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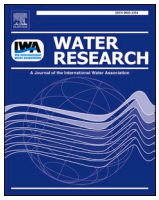

Longitudinal metagenomic analysis on antibiotic resistome, mobilome, and microbiome of river ecosystems in a sub-tropical metropolitan city

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ABSTRACT

Rivers play an important role as reservoirs and sinks for antibiotic resistance genes (ARGs). However, it remains underexplored for the resistome and associated mobilome in river ecosystems, and hosts of riverine ARGs particularly the pathogenic ones are rarely studied. This study for the first time conducted a longitudinal metagenomic analysis to unveil the resistome, mobilome, and microbiome in river water, by collecting samples from 16 rivers in Hong Kong over a three-year period and using both short-read and long-read sequencing. Results revealed that aminoglycoside, bacitracin, β-lactam, macrolide lincosamide-streptogramin, and sulfonamide were the predominant ARG types in the river water samples. Riverine ARGs exhibited high spatial variations in abundance and diversity. Environmental factors such as fecal coliform count, *Escherichia coli* count, 5-day biochemical oxygen demand (BOD_5), dissolved oxygen (DO), and total organic carbon (TOC) had a significant correlation to the absolute concentrations of ARGs. Nanopore sequencing was used to reveal the physical genetic linkage of mobile genetic elements (MGEs) with ARGs in river water samples. The results showed that qacEdelta, transposase, integrase, and Tn916 had a high prevalence in ARG-carrying long reads. Host tracking using ARG-carrying reads identified 23 pathogenic bacteria species that harbored ARGs. Some ARGs were shared by different bacterial groups. This study presented a nuanced insight of resistome in river water by a longitudinal metagenomic analysis and deepened our understanding of common and divergent riverine antimicrobial resistant risk across the regional patterns.

1. Introduction

Antibiotic resistance presents a growing risk to global public health. From the "One Health" perspective, the transmission of antibiotic resistance genes (ARGs) was considered under connections between human, animal, and environmental health (Hernando-Amado et al., 2019). The pathogenic antibiotic-resistant bacteria (ARB) has caused intractable infection and high mortality in clinics (Kern and Rieg, 2020). In 2017, WHO published the first list of antibiotic-resistant "priority pathogens", highlighting the pathogens that pose significant public health threats escaping antibiotic therapy (WHO, 2017). In addition to clinical ARB, environmental antibiotic resistance has gained increasing attention. In the environment, horizontal gene transfer (HGT) regulated by mobile genetic elements (MGEs) plays a crucial role in the proliferation of ARGs (Bengtsson-Palme et al., 2018; Zhou et al., 2021). Although bacteria could get ARGs *via* evolutionary events, it is rarer and more unpredictable than the acquisition of ARGs *via* HGT (Larsson and Flach, 2022). Therefore, besides the surveillance of the exposure level to ARGs in representative environments, quantification of MGEs and deciphering their association with ARGs are also vital for capturing the

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interactions between environmental resistome and mobilome as well as assessing the risk of ARGs (Moralez et al., 2021; Zhang et al., 2021a).

Metagenomic sequencing is an efficient method for resistome analysis in various environment habitats. As a high-throughput strategy, it provided fast and effective detection for a broad spectrum of ARGs, not limited by the availability of primer sets targeting known gene sequences and the failure of polymerase chain reaction (PCR) during amplification of the genes. Genetic contextual information could also be documented in metagenomic data, such as MGEs and microbial hosts, which provide insights into their association with ARGs (Qiu et al., 2022; Rice et al., 2020). For the global view of the antibiotic resistome, metagenomic analysis is rather promising because public metagenomic datasets enable comparisons among different studies or geographical settings (Hendriksen et al., 2019; Yin et al., 2023a). Pipelines have been developed for the interpretation of resistome from metagenomic data, such as the ARGs-OAP for ARG detection and quantification using short-reads sequencing data (Yin et al., 2023b) and the Melon and other tools for identification of ARG hosts and relevant neighboring genes using Nanopore sequencing data (Che et al., 2019; Chen et al., 2024). The assembled contigs of short-reads data were used for the identification of the contextual information in previous studies (Qiu et al., 2022), although the accuracy of the results could be challenged. Recently, long reads were considered to provide a direct evaluation to the contextual information and determine the linked MGEs and host taxa (Che et al., 2019; Dai et al., 2022).

River ecosystem is one of the most important reservoirs and routes of dissemination of antibiotic resistance in the environment. Rivers are essential for agriculture, fisheries, navigation, and sources of drinking water; however, susceptible to anthropogenic pollution. Rivers and streams receive antimicrobial compounds and ARGs from various sources, including effluents from wastewater treatment plants, livestock and other anthropogenic activities (Jia et al., 2017; Rodriguez-Mozaz et al., 2015; Sabri et al., 2020). As the environmental dimension of antibiotic resistance gained increasing attentions under the “One Health” concept, a large collection of studies has investigated the resistome in various environments to define the hotspots constituting the most severe risks and the routes facilitating the spread of ARGs (Tiedje et al., 2019; Yin et al., 2023a). There were sporadic studies revealing the resistome in rivers from different countries, highlighting the urgent need for comprehensive and long-term monitoring on the riverine ARGs (Gao et al., 2024; Jiang et al., 2022; Lee et al., 2020; Samson et al., 2023). Influences of anthropogenic activities on the river relating to the discharge of partially treated sewage, municipal drainage and agriculture runoff have been reported (Gao et al., 2024; Lee et al., 2021; Liang et al., 2020). MGEs and bacterial communities were found contributing to shaping the resistome in river water (Jia et al., 2017; Lee et al., 2020). However, more comprehensive studies focusing on the antibiotic resistome in river ecosystems are essential to enhance our understanding of the regional and global distribution of riverine ARGs, particularly by employing metagenomic sequencing to generate more comparable datasets. Furthermore, the related mobilome and host in river ecosystem has yet to be adequately addressed. To improve our understanding on the abundance, dissemination, and potential risk of ARGs and ARBs in river, an in-depth investigation of riverine resistome, mobilome, and microbiome is desperately required.

To explore the resistome, as well as the mobility and hosts of ARGs in river ecosystems of Hong Kong (HK), a metropolitan city in the subtropics, water samples from 16 rivers/streams over three years (12 quarterly time points) were analyzed by metagenomic analysis in this study. Located on the southeastern coast of China, HK is one of the most densely populated cities in the world, and has a typical subtropical climate characterized by hot and humid summers, and mild and relatively dry winters. Unlike large rivers in previous studies flowing through landscapes with varying degrees of urbanization, the rivers in HK are not very large and are characterized by the city’s unique geographical setting, which includes a dense urban development and a subtropical climate. Spatial distribution of ARG diversity and abundance in the river water was clarified and compared with the riverine resistome previously reported in other regions. Using long-read sequencing, MGEs in physical genetic linkage with ARGs, as well as the accurate identification of ARG hosts, enabled a better evaluation of risk caused by antibiotic resistome in the river ecosystem. This study represents the first longitudinal study to provide holistic and systematic insights into the resistome, mobilome, microbiome of multiple rivers in a metropolitan city, unveiled by Illumina and Nanopore metagenomic sequencing.

