



Original Research

Seasonal hydrological dynamics govern lifestyle preference of aquatic antibiotic resistome



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ABSTRACT

Antibiotic resistance genes (ARGs) are a well-known environmental concern. Yet, limited knowledge exists on the fate and transport of ARGs in deep freshwater reservoirs experiencing seasonal hydrological changes, especially in the context of particle-attached (PA) and free-living (FL) lifestyles. Here, the ARG profiles were examined using high-throughput quantitative PCR in PA and FL lifestyles during four seasons representing two hydrological phenomena (vertical mixing and thermal stratification) in the Shuikou Reservoir (SR), Southern China. The results indicated that seasonal hydrological dynamics were critical for influencing the ARGs in PA and FL and the transition of ARGs between the two lifestyles. ARG profiles both in PA and FL were likely to be shaped by horizontal gene transfer. However, they exhibited distinct responses to the physicochemical (e.g., nutrients and dissolved oxygen) changes under seasonal hydrological dynamics. The particle-association niche (PAN) index revealed 94 non-conservative ARGs (i.e., no preferences for PA and FL) and 23 and 16 conservative ARGs preferring PA and FL lifestyles, respectively. A sharp decline in conservative ARGs under stratified hydrologic suggested seasonal influence on the ARGs transition between PA and FL lifestyles. Remarkably, the conservative ARGs (in PA or FL lifestyle) were more closely related to bacterial OTUs in their preferred lifestyle than their counterparts, indicating lifestyle-dependent ARG enrichment. Altogether, these findings enhanced our understanding of the ARG lifestyles and the role of seasonal hydrological changes in governing the ARG transition between the lifestyles in a typical deep freshwater ecosystem.

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1. Introduction

Extensive antibiotic use has increased antibiotic resistance bacteria (ARB) and enhanced the spread of antibiotic resistance genes (ARGs) in human/animal guts [1,2]. Discharging municipal

and livestock effluents combined with agricultural run-off can increase ARG pollution levels via horizontal gene transfer (HGT) and vertical gene transfer (VGT) in rivers [3] and reservoirs [4]. The HGT is mainly mediated by mobile genetic elements (MGEs) and has been reported as the major mechanism for the spread of ARGs in aquatic ecosystems [3–5]. However, some studies have shown that VGT could be an essential mechanism to shape the ARG profiles in microbial communities under certain scenarios [6,7]. The enhanced ARG levels in the environment may harm aquatic ecosystem health and cause an increased risk of disease transmission [8,9]. Since freshwater reservoirs are key drinking water resources, a

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comprehensive understanding of ARG profiles and mechanisms associated with their dissemination in the reservoirs is very important for making suitable management policies to manage ARGs and associated health risks.

Recent studies have reported the occurrence of ARGs in freshwater reservoirs [4,10]. In one of the studies, ARGs exhibited seasonal variation in reservoir [4], where abiotic (e.g., antibiotics and nutrients) and biotic (e.g., MGEs and cyanobacterial blooms) factors played essential roles in influencing the abundance and composition of ARGs [10]. Albeit gaps exist in our understanding of the seasonal-vertical variations of ARGs, their dissemination mechanisms and the influencing factors in deep reservoirs experiencing both vertical mixing and thermal stratification. Unlike shallow reservoirs, deep reservoirs usually undergo a complete vertical mixing from winter to spring and a thermal stratification from summer to fall [11]. However, lack of water column vertical mixing at depths may result in a significant decrease in temperature and dissolved oxygen (DO) during stratified seasons (e.g., summer and fall) [12]. This phenomenon can subsequently result in dramatic changes in the composition and function of microbial communities between upper and deeper layers [13,14]. Therefore, similar depth-related variations in the ARG profiles could be anticipated, especially during the stratified seasons.

Planktonic microorganisms in aquatic environments can be empirically classified into particle-attached (PA, $> 3 \mu\text{m}$) and free-living (FL, between $3 \mu\text{m}$ and $0.22 \mu\text{m}$) fractions based on their lifestyle preferences [15,16]. The suspended particles have been considered the hotspot of microbial diversity, biomass, and activity due to the high adsorption of organic and inorganic nutrients on the particle surfaces [15,17]. Numerous studies have demonstrated that PA and FL fractions tend to have distinct microbial community compositions in diverse aquatic ecosystems [16,18,19]. This can be attributed to the different lifestyle preferences of most microbial phyla/classes (i.e., PA or FL lifestyle) [16]. Previous studies have shown that PA and FL bacterial communities respond differently to various environmental parameters [14,20]. Guo et al. (2018) found that ARGs in PA and FL lifestyles showed different responses (e.g., abundances, diversities, and compositions) to cyanobacterial bloom events in a subtropical reservoir [10]. These findings suggest that different abiotic and biotic factors may regulate ARG dynamics in these two lifestyles. Moreover, a few reports have also suggested that some microbial taxa can change their lifestyle preference (i.e., from PA to FL lifestyle or vice versa) under temporal-vertical scales to adapt to environmental conditions [18,19,21]. For instance, Mestre et al. (2017) reported non-conservative preferences of Rhodobacterales (Alphaproteobacteria), SAR11 (Alphaproteobacteria), and Sh765B (Deltaproteobacteria) along the water column in the northwestern Mediterranean Sea. Another study revealed that SAR11 could change its lifestyle from FL to PA during the cold season [22]. Despite this, little information is available regarding the lifestyle preference of ARGs for PA and FL fractions in aquatic ecosystems. More questions need to be answered: Does most ARGs prefer a non-conservative or a conservative lifestyle? Moreover, what are the environmental factors and the associated dissemination mechanisms for controlling this lifestyle pattern?

This study used high-throughput quantitative PCR (HT-qPCR) to comprehensively profile the ARGs in a deep reservoir (Shuikou Reservoir, SR) in Southern China. A total of 285 ARGs and ten MGEs, which cover the most common types and mechanisms of resistance, were examined in both FL and PA fractions along a water depth gradient during four seasons. We hypothesized that (i) the abiotic factors (e.g., temperature, DO, etc.): related to changes in seasonal-hydrological conditions (i.e., vertical mixing and thermal stratification) would influence the ARG profiles in PA and FL fractions as well as their transition between PA and FL fractions; (ii)

MGEs may play an important role in the spread of ARGs in PA and FL and the transition between the two fractions; PA rather than FL fraction may be the main HGT hotspot; and (iii) PA and FL fractions may have distinct co-occurrence network patterns consisting of non-conservative and conservative ARGs. Particularly, more associations between ARGs and bacterial taxa (as potential ARG hosts) with the same lifestyle preference (i.e., FL or PA lifestyle) are highly expected. This study will promote our understanding of the role of seasonal mixing and thermal stratification in the spread of ARGs in PA and FL fractions in deep aquatic ecosystems.

