

## Distribution characteristics of antibiotic resistance bacteria and related genes in urban recreational lakes replenished by different supplementary water source

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### ABSTRACT

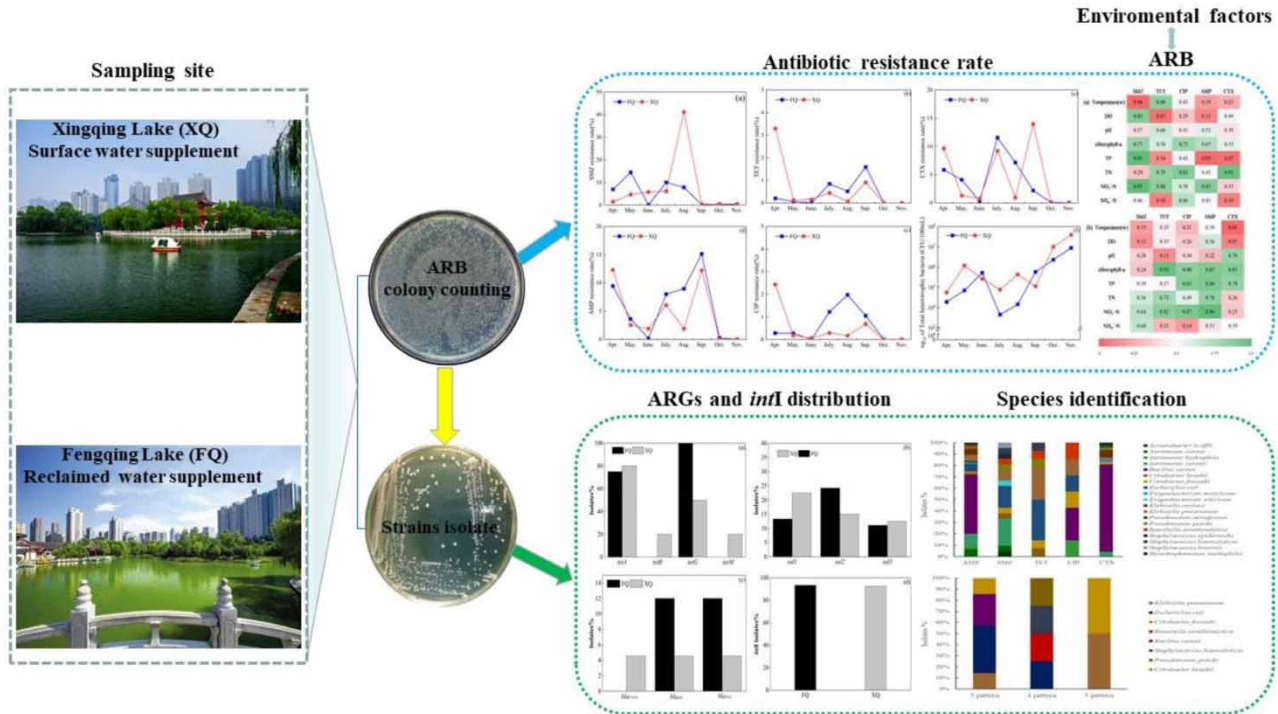
The distribution characteristics of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in urban recreational water from different water-supply sources might be different. In this study, water samples were collected to detect the antibiotic resistance of heterotrophic bacteria to five antibiotics, and the content, phenotype, gene type and species distribution of resistant bacteria were analyzed. The results showed that the changes of bacteria resistance rate in two lakes to five kinds of antibiotics were synchronous with time, and it would reach its maximum in autumn. The detection of ARGs and *int I* in 80 resistance strains showed that the detection rate of *tetG*, *tetA* and *int I* was high. Here, 51.25% of the bacteria were doubly resistant to AMP-CTX. The 80 isolate strains were of nine genera and 19 species, among which *Bacillus cereus*, *Escherichia coli*, *Aeromonas veronii*, *Aeromonas caviae* and *Raoultella ornithinolytica* were the common ARB species in two lakes. Correlation analysis showed that the water temperature was significantly correlated with the content of ARB in sulfamethoxazole (SMZ) and cefotaxime (CTX) ( $p < 0.05$ ), and the total phosphorus (TP) in FQ lake was significantly correlated with the content of AMP-resistant bacteria ( $p < 0.05$ ), while there were no other correlations between the changes of other water quality indexes and the content of ARB ( $p > 0.05$ ).

**Key words:** antibiotic resistance genes (ARGs), antibiotic-resistant bacteria (ARB), reclaimed water, surface water, urban recreational water

### HIGHLIGHTS

- Different water supplementary sources will not affect the content of ARB in two lakes.
- The ARB isolated from the two lakes were mainly double-resistance bacteria.
- With the same resistance patterns, the distribution of ARGs are different.
- *Bacillus cereus* and *Escherichia coli* were the dominant species in two lakes.
- Water quality parameters have little effect on the content of ARB.

## GRAPHICAL ABSTRACT



## INTRODUCTION

In recent years, antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) have attracted extensive attention as emerging water pollutant (Pruden *et al.* 2006). In fact, both of them have been detected in lakes (Low *et al.* 2016), aquaculture farms (Gao *et al.* 2012), soil (Tan *et al.* 2019), wastewater treatment plant (McConnell *et al.* 2018; Zhang *et al.* 2019; Collivignarelli *et al.* 2021), rivers and sediments (Guo *et al.* 2018), surface water biofilm (Winkworth-Lawrence & Lange 2016) and sea sediments (Chen *et al.* 2013). Compared with the widespread existence of ARB, ARGs are the fundamental reason for the wide spread of bacterial resistance. ARGs exist widely in a variety of environmental media, and exacerbate the spread of microbial antibiotic resistance through horizontal transfer, which poses a threat to the environment and human health. Water is a vital part of our life and constitutes an important part of human livelihood all over the world. However, recent studies have shown that the water environment is highly likely to become the source and storage of ARB (Dong *et al.* 2019) and may exacerbate the spread of antibiotic-resistant pathogens. (Fewtrell and Kay 2015; Pang *et al.* 2016). Previous studies have mainly focused on fecal indicators, enterovirus, and other several kinds of pathogenic bacteria such as *Salmonella* in rivers, lakes, and other water bodies (Hlavsa *et al.* 2015). Compared with rivers and lakes, in urban areas with high population density, recreational water such as fountains and landscape lakes provide excellent recreational facilities for urban residents' leisure and entertainment (Helena *et al.* 2015). Moreover, urban recreational water is closely related to residents' life, people will contact it by boating, swimming and other ways. Also, urban recreational water plays an important role in the urban water cycle, most of which are connected to the urban river and some of them use reclaimed water as its supply source. However, this might directly threaten public health if there are pathogenic bacteria or other dangerous sources of ARGs in water. Therefore, it is extremely necessary to analyze the distribution of antibiotic-resistant microorganisms in urban recreational water.

