RESEARCH ARTICLE

Occurrence of antibiotic resistance and integronase genes in Taihu Lake

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Abstract: Antibiotic resistance genes (ARGs), a potential threat to the health of humans and animals, have been widely detected in various environments. However, not much information about ARGs in freshwater lakes have been recorded. In this study, we investigated the occurrence of 17 kinds of ARGs and three types of integronase genes in Taihu Lake (China), an important drinking water source for local residents. Fecal coliforms were also isolated from the water and sediments for antimicrobial susceptibility tests and related ARGs detection. Results showed that tetracycline resistance gene *tet*C, sulfanilamide resistance genes *sul*1 and *sul*2, and class 1 integronase gene *int*1 were present in all water and sediment samples. *Tet*G was present in all water samples but was mainly distributed in sediment samples from the northern region of Taihu Lake. β-Lactam resistance gene bla_{OXA-1} was present in all water samples but was absent in the sediment samples. *Tet*M and *tet*O were found present in water and sediment samples from the western area of the lake. Remarkably, 95% of isolated fecal coliforms were resistant to trimethoprim and multi-drug resistant isolates were also observed. *Sul*1 and *tet*C genes were found to be carried by isolates resistant to corresponding antibiotics. This study provided baseline information about the occurrence of ARGs and integronase genes in Taihu Lake and the results may extend our knowledge about antibiotic resistance of microbial communities in the lake.

Keywords: antibiotic resistance genes, class 1 integrons, fecal coliforms, Taihu Lake, water environment

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1. Introduction

Within the past 70 years, antibiotics have been increasingly developed and applied to treat diseases and promote animal production after penicillin was first developed. The misuse of antibiotics has resulted in widespread antibiotic resistant bacteria (ARB) and resistance genes (ARGs) in the aquatic environment[1,2]. ARGs have the ability to transfer from the host bacteria to other species through conjunction, transduction, or transformation[3], which in turn changes the structure and activity of microbial communities especially in the presence of antibiotics[4]. Unremarkably, the acquisition of ARGs in pathogens leads to drug failure, posing serious potential health threats to humans and animals[5]. Among various kinds of antibiotics, tetracycline, sulfanilamide, and β-lactam are the most frequently used in animal production and disease treatment[6]. To date, more than 38 tetracycline resistance genes[7], 20 sulfanilamide resistance genes[8], and 200 β-lactam resistance genes[9] have been detected in the environment. Moreover, integrons carrying various ARGs have also been discovered in the environment[10]. These integrons are endogenous DNA sequences that can incorporate exogenous ARGs into their gene cassettes which play

important roles in horizontal transfer of ARGs[11].

As the third largest freshwater lake in China, Taihu Lake is an important drinking water source for millions of local residents. However, the lake has been suffering from serious water pollution in the past 30 years. Previous studies focused on the notorious algal bloom annually happening in the lake. Toxic microcystin[12], various organic pollutants[13], and heavy metals[14] have also been found present in the lake water. However, less attention has been paid to the occurrence of ARGs in Taihu Lake which can also pose great health threats to humans.

Amounts of treated or untreated domestic sewage is discharged into Taihu Lake through over 20 river branches[15]. Sewage is considered as an important environmental reservoir for a variety of ARB and ARGs[16]. Moreover, rapid development of animal breeding and aquaculture in the region may contribute to the emergence of ARB and ARGs in the lake[17]. Previous studies have revealed that tetracycline resistance genes *tet*A and *tet*C are widely distributed in Meiliang Bay of the lake[1] and *Escherichia coli* isolates from the lake's water are highly resistant to streptomycin, tetracycline and ampicillin[18]. However, a comprehensive study has not been conducted to investigate the occurrence of the ARB and ARGs in the lake water.

In this study, the occurrence of 17 kinds of ARGs (encoding resistance to tetracycline, sulfanilamide or β-lactam) and three types of integronase genes in Taihu Lake water and sediments were investigated using polymerase chain reactions (PCR). Fecal coliforms were also isolated from the lake water for antimicrobial tests and related ARGs detection. Results may extend our knowledge about antibiotic resistance of microbial communities in lakes undergoing disturbance from human activities.