2. Materials and methods

2.1. Sample collection and DNA extraction

River water samples were collected from 16 sampling sites from 16 rivers/streams in HK at 12 quarterly time points (T1-T12) spanning a period of 3 years (from May 2021 (T1) to February 2024 (T12), Fig. 1A, Fig. S1). One sampling site was selected at each river. These rivers include the long and meandering rivers, and small rocky streams through hilly terrain. For example, the sampling sites SM and LT were selected from the Shing Mun River and Lam Tsuen River which run through the densely populated urban area of Sha Tin and Tai Po, respectively; TM was selected from the Tuen Mun River which passes through rural areas in its upstream section; NHS was selected from the Ngau Hom Sha Stream, a small stream in the Lau Fau Shan mountainous area. Location of sampling sites were shown in Fig. 1A. Water samples were filtered through 0.45 μm cellulose nitrate membranes, and stored at -20 °C for DNA extraction. Total DNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany). DNA concentrations and quality were determined using microspectrophotometry (NanoDrop ND-1000; Wilmington, DE).

2.2. Illumina sequencing

Illumina sequencing was performed on the Illumina NovaSeq 6000 platform using PE150 strategy (Novogene Corporation, China). For Illumina metagenomic data analysis, raw reads were filtered to remove reads containing low-quality (Qscore $\leq$ 5) bases which were over 50% of the total base, ambiguous bases (N$>$ 10%), and adapters. In total, there were 1.85 Tera base (Tb) short-read sequencing data generated for 173 river water samples.

2.3. Quantification of ARGs, MGEs and bacteria using short-read data

ARGs were annotated and quantified using the pipeline ARGs-OAP with SARG database (Yin et al., 2023b). This pipeline normalized ARG quantitation into different units, the unit of ARG copies per cell was used for analysis. Furthermore, the absolute quantification of ARGs was calculated in the unit of copies of ARG per mL based on the mass conservation between extracted DNA and sequenced DNA according to our previous study (Yin et al., 2022). MGEs in short-read data were annotated by aligning to a published MGEs reference database including transposes, integrases, Tn916 and other important MGEs (Parnanen et al., 2018) using the ARGs-OAP pipeline. The MGEs were quantified in unit of copies of MGEs per cell. Kraken2 v2.1.2 was used for taxonomic classification based on the Genome Taxonomy Database (GTDB; release 207) (Parks et al., 2020). The results of kraken2 were summarized in the relative quantification of each taxon by Bracken 2.0.

To compare the resistome of samples from HK with those from other area worldwide, metagenomic datasets of river water were searched and downloaded from previous publications, including short-read metagenomic data from Mainland China (Gao et al., 2024; Liang et al., 2020), Korea (Lee et al., 2020), and Switzerland (Lee et al., 2021). The collected data were analyzed using the same pipeline described above for the quantification of ARGs.

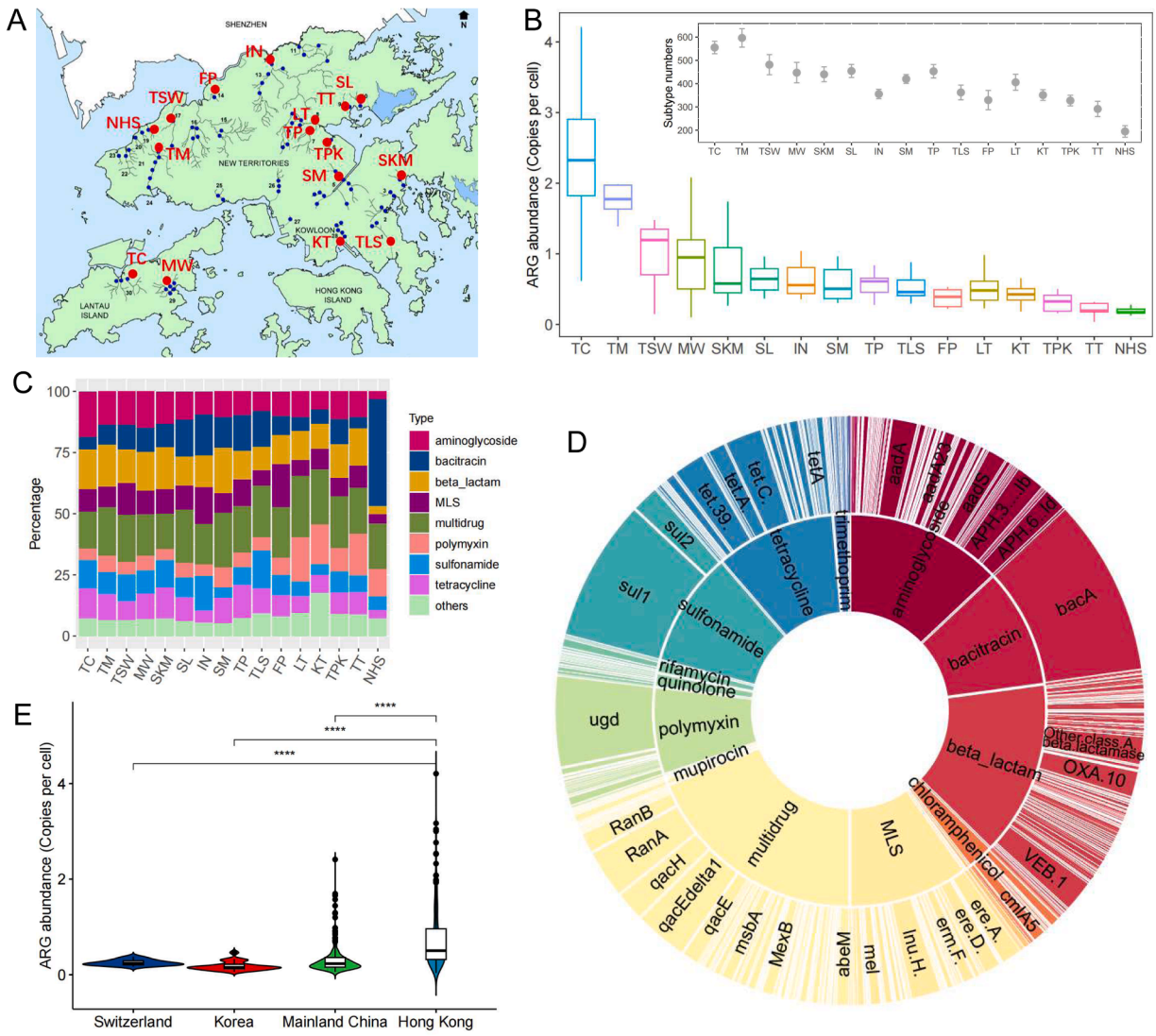

Fig. 1. Profile and distribution of antibiotic resistome in river water samples from Hong Kong (HK). (A) Sampling sites for river water samples in HK; (B) ARG abundance and subtype number at different sites; (C) Structure of ARG types; (D) Composition of core ARG subtypes, inner and outer circles represent ARG types and subtypes, respectively; (E) Total level of ARGs in river water samples from the four regions (**** indicates significant difference at $p<0.0001$).

2.4. Nanopore library preparation and sequencing

Library preparation for Nanopore sequencing was performed using SQK-LSK 110 and SQK-NBD112.24 ligation sequencing genomic DNA kit. GridION and PromethION sequencing were performed using R9.4.1 flowcells. A total of 137.16 Gb nanopore sequencing data for metagenomic analysis were obtained for 10 river water samples using 4 flowcells. Raw data were base-called by Guppy. For each sample, at least 10 Gb data were generated with N50 ranged from 1.28 kb to 8.15 kb (Table S1). The longest read was 272.98 kb obtained in the LT_T2 sample.

