



Deciphering the antimicrobial resistomes and microbiome landscape of open drain wastewater using metagenomics in a progressive Indian state



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ABSTRACT

Antimicrobial resistance (AMR) is a growing environmental and public health concern, with wastewater systems are acting as a critical reservoirs for resistant microorganisms and genes. Open drains in densely populated and industrialized regions can accelerate AMR dissemination into the environment. Despite Maharashtra's high urban density and industrial activity, comprehensive metagenomic surveillance of its wastewater resistome is lacking. This study applied high-throughput nanopore sequencing to 138 wastewater samples collected from 23 open-drain sites across three regions of Maharashtra (Western, Mumbai, and Central). Bioinformatic pipelines were used to characterize microbial communities, resistance genes, mobile genetic elements (MGEs), and resistome risk scores. Microbial composition varied significantly across regions, with Mumbai and Central regions explaining up to 13 % of variance at the family level. Thirty indicator taxa were identified through LEfSe analysis. Resistome profiling revealed 28 drug classes and 808 ARGs, dominated by multidrug (40.49 %), macrolide-lincosamide-streptogramin (15.84 %), beta-lactam (7.95 %), and tetracycline (6.52 %). WHO-priority pathogens such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* harbored high-abundance ARGs including *sul1*, *mdr(ABC)*, and *acrB*. Resistome risk scores were highest in Mumbai, indicating elevated ecological and human health risks. These findings underscore wastewater as a hotspot for AMR persistence and spread. Integrating wastewater-based surveillance within a One Health framework enables systematic tracking of resistance trends, comprehensive assessment of environmental risks, and evidence-driven regional interventions. This integrated approach supports the development of targeted mitigation strategies to curb the spread of antibiotic-resistant contaminants across ecosystems.

1. Introduction

The rise of antimicrobial resistance (AMR) represents one of the foremost challenges to public health worldwide, posing significant threats to the effective treatment of infectious diseases and impacting both human and environmental health (Zhu et al., 2022). The World Health Organization (WHO) has underscored the critical need to

monitor AMR globally, citing wastewater environments as hotspots for the emergence and transmission of resistance determinants. Wastewater particularly from urban centres, industrial sites, and healthcare facilities, serves as a convergence point for a diverse array of microbial communities exposed to antibiotics and other selective pressures, thereby facilitating the enrichment and dissemination of resistance genes (Berendonk et al., 2015; Manaia, 2017). As untreated wastewater

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flows through open drains into broader ecosystems, it provides a fertile environment for gene transfer among microbes via mobile genetic elements (MGEs), enhancing the risk of horizontal transfer of AMR traits across species and potentially introducing resistance genes into natural water systems and soil (Rizzo et al., 2013; Von Wintersdorff et al., 2016).

In densely populated and industrialized regions, such as Maharashtra, India's second-most populated state and home to extensive pharmaceutical manufacturing, wastewater systems may serve as significant reservoirs for AMR genes. Maharashtra's high urban density and industrial activity and Mumbai's role as a major economic centre highlight the need for comprehensive surveillance to monitor and understand AMR patterns in the region. Despite this pressing need, there is currently no in-depth study assessing the resistomes within Maharashtra's wastewater networks. A detailed AMR profile of this area could offer critical insights into the role of anthropogenic activity in shaping microbial resistance landscapes, facilitating early detection and targeted intervention strategies to mitigate AMR spread (Hendriksen et al., 2019). By providing a foundation of data on resistance patterns, such an investigation would not only fill a significant knowledge gap but also inform regional and national health policies aimed at AMR management.

While prior studies from India have provided valuable insights into AMR in wastewater, they are often restricted to local scales, hospital effluents, or culture-based and amplicon sequencing approaches, leaving a gap in large scale spatially resolved analysis. In contrast, this study addresses critical gaps in environmental antimicrobial resistance (AMR) surveillance by applying a high-throughput metagenomic approach to analyse wastewater from 23 open-drain sites across Maharashtra, India. These sites were strategically selected to represent a wide spectrum of population types including urban, rural, sub-urban, industrial, and hospital-dominated would reveal differential patterns in AMR abundance. Focusing on three representative regions – the Western Region (WR), the Mumbai Region (MR), and the Central Region (CR), we aimed to: (1) Compare microbial community structures and resistome profiles across regions; (2) Identify bacterial hosts associated with antimicrobial resistance genes (ARGs) and (3) Assess the prevalence and risk of ARGs in WHO-priority pathogens.

Recognizing the limitations of previous culture-based studies, we employed a high-throughput sequencing approach to capture uncultivable microbial populations and resistance patterns comprehensively (Brown et al., 2021; Zhao et al., 2023). Furthermore, our analysis also included the quantification of mobile genetic elements (MGEs), virulence factors, and resistome risk scores providing an overall perspective on AMR dissemination in wastewater environments (Bengtsson-Palme et al., 2018). Through this comprehensive characterization, the study generates novel insights into the environmental distribution of AMR and provides a robust data set to inform AMR risk assessment frameworks specific to Maharashtra. Ultimately, this work supports the broader public health mission of AMR management through environmental monitoring, delivering actionable data for regional interventions, and contributing to the global AMR surveillance and response network. This study not only provides insights to the regional landscape of antimicrobial resistance in Maharashtra but also contributes valuable insights for the development of environmental risk assessment frameworks and supports integration with India's national AMR action plan.

2. Material and methods

2.1. Sample collection

Wastewater samples were collected following the protocols set by the Centres for Disease Control and Prevention (CDC), USA, for COVID-19

wastewater surveillance. All procedures were approved by the Institutional Biosafety Committee (IBSC). Weekly samples were gathered from open drains in 23 cities across Maharashtra, India, starting from the fourth week of December 2022 and continuing through the fourth week of May 2023. The sampling aimed to analyse microbial diversity and antimicrobial resistance patterns. The WR included six cities: Pune (PMC), Satara (STR), Sangli (SMK), Ichalkaranji (IKZ), Kolhapur (KOP), and Pimpri Chinchwad (PCMC). The MR covered ten cities: Navi Mumbai (NM), Panvel (PNVL), Badlapur (BUD), Ambarnath (ABH), Ulhasnagar (ULNR), Kalyan-Dombivli (KYN), Bhiwandi Nizampur MC (BIRD), Thane (TNA), Mira Bhayandar (BY), and Vasai-Virar City MC (BSR). The CR region comprised six cities: Osmanabad (OBD), Jalna (JLN), Beed (BEED), Barshi (BRS), Solapur (SUR), Ahmednagar (AMN), and Aurangabad (AU) (Fig. S1). Approximately 500 mL of wastewater was collected from each site in sterile polypropylene bottles and transported in cold containers to the laboratory for analysis.

2.2. Isolation of genomic DNA and shotgun metagenomic sequencing

DNA was extracted from 250 mg of sludge samples using the DNeasy PowerSoil Pro Kit (Qiagen), following the manufacturer's protocol. DNA quality and concentrations were assessed using a Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific) and further quantified using the Qubit fluorometer (Thermo Fisher Scientific) with the Qubit Broad Range (BR) and Qubit High Sensitivity (HS) assay kits (Thermo Fisher Scientific, Q32853 and Q32851, respectively). The DNA was stored at -80 °C until further use. Only samples with a purity ratio of 1.8 (OD 260/280) were selected for further processing. Library preparation was carried out using the 1D Ligation Sequencing Kit SQK-LSK109, and the 24 samples were barcoded with Native Barcoding EXP-NBD104 and NBD114 according to the manufacturer's guidelines. The resulting library was quantified with the Qubit High Sensitivity (HS) assay kit, and approximately 500 ng of the library was loaded onto FLO-MIN 106D R10 flowcells.

