Impact of Wastewater Effluent on Surface Water Resistomes in a Small Lower-Income Community with Deteriorated Sewage Infrastructure

Gabriel E. Maldonado Rivera

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of
Master of Science
in
Environmental Science and Engineering

Amy J. Pruden-Bagchi, Chair
Peter J. Vikesland
Alasdair G. Cohen
Benjamin C. Davis

February 17, 2025

Blacksburg, VA

Keywords: Antimicrobial Resistance, Rivers, Wastewater Treatment Plants, Metagenomics, ESBL *Escherichia coli*

Impact of Wastewater Effluent on Surface Water Resistomes in a Small Lower-Income Community with Deteriorated Sewage Infrastructure

Gabriel E. Maldonado Rivera

Abstract

Antimicrobials are known for their lifesaving benefits, but their widespread use has increased selective pressure favoring the development of resistant bacteria and antibiotic resistance genes (ARGs). Consequently, this has led to a global antimicrobial resistance (AMR) crisis associated with an estimated 4.71 million deaths globally in 2021. Due to inputs of antimicrobials and antibiotics excreted and flushed down the drain, nutrient rich conditions, and high active microbial density, wastewater treatment plants (WWTPs) are hypothetical hotspots for the evolution and dissemination of AMR, but also function as a crucial checkpoint for restricting discharge of resistant bacteria, ARGs, and other pollutants into the environment. In this study, we evaluated the levels of ARGs and antibiotic-resistant bacteria in wastewater influent, wastewater effluent, and surface water samples collected upstream and downstream of a deteriorating WWTP serving a small, lower-income, community in a rural region of Virginia. Samples were collected monthly over a 12-month period. Multiple AMR indicators were assessed by bacterial culture, droplet digital polymerase chain reaction (ddPCR), and DNA-sequencing. A significant difference was observed in the relative abundance of total ARGs between upstream and effluent samples ($p = 1.9x10^{-5}$), whereas no significant differences were observed between effluent and downstream ($p = 0.16$ or upstream and downstream ($p = 0.21$). Across all sampling dates, the WWTP achieved a 56% reduction in relative abundance of total ARGs. Analysis of unique ARGs by sampling site revealed that, despite an overall reduction in distinct ARG types` across the treatment process, the downstream site still harbored substantially

more unique ARGs (n=402) compared to the upstream site (n=106). Furthermore, most of these downstream-specific ARGs (n=272) were also detected in the effluent. Positive trends between precipitation and ARG abundance in effluent and downstream river samples were observed, though none were statistically significant. This suggests that while precipitation may play a role in ARG composition, its influence varies across different water matrices and other environmental factors. ddPCR enumeration of *bla*CTXm, an ARG that encodes resistance to a broad spectrum of beta lactam antibiotics and is of key clinical concern, displayed the same trend as the total ARG relative abundance. Specifically, *bla*CTXm, was significantly higher downstream than upstream of the WWTP. Culture-based enumeration of cefotaxime-resistant *Escherichia coli* confirmed occasional elevated levels in the WWTP effluent discharged to the river, though there were also upstream sources. Whole genome sequencing of resistant *Escherichia coli* and carbapenem-resistant bacteria provided a means to assess the range of strains encountered and can be compared with putative sources and clinical data in future studies. These findings provide valuable insights into the role of a deteriorating WWTP in the dissemination of AMR within rural, low-income community. This study underscores the importance of maintaining the integrity of wastewater infrastructure for protecting water quality and public health.

Impact of Wastewater Effluent on Surface Water Resistomes in a Small Lower-Income Community with Deteriorated Sewage Infrastructure

Gabriel E. Maldonado Rivera

GENERAL AUDIENCE ABSTRACT

Antibiotics were discovered about 100 years ago and now are used widely to treat and prevent a wide range of potentially deadly bacterial infections, including urinary tract infections, pneumonia, skin infections, and blood infections. However, over time, bacteria have evolved the capability to resist antibiotics, and their use favors the survival of drug-resistant bacteria, making infections harder to treat. This global health crisis contributed to an estimated 4.71 million deaths globally in 2021. This has sparked scientific interest in trying to understand where antibiotic resistant bacteria come from and how they spread and cause infections in people.

One potential source of antibiotic-resistant infections is the environment, for example, contaminated soil, water and air. When humans or animals consume antibiotics, they are not broken down completely and ultimately are excreted into wastewater. Resistant gut bacteria that survive antibiotic treatment will also be released. Ultimately, wastewater is routed through sewer networks to wastewater treatment plants (WWTPs). WWTPs provide essential infrastructure for treating water before it is returned to the environment. This research was motivated by the need to assess the extent to which WWTPs help to remove antibiotic resistant bacteria treated effluent into prior to discharge to waterways. Further, it is important to evaluate WWTPs not only when they are functioning properly, but also when these systems are experiencing issues.

In this study, we examined how well a deteriorating WWTP serving a rural, low-income community was able to remove antibiotic resistant bacteria and their DNA from wastewater. We also assessed whether levels of antibiotic-resistant bacteria and their DNA were higher downstream of the WWTP discharge point than upstream. We collected water samples from the plant influent, effluent, and from a nearby river both upstream and downstream of the plant. Our findings showed that while the WWTP reduced the total number of antibiotic resistance genes (ARGs) found in the DNA sequences by 56.3%, the downstream river still contained significantly more unique ARGs than the upstream site. This suggests that the treated wastewater contributes to the dissemination of antibiotic resistance into the environment. We also explored whether rainfall influenced ARG levels, but no strong patterns were found. Finally, we sequenced the DNA of isolates of antibiotic-resistant bacteria to support future studies aimed at tracking their sources. The overall results highlight the importance of maintaining and upgrading WWTP infrastructure to help prevent the spread of antibiotic resistance. Addressing this issue would be crucial to safeguarding public health, particularly in underserved areas where resources for wastewater management may be limited.

ACKNOWLEDGEMENTS

This research was funded in part by the US National Science Foundation Research Traineeship (NSF NRT) 2125798, the Appalachian Regional Commission (ARC), the VT Post-baccaluareate Research and Education Program (PREP) NIH R25 grant to Virginia Tech and the Virginia Tech College of Engineering New Horizons Program.

To my advisor Dr. Amy Pruden- for the opportunity to be part of this amazing group of people and exciting research. Thank you, Amy, for your patience and guidance over the last 3 years.

To Amanda Darling, Clayton Markham, Madeline Deck, Amber Amaral-Torres, Thomas Byrne and Sarah Price- for the countless nights spent in the lab working together and collecting samples. A special group of people that made the extensive lab processing go by fast.

To my family- for always believing in me and giving me that much needed support through grad school.

TABLE OF CONTENTS

Table of Contents

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: Introduction

1.1 Antimicrobial Resistance

Modern medicine was transformed by Alexander Fleming's discovery of the first antibiotic, penicillin. This breakthrough led to a surge in the discovery and production of new antimicrobials as life-saving drugs for curing and preventing bacterial infections, improving survival rates worldwide. Consequently, increased production and use of antimicrobials also increases selective pressure, favoring the survival of microbes that are drug resistant. Correspondingly, an increase in the development of antimicrobial resistance (AMR) among microorganisms has been observed (Smalla et al., 2018). This rise of AMR has turned into a crisis, with an estimated 4.71 million reported human deaths associated with bacterial AMR infections in 2021. Antimicrobials include antifungals, antivirals, and antibiotics, with the latter specifically killing or inhibiting bacteria. AMR develops when an organism acquires genes, i.e., antimicrobial resistance genes (ARGs), that encode capabilities that allow them to thrive and spread in the presence of antimicrobials. This happens when the organism acquires an ARG from another microorganism via horizontal gene transfer (HGT), or through mutation and evolution in function of an existing gene. HGT is of special concern because it allows bacteria to share their ARGs with each other (Gaze et al. 2011), calling for strategies that not only contain resistant bacteria, but their genetic material as well.

1.2 Wastewater Treatment Plants and AMR

Wastewater treatment plants (WWTPs) in particular serve as potential hot-spots for the evolution and spread of AMR due to their nutrient rich and diverse microbial environment (Smalla et al., 2018), but also as an important node for controlling the release of AMR to the environment. Many of the treatment processes employed at WWTPs, including primary settling, activated sludge, and disinfection, also aid in reducing antibiotic resistant bacteria (ARB) and ARGs (Ashbolt et. al., 2019). However, removal rates can vary among WWTPs due to a variety of factors, including the wide range of treatment processes employed and whether they are functioning according to design. A fundamental concern is whether ARB of clinical relevance persist in WWTP effluent, are released into the environment, and serve as a source of exposure (Marutescu et al., 2023). Despite influent wastewater undergoing treatment processes for the removal of contaminants, the extent of ARB removal in effluents varies among WWTPs (Łuczkiewicz et. al 2010). While well-functioning WWTPs have been found to generally remove ARB and ARGs by one to several logs (Garner et al., 2024), concerns especially arise when the WWTP is not functioning as designed.

Table 1. Removal concentrations of AMR-relevant genes in wastewater matrices

Gene	*Function*	Log Removal						
		Sedimen-tation	**Anaerobic Tank**	**Anoxic Tank**	**Aerobic Tank**	**Primary Clarifier**	**Secondary Clarifier**	**Chlorination**
16s rRNA	Total bacteria marker	-0.20^{f}	-2.041^{d}	0.067^{d}	-0.319^{d}	-0.074^{d}	2.097^{d}	0.513^{b}
Sul	ARG enconding sulfonamide resistance	$-0.20^{f}*$	$0.362^{c}*$	$0.426^{e}*$	$-0.368^{e}*$	$0.171^{c}*$	$0.570^{c}*$	0.327^{b}
intI1	Class I integron that facilitates HGT of ARGs	0.625^{a}	0.468^{c}	0.359^{e}	-0.535^{e}	0.447^{c}	0.643^{c}	0.689^{a}

References: a. (Di Cesare et al., 2016), b. (Mao et al., 2015), c. (Li et al., 2016), d. (Luo et al., 2014), e. (Du et al., 2015), f. (Gao et al., 2012)

*sul1

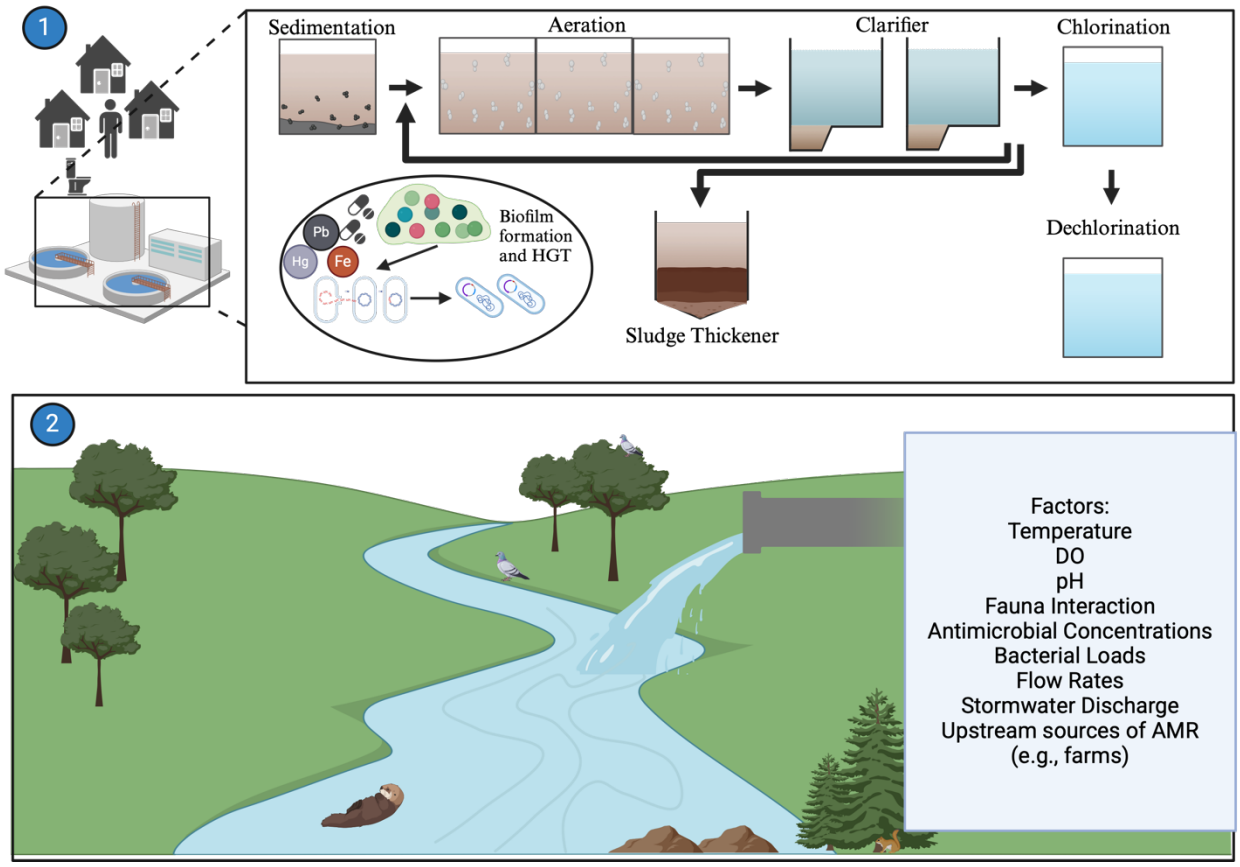


Figure 1 Fate of ARB and ARGs through a typical WWTP; 2; Surface water interactions and factors that affect ARB and ARG transport downstream. Created in BioRender. Maldonado Rivera, G. (2025) https://BioRender.com/l37w345

Proper operation of WWTPs is essential for protection of public and environmental health. However, not all WWTPs consistently operate as designed. In the U.S., the Environmental Protection Agency (EPA) reported in 2023 that approximately 10 % of WWTPs received a violation of the Clean Water Act National Pollutant Discharge Elimination System (NPDES) (EPA 2024). These violations occur due to overloading of the system, chemical imbalances, power failures, and other issues.

ARB in WWTP effluents can potentially be a source of dissemination of ARGs among interconnected aquatic systems (e.g., surface waters) (Allen et al., 2010). Small and rural

WWTPs are prone to higher rates of violations due to aging infrastructure and limited access to basic wastewater services. Among these violations, there is the widespread issue of discharge of untreated wastewater due to sanitary sewer overflows (SSOs) of WWTPs across the US (EPA NPDES Compliance). A primary driver of this problem is infiltration and inflow (I&I), which occurs when excessive rainfall and groundwater seep into the sewer system through cracks or poorly connected sewer lines, leading to an increased hydraulic load on the WWTP (EPA NPDES Compliance). Proper management and operation of the facilities, along with ensuring timely maintenance, are essential for preventing untreated discharge to the environment. However, sustaining proper WWTP operations in these communities is constrained by several factors, including financial resources, challenging rural and topographic conditions and deteriorating sewer systems. Therefore, measuring outputs of AMR from WWTPs that are struggling to meet discharge regulations can provide valuable information in terms of estimating potential exposures associated with the receiving aquatic environment.

1.3 One Health & AMR: Environmental Dimensions

AMR is increasingly being viewed as a One Health (i.e., people, animals, environment) challenge (Fig. 2), recognizing their essential use in human health and veterinary medicine (Topp et al., 2023), but also that their overuse and misuse can exert selective pressure favoring survival of resistant microbes and contribute to their spread in the environment (e.g., soil, air, water) (Larsson & Flach, 2022). Recent research has focused on the need to understand the evolution and spread in aquatic environments in particular (Bürgmann et al., 2018). This is especially important to consider because environmental bacteria, which can act as reservoirs for ARGs, which can eventually be transferred into pathogens capable of affecting humans and animals alike (Robinson et al., 2016). The rate that this occurs can be aggravated by anthropogenic

contamination, such as human waste, hospitals, pharmaceutical industry and livestock (Ben et al., 2017; Larsson et al., 2007; Reinthaler et al., 2003). In particular, accumulation of antimicrobials and other pollutants in the environment is thought to facilitate the spread of ARGs among bacteria. Antibiotic contamination in wastewater systems has the potential to promote mutations leading to the evolution of ARGs among affected bacteria (Kümmerer, 2009). Many antibiotic pollutants are resistant to biodegradation resulting in their persistence after wastewater treatment processes (Rodriguez-Mozaz et al., 2015). This results in low concentrations of antibiotics in treated waters, which prompts the production of metabolites that can facilitate horizontal gene transfer. Thus, aquatic environments, especially WWTPs, are an important environment to study (Hanna et al., 2023; Purohit et al., 2017).

Figure 2. One Health Dimensions Across Sectors: Human, Animal, and Environmental Health. Created in BioRender. Maldonado Rivera, G. (2025) https://BioRender.com/r19j175

1.4 Environmental Bacteria & AMR

Exposure to ARB and ARGs in the environment can occur by coming into direct contact with contaminated recreational waters through activities such as canoeing, kayaking, swimming, and fishing (Gaze et al 2017). Efforts are underway to begin to develop risk assessment models for quantifying AMR-associated risks of exposure to environmental waters, such as stormwater (Hamilton et al., 2020). Important to informing such risk assessment efforts is surveying AMR in surface water, determining concentrations, and identifying likely sources. Recently, a study was conducted by the US Environmental Protection Agency as part of their National Streamwater Assessment program, wherein the measured a suite of ARGs by quantitative polymerase chain reaction (qPCR), including: *sul*1, *tet*(W), *bla*TEM and the class 1 integron, *int*I (Keely et al., 2022). It was found that the magnitudes of these genes encountered in streams was strongly related to pollution sources, including upstream WWTPs and agricultural operations. Effective monitoring of this nature can aid in better understanding the factors contributing to the spread of AMR, as well as to inform early warning efforts and public health actions to benefit humans and animals (CDC 2019).

