İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

PHYLOGENETIC ANALYSIS OF 16S rRNA FOR DETERMINATION OF MICROBIAL COMMUNITY IN ANOXIC MARINE SEDIMENTS OF HALİÇ

M.Sc Thesis by

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Department : **Advanced Technologies**

Programme: **Molecular Biology-Genetics and Biotechnology**

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MİKROBİYAL KOMÜNİTENİN BELİRLENMESİ İLE HALİÇ ANOKSİK DENİZ SEDİMENTLERİNDE 16S rRNA'NIN FİLOGENETİK ANALİZİ

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TABLE OF CONTENTS

ABBREVIATIONS vı
LIST OF TABLES vıı
LIST OF FIGURES vıı
SUMMARY ıx
ÖZET x

1. INTRODUCTION 1

2. POLLUTION IN HALİÇ 5

2.1. Base Formation of Haliç 5

2.2. Pollutant Sources and Their Effects 5

2.2.1. Ecological View of the Estuary 7
2.2.2. Reclamation Projects and Works 7
2.2.3. Haliç Rehabilitation Project 7
2.2.4. Results of the Rehabilitation Project 8

2.3. Marine Sediments 9

2.3.1. General Characteristics and Importance of Marine Sediments 9
2.3.2. Microbial Ecology in Marine Sediments 10
2.3.3. Archael Community in Marine Sediments 13
2.3.4. Bacterial Diversity in Marine Sediments 14
2.3.5. Biological Role of Bacteria and Archae 15

2.4. Major Anaerobic Processes in Marine Sediments 16

2.4.1. Anaerobic Oxidation of Methane 16
2.4.2. Sulfate Reduction 17

2.5. Molecular Approaches to Molecular Ecology 18

2.5.1. The Need for Molecular Techniques 18
2.5.2. Advantages of Molecular Techniques 20
2.5.3. Advantages of DNA based Molecular Techniques 22
2.5.4. The Importance of 16S rRNA 23
2.5.5. Polymerase Chain Reaction (PCR) 24
2.5.6. Denaturant Gel Electrophoresis (DGGE) 25
2.5.7.Limitations of PCR and 16S rRNA based Molecular Methods 27
2.5.8. Clone Library or Cloning 30

3. MATERIALS AND METHODS 32

3.1. Sampling and Preservation 32

3.2. Chemical Analysis 33

3.3. Extraction of Genomic DNA from the Sediment Microbial Community 34

3.4. Polymerase Chain Reaction (PCR) 34

3.5. DGGE (Denaturant Gradient Gel Electrophoresis) 36

3.5.1. Preparation and Electrophoresis of the Denaturant Gradient Gel 37
3.5.2. Running the Gel 38

3.6. Generation of 16S rRNA clone libraries 40

3.7. Sequencing and Phylogenetic Analyses of 16S rRNA Gene Fragments 40

4. RESULTS 42

4.1. Chemical Analysis of the Sediments 42

4.2. Molecular Community Analyses Based on Clone Libraries 44

4.3. Genomic DNA Extraction and PCR Amplification 45

4.4. Archaeal Diversity of Adalar Sonrası Station (H1) 45

4.5. Bacterial Diversity of Adalar Sonrası Station(H1) 46

4.6. Archaeal Diversity of Eyüp Station (H2) 47

4.7. Bacterial Diversity of Eyüp Station (H2) 48

4.8. Archaeal Diversity of Valide Sultan Köprüsü Station (H3) 49

4.9. Bacterial Diversity of Valide Sultan Köprüsü Station (H3) 49

5.DISCUSSION 51

5.1. Evaluation of Microbial Community of Adalar Sonrası Station (H1) 52

5.2. Evaluation of Microbial Community of Eyüp Station (H2) 55

5.3. Evaluation of Microbial Community of Valide Sultan Köprüsü Station (H3) 57

6. CONCLUSION 59

REFERENCES 61

CURRICULUM VITAE 69

ABBREVIATIONS

DGGE	: Denaturant Gradient Gel Electrophoresis
TGGE	: Temperature Gradient Gel Electrophoresis
PCR	: Polymerase Chain Reaction
RFLP	: Restriction Fragment Length Polymorphism
ARDRA	: Amplified Ribosomal DNA Restriction Analysis
TS	: Total Solid
TVS	: Total Volatile Solid
TOC	: Total Organic Carbon
TC	: Total Carbon
EDTA	: Ethylene Diamine Tetra Acetic Acid
H3	: Valide Sultan Köprüsü
H2	: Eyüp
H1	: Adalar Sonrası

LIST OF TABLES

		Page No
Table 3.1	The primers and their annealing temperatures…….	35
Table 4.1	pH and electric potential of sediment samples……..	42
Table 4.2	Heavy metal concentrations of sediment sample……..	43
Table 4.3	Anion concentrations within the sediment……	43
Table 4.4	TS, TVS, TC, TOC, analysis…….	44