2.3. Bioinformatics analysis and visualization of the results

2.3.1. Bioinformatics analysis

Raw sequencing reads were basecalled and demultiplexed using Guppy (v6.5.7) (Wick et al., 2019). A qscore cut-off of 8 was selected for base calling. NanoPack2 [NanoPlot (v1.42.0) (De Coster and Rademakers, 2023) and NanoComp (v1.12.0) (De Coster and Rademakers, 2023) were used to examine and compare read statistics. Raw reads were filtered for quality and minimum length using the NanoFilt (v2.7.2) (De Coster et al., 2018). Post filtering, the quality of the filtered reads was again assessed using NanoStat and NanoComp.

The Kraken2 (v2.1.2) (Wood et al., 2019) pipeline was used to analyse the microbial community profiles of the samples. A Kraken2 standard collection database of Kraken, which provides a k-mer-based approach for fast taxonomic classification of metagenomic sequence data, was used for taxonomic classification. The report files from Kraken2 include OTUs and taxonomic classification that can be collated to obtain a feature table containing the abundance of OTUs across all the samples and their respective taxonomies. For only real time assessment during sequencing EPI2ME platform (v3.5.7) (Oxford Nanopore Technologies) using the WIMP (what's in my pot) (Juul et al., 2015) protocol to map the reads to taxonomies providing feature tables was also carried out.

The DeepARG tool (Arango-Argoty et al., 2018) is a deep learning-based approach to predicting antibiotic resistance genes (ARGs) from metagenomes, which were used with default parameters to predict the antimicrobial drug classes and associated genes. To compare

the abundance of antimicrobial resistance genes (ARGs) across different samples, normalization was performed using a method similar to those outlined by (Ma et al., 2016; Arango-Argoty et al., 2018). In this approach, the number of ARG copies in each sample was adjusted relative to the total gigabase pairs (Gbp) in the sample. This calculation allowed for the determination of relative ARG abundances, as described in the equation below.

$$\text{Normalized copy number of ARGs} = \frac{\text{Total ARG count}}{\text{Size of the dataset in Gb}}$$

1Gbp in the data corresponds to nucleotides in the library divided by 1×10^9 . A manual script was used to extract the host reads for antimicrobial resistance drug classes and genes to identify bacterial pathogens of concern, as classified by the World Health Organization (WHO) based on their high AMR potential (Tacconelli et al., 2018).

Mobile genetic elements (MGEs) were identified using mobileOG-db (Data Version: Beatrix 1.6 v1) (Brown et al., 2022), and were divided into five major mobileOG categories, which represent the key groups of MGE-associated molecular mechanisms: Replication/Recombination/Repair (RRR), Integration/Excision (IE), Stability/Transfer/Defence (STD), Inter-Organism Transfer (T), and Phage-related biological processes (P). The resistome risk score was predicted using the tool Metacompare2.0 (Rumi et al., 2024). A bioinformatic tool specifically designed to evaluate resistome risk in environmental samples by quantifying the abundance and transfer potential of ARGs, particularly those associated with mobile genetic elements (MGEs) and pathogens. This framework provides a relative risk scoring system that allows meaningful comparisons across different regions, identifying environmental compartments with higher potential for ARG dissemination. For computing the resistome risk score, reads were assembled into contigs using the flye tool (Kolmogorov et al., 2020) with -meta tag.

2.3.2. Statistical analysis and visualization of the result

For microbial diversity analysis, we rarefied data using the single_rarefaction.py script along with a rarefy even depth function implemented in both QIIME (Bolyen et al., 2019) and R (Chambers, 2008). Further downstream analysis of feature tables (BIOM formatted) generated from either approach was performed by importing them into R using statistical packages. The data was normalized by median sequencing depth and transformed to relative counts. Alpha diversity and beta diversity measures were obtained using Phyloseq (McMurdie and Holmes, 2013). Rarefaction analysis was performed with the vegan package (Oksanen et al., 2001) to assess the sufficiency of sequencing depth for each sample. Heatmaps were generated using the manual Python script.

To identify significant differences in bacterial taxa across regions, a Linear Discriminant Analysis Effect Size (LEfSe) method was used. This approach analysed the relative abundances of specific taxa in the samples. In this study, such differentially enriched groups are referred to as indicator taxa, as they highlight region-specific shifts in microbial community composition. Initially, the Kruskal-Wallis non-parametric rank sum test was employed to detect significant variations in taxa abundance among groups. Subsequently, Linear Discriminant Analysis (LDA) was used to classify the data and evaluate the impact of significantly different taxa, as indicated by the LDA score. For the analysis, the p-value threshold for group comparisons in the Kruskal-Wallis test was set at 0.05, while a minimum logarithmic (log10) LDA score of 2.0 was required to identify discriminative features, indicating that local environmental or anthropogenic factors may drive differences in bacterial hosts carrying ARGs. The findings were displayed through a cladogram and an LDA score distribution bar plot highlighting indicator taxa.

Normality of alpha-diversity indices (Shannon, Simpson, observed richness) was first evaluated using the Shapiro-Wilk test, because the distributions were non-normal, regional differences were assessed with the Kruskal-Wallis test, followed by Dunn's post-hoc test with Benjamini-Hochberg correction to identify significant pairwise correlations. For beta-diversity, PERMANOVA was applied to evaluate overall dissimilarity in resistome composition, while PERMDISP confirmed that significant differences were not due to unequal dispersion within group.

Additionally, the outcomes of Principal Coordinate Analysis (PCoA), Adonis (group differences), and PermDisp (dispersion differences) tests were visualized. Venn diagrams, scatter plots, and cladograms were generated using the ImageGP server (Chen et al., 2022).

3. Results

A six-month study was conducted to explore microbial diversity and assess the prevalence of antibiotic resistance (ARGs) in Maharashtra. A total of 138 wastewater samples were collected from open drains across the state from December 2022 to May 2023. Utilizing nanopore MinION sequencing technology, the study generated a total of 56.99 gigabases (Gbp) of sequencing data, comprising 39.83 million reads with an average quality score of 10.68.

3.1. Regional bacterial composition and key indicator taxa in wastewater samples

A total of 6214 - 1,036,544 reads were analysed, of which 165,262 - 1,036,544 reads remained after rarefaction. Rarefied reads were analysed to determine microbial biodiversity in WW samples. 17.9 %–74.1 % of the reads were classified into bacterial taxonomic category (Table S1; Fig. S1). The analysed WW samples were colonized by bacteria representing 47 phyla, 101 classes, 216 orders, 525 families, and 1983 genera.

The cladogram (Fig. 1a) represents the hierarchical relationships between significantly different taxa across the three regions. Each coloured branch corresponds to a bacterial taxon, with red for the CR, green for the MR, and blue for the WR, and the yellow nodes signify taxa that are not significantly different across regions. Linear discriminant analysis (LDA) effect size (LEfSe) approach to determine the differences in the prevalence of the classified bacterial taxa in WW samples across three different regions of Maharashtra. The three primary color-coded panels illustrate that thirty microbial taxa exhibited significant differences in prevalence when the Linear Discriminant Analysis (LDA) score for discriminative features was set at 2.0 (Fig. S2). These thirty bacterial taxa, which were identified in wastewater samples from the three regions, may be considered potential indicator taxa, as summarized in the table (Table S2).

