Spatial Distribution of Antibiotic Resistance Genes of the Zaohe-Weihe Rivers, China: Exerting a Bottleneck in the Hyporheic Zone

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Abstract

The hyporheic zone (HZ) is an active biogeochemical region where groundwater and surface water mix and a potential reservoir for antibiotic resistance genes (ARGs). In this paper, the relative abundance and spatial distribution of ARGs in the HZ media was investigated, taking into consideration both the speciation of 6 metals and the local characteristics. The samples of surface water, groundwater and sediment were collected from Zaohe-Weihe rivers of Xi'an City, which is representative cities with characteristics of the north-west region region of China. Of 271 ARGs associated with 9 antibiotics, 228 were detected, with a total detection rate of 84%. Sulfonamide and aminoglycoside ARGs were the dominant types of ARGs. The top 6 ARGs and mobile genetic elements (MGEs) in terms of abundance were *tnpA*-04, *cepA*, *su1*, *aadA*2-03, *su2* and *int1*. The results of principal component analysis (PCA) showed that the distribution characteristics of ARGs were not associated with the sampling sites but with the environmental medias. Similarity in the water phases and significant differences in the water and sediment phases were found. The redundancy analysis (RDA) identified the key factors controlling ARG pollution, including dissolved oxygen (DO) in surface water, total nitrogen (TN) in groundwater and total organic carbon (TOC) in sediments. In terms of the speciation of heavy metals, we further revealed the promotion effect between ARGs and heavy metals, especially the residual fraction of Ni. In terms of horizontal transfer mechanism, ARGs were significantly correlated with *tnpA*-03 in water phase, and were significantly correlated with *tnpA*-04 in sediment. In the three media, *int1* and ARGs, all show a significant correlation. These findings showed that hyporheic zone exerted a bottleneck effect on the distribution and transfer of ARGs.

1. Introduction

Many factors, such as extensive use, continuous discharge and incomplete degradation, have caused antibiotics to be widely distributed in a variety of environmental media (Hernando et al. 2006, Shen et al. 2020). Excessive dependence on antibiotics to control bacteria is considered to be related to the evolution of antibiotic resistance. The persistent residues of antibiotic resistance genes (ARGs) in the environment can transfer ARGs to microorganisms and pathogens that are symbiotic with humans. In recent years, novel resistance genes have spread globally, the treatment effect of common diseases has been greatly reduced, resulting in an increase in the hospitalization rate and mortality of patients (Martinez and L. 2008, WHO 2014). Therefore, to protect ecological safety and human health, it remains critical to further explore the spatial distribution of ARGs in key areas in the environment.

In association with rapid industrialization and urbanization, the population density, human activities, and medical development have promoted the emergence and spread of a diversity of ARGs (Oberle et al. 2012, Su et al. 2017). A large amount of antibiotics and other contaminants (i.e., metals, pesticides and nutrients) mix together in pharmaceutical factory effluents, wastewater treatment plants (WWTPs), estuary environments and livestock and aquaculture farms and is likely to cause complex pollution and lead to the area becoming an environmental reservoir for ARGs (Czekalski et al. 2015, Sabri et al. 2018, Sanganyado and G. 2019). Zhu et al. (2016) found that a total of more than 200 ARGs have been

detected in coastal estuary environments in China. Moreover, surface water, groundwater, sediment, leachate and air also have high ARG detection rates (Carlos et al. 2016, Xu et al. 2014, Chen et al. 2021). The abundance of ARGs in sediments was 120–2000 times that of ARGs in water (Luo et al. 2010). Therefore, Chen et al. (2019a) believed that environmental media can significantly affect the distribution and co-occurrence of ARGs in the aquatic environment. In addition, the types and abundance of ARGs are often affected by physical and chemical indicators, such as pH, temperature, salinity, sediment grain size and organic matter content (Guo et al. 2018, Lu et al. 2015, Mao et al. 2014). Zhao et al. (2017) demonstrated that the profile and dissemination of ARGs were mainly determined by nutrients, including the total nitrogen and organic carbon contents. Additionally, it is well known that the gene movement elements (MGEs, conjugative plasmids, transposons and integrons) are also the dominant factors driving the distribution and horizontal gene transfer (HGT) of ARGs (Cesare et al. 2016). Microorganisms that acquire resistance through HGT in the environment constitute a very large ARG repository. The significant correlations between heavy metals and ARGs was also occurred by co-selection and cross-selection processes (Chen et al. 2021). However, these metals were generally the total extractable fractions in sediments based on the digestion by strong acids. There are few studies involving the speciation of metal. Acid-exchangeable fraction of metals may further regulate the behavior of ARGs in sediments (Guo et al. 2018). Therefore, it is necessary to further explore how different speciation of heavy metals affect ARGs and MGEs.

