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Nanopore-based long-read metagenomics uncover the resistome intrusion by antibiotic resistant bacteria from treated wastewater in receiving water body

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ABSTRACT

Wastewater treatment plant (WWTP) effluent discharge could induce the resistome enrichment in the receiving water environments. However, because of the general lack of a robust antibiotic-resistant bacteria (ARB) identification method, the driving mechanism for resistome accumulation in receiving environment is unclear. Here, we took advantage of the enhanced ARBs recognition by nanopore long reads to distinguish the indigenous ARBs and the accumulation of WWTP-borne ARBs in the receiving water body of a domestic WWTP. A bioinformatic framework (named ARGpore2: https://github.com/sustc-xylab/ARGpore2) was constructed and evaluate to facilitate antibiotic resistance genes (ARGs) and ARBs identification in nanopore reads. ARGs identification by ARGpore2 showed comparable precision and recall to that of the commonly adopt BLASTP-based method, whereas the spectrum of ARBs doubled that of the assembled Illumina dataset. Totally, we identified 33 ARBs genera carrying 65 ARG subtypes in the receiving seawater, whose concentration was in general 10 times higher than clean seawater's. Notably we report a primary resistome intrusion caused by the revival of residual microbes survived from disinfection treatment. These WWTP-borne ARBs, including several animal/human enteric pathogens, contributed up to 85% of the receiving water resistome. Plasmids and class 1 integrons were reckoned as major vehicles facilitating the persistence and dissemination of ARGs. Moreover, our work demonstrated the importance of extensive carrier identification in determining the driving force of multifactor coupled resistome booming in complicated environmental conditions, thereby paving the way for establishing priority for effective ARGs mitigation strategies.

1. Introduction

Antibiotic resistance has been hailed as top global health concerned in 21st century by the WHO ("WHO | Antimicrobial resistance," n.d.). The increasing level of antibiotics in environment could form persistent selection pressure for antibiotic resistant bacteria (ARB) to develop resistance via obtaining antibiotic resistance genes (ARGs), contributing to proliferate antimicrobial resistance (AMR) in microbial communities (Tyers and Wright, 2019). Importantly, ARGs are frequently encoded on mobile genetic elements (MGEs), which facilitates its transfer and transformation between bacteria among phylogenetically distant lineages (Zhang et al., 2018). For instance, human bacterial pathogens might recruit ARGs from microbes colonized in soil and other environments (Forsberg et al., 2012) through horizontal gene transfer (HGT).

Abbreviations: WWTP, wastewater treatment plant; ARB, antibiotic-resistant bacteria; ARGs, antibiotic resistance genes; AMR, antimicrobial resistance; HGT, horizontal gene transfer; MGEs, mobile genetic elements; epicPCR, Emulsion, Paired Isolation and Concatenation PCR.

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Therefore, recognizing and identifying the host populations of ARGs, is crucial to reveal dynamic dissemination of ARGs and prerequisite to uncover the molecular mechanism for AMR accumulation in an environment.

The wastewater treatment plant (WWTP) is one of the typical hotspot for dissemination of ARGs and proliferation of ARBs (Hendriksen et al., 2019; Ju et al., 2018). Even though more than 98% of ARGs could be removed after the wastewater treatment process (Yang et al., 2014), the downstream receiving water body of the WWTP was observed as an important reservoir for transmission of pathogenic bacterial host with alarming resistance level (Luo et al., 2010). Existing knowledge regarding the risk of ARGs dissemination from WWTP was primarily derived from the observation of human-threat ARG genotypes, such as BlaNDM-1, KPC-2, etc., in receiving environments (Luo et al., 2010; Yang et al., 2017, 2016). Despite confirming the release of ARGs from WWTP, these qPCR-based or culture-based effort focusing on specific marker gene offered little quantitative information into the influence of WWTP discharge on natural environment. Currently, the available technique for accessing such influence is based on Bayesian source tracking analysis, which predicts the apportioned relative contribution of a community originating from a set of source environments (Chen et al., 2019). However, the ecological machinery underpinning the statistical results cannot be revealed without exhaustive association between ARGs and corresponding carrying species. Such resistance increment could be resulted from a combined effect of chemical and biotic factors. Excessive nutrient (Wang et al., 2017), residual antibiotics, heavy metals (Di Cesare et al., 2016; Lin et al., 2019) and non-antibiotic pharmaceuticals (Wang et al., 2018), introduced by the discharged effluent/reclaimed water all could induce endogenous resistome selection in the native natural community. Besides that, ARBs which had survived the disinfection treatment, could be activated in the receiving environments where the inhibiting effect of residual disinfectants is minimized, also attributing to the resistance accumulation (Zhu et al., 2021). Understanding the driving force shaping such resistome booming in receiving environments is essential to establish priority in mitigating ARGs spread and evaluate health risks. However, this question cannot be fully elucidated without the quantitative source tracking based on precise and extensive identification of the host populations of ARGs.

Given the pressing need to reveal the hosts of ARGs and their fates in diverse environmental settings, researchers have tried different approaches including isolation (Hu et al., 2008; Paiva et al., 2017), epicPCR (Emulsion, Paired Isolation and Concatenation PCR) (Hultman et al., 2018), high-throughput sequencing (HST) (Ma et al., 2016). Isolating ARB pure cultures coupled with whole-genome sequencing is the standard approach for clinical resistome investigations (especially focusing on pathogens). However, this method is not robust to study environmental resistome as only less than 1% of environmental microbes can be isolated in pure culture (Kaeberlein et al., 2002). Recent advances in epicPCR brought new possibility towards environmental bacterial resistance surveillance. However, the wide-application of this PCR-based method is constrained by the target primer sets, relative low throughput as well as delicate PCR condition to ensure the connection between ARGs and marker gene (Zhu et al., 2013). In recent years, HST-based metagenomics is gradually becoming the most robust approach to uncover the diversity and abundance of ARGs in environmental microbiomes (Feng et al., 2018; Ma et al., 2017b). However, even though the short reads generated by Illumina HST can be assembled into contigs to improve resolution in identifying ARGs and the neighbourhood genes, it is still challenging to associate ARGs to its host populations because of the fragmented assembly of environmental metagenomes.

