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Research article

Metagenomic analysis of antibiotic resistance across the wastewater process

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ABSTRACT

Bacterial resistance to antimicrobials is a global health threat. Within the One Health context, water from regions with high antibiotic usage, such as clinical and urban areas, collects at wastewater treatment plants (WWTPs). In the WWTP, the activated sludge becomes a complex environment where various antimicrobials and microorganisms converge. While significant research has focused on the influent, activated sludge, and effluent, upstream and downstream sectors around the WWTP are often neglected. We conducted a systematic analysis using five publicly available metagenomic datasets (n = 164) from different WWTP sectors and adjacent freshwater systems: upstream (n = 14), influent (n = 14), activated sludge (n = 109), effluent (n = 14), and downstream (n = 13) to identify and characterise the microbiome, resistome, and mobilome. Opportunistic pathogenic bacteria, such as *Pseudomonas*, *Aeromonas*, and *Acidovorax*, were found in all WWTP sectors, with abundances exceeding 9% in the influent. ESKAPE pathogens, including *Klebsiella pneumoniae* and *Enterobacter* species, were identified in the effluent with abundances over 1%. We detected 230 antibiotic resistance genes (ARGs) throughout the WWTP. FTU and CKO β -lactamase gene families dominated the upstream, effluent, and downstream sectors, while the OXA β -lactamase gene family was highly abundant in the influent and activated sludge. ARGs, such as the OXA β -lactamase gene family, were linked to plasmids. Class-1 integrons, associated with the *sul1* gene, a marker for anthropogenic pollution, were prevalent in the effluent and downstream sectors. Integrative elements (ICE_{clc}, Tn4371, and PGI2), linked to ARGs, were identified in all sectors, increasing AMR dissemination. These integrative elements conferred resistance to antibiotics, including sulfonamides, tetracyclines and carbapenems. Our findings highlight the presence of ARGs and mobile genetic elements in WWTPs and nearby freshwater systems, raising concerns about AMR transmission to humans, animals, and the environment. This study emphasises the need for effective AMR monitoring and strategies in wastewater treatment to protect public and environmental health.

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1. Introduction

Antimicrobial resistance (AMR) is responsible for at least 700,000 deaths annually worldwide. It is anticipated to rise to 10 million deaths per year by 2050, affecting all income regions [1]. The World Health Organisation has endorsed a global action plan to control antimicrobial resistance as infectious diseases caused by multidrug resistant (MDR) bacteria are one of the leading causes of death worldwide [2,3]. The proliferation of antibiotic resistant and MDR bacteria is directly correlated to the use of antibiotics in clinical and non-clinical settings [4]. According to the One Health concept, wastewater treatment plants (WWTPs) should act as a barrier between sewage and the receiving environments as sewage contains a full spectrum of human microbiome-associated bacteria, antibiotic resistant bacteria and associated antibiotic resistance genes (ARGs) released by anthropogenic factors [4,5].

WWTPs employ various treatment technologies and management practices. These practices can be broadly categorised into natural attenuation, advanced treatment processes and conventional practices which aim to remove solids, organic matter, nutrients and contaminants [6,7]. Natural attenuation uses processes such as dilution, biodegradation, and sorption, which are cost-effective and simple but inefficient in fully removing contaminants and microorganisms [7]. Advanced oxidation processes can break down antibiotics but may generate oxidation products that could introduce ARGs into microorganisms, particularly pathogenic bacteria [8,7,9]. Conventional treatment options, such as membrane treatments, act as robust yet costly barriers, producing brine water. In contrast, incorporating activated carbon is cost-effective but leads to sludge generation [5]. One of the most common biological processes in conventional WWTPs is the activated sludge process. This method is widely adopted for treating both municipal and industrial wastewater. However, it is concerning that despite the activated sludge process being effective in removing some ARGs, contaminants and microorganisms can persist in the treated water after the process [5,2].

At present, WWTPs act as primary barriers against the spread of emerging contaminants such as antibacterial drugs causing antibiotic resistant bacteria [7]. However, these contaminants cannot be removed at low concentrations and removal efficiency is not satisfactory [7]. This inefficiency can result in the release of antibiotic resistant bacteria with the effluent into receiving environments e.g. rivers and lakes [5]. Moreover, WWTPs combined with activated sludge may enhance the spread of antibiotic resistant bacteria and ARGs into these overlooked environments [10,11]. AMR surveillance has mainly focused on the influent, effluent, and activated sludge sectors, or their combinations. Che et al. [12] identified plasmids carrying ARGs conferring resistance to clinically relevant antibiotics e.g. tetracycline and macrolide-lincosamide-streptogramin in the aforementioned sectors increasing the risk of ARG-carrying bacteria spreading to rivers and lakes. Iwan et al. [13] found *E. coli* isolates increasing in resistance to antibiotic agents along the Tama River in Japan from the effluent of an urban WWTP to the downstream river. Similarly, sulfonamide levels were similar in upstream and downstream sectors of a feedlot WWTP however, the sulfonamide-resistant microorganisms and gene abundances were higher in the downstream river [14]. The presence of antibiotic agents, antibiotic resistant bacteria and ARGs present in the upstream and downstream sectors contributes to the rise of AMR and enhances horizontal gene transfer (HGT) via mobile genetic elements (MGEs) such as plasmids, integrons and transposons [15–18]. AMR surveillance should consider adjacent areas of the WWTP, such as the upstream freshwater system and downstream receiving environmental sectors, to fully address the AMR spread (Fig. 1).

This study aims to analyse publicly available metagenomic datasets from various sectors of wastewater treatment plants (WWTP) — including upstream, influent, activated sludge, effluent, and downstream — across different geographical locations. Using a standardised pipeline, this study aims to identify bacterial populations, antibiotic resistance genes, and mobile genetic elements in each sector and examine their role in the spread of AMR into receiving environments.

2. Methods and materials

2.1. Data sources

164 whole-metagenome sequences representing the wastewater treatment plant were selected for this study. These included upstream (n=14), influent (n=14), activated sludge (n=109), effluent (n=14), and downstream (n=13) [19–24]. All raw reads were publicly available and obtained from NCBI-SRA (<https://www.ncbi.nlm.nih.gov/sra>). A combination of the following keywords was used to find whole metagenome sequences, these included “upstream”, “influent”, “activated sludge”, “effluent”, “downstream”, “whole genome”, “metagenome”, “wastewater”, “wastewater treatment plant”. The identified samples were then filtered based on the sequencing technology used, specifically selecting only those generated using Illumina technology. Additionally, only samples with accompanying publications were included. Samples that did not meet these criteria were discarded.

Samples obtained were from different studies conducted in various geographical locations (Fig. 1). Influent and effluent samples were obtained from urban and industrial sources from Portugal, Colombia, China and South Korea, upstream and downstream samples were obtained from a river source in Spain and South Korea and lastly, activated sludge samples were obtained from municipal, urban and industrial sources from China and Colombia. Samples were categorised into five sectors: upstream, influent, activated sludge, effluent and downstream based on the sample source descriptions. Upstream samples were obtained from freshwater systems over 50 m away from the WWTP and in the opposite direction of water flow. Influent samples consisted of raw sewage samples taken before the treatment process whereas activated sludge samples were collected from reactors during biological treatment. The effluent samples consisted of treated water flowing out of the WWTP and the downstream samples were collected from freshwater systems more than 50 m away from the WWTP, following the flow of water (Fig. 1). Detailed information about the metagenomes retrieved from the database is included in Table S1.