At present, most studies have focused on a single system of lakes, reservoirs or rivers (Stange et al. 2019), while there are few studies on river-groundwater systems, such as hyporheic zone (HZ). As such systems represent complex geographic environments, a variety of pollutants, including ARGs, are enriched in multiple forms of media and multiple regions. The Zaohe River in the study area combines effluents from various ARG sources, such as domestic sewage and hospital wastewater. However, the existing sewage treatment unit cannot effectively remove ARGs, and even different biological treatment stages will exacerbate the occurrence frequency of ARGs in pathogenic bacteria and other bacteria (Cacace et al. 2019, McConnell et al. 2018). The effluents enriched in antibiotics, ARGs and MGEs take water as a carrier and continue to converge in the HZ of the Weihe River. As a special sedimentary layer involved in water movement, the HZ is an important interface linking rivers and groundwater systems and a vital link in the cycling of elements and materials in Earth's critical zone (Boano et al. 2014, Richter et al. 2009). At present, the most concerning issues associated with the HZ are nitrogen and heavy metals, such as the attenuation of N and P (Palumbo-Roe et al. 2017, Zhang et al. 2021). The chemical components and nutrients carried by the groundwater and surface water are mixed to form a strong chemical gradient and accelerate the transformation of elements (Fischer et al. 2005). The circulation of contaminants in the HZ may be faster than that in other water environments. Liu et al. (2000) found that the nitrogen conversion activity of sediments in the HZ was accelerated by water chemistry and hydrodynamic conditions. Unfortunately, research on ARGs in such an active biogeochemical area is almost completely absent. Exposure to antibiotics at low concentrations will inhibit or kill many microorganisms and cause damage to or the loss of the ecological function of the HZ over the long term.

Therefore, it is necessary to conduct a qualitative and quantitative analysis of ARGs in the HZ and further explore the effect of HZ on the spread and distribution of ARGs.

Taking the HZ in arid and semiarid regions in China as the research object, an ideal research area (the junction of the Zaohe and Weihe rivers) was selected to reveal the broader profiles of ARGs. This study considered three types of media (surface water, groundwater, and sediments) based on the understanding of the hydrogeochemical characteristics of the HZ and revealed the distribution characteristics of ARGs. The effects of the unique environmental factors of the HZ on ARGs were discussed, and gained a new understanding of the relationship between heavy metal speciation and ARGs. The key mechanisms of ARG transfer and diffusion under the long-term recharge of surface water and groundwater were also clarified.

2. Materials And Methods

2.1 Study area and sampling sites

The study area is the junction of the Weihe River and Zaohe River. It is located in the Guanzhong Basin of Shaanxi Province, China. The Weihe River is the largest tributary of the Yellow River, and the Zaohe River is its primary tributary. It is the main sewage and flood discharge channel in Xi'an City of Shaanxi Province. Sampling campaigns were performed at 6 sites (S0-S5) in October 2019. Detailed information on the sampling sites is provided in Fig. 1 and Table S1. All the sediment sampling sites corresponded with the water sampling sites, with the exception of S5. For the water sampling phase, 2 L surface water (0.3 m below the surface) and groundwater samples were collected. Sediment samples were collected with a gravity sediment sampler. After sampling, the samples were immediately transported back to the laboratory in a cooler. The water and sediment samples were stored at 4°C and – 20°C, respectively, and all the samples were analyzed within a week.

2.2 Pretreatment and sample analysis

The dissolved oxygen (DO), temperature, and pH of the water samples were analyzed with a multiparameter controller (Milti3430, WTW, Germany). Chemical oxygen demand (COD) was measured with a digestion instrument (5B-1B (V8), China), and total organic carbon (TOC) and total nitrogen (TN) were measured with an analyzer (Elementar Vario EL Ⅲ type, Germany). Parallel samples were used for each run and the average was taken as the result.

2.3 Extraction of total heavy metals and five fractions

Six heavy metals (Pb, Cr, Cd, Zn, Ni and Cu) in the sediment were measured.