1.5 Objective of this Thesis

The primary objective of this thesis was to quantify the concentrations of ARGs and ARB in wastewater influent, wastewater effluent, and surface water samples collected upstream and downstream of a deteriorating WWTP serving a rural, low-income community. This analysis aimed to measure the contribution of AMR to downstream surface waters under a hypothetical worst-case scenario. This was addressed via profiling various indicators of AMR in the influent

and effluent of the WWTP as well as upstream and downstream in the river where it discharges. Relative to prior studies wherein only a few ARGs were able to be examined via qPCR, a strength of this study is that both culture and DNA-sequencing based methods were applied. Specifically, we measured cefotaxime resistant *Escherichia coli* (cef-resistant) using recently published standard methods for AMR monitoring of aquatic environments (Calarco et al., 2024) and carbapemase-producing organisms using an exploratory method. Whole genome sequencing of isolates was carried out to examine evidence of WWTP effluent as a source of downstream ARB. Shotgun metagenomic sequencing was also applied to provide a comprehensive profile of ARGs carried across the microbial communities (i.e., the "resistome") representative of the influent, effluent, upstream, and downstream samples. To capture a range of variability in the dataset and influence of a range of factors (e.g., season, weather, WWTP operating conditions), samples were collected monthly over a 1-year sampling campaign.

The work presented in this thesis constitutes a part of a larger team project aimed at tracking pathogens and antibiotic resistance in a small, rural sewershed experiencing excessive levels of inflow and infiltration (I&I). Related works have been published elsewhere (e.g., Cohen et al., 2024; Darling et al., 2025). Given that the work presented in this thesis represents a team effort, it is envisioned that a multi-authored peer-reviewed manuscript will be adapted for publication based on this thesis. The following is the attribution statement of the co-authors of this manuscript in preparation representing the research presented in this thesis (alphabetical by last name):

Amber Amaral-Torres- PhD student supporting this research. Assisted in collecting and processing samples.

Thomas Byrne- PhD student supporting this research. Assisted in the collection and processing of samples.

Alasdair Cohen- PI and co-PI of sponsored projects supporting this research. Committee member of G. Maldonado Rivera. Established the study site and assisted in directing the research plan, advising analysis of data, reviewed and edited the thesis.

Amanda Darling- Lab member leading and supporting this research. Assisted in directing sampling plan, advising and collecting samples.

Benjamin Davis- Committee member of G. Maldonado Rivera. Assisted in directing the research plan, advising analysis of bioinformatic and other data, reviewed and edited the thesis.

Madeline Deck- MS student supporting this research. Assisted in the collection of samples and processing.

Gabriel Maldonado Rivera- Author of thesis

Clayton Markham- PhD student supporting this research. Assisted in the collection and processing of samples.

Sarah Price- PhD student supporting in the collection and processing of the samples.

Amy Pruden- PI and co-PI of sponsored projects supporting this research. Primary advisor of G. Maldonado Rivera. Assisted in directing the research plan, advised analysis of data, reviewed and edited the thesis.

Peter Vikesland- co-PI of sponsored projects supporting this research. Committee member of G. Maldonado Rivera. Assisted in directing the research plan, advising analysis of data, reviewed and edited the thesis.

CHAPTER 2: Materials and Methods

2.1 Sampling region, collection and preservation

To examine the impact of WWTP effluent on the resistome of receiving waters, a small (0.5 MGD) WWTP located in a low-income rural US community and the corresponding receiving river were subjected to a monthly sampling campaign over the course of 1-year. The WWTP was designed to have a sedimentation tank, an aeration tank consisting of an anaerobic/anoxic/aerobic system, two secondary clarifiers, chlorination, dechlorination and a sludge thickener tank. The system has experienced difficulty maintaining the aeration tank and keeping the primary clarifier working as designed. There were also I&I problems with heavy rain events due to the deteriorating infrastructure (Darling et al., 2025).

24-hour composite samples using an ISCO 6712 (Teledyne Inc. Louisville, KY) were collected for the 4 sites. For each sampling round, 3-L composite samples were collected for effluent discharge, river upstream and river downstream in a 24-hour period between 6 am and 8 am using 1-L sterile bottles. River samples were collected upstream and downstream of the wastewater effluent discharge point. After collection, all samples were placed on ice for preservation and transport. All samples were transported directly back to the laboratory, on ice, and processed within 12 hours of sampling. Temperature, pH, dissolved oxygen were measured on site with a wastewater multimeter for all samples. Precipitation was measured by the WWTP the day of and 5 days prior to sampling.

2.2 Enumeration of Total and Cefotaxime-resistant *E. coli*

Upon arrival at the laboratory, samples were immediately placed on ice and processed within 12 hours, well within the 48-hour window previously validated for this protocol (Liguori et al., 2023). Culture methods were adapted from the Tricycle protocol by the World Health Organization (WHO) and EPA Method 1603 for *E. coli* (Calarco et al., 2024). For culturing and isolating *E. coli*, vacuum filtration was performed using sterile Nalgene Reusable Filter funnels with a 0.45 µm nitrocellulose filter under a biosafety level II cabinet. Filters were manipulated using flame sterilized forceps and were placed on filter funnels grid side up. Filtration amounts/dilutions were guided following suggested volumes (Table#) and filtered until clogging. The actual volume filtered was recorded, given that the sampling volume and turbidity varied across sampling time point. Filters bearing the concentrated samples were placed on *E. coli* selective modified mTEC agar (BD Difco Cat No. B14880) and modified mTEC with 4 µg/mL cefotaxime (Millipore Sigma Product No. 219504; Fisher Sci Cat No. AC454950010). All samples were plated in technical duplicate and at three target dilutions. Subsequently, plates were incubated for 2 hours at 37 ℃ and moved to a water bath at 44.5 ℃ for 22 ± 2 hours. Once the incubation period concluded, magenta colonies were counted. Colony counts were averaged between replicates and CFU/100 mL was calculated (Equation 1).

$$\frac{E.coli}{100mL} = \frac{Average\ E.coli\ colonies\ at\ countable\ dilution}{Volume\ of\ sample\ filtered\ (mL)} \times 100 \qquad \textit{(Equation 1)}$$

Table 2 Target Filtration Amounts for E. coli and Cef-resistant E. coli for Each Sample

Sample	**Target**	**Filtration Amount & Dilutions** *(mL)*
WWTP Influent	*E. coli*	1 at $10^{-2}, 10^{-3}, 10^{-4}$
	Cef-resistant *E.coli*	1 at $10^{-1}, 10^{-2}, 10^{-3}$
WWTP Effluent	*E. coli*	500
	Cef-resistant *E.coli*	1000
Surface Water Upstream	*E. coli*	500
	Cef-resistant *E.coli*	1000
Surface Water Downstream	*E. coli*	500
	Cef-resistant *E.coli*	1000

2.3 Isolation on mSuperCARBA CHROMagar

To assess the presence of carbapenem-resistant Enterobacteriaceae (CRE) in our samples, an exploratory method was employed using mSuperCarba CHROMagar (CHROMagar^{TM}, La Plaine Saint-Denis, France). The method followed the same protocol as above for the enumeration of cefotaxime-resistant *E. coli,* but instead substituted the use of commercial MSuperCarba media, which is commonly used in clinical practice but not yet validated for environmental samples. MSuperCarba is formulated to target Enterobacteria exhibiting resistance to carbapenem antibiotics. After placing filters on agar plates, the plates were incubated at 36 ℃ for 22 ± 2 hours. Following the incubation period, colonies representing distinct morphological features were selected and re-streaked onto the same agar medium. This process was repeated until uniform and isolated colonies were observed. Bacteria glycerol stocks were prepared and stored following the same protocol described in the previous section.

2.4. Isolation of Pure Cultures

A subset of cef-resistant colonies were selected for isolation and were restreaked at least twice on fresh plates to obtain selected colonies. Well-isolated colonies were picked and transferred to 5 mL of LB Broth (Lennox) and incubated overnight at 37 ℃ while shaking. Isolates were presevered for storage by aliquoting 500 µL of bacterial culture into 500 µL of 50% sterile glycerol. The glycerol stock was stored at a -80 ℃ freezer until further processing.

2.5 DNA Extraction for Molecular Analyses

Aqueous samples were concentrated for DNA extraction via vacuum filtration using sterile Nalgene reusable filter units. Following the same 'filtration until clogging' protocol using 22-µm membrane filters noted above for the culture-based analysis of samples. With sterilized forceps, the filter containing the concentrated sample was folded and placed into 2-mL microcentrifuge tubes containing 100% ethanol. Filters were stored in -80 ℃ until further processing. All samples were DNA extracted using the FastDNA Spin Kit for Soil (MPBio, Solon, OH) following the manufacturer's instructions.

2.6 Digital droplet polymerase chain reaction (ddPCR)

ddPCR analyses were conducted using Bio-Rad's QX200^{TM} Droplet Digital PCR System (Bio-Rad, Hercules, CA, USA). Assays targeting the integrase gene of the class 1 integron, intI1, beta-lactamase-encoding gene, CTXM-1, and the universal bacterial 16S rRNA gene were conducted. Primers, probes (IDT, Coralville, IA, USA) and thermocycle optimized conditions used for each assay are presented in Supplementary Table S3. Generated droplets were then

placed on a QX200 Droplet Reader (Bio-Rad, Hercules, CA, USA). Droplets were deemed positive if there were at least three positive droplets. Amplitude thresholds for each assay were manually placed at one third of the distance between the positive and negative cluster.

2.7 Metagenomic Sequencing and Analysis

For molecular profiling of microbial population and resistomes across samples, we first quantified DNA using the Qubit dsDNA High Sensitivity Assay Kit on a Qubit Fluorometer (Invitrogen, Carlsbad, CA). Subsequently, the samples were overnight shipped on dry ice to the Duke Center for Genomic and Computational Biology. Library preparation was conducted using Nextera Flex (DNA Prep) 1/8 reactions and the resulting libraries were sequenced on the Illumina NovaSeq 6000 platform, generating approximately 18 Gb per sample. Upon receiving sequences, the paired fastqs were quality checked using FastQC (Andrew S.,2010) and trimmed and filtered using fastp (Chen, 2023). Clean reads were taxonomically annotated with Kraken2 (Wood et al., 2019) and the relative abundance at each taxonomic rank was estimated using Bracken (Lu et al., 2017). Sequences were then aligned to the Comprehensive Antibiotic Resistance (CARD v. 3.2.4) database using DIAMOND *blastx* (ID=80%, query coverage=80%, max-target-seqs=1, evalue=1e-10), to annotate ARGs in each sample (Alcock et al., 2022). The abundance of *rpob* was also determined using DIAMOND *blastx* (evalue=1e-5) to a pre-computed collection reference sequences (http://enve-omics.ce.gatech.edu/rocker/models).

2.8 Whole Genome Sequencing

To characterize the genetic composition of isolated colonies, pure cef-resitant *E. coli* (n=168) and CRE isolates (n=29) were subjected to further characterization by whole genome sequencing (WGS). Stored isolates were first re-streaked on their respective agars until uniform and isolated colonies were observed. A single colony was selected using a flame-sterilized bacterial loop and incubated in 5 mL of LB broth medium overnight at 37 °C, shaken at 130 rpm. After the incubation period, the broth was centrifuged at 5,000 g for 5 minutes, and the supernatant was discarded. The pellet was then resuspended in 1 mL of 1x PBS, and 300 µL of this suspension was added into lysing matrix tubes of the FastDNA Spin Kit for Soil (MPBio, Solon, OH) and extracted as per manufacturer's instructions. Subsequently, DNA was quantified using a Qubit Fluorometer (Invitrogen, Carlsbad, CA) and mass was normalized to 300 ng total input. Samples were then shipped on dry ice overnight to the Duke Center for Genomic and Computational Biology. There, library preparation and sequencing were performed using DNA Prep ¼ volume reaction for high DNA input, with a targeted 50x coverage. The prepared libraries were then sequenced on a single NovaSeq X Plus 1.5B flow cell.
Unassembled metagenomic sequencing reads were subjected to quality check and trimming using the Fastp tool (Chen, 2023b). Afterwards, *de novo* assembly of clean and quality genome sequences was performed using the SPAdes package (Prjibelski et al., 2020). The quality of the resulting assemblies was evaluated using QUAST (Mikheenko et al., 2018) and CheckM (Parks et al., 2015). Taxonomic classification was then carried out using GTDB-tk *classify_wf* (Chaumeil et al., 2020). Lastly, the RGI (Alcock et al., 2022) software was used on the assembled genomes to annotate ARGs from the CARD (v3.2.4) database, maintaining only "strict" or "perfect" alignments.

2.9 Statistical analyses and data visualization

All figures and statistical analyses were generated in R studio v2024.04.2+764. ARG abundances were analyzed using no-parametric tests due to the non-normality of the data, which was confirmed using Shapiro-Wilk test. The Kruskal-Wallis rank-sum test and Wilcoxon rank-sum test were performed to analyze the variability of ARGs abundance among the different sites (influent, effluent, upstream, and downstream) and dates. Spearman's rank correlation was used to assess relationships between ARG abundance and environmental variables. Non-metric multidimensional scaling (NMDS) was performed to assess the relationship between microbiome and resistome across water matrices. To evaluate the structural correlation of the resistome and microbiome, a Procrustes analysis was performed on NMDS results. The 'vegan' R package version 2.6-10 was used to perform all multivariate analyses.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Comparison of Total *E. coli* and Resistant *E. coli* across Sampling Sites and Dates

Out of the 82 presumptive cef-resistant *E. coli* colonies isolated across all sample types, 99% (81/82) were confirmed as *E. coli* through WGS. The single non-*E. coli* isolate was identified as *Citrobacter freundii*, which was recovered from wastewater influent. The presence of *C. freundii*, highlights the potential for misidentification of presumptive isolates when relying solely on phenotypic methods.

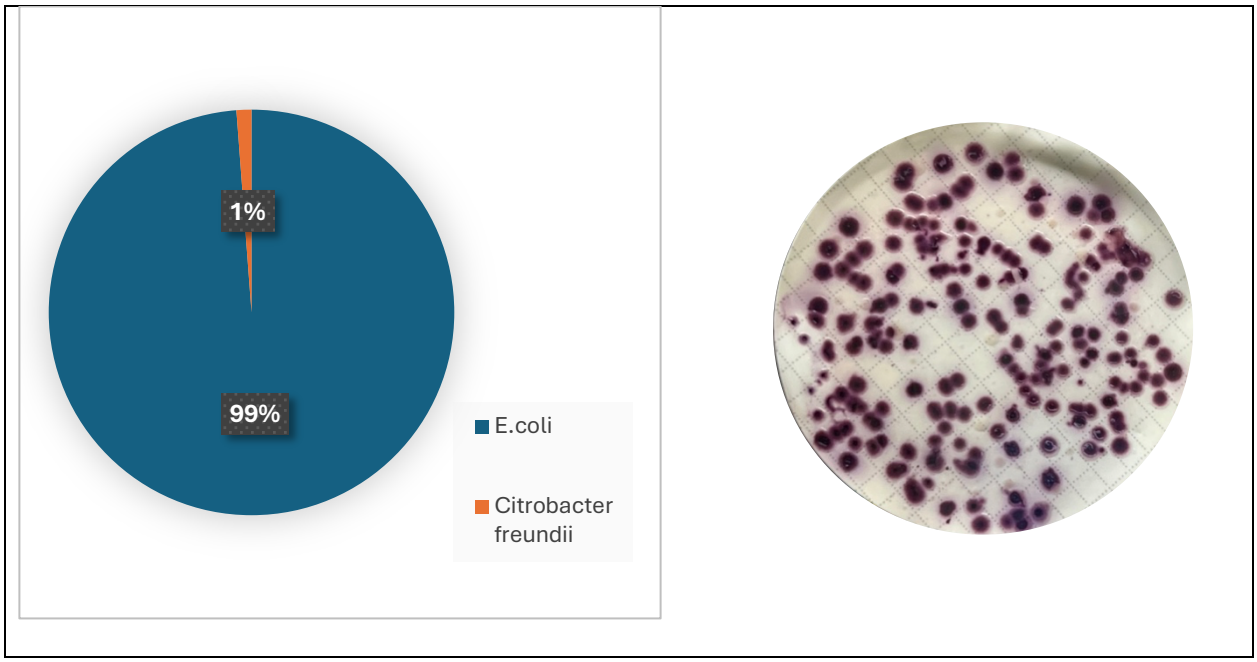


Figure 3. Cef-resistant E. coli confirmation by Whole Genome Sequencing and colony isolation

E. coli resistant to cefotaxime were detected across all sampling sites (Fig. 4). The total *E. coli* concentrations remained relatively stable over the sampling period, with values consistently ranging around 4-5 log in upstream and downstream sites, whereas wastewater effluent exhibited more pronounce fluctuations. In contrast, resistant *E. coli* counts varied significantly across sites and month. Peaks in resistant *E. coli* concentrations were observed in late spring and early summer across all sites, with effluent samples generally showing higher variability compared to upstream and downstream samples. The observed total *E. coli* concentrations across effluent, upstream, and downstream sites varied between months but the majority of the values remain above log 2.1(126 CFU/100 mL), which violates EPA's discharge values.

To assess the relative "cleanliness" of the whole system, we used the upstream site as a reference sample, as it represents the background microbial concentrations prior to discharge. We observed that the upstream, total *E. coli* concentrations remained consistently high throughout the sampling period, indicating that non-point sources may be contributing to *E. coli* concentrations before wastewater discharge. However, downstream site exhibited an increment in resistant *E. coli* following effluent discharge, supporting our hypothesis that the WWTP serves as a point-source of AMR in receiving waters.

