



Quantitative study of ESBL and carbapenemase producers in wastewater treatment plants in Seville, Spain: a culture-based detection analysis of raw and treated water

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ABSTRACT

Antibiotics can modify populations of multidrug-resistant microorganism (MDRO) in urban wastewater. Our objectives were to quantify the differences in MDR Gram-negative bacteria between influents and effluents of WWTPs of a Spanish city and to evaluate the influence of human antibiotic prescriptions, as well as the persistence of these bacteria after treatment and their genetic relatedness to clinical isolates. The mean count of ESBL producers and carbapenemase producers were 3.77 and 2.74 log₁₀ CFU/ml, respectively. The reduction achieved by water treatment of ESBL-producing organisms was 1.4-log (96.11 %), whereas a 1.8-log reduction (98.36 %) was obtained regarding carbapenemase producing organisms. *Aeromonas* spp. predominated among MDROs and *bla*_{KPC-2} was the main carbapenemase detected in the influent wastewater samples. Among *Escherichia coli* and *Klebsiella pneumoniae* influent isolates, 44 % and 30 %, respectively, belonged to high-risk clones. Regarding Enterobacteriaceae, 10.6 % matched clinical isolates and one strain from an ongoing hospital outbreak was identified among raw samples. New MDROs and persistence of certain strains were detected in effluent samples. Quinolone and third-generation cephalosporin prescriptions, flow rate and population density were associated with higher OXA-48 producer counts. Despite reductions, additional technologies should be implemented in WWTPs receiving hospital discharges. Given the prevalence of environmental species, culture-based and metagenomic approaches should be combined to distinguish between human and sewage sources for antibiotic resistance monitoring. Overall, this study shows that WWTPs with secondary treatment are effective at removing MDRO, and antibiotic stewardship is a potential strategy to reduce the release of MDROs.

1. Introduction

Strategies to control antimicrobial resistance (AMR) should include those aimed at the original sources where resistance determinants are produced. There are two main reasons why wastewater is an important driver of AMR. First, it can accumulate antibiotic and disinfectant

residues. Second, it represents a niche where human pathogens of intestinal origin interact with bacteria adapted to the environment. Wastewater treatment plants (WWTPs) aim to remove solids, organic matter, and nutrients from sewage through the use of different physicochemical and biological treatments before it is released into the environment (LaPara et al., 2011; Manaia et al., 2018). However, the

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effectiveness of these processes in removing multidrug-resistant microorganisms (MDROs) is less understood (Oliveira et al., 2016), despite being a topic of growing interest in recent years (Korzeniewska et al., 2013; Childress et al., 2014; Tesfaye et al., 2019). Sampling wastewater from WWTPs has been used to monitor community viral load (Rodríguez Raserio et al., 2022) and has been proposed as a means of estimating the prevalence of carbapenemase and extended-spectrum beta-lactamase (ESBL) producers in the community.

Both culture-based and molecular approaches have been developed to assess the presence of MDROs in WWTP samples (Rizzo et al., 2013). While the majority of global surveillance studies have used metagenomic approaches (Hendriksen et al., 2019), these approaches are unable to differentiate between human and environmental sources. Previous culture-based, while offering a direct association of MDROs and their resistance determinants, are scarce. There are several limitations of existing studies. Some previous studies often fail to track temporal variations: firstly because samples were collected over relatively short periods (Gomi et al., 2018), secondly because only two seasonal sample events were included (Pérez-Etayo et al., 2020), or there is no comparison of the results from different seasons (Conte et al., 2017; Pérez-Etayo et al., 2020), and others because a single sampling was collected (Picão et al., 2013). There are WWTPs analyses that could not assess the reduction of the treatment because they focus exclusively on influent samples without consideration of effluent samples (Huijbers et al., 2020). Studies carried out exclusively on effluents also lack information on reductions, such as the study carried out in 21 wastewater treatment plants in northern Spain (Ojer-Usoz et al., 2014). On the other hand, wastewater analyses focused mainly on resistant coliforms and the rationale for this type of bacteria is that coliform quantification methods are internationally accepted as indicators. This concept has been used in an international monitoring of 22 countries, where cefotaxime resistant coliforms were found to be ubiquitous in raw samples (Marano et al., 2020). In the case of acquired carbapenemases, the corresponding genes are vehiculated in a broader type of genetic platforms as well as in broader-host plasmids spectrum, such as *IncL* or *IncX* (Carattoli, 2013). In this case, the carbapenemase-producing coliforms could not accurately reflect the amount of carbapenemase producers in the waste, taking into account the interspecies transferability. *Aeromonas* could carry acquired carbapenemase genes that can be shared with Enterobacterales (Xu et al., 2022) and these bacteria have been previously associated to KPC-2 (Sekizuka et al., 2019). Very few do not integrate human antibiotic consumption and hospital-associated outbreaks (Sib et al., 2020; Zurfluh et al., 2017). At the same time, there have been studies that have attempted to establish a link between MDROs in WWTPs and human consumption of antibiotics. These studies have often been retrospective, based on historical records or aggregated national-level data rather than data within specific health districts (Hendriksen et al., 2019; Pazda et al., 2019).

To address these limitations, the main objectives of this study were to investigate the degree of reduction of MDROs in WWTPs, to quantify these microorganisms in discharges to surface waters, and to relate the impact of antibiotic prescription in the city and other factors already studied, such as population, temperature, seasonality and biological characteristics of the waters, on the MDRO counts.

2. Materials and methods

2.1. Wastewater treatment plant selection

Four WWTPs serving a resident population of 1.004.000, the total urban population in and around the city of Seville (including 681.184 in the metropolitan area in 2022) (<https://www.juntadeandalucia.es/institutodeestadisticaycartografia/sima/ficha.htm?mun=41091>) were selected for analysis. The characteristics, design, and hospital discharges to each collection basin are detailed in Fig. S1 and Table S1 of the supplementary materials.

2.2. Water sampling and bacteriological culture

Once a quarter, influent and effluent were taken on the same day from each WWTP, all four WWTPs on the same day. Sampling dates were 20/09/21, 14/12/21, 22/03/22 and 21/06/22.

Sampling was conducted using an automated 24-hour composite sampling device. All composite samples were transported to the laboratory at 4 °C and processed on arrival. Serial dilutions on influent (up to 10⁻⁵) and effluent (up to 10⁻³) samples were performed. For both influent and effluent samples, non-diluted and 10⁻² dilutions were plated on chromogenic media (ChromID® CARBA, OXA-48 and ESBL agar, bioMérieux, Marcy-L'Étoile, France). Additionally, 10⁻⁴ and 10⁻⁵ dilutions of influent samples, and 10⁻² and 10⁻³ dilutions of effluent samples were plated on tryptic soy agar (TSA) plates to count aerobic bacteria. In all cases, 50 µL were inoculated by using an Eddy Jet spiral plater (IUL Instruments, Barcelona, Spain). This plating device performs a logarithmic dilution and distributes it with decreasing concentration across the plate. The plating corresponds to 3 or 4 dilutions compared to the traditional method. The volume is calibrated and known at every point of the plate. All the plates were incubated for 18 ± 2 h at 37 °C under aerobic conditions. In the selective chromogenic agar, each different colony, based on differences in colour, morphology, size, texture and edge characteristics, was selected and counted. The count included all the colonies that exhibited the same appearance in each selective plate and this count is revised after sequencing. The results are expressed in log₁₀ CFU/ml. Each selected colony was identified by mass spectrometry (MALDI-TOF MS; Microflex® LRF; Bruker).

The screening for carbapenemase producer was carried out by testing ertapenem, imipenem and meropenem discs (10 µg) in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guideline for detection of resistance mechanisms (Giske et al., 2017). When inhibition zone of carbapenems were lower than screening cut-off values (Giske et al., 2017), hydrolysis of imipenem was sought by using the β-CARBA® test (Bio-Rad, Marnes-la-Coquette, France) as a first step. Once hydrolysis compatible with a carbapenemase was detected, the carbapenemase group was then identified using two complementary approaches: the NG-Test CARBA 5 immunochromatographic assay (NG Biotech, Guipry, France) and the disc method with meropenem and inhibitors (Rosco Diagnostica A/S, Taastrup, Denmark), as neither test can detect all carbapenemase variants for each group (Giske et al., 2017). For screening of potential ESBL producers, a comparison of the inhibition zone of ceftazidime and cefotaxime (30 µg) discs with ceftazidime/clavulanic and cefotaxime-clavulanic (30/10 µg) discs was performed on Mueller-Hinton agar (MHA) and MHA supplemented with 200 mg/L cloxacillin (Jarlier et al., 1988; Thomson and Sanders, 1992). When the differences of inhibition zone diameters were ≥5 mm the isolates were considered potential ESBL producers.

2.3. Sequencing, annotation and phylogenomic analysis

All potential acquired ESBL and/or carbapenemase producers identified through phenotypic tests were sequenced using a short-read strategy. DNA was extracted from purified cultures with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Libraries were prepared using the Nextera DNA Library Prep Kit (Illumina, San Diego/CA, USA) for sequencing on the Illumina MiSeq instrument. Fastq files were trimmed, and *de novo* assembly was performed using the CLC Genomics Workbench, v.9.5.2. Assembly quality was checked with QUASt v.5.0.2 (Table S2 of the supplementary materials); BWA-MEM v.0.7.17-r1188, and SAMtools v.1.11 were used to align reads to the assembled genome and assess coverage along the genome.

Multilocus sequence typing (MLST) was performed using MLSTFinder (<https://cge.food.dtu.dk/services/MLST/>) and the Institute Pasteur database (<https://bigsd.bpasteur.fr/klebsiella/>). ResFinder v.4.1 (<https://cge.food.dtu.dk/services/ResFinder/>) and the CARD database (<https://card.mcmaster.ca/analyze/rgi>) were used to identify

acquired resistance determinants.