2. Materials and methods

2.1. Study area, sample collection, and processing

Minjiang River is the largest river in Fujian Province, China, with a mainstream length of 562 km. It is the main water source of economic and cultural activities (e.g., agricultural, residential, and industrial uses) for ~12 million people [23]. In this study, water samples at different depths were collected from two sites (i.e., S4 and S7) ~8 and ~16 km apart in upstream of the SR dam during four seasons (i.e., April 2017, August 2017, November 2017, and January 2018) to represent vertical mixing (i.e., April and January) and thermal stratification seasons (i.e., August and November) (Fig. S1). The selected sites adequately represented the condition of the SR water column as indicated in a related work examining the SR hydrodynamics [12]. In each season, duplicate samples from each depth, including 0.5, 10, 20, and 40 m beneath the surface, were collected from both sites. Additional samples at a depth of 53 m (right above the sediment) were collected only in S4 to compensate higher water depth at this site. Due to technical reasons, sampling at a depth of 53 m was not possible during November 2017. Due to algal bloom and thermal stratification in SR from mid-July 2017 to November 2017, an oxygen gradient existed between highly oxygenated surface water to hypoxia/anoxia at the bottom. Meanwhile, water columns were well-oxygenated between April 2017 and January 2018. Details on reservoir seasonal-vertical hydrodynamics of this sampling campaign were described elsewhere [12]. Based on decreasing dissolved oxygen (DO) during stratified seasons, water columns were split into two zones: (i) the shallow zone (0–10 m) with average DOs higher than 3 mg L^{-1} and (ii) the deep zone (20–53 m) with the average DOs lower than 3 mg L^{-1} . Data of 16 physicochemical parameters and most frequently detected micropollutants ($>30\%$; 17 micropollutants) was included for further analysis (Table S1) [24]. Water samples (500–1000 mL) were filtered through $3 \mu\text{m}$ and $0.22 \mu\text{m}$ filters to collect DNA of PA and FL fractions, respectively.

2.2. HT-qPCR and 16S rRNA gene amplicon sequencing

DNA from a total of 70 samples from various depths (18 for April, August, and January and 16 for November) representing both PA and FL fractions were extracted by using a FastDNA SPIN kit for soil (Obiogene-MP Biomedicals, Irvine, CA, USA) following the manufacturer's protocol [14]. Subsequently, samples were analyzed via HT-qPCR (Wafergen SmartChip Real-time PCR system) with a total of 296 primer sets representing 285 ARGs, ten MGEs (eight transposase genes and two integrons), and a 16S rRNA gene (as internal control). HT-qPCR protocol is described in Supplementary Information S1. For the preceding analyses, the abundance of ARGs or MGEs is always referred to as the normalized abundance (i.e., the ratio of ARG or MGE copy number to 16S rRNA gene copy number) [25]. The ARGs with low abundances ($<0.01\%$ relative abundance of the total communities) were discarded. Also, three FL samples from January (at a depth of 20, 40, and 53 m from S4) and one FL sample

from November (at a depth of 53 m from S4) were not included in the study due to a lower number of ARG occurrences (≤ 5 ; the mean number of ARGs per sample = 58.53). The absolute copy number of 16S rRNA genes was determined using a LightCycler Roche 480 Real-time PCR system (Roche Inc., Basel, Switzerland). The detailed protocol was described in Supplementary Information S2.

The V4–V5 hypervariable region of the prokaryotic 16S rRNA gene was amplified by using 515F (5'-GTG YCA GCM GCC GCG GTA-3') and 907R (5'-CCG YCA ATT YMT TTR AGT TT-3') and subsequently sequenced by using Illumina HiSeq 4000 sequencing platform. The V4–V5 region of the prokaryotic 16S rRNA was applied for accurate classification and recognition of both bacteria and archaeal communities [14]. Generated raw reads were deposited in the NCBI short reads archive database under BioProject number PRJNA559031. Reads were clustered into operational taxonomic units (OTUs) with 97% sequence similarity. Taxonomic assignment was performed using the RDP classifier with SILVA database v132 [14]. The total reads of each sample were rarified to a total of 17,504 reads. Rare taxa with a relative abundance of less than 0.01% per sample and observed in less than ten samples in PA and FL were excluded.

2.3. Null model analysis

Numerous studies applied the null model analysis to check whether the difference in microbial community composition and structure in habitat was associated with the variations of alpha-, gamma-diversity, or randomization [26]. Here, a null model-based stochasticity ratio was used to explore the strength of stochastic processes in governing the assembly of PA and FL ARGs [5]. Initially, the model calculated the expected similarity community based on 1000 randomizations of original ARG community data, while the proportion of species occupancy was kept the same with the observed community. Subsequently, the relative importance of stochastic processes was calculated using equation (1) [5]:

$$\text{Stochasticity (\%)} = 1 - \frac{S_{\text{obs}} - E_{\text{exp}}}{S_{\text{obs}}} \quad (1)$$

where S_{obs} refers to the observed total similarity and E_{exp} refers to the mean of expected similarity from the null communities.

2.4. Particle-association niche (PAN) index

Previous studies have demonstrated that the particle-association niche (PAN) index can characterize microbial lifestyle preferences as PA and FL [16,19]. Here, the PAN index was adopted to define the lifestyle preferences of ARGs. ARG PAN index of each ARG subtype was calculated using equation (2) extracted from the R script provided by Salazar et al. (2015) [16]:

$$\text{ARG PAN index} = \frac{0 \times \sum_{i=1}^n FL_n + 1 \times \sum_{i=1}^n PA_n}{\sum_{i=1}^n FL_n + \sum_{i=1}^n PA_n} \quad (2)$$

where FL refers to the abundance of the ARG subtype in the FL fraction, PA refers to the abundance of the ARG subtype in the PA fraction, and n refers to the number of samples. The ARG abundance in the FL fraction was given a weight of 0, while the ARG abundance in the PA fraction was given a weight of 1 creating a continuous index from 0 (strictly FL ARGs) to 1 (strictly PA ARGs). This index was then compared against a null distribution of the PAN index generated from 1000 permutations [16]. ARGs with significant indices were referred to as conservative ARGs (for PA or FL fractions), while non-significant indices were defined as non-conservative ARGs (no lifestyle preferences). Besides ARGs, the

PAN index was also employed for bacterial communities to explore the potential links between ARGs and bacteria with the same lifestyle. In addition, the PAN index for each ARG type (ARGs conferring resistance to the same antibiotics) and total ARGs were also computed.

