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Functional resistomes in municipal wastewater treatment plants pose challenges to public health

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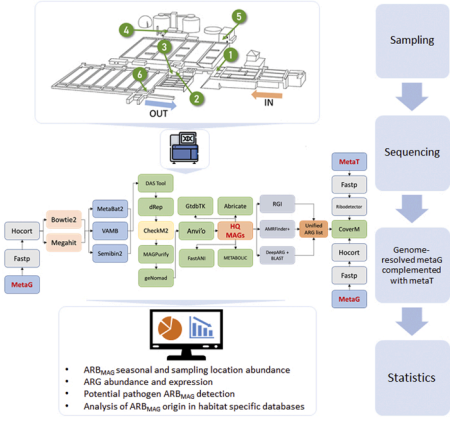
HIGHLIGHTS

- Human- and environment-derived ARB-$_{MAGs}$ show distinct seasonal resistance patterns.
- Anaerobic digestion decreases PP-H-ARB$_{MAGs}$ abundance.
- *adeF* and *van* gene homologues remain highly expressed across ARB$_{MAG}$ types.
- Fluoroquinolone resistance genes show ARB$_{MAG}$-type-specific expression patterns.
- Rare clinically relevant ARGs may pose latent risk in PP-H-ARB$_{MAGs}$.

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewater treatment plants (WWTPs) are essential for controlling antimicrobial resistance, but also serve as hotspots for resistance gene persistence and dissemination. In this study, a novel combinatory approach using genome-resolved metagenomics and metatranscriptomics was employed to examine the resistome of a full-scale municipal WWTP across treatment stages and seasons. Results reveal that although human-associated, potentially pathogenic, antibiotic resistance gene (ARG)-harbouring metagenome-assembled genomes (ARB$_{MAGs}$)

Abbreviations: ARGs, antibiotic resistance genes; ARB, antibiotic-resistant bacteria; WWTP, wastewater treatment plant; RWW, wastewater inlet; PS, primary sludge; BTS, biologically treated sludge; MS, mixed sludge; ADS, anaerobically treated sludge; TWW, treated wastewater; MAG, metagenome-assembled genome; SCG, single-copy core gene; VF, virulence factor gene; MGE, mobile genetic element; CPM, copy per million; FPM, fragment per million; TPM, transcript per million; IQR, interquartile range; PCoA, principal coordinate analysis; TAN, total ammonia nitrogen; VOAs, volatile organic acids; ARB$_{MAG}$, antibiotic-resistant bacterial metagenome-assembled genome; H-ARB$_{MAGs}$, human associated antibiotic-resistant bacterial metagenome-assembled genome; E-ARB$_{MAGs}$, environmental antibiotic-resistant bacterial metagenome-assembled genome; U-ARB$_{MAGs}$, antibiotic-resistant bacterial metagenome-assembled genome of unknown origin.

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declined in abundance during treatment, many ARGs remained transcriptionally active, particularly efflux, beta-lactam, and macrolide–lincosamide–streptogramin genes conferring resistance against fluoroquinolones, cephalosporins, and macrolides. Environment and treatment-adapted microbes become increasingly dominant, with plasmids identified as major vectors of mobile resistance. Dominant potential pathogenic human-associated ARB_{MAGs} were abundant during spring and summer, whereas environmental ARB_{MAGs} predominated in colder seasons. Fluoroquinolone resistance genes displayed varying expression levels across ARB_{MAG} types, with the lowest levels observed during the anaerobic phase of treatment. Although clinically relevant ARGs were detected at low relative abundance and expression levels, one of the carriers was *Citrobacter freundii* ARB_{MAG}, a human-associated potential pathogen. These findings underscore the value of integrating genomic and transcriptomic data to assess site-specific and ARB_{MAG}-type-specific resistance and to strengthen antibiotic resistance monitoring in engineered systems.

1. Introduction

The emergence of antimicrobial resistance predates human activity. Antibiotic resistance genes (ARGs) are embedded in environmental microbial communities and have evolved in response to compounds produced by competing microbes (D'Costa et al., 2011). However, widespread antibiotic use in human medicine and animal husbandry has accelerated ARG evolution and spread. This anthropogenic acceleration has intensified selective pressures on microbial populations, leading to the selection and increased prevalence of antibiotic-resistant bacteria (ARB). In response, the 'One Health' initiative proposed by the World Health Organisation addresses the interconnection of humans, animals, and environmental health, particularly regarding ARG transmission. This holistic approach requires integrated, cross-sectoral surveillance strategies (Aarestrup and Woolhouse, 2020; Djordjevic et al., 2023; Joakim Larsson and Flach, 2022).

Within this framework, monitoring ARGs in wastewater is essential. The European Union is considering implementing wastewater surveillance in treatment plants serving populations of 100,000 or more. To monitor ARGs in human populations effectively, it is valuable first to identify microbial diversity in these urban environments, including novel species and temporal changes (Larsson et al., 2023). A prior study, comprehensively using metagenomic datasets from multiple habitats, found that ~23 % of ARGs in wastewater pose a health risk based on human accessibility, mobility, presence in pathogens and clinical relevance (Zhang et al., 2022b). Therefore, when comparing microbial profiles and assessing risks in the wastewater microbiome, it is critical to consider temporal and spatial variations alongside environmental factors (Caucci et al., 2016; Chau et al., 2022; Ching et al., 2025; Newton et al., 2015). Understanding these influences is vital for drawing meaningful conclusions from wastewater analyses.

Wastewater treatment methods using activated sludge and anaerobic digestion show promise for reducing pathogens and their ARGs (Ju et al., 2016; J. Wang et al., 2020). Despite the strengths of such advanced technologies, the relevant literature reveals discrepancies in effectiveness (Miłobedzka et al., 2022; Ying Wang et al., 2022). Research has confirmed that wastewater treatment plants (WWTPs) are major reservoirs and potential dissemination points for antibiotic resistance, harbouring diverse ARGs (Guo et al., 2017; Yiqing Wang et al., 2022). Prior studies have demonstrated that although core resistance genes exist within WWTPs, their widespread dissemination to other environments, including human pathogens, appears limited (Munck et al., 2015). However, newer findings have indicated that the treatment process itself markedly influences the dynamics of ARGs and mobile genetic elements (MGEs). Various factors, including seasonality and treatment configuration, are crucial in shaping antimicrobial resistance across influent, sludge and effluent (de Nies et al., 2022; Honda et al., 2023). WWTPs are not only sites for treating human-origin wastewater (Newton et al., 2015); but also complex ecosystems where human-associated, and environmental microbes co-exist and interact at high densities (McLellan and Roguet, 2019; Roguet et al., 2022). Identifying the full spectrum of microbial populations, including potential novel species, in these environments is essential, as they may harbour unrecognised ARG sources. Moreover, as environmental bacteria naturally carry diverse resistance genes, distinguishing their origins is critical for targeted monitoring of ARGs associated with human activity. This underscores the need to investigate microbial community origins and their resistomes within WWTPs to develop effective strategies for mitigating ARG release protecting public health (Nguyen et al., 2021; Shi et al., 2023; Shuai et al., 2024).

Traditional culture-based methods and quantitative polymerase chain reaction targeting specific ARGs, although informative, offer limited scope and are biased by primer selection and low coverage of unknown or emerging resistance genes (Elbait et al., 2024; Keenum et al., 2022; Srathongneam et al., 2024). Conversely, shotgun metagenomics provides a powerful alternative for comprehensive resistome profiling, enabling the broad ARG detection (Davis et al., 2023; Rodríguez et al., 2021). Metagenomic studies have shown geographically-driven variations in ARG composition across global WWTPs, reflecting regional climate and socioeconomic factors (Hendriksen et al., 2019; Munk et al., 2022). A comprehensive trans-European investigation found that ARG profiles in WWTP effluents often reflect clinical antibiotic resistance patterns across regions, linking antibiotic use to ARG persistence in wastewater (Pärnänen et al., 2019). By assembling metagenomic reads into contigs and binning them into metagenome-assembled genomes (MAGs), we can identify ARG genomic contexts, microbial hosts and their localisation on MGEs (Becsei et al., 2024; Kim et al., 2023). This enables recovery of draft or complete genomes from metagenomes, offering rich, contextual genetic insights into resistance in complex microbial communities.

However, although genome-resolved metagenomics reveals ARG presence, it does not indicate their activity or expression. In this context, metatranscriptomics combined by metagenomic binning becomes relevant. By analysing microbial transcripts, metatranscriptomics shows which genes are actively expressed and clarifies ARG functional potential (Wang et al., 2020). ARG expression analysis across human, and animal guts as well as other ecological niches have revealed environment-specific (Versluis et al., 2015). One study found that only 65 % of ARGs show active expression in the activated sludge microbiome (Liu et al., 2019). A WWTP resistome study identified persistent ARGs with elevated transcriptional activity, especially on MGEs and in final effluent (Ju et al., 2019). This is particularly relevant in wastewater treatment where environmental conditions and process-related stresses can influence gene expression (Asante and Osei Sekyere, 2019). Therefore, integrating metatranscriptomics with genome-resolved metagenomics can provide a more comprehensive picture of ARG activity in wastewater microbiome (Liu et al., 2019). While this combined MAG-based approach reveals the microbes and their functions in a complex microbiome, these data are intrinsically compositional. Shotgun-sequencing yield the relative proportions of DNA or mRNA (i.e. cDNA) fragments assigned to different taxa rather than their absolute abundances or expression (Gloor et al., 2017; Nayfach and Pollard, 2016; Props et al., 2017).

This present study investigated the role of a local industrial-scale wastewater treatment plant as a mitigator and reservoir of antibiotic resistance. Using an integrated genome-resolved metagenomics and

metatranscriptomics approach, we analysed the microbial resistome of a conventional activated sludge system coupled with anaerobic digestion. This system common in modern treatment facilities integrates aerobic and anaerobic processes to improve organic matter removal while addressing sludge management (Semblante et al., 2014). The main objective was to assess not only ARG presence but also their expression, mobility and ecological context across treatment stages. We found that although most potentially pathogenic microbes and their high-risk ARGs were reduced, a notable proportion remained transcriptionally active, especially in treatment-adapted microbial populations, emphasising the need for expression-based surveillance to better assess resistance risks.

2. Methods

2.1. Sample collection

Samples were collected from an industrial-scale municipal WWTP located near Szeged, Hungary operated by Szeged Waterworks Ltd. The facility processes domestic sewage, industrial wastewater (including water from food processing) and rainwater runoff. Sampling locations were selected to represent key stages of the treatment process: including wastewater inlet (RWW), primary sludge (PS), biologically treated sludge (BTS), mixed sludge (MS), anaerobically treated sludge (ADS) and treated wastewater (TWW) (Supplementary fig. 1). Key operational data is summarised in Supplementary tab. 1. Sampling was performed during six seasonal time points in 2023: spring-summer: March, May and July; autumn-winter January, September and November. All samples were transported to the laboratory and processed immediately upon arrival.

