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Investigation of multiple resistance frequencies (antibiotic and heavy metal) of bacteria isolated from Gökçeada Island coastal marine sediment

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ABSTRACT

Marine sediments are important reservoirs for antibiotics and heavy metals. Bacteria play a key role in polluted sedimentary habitats. This study aimed to identify heavy metal and antibiotic resistance in marine sediment bacteria isolated from Gökçeada Island in Turkiye. The samples were collected seasonally from 10 different sampling stations in 2015. Ninety isolates determined by VITEK 2 were tested against seven antibiotics using the disk diffusion method. The minimum inhibitory concentration values were measured against four heavy metal salts. The antibiotic resistance frequency rates were ordered as sulphonamides compound (93.3%), cefotaxime (78.9), ampicillin (77.8%), oxacillin (67.8%), rifampicin (57.8%), imipenem (1.1%), and oxytetracycline (0%). The heavy metal resistance ratios against $ZnCl_2$, $CuSO_4$, $Pb(CH_3COO)_2$, and $HgCl_2$ were measured as 100%, 100%, 96.7%, and 73.3% respectively. The multiple heavy metal resistance index values were ranged from 0.75 (22.2%) to 1.0 (77.8%). The results show significant heavy metal and antibiotic contamination in the sediments of the Gökçeada Island. It is recommended that measures be taken against antibiotics and heavy metal pollution, as well as identifying and monitoring critical control points.

Keywords: Antibiotic resistance, Gökçeada island, Heavy metal resistance, Heterotrophic bacteria, Marine sediment

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Introduction

Marine sediments are the important habitats of the islands. Biogeochemical processes determine the sediments' nutrient cycles and organic matter degradation (Arndt et al., 2013). The sediments are highly dynamic environments with rich microbial diversity. Microorganisms may grow in such rich environments by using different energy sources. Although approximately 70% of the earth's surface is formed of marine sediments, the diversity and features of bacteria in the marine sediments have not been understood entirely (Jørgensen & Boetius, 2007; Hoshino et al., 2020). Coastal marine sediments in aquatic ecosystems are potential reservoirs of various antibiotics and heavy metals (Buccolieri et al., 2006; Matyar et al., 2008; Vignaroli et al., 2018). Those ecosystems act as a pool of resistance genes, and marine sediment bacteria within the sediments may effectively exchange the resistance globally (Yang et al., 2013).

Antibiotics are the main group of pharmaceuticals. They have been used for several reasons, both for human and veterinary health, but the environmental risk of the antibiotics remains unclear. Resistance of bacteria against antibiotics in aquatic environments is still poorly studied (Kümmerer, 2009). However, antibiotic pollution threatens marine sediments' bacterial diversity and ecosystem health (Näslund et al., 2008). Environmental bacteria may transfer antibiotic resistance to human pathogens, which may cause serious health problems. Antibiotic discharges must be monitored consistently to define potential risks (Larsson, 2014).

Heavy metals are toxic pollutants and may be considered the major contaminants of marine sediments. Heavy metal contamination in marine environments details the long-term negative effects on the ecosystem (Bryan & Langston, 1992; Gillan et al., 2005). Bacteria metabolisms are highly sensitive to heavy metal contamination. Bioaccumulation of heavy metals in marine environments becomes a crucial issue. Some bacteria can survive in highly contaminated environments and tolerate certain concentrations of heavy metals. Heavy metal-resistant bacteria could prove valuable for detoxifying harmful heavy metals (Iyer et al., 2005; Nithya et al., 2011). Bacteria may be useful as bioindicators of pollution factors in marine environments (Dell'Anno et al., 2003).

It is established that heavy metal exposure and antibiotic resistance in bacteria are connected. Heavy metal contamination can be a significant factor in selecting antibiotic resistance traits, with implications for both environmental and clinical settings. However, thoroughly exploring the relationship between heavy metals and antibiotics is necessary to grasp the mechanisms underlying antibiotic resistance (Baker-Austin et al., 2006).