The major potential indicator taxa for the WR include the class *Gammaproteobacteria* with average relative abundance of 21.6 %, which consists of *Aeromonas* (3.1 %) and *Moraxella* (1.4 %) genera. In the Mumbai region (MR), the class *Betaproteobacteria* emerged as a major indicator taxa, exhibiting an average relative abundance of 23.3 % consists of *Ideonella* (2.4 %) and *Alicycliphilus* (5.2 %) genera. *Komagataeibacter* (1.4 %) and *Erwinia* (1.1 %) genera are also the potential markers of MR. These genera belong to the phylum *Pseudomonadota* with an average relative abundance of 58.3 % is also among the most abundant phyla across all three regions. The major potential indicator taxa for the CR include the class *Epsilonproteobacteria* with an average relative abundance of 27.0 % consists of *Arcobacter* (6.8 %) and

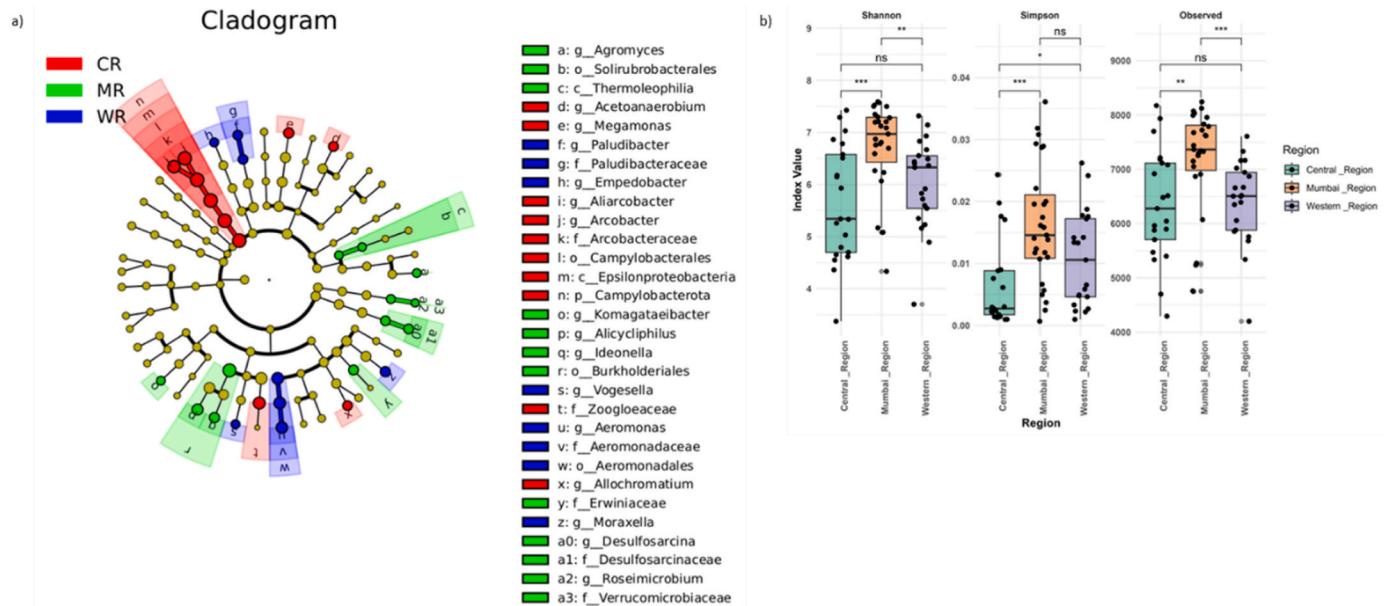


Fig. 1. Regional differences in microbiome community. a - Cladogram plotted based on the results of the LEfSe analysis. In the cladogram, circles radiating from the inside to the outside represent different classification ranks from the phylum to the genus. Each circle represents a distinct taxon at the corresponding taxonomic level, and its diameter is positively correlated with relative abundance. Taxa whose abundance did not differ significantly are marked in yellow, and indicator taxa ($p < 0.05$, LDS score > 2) are marked with different colours based on the corresponding groups. b - alpha diversity metrics (Shannon, Simpson, Observed) of bacterial communities at the genus level.

Aliarcobacter (18.3 %) genera (Fig. S3 and S4).

3.2. Statistical evaluation of taxonomic diversity and compositional variability across regions

Before conducting the comparative analysis, we assessed the normality of the diversity indices (Simpson, Shannon, and Observed

richness) using the Shapiro-Wilk test, none of the indices (Table S3) followed a normal distribution (all p -values < 0.05), so we opted for non-parametric methods, specifically the Kruskal-Wallis test used to compare groups. Simpson index ($\chi^2 = 14.335$, $p = 0.0007714$), Shannon index ($\chi^2 = 14.12$, $p = 0.00086$), and observed richness ($\chi^2 = 12.72$, $p = 0.00173$). These findings suggest that at least one region differs significantly in terms of taxonomic diversity and richness across all indices.

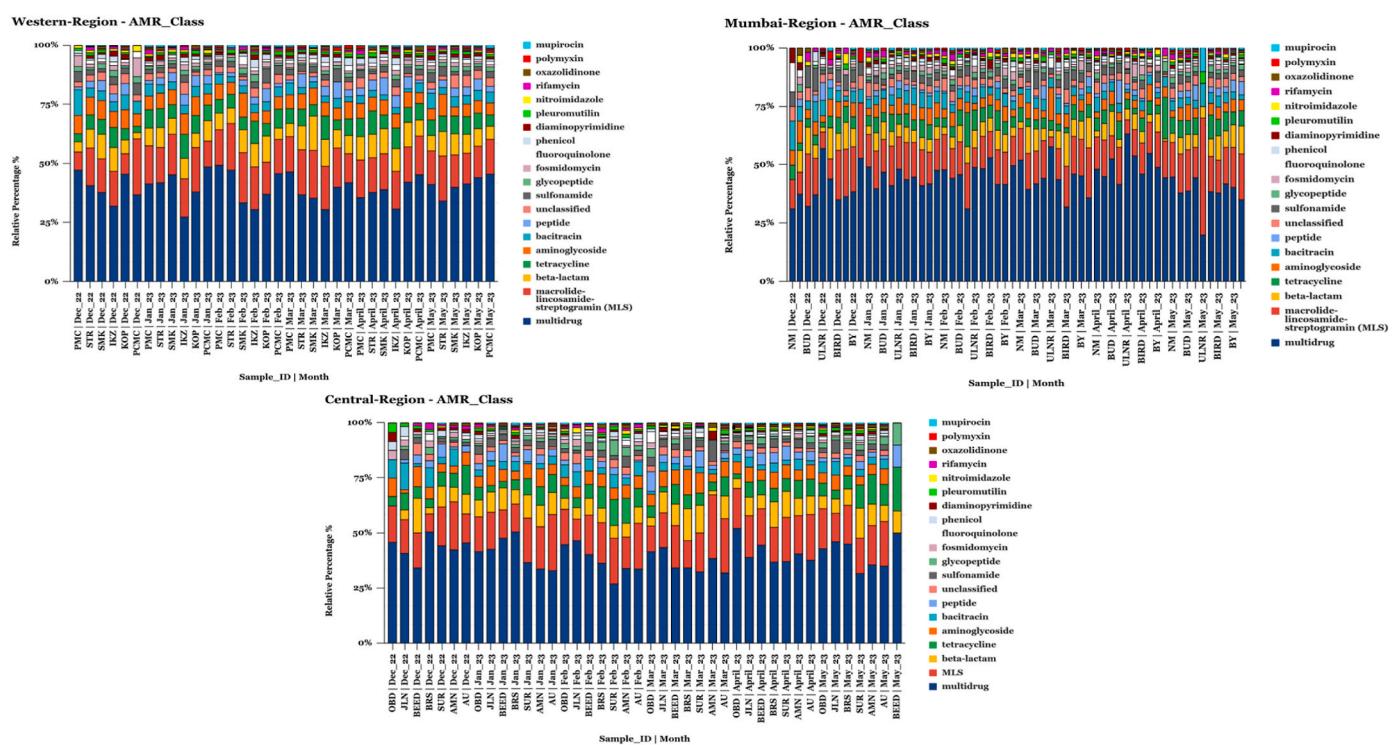


Fig. 2. Regional abundance of antibiotic resistance drug class. x-axis demonstrates sampling location and month, y-axis demonstrates the relative abundance of resistant drug classes.