Total extraction state: put 0.5g of sediments in a 50mL PTFE crucible, add 10ml of hydrochloric acid, heat it on a hot plate at low temperature, evaporate to about 3mL, add 5ml HNO₃, 5mL HF and 3mL

HClO₄, and heat until the black organic matter disappears and steam until it is viscous. Add 1 mL HNO₃ and a small amount of distilled water, cool and transfer to a 50ml volumetric flask, make the volume constant with distilled water. Heavy metals in the sediment were continuously extracted through Tessier method to obtain the five fractions (Tessier et al. 1979). Heavy metal standard samples and extracts were measured using inductively coupled plasma mass spectrometer and inductively coupled plasma spectrometer (ICP-MS, Agilent 7700, USA).

2.4 DNA extraction and quantitative real-time PCR (qPCR)

Five hundred milliliters of each water sample was filtered through a 0.22 µm filter. Then, the filter membrane and total DNA in the sediments were processed according to the manufacturer's protocols using a commercial DNA isolation kit (K718, Biocolor, China) and stored at -20°C before polymerase chain reaction (PCR) and quantitative PCR (qPCR) testing for the target genes. An ultramicro UV spectrophotometer (Quawell Q3000, USA) was used to measure the OD260/OD280 value to assess DNA quality. The details of the qPCR procedure are described in Text S1. All samples and standards were analyzed in duplicate. To minimize the difference in ARG abundance caused by the variation in background bacterial abundance and DNA extraction efficiency, gene concentrations were expressed as the relative abundance according to Eq. (1) (Livak and Schmittgen 2001).

$$F = 2^{- (\Delta Ct_1 - \Delta Ct_2)} \quad (1)$$

where ΔCt_1 is the difference between the Ct value of the target gene and that of the 16S rDNA of the sample, and ΔCt_2 is the difference between the Ct value of the target gene and that of the 16S rDNA of the control group.

2.5 Data analysis

Principal coordinate analysis (PCA), redundancy analysis (RDA) and Procrustes analysis were performed using Canoco 5.0 (Microcomputer Power, USA). One-way analysis of variance (ANOVA) was adopted to evaluate the significance of the differences in antibiotic and ARG concentrations with SPSS software. The correlations between the ARG concentration and environmental factors were analyzed with Pearson's rank correlation test. Diagrams were generated using Origin 2017 (OriginLab, USA), and calculations were performed in Microsoft Excel (2016).

3. Results And Discussion

3.1 Diversity and abundance of ARGs in the HZ

The mixed soil and water sample from S4-3, with a high antibiotic concentration, was used as the target pollutant sample. Of the 271 targeted ARGs associated with 9 antibiotics, a total of 228 were detected in the sample. The total detection rate of 84.1% obtained in our study was higher than that in the reservoir

system (78.9%) (Chen et al. 2019). This result indicates that the existence of more resistant bacteria in the microbial communities of the HZ and complex environmental incentives promoted the emergence of novel antibiotic resistance. The detection rates of ARGs associated with other antibiotics are shown in Fig. 2a. Among them, the highest and lowest relative abundances were obtained for *aadA2-03* and *tetV*, with values of 6.30×10^{-2} and 1.47×10^{-6} , respectively. There was only one vancomycin-related ARG (*vanSE*, 4.48×10^{-3}) in the top 100, and the rest were on the order of 10^{-6} . The 82% detection rate for vancomycin indicates the emergence of an increasing number of vancomycin-resistant bacteria. The concentration ratio of the top 150 ARGs was analyzed (Fig. 2b), and the ARG types followed the order aminoglycoside > beta lactamase > sulfonamide > MGEs > tetracycline > acrolides, lincosamides and type B streptogramin (MLSB) > fluoroquinolones-chloramphenicol (FCA) > vancomycin. Aminoglycosides and beta lactamase ARGs were the dominant types of ARGs, which was similar to the results found in the Wen-Rui Tang River in China (Zhou et al. 2017).