2 Materials and Methods

2.1 Sample Collection

Water and sediments were sampled from 23 locations in Taihu Lake (Figure 1), including three drinking water source stations for the surrounding cities (Sites N7, N10, and S7). Water samples (1000 mL each) were collected in sterilized bottles from the 23 selected sites at a depth of 50 cm below the water surface in April 2012. Soft sediment samples (around 100 g each) were collected in sterilized bags using bottom sampler from 19 of the 23 sites (the bottom sampler was unable to collect samples from hard solid bottoms at sites N6, N11, N12, and S9), simultaneously. Water and sediment samples were kept on ice and transported to the lab within 6 hrs.

2.2 DNA Extraction

For DNA extraction, water samples were filtered onto cellulose ester filters (pore size 0.45 μm, Millipore, USA) until the filter gets clogged. After filtration, filters were cut into pieces immediately for total DNA extraction using FastDNA Spin Kit for Soil (MP Biomedicals, USA). Similarly, the kit was also used for total DNA extraction from the sediments (500 mg). All DNA extractions were performed strictly following the manufacturer's recommended protocol (MP Biomedicals, USA). Purified DNA was stored in −20°C until further use.

2.3 Isolation and Identification of Fecal Coliforms

Each of the water samples (5 mL) from the different sites was diluted with 45 mL sterile saline solution (0.85% NaCl). Each sediment sample (5 g) was also mixed with 45 mL sterile saline solution (0.85% NaCl) and vibrated at a speed of 200 rpm/min at 28°C. After vibrating for 30 min, 1 mL of the sediment-water mixture was diluted with 45 mL sterile saline solution (0.85% NaCl) again. The diluted water and sediment samples were filtrated through the cellulose membranes (pore size 0.45 μm, Millipore, USA). The obtained membranes were stuck onto m-FC agar for fecal coliform incubation following the methods applied by Niemi *et al*.[19]. Sterile saline solution (45 mL) was used as a negative control. All the presumed fecal coliforms were further purified and identified by applying the analytical profile index (API) method from Adicon Clinical Laboratory (Nanjing, China). Total DNA was extracted from each of the fecal coliforms using the boiling method[20].

2.4 Antimicrobial Susceptibility Test

Antibiotic resistance of the fecal coliforms was determined using the Kirby-Bauer disk diffusion method at Adicon Clinical Laboratory (Certification: No. CNAS MT0061) for antimicrobial susceptibility tests. Resistance to 7 commonly used antibiotics including tetracycline, ampicillin, minocycline, trimethoprim, streptomycin, gentamycin, and ciprofloxacin were tested in this study. Each bacterial isolate was classified as susceptible (S), intermediate (M), and resistant (R) to antibiotics.