2.5. Identification of ARG-related MGEs and ARG hosts in long reads

Long reads were aligned to the nucleotide sequences of the SARG database to annotate ARGs using minimap2. The results were filtered by the cutoff of alignment length cover > 75% and similarity > 80%. The ARG-containing reads were extracted and PlasFlow was used to identify the ARGs carried by plasmids or chromosomes (Krawczyk et al., 2018). Other MGEs, such as transposes, integrases, and Tn916 were identified using minimap2 aligned to the MGE reference database (Parnanen et al., 2018). The results with alignment length cover > 50% and similarity > 80% were filtered for further analysis. The taxonomy of ARG-containing chromosomal reads was assigned by kraken2 with the GTDB Database (release 207); the classification results were visualized with Pavian (https://github.com/fbreitwieser/pavian). Potential species-level bacterial pathogen was identified by comparing the taxonomic results to a pathogen list (Yang et al., 2022).

2.6. Construction and taxonomic assignment of metagenomic assembled genomes (MAGs)

MAGs were retrieved by NanoPhase (v0.2.1) via meta mode and hybrid tag, using the long-read and the corresponding short-read datasets from the same sample. MAGs with completeness of more than 50% and contamination of less than 10% were retained for further analysis. The taxonomy of MAGs was classified by GTDB-Tk v2.1.1 with the database GTDB (release 207). The phylogenetic tree was generated by GTDB-Tk via the infer mode and presented by iTOL (https://itol.embl.de/).

2.7. Statistical analyses

To identify the predominant environmental factors modulating the

distribution patterns of ARGs absolute quantification, random forest model was constructed using "randomForest" package in R studio (Breiman, 2001). The environmental parameters were collected from the website of Environmental Protection Department of HK SAR Government (https://www.epd.gov.hk/epd/english/environmentinhk/water/hkwqrc/waterquality/river.html), including 5-day biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), dissolved oxygen (DO), fecal coliforms count, *Escherichia coli* count, and so on. Source attribution prediction was conducted using a microbial source tracking model with the parameter settings and training datasets from our previous study, which included the sources from 13 various environmental habitats and calculated the predictive proportions for the query sink samples (Yin et al., 2023a). Principal Coordinates Analysis (PCoA) was conducted based on Bray-Curtis distance, using R packages "vegan" and "BiodiversityR".

3. Results

3.1. Riverine antibiotic resistome across Hong Kong

The total ARG abundance (in the unit of copies per cell) of the river water was highly variable among the 16 sampling sites. The highest ARG abundance was detected in the TC samples, with 2.34 copies per cell on average, which is 12.3-fold higher than the lowest level in the NHS samples (Fig. 1B). TC and NHS samples also had the highest and the lowest subtype diversity of ARGs, respectively. The profiles of ARG at type level were similar among different sampling sites, as similar core ARG types were found, including aminoglycoside, β-lactam, macrolide lincosamide-streptogramin (MLS), sulfonamide, multidrug, tetracycline, bacitracin, and polymycin (Fig. 1C). These eight core ARG types accounted for more than 82.0% of total ARGs in each sampling site. While the ARG abundance was largely shaped by spatial heterogeneity, seasons did not have a significant impact on the resistome (Fig. S1). Although the ARG concentrations at a few sampling sites showed temporal variations, such variations seemed to be stochastic (Fig. S1).

The diversity of ARG subtypes decreased along with the decrease in total ARG abundance (Fig. 1B). A total of 1881 ARG subtypes from 30 ARG types were found in the river water samples, out of the total of 2843 ARG subtypes from 32 ARG types in the SARG database. Among these ARG types, β-lactam (1106 subtypes), aminoglycoside (147 subtypes), multidrug (118 subtypes), and MLS (114 subtypes) had higher subtype diversity (Fig. 1D). The subtypes of sul1, bacA, ugd and tetC had higher abundance compared with others, which belonged to the ARG types of sulfonamide, bacitracin, polymyxin and tetracycline, respectively. There were 28 subtypes commonly shared in all the river water samples, and the structure of these ARG subtypes in different samples was similar (Figure S2).

To compare the riverine resistome abundance and diversity with previous studies, metagenomic datasets of river water samples from Pearl River and Chaobai River from Mainland China (Gao et al., 2024; Liang et al., 2020), the Han River from Korea (Lee et al., 2020), and the Suze River from Switzerland (Lee et al., 2021) were downloaded and reanalyzed using the same pipeline and parameters. The total ARG abundance of river water samples from the previous studies was significantly lower than that of samples in this study ($p<0.01$) (Fig. 1E). The differences might be resulted from the selection of sampling sites. The rivers in this study were not as large as those in previous studies conducted in Mainland China, Switzerland, and Korea. Compared to large rivers flowing through pristine or sparsely populated areas and other areas with different degrees of urbanization, the majority of sampling sites in this study were located in regions with higher population density, where the resistome was subject to greater influence from human activities. The dissimilarity of the resistome in those samples from HK with others was obviously shown with the profile at type level (Figure S3A), and the principal coordinates analysis (PCoA) also demonstrated the distinction at subtype level, as samples from HK were clustered together and away from other datasets (Figure S3B). For the structure of the ARG types (Figure S3A), it worth noted that tetracycline accounted for 9.9% for those river water samples from HK, while it is 2.1% for that of Korea and Switzerland, and 5.7% for that of Mainland China. Bacitracin accounted for over 44.4% for Korea and Switzerland as the dominant ARG type, while in samples from HK bacitracin accounted for about 9.9% of riverine resistome.

3.2. Antimicrobial resistance (AMR) risk reflected by Rank I ARGs in the river

Rank I ARGs (ARGs posing the highest risk and classified as "current threats" considering human-associated-enrichment, gene mobility, and host pathogenicity) were identified by comparing to the list provided by the previous study (Zhang et al., 2021a). The list encompassed 122 ARGs classified as Rank I, referring to human-associated and mobile ARGs already present in ESKAPE pathogens. In total, 62 out of 122 Rank I ARGs were detected in samples of river water from HK. Consistent with total resistome, the level of Rank I ARGs were the highest in TC samples (Fig. 2A), reaching 0.031 copies per cell on average. However, it was noteworthy that some samples (LT and KT samples) had relatively low total resistome, but their level of Rank I ARGs was also high. The most abundant ARG variants were *ANT(2″)-Ia* (aminoglycoside resistance) and *VEB* (beta_lactam resistance), and both of these two were found with the highest level in TC samples (Fig. 2B). The high level of Rank I ARGs in LT and KT samples were predominantly composed by *qnrS* (quinolone resistance). The overall relative abundance of Rank I ARGs were significantly higher in those samples collected in HK rivers than that of previously reported in Mainland China, Switzerland and Korea (Fig. 2C).

3.3. Contributors to the variation of ARG absolute quantification

The absolute quantification of ARGs (copies per mL water) was calculated based on the Illumina sequencing data and sample volume. ARG concentration varied largely across different sampling sites (Fig. 3A). Higher absolute concentrations were detected in the samples from TM, FP, LT, TSW and TC, with an average concentration exceeding 10^6 copies per mL, although the relative abundance of ARGs in the FP and LT samples were not high. The difference between relative ARG abundance (copies per cell) and ARG absolute concentration (copies per mL) was mainly due to the variability in biomass among different river sites since the biomass in FP and LT samples were higher than in other sites (Fig. S4).

