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First records and microgeographical variations of culturable heterotrophic bacteria in an inner sea (the Sea of Marmara) between the Mediterranean and the Black Sea, Turkey

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Abstract: The microdiversity and composition of culturable heterotrophic aerobic bacteria were investigated in seawater samples taken from the Sea of Marmara (an important basin between the Mediterranean and the Black Sea) in different time periods throughout 2002–2010. The bacterial isolates were identified with the automated microidentification system VITEK 2 Compact 30 (bioMérieux, France). The compositions of identified bacteria according to their exposure to environmental factors in the areas from which they were isolated were compared. The primary hydrographic parameters (temperature, salinity, and dissolved oxygen) were recorded at the sampling station. The highest heterotrophic aerobic bacteria abundance was found in the coastal stations. It was possible to isolate enteric bacteria species from the lower and upper stratification of various marine localities, which possessed salinity values between 24.0 psu and 39.2 psu during the study. Six bacterial classes were determined: Gammaproteobacteria (49%), Bacilli (34%), Alphaproteobacteria (9.09%), Betaproteobacteria (3.03%), Flavobacteria (3.03%), and Actinobacteria (3.03%). This study increases the knowledge of the composition and biochemical response of bacteria isolated from eutrophic and oligotrophic areas. Twenty-three bacteria species belonging to 16 families are reported in this study as the first records for the Sea of Marmara.

Key Words: Bacterial diversity, Sea of Marmara, culturable bacteria, aerobic heterotrophic bacteria

1. Introduction

The Sea of Marmara separates Turkey's Asian and European regions, and the Turkish strait system connects the Sea of Marmara to the Black Sea and the Aegean Sea. The pollution levels in the Sea of Marmara have increased as a result of the effects of the Black Sea due to opposite water currents between the Black Sea and the Aegean Sea (1,2). Additionally, the Sea of Marmara is under the influence of various anthropological factors such as dwellings and domestic and industrial wastes. Furthermore, as an important water route between the Mediterranean and the Black Sea, the Sea of Marmara is under pressure from heavy marine transportation. The bacteria that come from ships' ballast water is another effective factor on the composition and abundance of bacteria in the Sea of Marmara.

The majority of bacteria present in domestic wastewater is composed of saprophyte bacteria of fecal or terrestrial origin and pathogenic bacteria such as *Salmonella*, *Shigella*, *Brucella*, *Mycobacterium*, *Escherichia coli*, *Leptospira*, *Campylobacter*, *Vibrio*, and *Yersinia* (3–5).

Although culture-independent studies have served as common applications in detecting bacterial diversity, there are also a number of studies in which it has been shown that cultured strains of marine bacteria can represent significant fractions of the bacterial biomass in sea water (6,7). Based on DNA–DNA hybridization of the genomic DNAs of isolates obtained with the traditional medium against community DNA, it has been suggested that readily culturable bacteria are abundant in the marine water column (6–9). There are still few studies on culturable bacterial abundance and indicator bacteria in the Sea of Marmara (10–14).

Due to its peculiar hydrodynamic characteristics and the various pollution factors mentioned above, the Sea of Marmara offers unique opportunities for researching bacterial composition under different, poorly described conditions. Although the Sea of Marmara is an important basin between the Mediterranean and the Black Sea, data concerning bacterial composition for an understanding of ecological roles of the most abundant bacterial species are still scant in the area. In this study, the composition of culturable heterotrophic bacteria was investigated in the Sea of Marmara. This paper also reports species of culturable heterotrophic aerobic bacteria as new records for the Sea of Marmara.

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2. Materials and methods

2.1. Sampling

The sea water samples were collected from the Sea of Marmara at different time periods between 2002 and 2011 (Figure 1). The samples used in the analysis were collected in a Nansen bottle that had been cleaned with acid (10% HCl in distilled water), sterilized with alcohol (50:50, v/v), and rinsed with sterile water. The samples were transferred into 250-mL sterile brown glass bottles under aseptic conditions and processed on board the İstanbul University research vessel YUNUS-S. Sampling areas, number of samples, number of stations, sampling type, and sampling periods are summarized in Table 1.