As urban recreational water has played a significant role in the continuous expansion of urbanization, the situation of microbial pollution in water should be paid sufficient attention. Although urban recreational water is developing rapidly at present, the source of water supply is an important problem in practice due to the constraint of its own characteristics. Some studies have found that, although the water source and sediment polluted by human beings have been further treated, it is still likely to play an important role in the re-pollution of water for recreational purposes (Elmahdy *et al.* 2016). In recent

years, due to the shortage of water resources, reclaimed water which has excellent recyclability is gradually being used to replace surface water as the supplementary water source of urban landscape water (Zhao *et al.* 2011). However, the current sewage treatment standards and technology make it hard to effectively treat ARB and ARGs in reclaimed water (Hwangbo *et al.* 2019; Posada-Perlaza *et al.* 2019; Verburg *et al.* 2019; Piotrowska *et al.* 2020; Zammit *et al.* 2020); this may lead to a considerable number of ARBs and ARGs in reclaimed water, which may aggravate the content of resistant bacteria and resistant genes in urban recreational water, and increase the public safety risk of urban residents. (Dong *et al.* 2019). With the process of urbanization and the improvement of people's living standards, the existing role of urban recreational water will become much more important (Cui *et al.* 2016), and the application of reclaimed water in recreational water is becoming more common. At the same time, its microbial pollution also needs further research, especially whether the different water-supply sources will lead to the increase of microbial risks such as resistant bacteria, and pose a threat to human health, which needs further exploration. Therefore, it is of great practical significance to study the occurrence of ARGs and ARB in urban landscape water from different water-supply sources.

Our previous study found that tetracycline (TET), sulfonamide, quinolone and other antibiotic resistance genes are mainly distributed in the urban landscape water of Xi'an (Zhang *et al.* 2021). Based on this, five kinds of common ARB patterns like: tetracycline (TET), sulfamethoxazole (SMZ), ampicillin (AMP), ciprofloxacin (CIP), and cefotaxime (CTX) antibiotic resistance bacteria were selected as the research target in this study and 10 kinds of ARGs and *intI* were selected to test their distribution in ARB. Two landscape lakes Xingqing Lake (XQ) and Fengqing Lake (FQ) with different water-supply sources in Xi'an were selected as the research object. This study focused on the characteristics of: (1) water quality indicators and distribution characteristics of ARB in two lakes; (2) ARGs carried by ARB; (3) the species distribution of ARB. This study will give information on the characteristics of ARB and the ARGs carried in urban landscape waters replenished by surface water and reclaimed water, which provides a scientific basis and the control of microbial risk for the safe use of reclaimed water in urban recreational waters.

## MATERIALS AND METHODS

### Sampling site and sample collection

The urban recreational water selected in this study were lakes XQ and FQ (Fig. S1, site 1: 108°59'24.04"N; 34°15'36.05"W; site 2: 108°54'24.44"N; 34°15'7.04"W). Both of them are located in the ancient city of Xi'an, which contains famous tourist attractions and a large amount of tourists visit these two lakes every day. Meanwhile, both lakes have a variety of water entertainment items, such as boating, and people have frequent contact with landscape water. XQ lake has an area of about 100,000 square meters and an average water depth of 2 meters, surface water is its source of water replenishment. The surface area of FQ lake is about 38,000 square meters, with an average depth of 1.5 meters, using reclaimed water as its supply source and received about 41,643 m<sup>3</sup> per year, the quality of the reclaimed water was according to Zhao *et al.* (2015).

There are five sampling sites in XQ and four in FQ. From April to November 2018, 16 water samples were collected from two lakes. For each lake, water samples were collected at each sampling sites at a depth of 0.5 m below the surface and then thoroughly mixed (Zhang *et al.* 2021). A total of 1 L water sample was collected from each lake and kept in the sterile plastic bottle. All the samples were transported to the laboratory in 6 h, stored at 4 °C and all experiments were carried out within 12 h.

### Water quality parameters

The contents of total nitrogen (TN) and total phosphorus (TP) in samples were detected by the method according to Guo *et al.* (2018). After the samples were filtered through the filter membrane, the content of chlorophyll *a* was determined by the thermal ethanol method (Chen *et al.* 2006). The content of dissolved oxygen, pH, and water temperature were measured according to Harnisz (2013).

### Content determination of heterotrophic bacteria and ARB

The heterotrophic bacteria in water samples were counted by the filter membrane method (Novo *et al.* 2013). After gradient dilution of the water sample, a mixed cellulose filter membrane with a diameter of 0.45 microns was used to filter the samples by vacuum extraction. The bacteria trapped in the filter membrane were closely attached to the plate count agar (PCA) and cultured at 30 °C for 24 h. Colony count was conducted on the culture plate with a number of 20–200 colonies. The average number of colonies was used to calculate the number of heterotrophic bacteria per 100 mL of the water samples. According

to the standards of the American Clinical and Laboratory Standards Institute (CLSI) (Fothergill 2012), AMP, SMZ, TET, CIP, and CTX were added into PCA plates to prepare resistance plates, in which the mass concentrations of corresponding antibiotics were 32 µg/mL, 512 µg/mL, 16 µg/mL, 4 µg/mL and 4 µg/mL respectively. Similarly, the content of five kinds of ARB in the water sample was calculated by using the filter membrane method to determine the antibiotic resistant bacteria in the water sample.

### Isolation and susceptibility testing of antibiotic-resistant strains

Single colonies were picked from the PCA and grown in LB medium overnight, after that the strains were streaked on LB agar plates and preserved. Five kinds of antibiotics as sulfamethoxazole (SMZ 300 µg), tetracycline (TET) 30 µg, ampicillin (AMP 10 µg), ciprofloxacin (CIP 5 µg) and cefotaxime (CTX 30 µg) were selected for drug susceptibility test using the paper diffusion method (K-B method) according to the American CLSI (Fothergill 2015).

### Detection of resistance, integron and strains sequencing analysis

DNA from the isolated ARB were extracted according to the TaKaRa MiniBEST Bacteria Genomic DNA Extraction Kit v. 3.0 instructions and stored at -20 °C. 16S rDNA PCR was performed using universal 27F and 1492R primers (Harnisz and Korzeniewska 2018). The PCR products were sent to Sangon Biotech (Shanghai) Co., Ltd for DNA sequencing. The sequencing results were checked by Bioedit v7.0.9 software, and the obtained 16S rDNA sequences were compared in the GenBank database in the National Center for Biotechnology Information (NCBI) to confirm the species.

According to literature reports, 10 kinds of ARGs were identified, including TET resistance genes *tetA*, *tetB*, *tetG*, and *tetM*; Sulfanilamide resistance genes *sul1*, *sul2*, *sul3*; β-lactam type of resistance genes *bla<sub>TEM</sub>*, *bla<sub>CTX-M</sub>*, *bla<sub>SHV</sub>*, and class I integron gene *int1*. References of the gene primer sequence (Luo *et al.* 2011; Zhang *et al.* 2014; Zhang *et al.* 2015; He *et al.* 2016) were synthesized by Sangon Biotech (Shanghai) Company. Sequences of resistance genes and reaction conditions shown in Table 1.