For comparative analysis within of the MLSTs of *Klebsiella pneumoniae* isolates, first a core genome multilocus sequence typing (cgMLST) scheme (based on 2358 genes) and then a whole genome scheme (4891 genes) were used with SeqSphere+ v.8.5.1 (Ridom© GmbH, Münster, Germany); for *E. coli* analysis, the Enterobase *E. coli* cgMLST scheme with 2513 genes was used. For phylogenetic assessment of *Aeromonas* spp. genomes, the MLST allelic profiles were used. For other species, the SNIPPY v.4.6.0 tool (<https://github.com/tseemann/snippy>) was used to identify single nucleotide polymorphisms (SNPs) among the different isolates. A gene-by-gene and a SNP approach was used to compare genomes with previous clinical isolates that had been voluntarily submitted to the Andalusian Reference Laboratory, as well as *E. coli* ST131 isolates from a previous colonisation study (Salamanca-Rivera et al., 2022). To identify clonal clustering, fewer than 10 cgMLST loci was used for *E. coli* (Muloi et al., 2022), fewer than 20 wgMLST loci for *K. pneumoniae* (David et al., 2019), and fewer than 20 SNPs (David et al., 2019) was used as the threshold for other Enterobacteriaceae. For *Aeromonas* spp. a threshold of <20 SNPs was also used for clonal clustering. High-risk (HR) clones were those that had previously been detected in human samples on an international scale (Table S3 of the supplementary materials) (Woodford et al., 2011). Isolates from the same plate that differed less than the above SNP/locus thresholds were considered equal, and their counts were combined for further study.

Two groups were defined according to their clinical epidemiological importance. The first, termed “Clinically associated (CA-) ESBL/carbapenemase producers” included beta-lactamase (*bla*) genes and species commonly associated with causing human infections and outbreaks. This group may also include those that produce infections in conjunction with *bla* genes that are rare in human infections. The second group, “Environmentally associated (E-) ESBL/carbapenemase producers”, includes *bla* genes (e.g. *bla*_{TLA} and *bla*_{VEB}) and species that are rarely reported to cause human infections (e.g. carbapenemase-producing *Aeromonas*) but have been detected in environmental samples. The species and *bla* genes classified as CA- or E-producers are detailed in Table S4 of the supplementary materials.

2.4. Physico-chemical parameters of the samples and assessment of antibiotic consumption

The following physico-chemical parameters of the wastewater samples of the sampling day were recorded: pH, Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD), Total Suspended Solids (SS), Total Nitrogen (Nt) and Total Phosphorus (Pt). The hydraulic retention time and the sludge age were also measured. Temperatures on the days of sampling were obtained from the Spanish Meteorological Agency (AEMET) (<https://www.aemet.es/>).

Data on antibiotic use was obtained from all public health centres and hospitals (covering 98.3 % of the population of Seville) discharging wastewater for each WWTP basin, using computerised pharmacy records of reimbursed and dispensed drugs authorised for prescription by the Andalusian Health Service. The data were used as a proxy for the consumption of antibiotics by the corresponding resident population. The data were expressed as DID: defined daily doses (DDD) per 1000 inhabitants for antibacterials for systemic use (Group JO1, WHO Anatomical Therapeutic Chemical Classification System) and the following antibiotics, which are mainly prescribed in the outpatient setting: amoxicillin-clavulanic, third-generation cephalosporins, cefuroxime, cefixime, total quinolones, ciprofloxacin, levofloxacin, and fosfomicin-trometamol.

2.5. Statistical analysis

Linear mixed model (LMM) regression analysis with random intercepts was used to assess differences between influent and effluent outcomes. First-order autoregressive (AR) or compound symmetry (CS)

covariance structures were used, as appropriate, to account for autocorrelation of repeated measures. Selection of the optimal model was based on the Akaike Information Criterion (AIC) (Twisk, 2011). Models were fitted using the restricted maximum likelihood estimation method with the Satterthwaite approximation to minimise type I error in studies with small samples (Nugent and Kleinman, 2021), using the SPSS MIXED procedure. To quantify differences in variables of interest between WWTPs, a pairwise comparison of the main effects was performed by post hoc analysis of the estimated marginal means using the Bonferroni correction to account for multiple comparisons. Seasonality among WWTPs was determined using the Friedman test (Ollech and Webel, 2023). Mann-Whitney U tests were used to compare overall counts. Pearson’s test was used to identify correlations between MDRO counts and physico-chemical parameters and antibiotic prescriptions. A p-value <0.05 (two-tailed) was considered significant. All analyses were performed using SPSS (v. 23) and GraphPad Prism (v. 9.1.1).

3. Results

3.1. Characterisation of ESBL /carbapenemase producers in WWTP influent samples

A total of 575 colonies grew on selective media, up to 62 % were selected due to preliminary phenotypic tests for sequencing and 35 % were found to carry ESBL/acquired carbapenemase-coding genes (Table S5 of the supplementary materials). In the influent samples, the mean count of ESBL producers was higher than that of carbapenemase producers (Table 1). A total of 15 out of 16 influent samples (93.8 %) yielded carbapenemase producers, while all samples (16/16) yielded ESBL-producing bacteria. A total of 152 isolates carrying acquired ESBL/carbapenemase genes were recovered. Fifty-five (36.2 %) isolates were exclusively carbapenemase producers, 74 (48.7 %) were ESBL-producing bacteria and 23 (15.1 %) co-produced at least one carbapenemase and one ESBL. The most common carbapenemase was KPC-2, followed by the OXA-48 group (Figs. 1 and 2; Table S4 of the supplementary materials). Eight (10.3 %) isolates carried both the *bla*_{KPC-2} and *bla*_{OXA-48} groups, and three (3.9 %) carried both the *bla*_{KPC-2} and *bla*_{OXA-48} groups in addition to *bla*_{ESBL} genes. Only two VIM-1-producing isolates were identified: one carried *bla*_{VIM-1}, *bla*_{OXA-48}, and *bla*_{CTX-M-15} genes and the other carried *bla*_{VIM-1} and *bla*_{CTX-M-15} genes; no other metallo-beta-lactamases were identified. Among ESBL producers, the primary determinant of resistance was the *bla*_{CTX-M-1} group ($n = 35$; 36.1 %), and 15 (15.5 %) isolates produced CTX-M-9 group enzymes. Only two isolates produced both clinical and environmental ESBL enzymes (*bla*_{CTX-M-15} with *bla*_{TLA-4}; *bla*_{CTX-M-15} with *bla*_{VEB-1}). The total count of CA-ESBL-producing Enterobacteriaceae was higher than that of E-ESBL-producing Enterobacteriaceae ($p = 0.002$). For carbapenemase producers, no significant differences were observed among Enterobacteriaceae ($p = 0.15$) (Fig. 1; Table S4 of the supplementary materials). In general, the proportion of E-producers was higher than that of CA-producers (ESBL producers: 59 % vs 41 %; carbapenemase producers: 93 % vs 7 %, respectively, of total bacterial counts).

Some differences in the influent content were found between the four WWTPs studied. The overall mean number of ESBL/carbapenemase producers was found to be higher in WWTP-A ($p = 0.011$). The count of carbapenemase producers was also higher when analysed separately ($p = 0.06$; Table S6 of the supplementary materials). WWTP-A influent receives waste from most of the hospitals in the area. Other factors also influenced the number of resistant bacteria in the influent. Higher hydraulic retention times are associated with higher CA-ESBL producer counts ($p = 0.03$), while higher activated sludge ages are correlated with lower CA-carbapenemase producers ($p = 0.03$) (Fig. 2 and Table S8 of the supplementary materials). In addition, both higher population counts, and flow rates significantly increased all ESBL producers ($p = 0.01$ and $p = 0.01$, respectively) and OXA-48 producers ($p = 0.03$ and $p = 0.04$, respectively).

Table 1

Average reduction of ESBL and carbapenemase producers counts observed across all four studied wastewater treatment plants (WWTPs). The log₁₀ CFU/ml of the means and their standard deviation was calculated based bacterial counts.

Variable	Influent mean Log CFU/ml (SD)		Effluent mean Log CFU/ml (SD)		Reduction		p-value	CI 95 %		
					Avg. reduction (SE)	Percentage of reduction (%)		Low	Upper	
Total aerobic bacteria	6.66	(0.30)	5.52	(0.81)	-1.14	(0.22)	92.76	<0.0001	0.67	1.61
Carbapenemase producers	2.74	(1.18)	0.96	(1.29)	-1.79	(0.38)	98.36	<0.01	0.90	2.67
CA-carbapenemase producers	1.67	(0.99)	0.49	(1.96)	-1.19	(0.30)	93.48	<0.01	0.52	1.86
E-carbapenemase producers	2.44	(1.47)	0.56	(1.11)	-1.88	(0.40)	98.68	<0.01	0.98	2.79
KPC producers	2.51	(1.24)	0.93	(1.26)	-1.58	(0.36)	97.39	<0.01	0.76	2.41
CA-KPC producers	1.31	(1.15)	0.47	(0.94)	-0.84	(0.29)	85.38	0.01	0.21	1.46
E-KPC producers	2.18	(1.39)	0.53	(1.07)	-1.65	(0.36)	97.76	<0.01	0.84	2.46
OXA-48 producers	1.69	(1.31)	0.28	(0.61)	-1.42	(0.39)	96.17	<0.01	0.50	2.33
CA-OXA-48 producers	1.34	(0.95)	0.12	(0.33)	-1.22	(0.29)	93.92	<0.01	0.52	1.91
E-OXA-48 producers	1.16	(1.45)	0.17	(0.54)	-0.99	(0.38)	89.81	0.03	0.12	1.86
VIM producers	0.23	(0.63)	0.07	(0.33)	-0.16	(0.15)	31.13	0.29	-0.16	0.48
CA-VIM producers	0.23	(0.63)	0.07	(0.33)	-0.16	(0.15)	31.13	0.29	-0.16	0.48
E-VIM producers	ND		ND		ND		ND	ND	ND	ND
Carbapenemase-producing Enterobacteriaceae	1.91	(1.20)	0.65	(1.00)	-1.27	(0.44)	94.6	0.03	0.18	2.36
Carbapenemase-producing Aeromonadaceae	1.82	(1.68)	0.44	(1.08)	-1.38	(0.34)	95.80	0.01	0.64	2.11
ESBL producers	3.77	(0.75)	2.36	(1.01)	-1.41	(0.26)	96.11	<0.0001	0.84	1.98
CA-ESBL producers	3.38	(0.58)	1.27	(1.22)	-2.11	(0.33)	99.23	<0.0001	1.37	2.85
E-ESBL producers	2.65	(1.84)	1.71	(1.33)	-0.94	(0.38)	88.44	0.03	0.11	1.76
CTX-M producers	3.26	(0.55)	1.16	(1.25)	-2.10	(0.34)	99.20	<0.0001	1.48	2.84
SHV producers	1.31	(1.57)	0.25	(0.52)	-1.06	(0.45)	91.35	0.04	0.07	2.06
VEB producers	1.32	(1.81)	1.28	(1.37)	-0.04	(0.41)	9.22	0.92	-0.83	0.91
GES producers	0.95	(1.37)	0.19	(0.50)	-0.77	(0.41)	82.98	0.09	-0.16	1.69
Other ESBL producers	1.59	(1.89)	0.89	(1.24)	-0.71	(0.42)	80.41	0.11	-0.19	1.61
ESBL-producing Enterobacteriaceae	3.38	(0.58)	1.35	(1.17)	-2.03	(0.30)	99.07	<0.0001	1.38	2.68
ESBL-producing Aeromonadaceae	2.55	(1.94)	1.72	(1.33)	-0.83	(0.40)	85.04	0.06	-0.03	1.68

Avg = Average. CA = clinical-associated. E = environmental-associated. ND = not detected. SD = standard deviation. SE = standard error. CI = confidence interval.

