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# Mapping antimicrobial resistance landscape at a city scale sewage network

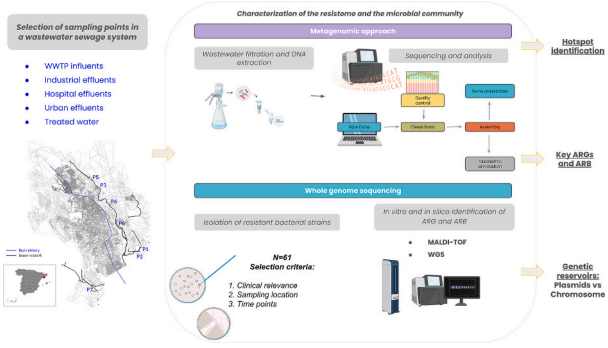
Clara Díaz-García [a,1], Miquel Sánchez-Osuna [b,c,1], Albert Serra-Compte [d], Ioanna Karakatsanidou [d], Inmaculada Gómez-Sánchez [b,c], Berta Fidalgo [e], César Barbuzana-Armas [a], Mariana Fittipaldi [a], Riccardo Rosselli [a,f], Jordi Vinyoles [g], Susana González [d], Oscar Q. Pich [b,c], Mateu Espasa [e,*], M. Adela Yáñez [a,*]

[a] LABAQUA, S.A.U., c/ Dracma, 16-18, Polígono industrial Las Atalayas, 03114 Alicante, Spain
[b] Laboratori de Recerca en Microbiologia i Malalties Infeccioses, Hospital Universitari Parc Taulí, Institut d'Investigació i Innovació Parc Taulí (I3PT-CERCA), Universitat Autònoma de Barcelona, Sabadell, Spain
[c] Institut de Biotecnologia i Biomedicina, Universitat Autònoma de Barcelona, Bellaterra, Spain
[d] Cetaqua, Water Technology Centre, Ctra. d'Esplugues, 75, 08940 Cornellà de Llobregat, Barcelona, Spain
[e] Servei de Microbiologia, Hospital Universitari Parc Taulí, Institut d'Investigació i Innovació Parc Taulí (I3PT-CERCA), Universitat Autònoma de Barcelona, Sabadell, Spain
[f] Department of Physiology, Genetics and Microbiology, University of Alicante, Alicante, Spain
[g] Aigües Sabadell, C. Concepció, 20, 08202 Sabadell, Spain

## HIGHLIGHTS

- Hospital effluent presented the highest amount and diversity of ARGs and ARB.
- Plasmids play a key role in ARG dissemination in wastewater.
- *blaOXA-1*, *blaKPC-2*, *blaTEM-1*, *msr(E)* and *erm(F)* were proposed as AMR tracking makers.
- On-site wastewater treatments can contribute to AMR mitigation strategies.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Wastewater is a valuable source for monitoring contaminants of biotic or abiotic origin. Antimicrobial resistance (AMR) has emerged as a public health threat that consists of the ability of microorganisms to resist the effects of antimicrobial compounds, rendering them very difficult or impossible to eradicate in case of infection. Considering the dissemination of antimicrobial resistance genes (ARGs) to a wide number of ecosystems, there is a need for the identification of hotspots that concentrate antimicrobial resistance determinants. A comprehensive investigation conducted at a city-scale in Sabadell (Barcelona, Spain) has integrated both phenotypic and genotypic methodologies, including metagenomics and culture-based techniques coupled with whole-genome sequencing (WGS), to monitor ARG presence in seven different spots of the sewage system. Metagenomics

* Corresponding authors.
*E-mail addresses:* mespasa@clinic.cat (M. Espasa), adela.yanez@labaqua.com (M.A. Yáñez).
[1] These authors contributed equally to this work.

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approach identified 262 ARG variants across analyzed sampling sites, grouped into 15 resistance categories. The most prevalent ARGs were macrolides-lincosamides-class B streptogramins ($MLS_B$) (35.1 %) and beta-lactams (28.7 %), including carbapenems (5.9 %) and cephalosporins (5.3 %). $MLS_B$ resistance featured dominant *msr (E)* and *mph(E)* genes, the most abundant ARGs in our study. ARGs conferring resistance to beta-lactam were dominated by *blaOXA-464*, *blaOXA-491*, and *blaNPS*. Key genes for carbapenem (*blaOXA-372, blaKPC-2*) and cephalosporin (*blaOXA-10, blaOXA-1*) resistance were identified. The hospital sector exhibited the highest relative abundance of ARGs, dominated by beta-lactams, $MLS_B$, and aminoglycosides. Wastewater treatment plant (WWTP) entrance points and residential areas displayed similar ARG profiles, while WWTP effluent and industrial zones had the lowest ARG levels. WWTP significantly reduced ARG presence (93.3 %). The characterization of antibiotic-resistant bacterial isolates found that most abundant ARGs were predominantly plasmid-borne, favoring ARG spread across bacterial genera. This finding confirmed the significant role of plasmids in ARG dissemination, increasing both diversity and prevalence within waterborne bacterial communities. City-scale surveillance programs can play a pivotal role in guiding effective measures to reduce the dissemination of AMR and mitigate their environmental impact.

## 1. Introduction

Overuse of antimicrobials promotes the emergence and spread of antimicrobial resistance genes (ARGs) and the concomitant selection of multidrug resistant (MDR) bacteria. This situation limits the therapeutic strategies available against human and animal infections, leading to higher morbidity, longer hospitalization time and a significant increase in the associated medical costs (Rizzo et al., 2013). O'Neill's report estimated that currently 700,000 people die worldwide annually from infections that no longer respond to antibiotics. However, it is predicted that this number will increase to 10 million by 2050, becoming the leading cause of death worldwide, with economic costs increasing to USD 100 trillion if no specific global action is undertaken (O'Neill, 2016).

Settings associated with high antibiotic usage (such as hospitals, nursery homes and densely populated areas, among others) are hotspots of antimicrobial resistance (AMR), which, in most cases, are discharged into the urban sewer network without any previous treatment (Antimicrobial Resistance Collaborators, 2022). In addition, traditional wastewater treatment facilities primarily focus on removing solids, degradable organic matter, and nutrients from water and only partially remove emerging contaminants such as antibiotics, ARGs or MDR bacteria (Ravasi et al., 2019). Wastewater presenting sub-inhibitory concentration of antibiotics apart from a wide variety of bacteria and other compounds such as metals, exert a pronounced selection pressure to ARG transmission within the resident bacterial communities (De Giglio et al., 2018; Murray et al., 2024). This allows their continuous spread into the environment when they are not completely removed during the wastewater treatment process (Aminov and Mackie, 2007).

Bacterial strains can develop AMR through the acquisition of mobile genetic elements (MGEs) and through de novo point mutations. However, even though both processes contribute to antibiotic resistance, mobile ARGs pose a greater threat due to their ability to rapidly spread between species through genetic platforms such as plasmids, transposons and integrons that confer an evolutionary beneficial trait to the bacteria under certain conditions (Partridge et al., 2018). In contrast, mutational resistances are rarely mobilized and disseminated among bacteria (Sánchez-Osuna et al., 2023). Consequently, mobile ARGs are the main responsible for the accumulation of MDR bacteria in the aquatic environment, increasing AMR reservoirs (Wang et al., 2020).

To reduce the spread and accumulation of AMR genes in the environment, it is necessary to establish control measures at the key hotspots (Kunhikannan et al., 2021). The wastewater drainage network plays a crucial role in addressing this challenge, and wastewater-based epidemiology (WBE) has emerged as a valuable tool for monitoring AMR, emerging contaminants, chemical exposure or human diseases. Its worldwide application during the SARS-CoV-2 pandemic has demonstrated WBE's ability to track viral circulation within the population as well as anticipating viral waves (Medema et al., 2020). Monitoring of AMR in wastewater has also been proposed as a resourceful surveillance tool (Mutuku et al., 2022). The monitoring of AMR in wastewater has been generally focused on the analysis of few wastewater treatment plants (WWTPs) influent (Tiwari et al., 2022), as well as the comparison between AMR occurrences in different cities and countries (Munk et al., 2022). However, there is a growing interest in the resistome characterization of ARGs reservoirs such as wastewater, sewage sludge, effluents from hospitals (Kunhikannan et al., 2021), residues generated from agriculture/livestock (Graham et al., 2019) or sewage from airplanes (Heß et al., 2019). City-scale monitoring, including the analysis of sewer network points, is much more scarce but it is proposed as a useful source of epidemiological information (Maestre-Carballa et al., 2024).

The analysis at the city scale can provide critical information for decision-making processes, identifying hotspots of AMR to implement in-situ advanced wastewater treatment options or awareness campaigns in areas with a high impact of AMR discharge. This is in line with the One-health perspective proposed by the World Health Organization (WHO), that promotes a balance between human, animal, and environmental health (Velazquez-Meza et al., 2022). These interconnected axes and the associated ecosystems are linked by water and therefore sewage systems within a city are decisive in safeguarding water quality.

In this context, the present study aims to comprehensively monitor AMR at different points of the sewer network of Sabadell city (N.E. Spain) to provide specific knowledge for sanitary and local decision-making to reduce the risk of AMR dissemination. The study combines different analytical techniques including phenotypic, metagenomic, and WGS for detailed antimicrobial characterization in wastewater.

## 2. Material and methods

### 2.1. Study site and sampling campaigns

The study was conducted in the drainage network of Sabadell city (N. E. Spain, Fig. 1). Sabadell collector system is divided into two basins; Riu Sec basin and Riu Ripoll basin, which convey wastewater to the two respective WWTPs. The different sampling sites have been selected along the Riu Ripoll basin which collects wastewater from the local hospital (Taulí Hospital) and allows a better sectorization of the sewer network. Parc Taulí University Hospital is a tertiary care hospital that covers an area with 400,000 inhabitants. It has a total of 730 beds, including 530 for acute care patients (80 of which are in intensive care) and 200 for chronic care patients. The selected sampling points were (P1) Riu Ripoll WWTP Entrance, which has a treatment capacity of 79,000 equivalent/inhabitants/day and treats all the wastewater drained by the Riu Ripoll basin; (P2) Riu Ripoll WWTP exit, corresponding to the treated wastewater from the Riu Ripoll WWTP, which has a biological process with nutrient removal (nitrogen and phosphorus); (P3) Industrial zone, that mainly collects wastewater from textile manufacturing in an industrial park.; (P4) Taulí Hospital Sector, mainly corresponding to the Hospital wastewater effluent (with no previous treatment) and few adjacent neighborhoods; (P5) Ca n'Oriac

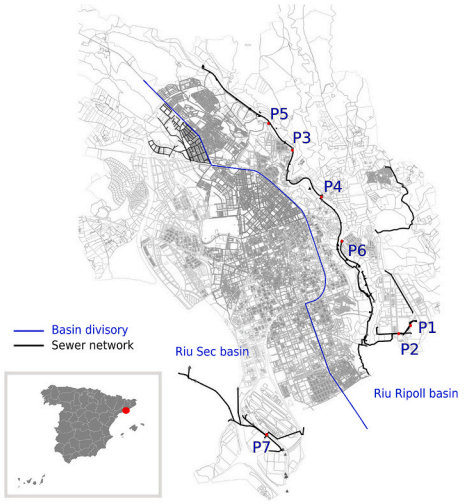


**Fig. 1.** Map of the sampling sites within the Sabadell city sewer system. The blue line depicts the basin divisory and the black line depicts the sewer network. Sampling sites are highlighted with a red dot and are enumerated from P1 to P7.

