water

MDPI

Article

Spatial Distribution and Seasonal Variation of Antibiotic-Resistant Bacteria in an Urban River in Northeast China

Qingshan Xiao †, Xin Wang †, Chongxin Xu, Wei Chen, Qianchi Huang and Xin Wang *

Engineering Research Center of Agricultural Microbiology Technology, Ministry of Education & Heilongjiang Provincial Key Laboratory of Ecological Restoration and Resource Utilization for Cold Region & Key Laboratory of Molecular Biology, College of Heilongjiang Province & School of Life Sciences, Heilongjiang University, Harbin 150080, China; xqs13263513817@126.com (Q.X.); 19154091621@163.com (X.W.); 18804535736@163.com (C.X.); chen6560sir@163.com (W.C.); qian030628@163.com (Q.H.)

* Correspondence: tianronghaise@hlju.edu.cn

† These authors contributed equally to this work.

Abstract: As the largest freshwater river flowing through Harbin, the Songhua River is a standby water source. It is very important to know the species and distribution of antibiotic-resistant bacteria (ARB) in the river. In this study, five antibiotics were selected to screen and identify ARB in spring and autumn. The results showed that the concentration of cefotaxime-resistant bacteria was the highest, and the maximum concentration at S6 in spring was up to 1.40×10^4 CFU/mL. In spring and autumn, bacteria resistant to three antibiotics were screened at S1 of the Songhua River, and bacteria resistant to five antibiotics were screened at S6. No multiple antibiotic-resistant bacteria (MARB) were screened in the other four sites in autumn, while MARB were screened in the other three samples except S2 in spring. In all sample areas in spring and autumn, the probability of screening MARB at S1 and S6 was the highest, reaching 100%. The identification results of 16S rDNA polymerase chain reaction (PCR) products of ARB showed that a total of 51 ARB strains from 15 bacterial genera were screened in the Songhua River, of which 20 ARB strains were from *Pseudomonas*. Among the 15 bacterial genera, bacteria from 8 bacterial genera have pathogenicity. The results of this study revealed the concentration, spatial distribution, and seasonal variation of culturable ARB in the Songhua River, providing data support for the remediation of antibiotic resistance gene (ARG) pollution in the river.

Keywords: antibiotic-resistant bacteria; river; antibiotics; seasonal changes; pathogenic bacteria

Citation: Xiao, Q.; Wang, X.; Xu, C.; Chen, W.; Huang, Q.; Wang, X. Spatial Distribution and Seasonal Variation of Antibiotic-Resistant Bacteria in an Urban River in Northeast China. *Water* **2024**, *16*, 1268. https://doi.org/10.3390/w16091268

Academic Editors: Abasiofiok Mark Ibekwe and Hodon Ryu

Received: 12 March 2024
Revised: 17 April 2024
Accepted: 26 April 2024
Published: 28 April 2024

1. Introduction

For decades, antibiotics have made great contributions to the treatment of human and animal diseases, but with the extensive and unreasonable use of antibiotics, a large number of antibiotics have entered the natural environment [1]. The degradation of antibiotics in the natural environment is very slow. Bacteria in the environment will acquire genomic mutations and obtain antibiotic resistance after exposure to sub-inhibitory or sub-lethal concentrations of antibiotics to cope with the survival crisis caused by this drug stress [2]. What is more serious is the vertical and horizontal transfer of antibiotic resistance genes (ARGs) carried by antibiotic-resistant bacteria (ARB) during bacterial reproduction [3]; that is, these ARGs will be passed to future generations of the same species, and even other species, of bacteria [4]. Especially when these ARGs are transferred to pathogenic bacteria, the antibiotic resistance of pathogenic bacteria makes it more difficult to treat the diseases caused by them, which seriously threatens human health. Therefore, ARGs have been identified as a new type of pollutant [5]. At present, a large number of studies have shown that ARGs and ARB are present in water [6], soil [7], air [8], and even in animals [9] and

plants [10], and the types and quantities of ARB in the environment have become very large and even include a large number of pathogenic bacteria.

Freshwater resources are indispensable for organisms living on the earth and are directly related to the continuation of life. As a freshwater resource on the ground, river water runs through human cities and agricultural development areas and has made great contributions to the development and continuation of human beings. However, with the acceleration of urbanization and the rapid development of agriculture, freshwater resources have been polluted to a certain extent [11]. Therefore, the intensification of human activities is often accompanied by a certain degree of negative impact on the environment [12]. Among them, a large number of antibiotics and ARB remaining in medical wastewater and domestic sewage continue to enter the natural environment, especially rivers [13], making the pollution of antibiotics and ARGs increasingly serious [14]. Urban river waters with a large number of ARB are often used for crop irrigation in agricultural development areas [15]. A large number of ARB in rivers adhere to the surface of crop leaves through irrigation, and some ARB enter and colonize the soil. ARB that enter the soil can transfer their ARGs to other bacteria in the soil through horizontal transfer, resulting in a large number of ARB in the rhizosphere soil of crops. What is more serious is that ARB in the rhizosphere of crops can enter the roots by absorption through crop roots, and then use the nutrient transport of crops to transfer to stems and leaves through vascular bundles [16]. Finally, they colonize in the roots, stems, and leaves of crops and become endophytic bacteria [17]. Moreover, the ARGs carried by these ARB are horizontally transferred so that other endophytic bacteria in crops will also obtain antibiotic resistance, which is a very terrible phenomenon. When humans and animals eat these crops, especially the frequently eaten raw crops [10], the colonization of ARB in the digestive tract and the transfer of ARGs to the intestinal flora [18] pose a serious risk to human health [19]. In addition, studies have found that when recreational activities are carried out in river waters with ARB, the ARB will attach to the body surface or even directly enter the body [20]. Moreover, the ARB attached to the body surface are generally difficult to be completely removed by simple cleaning and may enter the body with food after contact with food.

Therefore, the detection of ARB in urban river water has become very important and urgent, especially with the large number of antibiotic-resistant pathogens. The Songhua River is the largest river water body flowing through Heilongjiang Province, China. It is a standby drinking water source in Harbin and an important storage water body for sewage discharge. It is also used for irrigation and drainage of crops and flood control and discharge. Harbin is located in the upper reaches of the Songhua River Basin. The water body in this river section has large fluidity, and the water body in some areas is relatively turbid. The Songhua River runs through Harbin City. Hospitals, schools, and a large number of entertainment places are distributed on both sides of the river. The river also intersects with two other rivers that store sewage. Affected by sewage discharge and human activities, the river water body will be polluted by ARGs to a certain extent, accompanied by a large number of ARB [21]. In this study, cefotaxime, sulfadiazine, tetracycline, gentamicin, and ciprofloxacin were selected for the screening of ARB. These five antibiotics are commonly used in clinical medicine, animal husbandry, and aquaculture, and exposure to these antibiotics could make the surrounding microorganisms obtain antibiotic resistance. These antibiotics, ARB, and ARGs are usually directly discharged into the Songhua River through rainwater washing or indirectly discharged into the Songhua River through sewage treatment plants, which makes the pollution of ARGs in the Songhua River water serious. Therefore, it is very important to understand the pollution of ARGs in the Songhua River, especially the bacterial vectors carrying these ARGs. At the same time, the species of ARB were identified by using bacterial 16S rDNA polymerase chain reaction (PCR) products, and the relationships between the types, concentrations, and distributions of ARB and factors such as time, space, and environment were analyzed. At the same time, the health problems caused by antibiotic-resistant pathogens screened in this study were briefly described.

2. Materials and Methods

2.1. Collection of River Water Samples

In October 2022 (autumn) and April 2023 (spring), surface water samples were collected from the Harbin section of the Songhua River along the downstream site S1 to the upstream site S6. The locations are shown in Figure 1 and Table 1. Each sampling site was repeatedly sampled three times, and the three samples collected were mixed and stored in a 500 mL polypropylene, blue-mouthed brown glass bottle after sterile treatment and washing with water samples. Samples were then stored at 4 °C and transported back to the laboratory for ARB screening.

Figure 1. Regional location and Songhua River sampling site distribution. The symbol S denotes the Songhua River.

Table 1. Songhua River sampling site information.

Position Number	Positional Information	North Latitude	East Longitude
S1	Near Heilongjiang Shipyard Machinery Factory	45°83′31.31″	126°72′33.72″
S2	Songpu Bridge	45°80′44.89″	126°66′45.87″
S3	Songhua River Railway Bridge	45°79′13.18″	126°63′45.78″
S4	Near People's Square	45°78′10.61″	126°60′71.49″
S5	Songhua River Highway Bridge	45°76′94.94″	126°59′83.28″
S6	The intersection of Hejiagou River and Songhua River	45°76′10.44″	126°58′22.34″

2.2. Preparation of Antibiotics

Five antibiotics were selected in this study: tetracycline (TET), ciprofloxacin hydrochloride (CIP), sulfadiazine sodium salt (SDZ), gentamicin (GEN), and cefotaxime sodium (CTX) (Table 2). The five antibiotics were prepared into specific concentrations according to the standards established by the Clinical and Laboratory Standards Institute. The antibiotics were dissolved in water and then filtered with a sterile 0.22 μm filter membrane to remove bacteria, and the antibiotics were then stored in the refrigerator at −20 °C.

Table 2. Antibiotic information for the experiment.

Antibiotic	Reserve Solution Concentration	Final Concentration	Category
TET	50 mg/mL	16 μg/mL	Tetracycline class
GEN	16 mg/mL	16 μg/mL	Aminoglycosides
CIP	4 mg/mL	4 μg/mL	Quinolones
CTX	4 mg/mL	4 μg/mL	β-Lactamides
SDZ	512 mg/mL	512 μg/mL	Sulfonamides

In this study, ARB were screened on an LB solid medium containing one or more antibiotics, and the combination of multiple antibiotics was selected according to the growth of bacteria on the LB solid medium containing one antibiotic. The final results were the combination of two antibiotics CTX + SDZ, the combination of three antibiotics CTX + SDZ + CIP, the combination of four antibiotics CTX + SDZ + CIP + GEN, and the combination of five antibiotics CTX + SDZ + CIP + GEN + TET.

2.3. Screening of Culturable Bacterial Strains

The medium used in this study was the Luria–Bertani (LB) liquid medium and the LB solid medium containing 1.25% agar. The pH value was 7, and the medium composition was 10 g/L tryptone, 5 g/L yeast extract powder, and 10 g/L NaCl. A total of 200 μL of the sample (including water sample stock solution or samples appropriately diluted and concentrated by sterile water) was evenly coated in the LB solid medium without antibiotics and containing selected antibiotics, and three repeated experiments were carried out. The samples were coated on a sterile ultra-clean bench. The coated medium was placed in a constant temperature incubator and cultured at 30 °C for 24 h. The number of colonies in the medium was controlled between 10 and 300 for colony counting. The proportion of culturable ARB = the number of culturable ARB/the total number of culturable bacteria; the unit is CFU/mL. Single colonies with different sizes, shapes, transparency, smoothness, and colors on the solid medium were selected and cultured overnight at 30 °C in the LB liquid medium containing the same concentration of antibiotics. A small amount of cultured bacterial solution was taken for Gram staining and observed under a microscope. Strains with different sizes, shapes, and colors were selected for 16S rDNA PCR.