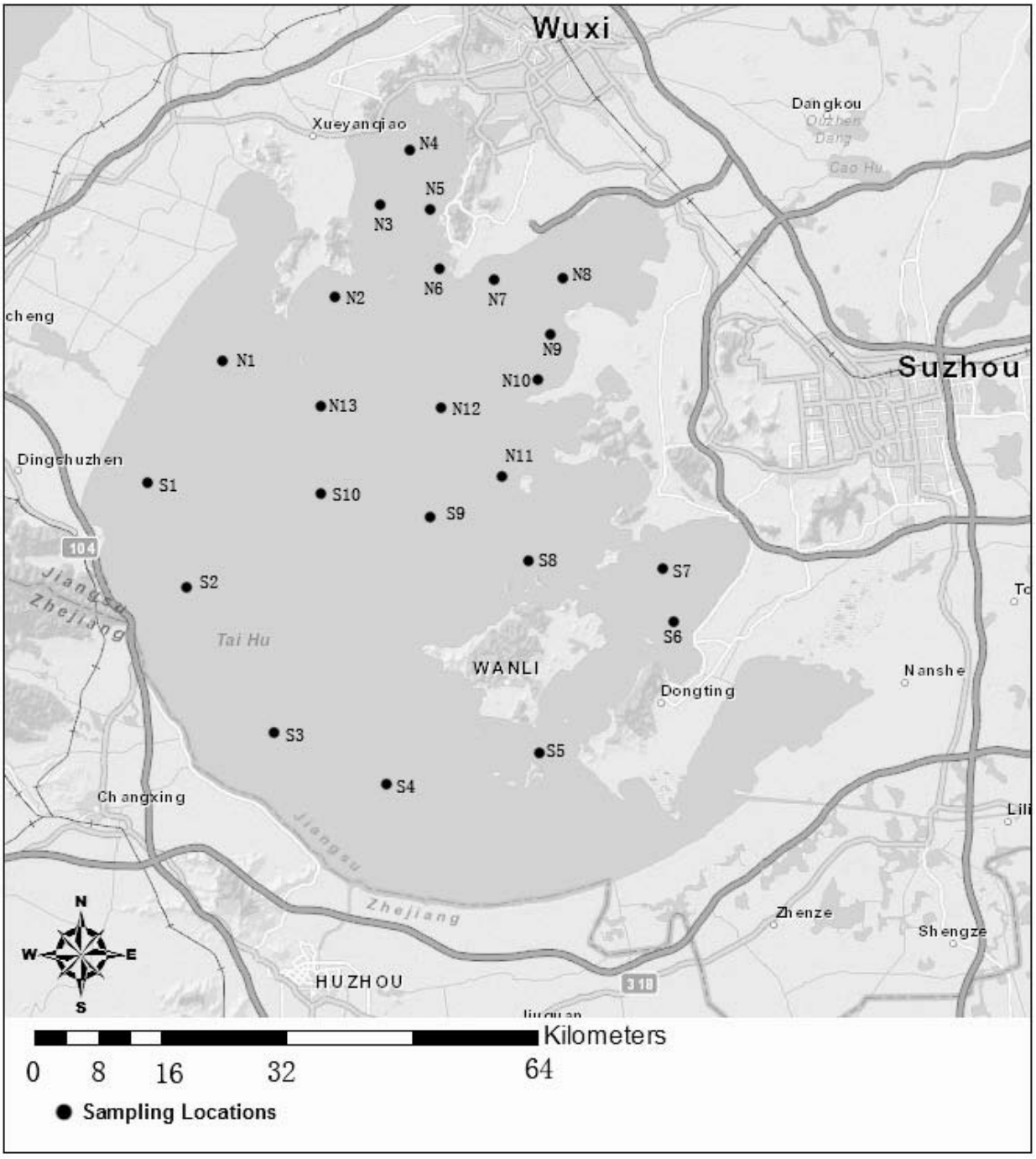

Figure 1. Sampling locations in Taihu Lake. Water and sediments were sampled from 23 locations in Taihu Lake, including three drinking water source stations for the surrounding cities (Sites N7, N10, and S7).

2.5 Detection of ARGs and Integronases Genes

We utilized the PCR technique to detect 17 kinds of ARGs and three types of integronase genes in each sample. Primer sequences used for PCR amplification of ARGs and integronase genes were listed in Table 1. The PCR system (30 μL) contained 15 μL of 2× EasyTaq PCR Supermix (TransGen Biotech, China), 1 μL of each primer (0.3 μM), 2 μL of template DNA, and 11 μL of ddH_2O. PCR was initiated by incubating the reaction mixture at 94°C for 3 min, followed by 30 cycles of 30 s at 94°C, 30 s at the annealing temperature (Table 1), and 1 min at 72°C for extension. The reaction was terminated after a final extension step for 7 min. Blank control (ddH_2O instead of template DNA)

