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Urban ponds as hotspots of antibiotic resistome in the urban environment

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ABSTRACT

The occurrence, dissemination and assembly processes of antibiotic resistance genes (ARGs) in urban water ecosystems are far from being understood. Here, we examined the diversity and abundance of ARGs in urban water ecosystems including landscape ponds, drinking water reservoirs, influents (IFs) and effluents (EFs) of wastewater treatment plants of a coastal city, China through high-throughput quantitative PCR. A total of 237 ARGs were identified, where multidrug, aminoglycoside and beta-lactamase resistance genes were the most abundant. Urban ponds had a comparatively high diversity and large numbers of shared ARGs with IFs and EFs. The average absolute abundance of ARGs (1.38×10^7 copies/mL) and mobile genetic elements (MGEs) (4.19×10^6 copies/mL) in ponds were only one order of magnitude lower than those of IFs, but higher than those of EFs and reservoirs. Stochastic processes dominated the ARG community assembly in IFs and ponds due to the random horizontal gene transfer caused by MGEs. These results imply that urban ponds are hotspots of ARGs. We further identified 25, 3, and 11 indicator ARGs for tracing the ARG contamination from IFs, EFs and ponds, respectively. Our study represents the first to highlight the role of urban ponds in the dissemination of ARGs.

1. Introduction

Urban water ecosystem plays an important role in a variety of ecological and economic services. These may include water sources, ecological habitats, recreational activities and urban landscape (Lundin and Morrison, 2002). Such ecosystems have been impacted heavily due to rapid urbanization, industrialization and microbial contamination, etc. In particular, antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) have been widely detected in various urban water habitats such as urban lakes (Yang et al., 2017b), wastewater treatment plants (WWTPs) (Guo et al., 2017; Lorenzo et al., 2018) and rivers/streams (Berglund et al., 2015). The spread of ARGs in urban water ecosystems has mainly caused by the excessive use of antibiotics that subsequently poses threat to human health and environment (Berendonk et al., 2015). More importantly, ARGs can be exchanged and transferred among environmental microorganisms. The transfer mainly takes place via horizontal gene transfer (HGT) carried out by mobile genetic elements (MGEs) including plasmids, transposons and integrons (Zhu et al., 2013). Urban water ecosystems serve as environment–human interfaces, where agricultural waste, domestic wastewater influents and effluents are significant reservoirs of both ARB and ARGs. They are then discharged into the receiving aquatic systems (e.g., rivers, lakes and ponds), and thus accelerating the spread and enrichment of ARGs (Lorenzo et al., 2018). Since there are possibilities that the city dwellers may get in direct contact with the ARGs in such aquatic environments, it is essential to investigate the distribution and dissemination of ARGs in

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urban water ecosystems.

Among different urban water habitats, WWTPs are well known hotspots for the spread of ARGs, as they create favorable conditions for ARB proliferation and the HGT of ARGs (Corno et al., 2019; Guo et al., 2017; Lee et al., 2017; Pazda et al., 2019). Earlier studies have mainly focused on the responses of ARGs and MGEs to WWTP treatments (Li et al., 2019; McConnell et al., 2018; Zheng et al., 2017b), while others have investigated the fate of ARGs and MGEs through WWTP, and occasionally the receiving environment (Corno et al., 2019; Lee et al., 2017; Quintela-Baluja et al., 2019). For example, it has been shown that WWTP effluents have the ability to accumulate the ARGs and MGEs in the receiving river (Berglund et al., 2015; Cacace et al., 2019; Lorenzo et al., 2018) and waste stabilization ponds (Neudorf et al., 2017). Similarly, ARGs conferring resistance against beta-lactam, macrolide, quinolone, tetracycline, sulfonamide, trimethoprim and multidrug (Pazda et al., 2019) as well as several types of MGEs (Di Cesare et al., 2016; Fiorentino et al., 2019; Guo et al., 2017) have frequently been detected in WWTPs worldwide.

Urban reservoirs, that are commonly utilized for the abstraction of drinking water and recreational activities, have recently been investigated for high-throughput profiles of ARGs and MGEs, as well as the associations between ARGs and microbial communities (Chen et al., 2019; Fang et al., 2019; Guo et al., 2018; Huerta et al., 2013). Some reservoirs exhibited high levels of ARGs and MGEs, with multidrug resistance as the most dominant ARG type (Fang et al., 2019; Guo et al., 2018). It has been suggested that multiple environmental factors, such as nutrients and precipitation, influence the composition and dynamics of ARGs in complex ways (Chen et al., 2019; Fang et al., 2019; Guo et al., 2018; Huerta et al., 2013). Urban landscape ponds play an important role in the human/environment-water interface by providing water for agriculture and aiding in habitat restoration (Hassall, 2014). However, too little attention has been paid to the occurrence and fate of ARGs and MGEs in such ponds. In our earlier studies, it was found that landscape ponds are constantly or sporadically impacted by domestic sewage, and thus they can harbor a significant proportion of sewage- and fecal-indicator taxa (Hu et al., 2018). This may imply that certain amount of ARGs from these taxa exist in landscape ponds that can further spread into the environment.

Although stochastic (i.e., speciation, birth, death and immigration) and deterministic (i.e., environmental filtering and bio-interactions) processes simultaneously remodel the microbial communities in various urban aquatic habitats (Hassell et al., 2019; Hou et al., 2019; Hu et al., 2018; Zhang et al., 2019), still the community assembly mechanisms of ARGs in urban ponds are largely unknown (Fang et al., 2019; Guo et al., 2018; Zhu et al., 2017). Stochastic processes (i.e., dispersal limitation) appeared to determine the prokaryotic community assembly in landscape ponds (Hu et al., 2018), and may also remodel their ARG communities. Moreover, deterministic processes such as abiotic (e.g., temperature, pH, nutrients and chemical pollutants) and biotic factors (e.g., MGEs, microbial biomass and composition) need to be considered (Hu et al., 2020; Peng et al., 2020). Understanding how these processes contribute to ARG community assembly is important to design mitigation strategies for controlling and reducing the dissemination of ARGs in urban water ecosystems.

Despite the considerable amount of research that has been carried out on ARGs and MGEs for some urban water habitats, a comprehensive study assessing their distribution and dissemination in urban water ecosystems at the city level is still lacking (Huang et al., 2019). Here, we comprehensively explore and compare the diversity and abundance of ARGs and MGEs among different urban water habitats by adopting seawater as a natural reference. We aimed to (i) characterize the ARG profiles and their controlling factors in major urban water habitats, especially for urban ponds; (ii) identify the indicator ARGs for different habitats; and (iii) assess the relative importance of deterministic and stochastic processes underlying ARG community assembly. It was hypothesized that urban ponds may be the potential hotspots for ARGs due to fecal/sewage pollution and the different assembly mechanisms may be responsible for remodeling the assembly of ARGs communities in different urban water habitats. Similarly, it was considered that stochastic processes might mainly shape the structure of ARGs communities of the urban ponds. This study provides fundamental data for evaluating the ecological risks of ARGs in urban water ecosystems and further enhances the understanding of the role of urban ponds in accelerating the spread of ARGs in the environment.

2. Materials and methods

2.1. Sampling area

Xiamen is a major coastal city located in southeast China, with an area of 1699 km^2 (Fig. S1). Due to rapid urbanization, the urban land area has nearly doubled in Xiamen, from 180 km^2 in 2007 to 334 km^2 in 2016, while the population of Xiamen increased by 29% from 3.05 million in 2007 to 3.92 million in 2016 (Hu et al., 2018). Similarly, the total wastewater volume increased rapidly from around 210 million tons in 2007 to 320 million tons in 2016 (Xiamen Environmental Protection Bureau, 2007–2016). Previous studies have showed that the increasing discharge of nutrients and chemical pollutants into urban water bodies of Xiamen led to eutrophication and deterioration of water quality (Hu et al., 2018; Huang et al., 2013; Yang et al., 2017a; Zhang et al., 2011). Likewise, the harmful microorganisms (e.g., sewage and fecal indicator microorganisms) and genes (e.g., ARGs and MGEs) were detected in considerable abundance in different habitats (i.e., drinking water reservoirs, urban ponds and WWTPs) of the urban water ecosystems of Xiamen (An et al., 2018; Guo et al., 2018; Hou et al., 2019; Hu et al., 2018; Wang et al., 2020).

