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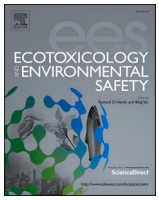

Antibiotic resistome in groundwater and its association with mountain springs and river

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ABSTRACT

The distribution of antibiotic resistance genes (ARGs) in water sources potentially threatens drinking water safety. However, the sources of antibiotic resistome in groundwater are still under-investigated. Here, we evaluated the profiles of antibiotic resistome in peri-urban groundwater and its associated water sources (river and mountain spring) to characterize the antibiotic resistome from natural water sources on groundwater resistome. A total of 261 antibiotic resistome were detected in groundwater, mountain spring, and river samples. The relative abundances of ARGs and mobile genetic elements (MGEs) were significantly higher in the river samples than in spring water and groundwater samples. The resistome profiles were similar between groundwater and spring water but differed from the river samples. According to source tracking results, the groundwater resistome was likely to be derived from springs (28.0%−50.0%) and rivers (28.6%−48.6%), which share the same trend for the source tracking of bacterial communities. Bacterial α-diversity, bacterial β-diversity, and MGEs directly or indirectly affected the ARGs in groundwater samples. Although the abundance of groundwater resistome was not elevated by river and spring water, groundwater resistomes were diverse and may be derived from both river and spring water. We highlight the importance of groundwater resistome and its association with potential water sources, providing a better understanding and basis for the effective control of the ARG proliferation and dissemination in groundwater from exogenous water bodies in the future.

1. Introduction

The proliferation and dissemination of antibiotic resistance threatens public health (Berendonk et al., 2015). Anthropogenic discharge accelerates the accumulation of partially degraded antibiotics (Kummerer, 2009), triggering the distribution of antibiotic resistome in the aquatic environment. Aquatic systems are considered an essential reservoir of ARGs, posing a high risk to human health (Zhou et al., 2022). Watersheds and aquifers in peri-urban areas are susceptible to intensive industrial and agricultural activity (Szekeres et al., 2018). For example, rivers receive ARGs and ARB from urban runoff, agricultural fertilization, and effluent of wastewater treatment plants (WWTPs); the groundwater resistome is frequently associated with adjacent landfills and farms (Hong et al., 2013; Huang et al., 2022). In addition, population and conurbation size has been reported to play a role in shaping ARGs profiles in watersheds (Peng et al., 2020; Zhou et al., 2022).

Groundwater accounts for 97% of the global freshwater and is an essential source of drinking water globally (Jurado et al., 2019; Szekeres et al., 2018). The physicochemical properties of groundwater are usually related to soil composition and permeability, adjacent aquifers, and agricultural activities (Ravindra et al., 2019). However, the anthropogenic input of contaminants with potentially bioactive properties (e.g., personal care products and antibiotics) negatively influences groundwater quality and therefore threatens groundwater safety (Lapworth

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et al., 2012). The sorption capacity of soils and sediments is determined by physicochemical properties of antibiotics, hydraulic properties, and environmental conditions (Lal and Shukla, 2004; Luneberg et al., 2018) and hence is a key factor affecting the input of antibiotic resistome and antibiotics to groundwater. In addition, the limited nutrient and anaerobic environment in aquifers help develop a distinct microbial community in groundwater, which slows down the biodegradation of antibiotics in groundwater (Ma et al., 2022; Wang et al., 2017). The prolonged persistence of antibiotics respectively selects and proliferates ARB and ARGs in groundwater (Zainab et al., 2020). Furthermore, horizontal gene transfer promotes the transport of genetic material within the microbial community, transforming bacteria from antibiotic-sensitive to antibiotic-resistant (Huang et al., 2021). Groundwater recharge has diverse sources in rural and urban areas, including precipitation, exchange of inter-aquifer flows, surface water, urban runoff, wastewater, sewer leakage, and agricultural irrigation (Barrett et al., 1999; Hendrickx, 1992; Yang et al., 2022). The status of the water sources may impact antibiotic resistome and microbial communities in associated groundwater. For example, rainfall may lead to an increased abundance of groundwater ARGs (Ji et al., 2022); and high levels of agricultural effluent infiltration cause an elevated level of resistome and pathogenic bacteria (Barrios et al., 2020; Sasakova et al., 2018). The hyporheic zone, an active area in which shallow groundwater and surface water mix, sustains the ecological balance of groundwater (Boulton et al., 1998). It has a higher chemical activity than surface water or deep groundwater and a lower seepage velocity, which on the one hand, supports the establishment of microbial communities and, on the other hand, increases the risk of external contamination (Hester et al., 2013; Ling et al., 2022). Thus, it is essential to understand the behavior of antibiotic resistome in this zone and track the potential sources of pollutants.

Spring water is a natural mechanism for groundwater discharge and is one of the major sources of drinking water (Toth et al., 2022). Although ARGs are ancient and long-standing, the occurrence of ARGs in spring water has been exacerbated by the impact of agricultural activity, leading to the ARG prevalence in springs (Stange and Tiehm, 2020). In general, the spring antibiotic resistome level is usually significantly lower than that in urban groundwater and rivers due to the reduced intensity of anthropogenic activities (Wu et al., 2020). On the one hand, upstream groundwater may facilitate groundwater flow and dilute various contaminants as it flows through urban areas (Laws et al., 2011; Wang et al., 2015). On the other hand, urban groundwater may be recharged by surface water such as rivers, bringing in ARGs and ARB and aggravating groundwater pollution (Ji et al., 2022; Junaid et al., 2022). However, the potential dissemination process and intrinsic linkage of antibiotic resistome between suburban rivers, groundwater (hyporheic zone), and springs have not been well studied.

