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Seasonal variations of microbial community and antibiotic resistome in a suburb drinking water distribution system in a northern Chinese city

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ABSTRACT

Antibiotic resistance genes (ARGs) are an emerging issue for drinking water safety. However, the seasonal variation of ARGs in drinking water distribution systems (DWDS) is still unclear. This work revealed the tempo-spatial changes of microbial community, ARGs, mobile genetic elements (MGEs) co-occurring with ARGs, ARG hosts in DWDS bulk water by means of metagenome assembly. The microbial community and antibiotic resistome varied with sampling season and site. Temperature, ammonia, chlorite and total plate count (TPC) drove the variations of microbial community structure. Moreover, environmental parameters (total organic carbon (TOC), chlorite, TPC and hardness) shifted antibiotic resistome. ARGs and MGEs co-occurring with ARGs showed higher relative abundance in summer and autumn, which might be attributed to detached pipe biofilm. In particular, ARG-bacitracin and plasmid were the predominant ARG and MGE, respectively. ARG hosts changed with season and site and were more diverse in summer and autumn. In winter and spring, *Limnohabitans* and *Mycobacterium* were the major ARG hosts as well as the dominant genera in microbial community. In addition, in summer and autumn, high relative abundance of *Achromobacter* and *Stenotrophomonas* were the hosts harboring many kinds of ARGs and MGEs at site in a residential zone (0.4 km from the water treatment plant). Compared with MGEs, microbial community had a greater contribution to the variation of antibiotic resistome. This work gives new insights into the dynamics of ARGs in full-scale DWDS and the underlying factors.

Introduction

Safe drinking water is pivotal resource for people life and public health. Although source water is purified by sedimentation, filtration and disinfection processes, the finished water leaving water treatment plant still deteriorates along the distribution system and threatens public health. It is necessary to pay attention to microbial contamination in drinking water distribution system (DWDS). On the one hand, some microbial taxa can still regrow in DWDS with disinfectant decaying. On the other hand, the DWDS is suitable for

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biofilm growth because of huge surface area, and biofilms are prone to detaching and go into bulk water, which causes contamination when the hydraulic condition changes. A lot of previous works have been performed to investigate the microbial community and its influential factors in DWDS. The DWDS microbial community usually show spatial or/and temporal variations (Bian et al., 2021; Jing et al., 2021; Ma et al., 2022; Vavourakis et al., 2020). Moreover, the water environmental parameters can affect bulk water and biofilm bacterial communities (Sevillano et al., 2020; Webster et al., 2021; Yu et al., 2022). In addition, from source to distribution, the microbial community can be affected by a variety of factors, such as drinking water source (Webster et al., 2021), treatment process (Lin et al., 2014), disinfectant type and dosage (Mi et al., 2015), pipe materials (Learbuch et al., 2021) and hydraulic condition (Douterelo et al., 2013). Pathogens (*Legionella* spp., *Mycobacterium* spp., *Mycolicibacterium* spp. and *Pseudomonas*, etc.) in DWDS also deserve concerns because they are frequently detected in DWDS (Bal Krishna et al., 2021; Thom et al., 2022).

The resistance of bacteria to antibiotics usually occurs and antibiotic resistance genes (ARGs) are viewed as emerging pollutants nowadays. If patients are exposed to potential pathogenic bacteria harboring ARGs in drinking water and the pathogens cannot be killed by antibiotics, it will put them at risk. Although microbial abundance and antibiotic pressure are relatively low in drinking water, ARGs still exist in drinking water all over the world according to a large-scale investigation (Ma et al., 2017). Moreover, mobile genetic elements (MGEs) such as plasmids, integrons and transposons can accelerate ARG transfer among bacteria and increase the ARG risks towards public health (Jia et al., 2019). However, information about the spatial and temporal variations of ARGs and MGEs and their hosts in DWDS is still scarce. The links of ARGs with MGEs, microbial community and environmental factors in DWDS still remain elusive.

The quantification of ARGs can be achieved by qPCR or metagenomics. Metagenomics can yield more comprehensive information about ARGs and avoid the primer bias. In addition, metagenome assembly can aid to discover MGEs co-occurring with ARGs in the same contigs and identify the host of ARGs more directly according to contig's taxonomy classification rather than statistical correlation by network analysis (Jia et al., 2019; Zhang et al., 2019). However, the ARGs in DWDS have been seldomly explored using metagenomes method.

In the present study, we collected tap waters in four seasons at two DWDS sites in a northern Chinese city. The ARGs in tap waters were analyzed using metagenomics. Our work aimed to (1) characterize the seasonal changes of microbial community, ARGs, MGEs and ARG hosts, (2) explore the links of environmental factors with microbial community and antibiotic resistome, (3) discern the opportunistic pathogenic bacteria harboring ARGs as well as MGEs, and (4) uncover the contributions of microbial community, MGEs and environmental factors to ARGs variation. This study may help to extend our knowledge about the seasonal variations of antibiotic resistome and the driving factors in full-scale DWDSs and to provide a guidance for drinking water antibiotic resistance risk assessment and the minimization of the health risks of ARGs in tap water.

1. Materials and Methods

1.1. Sampling and water quality analysis

The studied DWDS was located in the suburb of a northern Chinese city. The suburb DWDS was fed with the treated surface water (after a series of processes including sedimentation, sand filtration, activated carbon filtration and chlorine dioxide disinfection). Two sampling sites were selected, including one in the residential zone (site LH) (0.4 km from the water treatment plant), and another near a primary school (site XX) (1.5 km from the water treatment plant). The distribution pipe at site LH was made of ductile cast iron and had been used for 10 years. The pipe at site XX (2 years old) was made of PE (polyethylene).

Sampling activities were carried out in November 2020 (winter), March 2021 (spring), June 2021 (summer), and September 2021 (autumn). Before sampling, tap was disinfected with 75% ethyl alcohol and had been turned on for 5 min to drain the stagnant water. Sterile PE bucket was rinsed with tap water for three times and then water sample (20 L) was collected in triplicate. Sterilized sodium thiosulfate (1%) was used to quench chlorine dioxide in tap water (Jing et al., 2021). Each sample was transported to lab within 2 hr. Tap water was filtered by the peristaltic pump (BT100-2J, Longer) using a stainless-steel pressure filter (NO. XX4404700, Millipore, Billerica, MA, USA). The microbiota was retained by 0.22-μm EPS membrane (Millipore, Australia) until water could not pass the filter (Jing et al., 2021). The filters were preserved in 1.5-mL centrifuge tubes at -80 °C before DNA extraction.