2.2. Sampling design, collection and environmental factors

To investigate the distribution of ARGs across different habitats of urban water ecosystem of Xiamen, water samples were collected from drinking water reservoirs, urban ponds and WWTPs as explained by Hou et al. (2019) and Hu et al. (2018) (Fig. S1). In this study, drinking water reservoirs represent relatively well-managed water bodies, while urban ponds were likely under the influence of anthropogenic disturbances. For WWTPs, both influent (IF) and effluent (EF) samples were collected. The IF can represent a collection of local pollution sources (raw sewage), whereas EF was considered as a proxy of treated waters (Hu et al., 2018). Moreover, as the seawater might be little influenced by anthropogenic activities, therefore seawater samples were included as a natural reference.

Surface water samples were collected from three reservoirs (12 samples) and 16 urban landscape ponds (16 samples) from the rural and sub-urban developing areas of Xiamen, respectively, during spring 2017 (Hu et al., 2018) (Fig. S1). In addition, three days 24-h composite samples of IF and EF (3 IF and 3 EF samples per plant; 30 samples in total) were collected from five municipal WWTPs of Xiamen between February 28th and March 1st, 2016 (Hou et al., 2019) (Fig. S1). Surface seawater samples (14 samples) were collected by the RV YueZhanYuKe 10 from the open ocean areas of South China Sea between July 17th and 24th, 2019 (Fig. S1). Water samples were filtered through a 0.22 μm pore size Sterivex-GP filter (Millipore, Billerica, MA, USA) to collect microbial cells, and subsequently stored at − 80 °C until analysis.

Environmental factors including temperature, pH, conductivity (EC), salinity and dissolved oxygen (DO) of samples from reservoirs, ponds and WWTP were measured in situ using a portable probe (Hach HQ40d; Loveland, CO, USA). Additionally, for water samples collected from reservoirs and ponds, oxidation-reduction potential (ORP) was also found out using the same probe. Similarly, Chl-a concentration was determined for reservoir and ponds samples using a Phyto-PAM phytoplankton analyzer (HeinzWalz, Effeltrich, Germany). Nutrients (NH_4–N, NO_2–N, NO_3–N and total nitrogen (TN)) were measured using a Lachat

QC8500 Flow Injection Auto-analyzer (Lachat Instruments, Loveland, CO, USA) (Hu et al., 2018). Likewise, NH_4–N, TN and total phosphorus (TP) for WWTP samples were analyzed using a Lachat QC8500 Flow Injection Auto-analyzer (Lachat Instruments, Loveland, CO, USA) (Wang et al., 2020). The concentration of suspended solids (SS), chemical oxygen demand (COD) and 5-day biological oxygen demand (BOD_5) in IF and EF samples were analyzed using standard methods (China's State Environment Protection Agency, 2002). Temperature and salinity of seawater samples were measured in situ using a CTD48M (Sea & Sun Technology, Trappenkamp, Germany). NH_4–N, NO_2–N, NO_3–N, TN, PO_4–P and SiO_3–Si were measured colorimetrically by an AA3 Auto-analyzer (Seal, Norderstedt, Germany).

2.3. DNA extraction, PCR amplification and 16S rRNA gene amplicon sequencing

DNA was extracted from filters using the FastDNA SPIN Kit for Soil (Qbiogene-MP Biomedicals, Irvine, CA, USA) according to our previous modifications (Hu et al., 2018). The V4-V5 region of the bacterial 16S rRNA genes was amplified with primer pair 515F (5'-GTG YCA GCM GCC GCG GTA-3') and 907R (5'-CCG YCA ATT YMT TTR AGT TT-3') (Quince et al., 2011). A 25 μL PCR reaction volume was used, containing ~20 ng DNA template, 12.5 μL of the AmpliTaq™ Gold PCR Master Mix (2 ×) (Applied Bio-systems, CA, USA) and 0.4 μM of each primer. The PCR amplification procedure consisted of initial denaturation at 95 °C for 5 min, followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s, and a final extension at 72 °C for 10 min. The PCR products were gel purified and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Hu et al., 2018). The purified PCR products were sequenced on an Illumina HiSeq 2500 platform (Illumina Inc., San Diego, CA, USA) with paired-end approach (2 × 250 bp). The raw sequence data was deposited in the NCBI short reads archive database under BioProject numbers PRJNA407260, PRJNA545043 and PRJNA608982.

2.4. Sequence analysis

Raw 16S rRNA gene reads were denoised and assembled using DADA2 v1.1.3 (Callahan et al., 2016) by following the procedure described in Hu et al. (2019). The high-quality reads were then clustered into amplicon sequence variants (ASVs) with single-nucleotide resolution. The taxonomic assignment of each ASV was performed using the RDP classifier with the SILVA database v132 (Quast et al., 2013) with a confidence threshold of 80%. All samples used in this study were randomly resampled to the smallest library size (16,000 reads) in order to standardize the uneven sequencing effort.

2.5. High-throughput quantitative PCR (HT-qPCR) analysis of ARGs

HT-qPCR analysis was performed by using a SmartChip Real-time PCR System (WaferGen Inc., USA) with SYBR Green I® chemistry method. A total of 296 primer pairs were used, targeting 285 ARGs, eight transposase and two integrase genes as well as the 16S rRNA gene (Su et al., 2015; Zhu et al., 2017). Amplification was carried out in a 100 nL reaction system and the thermal cycle was set as follow: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s (Su et al., 2015; Zhu et al., 2017). A melting curve analysis was performed to check the specificity of the PCR products. All qPCR reactions were performed in triplicate and a negative control was included. The HT-qPCR data was analyzed by using the SmartChip qPCR software (V2.7.0.1), and the reactions with multiple peaks or with amplification efficiency beyond 90–110% were discarded (Su et al., 2015; Zhu, et al., 2017). A threshold cycle (Ct) of 31 was used as the detection limit, and only samples having more than two replicates were retained for further analysis. Relative copy number was calculated by using Eq. (1) (Looft et al., 2012):

$$Relative\ gene\ copy\ number = 10^{(31-Ct)/(10/3)} \quad (1)$$

The average copy number of 16S rRNA genes per bacterial cell is estimated at four based on the Ribosomal RNA Operon Copy Number Database (Stoddard et al., 2015). Accordingly, the relative abundance of ARGs (copies per bacterial cell) was estimated by dividing the ARGs concentration in each sample by its corresponding number of bacterial cells, which can be calculated by dividing the copy number of 16S rRNA gene by four (Zhu et al., 2017). The absolute abundance of ARGs was calculated by normalizing the absolute 16S rRNA gene copy number (Su et al., 2015; Wang et al., 2020; Zhu et al., 2017).

The absolute copy number of 16S rRNA gene was determined on a LightCycler® Roche 480 Real-time PCR instrument (Roche Inc., Basel, Switzerland). A 25 μL PCR reaction volume was used, containing 10 μL LightCycler® 480 SYBR Green I Master Mix (2 ×) (Applied Bio-systems, CA, USA), 0.5 μM of each primer, 1 μg/L bovine serum albumin (Sigma, Steinheim, Germany), 20 ng of template DNA and nuclease-free PCR-grade water. The amplification was performed as follow: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and extension 72 °C for 15 s. The standard curve was achieved by using tenfold serially diluted 16S rRNA gene incorporated plasmids. The assay efficiency of the bacterial 16S rRNA genes was ~99.3% with R^2 values > 0.998.

2.6. Statistical analysis

Nonmetric multidimensional scaling (NMDS) was used to display the structural patterns of the ARG and bacterial communities as based on Bray-Curtis dissimilarity matrices. Permutational multivariate analysis of variance (Adonis) and the analysis of similarity (ANOSIM) were used to test the significance of the difference of ARG communities among different habitats (i.e., seawater, drinking water reservoirs, urban ponds, IFs and EFs) (Hu et al., 2019).