2.5. Statistical analysis

The seasonal-vertical pattern of ARG communities in the SR was elucidated by multivariate analyses, including permutational multivariate analysis of variance (PERMANOVA) and principal components analysis (PCA). The normalized abundance of ARGs was centered log-ratio transformed prior to PCA [27]. PERMANOVA was conducted with the “adonis” function in the R package vegan [28]. Correlation analysis was done using Spearman correlation, and the P -values were adjusted by the Benjamini-Hochberg method. The significance test for two and multiple groups was achieved using the Wilcoxon and the Kruskal-Wallis test, respectively. Dunn test with Benjamini-Hochberg P -value correction was performed for pairwise comparison in multiple group comparisons.

The partial least squares path modeling (PLS-PM) [29] was used to explore the relationships between various factors and ARG abundances (in PA and FL fractions) or lifestyle transition (between PA and FL fractions as indicated by PAN index). The hypothetical causation model was built by grouping variables based on their traits as follows: environmental variables (EVs), micropollutants (MPs), MGEs, bacterial biomass (16S rRNA gene abundance), and bacterial communities (BC). The initial model showed that temperature had distinct effects compared to other EV parameters. Therefore, it was separated as an independent variable. Moreover, to investigate the effects of the assembly of ARG communities on the lifestyle transition of ARGs, the stochasticity ratios between each pair of PA and FL ARG communities (ARG stochasticity) were included in the PLS-PM model. BC, MGEs, and ARGs were expressed using their PAN indices in this model. Parameters with highly correlated variables (loading ≥ 0.7) were included in the final PLS-PM model. The significance of the path coefficient and R^2 in all PLS-PM were validated using 1000 bootstraps. In addition, the projection pursuit regression (PPR) based on MGEs was used to calculate the potential of HGT in PA and FL across seasons in SR. The principle behind PPR is projecting high-dimensional data into low-dimensional space to extract patterns that occurred within the data. This analysis was done with MATLAB 2013 [30]. Additionally, the random forest (RF) method was also used to identify variables contributing to the abundance of ARGs in PA and FL [3]. The significance of the models was determined with 999 permutations [31], and the importance of predictors was evaluated by the increase in the mean square error (Inc-MSE) with 999 permutations. Variables were subjected to variation inflation analysis (VIF) before RF to eliminate the multicollinearity effects [14].

Correlation-based networks were constructed to reveal the co-occurrence among bacterial OTUs and ARGs in PA and FL fractions. Only ARGs and bacterial OTUs detected in >10 samples (~30% of PA or FL samples) were included in the network analysis. The correlation cut-off was set at 0.6 with the adjusted P -value < 0.01. The dissimilarity between PA and FL networks was calculated as β_w (equation (3)) proposed by Poisot et al. [32]:

$$\beta_w = \frac{a + b + c}{(2a + b + c)/2} - 1 \quad (3)$$

where β_w is the dissimilarity between two networks, a is the number of shared edges, b is the number of unique edges for network one, and c is the number of unique edges for network two.

3. Results

3.1. Seasonal-vertical variations of ARGs in PA and FL fractions in the SR

HT-qPCR indicated that a total of 133 ARG and ten MGE subtypes were detected in PA and FL fractions. The mean normalized abundance of ARGs was significantly higher in the PA (0.07 copies per 16S rRNA gene) than in the FL fraction (0.05) (Wilcoxon test, $P < 0.05$) (Fig. 1a). The mean absolute abundance of ARGs was 1.9×10^5 and 5.9×10^5 copies mL^{-1} in the FL and PA fractions, respectively (Wilcoxon test, $P < 0.1$), respectively (Fig. S2). The normalized abundances of several ARG and MGE types, such as aminoglycoside, multidrug, chloramphenicol, sulfonamide resistance genes, and transposase genes, were significantly higher in the PA than in the FL fraction (Wilcoxon test, $P < 0.05$) (Fig. 1a).

Seasonal and vertical variations of the PA and FL ARGs were also investigated. PERMANOVA indicated that the ARG communities in SR were mainly subjected to seasonal variations (PA fraction: $R^2 = 0.373$, $P < 0.001$; FL fraction: $R^2 = 0.301$, $P < 0.001$) (Fig. 1b). Following PERMANOVA, PCA also revealed seasonal patterns for both PA and FL ARG communities (Fig. 1c). Interestingly, ARG types in PA and FL fractions showed unique seasonal patterns. For example, aminoglycoside resistance genes showed significant seasonal variations in FL fraction only (Kruskal-Wallis test, $P < 0.01$), while sulfonamide ($P < 0.01$) and chloramphenicol ($P < 0.01$) resistance genes showed significant seasonal differences in their normalized abundances in PA fraction (Fig. 1d and e).

Moreover, seasonal variations of transposase genes ($P < 0.05$) and the total MGEs ($P < 0.01$) occurred only in PA communities.

The vertical stratification in SR water columns was pronounced during the stratified seasons (August–November), as shown by the depletion of dissolved oxygen from surface to bottom water (Fig. 2a). PERMANOVA suggested that depth played a more important role in shaping the PA and FL ARG communities in stratified seasons (PA fraction: $P < 0.05$, FL fraction: $P < 0.05$ in August; FL fraction: $P < 0.05$ in November) than the seasons where water column was well-mixed (PA and FL fractions: $P > 0.05$ in April and January) (Fig. 2d). Although, ARG abundances in PA and FL fractions were comparable during the stratified period, the abundance of both ARGs fractions varied between shallow and deep water (Fig. 2b and Fig. S3). For example, the abundance of beta-lactamase resistance genes was higher in shallow water (Wilcoxon test, $P < 0.05$) (Fig. 2c), while that of multidrug-, sulfonamide-, vancomycin-resistance genes, and MGEs was higher in the deep-water PA and FL fractions (Wilcoxon test, $P < 0.05$). During well-mixed water column seasons (i.e., April and January), the vertical differences in the abundance of ARGs and MGEs were smaller.