Antibiotic and heavy metal pollution pose serious threats to human health. However, bacterial resistances influenced by anthropogenic pollution have not been studied extensively enough to understand their effects on aquatic ecosystems fully (Baquero et al., 2008; Marti et al., 2014; Sharifuzzaman et al., 2016).

Gökçeada Island, which has an area of 285.5 km^{2,} is the biggest in Turkiye, located between Samothrace Island, Lemnos Islands, and Gallipoli Peninsula (Balkıs et al., 2001). Previous studies have shown that Gökçeada Island has significant importance for marine life in terms of its rich biological diversity, and it has been suggested to set special regulations to protect against negative pollution effects (Keskin & Ünsal, 1998; Tarkan, 2000; Akmirza, 2013; Acarli et al., 2018; Altın & Ayyıldız, 2018; Gönülal & Güreşen, 2014; Güreşen et al., 2020).

Several studies have been conducted to assess the levels of heavy metal and antibiotic resistance among heterotrophic bacteria found in marine sediments in Turkiye (Altuğ ve Balkıs, 2009; Matyar et al., 2008; Kaçar et al., 2013; Kaçar & Koçyiğit, 2013; Tuncer & Bizsel, 2017; Altuğ et al., 2020). In previous studies, marine bacteria from both the seawater and sediment around Gökçeada Island were examined to investigate their metabolic characteristics and enzyme profiles (Altuğ et al., 2010; Cardak et al., 2013; Çiftçi-Türetken & Altuğ, 2016; Türetken, 2021). However, no research regarding antibiotic and heavy metal resistance in the vicinity of Gökçeada Island has been documented in the literature. The primary aim of this study was to analyse the prevalence of heavy metal and antibiotic resistance among marine heterotrophic bacteria isolated from coastal sediments to identify critical pollution sites around Gökçeada Island in Turkiye.

Materials and Methods

Sampling Area

The sediment samples were collected from 10 distinct sampling stations around the coastal regions of Gökçeada Island in Turkiye (see Figure 1). Stations have been chosen as recreational areas (St4-5-6-8-9), ports (St 2-3-7), bays (St1-5), and reference points (St 10) where there is no human activity. The study was conducted seasonally from April to November 2015. The collection of sediment samples was performed using an

Ekman Grab (Hydrobios), which was stored in sterile containers. Subsequently, the samples were refri-gerated and transported to the laboratory while maintaining a cold chain.

Identification of Bacterial Isolates

The sediment samples were aseptically weighed at one gram each. Serial dilutions were prepared using sterile phosphate-buffered distilled water. Marine Agar (Difco) served as the growth medium for heterotrophic bacteria. Petri dishes were then incubated at 22 ±0.1°C. Colonies that formed on the plates after 72 hours were monitored every 24 hours. Selected isolates were categorised based on gram reactions as GN (Gram-negative fermentative and non-fermentative rods), GP (Gram-positive cocci and non-spore-forming rod-shaped bacteria), and BCL (Gram-positive spore-forming rods). Colonies were identified using the VITEK 2 Compact 30 automatic micro-identification system (bioMerieux, France). Following identification, ninety bacterial isolates (comprising sixteen different species) were tested against various antibiotics and heavy metals (refer to Table 1).

