ANTIBIOTICS AND ANTIBIOTIC RESISTANCE GENES IN WATERS: POLLUTION, RISKS, AND CONTROL

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ANTIBIOTICS AND ANTIBIOTIC RESISTANCE GENES IN WATERS: POLLUTION, RISKS, AND CONTROL

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EDITORIAL
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Editorial: Antibiotics and Antibiotic Resistance Genes in Waters: Pollution, Risks, and Control

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Editorial on the Research Topic

Antibiotics and antibiotic resistance genes in waters: pollution, risks and control

INTRODUCTION

In 1928, British microbiologist Alexander Fleming discovered penicillin from *Penicillium* for the first time. Since then, human society has entered the golden age of antibiotics (Kümmerer, 2009; Zhang et al., 2015). Antibiotics play a great role in preventing and treating the disease, health maintenance, and life extension. However, it is also due to the extensive use and abuse of antibiotics that the continuous residue of antibiotics in the environment has been detected (Wang et al., 2017) resulting in their toxic effects on the ecosystem (Boxall et al., 2003; Sarmah et al., 2006). Moreover, long-term antibiotic exposure could induce the transfer, proliferation and diffusion of microbial antibiotic resistance genes (ARGs), and further, induce "super-resistant bacteria" in the environment or human body (Pruden et al., 2006; Martínez et al., 2015). Once these super-resistant bacteria invade humans, the corresponding diseases typically become incurable. According to the current development trend, it is predicted that "super-resistant bacteria" will cause about 10 million deaths worldwide every year by 2050 (de Kraker et al., 2016). Antibiotic resistance has been listed by the World Health Organization as one of the most urgent public health problems facing the world in the 21st century (WHO, 2014).

Antibiotics are poorly absorbed and metabolized by humans or animals. Studies revealed that up to 70% of antibiotics doses consumed are excreted through urine and faeces including primarily active substances or metabolites (e.g., Jutkina et al., 2018). Conventional sewage or sludge treatment facilities cannot remove antibiotics and resistance genes effectively, and a large number of antibiotics and ARGs eventually enter the natural environment (Berendonk et al., 2015; Cacace et al., 2019). Water is an important sink of antibiotic and resistance gene pollution. Antibiotics and ARGs have been widely detected in surface water, groundwater, and water supply systems (Ma et al., 2017; Wang et al., 2020; Zhang et al., 2022), which pose a great threat to both the ecosystem and human health. Due to the risk of antibiotics and resistance genes, the research on antibiotics and ARGs in the environment has become a hot issue during the past 20 years. Although several investigators have reported the distribution characteristics of antibiotics and ARGs in different environmental media, the understanding of pollution pathways, ecological and health risks, and the degradation and removal mechanisms of

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antibiotics and ARGs remain a challenge for the field of antibiotics and ARG research due to the complexity of the natural and artificial water systems.

CONTENTS OF THIS RESEARCH TOPIC

This topic mainly aims at the challenges in the current basic and technical research of antibiotics and ARGs in water systems. Finally, we selected 10 manuscripts for publishing after peer review. The main authors are from universities and scientific research institutes in China, America, India, Canada, Brazil, Japan, Ukraine, Switzerland, etc. Among ten published articles, one is a mini-review article, one is an opinion, and eight are original research articles. These published articles are all focused on the different aspects of antibiotics and/or antibiotic resistance genes in water.

Peng et al. introduced the method of analyzing antibiotic resistome in the environment by using metagenomic data and bioinformatics tools. They found that although SARG (Structured Antibiotic Resistance Gene database) is a good database, the application of two or more bioinformatics tools and databases could provide comprehensive information and an in-depth understanding of ARG transmission in the environment. In addition, Das et al. introduced HT-ARGfinder (horizontally transferred ARG finder), which is a pipeline for detecting and enumerating horizontally transferred ARGs in metagenomic data and can also estimate the directionality of transfer.

Gomes et al. investigated the pollution and risk assessment of 10 antibiotics in the river water of the Doce river watershed (Brazil). They found that the detection rate of acyclovir, amoxicillin, azithromycin, ciprofloxacin, enrofloxacin, fluoxetine, erythromycin, sulfadiazine and sulfamethoxazole was 100%; and the highest concentrations of ciprofloxacin and sulfamethoxazole was 4,854.6 ng/L and 9,640 ng/L, respectively. They found most of the investigated antibiotics were above concentrations that pose an ecotoxicological risk to aquatic biota. Guo et al. detected 12 target antibiotics in Dongting Lake in China and found that the antibiotic concentrations in the surface water and sediment were N.D. ~ 943.49 ng/L and N.D. ~ 177.43 ng/g, respectively. The risk assessment shows that ofloxacin, sulfamethoxazole, ciprofloxacin, enrofloxacin, roxithromycin and erythromycin have high ecological risks.

Duygan et al. systematically studied the biodegradation of 15 antibiotics such as ampicillin, chloramphenicol, erythromycin, penicillin and sulfamethoxazole under different sources of microorganisms and their effects on microbial communities. They found that growth linked biodegradation of antibiotics at low concentrations may be present among typical environmental microbes, but for a selected subset only, whereas for the majority of antibiotics negative effects prevail without any sign of productive growth.

The high concentration of antibiotics in wastewater can lead to the widespread of ARGs. Chen et al. studied the ARG distribution in cephalosporin production wastewater treatment plant (X-WWTP), subsequent municipal wastewater treatment plant (Y-WWTP) and receiving stream based on functional gene microarray technology. They found that the total abundance of ARGs in X-WWTP wastewater samples were significantly higher than that in Y-WWTP wastewater and stream samples, while the relative abundance of ARGs in river sediments did not change significantly with the distance from the Y-WWTP outlet. Kasuga et al. used a high-throughput quantitative polymerase chain reaction to characterize the profiles of ARGs and mobile gene elements (MGEs) in 24 urban rivers in Tokyo in Japan and their surrounding areas. They also found the effluent from the sewage treatment plant will affect the ARGs of the receiving river and *int*1could be used as a proxy for monitoring these ARGs and MGEs in urban rivers.

Yang et al. investigated the occurrence of ARGs and bacterial communities in the water and soil of the Ili River (in Xinjiang Province, China) using bacterial testing and metagenomic sequencing. They found fluoroquinolone, aminoglycoside, sulfonamide, and tetracycline resistanceenes were the most prevailing types in this river and pointed out that proteobacteria, bacteroidetes, and actinobacteria were the main potential hosts of ARGs. Guo et al. found *sul*1 and *sul*2 were the dominant ARGs in East Dongting Lake Basin; while in the sea ecosystem, Prekrasna et al. found that although the concentration of antibiotics in the Black Sea was below the detection limit, there were relatively high ARG pollution concentrations of *van*B, *bla*SHV, *bla*CMY and *mcr*-1.

In addition, Singh et al. published an article on the pollution, risk and control of antibiotics and resistance genes in water. They emphasized the pollution sources and health effects of ARGs in water.

FUTURE PROSPECTS

Due to the extensive use and abuse of antibiotics, it is predictable that residues of antibiotics will be present in the environment for the foreseeable future, and that will contribute to the amplification and transmission of ARGs. In the past half-century, the increasing prevalence of microbial antibiotic resistance has far exceeded the discovery and invention of new antibiotics. Antibiotic resistance has become a global health risk problem. The water environment is the main storage and transmission reservoir of antibiotics and ARGs. The pollution potential of antibiotics and ARGs are bound to have impacts on the ecosystems and human health. Although research on antibiotics and ARGs is increasing in many conunties, major scientific gaps and questions persist, including: 1) The environmental behaviour and ecological effects of antibiotics in different environmental media. What concentration and how long exposure can bring irreversible risks to the ecological environment and biosphere? 2) What are the influencing factors or limiting factors of ARG transmission and diffusion, what is the specific mechanism, and whether the purpose of ARG control can be achieved by regulating these factors? 3) How to quantify the risk of ARGs to the ecosystem and human health? 4) Can regulatory standards of ARGs in the water environment be established? 5) How should antibiotics and ARG pollution be managed and controlled in the

future, and how to suggest a balance between antibiotic use and control? All these issues warrent further research.

Finally, we sincerely thank the authors, editorial staff and reviewers who have made great efforts for this Research Topic.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Assessing Antibiotics Biodegradation and Effects at Sub-inhibitory Concentrations by Quantitative Microbial Community Deconvolution

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Antibiotics in the environment cause widespread concern as a result of their potent inhibitory action on microbial growth and their role in potentially creating selective conditions for proliferation of antibiotic resistant bacteria. Comprising a carbon skeleton, antibiotics should be amenable to microbial biodegradation, but this is still largely uncharted territory because of their simultaneous strong toxicity. In this study, we estimated potential antibiotics degradation by and effects on mixed microbial communities at concentrations sufficiently high to allow sensitive detection of biomass growth, but simultaneously, low enough to mitigate their toxic action. We used three different mixed inoculum sources freshly derived from freshwater, activated sludge or soil, and tested a series of 15 antibiotics from different classes at 1 mg C-carbon l^{-1} dosage. Consistent community growth was observed for freshwater and activated sludge with ampicillin, erythromycin and chloramphenicol, and with sulfomethoxazole for activated sludge, which was accompanied by parent compound disappearance. Community growth could be attributed to a few subclasses of recognized cell types by using supervised machine-learning-based classifiers. Most other tested antibiotics resulted in inhibition of community growth on background assimilable organic carbon, concomitant with altered composition of the resulting communities. We conclude that growth-linked biodegradation of antibiotics at low concentrations may be present among typical environmental microbiota, but for a selected subset only, whereas for the majority of antibiotics negative effects prevail without any sign of productive growth.

Keywords: antibiotics, biodegradation, microbial communities, flow cytometry, machine learning

INTRODUCTION

Antibiotics are ubiquitously used and have become widely distributed in the environment at low (ng—μg l^{-1}) concentrations (Kümmerer, 2009; Ma et al., 2014; Carvalho and Santos, 2016). Mostly being used to target pathogenic bacteria and infections, antibiotics act rather indiscriminately and inhibit any sensitive non-target bacterium residing in the same microbiome (Raymann et al., 2017; Grenni et al., 2018; Cycon et al., 2019). Even low (non-clinical) antibiotic concentrations are suspected to lead to selection and outgrowth of tolerant and resistant bacteria (Atashgahi et al., 2018; Cairns et al., 2018; Pärnänen et al., 2019; Yang et al., 2019), resulting in altered compositions and

diversity of microbiota (Grenni et al., 2018; Yang et al., 2019; Tong et al., 2020). Hence, there is wide concern about the environmental occurrence and fate of antibiotics. However, while most studies have focused on selection of resistance formation to antibiotics (Cairns et al., 2018; Li et al., 2010; Pärnänen et al., 2019; Paul et al., 2018), there is a clear lack of knowledge on their biodegradation (Cycon et al., 2019; Reis et al., 2020a; b). Having a better grasp on possible antibiotic biodegradation reactions would potentially permit to develop mitigation or treatment procedures that might alleviate some of their negative ecological consequences.

As a result of their toxicity, microbial degradation studies with antibiotics are difficult to carry out. Some studies have claimed widespread existence of bacterial subsistence on antibiotics at high (therapeutic, ~1 mg ml^{-1}) concentrations (Dantas et al., 2008; Barnhill et al., 2010). However, 'subsistence' is relatively poorly defined (a twofold increase of biomass or culturable colonies in typical minimal media with the dosed antibiotic) and not equivalent to compound mineralization. Other studies have failed to reproduce antibiotics degradation at those concentrations and, instead, have attributed observed growth to other carbon-containing compounds in the used media (Walsh et al., 2013), or managed to reproduce the effect but could not show compound transformation (Bello Gonzalez et al., 2015). As emphasized by some recent comprehensive review studies (Cycon et al., 2019; Reis et al., 2020a; b), except for a few compounds such as sulfomethoxazole or penicillin G, hardly any solid data demonstrating biodegradation of antibiotics by bacteria and fungi exists. Furthermore, although for some compounds individual isolates producing possible metabolic intermediates were found, in general most antibiotics appear very recalcitrant and "non-biodegradable" (Cycon et al., 2019; Reis et al., 2020a; b). In contrast, several antibiotic biotransformation reactions are well documented, some of which directly contribute to resistance mechanisms, as they inactivate the inhibitory action of the parent compound (Cycon et al., 2019; Reis et al., 2020a; b). There is thus a major gap in the assessment of antibiotic biodegradation due to the use of high (therapeutic) antibiotic concentrations for microbial growth studies, which leads to toxicity and inhibition of most microbes. This also causes selection for resistant or tolerant microbes and not necessarily degrader bacteria. As a result, it makes it difficult to determine any growth-linked biodegradation of antibiotics by environmental microbial communities.

The aims of our study were to design experimental conditions that would permit to quantify biomass growth at the expense of single dosed antibiotics at low concentrations, and to simultaneously study their effects on community compositions. These concentrations would need to be below the typical minimal inhibitory concentrations (MIC) (Obayiuwana et al., 2018) to improve the chances to observe growth of bacterial strains using antibiotics as sole carbon and energy source. However, considering that the fraction of specialist degrader bacteria in environmental microbiota is very low (Thouand et al., 2011; Johnson et al., 2015; Martin et al., 2017), detection of specific growth at low compound dosages becomes extremely challenging and can be confounded by many factors.

Our hypothesis, therefore, was that productive utilization of antibiotics by strains in the target inoculum may become detectable as a growth surplus compared to a no–carbon medium control. Liquid media frequently contain an estimated 100–150 µg assimilable organic carbon (AOC) l^{-1} (Özel Duygan et al., 2021), which would be sufficient for growth of ~10^6 cells ml^{-1} (Hammes and Egli, 2005; Vital et al., 2007; Vital et al., 2008). We used starting concentrations of antibiotics of 0.1 or 1 mg C-carbon l^{-1}, which in case of utilization for biomass formation could potentially lead to an increase of 2–20 times the background growth and, as such, become detectable. We tested three inoculum sources (freshwater community; activated sludge community and soil community), with a total of 15 antibiotics. Community growth was quantified by flow cytometry counting of fluorescent-stained cells in time incubation series. Potential inhibitory effects of antibiotics on community growth and compositional shifts were further analyzed by a previously developed supervised machine-learning-based classifier that classifies cell types from flow cytometry data (Özel Duygan et al., 2020), and, in one case, by 16S rRNA gene amplicon analysis. For a subset of antibiotics for which good analytical methods were available, we quantified the fate of the parent compound in the incubations compared to abiotic controls. Our results indicate a few distinct cases of parent compound disappearance concomitant with community growth that can be attributed to a few cell types. For most antibiotics, however, mostly inhibition and corresponding community compositional shifts were observed.

MATERIALS AND METHODS

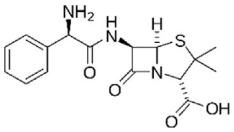
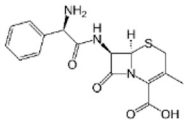
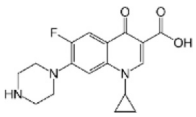
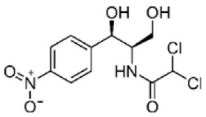
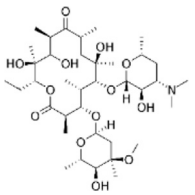
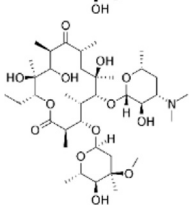
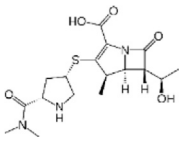
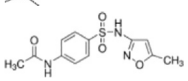
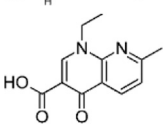
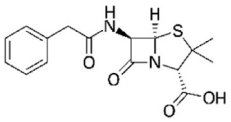
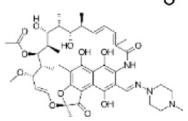
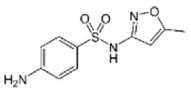
Sources of Microbial Communities

Three different samples were used as sources for the starting communities to be tested for antibiotics effects. As *freshwater inoculum* source, we sampled Lake Geneva water from nearby St. Sulpice, Switzerland. 10 L of sample was recovered from right below the water surface in a Teflon-carboy, transported to the laboratory within 20 min and processed immediately, as described previously (Özel Duygan et al., 2020).

Secondly, 1 L *activated sludge* was obtained from the effluent Vidy Wastewater Treatment Plant (Lausanne, Switzerland). The activated sludge samples were transferred in Teflon-containers to the laboratory within 20 min and processed immediately. First, the sample was aliquoted in 50 ml Falcon tubes, centrifuged at 3,000 rpm for 5 min and decanted, after which the cell pellets were resuspended in 50 ml PBS (1X phosphate-buffered saline), pooled and mixed in kitchen blender for 3 min to disrupt flocks. The homogenized cell suspension was centrifuged at 800 rpm for 6 min to remove larger particles, the supernatant was decanted and further centrifuged at 5,000 rpm for 5 min. The cell pellet was then resuspended in PBS.

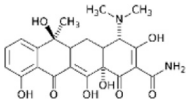
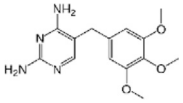
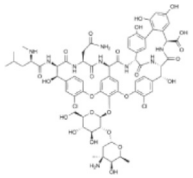
Finally, microbial cells were washed from 20 g of 2-mm sieved forest topsoil (−5−−10 cm), nearby Dorigny, Switzerland, following the protocol described by (Li et al., 2010). In short, cells were detached by mixing with 2 g l^{-1} sodium pyrophosphate solution at pH 7.5, then purified by sucrose density centrifugation

TABLE 1 | List of antibiotics used in this study with chemical formula, molecular structure, mode of action and class (PubChem, https://pubchem.ncbi.nlm.nih.gov).

Name of the antibiotic, (internal standard)	Chemical formula	Molecular structure	Mode of action	Antibiotic class
Ampicillin (AMP), (Lidocain-D10)	$C_{16}H_{19}N_3O_4S$		Inhibition of cell wall synthesis	Beta lactam antibiotics
Cephalexin (CEP)	$C_{16}H_{17}N_3O_4S$		Inhibition of cell wall synthesis	Beta lactam antibiotics
Ciprofloxacin (CIP)	$C_{17}H_{18}FN_3O_3$		Inhibition of bacterial DNA topoisomerase and DNA-gyrase	Fluoroquinolone antibiotics
Chloramphenicol (CHL), (N4acetylsulfamethoxazole-D4)	$C_{11}H_{12}Cl_2N_2O_5$		Blocks peptidyl transferase (protein synthesis)	Beta lactam antibiotics
Erythromycin (ERT), (Erythromycin-D3)	$C_{37}H_{67}NO_{13}$		Blocks transpeptidation and translocation by binding to 23S rRNA (protein synthesis)	Macrolide
Gentamycin (GEN)	$C_{21}H_{43}N_5O_7$		Irreversibly binds to 30S ribosomal subunit leading to misreading tRNA	Aminoglycoside
Meropenem (MER)	$C_{17}H_{25}N_3O_5S$		Components of cell wall synthesis impaired	Beta lactam antibiotics
N4-Acetylsulfamethoxazole (NSMX)	$C_{12}H_{13}N_3O_4S$		Metabolite of Sulfomethoxazole	Sulfonamide
Nalidixic acid (NAL), (Carbamazepin-D8)	$C_{12}H_{12}N_2O_3$		Inhibition of DNA gyrase subunit A	Quinolone
Penicillin G (PEN), (Carbamazepin-D8)	$C_{16}H_{18}N_2O_4S$		Inhibition of cell wall synthesis	Beta lactam antibiotics
Rifampicin (RIF)	$C_{43}H_{58}N_4O_{12}$		Transcription inhibition through binding to RNA polymerase	Rifamycin
Sulfamethoxazole (SMX), (Sulfamethoxazole-D4)	$C_{10}H_{11}N_3O_3S$		Interference with folic acid synthesis	Sulfonamide

(Continued on following page)

TABLE 1 | (*Continued*) List of antibiotics used in this study with chemical formula, molecular structure, mode of action and class (PubChem, https://pubchem.ncbi.nlm.nih.gov).

Name of the antibiotic, (internal standard)	Chemical formula	Molecular structure	Mode of action	Antibiotic class
Tetracycline (TET)	$C_{22}H_{24}N_2O_8$		Binds to 30S ribosomal subunit and blocks tRNA from binding	Tetracyclines
Trimethoprim (TRI)	$C_{14}H_{18}N_4O_3$		Inhibits dihydrofolate reductase (thymidine pathway)	Sulfonamide
Vancomycin (VAN)	$C_{66}H_{75}Cl_2N_9O_{24}$		Inhibition of cell wall synthesis	Glycopeptide

and Nycodenz centrifugation-concentration. Cells were finally washed and diluted in PBS.

For all community preparations, the cell suspensions were kept on ice, serially diluted, stained with SYBR Green I (see below) and measured by flow cytometry to determine the cell density. Three to five replicate samples of the starting material were stored at −80°C for later DNA isolation and 16S rRNA gene amplicon community sequencing (see below). Incubation experiments were then started immediately.

Glassware and Mineral Medium Preparation

In order to minimize interference of background AOC, all Schott borosilicate glassware (500 ml flasks) was rinsed with 6M HCl four times, then four times with MilliQ water to eliminate residual carbon from dishwasher cleaning. Air-dried flasks were covered with aluminium foil and autoclaved. Artificial lake water (ALW) medium was prepared in 5-L size HCl-treated and sterile glass bottles as previously described (Özel Duygan et al., 2021). ALW contains, per L: 36.4 mg $CaCl_2{\cdot}2H_2O$, 0.25 mg $FeCl_3{\cdot}6H_2O$, 112.5 mg $MgSO_4{\cdot}7H_2O$, 43.5 mg K_2HPO_4, 17 mg KH_2PO_4, 33.4 mg $Na_2HPO_4{\cdot}2H_2O$, and 25 mg NH_4NO_3, pH = 7.0. For each experiment, the ALW medium was prepared 1 day before starting the incubations and kept at 4°C; then filter-sterilized by passage through a 0.2–μm membrane.

Antibiotic Incubation Experiments

Washed and recovered freshwater, activated sludge and soil microbial community suspensions were diluted to a starting concentration of 10^5 cells ml^{-1} in 100 ml ALW in a 500 ml prepared Schott flask, closed with Teflon-lined septa and caps. A set of 14 antibiotics was selected on the basis of their mode of action (**Table 1**), which were dosed individually to each flask at a concentration of either 0.1 or 1 mg C L^{-1}. As positive control we included 1 mg C l^{-1} of phenol, whereas non-amended flasks with medium only functioned as no-carbon controls for background growth. Per antibiotic or control three replicate flasks were prepared, which were incubated in the dark at 150 rpm rotary movement and 21°C for a maximum of 8 days (**Table 2**). Samples (1 ml) were withdrawn each day in a sterile laminar flow hood without opening the caps, using a syringe and long hypodermic needle. Samples were used for community size measurements (flow cytometry) and antibiotic

TABLE 2 | Overview of the experiments.

Inoculum source	Antibiotics tested	Carbon dosage [mg C l^{-1}]	Initial cell density [cells m^{-1}]	Inoculum sampling date	End of experiment	Sampling days
Freshwater	All compounds in **Table 1**	0, 1	100,000	March 6, 2019	March 12, 2019	0, 1, 2, 3, 5, 6
Activated sludge	All compounds in **Table 1**	0, 1	100,000	March 20, 2019	March 25, 2019	0, 1, 2, 3, 5
Activated sludge	AMP, CHL, ERT, GEN, NAL, PEN, SMX	0, 0.1, 1	100,000	November 27, 2019	December 5, 2019	0, 2, 6, 8
Soil	AMP, CHL, ERT, GEN, NAL, PEN, SMX, PHE	0, 1	100,000	May 1, 2019	May 8, 2019	0, 1, 2, 5, 7
	Amplicon sequencing—inoculum preparation date					
Freshwater (3 replicates)	January 30, 2019					
Activated sludge (3 replicates)	November 27, 2019					
Soil (5 replicates)	February 19, 2019					

TABLE 3 | CellCognize classes.

Full name of CellCognize classes		Abbreviation
Acinetobacter johnsonii	Subpopulation 1	AJH1
	Subpopulation 2	AJH2
Acinetobacter tjernbergiae	Subpopulation 1	ATJ1
	Subpopulation 2	ATJ2
Arthrobacter chlorophenolicus	Subpopulation 1	ACH1
	Subpopulation 2	ACH2
	Subpopulation 3	ACH3
Bacillus subtilis	Subpopulation 1	BST1
	Subpopulation 2	BST2
Caulobacter crescentus	Subpopulation 1	CCR1
	Subpopulation 2	CCR2
Cryptococcus albidus		CAL
Escherichia coli DH5α-λpir		ECL
Escherichia coli MG1655	Exponential phase (in LB medium)	ECL_EXP3 (MG1)
	Stationary phase (in LB medium)	ECL_STAT_LB (MG2)
	Stationary phase (in Mineral Medium)	ECL_STAT_MM (MG3)
Lactococcus lactis		LLC
Pseudomonas knackmussii		PKM
Pseudomonas migulae		PMG
Pseudomonas putida		PPT
Pseudomonas veronii	Subpopulation 1	PVR1
	Subpopulation 2	PVR2
Sphingomonas wittichii		SWT
Sphingomonas yanoikuyae		SYN
0.2 µm bead		B02
0.5 µm bead		B05
1 µm bead		B1
2 µm bead		B2
4 µm bead		B4
6 µm bead		B6
10 µm bead		B10
15 µm bead		B15

concentrations (liquid chromatography mass spectrometry) as explained below.

Flow Cytometry and Cell Type Fingerprinting

Community samples (200 µL) were stained with 2 µL SYBR Green I (from a dilution of 10 µL original stock in 1 ml DMSO, Molecular Probes) and incubated in 96-flat-bottom-well-plates in the dark for 15 min. In case of counting dead cells, we additionally added 2 µL propidium iodide (from a dilution of 10 µL original stock in 1 ml DMSO, Molecular Probes). 20 µL of the stained samples were then aspired at 14 µL min^{-1} at a sample acquisition rate of (maximally) 35,000 events s^{-1} on a NovoCyte flow cytometer (ACEA Biosciences, Inc.), with thresholds as described previously (Özel Duygan et al., 2020). Seven parameters (FITC-A, FITC-H, FSC-A, FSC-H, SSC-A, SSC-H and Width) were recorded for each cell.

Data sets were exported as .csv files for analysis. Cells with negative values in any of the parameters were removed and only cells passing all thresholds were counted. Cells were additionally classified into a set of 32-predefined types (see **Table 3**) by using five independently acquired algorithms derived from a machine-learned pipeline for cell type classification (CellCognize) (Özel Duygan et al., 2020). For classification we used the maximum probability score for each cell of belonging to any of the 32 classes, as described previously (Özel Duygan et al., 2020).

Analytics of Targeted Antibiotics

Based on preliminary experiments, ampicillin, chloramphenicol, erythromycin, nalidixic acid, penicillin and sulfamethoxazole were selected for chemical analysis. Samples (1 ml) from community incubations were centrifuged for 5 min at 15,000 rpm to remove cells, and 1 ml supernatant was transferred to an amber glass vial, which was capped and stored at −20°C until analysis.

For analysis, samples were thawed and diluted 100 times with ALW medium, and then spiked with internal standard solution (0.1% v/v; final concentration in the samples 0.05 µg L^{-1}). The internal standard solution consisted of ^{13}C-isotope-labeled equivalents of the target antibiotics (see **Table 1**) with similar chromatographic retention time, dissolved in ethanol (stock concentration of 50 µg L^{-1}). Each targeted antibiotic was dissolved individually and diluted to prepare calibration curve standards (between 100 ng L^{-1} and 50 µg L^{-1}), which were also spiked with the same internal standard solution. Samples and calibration standards were analyzed by liquid chromatography coupled to high-resolution mass spectrometry, and for analysis, 20 µL were injected. Chromatographic separation was performed over a reversed-

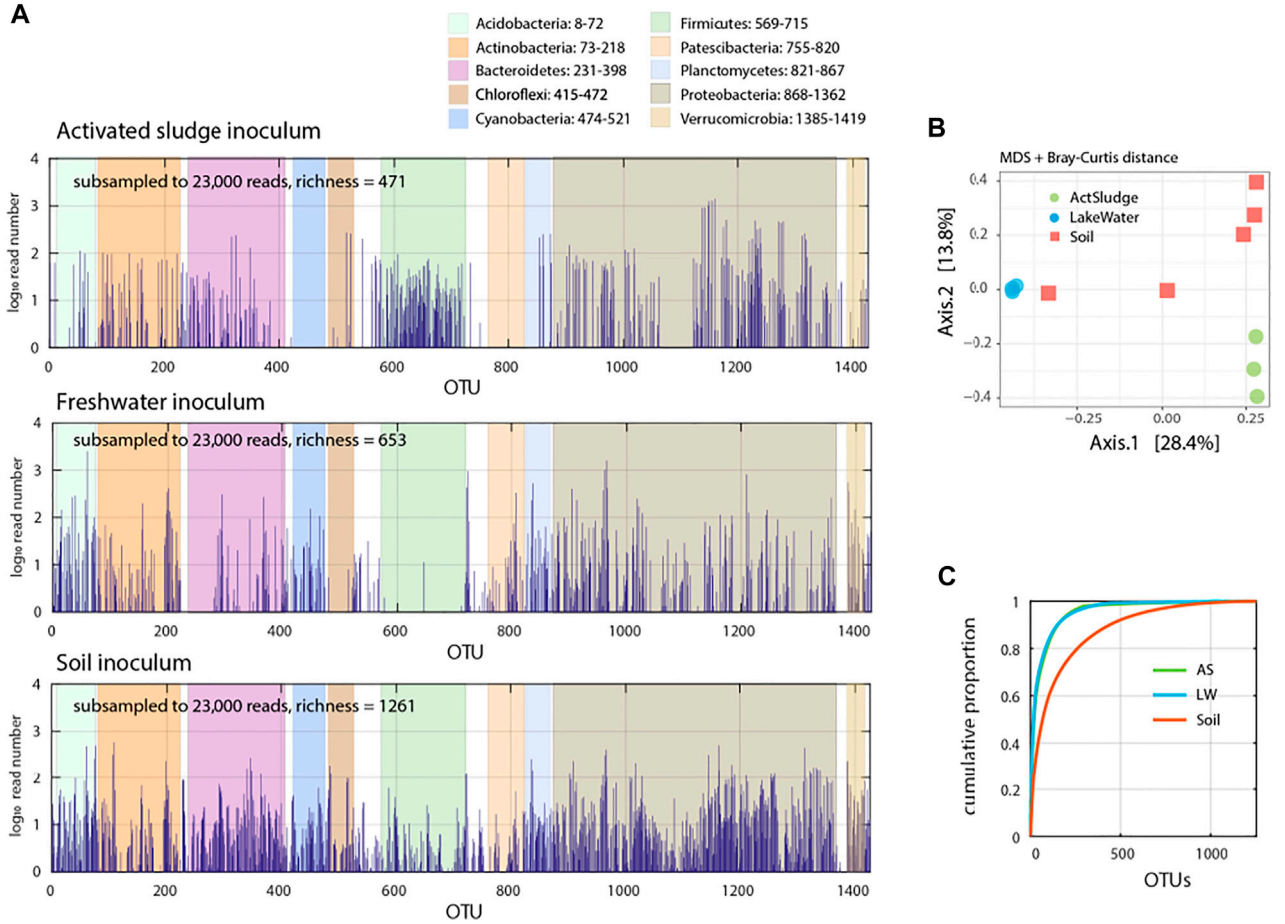


FIGURE 1 | Species abundance in the target microbial communities. **(A)** Species distribution in activated sludge (Vidy Wastewater Treatment Plant), freshwater (Lake Geneva water) and top soil (University of Lausanne forest) microbiota determined from 16S rRNA amplicon sequencing. Read numbers normalized from summed (activated sludge and lake water, n = 3; soil, n = 5 replicates) subsampled down to 23′000 reads and its distribution over determined operational taxonomic unit (OTU), shown in semi-log plot. **(B)** Multidimensional scaling (MDS) plot of microbial communities Bray-Curtis dissimilarity based on relative abundance of subsampled (23,000) sequence reads. Circles and squares show individual replicates. **(C)** Cumulative proportion of sequence reads per determined OTU in the target microbiota.

phase C18 Atlantis T3® column (3 mm × 150 mm, 3 µm particle size). Mobile phase comprised of nanopure water (solvent A) (18.2 MΩ-cm-resistivity) and methanol (solvent B), both acidified with 0.1% formic acid, and were used to generate the following binary gradient elution profile: 0/95/95/5% B at 0/18.5/28.5/29 min at a flow rate of 0.3 ml min^{-1}. HPLC eluates were introduced into a high-resolution mass spectrometer (Q Exactive Orbitrap, Thermo Scientific) via an electrospray ionization (ESI) probe operated in both positive and negative switching mode (4 kV/3 kV spray voltage, 325°C capillary temperature, 40 arbitrary units (AU) sheath gas, 15 AU auxiliary gas, 0 AU sweep gas, and 40°C auxiliary gas heater temperature). Mass spectra was acquired in full-scan mode at a mass resolution of 140,000 (FWHM at *m/z* 200) and the scan range was between 50 and 750 m/*z*. Data were analyzed using XCalibur software (Thermo Scientific, United States) for quantitative analysis with reference standards (the Quan Browser) and for the identification procedure (the Qual Browser).

Solid-Phase Microcolony Growth

Selected community enrichments on antibiotics were sampled and directly spreaded (1–5 µL) on MicroDish® platforms (Ingham et al., 2007) placed on 2 mm thick silica gel disks in ALW medium medium at pH 7, containing 1 mg C L^{-1} of the same target antibiotic (ampicillin, chloramphenicol, erythromycin, nalidixic acid, penicillin, or sulfamethoxazole). MicroDish-es were incubated for 3 days at 21°C, after which colonies were photographed on an inverted AF6000 LX epifluorescence microscope (Leica AG, Germany) equipped with a DFC350FXR2 camera at 40-fold magnification. Visible larger microcolonies were picked from the MicroDish surface using a MiBot mobile robot equipped with a stainless-steel sterilized needle (Imina Technologies, SA). Needles with colony material were transferred into 4 ml closed-cap teflon-lined glass vials with 1 ml ALW. After resuspending cells from the needles for 24 h at 150 rpm rotary movement, the cell density was measured by flow cytometry, after which the 'pre-cultures' were replicated to 10^4 cells ml^{-1} at start into three vials each, either

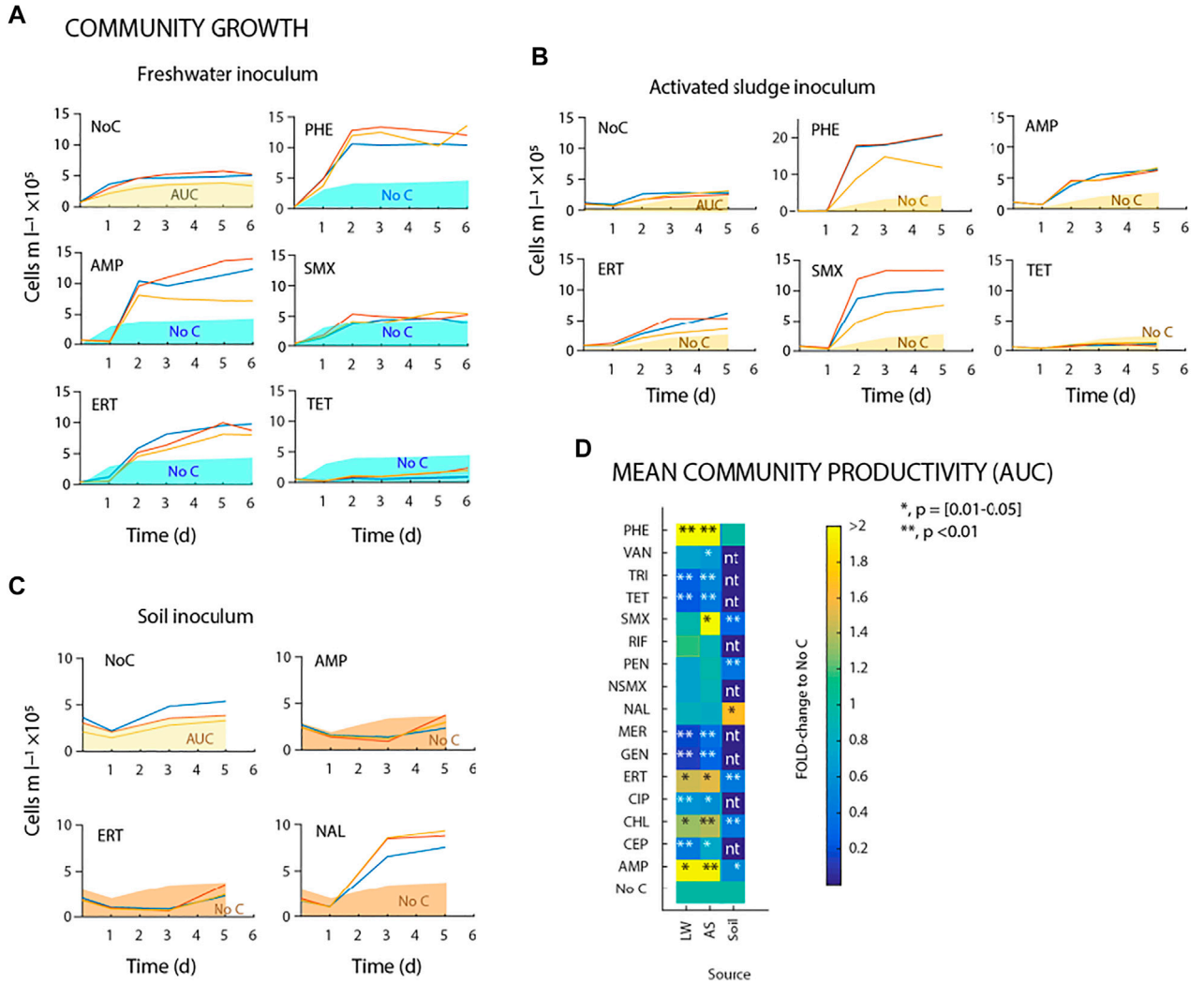


FIGURE 2 | Microbial community growth on antibiotics as sole carbon and energy source. **(A–C)** Freshwater, activated sludge and forest top soil microbial community growth by flow cytometric cell counts during incubations with antibiotics. Shaded areas (green, yellow or brown bars) correspond to average background growth on triplicate no-carbon controls (NoC). **(D)** Comparison of mean community productivity (Area Under Curve; AUC) during incubation experiments (up to 6 days) with freshwater, activated sludge and forest top soil microbial communities (n_{batch} = 3 for each condition). The gradient color scale shows fold changes compared to no-carbon control. *indicates significant difference to no-carbon control (paired *t*-test).

dosed with the same antibiotics as used for enrichment (1 mg C l^{-1}) or with medium only (no-carbon control). Vials were incubated and sampled as before for cellular growth.

Community Composition Analysis by 16S rRNA Gene Amplicon Sequencing

DNA was isolated from the time samples from one of the activated sludge incubation experiments, and from the freshwater (n = 3 replicates), activated sludge (n = 3 replicates) and soil inoculum suspensions (n = 5 replicates). Cells were collected from the samples by filtration on a Sartorius in-line filter holder (25 mm, polyethersulfone 0.22 µm, Sartorius), prewashed with 70% (*v/v*) ethanol in water, then rinsed three times with MilliQ water and air dried, UV-treated for 30 min and assembled to the vacuum line. Filters with cells were rolled with the help of sterile tweezers and stored at −80°C in 2-ml centrifuge tubes of the FastDNA ™ Spin Kit for Soil DNA extraction kit (MP Biochemicals) until processing. DNA was extracted using the protocol provided by the supplier (MP Biochemicals). Aliquots with 10 ng DNA were used to amplify the V3-V4 hypervariable region of the 16S rRNA gene using the 341f/785r primer set, Illumina adapters and barcodes, and PCR conditions, as indicated in the Illumina Amplicon sequencing protocol (https://supportillumina.com/documents/documentation/chemistry_documentation/16s/16smetagenomic- library-prep-guide-15044223-b.pdf). Equal amounts of amplified purified barcoded DNAs per sample were pooled and sent for bidirectional sequencing on the Illumina MiSeq platform at the Lausanne Genomic

Technologies Facilities. Raw sequences were separated by barcode, then quality-filtered, concatenated, verified for the absence of chimera, dereplicated and mapped to known bacterial taxonomy using QIIME2 on a UNIX platform (Bolyen et al., 2019) at 99% similarity to the SILVA taxonomic reference database.

Statistics

Multidimensional scaling plots based on Bray-Curtis dissimilarity of the sample replicates were derived from the relative abundance of either each taxon (i.e., 16S rRNA amplicon sequence reads at SILVA taxonomy level 6) or CellCognize class (i.e., FCM fingerprints deconvoluted into 32 classes) in the *phyloseq* package in R.

Significance of enriched taxa (i.e., at SILVA taxonomy level 3, Class) in an antibiotic-incubated sample versus the no-carbon control at the same incubation time point comparison of triplicate community was calculated using the *mattest* function (MatLab 2019a) under 1,000 permutations, with a fold change cut-off 10, a false-discovery rate of 0.05 and a q value of 0.05.

Significance of mean community productivity or cell type productivity in an antibiotic-incubated sample versus the no-carbon control comparison of the triplicates was calculated using *paired t-test* (0 for $p > 0.05$, 1 for $p < 0.05$).

RESULTS

Species Distribution in Targeted Microbial Communities

In order to address the capacity of resident microbial communities to subsist on antibiotics, we prepared communities of washed cells from freshwater, activated sludge or forest top soil. All three community inocula covered a wide phyla diversity (**Figure 1A**) but were distinct in multidimensional scaling plots based on relative abundances of assigned operational taxonomic units (OTU, 99% similarity to SILVA taxonomy of 16S rRNA genes) using Bray-Curtis distance (**Figure 1B**). Distributions of relative OTU abundances were skewed with 151 (activated sludge), 150 (freshwater) and 439 (soil) OTUs making up 90% of all detected taxa (**Figure 1C**). The proportion of potentially compromised or dead cells (i.e., propidium iodide positive in flow cytometry) in the washed communities varied from low (2%) in freshwater to high in soil (75–80%). This viability difference may have had effects on detectable community growth within the duration of our assays, as we explain below.

Microbial Community Growth on Antibiotics as Sole Carbon and Energy Source

In order to assess whether antibiotics can be used as substrates for growth, the recovered and washed microbial communities were diluted to 10^5 cells ml^{-1} at start in closed batch liquid suspensions, dosed with individual antibiotics, and quantified over time (**Table 1**, $n = 3$ replicates per antibiotic, dissolved to 0.1 or 1 mg C l^{-1} - calculation based on compound carbon mass). Community growth was quantified by flow cytometry counting of SYBR Green I-stained cells. Community size increase in presence of antibiotics was compared to that of a no-added carbon medium control, consisting of the used mineral salts and its inherent background AOC, and further to a positive control, consisting of the same medium with added 1 mg C l^{-1} phenol, a readily biodegradable reference compound (Özel Duygan et al., 2021).

As expected, the community size in the no-carbon control increased after 5–6 days of incubation to between 2–5 × 10^5 cells ml^{-1} depending on the source inoculum, indicative for utilisation of the medium-inherent AOC (**Figures 2A–C**; overview of all antibiotics shown in **Supplementary Figures S1–S3**). Incubations with phenol at 1 mg C l^{-1} led to significantly higher growth of freshwater or activated sludge bacteria to 1–2 × 10^6 cells ml^{-1} within 6 days (**Figures 2A,B**). This was interpreted as a measure for the inherent viability of the inoculum sources, and was subsequently contrasted to potential growth on antibiotics and/or their inhibitory action.

Addition of antibiotics frequently caused an initial decrease in the community sizes compared to those in the no-carbon controls, which may be due to cell lytic action (e.g. day 0 and day 1 in **Figures 2A–C**). In comparison to the no-carbon control, the freshwater community size increased more during incubation with ampicillin (AMP) or erythromycin (ERT) at 1 mg C l^{-1} (**Figure 2A**). In contrast, sulfamethoxazole (SMX) had no effect whereas tetracycline (TET) and most other antibiotics (**Supplementary Figure S1**) caused clear inhibition of the freshwater community growth compared to the no-carbon control (**Figure 2A**). With activated sludge inoculum, community sizes increased more with 1 mg C l^{-1} of AMP or ERT compared to the freshwater communities, and also showed increased growth with SMX compared to the no-carbon control (**Figure 2B**; for all compounds, see **Supplementary Figure S2**). Chloramphenicol (CHL) at 0.1 mg C l^{-1} yielded net growth above that of the no-carbon controls with both inocula (**Supplementary Figures S1–S2**). Finally, with soil inoculum, neither AMP nor ERT, but in this case nalidixic acid (NAL) yielded net community growth within 5 days, whereas most others resulted in community decline (**Figure 2C**; for all antibiotics, see **Supplementary Figure S3**). As discussed below, the starting soil inoculum showed reduced cell viability as inferred from propidium iodide staining and from the lack of growth on PHE. This impeded proper interpretation of growth with dosed antibiotics, and we did not further consider the soil community data in our results.

Given that changes in community size were different in rate and magnitude for dosed compounds (e.g. **Figures 2A–C**, **Supplementary Table S1**), we compared compound effects relative to a calculated cumulative "area-under-the-curve" (AUC) as in (Piccardi et al., 2019), rather than relative to maximum community "yield" or a community growth rate, as illustrated in **Figures 2A–C**. The fold-change normalized ratio of AUC for antibiotic-incubated communities in comparison to the no-carbon control, showed statistically significant increases for AMP, ERT or CHL for both freshwater and activated sludge inocula, and for SMX for the activated sludge inoculum (**Figure 2D**; **Supplementary Table S1**). Consistent neutral

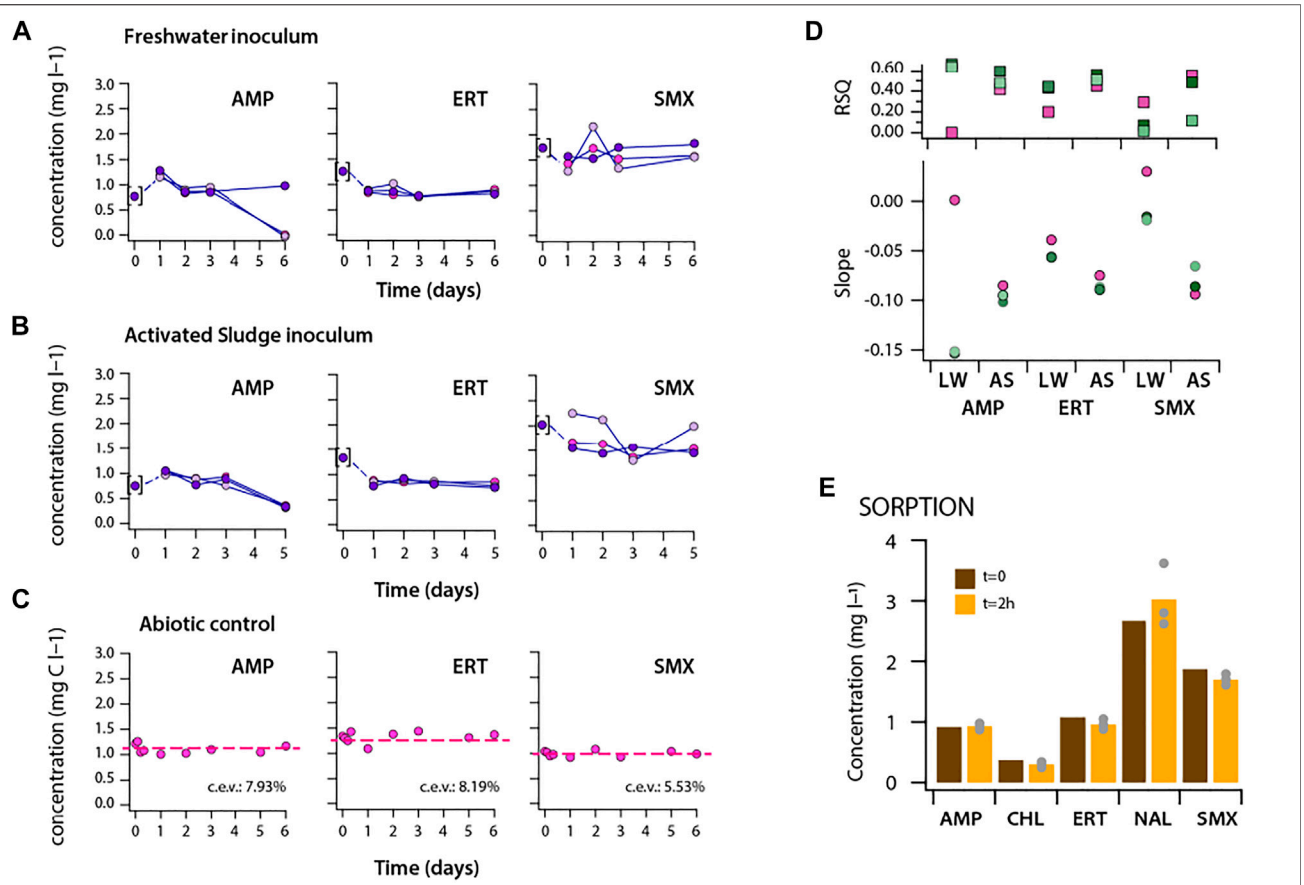


FIGURE 3 | Antibiotic concentrations during incubation experiments with **(A)** freshwater, **(B)** activated sludge microbial communities in triplicate batches. Measurements at first sampling points (t:0) were shown in brackets as the sampling was done from the working stock solutions instead of individual closed batches for each condition (which may have caused the apparent concentration "increase" for AMP between start and day 1 sample). **(C)** Abiotic control experiment with the test compounds in ALW medium. Antibiotic concentrations of the target antibiotics in biotic or abiotic conditions are given in **Supplementary Table S2**. **(D)** r-squared values and slope of the trendlines of the target antibiotics (shown in **(A–B)**) during incubation experiments including all sampling points. **(E)** Sorption control experiment with antibiotics at initial biomass density of 100,000 cells per ml.

effects (i.e., no statistically significant difference in AUC compared to that in the no-carbon controls) were observed upon addition of vancomycin, nalidixic acid, penicillin or rifampicin at 1 mg C l^{-1} (**Figure 2D**, **Supplementary Figures S1–S5**). In contrast, addition of trimethoprim (TRI), tetracycline (TET), meropenem (MER), cephalexin (CEP), ciprofloxacin (CIP) or gentamicin (GEN) at 1 mg l^{-1} reproducibly led to a significant decrease of community AUC across all experiments, indicative of inhibition of the growth of the microbial communities compared to the no-carbon controls (**Figure 2D**, $p < 0.05$, $n = 3$ biological replicates).

We attempted to isolate potential antibiotics-utilizing strains from the primary enrichment cultures. Although plating of the communities with increased AUC on silica-gel surface with artificial lake water (ALW) medium consisting of 1 mg C l^{-1} of the same antibiotics led to formation of colonies, none of those in isolation were capable of growing in ALW liquid medium with the same antibiotics. These colonies are therefore likely to have been false positives, opportunistically growing on traces of AOC in ALW medium. Alternatively, their growth may have been due to interdependent cooperative interaction among colonies (for details, see **Supplementary Text S1**). In conclusion, these results showed that few antibiotics led to consistent net community size increase compared to no-carbon controls, which is a first indication for biomass growth that can be attributed to antibiotic dosing. Most others showed inhibitory to neutral effects on community growth.

Disappearance of Antibiotics Dosed as Sole Carbon and Energy Source

The maximum fold-increase of AUC in incubations with dosed antibiotics was between 1.8 (CHL)—4.6 (SMX) (**Figure 2D**). In order to understand whether this increase could have been due to utilization of the dosed antibiotic as carbon or nutrient substrate, we measured concentrations of a selected number of antibiotics as a function of incubation time in the communities (**Figure 3**, **Supplementary Table S2**). In the freshwater community, the observed increase in AUC, compared to no-carbon controls, during incubation with AMP and ERT was accompanied by a

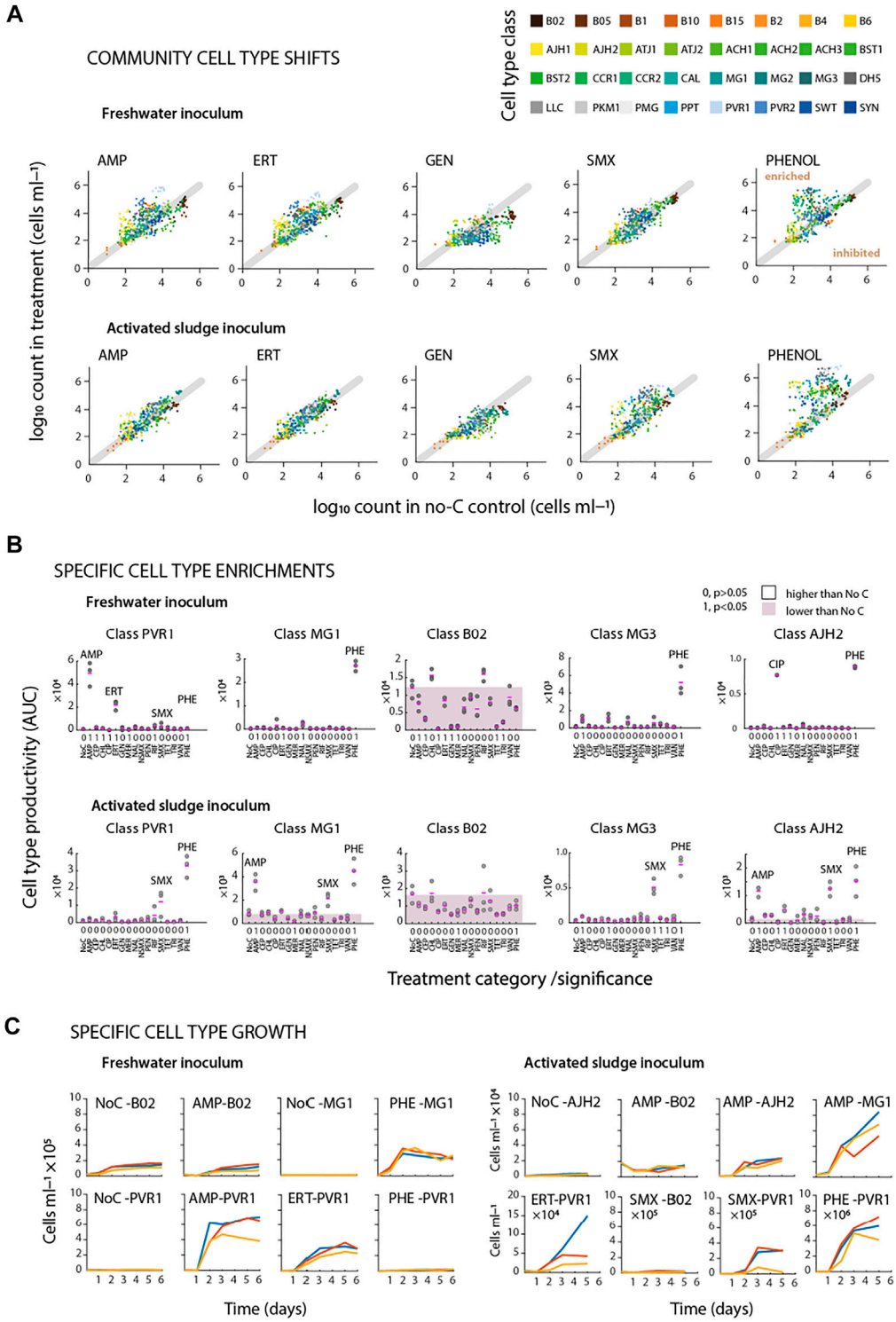


FIGURE 4 | Identifying community variations from flow cytometric fingerprints. **(A)** Community cell type comparisons based on predefined 32 cell types. Dots (paired between treatments and no added carbon control series) represent absolute cell type abundances per replicate (n_{batch} = 3). Cell types shifting upwards of the diagonal line show enrichment as a function of added substrates. **(B)** Specific cell type productivity (Area Under Curve; AUC) during incubations of freshwater (t = 6 days) or activated sludge (t = 5 days) microbial communities. Pink dash lines show average of triplicate flasks. Shaded area shows mean productivity of No C series. Significance values are presented as 0 or 1 (true, false for *p* values <0.05). **(C)** Specific cell type growth during incubations of freshwater (left) or activated sludge (right) microbial communities in triplicate batches.

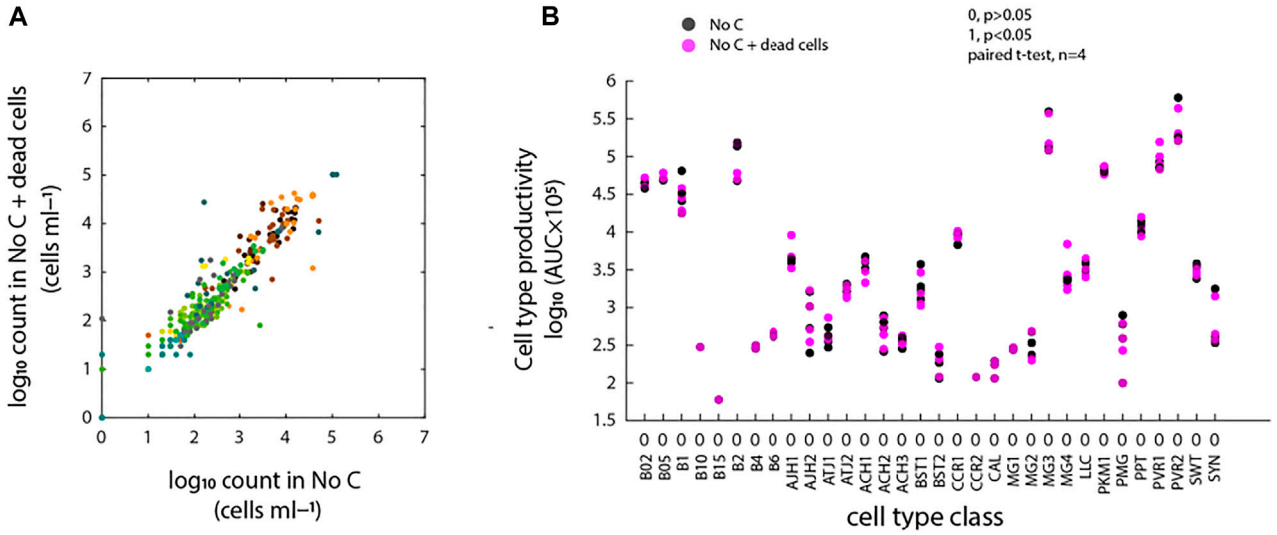


FIGURE 5 | Effects of dead cells on background microbial community growth. **(A)** Community cell type comparison based on deconvoluted FCM fingerprints of freshwater microbial community into 32 CellCognize classes between the two conditions (i.e., no-carbon controls with or without addition of 25% of pasteurized dead cell community). Scattered dots represent absolute cell type abundances per replicate (n_{batch} = 4). **(B)** Cell type productivity (AUC) comparison between the two conditions for each CellCognize class. Significance level was calculated based on paired *t*-test (n = 4). 0 and 1 indicate significance level of $p > 0.05$ and $p < 0.05$, respectively.

decrease in antibiotic concentration (**Figure 3A**), whereas SMX concentrations remained the same. In the activated sludge incubations, all three compounds (AMP, ERT and SMX) decreased in concentration (**Figure 3B**). Parallel abiotic controls in ALW did not show any decrease of antibiotics concentration (**Figure 3C**). Without assuming any kinetic model but taking into account all time points measured, the concentration decreases were statistically significant (i.e., negative slopes of trend lines and $r^2 > 0.5$, **Supplementary Table S3**) for AMP in both inocula, and for ERT and SMX in activated sludge (**Figure 3C**). The decrease was not due to sorption of the antibiotic to microbial biomass (**Figure 3E**), suggesting that it was due to biotransformation and may have led to the increase of community AUC. Also, the observed significant increase in community AUC with CHL (**Figure 2D**) was accompanied by its frequent disappearance in the medium (**Supplementary Table S2**), suggesting biotransformation.