The sea water samples were transported daily to the laboratory. However, because of the long distance between the sampling point and the laboratory, certain procedures (CFU analyses) on samples that were taken from the eastern part of the Sea of Marmara were carried out during cruising in the bacteriology laboratory of the research vessel YUNUS-S.

2.2. Bacteriological analyses

The sea water samples were collected in sterilized glass bottles, serial dilutions of 10^{-5} were prepared in 9-mL amounts of sterile seawater (artificial seawater, Sigma) and inoculated (0.2 mL) in duplicate on marine agar (Difco), and the plates were incubated for 5 days at 22 ± 0.1 °C (15). At the end of the incubating period, different colonies were picked and restreaked several times to obtain pure cultures. The pure isolates were Gram-stained and then identified using GN (gram-negative fermenting and nonfermenting bacilli), GP (gram-positive cocci and nonspore-forming bacilli), and BCL (gram-positive spore-forming bacilli) cards in the automated microidentification system VITEK 2 Compact 30 (bioMérieux, France). The identification cards are based on established biochemical methods and newly developed substrates. There are biochemical tests (46 tests for BCL, 43 tests for GP, 47 tests for GN) measuring carbon source utilization, enzymatic activities, inhibition, and resistance. Calculations are performed on raw data and compared to thresholds to determine reactions for each test. On the VITEK 2 Compact, test reaction results appear as "(–)" or "(+)". Reactions that appear in parentheses were evaluated as an indicator of weak reactions that are too close to the test threshold (16).

2.3. Physicochemical analyses

Temperature, dissolved oxygen, pH, and salinity values were measured in situ using the CTD SBE-19 SEACAT

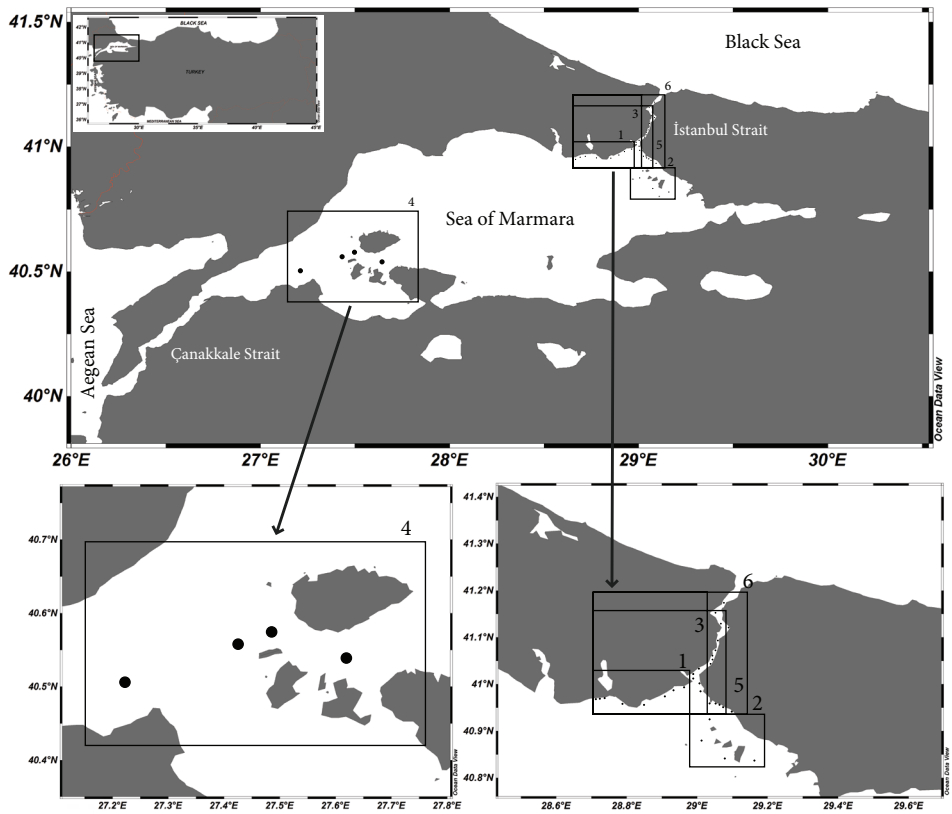

Figure 1. Study area 1: northwestern part of the Sea of Marmara, 2: eastern part of the Sea of Marmara, 3: northeastern coastal areas of İstanbul, 4: western part of the Sea of Marmara, 5: southern coastal areas of İstanbul, 6: northern coastal areas of İstanbul.