To reveal the impacts of environmental factors on ARG pollution, correlations between the ARGs absolute concentration and environmental factors were evaluated by the random forest model (Fig. 3B). The results showed that ARG absolute concentrations were significantly correlated to fecal coliform count, *E. coli* count, BOD_5, DO, and total organic carbon (TOC). Consistently, positive correlations were found between ARG absolute concentrations and fecal coliform, *E. coli*, BOD_5, and TOC, while negative correlations existed between ARGs and DO (Fig. 3C). The ARG concentrations were also closely related to water quality index (WQI), a comprehensive index for routine surveillance on water quality, which was calculated based on an integration of relative levels of DO, BOD_5, and ammonia-Nitrogen. These correlations likely indicated an association of the organic pollution in the river and the ARG abundance, as organic pollution would affect the microbial biomass density and proliferation. Then the proliferation of bacteria may directly increase ARGs by vertical gene transfer, and proximity of microorganisms to each other is likely to provide more opportunities for interspecific interactions, increasing the chance for HGT. Additionally, fecal coliforms and *E. coli* cells commonly reflected the fecal contamination (Devane et al., 2020). The high load of these bacteria indicated possible discharges into the river with deficient treatment. However, other factors in the routine monitoring of river water quality such as nitrite or nitrate nitrogen, temperature, and pH showed no significant

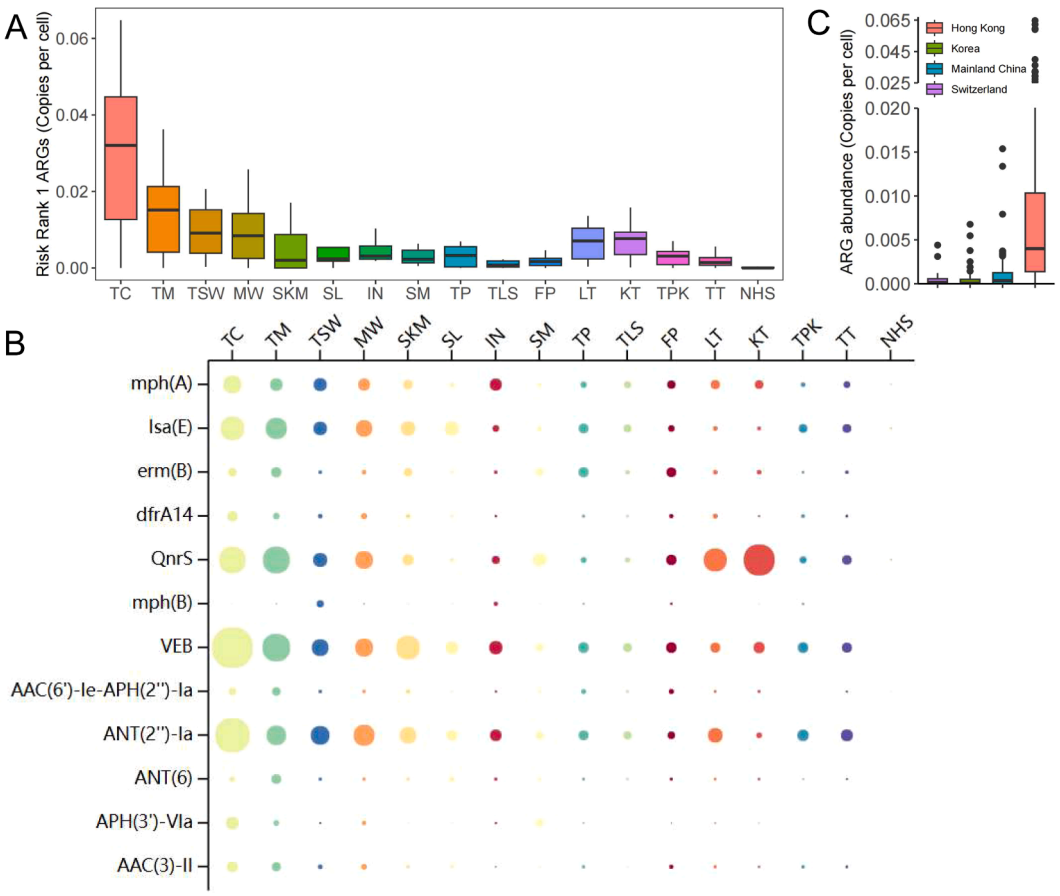

Fig. 2. Profile and distribution of Rank 1 ARGs in river water samples from HK. (A) Total abundance of Rank 1 ARGs in different sampling sites; (B) Abundance of dominant Rank 1 ARGs (with abundance > 0.002 in at least one sample); (C) Total level of Rank 1 ARGs in river water samples from the four regions.

correlation with the level of riverine ARGs (Fig. 3B). Furthermore, the PCoA analysis revealed a resemblance between the resistome of those samples of river water in HK and the resistome in samples from WWTP and sewage (Figure S5A). Consistently, the resistome of river water was mainly attributed to WWTP samples and sewage by the source tracking model (Figure S5B), indicating the potential impact of sewage discharge on the composition of ARG profile in the samples of river water.

3.4. MGEs abundance, diversity, and the association with ARGs

MGE abundance was firstly annotated and quantified based on the short-read sequencing data (Fig. 4A, 4B). The total abundance of MGEs ranged from 0.48 (NHS) to 13.96 (TM) copies per cell (Fig. 4A). The profiles of MGE types were similar among different sampling sites, as the transposase and insertion_element_IS91 were two dominant MGEs in all samples. Unsurprisingly, there existed a positive correlation between the total relative abundances (copies per cell) of ARG and MGE across the river water samples (Fig. 4B).

Samples from the sites of TM, FP, LT, TSW, and TC were selected for nanopore long-read sequencing as the total ARG concentration of these sites were higher than others. Totally, 7253 ARG-carrying long reads were obtained (Fig. 4C), and 14 ARG types were found in these reads. 57% of ARG-carrying long reads were originated from plasmids, while only 19% were originated from the chromosome. ARGs in plasmids were mainly resistance to beta_lactam, sulfonamide, and aminoglycoside. Furthermore, co-occurrence of ARGs and other MGEs were detected on 1641 reads, which were used for further analysis of their physical linkage. The total MGE profile resulted from all long reads was similar to that from Illumina data, as the transposase and insertion_element_IS91 contributed to 73.8% and 10.0%, respectively (Fig. 4D). However, the profile of ARG-related MGEs was apparently different from total MGEs, and the main differences were observed in the significant increase in the percentage of qacEdelta, integrase and Tn916 and the decrease in the transposase in ARG-related MGEs. Similarly, the profile of mobile ARGs was different from ARGs profile on all long reads. The most abundant mobile ARGs was sulfonamides (42.5%). The percentage of bata-lactam was significantly lower in mobile ARGs (14.7%), although it dominated in ARGs on all reads (37.2%) (Fig. 4D).

The ARG-related MGEs have a broad spectrum, and the most abundant ones were qacEdelta, transposase and integrases (Fig. 4C, 4D). There were 9 ARG types physically linked with qacEdelta and integrase, and 12 ARG types physically linked with transposase (Fig. 4C). These ARGs were mainly belonging to the ARG type of sulfonamide, aminoglycoside, and beta_lactam, indicating the high mobility of these types. Interestingly, although there were only 4 ARG types had physical linkage with Tn916, Tn916 showed specific enrichment in the reads containing tetracycline. The complex arrangement of ARGs and adjacent MGEs could easily be revealed by Nanopore long-read sequencing (Fig. 4E). Genetic analysis indicated the co-occurrence of ARGs from different resistant types on the same long reads, along with multiple MGEs.

