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The influence of urbanization and water reclamation plants on fecal indicator bacteria and antibiotic resistance in the Los Angeles River watershed: A case study with complementary monitoring methods

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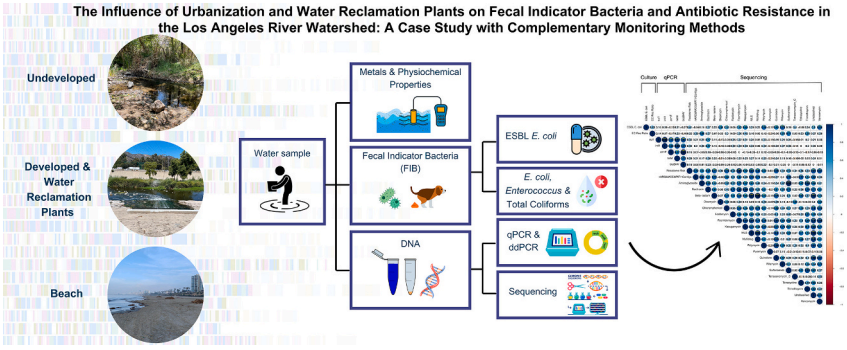
HIGHLIGHTS

- ARGs, fecal indicator bacteria, and human fecal markers increase in abundance through an urbanization gradient.
- Water reclamation plants increase ARGs, reduce fecal indicator bacteria, and increase resistant *E. coli* and *P. aeruginosa*.
- A low-cost culture based screening tool identified AR hotspots, confirmed by qPCR- and metagenomics-based techniques.

GRAPHICAL ABSTRACT



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ABSTRACT

Urban land use and water reclamation plants (WRPs) can impact fecal indicator bacteria (FIB) and antimicrobial resistance (AMR) in coastal watersheds. However, there is a lack of studies exploring these effects on the US West Coast. Additionally, there is limited research using a complementary approach across culture-, qPCR-, and metagenomics-based techniques for characterizing environmental AMR. In this study, sixteen locations were sampled in the Los Angeles River, encompassing both upstream and downstream of three WRPs discharging into the river. Culture-dependent methods quantified *Enterococcus*, total coliforms, *E. coli*, and extended spectrum beta-lactamase-producing *E. coli* as a low-cost screening tool for AMR, while qPCR measured selected antibiotic resistance genes (ARGs): *sul*1, *erm*F, *tet*W, *bla*SHV, along with *intI*1 and 16S rRNA genes. Bacteroides HF183 and crAssphage markers were quantified via ddPCR. All samples underwent shotgun sequencing to investigate gene abundance and mobility and an overall risk score for AMR. Results reveal downstream sites contain ARGs at least

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two orders of magnitude greater than upstream locations. Developed areas had the highest ARG sequence abundances and the most ARG classes as indicated by metagenomic analysis. WRP effluent exhibited elevated ARGs and co-location of ARGs, mobile genetic elements, and pathogens. A culture-based assessment of AR in *E. coli* and *Pseudomonas aeruginosa* revealed increased resistance ratios for most antibiotics from upstream to downstream a WRP discharge point. This study highlights the impacts of land use and WRPs on ARGs and FIB, offering a multi-pronged analysis of AMR.

1. Introduction

Antibiotic resistance is deemed a formidable challenge to global public health. Each year in the United States alone, over 2.8 million infections and over 35,000 deaths are attributed to antibiotic-resistant bacteria (ARB) (CDC, 2019). Despite the known risk of antibiotics causing resistance in clinical isolates in as little as a year (Ventola, 2015), antibiotics are still extensively used in humans and animals. Environmental factors such as the presence of antibiotics, detergents, and heavy metals, as well as temperature, pH, and nutrient loadings mediate the emergence, persistence, and dissemination of antibiotic resistance genes (ARGs) in bacteria (Bombaywala et al., 2021; Vikesland et al., 2017). ARGs can be transferred among bacteria through generations via vertical gene transfer (VGT) and through horizontal gene transfer (HGT) between different species of bacteria present in a location (Narciso-Da-Rocha et al., 2014). Mobile genetic elements (MGEs) such as integrons, plasmids, and transposons facilitate the transfer of ARGs among opportunistic microorganisms (Ma et al., 2017; Partridge et al., 2018). Due to the health threats to the public, research studies are quantifying and characterizing ARGs and ARB across various environmental compartments. However, despite the recognition of these compartments as hotspots for the proliferation and dissemination of antibiotic resistance, there is currently no standard routine monitoring method. Of these environmental compartments, studies on ARGs and ARB in surface waters, especially in populous coastal areas, are increasing (A. A. Hatha et al., 2020; Zheng et al., 2021).

Anthropogenic pollution plays a substantial role in the dissemination of antibiotic resistance in water bodies. Key contributors include discharge from wastewater treatment plants (WWTPs) (Eramo et al., 2019; Lee et al., 2022; Miłobedzka et al., 2022; Proia et al., 2018; Sabri et al., 2020; Tang et al., 2021; Wang et al., 2021), livestock operations, agriculture, and land applications of manure (Barrios et al., 2020; Chee-Sanford et al., 2009; Cira et al., 2021; He et al., 2020; Hung et al., 2022; Jacobs et al., 2019; West et al., 2011), and hospitals (Hassoun-Kheir et al., 2020; Hocquet et al., 2016; Narciso-Da-Rocha et al., 2014; Paulus et al., 2019; Petrovich et al., 2020). Larger and denser cities amplify the impacts of antibiotic resistance in surface waters (Xu et al., 2016), with urban runoff contributing significant amounts of chemical pollutants, bacteria, sediments, and nutrients in water (Almakki et al., 2019). Direct discharge sources from WWTPs were found to contribute to ARG loadings in urban rivers in Beijing, China (Xu et al., 2016). Sewage was found to greatly influence ARG reservoirs in coastal waters in the capital of Uruguay (Fresia et al., 2019). Human fecal pollution from municipal wastewater discharges was the dominant factor shaping ARG patterns along the Danube River (Schachner-Groehs et al., 2024). Though many studies have been conducted on quantifying antibiotic resistance in surface waters, few have been conducted in large cities in the coast of the western United States (Zheng et al., 2021) or tested existing frameworks and standardized methods for this purpose (Borchardt et al., 2021; Davis et al., 2020; Keenum et al., 2022). HF183 and crAssphage are commonly used microbial source tracking (MST) markers for human fecal pollution to improve detection of health-relevant microbial pollution. Current research demonstrated that crAssphage and HF183 associated well with broad fecal indicator bacteria in sewage and surface water, such as *E. coli* and enterococci (Ahmed et al., 2021; Stachler et al., 2019). Moreover, investigations have revealed the co-occurrence of crAssphage and antibiotic resistance genes (ARGs) in water bodies impacted by wastewater (Ahmed et al., 2018; Karkman et al., 2019).

A variety of techniques are employed to quantify and characterize antibiotic resistance. The most prevalent technologies for monitoring environmental antibiotic resistance in water include microbial source tracking, qPCR, whole genome sequencing, metagenomics, and culture-based methods (Ishii, 2020; Miłobedzka et al., 2022; Sanderson et al., 2016). Each method has its advantages and disadvantages. Culture-based methods allow for isolation of viable target organisms (McLain et al., 2016), but are time and labor intensive, and many organisms are unculturable in laboratory settings (Ishii, 2020; Sukhum et al., 2019). A potential exists for widespread surveillance of antibiotic resistance of *E. coli* in surface waters using a modified version of the IDEXX Colilert method with the use of added antibiotics (Hornsby et al., 2022). Amplification methods, such as qPCR, exhibit greater sensitivity compared to metagenomics; however, they demand more extensive effort to cover a broad range of genes and taxonomic markers (Ferreira et al., 2023). Based on the PCR technique and fluorescent signals, qPCR faces challenges such as PCR bias, complicating direct performance comparisons across different laboratories (Mao et al., 2024). Metagenomics is thought to be superior in identifying ARGs in complex environmental and clinical metagenomes using an array of databases, but is a costly method (Guitor et al., 2020; Vikesland et al., 2017). Some experts argue for antibiotic susceptibility tests for phenotypic data and suggest combining these data with molecular analysis from whole genome sequencing (Diallo et al., 2020; Sukhum et al., 2019). However, there is currently no standard method to quantify antibiotic resistance or standard ARG unit (Nguyen et al., 2021), and dose and response models for ARB are still being explored (Chandrasekaran and Jiang, 2019).

Wastewater (WW) is a well known source of AR to environmental receiving waters. Many WW-associated bacteria are not well-suited for growth in the environment; however, they can be a source of genes that can be transmitted by horizontal gene transfer (HGT). Thus, it is important to characterize the potential for propagation of ARGs to environmental microbial populations. *E. coli* is highly associated with WW but also grows well in the environment, so inputs of this bacterium involve the potential for long-term survival and propagation of AR. *P. aeruginosa* is not considered prevalent in the human gut but is nearly ubiquitous in surface waters and sediments. Changes to the resistance profile of environmental bacteria (not associated with WW) above and below WWTPs has been hypothesized as a method for indicating impact of the WWTP on environmental AR by HGT (Milligan et al., 2023).

