ASSESSMENT OF PHYSICOCHEMICAL AND MICROBIOLOGICAL WATER QUALITY AND BACTERIAL BIOFILM FORMATION IN THE COASTAL WATERS OF ÇANAKKALE, TURKEY

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Abstract. This study, it was aimed to determine water quality based on the physicochemical and microbiological parameters, as well as microbial indicators (in addition to antibiotic-heavy metal resistance and biofilm formation) in Çanakkale Coastal waters, Turkey. The results showed that the stations were within acceptable limit values in terms of general sanitation values, however, the isolated bacteria showed high antibiotic and heavy metal resistance and biofilm formation capacity. A total of 68 Gram negative bacterial isolates were collected from the Çanakkale coastline, and were recorded as Aeromonadaceae, Burkholderiaceae, Enterobacteriaceae, Plesiomonodaceae, Pseudomonadaceae, Shewanellaceae and Vibrionaceae. The highest antibiotic resistances were detected against cephalothin, erythromycin, cefoxitin antibiotics from all bacterial isolates. Frequency of resistance of isolated bacteria against heavy metals (Cu^{2+}, Cd^{2+}, Cr^{3+}, Pb^{2+} and Mn^{2+}) were detected as averages of 67.64%, 60.29%, 86.76%, 79.41% and 92.64%, respectively. Sixty bacterial isolates also exhibited strong biofilm formation. This study demonstrated that the coastal waters of Çanakkale represented a rich source of bacterial diversity and high incidence of antibiotic and heavy metal resistance and biofilm formation among isolated bacteria. It is expected that this study will lead to more comprehensive researches to investigate microbial quality in terms of public health in coastal waters and seafood in the region.
Keywords: *coastline, monitoring, bacterial indicator, antibiotic - heavy metal resistance, biofilm*

Introduction

Pollution is a major problem that has negative effects on all of the planet's ecosystems, including the marine waters. In many parts of the globe, economic development has been most active in coastal zones, putting enormous pressures on coastal ecosystems. Coastal water pollution has increased throughout the world, mainly due to direct discharges from rivers, increased surface run-off and drainage from expanding port areas, oil spills and other contaminants from shipping, and domestic and industrial effluent. Most of the world's wastes- around 20 billion tons per year- end up in the sea, often without any preliminary processing (Krishnakumar and Asokan, 2017). The microbial quality of water is typically determined by monitoring microbial presence, especially faecal coliform bacteria and physicochemical parameters (EPA, 1999). Along with faecal bacteria, also potentially pathogenic bacteria such as *Aeromonas hydrophila*, *Campylobacter* spp., *Clostridium perfringens*, *Klebsiella pneumoniae*, *Neisseria* spp., *Pseudomanas aeruginosa*, *Salmonella* spp., *Shigella* spp., *Staphylococcus aureus* and *Vibrio cholera* whose normal habitat is not the marine

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environment are introduced into the seawater. The accumulation of these and other pathogenic bacteria in the recreational seawater and the beach sand represents a hazard to bathers, i.e. leads to a potential disease (Zbigniew et al., 2014). In this case, microorganisms are the most appropriate objects for the studies, since they are distinguished by their unique capability of rapidly adjusting to the changing environment, can transform and utilize virtually all the organic substances existing in the environment, and feature high reproduction and growth rates (Buzoleva et al., 2008).

There is no report pertaining to exploration of periodically variation physicochemical and microbiological parameters in the Çanakkale (Turkey) coastal waters. The main aims of this study were to reveal (i) physicochemical and microbiological changes of 4 different stations representing the Çanakkale (Turkey) coastline (ii) identify important pathogen Gram negative bacteria and their virulence factors such as level of antibiotic-heavy metal resistance and biofilm formation.

Materials and methods

Study area and sample collection

Water samples were collected at four sites located at different places over the perimeter of the Çanakkale (Turkey) coastline; Station 1: Güzelyalı village (40°2'35" N, 26°20' 44" E); Station 2: Çınarlı village (40°08'18" N, 26°3'65" E); Station 3: Kepez waist (40º06'21" N, 26º22'41" E); and Station 4: Çanakkale center cord (40°9′0″N, 26°24′0″E) (*Fig. 1*).