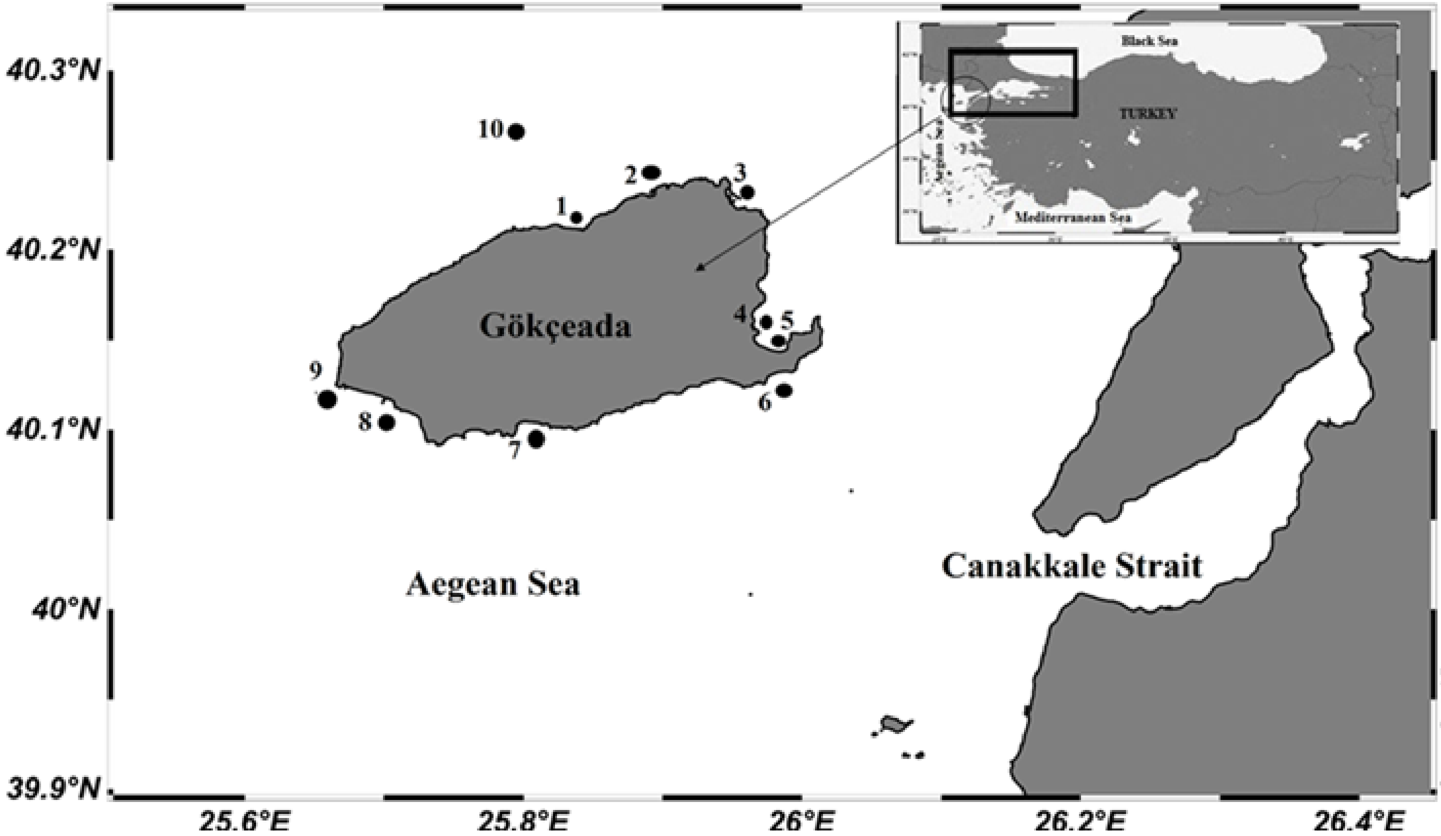

Figure 1. The sampling stations around the Gökçeada Island.

Table 1. The isolate codes and tested bacteria species.

Bacteria species	Isolation Frequency (%)	Station Code
Acinetobacter baumannii complex	2.2	St6 – St9
Acinetobacter calcoaceticus	3.3	St2 – St7 – St10
Acinetobacter haemolyticus	1.1	St5
Acinetobacter lwoffii	3.3	St3 – St6 – St8
Acinetobacter nosocomialis	2.2	St1 – St5
Acinetobacter pittii	2.2	St6 – St7
Aeromonas salmonicida	1.1	St5
Bacillus cereus/thuringiensis/mycoides	5.6	St3 – St4- St5 – St8- St9 – St10
Burkholderia mallei	3.3	St2 – St4 – St9
Enterococcus faecalis	1.1	St5
Kocuira kristinae	1.1	St5
Pasteurella canis	1.1	S4
Pseudomonas stutzeri	1.1	St4
Serratia marcescens	43.3	St1 – St2 – St3 – St4 – St5 – St6 – St7 – St8 – St9
Sphingomonas paucimobilis	15.6	St2 – St3 – St4 – St5 - St6 – St7 – St9
Stenotrophomonas maltophilia	4.4	St2 – St7 – St9 – St10