This study aimed to: 1) apply an existing framework for monitoring antibiotic resistance in watersheds (Davis et al., 2020) across a highly urbanized watershed; and 2) cross-validate between culture, qPCR, and metagenomic techniques along an urbanization gradient in Los Angeles, California—one of the most populous cities in the United States. The Los Angeles River (LAR) watershed was evaluated for ARGs, fecal indicator bacteria, and Extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* in different land use contexts. An additional sampling event took place at one of the WRPs during a wet weather event for a suite of antibiotic resistant bacteria. qPCR was used to quantify *sul*1, *tet*W, *intI*1, *erm*F, bla_{SHV}, and16S rRNA genes, while ddPCR was used to quantify HF183 and crAssphage. Samples were shotgun sequenced to compare them against relevant antibiotic resistance databases, calculate ARG abundances, and resistome risk scores. Water samples were taken in the river at points above and below three different water reclamation plants (WRPs), swimming and kayak sites, and from beaches near the

coastal pour point using a snapshot study approach (Davis et al., 2020). This study is the first of its kind in the LAR watershed and provides critical insights to antibiotic resistance dynamics in an urban watershed, informing future research and policy.

2. Materials and methods

2.1. Study area

The Los Angeles River (LAR) watershed is approximately 824 mi^2 in size, and is home to roughly 4.5 million people. The LAR is 51 miles long and is bounded by the San Gabriel, Santa Susana, and Santa Monica Mountains in the west and north, the San Gabriel River Watershed in the east, and the Pacific Ocean in the south. The river discharges at the San Pedro/Los Angeles and Long Beach Harbor complex, which has a semi-enclosed breakwater of 7.5 miles. The river's estuary is about three miles from where it meets Queensway Bay.

The land use within the LAR watershed is diverse with 324 mi^2 being open space and forest and the remaining being highly developed residential, industrial, and commercial areas. The land use distribution is as follows: 35 % residential, 5 % commercial, 8 % industrial, and 51 % open land (Ackerman et al., 2003). Most of the river in the developed area is lined with concrete, but some areas in the Glendale Narrows and Sepulveda Flood Control Basin are unlined to maintain riparian habitat.

Currently, the majority of the LAR's water volume stems from three WRPs, supplemented by additional inputs from runoff and groundwater upwelling. Approximately 27.1 million gallons per day (MGD) originate from the DC Tillman WRP, 7.8 MGD from the LA Glendale WRP, 4.5 MGD from the Burbank WRP, and 3.6 MGD from groundwater upwelling, with an additional 0.032 to 7.8 MGD from runoff (LA City Sanitation, 2018). All WRPs utilize tertiary treatment technologies to process municipal and industrial wastes before discharging treated effluent into the river. Notably, Burbank and LA Glendale WRPs discharge directly in the river, while the Tillman WRP partially discharges into a nearby garden and lake system before reaching the river.

The LAR has multiple recreational areas for kayaking, fishing, and swimming. Two recreation zones exist along the main stem of the river—the Elysian Valley and Sepulveda Basin LA River Recreation Zones (Mountains Recreation and Conservation Authority, n.d.). Reportedly, thousands of people swim in unpermitted and designated areas in the LAR [49]. Most recreational swimming sites are located in the upper LAR watershed in the Hansen Dam and the Angeles National Forest.

2.2. Sample collection and filtration

Sample collection was carried out at sixteen sites featuring a range of land uses as illustrated in Fig. 1. Five locations were above and below the three WRPs (Tillman Above, Tillman Below, Burbank Below, Glendale Above, Glendale Below), with exception of above the Burbank WRP due to no flow. Three kayaking sites (Sepulveda Dam, Rattlesnake Park,

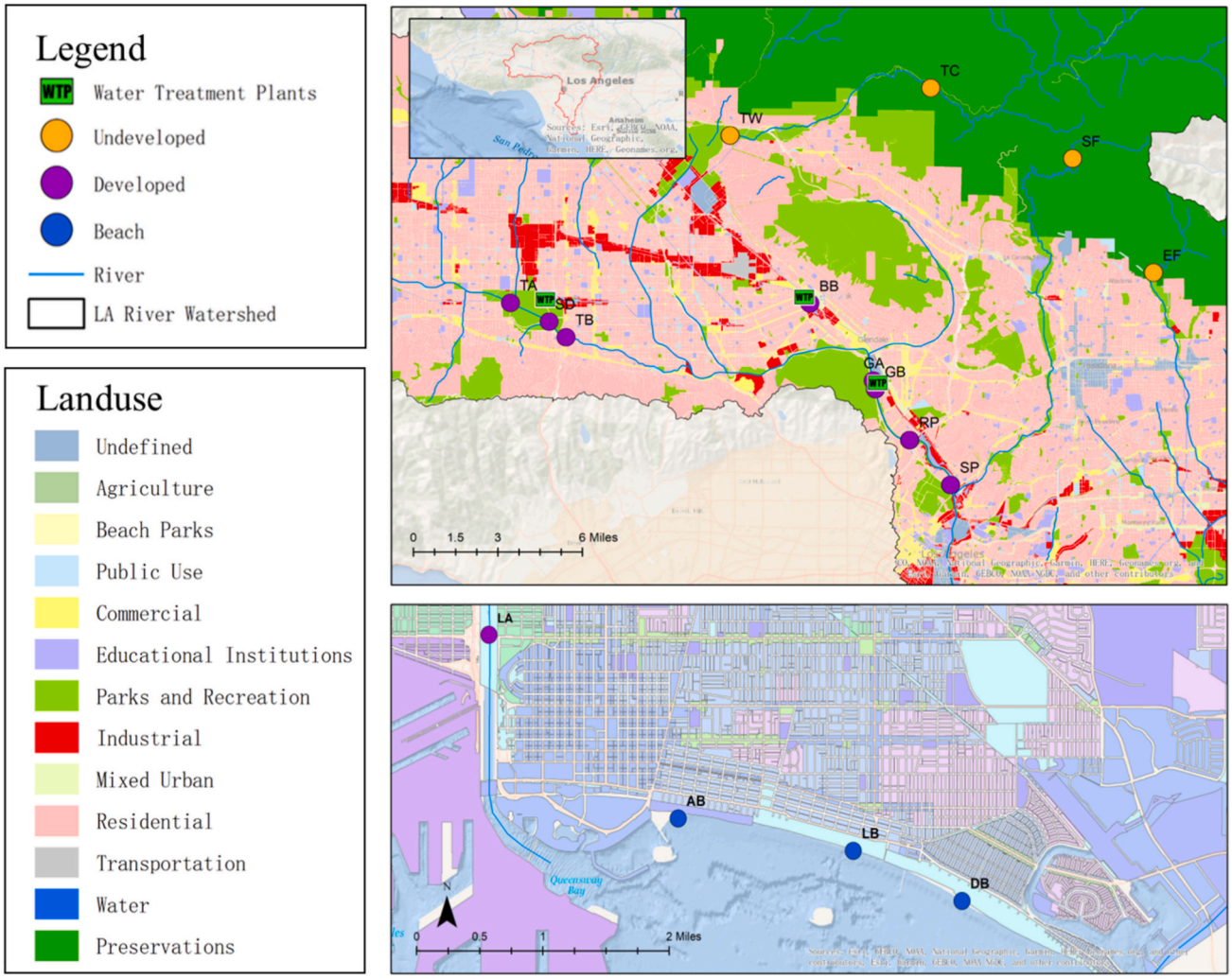

Fig. 1. Map of Land use in the Los Angeles River watershed and sampling locations.

Steelhead Park) and four swim sites (Eaton Canyon Falls, Switzer Canyon Falls, Big Tujunga Creek, and Tujunga Wash) were sampled. Three beaches (Alamitos Beach, Long Beach City Beach, and Rosie's Dog Beach) and one estuary/tidal site (LA River in Long Beach) were included where the LAR discharges to the ocean.

Each site was classified as "developed", "undeveloped," and "beach" based on a land use map of Los Angeles County. Coordinates, land use designations, and sampling dates for each location are provided in Table S1 (Supporting Information). Site locations were informed by water quality sample locations used by the Council for Watershed Health (Council for Watershed Health, 2020) and Heal the Bay for their river and beach report cards (Heal the Bay, 2020a, 2020b).

Sample collection occurred during a one-week timeframe between October and November 2021. Follow-up samples were collected in Glendale for further culture-based analysis on 11/18/22, 2/26/23, and 8/29/23. On October 25, 2021, the Burbank-Glendale weather station recorded 0.64 in. of rain and a total of 0.75 in. of rain in October 2021. These precipitation events were the first of the winter season and provided the opportunity to understand the impacts of runoff in the LAR watershed which is known to increase antibiotic resistance in surface waters (Carney et al., 2019; Lee et al., 2020). Water sample collection occurred during the early morning hours to ensure minimal UV solar radiation. At each site, four liters of water were collected in sterile polypropylene bottles, which were pre-rinsed three times with ambient water before collection. Samples were then transported on ice (4 °C) to the laboratory, and processed within six hours of collection. Water temperature, pH, conductivity, dissolved oxygen, and turbidity were measured for each location using a multiparameter sonde (Hydrolab HL4, OTT Hydromet, Loveland, CO).