During field sampling, turbidity (2100Q, HACH, USA), pH (HQ30D, HACH, USA), temperature (HQ30D, HACH, USA), free chlorine (PC II, HACH, USA), total chlorine (PC II, HACH, USA) were immediately measured using portable apparatuses. Moreover, 1 L tap water was used to analyze water parameters in the lab (including total organic carbon (TOC), ammonia-nitrogen, total phosphorus, nitrite-nitrogen, nitrate-nitrogen, chloride ion, sulfate, total dissolved solid, hardness, total plate count (TPC), color and chlorite). TOC was measured using TOC-LCPH (SHIMADZU, Japan). The levels of chloride (Cl^-), nitrate (NO_3^-), sulfate (SO_4^{2-}) and chlorite (ClO_2^-) were assayed by ion chromatography (Metrohm, Switzerland). Ammonia-nitrogen, total phosphorus, permanganate index (COD_{Mn}), nitrite-nitrogen, total dissolved solid, TPC, hardness and color were measured using standard methods according to China Environmental Protection Agency (2002).

1.2. DNA extraction and metagenomics sequencing

The filters were cut into pieces by sterile scissors and water DNA was extracted using Powersoil DNA extraction kit (Mobio Laboratories, USA). The concentration and purity of DNA were measured with Quantus Fluorometer (Picogreen) and NanoDrop2000 (Thermo Fisher Scientific, USA). The integrity of DNA was tested by agarose gel electrophoresis. DNA was then sent to quality testing (tested as level B) and shotgun sequencing (2 × 150 bp) at an Illumina novaseq 6000 platform (Shanghai Majorbio Bio-pharm Technology Co., Ltd.). The metagenomic sequencing data were firstly conducted to do quality

control using fastqc software (version 0.11.9). Then these data were preformed to separate contaminant reads using kneaddata software (version 0.6.1) (Chen et al., 2022b). Afterwards, the size of paired-end reads of high quality in FASTQ format ranged from 10.63 to 14.43 Gb. All sequencing data are publicly attainable in the NCBI Sequence Read Archive under accession number PRJNA826697.

1.3. Taxonomic classification, ARGs annotation and quantification

Kraken2 software (version 2.1.0) was used for microbial community taxonomic classification based on exact k-mer matches to achieve high accuracy. The clean reads were assembled by megahit software (version 1.2.9) to get longer contigs for each sample. Then the contigs were checked for quality by quast software (version 5.0.2). The contigs with length >500 bp were retained. Prokka software (version 1.12) was used for the open reading frames (ORFs) prediction of the contigs. The ORFs of triplicate samples were pooled together. The software cd-hit (version 4.8.1) was used to delete the redundant ORFs. The amount of non-redundant ORFs of every pooled sample varied from 734,839 to 1,003,709. The predicted ORFs sequences were translated into protein sequences, and then the protein sequences were aligned with SARG v2.2 database. If the identity and coverage was higher than 80% and 70% respectively, the ORFs were regarded as ARGs. Moreover, the contig with at least one ORF like ARG was assigned as antibiotic resistance contig (ARC). The relative abundance of ARGs (copies per cell, cpc) was calculated using the following equation (Jia et al., 2020).

$$\text{Relative abundance} = \sum_{i=1}^{n} \frac{\text{Ni}_{\text{mapped reads}} \times \text{Li}_{\text{read}}}{\text{Li}_{\text{reference sequence}} \times \text{N}_{\text{cell}}} \quad (1)$$

Where the n is the number of ARG subtype of the same type. $\text{Ni}_{\text{mapped reads}}$ can be obtained using salmon software (version 0.13.1). Li_{read} is 150 bp of short read length. $\text{Li}_{\text{reference sequence}}$ (bp) can be gotten by prokka results. The N_{cell} can be obtained from ARGs-OAP pipeline (Yin et al., 2018).

1.4. MGEs quantification and location with ARGs

A MGEs database was constructed by extracting sequences with keywords (plasmid, integrase, integron, transposase, transposon, recombinase, recombination, conjugative, conjugal or mobilization) from the non-redundant protein sequence database (NR). The ORFs protein sequences were searched against MGE database (identity≥80%, coverage≥70%). In order to uncover the MGEs co-occurring with ARGs, the contigs were picked out with both ARG and MGE. The details of quantification of MGEs co-occurring with ARGs can refer to previous studies (Chen et al., 2022b; Ma et al., 2016; Zhang et al., 2019).

1.5. ARG host identification

All ORFs of ARC were compared with NR database (e-value cutoff≤10^{-5}, identity≥80%, coverage≥70%) and then were taxonomically classified using MEGAN. If half of the ORFs on one ARC were affiliated to the same genus, the host of ARC (namely the ARG host) belonged to this genus. Moreover, the network analysis was further conducted to identify the potential ARG host. The ARC quantification could be performed according to previous studies (Chen et al., 2022b; Zhang et al., 2019).

1.6. Statistical analyses

The bar graphs of microbial community and ARG components were completed by Rstudio and R package ggplot2. The heatmap was also achieved by R package pheatmap. Principal co-ordinates analysis (PCoA) was used to reveal the seasonal variations of microbial community and antibiotic resistome. Redundancy analysis (RDA) and canonical correspondence analysis (CCA) were applied to illustrate the links of environmental parameters with microbial community and antibiotic resistome, respectively. In addition, PCoA, RDA and CCA were achieved using R package vegan. The influences of sampling season and site on microbial community and antibiotic resistome were tested by PERMANOVA and ANOSIM test using R package vegan. The network correlations (r>0.6, p<0.05) of ARGs with microbial community were carried out using RStudio (version 4.0.0) and the results were visualized by Gephi 0.9.2 (Ma et al., 2017).

2. Results

2.1. Seasonal and spatial changes of microbial community structure

Based on metagenomic sequencing, taxonomic classification was identified by kraken2 software using clean short reads. The top 15 taxa at phylum level and top 20 taxa at genus level in water samples were shown in Fig. 1. Metagenomic sequencing technology could unveil all kinds of taxa, including not only bacteria but also eukaryote, archaea and virus. Bacteria was the predominant domain in tap waters (98.8%), followed by eukaryota (0.9%), archaea (0.2%) and viruses (0.1%). Chordata (0.86%, within eukaryote) and Euryarchaea (0.17%, within archaea) were listed among the top 15 phyla. Proteobacteria (49.7%–93.8%, average 70.1%) was the largest bacterial phylum in all DWDS water samples. Proteobacterial proportion displayed a clear seasonal variation trend (average relative abundance: Autumn > Summer > Spring > Winter). Actinobacteria (18.1%) and Bacteroidetes (4.7%) were also the major bacterial phyla in tap waters. Bacteroidetes were more abundant in winter and spring. In addition, *Sphingopyxis, Sphingobium, Sphingomonas, Brevundimonas, Candidatus Planktophila, Pseudomonas* and *Limnohabitans* were the dominant genera in tap waters. *Sphingopyxis, Brevundimonas* and *Sphingobium* were more abundant in summer and autumn, especially at site XX, whereas *Limnohabitans* showed higher abundance in winter and spring.