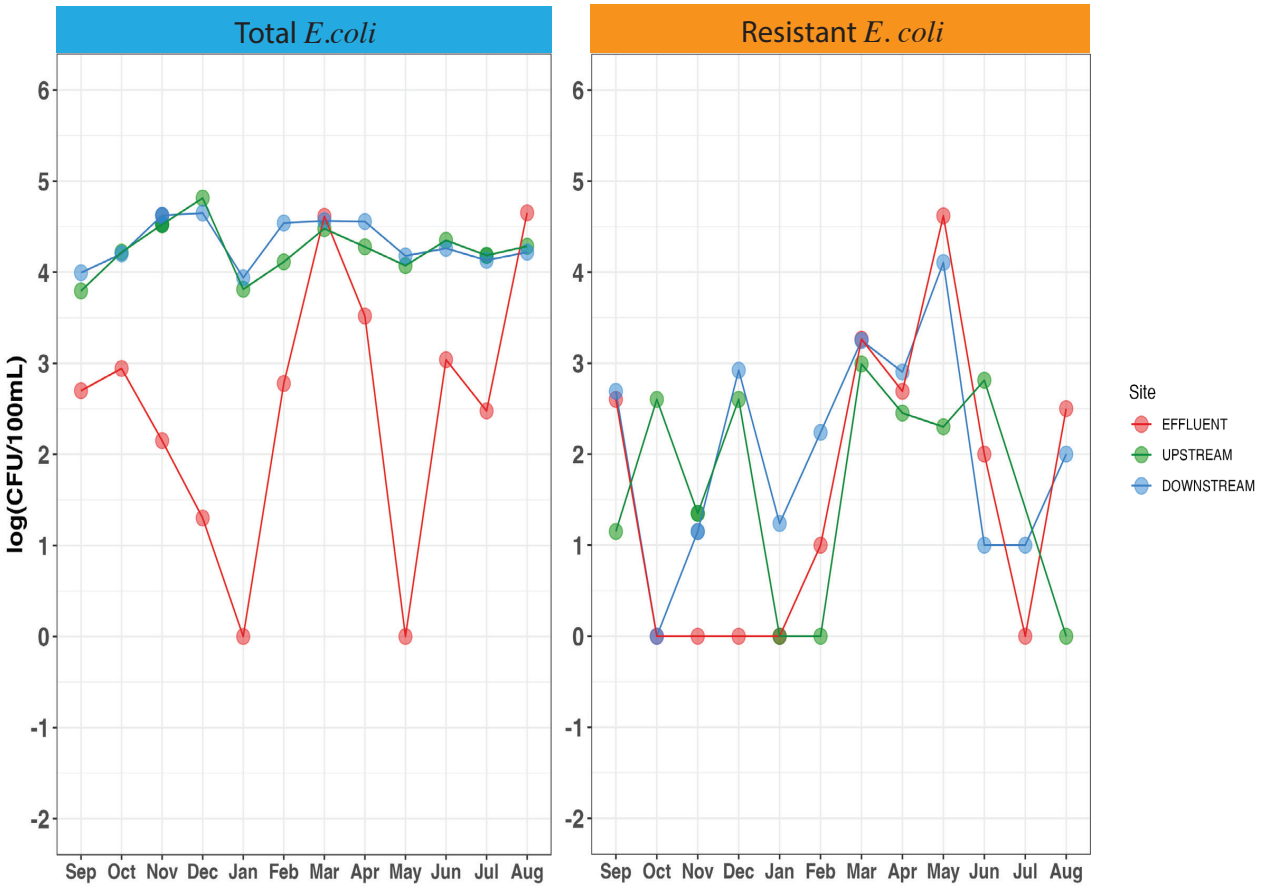


Figure 4. Average (n=2) of total and cef-resistant E. coli by sampling and month

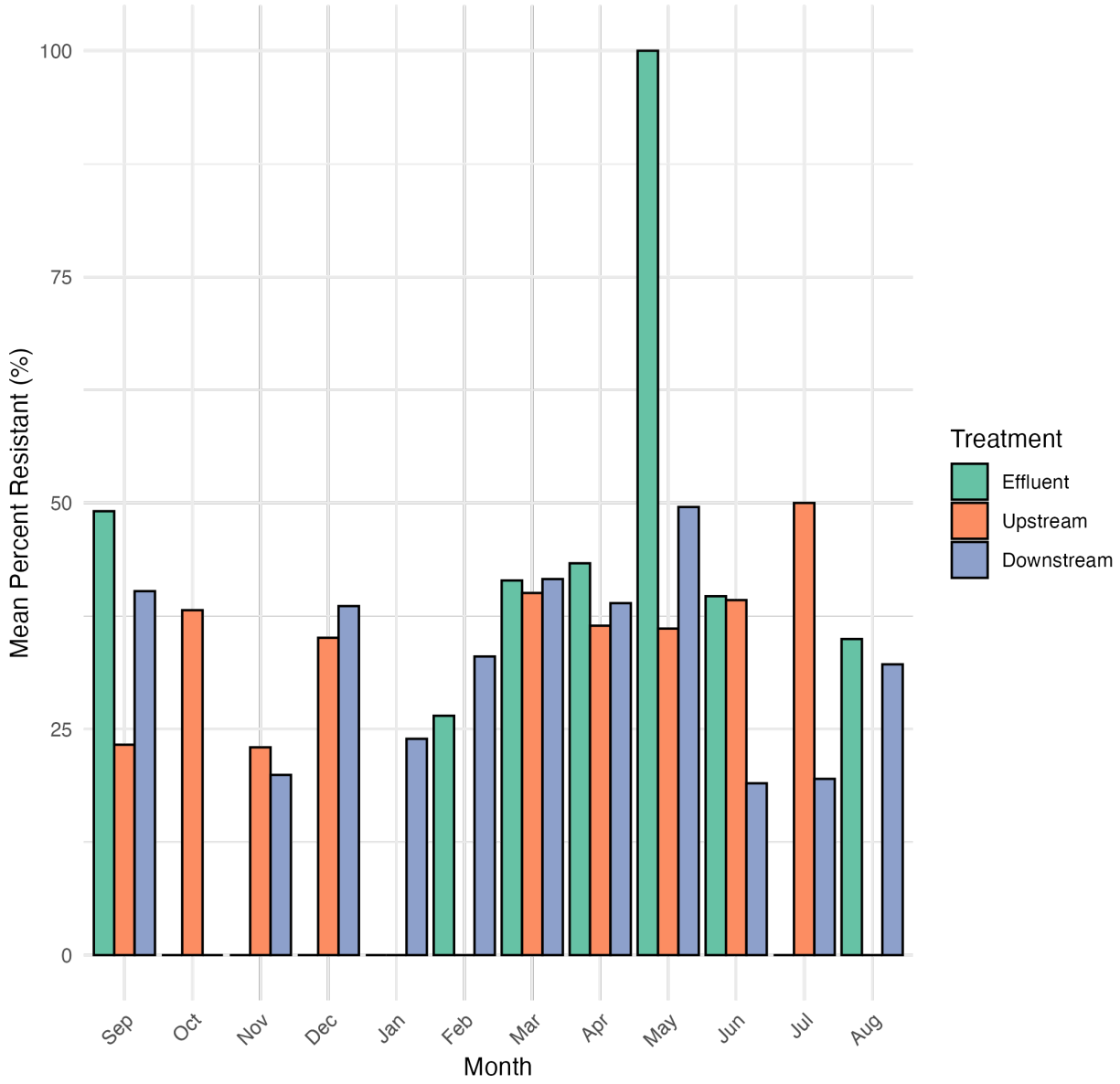
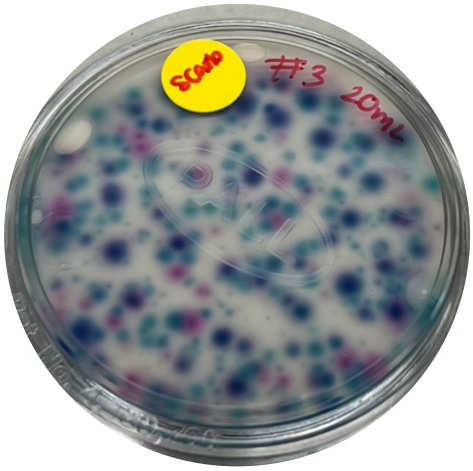


Figure 5. Percentages of resistant E. coli across wastewater influent, effluent, upstream, and downstream by month,

3.2 Carbapenem-resistant Enterobacteriaceae (CRE) and Carbapenemase producing organism (CPO) Environmental Isolation and Taxonomic Characterization

Table 3 shows the growth of bacterial colonies on mSuperCARBA agar. To characterize the bacteria, we followed the manufacturer color-based classification system. The classification categorizes bacterial growth by colony color, which allowed us to preliminarily classify the species of each isolate. This initial phenotypic screening of isolates indicated colonies in the samples classified as CPE *E. coli, Klebsiella, Enterobacter*, or *Citrobacter*. The observed colony diversity and crowding on the plates underscores the complexity of environmental microbial populations when compared to the clinical samples for which mSuperCARBA media were designed. To obtain genotypic resolution on the identity of isolates, we selected a subset of isolates from each sample type representing a range of morphologies and performed WGS (Table 3).

Table 3. Phenotypic characterization of colonies growing on mSuperCARBA plates.



Color	Organism
Dark pink to reddish	CPE *E. coli*
Metallic Blue	CPE *Klebsiella, Enterobacter, Citrobacter*
Translucent cream to blue	*CPO Pseudomonas*
Cream, opaque	CPO *Acinetobacter*

According to WGS, the detection of CRE isolates exhibiting ESBL and carbapenemase genes varied across species isolated (Figure 6). The classification of cabapenemase enzymes is commonly done according to the Ambler classification (Ambler RP., 1980). Notably, *Enterobacter roggenkampii* was identified carrying the KPC-18 gene and the MIR-10 in isolates for wastewater influent and wastewater effluent and downstream samples, respectively. The KPC-18 is considered a variant of KPC-2, which is associated with increased resistance to carbapenems and present in *Klebsiella pneumoniae (Maguire et al., 2024)*. No isolates of this species were recovered from upstream sites. Additionally, *Aeromonas caviae* was found to possess MOX-8 and OXA-504 genes. The OXA-504 gene which are a class D oxacillinases are commonly found in Aeromonas species (Janda & Abbott, 2010).The MOX family of enzymes are known for hydrolyzing extended-spectrum cephalosporins (Oguri et al., 2014). This species was found at both upstream and downstream sites, which was expected given that it is primarily an aquatic bacteria that naturally occurs in freshwater habitats and in soil (Galloway & Cohen, 2021).

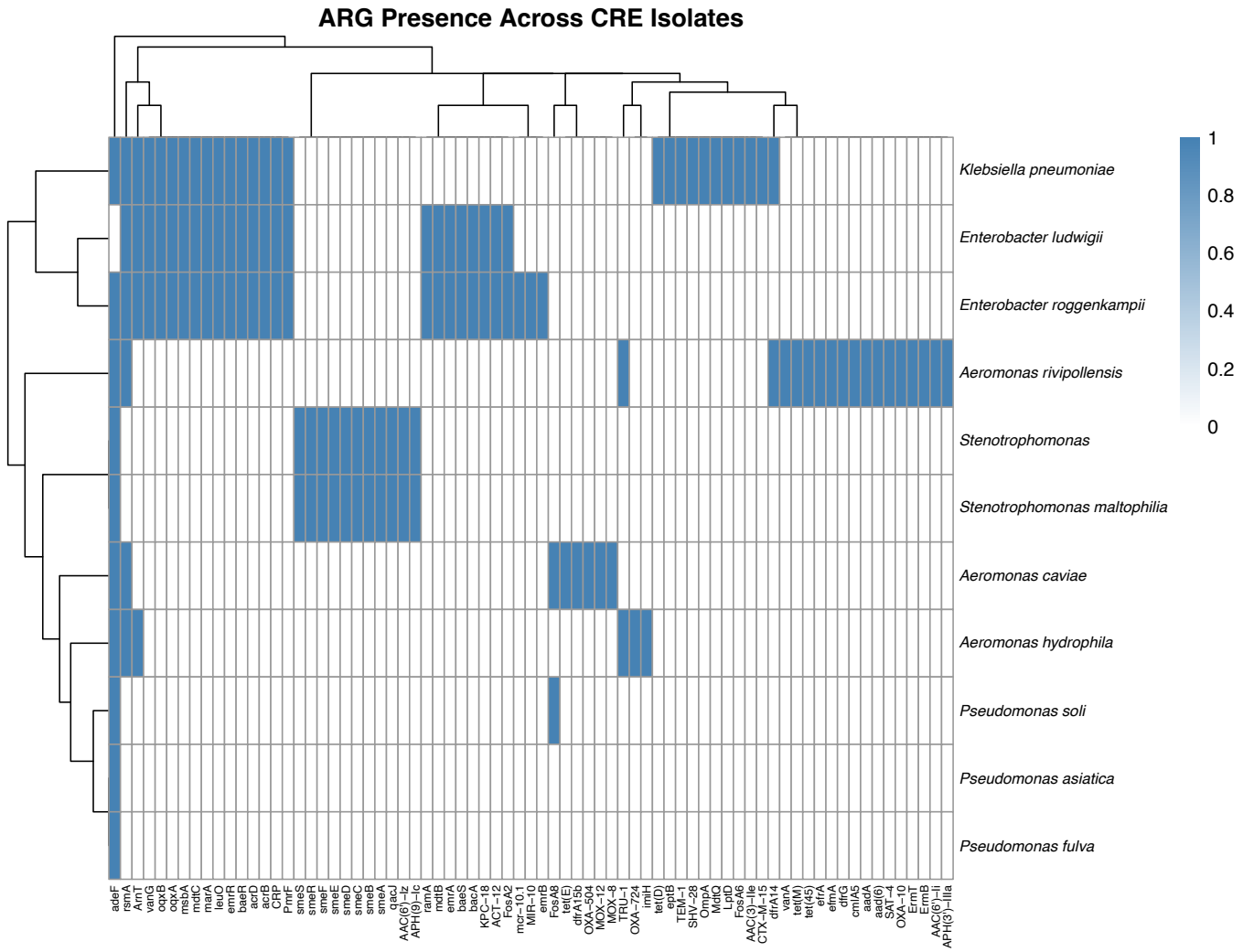


Figure 6. ARG Presence Across CRE Isolates from mSuperCARBA Plates. Each row represents a different species with each column corresponding to a specific ARG.

Despite the CRE isolates mainly being Gram-negative bacteria, we recovered a single strain of *Enterococcus faecium* in wastewater influent. Similar to *E. coli, E. faecium* is part of the normal flora of the human gastrointestinal tract and is considered a fecal indicators (Davis et al., 2022; Jang et al., 2017). The isolated E. faecium harbored multiple antibiotic-resistant genes (Table 4), including genes conferring vancomycin resistance. This particular resistance has been shown in E. faecium isolates responsible for central line-associated bloodstream infections and catheter-associated UTIs in the US (Weiner-Lastinger et al., 2020).

Table 4. Resistance Genes Present in Enterococcus faecium Isolated from mSuperCARBA Plates

Site	Bacterial species	Resistance Class	Genes	Resistance Mechanism
Influent	*Enterococcus faecium*	Vancomycin	vanA, vanH, vanR, vanS, vanX,vanY,vanZ	Antibiotic target alteration
		Aminoglycoside	aad(6), APH(3')-IIIA, SAT-4, AAC(6')-Ii	Antibiotic inactivation
		Macrolide	ErmB, ErmT	Antibiotic efflux
		Tetracycline	tet(M),tet(45)	Antibiotic target protection

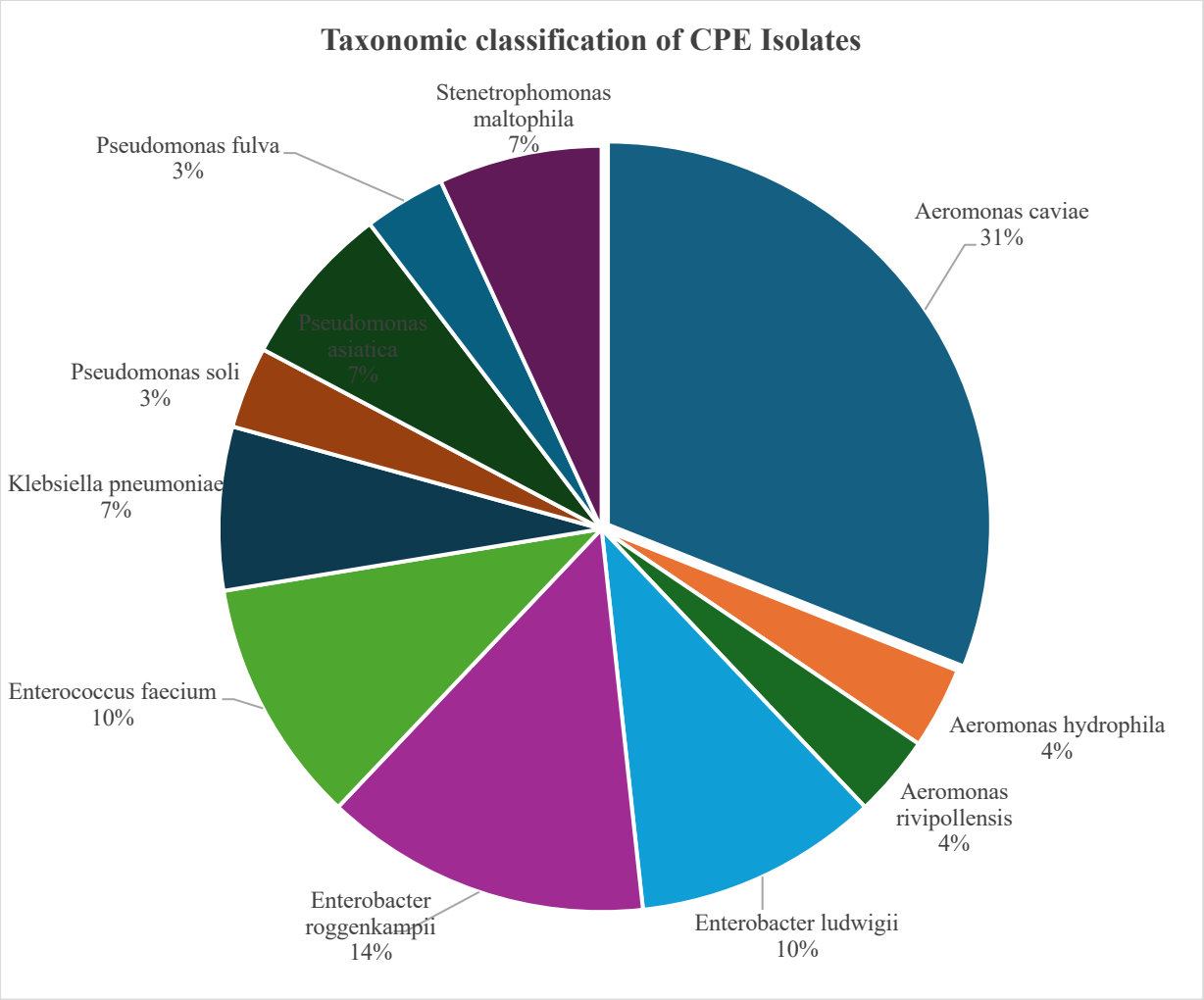


Figure 7. Overview of the taxonomic classification of isolates obtained on mSuperCARBA media across all samples (n=29). Isolates were selected to capture a range of phenotypes, with an equivalent total number sequenced across sample type (Influent n=16; Effluent n=4, Upstream n=4, Downstream n=5).