The indicator ARG subtypes for each habitat were identified by using the 'IndVal' function in R package labdsv (Roberts, 2007). Only ARG subtypes with highly significant indicator values (i.e., IndVal value > 0.70, $P < 0.01$) were considered as good indicators for their respective habitats. Moreover, to identify the most correlated variables (i.e., ARGs, MGEs and bacterial ASVs) involved in the discrimination between habitats, the N-integration algorithm data integration analysis for biomarker discovery using latent components (DIABLO) was conducted using the package mixOmics (Rohart et al., 2017). Briefly, an optimal number of variables, which can discriminate different sample groups (i.e., habitats) with the lowest possible error rate, were determined using 'tune.block.splsda' function in the package mixOmics (Adyari et al., 2020; Rohart et al., 2017). A relevance network graph showing strong correlations ($\geq |0.8|$) among variables was generated and visualized using Cytoscape v3.7.2 (https://cytoscape.org/cy3.html). The relative contribution of bacterial communities, MGEs, environmental variables and spatial factors to ARG community composition was assessed by using variation partitioning analysis (VPA) (Hu et al., 2017a). The first two axes of NMDS ordination of bacterial communities were included into VPA as the response variables. Moreover, the geographic coordinates were transformed into principal coordinates of neighborhood matrices (PCNM) using the 'PCNM' function in vegan (Oksanen et al., 2009). Then, the PCNM variables were used as spatial factors for VPA (Borcard and Legendre, 2002). Prior to VPA, multicollinearity between environmental variables was assessed with the variation inflation factor (VIF) using "vifstep" function in R package usdm (Naimi et al., 2014). Only variables with VIF < 10 were included into further analysis (Adyari et al., 2020). Forward selection was used to identify the best response variables explaining community variation with the 'ordiR2step' function in R package vegan (Oksanen et al., 2009).

All statistical analysis and visualization were performed using R v3.60 with the packages phyloseq (McMurdie and Holmes, 2013),

ggplot2 (Wickham, 2016) and ComplexHeatmap (Gu et al., 2016).

2.7. Null model analysis

The relative importance of deterministic and stochastic processes underlying the assembly of ARGs communities was assessed using null model analysis (Zhang et al., 2019). To conduct the analysis, R code that is available on http://mem.rcees.ac.cn/download/ was used. Briefly, the expected similarity (E_{exp}) of null expected communities was generated based on 1000 random shuffles of the original community data. During this procedure, community richness and the proportion of species occupancy were kept the same as the observed ones (Chase et al., 2011; Zhang et al., 2019). Then, the relative importance of stochastic processes was calculated using Eq. (2):

$$Stochasticity(\%) = 1 - \frac{S_{obs} - E_{exp}}{S_{obs}} \quad (2)$$

Where S_{obs} refers to the observed similarity of the actual communities, and E_{exp} refers to the average expected similarity of null expected communities (Zhang et al., 2019).

3. Results

3.1. Diversity and abundance of ARGs detected in urban water ecosystems

A total of 237 ARG subtypes were detected in the urban water ecosystems, including eight major ARG types (i.e., aminoglycoside, beta-lactamase, chloramphenicol, Macrolide-Lincosamie-Streptogamin B (MLSB), multidrug, sulfonamide, tetracycline and vancomycin). The average number of ARGs detected in different urban water habitats decreased in the following order: IFs > EFs > ponds > reservoirs, ranging from 87 to 20 per sample (Fig. 1A). The average number of ARGs detected in seawater samples (natural reference habitat; 24 per sample) was lower than in ponds (56 per sample) but higher than reservoirs (20 per sample). Multidrug, aminoglycoside and beta-lactamase resistance genes were the most frequently detected ARGs in urban water ecosystems, accounting for 24–40%, 17–32% and 12–20% of total detection frequency, respectively (Fig. 1A). Among the detected ARGs, 66 were shared among all habitats of the urban water ecosystem (Fig. 2). Notably, ponds shared a higher proportion of ARGs with IFs and EFs (151) than reservoirs (73). Additionally, seawater shared a proportion of ARGs with IFs (93), EFs (92), ponds (107) and reservoirs (70) (Fig. 2), respectively, which mainly consisted of multidrug, aminoglycoside and beta-lactamase resistance genes (Fig. 1A).

Compared with seawater, urban water ecosystems had a significantly higher abundance of ARGs (Kruskal-Wallis test, $P < 0.05$). The absolute abundance of ARGs in urban water ecosystems showed a similar pattern with the diversity of ARGs (Fig. S2). The abundance of ARGs in reservoirs was the lowest (average 7.84×10^4 copies/mL), while IFs had the highest (average 2.07×10^8 copies/mL). WWTPs processes significantly reduced the abundance of ARGs by two orders of magnitude from IFs to EFs (Kruskal-Wallis test, $P < 0.05$; Fig. S2). Moreover, the average absolute abundance of ARGs in ponds was 1.38×10^7 copies/mL, which was higher than that in EFs (Kruskal-Wallis test, $P < 0.05$).

As for the relative abundance of ARGs in different urban water habitats, reservoirs had the lowest value (average 0.07 copies per bacterial cell), while IFs harbored the highest value (2.27) (Fig. 1B), followed by EFs (1.66), and then ponds (1.40). All urban water ecosystems harbored a higher relative abundance of ARGs than that in seawater (0.01).

3.2. Relationship between the absolute abundance of ARGs and MGEs

IFs contained the highest absolute abundance of MGEs (3.26×10^7 copies/mL), followed by urban ponds (4.19×10^6 copies/mL), EFs (2.69×10^6 copies/mL) and then reservoirs (1.01×10^5 copies/mL) (Fig. S2). Overall, the absolute abundance of MGEs was significantly correlated with the total absolute abundance of ARGs in the investigated samples ($n = 72$, $r = 0.96$, $P < 0.001$) (Fig. 3), suggesting a strong co-occurrence relationship between ARGs and MGEs. Despite this, the strength of the correlation between the absolute abundance of ARGs and MGEs varied between habitats. For instance, the correlation in ponds ($r = 0.98$, $P < 0.001$) and IFs ($r = 0.95$, $P < 0.001$) were stronger than those in EFs ($r = 0.85$, $P < 0.001$) and reservoirs ($r = 0.69$, $P < 0.05$). Additionally, a weaker correlation between the absolute abundance of ARGs and MGEs in seawater was observed ($r = 0.63$, $P < 0.05$) (Fig. 3). Notably, Spearman correlation analysis indicated the absolute abundance of most ARG types (e.g., sulfonamide, multidrug, MLSB, chloramphenicol, beta-lactamase and aminoglycoside) was significantly correlated with those of integrons, transposases and total MGEs in ponds, IFs and EFs (Fig. S3). However, not all ARGs types were significantly correlated with different MGEs in seawater or reservoirs (Fig. S3).

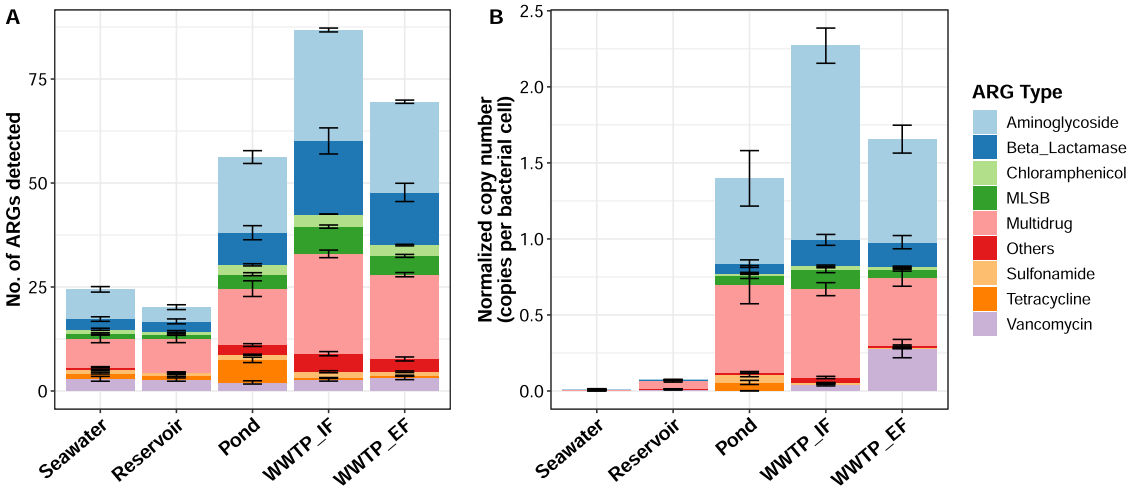

Fig. 1. Diversity (A) and abundance (B) of ARGs in surface seawater of South China Sea and in different habitats of the urban water ecosystem (i.e., drinking water reservoir, urban pond, and influent (IF) and effluent (EF) of WWTPs) of Xiamen, China. ARGs were classified into aminoglycosides, beta-lactamase, chloramphenicol, macrolide-lincosamide-streptogramin B (MLSB), multidrug, sulfonamide, tetracycline, vancomycin and others (other ARG types). The relative copy number of ARGs was normalized to copies per bacterial cell, based on the 16S rRNA gene copy number (Zhu et al., 2017).