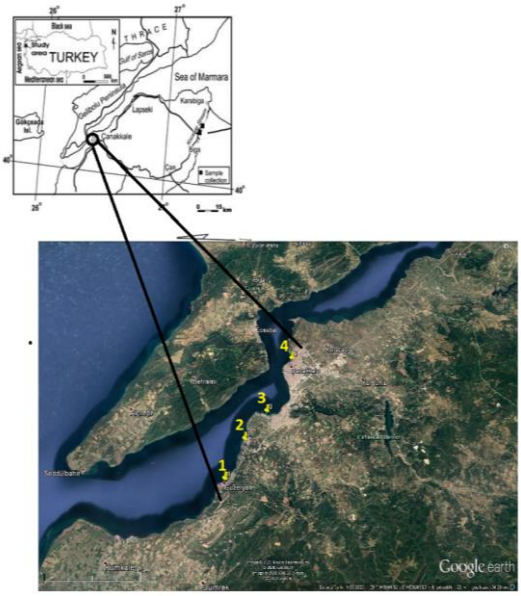

Figure 1. *Location of coastal sampling sites*

Sampling for water quality parameters were carried out in the four sites at seasonally intervals between December 2018 and September 2019 (1. sampling: December 2018; 2. sampling: March 2019; 3. sampling: June 2019; 4. Sampling: September 2019) covering dry and rainy seasons. Standard methods (APHA, 1998) were used during collection, preservation and estimation of different parameters. All samples were

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collected in 2 litres sterile bottles, kept at 4 ºC and analyzed within 3 h for microbiological and BOD_5 analysis. The collected water samples were analyzed for physicochemical parameters [temperature (T), dissolved oxygen (DO), biological oxygen demand (BOD), pH, electrical conductivity (EC), total suspended solid (TSS) and heavy metal rates] and microbiological parameters [total coliform (TC), faecal coliform (FC), faecal streptococci (FS)] by following the standard methods of American Health Association (APHA) (1998) in *Table 1*. The five heavy metal [Copper (Cu^{+2}), Cadmium (Cd^{+2}), Chromium (Cr^{+3}), Lead (Pb^{+2}), Manganese (Mn^{+2}), in the water samples were analyzed by Perkin Elmer ICPOES Optime 8000 through procurement of services.

Table 1. *Analytical method and standards of physicochemical parameters for analysis of Çanakkale coastline water quality*

Parameters	Unit	Analytical Methods	Instruments	Standards* (Anonymous, 2004)
T	ºC	Instrumental	Instrumental Hatche Lange pH meter	28-30
DO	mg/L	Instrumental	Instrumental Hatche Lange pH meter	>5
BOD	mg/L	Titrimetric (Winkler's)	Titration assembly and BOD incubator	20
pH	-	Instrumental	Instrumental Hatche Lange pH meter	6-9
EC	(µS/cm)	Instrumental	Instrumental Hatche Lange pH meter	n/a
TSS	g	Filtration technique	-	30 mg/L
Cu^{+2}	mg/L	Instrumental	EPA 200.7 method	0.01
Cd^{+2}	mg/L	Instrumental	EPA 200.7 method	0.01
Cr^{+3}	mg/L	Instrumental	EPA 200.7 method	0.1
Pb^{+2}	mg/L	Instrumental	EPA 200.7 method	0.1
Mn^{+2}	mg/L	Instrumental	EPA 200.7 method	0.01
TC	MPN/100 mL	Multiple tube technique	-	1000
FC	MPN/100 mL	Multiple tube technique	-	200
FS	MPN/100 mL	Multiple tube technique	-	100

*(Anonymous, 2004) State of Turkey, in Turkish Water Pollution Control Regulation, State of Turkey, 2004, Annexes, Table 4; n/a: not available; mg/L: miligram/Liter; MPN/100 mL: most probable number/100 mililiter; T: temperature; DO: dissolved oxygen; BOD: biological oxygen demand; EC: electrical conductivity; TSS: total suspended solid; Cu^{+2}: Copper; Cd^{+2}: cadmium; Cr^{+3}: chromium; Pb^{+2}: lead Mn^{+2}: manganese; TC: total coliform; FC: faecal coliform; FS: faecal streptococci

Bacterial isolation

Zobell Marine Agar (ZMA) and Mueller Hinton Agar (MHA) (Difco, USA) were used for the isolation of gram negative bacteria from water samples. Three to five colonies from each plate showing different colonial features were picked and inoculated to specific media (MacConkey agar (MAC); Eosin Methylene Agar (EMB); Thiosulfate citrate bile salts sucrose agar (TCBS), Glutamate starch phenol red agar (GSP), Ampicillin Aeromonas Agar, (AAA), Inositol brilliant green bile agar (IBG); Cetrimide Agar). All the plates were incubated at 25– 30 °C for 24–48 h. Biochemical tests (Murray et al., 1999) and Microgen ID-A Panel-Gram negative (MID-64) were performed to confirm the identity of obtained isolates.