The highest counts of both carbapenemase- and ESBL-producing *Aeromonas* were found in WWTP-A, WWTP-B and WWTP-C (Fig. 3). These bacteria were also the most frequently resistant Gram-negative bacteria detected, with 10/16 (62.5 %) and 11/16 (68.8 %) samples yielding carbapenemase- and ESBL-producing *Aeromonas*, respectively. ESBL genes associated with *Aeromonas* spp. belonged to environmental *bla* genes (Table S4 of the supplementary materials). The predominant carbapenemase detected in *Aeromonas* spp. was KPC-2. For *E. coli*, the numbers of SHV-12 and CTX-M-9 group producers were highest in WWTP-A, while those of CTX-M-1 group producers were highest in WWTP-D (Fig. 3). The prevalence of *K. pneumoniae* was higher among CTX-M-1 group producers (Table S4 of the supplementary materials). The numbers of other Enterobacteriaceae were lower, and predominantly carried environmental carbapenemases (Fig. 3 and Table S4 of the supplementary materials).

Seasonal differences were also observed. The mean MDRO count was lowest in summer, while mean MDRO's counts predominated in autumn and winter (Fig. 1). Despite the observed differences among mean counts, no seasonality was observed for ESBL/carbapenemase producers during the year of study ($p = 0.54$ and $p = 0.65$, respectively) (Fig. S2 of the supplementary materials). In general, the mean counts of carbapenemase producers decreased in autumn (85 % of the seasonal index), whereas the mean counts of ESBL producers decreased in winter and summer (94.6 % and 95.5 % of the seasonal index, respectively) (Fig. 1; Table S7 of the supplementary materials). However, the behaviour of MDRO counts differed between WWTPs, despite similar rainfall regime: while WWTP-A and WWTP-D had lower counts of carbapenemase producers in summer (74.8 % and 83.6 %, respectively), WWTP-B and WWTP-C had lower counts in autumn (0 % and 91.7 %, respectively). In the case of ESBL producers' counts, seasonal indexes widely varied among WWTPs (Table S7 of the supplementary materials).

Significant proportions of influent *E. coli* (44 %) and *K. pneumoniae* (30 %) counts were identified as belonging to HR clones (Table S3 of the supplementary materials). Nine (14 %) *E. coli* isolates belonging to four MLSTs (ST2179, ST2083, ST1434, and ST10) matched five clinical isolates: one from a patient in Seville, and the other four from patients in

different Andalusian provinces (Fig. S3 of the supplementary materials). *E. coli* ST1193 ($n = 4$) and ST131 ($n = 5$) isolates showed >10 loci differences from the clinical isolates. For *K. pneumoniae*, six (27.3 %) isolates belonging to four MLSTs (ST15, ST307, ST469 and ST4988) matched clinical isolates: 68 isolates were obtained in Seville, and 11 in other Andalusian provinces. The ST15 isolates from influent were associated with clinical isolates from an ongoing outbreak in one of the hospitals (Fig. S4 of the supplementary materials). Other Enterobacteriaceae belonged to different MLSTs from the clinical isolates.

3.2. Antibiotic prescriptions and their association with MDRO counts in influent

In general, antibiotic prescriptions were positively correlated with MDRO counts (Fig. 2), whereas the physicochemical parameters for wastewater testing showed no such correlation. Higher prescriptions of quinolones (levofloxacin) and third-generation cephalosporins were associated with higher counts of carbapenemase-producing Enterobacteriaceae, especially OXA-48 producers. In general, an alkaline pH reduced MDRO counts, especially for CA-KPC and OXA-48 producers, while both flow rate and population density increased the numbers of ESBL-and OXA-48-producing bacteria (Fig. 2 and Table S8 of the supplementary materials). Total antibiotic prescriptions showed seasonal variation ($p = 0.011$), with the lowest DID values observed during the summer months and the highest in spring season (Fig. S2 of the supplementary materials). Regarding individual antibiotic prescriptions previously described, also seasonal distribution was seen (except for levofloxacin ($p = 0.054$)).

3.3. Differences between influent and effluent samples

Overall, a 1.8 log reduction (98.36 %) in carbapenemase producers (2.74 vs 0.96 log₁₀ CFU/ml; $p < 0.01$) and a 1.4-log reduction (96.11 %) in ESBL producers (3.77 vs 2.36 log₁₀ CFU/ml; $p < 0.0001$) was found when comparing influent and effluent samples (Table 1). The reduction was statistically significant for both ESBL and carbapenemase producers

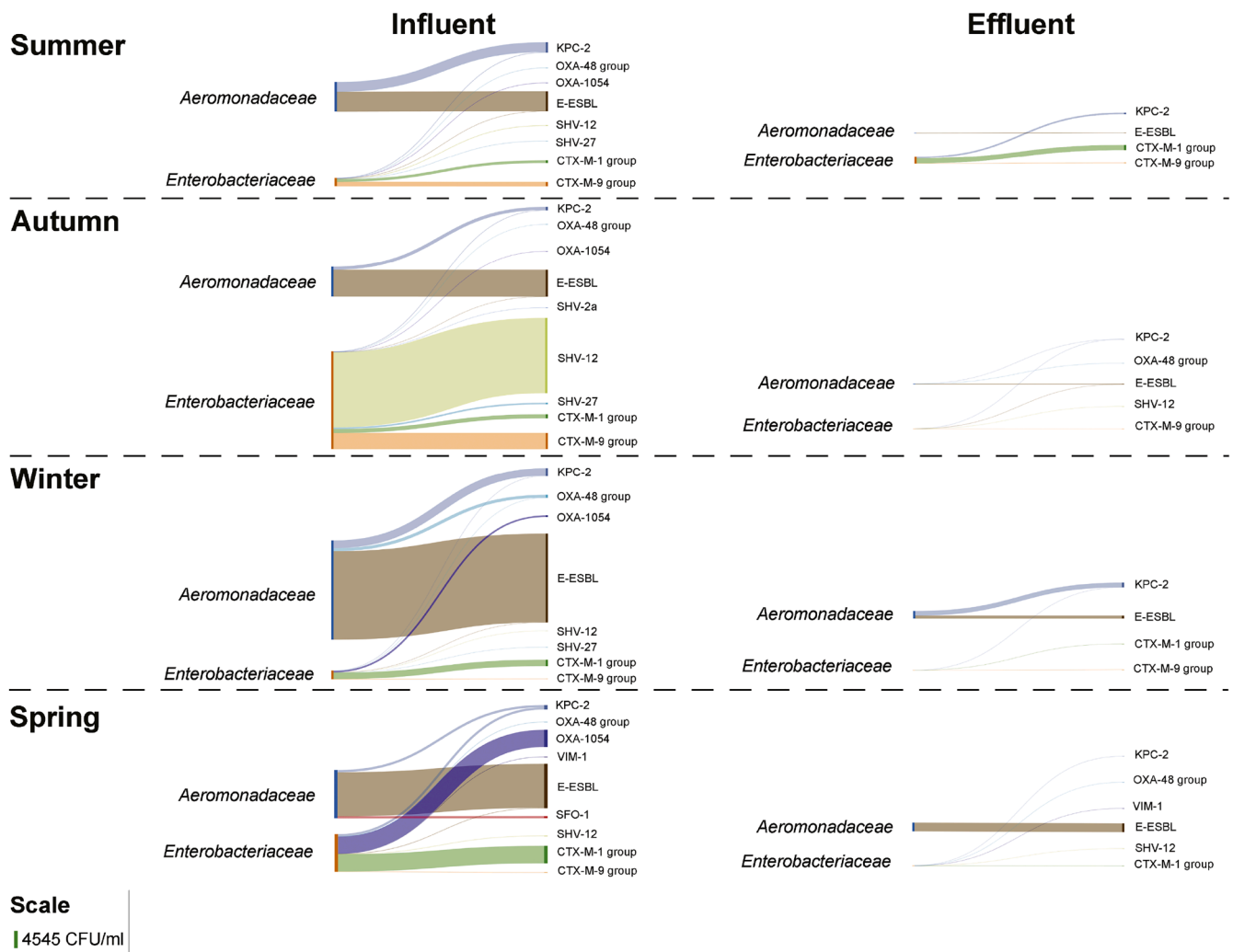


Fig. 1. Sankey diagram showing the difference in mean bacterial counts (CFU/ml) per quarter between aggregated wastewaters from WWTPs. Differences between influent and effluent are shown and counts have been aggregated across all sampled WWTPs. The width of each flow represents the mean bacterial counts and the *bla* genes are differentiated by colours. Aeromonadaceae and Enterobacteriaceae mean counts were differentiated. E-ESBL = environmental-associated ESBL.

(Fig. 4). No significant differences were found between the wastewater treatment plants (Table S6 of the supplementary materials), despite the different characteristic of WWTP-B, which has a biological tertiary treatment. E-producers predominated in treated wastewater (ESBL producers: 68 %, carbapenemase producers: 75 %); however, an increase in CA-carbapenemase producers was noted in effluent (from 7 % to 25 %; $p < 0.01$).