2.2. Nucleic acid extraction and sequencing

From each sampling site 1 and 6 L samples were collected (six replicates per season). Aliquots were prepared for nucleic acid extractions as follows: 3 × 0.5 L for RWW samples, 3 × 1 mL for PS, BTS, MS and ADS; and 3 × 6 L for TWW. Microbial biomass was collected by centrifugation at 13,000 rpm for 10 min. Total community DNA and RNA were extracted in triplicate pooled to ensure representative coverage. Extractions were performed using the ZymoBIOMICS DNA/RNA Miniprep Kit (R2002, Zymo Research, Irvine, CA, USA). Nucleic acid quality and integrity were assessed using the Agilent 4150 TapeStation (Agilent Technologies, Santa Clara, CA, USA). The metagenomics libraries were prepared from pooled DNA using the NEBNext Ultra II Library Prep Kit (New England Biolabs, Ipswich, MA, USA) and sequenced using paired-end reads on an NextSeq 550 platform (Illumina, San Diego, CA, USA) with the NextSeq High Output Kit v2 Sequencing Reagent Kit. For metatranscriptomic analysis, RNA libraries were prepared using the Zymo-Seq RiboFree Total RNA Library Kit, which includes a universal rRNA depletion. Paired-end mRNA sequencing was similarly performed on the NextSeq 550 sequencer using the NextSeq High Output Kit v2 Sequencing Reagent Kit. Base calling was conducted using bcl2fastq (v2.17.1.14; Illumina). Characteristic fragment parameters are summarised in Supplementary tab. 2.

2.3. Metagenome assembly and binning

Raw metagenomic reads were quality-filtered using fastp (v0.23.2; minimum length: 150 bp) and decontaminated against the *Homo sapiens* reference genome (GRCh38) using HoCoRT (v1.2.2). Read quality was verified using FastQC (v0.11.8) (Chen, 2023; Rumbavicius et al., 2023). Filtered reads were co-assembled per sample using Megahit (v1.2.9) with the following parameters: min contig length: 1500; min k-mer size: 21; max k-mer size: 141; presets = meta-large (Li et al., 2015) (Supplementary tab. 2). Contig coverage was determined by mapping filtered reads using Bowtie2 (v2.5.2 using -q and –fr) (Langmead and Salzberg, 2012). Resulting SAM files were converted and sorted to BAM format using Samtools (v1.19) (Danecek et al., 2021).

To enhance MAG recovery, we employed three binning strategies: two machine learning-guided tools, namely Semibin2, (v1.5.1) in self-supervised mode and VAMB (v4.1.3) in default variational autoencoder mode and one based on tetra-nucleotide frequency score and graph structure, namely MetaBat2 (v2.2.15) (Kang et al., 2019; Nissen et al., 2021; Pan et al., 2023). The mapped and sorted BAM files produced by Samtools were used to determine contig coverages with the jgi_summarize_bam_contig_depths script (MetaBat2). The jgi-depth table and assembled contig sequences were inputted into MetaBat2 (minimum contig length: 1500). The six sample-specific MAG sets produced through the three binning tools were refined using DASTool (v1.1.7) with the options write_bins and score_threshold 0.1 (Sieber et al., 2018). Dereplication of MAGs was achieved using dRep (v2.2.3) with the following parameters: dereplicate: comp 10, con 5, S_algorithm FastANI, sa 0.95 (Olm et al., 2017). The resulting MAGs were filtered based on completeness (>10 %) and contamination (<5 %), as estimated by CheckM2 (v1.0.1) (Bowers et al., 2017; Chklovski et al., 2023). A contig database was generated in Anvi'o (v8: 'Marie') from the dereplicated and filtered MAGs (Eren et al., 2021). Contamination was assessed using automated and manual approaches. First MAGPurify2 (v2.1.2) was used with the phylo-markers and known-contam options to identify potential contaminants (Antonio P. Camargo et al., 2023). Manual refinement was then performed in Anvi'o using the anvi-refine function, incorporating tetra-nucleotide frequency, GC content, single-copy core genes (SCGs) content and cross-sample coverage profiles.

Taxonomic assignment of the non-redundant MAGs was performed using the GTDB (Release 226) (Parks et al., 2021) with GTDB-Tk (v2.4.1) (Chaumeil et al., 2022). Phylogenetic trees were constructed based on 120 bacterial SCGs using GTDB-Tk in de_novo_wf mode as-well-as IQTree2 (v2.2.6) with the following parameters: number of bootstraps: 1000; maximum iterations: 1000; stopping rule: 100 (Minh et al., 2020). The final tree was visualised using the interactive Tree of Life (v6.7.3; https://itol.embl.de/).

For functional annotation, we applied METABOLIC (v4.0) at the bin level using genomic and metatranscriptomic input data (Zhou et al., 2022). MAG abundances were calculated with CoverM (v0.7.0) in genome mode, using transcript per million (TPM) method. Values are reported as copies per million reads (CPM) per genome. The following parameters were used: m TPM; min-covered-fraction 10; min-read-percent-identity 0.95; min-read-aligned-percent 0.75 (Aroney et al., 2025).

2.4. Identification of ARGs, VFs and MGEs

Open reading frames were predicted during contig database creation using Anvi'o with its built-in Prodigal programme (v2.6.3) (Hyatt et al., 2010). The anvi-summarise and anvi-get-sequences-for-gene-calls scripts were used to extract contig IDs, gene coordinated and amino acid sequences from the non-redundant MAGs. These sequences served as input for ARG prediction, using report-extended-deflines and get-aa-sequences. A combination of one deep-learning-based tool, DeepARG (v1.0.2) and two sequence alignment-based methods, RGI (v6.0.3) and AMRFinderPlus (v3.12.8), was used to maximise ARG detection accuracy (Alcock et al., 2020; Arango-Argoty et al., 2018; Feldgarden et al., 2021). RGI was run using DIAMOND mode, with protein input, retaining only Perfect and Strict matches. AMRFinderPlus was also run with protein input and default settings. DeepARG was executed with the LS model, protein input, a minimum probability of 0.8, alignment identity of 70 %, E-value of 1e-10, and 1000 alignments per entry, retaining only high-confidence ARGs validated by BLASTp. All ARGs were classified using annotations from the Comprehensive Antibiotic Resistance Database (CARD) and the NCBI Reference Gene Catalogue.

To estimate the potential health risks of the detected ARGs, we applied a combined approach. We used the quartile-based risk scoring

framework developed by Zhang et al. (Zhang et al., 2022b), this 'omics-based' method classifies ARGs into four categories from Q1 (highest risk) to Q4 (lowest risk). This was complemented by the risk scoring system proposed by Shuai et al. (Shuai et al., 2024), based on the importance of antibiotics as defined by the WHO, assigning ARGs to four categories: highest priority (P1), high priority (P2), highly important (P3), and important (P4).High risk/priority ARGs were selected as follows: 1.) listed as high-risk/important by one of the frameworks used. 2.) relatively abundant in RWW. 3.) harboured by potentially pathogenic, antibiotic resistance gene (ARG)-harbouring metagenome-assembled genomes (ARB_{MAGs}). Additionally, given the identification of ARGs previously associated with rapid resistance acquisition against traditional and post-2017 antibiotics in ESKAPE pathogens, we performed a comparative analysis. Nucleotide sequences of the identified ARGs were aligned against ARGs described by Daruka et al. (Daruka et al., 2025) using BLASTn (v2.13.0+) with the following parameters: sequence identity ≥99 %; query alignment coverage ≥95 %.

To identify potential pathogenicity features, we used Abricate (v1.0.1) with the virulence factor (VF) database (using vfdb, minid 80, mincov 50; https://github.com/tseemann/abricate) (Liu et al., 2022). To predict MGEs, including plasmids and prophages, geNomad (v1.7.4) was integrated into the workflow in end-to-end mode (Antonio Pedro Camargo et al., 2023). Detection was performed on the concatenated contig dataset of the reconstructed MAGs. Finally, identified ARGs, VFs and MGEs were mapped to their respective MAGs using genomic coordinates from the Anvi'o-based contig database. This allowed confident attribution of ARGs, VFs and MGEs to their microbial hosts.

2.5. Meta-omics data mapping and analysis

To minimise biases in metatranscriptomic quantification, residual ribosomal RNA sequences (small and large subunit rRNA) were removed from quality-filtered reads using Ribodetector programme (v0.3.1) (Deng et al., 2022) following initial filtering with fastp. The resulting quality-filtered and contamination screened metagenomic and metatranscriptomic reads were mapped to predicted coding sequences using CoverM in contig mode (parameters: min-covered-fraction 0; min-read-percent-identity 0.95; and min-read-aligned-percent 0.75). Gene abundances were quantified using both raw read counts and normalised transcript levels (TPM). As TPM and count-based quantifications yielded comparable patterns, metatranscriptomic gene expression (RNA) was represented using TPM, whereas metagenomics abundance (DNA) was shown in fragment per million (FPM).

We applied 'ARG copy per cell' normalization to facilitate cross-study comparisons of ARG differences in relative abundance and expression profiles (Yin et al., 2023). This was achieved by scaling ARG counts against average metagenomics and metatranscriptomic read counts mapped to 10 conserved non-ribosomal, single-copy phylogenic marker genes (KEGG ontology IDs: K06942, K01889, K01887, K01875, K01883, K01869, K01873, K01409, K03106, and K03110) (Milanese et al., 2019). This approach accounted for sequencing depth, ARG length and prokaryotic MAG abundance, providing a biologically meaningful normalisation framework.

2.6. Comparison of reconstructed MAGs with microbial catalogues

To assess the overlap between human microbiota and our reconstructed, non-redundant MAGs, we performed comparison using reference genomes obtained from two sources. First, we downloaded the Unified Human Gastrointestinal Genome (UHGG) catalogue (v2.0), which includes prokaryotic genomes clustered into representative species of the human gut microbiome (Almeida et al., 2021). Next, we retrieved the Searchable Planetary-scale mIcrobiome REsource (SPIRE), which aggregates MAGs from diverse habitats, including host-associated, aquatic, engineered and terrestrial environments (Schmidt et al., 2024). Additionally, metadata from GTDB was used to provide species-level context for MAGs (Parks et al., 2021).

