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Antibiotic resistomes in drinking water sources across a large geographical scale: Multiple drivers and co-occurrence with opportunistic bacterial pathogens

Ziming Han [a, d], Yu Zhang [a, d, *], Wei An [b], Junying Lu [a], Jianying Hu [c], Min Yang [b, d, **]

[a] State Key Laboratory of Environmental Aquatic Chemistry, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, 100085, China
[b] Key Laboratory of Drinking Water Science and Technology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, 100085, China
[c] Laboratory for Earth Surface Processes, College of Urban and Environmental Sciences, Peking University, Beijing, 100871, China
[d] University of Chinese Academy of Sciences, Beijing, 100049, China

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ABSTRACT

Antibiotic resistance genes (ARGs) can survive the water treatment process. However, the prevalence patterns, key drivers, and relationships with opportunistic pathogens of the antibiotic resistome harbored in drinking water sources remain unclear. Herein, 53 drinking water samples collected across a large geographical scale in China were characterized based on ARGs, mobile genetic elements (MGEs), bacterial communities, antibiotics, and opportunistic bacterial pathogens. A total of 265 unique ARGs and MGEs were detected by high-throughput quantitative polymerase chain reaction (HT-qPCR), and 101 genes were shared among over 50% of samples. ARG abundance was higher in rivers than in reservoirs or groundwater, and ARG similarity showed a distance-decay relationship at the >4 000 km scale. Four out of the five detected opportunistic pathogens (i.e., *Escherichia coli*, *Mycobacterium* spp., *Clostridium perfringens*, and *Bacillus cereus* group) were potential hosts of ARGs. Based on multivariate statistics, our results demonstrated that the factors influencing the antibiotic resistome in drinking water sources were multiple and interactive. The bacterial community greatly contributed to ARG structure, and antibiotic concentrations and MGEs also affected ARG proliferation. The structural equation model indicated that geographical location and sample types (i.e., river, reservoir, and groundwater) had indirect effects on ARGs by changing the bacterial community and antibiotic concentration. Holistic consideration of natural and anthropogenic factors is recommended to understand antibiotic resistome variation in drinking water sources at a large geographical scale. Furthermore, large-scale diverse samples are suggested to minimize the potential influence of accident or stochasticity. Our findings provide insight into water quality risks induced by drinking water antibiotic resistomes.

1. Introduction

Antibiotic resistance genes (ARGs) are regarded as emerging environmental pollutants and are of major public health concern (Pruden et al., 2006; Sanderson et al., 2016), especially when found in pathogens infecting humans (Jiang et al., 2017). Thus, their dissemination pathways and host pathogens within environmental media have drawn much attention (Pruden et al., 2018). Drinking water is an important medium for the propagation of ARGs and pathogens between the environment and humans (O'Flaherty et al., 2018).

Drinking water sources can host diverse ARGs and pathogens due to their links with pollution from manure-contaminated soil (Udikovic-Kolic et al., 2014), antibiotic production wastewater (Li et al., 2010), and urban and hospital sewage (Szekeres et al.,

* Corresponding author. State Key Lab. of Environmental Aquatic Chemistry, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, 100085, China.
** Corresponding author. Key Laboratory of Drinking Water Science and Technology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, 100085, China.
E-mail addresses: zhangyu@rcees.ac.cn (Y. Zhang), yangmin@rcees.ac.cn (M. Yang).

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2017). Our recent large-scale investigation reported that drinking water sources are bacterial community seeds for tap water, and this biological contribution may not be eliminated by drinking water treatment processes (Han et al., 2020). In fact, ARGs have been detected in tap water at an abundance of 2.8×10^{-2} to 4.2×10^{-1} copies/cell in 25 cities worldwide (Ma et al., 2017), at 2.25×10^{-2} copies/cell after O_3/Cl_2 disinfection (Zhang et al., 2019a), and at 4.0×10^{-2} to 1.0×10^{0} copies/cell in small-sized microbes (Ma et al., 2019). At the same time, waterborne pathogens, especially ARG-carrying opportunistic bacterial pathogens in drinking water systems, are a key source of waterborne diseases (Paul McClung et al., 2018). As ARGs and waterborne pathogens in drinking water sources can migrate to tap water, systematic depiction of antibiotic resistomes and their host pathogens in drinking water sources is essential for water source protection and optimization of drinking water biosafety. However, studies on the co-occurrence of ARGs and pathogens in drinking water microbiomes remain limited, which has hindered risk assessment of opportunistic pathogens in drinking water sources.

The complexity of drinking water sources also hampers our understanding of antibiotic resistomes. Bacterial communities and mobile genetic elements (MGEs) are associated with the vertical and horizontal dissemination of ARGs, respectively (Forsberg et al., 2014; Gillings et al., 2015). Concretely, ARGs vertically transfer during the reproduction of bacterial hosts, and the close links between bacterial community shifts and ARG dynamics indicate the contribution of vertical pathway of ARG dissemination. ARGs also horizontally transfer between different bacterial cells via transformation, transduction, or conjugation, and diverse MGEs have been reported to accelerate the horizontal pathway. Antibiotics can exert direct selection on antibiotic resistant bacteria (ARB) (Chen et al., 2018; Zhu et al., 2017). These variables may contribute to the considerable ARG variation found in drinking water sources. In addition, drinking water sources are highly diverse among the different types (e.g., river, lake/reservoir, and groundwater) and at different geographical scales. Recent investigations on single rivers and reservoirs revealed seasonal variation and anthropogenic promotion of ARGs (Dang et al., 2020; Zhang et al., 2019b; Zheng et al., 2018). Furthermore, a large-scale survey of ARGs in lakes/reservoirs from central and eastern China identified environmental selection and dispersal limitations or barriers as the ecological mechanisms of the biogeographical patterns (Liu et al., 2018). However, most previous investigations on the determinants of ARGs have been spatially limited or have only focused on a single type, often neglecting the common occurrences, indicators, and influencing factors of antibiotic resistomes in drinking water sources at large geographical scales.

To clarify the multiple drivers and co-occurrence with opportunistic pathogens of the antibiotic resistomes in drinking water sources, we carried out a systematic survey to profile the relationships among ARGs and sample types (e.g., river, reservoir, and groundwater), geographical positions, antibiotics, bacterial communities, MGEs, and opportunistic bacterial pathogens. In total, 53 water samples were collected from the inlet of drinking water treatment plants across China, and the above-mentioned variables were profiled with multivariate statistical approaches. The objectives of this study were to 1) explore the co-occurrence of ARGs, MGEs, and specific opportunistic pathogens in drinking water sources; 2) investigate variations in ARGs and MGEs across sample types and geographical location; and 3) disentangle the interactions among the multiple environmental factors (e.g., antibiotics, bacterial communities, and MGEs) shaping ARG structure at a large geographical scale. By collecting samples spanning across 4 000 km with highly diverse antibiotic resistomes and local conditions, our results could decrease stochastic variabilities and reveal universal patterns of antibiotic resistomes in drinking water sources.

