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The Municipal Sewage Discharge May Impact the Dissemination of Antibiotic-Resistant *Escherichia coli* in an Urban Coastal Beach

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Abstract: To determine the potential of the recreational marine environment as a dissemination vector of antibiotic-resistant microorganisms, the dissemination of antibiotic-resistant *E. coli* strains isolated from an urban coastal beach was studied. Sixty-nine and thirteen *E. coli* strains were isolated from the seawater and sand, respectively, in Fujiazhuang bathing beach, China. The average Antibacterial Resistance Index (ARI) value detected in the seawater is approximately three times that in beach sand. All the isolates from the sand were grouped into one cluster and only the isolates from the municipal sewage outlet were classified into three antibiogram clusters that were observed in the hetero-sites of the *E. coli* isolates. The *E. coli* strains with multiple antibiotic resistance (58% of total) were prevalent in the seawater, whereas the isolates from the sand were not detected with multiple antibiotic resistance. A significant association ($p < 0.05$) between all phenotypic and relative genotypic resistance profiles was observed in the isolates, except in the quinolones resistance genotype. The presence of a class 1 integron was significantly correlated with the resistance of *E. coli* to sulfonamides, streptomycin, and levofloxacin ($p < 0.01$). This study revealed that the municipal sewage discharge may impact the dissemination of antibiotic-resistant strains in the urban coastal beach, and that the class 1 integrons play an important role in mediating the resistance of *E. coli* to sulfonamide antibiotics.

Keywords: antibiotic-resistant *Escherichia coli*; urban coastal beach; antibiotic-resistant gene; dissemination

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1. Introduction

The World Health Organization has recognized antibiotic resistance as one of the serious threats to public health in the 21st century. In recent years, the abuse of antibiotics has accelerated the spread of resistant bacteria in the natural environment [1]. These resistant microorganisms may enter the human body through direct or indirect contact (such as the food chain) and increase the resistance of bacteria to antibiotics, thereby posing a threat to public health. It has been reported that more than 700,000 people die each year due to infections with drug-resistant bacteria, mainly in low- and middle-income countries [2]. It is estimated that by 2050, the problem of bacterial resistance will be even more serious than that of cancer, and 10 million people will lose their lives each year due to bacterial resistance. At the same time, it will cause huge economic losses to the world [3]. To promote the management of antibacterial agents and bacterial resistance, as well as to protect public health and guarantee harmonious economic and social development, in 2016, the National Health Commission of the People's Republic of China and 14 other departments jointly

formulated the National Action Plan to Contain Antimicrobial Resistance (2016–2020) to implement comprehensive governing strategies and measures at a national level [4].

The ocean has gradually become an important reservoir for resistant microorganisms and their resistance genes [5,6]. Due to the incomplete removal of micropollutants by conventional wastewater treatment plants (WWTPs), WWTP effluents are important point sources for antibiotics and other pharmaceutical products entering into the seawater [7–9]. There are three levels of antibiotic-resistant pollution sources and transmission vectors in the marine environment: drug-resistant bacteria, drug-resistant plasmids, and drug-resistant genes. The spread of each of these pollution sources and transmission carriers is achieved in an exponential form. In addition to the mobility of natural seawater, the spread of drug resistance in the ocean is fast, wide-ranging, and harmful. The diversity of bacteria in the ocean, the diversity of resistant plasmids, and the diversity of resistance genes determine the diversity and complexity of marine resistance mechanisms and pathways of the resistance spread. Moreover, environmental bacteria can serve as both the donor and recipient of drug-resistant genes, and then spread into humans [10]. More importantly, due to the direct interaction between land and ocean and the direct or indirect interaction between man and the ocean, all three levels of antibiotic-resistant pollution sources and transmission vectors in the ocean may cause drug-resistant pollution in the terrestrial environment and people.

The coastal zone area where the recreational marine environment is located is the boundary zone of the sea and land plate, and it has the world's most valuable and productive ecosystem. As a sea-land connection area, the bathing beach has more special physical and chemical properties. The specificity of the water quality environment and the selective pressure of external human activities can affect the evolution of microbial resistance. The resistant bacteria will be brought into the coastal tourist area near the shore with the rain, tide, and currents. Through the horizontal gene transfer between different strains, the antibiotic-resistant genes may be introduced into the human pathogens in the seawater environment. By monitoring the transfer of antibiotic resistance genes (ARGs) in the static beach environment and the comparison of the dynamic marine environment, it is possible to effectively analyze the route of their transmission in the recreational marine environment [11]. Since beach recreational waters gather tourists, these drug-resistant pathogens can cause fatal intestinal diseases in swimming populations by swallowing seawater, thus greatly increasing the possibility of the occurrence and prevalence of human-resistant pathogens. With the increasing development of coastal tourism resources and the increasing population of the coastal zone, the recreational marine environment may become another important mediator of microorganisms carrying antibiotic-resistant genes.

Escherichia coli is one of the most common enterobacteriaceae bacteria that can cause infectious diseases and is considered an important indicator of fecal pollution for seafood and the marine environment. The CHINET surveillance report of bacterial resistance in China in 2018 showed that the isolation rate of *Escherichia coli* was the highest among Gram-negative bacteria (28.9%) [12]. Pathogenic *E. coli* can also cause serious diarrhea and sepsis. As a pathogenic water-borne microorganism, *E. coli* may transfer antibiotic-resistant genes with indigenous flora after entering recreational seawater, which can cause the horizontal transfer of resistance elements with indigenous bacteria and opportunistic pathogens, resulting in the spread of resistance in the marine environment [13]. Antibiotic-resistant *Escherichia coli* and its antibiotic-resistant genes can be transmitted into humans through the consumption of seafood or swallowing seawater. The presence of AR strains of *E. coli* in coastal water represents a health issue in areas that are used for recreational activities [14]. Multiple antibiotic resistant (MAR) *E. coli* has been reported in different aquatic environments [15]. Integrons play an important role in the rapid spread of resistance genes in water-borne pathogens, further contributing to the development of multiple antibiotic resistance in several water-borne pathogens [16]. *E. coli*, through the integrons under the action of integrase, continuously captures drug-resistant genes from the surrounding environment, which are expressed through the promoter, making the bacteria

resistant or multiple-antibiotic-resistant. Classes 1, 2, and 3 integrons have been found in *E. coli*, of which class 1 integrons are the most prevalent. Many research studies concerning the distribution, the antibiotic-resistant phenotype, and the antibiotic-resistant pattern of *E. coli* harboring the important drug-resistant genes in the recreational marine environment have been carried out, but the antibiotic-resistance mechanism of *E. coli* in the recreational marine environment is less understood [17–19].