Since Oxford nanopore sequencing could generate long reads averaging 10 kb in length, which is long enough to span most repetitive regions on bacterial genome (Ashton et al., 2015), it provides an opportunity to bridge ARGs and their flanking regions to circumvent the limitations aforementioned. In the last five years, nanopore-based metagenomics has been applied to trace the ARGs dissemination in environmental settings. For example, Xun Qian et.al applied nanopore metagenomic sequencing to identify *Enterobacteriaceae* as the dominant hosts of antibiotic-resistant plasmids in cow feces samples (Qian et al., 2021), and our previous research had found the primary attribution of plasmid-enabled resistome in WWTP microbiomes (Che et al., 2019). Tools, such as NanoARG (Arango-Argoty et al., 2019) and NanoOK RT (Leggett et al., 2019), have been developed to enable ARGs identification in Nanopore-based metagenomic datasets. Although these pioneering works had demonstrated the feasibility of long-reads metagenomics in revealing the hosts and mobility of ARGs, numerous key aspects of nanopore sequencing application in environmental resistome study, such as similarity threshold for ARGs identification, remains to be evaluated and optimized at current stage.

Taken together, to elucidate the driving mechanism of resistome dissemination in receiving water body of WWTP, the performance of nanopore sequencing in identifying ARGs and corresponding host populations was rigorously evaluated in term of precision and recall in this study. The algorithms optimized was wrapped up in a package named ARGPore2 (github.com/sustc-xylab/ARGpore2) to provide a robust workflow to profile the ARGs and trace the ARBs in complicated environmental metagenomic samples. With the aid of this improved methods, chromosomal and non-chromosomal encoded ARGs were quantified in an unprecedent high throughput manner in receiving water body, WWTPs effluent and clean seawater to disentangle the cross-linked endogenous resistome selection and exogenous ARB intrusion introduced by WWTP discharge. The approach adopted in this study demonstrated the value of extensive ARB identification in revealing the driving machinery of multifactor coupled resistome booming in complicated environmental conditions, thereby paving the way for establishing priority in surveillance of ARG dissemination.

2. Materials and methods

2.1. Sampling site and collection

Receiving water samples were collected at the three daily resident water usage peaks namely, 9:00am, 14:00pm and 19:00pm. Coastal receiving water receiving effluent discharge from the FuTian full-scale wastewater treatment plant (WWTP) in the city of ShenZhen (22.540° N, 114.003°E) was selected as the study site. And the clean seawater, as control group, were collected from the study site that is 5 km far away from the receiving effluent discharge site (Fig. S5). The site locates in the Shenzhen Bay Park, which covers an area of 108.7 hectares in the south of Shenzhen City, representing an important natural reserve for migratory bird and providing an entertainment place for people. The mixture of seawater and WWTP effluents were collected in 10 L bucket and transported to the laboratory within 0.5 h after sampling. Additionally, the effluent of WWTP and clean seawater were also collected into 10.0 L bucket and transported to the lab for immediate processing (within 0.5 h). The water samples (10.0 L) were filtered through 0.45 μm cellulose nitrate filters (Millpore, Germany) using a vacuum filtration apparatus, and the filters fixed with three drops of 100% ethanol on each surface were stored in 50 ml centrifuge tubes. Then, all the fixed samples were stored at −20 °C before DNA extraction.

2.2. Cultivation of multidrug-resistant coliform bacteria

The collected receiving water samples, clean sea water samples and effluent samples were added 100 μL onto MacConkey agar and Lysogeny broth (LB) medium respectively supplemented with 100 mg/L ampicillin (A), 10 mg/L tetracycline (T), 50 mg/L kanamycin (K), 50 mg/L penicillin (P) and combination of "$A + T + K$" antibiotic (Fig. S1). After incubating overnight (16 h) at 37 °C, the multidrug resistant bacterial isolates were available to confirm the resistome phenotype for

downstream analysis.

2.3. General water quality and chemical analysis

General water quality parameters including pH, temperature, dissolved oxygen (DO), were measured in-situ using a pH and DO portable probe (Hach, USA). Chemical oxygen analysis (COD), total phosphate (TP), ammonium ($NH4^+$), hypochlorite (ClO^-) and nitrate ($NO3^-$) were measured using Hach kits (USA; LCK 1414, LCK 349, LCK 304 and LCK 349, respectively) for each triplicate sample. COD and total phosphorus were measured directly in the stored samples, whereas $NH4^+$ and $NO3^-$ were filtered using a 0.45 μm filter paper (Millpore, Germany), prior to analysis. All samples were processed within 2 days after sampling.

2.4. DNA extraction and purification

As for the collected water samples, two of the fixed filter papers were sliced and placed in one extraction tube of the DNeasy PowerSoil Kit (QIAGEN, Germany). Total genomic DNA of the membranes was extracted following the manufacturer's protocol. The extracted DNA was purified via AMPure XP beads (Beckman Coulter). The genomic DNA of 24 multidrug resistant bacterial isolates was extracted using DNeasy PowerSoil Kit (QIAGEN, Germany) following the manufacturer's instruction.

The concentration and purity of purified and original DNA samples were determined by spectrophotometer analysis (NanoDrop one, NanoDrop Technologies, Willmington). All the DNA samples matching the criteria of sufficient purity (OD 260/280 of 1.8 and OD 260/230 of 2.0~2.2) were used for library preparation.

2.5. Nanopore library preparation

To generate the metagenomic sequencing library for each sample, 2 mg purified DNA with high quality (A260/230 ≈ 1.8, A260/280 ≈ 2.0) was subject to end-pairing and dA-tailing (NEBNext Ultra II End-Repair/dA-tailing Module, New England Biolabs), and then cleaned with AMPure XP beads (Beckman Coulter, UK). The residual DNA was eluted in 31 μL of Nuclease-free water (ThermoFisher). The library was then prepared following the protocol of the ligation Sequencing Kit 1D (SQK-LSK108, ONT, UK). The end-repaired DNA was ligated with Adaptor Mix from Oxford Nanopore using Blunt/TA Ligation Master Mix (NEBNext, New England Biolabs). The resulting DNA was purified with AMPure XP beads (Beckman Coulter, UK) and the ABB buffer supplied in the kit. The concentration of purified-ligated DNA was measured using Qubit 2.0 Fluorometer through use of Qubit (ds)DNA HS Assay Kit (Thermo Fisher Scientific). Library was resuspended in 25 μL of elution buffer before loading on the flow cells for sequencing. The six independent water samples were performed using R9.4 flow cells (FLO-MIN 106) for 24 h individually.