2. Materials and methods

2.1. Sample collection and antibiotic analysis

A total of 53 water samples (including 26 reservoir water, 24 river water, and three groundwater samples) from 31 cities spanning 4 000 km across China (Table S1) were collected at the inlet of the water supply systems. Approximately five liters of water were collected for each sample. All samples were stored in car refrigerator at 4 °C within 24–48 h until filtration at nearest labs. To quantify the antibiotic concentrations in water samples, approximately 500 mL water sample was concentrated using SPE cartridges. The samples were extracted using Oasis hydrophilic-lipophilic balance (HLB) (500 mg/6 ml), and then purified using Sep-Pak Silica cartridge (500 mg/3 ml). Four antibiotic categories, including sulfanilamides (SAs), macrolides (MLs), fluoroquinolones (FQs), and tetracyclines (TCs), were determined using ultra-performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS, Waters Acquity UPLCTM, Milford, MA, USA) on the basis of previously developed protocols (Jia et al. 2009, 2011; Xiao et al., 2008). The list of tested antibiotics can be found in the Supplementary Information.

2.2. DNA extraction and bacterial 16S rRNA gene sequencing

Water samples of approximately 500 mL were filtered through 0.22-μm mixed cellulose ester membrane filters (Millipore, Australia), which were then stored at −20 °C in 2-mL centrifuge tubes until use. DNA extraction was performed using a FastDNA SPIN Kit (MP Biomedicals, CA, USA) according to the manufacturer's instructions, and DNA concentration and purity were measured by microspectrophotometry (NanoDropND-2000, NanoDrop Technologies, Wilmington, DE, USA). Primers (515F: GTGCCAGCMGCCGCGGTAA and 806R: GGACTACHVGGGTWTCTAAT) (Caporaso et al., 2011) with barcode sequences at both ends were applied for amplification of the 16S rRNA gene V4 region. High-throughput sequencing was conducted on an Illumina MiSeq platform (Illumina, San Diego, USA) at the Institute for Environmental Genomics, Department of Microbiology and Plant Biology, University of Oklahoma, Norman, Oklahoma, USA. The sequencing data were deposited in the NCBI BioProject database with accession number PRJNA563354. Raw sequencing data were pre-processed and analyzed using an in-house Galaxy Pipeline (http://mem.rcees.ac.cn:8080/) integrated with FLASH (Magoc and Salzberg, 2011), Btrim (Kong, 2011), Uparse (Edgar, 2013), and taxonomy assignment. The sequences were clustered by Uparse into operational taxonomic units (OTUs) at a 97% similarity cut-off, with singleton OTUs removed. One representative sequence of each OTU was assigned to one taxonomic identity using the Ribosomal Database Project (RDP) 16S rRNA classifier (Wang et al., 2007).

2.3. High-throughput quantitative polymerase chain reaction (HT-qPCR) of ARGs and MGEs

HT-qPCR was conducted using a SmartChip Real-Time PCR system (Wafergen Inc., Fremont, CA, USA) to characterize the composition and abundance of ARGs and MGEs in the drinking water samples. HT-qPCR included 296 primer pairs (one for 16S rRNA gene, eight for transposase genes, one for universal class I integron-integrase gene (*intI1*), one for clinical class 1 integron-integrase gene (*cintI1*), and 285 for ARGs) (Table S2) (Su et al., 2015). The HT-qPCR protocols followed those of Su et al. (2015). In brief, the HT-qPCR conditions were: 95 °C (10 min), 95 °C

(0.5 min), and 60 °C (0.5 min) for 40 cycles. We adopted 31 as a threshold cycle (CT) to detect ARGs. Each sample was tested three times (three technical replicates). The data were only adopted when the results from the three technical replicates were all positive, amplification efficiency was between 1.8 and 2.2, and r^2 was over 0.99. The relative abundances of ARGs and MGEs were calculated and transformed to absolute abundance by normalizing to 16S rRNA gene copy numbers, which were quantified separately by the same primer using qPCR (Zhu et al., 2017).

2.4. qPCR of five opportunistic bacterial pathogens

Five opportunistic pathogens (i.e., *Escherichia coli*, *Mycobacterium* spp., *Clostridium perfringens*, *Bacillus cereus* group, and *Aeromonas hydrophila*) were quantified using SYBR-Green Real-Time qPCR according to previously published methods (Bin Kingombe et al., 1999; Kaushik et al., 2012; Mendum et al., 2000; Priha et al., 2004; Rinttila et al., 2004). These five opportunistic pathogens are important and representative in drinking water microbiome, and have been detected frequently in previous studies (Hull et al., 2017; Wang et al., 2017). Standard plasmids carrying target genes were obtained using TA clones and extracted using a TIAN Pure Mini Plasmid Kit (Tiangen, China). A detailed description is provided in the Supplementary Information, with the primers and references listed in Table S3.

2.5. Statistical analysis

Differences in nonparametric grouped data were analyzed using Kruskal-Wallis (KW) analysis of variance (ANOVA) tests. Considering greatly different samples numbers of river, reservoir and groundwater, KW test could provide more robust results than parametric methods when comparing the ARG abundance and antibiotic concentration among sample types. Pearson's and Spearman's correlations were performed using the vegan package (Dixon, 2003). Principal coordinate analysis (PCoA), permutational multivariate analysis of variance (PERMANOVA), analysis of similarities (ANOSIM), and multi-response permutation procedures (MRPP) were used to investigate differences in the Bray-Curtis similarity of composition of the bacterial community, ARGs, and MGEs between samples (Anderson, 2001). Distance-decay relationships between geographical distances and Bray-Curtis similarities of the bacterial community, ARGs, and MGEs were calculated based on linear regression using vegan and SoDA packages. Mantel test, Procrustes test, redundancy analysis (RDA), and variation partition analysis (VPA) were used to explore the relationships among ARG profiles based on ARGs and potential influencing factors with the vegan, permute, and lattice packages (Forsberg et al., 2014). Spatial factors were generated using principal coordinates of neighbor matrices (PCNM) based on the geodetic coordinates from latitude and longitude with the vegan and SoDA packages (Vincenty, 1975). Data were normalized using log-transformation when necessary prior to statistical analysis. The threshold for significance was $P < 0.05$. The above-mentioned methods were conducted using R 3.4.3.

Network analysis has been used extensively in ecological and biological studies to visualize the underlying associations among microbial taxa, functional genes, and proteins in complex microbial communities (Gómez et al., 2010; Ju and Zhang, 2014). Here, a correlation matrix was constructed by calculating all pairwise Spearman's correlations among ARG and MGE subtypes using the Hmisc package in R 3.4.3 (Harrell and Frank, 2008). The ARGs and MGEs that occurred in <50% of all samples were discarded to reduce the complexity of computation and avoid spurious correlation bias (Han et al., 2018). A correlation was considered statistically robust with Spearman correlation coefficient >0.7 and *P*-value < 0.01 (Li et al., 2015). Networks were visualized on the interactive platform of Gephi v0.9.2 (Bastian et al., 2009).