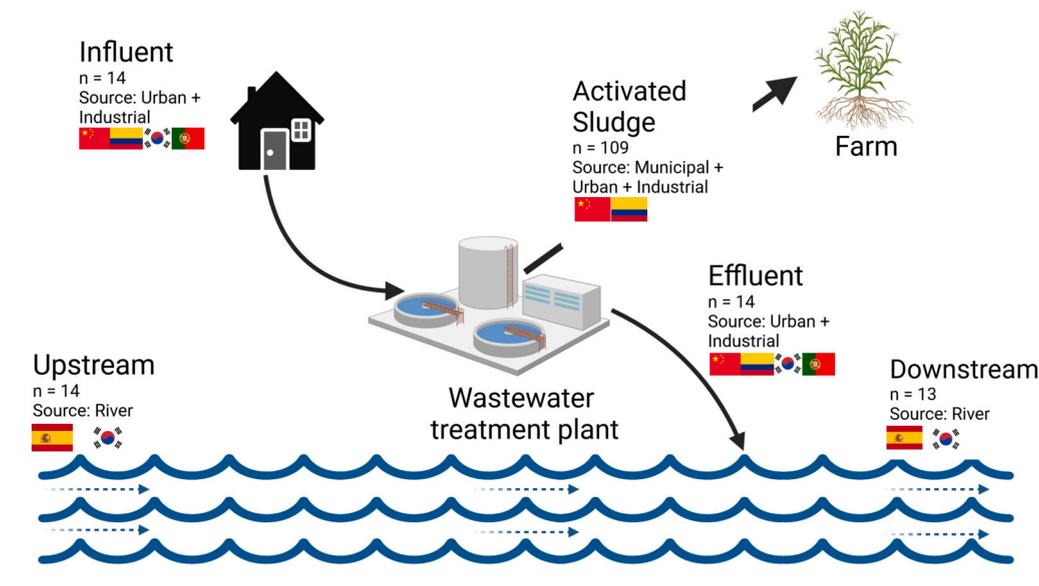


Fig. 1. The process of the wastewater treatment plant. The upstream sector includes freshwater systems such as streams, lakes and rivers that flow toward the wastewater treatment plant (dotted arrows). The wastewater treatment plant begins with the sewage (influent) from urban and municipal areas which flow to the wastewater treatment plant (black arrows). Sewage that flows into the wastewater treatment plant can be treated by the biological treatment process i.e. activated sludge, to treat the wastewater. Treated water is released as effluent which flows downstream areas such as rivers and lakes and can be used in farming. Each flag represents the geographical location from where the samples were taken, and the sample's source is indicated above the flags. Influent and effluent samples were obtained from urban and industrial sources from Portugal, Colombia, China and South Korea, upstream and downstream samples were obtained from a river source in Spain and South Korea and lastly, activated sludge samples were obtained from municipal, urban and industrial sources from China and Colombia.

2.2. Bioinformatic analysis

The quality of the reads was assessed using FastQC v.0.11.9 with default parameters [25]. Paired-end reads were trimmed and filtered by Trimmomatic v.0.39 [26] while specifying the ILLUMINACLIP: adapters.fa:2:30:10 LEADING:3 TRAILING:3 MINLEN:30 SLIDINGWINDOW: 4:20 parameters. The microbiome was profiled by Kraken2 v.2.1.0 [27] using the minikraken2 microbial database (2019, 8 GB). Species confirmation and the estimation of abundances were done by Bracken v.2.6.2 for each sample across all studies and sectors [28]. The average relative abundances of bacterial phyla, families, genera, and species were calculated across all studies within each sector to characterise the microbial populations present in each sector of the wastewater treatment plant (WWTP).

Average relative abundances of the bacterial species detected in each sector were used to calculate the beta-diversity between sectors of the WWTP using the R package vegan v.2.6-4. The beta-diversity of the microbiome between the different sectors of the WWTP was visualised using non-metric multidimensional scaling (NMDS) analysis with Bray-Curtis dissimilarity. Spearman's correlation analysis was used to analyse the correlation between the overall microbial communities and specifically ESKAPE pathogens found in each sector of the WWTP using the scipy.stats package in Python.

Filtered paired-end reads were *de novo* assembled using metaSPAdes v.3.15.2 [29] on meta mode with default parameters. The assembled metagenomic contigs were annotated to determine the antibiotic resistance gene families and drug classes using the Comprehensive Antimicrobial Resistance Database Resistance Gene Identifier (CARD-RGI) v.3.1.2 with default parameters including loose matches and the `-low_quality -clean` options [30]. Furthermore, the average relative abundances of the annotated antibiotic resistance genes in each sector were assessed. This data was normalised based on the total number of antibiotic resistance genes detected in each sector, indicating their respective proportions. Spearman's correlation analysis was used to analyse the correlation between antibiotic resistance gene families found in each sector of the WWTP using the scipy.stats package in Python.

Metagenomic contigs were aligned to detect integrons and integrative elements using the INTEGRAL database [31] and the ICEberg database [32]. Alignments were performed by BWA-mem v.0.7.10 with default parameters [33], generating a SAM file. The output SAM file was converted into a BAM file using SAMtools version v.1.9 [34]. Contigs that aligned to the databases were considered integrons and integrative elements. SAM output files containing the detected integrative elements were converted to .txt files and matched to the output .txt files generated with CARD-RGI from their respective WWTP sector. This process was conducted to determine which integrative elements carried antibiotic resistance genes. Contigs were classified as plasmid-originating using Plasclass v.0.1.1 [35]. To confirm that contigs originated from plasmids, a threshold was set with a minimum contig length of ≥ 1000 and probability of ≥ 0.75 . Detected plasmids were matched to the previously generated CARD-RGI output .txt files to determine if the detected plasmids were linked to the antibiotic resistance genes previously found.

3. Results and discussion

Publicly available metagenomic data representing different sectors (upstream, influent, activated sludge, effluent and downstream) of the WWTP were analysed to determine the differences in the microbial communities, antibiotic resistance genes and mobile genetic elements that facilitate the spread of AMR.

3.1. Opportunistic bacterial pathogens are present across the WWTP process

We analysed the bacterial taxonomic composition in each sector of the WWTP. The main goal of the WWTP is to reduce pollutants and pathogens through chemical, biological, or physical processes, releasing treated water as effluent into downstream systems [36]. Our study classified bacterial taxa at the phyla, family, genus, and species levels to assess the microbial composition and determine the average relative abundances of environmental, pathogenic, and emerging pathogenic bacteria in each sector of the WWTP. Data on the phyla, families, genera and species can be found in Supplemental Tables S2 - S5.