LIST OF FIGURES

Page No

Figure 3.1 : Locations of the Adalar Sonrası, Eyüp, Valide Sultan Köprüsü sampling points..... 32
Figure 3.2 : The Research ship ARAR, of İstanbul University and Van Ween grab sampler..... 33
Figure 3.3 : Components of the gel sandwich: gel clamps and spacers between two glass plates..... 37
Figure 3.4. : The gradient wheel..... 38
Figure 3.5. : Gel assembly with comb inserted..... 38
Figure 3.6. : Gels attached to the core assembly..... 39
Figure 3.7. : Bio-Rad DCodeTM system apparatus in action..... 39

PHYLOGENETIC ANALYSIS OF 16S rRNA FOR DETERMINATION OF MICROBIAL COMMUNITY IN ANOXIC MARINE SEDIMENTS OF HALİÇ

SUMMARY

Haliç has been the most polluted estuarine environment in Turkey that is exposed to textile, shipyard, pharmaceutical, domestic and gold processing wastewaters. The region is important because of its geological position that has been the most popular recreation area in İstanbul since Bizantium period.

The microbial community of the anoxic sediments of the three stations of Haliç was analysed for the first time in terms of chemical analyses and DNA-based molecular phylogenetic methods including Denaturing Gradient Gel Electrophoresis (DGGE) and performing Polymerase Chain Reaction (PCR) and cloning 16S rRNA genes amplified with *Bacteria* and *Archaea*-specific primers and sequencing.

The results indicated dominant archael clone sequences in Valide Sultan Köprüsü (H3) station belong to the orders uncultured archaeon (%29,5), Methanosarcinales (%16), uncultured Eucaryotha (%9,8), Methanomicrobiales (%8,1), in Eyüp (H2) station we identified members of uncultured archaeon (%26,6), uncultured eucaryotha (%13,3), Methanosaeta (%10), Methanomicrobiales (%10), Methanosarcina (%5), Methanogenic endosymbiont (%5), Methanosarcinales (%3,3), and Adalar Sonrası (H1) stations belong to order uncultured archaeon (%19,3), Methanosaeta (%16,1), Methanosarcinales (%11,2), Methanomicrobiales (%9,6), Methanospirillaceae (%9,6) were detected. Dominant bacterial clone types in H3 and H1 belonged to Epsilon and Delta-proteobacteria group. Chloroflexi was the dominant clone type in H1 station.

These results suggest that utilization and cycling of methane and sulfur compounds may play the major role in the energy production and maintainence of microbial communities in these highly polluted environments. Sequences of

organisms potentially associated with processes such as anaerobic methane oxidation and sulfate reduction were thus identified.

MİKROBİYAL KOMÜNİTENİN BELİRLENMESİ İLE HALİÇ ANOKSİK DENİZ SEDİMENTLERİNDE 16S rRNA'NIN FİLOGENETİK ANALİZİ

ÖZET

Haliç tekstil, altın işleme, evsel ve endüstriyel atıksuların deşarjı ve tersanenin bulunması sebebiyle Türkiye' deki nehir ağızları arasından en çok kirlenenidir. Bölge İstanbul ve hatta Bizans döneminden beri eğlence yeri olmasından dolayı jeolojik öneme sahiptir.

İlk defa olarak Haliç'in üç noktasından alınan anoksik deniz sedimentleri kimyasal analizler ve DNA'ya dayalı filogenetik metodlarla "Denaturing Gradient Gel Electrophoresis (DGGE)", arkea ve bakterilere spesifik oligonükleotid primerlerle Polymerase Chain Reaction (PCR) ve klonlama yöntemleriyle mikrobiyal kommünite analizi yapılmıştır.

Oluşturulan klon kütüphanesi sonuçları şöyledir: Valide Sultan Köprüsündeki dominant arkeal klonlar sırasıyla kültür edilmemiş arke % 29,5, , Methanosarsinales %16, kültür edilmemiş ökaryot %9.8, Methanomikrobiales %8.1, Eyüp bölgesinde kültür edilmemiş arke %26.6, kültür edilmemiş ökaryot %13.3, Metanosaeta %10, Metanomikrobiales %10, Metanosarsina %5, Methanogenik endosimbiont %5, Metanosarsinales %3.3, belirlendi ve Adalar Sonrası bölgesinde kültür edilmemiş arke %19.3, Metanosaeta %16.1, Metanosarsinales %11.2, Metanomikrobiales %9.6, Metanospirillase %9.6. Dominant bakteriyel klon tipleri Valide Sultan Köprüsü ve Eyüp bölgelerinde Epsilon ve Deltaproteobakteri grubuna aittir. Adalar Sonrası bölgesinde ise Klorofleksi dominant gruptur.