The library of each 12 bacterial isolates' DNA was performed using SQK-RBK004 rapid barcoding kit with Rapid Barcode (ONT) according to the manufacturer's protocol. Libraries were loaded onto flow cell versions FLO-MIN106 R9.4 SpotON and sequenced for 16 h.

2.6. MinION data processing

Raw MinION sequencing of fast5 files resulting from MinKNOW were base-called using Guppy (v4.2.2) transferred into fastq files, where adapters and multiple reads were trimmed and demultiplexed with PoreChop (0.2.4) (https://github.com/rrwick/Porechop) from the passed sequences. To estimate the error rate of MinION sequencing, the resulting 1D reads were mapped to the corresponding de novo assembly of Illumina sequencing (via Spades) using LAST (version 983) aligner. After the adapter-trimmed, the passed sequences of receiving water samples, clean seawater samples and WWTP effluent samples were polished with their respective Illumina sequencing datasets via Polypolish (Wick and Holt, 2021) for the following data analysis. General statistical analyses and plotting of resulting data was performed and visualized using NanoPlot (version 1.20.1) (De Coster et al., 2018). The passed sequences of 24 bacterial isolates were directly assembled using Unicycler (v0.4.8) (Wick et al., 2017) with self-polishing by Pilon (Walker et al., 2014). All the basecalled Nanopore datasets were deposited into the NCBI SRA database (PRJNA753671).

As for the identification of antibiotic resistance genes, the 1D reads after adaptor removal were aligned to the nucleotide sequences of the SARG database using the ARGPore2 (https://github.com/sustc-xylab/Argpore2) with cut-off of alignment length > 75% and similarity > 75% (Fig. S2). When the ARGs-containing regions of a nanopore read overlapped for > 75% of their length, only the ARG hit with higher bit score was kept for this region. The concentration of ARGs was determined as copy number (one read per copy) per million reads (in unit of ppm) by normalizing the number of ARGs-containing reads against that of total nanopore reads.

The taxonomy classification of the ARGs-carrying reads were conducted using Centrifuge (v1.0.4) (Kim et al., 2016) with the NCBI nonredundant nucleotide sequence database as reference. To identify the ARGs carried by plasmids from all the detected ARGs-carrying reads, the raw reads were aligned to PLSDB database (Galata et al., 2019) used with cut-off of alignment length > 70% and similarity > 70% to separate the chromosome, plasmids and unclassified sequences with LAST tool (version 983) to confirm plasmids identification.

Next, we used the frameshift sensitive mod (-s 2 -T 0 -Q 0 -a 1 -P 60) of LAST tool (version 983) to align ARGs-carrying reads against the protein database of the NCBI Reference Sequence Database (RefSeq) to identify the transposons and integron. And only the alignments matching 75% (Ruvindy et al., 2016) amino acid identity over more than 70% of the marker protein (transposase or integrase) length were kept.

The completed circular genome and "near-finished" circular genome of 24 bacterial isolates' taxonomy were classified using GTDB-Tk (Chaumeil et al., 2020) respectively. The genome assembly was annotated using Prokka (v 1.13) (Seemann, 2014) and the annotated position were aligned to the protein sequences of the CARD database (Alcock et al., 2020) using BLASTP with default parameter (minimum 95% sequence identity and 90% length cut off). For comparison, the completed genome of *E.coli* were aligned to each other via BRIG, displaying sequence matches with nucleotide identity ≥70% (Alikhan et al., 2011).

2.7. Illumina data processing

In addition to nanopore sequencing, the same DNA was also sent out for high throughput metagenomic sequencing on Illumina HiseqXten platform. 150 bp Paired-end reads were generated at the Novogen Coperation (Beijing, China). Each sample were targeted to generate 10 Gb fastq reads, and all the sequenced datasets were deposited into the NCBI SRA database (PRJNA753671).

For each Illumina dataset, the clean data after QC by fastp (v 0.20.1) (Chen et al., 2018) was de novo assembly via Spades with meta mode (Bankevich et al., 2012) with default parameter. Prodigal was used to call Open reading frames (ORFs) before the ARGs-like ORFs were determined via BLASTP against the SARG database at E-value cut-off of 1E-7 with a similarity cut-off of 80% (Yang et al., 2013). To compare the resistome profile with Nanopore datasets, in addition to the BLASP searching method, the assembled contigs were also subject to resistome annotation using ARGPore2.

2.8. Evaluation of ARGpore2

To validate the ARGs annotation precision of ARGpore2, 602,250 non-ARGs bacterial genes (as determined by KO annotation) were download from KEGG database (Kanehisa and Goto, 2000) (Table S1). Next, we randomly selected 10,000 nucleotide reads from non-ARGs

genes pool and 100 nucleotide reads from SARG-nt database. The combined dataset was used for evaluation of ARGpore2. Only hit with identical subtype were counted as valid ARGs identification. The validation process was repeated ten times to report the average precision value (Fig 1b).

2.9. Definition of ARGs and ARBs source

In this study, we focused on two types of source tracking analyses using SourceTracker (Knights et al., 2011). Since receiving water body is a mixture of clean seawater and WWTP effluent, we assume it can serve as a "sink" in our Bayesian algorithm, leaving other water types as "source". To test the sensitivity and performance of SourceTracker, ten artificial sink communities were generated containing defined proportions of different sources. The evaluation result of ten configuration were conducted in R using SourceTrakcer under default parameter settings (rarefaction_depth = 1000, alpha1 = 0.001, aplha2 = 0.01, burnin = 100, nrestarts = 10). Each genus-level community profiles were firstly sorted based on abundances, and then commonly shared information was extracted and reorganized into matrix format for all samples. Besides community source tracking we also took ARGs genotype profiling results in source tracking analysis. For each sample, predicted proportion for three potential sources (cleansea, WWTP and unknown) across five runs was averaged, and within the source tracking result of communities and resistome, relative standard deviation (RSD) was calculated across predicted proportions.