Table 1. Sampling areas, number of samples and stations, sampling type, and sampling periods.

Sampling area	Number of samples	Number of stations	Sampling type	Sampling period	Sampling depth
1	28	7	Seasonally	2002	0–30 cm
2	16	4	Seasonally	2003	0–30 cm 10 m 20 m
3	216	18	Monthly	2005	0–30 cm
4	48	4	Seasonally	2005–2006	0–30 cm 10 m 50 m
5	102	17	Monthly	2009–2010	0–30 cm
6	102	17	Monthly	2009–2010	0–30 cm
Total	512	67		2002–2010	0–30 cm 10 m 20 m 50 m

1: Northwestern part of the Sea of Marmara, 2: eastern part of the Sea of Marmara (around the Princes' Islands), 3: northeastern coastal areas of İstanbul, 4: western part of the Sea of Marmara (entrance of the Çanakkale Strait), 5: southern coastal areas of İstanbul, 6: northern coastal areas of İstanbul.

Profiler or a portable multiparameter tool (Hach Lange HQ 40D) at the stations.

3. Results and discussion

The diversity of culturable heterotrophic aerobic bacteria according to their isolated areas is shown in Table 2.

The minimum and maximum heterotrophic plate count and primary hydrographic values (salinity, temperature, dissolved oxygen, and pH) of the studied areas are shown in Table 3.

The total number of species was higher in the coastal areas than around the islands (sampling area 2) and the entrance of the Çanakkale Strait (sampling area 4). Depending on the trophic level of the environment, increases in culturable bacteria counts were observed in the sampling areas.

While the minimum and maximum pH values of the areas in which the bacteria were isolated were recorded to be 7.21–9.39, the mg/L values for dissolved oxygen were 5.38–11.2.

During the study period, 6 bacterial classes were recorded: Gammaproteobacteria (49%), Bacilli (34%), Alphaproteobacteria (9.09%), Betaproteobacteria (3.03%), Flavobacteria (3.03%), and Actinobacteria (3.03%). The percentage distributions of the isolated genera are shown in Figure 2.

The isolated strains obtained during the study period were evaluated with respect to their potential pathogenicity, metabolic characteristics, and microgeographical variations according to their exposure to environmental factors in the areas from which they were isolated.

Bacterial compositions exhibited differences according to the pollution status of the study areas from which they were isolated. The sampling areas are designated below as "polluted" or "unpolluted" according to analyses of nutrients, chlorophyll a (unpublished data), and indicator bacteria (13).

Bacteria isolated from the coastal areas of the Sea of Marmara displayed different compositions as compared to species isolated from around the islands (Station 2) and the western part of the Sea of Marmara (entrance of the Çanakkale Strait). For example, Gammaproteobacteria was the most common group in terms of species number in comparison to the other taxonomic groups in the study areas during the study period. While the members of the phylum Firmicutes were recorded as the second most common group, the members of the phylum Alphaproteobacteria followed them.

The species belonging to the family Enterobacteriaceae were determined to have the highest count in these regions, implying that abundance of enteric bacteria is a part of the anthropological pollution input of the coastal areas. Furthermore, modeling studies conducted in the İstanbul Strait and the Sea of Marmara indicate that under normal conditions, 11%–15% of the wastewater discharged from various points of the Sea of Marmara into

Table 2. Diversity of culturable heterotrophic aerobic bacteria according to their isolated areas.