3.2. Transition of ARGs between PA and FL lifestyles among different hydrological seasons

The PAN index analysis indicated 23 PA conservative ARGs, 16 FL conservative ARGs, and 94 ARGs with non-conservative lifestyles (no lifestyle preferences) (Fig. 3). However, the lifestyles of these

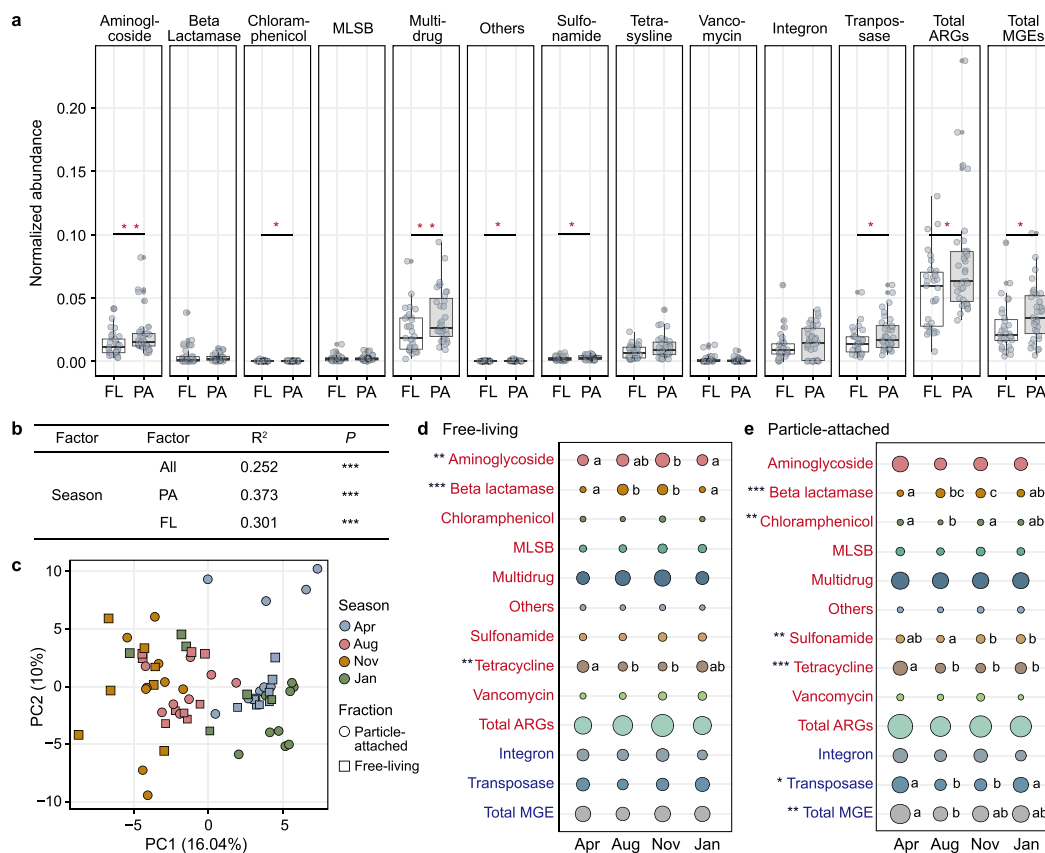


Fig. 1. a, Pairwise comparison of the normalized abundance of ARG types, total ARGs, MGE types, and total MGEs between PA and FL lifestyles. Significant differences were tested by using the Wilcoxon test ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$). b, PERMANOVA analysis based on Bray-Curtis dissimilarity distance of ARGs with 9999 permutations showing the variance of ARG communities in all (PA + FL), PA, and FL fractions explained by season. c, PCA plot showing the seasonal variation of the composition of ARG communities from PA and FL fractions in the SR. d–e, Seasonal differences in the normalized abundances of ARG types, total ARGs, MGEs (classified into integron and transposase), and total MGEs in FL (d) and PA fractions (e). Significant differences were tested by using the Kruskal test ($***P \leq 0.001$; $**P \leq 0.01$; $*P \leq 0.05$). Seasons with different letters indicate a significant difference (Dunn test, $P \leq 0.05$).

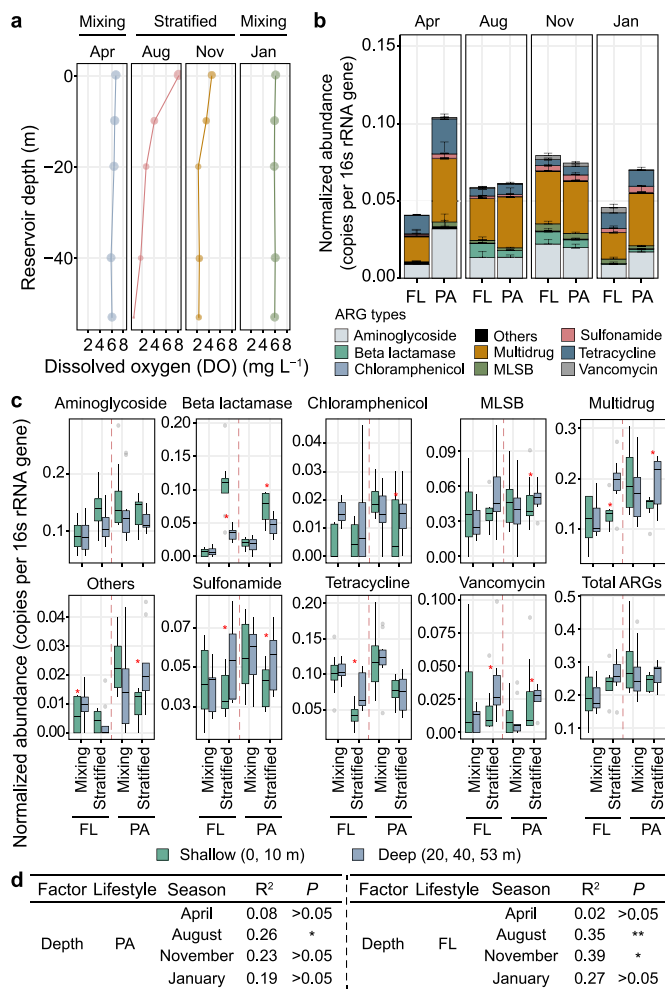


Fig. 2. a, Vertical profile of dissolved oxygen (DO) in the water column of SR at four seasons. b, Normalized abundances of ARG types including aminoglycosides, beta-lactamase, chloramphenicol, macrolide-lincosamide-streptogramin B (MLSB), multidrug, sulfonamide, tetracycline, vancomycin, and others (i.e., other ARG types) at different seasons with error bars indicate standard errors of samples. c, Pairwise comparisons between the normalized abundance of ARG types at the shallow and deep zones for PA and FL in seasons with well-mixed water columns (Mixing) and stratified seasons (Stratified) (Wilcoxon test, * $P < 0.05$). d, PERMANOVA analysis based on Bray-Curtis dissimilarity matrix explaining the variances of ARG communities of PA and FL lifestyles explained by depth during stratified periods (i.e., August and November) and mixing periods (i.e., April and January) (* $P < 0.05$, ** $P < 0.01$).

ARGs were not consistent among different seasons, suggesting that these ARG subtypes did not strictly maintain one lifestyle across different seasons. Nevertheless, there was a noticeable reduction in number of PA and FL conservative ARGs in stratified column (August: PA = 3, FL = 2; November: PA = 2, FL = 1) compared with well-mixed water columns (April: PA = 11, FL = 9; January: PA = 4, FL = 11) (Fig. 3b, Table S2). Furthermore, the total ARGs tended to be more abundant in the PA fraction (PAN index >0.5) in the seasons with well-mixed water columns than in the stratified seasons. Interestingly, the total ARGs became equally abundant in PA and FL fractions (PAN index \approx 0.5) during stratified seasons (i.e., August and November) ($P < 0.001$) to suggest ARG transition between PA and FL lifestyles (Fig. 3c and Fig. S3).