This exploratory application of mSuperCARBA agar for environmental sampling has demonstrated its utility as an effective preliminary screening tool. This approach facilitates subsequent microbiological and molecular analyses, contributing to a more comprehensive understanding of the distribution of taxa of bacteria known to contain pathogens of urgent concern from a clinical antibiotic resistance standpoint, particularly those conferring resistance to carbapenems. The observed distribution of CRE isolates highlights the potential role of WWTPs in the dissemination of AMT into aquatic environments.

3.3 ddPCR analyses

The observed *int*I1 concentrations, measured in log gene copies per mL, varied significantly across the different sampling locations. The influent samples exhibited the highest concentrations, with median values >6 logs. Effluent samples showed a noticeable reduction in *int*I1 concentrations compared to influent, with median values around 4.5 logs. Downstream samples show an increase in concentration compared to upstream, with median values around 3 log. These findings are consistent with the understanding of *int*I1 being an indicator of anthropogenic sources of ARGs.

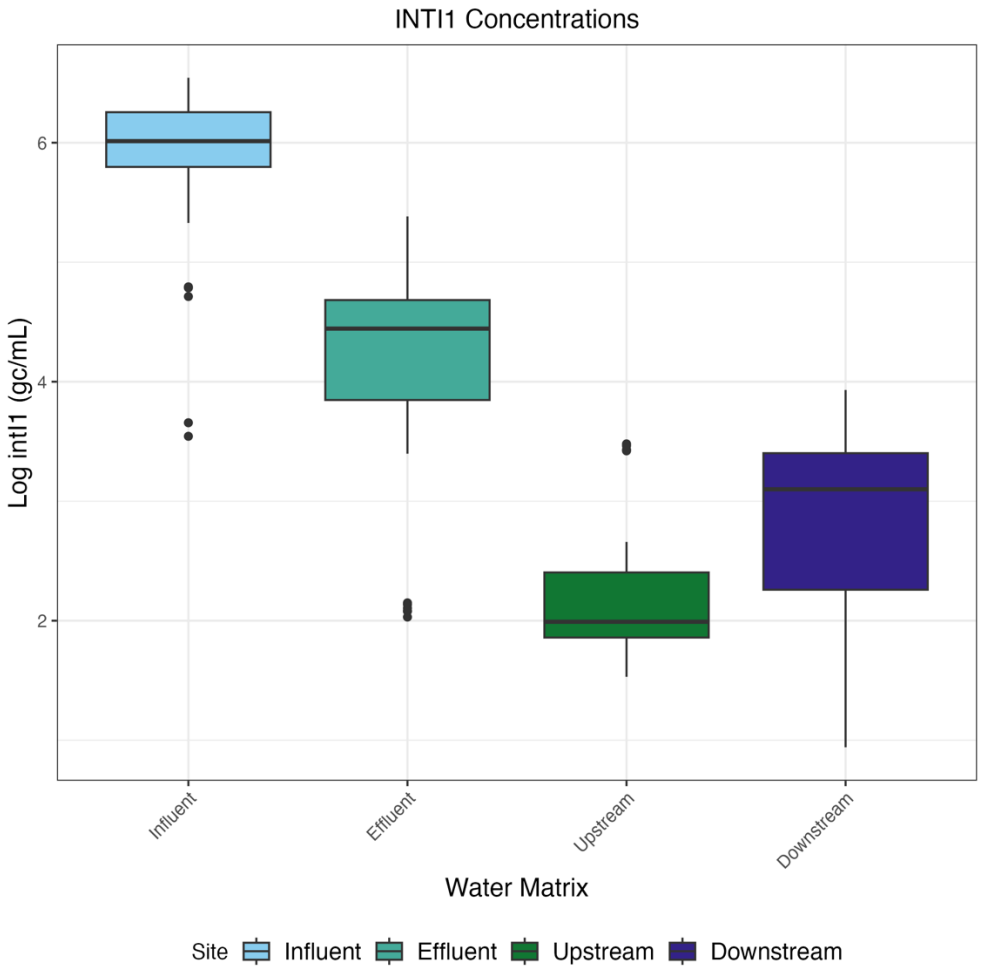


Figure 8. Concentrations of intI1, expressed in log gc/mL, across different water matrices: influent, effluent, upstream, and downstream.

Patterns in occurrence of *bla*CTX-M-1 mirrored those of *int*I1. The influent samples showed the highest concentrations of *bla*CTX-M-1, with a median value >3.5 logs. A notable reduction in *bla*CTX-M-1 concentrations is observed in the effluent samples, with median values around 1.5 logs. The downstream samples show a noticeable increase in *bla*CTX-M-1 concentrations compared to upstream, with median values around 1 log. Overall, the data suggests that wastewater treatment substantially reduced *bla*CTX-M-1 concentrations, but residual levels in effluent and downstream waters are elevated in *bla*CTX-M-1 as a result of wastewater discharge.

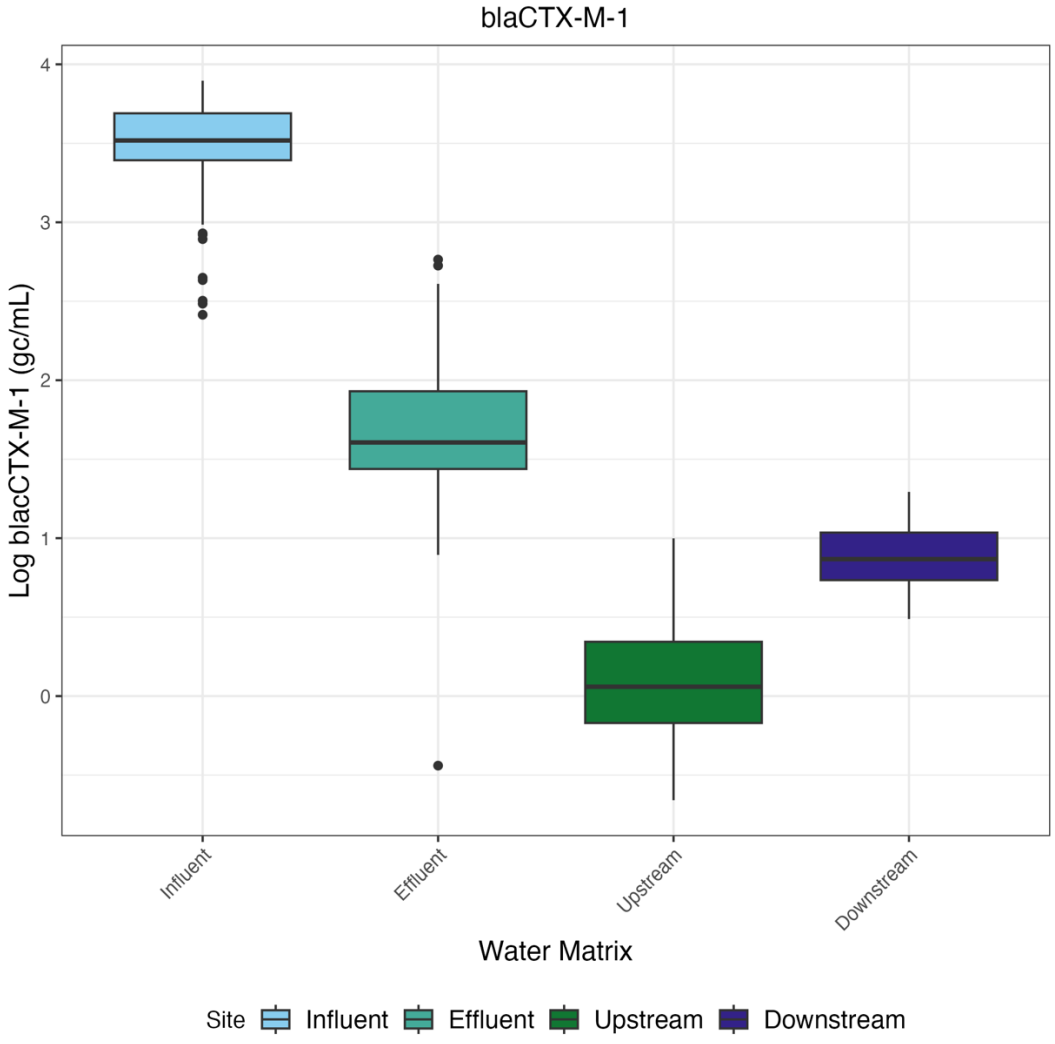


Figure 9. Concentrations of blaCTX-M-1, expressed in log gc/mL, across different water matrices: influent, effluent, upstream, and downstream.

Consistent with observations of intI1 and blaCTX-M-1 gene abundances, the 16S rRNA concentrations (Fig. 10) indicated that the wastewater effluent contributes to the total bacterial load in the receiving waters. Specifically, effluent discharge appears to increase the bacterial concentration downstream by approximately 1.5 log units relative to upstream levels. Elevation in total bacterial abundance downstream coincides with increased ARGs, suggesting that the WWTP discharge may serve as a source of not only resistant strains but also mobile genetic elements that facilitate ARG spread.

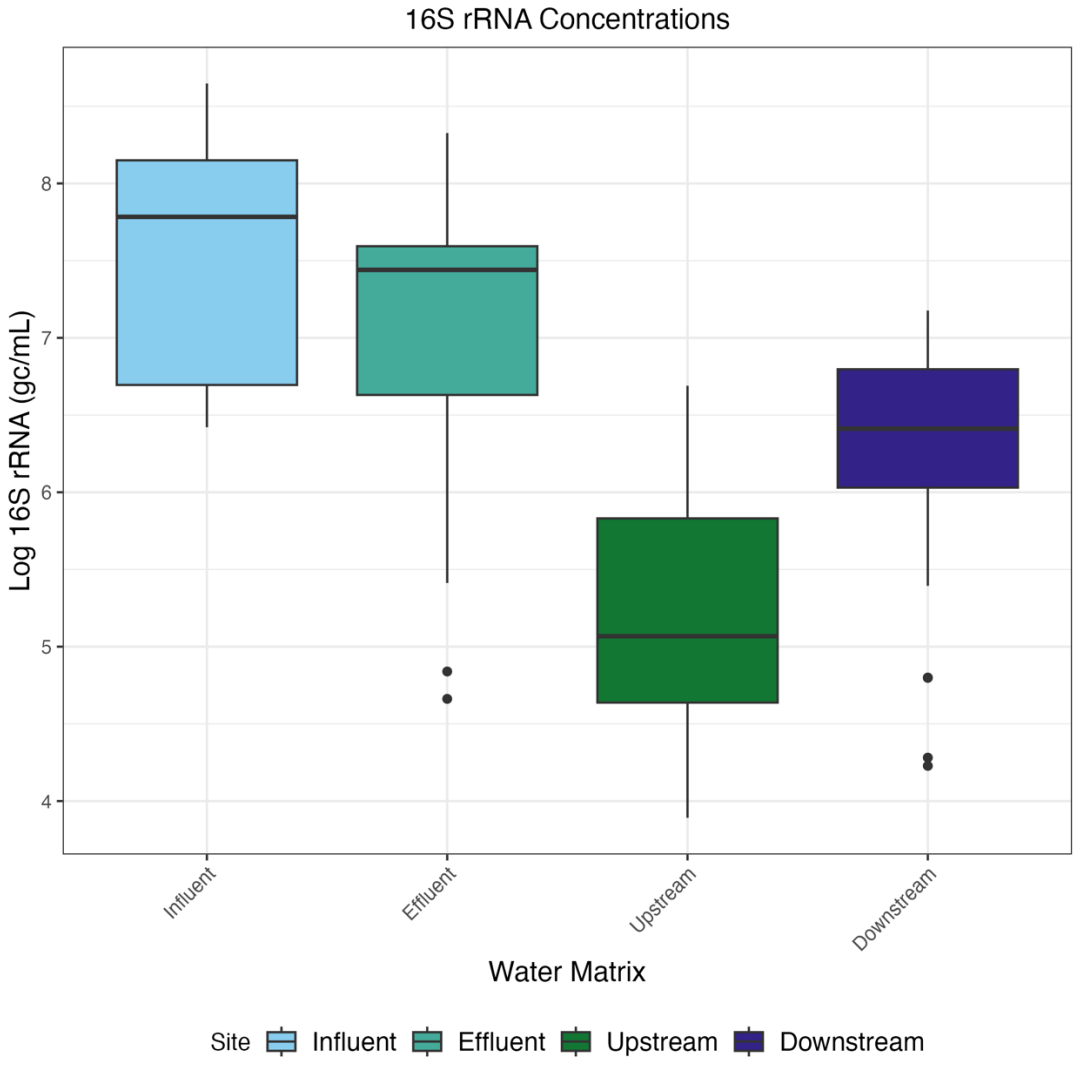


Figure 10. Concentrations of 16S rRNA gene (gene copies/mL) across wastewater Influent, wastewater Effluent, Upstream, and Downstream

3.4 Total ARG Relative Abundances in the WWTP Influent, WWTP Effluent, Upstream, and Downstream Across Sampling Dates

To calculate total ARG abundances, we performed deep sequencing on 45 samples representing wastewater influent (n=12), wastewater effluent (n=12), upstream (n= 10) and downstream (n=11) across the 12 sampling dates. Relative abundances of ARGs were determined by normalizing to the *rpo*B gene, which is a single copy housekeeping gene present in all bacteria, as an indicator of the proportion of bacteria in the community carrying ARGs. Relative abundance of ARGs was found to vary across the sampling dates (Fig. 11), with the highest average abundance found in wastewater influent (mean=10) during the November sampling event.

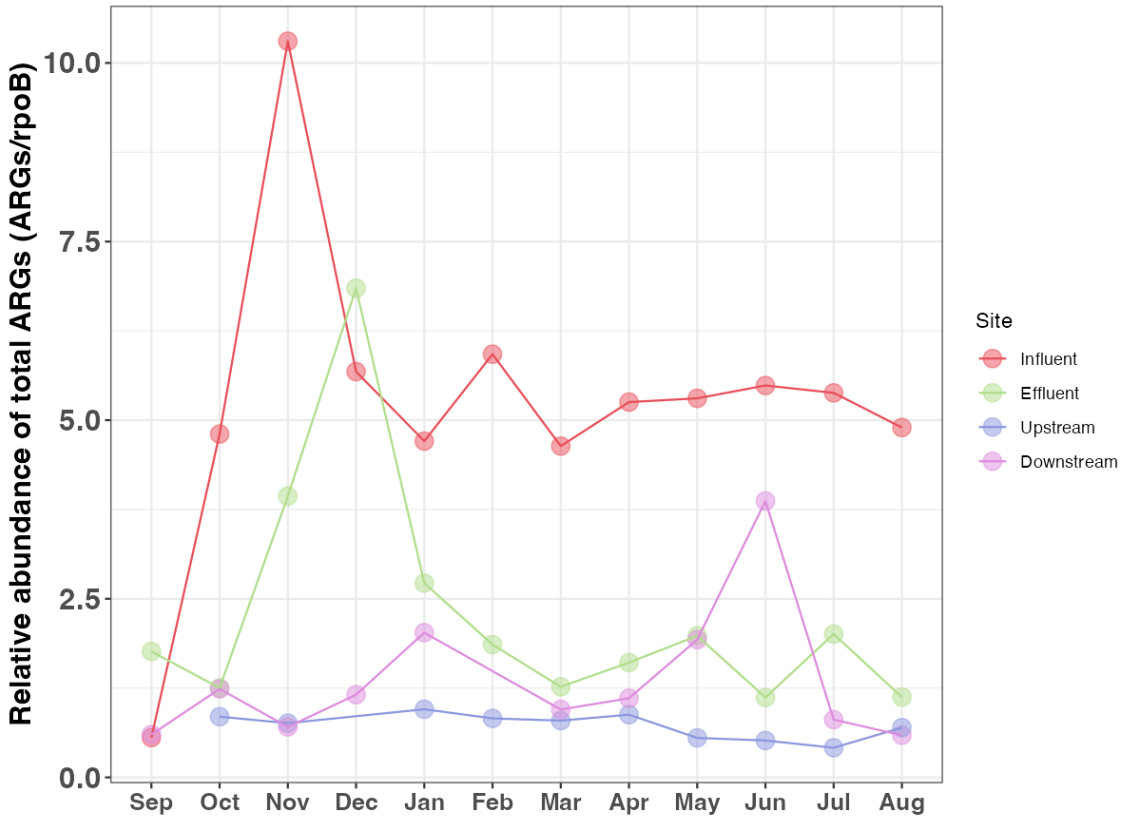


Figure 11. Total ARG relative abundances in the wastewater Influent and Effluent, Upstream, and Downstream across sampling dates determined via shotgun metagenomic sequencing. ARGs were annotated using CARD v. 3.2.4.

Relative abundance of total ARGs in influent, effluent, upstream and downstream, pooled in each category across 12 sampling dates (i.e., considering the sampling dates as replicates) was analyzed using Kruskal-Wallis. A Kruskal-Wallis rank sum test was performed and revealed a statistically significant difference in the relative abundance of total ARGs among the influent, effluent, upstream, and downstream sampling points when considering the sampling dates as replicates (p=6.186e-06). Pairwise comparisons between sites were performed using the Wilcoxon rank-sum test with Bonferroni adjustment. The results revealed that wastewater influent differed significantly from the other sites, with *p* values of 0.01748 (influent vs. effluent), 0.00084 (influent vs. upstream), and 0.00173 (influent vs. downstream). Similarly, a significant difference was observed between the upstream and effluent samples ($p = 1.9x10^{-5}$). However, no significant difference was observed between the effluent and downstream sites (p=0.16080) or between the upstream and downstream samples (p = 0.21420). This results have been previously reported, emphasizing the role of effluent in maintaining ARG loads in receiving water bodies (Mills et al., 2024; Read et al., 2024).