Pairwise genome comparisons were performed using FastANI (v1.33) with the following parameters: k-mer 16; fragLen 3000; minFraction 0.2; maxRatioDiff 10; ANI cut-off: >95 % (Jain et al., 2018). Although genome-centric metagenomics analysis enables these comparisons, there are some potential limitations. These include the completeness of the databases used, the sensitivity of the genome similarity threshold, and incompleteness or unknown biases in our reconstructed genomes.

2.7. Statistical analysis

All statistical analyses were performed in R (v4.4.1). Data visualisation was performed using the ggplot2 (v3.5.1). Relative microbial abundance (CPM), gene expression (TPM) and abundance (FPM) values were log-transformed using the formula $\log_{10}$ (CPM, TPM or FPM +1). Group comparisons were evaluated using the Wilcoxon rank-sum test implemented using ggpubr (version 0.6.0) (Wickham et al., 2019). Principal coordinates analysis (PCo) and Bray-Curtis distances of microbiomes were computed and visualised using microeco (v1.13.0) (Liu et al., 2021). To identify discriminative ARG-harbouring MAGs, we applied linear discriminant analysis by lefser within microeco, using a $p \leq 0.05$ threshold (and the following parameters: trans_diff: alpha: 0.05, p_adjust_method: 'fdr'; boots: 30; taxa_level: species; linear discriminant analysis (LDA): > 2) (Segata et al., 2011). Spearman correlation analysis, performed by ggpubr (v0.6.0), was used to assess relationships between ARB and ARG abundances, ARG abundance and expression and ARG and virulence factor (VF) abundances and expression. Differences between average ARG and ARB abundance and ARG abundance and expression were determined using interquartile range (IQR) calculation (outlier threshold is 1.5 times the IQR). To explore correlations between microorganisms and chemical parameters, the trans_env function from the microeco R package was implemented (parameters: use_data: phylum; cor_method: spearman; p_adjust_method: fdr). Differential ARG abundance and expression analyses were conducted using DESeq2 (v1.44.0) (Love et al., 2014), with input data filtered to retain counts ≥ 3 in at least 2 samples. ARG copy per cell-normalised metagenomics and metatranscriptomic data were used to stabilise dispersion estimates during analysis. Subsequently, DESeq2 was applied with default parameters to identify significantly differentially expressed ARGs ($\log_2$ FC ≥ 2.0 and $p \leq 0.05$), which were visualised using ggplot2.

3. Results

3.1. Microbes harbouring ARGs play key roles in wastewater treatment

This study investigates an industrial-scale wastewater treatment plant employing a conventional activated sludge system coupled with anaerobic digestion, processing wastewater from multiple sources. Six samples per key process points (i.e.: RWW wastewater inlet, PS primary sludge, BTS biologically treated sludge, MS mixed sludge, ADS anaerobically treated sludge, and TWW treated wastewater) were collected in seasonal intervals (i.e.: spring-summer and autumn-winter) yielding 36 metagenomic and 36 metatranscriptomic paired-end sequencing datasets. Deep sequencing generated over 2 billion metagenomics reads (mean: 30.5 million per sample) and 260 million metatranscriptomic reads (mean: 3.6 million per sample) passing filtering (Supplementary tab. 2). Following sample-specific co-assembly (six independent assemblies by location) a genome-centric metagenomics strategy was applied to each contig set. In this analysis, machine learning-guided tools were used in three key stages: (i) metagenomic binning, (ii) ARG detection, and (iii) gene localization analysis. The dereplicated, quality-filtered MAG dataset, comprising 623,109 contigs (N50: 6210) and 3.06 million genes, was used for further analysis.

Of the 1629 dereplicated, non-redundant MAGs generated, 56 % (n = 920) harboured ARGs (hereafter referred to as ARB_{MAGs}), accounting for

61 % CPM relative abundance in the microbiome (Fig. 1B and Supplementary tab. 3). Based on lineage-specific SCGs, 315 (34 %) of the non-redundant ARB_{MAGs} exhibited >90 % completeness and <5 % contamination, as assessed by CheckM2 (Fig. 1A and Supplementary tab. 4). Across the treatment process, these ARB_{MAGs} contained 194 distinct ARG sub-types from 17 ARG classes, comprising 0.07 % FPM and 0.05 % TPM of the total gene pool in relative abundance and expression, respectively. A significant positive correlation was observed (Spearman's rho = 0.76, $p < 2.2e^{-16}$) between the mean abundance of detected ARGs and that of their bacterial hosts (Fig. 1C). However, interquartile range (IQR) analysis revealed inconsistencies between microbial abundance and their corresponding ARGs, according as previously reported (Wirth et al., 2025). Six microorganisms, namely: *Desulfobacillus* sp016791205_1, *Escherichia coli*, HK-STAS-PATE-34_species, JAGNNG01 sp016794365, *Nitrospira_A* sp900170025_2, and *Vicinibacter* sp016714025 exhibited significantly different abundances compared with their associated ARGs (IQR > 1.5) (Supplementary fig. 2). This indicates that the observed correlation was not entirely uniform among ARB_{MAGs}.

PCoA and Bray-Curtis dissimilarity metrics revealed distinct ARB_{MAG} community structures throughout the treatment process, showing seasonal variation and sample location-specific metabolic activity (Fig. 1F and Supplementary fig. 3). Microbial profiles from PS most closely resembled RWW, whereas BTS and MS clustered more closely with TWW. ADS exhibited the most divergent ARB_{MAG} composition (Fig. 1G and F). Dominant phyla across most treatment stages included Pseudomonadota, Bacteroidota, and Bacillota, reflecting their key metabolic roles in carbon, nitrogen and sulphur cycling (Supplementary fig. 4). Seasonal dynamics were also evident ($n = 773$ ARB_{MAGs}; Fig. 1E), with the largest shifts in Pseudomonadota ($n = 181$) (Supplementary tab. 5). *Pseudomonas_E fluvialis* was most abundant in the RWW microbiome during autumn and winter, averaging 30 % CPM (LDA score = 5.17) (Fig. 1E). Similarly, members of the *Nitrospirota* genus, central to nitrogen cycling, were significantly enriched in BTS during warmer seasons (LDA score > 3) (Fig. 1D and E). In contrast, Chloroflexota, Desulfobacterota, and Spirochaetota were more abundant in ADS and exhibited lower seasonal variation, suggesting specialised roles in anaerobic processes, such as formate and sulphur metabolism and associations with hydrogenotrophic methanogens via hydrogen generation (Wirth et al., 2023) (Fig. 1E and Supplementary fig. 4). Notably, Pseudomonadota abundance was lowest in ADS (Fig. 1G). Correlation analyses revealed that ADS-specific ARB_{MAGs} abundance positively correlated with total ammonia nitrogen (TAN) and volatile organic acids (VOAs) (Spearman's rho > 0.7), whereas Pseudomonadota members showed a negative correlation (rho < −0.5) (Supplementary fig. 5). These results suggest that, beyond anaerobic conditions, elevated TAN

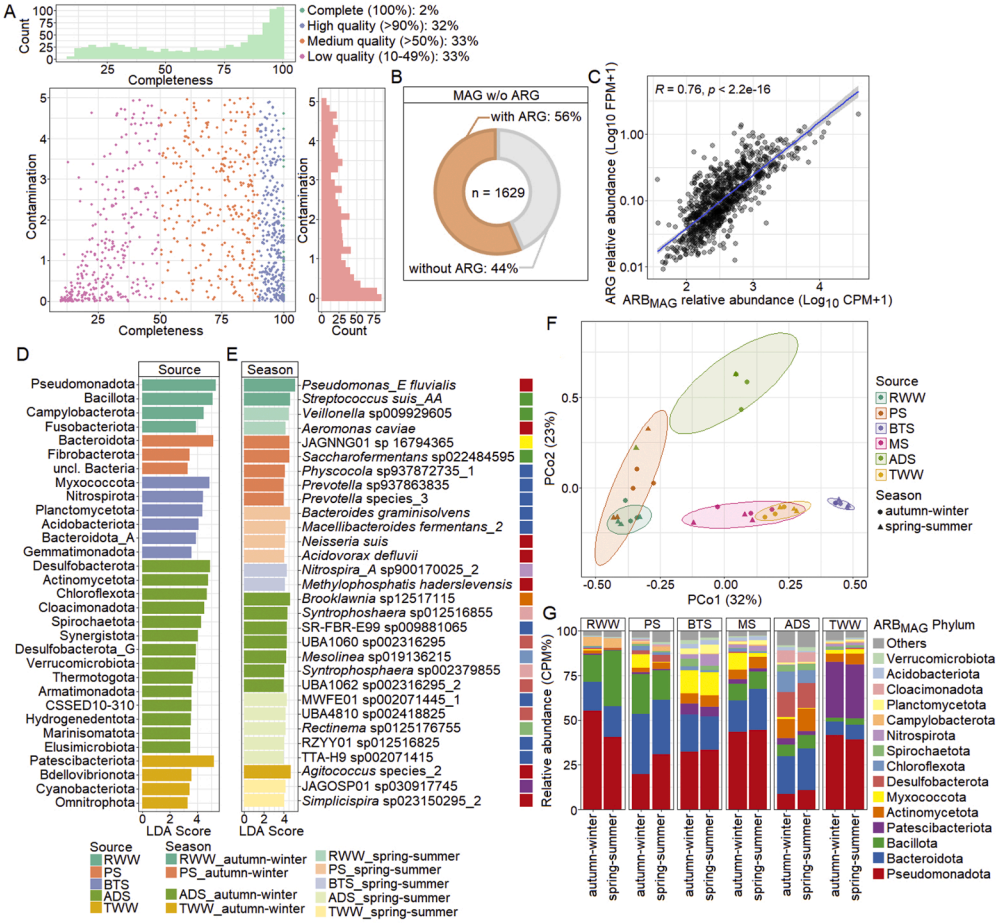

Fig. 1. Distribution of antibiotic-resistant bacterial metagenome-assembled genomes (ARB_{MAGs}) in the wastewater microbiome. (A) Distribution of reconstructed antibiotic-resistant bacteria (ARB_{MAGs}) based on genome completeness and contamination, as assessed via CheckM2. Colours indicate genome completeness levels. (B) Proportion of reconstructed MAGs with or without antibiotic resistance genes (ARGs). (C) Spearman correlation between the $\log_{10}$-transformed average abundance of ARB_{MAGs} in copies per million (CPM)and the $\log_{10}$-transformed of the ARGs they carry in fragment per million (FPM). (D) Significantly differentially abundant phyla across sample sources identified via linear discriminant analysis effect size (LefSE) analysis. (E) Significantly differentially abundant species across sources and seasons based on LefSE analysis (LDA: linear discriminant analysis). Phylum-level classifications are indicated by colour next to the species names (F) Principal coordinates analysis (PCoA) of wastewater microbiomes, based on species-level annotations. (G) Distribution of ARB_{MAG} abundances (CPM) across sampled locations.

and VOAs are important environmental drivers shaping ARB_{MAG} composition in the anaerobic digester microbiome and that ARG-harbouring microbes contribute essential functions throughout the treatment process.

