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ORIGINAL RESEARCH

Metagenomic Analysis of Urban Wastewater Treatment Plant Effluents in Tokyo

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Purpose: Urban wastewater treatment plant (WWTP) effluents, even with proper treatment, may cause antimicrobial resistance (AMR) burden, with a high frequency of acquired antimicrobial resistance genes (ARGs). The dissemination of ARGs into the environment increases the risk of infectious diseases; however, there is little direct evidence regarding their epidemiological effects. This study aimed to assess effluents from urban WWTPs around the Tama River and Tokyo Bay using metagenomic analysis of (AMR) genes (ARGs) and heavy-metal resistance genes.

Methods: Metagenomic DNA-seq analysis of water samples and resistome analysis were performed.

Results: The most prevalent ARG was the sulfonamide resistance gene, *sul1*, followed by the quaternary ammonium compound resistance gene, *qacE*, suggesting that basic gene sets (*sul1* and Δ*qacE*) in the class 1 integrons are the predominant ARGs. The aminoglycoside resistance genes, *aadA* and *aph*, and macrolide resistance genes, *msr*(E) and *mph*(E), were the predominant ARGs against each antimicrobial. bla_{OXA} and bla_{GES} were frequently detected, whereas the $bla_{\mathrm{CTX\text{-}M}}$ cluster was faintly detected. Non-metric multidimensional scaling plot analysis and canonical correspondence analysis results suggested that marked differences in ARGs could be involved in the seasonal differences; *qnrS2, aac(6')-Ib*, and *mef*(C) increased markedly in summer, whereas *msr*(E) was more frequently detected in winter. Heavy-metal (Hg and Cu) resistance genes (HMRGs) were significantly detected in effluents from all WWTPs.

Conclusion: We characterized a baseline level of the environmental ARG/HMRG profile in the overall community, suggesting that environmental AMR surveillance, particularly in urban WWTPs, is a valuable first step in monitoring the AMR dissemination of bacteria from predominantly healthy individuals carrying notable ARG/Bs.

Keywords: urban sewage, effluent, metagenomics, *Enterobacteriaceae*, ESBL, carbapenemase, heavy-metal resistance

Introduction

The World Health Organization has endorsed a global action plan for antimicrobial resistance (AMR) to adopt mitigation strategies based on the One-Health approach.[1] In particular, the properties of the microbial resistome in ecosystems dominated by humans and how to monitor these environmental factors to evaluate their potential risk for promoting AMR evolution have yet to be sufficiently characterized. Sewage AMR surveillance could highlight a broader picture of the global AMR burden, including the locally specific urban, suburban, industrial, and agricultural features described by the One-Health approach.

There is a growing concern for sludge management due to high contaminant levels. The design of current wastewater treatment plants (WWTPs) does not restrict the elimination of emerging contaminants and their metabolites. These contaminants are released into rivers or streams as sewage effluents. The prevalence of AMR bacteria (ARB) and AMR genes (ARGs) from WWTP effluents in rivers downstream is increasing.[2–4] Indeed, the high density of bacteria in WWTPs provides an optimum environment for horizontal gene transfer (HGT) between environmental bacteria and human pathogens.[5] Moreover, WWTP wastewater and active sludge act as reservoirs and environmental suppliers of AMR, which implies that WWTPs are hotspots for HGT under the selective pressure of antibiotics, disinfectants, and metals, even at low concentrations. Thus, WWTPs could facilitate the dissemination of antibiotic resistance genes among different bacterial species.

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Furthermore, there is a concern that hospital and community effluents comprise a considerable proportion of ARGs in WWTPs;[6] however, hospitals are not the only source of antibiotic resistance in the environment. According to the European Centre for Disease Prevention and Control, a significant proportion of antibiotics is consumed by humans in the community rather than in healthcare settings,[7] suggesting that outpatient therapy could be the most critical factor in increasing the proportion of antimicrobial resistance, selected ARGs, and ARB in WWTPs. Thus far, the number of healthy individuals carrying extended-spectrum β-lactamase (ESBL)-positive organisms (EPO) has been increasing worldwide, and Japan is no exception to this issue. In Japan, the detection rate of EPO is 12.2% in healthy adult volunteers[8] and 15.6% in healthy food handlers.[9] The ARBs can colonize humans without notable symptoms, resulting in an increased number of healthy carriers.[10] As colonization may be asymptomatic in most humans, this may cause further dissemination during frequent clinical antimicrobial use, leading to an underestimation of the extent of ARB transmission from the environment to humans and from humans to humans in the community. According to the Japan Nosocomial Infections Surveillance (JANIS) system, clinical reports of antibiotic-resistant gram-negative bacteria are increasing (https://janis.mhlw.go.jp/english/index.asp). Therefore, constantly monitoring ARB carriers could be effective in predicting the AMR burden in nosocomial infections in the overall AMR risk assessment. Further risk assessment regarding possible AMR vector transmission from the environment to humans cannot be adapted from a pathogen model because most AMR vectors are likely composed of non- or low-pathogenic bacterial species. Although nonpathogenic bacteria cannot colonize and infect humans, their proliferation and subsequent dissemination in the environment increase ARG abundance and diversity in vectors. Hence, it may increase the risk of transmission of pathogenic ARB, such as hypervirulent *Klebsiella*, to humans. Environmental AMR surveillance, particularly in urban WWTPs, is a valuable first step in monitoring the dissemination of bacterial flora from predominantly healthy individuals carrying notable ARG/Bs.

The Technical University of Denmark (DTU) has collaborated globally with the Global Sewage Surveillance Project (https://www.compare-europe.eu/Library/Global-Sewage-Surveillance-Project) to analyze untreated sewage from 60 countries revealed a clear geographic distinction in AMR levels, with countries in Asian, African, and South American having more abundance and variety of AMR genes from poor sanitation and public health than those in Europe, North America, and Oceania.[11] This novel study suggested that metagenomic analysis provides an affordable method of conducting global AMR surveillance.[11]

To estimate the risk of environmental exposure to ARB, it is necessary to collect quantitative data from extensive sampling locations. The risk of environmental AMR burden must be assessed based on ARG prevalence. The purpose of this study was to characterize the environmental AMR burden from the effluents of WWTPs around the Tama River and Tokyo Bay area using ARG metagenomic analysis. Here, we demonstrate that AMR monitoring can uncover the actual status of WWTP effluent, and it leads to proposing further studies to reduce the related AMR burden for becoming a potential health risk.

Materials and Methods

Sample Collection

Sewage effluent samples were collected from eight WWTPs (WP1–WP9; WP6 was treated as missing numbers because of incorrect sampling locations) along the Tama River and around Tokyo Bay (Table 1 and Figure 1). General information regarding WWTP location and treatment performance is summarized in Table 1. Surface water from a recreational beach (BEC1) was used as an environmental control sample (Figure 1). Sampling was conducted during the summer and winter seasons for 2 years, from August 2017 to February 2019 (Supplementary Table 1). All samples in this study were collected in 500 mL sterilized containers (Corning, NY, USA) on five consecutive days without recent rainfall, to exclude weather effects.