In the present study, 17 ARGs belonging to different categories (*aac(6')*-Ib-3, *aadA-01* and *aadA2-03* for aminoglycosides; *blaOXA1*, *blaOXA10-02* and *cepA* for beta-lactam; *su1* and *su2* for sulfonamides; *tetG-02*, *tetM-01* and *tetX* for tetracyclines; *catB3*, *msr(D)* and *mexF* for MLSB/FCA; *int1*, *tnpA-03* and *tnpA-04* for MGEs) were investigated comprehensively (Fig. 3). As shown in Fig. 3a, 15 of the 17 ARGs were successfully detected in the surface water; those that were not *blaOXA10-2* and *cepA*. The total relative abundances of ARGs ranged from 0.16 to 0.29, with a main focus on S2, S3, and S4. The distribution characteristics of ARGs at each sampling point were relatively similar, with an order of those associated with sulfonamide > aminoglycoside > MLSB/FCA > tetracycline > beta-lactamase. The distribution ratios of ARGs at each sampling point are shown in Fig. 3b. It is obvious that *tnpA-04* represented a large percentage of the total ARGs at each sampling point, up to 64%. The relative abundances of the top five other ARGs were in the order of *su1* > *su2* > *aadA2-03* > *int1* > *mexF*. The two sulfonamide ARGs reached a total amount of up to 5.06×10^{-2} and 2.57×10^{-2} , respectively. A high abundance of sulfonamide ARGs is commonly detected in water environments such as the Haihe River (Luo et al. 2011). This may be due to the long-term selective pressure caused by low levels of sulfonamide antibiotics and their relatively low hydrolysis rate (Wang 2019). 16 of the 17 ARGs were successfully detected during the groundwater (*blaOXA10-2* was not detected) (Fig. 3c). The order of the ARGs in terms of abundance was consistent with the pattern observed for the surface water, but the total abundance was slightly higher than that in the surface water. In contrast to the water samples, the 17 ARGs were successfully detected to varying degrees in the sediments (Fig. 3e). The distribution of the ARGs in the sediments followed the order MGEs > aminoglycoside > sulfonamide > tetracycline > beta-lactamase > MLSB/FCA. The relative abundances of the top five ARGs showed the order *tnpA-04* > *aadA2-03* > *su1* > *su2* > *aadA2-01* (Fig. 3f), and the total amounts of these ARGs were all above 10^{-1} . All types of ARGs expressed their maximum abundance at S5. This is because the sediments are mainly clay and contain a high TOC content. In the HZ, due to the periodic interaction, the overall difference in the abundance of ARGs in the media was not large, but the groundwater and sediment phases were prone to harbor more diverse ARGs and some high-abundance ARGs. Maybe the facultative anaerobic conditions of groundwater are particularly suitable for

the growth of resistant bacteria. More attention should be given to addressing this insufficiency in future research, especially in some unique and different types of HZs.

3.2 Distribution differences in ARGs and dominant ARGs

The abundance data for the 17 ARGs in three types of media are represented with box plots (Fig. 4). These ARGs were classified into two groups: One group contained the dominant ARGs, with the top 5 in terms of abundance being *tnpA*-04, *cepA*, *su1*, *aadA2*-03 and *su2*. Their abundances were 8.38×10^{-2} , 3.04×10^{-2} , 2.61×10^{-2} , 2.22×10^{-2} and 1.84×10^{-2} . The other group contained non-dominant ARGs, and their average values were below 10^{-3} . And the high outliers and great degree of dispersion (especially for *aadA*-01, *aadA2*-03 and *su1*) resulted from the differences in three types of media.

PCA based on the Bray-Curtis distance was applied to analyze the ordination plot of the ARG distribution patterns in association with the variation in media (Fig. 5). Overall, as summarized in Fig. 5a, the B1 site was distinct from the other sampling sites. The first two PCs explained 65.5% of the total ARG abundance variation, with PC1 explaining 43.2% of the variation. The 25 sites were divided into three groups: Class 1 mainly included sediment along the line (C1, C2, B2, C4-3 and C5), Class 2 mainly included groundwater and surface water (A2, A3, A4, A5, B3, B4-1, B4-2, B4-3, B4-4 and B4-5), and Class 3 mainly included lateral sediments (C0, C3, C4-1, C4-3, C4-4 and C4-5). It is worth noting that surface water and groundwater were in the same class, which suggests the existence of similarities in the water phase. The sampling points were randomly distributed, indicating that geographic location was not the main factor affecting the change in ARGs. The sampling sites in the sediments were clustered into Class 1 and Class 3, and there were obvious horizontal and lateral differences. Overall, the ARGs in water and sediment showed obvious differences. Antibiotic residues and physiochemical properties result in the creation of different microorganism habitats and cause the evolution of diverse antibiotic-resistant bacteria and ARGs. Zhou et al. (2017) showed that ARGs had little relationship with geographic location and were mainly affected by the composition of the bacterial colonies. The unique media of the HZ represent good habitats for resistant bacteria. The results also indicate that the media environments were a driving force affecting the distribution of ARGs. Figure 5b shows the results of the PCA in terms of MGE abundance. Class 3 included the three types of media in the distribution characteristics of the MGEs (Fig. 5b), which was consistent with the phenomenon that high-abundance MGEs exist in the three types of media in Sect. 3.1. This result indicates that the distribution of MGEs at the sampling sites showed a certain degree of randomness. In addition, filtering out ARGs with significant differences in abundance is of great significance for exploring the formation of dominant ARGs in different media (Fig. 6). The X-axis represents the logarithmic value of the fold change with 2 as the base ($\log_2$ (fold change)), and the Y-axis represents the negative logarithm of the p value ($-\log_{10}$ (p-value)). In the groundwater/surface water, only *aac*(6')-Ib-3 expression was significantly upregulated among the 15 ARGs, indicating that *aac*(6')-Ib-3 was most prevalent in the groundwater environment. In the sediments/surface water, the expression of 5 ARGs (*tnpA*-04, *catB*3, *tetG*-02, *tetM*-01, and *msr*(D)) was significantly upregulated. Differences in