Antibiotics Cause Distinguishable Population Variations Within Communities

Community growth as a whole (e.g. AUC) may mask smaller magnitude enrichments among microbial subgroups within the communities. In order to further quantify such small-magnitude enrichments in the freshwater and activated sludge microbial communities during antibiotic incubation, we classified microbial cell types from the multidimensional flow cytometry data by similarity to a set of predefined 32 cell standards using a supervised machine-learning classifier (CellCognize) (Özel Duygan et al., 2020). Although CellCognize has no *a priori* knowledge on the taxa distribution in the used source communities, it calculates for each cell in the flow cytometry data the probability of similarity to one of the predefined 32 classes. As a first approximation, we used and report here the best class attribution for any cell. By plotting all class attributions for antibiotic incubations at all time points and replicates versus the corresponding no-carbon control, one can easily distinguish whether aberrations occur (i.e., enrichment or inhibition) in specific cell type populations during an incubation. For example, in the freshwater community incubations, addition of AMP or ERT led to an absolute increase of a variety of distinct cell type populations in comparison to the no-carbon control. Various cell type populations also became enriched in incubations with phenol, indicating that such enrichments truly represent growth of specific populations in the community (PHE, **Figure 4A**). In contrast, SMX did not cause major changes in cell type populations in the freshwater community, whereas gentamycin (GEN) inhibited growth of a variety of classes but not all (**Figure 4A**). Similarly, with activated sludge as inoculum, addition of SMX caused the most drastic increase of different cell type populations, but less so with AMP or ERT, and not visible for GEN (**Figure 4A**). These results are in agreement with the total community size data and the conducted antibiotic concentration measurements.

Considered as the growth of individual cell type populations (e.g., as their AUC in comparison to that in the no-carbon controls), the major enrichments for phenol (PHE) in the freshwater community occurred in the classes represented by the cell standards MG1, AJH2, and MG3 (**Figure 4B**, **Supplementary Figure S4**). Class enrichments were selective, because in the activated sludge source community on phenol other classes appeared (i.e., PVR1, **Figure 4B**), whereas, for example, the abundant class B02 did not react to phenol at all (**Figure 4B**, for a full representation of all classes, see

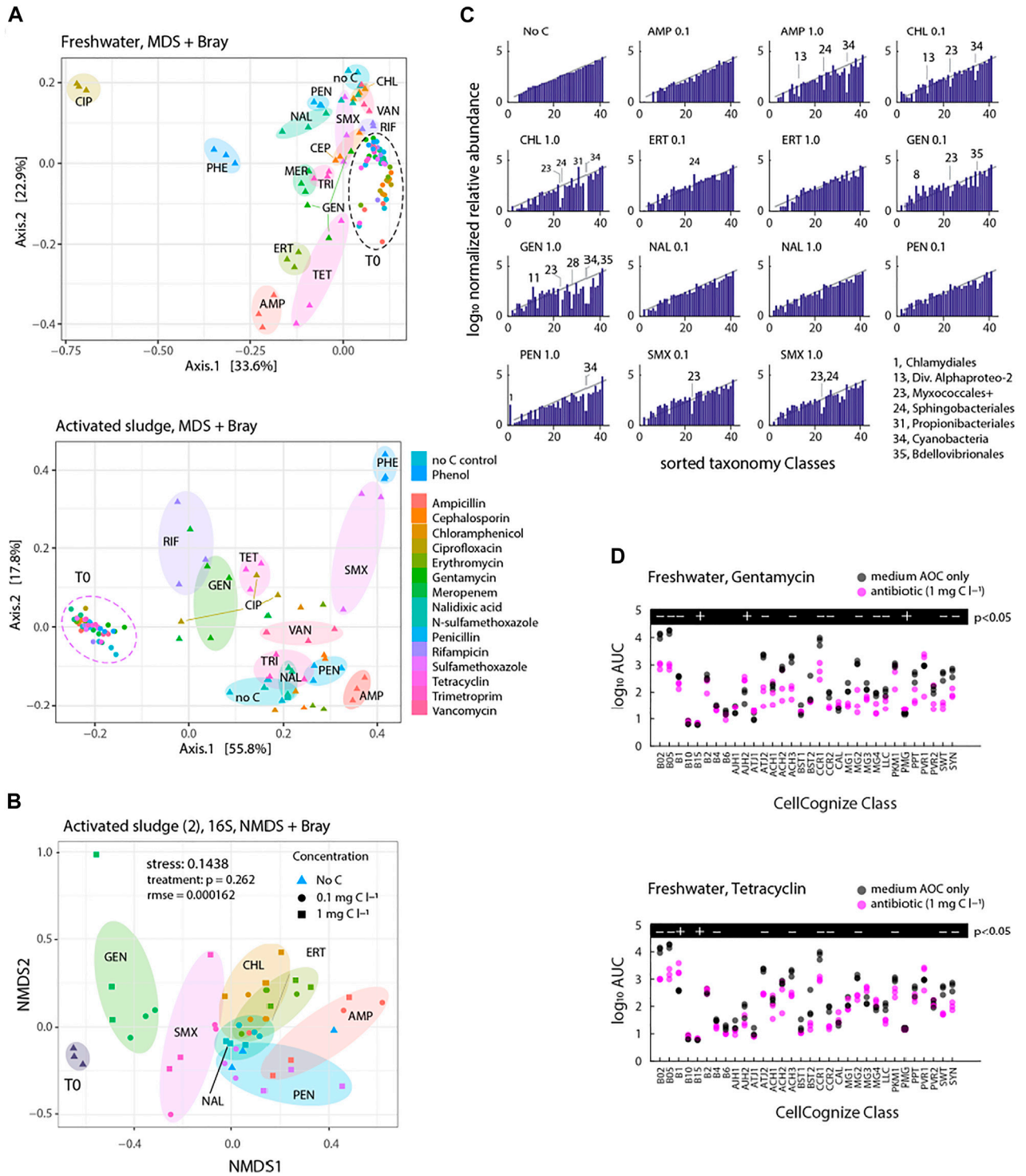


FIGURE 6 | Freshwater or activated sludge community composition after incubation with antibiotics at nominal initial concentrations of 0.1 and 1 mg C L^{-1}. **(A)** Community composition clustering based on Bray-Curtis dissimilarity distance (*phyloseq* package in R) of antibiotics or no added carbon control series (nbatch = 3) for 16S rRNA gene amplicon sequence SILVA-classified reads at taxonomy level 7 (stress 0.1438, rmse = 0.000162). Significance of treatment circumfered with transparently coloured ellipsoids is 0.262. **(B)** Community composition clustering based on Bray-Curtis dissimilarity distance (phyloseq package in R) of antibiotics or no added carbon control series for flow cytometric fingerprints deconvoluted into 32 cell types. Transparently coloured ellipsoids circumfere individual replicates per time points of incubations for treated activated sludge inoculum source. **(C)** Log_{10} normalized relative abundance of sorted Orders (taxonomy level 4) for each treatment condition compared to No C after 8 days incubation (the same activated sludge microbial community experiment as in **(B)**. **(D)** Comparison of cell type productivity (AUC) of the incubation of freshwater community with gentamycin (top) or tetracycline (bottom) to no-carbon control (n_{batch} = 3). Significance of treatment effect was calculated with paired *t*-test (+is for $p < 0.05$ and—is for $p > 0.05$).

Supplementary Figure S4). A single class enrichment (represented by the cell type population PVR1) stood out in freshwater community incubations with AMP or ERT (**Figure 4B**), with other minor ones detectable as well (**Supplementary Figure S6**), which was consistent with total community growth and antibiotic disappearance. Interestingly, other antibiotics such as ciprofloxacin (CIP) also led to significant increases of cell type populations (here AJH2, **Figure 4B**), but this was too small an effect to be observed at community size level. A different profile was observed for class enrichments with activated sludge inoculum, where AMP led to increases in cell type populations represented by the MG1 and AJH2 cell standards, and SMX enriching a variety of classes, suggesting implication of various different types of bacteria (**Figure 4B**, for a full representation of all classes, see **Supplementary Figure S5**).

Enrichment of the identified cell type populations was visible as specific subclass "growth" over time. For example, in comparison to freshwater community growth in the no-carbon controls, class B02 did not specifically enrich with AMP (**Figure 4C**, AMP-B02 versus NoC-B02), whereas class PVR1 increased rapidly (**Figure 4C**, AMP-PVR1, **Supplementary Figure S6**). Growth of the same cell type population also occurred with ERT but not with PHE, indicating selective response; despite the relatively coarse attribution of 32 cell type populations in our analysis (**Figure 4C**, ERT-PVR1 and PHE-PVR1). Freshwater community growth on phenol was again specific for class MG1 (**Figure 4C**, NoC-MG1 versus PHE-MG1). Growth of cell type populations within the activated sludge inoculum was different, with slower growth on AMP (e.g., cell type populations AJH2 and MG1), and variable growth for ERT and SMX (**Figure 4C**, note different abundance scales, **Supplementary Figure S7**). These examples thus indicate that the observed community growth in some cases (e.g., AMP and SMX) was due to distinguishable growth of a small number of cell type populations. In other cases (e.g., CIP), a single cell type population detectably and selectively grew but its size was insufficient to cause a significant effect on the total community size. In summary, the differentiation and quantification of cell type populations within the community strongly suggested selective responses to the antibiotics, potentially as a result of their biotransformation and usage as growth substrate.

Effects of Dead Cells

Addition of antibiotics to mixed communities could potentially lead to cell death, which then on its turn could provide sources of carbon and nutrients for growth of subpopulations of cells within the community. Therefore, we repeated incubations of freshwater community with ALW medium only or with ALW mixed with 25% of pasteurized dead cell community (assuming this would lead to nutrient release). Growth of the communities in these conditions was not significantly different, neither in selective enrichment or decrease of identifiable cell type populations (**Figure 5A**), nor in cell type productivities during 6 days of incubation (AUC, **Figure 5B**, $p > 0.05$). Therefore, we concluded that it is unlikely that released carbon from dead cells upon antibiotic dosing would cause the observed selective cell type population growth in antibiotic incubations.

Community Composition Changes Induced by Antibiotics

In order to better understand the effects of the dosed antibiotics on the communities as a whole (including potential inhibitory action), we further investigated community composition changes by CellCognize class attribution from flow cytometry data and 16S rRNA gene amplicon sequencing analysis on an independent activated sludge inoculum experiment.

Community compositions distinguished on multidimensional scaling (MDS) of CellCognize class attributions showed clear effects for most of the antibiotics in samples after 5–6 days, both for freshwater and activated sludge inoculum, explaining 57 and 74% of variance, respectively, in two dimensions (**Figure 6A**). Activated sludge inoculum contrasted to freshwater inoculum with apparent compositional MDS distances in incubations with SMX and RIF compared to their no-carbon and phenol controls (PHE). The MDS distance between CIP-incubated samples and the others was more striking for freshwater than activated sludge inoculum (**Figure 6A**). Addition of TET and AMP caused obvious community deviations for both inoculum sources, whereas the other antibiotics resulted in lesser variation compared to the no-carbon controls (**Figure 6A**). When shown on the same MDS plot, inoculum-dependent clustering of the antibiotics effects was significant (stress = 0.152664, rmse = 0.09952, $p = 0.003$ **Supplementary Figure S8**).

Based on 16S rRNA gene amplicon analysis of an independent activated sludge inoculum experiment, antibiotics amendment at two different concentrations (0.1 and 1 mg C l^{-1}) led to significant community composition changes after 8 days of incubation (stress 0.1438, rmse = 0.000162, **Figure 6B**), but partly overlapping individual antibiotic- and concentration-effects ($p = 0.262$ for "treatment"). GEN, SMX and AMP deviated the most from the corresponding no-carbon controls, whereas CHL, ERT, PEN and NAL deviated less (**Figure 6B**).

16S rRNA gene amplicon analysis further suggested 7 out of 51 detected Classes to have a significantly changed relative abundance upon antibiotic addition, in comparison to the no-carbon control, after 8 days (**Supplementary Figure S9**; false discovery rate <0.05, q-value < 0.05). Depending on the dosed antibiotic, both decreases and increases compared to the no-carbon control (considered as inhibition and enrichment, respectively) were found within the same Class (see, for instance, the contrasting effects of AMP and CHL in the Actinobacteria and Bacteroida in **Supplementary Figure S9**). Gammaproteobacteria had the highest abundance in the initial community as well as after incubation with antibiotics and in the no-carbon control (**Supplementary Figure S9**).

Since the community size data had also suggested inhibitory responses by addition of antibiotics compared to no-carbon controls as mentioned above, we further compared log normalized relative abundances of sorted Orders (taxonomy level 4) based on the means of no-carbon controls for each antibiotic treatment condition after 8 days incubation.

Members of the Myxococcales were frequently among the most inhibited, and in a concentration-dependent manner (CHL, GEN, SMX, **Figure 6C**). Also, members of the Sphingobacteriales, Cyanobacteria and Bdellovibriales were found specifically inhibited compared to no-carbon control (**Figure 6C**). In terms of the CellCognize differentiation, inhibition was frequently observed in the abundant B02, B05 and CCR1 classes (**Figure 6D**, examples GEN and TET for freshwater inoculum). These represent small cell sizes (e.g., B02 and B05) and curved bacteria (e.g., CCR1). These results thus showed that antibiotics cause selective inhibition on communities, which is inoculum source- and taxonomy level or cell type-dependent.

DISCUSSION

Our study aimed to answer to what extent antibiotics with varying modes of action may be used as growth substrate at sub-MIC clinical levels (0.1 or 1 mg C L^{-1}) (Obayiuwana et al., 2018) by diverse microbial community members. In conjunction, we studied how community composition is influenced by the exposure to the antibiotics, assuming both growth, neutral or inhibitory effects. We found some evidence for growth-linked biodegradation of antibiotics by regular environmental communities in fresh water, activated sludge or soil, even at low carbon concentrations. For ampicillin, erythromycin, chloramphenicol, and sulfomethoxazole, we found consistent community growth, which was accompanied by parent compound disappearance. Nalidixic acid also led to community growth but this was not verified by parent compound measurements. Our results were further supported by identification of specific cell type populations within communities for which we could demonstrate growth upon antibiotic amendment, suggesting these might consist of bacterial species that can profit from the antibiotics as carbon, nitrogen or energy substrates. For the remainder of the tested antibiotics, we found no evidence for community growth or specific increases of certain cell type populations, except for one case of distinct growth of a cell type similar to AJH2 with ciprofloxacin that was too small to lead to detectable community growth. Rather, most antibiotics led to inhibition of community growth on background AOC, and caused further clear changes in community composition.

Although biotransformation of selected antibiotics has been reported (Dantas et al., 2008) and individual strains using antibiotics as growth substrates have been isolated as recently summarized in (Reis et al., 2020a; b), there is still a lot of uncertainty about the fate of antibiotics in environmental microbial communities. Classical biodegradation studies and strain isolations are hampered and limited by the high toxicity of individual antibiotics, and have led to confusing and contradictory results (Walsh et al., 2013). Consequently, enrichment studies would have to be done at lower (0.1—1 mg C l^{-1}) or even environmentally relevant antibiotic concentrations (<1 μg C l^{-1}), but this leads to the difficulty of accurate detection of microbial growth. We tried to solve this conundrum here by quantifying microbial community cell numbers in closed batch system over time by means of flow cytometry coupled to a cell-type population recognition pipeline (i.e., CellCognize) (Özel Duygan et al., 2020). This allowed us to identify near real-time changes in absolute counts of cell-type populations within diverse microbial communities in comparison to no-carbon controls. Previously shown its usefulness for quantifying biodegradation of low concentrations of standard pollutants (e.g., phenol and 1-octanol) and fragrances in natural communities (Özel Duygan et al., 2021), this method was also valuable for assessing biodegradation of antibiotics. The observed specific community and subgroup increases, and corresponding chemical concentration measurements are strong indications that there is utilization of ampicillin, erythromycin, chloramphenicol, and sulfomethoxazole as growth substrates in the range of 0.1–1 mg C l^{-1} by members of natural environmental communities such as in freshwater or activated sludge.

Obviously, the difficulty with community growth studies at low substrate dosages is to provide a conclusive link between changes in cell population abundances and productive substrate utilization; in this case, the metabolism of the antibiotics. Instead of deducing cell population changes from relative abundances from typical 16S rRNA gene amplicon sequencing, the flow cytometry-based CellCognize pipeline used here gives absolute abundances of cell type populations. This makes it easier to discern growth of subpopulations within the community resulting from antibiotic addition compared to background growth in the no-carbon control. Comparison to no-carbon as "negative" and phenol as "positive" control showed unequivocally that there is selective enrichment of specific cell type populations in response to few select antibiotics (ampicillin, erythromycin, chloramphenicol or sulfomethoxazole) and inhibition in response to others, as one would intuitively expect. Antibiotics effects were also inoculum-dependent. For example, enrichment was observed with sulfamethoxazole in activated sludge but not in freshwater communities. The finding that multiple CellCognize-attributed cell types enriched simultaneously (e.g., ACH and PVR1 in ampicillin and erythromycin), suggested there may be multiple principal degraders or cooperating bacteria. In absence of a complete mass balance, we cannot completely ascertain that the antibiotics are indeed utilized for growth, but a rough calculation argues in favour of it. Chemical measurements suggested 30% (for erythromycin and sulfomethoxazole) to 100% (ampicillin, some freshwater replicates) parent compound disappearance, equivalent to a net community increase of 3–7 × 10^5 cells ml^{-1}, and equivalent in some cases to the community productivity on 1 mg C l^{-1} of added phenol. Previous measurements of phenol productivity in the same system showed mass carbon yields of 20% at 1 mg C l^{-1} dosage (Özel Duygan et al., 2021), which, when extrapolated, would indicate 6–20% yields for erythromycin or sulfomethoxazole and ampicillin, respectively. This would indicate at least partial utilization of the carbon structure of the parent compound for biomass formation. Consequently, although we did not focus on ciprofloxacin chemical measurements, the unique and selective enrichment of cell type AJH2 (and some others, Supp. Fig. 4) in freshwater

incubations with ciprofloxacin up to 10^4 cells ml^{-1} would be indicative for 2% carbon utilization from the parent compound (assuming similar yields as for phenol). Future studies should confirm this by investigating stable transformation products of these antibiotics in order to further elucidate their biodegradation by natural microbial communities.

Is subsistence on antibiotics widespread? Our results clearly indicate that this is not the case, at least not at the concentrations used here, which is similar to conclusions reached in earlier studies with mixed wastewater inocula (Alexy et al., 2004; Gartiser et al., 2007; Yang et al., 2019) or soil (Walsh et al., 2013). Our inocula covered a wide range of taxa from soil (439 OTUs), freshwater (150 OTUs) and activated sludge (151 OTUs). Even though degrader bacteria might be present at very low initial numbers, we anticipated we would be able to detect their growth if there were any. The example of ciprofloxacin in freshwater inoculum suggests that we would have been able to detect enrichment of a subpopulation to 10^4 cells ml^{-1} in the community mixture. Even though there was quite a bit of variation among the target inocula and no "pre-enrichment" of exposure to antibiotics, this should mean that the fraction of viable specialist antibiotic degraders (except for the four or five positive cases detected here) was too low or non-existent.

Of the cases where we detected growth-linked antibiotics biodegradation, sulfomethoxazole was the most expected, since its biodegradation is now widely established (Reis et al., 2020a). However, growth-linked degradation of ampicillin, chloramphenicol and erythromycin is less well-described, except in a few studies (Lai et al., 1995; Gartiser et al., 2007; Pan and Chu, 2016), and suggests that such capacity may be present more widely. It would be interesting to follow up on this and attempt to obtain pure cultures of organisms that would grow on these compounds at low antibiotics concentrations only. Although we tried and deployed different experimental strategies (e.g. use of MicroDish® culture chips (Ingham et al., 2007)), we were unable to isolate pure cultures from the primary enrichments that continued to grow with the same antibiotics and at similar dose of 1 mg C l^{-1}. Even subsequent dilutions of primary enrichment cultures into fresh ALW media did not lead to consistent further "enrichments", which is likely due to the insufficient net growth advantage of potential antibiotic-degraders upon community dilution in the renewed media AOC background. We tried to resolve this by looking for and separating microcolonies on low carbon solid growth media in presence or absence of the antibiotic. However, even here opportunistic community members seemed to profit from background AOC and produce (micro-)colonies, despite the selective presence of the antibiotic as sole carbon and energy source. Such opportunistic growth might have also been possible with synergistic effects of resistant cells protecting those sensitive cells from antibiotics (Sharma and Wood, 2021). Potential avenues to enrich further for antibiotics degraders at low concentrations might be to deploy slow sand filter columns as in earlier selection studies with chlorinated compounds in the μg l^{-1} range (van der Meer et al., 1987) or adapt enrichment under flow to specifically designed microfluidics platforms e.g. (Mahler et al., 2018) where cells can be retained while being exposed to low antibiotics concentrations as sole carbon and energy sources. Eventually, ^{13}C-labeled compound should be used to then confirm biomass incorporation and characterize transformation products.

Finally, most of the antibiotics caused selective inhibition of community growth and resulted in specific community composition changes, which were inoculum source- and cell type-dependent. This is not unexpected (Grenni et al., 2018; Cycon et al., 2019; Tong et al., 2020) but demonstrates anew the strong ecological effects exerted by the inhibitory action of the various antibiotics and thus the care society should be giving to avoid them entering into the various ecosystems in the first place.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI (accession: PRJNA744029). Raw flow cytometry data from all samples presented here are available from Flow Repository with experiment number FR-FCM-Z447 (https://flowrepository.org/id/ FR-FCM-Z447).

AUTHOR CONTRIBUTIONS

BÖD and CG provided experimental data. BÖD, KF and JvdM analysed the data. BÖD and JvdM wrote the main text. All authors corrected and approved the final text.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2021.737247/full#supplementary-material

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Utilizing Metagenomic Data and Bioinformatic Tools for Elucidating Antibiotic Resistance Genes in Environment

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Antibiotics resistance genes (ARGs) are mainly caused by the extensive use and abuse of antibiotics and have become a global public health concern. Owing to the development of high-throughput sequencing, metagenomic sequencing has been widely applied to profile the composition of ARGs, investigate their distribution pattern, and track their sources in diverse environments. However, the lack of a detailed transmission mechanism of ARGs limits the management of its pollution. Hence, it's essential to introduce how to utilize the metagenomic data to obtain an in-depth understanding of the distribution pattern and transmission of ARGs. This review provides an assessment of metagenomic data utilization in ARG studies and summarizes current bioinformatic tools and databases, including ARGs-OAP, ARG analyzer, DeepARG, CARD, and SARG, for profiling the composition of ARGs and tracking the source of ARGs. Several bioinformatic tools and databases were then benchmarked. Our results showed that although SARG is a good database, the application of two or more bioinformatic tools and databases could provide a comprehensive view of ARG profiles in diverse environmental samples. Finally, several perspectives were proposed for future studies to obtain an in-depth understanding of ARGs based on metagenomic data. Our review of the utilization of metagenomic data together with bioinformatic tools and databases in ARG studies could provide insights on exploring the profiles and transmission mechanism of ARG in different environments that mitigate the spread of ARGs and manage the ARGs pollution.

Keywords: antibiotics resistance genes, metagenomic data, bioinformatic tools, databases, source tracking

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INTRODUCTION

Since the discovery of penicillin, researchers have opened the modern era of the innovation, development, and application of antibiotics in human society. At present, antibiotics are used as medicine for humans and animals and widely applied in animal husbandry, agriculture, and aquaculture (Manage, 2018). However, with the intense use and abuse of antibiotics for human and agricultural purposes, antibiotics are continuously discharged into different environments, particularly those with limited sewage treatment capacity, resulting in a substantial increase of antibiotic residue in different environmental niches (Carvalho and Santos, 2016; Qiao et al., 2018). These residual antibiotics increase the risk of antibiotic resistance and produce antibiotic resistance

genes (ARGs) that could be transferred to various microorganisms. This phenomenon is not new (Yang et al., 2018) and has attracted global concern, particularly its spread and transmission mechanism (Holmes et al., 2016). To date, antibiotics and their effects on different environment niches, for example, the emergence and spread of ARGs, have become an urgent and growing global public health threat in environmental science (Sanderson et al., 2016; Yang et al., 2018; Iwu et al., 2020). Hence, many researchers paid attention to ARGs to investigate their distribution and transmission.

With the successes of investigation on ARGs, researchers have identified the composition of ARGs and explored their distribution in different environment niches. For example, a total of 139, 442, and 491 ARG subtypes were identified in sediments from the Yamuna River, sediments from an urban river in Beijing (Chaobai River), and activated sludge reactors, respectively (Chen et al., 2019a; Zhao et al., 2019; Das et al., 2020). Based on these published studies, we found that many studies have focused on the composition of ARGs and their dynamics; however, only a few studies investigated the transmission of ARGs; the transmission route for ARGs is poorly characterized (Zhou et al., 2018; Chao et al., 2019; Vrancianu et al., 2020). A comparison on the occurrence and abundance of ARGs and microbiota in healthy humans and sewage treatment systems in a Chinese village identified 53 ARGs and 28 bacteria genera in all samples; this result supports the idea that bacteria could carry and transfer several ARGs to humans and the environment (Zhou et al., 2018). Furthermore, different mechanisms of horizontal gene transfer, including conjugation, transduction, and transformation, were also found to contribute to the accumulation and transmission of ARGs in bacteria (Chen et al., 2019b; Li et al., 2019; Vrancianu et al., 2020). Given the scarcity of studies exploring the transmission of ARGs, their detailed transmission mechanism remains elusive.

Recently, many sequencing techniques have been developed and applied in ARG studies. Owing to its advantages, high-throughput sequencing has been widely applied in microbiome studies to detect ARGs and is expected to solve the problem of transmission and proliferation mechanism of ARGs in different environments. With the growing number of microbiome studies focusing on ARGs, many metagenomic datasets, bioinformatic tools, and associated ARG databases have been generated for ARG analysis. By using these tools and databases, researchers have profiled ARG composition in different environments and deepened the understanding of ARGs. However, some urgent scientific questions remain unanswered, such as which bioinformatic tools and ARG databases are suitable for detecting potential ARGs? In addition, the various equations for ARG abundance calculation make it impossible to directly compare the results of different ARG studies. Besides, details on the transmission and management of ARGs remain elusive. Therefore, given that antimicrobial resistance is still a crucial and urgent threat to human's health and the environment, a summary of the methods and prospects for ARG studies is essential. Therefore, this review first summarized the popular and latest bioinformatics methods for analyzing the metagenomic data generated by next-generation sequencing and third-generation sequencing, including bioinformatic tools, ARG databases, and MGE databases. And then several bioinformatic tools and databases were benchmarked to evaluate their benefits and drawbacks. Finally, several critical comments and perspectives were proposed for future ARG studies to obtain an in-depth understanding of ARGs based on metagenomic data.

METAGENOMIC DATA IN ANTIBIOTICS RESISTANCE GENE STUDIES

In past decades, researchers have proved that microbiota plays an important role in maintaining human health (Marchesi et al., 2016; Valdes et al., 2018) and participating in biogeochemical circulation (Carnevali et al., 2021). Owing to its advantages, high-throughput sequencing has been widely applied in ARG studies. With the successful investigation on microbial communities in diverse environments, massive metagenomic datasets have been produced to investigate the taxonomical and functional compositions of microbial communities, obtain an in-depth understanding of functional traits, such as nitrogen cycle (Jansson and Hofmockel, 2018; Miao and Liu, 2018) and ARGs (Stalder et al., 2019; Xiang et al., 2020), and explore the driving factors for the dynamic changes of functional traits (Pan et al., 2020). For example, based on metagenomic datasets, ARG profiles in different environments have been investigated and explored, such as activated sludge under high selective pressure with different antibiotics (Zhao et al., 2019) and seed activated sludge collected from a municipal wastewater treatment plant and five experiment groups with different antibiotics (Zhao et al., 2020) and a deep subtropical lake (Carnevali et al., 2021). These studies revealed that metagenomic sequencing creates an opportunity for capturing the majority of ARGs and their potential hosts. In addition, metagenomic analysis can reveal the transmission of ARGs and the risk of resistome (including ARGs) (Manaia, 2017; Yin et al., 2019; Qian et al., 2021a). In summary, proper utilization of metagenomic data can effectively provide an in-depth understanding of ARGs in the environment, particularly their transmission and risks.

BIOINFORMATIC TOOLS USED FOR DETECTING POTENTIAL ANTIBIOTICS RESISTANCE GENES BASED ON METAGENOMIC DATA

With the increasing of metagenomic datasets from next-generation sequencing and third-generation sequencing, many bioinformatic tools have been developed to conduct analyses at different aspects. In general, the methodological approaches of the whole metagenomic dataset can be divided into two types, namely, assembly-based and read-based (non-assembly, **Figure 1A**) (Boolchandani et al., 2019; Harris et al., 2019). With these strategies, several bioinformatics tools, including online tools, have been developed for identifying the ARGs and detecting new ARGs (**Figure 1**).

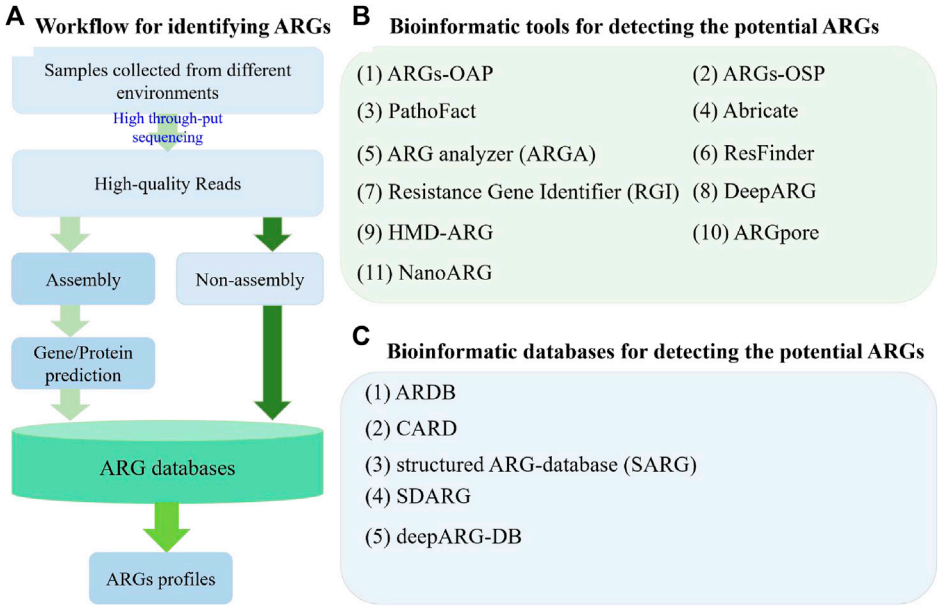

FIGURE 1 | Workflow and several bioinformatic tools and databases used in ARG studies for detecting potential ARGs. **(A)** Two different strategies used in ARG studies for profiling the composition of ARGs based on metagenomic data; **(B)** Information of several bioinformatic tools used for detecting potential ARGs. **(C)** Information of several bioinformatic databases used in ARG studies.

Specifically, on the basis of assembly-based strategy, ARGs-OAP (v1.0 and v2.0, http://smile.hku.hk/SARGs) (Yang et al., 2016; Yin et al., 2018), ARGs-OSP (http://smile.hku.hk/SARGs) (Zhang et al., 2020), PathoFact (https://git-r3lab.uni.lu/laura.denies/PathoFact/) (de Nies et al., 2021), ARG analyzer (ARGA, http://mem.rcees.ac.cn:8083/) (Wei et al., 2019), Resistance Gene Identifier (RGI, https://github.com/arpcard/rgi) (Alcock et al., 2020), ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/), DeepARG (https://bench.cs.vt.edu/deeparg) (Arango-Argoty et al., 2018), and HMD-ARG (http://www.cbrc.kaust.edu.sa/HMDARG/) (Li et al., 2021a) were developed and have been widely applied to detect potential ARGs from the gene datasets predicted from metagenomic contigs (**Figure 1B**). Together with the ARG database, ARGs-OAP was designed as an online pipeline to fast annotate and classify ARG-like sequences from metagenomic data (Yang et al., 2016). Compared with the version 1.0 of ARGs-OAP, the latest version was updated and added with the Hidden Markov Model algorithm for the enhancement characterization and quantification of ARGs in metagenomic datasets based on the 16S rRNA gene and the average coverage of essential single-copy marker genes (Yin et al., 2018). Similarly, to solve the most challenging topics and provide a guide for diverse research in ARG studies, including the risk, evolution, and emergence of ARGs, a comprehensive profile of the distribution of ARGs on an ARGs online searching platform (ARGs-OSP) was constructed based on the distribution of potential ARGs in 55,000 bacterial genomes, 16,000 bacterial plasmid sequences, 3,000 bacterial integrin sequences and 850 metagenomes (Zhang et al., 2020). Furthermore, PathoFact was designed and developed to solve the virulence factors (VFs) and ARGs of pathogenic microorganisms; this an easy-to-use, modular, and reproducible tool can predict VFs, bacterial toxins, and ARG from metagenomic data with high accuracy (de Nies et al., 2021). Moreover, on the basis of an updated database, ARGA was developed to assess the primer of ARGs and identify and annotate ARGs from environmental metagenomes (Wei et al., 2019). It should be noted that the identification of potential ARGs usually depends on the search results. The selection strategy is to choose the best hit among the search results; however, this strategy can produce a high rate of false negatives. As a solution, DeepARG with two deep learning models (Arango-Argoty et al., 2018) and HMD-ARG (Li et al., 2021a) were constructed for ARG detection.

In contrast, only a few read-based bioinformatic tools were developed for ARG detection. For example, one deep learning model of DeepARG, namely, DeepARG-SS, was designed to analyze the short-read sequences in metagenomes (Arango-Argoty et al., 2018). Moreover, it's well-known that Oxford Nanopore sequencing can produce ultra-long read sequencing reads; however, the identified ARGs can be analyzed at reads level. As a solution, ARGpore (Xia et al., 2017) and NanoARG (Arango-Argoty et al., 2019) were constructed (**Figure 1B**). Specifically, ARGpore was designed to detect ARGs and their hosts by utilizing BLAST, HMMER, and UBLAST (Xia et al., 2017). NanoARG was constructed as a web service to identify the ARGs from the long reads generated by Oxford Nanopore sequencing and provide the identification of metal resistance genes, mobile genetic elements (MGEs), and sequences with high similarity to known pathogens (Arango-Argoty et al., 2019).

In summary, diverse bioinformatic tools have been constructed and developed with different strategies. These bioinformatic tools can efficiently detect ARGs in different

environments to meet the requirements of ARG analysis. With the use of ARG profiles in environmental metagenomes, downstream analyses on co-occurrence patterns among ARGs, the arrangement of ARGs and MGEs, and host identification of ARGs can be performed to enhance the understanding of ARGs in diverse environments.

BIOINFORMATIC DATABASES USED FOR IDENTIFYING ANTIBIOTICS RESISTANCE GENES AND MGES

The identification of potential ARGs depends on the search results against the database. Therefore, ARG databases are very important because they determine the accuracy and completeness of ARGs in environmental metagenomes. To date, several ARGs databases have been constructed for ARG detection (**Figure 1C**), such as Antibiotic Resistance Genes Database (ARDB, http://ardb.cbcb.umd.edu/) (Liu and Pop, 2009), the Comprehensive Antibiotic Resistance Database (CARD, https://card.mcmaster.ca/) (McArthur et al., 2013), structured ARG-database (SARG, http://smile.hku.hk/SARGs) (Yang et al., 2016; Yin et al., 2018), sequence database of antibiotic resistance genes (SDARG, http://mem.rcees.ac.cn:8083/) (Wei et al., 2019), and deepARG-DB (Arango-Argoty et al., 2018). Specifically, ARDB was constructed in 2009 and contains the resistance information for 13,293 genes and 257 antibiotics (Liu and Pop, 2009). This database was widely applied to detect potential ARGs but is now abandoned because of the lack of updates. As a solution, CARD was rigorously constructed and developed in 2013. This database integrates disparate molecular and sequence data, provides a unique organizing principle (antibiotic resistance ontology and antimicrobial resistance gene detection models), and can quickly and effectively detect putative ARGs (McArthur et al., 2013). CARD is currently a bioinformatic database and a compressive platform for identifying resistance genes, including their products and associated phenotypes (https://card.mcmaster.ca/). In 2016, SARG was constructed with a hierarchical structure (type-subtype-reference sequence) by integrating the two most commonly used ARG databases ARDB and CARD, removing their redundant sequences, and re-selecting the query sequences based on the similarity of sequences; this database can identify ARG sequences through similarity search (Yang et al., 2016) and has been widely used in ARG studies (Zhao et al., 2019; Zhao et al., 2020). The latest version of SARG (v2.0) has tripled the sequences of the first version, improved the coverage of ARG detection, and annotated the high-throughput raw reads by using a similarity search strategy in diverse environmental metagenomes (Zhao et al., 2020). Based on ARDB, an updated SDARG, including 1,260,069 protein sequences and 1,164,479 nucleotide sequences from 448 types of ARGs belonging to 18 categories of antibiotics, was constructed and used in ARGA (Wei et al., 2019). Moreover, as a companion database to DeepARG, DeepARG-DB was designed to improve the quality of the model (Arango-Argoty et al., 2018). These ARG databases provide choices for researchers to comprehensively detect ARGs in environmental metagenomes.

Additionally, previous studies have demonstrated that the transmission of ARGs is associated with MGEs and the relationship between ARGs and MGEs is a hot topic in ARG studies (Wang et al., 2020a; Liu et al., 2021; Lin et al., 2021; Wang et al., 2020b). Therefore, the associated databases for MGE identification in metagenomic datasets must be constructed to address these issues. At present, several databases, including ACLAME (Leplae et al., 2004; Leplae et al., 2010), ISfinder Database (Siguier et al., 2006), ISsaga2 (Varani et al., 2011), INTEGRALL (Moura et al., 2009), *Gypsy* Database (GyDB) (Llorens et al., 2010), and a MGE database (https://github.com/KatariinaParnanen/MobileGeneticElementDatabase) (Pärnänen et al., 2018), have been constructed and applied to investigate the occurrence of ARGs and MGEs.

Together, these diverse ARG and MGE databases provide a powerful resource for identifying ARGs and MGEs, exploring the distribution of ARGs, investigating the relationship between ARGs and MGEs, and obtaining an in-depth understanding of ARG transmission that benefits their management.

BENCHMARKING OF THE BIOINFORMATIC TOOLS AND DATABASES USED FOR ANTIBIOTICS RESISTANCE GENE DETECTION IN DIVERSE ENVIRONMENTS

Several bioinformatic tools, including Abricate (https://github.com/tseemann/abricate), DeepARG, and Blastx, and ARG databases, including ARG-ANNOT, Resfinder, CARD, DeepARG-DB, and SARG (v2.0), were benchmarked to determine which bioinformatic tools and ARG databases are suitable to detect potential ARGs in diverse environmental samples. In particular, three metagenomic datasets were collected for each of the five kinds of environmental niches chosen for benchmarking, namely, the water of lake (SRR14578319, SRR14578320, and SRR14578321), the sediment of lake (SRR14887730, SRR14887731, and SRR14887732), activated sludge (SRR14610228, SRR14610229, and SRR14610230), ocean (SRR14865845, SRR14865846, and SRR14865847), and human feces (SRR15032192, SRR15032193, and SRR15032194). Finally, 15 metagenomic datasets were downloaded from NCBI. The quality of these metagenomic datasets was controlled with Trimmomatic (Bolger et al., 2014) using the following parameters: TruSeq3-PE.fa:2:30:10, LEADING:3, TRAILING:3, SLIDINGWINDOW:5:20, and MINLEN:25, to obtain high-quality reads. High-quality reads were assembled by MEGAHIT (Li et al., 2016) with the following parameters: -meta-large and a *k*-mer list of 27, 37, 47, 57, 67, 77, 87, 97, 107, 117, and 127, to obtain the metagenomic contigs (contigs of length >500 bp were kept), and then the gene sequences and protein sequences were predicted with Prodigal (Hyatt et al., 2010). The nucleic acid sequences obtained from different environmental samples were searched against the ARG-ANNOT, Resfinder, and CARD databases by using Abriate and the potential ARGs were selected

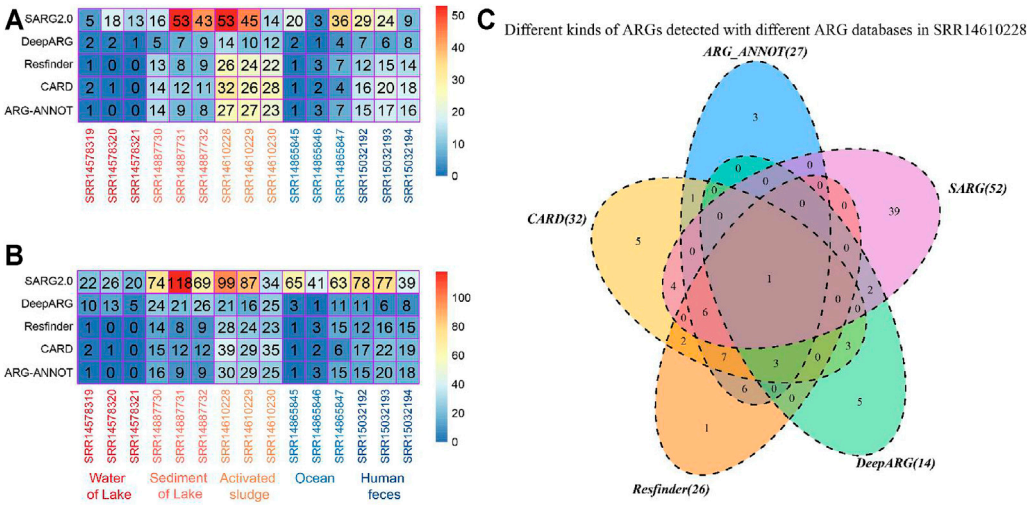

FIGURE 2 | Benchmark of the bioinformatic tools and databases for ARG detection in various environments. Distribution of **(A)** kinds of ARGs and **(B)** the total number of ARGs identified in various environmental niches with different bioinformatic tools and databases. **(C)** Different kinds of ARGs detected with different ARG databases in sample SRR14610228.

with similarity ≥80% and coverage ≥70%. Whereas, the potential ARGs identified with DeepARG (v2.0) were selected with default settings (similarity ≥80%), and the ARG candidates identified with Blastx against SARG databases (v2.0, protein sequences) were selected with similarity ≥80% and coverage ≥70%.

Comparison of ARG profiles in various environmental niches identified with different bioinformatic tools and databases revealed inconsistency in the kinds and total number of ARGs (**Figure 2**). For the sediment of lake and activated sludge samples, the number of ARG types identified with ARG-ANNOT, CARD, and Resfinder was higher than that with DeepARG-DB but fewer than that with SARG (v2.0, **Figure 2A**). Similarly, the total number of ARGs identified with SARG (v2.0) was the highest among all databases (**Figure 2B**). Further comparison of ARG profiles in sample SRR14610228 revealed the differences in the intersections of ARG profiles detected with two, three, four, and five bioinformatic tools and databases (**Figure 2C**). All these results suggested that although SARG (v2.0) is a good database for identifying potential ARGs, the application of two or more bioinformatic tools and databases could provide comprehensive ARG profiles in different environmental samples.

BIOINFORMATIC TOOLS FOR TRACKING THE ANTIBIOTICS RESISTANCE GENE SOURCE

Considering the tight linkage between ARGs in the environment, the ARG source is important to their transmission and management. Therefore, an ARG source tracking platform must be urgently developed. In the past 2 decades, many researchers have realized the importance of tracking the source of ARG and thus developed many bioinformatic tools or frameworks. Among which, a series of bioinformatic tools or frameworks were developed and proposed for tracking ARG pollution from different sources, such as SourceTracker (Knights et al., 2011) and its application in metagenomic datasets (Meta-SourceTracker) (McGhee et al., 2020), Microbial Source Tracking (MST) (Li et al., 2018), Meta-Prism (Zhu et al., 2021), and FEAST (Shenhav et al., 2019). However, only a few tools and applications were used to track the genetic location of the host of ARGs (host-tracking of ARGs), such as PlasFlow (Krawczyk et al., 2018). Specifically, among these source-tracking tools, some including SourceTracker and MST can be used to precisely track ARG pollution from different sources. For example, based on deep-sequencing marker genes, such as 16S rRNA, SourceTracker was designed and constructed with a Bayesian classification model; this tool uses Gibbs sampling to determine the possibility and predict the source of samples (Knights et al., 2011) and has been widely applied to determine the source of ARG pollution in diverse environment samples (Hu et al., 2020; Chen et al., 2019c). Moreover, on the basis of a machine-learning classification strategy with ARG abundance profiles, MST was developed and constructed as a source-tracking platform that can precisely track ARG pollution from different sources, such as feces of humans and animals, wastewater treatment plants, and other natural environments (Li et al., 2018; Li et al., 2020), which is available at https://smile.hku.hk/SARGs/. Additionally, based on the genome signatures of sequences from 9,565 bacterial plasmid and chromosomes, PlasFlow with a deep neural network model was developed to predict the bacterial plasmid sequences or chromosomes in metagenomic contigs with high classification accuracy (Krawczyk et al., 2018) and then assist in the tracking of the genetic location and taxonomy of ARG host. To date, the accurate host-tracking of ARGs remains a challenge in ARG studies. Nevertheless, numerous studies using these bioinformatic tools have been conducted to determine the source of ARG pollution

and host-tracking of ARGs and explore the distribution pattern of ARGs in diverse environment samples (Ma et al., 2017; Chen et al., 2019c; Dang et al., 2020; Raza et al., 2021; Zhou et al., 2021). Undoubtedly, tracking the source of ARG, including the source of ARG pollution and the host-tracking of ARGs, is important to obtain an in-depth understanding of ARG transmission and provide suggestions for managing ARGs in natural environments.

FUTURE PERSPECTIVES IN ANTIBIOTICS RESISTANCE GENE STUDIES

ARG pollution caused by the overuse of antibiotics has increased in diverse natural environments and has become a global concern about human health. At present, the metagenomic dataset produced by high-throughput techniques was popularly applied in ARG studies. Numerous investigations have been conducted to explore the distribution, transmission, source of ARG, and the key factors driving ARGs (Li et al., 2015; Zhao et al., 2020; Li et al., 2021b). Although an increasing number of studies have been conducted, the lack of in-depth understanding of ARGs limits the management and elimination of ARG pollution. Hence, we provide several critical perspectives about research methods and data analysis in ARG studies to deep mining the knowledge of ARGs.

First, the deep mining of metagenomic datasets is essential, especially in studying the transmission and host-tracking of ARGs. Current metagenomic analyses are mainly focused on the detection of ARGs, the co-occurrence network among ARGs, and the relationship between ARGs and MGEs. However, the content of the analysis is nearly ending, and the metagenomic dataset is not fully utilized. Hence, a comprehensive analysis of metagenomic data is essential to expand the understanding of ARGs. For example, investigating the arrangement of ARGs and their relationship with adjacent genes and MGEs is a potential approach to reveal the transmission of ARGs. Moreover, on the basis of metagenome binning results, the taxonomy of ARG-carrying contigs can be accurately identified, and the key challenge of annotating the taxonomical source of ARG can be solved, thereby benefiting the host-tracking of ARGs.

Second, a formula or standard for calculating the ARGs abundance should be unified. Recent calculation methods of ARGs abundance are diverse, such as the transcripts per kilobase of exon model per million mapped reads (TPM) (Jing and Yan, 2020), reads per kilobase of exon per million mapped reads (RPKM) (Sekizuka et al., 2020), one read in one million reads (parts per million, ppm) (Zhang et al., 2015), (number) copy of ARG per copy of 16S rRNA gene (Li et al., 2015), and abundance (coverage, ×/Gb) (Zhao et al., 2020). This condition limits the intuitive comparison of the profiles and risks of ARGs in various environments. Designing and appointing a unified formula for calculating ARG abundance are necessary to estimate ARG pollution and its risks in different environments.

Third, the application of third-generation sequencing in ARG studies can expand the understanding of ARGs, especially their genetic location and hosts. Third-generation sequencing techniques, such as Pacific Biosciences (PacBio) and Nanopore sequencing techniques, should be applied to profile ARG composition in diverse environments, explore the occurrence pattern of ARGs, and track their source. These techniques can generate long reads and obtain large genomes that can span most repetitive sequences and benefit the taxonomical identification of ARGs (Ye et al., 2016; Qian et al., 2021b). For example, the profile, genetic location, and hosts of ARGs, particularly the potential ARG-carrying pathogens, were investigated and explored throughout the wastewater treatment process by using the combination of Nanopore and Illumina sequencing; this work established a baseline analysis framework to explore ARGs in environmental niche and expanded the knowledge of resistome in wastewater treatment plants (Che et al., 2019). Several shortcomings, such as the cost of sequencing and the extract method of high-quality DNA, limit the use of third-generation sequencing in current ARG studies.

Finally, the findings should be verified in a wet laboratory. Current ARG studies mainly collected samples from natural environments, and the results or conclusions are untested and un-verified in a wet laboratory. Hence, proper experimental works should be designed and conducted to simulate the natural environment in the laboratory and verify the pattern of ARGs under these conditions. The results will have substantial implications for estimating ARG pollution and managing the related risks.

CONCLUSION

This review summarized current bioinformatic approaches and databases for identifying potential ARGs in metagenomic data. In particular, several bioinformatic tools and databases were benchmarked to estimate their advantages in detecting ARGs in different environmental niches. Several suggestions were also proposed to expand the analysis content of ARG studies. Together, by accumulating and updating current bioinformatic tools for analyzing metagenomic datasets and ARG and MGE databases, source-tracking tools for ARGs, and providing perspectives for future ARG studies, this comprehensive review provides a holistic assessment of the application of metagenomic data in ARG studies. The findings provide insights into the transmission of ARGs and pave the way for establishing priority in managing ARG pollution.

AUTHOR CONTRIBUTIONS

MH and ZW designed the study. ZP, YM, MH, and ZW wrote the initial draft of the manuscript. All authors read, modified, and approved the final manuscript.

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Behaviors of Homologous Antibiotic Resistance Genes in a Cephalosporin WWTP, Subsequent WWTP and the Receiving River

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High concentrations of antibiotics in antibiotic production wastewater can cause the widespread transmission of antibiotic resistance genes (ARGs). Here, we collected a set of time series samples from a cephalosporin production wastewater treatment plant (X-WWTP), the subsequent municipal WWTP (Y-WWTP) and the receiving stream. Using a functional gene microarray, GeoChip 5.0, which contains multiple homologous probes for 18 ARG and 13 antibiotic metabolism gene (AMG) families, we found that more than 50% of homologous probes for 20 gene families showed a relative abundance higher in X-WWTP, while only 10–20% showed lower relative abundance. The different response patterns of homologous ARG (hARGs) within the same ARG family imply environmental selection pressures are only responsible for the ARG enrichment and spread of some specific instead of all ARG-containing microorganisms, which contradicted the traditionally held belief that environmental selection pressures, especially antibiotic concentration, select for all ARG-containing microorganisms thereby selecting different hARGs in the same ARG family in an undifferentiated way. Network results imply that hARGs from three β_lactamase families enriched under the selection pressure of high cephalosporin antibiotic concentrations in X-WWTP formed positively correlated homologous ARG clusters (pohARGCs). The pohARGCs were also enhanced in the sediment of the receiving stream. The enrichment of hARGs from three β_lactamase families was likely through microorganisms belonging to the *Betaproteobacteria* genus.

Keywords: pharmaceutical wastewater, antibiotic resistance genes, homologous ARG, GeoChip, receiving river

INTRODUCTION

The biological toxicity and bacterial antibiotic resistance of antibiotic production wastewater is increasingly causing environmental issues (Arslan-Alaton and Caglayan, 2006; Guo N. et al., 2018; Hou et al., 2019; Klavarioti et al., 2009; Zhang et al., 2013). Antibiotic production wastewater contains high concentrations of organic compounds, such as heterocyclic compounds and active pharmaceutical intermediates, with substantial biological toxicity (Arslan-Alaton and Caglayan,

2006; Klavarioti et al., 2009; Wei et al., 2019). Pharmaceutically active intermediates, including antibiotic residues, and antibiotic resistant bacteria (ARB) in antibiotic containing pharmaceutical wastewater can intensify bacterial antibiotic resistance (N. Guo et al., 2018a; Hou et al., 2019; Y. Zhang et al., 2013). As a key indicator reflecting the risk of antibiotic resistance, antibiotic resistance genes (ARGs) have been frequently detected in various environmental media (Martínez, 2008; Allen et al., 2010; Che et al., 2019). The fate and removal of ARGs and factors influencing these processes have been the focus of recent research (Chen et al., 2015; Guo et al., 2017; Pallares-Vega et al., 2019).

In China, the United States, and many other countries, antibiotic production wastewater is treated at specialized antibiotic production wastewater treatment plants (WWTPs) to remove antibiotics and then at subsequent municipal WWTPs to reduce conventional biochemical indicators such as BOD, TN and TP in the wastewater before discharging the effluent to natural water bodies (SEPA, 2008; Klavarioti et al., 2009). Unlike conventional biochemical indicators, regulations have not been established for the amount of allowable ARBs or ARGs in WWTP effluent (Chokshi et al., 2019; Hobæk and Lie, 2019). Although it is known that antibiotic production WWTPs reduce the total number of ARB and ARGs in the effluent wastewater (Jäger et al., 2018; Faleye et al., 2019), how this reduction influences downstream microbial populations at subsequent WWTPs and in the receiving waters is not well researched.

Most studies of ARGs are based on the abundance of ARG subtypes or ARG families. For example, Alcock et al. used ARG families to classify ARGs in the Comprehensive Antibiotic Resistance Database (Alcock et al., 2020) and Yin et al. used ARG subtypes to classify ARGs in the Integrated Structured ARG-database (Yin et al., 2018). According to ARG classifications among the GeneBank Database (https://www.ncbi.nlm.nih.gov/genbank/), the Comprehensive Antibiotic Resistance Database (Alcock et al., 2020) and the Integrated Structured ARG-database (Yin et al., 2018), Gene families are groups of related genes that share a common ancestor. Members of gene families may be paralogs or orthologs. Paralogs are genes with similar sequences in the same species while orthologs are genes with similar sequences in different species (Ohta, 2001; Demuth and Hahn, 2009). Resistance "types" represent the class of antibiotics to which a given ARG confers resistance, and resistance "subtypes" represent individual ARGs that share a common ancestor (Yang et al., 2016). ARG subtype and ARG family are the same concept for ARGs. Sequences from the same ARG family in different hosts are called homologous ARGs (hARGs) and include orthologous ARGs and paralogous ARGs and are not always identical (Michael, 2005). The abundance of different hARGs presented different response patterns depending on their hosts (Brochier et al., 2004), necessitating the need to examine individual hARGs rather than ARG families.

In this study, we examined samples from a cephalosporin antibiotic production WWTP, the subsequent municipal WWTP, and the receiving water bodies. Statistical analysis of the microbial community composition and functional genes including hARGs were performed to find the types, pathways and environmental influence of the ARGs enriched in the antibiotic production WWTP.

MATERIALS AND METHODS

Study Site Description

This study examined two WWTPs located in a pharmaceutical industrial park in Hebei Province, China. The industrial park contains more than 20 facilities that manufacture a variety of antibiotics, including amoxicillin, penicillin, ampicillin, cephalosporin, lincomycin, clindamycin, streptomycin, gentamicin, avermectin, ivermectin, doxycycline, neomycin, and minocycline, among others. The cephalosporin antibiotic production facility has a specialized WWTP (X-WWTP) to treat antibiotic production wastewater. The effluent from X-WWTP is then transported to a dedicated municipal WWTP (Y-WWTP) (approximately 1.5 km distance away) that treats effluent from all the specialized pharmaceutical production WWTPs as well as sewage within the industrial park (**Figure 1**). Additional information on the WWTPs is shown in **Table 1**.

X-WWTP mostly accepts cephalosporin antibiotic production wastewater, including 7-aminocephalosporanic acid, cefuroxime (CXM), ceftriaxone (CTR), and other cephalosporins. X-WWTP effluent comprises a majority of Y-WWTP influent (80%). Effluent from Y-WWTP is subject to the Grade A discharge standard of Pollutants for Municipal Wastewater Treatment Plant (GB18918-2002). After discharge, Y-WWTP effluent is transported to a receiving stream through a culvert. The upstream section of the receiving stream is a dry stream bed, so the main water source for the receiving stream is the effluent from Y-WWTP. Our study focused on the influent and effluent of X- and Y-WWTPs and the receiving stream.

Sample Collection and Pretreatment

Wastewater samples were collected in December 2015, January 2016, February 2016, March 2016, April 2016, July 2016, October 2016 and January 2017. Sampling from X-WWTP and Y-WWTP and the receiving stream were performed on the same day. At X-WWTP, samples were collected from the influent of the hydrolysis acidification tank (Xinf) and the effluent of the secondary sedimentation tank (Xeff). At Y-WWTP, samples were collected from the influent of the regulation tank (Yinf) and the final effluent (Yeff) of Y-WWTP. Three wastewater samples were collected from each sampling site at 8 h intervals into 2 L sterile bottles at each sampling time. The triplicate samples were combined by mixing equal volumes of each replicate into one sample. Samples were transported back to the laboratory within 2 h using an insulated case with a built-in ice pack.

River water samples were collected in December 2015, January 2016, February 2016 and March 2016. Samples were collected at 0.2 km (Ra), 1 km (Rb), 3.5 km (Rc), 4.5 km (Rd) and 6.5 km (Re) distance from the effluent culvert of Y-WWTP in the receiving stream and were labeled a, b, c, d and e, respectively. At each sampling location, 2 L of water samples were collected from three

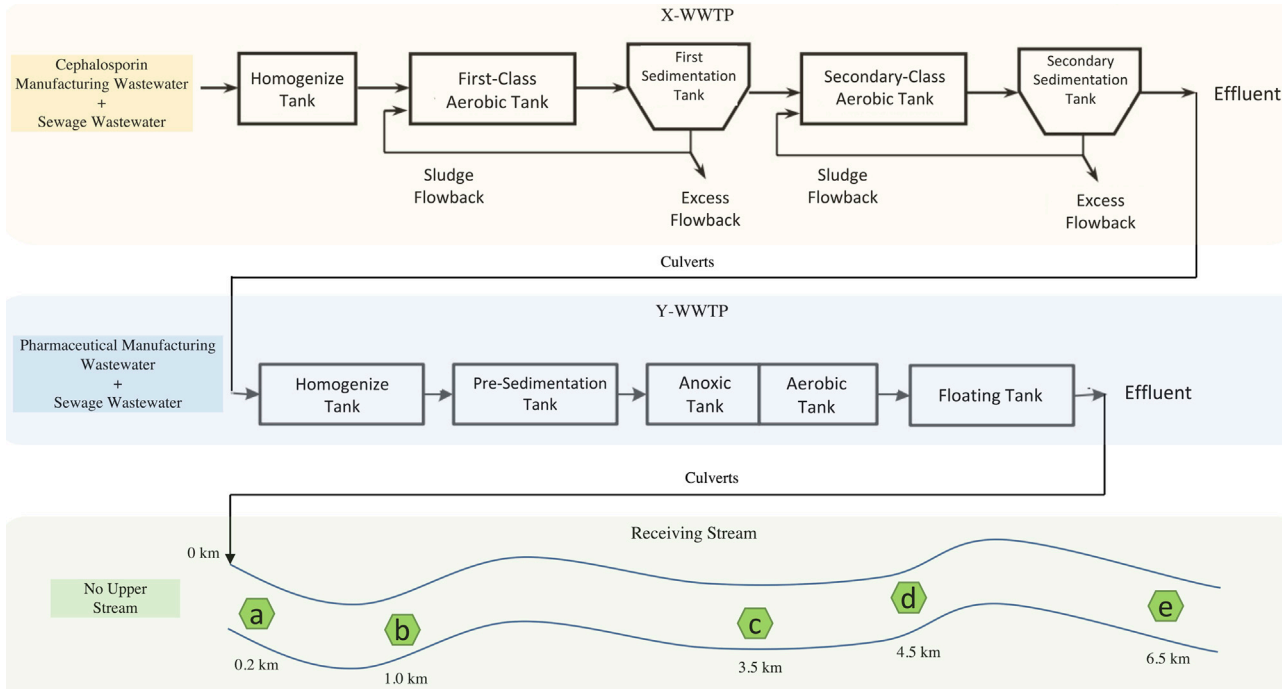

FIGURE 1 | Sampling map.