Certain combinations of *bla* and bacterial species were only found in effluent after treatment. VIM-1- and SHV-12-producing *E. cloacae*, BEL-1- and GES-5-producing *R. ornithinolytica*, VEB-1-producing *Aeromonas* spp., and KPC-2- and OXA-917-producing *Aeromonas* spp. were identified in effluent (Table S2 of the supplementary materials). With regard to the likely persistence of the same strain after treatment, a matched pair of CTX-M-65-producing *E. coli* was found in pre-treatment and post-treatment samples from WWTP-D, and a matched pair of TLA-4-producing *A. caviae* was found in WWTP-A. Two matching CTX-M-65 *E. coli* were found in two influent samples from WWTP-D taken in different seasons, and two matching CTX-M-14 *E. coli* were found in two effluent samples from WWTP-C, also taken in different seasons (Figs. S5 and S6 of the supplementary materials). No persistence of *Klebsiella* spp. was observed (Fig. S7 of the supplementary materials).

Other antimicrobial resistance genes (ARG) were identified in ESBL /carbapenemase producers. One ESBL-producing *E. coli* was found to co-produce CMY-42-like (Fig. S5 of the supplementary materials) and two

KPC-2-producing *K. oxytoca* carried *bla*_{FOX-8}. Among other Enterobacteriaceae, five (33 %) ESBL-producing Enterobacteriaceae and 15 (32 %) carbapenemase-producing Enterobacteriaceae, 11 (26 %) ESBL-producing *Aeromonas* and 11 (39 %) carbapenemase-producing *Aeromonas* carried *pAmpC* genes (Fig. S6 of the supplementary materials). With regard to resistance to other antibiotic families, 77 %, 60 %, 43 %, 40 %, and 39 % of isolates carried some resistance gene resistant to aminoglycosides, sulfonamides, quinolones, amphenicols, and tetracyclines, respectively (Table S9 of the supplementary materials). One hundred and sixty-seven isolates carried at least one *rep* gene, and in 37 isolates (36 *Aeromonas* spp. and one *E. coli* isolate) no *Inc* plasmid was detected (Fig. S8 of the supplementary materials).

4. Discussion

This study adds to evidence that wastewater treatment is effective in reducing the levels of MDROs in wastewater. However, the reduction achieved with secondary treatment was not enough to prevent the presence of environmental MDROs and ESBL-producing bacteria in the effluent. To our knowledge, this is the first study to investigate the association between ESBL- / carbapenemase-producing bacteria in WWTPs and population colonisation rates and antibiotic use over a 12-month period. The study included the entire population of a single city and compared reductions in the burden of MDROs achieved between

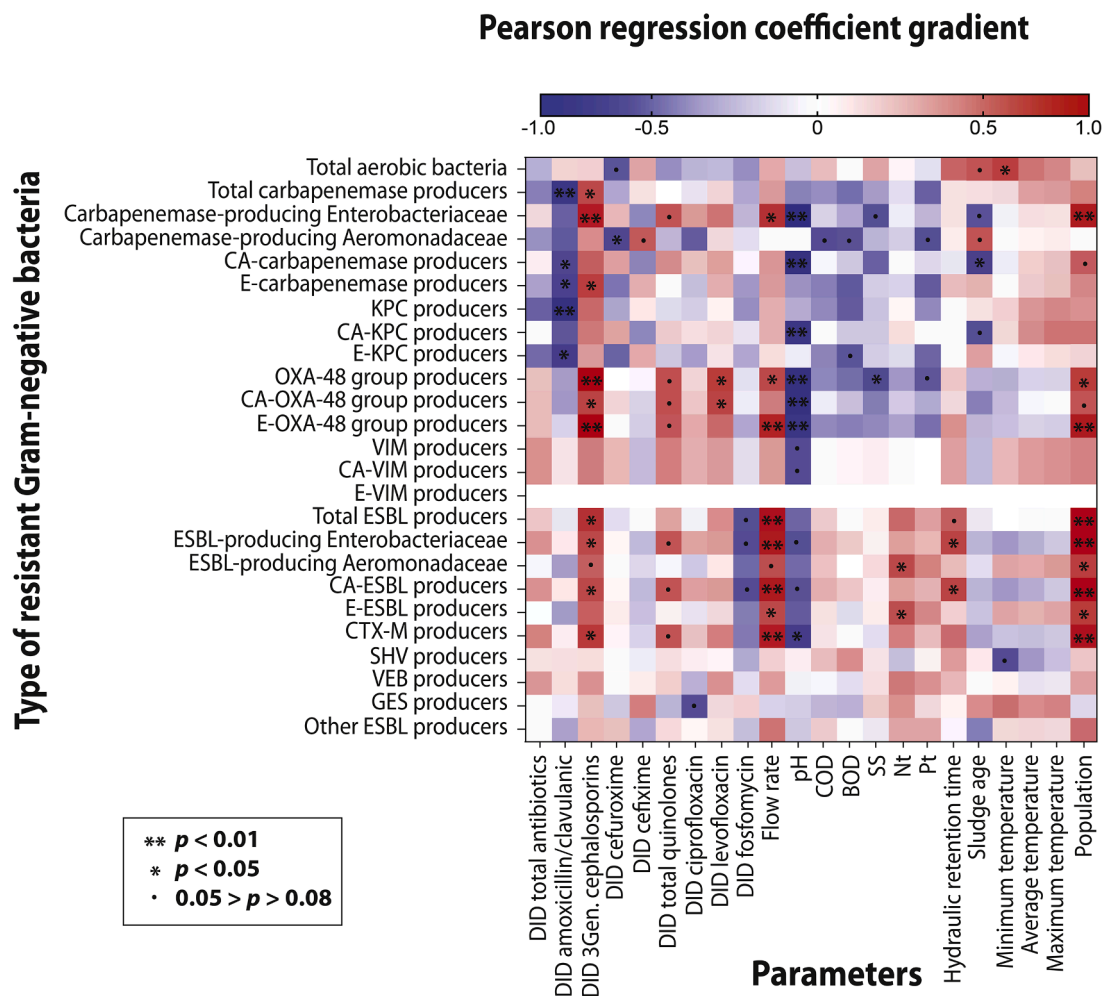


Fig. 2. Heat map of the association between resistant bacteria count and antibiotic consumption, physico-chemical parameters, and population. Red colour refers to a directly proportional correlation, and blue colour refers to an inversely proportional correlation. Significant p values are indicated as (·) close to significance (0.05 to 0.08); (*) < 0.05 ; (**) < 0.01 . Antibiotic use is assessed as DID: defined daily doses (DDD) per 1000 inhabitants. Bacterial count is assessed as \log_{10} CFU/ml. Flow rate is assessed in m^3/day . COD and BOD are assessed as $\text{mg O}_2/\text{L}$. SS is assessed as mg/L . Nt and Pt are assessed as mg N/L and P/L , respectively. Hydraulic retention time is assessed in hours. Sludge age is assessed in days. Temperature is assessed in Celsius degrees. Population is assessed as population equivalent (p.e.). CA = clinical-associated. E = environmental-associated. COD = chemical oxygen demand. BOD = biological oxygen demand. SS = suspension solids. Nt = total nitrogen. Pt = total phosphorus.

different basins and seasons.

Few studies have reported quantitative cultures of MDROs at wastewater inlets, with most results being based on metagenomics (Hendriksen et al., 2019). Some differences in ESBL producers were found compared with three previous culture-based surveys: one conducted in a single Polish WWTP, another in seven Croatian WWTPs and the last in 20 WWTPs in the East of England. For ESBL producers in the European surveys, mean counts were 2–3 \log_{10} CFU/ml (Makowska et al., 2020; Puljko et al., 2022; Raven et al., 2019). These previous studies found lower averages than we found; however, when only ESBL-producing Enterobacteriaceae (excluding *Aeromonas* spp.) were considered, the numbers were comparable, except for the WWTP that received the majority of hospital wastewater discharges. For carbapenemase-producing Enterobacteriaceae, our mean counts were similar to those found in the Polish and Croatian studies. It would be reasonable to expect differences in counts using different methods of sampling (24-hour composite vs point sampling), seeding (with/without filtration) and selective media, but despite the differences between studies, the counts for Enterobacteriaceae were similar. Acquired ESBL- and carbapenemase-producing *Aeromonas* spp. predominated in our samples. In contrast, previous studies differed in study scope and they have primarily focused on Enterobacteriaceae (Makowska et al., 2020;

Puljko et al., 2022; Raven et al., 2019). The fact that we also study *Aeromonas* has allowed us to discover that a large proportion of acquired resistance genes detected in wastewater are carried by this group. Most of these genes are shared between Enterobacteriaceae and *Aeromonas*. If the aim of the new European Directive (EU) 2024/3019 is to regularly monitor antimicrobial resistance in the effluents of urban WWTPs, bacteria other than Enterobacteriaceae should be included. In our study, KPC-2 producers accounted for almost 90 % and 100 % of *Aeromonas* spp. in influent and effluent, respectively, which is consistent with the species/determinant combination found in previous studies in Asian countries (Sekizuka et al., 2019; Zhang et al., 2020). This is a limitation for using influent to estimate population colonisation with MDROs, although it could serve as an early warning of HR clones associated with hospital spread. Metagenomic-based surveys did not trace persistent strains or measure the impact of acquired ESBL/carbapenemase-producing *Aeromonas* spp. in WWTP influents (Chu et al., 2018). Molecular approaches may overestimate the ESBL-/carbapenemase load attributable to a direct human source by detecting genes that could be of both human and environmental origin. A recent metagenomic-based global monitoring analysis could not fully explain MDROs (Hendriksen et al., 2019), probably because of the mixing of environmental and human-derived bacteria.