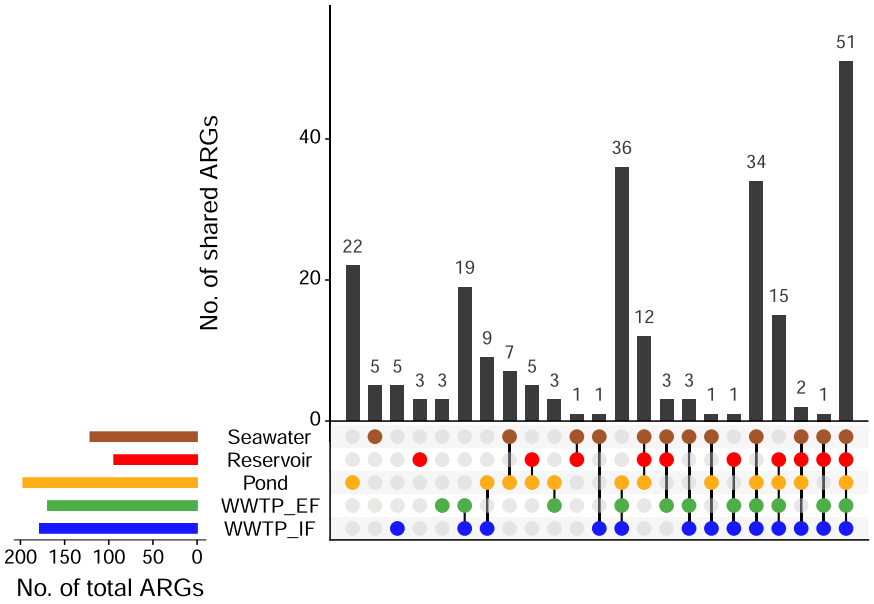

Fig. 2. Upset plot showing overlap of ARGs among different environmental groups (i.e., seawater, drinking water reservoir, urban pond, influent (IF) and effluent (EF) of WWTPs). The bar chart on the bottom left indicates the total number of ARGs for each environmental group. Number of ARGs shared among different environmental groups is indicated in the upper bar chart. Circles in the matrix on the bottom chart indicate sets of groups that intersect.

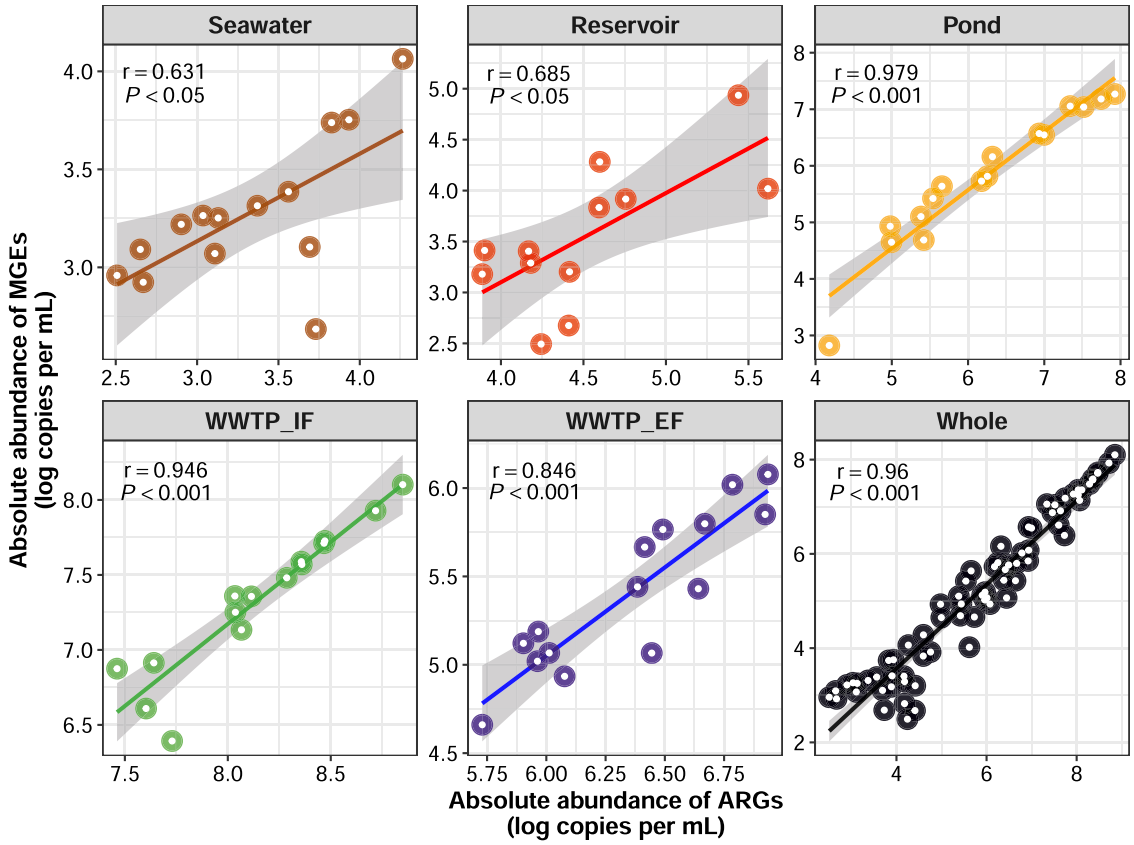

Fig. 3. Correlation analysis of the absolute abundance of ARGs and MGEs within each environmental group or in the whole dataset.

3.3. Patterns and processes of the assembly of ARG communities

PCoA ordination analysis showed that ARG communities from the same habitats tended to cluster together, revealing distinct ARG community compositions among different habitats (Adonis and ANOSIM tests, $P < 0.05$) (Fig. 4A). However, pond samples tended to gather with IFs/EFs samples, matching the previously observed pattern of ponds and IFs/EFs sharing a high proportion of ARGs (Fig. 2). Moreover, Procrustes analysis indicated that bacterial community composition was significantly correlated with ARGs composition ($M^2 = 0.56$, $P < 0.001$) (Fig. S4).