Upon arrival at the laboratory, water samples were filtered in at least triplicates on 0.2 μm polycarbonate filters (MilliporeSigma, Burlington, MA). The volume of water necessary to clog the filter ranged from 100 to 600 mL per sample and was recorded for each replicate. Filters were stored in 2 mL screw cap tubes with flame sterilized tweezers and fixed with 50 % ethanol. Samples were stored at −20 °C prior to DNA extraction. A phosphate buffer solution blank was filtered and stored during each sampling event.

2.3. DNA extraction, qPCR, and ddPCR

Ethanol-fixed filters were cut into approximately 1 cm^2 pieces with flame-sterilized scissors and transferred into lysing matrix tubes from the FastDNA SPIN Kit for Soil (MP Biomedicals, Irvine, CA). The residual ethanol solution was subjected to centrifugation at 5000 x*g* for 10 min before being resuspended with the sodium phosphate buffer provided in the DNA kit, and then added to the lysing matrix tube. Extraction procedures were carried out according to manufacturer's instructions opting for the longest recommended times for incubation, mixing, and settling. An extraction blank was processed with each extraction batch. Total DNA concentrations and 260/280 absorbance ratios were determined through spectrophotometry via a NanoDrop 2000c (Thermo Scientific, Waltham, MA).

All samples were analyzed for ARG abundance of *sul*1 (resistance to sulfonamides), *tet*W (resistance to tetracyclines), bla_{SHV} (resistance to beta-lactams), and *erm*F (resistance to macrolides). The *intI*1 gene was quantified as a proxy for anthropogenic pollution and the 16S rRNA gene served as a surrogate for total bacteria. Gene target sequences and cycling conditions are available in Table S2, Supporting Information. qPCR amplification was performed using the StepOnePlus system (Applied Biosystems, Foster City, CA) in 25 μL reaction volumes containing 12.5 μL PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, CA), 1.25 μL of each forward and reverse primer, 2 μL of template DNA, and molecular grade water for the remaining volume for all genes except 16S rRNA. The 16S rRNA gene was performed in a 20 μL reaction volume with 10 μL of PowerUp SYBR Green Master Mix, 1 μL of each forward and reverse primer, 3 μL of template DNA, and molecular grade water for the remaining volume. All assays were performed in 96-well plates. At least a five-point standard curve was run with each plate utilizing double-stranded gBlock gene fragments resuspended according to manufacturer instructions (IDT, Coralville, IA) and quantified on the NanoDrop 2000c (Thermo Scientific, Waltham, MA). Ten-fold dilutions were carried out for the 16S rRNA gene to uncover the presence of PCR inhibitors. The accepted minimum qPCR efficiency was 83 % and the lowest R^2 value was 0.997. The limit of detection was based on the lowest standard per assay (Borchardt et al., 2021; Bustin et al., 2009). The data for regular qPCR were analyzed in StepOne Software v2.3. The MIQE guidelines for the qPCR assays are located in Table S3, Supporting Information.

Molecular quantification of human associated fecal maker targets was conducted using a Bio-Rad QX200 Droplet Digital PCR System (Bio-Rad Laboratories, Hercules, CA). The assay primers targeting crAssphage and HF183/BacR287 molecular indicator regions were adapted and performed (Table S4, Supporting Information). 20-μL reactions were made using 4× Supermix of Probes (Bio-Rad Laboratories, Hercules, CA). Targets were analyzed in duplex. Droplets were generated with a Bio-Rad QX200 Auto Droplet Generator, and amplification was performed using a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's recommendations: 94 °C for 10 min, followed by 40 cycles of 94 °C for 30 s, then 60 °C for 1 min. At least two no template control (NTC) assays were performed for each assay by replacing the DNA with molecular grade water. Droplets were read on a QX200 Droplet Reader (Bio-Rad Laboratories, Hercules, CA). QA/QC was performed for all samples. Samples that yielded <10,000 accepted droplets were excluded from data analysis. Copy number concentrations were calculated by normalizing the data to 100 mL of sampled water to compare all markers on the same scale. The MIQE guidelines for the ddPCR assays are located in Table S5, Supporting Information.

2.4. FIB, heavy metal analysis, and ESBL E. coli enumeration

Enumeration of fecal indicator bacteria (FIB) involves the quantification of total coliforms, *Escherichia coli* (Colilert-18, IDEXX), and *Enterococci* (Enterolert, IDEXX) bacteria using standard methods and kits (IDEXX Laboratories, Westbrook, ME). Final concentrations were reported in Most Probable Number (MPN) per 100 mL. Marine samples and samples along the main stem of the river were diluted 10-fold and up to 1000-fold, respectively, according to manufacturer recommendations.

For quantifying extended-spectrum beta-lactamase (ESBL) *E. coli*, 100 μL of 1 mg/mL cefotaxime was added to each prepared 100 mL IDEXX bottle with Colilert-18 media (Hornsby et al., 2022). Samples were diluted at most 100-fold.

Heavy metals were quantified in the water samples using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) for Copper (Cu), Nickel (Ni), Chromium (Cr), Manganese (Mn), Iron (Fe), Vanadium (V), and Aluminum (Al), with the concentrations expressed in mg/L.

2.5. Percent antibiotic resistant for Pseudomonas aeruginosa, E. coli, and enterococci

Follow-up samples were taken in Glendale during dry weather (2/26/23, and 8/29/23) and wet weather (11/18/22) periods. Dry weather reflected the usual state of the Los Angeles River with impact from the WWTP effluent, while the wet weather sampling took place during a severe storm event, where the WWTP effluent impact was likely insignificant due to the high level of flood waters in the river. Colilert, Enterolert, and Pseudolert (IDEXX) were used both as directed and modified with tetracycline (30 mg/mL & 3 mg/mL), penicillin (100 mg/mL), and erythromycin (50 mg/mL). The MPN/100 mL in the presence of each antibiotic was divided by the total MPN/100 mL (absence of antibiotic) to determine the percent that is resistant for each antibiotic.

2.6. Sequencing and bioinformatics

Approximately 100 ng of DNA from each of the 16 samples were sequenced via 2 × 150 bp paired-end shotgun metagenomic sequencing using an Illumina NovaSeq 6000 facilitated by Mr. DNA (Shallowater, TX). Each library yielded between 10 and 30 million paired-end reads. All sequence data were deposited into the public NCBI Short Read Archive (SRA) database under BioProject ID PRJNA1061832. The raw sequences were uploaded into the Galaxy platform (https: //usegalaxy.org) for processing and assembly. Trimmomatic (Galaxy Version 0.38.0) was employed to remove low-quality reads from our pair-end data (Bolger et al., 2014). The Trimmomatic operation SLIDINGWINDOW was set to 4 bases and a quality score of 20, the MINLEN operation was set to a length of 50 bases, and AVGQUAL operation was set to a score of 20.

The paired data was assembled using de novo metagenomic assembly by MEGHIT (Galaxy Version 1.2.9) using the default settings of 2 for minimum multiplicity and 200 bp for the minimum length of output contigs (Li et al., 2015). FastQC (Galaxy Version 0.73) and Fasta Statistics (Galaxy Version 2.0) were used for quality control. Open reading frames (ORFs) on the contigs were predicted using Prodigal v2.6.3 (Hyatt et al., 2010).

ARGs were characterized using the ARGs-OAP pipeline (v2.3) which employs the Structured Antibiotic Resistance Genes database to quantify ARG subtypes by cell number and 16*S* rRNA (Yin et al., 2018). Environmental resistome risk scores were calculated using the MetaCompare pipeline, which assigns a risk score based on the co-occurrence of ARGs, MGEs, and pathogens on assembled contigs (Oh et al., 2018). Percent mobility is calculated as the percentage of co-occurring ARGs and MGEs on assembled contigs. Read-based taxonomic classification was performed via the Kraken2 (v2.0.8) software on the National Microbiome Data Collaborative (NMDC) EDGE bioinformatics platform (https://nmdc-edge.org/).

2.7. Statistical analysis

Data analysis and visualization were conducted in RStudio (v4.0.2). ARG data were categorized by land use and assessed for statistical significance through the Wilcoxon test at an alpha level of 0.05. Resistome risk score statistical significance was assessed using the non-parametric Kruskal-Wallis H test to compare multiple groups, followed by Dunn's test for post-hoc pairwise comparisons, with an alpha level of 0.05. Correlation plots were created with the "corrplot" (v0.90) and "Hmisc" (v4.6) packages. The principal component analysis plot was generated using the "prcomp" function and "ggbiplot" (v0.55) package. The "ggplot2" (v3.3.6) package was used to create all bar plots. The three-dimensional plot of resistome risk factors was generated using the "plot3D" (v1.4) package. Heatplots and chord plots were created using the "pheatmap" (v1.0.1) and "circlize" (v0.4.1) package, respectively.