2.4. Identification of Culturable Antibiotic-Resistant Strains

A pair of universal primers, 27F (5′-AGAGTTTGATCCTGGCTCAT-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′), targeting the 16S rDNA gene were selected for the PCR. Two PCR systems were used in this study. The first reaction system comprised 1 μL bacterial liquid sample, 0.5 μL forward and reverse primers, 10 μL 2 × Taq PCR Master Mix II (with dye), and 8 μL sterile water, making up a total of 20 μL in the reaction system. The second reaction system comprised 1 μL bacterial liquid sample, 2.5 μL 10 × Ex Taq Buffer (Mg^+ free), 0.125 μL Takara Ex Taq (5 U/μL), 2 μL $MgCl_2$ (25 mM), 2 μL dNTP Mixture (2.5 mM), 1.25 μL forward and reverse primers, and 14.875 μL sterile water, making up a total of 25 μL in the reaction system. The amplification procedure included heating at 94 °C for 5 min, followed by 30 cycles of heating at 94 °C for 20 s, 55 °C for 30 s, 72 °C for 90 s, and finally extension at 72 °C for 5 min. The PCR products were detected by 1% agarose gel electrophoresis, and the PCR products with positive results were sent to the Shanghai Bioengineering Co., Ltd. (Shanghai, China). for sequencing. The nucleotide sequences of the corresponding antibiotic-resistant strains were compared in the Nucleotide BLAST of the National Center for Biotechnology Information (NCBI), and the sequences of the comparison results and the sample sequences were downloaded and saved in the FASTA format. The sequences saved as FASTA files were aligned using Clustal W in the MEGA11 version 11.0.13 software. The alignment results were used to construct a phylogenetic tree using the MEGA11 version 11.0.13 software to complete the identification of ARB.

2.5. Statistical Analysis

Statistical analyses were performed by IBM SPSS Statistics 20. An analysis of variance test was used to estimate statistically significant differences with a significance level of 5% ($p < 0.05$). Duncan's test was used to analyze the seasonal differences in the total number of culturable bacteria and ARB at different sampling sites. The chart was produced by WPS Office Excel and Origin 2019b.

3. Results

3.1. Distribution of Culturable Bacteria

From Figure 2, it can be seen that in the Harbin section of the Songhua River, the concentration of culturable bacteria at the upstream S6 is the highest, and can reach more than 1.0×10^4 CFU/mL in spring and autumn. In the Songhua River in autumn, there was no significant difference in the total number of culturable bacteria at S3, S4, and S5 ($p > 0.05$), but there was a significant difference compared with the total number of culturable bacteria at S1, S2, and S6 ($p < 0.05$). The total number of culturable bacteria at S1 was significantly different from that at S2 and S6 ($p < 0.05$). The total number of culturable bacteria at these three sampling sites showed a trend of S6 > S1 > S2. In the Songhua River water in spring, there was no significant difference in the total number of culturable bacteria at S1, S2, S3, and S4 ($p > 0.05$), but there was a significant difference compared with the number of culturable bacteria at S6 ($p < 0.05$). Except for S6, the total number of culturable bacteria at S5 was the highest, which was significantly different from that at S6 ($p < 0.05$), and significantly different from that at S2, S3, and S4 ($p < 0.05$). Comparing the total number of culturable bacteria at each sample point in spring and autumn, the total number of culturable bacteria in autumn at sample S1 was higher than that in spring, and the difference was extremely significant ($p < 0.001$). At sample S2, the total number of culturable bacteria in autumn was also higher than that in spring, and the difference was significant ($p < 0.01$). There was no seasonal difference in the total number of culturable bacteria at S3 and S4, but at S5 and S6, the total number of culturable bacteria in spring was higher than that in autumn, and the difference was extremely significant ($p < 0.001$).

ARB were widely distributed in various sample areas of the Songhua River (Figure 3). Among them, tetracycline-resistant bacteria at sampling sites S1, S3, and S4 were more prevalent in autumn than in spring, and there was a significant difference ($p < 0.05$). The concentration of gentamicin-resistant bacteria was higher in spring than in autumn, with a significant difference ($p < 0.01$). There was no significant difference in the concentration of ciprofloxacin-resistant bacteria at S1, S2, and S4 ($p > 0.05$), but there was a significant difference at S3, S5, and S6 ($p < 0.01$), and the concentration in spring was higher than that in autumn. Except for S6, the concentration of cefotaxime-resistant bacteria in autumn was higher than that in spring, with a significant difference ($p < 0.01$). The concentration of sulfadiazine-resistant bacteria was significantly different at S1 and S2, and more in autumn than in spring ($p < 0.01$). There was no significant difference between S3 and S4 ($p > 0.05$). There was a significant difference between S5 and S6, and more in spring than in autumn ($p < 0.001$). At the sampling site S6, the concentrations of various ARB in spring were higher than those in autumn, and there was a significant difference ($p < 0.01$).

At S6 of the Songhua River in spring, the sum of the five types of ARB could reach more than 85% of the total number of culturable bacteria. In the Songhua River in autumn, except for the sample S6, the sum of the five types of ARB in the remaining five sample areas could reach more than 60% of the total number of culturable bacteria in each sampling site. It can be seen from Figure 4 that the ARB in the Songhua River in spring and autumn were mainly cefotaxime-resistant bacteria and sulfadiazine-resistant bacteria, and the concentration of cefotaxime-resistant bacteria was the highest. In spring, the proportion of cefotaxime-resistant bacteria in the Songhua River reached 38.8% at S6 (Figure 5). In autumn, except for S6, the proportion of cefotaxime-resistant bacteria in the other five sampling sites exceeded 50%, and even reached 72.31% at S3 (Figure 6). Secondly, the proportion of sulfadiazine-resistant bacteria at S6 in spring can also reach 26% (Figure 5).

Ciprofloxacin-resistant bacteria were screened at all sampling sites in the Songhua River, with the maximum concentration at S6. In autumn, gentamicin-resistant bacteria were not screened at S1 and S2 and tetracycline-resistant bacteria were not screened at S2 and S5. Tetracycline-resistant bacteria were not detected at S2 and S4 in spring (Figure 5). In autumn, the concentration of tetracycline-resistant bacteria and cefotaxime-resistant bacteria at S1 of the Songhua River was the highest, and the concentration of other types of ARB had the maximum value at S6. In spring, the concentration of various types of ARB had the maximum value at S6. It is worth noting that in autumn, multi-antibiotic-resistant bacteria (MARB) were screened only at sampling sites S1 and S6—bacteria resistant to three antibiotics were screened at S1, and bacteria resistant to five antibiotics were screened at S6. In spring, except for the bacteria resistant to two antibiotics at S2, MARB were screened at the other sampling sites, especially at S5 where bacteria resistant to four antibiotics were screened. Bacteria resistant to five antibiotics were still screened at S6 in spring, and the concentration of MARB was higher than that in autumn.

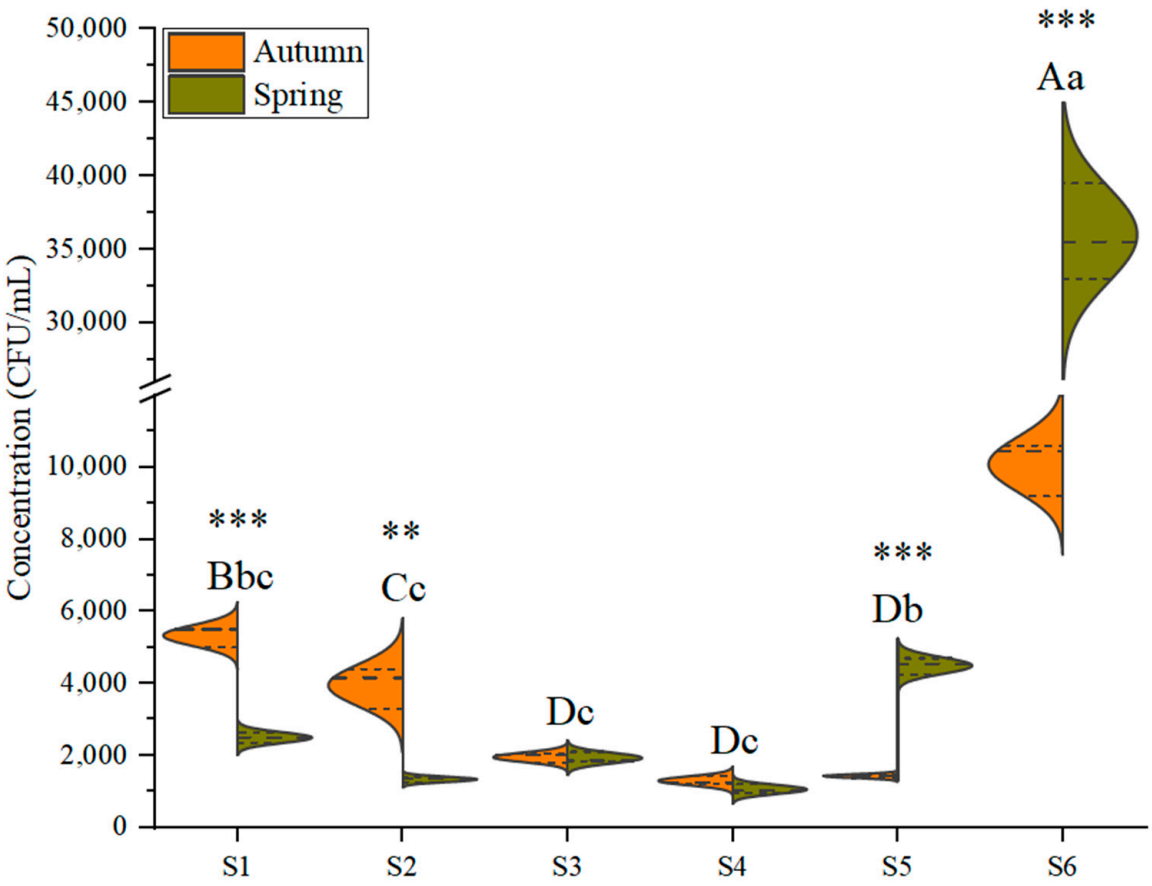

Figure 2. The total number of culturable bacteria at each sampling site in autumn and spring was compared by Duncan's test. Different letters indicate a significant difference ($p < 0.05$) among different treatments. The difference of the total number of culturable bacteria at each sampling site in autumn was indicated by uppercase letters and the difference of the total number of culturable bacteria at each sampling site in spring was indicated by lowercase letters. The seasonal difference of the total number of culturable bacteria at each sampling site was indicated by asterisks (*), where ** ($p < 0.01$), and *** ($p < 0.001$). The lines in the figure represent the range of the quartiles.