The seasonality effect on microbial community in tap water was obvious, according to the result of PCoA (Appendix A **Fig. S2**). Based on bray-curtis distance, season had a significant impact on the microbial community structure (Adonis test, R^2=0.536, p=0.001). In addition, sampling site could also govern the microbial community structure (Adonis test, R^2=0.087,

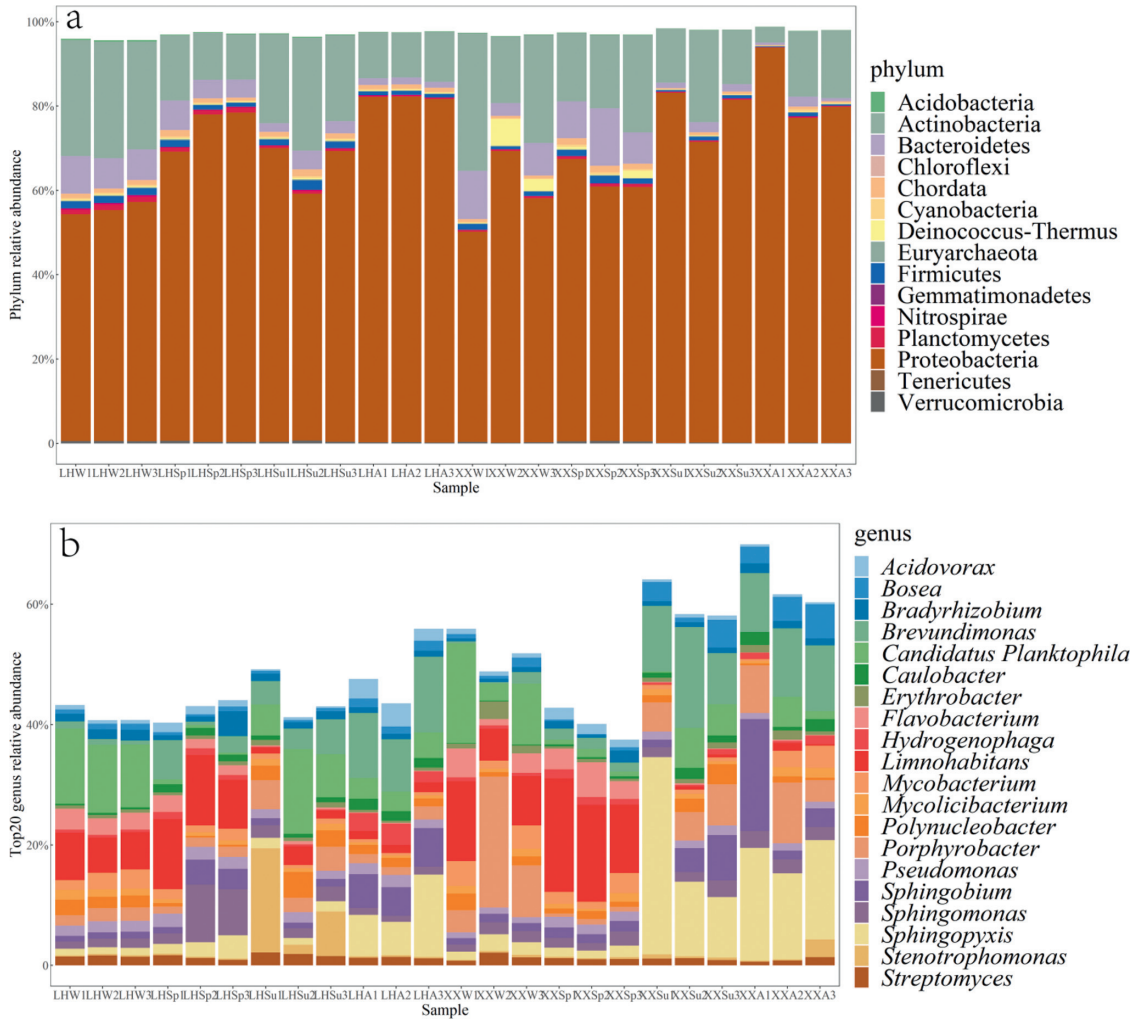

Fig. 1 – Relative abundance of top 15 phyla (a) and top 20 genera (b) in tap waters in four seasons and at two sites. Samples are coded with sampling site followed by season and replicate. LH and XX indicate two sampling sites. W, Sp, Su and A represent winter, spring, summer and autumn, respectively. Digits (1, 2 and 3) represent replicate number.

p=0.002). Furthermore, site effect was coupled with season effect to influence microbial community structure (Adonis test, R^2=0.151, p=0.004). Microbial communities at sites XX and LH were clearly separated in summer, but closely clustered in winter.

2.2. *Seasonal and spatial changes of ARGs*

In total, 35 ARG subtypes belonging to 11 ARG types were detected in tap waters according to metagenome assembly and SARG database annotation. The relative abundance of total ARGs was higher in summer and autumn than in other two seasons at both two sites (Fig. 2a). The peak ARG relative abundance reached 0.133 copies per cell (cpc) in summer at site LH. The dominant ARG type in summer at site LH was multidrug (0.098 cpc) followed by beta-lactam (0.01 cpc). Average ARG relative abundance in autumn at site LH was also high (0.057 cpc), with bacitracin and sulfonamide as the main types. Bacitracin was the major ARG type in spring and winter, while sulfonamide was more abundant in summer and autumn.

Among the 35 identified ARG subtypes, the *bacA* belonging to bacitracin type ARG was a generalist in all samples (Fig. 2c). Some ARG subtypes belonging to multidrug type including *emrE, mexE, multidrug ABC transporter, multidrug transporter, smeC, smeD* and *smeF* were mainly observed in summer at site LH. *aph(3)-IIb* belonging to aminoglycoside type appeared only in summer at site LH. *Sul1* and *sul2* mainly existed in summer and autumn. More subtypes of ARGs appeared at site LH than at site XX. *aac(2)-I* was mainly detected at site XX. *aac(3)-IIIa, rifampin monooxygenase, tetracycline resistance protein, tetV and vanR* appeared at site XX in summer or autumn. The PCoA results of antibiotic resistome based on bray-curtis distance illustrated a considerable shift in antibiotic resistome with season (Fig. 2b). Adonis test revealed that season factor (R^2=0.3181, p=0.001), site factor (R^2=0.0688, p=0.003) and their joint effect (R^2=0.2784, p=0.001) contributed to antibiotic resistome change. The PCoA result indicated that resistomes at