Sector, urban area with households, geriatric center, schools and sports center; (P6) Torre Romeu Sector, urban area with households, educational center and public market; and (P7) Riu Sec WWTP entrance, which has a treatment capacity of 170,000 equivalent/inhabitants/day and collects wastewater from the Riu Sec watershed in Sabadell, as well as a significant portion of a nearby municipality.

Grab samples were collected from the seven selected points during four sampling campaigns (July, October, November 2022 and February 2023). Samples were taken under representative seasonal weather conditions, avoiding days with relevant rainfall that could provoke potential dilution of wastewater.

Further information regarding sampling sites description, sampling procedure and sample physicochemical monitoring can be found at supporting information (Data S1, Table S1).

### 2.2. Metagenomic analysis

Sample pretreatment for metagenomic analysis typically filtered 50 mL of non-treated wastewater (sampling points P1, P3, P4, P5, P6 and P7) and 200 mL of treated water (sampling point P2) through 0.22 μm polycarbonate (PC) membranes (Sartorius UK Limited, Surrey, UK, ref. ES47020100) by using a vacuum pump unit. PC membranes were kept at −20 °C prior to DNA extraction.

The total DNA was extracted from the biomass collected in the membrane using Dneasy PowerWater Kit as per the manufacturer's instructions (Qiagen, Crawley, UK). DNA concentration was measured using Qubit dsDNA HS Assay Kit (Life Technologies, UK) in a Qubit 4 Fluorometer (Thermo Fisher Scientific, USA).

After DNA extraction, total DNA was sequenced using Illumina NovaSeq 6000 Device (150 bp, paired-end read) (Novogene, United Kingdom) following construction of a Nextera XT library as per the manufacturer's protocol. Each sample was analyzed in duplicate and for each one, approximately 6 Gb were sequenced.

The raw Illumina sequencing reads were processed to remove low-quality bases and adapter sequences using Trimmomatic v0.39 (Bolger et al., 2014). The following parameters were used for trimming: ILLUMINACLIP:adapter.fa:2:30:10, LEADING:3, TRAILING:3, SLIDINGWINDOW:4:15, and MINLEN:36. The resulting high-quality reads were assembled into contigs using MEGAHIT v1.2.9 (Li et al., 2016). The assembly was performed with the default parameters, optimized for metagenomic datasets. The --min-contig-len 1000 flag was set to filter out contigs shorter than 1000 bp. Open reading frames (ORFs) were predicted from the assembled contigs using Prokka v1.14.6 (Seemann, 2014) with default settings. Next, predicted proteins were annotated against the non-redundant (NR) protein database using DIAMOND v2.0.13 (Buchfink et al., 2015), operating in BLASTp mode. The following parameters were applied for the annotation: --max-target-seqs 1 and --evalue 1e-5, ensuring the best possible match for each predicted protein. Taxonomic classification was assigned based on the taxonomy information associated with the best protein hit for each ORF.

High-quality reads were aligned against the assembled contigs using BWA v0.7.17 (Li and Durbin, 2009). The alignment was performed in paired-end mode using the mem algorithm. The abundance of contigs (representing the taxa identified) was quantified by calculating counts per million (CPM) of reads. CPM was computed by normalizing the number of reads aligned to each contig relative to the total number of aligned reads. Additionally, CPM values were also evaluated for gene quantification, using predicted ORFs from Prokka to assess gene abundance across samples. To predict ARGs, two tools were used: ResFinder v4.1 (Bortolaia et al., 2020) (with options -s Other, −l 0.4, −t 0.6) and deepARG v2.0 (Arango-Argoty et al., 2018) (with options --model LS, --type prot, --min-prob 0.8, --arg-alignment-identity 50, --arg-alignment-evalue 1e-10). The programs were run on predicted ORFs and proteins from all samples to identify potential AMRs. After obtaining the predictions from both tools, redundant AMR annotations, due to overlapping or identical annotations, were removed to avoid duplication. This was done by comparing the gene annotations and retaining unique predictions.

The Grubbs test was applied to detect ARGs with biased distribution, identifying those with significantly different abundances that diverged from the overall trend. A *p*-value threshold of 0.05 was considered as significant.

### 2.3. Bacterial culturing and whole-genome sequencing

A total of 600 mL of each wastewater sample (1200 mL in the case of WWTP effluent) were pre-filtered through a 6 μm filter to remove large particles and debris. The filtered wastewater was diluted to 1:10000 and 1:100000 to avoid overwhelming bacterial growth and obtain countable colonies. The diluted filtered wastewater was collected and passed through a second filter, in this case, 100 mL (200 mL for WWTP effluent samples) of each dilution were filtered through a 0.45 μm cellulose filter (Whatman® MicroPlus STL cellulose nitrate) to capture bacteria (APHA, 2017). This process was repeated 5 times for each sample, resulting in 5 filters per sample. The filters were placed in culture plates and were incubated at 36 °C to be read at 24 and 48 h. Culture media were selective for the isolation of clinically relevant resistant bacteria to; i) third-generation cephalosporins (CHROMID® ESBL, bioMérieux), ii) carbapenems (CHROMID® CARBA, bioMérieux), and iii) methicillin/cloxacillin (CHROMID® MRSA, bioMérieux). Blood agar plate with nalidixic acid and colistin (CNA Columbia agar +5 % sheep blood®, Becton-Dickinson) and ii) MacConkey agar plate® (Becton-Dickinson). The last two plates were used as positive growth control for Gram-positive and Gram-negative bacteria, respectively. Bacterial colonies from the selective media were isolated for identification by mass spectrometry (MALDITOF®, Bruker) and antibiotic sensitivity testing by microdilution system (Phoenix®, Becton-Dickinson). Immunochromatography tests for carbapenemase detection (NDM, VIM, OXA, and KPC, NG-Test® CARBA-5, NG-Biotech) and extended spectrum Beta-lactamase (CTX-M, NG-Test® ESBL, NG-Biotech) were used for analyzing carbapenem-resistant and cephalosporin-resistant bacteria.

A selection of isolated bacteria was studied by WGS. The criteria of selection to avoid duplicate isolates by date and point collection were: carbapenem-resistant and/or third-generation cephalosporin-resistant, one bacteria species by collection-point and date sampling. DNA was purified using the DNeasy Blood & Tissue Purification Kit (Qiagen) following the manufacturer's instructions. Libraries for sequencing were prepared with the Nextera XT DNA Sample Preparation Kit (Illumina).

WGS was performed using paired-end sequencing on a NovaSeq 6000 device (Illumina) available at the laboratories of the Centre de Regulació Genòmica (CRG, Barcelona). Read pre-processing (sequencing adapter removal) and filtering (Phred score ≥20) were performed with Trim-Galore (https://github.com/FelixKrueger/TrimGalore, accessed in July 2023). Trimmed paired-end reads were assembled using shovill (https://github.com/tseemann/shovill, accessed in July 2023) and annotated with Prokka (Seemann, 2014) against the COG (Galperin et al., 2021), HAMAP (Pedruzzi et al., 2015) and Pfam (Mistry et al., 2021) databases. CheckM2 was used for assessing the quality of assemblies (Chklovski et al., 2023) and 16S rRNA gene analysis for microbial classification were evaluated with the SILVA online service (Quast et al., 2013). Putative ARG were predicted with ABRicate (https://github.com/tseemann/abricate, accessed in July 2023) and plasmid scaffolds were identified using the mob_recon module available at the MOB-suite (Robertson and Nash, 2018).

### 2.4. Data availability

Sequence data from this study have been submitted to the NCBI database and are available under the BioProject PRJNA1168082.

## 3. Results

### 3.1. Metagenomic analysis

#### 3.1.1. Bacterial species diversity

Considering the mean CPM across all sampling campaigns, the 20 most abundant genera were identified (Fig. 2, Table S2). The microbial composition and abundance varied across the sampling sites, with the Riu Ripoll WWTP entrance (P1) exhibiting the highest total relative abundance ($1.35{\cdot}10^6$ CPMs). The microbial community was dominated by *Aliarcobacter* (786,406 CPMs), *Acinetobacter* (165,656 CPM), and *Aeromonas* (44,608 CPM), reflecting its microbial richness and complexity before treatment. Conversely, Riu Ripoll WWTP exit (P2) exhibited the lowest relative abundance of the 20 most abundant genera ($9.20{\cdot}10^4$ CPMs), indicating a marked reduction in microbial load likely due to the biological treatment process. Despite this, resilient genera such as *Flavobacterium* (37,339 CPMs) and *Pseudomonas* (16,437 CPMs) persisted, highlighting their adaptation to post-treatment conditions. The Industrial zone (P3) displayed a distinct profile, with *Aeromonas* (192,539 CPMs) and *Pseudomonas* (347,232 CPMs) dominating, reflecting the influence of industrial discharges and the metabolic versatility of these genera in such environments.

The microbial community in Taulí Hospital sector (P4) was dominated by *Aliarcobacter* (483,974 CPMs), *Acinetobacter* (140,307 CPMs) and *Bacteroides* (125,245 CPMs) but it also stands out for the elevated levels of other clinically relevant taxa such as *Prevotella* (73,173 CPMs), *Klebsiella* (41,227 CPMs), *Pseudomonas* (31,824 CPM) and *Enterobacter* (7205 CPMs), indicative of human and medical practices contributing to this site.