TABLE 1 | Additional information of Two WWTPs.

Information	X-WWTP	Y-WWTP
Main treatment process	Hydrolysis acidification +A/O	Hydrolysis acidification + oxidation ditch + activated carbon filter
Design processing capability	4500 m3/d	50000 m3/d
COD of influent	2000–6000 mg/L	100–300 mg/L
COD of effluent	100–400 mg/L	30–100 mg/L

points in the stream, approximately 0.5 m apart and 0.5 m below the surface with a water collector. The three samples were then pooled to make one water sample.

Sediment samples were collected in December 2015, January 2016, March 2016, April 2016, July 2016, October 2016 and January 2017. Sediment samples were collected at 0.2 km (SD1), 1 km (SD2), 3.5 km (SD3) and 4.5 km (SD4) away from the effluent culvert of Y-WWTP in the receiving stream. The grab-type sediment collector (HAD-QNC6-1, Hengde Instrument Co., Ltd., Beijing) was used to collect the sediment samples. At each sampling location, sediment samples were collected from three points in the stream, approximately 0.5 m apart. The sediment was collected using a quartet sampling method. An area (20 × 20 cm) was selected at each sampling location and then divided into quadrents by connecting the diagonal lines. Sediment samples were collected from each quadrent and then mixed to make one sediment sample.

After being transported to the laboratory, the 6 L wastewater and stream water samples were divided and 2 L was stored at 4°C for immediate physical and chemical indicator analysis. The remaining 4 L of each sample was suction filtered immediately using 0.22-μm-pore-size filter membranes (GSWP04700 filters, Millipore, United States). The filter membranes were stored at −80°C for DNA extraction. The sediment samples were stored at 4°C for immediate physical and chemical indicator analysis. At the same time, a portion of the sediment samples were centrifuged (15000 r/min) for 10 min and the supernatant was discarded. The sediment pellets were stored at −80°C for DNA extraction. All physical and chemical analyses were completed within 24 h.

The sampling date is designated by a four digit number indicating the year and month. The number "_1, _2, and _3" after sampling date meant parallel samples. For example, Xinf16.01 refers to the influent sample of X-WWTP, which was sampled in January 2016.

DNA Extraction, Purification, and Quantification

DNA was extracted from the filtered membrane using a HiPure Water DNA Kit (D3145-02, Magen, again, city name, province

TABLE 2 | The detail of ARG families on GeoChip 5

ARG family	Probe quntity	Detailed information
ß_lactamase	183	Beta-lactamases
ß_lactamase_A	267	Beta-lactamase classA
ß_lactamase_B	8	Beta-lactamase classB
ß_lactamase_C	309	Beta-lactamase classC
fosa	33	Glutathione transferase fosA
fosb	28	Metallothiol transferase fosB
fosx	8	Fosfomycin resistance protein fosX
tetx_resistance	3	tetX monooxygenase
vgb	91	Virginiamycin Blyase
qnr	8	Quinolone resistance protein
Van	48	D-alanine--D-lactateligase/Vancomycin/teicoplanin resistance protein
—	—	
ABC_antibiotic_transporter	1,104	ABC antibiotic transporter
ABC_multidrug_fungi	378	ABC antibiotic transporter
MATE_antibiotic	392	Multi-drug efflux transporter
Mex	3,724	Multi-drug efflux protein
MFS_antibiotic	7,977	Multidrug efflux system protein
SMR_antibiotics	1,015	Small multidrug resistance efflux transporter
Tet	276	Tetracycline/Oxytetracycline resistance protein

name, China) following the manufacturer's instructions and cleaned by ethanol purification. DNA quality was assessed based on absorbance ratios [260–280 nm (>1.8); 260 to 230 nm (>1.7)] detected by a NanoDrop 2000 (ThermoFisher, America). Qualified DNA samples were stored at −80°C. Samples that did not reach the quality cutoff were not used.

PCR Amplifiation, Sequencing, and Data Processing

A two-step PCR amplification method was used for PCR product library preparation as described previously (Zhou et al., 2011). Standard primers were used to amplify the V4 region of prokaryotic 16S rRNA genes [515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′)]. Sample libraries were then sequenced on an Illumina MiSeq (San Diego, CA, United States) as described previously (X. Guo et al., 2020).

16S rRNA gene sequencing data generated from MiSeq were processed by demultiplexing, triming primers, combining paired-end reads, removing sequences containing N's, and detecting and removing chimeras using a Galaxy-based pipeline http://zhoulab5.rccc.ou.edu:8080 (Zhou et al., 2016). OTUs were generated by QIIME II with a 97% similarity threshold. The Sliva (http://www.arb-silva.de/) 16S reference database was used (Quast et al., 2012). OTUs were identified taxonomically using the QIIME II classifier (Bolyen et al., 2019). Only OTUs with greater than two reads were retained as valid data.

Functional Structure Analysis With GeoChip 5.0

The latest generation of functional gene array, GeoChip 5.0 (180K) (Zhou et al., 2015), was used to detect ARGs in the samples. GeoChip 5.0 contains 167,044 probes targeting 395,894 coding sequences from 1,593 functional gene families. A total of 15,474 probes on the GeoChip 5 are associated with antibiotic resistance, accounting for approximately 10% of the total number of gene probes. Each probe in GeoChip has its own microbial annotation which can be found at www.ou.edu/ieg. The source of the microbial annotation is the Genebank information in the NCBI database. Additional information is shown in **Table 2**.

In our study, 500 ng of DNA from each sample were hybridized to GeoChip at the Institute for Environmental Genomics, University of Oklahoma, as described previously (X. Guo et al., 2020). The microarray data was preprocessed using the microarray data manager pipeline on the Institute for Environmental Genomics' website (www.ou.edu/ieg/tools/data-analysis-pipeline) as described previously (He et al., 2010; Tu et al., 2014).

Statistical Analyses

Alpha diversity analysis, beta diversity analysis, core microbiome analysis, and linear discriminant analysis effect Size (LEfSe), which is used to find the gene or functional characteristics that can best explain the differences between groups in two or more groups of samples under different biological conditions or environments and the influence of these characteristics on the differences between groups, and correlation feature pattern search analysis of 16S rRNA genes in all samples were performed on https://www.microbiomeanalyst.ca/. The alpha diversity analysis used the fragrance index. Beta diversity analysis is principal co-ordinates analysis (PCoA) based on the Bray-Curtis index. Core microbiome analysis selected OTUs that appear in more than 50% of samples and have a relative abundance greater than 0.01%. The LEfSe analysis, which compared the 16S rRNA gene abundance profiles between samples in different state, had a *p*-value cutoff of 0.1 and a log LDA score of 2.0. Pattern Search analysis, which searched for a pattern based on correlation analysis on defined pattern, had a *p*-value cutoff of 0.05 in pearson relevance.

Before GeoChip data statistical analysis, logarithmic transformation was carried out on the original data, then

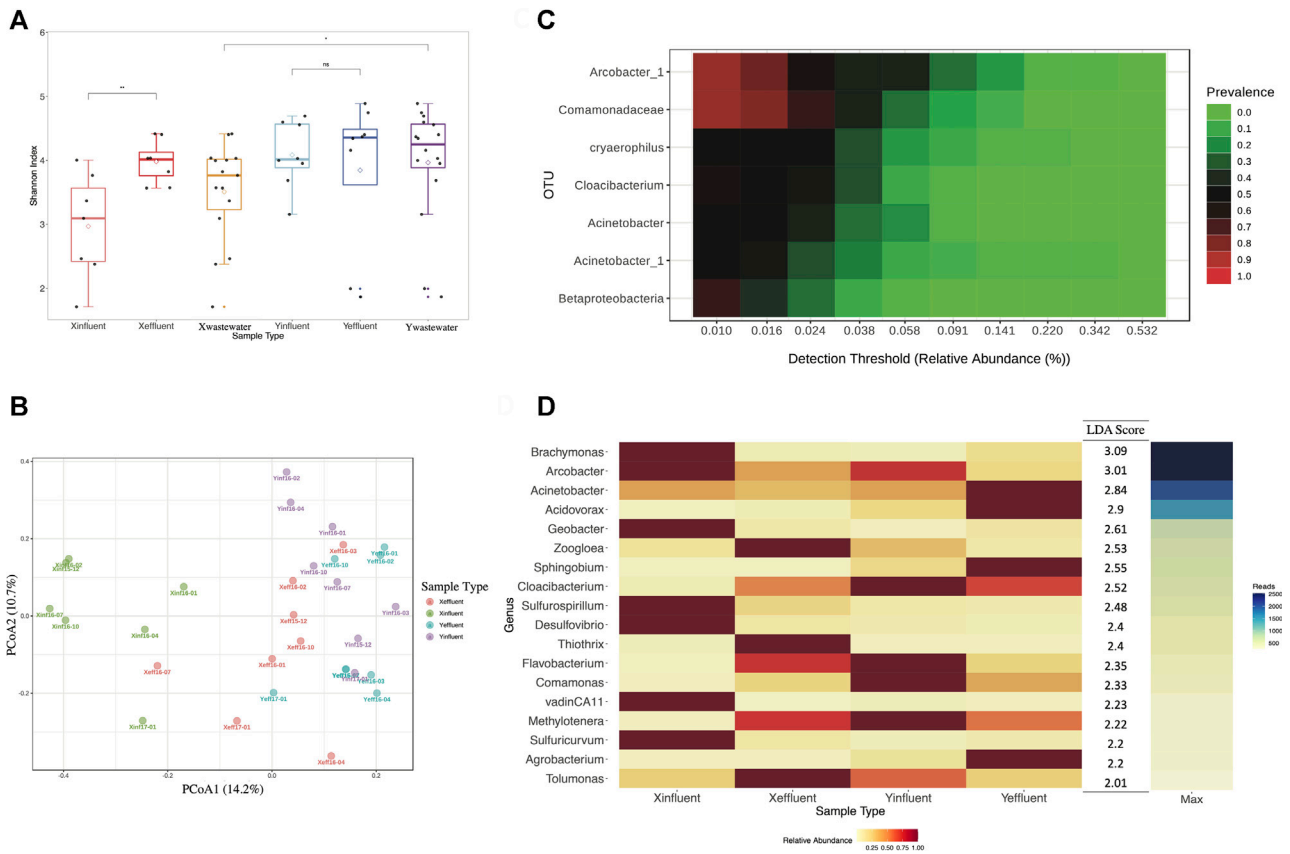

FIGURE 2 | Microbial community composition analysis of wastewater samples at OTU level. **(A)** SHDI of wastewater samples. **(B)** PCoA of wastewater samples based on Bray-Curtis Index. **(C)** Core Microbiome analysis of wastewater samples. **(D)** LEfSe analysis of wastewater samples.

transferred into relative abundances. Probes detected in at least 6 of 29 samples were retained as valid data. The GeoChip data analysis of all samples was completed in R. We summarized all gene categories of each sample and analyzed the alpha, beta diversity, response ratio and abundance variation ratio analysis at the levels of ARG and AMG probes. The alpha diversity analysis used the fragrance Index. Detrended Correspondence Analysis (DCA) based on the Bray-Curtis index was used for beta diversity analysis. The response ratio of ARGs was calculated using the formula described previously (Y. Luo et al., 2006). The total abundance of all probes in every ARG family was taken for response ratio analysis. A response ratio is considered significant when $p < 0.05$. Network analysis of hARGs was performed using the Molecular Ecological Network Analysis platform at http://ieg4.rccc.ou.edu/mena.

RESULTS

Microbial Community in Wastewater of WWTPs

16S rRNA gene sequencing was performed on 31 wastewater samples from X-WWTP and Y-WWTP and 18 stream water samples from the receiving stream. A total of 1,699,901 effective reads and 2,260 OTUs were obtained from wastewater and stream water samples. The maximum number of effective reads per sample was 86,766 and the minimum was 14,222. The microbial diversity of X-WWTP wastewater was lower than that in Y-WWTP (**Figure 2A**). The microbial diversity of X-WWTP effluent was higher than that of the influent, while there was no difference in the microbial diversity between the influent and effluent of Y-WWTP. The microbial community from the influent in X-WWTP was distinguisable from all other wastewater samples (PCoA, **Figure 2B**). Samples did not separate based on time, indicating that there was no variation in the wastewater microbial community over the time period examined.

A total of 7 OTUs from all the wastewater samples were identified as members of the core microbiome (**Figure 2C**). OTUs from the Acinetobacter, Cloacibacterium and Arcobacter genera, which can cause human diarrhea, were common in wastewater from two WWTPs. LEfSe analysis of all wastewater samples selected 18 differentiated genera that best explained the differences among X-WWTP influent, X-WWTP effluent, Y-WWTP influent and Y-WWTP effluent (**Figure 2D**). OTUs belonging to 10 genera, including *Sulfurospirillum*, *Desulfovibrio_1*, *kujiense*, and *Methanomethylovorans*, were more abundant in X-WWTP, while OTUs belonging to 8 genera, including *Acidovorax*, and *Comamonas_1*,

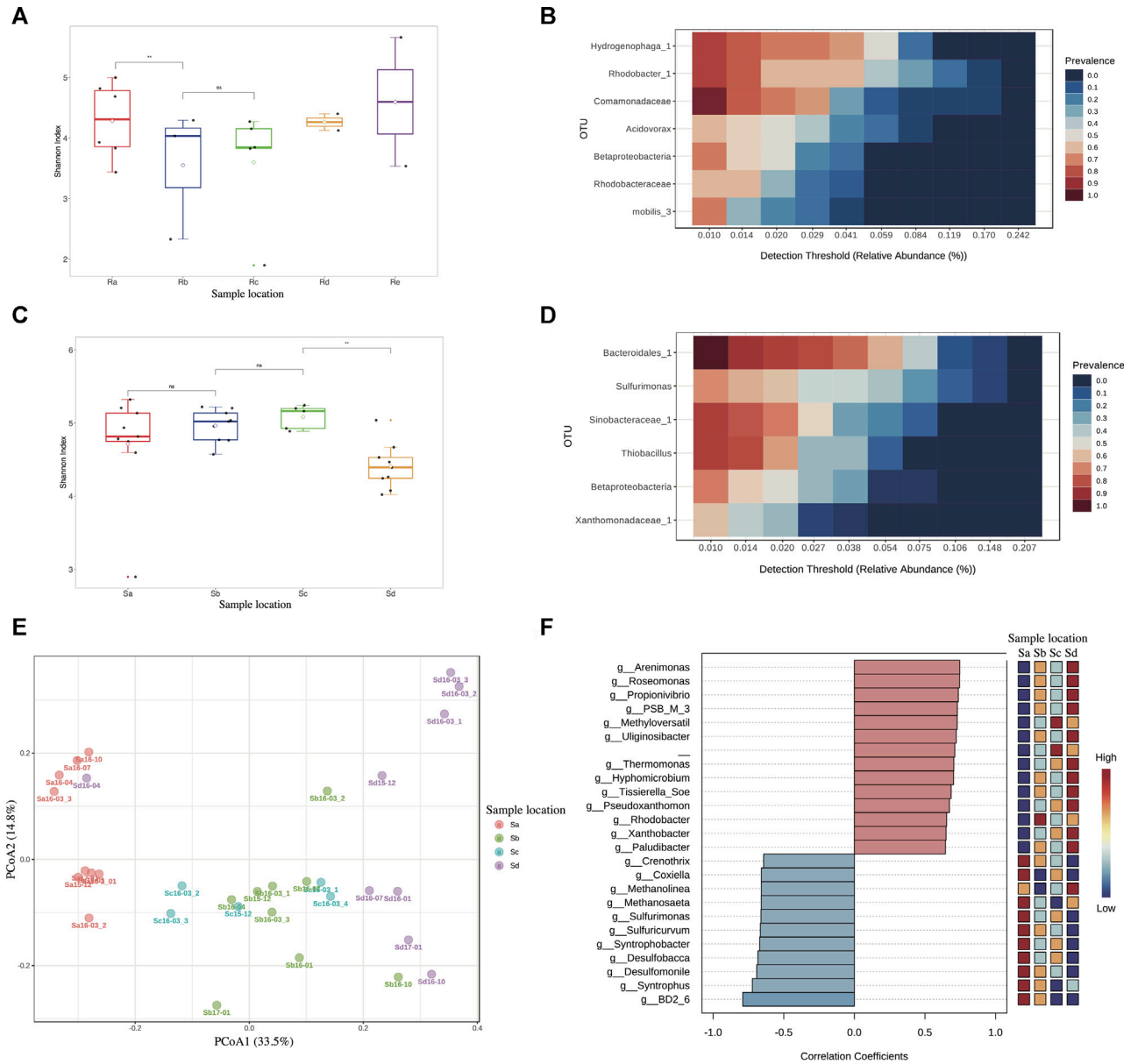

FIGURE 3 | Microbial community composition of stream water and sediment samples at OTU level. **(A)** SHDI of stream water samples. **(B)** Core Microbiome analysis of stream water samples. **(C)** SHDI of sediment samples. **(D)** Core Microbiome analysis of sediment samples. **(E)** PCoA of sediment samples based on Bray-Curtis Index. **(F)** Pattern Search analysis of sediment samples.

Sphingobium, were more abundant in Y-WWTP. In X-WWTP influent, seven of the 18 genera were in lowest abundance while 10 were in highest abundance. Most of these microorganisms were in low abundance in the effluent of Y-WWTP.

Microbial Community Composition in Stream Water and Sediment

16S rRNA gene sequencing was performed on 32 sediment samples from the receiving stream. A total of 1,545,069 effective reads and 2,095 OTUs were obtained. The maximum number of effective reads per sample was 83,531; the minimum number was 22,039. The bacterial diversity of the river water samples (**Figure 3A**) and river sediment samples (**Figure 3C**) indicated that the bacterial diversity of the river water at point a was close to the wastewater, since point a was where the WWTP effluent discharged into the stream. The bacterial diversity of the river water decreased downstream and then began to increase further downstream, while the bacterial diversity of the sediment was relatively similar from points a to c and then decreased sharply at point d. Core microbiome analysis of the stream water (**Figure 3B**) and sediment samples (**Figure 3D**) showed that only one OTU, belonging to the Betaproteobacteria class, was observed to occur frequently in each sample. PCoA of OTUs from all

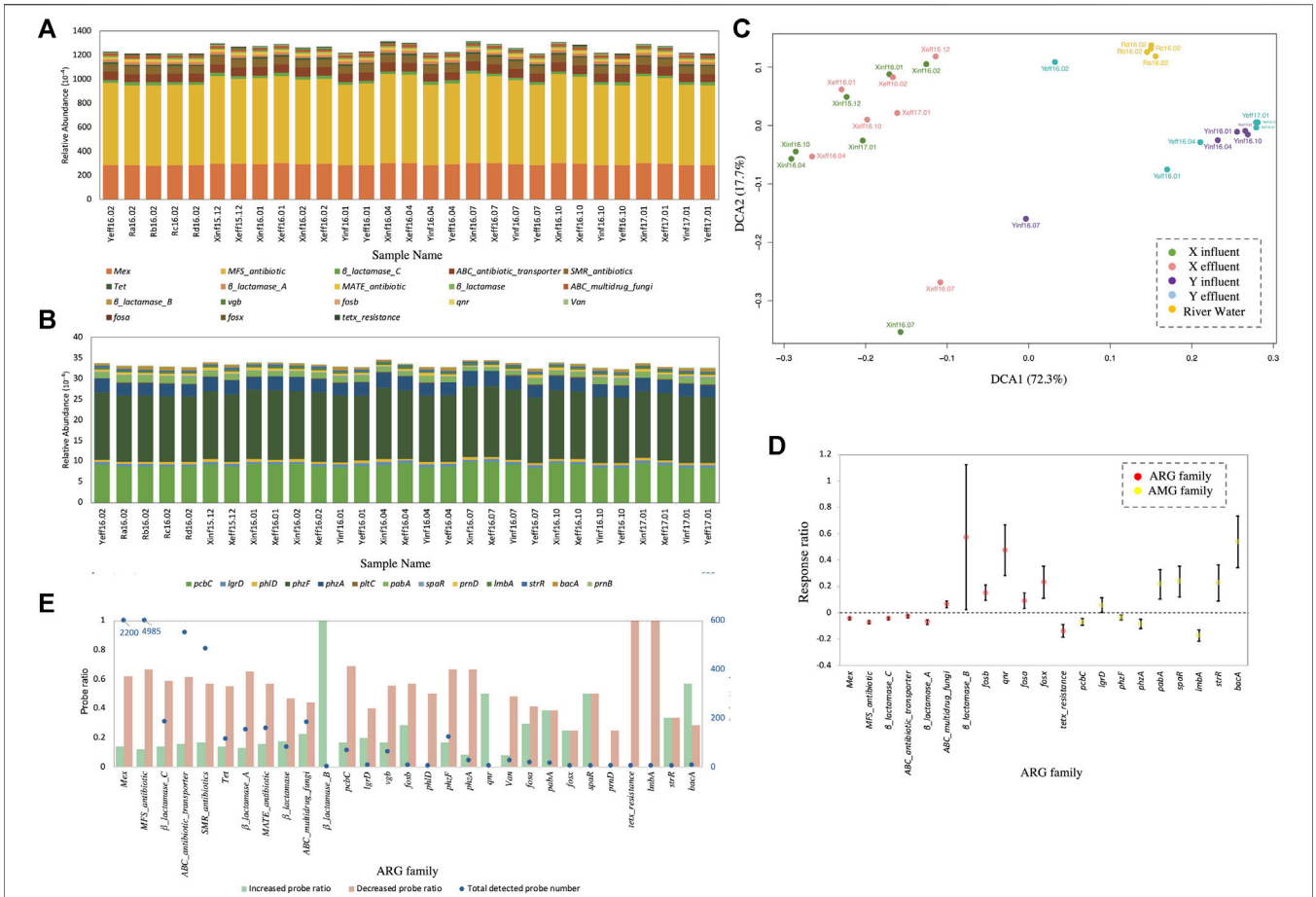

FIGURE 4 | ARGs and AMGs of wastewater samples. **(A)** Relative abundance of ARGs at the gene family level. **(B)** Relative abundance of AMGs at the gene family level. **(C)** DCA at the Probe level. **(D)** Response ratio analysis on the gene family level of ARGs and AMGs in Y-WWTP compared with X-WWTP. **(E)** Probe quantities detected in each gene family and ratio of probes which increased or decreased in relative abundance detected in Y-WWTP compared with those detected in X-WWTP.

sediment samples indicated microbial communities from sediment at different sampling positions were different, except those between Points b and c. Pattern search analysis (**Figure 3F**) searched 25 differentiated OTUs based on person correlation analysis on among sediment samples from different positions. The results of Pattern Search analysis showed that the relative abundance of the top 14 genera, including *Sulfurimonas*, *kujiense*, and *syntrophus_1*, increased from point a to d, while 11 genera, including *SB_1*, *Sinobacteraceae_1*, and *Flavobacteriaceae_1*, decreased from point a to d.

ARGs and AMGs in Two WWTPs

To determine which ARGs were present in the samples, GeoChip 5.0 analysis was performed on 25 wastewater samples from the two WWTPs, 2 sludge samples from Y-WWTP, 4 stream water samples and 27 stream sediment samples. A total of 80,166 functional genes represeting 1,110 gene families, 15 functional groups, and 129 subgroups were detected.

The relative abundances of ARGs (**Figure 4A**) and antibiotic synthesis genes (**Figure 4B**) were quantified at the gene family level in each wastewater sample. The total relative abundance of 18 types of ARGs in all samples was about 12%, and the total relative abundance of AMGs was about 0.32%. The total abundance of ARGs and AMGs in X-WWTP wastewater samples was significantly ($p < 0.01$) higher than that in Y-WWTP wastewater and the stream water samples. The DCA (**Figure 4C**) of all wastewater and stream water samples at the probe level indicated X-WWTP samples were separated from Y-WWTP and stream samples. None of the samples showed significant seasonal variation. The response ratio analysis of ARGs and AMGs from the two WWTPs (**Figure 4D**) indicated a greater relative abundance of Mex, MFS_antibiotic, β_lactamase_C, ABC_antibiotic_transporter, β_lactamase_A, tetx_resistance, pcbC, and lmbA genes in X-WWTP than in Y-WWTP, while the relative abundance of ABC_multidrug_fungi, β_lactamase_C, fosb, qnr, fosa, and fosx was richer in Y-WWTP.

Most ARG families have many different probes which stand for different hARGs, and each hARG has its own host bacteria according to the Genebank imformation. **Figure 4E** shows the details of each ARGs family at the probe level, including the number of probes detected in each gene family and ratio of probes which increased or decreased in relative abundance detected in Y-WWTP compared with those detected in X-WWTP. More

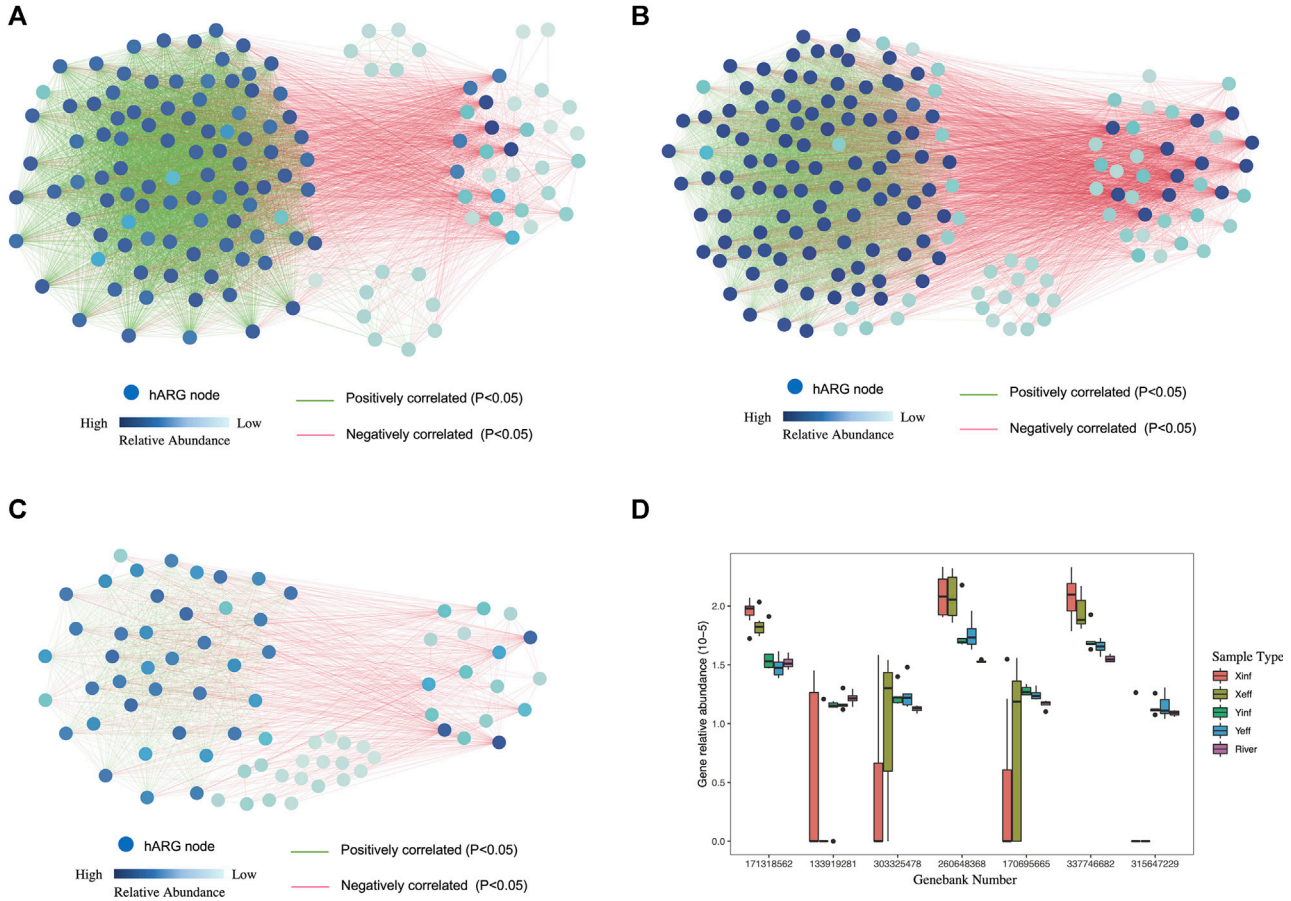

FIGURE 5 | Network analysis of homologous genes of the **(A)** β_lactamase_A, **(B)** β_lactamase_C and **(C)** β_lactamase ARG families in wastewater samples. **(D)** Relative abundance of representative homologous genes of four β_lactame ARG families in wastewater and river samples.

than 50% of probes from 22 ARG families, including Mex, MFS_antibiotic and β_lactamase_C, showed relative decreases, while only 10–20% of the probes showed relative increases. Five ARG families had a higher ratio of probes with relative increases, but each of these ARG families had less than 3 probes.

Network Analysis of hARGs

Network analysis of β_lactamase_A (**Figure 5A**), β_lactamase_C (**Figure 5B**) and β_lactamase (**Figure 5C**) hARG families from the WWTP wastewater samples showed correlations among the hARGs of these families in the β_lactamase_B was not included since it only had one hARG. Each β_lactamase ARG family had one cluster in which the hARGs were positively correlated (indicated by green linkages in **Figure 5**). In contrast, a proportion of hARGs that were negatively correlated (indicated by red linkages in **Figure 5**) with the hARGs in the positively correlated homologous ARG cluster (pohARGC). We selected seven representative hARGs from the four β-lactamase families to compare the relative abundance (**Figure 5D**). GB171318562, GB260648368, and GB 337746682 represented the hARGs in the pohARGCs of β_lactamase_A, β_lactamase_C, and β_lactamase ARG families and had higher abundance in X-WWTP. GB133919281, GB303325478, GB170695665, and GB315647229 represented the hARGs that were either negatively or not correlated with the pohARGCs of β_lactamase_A, β_lactamase_B, β_lactamase_C, and β_lactamase ARG families and had higher abundance in Y-WWTP.

ARGs and AMGs in Sediment

The relative abundance of ARGs (**Figure 6A**) and AMGs (**Figure 6B**) were also calculated at the gene family level in stream water and sediment samples. The total relative abundance of each gradually increased in stream sediment over time, but no dicernible variation was observed with distance from the Y-WWTP effluent discharge. The results of the DCA (**Figure 6C**) indicated a seasonal variation across the secondary axis for the sediment samples, but it only explained 11.10% of the observed variation. Therefore, seasonal variation was not a major factor in the diversity variation. No changes with increasing distance were observed.

We also selected representative hARGs from the four β-lactamase families in sediments to compare the relative abundance, with only one homologous ARG for

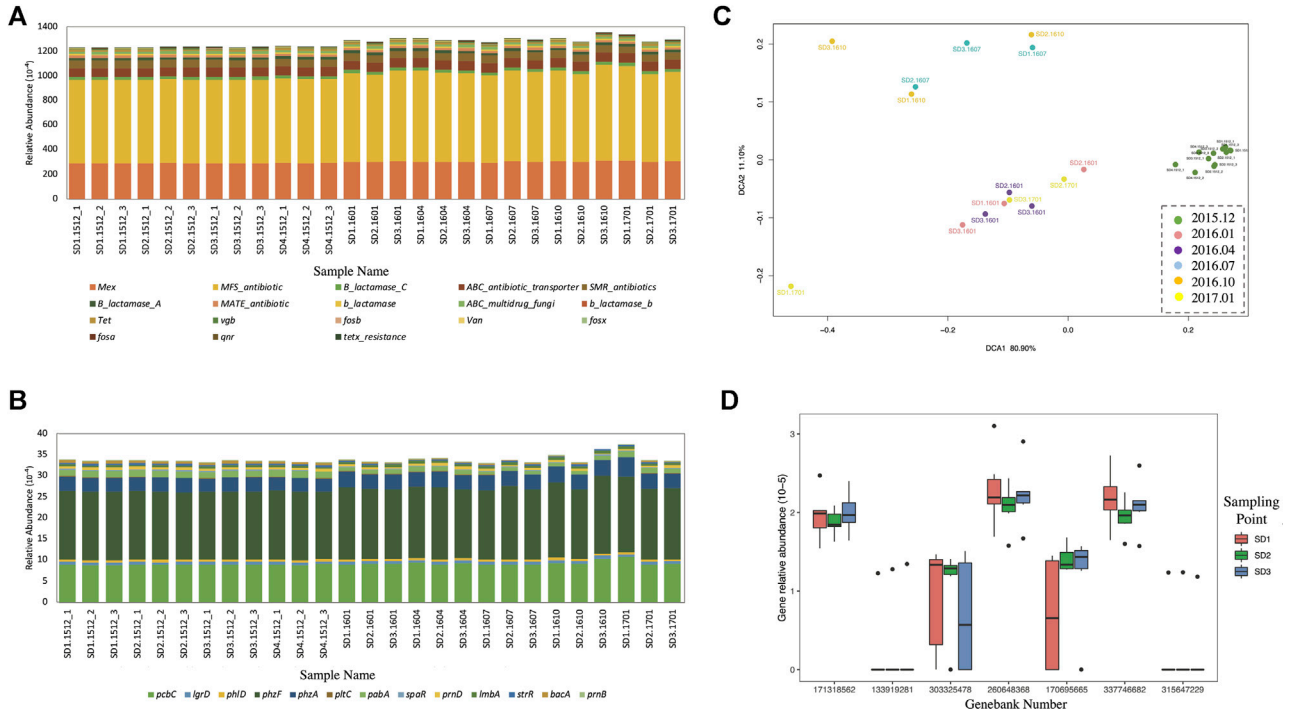

FIGURE 6 | ARGs and AMGs of sediment samples. **(A)** Relative abundance of ARGs at the gene family level. **(B)** Relative abundance of AMGs at the gene family level. **(C)** DCA at the Probe level. **(D)** Relative abundance of representative homologous genes of four β_lactame ARG families.

β_lactamase_B (**Figure 6D**). GB171318562, GB 260648368, and GB 337746682 were abundant in all sediment samples, while GB 133919281, GB 303325478, GB 170695665, and GB 315647229 had little or no abundance in sediment samples.

DISCUSSION

Previous studies have often examined pharmaceutical manufacturing or municipal WWTPs in isolation (X. Guo et al., 2018b; Tong et al., 2019; Wang et al., 2015). However, the continuous wastewater flow from pharmaceutical manufacturing WWTP to municipal WWTP and then into the local receiving streams has been rarely addressed in previous studies. This study revealed some essential variation in community responses and ARGs in this particular situation. Since the ARGs we detected were intracellular ARGs (Yuan et al., 2019), this study examined the variation in microbial community structure first.

In this study, we observed that the microbial diversity of X-WWTP was significantly ($p < 0.01$) lower than that of Y-WWTP, and that the X-WWTP influent had the lowest microbial diversity of all sampling sites. In a study of activated sludge from 12 full-scale pharmaceutical WWTPs, a low bacterial diversity was also observed for pharmaceutical production WWTPs compared to that of municipal WWTPs (F. Zhao et al., 2019a). High concentrations of organic compounds with strong biological toxicity, such as heterocyclic compounds and antibiotic active intermediates in antibiotic-containing pharmaceutical production wastewater, are the primary cause of lower microbial diversity in pharmaceutical production WWTPs (Arslan-Alaton and Caglayan, 2006; Klavarioti et al., 2009; Wei et al., 2019). β-diversity analysis (**Figure 2B**) showed that the X-WWTP influent microbial community was unique, while the X-WWTP effluent microbial community was more similar to that of the Y-WWTP wastewater (influent and effluent. The abundance of 21 OTUs in the X-WWTP effluent showed polarity, indicating that the treatment process in the X-WWTP had a significant remodeling effect on the microbial community. The OTUs that had extremely high abundance in X-WWTP effluent also appeared in the Y-WWTP influent, indicating that the microorganisms selected in X-WWTP were spreading to subsequent treatment process With the sewage flow.

The microbial communities in both the sediment and water of the receiving stream were initially influenced by the Y-WWTP effluent. The microbial communities from the water and sediment in the stream showed different trends in alpha diversity. Differences in the water community could be because of Y-WWTP effluent discharge with the oxidation, reduction and sunlight decomposition of pollutants and the sedimentation of pollutants and microorganisms of streams (Li et al., 2010; Price et al., 2018; Zhang et al., 2016). The Y-WWTP effluent discharged to the receiving stream had both a large number of conventional biochemical contaminants and a complex microbial community, which determined both the physiochemical and microbiological composition of the

receiving streams (Price et al., 2018). The microbial diversity in the water decreased around 1 km downstream from the discharge site, indicating the microorganisms that had adapted to the environment in Y-WWTP gradually disappeared as the distance from the Y-WWTP increased because the stream environment was very different from Y-WWTP environment. Microbial diversity began to increase around 4.5 km downstream from the discharge site, reflecting a change to a more natural state in which the microorganisms had adapted to the stream environment.

Microbial communities in the sediment of receiving streams were previously found to be influenced by the effluent of WWTP discharge and stream water composition (Milaković et al., 2019). From the point of discharge to around 3.5 km downstream, the microbial diversity of the sediment remained relatively stable because most of the microorganisms in the stream water were still adapted to the Y-WWTP environment. The microbial diversity of the sediment at point d (around 4.5 km distant from the discharge point) was lowest because the microorganisms had adapted to the new environment and the phychemical properties of the stream water at this point were very different from the Y-WWTP effluent. So, the Y-WWTP effluent discharge no longer influenced the microbial community after 3.5–4.5 km downstream.

While the microbial community results of this study were similar to some previous studies, the results of the ARG and homologous ARG studies disagreed with previous findings. ARG studies have been a hot topic in the environmental field in recent years, but almost all of them focused on the abundance of ARG subtypes (J. Guo et al., 2017; F. Zhao et al., 2019a; Zhao et al., 2018). For example, qPCR provides information on the abundance of representative sequences of particular ARG subtypes (Auerbach et al., 2007; Czekalski et al., 2014), while metagenomics provides information on the abundance of all representative sequences of all ARG subtypes (Ju et al., 2019; Zhao F. et al., 2019). A limitation of these previous studies is that ARGs belonging to the same subtype (hARGs) may exhibit different responses because they are in different microorganisms (Brochier et al., 2004; Michael, 2005; Alcock et al., 2020). Here we investigated the response patterns of individual hARGs using GeoChip 5.0.

The X-WWTP and Y-WWTP wastewater were very different in terms of ARGs. We analyzed the change regularity of ARGs detected by GeoChip 5.0 at three levels: the total relative abundance of ARGs, the relative abundance of each ARG family and the relative abundance of each hARG. The total relative abundance of ARGs in X-WWTP was significantly ($p < 0.01$) higher than in Y-WWTP, which is consistent with previous findings (X. Guo et al., 2018b; Wang et al., 2015). Although X-WWTP contained mainly cephalosporin, a beta-lactam antibiotic, production wastewater, X-WWTP had 8 of 18 ARG families with higher relative abundance than Y-WWTP, the relative abundance of β_lactamase_B was lower than that in X-WWTP. These unexpected results indicated that the relative abundance of ARG families did not truly reflect the response pattern of ARGs so we further analyzed individual ARGs from each family.

More than 50% of probes from 18 ARG families were more abundant in X-WWTP than in Y-WWTP, which indicated hARGs' change regularity was different from the ARG families. In order to study the change regularity of the hARGs further, hARGs from three β_lactamase families were selected for network analysis. Each family had some members that formed a positively correlated hARG cluster (pohARGC) and some that were negatively correlated with the hARGs in pohARGC (**Figures 5A–C**). According to the abundance analysis of representive probes, pohARGCs were composed of the hARGs that were richer in X-WWTP. Since different hARGs belonging to the same ARG family often have different microbial annotations based on GeoChip, the reason for the enrichment of pohARGCs is probably due to the enrichment of strains that carry them in X-WWTP. In other words, it is inferred that the enrichment of specific strains in X-WWTP led to the enrichment of pohARGCs. This is consistent with the specificity of the microbial community in X-WWTP in the 16S rRNA gene sequencing analysis. According to the same reason, the hARGs that were negatively correlated or not correlated with the pohARGCs may be contained by the environmental strains or from strains that were enriched in domestic sewage conditions of Y-WWTP.

The hARGs from the same ARG family have different response patterns based on their homology, indicating that changes in ARG abundance are closely related to changes within the microbial community (Klümper et al., 2019; Luo et al., 2017; Wu et al., 2017). As such, previous studies that focused on ARG families as a whole or ARGs independent of the host microorganism may not provide accurate results because some hARGs from the same ARG family exhibited different response patterns due to changes of the host microorganism or due to horizontal transfer (Oliveira et al., 2017).

It was suggested that the horizontal transfer of ARGs in two WWTPs was frequently occurring, and the horizontal transfer of ARGs in X-WWTP was more frequent than Y-WWTP (**Supplementary Figure S2**), which indicated that some hARGs from the same ARG family exhibited different response patterns may be due to horizontal transfer. But we can't compare the difference in contribution between changes of the host microorganism and horizontal transfer.

Correlation networks and Variance Partitioning Analysis have often been used to look for relationships between ARG families and environmental factors. However, these methods often show spurious or indirect correlations or suggest that changes in the bacterial communities due to environmental factors are comparable to ARGs response patterns, which is hard to explain (Carr et al., 2019; Wu et al., 2019). This study indicated that environmental selection pressures are only responsible for the ARG enrichment and spread of some specific instead of all ARG-containing microorganisms, which contradicted the traditionally held belief that environmental selection pressures, especially antibiotic concentration, select for all ARG-containing microorganisms and cause the same change pattern to hARGs from the same ARG family (Durão et al., 2018; Guo X. et al., 2018; Zhao R. et al., 2019). The reason

why some ARG-containing microorganisms were not enriched under environmental selection pressures may be because the hARGs in their bodies were hard to expressed.

More importantly, the ARGs in the pohARGCs that were enriched under the antibiotic selection pressure in X-WWTP were abundant in the receiving stream sediments, while background ARGs or those enriched under domestic sewage conditions did not occur in the sediments. The enrichment of ARGs in the sediments causes long-term and long-distance hazards. The enrichment of hARGs of 3 β_lactamase families was likely through *Betaproteobacteria*, a member of the core microbiomes of both the wastewater and sediment, which is one of the most abundant and ubiquitous ARB genera in water environment (Suvorova and Gelfand, 2019; Petrovich et al., 2020). Other bacteria and horizontal transfer of ARGs could also contribute to this enrichment, but further study is needed to confirm this.

CONCLUSION

This study examined the microbial community structure and ARGs (especially hARGs) in wastewater from a cephalosporin antibiotic production WWTP (X-WWTP) and the subsequent municipal WWTP (Y-WWTP), and water and sediment from the receiving stream using GeoChip 5.0. We found that the hARGs of three β_lactamase ARG families enriched under the selection pressure of high antibiotic concentration in X-WWTP formed pohARGCs. The pohARGCs were also enhanced in the sediment of the receiving stream, suggesting that these enriched ARGs likely pose a more permanent public health risk. The enrichment of pohARGCs of three β_lactamase families was likely through *Betaproteobacteria* genus. The different response patterns of homologous ARG (hARGs) within the same ARG family imply environmental selection pressures are only responsible for the ARG enrichment and spread of some specific instead of all ARG-containing microorganisms. This study also represents a new gradation of ARG study, the results of which indicate examining individual hARGs can reveal a more nuanced response pattern of ARGs.

DATA AVAILABILITY STATEMENT

DNA sequences of 16S rRNA gene were available in NCBI Sequence Read Archive under project PRJNA767527.

AUTHOR CONTRIBUTIONS

LC, MZ, and JZ contributed to conception and design of the study. LC, MZ, and DN organized the experiments. LC performed the statistical analysis. LC wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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SUPPLEMENTARY FIGURE S1 | Microbial community composition of stream water samples at OTU level. **(A)** PCoA of stream water samples based on Bray-Curtis Index. **(B)** Pattern Search analysis of stream water samples.

SUPPLEMENTARY FIGURE S2 | The abundance of gene transfer element (Int1) in different wastewater samples.

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Emerging Contaminants in Streams of Doce River Watershed, Minas Gerais, Brazil

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This study investigated the occurrence and risk assessment of ten pharmaceutical products and two herbicides in the water of rivers from the Doce river watershed (Brazil). Of the 12 chemicals studied, ten (acyclovir, amoxicillin, azithromycin, ciprofloxacin, enrofloxacin, fluoxetine, erythromycin, sulfadiazine, sulfamethoxazole, glyphosate and aminomethylphosphonic acid) had a 100% detection rate. In general, total concentrations of all target drugs ranged from 4.6 to 14.5 μg L^{-1}, with fluoroquinolones and sulfonamides being the most representative classes of pharmaceutical products. Herbicides were found at concentrations at least ten times higher than those of the individual pharmaceutical products and represented the major class of contaminants in the samples. Most of the contaminants studied were above concentrations that pose an ecotoxicological risk to aquatic biota. Urban wastewater must be the main source of contaminants in waterbodies. Our results show that, in addition to the study of metal in water (currently being conducted after the Fundão dam breach), there is an urgent need to monitor emerging contaminant in waters from Doce river watershed rivers, as some chemicals pose environmental risks to aquatic life and humans due to the use of surface water for drinking and domestic purposes by the local population. Special attention should be given to glyphosate, aminomethylphosaphonic acid, and to ciprofloxacin and enrofloxacin (whose concentrations are above predicted levels that induce resistance selection).

Keywords: antimicrobials, herbicide, aquatic toxicology, ecological risk assesment, glyphosate

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INTRODUCTION

On the 5th of November 2015, one of the biggest environmental tragedies in the world occurred in the municipality of Mariana, MG (Brazil): the collapse of the Fundão dam, belonging to Samarco (a joint venture of Brazilian Vale and Anglo-Australian BHP Billiton), was responsible for releasing about 50 million m^3 of mining waste into the environment (Porto, 2016). The disaster, classified as very large and sudden (due to the severity of negative impacts caused), directly affected about 663.2 km of one of the most important Brazilian rivers (Doce river), which stretches between the states of Minas Gerais and Espírito Santo (IBAMA, 2015). Among the environmental impacts caused by the silt wave of tailings, the destruction of permanent

FIGURE 1 | Sampling sites along the Rio Doce watershed, Minas Gerais, Brazil. Site 1 in the Carmo River in the Mariana district, characterized by urban discharges; Site 2 in the Carmo River near its surrounding agricultural fields (mainly extensive cattle ranching and eucalyptus plantations); Site 3 in the Gualaxo do Norte River, surrounded by agricultural fields (mainly arable), into which wastewater in SAMARCO iron ore mining; and Site 4 in the Doce river which is formed by the junction of the Piranga and Carmo Rivers, also receiving urban discharges from the city of Ponte Nova through the Piranga River.

protected areas and native vegetation of the Atlantic Forest and above all, the impact on aquatic ecosystems should be highlighted.

The spoil heaps of the Fundão dam quarry flooded the district of Bento Rodrigues, however, it was dammed by the Risoleta Neves hydroelectric power plant. This was practically the only area of floodplain affected by the disaster. The material deposits in the area were considered an ecological time bomb due to their potential to release metals into the environment, including water—although this remains controversial in the literature (Queiroz et al., 2018). After the disaster, monitoring the water quality of the Doce river became a priority to track the potential impact on the disaster on aquatic environment. In addition, this study is important because the Doce river water is used to supply several cities in the states of Minas Gerais and Espírito Santo. However, the main focus of these studies was to evaluate the metal concentrations in the water. As far as we know, there have been no studies that have evaluated organic contamination of the water by emerging contaminants such as pesticides and personal and pharmaceutical products. In addition to mining activities, the Doce river watershed experiences continuous discharges of untreated wastewater, as well as contamination from agriculture (e.g., fertilizers and pesticides) and inadequate disposal of municipal waste (ANA, 2015) which are inevitably reflected in the presence of these emerging contaminants in the water. Once in the aquatic environment, drug and pesticide

residues can cause potential environmental risks by affecting aquatic organisms and, in the case of antibiotics, promoting the spread of antibiotic-resistant genes (Gomes et al., 2017; Gomes et al., 2019; Mendes et al., 2021). In addition, the use of contaminated water for crop irrigation can lead to the accumulation of pesticides and pharmaceuticals in crops and their uptake into the food web (Gomes et al., 2019; Gomes et al., 2020b). Here, we tracked concentrations of pharmaceuticals and pesticides in water from rivers in the Doce river Watershed from 2018 to 2019. We wanted to draw attention to the need to focus water investigations on the presence of novel contaminants that may affect water and environmental safety, in addition to metals.

MATERIALS AND METHODS

Study Area

The study area includes the Doce river drainage watershed, in the state of Minas Gerais (Brazil) (**Figure 1**). The region has approximately 199,000 inhabitants, mainly located in the urban area of the cities of Ouro Preto (74,558 inhabitants) and Mariana (61,228 inhabitants) (IBGE, 2020). Samples were collected from four sites with pronounced human activities in vicinity of the collecting point (**Supplementary Tables S1, S2**): In the Carmo River, samples were collected in the Mariana downtown area, typically characterized by urban discharges (**Figure 1**, site 1), and near the small town of Acaiaca (3,994 inhabitants) (**Figure 1**, site 2), which is surrounded by some agricultural fields with extensive livestock (mainly) and eucalyptus plantations. In the Gualaxo do Norte River, the samples were collected in an area surrounded by agricultural fields (mainly arable); the water also receives effluents from mining in SAMARCO iron ore mining (**Figure 1**, site 3). Finally, samples from the Doce river were collected near Risoleta Neves dam (**Figure 1**, site 4). The Doce river is formed by the confluence of the Piranga and Carmo rivers and, also receives urban runoff from the city of Ponte Nova *via* the Piranga river (**Figure 1**; **Supplementary Tables S1, S2**).

Selection of the Studied Chemicals

Pharmaceuticals (acyclovir, amoxicillin, azithromycin, ciprofloxacin, doxycycline, enrofloxacin, fluoxetine, erythromycin, sulfadiazine and sulfamethoxazole) were selected based on their abundance in on surface waters worldwide (Grill et al., 2016; Bertram et al., 2018; Beatriz et al., 2020; Gupta et al., 2021). Antibiotics such as amoxicillin (β-lactam), azithromycin and erythromycin (macrolides), ciprofloxacin and enrofloxacin (fluoroquinolones), doxycycline (tetracyclines), sulfamethoxazole and sulfadiazine (sulfonamides) are among the most commonly used in human and animal treatment, aquaculture and as feed additives (Giang et al., 2015). Acyclovir is one of the most effective and widely used anti-herpes agents (Mucsi et al., 1992; Gupta et al., 2021) and, fluoxetine is one of the most commonly prescribed antidepressants (Bertram et al., 2018). Glyphosate, on the other hand is the most commonly used herbicide in the world (Gomes et al., 2014) and is frequently used in the fields surrounding the sampling sites. Conversely, aminomethylphosphonic acid (AMPA) is the major metabolite of glyphosate, which is formed in the environment mainly through the degradation of the herbicide by microbes (Brock et al., 2019). In addition, organic phosphonates used in both industrial and domestic applications (detergents, flame retardants, corrosion inhibitors, anti-limescale agents and in the textile industry) are also sources of AMPA in aquatic ecosystems (Levine et al., 2015; Grandcoin et al., 2017).

Sampling Campaign and Preparation

Sampling was conducted in June 2018 (total precipitation from 0.2 to 0.6 mm/average flow rate 3.98 to 48.54 m^3/s), November 2018 (total precipitation from 146.1 to 210.1 mm/average flow rate 9.73 to 118.26 m^3/s), and April 2019 (total precipitation from 108.0 to 142.8 mm/average flow rate 5.95 to 102.75 m^3/s) (**Supplementary Tables S3, S4**). All sampling equipment was thoroughly cleaned with 70% ethanol before fieldwork and then washed with deionized water. Three water samples (5,000 ml) were collected at 50 m intervals at each point. The surface water samples were collected in sterile amber glass bottles. Samples were stored in ice (4°C) until arrival at the laboratory and then filtered through glass fiber membranes (0.45 µm, Millipore). Samples were then separated for evaluation of drugs (acyclovir, amoxicillin, azithromycin, ciprofloxacin, doxycycline, enrofloxacin, fluoxetine, norfloxacin, erythromycin, sulfamethoxazole, and sulfadiazine) and herbicides [glyphosate and aminomethylphosphonic acid (AMPA)]. The pH of the samples was adjusted to 6.5 and 2.5 for the drug and herbicide analyses, respectively.

For the drug analyses, the filtered water samples (500 ml) were concentrated by solid-phase extraction (SPE) using a Visiprep™ SPE Vaccum manifold (Sigma-Aldrish, Brazil) with 200 mg, 3 ml^{-1} Phenomenex Strata-X® cartridges (Torrance, California, United States). SPE conditions were the same as those described by to Beatriz et al. (2020). Cartridges were conditioned with 4 ml methanol followed by 6 ml ultrapure water and analytes were eluted in 4 ml methanol. For herbicide evaluation, samples were concentrated using C18 cartridges (500 mg/6 ml; Applied Separations, United States) previously conditioned with 15 ml of acidified water (pH 2.5) and 5 ml of methanol (Mendes et al., 2021). The cartridges containing the samples were eluted with 3 ml of 50% methanol in water (v/v). For both antibiotics and herbicides, the eluate was dried in a SpeedVac device (RC1010, Thermo), and the residues resuspended in the mobile phase (water and acetonitrile in a 50:50 v/v ratio with 0.1% formic acid and 5 µM ammonium formate for antibiotics and 5 mM ammonium acetate in water for herbicides).

Chromatographic Analyses

Analyses were performed using a LC-MS/MS system consisting of a Xevo TQD triple quadrupole mass spectrometer (Waters) with electrospray (ESI) ionisation source and an HPLC Varian SYS-LC-240-E with autosampler. Drugs were evaluated following (Beatriz et al., 2020), while glyphosate and AMPA were evaluated following (Gomes et al., 2015). For the drugs, chromatographic separations were performed using a 4.6 mm × 150 mm, 5 µm particle size Zorbax Eclipse XDB-C8 column (Agilente, Milford, United States) using water as phase A and acetonitrile/water (95:5 v/v) as phase B, both containing 0.1%

TABLE 1 | Limit of detection (LOD) and limit of quantification (LOQ) of drugs and herbicides evaluated using a LC-MS/MS (Gomes et al., 2015; Beatriz et al., 2020).

Chemical	LOD (ng L^{-1})	LOQ (ng L^{-1})
Pharmaceutical		
ACY	10	20
AMX	100	200
AZI	14	20
CIP	10	20
DOX	100	200
ENR	10	20
ERY	20	40
FLX	100	200
SDZ	10	20
SMX	10	20
Herbicide		
AMPA	10	20
GLY	10	20

Acyclovir (ACY), amoxicillin (AMX), aminomethylphosphonic acid (AMPA), azithromycin (AZI), ciprofloxacin (CIP), doxycycline (DOX), enrofloxacin (ENR), erythromycin (ERY), fluoxetine (FLX), glyphosate (GLY), sulfadiazine (SDZ) and sulfamethoxazole (SMX).

formic acid and 5 mM ammonium formate. For the herbicides, chromatographic separations were performed using an Ascentis® C18 column (Sigma-Aldrich, Brazil) with a mobile phase consisting of 5 mM ammonium acetate in water (phase A) and 5 mM ammonium acetate in methanol (phase B), both pH 7.0. Mass spectrometry analyses were performed in positive and negative ion modes for antibiotics and herbicides, respectively. Acyclovir (ACY), amoxicillin (AMO), azithromycin (AZI), ciprofloxacin (CIP), doxycycline (DOX), enrofloxacin (ENR), fluoxetine (FLU), erythromycin (ERY), sulfadiazine (SDZ), sulfamethoxazole (SMX), glyphosate (GLY) and AMPA (Sigma-Aldrich, Canada) in analytical grade were used to construct the calibration curves. Standard stock solutions (1,000 μg ml^{-1}) of these compounds were prepared using different compositions of methanol, water and acetonitrile, with formic acid and ammonium formate, depending on solubility. The six-point calibration curves showed good linearity for the analytes ($r^2 \geq 0.95$; $p < 0.0001$). Each sample batch included three blanks, three standards, and three fortified samples (for quality control). The recoveries for all compounds were greater than 87%. The limit of detection (LOD) and limit of quantification (LOQ) of each analyte are listed in **Table 1**.

Ecological Risk Assessment

The predicted no effect concentration (PNEC) was estimated using the ecological structure-activity relationships (ECOSAR) model (Moore et al., 2003) and was calculated by dividing the no-observed effect concentration (NOEC) found in the literature by an assessment factor (AF) of 1,000, which represents chronic toxicity (Ikem et al., 2017).

Hazard quotient (HQ) was used to assess the environmental risk of chemicals and their potential to cause adverse effects in the environment (Carlsson et al., 2006) and was calculated as follows:

$$HQ = \frac{MEC}{PNEC}$$

Where PNEC is the predicted no-effect concentration (from literature) and MEC is the measured environmental concentration. For MEC, the mean of the concentrations found over time ($n = 9$) for each collection site was used.

Statistical Analyses

Results were expressed as the average of three replicates. Statistical analyses were performed using JMP 10.0 software (SAS Institute Inc.). Results were subjected to normality (Shapiro–Wilk) and homogeneity (Bartlett) tests and then statistically analyzed. Univariate repeated measures ANOVA, with time as a within-subject factor and sites as the main effect, were used to analyze differences in chemical concentrations during the sampling period. The sphericity of the data was tested using Mauchly's criteria to determine if the univariate F-tests were valid for within-subject effects. If F-tests were invalid, the Greenhouse-Geisser test was used to estimate epsilon (ε). Contrast analysis was used when there were significant differences in the variables examined.

RESULTS

Occurrence of Pharmaceutical Products on Surface Waters

With the exception of DOX, all surface water samples were contaminated with the tested drugs (**Figure 2**). The highest concentrations of antibiotics were found in CIP (up to 4,854.6 ng L^{-1}), followed by SMX (up to 9,640 ng L^{-1}). The concentrations of DOX (up to 2.25 ng L^{-1}) were lower (or were not detected) compared to the other drugs (**Table 2**). With the exception of ERY and FLX ($p > 0.05$), a significant interaction ($p < 0.05$) between time and site of sampling was observed for the drugs (**Table 2**). Regardless of the sampling date, the concentrations of ACY, AMX, CIP, SDZ, SMX (except for the last sampling date) were higher and ENR concentration was lower at site one than at the other sampling sites (**Figure 2**). High concentrations of ACY, AZI, CIP, SDZ and SMX were detected in the water samples from site one on the first sampling date and lower concentrations of ACY, AMX, ENR, ERY, and SDZ were detected on the second sampling date compared to the other sampling dates (**Figure 2**). CIP concentrations decreased and ENR concentrations increased over time in the site 2 water samples (**Figure 2**). In addition, ERY, SDZ, and SMX concentrations were lower in site 2 water samples at the second time point compared to the other sampling time points (**Figure 2**). With the exception of ENR, whose concentration decreased at the second sampling time point, and ACY, whose concentration increased at the last sampling time point, the concentrations of the other drugs in the water samples from Site three did not differ significantly ($p < 0.05$) over time (**Figure 2**). When compared over time of collection, the concentrations of ACY, AZI, and SDZ were higher in the water samples from Site four on the first sampling date and the concentrations of AMX and ERY were lower on the second sampling date (**Figure 2**).