Structural equation models (SEMs) were adopted to evaluate the direct and indirect effects of space (i.e. geographical location), sample type, bacterial abundance, bacterial diversity, antibiotic concentration, and MGEs on ARG patterns. SEM is an *a priori* approach with the capacity to identify casual relationships between variables by fitting data to the models representing causal hypotheses (Eisenhauer et al., 2015). SEMs are tested based on robust maximum-likelihood evaluation with AMOS (Byrne, 2001). The models should meet multiple goodness-of-fit criteria: i.e., nonsignificant $\chi 2$ test ($P > 0.05$), root mean square error of approximation (RMSEA) < 0.08, goodness-of-fit index (GFI) > 0.90, and Akaike information criterion (AIC) from the default model lower than that from the saturated and independent models (Schermelleh-Engel et al., 2003).

3. Results

3.1. Overview of ARGs and MGEs in different types of drinking water sources

A total of 255 unique ARGs from the 285 target ARGs in the HT-qPCR assay were detected in at least one sample, and the detection frequencies ranged from 47 to 165 (Fig. S1c). Among sample types, ARG diversity exhibited significant differences, with the average number of detected genes in rivers (115.0 ± 25.1) being higher than that found in reservoirs (83.8 ± 22.9) or groundwater (68.0 ± 5.0) (KW test, $P < 0.01$, Fig. S2). ARG abundance was also highly diverse, ranging from 0.001 to 0.676 copies/16S rRNA gene (Fig. S1b). Multidrug resistance genes showed the highest average relative abundance (40.33%), followed by aminoglycoside (19.98%) and betalactamase (16.30%) (Fig. 1a). Dissimilarity tests revealed that the ARG structures were distinct among the river, reservoir, and groundwater samples (PERMANOVA, $F = 2.075$, $P = 0.005$) (Table S4). The absolute abundance of ARGs ranged from 1.93×10^3 to 3.95×10^6 copies/mL (Fig. S1a), and multidrug resistance genes showed the highest average absolute abundance (13.85%), followed by aminoglycoside (12.65%) and betalactamase (12.42%) (Fig. 1b). Average absolute abundance of ARGs in river samples (6.18×10^5 copies/mL) was higher than that in reservoir (4.84×10^5 copies/mL) and groundwater samples (8.05×10^4 copies/mL) (KW test, $P < 0.05$, Fig. S3).

A total of 10 targeted MGEs (two *intI* genes and eight transposase genes) were detected in at least one sample, and the detection frequency ranged from 3 to 10. Both MGE diversity and abundance showed similar trends in variation among the different sample types with ARGs (Fig. 1, S2, and S3, Table S4). In total, 101 genes were shared among >50% samples, which accounted for 98.21% of total abundance of the resistome. These frequently occurring genes in drinking water sources, which were share among >50% samples, were defined as the common resistome by previous study (Yan et al., 2019) as shown in Fig. 1c. A total of 28 genes were shared among >80% samples (Fig. S4), and *aadA*, *aadA2*, *qacEΔ1*, and *tnpA* were found in all samples (Fig. S4).

3.2. Characterization of bacterial community

Bacterial abundance, measured by the copy number of the 16S rRNA gene, ranged from 3.16×10^5 to 3.39×10^7 copies/mL in the drinking water samples. Average bacterial abundance followed the order: river (8.48×10^6 copies/mL) > reservoir (4.47×10^6 copies/mL) > groundwater (9.88×10^5 copies/mL) (KW test, $P < 0.05$, Fig. S3).

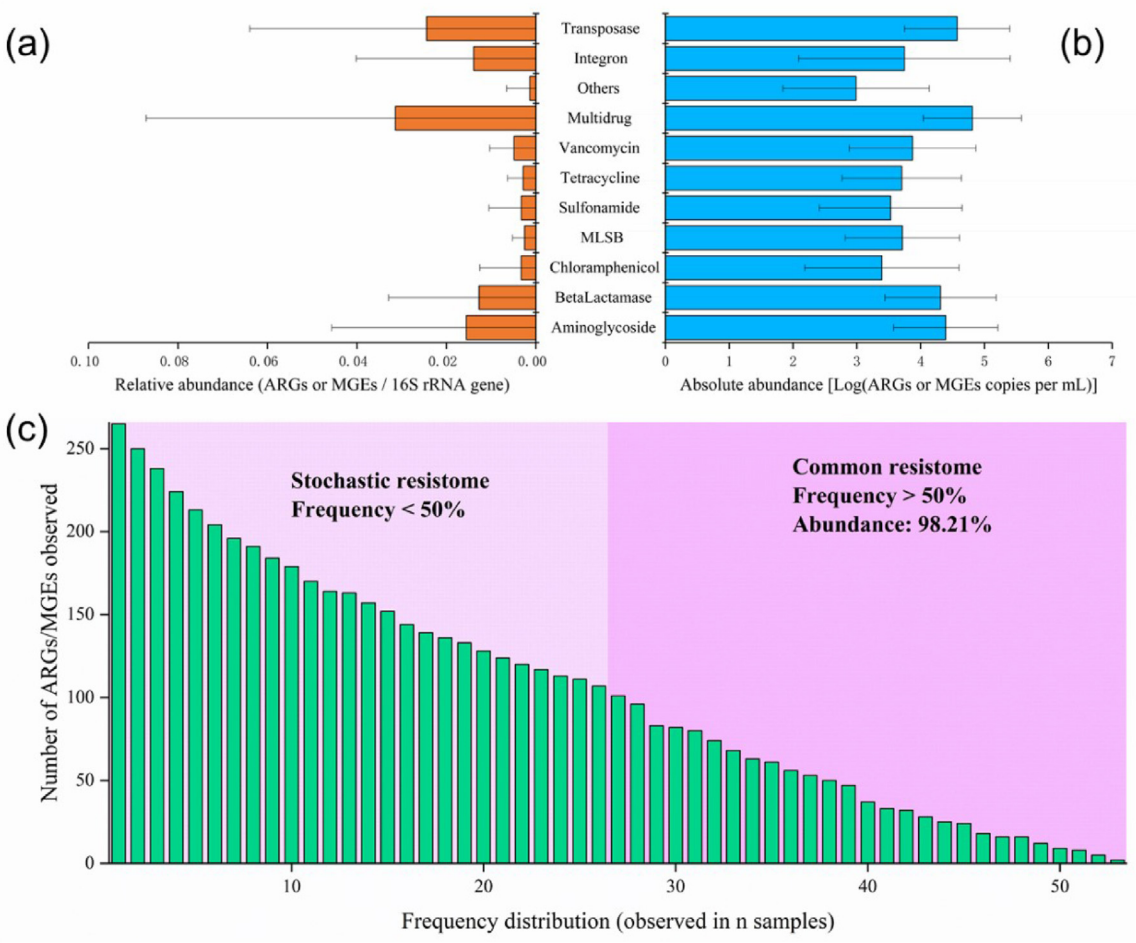

Fig. 1. Classification and abundance of the detected antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) in drinking water source samples. ARGs are classified based on the antibiotics to which they conferred resistance: aminoglycosides, betalactams, chloramphenicol, macrolide-lincosamide-streptogramin B (MLSB), sulfonamides, tetracycline, vancomycin, multidrug and others. MGEs are classified into integron and transposase.
(a) Relative abundances of ARGs and MGEs are copies of ARGs and MGEs divided by copies of 16S rRNA gene. (b) Absolute abundances of ARGs and MGEs, expressed as copies per mL water, are plotted on a log scale. (c) Frequency distribution of ARGs and MGEs in drinking water source samples.
Error bars represent standard deviation (s.d.) of all samples (n = 53).