The relative importance of deterministic and stochastic processes in

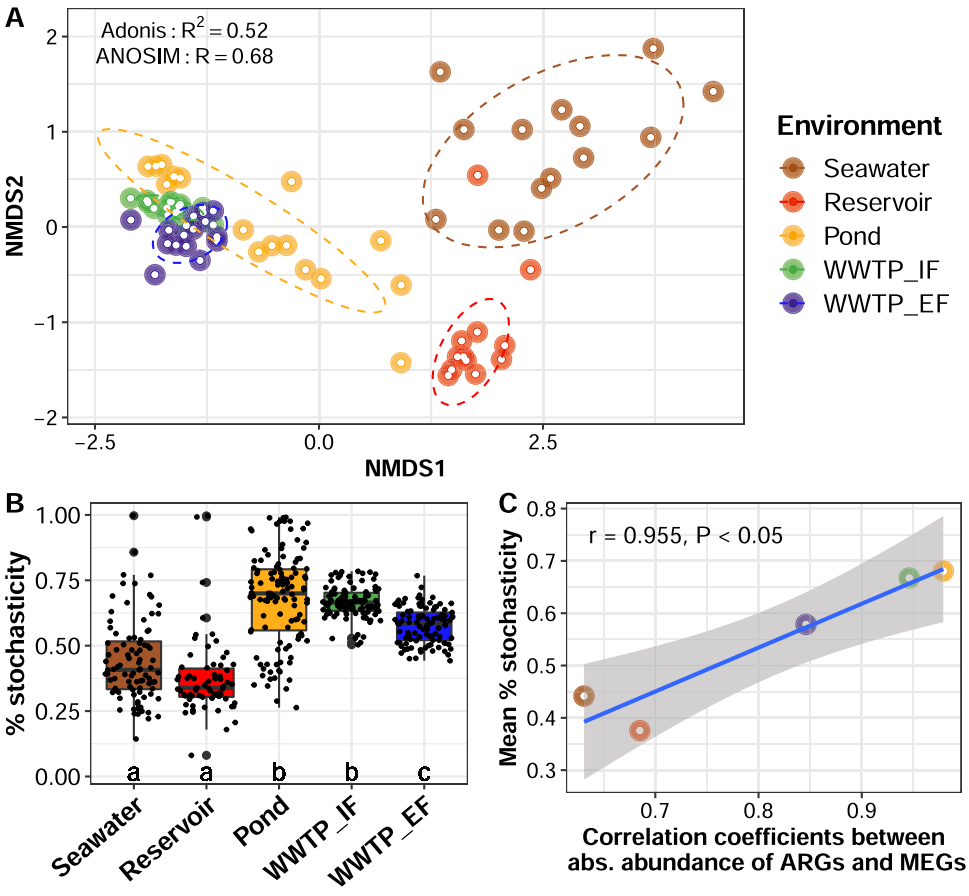

Fig. 4. PCoA ordination analyses based on Bray-Curtis distance matrix in terms of the composition of ARG communities from different environmental groups (i.e., seawater, drinking water reservoir, urban pond, and influent (IF) and effluent (EF) of WWTPs) (A). The relative importance of stochasticity in governing the assembly of ARG communities from different environments. The different letters indicate significant difference among environments (B). Correlation analysis of the relative importance of the compositional stochasticity and correlation coefficients between the absolute abundance of ARGs and MGEs (C).

governing the assembly of ARGs communities was assessed using null model analysis (Fig. 4B). It was observed that stochastic processes played a dominant role in shaping ARG communities in ponds (average 68.1%) and IFs (66.7%) compared to EFs (57.9%) and reservoirs (37.6%) (Kruskal-Wallis test, $P < 0.01$). The correlation analysis suggested that the relative importance of the compositional stochasticity is positively correlated with the correlation coefficients between the absolute abundance of ARGs and MGEs (Fig. 4C).

3.4. Influence of abiotic and biotic factors on ARG communities

VPA showed that both abiotic (environmental and spatial factors) and biotic factors (bacterial communities and MGEs) had significant effects on the β-diversity variation of whole ARG communities (including seawater) (Fig. 5B). Overall, among the urban water ARG communities, spatial factor (8.3%) was the most significant-controlling factor, followed by MGEs (5.7%), environmental variables (2.1%) and bacterial community composition (1.4%) (Fig. 5C). Moreover, the key controlling factors in shaping ARG communities in different urban water habitats varied. For example, environmental variables (9.1%) and MGEs (8.5%) significantly affected ARG composition in urban ponds (Fig. 5D). In IFs, MGEs (24.6%) had the most significant effect on ARGs composition, followed by environmental variables (6.1%), bacterial community composition (2.4%) (Fig. 5E). While in EFs, bacterial community composition (8.9%), was the only significant factor affecting ARGs composition (Fig. 5F). Moreover, no significant factors for reservoir ARG communities were found.

3.5. Indicator ARGs for different habitats of urban water ecosystems

Based on the occurrence and abundance of ARGs across the four urban water habitats, 40 ARG subtypes were identified as indicators (Fig. 6). 25, 3 and 11 ARG indicators were selected for IFs, EFs and ponds, respectively. Considering a much lower abundance profile of ARGs in the reservoir, only one ARG indicator (i.e., *pik*R2 (MLSB)) was identified. No ARG indicator was observed for seawater. IFs indicators were varied and mainly consisted of multidrug, beta-lactamase, MLSB and aminoglycoside resistance genes (Fig. 6). Notably, similar results were observed for the best discriminant ARGs identified in IFs through DIABLO analysis (Fig. 7A). EFs indicators were mainly comprised of beta-lactamase and vancomycin resistance, such as bla_{TEM} and *van*B_01. However, the indicator ARG subtypes of urban ponds were quite different from those of IFs or EFs. For urban ponds, both the indicator ARGs (Fig. 6) and the discriminant ARGs discovered through DIABLO analysis (Fig. 7B) were mainly composed of resistance genes against tetracycline and sulfonamide, which strongly associated with several transposases belong to the TnpA family (Fig. 7B). Moreover, the relevance network in IFs was primarily composed of Firmicutes (e.g., *Faecalibacterium* and *Lactococcus*) and Bacteroidetes (e.g., *Parabacteroides* and *Bacteroides*) (Table S1 and S2). Most of the corresponding taxa in the relevance network of urban ponds were composed of Bacteroidetes (e.g., *Bacteroides* and *Anaerocella*), Epsilonbacteraeota (e.g., *Arcobacter* and *Sulfurospirillum*), Firmicutes (e.g., *Anaerovorax*) and Gammaproteobacteria (e.g., *Chromatium* and *Macromonas*).

4. Discussion

With rapid urban development, and the associated promotion of vegetation and water areas within the urban matrix, the river-reservoir-ponds system has become a representation of the urban aquatic environment. However, little attention has been given to ARGs abundance or the associated processes or factors driving their dynamics in urban ponds. In the present study, HT-qPCR was utilized to comprehensively characterize the distribution, abundance, and diversity of ARGs in urban