3.2. Abundance of human-associated ARB_{MAGs} is reduced by wastewater treatment

Wastewater harbours a complex microbiome from diverse inputs, including human-associated and environmental sources. To assess the contribution of human-associated microbes to the ARG-harbouring microbiome, we compared our non-redundant ARB_{MAGs} set to the Unified Human Gastrointestinal Genome (UHGG) catalogue (v2.0), representing the human gut microbiome (Almeida et al., 2021) and the Searchable Planetary-scale mIcrobiome REsource (SPIRE) database, which catalogues MAGs from global environments (Schmidt et al., 2024). This allowed us to classify ARB_{MAGs} as human-associated ($H\text{-}ARB_{MAGs}$), environmental ($E\text{-}ARB_{MAGs}$) or unknown origin ($U\text{-}ARB_{MAGs}$) (Supplementary tab. 6).

In influent wastewater (i.e., RWW), ~12 % (CPM) of ARB_{MAGs} originated from the human microbiome, whereas 43 % CPM were associated with environmental and unknown origins [average nucleotide identity (ANI) cut-off: 95 %] (Fig. 2C). Treatment significantly reduced $H\text{-}ARB_{MAGs}$ abundance in final effluent (Wilcoxon test $p < 0.001$) (Fig. 2C), whereas $E\text{-}ARB_{MAGs}$ exhibited significant increase throughout the process compared with untreated wastewater (Wilcoxon test $p < 0.001$) (Fig. 2D and Supplementary fig. 6). Based on SPIRE database 'microntology' and Genome Taxonomy Database (GTDB) metadata, the most common $E\text{-}ARB_{MAGs}$ were previously found in wastewater environments ($n = 280$; 37 % CPM mean abundance of ARB_{MAGs} community), followed by those from anaerobic digesters ($n = 144$; 19 % CPM) (Fig. 2B). These findings support the notion that the treatment plant fosters a distinct microbiome beyond human inputs (LaMartina et al., 2021; McLellan and Roguet, 2019; Roguet et al., 2022).

The origin of 401 ARB_{MAGs} (24 % CPM relative abundance in ARB_{MAG} community) could not be resolved (i.e., $U\text{-}ARB_{MAGs}$; UHGG and SPIRE ANI: > 95 %) (Fig. 2B). While incomplete genome reconstruction may partially explain this, we identified 69 ARB_{MAGs} among $U\text{-}ARB_{MAGs}$ with genome completeness exceeding 95 % (based on CheckM2 analysis) that could not be classified at species-level using GTDB (Supplementary tab. 7). These unknown ARB_{MAGs} –covering 5 % CPM among the ARB_{MAG} community– were mainly affiliated with the Patescibacteria ($n = 30$) and Pseudomonadota ($n = 21$) phyla. They harboured 30 distinct ARG subtypes primarily from the glycopeptide resistance class (4 % of total ARG FPM; 3 % of total ARG TPM). Two high-quality $U\text{-}ARB_{MAGs}$, contained multiple ARGs (*Acetobacterium* species_1: $n = 7$; CAISKJ01_species: $n = 6$; Supplementary tab. 8). These novel genomes, which are either unclassified at the species level in GTDB (release 226) or do not share ≥95 % ANI with genomes in the UHGG and SPIRE databases, likely represent underexplored microbial lineages that may serve as reservoirs of ARGs.

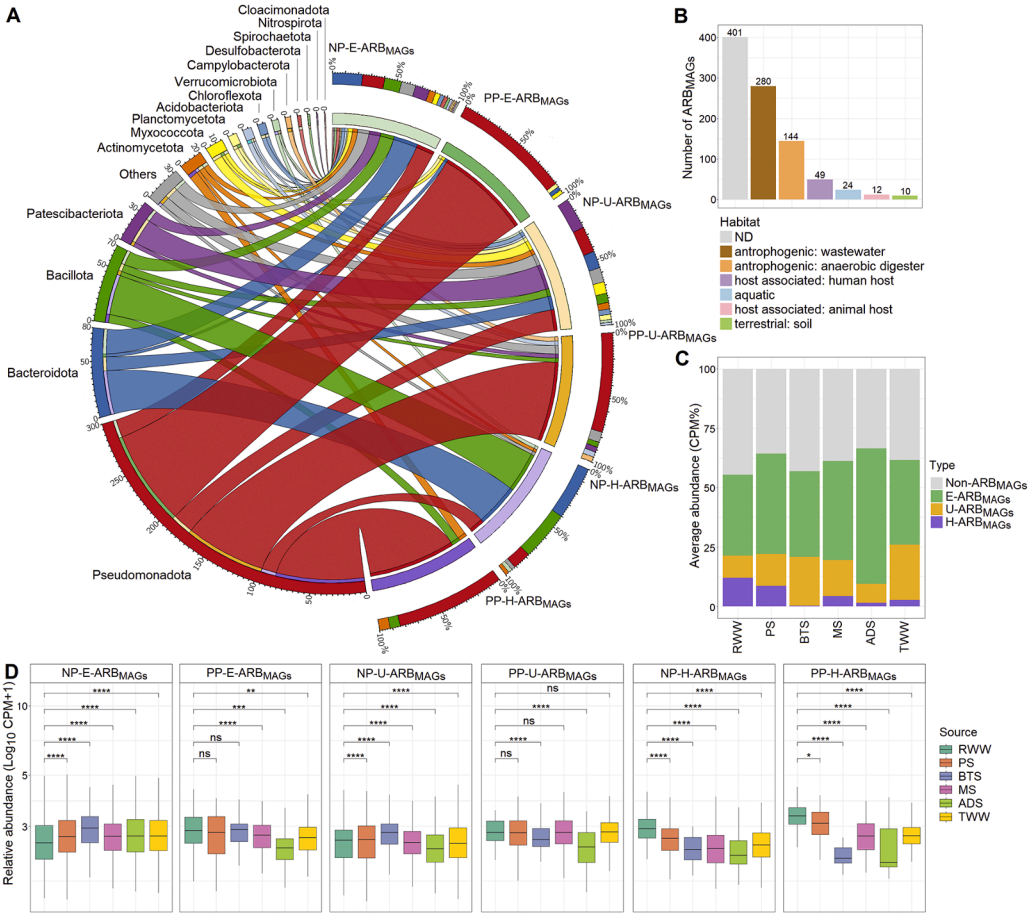

Fig. 2. Distribution of antibiotic-resistant bacterial metagenome-assembled genome (ARB_{MAG}) types in the wastewater microbiome. (A) Circos plot showing the distribution of ARB_{MAG} types across major phyla. (NP and PP: non-pathogenic and potentially pathogenic, respectively; E: environmental; U: unknown origin; H: human-associated ARB_{MAGs}) (B) Number of ARB_{MAGs} associated with the specific habitats, based on UHGG and SPIRE database classification. (C) Distribution of MAG type abundances in copies per million (CPM) across sampled locations. (D) Comparison of ARB_{MAG} abundances across locations grouped by ARB_{MAG} type. Statistical significance is indicated as follows: $p < 0.05$ (*), $p < 0.001$ (**), $p < 0.0001$ (***), $p < 0.00001$ (****), ns = not significantly different.

Beyond origin, virulence potential is a key metric for ARG-carrying microbes. Thus, analysing VF genes highlights potentially pathogenic ARB_{MAGs} (PP-ARB_{MAGs}) and the genetic elements they harbour. Metagenomic analysis revealed strong correlations (Spearman's rho > 0.7) between ARG classes and aggressive VFs linked to host invasion and competition, whereas associations with defensive traits, such as stress survival mechanisms [e.g., *kat(A)*: catalase] and biofilm formation were weaker (Spearman's rho > 0.5). In contrast, metatranscriptomic data showed weak correlations between ARGs classes and either aggressive or defensive VF types (Supplementary fig. 7). This aligns with previous findings that ARG and VF gene abundance often co-occur in wastewater metagenomes (Zhang et al., 2016, 2022a) but may not be co-expressed, implying that environmental and evolutionary factors shape these patterns (Pan et al., 2020; Waseem et al., 2017).

Taxonomic and seasonal profiling showed that Pseudomonadota dominated the PP-ARB_{MAGs}, thereby posing an elevated risk in wastewater microbial communities (Fig. 2A). Among H-ARB_{MAGs}, 12 were classified as PP-ARB_{MAGs}, with *Aeromonas* (mean 3 % CPM of ARB_{MAG} community) being most abundant, followed by *Acinetobacter* (1.6 % CPM), *Citrobacter* (0.6 % CPM) and *Escherichia* (0.4 % CPM) in RWW. These PP-H-ARB_{MAGs} were significantly abundant in untreated wastewater, particularly during spring and summer (mean LDA score = 3.6). However, their relative abundances were lowest in BTS and ADS (Fig. 2D and Supplementary tab. 5). Among U-ARB_{MAGs}, 25 were identified as PP-ARB_{MAGs}, with dominant genera including *Competibacter_A* (0.5 %), JAGOXX01 (0.4 %) and *Acetoanaerobium* (0.3 %). Their relative abundances were highest in PS and BTS across seasons, (mean LDA score = 3.6). Conversely, 56 E-ARB_{MAGs} were classified as PP-ARB_{MAGs} (i.e., PP-E-ARB_{MAGs}), with predominant genera including *Pseudomonas_E* (mean 20 % CPM of ARB_{MAG} community), JAGOXX01 (1.4 % CPM), *Azotobacter* (0.7 % CPM), and *Azomonas* (0.6 % CPM). Although overall PP-E-ARB_{MAGs} abundance was higher in RWW, particularly in spring and summer (mean LDA score = 2.97). *Azotobacter* and *Azomonas* showed no significant variation throughout the treatment process. These patterns indicate that anaerobic process are less effective at reducing the ARG-carrying environmental pathogens, emphasising the need for further monitoring non-human microbial sources of resistance.

3.3. *Most of the high-risk ARGs are harboured by Escherichia coli and Citrobacter freundii in the influent wastewater*

Mapping meta-omics reads to reconstructed ARB_{MAGs} enabled quantification of ARG abundance and expression within microbial hosts, as well as characterization of their chromosomal and plasmid-borne profiles. This enables us to conduct detailed surveillance of ARGs present in the wastewater microbiome. Machine learning-based analysis identified 82 plasmid sequences carrying 61 distinct ARGs, accounting for 10 % of total ARG abundance and 7 % of overall ARG expression. No ARGs were detected on prophage sequences (Fig. 3A and Supplementary tab. 8).