**Table 1** | Primer sequences of the relative resistance genes and reaction conditions

Genes	Primer sequences(5'-3')	Annealing temperature (°C)	Length of fragment (bp)
<i>tetA</i>	F:GCTACATCCTGCTTGCCTTC R:CATAGATCGCCGTGAAGAGG	55	210
<i>tetB</i>	F:TACGTGAATTTATTGCTTCGG R:ATACAGCATCCAAAGCGCAC	61	206
<i>tetG</i>	F:GCACGCTGGTTTGGCTACA R:TGGCTGTGATTAGTCTCCTTGA	56	176
<i>tetM</i>	F:ACAGAAAGCTTATTATATAAC R:TGGCGTGTCTATGATGTTTAC	45	171
<i>sul1</i>	F:CGCACCGGAAACATCGCTGCAC R:TGAAGTTCCGCCGCAAGGCTCG	56	163
<i>sul2</i>	F:TCCGGTGGAGGCCGGTATCTGG R:CGGGAATGCCATCTGCCTTGAG	56	191
<i>sul3</i>	F:CCCATACCCGGATCAAGAATAA R:CAGCGAATTGGTGCAGCTACTA	57	143
<i>bla<sub>TEM</sub></i>	F:AAGCTTATTAGCGTGT R:TGTCTATGATCTTGT	55	531
<i>bla<sub>CTX-M</sub></i>	F:GCTGGTTTCAGAA R:TGTGATTAGCTGT	55	869
<i>bla<sub>SHV</sub></i>	F:TGAATACGTTTTTAC R:TACCTTGTTATTAC	55	295
<i>int1</i>	F:GGCTTCGTGATGCCTGCTT R:CATTCTGGCCGTGGTTCT	57	146
16S rDNA	27F:AGAGTTTGATCCTGCCTCAG 1492R:TACGGYTACCTTGTTACGACTT	56	1465

The antibiotic resistance genes were detected by PCR with the 25  $\mu$ L Reaction system: template DNA 2  $\mu$ L, 10 $\times$  PCR buffer 2.5  $\mu$ L, dNTP mixture 2  $\mu$ L, TaKaRa *Taq* 0.2  $\mu$ L, upper/lower primers 1  $\mu$ L each, RNase-free water 16.3  $\mu$ L. Reaction conditions: 95  $^{\circ}$ C 8 min, 95  $^{\circ}$ C 30 s, annealing 30 s, 72  $^{\circ}$ C 5 min. The product was detected by 1% agarose gel electrophoresis (AGE).

### Data analysis

Using Microsoft Excel 2016 and SPSS 23.0 to process and analyze the data, and origin 8.5 was used to process and optimize the data images. Two independent sample tests (Mann–Whitney *U*-test) were used to analyze the difference between different samples, and Spearman correlation analysis was used to test the correlation between water quality index and antibiotic resistance rate of samples.

## RESULTS

### Water quality indicators in two lakes

Eight water physical and chemical properties of the two lakes were tested, including water temperature, dissolved oxygen, pH, chlorophyll *a*, TN, TP, nitrate nitrogen, and ammonia nitrogen, and the test results are shown in Table 2.

### Distribution of antibiotic resistance rate

The temporal variation of antibiotic resistance rate in two lakes are shown in Figure 1. The variation trend of heterotrophic bacteria content in two lakes is similar, showing an increasing trend with season. The changing trend of antibiotic resistance rate of TET and AMP in FQ was similar over time, and the number of CTX resistance bacteria had a larger change trend over time, while the change of antibiotic resistance rate of SMZ was less obvious, and the fluctuation of the antibiotic resistance rate of AMP was more obvious. The change in trend of AMP and CTX resistance rates in XQ with time was similar (Figure 1(d) and 1(e)), and the fluctuation was also obvious, both the resistance rates reached the maximum in July and September. The antibiotic resistance rate of SMZ was stable and reached the peak in September. By contrast, CIP resistance rate tended to be stable, and it was higher in autumn than that in other seasons. In this study, the overall trend of the changes for bacteria resistance rate in FQ and XQ was similar, among which the content of AMP resistance bacteria in FQ was higher, and AMP resistance rate fluctuated greatly with time.

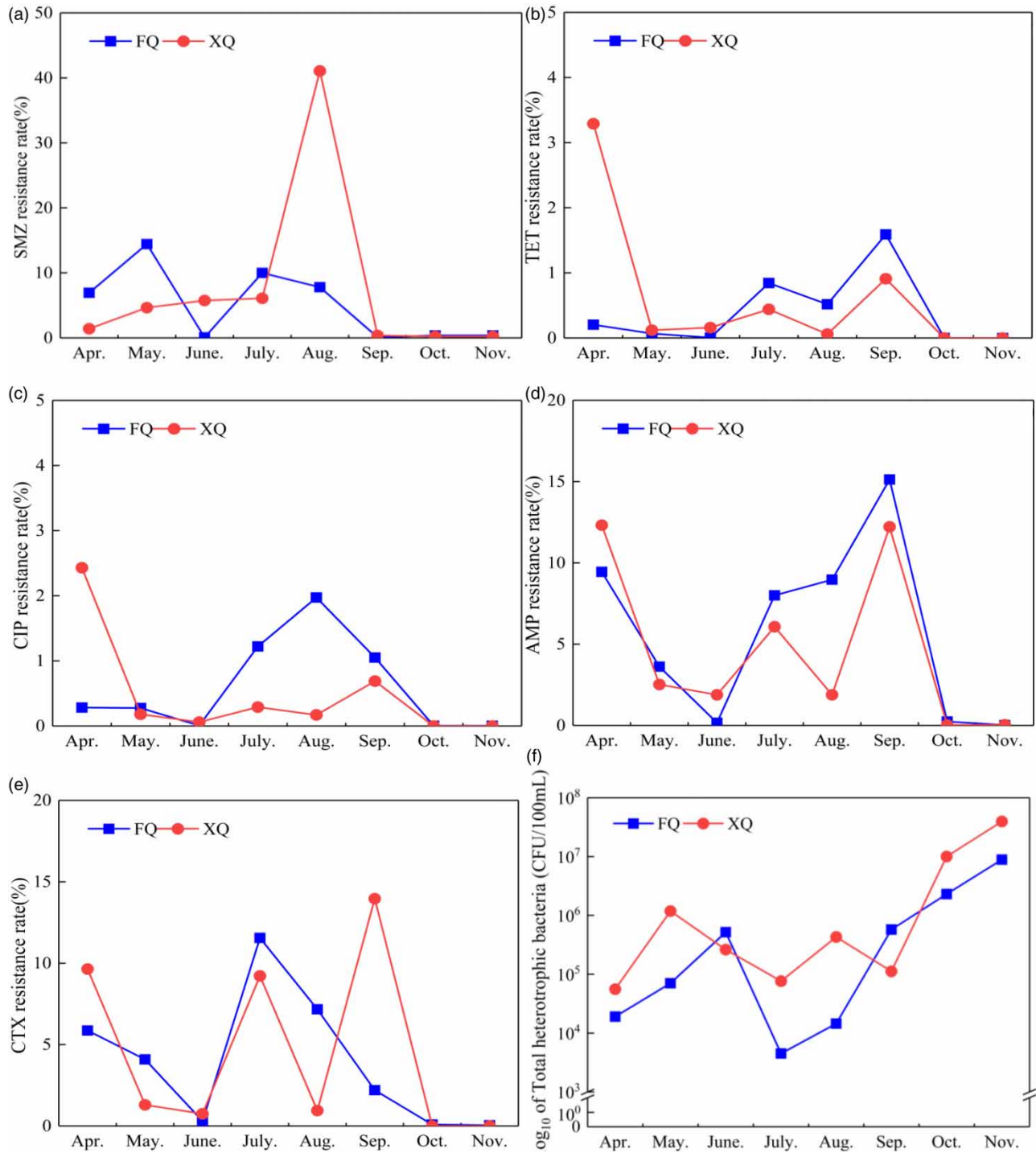
The antibiotic resistance rate of AMP in FQ reached highest in September at 15.13%, the resistance rate of CTX and CIP reached the maximum values in July and August, which were 11.56% and 1.97% respectively. Instead, the detection rates of SMZ and TET resistance bacteria were lower. The antibiotic resistance rate of SMZ in XQ varied greatly, in summer it was higher than that in winter, reaching a maximum of 41.07% in September. Compared with FQ, the bacteria resistance to CTX and AMP in XQ was relatively equal, with the highest antibiotic resistance rates of 13.96% and 12.32%, respectively.