Bu sonuçlar gösteriyor ki metan ve sülfür bileşiklerinin bu yoğun kirli bölgelerdeki mikrobiyal kommünite tarafından kullanılması enerji üretiminin temelini oluşturur. Ortaya çıkartılan organizmaların sekans sonuçları potansiyel olarak anaerobik metan oksidasyonuna ve sülfat redüklenmesiyle alakalıdır.

1. INTRODUCTION

Estuaries are transition zones from the freshwater to the sea, serving as habitat, breeding reproducing grounds or nursery areas for many species (Okuş et al., 2006). They are special semi-enclosed systems displaying a wide range of physical and chemical properties (Apaydın et al., 2004).

Conditions in estuaries are highly variable. By the result of rainfall, overland runoff and other meteorological conditions, salinity fluctuates in a wider range and anthropogenic effects are stronger at the estuaries since water circulation is much more limited than the coastal ecosystem (Okuş et al., 2006).

Coastal marine environments such as estuaries represent an important link between land and the open sea. They receive nutrient input, as a result, they are characterized by intense primary production and heterotrophic activity (Saas et al., 2006). Haliç is a highly used estuary in Turkey and has long been important to civilizations of Istanbul. Haliç is located within an area whose topography is dipping towards South. Alibey and Kağıthane streams, flowing southwards, reach Haliç at its north end, where it is about 450 m wide. Below this location, Haliç extends first in a southwest direction to Eyüp and from that point on continues in southeast direction towards Bosphorus. On both shores of Haliç, flat lands of about 150-m width with a few meters elevation from sea level are observed. Western shores of Haliç gently slope inclining to heights of 40-60 m, whereas eastern shores have higher slopes reaching to 80-140 m elevations. Bottom elevations in Haliç are higher than those in Bosphorus and therefore Haliç can be considered as a suspended valley. The rise due to tectonic movements caused formation of three ridges in the sea bottom. It is postulated that the third ridge sets a boundary for rapid deposition and filling, and the sections of Haliç beyond this point are under the influence of active marine and current environment (Kınacı et al., 2005).

Haliç is surrounded by Black Sea, Bosphorus Strait (İstanbul) and Marmara Sea and is close to Bosphorus strait-Marmara Sea junction, extending in northwestsoutheast

direction. It is 7.5 km long, water surface area is 2,600,000 m^2 (Okuş et al., 2004) ,150–900 m width. The deepest point of the estuary is 40 m at the entrance and decreases below 10 m at inner parts where a 3–4 km zone was completely filled with runoff carried by two small streams until the early 1990's (Kouzimi et al., 2004; Avşar et al., 2004).

It is generally accepted that the formation of Haliç started approximately 8000 years ago with the flooding of ancient river valleys by waters of Mediterranean (Kınacı et al., 2005). It has been a favorite area of Istanbul's cultures for centuries due to its magnificent view throughout ages thus it plays an important role in social, touristic recreational activities (Okuş et al., 2004). Meanwhile its ecosystem faced pollution over centuries (Apaydın et al., 2004). Literature shows that the regulations concerning the control of pollution around Haliç date back to Byzantine period. Fatih Sultan Mehmet prepared a decree to prevent the filling and pollution of the estuary in the 1450s. On the other hand, the turning point for Haliç was 1950s. Yet rapid growth of population in Haliç area due to industrialization starting from the early 1950s resulted in uncontrolled and untreated discharges of pharmaceutical, detergent, dye and leather industries and domestic wastewaters (Okuş et al., 2006). These have led to an unacceptable pollution levels in the estuary (Kınacı et al., 2005).

Sediments are excellent indicators of environmental pollution, as they accumulate pollutants to the levels that can be measured reliably by a variety of analytical techniques. Since sedimentation is a continuous process, sediments also store records about the pollution history of given water body (Tuncer et al., 2001). Therefore sediments are excellent indicators of environmental pollution, as they accumulate pollutants (Apaydın et al., 2005). Slow sedimentation rates in deep-sea basins do not allow one to study evolution of pollution in recent history (but such core samples do provide information variation in concentrations over geological time-scales occurring through natural processes). Sediments with faster sedimentation rates in bays and estuaries are more suitable for investigating pollution history in the twentieth century as they provide higher resolution (Tuncer et al., 2001).

Elements are excellent tracers of industrial and domestic pollutions as they have fairly well-known and relatively simple chemistry in the marine environment. Levels of elements in dated core-slices give information on how discharges to a particular marine environment change in time. Since elemental compositions of discharges of

different industries are different, analysis of collected samples for a host of elements can allow one to differentiate between different sources. Source apportionment studies are extensively used in air pollution studies due to the conservative nature of trace elements in the atmosphere. However, source apportionment applications in the marine environment are more limited, because trace elements do undergo adsorption and redox processes and hence are not conservative. Sediment accumulation rate in Haliç, which was calculated as 3.5 cm yr^{-1} using ^{210}Pb dating technique, is significantly higher than those reported for various bays and estuaries in the literature, which are typically close to, but smaller than 1.0 cm yr^{-1} (Tuncer et al., 2001).