Antibiotic Resistance

As Bauer et al. (1966) described, the disk diffusion method used Mueller-Hinton agar to assess the antibiotic resistance of bacteria isolated from the sediment samples. Ninety different bacterial isolates were tested against seven distinct antibiotic discs, including Ampicillin 10 µg (AMP), cefotaxime 30 µg (CTX), imipenem 10 µg (IPM), rifampicin 2 µg (RD), oxacillin 5 µg (OX), oxytetracycline 30 µg (OT), and sulphonamides compound 300 µg (S3). Blank sterilised discs (Oxoid, UK) were also negative controls. After preparing pure cultures, the bacterial cell density was adjusted to 0.5 McFarland. Each isolate was spread onto Mueller-Hinton agar plates, and antibiotic discs were carefully placed. Incubation occurred for 24 hours (species-dependent) at 37 ±0.1°C. The diameters of the inhibition zones (in mm) were measured and interpreted by comparing them to the breakpoint diameters specified in The Clinical and Laboratory Standards Institute's (CLSI) tables. Tested isolates were categorised as resistant, intermediate, or susceptible. Quality control organisms recommended by CLSI were also tested to ensure the accuracy of the experiment (CLSI, 2018). The multiple antibiotic resistance (MAR) indexes were calculated based on the number of resistant isolates and the number of antibiotics tested, following the method outlined by Krumperman (1983).

Heavy Metal Resistance

The minimum inhibitory concentration (MIC) method assessed heavy metal resistance against four metal salts. Solutions of zinc sulfate ($ZnCl_2$), mercury chloride ($HgCl_2$), copper sulfate ($CuSO_4$), and lead (II) acetate ($Pb(CH_3COO)_2$) were prepared and sterilised using a 0.45 µm pore-sized filter (Sartorius). Fifty microliters of each heavy metal solution were inoculated into microplate wells using serial dilution with sterilised water. The initial and final molar concentrations ranged from 12 mM to 0.01 mM. The McFarland value for bacterial cell density was set at 0.5, and 50 µL of bacterial suspensions were added to each well. The control well consisted of bacterial solution and sterilised water. MIC values were determined as the lowest concentration of metal salt at which no visible bacterial growth occurred. *Escherichia coli* (ATCC 25922™) served as the reference strain. Isolates exhibiting higher resistance than the reference strain were classified as resistant strains (Matyar, 2012; Gillard et al., 2019). The multiple heavy metal resistance (MHMR) indexes were calculated based on the number of resistant isolates, similar to the MAR index (Krumperman, 1983).

Results and Discussion

Antibiotic Resistance

The antibiotic resistance frequencies of the bacterial isola-tes against 7 different antibiotic types were determined (Table 2). The maximum frequency percentage obtained from each group (R: resistant, S: susceptible, I: intermediate) is shown in bold in the table. The highest resistance frequency was detected at 93% against the sulphonamide compound (S3). There was no resistance against oxytetracycline among all isolates. The highest and the lowest intermediate frequencies were measured as 41% and %1 against rifampicin and imipenem, respectively. The highest susceptible frequency was recorded as 97% against imipenem. The lowest susceptible frequencies were calculated as 1% against cefotaxime, rifampicin, oxacillin, and sulphonamides compound.

Heavy Metal Resistance

The heavy metal resistance frequencies of the bacterial isolates against 4 different heavy metal salts were determined (Table 3). The minimum inhibitory concentrations ranged from 0.38 mM to 12 mM for $ZnCl_2$ and $CuSO_4$, from 0.05 mM to 12 mM for Pb $(CH_3COO)_2$, and 3 mM to 12 mM for $HgCl_2$. The most tolerated and toxic heavy metal salts were determined as zinc sulfate and mercury chloride, respectively. The heavy metal resistance ratios against $ZnCl_2$, $CuSO_4$, $Pb(CH_3COO)_2$, and $HgCl_2$ were measured as 100%, 100%, 96.7%, and 73.3% respectively.