ARG abundance and expression patterns varied across the three ARB_{MAG} types (E- U- and H-ARB_{MAGs}) and between chromosomes and plasmids in RWW (Fig. 4A). Efflux pump-related resistance genes dominated across all categories, particularly in potential pathogens. Beta-lactam resistance was prevalent in U- and H-ARB_{MAGs}, with relative expression differing by genetic location. Glycopeptide (mean 57 % FPM and 78 % TPM) and aminoglycoside (mean 58 % FPM and 50 % TPM) resistance genes showed elevated expression in non-pathogenic (NP)-E-ARB_{MAGs} on both chromosomes and plasmids compared with their human-associated counterparts (Fig. 4A). Conversely, PP-H-ARB_{MAGs} exhibited higher expression of fluoroquinolone resistance genes relative to their abundance, primarily on plasmids (19 % FPM and 35 % TPM).

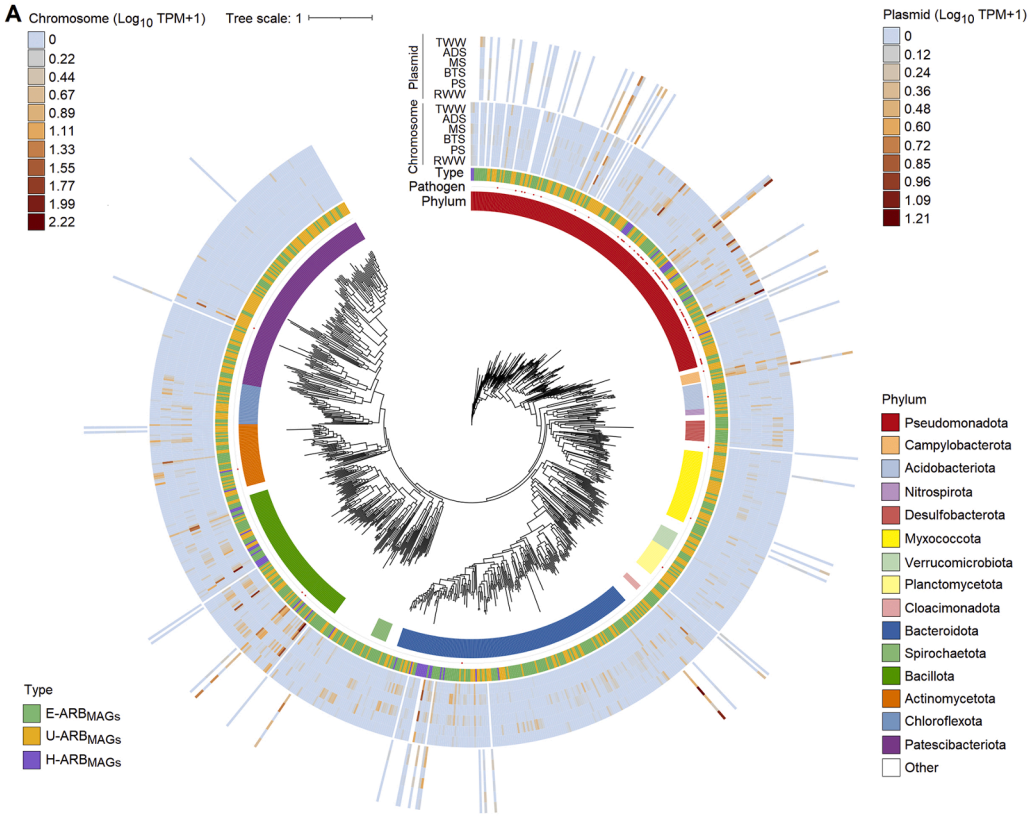

Fig. 3. Phylogenetic tree of reconstructed antibiotic-resistant bacterial metagenome-assembled genome (ARB_{MAGs}) and their antibiotic resistance gene (ARG) expression profiles. (A) Phylogenetic tree of ARB_{MAGs}, reconstructed from bacterial single-copy core genes (SCGs). From inner to outer rings: phylum level taxonomy; red dots in the next ring indicate potentially pathogenic ARB_{MAGs} (PP-ARB_{MAGs}); ARB_{MAGs} classification type. The first and second heat map rings show average expression levels of ARGs located on chromosomes and plasmids, respectively.

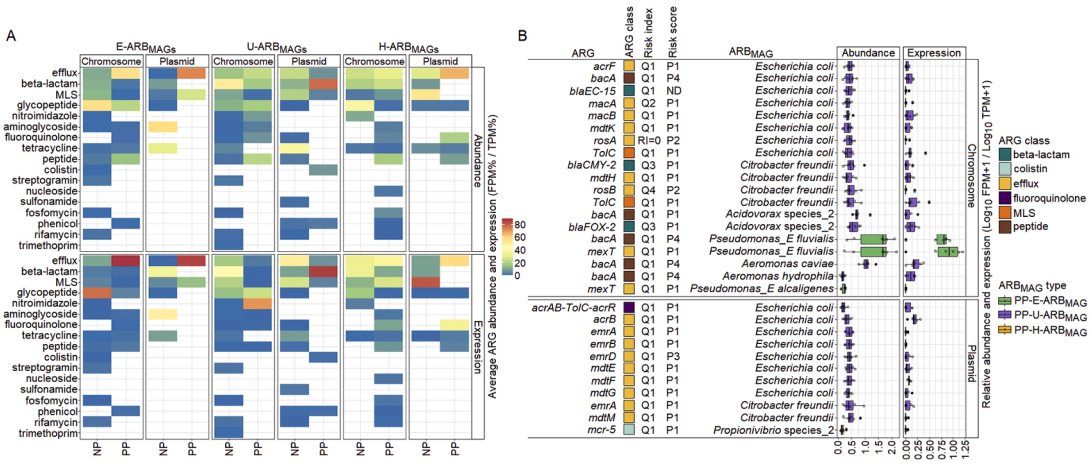

Fig. 4. Distribution of antibiotic resistance genes (ARGs) across antibiotic-resistant bacterial metagenome-assembled genome (ARB_{MAG}) types in the influent wastewater. (A) Average expression levels of ARG classes in ARB_{MAG} types, separated by chromosomal and plasmid locations. Blank spaces indicate ARG classes that are absent in the given ARB_{MAG}. (B) Relative abundance and expression of high-risk ARGs linked to their host ARB_{MAGs}. High-risk ARGs were determined using a combined risk assessment framework based on Zhang et al. and Shuai et al. (references in the text; ND: not determined).

Both NP- and PP-H-ARB_{MAGs} showed elevated expression of macrolide-lincosamide-streptogramin (MLS) resistance genes on chromosomes and plasmids (Fig. 4A). Tetracycline resistance genes were more abundant on plasmids of NP-U-ARB_{MAGs} (38 % FPM and 9 % TPM), whereas nitroimidazole resistance was highly expressed on chromosomes of PP-U-ARB_{MAGs} (8 % FPM and 71 % TPM), compared to E- and U-ARB_{MAGs}.

Using the combined risk assessment framework of Zhang et al. (Zhang et al., 2022b) and Shuai et al. (Shuai et al., 2024), we characterised the risk levels of ARGs in the microbiome of RWW. The most abundant high-risk ARGs associated with PP-ARB_{MAGs} were affiliated with efflux, peptide, beta-lactam, and MLS classes, consistent with previously observed abundance and expression profiles (Fig. 4A and B). Most high-risk ARGs were carried by PP-H-ARB_{MAGs}. Among these, *E. coli* harboured 16, while *Citrobacter freundii* carried 6 high priority

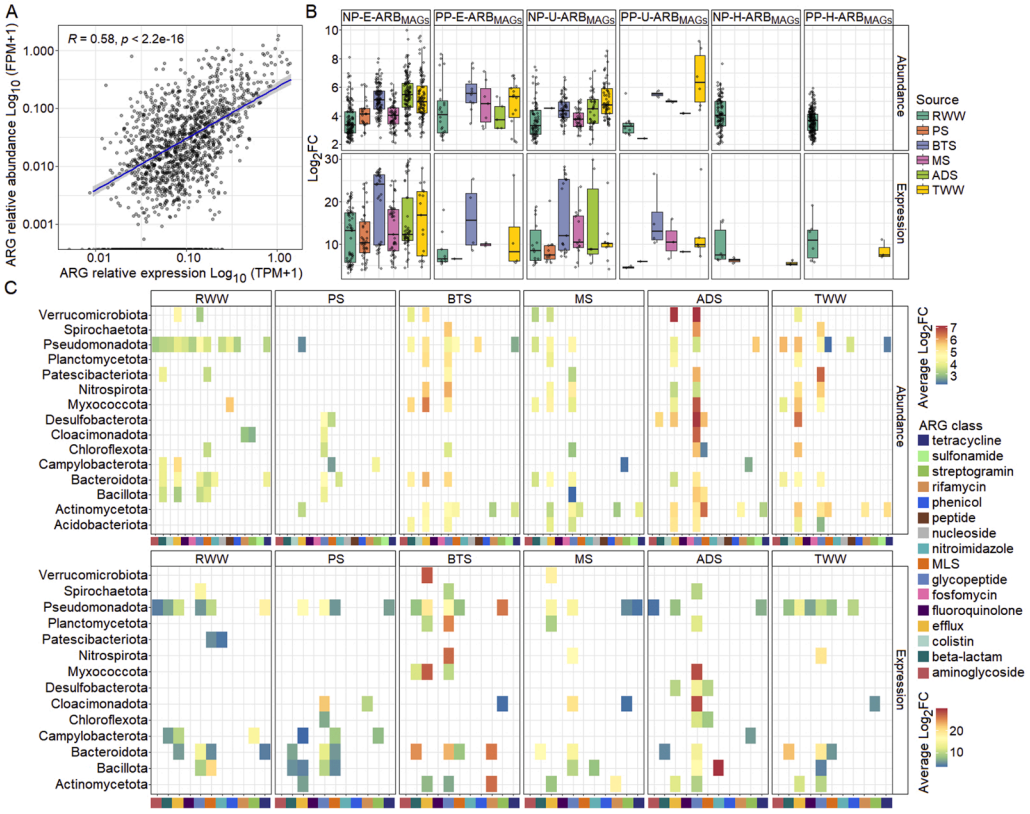

Fig. 5. Antibiotic resistance genes (ARGs) abundance and expression across antibiotic-resistant bacterial metagenome-assembled genome (ARB_{MAG}) types and sample locations. (A) Spearman correlation between log_{10}-transformed average abundance (FPM + 1) and log_{10}-transformed average expression (TPM + 1) of ARGs. (B) Genes significantly different in abundance and or expression across ARB_{MAG} types and locations. (C) Average log_2 fold change (Log_2FC) of significantly different (DNA-level) and differentially expressed (RNA-level) ARGs across bacterial phyla and sample locations. ARG classes are indicated by colour. Only genes with $Log_2FC > 2$ and $p \leq 0.05$ calculated via DESeq2 are considered statistically significant and shown.