In this study, we analyzed the antibiotic resistome in rivers, groundwater, and mountain springs in a suburban watershed. We tracked the origin of ARG and bacterial communities in groundwater using high-throughput qPCR (HT-qPCR) and source tracking methods. The aims of the present study include (1) characterizing the ARGs profiles in three different water sources and evaluating the anthropogenic impact on the aquatic environmental resistome; (2) analyzing the contribution of the river and springs antibiotic resistome and bacterial communities on groundwater; and (3) decipher the driving mechanism of aquatic ARGs and assess the relationship between the antibiotic resistome and potential bacterial hosts.

2. Material and methods

2.1. Sample collection and DNA extraction

The study area is located in a peri-urban watershed in Quanzhou City (25°24'34.7"N 118°01'19.6"E), Fujian Province, in Southeast China. Water samples were collected in the three (upstream, midstream, and downstream) mountain spring-groundwater-river continuums along the Kengzikou Stream, a tributary of the Jinjiang River (Fig. 1). Sterile plastic bottles were loaded with in situ water samples. To minimize potential sample variability, four replicates of each water sample were collected at each sampling site (36 water samples in total), on March 2022. Water samples were transported to the laboratory and on arrival filtered using a 0.22 μm mixed cellulose ester membrane (Bandao Co, Ltd, Shanghai). The filtered water volumes were 6 L, 4 L, and 0.3 L per sample for spring water, groundwater, and river water, respectively. Membranes were cut into pieces using sterile scissors and loaded into oscillation tubes (FastDNA® SPIN Kit for Soil; MP Biomedicals, USA) for DNA extraction, which followed the protocol provided by the manufacturer. The concentration and purity of sample DNA was analyzed by microspectrophotometry (NanoDrop ND 1000, Thermo Scientific, USA). All DNA samples were stored at − 80 °C until processing.

2.2. Quantification of antibiotic resistome

Antibiotic resistomes were quantified using high-throughput quantitative PCR following protocols described as previously (Zhu et al., 2013). Primer sets for Antibiotic resistome and MGE were reported previously (Yang et al., 2020). Three technical replicates were conducted for each primer set and only considered successful when each of the three replicates had successful amplification. The relative abundance of antibiotic resistome was calculated according to the formula reported in previous study (Zhou et al., 2019).

2.3. Analysis of bacterial community

The V4-V5 region (515 F: GTGCCAGCMGCCGCGG, 907 R: CCGTCAATCMTTTRAGTTT) of the 16 S rRNA gene was sequenced to analyze the structure and composition of the water bacterial community. The reaction volume, conditions, and product processing of PCR were described as previously (Zhou et al., 2019). Purified PCR products were sent to GENE DENOVO (Guangzhou, China) for high-throughput sequencing using an Illumina platform. We subsequently filtered low-quality reads using Quantitative Insights Into Microbial Ecology (QIIME)(Caporaso et al., 2010). A 97% similarity threshold was used to identify bacterial operational taxonomic units (OTUs). Taxonomic classification of bacterial taxa was performed using the Greengenes database (V13.8) (McDonald et al., 2012). Sequence data are available at the National Center for Biotechnology Information (NCBI) under Bioproject number: PRJNA879895.

2.4. Statistical analysis

A preliminary analysis of the raw data was performed using Excel 2021, including the calculations of mean and standard deviations. Analysis of Variance (ANOVA) was performed using SPSS, and results were considered statistically significant only when $p < 0.05$. Fast Expectation Maximization Microbial Source Tracking (FEAST) (Shenhav et al., 2019) was used to analyze the potential sources of antibiotic resistome and bacterial communities in groundwater samples. Principal Coordinate Analysis (PCoA), Procrustes analysis, and Mantel tests were calculated based on Bray-Curtis dissimilarity distances using the R package "vegan" (Oksanen et al., 2019) and were visualized by "ggplot2" (Wickham et al., 2020). Venn diagrams were calculated and depicted by Venny (2.1). Bacterial α-diversity, β-diversity, and mobile genetic elements (MGEs) were used for establishing Structural equation models (SEMs) (AMOS Graphic, IBM). We assumed that: (1) bacterial α-diversity, β-diversity and MGEs directly affect ARGs in water samples; (2) bacterial β-diversity indirectly and negatively affects ARGs by directly affecting water MGEs. The model must meet the following conditions to be valid: (1) Probability level ($P > 0.05$); (2) the root mean square error of approximation (RMSEA < 0.05); and (3) high goodness of fit index (GFI > 0.9).