3.3. Relative impacts of abiotic and biotic factors on ARGs in PA and FL fractions

PLS-PM was used to decipher the effects of abiotic and biotic factors regulating the abundances of ARGs in PA and FL fractions in

the SR. The results indicated that MGEs were the main factor shaping the abundances of PA (total effect = 0.79) (Fig. 4a and d) and FL (total effect = 0.5) ARGs (Fig. 4b and e). Although the total effect of the bacterial communities on the ARG abundances was as high as MGEs (total effect = 0.74) in PA, the bacterial community effects on the ARG abundances were less than that of MGEs (total effect = 0.26) in FL (Fig. 4d and e). Environmental variables negatively affected (total effect = -0.44), while MGEs positively affected the FL fraction, while environmental variables had lower positive total effects than MGEs on the PA fraction (total effect = 0.38). It is worth noting that micropollutants played a less important role in shaping the PA (total effect = 0.27) than FL (total effect = 0.37) ARG abundances. PLS-PM analysis also showed that MGEs were the main factor influencing the lifestyle shift of ARGs (total effect = 0.62), followed by the bacterial communities (total effect = 0.46) (Fig. 4c and f). In the case of abiotic factors, the environmental variables (e.g., DO, NO₂-N, and DOP) had a similar impact on the lifestyle shift of ARGs, like the bacterial communities (total effect = 0.41) (Table S3). Remarkably, the temperature negatively affected the lifestyle shift of ARGs (total effect = -0.34).

3.4. Co-occurrence associations between ARGs and bacterial taxa

A total of 409 and 402 ARG-bacterial OTU associations were found in PA and FL fractions, respectively. The high number of unique associations between ARGs and bacterial OTUs in each network (unique associations for PA = 336; unique associations for FL = 329) revealed high dissimilarity ($\beta_w = 0.82$) between the two networks (Fig. 5). Interestingly, the shared ARG-bacterial OTU associations between PA and FL networks were mainly related to the non-conservative ARGs (\approx 66%) and FL conservative ARGs (\approx 34%), but not the PA conservative ARGs. Additionally, there was a high proportion of non-conservative ARGs in PA (24 of 37 ARGs and 300 of 409 associations) and FL (26 of 35 ARGs and 304 of 402 associations) networks. No significant differences were observed between the number of associations of these non-conservative ARGs and bacterial OTUs in the PA and FL networks ($P > 0.05$). However, some PA conservative ARGs had significantly higher associations with bacterial OTUs in the PA network than those in the FL network ($P < 0.05$). For example, one of the PA conservative ARGs, *ermT-02* (macrolide-lincosamide-streptogramin B (MLSB) resistance), had 16 associations in the PA network but 0 associations in the FL network. The same pattern was also found for the FL conservative ARGs in the FL network. For instance, *aac(6')-II* (aminoglycoside) and *vanXD* (vancomycin) that belonged to FL conservative ARGs had higher associations in the FL network (*aac(6')-II*: 13; *vanXD*: 8) than in the PA network (*aac(6')-II*: 1; *vanXD*: 3). Furthermore, the number of associations between conservative ARGs and conservative bacteria was higher in the FL network (16 associations) than in the PA network (one association).

4. Discussion

ARGs are recognized as emerging contaminants at a global scale [33]. The pathways and mechanisms of ARG dissemination in deep freshwater reservoirs have not been investigated thoroughly, especially for PA and FL ARGs. Our results indicated that seasonal change was the major driving force for the ARG communities (in PA and FL) in the deep-water reservoir. Similar seasonal variations of ARGs were observed in different aquatic environments [3,34,35]. However, the dynamics of PA and FL ARGs were controlled by different physico-chemical parameters of water induced by vertical mixing and thermal stratification. PLS-PM result indicated that the abundances of PA ARGs were mainly influenced by nutrients such as NH₄-N, DTP, and DOP, which were highest during April (the