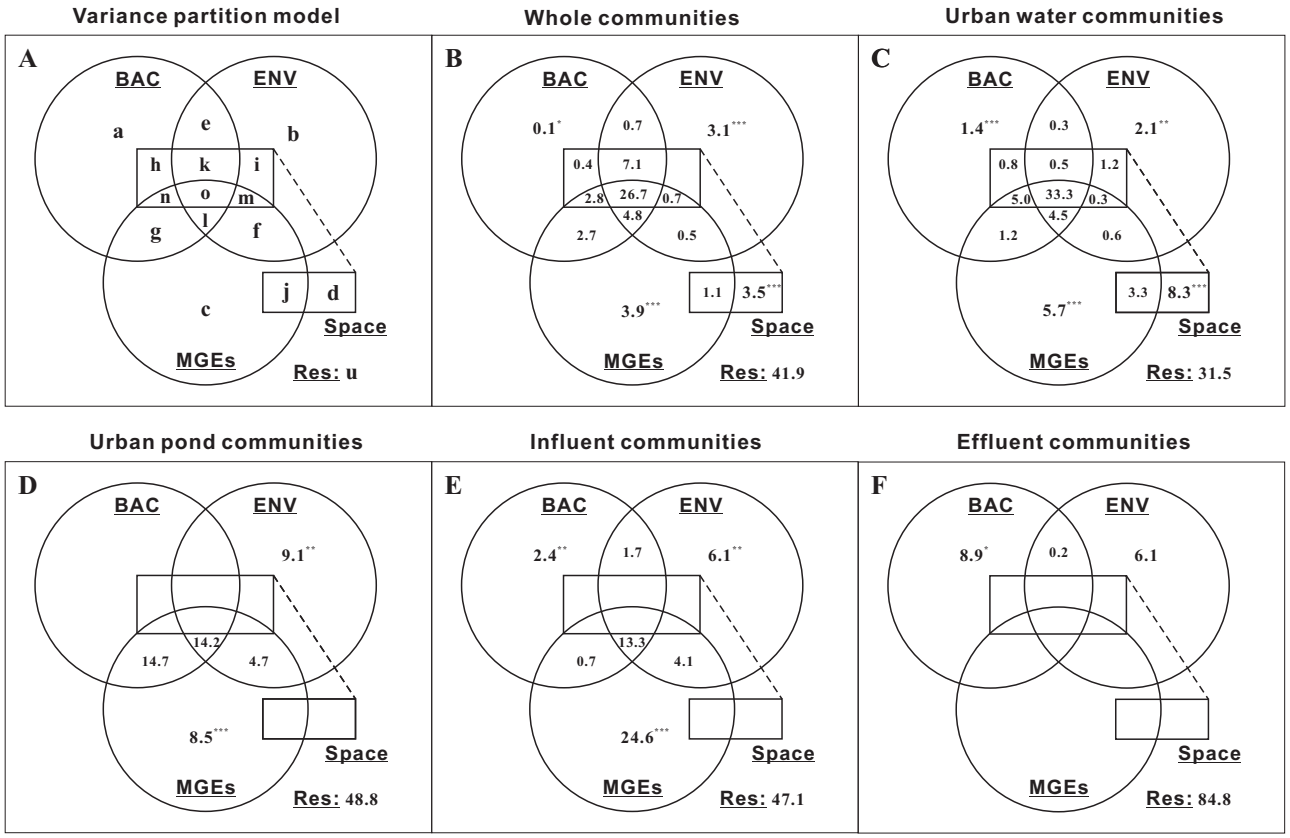

Fig. 5. Variation partitioning analysis of β-diversity variation of ARG communities among bacterial community composition (*BAC*), environmental variables (*ENV*), mobile genetic elements (*MGEs*) and spatial factors (*Space*) as well as their interactions. (A) General variation partitioning model. Within the model, a–d refer to the pure effect of each predictor; e–j refer to the joint effect of two predictors, k–n refer to the joint effect of three predictors; o relates to the joint effect of all four predictors, and u (Res, residuals) is the residual variance; (B)-(F) variance explained by each term in the whole communities (i.e., seawater plus urban water communities), urban water, urban pond, influent and effluent communities. Significance codes: ***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$.

water ecosystems, with emphasis on urban ponds.

4.1. *Urban landscape ponds serve as hotspots for ARGs*

Among the ARGs examined in this study, 237 subtypes (83.2%) were detected in various urban water habitats (Figs. 1A and 2). The average number of ARGs (20–87) in different urban water habitats is in the range of average ARGs reported in other urban water systems (Huang et al., 2019; Liu et al., 2019). Not surprisingly, the occurrence of ARGs varied across urban water ecosystems due to anthropogenic activities (Fig. 2). The ARGs might be released from humans to wastewater and transmitted directly or indirectly to urban water bodies, resulting in the difference of ARGs abundance and occurrence in various urban water habitats (e.g., reservoirs and ponds) (Lu et al., 2020; Vaz-Moreira et al., 2014). Although WWTPs removed certain amount of ARGs, still sulfonamide and macrolide resistance genes were detected in different water bodies of Beijing, including groundwater and Wenyu River (Liu et al., 2019). Moreover, the discharge of heavy metals and antibiotics from untreated urban sewages and hospital effluents could promote the propagation and spread of ARGs in the receiving waters (Laffite et al., 2020). ARGs have been widely detected in urban lakes, which often have similar characteristics but larger than ponds. For instance, ARGs that were resistant to sulfonamide, tetracycline and quinolone were widespread in six urban lakes in Wuhan, China, they were closely related to antibiotics and heavy metals (Yang et al., 2017b).

Previous studies have shown that a certain level of ARGs is always present in drinking water sources (Chen et al., 2019), water plants (Xu et al., 2016) and water supply systems (Huang et al., 2019; Su et al., 2018). The average detected number for some types (e.g., multidrug and aminoglycoside) of ARGs in the reservoirs fell within the range reported by previous studies (Fang et al., 2019, Guo et al., 2018). Although reservoirs are less contaminated by ARGs than WWTPs and ponds, they could still cause an increased risk of ARGs in the water supply network (Han et al., 2020). Normally, in the modern cities, drinking water from nearby surface water or reservoirs is treated rigorously in drinking water treatment plants and then supplied to the residents. However, drinking water treatment processes cannot eliminate ARGs entirely (Han et al., 2020). Furthermore, due to human antibiotic use, the diversity of ARGs would increase in wastewater streams and contribute to the ARGs in WWTPs (Quintela-Baluja et al., 2019). In this study, ARGs diversity decreased from IFs to EFs (Figs. 1A and 2), which may be due to effective ARGs reduction through WWTPs processes (An et al., 2018; Pallares-Vega et al., 2019). Many studies have demonstrated the increased numbers of ARG types in the downstream receiving waters of WWTPs (Huang et al., 2019; Quintela-Baluja et al., 2019). Although not as high as WWTPs, urban ponds still had a large number of ARGs types (Fig. 1A), some of which may have originated from WWTPs (Figs. 2 and 4). This indicated that urban ponds were significantly impacted by

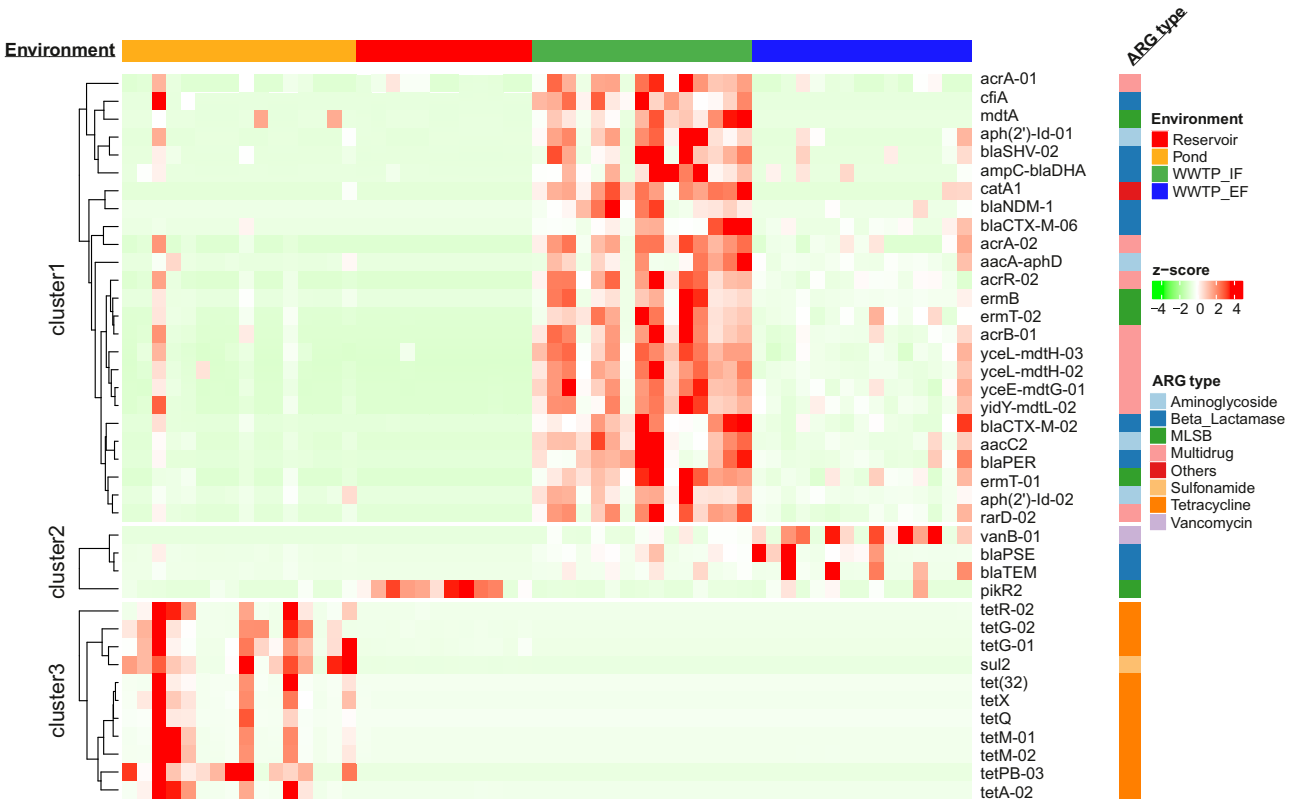

Fig. 6. Heatmap diagram showing the distribution of 40 ARG indicator subtypes in different units of urban water ecosystem (i.e., drinking water reservoir, urban pond, and influent (IF) and effluent (EF) of WWTPs) of Xiamen, China. Each row and column of the heat map diagram corresponds to a single indicator subtype and sample, respectively. The row data for each indicator was z-score transformed. The row dendrogram was generated based on Spearman correlation clustering. The orange, red, dark green and blue colors in the column annotations indicate the reservoir, pond, influent and effluent samples, respectively. The row annotation on the right-hand side indicates the ARG type of each subtype. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

anthropogenic pressure, especially municipal wastewater systems, which support our previous findings that urban ponds have a high proportion of sludge/sewage, human/animal gut and terrestrial-related microbes (Hu et al., 2018). Additionally, although seawater samples in open ocean areas were collected as a natural reference, certain proportion of shared ARGs between seawater with IFs/EFs was observed (Fig. 2), suggesting that the investigated marine area may be polluted by the raw or treated wastewater from the nearby mega cities (e.g., Guangzhou and Hong Kong).