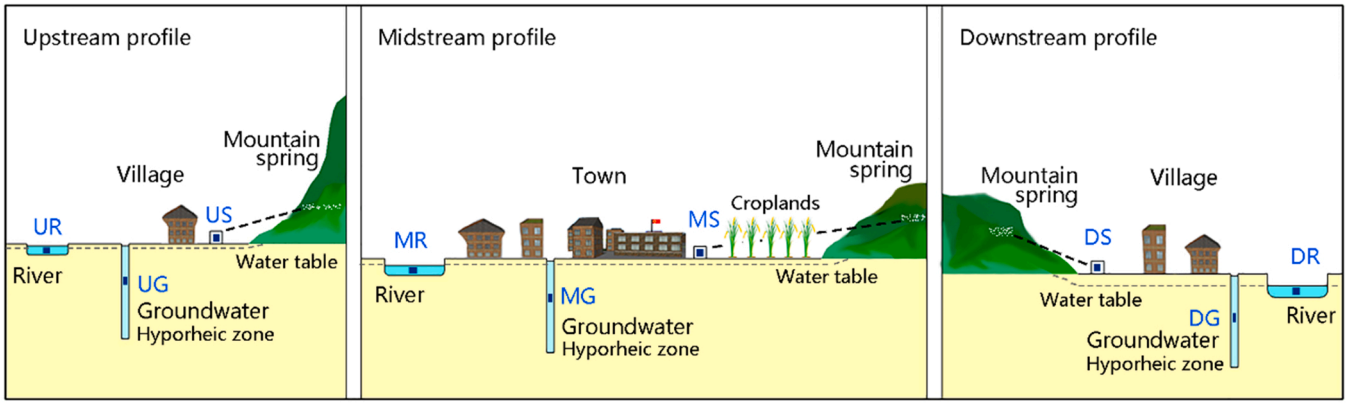

Fig. 1. Schema of the current study.