Haliç sediments, which consist mainly of clay, organic substances and heavy metals, are formed with the contribution of industrial and domestic wastes released in Haliç estuary (Çelik and Elbeyli 2004).

There are many studies focused on determination of microbial diversity by molecular phylogenetic tools (Barns et al., 2005; Fry et al., 2006; Lehours et al., 2007; Sjöling and Cowan 2003; Bowman and Cuaig 2003). The studies that are taken in Haliç are evaluation of metal pollution, heavy metal concentrations (Tuncer et al., 2001), toxic cyanobacterium Microcystic species that occur due to high nutrient inputs (Okus et al., 2004), anaerobic microbial diversity (Akarsubaşı et al., 2006) and geochemical characteristics of sediments of Haliç (Bodur and Ergin, 2003; Özger et al., 2007). This is the first detailed study that exposures microbial diversity in anoxic marine sediments by using molecular phylogenetic methods.

A significant number of studies dealing with microbial biodiversity involve the use of molecular tools. Conventional culture dependent methods are inadequate for analysis of natural environments because of their small size, the absence of distinguishing phenotypic characters, and the fact that most of these organisms cannot be cultured are the most important factors that limit the evaluation of their biodiversity (Dorigo et al., 2005). Only a small fraction of microorganisms can currently be cultured from environmental samples, and even if a microorganism is cultured, its role in a community and contribution to ecosystem function are not necessarily revealed (Hamme et al., 2003). Recently characterization of natural microbial communities by molecular methods based on 16S rRNA has been widely used to reveal intrinsic genetic diversity. In particular fingerprinting methods such as

DGGE, PCR amplified 16S rDNA fragments that encodes 16S ribosomal RNA (rRNA) gene have been found suitable for monitoring the microbial communities as their community structure can be disclosed by the motility of DGGE bands (Kouzumi et al., 2004; Mahmood et al., 2006).

The potential benefits of exploration of microbial diversity in anoxic marine sediments of Haliç derivate from future biotechnological exploitation of the Marmara Sea's gene pool and from new insights into the biological mechanisms of adaptation to and tolerance of extreme environments by microorganisms (Sjöling and Cwann, 2003). After the determination of bacterial and archaeal population, a bioremediation strategy could possibly be designed.

The objective of the study is determination of archaeal and bacterial communities by analysing 16S rDNA clone libraries. The analysis of clone libraries created from 16S rDNA amplified from environmental DNA can be used to assess community structure and diversity at the highest possible resolution, with 16S rDNA gene data sets providing a qualitative guide to the microbial composition of a given sample (Bowman and Cuaig, 2003). In this study molecular phylogenetic approaches (DGGE and 16 S rRNA clone library analysis) were used to examine the bacterial and archaeal composition of the microbial communities in the sediments of Haliç.

2. POLLUTION IN HALİÇ

2.1. Base Formation of Haliç

The base formation around Haliç area consists of greywacke containing sandstone-siltstone-claystone rocks. Young Haliç deposits (Holocene) are directly seated on graywacke rocks with a thickness reaching 50-60 m, and they are comprised of mainly dark gray colored silty clays and clayey silts. Sands and sandy gravel pockets are also encountered within these alluvial deposits. The upper levels of young alluvial deposits are highly polluted and rich in organic content because of municipal and industrial wastes discharged into this low energy environment. The shores of Haliç are covered with a thick artificial fill layer seated on these young deposits (Berlgen et al., 2006).

2.2. Pollutant Sources and Their Effects

The major pollution sources of Haliç can be listed as (i) debris carried by surface runoff stated that 60.000 to 80.000 m^3/year amount of alluvinium is entering Haliç that resulted in increasing of bottom level 6 to 10 cm every year, (ii) domestic waste waters in 1995 average flow rate of domestic waste water discharge was about 125.000 m^3 each day and as for the year 1997 no domestic waste water is discharged, (iii) industrial waste waters pharmaceutical, leather etc also effects of accumulated pollutants in the bottom sediments are still a major problem, (iv) effluents from ships, pollutant transport through surface runoff, (v) bottom sediment contains high amounts of organic and inorganic pollutants carried by untreated discharges (Kınacı et al., 1998).

