Distribution of antibiotic resistance genes in Bosten Lake, Xinjiang, China

Ting Zhou, Jianjiang Lu, Yanbin Tong, Shanman Li and Xiaolong Wang

ABSTRACT

The occurrence of antibiotic resistance genes (ARGs) and resistant bacteria was quantified in 17 water samples collected across Bosten Lake, Xinjiang, China. The heterotrophic plate count method was used to detect the levels of sulfonamide- and tetracycline-resistant bacteria, which have mean concentrations of 2.50×10^5 and 4.63×10^3 CFU/mL, respectively. The resistance genes of sulfonamide (*sul1*, *sul2*) and tetracycline (*tetM*, *tetO* and *tetW*) were detected, and results showed that all other ARGs except the *tetO* gene were obtained from all samples. Four of the obtained ARGs were further quantified, and results showed that the sulfonamide resistance genes were prevalent. The relative abundance was in the range of 2.81×10^{-5} to 3.33×10^{-3} for the *sul1*/16s-rRNA and 1.04×10^{-5} to 3.80×10^{-3} for the *sul2*/16s-rRNA. For the *tet* genes, the relative concentrations of *tetM*/16s-rRNA and *tetW*16s-rRNA ranged from 1.18×10^{-5} to 2.46×10^{-4} and 1.58×10^{-6} to 4.19×10^{-4}, respectively. The concentration divergence among ARGs may be related to the different characteristics of each gene. This study validated that Bosten Lake was affected by ARGs and resistant bacteria, thus turning the lake into an important reservoir for the transfer of ARGs and resistant bacteria.

Key words | antibiotic-resistant bacteria, antibiotic resistance genes, quantification, sulfonamides, tetracyclines

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INTRODUCTION

Antibiotics are widely used in concentrated animal feeding operations to prevent infectious diseases and promote growth. Furthermore, antibiotics are often used in human medicine to treat infectious diseases. However, antibiotics are incompletely metabolised by humans and animals, with an estimated metabolic rate of around 30% (Kümmerer & Henninger 2003; Gao *et al.* 2012). In recent years, the extensive use of antibiotics has accelerated the propagation of resistance genes among different bacteria. Studies have proven that antibiotic use and the emergence of antibiotic-resistant bacteria are correlated (Aminov *et al.* 2001; Gao *et al.* 2012). The increase in antibiotic resistance is considered closely linked with the use of antibiotic pharmaceuticals in humans and animals (Pruden *et al.* 2006). The presence of antibiotic resistance genes (ARGs) mainly causes bacterial resistance. Ever since Pruden *et al.* (2006) proposed ARGs as a new type of environmental pollutant, reports on the propagation and pollution of ARGs have steadily increased (Peak *et al.* 2007; Luo *et al.* 2010).

ARGs are usually found in mobile genetic elements (plasmids, transposons or integrons), thereby facilitating the diffusion of these ARGs into various environmental compartments through horizontal gene transfer (Pruden *et al.* 2006; Stoll *et al.* 2012). Several methods have been developed to detect ARGs, which have long persistence times in the environment and easy migration and transformation among different floras; thus, ARGs cause greater harm than antibiotic residues in the environment (Bertolla *et al.* 2000; Dantas *et al.* 2008). Upon the death of bacteria carrying ARGs, naked DNA-carrying ARGs can exist in the environment for long periods even after the completion of selection pressure (Hill & Top 1998). Most reports show that high concentrations of antibiotic-resistant bacteria and ARGs are relevant to human or agricultural activities (Chee-Sanford *et al.* 2001; Pruden *et al.* 2006).

Most previous studies focused on the presence of ARGs in inland rivers and river tributaries (Stoll *et al.* 2012), aquaculture facilities (Ji *et al.* 2011), river basins (Pei *et al.* 2006; Luo *et al.* 2010), swine farms (Selvam *et al.* 2012) and wastewater utilities (Zhang & Zhang 2011). However, few studies have focused on inland freshwater bodies close to richly populated areas. Bosten Lake is located in the southern region of Yanqi

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Basin in Korla City, Xinjiang, China. The lake plays an irreplaceable function in the economic development of the South Xinjiang region. In recent years, because of the excessive development of the basin, industrial wastewater and domestic sewage have been discharged uncontrollably into canals, thus causing serious pollution in Bosten Lake, destroying the ecological environment, and increasing lake salinity. Large-scale urbanisation has affected the environment of Bosten Lake in various aspects. Therefore, examining the ARG concentration in Bosten Lake is necessary.