In addition to the "WWTP-borne" group, the seawater populations were categorised into three different sub-groups based on their shifts in prevalence after receiving WWTP effluent. Firstly, the microbial population of receiving water increased after WWTP effluent discharge, namely "seawater-dominant enriched", containing dominant populations (higher than 75% populations) of clean seawater, showed evident (> 2 fold change) increase after receiving WWTP discharge. Secondly, the microbial population of receiving water was decrease after WWTP effluent discharge, namely "seawater-dominant reduce", containing higher than 75% populations of clean seawater, but dramatic decline by WWTP discharge. Thirdly, the minority population of receiving seawater, namely "seawater minor" for other ARBs populations that either showed low abundance in clean seawater community or no strong variation after receiving WWTP discharge.

3. Results and discussions

3.1. Error estimation and statistics of nanopore reads

Nine independent 24-hour MinION sequencing runs resulted in a total of 43.8 Gb effective base-called data (fastq format) for the nine metagenomics libraries sequenced with N50 range from 2276 to 9184 bp, average median length of 1804 bp, maximum length of 69.3 kbp (Fig. S3). Based on error-profile obtained by mapping nanopore reads to Illumina assembly, we noticed an indel size of 183.07Mb in mapped 1D reads equivalent to an indel frequency of 2.17% for R9.4.1 flow cell chemistry applied in this study. Averagely, 38.3% of these indels were deletions (70.07 Mb), while the insertions (112.95Mb) account for 61.7% indel errors. Despite the overall improved indel frequency compared to previous nanopore chemistry (Ashton et al., 2015), short palindromes 6-kmer of "GCGCGC" showed exceptionally high insertion frequency (z-score ≈ 9) (Fig. S4). The taxonomic comparison of two biological duplicates of clean seawater samples confirmed that MinION-based metagenomic sequencing has outstanding stability (Pearson's R of 0.99) for community and resistome profiling (Fig. S2).

3.2. ARGs and ARBs identification

To identify ARGs and ARBs in nanopore datasets, a bioinformatics framework – ARGPore2 (github.com/sustc-xylab/ARGpore2) was constructed and evaluated (Fig. 1a). The integrated database of ARGs was collected from nt version of SARG database v2 (Yin et al., 2018), in which the nucleotide reads belonging to same type of ARGs were clustered by CD-hit (Fu et al., 2012) based on the similarity of 90% (parameter -c 0.9 -S 0.9 -d 0) with subtypes of clustered queries renamed (Table S2). ARGs were identified through aligning the raw nanopore reads manually integrated ARGs database with LAST (version of 983) (Frith et al., 2010), whose adaptive seeds algorithm had shown the highest sensitivity for nanopore reads alignment (Ashton et al., 2015). A combined dataset of randomly selected non-ARGs genes downloaded from KEGG database and sequences from the SARG-nt database were used for the evaluation of ARGpore2. As shown in Fig. 1b, ARGs identification with a similarity cut-off of 75% showed precision stably around 95%, which indicates effective filtering of false-positive hits at this cut-off. Notably, the value of similarity cut-off increasing greater than 75% showed a minimum effect on improving precision. Thereafter, ARGpore2 adopted a similarity cut-off of 75% over 90% of the subject ARGs length for ARGs identification. Although similarity cut-off used to determine homology is critical in resistome prediction, assessment like the above is rare. Consequently, most prior nanopore long reads based ARGs studies applied an overly stringent similarity cut-off of 90% or higher to minimize false-positive rate (Che et al., 2019), which may in return makes their conclusions suffer from biased resistome profile caused by the skewed view of resistome diversity within the community. Noteworthy, resistome prediction based on protein sequences (amino acids) alignment could reach a precision more than 97% with recall around 30% at BLASTP similarity cut-off of 80% (Chen et al., 2021). The evaluation of ARGPore2 demonstrated that nanopore-based DNA

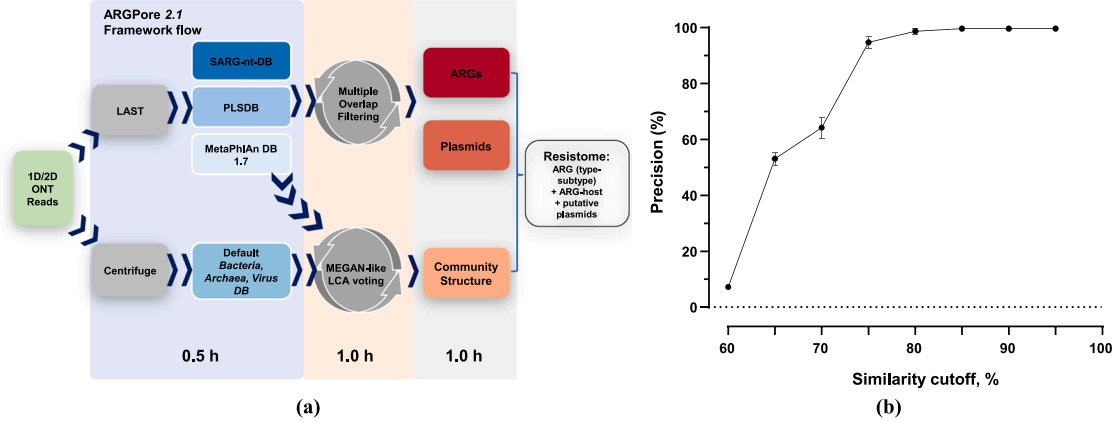


Fig. 1. Workflow and performance of ARGPore2.0 (a) The analysis workflow of ARGPore2 pipeline; (b) Precision of ARGs identification at different similarity cut-offs by ARGpore2.

alignment is capable to predict ARGs at an accuracy comparable to that of this widely adopted BLASTP method. Therefore, the appraisal of limited sequencing accuracy of nanopore sequencing, should not diminish its importance as ground-breaking framework to overcome the culture and short-read assembly barrier for reliable antibiotic resistance identification and host tracking for environmental resistome investigations.