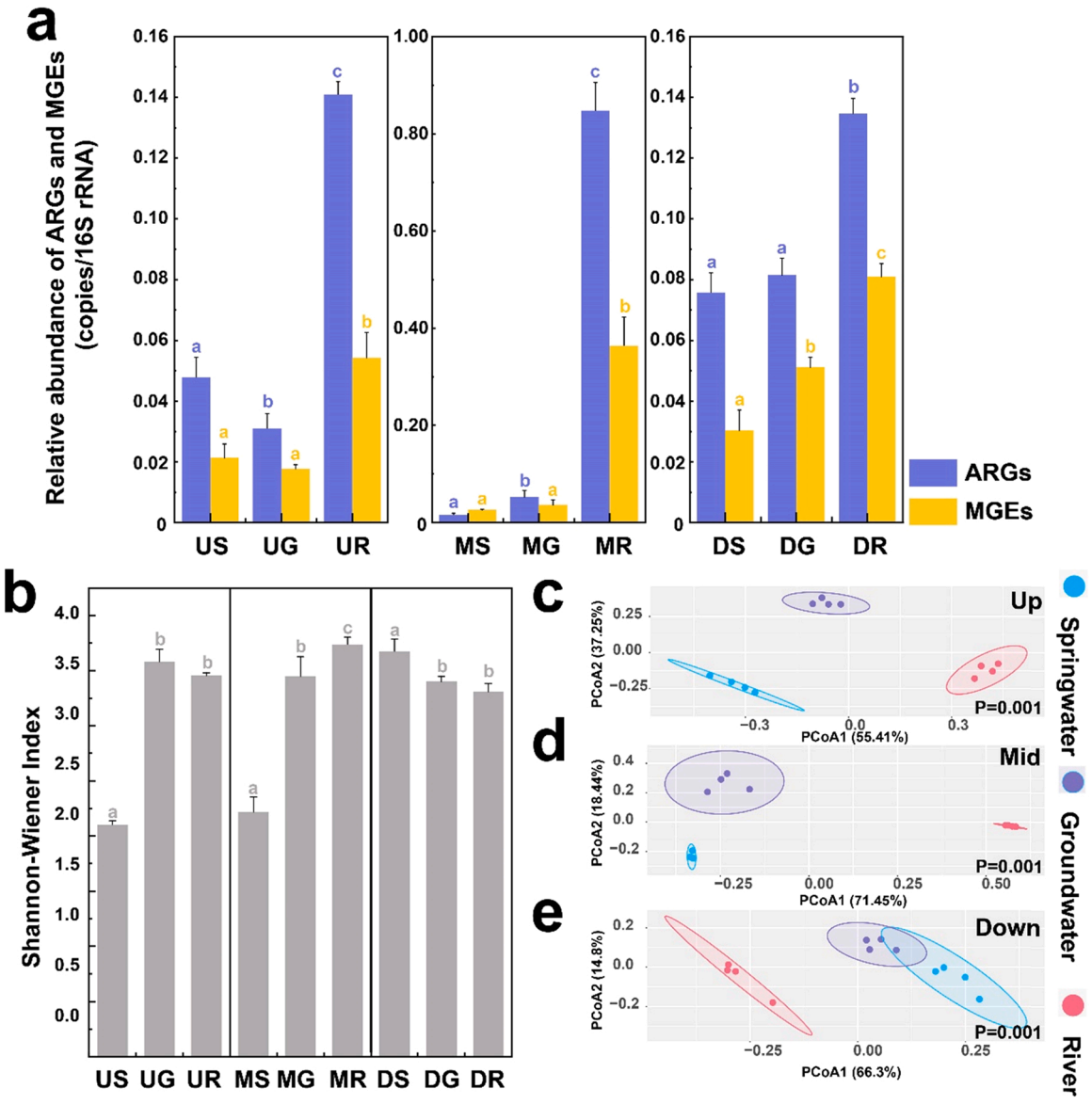

Fig. 2. Profiles of antibiotic resistome in spring water, groundwater and river. (a) Relative abundance of ARGs and MGEs in each sampling site. Significant differences at $P < 0.05$ level (ANOVA) are indicated by different letters. (b) Shannon-Wiener index of antibiotic resistome. (c-e) Principal Coordinate Analysis (PCoA) is conducted based on Bray-Curtis dissimilarity metrics, to evaluate the resistome pattern in spring water, groundwater and river in up- mid- and downstream, respectively. The letters in the bar chart indicate the significant difference between each bar.

3. Results

3.1. Overview of the antibiotic resistome

A total of 261 ARGs (identified as aminoglycoside, beta-lactams, chloramphenicol, macrolides lincosamides and streptogramin B (MLSB), multidrug, sulfonamide, tetracycline and vancomycin resistance) and 12 MGEs were detected in spring water, groundwater, and river samples. The detected number of ARGs in river samples was significantly higher than in spring water and groundwater samples ($P < 0.05$).

The relative abundances of ARGs and MGEs from river samples were significantly higher than those in spring and groundwater at all sample sites (upper, mid and downstream) (Fig. 2a). Upstream, the relative abundance of ARGs was higher in spring than in groundwater, with the opposite observed midstream ($P < 0.05$). The relative abundance of MGEs in spring and groundwater samples did not differ at both upper and midstream (Fig. 2a). Shannon-Wiener indices for the antibiotic resistome of groundwater and river samples were significantly higher than those of the spring water samples in upstream and midstream. In contrast, the opposite situation was observed downstream (Fig. 2b). The composition of antibiotic resistome in river samples was distinct from groundwater and spring along the PCoA1 axis, whereas the composition of antibiotic resistome in spring and groundwater water samples was distinct along the PCoA2 asix in up- and midstream (Fig. 2c, d). The composition of antibiotic resistome was similar in spring water and groundwater but distinct from the river water sample in downstream (Fig. 2e).

A total of 90, 129, and 167 resistomes were shared in spring water, groundwater, and river in upstream, midstream, and downstream, respectively (Fig. 3a). In the upstream, spring water and groundwater, river and groundwater shared 110 and 140 resistomes, respectively. In the midstream, groundwater and spring shared lower resistance genes than groundwater and river water ($P < 0.05$). In the downstream, spring and groundwater, river and groundwater shared the same number of antibiotic resistomes. The majority of shared antibiotic resistomes were