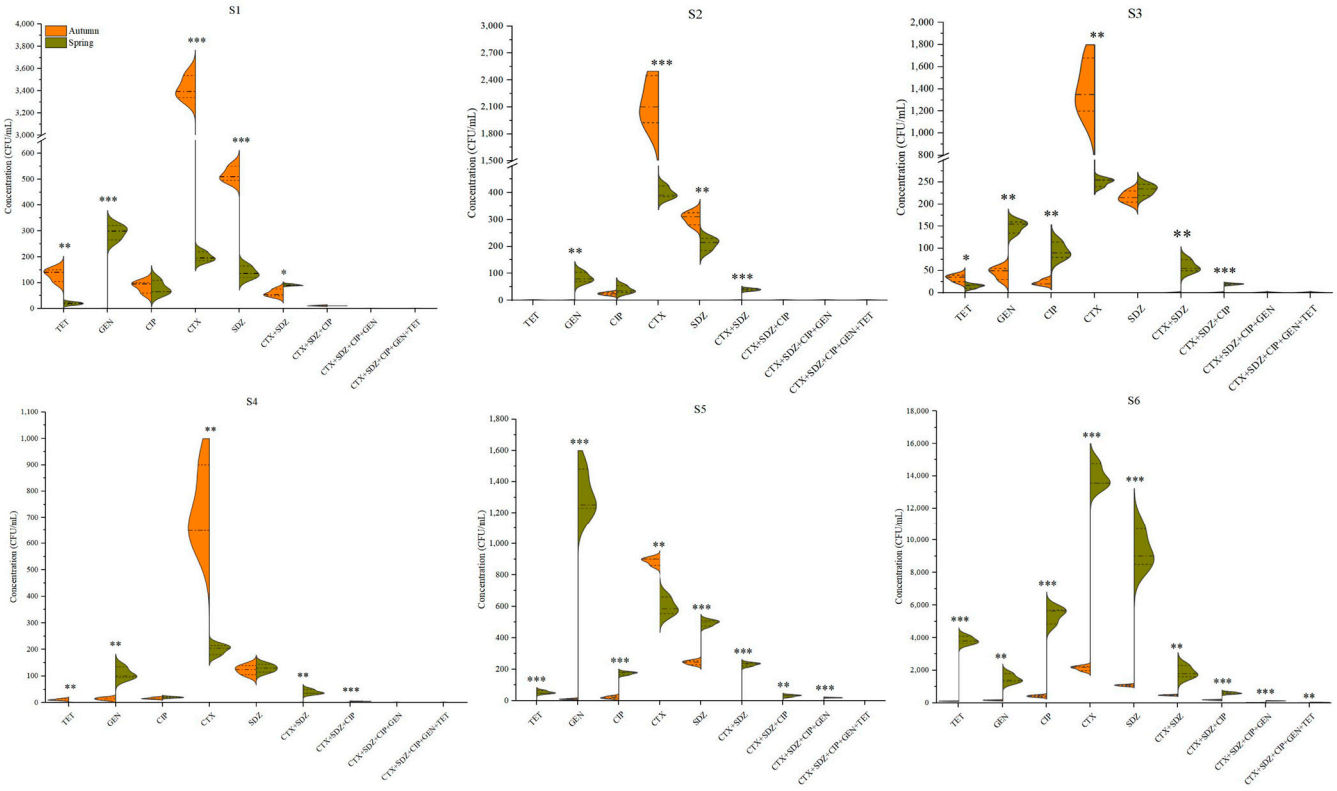

Figure 3. The seasonal differences in ARB at different sampling sites. Seasonal differences among ARB were tested using Duncan's test, indicated with * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). The unit is CFU/mL. The lines in the figure represent the range of the quartiles.

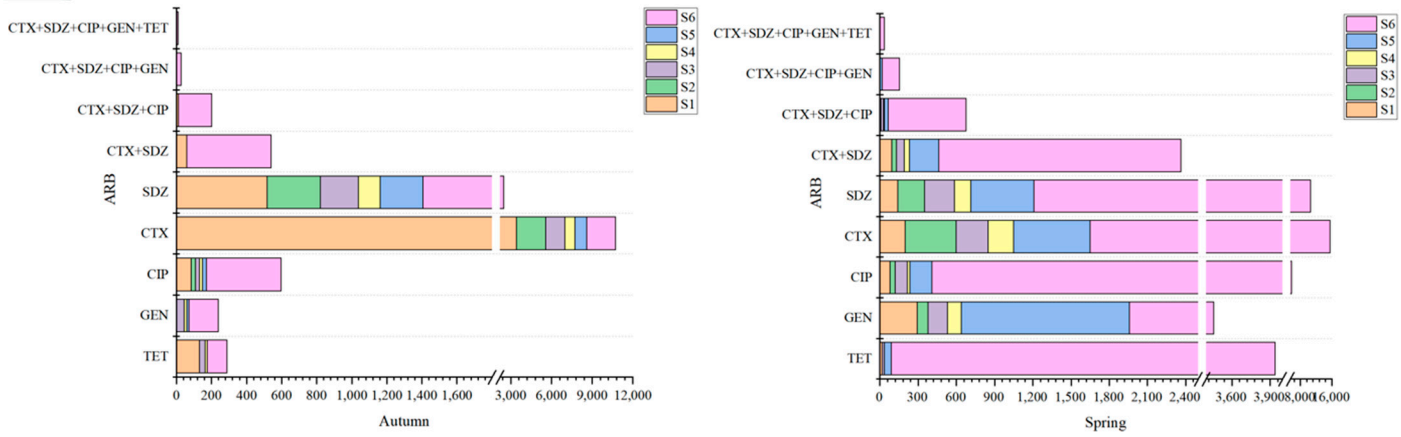

Figure 4. The concentration of various ARB at each sampling site in the Songhua River in spring and autumn.

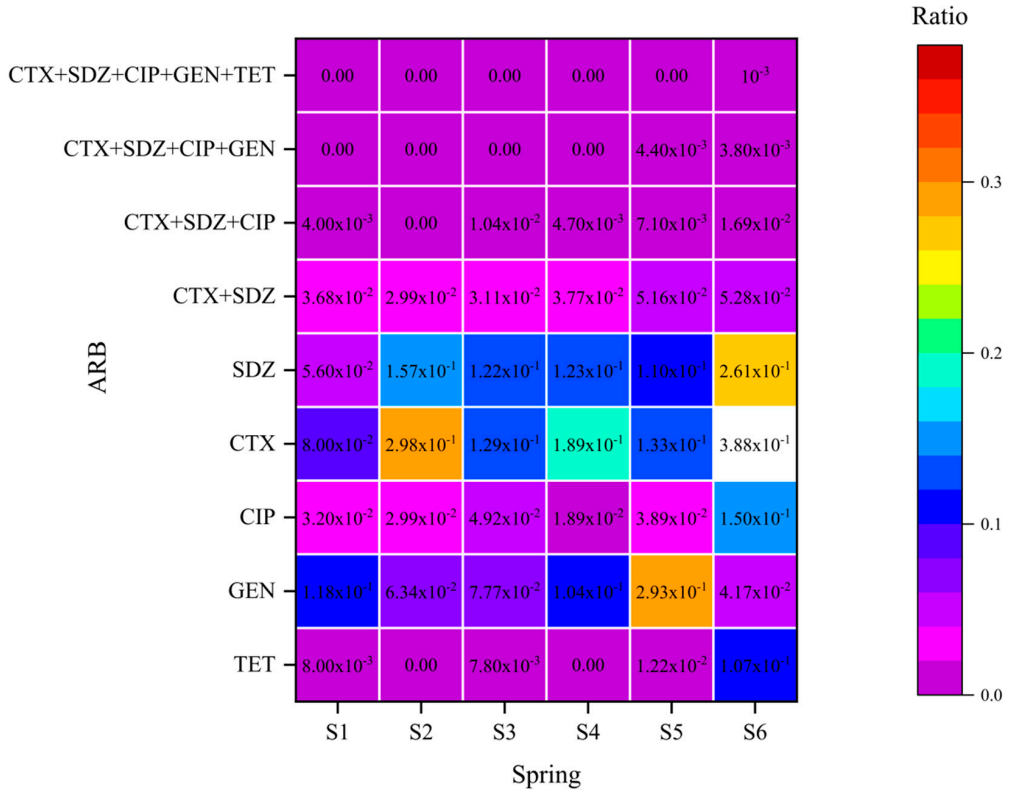

Figure 5. Proportion of ARB at each sampling site in spring. The white part is the maximum value of the data, which is outside the gradient range.

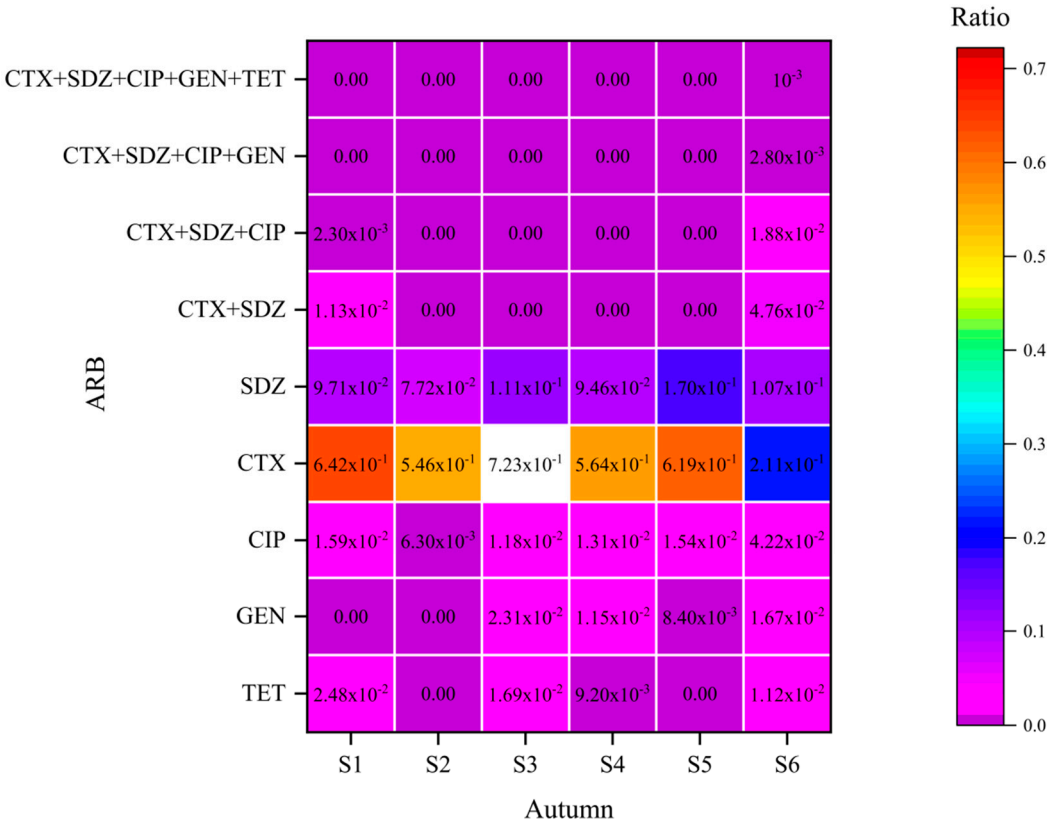

Figure 6. Proportion of ARB at each sampling site in autumn. The white part is the maximum value of the data, which is outside the gradient range.

3.2. Identification Results of ARB

In this study, the sequencing results of the bacterial PCR products were compared on NCBI. The selected bacteria were identified according to the sequence alignment results, and all the selected strains were classified. The detailed information is shown in Table 3. A total of 51 ARB strains were screened in the Songhua River in spring and autumn, including 15 genera. The 15 genera were *Acinetobacter*, *Pseudomonas*, *Priestia*, *Bacillus*, *Arthrobacter*, *Paenarthrobacter*, *Brevundimonas*, *Flavobacterium*, *Peribacillus*, *Exiguobacterium*, *Escherichia*, *Shigella*, *Aeromonas*, *Citrobacter*, and *Lysinibacillus*. Among them, there were 20 strains from *Pseudomonas*, which was the most prevalent bacterial genus of ARB strains screened in Songhua River water. Secondly, there were 6 strains from *Bacillus*, 5 strains from *Acinetobacter*, 4 strains from *Aeromonas*, 2 strains each from *Priestia*, *Arthrobacter*, *Flavobacterium*, *Peribacillus*, and *Escherichia*, and 1 strain from each of the other six bacterial genera. In particular, strains with less than 95% sequence similarity to *Bacillus* and *Aeromonas* were screened from the water sample. The sequence similarity of one strain with *Aeromonas sanarelli* was only 84.71% (Figure 7), and the sequence similarity of the other strain with *Bacillus fungorum* was 88.32% (Figures 8 and 9). These two strains may be new bacterial strains. The sequence data of all the strains screened by the test have been uploaded to GenBank; GenBank number: OR660250-OR660298.

Table 3. Information of the 51 ARB in the Songhua River.