The sequences obtained using the MiSeq platform were clustered into 10 886 OTUs based on a 3% cutoff dissimilarity level. *Proteobacteria* (60.60%) was the most dominant phylum, followed by *Actinobacteria* (13.95%), *Bacteroidetes* (5.43%), *Firmicutes* (4.82%), *Verrucomicrobia* (3.54%), *Planctomycetes* (3.27%), *Acidobacteria* (1.94%), *Cyanobacteria* (1.39%), and *Chloroflexi* (1.17%) (Fig. S6). Bacterial diversity (Shannon index) in river samples (5.44 ± 0.83) was higher than that in reservoir (4.05 ± 1.09) or groundwater samples (3.42 ± 0.51) (KW test, $P < 0.01$, Fig. S6a-c). Dissimilarity tests revealed that the bacterial community structure was distinct among the three sample types (PERMANOVA, $F = 2.968$, $P < 0.001$, Table S4).

3.3. Geographical distribution of bacterial communities, ARGs, and MGEs

The collected drinking water source samples spanned 4 000 km across China, thus providing an opportunity to explore the geographical distribution of bacterial communities and resistomes. Both ARGs and MGEs showed distance-decay relationships, but significance was weaker than that of bacterial communities (Fig. 3a–c). The bacterial community, ARG, and MGE diversities were also negatively correlated with latitude ($P < 0.05$, Fig. 3d–f), indicating the important effects of spatial factors.

3.4. Co-occurrence of opportunistic pathogens, ARGs, and MGEs

Escherichia coli, *Mycobacterium* spp., *Clostridium perfringens*, *Bacillus cereus* group, and *Aeromonas hydrophila* were quantified in the drinking water samples. Abundance of *Mycobacterium* spp. (0.0013 copies/16S rRNA gene on average) was higher than that of the other four detected opportunistic pathogens ($P < 0.05$, Fig. S6d). Significant correlations between pathogens and ARGs and MGEs were found, except for *Aeromonas hydrophila* (Fig. 2). In total, 19 ARGs and one MGE were positively correlated with *Clostridium perfringens*, indicating that this pathogen may carry multiple ARGs at the same time (Fig. 2).

The co-occurrence patterns of ARGs and MGEs were visualized using network analysis, and the modularity features were obtained. ARGs *blaVEB* (beta-lactamase resistance gene), *vanTG* (vancomycin resistance gene), and *ereA* (MLSB resistance gene) were identified as hub genes for three largest modules (modules I, II, and III, respectively) and consequently could be used as indicators for co-occurring ARGs in each module (Fig. S5, Table S5). ARGs with similar resistome phenotypes (tetracycline in module I, vancomycin in module II, and multidrug in module III) were also clustered together (Fig. S5).

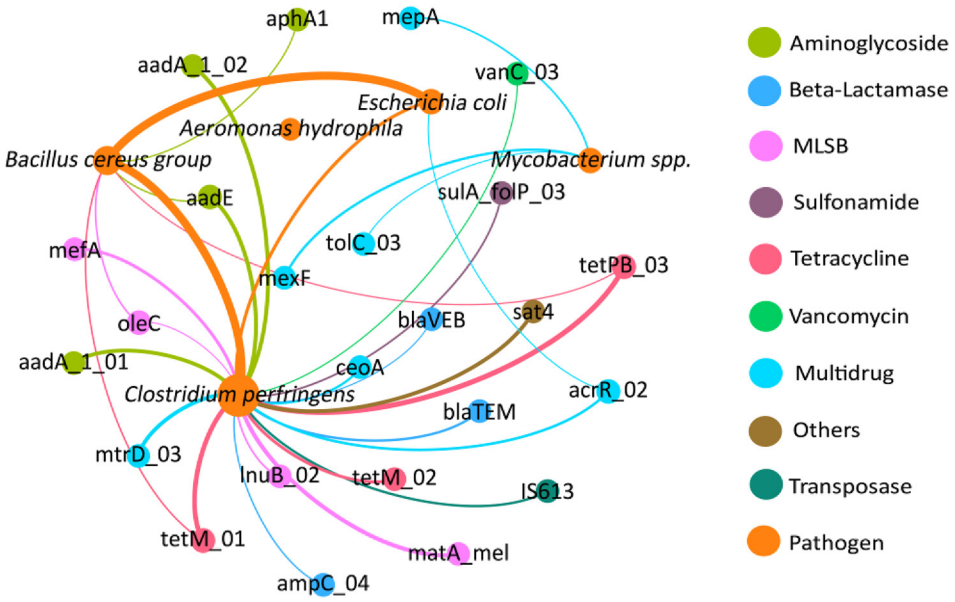

Fig. 2. The co-occurrence patterns between detected opportunistic pathogens and ARGs, MGEs. The nodes are colored according to pathogens or ARGs, MGEs types. An edge represents a significant correlation between two nodes ($P < 0.05$). The edges are weighted according to the correlation coefficient and node size is weighted according to the number of connections (i.e., degree).

