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Antibiotic resistance profiles of *Escherichia coli* isolated from the floating islands and water of Çat Dam Lake, Adiyaman, Turkey

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Antibiotics, metabolites of the antibiotics, and resistant bacteria reach the aquatic environment through treated and untreated sewage, hospital waste, aquaculture discharges and agricultural irrigation. Therefore, aquatic environments have a significant role in spreading the antibiotic-resistance genes. The current study demonstrates the prevalence of antibiotic-resistant bacteria in surface water and floating islands of Çat Dam Lake, Adiyaman, Turkey. A total of 79 *E. coli* colonies were isolated from Çat Dam Lake water samples and the floating islands, of which 36 were from the first period (August 2021), 28 were from the second period (November 2021), and 15 were from the third period (May 2022), which were also confirmed as *E. coli* by polymerase chain reaction (PCR). The confirmed isolates were tested for susceptibility using the EUCAST protocol. The results showed that the prevalence of resistance to erythromycin (E), ceftaroline (CPT) and cefazolin (CZ) was significantly higher than other tested antibiotics. In total, 96.2% of the isolated bacteria from all three periods were resistant to E, 77.21% to CPT and 48.1% to CZ, 12.65% to tetracycline (TE), 8.86% to cefuroxime (CXM), 6.32% to chloramphenicol (C) and cefotaxime (CTX), 2.53% to cefepime (FEP) and 1.26% to imipenem (IPM) and gentamicin (CN). Thirteen (16.5%) isolates were found with a high multiple antibiotic resistance (MAR) index. The observed MAR index could be due to the contamination of water sources with antibiotics used in the surrounding areas. It throws a potential risk to the local population from antimicrobial-resistant infections that can lead to serious side effects such as organ failures.

Keywords: Antibiotic-resistance genes, Aquatic pollution, Artificial floating ecosystem, Bacterial identification, Multiple antibiotic resistance (MAR), Soil

Exposure of aquatic ecosystems to anthropogenic industrial, agricultural and sewage wastes pose a potential hazard in the surrounding areas as a carrier of pathogenic microorganisms[1,2]. Bacteria such as *Escherichia coli*, *Salmonella* spp., *Campylobacter* spp., *Staphylococcus* spp., *Clostridium* spp. and *Pseudomonas* spp. are the main pathogens that cause water pollution and related diseases[3,4]. Overuse of antibiotics against bacterial infections has caused potentially direct toxic effects on aquatic organisms, and thereby the spread of resistance among bacteria[5-7]. In general, antibiotics are poorly absorbed in the target organisms and released with feces or urine as metabolites[8]. Antibiotic residues mixed with aquatic environments contribute to the spread of multi-antibiotic resistance in bacteria[9,10]. Antibiotic resistance that develops in pathogenic bacteria with horizontal gene transfer is a huge concern for human health[11]. Lykov & Volodkin[12] have cautioned that the aquatic environments are potential reservoirs of antibiotic-resistant microorganisms capable of passing resistance genes to other members of the bacterial community.

In microbial ecology, especially sewage-related pollution indicates the presence of coliform bacteria and microbial contamination of water. Among coliforms, *Escherichia coli* is an important indicator of fecal contamination in aquatic ecosystems, as it is commonly found in the large intestines of humans and other warm-blooded animals[13]. Antibiotic resistance, a potential source of pollution in *E. coli*, is a growing concern worldwide[14-17]. *E. coli* acts as the primary reservoir of antibiotic-resistance genes which can easily be transmitted among bacteria[18]. Furthermore, *E. coli* is an indicator microorganism used to assess the waterbodies' microbiological safety and quality[19,20]. Therefore, the presence of *E. coli* in the aquatic environment and the high prevalence of antibiotic resistance are important indicators in terms of revealing the degree of antibiotic contamination[7].