3.5. Microbiome diversity and ARG host identification

The relative abundance of microbial community in river water was revealed by Illumina sequencing (Fig. 5A). At class level, most reads were assigned to *Gammaproteobacteria*, followed by *Alphaproteobacteria, Actinomycetia, Bacteroidia*. As indicated by the identification of ARG hosts by Nanopore long reads, a majority of ARG-containing reads were assigned to the phyla of *Proteobacteria* and the class of

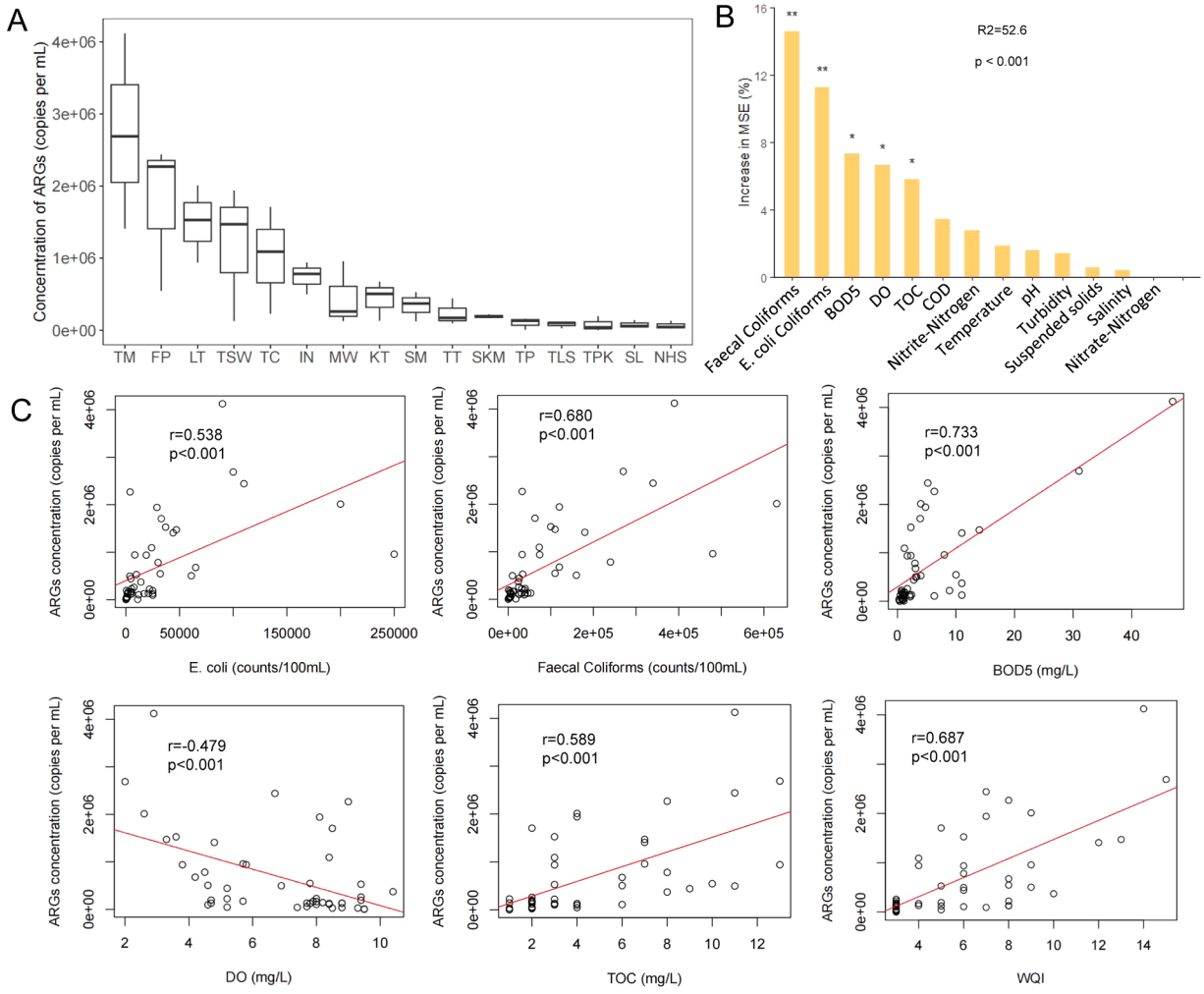

Fig. 3. Variations and contributors of ARG absolute concentrations. (A) Spatial variability of absolute concentrations; (B) Effects of environmental factors to ARG absolute concentrations evaluated by random forest (* and ** indicate a significant factor at $p<0.05$ and $p<0.01$, respectively); (C) Correlations between ARG absolute concentrations and environmental factors.

Gammaproteobacteria (Fig. 5B). This result was likely due to the high abundance of this class in the whole microbial community in river water, as demonstrated by the short-read data. Among the top 15 dominant species of the hosts of river ARGs shown in Fig. 5B, there were three pathogenic species, namely *E. coli, Klebsiella pneumoniae,* and *Enterobacter roggenkampii*. Nanopore sequencing generated more ARG-carrying long reads than contigs assembled from Illumina short reads with similar sequencing depth, and the length was longer (Figure S6). In this study, 7253 ARGs-carrying long reads with an N50 length of 8.4 kb were identified, while 196 ARGs-carrying contigs with an N50 length of 3.5 kb were assembled from corresponding short-read data, indicating the advantage on assessing the genetic context of ARGs by long-read sequencing. Using the long reads located on the chromosome, in total 23 pathogenic species were identified carrying ARGs among 9 types (Fig. 5C, 5D), which was much more than 2 pathogen hosts founded using contigs (Fig. S6). *K. pneumoniae, E. coli,* and *E. roggenkampii* harbored high ARG diversity at both subtype level and type level. At ARG type level, the pathogenic hosts of beta_lactam and macrolide-lincosamide-streptogramin resistant genes had wider taxonomic distribution, followed by multidrug and aminoglycoside (Fig. 5D).

From the hybrid assembly and binning using both long- and short-reads, 277 MAGs were obtained. The phylogenetic tree of these MAGs is shown in Fig. 6, along with the number of ARGs in these MAGs. Overall, there were 40 subtypes of ARGs from 8 types that were identified in these MAGs (Table S2). Some ARGs were shared in a high taxonomy level. *OXA, aadA22*, and *AAC(6′)-Ib7–9-10* were shared by the MAGs across different phyla, while *sul1* was shared by cross-class MAGs in the same phylum (*Pseudomonadota*). Among these MAGs, 4 MAGs were assigned to pathogenic host at species level, and ARGs were also founded in these MAGs. Especially, one MAG of *E. coli* carried 11 ARGs, including 1 polymyxin resistance gene (*arnA*), 1 beta lactam resistant gene (*ampC*), and 9 multidrug resistance genes (*mdtA, mdtG, mdtH, mdtL, mdtM, emrA, emrB, emrD*, and *CRP*), which was the highest ARG number in the sole MAG. Among these genes, *emrA* was shared with a MAG of another pathogenic species, *E. roggenkampii*. Besides, *mdtH* and *emrB* were shared with the non-pathogenic MAGs of *Klebsiella quasipneumoniae*.