illumination and temperature can affect the expression of ARGs. Compared with those in the surface water, the steadier temperatures and low light availability in the sediments resulted in a higher abundance of ARGs. In the sediments/groundwater, *catB3* was significantly upregulated, while *tnpA-04* and *mexF* were significantly downregulated. Han et al. (2017) found that *mexF* was amplified by 105 times in sediments due to the addition of fishmeal. Therefore, there may be other factors that cause *mexF* to become more prevalent in groundwater. It is worth mentioning that although have different abundances. The difference of *catB3* and *mexF* in sediments and groundwater may be due to the expression of different resistance mechanisms. Overall, more than half of the total detected ARGs were shared among the environmental compartments, indicating that these ARGs are widespread and persistent in the HZ environment. The existence of dominant ARGs in the different phases (9 ARGs) suggests that these genes might be primordial genes in their corresponding phase, which is important for understanding the production of ARGs in the HZ.

3.3 Environmental factors influencing the profiles of ARGs

In general, residues of antibiotics and environmental factors may cause the potential risk of promoting the development of bacterial resistance genes through genetic mutation and HGT (Bouki et al. 2013). Research in recent years has found that the correlation between ARGs and antibiotics is not consistent (Guo et al. 2018, Luo et al. 2011). The contribution of antibiotics to ARGs in the HZ needs to be further addressed. The correlations among physicochemical parameters, antibiotics and ARGs were evaluated with RDA (Fig. 7). In the surface water (Fig. 7a), antibiotics provided a smaller contribution to the overall variance than environmental factors and were filtered out in the forward selection results. Of the total variation, 88.6% could be explained by axis 1 and 7.7% could be explained by axis 2. The order of the sampling sites projected to the line of ARGs indicated that the ARGs at sites 1, 2 and 4 were greatly affected by COD. A positive correlation was still found between ARGs and most environmental factors (temperature and TN). However, DO ($p < 0.05$) showed a negative correlation with the ARG levels. In the surface water, antibiotic residues may only exert a small amount of selective pressure influencing the occurrence of ARGs. Bengtsson-Palme and Larsson (2016) consider that the low concentrations of antibiotics in the water environment are not sufficient to promote the proliferation and spread of ARGs. Other residual chemicals may provide selection or co-selection effects, such as heavy metals and polycyclic aromatic hydrocarbons (Wang et al. 2017). In contrast to the relationships in the surface water, SMZ and OTC were positively correlated with most ARGs in the groundwater, especially *su1* and *su2* ($p < 0.05$) (Fig. 7b). Of the total variation, 78.0% could be explained by axis 1 and 18.5% could be explained by axis 2. Sulfonamide antibiotics have a certain inducing effect on their corresponding ARGs, and it have been reported to occur in landfills in China (Wu et al. 2017). At S1, *cepA*, *tetM-01*, *aadA2-03* and *tnpA-03* were most affected by OTC and TN ($p < 0.05$). All the sampling sites except for S1 were arranged in clusters, indicating that the expression of ARGs at these sites was affected by similar environmental factors. The sampling sites along the line were most affected by DO, while the lateral sampling sites in the HZ showed greater influences of COD, TOC, and temperature. Compared with the observations in the water phase, the antibiotics in sediments were found to contribute more to the total variance in ARGs, especially for SMZ (Fig. 7c). Of the total variation, 62.6% could be explained by axis 1 and 7.7% could be