Fig. 2 illustrates the presence of opportunistic pathogens throughout the WWTP process. In our study, the *Pseudomonas*, *Aeromonas* and *Acidovorax* genera were present in each sector of the WWTP with abundances exceeding 9% in the influent (Table S4 and Fig. 2). Specifically, we identified the presence of *Aeromonas caviae* (Fig. 2), a human pathogenic bacterium causing gastrointestinal infections, in the influent (2%), effluent (1%) and downstream (1%) sectors (Table S5). Other pathogenic bacterial species detected included *Acinetobacter johnsonii*, *Aliarcobacter skirrowii* and *Aliarcobacter trophiarum* which had an abundance of 2% in the influent and less than 1% in the effluent and downstream sectors. Despite low abundances downstream, these bacterial species still pose a zoonotic potential as studies have linked them to meningitis and gastrointestinal infections in humans [7,37–40].

In the activated sludge sector, we observed a blend of pathogenic and non-pathogenic bacteria, including those crucial for biological treatment. In our study, the *Pseudomonas aeruginosa* (1.61%), *Thauera sp. MZ1T* (3%) and *Acidovorax sp. KKS102* (12%) species dominated the activated sludge sector (Fig. 2). Phyla such as Proteobacteria, Bacteroides, Actinobacteria, Acidobacteria, Chloroflexi, and Planctomycetes are commonly found in activated sludge further confirming the mixture of bacteria in the activated sludge with bacteria commonly found in the influent (Table S2) [41–44]. Given that bacteria play a vital role in biological treatment, the microbial community within the activated sludge is a key factor in evaluating WWTP efficiency. This is particularly significant as the efficiency depends on the microbial community's capacity to degrade pollutants and xenobiotic compounds like pesticides [45].

We identified ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) pathogens in the different sectors of the WWTP process in Fig. 3A. *Klebsiella pneumoniae* was found at an abundance of over 1% in the effluent with *Pseudomonas aeruginosa* exceeding 1% in the activated sludge sector. *Enterobacter* spp. showed an abundance exceeding 1% in both the influent and effluent sectors with a high correlation between both sectors (Fig. 3B). In all remaining sectors, these bacteria were present at abundances below 1%. Other ESKAPE pathogens, including *Staphylococcus aureus*, *Acinetobacter baumannii*, and *Enterococcus faecium*, were also present in all WWTP sectors ($\leq 1\%$).

A high correlation between ESKAPE pathogens was observed across sectors of WWTP, e.g. influent and effluent (1.00), upstream and influent (0.97), and effluent and downstream (0.88), indicating that ESKAPE pathogens in the upstream and influent sectors could predict their presence in effluent and downstream sectors. Correlations of all sectors with activated sludge were lower with higher variability. Treatment seems to reduce the abundance of pathogens, but it does not eliminate them as seen in the correlation between upstream-to-effluent (0.97). Moreover, the strong effluent-to-downstream correlation highlights the environmental and public health risks posed by residual ESKAPE pathogens in the treated effluent. Despite the microbial load reduction achieved by WWTPs, previous studies have highlighted the release of ESKAPE pathogens in the effluent [46]. These bacterial pathogens can harbour multiple ARGs, conferring resistance to a broad spectrum of antibiotics, including last-line drugs such as carbapenems and glycopeptides. The potential for these pathogens to spread to other environments, animals, and humans poses a significant threat to public and environmental health [47,48,17,49].

Lastly, *Escherichia coli* (*E. coli*), a key microbial indicator of water quality, was detected in both the influent and effluent ($\geq 1\%$). *E. coli* may enter freshwater systems such as upstream sectors through sources such as wildlife or agricultural runoff indicating low water quality however, its presence in the influent is expected. Unfortunately, its detection in the effluent (1.42%) and downstream ($\leq 1\%$) is concerning [50]. Both *E. coli* and *Enterococcus faecium* (*E. faecium*), also detected in this study, are recognised as indicators of faecal contamination [51]. Previous research suggests that *E. coli* levels in the effluent can vary depending on the geographic location of the samples. WWTPs treating hospital waste may discharge higher levels of *E. coli* compared to those handling urban or municipal effluent, as analysed in our study [52]. In untreated sewage, *E. coli* and *Enterococci* signal potential health risks due to their high concentrations in mammalian faeces [53]. Effective WWTPs should reduce such contaminants, minimising risks to human health and the environment. However, operational issues such as poor maintenance, design flaws, environmental factors, and chemical or biological conditions can enable *E. coli* to survive in the effluent [54,55].

It should be noted that variations in bacterial communities and abundances across different sectors of the WWTP process can stem from various selection pressures or the use of public data from various studies. Environmental factors such as heavy metals, organic compounds, disinfectants, as well as the presence of diverse bacterial types: environmental, pathogenic, non-pathogenic, human, and animal commensal bacteria can play a role in determining the bacterial communities present at different sectors [56,57]. Additionally, data sourced from different urban and municipal WWTPs introduces variability due to factors like geographic location, climate, industrial practices, local regulations, and the distinct sample collection and processing methods used in various studies. These influences can impact microbial communities and may support the emergence of antibiotic resistant bacteria and genes, as observed with pathogens like *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* detected in our study.