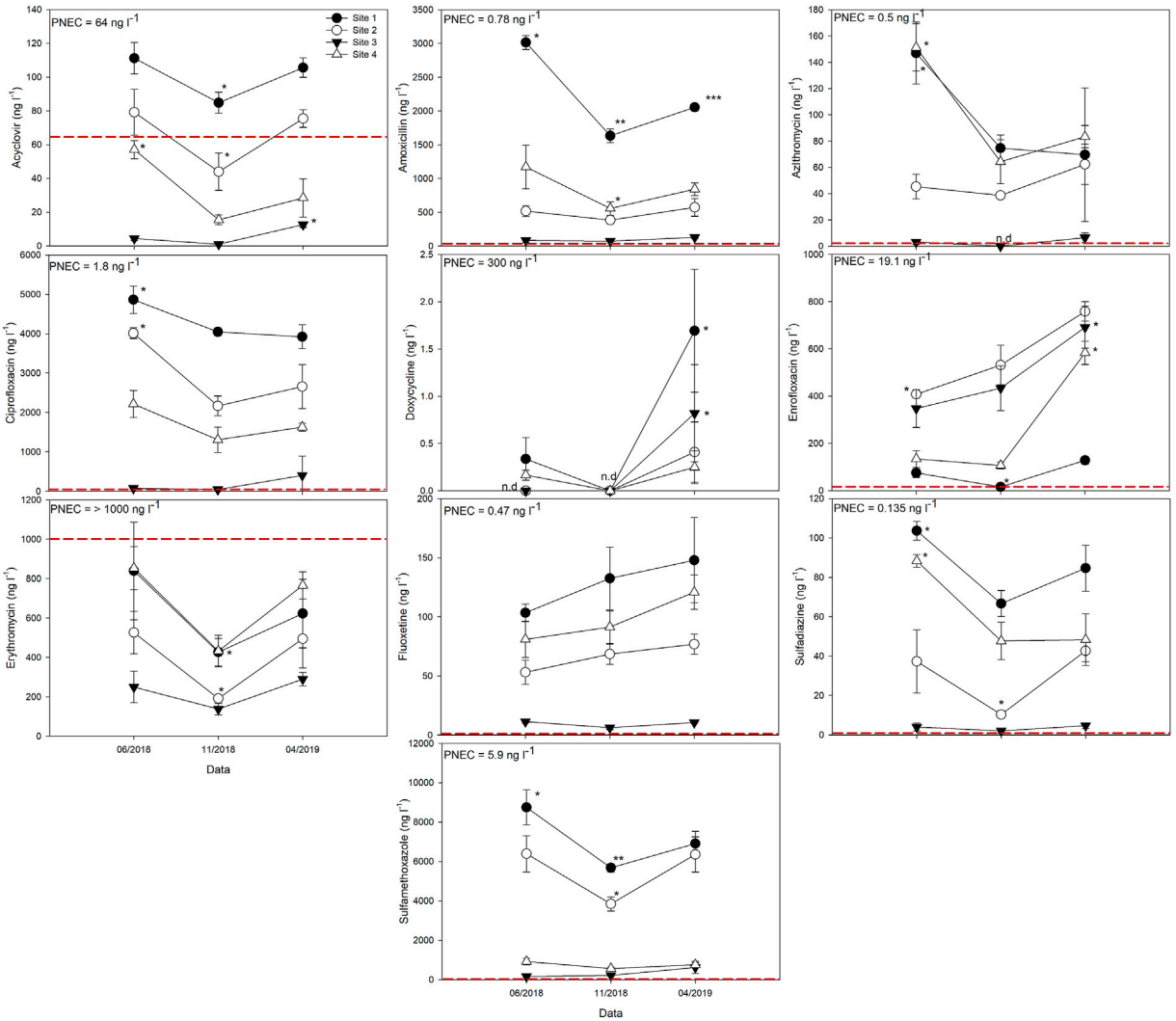

FIGURE 2 | Concentrations of pharmaceutical target products in surface water samples from rivers of the Doce river watershed, Minas Gerais, Brazil. Values are means ± SD of three replicates. Values marked with * differ significantly ($p < 0.05$) within the same sampling site by the contrast test. Predicted no effect concentration (PNEC) is shown as dotted red line. NOEC = concentration with no observed effect.

TABLE 2 | Repeated-measure ANOVA for the effects of the site of sampling and time on the concentration of chemicals in surface water of rivers of Rio Doce Basin.

Source of variation	D.F	ACY	AMX	AZI	CIP	DOX	ENR	ERY	FLX	SDZ	SMZ	GLY	AMPA
Site	3	337.59***	671.57***	86.89***	375.44***	7.05*	61.34***	27.11***	46.87***	127.58***	482.75***	30.22***	9.92**
Time	2	41.05***	28.38***	19.13**	71.15***	18.02**	898.29***	56.60***	12.94***	30.31***	21.39***	7.86*	12.20**
Site x time	6	3.92*	6.31*	3.99*	11.69***	4.21*	32.57***	1.96	1.03	5.38*	5.96***	3.36*	3.10*
Mean concentration (ng L^{-1})[a]													
Site 1	—	100.56	2,234.66	97.11	3,993.61	0.67	73.20	629.33	127.97	85.00	7,112.44	71,170	23,460
Site 2	—	66.20	491.88	48.77	3,228.71	0.13	566.00	404.00	66.12	30.11	5,532.55	109,640	38,380
Site 3	—	6.01	95.29	3.18	171.93	0.27	491.11	225.55	9.34	3.55	332.78	117,070	41,250
Site 4	—	33.66	857.11	99.66	1716.20	0.13	274.77	683.77	97.72	61.44	746.34	51,880	30,050
Frequency (%)	—	100	100	91.6	100	41.6	100	100	100	100	100	100	100

[a]*Mean of concentration found overtime (n = 9). D.F., degrees of freedom.*

, **, * Significant at 0.05, 0.01 and 0.001, respectively. RSD, relative standard deviation.*

Acyclovir (ACY), amoxicillin (AMX), aminomethylphosphonic acid (AMPA), azithromycin (AZI), ciprofloxacin (CIP), doxycycline (DOX), enrofloxacin (ENR), erythromycin (ERY), fluoxetine (FLX), glyphosate (GLY), sulfadiazine (SDZ) and sulfamethoxazole (SMZ).

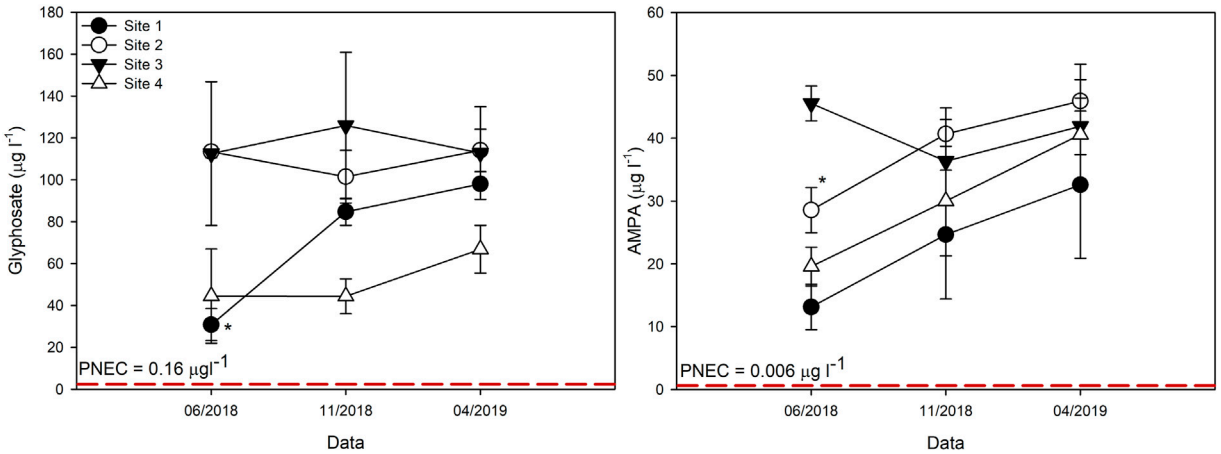

FIGURE 3 | Concentrations of glyphosate and AMPA in surface water samples from rivers of the Doce river watershed, Minas Gerais, Brazil. Values are means ± SD of three replicates. Values marked with *, differ significantly ($p < 0.05$) within the same sampling site by the contrast test. Predicted no effect concentration (PNEC) is shown as dotted red line. NOEC = concentration with no observed effect.

TABLE 3 | No-observed effect concentration (NOEC), the predicted no effect concentration based on ecotoxicological data [$PNEC_{(ecotox)}$], predicted no effect concentration for antibiotic-resistance selection [$PNEC_{(resi.\ sel.)}$] and hazard quotient (HQ) (in ngl^{-1}).

Chemical	NOEC[a]	References	Test species	End point	Test duration	$PNEC_{(ecotox)}$	$PNEC_{(resi.\ sel.)}$[b]	HQ[c]			
								Site 1	Site 2	Site 3	Site 4
Pharmaceutical											
ACY	64,120	Minguez et al. (2016)	*Daphnia magna*	EC_{50}	48 h	64.12	n.a	1.56	1.03	0.09	0.52
AMX	780	Andreozzi et al. (2004)	*Synechococcus leopolensis*	NOEC	96 h	0.78	250	2,864.95	630.62	122.16	1,098.86
AZI	500	Minguez et al. (2016)	*Pseudokirchneriella subcaptata*	EC_{50}	72 h	0.5	250	194.22	97.55	6.37	199.33
CIP	1800	Martins et al. (2012)	*D. magna*	NOEC	96 h	1.8	64	2,218.67	1793.72	95.51	953.44
DOX	300,000	Brain et al. (2004)	*Lemna gibba*	LOEC	7 days	300	2000	0	0	0	0
ENR	19,100	Ebert et al. (2011)	*Anabaena flos-aquae*	NOEC	72 h	19.1	64	3.83	29.63	25.71	14.38
ERY	106	Brain et al. (2004)	*L. gibba*	LOEC	7 days	1,000	1,000	0.62	0.40	0.22	0.68
FLX	470	Nentwig (2007)	*Potamopyrgus antipodarum*	NOEC	65 days	0.47	n.a	272.29	140.69	19.88	207.92
SDZ	135	Lützhøft et al. (1999)	*Microcystis aeruginosa*	EC_{50}	21 days	0.135	n.d	629.62	223.04	26.33	455.14
SMZ	5,900	Ferrari et al. (2004)	*Synechococcus leopoliensis*	NOEC	96 h	5.9	16,000	1,205.49	937.72	56.40	126.49
Pesticides											
GLY	16,000	Mendes et al. (2021)	*Salvinia molesta*	EC_{10}	14 days	16	n.a	4,448.68	6,852.63	7,317.36	3,243.05
AMPA	6,100	Mendes et al. (2021)	*S. molesta*	EC_{10}	14 days	6.1	n.a	3,847.35	6,291.80	4,927.14	4,927.14

[a]*The NOEC, column also represents LOEC (lowest-observed effect concentration), EC_{10}, EC_{50}, and EC10 data when NOEC, data was not available.*

[b]*Correspond to the size-adjusted lowest minimum inhibitory concentration (MIC) divided by an assessment factor of 10 (Bengtsson-Palme and Larsson, 2016).*

[c]*$MEC/PNEC_{(ecotox)}$.*

n a, not applicable; n.d, not determined.

Occurrence of Glyphosate and AMPA in Surface Waters

All surface water samples were contaminated with GLY and AMPA (**Figure 3**). For GLY and AMPA, a significant interaction ($p < 0.05$) was observed between time and site of sampling (**Table 2**). Higher concentrations of these chemicals were observed in samples from sites 2 and three compared to samples from sites one and 2 on the first day of sampling. Glyphosate and AMPA concentrations increased over time in samples from sites 1 and 2, respectively (**Figure 3**). At site 3, AMPA concentrations were lower on the second sampling date (**Figure 3**). Glyphosate and AMPA concentrations in samples from site four did not differ over time (**Figure 3**).

Ecological Risk Assessment

With the exception of ACY, DOX, ERY, the observed concentrations of the chemicals were higher than their calculated $PNEC_{(ecotox)}$ (**Figures 2**, **3** and **Table 3**). For ACY, all concentrations observed at site one were greater than the calculated $PNEC_{(ecotox)}$; for site 2, only the concentrations found on the first and last sampling dates were greater than the calculated $PNEC_{(ecotox)}$. At all sites, the observed concentrations of AZI, DOX, ERY, and SMZ were lower than the $PNEC_{(resi.\ sel)}$ (**Table 3**). Only the HQ of DOX and ERY were lower than one for all sites. At sites 3 and 4, HQ < 1 was also observed for ACY (**Table 3**).

DISCUSSION

Of the ten pharmaceutical products studied, all had a 100% detection rate, except for AZI (91.6%) and DOX (41.6%) (**Table 3**). In general, the total concentration of all target pharmaceutical ranged from 4,595.40 to 14,478.59 ng L^{-1}, with fluoroquinolones (CIP + ENR) and sulfonamides (SDZ + SMX) accounting for 28.08–49.42% and 17.57–53.28%, respectively. Based on the average proportion at all sites, the proportion of different pharmaceuticals was as follows: Fluoroquinolones ≥ Sulfonamides > Macrolides (AZI + ERY) (5.01–14.05%) > β-Lactam (AMX) (4.05–18.65%) > antiretrovirals (ACY) (1.5–1.9%) antidepressants (FLU) (0–4.6%) > tetracyclines (DOX, <0.1%).

Among the fluoroquinolones, CIP was the most frequently detected antibiotic, regardless of the site and time of sampling (**Figure 2**), which is not surprising since CIP is the most commonly prescribed fluoroquinolone worldwide (Andreu et al., 2007) whose bactericidal effect is based on inhibition of DNA replication by inhibition of bacterial DNA topoisomerase and DNA-gyrase. CIP has been detected in milligram amounts in sewage sludge (Golet et al., 2003; Martínez-Carballo et al., 2007). However, in water samples, the detected concentrations are lower. In untreated hospital wastewater, CIP concentrations ranged from 1,100 to 44,000 ng L^{-1} in Vietnam and 388–578 ng L^{-1} in Malaysia (Duong et al., 2008; Thai et al., 2018). In urban wastewater, CIP was previously detected at concentrations ranging from 242 to 415 ng L^{-1} in China (Low et al., 2021) and in municipal landfills, concentrations ranged from 60.2 to 4,482 ng L^{-1} (Wu et al., 2015). In Brazilian surface waters, CIP concentrations were below 0.41 ng L^{-1} in the Atibaia River (São Paulo) (Locatelli et al., 2011) and ranged from 180 to 340 ng L^{-1} in rivers from the four largest hydrographic catchments of the city of Curitiba (Paraná) (Beatriz et al., 2020). The higher CIP concentration in surface waters observed in our study must be related to the lack of wastewater treatment (present in the other Brazilian cities cited) and the direct discharge of urban wastewater into the waters of Doce river watershed rivers. Similarly, a CIP concentration of 15,000 ng L^{-1} was observed in surface water in South Africa (Agunbiade and Moodley, 2014). Indeed, samples from sites under the influence of direct urban discharges (sites 1, 2 and 4) had high concentrations of the antibiotics compared to sites without urban proximity (site 3). Interestingly, ENR concentrations were lower in samples from site one compared to the other sites (**Figure 2**). This antibiotic is used in veterinary medicine (Rusu et al., 2015), and indeed high ENR concentrations were found at sites near livestock (sites 2 and 3). It is important to note that ENR can be degraded to CIPRO through biotransformation (Walters et al., 2010), which could contribute to the CIP concentrations in water at sites 2 and 3.

In the group of sulfonamides, SMX were detected higher concentrations (from 332.78 to 7,112.44 ng L^{-1}) than SDZ (from 3.55 to 61.44 ng L^{-1}) (**Figure 2**; **Table 3**). In rivers near the city of Curitiba (Brazil), SXM was found at concentration of 1859 ng L^{-1}, while SDZ were reported at concentration of 27 ng L^{-1} (Beatriz et al., 2020). In South Africa, SMX was detected at concentrations of 7,300 (Matongo et al., 2015) and 14,000 ng L^{-1} in surface waters (Ngumba et al., 2016). In Kenya, concentrations of up to 40,000 ng L^{-1} have been observed in river waters (K'oreje et al., 2016) while SDZ has been detected in concentrations of up to 40 ng L^{-1} in rivers from Nigeria (Oluwatosin et al., 2016). In China, up to 764.6 ng SMX l^{-1} has been detected in rivers (Chen and Zhou, 2014). Sulfonamides are bacteriostatic antibiotics that interfere with folic acid synthesis and are mainly used for acne and urinary tract infections—justifying their high concentrations in rivers near cities (sites 1 and 2). In China, sulfonamides were the major class of antibiotics found in rivers. SDZ and SMZ were detected at 100% and had mean concentrations of 259.6 and 7.6 ng L^{-1}, respectively (Chen and Zhou, 2014).

Macrolides such as AZI and ERY inhibit bacterial protein biosynthesis, while the β-lactam AMX acts by binding to penicillin-binding proteins, resulting in the activation of autolytic enzymes in the bacterial cell wall. These antibiotics are used for both human and veterinary purposes. This may explain why macrolide and β-lactam concentrations were lowest at site 4, where there are no direct urban discharges and where crop cultivation is the main activity in the environment (**Figure 2**). Among macrolides, ERY was observed at high concentrations in our study, regardless of time and sites of collection (**Table 3**; **Figure 2**). ERY concentrations up to 20,000 ng L^{-1} and 1,000 ng L^{-1} were observed in surface waters in South Africa (Matongo et al., 2015) and Nigeria (Oluwatosin et al., 2016), respectively. AZI concentrations

ranged up to 650 ng L^{-1} in Brazilian rivers (Beatriz et al., 2020) and up to 30 ng L^{-1} in South African rivers (Módenes et al., 2017), while it was not detected in Nigerian rivers (Oluwatosin et al., 2016). As for β-lactam, AMX has been detected in concentrations up to 99.4 mg L^{-1} in wastewater in Egypt (Abou-Elela and El-Khateeb, 2015). In Brazil, this antibiotic has been detected at concentrations up to 1,570 ng L^{-1} in rivers from the Curitiba region (Beatriz et al., 2020) and up to 1,284 ng L^{-1} in rivers from the state of S o Paulo (Locatelli et al., 2011).

Data on the concentrations of ACY and FLX are scarce in the literature. These drugs are used to treat human, which justifies their high concentrations in areas with urban runoff (**Figure 2**). ACY is generally used as the first choice in the treatment of viral infections such as herpes simplex, *Varicella* zoster, herpes zoster, herpes labialis and acute herpetic keratitis (O'Brien and Campoli-Richards, 1989). In Brazilian rivers (Curitiba, Paraná), concentrations ranged from ACY to 990 ng L^{-1} (Beatriz et al., 2020). In Germany, ACY concentrations in river water ranged up to 190 ng L^{-1} (Prasse et al., 2010). Average concentrations of FLX in surface waters concentrations ranged from 12 to 1,400 ng L^{-1} worldwide (Kolpin et al., 2002; Christensen et al., 2009). In Brazil, FLX concentrations in streams of Curitiba were as high as 620 ng L^{-1} (Beatriz et al., 2020). FLX is primarily used to treat depression, but also helps treat other mental disorders such as obsessive-compulsive disorder, bulimia nervosa, and panic syndrome, and is one of the most commonly prescribed psychotropic drugs in Brazil (Quintana et al., 2015). As with some other drugs studied, we found relatively high concentrations of pharmaceuticals in the waters of the Doce river watershed (**Figure 2**). Seasonal aspects could have influenced the results obtained. For example, the concentrations of ACY, AMX, AZI, CIP, ERY, SDZ and SDX were high at some sites on the first sampling date which corresponding to the dry season. During a low-precipitation period, water flow decreases and, assuming that pollution sources are constant, dilution effects must play a central role in the occurrence and concentrations of pharmaceuticals in the water samples sampled (Locatelli et al., 2011).

The flow of a river is the result of complex natural processes that occur at the catchment scale and are largely influenced by precipitation (Yunus and Nakagoshi, 2004). Changes in streamflow affect water quality (Caruso, 2001) and pollution of rivers increases when streamflow is low, due to low dilution capacity (Yunus and Nakagoshi, 2004). We clearly observed the influence of rainfall on flow (**Supplementary Table S4**) and concentration of the studied drugs (**Figure 1**). At least for two of the sampling sites, the concentrations of the analyzed drugs (except for DOX and ENR) were higher when precipitations were the lowest (June 2018). The higher concentrations of DOX and ENR at the higher rainfall levels (294 mm in November 2018 and 108 mm in April 2019), indicate that the source of these drugs increased during rainy season. This could be due to increased seepage and runoff (Yunus and Nakagoshi, 2004) or to the increased use of these drugs during the rainy season.

Although high concentrations of pharmaceuticals were found in the water samples, the most worrying results are associated with the observed GLY and AMPA concentrations (**Figure 3**). GLY and AMPA contamination levels exceeded those observed for pharmaceutical products by several times. These contaminants were observed at concentrations ranging from 51.88 to 117.07 and 23.46–41.25 μg L^{-1}, respectively, indicating that herbicides are the main source of contamination in the rivers studied. In the Paraná River watershed (Brazil), GLY concentrations ranged from 0.4 to 91.91 μg L^{-1} (Mendonça, 2018), while AMPA was detected at concentrations up to 14.78 μg L^{-1} (Da Silva et al., 2003). In another study, glyphosate concentrations up to 100 μg L^{-1} and AMPA concentrations up to 50 μg L^{-1} were detected in the Arroio Passo do Pilão watershed (Brazil). GLY is not only used in crops and eucalyptus plantations but is also widely used for weed control in Brazilian cities, and its use is often unregulated. Therefore, it is possible that GLY concentrations >80 μg L^{-1} at sites under urban areas (**Figure 3**). In addition to GLY, the observed concentrations of AMPA in water samples must be derived from its use in industry and household products (such as detergents). (Levine et al., 2015; Grandcoin et al., 2017). Unlike pharmaceuticals, concentrations of GLY and AMPA were not affected by pluviosity (except for sites one and 2 for glyphosate and AMPA, respectively). Considering the dilution effect of high pluviosity and river flow on river pollutants (Yunus and Nakagoshi, 2004), we hypothesize that herbicide use was increased during rainy season. In fact, glyphosate uses in Brazil is declining from April to September, as the herbicide is mainly used during the rainy season, when crops are growing (Dias et al., 2021). At concentrations as low as 5 μg L^{-1}, the herbicide glyphosate reduced algal diversity in phytoplankton communities of freshwater streams (Smedbol et al., 2018) and the EC10 value for GLY and AMPA in the macrophyte *Salvinia molesta* was 16 and 6.1 μg L^{-1}, respectively (Mendes et al., 2021). Therefore, it is reasonable to assume that the concentrations of these herbicides found in the Doce river watershed could trigger an alteration of aquatic life, and to assess the potential risk of these chemicals (along with the pharmaceutical products evaluated), we conducted a risk assessment.

PNEC values are based on toxicological data from the literature. In this study, we selected the NOEC of species representative of the those found in Brazil to calculate the $PNEC_{(ecotox)}$, using an assessment factor of 1,000, which represents chronic toxicity (Ikem et al., 2017). If the reported concentrations in the environment are higher than the PNEC, there is a toxicological risk to the environment. With the exception of ACY, DOX, ERY, the concentration of all other chemicals studied poses a potential toxicological risk. In the case of ACY, the observed concentrations at site one are also of toxicological concern. The risk level is generally classified into four groups: no risk (HQ < 0.01), low risk (0.01 ≤ HQ ≤ 0.1), medium risk (0.1 ≤ HQ ≤ 1), and high risk (HQ > 1) (Rodriguez-Mozaz et al., 2020). Only DOX had a HQ < 0.01, and did not pose an ecotoxicological risk to the aquatic environment. For the site 3, ACY poses a low risk (HQ = 0.09), and for site 4, ACY poses a moderate risk (HQ = 0.52). Similarly, ERY poses a medium risk (HQ < 1) to aquatic life, regardless of sampling locations. However, for all other chemicals sampled, HQ was greater than 1 (and can reach 7,317.36), representing a high

ecotoxicological risk to the aquatic environment. The mean value of HQ for Cd, Pb, Cr, Zn, Cu and As in the Doce river ranged from 226.30 to 841.60 (Gabriel et al., 2020). Although the HQ indices for these metals and metalloids represent a high ecotoxicological risk, they are lower than the HQ calculated here for some chemicals (i.e., AMX, CIP, GLY, and AMPA) (**Table 3**). These results demonstrate the urgent need to consider emerging contaminants (and not just metals) in risk assessment, given the importance of these chemicals to aquatic ecosystems. It is also important to note that concentrations of AMX, CIP, and ENR are high than the proposed PNEC for resistance selection. Antimicrobial resistance is an emerging concern, as the spread of resistance genes is a global problem with direct detrimental effects on the economy and public health (Kent et al., 2020). Moreover, very few studies have investigated the toxicity of drug mixtures in natural water samples. For example, Gomes et al. (2020a) observed interactive effects of AMX, ENR, and oxytetracycline on *Lemna minor* plants, which demonstrates the importance of evaluating both the isolated and integrative toxic effects of chemicals. Clearly, toxicological testing involving exposure to a cocktail of multiple drugs is needed, especially in highly contaminated surface water (Anh et al., 2021), as noted here.

The main objective of this study was to draw attention to the presence of considerable amounts of emergent contaminants in the waters of the Rio Doce basin, which, among other contaminants, such as trace elements, can limit aquatic life. Our data also suggest that environmental factors, especially pluviosity (and its effect on water flow), play an important role in the concentrations of chemicals found in the water. The fate of organic contaminants is influenced by the physicochemical and biological properties of the water and sediments. Indeed, temperature, pH, microbial activity and light conditions may affect the availability of the contaminants (Moncmanová, 2007) and alter their rates of degradation, sorption, and bioaccumulation. Therefore, we cannot comment on the exact contribution of an upstream source to the concentration of chemicals along the river (downstream sites). To this end, studies with isotopically labeled chemicals would permit to elucidate the fate as well as the specific role of anthropogenic activities on concentrations of emerging contaminants in the Rio Doce basin rivers. However, in a climate change scenario, we pointed out the possible increased of toxicological impacts of contaminants. As a result of rising temperatures, increased drought, El-Niño Southern Oscillation, and reduced pluviosity (Caruso, 2001), there may be low water flow and increased concentrations and harmful effects of chemicals on aquatic ecosystems.

CONCLUSION

Through sampling and analysis, the concentrations and distribution of 12 contaminants (pharmaceutical products and herbicides) were determined at four different sites in rivers of the Doce river watershed. Although the concentrations detected were within the range of those observed in other emerging countries, the sampled waters were highly contaminated, especially by the herbicide GLY and its metabolite AMPA. The risk assessment analysis conducted here shows that most of the chemicals assessed are present at concentrations above the $PNEC_{(ecotox)}$ level, posing a potential threat to the aquatic environment. In addition, several antibiotic concentrations are higher than those known to cause antibiotic resistance, particularly those in the fluoroquinolone class. The concentrations of chemicals studied were related to human activities in vicinity of the sampling sites, but the lack of water treatment in urban areas could be the main cause of river contamination. Based on the HQ index, the risk assessment approach provided useful guidance on which chemicals needs to our priority attention for future control and remediation. In this context, particular attention needs to be give to GLY, AMPA, fluoroquinolones and sulfonamides. The results show that there is an urgent need to monitor the presence of emerging contaminants in water, which, in addition to metals (the main target in the study of water quality in the rivers of the Doce river watershed), may pose risk to the environment and humans due to the frequent use of surface water for drinking and domestic purposes by the local population.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

MG, FV, and PJ conceived and designed the experiments, gave technical support and conceptual advice. MG, JB, RK, and PJ performed chemical analysis. MG, JB, and PJ wrote manuscript. FV provided technical and editorial assistance. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2021.801599/full#supplementary-material

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High-Throughput Screening of Antimicrobial Resistance Genes and Their Association With Class 1 Integrons in Urban Rivers in Japan

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Antimicrobial resistance (AMR) is a serious public health concern. Many countries have implemented AMR surveillance programs for humans and animals, but a scheme for monitoring AMR in the environment has not yet been established. Class 1 integrons, which can acquire antimicrobial resistance genes (ARGs) to gene cassettes, were proposed as a candidate to evaluate the anthropogenic impacts on AMR. However, the association between class 1 integrons and ARGs in aquatic environments is less studied and requires further elucidation. This study used high-throughput quantitative polymerase chain reaction (HT-qPCR) to characterize the pollution profiles of ARGs and mobile gene elements (MGEs) in 24 urban rivers in Tokyo and its surrounding area. The abundance of class 1 integron-integrase gene (*intI1*) and the array of class 1 integron gene cassettes were also determined. In total, 9–53 target genes were detected per sample, and their abundances increased following effluent discharge from wastewater treatment plants. The river and wastewater samples were categorized based on their HT-qPCR profiles, indicating that this method was useful for characterizing the pollution status in aquatic environments. The prevalence of *intI1* in the rivers was observed. Some ARGs and MGEs were positively correlated with *intI1*, indicating that *intI1* could be used as a proxy for monitoring these ARGs and MGEs in urban rivers. Long-read sequencing of class 1 integron gene cassettes revealed that one to three ARGs were present in the gene cassettes. Regardless of the sample type, $bla_{\text{GES-24}}$, *aadA2*, and *qacH* were dominant in the gene cassettes. The source and spread of class 1 integrons carrying these ARGs in aquatic environments should be further monitored.

Keywords: Antimicrobial resistance gene, class 1 integron, gene cassette, high-throughput quantitative PCR, urban river

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INTRODUCTION

The health burden of antimicrobial resistance (AMR) is a crucial issue across the world (O'Neill, 2016). While the overuse or abuse of antimicrobial agents for humans and animals has led to the emergence of antimicrobial resistant bacteria (ARB) and antimicrobial resistance genes (ARGs), the recipient environments could serve as their reservoirs (Nnadozie and Odume, 2019). Therefore, One Health, which is a comprehensive and multisectoral approach to address AMR issues in humans,

animals, and the environment, serves as an essential initiative to mitigate the spread of AMR in the society. Many countries have implemented surveillance programs for pathogenic ARB in humans and animals (WHO, 2021a). On the other hand, the dimension of environmental AMR remains unknown (Larsson et al., 2018; Samreen et al., 2021; Zhuang et al., 2021), and the interaction between pathogens and environmental bacteria carrying ARGs could be facilitated by horizontal gene transfer (Martínez, 2019). WHO recently launched the Tricycle project, which is aimed at One Health surveillance by focusing on extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* (Pruden et al., 2021; WHO, 2021b). However, monitoring targets and goals to control AMR in the environment is still challenging owing to the lack of basic information such as ARG abundance and diversity.

Metagenomic analysis and high-throughput quantitative polymerase chain reaction (HT-qPCR) are promising tools for the comprehensive surveillance of ARGs and mobile gene elements (MGEs) in the environment. Metagenomic analysis is a non-target screening method in which no preliminary information of genes is known (Chen et al., 2019b; Hendriksen et al., 2019; Liang et al., 2020; Lira et al., 2020). HT-qPCR, which enables the simultaneous quantification of hundreds of target genes, is generally more sensitive than metagenomic analysis for ARGs and MGEs surveillance (Waseem et al., 2019). Although HT-qPCR provides semiquantitative data, it is still rapid and inexpensive for screening complex AMR profiles in the environment. Therefore, HT-qPCR has been employed to evaluate ARGs and MGEs in various aquatic environments, including rivers (Khan et al., 2019; Lai et al., 2021; Yu et al., 2021), water sources (Han et al., 2020), drinking water (Xu et al., 2016), urban sewage (Huang et al., 2019; Pärnänen et al., 2019; Quintela-Baluja et al., 2019), and aquaculture systems (Muziasari et al., 2016). HT-qPCR provides information to help determine factors that shape environmental resistome, such as bacterial community, antibiotic concentration (Han et al., 2020), and ARG sources in the environment (Khan et al., 2019).

In urban rivers, wastewater treatment plants (WWTPs) are hotspots that release ARB and ARGs as well as residual antibiotics (Michael et al., 2013; Mao et al., 2015; Guo et al., 2017; Amarasiri et al., 2019). In a previous study, HT-qPCR based on 384 primer sets was used for the comprehensive surveillance of ARGs and MGEs in WWTP influent and effluent in seven European countries, and 289 primer sets showed positive results (Pärnänen et al., 2019). HT-qPCR revealed that the total abundances of ARGs in recipient surface water bodies were higher than those at upstream sites, suggesting that WWTP effluent was a major source of ARGs in urban aquatic environments (Huang et al., 2019; Quintela-Baluja et al., 2019; Lai et al., 2021).

Representative indicators are useful for the efficient and routine monitoring of various ARGs and MGEs. Class 1 integrons have been proposed as an anthropogenic pollution marker for AMR (Amos et al., 2015; Gillings et al., 2015; Zheng et al., 2019; Li et al., 2020). Integrons are bacterial genetic elements that can incorporate multiple exogenous genes, including ARGs, into gene cassettes (GCs) by the site-specific recombination function of integrase (Gillings, 2014). Because the incorporated genes can be expressed through the integron-associated promoter (Collis and Hall, 1995), integron GCs containing ARGs can spread multidrug resistance (Gillings, 2014). Five classes of mobile integrons (class 1–5) are involved in the spread of ARGs as they are frequently associated with transposons and conjugative plasmids (Gillings, 2014). The class 1 integron-integrase gene (*intI1*) was found to be prevalent in wastewater and river water (Ma et al., 2017). A strong correlation was observed between *intI1* and ARGs, such as aminoglycoside resistance genes and sulfonamide resistance genes, in aquatic environments (Gillings, 2014; Ma et al., 2017; Dong et al., 2019; Zheng et al., 2019; Agramont et al., 2020; Nguyen et al., 2021). Previous studies further investigated class 1 integron GCs in wastewater using clone library analysis or next-generation sequencing (Gatica et al., 2016; Ma et al., 2017; An et al., 2018). In wastewater, many ARGs conferring resistance to aminoglycoside, beta-lactam, and trimethoprim were often detected in class 1 integron GCs (Ma et al., 2017; An et al., 2018). As there is a large diversity in the types of GCs (Moura et al., 2009), they can be regarded as fingerprints of AMR in the environment.

In Japan, several studies have reported the presence of antimicrobial-resistant *E. coli* in rivers (Ham et al., 2012; Urase and Sato, 2016; Gomi et al., 2017; Yamashita et al., 2017; Suzuki et al., 2019; Tsutsui and Urase, 2019). Although some studies investigated specific ARGs in rivers (Nguyen et al., 2019; Liu et al., 2020), to the best of our knowledge, no comprehensive profiles of ARGs and MGEs in urban rivers have been reported in Japan. In the present study, HT-qPCR was performed to determine the prevalence of 67 ARGs and MGEs in 24 urban rivers in Tokyo and surrounding prefectures. Based on the results of HT-qPCR, the applicability of *intI1* as a surrogate marker was assessed. The arrays of class 1 integron GCs were determined by using nanopore long-read sequencing to further evaluate the association between class 1 integrons and ARGs in urban rivers.

MATERIALS AND METHODS

Sampling

From September 2019 to February 2020, river water samples (n = 30) from 24 rivers in Tokyo and its surrounding prefectures (Kanagawa, Chiba, Saitama, and Ibaraki prefectures) were collected. According to Ministry of Land, Infrastructure, Transport and Tourism in Japan, sewage coverage rates in Tokyo Metropolis, Kanagawa, Chiba, Saitama, and Ibaraki prefectures in 2019 were 99.6%, 96.9%, 75.5%, 81.9%, and 63.0%, respectively. The sampling sites are summarized in **Figure 1** and **Table 1**. They were categorized into three groups based on the rough estimation of the percentages of treated effluent from WWTPs to river flow rates (annual average) at the sampling sites (**Table 1**). Group A included sampling sites with no WWTPs located upstream. Group B

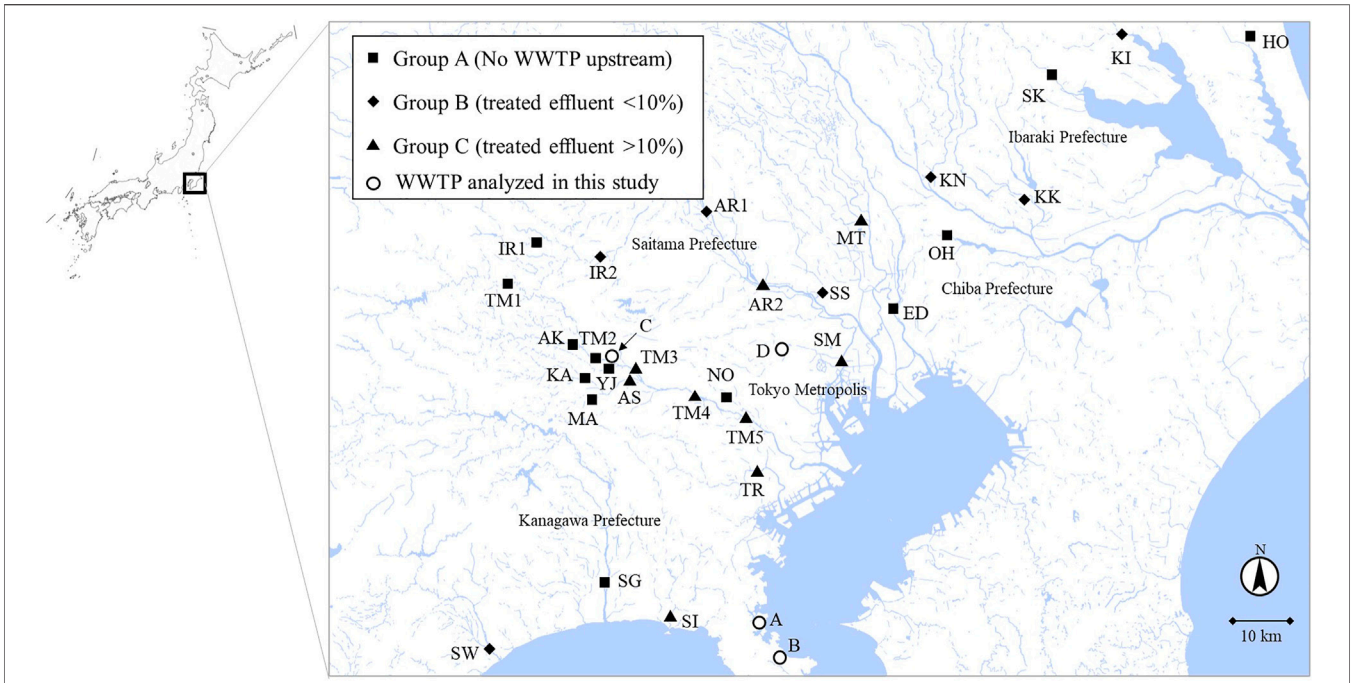

FIGURE 1 | River water sampling sites. The sampling sites were categorized into Group A (no WWTP upstream), Group B (effluent percentage <10%), and Group C (effluent percentage >10%) according to the percentages of treated effluent from WWTPs to annual average river flow rates. The map was adapted from the original data of Geospatial Information Authority of Japan (https://maps.gsi.go.jp/).

included sampling sites in which the percentages of effluent were estimated to be <10% (1–6%, average: 2%). Group C included sampling sites in which the percentages of effluent could be >10% (11–100%, average: 43%). Additional pollution sources such as the livestock industry and decentralized wastewater treatment facilities were not considered in the grouping. In Tamagawa River, Iruma River, and Arakawa River, water samples were collected from upstream (TM1, TM2, IR1, and AR1) and downstream sites (TM3, TM4, TM5, IR2, and AR2) to evaluate the impact of treated effluent discharged between two sites. Influent (Group INF) and treated effluent (Group EFF) samples (n = 8) were collected from four municipal WWTPs (WWTP A–D) in the region. All plants use a conventional activated sludge process followed by chlorine disinfection to treat domestic wastewater. The treatment capacities of WWTP A–D were 20,000 m^3/day, 140,000 m^3/day, 290,000 m^3/day, and 450,000 m^3/day, respectively.

Water Quality Analysis

The water temperature, pH, and conductivity were measured onsite using a portable Combo meter (Hanna Instruments, RI, United States). Total coliforms and *E. coli* were cultured on Chromocult coliform agar (Merck Millipore, MA, United States) at 37°C for 24 h. The water samples were filtered through 0.2-μm mixed cellulose ester membranes (DISMIC-25 AS, Advantec, Japan) and subjected to ammonium analysis. Ammonium concentrations were measured using a salicylate method with a spectrophotometer (TNT830A, DR2800-01B, Hach, CO, United States). Total cell counts (TCC) were determined using a flow cytometer (Accuri C6, BD, NJ, United States) by staining samples with SYBR Green I (Thermo Fisher Scientific, MA, United States) (Kasuga et al., 2020).

DNA Extraction

The water samples (100 ml) were filtered through 0.22-μm Isopore polycarbonate membrane filters (Merk Millipore, MA, United States) to harvest bacteria. The filters were dissolved in phenol:chloroform:isoamyl alcohol solution (25:24:1) (Nippon Gene, Japan) and treated by bead beating using a FastPrep 24 Instrument (MP Biomedicals, CA, United States). DNA extraction was performed by using the FastDNA SPIN Kit for Soil (MP Biomedicals) (Kasuga et al., 2020). DNA concentrations were measured with a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

Quantitative Polymerase Chain Reaction

Bacterial 16S rRNA genes, *intI1*, *sul1* (sulfonamide resistance gene), and *tetA* (tetracycline resistance gene) were quantified using LightCycler 480 SYBR Green I Master (Roche, Switzerland) to compare the results of HT-qPCR, and human-specific cross-assembly phage (crAssphage) (Dutilh et al., 2014; Stachler et al., 2019) was quantified using LightCycler 480 Probe Master (Roche). The primers and probes used in the analysis are listed in **Supplementary Table S1**. PCR was performed in triplicate using a LightCycler 480 II (Roche). The PCR conditions for 16S rRNA genes, *intI1*, *sul1*, and *tetA* comprised of 95°C for 5 min, followed by 45 cycles of 95°C for

TABLE 1 | Sampling sites.

River		Code	Location	Date	Group category (average percentage of treated effluent from WWTPs to river flow rates) Group A: no WWTPs Group B: treated effluent <10% Group C: treated effluent >10%
Tamagawa River		TM1	N 35.8038, E 139.1941	1 September 2019	A
		TM2	N 35.6975, E 139.3463	1 September 2019	A
		TM3	N 35.6832, E 139.4125	1 September 2019	C (37%)
		TM4	N 35.6438, E 139.5250	1 September 2019	C (38%)
		TM5	N 35.6097, E 139.6246	1 September 2019	C (41%)
Tributaries of	Akikawa River	AK	N 35.7173, E 139.3172	1 September 2019	A
Tamagawa River	Yaji River	YJ	N 35.6867, E 139.3787	1 September 2019	A
	Kitaasa River	KA	N 35.6803, E 139.3004	1 September 2019	A
	Minamiasa River	MA	N 35.6622, E 139.3108	1 September 2019	A
	Asakawa River	AS	N 35.6675, E 139.4199	1 September 2019	C (17%)
	Nogawa River	NO	N 35.6238, E 139.6073	1 September 2019	A
Iruma River		IR1	N 35.9117, E 139.1456	25 September 2019	A
		IR2	N 35.8413, E 139.3685	25 September 2019	B (6%)
Arakawa River		AR1	N 35.8923, E 139.5624	3 December 2019	B (1%)
		AR2	N 35.8004, E 139.6471	3 December 2019	C (34%)
Hokota River		HO	N 36.1519, E 140.5123	5 February 2020	A
Koise River		KI	N 36.1634, E 140.2838	5 February 2020	B (3%)
Sakura River		SK	N 36.1131, E 140.1442	5 February 2020	A
Kokai River		KK	N 35.9268, E 140.1280	5 February 2020	B (1%)
Kinugawa River		KN	N 35.9674, E 139.9508	5 February 2020	B (1%)
Ohori River		OH	N 35.8731, E 139.9848	10 February 2020	A
Edogawa River		ED	N 35.7682, E 139.8809	10 February 2020	A
Sumida River		SM	N 35.6943, E 139.7888	10 February 2020	C (60%)
Nakagawa River		NK	N 35.7488, E 139.8625	10 February 2020	B (4%)
Shinshiba River		SS	N 35.7867, E 139.7485	10 February 2020	B (1%)
Motoara River		MT	N 35.8870, E 139.8362	10 February 2020	C (11%)
Sakawa River		SW	N 35.2746, E 139.1632	19 February 2020	B (2%)
Sagami River		SG	N 35.3738, E 139.3707	19 February 2020	A
Sakai River		SI	N 35.3226, E 139.4863	19 February 2020	C (52%)
Tsurumi River		TR	N 35.5347, E 139.6347	19 February 2020	C (100%)

10 s, 55°C for 20 s, and 72°C for 20 s (detection). Melting curve analysis was performed by increasing the temperature from 65 to 95°C to check for nonspecific amplification. The PCR conditions for crAssphage comprised of 95°C for 5 min, followed by 45 cycles of 95°C for 10 s, 60°C for 50 s, and 72°C for 1 s (detection). A 10-fold dilution series (5.0×10^1 to 5.0×10^6 gene copies/μl) was prepared for standard curves using an artificially synthesized plasmid containing the target genes. The average PCR amplification efficiencies of 16S rRNA genes, *intI1*, *sul1*, *tetA*, and crAssphage were 99.3%, 93.7%, 98.5%, 92.5%, and 97.8%, respectively.

High-Throughput-Quantitative Polymerase Chain Reaction

The DNA extracts of 38 samples were sent to Resistomap Oy (Helsinki, Finland) for HT-qPCR analysis using a SmartChip Real-time PCR system (TaKaRa Bio, Japan) (Stedtfeld et al., 2018). The target genes, including ARGs, MGEs, and 16S rRNA genes, were analyzed using 68 primer sets validated by Primer Set 2.0 (Stedtfeld et al., 2018) (**Supplementary Table S2**). The PCR reaction mixture (100 nL) was prepared using 1× SmartChip TB Green Gene Expression Master Mix (TaKara Bio, Japan), nuclease-free PCR-grade water, 300 nM of each primer, and 2 ng/μL DNA template. After initial denaturation at 95°C for 10 min, PCR comprised 40 cycles of 95°C for 30 s and 60°C for 30 s, followed by melting curve analysis for each primer set (Wang et al., 2014). The threshold cycle (C_T) of 27 was selected as the detection limit (Muziasari et al., 2016; Muziasari et al., 2017). Amplicons with nonspecific melting curves and multiple peaks were excluded. The mean C_T of three technical replicates in each reaction was used to calculate the ΔC_T values (C_T of detected gene–C_T of 16S rRNA gene). The relative abundances of the detected gene to 16S rRNA gene were estimated using the ΔC_T method (Schmittgen and Livak, 2008).

Amplicon Sequencing of Class 1 Integron Gene Cassettes

Class 1 integron GCs were amplified from all DNA extracts of rivers and WWTPs samples using 5′CS (5′-GGCATCCAAGCA GCAAG-3′) and 3′CS (5′-AAGCAGACTTGACCTGA-3′), which are specific to the conserved segments of both ends of class 1 integron GCs (Levesque et al., 1995; Ma et al., 2017; An et al., 2018). The thermal conditions of the first PCR were as follows: 25 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2.5 min, with a final extension at 72°C for 10 min. TaKaRa EX Taq Hot Start version (TaKaRa Bio) was used for PCR. The first PCR

product was purified using the MinElute PCR Purification Kit (Qiagen, Germany). The second PCR was performed using the same primers with nanopore sequencing adapters (underlined): 5′CS-adp (5′-TTTCTGTTGGTGCTGATATTGCGGCATC CAAGCAGCAAG-3′) and 3′CS-adp (5′-ACTTGCCTGTCG CTCTATCTTCAAGCAGACTTGACCTGA-3′). The thermal conditions of the second PCR were as follows: 15 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2.5 min. A final extension at 72°C for 10 min was added. The second PCR products were purified using the MinElute PCR Purification Kit and checked by electrophoresis on 1.0% agarose gel at 100 V for 20 min.

For multiplex nanopore sequencing, barcoding adapters were attached to the second PCR products using PCR Barcoding Expansion Pack 1-96 (Oxford Nanopore Technologies, United Kingdom) and LongAmp Taq 2× Master Mix (New England BioLabs, MA, United States). The barcoding PCR involved the following steps: initial denaturation at 95°C for 3 min, followed by 15 cycles of 95°C for 15 s, 62°C for 15 s, and 65°C for 5 min. A final extension step at 65°C for 5 min was added. The products were purified using AMPure XP (Beckman Coulter, CA, United States). Finally, equal amounts of the barcoded PCR products were pooled and mixed with DNA CS (Ligation Sequencing Kit 1D, Oxford Nanopore Technologies), NEBNext FFPE DNA Repair Buffer (New England BioLabs), NEBNext FFPE DNA Repair Mix (New England BioLabs), Ultra II End-prep reaction buffer (New England BioLabs), and Ultra II End-prep enzyme mix (New England BioLabs). They were incubated at 20°C for 5 min and at 65°C for 5 min. After purification, adapter ligation was performed using the Ligation Sequencing Kit 1D (SQK-LSK109) (Oxford Nanopore Technologies). The prepared library was loaded onto an FLO-MIN106D flow cell (R9.4.1) (Oxford Nanopore Technologies) and sequenced on a MinION (Oxford Nanopore Technologies). Base-calling and debarcoding were then performed using Guppy (version 5.0.16) (Oxford Nanopore Technologies) with the super-accuracy mode. Reads with shorter than 500 bp of sequence length and lower than Q10 of mean quality were excluded using Filtlong (version 0.2.0) (https://github.com/rrwick/Filtlong) from further analysis. Error correction of the filtered reads was performed using Canu (version 2.1.1) (Koren et al., 2017) with default parameters. ARGs were detected using Staramr (version 0. 7.2) (https://github.com/phac-nml/staramr) with the setting of identity ≥90% and overlap ≥60%. The nucleotide sequence data are available at the DDBJ Sequence Read Archive under the accession number DRA013066.

Microbial Community Analysis

Microbial community structures were analyzed for all samples from rivers and WWTPs by amplicon sequencing of V4 regions of 16S rRNA genes. A primer set of 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) with the adapter sequence was used (Caporaso et al., 2011). Paired-end sequencing analysis was performed on the Miseq platform (Illumina, CA, United States) using MiSeq Reagent Kit v3 kit (2 × 300 bp) at Bioengineering Lab (Japan). Quality filtering was conducted using the FASTX-Toolkit (version 0.0.14) (http://hannonlab.cshl.edu/fastx_toolkit/) to extract reads, which showed a perfect match with the primer sequences. Chimeric sequences and noise were removed in DADA2 in QIIME 2.0 pipeline (version 2021.4) (Bolyen et al., 2019). Phylogenetic analysis was performed using the q2 feature-classifier plugin of QIIME 2.0 with reference sequences in Greengene (version 13_8) (DeSantis et al., 2006). Operational taxonomic units were defined by a sequence similarity threshold of 97%. The nucleotide sequence data are available at the DDBJ Sequence Read Archive under the accession number DRA013028.

Statistical Analysis

Statistical tests were performed using BellCurve for Excel (version 3.21) (Social Survey Research Information Co., Ltd., Japan). Cluster analysis based on the Ward method and principal coordinate analysis based on the Bray–Curtis dissimilarity index was performed using R (version 4.0.5) with the vegan package. Network analysis was performed based on Spearman's rank correlation coefficient between the relative abundances of the target genes and the genus-level abundances of the microbial community. Spearman's rank correlation coefficient was calculated using the psych package in R. Associations with a correlation coefficient of >0.600 ($p < 0.05$) were visualized using Gephi (version 0.9.2) (Bastian et al., 2009).

RESULTS

Comparison Between Quantitative Polymerase Chain Reaction and High-Throughput-Quantitative Polymerase Chain Reaction

The ratio of target genes against 16S rRNA gene such as *intI1*/16S rRNA gene, *sul1*/16S rRNA gene, and *tetA*/16S rRNA gene assessed using conventional qPCR was compared with the results of HT-qPCR to evaluate the quantitative performance of HT-qPCR (**Supplementary Figure S1**). Both data showed significantly positive correlations (Pearson's correlation coefficient $r = 0.876$ for *intI1*, $r = 0.827$ for *sul1*, and $r = 0.613$ for *tetA*; $p < 0.05$). However, the conventional qPCR results were 6.2, 2.6, and 5.0 times higher than those of HT-qPCR for *intI1*, *sul1*, and *tetA*, respectively.

Detection Frequency of Antimicrobial Resistance Genes and Mobile Gene Elements in Rivers

Water samples were collected from 30 sites from 24 rivers. They were categorized to Group A–C based on the percentages of treated effluent from WWTPs to river flow rates. The water quality parameters are summarized in **Supplementary Table S3**. The abundance of *E. coli* in Group C (mean ± SD = $7.5 \times 10^2 \pm 7.5 \times 10^2$ CFU/100 ml, $n = 9$) was significantly higher than that in Group A ($2.2 \times 10^2 \pm 3.1 \times 10^2$ CFU/100 ml, $n = 13$) and Group B ($1.9 \times 10^2 \pm 3.0 \times 10^2$, $n = 8$) (Steel–Dwass test, $p < 0.05$). In addition, the

TCC of Group C ($1.3 \times 10^7 \pm 1.1 \times 10^7$ cells/ml, $n = 9$) was significantly higher than that of Group A ($2.6 \times 10^6 \pm 1.7 \times 10^6$ cells/ml, $n = 13$) (Steel–Dwass test, $p < 0.05$).

Among 67 target ARGs and MGEs, 9–37, 21–37, and 23–53 genes were detected in Groups A, B, and C, respectively. There was a significant difference in the number of detected genes between Groups A and C (Steel–Dwass test, $p < 0.05$). Furthermore, 56–60 and 28–42 genes were detected in Groups INF and EFF, respectively. After the entry of WWTP effluent, the number of ARGs and MGEs increased from 21 (TM2) to 41 (TM3), from 14 (IR1) to 36 (IR2), and from 37 (AR1) to 53 (AR2) in Tamagawa River, Iruma River, and Arakawa River, respectively.

The genes that were frequently detected from >70% of river water samples are listed in **Table 2**. ARGs conferring resistance to aminoglycoside, beta-lactam, phenicol, multidrug, macrolide-lincosamide-streptogramin B (MLSB), and sulfonamide were included. For MGEs, class 1 integrons (*intI1*), transposons (*tnpA*), and insertion sequences (*ISPps* and *IS26*) were included. *qacEdelta1* (multidrug resistance), *aadA* (aminoglycoside resistance), and *sul1* were detected in >90% of the samples. These genes were also detected in most of the influent and effluent samples. The average detection frequencies of the top 20 genes were 71% (Group A), 85% (Group B), 85% (Group C), 85% (Group INF), and 80% (Group EFF).

Profiles of River Water Resistome

The relative abundances of the ARGs and MGEs in the samples are shown in **Figure 2**. There was a positive correlation between total ARGs/16S rRNA genes and total MGEs/16S rRNA genes (Pearson's correlation coefficient $r = 0.840$, $p < 0.05$) (**Supplemetary Figure S2**). The total relative abundances of the ARGs and MGEs were not significantly different among Groups A, B, and C (Steel–Dwass test, $p > 0.05$). ARGs conferring resistance to aminoglycoside, multidrug, and sulfonamide were dominant among the analyzed ARGs in the river water samples. No general relationship was noted between the relative gene abundances and group category. The total relative gene abundances of ARGs and MGEs at the downstream sites of Tamagawa River, Iruma River, and Arakawa River were 2.4–4.7 times higher than those at the upstream sites. In particular, the downstream site of Arakawa River (AR2), which was located immediately after the effluent discharge of a large WWTP in Arakawa River, showed relative gene abundances equivalent to those of the influent samples. The relative gene abundances of the influent samples were generally higher than those of the other groups, whereas wastewater treatment reduced the relative gene abundances by 30–62%. The abundances of MLSB and tetracycline resistance genes in the influent samples decreased following treatment. In contrast, the relative abundances of multidrug resistance genes as well as aminoglycoside and sulfonamide resistance genes increased or remained constant in the effluent samples. For MGEs, the samples with higher abundances of ARGs/16S rRNA genes also showed higher abundances of MGEs/16S rRNA genes. Extraordinary higher abundances of MGEs/16S rRNA genes were observed at the sampling site in Yaji River (YJ in Group A) than those at the sites in the other tributaries of Tamagawa River.

The river and wastewater samples were grouped based on the relative abundances of ARGs and MGEs by Cluster analysis, as shown in **Figure 3**. They were categorized into three major clusters: Clusters 1–3. Cluster 1 included wastewater samples. Sub-clusters 1a and 1b contained effluent and influent samples, respectively. The sampling sites of Sakai River (SI) and downstream of Arakawa River (AR2) in Group C, which were considerably affected by effluent, were also included in sub-cluster 1a. The river samples in Groups A–C were mixed in Cluster 2. Cluster 3 was separated into sub-clusters 3a, 3b, and 3c. Sub-clusters 3a and 3b included the upstream sites of Tamagawa River (TM1 and TM2 in Group A) and its tributaries, and the downstream sites of Tamagawa River (TM3–TM5 in Group C) and several other rivers were included in sub-cluster 3c.

As compositional changes in ARGs and MGEs were observed between upstream and downstream sites in Tamagawa River, the impact of effluent was also observed in cluster analysis for Iruma River (IR1: Cluster 3a, IR2: Cluster 2a) and Arakawa River (AR1: Cluster 3c, AR2: Cluster 1a). Furthermore, after the entry of WWTP effluent into the river, the emergence or increase of ARGs such as ESBL genes (bla_{VEB}, *cfxA*, and bla_{GES}), MLSB genes (*ermF* and *mphA*), and tetracycline resistance genes (*tetQ*, *tetX*, and *tetE*), as well as MGEs (*Tp614* and *Tn3*), were observed.

Microbial Community Structures

The microbial community structures were analyzed by amplicon sequencing of 16S rRNA genes. Comamonadaceae, *Flectobacillus*, and *Flavobacterium* were dominant in the river water samples. *Arcobacter*, *Acinetobacter*, and *Bacteroides* were dominant in the influent samples (**Supplementary Figure S3**). The community structures were compared using principal coordinate analysis (**Figure 4**). Axis 1 distinguished river water and wastewater samples, whereas axis 2 distinguished influent and effluent samples. The microbial community structures in Tamagawa River (upstream and downstream) and its tributaries were similar. However, the community structures in Ohori River (OH) and Hokota River (HO) in Group A were different from those in the other sites in Group A. The community structures in Sakai River (SI), Tsurumi River (TR), Sumida River (SM), and downstream of Aarakawa River (AR2) in Group C were more related to those in effluent or influent features. Although a clear transition of community structure driven by effluent discharge was observed in Iruma River (IR1 and IR2) and Arakawa River (AR1 and AR2), the difference was not clear in Tamagawa River (TM1–2 and TM3–5).

The co-occurrence of microbial taxa with ARGs and MGEs was visualized by network analysis, as shown in **Figure 5**. Four major modules (Modules 1–4) were identified. Module 1 was composed of taxa that were abundant in the intestinal flora, such as Enterobacteriaceae, Clostridiales, Aeromonadaceae, *Streptococcus*, *Bifidobacterium*, *Prevotella*, and *Faecalibacterium*. They were correlated with genes conferring resistance to tetracycline (*tetA*, *tetE*, *tetQ*, and *tetX*), aminoglycoside (*aadA*), beta-lactam (*cfxA* and bla_{VEB}), MLSB (*ermF*), and phenicol (*catB3*). Transposons (*Tp614* and *Tn3*) were also related to Module 1. Module 2 was composed of *Bacteroides*, *Arcobacter*, and *Acinetobacter*, which were correlated with ARGs such as aminoglycoside resistance genes (*aadA1*, *aadA2*, and *strB*) as well as an insertion sequence (*ISCR1*). In Module 3, class 1 integrons and genes frequently associated with class 1 integron GCs

TABLE 2 | Target genes detected by HT-qPCR from >70% of river water samples. The percentages of positive samples in each group are shown.