ARG subtypes, distributed across their chromosomes and plasmids (Fig. 4B). High-risk efflux ARGs were predominantly liked to fluoroquinolone and macrolide resistance, while beta-lactam genes encoded by H-ARB$_{MAGs}$ conferred cephalosporin, carbapenem and cephamycin resistance, reflecting their clinical relevance. Among E-ARB$_{MAGs}$ *Pseudomonas_E fluvialis* has the most high-risk ARGs on chromosomes. Notably, *Propionivibrio* species_2 (PP-U-ARB$_{MAG}$) carried plasmid-borne *mcr-5* gene conferring colistin resistance. Together, these findings underscore the contribution of different PP-ARB$_{MAG}$ types, particularly the members of *Enterobacteriaceae*, to the potential dissemination of high-priority ARGs in wastewater.

3.4. Contextual insights into ARG abundance and expression during wastewater treatment

To assess ARG behaviour across wastewater treatment, we examined individual gene-level patterns in abundance and expression within E-, U-, and H-ARB$_{MAGs}$ at each treatment stage. For consistency with previous studies, we adopted the 'ARG copy per cell' metric to access significant differences, as recommended by Yin et al. (Yin et al., 2023).

We found that 72 % of identified ARGs were transcriptionally active, but their abundance and expression varied across treatment stages. The lowest relative ARG expressions were observed in the BTS and ADS treatment stages, where most ARGs were transcriptionally silent (Fig. 3A). A moderate but significant correlation was observed between ARG relative abundance and expression (Spearman's rho = 0.58, $p < 2.2e^{-16}$) (Fig. 5A). IQR analysis identified 224 ARGs with disproportionate expression relative to abundance (1.5 × IQR) (Supplementary fig. 8). Among these, the most notable differences were observed in the efflux ARG class genes *adeF* (observed 22 times), and *rsmA* ($n = 10$), as well as in glycopeptide class clusters: *vanT in vanG* ($n = 41$), *vanW in vanI* ($n = 20$), and *vanY in vanB* ($n = 10$) (Supplementary tab. 9).

The most pronounced shifts in abundance and expression were observed in two glycopeptide resistance genes (*vanT* in *vanG* and *vanW* in *vanI*), and one efflux gene (*adeF*), which exhibited the strongest differential patterns [average log2 fold change (FC) > 5, $p < 0.05$ compared with RWW]. The *adeF* and *vanW in vanI* genes were common across E-, U-, and H-ARB$_{MAGs}$ in the wastewater resistome; therefore, were considered these as core ARGs (Supplementary fig. 9). Interestingly, *vanT* in *vanG* and *vanW* in *vanI* exhibited significant expression changes only in NP-ARB$_{MAGs}$, particularly during the BTS and ADS treatment stages, where higher expression levels were observed compared with RWW (*vanT* in *vanG* in BTS: average $\log_2FC = 11$, $p = 0.006$, in *vanT* in *vanG* ADS: mean $\log_2FC = 17$, $p = 0.009$; *vanW* in *vanI* in BTS: mean $\log_2FC = 13$, $p = 0.030$, in *vanW* in *vanI* ADS: mean $\log_2FC = 13$, $p = 0.013$). Similarly, *adeF* showed increased expression, although levels were elevated in all ARB$_{MAG}$ types and sampling locations (Supplementary tab. 10). These results underscore the importance of contextual data for identifying habitat-specific ARG dynamics during wastewater treatment.

Overall, wastewater treatment significantly reduced ARG abundance in H-ARB$_{MAGs}$ (Fig. 5B), whereas E-ARB$_{MAGs}$ and U-ARB$_{MAGs}$ typically showed increased ARG abundance and expression compared with RWW ($\log_2FC > 2$, $p < 0.05$). Most ARG changes occurred in Pseudomonadota with microbes this phylum showing location-specific and ARB$_{MAG}$-type specific patterns in the abundance and expression of *patA, mexT*, and *CRP*, which are associated with fluoroquinolone resistance. Among Pseudomonadota representatives classified as PP-E-ARB$_{MAGs}$, the *mexT* gene showed decreased abundance and expression at the anaerobic digestion stage (i.e., ADS) ($\log_2FC > 3$, $p = 0.05$ compared with RWW). For Pseudomonadota considered PP-H-ARB$_{MAGs}$, 28 ARGs showed decreased abundance in ADS. Among these, *patA* and *mexT* exhibited significantly reduced abundance ($\log_2FC > 2$, $p < 0.05$ compared with RWW), although their expression levels were not notably altered during this treatment stage. Conversely, TWW samples displayed increased expression of *mexT, CRP*, and *patA* ($\log_2FC > 6$, $p < 0.02$ compared with RWW) (Fig. 5C). However, the abundance and expression of these genes remained unchanged in PP-U-ARB$_{MAGs}$ across sampling locations.

Genome-resolve metagenomics enable MAG-level analysis of ARG abundance and expression. We identified the top 15 ARB$_{MAGs}$ with the most significant variations (Figs. 6 and 7). ARGs typically varied more in abundance than in expression ($\log_2FC > 2$, $p < 0.05$ compared to RWW) (Figs. 4A and 6 and 7) (Liu et al., 2019; Wirth et al., 2025). For example, while all high-risk ARGs in *E. coli*—including efflux, beta-lactam, MLS, and peptide ARG classes—showed a significant decrease in abundance, only acrB exhibited a change in expression ($\log_2FC > 8$, $p < 0.01$) (Fig. 7). In contrast, Citrobacter freundii showed a significant decrease in ARG abundance ($\log_2FC > 2$, $p < 0.05$), but no change in expression compared with RWW. Interestingly, patA (associated with fluoroquinolone resistance) was strongly upregulated post-treatment in *E. coli* ($\log_2FC > 9$, $p < 0.01$ in TWW compared with RWW). Similarly, in ADS, patA expression remained steady despite declining abundance in C. freundii and *E. coli*. Pseudomonas_E fluvialis also showed significantly reduced mexT abundance and expression in ADS compared with RWW ($\log_2FC > 5$, $p < 0.01$). Collectively, these results suggest that although ARG abundance declined post-treatment, expression did not always follow this trend, highlighting decoupled dynamics between gene presence and activity.

3.5. Clinically relevant ARGs and wastewater treatment efficiency

A prior study using multiple methodologies identified a wide range of resistance mechanisms against both traditional and newly developed antibiotics in clinically relevant ESKAPE pathogens (Daruka et al., 2025; Maharramov et al., 2025). Sequencing of multiple resistant isolates revealed diverse resistance mechanisms, some already present in clinically relevant microbiome genomes, highlighting their potential impact on therapeutic outcomes. Building on these observations, we examined wastewater treatment systems as key environments for the emergence and spread of clinically relevant ARGs. We compared our omics data to previously reported resistance gene sequences to assess ARG diversity and activity across treatment stages, with a particular focus on their dissemination potential.

We identified four ARGs with high sequence identity to known clinical resistance determinants (>99 % identity, average e-value: $5e^{-128}$): *cblA* (beta-lactam), *ArnT* (peptide), *tet(A)* (tetracycline) and *ramA* (efflux) (Supplementary tab. 10). Of these, *tet(A)* had the highest relative abundance and expression (0.56 FPM % and 0.16 TPM % of total ARGs). *ArnT* and *cblA* exhibited lower levels (*ArnT*: 0.061 FPM %, and 0.002 TPM %; *cblA*: 0.057 FPM % and 0.009 TPM %) and *ramA* was the least prevalent (0.027 FPM % and 0.01 TPM % of total ARGs) (Supplementary tab. 8).

Two of these genes were detected in H-ARB$_{MAGs}$: *ArnT* in *C. freundii* (PP-H-ARB$_{MAG}$) and *cblA* in *Bacteroides uniformis* (NP-H-ARB$_{MAG}$) (Fig. 8A). Both were chromosomally encoded, indicating vertical inheritance and reduced likelihood of immediate horizontal gene transfer. The remaining two ARGs, *tet(A)* and *ramA*, were found in NP-U-ARB$_{MAG}$ hosts. Specifically, *tet(A)* was harboured by *Azonexus* species 5 (semibin2_5_62_sub) and *ramA* by J088 species 4 (semibin2_6_400_sub), with both genes located on plasmids (Fig. 8A and B).

Notably, *tet(A)* in *Azonexus* species 5 (semibin2_5_62_sub) showed significant reductions in abundance ($\log_2FC = 2.43$, $p = 0.002$) and transcription ($\log_2FC = 6.01$, $p = 0.024$) compared with RWW. Conversely, *cblA* and *ArnT*, associated with human-related MAGs, e.g. *B. uniformis* and *C. freundii*, exhibited significantly decreased abundance across the treatment stages with no observed differences in expression levels (*cblA* abundance: $\log_2FC = 4.15$, $p = 0.011$; *ArnT* abundance: $\log_2FC = 3.38$, $p = 0.027$ compared with RWW) (Supplementary tab. 9). This likely reflects their already low transcriptional activity in untreated influent (Fig. 8B). Similarly, *ramA* in J088 species 4 (semibin2_6_400_sub) showed no significant changes in abundance or expression, consistent with persistently low ARG transcription