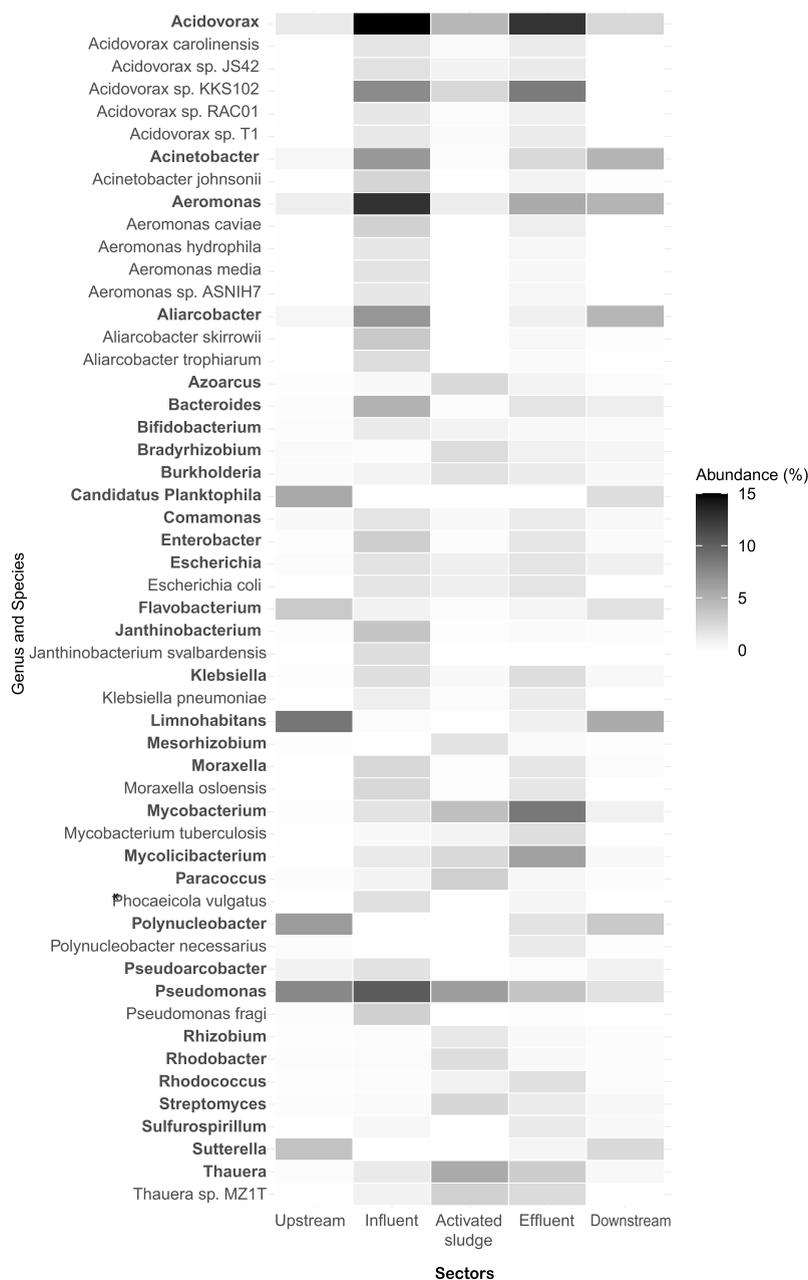


Fig. 2. Heatmap of the relative abundances (over 1%) of all bacterial genera and species identified in each sector of the wastewater treatment plant. Bacterial genera are indicated in bold text. Respective species belonging to its genus that have an abundance of over 1% are listed below the genus in plain text. Abundances are indicated in greyscale, low = white and high = black. **Phocaeicola vulgatus* has previously been identified as *Bacteroides vulgatus* in the *Bacteroides* genus.

3.2. Similar bacterial species present in the influent, activated sludge and effluent

We assessed differences in microbial communities within each sector by measuring the Beta-diversity using the Bray-Curtis dissimilarity index. This index quantifies the “distance” between microbial communities based on the species abundances. Visualised with non-metric multidimensional scaling (NMDS), a smaller distance between samples within the same sector indicates greater similarity between microbial communities and their respective sectors [58]. Furthermore, we used Spearman’s correlation analysis to validate the similarity in the microbial communities between each sector of the WWTP and identified the common bacterial species (Table S6).

Fig. 4A, shows the NMDS plot representing the microbial community similarities within each WWTP sector. Each colour represents the samples from their respective WWTP sector. Samples from the influent (purple) and effluent (green) relatively cluster together while samples from the activated sludge (red) form a cluster close to the effluent.

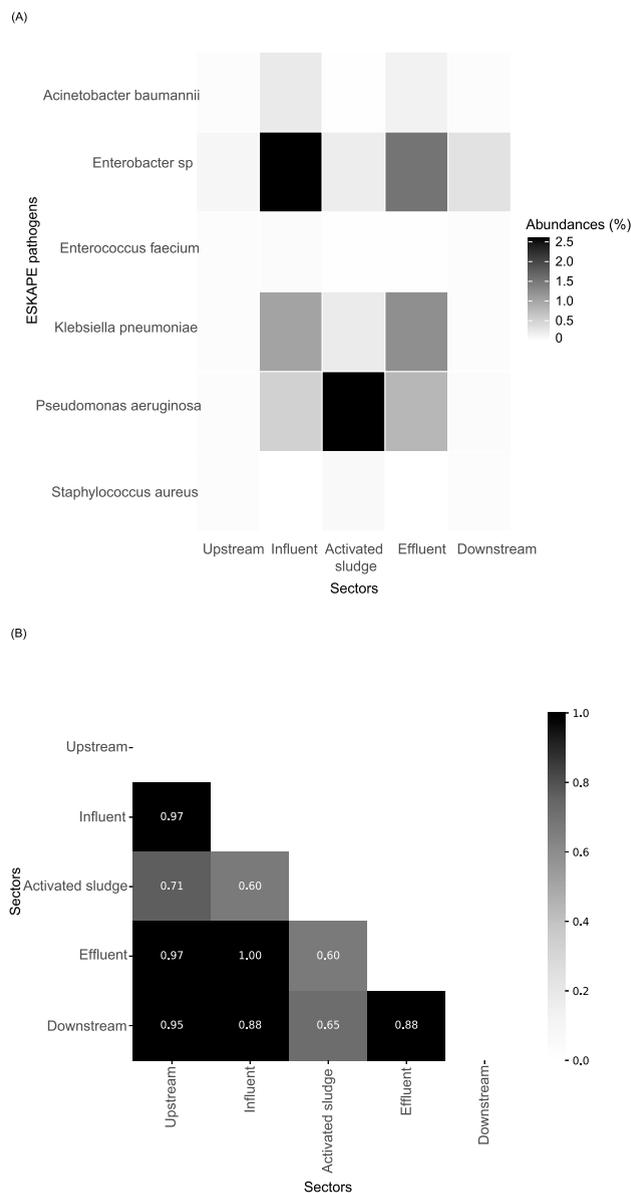


Fig. 3. (A) Heatmap of the relative abundances of ESKAPE pathogens identified in each sector of the wastewater treatment plant. Abundances are indicated in greyscale, low = white and high = black. (B) The Spearman's correlation analysis of the ESKAPE pathogens present was conducted between each sector of the wastewater treatment plant. The correlation rank values are depicted in text and indicated by the intensity of grey.

Similar bacterial species are found in the influent, activated sludge and effluent. Firstly, the clustering of the activated sludge samples suggests its microbial community is determined by the biological treatment processes such as operational parameters of bioreactors, characteristics of the influent and the environment [59]. Additionally, the effluent (green) and influent (purple) cluster closely together with the activated sludge (red) indicating similarity between groups of bacteria in each sector. This suggests that urban and municipal areas can share common microbial communities regardless of the geographical location from which the samples are obtained. Downstream (blue) and upstream (orange) samples cluster further away from the influent, activated sludge and effluent clusters, suggesting that these microbial communities are likely shaped by the freshwater taxa and or the exposure to human activity [60].