Despite the deteriorating WW system, a 56.3% reduction in total ARGs was achieved during wastewater treatment. This result is comparable to the 65.6% removal reported by (Ping et al., 2022), leaving a difference of 9.27%. These results suggest that the WWTP is removing ARGs, at least to some extent, comparably to municipal WWTP.

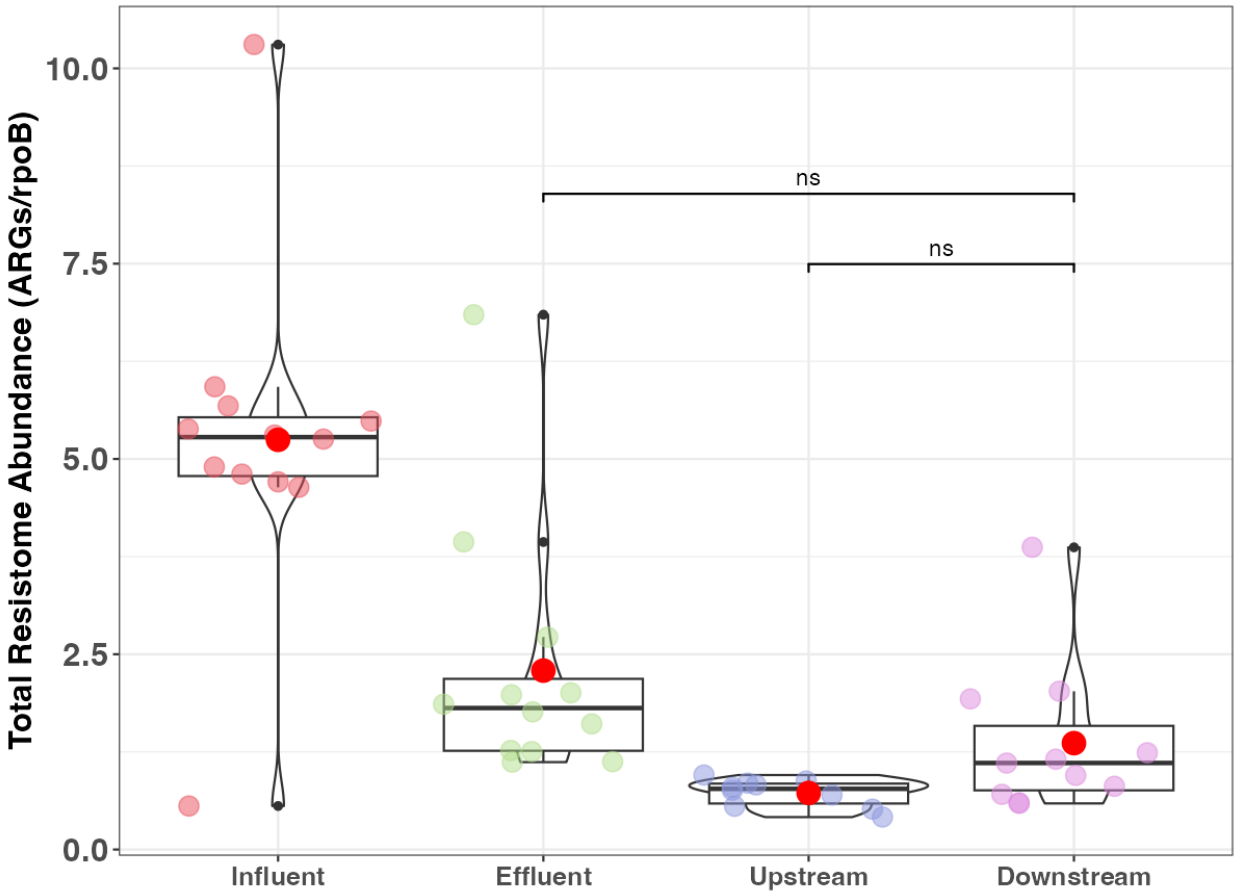


Figure 12. Total relative ARG abundance (normalized to rpoB) in wastewater Influent, wastewater Effluent, Upstream, and Downstream averaged by sampling Date. Averaged concentrations are shown as colored circles, and red circles indicate the mean. Brackets denote no statistical difference (p >0.05) between sites.

Figure 13 shows the monthly distribution of ARGs, normalized to *rpoB*, across sites. Overall, wastewater influent showed the highest total ARG abundance, dominated by multidrug and beta-lactam classes. Wastewater effluent ARG composition showed a decline but still diversity, indicating that treatment reduces but does not eliminate ARGs of multiple classes. Upstream showed relatively low ARG abundance, whereas downstream occasionally showed elevated ARG levels.

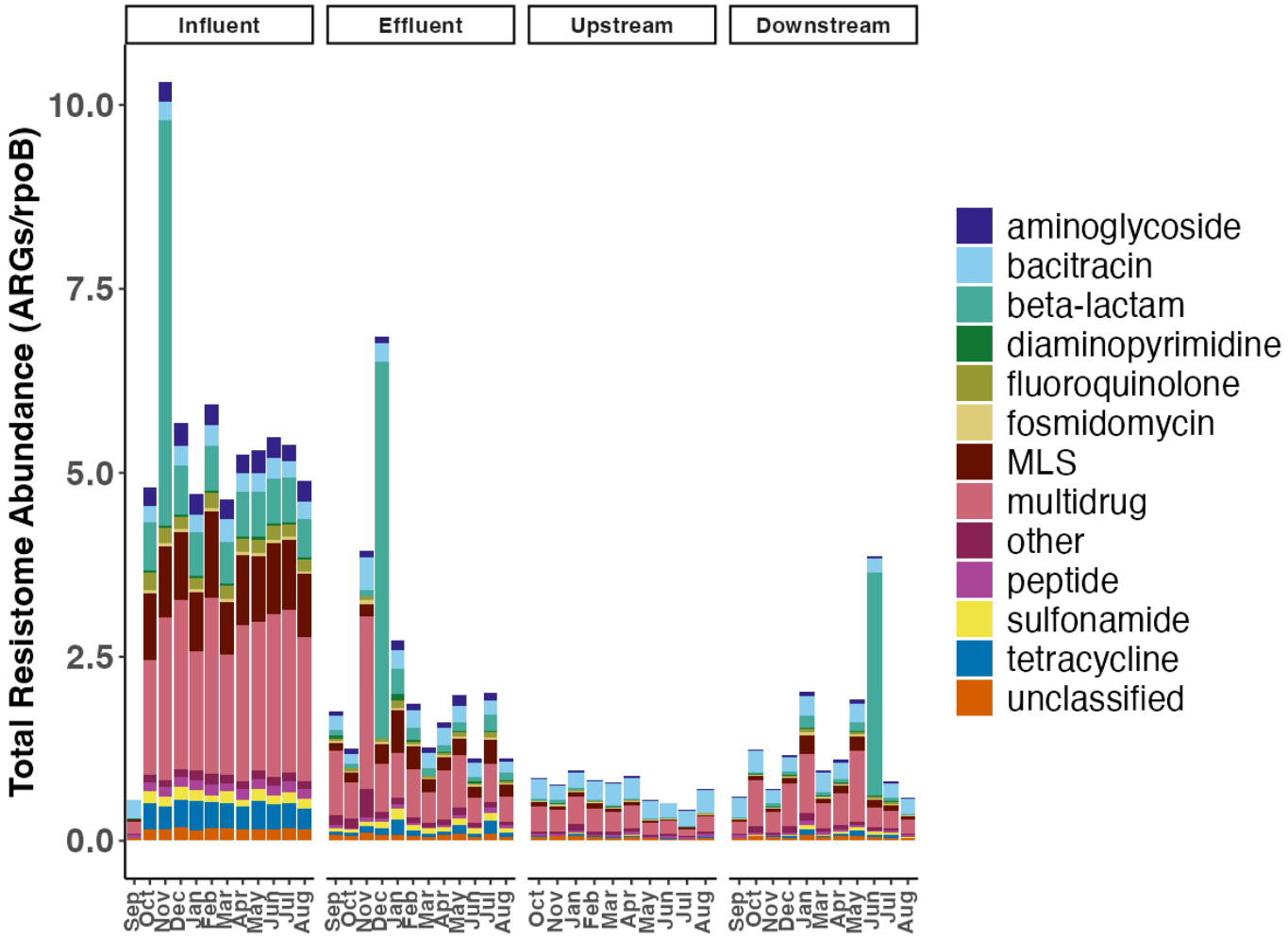


Figure 13. Relative abundances of ARGs observed at each sampling sites over a 1-year sampling period for each sampling date. ARG copies were normalized to the rpoB gene using the CARD model and database.

3.5 Unique ARGs across sampling sites

The number of shared and unique ARGs detected in influent, effluent, upstream and downstream sample categories are shown in Fig. 14, with specific ARGs in the effluent and downstream listed. ARGs unique to each site are listed in the Supplementary Information (Table S1). As expected, wastewater influent had the highest number of unique ARGs (364), which accounts to 20.9% of the total number of distinct ARG types observed across all samples. This was followed by downstream (57), effluent (50) and finally upstream (43). These results suggest that there was a reduction in the number of distinct types of ARGs during the wastewater treatment processes, but there are other sources contributing to ARGs observed in the river. Nonetheless, a striking finding was that there were far more types of ARGs unique to the downstream (402) versus the upstream (106) and that the vast majority of these unique downstream ARGs were also found in the effluent (272). Of these 272 ARG types 253 were also found in the effluent, suggesting that they pass through treatment and remain detectable in receiving waters, a trend similarly reported by Read et al., 2024.

Furthermore, an additional 73 ARG types only shared between the influent and downstream. For this latter category of 73 genes, they might have been below the metagenomic detection limit in the effluent but were amplified downstream. . This has been demonstrated by (Davis et al., 2023), who reported that metagenomic sequencing exhibits elevated detection limits, which can lead to an underestimation of ARG prevalence. By contrast, there were relatively few ARGs types in the upstream samples that only overlapped with the effluent samples (n=7) or only with the downstream samples (n=28). The overall findings suggests that a substantial portion of ARGs in the influent of this WWTP made their way through to the effluent and remained detectable in the downstream river samples.

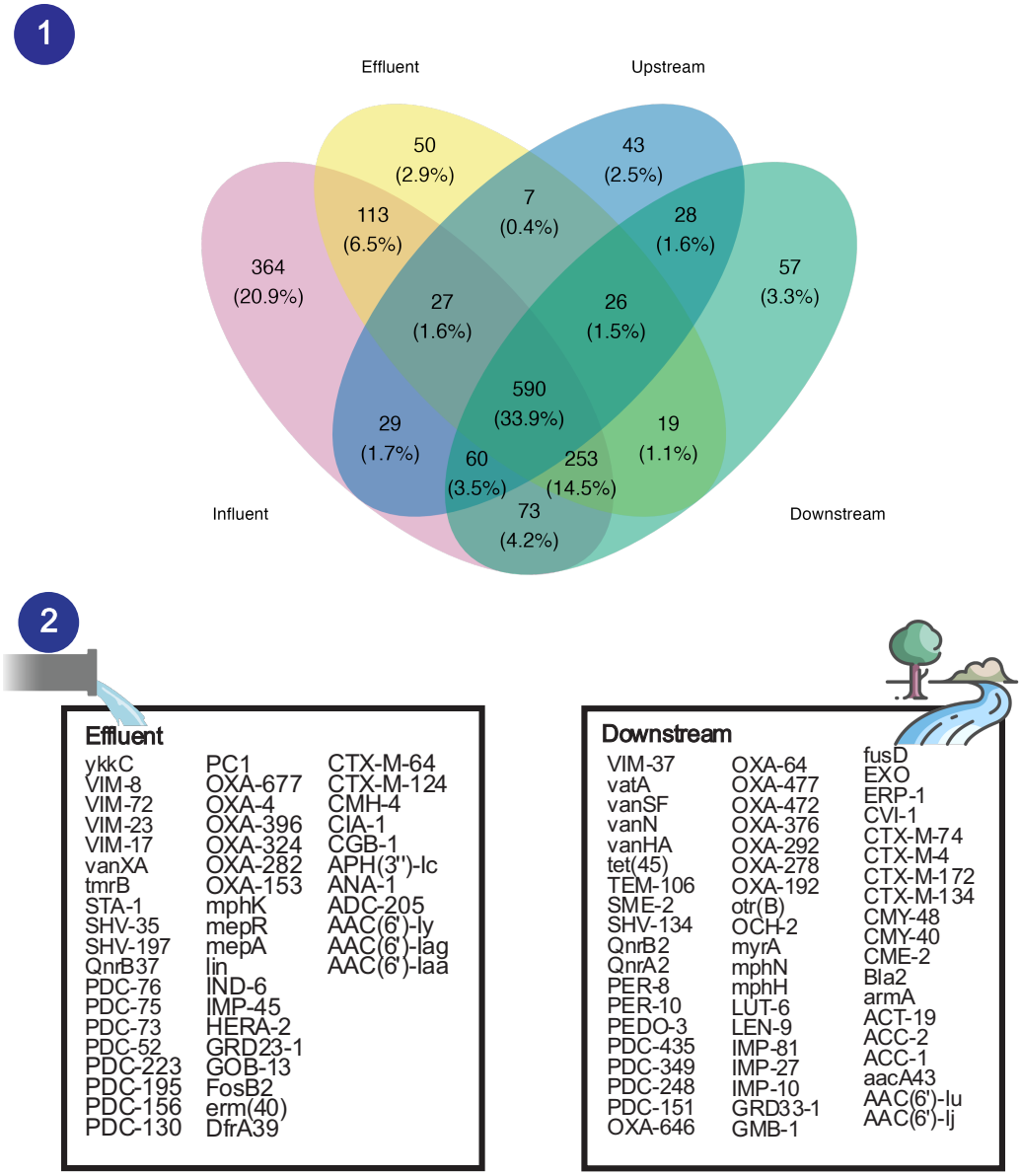


Figure 14. 1; Unique and shared ARGs across sampling sites and dates. 2; Specific ARGs unique to wastewater effluent (n=50) and downstream of the river (n=57).

3.6 Associations of ARG occurrence patterns with precipitation

We assessed the relationship between ARG abundance and rainfall events by performing a linear regression analysis with Spearman's rank correlation. Precipitation was calculated by averaging the cumulative precipitation for the 6 days preceding sampling. For the wastewater influent samples, the regression model indicated no relationship between precipitation and an increase in ARG abundance (rho = -0.067, p=1). This suggests that ARG levels in wastewater influent were not significantly altered by rainfall events, either by dilution or concentration. In contrast, wastewater effluent showed a weak positive correlation (rho =0.289, p = 0.54) between precipitation and ARG abundance. While this suggests a possible increase in ARG levels due reduced hydraulic residence time, the relationship was not statistically significant. Upstream river samples, show a slight negative correlation with rainfall (rho = -0.097, p= 1) suggesting a potential decrease in ARG abundance with higher rainfall. Similarly, the downstream site displays a moderate increase in ARG abundance with rainfall (rho=0.34, p =0.31), suggesting potential contributions from effluent discharge to downstream river sampling points.

While rainfall events may influence ARG dynamics in effluent and downstream, the relationships observed were weak to moderate and proved to not be statistically significant. Previous studies (Ahmed et al., 2018; Di Cesare et al., 2017) have reported that the effects of precipitation on ARG transport are highly site-specific. Additionally, the study by Darling et al., 2025 would lead us to expect a greater variation in AMR load at the influent with rain events due to I&I, yet we did not observe this in our results. The absence of such pattern suggests that the baseline resistome may be buffering overall fluctuation in total resistome abundance, potentially diminishing the observable impact of rain events.