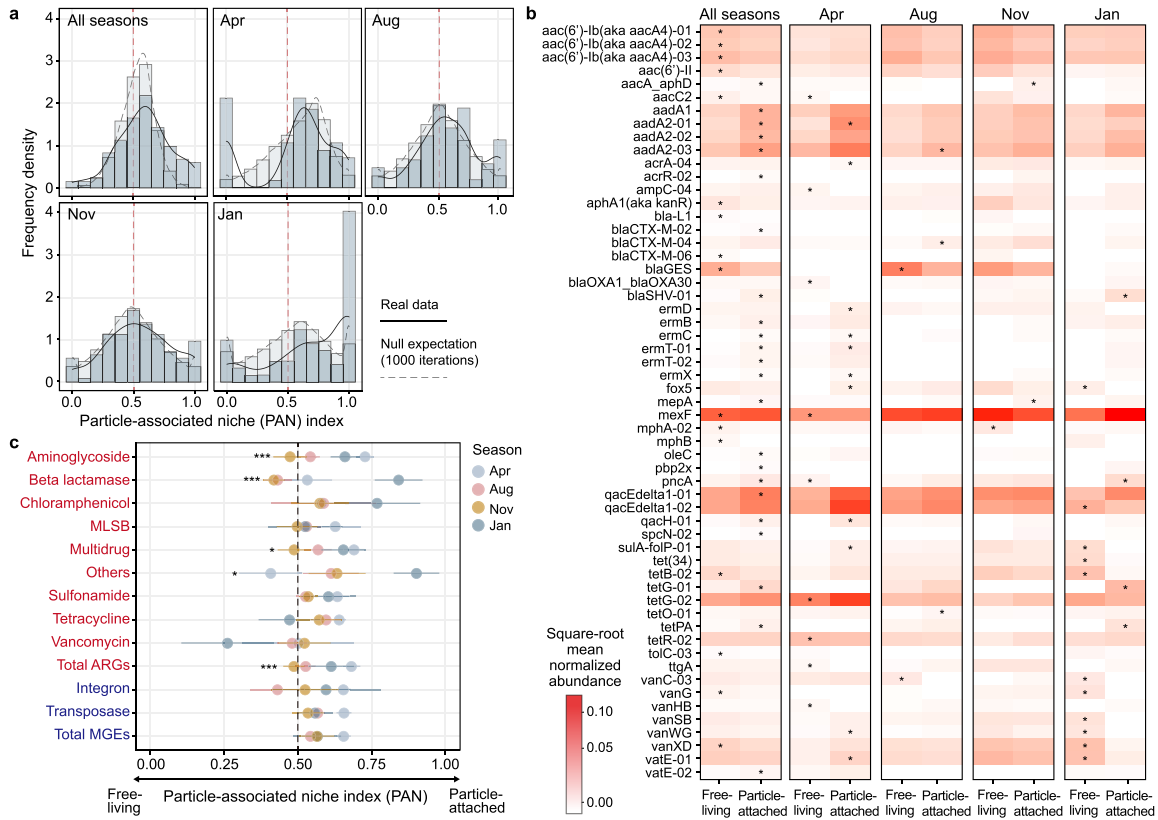


Fig. 3. **a**, The histogram of the observed PAN index distribution for each ARG subtype compared to the expected PAN index from the null model (1000 permutations) in the whole dataset and each season. **b**, The heatmap of ARG subtype with significant lifestyle (in PA or FL lifestyle) in all and individual seasons. **c**, The seasonal variations of the lifestyle shift (i.e., PAN index) of ARG types, total ARGs, MGE types, and total MGEs (Kruskal Wallis test: $***P < 0.001$; $**P < 0.01$; $*P < 0.05$).

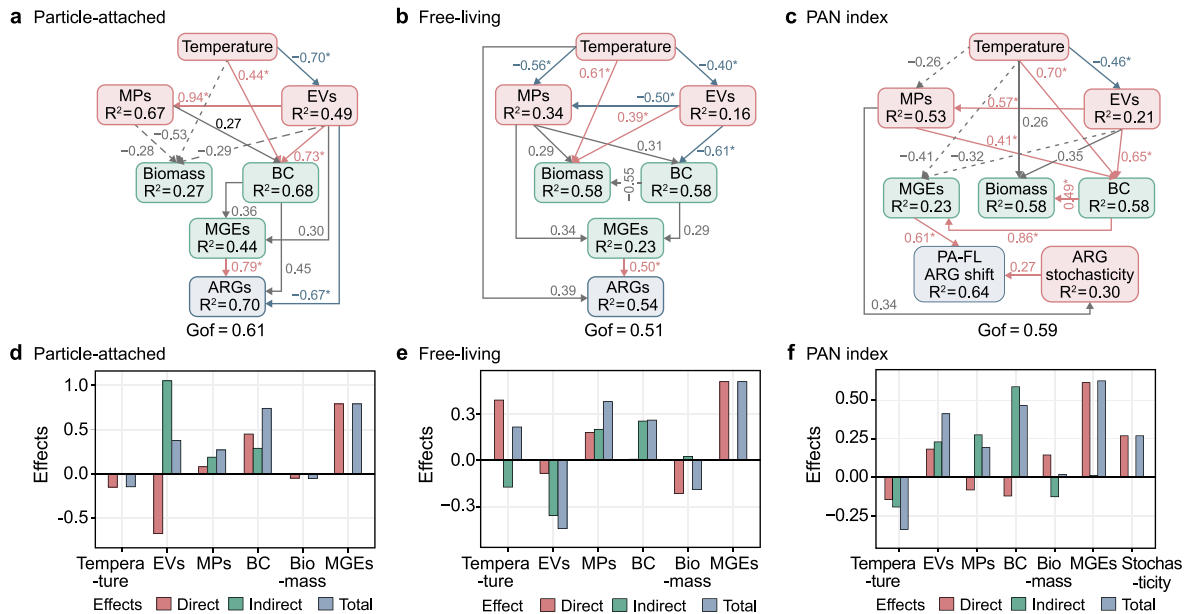


Fig. 4. **a–c**, PLS-PM shows the effects of different factors on the total normalized abundance of ARGs in PA (**a**) and FL fractions (**b**) and the lifestyle shift of ARGs (i.e., PAN index) (**c**). Variables included for the PA and FL models were bacterial communities (BC), micropollutants (MPs), environmental variables (EVs), MGEs, and temperature. For the PAN model, ARG community stochasticity was included, and the PAN index of bacterial communities (BC), FL bacterial abundance, and the PAN index of MGEs were used. Dashed and solid lines represent the negative and positive paths. Significant paths are indicated by the asterisk ($*P < 0.05$) and colors (pink: positive; blue: negative). The path coefficients (R^2) and total standardized effects were calculated after 1000 bootstraps. Models were assessed with goodness of fit (GoF) values. **d–f**, Total standardized effects from each latent variable for PA ARGs (**d**), FL ARGs (**e**), and lifestyle shifts of ARGs (**f**) were shown in the bar chart.