Multiple Antibiotic Resistance and Multiple Heavy Metal Resistance

Figure 2 summarises bacterial isolates' multiple antibiotic resistance (MAR) and multiple heavy metal resistance (MHMR) indexes.

Table 2. The antibiotic resistance frequencies of the bacterial isolates.

Antibiotic derives	Number of isolates			Frequency (%)		
	R	S	I	R	S	I
Ampicillin (10 µg)	70	2	18	77.8	2.2	20.0
Cefotaxime (30 µg)	72	1	17	78.9	1.1	18.9
Imipenem (10 µg)	1	88	1	1.1	**97.8**	1.1
Rifampicin (2 µg)	52	1	37	57.8	1.11	**41.1**
Oxytetracycline (30 µg)	0	63	27	0.00	70	30.0
Oxacillin (5 µg)	61	1	28	67.8	1.1	31.1
Sulphonamides compound (300 µg)	84	1	5	**93.3**	1.1	5.6

R: resistant, S: susceptible, I: intermediate

Table 3. The heavy metal resistance frequencies of the bacterial isolates.

	Metal concentrations (mM)												
Heavy metal salts	**12**	**6**	**3**	**1.5**	**0.75**	**0.38**	**0.19**	**0.09**	**0.05**	**0.02**	**0.01**	**Resistant Isolates**	**%**
$ZnCl_2$	61	18	3	6	1	1	-	-	-	-	-	**90**	**100**
(Reference strain)										*			
$CuSO_4$	5	1	4	28	41	11	-	-	-	-	-	90	**100**
(Reference strain)										*			
Pb $(CH_3COO)_2$	14	9	1	41	14	4	2	2	3	-	-	87	**96.7**
(Reference strain)									*				
$HgCl_2$	5	6	3	-	-	-	-	-	11	41	20	66	**73.3**
(Reference strain)											*		