No.	Species	Length (bp)	Coverage	Identity	Accession
1	*Acinetobacter bouvetii*	1530	100%	98.34%	NR_117628.1
2	*Acinetobacter movanagherensis*	1331	100%	99.86%	NR_145841.1
3	*Acinetobacter kyonggiensis*	1395	100%	99.17%	NR_116714.1
4	*Acinetobacter piscicola*	1501	99%	96.32%	NR_159919.1
5	*Acinetobacter oryzae*	1499	100%	99.72%	NR_180005.1
6	*Pseudomonas vancouverensis*	1492	100%	99.72%	NR_041953.1
7	*Pseudomonas silesiensis*	1539	100%	100%	NR_156815.1
8	*Pseudomonas oryzihabitans*	1527	100%	99.31%	NR_025881.1
9	*Pseudomonas mohnii*	1459	100%	98.89%	NR_042543.1
10	*Pseudomonas paracarnis*	1431	100%	99.31%	NR_178976.1
11	*Pseudomonas alloputida*	1464	100%	99.86%	NR_179595.1
12	*Pseudomonas umsongensis*	1455	100%	100%	NR_025227.1
13	*Pseudomonas qingdaonensis*	1525	100%	100%	NR_169411.1
14	*Pseudomonas kielensis*	1537	100%	100%	NR_181570.1
15	*Pseudomonas kilonensis*	1528	100%	100%	NR_028929.1
16	*Pseudomonas peli*	1497	100%	99.31%	NR_042451.1
17	*Pseudomonas promysalinigenes*	1331	100%	99.86%	NR_178291.1
18	*Pseudomonas petroselini*	1494	100%	99.86%	NR_179384.1
19	*Pseudomonas mandelii*	1518	100%	100%	NR_024902.1
20	*Pseudomonas lactis*	1428	100%	100%	NR_156986.1
21	*Pseudomonas chengduensis*	1529	99%	97.49%	NR_125523.1
22	*Pseudomonas laurylsulfativorans*	1499	100%	99.72%	NR_179728.1
23	*Pseudomonas arcuscaelestis*	1567	100%	99.86%	NR_181857.1
24	*Pseudomonas defluvii*	1532	100%	100%	NR_179168.1
25	*Pseudomonas persica*	1472	100%	98.34%	NR_179596.1
26	*Priestia qingshengii*	1455	100%	99.31%	NR_133978.1
27	*Priestia megaterium*	1495	100%	99.86%	NR_117473.1
28	*Bacillus mycoides*	1477	100%	99.86%	NR_113996.1
29	*Bacillus thuringiensis*	1544	100%	99.86%	NR_121761.1
30	*Bacillus proteolyticus*	1509	100%	99.86%	NR_157735.1
31	*Bacillus altitudinis*	1506	100%	100%	NR_042337.1
32	*Bacillus zhangzhouensis*	1513	100%	99.86%	NR_148786.1
33	*Bacillus fungorum*	1576	87%	88.32%	NR_170494.1
34	*Arthrobacter oryzae*	1465	100%	99.72%	NR_041545.1
35	*Arthrobacter ginsengisoli*	1454	100%	99.31%	NR_178602.1

Table 3. *Cont.*

No.	Species	Length (bp)	Coverage	Identity	Accession
36	*Paenarthrobacter nicotinovorans*	1468	100%	99.58%	NR_026194.1
37	*Brevundimonas vesicularis*	1386	100%	99.72%	NR_113586.1
38	*Flavobacterium tructae*	1458	100%	99.86%	NR_133749.1
39	*Flavobacterium suzhouense*	1477	100%	99.31%	NR_178734.1
40	*Peribacillus frigoritolerans*	1503	100%	98.89%	NR_117474.1
41	*Peribacillus simplex*	1522	100%	100%	NR_042136.1
42	*Exiguobacterium undae*	1550	100%	99.86%	NR_043477.1
43	*Escherichia marmotae*	1504	100%	99.17%	NR_136472.1
44	*Escherichia fergusonii*	1542	100%	99.45%	NR_074902.1
45	*Shigella flexneri*	1488	100%	99.86%	NR_026331.1
46	*Aeromonas media*	1460	100%	99.86%	NR_119041.1
47	*Aeromonas hydrophila subsp. ranae*	1350	100%	99.72%	NR_042518.1
48	*Aeromonas hydrophila*	1460	100%	100%	NR_119039.1
49	*Aeromonas sanarellii*	1503	85%	84.71%	NR_116584.1
50	*Citrobacter pasteurii*	1492	100%	99.86%	NR_178769.1
51	*Lysinibacillus composti*	1475	100%	99.86%	NR_126171.1

S6-CIP:
GGCCGAAGGGCGGCCCTATACATGCTAGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGCCTGGGAAATTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTA
ATACCGCATACGCCCTACGGGGGAAAGCAGGGGACCTTCGGGCCCTGCGCGATTGGTATGCCCAGGTGGGATTAGTTGTTGGTGAGGTAATGGTTCACCAAGGCGACAACCCCTACTTGGCCTGAAAGGATGACCACCCC
CCTGGGACTTGAACCCCGGCCCAAATCCCTCCGGAAGGCACCAGGGGGAATTTTTGCCAATGGGGGGAAACCCTGATGCACCCTTGCCCCGTGGGTGAAAAAGGCCTTCGGGTTGAAAACCACTTTCACCAAGGAGGAAA
GGAGAAACCCTTTCCCTGCTGTGTTGGACGCCCCTAAAAAAAGAACCCCGGCTCTCTCCGTGCCCCCCGCCGCGGTAATACGGAGGGCGCAAGCGTTACTCAAATTCACTGGGCGTAAAGCGCACACAGGCGATTGGATA
ATTATGATGTGACCCCCCCGGCCTCCCCTTGGTATTGTTTTCAAATCTCTCCTAGTATCCTCTTGTAGAGGGGGGGAATTCCTCCCCGTGCCCTGCCATTCATGTGCATCTATCTGGAAGAACAGTGGCTGGAGAATGCC
CCCTCTCCGTAGAATGACTGCTCGATCGCGCAGAGACTGCGTGCCTAACACACTATGATTACACTACATAGGCCACTCACTCCACTATATCTATTTATGTACGCTGTCCTGTAGAGCCCTCTTTTCCACCTTACCCATCA
CAAACACTGCTAACGCGAGTAGATTGCGACGTACAGAAATCACATTTAATACTTGTGTGGCTCTCCACGCGGTACCGCACGTGCGTGAGATCATGTAATCCAATAGCTTACCCTCCACTACCTCTTATCTGGTCCTGAAA
CATCGGAATTTTCTGA

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Aeromonas sanarellii strain A2-67 16S ribosomal RNA, partial sequence	Aeromonas sanarellii	606	606	85%	6e-173	84.71%	1503	NR_116584.1
Aeromonas hydrophila strain DSM 30187 16S ribosomal RNA, partial sequence	Aeromonas hydrophila	601	601	85%	3e-171	84.55%	1538	NR_119190.1
Aeromonas caviae strain ATCC 15468 16S ribosomal RNA, partial sequence	Aeromonas caviae	601	601	85%	3e-171	84.55%	1460	NR_029252.1
Aeromonas caviae strain CECT 4221 16S ribosomal RNA, partial sequence	Aeromonas caviae	601	601	85%	3e-171	84.55%	1494	NR_104824.1
Aeromonas media strain ATCC 33907 16S ribosomal RNA, partial sequence	Aeromonas media	595	595	85%	1e-169	84.39%	1460	NR_119041.1
Aeromonas media strain RM 16S ribosomal RNA, partial sequence	Aeromonas media	595	595	85%	1e-169	84.39%	1503	NR_036911.2
Aeromonas enteropelogenes strain CECT 4487 16S ribosomal RNA, partial sequence	Aeromonas enteropelogenes	595	595	85%	1e-169	84.37%	1506	NR_116026.1
Aeromonas taiwanensis strain A2-50 16S ribosomal RNA, partial sequence	Aeromonas taiwanensis	595	595	85%	1e-169	84.37%	1503	NR_116585.1
Aeromonas dhakensis strain P21 16S ribosomal RNA, partial sequence	Aeromonas dhakensis	592	592	85%	2e-168	84.24%	1498	NR_042155.1
Aeromonas rivipollensis strain P2G1 16S ribosomal RNA, partial sequence	Aeromonas rivipollensis	590	590	85%	6e-168	84.19%	1464	NR_144574.1
Aeromonas hydrophila subsp. ranae strain Au-1D12 16S ribosomal RNA, partial sequence	Aeromonas hydrophila subsp. ranae	590	590	85%	6e-168	84.24%	1350	NR_042518.1
Aeromonas hydrophila strain ATCC 7966 16S ribosomal RNA, partial sequence	Aeromonas hydrophila	584	584	85%	3e-166	84.08%	1537	NR_074841.1
Aeromonas hydrophila strain ATCC 7966 16S ribosomal RNA, partial sequence	Aeromonas hydrophila	584	584	85%	3e-166	84.08%	1460	NR_119039.1
Aeromonas hydrophila strain JCM 1027 16S ribosomal RNA, partial sequence	Aeromonas hydrophila	584	584	85%	3e-166	84.08%	1467	NR_113342.1
Aeromonas hydrophila strain ATCC 7966 16S ribosomal RNA, partial sequence	Aeromonas hydrophila	584	584	85%	3e-166	84.08%	1503	NR_118944.1
Aeromonas hydrophila strain CCM 7232 16S ribosomal RNA, partial sequence	Aeromonas hydrophila	584	584	85%	3e-166	84.08%	1542	NR_043638.1
Aeromonas hydrophila subsp. ranae strain LMG 19707 16S ribosomal RNA, partial sequence	Aeromonas hydrophila subsp. ranae	575	575	85%	2e-163	83.60%	1497	NR_114883.1
Aeromonas enteropelogenes strain DSM 6394 16S ribosomal RNA, partial sequence	Aeromonas enteropelogenes	571	571	83%	2e-162	83.77%	1490	NR_044846.1
Aeromonas hydrophila strain ATCC 7966 16S ribosomal RNA, partial sequence	Aeromonas hydrophila	564	564	85%	3e-160	83.60%	1357	NR_115183.1
Aeromonas allosaccharophila strain CECT 4199 16S ribosomal RNA, partial sequence	Aeromonas allosaccharophila	562	562	85%	1e-159	83.47%	1503	NR_025945.2
Aeromonas lusitana strain MDC2473 16S ribosomal RNA, partial sequence	Aeromonas lusitana	556	556	85%	6e-158	83.28%	1503	NR_178472.1

Figure 7. A strain sequence with a homologous similarity of 84.71% to *Aeromonas*.