explained by axis 2. The order of the sampling sites projected to the line of ARGs indicated that S2 and S5 had the highest abundances of ARGs, which were driven by TOC and clay overall. Nutrients are an essential factor allowing antibiotic-resistant bacteria to maintain normal life activities. The results for all three types of media in the HZ suggested that carbon energy sources and major nutrients (TN) were key factors driving the microbial community composition. Most ARGs were found at sampling sites S1 and S2, which was related to the pollution source. External pollutants were imported from the Zaohe River into the Weihe River and then were easily absorbed or bound by small molecules such as clay minerals, hydrated oxides, and dissolved organic matter after being recharged by the surface water and groundwater. The combined effect of these pollutants may become a potential incentive for the development of ARGs. Furthermore, the TOC in the clay can regulate the production and spread of ARGs. The plasmid DNA adsorbed onto the clay particles can escape the risk of transformation and degradation due to the physical protection of TOC, thereby regulating the occurrence and permanent dissemination of ARGs in the environment (Mao et al. 2014). In addition, the low concentration of DO indicated serious water pollution and the proliferation of anaerobic bacteria (Jia et al. 2018). The microbial community structure in the HZ was influenced by the response to nitrogen speciation and DO for microbial-mediated reactions, which were jointly controlled by the chemistry of the solution and sediment and fluid residence time.

As an urban river, the Weihe River is particularly susceptible to the effects of human activities. In addition, WWTPs also typically represent anthropogenic activity. The continuous discharge of wastewater has significantly altered the quality of urban river habitats. The unique environmental characteristics created by the periodic interaction of surface water and groundwater in the HZ and the environmental factors (TOC, TN, DO, and particle size) greatly contribute to the distribution and diversity of ARGs as a result of coregulatory effects. In fact, shifts in the composition of the microbial community under the influence of environmental factors represent the internal driving force. Nevertheless, the weak effects of antibiotics on ARGs should not be overlooked. With the rise in antibiotic accumulation, the role of antibiotics in the spread of ARGs is increasingly important.

3.4 Heavy metals and ARGs

Analyzing the six heavy metals in the sediments of HZ, the concentrations of heavy metals at the 10 sampling points showed similar characteristics. (Fig. S4) From the average point of view, the order is: Zn > Cr > Cu > Pb > Ni > Cd; The lower Cd may be due to their low adsorption capacity and accumulation rate in sediments (Li et al. 2018). The Zn and Cr with highest concentrations will show a certain degree of toxicity to the entire biological group. In addition, the speciation of heavy metals is more worthy of attention. The occurrence, valence and speciation of heavy metals is an important basis for understanding the impact of heavy metals on microbial communities and ARGs. In Fig. 8, there is little difference in the distribution of heavy metal at different sampling points, but the composition of different metal is slightly different. The composition of Cd and Zn is relatively uniform, and The other metals are mainly distributed in the residual fraction (F5) and organically bound fraction (F4). The exchangeable fraction (F1) of Cd, Zn and Ni have a significantly higher proportion. The larger proportion of "unsteady

states" indicates that these metals are more likely to migrate and cause harm to the environment and human health.