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Çat Dam Lake, in Adıyaman province of Turkey, is used for various purposes, such as the generation of electricity, aquaculture, irrigation of agricultural fields and potable water source. It is also an important lake in terms of tourism due to the floating islands (FI) on its surface. Floating islands are formed by the growth of materials from the organic matter-rich areas filled by the natural cover of swamps and lakes, by clinging to suspended aquatic plants[21].

Zhou *et al.*[22] tried to remove antibiotics and antibiotic-resistance genes (ARGs) from surface water by combining membrane technology with a novel river water purification technology named as the artificial floating ecosystem (AFES), developed by their group. This was done in response to the ARGs and significant contamination of antibiotics in surface water and the challenges with traditional treatments. The AFES was a novel kind of mobile ecosystem that could move the floating island when necessary and mingle plants and algae. Nevertheless, not all antibiotics and resistance genes were completely removed by plants and algae, and some of the debris still remained in the effluent. As a result, one of the promising approaches for solving these difficulties would be to combine an artificial floating ecosystem with membrane technology, which is a membrane ecosystem[22].

To the best of our knowledge, there has been no study conducted on microbial pollution and antibiotic resistance in the Çat Dam Lake. Therefore, the present study explores antibiotic resistance profiles and multiple antibiotic resistance rates in *E. coli* isolated from water of Çat Dam Lake and soil of the floating islands.

Material and Methods

Sample collection

Water and soil samples were collected in August 2021, November 2021, and May 2022 from 5 selected waters and soils each taken from the floating island representative sections on the Çat Dam Lake (Fig. 1). Water samples were collected from 10 to 15 cm below the water's surface using a sterile glass wide-mouthed bottle (500 mL). Soil samples were taken 2-5 cm below the surface. All samples were brought in an icebox to the laboratory and processed within 2-4 h[23]. The protocol of collection water and soil samples were carried out as described by Eaton[23] and Gupta *et al.*[24].

Bacteriological analyses

A standard membrane filtration technique was used to analyze water samples as described by APHA 2005[23]. Briefly, 250 mL of each water sample was filtered using a 47 mm membrane filter (Cellulose Nitrate filter, Sartorius, Germany) with a pore size of 0.45 µm using a vacuum filtration system. Then the filtrate was diluted using sterile physiological saline (0.9%) and inoculated to Harlequin *Escherichia coli*/Coliform agar (Neogen) and incubated at 44°C for 12-24 h.

The soil microorganisms were isolated by serial dilution technique on nutrient agar medium. One gram of soil from the sample was suspended in 10 mL of distilled water and vortexed for 15 min. Each suspension was diluted serially from 10^{-1} to 10^{-6}. To isolate the organism from the diluted sample, the spread plate technique was used. Pipette 0.1 mL onto nutrient agar plates, spread with a one-time use plastic L shape spreader, and incubated at 37°C for 24 h. The most prominent colonies were isolated and inoculated initially on Mueller Hinton agar then on Harlequin *E. coli*/Coliform agar (Neogen) and incubated at 44ºC for 12-24 h.