China has a long coastline and abundant resources for marine tourism and leisure such as bathing beaches. Therefore, in this study we focused on the environmental behavior of the emerging MAR water-borne pathogens in the recreational marine environment in China and the potential of bathing beaches to serve as a potential dissemination vector of multiple antibiotic-resistant microorganisms and their resistance genes. To achieve this aim, the antibiotic susceptibility patterns of *Escherichia coli* isolated from seawater and the beach were characterized, and the occurrences of class 1 and 2 integrons in the selected marine bathing beach in China were determined. This study will expand our understanding on the spread mechanism of bacterial resistance in the bathing beach and provide basic data on the human-health-related risk assessment of the recreational water for the local administrative department.

2. Materials and Methods

2.1. Study Site

Fujiazhuang bathing beach is one of the high-traffic marine bathing beaches in Dalian during the swimming season. The total area of the beach is 26,000 m^2. It is an exposed sandy beach with a 450 m long coastline, and the average width of the beach is 32 m, with a slope of 8.9%. The offshore of the beach is gravel, and rocks are approximately 100 m away. A small urban sewage treatment plant is located near the beach, which processes 10,000 tons of sewage every day. Foreign tourists and local inhabitants intensively visit this beach for recreational activities during the summer months. During peak seasons, the average daily passenger flow of the beach can reach 50,000 people, and it is the main place for tourists among the beaches in Dalian.

2.2. Sample Collection

Three nearshore seawater samples (approximately 0.2 m below the surface), one offshore wet sand sample from the wave-washed area and one dry sand samples at 2 m from the high-water line were collected in triplicate at Dalian Fujiazhuang bathing beach during the swimming season. Sites 1, 2, and 3 (S1, S2, and S3) were set every 100 m nearshore to collect seawater samples, and site 1 (S1) was near the municipal sewage outlet to the sea. S2 and S3 were set in densely populated areas. Site 4 (S4) and site 5 (S5) were set to collect wet sand and dry sand, respectively (Figure 1). The surface seawater samples were stored in sterile glass bottles, and the sand samples were stored in sterile bag at 4 °C and transported to the laboratory for analysis within 24 h.

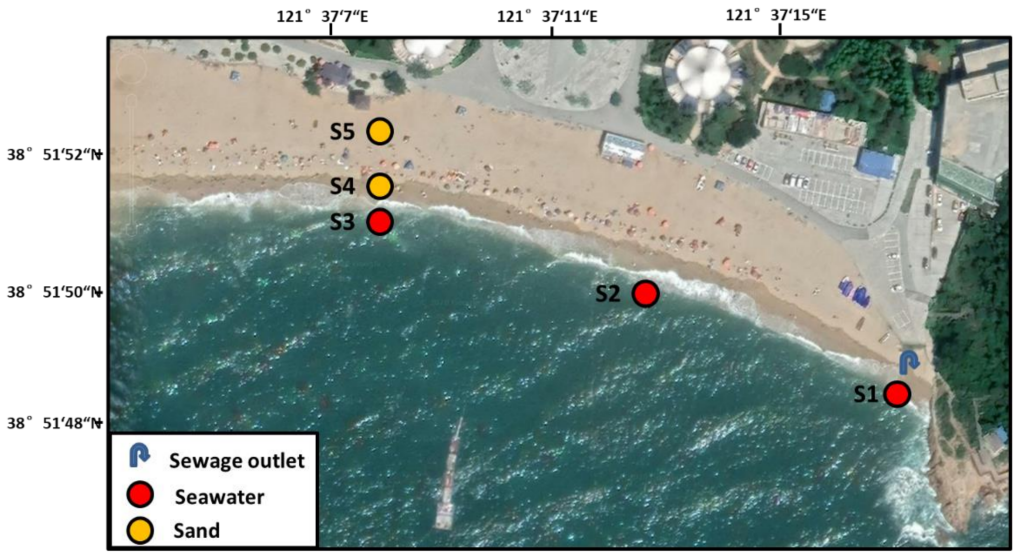

Figure 1. Sampling locations in Fujiazhuang bathing beach in Dalian, China.

2.3. Isolation and Identification of E. coli

Approximately 2.0 L of seawater sample from each site was filtered through a 0.45 μm pore size filter according to US Environmental Protection Agency specifications. The filters were placed on membrane thermotolerant *E. coli* (m-TEC) agar plates (Qingdao Hope Bio-Technology Co., Ltd., Qingdao, China) with incubation at 35 °C for 2 h and then moved into a 44.5 °C incubator for 22 h for the isolation of *E. coli*, according to EPA guidelines [20]. Yellow colonies grown on m-TEC agar plates were isolated and further subjected to the Gram staining test [21] and biochemical tests, including sugar fermentation tests, an indole production test, a methyl-red test, and Voges-Proskauer (IMIVC) tests, following the standard methods described by Cowan [22].

2.4. Antibiotic Susceptibility Assay

The antimicrobial resistances of *E. coli* isolates were determined on Mueller–Hinton agar (Oxoid) by the disk diffusion method, according to Clinical and Laboratory Standards Institute guidelines [23]. The isolates were tested for susceptibility to 10 antimicrobials of 6 classes: tetracycline (TCY, 30 μg), sulfamethoxazole-trimethoprim (SXT, 23.75–1.25 μg), trimethoprim (TMP, 5 μg), gentamicin (GEN, 10 μg), streptomycin (STR, 10 μg), ciprofloxacin (CIP, 5 μg), levofloxacin (LVX, 5 μg), cephalothin (CEP, 30 μg), aztreonam (ATM, 30 μg), and chloramphenicol (CHL, 30 μg). The antimicrobial susceptibility of isolates to different antimicrobials was measured by breakpoints of the inhibition zone diameters. The results of isolates were interpreted with reference to the CLSI standards as susceptible (S), intermediate (I), and resistant (R) after 24 h of incubation at 37 °C. *E. coli* ATCC 25,922 was used as a quality control susceptible bacteria. A bacterium showed resistance to three or more antibiotics, which was defined as 'multiple antibiotic resistant' (MAR) strain [24]. Antibacterial resistance index (ARI) was used to analyze the prevalence of resistant isolates from bathing beach and calculated for each sampling site [25].

2.5. Antibiotic-Resistant GENE Detection

Genome DNA from *E. coli* cells was extracted using a TIANamp Bacteria DNA Kit. All the isolates displaying resistance phenotypes were screened by PCR to detect genes conferring resistance to tetracycline (*tet*A, *tet*B), β-lactams (bla_{TEM}), sulfonamides (*sul*1), and quinolones (*qnr*A, *qnr*B, *qnr*C, *qnr*D, *qnr*S). The primers and PCR conditions are presented in Table S1.