The Simpson and Shannon indices reveal stronger differentiation between the regions than the observed richness. Dunn's post-hoc tests with Benjamini-Hochberg correction to identify which specific regions contributed to these differences. These findings indicate that the CR and WR regions share the most comparable microbial communities, while the MR stands out in terms of diversity and evenness. Significant p-values support these observations, underscoring the distinct taxonomic compositions of these geographical areas (Fig. S4).

The PERMANOVA results and the PERMDISP analysis provide insights into both compositional shifts and potential influences of variability on the observed patterns in three different regions of Maharashtra. The analysis revealed significant differences in microbial community composition across the three regions from the phylum to the genus level, with the strongest differences consistently observed for the family and genus level. The genus level comparison between MR and CR explained 11 % of the variation ($R^2 = 0.112$, p.adj <0.01), while the family level explained 13 % of the variance ($R^2 = 0.126$, p.adj <0.01). In WR and CR, variance in composition (genus - $R^2 = 0.056$, p.adj <0.01; family- $R^2 = 0.060$, p.adj <0.01) is lower (Table S4).

3.3. Regional profiling of antibiotic resistance drug classes and associated ARGs

The antibiotic resistance profile of WW samples was analysed using the deepARG model. 28 different drug classes associated with 808 genes were present in the WW samples. Among these, 452 ARGs were dominant, with a relative abundance of more than 1 %. Multidrug resistance genes were the most prevalent, comprising 40.49 % of the total ARGs

detected, and were primarily associated with efflux pump mechanisms involving genes such as *rpoB2*, *mdr(ABC)*, and *acrB*. Macrolide-lincosamide-streptogramin (MLS) resistance accounted for 15.84 %, with representative genes including *macB*, *msrE*, and *mphD*. Beta-lactam resistance made up 7.95 % of the ARGs, with dominant genes such as *VEB-1*, *PBP-1A*, and *OXA-10*. Tetracycline resistance represented 6.52 %, linked to genes like *tetA*, *tetM*, and *tetP*. Bacitracin resistance contributed 3.87 %, associated with *bacA*, *bcrA*, and *bahA*. The most abundant genes within each class were selected based on their normalized relative abundance across all samples, with detailed data provided in Supplementary Table S7. These distributions are illustrated in Figs. 2a and 3a,b. The majority of antibiotic resistance genes (ARGs) were shared across the three regions (n = 444), while a subset of ARGs (n = 65–96) was unique to each individual region (Fig. S5).

The antimicrobial drug resistance patterns across the three regions were analysed using non-parametric statistical methods due to non-normal distribution (Shapiro-Wilk test). Resistance pattern varied significantly between regions, as shown by statistical tests (Simpson $\chi^2 = 8.22$, p = 0.0164; Shannon $\chi^2 = 10.27$, p = 0.0059), while differences for observed richness were marginal $\chi^2 = 5.79$, p = 0.0553). This indicates that pattern of resistance genes differed across regions. Post-hoc pairwise comparisons using Dunn's test showed a significant difference between the CR and MR for the Simpson index (p.adj = 0.0125) but not between other regional pairs. For the Shannon index, diversity differed significantly between regions, with the WR exhibiting higher diversity compared to both the CR (WR > CR, p.adj = 0.0092) and the MR (WR > MR, p.adj = 0.0097), while the CR and MR did not differ significantly (Fig. S6).

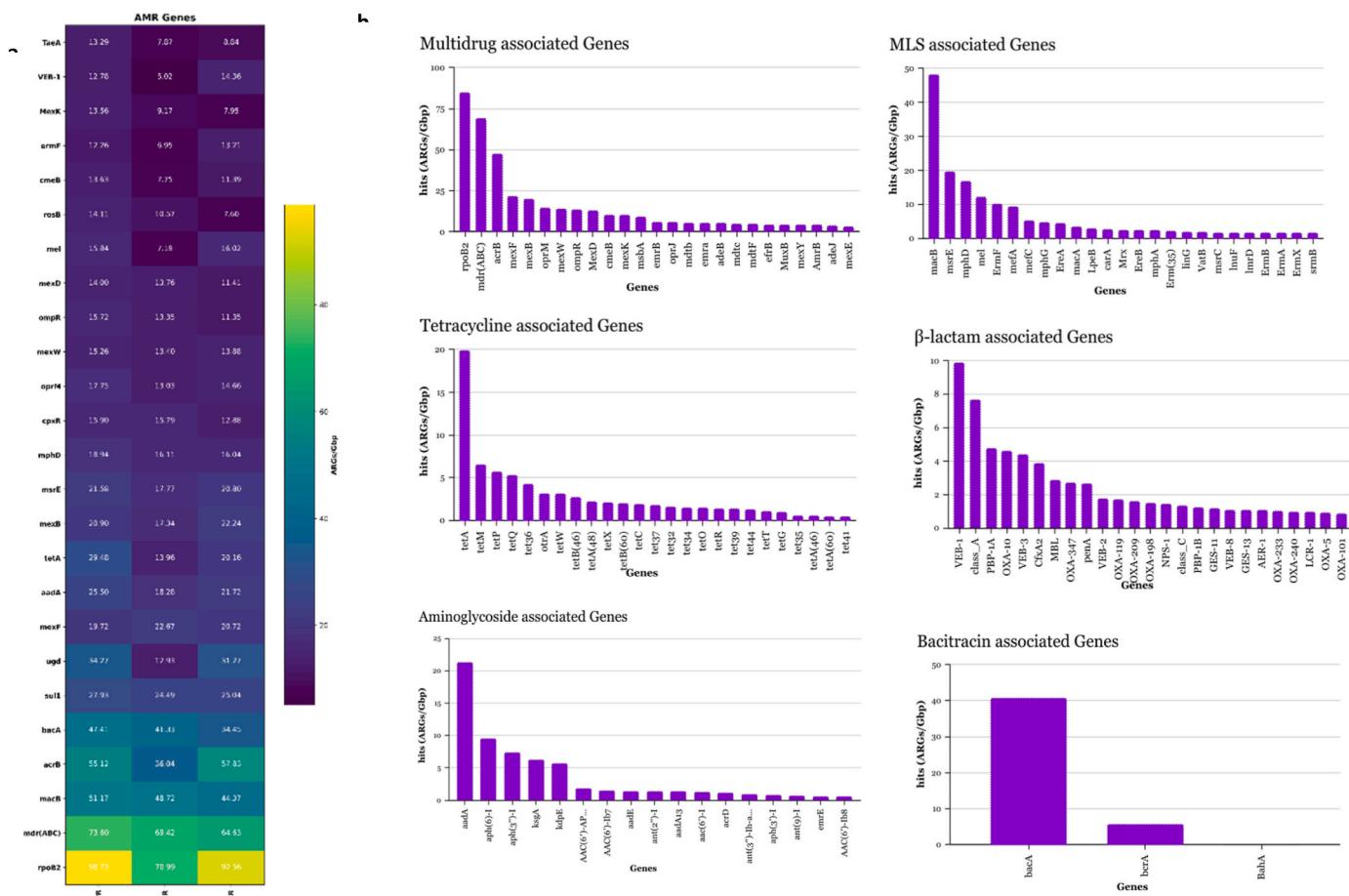


Fig. 3. Occurrence of specific ARGs. a-heatmap showing the 50 most frequently detected ARGs (ARG reads/Gbp) in WW samples (WR- Western Region, MR- Mumbai Region, CR- Central Region); b- ARGs were grouped based on resistance to a given class of antibiotics (ARGs/Gbp (log10)). c- Venn diagram represents the number of ARGs unique to each site (side values) and shared between sites (middle value). Circle colours represent different regions.