Further analysis into the microbial communities and the WWTP sectors was done using Spearman's correlation (Fig. 4B). A strong correlation was found between the microbial communities of the influent, activated sludge and effluent (≥ 0.5) indicating that these three sectors contain similar bacterial species irrespective of the geographical location and sampling times (Table S1). Additionally, a strong correlation of 0.65 was observed between the upstream and downstream sectors which can be attributed to similar bacterial species present between the two sectors due to inefficient removal as the samples originated from the same studies (Table S1 and S6). We identified 42 common bacterial species with an abundance of over 0.01% in the influent, activated sludge and effluent with

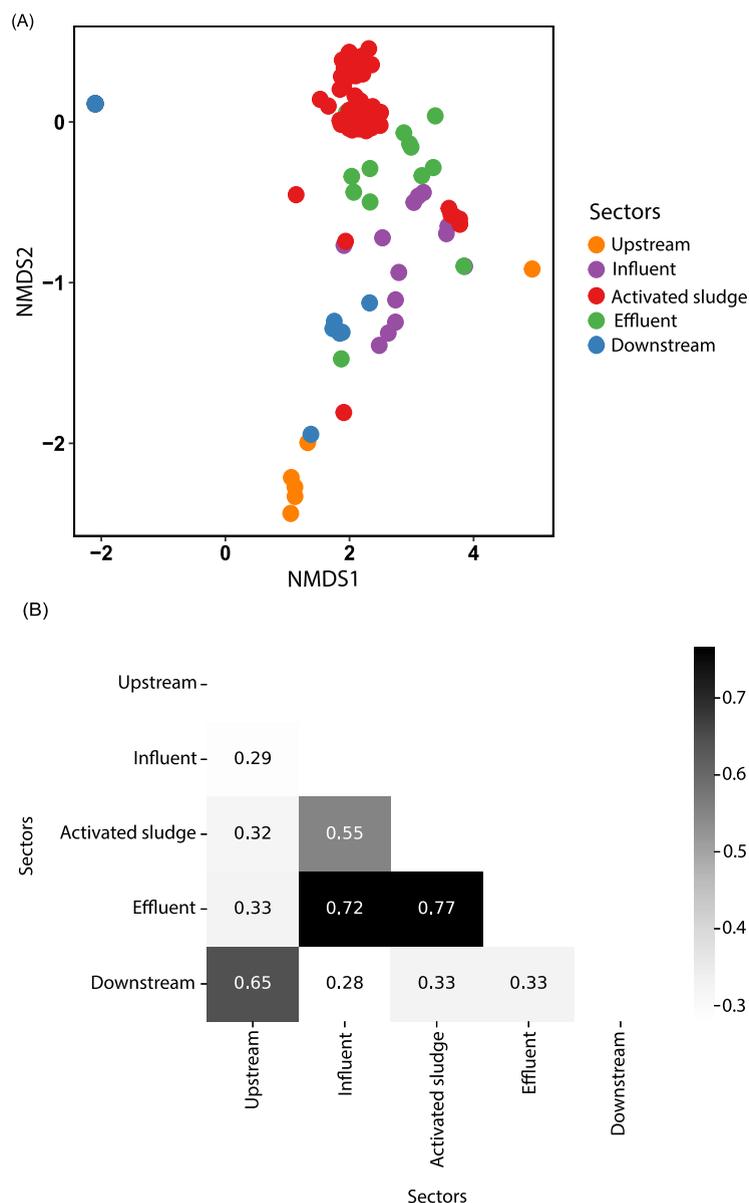


Fig. 4. (A) Nonmetric multi-dimensional scaling ordination (NMDS) of Bray-Curtis dissimilarities of the microbial community between the different sectors of the wastewater treatment plant. The samples for each sector of the wastewater treatment plant are shown in different colours, upstream = orange, influent = purple, activated sludge = red, effluent = green, and downstream = blue. (B) Spearman's correlation analysis of the microbial communities present was performed between each sector of the wastewater treatment plant. The correlation rank values are depicted in text and indicated by the intensity of grey.

Acidovorax sp. *KKS102* being dominant ($\geq 1\%$ abundance). A list of the common bacterial species are present in Table S6. The similar bacterial species identified in the influent, activated sludge and effluent are due to the mixture of aerobic, anaerobic, and facultative bacteria. Even in low abundances, these bacteria can be released with the effluent, contributing to the potential spread of AMR [61].

3.3. Various β -lactamase gene families present in the WWTP

We investigated the average relative abundances of AMR gene families and identified similar AMR gene families throughout the WWTP process. These genes can exist in low abundances, even in treated water, conferring resistance against single or multiple antibiotic drug classes. We highlight two major examples observed in our data i.e., the β -lactam and efflux pump gene families (Fig. 5 and Table S7).

Firstly, β -lactam antimicrobial agents are widely used to treat bacterial infections, but their extensive use has significantly contributed to the global rise of resistance to these antibiotics in bacteria. β -lactamase gene families, including those encoding ESBLs

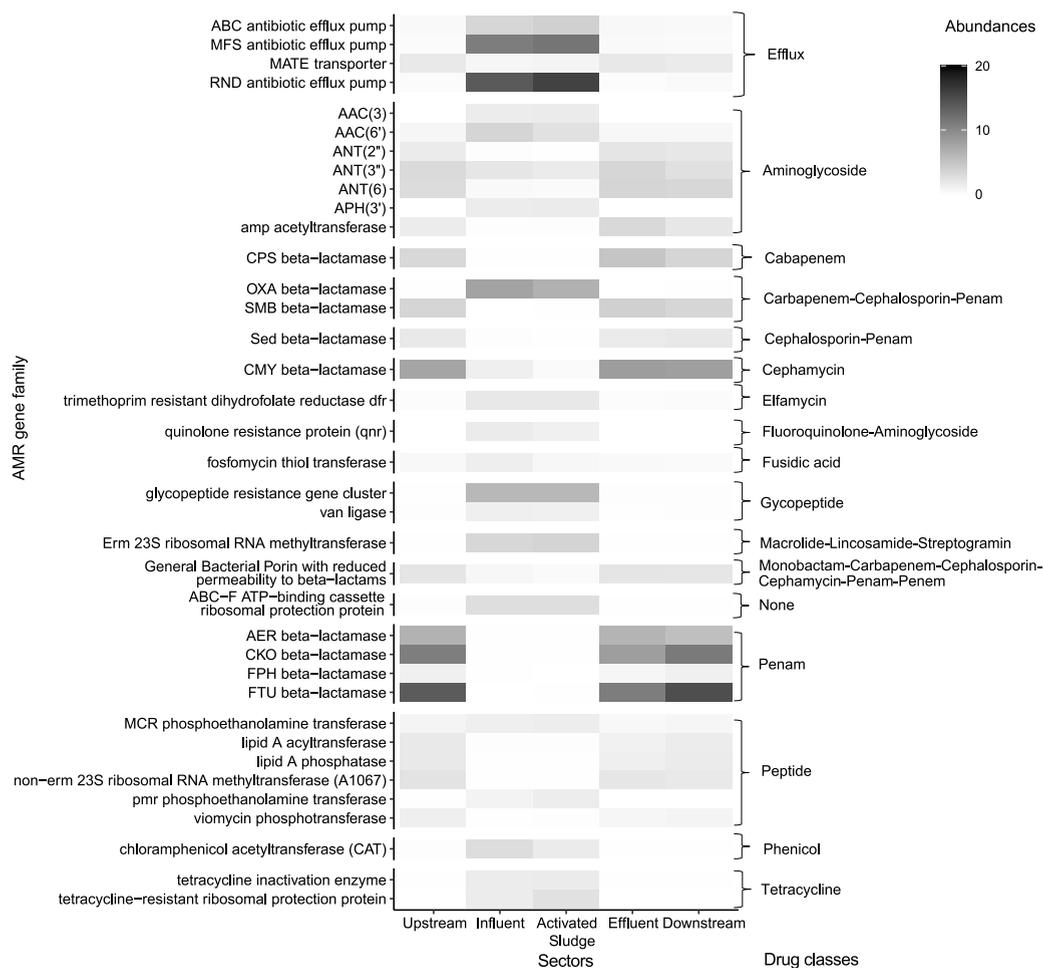


Fig. 5. Heatmap of the abundances of antimicrobial resistance (AMR) gene families over 1% in each sector of the wastewater treatment plant (upstream, influent, activated sludge, effluent, downstream) and corresponding drug classes. Abundances are indicated by greyscale, low = white and high = black.