Metals and antibiotics which be adsorbed in sediments will accelerate the selection pressure of ARGs via cross-selection and co-selection mechanisms, leading to the rapid spread of ARGs in bacterial communities (mediated by HGT and VGT). Therefore, it is of great significance to analyze the relationships of ARGs, heavy metals and their speciation (Fig. 9). In terms of total amount, except for Cr, the other 5 heavy metals are negatively correlated with ARGs and MGEs. Among them, *aadA-01*, *su1*, *su2*, *tetX*, *tnpA-03* and *tnpA-04* are significantly positively correlated with Cr ($r > 0.6$, $p < 0.05$), indicating that Cr can promote the production and proliferation of ARGs. The content of Cr in sediments ranges from 27.04 to 94.19 mg/kg. Due to the non-biodegradable, the high concentration of Cr in the sediment can provide long-term selective pressure for bacteria. According to the degree on the selection of ARGs, the six metals roughly behave in the order: Cr > Pb > Cd > Ni > Cu > Fe. However, Ohore et al. (2020) found that the degree of influence of metals on ARGs is Cd > Ni > Cu > Zn > Cr. This different research result shows that the impact of heavy metals on soil and sediments is more dependent on specific environmental characteristics. In terms of the speciation of heavy metals, there is no significant correlation between the exchangeable fraction (F1) and ARGs ($p > 0.05$). The carbonate binding fraction (F2) of Cr and Zn has a significant positive correlation with *mexF*. The host bacteria of *mexF* are generally multi-drug resistant bacteria. The F2 fraction is more likely to be released into the environment and compared with total extractable state, especially bioavailable metals are more effective in regulating the occurrence and distribution of ARGs (Guo et al. 2018). The iron-manganese oxidation fraction (F3) shows a significant positive correlation with *aac(6)-Ib-3* and *aadA-01* in Cr, and a significant negative correlation with *tetM-01* in both Cd and Pb. The organic binding fraction (F4) only showed a significant positive correlation with *aadA-01* and *su1* in Pb. The residual fraction (F5) has a certain significant correlation in other metals except Cu, especially the Ni and *su1*, *su2*, *tetX*, *tnpA-03*, *tnpA-04* all show a very significant positive correlation ($r > 0.5$, $p < 0.01$). On the one hand, the residual Ni is relatively stable with a relatively large proportion in the environment. On the other hand, it also promotes the production of two transposases (*tnpA-03* and *tnpA-04*) and *int1*, and accelerates the frequency of horizontal transfer of ARGs. In recent research, Ohore et al. (2020) have found that Ni has a strong influence on the selection of ARGs. However, the analysis of the occurrence speciation has not been carried out. From the results of this study, it is more likely that the residual fraction of Ni plays a leading role in ARGs. The factors that affect the occurrence of ARGs are not single, and follow-up studies need to pay more attention to antibiotics, MGEs and heavy metals speciation, especially the synergy between the environmental factors of production and transmission

3.5 The roles of MGEs in the HGT of ARGs in different media

To explore the relationship between ARGs and MGEs in different media, a correlation analysis of 13 ARGs and 3 MGEs with a high detection rate was carried out (Fig. 10, Table S3). According to the results, *int1* had a good correlation with ARGs in all media. The *int1*, *aadA-01*, *blaOXA-1* of water and three

tetracycline ARGs (*tetG*-02, *tetM*-01 and *tetX*) of sediments had a significant positive correlation. Research has suggested that *aadA* can form a gene cassette with other ARGs and then generate multidrug resistance genes, which are easily captured by integrons. The resistance gene cassette carried by *int11* mainly encodes resistance genes related to aminoglycosides, β -lactams and chloramphenicol resistance (Zheng et al. 2018). Tetracyclines are widely used in humans and animals, and gram-negative bacteria are sensitive to them. Regardless of whether the water phase, sediment phase or both phases were considered, four ARGs (*aadA*-01, *su1*, *su2*, and *tetX*) showed a significant positive correlation with *int11*, among which *su1* and *su2* were extremely significantly correlated ($r = 0.880$, $p = 0.000$; $r = 0.821$; $p = 0.000$, respectively). This is because most of the *int11* contains *su1* at the 3' end. The *int11* in proteobacteria is captured by the transposon Tn402, and the transposon and integrons integrate into a sulfa-resistant gene, such as *su1* (Rosewarne et al. 2010). There was a high correlation between *int11* and the abundance of all ARGs except for *aac*(6')-Ib-3, *aadA*-03, *catB3*, *msr*(D), and *mexF*, indicating that the spread of ARGs was mainly attributed to the transfer of the integron in the HZ. Research shows that *int11* is a very important pathway for the proliferation of ARGs, especially under certain selective pressure (Cesare et al. 2016). Therefore, the type of integron may reflect the interference intensity of human activities on the microbial community. In addition to being the genes necessary for transposition, transposons also carry other specialized genes, including ARGs and metal-resistance genes (Fig. 10). In the water phase, the tetracycline ARGs (*tetM*-01, *tetX*) showed a very significant correlation with *tnpA*-03 ($r = 0.763$, $p = 0.000$; $r = 0.589$, $p = 0.004$). *tetM* and *tetW* are always present in transposons and other MGEs (Gao et al. 2012). In the sediment, most ARGs, such as *aac*(6')-Ib-3, *aadA*-01, *su1*, *su2* and *tetG*-02, were significantly correlated with *tnpA*-04. Wang et al. (2014) found the same positive correlation and that *tnpA*-04 was the most enriched transposase gene in reclaimed water irrigation samples. This result indicated that WWTP effluent surroundings may be particularly likely to produce *tnpA*-04. In both phases together, ARGs were significantly correlated with *tnpA*-03 ($p < 0.05$), except for *mexF* and *su1*. It is worth noting that the four ARGs of *aac*(6')-Ib-3, *catB3*, *msr*(D) and *mexF* were correlated only with transposase genes. Aminoglycosides and chloramphenicol ARGs (*aac*(6')-Ib-3, *catB3*) mostly appear in the form of gene cassettes. The macrolide resistance genes *msr*(D) and *mexF* are resistant to antibiotics through the action of the efflux pump. A previous study showed that bacteriophages can integrate *msr*(D) into the *Streptococcus suis* genome, supporting the horizontal transmission of *mefA/msr*(D) in different strains as well as clonal propagation (Daly et al. 2004). This is more likely to indicate that the behaviors of these four ARGs in the HZ relate to other MGEs, such as transposons and bacteriophages.