Metagenomic DNA-Seq Analysis of Water Samples

The brief experimental procedure is shown in Supplementary Figure 1. Collected samples were transported to the lab at room temperature (approximately 23°C), followed by filtration within 6 h. To collect organisms larger than bacteria, water samples were passed through TPP Rapid Filtermax Vacuum Filtration systems (TPP, Trasadingen, Switzerland) in

Table 1 General Information on Wastewater Treatment Plants and Sampling Sites

Location ID	Sampling Site (GPS) Coordinates	Population Estimate	Area (ha)	Sewage Treatment (m^3/Day)	A_2O Process	Sand Filtration/ Membrane Filtration	Chlorination	BOD Effluent (mg/L)*	COD Effluent (mg/L)*
Wastewater treatment plant (WWTP)									
WP1	35°41′06.8″ N 139°24′37.2″ E	130,000	1134	63,300	+	–	+	3	N/A
WP2	35°39′50.5″ N 139°26′16.0′ ′E	230,000	2744	48,296	+	–	+	3	8
WP3	35°40′06.1″ N 139°25′40.0′ ′E	263,000	3902	78,245	+	–	+	3	8
WP4	35°39′02.7″ N 139°28′45.6″ E	360,000	5900	111,978	+	–	+	3	8
WP5	35°39′13.8″ N 139°30′45.2″E	489,000	5124	200,813	+	–	+	5	7
WP7	35°33′42.9″N 139°45′11.0″E	2,127,000	14,675	1,138,446	+	–	+	3	7
WP8	35°37′53.8″ N 139°44′45.6″ E	705,000	6440	607,162	+	–	+	16	12
WP9	35°37′49.9″ N 139°47′02.9″ E	N/A	N/A	15,968	+	–	+	1	9
Recreational beach									
BEC1	35°37′50.0″ N 139°46′31.3″ E	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Note: *Average value as reported in April 2018.

Abbreviations: WP, wastewater treatment plant; BEC, recreational beach; GPS, Global Positioning System; N, north latitude; E, east longitude; ha, hectare; N/A, not available; A_2O process, anaerobic–anoxic–oxic process, BOD, biological oxygen demand; COD, chemical oxygen demand.

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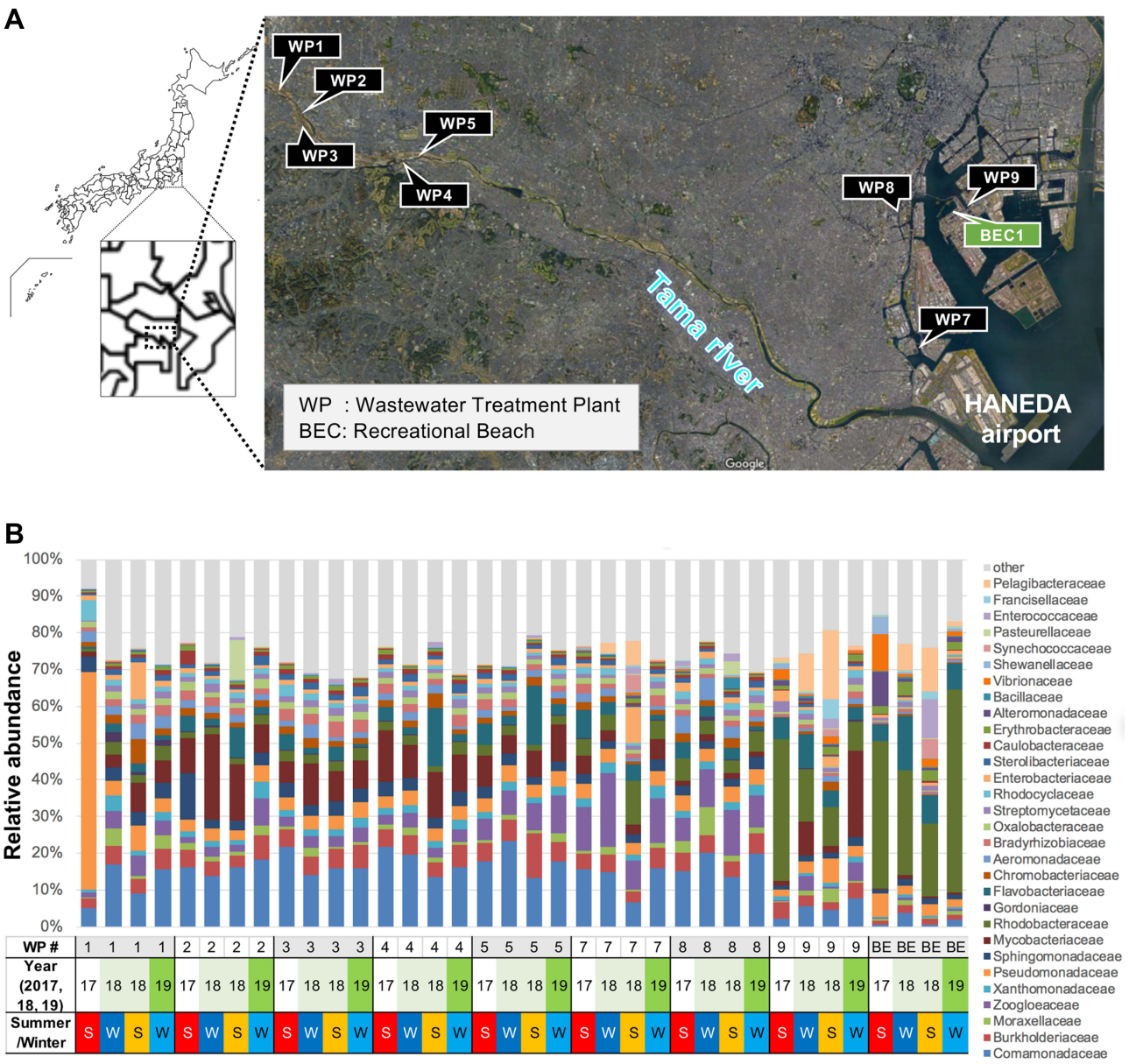

Figure 1 Map of water sampling sites along the Tama River and around Tokyo Bay area. (**A**) WWTP (WP) effluents and surface water from a recreational beach (BEC) were obtained for AMR investigations in this study. (**B**) Taxonomic classification of the domain rank of the effluents from WPs (WP1–9) and a recreational beach (BE) based on metagenomic DNA-Seq analysis results.
Abbreviations: S, summer; W, winter.

500-mL bottles fitted with 49 cm^2 PES 0.2-µm membranes. The membranes were removed from the bottles and stored at −30°C until DNA extraction. One-fourth of the collected membrane was cut into small pieces and placed into ZR-96 BashingBead Lysis Tubes (0.1 and 0.5 mm; Zymo Inc., Irvine, CA, USA). Bacterial lysis buffer (800 µL; Roche, Basel, Switzerland) was added to a bead tube, which was frozen at −30°C and thawed at 23°C. The tube was subjected to bead-beating (1500 rpm for 10 min) with a GenoGrinder 2010. After brief centrifugation (8000 × g for 3 min), 400 µL of the upper supernatant was collected. The DNA in the supernatant was purified using a Roche MagNa Pure Compact instrument (DNA_Bacteria_v3 protocol; Elution: 50 µL). DNA concentrations and purity were measured using the Qubit DNA HS kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Metagenomic DNA-seq libraries were prepared using the QIAseq FX DNA library kit (Qiagen, Hilden, Germany), followed by short-read sequencing using a NexSeq 500 platform (2 × 150-mer paired-end) (Illumina, San Diego, CA,

USA). Adapter and low-quality sequences were trimmed using Sickle version 1.33 (https://github.com/najoshi/sickle) with the following parameters: average quality threshold "-q 20" and minimum length threshold "-l 40". Metagenomic DNA-seq analysis was performed using cleaned reads for homology search without *de novo* assembly in all following analyses. Detailed scripts and databases are described below.