Bosten Lake was selected as the research area in this study to investigate the abundance and distribution of sulfonamide and tetracycline resistance genes in freshwater lake systems, which are the main sources of water resources to locals. Real-time polymerase chain reaction (PCR) was applied to quantify the ARG pollution. This investigation could provide direct field evidence of ARG pollution in Bosten Lake.

MATERIALS AND METHODS

Sampling sites

Bosten Lake (86° 26′ E–87° 40′ E, 41° 56′ N–42° 14′ N) in Southern Xinjiang has many unique attributes. Bosten Lake is considered the largest inland freshwater lake in China and the largest organic aquaculture in Xinjiang because of its lush reed resources and precious fish species. The lake is characterised by picturesque natural scenery, which attract many tourists. Bosten Lake is also known as the 'Hawaii' of Xinjiang. Furthermore, the lake is the main source of water for the local populace and is the direct water source of the downstream Tarim River. Surface water samples were collected at various sites in Bosten Lake in July 2012.

Sample collection and processing

Surface water samples (2.0 L) from the top 0.5 m layer were collected and decanted into sterile containers. In total, 17 samples were obtained from different locations; the samples were frozen and kept in the dark prior to analysis. Each 1.5 L sample was stored at −80 °C in the laboratory for subsequent molecular analysis. The remaining volume was processed within 24 h for viable culturing.

Screening for antibiotic-resistant bacteria

To obtain the concentrations of sulfonamide- and tetracycline-resistant bacteria in the samples, the heterotrophic plate count (HPC) method was used to evaluate the colony-forming units (CFUs). A 10-fold serial dilution was made with a 10^{-1} dilution (1 mL of water sample diluted in 9 mL of sterilised phosphate buffered saline (pH = 7.4)). The diluted water samples (0.1 mL) were directly plated on an R2A agar medium with and without added antibiotics. Antibiotic concentrations of 281.8 mg/L sulfamethazine (Sigma Aldrich) and 16.0 mg/L tetracycline (Sigma Aldrich) were used on the basis of the suggestions indicated in literature (Aminov *et al.* 2001; Pei *et al.* 2006). The plates were then incubated at 37 °C for 2 d and at 27 °C for another 5 d (Brooks *et al.* 2007). The CFUs were calculated with 30-well to 300-well separated colonies per plate. The screening process was performed in duplicate.

DNA extraction

Water samples (0.5–1 L) were concentrated by filtration with a 0.22 μm nitrocellulose membrane (Millipore, USA). The filters with the trapped biomass were collected via reusable filter funnels, and then placed in the extraction tubes provided in the Power Water DNA Isolation Kit (MoBio Laboratories, Inc.). DNA was extracted according to the method indicated by the manufacturer. The extracted DNA was further purified by using a universal DNA Purification Kit (Tiangen, Beijing, China) to minimise PCR inhibition. The concentration and quality of the extracted DNA were determined by using a spectrophotometer (NanoDrop ND-1000, NanoDrop, USA) and 0.8% agarose gel electrophoresis. Thereafter, the extracted DNA was stored at −20 °C for subsequent analysis.

Qualitative PCR assays for the detection of ARGs

Qualitative PCR assays were used to detect the presence of sulfonamide ARGs (*sul1* and *sul2)* and tetracycline ARGs (*tetM*, *tetO* and *tetW*) in the surface water samples. PCR amplification was performed by using an ABI PCR System 9700 (ABI, USA). The reaction mixture (25 μL total volume) for the PCR contained 2.5 μL 1× *Taq* reaction buffer, 2 μL of 0.2 mM dNTPs, 0.2 mM primers, 1.75 U of *Taq* DNA polymerase and 1–2 μL of template. Table 1 lists the primers that target ARGs with their conditions. The temperature programme consisted of the following: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 15 s at 95 °C, 30 s at the annealing temperature (Table 1 for *sul* and *tet* genes), 30 s at 72 °C and a final extension step for 7 min at 72 °C. The PCR products were analysed by electrophoresis in a 2% (w/v) agarose gel in a 1× TAE buffer. Positive PCR products were further identified by cloning and sequencing.