On the other hand, urban sectors showed a similar microbial distribution between them. Ca n'Oriac sector (P5) community was dominated by *Aliarcobacter* (752,240 CPMs), followed by *Acinetobacter* (221,018 CPMs) and *Prevotella* (33,137 CPMs). Similarly, Torre Romeu sector (P6) was characterized by high levels of *Aliarcobacter* (649,605 CPMs), Acinetobacter (180,369 CPMs), and Aeromonas (91,793 CPMs). At Riu Sec WWTP entrance (P7), *Aliarcobacter* again dominated (759,968 CPMs), with *Acinetobacter* (171,342 CPMs) and *Aeromonas* (50,708 CPMs) contributing significantly to the microbial community. This gradient of

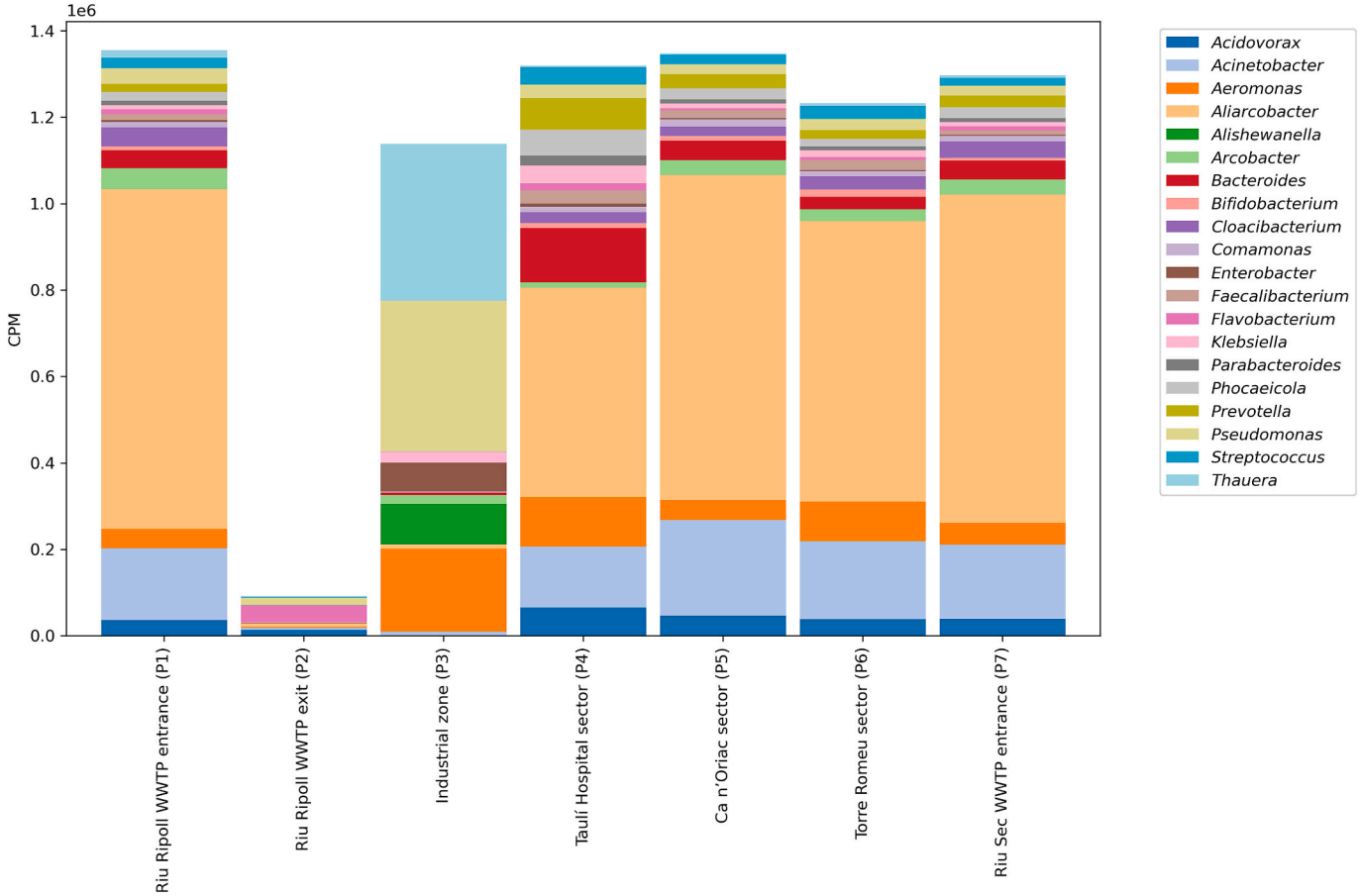


**Fig. 2.** Top 20 bacterial genera classified at the different sampling sites of the study (P1-P7).

microbial diversity across the sampling sites demonstrates the interplay of environmental, industrial, and human activities in influencing microbial compositions.

### 3.1.2. ARG profiling

ARGs annotation across the different sampling campaigns identified 262 ARG variants, which were classified in 15 groups based on the antimicrobial agent they confer resistance to (Fig. 3, Table S3). Genes conferring resistance to macrolides, lincosamides and type B streptogramins ($MLS_B$) were the most abundant (35.1 %), followed by beta-lactams (28.7 %), including carbapenems (5.9 %) and cephalosporins (5.3 %), aminoglycosides (12.6 %), tetracyclines (8.6 %), sulfonamides (4.6 %), fluoroquinolones (3.3 %), and phenicols (2 %). Less abundant ARGs (<1 %) conferring resistance to colistin (0.8 %), trimethoprim (0.7 %), rifampicin (0.6 %), fosfomycin (0.3 %), and nitroimidazole (0.1 %) were also identified. Genes conferring resistance to disinfectants were also predicted (2.6 %).

Genes conferring resistance to beta-lactams, carbapenems and third-generation cephalosporins included up to 111 different variants. Among those, third-generation cephalosporins and carbapenems were represented by 34 and 22 different resistance genes variants, respectively. Some diversity was also observed for other ARG groups: aminoglycosides (42 genes), $MLS_B$ (30 genes), tetracyclines (23 genes), phenicols (13 genes), trimethoprim (10 genes), colistin (8 genes), fluoroquinolones (7 genes), disinfectant (5 genes), fosfomycin (4 genes), sulfonamides (4 genes), nitroimidazole (3 genes) and rifampicin (2 genes).

The percentage of relative abundance for each ARG variant across all sampling campaigns was calculated to identify dominant genes (>5 % CPMs) (Fig. 4). Genes conferring resistance to beta-lactams were characterized by their abundance and high diversity. Specifically, the most abundant ARGs within this class were *blaOXA-464* (19.7 %), *blaOXA-491* (11.7 %) and *blaNPS* (8.3 %). Within these genes, resistance to third-generation cephalosporins was represented by *blaOXA-10* (40.1 %) and *blaOXA-1* (25.7 %) and resistance to carbapenems was represented by *blaOXA-372* (53.5 %), *blaOXA-333* (8.1 %) and *blaOXA-24* (5.2 %), but also *blaKPC-2* gene (11.5 %) and *blaGES-5* gene (10.4 %). Aminoglycosides-resistant genes were primarily represented by *aph(6)-Id* (18.3 %) and *aph(3″)-Ib* (18.2 %). However, they also exhibited notable diversity of *aad* and *ant* genes, including *aadA1* (8.3 %), *aadA11* (5.2 %), *aadA13* (5.0 %) and *ant(6)-Ia* (5.0 %). Determinants conferring resistance to the $MLS_B$ family stood out for the dominance of *msr(E)* (31.3 %) and *mph(E)* (27.9 %) genes, which were present at comparable abundances. These two genes were the most abundant ARGs in this study. The *erm(F)* (8.1 %) and *erm(B)* (8.0 %) genes conferring resistance to $MLS_B$ were also found at notable abundances. The most prevalent genes predicted to confer tetracycline resistance were *tet(Q)* (31.1 %), *tet(39)* (18.9 %), *tet(X)* (11.0 %), *tet(C)* (9.2 %), *tet(W)* (6.8 %) and *tet(A)* (5.5 %). Sulfonamide resistance was mainly mediated by *sul1* (81.4 %), and to a lesser extent by *sul2* (17.8 %). Similarly, the most abundant genes predicted to confer trimethoprim resistance were *dfrA14* (42.6 %), *dfrB4* (15.6 %), *dfrA1* (15.4 %), *dfrA12* (9.1 %), *dfrA7* (6.3 %). Fluoroquinolone resistance was mediated mainly by *aac(6′)-Ib-cr* (43.9 %), *qnrS2* (41.2 %), and *qnrVC4* (11.8 %) genes. Phenicol resistance was primarily governed by *catB3* (31.0 %), *cmlA1* (28.2 %), *floR* (10.0 %), and *catQ* (9.4 %) variants. Finally, several ARGs clearly dominated resistance to a specific antimicrobial group: *mcr-7.1* (88.9 %) for colistin resistance, *fosA* (91.7 %) for fosfomycin, *arr-3* (90.8 %) for rifampicin, and *qacE* (79.7 %) for disinfectants.

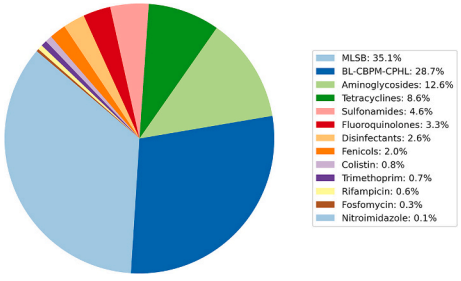


**Fig. 3.** Distribution of the ARG variants in groups based on the antimicrobial agent they confer resistance to. The abbreviations $MLS_B$ refers to the group of genes resistant to macrolides, lincosamides and type B streptogramins, while BL-CBPM-CPHL refers to the group of genes resistant to beta-lactams, carbapenems and cephalosporins.

### 3.1.3. Distribution of ARGs among different sampling sites from Sabadell's city sewage

Relative abundance of gene classes varied substantially between the different sampling points (Fig. 5). The total relative abundances of ARGs for each category or gene class were calculated to identify the most prevalent classes of resistance genes. The hospital sector (P4) showed the highest mean ARG relative abundance across the four sampling campaigns, with an average of 1753.8 ± 446.7 CPMs, being beta-lactams (5.6 %), carbapenem (4.9 %) and cephalosporin (2.1 %), $MLS_B$ (9.8 %), aminoglycosides (4.2 %), tetracyclines (2.8 %), fluoroquinolones (1.8 %) and sulfonamides (1.5 %) the most abundant gene classes at this sampling site. The mean ARG relative abundance was similar in the two WWTPs entrance points (P1: 738.7 ± 93.4 CPM and P7: 701.2 ± 216.0 CPM) and the two residential sectors (P5: 744.1 ± 101.9 CPM and P6: 918.4 ± 54.0 CPM). Riu Ripoll WWTP Entrance (P1) showed high relative abundance of genes conferring resistance to $MLS_B$ groups (5.5 %), resistance to beta-lactams, carbapenems and cephalosporin group (3.2 %), aminoglycosides (1.8 %) and tetracyclines (1.4 %). Similarly, Riu Sec WWTP Entrance (P7) exhibited a high mean relative abundance of genes conferring resistance to $MLS_B$ groups (5.7 %), resistance to beta-lactams, carbapenems and cephalosporin group (2.8 %), aminoglycosides (1.6 %) and tetracyclines (1.3 %). Regarding the residential areas, Ca n'Oriac sector (P5) showed a high mean relative abundance of genes conferring to $MLS_B$ groups (6.6 %), aminoglycosides (2.1 %), resistance to beta-lactams, carbapenems and cephalosporin group (1.9 %) and tetracyclines (1.5 %); and Torre Romeu sector (P6) also revealed high mean relative abundance of genes conferring resistance to $MLS_B$ groups (7.0 %), resistance to beta-lactams, carbapenems and cephalosporin group (3.1 %), aminoglycosides (2.5 %) and tetracyclines (1.5 %). This suggests that both WWTP entrances and residential areas exhibit high quantities of similar gene classes. In contrast, the industrial zone (P3) and the exit of the WWTP (P2) exhibited the lowest relative abundances, with 131.3 ± 131.1 CPM and 49.7 ± 22.5 CPM, respectively. The industrial zone (P3) only showed high mean relative abundance of gene conferring resistance to resistance to beta-lactams, carbapenems and cephalosporin group (1.1 %). Considering the relative abundance of ARGs at the WWTP entrance (P1), the effluent reduced the presence of ARGs by 93.3 %, and this sampling site (P2) did not show any gene classes at a percentage >1 %.