The overall trend of antibiotic resistance rate in the two lakes was similar, and all showed that the antibiotic resistance rate in winter was lower than that in summer. Except for CTX resistance bacteria in FQ that had the highest detection rate in July, the other four antibiotic resistant bacteria in the two lakes all reached the maximum detection rate in autumn. It was found that the antibiotic resistance rates of TET and AMP were lower in the two lakes, while the resistance rates of the other three antibiotics were higher. The antibiotic resistance rates in summer and autumn were higher than that in winter. Moreover, the antibiotic resistance rate of bacteria in the two lakes was higher in August and September than in other months, presenting a significant upward trend.

**Table 2** | Physical and chemical properties of water samples from FQ and XQ lake (mean values are in parentheses)

Lake	Temperature (w) ( $^{\circ}$ C) <sup>a</sup>	DO (mg/L)	pH	Chlorophyll <i>a</i> ( $\mu$ g/L)	TN (mg/L)	TP (mg/L)	NO <sub>3</sub> <sup>-</sup> -N (mg/L)	NH <sub>4</sub> <sup>-</sup> -N (mg/L)
FQ	9.30–31.80 (21.59)	7.88–11.37 (9.91)	8.14–9.06 (8.66)	0.00–21.39 (7.08)	1.08–5.47 (3.23)	0.02–0.06 (0.03)	0.58–1.24 (0.93)	0.02–0.78 (0.29)
XQ	14.40–33.90 (24.43)	6.32–9.58 (8.14)	7.67–8.53 (8.19)	0.00–24.65 (9.42)	0.59–2.93 (2.14)	0.04–0.09 (0.07)	0.27–2.19 (0.88)	0.00–0.25 (0.12)

<sup>a</sup>Temperature (w): water temperature.



**Figure 1** | Temporal changes of five kinds of antibiotic resistance bacteria rate and total heterotrophic bacteria changes in water taken along the lakes FQ and XQ (a) SMZ: sulfamethoxazole. (b) TET: tetracycline. (c) CIP: ciprofloxacin. (d) AMP: ampicillin. (e) CTX: cefotaxime. (f) Total heterotrophic bacteria.

**Analysis of antibiotic resistance pattern and antibiotic susceptibility testing**

Based on the detection of antibiotic resistance rate in two lakes, 5–8 strains were isolated and purified from each sample, and 125 strains of heterotrophic bacteria were isolated, including 63 strains in FQ and 62 strains in XQ. Then 80 antibiotic resistance strains were screened out from heterotrophic bacteria according to judgment criteria (Fothergill 2015), including 41

strains in FQ and 39 strains in XQ. The resistance of 80 strains to five kinds of antibiotics was shown in Table 3. The result showed that the isolated ARB are different in degrees of resistance to five different antibiotics, among which the resistance to AMP and CTX accounted for 93.75% (75/80) and 58.75% (47/80), respectively. However, the sensitivity to SMZ and TET was higher, with the proportion of antibiotic resistant bacteria being 26.25% (21/80) and 17.50% (14/80), respectively, with the lowest proportion of antibiotic resistant bacteria being 8.75% (7/80) to CIP.

The analysis of the antibiotic resistance pattern is shown in Table 4. The experimental analysis indicated that some ARB had single-resistance pattern, accounting for 13.60% (17/125) and 21.25% (17/80) of the isolated heterotrophic and ARB. In total, 70% of the single-resistance pattern was AMP, followed by SMZ. Most of ARB showed resistance to two antibiotics, accounting for 40% (50/125) and 62.50% (50/80). The main double-resistance pattern was AMP–CTX, and 41 strains were detected, accounting for 82% of all the double-resistance strains. There were 13 strains showed multiple resistant patterns and five of them had the AMP–SMZ–TET resistance pattern. Among which, four strains are resistant to four antibiotics, and two resistant to all five antibiotics. Multiple resistant bacteria resistant to three, four, and five antibiotics were 8.75% (7/80), 5.00% (4/80), and 2.50% (2/80), respectively, accounting for the proportion of the total number of heterotrophic screened out being 5.60% (7/125), 3.20% (5/125), and 1.60% (2/125). Among 80 ARB, AMP–CTX was the most common resistance pattern, more than half of the isolates of ARB had this phenotype. On the whole, the number of resistant patterns in FQ was

**Table 3** | Antibiotic resistance phenotype of 80 strains

Antimicrobial agents	Sensitive		Antibiotic resistance	
	Number of strains	Isolates%	Number of strains	Isolates%
Ampicillin	5	6.25	75	93.75
Sulfamethoxazole	59	73.75	21	26.25
Tetracycline	66	82.50	14	17.50
Ciprofloxacin	73	91.25	7	8.75
Cefotaxime	33	41.25	47	58.75

**Table 4** | Antibiotic resistance patterns for 80 resistance strains in two lakes

Resistance to multiplicity	Resistance phenotype	Source and quantity of samples (strains)		Total (plant)
		FQ	XQ	
#1 <sup>a</sup>	AMP <sup>b</sup>	6	6	12
	SMZ	3	2	5
	TET	ND	ND <sup>c</sup>	0
	CIP	ND	ND	0
	CTX	ND	ND	0
#2	AMP, SMZ	4	1	5
	AMP, TET	1	2	3
	AMP, CIP	ND	1	1
	AMP, CTX	24	17	41
#3	AMP, SMZ, TET	2	3	5
	AMP, CIP, CTX	ND	2	2
#4	AMP, SMZ, TET, CIP	ND	2	2
	AMP, SMZ, TET, CTX	1	1	2
#5	AMP, SMZ, TET, CIP, CTX	ND	2	2

<sup>a</sup>#1: single-resistance phenotype, #2:2 heavy resistance phenotype, #3:3 heavy resistance phenotype, #4:4 heavy resistance phenotype, #5:5 heavy resistance phenotype.