Table 1 | PCR primers used in this study

Target	Primers	Sequences (5′-3′)	Annealing temperature (°C)		Amplicon size (bp)
			PCR	qPCR	
sul1	Sul1-FW	CGCACCGGAAACATCGCTGCAC	55.9	65	163
	Sul1-RV	TGAAGTTCCGCCGCAAGGCTCG			
sul2	Sul2-FW	TCCGGTGGAGGCCGGTATCTGG	60.8	57.5	191
	Sul2-RV	CGGGAATGCCATCTGCCTTGAG			
tetM	Tet M-FW	ACAGAAAGCTTATTATATAAC	55	55	171
	Tet M-RV	TGGCGTGTCTATGATGTTCAC			
tetO	Tet O-FW	ACGGARAGTTTATTGTATACC	60	50	171
	Tet O-RV	TGGCGTATCTATAATGTTGAC			
tetW	Tet W-FW	GAGAGCCTGCTATATGCCAGC	64	60	168
	Tet W-RV	GGGCGTATCCACAATGTTAAC			

Real-time quantitative PCR

The quantitative PCR (qPCR) method and 16s-rRNA using the SYBR Green approach were applied to quantify the ARGs. The primer sequences and targets, along with the annealing temperature used for the sulfonamide and tetracycline ARGs, were previously designed (Aminov *et al.* 2001; Pei *et al.* 2006) (Table 1). The annealing temperature for some primers was different from the temperature used in traditional PCR because qPCR is more sensitive and accurate than traditional PCR. The qPCR method requires high quality primer and is susceptible to DNA extraction matrix inhibition; thus, the genes were detected by traditional PCR but could not be quantified by qPCR (Pei *et al.* 2006). The primer sets for the 16s-rRNA genes were reported in the study of Peak *et al.* (2007). All qPCR analyses were performed by using an ABI 7300 apparatus (ABI, USA) in a 20 μL reaction mixture comprising 2× *TransStart* Top Green qPCR SuperMix (Transgene, China), 0.2 mM primers and a 1 μL template. The procedure used was as follows: 95 °C for 15 min followed by 45 cycles at 95 °C for 15 s, 30 s at the annealing temperature (Table 1), 72 °C for 30 s, and a final melt curve stage with temperatures ranging from 60 to 95 °C.

Data analysis

DNAMAN and BLAST were used to analyse the accession number of the ARGs. The data analysis of the relative concentrations of the ARGs/16S genes was performed by using Origin 8.5 (Origin Lab Corporation, USA). The average and standard deviation of all the data were determined by using Microsoft Excel 2007.

RESULTS AND DISCUSSION

Sulfonamide- and tetracycline-resistant bacteria concentrations in Bosten Lake

The HPC method was used to detect the occurrence of sulfonamide- and tetracycline-resistant bacteria in Bosten Lake. The results showed that the mean concentrations of sulfonamide- and tetracycline-resistant bacteria and the total HPC in the water samples were 2.55×10^5, 4.63×10^3 and 8.43×10^5 CFU/mL, respectively. The concentration ratios of the sulfonamide- and tetracycline-resistant bacteria normalised with the total heterotrophic cultured bacterial counts were 0.30 and 0.005, respectively. The CFU of the sulfonamide-resistant bacteria was more than that of the tetracycline-resistant bacteria. Thus, a higher level of sulfonamide-resistant bacteria than tetracycline-resistant bacteria was present in Bosten Lake. Munir *et al.* (2011) and Gao *et al.* (2012) studied the mean concentrations of sulfonamide- and tetracycline-resistant bacteria in the final effluent of wastewater utilities, with values of 6.10×10^3 and 3.09×10^3 CFU/mL, respectively. The ratio of the sulfonamide-resistant CFU that was normalised with the total control was 0.30, which was higher than the ratio obtained by Pei *et al.* (2006). The mean concentration of the tetracycline-resistant bacteria in the final effluent of sewage treatment plants was 1.05×10^1 CFU/mL (Gao *et al.* 2012), which is about 2 logs less than our result. The antibiotic-resistant bacteria concentrations in the current study are relatively higher than the levels found in wastewater treatment plant (WWTP) effluent (Table 2), thus indicating that sulfonamide- and tetracycline-resistant bacteria are widely distributed in Bosten Lake.