Thanks to the long-read length of nanopore sequencing, genomic fragments containing ARGs could be phylogenetically annotated with ease using modern tools. To balance the trade-off between computational cost (including database index size, RAM requirement, and run speed) and the priority of annotating ARBs down to strain level, ARGpore2 combines taxonomic results from tools of Centrifuge (Kim et al., 2016) against its NCBI nonredundant nucleotide sequence database and LAST against MetaPhlan marker gene database v2 (Truong et al., 2017) for phylogenetic assignment of ARBs. In contrast to the robust phylogenetic annotation of nanopore long reads, the inherent repetitive nature of flanking regions of exogenous ARGs obtained by horizontal gene transfer will leave numerous gaps in the assembled genomes, resulting in ARGs-containing contigs too short to be phylogenetically annotated in short-reads datasets. In our Illumina-based datasets, only 18 ARGs-containing contigs are identified in 10 bacterial genera affiliated to 3 Classes by metagenomic assembly accounting for 0.04% of the receiving water bacterial community, which constituted a very small fraction of resistome detected by unassembled short reads (Fig. 2a). In contrast, nanopore datasets detect 380 ARBs with phylogenetic spectrum enlarged to 33 genera affiliated to 12 Classes, which was enlarged at least for 2-fold. Additionally, culture-based validation demonstrated a high degree of congruence with ARBs profile revealed by nanopore sequencing, which revealed that *Escherichia* and *Klebsiella* were predominant resistant populations, whereas the *Escherichia* carrying diverse ARGs against beta-lactam were overlooked by Illumina-assembly (Fig. 2a,b). Notably, such mis-leading ARBs detection by Illumina-assembly in our water sample is likely the norm rather than an exception in environmental communities. In feces samples of adult chicken over a full-treatment course of chlortetracycline at a therapeutic dose, only 160 ARGs-containing contigs were recovered from assembly, whereas the ARGs level was as high as 7762 × /Gb (Ma et al., 2016). Therefore, these results indicate the assembly-free approach by nanopore sequencing could disclose a more thorough and unbiased ARBs composition, which is critical to understand the environmental spread of resistance. As will be put forward, the importance of exhaustive ARBs detection was demonstrated in tracking the source of ARGs pollution by identifying the key resistant groups in subsequent sections.

3.3. Enrichment of resistome in WWTPs receiving waterbody

Nanopore-based metagenomic sequencing was applied on three types of water samples, namely three receiving seawater samples collected at three different time points (7am, 14pm and 19pm) of one day, three parallel clean seawater samples collected 5 km upstream of the WWTP discharging point and three parallel effluent samples of the corresponding WWTP (Fig. S5). Such extensive nanopore-based metagenomics detected totally 780 ARGs-carrying nanopore long reads with average length of 4471 bp (Table S3). And about 74% of them could be phylogenetically assigned at genus level. As shown in Fig. 3a, totally 65 subtypes belonging to 14 types of ARGs were detected. Microbial resistance against 12 classes of antibiotics were detected in receiving

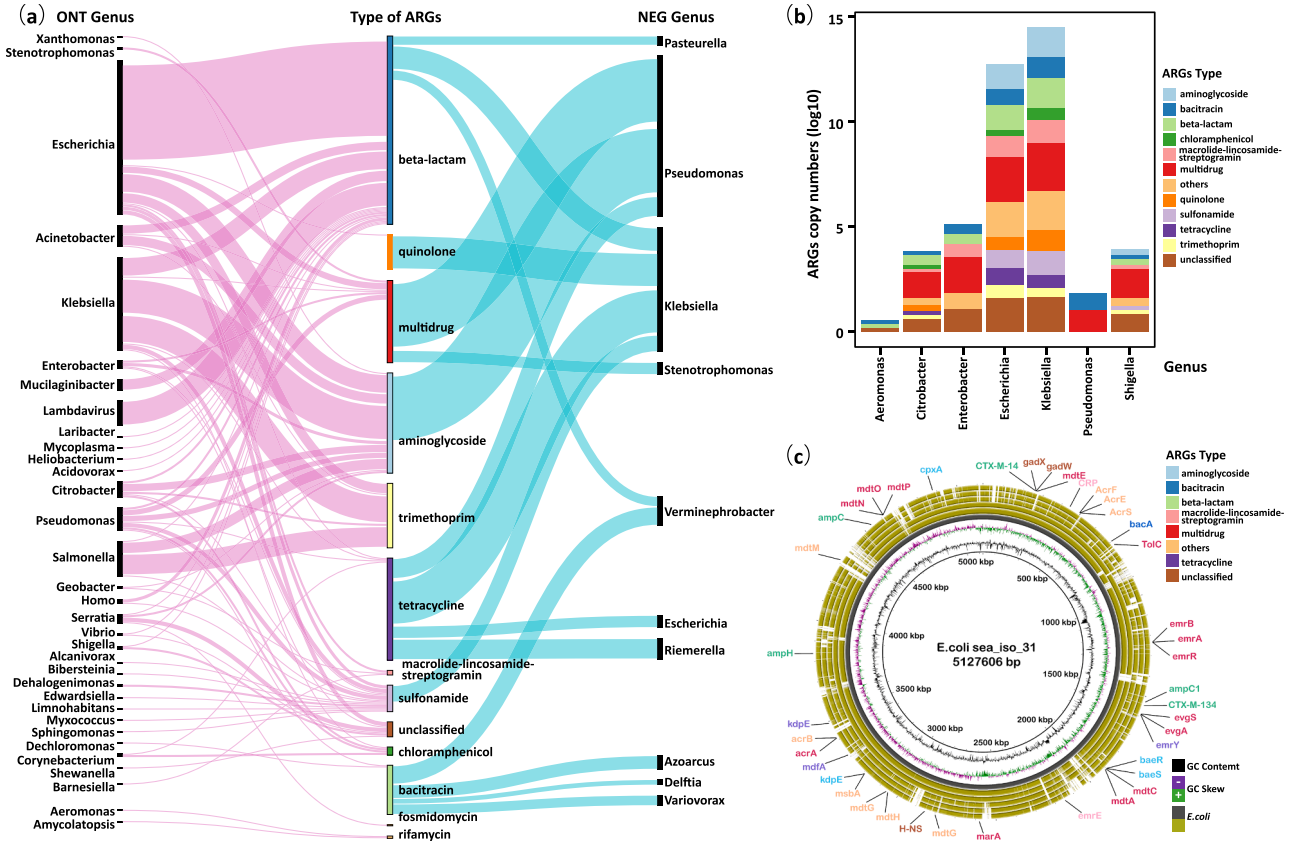
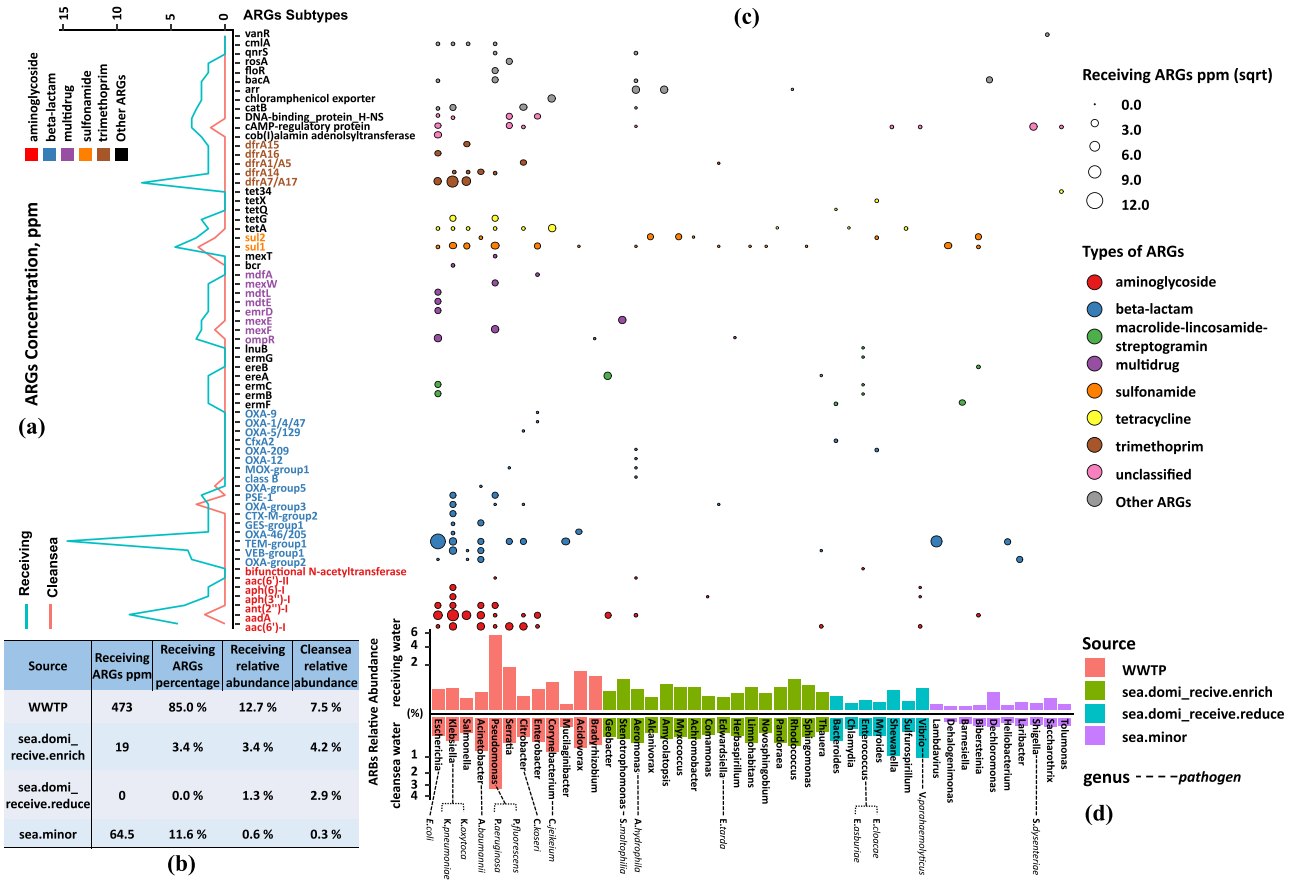