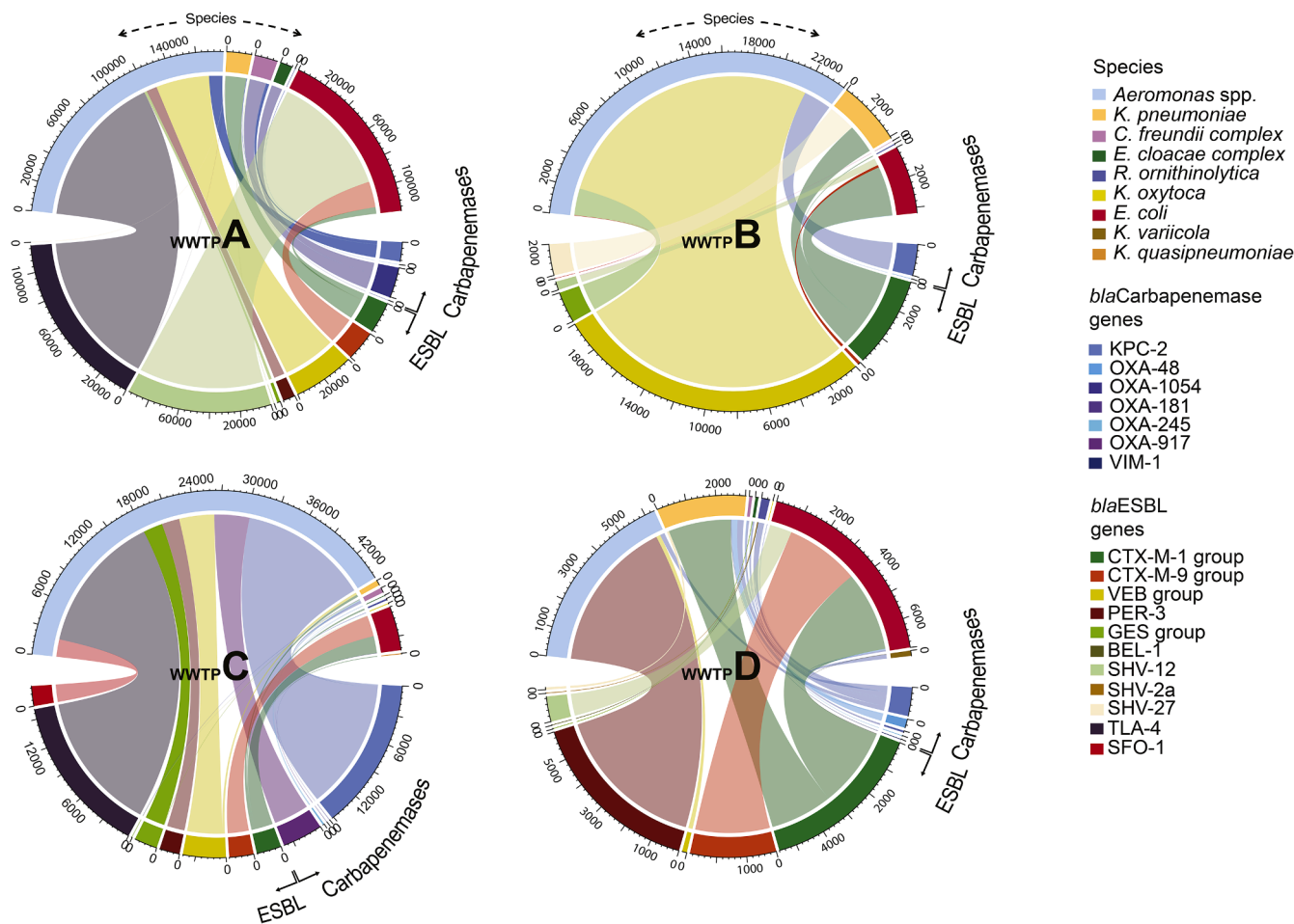


Fig. 3. Chord diagram of the aggregated influent samples of the WWTPs under study that correlates the species and their *bla* genes. Each diagram shows the bacteria count (CFU/ml) harbouring different resistance genes per WWTP. The upper half of the diagrams shows the species, and the lower half shows *bla*Carbapenemase and *bla*ESBL genes. Each segment is numerically graduated from 0 to total bacterial count (CFU/ml). The colours of the flows refer to the determinants of resistance and their width indicates the total count of producers; a greater width corresponds to greater counts.

The correlation between community antibiotic use and MDRO counts in wastewater was one of the most interesting findings of our study. There is currently limited evidence on how human antibiotic use affects the microbiological composition of raw wastewater, partly because few wastewater analyses collect this information. Some studies have measured antibiotics in wastewater directly using antibiotic concentration values (Voigt et al., 2020), or by estimating DID from antibiotic concentrations (Hendriksen et al., 2019). The disadvantage of measuring antibiotic concentrations in wastewater is that it does not account for the pH-induced degradation, particularly of beta-lactams (Ribeiro et al., 2018), that occurs between the point of excretion from the patient and the influent. To date, the only association found has been with quinolones, which are more stable molecules in the environment (Joadas et al., 2024; Voigt et al., 2020). This may explain why MDROs in raw sewage have not yet been linked to cephalosporin prescribing. Other studies have used national data from the ECDC database (Hendriksen et al., 2019), but have not taken into account the data corresponding to each basin affected by the presence of hospitals or high population densities. To the best of our knowledge, this is the first study to compare MDROs with the DIDs of the population served by WWTPs and to show the significant correlation with third-generation cephalosporins and quinolones. This association was probably detected because the amount of antibiotics prescribed per 1000 inhabitants was calculated as an estimate of consumption.

We were unable to find statistically significant seasonal patterns in the mean counts of ESBL/carbapenemase producers, as the WWTPs have

different behaviours. However, it was observed that the overall numbers were higher in the autumn and winter months. An analysis of a WWTP in a rural area in Germany found lower MDRO counts during the warmer months (Schages et al., 2020), but lacked information on antibiotic use. Caucci et al. showed higher levels of ARGs with a qPCR approach in autumn and winter seasons during a 2-years study (Caucci et al., 2016). However, in our study significant seasonal variation was observed for systematic antibiotic prescriptions in agreement with many other previous studies. Interestingly, prescriptions were higher in spring in our city, coinciding with the peak period for respiratory infections, and this does not align with the higher bacterial counts in autumn and winter. However, the bacterial counts of the four basins investigated did not behave in a similar way. For example, in the WWTP-A basin, most of the antibiotic prescriptions were slightly predominant in the winter season, when the total counts are high, in contrast with the others WWTPs. Many previous studies have reported the role of antibiotics in WWTP as selective agents (Gao et al., 2012), but it is still unclear whether the increase of ARG in wastewater follows a seasonal pattern similar to that of antibiotic prescription and consumption.

It is important to note that other factors beyond antibiotic prescriptions may influence seasonal trends or municipal district size, such as flow rates, sewerage network and WWTP design and population density. In our study, the correlations found suggested that increased flow rates, increased hydraulic retention times or higher population densities may contribute to higher counts of ESBL producers and sludges age may reduce CA-carbapenemase producers. Previous studies using

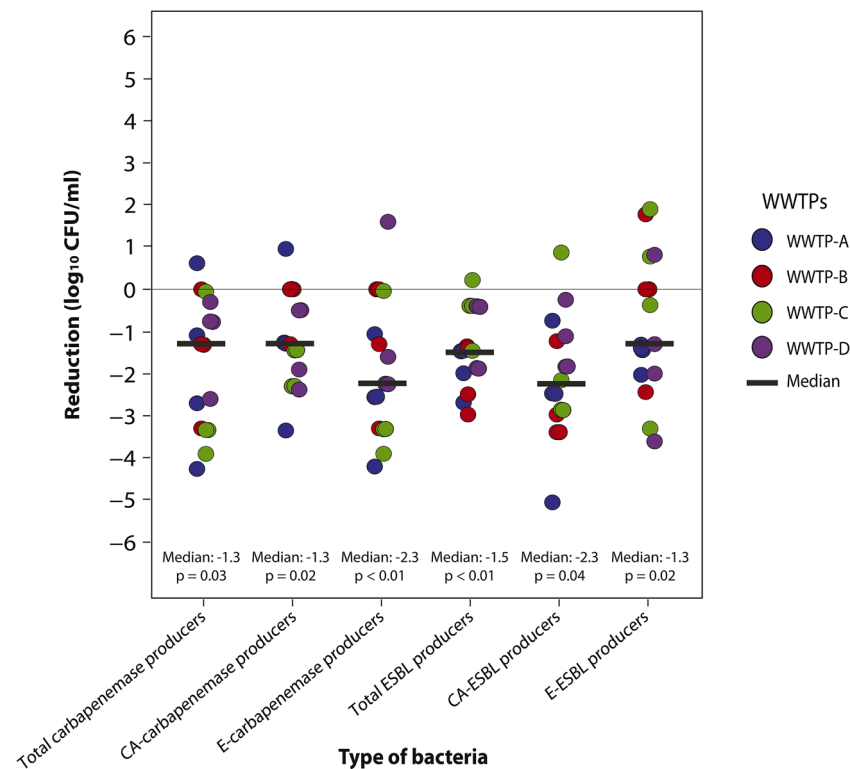


Fig. 4. Reduction counts (CFU/ml) of multi-drug resistant bacteria after wastewater treatment. Coloured dots above the 0 line refers to increase in bacterial counts in effluent samples after the treatment, while dots below the 0 line refers to a reduction in bacterial counts in effluent samples. CA = clinical-associated. E = environmental-associated.

reactors have shown conflicting results with hydraulic retention times, achieving a decrease (Iakovides et al., 2019) or increase (Liao et al., 2019) in the relative abundance of *tet*, *aad* or *sul* genes. In the case of sludge ages, other studies showed that they could help to decrease MDROs populations (Chiemchaisri et al., 2022; Kang et al., 2022).

Previous comparative studies based on collection of hospital discharge have shown that some carbapenemase genes are more frequently found in the clinical wastewater system (Müller et al., 2018). Similarly, in our study, influents receiving hospital discharges contained more carbapenemase producers, although the differences were not significant. Our results showed that wastewater treatment was effective in reducing most levels, in agreement with previous analyses (Makowska et al., 2020; Puljko et al., 2022; Raven et al., 2019). In contrast, in a Czech Republic study, an increase of CTX-resistant *E. coli* and other resistant coliform bacteria in the effluent after using a secondary treatment was observed (Kutilova et al., 2021). It is not clear whether additional treatments could achieve a better reduction of MDROs, as we observed in the only WWTP with a biological nutrient removal process step. The median 2.75 log and 5 log reductions in ESBL-producing *E. coli* achieved in English (Raven et al., 2019) and Spanish (Oliveira et al., 2023) WWTPs after a UV irradiation, respectively, were greater than those achieved with a secondary treatment in our study. However, other works provide evidence that ARGs can be found despite this third treatment, such as relative abundance of *tet* after UV disinfection (Auerbach et al., 2007) or *tet* and *sul* genes after chlorinated-disinfection (Gao et al., 2012; LaPara et al., 2011; Liu et al., 2018). Liu et al. also suggested that faecal bacteria could be the source of these ARGs after chlorine disinfection (Liu et al., 2018), suggesting the survival of these bacteria in this type of treatments. In addition, ESBL- and carbapenemase-producing Enterobacteriaceae have been previously reported in surface waters after tertiary treatment (Pérez-Etayo et al., 2020).