Overall, the ARGs in water were significantly related to *int11* and *tnpA*-03, the ARGs in the sediments were significantly related to *int11* and *tnpA*-04, and the ARGs in three types of media were significantly related to *int11* and *tnpA*-03. The transposases of *tnpA*-03 and *tnpA*-04 belong to different gene families. As the carrier of ARGs, the microbial community also plays an important role in their proliferation and transmission. Based on the Ct value of the abundance of 16S rRNA in the different phases, the comparison of the results among the phases indicated that the sediment phase had the most bacterial biomass, followed by the surface water phase and finally the groundwater phase. The HZ can introduce transposons or other MGEs harboring ARGs into the microbiome in other phases, and the high mobility

and versatility of these elements may contribute to ARG propagation among the many native bacteria of the different phases. Martínez et al. (2015) considered that the ARGs residing in the MGEs harbored in human bacterial pathogens have the highest risk level. In our study, we observed significantly positive correlations between ARGs and transposase abundance. More factors driving resistance in the HZ environment should be considered to understand how resistance emerges and is disseminated to control the persistent pollution caused by ARGs.

4. Conclusions

In summary, this study provides important insights for understanding the spatial distribution and transfer of ARGs in the HZ. The main conclusions obtained from the present work have some reference value for evaluating the profiles of ARGs in HZs of other types and in special areas.

- (1) The distribution characteristics of ARGs followed the order sulfonamide > aminoglycoside > MLSB/FCA > tetracycline > beta-lactamase. Compared with the values obtained for the surface water, most ARGs were enriched in the groundwater and sediments. The facultative anaerobic conditions of the sediments and groundwater in the HZ are particularly suitable for the growth of resistant bacteria.
- (2) Compared with the geographical location of the sampling point, the environmental media significantly affected the distribution characteristics of ARGs, and the water and sediment phases showed significant differences.
- (3) The RDA results showed that the basic properties of the HZ, including the DO in surface water, nutrients in groundwater and TOC in sediments were the dominant factors controlling ARG pollution. In addition, the residual fraction of Ni plays a role in the production and proliferation of ARGs.
- (4) A high abundance of MGEs plays a vital role. In particular, *tnpA*-03 and *tnpA*-04 were significantly positively correlated with the water and sediment phases, respectively, accelerating the horizontal transfer of ARGs between different bacteria and media. To a certain extent, these results show that hyporheic zone exert a bottleneck effect on the distribution and transfer of ARGs. It is of great significance for the precise regulation of ARGs in different media in the future.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Author contribution

Siqi Shen: Visualization, Formal analysis, Writing - original draft. Shengke Yang: Investigation, Funding acquisition, Validation. Dan Zhang: Methodology, Software. Yang Jia: Investigation. Fanfan Zhang: Data curation. Yanhua Wang: Writing - review & editing. Wenke Wang: Resources.

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Figures

Figure 1

Overview of the study area and sampling points in Zaohe-Weihe Rivers

Figure 2

Diversity (a) and abundance ratio (b) of ARGs in the hyporheic zone.

Figure 3

The relative abundance and percentage of ARGs in hyporheic zone. (a,b) surface water samples; (c,d) groundwater samples; (e,f) sediment samples.

Figure 4

Box plots of the abundances of ARGs in water and sediment phase.

Figure 5

Principal coordinate analysis (PCA) showed the overall distribution pattern of ARGs (a) and MGEs (b) profiles among the hyporheic zone samples. (A represents surface water, B represents groundwater, and C represents sediment samples).

Figure 6

Volcano plot to screen differentially expressed genes in three types of media.

Distribution of speciation of six heavy metals in sediments.

Figure 9

Correlation between the speciation of heavy metals and ARGs in sediments.

Pearson Correlation

Figure 10

Correlation between MGEs and ARGs in different media.

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