Community-level analysis supported these findings, PERMANOVA (a test for overall community dissimilarity) showed that resistome composition differed significantly between the WR and CR ($F = 9.87$, $R^2 = 0.095$, $p.\text{adj} = 0.001$) and the MR and CR ($F = 3.76$, $R^2 = 0.036$, $p.\text{adj} = 0.0188$), but not between the WR and CR ($F = 2.49$, $R^2 = 0.032$, $p = 0.0657$). Importantly, a PERMDISP test confirmed that these differences were due to true compositional variation rather than unequal variability within groups. These results indicate that the western region harbours a more diverse resistomes, while MR exhibits a distinct but less diverse resistance profile.

On average, antibiotic efflux 23.23 % (25.0 %–44.7 %), antibiotic target alteration 6.83 % (12.5 %–8.0 %), and antibiotic inactivation 7 % (12.5 %–8.5 %) were the most prevalent resistance mechanisms in WW samples in the three regions of Maharashtra (Fig. S7).

3.4. Identification of key bacterial taxa with high ARG load

In this study, we assessed the dynamics of antibiotic-resistant bacterial communities, identified ARG-containing reads were extracted, and these ARG-positive reads were taxonomically classified, enabling the association of resistance genes with their potential bacterial hosts. Reads containing ARGs from unidentified hosts and those not associated with unicellular organisms (e.g., Eukaryotes), Archaea, and viruses were excluded from the analysis. Based on the above criteria, 89.55 % of all reads containing ARGs in WW samples were successfully associated with bacterial taxa. Not all ARG hosts were identified at the same taxonomic level. The distribution of antimicrobial resistance (AMR) at the class level was analysed across six major bacterial classes, *Gammaproteobacteria* (38.27 ± 4.20 %), *Betaproteobacteria* (17.77 ± 3.68 %), *Epsilonproteobacteria* (9.77 ± 3.85 %), *Alphaproteobacteria* (7.17 ± 3.06 %), *Flavobacteriia* (4.67 ± 0.49 %), and *Bacilli* (4.26 ± 1.25 %) with comparison to three different regions (Fig. S8a). Multidrug, MLS,

beta-lactam, and tetracycline resistance was present in considerable amounts across all taxonomic classes in all three regions. The *Gammaproteobacteria* class demonstrated pronounced resistance to sulfonamide (90.17 ± 2.55 %), aminoglycoside (74.09 ± 3.50 %), and beta-lactam (63.57 ± 3.17 %) drug classes. Similarly, the *Betaproteobacteria* class showed notable resistance to bacitracin (50.31 ± 2.93 %), multi-drug (26.86 ± 4.15 %), and glycopeptide (24.53 ± 14.24 %) drug classes, while the *Epsilonproteobacteria* class exhibited significant resistance to glycopeptide (56.48 ± 17.84 %) and peptide (40.44 ± 17.87 %) drug classes. Each of these classes displayed unique resistance profiles relative to other taxonomic groups. *Bacilli*, *Flavobacteriia*, and *Alphaproteobacteria* classes exhibited relatively low AMR drug class abundance across all categories (Fig. S9).

The AMR distribution across bacterial genera was comprehensively visualized using a combination of bar charts and bubble plots, revealing both the prevalence of resistance drug classes and their association with specific genera (Fig. 4). The bubble plot further illustrates the genus-specific distribution of AMR classes, where the size and colour of the bubbles correlate with the abundance of AMR genes within a genus. The antimicrobial resistance (AMR) distribution at the genus level was analysed across the top 15 major bacterial genera. *Pseudomonas* (8.3 %), *Klebsiella* (7.19 %), *Aliarcobacter* (6.64 %), *Escherichia* (6.21 %), and *Acinetobacter* (3.32 %) were the top abundant taxa possessing AMR genes. The multidrug (45.31 %) class was primarily associated with *Pseudomonas* (10.33 %), *Aliarcobacter* (7.64 %), and *Acinetobacter* (4.02 %). MLS (17.27 %) resistance was predominantly linked to *Klebsiella* (10.58 %), *Aliarcobacter* (8.39 %), and *Escherichia* (5.28 %), while beta-lactam (7.69 %) resistance was contributed by *Pseudomonas* (15.5 %), *Klebsiella* (11.71 %), and *Escherichia* (8.9 %). Tetracycline (6.78 %) resistance was mainly driven by *Escherichia* (12.1 %) and *Klebsiella* (4.58 %). Aminoglycoside (6.45 %) resistance was observed primarily in *Escherichia* (19.95 %), *Klebsiella* (17.38 %), and *Pseudomonas* (8.54 %).



Fig. 4. Distribution of bacterial hosts based on the quantification of reads. The bubble chart displays the top 15 genera with the highest ARG read counts, along with their relative share of total ARG hosts. The x-axis bar plot shows the relative abundance of different drug classes, while the y-axis bar plot represents the relative abundance of bacterial taxa.