S6-CTX+SDZ:
CATAAGGCCGGGGGCGCCTATAATGCTAGTCGAGCGACTGGATTAAGAGCGTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAAT
ACCGGATAACATTTTGACCTGCATGGGACAAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGTCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCCTAGCCGACCTGATAGGGT
GATCGGCCACACTGGGACTGACACACGGCCCATACTCCTACGGGAGGCAGCAGTGGGTAATCTTCCGAATTGGACAAACTTCTGACGCACCACCCCGGGGAAAAAAAAAAGGCTTTTCGGTCCTAATAACTCTGTTTTAA
GGTGGAATCAGGACGGGAGTAACTGCTGGTACCTTGACGGTTCCTAACCAGAAAGCCACGGCTAACTACTTGCCCGCAGCCGCGGTAATCCGTAAGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCACGCAGG
CGGTTTCTTAAGTCTGATGTTAAAGCCCACGGCCCCACCCTGGAAGGGTCCTTGGAAACTGGGGAACTTGGATGCAAGATAGAGAAGCGGAATTCCCAGTGTAACGTTCAAGTGCTCACATATAAATTGAAGTACCACTG
GGGCAGTGGGGCTTTTTGTTCTGGAATGGATCCTGATTTTGAGAAGACGTGTGGAGGACCCAAGCCTAGATACCCTGCTTAGCCACGCCCGGACACGATCAATGCTGAATGATATTCGGTTTCCACCTCTATCGCTAGCT
GCTAATCATCACGCACTCCCTCCAGTTAGTGCGGTCTCTCAACGAGATCTTGAATAATTGGAAGGGGCCCCGGCCACGGTTTAAGATTGATGTTTAAATTCTAAAAAAGACGATAATCCTTACGGA

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Bacillus wiedmannii strain FSL W8-0169 16S ribosomal RNA, partial sequence	Bacillus wiedmannii	756	756	87%	0.0	88.32%	1540	NR_152692.1
☑	Bacillus proteolyticus strain MCCC 1A00365 16S ribosomal RNA, partial sequence	Bacillus proteolyticus	756	756	87%	0.0	88.32%	1509	NR_157735.1
☑	Bacillus sanguinis strain BML-BC004 16S ribosomal RNA, partial sequence	Bacillus sanguinis	756	756	87%	0.0	88.32%	1555	NR_175555.1
☑	Bacillus fungorum strain 17-SMS-01 16S ribosomal RNA, partial sequence	Bacillus fungorum	756	756	87%	0.0	88.32%	1576	NR_170494.1
☑	Bacillus tropicus strain MCCC 1A01406 16S ribosomal RNA, partial sequence	Bacillus tropicus	750	750	87%	0.0	88.16%	1509	NR_157736.1
☑	Bacillus paramycoides strain MCCC 1A04098 16S ribosomal RNA, partial sequence	Bacillus paramycoides	750	750	87%	0.0	88.16%	1509	NR_157734.1
☑	Bacillus nitratireducens strain MCCC 1A00732 16S ribosomal RNA, partial sequence	Bacillus nitratireducens	750	750	87%	0.0	88.16%	1509	NR_157732.1
☑	Bacillus luti strain MCCC 1A00359 16S ribosomal RNA, partial sequence	Bacillus luti	750	750	87%	0.0	88.16%	1509	NR_157730.1
☑	Bacillus albus strain MCCC 1A02146 16S ribosomal RNA, partial sequence	Bacillus albus	750	750	87%	0.0	88.16%	1509	NR_157729.1
☑	Bacillus cereus ATCC 14579 16S ribosomal RNA (rrnA), partial sequence	Bacillus cereus ATCC 14579	750	750	87%	0.0	88.16%	1512	NR_074540.1
☑	Bacillus cereus strain IAM 12605 16S ribosomal RNA, partial sequence	Bacillus cereus	750	750	87%	0.0	88.16%	1486	NR_115526.1
☑	Bacillus cereus strain JCM 2152 16S ribosomal RNA, partial sequence	Bacillus cereus	750	750	87%	0.0	88.16%	1474	NR_113266.1
☑	Bacillus cereus strain CCM 2010 16S ribosomal RNA, partial sequence	Bacillus cereus	750	750	87%	0.0	88.16%	1535	NR_115714.1
☑	Bacillus cereus strain NBRC 15305 16S ribosomal RNA, partial sequence	Bacillus cereus	750	750	87%	0.0	88.16%	1476	NR_112630.1
☑	Bacillus cereus ATCC 14579 16S ribosomal RNA, partial sequence	Bacillus cereus ATCC 14579	750	750	87%	0.0	88.16%	1482	NR_114582.1
☑	Bacillus clarus strain ATCC 21929 16S ribosomal RNA, complete sequence	Bacillus clarus	745	745	87%	0.0	88.01%	1552	NR_180213.1
☑	Bacillus pseudomycoides strain NBRC 101232 16S ribosomal RNA, partial sequence	Bacillus pseudomycoides	745	745	87%	0.0	88.02%	1477	NR_113991.1
☑	Bacillus toyonensis strain BCT-7112 16S ribosomal RNA, partial sequence	Bacillus toyonensis	739	739	87%	0.0	87.85%	1544	NR_121761.1
☑	Bacillus bingmayongensis strain FJAT-13831 16S ribosomal RNA, partial sequence	Bacillus bingmayongensis	739	739	87%	0.0	87.87%	1443	NR_148248.1
☑	Bacillus thuringiensis strain IAM 12077 16S ribosomal RNA, partial sequence	Bacillus thuringiensis	739	739	87%	0.0	87.85%	1486	NR_043403.1
☑	Bacillus thuringiensis strain ATCC 10792 16S ribosomal RNA, partial sequence	Bacillus thuringiensis	739	739	87%	0.0	87.85%	1482	NR_114581.1

Figure 8. A strain sequence with a homologous similarity of 88.32% to *Bacillus*.

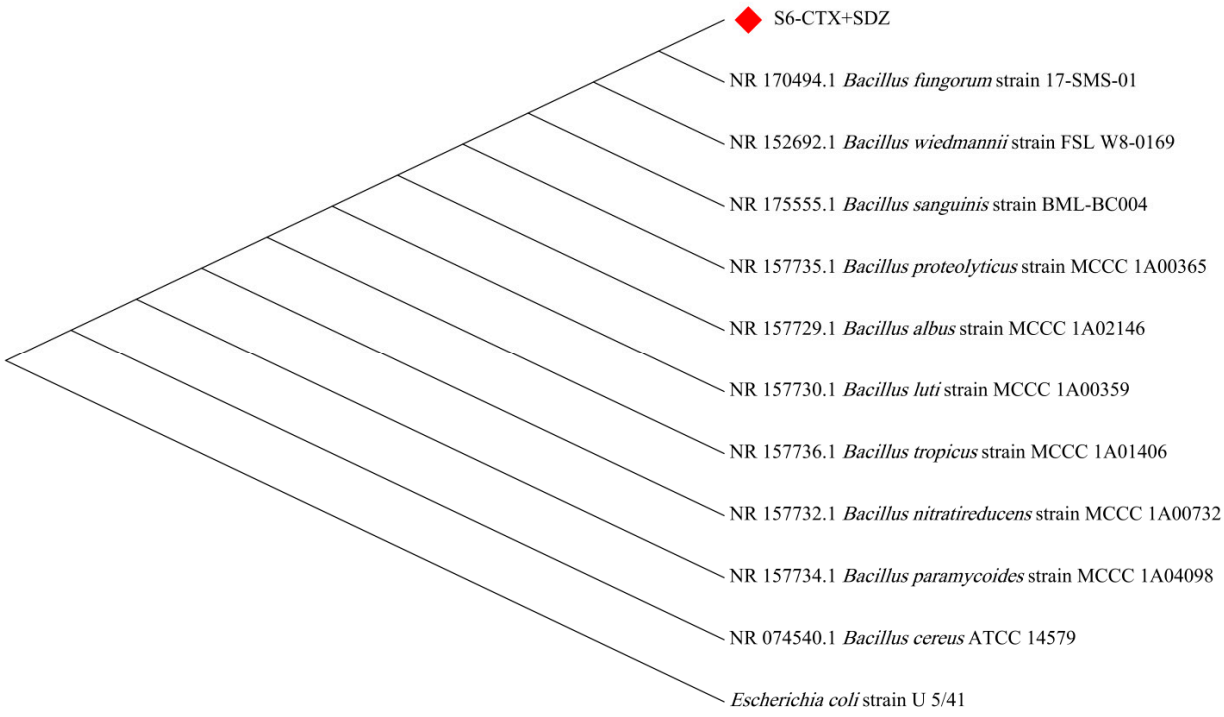

Figure 9. The phylogenetic tree constructed by the sequence of a strain with a homologous similarity of 88.32% to Bacillus. The red diamond pattern represents the bacterial strain used for sequence alignment.

3.3. Distribution of Antibiotic-Resistant Strains

In this study, ARB from the Songhua River were screened and identified. As shown in Table 4, the antibiotic resistance and distribution of these 51 ARB strains from 15 genera

were related to the environment and would be affected by environmental factors. The results showed that *Pseudomonas* were screened out from six sampling sites in Songhua River, and the number was much higher than that of other bacteria. It can be seen that the main ARB in the Songhua River in spring and autumn were from *Pseudomonas*. Among them, *Pseudomonas silesiensis* strain A3 was screened in all five sampling sites except for sampling site S6, and the strain only showed resistance to cefotaxime in these five sampling sites. Meanwhile, research has found that strains of *Pseudomonas* were screened in five single antibiotic culture media. In S3, S4, and S5, *Arthrobacter* strains resistant only to gentamicin were screened, *Paenarthrobacter* and *Brevundimonas* strains resistant to ciprofloxacin were screened at S3, *Flavobacterium* strains resistant only to gentamicin were screened at S4 and S5, and *Exiguobacterium* strains resistant to sulfadiazine were screened at S5. In addition, strains of *Escherichia*, *Shigella*, *Aeromonas*, *Citrobacter*, and *Lysinibacillus* were only screened at S6. Figure 10 shows that 14 strains of *Pseudomonas* were resistant to cefotaxime, 7 strains of *Pseudomonas* were resistant to ciprofloxacin, 7 strains of *Pseudomonas* were resistant to sulfadiazine, and 3 strains of *Pseudomonas alloputida* strain Kh7, *Pseudomonas persica* strain VKh13, and *Pseudomonas defluvii* strain WCHP16 were even resistant to three antibiotics, and thus belonged to the MARB group. Among the *Aeromonas* strains screened at S6, one strain was resistant to five antibiotics at the same time. Figure 11 is a phylogenetic tree constructed based on the alignment results of the strain sequence on NCBI, and it can be determined that the strain is the *Aeromonas media* strain ATCC 33907.

Table 4. Distribution of antibiotic-resistant strains in the Songhua River.

ARBs	Antibiotics
Acinetobacter bouvetii strain DSM 14964	S1-TET
Acinetobacter movanagherensis strain Movanagher 4	S1-TET, CIP
Acinetobacter kyonggiensis strain KSL5401-037	S1-TET
Acinetobacter piscicola strain LW15	S1-TET
Acinetobacter oryzae strain B23	S6-TET
Acinetobacter movanagherensis strain Movanagher 4	S6-TET
Pseudomonas vancouverensis strain DhA-51	S1-TET
Pseudomonas alloputida strain Kh7	S1-CIP, SDZ, CTX
Pseudomonas umsongensis strain Ps 3-10	S1-SDZ, CTX
Pseudomonas qingdaonensis strain JJ3	S1-SDZ, CTX
Pseudomonas silesiensis strain A3	S1-CTX
Pseudomonas oryzihabitans strain L-1	S1-CTX
Pseudomonas mohnii strain IpA-2	S1-CTX
Pseudomonas paracarnis strain V5/DAB/2/5	S1-CTX
Pseudomonas alloputida strain Kh7	S1-CTX + SDZ
Pseudomonas umsongensis strain Ps 3-10	S1-CTX + SDZ
Pseudomonas qingdaonensis strain JJ3	S1-CTX + SDZ
Pseudomonas alloputida strain Kh7	S1-CTX + SDZ + CIP
Pseudomonas kielensis strain MBT-1	S2-CTX
Pseudomonas kilonensis strain 520-20	S2-CTX
Pseudomonas silesiensis strain A3	S2-CTX
Pseudomonas peli strain R-20805	S2-CTX, CIP
Pseudomonas promysalinigenes strain RW10S1	S2-CIP
Pseudomonas petroselini strain MAFF 311094	S2-CIP
Pseudomonas vancouverensis strain DhA-51	S3-TET, GEN
Pseudomonas umsongensis strain Ps 3-10	S3-TET
Pseudomonas silesiensis strain A3	S3-CTX
Pseudomonas mandelii strain CIP 105273	S3-CTX
Pseudomonas lactis strain DSM 29167	S3-SDZ
Pseudomonas chengduensis strain MBR	S4-CTX
Pseudomonas silesiensis strain A3	S4-CTX
Pseudomonas vancouverensis strain DhA-51	S4-GEN