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Antibiotic sensitivity testing

Susceptibility testing was performed by an agar diffusion test, using MHA (Matyar et al., 2008) and 14 different of antibiotics: Trimethoprim (TR5 µg/mL), Tobramycin (TB10 µg/mL), Kanamycin (K30 µg/mL), Amoxycillin (AM10 µg/mL), Oxytetracycline (O30 µg/mL), Cephalothin (CH30 µg/mL), Cefmetazole (CMZ30 µg/mL), Gentamicin (G120 µg/mL), Furazolidone (FR50 µg/mL), Erythromycin (E15 µg/mL), Cefoxitin (CN30 µg/mL), Ampicillin (A10 µg/mL), Cefotaxime (CE30 µg/mL) and Chloramphenicol (C30 µg/mL).

Determination of the minimal inhibitory concentration (MIC) of heavy metals

MIC for each bacterial isolate for five heavy metals was determined by using MHA which is containing Cu^{2+}, Cd^{2+}, Cr^{3+}, Pb^{2+} and Mn^{2+} at concentrations ranging from 100 to 12800 µg/mL. The metals were added as $CuSO_4.5H_2O$, $CdCl_2.2H_2O$, $K_2Cr_2O_7$, $Pb(NO_3)_2$ and $MnCl_2.2H_2O$. The isolates were considered resistant if the MIC values exceeded that of the *E. coli* K-12 strain which was used as the control (Matyar et al., 2008).

Analysis of biofilm formation

Bacterial biofilm formation activity of isolates was using the crystal violet technique adapted from (Julistiono et al., 2018) by using polystyrene 96-well microplate. Bacterial isolates were grown on seawater agar media for 48 h at 37 °C. Each colony was picked up by sterile tip needle and dipped into (inoculated) in three parallel wells of a 96 well microtiter plate containing 200 µl seawater media and incubated for 6 d at 37 °C. After the incubation period, the wells were rinsed with physiological saline and fixed with 2 µL of 99.99% ethanol for 10 min. The attached bacterial material was then stained by adding 2 µL of crystal violet (2%) for 20 min. The plate was rinsed with tap water gently and the attached biomass was measured using a microplate reader at 570 nm. The experiment was performed in triplicate and the mean of OD value is presented.

Statistical analysis of data

Mean and SE mean of physicochemical and microbiological analysis data were used to present seasonally values for these parameters. Statistical parameters of physicochemical and microbiological analyses data were used to present the values of these water quality characteristics. Pearson's correlation coefficient (r) was used to show correlation between the all parameters data using the MINITAB Statistical Software 13.20. The Student's t-test was used to determine the statistical significance. Probability was set at $p < 0.05$.

Results

Water quality

The results of field measurements and laboratory analysis on the quality of Çanakkale coastline waters were presented in *Table 2*. Based on results of comparison of data with Anonymous (2004) (*Table 1*), it is seen that Çanakkale coastline waters in all station was acceptable limits for physicochemical parameters (T, DO, BOD, pH, EC, TSS and heavy metal contents) (*Table 2*).

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Table 2. *The physicochemical and microbiological parameters in coastal waters of Çanakkale, Turkey*

Parameters	Stations				Average
	St.1	St.2	St.3	St.4	
T	12.6±1.83*	13.2±2.40	13.45± 0.35	11.2±1.97	12.61± 1.65
DO	10.525±0.40	10.035±0.07	9.335± 0.021	10.62±0.86	10.12±0.65
BOD	7.2±8.20	5.25±1.06	1.8±0.424	5.7±3.81	4.76±4.26
pH	7.95±0.148	7.98±0.084	7.93±0.035	8.07± 0.01	7.98±0.06
EC	51.8±0.84	52.6±0.14	35.7±0.28	45.5±0.28	46.4±7.80
TSS	0.032±0.012	0.0479±0.018	0.0326±0.022	0.041±0.014	0.038±0.007
Cu^{+2}	<0.01	<0.01	<0.01	<0.01	<0.01
Cd^{+2}	<0.01	<0.01	<0.01	<0.01	<0.01
Cr^{+3}	<0.1	<0.1	<0.1	<0.1	<0.1
Pb^{+2}	<0.01	<0.01	<0.01	<0.01	<0.01
Mn^{+2}	<0.01	<0.01	<0.01	<0.01	<0.01
TC	600±424.26	300±519.61	577500±120208.2	535±28.28	578260±449452.2
FC	685±162.34	610±0	1114000±766503.8	797.5±100.16	279023.12±365929.3
FS	462.5±0	11790±162.63	55325±714177.2	567.5±1032.37	17036.25±273377.7