Table 1. Primers used to detect ARGs and integronase genes

Gene	Primer pair[a]	Sequences (5'–3')	Annealing temp (°C)	Amplicon size (bp)	Reference
*tet*A	*tet*A-F	GCTACATCCTGCTTGCCTTC	55	210	[43]
	*tet*A-R	CATAGATCGCCGTGAAGAGG			
*tet*B	*tet*B-F	TTGGTTAGGGGCAAGTTTTG	55	659	[43]
	*tet*B-R	GTAATGGGCCAATAACACCG			
*tet*C	*tet*C-F	CTTGAGAGCCTTCAACCCAG	55	418	[43]
	*tet*C-R	ATGGTCGTCATCTACCTGCC			
*tet*D	*tet*D-F	AAACCATTACGGCATTCTGC	55	787	[43]
	*tet*D-R	GACCGGATACACCATCCATC			
*tet*G	*tet*G-F	CAGCTTTCGGATTCTTACGG	55	844	[43]
	*tet*G-R	GATTGGTGAGGCTCGTTAGC			
*tet*M	*tet*M-F	GTGGACAAAGGTACAACGAG	55	406	[43]
	*tet*M-R	CGGTAAAGTTCGTCACACAC			
*tet*O	*tet*O-F	AACTTAGGCATTCTGGCTCAC	55	515	[43]
	*tet*O-R	TCCCACTGTTCCATATCGTCA			
*tet*Q	*tet*Q-F	TTATACTTCCTCCGGCATCG	55	904	[43]
	*tet*Q-R	ATCGGTTCGAGAATGTCCAC			
*sul*1	*sul*1-F	CGGCGTGGGCTACCTGAACG	55	433	[44]
	*sul*1-R	GCCGATCGCGTGAAGTTCCG			
*sul*2	*sul*2-F	TCCGGTGGAGGCCGGTATCTGG	60.8	191	[45]
	*sul*2-R	CGGGAATGCCATCTGCCTTGAG			
*sul*3	*sul*3-F	TCCGTTCAGCGAATTGGTGCAG	60	128	[45]
	*sul*3-R	TTCGTTCACGCCTTACACCAGC			
*sul*A	*sul*A-F	TCTTGAGCAAGCACTCCAGCAG	60	299	[45]
	*sul*A-R	TCCAGCCTTAGCAACCACATGG			
*amp*C	*amp*C-F	CCTCTTGCTCCACATTTGCT	58	189	[46]
	*amp*C-R	ACAACGTTTGCTGTGTGACG			
bla_{TEM}	bla_{TEM}-F	ATAAAATTCTTGAAGACGAAA	56	1086	[25]
	bla_{TEM}-R	GACAGTTACCAATGCTTAATC			
bla_{SHV}	bla_{SHV}-F	GGGTTATTCTTATTTGTCGC	58	567	[25]
	bla_{SHV}-R	TTAGCGTTGCCAGTGCTC			
bla_{CTX-M}	bla_{CTX-M}-F	CGCTTTGCGATGTGCAG	54	550	[25]
	bla_{CTX-M}-R	ACCGCGATATCGTTGGT			
bla_{OXA-1}	bla_{OXA-1}-F	ACACAATACATATCAACTTCGC	56	885	[25]
	bla_{OXA-1}-R	AGTGTGTTTAGAATGGTGATC			
*int*1	*int* Ⅰ-F	ACGAGCGCAAGGTTTCGGT	52	565	[25]
	int Ⅰ-R	GAAAGGTCTGGTCATACATG			
*int*2	*int* Ⅱ-F	GTGCAACGCATTTTGCAGG	52	403	[25]
	int Ⅱ-R	CAACGGAGTCATGCAGATG			
*int*3	*int*Ⅲ-F	CATTTGTGTTGTGGACGGC	52	717	[25]
	*int*Ⅲ-R	GACAGATACGTGTTTGGCAA			

[a] F, forward; R, reverse

was included in all the PCRs. We performed agarose gel electrophoresis to detect target PCR products. Furthermore, PCR positive products were randomly selected and purified using QIAquick PCR purification kit for Sanger sequencing (BGI, China). The obtained sequences were subjected to BLAST against

NCBI Nuclease Database to confirm the target genes. All sequences have been deposited in GenBank under accession numbers (Table 2).

Table 2. BLAST results of PCR-positive isolates. Results were obtained by comparing the sequence results of PCR-positive products with target gene sequences in the NCBI Nuclease Database

Target genes	Sequence results	BLAST results	
		Accession No.	Similarity (%)
tetO	Successful	GQ240298.1	99%
tetM	Successful	JN846698.1	98%
tetG	Successful	AF133140.1	99%
tetC	Successful	EU751610.1	99%
sul2	Successful	HQ441169.1	99%
sul1	Successful	FJ711652.1	100%
int1	Successful	JQ407409.1	99%
bla_{OXA-1}	Successful	GQ896560.1	99%

2.6 Statistical analyses

Microsoft Office Excel 2007 was used to analyze the occurrence of ARGs and integronase genes in both water and sediment samples.