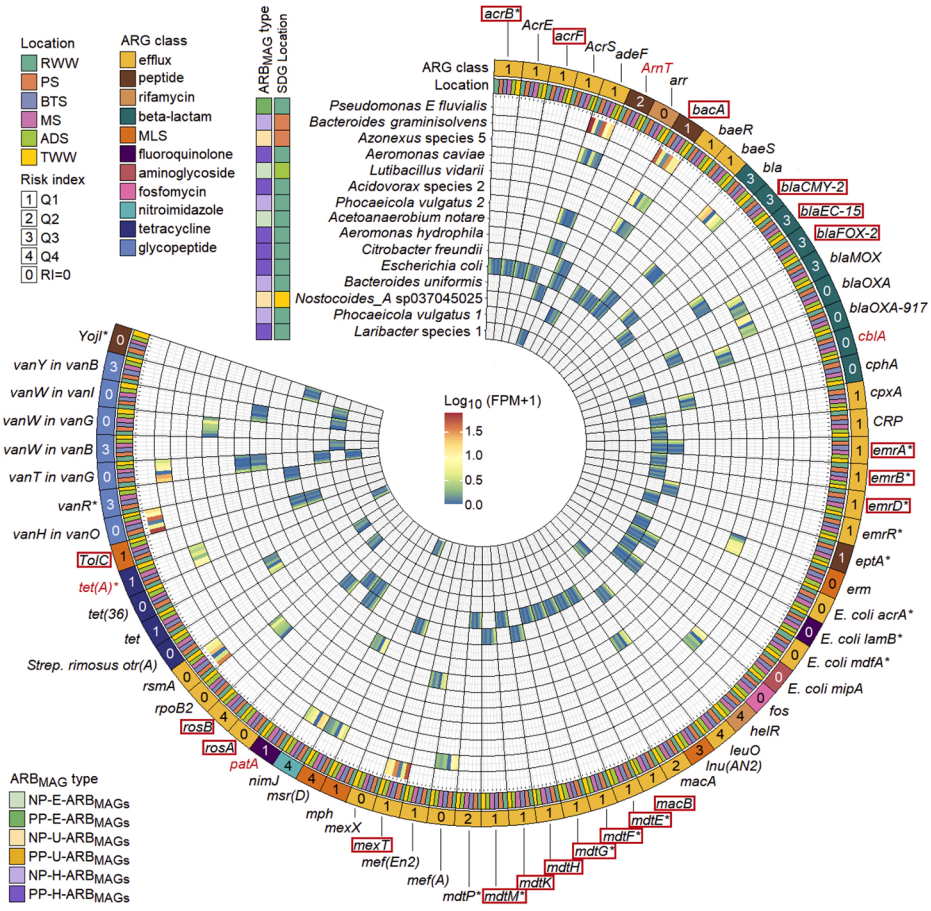

Fig. 6. Significantly different antibiotic resistance genes (ARGs) in selected antibiotic-resistant bacterial metagenome-assembled genomes (ARB_{MAGs}). (A) From outer to inner rings: specific ARG names (red box around the name: clinically relevant, red name: additional clinical relevance); * plasmid-borne; ARG class and risk index; and sample locations. Inner heat-map displays the average abundance of each ARG in specific sample locations. Colours next to ARB_{MAG} names donate locations where those ARGs are significantly more abundant. ARB_{MAG} classification types are shown to the left.

throughout treatment. These findings suggest that wastewater treatment can reduce the abundance of clinically relevant but relatively rare ARGs. However, gene expression levels appeared to be more influenced by initial transcription activity than by treatment effects (Fig. 8B). Although their reduced abundance and low expression may limit immediate risk, the persistence of genes such as *ramA* at the DNA level poses a latent threat, particularly if mobilized under different environmental conditions downstream.

4. Discussion

In this study, we investigated the resistome of an industrial-scale municipal WWTP using an activated sludge system combined with anaerobic digestion, two commonly implemented treatment strategies. By integrating genome-resolved metagenomics with metatranscriptomics, we characterised the resistome at taxonomic, functional and transcriptional levels. This combined approach enabled us to move beyond ARG detection and evaluate potential risk linked to their active expression across both chromosomal and plasmid elements.

We observed notable discrepancies between microbial and ARG abundances, indicating that ARG presence does not uniformly correlate with bacterial population size. This mismatch may be due to gene duplication events (Maddamsetti et al., 2024) (e.g. *adeF)* or to strain-level variation within MAG populations, where not all representatives carry the same set of genes, including ARGs. Our findings support the latter explanation as more common, consistent with previous reports (Albertsen et al., 2013; Sangwan et al., 2016). This highlights the value of genome-centric metagenomics over correlation-based methods commonly used to assign ARGs to microbial hosts (Lal Gupta et al., 2020; Sun et al., 2021).

Analysis across treatment stages revealed distinct ARB_{MAG} community structures, with observable seasonal variation. Metabolic profiling showed that most ARB_{MAGs} were involved in carbon, nitrogen and sulphur cycling and supported syntrophic interactions essential for efficient and stable wastewater treatment (Wirth et al., 2025). Members of phylum Pseudomonadota varied by treatment stage and season (Chobert et al., 2025). For example, *Pseudomonas_E fluvialis* was dominant ARB_{MAG} in influent samples during autumn and winter. Although this genus is commonly found in wastewater microbiomes, seasonal trends are not consistently observed across geographic regions with diverse climates (Becsei et al., 2024). However, in our study its increased prevalence in colder months aligns with prior observations reinforcing the importance of accounting for local climate when designing wastewater sampling strategies.

To determine the contribution of human-associated microbes to the wastewater microbiome, we compared our reconstructed, non-redundant ARB_{MAGs} against reference genome datasets (UHGG and SPIRE) (Almeida et al., 2021; Schmidt et al., 2024). This allowed us to infer microbial origin and link wastewater-derived taxa to known human or environmental sources, although such inferences remain constrained by methodological limitations (Jain et al., 2018). Prior studies have reported a relatively low abundance of human-associated microbes in wastewater (e.g. an average normalised read count of 14 %), albeit with variation among geographic locations (Becsei et al.,

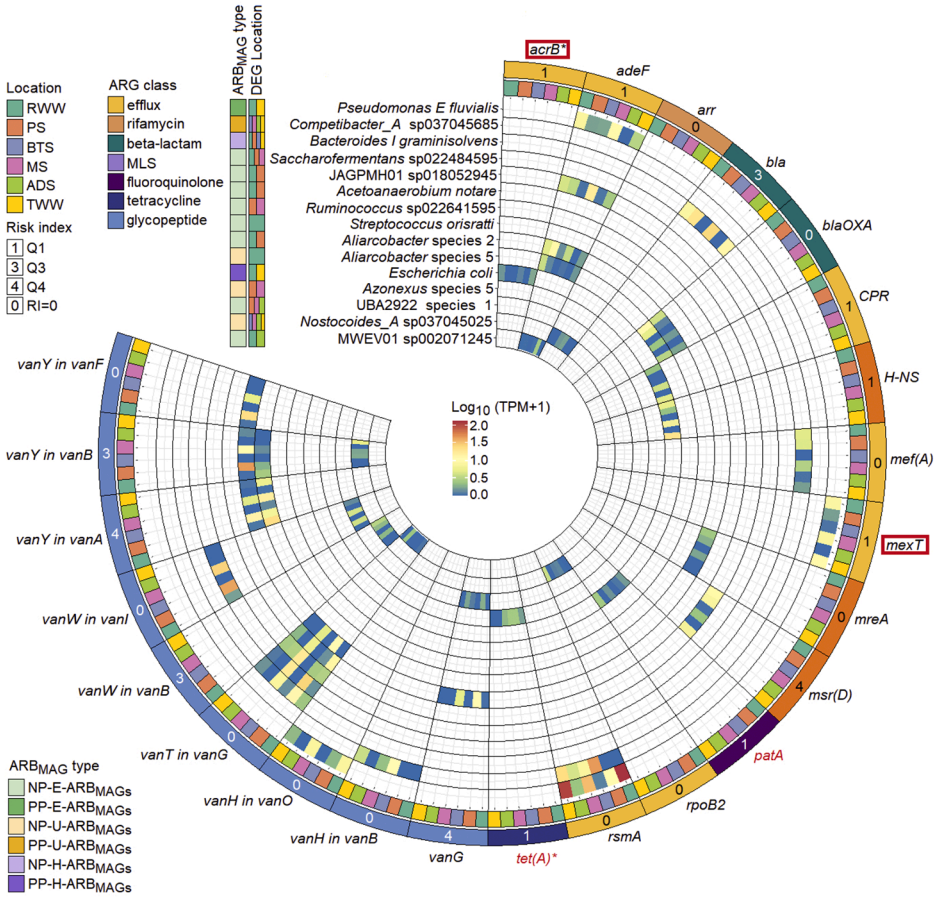

Fig. 7. Differentially expressed antibiotic resistance genes (ARGs) in selected antibiotic-resistant bacterial metagenome-assembled genomes (ARB_{MAGs}). (A) From outer to inner rings: specific ARGs names (red box around the name: clinically relevant, red name: additional clinical relevance); * plasmid-borne; ARG class and risk index; and sample locations. Inner heat-map displays average expression levels for each ARG in specific sample locations. Colours next to ARB_{MAG} names indicate locations where ARGs are significantly differentially expressed. Classification type is shown to the left.

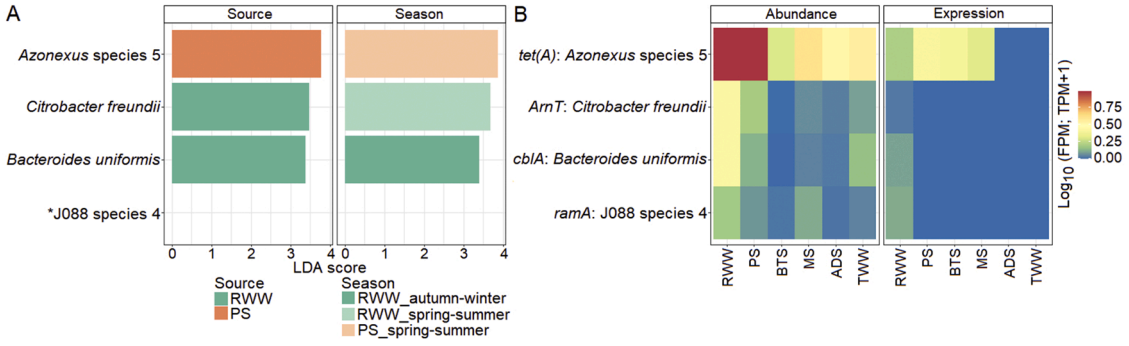

Fig. 8. Clinically relevant antibiotic resistance genes (ARGs) and their antibiotic-resistant bacterial metagenome-assembled genome (ARB_{MAG}) hosts. (A) Significantly enriched ARB_{MAG} species across sample locations and seasons, as identified using linear discriminant analysis effect size (LEfSe). *: ARB_{MAG} not identified as a biomarker in any sample location. (B) Heat-map showing the average abundance (log_{10} FPM) and expression (log_{10} TPM) of clinically relevant ARGs and their corresponding ARB_{MAG} hosts across sampled locations.

2024). This aligns with our findings (an average CPM of 12 %) providing context for interpreting microbial shifts during wastewater treatment.