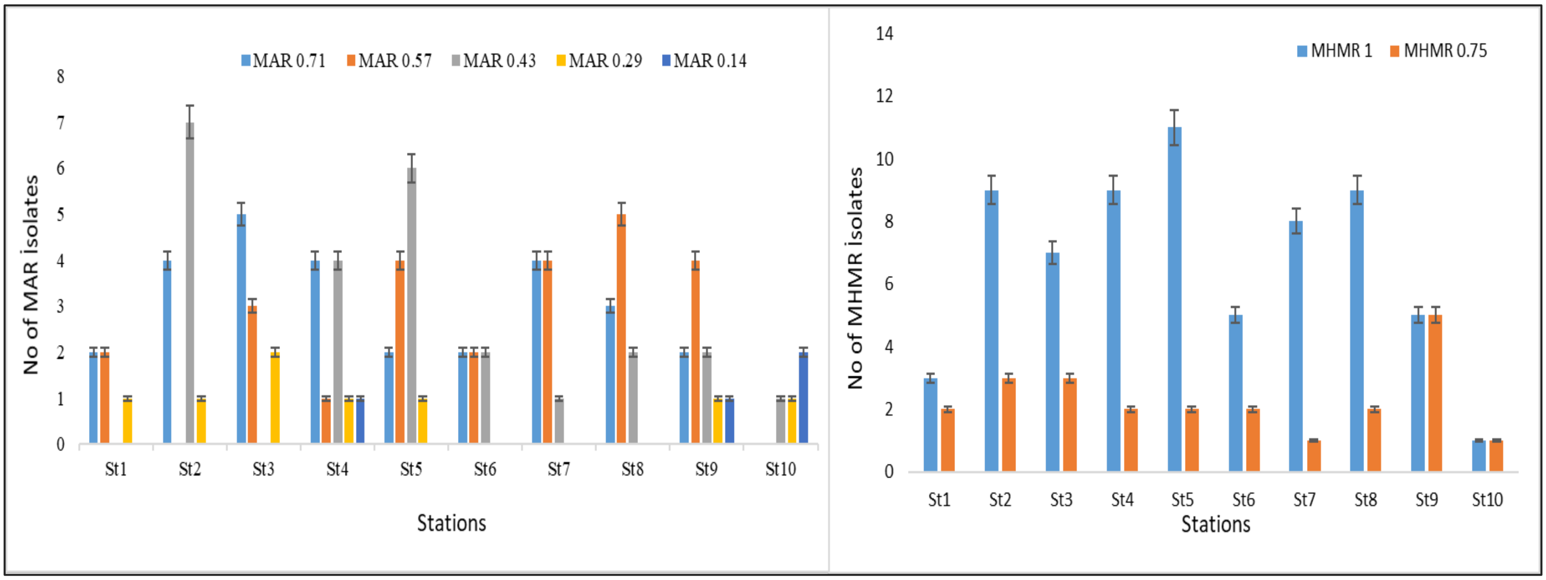

Figure 2. Distribution of multi-resistant bacterial isolates according to stations

The multiple antibiotic resistance (MAR) index values of the bacterial isolates were recorded as 0.71 (33.1%), 0.57 (33.3%), 0.43 (22.2%), 0.29 (8.9%) and 0.14 (4.4%). The multiple heavy metal resistance (MHMR) index values of the bacterial isolates ranged from 0.75 (22.2%) to 1.0 (77.8%). It was noted that 75.5% of the isolates were resistant to the concentrations of all heavy metal salts.

Studies aimed at determining bacterial diversity in marine ecosystems are crucial for comprehending ecosystem dynamics and identifying pollution types. The highly dynamic nature of marine environments, in contrast to terrestrial ones, leads to the development of resistance mechanisms in bacteria as they adapt to environmental conditions. The identification of bacterial isolates exhibiting resistance to encountered pollutants indicates ongoing exposure of the environment to these contaminants (Zeglin, 2015; Delgado-Baquerizo, 2016).

The misuse of antibiotics poses a significant threat to human and ecosystem health. Aquatic environments, heavily impacted by anthropogenic activities, serve as ideal settings for disseminating antibiotic resistance. The emergence of antibiotic resistance has spurred the search for new classes of antibiotics. However, developing novel antibiotic derivatives is economically unfeasible for the pharmaceutical industry. Therefore, it is imperative to regulate and control the unnecessary use of antibiotics through important regulatory measures. Antibiotics enter marine environments via anthropogenic, industrial, and clinical pathways, facilitating the spread of resistance mechanisms among bacteria through horizontal gene transfer. Given bacteria's affinity for surface attachment, the littoral region acts as a dynamic environment conducive to the exchange of resistance traits developed against pollutants such as antibiotics and heavy metals (Sabatino et al., 2020; Zhang et al., 2020; Marti et al., 2014).

Several researchers have documented that marine sediment bacteria isolated from Turkish marine environments exhibit a high antibiotic and heavy metal resistance prevalence. Matyar et al. (2008) reported that bacteria isolated from Iskenderun Bay sediments displayed resistance to ampicillin (94.4%) and imipenem (4.4%). Altuğ et al. (2020) found that sediment bacteria from Güllük Bay exhibited resistance to sulfonamide (100%), rifampicin (100%), tetracycline (100%), ampicillin (100%), nitrofurantoin (98%), and oxytetracycline (98%). Kacar and Kocyigit (2013) demonstrated that bacteria isolated from sediment in the Aliaga ship dismantling zone in the Eastern Aegean Sea were resistant to gentamicin and tobramycin. Additionally, Çardak et al. (2016) indicated that bacterial isolates from the Marmara Sea and the Turkish Straits displayed resistance to kanamycin (82%), vancomycin (78%), and ampicillin (60%). The findings of the present study also reveal high resistance rates among bacterial isolates against commonly used antibiotics, such as sulphonamides compound (93.3%), cefotaxime (78.9%), ampicillin (77.8%), oxacillin (67.8%), rifampicin (57.8%), imipenem (1.1%), and oxytetracycline (0%). These results suggest that islands typically considered less polluted, such as Gökçeada Island, may be more contaminated than anticipated concerning antibiotics. Therefore, monitoring studies are recommended, especially at ports and recreational areas.