The pollution in Haliç is one of the most important environmental problems of Istanbul (Kınacı et al., 1998). The rapid growth of population in Haliç area due to industrialization starts from the early 1950s and uncontrolled and untreated discharge of municipal and industrial waste until recent years have led to an unacceptable situation. The upstream part of Haliç became almost completely filled. Water depth

in this region was reduced to below 1.0 m, and large areas were turned into swamps.Therefore, Alibey and Kağıthane streams could no longer flow freely into Haliç, navigation became impossible even for small vessels, and due to lack of sufficient oxygen, a highly polluted environment with a dense odor was created (Berlgen et al., 2006). Until 1980's, most of the industrial plants located at both sides of Haliç were discharging their wastewaters and solid wastes. In that period, Alibey and Kagithane creeks were carrying heavy domestic and industrial pollution loads to Haliç. In the upstream, a stretch of 3–4 km length was almost filled of debris and organic solids. Odors produced as a result of anaerobic decomposition of organic matter in the sediment have created severe public nuisance (Apaydın et al., 2006). At the end of 1990's, the estuary became anoxic towards the upper parts (Kouzimi et al., 2004).

It is revealed that the metal pollution due to anthropogenic disturbance altered significantly within the second half of the century. In addition, the building of a dam on the stream weakened freshwater renewal. Furthermore, bridges, floating on large buoys and shipyards with large buoyant dry docks blocked circulation of upper layer and strengthen the pollution effect. Poor renewal of estuarine water and heavy nutrient load including numerous types of organic and inorganic effluents resulted in decreased biodiversity, with some pollution resistant macroalgae species (e.g Enteromorpha intestinalis) and planktonic organisms such as Ceratium spp. and Dinophysis caudata at the outer part of the estuary. The inner part, on the other hand, had only anaerobic life characterised by hydrogen sulfide formation. The anthropogenic pollution at the estuary not only adversely affected the communities living in the estuary but also human life, giving a heavy odor of hydrogen sulfide and an unaesthetic appearance to this once-recreational area (Avşar et al., 2004).

There are two main streams, Alibeyköy and Kağıthane, flowing into Haliç. These streams have a total drainage area of 380 km^2 and discharge approximately 50×10^6 m^3 of water and 59×10^3 m^3 of sedimentary material every year. In addition to river discharges, approximately 1.9×10^6 tons of liquid and 4.9×10^4 tons of solid wastes are discharged to Haliç from a total of 364 small and large scale industrial of pharmaceutical, detergent, dye, leather industries and domestic discharges. (Avşar et al., 2004). The accumulation rate was so high that, deposits started to emerge in the

inner parts of Haliç, and the whole watershed had to be dredged several times for the safe operation of marine vessels (Tuncer et al., 2001).

2.2.1. Ecological View of the Estuary

Phytoplantone: In 1986 Phytoplankton distribution was very weak and almost no plankton existed in the upper layer. Plankton studies later than 1998 clearly reflected the changing ecosystem of Haliç. The number of phytoplankton species increased from 44 species in 1998 to 60 in 1999, 81 in 2000 and finally to 92 in 2001 (Okuş et al., 2006).

Macroalgae: One of the earliest records of macroflora in Haliç dates back to the studies of Fritsch (1899) pointing out to a eutrophic estuary a century ago (Okuş et al., 2006).

Macrozoobenthos: Distributions of species clearly reflected the level of organic pollution at the estuary, particularly in the upper parts, first ichthyoplankton samplings was started in 1999 and all eggs and larvae collected appeared to be dead before sampling. However, ichthyoplankton rapidly diversified in the estuary parallel to the recovery of the upper layer. Mortality rates decreased from 100% in 1999 to 40% in 2002 (Okuş et al., 2006).

Fish: In 1996 fish distribution was limited to the lowermost region (around Galata Bridge). Nowadays the fish community of Haliç is once again significantly diversed (Okuş et al., 2006).

2.2.2. Reclamation Projects and Works

After 1980's, most of the industrial factories were moved from that area to newly built industrial regions. In 1999, after the construction of Northern and Southern sewerage trunk systems, pollution of the estuary was prevented (Apaydın et al., 2006).

2.2.3. Haliç Rehabilitation Project

In the beginning of 1997, Haliç Rehabilitation Project was initiated. Rehabilitation works includes the dredging of the sea bottom sediments at the upstream half to provide a minimum water depth of 5 m only wide channels should be dredged along

the courses of Alibeyköy and Kağıthane streams. Below these areas, the whole surface was dredged to reach a 5-m water depth (Berlgen et al 2006).

Haliç Project was contracted to a Turkish-American joint venture and the dredging was completed during the time scale planned in the contract. After the preparation of the disposal site at the rock quarry, dredging operations started in April 1997 and continued for about 12 months. In situ volume of the material to be dredged was estimated as 5.000.000 m^3 of the soft bottom sediments. The bulk of the dredged material was transferred via a pipeline to a quarry pit for disposal and land reclamation (Berlgen et al 2006).

Later, in May 2000, freshwater was released from the closest dam to the estuary for rapid oxygenation of the anoxic water body. Meanwhile, most of the domestic discharges were gradually connected to a collector system discharging deep into the lower layers of the strait, reaching deep water in the Black Sea. Finally, in May 2000, the floating bridge opened to ease water circulation (Avşar et al., 2004).