Table 2 | Antibiotic-resistant bacteria concentrations in the different samples detected through the HPC method

Type of water sample	Antibiotic targeted	CFU/mL	References
WWTP effluent	Sulfonamide	$10^{3.49}$ (mean)	Gao *et al.* (2012)
	Tetracycline	$10^{1.02}$ (mean)	
WWTP influent	Sulfonamide	$10^{5.23}$–$10^{7.08}$	Munir *et al.* (2011)
	Tetracycline	$10^{4.18}$–$10^{5.36}$	
WWTP effluent	Sulfonamide	$10^{2.02}$–$10^{3.79}$	
	Tetracycline	$10^{0.70}$–$10^{2.48}$	
Freshwater lake	Sulfonamide	$10^{5.40}$–$10^{5.42}$	The current study
	Tetracycline	$10^{3.64}$–$10^{3.70}$	

Occurrence of ARGs in Bosten Lake

Data on the qualitative occurrence of sulfonamide and tetracycline resistance genes obtained by using PCR assays are listed in Table 3. Sulfonamide and tetracycline resistance genes were detected in all 17 samples collected in Bosten Lake. However, *tetO* was not detected by conventional PCR analysis. The widespread prevalence of two *sul* genes, namely, *sul1* and *sul2*, in the aquatic ecosystems in Bosten Lake (100%) was most likely caused by the easy dissemination of these genes via mobile genetic elements. The *sul1* and *sul2* encoding forms of the enzyme (dihydropteroate synthase; DHPS) are two alternative sulfonamide-resistant DHPS genes and are uninhibited by antibiotic sulfonamides. These two genes are commonly found in Gram-negative bacteria and in mobile genetic elements, such as class 1 integrons or plasmids (Skold 2000; Antunes *et al.* 2005). Furthermore, sulfonamides and sulfonamide resistance genes may be correlated, thus accelerating the dissemination of sulfonamide resistance genes in the aquatic environment. The extremely high prevalence of the *sul1* and *sul2* genes in Bosten Lake suggests that the use of sulfonamides may not be limited to aquatic ecosystems because antibiotics are highly important broad-spectrum antimicrobial agents.

Sulfonamide and tetracycline resistance genes are the most frequently detected ARGs in aquatic environments according to several published studies. Chopra & Roberts (2001) indicated that bacterial resistance to tetracycline is mainly mediated by the energy-dependent efflux pump (efflux proteins) and ribosomal protection proteins (RPP). The *tetO*, *tetM* and *tetW* genes were chosen to represent tetracycline resistance because of their high frequency of prevalence and detection relative to the efflux protein genes. In the current study, *tetM* and *tetW* genes, but not the *tetO* gene, were detected in all samples. The results showed that Bosten Lake was polluted with tetracycline-resistance genes; thus, further analysis must be performed to quantify the extent of these genes. Tetracycline resistance genes are often carried by conjugative plasmids or transposons, which allow the mobilisation of these genes via horizontal gene transfer. The *tetO* gene was found by Roberts (1997) in both Gram-positive and Gram-negative bacteria. The *tetM* gene encodes a protein with a significant sequence, such as cytoplasmic proteins, which confer a ribosomal protection tetracycline-resistance mechanism. The RPP gene *tetM* has been reported to occur in the microbial communities of lagoons, WWTPs (Chee-sanford *et al.* 2001; Zhang & Zhang 2011) and natural environments (Mackie *et al.* 2006). Therefore, the *tetM* gene is widely distributed in different microbial environments and can be transferred between various bacterial species. The *tetW* gene has recently been identified and associated with conjugative transposons, which can be potentially transferred into other genera (Aminov *et al.* 2001; Melville *et al.* 2004).

PCR products were further sequenced, and the results showed that the *sul1*, *sul2*, *tetM* and *tetW* sequences were mostly identical with the *Escherichia coli* strain S1 sulfonamide resistance protein (*sul1*) gene (KF240816), *E. coli* strain plasmid DHPS type-2 (*sul2*) gene (EU653289), uncultured bacterium clone TJ3-8 tetracycline resistance protein (*tetM*) gene, partial cds (GU474976.1) and uncultured organism clone SCDw-50 tetracycline resistance protein (*tetW*) pseudogene

Table 3 | PCR analysis of ARGs in water samples of Bosten Lake

	July 2012 Bosten Lake sampling site																	
ARG	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	+ Control
sul1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
sul2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
tetM	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
tetO	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
tetW	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+: present; −: absent.

with partial sequence (GU116963.1), respectively. Each PCR product had a sequence identity of more than 98%.