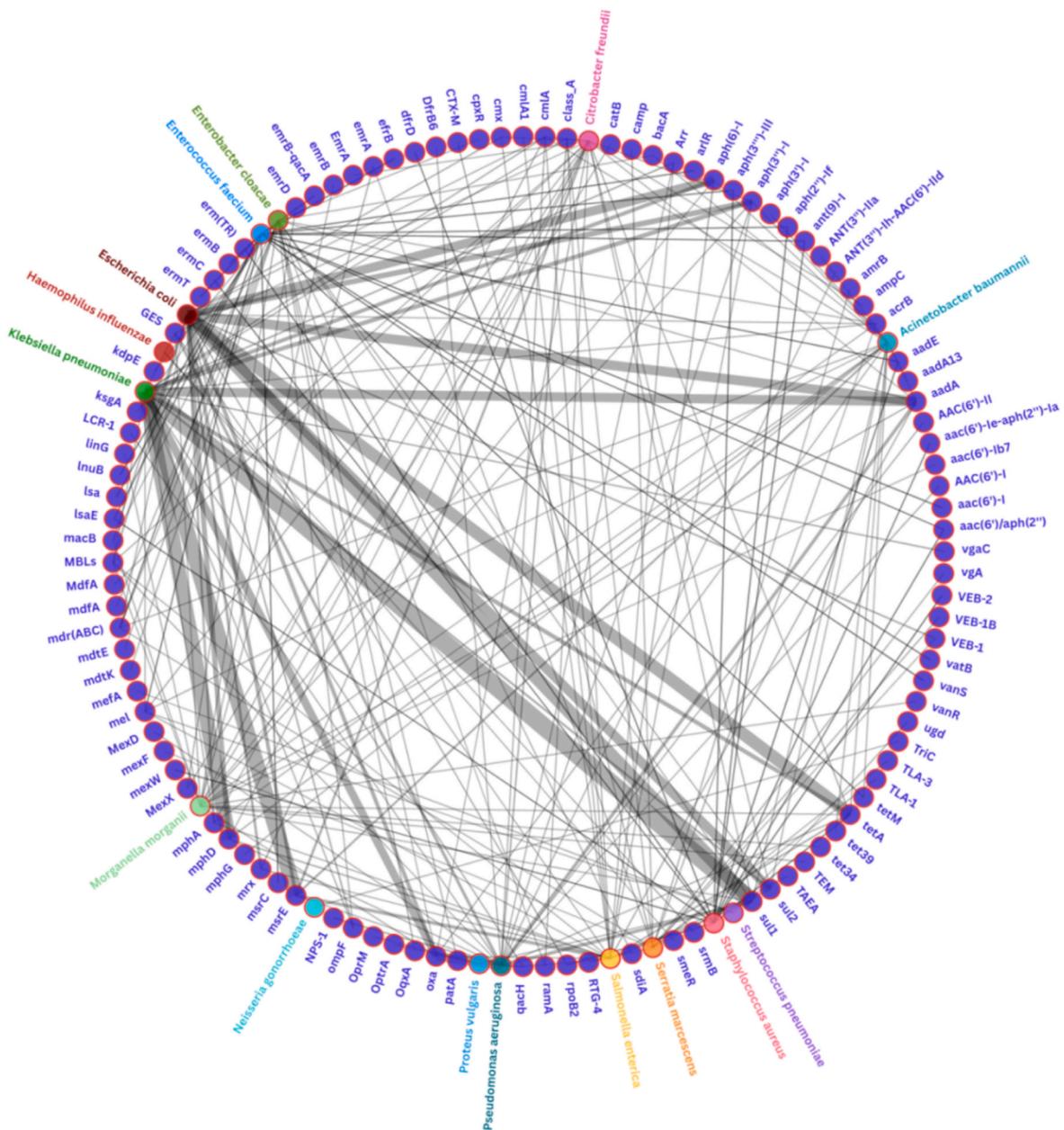


Fig. 5. Network analysis of WHO priority pathogens. Different coloured nodes represent the priority pathogens and ARGs related to the main antibiotic drug classes. Thickness of edges corresponds to the abundance of particular ARGs (reads/Gbp).

Bacitracin (4.2 %) was largely contributed by *Pseudomonas* (9.26 %) and *Thauera* (6.73 %). Peptide (3.56 %) resistance by *Aliarcobacter* (26.83 %) and *Arcobacter* (4.93 %), sulfonamide (3.64 %) resistance was predominantly linked to *Klebsiella* (29.82 %), *Escherichia* (29.66 %), and *Enterobacter* (7.22 %).

3.5. Prevalence of antibiotic resistance genes among WHO priority pathogens

The network plot (Fig. 5) visualizes the relationships between antimicrobial resistance (AMR) genes and WHO-priority bacterial pathogens. Extracted ARG reads were classified to identify their bacterial hosts, with a focus on WHO-priority pathogens. These host-ARG associations were then represented as a network where, the outer ring represents various bacterial species, each color-coded and connected to specific antimicrobial resistance genes (ARGs) depicted as blue nodes. The connections (edges) between the species and ARGs highlight the

prevalence and intensity of associations; thicker edges indicate a higher frequency of occurrence. This visual emphasizes the crucial role these bacterial species play in harbouring and disseminating AMR genes, which pose a significant challenge to global public health.

Upon analysing genes with an abundance greater than 5 %, we observe that 91.05 % of ARGs are concentrated in four opportunistic pathogens: *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Salmonella enterica*. These species are responsible for a wide range of severe infections and are notorious for their role in AMR spread. *Escherichia coli* exhibited multiple resistance genes including *sul1* (9.76 %), *tetA* (8.90 %), *aadA* (6.89 %), *sul2* (6.75 %), and *aph(6)-I* (6.39 %), each representing their relative abundance with the *E.coli* community. Similarly, *Klebsiella pneumoniae* showed high levels of *sul1* (13.52 %), *msrE* (11.38 %), *mphD* (10.75 %), *aadA* (6.8 %), and *OXA* (5.76 %). *Pseudomonas aeruginosa* exhibits a particularly concerning ARG profile, with high frequencies of carbapenem resistance genes such as *OXA* (23.83 %) and *GES* (7.59 %), alongside other ARGs like *sul1* (8.66 %)

and *aadA* (8.66%). *Salmonella enterica*, another critical pathogen, shows high levels of *msrE* (17.02%), *tetA* (8.78%), *mphD* (8.51%), *sul1* (7.71%), *aadA* (7.44%), *aph(6')-I* (7.18%), and *aph(3')-I* (6.64%). Additionally, *Enterobacter cloacae* carry *acrB* (5.22%). In *Staphylococcus aureus* and *Enterococcus faecium*, aminoglycoside resistance genes *AAC* (acetyltransferase) and *APH* (phosphotransferase) collectively accounted for approximately 30% of the ARGs within their respective communities.

Citrobacter freundii exhibited a high proportion of *msrE* (39.47%), while *Serratia marcescens* showed notable prevalence of *TLA-3* (19.04%). *Streptococcus pneumoniae* harbored *mef(A)* at 77.77%, indicating significant macrolide resistance. In *Proteus vulgaris*, genes such as *sul1*, *qacH*, *oprM*, and *optrA* were each present at 25%. *Haemophilus influenzae* contained *smeR* and *srmB*, both at 50%. *Neisseria gonorrhoeae* was found to carry only the *tric* gene (Fig. S10a and b).

3.6. Distribution and abundance of mobile genetic elements and virulence factors

The comparative analysis of mobile genetic elements (MGEs) and virulence factor genes (VFGs) across three distinct regions of Maharashtra WR, MR, and CR provides critical insights into the distribution of antimicrobial resistance (AMR) and pathogenicity determinants in environmental bacterial communities. For MGEs, genes that were predominantly present are site-specific recombination (integration/excision, IE) (43.68% on average), followed by elements related to replication, recombination, and repair (RRR) of nucleic acids (22.37% on average), and stability/transfer/defence mechanisms (STD) (13.17% on average) were the most frequent elements in all the regions. The least frequently identified elements were phage-mediated biological processes (phage, P) (11.99% on average) and conjugation and natural transformation mechanisms (transport, T) (8.85% on average) (Fig. S11a).

Virulence factor analysis revealed that motility, accounting for an average of 28.65%, was a major contributor, with *cheD* (60.19 reads/Gbp), *cheV3* (38.73 reads/Gbp), and *tsr* (26.38 reads/Gbp) being the most abundant genes associated with this factor. Immune modulation, averaging 25.56%, was also predominantly present in wastewater (WW) samples, with *wblL* (27.13 reads/Gbp), *gmd* (26.44 reads/Gbp), and *ugd* (20.38 reads/Gbp) as the leading genes linked to this mechanism. Adherence factors were likewise prominent, with *tufA* (121.17 reads/Gbp), *htpB* (41.37 reads/Gbp), and *pilT* (19.56 reads/Gbp) playing a significant role. Additionally, biofilm formation, which is closely

linked to antimicrobial resistance, constituted an average of 3.99% in the samples. Key genes involved in biofilm production included *algW* (10.62 reads/Gbp), *luxS* (10.31 reads/Gbp), and *mucD* (8.53 reads/Gbp), highlighting their role in biofilm development and potential contributions to antimicrobial resistance (Fig. S11b and c).

3.7. Risk assessment of the resistome in ecological and human health contexts

The resistome risk score was predicted using the MetaCompare 2.0 bioinformatic pipeline. The 3D hazard space plot presents an advanced assessment of ERR (ecological resistome risk) and HHRR (human health resistome risk) using MetaCompare 2.0, focusing on the abundance and transferability of antimicrobial resistance genes (ARGs). The analysis was based on contigs assembled from filtered reads. The x-axis ($Q(\text{ARG})$) shows the general abundance of ARGs, while the y-axis ($Q(\text{ARG_MGE})$) indicates the proportion of ARGs localized on MGEs. The z-axis ($Q(\text{ARG_MGE_PAT})$) further refines this by measuring ARGs within mobile genetic elements (MGEs) associated with pathogens, which increases the likelihood of gene transfer to pathogens. Normalized value obtained as ($Q(\text{ARG})$, $Q(\text{ARG_MGE})$, $Q(\text{ARG_MGE_PATH})$) (Table S5). ERR and HHRR were highest in the MR as compared with other regions. The average risk score for the environment was 71.76 ± 15.35 , and for human health was 5.58 ± 0.57 (Fig. 6) (Fig. S6).