Although 262 different ARGs variants were identified in the metagenomic analysis, their distribution diverged across the different sampling sites (Fig. 4). Typically, >140 different ARGs were found per sampling area, except in the Riu Ripoll WWTP exit (P2) and in the industrial zone (P3), in which 54 and 46 different ARG classes were predicted, respectively.

Out of 262 predicted ARGs variants, 16 (6.1 %) were present in all sampling sites (Fig. S1), including *aadA1*, *aph(3″)-Ib*, *aph(6)-Id* (aminoglycosides); *blaOXA-129*, *blaOXA-2*, *blaOXA-464* (beta-lactams); *mcr-7.1* (colistin); *qacE* (disinfectants); *erm(B)*, *erm(F)*, *mph(E)*, *msr(E)* ($MLS_B$); *cmlA1* (phenicols); *sul1*, *sul2* (sulfonamides); and *tet(39)* (tetracycline) genes. However, Grubbs' test revealed the relative abundance of *aadA1, blaOXA-2, blaOXA-129* and *sul1* genes were higher in the hospital sector (P4) and *mcr-7.1* in the industrial zone (P3) when compared to the rest of sampling sites.

Concerning the 160 (61.1 %) ARG variants found in at least two

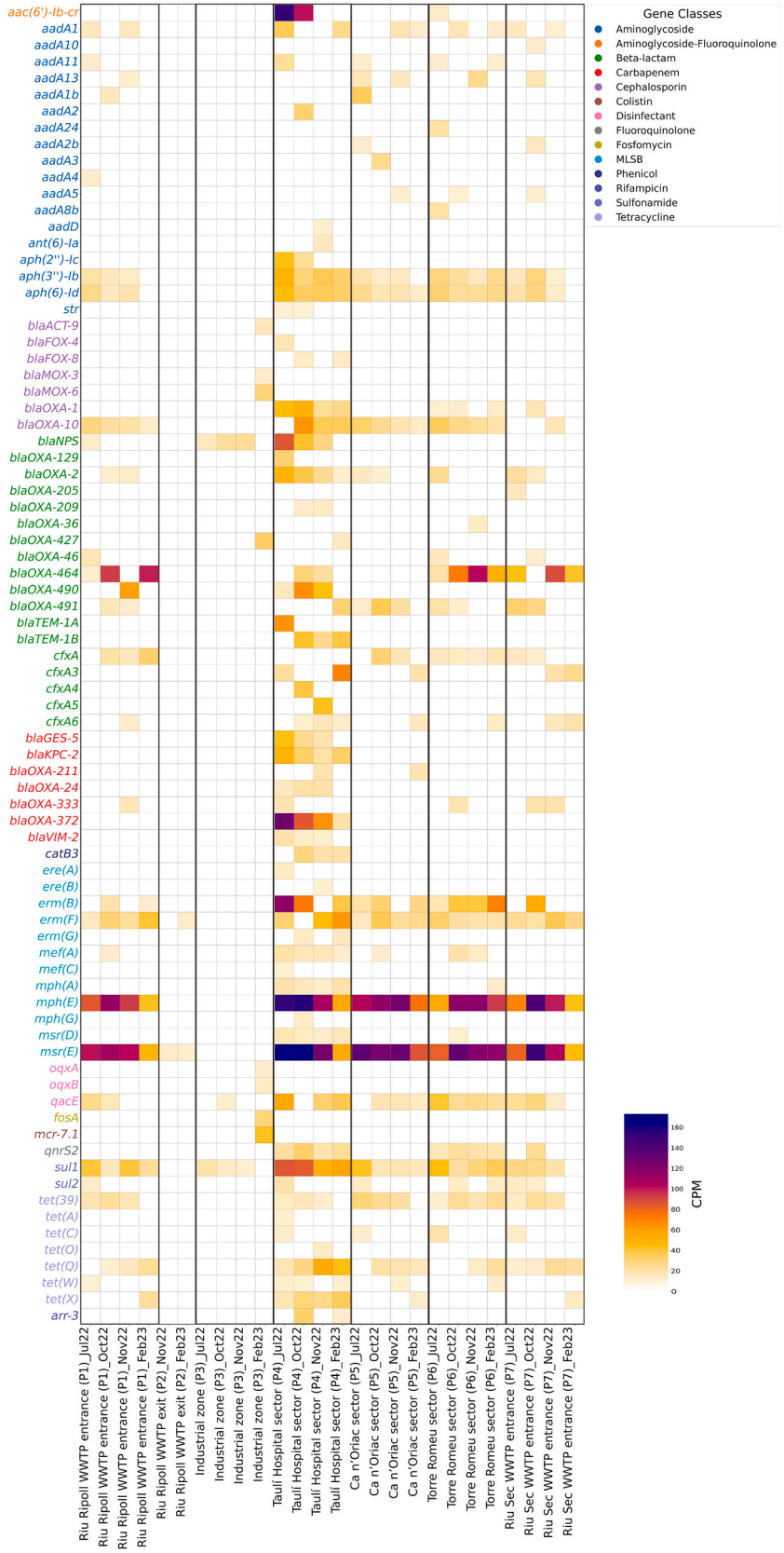


**Fig. 4.** Distribution of the ARGs identified in the study across various sampling points and sampling campaigns. Only ARGs with a relative abundance >10 CPMs are represented, hence the two sampling campaigns in July and October at the Riu Ripoll WWTP exit are not shown in the figure.

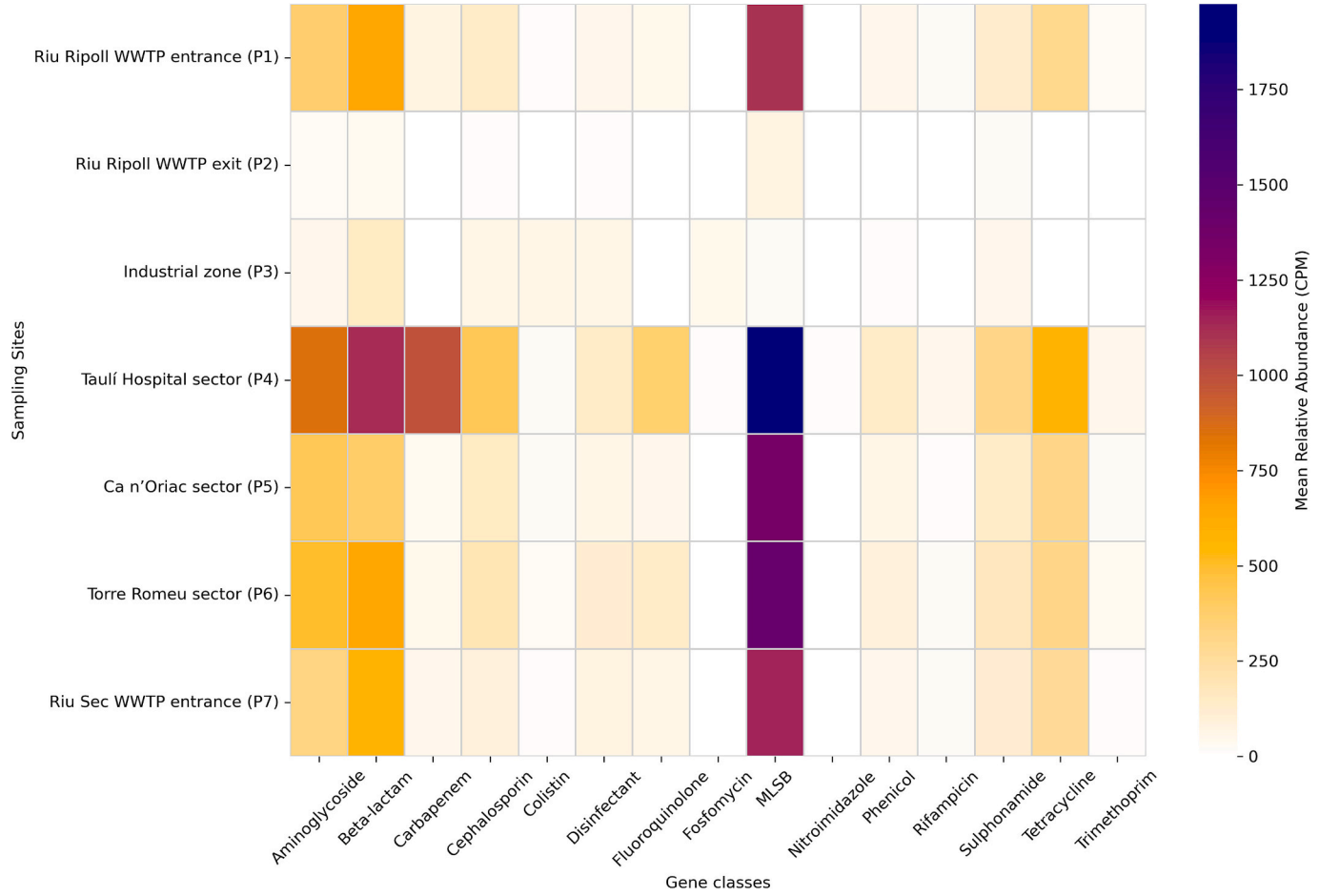


**Fig. 5.** Distribution of the relative abundance of ARGs grouped by the antimicrobial class confer resistance to (gene classes) in the seven selected sampling points from the Sabadell's city (Barcelona, Spain) drainage network (P1-P7).

sectors, 55 exhibited biased distributions regarding the sampling location (Fig. S1). Of note, particular ARGs were predicted almost exclusively (>75 %) in a specific sector, including *blaOXA-372* (carbapenems) *blaTEM-1 A, blaTEM-1B* (beta-lactams)*; aac(6′)-Ib-cr, aac(6′)-aph(2″)*, *aph(2″)-Ic* (aminoglycosides)*;* and *dfrA12* (trimethoprim) genes in the hospital sector (P4). Among them, *blaOXA-372* (92.9 % in the hospital sector) and *aac(6′)-Ib-cr* (87.0 % in the hospital sector) were of great interest because of their high CPM values. The *blaACT-9* (cephalosporins) and *blaOXA-21* (beta-lactams) genes were significantly more prevalent in the industrial zone (P3).