Taxonomic classification of every single read for from metagenomic analysis was performed using KrakenUniq version 0.5.8[12] with default parameters against a customized database that included the complete genome sequences of bacterial (n = 13,737), archaea (n = 295), and viral species (n = 8972); human genome sequences (GRCh38.p13); and the nucleotide database of bacteria (n = 7,041,584), archaea (n = 355,027), viruses (n = 2,386,054), fungi (n = 4,520,379), and protozoa (n = 1,670,542). The database containing these sequences was built using the KrakenUniq build program with a K-mer length of 31 bp. All metagenomic data were summarized with Pavian version 1.0.0.[13] Rare genera with one sequence count in one sample were excluded.

Alpha diversity indices and InvSimpson diversity were calculated with the R package "vegan" version 2.5.6 (https://CRAN.R-project.org/package=vegan). Non-metric multidimensional scaling (NMDS) ordination of the relative abundance was generated at the genus level. NMDS plots were visualized with metaMDS from the "vegan" package using Bray–Curtis distances. Canonical correspondence analysis (CCA) was performed in R v3.6.0 (R Foundation for Statistical Computing, Vienna, Austria) using the "vegan" package v2.5.6. To identify metagenomic biomarkers, linear discriminant analysis effect size (LEfSe) analysis was performed with relative abundance at the genus level in bacteria.[14] $p < 0.05$ was considered significant. Detailed information on the samples, sequencing reads, and short read data archives is summarized in Supplementary Table 1.

Resistome Analysis

Prior to resistome analysis, an ARG and heavy-metal resistance gene (HMRG) database was constructed. DNA sequences of ARGs and 16S rRNA genes were obtained from the Bacterial Antimicrobial Resistance Reference Gene (NCBI BioProject ID, PRJNA313047), ResFinder (https://bitbucket.org/genomicepidemiology/resfinder_db/src/master/),[15] and SILVA databases (https://www.arb-silva.de/no_cache/download/archive/current/Exports/).[16] The study database was constructed using Makeblastdb in the BLAST+ program. The HMRG protein sequences (confirmed and predicted genes) and RNA polymerase β subunit RpoB were downloaded from the BacMet version 2.0 (http://bacmet.biomedicine.gu.se/download_temporary.html)[17] and InterPro databases (http://www.ebi.ac.uk/interpro/entry/tigrfams/TIGR02013/protein/UniProt/#table).[18] The protein database was constructed using RAPSearch v2.24.[19] Operational taxonomic units (OTUs) in the ARG and HMRG database were created by clustering at ≥90% sequence identity and ≥80% coverage using vsearch version 2.10.4.[20]

The nucleotide and coding sequences from the metagenomic DNA-seq reads were searched using mega-BLAST (e-value threshold, 1E-20; identity threshold, 95%) and RAPSearch (e-value threshold, 1E-10; identity threshold, 90%), respectively, against the customized ARG and HMRG database described above. The detected genes were summarized for each ARG and HMRG OTU. The Reads Per Kilobase of gene per Million (RPKM) counts were calculated using the following formula for normalization: RPKM = number of detected reads against OTUs/[average gene length of detected OTUs (bp) × total number of trimmed reads] × 10^9.

Prior to ARG evaluation, we assessed whether the metagenomic DNA-Seq short reads properly characterized the bacterial population size using RPKM normalization for 16S rRNA genes and *rpoB* RNA polymerase β-subunit orthologs (Supplementary Figure 2B). ARG ontology was also analyzed using the Comprehensive Antibiotic Resistance Database CARD (https://card.mcmaster.ca/ontology/) to determine ARG prevalence among bacterial species.

NMDS plots for resistome analysis were visualized as described in the previous section.

Statistical Analysis

Statistical analysis was performed with exact Wilcoxon rank–sum test using the R package "exactRankTests" version 0.8.30. Differences were considered significant at $p < 0.05$ and $q < 0.4$.

Results

Bacterial Proportions in WWTP Effluents

Sewage effluents at eight WWTP sites (WPs) and BEC1 (Figure 1) were investigated via metagenomic DNA-seq analysis (Supplementary Figure 1). Taxonomic classification of the domain rank (Supplementary Figure 2A) suggested that the WWTP effluents (WP1–8) were mostly composed of bacterial sequences, whereas those of brackish water (WP9 and BEC1) were rich in eukaryotic organisms, such as mussels (*Mytilus edulis*) and diatoms (*Chaetoceros* spp.), found in seawater habitats. Further taxonomic classification of the family rank (Figure 1B) suggested that WWTP effluents (WP1–8) predominantly contained Comamonadaceae, which are aerobic gram-negative β-proteobacteria found in sewage active sludge digesters,[21] and Mycobacteriaceae, which are abundant in sewage active sludge.[22] The brackish water effluents (WP9 and BEC1) were rich in Rhodobacteriaceae, which mostly inhabit aquatic environments.

NMDS based on genus taxonomic classification showed that the WWTP effluents (WP1–8) were closely clustered, but the plots were markedly divided between summer and winter in a season-dependent manner (Figure 2A). In addition to NMDS plot analysis, we performed CCA, revealing seasonal trends based on taxonomic classification at the genus level (Supplementary Figure 3).

LEfSe revealed that multiple aquatic bacteria were mostly observed in the winter, whereas the abundance of some members of human gut flora (*Streptococcus, Escherichia*, and *Anaerococcus*), Cyanobacteria (*Dolichospermum*), and