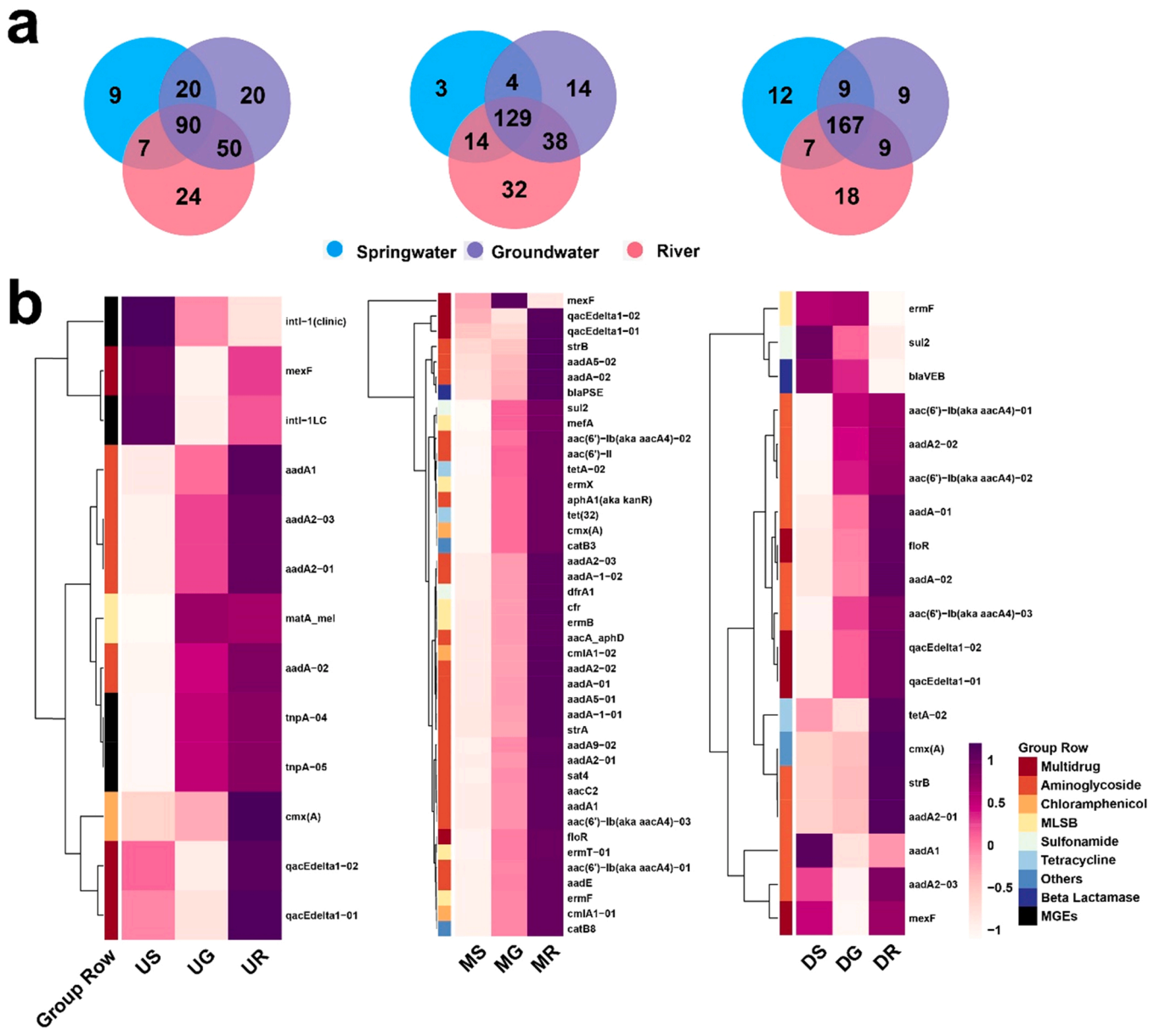

Fig. 3. Shared pattern of antibiotic resistome. (a) The total number of shared ARGs and MGEs across spring water, groundwater, and river. (b) Abundance heatmap analysis of shared ARGs and MGEs (abundance > 0.1%) in each site.

more significant in river samples than either spring or groundwater samples ($P < 0.05$) for all sampling sites (Fig. 3b). For example, the relative abundances of *aadA1* (aminoglycoside resistance), *cmx (A)* (chloramphenicol resistance) and *qacEdelta1–02* (multidrug resistance) were significantly higher (p value) than those of groundwater and spring water in the upstream. However, the relative abundances of *intl-1 (clinic)*, *intl-1LC* and *mexf* were significantly higher in spring water. In the midstream, the abundance of all shared genes, except for the *mexf*, was significantly greater in the river water samples.

3.2. The bacterial community in water samples

A total of 3, 962,288 high-quality bacterial sequences were generated across all water samples, ranging from 100,117 to 115,559 per sample. *Novosphingobium*, *Arthrobacter*, *Flavobacterium*, *Pseudarcicella*, *Limnohabitans*, *Duganella*, *Aquabacterium*, *Pseudorhodobacter*, *Undibacterium,* and *hgcl_clade* were the most abundant identified genera detected in analysed samples (Fig. 4a). In both upstream and midstream, the relative abundances of *Novosphingobium* in spring were significantly higher than that of groundwater and river ($P < 0.05$). In the whole watershed of the river, *Arthrobacter* had the highest abundance ($P < 0.05$) in upstream and was the most dominant genus among known microbial taxa. The composition of the bacterial community in spring, groundwater and river water was significantly distinct from each other (Fig. 4b-d).

3.3. Source tracking of antibiotic resistome and bacteria in groundwater

The potential contribution of resistome and microbiome in sources (spring water and river) to that in the sink (groundwater) were quantified using the source tracking method (Fig. 5a, b). In the upstream, river resistome contributed significantly a higher proportion to the antibiotic resistome in groundwater than spring water resistome ($P < 0.05$). The contribution of the resistome sources to downstream groundwater (DG) explained over 90% of the variation. There was no significant difference between the contribution of resistomes in spring and river water to resistomes in groundwater from the midstream. For the contribution of individual sources to the sink in each sampling site, bacteria shared the same trend as resistome. For example, the contribution of the bactieral community in UR was significantly higher than in US ($P < 0.05$). In comparison, the contribution of the river was lower than spring water downstream ($P < 0.05$).

3.4. Biotic and abiotic drivers of resistome

Procrustes analysis and Mantel test revealed a strong association between ARG matric and bacterial community at OTU level for all water samples ($M^2 = 0.53$, Permutations = 9999, $P < 0.001$, $r = 0.46$) (Fig. 6a). A co-occurrence network between bacterial genera and antibiotic resistome identified five potential hosts for antibiotic resistome (Fig. 6b). For example, *Arthrobacter* was significantly correlated with *intl3* (MGE), *tetD-02* (tetracycline RG), *vanXD* (vancomycin RG), *mtrC-02* (multidrug RG) and *vatB-02* (MLSB). The direct and indirect effects of bacterial communities and MGEs on the ARG profile were revealed by SEM (Fig. 6c). Bacterial α-diversity and MGEs had direct and positive effects on ARGs profiles, respectively ($\lambda = 0.51$, $P < 0.001$; $\lambda = 0.62$, $P < 0.001$), while bacterial β-diversity had direct and negative effects on ARGs ($\lambda = -0.94$, $P < 0.001$) (Fig. 6d).

