Puljko: Methodology, Investigation, Formal analysis, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Louis Weisse:** Investigation, Formal analysis, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Jurica Štiglic:** Investigation, Formal analysis, Writing – review & editing. **Ana Maravić:** Investigation, Writing – review & editing. **Magdalena Ujević Bošnjak:** Conceptualization, Methodology, Resources, Writing – review & editing. **Nikolina Udiković Kolić:** Conceptualization, Methodology, Resources, Visualization, Supervision, Project administration, Funding acquisition, Writing – review & editing.

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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2026.143725.

Data Availability

Data will be made available on request.

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