In contrast, 86 (32.8 %) ARG variants were unique to specific sampling sites (Fig. S1). However, most of them (79) had an abundance lower than 10 CPM. Considering ARGs with higher CPM (> 10 CPM) values, *blaMOX-3* (cephalosporins) were exclusive of the industrial zone (P3); *blaOXA-334* (carbapenems) of Riu Ripoll WWTP entrance (P1); and *blaOXA-235* (carbapenems), *blaFOX-4* (cephalosporins), *cfxA4* (beta-lactams) and *str* (aminoglycosides) of the hospital sector (P4).

Finally, when the same analysis was conducted considering the antimicrobials to which resistance is conferred, biased distributions across different sampling sites were again observed using Grubbs' test. Genes conferring resistance to beta-lactams, carbapenems, third-generation cephalosporins, sulfonamides, fluoroquinolones, rifampicin and nitroimidazole were more prevalent in the hospital zone (P4) when compared to the other sampling sites. Predicted genes conferring resistance to fluoroquinolones were also significantly more abundant in the Torre Romeu sector (P6). The industrial sector (P3) was characterized by a high abundance of colistin and fosfomycin resistance genes.

### 3.1.4. Distribution of ARGs in wastewater at independent times points

Sampling campaigns at different locations were carried out in four distinct time points to evaluate temporal changes in ARG variation (Fig. 6). The selected months were July-22, October-22, November-22 and February-23. Grubbs' test revealed that CPM values obtained at the different time points were stable across the studied locations, except for the industrial zone (P3), which experienced a significant increase in February-23. Conversely, although not significant, the hospital sector (P4) exhibited a reduction of 32.0 % and 44.1 % in CPM abundances in the last two campaigns when compared to the first one, respectively.

Relative abundance of some ARG variants showed stational dependence, being detected mainly (>50 %) in a particular sampling campaign. Specifically, *aadA11, aph(2″)-Ic, blaBEL-1, blaTEM-1 A, blaOXA-46* and *blaOXA-129* genes were more prevalent in July-22; *aadA2, aadA3, blaBRO-2, blaOXA-21* and *blaVEB-3* in October-22; and *blaMOX-6, cfxA3, fosA, mcr-7.1, oqxA* and *oqxB* in February-23. Among them, *aadA11, cfxA3* and *mcr-7.1* genes were of notable interest due to their abundance (>100 CPM). Some of these genes, in turn, were more prevalent in a single location during these months, including *aadA2, aph (2″)-Ic, blaOXA-129* and *blaTEM-1 A* in the hospital sector (P4); and *blaMOX-6, blaOXA-21*, *fosA*, *oqxA* and *oqxB* in the industrial sector (P3). Sampling campaigns conducted at the Riu Ripoll WWTP exit showed low relative abundances of ARGs. Specifically, in July and October-22, ARGs remained below 10 CPM, whereas in the subsequent sampling campaigns, two ARGs exceeded this threshold: *msr(E)* gene in November-22, and both *erm(F)* and *msr(E)* genes in February-23.

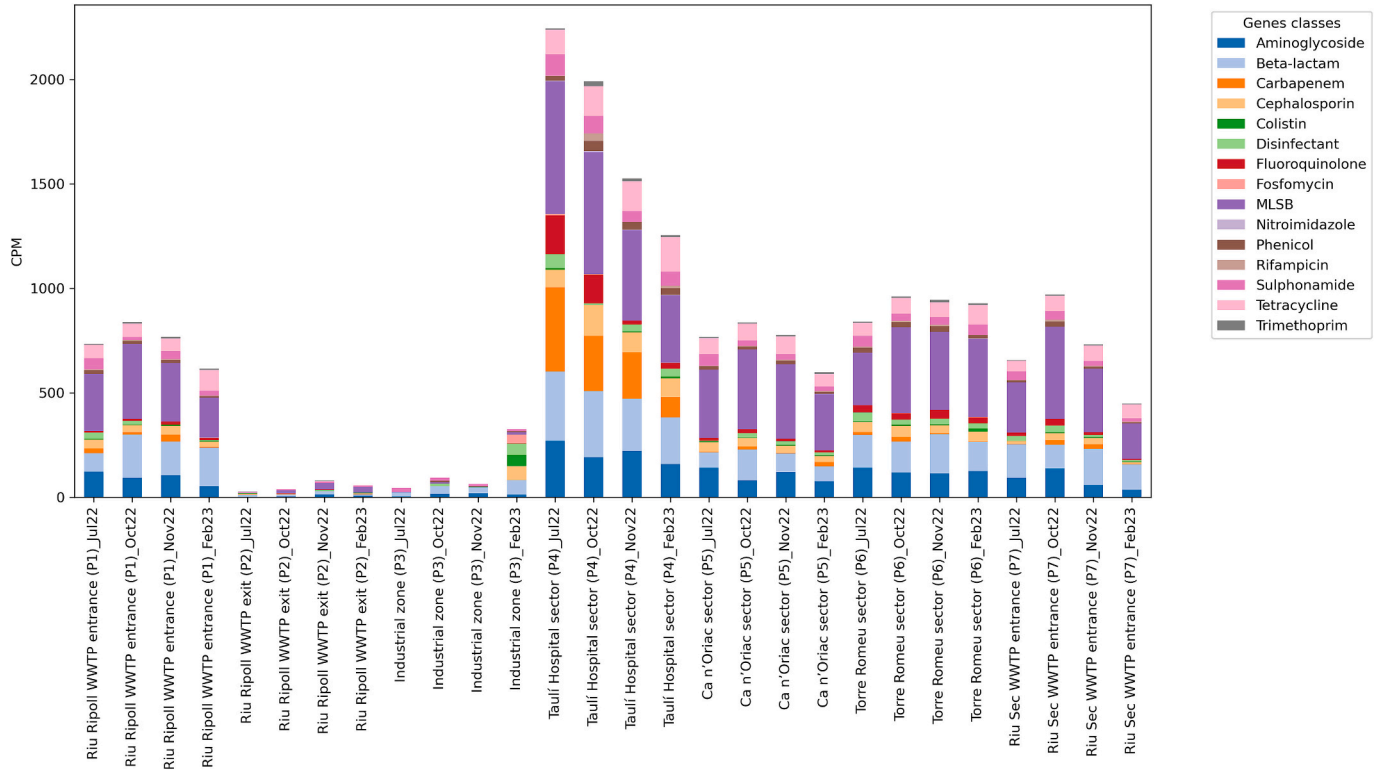


**Fig. 6.** Temporal evolution of the ARGs grouped by the antibiotic to which they confer resistance to across the different sampling campaigns. The resistance profiles remain consistent across different geographic regions over four sampling times.

### 3.2. Phenotypic analysis

To get further knowledge on the ARG genes and antibiotic resistant bacteria (ARB) present in the wastewater samples, organic fractions retained on 0.45 μm filters were cultured on plates selecting for methicillin-resistant *Staphylococcus aureus*, third-generation cephalosporin or carbapenem-resistant bacteria, due to its clinical relevance in hospital infections. These analyses were done at different points of time at all the selected sites.

As shown in Fig. 7 and in Table S4, third-generation cephalosporin-resistant bacteria were detected in all the sampling sites during at least one of the sampling campaigns. Carbapenem-resistant bacteria were also widespread, but they were never observed in the industrial sector. Of note, third-generation cephalosporin-resistant bacteria were only detected in the industrial sector in one sampling campaign (February 2023). Therefore, the industrial sector was the sampling spot with the lowest load of the ARB under study. In acute contrast, the hospital sector showed the highest occurrence of the characterized ARB. Specifically, third-generation cephalosporin and carbapenem-resistant bacteria were detected in all the sampling campaigns at this location. Moreover, the number of third-generation cephalosporin and carbapenem-resistant bacteria present in the hospital sewage was always substantially higher as compared to the other sampling spots.

The remaining sampling points showed comparable log-counts of third-generation cephalosporin and carbapenem-resistant bacteria. Slight differences were observed between the two WWTPs entrances (Riu Ripoll and Riu Sec). Specifically, the occurrence of carbapenem-resistant bacteria was higher in Riu Ripoll inlet (P1), which receives the wastewater effluent from the hospital, when compared to the inlet of the Riu Sec WWTP (P7), where there is no hospital input. Finally, it is important to note that no seasonal trend was observed in any sampling point nor for the characterized ARB along the four sampling campaigns carried out. Of note, we did not detect the presence of methicillin resistant staphylococci in our study. In addition to third-generation cephalosporin and carbapenem resistance, other antibiotics resistance phenotypes were characterized (Table S4).

### 3.3. Genetic analysis of selected isolates recovered from the Sabadell's city sewage

To get further knowledge on the ARG variants detected in our study, 61 sewage isolates were selected for WGS analysis according to their clinical relevance. Selected genera included *Klebsiella* ($n = 22$), *Escherichia* ($n = 15$), *Enterobacter* ($n = 8$), *Citrobacter* ($n = 7$), *Aeromonas* ($n = 3$), *Raoultella* (n = 3), *Acinetobacter* (n = 2) and *Serratia* (n = 1). Among these, 18 isolates were resistant to carbapenems and 52 exhibited resistance to third-generation cephalosporins.