<sup>b</sup>AMP, Ampicillin; SMZ, Sulfamethoxazole; TET, Tetracycline; CIP, Ciprofloxacin; CTX, Cefotaxime.

<sup>c</sup>ND, none detected.

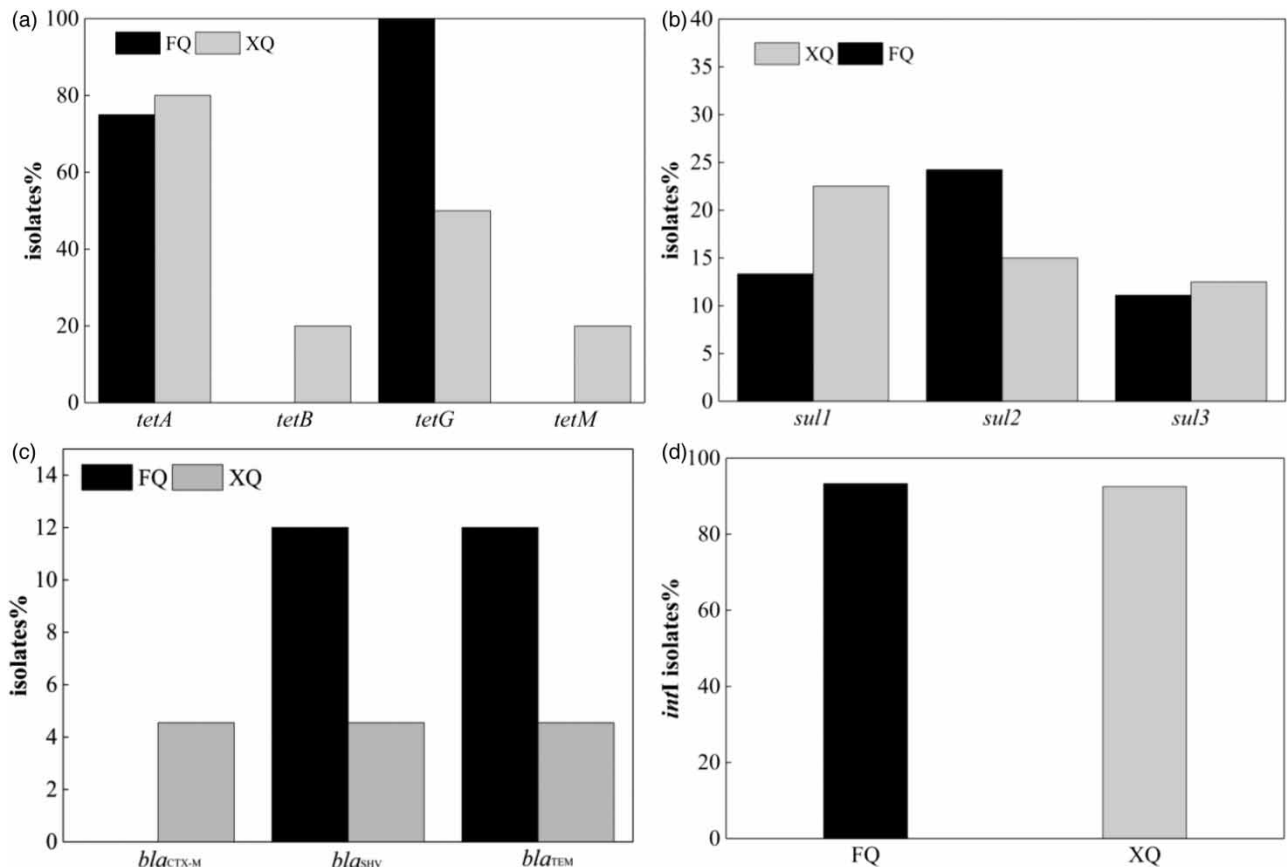
higher than XQ, but the proportion of multiple resistant bacteria was opposite. Moreover, it was found that the detection amount of multiple antibiotic-resistant bacteria decreased with the increase in antibiotic types.

### Occurrence of ARGs and integron in strains

The results of resistance genes detection of 80 ARB isolated from two lakes were shown in Figure 2. In total 14 TET ARB were isolated from the two lakes, of which four belonged to FQ and the rest came from XQ. It was found that *tetG* had a high detection rate in FQ (100% (4/4)) than XQ (50% (5/10)), indicating that *tetG* is the dominant TET resistance gene in FQ. In contrast, the detection rate of *tetA* in XQ was slightly higher (80% (8/10)) than that in FQ lake 75% (3/4). The detection rate of *tetB* and *tetM* was relatively low, both of the gene detection rates were 20% (2/10) in XQ, while neither of them were detected in FQ. All of the TET resistant bacteria in FQ detected the corresponding resistance gene, while there was one strain in XQ that did not contain any corresponding resistance gene.

Among the three  $\beta$ -lactam resistance genes, FQ detected two types of genes as *bla*<sub>SHV</sub>, and *bla*<sub>TEM</sub> (Figure 2(c)), with the same detection rate of 12% (3/25). There were three strains from 22 isolated  $\beta$ -lactam ARB in XQ for which we detected the related ARGs, in addition to the above 2 genes, *bla*<sub>CTX-M</sub> has also been detected. The overall detection rates of *bla*<sub>SHV</sub>, *bla*<sub>TEM</sub>, and *bla*<sub>CTX-M</sub> in the two lakes were low.

The detection results of three SMZ resistance genes are shown in Figure 2(b). It was found that *sul2* in FQ had the highest detection rate at 26.83% (11/45), while *sul1* and *sul3* were the same at 12.20% (5/41). The detection rate of *sul1* in XQ was 23.07% (9/39), and that of *sul2* was 15.38% (6/39), slightly higher than *sul3* (12.82% (5/39)). FQ and XQ screened out 10 and 11 strains of SMZ ARB, respectively, and the corresponding detection rates of ARGs were 100% (10/10) and 90.90% (10/11), respectively. Compared with antibiotic resistance genes, the detection rate of *int I* in two lakes was the highest, over 90%.