2.2.4. Results of the Rehabilitation Project

Following the onset of rehabilitation studies, the surface dissolved oxygen (DO) concentrations have raised significantly throughout the estuary. Minimum values were 3 mg l^{-1} in 1998 at Galata Bridge and rose to 5 mg l^{-1} in 2000. The heavy H_2S formation detected at the onset of the study disappeared in the following years and maximum DO reached hyper-saturation values after the partial opening of the bridge at the innermost parts, as a result of increased primary production. Total suspended sediment (TSS) values decreased from 80 mg l^{-1} to 10 mg l^{-1} in 2000 at mid-estuary, but remained around 25 mg l^{-1} in more upper parts due to high phytoplankton densities and influxes, particularly those originating from rainfall (Okuş et al., 2006).

Excessive nutrient concentrations throughout the estuary in 1998 were indications of intense pollution. Following the connection of numerous discharges to the collector system, the semi-opening of Valide Sultan Köprüsü (VSB) and release of freshwater from a dam located on the Alibeyköy Stream resulted in rapid renewal of the water body and the nutrient concentrations at the lower estuary approached the concentrations of the Strait of Istanbul. The level of change in the water quality can be best followed from the bacteriological data. High bacterial counts of 1998 approaching 10^7 CFU 100 ml^{-1} gradually decreased during the rehabilitation studies.

Bacteriological values decreased to 10^3 CFU 100 ml^{-1} by the end of 2001, with the exceptions in rainy periods and therefore it is can be concluded that rainfall became the dominant factor influencing the water quality within the estuary following the rehabilitation studies (Okuş et al., 2006).

The water quality has been highly changed as the pollutant loads have been sent out to this region by the existing discharge points and throughout the creeks functioning as open channels previously. At present, freshwater input to the estuary comes mainly from rainfall and its overland runoff (Okuş et al., 2002).

2.3. Marine Sediments

2.3.1. General Characteristics and Importance of Marine Sediments

Marine sediments cover more than two-thirds of Earth. Microbial cells and prokaryotic activity appear to be widespread in those sediments (Teske, 2006). Sediments play an important role in the remineralization of deposited organic matter in highly productive continental shelf areas (Amann et al., 2005).

There are two main classifications for marine sediments, terrigenous and pelagic. Terrigenous sediments are derived from land and found near shore. They are normally delivered by rivers to coastal regions. Pelagic sediments settle slowly out of the water column and are deposited all over the ocean. Pelagic sediments are deposited at such low rates that they tend to be overwhelmed near shore by terrigenous deposits from land. So, pelagic sediments are normally associated with deep sea regions (Berner, 1982). Microscopic analysis indicates that marine sediments are a heterogeneous environment of organic and inorganic particles of various sizes, bridged, or encompassed, by an organic matrix. Marine sediments ranging from sand to fine muds all consist of loose particles and interstitial spaces variously filled with water, air, detritus and organisms (Findlay et al., 1990).

As being the sub-seafloor biosphere is the largest prokaryotic habitat on Earth but also a habitat with the lowest metabolic rates that means microbial activity rates are very low, indicating that most prokaryotes may be inactive or have extraordinarily slow metabolism (Parkes et al., 2005).

Marine sediments play a significant role in the global cycling of carbon and nutrients. Organic matter from primary production settles to the sea floor, where a

major part is remineralized by microorganisms that colonize the sediments. Step redox potentials provide niches for a wide variety of metabolically diverse microorganisms (Ravenschlag et al., 2000). More than 85% of the ocean's organic carbon is deposited in shallow, anoxic marine sediments (Kendall et al., 2007). Sediments probably represent some of the most complex microbial habitats on Earth (Urakawa et al., 1999). Sulfate reduction is the major bacterial process in marine sediments, accounting for up to 50% of the total organic carbon remineralization (Ravenschlag et al., 2000).

The oxygen deficit is very close to the surface and anoxic conditions in the deepest regions, which favors the development of anaerobic communities that supplement the system with reduced gases such as methane and hydrogen sulfide (Briee et al., 2007).

2.3.2. Microbial Ecology in Marine Sediments

The prokaryotic species evolved more than 3.8 billion years ago and have continued to evolve to occupy almost any niche on the Earth (Muyzer, 1999). Microbial life is widespread in the marine sediments that cover more than two-thirds of Earth's surface and the prokaryotes of subseafloor sediments have been estimated to constitute as much as one-third of Earth's total living biomass (D'Hondt et al., 2004). Marine microbial communities were among the first microbial communities to be studied using cultivation independent genomic approaches (Lai et al., 2006).