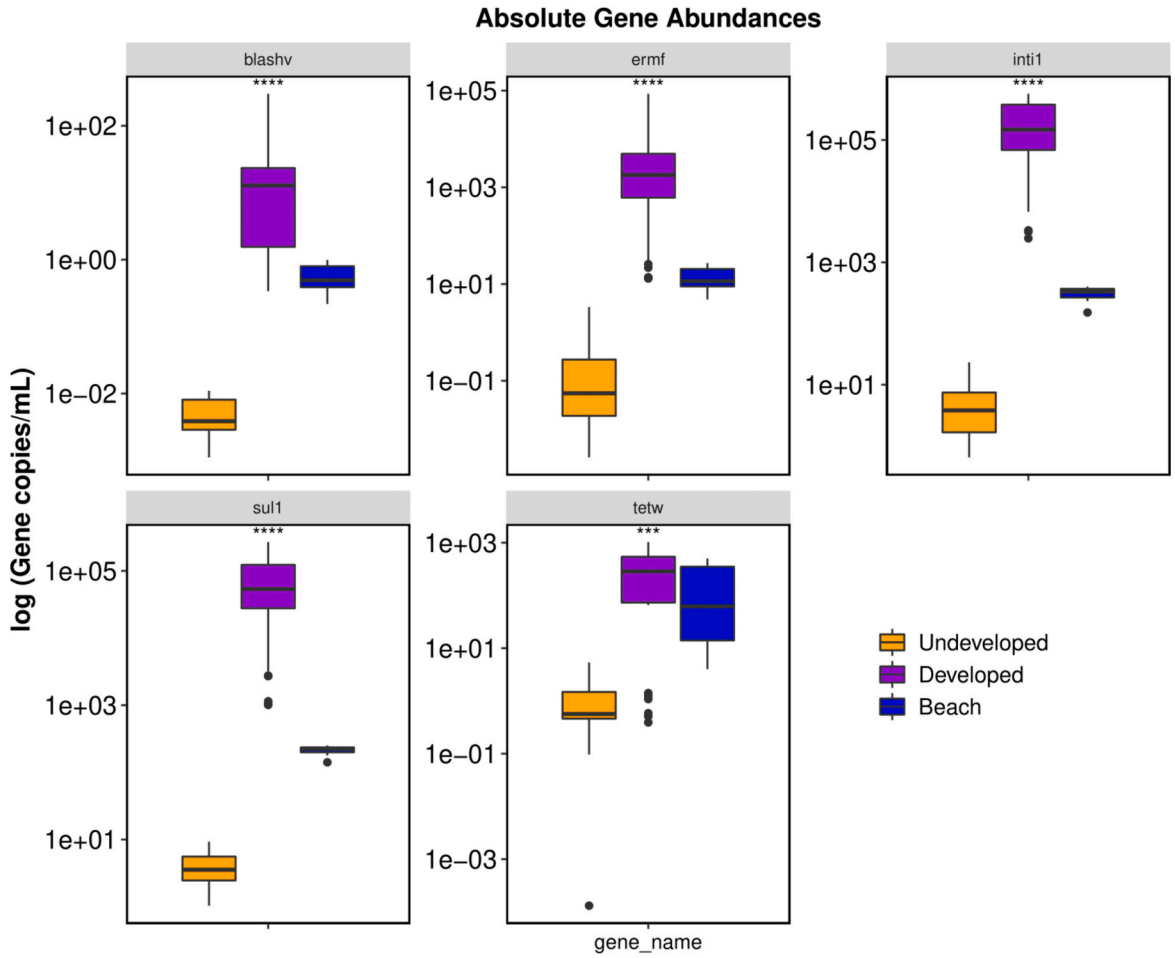

Fig. 2. Absolute gene abundances per land use type (undeveloped, developed, and beaches) for blaSHV, ermF, intI1, sul1, and tetW as gene copies per mL of water sampled. Undeveloped samples were taken in forested land preservation, parks, and recreational areas. Developed areas were collected in mixed urban, residential, commercial, and industrial areas. The sampling points above and below water reclamation plant effluent points are also encompassed in the developed category. The beach classification includes samples collected at public beaches near the Los Angeles River pourpoint. Asterisks denote the statistical significance between the categories with the following convention: *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$.

3. Results and discussion

3.1. Absolute gene abundances of ARGs and intI1

In this study, four ARGs (*sul*1, *erm*F, *bla*SHV, and *tet*W), 16S rRNA gene, and *intI*1 gene were quantified using qPCR in all water samples. The absolute abundances of all genes per land classification are displayed in Fig. 2. The undeveloped areas consistently had the lowest gene values, while sites in developed areas had the highest. Beaches had moderate levels for all genes, except *tet*W where beaches had similar values to developed sites. On average, developed sites exhibited median gene values 1–3 orders of magnitude greater than undeveloped sites, and 1–2 orders of magnitude greater than beach sites, except for *tetW*. LA River in Long Beach (LA), the most downstream developed area, consistently had the highest average absolute gene value. Big Tujunga Creek (TC) had the lowest average relative abundance for *sul*1, *intI*1, and *tet*W, and Switzer Canyon Falls (SF) had the lowest for *bla*SHV and *erm*F. Absolute gene abundances were statistically significant between developed and undeveloped land uses for all genes ($p < 0.05$). Developed gene abundances for *sul*1, *intI*1, and *erm*F were significantly different compared to beaches ($p < 0.05$). Beach and undeveloped gene abundances were not statistically significant.

Our findings align with a study that reported an increase in downstream concentrations of ARGs, except *tet*W, in a wastewater effluent receiving river in the Netherlands (Sabri et al., 2020). Comparing the same gene targets (*sul*1, *intI*1, and *tet*W), the values in the Netherlands

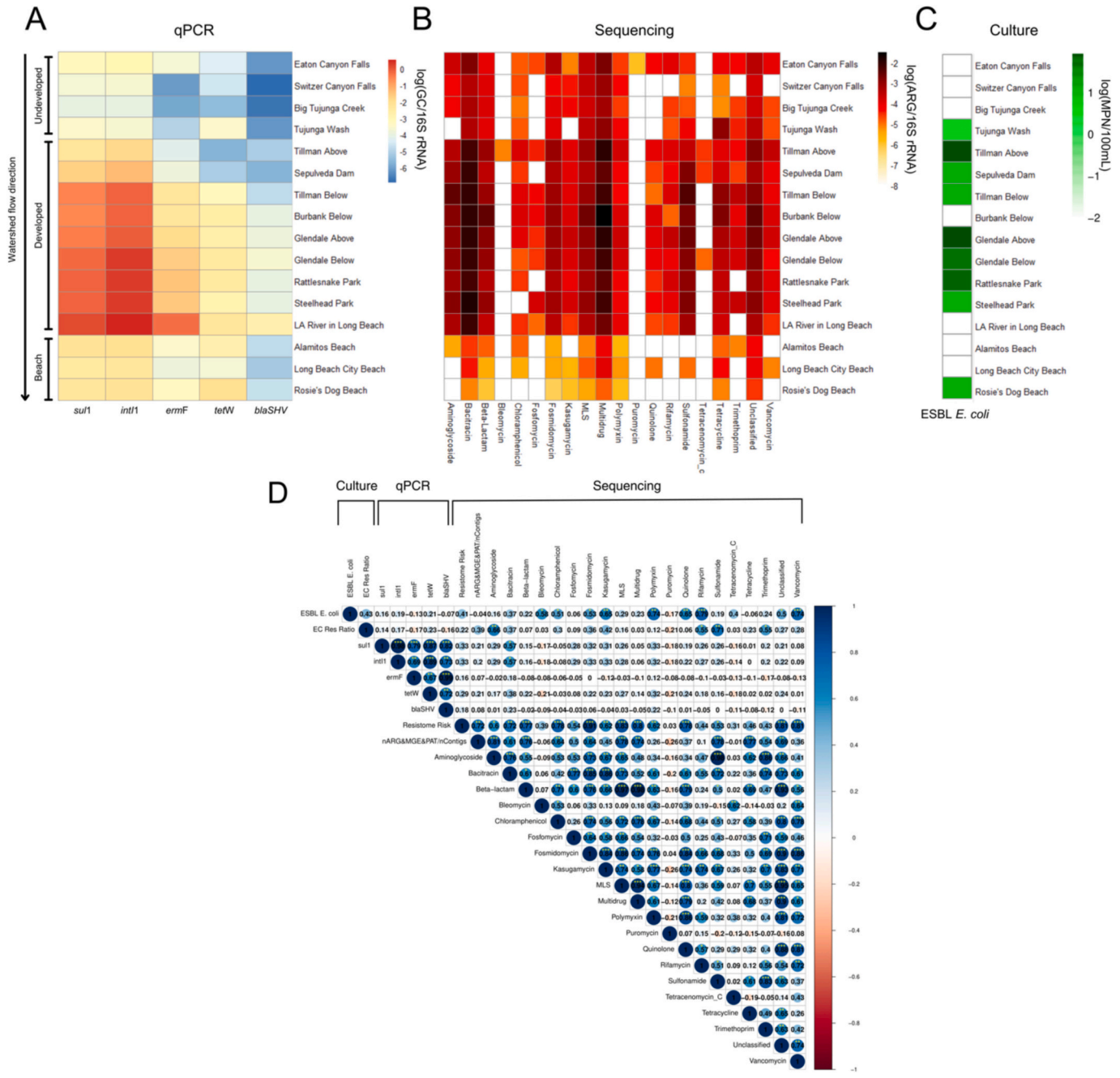

Fig. 3. Heat plots of relative gene abundances through the watershed via (A) qPCR, (B) metagenomic data., and (C) ESBL *E. coli* levels. Sites are oriented from undeveloped (top), to developed (middle), to beaches (bottom). (D) Correlogram between culture-, qPCR- (absolute abundances), and sequencing-based AR determinants. One star ('*'), Two stars ('**'), Three stars ('***') denote *p*-values <0.05, 0.01, and 0.001, respectively. Developed sites encompass the kayaking locations and sites above and below the water reclamation plants.

were akin to those from our developed locations, though our *tet*W values were lower. Notably, our *sul*1 gene values in developed areas were the most comparable to that in the Funan (Yang et al., 2018) and Zenne Rivers, both of which are impacted by wastewater. We had significantly less *tet*W levels which may be due to tetracycline being prevalently detected in the Zenne River (Proia et al., 2018). Our absolute *sul*1 and *intI*1 values were also similar to the Murg River in Switzerland which receives WWTP effluent with increased values during bypass events (Lee et al., 2021).