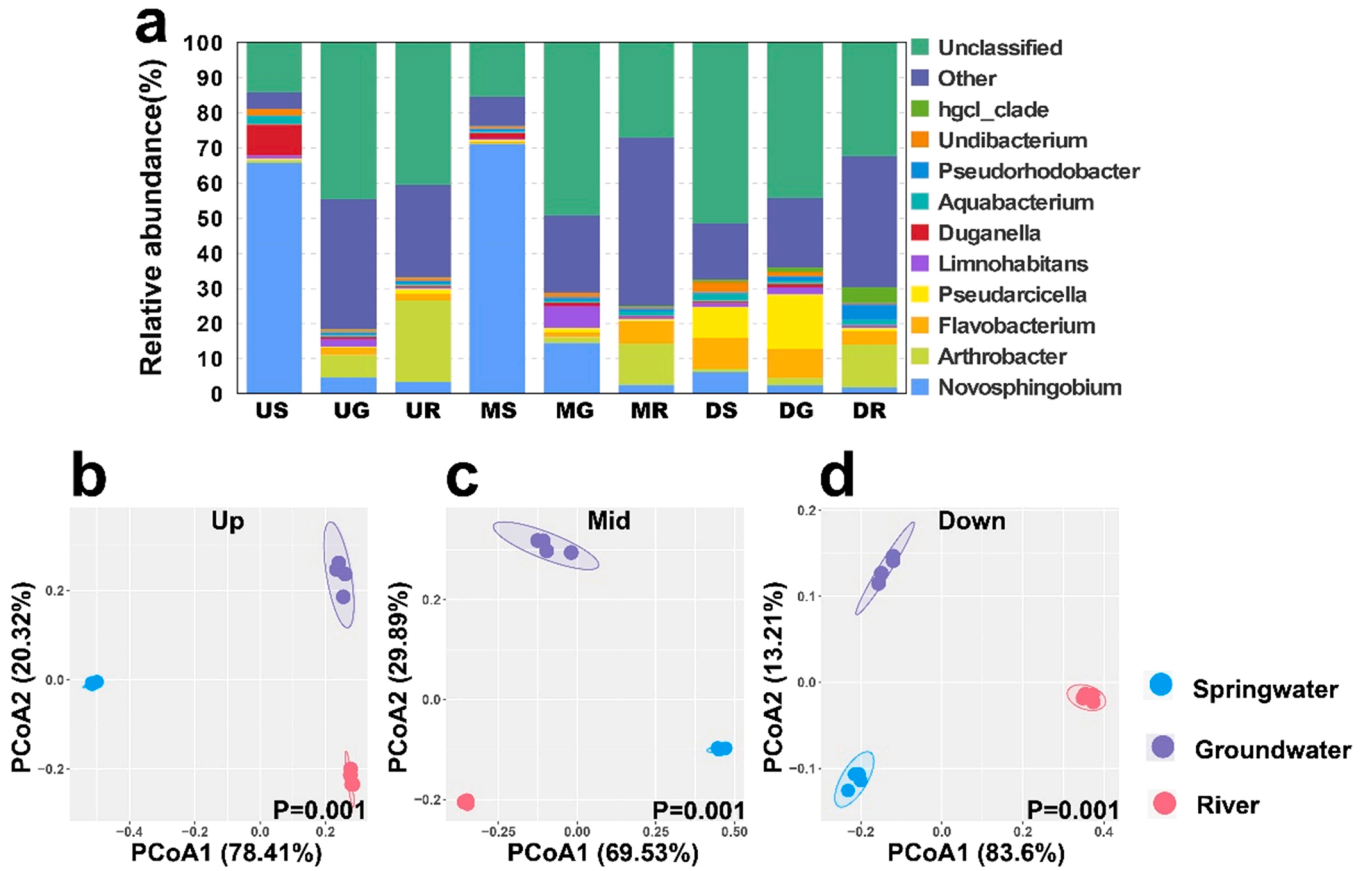

Fig. 4. Profiles of bacterial communities in water samples. (a) Relative abundance of bacterial communities at the genus level. (b-d) Principal Coordinate Analysis (PCoA) indicates different patterns of bacterial communities in spring water, groundwater and river, respectively.

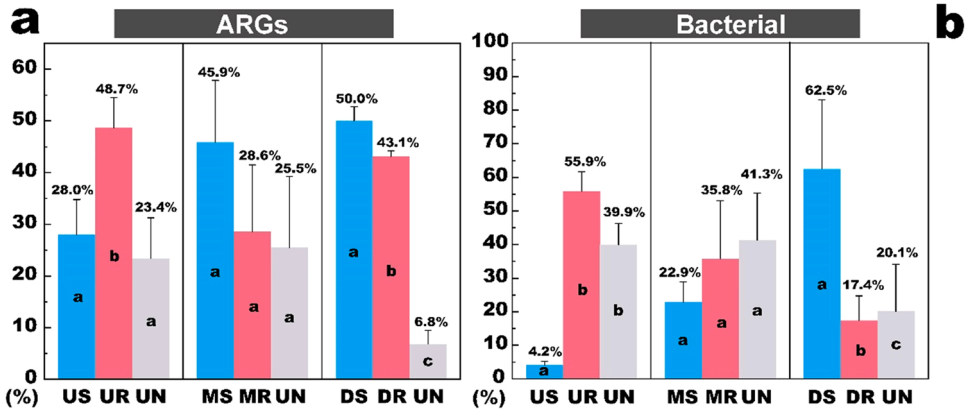

Fig. 5. Source tracking of antibiotic resistome and microbes. Fast expectation maximization microbial source tracking (FEAST) showing potential ARGs (a) and bacterial (b) sources of groundwater. (b) UN represents an unknown source.