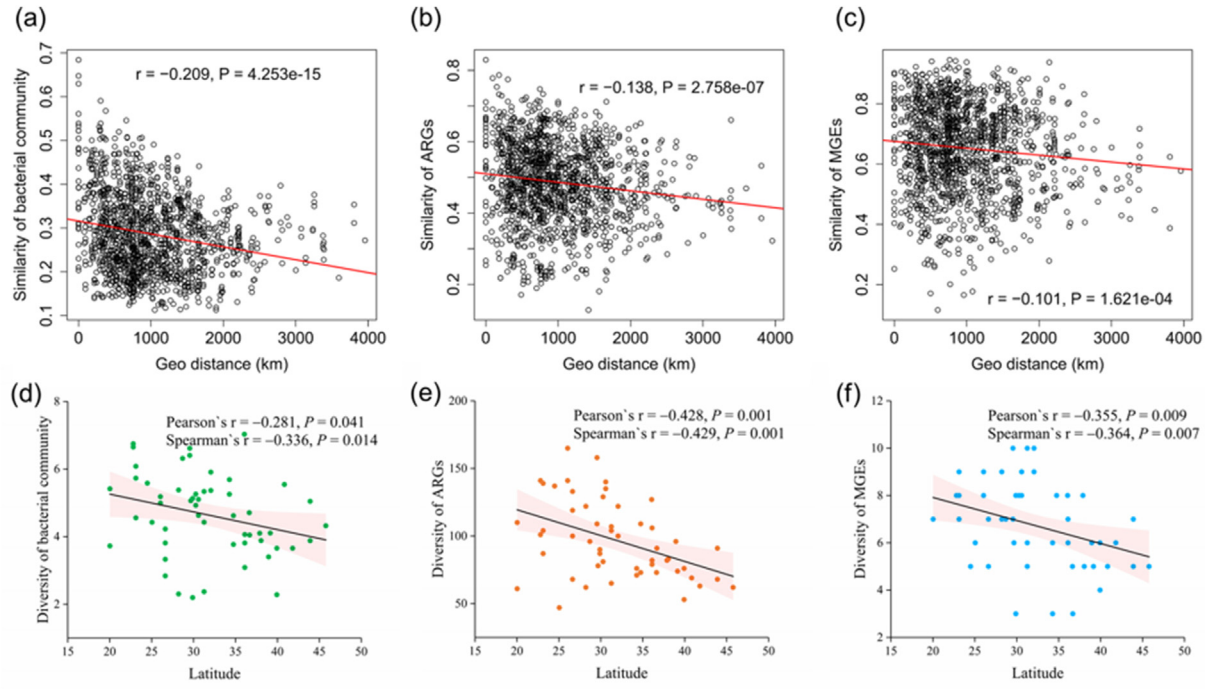

Fig. 3. Geographical distribution of bacterial community, ARGs, and MGEs in drinking water source samples at a large geographical scale.
(a-c) Distance-decay analysis of similarity of bacterial community (a), ARGs (b), and MGEs (c) among samples based on Bray-Curtis distance. The line through the graph indicates the linear regression model.
(d-f) Correlation between latitude and diversity of bacterial community (i.e., Shannon index) (d), ARGs (i.e., number of ARGs detected) (e), and MGEs (i.e., number of MGEs detected) (f). The line through the graph indicates the linear regression model, and the shaded area represents its 95% confidence limits.

3.5. Relationships among ARGs, MGEs, bacterial communities, and antibiotics

The absolute abundance, relative abundance normalized by 16S rRNA gene copy number, and diversity of ARGs were significantly correlated with those of MGEs ($P < 0.001$, Fig. 4a and S7). Positive correlations between ARG and MGE types were also widespread (Fig. 4b), indicating the important role of horizontal transfer during ARG dissemination.

The Procrustes tests depicted significant correlations between ARG structure and bacterial community structure based on the Bray-Curtis dissimilarity matrix (sum of squares $M^2 = 0.818$,

r = 0.426, $P < 0.001$, 9 999 permutations, Fig. 4c), as confirmed by the Mantel test (Spearman's r = 0.230, $P = 0.002$). A network consisting of 38 nodes (27 bacterial genera and 11 ARGs/MGEs) and 65 edges was visualized to explore the interactions among ARGs, MGEs, and bacterial taxa (Fig. 4d, Table S6). A total of 11, 3, 3, 3, and 2 genera affiliated with *Proteobacteria*, *Actinobacteria*, *Chloroflexi*, *Firmicutes*, and *Bacteroidetes*, respectively, were positively related to specific ARG/MGE subtypes (Spearman's r > 0.7, $P < 0.001$), suggesting these bacterial taxa were potential hosts of the corresponding ARGs or MGEs. In addition, absolute abundance of bacteria was significantly correlated with absolute abundance of ARGs and MGEs (both total and type) (Fig. S8), hinting that antibiotic resistome abundance may be in scale with bacterial cell number.

Antibiotics in drinking water sources were diverse (Fig. S9a), with total concentrations of SAs, MLs, FQs, and TCs ranging from 0.10 to 1 454.60 ng/L, and total concentrations differing among the three sample types (KW test, $P < 0.01$, Fig. S9b). The average concentrations of the four antibiotic categories were SAs (115.76 ng/L) > MLs (86.92 ng/L) > FQs (9.45 ng/L) > TCs (0.51 ng/L) (Fig. 4e). Correlation analysis indicated that the concentrations of SAs, MLs, and Σantibiotics (i.e., the sum of detected antibiotics) were correlated with abundance and diversity of ARGs, MGEs, and some specific ARGs and MGEs types ($P < 0.05$), but the correlations between FQs and TCs with ARGs and MGEs were weak (Fig. 4f), suggesting that the selective pressure of antibiotics on organisms carrying ARGs in water was dose dependent.

3.6. Multiple factors influenced ARG profiles interactively

Redundancy analysis (RDA) was performed to better understand the effects of environmental factors on ARG structure (Fig. 5a). In addition, RDA-based variation partitioning analysis (VPA) further differentiated the contributions of space, MGEs, bacterial communities, and antibiotics on ARG variation (Fig. 5b). The bacterial community (14.60%) showed greater contribution to ARGs, followed by antibiotics (11.60%), MGEs (9.26%), and space (5.20%); the joint effects of multiple factors explained 5.85% of ARG variation, leaving 53.49% of ARG variation unexplained.

The SEMs quantified the indirect and direct effects of space, sample type, bacterial abundance, bacterial diversity, antibiotics, and MGEs on ARG profiles from a holistic view. All variables explained 62% of ARG structure variance (Figs. 5c) and 69% of ARG abundance variation (Fig. S10). Importantly, although space and sample type did not affect ARG profiles directly when accounting for multiple factors, they exerted an indirect influence based on changing bacterial communities, antibiotics, and MGEs. Space influenced bacterial abundance ($\lambda = -0.32$, $P < 0.01$), bacterial diversity ($\lambda = -0.55$, $P < 0.001$), and MGEs ($\lambda = 0.52$, $P < 0.001$) in the SEMs, in line with the geographical distribution patterns of the bacterial communities and MGEs (Fig. 3). Sample type showed a significant influence on bacterial abundance ($\lambda = 0.42$, $P < 0.01$), bacterial diversity ($\lambda = 0.29$, $P < 0.01$), and antibiotic concentration ($\lambda = 0.36$, $P < 0.01$), as confirmed by comparison tests (Figs. S2, S3, and S9b). Both the VPA and SEM results demonstrated that potential causal relationships may occur among ARGs and their multiple influencing factors, and these factors shape ARG profiles interactively.

4. Discussion

Drinking water treatment processes cannot eliminate ARGs from drinking water sources absolutely, and thus, drinking water supply systems may be a primary dissemination route of ARGs from the environment to host, causing potential risks to human health. Understanding the antibiotic resistomes in drinking water sources could provide important information to improve drinking water management strategies. Given the great variation and multiple drivers of ARGs in drinking water sources, systematic data mining of ARGs and related variables is urgent. To the best of our knowledge, this study presents the most comprehensive investigation on antibiotic resistomes in different drinking water sources with consideration of biotic factors (vertical and horizontal transfer potential), abiotic factors (antibiotic pollution, spatial distribution), sample types (river, reservoir and groundwater), and relationships with opportunistic pathogens. Due to the 4 000-km scale variations among samples, we collected diverse bacterial community and ARG data to be random and not biased.