The microorganisms classified in the two prokaryotic domains of the tree of life, Bacteria and Archaea, possess immense metabolic diversity, and their activities are critical in processes ranging from sewage treatment to regulating the composition of the atmosphere (Ward, 2002). Bacteria and Archaea in sub-seafloor sediments make up about 70% of the global number of prokaryotes, but these have not been extensively studied. Prokaryote populations are highest in shallow water, high productivity sites where the organic carbon content of the sediment is high, and lowest in deep water, low productivity sites. Despite lower population sizes in deeper layers prokaryotic activity can be measured and can account for the majority of depth integrated activity (Newberry et al., 2004). Microbial community structure analysis can be extended to give us an understanding of functional and biogeographical relationships and such data are vital for an improved understanding of sediment ecosystem processes and the role that the microbial community plays in overall sediment processes (Bowman and Cuaig, 2003).

Despite global dispersal of microorganisms, this number is probably rather large. The total biodiversity of an ecosystem is composed of two elements: first, a set of abundant taxa that carry out most ecosystem functions, grow actively and suffer intense losses through predation and viral lysis. These taxa are retrievable with molecular techniques but are difficult to grow in culture. Second, there is a seed bank of many rare taxa that are not growing or grow extremely slowly, do not experience viral lysis and predation is reduced. Such taxa are seldom retrieved by molecular techniques but many can be grown in culture, which explains the dictum ‘’everything is everywhere’’ (Pedros-Alios, 2006).

What we have learned from recent analyses of samples from environmental samples can be summarized as follows: (i) the cultured microorganisms represent only a small fraction of natural microbial communities and hence the microbial diversity in terms of species richness and species abundance is grossly underestimated, (ii) our understanding of microbial diversity is not represented by the cultured fraction of the diversity (Wintzingerade et al., 1997).

For seawater, the cultivable prokaryotic population size was a few hundred cells per milliliter. That was an almost inconsequential number relative to the thousands or tens of thousands of planktonic algal cells that could be seen (literally, with a microscope) in the same milliliter of water. So the answer to the question ‘‘how many are there?’’ led to intense interest in ‘‘who are they?’’ and ‘‘what are they doing?’’ When beginning from the last question prokaryotes play essential roles in the primary production and consumption of organic matter and the cycling of nutrient elements in the modern environment and were perhaps even more important in the evolution of the atmosphere and hydrosphere before the appearance of eukaryotes was provided by sensitive stable isotope and radiotracer methods: Woese introduced the now widely accepted three domain tree of life in which prokaryotes constitute two branches (Bacteria and Archaea) and Eukarya the third. ‘’Who are they?’’ became synonymous with ‘‘Where do they fall in the 16S rRNA-based tree of life?’’ because to determine the uncultivetad organisms the first ‘‘universal’’ primers for use in the polymerase chain reaction (PCR) were designed and used to pluck the 16S ribosomal RNA genes right out of the soil and water of their natural environments. Once those genes were sequenced, their evolutionary relationship is highlighted (Ward, 2002). There are now more than 40 major bacterial divisions

recognized and 12 or more in the Archaea and the tree is still growing (Tuncer et al., 2001). Approximately 6000 species of prokaryotes and 100.000 species of protists have been formally described (Pedros-Alio, 2006).

Microbial biodiversity has also received particular attention in areas where industrial applications are evident such as for marine, medical, and food biotechnology and where microbial activity has important implications for Earth's climate and for the bioremediation of polluted sites (Morris et al., 2002).

The difference of diversity and biodiversity is proposed by Marganef that biodiversity would be the total genetic information on Earth or in any part of it. Diversity, in turn, would be the components that are active and abundant at one particular time and place. A literary analogy would equate biodiversity with a dictionary of all the words in a language, whereas diversity would be the particular set of words. Some species were present every year and their abundance was high. They called these 'core' species. The remaining species had low abundance and were not found every year. These were 'occasional' species. Magurran and Henderson proposed that core species are responsible for carbon and energy flow in the ecosystem whereas occasional species wander sporadically to areas at their limits of tolerance and eventually disappear (Pedros-Alio, 2006).

The complexity of microbial communities may differ significantly, ranging from a single species, e.g. the sulfur-oxidizing bacterial ectosymbiont associated with the marine nematode Laxus sp., few species communities, e.g., iron-leaching species of the genus Thiobacillus to highly diverse communities like those of agricultural soils, municipal waste-treatment plants or peat. The range of microbial diversity can be initially estimated from:

(i) The number of different morphotypes determined by fluorescent microscopy following staining with specific dyes that bind DNA of living cells only, (ii) physical and chemical parameters of the sampling site like pH, temperature or supplied substrates, and (iii) prescreening of diversity by denaturing gel electrophoresis of amplified 16S rDNA (Wintzingerade et al., 1997).