Table 4. *Cont.*

ARBs	Antibiotics
Pseudomonas peli strain R-20805	S4-TET, CIP
Pseudomonas laurylsulfativorans strain AP3_22	S5-SDZ
Pseudomonas silesiensis strain A3	S5-CTX
Pseudomonas kielensis strain MBT-1	S5-CTX
Pseudomonas umsongensis strain Ps 3-10	S5-CTX
Pseudomonas peli strain R-20805	S5-CIP
Pseudomonas arcuscaelestis strain P66	S6-CIP
Pseudomonas defluvii strain WCHP16	S6-CIP, CTX, SDZ
Pseudomonas persica strain VKh13	S6-CIP, CTX, SDZ
Pseudomonas defluvii strain WCHP16	S6-CTX + SDZ
Pseudomonas persica strain VKh13	S6-CTX + SDZ
Pseudomonas persica strain VKh13	S6-CTX + SDZ + CIP
Pseudomonas defluvii strain WCHP16	S6-CTX + SDZ + CIP
Priestia qingshengii strain G19	S1-SDZ
Priestia megaterium strain ATCC 14581	S4-SDZ
Priestia megaterium strain ATCC 14581	S6-CTX
Bacillus mycoides strain NBRC 101238	S1-CTX, SDZ
Bacillus mycoides strain NBRC 101238	S1-CTX + SDZ
Bacillus thuringiensis strain IAM 12077	S2-CTX, SDZ
Bacillus proteolyticus strain MCCC 1A00365	S3-SDZ
Bacillus altitudinis 41KF2b	S5-CTX
Bacillus zhangzhouensis strain MCCC 1A08372	S5-CTX
Bacillus thuringiensis strain IAM 12077	S6-CTX, SDZ
Bacillus fungorum strain 17-SMS-01	S6-CTX, SDZ
Bacillus thuringiensis strain IAM 12077	S6-CTX + SDZ
Bacillus fungorum strain 17-SMS-01	S6-CTX + SDZ
Arthrobacter ginsengisoli strain DCY81	S3-GEN
Arthrobacter oryzae strain KV-651	S4-GEN
Arthrobacter oryzae strain KV-651	S5-GEN
Paenarthrobacter nicotinovorans strain DSM 420	S3-CIP
Brevundimonas vesicularis strain NBRC 12165	S3-CIP
Flavobacterium tructae strain 435-08	S4-GEN
Flavobacterium suzhouense strain XIN-1	S5-GEN
Peribacillus frigoritolerans strain DSM 8801	S4-SDZ
Peribacillus simplex NBRC 15720 = DSM 1321	S5-SDZ
Peribacillus frigoritolerans strain DSM 8801	S6-CTX
Exiguobacterium undae strain DSM 14481	S5-SDZ
Escherichia marmotae strain HT073016	S6-TET
Escherichia fergusonii ATCC 35469	S6-TET
Shigella flexneri strain ATCC 29903	S6-TET, CIP
Aeromonas media strain ATCC 33907	S6-TET, GEN, CTX, CIP, SDZ
Aeromonas hydrophila subsp. ranae strain Au-1D12	S6-GEN, CTX
Aeromonas hydrophila strain ATCC 7966	S6-GEN
Aeromonas sanarellii strain A2-67	S6-CIP
Aeromonas media strain ATCC 33907	S6-CTX + SDZ
Aeromonas media strain ATCC 33907	S6-CTX + SDZ + CIP
Aeromonas media strain ATCC 33907	S6-CTX + SDZ + CIP + GEN
Aeromonas media strain ATCC 33907	S6-CTX + SDZ + CIP + GEN + TET
Citrobacter pasteurii strain CIP55.13	S6-CIP
Lysinibacillus composti strain NCCP-36	S6-SDZ

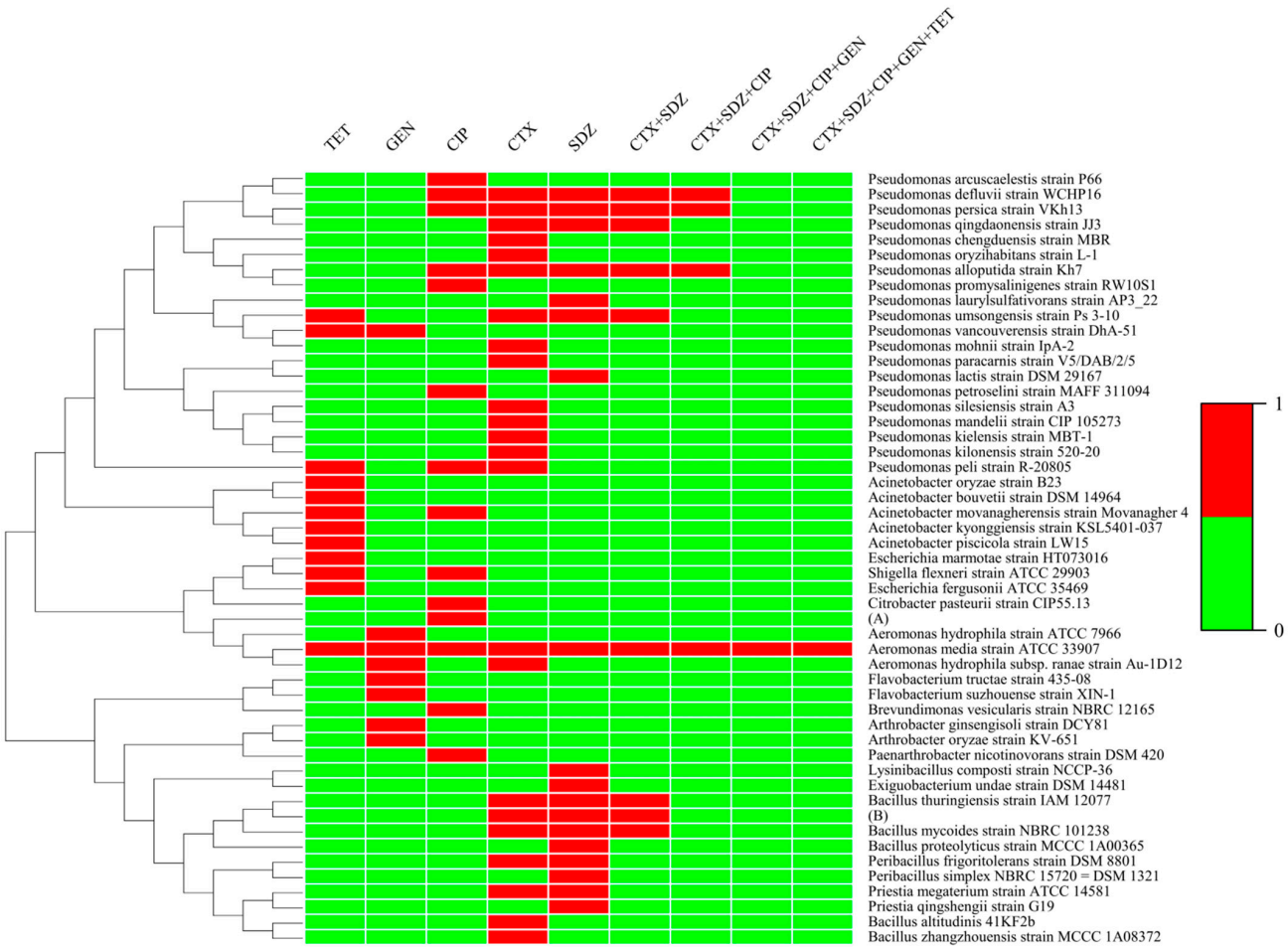

Figure 10. Antibiotic resistance and genetic relationship of the 51 ARB. (A) represents the strain with 84.71% similarity to *Aeromonas sanarelli*, and (B) represents the strain with 88.32% similarity to *Bacillus fungorum*.

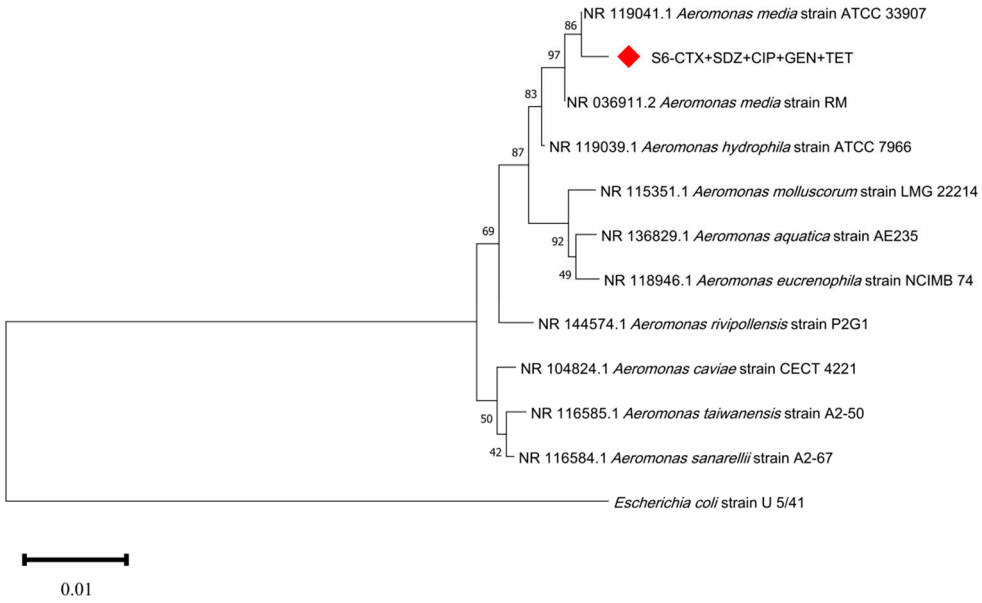

Figure 11. Phylogenetic tree constructed by nucleotide sequences of bacteria resistant to five antibiotics. The red diamond pattern represents the bacterial strain used for sequence alignment.

4. Discussion

The screening results of ARB in the Songhua River in different seasons showed that the number of ARB in spring was higher, and the concentration of ARB was generally larger than that in autumn. Part of the reason for the above results may be due to the influence of the seasonal climate, in which the special climate in winter may increase the probability of bacteria in Songhua River obtaining antibiotic resistance. Calero-Cáceres et al. found that the lower irradiance and temperature in winter and increased total organic carbon will lead to an increase in the number of ARB in the water body [22,23]. The probability of horizontal transfer of ARGs also increases under the conditions of contact with various high concentrations of ARB, resulting in bacteria more likely to acquire antibiotic resistance. Secondly, due to the influence of human activities, the water surface of the Songhua River is frozen in winter and the number of recreational activities increases, which leads to the intensification of human activities. A large amount of domestic garbage and drugs left on the ice surface and river bank enter the water body after the ice and snow melt, which may also be one of the reasons why bacteria in the water body after the thawing of the river water in spring obtain more antibiotic resistance [24]. Compared with winter, the higher irradiance and temperature in summer and autumn lead to the degradation of ARGs in the water, which increases the loss of ARGs in the water environment and reduces the number of ARB [25,26]. Moreover, previous studies have confirmed that the degradation of bacteria by sunlight is carried out by photoactivation. After sunlight irradiation, it was observed that *Shigella flexneri* and *Escherichia coli* were degraded [27,28]. In addition, the flow state of water can also affect the concentration and distribution of ARB [18]. Previous studies have found that in rivers with a high flow, ARB and ARGs are rapidly diluted by water flow, which is the dilution effect of water flow [29,30]. In areas with large water bodies, faster water flow and strong tides will rapidly dilute and disperse ARB and ARGs in water, resulting in a decrease in the number of ARB in local water bodies. This should be one of the reasons for the small number of ARB in the Songhua River in autumn. However, after entering winter, the flow of water below the ice surface of the Songhua River slowed down, and the dilution effect of water flow on ARB became smaller, which increased the number of ARB in the local range. Moreover, a large number of ARB in the upstream water body was likely to accumulate slowly in the downstream water body, which increased the number of ARB in the downstream water body compared with that in autumn. However, with the increase of distance, it was affected by the filtration of riverbanks and islands in rivers, and the degree of influence became smaller. In this study, the concentration of MARB in the water environment of the Songhua River in spring and the probability of bacteria obtaining multiple antibiotic resistance decreased gradually from upstream S6 to downstream S2, which well explained the influence of water flow state on ARB.