3 Results

3.1 ARGs and Integrons in Taihu Lake

Among the 17 kinds of ARGs tested in this study, tetracycline resistance genes *tet*C, and sulfanilamide resistance genes *sul*1 and *sul*2 were found in all 42 locations (23 water samples and 19 sediment samples) (Figure 2, Table 3). In the water samples, *tet*M was found at sites S2, S3, and S4, while *tet*O was found at sites N1, S3, and S4. Both of these two genes were mainly detected in the western region of Taihu Lake. β-Lactam resistance gene bla_{OXA-1} was present in all water samples but was undetected in each of the sediment samples. *Tet*G was present in all water samples but was only detectable in sediments of six sites (N1, N2, N3, N4, N5, and N8). *Tet*M was found in the sediments of five sites (N1, N3, N4, N5, and N7). Both *tet*G and *tet*M were mainly distributed in the sediments of the northern region of Taihu Lake. The other 10 kinds of ARGs (*tet*A, *tet*B, *tet*D, *tet*Q, *sul*3, *sul*A, ampC, bla_{TEM}, bla_{SHV}, and bla_{CTX-M}) were absent in both water and sediment samples. Class 1 integrons were present in all water and sediment samples, while classes 2 and 3 integrons were not detected in Taihu Lake.

3.2 Antimicrobial Susceptibility of Fecal Coliforms

A total of 82 presumed fecal coliform strains were isolated from water samples and 20 were identified as fecal coliforms via the ABI test. Antibiotic susceptibility analysis was conducted for all the fecal coliform isolates. Figure 3 showed resistance frequencies of the 7 tested antibiotics for all 20 strains. Most of the isolated fecal coliforms (95%) were found resistant to trimethoprim. The resistance frequency of ampicillin, gentamycin, streptomycin, and ciprofloxacin was 20% (4 isolates), 10% (2 isolates), 5% (1 isolate), and 5% (1 isolate), respectively. One isolate (5%) had intermediate resistance to minocycline (excluded in Figure 3) but no isolate was found resistant to tetracycline. Remarkably, 5 isolates were found to be multi-resistant (resistant to at least 2 kinds of antibiotics) and one isolate was even resistant to 6 kinds of tested antibiotics.

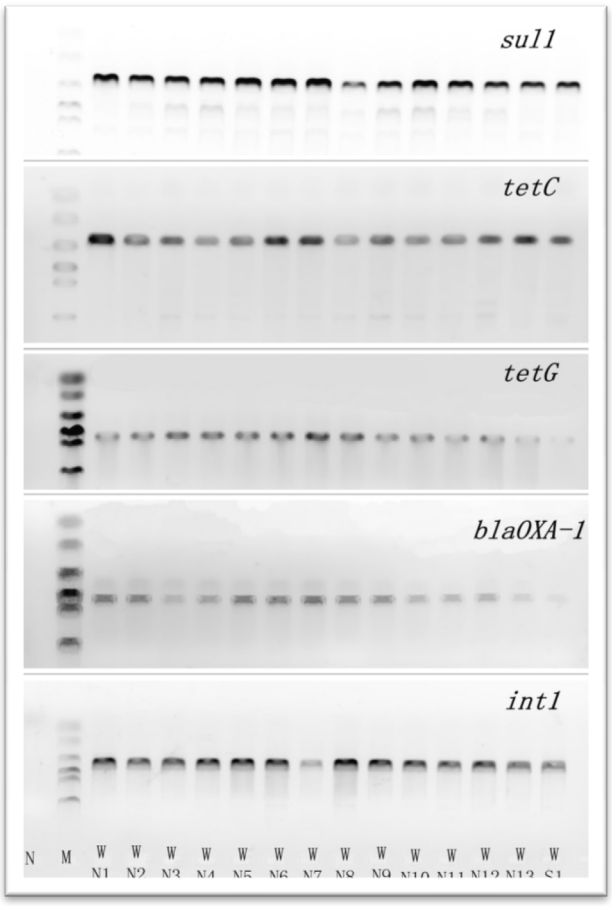

Figure 2. Occurrence patterns of *sul*1, *tet*C, *tet*G, bla_{OXA-1}, and *int*1 genes in water samples analyzed via PCR product electrophoreses. N: blank control with sterilized ddH_2O used as PCR template; M: DNA Marker (DL2000); W: water.