Fig. 2. Comparison of ARGs host identification between nanopore-based and Illumina-assembled metagenomics. ARGs are coloured according to their resistance types. **(a)** The Sankey plot represents ARGs host populations identified based on nanopore and Illumina-assembly at genus level. The width of the ribbon is proportional to the relative abundance of host populations in corresponding dataset. **(b)** The ARGs profile of culture-based validation based on 24 beta lactams resistant bacterial isolates. **(c)** BRIG genome map depicting resistome of 6 isolated strains of *E. coli*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



Source	Receiving ARGs ppm	Receiving ARGs percentage	Receiving relative abundance	Cleansea relative abundance
WWTP	473	85.0 %	12.7 %	7.5 %
sea.domi_recive.enrich	19	3.4 %	3.4 %	4.2 %
sea.domi_receive.reduce	0	0.0 %	1.3 %	2.9 %
sea.minor	64.5	11.6 %	0.6 %	0.3 %

Fig. 3. Profile of resistome of receiving seawater and clean seawater. (a) The comparison of mean concentration of ARGs in receiving seawater and clean seawater samples; (b) The host range of ARGs and the fate of antibiotic resistance pathogens in costal water systems; (c) Heatmap indicated the concentration of ARGs in receiving seawater samples; (d) Table showed that relative abundance and total concentration of ARGs of four source groups.

seawater samples, while we only identified 6 types in the clean seawater samples. The strong antimicrobial resistance in the receiving water could also be evidenced by prevalent of multidrug resistant isolates in the culturing test (Fig. S1). The overall resistome concentration of receiving water community was roughly 10 times higher than of clean seawater, with subtypes of *TEM-group1, OXA-group2, aac(6′)-I, aadA* and *dfrA7/A17* being the most sharply increased (Chen et al., 2019; Ju et al., 2018; Luo et al., 2010; Yang et al., 2019). Beta-lactam resistance genes were the most dominant resistance type in receiving water samples, primarily composed of *TEM*-group and *OXA*-group. Furthermore, *TEM-group* beta-lactamases, *aadA* aminoglycoside, *dfrA7/A17* trimethoprim which had shown evident clinical relevancy (Cameron et al., 2018; Liu et al., 2008; White et al., 2000), were among the most prevalent resistance genotypes in receiving water samples.