Family	Species	Sampling areas						Phylum/Class
		1	2	3	4	5	6	
Enterobacteriaceae	**Klebsiella pneumoniae* subsp. *pneumoniae* (Schroeter 1886), Trevisan 1887	+	-	-	-	+	+	Proteobacteria/ Gammaproteobacteria
	**K. oxytoca* (Flügge 1886) Lautrop 1956	+	-	+	-	+	+	
	**Citrobacter freundii*	+	-	+	-	+	+	
	**Serratia fonticola* (Gavini et al. 1979)	+	-	+	+	+	+	
	S. liqefaciens (Grimes and Hennerty 1931) Bascomb et al. 1971	+	-	+	+	-	-	
	Escherichia coli (T. Escherich, 1885)	+	+	+	+	+	+	
	Enterobacter cloacae (Jordan 1890) Hormaeche and Edwards 1960	+	-	+	-	+	+	
	E. sakazaki (Farmer et. al., 1980)	+	-	+	-	+	+	
	E. aerogenes Hormaeche and Edwards 1960	+	-	+	-	-	-	
	**Salmonella enterica* subsp. *arizonae* (Borman 1957) Le Minor and Popoff 1987	+	-	+	-	-	-	
Pseudomonadaceae	*Pseudomonas luteola* Kodoma, et al. 1985	+	-	+	-	+	+	
	**P. putida* Trevisan, 1889	+	-	+	+	-	-	
	P. aeruginosa (Schroeter 1872) Migula 1900	+	-	+	+	-	-	
Xanthomonadaceae	**Stenotrophomonas maltophilia* Palleroni and Bradbury 1993	+	-	+	-	+	+	
Shewanellaceae	**Shewanellla algae* Simidu et al. 1990	-	+	-	+	-	-	
	S. putrefaciens (Lee et al. 1981), MacDonell and Colwell 1986	+	-	+	-	+	+	
Brucellaceae	**Brucella melitensis* (Hughes 1893), Meyer and Shaw 1920	-	-	-	-	+	-	
Sphingomonadaceae	**Sphingomonas paucimobilis* (Holmes et al. 1977), Yabuuchi et al. 1990	+	-	+	-	+	+	Proteobacteria/ Alphaproteobacteria
Caulobacteraceae	**Brevundimonas vesicularis* (Büsing et al. 1953) Segers et al. 1994	+	-	+	-	+	+	
Aeromonadaceae	*Aeromonas hydrophila* (Chester, 1901) Stanier, 1943	+	-	+	-	+	+	
	**A. caviae* Eddy 1962, Popoff 1984	+	-	+	-	-	-	Firmicutes/ Bacilli
Alicyclobacillaceae	**Alicyclobacillus acidoterrestris* (Deinhard et al. 1988) Wisotzkey et al. 1992	+	-	+	-	+	+	
Bacillaceae	**Bacillus cereus* Frankland and Frankland 1887	+	-	+	+	+	+	
	**B. mycoides* Flügge 1886	+	-	+	-	+	+	
	**B. pumilus* Meyer and Gottheil 1901	+	-	+	-	+	+	
	**B. thuringiensis* Berliner 1915	+	-	+	-	+	+	
Streptococcaceae	**Streptococcus pneumoniae* (Klein 1884) Chester 1901	+	-	+	-	-	+	
	E. faecalis (Andrewes and Horder 1906) Schleifer and Kilpper-Balz 1984	+	-	+	-	+	+	
Staphylococcaceae	**Staphylococcus hominis* Kloos and Schleifer 1975 emend. Kloos et al. 1998	-	+	-	+	-	-	
Noctuoidea	**Virgibacillus pantothenticus* (Proom and Knight 1950) Heyndrickx et al. 1998	+	-	-	-	+	+	
Flavobacteriaceae	**Chryseobacterium indologenes* (Yabuuchi et al. 1983) Vandamme et al. 1994	+	-	+	-	+	+	Bacteroidetes/ Flavobacteria
Micrococcaceae	**Micrococcus luteus* Lehmann and Neumann 1896	+	+	+	-	+	+	Actinobacteria/ Actinobacteria
Alcaligenaceae	**Alcaligenes faecalis* subsp. *faecalis* (King 1959) Kim et al. 2005	+	+	-	+	-	+	Proteobacteria/ Betaproteobacteria
Gram-negative fermenting and nonfermenting bacteria	23	23	3	3	7	15	15	
Gram-positive cocci and nonspore-forming bacilli	4	3	2	2	0	2	3	
Spore-forming gram-positive bacilli	6	6	0	5	1	6	6	
Total number of species	33	30	5	27	9	23	24	

1: Northwestern part of the Sea of Marmara, 2: eastern part of the Sea of Marmara, 3: northeastern coastal areas of İstanbul, 4: western part of the Sea of Marmara, 5: southern coastal areas of İstanbul, 6: northern coastal areas of İstanbul. *First record for the Sea of Marmara.