Quantification of ARGs at the sites

A relative abundance of the four resistance genes was directly detected by qPCR in all samples. The relative gene expression of the sample represents the number of ARGs contained in the microbial community, which could reveal the distribution of genes in the samples (Figures 1 and 2). To assess the level of resistance relative to the overall population size, the normalised number of copies of 16S rRNA genes was chosen as the mean. Figure 1 shows that the total concentrations of the two *sul* genes (*sul1* and *sul2*) ranged from 2.81×10^{-5} to 3.33×10^{-3} and 1.04×10^{-5} to 3.80×10^{-3} (copy of *sul* genes/copy of 16S genes), respectively. However, for the *tet* genes, the ratio of the concentration of ARGs normalised with the 16 s rRNA genes ranged from 1.18×10^{-5} to 2.46×10^{-4} for the *tetM* gene and 1.58×10^{-6} to 4.19×10^{-4} for the *tetW* gene. Bosten Lake is the largest fish production base in Xinjiang. The fish feed used in the area contains antibiotics that partially enter into the water column. Thus, these antibiotics contribute to the abundance of ARGs. Our research group also confirmed the existence of corresponding antibiotics in the lake.

For the *sul* genes, the level of the *sul1* gene was similar to that in the Poudre River of northern Colorado, with a relative concentration of 10^{-3} in the most polluted regions of the river. However, the *sul2* gene had a concentration of 10^{-4}, which is lower than that in the Poudre River (Pruden *et al.* 2006). The concentrations of the *sul* genes in the area were evidently affected by urban and agriculture activities (Pei *et al.* 2006); this result was in accordance with the results obtained in the current study. Our results also conformed to the aquaculture environment of Tianjin with the relative concentrations of the *sul* genes at 3.0×10^{-5} to 3.3×10^{-4} for the *sul1*/16S rRNA and 2.0×10^{-4} to 1.8×10^{-3} for the *sul2*/16S rRNA (Gao *et al.* 2012). However, the relative abundance of these genes was lower than the levels reported in the Haihe River (Luo *et al.* 2010). The relative abundance of *tet* genes in Bosten Lake was comparable with the levels reported in wastewater lagoons (Peak *et al.* 2007). The concentration of *tetW* genes was more than that of the result obtained by Pei *et al.* (2006), and the level of the *tetM* gene was consistent with the result reported by Zhang & Zhang (2011). *tetM* and *tetW* gene concentrations were lower than those of the *sul1* and *sul2* genes in different samples, which were similar to previous observations (Pei *et al.* 2006; Luo *et al.* 2010; Munir *et al.* 2011; Gao *et al.* 2012). The study also showed a dramatic variation of ARG levels among the different sample sites. The high concentrations of ARGs obtained from various studies indicated that Bosten Lake was considered an important reservoir for ARGs.

In the 17 sites, the normalisation of the ARG concentrations with that of the total 16 s rRNA genes varied greatly among the different samples. The following overall trend was observed with respect to the ARG concentrations: *sul1* > *sul2* > *tetM* > *tetW*. *sul1* and *tetW* have the highest and lowest concentrations. Such differences in the prevalence of specific ARGs might be attributed to different patterns of

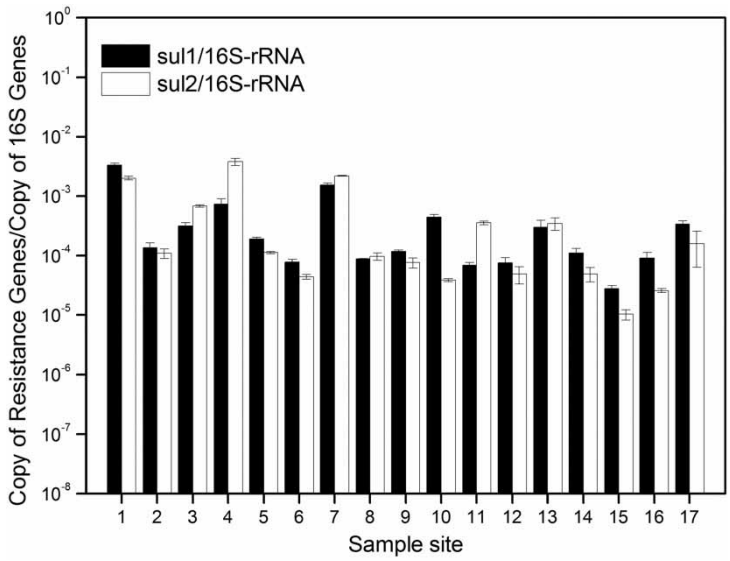

Figure 1 | Copies of resistance genes (*sul1* and *sul2*) normalised with the number of bacterial 16 s rRNA genes at different sites. The error bars indicate three independent qPCR runs in duplicates.