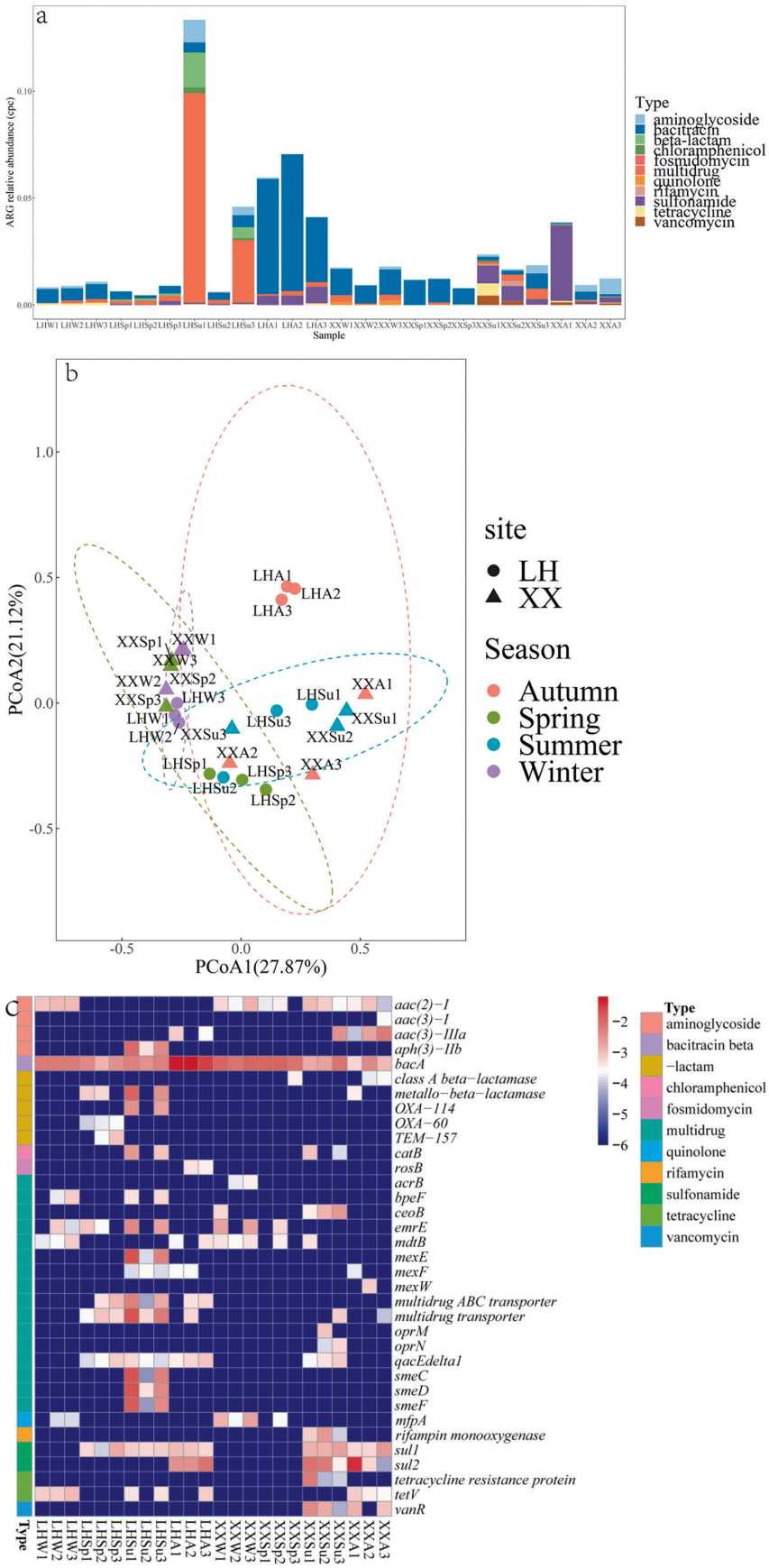

Fig. 2 – The occurrence of ARG types (a) and PCoA of ARG subtypes (b), and the heatmap of ARG subtypes (c).

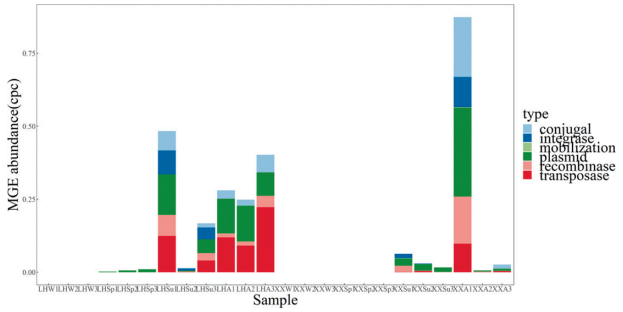

Fig. 3 – Types and relative abundance of MGEs co-occurring with ARGs at the same contigs.

sites LH and XX were separated in autumn but closely clustered in winter.

2.3. *ARGs and MGEs co-occurrence*

If the ARGs and MGEs occur in the same contig, ARGs will have a stronger migration ability. In this study, the MGEs located at the contigs with ARGs (named ARC-MGE) were also quantified. The ARC-MGEs mainly appeared in summer and autumn and exhibited a seasonal change (Fig. 3). ARC-MGEs ranged from 0 to 0.873 cpc. The highest abundance of ARC-MGEs appeared in autumn at site XX (0.873 cpc). The major components of ARC-MGEs were conjugal, integrase, plasmid, recombinase and transposase, and plasmid showed the highest detection frequency (62.5%).

According to the constructed ARC (antibiotic resistant contig) with MGEs (Appendix A **Fig. S3**), some ARCs had the ORFs that were identified as both ARGs and MGEs. For example, the ARGs (*sul1, TEM-157* or *qacEdelta I*) and plasmid were annotated in the same ORFs in spring at site LH. As for the tap water samples at site LH in summer, they were detected with the largest number of ARCs co-occurring with MGEs (8 ARCs with MGEs). LHSu1_695155 contig had two ARGs (*smeC* and *mexE*) belonging to multidrug type and two MGEs (plasmid and transposase), with contig relative abundance of 0.018 cpc. LHSu1_379126 contig had a greater migration potential because it had 8 ORFs identified as MGEs (including plasmid, integrase, transposase and recombinase) with one ARG (*multidrug ABC transporter*). Moreover, LHSu1_753047 (relative abundance, 0.0138 cpc) also contained 7 ORFs belonging to MGEs (including plasmid, conjugal and transposase) and 1 ARG (*metallo-beta-lactamase* belonging to beta-lactam type). LHSu1_765411 (0.0168 cpc) harbored multiple ARGs belonging to multidrug type (*smeD, smeF* and *multidrug transporter*) and MGEs (recombinase). The ARC's ARGs in summer at site LH was commonly multidrug type. For the tap water in autumn at site LH, LHA1_484796 (0.0051 cpc) contained *sul2* and 10 MGEs with a strong ability to transfer ARGs. LHA1_495746 (0.0607 cpc) was the most abundant contig with ARG (*bacA*) and MGE (plasmid). *Sul1-qacEdelta1*-plasmid co-occurrence appeared in LHA2_42062 and XXSu1_520235 contigs. For the tap water in autumn at site XX, XXA1_270419 contig (0.0315 cpc) possessed 9 MGEs (including integrase, plasmid, conjugal, recombinase and transposase) and *sul2*. To sum up, multidrug type ARGs (e.g. *multidrug transporter* and *qacEdelta1*) and sulfonamide type ARGs (e.g. *sul1* and *sul2*) usually co-occurred with multiple MGEs, which suggested that these ARGs owned a greater ability to transfer.