Table 3. Minimum–maximum heterotrophic plate count and salinity, temperature, dissolved oxygen (DO), and pH values of the stations during the study periods

Areas	Season	HPC (CFU/100 mL)	Salinity (psu)	Temp. (°C)	DO (mg/L)	pH	Depth
1	Spring	18×10^6 to 21×10^7	11.8–20.4	20.1–22.0	7.01–7.42	8.71–9.11	0–30 cm
	Summer	21×10^6 to 17×10^8	20.3–22.1	22.1–23.4	6.62–7.73	8.31–8.33	
	Autumn	12×10^6 to 11×10^7	17.6–18.1	17.1–17.4	6.23–7.55	7.82–8.01	
	Winter	2.0×10^4 to 2.5×10^5	18.2–19.7	8.5–8.6	6.72–7.45	8.82–9.22	
2	Spring	10×10^2 to 10×10^5	22.8–38.9	18.5–23.7	7.07–8.10	8.27–8.39	Water column 0–30 cm 10 m 20 m
	Summer	10×10^2 to 10×10^5	23.1–38.9	21.2–25.5	7.65–8.25	8.11–8.44	
	Autumn	10×10^2 to 10×10^4	22.4–38.7	15.7–21.3	7,66–8.15	8.35–8.75	
	Winter	10×10^2 to 10×10^3	25.6–38.7	8.5–14.5	8.04–9,37	8.62–9.01	
3	Spring	18×10^5 to 19×10^7	12.7–21.0	21.3–22.6	10.1–11.2	9.15–9.22	0–30 cm
	Summer	82×10^6 to 25×10^8	21.3–22.0	22.3–23.1	9.25–9.46	8.76–9.21	
	Autumn	65×10^6 to 82×10^7	18.5–22.1	18.3–18.8	8.76–9.32	9.01–9.11	
	Winter	12×10^4 to 21×10^5	18.1–19.2	8.01–9.12	8.48–9.01	8.5–9.10	
4	Spring	66×10^3 to 47×10^5	23.0–39.1	16.6–24.1	6.07–6,94	9.22–9.39	Water column 0–30 cm 10 m 50 m
	Summer	48×10^5 to 89×10^5	21.8–38.8	21.2–25.4	7.00–7.67	8.01–8.34	
	Autumn	20×10^3 to 21×10^5	22.5–38.9	14.7–20.3	5.38–7,69	8.97–9.15	
	Winter	6×10^2 to 25×10^4	25.8–38.8	8.9–14.3	6.04–9,37	9.12–9.33	
5	Spring	28×10^5 to 12×10^7	12.4–19.5	21.7–22.8	7.01–7.72	7.71–8.01	0–30 cm
	Summer	24×10^6 to 27×10^7	18.4–19.3	23.7–25.4	7.35–8.03	7.21–7.86	
	Autumn	22×10^6 to 41×10^7	17.2–18.5	16.3–19.2	6.83–7.25	7.22–8.01	
	Winter	36×10^5 to 44×10^5	18.2–19.7	8.7–9.4	6.82–7.37	8.52–9.02	
6	Spring	38×10^5 to 32×10^7	17.0–19.2	18.4–20.2	7.36–8.15	8.25–8.36	0–30 cm
	Summer	44×10^6 to 37×10^7	18.5–19.6	24.5–26.2	7.25–8.02	7.37–8.52	
	Autumn	28×10^6 to 43×10^7	17.5–18.2	18.4–20.6	7.83–8.11	7.88–8.42	
	Winter	66×10^5 to 84×10^5	18.6–18.9	8.9–11.2	7.22–7.77	8.12–8.65	

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the İstanbul Strait returns to the Sea of Marmara (2,17). The hydrodynamic characteristics of the Sea of Marmara mentioned above also complicate the elimination of the presence of enteric bacteria.