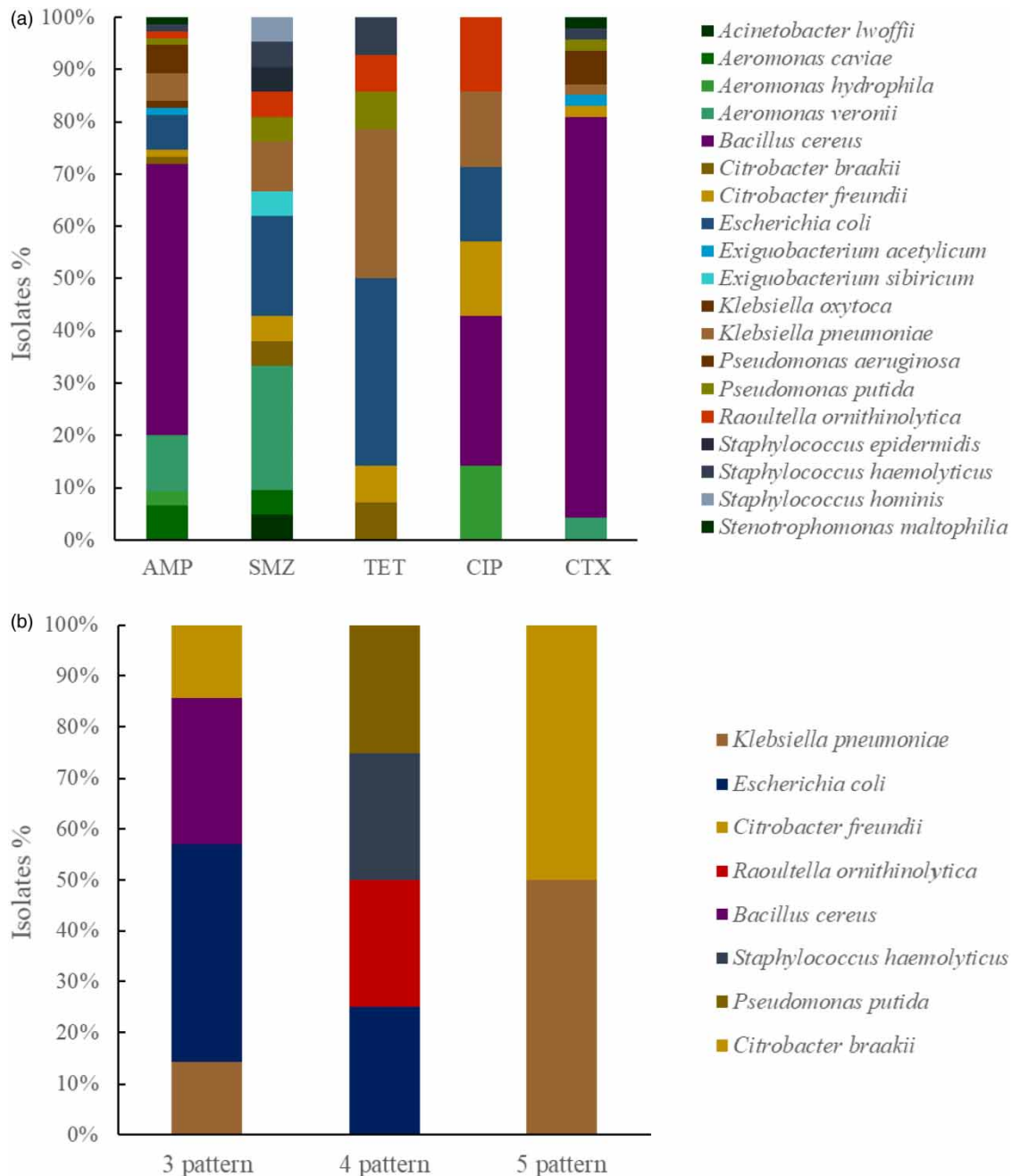


**Figure 2** | ARGs and class I integron distribution of 80 antibiotic resistant bacteria isolated from FQ and XQ lake. The prevalence of (a) tetracycline resistance genes; (b) sulfa resistance genes; (c)  $\beta$ -lactam resistance genes; (d) class I integron.



**Result of species identification**

The identification of bacteria species is shown in Figure 3(a). The result showed that the 80 ARB can be divided into nine genera and 19 species, and there are differences in the species distribution of different types of ARB. The dominant ARB of AMP and CTX is *Bacillus cereus*, the common species of TET-resistant bacteria is *Escherichia coli*. It was found that *Bacillus cereus*, *Escherichia coli*, *Aeromonas veronii*, *Aeromonas caviae*, and *Raoultella ornithinolytica* are the common resistant species in the two lakes. Among the antibiotic resistant bacteria screened from the sample, *Bacillus cereus* occupied a certain number advantage over other bacteria, which showed that this kind of species belongs to the main antibiotic resistant bacteria in landscape water, indicating that this species may play a role in promoting the transmission, migration and transformation



**Figure 3** | (a) Identified genera containing 80 antibiotic resistance bacteria. (b) Identified genera of multiple antibiotic resistance bacteria. 3 pattern: 3 antibiotic resistance patterns; 4 pattern: 4 antibiotic resistance patterns; 5 pattern: 5 antibiotic resistance patterns.

of ARGs. The identification of multiple resistant bacteria is shown in Figure 3(b). The results showed that *Escherichia coli* was the main strain of triple antibiotic resistant bacteria, accounting for 42.86%. *Raoultella ornithinolytica* was the main resistant strain. Among all multiple resistant bacteria, *Escherichia coli* has a higher detection rate, and it was detected in triple and quadruple antibiotic resistant bacteria.

### Correlation between ARB and environmental variables

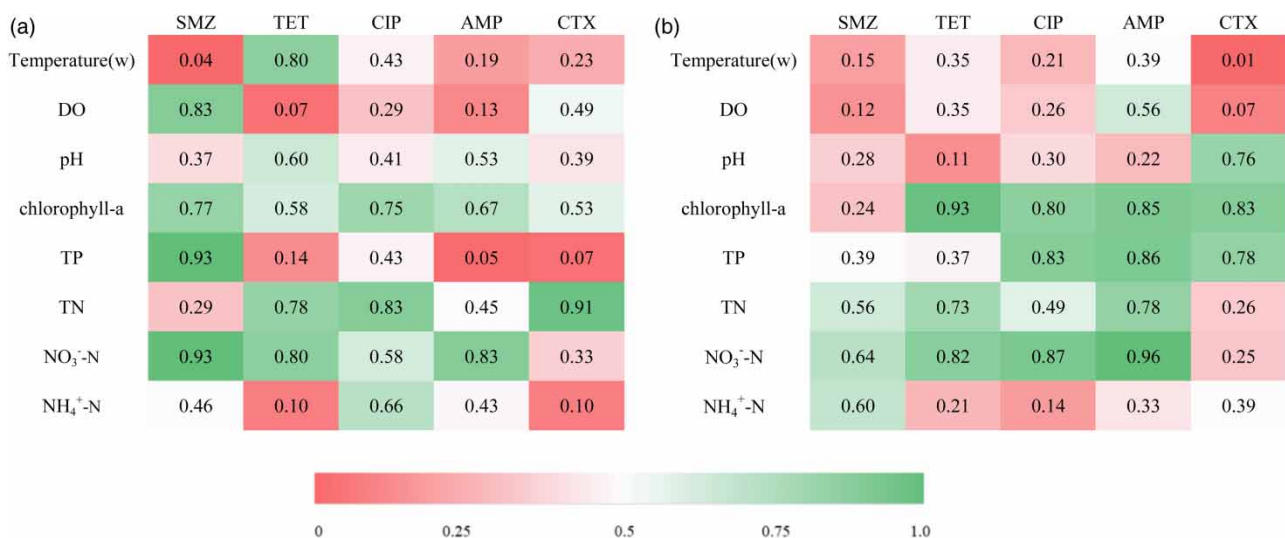
Correlation analysis was conducted between the detected concentration of antibiotic resistant bacteria and the synchronized water quality detection indexes of water temperature, dissolved oxygen, pH, chlorophyll *a*, TP, TN, nitrate nitrogen, and ammonia nitrogen. The results are shown in Figure 4, where  $p < 0.01$  indicated significant correlation and  $p < 0.05$  indicated significant correlation. As can be seen from the figure, the water temperature was significantly correlated with the content of SMZ-resistant bacteria and CTX-resistant bacteria in two lakes ( $p < 0.05$ ), the TP content (Figure 4(a)) in FQ lake was significantly correlated with the content of AMP-resistant bacteria ( $p < 0.05$ ), while the other remaining water quality indexes were not significantly correlated with the concentration of ARB ( $p > 0.05$ ).