2.4. *Hosts of ARCs*

By using BLASTP with the NR database and MEGAN annotation, the hosts of ARGs could be determined at the genus level from contigs' scope. The occurrence frequency and relative abundance of *Limnohabitans* and *Mycobacteria* were the highest among ARG hosts, followed by *Polynucleobacter, Mycolicibacterium, Bosea* and *Polaromonas* (Fig. 4**, Appendix A Fig. S5**). The occurrence frequency of *Limnohabitans* and *Mycobacteria* were both 41.7%, and their proportions were 0–87.79% and 0–53.2%, respectively. In winter and spring, the ARG hosts in tap waters were mainly *Limnohabitans* and *Mycobacterium*. *Limnohabitans* was the host of ARG-bacitracin, but *Mycobacterium* carried multiple ARGs such as aminoglycoside, multidrug, beta-lactam and quinolone. Compared with the samples in winter and spring, the ARG hosts in tap waters in summer and autumn were more diverse. The majority of ARG hosts in summer at site LH were *Stenotrophomonas, Achromobacter, Delftia, Paraburkholderia* and *Mycobacterium*. *Stenotrophomonas* was the host of aminoglycoside, beta-lactam and multidrug type ARGs. *Achromobacter* was also the host of various ARGs (bacitracin, beta-lactam, chloramphenicol and multidrug). *Delftia, Paraburkholderia* and *Mycobacterium* were the host of bacitracin, multidrug and aminoglycoside, respectively. In addition, as ARG-bacitracin host, *Curvibacter* constituted 83.4% in one of the triplicate water samples in autumn at site LH. *Polynucleobacter* and *Mycolicibcterium* were the major ARG hosts in water samples in autumn at site LH, carrying bacitracin and tetracycline, respectively. *Microbacterium* was abundant in summer at site XX, and it was the host of many ARGs (aminoglycoside, tetracycline and vancomycin). *Rhodococcus* and *Polynucleobacter* were the host of rifamycin and bacitracin, respectively. *Bosea* existed in samples at site XX in both summer and autumn, carrying the ARG of aminoglycoside. *Limnohabitans* carrying ARG-bacitracin exited in the sample at site XX in both summer and autumn.

For the contigs carrying both ARG and MGE, it was of great importance to detect the hosts of these particular contigs, although the major hosts were taxonomically unclassified (Appendix A **Fig. S3**). In detail, in spring at site LH, the only identified host carrying both ARG (*sul1*) and MGE (plasmid) was *Salmonella*. In summer at site LH, the classified hosts of ARC with MGEs were *Stenotrophomonas, Achromobacter* and *Paraburkholderia*. In addition, *Curvibacter* and *Acinetobacter* were found to be the hosts of ARCs with MGEs for the samples in autumn at site LH. LHA1_495746 was the most abundant ARC with MGEs, with *Curvibacter* as its host. *Paracoccus, Acinetobacter* and *Nocardiaceae* were the hosts of the ARCs with MGEs in summer at site XX. *Tabrizicola* and *Bosea* carried ARCs with MGEs in autumn at site XX. Therefore, the hosts of ARCs with MGEs varied with season and site.

Since there were many ARCs with taxonomically unclassified hosts, it is necessary to resort to network analysis of ARGs and microbial community to identify more ARG hosts. If the correlation of ARG with a certain bacterial genus was

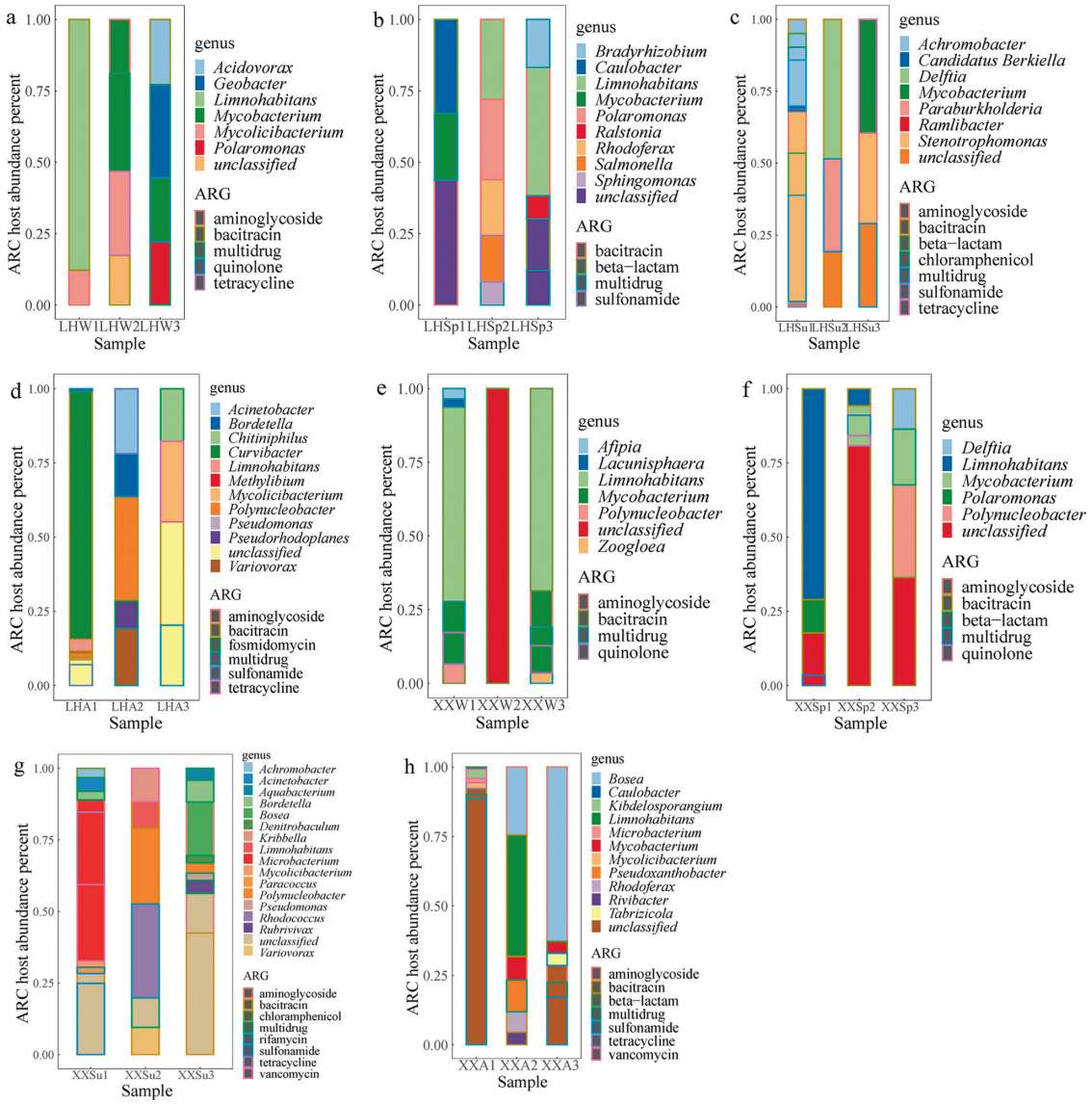

Fig. 4 – The ARG hosts' relative abundance in each water sample (a-h). The fill color represents the ARG hosts at the genus level, while the frame color represents the ARG type harbored by hosts.