Abricate software predicted a total of 113 distinct ARGs, which were identified across various resistance mechanisms (Fig. 8A, Fig. S2). Specifically, genes conferring resistance to clinically relevant antibiotics were predicted: beta-lactams (100 % of analyzed isolates), including third-generation cephalosporins (90.2 %) and carbapenems (34.4 %); quinolones (82.0 %), aminoglycosides (73.8 %), sulfonamides (52.5 %), fosfomycin (54.1 %), macrolides (49.2 %) and trimethoprim (45.9 %) (Table S5). Third-generation cephalosporin resistance was predicted to be mostly mediated by the *blaCTX-M-15* ($n = 21$) and *blaOXA-1* (n = 21) variants, and carbapenem resistance by *blaKPC-2* (n = 15). The β-lactamase-encoding *blaTEM-1B* (n = 15) gene was also abundant in this collection. Aminoglycoside resistance was predicted to be mainly achieved by *aac(6′)-Ib-cr* ($n = 23$), *aph(3″)-Ib* ($n = 14$), *aph(6)-Id* (n = 14), *aac(3)-IIa* ($n = 10$) and *aadA2* (n = 10) genes. Quinolone resistance was predicted to be predominantly mediated by *oqxB* ($n = 32$) and *oqxA* ($n = 31$) genes. The *sul1* ($n = 20$), *sul2* ($n = 13$) and *dfrA14* (n = 13) variants were the most predicted genes conferring resistance to sulfonamides and trimethoprim, respectively. Fosfomycin resistance was predicted to be mainly mediated by *fosA* ($n = 26$) variants (Table S5). These results are in agreement with the phenotypic data obtained by antibiotic susceptibility tests (Fig. 8B, Table S6). Notably, >80 % of the strains predicted

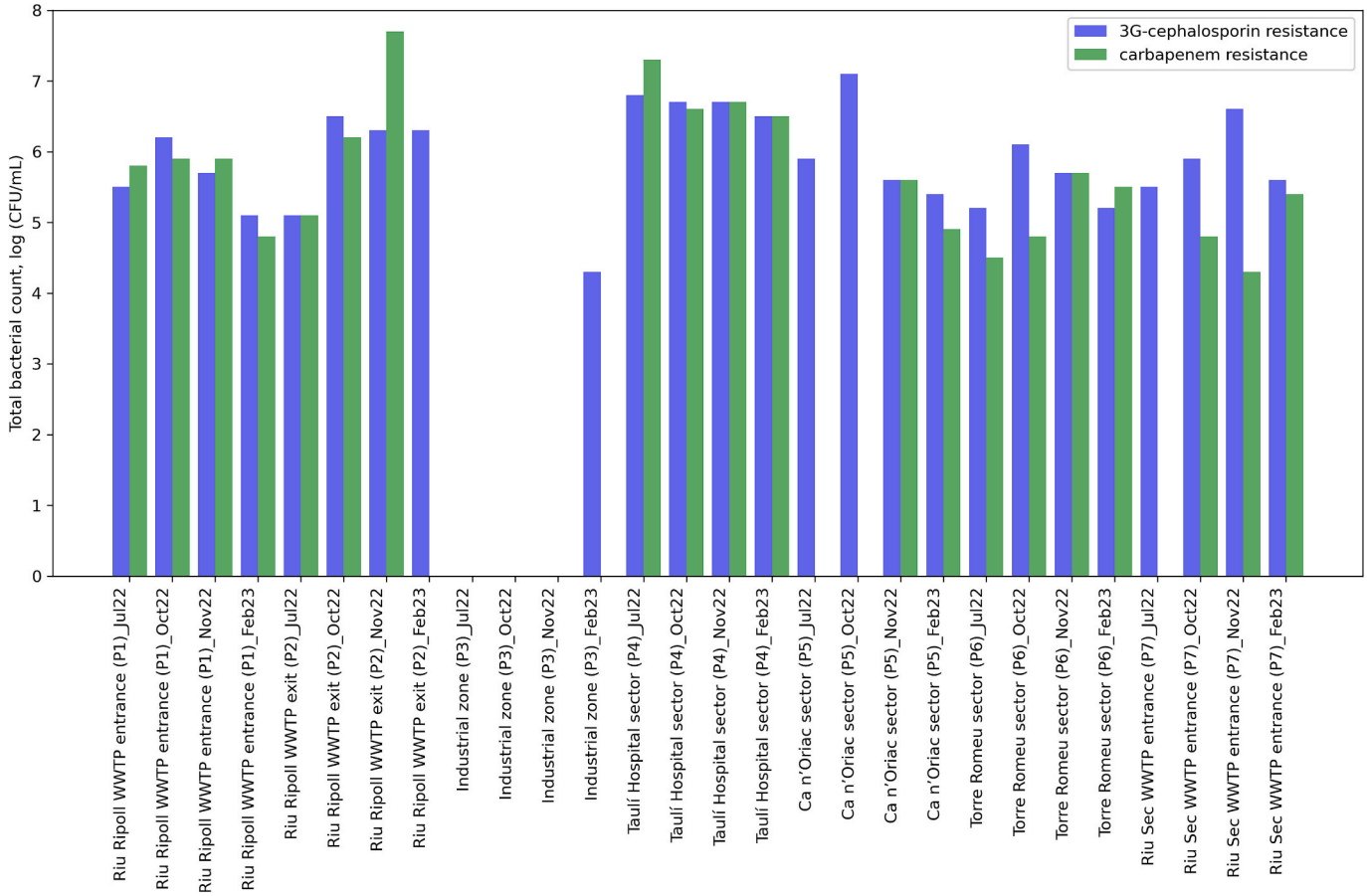


**Fig. 7.** Total 10-log colony-forming unit/mL (CFU/mL) count for each sampling point. Blue bars represent third-generation cephalosporin-resistant bacteria and green bars represent carbapenem-resistant bacteria.

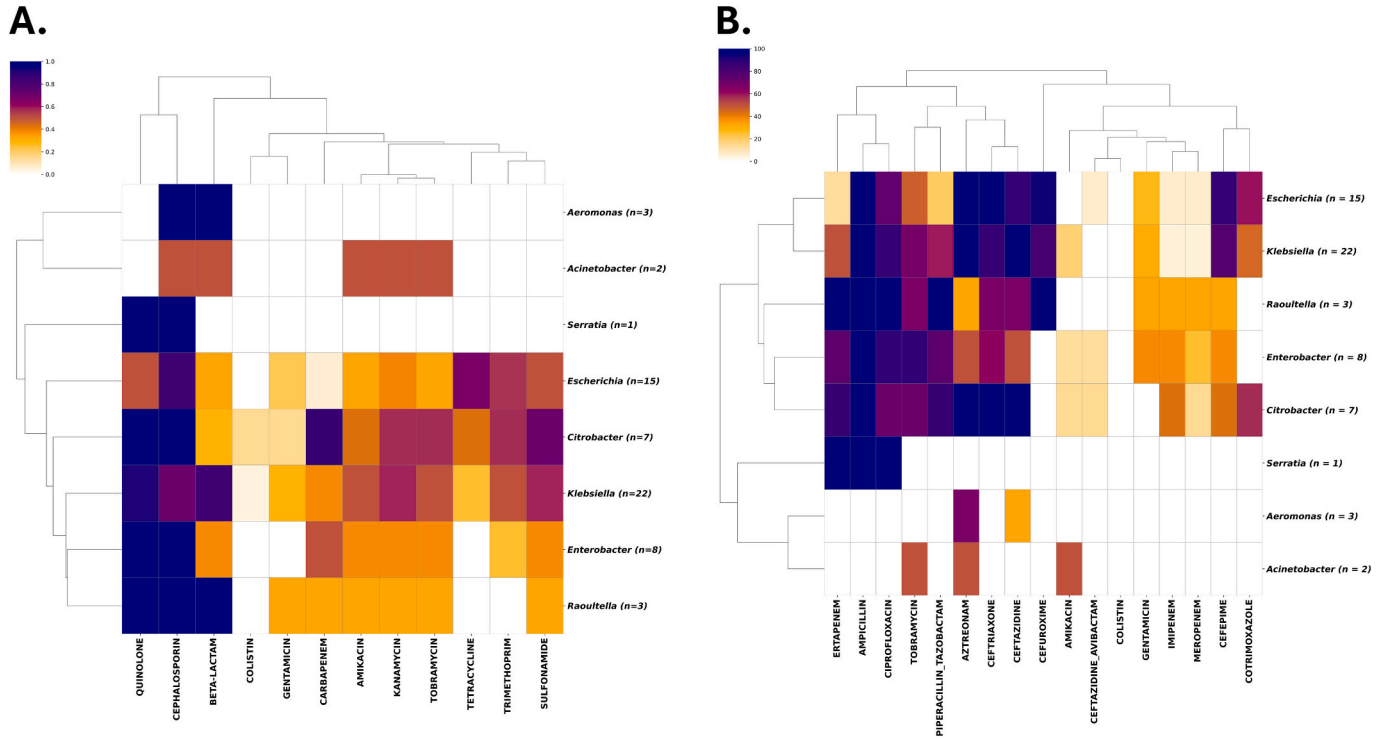


**Fig. 8.** Clustered heatmaps showing the frequency distribution of predicted (A) and experimentally confirmed (B) resistant strains.

to be resistant to carbapenems, tobramycin, third-generation cephalosporins, gentamycin, and quinolones were confirmed to be resistant. However, the prediction accuracy for resistance to amikacin (11.5 %) was remarkably lower.

### 3.4. Impact of plasmids in ARG dissemination among bacteria

To study the role of plasmids on antimicrobial resistance dissemination in water environments, plasmid sequences were predicted on all the selected strains with the MOB_suite. The location of ARGs was then visualized as a bipartite network using Gephi. The network analysis uncovered two clusters (Fig. 9A), with one comprising ARGs encoded in chromosomes and the other mainly consisting of plasmid-borne ARGs. The correlation between the total number of each predicted ARG in all genomes and its number in plasmids was depicted as a scatter plot. The results (Fig. 9B) indicated that most abundant genes in the selected collection were plasmid-borne (Pearson Correlation Coefficient (PCC) = 0.64). Among these, *sul1, qacE, aac(6′)-Ib-cr, blaOXA-1, blaTEM-1B, aph (3″)-Ib, aph(6)-Id, blaCTX-M-15, blaKPC-2, mph(A), tet(A), dfrA14* and *sul2* genes showed to be most prevalent in plasmids (Fig. 9B). Likewise, ARG predicted in different bacterial genera were preferentially plasmid-encoded (Mann-Whitney *U* test $p < 0.05$) (Fig. 9C), showing that these mobile elements favor the spread of ARG. Overall, these results pointed out that plasmids play a role in the dissemination of ARGs and contribute to the quantity and diversity of ARGs in bacterial communities.

## 4. Discussion

### 4.1. Hospital sector is the key contributor to ARG abundance and diversity

The present study has characterized the resistome at seven points in the sewage network of Sabadell city (Barcelona, Spain) across four sampling campaigns. Using a comprehensive approach, including metagenomics, bacterial culture for specific ARB isolation, and WGS of resistant isolates, key hotspots in the sewage network were identified at the city scale, revealing ARG variants and ARB of critical concern.