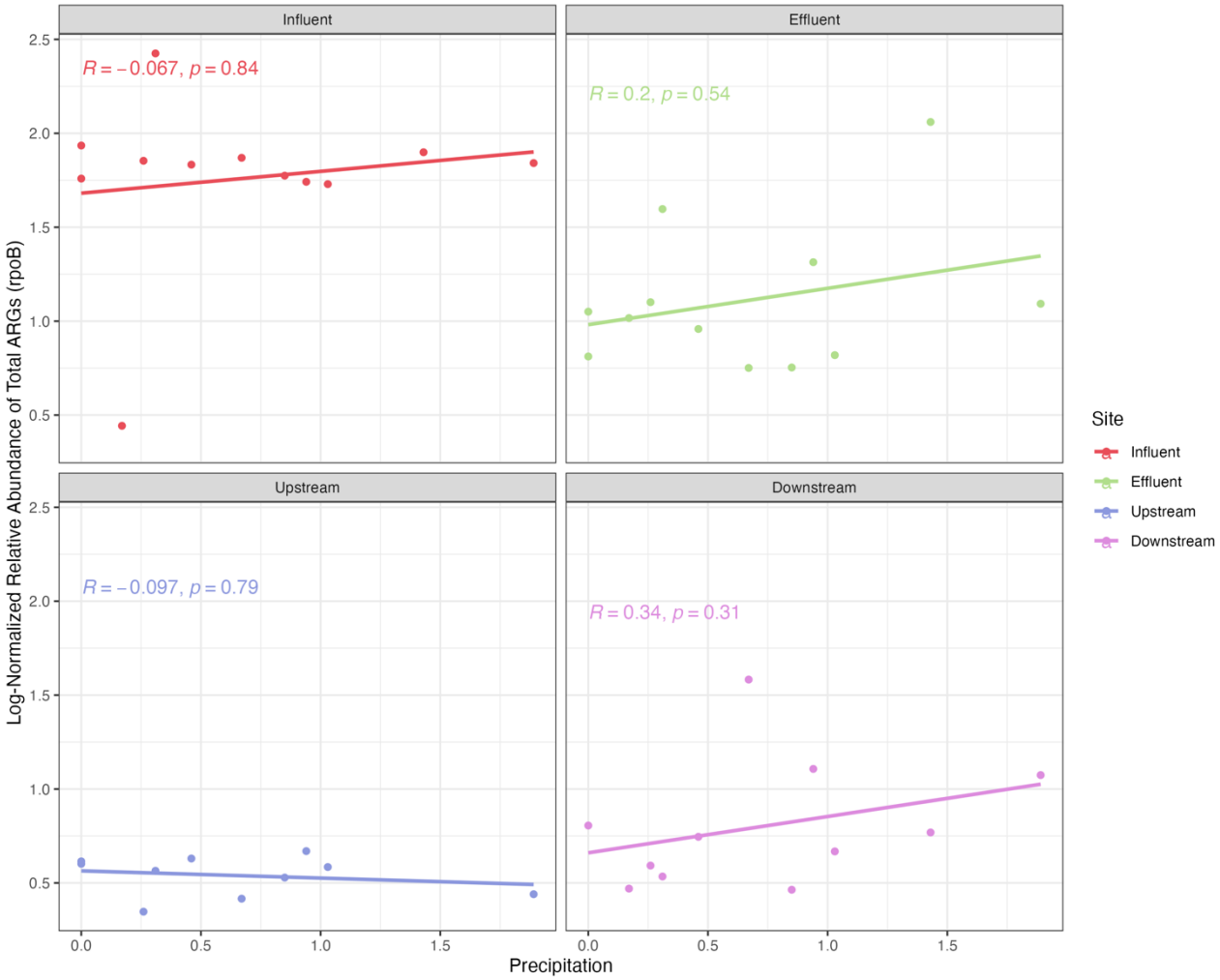


*Figure 15. Relative abundance of total ARGs (normalized to rpoB), estimated via metagenomic sequencing, versus precipitation, plotted as cumulative precipitation (inches) across 6 days before sampling. *R=rho*

3.7 Microbiome and Resistome Composition Across Different Aquatic Matrices

Taxonomic analysis of the metagenomic data sets provided insights into overlap in the composition of the microbiomes, i.e., bacterial composition, of the sampling locations. Beta diversity analysis confirmed that the influent sewage microbiome was distinct, as the samples clustered away from the effluent, upstream, or downstream samples (Fig. 16). This is consistent with activated sludge fostering a distinct microbiome, relative to the influent, that is highly efficient at consuming organic carbon and other nutrients in the wastewater (Dai et al., 2022). Relative to the research questions guiding the present study, a striking observation was that the effluent samples formed a distinct cluster relative to the upstream samples but overlapped substantially with the downstream samples. This suggests that effluent contributes measurably to bacteria measured in the river downstream, which could also contribute to the differences in the resistome patterns, depending on which ARGs are carried by the effluent bacteria. Notably, there is a veterinary clinic located close to the sewer shed, which may contribute to the microbial communities observed. It was also apparent from the beta diversity analysis that there was the greatest variability in the composition of the upstream microbiome and the least variability in the influent sewage microbiome, with the effluent and downstream microbiomes falling somewhere in the middle. Thus, the relatively more consistent microbial composition of the effluent could be dominating the more consistent composition of the downstream relative to the upstream microbiome.

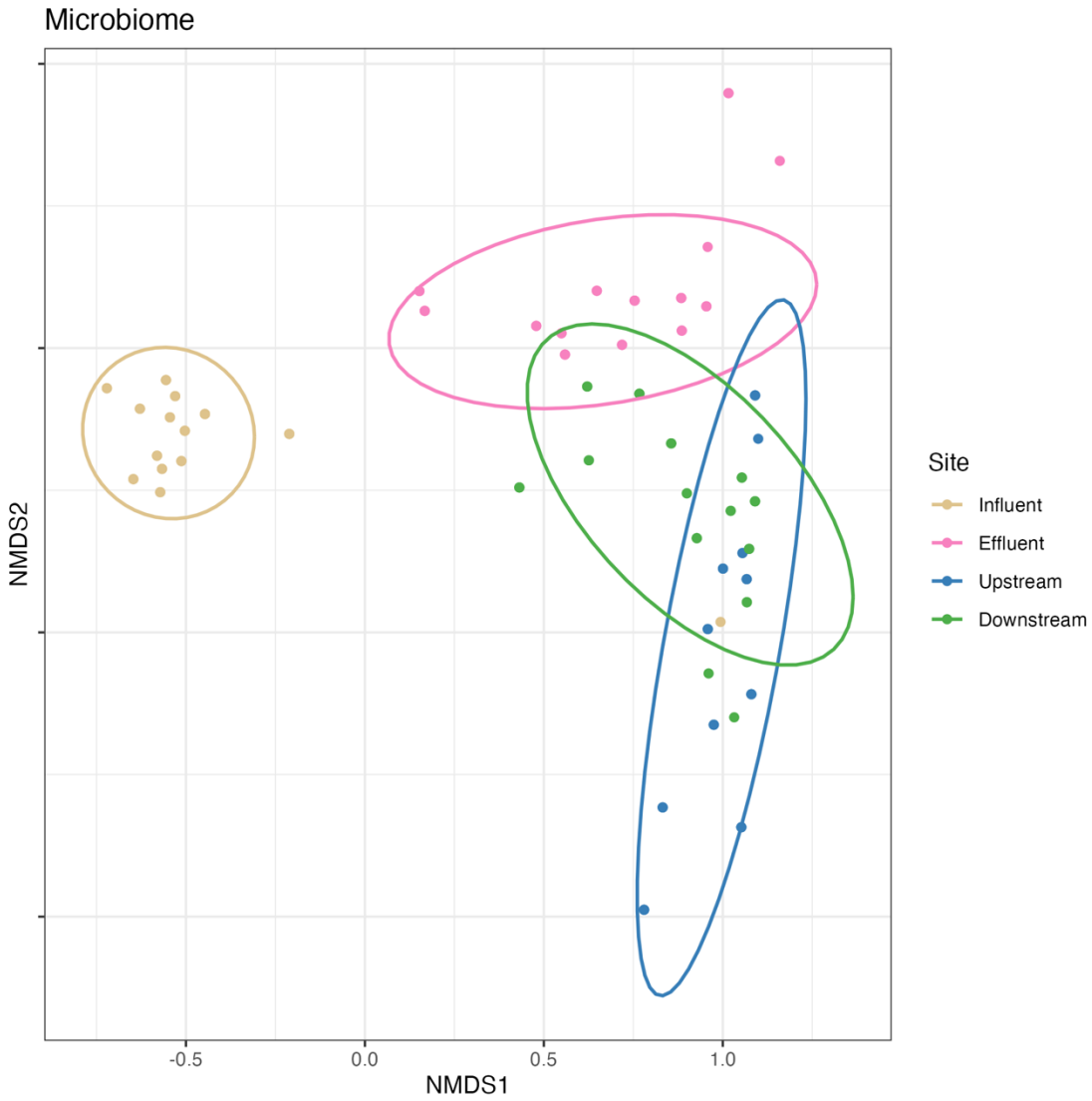


Figure 16. Non-metric Multidimensional Scaling (NMDS) Analysis of the Bacterial Composition Across Sampling Sites and Dates.

Beta diversity analysis of the resistome composition across the four-sampling sites is shown in Figure 17. As was seen in the microbiome analysis, the influent resistome was distinctly separated from the other sampling locations, indicating a unique resistome profile. Also as was observed in the microbiome analysis, the effluent and downstream sites overlapped in the composition of the resistome. The overall beta diversity clustering pattern of the resistome was consistent with that of the microbiome, with the upstream ARGs overlapping completely with the downstream ARGs, and the downstream ARGs overlapping partially with the effluent ARGs.

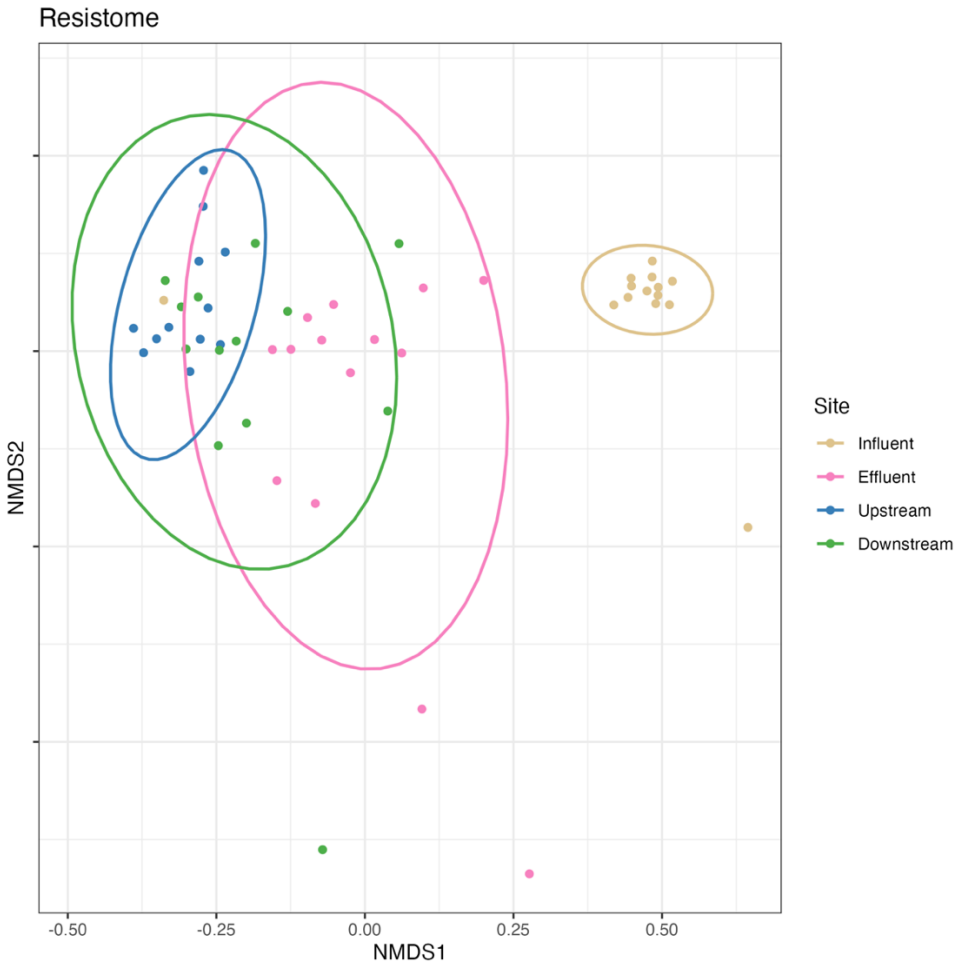


Figure 17. Non-metric Multidimensional Scaling (NMDS) Analysis of the Resistome Across Sampling Sites and Dates.

3.8 Comparing Resistome and Microbial Composition

Procrustes analysis was carried out to assess the degree to which the microbiome composition aligned with the ARG composition across water matrices (Fig. 18). According to the Procrustes sum of squares for wastewater influent samples, there was a relatively high degree of misalignment between the microbiome and resistome, yielding a sum of squares (m12 square) of 0.9475 and a Procrustes correlation of 0.2292 with a *p* value of 0.1573. For wastewater effluent, the relationship between the resistome and microbiome was stronger (0.5186, $p = 0.0394$, m12 square = 0.7311), suggesting that shifts in ARGs in the effluent partially mirror shifts in bacterial composition of the effluent with time. Similarly, we observed a correlation between the microbiome and resistome in the upstream site (0.6551) with significant *p* value at 0.0259 and a sum of squares of 0.5708. The downstream site presented a Procrustes correlation of 0.4808 and a sum of squares of 0.7688. However, the p value (0.0926) showed no statistical significance in this correlation. This suggest that there is a moderate correlation between the microbiome and the resistome, indicating that there's additional factors that could be influencing the microbiome. The general results suggest that changes in the microbial composition might be a driver for changes in concentration of ARGs in water matrices.

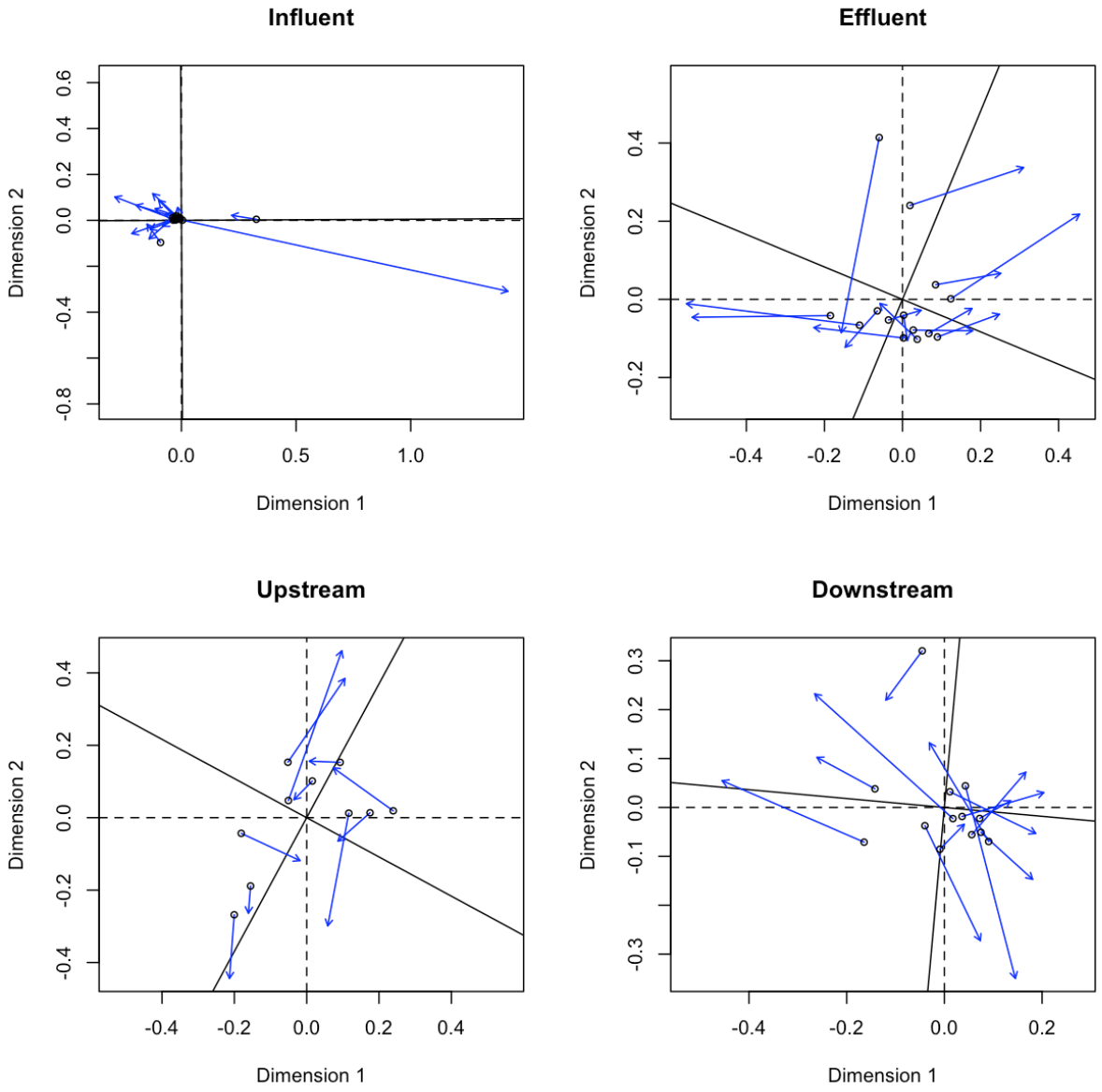


Figure 18. Procrustes analysis of the relationship between resistome and microbiome compositions of the influent, effluent, upstream and downstream samples

CHAPTER 4: Conclusion

WWTPs play a crucial role in removing a vast of potentially harmful substances in waste that would otherwise affect the environment and public health. Thus, this study aimed to understand the influence of a rural WWTP on the receiving river and how can this affect the dissemination of ARBs and ARGs.

The overarching conclusion of this research is that the WWTP effluent examined in this study is a contributor of ARBs and ARGs observed downstream in the receiving river. Key evidence to support this conclusion include:

- Relative abundance of total ARGs ranked as effluent > downstream > upstream. This suggests that the effluent is enriched in ARGs and is diluted by the upstream water, but levels still remain measurably elevated in the downstream relative to the upstream.
- There were more specific ARG types shared between the effluent and downstream samples than between the effluent and upstream samples.
- Beta diversity analysis of both taxonomy and resistomes indicated more overlap between effluent and downstream samples than between effluent and upstream samples.
- *E. coli* with the same genotype identified by WGS in effluent and downstream samples that were not found in upstream samples.

The study also provides insights into mechanisms driving the dissemination of effluent ARGs into the receiving river. As was noted in the Introduction, the WWTP that was the focus of this study is known to suffer from extreme I&I during rainfall events and the WWTP does not always operate as designed. Correspondingly, it was noted that the vast majority of ARGs identified to overlap between effluent and downstream samples were also found in the influent, suggesting

that many ARGs pass through without treatment. Correlation analysis with the amount of precipitation in the previous six days indicated a weak relationship for ARG relative abundances in downstream samples, but not in upstream samples. This suggests that precipitation could be a factor, but is probably not the sole driver of shared ARGs between downstream and effluent samples.

Taxonomic analysis indicated that the WWTP effluent was also a major contributor of the bacterial microbiome observed downstream versus upstream, and therefore bacteria emitted from the WWTP could be the primary carriers of the distinct ARGs observed downstream. Consistent with this, ddPCR enumeration of bacterial 16S rRNA genes revealed 1.5 log higher bacterial numbers downstream from the WWTP relative to upstream. Additionally, ddPCR of genes that have been proposed as environmental monitoring targets for AMR, intI1 and blaCTXm-1, revealed the exact same pattern of effluent>downstream>upstream.