positive and significant ($r>0.6$, $p<0.05$), we speculated that the bacterial genus was the potential host of ARG (Appendix A **Fig. S4**). *Sphingopyxis*, *Brevundimonas*, *Solimonas*, *Caulobacter* and *Bosea* were likely the hosts of *sul2*. *Sphingopyxis*, *Brevundimonas*, *Phenylobacterium* and *Caulobacter* were assigned as the hosts of *sul1*. Because both *Sul1* and *Sul2* genes were sulfonamide type, their hosts were almost the same. *Bosea* and *Sphingopyxis* were the potential hosts of *aac(3)-IIIa*. *Sphingopyxis* was the possible host of *vanR*. Moreover, the hosts of *bacA* or *tetV* were diverse. *BacA*'s hosts were *Rhodoferax*, *Comamonas*, *Verminephrobacter*, *Ottowia*, *Melaminivora*, *Acidovorax*, *Serpentinomonas* and *Simplicispira*. *TetV*'s hosts also consisted of *Phreatobacter*, *Ensifer*, *Rhodopseudomonas*, *Chelatococcus*, *Nitrobacter*, *Microvirga*, *Rhizobium* and *Paracoccus*. The results of network analysis tended to be consistent with ARC host taxonomy classification. However, the network analysis could yield more hosts of ARGs for unclassified ARCs.

2.5. *Environmental factors associated with microbial community and antibiotic resistome*

For the relationships of environmental factors with microbial community, a statistically significant RDA model ($p=0.001$) was obtained by a stepwise regression method based on AIC with adjusted R^2 of 0.552 (Fig. 5a). This RDA model screened temperature, ammonia, chlorite, and TPC as explanatory factors to decipher the microbial community shift by forward and backward selection. The first two RDA axes explained 35.87% and 11.18% of the variation of microbial community.

CCA model ($p=0.001$) was achieved with adjusted R^2 of 0.472 to explain the variation of antibiotic resistome by selecting TOC, chlorite, TPC, and hardness (Fig. 5b). The first two CCA axes respectively explained 21.49% and 14.55% of the variation of antibiotic resistome. Apart from environmental factors (e.g. TOC), microbial community (41.8%) contributed to the largest

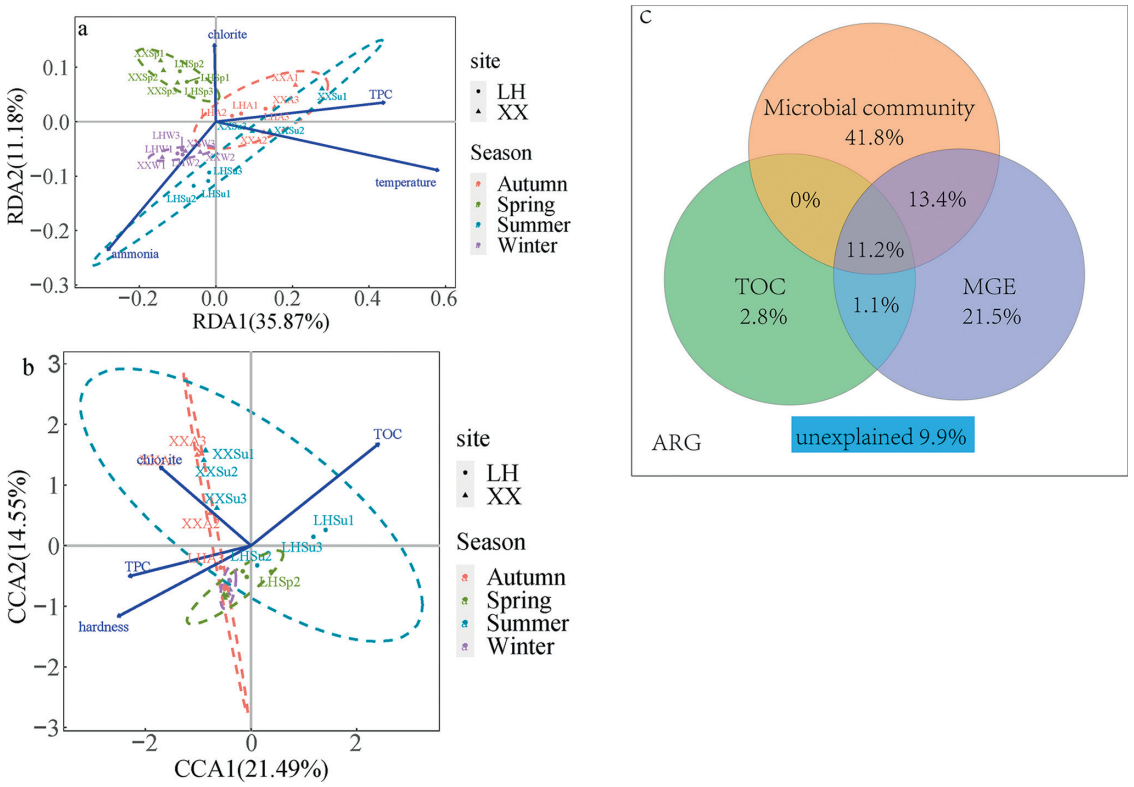

Fig. 5 – Redundancy analysis of microbial community (at genus level) with environmental factors (a). Canonical correlation analysis of antibiotic resistome (at ARG subtype level) with environmental factors (b). Variation partitioning analysis distinguishing the effects on antibiotic resistome of microbial community, MGEs and temperature (c).

influence on the antibiotic resistome based on VPA analysis, followed by MGEs (21.5%) (Fig. 5c). These three factors (microbial community, MGEs and TOC) totally contributed to 11.2% of the variation of antibiotic resistome.