(extended-spectrum β lactamases), contribute to this resistance by conferring single and multidrug resistance to commonly used penicillins, as well as potent antibiotics such as carbapenems, cephalosporins and penams [62].

Fig. 5 shows several β -lactamase gene families throughout different sectors of the WWTP process. Among these, the OXA β -lactamase gene, associated with the ESBL class D group, was detected across all sectors, constituting 7% of AMR gene families in the influent and 6% in the activated sludge, but less than 0.05% in upstream, effluent, and downstream sectors. Its prevalence was notably high in 100% of upstream samples, over 95% in influent and activated sludge and over 60% in effluent and downstream samples. This variation can be attributed to several factors such as the efficiency of the WWTP in reducing the concentration of β -lactamase gene families therefore lowering its prevalence in the effluent and downstream samples. Environmental factors, such as pH and temperature, may also affect the survival and detectability of these genes. Additionally, differences in sampling methods and detection sensitivity across studies might contribute to the observed variations in the gene prevalence [54,63]. The OXA β -lactamase gene family can be detected in non-pathogenic and environmental bacteria present in aquatic areas and may behave as a reservoir for emerging antibiotic resistance [64]. The OXA β -lactamase gene family can inactivate a variety of β -lactam antibiotics including penicillins, cephalosporins, carbapenems and extended-spectrum penicillins such as piperacillin [65,66]. Additionally, the OXA β -lactamase gene family has been identified in various bacterial species, particularly within the Enterobacteriaceae family and non-fermenting Gram-negative bacteria such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa* [65]. Furthermore, we detected the OXA β -lactamase gene family in the *Pseudomonas*, *Enterobacter*, *Klebsiella* and *Acinetobacter* genera in all sectors of the WWTP (Figure S2).

Conversely, the FTU and CKO β -lactamase gene families, known for conferring resistance to penam antibiotics, were detected predominantly in the upstream, effluent, and downstream sectors. The FTU β -lactamase gene family showed an abundance of over 9% in these sectors, present in more than 14% of upstream and downstream samples and 35% of effluent samples. Similarly, the CKO β -lactamase gene family was present in 45% of effluent samples and less than 14% in upstream and downstream samples. The FTU β -lactamase gene families are categorised as class A β -lactamase which has been identified in *Francisella tularensis* subsp. *holartctica* LVS and produces the FTU-1 enzyme that functions to hydrolyse various β -lactam antibiotics (penicillins and cephalosporins)

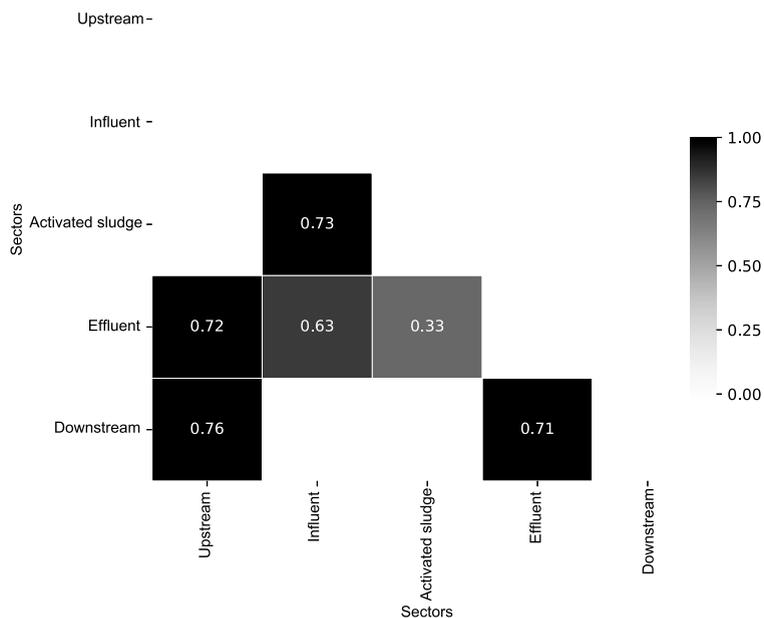


Fig. 6. Visualisation of the Spearman's correlation analysis of the AMR gene families present was performed between sectors that shared samples of the wastewater treatment plant. The correlation rank values are depicted in text and indicated by the intensity of grey.

[67,68]. Similarly, the CKO β -lactamase gene family, identified in *Citrobacter koseri*, produces the CKO-1 enzyme [69]. The presence of both FTU and CKO β -lactamase gene families allows for bacteria to survive by breaking down β -lactam antibiotics rendering them ineffective. Overall, the detection of various β -lactamase gene families across different sectors is concerning as it facilitates the dissemination of multidrug resistance, posing a threat to both clinical treatment effectiveness and environmental health.

Secondly, efflux pump gene families, including major facilitator superfamily (MFS) and resistance-nodulation cell division (RND), play crucial roles in multidrug resistance among pathogens like *Pseudomonas* and *Streptococcus* [70,71,17,72]. The presence of these gene families in the effluent can increase the incidence of bacteria selecting them. Efflux pump gene families can be located on MGEs and can efflux clinically important antibiotics such as tetracycline out of the bacterial cell wall [73,74,17,75].

We identified the MFS and RND efflux pump gene family in every sector of the WWTP (Fig. 5 and Table S7). In our study, the MFS and RND efflux pump gene families comprised 10% - 12% of the total AMR gene families in the influent, 10% - 14% in the activated sludge and less than 0.5% of the total AMR gene families in the upstream, effluent and downstream sectors. The MFS and RND gene families were detected in all upstream samples and 90%, 98%, 70% and 60% of the influent, activated sludge, effluent and downstream samples, respectively. The MFS and RND gene families present in all sectors of the WWTP are common as these genes exist naturally in the chromosomes of bacteria. The MFS efflux pump functions to remove substrates such as antibiotics, sugars and ions out of the bacterial cell leading to cellular homeostasis and antibiotic resistance. Similarly, the RND efflux pump can expel antibiotics, dyes and detergents from the bacterial cell and aids in detoxifying the cell from harmful substances [76,77].

Similar AMR gene families were present in the upstream, effluent and downstream sectors and in the influent and activated sludge sectors as illustrated in Fig. 5 and 6. Overall, the AMR gene families identified in these sectors were positively correlated (Fig. 6). The similarity between sectors could be due to the inefficient treatment of wastewater. However, the correlation had to be calculated within studies potentially confounding regional effects with sectorial effects. Furthermore, the presence of similar AMR gene families that can be found in environmental freshwater systems such as the CMY β -lactamase gene family, leading to high similarity between the AMR gene families found between upstream and effluent samples (0.72) [78]. A full Spearman correlation of sectors that share studies can be found in Figure S3.