4.1. Occurrence and biogeography of antibiotic resistomes in drinking water sources

Antibiotic resistomes were widely found in the tested samples (Fig. 1 and S1). However, the abundance of ARGs detected in the current study was lower than that reported in manure (Han et al., 2018) and estuarine sediment (Zhu et al., 2017) using the same HT-qPCR platform. It is not surprising that ARGs were prevalent in drinking water sources as intrinsic antibiotic resistomes also occur in natural ecosystems such as forest biomes with low human disturbance (Hu et al., 2018), caves isolated from the surface for over four million years (Pawlowski et al., 2016), and 30 000-year-old Beringian permafrost sediment (D'Costa et al., 2011). Although it is accepted that ARGs are ancient and widespread, each ecosystem contains different bacterial community, and thus contains different dominate ARGs. On the other hand, drinking water sources are disturbed by human activities, the influencing factors of antibiotic resistome might be more complex and multiple.

By large-scale sampling, we identified a more universal pattern of ARGs in drinking water sources. Similar to previous research (Chen et al., 2018; Liu et al., 2018), our study revealed that multidrug, aminoglycoside, and betalactamase resistance genes were dominant in drinking water sources, with multidrug resistance genes also dominant in treated drinking water (Ma et al., 2019). Despite the great diversity in ARG composition, a common resistome was identified in the drinking water samples (Fig. 1c, S4). We found 101 and 28 ARGs were shared among >50% and >80% of all samples, respectively (Fig. 1c, S4), indicating dominant and stable ARGs shared by drinking water sources spanning the 31 cities (over 4 000 km) across China. Notably, aminoglycoside resistance gene *aadA*, multidrug resistance gene *qacEΔ1*, and transposase gene *tnpA* were found in all samples (Fig. S4). The *aadA* and *qacEΔ* genes are known components of integron gene cassettes (Gillings, 2014; Partridge et al., 2009), thus hinting the potential of horizontal gene transfer during ARG dissemination in natural waters. Transposon is a kind of common mobile element in bacteria (Aziz et al., 2010). Transposon could carry diverse ARGs including *tet*(A), *tet*(B), *tet*(E) and *tet*(31) (Shi et al., 2020), and mediate efficient dissemination of antibiotic resistance among bacterial communities (Partridge, 2011). The widespread *tnpA* genes in drinking water sources could accelerate the dissemination of ARGs through horizontal gene transfer. In addition, considerable ARG diversity and abundance was found in the drinking water sources, suggesting that different risk levels of antibiotic resistomes in drinking water sources require different treatment strategies to improve drinking water biosafety.

Biogeographical distribution is a common phenomenon in natural ecosystems (Martiny et al., 2006) and engineered systems (Wu et al., 2019). The distance-decay relationship, i.e., decrease in similarity with increasing geographical distance, can be used to confirm biogeographical patterns (Hanson et al., 2012). Dispersal limitation of microbe is the reason of distance-decay relationship,