In sum, this study provides important confirmation that WWTPs can be a source of ARGs to riverine environments. It further provides quantitative estimates of the input of key genes that have been proposed for AMR monitoring (intI1 and blaCTXm-1) as well as comprehensive metagenomic profiles of ARGs. Analysis of shared ARGs across the four environments (influent, effluent, upstream, downstream) can further help to focus monitoring efforts going forward to genes of clinical concern that are most likely to persist from influent to effluent to downstream.

A useful follow-up study will be to conduct phylogenetic analysis of the sequenced isolates and to compare them to clinical isolates available in the region.

References

Ahmed, W., Zhang, Q., Lobos, A., Senkbeil, J., Sadowsky, M. J., Harwood, V. J., Saeidi, N., Marinoni, O., & Ishii, S. (2018). Precipitation influences pathogenic bacteria and antibiotic resistance gene abundance in storm drain outfalls in coastal sub-tropical waters. *Environment International*, *116*, 308–318. https://doi.org/10.1016/j.envint.2018.04.005

Alcock, B. P., Huynh, W., Chalil, R., Smith, K. W., Raphenya, A. R., Wlodarski, M. A., Edalatmand, A., Petkau, A., Syed, S. A., Tsang, K. K., Baker, S. J. C., Dave, M., McCarthy, M. C., Mukiri, K. M., Nasir, J. A., Golbon, B., Imtiaz, H., Jiang, X., Kaur, K., … McArthur, A. G. (2022). CARD 2023: Expanded curation, support for machine learning, and resistome prediction at the Comprehensive Antibiotic Resistance Database. *Nucleic Acids Research*, *51*(D1), D690–D699. https://doi.org/10.1093/nar/gkac920

Barraud, O., Baclet, M. C., Denis, F., & Ploy, M. C. (2010). Quantitative multiplex real-time PCR for detecting class 1, 2 and 3 integrons. *Journal of Antimicrobial Chemotherapy*, *65*(8), 1642–1645. https://doi.org/10.1093/jac/dkq167

Ben, W., Wang, J., Pan, X., & Qiang, Z. (2017). Dissemination of antibiotic resistance genes and their potential removal by on-farm treatment processes in nine swine feedlots in Shandong Province, China. *Chemosphere*, *167*, 262–268. https://doi.org/10.1016/j.chemosphere.2016.10.013

Bürgmann, H., Frigon, D., H Gaze, W., M Manaia, C., Pruden, A., Singer, A. C., F Smets, B., & Zhang, T. (2018). Water and sanitation: An essential battlefront in the war on antimicrobial resistance. *FEMS Microbiology Ecology*, *94*(9), fiy101. https://doi.org/10.1093/femsec/fiy101

Calarco, J., Pruden, A., & Harwood, V. J. (2024). Comparison of methods proposed for monitoring cefotaxime-resistant Escherichia coli in the water environment. *Applied and Environmental Microbiology*, *90*(5), e02128-23. https://doi.org/10.1128/aem.02128-23

Chaumeil, P.-A., Mussig, A. J., Hugenholtz, P., & Parks, D. H. (2020). GTDB-Tk: A toolkit to classify genomes with the Genome Taxonomy Database. *Bioinformatics*, *36*(6), 1925–1927. https://doi.org/10.1093/bioinformatics/btz848

Chen, S. (2023a). Ultrafast one-pass FASTQ data preprocessing, quality control, and deduplication using fastp. *iMeta*, *2*(2), e107. https://doi.org/10.1002/imt2.107

Chen, S. (2023b). Ultrafast one-pass FASTQ data preprocessing, quality control, and deduplication using fastp. *iMeta*, *2*(2), e107. https://doi.org/10.1002/imt2.107

Cohen, A., Vikesland, P., Pruden, A., Krometis, L.-A., Lee, L. M., Darling, A., Yancey, M., Helmick, M., Singh, R., Gonzalez, R., Meit, M., Degen, M., & Taniuchi, M. (2024). Making waves: The benefits and challenges of responsibly implementing wastewater-based surveillance for rural communities. *Water Research*, *250*, 121095. https://doi.org/10.1016/j.watres.2023.121095

Dai, D., Brown, C., Bürgmann, H., Larsson, D. G. J., Nambi, I., Zhang, T., Flach, C.-F., Pruden, A., & Vikesland, P. J. (2022). Long-read metagenomic sequencing reveals shifts in associations of antibiotic resistance genes with mobile genetic elements from sewage to activated sludge. *Microbiome*, *10*(1), 20. https://doi.org/10.1186/s40168-021-01216-5

Darling, A., Davis, B., Byrnex, T., Deck, M., Rivera, G. M., Price, S., Amaral-Torres, A., Markham, C., Gonzalez, R., Vikesland, P., Krometis, L.-A., Pruden, A., & Cohen, A. (2025). Subsewershed Analyses of the Impacts of Inflow and Infiltration on Viral

Pathogens and Antibiotic Resistance Markers Across a Rural Sewer System. *Water Research*, 123230. https://doi.org/10.1016/j.watres.2025.123230

Davis, B. C., Brown, C., Gupta, S., Calarco, J., Liguori, K., Milligan, E., Harwood, V. J., Pruden, A., & Keenum, I. (2023). Recommendations for the use of metagenomics for routine monitoring of antibiotic resistance in wastewater and impacted aquatic environments. *Critical Reviews in Environmental Science and Technology*, *53*(19), 1731–1756. https://doi.org/10.1080/10643389.2023.2181620

Davis, B. C., Keenum, I., Calarco, J., Liguori, K., Milligan, E., Pruden, A., & Harwood, V. J. (2022). Towards the standardization of *Enterococcus* culture methods for waterborne antibiotic resistance monitoring: A critical review of trends across studies. *Water Research X*, *17*, 100161. https://doi.org/10.1016/j.wroa.2022.100161

Di Cesare, A., Eckert, E. M., D'Urso, S., Bertoni, R., Gillan, D. C., Wattiez, R., & Corno, G. (2016). Co-occurrence of integrase 1, antibiotic and heavy metal resistance genes in municipal wastewater treatment plants. *Water Research*, *94*, 208–214. https://doi.org/10.1016/j.watres.2016.02.049

Di Cesare, A., Eckert, E. M., Rogora, M., & Corno, G. (2017). Rainfall increases the abundance of antibiotic resistance genes within a riverine microbial community. *Environmental Pollution (Barking, Essex: 1987)*, *226*, 473–478. https://doi.org/10.1016/j.envpol.2017.04.036

Du, J., Geng, J., Ren, H., Ding, L., Xu, K., & Zhang, Y. (2015). Variation of antibiotic resistance genes in municipal wastewater treatment plant with A(2)O-MBR system. *Environmental Science and Pollution Research International*, *22*(5), 3715–3726. https://doi.org/10.1007/s11356-014-3552-x

Galloway, D., & Cohen, M. B. (2021). 38—Infectious Diarrhea. In R. Wyllie, J. S. Hyams, & M. Kay (Eds.), *Pediatric Gastrointestinal and Liver Disease (Sixth Edition)* (pp. 398-415.e5). Elsevier. https://doi.org/10.1016/B978-0-323-67293-1.00038-4

Gao, P., Munir, M., & Xagoraraki, I. (2012). Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. *The Science of the Total Environment*, *421–422*, 173–183. https://doi.org/10.1016/j.scitotenv.2012.01.061

Garner, E., Maile-Moskowitz, A., Angeles, L. F., Flach, C.-F., Aga, D. S., Nambi, I., Larsson, D. G. J., Bürgmann, H., Zhang, T., Vikesland, P. J., & Pruden, A. (2024). Metagenomic Profiling of Internationally Sourced Sewage Influents and Effluents Yields Insight into Selecting Targets for Antibiotic Resistance Monitoring. *Environmental Science & Technology*, *58*(37), 16547–16559. https://doi.org/10.1021/acs.est.4c03726

Hamilton, K. A., Garner, E., Joshi, S., Ahmed, W., Ashbolt, N., Medema, G., & Pruden, A. (2020). Antimicrobial-resistant microorganisms and their genetic determinants in stormwater: A systematic review. *Current Opinion in Environmental Science and Health*, *16*, 101–112. https://doi.org/10.1016/j.coesh.2020.02.012

Hanna, N., Tamhankar, A. J., & Lundborg, C. S. (2023). Antibiotic concentrations and antibiotic resistance in aquatic environments of the WHO Western Pacific and South-East Asia regions: A systematic review and probabilistic environmental hazard assessment. *The Lancet Planetary Health*, *7*(1), e45–e54. https://doi.org/10.1016/S2542-5196(22)00254-6

Janda, J. M., & Abbott, S. L. (2010). The genus Aeromonas: Taxonomy, pathogenicity, and infection. *Clinical Microbiology Reviews*, *23*(1), 35–73. https://doi.org/10.1128/CMR.00039-09

Jang, J., Hur, H.-G., Sadowsky, M. j., Byappanahalli, M. n., Yan, T., & Ishii, S. (2017). Environmental Escherichia coli: Ecology and public health implications—a review. *Journal of Applied Microbiology*, *123*(3), 570–581. https://doi.org/10.1111/jam.13468

Keely, S. P., Brinkman, N. E., Wheaton, E. A., Jahne, M. A., Siefring, S. D., Varma, M., Hill, R. A., Leibowitz, S. G., Martin, R. W., Garland, J. L., & Haugland, R. A. (2022). Geospatial Patterns of Antimicrobial Resistance Genes in the US EPA National Rivers and Streams Assessment Survey. *Environmental Science & Technology*, *56*(21), 14960–14971. https://doi.org/10.1021/acs.est.2c00813

Kümmerer, K. (2009). Antibiotics in the aquatic environment – A review – Part I. *Chemosphere*, *75*(4), 417–434. https://doi.org/10.1016/j.chemosphere.2008.11.086

Larsson, D. G. J., de Pedro, C., & Paxeus, N. (2007). Effluent from drug manufactures contains extremely high levels of pharmaceuticals. *Journal of Hazardous Materials*, *148*(3), 751–755. https://doi.org/10.1016/j.jhazmat.2007.07.008

Larsson, D. G. J., & Flach, C.-F. (2022). Antibiotic resistance in the environment. *Nature Reviews Microbiology*, *20*(5), 257–269. https://doi.org/10.1038/s41579-021-00649-x

Li, J., Cheng, W., Xu, L., Jiao, Y., Baig, S. A., & Chen, H. (2016). Occurrence and removal of antibiotics and the corresponding resistance genes in wastewater treatment plants: Effluents' influence to downstream water environment. *Environmental Science and Pollution Research International*, *23*(7), 6826–6835. https://doi.org/10.1007/s11356-015-5916-2

Liguori, K., Calarco, J., Maldonado Rivera, G., Kurowski, A., Keenum, I., Davis, B. C., Harwood, V. J., & Pruden, A. (2023). Comparison of Cefotaxime-Resistant Escherichia coli and sul1 and intI1 by qPCR for Monitoring of Antibiotic Resistance of Wastewater,

Surface Water, and Recycled Water. *Antibiotics*, *12*(8), Article 8. https://doi.org/10.3390/antibiotics12081252

Lu, J., Breitwieser, F. P., Thielen, P., & Salzberg, S. L. (2017). Bracken: Estimating species abundance in metagenomics data. *PeerJ Computer Science*, *3*, e104. https://doi.org/10.7717/peerj-cs.104

Luo, Y., Yang, F., Mathieu, J., Mao, D., Wang, Q., & Alvarez, P. J. J. (2014). Proliferation of Multidrug-Resistant New Delhi Metallo-β-lactamase Genes in Municipal Wastewater Treatment Plants in Northern China. *Environmental Science & Technology Letters*, *1*(1), 26–30. https://doi.org/10.1021/ez400152e

Maguire, M., Serna, C., Montero Serra, N., Kovarova, A., O'Connor, L., Cahill, N., Hooban, B., DeLappe, N., Brennan, W., Devane, G., Cormican, M., Morris, D., Coughlan, S. C., Miliotis, G., Gonzalez-Zorn, B., & Burke, L. P. (2024). Spatiotemporal and genomic analysis of carbapenem resistance elements in Enterobacterales from hospital inpatients and natural water ecosystems of an Irish city. *Microbiology Spectrum*, *13*(1), e00904-24. https://doi.org/10.1128/spectrum.00904-24

Mao, D., Yu, S., Rysz, M., Luo, Y., Yang, F., Li, F., Hou, J., Mu, Q., & Alvarez, P. J. J. (2015). Prevalence and proliferation of antibiotic resistance genes in two municipal wastewater treatment plants. *Water Research*, *85*, 458–466. https://doi.org/10.1016/j.watres.2015.09.010

Marutescu, L. G., Popa, M., Gheorghe-Barbu, I., Barbu, I. C., Rodríguez-Molina, D., Berglund, F., Blaak, H., Flach, C.-F., Kemper, M. A., Spießberger, B., Wengenroth, L., Larsson, D. G. J., Nowak, D., Radon, K., De Roda Husman, A. M., Wieser, A., Schmitt, H., Pircalabioru Gradisteanu, G., Vrancianu, C. O., & Chifiriuc, M. C. (2023). Wastewater

treatment plants, an “escape gate” for ESCAPE pathogens. *Frontiers in Microbiology*, *14*, 1193907. https://doi.org/10.3389/fmicb.2023.1193907

Mikheenko, A., Prjibelski, A., Saveliev, V., Antipov, D., & Gurevich, A. (2018). Versatile genome assembly evaluation with QUAST-LG. *Bioinformatics*, *34*(13), i142–i150. https://doi.org/10.1093/bioinformatics/bty266

Mills, M., Mollenkopf, D., Wittum, T., Sullivan, M. P., & Lee, J. (2024). One Health Threat of Treated Wastewater Discharge in Urban Ohio Rivers: Implications for Surface Water and Fish Gut Microbiome and Resistome. *Environmental Science & Technology*, *58*(30), 13402–13414. https://doi.org/10.1021/acs.est.3c09070

Oguri, T., Furuyama, T., Okuno, T., Ishii, Y., Tateda, K., Bonomo, R. A., & Shimizu-Ibuka, A. (2014). Crystal Structure of Mox-1, a Unique Plasmid-Mediated Class C β-Lactamase with Hydrolytic Activity towards Moxalactam. *Antimicrobial Agents and Chemotherapy*, *58*(7), 3914–3920. https://doi.org/10.1128/AAC.02363-13

Parks, D. H., Imelfort, M., Skennerton, C. T., Hugenholtz, P., & Tyson, G. W. (2015). CheckM: Assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Research*, *25*(7), 1043–1055. https://doi.org/10.1101/gr.186072.114

Pholwat, S., Liu, J., Taniuchi, M., Chinli, R., Pongpan, T., Thaipisutikul, I., Ratanakorn, P., Platts-Mills, J. A., Fleece, M., Stroup, S., Gratz, J., Mduma, E., Mujaga, B., Walongo, T., Nshama, R., Kimathi, C., Foongladda, S., & Houpt, E. R. (2019). Genotypic antimicrobial resistance assays for use on E. coli isolates and stool specimens. *PLOS ONE*, *14*(5), e0216747. https://doi.org/10.1371/journal.pone.0216747

Ping, Q., Zhang, Z., Ma, L., Yan, T., Wang, L., & Li, Y. (2022). The prevalence and removal of antibiotic resistance genes in full-scale wastewater treatment plants: Bacterial host, influencing factors and correlation with nitrogen metabolic pathway. *Science of The Total Environment*, *827*, 154154. https://doi.org/10.1016/j.scitotenv.2022.154154

Prjibelski, A., Antipov, D., Meleshko, D., Lapidus, A., & Korobeynikov, A. (2020). Using SPAdes De Novo Assembler. *Current Protocols in Bioinformatics*, *70*(1), e102. https://doi.org/10.1002/cpbi.102

Purohit, M. R., Chandran, S., Shah, H., Diwan, V., Tamhankar, A. J., & Stålsby Lundborg, C. (2017). Antibiotic Resistance in an Indian Rural Community: A 'One-Health' Observational Study on Commensal Coliform from Humans, Animals, and Water. *International Journal of Environmental Research and Public Health*, *14*(4), Article 4. https://doi.org/10.3390/ijerph14040386

Read, D. S., Gweon, H. S., Bowes, M. J., Anjum, M. F., Crook, D. W., Chau, K. K., Shaw, L. P., Hubbard, A., AbuOun, M., Tipper, H. J., Hoosdally, S. J., Bailey, M. J., Walker, A. S., & Stoesser, N. (2024). Dissemination and persistence of antimicrobial resistance (AMR) along the wastewater-river continuum. *Water Research*, *264*, 122204. https://doi.org/10.1016/j.watres.2024.122204

Reinthaler, F. F., Posch, J., Feierl, G., Wüst, G., Haas, D., Ruckenbauer, G., Mascher, F., & Marth, E. (2003). Antibiotic resistance of E. coli in sewage and sludge. *Water Research*, *37*(8), 1685–1690. https://doi.org/10.1016/S0043-1354(02)00569-9

Robinson, T. P., Bu, D. P., Carrique-Mas, J., Fèvre, E. M., Gilbert, M., Grace, D., Hay, S. I., Jiwakanon, J., Kakkar, M., Kariuki, S., Laxminarayan, R., Lubroth, J., Magnusson, U., Thi Ngoc, P., Van Boeckel, T. P., & Woolhouse, M. E. J. (2016). Antibiotic resistance is

the quintessential One Health issue. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *110*(7), 377–380. https://doi.org/10.1093/trstmh/trw048

Rocha, J., Fernandes, T., Riquelme, M. V., Zhu, N., Pruden, A., & Manaia, C. M. (2019). Comparison of Culture- and Quantitative PCR-Based Indicators of Antibiotic Resistance in Wastewater, Recycled Water, and Tap Water. *International Journal of Environmental Research and Public Health*, *16*(21), Article 21. https://doi.org/10.3390/ijerph16214217

Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sànchez-Melsió, A., Borrego, C. M., Barceló, D., & Balcázar, J. L. (2015). Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. *Water Research*, *69*, 234–242. https://doi.org/10.1016/j.watres.2014.11.021

Smalla, K., Cook, K., Djordjevic, S. P., Klümper, U., & Gillings, M. (2018). Environmental dimensions of antibiotic resistance: Assessment of basic science gaps. *FEMS Microbiology Ecology*, *94*(12), fiy195. https://doi.org/10.1093/femsec/fiy195

The structure of β-lactamases. (1980). *Philosophical Transactions of the Royal Society of London. B, Biological Sciences*, *289*(1036), 321–331. https://doi.org/10.1098/rstb.1980.0049

Weiner-Lastinger, L. M., Abner, S., Edwards, J. R., Kallen, A. J., Karlsson, M., Magill, S. S., Pollock, D., See, I., Soe, M. M., Walters, M. S., & Dudeck, M. A. (2020). Antimicrobial-resistant pathogens associated with adult healthcare-associated infections: Summary of data reported to the National Healthcare Safety Network, 2015–2017. *Infection Control & Hospital Epidemiology*, *41*(1), 1–18. https://doi.org/10.1017/ice.2019.296

Wood, D. E., Lu, J., & Langmead, B. (2019). Improved metagenomic analysis with Kraken 2. *Genome Biology*, *20*(1), 257. https://doi.org/10.1186/s13059-019-1891-0

Andrews S. (2010). FastQC: a quality control tool for high throughput sequence data. Available online at:

Ashbolt, N. J., Pruden, A., Riquelme, M. V., Maile-Moskowitz, A., & Miller, J. H. Antibiotic resistance as a global sanitation "Pathogen". In *Global Water Pathogens Project (GWPP)*. 2, ebook.