Bacteria isolated from the marine environment play crucial roles in ecosystem functioning and biotechnological applications. They acquire various characteristics through adaptation to pollutants, making them valuable for human health and environmental management. Heavy metals, elements naturally occurring in the earth's crust with high weight-to-volume ratios, are present in the marine environment due to both natural processes (such as river runoff, erosion, atmospheric and volcanic activity) and human activities (including the use of fossil fuels, pesticides, mining, wastewater discharge, and the disposal of domestic and industrial waste).

While limited research specifically compares heavy metal resistance among sediment bacteria in Gökçeada Island, several studies have focused on heavy metal levels and environmental contamination in the region. Kahraman et al. (2009) found elevated levels of zinc (Zn), lead (Pb), and other heavy metals in various lichen species. Aslan et al. (2021) reported higher levels of Zn, Pb, and copper (Cu) in sediment samples from the Salt Lake lagoon of Gökçeada Island, attributing this to environmental issues linked to population growth, anthropogenic waste, and sewage. Yılmaz and Tuncer (2021) identified elevated concentrations of heavy metals, with iron (Fe), Zn, Cu, Pb, and cadmium (Cd) being ranked highest in sea urchin species, recommended as pollution biomonitors. Belivermis et al. (2019) found high mercury (Hg) concentrations in cephalopod muscular tissues. Sarı and Cagatay (2001) investigated heavy metal concentrations in surface sediments near Gökçeada Island, reporting it as comparatively unpolluted but affected by anthropogenic and natural inputs, especially Cu and Pb. In the present study, bacterial resistance against heavy metals was found to be highest for Zn, followed by Cu, Pb, and Hg, with mercury being the most toxic. These findings suggest that Gökçeada Island is exposed to Zn, Cu, Pb, and Hg, with heavy metal effects in sediments expected to increase due to rising maritime traffic and pollution levels.

It is recognised that heavy metals' toxicity varies depending on their cycling between components such as water, sediment, flora, and fauna in the marine ecosystem. Heavy metals that remain without dissolution or bacterial degradation accumulate in sediment, persisting as fixed pollutants.

The Multiple Antibiotic Resistance (MAR) and Multiple Heavy Metal Resistance (MHMR) index ratios exceeding a value of 0.2 indicate a potential risk of antibiotic and heavy metal pollution in environments (Krumperman, 1983). Matyar et al. (2008) found that the MAR index of sediment bacteria from İskenderun Bay exceeded the 0.2 thresholds. Conversely, Vignaroli et al. (2018) reported that the MAR index of enterococci isolated from the sediment of the Adriatic Sea remained below the 0.2 threshold, indicating a lower risk to public health. Ab Rahman et al. (2015) demonstrated that a high percentage of sediment bacteria isolated from coastal waters in Malaysia had MAR index values above 0.2.

In the present study, 95.5% of the MAR index values for antibiotic resistance exceeded the threshold of 0.2. This evidence suggests significant antibiotic contamination in the sediment of Gökçeada Island.

Conclusion

With this study, we aimed to elucidate the antibiotic and heavy metal resistance levels among bacteria isolated from sediment samples collected from Gökçeada Island. The high resistance against common antibiotics may signal significant anthropogenic impacts and excessive antibiotic pollution in the sediments. The findings of this study will be instrumental in highlighting the risks associated with antibiotics in the marine environments of Gökçeada Island. Strong measures to control antibiotic pollution and the need for further advanced studies are advised.

Moreover, the high resistance frequencies against heavy metal salts indicate that bacteria have adapted to cope with these metals over a prolonged period, suggesting longstanding heavy metal contamination in the sediment structure of Gökçeada Island. Other studies conducted in the area also indicate heavy metal contamination. Further analysis is warranted to detect heavy metal accumulation in sediment using advanced methods precisely. Additionally, certain bacterial species may have evolved to thrive in high concentrations of heavy metals. Their abilities could be harnessed through experimental methods, including proteomic and transcriptomic studies, for applications in wastewater treatment and other biotechnological endeavours.

Compliance with Ethical Standards

Conflict of interest: The author(s) declare no actual, potential, or perceived conflict of interest for this article.

Ethics committee approval: This study does not require ethics committee permission or any special permission.

Data availability: Data will be made available on request.

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Disclosure: -

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