3.2. ARG trends in the watershed captured by amplification-, metagenomic-, and culture-based methodologies

Comparing normalized ARG abundances between qPCR data (Fig. 3A) and metagenomic data (Fig. 3B), alongside culture-based ESBL *E. coli* levels (Fig. 3C), reveals similar trends through the LAR watershed. Each method captures different nuances of antibiotic resistance trends across various land uses and water types. In the qPCR and metagenomic data (Fig. 3A and B), the four undeveloped sites display low ARG abundances with Eaton Canyon Falls (EF), a popular hiking location, displaying higher values with metagenomic data but not by qPCR. ESBL *E. coli* is not detected in the undeveloped sites until the Tujunga Wash (TW) (Fig. 3C), a site previously known to have high levels of FIB. Both sequencing and culture-based methods (Fig. 3B and C) capture increases in antibiotic resistance in developed regions beginning at Tillman Above (TA), while qPCR registered amplified levels of *sul*1 and *intI*1 at Tillman Below (TB). High ARG values (Fig. 3A) persist and increase through the developed main stem until diluted by the Pacific Ocean as evidenced by the lower values at the three beaches.

On the other hand, ESBL *E. coli* (Fig. 3C) fluctuates within the developed main stem of the LAR and its presence is detected at one of the beaches (Rosie's Dog Beach). In the qPCR data (Fig. 3A), higher ARG values are retained at beaches compared to the undeveloped sites, whereas in the metagenomic data (Fig. 3B) the beach ARG values are lower than the undeveloped sites.

These differences in sensitivity between qPCR and metagenomic data were similarly studied in wastewater, animal feces, and wastewater impacted rivers in Portugal (Ferreira et al., 2023). In Ferreira et al., qPCR and sequencing methodologies successfully detected gradient dilutions between different samples, with qPCR having greater sensitivity in detecting stepwise gradual changes in water samples versus animal feces. In our study, both methodologies successfully detected gradient dilutions across different land uses with differences where the watershed transitions from undeveloped to developed. Here, due to the large number of genes analyzed, metagenomic data demonstrated superior ability to detect and quantify stepwise changes in ARGs and offered a more comprehensive coverage of ARG classes and complex patterns. However, qPCR, which has been shown to have a higher sensitivity per gene than metagenomic techniques (Ferreira et al., 2023) consistently detected all genes tested, while the metagenomic data showed many non-detects in marine water. However, qPCR had a better sensitivity with marine samples compared to metagenomic data, comparing Fig. 3A and B. Culture-based and sequencing methods were able to detect areas with elevated antibiotic resistance, such as Tillman Above, while qPCR did not. These differences highlight the complexities in antibiotic resistance trends in the environment and the need to combine multiple techniques to avoid underestimation and overestimation of AR determinants. Similar variations across these methods were also seen in antibiotic resistance trends in agricultural soils (Wind et al., 2021).

Across study sites, *intI*1 was highly correlated with all resistance genes tested ($p < 0.001$ for *sul*1, $p < 0.01$ for *erm*F, $p < 0.001$ for *tet*W, and $p < 0.01$ for bla_{SHV}, Fig. 3, Panel D), suggesting human influence for sulfonamides, macrolides, tetracyclines, and beta-lactamases, and in the LA River. The relative abundance of *intI*1 is considered to be an indication of microbial ability to acquire foreign DNA (Suhartono et al., 2018), leading to enhanced transmission rates in water (Yang et al., 2017). Numerous studies have used correlation with *intI*1 to indicate likely anthropogenic sourcing of ARGs (Gillings et al., 2015). For example, in an impacted lake water in China, only *tet*A and *tet*W were correlated with *intI*1, suggesting anthropogenic sources were particularly important for those genes (Yang et al., 2017).

3.3. Diversity of ARGs through metagenomic data

The use of sequencing data enables the search of a greater diversity of ARGs from different classes. 20 out of the 24 antibiotic classes within the ARGsOAP pipeline were found in the water samples. Based on the relative abundance ARG data from the pipeline, there is a greater diversity of antibiotic classes represented in the developed sites (Fig. 4A). Burbank Below (BB) had the highest relative abundance mainly stemming from multidrug resistance ARG types. Fig. 4B, depicts the top 10 ARG classes among all samples. Multidrug resistant ARG types were the most abundant followed by bacitracin, unclassified, MLS, sulfonamide, aminoglycoside, beta-lactam, fosfomycin, tetracycline, and vancomycin. Multidrug resistant ARGs were also the largest proportion of ARGs in an urban stream in Nebraska, USA (Baral et al., 2018). Lee et al., 2022, also found high abundance of MLS, aminoglycosides, beta-lactam, sulfonamide, and tetracycline resistance classes in metagenomic data in WWTP effluent and bypass in a Swiss river (Lee et al., 2022). Multidrug, MLS, and beta-lactam ARG types were also most abundantly found in a WWTP in Virginia, USA (Majeed et al., 2021). Developed land uses have the highest portion of ARGs, followed by undeveloped and beaches.

Previous work has assessed environmental antibiotic resistance by both high-throughput qPCR (HT-qPCR) and shotgun metagenomic sequencing (SMS). Habibi et al. (2023) mapped ARG elements (ARGEs) in pollution-impacted coastal sediments off Kuwait (Habibi et al., 2023). HT-qPCR identified 100 ARGEs while SMS showed 402, leading the authors to conclude that SMS was preferable for monitoring due to being both more comprehensive and less expensive than HT-qPCR (which was roughly double the SMS cost). In the Kuwaiti sediments, HT-qPCR and SMS gave similar results for the relative abundance of various gene classes: beta-lactams were the dominant ARG class (37 %), followed by macrolides (19 %), tetracyclines (7 %), and fluoroquinolones (4 %).

3.4. Bacterial diversity

After comparing the top three taxonomies for each site, there were nineteen unique species and *Limnohabitans* sp. 63ED37–2 appeared in half of the sites. This organism is commonly found in freshwater habitats (Lee et al., 2021), and was found in LA, SP, RP, GB, TB, GA, SD, and TA. *Limnohabitans* sp. 63ED37–2 was the most predominant species in six of their taxonomies. The eight sites that lacked *Limnohabitans* sp. 63ED27–2 in their top ten taxonomies were the three beach sites (AB, LB, DB), the four undeveloped sites (TW, TC, SF, EF), and one developed site, Burbank Below (BB). BB was the closest site downstream from the undeveloped sites, which could explain why its taxonomy differs from the other eight developed sites, in that it lacks an organism that typically features in the taxonomies of anthropogenically-influenced freshwater sites. 12 out of the 16 samples had *Homo sapiens* as a top contributor which was commonly found in large US rivers (Linz et al., 2023).