Due to different WWTP processes and wastewater characteristics, the variation of the relative abundance of ARGs from IFs to EFs is controversial (Lee et al., 2017). In this study, WWTPs of Xiamen adopted various treatment processes and treated different types of wastewaters (Table S3). Our results showed that the relative abundance of ARGs decreased from IFs to EFs, and the relative abundance of ARGs in EFs (1.67) was higher than those reported by Huang et al. (2019). This may be due to a higher removal efficiency of microbial cells using membrane bioreactor techniques in Huang's study as compared to the sedimentation processes in current study. Moreover, the relative abundance of ARGs in urban ponds was 1.40, indicating that each microorganism in EFs and urban ponds likely carried multiple sets of ARG elements. The average absolute abundance of ARGs in ponds in current study was higher than some urban lakes, which had ARG concentrations ranging from 4.58×10^0 to 5.0×10^5 copies/mL (Dong et al., 2019). Among the absolute abundances of ARGs in the various urban water habitats, reservoirs exhibited the lowest level (7.84×10^4 copies/mL; Fig. S2), which was lower than the previously reported values (4.55×10^5–4.55×10^8 copies/mL) (Chen et al., 2019; Fang et al., 2019). This may be attributed to the good management and protection of the studied reservoirs (Hu et al., 2018). Consistent with previous studies, the absolute abundance of ARGs in WWTPs from IFs to EFs decreased (Fig. S2) (An et al., 2018; McConnell et al., 2018; Pallares-Vega et al., 2019). Remarkably, urban ponds showed a much higher average absolute abundance of ARGs and MGEs than EFs, and were only one order of magnitude lower than in IFs (Fig. S2). Previous researches have demonstrated that receiving rivers also had a relatively higher absolute abundance of ARGs than EFs (Huang et al., 2019; Neudorf et al., 2017). A potential explanation is that the ARG-carrying microorganisms may adapt to the pond environment and carry on reproduction as well as conduct horizontal gene transfer resulting in enriched and persistent ARGs in urban ponds (Yang et al., 2014). Although the relative abundances of ARGs in seawater (0.014 copies per 16S rRNA) were low compare to the average relative abundances of ARGs in other seas (0.027) (Yang et al., 2019), the resistance risk should not be ignored since their absolute abundance was approximately 3.98×10^3 copies/mL.

Several studies have previously reported on the accumulation of ARGs in agricultural ponds receiving various wastewaters from households and aquaculture facilities (Huang et al., 2017; Zhang et al., 2013). Some urban lakes were found to be contaminated by ARGs against sulfonamide (*sul1*, *sul2*), tetracycline (*tetW*, *tetX*), and beta-lactamase (bla_{TEM}, bla_{SHV}), and one MGE (*intI1*) (Dong et al., 2019). However, urban landscape ponds have been overlooked for ARGs and MGEs pollution. Here, we suggest that urban ponds are hotspots for the occurrence of ARGs in urban water ecosystems, and are a potential vector for the transmission of ARGs. Firstly, urban ponds had a relatively high abundance and diversity of ARGs (Fig. 2 and S2). Secondly, these ponds also possessed a high level of MGEs, which were significantly

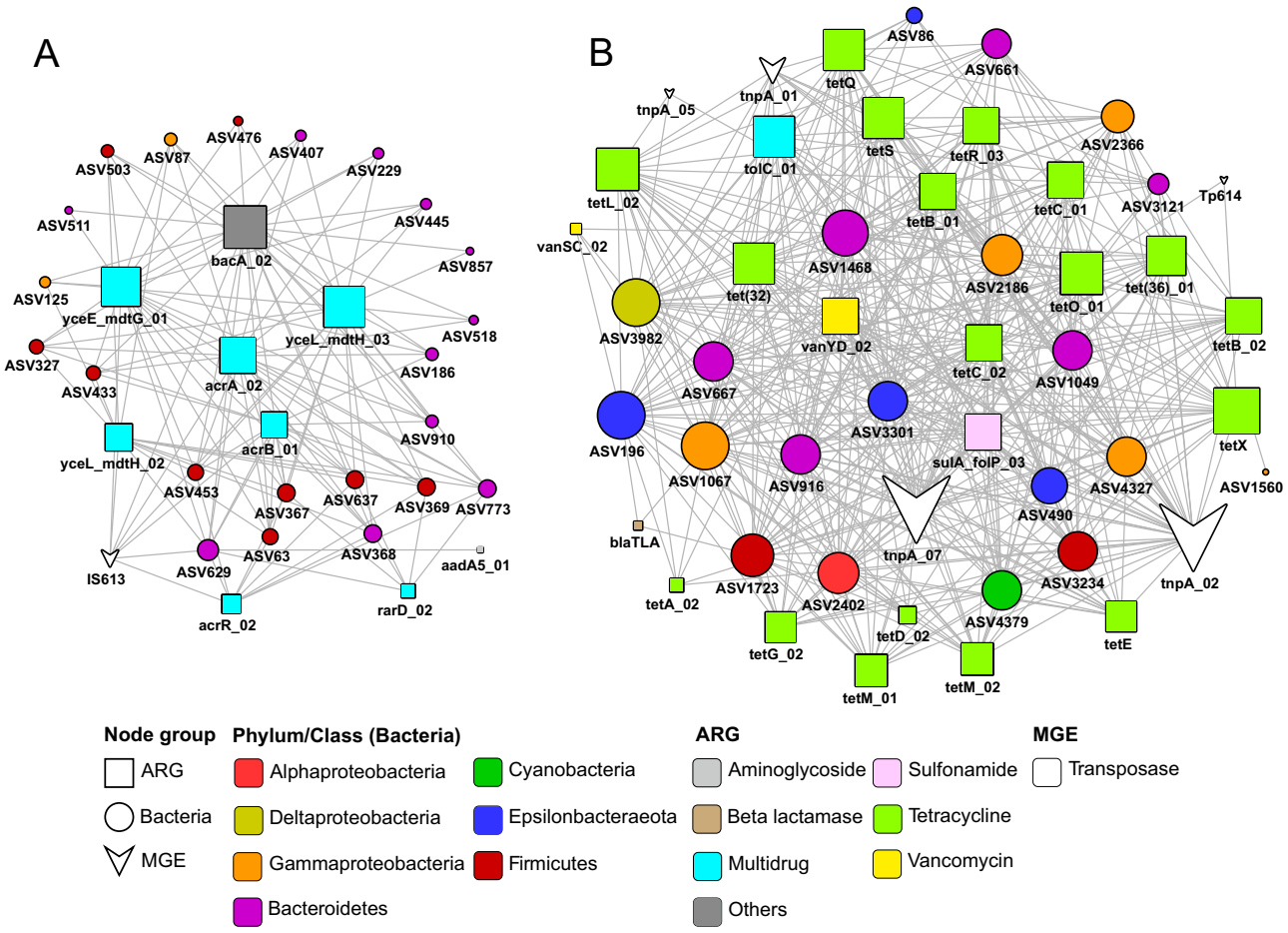

Fig. 7. Relevance network showing covariations among the identified best discriminant ARGs, MGEs and bacterial ASVs in the influent of WWTPs (A) and urban ponds (B). Only the strongest positive associations ($r > 0.8$) are displayed, since no negative associations were identified here. Node shapes represent different classes of variables (i.e., ARGs, MGEs and bacterial ASVs). The size of nodes indicates the degree of vertices in the network.

correlated to the ARGs (Figs. S2 and 3). The transfer of ARGs in urban ponds might be promoted through the horizontal exchange of MGEs (Rizzo et al., 2013; Stalder et al., 2012). Previous studies demonstrated that MGEs, forming the mobilome, could move ARGs across species, or even phylum boundaries (Gonzalez-Plaza et al., 2019; Guo et al., 2017). This could easily lead to a higher public health risk where ARGs are transferred by MGEs from urban ponds to other habitats that are in close proximity to human activities (Koike et al., 2010).