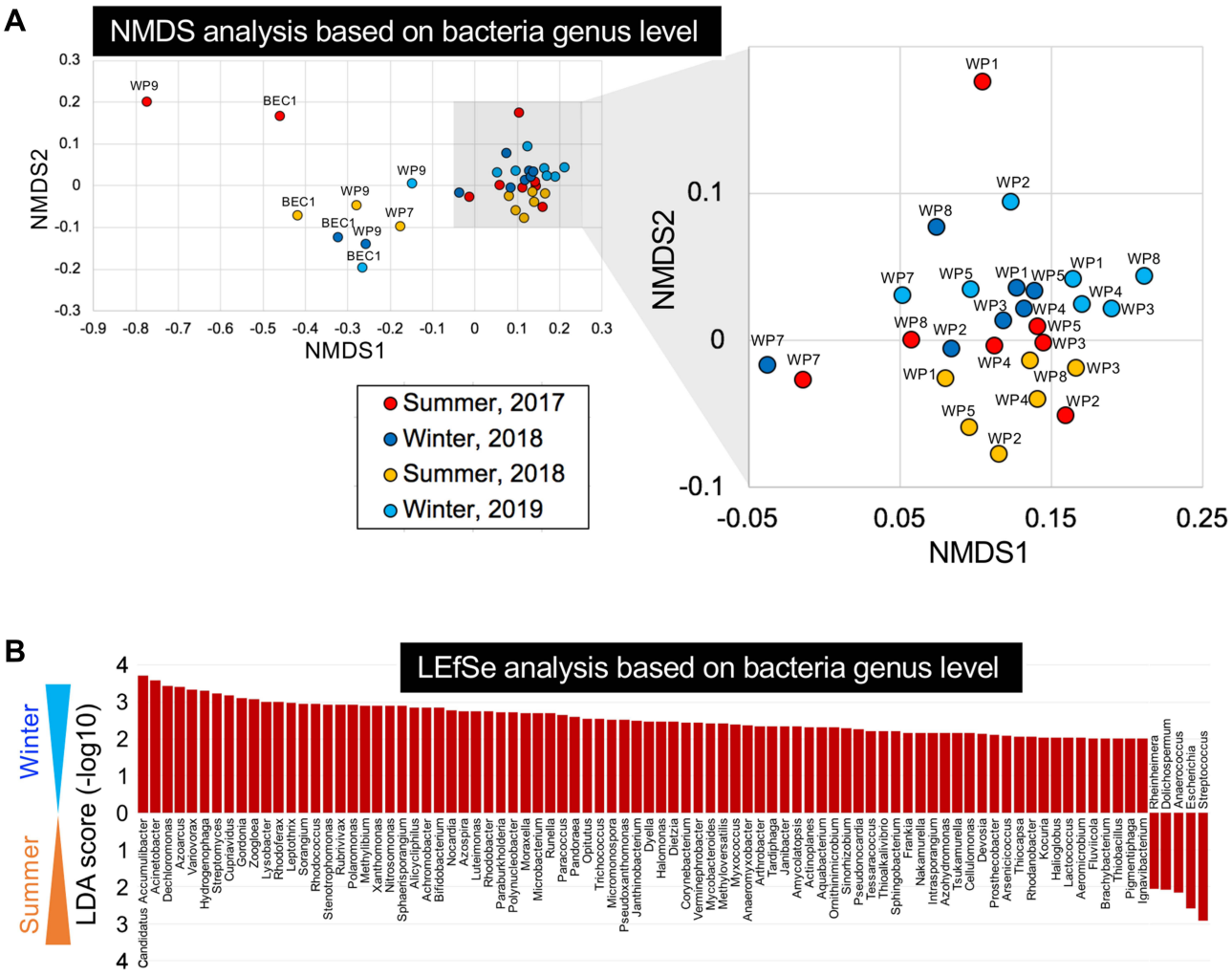

Figure 2 Metagenomic analysis of bacteria in WWTP effluents. (**A**) NMDS plots of metagenomic sequencing reads classified at the genus level for WWTP (WP1–9) effluents and a recreational beach sample (BEC1). Freshwater areas in WWTPs (WP1–8, see Figure 1) were clustered (gray-shaded area). Clusters were separated in a season-dependent manner but were not WWTP dependent. (**B**) Seasonality was observed in multiple bacterial genera using LEfSe analysis (score threshold: 2.0).

purple sulfur bacteria (*Rheinheimera*) increased in summer (Figure 2B). These results demonstrate typical seasonal features.

ARG Proportions in WWTP Effluents

RPKM estimation is an effective approach to characterize the relative ARG abundance. No significant differences in the copy ratio were observed between 16S rRNA and *rpoB* in any samples, except for BEC1 (Supplementary Figure 2B). Upon comparing with RPKM values from BEC1, those of WWTP effluents (WP1-8) showed 10- to 80-fold-increased RPKM values (Figure 3A), indicating that the original effluents contained abundant ARB before dilution in downstream

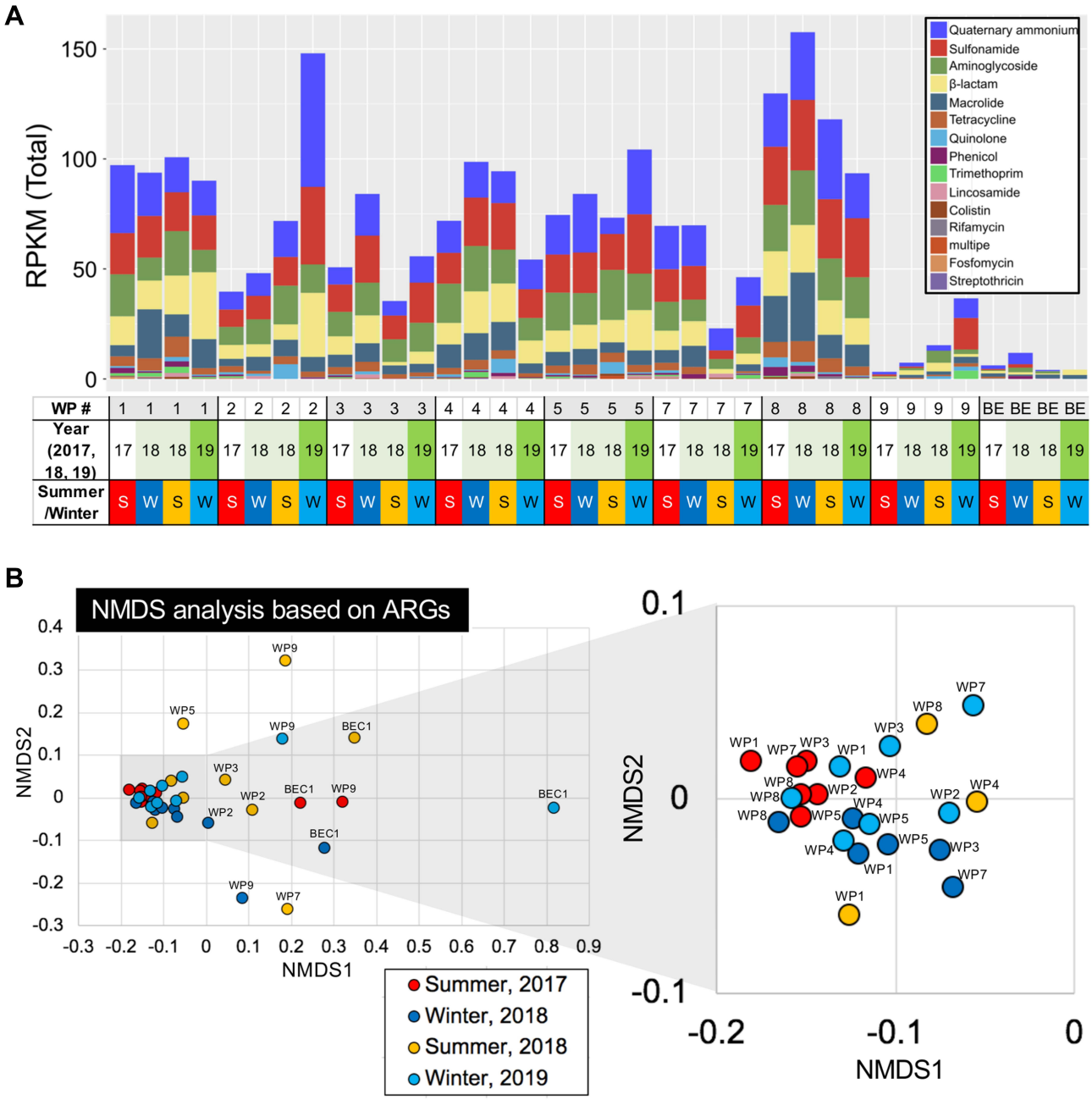

Figure 3 Metagenomic analysis of ARGs in WWTP effluents. (**A**) ARG detection in the effluents from WWTP (WP) and beach (BE) samples. Metagenomic DNA-seq short reads were analyzed for homology using the ResFinder ARGs database, followed by RPKM normalization. See Supplementary Table 2 for the detailed list of detected ARGs. (**B**) NMDS plots of metagenomic sequencing reads classified for ARGs in effluents from WWTPs (WP1–9) and a recreational beach (BEC1). Freshwater areas in WWTPs (WP1–8, see Figure 1) were clustered (gray-shaded square), but the plots were separated in a sampling time-dependent manner with partial WWTP-dependency for WP8. **Abbreviations**: S, summer; W, winter.