Based on our results, sampling sites can be categorized into high-risk, medium-risk and low-risk spots. The high-risk sector identified in this study was the Taulí Hospital Sector (P4), which exhibited the highest diversity and relative abundance of ARG variants together with a microbial community characterized by bacteria typically associated with hospital settings, such as *Enterobacter*, *Acinetobacter*, *Klebsiella* or *Pseudomonas*. These genera may include species belonging to the ESKAPE group, which is characterized by its significant role in nosocomial infections and its resistance to different clinically relevant antibiotics (Mancuso et al., 2021). In this way, the hospital area was characterized

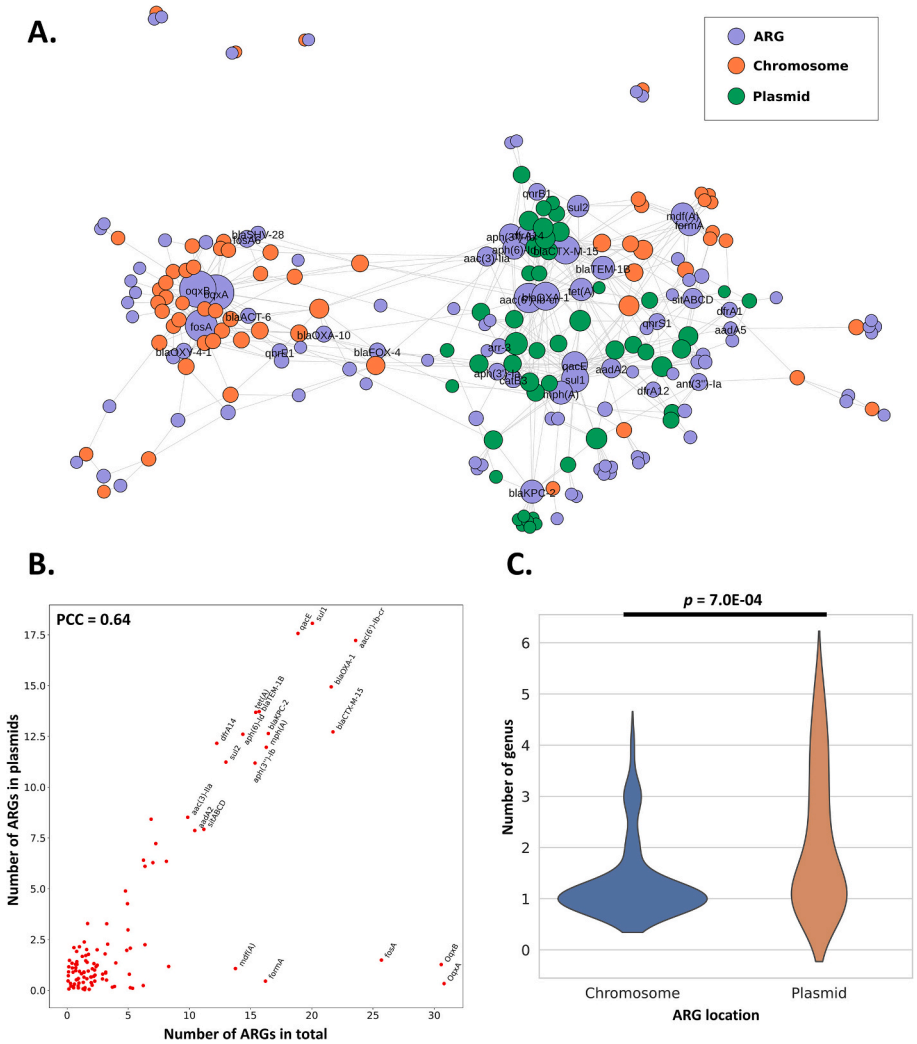


**Fig. 9.** Role of plasmids in ARG dissemination. (A) Bipartite network illustrating the association between ARGs and their genomic location, namely chromosome or plasmid. (B) Scatter plot highlighting the relationship between the total number of ARGs identified in our isolates and those specifically located on plasmids. (C) Violin plots representing the distribution of species in which each ARG is found depending on its genomic location.

by the identification of the highest number of unique genes not detected at any of the other sampling points (Fig. S3). Among these, ARG variants conferring resistance to last resort antibiotics such as carbapenems (*blaOXA-48*) or third-generation cephalosporins (*blaSHV-12* and *blaSHV-28*) stood out in the analysis. These ARG variants represent a significant concern due to the limited therapeutic alternatives. These types of antibiotics are often prevalent in hospital settings highlighting the need of establishing surveillance measures in selected hotspots (Codjoe and Donkor, 2017; Nasri et al., 2017; Markkanen et al., 2023). Considering the predominant antibiotic resistance groups detected in P4 (beta-lactams, carbapenems, third-generation cephalosporins, $MLS_B$, aminoglycosides, tetracyclines, fluoroquinolones, and sulfonamides), key resistance genes are highlighted: *blaOXA-1* (third-generation cephalosporins), *blaKPC-2* (carbapenems), *blaTEM-1B* (beta-lactams), *aac(6′)-Ib-cr* (aminoglycosides/fluoroquinolones), *tetQ* (tetracyclines), *oqxB* (fluoroquinolones), and *sul1* (sulfonamides), as these genes were further confirmed through phenotypic analysis. Nevertheless, the most abundant gene found in the metagenomic analysis, *blaOXA-372,* was not identified in the isolated ARB. The finding of this gene at P4 is consistent with its first identification of *blaOXA-372* in a *Citrobacter freundii* isolate retrieved from a hospital wastewater treatment plant (Antonelli et al., 2015).

Both metagenomics and culture analysis showed the presence of third-generation cephalosporins and carbapenem resistance genes in wastewater at different collection points. The occurrence of carbapenem-resistant bacteria was higher in Riu Ripoll inlet (P1), which receives the wastewater effluent from the hospital, when compared to the inlet of the Riu Sec WWTP (P7), without the hospital input. These data are similar to previous studies (Rozman et al., 2020; Zhang et al., 2020; Majumder et al., 2021) and confirm that hospitals are a hotspot of antibiotic major use and selection of resistant bacteria. Infections caused by third-generation cephalosporin and carbapenem-resistant bacteria are a major concern in nosocomial infections due to their reduced antibiotic treatment options. So, this wastewater surveillance could be very useful to detect the emergence of newly resistant bacteria in these settings. The chemical detection of key antibiotics in P4 (Fig. S4 and Data S1) together with the ARG variants identified in the metagenomic analysis and the ARB isolated from the phenotypic analysis underscores the selective pressure exerted on hospital effluents that drives resistance mechanisms (Hassoun-Kheir et al., 2020). Those ARGs and ARB may be discharged into the environment and have the potential threat to spread in other settings, being a problem for humans, animals and the environment (Serna and Gonzalez-Zorn, 2022). Therefore, hospital effluent is identified as a hotspot for antimicrobial resistance dissemination, particularly carbapenem and third-generation cephalosporin resistance, highlighting the critical need for pre-treatment of these discharges before the release into the sewage network. In contrast, MRSA was not detected in the analyzed wastewater samples. MRSA colonizes the human skin and mucous membranes, but it is not a commensal of the intestinal tract; hence, its presence in wastewater is indirect. While some studies have reported the presence of MRSA in wastewater samples (Rosenberg Goldstein et al., 2012), the low prevalence of MRSA in our hospital is in agreement with the absence of these bacteria in our study (Sánchez-Osuna et al., 2024).

Urban effluents originated in Ca n'Oriac sector (P5) and Torre Romeu sector (P6), together with input water at the studied WWTPs, Riu Ripoll WWTP entrance (P1) and Riu Sec WWTP entrance (P7) can be classified as medium-risk zone due to a more heterogeneous pattern, with a greater variety of ARG variants and bacterial taxa. This diversity reflects the influence of multiple inputs from the drainage network. These sampling points exhibited a similar pattern in terms of microbial community composition and the antibiotic groups representing the detected ARG variants, including resistance to $MLS_B$, beta-lactams, carbapenems, third-generation cephalosporins, aminoglycosides and tetracyclines. The relative abundance of ARG variants detected in these urban sites was lower than in the hospital sector. Notwithstanding this, urban wastewater has been identified as a critical point for monitoring due to its potential role in the ARG dissemination into the environment (Triggiano et al., 2020).

The low-risk zones identified in this study include the Riu Ripoll WWTP exit (P2) and the Industrial zone (P3). Our results revealed that the effluent from P2 and the adjacent P3 exhibit the lowest abundance of ARG variants compared to other sampling points. This suggested that the biological treatment applied at the treatment plant (P2) was effective in reducing the abundance of ARGs and ARB. The low relative abundance of ARGs at P2 was consistent with the low relative abundance of the classified taxa. The diversity and evenness observed in the microbial community of P2, which corresponds to the treated water after biological process, are in agreement with previous results (Gad et al., 2023; Wardi et al., 2023). Biological treatments, such as activated sludge processes, are designed for nutrient removal, organic matter degradation, and pathogen control. Consequently, the treated effluent at P2 exhibits a different microbial profile from the influent water, as the water treatment enriches a wide range of microorganisms. The microbial community observed at P2 included genera that can be associated with potentially pathogenic species, such as *Pseudomonas*, which are frequently found in wastewater samples and may contain *Pseudomonas aeruginosa* (Luczkiewicz et al., 2015). However, metagenomics does not provide information on the viability of the bacterial community, as it detects all DNA present in a sample, including that from dead cells and extracellular DNA. Therefore, it is essential to complement the metagenomics approach with techniques that assess bacterial viability (Nowrotek et al., 2019), as performed in this study. Also techniques with lower limits of detection and quantification, such as qPCR, constitute a rapid diagnostic tool that facilitates the precise and continuous monitoring of these contaminants (Ferreira et al., 2023).

On the other hand, the different anthropogenic water origin of the industrial zone (P3) compared to the rest of the points contributed to a distinct genetic diversity, resulting in a specific ARG profile characteristic of this environment, being the *mcr-7.1* gene, which confers resistance to colistin, one of the most abundant genes identified in metagenomic analysis. While in the high- and medium- risk sites, the genera dominating the microbial community were *Aliarcobacter* and *Acinetobacter*, the microbial community of P3 was primarily characterized by the dominance of *Aeromonas*, *Pseudomonas* and *Thauera*. These genera may reflect the unique environmental conditions of the industrial zone, which likely select for a distinct microbial community. These results are in agreement with a distinctive ARG profile of P3 compared to the other sampling sites.

Considering the global resistome described after metagenomic analysis, it is striking that *msr(E)* and *mph(E)* genes conferring resistance to $MLS_B$ group were detected across all sampling sites. This is in agreement with previous studies (Sekizuka et al., 2022; Begmatov et al., 2024), where it was observed that these genes persisted even after wastewater treatment, as observed in P2. Metagenomic analyses revealed similar high relative abundances for these genes (Fig. 3), which align with previous studies indicating their frequent co-occurrence in tandem on discrete plasmid modules. This genomic arrangement facilitates their tandem spread, further emphasizing their role in antimicrobial resistance dissemination (Blackwell and Hall, 2017). Particularly, *msr(E)* and *mph(E)* exhibited high concentrations in the high- and medium-risk zones constituting a major reservoir in non-treated wastewater.