Most of the carbapenemase producers detected in treated wastewater

in our study were environmental producers, although an increase in CA-carbapenemase producers and the appearance of new ones in the effluent was detected. An increase in VIM, OXA-48 and KPC-2-producing coliforms in effluent has previously been documented (Makowska et al., 2020; Yang et al., 2017). These increases or *de novo* detections in treated wastewater could indicate that the treatment plant itself probably plays a role in facilitating genetic exchanges and generating MDROs. However, other factors should be considered in the contribution of new MDROs detections, such as methodological limitations processes, selective pressure from the treatment process or different survival rates. Further analyses are required to elucidate the competitive dynamics of bacterial populations in sludge— where fungi, protists, phages, and arthropods may also interact— and to identify the optimal treatment strategy for improved MDRO reduction.

Our study has several limitations. First, the limited number of WWTPs studied does not allow us to draw general conclusions, as the accuracy of between-WWTP population variance may be compromised (Harrison et al., 2018). To minimise this limitation, our LMM regression included a Satterthwaite degrees-of-freedom correction for small samples, which allowed for improved accuracy in parameter estimation; our study can therefore be considered exploratory (Leyrat et al., 2018). Further studies involving >20 (preferably >40) WWTPs would be necessary to confirm our findings. Furthermore, to generalise the conclusions of the study, different climatology, population densities, sewer designs and ages, and flow rates would have to be included. Although one-year study has allowed us to calculate quarterly seasonal indexes, it would be necessary to extend the number of annual cycles to be investigated (i.e. >4 years) to verify more precisely the effect of cycles, trends, and seasonal variation indexes. Second, it is likely that certain MDROs were not detected, either because of phenotypic similarity or because the bacteria were non-culturable (it has been estimated that the culturable fraction of environmental bacteria is <1 %) (Pazda et al., 2019). By way of comparison, metagenomic studies also have

limitations; it is difficult to obtain information from complex environmental samples using this method, as high sequencing depth is required to ensure detection of species that are less abundant than waterborne species such as Enterobacteriaceae. In China, use of culture-enrichment metagenomics in a municipal WWTP and its receiving river resulted in increased enrichment of *bla* genes from 29 to 239, mainly from the NDM/KPC families (Zhang et al., 2022).

5. Conclusion

In conclusion, our study shows that a significant proportion of the MDROs present in untreated wastewater belong to environmental species that may be unrelated to human disposal. Use of wastewater as a means of estimating the prevalence of MDROs in a population should be based on methods capable of differentiating between bacteria of human origin and those derived from sewage ecosystems. Our data indicated that third-generation cephalosporins used in humans also have an important environmental impact on the number of OXA-48 and ESBL producers, previously attributed only to quinolones. Finally, current WWTPs without additional steps after secondary treatment are able to reduce the load of MDROs. Nevertheless, these microorganisms are not completely eliminated from the treated wastewater. The new European Directive (EU) 2024/3019 states that the aim of wastewater monitoring is to provide accurate data for public health decisions. For this monitoring to be useful for public health, further studies are needed to analyse whether the bacterial load of MDROs in wastewater correlates in any way with antibiotic use.

Statement

During the preparation of this work the author(s) did not use any AI tool.

CRedit authorship contribution statement

Laura Monge-Olivares: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Germán Peñalva:** Writing – review & editing, Validation, Software, Formal analysis. **Marina R Pulido:** Writing – review & editing, Visualization, Software. **Lara Garrudo:** Writing – review & editing, Investigation. **Miguel Ángel Doval:** Writing – review & editing, Investigation. **Sofía Ballesta:** Writing – review & editing, Resources, Project administration. **Nicolás Merchante:** Writing – review & editing, Investigation. **Pablo Rasero:** Writing – review & editing, Methodology. **Lucila Cuberos:** Writing – review & editing, Methodology. **Graciano Carpes:** Writing – review & editing, Methodology. **Lorena López-Cerero:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

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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microbianas productoras de carbapenemasas desde una perspectiva One-Health (MicroCarbaFlux, CC23140547)”.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2025.123706.

Data availability

All genomes are available from NCBI (BioProject accession PRJNA1026089; <https://dataview.ncbi.nlm.nih.gov/object/PRJNA1026089?reviewer=ar8dlou3b5u2q50s029jbnclntu>). De-identified study metadata and statistics can be accessed by accredited researchers in <https://idus.us.es/items/3d72f616-891a-490e-9710-2d3bafda65e3>.

References

- Auerbach, E.A., Seyfried, E.E., McMahon, K.D., 2007. Tetracycline resistance genes in activated sludge wastewater treatment plants. *Water Res.* 41, 1143–1151. <https://doi.org/10.1016/j.watres.2006.11.045>.
- Carattoli, A., 2013. Plasmids and the spread of resistance. *Int. J. Med. Microbiol.* 303, 298–304. <https://doi.org/10.1016/j.ijmm.2013.02.001>.
- Caucci, S., Karkman, A., Cacace, D., Rybicki, M., Timpel, J., Voolaid, V., Gurke, R., Virta, M., Berendonk, T.U., 2016. Seasonality of antibiotic prescriptions for outpatients and resistance genes in sewers and wastewater treatment plant outflow. *FEMS Microbiol. Ecol.* 92, fiw060. <https://doi.org/10.1093/femsec/fiw060>.
- Chiemchaisri, W., Chiemchaisri, C., Wittthayaphrom, C., Saengam, C., Mahavee, K., 2022. Reduction of antibiotic-resistant-*E. coli*, -*K. pneumoniae*, -*A. baumannii* in aged-sludge of membrane bioreactor treating hospital wastewater. *Sci. Total Environ.* 812, 152470. <https://doi.org/10.1016/j.scitotenv.2021.152470>.
- Childress, H., Sullivan, B., Kaur, J., Karthikeyan, R., 2014. Effects of ultraviolet light disinfection on tetracycline-resistant bacteria in wastewater effluents. *J. Water Health* 12, 404–409. <https://doi.org/10.2166/wh.2013.257>.
- Chu, B.T.T., Petrovich, M.L., Chaudhary, A., Wright, D., Murphy, B., Wells, G., Poretsky, R., 2018. Metagenomics reveals the impact of wastewater treatment plants on the dispersal of microorganisms and genes in aquatic sediments. *Appl. Environ. Microbiol.* 84, e02168. <https://doi.org/10.1128/AEM.02168-17>.
- Conte, D., Palmeiro, J.K., da Silva Nogueira, K., de Lima, T.M.R., Cardoso, M.A., Pontarolo, R., Degaut Pontes, F.L., Dalla-Costa, L.M., 2017. Characterization of CTX-M enzymes, quinolone resistance determinants, and antimicrobial residues from hospital sewage, wastewater treatment plant, and river water. *Ecotoxicol. Environ. Saf.* 136, 62–69. <https://doi.org/10.1016/j.ecoenv.2016.10.031>.
- David, S., Reuter, S., Harris, S.R., Glasner, C., Feltwell, T., Argimon, S., Abudahab, K., Goater, R., Giani, T., Errico, G., Aspbury, M., Sjunnebo, S., Group, the EuSCAPE Working, Koraqi, A., Lacey, D., Apfalter, P., Hartl, R., Glupczynski, Y., Huang, T.-D., Strateva, T., Marteva-Proevska, Y., Andrasevic, A.T., Butic, I., Pieridou-Bagatzouni, D., Maikanti-Charalampous, P., Hrabak, J., Zemlickova, H., Hammerum, A., Jakobsen, L., Ivanova, M., Pavelkovich, A., Jalava, J., Osterblad, M., Dortet, L., Vaux, S., Kaase, M., Gatermann, S.G., Vatopoulos, A., Tryfinopoulou, K., Tóth, Á., Jánvári, L., Boo, T.W., McGrath, E., Carmeli, Y., Adler, A., Pantosti, A., Monaco, M., Raka, L., Kurti, A., Balode, A., Saule, M., Miculeviciene, J., Mierauskaite, A., Perrin-Weniger, M., Reichert, P., Nestorova, N., Debattista, S., Mijovic, G., Lopicic, M., Samuelsen, Ø., Haldorsen, B., Zabicka, D., Literacka, E., Caniga, M., Manageiro, V., Kaftandzieva, A., Trajkovska-Dokic, E., Damian, M., Lixandru, B., Jelesic, Z., Trudic, A., Niks, M., Schreterova, E., Pirs, M., Cerar, T., Oteo, J., Aracil, B., Giske, C., Sjöström, K., Gür, D., Cakar, A., Woodford, N., Hopkins, K., Wiuff, C., Brown, D.J., Group, the ESGEM Study, Feil, E.J., Rossolini, G. M., Aanensen, D.M., Grundmann, H., 2019. Epidemic of carbapenem-resistant *Klebsiella pneumoniae* in Europe is driven by nosocomial spread. *Nat. Microbiol.* 4, 1919–1929. <https://doi.org/10.1038/s41564-019-0492-8>.
- Gao, P., Munir, M., Xagorarakis, I., 2012. Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. *Sci. Total Environ.* 421–422, 173–183. <https://doi.org/10.1016/j.scitotenv.2012.01.061>.
- Giske, C., Martínez-Martínez, L., Cantón, R., Stefani, S., Skov, R., Glupczynski, Y., Nordmann, P., Wootton, M., Miriagou, V., Simonsen, G., Zemlickova, H., Cohen-Stuart, J., Gniadkowski, M., 2017. EUCAST Guidelines for Detection of Resistance Mechanisms and Specific Resistances of Clinical and/or Epidemiological Importance, 2a ed.
- Gomi, R., Matsuda, T., Yamamoto, M., Chou, P.-H., Tanaka, M., Ichiyama, S., Yoneda, M., Matsumura, Y., 2018. Characteristics of carbapenemase-producing enterobacteriaceae in wastewater revealed by genomic analysis. *Antimicrob. Agents Chemother.* 62, e02501–e02517. <https://doi.org/10.1128/AAC.02501-17>.
- Harrison, X.A., Donaldson, L., Correa-Cano, M.E., Evans, J., Fisher, D.N., Goodwin, C.E. D., Robinson, B.S., Hodgson, D.J., Inger, R., 2018. A brief introduction to mixed effects modelling and multi-model inference in ecology. *PeerJ* 6, e4794. <https://doi.org/10.7717/peerj.4794>.
- Hendriksen, R.S., Munk, P., Njage, P., Pevs, B., Bunnik, B., McNally, L., Lukjancenko, O., Röder, T., Nieuwenhuijse, D., Pedersen, S.K., Kjeldgaard, J., Kaas, R.S., Clausen, P.T. L.C., Vogt, J.K., Leekitcharoenphon, P., Van De Schans, M.G.M., Zuidema, T., De