*: mean± std. deviation; St.1: station 1

In the coastline water, TC, FC, FS counts varied from 300±519.61 MPN/100 mL (St.2) to 577500±120208.2 MPN/100 mL (St.3); 610±0 MPN/100 mL (St.2) - 1114000±766503.8 MPN/100 mL (St.3); 462.5±0 MPN/100 mL (St.1) - 55325±714177.2 MPN/100 mL (St.3), respectively. St. 3 (Kepez waist) was recorded the highest densities for microbial quality indicator bacteria (*Table 2*). *Table 3* provides the correlation matrix of the water quality parameters and also showed significant positive-negative correlation which is indicated by asterisk. There was a positive correlation between DO and EC (r = 0.577), pH and TSS (r = 0.578), TC and FC (r = 0.734), TC and FS (r = 0.778), FC and FS values (r = 0.997). On the contrary, there was a negative correlation between T and pH (r = - 0.622), T and TSS (r = - 0.914), T and FC (r = - 0.502), DO and TC (r = - 0.745), DO and FS (r = - 0.527), BOD and TC (r = - 0.560) EC and TC (r = - 0.909), EC and FC (r = - 0.617), and EC and FS values (r = - 0.622).

Table 3. *Correlation coefficients between the parameters in coastal waters of Çanakkale*

Parameters	T	DO	BOD	pH	EC	TSS	TC	FC	FS
T	1	-0.112	0.453	-0.622*	0.281	-0.914*	-0.193	-0.502*	-0.484
DO		1	0.452	0.219	0.577*	0.328	-0.745*	-0.489	-0.527*
BOD			1	0.312	0.487	-0.236	-0.560*	-0.398	-0.425
pH				1	0.146	0.578*	-0.341	-0.123	-0.146
EC					1	0.122	-0.909*	-0.617*	-0.662*
TSS						1	-0.153	0.310	0.272
TC							1	0.734*	0.778*
FC								1	0.997*
FS									1

*Correlation is significant at the 0.05 level

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Bacterial diversity

During the study period, 7 bacterial families were recorded: Aeromonadaceae (8.82%), Burkholderiaceae (1.47%), Enterobacteriaceae (55.88%), Plesiomonodaceae (8.82%), Pseudomonadaceae (16.17%), Shewanellaceae (1.47%) and Vibrionaceae (7.35%). A total of 68 bacterial isolates were defined from Çanakkale coastline. The species belonging to Enterobacteriaceae family was the most common taxonomic group in the coastal areas of Çanakkale. Pseudomonadaceae family was the second most common group (*Table 4*).

Table 4. *Diversity of Gram negative bacteria according to their isolated stations*

Familia	Species	Stations			
		St.1 (n=17)	St.2 (n=15)	St.3 (n=16)	St.4 (n=20)
Aeromonadaceae	*Aeromonas hydrophila*	+	+	+	-
Burkholderiaceae	*Burkholderia pseudomallei*	-	+	-	-
Enterobacteriaceae	*Enterobacter aerogenes*	+	+	+	+
	E. cloacae	+	-	-	-
	Escherichia coli	+	+	+	+
	Klebsiella oxytoca	+	+	+	+
	K. pneumoniae	-	+	+	+
	Pantoea agglomerans	+	+	+	-
	Proteus vulgaris	-	+	-	-
	Salmonella typhimurium	-	-	-	+
Plesiomonodaceae	*Plesiomonas shigelloides*	+	+	-	+
Pseudomonadaceae	*Pseudomonas aeruginosa*	+	+	+	+
Shewanellaceae	*Shewanella putrefaciens*	+	-	-	-
Vibrionaceae	*Vibrio alginolyticus*	+	-	-	-
	V. parahaemolyticus	-	+	+	+

St.1: station 1; n: Isolate number

Antibiotic susceptibility

The results of the antibiotic susceptibility test revealed 0% (TB10-C30) - 100% (E15-CN30), 6.66% (TB10) - 80% (CH30), 6.2% (TB10-G120) – 87.5% (CN30) and 15% (G120)- 95% (CH30) for the bacterial isolates of four sites, respectively (*Fig. 2*).