Studies have confirmed that the discharge of wastewater from wastewater treatment plants and hospitals is globally recognized as the main source of ARB and ARGs in the river water environment [31], and the proportion of ARB in river systems is extremely high, up to 98% of the total number of detected bacteria [18]. At S6 of the Songhua River in spring, the proportion of bacteria resistant to the five antibiotics selected in this study reached more than 85% of the total number of culturable bacteria, which also showed this high proportion. According to previous studies, the number of cefotaxime-resistant bacteria [32] and sulfadiazine-resistant bacteria [31] in urban rivers is directly related to the discharge of urban wastewater. The results of this study also directly reflect this relationship. The results of this study showed that the concentration of cefotaxime-resistant bacteria was the highest in the Songhua River in spring and autumn, followed by sulfadiazine-resistant bacteria, which may be affected by the discharge of urban wastewater in Harbin. It can be seen from the geographical location that the sampling site S6 is located at the intersection of the Songhua River and the Hejiagou water body. The Hejiagou water body runs through the main urban area of Harbin, flows through schools and hospitals, and the water body contains a large amount of urban wastewater [33]. This polluted river water is collected at S6 and greatly affects the water quality at S6 [34], resulting in a sharp increase in the

amount of cefotaxime-resistant bacteria [32] and sulfadiazine-resistant bacteria [31] in the water. At the same time, a large amount of N, P [35], antibiotics [36], heavy metals [37], and other pollutants in the wastewater also promote the horizontal transfer of ARGs carried by ARB in the water [38], resulting in the concentration of ARB in the water to be significantly higher than that in other sample areas, and a larger number of MARB to be created. In this study, a *Aeromonas media* strain ATCC 33907 resistant to five antibiotics was screened, which was screened at the S6. This strain may also have other antibiotic resistance, which poses a great threat to human health. In addition, between the sampling sites S1 and S2, the Majiagou River water body and the Songhua River water body converge. The Majiagou River water body also flows through hospitals, schools, and residential areas in the main urban area of Harbin. Similar to the Hejiagou River, the river also receives a large amount of urban sewage, which increases the number of ARB in the Songhua River after confluence with the Songhua River, resulting in the emergence of MARB. Therefore, in the Songhua River in autumn, sample S1 is the area where MARB is screened out, except at sample S6. Therefore, the discharge of urban wastewater will lead to an increase in the number and type of ARB in the water environment, which is more likely to lead to the emergence of MARB, and even the emergence of pathogenic bacteria with multiple antibiotic resistance. In addition, the reason why ARB from *Acinetobacter* was only screened at S1 and S6 may be directly related to these two rivers.

Antibiotic-resistant pathogens are widely distributed in river water bodies and pose a threat to human health. In this study, ARB from 15 bacterial genera were screened from the Songhua River, and the pathogenic ARB were identified from 8 bacterial genera including *Pseudomonas*, *Acinetobacter*, *Brevundimonas*, *Flavobacterium*, *Escherichia*, *Aeromonas*, *Citrobacter*, and *Shigella*. Among them, *Pseudomonas* is often distributed in water, air, soil, and plants. It can cause infections of human and plant wounds, and even cause infections of the human urethra and respiratory tract [39]. The pathogenicity of *Acinetobacter* is to cause infection when the body's resistance is reduced, which can cause respiratory and wound infections, and in severe cases can lead to death. It is often distributed in hospitals, water, and soil [40]. A strain of *Brevundimonas vesicularis* screened in this study is a rare opportunistic pathogen in humans. It is usually distributed in soil and rivers and can infect people with low immunity. After infection, it can cause bacteremia [41]. *Flavobacterium* is often distributed in water, soil, and plants. It is also one of the common opportunistic pathogens causing hospital infections. When the body's immunity declines, it may cause infection, and the most susceptible population is newborns [42]. *Escherichia* is mainly distributed in the intestinal tract and natural environment of humans and animals. When the body's immunity is low, certain serotype strains can cause various inflammations. For example, the strain *Escherichia fergusonii* screened in this study can cause infections in humans and animals [43], including traumatic and urinary tract infections. *Aeromonas* is mainly distributed in the water environment, and strains with virulence factors have pathogenic ability. For example, the strain *Aeromonas hydrophila* screened in this study can produce cytotoxins, enterotoxins, and hemolysins, which can cause diarrhea and wound and soft tissue infections. *Aeromonas hydrophila* is also a common pathogen of aquatic animals, especially fish [44]. *Citrobacter* is mainly distributed in water, soil, and food. It is a common flora in humans and animals, and it is also an opportunistic pathogen [45]. When the body's resistance is reduced, it can cause a series of infections involving the body's intestine, respiratory tract, and blood. *Shigella* is the most common pathogen, which can easily spread rapidly between people. It can be divided into four serum groups: *Shigella flexneri*, *Shigella dysenteriae*, *Shigella boydii*, and *Shigella sonnei* [46]. The strain of *Shigella flexneri* screened in this study is a major epidemic pathogen in China, which can lead to invasive infection [47]. The above eight pathogenic bacteria screened from the Songhua River water body pose a great threat to human health. Most frighteningly, these bacteria have developed resistance to multiple antibiotics.

Bacteria are the carriers of antibiotic resistance genes. The concentration of ARB in Songhua River water can reflect the degree of pollution of ARGs in water. The results of

this study showed that the pollution of ARGs in the Songhua River was serious, especially for cefotaxime resistance genes and sulfadiazine resistance genes. The presence of a large number of cefotaxime-resistant bacteria and sulfadiazine-resistant bacteria has aggravated the spread of these two ARGs. In particular, these two antibiotics are commonly used antibiotics in clinical medicine. The high concentration and wide spread of these two ARB and ARGs have seriously threatened human health. In addition, the presence of other types of ARB also poses certain ecological risks. Although the concentration of these ARB in the water is very low, a large number of microorganisms in the water also provide carriers for these types of ARGs. Under the influence of natural and human factors for a certain period of time, these ARB and ARGs will also be produced and widely spread.

5. Conclusions

In this study, ARB were screened at six sampling sites in the Songhua River in spring and autumn. The results showed that seasonal changes and human activities had certain effects on the concentration and distribution of ARB. Among them, the concentration of MARB was higher and the distribution was wider in spring. The concentration of ARB at the sampling site S6 was generally higher than other sampling sites in spring and autumn. Geographical factors also had a certain correlation with the distribution of ARB. The water quality of river water throughout the urban development area was easily affected by wastewater discharge from factories, schools, hospitals, and residential areas, which directly promoted the spread of ARGs and the emergence of MARB. Through the identification of the screened ARB, it was found that there were antibiotic-resistant pathogenic bacteria threatening human health in the Songhua River, especially at sampling site S6. The results of this study show that there is a great risk of direct contact with urban rivers and the use of rivers for crop irrigation. For the sake of human health, reasonable measures should be taken to eliminate ARG pollution in urban rivers and ARB in water bodies, and to protect freshwater resources.

Author Contributions: Conceptualization, Q.X. and X.W. (Xin Wang 2); methodology, Q.X. and X.W. (Xin Wang 2); software, Q.X.; validation, Q.X. and X.W. (Xin Wang 1); investigation, Q.X., X.W. (Xin Wang 1), C.X., Q.H. and W.C.; resources, X.W. (Xin Wang 2); data curation, Q.X. and X.W. (Xin Wang 1); writing—original draft preparation, Q.X.; writing—review and editing, Q.X.; visualization, Q.X. and X.W. (Xin Wang 2); project administration, X.W. (Xin Wang 2); acquisition, X.W. (Xin Wang 2). All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by the Natural Sciences Foundation of Heilongjiang Province (HL2023C089) and the National Innovative Entrepreneurship Training Program For Undergraduates (2023102120285).

Data Availability Statement: Data are contained within the article.

Conflicts of 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.

References

1. Wang, J.; Xu, S.; Zhao, K.; Song, G.; Zhao, S.; Liu, R. Risk control of antibiotics, antibiotic resistance genes (ARGs) and antibiotic resistant bacteria (ARB) during sewage sludge treatment and disposal: A review. *Sci. Total Environ.* **2023**, *877*, 162772. [CrossRef] [PubMed]
2. Allen, H.K.; Donato, J.; Wang, H.H.; Cloud-Hansen, K.A.; Davies, J.; Handelsman, J. Call of the wild: Antibiotic resistance genes in natural environments. *Nat. Rev. Microbiol.* **2010**, *8*, 251–259. [CrossRef] [PubMed]
3. Lerminiaux, N.A.; Cameron, A.D.S. Horizontal transfer of antibiotic resistance genes in clinical environments. *Can. J. Microbiol.* **2018**, *65*, 34–44. [CrossRef]
4. Kumar, M.; Ram, B.; Sewwandi, H.; Sulfikar; Honda, R.; Chaminda, T. Treatment enhances the prevalence of antibiotic-resistant bacteria and antibiotic resistance genes in the wastewater of Sri Lanka, and India. *Environ. Res.* **2020**, *183*, 109179. [CrossRef] [PubMed]
5. Pruden, A.; Pei, R.; Storteboom, H.; Carlson, K.H. Antibiotic Resistance Genes as Emerging Contaminants: Studies in Northern Colorado. *Environ. Sci. Technol.* **2006**, *40*, 7445–7450. [CrossRef] [PubMed]