3. Discussion

3.1. *Profile of DWDS microbial communities and influential factors*

Proteobacteria was the dominant bacterial phylum in DWDSs (Chen et al., 2022a; Liang et al., 2022; Ma et al., 2019; Sevillano et al., 2020; Siedlecka et al., 2021; Zhang et al., 2021), which was in line with the present study. Moreover, *Sphingomonas* was the generalist in the studied tap waters. *Sphingomonas* is ubiquitous in DWDSs (Ma et al., 2017; Tang et al., 2021), because it can be adapted to oligotrophic environment (Bal Krishna et al., 2021) and has a strong resistance to disinfectants (e.g. chlorine) (Sun et al., 2013). This genus is generally viewed as a pioneer in biofilm formation of pipes (Ren et al., 2015). Therefore, it might originate from distribution pipes biofilm. *Pseudomonas* and *Mycobacteria* were also the important bacterial components in the studied tap waters. Moreover, these two genera contained some species as known pathogens and might come from detached biofilms (Douterelo et al., 2018b; Learbuch et al., 2021). They deserved more attention because they raised higher health concerns as opportunistic pathogens and were frequently detected in DWDS.

In this study, microbial community structure illustrated an obvious seasonal shift, consistent with numerous previous studies (Potgieter et al., 2018; Vavourakis et al., 2020; Yu et al., 2022; Zhang et al., 2021). The major environmental factors regulating microbial community structure were found to be temperature, ammonia, chlorite and TPC based on RDA (Fig. 5a). Previous studies indicated that temperature affected alpha diversity of DWDS bulk water according to a long-term sampling campaign (Potgieter et al., 2018) as well as microbial community structure in DWDS (Nagymate et al., 2016; Yu et al., 2022). In addition, nutrients including ammonia had a positive link with the bacterial diversity in drinking water (Yu et al., 2022). In this study, ammonia was found to have a significant impact on microbial community on account of RDA result. Nutrients are important for microbial growth especially in the oligotrophic environment like drinking water. Furthermore, in this study, chlorite as a disinfectant could also cause a shift in microbial community structure by selective inactivation of microbiota. Different disinfectant types can determine microbial community structure of DWDS bulk water or biofilms (Mi et al., 2015; Potgieter et al., 2018; Wang et al., 2014; Zhang et al., 2021). Moreover, previous researches usually focused on the effects of physical-chemical parameters on microbial community, while the correlation between microbial absolute abundance and microbial community structure was less known. In this study, TPC, representing the number of

culturable cells, was found to shape microbial community structure.

The PCoA and RDA results revealed that sampling site also determined DWDS microbial community structure. The difference in microbial community at two sites might be attributed to different water transportation distances and pipe materials. Site LH was near drinking water treatment plant (0.4 km), while site XX was farther (1.5 km) (Appendix A **Fig. S1**). A previous study found that transportation distance greatly affected the bacterial community (Bian et al., 2021). Furthermore, transportation pipes at sites LH and XX had different materials. The pipes at sites LH and XX were nodular cast iron and PE, respectively. Pipe materials could impact biofilm bacterial community assembly due to surface roughness and reaction activity (Tang et al., 2021; Wang et al., 2014; Zhang et al., 2016). Zhang et al. (2016) found that microbial community on PE pipe had higher diversity than that on ductile iron pipe. PE pipe could release organic matter into DWDS bulk water and thus accelerate bacterial growth (Learbuch et al., 2021), whereas cast iron contained corrosion-related microorganisms like *Desulfovibrio* (Tang et al., 2021). When water flushes in DWDS pipes, biofilms may detach from the pipes and enter into bulk water. Therefore, pipe material could regulate planktonic bacterial community structure by biofilm detaching.

3.2. *Profile of DWDS ARGs and influential factors*

In this study, *bacA* subtype belonging to bacitracin almost existed in all tap waters. *bacA* gene is the dominant persistent ARGs in drinking water all over the world and is regarded as the intrinsic gene of bacteria, existing in many genera (Ma et al., 2017). Moreover, the present study found that the multidrug and aminoglycoside type ARGs occurred mainly in summer tap water at site LH. Multidrug resistance gene and aminoglycoside resistance gene were also dominant in drinking water and household activated carbon water purifier's biofilms (Zhou et al., 2021). The antibiotic resistance level of drinking water was at level I (<0.1 cpc) according to the standard proposed by a previous study (Ma et al., 2017). Therefore, the antibiotic resistance risk in the studied suburb DWDS waters was low.

In this study, the ARG composition revealed an obvious seasonal change. The relative abundance of ARGs was higher in summer and autumn than in winter and spring. Hao et al. (2019) also found that extracellular and intracellular ARGs presented an evident seasonal change pattern, and ARGs were the most abundant in summer. The ARGs in tap water were the lowest in winter, which might be caused by sedimentation step in winter (Yu et al., 2022).The multidrug and bacitracin resistance genes were much more abundant in summer and autumn, especially at site LH. These increased ARGs in bulk water might come from detached biofilms of pipes (Zhou et al., 2021). Generally, the ARGs in biofilm were more abundant than in bulk water and were the important reservoir of ARGs (Chen et al., 2022a; Chen et al., 2020; Douterelo et al., 2018a; Zhou et al., 2021). When we turn on the tap faucet, the biofilm detached from pipe into water can increase ARGs in drinking water. A previous study demonstrated that biofilm detachment changed bacterial antibiotic resistance in drinking water in an annular bioreactor (Zhang et al., 2018). In DWDS pipe biofilms, there are numerous bacterial cells under viable but non-culturable (VBNC) condition in order to resist the oligotrophic environment in tap water and they show a stronger resistance towards antibiotic pressure. The number of VBNC cells is usually positively correlated with ARGs. When chlorine disinfectant increased, the detached biofilm provided VBNC for tap water and promoted the ARGs in tap water (Zhang et al., 2018). Moreover, the detached biofilms had higher relative abundance of ARGs than adherent biofilms in drinking water, especially with starvation treatment (Chen et al., 2022a). Hence, the detached biofilms could have more ARGs in more oligotrophic environments. A previous study found that intracellular ARGs increased along the distribution system and biofilm detachment accounted for it (Liang et al., 2022). In addition, a full-scale investigation pointed out that season, location and pipe material affected the biofilm's ARGs distribution (Siedlecka et al., 2021), which might further regulate the ARGs distribution in bulk water. In the future work, it is necessary to detect the ARGs of bulk water and biofilms simultaneously and elucidate the contribution of detached biofilm to bulk water with respect to ARGs.

Based on CCA, the present study suggested that TOC and chlorite had tight correlations with antibiotic resistome. Yu et al. (2022) reported that TOC exhibited positive correlations with many ARGs in drinking water. Hao et al. (2019) noticed that residual chlorine showed negative correlations with intracellular macrolide resistance genes. Chlorine could promote the relative abundance of ARGs and change antibiotic resistome by enriching the ARG hosts and MGEs co-existing with ARGs (Jia et al., 2019). A previous study also indicated that total chlorine and TOC could also account for the change of antibiotic resistome in DWDS waters in light of dbRDA model (Sevillano et al., 2020). Nevertheless, to date, the effects of TPC and hardness on antibiotic resistome are still unclear. The present study further suggested that TPC and hardness were tightly correlated with antibiotic resistome.