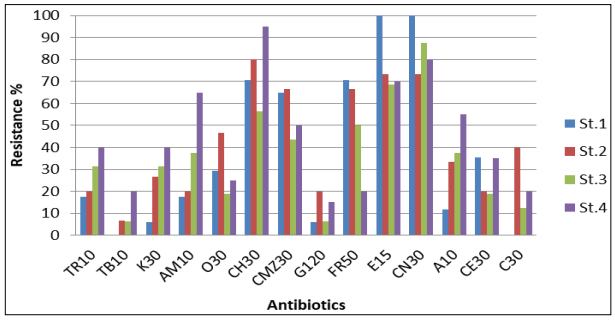

Figure 2. *Antimicrobial resistant profile of isolated bacteria*

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Heavy metal resistant

The results were shown as the frequency of resistance of these bacteria in *Fig. 3*. A hundred percent of all isolates were resistant to manganese. However, the higher frequencies of resistant bacteria were found in station 4, except copper. Frequency of resistance of Cu^{2+}, Cd^{2+}, Cr^{3+}, Pb^{2+} and Mn^{2+} were detected as an average of 67.64%, 60.29%, 86.76%, 79.41% and 92.64%, respectively in a total of 68 bacteria isolated from Çanakkale coastline.

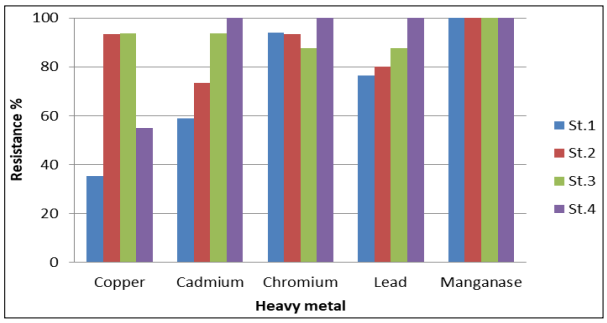

Figure 3. *Heavy metal resistance isolated bacteria*

Results of biofilm formation

Out of eight, sixty bacterial isolates exhibited biofilm formation. Most of them formed moderate to strong biofilms. OD570>0.2 indicated significant biofilm formation whereas that in the range of 0.1-0.2 indicated moderate biofilm formation. The results of biofilm formation in isolates were as tabulated (*Table 5*).

Table 5. *Biofilm formation rates of isolated bacteria*

Isolates (St.1)	Biofilm rates	Isolates (St.2)	Biofilm rates	Isolates (St.3)	Biofilm rates	Isolates (St.4)	Biofilm rates
A1	+++	**B1**	+	**C1**	+++	**D1**	+++
A2	+	**B2**	+++	**C2**	++	**D2**	+++
A3	+++	**B3**	+++	**C3**	++	**D3**	+
A4	+++	**B4**	-	**C4**	++	**D4**	+++
A5	+++	**B5**	+	**C5**	++	**D5**	+
A6	+++	**B6**	+++	**C6**	+++	**D6**	+++
A7	+	**B7**	-	**C7**	-	**D7**	+++
A8	+++	**B8**	+++	**C8**	+++	**D8**	+++
A9	++	**B9**	+++	**C9**	+++	**D9**	-
A10	++	**B10**	+++	**C10**	+++	**D10**	-
A11	+	**B11**	-	**C11**	+++	**D11**	+++
A12	+++	**B12**	-	**C12**	++	**D12**	+++
A13	+++	**B13**	+++	**C13**	-	**D13**	+++
A14	+++	**B14**	+++	**C14**	+++	**D14**	+
A15	+	**B15**	+	**C15**	+++	**D15**	+++
A16	+++			**C16**	+	**D16**	++
A17	+					**D17**	++
						D18	+++
						D19	+++
						D20	+++

St.1: station 1; A1: Number of isolated bacteria (A: St. 1, B: St. 2, C: St. 3, D: St. 4); (+++) = strong, (++) = moderate, (+) = weak, (-) = Not detected

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Discussion

There is no study has been found that extensively investigates the water quality of the Çanakkale coastline in terms of physicochemical and microbiological parameters. For this reason, the present study was designed in which revealed these parameters and antibiotic-heavy metals resistance patterns and biofilm formation capacity of bacterial isolates.