Table 3. Occurrence of ARGs and integronase genes in water and sediment samples

Sample type	% of detection																			
	*tet*A	*tet*B	*tet*C	*tet*D	*tet*G	*tet*M	*tet*O	*tet*Q	*sul*1	*sul*2	*sul*3	*sul*A	*amp*C	bla_{TEM}	bla_{SHV}	bla_{CTX-M}	bla_{OXA-1}	*int*1	*int*2	*int*3
Water	0	0	100	0	100	13.04	13.04	0	100	100	0	0	0	0	0	0	100	100	0	0
Sediment	0	0	100	0	31.58	26.32	0	0	100	100	0	0	0	0	0	0	0	100	0	0

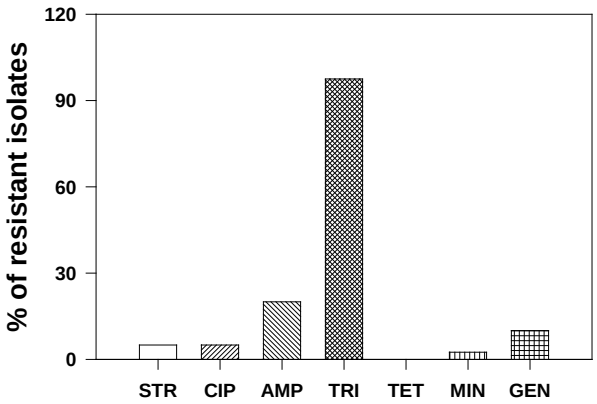

Figure 3. Frequencies of antibiotic resistance to the fecal coliforms (n = 20) in water and sediment samples in Taihu Lake. STR: streptomycin; CIP: ciprofloxacin; AMP: ampicillin; TRI: trimethoprim; TET: tetracycline; MIN: minocycline; GEN: gentamycin.

3.3 ARGs and Class 1 Integrons in Fecal Coliforms

We conducted PCRs to detect *sul*1 and *sul*2 in the bacterial isolates resistant to trimethoprim, bla_{OXA-1} in the isolates resistant to ampicillin, and *tet*C and *tet*G in the isolates resistant to minocycline. As shown in Table 4, among the 20 trimethoprim resistant isolates, 8 were positive for *sul*1. The minocycline resistant isolate was found to carry *tet*C. Additionally, all 20 isolates were subjected to class 1 integronase gene *int*1 detection, in which it was present in 2 isolates. However, *sul*2, bla_{OXA-1}, and *tet*G were undetected in the corresponding antibiotic resistant bacterial isolates. Additionally, no isolate was found to host multiple ARGs (more than 2 kinds of ARGs) in this study.

4 Discussion

Currently, tetracycline resistance genes have been widely selected as an indicator to evaluate ARGs contamination[21–23]. Among the four kinds of tetracycline resistance genes (*tet*C, *tet*G, *tet*M, and *tet*O) tested in this study, *tet*C was detected in all sampling sites (both water and sediment) and *tet*G was also detected in all water samples which was consistent with previous studies[1,22,24–26]. Therefore, *tet*C and *tet*G may appear as the dominant tetracycline resistance gene types in Taihu Lake. Additionally, *tet*G were mainly present in the sediments of the northern parts of the lake (mainly in Meiliang Bay and Gonghu Bay), and *tet*M and *tet*O were mainly distributed in the western region. This was similar to the distribution pattern of organic pollutants and heavy metals in Taihu Lake which was presumably due to the comparatively greater impacts of anthropogenic activities in the northern and western regions[14,27].