4. Discussion

Rivers are one of the reservoirs of ARGs and play as critical connection points between environment and human health. Previous studies have showed that some antibiotics were frequently detected in river water in HK, especially sulfonamides (sulfadimidine and sulfamethoxazole) and tetracycline (Deng et al., 2018). However, the data of antibiotic resistome and the related genetic context was still deficient. Some historical data of the exposure level of typical ARG targets in river water were generated by qPCR method (Zhang et al., 2020), which was limited in both the detection range of ARG variants and the comparison with other studies. To our knowledge, this is the first study to

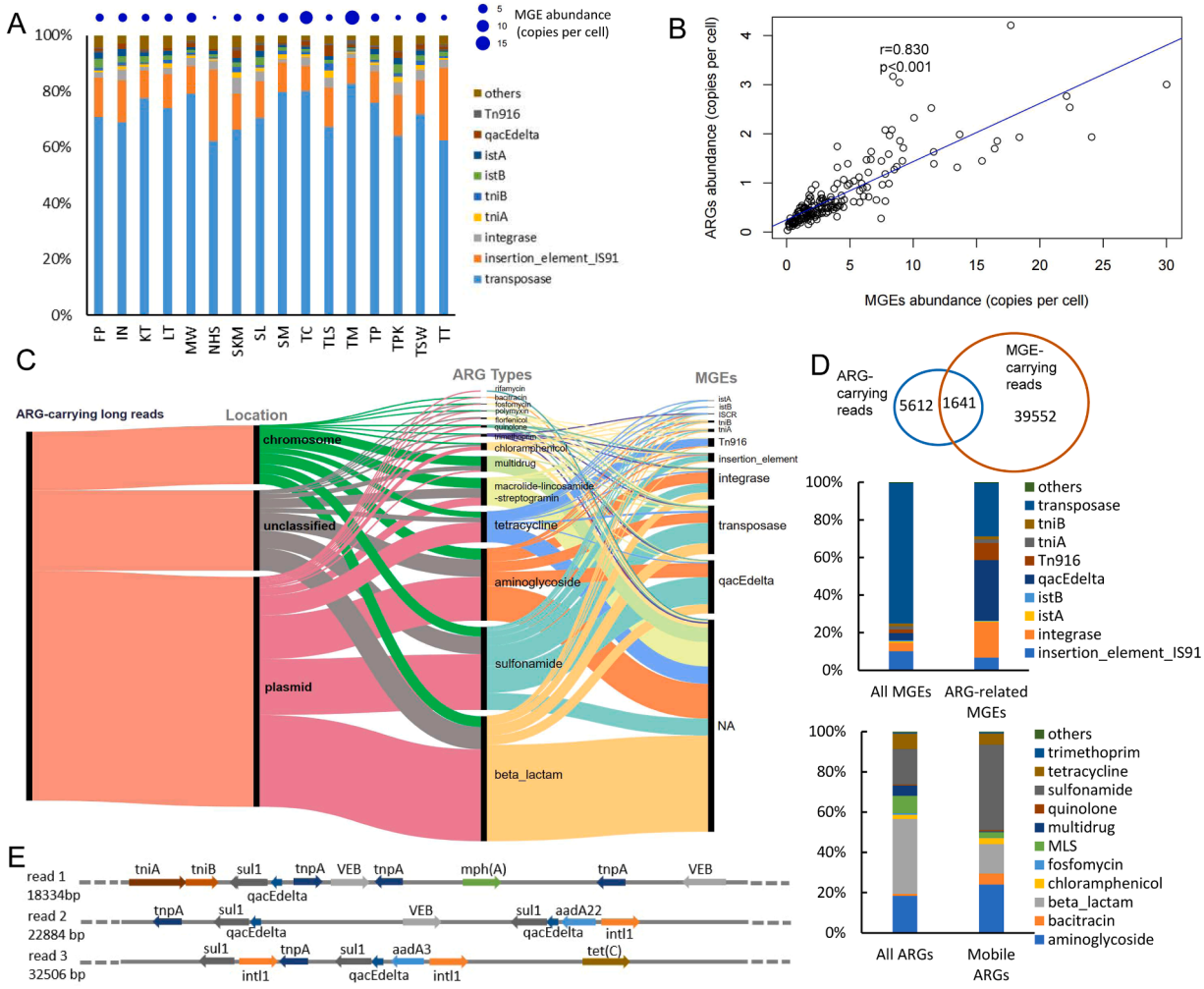

Fig. 4. MGEs in river water samples from HK revealed by Illumina (A-B) and Nanopore sequencing (C-E). (A) Abundance and structure of mobilome from short reads; (B) The correlation analysis of ARGs and MGEs level; (C) The co-localization of ARGs and MGEs on long reads; (D) Diversity of ARGs and MGEs on long reads; (E) Schematic of the genetic organization of ARGs and MGEs on the same long reads.

comprehensively decipher the resistome profile using metagenomic sequencing in water samples collected from 16 rivers/streams across a sub-tropical metropolitan city spanning a three-year longitudinal period. The gathering information for multiple rivers in this regional study allows us to compare different river systems, identify common AMR challenges across the regional river ecosystems, and highlight specific ARG hotspots, which could not be observed when studying individual rivers in a short term. In this study, spatial variations of resistome were observed from the in-depth investigation of samples from 16 rivers. High ARG levels were founded at TC and TM sampling site regarding to the ARG relative abundance, Rank 1 ARGs, and subtype diversity. The lowest ARG relative abundance, Rank 1 ARGs, and subtype diversity were detected at NHS. By the gathering information for multiple rivers, ARG hotspots such as TC and TM were identified for further surveillance and management. In spite of the high spatial heterogeneity in the ARG exposure level, common AMR challenges in the regional river ecosystem were also revealed such as the core ARG types in all rivers and potential sources. The structures of ARG types were similar among different sampling sites in HK with the core resistome constituting by aminoglycoside, bacitracin, β-lactam, MLS, and sulfonamide (Fig. 1B). Longitudinal metagenomic data over three years provided more solid evidence for these findings on the regional patterns, confirming a consistent and stable pattern across a long term. Compared with the river resistome previously reported in other regions, it was worth noting that the antibiotic resistome in those samples from HK was significantly distinct from those in Mainland China, Korea and Switzerland. The total relative abundance (in the unit of copies per cell) of ARGs in those water samples collected in this study from HK rivers was higher than those in the Pearl River and Chaobai River from Mainland China, the Han River from Korea, and the Suze River from Switzerland (Fig. 1E). Particularly, the abundance of Rank 1 ARGs were higher in those HK river water samples especially in the samples from the sites of TC and TM (Fig. 2C), furtherly indicating a relatively higher risk, as also indicated by other water quality parameters, like *E. coli* count and fecal coliform count. The ARG types profile and clustering results at subtype level both demonstrated the separation of resistome structures in the river water from different geographical areas (Figure S3). These results suggested that tailored management strategies towards resistome surveillance and risk control were required to effectively address the varying needs of different regions.