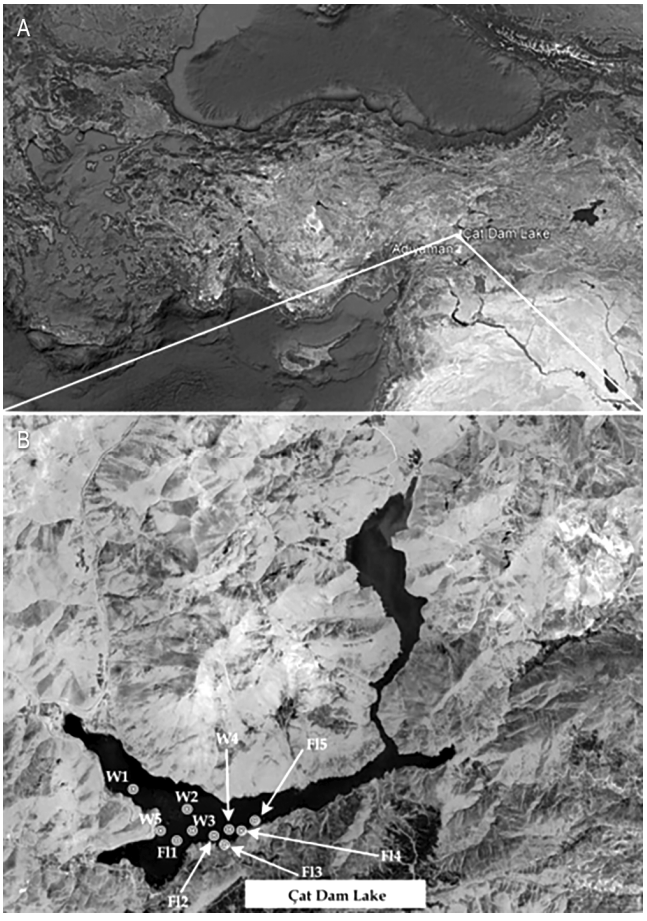

Fig. 1 — Location of sample collection sites (W: denotes for water samples; FI: denotes for floating islands)

At the end of the incubation period, blue-violet colonies were selected as *E. coli* suspects. Each one of the blue-violet colonies was grown on EMB (Eosin Methylene-blue Lactose Sucrose) (Merck) agar by streak inoculation and those that gave green bright zones were evaluated using the IMViC (indole, methyl red, Voges-Proskauer and citrate utilization) test. This test was applied to the isolates for the confirmation of *E. coli*, which were also confirmed by a molecular method. Stock cultures were also cultured on tryptic soy agar (TSA) (Merck) and tryptic soy broth (TSB) for further analyses. All isolates were kept both in TSB broth with 10% glycerol at –80°C in a deep freezer for future use and TSA at +4°C for immediate use.

Confirmation of *E. coli* strains using PCR

A total of 13 bacterial strains, which presented high resistance rates, were selected from different three periods. All isolates were incubated in tryptic soy broth for 18 h at 37°C. At the end of the incubation period, to expose the genomic DNA of these isolates a standard boiling method (at 95°C for 5 min) was applied as described by Chapman *et al*.[25]. After the heat treatment, bacterial cultures were centrifuged and supernatants were taken and used for confirmation. The heat-treated *E. coli* isolates were confirmed by polymerase chain reaction (PCR) by amplification of the universal stress protein (*uspA*) gene, which is species-specific for *E. coli*[26]. The primer sequences used were (sense) 5′-CCGATACGCTGCCAATCAGT-3′ and (antisense) 5′-ACGCAGACCGTAGGCCAGAT-3′, generating an 884-bp fragment. For a PCR reaction in a PCR tube, 2.5 µL of 10× PCR buffer, 0.5 µL of 50 ng/µL each primer, 2 µL of 3 mM $MgCl_2$, 1 µL of 5 u/µL *Taq* polymerase, 3 µL of 2.5 mM dNTPs, 4 µL of template DNA and 11.5 µL dH_2O. PCR procedure carried our as follows: initial denaturation at 94°C for 5 m; 30 cycles of secondary denaturation at 94°C for 30 s, annealing at 56°C for 30 s, elongation at 72°C for 30 s; and final elongation at 72°C for 5 m. Amplified PCR products (amplicons) were electrophoresed on 1.5% agarose (Thermo Fisher Scientific, USA) gel in 1 × Tris base-EDTA (TBE) buffer and afterward ethidium bromide was used to stain it. Then, the gel was visualized under the UV light as shown in Fig. 2. As presented in the figure any isolate that presented a product with an 884 bp amplicon was confirmed as *E. coli*.