A list of common AMR gene families can be found in Table S9. Differences in the abundance and presence of AMR gene families in each sector of the WWTP can be influenced by the sector-specific microbial community and environmental factors i.e., pollutants and organic matter. Additionally, samples from various urban and municipal studies, geographical locations, and time points could have introduced differences in the AMR gene families detected within the WWTP process sectors. However, sectors such as the influent and the activated sludge consist of a mixture of bacteria and pollutants therefore AMR gene families like the OXA β -lactamase gene family may be present in the bacterial genome. This can potentially be exchanged within the microbial population contributing to their high abundance [79]. Bacteria carrying these ARGs have the potential to survive the WWTP and spread AMR to different bacteria in different sectors [80,81,79].

A total of 230 AMR gene families were identified in the WWTP process, providing resistance to 25 single antibiotic drug classes and 32 combination/multiple drug classes. Additionally, the *Pseudomonas* genus harboured numerous AMR gene families including various β -lactamase gene families, yet no single ARG exceeded a 1% abundance. (Refer to Table S7 - S9 and Figures S1 - S2 for detailed data).

Table 1

The average relative abundance (%) of the different classes of integrons in each sector of the WWTP. Integrons were classified as class 1, class 2, class 3, unknown and unclassified in the upstream, influent, activated sludge, effluent and downstream sectors of the WWTP.

Classes of integrons	Upstream	Influent	Activated sludge	Effluent	Downstream
Class 1	17.37%	16.94%	17.39%	19.18%	15.12%
Class 2	2.08%	2.32%	174%	1.92%	1.74%
Class 3	2.87%	3.57%	2.17%	3.92%	2.59%
Unknown	0.00%	0.06%	0.00%	0.00%	0.05%
Unclassified	77.68%	77.11%	78.71%	74.98%	80.50%

3.4. Plasmids with ARGs are released into the environment

We analysed metagenomic assembled contigs from different sectors of the WWTP to determine the percentage of contigs originating from plasmids and their association with AMR gene families. Plasmids, crucial for acquiring and accumulating ARGs, maintain stability, persisting even after wastewater treatment [82].

In each sector of the WWTP, 1% of contigs were classified as plasmid originating. Further analysis showed that AMR gene families were found in 0.008% - 0.15% of plasmid-contigs. More specifically, we observed a high average relative abundance of plasmid-contigs associated with the previously mentioned OXA β -lactamase gene family conferring multidrug resistance. Four other AMR gene families conferring resistance to single antibiotic drug classes were detected in a high abundance (Table S10).

Plasmids associated with AMR gene families like the OXA β -lactamase have a greater chance of spreading AMR from the effluent to freshwater systems and capturing new ARGs. This is a potential route for transmission of multidrug resistant plasmids to other environments such as soil and animals, therefore, leading to resistance to veterinary and clinically significant antibiotics [83,82,84].

In our study, the OXA β -lactamase gene family represented more than 6% of the total plasmids associated with AMR gene families in every sector of the WWTP (Table S10). Plasmids associated with the OXA β -lactamase gene were present in 76%, 71%, 72%, 100%, and 46% of samples from upstream, influent, activated sludge, effluent and downstream, respectively, suggesting that plasmids associated with the OXA β -lactamase gene are released in the effluent and can be detected downstream.

While the presence of plasmids conferring resistance to multiple drug classes is concerning, plasmids associated with a singular antibiotic drug class can spread AMR. In this study, plasmids were associated with AMR gene families of a single antibiotic drug class (tetracycline resistant ribosomal protein (*Tet(O)*), aminoglycoside resistance gene (*ANT(3'')*), trimethoprim resistant dihydrofolate reductase (*dhfr*) and the sulfonamide resistance gene (*sulI*) were detected. While the abundances of plasmids associated with these AMR gene families vary in each sector of the WWTP, their presence can stimulate HGT (Table S10) [85–88,41,89].

Plasmids carrying ARGs can nullify the effectiveness of commonly used antibiotics. The presence of antibiotic residues in the effluent and downstream freshwater environments further enhances the mobility and stability of plasmids. This promotes induced resistance and genetic exchange among bacterial communities through plasmids and other MGEs as documented in studies by Rahube et al. [90], Wang et al. [81] and Calderón-Franco et al. [91].

3.5. Class 1 integrons reveal that AMR pollution is present in each sector around the WWTP

We investigated the presence of integrons and their association with ARGs in each sector of the WWTP. Integrons are responsible for AMR dissemination via HGT, which can occur between bacteria in all environments. In this study, we searched for integrons in contigs from each sector of the WWTP and classified them as classes 1, 2, 3, unknown, and unclassified (Table 1).

In our study, less than 1% of contigs (upstream: n = 1779, influent: n = 6858, activated sludge: n = 16137, effluent: n = 4104, downstream: n = 2010) were integrons. We classified the integrons into class 1, class 2, class 3, unknown and unclassified. Almost 80% of detected integrons remained unclassified. Class 1 and 3 integrons, commonly found in Proteobacteria across freshwater, soil, and biofilm environments, hold clinical significance, while class 2 integrons are prevalent in marine Gamma-Proteobacteria. Table 1 shows class 1 integrons were the most abundant, ranging from 17-19% in each sector of the WWTP, while class 2 and 3 integrons accounted for 2-4%. All classes were present in over 70% of samples in all sectors. The presence of class 1 integrons in all sectors of the WWTP is similar to the findings of Makowska et al. [92].

Class 1 integrons are associated with disinfecting agents and sulfonamide resistance [93]. We detected class 1 integrons with *qacE*-related disinfectant resistance genes throughout the WWTP sectors, whereas those carrying *sulI* and *sul3* genes for sulfonamide resistance were more abundant (Table S11). Specifically, 31% of class 1 integrons in the upstream sector were linked to the *sul3* gene. The *sulI* gene was absent in the upstream sector but made up more than 25% of the class 1 integrons present in the influent, activated sludge, effluent, and downstream sectors. The *sulI* and *sul3* genes, associated with class 1 integrons, were detected in over 90% of samples from the influent, activated sludge, and effluent sectors. In the downstream sector, approximately 60% of samples contained the *sulI* gene and 20% contained the *sul3* gene.

Class 1 integrons in combination with *qacE* and *sulI* act as a pollution marker and are commonly present in sewage areas like the influent [94]. In our study, sulfonamide resistance associated with class 1 integrons was high in the influent, activated sludge, effluent and downstream sectors. These sectors are prone to increased antibiotic resistance due to various factors, such as pollutants, disinfectants, organic matter, and the bacterial community present. Since sulfonamide antibiotics can persist in the environment for extended periods, the *sulI* gene will be frequently detected in the effluent, activated sludge, influent, and receiving waters (Table S11).