4. Discussion

4.1. Distinct taxonomic indicators across regions: Insights into AMR sources

Regional differences in microbial community structure and resistome composition are more likely driven by human activities and environmental pressures. Although our multivariate analyses revealed clear compositional separation between regions, these patterns are better understood when considering the combined influence of region-specific indicator taxa, dominant resistance mechanisms, mobile genetic elements (MGEs), and resistome risk scores. In the WR, *Gammaproteobacteria*, including *Aeromonas* and *Moraxella*, were identified as indicator taxa. Both genera are frequently detected in healthcare-impacted waters and are known to harbour beta-lactams, macrolides, aminoglycosides, and fluoroquinolones resistance determinants (Raveendran et al., 2020; Varela et al., 2016; Z. Zhang et al., 2022). This taxonomic signal aligns with the observed prominence of

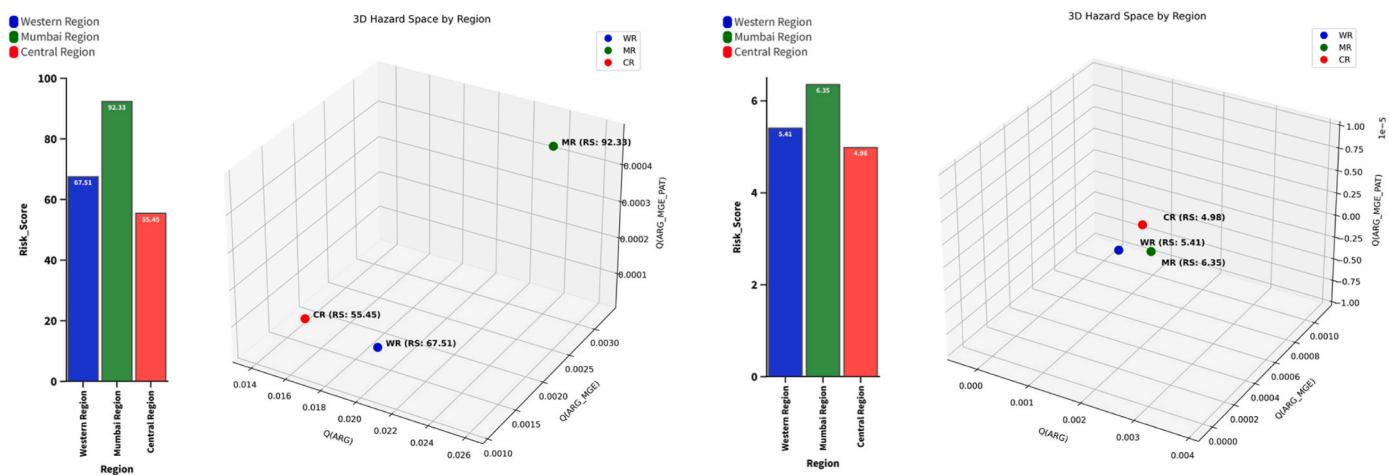


Fig. 6. Bar graphs represent the risk scores in the samples. 3D hazard space created considering relative frequency of critical components in samples: ARGs ($Q(\text{ARG})$), ARGs localized on MGEs ($Q(\text{ARG_MGE})$) and ARGs localized on MGEs in pathogens ($Q(\text{ARG_MGE_PAT})$). The points in 3D hazard space denote samples from different regions (marked in different colours).

efflux-mediated multidrug resistance, beta-lactams and MLS classes, and with literature identifying hospital effluents and downstream urban wastewater as hotspots for ARG selection and transfer (Ahmed et al., 2023; Hutinel et al., 2022). Together with the measurable burden of MGEs, these patterns suggest sustained antibiotic selection and a high potential for horizontal gene transfer in WR drains. In the MR, shows indicator taxa typical of industrial and mixed urban-aquatic settings, including *Ideonella*, *Alicycliphilus* and *Komagataeibacter*. These genera are repeatedly associated with xenobiotic metabolism and plastic/solvent exposure, conditions common in industrial catchments and peri-estuarine network (Al-Tohamy et al., 2022; Solís-González and Loza-Tavera, 2019). *Komagataeibacter* strains have demonstrated resistance to chloramphenicol, suggesting their potential to harbour resistance mechanisms (Cepec and Trček, 2022), while *Erwinia* species are known to exhibit resistance to aminoglycosides (Miller et al., 2022). Such matrices often co-occurring with metals and biocides can impose co-selection for ARGs and MGEs, thereby amplifying multidrug and β-lactam resistance even in the absence of direct antibiotic inputs. The highest ecological and human-health resistome risk scores in MR are coherent with this scenario, indicating not only abundance of ARGs but also their transfer potential via MGEs and pathogenic hosts. This converges with prior evidence that urban-industrial wastewater systems sustain dense resistomes through chemical co-selection and gene mobility (Abdulkadir et al., 2024; Rizzo et al., 2013). In the CR, indicator taxa such as *Arcobacter* and *Aliarcobacter* widely reported in wastewater, animal faeces, foodborne waste and contaminated surface waters with fecal contamination as the dominant driver (Kristensen et al., 2020; Vandenberg et al., 2004). These genera often carry MLS, fluoroquinolones, ampicillin, chloramphenicol and tetracycline resistance features and thrive at human-animal-environment interfaces, mirroring agro-rural inputs and sanitation gaps more than industrial footprints (Disli et al., 2024; Mahmud et al., 2023; Müller et al., 2020; Uljanovas et al., 2021). The comparatively distinct resistome composition in CR, is therefore consistent with diffuse fecal sources, enteric bacteria, and community-level antibiotic use, which are known to seed drains with mobile, clinically relevant ARGs.

In conclusion, The regional microbiome “fingerprints” and resistome structures reflect distinct environmental and human-driven pressures. In the WR, clinical and urban antibiotic use appears to sustain multidrug resistance and β-lactam/MLS determinants. The MR shows strong influence from industrial pollutants and chemical exposure, which promotes co-selection and facilitates gene transfer through mobile genetic elements, resulting in the highest risk scores. In contrast, the CR is shaped by fecal contamination and enteric bacterial dynamics, enriching taxa and resistance genes commonly associated with polluted waters. These interpretations align with global evidence on hospital and urban wastewater hotspots, industrial co-selection, and fecal contamination pathways providing an explanation for regional differences (Abdulkadir et al., 2024; Ahmed et al., 2023; Al-Tohamy et al., 2022; Cepec and Trček, 2022; Disli et al., 2024; Hutinel et al., 2022; Kristensen et al., 2020; Mahmud et al., 2023; Müller et al., 2020; Rizzo et al., 2013; Solís-González and Loza-Tavera, 2019; Uljanovas et al., 2021; Vandenberg et al., 2004).

4.2. Insights into antimicrobial resistance: Efflux systems, mobile elements, and regional hotspots

Across Maharashtra, resistance is shaped most strongly by efflux pump mediated mechanism, consistent with the dominance of the multidrug class in the resistome (Fig. 2). At the community level, efflux pump followed by target alteration and antibiotic inactivation, majorly contributed in resistance mechanisms (Fig. S7). These patterns align

with the taxonomic backbone of the wastewater microbiome. *Pseudomonas*, *Klebsiella*, *Escherichia*, and *Salmonella* were the major ARG hosts (Fig. S8b) and known for broad efflux capacity and diverse intrinsic and acquired resistomes. This finding aligns with studies by (Rajput et al., 2021), which reported genes encoding for multidrug resistance were the most prevalent ARG type in wastewater, riverine systems, and other anthropogenic environments. The results has a resistome pattern that is both polypharmacological (impacting many drug classes) and regionally modulated by selective pressures.

Efflux pump emerged as a dominant feature of the wastewater resistome, with multiple genes contributing to multidrug resistance across gram-negative hosts. Genes such as *acrB*, *macB*, *mdr(ABC)*, and *mexF* were broadly detected across *Enterobacteriales* (e.g., *Escherichia*, *Klebsiella*, *Enterobacter*) and *Pseudomonas*, reflecting the regional prevalence of these taxa (Fig. 4). These efflux systems are known to reduce susceptibility to a wide range of antibiotics including β-lactams, macrolides, tetracyclines, chloramphenicol, and fluoroquinolones (Orelle et al., 2019).