Taxonomic classifications at the phylum level are presented with a dynamic pattern across all samples in Fig. S1 (Supporting Information). Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, and Cyanobacteria were present in all of the samples in different proportions. Proteobacteria emerged as the dominant phylum in all samples, with a relative abundance ranging from 45 % to 81 %. Notably, its prevalence was comparatively lower in undeveloped and beach sites in contrast to developed sites. Similar results were found in fish ponds affected by aquaculture in China and rivers influenced by wastewater treatment plants in Spain (Marti et al., 2013; Xiong et al., 2015). The distribution of Firmicutes and Cyanobacteria exhibited divergence, presenting higher percentages at undeveloped and beach sites compared to

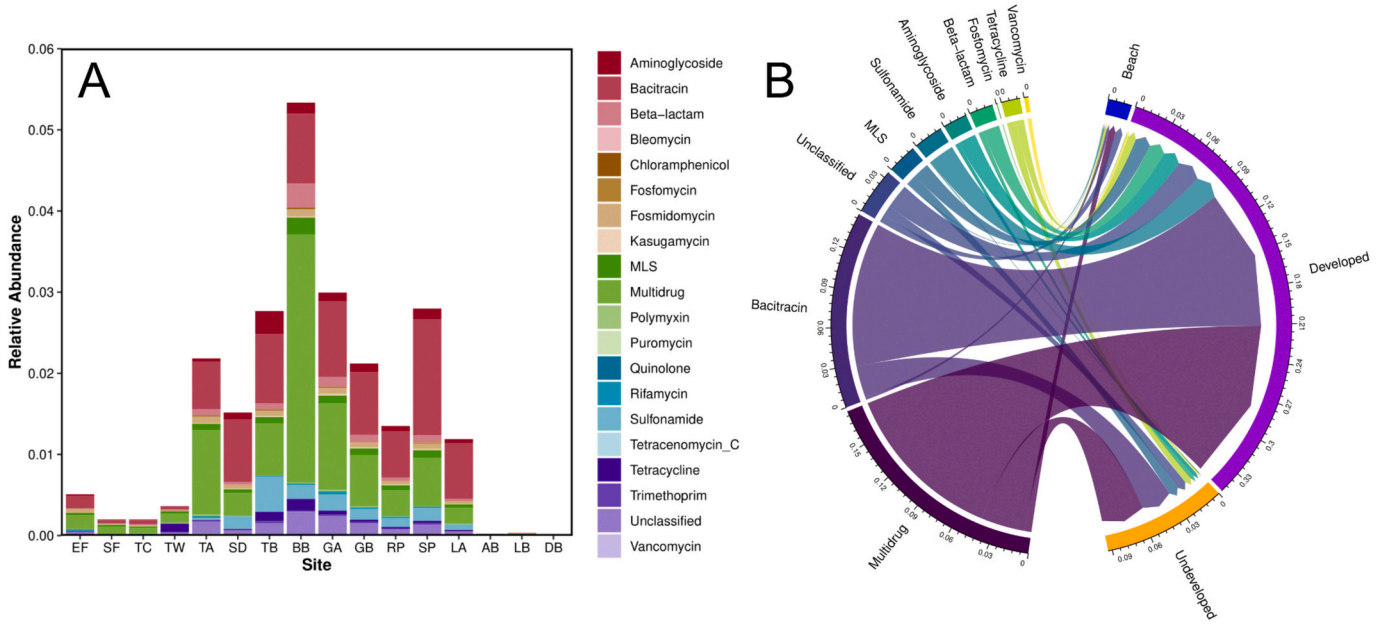

Fig. 4. (A) Total ARG relative abundance in water samples (upstream to downstream) annotated through the ARGsOAP v.2.3 pipeline. Each stacked bar represents one shotgun metagenomic sequence. (B) Chord plot of the top 10 ARG classes across sites partitioned by land use also annotated by the ARGsOAP v.2.3 pipeline.

developed sites. This shift parallels observations in coastal marine samples (Li et al., 2020), where Cyanobacteria, Proteobacteria, and Bacteroidetes were identified as prevalent phyla. In the current study, however, Proteobacteria, Actinobacteria, and Bacteroidetes are the dominant bacteria in coastal samples.

3.5. Resistome risk

Resistome risk scores were calculated for all samples from assembled contigs containing ARGs with the potential for mobility and presence in pathogens (Fig. 5A). The MetaCompare results were then plotted in a 3-dimensional hazard space based on the hits from the Comprehensive Antibiotic Resistance Database (CARD), ACLAME, and PATRIC databases (Fig. 5B). A Kruskal-Wallis H test revealed a statistically significant difference in resistome risk scores across the three site types ($H = 10.49$, $p = 0.005$). Resistome risk scores are greater in developed areas with the highest being at the Burbank Below (BB) location (RR = 27.94). Beach and undeveloped land use sites had similar resistome risk scores ($18.80 < RR < 22.80$), where Switzer Canyon Falls (SF) had the lowest score. Post-hoc analysis using Dunn's test with Bonferroni correction showed that developed sites had significantly higher resistome risk scores compared to undeveloped sites ($p = 0.013$). No significant differences

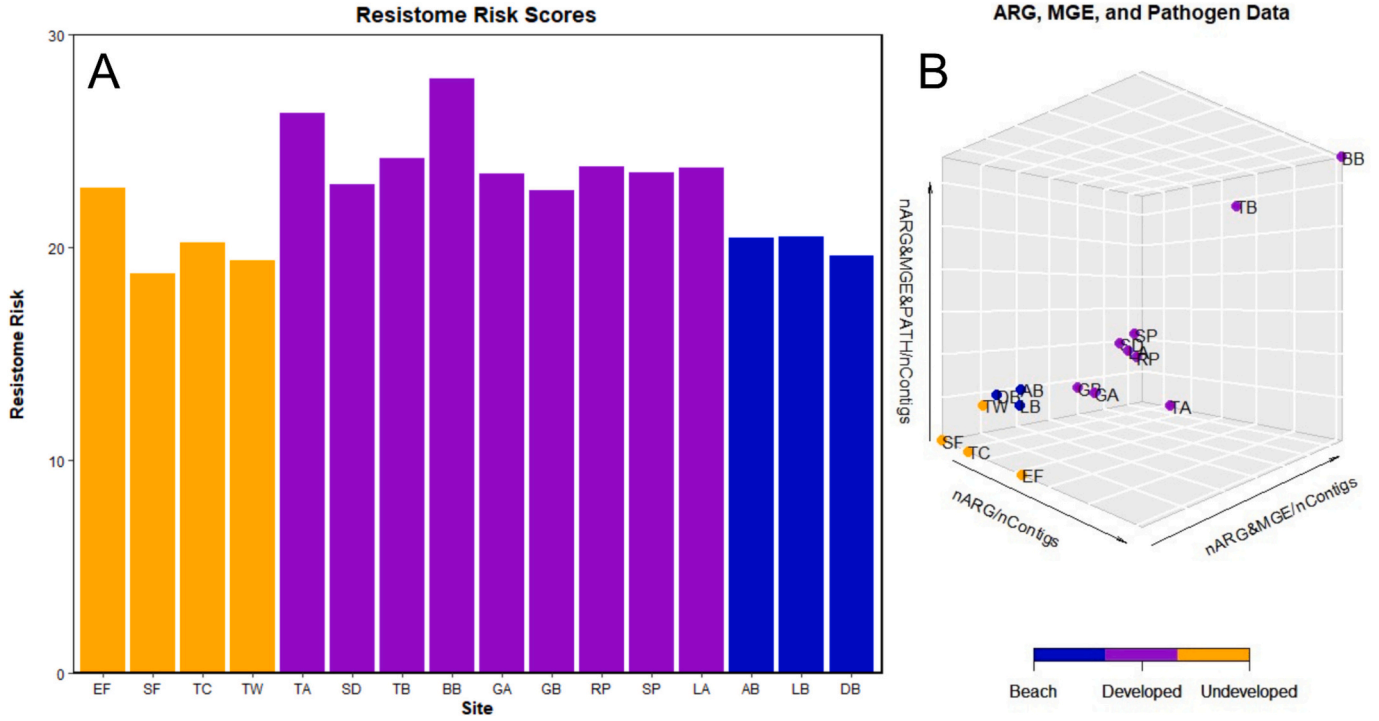

Fig. 5. (A) Resistome risk scores and (B) co-occurrence of ARGs, MGEs, and pathogens in a 3D hazard space.

were found between beach sites and the other two groups ($p > 0.05$). Co-occurrence of ARGs, MGEs, and pathogens increase from undeveloped to the developed sites and decrease at the beaches though not to the extent on the undeveloped sites (Fig. 5B). Among all undeveloped sites, only one location (TW) had one instance of co-location of an ARG and MGE among its 127,517 assembled contigs, resulting in a mobility percentage of 0.0007 %. BB had the highest mobility percentage (0.006 %) which had a total of 36 ARG and MGE co-locations among its 547,279 assembled contigs. Undeveloped, developed, and beaches had mobility percentages of 0.00019 %, 0.0029 %, and 0.0011 %, respectively. Notably, the sites below water reclamation plants (TB) have higher instances of co-occurrence than the sites above the respective plants (TA). Here the highest score was a river sample impacted by treatment plant water which was also seen in a study in Puerto Rico (Davis et al., 2020). The resistome risk scores in the developed areas were similar to those of secondary effluent of a US-based treatment plant (Majeed et al., 2021). Compared to European samples, our beach and undeveloped resistome risk scores were similar to WWTP effluent ($18.42 < RR < 22.77$) and the developed sites were similar to that of dairy lagoons ($22.71 < RR < 29.02$) but lower than hospital sewage ($RR > 34.47$) (Oh et al., 2018). The EU dairy lagoon values may be lower due to samples being taken after 2 months of operation. Longitudinal studies on the effects of manure on ARGs show that ARGs are detected in lower quantities in manured fields and receiving water after a few weeks (Barrios et al., 2020; Garder et al., 2014; Muurinen et al., 2017), with ARGs returning to background levels after manure application after 2 months (Van den Meersche et al., 2020).