### 4.2. Plasmids are the drivers of ARG dissemination in wastewater environments

To complement the metagenomic analysis, the whole genomes of several representative bacterial isolates recovered from different sampling points and periods were sequenced. Most of the isolates analyzed were *Enterobacteriaceae*, as the species from this family were enriched in our selection strategy for carbapenem and third-generation

cephalosporin-resistant bacteria. The presence of *Klebsiella* in treated wastewater is attributed to human emissions and the resilience of these bacteria to wastewater treatment processes. Its ability to survive and sometimes thrive in these environments raises concerns, especially given the increasing prevalence of virulent, MDR strains (Di Cesare et al., 2024). Previous studies have shown that wastewater *Klebsiella pneumoniae* isolates are indistinguishable from those of clinical origin based on phenotypic and genotypic characterization (Rocha et al., 2022). In our *Klebsiella* isolates, the most frequently detected resistance gene was *blaCTX-M-15* (45.8 %), primarily conferring resistance to third-generation cephalosporins. Notably, carbapenemase genes like *blaKPC-2* (20.8 %) and *blaFOX-4* (20.8 %) posed a significant concern for their potential to undermine last-resort carbapenem treatments. Noteworthy, some of the MDR *Klebsiella* isolates analyzed in our study harbored genes conferring resistance to other last-resort antibiotics such as colistin. The colistin resistance gene *mcr-9* was identified in some *Klebsiella* and *Citrobacter* isolates, consistent with its previous detection in wastewater isolates (Hem et al., 2024; Song et al., 2024). *Escherichia coli* ST131 has been widely reported as the predominant lineage in rivers, hospital sewage, and wastewater treatment plants. Other pathogenic lineages, including ST10, ST38, and ST69, are also frequently identified, particularly in hospital sewage, though they are also present in river water (Davidova-Gerzova et al., 2023). Consistent with these findings, our study detected ST131 at a notable frequency (27.8 %) and ST10 (11.1 %), while ST38 and ST69 were not detected (Table S7). In agreement with previous reports (Park et al., 2024), we found a very low prevalence of carbapenem (5.5 %) and colistin resistance (0 %) among the sequenced *Escherichia* isolates. However, we observed a remarkable presence of genes conferring resistance to tetracyclines (66.7 %). *Aeromonas* isolated from hospital sewage were suggested to act as a reservoir of genes resistant to last-line antibiotics (Wu et al., 2023). In our study, *Aeromonas* were particularly resistant to aztreonam and ceftazidime-avibactam, which are widely used in combination to treat infections caused by carbapenem-resistant bacteria (Falcone et al., 2021).

Our WGS analysis of resistant bacterial isolates also highlights the critical role of plasmids in the dissemination of ARGs within water environments. The ability of plasmids to move between bacteria makes them powerful platforms for transferring resistance genes (Sánchez-Osuna et al., 2023), thereby complicating efforts to control the rise of antibiotic resistance in environmental and clinical settings. In line with our results, previous studies showed that plasmids were a remarkable part of the resistome of wastewater samples (Che et al., 2019) and that they tend to encode most of the prevalent ARGs (Sentchilo et al., 2013). As mentioned, many of the most relevant resistance genes were predominantly plasmid-borne in our study. A similar global study of sewage water across 101 countries reported comparable findings, with *blaCTX-M-15* (100 %), *tet(A)* (92.7 %), *blaKPC-2* (85.7 %), *sul1* (83.3 %), *aph (3″)-Ib* (80.9 %), *blaTEM-1B* (71.9 %), *aph(6)-Id* (60.0 %), *sul2* (58.2 %), and *mph(A)* (52.0 %) primarily associated with plasmids. Contrasting our results, the *blaOXA-1* gene (33.3 %) was predominantly found on chromosomes in that study (Munk et al., 2022). The location of the *blaOXA-1* gene has been reported both on plasmids and chromosomes, though its localization can vary depending on the bacterial species and environmental context (Colom et al., 2003). It is noteworthy that previous reports also found regional patterns in plasmid resistomes (Munk et al., 2022; Teudt et al., 2022), so conducting similar studies in other cities would be of great interest.

Metagenomic sequencing cannot easily pinpoint plasmid hosts due to plasmid mobility across species, but WGS provides a clearer link between plasmids and their bacterial hosts (Che et al., 2019). Thus, we observed that plasmid-borne ARGs are found more often in different bacterial genera, reinforcing the plasmid role as a key vector for the spread of resistance, even crossing phylogenetic barriers. A previous analysis of over ten thousand plasmids showed that 60 % have host ranges beyond the species barrier and up to 10 % order barriers (Redondo-Salvo et al., 2020). In a recent study analyzing wastewater samples, authors noticed that plasmids carrying ARG were found in a broad range of hosts, reflecting their significance in connecting microbial networks and facilitating resistance transfer among different taxa (Risely et al., 2024). These findings underscore the significance of plasmids in shaping resistance dynamics in wastewater communities, contributing not only to the spread of AMR within bacterial populations but also to its overall diversity. The critical role of wastewater environments in mobile resistance has been highlighted in previous research, which identifies them as key sites for the origin of multiple mobile ARGs (Berglund et al., 2023).

### 4.3. ARG markers for AMR surveillance

The comprehensive characterization of AMR in this study, using various analytical methodologies and data processing approaches, enabled the identification of ARGs with key characteristics that support their proposal as markers for AMR tracking. These characteristics include abundance, clinical significance (determined according to both, literature review and ARGs predicted in ARB isolates), ARGs associated with plasmids, and ARGs detected in WWTP effluents. The ARGs that were included in most of the categories were *blaOXA*-related genes, highlighting the high concern of this group of ARGs as stated in previous studies (Hubeny et al., 2021). Specifically, *blaOXA-1*, *blaKPC-2*, and *blaTEM-1* met three key criteria: they are clinically relevant, predicted in ARB isolates, and located on plasmids. Additionally, monitoring *msr(E)* and potentially *erm(F)* will provide valuable insights into WWTP efficiency, as *msr(E)* was among the most abundant genes identified in this study, aiding in further AMR characterization. Other key genes of clinical concern are those related to carbapenem resistance, including *blaOXA-48*, *blaKPC*, *blaGES*, *blaIMP* and *blaVIM*, as pointed out by other authors in similar studies (Makowska et al., 2020).

Previous studies have proposed ARG markers for various purposes, including estimating the total ARG load, assessing WWTP efficiency, and identifying hotspots of clinically relevant ARGs. Some studies correlated the abundance of some specific ARG indicators with the total load of ARGs (Pärnänen et al., 2019; Tavares et al., 2024). Pärnänen and coworkers found a strong correlation between the abundance of two ARGs (*aadA* and *ermF*) and *intI1* with the total ARG abundance; and similarly Tavares and collaborators proposed using two ARGs (*blaGES*, and *qacEΔ1*) and also *intI1* to estimate the total ARG load in a given sample. These studies identified ARGs similar to those in the present work, such as *ermF*, although some were not included in our analysis. This underscores potential differences among study sites and the need for review studies to select the most broadly representative ARG indicators. The choice of specific antibiotic resistance as markers will depend on the prevalence in the local environment, broad-spectrum versus narrow-spectrum antibiotics, antibiotic associated with high resistance and the antibiotic used in different sectors.

ARG markers have also been identified to assess the efficiency of WWTPs in removing these contaminants. Recent studies have reported that ARG levels significantly decreased during wastewater treatment, although some ARGs, like *mphE, msr(E), tet39*, and *erm(F)*, were still detectable downstream (Teixeira et al., 2023; DG Environtment, 2024). Our findings align with this, as WWTP using conventional treatment significantly reduced ARG abundance by 93.3 %, yet some ARGs, including *msr(E)* and *erm(F)*, were still present at the outlet. These results support the use of these genes as markers for WWTP efficiency in reducing ARG loads. This may be explained by the fact that this WWTP did not account for a disinfection treatment, and hence no specified technology is applied for bacterial reduction, nevertheless further research would be needed in order to understand the different behavior of the monitored ARG and ARB along the wastewater treatment process. Quaternary treatments could represent a viable strategy for the comprehensive removal from the outlet as is mentioned in the Directive (EU) 2024/3019 of the European Parliament and of the Council of 27 November 2024 concerning urban wastewater treatment. Its use will be

mandatory for all plants over 150,000 p.e. (and over 10,000 p.e. based on a risk assessment) by 2045. For all agglomerations of 100,000 p.e. and more, the monitoring of AMR should be conducted at the inlets and outlets of WWTPs at least twice/year to increase knowledge/support further action to prevent its dispersion.

A particularly important area of interest is the use of clinically relevant ARG markers to monitor potential hotspots of dissemination, primarily from hospital effluents and using genes such as *blaOXA-48, blaCTX-M, blaIMP, blaTEM*, and *blaKPC-2* (Al Salah et al., 2020; Shuai et al., 2024). These ARGs were also found in the current study, highlighting their widespread distribution in hospital-related waters and reinforcing the need to include some of them in the final list of ARG indicators. The use of on-site hospital wastewater treatment would reduce the presence of ARGs before its entry to the drainage network in the general discharge reducing their transference and dissemination (Paulus et al., 2019).

In conclusion, this study underscores the importance of environmental analysis, such as wastewater monitoring from hospitals and urban areas, for AMR surveillance, aiding clinical decisions and policymaking. The results confirmed that hospitals are key hotspots for antibiotic resistant gene dissemination, with their wastewater containing high ARG diversity and abundance. By evaluating resistance patterns in relation to antibiotic consumption, WBE can provide a broader perspective that complements surveillance systems. Furthermore, this study reinforces that plasmids play a crucial role in the spread of ARG in wastewater. The comprehensive characterization of AMR in this study allowed to propose ARG markers for AMR tracking, however, further harmonization of AMR indicators and thresholds are needed to better characterize and compare results from different studies. Finally, on-site hospital wastewater treatment is proposed as a solution to limit environmental ARG spread.

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## CRediT authorship contribution statement

**Clara Díaz-García:** Writing – original draft, Methodology, Investigation. **Miquel Sánchez-Osuna:** Writing – original draft, Methodology, Investigation, Formal analysis. **Albert Serra-Compte:** Writing – original draft, Project administration, Investigation. **Ioanna Karakatsanidou:** Project administration, Investigation. **Inmaculada Gómez-Sánchez:** Methodology. **Berta Fidalgo:** Methodology. **César Barbuzana-Armas:** Methodology, Formal analysis. **Mariana Fittipaldi:** Investigation. **Riccardo Rosselli:** Formal analysis, Data curation. **Jordi Vinyoles:** Resources, Methodology, Funding acquisition, Conceptualization. **Susana González:** Supervision, Conceptualization. **Oscar Q. Pich:** Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Mateu Espasa:** Writing – original draft, Supervision, Funding acquisition, Conceptualization. **M. Adela Yáñez:** Writing – original draft, Supervision, Conceptualization.

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Both entities have contributed their human and material resources to the development of the project. Aigües Sabadell has led the definition of the sampling points, has participated in the carrying out of the sampling campaigns and has provided data on the drainage network and the operation of the WWTPs, collaborating in the writing of the report. The Corporació Sanitària Parc Taulí, along with the Institut d'Investigació i Innovació Parc Taulí (I3PT), have led the design of the analytical campaign, the analysis and interpretation of data and the writing of the report. The decision to submit the article for publication was joint between the funding sources and the other participating entities.

## Declaration of competing interest

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

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## Data availability

Data will be made available on request.

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