The presence of a negative relationship between salinity concentration and the number of enteric bacteria in sea medium has been previously reported (18–20). However, enteric bacteria of sewage origin undergo a sudden osmotic shock when they enter sea water and may adapt their metabolism to the new medium by means of their osmoregulation systems. This ability of enteric bacteria aids them in gaining resistance to salt in sea environments and increases their probability of survival (21). The less saline waters of the Black Sea (22–26 psu) reach the Mediterranean via upper currents while the concentrated saline waters of the Mediterranean (38.5–38.6 psu) reach the Black Sea via the undercurrents of the Çanakkale and İstanbul Straits (22, 23). It was possible during the study to isolate enteric bacteria species from the lower and upper stratification of various marine localities, which possessed salinity values between 24.0 psu and 39.2 psu. Among all the strains, the percentages of the gram-positive bacteria in the coastal areas and the northwestern part of the Sea of Marmara (sampling area 1) were 90% and 70%, respectively.

Heterotrophic bacteria play a key role in marine biogeochemical cycling and food webs because of the wide diversity of their metabolic properties. The bacteria classes found in this study include species that are able to secrete large quantities of ectoenzymes. This situation suggests that those particular species have potential importance in organic matter turnover in these areas. In addition,

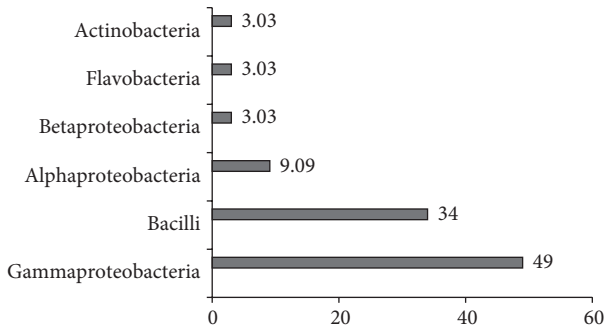

Figure 2. The percentage distributions of the isolated genera.

the composition of bacteria species displayed differences according to their exposure to environmental factors in the areas from which they were isolated. For example, *S. hominis* was reported as a less common species in clinical specimens and dominant in water samples collected from unpolluted regions (24,25). In this study, *S. hominis* was isolated from relatively unpolluted areas (sampling areas 2 and 4).

S. putrefaciens, which was isolated around the Princes' Islands (sampling area 2) of the Sea of Marmara, is one of the organisms associated with the odor of rotting fish, as it is a marine organism that produces trimethylamines (26). *M. luteus*, belonging to Actinobacteria, can play a vital role in organic matter decomposition and the carbon cycle, and it can also survive in oligotrophic environments for extended periods of time. In this study, *M. luteus* was isolated in the samples that were taken from both polluted and unpolluted areas.

S. paucimobilis is a soil bacillus and it is able to degrade lignin-related biphenyl chemical compounds (27). *P. putida* is a common degrader showing a very diverse metabolism, including the ability to degrade organic solvents such as toluene (28). In this study, *S. paucimobilis* and *P. putida* were isolated in the samples that were taken from polluted coastal areas. A few bacilli of marine origin have been reported to produce unusual metabolites different from those isolated from terrestrial bacteria (29). Due to *Bacillus* species' ubiquity and ability to survive under difficult circumstances (30,31), heterotrophic *Bacillus* strains are hardly considered to be species of certain habitats. In this study *B. pumilus*, *B. thuringiensis*, *B. mycoides*, and *B. cereus* were isolated from polluted coastal areas, and they have been stocked for further studies.

In this study, the presence of 23 culturable aerobic heterotrophic bacteria species belonging to 16 different families from the Sea of Marmara is reported for the first time. Although these bacteria have not previously been reported from these areas, they may be ubiquitous in aquatic environments. The occurrence of these species for the first time in this study may be related to the lack of bacterial diversity studies in this region. The study increases the knowledge of the composition and biochemical response of bacteria isolated from polluted and unpolluted areas. However, there is a need for long-term monitoring studies for the purpose of detecting bacterial composition and understanding their ecological roles in these areas.

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