## DISCUSSION

The major supplement source of urban recreational water is surface water, however, with the increasing shortage of water resources in recent years, many areas in China have gradually started to use reclaimed water from sewage treatment plants as the source of water replenishment for recreational water (He *et al.* 2016) to alleviate the increasingly exhausted water resources. However, at the same time, the removal of ARB and ARGs in the regenerated water is not required for treatment. If it is directly injected into the landscape water as supplementary water, it may cause pollution to the landscape water and thus affect human health.

### Distribution characteristics of antibiotic resistant bacteria and resistant pattern

In the case of seasonal variation, except that the resistance rates of AMP and CTX fluctuate clearly, the overall change trend of the resistance rates of the other three antibiotics is basically the same, and a similar situation also occurs in XQ (Figure 1). There is a special phenomenon in July to September, during this time, except for SMZ and CTX in FQ, for the remaining antibiotic resistance rates in the two lakes all percentages increased greatly, the maximum resistance compared with the baseline all appeared during this time, especially concentrated in September. This phenomenon may be related to the rainfall in Xi'an during that period. Rainfall is likely to help ARB and ARGs to enter recreational water, it was found that rainfall will increase the abundance of antibiotic resistant bacteria and antibiotic resistance genes in a storm drain (Ahmed *et al.* 2018), thereby causing microbial pollution to downstream water (Newton & McClary 2019). In addition, reclaimed water is used to irrigate the soil around the recreational water, resulting in certain biological antibiotic resistance pollution in the soil (Wang



**Figure 4** | Spearman correlations of antibiotic resistance bacteria abundance and water quality parameters in two lakes. (a) FQ Lake. (b) XQ Lake.

*et al.* 2014; Han *et al.* 2016), following rainfall scouring, these soil pollutants will enter the recreational water, thus increasing the content of ARB in the water. On the detection of resistance rate, FQ and XQ also produced a similar situation, SMZ, AMP and CTX resistant bacteria detection rates were higher than that of TET and CIP, the AMP and CTX resistant bacteria detection rate is the highest, the whole detection rate order from high to low was AMP > CTX > SMZ > TET > CIP. The test for water resistance phenotype from 80 isolated strains also confirmed the results. The existence of antibiotics in water will lead to resistance of bacteria in water under pressure selection (Xu *et al.* 2016). In addition, the antibiotic resistance rates in the two lakes were found to be high in summer and winter on the whole, which was similar to the situation found previously in the other study (Huang *et al.* 2019). In this study, a content difference test of five types of ARB were conducted and the results found that three kinds of antibiotic resistance bacteria content (AMP, CIP and SMZ) did not have relatively differences.

Based on the detection of antibiotic resistance rate in the early stage, 80 strains of antibiotic resistant bacteria were screened out from two lakes through an antibiotic sensitivity test, and the distribution of antibiotic resistant patterns of these antibiotic resistant bacteria was very similar. The phenotype resistant to AMP-CTX was found the most (Table 4), accounting for more than half of the antibiotic-resistant phenotypes, which is a very interesting phenomenon. In previous reports, resistance to one antibiotic was more common (Ham *et al.* 2012), while such a wide range of double resistance was rare. Moreover, in this study, the AMP-resistant phenotype was common and detected in almost all antibiotic resistant bacteria, which indicated that AMP-resistant phenotype was dominant in two lakes.

### Distribution characteristics of ARGs and class I integron in 80 antibiotic-resistant strains

PCR was performed on 10 antibiotic resistance genes and class I integron. Tetracycline resistance genes had a high detection rate, among which *tetA* and *tetG* genes were the main coding genes (Figure 2(a)). Bacterial resistance to antibiotics mainly develops through changing the target of antibiotic action, efflux pump antibiotic mechanisms, inactivating or inactivating enzymes of antibacterial antibiotics, changing pore protein to prevent antibiotic penetration, and changing antibiotic metabolic pathways (Tong and Wei 2012). However, the occurrence of tetracycline resistance is generally due to the efflux pumps shielding the target or the enzyme inactivation required by antibiotics. The mechanisms of the 2 TET resistance genes with the highest detection rate are both efflux pumps mechanism, and efflux pumps mechanism is the most common antibiotic resistance in tetracycline resistance. The recharge sources of recreational water in this study are surface water or reclaimed water, which may contain corresponding ARGs, studies on winter rivers in Shijiazhuang, China found that *tetA* and other genes were detected in natural water (Luo *et al.* 2019), and the detected amounts in rivers and sediments were different. Some research has found that sulfonamides, tetracycline and integron genes are ubiquitous in the sediments in the estuary and coastal areas of the Yangtze river (Lin *et al.* 2015), and the content of sulfonamides, tetracycline and integron genes showed a trend of decline from the estuary to the coast. It can be found that tetracycline resistance genes are quite widespread, and efflux pumps as tetracycline resistance genes are dominant. After being added to the recreational water, this may lead to the spread of bacterial antibiotic resistance through horizontal transfer. The detection rate of  $\beta$ -lactam resistance genes was relatively low, except for a small number of detected genes, most antibiotic resistance patterns strains did not detect the corresponding genes, this may be due to the difference of ARGs carried by some resistant bacteria. Lepuschitz *et al.* (2019) found that not all new extended spectrum beta-lactamases (ESBL) producing strains in Austrian rivers carry *bla*<sub>TEM</sub>, and the genes with high detection rate are mainly OXA-1, which was consistent with other studies (Jiang *et al.* 2013). It is speculated that in addition to the tested genes, there may be other corresponding resistance genes (Knapp *et al.* 2012). However, in this study, only three types of  $\beta$ -lactam resistance genes were selected, which means that the detection has certain limitations. It was also found that although tetracycline and sulfonamides resistance genes were detected in both antibiotic resistance bacteria in two lakes, the prominent points of these two antibiotic resistance genes were different. *tetG* had the highest detection rate for tetracycline in the FQ lake, and sulfonamides had the highest *sul2* rate, while the XQ lake had *tetA* and *sul1*. This may be related to a genetic original mobile *int I* higher detection rate, *int I* as the carrier, in the dissemination of resistance genes plays an important role (Uyaguari *et al.* 2013). Researchers have found that *int I* in natural waters such as the river has a high correlation with antibiotic resistance gene detection rate and a low correlation in the wastewater treatment plant (Ma *et al.* 2017). Although suspended substances in water were not detected in this study, suspended substances in landscape water, such as algae and microplastics, might influence the spread of antibiotic resistance genes together with integron (Fang *et al.* 2018a, 2018b). The *int I* detection rate was more than 90%, it is highly possible that rainfall also led to an increase in the content of integrons in lake, leading to the difference in the major antibiotic resistance genes found in the two lakes, which is similar to some studies (Chen *et al.* 2019), and the detailed reasons need to be further studied.