- Roda Husman, A.M., Rasmussen, S., Petersen, B., Bego, A., Rees, C., Cassar, S., Coventry, K., Collignon, P., Allerberger, F., Rahube, T.O., Oliveira, G., Ivanov, I., Vuthy, Y., Sophaek, T., Yost, C.K., Ke, C., Zheng, H., Baisheng, L., Jiao, X., Donado-Godoy, P., Coulbaly, K.J., Jergovic, M., Zhenovic, J., Karpiskova, R., Villacis, J.E., Legesse, M., Egualde, T., Heikinheimo, A., Malania, L., Nitsche, A., Brinkmann, A., Saba, C.K.S., Kocsis, B., Solymosi, N., Thorsteinsdottir, T.R., Hatha, A.M., Alebouyeh, M., Morris, D., Cormican, M., O'Connor, L., Moran-Gilad, J., Alba, P., Battisti, A., Shakenova, Z., Kiiyukia, C., Ng'eno, E., Raka, L., Avsejkeno, J., Bērziņš, A., Bartkevičs, V., Penny, C., Rajandas, H., Parimannan, S., Haber, M.V., Pal, P., Jeunen, G.-J., Gemmell, N., Fashae, K., Holmstad, R., Hasan, R., Shakoor, S., Rojas, M.L.Z., Wasył, D., Bosevska, G., Kochubovskij, M., Radu, C., Gassama, A., Radosavljević, V., Wuertz, S., Zuniga-Montanez, R., Tay, M.Y.F., Gavačová, D., Pastuchova, K., Truska, P., Trkov, M., Esterhuysen, K., Keddy, K., Cerdà-Cuellar, M., Pathirage, S., Norrgren, L., Örn, S., Larsson, D.G.J., Heijden, T.V.D., Kumburu, H.H., Sannet, B., Bidjada, P., Njanpop-Lafourcade, B.-M., Nikiema-Pessinaba, S.C., Levent, B., Meschke, J.S., Beck, N.K., Van, C.D., Phuc, N.D., Tran, D.M.N., Kwenda, G., Tabo, D., Wester, A.L., Cuadros-Orellana, S., Amid, C., Cochrane, G., Sicheritz-Ponten, T., Schmitt, H., Alvarez, J.R.M., Aidara-Kane, A., Pamp, S.J., Lund, O., Hald, T., Woolhouse, M., Koopmans, M.P., Vigre, H., Petersen, T.N., Aarestrup, F.M., The Global Sewage Surveillance project consortium, 2019. Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. *Nat. Commun.* 10, 1124. <https://doi.org/10.1038/s41467-019-08853-3>.
- Huijbers, P.M.C., Larsson, D.G.J., Flach, C.-F., 2020. Surveillance of antibiotic resistant *Escherichia coli* in human populations through urban wastewater in ten European countries. *Environ. Pollut.* 261, 114200. <https://doi.org/10.1016/j.envpol.2020.114200>.
- Iakovides, I.C., Michael-Kordatou, I., Moreira, N.F.F., Ribeiro, A.R., Fernandes, T., Pereira, M.F.R., Nunes, O.C., Manaia, C.M., Silva, A.M.T., Fatta-Kassinos, D., 2019. Continuous ozonation of urban wastewater: removal of antibiotics, antibiotic-resistant *Escherichia coli* and antibiotic resistance genes and phytotoxicity. *Water Res.* 159, 333–347. <https://doi.org/10.1016/j.watres.2019.05.025>.
- Jarlier, V., Nicolas, M.-H., Fournier, G., Philippon, A., 1988. Extended broad-spectrum -lactamases conferring transferable resistance to newer -lactam agents in enterobacteriaceae: hospital prevalence and susceptibility patterns. *Clin. Infect. Dis.* 10, 867–878. <https://doi.org/10.1093/clinids/10.4.867>.
- Joadas, A., Nicolau, B., Maia E Silva, A., Barroso, H., Duarte, A., 2024. Residual waters vs. multiresistant bacteria. *Ann. Med.* 51, 91. <https://doi.org/10.1080/07853890.2018.1561841>–91.
- Kang, M., Yang, J., Kim, S., Park, J., Kim, M., Park, W., 2022. Occurrence of antibiotic resistance genes and multidrug-resistant bacteria during wastewater treatment processes. *Sci. Total Environ.* 811, 152331. <https://doi.org/10.1016/j.scitotenv.2021.152331>.
- Korzeniewska, E., Korzeniewska, A., Harnisz, M., 2013. Antibiotic resistant *Escherichia coli* in hospital and municipal sewage and their emission to the environment. *Ecotoxicol. Environ. Saf.* 91, 96–102. <https://doi.org/10.1016/j.ecoenv.2013.01.014>.
- Kutilova, I., Medvecký, M., Leekitchaerophon, P., Munk, P., Masarikova, M., Davidova-Gerzova, L., Jamborova, I., Bortolaia, V., Pamp, S.J., Dolejska, M., 2021. Extended-spectrum beta-lactamase-producing *Escherichia coli* and antimicrobial resistance in municipal and hospital wastewaters in Czech Republic: culture-based and metagenomic approaches. *Environ. Res.* 193, 110487. <https://doi.org/10.1016/j.envres.2020.110487>.
- LaPara, T.M., Burch, T.R., McNamara, P.J., Tan, D.T., Yan, M., Eichmiller, J.J., 2011. Tertiary-treated municipal wastewater is a significant point source of antibiotic resistance genes into Duluth-superior harbor. *Environ. Sci. Technol.* 45, 9543–9549. <https://doi.org/10.1021/es202775v>.
- Leyrat, C., Morgan, K.E., Leurent, B., Kahan, B.C., 2018. Cluster randomized trials with a small number of clusters: which analyses should be used? *Int. J. Epidemiol.* 47, 321–331. <https://doi.org/10.1093/ije/dyx169>.
- Liao, J., Liu, C., Liu, L., Li, J., Fan, H., Ye, J., Zeng, Z., 2019. Influence of hydraulic retention time on behavior of antibiotics and antibiotic resistance genes in aerobic granular reactor treating biogas slurry. *Front. Environ. Sci. Eng.* 13, 31. <https://doi.org/10.1007/s11783-019-1115-6>.
- Liu, S.-S., Qu, H.-M., Yang, D., Hu, H., Liu, W.-L., Qiu, Z.-G., Hou, A.-M., Guo, J., Li, J.-W., Shen, Z.-Q., Jin, M., 2018. Chlorine disinfection increases both intracellular and extracellular antibiotic resistance genes in a full-scale wastewater treatment plant. *Water Res.* 136, 131–136. <https://doi.org/10.1016/j.watres.2018.02.036>.
- Makowska, N., Philips, A., Dabert, M., Nowis, K., Trzebny, A., Koczura, R., Mokracka, J., 2020. Metagenomic analysis of β -lactamase and carbapenemase genes in the wastewater resistome. *Water Res.* 170, 115277. <https://doi.org/10.1016/j.watres.2019.115277>.
- Manaia, C.M., Rocha, J., Scaccia, N., Marano, R., Radu, E., Biancullo, F., Cerqueria, F., Fortunato, G., Iakovides, I.C., Zammit, I., Kampouris, I., Vaz-Moreira, I., Nunes, O.C., 2018. Antibiotic resistance in wastewater treatment plants: tackling the black box. *Environ. Int.* 312–324.
- Marano, R.B.M., Fernandes, T., Manaia, C.M., Nunes, O., Morrison, D., Berendonk, T.U., Kreuzinger, N., Tenson, T., Corno, G., Fatta-Kassinos, D., Merlin, C., Topp, E., Jurkevitch, E., Henn, L., Scott, A., Heß, S., Slipko, K., Laht, M., Kisand, V., Di Cesare, A., Karaolia, P., Michael, S.G., Petre, A.L., Rosal, R., Pruden, A., Riquelme, V., Agüera, A., Esteban, B., Luczkiewicz, A., Kalinowska, A., Leonard, A., Gaze, W.H., Adegoke, A.A., Stenstrom, T.A., Pollice, A., Salerno, C., Schermer, C. U., Krzeminski, P., Guilloteau, H., Donner, E., Drigo, B., Libralato, G., Guida, M., Bürgmann, H., Beck, K., Garelick, H., Tacaó, M., Henriques, I., Martínez-Alcalá, I., Guillén-Navarro, J.M., Popowska, M., Piotrowska, M., Quintela-Baluja, M., Bunce, J. T., Polo-López, M.I., Nahim-Granados, S., Pons, M.-N., Milakovic, M., Udikovic-Kolic, N., Ory, J., Ousmane, T., Caballero, P., Oliver, A., Rodriguez-Mozaz, S., Balcazar, J.L., Jäger, T., Schwartz, T., Yang, Y., Zou, S., Lee, Y., Yoon, Y., Herzog, B., Mayrhofer, H., Prakash, O., Nimonkar, Y., Heath, E., Baraniak, A., Abreu-Silva, J., Choudhury, M., Munoz, L.P., Krizanovic, S., Brunetti, G., Maile-Moskowitz, A., Brown, C., Cytryn, E., 2020. A global multinational survey of cefotaxime-resistant coliforms in urban wastewater treatment plants. *Environ. Int.* 144, 106035. <https://doi.org/10.1016/j.envint.2020.106035>.
- Müller, H., Sib, E., Gajdiss, M., Klanke, U., Lenz-Plet, F., Barabasch, V., Albert, C., Schallenberg, A., Timm, C., Zacharias, N., Schmithausen, R.M., Engelhart, S., Exner, M., Parcina, M., Schreiber, C., Bierbaum, G., 2018. Dissemination of multi-resistant Gram-negative bacteria into German wastewater and surface waters. *FEMS Microbiol. Ecol.* 94. <https://doi.org/10.1093/femsec/fiy057>.
- Muloi, D.M., Wee, B.A., McClean, D.M.H., Ward, M.J., Pankhurst, L., Phan, H., Ivens, A. C., Kivali, V., Kiyong'a, A., Ndinda, C., Gitahi, N., Ouko, T., Hassell, J.M., Imboma, T., Akoko, J., Murungi, M.K., Njoroge, S.M., Muinde, P., Nakamura, Y., Alunasa, L., Furuma, E., Kaitho, T., Öhngren, E.M., Amanya, F., Ogendo, A., Wilson, D.J., Bettridge, J.M., Kiiru, J., Kyobutungi, C., Tacolli, C., Kang'ethe, E.K., Davila, J.D., Kariuki, S., Robinson, T.P., Rushton, J., Woolhouse, M.E.J., Fèvre, E.M., 2022. Population genomics of *Escherichia coli* in livestock-keeping households across a rapidly developing urban landscape. *Nat. Microbiol.* 7, 581–589. <https://doi.org/10.1038/s41564-022-01079-y>.
- Nugent, J.R., Kleinman, K.P., 2021. Type I error control for cluster randomized trials under varying small sample structures. *BMC Med. Res. Methodol.* 21, 65. <https://doi.org/10.1186/s12874-021-01236-7>.
- Ojer-Usoz, E., González, D., García-Jalón, I., Vitas, A.I., 2014. High dissemination of extended-spectrum β -lactamase-producing Enterobacteriaceae in effluents from wastewater treatment plants. *Water Res.* 56, 37–47. <https://doi.org/10.1016/j.watres.2014.02.041>.
- Oliveira, M., Serrano, I., Van Harten, S., Bessa, L.J., Bernardo, F., Da Costa, P.M., 2016. Fecal contamination of wastewater treatment plants in Portugal. *Environ. Sci. Pollut. Res.* 23, 14671–14675. <https://doi.org/10.1007/s11356-016-6962-0>.
- Oliveira, M., Truchado, P., Cordero-García, R., Gil, M.I., Soler, M.A., Rancano, A., García, F., Álvarez-Ordóñez, A., Allende, A., 2023. Surveillance on ESB-*Escherichia coli* and indicator ARG in wastewater and reclaimed water of four regions of Spain: impact of different disinfection treatments. *Antibiotics* 12, 400. <https://doi.org/10.3390/antibiotics12020400>.
- Ollech, D., Webel, K., 2023. A random forest-based approach to combining and ranking seasonality tests. *J. Econom. Methods* 12, 117–130.
- Pazda, M., Kumirska, J., Stepnowski, P., Mulkiewicz, E., 2019. Antibiotic resistance genes identified in wastewater treatment plant systems – a review. *Sci. Total Environ.* 697, 134023. <https://doi.org/10.1016/j.scitotenv.2019.134023>.
- Pérez-Etayo, L., González, D., Leiva, J., Vitas, A.I., 2020. Multidrug-resistant bacteria isolated from different aquatic environments in the North of Spain and South of France. *Microorganisms* 8, 1425. <https://doi.org/10.3390/microorganisms8091425>.
- Picão, R.C., Cardoso, J.P., Campana, E.H., Nicoletti, A.G., Petrolini, F.V.B., Assis, D.M., Juliano, L., Gales, A.C., 2013. The route of antimicrobial resistance from the hospital effluent to the environment: focus on the occurrence of KPC-producing *Aeromonas* spp. and Enterobacteriaceae in sewage. *Diagn. Microbiol. Infect. Dis.* 76, 80–85. <https://doi.org/10.1016/j.diagmicrobio.2013.02.001>.
- Puljko, A., Milaković, M., Krizanović, S., Kosić-Vukšić, J., Babić, I., Petrić, I., Maravić, A., Jelić, M., Udiković-Kolić, N., 2022. Prevalence of enteric opportunistic pathogens and extended-spectrum cephalosporin- and carbapenem-resistant coliforms and genes in wastewater from municipal wastewater treatment plants in Croatia. *J. Hazard. Mater.* 427, 128155. <https://doi.org/10.1016/j.jhazmat.2021.128155>.
- Raven, K.E., Ludden, C., Gouliouris, T., Blane, B., Naydenova, P., Brown, N.M., Parkhill, J., Peacock, S.J., 2019. Genomic surveillance of *Escherichia coli* in municipal wastewater treatment plants as an indicator of clinically relevant pathogens and their resistance genes. *Microb. Genom.* 5. <https://doi.org/10.1099/mgen.0.000267>.
- Ribeiro, A.R., Sures, B., Schmidt, T.C., 2018. Cephalosporin antibiotics in the aquatic environment: a critical review of occurrence, fate, ecotoxicity and removal technologies. *Environ. Pollut.* 241, 1153–1166. <https://doi.org/10.1016/j.envpol.2018.06.040>.
- Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. *Sci. Total Environ.* 447, 345–360. <https://doi.org/10.1016/j.scitotenv.2013.01.032>.
- Rodríguez Raserio, F.J., Moya Ruano, L.A., Raserio Del Real, P., Cuberos Gómez, L., Lorusso, N., 2022. Associations between SARS-CoV-2 RNA concentrations in wastewater and COVID-19 rates in days after sampling in small urban areas of Seville: a time series study. *Sci. Total Environ.* 806, 150573. <https://doi.org/10.1016/j.scitotenv.2021.150573>.
- Salamanca-Rivera, E., López-Cerero, L., Rodríguez-Martínez, J.M., Pascual, A., Rodríguez-Baño, J., 2022. Prevalence, incidence, and risk factors for intestinal colonization due to fluoroquinolone-resistant ST131 *Escherichia coli*: a longitudinal study in highly dependent, long-term care facility residents. *Microbiol. Spectr.* 10, e01673. <https://doi.org/10.1128/spectrum.01673-22>.
- Schages, L., Wichern, F., Kalscheuer, R., Bockmühl, D., 2020. Winter is coming – impact of temperature on the variation of beta-lactamase and mcr genes in a wastewater treatment plant. *Sci. Total Environ.* 712, 136499. <https://doi.org/10.1016/j.scitotenv.2020.136499>.
- Sekizuka, T., Inamine, Y., Segawa, T., Hashino, M., Yatsu, K., Kuroda, M., 2019. Potential KPC-2 carbapenemase reservoir of environmental *Aeromonas hydrophila* and *Aeromonas caviae* isolates from the effluent of an urban wastewater treatment plant in Japan. *Environ. Microbiol. Rep.* 11, 589–597. <https://doi.org/10.1111/1758-2229.12772>.
- Sib, E., Lenz-Plet, F., Barabasch, V., Klanke, U., Savin, M., Hembach, N., Schallenberg, A., Kehl, K., Albert, C., Gajdiss, M., Zacharias, N., Müller, H., Schmithausen, R.M.,