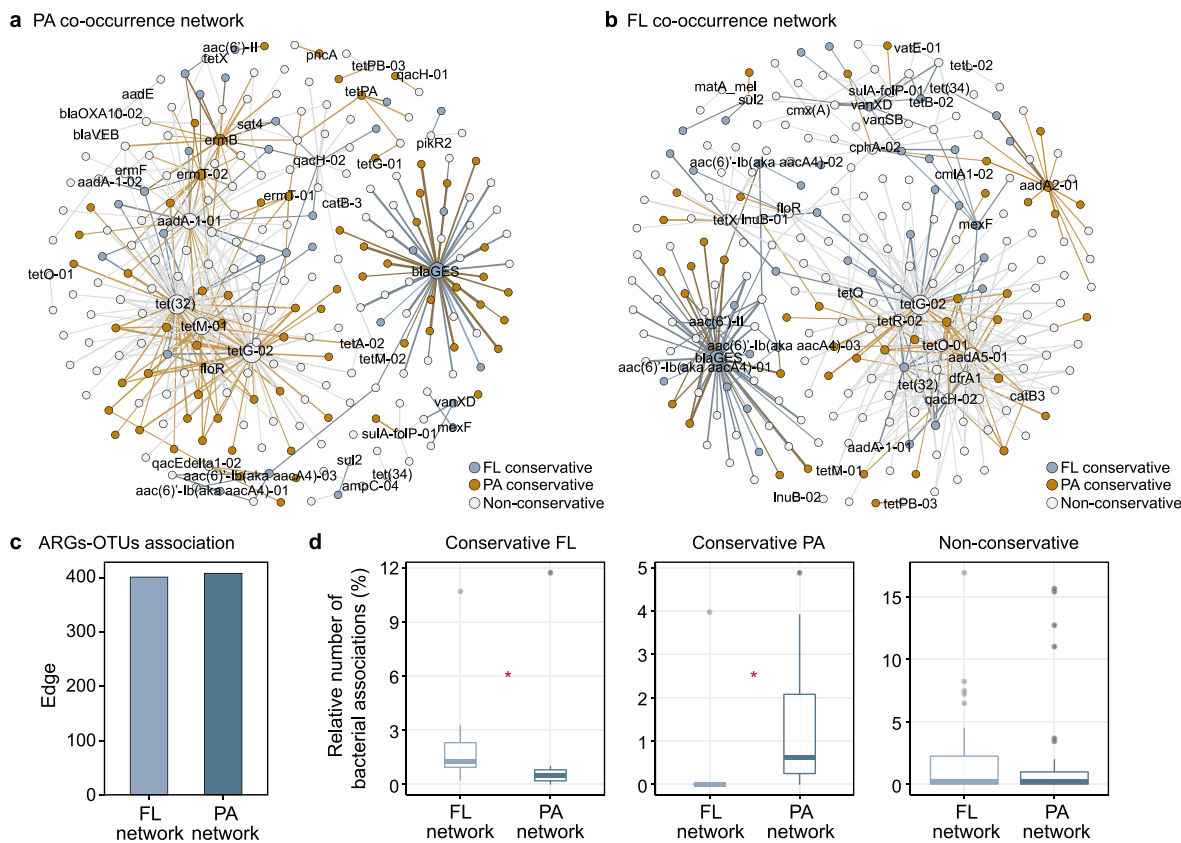


Fig. 5. a–b, Associations between ARGs and bacterial OTUs in PA (a) and FL (b) networks. c, The number of ARGs-OTUs associations in the PA and FL network. d, A comparison of degree numbers between conservative and non-conservative ARGs in different lifestyles. Significant test was done using the Wilcoxon test (* $P < 0.05$).

season with well-mixed water columns) (Fig. S4). Nutrients were found to correlate with the abundances of certain ARG types (e.g., tetracycline, MLSB, and sulfonamide) in a riverine ecosystem [35] to indicate their function in enhancing cell metabolisms and activities [36]. The PA ARGs in water columns may come from sediment resuspension in well-mixed water columns (i.e., January and April) [12] or via upstream river deposits. Additionally, positive correlations between ARGs and different physicochemical parameters, such as between sulfonamide ARGs and $\text{NO}_3\text{-N}$ or soluble reactive phosphorus, between tetracycline ARGs and DO might be due to the seasonal variations in the microbial community carrying these ARGs [37].

In addition, the abundances of FL ARGs in hypoxia water columns during the stratified columns were significantly higher than those of well-mixed water columns (Fig. S3). This phenomenon could be related to the seasonal transition of ARGs between PA and FL lifestyles (see discussion below). Moreover, the positive correlations between certain FL ARGs (e.g., tetracycline and sulfonamide resistance genes) and nutrients were like the ones for PA ARGs. This could be due to the detachment of ARG-carrying microbes from particles to live freely [38].

As a typical subtropical deep freshwater ecosystem, the SR experienced a seasonal vertical stratification in the water column starting from summer to fall [12]. Similarly, vertical differentiation of the ARG profile from the surface to bottom water was also observed (Fig. 2c). The increased abundance of multidrug-, sulfonamide-, and vancomycin-related ARGs towards hypoxic zones at the bottom of the SR might be due to the sinking and/or resuspension of particles containing ARGs from the sediments [39]. Similarly, a previous study reported an increasing trend of tetracycline and integron abundance with depth in a subalpine lake

during stratification periods [39]. Suspended particles were vectors for transporting surface microbiome to deep water in the marine environment [19]. Our results hereby suggested that ARGs might be transported by the same mechanism.

Correspondingly, beta-lactamase resistance genes were higher in the shallow water compared to the bottom water of SR (Fig. 2c). Its strong significant correlations with temperature and chlorophyll-a (Fig. S5) indicate that the phytoplankton bloom might have a role in beta-lactamase resistance gene dissemination [40]. Overall, these results highlight that the reservoir undergoing seasonal vertical mixing and thermal stratification may regulate ARGs in PA and FL differently through the changes of abiotic (e.g., physico-chemical parameters) factors. RF results indicated that Sulfamethoxazole was one of the important MPs influencing the total ARGs in PA and FL (Fig. S6). Additionally, specific significant positive associations between sulfonamide resistance genes and the concentration of sulfamethoxazole in PA and FL fractions were also found (Fig. S5). However, the overall impact of micropollutants on the dissemination of whole ARGs may be insignificant due to their low concentrations in the SR [14]. Regardless, the impact of MPs was found to be comparably stronger in FL than PA, according to PLS-PM, which could be due to a higher concentration of MPs in bulk water than in the particles. The discrepancy between MP concentration in bulk water and particles has been reported earlier [41]. In order to get a better understanding, MP concentrations in the particles need further investigations in future studies.

Mounting evidence suggests that PA and FL fractions have distinct microbial communities in various aquatic environments [16,19]. However, some recent evidence showed that PA and FL microbial communities were connected and could change lifestyles in response to environmental changes [21,38,42]. Although some

studies have explored PA and FL resistome in various environments [10,43,44], this study represents the first investigation into the ARG dynamic in an aquatic system by considering the connectivity between PA and FL lifestyles. Using the PAN index as a proxy of lifestyle preference, it was found that most ARGs had a non-conservative lifestyle in the SR. Despite that, several conservative ARG subtypes in both PA and FL fractions tended to have significantly higher associations with bacterial OTUs in their preferred lifestyle (Fig. 5d). This phenomenon may be due to a better adaptation of microbial communities carrying these ARGs in their preferred lifestyle than in the corresponding lifestyle [16,45]. In this context, it also indicates that lifestyle could be important in determining the dissemination of specific ARGs. Additionally, the associations between conservative ARGs and conservative bacterial OTUs with the same lifestyle preference were also found (one association in the PA network; 16 associations in the FL network).

Apart from unique host-ARGs relationships in PA and FL, some host-ARG relationships were found in both fractions. For example, OTU 420 and OTU 274 from Family Chitinophagaceae were correlated with *tetG-02* (tetracycline resistance gene). OTU 53 and OTU 38 from the same family (i.e., Chitinophagaceae) were associated with *blaGES* (beta-lactamase resistance gene). Interestingly, this bacterial family was reported to correlate with many ARGs such as vancomycin, tetracycline, MLSB, multidrug, and aminoglycoside resistance gene in the freshwater waterbody [46,47]. In addition, eight OTUs from phylum Cyanobacteria were found to be significantly correlated with *blaGES*, indicating their importance as reservoirs of ARGs in SR. Bacteria from Family Burkholderiaceae, which had been reported as the reservoir for multiple ARGs [48], were significantly associated with *qacH-02* (multidrug resistance gene) and *tetG-02* (tetracycline resistance gene) in both fractions in SR. All these results indicate that ARGs carrying bacteria might be detached from particles or have shifted lifestyles (see discussion below).