bays or the ocean. These results also show that ARGs to quaternary ammonium compounds (QAC), sulfonamide (SA), aminoglycoside (AMG), β-lactam, and macrolide (MAC) were present in all WWTP effluents except for WP9 (Figure 3A). NMDS plots suggested that most of the WWTP effluents were closely clustered, with no notable seasonal differences, based on the ARG profiles among the WWTP effluents (Figure 3B), indicating that ARG composition was similar among the WWTP effluents.

The detected ARGs in each AMR category were subsequently classified into orthologous genes (or gene families), followed by summarizing of the RPKM values of ARGs in Supplementary Table 2, and visualization in a graph (Figure 4A). The most commonly detected ARG was the SA resistance gene *sul1* (440.5 RPKM). *sul2* was detected at low levels, while *sul3* was not detected. The second most commonly detected ARG was the QAC resistance gene *qacE* (203.8 RPKM); however, our analysis could not distinguish between intact and deleted *qacE* (Δ*qacE*) in the class 1 integrons. The detection of both *sul1* and *qacE* suggested that basic gene sets (*sul1* and Δ*qacE*) in the class 1 integrons[23] were the predominant ARGs. Regarding clinically important ARGs, multiple AMG- and MAC-resistant genes were identified (Figure 4A). The seven predominant AMG resistance genes belonged to the *aadA* (in total 240.2 RPKM) and *aph* (in total 79.9 RPKM) gene families, which mainly contribute to streptomycin resistance. The two predominant MAC resistance genes were *msr*(E) (in total 81.7 RPKM) and *mph*(E) (in total 71.9 RPKM), which are primarily located on the chromosomes of *Acinetobacter* and *Proteus* species [*msr*(E) https://card.mcmaster.ca/ontology/39685; *mph*(E) https://card.mcmaster.ca/ontology/40396].

Regarding β-lactamase genes, bla_{OXA}, bla_{GES}, and bla_{IMP} variants were detected at >15.0 RPKM, whereas $bla_{CTX\text{-}M}$, which is a clinically important ESBL gene, was observed at only 0.82 RPKM. bla_{OXA}, bla_{GES}, and bla_{IMP} variants may be present at baseline levels, since these variants are often identified from environmental bacteria such as *Acinetobacter, Aeromonas, Pseudomonas* species, but not pathogenic bacteria ($bla_{OXA\text{-}10}$ https://card.mcmaster.ca/ontology/37805; $bla_{GES\text{-}1}$ https://card.mcmaster.ca/ontology/38730; $bla_{IMP\text{-}1}$ https://card.mcmaster.ca/ontology/38592).

In addition, NMDS plots did not exhibit the seasonal differences in the overall ARG profiles among the WWTP effluents, as described above (Figure 3B); however, ARG-specific resolution suggested that several ARGs could be involved in the seasonal differences. For instance, the levels of $bla_{GES\text{-}1}$, *qnrS2, qnrS6, aac(6')-Ib*, and *mef*(C) were markedly increased in summer (Figure 4B), whereas *msr(E)* and *aadA13* were more frequently detected in winter (Figure 4C). Next, WWTP-dependent differences in ARGs were examined using fold differences ($\log_2$ ratio) in RPKM values (Figure 5A). Season-dependent ARGs and ARGs with steadily increasing levels were observed in WP-specific features (Figure 5B). The analysis of WP2 effluents revealed that the levels of the major ARGs [AMG, *aadA2* and *aadA4*; MAC, *msr*(E) and *mph*(E); SA, *sul1*; and QAC, *qacF*] increased steadily (Figure 5B), suggesting that major ARB species may predominantly increase.

HMRGs in WWTP Effluents

Genes that confer resistance to mercury (Hg), copper (Cu), and arsenate (As) were detected in effluents from all WWTPs (Figure 6A and Supplementary Table 3). Seasonal differences were not observed in HMRGs (Figure 6B). The Mer and Cop transport systems were significantly observed for Hg and Cu resistance, respectively (Figure 7). These transport systems are mainly present in proteobacteria, including *Enterobacterales* and *Pseudomonadales* [detailed taxonomy can be reviewed at InterProscan ID: IPR001802 (mercuric transport protein periplasmic component/copper chaperone MerP/CopZ)]. Arsenite methyltransferase (ArsM; InterProscan ID: IPR026669) and chromate (Cr) transporter (ChrA; InterProscan ID: IPR014047) are the most potent determinants for As and Cr resistance, respectively, and these transport systems are mainly present in *Bacillales, Burkholderiales* and *Pseudomonadales*.

Discussion

In this study, we investigated how urban WWTP effluent is a potential source of AMR burden by detecting ARGs and HMRGs using a metagenomic DNA-seq approach. Metagenomic DNA-seq analysis showed that *Commamonadaceae* and *Mycobacteriaceae* (Figure 1B), which are among the most abundant bacteria in WWTP active sludge, were predominant in most WWTP effluents, suggesting that wastewater is sufficiently treated by general anaerobic–anoxic–oxic (A_2O) water treatment systems. However, bacterial proportions at the genus level exhibited season-dependent