Antibiotic resistance

Antibiotic resistance testing was performed using Mueller Hinton Agar (MHA) medium with the Kirby-Bauer disc diffusion test according to the Clinical Laboratory Standards Institute (CLSI) guidelines[27]. The turbidity of the 12-24 h bacterial cultures was set to the 0.5 McFarland standard reference range. After inoculating (50 µL) on Mueller Hinton agar with a sterile swab, the agar plates were incubated at 37°C for 16-18 h. At the end of the incubation period, the diameters of the formed inhibition zones were measured and the results were evaluated as susceptible, moderately resistant, or resistant, taking into account EUCAST[27] standard reference values for cefazolin and ceftaroline antibiotics and CLSI[28] standard reference values for other antibiotics. Intermediate resistant results are included in the resistant class. *Escherichia coli* strain ATCC 25922 was used as a control strain for antibiotic resistance testing. In our study, Bioanalyse brand cefazolin (CZ 30 µg), cefuroxime (CXM 30 µg), cefotaxime (CTX 30 µg), cefepime (FEP 30 µg), ceftaroline (CPT 5 µg), erythromycin (E 15 µg), Gentamicin (CN 10 µg), imipenem (IPM 10 µg), tetracycline (TE 30 µg) and chloramphenicol (C 30 µg) antibiotic discs were used.

Multi-antibiotic resistance (MAR) index value was obtained by dividing the number of antibiotics to which the bacteria were resistant by the total number of antibiotics exposed. A MAR index value higher than 0.2 was interpreted as an indication of high-risk source contamination where antibiotics are frequently used[29].

Statistical analyses

The normality of the data for Floating Island (FI) was investigated with the Kolmogorov-Smirnov test. Since the data were not normally distributed, Kruskal-Wallis and later Mann-Whitney U post-hoc test (or each data was evaluated separately with paired samples t-test. The same differences are obtained). Data are presented as the arithmetic mean ± standard error (N = 15-32). The data with different letters show a statistical difference (P <0.05).

The statistical separation between W data was analyzed with the paired samples t-test (or with the independent samples t-test, neither showing any

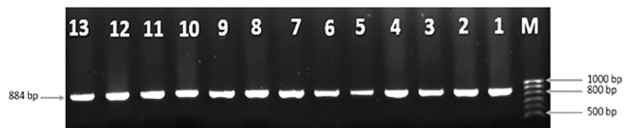

Fig. 2 — Species confirmation was done by the presence of the *uspA* gene highly specific for *E. coli* by a PCR assay as described previously (Chen & Griffiths, 1998). M: Marker, 1 – 13 *E. coli* with high MAR index

difference). Data are presented as arithmetic mean ± standard error (N = 3-4). No statistical difference was found between the data (P >0.05).

Results and Discussion

Through hospital waste, treated and untreated sewage, discharges from aquaculture, agricultural irrigation, and hospital waste, antibiotics, their metabolites, and resistant bacteria enter the aquatic environment. Therefore, aquatic environments may have a significant role in the spread of antibiotic resistance genes[30].

The current study demonstrated the significant prevalence of antibiotic-resistant bacteria in the surface water and floating islands of Çat Dam Lake. To the best of our knowledge, the presented data is the first data on antibiotic resistance against some commonly used antibiotics in this lake region. A total of 79 *E. coli* colonies were isolated from Çat Dam Lake water samples and floating islands. Of these samples, 6 were from water and 73 were from soil samples, 36 (32 from floating islands and 4 from water) from the 1st period, 28 (26 from floating islands and 2 from water) from the 2nd period, and 15 (all from floating islands) from the 3rd period. There are differences in precipitation between periods. Considering the seasonal differences, especially the summer season is very dry. Therefore, there are differences in the number of bacteria isolated, accordingly. Sampling could not be done due to snowfall and frost in winter. However, no academic study has been found on this issue in terms of different seasons. Moreover, no evaluation has been made in terms of anthropogenic pressure.

The obtained result for the antimicrobial susceptibility test is shown in Fig. 2. For the present study, the prevalence of resistance to E, CPT and CZ was significantly higher than other tested antibiotics.