3.6. Correlation of ARGs and water quality parameters

Fig. 6A depicts the correlation coefficients between normalized ARGs, 16S rRNA, *intI*1, FIB, heavy metals, and other physicochemical properties. There were statistically significant positive correlations among turbidity and ESBL *E. coli* ($p < 0.01$), Fe ($p < 0.05$), total coliforms ($p < 0.05$). There were also positive correlations between 16S rRNA, total coliforms, ESBL *E. coli*, and *E. coli*. All ARGs and *intI*1 correlated positively with each other and with pH. There are statistically significant negative correlations between dissolved oxygen and ESBL *E. coli* ($p < 0.05$), *E. coli* ($p < 0.001$), total coliforms ($p < 0.001$), and 16S rRNA ($p < 0.01$). There were strong negative correlations between 16S rRNA and Ni ($p < 0.05$) and Mn ($p < 0.05$) concentrations. Ni concentrations also correlated negatively with ESBL *E. coli* ($p < 0.05$) and the ESBL *E. coli* resistance ratio ($p < 0.01$).

The principal component analysis (PCA) displayed sample separation based on land use classification where the principal components together accounted for 63 % of the total variation (Fig. 6B). The first PCA axis correlates best with *Enterococcus* and the *sul*1 and *intI*1 genes. The second PCA axis generally separated the developed sites from beach and undeveloped samples. Within the developed sites, the most upstream (TA) and downstream developed sites (LA) were outliers. All genes and FIB were driving factors in developed sites and dissolved oxygen and conductivity were big drivers for beach sites.

3.7. FIB and ESBL E. coli

Fig. S2 (Supporting Information) depicts the results from FIB, ESBL *E. coli* for the sampling campaign. In the undeveloped areas in the Angeles National Forest, there were low levels of FIB and ESBL *E. coli*. In the developed areas of the river where the kayaking sites and WRPs are located, levels of FIB and ESBL *E. coli* increase and remain high until the tidal/estuarine area. Most areas exceed their respective recreational limits. Levels of FIB and ESBL *E. coli* decrease at the beaches near the LAR pour point. WRPs mostly have a dilution effect on FIB and ESBL *E. coli* due to high treatment standards for the effluent before it is discharged to the river.

Our *E. coli* levels were comparable to those in Funan River in Chengdu, China, where higher levels were found downstream from residential areas, a hospital, and a WWTP (Yang et al., 2018). A study on antibiotic resistant *E. coli* in a wastewater-impacted Belgian river also reported higher levels of bacteria downstream compared to upstream sampling sites (Proia et al., 2018). However, the bacterial levels and resistance were amplified by inputs from the wastewater treatment plants. These contrasting results may be due to their treatment plants encompassing processes equivalent to secondary treatment in the US,

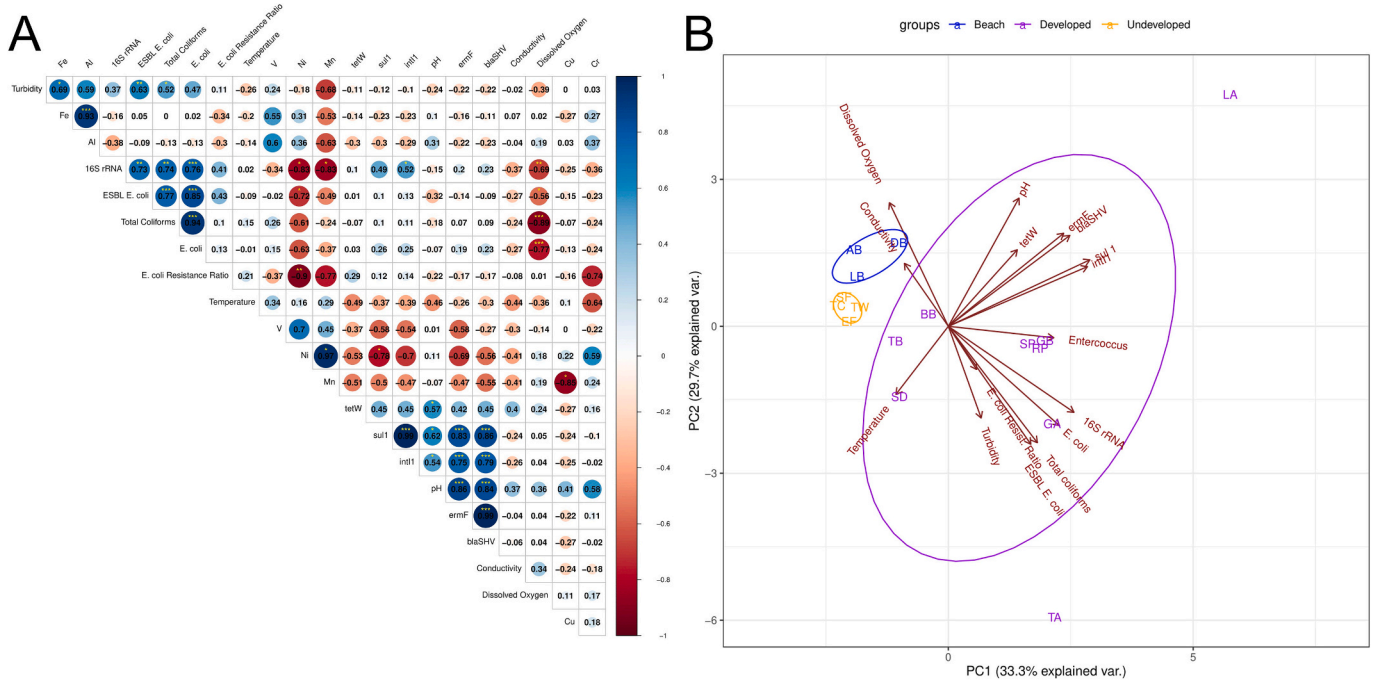

Fig. 6. (A) Correlation plot of relative ARGs, heavy metals, FIB, and physicochemical properties. One star ('*'), Two stars ('**'), Three stars ('***') denote *p*-values <0.05, 0.01, and 0.001, respectively. (B) PCA plot of ARGs, FIB, and physicochemical properties by land use. Data are shown by the abbreviations for the locations.

whereas the WRPs discharging in the LAR have tertiary treatment technologies.

3.8. HF183 and crAssphage

A total of sixteen field samples were analyzed for HF183 and crAssphage targets using the ddPCR method (Fig. 7). The average concentrations of crAssphage were 0.32, 3.62, and 1.62 log10 copies/100 mL for undeveloped, developed, and beach sites, respectively, while HF183 concentrations were 0, 3.062, and 0.92 log10 copies/100 mL for undeveloped, developed, and beach sites, respectively. Overall, the levels of HF183 and crAssphage were highest in developed areas, followed by beach sites. The Burbank Below (BB) site exhibited the highest concentration of HF183, which corresponds to the resistome risk scores obtained. The concentrations measured in our study were comparable to or greater than concentrations measured in other studies. For instance, in an impacted urban watershed in Pittsburgh, PA, USA, crAssphage concentrations ranged from 3.0 to 5.2 log10 copies/100 mL, which is similar to the average concentrations of crAssphage observed in our developed area sites (Stachler et al., 2019). Ahmed et al. reported HF183 ranging from 2.18 to 4.83 log10 copies/100 mL and crAssphage ranging from 2.17 to 3.80 log10 copies/100 mL for crAssphage, respectively, in the stormwater collected from urban sites and peri-urban sites in Australia. The co-occurrence of the two human fecal markers is more obvious at the developed sites. Moreover, the crAssphage and HF183 concentrations in our study sites were strongly correlated ($r = 0.9$, $p < 0.05$), suggesting that both markers may originate from similar sources and undergo similar environmental fates within the time scale required for water to pass through the Los Angeles River watershed. These findings contribute to our understanding of human fecal markers and microbial source marker dynamics in the study areas.

3.9. Percent antibiotic resistant for Pseudomonas aeruginosa, E. coli, and enterococci

Milligan et al. (2023) proposed that changes in levels of resistance seen in environmental bacteria (rather than wastewater borne bacteria) may indicate horizontal gene transfer at a certain location in the environment, such as downstream of an outfall. *P. aeruginosa* is known to be particularly susceptible to multi-drug resistance via HGT and has a tendency to grow and thrive in impacted aquatic environments (Milligan et al., 2023). In dry weather, levels of *P. aeruginosa* in the absence of antibiotics decreased, potentially due to dilution with disinfected wastewater (Table 1). Notably the percent of *P. aeruginosa* resistant to antibiotics increased downstream of the outfall (3 to 82 % for erythromycin, 63–97 % for penicillin, and 54–159 % for tetracycline at the lower dose).

We also assessed resistance of fecal indicator bacteria. The levels of antibiotic resistant bacteria were calculated above and below the outlet for the Glendale Water Reclamation Plant. For *E. coli* during dry weather, the level of this bacterium in the absence of antibiotics decreased after the outlet, as expected due to dilution with disinfected wastewater. However, the concentration of ESBL *E. coli* increased after the outfall, and the % of EC that were ESBL increased from 2 to 10 %. For wet weather, less of a change was observed from upstream to downstream of the outfall, likely due to the very high flow rate of the river. Similar to *E. coli* during dry weather, the levels of total coliform decrease and the percent resistant increased from 0.4 to 0.8 %.