2.6. Detection of the Integrase Gene

MAR *E. coli* isolates were tested for the presence of integrons by PCR amplification of the *intI*1 gene encoding the integrase of class 1 integrons, and *intI*2 gene encoding the integrase of class 2 integrons. Primers used for *intI*1 gene and *intI*2 gene amplification are shown in Table S1.

2.7. Amplification and Sequencing of the Gene Cassette Region

The intI1-positive isolates were further detected for the variable gene cassette region of class 1 integrons by the PCR assay with primer set *int*1F-*int*1R (Table S1). The thermocycling reaction was as follows: 94 °C annealing for 5 min; 30 cycles of 94 °C for 30 s, 56 °C for 40 s, 72 °C for 30 s, and 72 °C for 10 min. The PCR product of the gene cassette insertion region was purified and sequenced by Sangon Biotech. The sequencing results were analyzed by DNAStar software and compared with the GenBank database to determine the gene cassettes carried in the integron.

2.8. Statistical Analyses

Hierarchical cluster analysis of antibiotic susceptibility assay results was carried out using Complex Heatmap r package [26,27] in R version 3.6.2. All statistical analyses were conducted with SPSS version 23 (IBM, Armonk, NY, USA). Fisher's exact tests were used to compare the antibiotic resistance phenotype and antibiotic resistance genes phenotype among resistant isolates. Pearson's correlation analysis was implemented to evaluate the relationship between the antibiotic resistant phenotypes. p value < 0.05 was considered statistically significant.

3. Results

3.1. Prevalence of E. coli in Seawater and Sand

A total of 373 isolates (seawater 248 and sand 125) were presumptive by the culture on the m-TEC medium. Twenty-eight percent (69/248) of the isolates from the seawater and 10% (13/125) of the isolates from the sand were confirmed as *E. coli* using biochemical tests. Of the 69 confirmed *E. coli* isolates from the seawater, 46 of the isolates were from sampling site 1, which is near the sewage outlet. Of the 13 confirmed *E. coli* isolates from the sand, 8 isolates were from the wet sand and 5 were from the dry sand, respectively. Therefore, a total of 82 *E. coli* isolates were identified from Dalian Fujiazhuang bathing beach.

3.2. Cluster Analysis between Isolates' Antibiogram Profiles

The ARI values from all sampling sites were calculated to be lower than the threshold value of 0.2, reflecting low exposure to antibiotics in Fujiazhuang bathing beach. The average ARI value detected in the seawater was approximately three times the value in the sand in Fujiazhuang bathing beach. Fifteen MAR *E. coli* (22%) were isolated from the seawater, but not in the beach sand (Figure 2). Seventeen isolates (40%), eight isolates (44%), and one isolate (20%) from the seawater samples at the S1, S2, and S3 sites showed resistance to one of the tested antibiotics. Among the 15 multiple antibiotic-resistant isolates from the seawater, 13 isolates were resistant to three to five antibiotics and 2 isolates were resistant up to 8 antibiotics. One *E. coli* isolate resistant to 8 antibiotics was isolated from site 1 near the sewage outlet to the sea, and another *E. coli* isolate resistant to 8 antibiotics was found at site 2, which was 100 m nearshore to site 1. Multiple antibiotic-resistant isolates exhibited 10 multiple antibiotic resistance patterns in total.

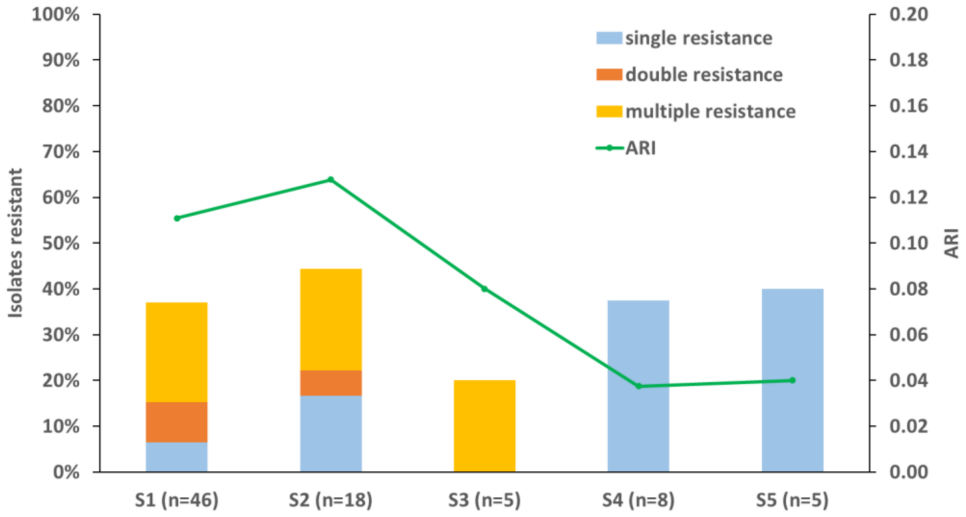

Figure 2. Distribution of resistant *E. coli* and antibiotic resistance index (ARI) across sampling sites in Fujiazhuang bathing beach. Single resistance: resistance to one antibiotic; Double resistance: resistance to two antibiotics; Multiple resistance: resistance to three or more antibiotics.

Cluster analysis of antibiogram profiles of *E. coli* isolates against 10 tested antibiotics are shown in Figure 3. Two clusters of an antibiogram profile, column-wise, were observed, mainly based on the antibiotic resistance and antibiotic susceptibility of the *E. coli* isolates. Three antibiogram clusters of the hetero-sites of *E. coli* isolates were noticed row-wise. All the isolates originated from the sand were grouped into one cluster and isolates from site 1 have been disseminated into 3 clusters.