Four sulfanilamide resistance genes (*sul*1, *sul*2, *sul*3, and *sul*A) were chosen for PCR detection in this study. Among the four ARGs, *sul*1 and *sul*2 were present in all sampling sites while *sul*3 or *sul*A was absent in each sample. Similarly, *sul*1 and *sul*2 were also frequently found in other water environments including wastewater treatment plants[28], aquaculture ponds[29], and livestock lagoons[30]. High concentrations

Table 4. PCR results of ARGs and *int1* gene in fecal coliforms

Gene type	No. of strains																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
*sul*1	+[a]	+	+	–	–	–	+	+	–	–	–	+	–	–	–	–	–	–	+	+
*int*1	–[b]	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
*sul*2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
tetC	ND[c]	ND	ND	ND	ND	ND	ND	ND	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
tetG	ND	ND	ND	ND	ND	ND	ND	ND	–	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
bla_{OXA-1}	ND	ND	ND	ND	ND	ND	ND	–	ND	–	ND	ND	ND	ND	ND	–	ND	ND	ND	–

[a] +: PCR positive; [b] –: PCR negative; [c]Not detected.

of tetracycline and sulfanilamide have been detected in different types of aquaculture ponds around the Taihu Lake basin[31] and the practice of aquaculture in Taihu Lake has reached 10647.02 hm^2 in 2003[32]. Therefore, massive use of tetracycline and sulfanilamide in aquaculture in Taihu Lake basin may play an important role in the wide distribution of tetracycline and sulfanilamide resistant genes.

Among the five kinds of β-lactam resistance genes (*amp*C, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{OXA-1}) tested in this study, bla_{OXA-1} was present in all water samples revealing the wide dissemination of bla_{OXA-1} in Taihu Lake. However, other ARGs were absent in the water or sediment samples. Bla_{OXA-1} has a broad host range including clinical isolates such as *Klebsielia*[33], *Salmonella*[34], and *Pseudomonadaceae*[35]. On the other hand, this gene was seldom reported to be present in environmental samples. Chen *et al*. observed the absence of bla_{OXA-1} in the waters of Yangtze River (Chongqing Basin)[25] but Zhang *et al*. recently reported high abundance of bla_{OXA-1} in Jiulong River (Fujian Province, China) and the abundance was highly related to human activities[36].

Among the tested integronase genes, results showed that only *int*1 was present in all sampling locations in Taihu Lake while both *int*2 and *int*3 were undetected in each of the samples. Recently, *int*1 had been detected in various water environments especially in wastewater treatment plants serving as a potential *int*1 pool[37]. The high population density in the region surrounding Taihu Lake may account for the wide dissemination of *int*1. Furthermore, *int*1 usually carries one or more gene cassettes, each of which containing one ARGs coding resistance to different antibiotics[38]. Thus, the *int*1 gene cassettes found in Taihu Lake still require further investigation.

Antibiotic resistant fecal coliforms were already found widely disseminated in the environment[39], even in drinking water in India[40]. As a very important drinking water source for the surrounding cities, Taihu Lake deserves more public health attention concerning fecal coliform contamination. Only 20 fecal coliforms were isolated from both water and sediment samples in this study which may be due to the low temperature during sampling time (water temperature around 10°C). Low environmental temperature is unfavorable to the survival of fecal coliforms[41]. However, resistance to trimethoprim was found ubiquitous (95%) among all the tested isolates. These results were in accordance with the high detection frequency of *sul*1 and *sul*2 genes in the water and sediment samples. However, genotype detection showed only 40% of fecal coliforms hosted *sul*1 gene. The reason may be due to the ability of many kinds of ARGs including dihydropteroate synthases genes *sul* and dihydrofolate reductase genes *drf* to code for trimethoprim resistance[42]. Similarly, the frequently detected bla_{OXA-1} in the water samples was not present in ampicillin resistant strains. It is known that more than 400 kinds of β-lactam resistance genes have been discovered[9], so a more comprehensive investigation has to be conducted to determine the occurrence of different ARGs in bacterial isolates.

In conclusion, this study provided baseline information about the occurrence of ARGs in Taihu Lake. *Tet*C, *tet*G, *sul*1, *sul*2, and bla_{OXA-1} are widely distributed in Taihu Lake and the abundance of ARGs in the northern and western parts of the lake are relatively higher than the other parts. Nearly all fecal bacterial coliforms isolated were found to be resistant to trimethoprim and multi-antimicrobial resistance isolates were also observed which deserve special attention. *Int*1 is ubiquitous in Taihu Lake and future work should include identification of the ARGs contained in its gene cassettes. More comprehensive and in-depth studies have to be conducted to determine the occurrence, abundance, and diversity of the ARGs as well as the temporal and spatial variations and its relationship with environmental conditions in Taihu Lake.

Conflict of Interest and Funding

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