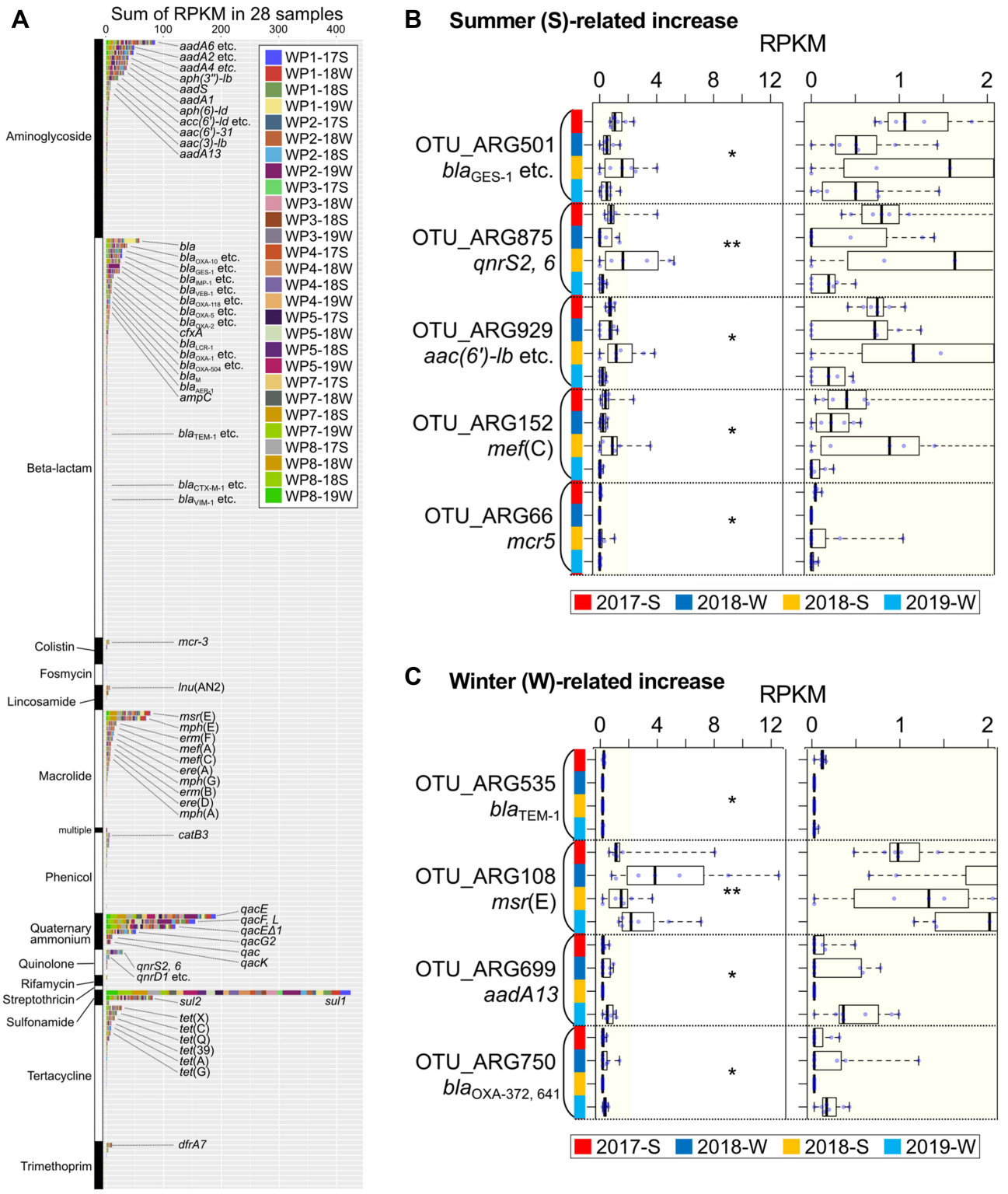

Figure 4 ARG profiling based on AMR categories. (**A**) Trends in ARG variations, the detected ARGs in each AMR category at every sampling site, and a unique season-dependent ARG in (**B**) summer and (**C**) winter. *p<0.05, **p<0.01. WP1-17S stands for the sample at WP1 site in 2017 summer.

patterns to some extent, including increased *Escherichia* species, which are pivotal pathogenic bacteria, in the summer season (Figure 2A). Thus, the varying bacterial populations might have affected ARG variations at all sampling and WWTP sites, since ARGs were affected by seasonal elements, particularly in a temperature-dependent manner (above

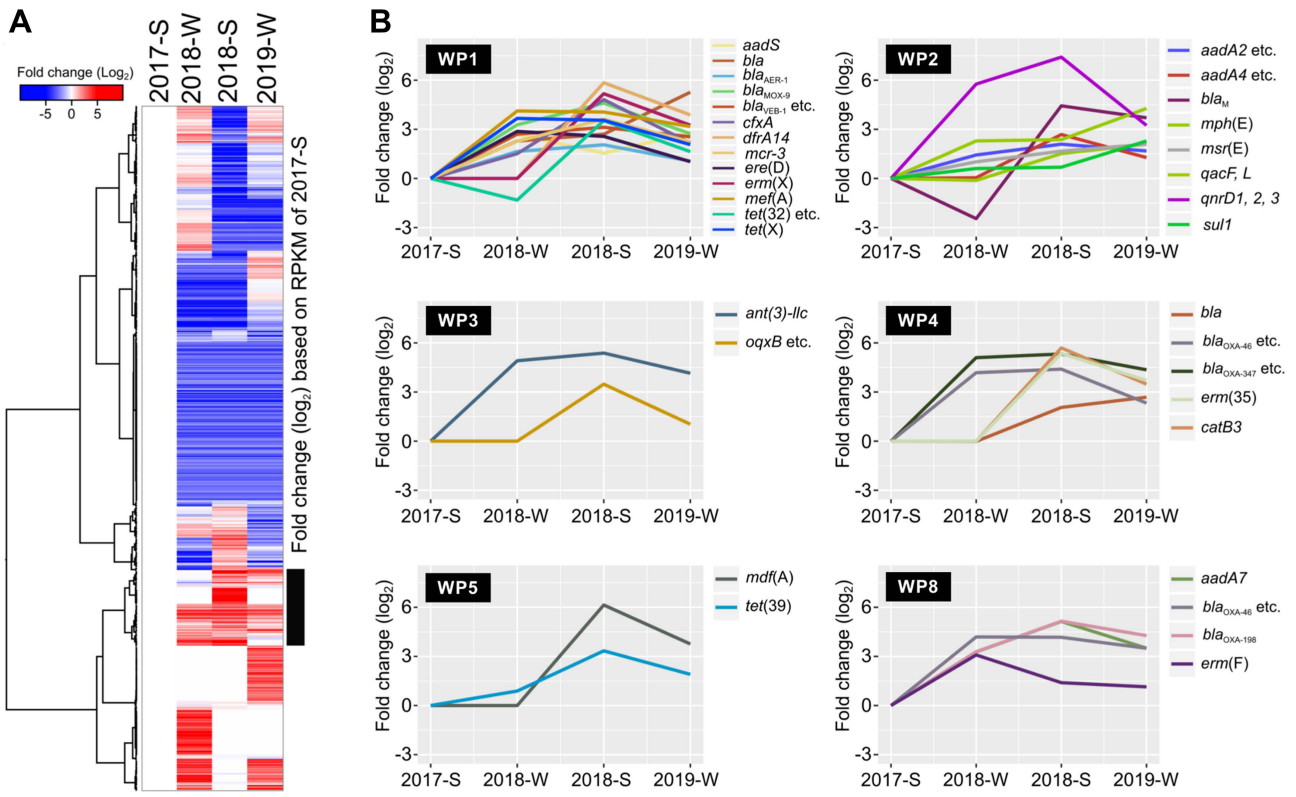

Figure 5 Seasonal or temporal trends of ARG profiles from the WWTP-dependent treated area. (**A**) Seasonal (or temporal) or (**B**) WWTP-dependent ARG differences shown in RPKM fold change (log_2 ratio).
Abbreviations: S, summer; W, winter.

30°C in summer and below 10°C in winter; Supplementary Table 1), for ARB growth in active sludge in anaerobic–anoxic–oxic (A_2O) water treatment systems.