4.2. Indicator ARGs for wastewater contamination in urban water ecosystems

We identified representative ARGs belonging to each urban water habitat to assist the tracing ARG sources and dissemination routes within urban water ecosystems in the studied area. IFs indicators were numerous and mainly composed of ARG subtypes conferring resistance to multidrug, beta-lactamase, MLSB and aminoglycoside (Figs. 6 and 7), which is consistent with ARGs reported to be dominant in WWTPs' IFs in other regions in Asia (Ng et al., 2018; Zheng et al., 2020). The DIABLO network indicated that these genes mainly derived from human feces since they were strongly correlated with common human gut microbiota like *Firmicutes* (e.g., *Faecalibacterium* and *Lactococcus*) and *Bacteroidetes* (e.g., *Bacteroides* and *Parabacteroides*) (Ma et al., 2016). Among these indicator ARGs, $bla_{\text{NDM-1}}$ was widely detected in the IF of WWTPs in this study, as it has been detected in a few enteric pathogens from Xiamen, China (Hu et al., 2017b). These indicate that $bla_{\text{NDM-1}}$ is spreading widely among residents in Xiamen, which poses a great threat to human and animal health in this area since $bla_{\text{NDM-1}}$ is effective against almost all antibiotics with beta-lactam rings (Khan et al., 2017). What is more, as one of three ARG indicators for EFs, bla_{TEM} is resistant to beta-lactamase, which has shown to be persistent in hospital–urban wastewater treatment processes (Laffite et al., 2016; Narciso-da-Rocha et al., 2014).

Indicator ARGs to evaluate the antimicrobial resistance status of urban ponds were mainly against tetracycline (e.g., seven efflux pump genes (*tet*A, *tet*B, *tet*C, *tet*D, *tet*E, *tet*G, *tet*L and *tet*R)), four ribosomal protection protein genes (*tet*M, *tet*O, *tet*Q and *tet*S), and one enzymatic modification gene (*tet*X) (Figs. 6 and 7). These observations are in agreement with published literature regarding the predominance of tetracycline resistance genes in some aquaculture ponds, which may be caused by the input of wastewater (Zhang et al., 2013; Zhou et al., 2019). For instance, high levels of *tetG*, *tetM* and *tetX* have been observed in swine and beef cattle wastewater (Sui et al., 2016). Moreover, the migratory birds could also contribute to the spread of genes against tetracycline by introducing their feces to urban ponds (Cao et al., 2020). These genes may be taken up by chemoautotrophic bacteria (Du et al., 2019), which in turn enhance the enrichment and domination of these genes in ponds. Vancomycin and multidrug resistance genes were also presented in the urban ponds, which may be from the hospital wastewater effluent or industrial pollution (Yan et al., 2019). The DIABLO network clearly showed that these genes were frequently associated with both transposons (i.e., *tnpA*) and gut-related (*Faecalibacterium*, *Lactococcus*, *Parabacteroides*, and *Bacteroides*) and chemolithoautotrophic microbial taxa (Fig. 7). These taxa were also reported as the most prevailed functional groups of ponds in our previous study (Hu et al., 2018). Chemolithoautotrophic microbes, such as

microaerobic Gammaproteobacteria (e.g., *Chromatium* and *Macromonas*) and anaerobic Epsilonbacteraeota (e.g., *Arcobacter* and *Sulfurospirillum*), capable of carbon, sulfur and nitrogen metabolic pathways (Li et al., 2018), were over-represented in the ponds compared to other habitats. It was observed that tetracyclines significantly selected Gammaproteobacteria in manure-polluted aquatic environments (Xiong et al., 2015). This implied that urban ponds were under high anthropogenic selective pressures (e.g., sewages and migratory bird feces) and thus may have an elevated risk for the dissemination of ARGs and MGEs.

4.3. Stochastic assembly of ARG communities in the urban water ecosystems

Previous studies have delved into the assembly mechanisms of ARG communities from a single urban water habitat, such as drinking water reservoirs (Fang et al., 2019; Guo et al., 2018) or WWTP-influenced water bodies (Corno et al., 2019). Here, we investigated multiple urban water habitats and found that, except for reservoirs, stochastic processes played a more important role in the structuring of ARG profiles in an urban ecosystem (compositional stochasticity > 50%) than deterministic processes (Fig. 4B). In contrast, Fang et al. (2019) reported that stochastic processes explained only a minor part of the variation in ARG profiles in an urban reservoir under subtropical conditions. However, in an urbanizing subtropical watershed, stochastic processes were not an important mechanism driving the ARG community assembly (Peng et al., 2020). As can be seen from Fig. 5, ARGs profiles in the urban water ecosystems of this area were determined by complex factors (e.g., the spatial factors, MGEs, bacterial community and environmental factors). The high abundance and ubiquitousness of MGEs (Figs. 3 and 4C) causing massive HGT with stochastic nature in the urban water ecosystems might be the main contributing agents to the stochastic processes (Hu et al., 2018; Li et al., 2018). Additionally, the scale effect (small space scale, like ponds) of these urban water habitats appear to favor stochastic processes at spatial scale (Peng et al., 2020).

Consistent with our previous study (Hou et al., 2019), IFs and EFs bacterial communities were shaped mainly by deterministic and stochastic processes, respectively (Fig. S5). Although the bacterial community assembly affected ARG communities in IFs (Fig. 5), it can be said that stochastic processes mainly influenced them (Fig. 4B). Similarly, in the urban ponds, both bacterial and ARG communities were primarily shaped by stochastic processes (Figs. S5 and 4B). However, the stochastic processes shaping the bacterial community are distinct from the stochastic processes shaping the ARG communities. Dispersal limitation played an important role in the bacterial community assembly (Hu et al., 2018), but played a negligible role in the ARG community assembly (Fig. S5). Due to the relatively high abundance of MGEs and their stronger correlation with ARGs in ponds (Fig. 4C), the dispersal of MGEs contributed more to the ARG community assembly.

For different urban water habitats, the selective forces driving ARGs communities varied greatly. Previous studies have suggested that the bacterial community was one of the main forces impacting ARG profiles in antibiotic-rich environments (Su et al., 2015; Zheng et al., 2017a). Due to higher concentrations of antibiotics in WWTPs compared with receiving rivers (Huang et al., 2019), bacterial community composition has been identified as the key driver shaping the ARG communities in IFs and EFs, but not in ponds. Environmental factors played a greater role in IFs and ponds than EFs (Fig. 5). For example, salinity, temperature, total carbon, nitrate-nitrogen and total phosphorus may foster the proliferation of microbial taxa that carry ARGs or improve the growth of non-resistant microorganisms in IFs and ponds, as similar results have been reported in a previous study (Su et al., 2017).

5. Conclusions

In this study, a broad profile of ARGs and MGEs was revealed and different indicator ARGs for various urban water habitats were identified. Among different urban water habitats, IFs had the highest absolute abundance ARGs, followed by urban ponds, EFs and then drinking water reservoirs. Multidrug, aminoglycoside and beta-lactamase resistance genes were the dominant ARGs in these urban water ecosystems. Urban ponds showed the strongest co-occurrence relationship between ARGs and MGEs and exhibited more exogenous pollution caused by anthropogenic activities. These results suggest that ponds are hotspots for the spread of ARGs. Stochastic processes played a more important role in shaping ARGs communities in the urban ecosystem (i.e., IFs/EFs and urban ponds), which may be attributed to one of the main controlling factors, MGEs, which can enable the HGT of ARGs among different taxa. High frequency and long-term time series studies are needed to conduct a comprehensive analysis of the interaction among antibiotics, ARGs and ARBs in all types of urban water habitats.

CRediT authorship contribution statement

Liyuan Hou: Conceptualization, Formal analysis, Validation, Writing – original draft, Visualization. **Lanping Zhang**: Investigation, Methodology, Data curation. **Furun Li**: Investigation, Methodology, Data curation. **Sijun Huang**: Investigation, Methodology, Resources. **Jun Yang**: Investigation, Methodology, Resources. **Cong Ma:** Investigation, Resources. **Duanxin Zhang:** Investigation, Resources. **Anyi Hu**: Conceptualization, Methodology, Data curation, Software, Validation, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Chang-Ping Yu**: Resources, Supervision, 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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2020.124008.

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