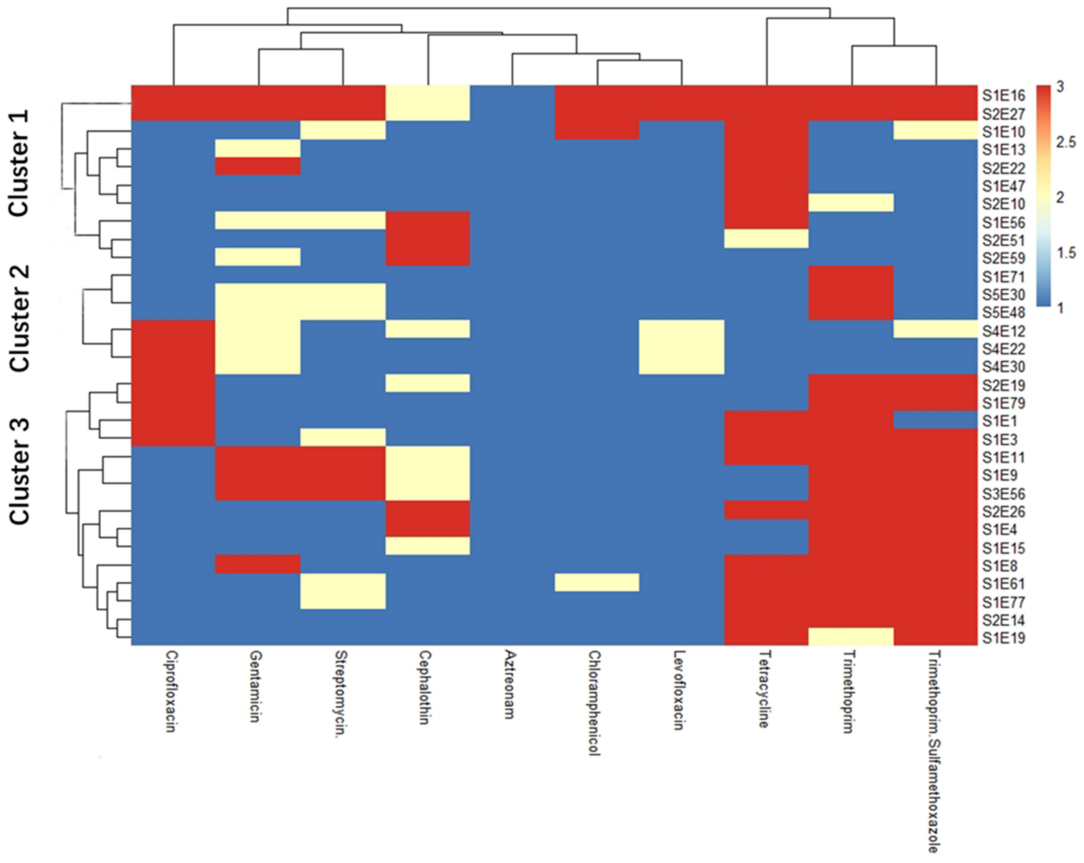

Figure 3. Heatmap cluster analysis of *E. coli* isolates' antibiogram profiles. Color interpretation, blue (1): susceptible, yellow (2): intermediate, and red (3): resistant.

Overall, resistance was detected for all antibiotics tested except aztreonam, and 38% (26/69) of the isolates from the seawater were resistant to at least one of the antibiotics. The most prevalent resistances were toward tetracycline (25%), trimethoprim (25%), and sulfamethoxazole-trimethoprim (23%), followed by gentamicin (10%), ciprofloxacin (9%), streptomycin (7%), and cephalothin (7%) in the seawater. The frequencies of antibiotic resistance to chloramphenicol and levofloxacin (3–4%) were at the lowest levels. Five isolates (39%) from the bathing beach sand were resistant to at least one of the tested antibiotics. Only three isolates (37.5%) from the wet sand showed resistance and were single resistant to ciprofloxacin. Two isolates (40%) from the dry sand were resistant and showed single resistance to trimethoprim (Table 1). Among the multiple antibiotic-resistant beach *E. coli* isolates, the antibiograms observed most frequently were TMP-SXT-TCY (three isolates.) Meanwhile, three antibiograms were seen each in two different isolates: TMP-SXT-CIP, TMP-SXT-GEN-STR, and TMP-SXT-TCY-GEN-STR-CIP-CHL-LVX (Table S2).

Table 1. Antibiotic susceptibility profiles of *E. coli* isolates from seawater and beach sand.

Antibiotics	% of Resistance (No. of Isolates)				
	S1 (n = 46)	**S2 (n = 18)**	**S3 (n = 5)**	**S4 (n = 8)**	**S5 (n = 5)**
Tetracycline (TCY)	26.1 (12)	27.8 (5)	0	0	0
Sulfamethoxazole-Trimethoprim (SXT)	23.9 (11)	22.2 (4)	20 (1)	0	0
Trimethoprim (TMP)	26.1 (12)	22.2 (4)	20 (1)	0	40 (2)
Gentamicin (GEN)	8.7 (4)	11.1 (2)	20 (1)	0	0
Streptomycin (STR)	6.5 (3)	5.6 (1)	20 (1)	0	0
Ciprofloxacin (CIP)	8.7 (4)	11.1 (2)	0	37.5 (3)	0
Levofloxacin (LVX)	2.2 (1)	5.6 (1)	0	0	0
Cephalothin (CEP)	4.3 (2)	16.7 (3)	0	0	0
Aztreonam (ATM)	0	0	0	0	0
Chloramphenicol (CHL)	4.3 (2)	5.6 (1)	0	0	0

The Pearson correlation was used to analyze the association between antibiotic resistance phenotypes of *E. coli* isolates from the bathing beach (Table S3). Apart from co-resistance to the same class of antibiotics, resistance to aminoglycosides (gentamicin, streptomycin) was significantly correlated with sulfamethoxazole-trimethoprim ($p < 0.05$) or levofloxacin ($p < 0.001$). Levofloxacin resistance is closely associated with chloramphenicol resistance ($p < 0.001$).

3.3. Correlation between Phenotypic Resistance Profile and Relative Genotypic Resistance Profile

The resistance genes chosen in this study were detected in 41 isolates displaying a resistance phenotype. Generally, a total of six ARGs tested were detected in one or more resistant isolates. The genes with higher detection rates were *tet*A, *tet*B, *sul* 1, and bla_{TEM}, which were more prevalent in the isolates from the seawater than in the isolates from the sand. Among the 17 isolates of tetracycline-resistant *E. coli* from the seawater, the tetracycline-resistant gene was detected in the genomic DNA of 15 isolates (88.2%), of which 65% (11/17) carried only the *tet*A gene and 41% carried only the *tet*B gene (7/17), while 18% (3/17) of the strains carried the *tet*A +*tet*B genes. There was a significant correlation ($p < 0.05$) between all phenotypic and relative genotypic resistance profiles (*tet*A, *tet*B, bla_{TEM}, and *sul*1) in all *E. coli* isolates (Table S4).

3.4. Occurrence of Class 1 Integrons in the MAR Strain

To investigate the occurrence of class 1 and 2 integrons in the 15 multiple antibiotic-resistant *E. coli* isolates, the integrase genes intI1 and intI2 were detected, respectively. The class 1 integrase gene was detected in the 11 multiple antibiotic-resistant isolates (73%). No class 2 integrase gene was detected among the 15 multiple antibiotic-resistant isolates. The Pearson correlation (Table 2) demonstrated that the presence of the class 1 integrase gene was closely correlated with sulfonamides, streptomycin, and levofloxacin

resistance detected in the 15 multiple antibiotic-resistant *E. coli* isolates ($p < 0.01$). Significant correlations existed between the class 1 integrase gene and the tetracycline resistance ($p < 0.05$).