- Exner, M., Kreyenschmidt, J., Schreiber, C., Schwartz, T., Parčina, M., Bierbaum, G., 2020. Bacteria isolated from hospital, municipal and slaughterhouse wastewaters show characteristic, different resistance profiles. *Sci. Total Environ.* 746, 140894. <https://doi.org/10.1016/j.scitotenv.2020.140894>.
- Tesfaye, H., Alemayehu, H., Desta, A.F., Egualé, T., 2019. Antimicrobial susceptibility profile of selected Enterobacteriaceae in wastewater samples from health facilities, abattoir, downstream rivers and a WWTP in Addis Ababa, Ethiopia. *Antimicrob. Resist. Infect. Control* 8, 134. <https://doi.org/10.1186/s13756-019-0588-1>.
- Thomson, K.S., Sanders, C.C., 1992. Detection of extended-spectrum beta-lactamases in members of the family Enterobacteriaceae: comparison of the double-disk and three-dimensional tests. *Antimicrob. Agents Chemother.* 36, 1877–1882. <https://doi.org/10.1128/AAC.36.9.1877>.
- Twisk, J.W.R., 2011. *Applied Multilevel analysis: a Practical guide*, 5. print. ed, *Practical Guides to Biostatistics and Epidemiology*. Cambridge University Press, Cambridge.
- Voigt, A.M., Zacharias, N., Timm, C., Wasser, F., Sib, E., Skutlarek, D., Parčina, M., Schmithausen, R.M., Schwartz, T., Hembach, N., Tiehm, A., Stange, C., Engelhart, S., Bierbaum, G., Kistemann, T., Exner, M., Faerber, H.A., Schreiber, C., 2020. Association between antibiotic residues, antibiotic resistant bacteria and antibiotic resistance genes in anthropogenic wastewater – An evaluation of clinical influences. *Chemosphere* 241, 125032. <https://doi.org/10.1016/j.chemosphere.2019.125032>.
- Woodford, N., Turton, J.F., Livermore, D.M., 2011. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. *FEMS Microbiol. Rev.* 35, 736–755. <https://doi.org/10.1111/j.1574-6976.2011.00268.x>.
- Xu, Z., Shen, W., Zhang, R., Cai, J., 2022. Clonal dissemination of aeromonas hydrophila with binary carriage of blaKPC-2-bearing plasmids in a Chinese hospital. *Front. Microbiol.* 13, 918561. <https://doi.org/10.3389/fmicb.2022.918561>.
- Yang, F., Huang, L., Li, L., Yang, Y., Mao, D., Luo, Y., 2017. Discharge of KPC-2 genes from the WWTPs contributed to their enriched abundance in the receiving river. *Sci. Total Environ.* 581–582, 136–143. <https://doi.org/10.1016/j.scitotenv.2016.12.063>.
- Zhang, L., Ma, X., Luo, L., Hu, N., Duan, J., Tang, Z., Zhong, R., Li, Y., 2020. The prevalence and characterization of extended-spectrum β -Lactamase- and carbapenemase-producing bacteria from hospital sewage, treated effluents and receiving rivers. *Int. J. Environ. Res. Public Health* 17, 1183. <https://doi.org/10.3390/ijerph17041183>.
- Zhang, Z., Zhang, G., Ju, F., 2022. Using culture-enriched phenotypic metagenomics for targeted high-throughput monitoring of the clinically important fraction of the β -lactam resistome. *Environ. Sci. Technol.* 56, 11429–11439. <https://doi.org/10.1021/acs.est.2c03627>.
- Zurfluh, K., Bagutti, C., Brodmann, P., Alt, M., Schulze, J., Fanning, S., Stephan, R., Nüesch-Inderbinen, M., 2017. Wastewater is a reservoir for clinically relevant carbapenemase- and 16s rRNA methylase-producing Enterobacteriaceae. *Int. J. Antimicrob. Agents* 50, 436–440. <https://doi.org/10.1016/j.ijantimicag.2017.04.017>.