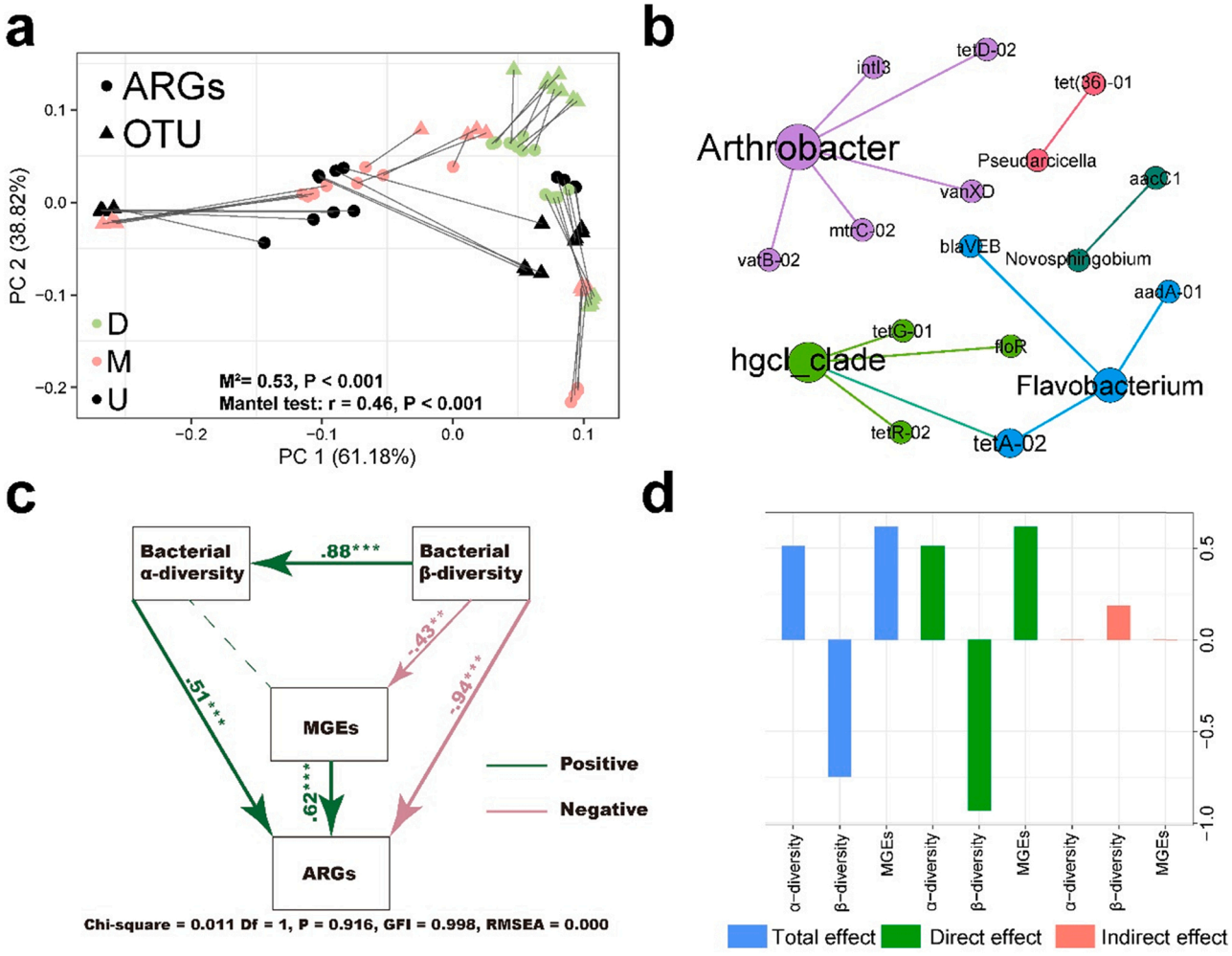

Fig. 6. Correlation and contribution of microbial and environmental factors to antibiotic resistome. (a) Procrustes analysis and Mantel test between bacterial and antibiotic resistome. D, M, and U represent the sampling sites, respectively. (b) Co-occurrence network between bacterial genera and ARGs (Spearman's $\rho > 0.8$, $P < 0.01$). (c) Structural equation modelling suggests that bacterial alpha-diversity, bacterial beta-diversity and MGEs have direct and indirect effects on the composition of ARG in water samples (Chi-square = 0.011, P = 0.916, df = 1, GFI = 0.998, and RMSEA = 0.000). Green and red arrows indicate the positive and negative effects of each factor, respectively. Solid lines with path coefficients indicate the significant effects on the targets, while dotted lines indicate insignificant paths (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$). (d) Bar charts indicate the total, direct and indirect effect of each factor on the target respectively.