Table 2

The total number of each integrative element detected in each sector of the wastewater treatment plant including the total number and percentage (%) of each integrative element carrying antibiotic resistance genes (ARGs). Integrative elements include integrative and conjugative elements (ICE), integrative and mobilizable elements (IME) and cis-integrative and mobilizable elements (CIME).

Integrative elements	Upstream	Influent	Activated sludge	Effluent	Downstream
Total ICE	4830	27796	7585	14129	6129
ICE with ARG	544 (11%)	723 (3%)	924 (12%)	597 (4%)	165 (3%)
Total IME	4101	31190	35919	15930	4313
IME with ARG	312 (8%)	871 (3%)	1645 (5%)	390 (2%)	217 (5%)
Total CIME	487	3400	1458	264	146
CIME with ARG	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

While we observed the presence of class 1 integrons in the downstream sector, these integrons are naturally prevalent in freshwater systems as well [93,95,96].

3.6. Integrative elements are linked to ARGs in each sector of the WWTP

We identified integrative elements i.e., integrative and conjugative elements (ICEs), integrative and mobilisable elements (IMEs) and cis-integrative and mobilisable elements (CIMEs) in each sector of the WWTP. Since these self-transmissible elements can carry ARGs, we also determined if the integrative elements detected were linked to the ARGs previously identified in this study.

Integrative elements, comprising approximately 1% of contigs, were detected across all sectors of the WWTP, with varying average relative abundances ranging from 16.87% in activated sludge to 57.89% downstream (Table 2). Contrary to Calderón-Franco et al. [97], in our study, 80% of the integrative elements in activated sludge were IMEs.

ICE families linked to bacterial adaptation were identified in the WWTP. The ICE_{clc} and Tn4371 families were prevalent in over 95% of all samples across WWTP sectors, with similar abundances observed in each sector (Table S12). The ICE_{clc} family facilitates bacterial adaptation by transporting and transferring ARGs between bacterial species including *Aeromonas* and *Pseudomonas*, as detected in this study. ICE_{clc} utilises compounds like chlorocatechols, present in both polluted and freshwater environments to enhance bacterial proliferation, thereby promoting horizontal gene transfer [98]. Similarly, Tn4371 elements were widespread throughout the WWTP process, constituting approximately 40% of the ICE families in each sector. Tn4371 is commonly found in aquatic environments and man-made settings like sewage and industrial waste. Following processing in wastewater treatment plants, Tn4371 continues to harbour a significant load of ARGs [99,100]. Both ICE families, prevalent in clinical and veterinary sectors, maintain the stability of accessory genes like ARGs within-host bacterial species.

IMEs were detected in every sector of the WWTP, and PGI2 was present in over 90% of samples across all sectors, showing consistent abundances (Table S14). Like other MGEs found previously, IMEs can carry ARGs post-WWTP processing, contributing to bacterial multidrug resistance. PGI2, a genomic island characterised in a previous study, can harbour 14 different resistance genes, highlighting its role in promoting bacterial evolution towards multidrug resistance [101].

Lastly, we assessed if the integrative elements are associated with previously detected ARGs. In the upstream and activated sludge sectors, ICEs were found to carry ARGs in 11% and 12% of detected ICEs respectively. Additionally, Table 2 shows that 8% of detected IMEs are linked to ARGs. Bacteria carrying both integrative elements and ARGs have a greater potential to spread infection and can survive high concentrations of antibiotics. This leads to the spread of AMR to other environments from the downstream sector, potentially causing untreatable infections when reaching animal and human communities [96,12,17]. Details on the specific ICE families and IMEs carrying ARGs can be found in Tables S13 and S15.

4. Conclusion

We conducted a comprehensive analysis of publicly available metagenomes representing the different sectors of the WWTP (upstream, influent, activated sludge, effluent and downstream) to identify the microbial composition, antibiotic resistance genes and mobile genetic elements. Data from different geographical locations (Portugal, China, Spain, South Korea and Colombia) were integrated to provide a more holistic view of AMR in the urban/municipal WWTP.

Our study revealed the persistent presence of environmental bacterial genera across the WWTP, identifying *Pseudomonas*, *Aeromonas*, and *Acidovorax*. These genera include (opportunistic) pathogens that pose health risks to humans and animals. Additionally, we detected the presence of all six ESKAPE pathogens across the sectors. Specifically *Klebsiella pneumoniae* and *Enterobacter* sp. was detected with a higher abundance in the effluent sector. *Escherichia coli*, a faecal contaminant indicator, had a higher abundance in the effluent compared to other sectors.

Analysis of ARGs revealed various β -lactamase gene families throughout the sectors of the WWTP however, the OXA β -lactamase gene family is particularly prominent in the influent and activated sludge sectors while lower in the effluent and downstream suggesting that the reduction of ARGs is possible. The OXA β -lactamase gene family was also linked to more than 5% of the ARG-carrying plasmids detected indicating that ARGs can be selected for and transferred to other bacterial species. Integrons and integrative elements were identified in every sector of the WWTP process and linked to the previously detected ARGs further highlighting their role in the persistence and spread of AMR within microbial communities within the WWTP and its receiving environments, humans and animals.

This study underscores the significance of exploring the spread of MGEs and ARGs alongside associated bacterial species in non-clinical environments like WWTPs. Understanding these connections between environmental reservoirs, pollution sources, and human/animal exposure is crucial. However, the current study is limited by using public data from various WWTP studies. Although studies were based on sequencing technology, the type of WWTP, such as urban and municipal, as well as the sampling locations — upstream, influent, activated sludge, effluent, and downstream — differences in the use of public data can introduce biological variation in the types of bacteria and AMR genes detected.

Future research should take into account that upstream and downstream sectors are essential for the analysis of AMR in the WWTP and can provide information on HGT from receiving waters to other non-clinical environments. Additionally, geographical factors such as climate, local microbiome, industrial practices, and local regulations can influence the growth of microorganisms, types of AMR gene families detected, and MGEs and should be included in AMR research. Variations in influent and effluent may arise due to anthropogenic activities such as chemical and antibiotic usage, animal populations, vegetation, and pollutant discharge. Accounting for these factors is important in determining the full effect of WWTPs in managing AMR and environmental pollution [102–104].

CRedit authorship contribution statement

Stephanie Pillay: Writing – review & editing, Writing – original draft, Investigation, Data curation, Conceptualization. **Ramin Shiral Hosseini Zade:** Writing – review & editing. **Paul van Lent:** Writing – review & editing. **David Calderón-Franco:** Writing – review & editing. **Thomas Abeel:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization.

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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.

Appendix A. Supplementary material

Supplementary material related to this article can be found online at <https://doi.org/10.1016/j.heliyon.2025.e42919>.

Data availability

These datasets generated and/or analysed during the current study are available in the NCBI SRA repository.

- PRJNA532515: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA532515/>
- PRJNA505617: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA505617/>
- PRJNA648659: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA648659/>
- PRJNA432264: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA432264/>
- PRJNA377521: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA377521/>
- PRJNA298467: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA298467/>
- PRJNA506137: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA506137/>

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