Antimicrobial category	Gene	HT-qPCR Assay ID	River				WWTP	
			Total (n = 30)	G (%)roup A (n = 13)	G (%)roup B (n = 8)	G (%)roup C (n = 9)	G (%)roup INF (n = 4)	G (%)roup EFF (n = 4)
M (%)DR	*qacEdelta1*	AY236	97	92	100	100	100	100
AMG	*aadA*	AY10	93	85	100	100	100	100
SUL	*sul1*	AY245	90	92	88	89	100	75
MGE	*ISPps*	AY309	87	85	88	89	100	100
AMG	*aadA2*	AY331	83	69	88	100	100	100
BLA	bla_{GES}	AY125	83	62	100	100	100	75
AMG	*strB*	AY24	80	69	75	100	100	100
PHE	*cmlA*	AY41	80	77	100	67	75	75
MLSB	*ermF*	AY46	77	46	100	100	100	100
MLSB	*ermX*	AY546	77	54	88	100	50	50
PHE	*cmlA*	AY35	77	62	75	100	100	75
MGE	*intI1*	AY293	77	77	88	67	100	75
MGE	*tnpA*	AY300	77	77	88	67	75	75
AMG	*aadA1*	AY395	73	69	88	67	100	100
MDR	*qacEdelta1*	AY218	73	77	50	89	100	75
MLSB	*ereA*	AY528	73	54	88	89	50	75
MGE	*intI1*	AY289	73	77	75	67	100	50
MGE	*tnpA*	AY299	73	77	63	78	100	75
BLA	bla_{VEB}	AY105	70	38	100	89	100	50
MDR	*emrD*	AY208	70	77	75	56	75	50
MGE	*IS26*	AY512	70	77	63	67	75	100

Note: AMG: aminoglycoside, BLA: beta-lactam, MDR: multidrug resistance, MGE: mobile gene elements, MLSB: macrolide-lincosamide-streptogramin B, PHE: phenicol, SUL: sulfonamide.

(*sul1* and *qacEdelta1*) were clustered with several taxa such as Bacteroidales and *Zoogloea*. In Module 4, aminoglycoside resistance gene (*aadA6*) and multidrug resistance gene (*pcoA*), as well as MGEs (*tnpA* and *IS26*), were associated with several taxa such as *Pseudomonas* and *Parabacteroides*.

Relationship Between Class 1 Integrons and Other Genes

Two primer sets for class 1 integrons (AY293 and AY289) were employed in HT-qPCR. These primer sets were validated for use in HT-qPCR analysis (Stedtfeld et al., 2018). AY293 was originally designed to target clinical class 1 integrons (Gillings et al., 2015), and AY289 was designed for optimizing qPCR (Muziasari et al., 2014). Both primer sets demonstrated almost consistent results (**Supplementary Figure S4**). Based on the HT-qPCR data, the genes, that exhibited a positive correlation coefficient with class 1 integrons (AY293 and AY289) were screened (Pearson's correlation coefficient $r > 0.400$, $p < 0.05$) (**Figure 6**). The relative abundances of ARGs and MGEs such as *sul1*, *qacF/H* (multidrug resistance), *dfrA27* (trimethoprim resistance), *tnpA*, and *qacEdelta1*, were highly correlated with class 1 integrons. Although most genes were correlated with *intI1* determined by both AY293 and AY289, some genes, such as *tetR* (tetracycline resistance), *dfrA1* (trimethoprim resistance), *ereA* (MLSB resistance), *IS26*, and bla_{GES} (beta-lactamase), demonstrated significant correlation with either *intI1* (AY293) or *intI1* (AY289). In addition to ARGs and MGEs, qPCR analysis also showed that *intI1* was correlated with crAssphage (**Supplementary Figure S5**, $r = 0.600$, $p < 0.05$).

Characterization of Class 1 Integron GCs in River Samples

Class 1 integron GCs were analyzed by amplicon sequencing by a MinION nanopore sequencer. Amplicon sequencing depths of 12.7–29.1 Mb with average raw read lengths of 512–1,080 bp were obtained (**Supplementary Table S4**). The lengths of the most GCs were found to range from <500 bp to 2000 bp after quality filtering (**Supplementary Figure S6**). Then, 571–1,228 contigs ranging from 500 to 4,580 bp detected in each sample were analyzed (**Supplementary Table S5**). The median contig size ranged from 593 to 1,489 bp (**Supplementary Table S5**). While 35% of the contigs did not contain ARGs, 57%, 8%, and 1% of the contigs contained one, two, three ARGs, respectively. Overall, 148 GCs carrying ARGs were detected, including 65 GCs with one ARG, 76 with two ARGs, and 7 with three ARGs (**Supplementary Table S6**). The percentages of GCs without ARGs were >65% in TM1, TM2, AK, KA, MA (upstream of Tamagawa River and its tributaries), and IR1 (upstream of Iruma River) in Group A (**Supplementary Figure S7**). Conversely, >90% of the contigs in some rivers in Groups A–C and Group INF carried ARGs (**Supplementary Figure S7**). In the upstream and downstream sites of Tamagawa River and Iruma River, the percentages of GCs with ARGs increased from 35% (TM2) to 63% (TM3) and from 22% (IR1) to 68% (IR2), respectively (**Supplementary Figure S7**). **Figure 7** shows the relative abundances of contigs of class 1 integron GCs containing ARGs. The information of representative class 1 integron GCs containing ARGs is summarized in **Table 3**. While ARGs encoding resistance to aminoglycoside, beta-lactam, and

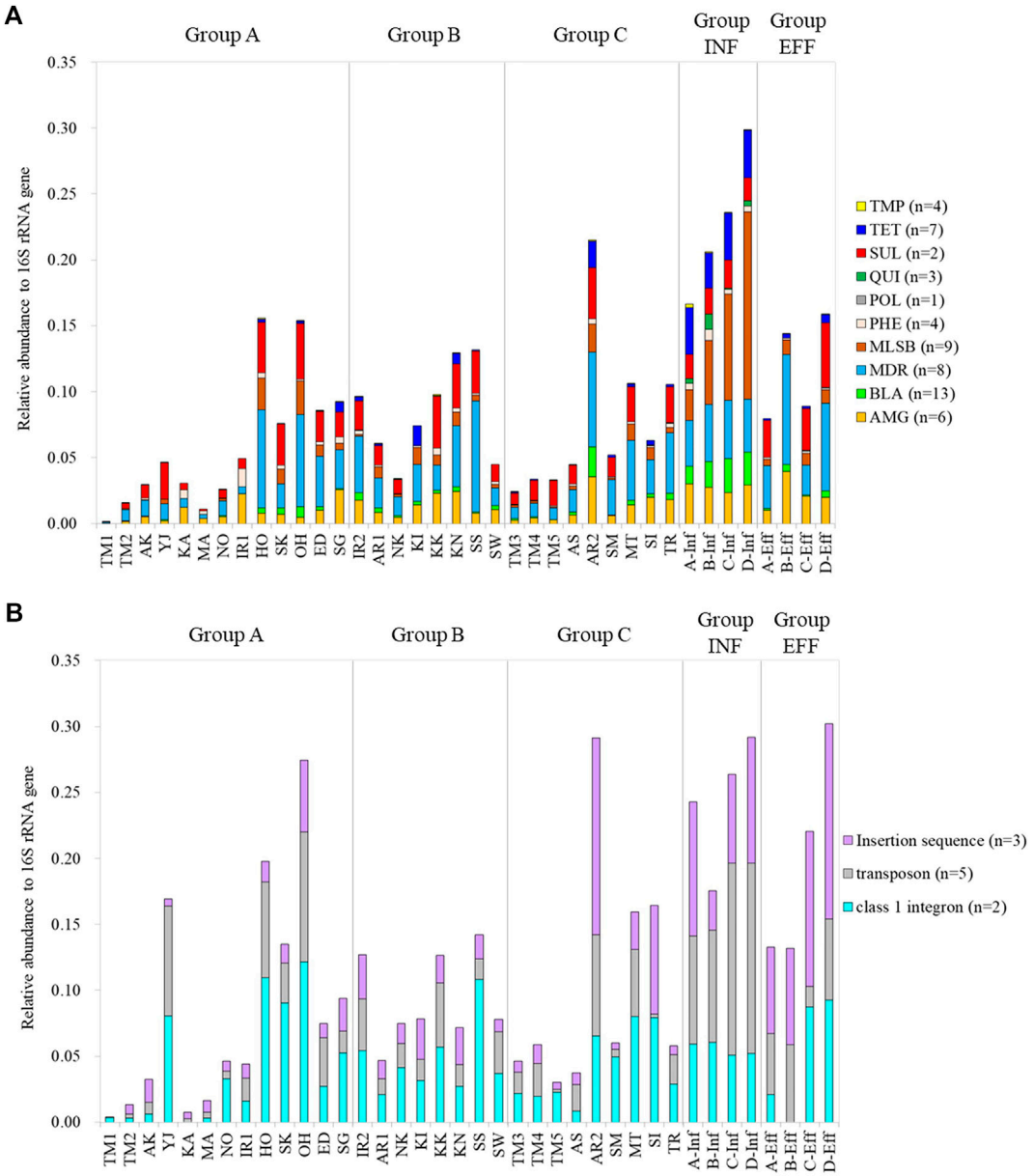

FIGURE 2 | Relative gene abundances of ARGs and MGEs based on gene category: **(A)** ARGs and **(B)** MGEs. AMG: aminoglycoside, BLA: beta-lactam, MDR: multidrug resistance, MLSB: macrolide-lincosamide-streptogramin B, PHE: phenicol, POL: polymyxin, QUI: quinolone, SUL: sulfonamide, TET: tetracycline, TMP: trimethoprim.

multidrug were dominant, specific features were also observed in different samples. $bla_{\text{GES-24}}$ (beta-lactam resistance), *aadA2*, and *qacH* (multidrug resistance) were prevalent in the GCs in most of the samples. *aac(6′)-31* and *aadA1* (both aminoglycoside resistance) were more abundant in the samples other than some rivers in Group A. *ere(A)* (MLSB resistance) was frequently detected in the river samples. At the same time, it was not dominant in Groups INF and EFF. The GC containing two ARGs (*aadA2-qacH*) was found in rivers and effluent, but it was rare in the influent samples. Tandem array of *aac(6′)-IIa-*$bla_{\text{OXA-21}}$*-catB3* (aminoglycoside resistance, beta-lactam resistance, and phenicol resistance) was only detected in influent samples of WWTP A and B.

DISCUSSION

AMR surveillance in wastewater and aquatic environments is required to fill the gap of One Health. The present study was the first to employ HT-qPCR to determine the prevalence of ARGs and MGEs in urban rivers in Japan. Among the genes detected from >70% of the river water samples, *sul1* and *qacEdelta1* were

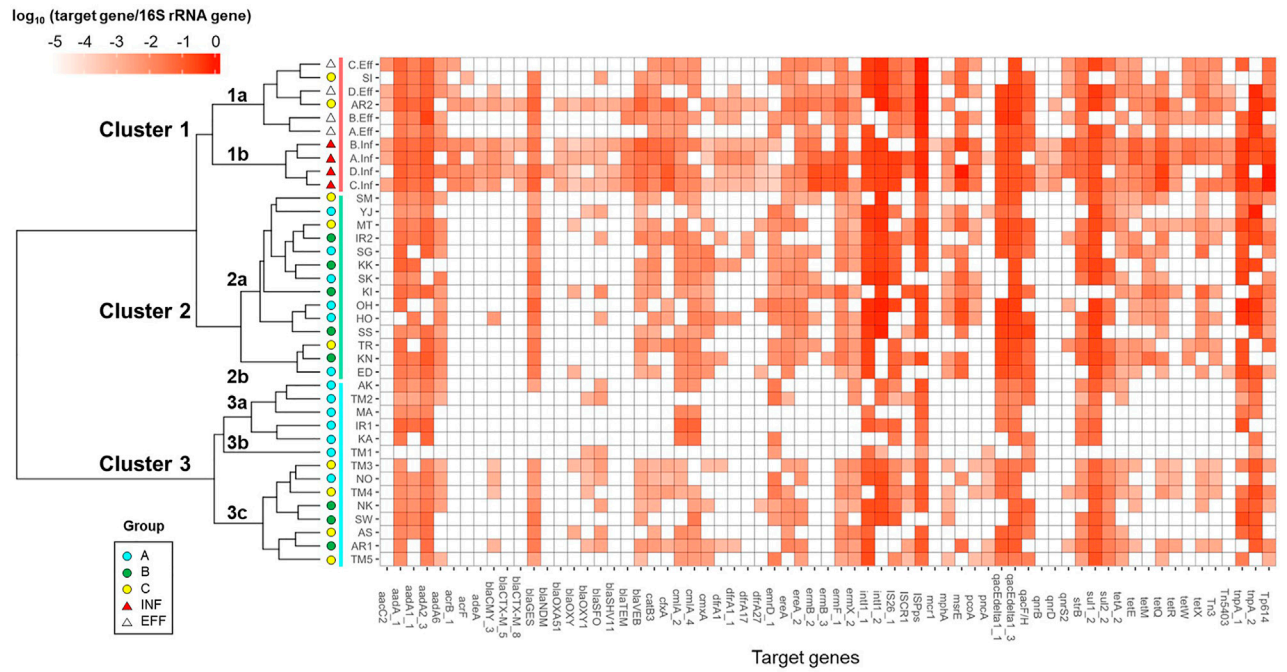

FIGURE 3 | Cluster analysis of the ARG and MGE profiles.

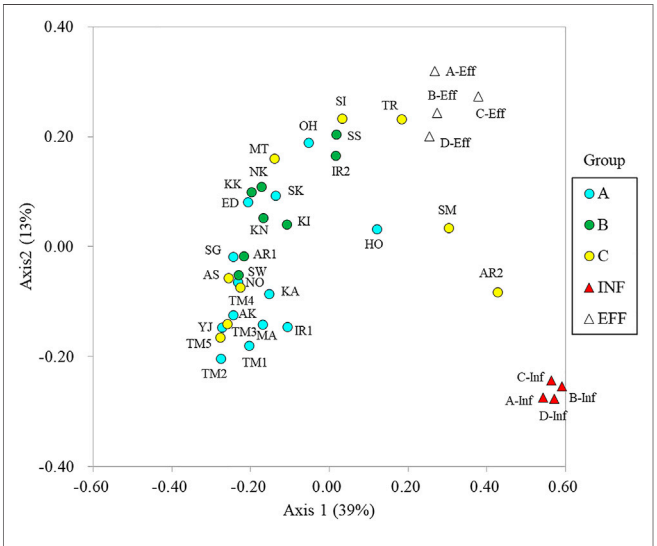

FIGURE 4 | Principal coordinate analysis of the microbial community structures.

also reported as "core wastewater ARGs and MGEs", which were present in all influent and effluent samples of 12 WWTPs in Europe (Pärnänen et al., 2019). Moreover, ARGs and MGEs, such as *aadA*, *strB*, *ermF*, *intI1*, *tnpA*, and *ISPps*, were also categorized as "persistent ARGs and MGEs", which remained in >90% of the effluent samples (Pärnänen et al., 2019). As these genes were detected in most of the influent and effluent samples in the present study, the core/persistent ARGs and MGEs associated with wastewater could be prevalent in aquatic environments in Japan. Because *intI1* and *ISPps* were even detected at upstream sampling sites of Tamagawa River (TM1) and Iruma River (IR1) with lower human activity impact, these genes could possibly serve as sensitive markers of anthropogenic pollution. Further study is necessary to identify the prevalence and sources of these genes in upstream area.

Effluent from WWTPs affect the resistome in recipient rivers (Rodriguez-Mozaz et al., 2015; Cacace et al., 2019; Khan et al., 2019; Pärnänen et al., 2019; Lai et al., 2021). The significantly positive correlation between *intI1* and crAssphage in the studied rivers suggests that class 1 integrons and the associated ARGs and MGEs could be originated from human feces (Chen et al., 2019a; Karkman et al., 2019; Agramont et al., 2020; Nguyen et al., 2021). Clear shifts in the relative abundances and profiles were observed after the entry of treated effluent in Tamagawa River, Iruma River, and Arakawa River, which is consistent with the qPCR results in Tamagawa River (Liu et al., 2020). Drastic changes in the microbial community were also observed from upstream to downstream in Iruma River and Arakawa River, suggesting that the microbial community in the effluent could mostly determine the resistome in these rivers. The resistomes in downstream of Arakawa River (AR2) and Sakai River (SI), which had higher percentages of effluent in river flow (AR2: 34%, SI: 52%), were clustered with the effluent resistome. The similarity in resistome profiles between effluent and recipient rivers in urban areas was also demonstrated using HT-qPCR (Huang et al., 2019; Khan et al., 2019). Conversely, the classification of sampling sites based on the ratio of effluent to river flow rates (Groups A–C) was not always associated with the relative abundance and profiles of

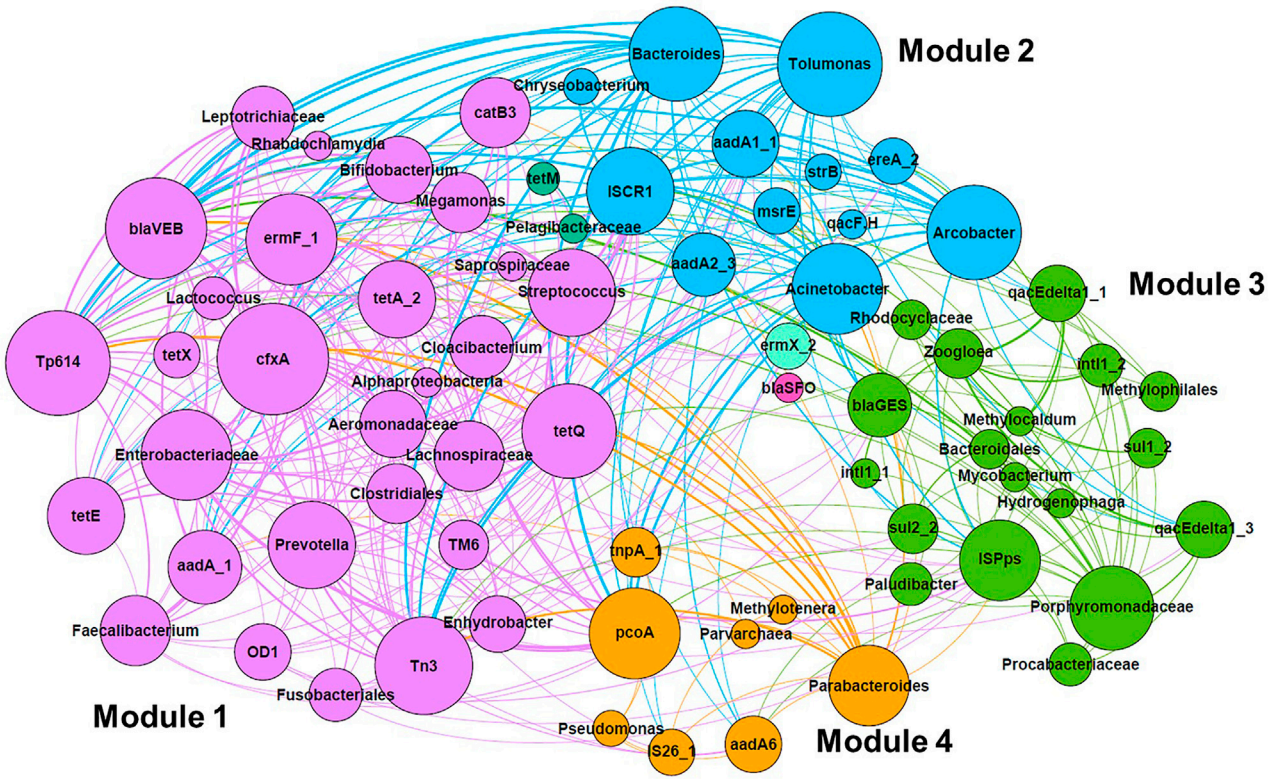

FIGURE 5 | Network analysis of co-occurrence pattern among microbial taxa, ARGs, and MGE based on Spearman's rank correlation coefficient. Associations with Spearman's rank correlation coefficient of >0.600 ($p < 0.05$) were selected. Different modules are shown in different colors. The size of nodes represents the number of associations, and the width of lines is proportional to Spearman's rank correlation coefficient.

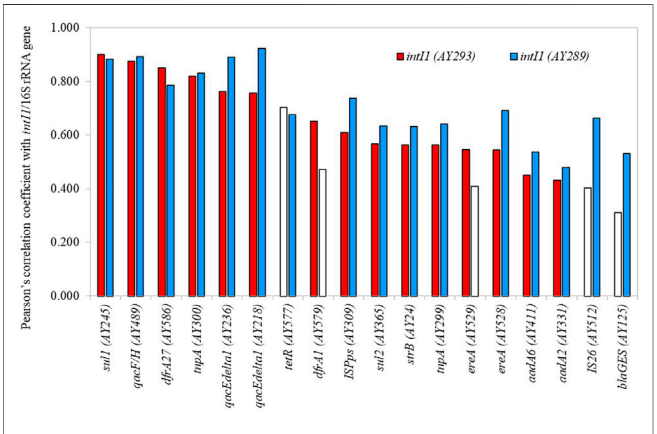

FIGURE 6 | Genes with positive correlation coefficient with class 1 integron-integrase genes. Open bars indicate $p > 0.05$.

ARGs and MGEs. Even in Group A, exceptionally higher abundances of ARGs and MGEs were observed in Yaji River (YJ), Hokota River (HO), Sakura River (SK), and Ohori River (OH). As categorization of the sites in this study was simple, the actual contribution of effluent at the sampling occasion could be different from the estimated percentages. The performance of WWTPs in removing ARB and ARGs can fluctuate (Harnisz et al., 2020), and additional pollution sources such as livestock industry and decentralized treatment facilities in rural areas should be considered, especially in rivers in rural area. More intensive sampling considering watershed characteristics is necessary to demonstrate the specific resistome profiles in these rivers.

It is important to know the hosts of ARGs and MGEs to determine the health risks. While single-cell sorting (Chijiiwa et al., 2020; Wang et al., 2020); emulsion, paired isolation, and concatenation PCR (Hultman et al., 2018); and high-throughput chromosome conformation capture (Stalder et al., 2019) can more directly identify the hosts of ARGs and MGEs, co-occurrence of specific taxa and these genes is also informative to explore potential hosts (Quintela-Baluja et al., 2019; Han et al., 2020; Yu et al., 2021). Interestingly, popular taxa related to some ESKAPE (Enterobacteriaceae, *Acinetobacter*, Aeromonadaceae, and *Pseudomonas*) were screened by network analysis. The associations observed in Enterobacteriaceae and Aeromonadaceae (*tetA*, *aadA*, and *catB3*), *Acinetobacter* (*aadA1*, *aadA2*, and *strB*), and *Pseudomonas* (*aadA6*) were endorsed by the comprehensive antibiotic resistance database (Alcock et al., 2020). Although network analysis showed that class 1 integrons and the related genes (*intI1*, *sul1*, and *qacEdelta1*) were associated with miscellaneous taxa, the whole genome database revealed that class 1 integrons are mostly carried by

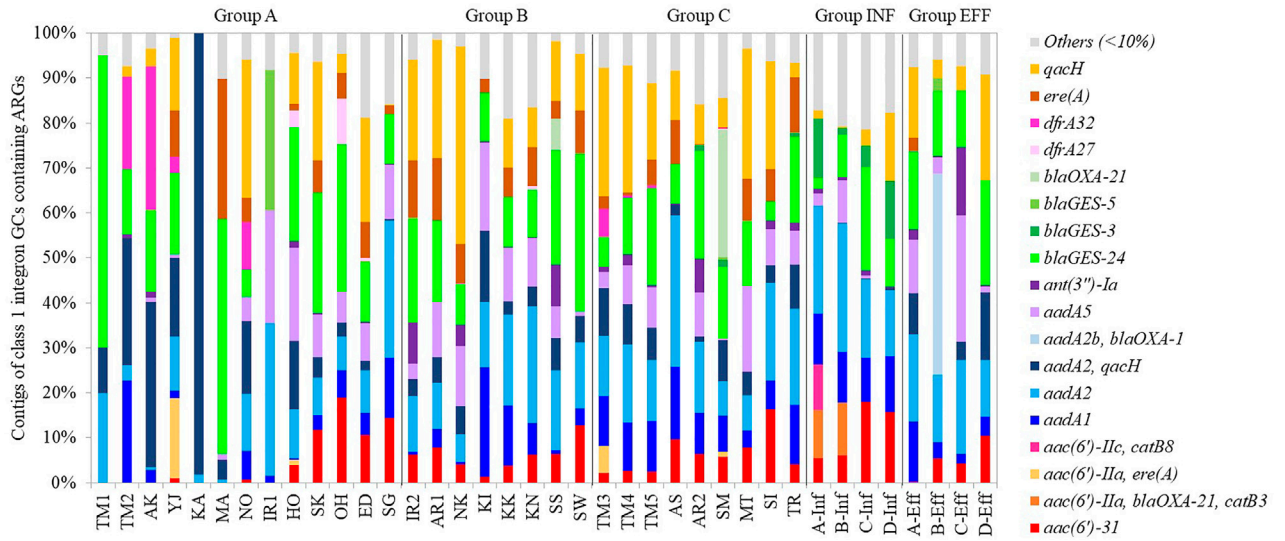

FIGURE 7 | Composition of contigs of class 1 integron GCs containing ARGs.

TABLE 3 | Representative ARGs present in class 1 integron GCs.

ARGs in GC (Phenotype)		ResFinder search		
		Identity (%) (average)	Overlap (%) (average)	Accession number of References sequence (length)
bla$_{GES-24}$ (BLA)		99.6	99.9	AB914515 (864 bp)
aadA2 (AMG)		99.5	99.2	NC010870 (819 bp)
qacH (MDR)		99.4	62.7	FJ172381 (945 bp)
aadA2, *qacH* (AMG, MDR)	*aadA2*	99.8	100	JQ364967 (792 bp)
	qacH	99.3	62.8	FJ172381 (945 bp)
aadA5 (AMG)		99.6	99.8	AF137361 (789 bp))
aadA1 (AMG)		99.7	99.7	FJ591054 (792 bp)
aac(6')-31 (AMG)		99.6	99.7	AM283489 (519 bp)
ere(A) (MLSB)		99.6	99.9	DQ157752 (1,221 bp)
dfrA32 (TMP)		99.5	100	GU067642 (474 bp)
ant(3")-Ia (AMG)		99.3	91.1	X02,340 (972 bp)
aadA2b, *bla*$_{OXA-1}$ (AMG, BLA)	*aadA2b*	99.7	100	D43625 (780 bp)
	bla$_{OXA-1}$	99.8	100	HQ170510 (831 bp)
bla$_{GES-5}$ (BLA)		99.5	98.0	DQ236171 (864 bp)
bla$_{GES-3}$ (BLA)		99.5	100	AB113580 (864 bp)
bla$_{OXA-21}$ (BLA)		99.5	99.5	AB626885 (828 bp)
aac(6')-IIa, *ere(A)* (AMG, MLSB)	*aac(6')-IIa*	99.0	99.7	M29695 (555 bp)
	ere(A)	99.4	100	DQ157752 (1,221 bp)
aac(6')-IIa, *bla*$_{OXA-21}$, *catB3* (AMG, BLA, PHE)	*aac(6')-IIa*	99.1	100	M29695 (555 bp)
	bla$_{OXA-21}$	99.6	100	AB626885 (828 bp)
	catB3	99.9	100	U13880 (633 bp)
dfrA27		99.4	100	FJ459817 (474 bp)
aac(6')-IIc, *catB8* (AMG, PHE)	*aac(6')-IIc*	99.5	100	NC012555 (582 bp)
	catB8	99.7	100	AF227506 (633 bp)

AMG: aminoglycoside, BLA: beta-lactam, MDR: multidrug resistance, MLSB: macrolide-lincosamide-streptogramin B, PHE: phenicol, TMP: trimethoprim.

three families: Enterobacteriaceae, Pseudomonadaceae, and Moraxellaceae in Gammaproteobacteria (Zhang et al., 2018). Spearman's rank correlation coefficients between class 1 integrons and these taxa, such as Enterobacteriaceae, *Pseudomonas*, and *Acinetobacter*, were only 0.108–0.363, which suggests that other methods should be used to validate the result of network analysis.

Many studies reported that class 1 integrons are a promising indicator of anthropogenic pollution of ARGs (Gillings et al., 2015; Pärnänen et al., 2019; Zheng et al., 2019). The high prevalence and correlation with other ARGs and MGEs in the river samples suggests that *intI1* is a representative target in rivers. Some ARGs and MGEs that showed stronger correlation

with *intI1* could be genetically associated with class 1 integron GCs. For instance, *sul1* and *qacEdetlta1* are typically fused genes in the 3′ conserved segment of class 1 integron GCs (Gillings, 2014). The other selected ARGs, such as *dfrA27* (Wei et al., 2008), *dfrA1* (Zhao et al., 2020), *strB* (Le-Vo et al., 2019), *ereA* (Malek et al., 2015), *aadA6* (Mirahsani et al., 2016), *aadA2* (Ahmed and Shimamoto, 2004), and bla_{GES} (Maehana et al., 2021), were also detected from class 1 integron GCs of Gram-negative bacteria. For MGEs, class 1 integron-*dfrA5*-IS26 element was found in *E. coli* (Dawes et al., 2010), and transposition genes such as *tnpA* were associated with class 1 integrons (Ghaly et al., 2017). These reports were consistent with the results of HT-qPCR, demonstrating that HT-qPCR can dissect the relationship between class 1 integrons and other ARGs/MGEs in aquatic environments.

ARGs conferring resistance to aminoglycoside, beta-lactam, multidrug, MLSB, phenicol, and trimethoprim were frequently acquired in class 1 integron GCs in urban rivers and wastewater samples analyzed in the present study, which is consistent with previous reports (Ma et al., 2017; An et al., 2018; Gatica et al., 2019). The acquisition of specific ARG types by class 1 integrons was demonstrated by whole-genome database analysis (Zhang et al., 2018). Although various GC types were detected in the present study, major GCs were not found in other studies analyzing class 1 integron GCs in wastewater by amplicon sequencing using the same primer set (Ma et al., 2017; An et al., 2018). As the composition of class 1 GCs was found to be different in river water, sewage, feces, and livestock wastewater (Ma et al., 2017), the diversity of class 1 integron GCs in aquatic environments could likely depend on geographical and socioeconomic settings.

The percentage of GCs containing ARGs was lower in upstream rivers in Group A than that in the other groups. Moreover, the composition of GCs containing ARGs was not necessarily identical among different rivers. Thus, GC profiles in aquatic environments may indicate AMR fingerprints in each watershed. Quantitative monitoring of class1 integrons and qualitative features of its GCs can be integrated for efficient resistome monitoring in aquatic environments. For example, *aac (6′)-31* and *aadA1* in GCs were more abundant in the samples other than upstream rivers, indicating the impact of wastewater effluent. *aadA2-qacH* was present in rivers and effluent but not in influent, suggesting that class 1 integron GCs containing *aadA2-qacH* could be enriched in wastewater treatment. While *ere(A)* was frequently detected in rivers and wastewater samples, *ere(A)* acquired by class 1 integrons was more abundant in rivers than influent and effluent samples. This gap suggests that the genetic context of *ere(A)* could be different in rivers and wastewater.

Common GC types containing bla_{GES-24}, *aadA2*, or *qacH* were observed in river (Groups A–C) and wastewater (Groups INF and EFF) samples. HT-qPCR also revealed a significantly stronger correlation between class 1 integrons and ARGs such as bla_{GES}, *aadA2*, and *qacF/H*. Class 1 integron GCs containing *aadA2* or *qacH* were previously reported in a riverine system (Amos et al., 2018). The prevalence of bla_{GES} in class 1 integron GCs in wastewater effluent in Cyprus and Israel was demonstrated, while bla_{OXA} associated with class 1 integron GCs was dominant in effluent samples in other European countries (Gatica et al., 2016). Although the GCs containing *aadA2* or *qacH* were present in the database of integron GCs (INTEGRAL) (Moura et al., 2009), the GC containing bla_{GES-24} has not yet been registered in the database. bla_{GES-24} encodes a variant of GES, which is class A beta-lactamase. GES-4, -5, -6, -14, which are characterized by a substitution of Gly170Ser, show carbapenem hydrolysis activity (Bontron et al., 2016). As GES-24 has the same substitution, it has potential to hydrolyze carbapenem. bla_{GES-24} was carried by bacteria, such as *Aeromonas hydrophila*, *Citrobacter freundii*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (Alcock et al., 2020). Four tandem copies of bla_{GES-24} were detected from class 1 integron GCs on the plasmids of *A. hydrophila*, which was isolated from clinical wastewater in Japan (Maehana et al., 2021). Although *Aeromonas* spp. in aquatic environments could be the key host of bla_{GES-24}, it is also possible that a wide range of bacteria can carry class 1 integron GCs containing bla_{GES-24}. This ARG was prevalent in rivers in urban areas and wastewater as well as in upstream rivers. More consideration should be given to the dissemination and evolution of bla_{GES} variants associated with class 1 integrons in aquatic environments.

As revealed using conventional qPCR, the relative abundances of the tested genes were 2.six to six times higher than those revealed using HT-qPCR. As the same thermal conditions were employed for all target genes in HT-qPCR, the amplification efficiency may not always be optimized for each gene (Waseem et al., 2019). Although the results of both methods were highly correlated, the quantified values should be carefully interpreted when they are compared with other studies. Moreover, a hydrolysis probe-based HT-qPCR protocol should be compared for more specific quantification (Khan et al., 2019). The technical limitations of class 1 integron GCs analysis include primer coverage of class 1 integron GCs and the accuracy of long-read sequencing. The primer set for class 1 integron GCs (5′CS and 3′CS) that were used in this study was also used in other studies on amplicon sequencing of class 1 integron GCs in aquatic environments (Ma et al., 2017; An et al., 2018). However, the coverage of this primer set was 23.6% of 2,153 integrons in the database (Zhang et al., 2018), which suggests a greater diversity of class 1 integron GCs in the environment. Novel ARGs have been discovered from class 1 integron GCs such as *sul4* (sulfonamide resistance) (Razavi et al., 2017) and *gar* (garosamine-specific aminoglycoside resistance) (Bohm et al., 2020). Therefore, a comprehensive approach such as metagenomic analysis and the amplicon sequencing approach are necessary to reveal the whole picture of class 1 integron GCs in aquatic environments. As co-occurrence of class 2 and 3 integrons with specific ARGs have been reported (Lai et al., 2021), the different integrons could contribute to the spread of specific ARGs in aquatic environments (Gillings, 2014; Deng et al., 2015; An et al., 2018). Regarding long-read sequencing, nanopore technology can circumvent the assembly errors of short reads, while the sequencing error rates of long-read sequencing are generally higher (Weirather et al., 2017). Although error correction of raw reads was applied and ARGs with high identity and overlap values in the polished

reads were explored in this study, the validation by short-read sequencing with higher accuracy could compensate for the limitations of long-read sequencing.

DATA AVAILABILITY STATEMENT

The nucleotide sequence data are available at the DDBJ Sequence Read Archive under the accession numbers DRA013066 and DRA013028.

AUTHOR CONTRIBUTIONS

IK and KN conducted sampling. IK, KN, and MS provided experimental data. IK, KN, and MS analyzed data. IK and KN wrote the main text. All authors corrected and approved the final text.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2022.825372/full#supplementary-material

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Antibiotic Resistance in Black Sea Microbial Communities

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Background: Antibiotic resistance genes (ARGs) are considered as pollutants and are found in natural and anthropogenically impacted environments. Distribution of ARGs in marine environment poses a threat to human health turning the water body into a pool for the ARGs' transmission.

Objectives: A large-scale study of antibiotic resistance in microbial communities has been performed in the Black Sea, both in the coastal and offshore regions.

Methods: The quantitative distribution of the genes responsible for the inactivation of the beta-lactam (bla_{CMY}, bla_{SHV}), vancomycin (*vanA*, *vanB*), macrolides (*ermB*) and colistin (*mcr-1*) was assessed with real-time quantitative PCR. Concentrations of the antibiotics belonging to the classes of beta-lactam/cephalosporin/carbapenem, macrolides and glycopeptides were determined by LC-ESI-QTOF-MS.

Results: The present study revealed the distribution of antibiotic resistance genes targeting the response to all antibiotics included in our analysis at various locations across the Black Sea. According to the ARGs copy number normalized to the 16S rRNA, *vanB* ($2 \times 10^{-1} \pm 1 \times 10^{-1}$) and bla_{SHV} ($4 \times 10^{-2} \pm 1 \times 10^{-2}$) were the most numerous genes, followed by bla_{CMY} ($1 \times 10^{-2} \pm 3 \times 10^{-3}$) and *mcr-1* ($3 \times 10^{-2} \pm 2 \times 10^{-2}$). The less abundant gene was *ermB* ($1 \times 10^{-3} \pm 5 \times 10^{-4}$) and *vanA* ($1 \times 10^{-5} \pm 5 \times 10^{-4}$). The *mcr-1*, bla_{CMY} and bla_{SHV} had moderate positive correlation with markers of ruminant faecal pollution. The concentration of antibiotics in seawater was below the detection limit. The abundance of all ARGs included in the study was significantly higher (p-value<0.05) within the northwest coastal area when compared to the offshore stations. The results clearly indicate an alarming antibiotic resistance problem in the region and call for a regular monitoring of ARGs abundance in the Black Sea and its major freshwater tributaries.

Keywords: ARGs, beta-lactam, macrolide, colistin, vancomycin, qPCR, Black Sea

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INTRODUCTION

The Black Sea is a large semi-closed European sea with an area of 436,400 km^2. It is virtually isolated from other seas and ultimately drains into the Mediterranean Sea via the narrow Bosphorus channel. The drainage basin of the Black Sea is ca. 2,000,000 km^2 and covers regions with intensive agriculture and industrial activities. Riverine input, especially of the major rivers Danube, Dnieper and Dniester,

is a considerable source of the Black Sea pollution (Shimkus and Trimonis, 1974; Alygizakis et al., 2019; Diamanti et al., 2020). Ports and coastal cities contribute to the pollution as well; persistent organic pollutants, metals and pharmaceuticals were frequently found in the Black Sea water, sediment and biota compartments (Bakan and Ariman, 2004; Ozkoc et al., 2007; Stoichev et al., 2007). Considering the overall pollution pattern, contamination of the Black Sea with antibiotic resistance genes (ARG) can be expected (Kümmerer, 2009; Kraemer et al., 2019; Zheng et al., 2021).

Antibiotic resistance is one of the biggest threats to global health, emerging as one of the major causes of death in the coming decades (https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance). ARGs have burst outside the clinic environment long ago (Kraemer et al., 2019), and numerous studies report distribution of ARGs in different water environments (Tang et al., 2015; Szekeres et al., 2018; Griffin et al., 2019) etc. Dissemination of ARGs in the natural environment implies a direct hazard to human health due to the possibility of ARGs and antibiotic resistance bacteria (ARBs) intake with food or by direct contact (Amarasiri et al., 2020; Zheng et al., 2021).

The Black Sea region has already been in focus in terms of the antibiotic resistance., however, most of the studies imply antibiotic resistant testing or ARG profiling of bacteria isolated from fish farms, marine biota, seawater and freshwater from the Danube river (Kayis et al., 2009; Kittinger et al., 2016a; Kittinger et al., 2016b; Türe and Alp, 2016; Terzi and Isler, 2019; Kayis et al., 2021). ARGs distribution in the Black Sea water was studied by Sabatino et al. (2020) in the eastern and western part of the Black Sea. bla_{CTXM}, *sul2*, and *tetA* were the most abundant ARGs, being present in at least 43% of the samples, while *ermB* and *qnrS* were not detected. A wide-scope chemical screening together with analysis of ARGs was carried out in the effluents of wastewater treatment plants (WWTPs) in the Danube river basin (Alygizakis et al., 2019). According to the study, *aph (III)a*, bla_{OXA}, *ermB*, *ermF*, *sul1* and *tetM* had widespread occurrence, while bla_{SHV}, *mecA*, *qnrS*, *tetB* and *vanA* were detected sporadically. In general, there is a lack of data on the ARGs distribution in the Black Sea environment.

Antibiotics are considered as important driving factors for ARG proliferation, as they pose selective pressure on microbes and enrich ARGs in estuarine and coastal environments (Zheng et al., 2021). Moreover, antibiotics may encourage the horizontal gene transfer (HGT), which facilitates the dissemination of ARGs (Szczepanowski et al., 2009). Faecal pollution is another factor of ARGs distribution (Khan et al., 2019). According to the publicly available metagenomic data, the presence of ARGs can simply result from faecal contamination with resistant bacteria (Karkman et al., 2019). Poorly treated or untreated sewage discharge containing faecal bacteria may fuel the pool of ARGs in anthropogenically impacted environments, especially in low-income countries.

To facilitate an overview of ARGs distribution in the Black Sea environment, 12 water samples were collected in the eastern, central and northwestern parts of the Black Sea in the course of the EU/UNDP EMBLAS-Plus project (https://emblasproject.org/).

TABLE 1 | Coordinates and regions of the sampling sites.

Station	Longitude	Latitude	Region
1A	30,2518	45,3386	Northwest shelf
2A	31,0021	46,3333	Northwest shelf
2	31,2350	45,2159	Northwest shelf
3	31,3041	44,2162	Northwest shelf
4	31,5632	44,1216	Open waters
5	31,8195	43,3998	Open waters
7	34,7733	43,3697	Open waters
8	36,0586	43,5384	Open waters
9	39,8810	42,2357	Eastern shelf
10	40,3369	42,1137	Eastern shelf
11	40,8367	41,9331	Eastern shelf
12	41,2196	41,7836	Eastern shelf

The research aimed at studying the spatial distribution of ARGs and associated factors contributing to their persistence in the Black Sea. The objectives of this study were to estimate whether: 1) ARGs targeting the antibiotics of wide usage and last-resort antibiotics are present in the Black Sea; 2) there is a difference in ARGs number in the open water and anthropogenically impacted shelf zone of the Black Sea; 3) the number of ARGs is associated with concentration of antibiotics in the water or with the markers of the faecal contamination.

MATERIALS AND METHODS

Sampling

The samples were collected during the Joint Black Sea Survey on the RV "Mare Nigrum" carried out within the EMBLAS-Plus project in July and August 2019. Altogether 12 sampling stations were investigated on a transect from Constanta (Romania) to Odessa (Ukraine) and Batumi (Georgia) (**Table 1**; **Figure 1**). 2 L of seawater were taken from the surface and passed through the Isopore PC 0.22 μm filters (Millipore, United States) using Microsart e. jet vacuum pump (Sartorius Stedim, Germany). Filters with bacterial biomass on them were immediately frozen at −180°C after filtration.

DNA Extraction

DNA was extracted from seawater using DNAeasy PowerSoil® Kit (Qiagen, Germany) according to the manufacturer's instructions with the adaptations to the material used. Filters were cut with sterile scissors prior to the lysis step. The DNA quantity and quality were estimated using NanoDrop Spectrophotometer 2000 (Thermo Fisher Scientific, United States). All samples had sufficient DNA concentration (with most above 30 ng/μL) and A260/280 ratio ~1.8.

Quantitative Real-Time PCR

ARGs that encode resistance to beta-lactams/cephalosporins (bla_{CMY}, bla_{SHV}), vancomycin (*vanA* and *vanB*), macrolide-lincosamide (*ermB*) and colistin (*mcr-1*) were enumerated by RT qPCR. Primers CMY_fwd/CMY_rev, SHV_fwd/SHV_rev (Roschanski et al., 2014), vanAF/vanAR, vanBF/vanBR (Mirzaei et al., 2015), erm (B)-91f/erm (B)-454r and mcr1FP/

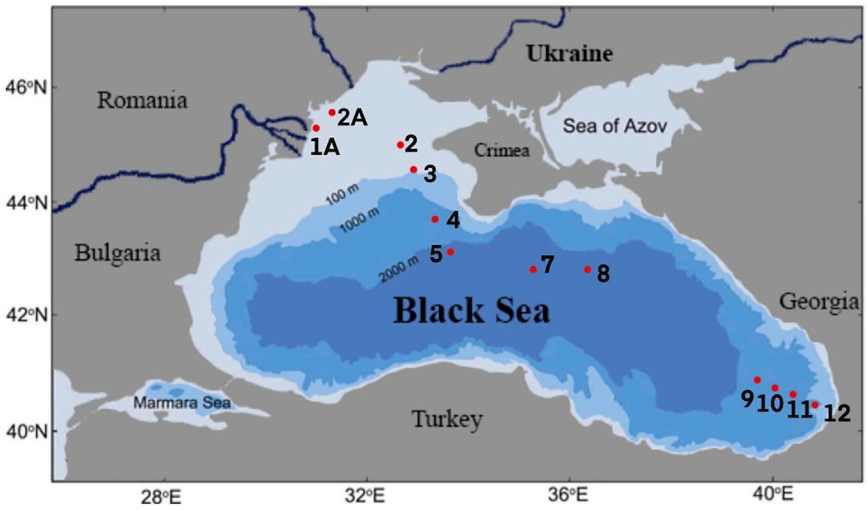

FIGURE 1 | Locations of the sampling sites.

mcr1RP (Hembach et al., 2017) were used correspondingly (**Supplementary Table S1**).

The list of targeted genes was based on the antibiotic resistance prevalence data from the European Resistance Surveillance Network (EARS-NET, 2019) and Joint Danube Survey 3 reports (Zarfel et al., 2015).

Gut host-specific bacteria of the *Bacteroidetes* phylum were used as markers of faecal pollution. To quantify human *Bacteroidetes* primers HF183 and BacR287 were used (Epa and of Science, 2019). BacR/BacF (Reischer et al., 2006) and Pig-2-Bac41F/Pig-2-Bac163Rm (Mieszkin et al., 2009) primers were used to quantify ruminant and pig *Bacteroidetes* respectively. Primers S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21 were used to estimate the quantity of the 16S rRNA gene (Klindworth et al., 2013). Primer sequences and annealing temperatures are presented in **Supplementary Table S1**.

RT qPCR was set using the QuantiFast SYBR Green PCR Kit (Qiagen, Germany) according to the standard manufacturer's procedures. The standards were created for each of the targeted genes using the DNA from the samples. Each 25 µL PCR reaction contained the following components: 2x QuantiFast SYBR Green PCR Master Mix—12.5 µL, Primer Reverse −2.5 µL, Primer Forward −2.5 µL, template DNA −1 µL, RNase-free water −6.5 µL. The initial concentration of DNA in each reaction was 5 ng/µL. The thermal conditions were different for all primers and are presented in **Supplementary Table S1**. Negative controls contained no DNA template.

The standards for each gene analyzed were purified with QIAquick PCR Purification Kit (Qiagen, Germany) and 10-fold serially diluted ranging from 1.0×10^3 to 1.0×10^7 to be used for standard curve generation in quantitative PCR. The threshold value (Ct) was used to determine the copy numbers of targeted genes in the environmental subsamples based on the standard curves.

Melting curve analysis was performed at the end of the amplification cycles in order to assess primer specificity and to ensure proper amplification of all target fragments. The PCR reactions were performed on the Qiagen Rotor-Gene Q (Qiagen, Germany). All reactions were performed in triplicate.

Concentrations of Antibiotics

Concentrations of the antibiotics belonging to the classes of β-lactam/cephalosporin/carbapenem (Amoxicillin, Ampicillin, Cefaclor, Cefadroxil, Cefalonium, Cefazolin, Cefoperazone, Cefquinome, Ceftazidime, Ceftiofur, Dicloxacillin, Meropenem, Oxacillin, Penicillin V), macrolides (Azithromycin, Clarithromycin, Erythromycin, Rifaximin, Roxithromycin, Tilmicosin, Tylosin), glycopeptides (Vancomycin), were determined by liquid chromatography-electrospray ionization-quadrupole time of flight-mass spectrometry (LC-ESI-QTOF-MS). All seawater samples were extracted on board using HORIZON SPE-DEX 4790 device (United States). The samples were spiked with internal standards for quality assurance and quality control purposes and were concentrated on Atlantic HLB-M Disk with 47 mm disk holder according to an automated extraction programme (Alygizakis et al., 2019). The extracts were evaporated using nitrogen and reconstituted in methanol:water (50:50 v/v) to the final volume of 500 µL achieving concentration factor of 4,000. The samples were filtered through 0.2 µm RC syringe filter before the LC-ESI-QTOF-MS analysis. The instrumental setup and gradient programme can be found elsewhere (Gago-Ferrero et al., 2020). The method was previously validated for linearity, accuracy using recovery experiments, repeatability and sensitivity (Gago-Ferrero et al., 2020).

Statistical Analysis

The obtained data was analyzed in the RStudio (version 3.6.0, packages: vegan, tidyverse, ade4, geosphere, corrplot, ggplot2). The data on the ARG abundance was tested for the normality by the Shapiro-Wilk normality test, which revealed non-normal distribution (p-value <0.05). Wilcoxon rank sum test was

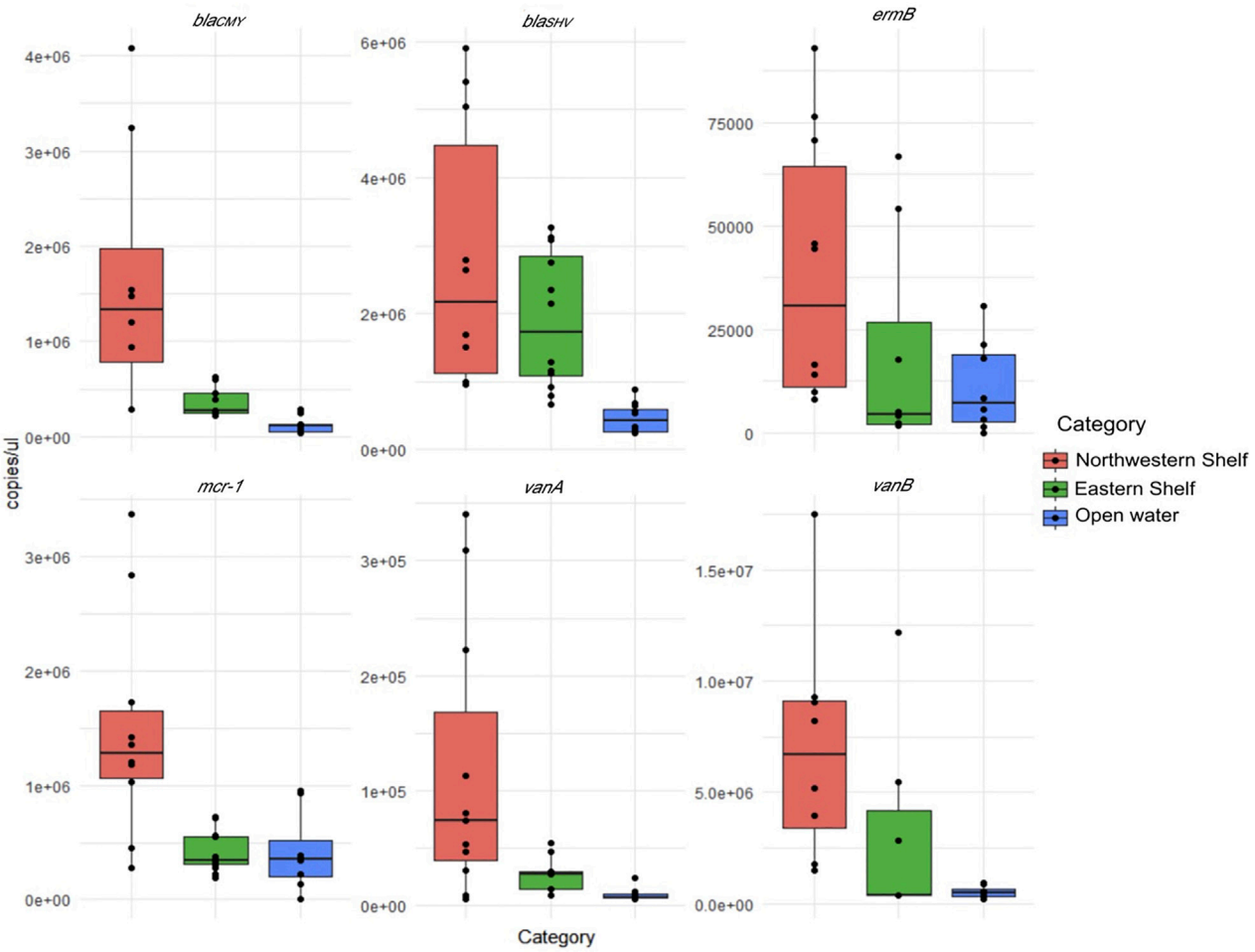

FIGURE 2 | Absolute number of ARGs in northwest shelf (stations 1A, 2A, 2, and 3), eastern shelf (stations 9, 10, 11, 12) and open waters (stations 4, 5, 7 and 8).

applied (q-value = 0.05), *p*-value adjustment method: fdr (q-value <0.05), to test for the difference in the genes' abundance in the water from different Black Sea locations. Spearman correlation was used to test the correlation between ARGs' abundance and number of the faecal pollution markers. Mantel test was performed with the purpose to infer whether the variance in genes' number is explained by geographical distance, using the abundance matrix (based on Bray–Curtis measure) and the geographical distance matrix (based on Haversine distance).

RESULTS

Number of Antibiotic Resistance Genes in the Black Sea Water Samples

All target antibiotic resistance genes were found in all Black Sea water samples (**Figure 2**). Absolute values of the ARGs in each sample are presented in **Supplementary Table S2**.

The average values normalized to the 16S rRNA gene number indicate that the *vanB* ($2 \times 10^{-1} \pm 1 \times 10^{-1}$) was the most numerous gene followed by bla_{SHV} ($4 \times 10^{-2} \pm 1 \times 10^{-2}$), *mcr-1* ($5 \times 10^{-2} \pm 3 \times 10^{-2}$) and bla_{CMY} ($1 \times 10^{-2} \pm 3 \times 10^{-3}$). *ermB* ($1 \times 10^{-3} \pm 5 \times 10^{-4}$) and *vanA* ($1 \times 10^{-5} \pm 5 \times 10^{-4}$) were the least numerous genes.

Considering the absolute values, differential abundance of ARGs in the waters of eastern, northwest shelf and open waters was pronounced. The higher number of ARGs in the waters of the northwest shelf was especially notable (**Figure 2**). Open waters contained the least number of all ARGs. Wilcoxon pairwise comparison proved that blaCMY, vanA and mcr-1 had significantly higher abundance in the northwest shelf coastal area compared to the open waters and the eastern shelf (Wilcoxon, $p < 0.05$—**Table 2**). Bla_{SHV} and bla_{CMY} had significantly higher estimates in the eastern shelf compared to the open waters, while bla_{CMY} and *mcr-1* significantly varied in their abundance in the eastern and northwestern shelf.

The data normalized to the 16S rRNA did not exhibit a pronounced difference in the three Black Sea areas due to the uneven distribution of 16S rRNA gene. It had the highest estimates in the northwest shelf ($3 \times 10^{8} \pm 6 \times 10^{7}$ copies/μL) and the lowest in the open waters ($3 \times 10^{7} \pm 8 \times 10^{6}$ copies/μL).

TABLE 2 | Wilcoxon rank sum test comparison of the AGS' absolute number in eastern shelf, open waters, northwest shelf. *p*-value adjustment method: fdr (q-value<0.05).

Gene/Target	Groups of comparison		
	Northwest shelf vs. Open waters	Eastern shelf vs. Northwest shelf	Eastern shelf vs. Open waters
bla_{SHV}	3.1×10^{-6}	0.38	5.2×10^{-6}
bla_{CMY}	8.2×10^{-5}	0.005	0.002
vanA	0.05	0.04	0.05
vanB	0.01	0.28	0.63
mcr-1	0.005	0.002	0.77
ermB	0.04	0.07	0.95
Human *Bacteroidetes*	0.03	0.11	0.003
Pig *Bacteroidetes*	0.63	0.51	0.18
Rumen *Bacteroidetes*	0.03	0.16	0.26
16S rRNA	6.0×10^{-4}	0.02	0.05

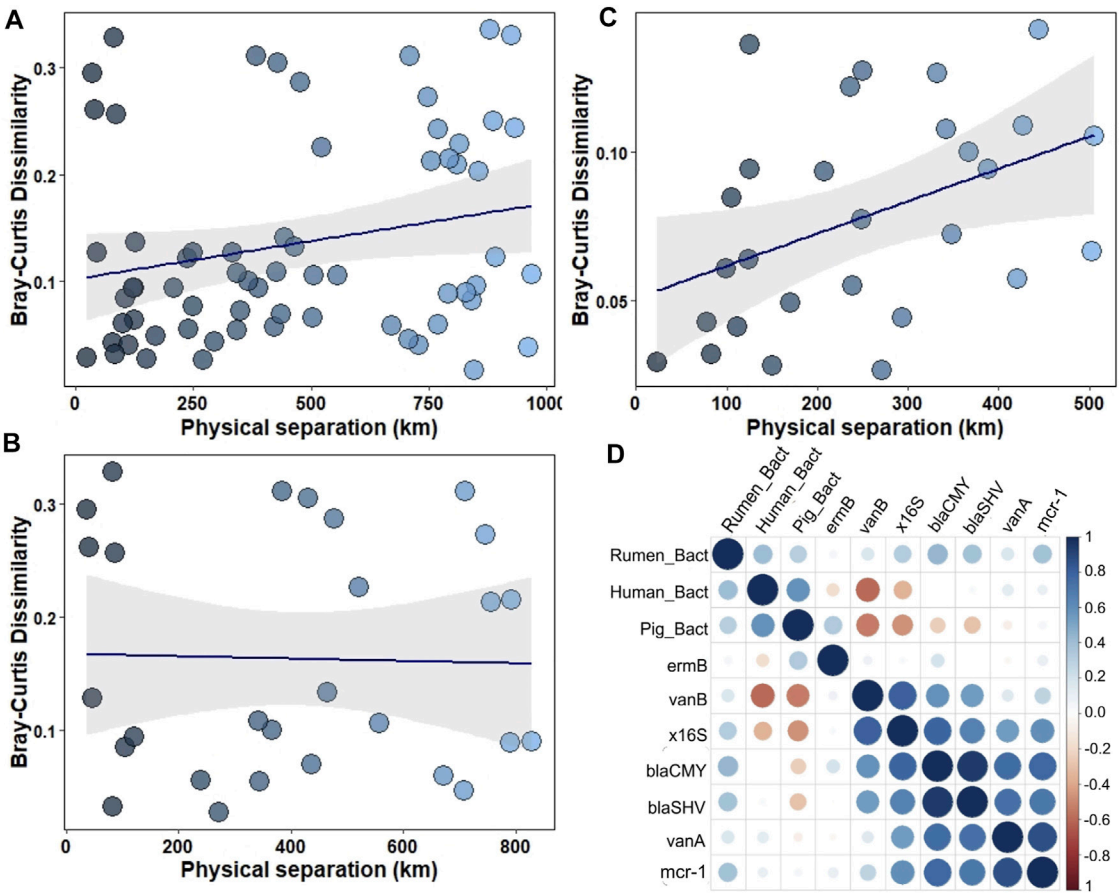

FIGURE 3 | (A–C) Variance of faecal pollution dependent on the physical separation of the samples: **(A)** within the northwest shelf—eastern shelf segment (from station 1A to station 12); **(B)**—within the open waters—eastern shelf segment (from station 4 to station 12); **(C)**— within the northwest shelf—open waters segment (from station 1A to station 8); **(D)**—Spearman correlation between ARGs' number and number of faecal pollution markers.