3.4. Microbial mechanism driving the resistance booming in receiving water body

The real-time and long-length capability of nanopore sequencing is substantial to rapidly identify and trace the potential antimicrobial-resistant pathogens (ARPs) in receiving water. According to the Bayesian source tracking results, WWTP showed major impact on the overall resistome (Prediction > 85%, RSD = 1.98%) and community (Prediction > 70%, RSD = 3.90%) of receiving water (Table S8), evidencing the strong influence by the discharge of WWTP (Chen et al., 2019; Ju et al., 2018; Luo et al., 2010; Yang et al., 2019). Despite the relatively small source library size, the trained model showed satisfactory prediction performance with significant correlation ($p < 0.01$) observed between predicted and expected contribution in artificial configurations (Fig. S6 and Fig. S7). To further disentangle the cross-linked impact of endogenous selection of local ARBs in seawater and exogeneous ARBs input by WWTPs discharge, the major source of ARBs was determined based on their shift of relative abundance in receiving water and clean seawater community. If ARB contained in the WWTP effluent, showed extremely low abundance (lower than 25% populations) in clean seawater community, while evidently dominated the receiving water community (abundance higher than 75% populations), it would be considered as WWTP-borne in this study. As shown in Fig. 3d, despite the nonnegligible ARGs background of clean seawater, above 85.0% resistome of receiving seawater were attributed by the ARBs within the WWTP-borne group, indicating the clear attribution of WWTP discharged exogenous resistome, whose enrichment could be partly facilitated by the minimized disinfectant concentration in the receiving water (Table S4).

Worth to note is that 187 putative pathogen species were detected in the receiving seawater community, among them 98 ARGs-carrying pathogens were detected with total relative abundance of 0.13% (Fig. 3d). Among them, four ESKAPE pathogen species of *E.coli, K. pneumoniae, A.baumannii* and *P.aeruginosa* were identified in the receiving water community. *TEM-group1, sul1* and *VEB-group1* were mainly presented in *E.coli* and *K.pneumoniae,* which is in consistency to the resistance genotypes encoded by clinically isolated ESKAPE strains (Zhang et al., 2021), suggesting their phenotypic relevance to pathogenicity and antibiotic resistance. These results reinforced the high risk of receiving water body as a reservoir and vehicle for ARPs proliferation in environment and for the first time allowed elucidation of to what extent the accumulation of exogenous ARBs could drive the resistome of local natural communities.

3.5. Distribution of mobile ARGs in receiving water body

The potential mobility of identified ARGs were investigated by identifying the MGEs associated genes localized on the same genetic fragment (long nanopore read). Results showed that ARGs identified in all clean seawater and receiving seawater were mostly located on bacterial chromosomes (74%) rather than on plasmids, which was contradictory to that observed in WWTP-associated resistome including influent, activated sludge and effluent (Che et al., 2019). As for the general trend, the ARGs encoded on plasmids and chromosomes were more prevalent in receiving water samples than in clean seawater samples. ARGs types of fosmidomycin, chloramphenicol, MLS, quinolone, bacitracin, trimethoprim and tetracycline were only presented in receiving seawater samples (Fig. 4). Nevertheless, more than half proportion (54%) of ARGs were encoded on chromosomes rather than plasmids in the receiving water samples, which showed the similar trend of the whole resistome distribution in all samples. It is worthy to notice that ARGs against fosmidomycin, widely disseminated via plasmids in environmental and clinical community (Arca, 1997; Martini et al., 2016), were only identified on the plasmids. Additionally, the ARGs-containing plasmids are extensively associated with clinically relevant pathogens like *E.coli, K.oxytoca, K. pneumoniae, P.aeruginosa, S. marcescens* and *B.megaterium*.

In addition to plasmids, a variety of MGEs were identified on the chromosomes, indeed, 16 subtypes belonging to 6 ARG types showed close genetic association with integrons, transposons and recombinase, implying their high tendency of HGT (Fig. 5). The genetic organization revealed by nanopore long reads showed strong connections between *aac-families, aad-families* and *sul*1 (Fig. 5), which are important components of gene cassettes of class 1 integron (CL1) (Ma et al., 2017a). Since CL1 had been reported as clinically relevant genetic elements with the ability to transfer ARGs conferring resistance to multiple antibiotics (Gillings et al., 2015), these findings, plus a widespread of class 1 integron-integrase gene (*intI* 1) in all receiving water samples, demonstrated that CL1 have played an important role in enabling HGT of chromosomal ARGs among bacteria of different phylogenetic lineages. Interestingly, no MGEs could be detected on ARGs-carrying plasmids, indicating a generally distinct transmission machinery between chromosomal and non-chromosomal resistome in receiving water community.

4. Conclusion

To understand the mechanism arousing the environmental resistome reservoir of costal receiving water communities, this study audaciously applied nanopore sequencing technologies to comprehensively investigate the dissemination and enrichment of ARBs. A robust bioinformatic framework (ARGpore2) was constructed and carefully tested to predict resistance and establish plasmids/MGE-associated mobility. An overall consistent resistome was observed between Illumina-based and nanopore-based metagenomic approaches. However, compared to Illumina-assembled contigs, nanopore sequencing datasets generated more reliable ARB profile that resistome of beta-lactamase carried by *Escherichia* overlooked by Illumina-assembled approach was accurately detected by nanopore sequencing, highlighting the power of assembly-free long-reads metagenomic approaches for assessing the mobile proportion of the resistome. Our results suggested the receiving costal system might be considered as a reservoir for ARGs mediated by the robust installation of WWTP-borne ARB community coupled with recombination and HGT enabled by plasmids and class 1 integrons. Consequently, in order to mitigate resistance pollution in receiving environments, advanced filtration or oxidative process should be included in the disinfection step of WWTPs to enable more complete disruption of pathogen cells and endospores during treatment.

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.