4. Discussion

4.1. Rivers harbor a higher level of antibiotic resistome

The prevalent occurrence of antibiotic resistome in the watershed, groundwater, and spring water has been reported previously (Huang et al., 2021, 2019; Zhou et al., 2022). The relative abundances of ARGs and MGEs were significantly higher in the midstream river than that in the groundwater and spring, where town is located, indicating that anthropogenic factors may play an active role in affecting river water resistome (Zhou et al., 2022). On the one hand, groundwater pollution of antibiotic resistance is exacerbated by the input of anthropogenic activities, such as irrigation of agricultural land, urban domestic water discharges, and the breakdown of urban pipe networks (Li et al., 2014; Manaia et al., 2016; Wolf et al., 2004). On the other hand, groundwater is closely linked to surface aquatic systems including river water, in which contaminants seepage into groundwater, posing a potential risk to groundwater quality (Saha et al., 2017; Vázquez-Suñé et al., 2010). The relative abundance of resistome in groundwater in the midstream basin was significantly lower, possibly due to the filtration and suffusion of microbes during underground transport (Knabe et al., 2021; Matthess and Pekdeger, 1981), suggesting that they could be better sources of drinking water. However, we found that groundwater and river samples in up- and midstream shared a higher number of ARGs than that shared by groundwater and springs, indicating river resistome may be an important potential source of groundwater resistome, especially in urbanized areas (Wu et al., 2020).

4.2. Microbial migration leads to the spread of resistance genes

We demonstrated the cumulative and spread of overall antibiotic resistomes in river, groundwater and mountain water. One interesting finding in this study is that the source tracking patterns of antibiotic resistome and microbiome in groundwater revealed by FEAST shared the same trend. For example, in the upstream region, the river contributed significantly higher in shaping groundwater resistome than spring water. Moreover, rivers also contributed a much higher proportion of groundwater microbiome than spring water. These provide further evidence that the antibiotic resistome in water samples, including groundwater, is closely linked to the microbial community (Ouyang et al., 2015; Su et al., 2015). The significant correlation between water resistome and microbes further supports the FEAST results that the transmission of antibiotic resistance genes in water may be closely linked to the transmission of host bacteria. In particular, pathogenic bacteria with antibiotic resistance enter water bodies and pose higher risks to human health via drinking water. *Novosphingobium*, previously isolated from spring water (Fujinami et al., 2020; Sheu et al., 2016), dominated in spring samples both up- and midstream regions. In contrast, the potential pathogen *Flavobacterium* was found in springs and groundwater from downstream areas, and *Pseudarcicella* from external animals (Zhao et al., 2021) was also detected in both water bodies, indicating a potential risk to the safety of drinking water (de Victorica and Galván, 2001). Notably, each of these opportunistic pathogen was associated with a certain antibiotic resistome according to the co-occurrence network in this study. For example, *Flavobacterium* may be the potential host of a beta-lactamase resistance gene *blaVEB*, which is consistent with a Danish study that found *Flavobacterium* in water samples to be resistant to amoxicillin (Bruun et al., 2000). In particular, the *Flavobacterium* were predominant in groundwater and springs in the downstream regions (Fig. 4a), suggesting improved disinfection might be needed before use as drinking water to avoid the potential infection of antibiotic-resistant pathogens (Li and Gu, 2019). A vancomycin resistance gene, *vanXD*, was linked to *Arthrobacter*, indicating a potential risk of failure of the "last line of defense" (Schäfer et al., 1996). SEM results revealed a direct effect of bacterial diversity (both α- and β-diversity) on ARG profiles, indicating that increased bacterial diversity could regulate the spread of antibiotic resistome (Chen et al., 2018). Besides the direct effect, bacterial β-diversity may also indirectly impact aquatic ARGs via its direct effect on MGEs, which was in line with our previous study (Zhou et al., 2021).

5. Conclusion

Collectively, higher levels of antibiotic resistomes were found in the rivers than that in spring and groundwater. FEAST revealed the groundwater resistome and microbiome likely both originated from river and spring water. Strong co-occurrence relationships were found between resistomes and microbes including potential human pathogens. SEMs further indicated that an increased β-diversity might result in a decreased abundance of antibiotic resistome in water samples. These findings suggested that groundwater and spring water as drinking water sources in suburban areas harbored lower levels of antibiotic resistome. With a limited impact of anthropogenic activity, groundwater microbiome and resistomes may originate from background geomicrobial processes and exogenous microbial penetration.

CRediT authorship contribution statement

Fu-Yi Huang: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft,Writing – review & editing. **Yi Zhao:** Conceptualization, Methodology, Writing - review & editing. **Roy Neilson:** Investigation, Writing – review & editing. **Xin-Yuan Zhou:** Investigation, Writing – review & editing. **Hu Li:** Funding acquisition, Writing – review & editing. **Lei Ding:** Methodology, Writing – review & editing. **Shu-Yi-Dan Zhou:** Conceptualization, Formal analysis, Investigation, Funding acquisition, Writing – review & editing. **Jian-Qiang Su:** Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

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

Data Availability

Data will be made available on request.

Acknowledgements

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.114603.

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