The 16S rRNA copy number was $1{\times}10^8 \pm 5 \times 10^7$ copies/μL in the eastern shelf.

Pollution by Faecal Markers

Genetic markers for human-, rumen- and pig-specific faecal members of the *Bacteroidetes* phylum were quantified to identify the sources of faecal contamination. Anaerobic bacteria belonging to *Bacteroidetes* phylum inhabit the faeces and exhibit host adaptation on the genetic level (Dick et al., 2005). For this reason *Bacteroidetes'* markers (specific regions of the 16S rRNA gene) are used as indicators that can discriminate between human and nonhuman sources of faecal

contamination (Reischer et al., 2006; Mieszkin et al., 2009; Epa and of Science, 2019).

Gene sequences of human-associated *Bacteroidetes* are usually found in the humans' faeces and environments polluted with human faeces. Human-associated *Bacteroidetes* can be present in other animals' sources (Layton et al., 2013). Similarly, pig-associated or cow-associated *Bacteroidetes* can have cross-reaction in other types of faeces due to the common omnivorous diet and similar digestive tract (Dick et al., 2005; Mieszkin et al., 2009). Nevertheless, presence of human-specific *Bacteroidetes* in environmental samples is usually associated with human faecal pollution (Layton et al., 2013).

Number of human associated *Bacteroidetes* (per μL) was $7 \times 10^3 \pm 9 \times 10^2$ in the northwest shelf, $7 \times 10^3 \pm 1 \times 10^2$ in the open water and $6 \times 10^3 \pm 2 \times 10^3$ in the eastern shelf. Number of pig associated *Bacteroidetes* did not vary in samples taken from different locations, presenting $1 \times 10^2 \pm 5 \times 10^1$, $2 \times 10^2 \pm 1 \times 10^2$, $1 \times 10^2 \pm 5 \times 10^1$ marker copy/μL in the northwest shelf, open waters and eastern shelf respectively. Number of rumen-specific *Bacteroidetes* was $6 \times 10^2 \pm 2 \times 10^2$, $4 \times 10^2 \pm 4 \times 10^2$, $4 \times 10^2 \pm 2 \times 10^2$ copies/μL in the three respective studied areas.

Human-specific and rumen-specific *Bacteroidetes* differed in quantity in the northwest coastal waters and open waters according to the Wilcoxon rank sum test (**Table 2**). Mantel test revealed moderate positive correlation (r = 0.52, *p*-value = 0.0033) between the faecal markers and physical separation within the northwest shelf—open waters segment (from station 1A to station 8). There was no correlation if the whole distance (from station 1A to station 12) or open water—eastern shelf segment (from station 4 to station 12) was considered (**Figure 3**). This finding suggests that markers of the faecal pollution mainly enter the Black Sea environment from the northwest coast.

Rumen-specific *Bacteroidetes* had moderate positive correlation (0.4, $p < 0.05$) with bla_{CMY}, bla_{SHV} and *mcr-1* genes (**Figure 3D**). The 16S rRNA gene had strong correlation with bla_{CMY}, bla_{SHV}, *van*A and *mcr-1* genes (**Figure 3D**).

Concentrations of Antibiotics

None of the tested antibiotics exceeded the screening detection limit (1.25 ng/L) possibly due to the high dilution of the analytes in seawater.

DISCUSSION

The distribution of ARGs in the Black Sea was evaluated on the sea region-wide scale involving sampling locations in the eastern shelf, open waters and northwest shelf. The list of genes included bla_{CMY} and bla_{SHV} genes that are responsible for the inactivation of beta-lactam antibiotics, *ermB* targeting macrolides, *vanA* and *vanB* genes that inactivate glycopeptides and *mcr-1* that compromises effectiveness of colistin.

All studied ARGs were found in the seawater samples. *vanB*, bla_{SHV} and bla_{CMY} were among the most numerous ARGs. *mcr-1* had also high estimates, which is of particular interest since, to our knowledge, this is the first report of the *mcr-1* distribution in the Black Sea. Results of the study reveal that *vanA* and *ermB* were the least numerous genes among the tested ones, but they were also found in all analysed samples.

The obtained data was compared to ARGs distribution in other aquatic environments summarized in the holistic analysis of the 122 metagenomic sets from lakes and seas (Yang et al., 2019). According to Yang et al. (2019) the relative abundance of beta-lactams' and aminoglycosides' resistance genes was in the range from 1×10^{-3} to 2×10^{-2} copies normalized to the 16S rRNA. The copy number of polymyxin and vancomycin resistance genes was on average by one order of magnitude lower (up to 3×10^{-3}). Our data on genes coding for aminoglycosides' resistance is in consistence with that of Yang et al. (2019). The sum of bla_{SHV} and bla_{CMY} varied from 7×10^{-3} to 2×10^{-1} copies that is higher compared to the sum of beta-lactam resistance genes presented by Yang et al. (2019). The copy number of beta-lactam resistance genes (bla_{OXA}) in the Danube River Basin had wider variation compared to our data: 2.4×10^{-8}–3.1×10^{-2} (Alygizakis et al., 2019). bla_{CTXM} was detected in the Black Sea water by Sabatino et al. (2020), but its quantity was not estimated.

The present study revealed higher *vanB* and *mcr-1* copy number compared to the estimates of vancomycin and polymixyn resistance genes presented by Yang et al. (2019). According to our data *vanB* reached $2 \times 10^{-1} \pm 1 \times 10^{-1}$ copies normalized to 16S rRNA in the Black Sea water. This gene was not detected in the effluents of WWTPs in the Danube River Basin, while *vanA* occurred sporadically (Alygizakis et al., 2019). That indicates that the Danube river is likely not a source of the *vanB* in the Black Sea, and the other rivers (Dnieper, Dniester or Western Bug) on the northwest site should be considered in this regard. The number of *mcr-1* genes varied from 2×10^{-3} to 3×10^{-2} copies normalized to 16S rRNA.

The copy number of *ermB* (1.8×10^{-5}–4.9×10^{-3}) in the effluents of WWTPs in the Danube River Basin (Alygizakis et al., 2019) may indicate the origin of this gene distribution. Contrasting with our data, no *ermB* was detected in the Black Sea water samples according to (Sabatino et al., 2020). This indicates that *ermB* dissemination in the Black Sea might be possibly on its rise.

High number of ARGs in the Black Sea is likely associated with prescription rates and poorly controlled antibiotic consumption in the riparian countries. Amoxicillin, ceftriaxone and cefuroxime were the most consumed antibiotics during 2013–2018 in Ukraine (Yakovlieva and Bahlai, 2019), which is in parallel with the increase in macrolides consumption in this region (Matyashova and Iakovlieva, 2015). According to (Tarcea Bizo et al., 2015), vancomycin is the most prescribed antibiotic in a university tertiary hospital in Cluj-Napoca, Romania. Meanwhile, European Resistance Surveillance Network (EARS-NET, 2019) reports distribution of hospital isolates resistant to beta-lactams and vancomycin in Black Sea countries. About 63% of *E. coli* isolates were resistant to amoxicillin/ampicillin in hospitals of Romania and Bulgaria. From 64.1 to 75.7% of *K. pneumoniae* isolates were resistant to cefotaxime/ceftriaxone/

ceftazidime in these two countries respectively. About 35.7% isolates of *E. faecium* from hospitals of Romania were resistant to vancomycin. Joint Danube Survey 3 results indicate the distribution of the beta-lactam and carbapenem resistant *Pseudomonas* spp., *Klebsiella* spp. and *E. coli* in the Danube water (Kittinger et al., 2016a; Kittinger et al., 2016b). Isolates with resistance patterns normally associated with intensive care units were also found in the Danube river water (Kittinger et al., 2016a). Unfortunately, data reporting the colistin resistance is not presented in the EARS-NET Report. Though, besides the human treatment, colistin is widely applied in veterinary medicine, which fuels the development of the resistance to this antibiotic (Kempf et al., 2016).

The differential abundance of all tested ARGs according to the location of the sampling stations is notable. ARGs were significantly higher (Wilcoxon rank sum test, $p < 0.05$) in number in the northwest shelf compared to the open waters. bla_{CMY} and bla_{SHV} were considerably more abundant in the eastern shelf than in the open waters. It goes in line with the data on *sul1* distribution in the coastal waters of the Black Sea (Sabatino et al., 2020). Similar effect was observed in coastal and open waters of the Bohai Sea and Yellow Sea areas (Lu et al., 2019). The difference in ARG number in the open and coastal waters clearly evidences the anthropogenic impact on the ARGs' distribution in the Black Sea environment.

The main sources of the coastal and estuarine pollution with ARGs are riverine runoffs, WWTPs, aquaculture and untreated sewage (Zheng et al., 2021). Big cities such as Odessa (Ukraine) and Constanta (Romania) along with a number of smaller towns (total population more than 1.5 million) are located on the northwest bank of the Black Sea. Moreover, Dnieper, Western Bug, Dniester and Danube rivers (total basin area is 1,437,790 km^2) fall into the Black Sea on this side providing input of ARGs and other pollutants. Analysis of effluent wastewater from WWTPs in the Danube River Basin has shown the abundance of ARGs (*aph(III)a*, *blaOXA*, *ermB*, *tetM* etc.,) in the wastewater released into the Danube river and its tributaries (Alygizakis et al., 2019). Similar accumulation of the ARGs is likely to occur in the other rivers. The eastern bank of the Black Sea is less populated with Batumi (Georgia) as the biggest city. Rivers Chorokhi and Rioni fall into the Black Sea on the eastern side and may provide input of the ARGs collected along their basins (total area of basins is 35,500 km^2). The higher number of the *vanA* and *mcr-1* in the northwest shelf compared to the eastern shelf is worth noting. Wider area of river basins falling on the north in the Black Sea, extensive agriculture and peculiarities of antibiotic consumption in the region can cause the differences in ARGs' abundance.

ARGs can persist in the environment due to the presence of antibiotics or disinfectants (Zheng et al., 2021), which provide the selective pressure and facilitate the HGT. On the other hand, faecal pollution is a straightforward source of ARGs dissemination in the environment since gut microbiota is a reservoir of antibiotic-resistant bacteria (Karkman et al., 2018; Khan et al., 2019). The concentrations of 22 targeted antibiotics were below the detection limit (1.25 ng/L) in all tested samples. This excludes the influence of antibiotics on the ARGs proliferation, while the ARGs' number decrease in the open waters evidences the elimination of the ARGs from the environment.

Previous studies indicated that faecal pollution (from urban areas, animal farms and pasture) was a crucial problem throughout the Danube River Basin (Kirschner et al., 2009). Obviously, the Black Sea is impacted by faecal polluted water from the Danube river and other tributaries, which is reflected in elevated concentration of the markers of faecal pollution in the northwest shelf area. bla_{CMY}, bla_{SHV} and *mcr-1* had moderate positive correlation with the *Bacteroidetes* common to the ruminant microbiome. The positive moderate correlation may evidence the impact of the beta-lactams and colistin use in the veterinary pharmaceuticals on the dissemination of these ARGs in the Black Sea. However, the faecal pollution is likely not the major reason for increased concentration of the ARGs in the coastal waters. Among the other reasons can be pollution with ARBs harbouring the corresponding ARGs since all ARGs had a strong positive correlation with 16S rRNA gene. It can be noted that ARGs could be selected for and transmitted to the environmental bacteria before entering the Black Sea (*via* WWTPs, sewage, etc.,), while the Black Sea receives the ARBs together with riverine and sewage input.

CONCLUSION

The distribution of ARGs targeting a wide spectrum of antibiotics (beta-lactams, macrolides, glycopeptides, colistin) including both first-line and last-resort antibiotics was investigated in the Black Sea water samples at a region-wide scale, including shelf and offshore areas. All studied ARGs were detected in all of the studied samples. The number of ARGs inactivating beta-lactams, vancomycin and polymyxin was higher compared to the previous studies performed in the region. The elevated number of colistin resistance *mcr-1* gene is of particular interest, as this gene was reported in Black Sea microbial communities for the first time.

ARGs were significantly higher in number in the shelf areas compared to the open waters, which signals involvement of the anthropogenic impact on the natural resistome of the water ecosystem.

The ARGs targeting beta-lactams, vancomycin and last resort antibiotic colistin were of the highest abundance in the northwest shelf, which might be attributed to poorly controlled antibiotic consumption in the riparian countries.

The observed correlation between bla_{CMY}, bla_{SHV} and *mcr-1* with the *Bacteroidetes* common to the ruminant microbiome indicates that the potential sources of ARGs in the Black Sea are the riverine and sewage inflow and, to the lesser extent, application of veterinary pharmaceuticals.

The results are of high environmental concern, highlighting an alarming presence of antibiotic resistance in the Black Sea. An inclusion of ARGs into regular legacy monitoring of the Black Sea and major inflowing rivers is recommended.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

IP: Formal analysis (lead); Investigation (equal); Methodology (equal); Visualization (equal); Writing—original draft (lead). MP: Investigation (equal); Methodology (equal); Validation (equal); Visualization (supporting); Writing—original draft (supporting). AD: Investigation (supporting); Validation (supporting); Visualization (supporting). ED: Investigation (supporting); Project administration (equal); Supervision (equal). NA: Formal analysis (supporting), Methodology (supporting), Writing—original draft (supporting). JS: Formal analysis (supporting); Project administration (equal); Resources (lead); Writing—original draft (supporting).

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SUPPLEMENTARY MATERIAL

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The Occurrence and Distribution Pattern of Antibiotic Resistance Genes and Bacterial Community in the Ili River

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The emergence of antibiotic resistance genes (ARGs) is a great risk to the ecosystem and human health; however, there are rare systematic studies about the characterization and source identification of ARGs in continental rivers. This study examined the occurrence of bacterial communities and ARGs in the water and soil of the Ili River using bacterial testing and metagenomic sequencing. Total dissolved solids (TDSs) and total bacterial count significantly increased from upstream to downstream sites. *Enterococcus* showed the highest abundance in Cockdara. Metagenomic sequencing revealed that the bacterial communities of surface water were different from those of nearshore soils. Among the top 10 ARGs, fluoroquinolone and aminoglycoside resistance genes exhibited dominant relative abundance, but only the multidrug resistance gene *adeF* was common in all water and soil samples. Proteobacteria carried almost 61% of ARGs, suggesting that these could be the main antibiotic-resistant bacteria (ARB) in the Ili River. Proteobacteria and ARB were mainly distributed in Yining city and Cockdara. Furthermore, the distribution pattern among the five sampling sites indicated that human activity and animal husbandry greatly contributed to the ARB and ARG contamination. This study first investigated the occurrence and distribution pattern of ARGs in the Ili River, demonstrating a clear correlation between bacteria and ARGs, and ARGs and mobile genetic elements (MGEs).

Keywords: bacterial community, antibiotic resistance genes, indicator bacteria, metagenomic analysis, antibiotic-resistant bacteria

INTRODUCTION

Worldwide, deteriorating water quality has become a critical environmental problem, causing a great threat to human health, ecological biodiversity, and even social stability (Landrigan et al., 2020). The river system, a precious fundamental resource for living species, plays critical roles as was for drinking, agricultural irrigation, traffic channel, and recreational activities. However, the river system can be a pool of various pollutants, including physical, chemical, and biological pollutions (Hu et al., 2017). The pollution of surface water is the most severe environmental concern causing diseases (Landrigan et al., 2020). Several studies showed a close link between river water pollution and the increasing incidence of gastrointestinal diseases (Rodriguez-Tapia and Morales-Novelo, 2017), infectious diseases (Johnson et al., 2012), waterborne endemic fluorosis, and arsenic poisoning (Boelee et al., 2019; Karkman et al., 2019).

Environmental microbiota, an important ingredient of the river ecosystem, carries out various essential biogeochemical processes, including nutrient cycling and pollutant degradation (Guan et al., 2018). However, the ever-growing economics and human activities have increased wastewaters discharged to the rivers, increasing community pathogens from hospitals, pharmaceutical industries, and animal husbandry (Molina et al., 2014; Stachler et al., 2017; Jennings et al., 2018). Fecal indicator bacteria, referred to as *Escherichia coli* and *Enterococci*, are routinely used to assess bacterial pollution of water systems (Boehm et al., 2018; Aydin et al., 2019). Some polluted waters such as the Haihe River and Taihu Lake contain high density of fecal indicator bacteria (Wang et al., 2017). A study showed the presence of seven potentially pathogenic genera including *Acinetobacter*, *Enterococcus*, and *Streptococcus* in Taihu Lake (Vadde et al., 2019). Bacterial pathogens, with low-dose infection and the possibility of secondary spread, are one of the most serious pollutants.

Antibiotics are widely used in medicine, animal husbandry, and agriculture to treat bacterial infections (Liu et al., 2019). However, the misuse/overuse of antibiotics has induced the emergence of ARGs and ARB in the natural environment, posing potential threats to human health and ecosystems (Stange et al., 2019; Uprety et al., 2020). The transmission and dissemination of ARGs induce ecological toxicity to aquatic ecosystems and severe infection to humans and livestock (Guo et al., 2019). ARGs have been identified as emerging environmental pollutants and one of the most serious threats to human health by the World Health Organization (Zheng et al., 2017; Yang et al., 2018). ARGs have been detected in various environments, such as surface water, sediments, soils, and underground water. Among these, surface water is an important reservoir of ARGs (Zhang et al., 2020). Previous investigations demonstrated that ARG spread is strongly related to human activities, like stock farming, and environmental factors, such as antibiotics, heavy metals, environmental estrogens, and MGEs (Zheng et al., 2017; Stange et al., 2019). Surface water such as the riverine system is more susceptible to human activities and therefore is an ideal setting for the prevalence of ARGs.

The abundance and distribution of ARGs in the global ocean suggest that quinolone, bacitracin, and fosmidomycin ARGs cause great environmental pressure (Cuadrat et al., 2020). In Italy, *Sul1*, *ermB*, *blatem*, *tetw*, and *qnrs* resistant *E. coli* were isolated from the Tiber and Arrone rivers (Pantanella et al., 2020). In China, there have been numerous investigations into ARGs; a study indicated that the most prevalent ARGs in the Haihe River were sulfonamide and tetracycline resistance genes (Dang et al., 2017). In the Bohai Sea, sulfonamide resistance genes (sul1 and sul2) are highly abundant, while other ARGs are present in low amounts. In 2021, 11 types of ARGs including eight tetracycline resistance genes, two sulfonamide resistance genes, and one β-lactam resistance gene were detected in the Yangtze River (Zhang et al., 2021). Likewise, in the Ba River in Xi'an, *tetC*, bla_{TEM}, *ermF*, *sul1*, *cmlA*, and *gyrA* were found to be the predominant ARGs. Most published studies are about the ARB and ARG pollution in outflow rivers, while there are rare studies on ARGs in inner rivers.

Inner rivers play important roles in regulating the local climate and biological diversity. Particularly, due to drought and scarce precipitation, the ARB and ARG pollution of inner rivers is more vulnerable to human activities. The Ili River, a typical inner river, originates in the middle of the Tian Shan Mountains, and it is an international river covering about 1,237 km across China and Kazakhstan. This river is a critical source of water for agriculture, husbandry, and industrial production in Xinjiang Province, China. In addition, the Ili River flows through several cities in Xinjiang, thereby receiving significant sewage discharge. So far, there are only few studies about the bacterial and ARG contamination of the Ili River.

The present study selected different sampling points close to the towns with low-population density areas, urban areas with frequent human activities, and grazing areas to investigate the bacterial pollution, and the distribution and abundances of bacterial community and ARGs in the Ili River using metagenomics, bacteriological analysis, and antibiotic susceptibility testing. The objectives of this study were to illustrate the effective factors related to the occurrence and distribution of ARGs and explore the relevance of bacterial community, MGEs, and ARGs.

MATERIALS AND METHODS

Sampling Sites and Sample Collection

The detailed sampling sites are shown in **Figure 1**. According to the climate, ecology, and geographical characteristics of the Ili River, five sampling sites were selected in the mainstream and upstream tributary of the river, namely, upstream tributary Kashi River (BGC), Yining city (YN), Cockdara (KKDL), Huiyuan city (HY), and the National Highway 219 (B219), respectively. At each site, triplicate water samples were collected in sterile 10-L polythene bottles from the selected sampling sites, 100 m upstream and 100 m downstream from the sampling point. At the time of sampling, water temperature (T), pH, and total dissolved solids (TDSs) were measured with a handheld pH meter (pH808, SMART SENSOR, China) and a TDS-measuring instrument (AR8011, SMART SENSOR, China), respectively. Two liters of water from each sample was filtered through a 0.22-μm filter membrane (Millipore, United States), and the membranes containing bacterial filtrates were stored in centrifuge tubes containing phosphate-buffered saline (PBS) at 4°C for the following experiment. In addition to the five water samples, four soil samples, except for the sampling site B219, were collected from the respective riverbank sites.

Detection of Total Culturable and Indicator Bacteria

The total count of culturable and indicator bacteria (total *coliforms*, *E. coli*, and *Enterococcus*) was estimated for each water sample after leaving the samples for more than 30 min. The numbers of total culturable bacteria (CFU/ml) were counted using the 3M Petrifilm Aerobic Count plate (3M, MN, Unites States) (Sinclair et al., 2021). The indicator bacteria (MPN/

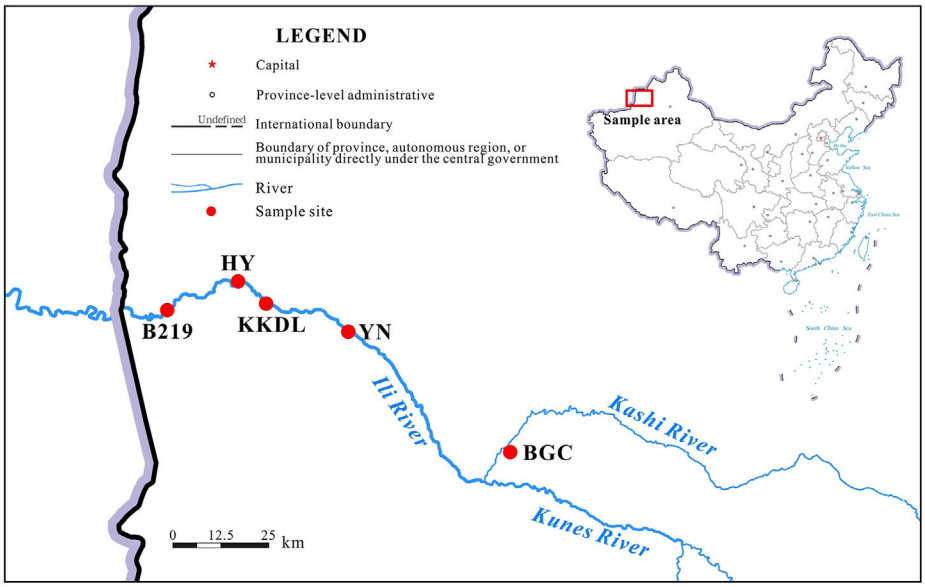

FIGURE 1 | Sampling sites of the Ili River.

100 ml), including total *coliforms*, *E. coli*, and *Enterococcus* were enumerated for each sample using the Colilert and Enterolert kits according to the manufacturer's instructions (IDEXX Laboratories, Westbrook, ME) (Sercu et al., 2011; Ledwaba et al., 2019).

Isolation and 16S rDNA Gene Identification of Pathogenic Bacteria

Bacteria in water samples (2 L each) were collected through filtration using a 0.22-μm microporous membrane (Millipore, United States). Then, the trapped bacteria on the microporous membrane surface were resuspended in sterile phosphate-buffered saline (PBS). The bacterial suspension was streaked on the respective selective medium plate to grow *Enterococcus* (*Enterococcus* chromogenic medium, Hopebio, Qingdao, China), *Staphylococcus aureus* (*Staphylococcus* chromogenic medium, Hopebio, Qingdao, China), *Salmonella* (*Salmonella* chromogenic medium, Hopebio, Qingdao, China), and *Coliform* (*E. coli/Coliform* Chromogenic Medium, Hopebio, Qingdao, China) at 37°C for 24 h. Typical colonies were picked up according to the color, shape, and properties of the colony as described in the manufacturer's instructions. Streak plate isolations on the respective selective medium plate were repeated at least three times.

After bacterial isolation, the DNA was extracted from bacterial cell pellets using the TIANamp Bacteria DNA Kit (Tiangen, Beijing, China). DNA samples were sent to the Allwegene Technologies (Beijing, China) for Sanger sequencing. The sequencing results were matched with the NCBI database. The preselected bacterial 16S rRNA sequence and the bacterial sequence were aligned by ClustalW in MEGA 5. After deleting non-aligned bases, the neighbor-joining method was used to construct a phylogenetic tree with 1,000 bootstrap replicates.

Antibiotic Susceptibility Testing

After incubation for 24 h, the bacterial suspension was soaked in Mueller–Hinton agar (MH). Then, the antibiotic susceptibility was tested using the disc diffusion method (Kirby–Bauer). The 13 types of antibiotic disc used in this study are listed in **Table 2**. The antibiotic discs were placed on the plate at a distance of no less than 25 mm, and the results were analyzed after incubation for 18–24 h at 30°C.

DNA Extraction and Metagenomic Sequencing

The genomic DNA of the soil samples was extracted using the TIANamp Soil DNA Kit (Tiangen Biotech, Beijing, China) following the manufacturer's instructions. The DNA concentration was measured using the Qubit dsDNA Assay Kit in a Qubit 4.0 Fluorometer (ThermoFisher Scientific, Waltham, MA, United States). To examine the diversity and abundance of bacteria and ARGs, a total amount of 1 μg DNA per sample was used as an input material. Sequencing libraries were generated using the NEBNext® UltraTM DNA Library Prep Kit for Illumina (NEB, United States) following the manufacturer's recommendations, and index codes were added to attribute the sequences to each sample. The raw data obtained from the Illumina HiSeq sequencing platform was processed using Readfq (V8, https://github.com/cjfields/readfq) to acquire the clean data for subsequent analysis. The specific processing steps were as follows: a) reads containing low-quality bases (default quality threshold value ≤38) above a certain portion (default length of 40 bp) were removed, b) reads with a certain percentage of N bases (default length of 10 bp) were removed, and c) reads sharing an overlap with the adapter above a certain portion (default length of 15 bp) were also removed.

Gene Prediction and Abundance Analysis

The scaftigs (≥500 bp) assembled from both single and mixed were all predicted the open reading frame (ORF) by MetaGeneMark (V2.10, http://topaz.gatech.edu/GeneMark/) software, and length information was filtered to be shorter than 100 nt from the predicted result with default parameters. For ORF prediction, CD-HIT software (V4.5.8, http://www.bioinformatics.org/cd-hit) was adopted to avoid redundancy and obtain the unique initial gene catalog (the genes here refer to the nucleotide sequences coded by unique and continuous genes), and the parameter options were −c 0.95, −G 0, −aS 0.9, −g 1, and −d 0.

Bacterial Community, ARGs, and MGEs Annotation

For bacterial community identification, the bacteria, fungi, archaea, and virus sequences were searched against the NR database (version: 2018-01-02, https://www.ncbi.nlm.nih.gov/) of the NCBI using DIAMOND blastp, with a cutoff e-value of $1e^{-5}$. In case a sequence was aligned to multiple results, the result with the *E*-value ≤ the smallest *E*-value *10 was selected to help the LCA algorithm to ensure the species annotation of sequence. A table containing the number of genes and the abundance information of each sample in the taxonomic hierarchy (kingdom, phylum, class, order, family, genus, and species) was prepared based on the LCA annotation results. The abundance of a species is equal to the sum of the gene abundance annotated for that species; the gene number of a species in a sample equals the number of genes whose abundance is non-zero.

Resistance Gene Identifier (RGI) software was used to align the Unigenes to CARD database (https://card.mcmaster.ca/) with the parameter setting are blastp and *E*-value ≤ $1e^{-30}$. Based on the aligned result, the relative abundance of ARO, the resistance genes' abundance distribution in each sample, and the species attribution analysis of resistance genes were conducted, and the "relative abundance" (ppm, parts per million) was defined as one hit in one million aligned sequencing genes.

Mobile genetic elements (MGEs) were identified using BLASTP to compare the ARG-carrying genes to A CLAssification of Mobile genetic Elements (ACLAME) amino acid database for plasmids, using an e-value ≤ 10^{-5} and a cutoff of ≥50% query coverage. The ISfinder database (Siguier, 2006) was used to find insertion sequences (ISs) with an e-value ≤ 10^{-5} and a cutoff of ≥45% query coverage. In addition, the IntegrALL nucleotide database (Moura et al., 2009) was used to identify integrons, with an e-value ≤ 10^{-5} and a cutoff of ≥45% query coverage.

Network Analysis

The networks were created as follows: a correlation matrix was constructed using ARGs and metagenomic data to explore the potential correlations among ARG subtypes, MGEs, and taxonomy (at the genus level) that occurred in at least 70% of samples, using R (psych package, v3.4.0). A correlation between two nodes was considered statistically significant if r ≥ 0.85 and $p \leq 0.05$ and was adjusted using the Benjamini–Hochberg method. The correlation between two nodes was termed the edge file, and a description was added to the node file comprising the phylum taxonomy at the genus level and the ARG type or the ARG subtype. The network analysis was visualized using the interactive platform Gephi (v 0.9.2) (Bastian et al., 2009).

Statistical Analysis

Data were presented as mean ± SD and analyzed using one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls test (SNK) and Spearman's correlation analysis using SPSS 22 statistical software. PCA analysis was performed using R (ade4 package, version 2.15.3), showing the different distribution of bacterial community between water and soil samples. Diversity analysis was used to demonstrate the diversities of ARGs and bacterial communities in different sampling sites. Simpson, Shannon, and inverse Simpson indices were calculated using R (vegan package, version 2.15.3). A p value < 0.05 was considered statistically significant.

RESULTS

Overview of Ili River Quality

The physical and biological characteristics of surface water samples are shown in **Table 1**. The sample pH ranged from 8.1 to 8.3, showing a significant difference among the five sampling sites. TDSs and total bacterial count showed a significant increase from upstream to downstream, that is, the total number of culturable bacteria was the lowest at the sampling site BGC and the highest at B219 ($p < 0.05$). Concerning the indicator bacteria, the amounts of *E. coli* showed no significant difference, ranging from 21 to 50 MPN/100 ml (**Figure 2**). *Enterococcus* amounts ranged from 18 to 183 MPN/100 ml, and sampling sites BGC and HY had the least amount of *Enterococcus*, followed by YN, B219, and KKDL. Notably, the KKDL site showed significantly high numbers of *Enterococcus* compared to the other four sampling sites. After 24-h incubation of the supernatant, *Coliform* and *Enterococcus faecalis* were cultured from all five samples, and *Staphylococcus aureus* was found except for the BGC site (**Table 2**). These results were proved by 16S rDNA gene identification and phylogenetic analysis (**Supplementary Figure S1**).

Bacterial Community Structure in the Ili River

In total, 15 water samples and four soil samples were analyzed. The results of metagenomic sequencing showed a significant difference between the community composition of soil and surface water samples (**Figure 4B**). Proteobacteria was the most abundant phylum in water samples followed by Bacteroidetes (**Figure 3A**). In addition to BGC, Cyanobacteria and Planctomycetes were also found in water samples, Proteobacteria showed a higher abundance (75.3%) in BGC samples than others ($p < 0.05$). Importantly, Acidobacteria,

TABLE 1 | Physicochemical properties in the Ili River.

Parameter	Sampling sites				
	BGC	YN	KKDL	HY	B219
pH	8.3	8.1	8.1	8.2 ± 0.1	8.1
TDS	302.7 ± 8.5	336.3 ± 2.1	343.3 ± 1.2	358.0 ± 2.7	391.3 ± 2.3
Bacteria (CFU/ml)	30.0 ± 4.4	198.0 ± 24.6	212.0 ± 31.8	294.0 ± 65.1	305.0 ± 13.0

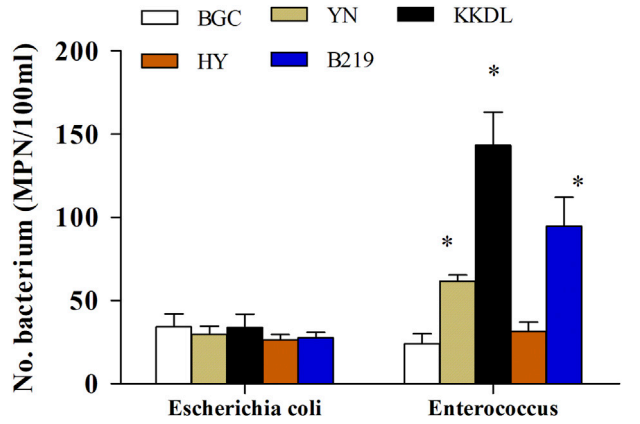

FIGURE 2 | Concentration of fecal indicator bacteria (FIB) in five sampling sites.

TABLE 2 | Results of purification of bacteria.

Sampling site	Identification of strains		
	Coliform	*Enterococcus*	*Staphylococcus aureus*
BGC	+	+	−
YN	+	+	+
YN	+	+	+
HY	+	+	+
B219	+	+	+

"+" means detected, and "–" means not detected.

Thaumarchaeota, Gemmatimonadetes, and Chloroflexi were also detected in the soil samples, which showed higher species abundance than water samples (**Figure 3A**).

As for the genus level, the bacterial community in the water samples of BGC showed a higher α-diversity than other sites (**Table 3**). *Pseudomonas* (most abundant), *Hydrogenophaga*, *Limnobacter*, and *Flavobacterium* were the dominant bacteria in water samples (**Figure 3B**). This was completely different from the soil samples, which showed a higher abundance of *Nocardioides* and *Sphingomonas*. *Pseudomonas* in BGC and YN water samples showed higher relative abundance than the other sampling sites, while B219 had the highest abundance of *Flavobacterium* (**Supplementary Figure S2**). The relative abundance of *Streptomyces* (almost 5.3%) was higher in BGC samples than in other soil samples. All these indicated the different relative abundance of species at different sampling sites. In addition, the species abundance of water and soil samples varied within the same sampling site. These results agreed with PCA of the water and soil samples (**Figure 4A**).

Diversity and Abundance of ARGs in the Ili River

As shown in **Figure 5A**, the composition of ARGs in water samples is more complex than that in soil samples. Also, almost all soil ARGs were present in water samples. Among the top 20 ARGs, 13 belong to antibiotic efflux genes that were extensively present in both water and soil samples. The top 20 ARGs were classified into multidrug resistance, fluoroquinolone, aminoglycoside, and tetracycline resistance genes. The relative abundance of multidrug resistance gene *adeF* was highest (40–95 ppm) among all target ARGs and distributed in all the water and soil samples. This indicated fluoroquinolones, tetracyclines, or ARB pollution in the whole Ili River.

Notably, the relative abundance of ARGs at the BGC site was higher than that at the other sampling sites, and the overall trend was BGC > YN > KKDL ≈ HY > B219. Diversity analysis also showed a higher α-diversity of ARGs in BGC samples (**Figure 5B**). The target ARGs in riverbank were approximately the same (**Figure 6**). Among the top 10 selected ARGs in water samples, the relative abundance of multidrug ARGs *smeF* was higher in the YN samples than in the other sampling sites (**Supplementary Figure S3**), but the other seven ARGs (*adeF*, *ANT2-Ia*, *APH3-Ib*, *APH6-Id*, *qacH*, *sul1*, and *tet39*) were higher in BGC samples (**Supplementary Figure S3**). *ACC-3* was mainly found in the soil samples of KKDL (35 ppm) while remaining undetected in other soil samples and water samples. Also, the bacteria of BGC sites primarily carried fluoroquinolone, tetracycline, aminoglycoside, and sulfonamide ARGs, while multidrug ARGs *TEM-116* was mainly carried by the bacteria of the KKDL site.

Correlation Among ARGs, MGEs, and Bacteria

Antibiotic susceptibility testing of the total bacteria suggested that YN showed the utmost variety of culturable ARB, while BGC and B219 showed least diversity (**Figure 7A**). In addition to ampicillin, all the water samples were resistant to vancomycin, which is considered the last line of defense (**Figure 7B**; **Supplementary Tables S1–S4**). *Enterococcus faecalis*, purified from water samples of YN, HY, and B219, was resistant to several antibiotics, suggesting the serious pollution of antibiotic-resistant

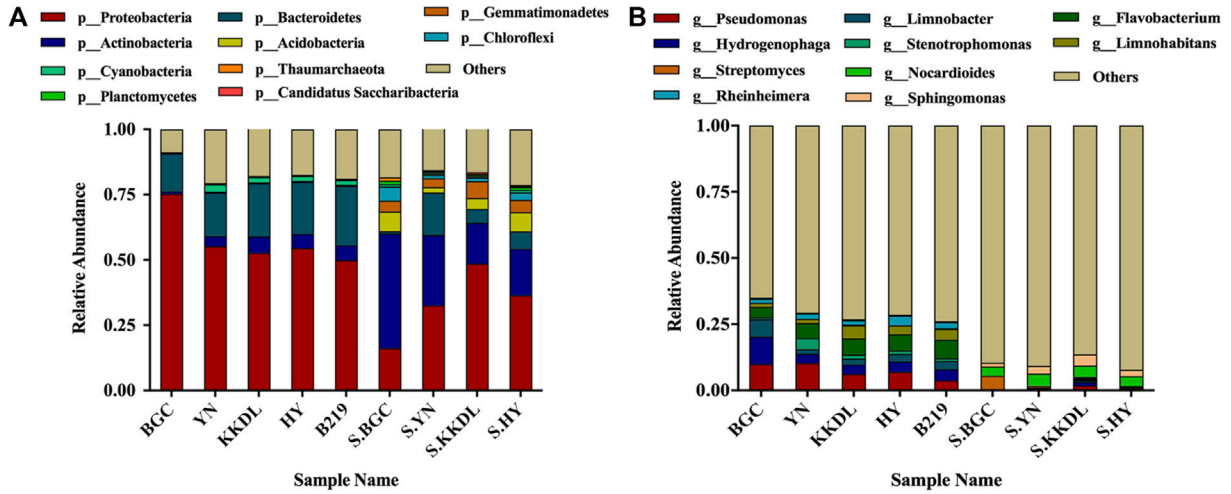

FIGURE 3 | Relative abundance of bacterial groups of top 10 genera. Phylum level **(A)**; genus level **(B)**. BGC, YN, KKDL, HY, and B219 refer to the relative abundance of bacterial community in water samples, S. BGC, S.YN, S. KKDL, and S.HY refer to the relative abundance of bacterial community in soil samples.

TABLE 3 | Diversity index at genus level.

Sample	Shannon	Inverse Simpson
BGC	3.165 ± 0.02	6.103 ± 0.32
YN	3.058 ± 0.06	5.021 ± 0.14
KKDL	3.058 ± 0.03	4.954 ± 0.02
HY	3.091 ± 0.01	5.086 ± 0.03
B219	3.084 ± 0.04	4.875 ± 0.05

Enterococcus faecalis. *Enterococcus* strains were resistant to Streptomycin (**Supplementary Table S2**). Antibiotic-resistant *Staphylococcus aureus* was detected only in YN and KKDL samples, while *Staphylococcus aureus* from HY and B219 was not antibiotic-resistant (**Supplementary Table S3**). *Coliform* acquired from the water samples of YN was resistant to multiple antibiotics, especially at downstream sampling sites (**Supplementary Table S4**). In general, *Enterococcus faecalis* and *E. coli* were resistant to a wider variety of antibiotics. The ARB pollution was more serious at YN and KKDL sites, which can be attributed to crowd activities.

To investigate the association between ARGs and the bacterial community, a correlation analysis was carried out between the relative abundance of ARGs (based on CARD analysis) and bacteria. The results indicated a significant correlation between ARGs and bacteria in the Ili River. As shown in **Supplementary Figure S4**, Proteobacteria carried the most multidrug ARGs (61%), and Bacteroidetes mostly carried fluoroquinolone ARGs (such as *mfpA* and *mdtk*), macrolide, and glycopeptide ARGs. *AxyY* and *pgpB* negatively correlated with Actinobacteria (**Figure 8A**), indicating their low transfer risk. Also, aminoglycoside *APH3-Ib* and sulfonamide *sul1* positively correlated with many kinds of MGEs (**Figure 8B**), indicating a high risk of horizontal transfer.

DISCUSSION

The Ili River is important for agriculture, husbandry, and industrial activities in Xinjiang Province, China. Drought and scarce precipitation promote the accumulation of ARB and ARGs in the Ili River, causing serious challenge to humans, agriculture, and animal husbandry.

The present study examined the bacterial community and ARG profile in the Ili River. The concentration of fecal indicator bacteria (FIB) is routinely used to report the bacterial pollution in the water environment. This study showed that the total bacteria, *E. coli*, and *Enterococcus* were significantly lower in the Ili River than in other rivers with frequent human activities, such as the Haihe and the Songhua rivers (Lee et al., 2013; Ren et al., 2020; Wang et al., 2017). It can be attributed to relatively low population density along the Ili River (Ili Kazakh Autonomous Prefecture 10 people/km^2, Haihe River Basin 384 people/km^2), which emphasizes that bacterial pollution in rivers is potentially affected by human activities. Similarly, the number of non-redundant genes in the sparsely populated town (BGC) was considerably lower than that in crowded cities (YN and KKDL) and grazing areas (HY and B219) (**Supplementary Figure S5**). This again suggested that the bacterial pollution in rivers probably originating from human activities or animal husbandry pollution. Also, the higher levels of indicator *Enterococcus* in KKDL samples (urban areas) indicated that this river environment might be extensively affected by urban sewage discharge (Lutterodt et al., 2012; Lu et al., 2018). In addition, TDSs and total bacterial count were significantly higher at downstream sites, suggesting the poorer water quality downstream than upstream regions.

Analyzing the types of ARGs, fluoroquinolone, aminoglycoside, sulfonamide, and tetracycline resistance genes were the most prevailing types in the Ili River. The antibiotic susceptibility testing also indicated that isolates from the Ili River

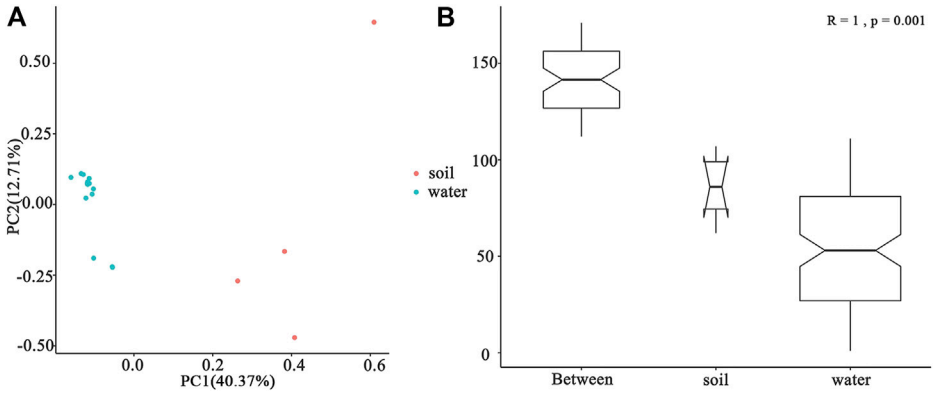

FIGURE 4 | Bacterial diversity between water and soil at the genus level. **(A)** PCA and **(B)** ANOSIM.

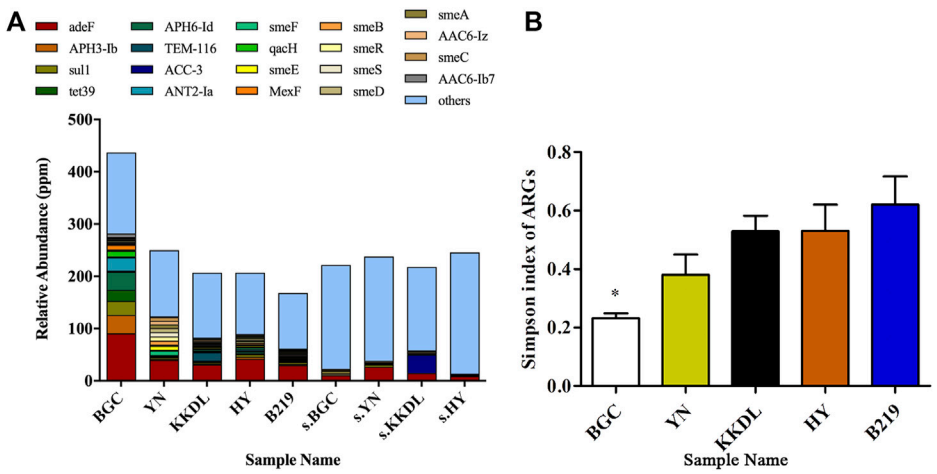

FIGURE 5 | Diversity and relative abundance of ARGs. **(A)** Diversity analysis of ARGs in all water samples; **(B)** relative abundance of ARGs of top 20.

displayed resistance to fluoroquinolone, aminoglycoside, sulfonamide, chloramphenicol, polypeptide, and tetracycline antibiotics. Previous studies report that tetracycline and sulfonamide resistance genes were prominent in livestock manures (Ji et al., 2012); fluoroquinolone, sulfonamide, and tetracycline are also frequently used in animal husbandry (Zhao et al., 2010), while aminoglycoside, macrolides, and lactams are used in a hospital setting (Jia et al., 2014). Therefore, the ARG pollution in the Ili River might result from animal manures, community sewage, or medical waste. Getahun et al. (Agga et al., 2019) found that the concentration of ARGs remained consistently higher in the feeding area than in the grazing area in the pasture–feedlot-type setting for beef cattle cultivation. Likewise, the free grazing farming in Xinjiang may alter the distribution of ARGs elsewhere. Compared with traditional husbandry (Ji et al., 2012), the ARGs in the Ili River showed significantly lower relative abundance such as for sulfonamide (approximately 10^{-6} to 10^{-5} in Ili River, 10^{-5} to 10^{-2} in animal husbandry) and tetracycline resistance genes (approximately 10^{-7} to 10^{-5} in the Ili River, 10^{-6} to 10^{-3} in animal husbandry). Accordingly, the four downstream sites showed no significant difference in the ARGs. Therefore, in contrast to traditional animal husbandry (Ji et al., 2012; Zhu et al., 2013; Agga et al., 2019), we speculate that free grazing did not cause severe pollution of ARGs in the Ili River.

Magnanimous evidence indicates that human activities exacerbate the spread of ARGs (Chen et al., 2019). The present study also suggested the same. This raises the question of whether the presence of an abundant bacterial community would be a potential reservoir for ARGs. This study found that the upstream sampling site BGC had the lowest total bacterial count but the highest abundance of ARGs. The phenomenon can be explained as follows: 1. Proteobacteria showed the highest relative abundance in BGC samples, which carried the most ARGs (61%); therefore, it led to the highest abundance even with a low total bacterial count. 2. Proteobacteria showed a significant relation with *adeF*, which accounted for almost 15% of the total ARGs, *adeF*, and thus an antibiotic efflux-related gene might induce

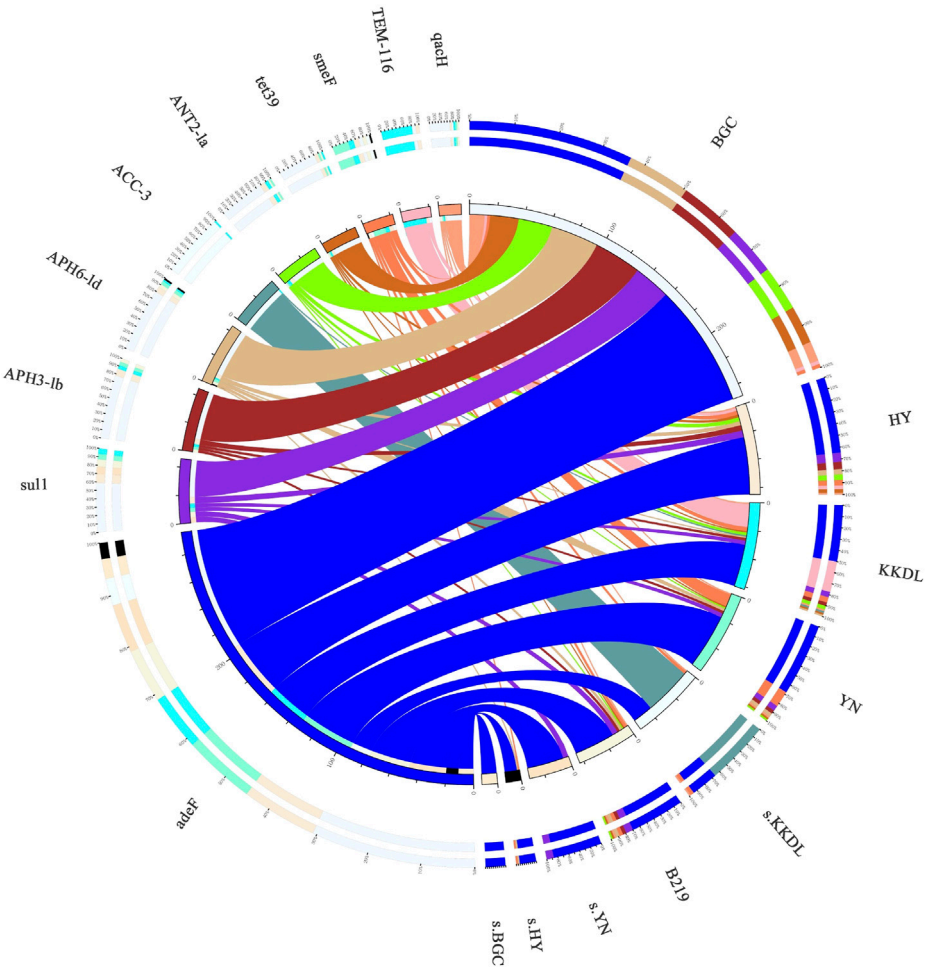

FIGURE 6 | Distribution of ARGs in every sampling sites. The ARG classes on the left and the sampling locations on the right side are presented as colored bands. Bar lengths on the outer ring represent the percentage of ARGs in each sample. The inner layer numbers indicate the relative abundances of ARG classes from a given sample (left) and the relative abundance of specific ARG classes in samples (right).

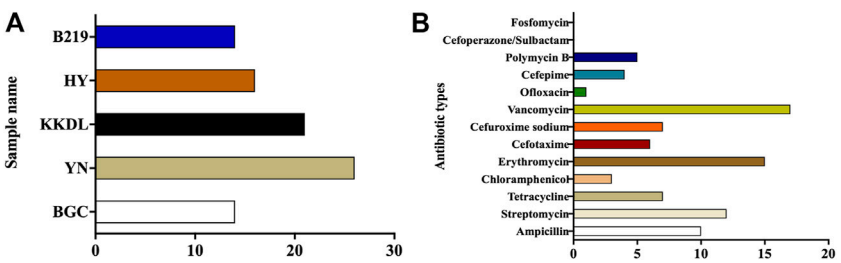

FIGURE 7 | Numbers of antibiotic-resistant bacteria. **(A)** The numbers of antibiotic-resistant bacteria in different sampling sites. **(B)** The numbers of bacteria resistant to different antibiotics.

other multidrug resistance genes. 3. Much evidence indicates that the spread of ARGs greatly contributes to severe pollution situations (Munro, 2015; Han et al., 2018); apart from *adeF*, other ARGs such as *APH3-Ib*, *sul1*, *tet39*, *APH6-Id*, *and ANT2-Ia* accounted for about 30.5% abundance in BGC samples, which positively correlated with many kinds of MGEs that possibly aggravated the spread of ARGs.

Normally, the abundance of the total ARGs reduces with water flow (Xu et al., 2015; Liu et al., 2019). This study also supported this as the total and relative abundances of seven types of ARGs (adeF,

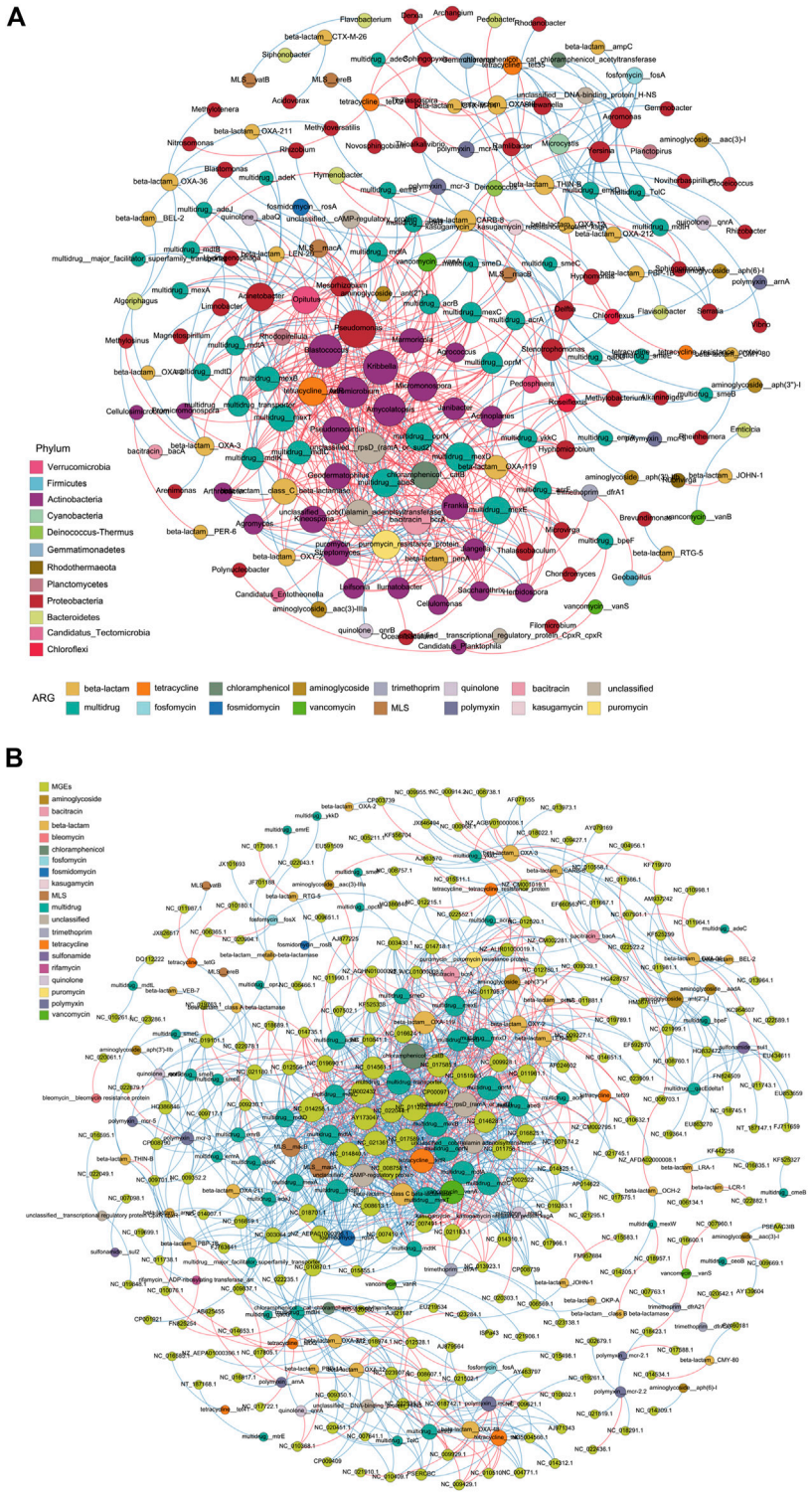

FIGURE 8 | Correlation between ARGs and bacteria, and ARGs and MGEs based on network analysis. Co-occurrence patterns between the bacteria and ARG subtypes **(A)**; ARG subtypes and MGEs **(B)**. The red line indicates a positive correlation between two nodes. The blue line represents a negative correlation. The size of each node is proportional to its number of connections.

ANT2-Ia, APH3-Ib, APH6-Id, qacH, sul1, and tet39) were significantly higher in BGC samples than in the downstream sites, and the diversity analysis indicated that a higher α-diversity of ARGs was observed in BGC samples than in other sites. The other possible cause might be the inflow of additional tributaries. As the bacteria are critical hosts of ARGs (Zhou et al., 2017), similar composition of the bacterial community in the four downstream sampling sites led to a similar occurrence and distribution of ARGs.

The potential main hosts of ARGs in the Ili River were Proteobacteria, Bacteroidetes, and Actinobacteria, which is consistent with other findings in the Yellow River Delta, Chongqing municipality, and the Karst River (Li et al., 2020; Wang et al., 2020; Xiang et al., 2020); however, the distribution of ARGs was different in different studies. It is reported that human activity promoted the spread of sulfonamide and tetracycline resistance genes in many rivers like the Yangtze River, Liaohe River, and the northern Yellow Sea, while this study found the fluoroquinolone, tetracycline, and aminoglycoside resistance genes as the most abundant ARGs. This could be due to different antibiotic usage in this area. The frequently reported antibiotics in both influents and effluents include sulfonamides and tetracyclines ranged from a few ng/L to 10 μg/L (Qiao et al., 2018), while some studies in Xinjiang showed that quinolone was present in the highest concentration with hundreds to thousands ng/L, followed by sulfonamides and tetracyclines ranging from tens to hundreds ng/L along the sewage treatment process (Liu et al., 2017). Previous studies demonstrated the correlation between ARGs and other pollutants induced by human activity, such as the concentration of antibiotics, heavy metals, sewage, and livestock (Zhou et al., 2017; Zhang et al., 2018; Ma et al., 2019; Ohore et al., 2020). Therefore, lower antibiotic discharge may alleviate the pressure of ARG pollution. The relative abundance of sulfonamide resistance genes ranged from 4.12×10^{-5} to 2.47×10^{-2} in the Ba River, Xi'an (Guan et al., 2018); however, the most abundant ARGs (*adeF*) in the Ili River ranged from 9.49×10^{-6} to 1.09×10^{-4}. These results indicated that the relative abundance of ARGs in the Ili River was much lower than that in outflow rivers due to the low population density and lesser use of antibiotics in this region. Also, the different usage of antibiotics between free grazing and intensive farming areas seems to be a critical factor. Overall, the humanities and environmental factors that caused the different discharge of ARGs must be further explored. The concentration of antibiotics, heavy metals, physicochemical properties of the river water, season, and rainfall should be evaluated to examine the source identification, prevalence, and subduction of the bacterial community and ARGs in the Ili River.

CONCLUSION

This study showed that TDSs, total bacterial count, *E. coli*, and *Enterococcus* were significantly lower in upstream sampling sites than in the downstream, indicating a deteriorating river environment with increased human and animal husbandry activities. However, the relative abundance of ARGs in the Ili River was much lower than that in outflow rivers; free grazing did not cause severe pollution of ARGs in the Ili River. Fluoroquinolone, aminoglycoside, sulfonamide, and tetracycline resistance genes were the most prevailing types in the Ili River, and the ARGs in crowded cities and grazing area showed no significant difference. The present study first investigated the occurrence and distribution pattern of ARGs in the Ili River and demonstrated the potential risk of ARGs, which will provide a profitable reference to the treatment of water environment of Ili River.

The sequencing data have been uploaded to https://www.ebi.ac.uk/ena/browser/view/PRJEB49834? Accession: PRJEB49834.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena/browser/view/PRJEB49834?

AUTHOR CONTRIBUTIONS

XY and LY completed the experiments and manuscript. YY, HZ, and SW collected all samples. YC, BX, and CL analyzed the data. ZS, JW, and ZQ designed this study.