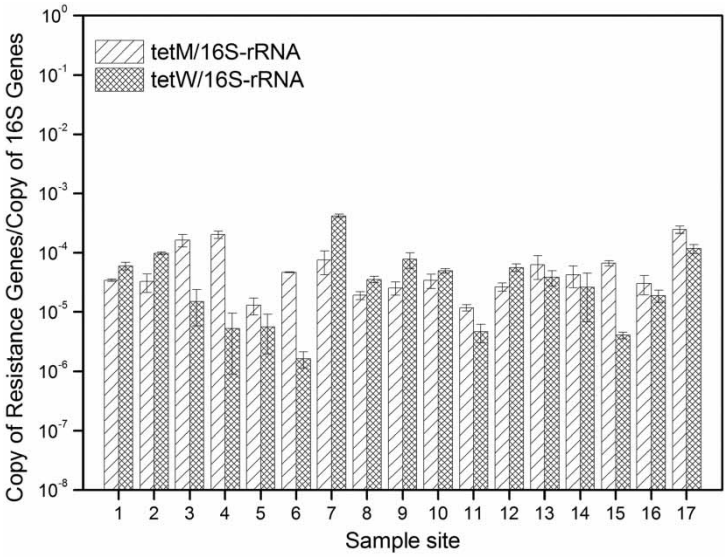

Figure 2 | Copies of resistance genes (*tetM* and *tetW*) normalised with the number of bacteria 16 s rRNA genes at different sites. The error bars indicate three independent qPCR runs in duplicates.

antibiotic use, i.e., the use of sulfonamides is more prevalent than the use of tetracyclines. For the *sul* genes, *sul1* was slightly higher than that of *sul2*. This result was attributed to the location of the *sul* genes: the *sul1* gene was normally found in class 1 integrons and *sul2* was usually located in small non-conjugated plasmids or large transmissible multi-resistant plasmids (Skold 2000; Enne *et al.* 2002). *sul1* and *sul2* genes could also occur in class 2 integrons (Sunde 2005). In the current study, we discovered that the *sul1* gene was the most abundant gene in the lake. This result was consistent with the result of Pei *et al.* (2006) and Pruden *et al.* (2006). However, for *tet* genes, the relative level of *tetM* was more abundant than that of *tetW* because *tetM* had the widest host range among the *tet* genes and was often carried in various environmental genera (i.e., *tetM* could be seen equally in both Gram-positive and Gram-negative bacteria). Thus, the *tetM* gene can be transferred between bacteria in at least 42 different genera (Roberts 2005; Zhang *et al.* 2009). The level of the *tetW* gene showed the second widest host range among the *tet* genes. However, a previous study showed otherwise, i.e., the concentration of the *tetM* gene was greater than that of the *tetW* gene and was often carried in various environmental genera (Gao *et al.* 2012). This difference in the concentrations of the two *tet* genes may be caused by different environmental conditions.

CONCLUSIONS

High concentrations of sulfonamide and tetracycline resistance genes and resistant bacteria were found in Bosten Lake. This finding was attributed to the possible prolonged exposure of the lake to antibiotics. This study clearly demonstrated that aquatic ecosystems were a major reservoir for the evolution and propagation of ARGs and antibiotic-resistant bacteria. Bosten Lake is affected by the increasing amount of ARGs and antibiotic-resistant bacteria, and the effect of this phenomenon on the local ecological environment needs to be investigated. Further studies are required to unravel the relationship between ARGs and their corresponding antibiotics. Other water environments in Xinjiang should also be analysed for the presence of ARGs and antibiotic-resistant bacteria. Thus, the distribution and propagation of ARGs must be carefully understood. Furthermore, an investigation on various ARGs in the environment under some conditions, such as pH, temperature and presence of heavy metals, is necessary. Obtaining this information will help us further assess the risks posed by ARGs and develop appropriate mitigation and control strategies.

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