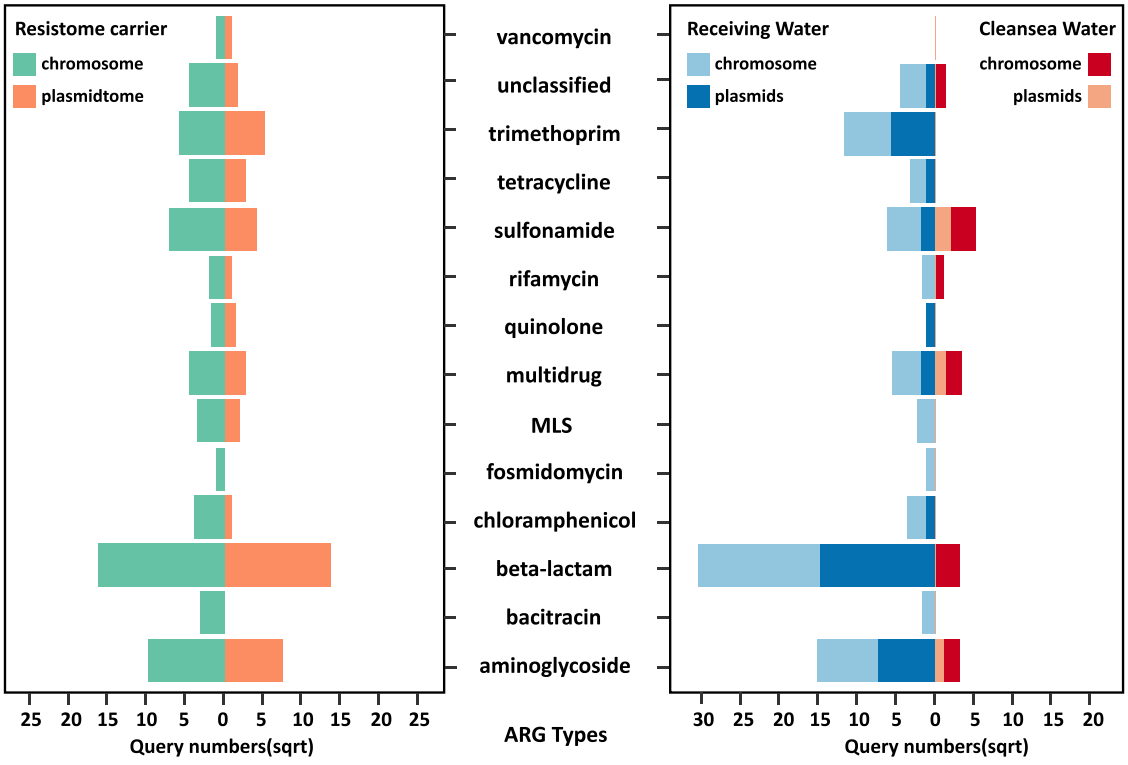


Fig. 4. The distribution of ARGs carried by plasmids, and chromosomes in receiving seawater samples and clean seawater samples. The left subfigure shows the overall distribution, while the differences between receiving water and clean seawater is shown in the right subfigure.

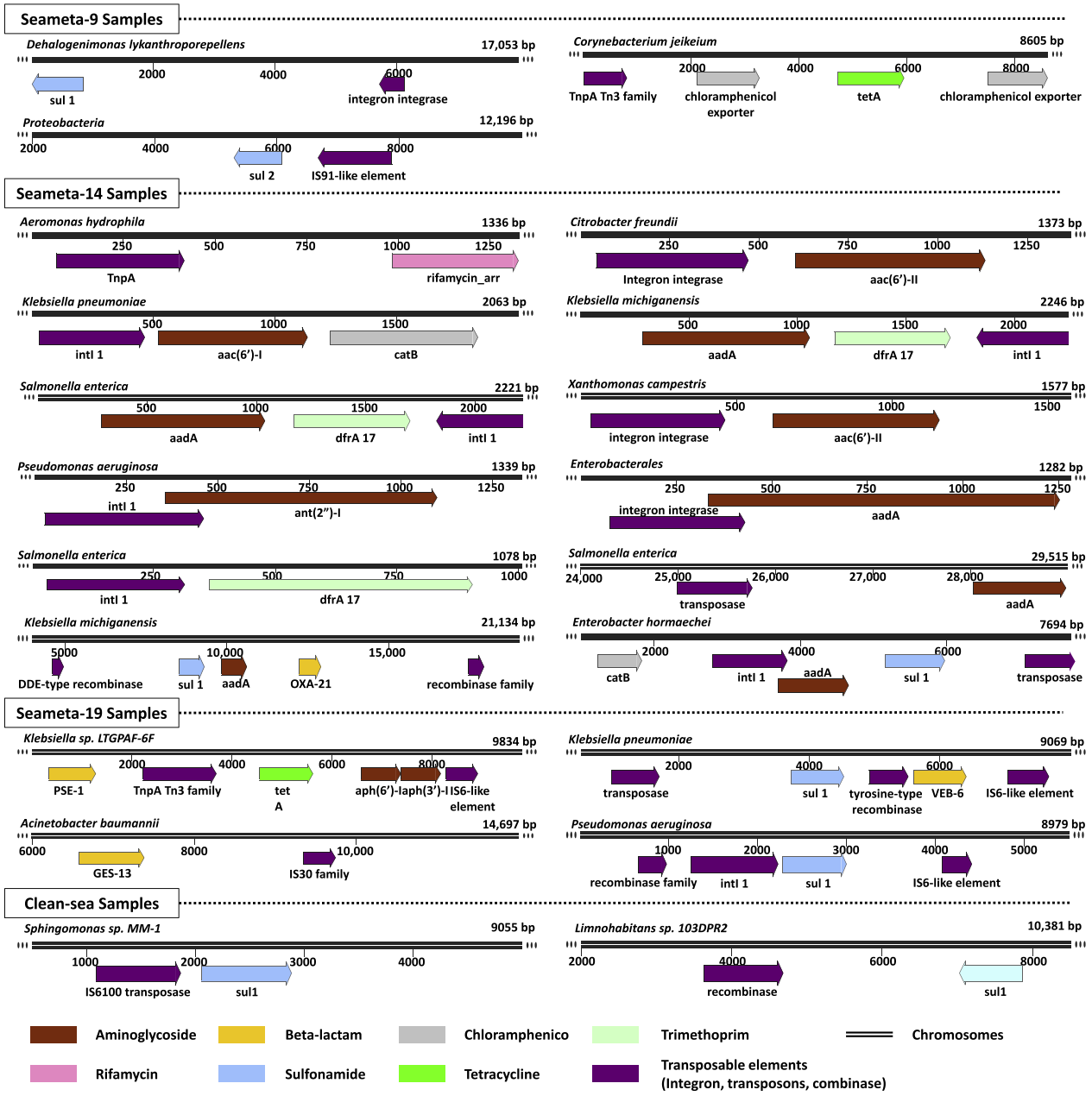


Fig. 5. Schematic of the genetic organization of antibiotic resistance gene clusters identified on nanopore reads.

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Data availability

Data will be made available on request.

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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.2022.119282.

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