Using ARG profiling to analyze the resistome, notable ARG levels were detected in urban WWTP effluents (Figure 3A) compared with those in a sea-water sample, although the effluent was released to environment after treatment. Notably, ARG-specific identification revealed that basic gene sets (*sul1* and Δ*qacE*) in the class 1 integron were predominant in the detected ARGs (Figure 4A), suggesting that the class 1 integron plays a pivotal role for HGT among environmental bacteria and human pathogens.[24]

Here, we did not completely investigate all the cassette genes in possible class 1 integrons. However, most AMG resistance genes (*aadA* and *aph* gene families), MAC resistance genes [*msr*(E) and *mph*(E)], and some β-lactamase genes, such as OXA-type carbapenemase, are cassette gene components in class 1 integron. It remains to be elucidated whether a detailed gene structure of each class 1 integron can be determined using long-read sequencing. The complete sequence of each class 1 integron would provide novel insights into the mobile genetic element (MGE)-dependent HGT among bacteria in WWTP active sludge.[5] Furthermore, such short read sequencing (150-mer paired-end) is not an appropriate method for determining whether ARG is located on a plasmid or chromosome.

We speculated that trends in ARB dissemination may affect ARG variations at all sampling and WWTP sites (Figure 5). Our analysis showed that increased ARG levels were uniquely different at each WWTP. We could not determine why this difference was observed in ARG-based resistome analysis. This difference may be particularly prevalent when WWTPs primarily treat urban wastewater influents from dense habitation that lacks significant influents from agricultural and industrial sources. Further, the treatment performance of each WWTP (Table 1) may be involved in WWTP-dependent differences in resistome profiles, which suggests that the resistome profiles in each WWTP could reflect the structure and diversity of resistant bacteria in the urban residents within the WWTP catchment areas (Figure 1A). Therefore, in addition to conventional standard treatment (biological treatment followed by A_2O and chlorination for disinfection), advanced

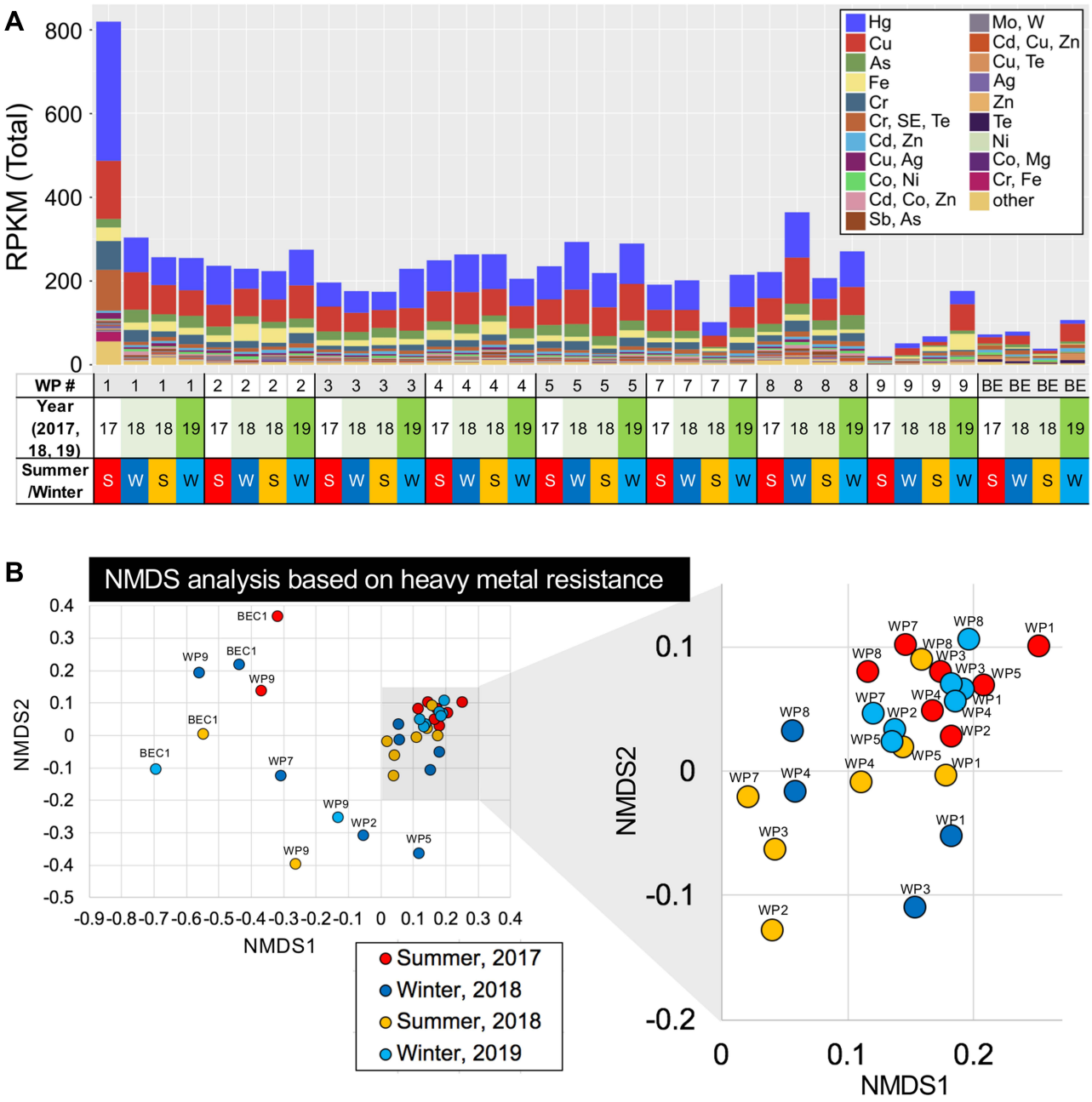

Figure 6 Metagenomic analysis of HMRGs in WWTP effluents. (**A**) HMRGs in the effluents from WWTPs (WP) and a beach sample (BE). Metagenomic DNA-seq short reads were analyzed for homology using the BacMet2 database (bioinformatics resource of antibacterial biocide- and metal resistance genes; http://bacmet.biomedicine.gu.se/), followed by RPKM normalization. See Supplementary Table 3 for the complete list of metal resistance genes. (**B**) NMDS plots of metagenomic sequencing reads classified by heavy metal resistance for effluents from WWTPs (WP1 to 9) and BEC1. Freshwater areas in WWTPs (WP1 to 8, see Figure 1) were clustered (gray-shaded square), but the clusters were separated in a sampling time-dependent manner that was partially WWTP-dependent.
Abbreviations: S, summer; W, winter.

treatment technologies, such as photocatalysis, membrane filtration, activated carbon adsorption, and advanced oxidation processes (AOPs), should be implemented to remove the emerging contaminants from wastewater.[25–27]

Additionally, remaining heavy metals are among the most important contaminants that drive selective pressure to ARB.[28–30] Unlike antibiotics, metals are not easily degraded and can represent prolonged selective pressure. Overall, heavy metals may be a dominant factor in estuaries and marine environments. Furthermore, heavy metals also play an important role in ARG maintenance and proliferation when antibiotic-selective pressure is weak. In this study, significant levels of HMRGs (in particular, Hg, Cu, and As) were detected (Figure 6). The presence of heavy metals in WWTP