We found that wastewater treatment effectively reduced the relative abundance of H-ARB_{MAGs}, including potentially pathogenic taxa. Conversely, E-ARB_{MAGs}, particularly those adapted to wastewater and anaerobic digester conditions, became increasingly dominant. Selective pressures likely favoured these treatment-adapted microbial populations, a trend that may have been overlooked in earlier studies of antibiotic resistance of microorganisms (Manaia et al., 2018). Additionally, a substantial proportion of previously unclassified ARB_{MAGs}, many carrying diverse ARGs and affiliated with various Pseudomonadota taxa, highlighting the complexity of wastewater microbial ecosystems. This is notable given that most potentially pathogenic microbes belonged to the Pseudomonadota phylum (Liang et al., 2020; Wang et al., 2024; Zhang et al., 2018). ARGs often co-coexist with VFs in PP-ARB_{MAGs} and their independent expression patterns suggest heterogeneous regulatory mechanisms across populations (Pan et al., 2020; Waseem et al., 2017). Overall, our findings show that although

wastewater treatment reduces ARG-carrying pathogen levels, and mitigates immediate public health risks, it also maintains a unique, environmentally shaped resistome influenced by treatment conditions (Honda et al., 2023).

WWTP also serves as active reservoirs for mobile ARGs, a large proportion of which were plasmid associated in our dataset. These ARGs frequently encoded efflux pumps, MLS and beta-lactam resistance, underscoring the broad-spectrum potential of plasmid-mediated resistance. The concentration of high-risk ARGs on plasmids raises concerns regarding interspecies transfer, especially between human-associated, environmental, or unclassified microbes (Redondo-Salvo et al., 2020). In line with this, we identified the *mcr-5* gene, conferring resistance to colistin, located on a plasmid sequence from a U-ARB_{MAG}, highlighting the potential for unrecognized environmental reservoirs to act as sources of clinically relevant resistance determinants. Notably, no ARGs were detected on prophage-associated sequences, suggesting that plasmids, rather than prophages, serve as the primary vectors of resistance in this system (Coluzzi and Rocha, 2025). However, identifying MGEs in MAGs remains technically challenging (Maguire et al., 2020). Future studies incorporating complementary approaches, such as long-read sequencing, may markedly improve the resolution of ARG context and mobility in wastewater microbiomes (Dai et al., 2021).

Although wastewater treatment significantly reduced the abundance of ARGs (n = 94 ARG sub-types), our metatranscriptomic analysis revealed that this decrease did not consistently correspond with reduced gene expression. Indeed, 72 % of ARGs were transcriptionally active across treatment stages, underscoring the need to incorporate expression-based metrics into risk assessments. Particularly in BTS and ADS, several resistance ARGs, especially those conferring glycopeptide and efflux resistance exhibited disproportionately high expression relative to their genomic abundance. Among these, *vanT* and vanW, both part of the *vanG* cluster for glycopeptide resistance, as-well-as the efflux pump gene adeF were consistently and highly expressed across E-, U- and H-ARB_{MAGs}. These ARGs have been reported in high abundance in previous WWTP studies (Zhu et al., 2025). Notably, *van* resistance gene homologues have been found on the chromosomes of permafrost soil bacteria and in environmental NP microbes (D'Costa et al., 2011; Wright, 2010). In our study, *adeF* emerged as a core ARG, consistently detected across all ARB_{MAGs} types (Supplementary fig. 8) (Munck et al., 2015; Xu et al., 2023). Its sustained high expression along with that of *van* homologues, indicates their potential role in preserving antimicrobial resistance in the wastewater microbiome (Rizzo et al., 2013).

Despite the persistence of some highly expressed ARGs (i.e., *adeF* and *van* ARG homologs), our results, consistent with earlier findings, demonstrate that anaerobic treatment (AD) effectively reduces the prevalence of PP-H-ARB_{MAGs} and their associated high-risk resistance genes (Wirth et al., 2025). The most significant declines in high-risk ARGs were observed in ADS relative to RWW. Furthermore, we identified a moderate negative correlation between the abundance of Pseudomonadota representatives and levels of TAN and VOAs during AD (Spearman's rho < 0.5), suggesting a role for AD in suppressing human-associated, potentially pathogenic microbes (Ma et al., 2022; Zhao and Liu, 2019).

According to previous findings, ARG profiles of H-ARB_{MAGs} in wastewater are often consistent with national antibiotic sales or consumption patterns (Lee et al., 2023; Pärnänen et al., 2019). In our study, this tendency was also observed when comparing national antibiotic consumption records with the ARG distribution patterns (Supplementary fig. 10). Additionally, ARG profiles also displayed location-specific and ARB_{MAG}-type-specific trends. High-risk ARGs, such as efflux pumps, beta-lactamases, and MLS genes (conferring resistance to fluoroquinolone, cephalosporins and macrolides), generally declined in abundance, but most remained unchanged in expression in emerging and clinically relevant *Enterobacteriaceae* species (Jabeen et al., 2023; Xu et al., 2025). Notably, *patA, mexT* and *CRP* ARGs followed distinct abundance and expression trajectories. Among PP-E-ARB_{MAGs}, *mexT* declined in both abundance and expression during AD, but remained stable in TWW. In contrast, PP-H-ARB_{MAGs} showed decreased abundance of *patA, mexT* and *CRP* in ADS, whereas their expression remained in TWW. PP-U-ARB_{MAGs} exhibited no significant changes in either abundance or expression across treatment locations. Both *patA* and *mexT* have been linked to fluoroquinolone resistance (Garvey et al., 2011; Llanes et al., 2011), whereas *CRP* is a transcriptional regulator involved in stress response and efflux regulation (Nishino et al., 2008); its role in modulating fluoroquinolone resistance has previously been reported (Kary et al., 2017). Given the widespread use of fluoroquinolones for treating urinary infections in Central and Eastern Europe (Benko et al., 2019), moreover their persistence ability in WWTP effluent likely exerts selective pressure on microbial communities (Gregova et al., 2021; Janecko et al., 2016). This selective pressure likely promotes the upregulation of resistance genes, such as *CRP, patA*, and *mexT*. These findings reinforce the value of ARB_{MAG}-type-specific transcriptomic monitoring for more accurately assessing functional resistance potential beyond simple gene presence.

Tracking the emergence and persistence of resistance to clinically used and investigational antibiotics offers valuable insights for public health and clinical management. Our study identified additional clinically relevant ARGs in wastewater microbiome that exhibited differing patterns of relative abundance and expression throughout the treatment process. These ARGs have been associated with resistance to widely used antibiotics (e.g. doxycycline, omadacycline, and eravacycline) as-well-as drug candidates (i.e., SPR-206) in clinically important ESKAPE pathogens (Daruka et al., 2025). Resistance mechanisms observed included enzymatic degradation, membrane modification, and efflux activation. Importantly, the detection of an ARG does not automatically imply functional resistance, especially for regulatory genes, such as *ramA* (Mariscotti and Véscovi, 2021). For instance, in *Citrobacter freundii* PP-H-ARB_{MAGs} representative, we detected multiple high-risk ARGs co-occurring with *arnT*, indicating a greater resistance potential compared with NP-ARB_{MAGs} carrying similar clinically relevant ARGs but lacking co-localisation. Overall, our results suggest that wastewater treatment can effectively reduce clinically relevant ARGs. However, genes with low expression in the WWTP environment may still pose latent threats if reactivated under different environmental or selective conditions, especially in PP-H-ARB_{MAGs}.

The compositional data presented in this study reflect proportional variances among sampling sites and seasons. Currently, genome or gene copies per million as well as the recently proposed 'ARG copies per cell' are widely used as relative quantification metrics, and these different units help elucidate distinct aspects of ARG-related issues (Yin et al., 2023). Although these metrics are based on the normalisation of sequencing depth, ARG lengths, and prokaryotic genome sizes in the dataset, they pose a limitation for the present study, as absolute quantifications may provide a different picture of microbial density and the ARGs they harbour (Supplementary fig. 11) (Shi et al., 2025).

5. Conclusion

This study provides a genome- and transcriptome-resolved view of the antibiotic resistome in a full-scale municipal wastewater treatment plant. We observed that, while treatment effectively reduced the relative abundance of PP-H-ARB_{MAGs}, a subset of ARGs remained transcriptionally active throughout the process, underscoring the limitations of abundance-based risk assessments. Notably, efflux pump and glycopeptide resistance genes were highly expressed across ARB_{MAG} types, indicating their key role in maintaining antimicrobial resistance potential. Anaerobic digestion was effective in reducing the relative abundance of high-risk ARGs and in suppressing potentially pathogenic populations. Seasonal shifts and local environmental conditions influenced ARB_{MAG} structure, reinforcing the need for spatiotemporal surveillance strategies. Fluoroquinolone resistance genes exhibited type- and location-specific expression patterns, with persistence observed

even in treated effluent, highlighting potential downstream risks. Clinically relevant ARGs were detected at low abundance but were co-localised with virulence factors in PP-H-ARB$_{MAGs}$, pointing to a potential reservoir of latent resistance with pathogenic potential. Overall, this study emphasises the importance of integrating gene expression and microbial context when assessing the resistome in WWTPs. It also underscores the need to monitor the persistence of emerging human-associated pathogens (like *Citrobacter freundii*), particularly in municipal wastewater systems.

CRediT authorship contribution statement

Roland Wirth: Writing – original draft, Visualization, Validation, Methodology, Investigation, Data curation, Conceptualization. **Bernadett Pap:** Formal analysis. **Márk Szuhaj:** Formal analysis. **Zoltán Bagi:** Writing – review & editing, Supervision, Conceptualization. **Zoltán Farkas:** Methodology, Formal analysis, Data curation. **Kornél L. Kovács:** Writing – review & editing, Supervision. **Gergely Maróti:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dr. Roland Wirth reports financial support was provided by National, Research, Development and Innovation Office. Dr. Roland Wirth reports financial support was provided by Hungarian Academy of Sciences. Dr. Zoltan Bagi reports financial support was provided by National, Research, Development and Innovation Office. Dr. Gergely Maroti reports financial support was provided by National, Research, Development and Innovation Office. Dr. Gergely Maroti reports financial support was provided by Hungarian Academy of Sciences. Dr. Zoltan Bagi reports financial support was provided by National, Research, Development and Innovation Office. If there are other authors, they 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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2025.124663.

Data availability

The raw metagenome and metatranscriptome sequences generated and analysed during the current study were deposited in the NCBI SRA under accession number PRJNA1282049. Potentially novel high-quality (compl. >90 % cont. <5 %) ARB$_{MAGs}$ ($n = 69$) are deposited in the NCBI SRA under accession numbers from SAMN49656043 to SAMN49656111. The main data generated and analysed in this study are included in this published article and its supplementary information files. Workflows and R scripts are available from the first author on reasonable request.

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