### Distribution characteristics of antibiotic resistance strains genera

The identification of 80 antibiotic resistant bacteria showed that *Bacillus cereus* and *Escherichia coli* were the main strains (Figure 3(a)). *Bacillus cereus* as a kind of conditionally pathogenic bacteria can cause food poisoning. It can be detected in food and clinical scenarios, but little documentation has reported it in the water environment (Zhuang *et al.* 2013). The reports on the distribution of *Bacillus cereus* in the water-supply plant were studied and it was found that conventional technology has a better removal effect (Jiang *et al.* 2018). Other studies tested the antibiotic resistance of 60 strains of food-borne *Bacillus cereus* in Guizhou, China, and found that they were resistant to ampicillin, trimethoprim, and cefepime, which could not be used as clinical therapeutic antibiotics for food-borne diseases caused by *Bacillus cereus* (Zhou *et al.* 2019). Humans will be exposed to these landscape water to a large extent, and may even inhale aerosols containing antibiotic resistance bacteria (Pal *et al.* 2016), which may increase the possibility of human infection with pathogenic bacteria. As an intestinal microorganism, *Escherichia coli* has been monitored as a water pollution indicator, and excessive drainage in sewage plants will lead to the increase in antibiotic-resistant *Escherichia coli* in water (O'Flaherty *et al.* 2019). Recent studies have shown that the spread of antibiotic resistance genes in water may be mainly caused by faeces, rather than the environmental pressure selection (Karkman *et al.* 2019), so the most common type of *Escherichia coli* found in excrement and urine, is thought to be the most likely to spread as a source of bacteria. *Escherichia coli* exist in the human intestinal tract, its resistance can be transferred to those symbiotic intestinal bacteria that were originally sensitive to antibiotics (Ham *et al.* 2012). In this study it was found that, in the detection of multiple resistance, *Escherichia coli* were resistant to AMP and three other kinds of antibiotics, but only sensitive to CTX (Figure 3(b)), no CTX-resistant *Escherichia coli* existed. This may signal that *Escherichia coli* has a high sensitivity to CTX. It may also be the reason that CTX resistance gene detection rate was low in the two lakes leading to a low shifting level, the detailed reason needs to be further exploration. Most of the remaining identified antibiotic resistant bacteria are not pathogenic bacteria, but they can transfer antibiotic resistance genes to pathogenic bacteria through horizontal gene transfer. Tests also found a small amount of other pathogenic bacteria such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, they exist in landscape water, providing opportunities in which the pathogenic bacteria may be possible to acquire resistance genes. Conditional pathogenic bacteria and pathogenic bacteria, after entering the human body, can increase the use of antibiotics for treatment, cause multiple antibiotic resistance superbug to appear (Alicia *et al.* 2018), and pose a potential health risk for humans. Bacterial resistance can be divided into intrinsic resistance and acquired resistant. In this study, among 80 antibiotic resistant bacteria, most of them were acquired resistance, few of them were intrinsic resistance. Nine strains were identified as intrinsic resistance, including three genera like *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia*, which needs to be further explored by subsequent studies.

### Effects of water quality indexes on the content of antibiotic resistant bacteria in landscape water

The physical and chemical properties of water as a routine indicator may affect the situation of antibiotic resistant bacteria in water. However, in this analysis of the correlation between water quality detection indexes and antibiotic resistant bacteria, except that the change of water temperature correlated with the concentration of antibiotic resistant bacteria in SMZ and CTX in two lakes, only the TP content had a significant impact on the concentration of AMP-resistant bacteria in lake FQ (Figure 4(a)), while the remaining water quality indexes did not affect the other concentrations of antibiotic resistant bacteria in the two lakes, This may be due to the synergistic effect of TP content and corresponding antibiotics in water. Some studies have found that the high-strength occurrence of antibiotics and antibiotic resistance genes might induce the phosphorus recovery process (Cai *et al.* 2020), thus TP may also have an effect on antibiotics and drug resistance genes, and then affect the content of drug-resistant bacteria, therefore, it might be of great significance to reduce the content of AMP-resistant bacteria in recreational water by controlling the content of TP in water.

## CONCLUSION

The distribution characteristics of ARB concentration and related ARGs in the two lakes replenished by different water source in Xi'an were analyzed in the present study, the results found were: (1) Five kinds of ARB were all detected in two lakes, most of the antibiotic resistant rates showed the characteristic of changing with season and reached the maximum in autumn. The results of resistance patterns showed that 51.25% the ARB were AMP-CTX, indicating that the ARB in the two lakes were mainly resistant to  $\beta$ -lactam antibiotics, while the main multiple resistance pattern was AMP-SMZ-TET. (2) Whether reclaimed water or surface water were used as the supplementary water source, tetracycline resistance genes (*tetA*

and *tetG*) and class I integron gene *int I* were the major ARGs that ARB carried, however there was a difference that *tetA* was widely distributed in XQ while *tetG* was mainly found in FQ. (3) Species identification showed that *Bacillus cereus* and *Escherichia coli* were the dominant species in the two lakes. *Escherichia coli* was the main strain of triple antibiotic resistant bacteria among all multiple resistant bacteria (42.86%). (4) Correlation analysis showed that water quality indexes in the two lakes had no significance relationship with the content of ARB, except water temperature and TP ( $p > 0.05$ ).

In conclusion, the choice of replenishment either by reclaimed water or surface water may not affect the distribution characteristics of ARB in urban recreational water, indicating that reclaimed water can be used as a safe and effective replenishment source. Our findings help to better understand the effect of reclaimed water reuse, and to improve the management and utilization of reclaimed water.

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## AUTHORS CONTRIBUTIONS

YQL sorted out all the data and wrote the manuscript; CMZ provided the idea for this study and was a major contributor to writing and modifying the manuscript; XM analyzed the water samples.; PPZ collected the water samples; JL helped the investigation; ZW helped the investigation. All authors read and approved the final manuscript.

## DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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