Table 2. Pearson correlation analysis of Class 1 integron and antibiotic resistance phenotype.

Gene	Tetracyclines	Sulfonamides		Aminoglycosides		β-Lactams	Quinolones		Phenicols
	TCY	TMP	SXT	STR	GEN	CEP	CIP	LVX	CHL
Class 1 integrase	0.921 *	1.000 **	1.000 **	0.974 **	0.860	0.040	0.585	0.967 **	0.262

Note: ** $p < 0.01$; * $p < 0.05$.

Only three of those isolates harboring the class 1 integrase gene could be amplified for the variable regions (1.5 to 1.9 kbp). The variable regions of these three isolates were sequenced to compare the content of class 1 integrons with associated resistance profiles. Sequencing of the variable region showed three different gene cassette arrangements: *dfrA12-orfF-aadA2* in the genome of one strain (A16, B14), *dhfr12-orfF-aadA2* in the genomic DNA of one strain (A16, B14), and *aadA2-linF* in the genomic DNA of one strain (B27). An aminoglycoside-adenyltransferase gene (*aadA*), conferring resistance to streptomycin/spectinomycin, was detected in the variable regions of all the three isolates harboring the class 1 integrase gene. The variable regions of the class 1 integron sequence primarily carried the gene *dfr* or *dhfr*, which both encode dihydrofolate reductases, conferring resistance to trimethoprim (Table 3). Sequencing data revealed that two isolates harbored the gene of *orf*F, encoding an unknown function.

Table 3. Characteristics of class 1 integrons identified in *E. coli* from seawater.

E. coli Strain	Pattern	Size of Amplicon(bp)	Gene Cassette Array
A16	TCY-TMP-SXT-GEN-STR-CIP-CHL-LVX	1800	*dfrA12-orfF-aadA2*
B14	TCY-TMP-SXT	1500	*dhfr12-orfF-aadA2*
B27	TCY-TMP-SXT-GEN-STR-CIP-CHL-LVX	1900	*aadA2-linF*

4. Discussion

Our study area is mainly covered with large gravel. Meanwhile, the small beach gravel has more surface area than the large beach gravel for bacterial attachment. This may explain the lower frequency (10.4%) of isolation from the beach sand than that observed in other bathing beach sand. A higher ARI value is detected in the seawater sites compared to the sand sites, indicating the higher exposure to antibiotics in the seawater as compared to the sand in Fujiazhuang bathing beach. Overall, a similar detection rate (38%) of resistant *E. coli* was observed in both the beach seawater and sand environment in our study. A high level (more than 50%) of drug-resistant *E. coli* was observed in the wet sand, dry sand, and seawater of the bathing beach [28]. The discrepancy in the resistance level of *E. coli* may be the result of the environmental heterogeneity among bathing places. Hierarchical cluster techniques have been reported to compare antibiogram profiles' similarities and the antibiotic disturbance of pathogens [27,29]. Cluster analysis of antibiogram profiles revealed the difference of resistance phenotypes between the seawater and beach sand. All the isolates which originated from the sand were grouped into one cluster and isolates from the sewage outlet have been disseminated into 3 clusters. Only the isolates from the sewage outlet were disseminated into each cluster, suggesting that the resistant strains may spread into the bathing beach through sewage discharge.

The major resistances of the *E. coli* isolates from the bathing beach were to tetracycline and sulfonamide, and resistance to tetracyclines was common among the seawater-isolated *E. coli*. Tetracyclines and sulfonamides were found to be the predominant antibiotics in the coastal water environment and coastal sediment environment of Dalian, respectively [30]. As broad-spectrum antibiotics, these two antibiotics, have been extensively used in China for clinical treatment, animal husbandry, and aquaculture. In China, the total

antibiotic usage for 2013 was approximately 162,000 tons, and the usage of tetracyclines and sulfonamide accounted for approximately 7% and 5%, respectively [31]. The high prevalence of *E. coli* isolates resistant to tetracycline in our study was also found in other aquatic systems [32]. The antibiotic resistance of coliform bacteria to sulfamethoxazole and trimethoprim in the Yitong River was approximately 9–40%, which was similar to the resistance of *E. coli* isolates to sulfonamides found in our study [33]. Various studies indicate that solar radiation can inhibit some antibiotic-resistant *E. coli*, and therefore affect their dispersion [34,35]. The risk of the development of resistance to sulfamethoxazole in surface water was found to be higher compared to ciprofloxacin, which may explain the high prevalence of SXT-resistant *E. coli* found in our study [35]. Overall, the high prevalence of resistance to tetracyclines and sulfonamides found in site 1 indicates that outlets may serve as large reservoirs of antibiotic-resistant bacteria, and they may play an important role in transferring antibiotic resistance. Significant correlations have been found among the resistance rates of quinolones and gentamicin [36]. Similarly, we also found significant correlations between the resistance to aminoglycosides (gentamicin, streptomycin) and quinolones (levofloxacin) or Sulfonamides (sulfamethoxazole-trimethoprim) which may be due to the cross-selection or co-selection for resistance among different antibiotics [37,38].

More than 40 classes of tetracycline-resistant (*tet*) genes with three main resistance mechanisms (efflux pump proteins, ribosomal protection proteins (RPPs), and inactivating enzymes) have been found in aquatic and soil environments [39]. *tet*A and *tet*B, which code for efflux pump proteins, were the prevalent tet genes in this study. This is also in agreement with previous studies on tetracycline-resistant *E. coli* isolates from other regions of China [32,40,41]. All the tet-resistant isolates from the surface water in Taihu Lake harbored at least two *tet* genes, and *tet*(A + E), *tet*(A + B), and *tet*(A + D) were detected more frequently than other combinations [42]. In our study, the combination *tet*(A + B) was detected in three isolates from bathing seawater.

Sulfonamides have been widely used as efficient and inexpensive antibacterial drugs for the treatment of both Gram-positive and Gram-negative pathogens. Sulfamethoxazole-trimethoprim, as a trimethoprim-sulfonamide combination, has been detected in surface seawater and freshwater [43–46]. It was found that *sul*1 was more abundant than other ARGs in all refuse samples ($p < 0.05$). The high prevalence of the *sul*1 gene detected in our study is consistent with the results of previous studies [33].