Ed Topp, David W. Graham, Teresa Coque, Anthony So, and Amy Pruden. (2023). United Nations Environment Programme. Bracing for Superbugs: Strengthening environmental action in the One Health response to antimicrobial resistance. Geneva. https://www.unep.org/resources/superbugs/environmental-action

Soto Gonzalez, S. (2024). One health: How to achieve optimal health for people, animals and our planet. [Blog post] ISGlobal https://www.isglobal.org/en/healthisglobal/-/custom-blog-portlet/one-health-una-sola-salud-o-como-lograr-a-la-vez-una-salud-optima-para-las-personas-los-animales-y-nuestro-planeta/90586/0

Supplemental Information

Table S1: Unique ARGs for Each Sampling Site

Sampling Site	Unique ARGs	Resistance Mechanism
	vgaC, vgaALC	antibiotic target protection
	VEB-26, VEB-17	antibiotic inactivation
	vatD	antibiotic inactivation
	vanYF, vanXYG, vanXB, vanTC, vanSB	antibiotic target alteration
	tva(A)	antibiotic target protection
	tetU, tet(J), tet(57)*	antibiotic efflux, antibiotic target protection*
	TEM-88, TEM-81, TEM-8, TEM-225, TEM-177, TEM-163, TEM-142, TEM-132, TEM-122	antibiotic inactivation
	tcr3	antibiotic efflux
	SLB-1	antibiotic inactivation
	SHV-9, SHV-73, SHV-70, SHV-6, SHV-52, SHV-22, SHV-199, SHV-187, SHV-182, SHV-180, SHV-16, SHV-156, SHV-152, SHV-126, SHV-125, SHV-124, SHV-123, SHV-100	antibiotic inactivation
	QnrS7, QnrS12, QnrB72, QnrB67, QnrB58, QnrB56, QnrB55, QnrB49, QnrB30, QnrB28, QnrB11	antibiotic target protection
	qacB, qacA	antibiotic efflux
	pmrA	antibiotic efflux
	PLA-6, PLA-3	antibiotic inactivation
	PDC-386, PDC-295	antibiotic inactivation
	OXY-6-4, OXY-6-3, OXY-6-1, OXY-5-2, OXY-5-1, OXY-2-9, OXY-2-8, OXY-2-3, OXY-2-15	antibiotic inactivation
	OXA-948, OXA-935, OXA-932, OXA-919, OXA-907, OXA-844, OXA-842, OXA-836, OXA-835, OXA-827, OXA-826, OXA-820, OXA-801, OXA-778, OXA-761, OXA-749, OXA-74, OXA-737, OXA-736, OXA-72, OXA-692, OXA-679, OXA-676, OXA-665, OXA-661, OXA-651, OXA-60, OXA-59,	antibiotic inactivation

	OXA-563, OXA-558, OXA-546, OXA-544, OXA-537, OXA-527, OXA-512, OXA-510, OXA-500, OXA-496, OXA-443, OXA-43, OXA-422, OXA-418, OXA-401, OXA-377, OXA-371, OXA-368, OXA-362, OXA-349, OXA-330, OXA-329, OXA-316, OXA-307, OXA-304, OXA-297, OXA-295, OXA-294, OXA-287, OXA-286, OXA-285, OXA-279, OXA-272, OXA-271, OXA-27, OXA-267, OXA-25, OXA-243, OXA-225, OXA-207, OXA-19, OXA-183, OXA-182, OXA-166, OXA-148, OXA-143, OXA-134, OXA-133, OXA-125, OXA-111, OXA-11, OXA-103, OXA-1	
	OKP-B-3, OKP-B-24, OKP-B-12, OKP-A-9	antibiotic inactivation
	OHIO-1	antibiotic inactivation
	OCH-7, OCH-1	antibiotic inactivation
	MUS-1	antibiotic inactivation
Influent	mphC	antibiotic inactivation
	MOR-2	antibiotic inactivation
	MOC-1	antibiotic inactivation
	MIR-6, MIR-18, MIR-14, MIR-12	antibiotic inactivation
	mecA	antibiotic target replacement
	MCR-6.1, MCR-3.8, MCR-3.7, MCR-3.41, MCR-3.2, MCR-3.11	antibiotic target alteration
	MAL-2	antibiotic inactivation
	lsa(D)	antibiotic target protection
	lnuE, lnuB	antibiotic inactivation
	LHK-3	antibiotic inactivation
	LEN-21	antibiotic inactivation
	LAT-1	antibiotic inactivation
	LAP-2	antibiotic inactivation
	KPC-81, KPC-66, KPC-65, KPC-22, KPC-10	antibiotic inactivation
	KLUC-5	antibiotic inactivation

IND-5, IND-10	antibiotic inactivation
IMP-51, IMP-26	antibiotic inactivation
IMI-13	antibiotic inactivation
hmrM	antibiotic efflux
GOB-4, GOB-31, GOB-23, GOB-2, GOB-12, GOB-10, GOB-1	antibiotic inactivation
GES-8, GES-42, GES-41, GES-37, GES-36, GES-30, GES-3, GES-28, GES-25, GES-23, GES-2, GES-15, GES-13 GES-10	antibiotic inactivation
FOX-17, FOX-1	antibiotic inactivation
FosD	antibiotic inactivation
FONA-2	antibiotic inactivation
FIM-1	antibiotic inactivation
FAR-1	antibiotic inactivation
ErmY, ErmA, Erm(33), Erm(31)	antibiotic target alteration
EC-18	antibiotic inactivation
EBR-4, EBR-3	antibiotic inactivation
DHA-2, DHA-19, DHA-18, DHA-17, DHA-16	antibiotic inactivation
dfrG, dfrD, dfrA8, dfrA27, dfrA25	antibiotic target replacement
CTX-M-94, CTX-M-84, CTX-M-82, CTX-M-8, CTX-M-78, CTX-M-76, CTX-M-68, CTX-M-60, CTX-M-58, CTX-M-42, CTX-M-38, CTX-M-34, CTX-M-31, CTX-M-30, CTX-M-241, CTX-M-226, CTX-M-219, CTX-M-205, CTX-M-161, CTX-M-151, CTX-M-137, CTX-M-132, CTX-M-107, CTX-M-106, CTX-M-102, CTX-M-101	antibiotic inactivation
CSA-1	antibiotic inactivation
CMY-84, CMY-66, CMY-6, CMY-51, CMY-50, CMY-5, CMY-41, CMY-35, CMY-20, CMY-136, CMY-135, CMY-116, CMY-115, CMY-112, CMY-110, CMY-100	antibiotic inactivation
cmlB	antibiotic efflux
CMH-6, CMH-5	antibiotic inactivation

CMA-2, CMA-1	antibiotic inactivation
clbA	antibiotic target alteration
CKO-1	antibiotic inactivation
CIA-4, CIA-3	antibiotic inactivation
cfrA	antibiotic target alteration
CFE-1	antibiotic inactivation
CARB-8, CARB-51, CARB-50, CARB-4, CARB-14, CARB-10	antibiotic inactivation
BPU-1	antibiotic inactivation
blaR1	antibiotic inactivation
BlaB-7, BlaB-16, BlaB-10, BlaB-1	antibiotic inactivation
BEL-1	antibiotic inactivation
APH(3")-Ia, APH(2")-Iva, APH(2")-If, ANT(3")-Ii-AAC(6')-IId	antibiotic inactivation
ADC-85, ADC-84, ADC-68, ADC-6, ADC-58, ADC-30, ADC-243, ADC-209, ADC-203, ADC-202, ADC-2, ADC-19, ADC-189, ADC-18, ADC-172, ADC-156, ADC-140, ADC-14, ADC-136, ADC-135, ADC-133, ADC-131, ADC-130, ADC-120, ADC-104	antibiotic inactivation
ACT-87, ACT-8, ACT-78, ACT-77, ACT-68, ACT-64, ACT-63, ACT-62, ACT-6, ACT-57, ACT-55, ACT-48, ACT-41, ACT-35, ACT-34, ACT-20, ACT-13	antibiotic inactivation
ACC-5, ACC-1d	antibiotic inactivation
aadA22	antibiotic inactivation
AAC(6')-Iid, AAC(6')-Ih, AAC(6')-Ig, AAC(6')-Ib9, AAC(6')-Ian, AAC(6')-Iaj, AAC(6')-Ia, AAC(6')-29b, AAC(6')-29a, AAC(3)-Via, AAC(3)-IIb	antibiotic inactivation
ykkC	antibiotic efflux
VIM-8, VIM-72, VIM-23, VIM-17	antibiotic inactivation
vanXA	antibiotic target alteration

	tmrB	reduced permeability to antibiotic
	STA-1	antibiotic inactivation
	SHV-35, SHV-197	antibiotic inactivation
	QnrB37	antibiotic target protection
	PDC-76, PDC-75, PDC-73, PDC-52, PDC-223, PDC-195, PDC-156, PDC-130	antibiotic target protection
	PC1	antibiotic inactivation
	OXA-677, OXA-4, OXA-396, OXA-324, OXA-282, OXA-153	antibiotic inactivation
	mphK	antibiotic inactivation
	mepR, mepA	antibiotic efflux
	lin	antibiotic inactivation
	IND-6	antibiotic inactivation
	IMP-45	antibiotic inactivation
Effluent	HERA-2	antibiotic inactivation
	GRD23-1	antibiotic inactivation
	GOB-13	antibiotic inactivation
	FosB2	antibiotic inactivation
	erm(40)	antibiotic target alteration
	DHA-7	antibiotic inactivation
	DfrA39	antibiotic target replacement
	CTX-M-64, CTX-M-124	antibiotic inactivation
	CMH-4	antibiotic inactivation
	CIA-1	antibiotic inactivation
	CGB-1	antibiotic inactivation
	APH(3")-Ic	antibiotic inactivation
	ANA-1	antibiotic inactivation

ADC-205	antibiotic inactivation
AAC(6')-Iy, AAC(6')-Iag, AAC(6')-Iaa	antibiotic inactivation

	YEM-1	antibiotic inactivation
	vanJ, vanE	antibiotic target alteration
	SIM-1	antibiotic inactivation
	SHV-137	antibiotic inactivation
	RSA-2	antibiotic inactivation
	PFM-2	antibiotic inactivation
	PDC-90, PDC-77, PDC-385, PDC-13, PDC-115	antibiotic inactivation
	OXA-914, OXA-899, OXA-574, OXA-573, OXA-550, OXA-54, OXA-363, OXA-283, OXA-22	antibiotic inactivation
	OKP-C-1	antibiotic inactivation
	LRA-5	antibiotic inactivation
Upstream	KPC-16	antibiotic inactivation
	IMP-42, IMP-25	antibiotic inactivation
	GOB-5	antibiotic inactivation
	ErmE, Erm(41), Erm(34)	antibiotic target alteration
	dfrK, dfrA18	antibiotic target replacement
	CTX-M-233, CTX-M-123	antibiotic inactivation
	CRH-3	antibiotic inactivation
	CMY-159	antibiotic inactivation
	apmA	antibiotic inactivation
	ADC-128	antibiotic inactivation
	AAC(6')-Ik, AAC(6')-I30, AAC(3)-VIIIa, AAC(3)-VIIa, AAC(3)-IIIa	antibiotic inactivation

	VIM-37	antibiotic inactivation
	vatA	antibiotic inactivation
	vanSF, vanN, vanHA	antibiotic target alteration
	tet(45)	antibiotic inactivation
	TEM-106	antibiotic inactivation
	SME-2	antibiotic inactivation
	SHV-134	antibiotic inactivation
	QnrB2, QnrA2	antibiotic target protection
	PER-8, PER-10	antibiotic inactivation
	PEDO-3	antibiotic inactivation
	PDC-435, PDC-349, PDC-248, PDC-151	antibiotic inactivation
	OXA-646, OXA-64, OXA-477, OXA-472, OXA-376, OXA-292, OXA-278, OXA-192	antibiotic inactivation
	otr(B)	antibiotic efflux
Downstream	OCH-2	antibiotic inactivation
	myrA	antibiotic target alteration
	mphN, mphH	antibiotic inactivation
	LUT-6	antibiotic inactivation
	LEN-9	antibiotic inactivation
	IMP-81, IMP-27, IMP-10	antibiotic inactivation
	GRD33-1	antibiotic inactivation
	GMB-1	antibiotic inactivation
	fusD	antibiotic target protection
	EXO	antibiotic inactivation
	ERP-1	antibiotic inactivation
	CVI-1	antibiotic inactivation

	CTX-M-74, CTX-M-4, CTX-M-172, CTX-M-134	antibiotic inactivation
	CMY-48, CMY-40	antibiotic inactivation
	CME-2	antibiotic inactivation
	Bla2	antibiotic inactivation
	armA	antibiotic target alteration
	ACT-19	antibiotic inactivation
	ACC-2, ACC-1	antibiotic inactivation
	aacA43	antibiotic inactivation
	AAC(6')-Iu, AAC(6')-Ij	antibiotic inactivation

Table S2: Physical chemical parameters

Sampling site	Month	Temperature	DO	Conductivity	pH	Cumulative precipitation
Influent	9	12.4	4.7	558	7.87	0.17
Effluent	9	16.7	7.6	591	7.44	0.17
Upstream	9	MD	MD	MD	MD	0.17
Downstream	9	14.8	8.49	1500	8.19	0.17
Influent	10	4.9	4.9	546	4.9	0
Effluent	10	13.4	8.6	656	8.6	0
Upstream	10	9.7	10.79	1087	10.79	0
Downstream	10	10.4	10.56	709	10.56	0
Influent	11	13.9	4.2	746	7.04	0.31
Effluent	11	13.4	9.2	664	6.66	0.31
Upstream	11	9	11.3	1075	7.7	0.31
Downstream	11	9.4	11.1	1671	7.8	0.31
Influent	12	13.2	4.5	659	7.01	1.43
Effluent	12	12.7	12.5	662	7.02	1.43
Upstream	12	10.5	12.3	391.1	7.58	1.43
Downstream	12	10.8	11.2	400.1	7.61	1.43
Influent	1	10.6	8.2	547	6.98	0.94
Effluent	1	9.7	12.1	509	6.97	0.94
Upstream	1	6.2	16	711	7.83	0.94
Downstream	1	6.4	10.95	841	7.95	0.94
Influent	2	11	4.7	526	7.23	0
Effluent	2	9.9	9.4	459.7	6.94	0
Upstream	2	8	13.3	527	7.75	0
Downstream	2	7.6	10.3	514	7.63	0
Influent	3	12.3	9.8	367.8	7.15	1.03
Effluent	3	12.1	7.76	371.8	6.72	1.03
Upstream	3	11	10.07	465.3	7.6	1.03
Downstream	3	11.2	10.08	466.9	7.63	1.03
Influent	4	11	5.5	357	7.29	0.46
Effluent	4	MD	7.7	398	7.24	0.46
Upstream	4	MD	9.7	763	8.03	0.46
Downstream	4	MD	9.6	753	7.96	0.46
Influent	5	16.7	5.5	539	7.52	1.89
Effluent	5	16.5	7.11	MD	7.03	1.89
Upstream	5	15.1	10.52	MD	7.74	1.89
Downstream	5	15.5	10.17	MD	7.7	1.89

Influent	6	5.6	9.6	652.3	6.87	0.67
Effluent	6	16.1	8	501	7.16	0.67
Upstream	6	8.9	12.9	765	7.75	0.67
Downstream	6	15.6	8.7	833	7.78	0.67
Influent	7	18.4	2.7	680	7.28	0.26
Effluent	7	18.7	7.1	638	7.61	0.26
Upstream	7	18.7	7.7	1187	8.11	0.26
Downstream	7	17.9	7.5	1152	8.17	0.26
Influent	8	9.5	4.6	563	7.76	0.85
Effluent	8	19	4.3	751	7.78	0.85
Upstream	8	12.1	11	1022	8.24	0.85
Downstream	8	19.9	8.1	1125	8.33	0.85

*MD: missing data

Table S3: Primers/Probes and Annealing Temperature for targeted genes

Target Gene	Primer and Probes	Annealing Temperature	Reference
intiI	F: GCC TTG ATG TTA CCC GAG AG R: GAT CGG TCG AAT GCG TGT P: (6-FAM) ATT CCT GGC CGT GGT TCT GGG TTT T (BHQ1)	60 ℃	(Barraud et al., 2010)
blaCTX-M-1	F: CCGTCACGCTGTTRTTAGGA R: AATGCCACMCCCAGYCKKCC P: FAM-CAGCAAAAACTTGCCGRATT-MGB	59 ℃	(Pholwat et al., 2019)
16S rRNA	F: CGGTGAATACGTTCYCGG R: GGWTACCTTGTTACGACTT	51℃	(Rocha et al., 2019)