In total, 96.2% of the bacteria isolated in three periods were resistant to E, 77. 21% to CPT and 48.1% to CZ, 12.65% to TE, 8.86% to CXM, 6.32% to C and CTX, 2.53% to FEP and 1.26% to IPM and CN (Fig. 3A).

In the first period, 100% of the *E. coli* was resistant to erythromycin and 55% to CPT. The resistance rates to other antibiotics are 11.1% to CZ, 8.33% to CTX, 5.55% to FEP and 2.77% to TE, CN and CXM, respectively. All bacteria were sensitive to IPM and C. In the 2nd period, all bacteria were resistant to E and CPT. The resistance rates against other antibiotics were 96.42% for CZ, 32.14% for TE, 17.85% for C and CTX, 7.14% for CTX and 3.57% for IPM. All bacteria were sensitive to FEP and CN. In the last period, 86.66% of bacteria were resistant to CPT, 80% to E, 46.66% to CZ and 6.66% to CXM. In this period, all bacteria were sensitive to C, CTX, FEP, CN, IPM and TE (Fig. 3B). In Turkey, empirical antibiotic use is frequently started against *E. coli* infections. The most commonly preferred antibiotics are quinolones, aminopenicillins, beta-lactam/beta-lactamase inhibitor combinations, trimethoprim-sulfamethoxazole (SXT), fosfomycin, nitrofurantoin, aminoglycosides and second and third generation oral cephalosporins[31]. However, increasing resistance to these antibiotics is reported[31]. Antibiotic resistance profiles were studied in samples taken from surface water systems in different regions of our country. Matyar *et al*.[32] determined that 286 Gram (−ve) bacteria isolated from Seyhan Dam Lake and Seyhan River had a high resistance rate against ampicillin (80.2%), streptomycin (71.6%) and cefazolin (60.4%). Kayış[32] stated that *E. coli* isolated from Atatürk Dam Lake were resistant to erythromycin (95%), ceftaroline (31%) and cefazolin (30%), and all bacteria were sensitive to imipenem and gentamicin. Mercimek Takci *et al*.[34] showed that all 21 *E. coli* isolated from the Seve Dam and Konak Pond were

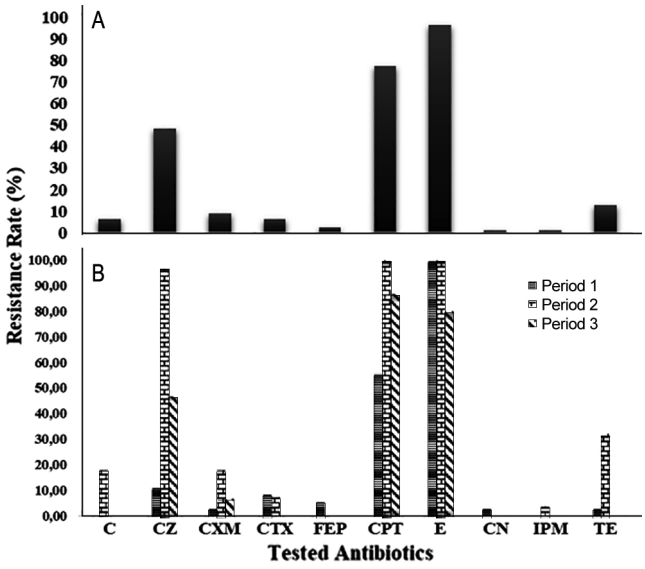

Fig. 3 — (A) Total resistance rate of isolates, collected on all three different times, to tested antibiotics; and (B) Resistance rate of isolates to tested antibiotics given for each collection period. [C: chloramphenicol; CZ: cefazolin; CXM: Cefuroxime; CTX: cefotaxime; FEP: cefepime; CPT: ceftaroline; E: erythromycin; CN: gentamicin; IPM: imipenem; and TE: Tetracycline]

resistant to erythromycin, clindamycin and metronidazole. In another study, high resistance was determined against streptomycin, ampicillin, tetracycline and chloramphenicol in a study conducted in Tau Lake[35].Giri *et al*.[36] determined that 20 *E. coli* isolates isolated from Nainital lake (Uttarakhand State, India) showed 100% resistance to penicillin G and 80% resistance to erythromycin, while all isolates (100%) were sensitive to gentamicin. Wambugu *et al*.[37] showed that high resistances for ampicillin (63.8%), cefoxitin (46.9%), amoxicillin/clavulanic acid (46.2%) and sulfamethoxazole (44%) in *E. coli* isolated from surface water samples.