Compared with above-mentioned environmental variables (e.g. TOC), in the present study, microbial community structure was the most important factor determining antibiotic resistome followed by MGEs in light of variance partitioning analysis. Microbial community structure determined the response of antibiotic resistome to chlorine disinfection (Jia et al., 2015, 2019). However, Yu et al. (2022) pointed out that MGEs outweighed microbial community with regard to the explanation of the variation of antibiotic resistome in tap waters. Therefore, the relative significance of microbial community and MGEs to antibiotic resistome was still controversial.

3.3. *MGEs co-occurring with ARGs in DWDS*

In this study, the MGEs that co-occurred with ARGs were mainly detected in summer and autumn. The MGEs can lead to ARGs transfer among different bacteria, causing the change of ARG hosts, which might contribute to the diversity of ARG hosts in summer and autumn. The major constitutes of MGEs were conjugal, integrase, plasmid, recombinase and transposase. These MGEs ranged from 0 to 0.87 cpc, and the plasmids had higher detection frequency compared with other MGEs. In household purifier's biofilms, the plasmids had the

highest detection frequency and abundance (Zhou et al., 2021). In addition, the present study further found that the plasmids mainly co-existed with sulfonamide resistance genes like *sul1* and *sul2* as well as multidrug resistance genes like *qacEdelta1*. Disinfected water in a full-scale drinking water treatment plant was also found to have contigs carrying ARG (*mexH*) and MGE (*recR*) (Zhang et al., 2019). Chlorination could increase the MGEs (transposon and plasmid) co-occurring with ARGs (Jia et al., 2019). After chlorination, the multiple transposons and multiple ARGs (*mex*E and RND) co-existed in one contig (Jia et al., 2019) . Metagenomic technology contributes to the identification of ARGs carrying MGEs and helps to monitor the mobile ARGs, and thus provides basic data for assessing the risks of mobile ARGs to human health in drinking water.

3.4. *ARG hosts and risk in DWDS*

ARG hosts altered with season. They were more diverse in summer and autumn than in winter and spring. Opportunistic pathogenic bacteria widely exist in DWDSs. If they are the ARG hosts, they can pose a great human health risk. In this study, *Mycobacterium* was found to carry many ARGs (aminoglycoside, multidrug, beta-lactam and quinolone) and it appeared mainly in winter and spring. *Mycobacterium* is frequently detected in biofilms and bulk waters of DWDSs because of its resistance to disinfectants in the oligotrophic environment (Sevillano et al., 2020; Webster et al., 2021; Zhou et al., 2021). *Mycobacterium* is a trouble in drinking water because some species from *Mycobacterium* like *M. avium* can infect people with weak immune ability and they are viewed as opportunistic pathogens (Yu et al., 2022). Meanwhile, if *Mycobacterium* owns resistance towards antibiotics, it becomes very difficult to cure *Mycobacterium*-related disease, which leads to enormous risk for patients. Notably, the chlorine-resistant bacteria are prone to tolerating antibiotic stress (Zhang et al., 2019). Antibiotic resistant bacteria had higher survival rates under chlorine pressure. The cross-resistance of disinfectants and antibiotics might be attributed to multidrug efflux pumps (Jia et al., 2019). Based on metagenome assembled genomes, the nontuberculous *Mycobacterium* harbored multiple antibiotic resistance genes (*RbpA, mtrA, murA*) and appeared only in disinfected drinking water (Sevillano et al., 2020). After chlorination, *Pseudomonas alcaligenes* was identified in drinking water, carrying ARGs belonging to RND and ABC antibiotic efflux classes (Jia et al., 2019). *Mycobacterium* was found to be the host of ARGs in tap water in a large-scale survey (Ma et al., 2017). Network analysis revealed that *Mycobacterium* was associated with many ARGs (*efpA, mtrA, murA, RbpA, aac(2')-Ib*) and carried MGEs like plasmids (pMKMS02, pMFLV01) and inserted sequence (ISMysp3) in household purifier's biofilms (Zhou et al., 2021). Moreover, metagenomics assembly method uncovered three opportunistic pathogens (*Pseudomonas alcaligenes, Pseudomonas aeruginosa* and *Mycobacterium gordonae*) harboring ARGs (*mexW, aph*(3')-I and *aac*(2')-I) in drinking water (Ma et al., 2019).

The present study also revealed that *Limnohabitans* harbored bacitracin resistance genes and mainly occurred in winter and spring as a general genus with high relative abundance. That indicated that microbial community could affect the specie of ARG hosts and further resistance resistome. *Limnohabitans* was also previously reported as the major host of ARGs (e.g. *mexH*) in disinfected drinking water (Zhang et al., 2019). In addition, in the studied suburb DWDS, the hosts harboring both ARGs and MGEs mainly appeared in summer and autumn, including *Achromobacter, Stenotrophomonas, Curvibacter, Acinetobacter* and *Bosea*. Among them, *Stenotrophomonas* as opportunistic pathogen should deserve more attention, because it harbored many ARGs and MGEs and had high relative abundance in summer. This genus (*Stenotrophomonas*) was poorly addressed in previously DWDS studies and its regrowth needs to be controlled because the co-occurrence of ARGs and MGEs existed in this genus with opportunistic pathogenic characteristics. Because of the limitation of culture methods, these pathogens containing ARGs and MGEs have not been monitored routinely on account of drinking water safety. This taxonomic annotation of ACCs provides an effective tool to identify possible ARG hosts with MGEs (especially pathogens harboring ARGs and MGEs) as for drinking water safety. Furthermore, this technique offers a reference for controlling and managing ARGs and ARB in drinking water. In the future, efforts should be made to construct a set of appropriate risk assessment systems based on ARGs, ARB and horizontal transfer among pathogens in drinking water.

4. Conclusion

The present study explored the microbial communities and antibiotic resistomes of tap waters in a suburb DWDS using metagenomic sequencing. Both microbial community structure and antibiotic resistome displayed considerable seasonal and spatial changes. Temperature, ammonia, chlorite and TPC were the environmental factors driving the variations of microbial community, while TOC, chlorite, TPC and hardness affected antibiotic resistome. *Limnohabitans, Spingobium, Spingopyxis,* and *Brevundimonas* etc. were major bacterial genera in tap water. ARG-bacitracin, -multidrug and -sulfonamide were the major ARGs. The ARGs and ARC-MGEs (mainly plasmids) were more abundant at higher temperatures (in summer and autumn). Moreover, ARG hosts also varied with season and site. ARG hosts were more diverse in summer and autumn. *Limnohabitans* and *Mycobacterium* were the major ARG hosts in winter and spring. *Stenotrophomonas* and *Achromobacter* harboring both multiple ARGs and MGEs were abundant in summer at site 0.4 km from water treatment plant. The variation of ARGs was mainly attributed to microbial community as well as MGEs.

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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Appendix A Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2022.07.001.

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