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SUPPLEMENTARY MATERIAL

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Multi-Media Occurrence of Antibiotics and Antibiotic Resistance Genes in East Dongting Lake

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With the extensive use of antibiotics, antibiotics and their induced resistance genes (ARGs) have become new types of pollutants widely distributed in a variety of environmental media. The contamination of antibiotics and ARGs occurring in important living and agricultural regions has aroused wide concern worldwide, especially in lake basins. The Dongting Lake basin is one of the important aquaculture and livestock areas in China, which is accompanied by a large amount of antibiotic use and discharge. However, the occurrence characteristics of antibiotics and ARGs in a multi-environment medium are still unclear. In this study, surface water and sediment samples in East Dongting Lake were collected by season, antibiotics and ARGs were quantitatively analyzed, and the risk quotient method was used to evaluate the ecological risk of antibiotics in surface water. 1) The concentration of antibiotics in the surface water of East Dongting Lake ranged from ND to 943.49 ng/L, with the maximum average concentration of 20.92 ng/L in spring. The concentration of antibiotics in sediments ranged from ND to 177.43 ng/g, with the maximum average concentration of 16.38 ng/g in fall. Ofloxacin (OFL) and sulfamethoxazole (SMX) were the main antibiotic pollutants in East Dongting Lake Basin. 2) *sul1* and *sul2* were the dominant ARGs in East Dongting Lake Basin. For spatial change, the total abundance of ARGs upstream was higher than that downstream. For seasonal change, the surface water and sediment were characterized by spring > summer > fall. 3) OFL and sulfamethoxazole might pose a significant high risk to aquatic organisms both in three seasons, and the ecological risk of antibiotics in East Dongting Lake is more significant at low temperatures than high. This study could provide important data information of the occurrence and concentration of antibiotics and ARGs in East Dongting Lake Basin.

Keywords: East Dongting Lake, antibiotics, antibiotic resistance genes, surface water, sediment, ecological risk

1 INTRODUCTION

Since the 1930s, antibiotics are widely used in areas such as health care, breeding, and agricultural. However, only 25–75% of antibiotics can be absorbed by the body, and large amounts of antibiotics are released into the environment through human activities, such as clinical treatment, livestock and poultry production, and pharmaceutical (Luo et al., 2010). Although antibiotics can be degraded by light, heat, and biodegradation under natural conditions (Ohore et al., 2020; Zhang et al., 2020), some residual antibiotics and antibiotic resistance can persist in the natural environment through the propagation and transmission of microorganisms (Xiong et al., 2018). Selective pressure caused by antibiotic residues causes some susceptible bacteria to develop antibiotic resistance genes (ARGs) (Kim, 2004), which can be transferred horizontally between microorganisms, further induce the transformation between different environmental media, along the food chain, and finally enter the human body to increase human antibiotic resistance (Ghosh et al., 2009; Stoll et al., 2012; Sengupta et al., 2013). According to data from the Global Antibiotic Resistance and Use Surveillance System in 2020, a high proportion of resistance gene problems have appeared in 66 countries, and more and more bacteria have developed resistance to available antibiotics. Therefore, the risks of antibiotics and ARGs need to be taken seriously (Sarmah et al., 2006).

The aquatic environment has been identified as an important reservoir of antibiotics and antibiotic resistance genes (ARGs) (Liu et al., 2018c; Nguyen et al., 2019). Every year in China, at least 5 tons of antibiotics enter the water environment in various forms. As a result, antibiotics and their ARGs have been widely detected in Hongze Lake (Liu L et al., 2018), Poyang Lake (Pan et al., 2018), Chaohu Lake (Liu et al., 2018c), Honghu Lake (Yang et al., 2016), Taihu Lake (Wu et al., 2016), and other water bodies in China. Exogenous inputs such as medical wastewater, livestock and poultry breeding wastewater, aquaculture wastewater, and effluent from sewage treatment plants (Kolar et al., 2014; Di Cesare et al., 2015; Zhang et al., 2015) are important sources of ARGs in the water environment. In addition, sediments might act as a sink but also as a secondary source of various contaminants including antibiotics and ARGs and even pose higher potential risk to aquatic organisms (Su et al., 2014; Lu et al., 2018). Studies have shown that ARGs have also been detected in the atmosphere near livestock farms, hospitals, and sewage treatment plants (Xu et al., 2014). Hence, the study on the pollution state and risk of antibiotics and ARGs in the lake basin, especially in important aquaculture and livestock areas, has been paid more and more attention.

As the second largest freshwater lake in China, Dongting Lake is the link connecting the Yangtze River with a strong flood storage capacity, which plays a very important role in regulating the flood runoff and environmental protection of the Yangtze River. Meanwhile, Dongting Lake is the birthplace of traditional Chinese agriculture and also is one of the important habitats for birds. Studies have shown that the emissions of antibiotic (3440t/a) in the Dongting Lake basin rank the first in China, among which the pollution level of East Dongting Lake Basin is the most prominent (Liu et al., 2018a, b). The research results of the contribution of the PCA–MLR model to antibiotics in East Dongting Lake show that livestock and poultry breeding is the main source, with the contribution rate of more than 70% (Di Cesare et al., 2015; Liu L et al., 2018). The pollution levels of antibiotics were higher in the eastern part than those in southern and western parts in Dongting Lake (Wang et al., 2019). Yang et al. (2016) detected ARGs in the sediments of East Dongting Lake, showing the highest relative abundance of *sul2*. In contrast, at present, the multi-media occurrence characteristics of antibiotics and their ARGs in the water and sediment environment of East Dongting Lake have not been systematically studied yet, especially under different hydrological periods, which underscores the need for complementary studies.

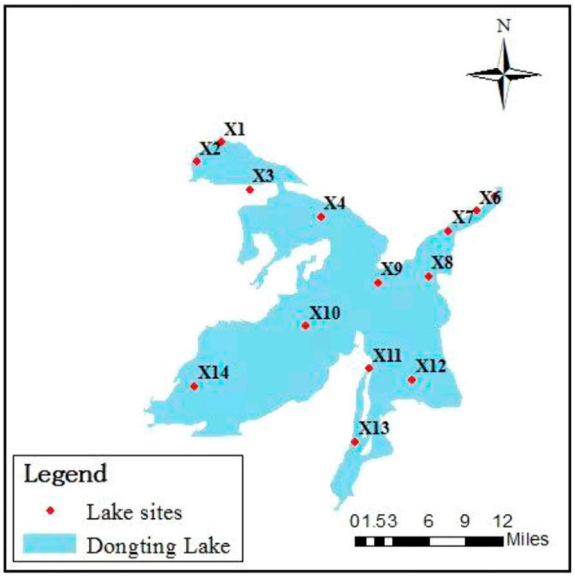

FIGURE 1 | Sampling sites in East Dongting Lake.

In this study, the pollution levels and spatial characteristics of four commonly used antibiotics (sulfonamides, macrolides, tetracyclines, and fluoroquinolones) and nine target ARGs in surface water and sediments of East Dongting Lake Basin were quantitatively studied, and the variation characteristics in different seasons were explored. Moreover, the risk quotient (RQ) method was used to evaluate the ecological risk of antibiotics in surface water. This work could provide reliable data information for further assessment of antibiotics and ARG pollution and ecological protection in East Dongting Lake Basin.

2 MATERIALS AND METHODS

2.1 Study Area and Sample Collection

East Dongting Lake (28°59′~29°38′N, 112°43′~113°15′E), located in the northeast of Hunan Province, has a total area of 1328 km^2 and an average water depth of 6.39 m (Liu et al., 2018a). As an important reservoir lake in the Yangtze River Basin and one of the important aquaculture and livestock areas in China, it plays a great role in regulating the flood runoff, ensuring ecological security, and promoting economic and social development (Wang et al., 2019).

By investigating the pollution source distribution and the site accessibility, surface water and sediment samples were collected from 14 sampling sites (**Figure 1**) at the entrance, outlet, and national nature reserve areas in spring, summer, and fall 2019, respectively, for the determination of antibiotics and ARGs in the lake body. At each sampling site, 1 L surface water at a depth of 0–1 m was collected by stainless steel drums and stored in 1 L brown sampling bottles with 5 ml methanol at 4°C. Simultaneously, sediment at a depth of 0–10 cm was collected by a mud bucket and stored at −80°C before analysis.

2.2 Pretreatment and Detection of Antibiotics

2.2.1 Water Sample Pretreatment

1 L water samples were filtered using a 0.22 μm aperture mixed cellulose micropore membrane (Millipore, United States), and then the solutions were purified and enriched using solid phase extraction (SPE, Oasis HLB cartridges, Waters, United States, 500 mg, 6 ml). After eluting with 6 ml methanol and 6 ml ammonia–methanol (5%) solution, the eluents were concentrated under nitrogen gas condition. The resulting residues were further redissolved by using 10% methanol (1 ml), filtered through a 0.22 μm membrane, and transferred to amber glass for quantitative analysis.

2.2.2 Sediment Sample Pretreatment

2 g dry sediment samples were extracted by 15 ml EDTA buffer solution and 15 ml acetonitrile; after oscillation (10 min, 200 rpm), ultrasonication (15 min), centrifugation (5 min, 6000 rpm), and concentration, 500 ml ultrapure water was added to dissolve the target antibiotics, and 0.25 g EDTA-2Na, with 1 mol/L hydrochloric acid, was used to adjust pH to 3.0 ± 0.05. The redissolved solutions were concentrated with SPE (Oasis SAX, HLB cartridges, Waters, United States) and then treated following the same steps as those for the water sample.

2.2.3 Detection of Antibiotics

The quantitative analysis of 12 target antibiotics belonging to four different classes: sulfonamides (SAs), including sulfamethoxazole (SMX), sulfamethazine (SMZ), and sulfadiazine (SDZ); macrolides (MLs), including erythromycin (ERM) and roxithromycin (ROM); tetracyclines (TCs), including oxytetracycline (OTC), chlortetracycline (CTC), and tetracycline (TC); and fluoroquinolones (FQs), including ofloxacin (OFL), norfloxacin (NOR), enrofloxacin (ENR), and ciprofloxacin (CIP), was performed by using the ultra-high–performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) method (Ultimate3000 HPLC system coupled with API3200, United States). The detailed chromatographic conditions were as follows: chromatographic column, Waters BEH-C18 column (3.0 × 150 mm, 3.5 μm); column temperature, 40°C; mobile phase, 0.01% formic acid (formic acid/ultrapure water, V/V) (A) and acetonitrile (B); flow rate, 0.3 ml min^{-1}; injection volume, 5 μL; and gradient program, 0–7 min, 3–15% B; 7–9 min, 15% B; 9–12 min, 15–30% B; 12–13 min, 30% B; 13–18 min, 30–42% B; 18–19 min, 42% B; 19–21 min, 42–3% B; and 21–29 min, 3% B.

2.3 Ecological Risk Assessment

Currently, studies on the risk assessment of antibiotics to aquatic organisms including bacteria, algae, invertebrates, and fish are extensive, but there is a lack of toxicological data on antibiotics in sediments. Therefore, this study only evaluated the ecological risk of antibiotics in surface water. RQs (risk quotients) can be calculated according to the following formula:

$$RQs = MEC/PNEC, \quad (1)$$

where RQs represent risk quotients, MEC represents the maximum measured antibiotic concentration, and PNEC represents no effective concentration in water. Ecological risks are classified according to RQ values: RQs < 0.01, 0.01 ≤ RQs < 0.1, 0.1 ≤ RQs < 1, and RQs ≥ 1 indicating no risk, low risk, medium risk, and high risk, respectively.

2.4 Detection of Antibiotic Resistance Genes

2.4.1 DNA Extraction

After water sample filtration, the 0.22 μm aperture mixed cellulose micropore membrane was used for DNA extraction by Water DNA Kit (Omega, United States). The DNA extraction of sediment samples was conducted following the manufacturer's protocols of Soil DNA Rapid Extraction Kit (MP Biomedicals, France). All extracted DNA samples were stored at −20°C for analysis.

2.4.2 Real-Time Quantitative PCR Analysis

Nine target ARGs (*sul1*, *sul2*, *tetA*, *tetM*, *tetW*, *qnrS*, *ermA*, *ermB*, *int1*) and 16S rRNA (total bacterial population) were analyzed by qPCR. The primer sequences of the selected ARGs are shown in **Supplementary Table S1**. SYBR Green kits (Takara, Dalian, China) were used for the preparation of PCR solution according to the manufacturer's protocols. The thermal cycle was set at 95°C for 3 min pre-degradation which was followed by 35 cycles at 95°C for 30 s, annealing for 30 s (the required annealing temperatures are shown in **Supplementary Table S1**), 72°C for 1 min, and extension for 40 s under 72°C.

2.5 Statistical Analysis

IBM SPSS Statistics 25.0 software (United States) was used for statistical analysis. Origin 9.0 was used for the production of the distribution map of antibiotics and ARGs.

3 RESULTS AND DISCUSSION

3.1 Occurrence of Antibiotics in Surface Water

Twelve antibiotics belonging to four classes, including macrolides, quinolones, sulfonamides, and tetracycline, were detected in the surface water of East Dongting Lake, and their

TABLE 1 | Concentration of antibiotics in the surface water of East Dongting Lake.

Antibiotics	Spring (n = 11, ng/L)				Summer (n = 8, ng/L)				Fall (n = 14, ng/L)			
	Min.	Max.	Mean	Rate (%)	Min.	Max.	Mean	Rate (%)	Min.	Max.	Mean	Rate (%)
SDZ	ND	2.82	0.66	90.9	0.17	0.57	0.26	100	0.13	2.6	0.99	100
SMZ	ND	0.32	0.13	72.7	ND	0.09	0.04	75	ND	2.8	0.59	64.3
SMX	3.03	163.52	36.10	100	7.17	244.8	79.28	100	ND	220.95	20.73	50
OTC	ND	ND	ND	0	ND	ND	ND	0	ND	45.73	22.12	50
TC	ND	ND	ND	0	ND	ND	ND	0	ND	30.8	3.45	14.3
CTC	ND	ND	ND	0	ND	ND	ND	0	ND	50.32	16.66	71.4
OFL	18.94	943.49	176.73	100	6.77	49.82	21.31	100	15.44	486.59	63.41	100
NOR	ND	ND	ND	0	ND	ND	ND	0	ND	12.17	0.87	7.1
CIP	ND	ND	ND	0	ND	ND	ND	0	ND	27.02	1.93	7.1
ENR	ND	0.45	0.04	9.1	ND	ND	ND	0	ND	63.75	8.09	50
ROM	4.60	17.98	9.98	100	0.16	0.35	0.26	100	1.22	110.66	15.75	100
ERM	11.14	54.12	27.40	100	ND	12.56	3.98	62.5	ND	ND	ND	0

"Rate" represents the detection rate; "ND" represents not detected.

concentrations are shown in **Table 1**. The detection rates of monomer varied from 0 to 100%, with an average detection rate of 47.9%. The detection rate of OFL and ROM in three seasons was 100%, and the detection rate of SDZ, SMZ, and SMX in three seasons was higher than the average level. The 12 target antibiotics detected in the surface water of East Dongting Lake were generally in ng/L, and their concentrations ranged from ND to 943.49 ng/L. The order of average concentration of antibiotics was quinolones (23.90 ng/L) > sulfonamides (13.68 ng/L) > macrolides (10.08 ng/L) > tetracycline (4.41 ng/L). The OFL concentration was the highest (170.45–1944.04 ng/L), followed by SMX (290.27–634.24 ng/L), ERM (0–301.41 ng/L), and ROM (2.04–220.47 ng/L). These four antibiotics are the main antibiotic compounds in the surface water, contributing 89% of the antibiotics in East Dongting Lake.

The order of average concentration of antibiotics in different seasons was as follows: spring (20.92 ng/L) > fall (11.96 ng/L) > summer (8.76 ng/L). Antibiotic concentrations are lowest in summer, partly because of the dilution of the antibiotics by increased rainfall. And on the contrary, higher temperatures in summer promote the biodegradation and non-biodegradation of antibiotics. For spring and fall, the frequent weather changes, high incidence of diseases, and increased use of antibiotics in these two seasons would lead to the increase of antibiotics entering the water environment (Stoll et al., 2012; Cheng et al., 2014). Moreover, the reduced temperature, hydrodynamic conditions, microbial activity, and dissolved oxygen could enhance the persistence of antibiotics in the water body (Kim and Carlson, 2007b; Loftin et al., 2008; Yan et al., 2013; Lu et al., 2018).

The cumulative concentration of antibiotics in the surface water of East Dongting Lake is shown in **Figure 2**. It can be seen that there are significant spatial differences in antibiotic concentration levels. The cumulative concentrations of antibiotics in spring, summer, and fall are 60.47–1166.26 ng/L, 16.10–289.33 ng/L, and 23.02–754.13 ng/L, respectively. The spatial distribution of antibiotics is closely related to the distribution of surrounding pollution sources. The

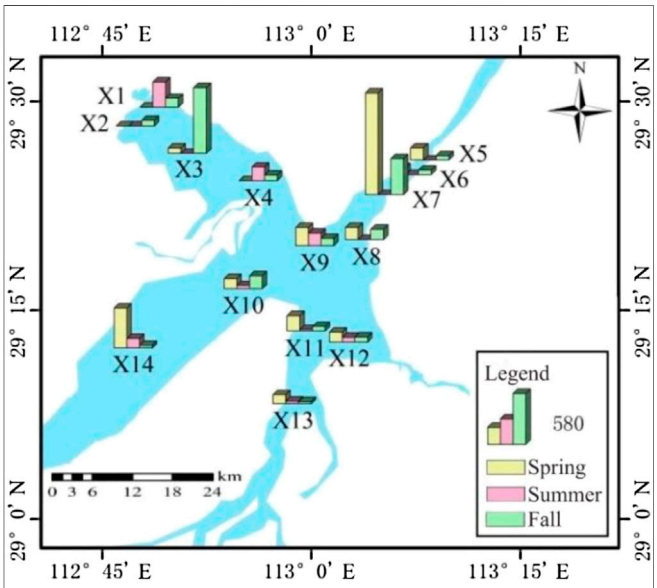

FIGURE 2 | Cumulative concentration of antibiotics in the surface water of East Dongting Lake in different seasons.

sampling site X7 is close to Yueyang Tower District, Yueyang City. With frequent human activities, the widespread use of antibiotics in the prevention and treatment of human diseases will lead to a high concentration of antibiotics in this area. Moreover, the sampling site X7 monitors the water quality at the outlet of East Dongting Lake, and the higher concentration at the outlet will affect the downstream lake. The aquaculture is developed in Huarong County with an aquaculture area of 8×10^8–1.2×10^9 m^2, which is close to sites X1 and X3. Seasonal drug use in the aquaculture industry may lead to seasonal differences in the concentration of antibiotics at this site. Low concentrations of antibiotics were detected at sites X11 and X13 in the national nature reserve, mainly due to their distance from densely populated areas and aquaculture

TABLE 2 | Contents of antibiotics in the surface sediment of East Dongting Lake.

Antibiotics	Spring (n = 6, ng/L)				Summer (n = 6, ng/L)				Fall (n = 13, ng/L)			
	Min.	Max.	Mean	Rate (%)	Min.	Max.	Mean	Rate (%)	Min.	Max.	Mean	Rate (%)
SDZ	0.03	0.67	0.18	100	0.02	0.12	0.06	100	0.21	2.42	0.54	100
SMZ	ND	0.03	0.02	83.3	ND	0.02	0.01	33.3	0.04	0.42	0.18	100
SMX	6.27	51.58	18.63	100	9.47	177.43	55.97	100	44.58	164.4	125.11	100
OTC	ND	54.78	9.6	33.3	ND	3.81	1.47	50	ND	30.62	5.93	92.3
TC	ND	5.98	0.98	16.7	ND	0.98	0.28	33.3	ND	8.92	1.78	23.1
CTC	ND	19.8	3.55	50	ND	3.91	1.01	33.3	ND	28.15	5.62	53.8
OFL	10.61	30.78	9.16	100	16.66	42.57	24.10	00	24.85	109.46	51.29	100
NOR	ND	0.49	0.32	83.3	ND	2.58	1.27	66.7	ND	3.41	1.34	46.2
CIP	ND	0.98	0.16	16.7	ND	ND	ND	0	ND	ND	ND	0
ENR	ND	0.28	0.12	50	ND	ND	ND	0	ND	4.76	3.44	92.3
ROM	2.55	35.22	12.61	100	0.11	0.71	0.38	100	0.64	2.46	1.16	100
ERM	0.72	7.12	3.13	100	ND	0.58	0.1	16.7	ND	1.09	0.13	15.4

"Rate" represents the detection rate; "ND" represents not detected.

areas, indicating that human activity intensity is also one of the important factors affecting the occurrence of antibiotics. Different from the research results in Nansi Lake (Zhang et al., 2020), the total antibiotic concentration downstream (X5–X9) was higher than that upstream with agriculture and aquaculture (X10–X14) in East Dongting Lake. It may be related to the fact that several sites of the lower lake are close to Yueyang Tower District and are greatly affected by urban sewage discharge.

3.2 Occurrence of Antibiotics in Surface Sediments

As were reported in existing studies, sediment could act as a crucial enrichment medium of pollutants including antibiotics and could increase the pollution levels and duration through second release effect (Chen and Zhou, 2014; Cheng et al., 2014). The concentrations of 12 antibiotics in the surface sediments of East Dongting Lake Basin are shown in **Table 2**. All the 12 antibiotics were detected. The detection rate of monomer varied from 0 to 100%, and the average detection rate was 63.6%, which was higher than the average detection rate in surface water (47.9%). The detection rate of SDZ, SMX, OFL, and ROM in three seasons was 100%. The 12 target antibiotics detected in the surface sediments of East Dongting Lake were generally in ng/g, and the concentrations ranged from ND to 177.43 ng/g. The order of average concentration was sulfonamide antibiotics (27.80 ng/g) > quinolone antibiotics (10.00 ng/g) > tetracycline antibiotics (3.66 ng/g) > macrolides (2.28 ng/g). The total concentration of SMX was the highest (111.76–1626.48 ng/g), followed by OFL (114.93–666.71 ng/g) and OTC (8.85–77.05 ng/g). SMX, OFL, and OTC were the main antibiotics in the sediments, and the contribution rate of antibiotics in East Dongting Lake reached 90.5%. Compared with the results of surface water, OFL and SMX are the main antibiotic compounds in water samples and sediments, indicating that these two classes of antibiotics are the main pollutants in East Dongting Lake Basin. Obviously, by natural sedimentation, the antibiotics in the water body could be absorbed, accumulated, and stored in sediments, which might lead to potential risk to aquatic organisms continuously.

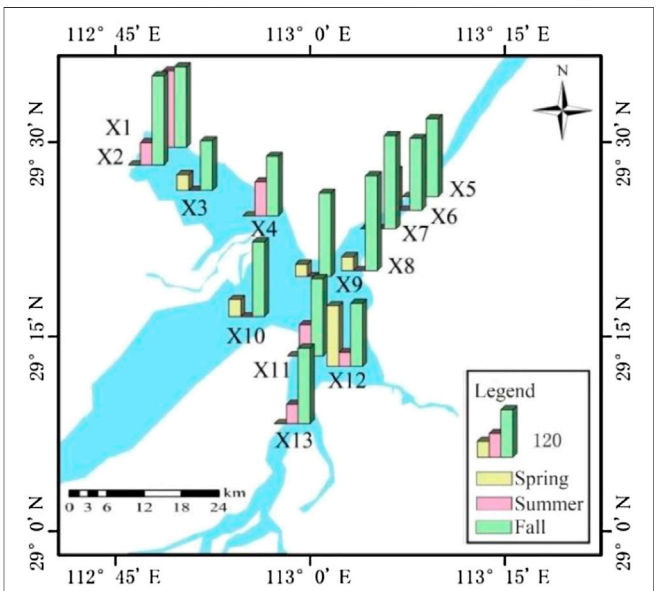

FIGURE 3 | Spatial distribution of antibiotic content in the sediment of East Dongting Lake in different seasons.

The average contents of antibiotics in the sediments of different seasons were in the order of fall (16.38 ng/g) > summer (7.05 ng/g) > spring (5.71 ng/g), which could be attributed to the consumption of antibiotics in different seasons (e.g., tetracyclines were commonly used in low-temperature seasons to prevent and treat respiratory infection and diarrhea) and also some environmental factors such as temperature-induced inhibited biodegradation and water flow rate (Kim and Carlson, 2007b; Loftin et al., 2008; Lu et al., 2018). In addition, the physical and chemical properties of the sediments, such as pH, C/N ratio, total organic carbon content, and particle size, can affect the adsorption behavior of antibiotics.

TABLE 3 | Ecological risk assessment parameters and RQs of antibiotics in East Dongting Lake water.

Antibiotics	PNEC (ng/L)	References	ME (g/L)			RQs in this study		
			Spring	Summer	Fall	Spring	Summer	Fall
SDZ	2220	González-Pleiter et al. (2013)	2.82	0.57	2.6	0.0013	0.0002	0.0012
SMZ	1277	Li et al. (2012)	0.32	0.09	2.8	0.0003	0.0001	0.0022
SMX	27	Pruden et al. (2012)	163.52	244.8	220.95	6.0563	9.0667	8.1833
OTC	1040	Liu et al. (2018a)	ND	ND	45.73	0	0	0.0440
TC	3310	González-Pleiter et al. (2013)	ND	ND	30.8	0	0	0.0093
CTC	5790	Liu et al. (2018a)	ND	ND	50.32	0	0	0.0087
OFL	21	Robinson et al. (2005)	943.49	49.82	486.59	44.9281	2.3724	23.1710
NOR	50,180	González-Pleiter et al. (2013)	ND	ND	12.17	0	0	0.0002
CIP	5	Robinson et al. (2005)	ND	ND	27.02	0	0	5.404
ENR	49	Robinson et al. (2005)	0.45	ND	63.75	0.0092	0	1.3010
ROM	100	Robinson et al. (2005)	17.98	0.35	110.66	0.1798	0.0035	1.1066
ERM	20	González-Pleiter et al. (2013)	54.12	12.56	ND	2.706	0.628	0

"ND" represents not detected.

Spatial distribution characteristics of antibiotic contents of surface sediments in East Dongting Lake are shown in **Figure 3**. It can be seen that there are significant spatial differences in antibiotic content levels. The cumulative content of antibiotics in spring, summer, and fall ranged from 31.46 ng/g to 156.23 ng/g, 35.69 ng/g to 197.15 ng/g, and 128.17 to 244.46 ng/g, respectively. The sampling site X12 monitors the water quality of input water from the Xinqiang River with the highest cumulative content of antibiotics in spring and the lowest in summer, which may be related to the seasonal use of antibiotics in this area, especially for respiratory infection and diarrhea treatment at low temperature (Matsui et al., 2008). The sampling site X1 is close to the area where aquaculture is more developed, and the widespread use of antibiotics may affect the content level here. The sampling site X8 monitors the pollutant content of the entry section in the main urban area of Yueyang City, which is affected not only by the water quality of the upper input Xinqiang River but also by the backwater quality of the Yangtze River. The antibiotic contents in the national nature reserve (site X3) and X9 are at a low level, which may be because they are far away from the exogenous input pollution source. Similar to the trend in the surface water, the total content of antibiotics downstream was higher than that upstream, which might be due to the sewage discharge in Yueyang City, water quality of the backwater of the Yangtze River, and enrichment of sediments.

3.3 Ecological Risk of Antibiotics

According to the PNEC estimation in the literature and the maximum measured antibiotic concentration in surface water, the ecological risk quotients of antibiotics in East Dongting Lake are evaluated (shown in **Table 3**). The results showed that OFL and SMX both have significantly high risk in three seasons, CIP, ENR, and ROM have high ecological risk in fall, and the risk level of ERM is high in spring and medium in summer. Algae play an important role in aquatic ecosystems and provide nutrients for other organisms. The high ecological risk of antibiotics can affect the growth of algae, worsen water quality, and disrupt the balance of aquatic ecosystems. Seasonal variations in antibiotic concentrations may cause seasonal variations in ecological risk. SDZ, SMZ, OTC, TC, CTC, and NOR show no or low risk to algae. Although the risk is low, they might induce the generation of drug-resistant bacteria or resistance genes in the water environment, enter the human body through the food chain, and pose a threat to human health. According to previous studies, SMX presented moderate risk in Gonghu Bay, Taihu Lake (Xu et al., 2014), while OFL presented low risk in Datong Lake (Liu and Lu, 2018). More attention should be paid to the high risk of antibiotics in the Dongting Lake basin such as OFL and SMX.

3.4 Occurrence of Antibiotic Resistance Genes in Surface Water

The absolute abundance of ARGs and *int1* in the surface water of East Dongting Lake in spring, summer, and fall is shown in **Figure 4**. The absolute abundance ranges from 0 to 4.88×10^4 copies/ml. The order of mean absolute abundance is as follows: sulfonamide ARGs (5.91×10^3 copies/ml) > integron *int1* (5.22×10^3 copies/ml) > tetracycline ARGs (4.59×10^2 copies/ml) > quinolone ARGs (3.54×10^2 copies/ml) > macrolide ARGs (5.22×10^3 copies/ml). The detection concentration of different types of ARGs is different, which may be the result of the combined action of various factors such as antibiotic residue concentration positive pressure (Caucci et al., 2016; Sui et al., 2017), microbial content, aquatic animals, and plants. The average absolute abundance of *sul1* was 9.78×10^3 copies/ml, followed by *int1* (5.22×10^3 copies/ml) and *sul2* (2.03×10^3 copies/ml). The average absolute abundance of *ermB* was only 2.73×10^1 copies/ml. Therefore, *sul1* and *sul2* were the dominant ARGs in the surface water of East Dongting Lake. Compared with other resistance genes, *sul1* and *sul2* genes might have a wider range of sources, which are generally located on large transmissible multi-resistant or small non-conjugative plasmids, and they are found in various plasmids, showing increased risk to ecological health (Frank et al., 2007; Gao et al., 2012).

The characteristics of the bacterial abundance were analyzed based on 16S rRNA. The absolute abundance of 16S rRNA in the surface water of East Dongting Lake Basin in spring, summer, and fall is shown in **Figure 5**. The absolute abundance of 16S rRNA ranged from 6.72×10^5 to 1.32×10^7 copies/mL in spring and 9.18×10^4 to 1.50×10^7 copies/ml in summer. The absolute abundance

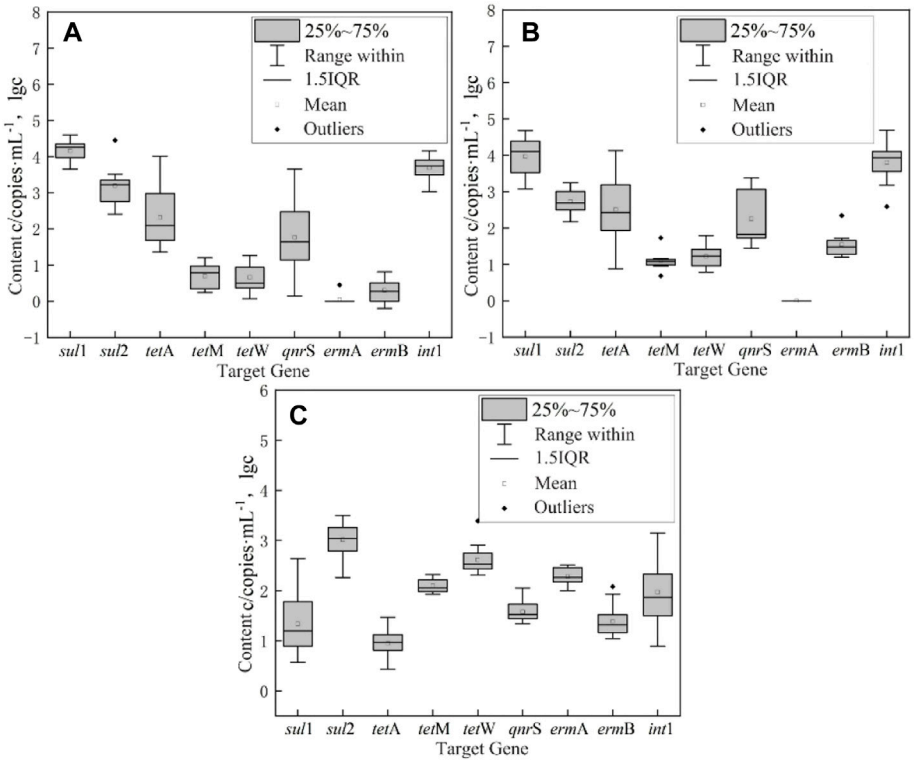

FIGURE 4 | Box-plot of absolute abundance of ARGs and integron *int1* in the surface water of East Dongting Lake in spring **(A)**, summer **(B)**, and fall **(C)**.

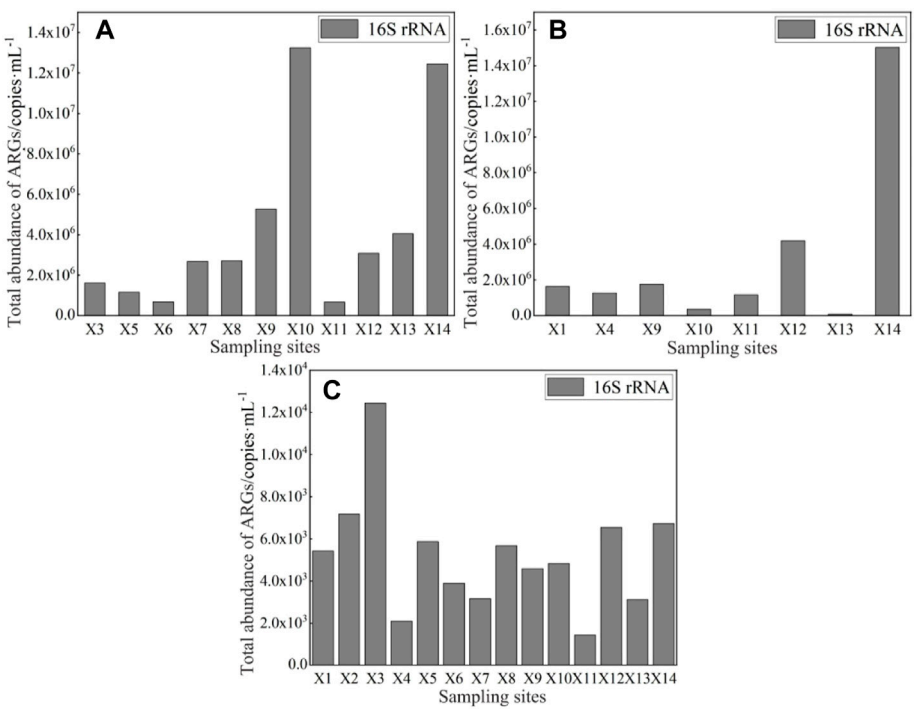

FIGURE 5 | Absolute abundance of 16S rRNA in the surface water of East Dongting Lake in spring **(A)**, summer **(B)**, and fall **(C)**.

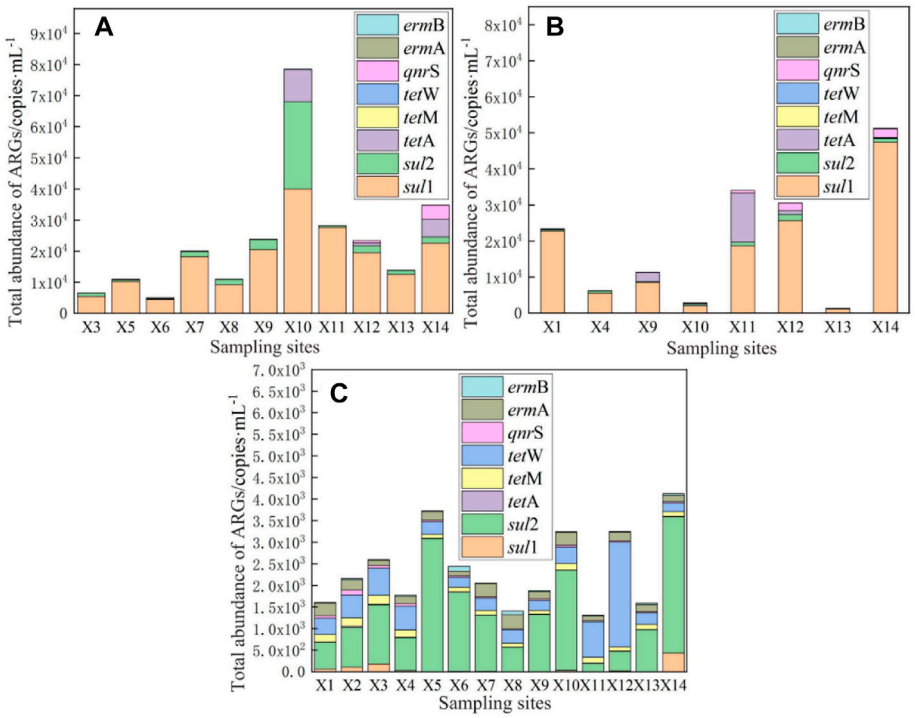

FIGURE 6 | Total abundance of ARGs in the surface water of East Dongting Lake in spring **(A)**, summer **(B)**, and fall **(C)**.

of 16S rRNA in fall ranged from 1.43×10^3 to 1.24×10^4 copies/ml. The average absolute abundance of 16S rRNA was 4.33×10^6 copies/ml in spring >3.19×10^6 copies/ml in summer >5.21×10^3 copies/ml in fall. The differences in the absolute abundance of 16S rRNA in different seasons may be influenced by various factors, including water temperature, flow conditions, and human activities. The absolute abundance of 16S rRNA in spring and summer was significantly higher than that in fall, which partly may be related to the high temperature and nutrient conditions in spring and summer, which are conducive to the growth and reproduction of microorganisms in the water environment and thus result in a greater number of copies of ARGs (Jiang et al., 2011; Knapp et al., 2012; Calero-Caceres et al., 2017).

The total abundance of ARGs in the surface water of East Dongting Lake is shown in **Figure 6**. It can be seen that the absolute abundance of ARGs has an obvious spatial difference. The total abundance of ARGs in spring, summer, and fall ranged from 5.08×10^3 to 7.85×10^4 copies/ml, 1.38×10^3 to 5.13×10^4 copies/ml, and 1.31×10^3 to 4.12×10^3 copies/ml, respectively. The spatial distribution of ARGs in East Dongting Lake was closely related to antibiotic pollution levels, pollution sources, and environmental factors. The sampling site X1 was located in the East Dongting Lake Bird Nature Reserve, and the total abundance of ARGs was at a medium level in both spring and summer. The migration of birds will carry ARGs from the external environment into East Dongting Lake, which will affect the abundance level of ARGs in the lake. Therefore, the influence of biological transport by birds on ARGs in the lake basin should be a cause for concern. Sampling sites X7, X1, and X3 were the points with the highest cumulative concentrations of antibiotics in the surface water of East Dongting Lake in spring, summer, and fall, respectively, while medium or low levels of ARGs were detected there. The opposite phenomena were also reported in other studies (Jiang et al., 2011, 2013; Knapp et al., 2012; Calero-Caceres et al., 2017), indicating that the seasonal distribution relationship of antibiotics and ARGs was complex and the antibiotic exposure level was not the only factor affecting the occurrence of ARGs.

3.5 Occurrence of Antibiotic Resistance Genes in Surface Sediments

The detection rates of eight ARGs and *int1* integron gene in the surface sediments of East Dongting Lake in three seasons were as follows: the detection rate was 17 and 33% for *ermA* in spring and summer, respectively, and 100% for other seven ARGs. The detection rate of all target genes was 100% in fall.

The absolute abundance of ARGs and *int1* in the surface sediments of East Dongting Lake in spring, summer, and fall is shown in **Figure 7**. The absolute abundance ranges from 0 to 2.74×10^9 copies/g. The order of average absolute abundance was sulfonamide ARGs (5.67×10^7 copies/g) > integron *int1* (1.40×10^7 copies/g) > tetracycline ARGs (3.80×10^5 copies/g) > quinolone ARGs (3.14×10^5 copies/g). The average absolute abundance of *sul2* was 1.08×10^8 copies/g, followed by *int1* (1.40×10^7 copies/g) and *sul1* (5.76×10^6 copies/g), which was the same trend as that in surface water. The water body and sediment are two closely related parts of the lake water environment. ARGs have dynamic equilibrium between water and sediment and can be transferred

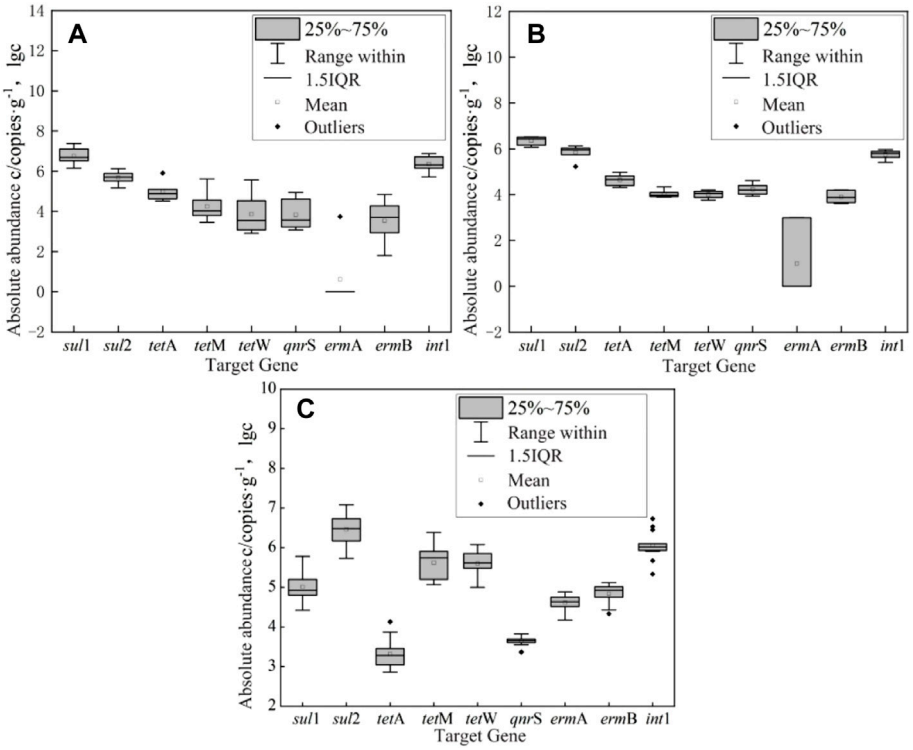

FIGURE 7 | Box-plot of absolute abundance of ARGs and integron *int1* in sediments of East Dongting Lake in spring **(A)**, summer **(B)**, and fall **(C)**.

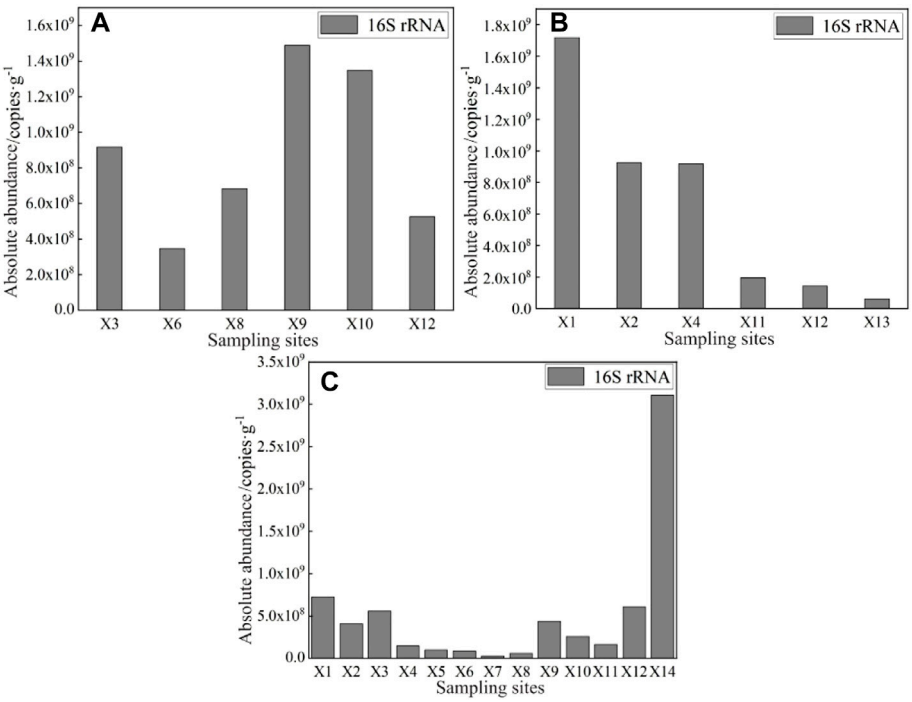

FIGURE 8 | Absolute abundance of 16S rRNA in surface sediments of East Dongting Lake in spring **(A)**, summer **(B)**, and fall **(C)**.

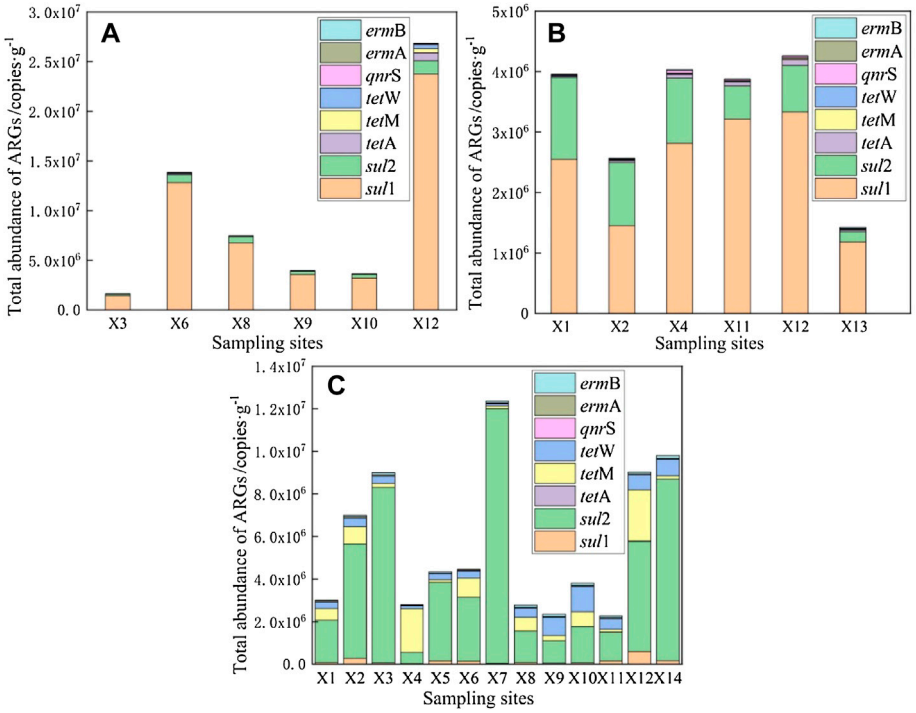

FIGURE 9 | Total abundance of ARGs in surface sediments of East Dongting Lake in spring **(A)**, summer **(B)**, and fall **(C)**.

to each other. Combined with the results of surface water and sediment detection, *sul1* and *sul2* were the dominant ARGs in East Dongting Lake Basin, which was in agreement with previous studies in Poyang Lake (Liang et al., 2020) and Huangpu River (Jiang et al., 2013). *Int1* was reported to act as an environmental marker for anthropogenic pollution and also could promote the widespread antibiotic resistance through horizontal gene transfer (Zhu et al., 2013; Gillings et al., 2015; Liu et al., 2018c). The high *int1* abundance in both water and sediment in our study revealed potential horizontal gene transfer in multi-media in East Dongting Lake.

The absolute abundance of 16S rRNA in surface sediments of East Dongting Lake Basin in spring, summer, and fall is shown in **Figure 8**. The absolute abundance of 16S rRNA ranged from 3.47×10^8 to 1.49×10^9 copies/mL in spring and 6.05×10^7 to 1.72×10^9 copies/mL in summer. The abundance range of 16S rRNA in fall was 2.62×10^7 to 3.10×10^9 copies/g. The average abundance in spring (8.85×10^8 copies/g) > summer (6.60×10^8 copies/g) > fall (5.14×10^3 copies/g) was the same as that in surface water. The absolute abundance of 16S rRNA in the sediments was 2–5 orders of magnitude higher than that in surface water. Generally, it was due to the adsorption of flowing components containing ARGs into suspended particles and accumulation in the sediments. On the contrary, the higher nutrient, temperature, and antibiotic concentration selective pressure in the sediments could promote the growth of microorganisms and induction of resistance genes.

The total abundance of ARGs in the surface sediments of East Dongting Lake is shown in **Figure 9**. The spatial difference of absolute abundance of ARGs was obvious. The total abundance of ARGs in spring, summer, and fall ranged from 1.65×10^6 to 2.68×10^7 copies/g, 1.42×10^6 to 4.26×10^6 copies/g, and 2.27×10^6 to 1.24×10^7 copies/g, respectively. The abundance of ARGs in the sediments of East Dongting Lake was affected not only by the concentration of antibiotics but also by the surrounding pollution sources. The total abundance of ARGs at X12 was at the highest level in both spring and summer. The highest level in spring may be because of the selective pressure by cumulative concentration of antibiotics at this site, which would induce the production of ARGs and increase the abundance of ARGs, while the cumulative concentration of antibiotics was the lowest here in summer. We speculate that the good temperature and flow conditions are beneficial to the biodegradation and migration of antibiotics, leading to the low pollutant content but high ARG abundance at this site. The sampling site X7 monitors the water quality at the outlet of East Dongting Lake. A higher concentration of pollutants will affect the water quality of downstream lakes. The total ARG abundance upstream was greater than that downstream, which was the same as that detected in surface water, indicating that the ARG abundance level in the sediments may also decrease with the pollutant migration process. In addition, for close to the outlet of the lake, the fast water flow rate led to a degree of dilution of the antibiotics and resistance genes. Furthermore, anthropogenic activities might lead to the different characteristics of the upstream and downstream sites (Li et al., 2017; Lu et al., 2018).

4 CONCLUSION

This study quantitatively analyzed the occurrence and distribution of antibiotics and ARGs in surface water and

sediment samples in East Dongting Lake by season. The results revealed that the concentrations of antibiotics were relatively low in East Dongting Lake, while their high detection rates and potential ecological risks were worthy of attention. Moreover, the concentration variation in antibiotics could produce a positive pressure in the abundance of ARGs. There exists significant media exchange between surface water and sediment in antibiotics and ARGs. The differences in hydrological conditions, temperature, rainfall, surrounding human activities, and environmental conditions probably led to different distribution characteristics and seasonal variations. It is necessary to analyze the main pollution sources, e.g., typical land pollution source (livestock and poultry breeding area, aquaculture area, and sewage treatment plant) and atmospheric subsidence source, for antibiotics and ARGs in East Dongting Lake Basin to identify the main pollution sources and control areas accurately.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

XG, SL and XL conceived and designed the study. RS performed the experiments. XG, RS and JC analyzed the data and wrote the paper. All authors contributed to the editing of the manuscript.

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SUPPLEMENTARY MATERIAL

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OPINION
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Antimicrobials and Antibiotic Resistance Genes in Water Bodies: Pollution, Risk, and Control

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The manuscript endeavors to provide a perspective on the role of water bodies in the spread of antimicrobial (antibiotic) resistance (AMR), antimicrobial resistant bacteria (ARB), and antimicrobial resistance genes (ARGs) among pathogens, animals, and humans. We briefly indicate how the AMR problem is globally affecting public health, along with strategies and mechanisms to combat the dissemination of ARB and ARGs. A brief systematic survey of the literature (2015-onwards) for the presence of antimicrobial residues and the occurrence of ARGs and antimicrobial resistant microorganisms in different water bodies/sources indicates the gravity of the situation and suggests their important role in the occurrence and spread of AMR, ARB, and ARGs. The prevalent water treatment methods which tend to reduce ARB and ARGs from water resources are unable to remove them completely, allowing the problem of AMR to continue and spread to organisms of concern. In this opinion article, we attempt to underline the key role of controlling the release/discharge of antimicrobial contaminants in water bodies and their buildup in checking the development and spread of AMR. The reduction in the release of antibiotic residues in the environment, especially water bodies, combined with the development of improved surveillance means and efficacious treatment/removal/decomposition methods could help curb the menace of AMR effectively. We suggest the expansion of the ambit of 'One Health Approach to AMR crises proposed by the World Bank, 2021 to include the 'reduction of antimicrobial contamination of the environment' as the 'seventh domain' of activity to effectively achieve its objective.

Keywords: antibiotic misuse, antimicrobial resistance genes, antimicrobial resistance, pollution, environment, wastewater, water bodies

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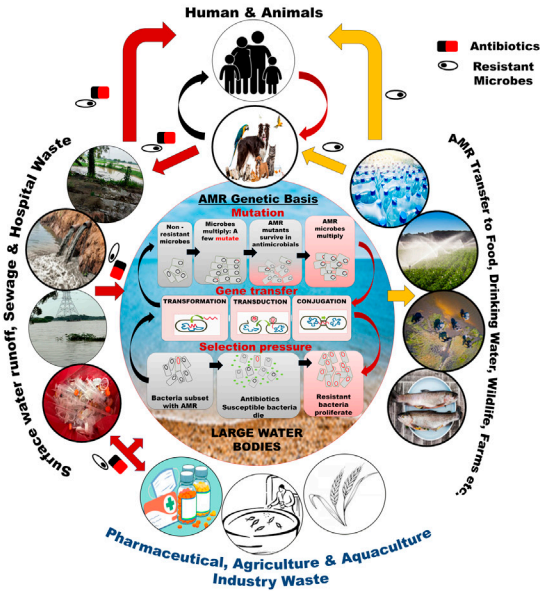
INTRODUCTION

Aquatic ecosystems are very important to maintain the high levels of biodiversity, livelihood, and productivity of the biosphere (Hossain et al., 2018; Vilca and Angeles, 2018; Irfan and Alatawi, 2019; Hassan et al., 2020). The presence of antimicrobials (antibiotics etc.), antimicrobialresistant bacteria (ARB), and antimicrobial resistance genes (ARGs) in the aquatic environment is becoming a cause of great concern as the possibility of development of antibiotic-resistant pathogens, even superbugs, is increasingly posing problems to the environment and human health (Ma et al., 2015; Wang et al., 2020; Zhuang et al., 2021). It is recognized that aquatic environments are one of the key reservoirs and transmission routes for the spread of antimicrobial/antibiotic resistance (AMR/AR) (Amarasiri et al., 2020). Antibiotics reach the environment *via* feces and urine of humans and animals, inappropriate disposal of unused drugs, and direct environmental contamination by waste material from antibiotic

A



B

S. No.	Domains	Activity
1	Awareness Raising	Improve awareness, understanding, and knowledge of AMR through communication, education, and training at local, national, and regional levels.
2	Antimicrobial Stewardship	Promote the optimal use of antimicrobial agents through appropriate prescribing in diverse health care settings, especially in primary care.
3	Reduction of Antimicrobial Contamination of the Environment #	Reduce antimicrobial contamination of environments which is potentially driving AMR selection and spread through identification, monitoring, regulation and implementation of activities and products/innovations that could help minimize antimicrobial residues in the environment (soil, air, water)
4	Surveillance, monitoring, and research for national surveillance systems with a One Health approach	Under a One Health approach, strengthen knowledge and evidence through AMR and associated public health surveillance, monitoring, and research for national systems.
5	Infection prevention and control in human and animal health	Reduce infections (including hospital- and community-acquired infections) in humans and animals.
6	Reduction of the spread of pathogens into the environment	Identify, monitor, and regulate environmentally related activities or products that result in the spread of AMR pathogens
7	Operational Research Agenda	Strengthen and sustain operational research capacity for evidence-based decision-making to improve program performance.

Notes:1. #Domain "Reduction of Antimicrobial Contamination of the Environment" is suggested by us.
2. As per World Bank Group (2018), "a One Health approach is: a collaborative approach for strengthening systems to prevent, prepare, detect, respond to, and recover from primarily infectious diseases and related issues such as antimicrobial resistance that threatens human health, animal health, and environmental health collectively, using tools such as surveillance and reporting with an endpoint of improving global health security and achieving gains in development" [WHO, 2018].

FIGURE 1 | (A) Antimicrobial resistance development and transmission of ARGs and ARBs. **(B)** One health approach to AMR crises: Seven domains and activity for guiding the data collection, analyses, and action.

production units (Amaya et al., 2012; World Health Organization, 2017a; United Nations Environment Programme, 2022). Antimicrobials/antibiotics exert selection pressure, accelerating the development of ARB resistant to the used antimicrobial and related compounds (Kolář et al., 2001; Ayukekbong et al., 2017; Serwecinska, 2020). All antibiotics put to use consistently end up in the environment, further accelerating the pace of AMR development (Larsson and Flach, 2021). Globally, AR has been frequently reported from freshwater sources (Kumar et al., 2013; Abdel Rahim et al., 2015; Jabbar Ibrahim and Kareem Hameed, 2015; Guzman-Otazo et al., 2019; Singh et al., 2020; Subbiah et al., 2020), wastewater systems including but not limited to pharmaceutical industries, and wastewater treatment plants (WWTPs) (Ferreira Da Silva et al., 2007; Tesfaye et al., 2019; Adegoke et al., 2020; Obayiuwana and Ibekwe, 2020; Praveenkumarreddy et al., 2020). AMR has emerged as one of the key public health problems of the 21st century that overshadows the efficacy of available effective treatments against a large number of pathogens which are increasingly no longer susceptible to common antimicrobials (Prestinaci et al., 2015). The AMR problem is increasing rapidly and becoming more critical with each passing day. Pathogens causing different common infections have been consistently acquiring and displaying a varying degree of resistance to most of the new antibiotics within <5–10 years of their introduction into the market (**Supplementary Table S1**). AMR is observed in bacteria, fungi, viruses, and parasites as they get adapted to multiply in the presence of antimicrobials (Founou et al., 2017; Dadgostar, 2019). The infection with AMR pathogens is supposed to escalate healthcare costs and treatment failures and cause up to 10 million more deaths annually by 2050 (Dadgostar, 2019; Amarasiri et al., 2020). The World Health Organization (WHO) has declared that due to increasing AR, we are almost out of treatment options (World Health Organization, 2017b; World Health Organization, 2021). Seeing the growing threat of AMR, the WHO has proposed a six-point plan, that is, "One Health Approach" (OHA) (World Bank Group, 2018; Mazimba et al., 2021). The OHA is envisaged as "involvement of human health, animal health, and environmental health and focus on those infectious disease-related issues (including AMR) that undermine overall health and well-being" (World Bank Group, 2018; Mazimba et al., 2021).

Numerous studies across the globe have reported the prevalence of ARB in different water bodies (Kumar et al., 2013; Abdel Rahim et al., 2015; Jabbar Ibrahim and Kareem Hameed, 2015; Guzman-Otazo et al., 2019; Singh et al., 2020; Subbiah et al., 2020) and wastewater systems (Ferreira Da Silva et al., 2007; Tesfaye et al., 2019; Adegoke et al., 2020; Obayiuwana and Ibekwe, 2020; Praveenkumarreddy et al., 2020). The inability of different drinking water treatments and WWTPs to completely remove ARGs and ARBs from water allows for their buildup in large water bodies (Alexander et al., 2020; Amarasiri et al., 2020). Considering the central role of water bodies in the development and spread of AMR (**Figure 1A**), the active monitoring of antimicrobial residues in the environment and control of disposal into the environment are suggested to help reduce the rate of AMR emergence/development (**Figure 1A, B**).

ANTIMICROBIAL RESISTANCE EMERGENCE AND SPREAD

Microbial genome plasticity supported by numerous genetic mechanisms such as conjugation, transformation, and

transduction enables them to evolve, adapt, and survive in environments contaminated with antibiotics. The development of AMR in microbes results from selection pressure, mutation, and gene transfer (Serwecinska, 2020; Larsson and Flach, 2021; Michael et al., 2014; Caniça et al., 2019; Van Hoek et al., 2011; Samreen et al., 2021; Kunhikannan et al., 2021; Amábile-Cuevas, 2021; Sriram et al., 2021; von Wintersdorff et al., 2016) (**Supplementary Table S2**), whereas conjugation supposedly remains the most frequently used mode of ARG transmission (**Figure 1A**) (von Wintersdorff et al., 2016).