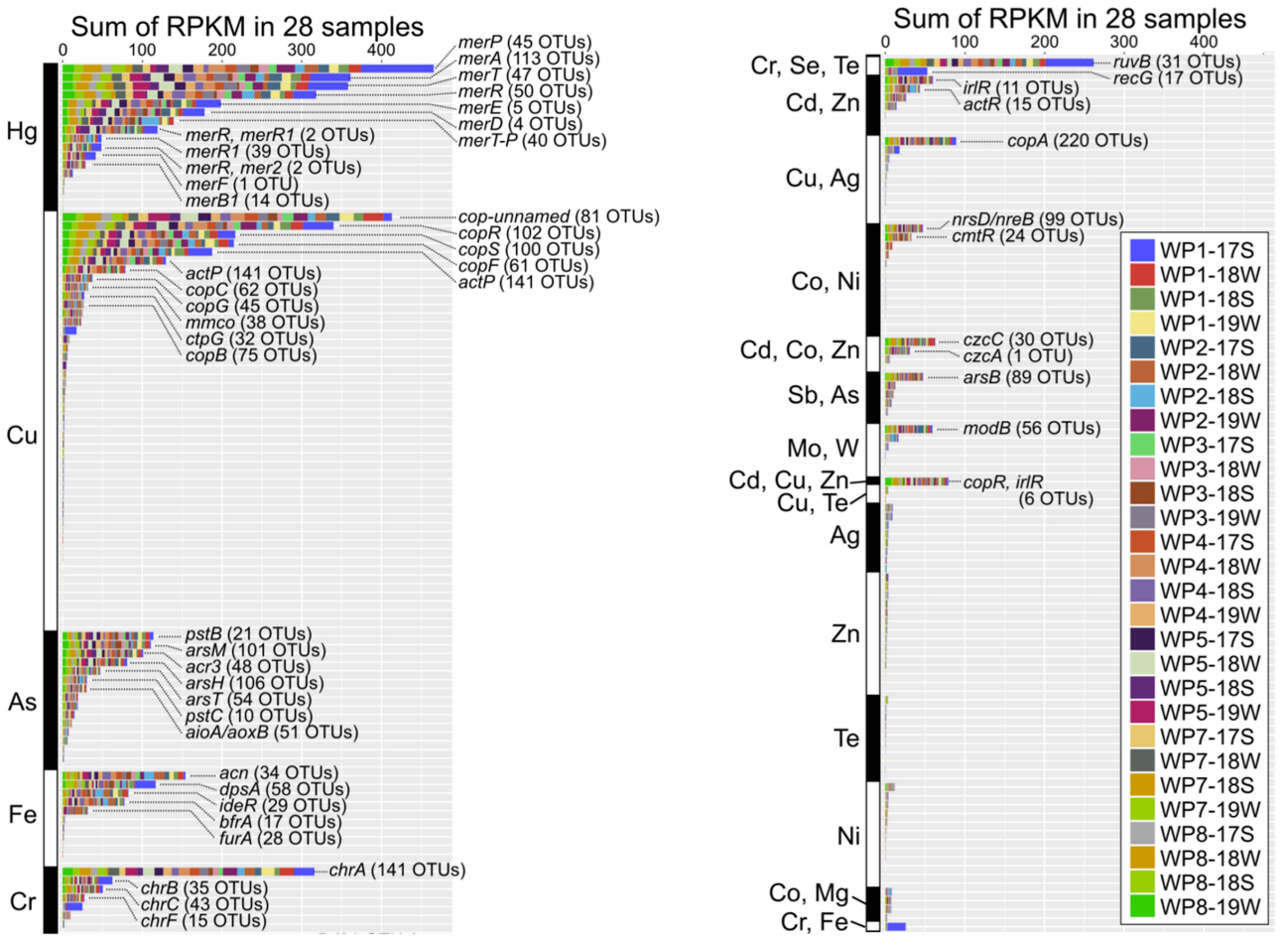

Figure 7 HMRG profiling. A Trends of metal resistance gene variations, the detected metal resistance genes in each metal at every sampling and WP site. WP1-17S stands for the sample at WP1 site in 2017 summer.
Abbreviations: S, summer; W, winter.

sediment provides another co-selection pressure for ARGs.[31,32] Such co-selection would generate a co-resistant phenotype on the same genetic determinant responsible for resistance to antibiotics and other contaminants.[33] In particular, even short-term Cu stress significantly enhances ARGs and MGE abundance as the Cu concentration increases, which can considerably change the potential of soil ARGs.[28,34] Thus, chemical pollution (eg, that caused by Cu) in the sediment could be involved in ARG contamination.

Diverse bacterial populations from household, hospital, and industrial wastewaters reach WWTPs. Therefore, urban WWTPs are among the main sources of ARB and ARGs released into the environment.[35] Moreover, wastewater and active sludge in WWTPs can act as AMR reservoirs and environmental sources, implying that WWTPs are HGT hotspots under selective pressure from antibiotics, disinfectants, and metals, even at low concentrations, enabling ARG dissemination among different bacterial species.[36] Even low antibiotic concentrations can cause ARG selection.[37] For instance, the fluoroquinolone antibiotic ciprofloxacin induces reactive oxygen species (ROS), leading to a mutant-generating cell subpopulation and homologous recombination.[38] Thus, it is very difficult to standardize the upper limit of antibiotic concentrations in wastewater. When the antibiotic concentration is reduced by dilution and active WWTP processing, non-corresponding contaminants (eg, heavy metals, organic pollutants, and physical and chemical factors) may play similar roles to antibiotics or may replace antibiotics, thus contributing to ARG propagation in the environment. In particular, heavy metals are used as feed supplements, which results in their accumulation in manure, indicating the potential for ARG co-selection.[31] The main route of ARG dissemination may switch from active transmission by antibiotic selection to passive transmission by non-corresponding contaminants such as heavy metals.[39]

Mainly, ARGs are located in multidrug-resistant (MDR) plasmids, which were possibly transferred to broad recipient targets among different Proteobacteria strains. The active potential bacteria that act as carriers and vectors appear to be γ-proteobacteria and β-proteobacteria, which are members of the phylum *Actinobacteria* and Firmicutes.[40] Members of the family *Enterobacteriaceae* and genera such as *Aeromonas*,[41] *Acinetobacter, Pseudomonas, Enterococcus*, and *Staphylococcus*[42] act as carriers in wastewater samples.[30] Thus, some of these species may play roles as HGT vector. Therefore, selective pressure from residual antibiotics and trace levels of heavy metals should be continuously monitored to avoid possible HGT among bacteria in wastewater.[43,44]

Conclusion

This study analyzed the resistome in WWTP effluents. The results suggested that urban communities, including hospitals, healthy carriers, and travelers, are potential ARG sources. We characterized a baseline level of the environmental ARG/HMRG profile in the overall community in Tokyo. The findings suggested that class 1 integron, including prevalent ARGs (*sul1* and Δ*qacE*), is essential in AMR surveillance in environments. In addition, seasonal differences in the ARG profile were observed in the present study; consequently, constant monitoring of the resistome in WWTP effluents is recommended for determination of the presence of notable ARGs and ARB in communities.

Data Sharing Statement

All raw read sequence files are available from the DRA/SRA database [accession numbers DRR198489–DRR198524] (metagenomic data, see Supplementary Table 1).

Ethical Statement

Ethical approval for this study is waived because the research was not conducted with identifiable biospecimen.

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Disclosure

The authors declare that they have no competing interests.

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