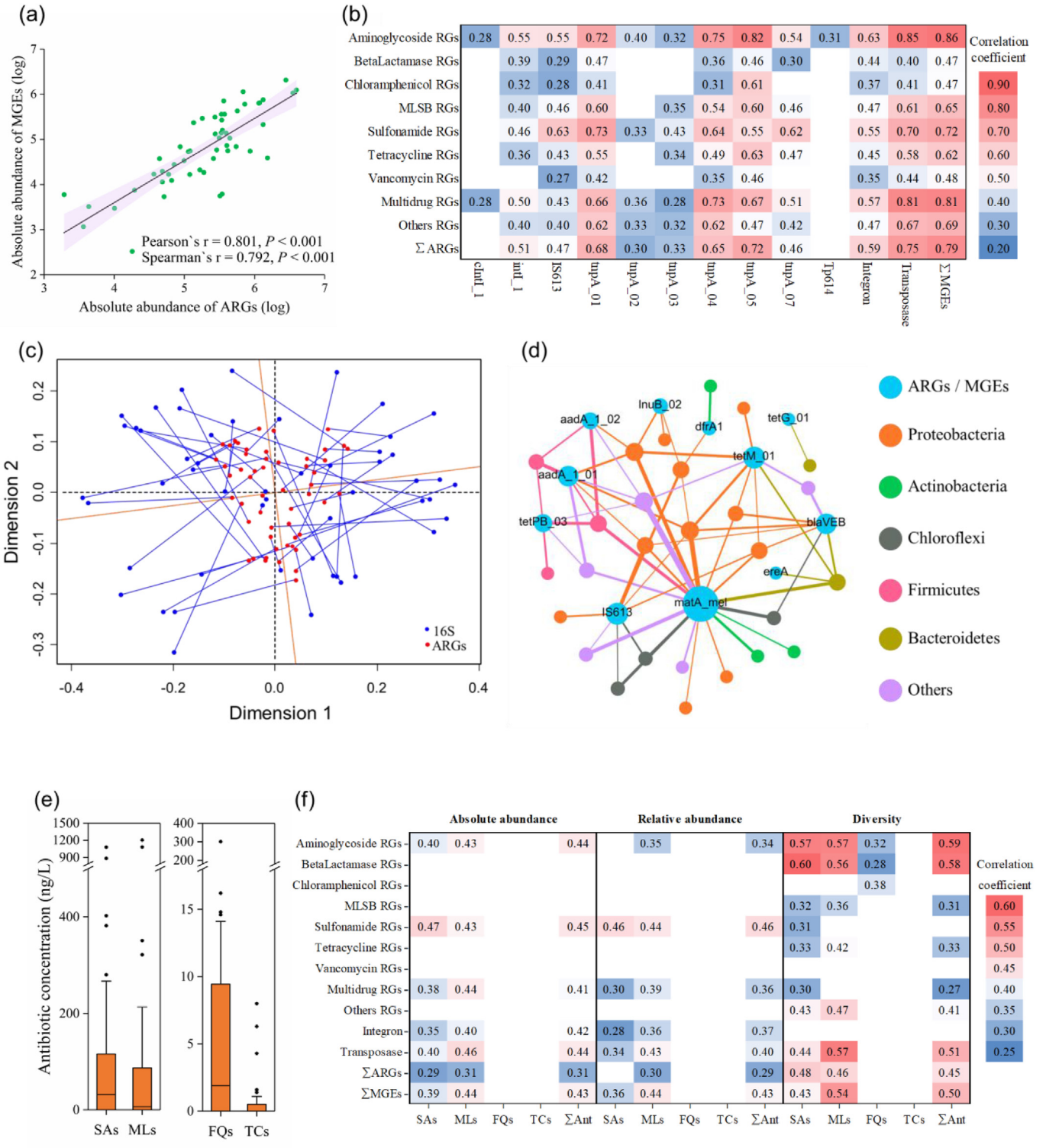

Fig. 4. Relationships between ARGs and MGEs, bacterial community, antibiotic concentrations in drinking water sources.
(a-b) Relationship between ARGs and MGEs. (a) Linear regression model reveals the correlation of total abundance of ARGs and total abundance of MGEs, and the shaded area represents its 95% confidence limits. (b) Heatmap shows the spearman correlation of abundance of ARG types and abundance of MGE types, and only significant results ($P < 0.05$) are shown.
(c-d) Relationship between ARGs, MGEs and bacterial community. (c) Procrustes test depicting the significant correlation between ARG structure and bacterial community structure based on Bray-Curtis dissimilarity matrix (sum of squares $M^2 = 0.818$, $r = 0.426$, $P < 0.001$, 9999 permutations). (d) The network depicting the close relationship between the bacterial genera and detected ARGs, MGEs. The nodes are colored by different phylum of bacteria. An edge indicates strong (Spearman's $r > 0.7$) and significant ($P < 0.001$) correlation between unique ARG and bacterial genera. The edges are weighted according to the correlation coefficient and node size is weighted according to the number of connections (i.e., degree).
(e-f) Relationship between ARGs, MGEs and antibiotic concentrations. (e) Box diagram shows antibiotic concentrations. (f) Heatmap shows the spearman correlation of abundance and diversity of ARGs, MGEs and antibiotic concentrations, and only significant results ($P < 0.05$) are shown. SAs, Sulfanilamides; MLs, macrolides; FQs, fluoroquinolones; TCs, tetracyclines; ∑ARGs, sum of detected ARGs; ∑MGEs, sum of detected MGEs; ∑Ant, sum of detected antibiotics.

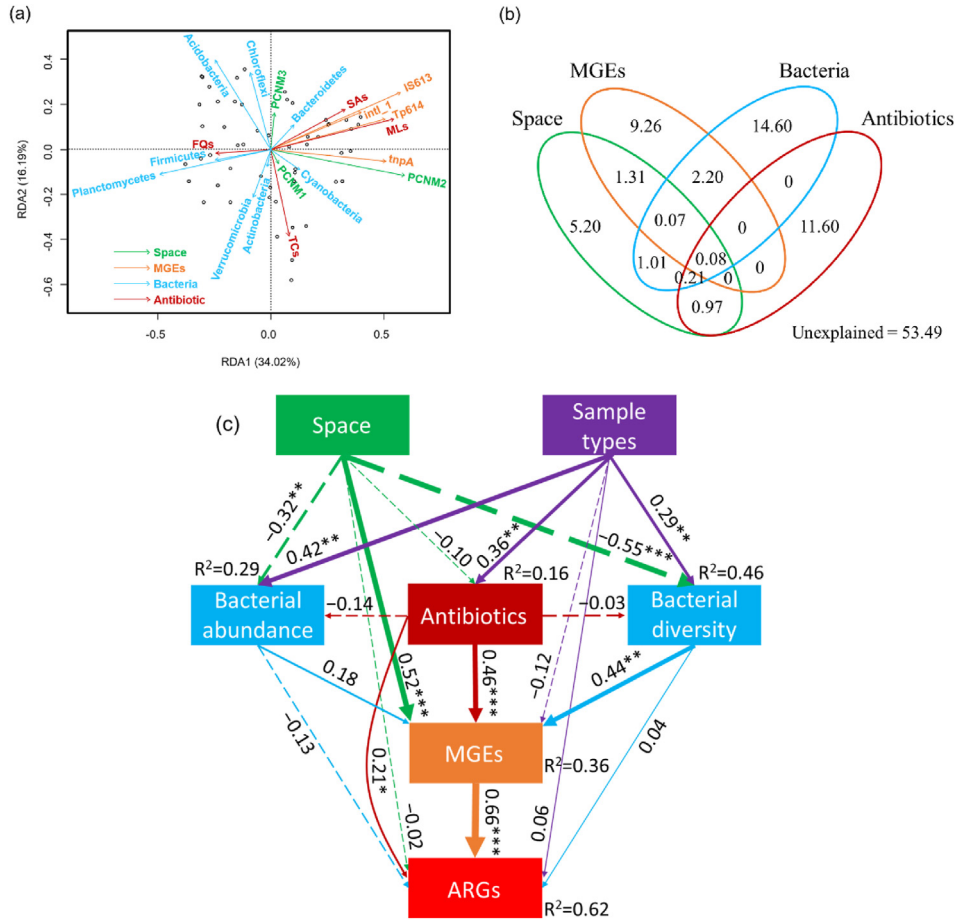

Fig. 5. Contribution of interactive drivers (space, sample types, bacteria, antibiotic and MGEs) on ARG structure.
(a) Redundancy analysis (RDA) between space (PCNM 1 to PCNM3), MGEs (detected integrons and transposons), bacterial community (phylum which relative abundance > 1%), and antibiotic (SAs, MLs, FQs and TCs) on ARG structure. Effective variables are chosen according to the variance inflation factor (VIF).
(b) Variation partitioning analysis (VPA) differentiating the effects (%) of space, MGEs, bacterial community, and antibiotic on ARG structure.
(c) Structural equation model (SEM) quantifying the indirect and direct effects of space, sample types, bacterial abundance, bacterial diversity, antibiotic and MGEs on ARG structure ($\chi^2 = 0.755$, $P = 0.385$, CMIN/DF = 0.755, GFI = 0.996, RMSEA < 0.001). The width of the arrows indicate the strength of the standardized path coefficient (λ). The solid lines indicate positive path coefficients while dashed lines indicate negative path coefficients. R^2 values represent the proportion of the variance explained for each variable. *, **, and *** indicate *P*-value < 0.05, <0.01, and <0.001, respectively.

and has been considered as a basic rule of bacterial community assembly (Martiny et al., 2006). The spatial distribution of ARGs has been studied in sediments along the Yangtze Estuary, China (Guo et al., 2018), and in lakes in eastern China (Liu et al., 2018). Our study further verified this in drinking water sources at the national scale. There might be several reasons behind this phenomenon. Firstly, the hosts of ARGs, i.e., the bacterial community, showed clear geographical patterns (Fig. 3a and d). Secondly, antibiotic concentrations also showed geographical patterns (Spearman's correlation between antibiotic concentration and latitude, $r = -0.331$, $P = 0.015$), which might be correlated with human activity density in different areas in China (Zhang et al., 2015). Thirdly, rapid spatial turnover of the integron gene was found, which could be a contributor of ARG biogeographical distribution (Ghaly et al., 2019). In addition, the significance of distance-decay relationship of ARGs and MGEs was weaker than that of bacterial community, indicating the contributions of geographical location to the ARG variation, MGE variation were weaker than bacterial community variation. ARGs and MGEs might be shaped by more complex environmental variables than bacterial community.

4.2. Potential pathogenic hosts of ARGs

Opportunistic pathogens in bulk water (Wang et al., 2012), biofilms (Gomez-Smith et al., 2015), cooling towers (Pereira et al., 2017), and water storage tanks (Li et al., 2018) have been profiled, and qPCR protocols targeting them are well established (Wang et al., 2017). Drinking water sources are important origins of opportunistic pathogens in tap water. Opportunistic pathogens in drinking water sources, such as *Mycobacterium*, do not disappear after water treatment, and can even increase after disinfection (Dias et al., 2020; Hull et al., 2017). ARG-carrying pathogens are among the most important risk factors to drinking water safety (Wang et al., 2017), and thus, the occurrence and co-occurrence of ARGs with opportunistic pathogens were explored in the present study.