Selection pressure determines the occurrence, amplification, and dissemination of ARGs in the environment and pathogens. Even low concentrations of antimicrobials/antibiotics can result in the selection of ARGs—making the establishment of a safe concentration of any antimicrobial compound in the environment a challenging task (Wang et al., 2020; Zhuang et al., 2021; Stanton et al., 2020; Yang et al., 2018). Several culture-independent studies on animals, food, humans, and environmental samples had shown the presence of huge reservoirs of ARGs (i.e., resistome) that could be potentially mobilized and transferred to other organisms (Abdel Rahim et al., 2015; Subbiah et al., 2020; Obayiuwana and Ibekwe, 2020; Adzitey, 2020; Forsberg et al., 2012; Hu et al., 2016; Abdel-Rahman et al., 2020; Meng et al., 2020; Morris and Cerceo, 2020; Balakrishna et al., 2017; D'Costa et al., 2011). Aquatic environments are identified as ideal settings for the acquisition and dissemination of AMR/AR. Human exposure to ARB and ARGs from aquatic environments poses an additional health risk (Karkman et al., 2018; Suzuki et al., 2017; Wellington et al., 2013; Leonard et al., 2018; Søraas et al., 2013; Leonard et al., 2015; O'Flaherty et al., 2018). Drinking water and wastewater treatment processes are mostly inadequate to remove ARGs (Li et al., 2015; Rodriguez-Mozaz et al., 2015; McGowan, 2007). The WWTP effluents, agriculture runoffs, *etc.* comprising ARB and ARGs can end up in aquatic environments such as lakes and rivers (**Figure 1A**). Usage of domestic wastewater in agricultural irrigation and recreational activities can introduce new ARB and ARGs to the specific environment (Leonard et al., 2018; Søraas et al., 2013; Leonard et al., 2015; O'Flaherty et al., 2018; Rodriguez-Mozaz et al., 2015; McGowan, 2007; Ben et al., 2017). In addition to drinking water, humans can be exposed to ARB and ARGs *via* different activities such as aquatic sports, bathing, occupational exposure during agricultural irrigation, and consumption of food produce from fields irrigated with reclaimed water (Leonard et al., 2018; Søraas et al., 2013; Leonard et al., 2015; O'Flaherty et al., 2018). However, the extent of human health risk resulting from exposure to ARB and ARGs present in aquatic environments remains poorly understood. It is primarily due to specific information such as the dose–response curves and exposure assessment data related to ARB and ARGs in different water usage scenarios being a prerequisite to conducting a quantitative microbial risk assessment (Ashbolt et al., 2013; Pepper et al., 2018).

ANTIMICROBIAL RESISTANCE IN WATER BODIES: CAUSE AND IMPACT

Antimicrobial Resistance in Aquaculture

The aquaculture field heavily relies on the application of antibiotics either directly in water or mixed with fish food to control infections, causing explosive growth of ARGs in farmed aquatic animals and environments (Heuer et al., 2009; Hinchliffe et al., 2018; Preena et al., 2020). Many antibiotics used in aquaculture are critically important for human treatment, for example, tetracycline, macrolides, and aminoglycosides (World Health Organization, 2021). Several studies indicated the indirect transfer of ARGs from fish origin antibiotic-resistant microbes to human pathogens such as *E. coli, Salmonella spp*., and *Aeromonas* spp through culture-independent studies (West et al., 2008; Heuer et al., 2009; Zou et al., 2012; Amarasiri et al., 2020). The presence of ARGs in *S. enterica* serotype *Typhimurium* DT104 isolates that caused salmonellosis outbreaks in Europe and the United States is also suspected to originate from the aquaculture system (Zou et al., 2012). The incidence of MDR in *Vibrio alginolyticus* and *Vibrio parahaemolyticus* isolates from farmed fishes in Korea further reaffirms the prevalent gene transfer phenomenon (Oh et al., 2011). Metagenomics or culture-independent studies have reported the occurrence of ARGs of different classes in marine sediments, suggesting a vital role for them in lateral gene transfer (Yang et al., 2013; Jiang et al., 2017; Karkman et al., 2018; Lerminiaux and Cameron, 2019; Sun et al., 2019).

Antimicrobial Resistance in Fresh and Wastewater

Freshwater bodies such as rivers, streams, springs, and lakes continuously receive antimicrobials/antibiotics, ARBs, and ARGs through different sources such as effluents from WWTPs, chemical manufacturing plants, animal husbandry, aquaculture, etc. Several studies had reported the presence of different antibiotics and ARGs in surface and groundwater sources (Liang et al., 2013; Shimizu et al., 2013; Ma et al., 2015; Matongo et al., 2015; Deng et al., 2016; Fernando et al., 2016; Madikizela et al., 2017; Danner et al., 2019) (**Supplementary Table S3A**). Even the bacterial communities of *Pseudomonas, Acinetobacter, Bacillus, Arthrobacter, Xanthomonas,* and *Flavobacterium* isolated from Eastern Siberian permafrost sediments had been shown to harbor several ARBs and ARGs by culture-dependent methods (Mindlin et al., 2008). Similarly, ARBs showing resistance to different classes of antibiotics had also been reported from freshwater samples of Antarctica and Siberian lakes (Lobova et al., 2011; Jara et al., 2020).

Wastewater remains a major reservoir of AMR in the environment as it allows ARBs with ARGs to persist and transfer the ARGs in the environment *via* different mechanisms (Rizzo et al., 2013; Fouz et al., 2020) (**Figure 1A**; **Supplementary Table S2**). The prevalent wastewater treatment methods that decrease the ARBs only have a limited impact on ARGs present in the environment (Ben et al., 2017; Hiller et al., 2019a; Singh, 2020). The ARGs present in the environment can get transmitted through horizontal gene transfer (HGT) to different organisms, including the medically important ones (Singh, 2020; Woolhouse et al., 2015). The environment assists the transfer of ARGs from one component to another, *viz.*, animals, soil, water, sediments, and sewage (**Figure 1A**)

(Wellington et al., 2013; Balcazar, 2014; Berglund, 2015; Fouz et al., 2020). ARGs are supposedly ubiquitous. However, their concentration may vary in different environments. The transfer of ARGs is not only limited to closely related species or genera but also occurs among phylogenetically distant species (Jiang et al., 2017). It leads to the ceaseless emergence of new variants of AMR organisms (Bouki et al., 2013; Hocquet et al., 2016; McKinney et al., 2018). The presence of ARGs in the environment represents a more complex and challenging problem concerning containment as they are not degradable and can be easily transmitted (Treangen and Rocha, 2011; Stecher et al., 2012; Beceiro et al., 2013; Hiller et al., 2019b; Ibrahim et al., 2019; Galhano et al., 2021; Koch et al., 2021; Zhuang et al., 2021; Grenni, 2022).

The major source of ARB and ARGs are human and veterinary clinical settings where intestinal bacteria encounter a high concentration of antibiotics, along with the associated WWTPs and land wastes (Karkman et al., 2018; Salyers et al., 2004; Ishikawa et al., 2018; Hendriksen et al., 2019). Bacteria passing *via* the intestinal tract can acquire AR through conjugation and transformation before ending up in human and animal feces (**Figure 1A**) (Salyers et al., 2004; Anderson et al., 2006). The effluent discharges from WWTPs to different environments where the environmental microorganisms can interact with ARB and ARGs may act as primary places of AMR development (Ben et al., 2017; Lee et al., 2017; Fouz et al., 2020; Manoharan et al., 2021).

Antimicrobial Resistance in Marine Environments

The mechanisms responsible for the occurrence of ARBs and ARGs in marine environments can be different from those of fresh water and wastewater. As per a report, marine environments contain about 28% of the ARGs (Hatosy and Martiny, 2015). The major source of increased AMR occurrence in the marine environment is the coastal runoff of the ARBs from the terrestrial environment (Hatosy and Martiny, 2015). In addition, anthropogenic activities are causing direct antibiotic residue outpourings into marine systems, for example, Chilean marine salmonid farms alone had used about 363.4 tons of antibiotics in 2016 that can act as a selection pressure for the development of AR in marine environments (Miranda et al., 2018). Metagenomics studies had reported the same ARGs in the intestines of Baltic Sea farm fishes and farm sediments; the possible reason suggested behind the observation is the usage of antibiotics during the hatching and rearing of juvenile fish or the acquisition of the ARGs by fishes from marine microorganisms in the farms (Rosenfeld and Zobell, 1947; Baam et al., 1966; Miranda et al., 2018; Tortorella et al., 2018). The potential bidirectional transfer of ARGs between these aquatic environments and humans cannot be ruled out.

Studies using both culture-dependent and culture-independent approaches suggest global contamination of the water environments including open oceans and widespread presence of ARBs (Shimizu et al., 2013; Hatosy and Martiny, 2015; Segura et al., 2015; Fekadu et al., 2019). In the natural aquatic environment, bacteria can develop AR due to induced mutagenesis at a low concentration of antibiotics (Kohanski et al., 2010). Although the fraction of resistant mutants is very low, the accelerated selection of ARBs could occur over generations (Gullberg et al., 2011) due to continued antimicrobial presence. Accordingly, attention should be paid to water environments as a key to the origin and spread of ARBs and ARGs.

ANTIMICROBIAL RESISTANCE IN WATER AND PUBLIC HEALTH

It was observed that AMR, including multiresistance and pan-resistance, is rapidly spreading in bacteria, leading to severe infections untreatable with current antimicrobials (World Health Organization, 2014; World Health Organization, 2015; George, 2019). The spread of AMR in the environment had received comparatively less attention as compared to the spread of AMR pathogens in animals and humans (Baekkeskov et al., 2020). There are two types of AMR in bacteria, that is, acquired AMR and intrinsic AMR (World Health Organization, 2015; Baekkeskov et al., 2020) (**Supplementary Table S2**). The release of antimicrobial compounds into the environment allows it to come in direct contact with the naturally occurring microbes and act as a driving force for microbial evolution and the emergence of more resistant strains (European Centre for Disease Prevention and Control, 2019; Graham et al., 2019; Taneja and Sharma, 2019; Singh, 2020).

AMR is beginning to endanger public health worldwide (Prestinaci et al., 2015; Founou et al., 2017; Centers for Disease Control and Prevention, 2019; World Health Organization, 2021; The World Bank, 2021). Infection with AMR pathogens causes serious illnesses requiring longer hospital stays and increased healthcare costs due to the higher cost of second-line drugs and sometimes treatment failure (Llor and Bjerrum, 2014; Prestinaci et al., 2015; Centers for Disease Control and Prevention, 2018; Shrestha et al., 2018; European Centre for Disease Prevention and Control, 2019). AMR in common infections heavily impacts immunocompromised individuals and those undergoing treatments such as chemotherapy, dialysis, joint replacement, surgery, *etc.* (Centers for Disease Control and Prevention, 2018; Centres for Disease Control and Prevention, 2021).

Globally, the frequent incidences of infection with multidrug-resistant Gram-negative bacteria (MDR-GNB) and Gram-Positive bacteria are posing treatment challenges (Llor and Bjerrum, 2014; Bassetti et al., 2019; Ramírez-Castillo et al., 2018; Annavajhala et al., 2019; Centers for Disease Control and Prevention, 2019; World Health Organization, 2017a; Viney et al., 2021). The MDR cases are projected to become a serious issue by 2040 (Friedrich, 2017; Salvatore et al., 2019; Viney et al., 2021). Common pathogens of concern, namely, *Enterococcus faecium, Helicobacter pylori, Neisseria gonorrhoeae, Campylobacter* spp, *etc.* are currently included in the list of priority pathogens by the WHO for the development of new

antibiotics due to rapid development of AMR in these pathogens (World Health Organization, 2017b).

The municipal wastewaters contain a high concentration of organic and inorganic matter that supports the growth of AMR microorganisms, further promoting the spread of ARGs and AMR (Da Silva et al., 2006; Baquero et al., 2008; Exner et al., 2017; Karkman et al., 2018). Unrestricted discharge of untreated urban waste had been contributing to an overall rise of ARBs and ARGs in the environment (Da Silva et al., 2006; Moura et al., 2009; Osińska et al., 2016). The WWTPs are the meeting point of most of the ARBs, especially in those processes in which activated sludge or percolator biological filter are used for biological treatment (Baquero et al., 2008; Karkman et al., 2018; Kumar and Pal, 2018). Some studies had shown a higher percentage of MDR bacteria in the effluent than the effluent of treated wastewater (Osińska et al., 2016; Exner et al., 2017). Generally, wastewater treatment regulates the level of bacterial count, but due to differences in treatment plant designs and operations, the fate of ARBs and ARGs may remain unaffected or amplified.

The bacterial communities proliferate in drinking water distribution system pipes even after chlorination (Korzeniewska et al., 2013; Razavi et al., 2017). As the process of chlorination initially lowers the total load of microbes, it may significantly increase the level of ARBs. Expectedly, the effect of chlorination on the secondary effluent of WWTPs had been found to cause reactivation of ARBs (Zhang et al., 2009). The plausible reason for ARB increase could be a decline of antibiotic-susceptible bacteria or the selective rise of the ARB population in wastewater. However, the potential threat to public health from ARBs, whether from reactivation or regrowth, calls for more intensive research on the phenomenon (Blasco et al., 2008; Martinez, 2009; Munir et al., 2011; Huang et al., 2012; Harnisz, 2013; Klanicova et al., 2013). A crucial point of intervention for environmental AMR management could be the removal of ARB and ARG contaminants from wastewater effluents that pose a direct threat to negatively impact other water resources. The enhancement of wastewater treatment technologies and rational use of antibiotics should be promoted to minimize the threat of pathogenic ARB emergence and infection.

The regular uptake of antibiotics through several environmental sources changes the composition of gut microbiota composition and induces the growth of ARB in human and animal gut (Cho and Blaser, 2012; Francino, 2016). This gut microbiota inequity leads to the growth of several AR pathogenic and opportunistic bacteria with the possibility of them evolving into superbugs whose infection could not respond to treatments and lead to untimely death (Cho and Blaser, 2012; Ben et al., 2019). There is a growing need to understand the relationship between antimicrobial/antibiotic exposure and the human microbiome, and its functional aspect related to health (Ben et al., 2019).

The extent of the growing global AMR problem can be gaged by a systematic search of the databases, *viz.,* Web of Science, JSTOR, and PubMed using a combination of pertinent keywords such as antimicrobial, antibiotic resistance gene, water, environmental factors, antibiotics, heavy metals, water bodies, pollutants, etc. for the original research article and review articles. A brief systematic literature search performed for the antibiotic levels, ARBs, and ARGs in fresh and marine water on 17th February 2022 for articles published 2015 onwards (**Supplementary Table S3B, S4**) indicates the widespread presence of the residues of different antimicrobials (amoxicillin, penicillin tetracycline, ofloxacin, ciprofloxacin, *etc.*), a large number of ARGs (tetA, tetB, sulI, qnr, aadA, tetO, ampC, etc), and ARBs of concern (*E. coli, Enterococcus, Salmonella, Shigella, Aeromonas, Vibrio,* etc) in the water bodies (aquaculture, freshwater, wastewater, marine water) as presented in tabular form in **Supplementary Table S3A**, highlighting the severity of the AMR problem in water bodies.

The infections caused by ARBs increase the economic burden in terms of healthcare and associated costs. The infection caused by ARBs drastically inflates the cost of treatment and increases the chances of adverse outcomes, as compared to that caused by antibiotic-susceptible bacteria (World Health Organization, 2014; Centers for Disease Control and Prevention, 2018). The estimated deaths caused by AMR could rise from the current rate of about 0.7 million to 10 million annually by 2050, if comprehensive actions are not taken (World Health Organization, 2019; Samreen et al., 2021). It would further cause a 3–4% reduction in the annual gross domestic product (GDP) globally—translating into an economic cost of 1–6 trillion yearly from 2030–2050 onwards, based upon AMR (low to high) scenarios encountered and depending upon the measures undertaken now (World Bank, 2017).

CONTROL OF ANTIMICROBIAL RESISTANCE EMERGENCE AND DISSEMINATION IN WATER BODIES

Removal of Antimicrobial Resistant Bacteria and Antimicrobial Resistance Genes From Water Supply Systems and Wastewater Treatment Units

The WWTPs were designed for the removal of organic matter, nutrients, and solids, but now they need to be able to remove antimicrobials/antibiotics, ARBs, and ARGs as well. So far, very little is known about the effectiveness of the treatments in the removal of ARB and ARGs. Research is needed to fill a huge knowledge gap in this area to help improve the design of WWTPs and the used methodologies. Water supply systems and WWTPs use a single or a combination of different treatment processes to achieve many log reductions in the number of specific target microbes which show intra- and inter-process variations (Sano et al., 2016; Amarasiri et al., 2017). Specific comprehensive guidelines suggesting minimum reductions for antibiotics, ARB, and ARGs in water/wastewater are desired (Hong et al., 2018).

The membrane bioreactor treatment plants had achieved significantly higher reductions of ARB and ARGs (log reduction range: 2.57–7.06) than conventional treatment methods such as sand filtration, sedimentation, activated

sludge, and rotating biological contactor or oxidation ditch (log reduction range: 2.37–4.56; $p < 0.05$) (Munir et al., 2011; O'Flaherty and Cummins, 2017; Su et al., 2018). The retention in the sand filter medium with low nutrient conditions is supposed to cause ARB starvation, leading to plasmid degradation and permanent loss of antibiotic resistance (Griffiths et al., 1990; Tan et al., 2019). Drinking water treatment by conventional methods had achieved variable ARG log reductions (0.03–2.4) that differed with the types of ARGs evaluated (Hu et al., 2019; Zhang et al., 2019). Sulfamethoxazole had killed ARBs immediately, but a delay in ARGs reduction was reported, possibly resulting from a competitive consumption of free radicals by sulfamethoxazole and ARGs (Hu et al., 2019).

Several studies indicate the inability of the water treatment processes to eliminate ARB and ARGs. There had been increased incidences of specific ARGs in the WWTP effluents (Rizzo et al., 2013; Xu et al., 2015; Chu et al., 2018; Karkman et al., 2018). A study revealed no significant differences in the ARGs present in potable source water and treated water, indicating the nonremoval of ARGs during the process (Garner et al., 2018). The activated granular carbon filtration method even enhanced the abundance of ARGs in the filtered water due to the formation of biofilms on the biological activated carbon surfaces where ARB can adhere and grow (Xu et al., 2016; Su et al., 2018; Hu et al., 2019; Tan et al., 2019). The significant correlations ($p < 0.05$) between the effluent ARG concentration (2 μg/l) and residual antibiotic concentration (0.5–0.22 μg/l) suggest a role for selection pressure on ARG enrichment (Mao et al., 2015; Hu et al., 2019).

The search for local solutions to avoid environmental dissemination of these pollutants requires prior information on the specific residues and the AR determinants present in wastewater. There is an urgent need for public health research to increase its pace to keep up with water sustainability technologies and even go beyond. In addition, more research work is essential for the application of effective treatment and disinfection approaches for the complete removal of ARB in WWTPs as the associated immediate environmental and public health risks are high.

Tracking the Sources of Antimicrobial Resistance in Organisms

The modern molecular techniques for the characterization of bacterial organisms can readily increase our ability to track the source of AMR and ARGs. These could provide useful insights, including but not limited to a comprehensive understanding of the population biology of organisms and the genetic diversity of organisms entering water (Olivas and Faulkner, 2008). These techniques could provide fast and accurate AMR source tracking and other genetic mobile platforms involved in AMR dissemination, providing a much more accurate image of the real diversity and complexity of AR in water-borne bacteria, unlike cultivation-dependent approaches (Henriques et al., 2006).

Revision of Domains of "One Health Approach" for Tackling Antimicrobial Resistance

The "One Health Approach" suggested by the WHO that currently focuses on activities to reduce the contamination and usage of antimicrobials and ways to minimize the development and spread of AMR pathogens (World Bank Group, 2018; Mazimba et al., 2021) should consider including active pursuance of the reduction of antimicrobial contamination of the environment as the seventh domain of the OHA for AMR crises to curb the development and spread of AMR (See **Figure 1B**).

DISCUSSION

Antimicrobials/antibiotics are used as both preventive and therapeutic agents in the treatment of animal diseases, human infections, aquaculture, agriculture, and the livestock industry (Baquero et al., 2008; Forsberg et al., 2012; Ben et al., 2019; Dadgostar, 2019; Schar et al., 2020; Schar et al., 2021). Antibiotic residues reach different environments through excretions (stool and urine of animals and human), improper disposal of unused drugs, waste stream from the antibiotic production unit, antibiotics used for plant production, etc. (Baquero et al., 2008; Ishikawa et al., 2018; Karkman et al., 2018; Pepper et al., 2018; Dadgostar, 2019; Larsson and Flach, 2021). Water bodies get contaminated by municipal sewage discharges, animal husbandry, landfill leachates of antibiotic disposal, manufacturing industries, and agricultural runoff (Hernando-Amado et al., 2019; Serwecinska, 2020). The increased frequency of ARGs in various ARBs of different environments is one of the concerning consequences of antimicrobial/antibiotic misuse and subsequent pollution (Kraemer et al., 2019). Studies indicate that aquatic environments act as a key reservoir and means of antibiotic resistance spread (Zhang et al., 2013; Matongo et al., 2015; Binh et al., 2018; Yang et al., 2018; Danner et al., 2019; Kraemer et al., 2019; Amarasiri et al., 2020; Schar et al., 2020; Larsson and Flach, 2021; Liyanage et al., 2021). In an aquatic environment, wastewater and WWTPs are considered one of the key potential hot spots for the spread of AR and transfer of ARGs (Matongo et al., 2015; Amarasiri et al., 2020; Ali et al., 2021; Buriánková et al., 2021; Guo et al., 2021; Markkanen et al., 2021; Obayiuwana et al., 2021; Yoo and Lee, 2021; Zhang et al., 2021). It was estimated that for the production of aquaculture animals, the global consumption of antimicrobials which was 10,259 tons in 2017 is projected to register an increase of 33% to 13,600 tons by 2030 (Schar et al., 2020). Different ARBs and ARGs had been frequently detected in groundwater (Singh et al., 2020; Kunhikannan et al., 2021), surface water (Deng et al., 2016; Binh et al., 2018), wastewater (Karkman et al., 2018; Nguyen et al., 2021), sediments (Liang et al., 2013; Xu et al., 2014; McInnes et al., 2021) and marine water (Buschmann et al., 2012; Shimizu et al., 2013; Vilca and Angeles, 2018). A brief systematic review of the literature aptly highlights the growing menace of ARBs and ARGs in fresh and marine water environments along with the contamination of different antibiotics (**Supplementary Table S3A**).

Both culture-dependent and culture-independent (metagenomics) studies have contributed to our understanding of the AMR problem. The combination of culture-dependent and culture-independent metagenomic techniques is reported to provide better retrieval of ARGs than either method alone (Korzeniewska and Harnisz, 2012; Fenske et al., 2020). Metagenomic studies provide an avenue to study the uncultivable total microorganisms (Forbes et al., 2017). A metagenomics study of municipal wastewater and hospital wastewater revealed the presence of tetracycline, beta-lactam, macrolide–lincosamide–streptogramin resistance gene and multidrug resistance genes ranging from 0.06–0.98 copy/cell, and biocide/metal resistance gene ranging from 0.30–1.99 copies/cell (Zhang et al., 2021). One of the studies reported the presence of different ARGs in the seawater sample at 1.7×10^2 copies/giga base (Zeng et al., 2019). The highest ARG levels of $1.57–700.58 \times 10^2$ copy/ml for penicillin were reported from surface water, whereas $0.37–312.7 \times 10^2$ copy/ml was reported from the groundwater of Sri Lanka. Among the penicillin resistance genes, the highest percentage of bla_{TEM} (700.58×10^2 copy/ml) followed by ampicillin ($0.37–371.7 \times 10^2$ copy/ml) and OPR D (1.57×10^2 copy/ml) resistance genes were reported from aquatic samples, whereas tetM and tetA resistance genes at the levels of $1.35–439.88 \times 10^2$ copy/ml were reported from the surface water samples. Only the tetM resistance gene was reported at 215.99×10^2 copy/ml from the groundwater sample of Sri Lanka (Liyanage et al., 2021). Zhang et al., 2021 reported that the number of ARGs is strongly correlated with the number of biocide/metal resistance genes in the WWTPs with more chemicals (Zhang et al., 2021). The municipal wastewaters had more abundant and diverse ARGs than hospital wastewater. From the urban canals and lakes of Vietnam, different levels of erythromycin, amoxicillin, sulfamethoxazole, ampicillin, clindamycin, tylosin, vancomycin, tetracycline, chloramphenicol, *etc.* were frequently detected (Tran et al., 2019). Metagenomics study of rural and urban water and sediments of Bangladesh reported a significant correlation between ARGs and human origin bacteria ($R^2 = 0.73$; $P = < 0.01$), suggesting that the release of untreated sewage could act as a driver for the transmission of ARGs in the environment (McInnes et al., 2021). A recent metagenomics study of 79 WWTPs situated in 60 countries reported the differences in diversity and abundance of ARGs among Africa, Asia, North America, South America, Oceania, and Europe (Strange et al., 2021). The Oceanic cluster reported a limited number of ARGs encoding macrolides in high number, whereas Africa, Asia, and South America clusters harbored ARGs representing sulfonamides and chloramphenicol. A study has also reported Vietnam, India, and Brazil to have the most divergent ARG distribution and suggested them as possible hotspots for the emergence of new antibiotic resistance mechanisms (Hendriksen et al., 2019). Several studies reported the prevalence of antibiotics, ARBs, and ARGs in the aquatic environment that was correlated with environmental factors (**Supplementary Table S3A**) (Binh et al., 2018; Tran et al., 2019; Hanna et al., 2020; Anh et al., 2021; Azanu et al., 2021; Duong et al., 2021; Guo et al., 2021; Lai et al., 2021; McInnes et al., 2021; Zhang et al., 2021; Zhuang et al., 2021). A metagenomics study of wastewater in Benin and Burkina showed the prevalence of resistance genes van, blaOXA, blaGES, blaIMP, blaKPC, blaNDM, blaOXA, blaVIM, qnr, and mcr (Markkanen et al., 2021). Recently, a large number of ARGs subtypes, *viz.,* blaNDM-1, blaCTX-M-15, mecA, blaTEM-1, sul1, vanA, blaKPC-2, sul2, blaCTX-M-14, and blaOXA-48 had also been reported in decreasing order from Asia, Europe, Africa, and North and South America (Zhuang et al., 2021).

The major problem to tackle the issue of AMR is existing knowledge gaps comprising incomplete knowledge or information and misperceptions about the use of antibiotics and the relative contribution of the release of ARB or ARGs in the environment from different sources (McCullough et al., 2016; Singh, 2020). Health professionals can play a major role in the prevention and spread of AMR by educating people about the possible risks of inappropriate usage and disposal of antibiotics and contaminated material containing ARBs with ARGs. The effective control of AMR development and spread of ARGs and ARBs can be facilitated by promoting the development of self-contained local wastewater treatment modules, use of antibiotics/antimicrobial degrading contraptions, and implementing strategies to minimize the concentration of antibiotics required for treatment, including the use of nanotechnology (Malakootian et al., 2019; Singh, 2020; Kaur et al., 2021; Singh et al., 2022).

AMR/AR has the potential to threaten human health and inflict huge blows to the economies of both developed and developing countries (Ventola, 2015; World Health Organization, 2021). Estimates for Europe, the United Kingdom, Thailand, and the United States, the project substantial increase in health costs from antibiotic-resistant bacterial infections (Da Silva et al., 2006; World Health Organization, 2015; Osińska et al., 2016; Abdel-Rahman et al., 2020; Obayiuwana and Ibekwe, 2020; Yoo and Lee, 2021). The waterborne AMR is causing an economic impact of $340-$680 billion annually on the health care system (World Economic Forum, 2021). The waterborne AMR is contributing about $1 to $5 billion per year in additional health care expenditure, and it is expected to increase as resistance develops further. The waterborne AMR could be responsible for about 3.5 million additional sicknesses annually at the cost of $300 million (World Economic Forum, 2021). The alarm of AMR crisis raised in recent times by various bodies such as WHO, FAO, CDC, World Bank, etc. (World Health Organization, 2018; Mulani et al., 2019; Centers for Disease Control and Prevention, 2019; Centres for Disease Control and Prevention, 2021) also calls for the strengthening of the synthesis and discovery pipeline of new antibiotics with better activities or activity against various antibiotic-resistant pathogens (**Supplementary Table S5**). Development of new more potent antibiotics with different or multiple modes of action, along with focused steps to curb AMR development and dissemination to pathogens, is required (Mulani et al., 2019; Singh, 2020; Léger et al., 2021). AMR, being a multidimensional problem, requires a proactive holistic, constructive, collaborative, and synergistic strategy and action by different stakeholders to comprehensively implement a One Health Approach to overcome the unfolding AMR crises.

CONCLUSION

The continued antimicrobial overuse, misuse, and uncontrolled contamination of the environment throughout the world are turning the AMR issue into a global health crisis. The tackling of

the AMR situation requires implementation of new policies that limit the release of antimicrobial residues into the environment and support appropriate monitoring to minimize their buildups and timely removal. More research efforts are needed toward understanding the extent and mechanistic underpinnings of AMR development and ARG transfer to other pathogenic bacteria to develop better control strategies. The involvement of the public to locally manage and dispose of the antimicrobials and AMB remains a potential area of collaboration and policy development to control the AMR crisis as it could promote both a sense of responsibility and awareness.

AUTHOR CONTRIBUTIONS

SS conceptualized, supervised, prepared, and finalized the manuscript. RK, AKS, and SS prepared the first draft. SV provided critical inputs. RK, AKS, SV, and SS together revised the manuscript. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2022.830861/full#supplementary-material

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HT-ARGfinder: A Comprehensive Pipeline for Identifying Horizontally Transferred Antibiotic Resistance Genes and Directionality in Metagenomic Sequencing Data

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Antibiotic resistance is a continually rising threat to global health. A primary driver of the evolution of new strains of resistant pathogens is the horizontal gene transfer (HGT) of antibiotic resistance genes (ARGs). However, identifying and quantifying ARGs subject to HGT remains a significant challenge. Here, we introduce HT-ARGfinder (horizontally transferred ARG finder), a pipeline that detects and enumerates horizontally transferred ARGs in metagenomic data while also estimating the directionality of transfer. To demonstrate the pipeline, we applied it to an array of publicly-available wastewater metagenomes, including hospital sewage. We compare the horizontally transferred ARGs detected across various sample types and estimate their directionality of transfer among donors and recipients. This study introduces a comprehensive tool to track mobile ARGs in wastewater and other aquatic environments.

Keywords: antibiotic resistance gene, horizontal gene transfer, metagenomics, urban conventional activated sludge, hospital sewage, urban sewage

1 INTRODUCTION

The ability of pathogens to resist antibiotic treatments significantly increases both morbidity and mortality (Bush et al., 2011). As hosts and pathogens have coevolved over millions of years, bacterial pathogens have adjusted their virulence to adapt to host defensive mechanisms (Beceiro et al., 2013). Antimicrobial-resistant infections kill at least 700,000 people worldwide each year, and, within 30 years, resistant infections are expected to kill 10 million people annually, far outnumbering cancer mortality (O'neill, 2014). There is growing recognition that problematic pathogens encountered in the clinic originally acquired their ability to resist antibiotics from the broader pool of bacteria inhabiting natural environments. Once a resistant pathogen becomes endemic in a hospital, it can create a source of nosocomial infection and elevate the overall risk of standard medical procedures (Davies and Davies, 2010; Cheong et al., 2017).

Horizontal gene transfer (HGT) is a natural process that contributes to the evolution of resistant bacterial strains through the physical passage of DNA from one cell to another (Khan and Rao, 2019). HGT occurs through three fundamental mechanisms: transformation (uptake of naked DNA by

competent cells), transduction (uptake of foreign DNA mediated by a phage), and conjugation (bacterial mating) (Amarasiri et al., 2020). This transfer of genetic material can cause both beneficial as well as adverse consequences from a human standpoint (Kunhikannan et al., 2021). HGT is commonly harnessed for industrial biotechnological purposes and generally serves to expand genetic diversity (Le Roux and Blokesch, 2018). However, HGT is also primarily responsible for mobilizing multidrug resistance (MDR) among strains. In particular, multi-antibiotic resistant superbugs, which can be resistant to all available antibiotic therapies, have arisen mainly as a result of HGT (Mathers et al., 2015; Wang and Sun, 2015; Malhotra-Kumar et al., 2016). The transfer of plasmids carrying multiple ARGs is one primary driver of the superbug phenomenon (Sun et al., 2019).

A growing body of research has brought to light the importance of aquatic environments as a hub for the dissemination of ARGs. Aquatic environments typically receive multiple waste streams, treated and untreated, containing various mixtures of contaminants. As such, aquatic environments have been identified as ideal settings for HGT of ARGs, while human exposure to antibiotic resistance bacteria, and ARGs in aquatic environments may pose an additional health risk (Amarasiri et al., 2020). The evolution and spread of ARGs in aquatic environments have been documented across several studies conducted in various locales across the globe. Hundreds of different ARGs have been discovered in bacteria found not just in hospitals, livestock, and meatpacking wastewater but also in sewage, effluent treatment facilities, surface water, groundwater, and even drinking water (Zhang et al., 2009).

Convenient, efficient, and accurate means of identifying ARGs subject to HGT would be highly valuable to the research community and to fledgling efforts to advance environmental monitoring of antibiotic resistance. This would allow the ability to focus on ARGs that are of most concern in terms of ability to mobilize to pathogens instead of intrinsic, non-mobile counterparts. Advances in shotgun metagenomic sequencing of environmental samples holds substantial promise for this purpose. The ability to recover millions of DNA sequences from a given sample affords the opportunity to correspondingly search for ARGs and mobile genetic elements (MGEs), including plasmids, transposons, and integrons. Recently, MetaCHIP was introduced as a tool to specifically identify genes subject to HGT across a microbial community (Song et al., 2019). MetaCHIP identifies HGT in assembled metagenomic data through a combination of best hit and phylogenetic approaches, which further serves to inform estimates of the directionality of the horizontally transferred genes (i.e., the donor and recipient of the HGT). LEMON (Li et al., 2019) is a similar tool that was also recently introduced and, like MetaCHIP, works best with nearly complete metagenome-assembled genomes. However, these existing tools are not explicitly tailored to identify and track the directionality of horizontally transferred ARGs.

We propose a new pipeline, HT-ARGfinder, which detects horizontally transferred ARGs from metagenomic data derived from complex environmental microbial communities. The pipeline was applied to metagenomes obtained from a range of wastewater samples, representing presumed hotspots for ARG evolution and mobilization. Two ARG databases were applied and compared: CARD, the most well-curated and up-to-date database of known ARGs, and DeepARG-DB, which comprises 14,933 genes including the three ARG databases: CARD, ARDB, and UNIPROT, to include putative new ARGs discovered based on deep learning Arango-Argoty et al. (2018). The specific objectives of this study were to: [1]) develop HT-ARGfinder as a comprehensive and user-friendly pipeline for identifying horizontally transferred ARGs in metagenomic data sets and inferring directionality of their transfer, 2) apply the pipeline for identifying and comparing horizontally transferred ARGs detected across a range of representative wastewater environments, and 3) infer the directionality of ARG transfer among various bacterial classes in several metagenomic data sets.

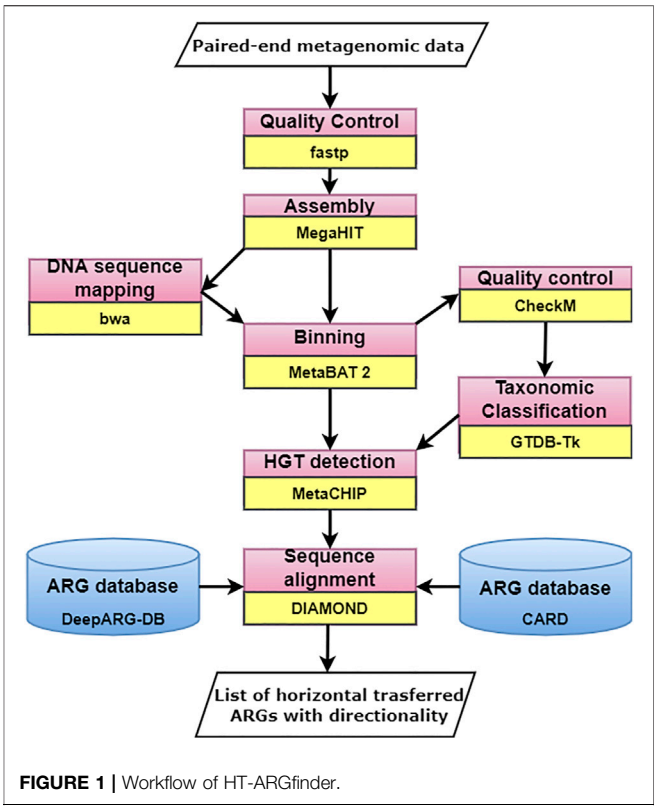

FIGURE 1 | Workflow of HT-ARGfinder.

2 MATERIALS AND METHODS

2.1 Workflow

HT-ARGfinder combines a series of bioinformatics tools to process metagenomic data, starting with quality control and ending with ARG detection and directionality inference. The workflow of HT-ARGfinder is shown in **Figure 1**.

[1]Environments were re-classified based on information reported by the authors for the consistency across this study

TABLE 1 | Summary of data sets used to demonstrate HT-ARGfinder.

Environment[1]	Accession no. (NCBI SRA)	Classification according to original study
Hospital sewage	ERX3538875, ERX3538874, ERX3538873, ERX3538872, ERX3538871, ERX3538870, ERX3538869, ERX3538868	Hospital wastewater
	SRX7901963, SRX7901962, SRX7901961, SRX7901960, SRX7901959, SRX7901958	Hospital sewage
Urban conventional activated sludge	SRX3720490, SRX3720489, SRX3720488, SRX3720487, SRX3720486, SRX3720481, SRX3720480, SRX3720479	Activated sludge
Urban sewage	ERX1795927, ERX1783568, ERX1795930, ERX1783571, ERX1795935, ERX1783576, ERX2608526, ERX2608605, ERX1783583, ERX1783584	Urban sewage

TABLE 2 | Total number of horizontally transferred genes (HGTs), ARGs according to CARD database, and ARGs according to DeepARG-DB.

Environment	Data set	HGTs	ARGs (CARD)	ARGs (DeepARG-DB)
Hospital sewage	ERX3538875	98	6	7
	ERX3538874	61	2	3
	ERX3538873	264	21	20
	ERX3538872	4	1	1
	ERX3538871	9	0	0
	ERX3538870	4	0	0
	ERX3538869	20	2	3
	ERX3538868	9	3	3
	SRX7901963	30	2	3
	SRX7901962	14	3	3
	SRX7901961	31	2	2
	SRX7901960	9	0	0
	SRX7901959	0	—	—
	SRX7901958	3	0	0
Urban conventional activated sludge	SRX3720490	20	1	1
	SRX3720489	11	1	1
	SRX3720488	19	3	3
	SRX3720487	44	2	2
	SRX3720486	8	2	2
	SRX3720481	14	2	2
	SRX3720480	11	1	2
	SRX3720479	24	0	1
Urban sewage	ERX1795927	12	3	3
	ERX1783568	8	0	0
	ERX1795930	1	1	1
	ERX1783571	4	1	1
	ERX1795935	11	1	1
	ERX1783576	18	1	1
	ERX2608526	11	0	0
	ERX2608605	11	0	0
	ERX1783583	12	1	1
	ERX1783584	9	0	0

2.1.1 Quality Control of Reads

Fastp (Chen et al., 2018), an ultra-fast FASTQ preprocessor with quality control, was incorporated for quality control and preprocessing of the paired-end reads. This tool is developed in C++ and has multi-threading support. Fastp is 2–5 times faster than other FASTQ preprocessing tools such as Trimmomatic (Bolger et al., 2014), Cutadapt (Martin, 2011), FASTQC (Brown et al., 2017), and AfterQC (Chen et al., 2017).

2.1.2 Metagenomic Assembly

MegaHIT (Li et al., 2015) was used for assembling metagenomes, which we previously found to provide the most accurate and extensive assemblies of short-read metagenomic data from complex environmental samples. MegaHIT is a *de novo* assembler that uses a succinct de Bruijn graph to assemble short reads (Bowe et al., 2012).

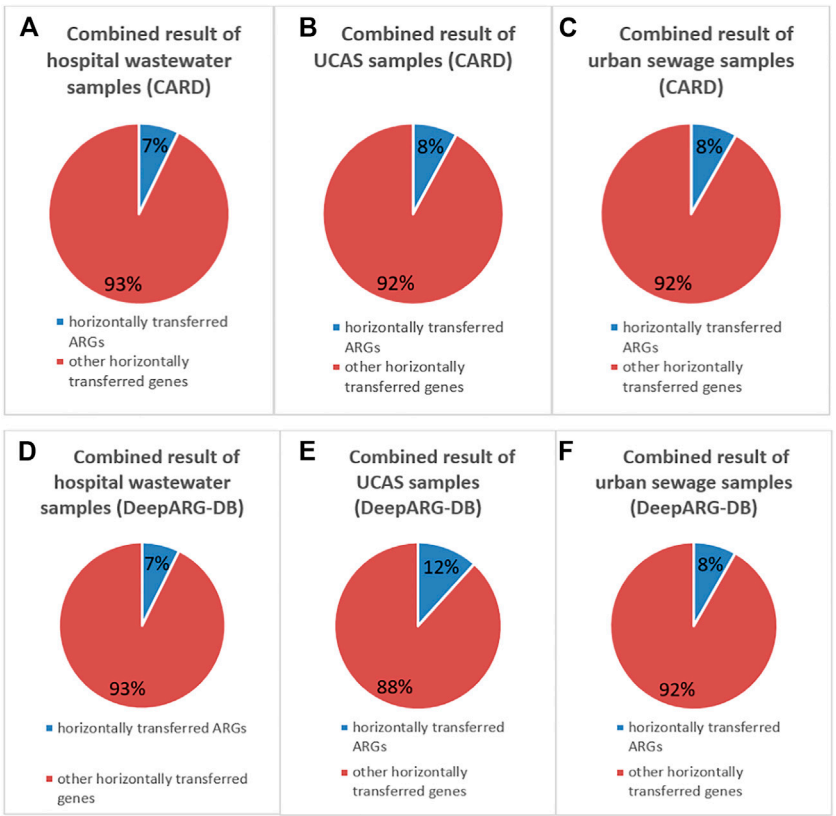

FIGURE 2 | Percentage of horizontally transferred ARGs detected [according to CARD **(A–C)**; according to DeepARG-DB **(D–F)**].

2.1.3 Binning

MetaBAT 2 (Kang et al., 2019) is incorporated for the purpose of reconstructing single genomes from microbial communities. In comparison to other binning tools such as MaxBin2 (Wu et al., 2016), CONCOCT (Alneberg et al., 2013), and MyCC (Lin and Liao, 2016), MetaBAT 2 shows better performance.

2.1.4 Quality Control of Bins

For quality control of the bins, CheckM (v1.1.2) (Parks et al., 2015) was employed. CheckM is an automated method for evaluating the quality of a genome using a broader set of marker genes specific to the position of a genome within a reference genome tree and information about the collocation of these genes. The bins were filtered based on completeness and contamination reported by CheckM. Bins having *completeness* ≥ 40% and *contamination* ≤ 5% were selected for the next step. According to Bowers et al. (2017), "Medium-quality drafts" SAGs and MAGs are those genomes with completeness estimates of ≥50% and with contamination of ≤10%. All the other SAGs and MAGs (<50% completeness or >10% contamination) should be reported as "low quality drafts." MetaCHIP has chosen ≤40% completess and 0% contamination for their pipeline Song et al. (2019). We have used ≤5% contamination since its less than the recommended 10% for medium quality drafts but slightly less stringent than MetaCHIP.

2.1.5 Taxonomic Classification

After binning, the genomes are taxonomically classified using GTDB-Tk (v1.6.0), which provides objective taxonomic assignments for bacterial and archaeal genomes based on the Genome Taxonomy Database (GTDB) (Chaumeil et al., 2020).

2.1.6 HGT Detection

MetaCHIP, a pipeline for reference independent HGT identification in metagenomic data (Song et al., 2019), is used for detecting HGT at the community level. MetaCHIP implements a combination of both best match and phylogenetic approaches. First, it uses sequence similarity comparison to identify putative open reading frames (ORFs) horizontally transferred for user-defined taxonomy ranks (e.g., class, order family, or genus). Then phylogenetic trees are constructed for the putative HGT gene candidates and compared to the species trees determined by single-copy genes. Finally, reconciliation of the gene trees and species trees is performed using Ranger-DTL v2.0 (Bansal et al., 2018) to identify HGTs and donors and recipients. MetaCHIP allows users to specify the taxonomy ranks for which HGTs are identified. The default is "class," and HT-ARGfinder keeps the default one.

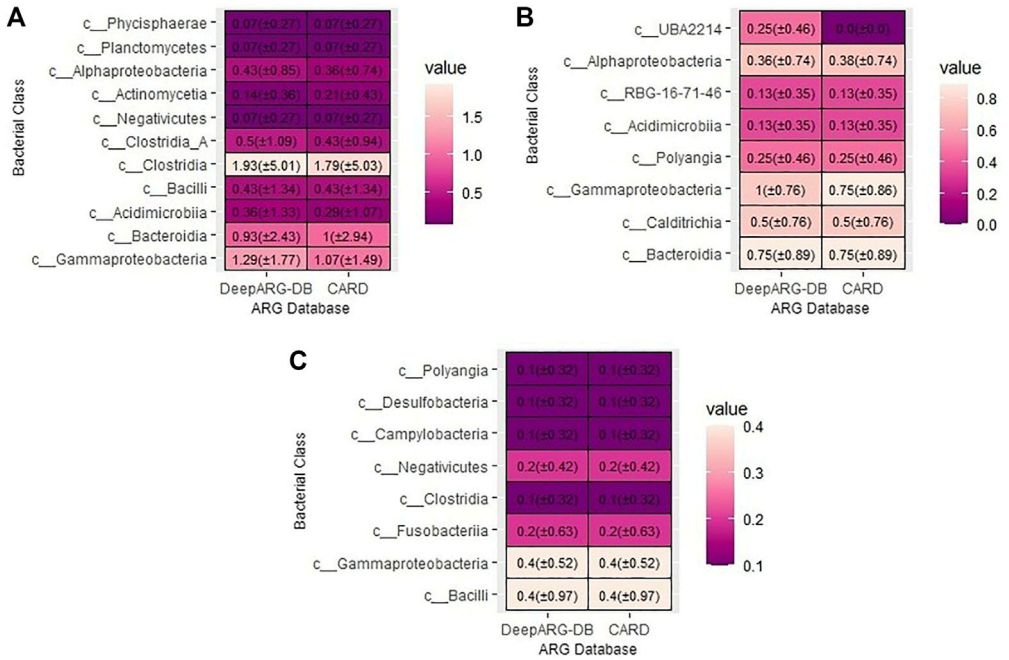

FIGURE 3 | Heatmap of the average number of ARGs per class according to both DeepARG-DB and CARD databases in **(A)** hospital sewage, **(B)** urban conventional activated sludge and **(C)** urban sewage samples. Each cell holds average (±standard deviation).

2.1.7 Databases and Sequence Alignment

The detected horizontally transferred genes are finally aligned against two reference databases, DeepARG-DB (Arango-Argoty et al., 2018) and CARD (Alcock et al., 2020) using DIAMOND (Buchfink et al., 2015) to determine ARGs. CARD represents a highly-curated database of previously described ARGs that is updated periodically. In contrast, DeepARG-DB is a larger database that encompasses the ARGs included in CARD and additional ARGs detected from a deep learning algorithm. There is an option to use new databases in our pipeline. Users can easily execute the command and include new databases to check ARGs against those databases.

2.2 Wastewater Metagenomes Included in This Study

We selected publicly-available metagenomic data from a range of wastewaters for inclusion in this study; see **Table 1**. We were particularly interested in data sets, such as hospital sewage, urban conventional activated sludge, likely to contain a diverse array of ARGs that had been subject to HGT. All data used in this study are open source and are available at NCBI Sequence Read Archive (SRA) under the Bioproject accession numbers reported in **Table 1**. These included hospital sewage, urban conventional activated sludge and urban sewage samples.

Table 1 provides a summary of these samples, including the environments analyzed as named by the original researchers and generalized categories based on common terminology used in this paper (hospital sewage, urban conventional activated sludge, and urban sewage). These datasets included Illumina HiSeq 4000 paired-end sequencing metagenomic data, MiSeq paired-end sequencing metagenomic data, Illumina HiSeq 2500, and Illumina HiSeq 3000 paired-end metagenomic data.

2.3 Demonstrating HT-ARGfinder

We demonstrated HT-ARGfinder using the data summarized in **Table 1**. The HT-ARGfinder output consisted of two lists of horizontally-transferred ARGs according to CARD and DeepARG-DB databases and their corresponding estimated directionalities. The number of detected ARGs from these two databases was compared. We also analyzed the abundances of different bacterial classes associated with the HGT events in the samples of different environments according to CARD and DeepARG-DB. MetaCHIP was applied to identify the bacterial classes corresponding to the detected horizontally-transferred genes. We filtered out the ARGs detected using both CARD and DeepARG-DB from that list. Finally, we mapped the output lists of HT-ARGfinder to directed graphs where the nodes represent different bacterial classes involved in HGT, and the directed edges represent the direction of the HGT. This graph provides visualization of the whole HGT scenario of a metagenomic sample.

3 RESULTS

3.1 ARG Detection

HT-ARGfinder was found to be a robust tool for the detection of horizontally transferred ARGs in complex environmental metagenomic data sets. **Table 2** presents the total number of

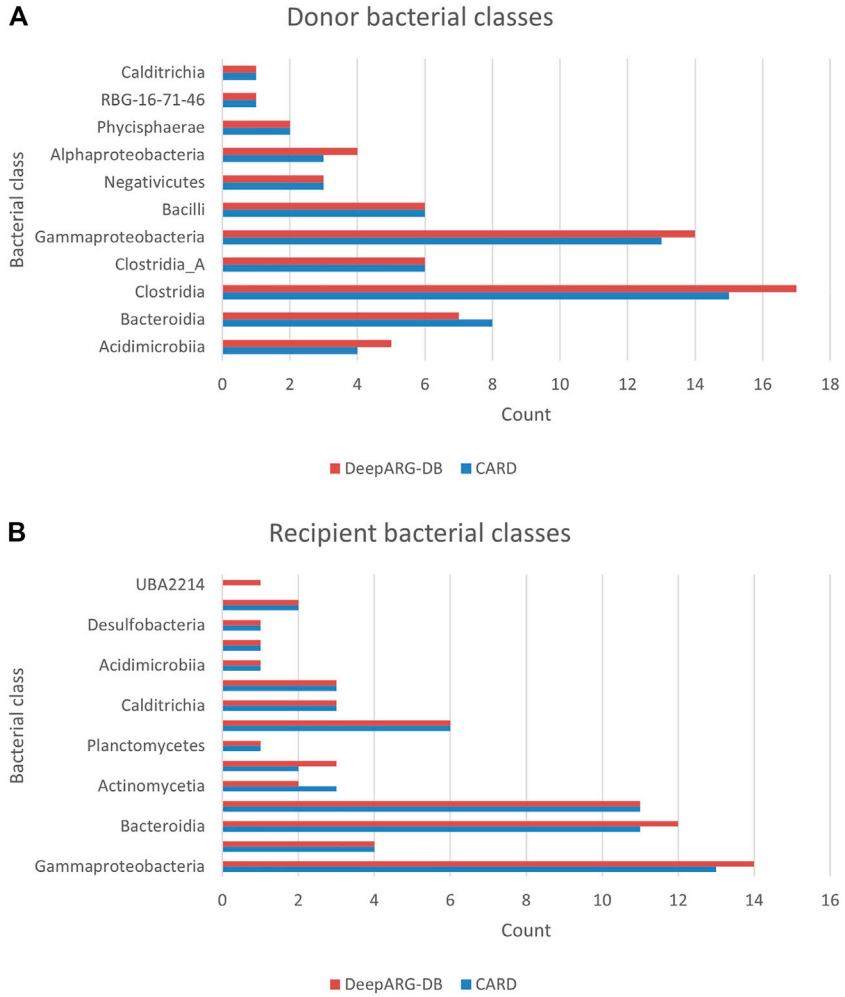

FIGURE 4 | Bacterial classes and their abundances as **(A)** donor and **(B)** recipient during horizontal ARG transfers in all samples.

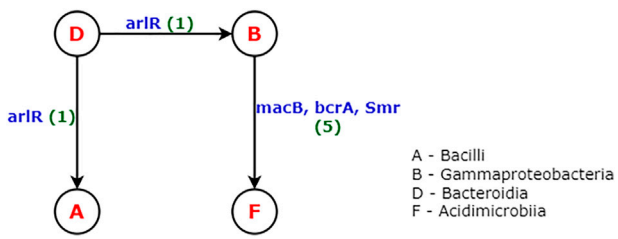

FIGURE 5 | Example directed graph illustrating the relationship among the bacterial classes in a hospital sewage sample (ERX3538875) according to DeepARG-DB. The vertices of the graph represent the bacterial classes associated with ARG transfer and the edges indicate the names and counts of the ARGs transferred.

horizontally transferred genes and the total number of ARGs according to CARD and DeepARG-DB databases. The table shows that raw hospital wastewater and urban conventional activated sludge have many HGT events. Still, fewer horizontally transferred genes were detected in the urban wastewater treatment plant samples. Based on these trends, raw hospital wastewater, and urban conventional activated sludge samples focused on the remainder of the HT-ARGfinder demonstration.

As can be observed from **Table 2**, ARGs represent a remarkably consistent portion of the total horizontally transferred genes detected (*10%* estimated for both types of environments). As expected, DeepARG-DB tended to detect a greater number of horizontally transferred ARGs than CARD. Still, the percent of the horizontally transferred ARGs were similar (**Figure 2**). This suggests that the relative proportion of mobile ARGs does not vary substantially across the environments tested, although the overall transfer rates may vary.

3.2 Identification of Donor and Recipient Bacterial Classes

MetaCHIP provides a prediction of the donor and recipient bacteria involved in each HGT event. HT-ARGfinder

improves upon this feature by filtering events related explicitly to ARG transfers. This yielded valuable information concerning the classes of bacteria actively engaged in the horizontal transfer of ARGs. Further, the abundance of these classes and ARGs can be estimated in each environment. Heatmaps were generated to illustrate the average number of bacterial classes estimated to be engaged in ARG transfer across the samples analyzed in this study (**Figure 3**).

The hospital sewage samples were found to be enriched with Actinomycetia, Negativicutes, Clostridia_A, Clostridia, Bacilli, Acidimicrobiia, Bacteroidia, Gammaproteobacteria, Phycisphaerae, Planctomycetes, and Alphaproteobacteria. **Figure 3** shows their average abundance (**Supplementary Tables** show the number of detected ARG classes in each sample). In urban conventional activated sludge samples, we found UBA2214, Alphaproteobacteria, RBG-16-71-46, Acidimicrobiia, Polyangia, Gammaproteobacteria, Calditrichia, and Bacteroidia (**Figure 3**). In urban sewage samples, Bacilli, Gammaproteobacteria, Fusobacteriia, Clostridia, Negativicutes, Campylobacteria, Desulfobacteria, and Polyangia were found (**Figure 3**). **Figure 4** shows all the bacterial classes and their abundances as donor and recipient in these metagenomic samples according to both DeepARG-DB and CARD databases. We can see that Clostridia and Gammaproteobacteria were higher in counts as the donor, whereas Gammaproteobacteria, Bacteroidea, and Clostridia were higher in counts as the recipient.

3.3 Comparison Between CARD and DeepARG-DB

Implementation of CARD versus DeepARG-DB in the pipeline resulted in different numbers of horizontally transferred ARGs detected in some samples (**Table 1**; **Figures 2–4**). For the cases in which the discrepancy was the greatest, DeepARG-DB has consistently yielded the higher count. This is consistent with DeepARG-DB being a larger database than CARD, encompassing the ARGs included in CARD and additional ARGs identified via the deep learning module.

3.4 Relationship Among Bacterial Classes in Samples

HT-ARGfinder provides additional value in inferring the directionality of ARG transfer among bacterial classes. This can be visualized in directed graphs that illustrate the various transfer relationships among the bacterial classes represented in a sample. For example, for a metagenomic sample, let $G = (V, E)$ be a directed graph where each vertex represents a bacterial class, each directed edge between two vertices indicates the directionality of the ARG transfer between the two bacterial classes, and the edge weight indicates the number of ARGs transferred. A hospital sewage sample (ERX3538875) was used to visualize the relationship among the bacterial classes (**Figure 5**). Four bacterial classes involved in HGT of ARGs were identified in this sample: Bacilli, Gammaproteobacteria, Bacteroidia, and Acidimicrobiia. The possible ARGs that are transferred between Gammaproteobacteria and Alphaproteobacteria encoded resistance to macrolides (macB), peptide (bcRA), and polymyxins (arnA).

4 DISCUSSION AND CONCLUSION

HT-ARGfinder provides a comprehensive and user-friendly tool specifically directed toward identifying mobile ARGs in metagenomic data sets and estimating the directionality of their transfer among bacterial classes. This pipeline can provide substantial value in targeting environmental monitoring efforts towards tracking mobile ARGs and help inform better and direct efforts to stop the spread of antibiotic resistance. Here we demonstrated the tool's utility for a spectrum of wastewater samples. We have used CARD and DeepARG-DB as the ARG databases, but this pipeline can be modified easily by replacing these databases with some recent ones, for example, HMD-ARG-DB (Li et al., 2021). It is composed of 17, 282 sequences, gathered and cleaned from 7 published ARG databases: CARD, AMRFinder (Feldgarden et al., 2019), ResFinder (Zankari et al., 2012), ARG-ANNOT (Gupta et al., 2014), DeepARG, MEGARes (Doster et al., 2020) and Resfams (Gibson et al., 2015). We have shown results using CARD and DeepARG-DB because these are well-known ones. Users can modify the pipeline by adding other databases easily.

Our tool detects all the ARGs that are transferred horizontally. These ARGs are horizontally transferred through the mobile genetic elements such as plasmids, transposons, integrons, and genomic islands. (Khezri et al., 2020) detects only the plasmid-mediated ARGs by first identifying plasmids. Since our tool detects all the ARGs transferred through HGT, it detects ARGs mediated through potential mobile genetic elements.

The analysis was consistent with the expectation of high abundances of mobile ARGs in hospital wastewater and urban conventional activated sludge samples. While the focus was on wastewater samples, the pipeline can readily be applied to various other complex metagenomes, including different aquatic and wastewater samples and gastrointestinal microbiota, broadly of interest for ARG surveillance and research. Based on this study, it was apparent that ARGs represent a substantial fraction (~ 10%) of genes actively transferred in a given wastewater microbiome. It would be interesting to examine further the remaining genes subject to HGT, which might include a novel, yet to be discovered ARGs. This is apparent in recognizing that many horizontally transferred ARGs were found when the pipeline queried DeepARG-DB. Additionally, such genes could encode other relevant functions, such as metal resistance or processes related to gene mobility. Further analysis, such as this, of the context of the ARGs, can help resolve the driver of their mobility. The directionality analysis is further of significant value in tracking the movement of ARGs across bacterial hosts. Such information could provide value in identifying which bacteria

should be targeted in mitigation efforts aimed at stemming the spread of antimicrobial resistance.

DATA AVAILABILITY STATEMENT

The source code of HTARGfinder can be found at https://github.com/Badhan023/HTARGfinder. All the data sets that have been used are publicly available in the NCBI Short Read 235 Archive (SRA).

AUTHOR CONTRIBUTIONS

LZ conceived the original idea for the pipeline. BD, ME, and NM accordingly planned and executed the study. AP provided consultation in the application of the pipeline and interpretation of results. BD designed and implemented the pipeline, wrote the initial draft of the manuscript. All authors read, modified, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2022.901917/full#supplementary-material

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