In this study, the absolute quantification in the unit of copies per mL was also adopted for ARG assessment along with the relative abundance in the unit of copies per cell. The difference between the results of relative abundance and absolute quantification was obvious (Fig. 1B, Fig. 3A). The most prominent difference was that the absolute concentrations in FP and LT were higher than most of other rivers, while the relative abundances were relatively low. The discrepancy between the absolute and relative abundances was reasonable because the cell density in the samples varied, while the composition of ARGs (in the percentage form) was consistent using different units. The absolute

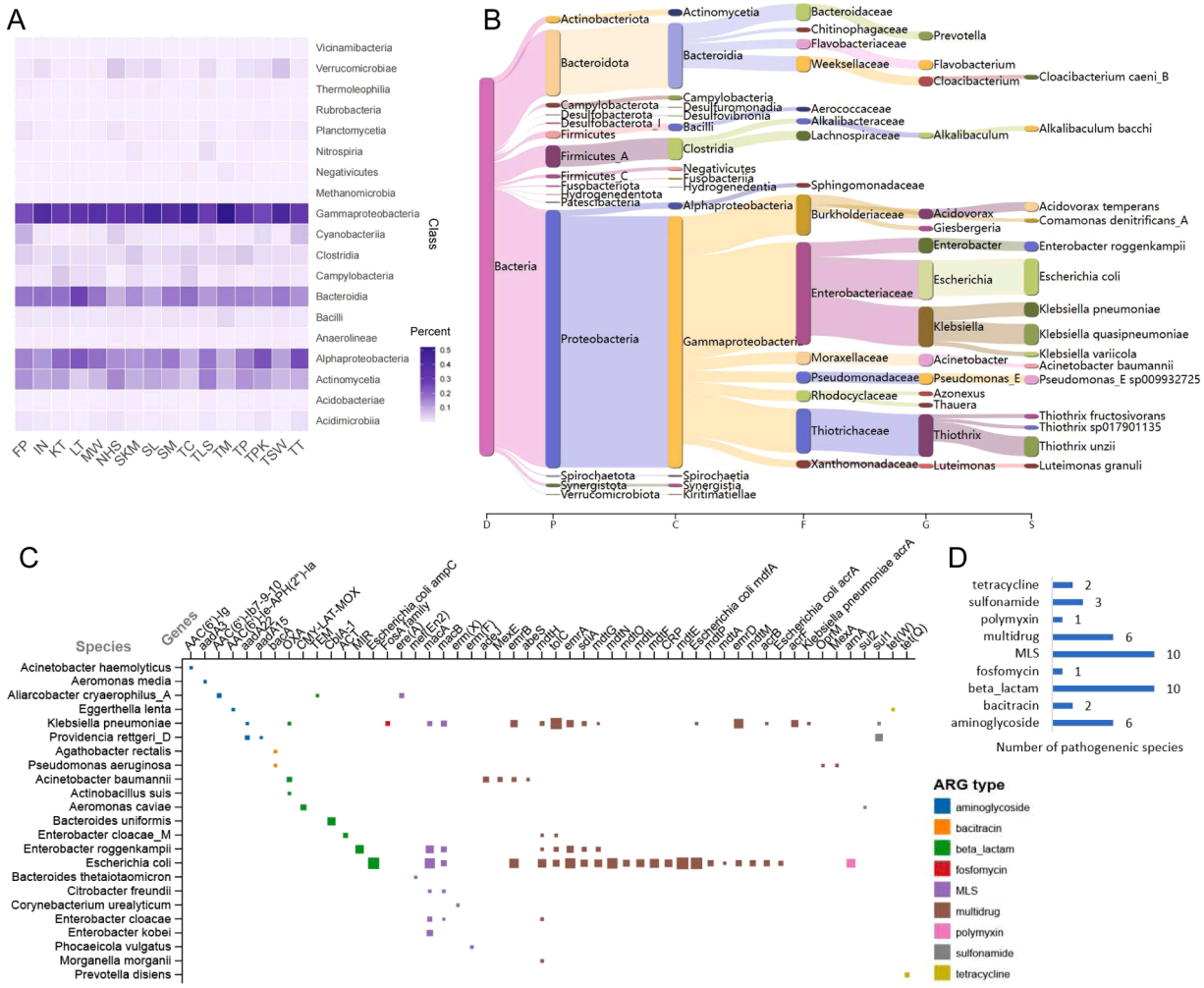

Fig. 5. Microbial community and ARBs in river water samples from HK revealed by Illumina (A) and Nanopore sequencing (B-D). (A) Microbial composition revealed by short reads at class level (only showing class with abundance > 0.02 at least in one sample); (B) Predominant ARG hosts revealed by long-reads; (C) ARG diversity in the pathogenic hosts; (D) The number of pathogenic species carrying the respective ARG types.

quantification for resistome analysis has more important scientific meaning in environmental ARG surveillance as it takes microbial cell density into consideration and reveals the real exposure dose in the environmental samples, which facilitates the comparison among different ARG habitats and the identification of hotspots (Yin et al., 2022). In addition to focusing on sites with a high relative abundance of ARGs, it was also important to pay close attention to samples with high ARG absolute concentration or the presence of high risk ARGs, such as FP and LT samples in this study. The absolute concentration of ARGs offered a more concrete measure of the exposure level to human in river water, which was more meaningful for understanding the AMR risk to human health and the correlation of ARGs with the environmental factors. However, the application of absolute concentration based on the mass conservation should be adopted with an extra care, as its calculation depends on the correct estimation of DNA extraction efficiency and the accurate metadata including sampling volume and the concentration of extracted DNA. At the current stage, parallel comparisons of global datasets could hardly be conducted by absolute quantification because of the limited availability of accurate sample volume and DNA concentration. Therefore, global efforts and cooperations were still required to further expand the collection of these sampling metadata to facilitate the real exposure ranking and hotspot identification by absolute concentration. In this study, we set a pioneer example of using absolute concentration to compare ARG levels in the river ecosystems, and strongly recommended to combine accurate sampling metadata with metagenomic analysis while conducting wide-range and long-term ARG surveillance in the environment. Another alternative absolute quantification method was based on the cellular spike-in (Wang et al., 2024; Yang et al., 2022), which could address the challenge of limited or inaccurate sampling metadata. However, the cost of incorporating spike-ins in the pretreatment process of all samples should be considered, especially in the large-scale surveillance.

ARGs with the high mobility and pathogenicity were regarded as the high-risk ones, which may cause severe threats to the human health (Zhang et al., 2021a; Zhang et al., 2022). The potential health risks caused by riverine ARGs have not been completely evaluated yet, because information of ARG exposure dose, the transfer risk and the presence in human pathogens were insufficient for risk assessment. This study provided valuable data for the risk assessment of riverine antibiotic resistome considering not only the exposure level of ARGs, but also the mobility and pathogenicity. MGEs were reported as the major driver to disseminate ARGs (Zhang et al., 2021b). In this study, a positive correlation between the total abundance of ARG and MGE was found based on the longitudinal metagenomic analysis of 173 river water samples collected from 16 rivers. Using long-read sequencing, this study revealed the high mobility of riverine ARGs, especially for the ARG types of sulfonamides, aminoglycoside and beta_lactam as most of these genes were co-localized to plasmids and other MGEs. Therefore, riverine ARGs poses high risk of dissemination via HGT. Consistent with a previous study, transposase was the most dominant MGE detected by