Life of all marine organisms depends on water quality of the coastal environment. Water quality indices are the basic tools to sketch out the environmental or ecological condition of a water body and to simplify the presentation of results. Most considerable physical and chemical factors which greatly influence the aquatic environment are temperature, pH, carbon dioxide, dissolved oxygen and heavy metal concentration etc. (Zafar et al., 2018). In the present study these physicochemical parameters were found acceptable limits contrast to findings about different coastal waters (Türkoğlu, 2010; Türkoğlu and Oner, 2010; Sreenivasulu et al., 2015; Zafar et al., 2018; Tanjung et al., 2019).

In recent years, the increase of bacterial contamination in coastal waters has become a global problem that limits recreation functions and can create negative presentations for public health (Sreenivasulu et al., 2015). The use of TC, FC and FS, which is share the same environment with the pathogenic bacteria, is much routine analyses as the indicator of the presence of the pathogenic bacteria. In our study, especially FC and FS levels were above the limit values in all sites. This is a proof that the Çanakkale coastline is significantly pressured in terms of faecal pollution. In this sense, our findings have correlated with previous studies carried out on the Çanakkale coastline and other sea coasts (Çardak and Altuğ, 2010, 2014; Çardak et al., 2016; Çiftçi Türetken and Altuğ, 2016; Hulyar et al., 2020).

Coastlines are becoming the hotspots of some new emerging diseases. Many opportunistic pathogens, including *Aeromonas*, *Clostridium*, *Klebsiella*, *Legionella*, *Listeria*, *Pseudomonas*, and *Vibrio*, are naturally enterprising in sea waters. When present in coastal waters, these opportunistic pathogens can persist and infect humans through recreational exposures or consumption of contaminated sea-food (Mishra et al., 2018). Though there have been reports investigating the distribution of fecal, supplemental-indicator, human pathogenic bacteria in the Canakkale coastline (Çardak et al., 2016), no studies have been done so far which put forth antibiotic – heavy metal resistance and biofilm formation of these bacteria. In this regard, this investigation would be the first report to isolate biofilm-forming bacteria from Canakkale coastline and deduce their antibiotic-heavy metal susceptibility patterns.

The coastal sites of Çanakkale represented a rich source of bacterial diversity. High incidence of antibiotic and heavy metal resistance among isolated bacterial strains is captivating and might be a consequence of increased number of antibiotic and heavy metal resistance genes in the coastal area and their cross-generational transfer. Environment may be recognized as a giant repository of resistance agents and the propensity of environmental bacteria to form biofilms may be implicated as a selective advantage to survive in the natural environments and spawn infectious diseases.

Conclusion

The correlation analysis on water quality parameters revealed that all parameters are more or less correlated with each other Person's Correlation matrix. It is observed that

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some of the parameters do not have significant correlation between them indicating the different origin source of pollution. From correlation analysis, the positive relationship between TC, FC, and FS each other reveals the high organic pollution with anthropogenic activities on the Çanakkale coastline. Thus it can be concluded that the water of the Çanakkale coastline represents serious threat to the ecosystem due to anthropogenic pollution. Since Çanakkale coastline is lifeline for people of Çanakkale who use its water for swimming, fishing or recreation are at risk. The correlation study and correlation coefficient values can help in selecting a few parameters which could be frequently measured to determine the status of water quality regularly. This will help the regulatory bodies to issue a warning on deteriorating water quality and taking steps to implement control measures so that proper treatment of effluent could be done to minimize contaminants in Çanakkale coastline. However, the isolated bacteria showed high antibiotic and heavy metal resistance and biofilm formation capacity. Our study is the first report studying on marine biofilms isolated in the Çanakkale coastline and therefore it can serve as an elementary data to several future studies aspiring to understand the ecology and quality of marine habitats. This study also demonstrated that anthropogenic effects of people significantly affected water quality of the Çanakkale coastline. So, coastline should be followed and pollution sources must be taken under control. Hence, the main goals are to reduce the number of pollution incidents in watercourses, eliminate the sources of pollutants, and minimize the consequences of accidental discharges on marine ecosystem. In order to prevent the local marine pollution in the coastal cities drainage and treatment/discharge systems should be built.

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