6. Pérez, J.I.; Álvarez-Arroyo, R.; Arrieta, J.; Suescun, J.M.; Paunero, S.; Gómez, M.A. Occurrence of antibiotics and antibiotic-resistant bacteria (ARB) in the Nervión river. *Chemosphere* **2022**, *288*, 132479. [CrossRef] [PubMed]
7. Scott, L.C.; Wilson, M.J.; Esser, S.M.; Lee, N.L.; Wheeler, M.E.; Aubee, A.; Aw, T.G. Assessing visitor use impact on antibiotic resistant bacteria and antibiotic resistance genes in soil and water environments of Rocky Mountain National Park. *Sci. Total Environ.* **2021**, *785*, 147122. [CrossRef] [PubMed]
8. Mao, Y.; Ding, P.; Wang, Y.; Ding, C.; Wu, L.; Zheng, P.; Zhang, X.; Li, X.; Wang, L.; Sun, Z. Comparison of culturable antibiotic-resistant bacteria in polluted and non-polluted air in Beijing, China. *Environ. Int.* **2019**, *131*, 104936. [CrossRef] [PubMed]
9. Swift, B.M.C.; Bennett, M.; Waller, K.; Dodd, C.; Murray, A.; Gomes, R.L.; Humphreys, B.; Hobman, J.L.; Jones, M.A.; Whitlock, S.E.; et al. Anthropogenic environmental drivers of antimicrobial resistance in wildlife. *Sci. Total Environ.* **2019**, *649*, 12–20. [CrossRef]
10. Srichamnong, W.; Kalambaheti, N.; Woskie, S.; Kongtip, P.; Sirivarasai, J.; Matthews, K.R. Occurrence of antibiotic-resistant bacteria on hydroponically grown butterhead lettuce (*Lactuca sativa* var. *capitata). Food Sci. Nutr.* **2021**, *9*, 1460–1470. [CrossRef]
11. Li, S.; Ondon, B.S.; Ho, S.-H.; Zhou, Q.; Li, F. Drinking water sources as hotspots of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs): Occurrence, spread, and mitigation strategies. *J. Water Process Eng.* **2023**, *53*, 103907. [CrossRef]
12. Cerqueira, F.; Matamoros, V.; Bayona, J.; Piña, B. Antibiotic resistance genes distribution in microbiomes from the soil-plant-fruit continuum in commercial *Lycopersicon esculentum* fields under different agricultural practices. *Sci. Total Environ.* **2019**, *652*, 660–670. [CrossRef] [PubMed]
13. Chauhan, N.S.; Punia, A. Chapter 8—Antibiotic pollution and antibiotic-resistant bacteria in water bodies. In *Degradation of Antibiotics and Antibiotic-Resistant Bacteria from Various Sources*; Singh, P., Sillanpää, M., Eds.; Academic Press: Cambridge, MA, USA, 2023; pp. 179–201.
14. Fletcher, S. Understanding the contribution of environmental factors in the spread of antimicrobial resistance. *Environ. Health Prev. Med.* **2015**, *20*, 243–252. [CrossRef] [PubMed]
15. Peng, F.; Guo, Y.; Isabwe, A.; Chen, H.; Wang, Y.; Zhang, Y.; Zhu, Z.; Yang, J. Urbanization drives riverine bacterial antibiotic resistome more than taxonomic community at watershed scale. *Environ. Int.* **2020**, *137*, 105524. [CrossRef] [PubMed]
16. Xiao, R.; Huang, D.; Du, L.; Song, B.; Yin, L.; Chen, Y.; Gao, L.; Li, R.; Huang, H.; Zeng, G. Antibiotic resistance in soil-plant systems: A review of the source, dissemination, influence factors, and potential exposure risks. *Sci. Total Environ.* **2023**, *869*, 161855. [CrossRef] [PubMed]
17. Zhang, Y.-J.; Hu, H.-W.; Chen, Q.-L.; Singh, B.K.; Yan, H.; Chen, D.; He, J.-Z. Transfer of antibiotic resistance from manure-amended soils to vegetable microbiomes. *Environ. Int.* **2019**, *130*, 104912. [CrossRef] [PubMed]
18. Nnadozie, C.F.; Odume, O.N. Freshwater environments as reservoirs of antibiotic resistant bacteria and their role in the dissemination of antibiotic resistance genes. *Environ. Pollut.* **2019**, *254*, 113067. [CrossRef]
19. Scaccia, N.; Vaz-Moreira, I.; Manaia, C.M. The risk of transmitting antibiotic resistance through endophytic bacteria. *Trends Plant Sci.* **2021**, *26*, 1213–1226. [CrossRef] [PubMed]
20. Leonard, A.F.C.; Zhang, L.; Balfour, A.J.; Garside, R.; Gaze, W.H. Human recreational exposure to antibiotic resistant bacteria in coastal bathing waters. *Environ. Int.* **2015**, *82*, 92–100. [CrossRef]
21. Omotade, I.F.; Lasisi, K.H.; Ajibade, F.O.; Ajibade, T.F.; Adelodun, B.; Kumar, P.; Nwogwu, N.A.; Adeoye, I.A.; Olanrewaju, O.O.; Adewumi, J.R. Chapter 9—Antibiotic-resistant bacteria in natural water bodies: Causes, routes, and remedies. In *Degradation of Antibiotics and Antibiotic-Resistant Bacteria from Various Sources*; Singh, P., Sillanpää, M., Eds.; Academic Press: Cambridge, MA, USA, 2023; pp. 203–229.
22. Calero-Cáceres, W.; Méndez, J.; Martín-Díaz, J.; Muniesa, M. The occurrence of antibiotic resistance genes in a Mediterranean river and their persistence in the riverbed sediment. *Environ. Pollut.* **2017**, *223*, 384–394. [CrossRef]
23. Calero-Cáceres, W.; Muniesa, M. Persistence of naturally occurring antibiotic resistance genes in the bacteria and bacteriophage fractions of wastewater. *Water Res.* **2016**, *95*, 11–18. [CrossRef] [PubMed]
24. Czekalski, N.; Sigdel, R.; Birtel, J.; Matthews, B.; Bürgmann, H. Does human activity impact the natural antibiotic resistance background? Abundance of antibiotic resistance genes in 21 Swiss lakes. *Environ. Int.* **2015**, *81*, 45–55. [CrossRef] [PubMed]
25. Strickler, K.M.; Fremier, A.K.; Goldberg, C.S. Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. *Biol. Conserv.* **2015**, *183*, 85–92. [CrossRef]
26. Anyaduba, T. *Fate and Transport of Antibiotic Resistance Genes in Aquatic Ecosystem*; Imperial College London: London, UK, 2016.
27. Scoullos, I.M.; Lopez Vazquez, C.M.; van de Vossenberg, J.; Hammond, M.; Brdjanovic, D. Effect of Artificial Solar Radiation on the Die-Off of Pathogen Indicator Organisms in Urban Floods. *Int. J. Environ. Res.* **2019**, *13*, 107–116. [CrossRef] [PubMed]
28. Mcclary, J.S.; Sassoubre, L.M.; Boeh, A.B. *Staphylococcus aureus* Strain Newman Photoinactivation and Cellular Response to Sunlight Exposure. *Appl. Environ. Microbiol.* **2017**, *83*, e01052-17. [CrossRef]
29. Jerde, C.L.; Olds, B.P.; Shogren, A.J.; Andruszkiewicz, E.A.; Mahon, A.R.; Bolster, D.; Tank, J.L. Influence of Stream Bottom Substrate on Retention and Transport of Vertebrate Environmental DNA. *Environ. Sci. Technol.* **2016**, *50*, 8770–8779. [CrossRef] [PubMed]
30. LaPara, T.M.; Madson, M.; Borchardt, S.; Lang, K.S.; Johnson, T.J. Multiple Discharges of Treated Municipal Wastewater Have a Small Effect on the Quantities of Numerous Antibiotic Resistance Determinants in the Upper Mississippi River. *Environ. Sci. Technol.* **2015**, *49*, 11509–11515. [CrossRef]

31. Devarajan, N.; Laffite, A.; Mulaji, C.K.; Otamonga, J.-P.; Mpiana, P.T.; Mubedi, J.I.; Prabakar, K.; Ibelings, B.W.; Poté, J. Occurrence of Antibiotic Resistance Genes and Bacterial Markers in a Tropical River Receiving Hospital and Urban Wastewaters. *PLoS ONE* **2016**, *11*, e0149211. [CrossRef]
32. Sta Ana, K.M.; Madriaga, J.; Espino, M.P. β-Lactam antibiotics and antibiotic resistance in Asian lakes and rivers: An overview of contamination, sources and detection methods. *Environ. Pollut.* **2021**, *275*, 116624. [CrossRef]
33. Shahid, A.; Muzammil, S.; Aslam, B.; Ashfaq, U.A.; Hayat, S.; Bilal, M.; Rajoka, M.S.R.; Nisar, M.A.; Khurshid, M. Chapter 2—Antibiotics and antibiotic-resistant bacteria in the environment: Sources and impacts. In *Degradation of Antibiotics and Antibiotic-Resistant Bacteria from Various Sources*; Singh, P., Sillanpää, M., Eds.; Academic Press: Cambridge, MA, USA, 2023; pp. 39–65.
34. Nelson, K.L.; Boehm, A.B.; Davies-Colley, R.J.; Dodd, M.C.; Kohn, T.; Linden, K.G.; Liu, Y.; Maraccini, P.A.; McNeill, K.; Mitch, W.A.; et al. Sunlight-mediated inactivation of health-relevant microorganisms in water: A review of mechanisms and modeling approaches. *Environ. Sci. Process. Impacts* **2018**, *20*, 1089–1122. [CrossRef]
35. Guo, M.-T.; Yuan, Q.-B.; Yang, J. Distinguishing Effects of Ultraviolet Exposure and Chlorination on the Horizontal Transfer of Antibiotic Resistance Genes in Municipal Wastewater. *Environ. Sci. Technol.* **2015**, *49*, 5771–5778. [CrossRef] [PubMed]
36. Lu, J.; Wang, Y.; Li, J.; Mao, L.; Nguyen, S.H.; Duarte, T.; Coin, L.; Bond, P.; Yuan, Z.; Guo, J. Triclosan at environmentally relevant concentrations promotes horizontal transfer of multidrug resistance genes within and across bacterial genera. *Environ. Int.* **2018**, *121*, 1217–1226. [CrossRef] [PubMed]
37. Zhao, Z.; Wang, J.; Han, Y.; Chen, J.; Liu, G.; Lu, H.; Yan, B.; Chen, S. Nutrients, heavy metals and microbial communities co-driven distribution of antibiotic resistance genes in adjacent environment of mariculture. *Environ. Pollut.* **2017**, *220*, 909–918. [CrossRef] [PubMed]
38. Jiang, Q.; Feng, M.; Ye, C.; Yu, X. Effects and relevant mechanisms of non-antibiotic factors on the horizontal transfer of antibiotic resistance genes in water environments: A review. *Sci. Total Environ.* **2022**, *806*, 150568. [CrossRef]
39. Hossain, Z. Pseudomonas. In *Encyclopedia of Food Safety*, 2nd ed.; Smithers, G.W., Ed.; Academic Press: Oxford, UK, 2024; pp. 236–251.
40. da Silva, M.E.P.; Gomes, M.A.d.S.; Rodrigues, R.S.; Lima, N.C.d.S.; Carvalho, A.G.; Taborda, R.L.M.; Matos, N.B. Multidrug-resistant *Acinetobacter* spp. from hospital intensive care units in Brazilian Amazon. *Braz. J. Infect. Dis.* **2023**, *27*, 103687. [CrossRef] [PubMed]
41. Lin, C.-Y.; Chen, Y.-H. Bacteremia due to *Brevundimonas vesicularis*. *J. Microbiol. Immunol. Infect.* **2013**, *46*, 143. [CrossRef]
42. Waśkiewicz, A.; Irzykowska, L. *Flavobacterium* spp.—Characteristics, Occurrence, and Toxicity. In *Encyclopedia of Food Microbiology*, 2nd ed.; Batt, C.A., Tortorello, M.L., Eds.; Academic Press: Oxford, UK, 2014; pp. 938–942.
43. Gaastra, W.; Kusters, J.G.; van Duijkeren, E.; Lipman, L.J.A. *Escherichia fergusonii*. *Vet. Microbiol.* **2014**, *172*, 7–12. [CrossRef] [PubMed]
44. Li, X.; Shang, B.; Zhang, X.; Zhang, H.; Li, Z.; Shen, X.; Chen, F.; Tao, S. Complete genome sequence data of multidrug-resistant *Aeromonas hydrophila* Ah27 isolated from intussusception channel catfish (*Ictalurus punctatus*). *Gene Rep.* **2023**, *33*, 101807. [CrossRef]
45. Adegun, B.R.; Oluduro, A.O.; Aregbesola, O.A. Isolation and molecular characterization of citrobacter species in fruits and vegetables sold for consumption in ILE-IFE, Nigeria. *Sci. Afr.* **2019**, *6*, e00173. [CrossRef]
46. Mumy, K.L. Shigella. In *Encyclopedia of Toxicology*, 4th ed.; Wexler, P., Ed.; Academic Press: Oxford, UK, 2024; pp. 501–503.
47. Zhang, J.; Liu, Y.; Wu, H.; Zhou, L. Is Shigella an under-recognized pathogen? A case of pyogenic cervical spondylitis caused by *Escherichia coli* and *Shigella flexneri* infection. *IDCases* **2024**, *35*, e01930. [CrossRef]