The antibiotic resistance of bacteria isolated from aquatic ecosystems is closely related to the use of antibiotics in the terrestrial ecosystem within the aquatic ecosystem's impact area[14]. The high resistance (95%) against erythromycin in our study suggests that this antibiotic is used extensively in the treatment of infectious diseases in the region.

A high multi-antibiotic resistance rate in lakes fed by rivers indicates that domestic wastes, animal excrement, and various pesticides used in agriculture are mixed with the aquatic ecosystem through rivers. In our study, the MAR index of 58.33% of bacteria isolated in the first period is between 0.2 and 0.6 (Fig. 4A). In the second period, the MAR index of 96.42 % of bacteria is between 0.2 and 0.7 (Fig. 4B). In the last period, the MAR index of 80% of the bacteria was found to be between 0.2 and 0.3 (Fig. 4C). Various studies have shown the presence of high multi-antibiotic resistance in bacteria isolated from surface waters. A study in the Karasu River found that the MAR index values of isolated *E. coli* strains were between 0.4 and 0.7[38]. In a previous study, which was conducted by Kurekci *et al*.[39] in the Orontes River and urban wastewaters the presence of extended spectrum β-lactamase (ESBL)-producing *E. coli* was investigated. The authors found that 54 *E. coli* strains were resistant to cefotaxime in the river waters and nearby waste water treatment plant. This study indicated a widespread distribution of CTX-M-15 producing *E. coli* strains in the surface waters in part of Turkey, suggesting an aquatic reservoir for ESBL genes. In another study, which was carried out on the Enterobacteriaceae isolated from the Giresun (a province in Turkey) coast, the researchers discovered that the multiple antibiotic resistance (MAR) index values of 91% of all isolates were greater than 0.2. Matyar *et al*.[32] reported that the MAR index was between 0.2 and 0.81 for Gram (−ve) bacteria isolated from Seyhan Lake Dam and Seyhan River. According to Giri *et al*. [36] found that the MAR index of *E. coli* bacteria isolated from Lake Nainital (Uttarakhand State, India) was the highest with a value of 0.73 in 1 isolate, and MAR index values higher than 0.2 in 13 of 20 isolates. Odonkor *et al*.[40] found that 63% of the multidrug-resistant *E. coli* strains isolated from samples taken from six different water sources including dams, boreholes, stream springs, rivers, canals and hand-made wells showed multi-antibiotic resistance (MAR) index value as >0.2. Toroglu *et al*.[41] showed that 40% of the bacteria they isolated from the Aksu river were resistant to five or more antibiotics.