For instance, *acrB* and *macB* are components of the AcrAB-TolC and MacAB-TolC systems, respectively, while *mdr(ABC)* represents ATP-binding cassette transporters, and *mexF* is part of the MexEF-OprN system in *Pseudomonas aeruginosa*. This gene is implicated in resistance to β-lactams, aminoglycosides, and fluoroquinolones, highlighting the adaptability of *P. aeruginosa* in clinical and environmental settings (Dai et al., 2024; Sterenczak et al., 2020; Terzi et al., 2014). Regionally, WR (with stronger clinical and urban signatures) exhibited a richer diversity of resistance classes, consistent with the activity of these efflux systems in hospital-associated lineages. In contrast, MR (characterized by industrial and xenobiotic exposure) showed a distinct but less diverse resistome, where efflux mechanisms remained central to resistance dynamics (Sections 3.2 - 3.3). Collectively, these genes illustrate how efflux-based resistance connects host composition, environmental selection, and class-level patterns, for the prominence of multidrug resistance across sites.

In addition to efflux-mediated resistance, the persistence and mobility of ARGs are shaped by integron-associated genes and stress-adaptive mechanisms. The *sul1* gene, a key marker of sulfonamide resistance, exemplifies the mobility potential of ARGs. Although not a mobile genetic element itself, *sul1* is frequently embedded within class 1 integrons, which are capable of capturing and transferring resistance genes across bacterial hosts. Its high abundance in *Escherichia coli* and *Klebsiella pneumoniae* (Fig. 4) reflects strong selective pressure from antibiotic use and supports its role as a sentinel for horizontal gene transfer. Previous studies have confirmed its environmental resilience, with *sul1* persisting in dairy-irrigated soils (Dungan et al., 2018) and surviving wastewater treatment processes (Aali et al., 2019). The *bacA* gene contributes to bacitracin resistance and bacterial persistence by evading antimicrobial peptides (Abdulkadir et al., 2024; Yu et al., 2019), while *ugd* enhances resistance through lipopolysaccharide modification, reducing membrane permeability to peptide antibiotics (Awori et al., 2023; Li et al., 2024).

These mechanisms enable bacteria to survive in hostile environments and maintain ARGs within microbial communities. The *mexF* gene further reinforces the adaptability of high-risk hosts, particularly *Pseudomonas aeruginosa*, which our study identifies as a critical reservoir of β-lactam resistance, reinforcing the importance of targeted surveillance for this genus (Fig. 4).

Mobile genetic elements (MGEs) play a central role in the evolution and dissemination of both resistance and virulence traits. The prevalence of site-specific recombination elements in our dataset highlights their role in facilitating horizontal gene transfer, particularly for genes like *sul1* and other cassette-borne ARGs. This observation aligns with

(Frost et al., 2005), who identified recombination systems as key facilitators of genetic exchange across microbial populations. MGEs also promote the integration of virulence factor genes (VFGs), including those associated with motility (*cheD*, *cheV3*, *tsr*), adherence (*tufA*, *htpB*, *pilT*), and biofilm formation (*algW*, *luxS*, *mucD*). Although biofilm-associated genes were less abundant, they contribute to AMR persistence by shielding microbial communities from antibiotics and environmental stressors, as reported by (A. Zhang et al., 2013). The co-occurrence of MGEs and VFGs with ARGs suggests a resistome architecture that supports both ecological persistence and transferability.

These findings provide a comprehensive view of ARG abundance, mobility, and ecological integration in wastewater systems. The interplay between efflux pumps, resistance enzymes, and mobile genetic contexts underscores the complexity of AMR dissemination. Our findings have clear implications for targeted interventions. The Mumbai Region (MR), which showed the highest resistome risk scores and strong linkage of ARGs to MGEs, should be prioritized for wastewater treatment upgrades and stricter antibiotic stewardship. Measures such as tertiary disinfection, advanced oxidation, and improved industrial effluent control could help reduce ARG persistence and transfer. Clinical surveillance should also be strengthened in MR and major hospitals in WR, focusing on high-risk pathogen–gene combinations such as *OXA* and *GES* carbapenemases in *Pseudomonas aeruginosa* and integron-associated markers in *Klebsiella* and *Escherichia*. Wastewater-based monitoring should routinely track sentinel genes like *sul1* (for mobility) and *acrB* (for efflux-mediated resistance), with higher sampling frequency in MR and baseline monitoring in WR and CR. In WR, hospital effluent pre-treatment and sewer connectivity should be prioritized, while CR requires improved sanitation and decentralized treatment to mitigate fecal contamination. Linking these environmental signals with clinical data will enable early warning systems and region-specific strategies to curb antimicrobial resistance. Embedding wastewater surveillance within One Health strategies would strengthen India's capacity to track, prevent, and manage AMR at the human–environment interface, directly aligning with WHO's call to integrate environmental monitoring into global AMR strategies.

5. Conclusion

This study presents a regional-scale assessment of microbial diversity and antimicrobial resistance (AMR) patterns in wastewater across Maharashtra, India, using metagenomic sequencing and resistome profiling. The analysis revealed distinct microbial community structures across urban centres, with notable enrichment of pathogenic and opportunistic taxa in densely populated regions. These microbial profiles were closely linked to the resistome composition, indicating that local environmental and anthropogenic factors shape both microbial and resistance landscapes. Efflux pump-mediated resistance emerged as the dominant mechanism across all sites, underscoring its role in multidrug resistance among Gram-negative bacteria. The consistent detection of genes associated with efflux systems, particularly those conferring resistance to multiple antibiotic classes. This highlights their ecological persistence and potential for widespread dissemination. Moreover, the study identified high-risk associations between antimicrobial resistance genes and clinically relevant hosts such as *Escherichia* and *Klebsiella*, suggesting a strong potential for environmental reservoirs to contribute to clinical resistance burdens. Mobile genetic elements, including integrons and plasmids, were prevalent across samples and played a key role in shaping the resistome architecture. Their occurrence of virulence factors further emphasizes the risk of horizontal gene transfer and the emergence of transmissible and pathogenic strains in wastewater environments. Despite these insights, the study is limited by its cross-sectional design and reliance on metagenomic correlations, which restrict direct conclusion and phenotypic validation. Future work should incorporate longitudinal sampling to capture seasonal dynamics, and integrate culture-based assays to confirm resistance phenotypes.

Expanding surveillance to include hospital and industrial effluents, as well as rural sanitation systems, will provide a more comprehensive understanding of AMR dissemination. Overall, this study demonstrates the utility of wastewater-based surveillance in identifying regional AMR hotspots and supports its integration into India's national AMR strategy under the One Health framework.

CRediT authorship contribution statement

Shubham Kumar: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sejal Matra:** Methodology, Formal analysis, Data curation, Conceptualization. **Vinay Rajput:** Writing – review & editing, Investigation. **Harshada Ghode:** Methodology, Investigation, Data curation. **Deepak Rathore:** Methodology. **Shailendra Kumar:** Methodology. **Sanjay Kamble:** Resources. **Syed Dastager:** Resources. **Abhay Bajaj:** Supervision, Conceptualization. **Asifa Qureshi:** Conceptualization. **Atya Kapley:** Supervision, Project administration, Funding acquisition, Conceptualization. **Mahesh Dharne:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Ethical approval

Ethical approval was not required since the study was related to community-based wastewater surveillance in environmental samples.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dr Mahesh Dharne reports financial support was provided by Council for Scientific and Industrial Research. 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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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envres.2025.123287>.

Data availability

Data will be made available on request.

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