We observed the prevalence of five opportunistic pathogens in drinking water sources. Based on network analysis, the abundances of *Escherichia coli*, *Mycobacterium* spp., *Clostridium perfringens*, and *Bacillus cereus* group were significantly correlated with ARGs, with 19 ARGs and one MGE found to be positively correlated with *Clostridium perfringens*. Previous research has reported resistance rates

of *Clostridium perfringens* isolated from diarrheal neonatal piglets for erythromycin and lincomycin of over 50%, with 82% of resistant isolates resistant to at least two antibiotics (Ngamwongsatit et al., 2016). *Mycobacterium gordonae* has also been detected as an ARG-carrying pathogen in drinking water (Ma et al., 2019). Our results suggested that the four opportunistic pathogens might act as hosts of ARGs (Fig. 2), highlighting the importance of considering multi-drug resistance in opportunistic bacterial pathogens in drinking water sources. It should be noted that our results on the co-occurrence of ARGs and opportunistic pathogens relied on correlation methods, and additional molecular detection of ARG-host pathogens needs to be done in the future. Although it has been reported that *Aeromonas hydrophila* acquired resistance to oxytetracycline (Hatha et al., 2005) and carried tetracycline efflux protein gene *tet*(E) (Shi et al., 2020), it was not found as potential host of ARGs in the present study. The limitation of correlation analysis, the limited amount of tested ARGs, and low selective pressure of tetracycline might explain this phenomenon, and future studies are needed.

4.3. Integrated effects of multiple factors on antibiotic resistomes in drinking water sources

From a holistic perspective, this study revealed the integrated influences of diverse variables on the antibiotic resistomes of drinking water sources, which were multiple and interactive. These results reflected actual environmental processes as much as possible and suggested the need for compositive monitoring systems for drinking water sources.

The close relationships among bacterial communities, ARGs, and MGEs were revealed by VPA and SEM analyses, as reported in previous study (Forsberg et al., 2014). From the SEM results, the concentration of antibiotics did not exert a significant influence on bacterial abundance and diversity ($\lambda = -0.14$, $P > 0.05$ and $\lambda = -0.03$, $P > 0.05$, respectively); however, it still showed potential selective pressure on ARGs ($\lambda = 0.21$, $P < 0.05$) and MGEs ($\lambda = 0.46$, $P < 0.001$) (Fig. 5c). Similar trends have also been reported in river-reservoir systems with anthropogenic activities (Chen et al., 2018), in the Pearl River in southern China (Chen et al., 2015), and in sediment samples along the Chinese coastline (Zhu et al., 2017). Here, antibiotic concentrations followed the trend SAs (115.76 ng/L) > MLs (86.92 ng/L) > FQs (9.45 ng/L) > TCs (0.51 ng/L) (Fig. 4e), and only two antibiotic groups with higher concentrations (i.e., SAs and MLs) showed close correlation with ARGs (Fig. 4e and f). Waterbodies act as the sink of pollution from human activities, and the relative high concentration of SAs (115.76 ng/L) in drinking water sources might be explained by the wide use of SAs in clinical and animal environment (Zhang et al., 2015). Compared to antibiotic production wastewater (Bengtsson-Palme et al., 2019), the concentrations of each antibiotic in the current study were very low (Fig. S9), however, the correlation between ARGs and antibiotics was still significant and dose-dependent. The possible reasons were, firstly, the complex ARG co-selection features of diverse antibiotics, which could be also called as the joint selective pressure of diverse antibiotics, may exist (Kovacevic et al., 2013); secondly, antibiotic concentrations may reflect the human activity density and anthropogenic factors, which could promote the widespread dissemination of ARGs (Zhang et al., 2015; Zhu et al., 2017).

The VPA results implied that the joint effects of multiple factors contributed to ARG variation, with the SEM results disentangling the causal relationships among the multiple variables to a certain extent (Fig. 5). Space and sample type of drinking water sources showed indirect effects through bacterial communities, antibiotics, and MGEs on ARGs. Concretely, the differences in bacteria and antibiotics among sample type were the main reason for their influence on antibiotic resistome, and the shift in bacterial abundance, diversity, and MGEs along the geographical gradient was the main reason for the spatial variable structuring of the antibiotic resistome (Fig. 5c). In brief, overall variation of the antibiotic resistome was the result of interactions among natural and anthropogenic factors.

4.4. Implications for drinking water source management on controlling ARGs

Selection and protection of drinking water sources is a key issue in drinking water safety. Our results showed that the bacterial community was a major driver of the antibiotic resistome, ARG abundance was associated with abundance of several opportunistic pathogens, and antibiotics were linked to antibiotic resistome trends (Fig. 5b). Therefore, protecting drinking water sources from antibiotic and biological contamination by strict policies may be practical actions toward future drinking water source management. Livestock and poultry breeding wastewater (Awad et al., 2014), clinical wastewater (Hocquet et al., 2016), and antibiotic production wastewater (Li et al., 2010) are priority targets. Although limited groundwater samples were studied here, our research showed that drinking water from groundwater sources contained lower antibiotic concentrations, bacterial biomass, and ARG abundance and diversity, suggesting that groundwater may be a safer and healthier drinking water source. In the future, more groundwater samples need to be collected at large-scale to obtain more understanding on microbiome and ARGs in groundwater.

Opportunistic pathogens, especially ARG-host pathogens, may be more important monitoring objectives than ARGs themselves. We found that ARGs co-occurred with four opportunistic pathogens, and qPCR and high-throughput sequencing protocols could be used for daily monitoring and risk assessment of drinking water sources in the future. Because ARGs can survive treatment, such monitoring and assessment are also essential for tap water. In addition, as natural conditions also affected bacterial biomass and ARG abundance, the background values of the antibiotic resistomes may be a function of the climatic region and waterbody type.

Assessment and optimization of techniques for antibiotic and ARG removal are essential. Disinfection can degrade and deactivate ARGs, especially extracellular ARGs (He et al., 2019), and integrated pre-coagulation and microfiltration can effectively reduce ARGs (Li et al., 2019). Because of the great diversity in antibiotic contamination in drinking water sources, stringent purification strategies in drinking water treatment plants should be considered. Monitoring of drinking water sources could help us assess the potential health risks of ARGs and pathogens, and drinking water sources with high risk of biological pollution need stricter disinfection or membrane treatment in addition to conventional water treatment processes.

5. Conclusions

This study performed a comprehensive survey of the antibiotic resistomes in drinking water sources at a large geographical scale. Drinking water sources were found to be reservoirs of ARGs, which may be hosted by some opportunistic bacterial pathogens. Based on large-scale sampling, our results indicated that the determinants of ARG patterns were multiple and interactive. Bacterial communities, antibiotic concentrations, and MGEs were directly associated with ARGs, and these direct factors were restricted by geographical location and sample type. These findings extend our knowledge regarding the occurrence and distribution of ARGs in drinking water sources and have implications for the optimization of water source protection and drinking water treatment to mitigate the dissemination of environmental ARGs to hosts.

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 data related to this article can be found at https://doi.org/10.1016/j.watres.2020.116088.

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