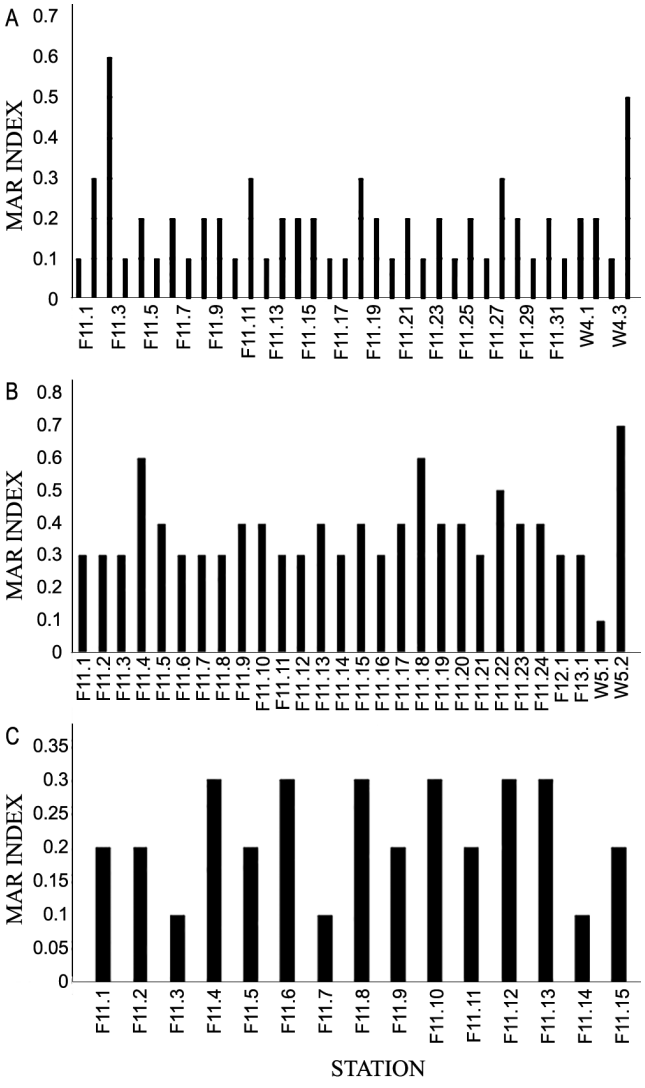

Fig. 4 — Multiple antibiotic resistance (MAR) index of collected samples from (A) 1st; (B) 2nd; and (C) 3rd period.

Table 1 —MAR index of *E. coli* isolated from floating islands (FI) and water (W) in different periods

Collection Place and Period	Multiple Antibiotic Resistance (MAR) Index (arithmetic mean ± standard error)
FI, 1st period	0.1813 ± 0.0182[a]
FI, 2nd period	0.3692 ± 0.0173[b]
FI, 3rd period	0.2200 ± 0.0147[a]
W, 1st period	0.2500 ± 0.0866[x]
W, 2nd period	0.4000 ± 0.1732[x]

A diverse mixture of antibiotics and other pollutants, their metabolites, and resistant bacteria reaches and enters the aquatic environment via treated and untreated sewage, hospital waste, aquaculture discharges, and agricultural runoff. As a result, aquatic compartments such as water and sediment may play an important role in Antibiotic resistance genes (ARG) transfer, ecology and evolution[30].

The MAR index of *E. coli* isolated from floating islands (FI) is significantly higher in the 2nd period than in the other periods. No significant change was observed in bacteria isolated from water samples depending on the periods (Table 1).

No previous studies were found on whether it received waste from anthropogenic source, pollution, treated or untreated sewage. However, since it is close to human settlements and activities such as fish farming, it is likely to be polluted by such pollution sources. Although there is no previous study on antibiotic resistance profiles in naturally formed floating islands, it has been stated that artificially created floating islands absorb resistance genes in the aquatic environment[21].The fact that the number of bacteria isolated from the samples taken from the floating islands in this study is higher than the samples taken from water in terms of both the number of bacteria and the high resistance rates. The current study results are in parallel with the study of Zhou *et al*.[22]. It showed that artificially created floating islands reduce water pollution by absorbing resistance genes in the water.

Conclusion

Aquatic environments are potential reservoirs of antibiotic-resistant microorganisms capable of passing resistance genes to other members of the bacterial community. The high MAR index found in this study relates to the possibility that the water sources were highly contaminated with antibiotics due to the widespread use of these chemicals in the areas surrounding the various water sources. It can be a potential risk to human and animal health and also results in financial loss. In addition, *E. coli* isolated from natural floating islands in aquatic ecosystems showed a higher antibiotic resistance rate than those in water, which supports the idea that floating island systems reduce water pollution by absorbing resistance genes in the water.

Conflict of Interest

Author declares no competing interests.

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