On comparison across different sample dates and bacteria types, there was a consistent increase in resistance above and below the WWTP outlet in the Glendale river. This increase is in agreement with the concept that resistance genes can transfer from dead bacteria in municipal wastewater to susceptible fecal bacteria, pathogens, and autochthonous bacteria in the river (Milligan et al., 2023).

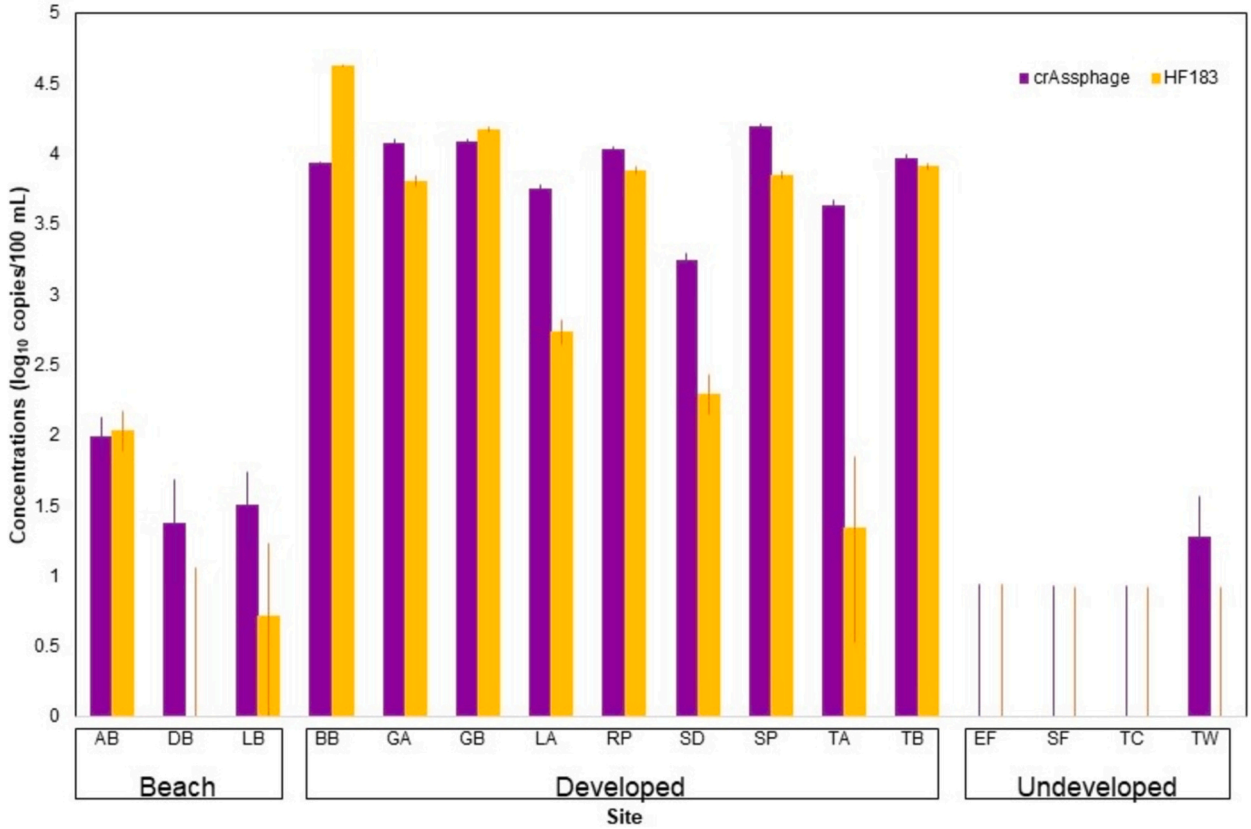

Fig. 7. ddPCR results for HF183 and crAssphage expressed in log copies per 100 mL of water filtered.

Table 1
MPN/100 mL concentrations and antibiotic resistance ratios (RR) for *P. aeruginosa, E. coli,* and total coliforms calculated for dry vs. wet weather days above and below the Glendale wastewater treatment outlet. ESBL-quantifying antibiotic cefotaxime (1 mg/mL), tetracycline (30 mg/mL & 3 mg/mL), penicillin (100 mg/mL), and erythromycin (50 mg/mL) are labeled as ESBL, tet30, tet3, pen100, and ery50, respectively.

Dry vs Wet	Antibiotic	GA MPN/100 mL	GA SD (±) MPN/100 mL	GB MPN/100 mL	GB SD (±) MPN/100 mL	RR % GA	RR % GB
P. aeruginosa							
Dry	No antibiotic	**886**	133.5	**323**	65.3	--	--
	ery50	**21.1**	5	**266**	55.8	3.4	82.4
	pen100	**565**	98.5	**313**	63.8	63.8	96.9
	tet3	**474**	81.5	**512**	88	53.5	158.5
	tet30	**182.9**	24.5	**60.2**	10.0	20.6	18.6
Wet	No antibiotic	**683**	110.8	**717**	116	--	--
	ery50	**579.4**	117.0	**648.8**	129.3	90.5	90.5
	tet30	**51.2**	7.8	**56**	8.5	8.6	7.8
E. coli							
Dry	No antibiotic	**510**	222.5	**200**	170	--	--
	ESBL	**10**	13.5	**20**	17	2.0	10.0
Wet	NA	**2495**	407.3	**2603**	474.5	--	--
	ery50	**41**	19.8	**97**	31.75	1.6	3.7
	ctx1	**63**	25.5	**63**	25.5	2.5	2.4
	tet30	**109**	34.8	**259**	56.75	4.4	10.0
Total Coliforms							
Dry	No antibiotic	**21870**	4070	**11870**	1950	--	--
	ESBL	**86**	31	**97**	31.8	0.4	0.8
Wet	No antibiotic	**68670**	13127.5	**92080**	16537.5	--	--
	ery50	**12997**	2615.5	**24196**	7714.3	18.9	18.9
	ESBL	**97**	31.8	**134**	37.25	0.1	0.2
	tet30	**789**	131.3	**712**	119.25	1.1	0.8

4. Recommendations

In this study, all three categories of methods for assessing environmental ARB were employed, which requires a great deal of work and expense. The goal of this work was not so much to advocate for the necessity of performing all types of analyses, but rather to explore the strengths and weaknesses of each approach. In this work, the very simple and accessible method, ESBL-EC, did an excellent job of identifying locations with elevated environmental antimicrobial resistance; this, it holds promise as a screening tool for further analysis.

Metagenomic techniques showed greater ARG abundances at all of the locations standing out as elevated identified by qPCR, albeit at much greater expense (and lesser workload) than qPCR. If funding is not an issue, metagenomics are an excellent approach for observing environmental AR. However, in lieu of metagenomics, qPCR was able to identify almost all of the elevated locations observed by metagenomics and was able to show likely human influence through correlation between the genes.

5. Conclusion

This study compared ARG and FIB trends in the Los Angeles River watershed and coastal water based on urbanization classification and influence of water reclamation plants. Metagenomic and amplification-based analysis of ARGs revealed increased loadings of ARGs in the river in the developed areas compared to undeveloped sites and beaches. Viability-based methods for FIB display similar trends where there are larger FIB concentrations in developed sites compared to beaches and undeveloped places exceeding recreational limits. Though WRPs in general diluted FIB due to high water quality standards in effluent water, ARG loadings were higher and downstream of WRPs and there were greater instances of ARGs being co-located on MGEs and pathogens. In accord with this finding, the percent resistance to the tested antibiotics generally increased in *P. aeruginosa*, *E. coli*, and total coliform from upstream to downstream of the WRP outfall. Our work shows that both qPCR and metagenomics are comparable in elucidating ARG trends based on land use and anthropogenic pollution which can be supplemented by viability-based methods. This work serves to compare various methods in monitoring ARGs and FIB in one of the most populated cities in the United States in the hopes of standardizing methods for monitoring these contaminants in aquatic environments.

CRediT authorship contribution statement

Ileana A. Callejas: Writing – original draft, Methodology, Conceptualization. **Yuwei Kong:** Writing – original draft, Investigation. **Katie Osborn:** Writing – review & editing, Methodology, Investigation. **Wei-Cheng Hung:** Writing – review & editing, Methodology, Investigation. **Marisol Cira:** Writing – review & editing, Methodology. **Taylor Cason:** Writing – review & editing, Investigation. **Ashlyn Sloane:** Writing – review & editing, Investigation. **Alexis Shenkiryk:** Writing – review & editing, Investigation. **Aaron Masikip:** Writing – review & editing, Investigation. **Akshyae Singh:** Writing – review & editing, Investigation. **Adriane Jones:** Writing – review & editing, Methodology. **Joshua A. Steele:** Writing – review & editing, Resources. **Jennifer A. Jay:** Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

The Supporting Information includes bacteria phylum distribution and percentages, fecal indicator bacteria concentrations, ddPCR concentrations, sampling locations and dates, qPCR and ddPCR assay information and respective MIQE guideline tables, physiochemical water data, and metagenomic data results. Supplementary data to this article can be found online at doi:https://doi.org/10.1016/j.scitotenv.2024.177577.

Data availability

Data is available on https://github.com/iacallejas/LA_River_ARG.

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