The PAN indices were further incorporated into PLS-PM to study the underlying factors governing the ARGs' lifestyle transition. The result suggested that temperature (total effects = -0.34) and DO (total effects = 0.4) might be critical factors indirectly driving the transition from PA to FL lifestyle by influencing the lifestyle transition of MGEs and bacteria (Fig. 4c and f). Noticeably, MPs may pose a weaker, but non-negligible force in lifestyle transition than in the individual lifestyle since the total effects of MPs to ARGs transition (0.19) was smaller than that in PA and FL (0.27 and 0.37, respectively) in PLS-PM results. Many previous studies have demonstrated the relationships between micropollutants and ARGs, especially between antibiotics and ARGs [49,50]. During stratified seasons, the SR experienced phytoplankton blooms (chlorophyll-a > 10 µg L⁻¹) and vertical stratification leading to hypoxia conditions in the deep layer of water column [12]. The

micro-patches with low oxygen levels could be the critical factor regulating the PA microbial communities [38,51]. Moreover, hypoxia conditions could reduce the oxygen diffusion within the particles more than under ambient oxygen conditions [52]. Therefore, microbial taxa carrying ARGs and/or MGEs may shift their lifestyle (i.e., from PA to FL lifestyle) timely to maintain their metabolisms [53]. In a relatively different environment, Yu et al. (2020) found a correlation between oxygen and size-fractionated ARGs abundance (PA and FL) in full-scale wastewater treatment plants (WWTPs) [43]. Alternatively, the transition of ARGs between the PA and FL lifestyles could be related to microbial responses to the increasing dissolved organic carbon produced during phytoplankton blooms [54]. A previous study found that the extensive bacterial exchange between attached and free-living communities during phytoplankton bloom could lead to an increased microbial community similarity between PA and FL fractions [21]. However, more complex abiotic and biotic interactions might play a role in governing this phenomenon. Thereby, a controlled experiment is needed to further evaluate the factors contributing to PA and FL ARGs community transition.

In this study, some evidence indicated that the PA rather than FL fractions might be more suitable micro-environments for ARG dissemination. Firstly, PLS-PM suggested that MGEs had a higher total effect on the abundance of ARGs in the PA fraction than that in the FL fraction (Fig. 4). In addition, the Spearman correlation analysis revealed a higher number of significant positive correlations between ARG and MGE types in the PA fraction (38) than in FL fraction (28) (Fig. S7). Secondly, PPR analysis showed a higher potential of HGT rate in the PA fraction than FL fraction across seasons (Fig. S8). Thirdly, the null model analysis indicated that the assembly of PA ARG communities had significantly higher stochasticity than that of FL ARG communities (Fig. S9). These findings are consistent with the report of Yu et al. (2021), demonstrating that the PA ARG communities exhibit higher stochasticity than the FL ARG communities in the surface waters of the Yellow River [44]. This phenomenon suggests a higher probability of HGT occurred in the PA than in FL fraction [5]. Furthermore, the higher abundances of ARGs and MGEs in the PA than in the FL fraction (Fig. 1a) may favor ARG exchange in the PA fraction [55,56] due to the limited space and close contact between cells on particle [55]. A shotgun metagenomics-based study in the marine environment reported that higher abundances of ARGs and MGEs were responsible for social interactions and cell-to-cell transfer in the PA fraction rather than the FL fraction [57]. This indicated that HGT may be a more common life strategy for microorganisms living on suspended particles. Intriguingly, the transition of ARGs from PA to FL fraction during this stratified season might not only lead to an increase in the ARG abundances (Fig. S3) but also enhance the HGT probability in FL fraction (Fig. S10).

Taken together, this study showed that size-fractionation of ARGs into PA and FL ARGs could be useful to model the lifestyle transition pattern of ARGs in a freshwater reservoir (Fig. 6). Environment variables (e.g., DO and nutrients) changing on a seasonal basis might be responsible for this phenomenon. It is noteworthy that this study mainly targeted the internal ARGs, while external ARGs can be abundant in similar environments [43,58] and may act as a potential source to foster different ARG dissemination pathways [58].

5. Conclusion

In this study, the influences of the seasonal hydrological dynamics (i.e., vertical mixing and thermal stratification) on the ARG profiles in individual PA and FL fractions but also in the transition between the two lifestyles (e.g., from PA to FL lifestyle) were

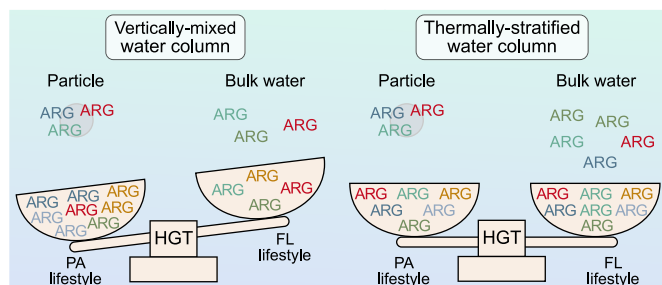


Fig. 6. Conceptual diagram of inter-lifestyle ARGs transition (between PA and FL) under the influence of vertically-mixed and thermally-stratified water columns in the deep freshwater reservoir. The changes in physico-chemical parameters of the water column indirectly modulate ARG transition through MGEs (HGT).

revealed in a deep freshwater reservoir. The main conclusions are as follows:

- (1). PA and FL ARGs responded differently to the changes in physicochemical parameters induced by the seasonal-hydrological pattern (i.e., vertical mixing vs. thermal stratification). The abundance of PA ARGs increased in the mixing period characterized by high nutrient loads, while the abundance of FL ARGs increased in the stratified period characterized by severe DO depletion in the deep-water column.
- (2). The seasonal-hydrological pattern indirectly shaped the ARGs' transition between the PA and FL lifestyles through the changes in water temperature and DO.
- (3). Despite a larger proportion of non-conservative ARGs, a fraction of conservative ARGs were found to be more dominant within their preferred lifestyle based on network analysis. Additionally, there was more free-living ARG-bacteria association in the FL network than the particle-attached ARG-bacteria association in the PA network.
- (4). Although the HGT was the main mechanism driving the dissemination of ARGs in PA and FL fractions and between the PA and FL fractions, a higher potential for HGT was found in the PA than in FL fractions.

CRediT author statement

Bob Adyari: Conceptualization, Investigation, Methodology, Formal analysis, Software, Writing - original draft, Visualization. Liyan Hou: Formal analysis, Validation, Writing - review & editing. Lanping Zhang: Investigation, Methodology, Data curation, Software. Nengwang Chen: Investigation, Methodology, Resources, Funding acquisition. Feng Ju: Methodology, Writing - review & editing. Longji Zhu: Methodology, Resources. Chang-Ping Yu: Resources, Supervision, Funding acquisition. Anyi Hu: Conceptualization, Data curation, Software, Validation, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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 to this article can be found online at <https://doi.org/10.1016/j.ese.2022.100223>.

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