In this study, a high multiple-antibiotic resistance level occurred in the seawater of Fujiazhuang bathing beach (58%). Site 1, where 10 of the 15 MAR isolates were recovered, was near the sewage outlet to the sea. The number of multiple-antibiotic-resistant strains is in decline from the outlet position to the farther distance. This may be due to the large number of antibiotic-resistant bacteria induced by the residues of antibiotics in the sewage outlet, and the number of resistant bacteria gradually decreased with the flow and erosion of the ocean. Therefore, the discharge of treated wastewater may broaden the dissemination of multiple-antibiotic-resistant *E. coli* in Fujiazhuang bathing beach. The multi-antibiotic resistance of the *E. coli* isolates involving more than 8 types of resistance cannot be explained from only one resistance mechanism. The *E. coli* isolates from the beach showed only single resistance. The isolates from the wet sand are resistant to only ciprofloxacin, while the isolates from the dry sand are resistant to only trimethoprim. The frequency and bacterial antibiotic resistance in marine recreational waters and sands varied from beach to beach, which may be related to the degree of local beach contamination, swimming population density, and rainfall [28].

Chromosomal integrons have been identified as one of the crucial factors for the development of multiple-antibiotic resistance in enterobacteriaceae [47]. In this study, the class 1 integron was detected in the genome DNA of 15 multiple-antibiotic-resistant *E. coli*, and there was no class 2 integron detected. The class 1 integron is reported as the most ubiquitous class of integrons among water-borne bacteria, such as *E. coli*, *Salmonella*, *Shigella*, *Vibrio*, *Campylobacter*, and opportunistic pathogens [48]. Of the 6 integrons that contained inserted gene cassettes, the gene *aadA* (*aadA2*, *aadA5*), that confers resistance to

streptomycin/spectinomycin, was found to be the most prevalent (100%) in our study, as previously described in other studies [49–51]. The sequence results also revealed that all the isolates carrying the gene cassette *dfr* or *dhfr*, both conferring resistance to trimethoprim, recovered the corresponding resistance phenotype. This indicates that the gene cassettes carried by the class 1 integron play an important role in mediating the resistance of *E. coli* to sulfonamide antibiotics. However, several resistant gene cassettes detected in the *E. coli* isolates cannot fully explain the extent of their resistance profiles. For example, the tetracycline resistance phenotype frequently found in *E. coli* isolates carrying an integrin, such as resistance, was not identified, and the corresponding gene among the variable regions was not sequenced.

Our findings also indicate that the removal efficiency of antibiotic-resistant bacteria and ARGs may be limited by traditional WWTPs. To reduce the spread of antibiotic resistance in WWTP effluents, it is necessary to improve the efficiency of WWTPs to eliminate antibiotic-resistant bacteria and ARGs. Furthermore, the regular and continuous antibiotic resistance surveillance in recreational waters should be established to assess the risk of antibiotic resistance to human health.

5. Conclusions

The ARI value based on the antibiotic resistance results indicated a higher exposure to antibiotics in the seawater as compared to the beach sand in Fujiazhuang bathing beach. Three clusters observed in hetero-site *E. coli* isolates are reflective of the antibiogram profile difference between the seawater and sand. Only the isolates from the sewage outlet were disseminated into each cluster, suggesting that the resistant strains may spread into the bathing beach through sewage discharge. There was a significant correlation between all phenotypic (tetracyclines, sulfonamides, and β-lactams) and relative genotypic resistance profiles in all *E. coli* isolates, except for the quinolone resistance genotype detected in this study. The multiple antibiotic resistance of *E. coli* is prevalent in seawater, whereas multiple-antibiotic resistance was not detected in the resistant *E. coli* identified in the beach sand. The presence of a class 1 integron was significantly associated with the resistance of *E. coli* to sulfonamides, streptomycin, and levofloxacin. The multiple antibiotic resistance is prevalent, and the anthropogenic sources may impact the dissemination of resistant strains in an urban coastal beach. Therefore, to effectively control the spread of antimicrobial resistant bacteria in the environment, it is suggested to improve the removal efficiency of antimicrobial resistant bacteria in the sewage treatment plant and reduce their discharge into the environment.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w14101639/s1, Table S1: Sequences of PCR primers; Table S2: Antibiogram of multidrug-resistant *E. coli* in seawater; Table S3: Correlation analysis between antibiotic resistance phenotypes of *E. coli* isolates from bathing beach; Table S4: Prevalence of resistance genes detected in the *E. coli* strains from seawater and beach sand, and Fisher's exact tests correlation analysis of phenotypic resistance profile and relative genotypic resistance profile.

Author Contributions: J.F. and J.S. designed the study; J.S., H.M. and S.Z. set up and conducted the sampling; J.S. and S.Z. performed the experiments of bacterial isolation, DNA extraction, the antibiotic susceptibility assay, and the detection of antibiotic resistance genes; J.S., X.Z. and Q.C. analyzed the data; J.S. and J.F. wrote the manuscript; and G.G., Y.F., D.G., Y.J. and T.S. revised it. All authors have read and agreed to the published version of the manuscript.