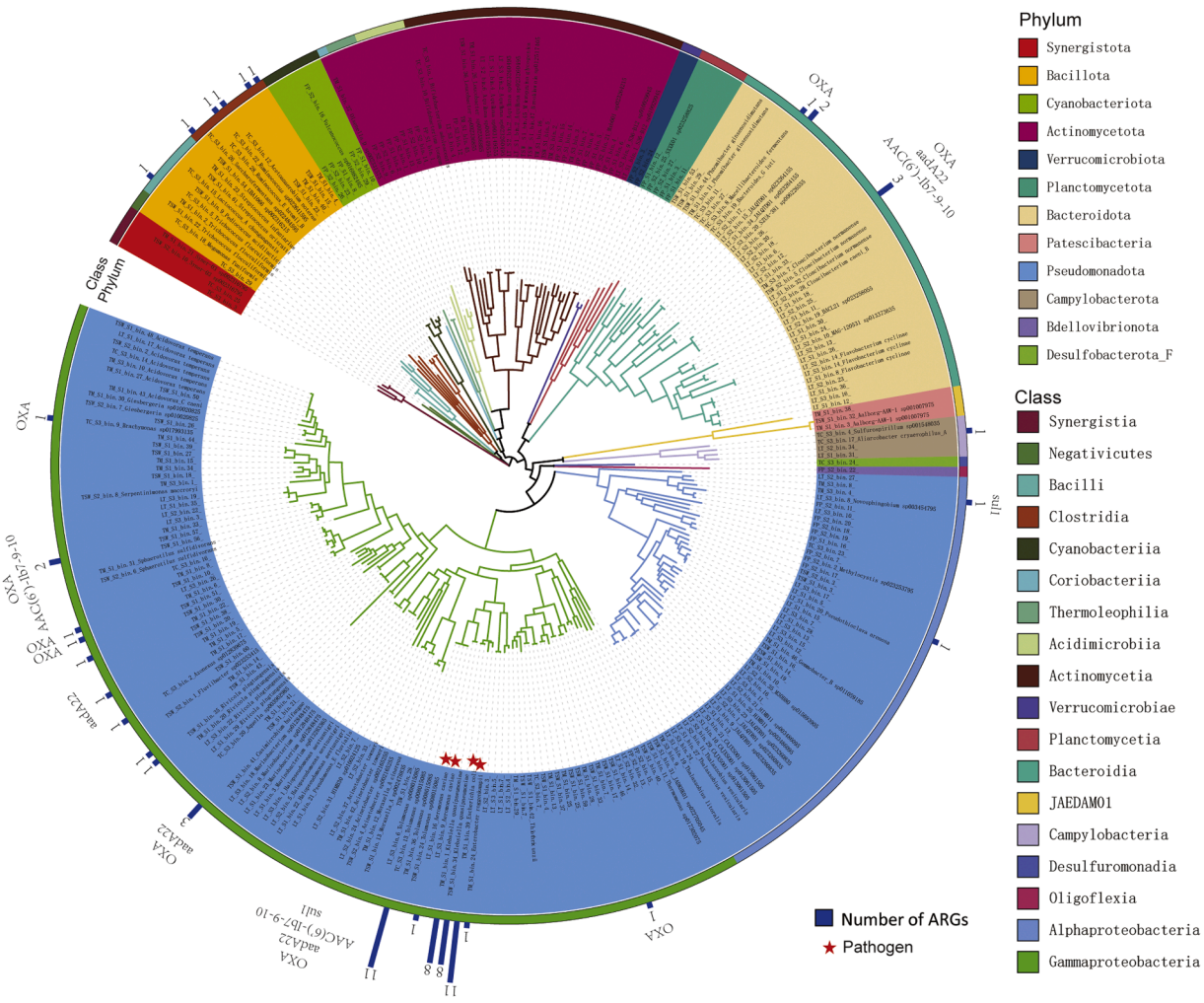

Fig. 6. ARGs shared across taxonomy ranks of MAGs. The bar indicates the number of ARGs in the genome. The colors of the clades and the strip of outer layer show the taxonomy at the class level.

Illumina sequencing in river water (Samson et al., 2023). The results of Nanopore sequencing also support the high abundance of transposase in ARG-carrying long reads, along with other MGEs including qacEdelta, integrase, and Tn916. Thus, transposase, qacEdelta, integrase and Tn916 were likely playing important roles in the dissemination of riverine ARGs. Interestingly, the Tn916 showed specific enrichment in the reads containing tetracycline, which was rarely reported in previous studies of riverine resistome.

The diversity of ARG hosts and the sharing of ARGs across different taxonomic ranks were demonstrated by long reads and MAGs (Fig. 5C, Fig. 6). Various bacteria pathogens in river water samples from HK were found harboring ARGs, which included *K. pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species, known as clinical pathogens as part of the "ESKAPE" (De Oliveira et al., 2020). There were six pathogen species each of which was previously identified as responsible for more than 250,000 deaths associated with AMR in 2019 (Antimicrobial Resistance Collaborators, 2022). Four out of these six species were detected as riverine ARG hosts in the study, namely *E. coli, K. pneumoniae, Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. This finding reflected the AMR risks in river water. ARGs including *OXA* (a beta_lactam resistance gene), *aadA22*, and *AAC (6′)-Ib7–9-10* (two aminoglycoside resistance gene) were found being shared across different phyla, while some ARGs were only found in one species such as *MIR* (a beta_lactam resistance gene harbored by *E. roggenkampii*) and *Cbla-1* (a beta_lactam resistance gene harbored by *Bacteroides uniforms*). These findings underscore the role of rivers as a reservoir for antibiotic-resistant pathogens and the potential risks they pose in transmitting ARGs from the environment to public health. This is the first study to reveal the mobility of riverine ARGs and candidate pathogenic ARBs by the combination of short-read and long-read metagenomic sequencing in the river water, providing an integrated assessment of the risk of riverine resistome. The current results offered an understanding of the spatial variation and interactions of riverine resistome, mobilome and microbiome, which is crucial for making location-specific monitoring and mitigation strategies for ARGs and pathogenic ARBs. Hence, incorporating ARGs surveillance into the routine water quality monitoring should be considered.

One of the limitations of this study is that we did not specially study the viability of host cells carrying ARGs. Future work on culture-based isolation of ARBs from rivers will yield vital evidence for assessing the human exposure risk. The data obtained in this study could provide guidance for designing the labor-consuming isolation process. Additionally, only one site was selected from each river in this study. A more comprehensive monitoring scheme of ARGs will provide a clearer and more accurate profile of resistome. The results in this study may serve as a guide for the design and implementation of such monitoring scheme.

5. Conclusions

The resistome, mobilome and microbiome in 173 water samples collected from 16 rivers/streams spanning three years were thoroughly studied by the combination of short-read and long-read metagenomic

sequencing. The resistome of the river water exhibited significant spatial variation regarding the total relative abundance of ARGs, Rank 1 ARGs and subtype diversity. This study presented the pioneering documentation of the absolute concentration of riverine ARGs and the correlation between ARGs and environmental parameters such as total fecal coliform count, *E. coli* count, BOD_5, TOC, and DO. MGE types of qacEdelta, transposase, integrase, and Tn916, were major contributors to the mobility of riverine ARGs unveiled by the Nanopore long reads. In addition, 23 pathogenic bacterial species were identified as hosts of ARGs in the rivers, highlighting the necessity of increased awareness and prioritization in future ARB surveillance efforts. Overall, this study enriched our understanding of the resistome and its connection to MGEs and pathogens through a longitudinal metagenomic analysis in river ecosystems.

CRediT authorship contribution statement

Xuemei Mao: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Xiaole Yin:** Writing – review & editing, Methodology. **Yu Yang:** Writing – review & editing, Methodology. **Fangzhou Gao:** Writing – review & editing, Validation. **Shuxian Li:** Investigation. **Xianghui Shi:** Investigation. **Yu Deng:** Writing – review & editing, Methodology. **Liguan Li:** Writing – review & editing, Methodology. **Kenneth M.Y. Leung:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Tong Zhang:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2025.123102.

Data availability

Data will be made available on request.

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