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References

1. Marti, E.; Variatza, E.; Balcazar, J.L. The role of aquatic ecosystems as reservoirs of antibiotic resistance. *Trends Microbiol.* **2014**, *22*, 36–41. [CrossRef] [PubMed]
2. The Lancet Infectious Diseases. Time for global political action on antimicrobial resistance. *Lancet Infect. Dis.* **2016**, *16*, 1085. [CrossRef]
3. O'neill, J.I.M. Antimicrobial resistance: Tackling a crisis for the health and wealth of nations. *Rev. Antimicrob. Resist.* **2014**, *20*, 1–16.
4. Xiao, Y.; Li, L. China's national plan to combat antimicrobial resistance. *Lancet Infect. Dis.* **2016**, *16*, 1216–1218. [CrossRef]
5. Satoru, S.; Amy, P.; Marko, V.; Zhang, T. Editorial: Antibiotic resistance in aquatic systems. *Front. Microbiol.* **2017**, *8*, 14.
6. Hatosy, S.M.; Martiny, A.C. The ocean as a global reservoir of antibiotic resistance genes. *Appl. Environ. Microb.* **2015**, *81*, 7593–7599. [CrossRef]
7. Pérez, D.M.G.; Pérez, J.I.; Nieto, M.G. Carbamazepine behaviour and effects in an urban wastewater MBR working with high sludge and hydraulic retention time. *J. Environ. Sci. Health Part A* **2016**, *51*, 855–860. [CrossRef]
8. González-Pérez, D.; Pérez, J.; Gómez, M. Behaviour of the main nonsteroidal anti-inflammatory drugs in a membrane bioreactor treating urban wastewater at high hydraulic-and sludge-retention time. *J. Hazard. Mater.* **2017**, *336*, 128–138. [CrossRef]
9. Cornejo, J.; González-Pérez, D.M.; Pérez, J.I.; Gómez, M.A. Ibuprofen removal by a microfiltration membrane bioreactor during the startup phase. *J. Environ. Sci. Health Part A* **2019**, *55*, 374–384. [CrossRef]
10. Mançano, S.M.C.N.; Campana, E.H.; Felix, T.P.; Barrueto, L.R.L.; Pereira, P.S.; Picão, R.C. Frequency and diversity of *Stenotrophomonas* spp. carrying bla_{KPC} in recreational coastal waters. *Water Res.* **2020**, *185*, 116210. [CrossRef]
11. Furlan, J.P.R.; Ramos, M.S.; dos Santos, L.D.R.; Gallo, I.F.L.; Lopes, R.; Stehling, E.G. Appearance of mcr-9, blaKPC, cfr and other clinically relevant antimicrobial resistance genes in recreation waters and sands from urban beaches, Brazil. *Mar. Pollut. Bull.* **2021**, *167*, 112334. [CrossRef] [PubMed]
12. Hu, F.; Guo, Y.; Zhu, D.; Wang, F.; Jiang, X.; Fu, Y.; Xiaolong, Z. CHINET surveillance of bacterial resistance in China: 2018 report. *Chin. J. Infect. Chemother.* **2020**, *20*, 1–10.
13. Alm, E.W.; Zimbler, D.; Callahan, E.; Plomaritis, E. Patterns and persistence of antibiotic resistance in faecal indicator bacteria from freshwater recreational beaches. *J. Appl. Microbiol.* **2014**, *117*, 273–285. [CrossRef]
14. Alves, M.; Pereira, A.; Araújo, S.M.; Castro, B.; Correia, A.; Henriques, I. Seawater is a reservoir of multi-resistant Escherichia coli, including strains hosting plasmid-mediated quinolones resistance and extended-spectrum beta-lactamases genes. *Front. Microbiol.* **2014**, *5*, 426. [CrossRef]
15. Pan, M.; Chu, L. Occurrence of antibiotics and antibiotic resistance genes in soils from wastewater irrigation areas in the Pearl River Delta region, southern China. *Sci. Total Environ.* **2018**, *624*, 145–152. [CrossRef]
16. Kaushik, M.; Kumar, S.; Kapoor, R.K.; Gulati, P. Integrons and antibiotic resistance genes in water-borne pathogens: Threat detection and risk assessment. *J. Med. Microbiol.* **2019**, *68*, 679–692. [CrossRef]
17. Leonard, A.F.; Zhang, L.; Balfour, A.J.; Garside, R.; Hawkey, P.M.; Murray, A.K.; Ukoumunne, O.C.; Gaze, W.H. Exposure to and colonisation by antibiotic-resistant E. coli in UK coastal water users: Environmental surveillance, exposure assessment, and epidemiological study (Beach Bum Survey). *Environ. Int.* **2018**, *114*, 326–333. [CrossRef] [PubMed]
18. Turgeon, P.; Michel, P.; Levallois, P.; Chevalier, P.; Daignault, D.; Crago, B.; Irwin, R.; McEwen, S.A.; Neumann, N.F.; Louie, M. Antimicrobial-resistant *Escherichia coli* in public beach waters in Quebec. *Infect. Dis. Med. Microbiol.* **2012**, *23*, e20–e25. [CrossRef]
19. Kotlarska, E.; Łuczkiewicz, A.; Pisowacka, M.; Burzyński, A. Antibiotic resistance and prevalence of class 1 and 2 integrons in Escherichia coli isolated from two wastewater treatment plants, and their receiving waters (Gulf of Gdansk, Baltic Sea, Poland). *Environ. Sci. Pollut. Res.* **2014**, *22*, 2018–2030. [CrossRef] [PubMed]
20. *EPA-821-R-14-010*; Method 1603: *Escherichia coli* (*E. coli*) in Water by Membrane Filtration Using Modified Membrane-Thermotolerant *Escherichia coli* Agar (Modified mTEC). U.S. Environmental Protection Agency: Washington, DC, USA, 2014.
21. Merchant, I.A.; Packer, R.A. Veterinary bacteriology and virology. *Q. Rev. Biol.* **1950**, *4*, 211–305.
22. Barrow, G.I.; Feltham, R.K. *Cowan and Steel's Manual for the Identification of Medical Bacteria*, 3rd ed.; Cambridge University Press: Cambridge, UK, 1993.
23. *CLSI Document M100-S25*; Performance Standards for Antimicrobial Susceptibility Testing. Twenty-Fifth Informational Supplement; Clinical and Laboratory Standard Institute: Malvern, PA, USA, 2015.
24. Heinrich; Manjusha, S.; Sarita, G.B.; Elyas, K.K.; Chandrasekaran, M. Multiple Antibiotic Resistances of Vibrio Isolates from Coastal and Brackish Water Areas. *Am. J. Biochem. Biotechnol.* **2005**, *1*, 193–198. [CrossRef]
25. Mohanta, T.; Goel, S. Prevalence of antibiotic-resistant bacteria in three different aquatic environments over three seasons. *Environ. Monit. Assess.* **2014**, *186*, 5089–5100. [CrossRef] [PubMed]
26. Gu, Z.; Eils, R.; Schlesner, M. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics* **2016**, *32*, 2847–2849. [CrossRef]
27. Ekundayo, T.C.; Okoh, A.I. Antimicrobial resistance in freshwater Plesiomonas shigelloides isolates: Implications for environmental pollution and risk assessment. *Environ. Pollut.* **2020**, *257*, 493. [CrossRef] [PubMed]

28. Andrade, V.D.C.; Zampieri, B.D.B.; Ballesteros, E.R.; Pinto, A.B.; De Oliveira, A.J.F.C. Densities and antimicrobial resistance of Escherichia coli isolated from marine waters and beach sands. *Environ. Monit. Assess.* **2015**, *187*, 342. [CrossRef]
29. McCusker, M.P.; Ferreira, D.A.; Cooney, D.; Alves, B.M.; Fanning, S.; Pagès, J.-M.; Martins, M.; Davin-Regli, A. Modulation of antimicrobial resistance in clinical isolates of Enterobacter aerogenes: A strategy combining antibiotics and chemosensitisers. *J. Glob. Antimicrob. Resist.* **2018**, *16*, 187–198. [CrossRef]
30. Na, G.; Fang, X.; Cai, Y.; Ge, L.; Zong, H.; Yuan, X.; Yao, Z.; Zhang, Z. Occurrence, distribution, and bioaccumulation of antibiotics in coastal environment of Dalian, China. *Mar. Pollut. Bull.* **2013**, *69*, 233–237. [CrossRef]
31. Zhang, Q.; Ying, G.; Pan, C.; Liu, Y.; Zhao, J. Comprehensive evaluation of antibiotics emission and fate in the river basins of China: Source analysis, multimedia modeling, and linkage to bacterial resistance. *Environ. Sci. Technol.* **2015**, *49*, 6772–6782. [CrossRef]
32. Hu, J.; Shi, J.; Chang, H.; LI, D.; Yang, M.; Kamagata, Y. Phenotyping and genotyping of antibiotic-resistant *Escherichia coli* isolated from a natural river rasin. *Environ. Sci. Technol.* **2008**, *42*, 3415–3420. [CrossRef]
33. Yu, Y.; Wu, G.; Wang, C.; Lu, N.; Yuan, X.; Zhu, X. Pollution characteristics of antibiotics and antibiotic resistance of coliform bacteria in the Yitong River, China. *Environ. Monit. Assess.* **2019**, *191*, 516. [CrossRef]
34. Rizzo, L.; Fiorentino, A.; Anselmo, A. Effect of solar radiation on multidrug resistant *E. coli* strains and antibiotic mixture photodegradation in wastewater polluted stream. *Sci. Total Environ.* **2012**, *427*, 263–268. [CrossRef] [PubMed]
35. Américo-Pinheiro, J.H.P.; Bellatto, L.C.; Mansano, C.F.M.; Vilar, D.D.S.; Ferreira, L.F.R.; Torres, N.H.; Bilal, M.; Iqbal, H.M.N. Monitoring microbial contamination of antibiotic resistant *Escherichia coli* isolated from the surface water of urban park in southeastern Brazil. *Environ. Nanotechnol. Monit. Manag.* **2021**, *15*, 100438. [CrossRef]
36. Chen, Z.; Yu, D.; He, S.; Ye, H.; Zhang, L.; Wenhui, Z.; Zhang, W.; Shuchang, C.; Chen, S. Prevalence of Antibiotic-Resistant *Escherichia coli* in Drinking Water Sources in Hangzhou City. *Front. Microbiol.* **2017**, *8*, 1133. [CrossRef]
37. Dang, H.; Song, L.; Chen, M.; Chang, Y. Concurrence of cat and tet Genes in Multiple Antibiotic-Resistant Bacteria Isolated from a Sea Cucumber and Sea Urchin Mariculture Farm in China. *Microb. Ecol.* **2006**, *52*, 634–643. [CrossRef] [PubMed]
38. Zhang, W.R.; Na, G.S.; Lu, Z.H.; Gao, H.; Li, R.J.; Wu, X.; Zu, G.R.; Yao, Z. Distribution of sulfonamides and sulfonamide-resistant *Escherichia coli* in the coastal marine environment of northern yellow sea, China. *Chin. J. Appl. Environ. Biol.* **2014**, *20*, 401–406.
39. Wu, N.; Qiao, M.; Zhang, B.; Cheng, W.-D.; Zhu, Y.-G. Abundance and Diversity of Tetracycline Resistance Genes in Soils Adjacent to Representative Swine Feedlots in China. *Environ. Sci. Technol.* **2010**, *44*, 6933–6939. [CrossRef]
40. Wang, Y. Study on Drug-Resistance and Resistance Gene of Integron Mediated in *Escherichia coli* from Seawater. Master's Thesis, Dalian Ocean University, Dalian, China, 2015.
41. Wang, C.; Gu, X.; Zhang, S.; Wang, P.; Guo, C.; Gu, J.; Hou, J. Characterization of Antibiotic-Resistance Genes in Antibiotic Resistance *Escherichia coli* Isolates from a Lake. *Arch. Environ. Contam. Toxicol.* **2013**, *65*, 635–641. [CrossRef]
42. Zhang, S.H.; Lv, X.; Han, B.; Gu, X.C.; Wang, P.F.; Wang, C.; He, Z.L. Prevalence of antibiotic resistance genes in antibiotic-resistant *Escherichia coli* isolates in surface water of Taihu lake basin, China. *Environ. Sci. Pollut.* **2015**, *22*, 11412–11421. [CrossRef]
43. Standley, L.J.; Rudel, R.A.; Swartz, C.H.; Attfield, K.R.; Christian, J.; Erickson, M.; Brody, J.G. Wastewater-contaminated groundwater as a source of endogenous hormones and pharmaceuticals to surface water ecosystems. *Environ. Toxicol. Chem.* **2008**, *27*, 2457–2468. [CrossRef]
44. Yang, Y.-Y.; Toor, G.S.; Williams, C.F. Pharmaceuticals and organochlorine pesticides in sediments of an urban river in Florida, USA. *J. Soils Sediments* **2015**, *15*, 993–1004. [CrossRef]
45. Kumar, A.; Xagoraraki, I. Pharmaceuticals, personal care products and endocrine-disrupting chemicals in U.S. surface and finished drinking waters: A proposed ranking system. *Sci. Total Environ.* **2010**, *408*, 5972–5989. [CrossRef]
46. Kolpin, D.W.; Furlong, E.; Meyer, M.; Thurman, E.M.; Zaugg, S.D.; Barber, L.B.; Buxton, H.T. Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in U.S. Streams, 1999−2000: A National Reconnaissance. *Environ. Sci. Technol.* **2002**, *36*, 1202–1211. [CrossRef] [PubMed]
47. Emilie, L.; Barbara, P.; Thierry, B.; David, S.; Fabienne, P. Occurrence of antibiotic resistance and class 1, 2 and 3 integrons in *Escherichia coli* isolated from a densely populated estuary (seine, france). *FEMS Microbiol. Ecol.* **2009**, *1*, 118–130.
48. Rosser, S.J.; Hilary-Kay, Y. Identification and characterization of class 1 integrons in bacteria from an aquatic, environment. *Antimicrob. chemother.* **1999**, *44*, 11–18. [CrossRef] [PubMed]
49. Tennstedt, T.; Szczepanowski, R.; Braun, S.; Pühler, A.; Schlüter, A. Occurrence of integron-associated resistance gene cassettes located on antibiotic resistance plasmids isolated from a wastewater treatment plant. *FEMS Microbiol. Ecol.* **2003**, *45*, 239–252. [CrossRef]
50. Skurnik, D.; Le Menac'h, A.; Zurakowski, D.; Mazel, D.; Courvalin, P.; Denamur, E.; Andremont, A.; Ruimy, R. Integron-associated antibiotic resistance and phylogenetic grouping of *Escherichia coli* isolates from healthy subjects free of recent antibiotic exposure. *Antimicrob. Agents Chemother.* **2005**, *49*, 3062–3065. [CrossRef]
51. Silva, M.; Vaz-Moreira, I.; Gonzalez-Pajuelo, M.; Nunes, O.C.; Manaia, C.M. Antimicrobial resistance patterns in Enterobacteriaceae isolated from an urban waste water treatment plant. *FEMS Microbiol. Ecol.* **2007**, *60*, 166–176. [CrossRef]