It is essential to understand how microbial communities affect ecosystem functioning and how human activities, such as agriculture, waste management, and climate modification, affect microbial communities. Thus discovering and understanding the

diversity of microbial communities (the number of species and their relative abundances) is a high priority in ecology (Ward, 2002). However, the relationships between microbial communities and geological conditions in sub-seafloor environments remain poorly defined. Defining the diversity and distribution of natural microbial communities in deep-sea sediments has been a long-standing challenge in the field of microbial ecology and evolution (Lai e al., 2006). It has already been argued that because of their small size and large numbers, microbes should be easily dispersed everywhere; thus, immigration rates are expected to be large (Pedros-Alio, 2006).

2.3.3. Archaeal Community in Marine Sediments

Archaea are primarily a diverse and widespread group on Earth, which are found in our gardens and forests, in the ocean's plankton and sediment, in freshwater lakes and deep down in the subsurface. By contrast, archaea domains are the least understood in terms of their diversity, physiology, genetics, and ecology (Kim et al., 2005; Robertson et al., 2005).

Those organisms cultivated in the laboratory and studied in detail such as methanogens, thermophiles and halophiles represent only a minority of phylotypes and phenotypes (Robertson et al., 2005). The early overview of archaeal diversity was examplified by a phylogenetic tree that had three main branches, the Euryarchaeota, Korarchaeota and the Crenarchaeota. This viewpoint was established in the late 1970's and was based on cultured organisms (Kim et al., 2005; Robertson et al., 2005). As it is currently impossible to construct culture-based phenotypic characterizations of many environmental Archaea, the physiological significance of Archaea in nature has remained unknown for a long time (Kim et al., 2005). Among the first discoveries of PCR-based molecular ecological surveys was the detection of 16S rRNA of members of the Crenarchaeota in the marine plankton. Since then, novel archaeal 'phylotypes' (bacterial phylogenetic types) have been detected in most environmental surveys that have targeted archaeal sequences. The broad distribution and abundance of archaea in soils and oceans implies that they contribute to global energy cycles. However, these organisms have been predicted solely from PCR-based surveys and no representatives have been cultivated in the laboratory (Schleper et al., 2005).

At present, 77% of archaeal rRNA sequences are derived from environmental samples. Representatives of Archaea were found in wide variety of locations, in samples from ocean water, ocean sediments, solid gas hydrates, tidal flat sediments (Apaydın et al., 2006; Kim et al., 2005), freshwater lakes, soil, plant roots, peatlands, petroleum-contaminated aquifers (Harayama et al., 2004), to cite just a few reports. A common theme among known Archaea is use of hydrogen-based energy metabolism. Thus, such organisms are likely to be encountered in anoxic environments, often in syntrophic association with other organisms (for instance engaged in interspecific hydrogen transfer) (Robertson et al., 2005).

When the phylogenetic features intrinsic to archaeal communities are related to the environment, they may provide important insights into the physiological functions and ecological roles of communities (Kim et al., 2005).

2.3.4. Bacterial Diversity in Marine Sediments

Only an estimated 20% of the naturally occurring bacteria have been isolated and characterized so far (Muyzer et al., 1992). Sedimentary bacteria live in a physically and chemically complex environment and numerous factors have been shown to influence their distribution. Microscopic sediment grain size, the mineral nature of the grains, and the microtopography of the grains have been shown to influence the bacterial biomass in marine sediments major. Early diagenetic reactions result from microbiological decomposition of organic matter and this activity produces many complex gradients within the sediments which in turn affect the distribution and metabolic activities of the bacteria there (Findlay et al., 1990).

The estimation of bacterial diversity is required for understanding of microbial biogeography, community assembly, and ecological processes (Stach et al., 2003). Sediment bacteria also play a significant ecological and biogeochemical role in marine ecosystems for they are instrumental in the marine food web, where they are the key players for recycling of nutrients and degradation of pollutants. This is largely a result of their high abundance relative to the overlaying water column and their key function in mediating and regulating the transformation and speciation of major bioactive elements (e.g. carbon, nitrogen, phosphorus, oxygen, and sulphur) in these environments (Polymenakou et al., 2005). Sediment bacteria also represent a major genetic variability with a local diversity equal to soil systems (Torsvik and Ovreas, 2002). Enrichments from deep sediments have enabled novel organisms to

be isolated, but, most probable, number counts of bacteria have been very low, with a low viability. (Newberry et al., 2004).

2.3.5. Biological Role of Bacteria and Archaea

Understanding the factors that control the composition and diversity of microbial communities is important to understand how bacterial populations function to facilitate biogeochemical processes (Humayoun et al., 2003).

Many of the microorganisms are thought to be key components in the biogeochemical cycling of elements such as carbon and nitrogen and, therefore, to play a significant role in the biosphere. Furthermore, they intervene in the decontamination of wastewaters released into the environment from urban, industrial, and agricultural activities, thus contributing to maintaining water quality (Briee et al., 2007). The microorganisms in the sediment play a significant role in remineralization of organic matter within the aquatic ecosystem. For example, bacterial populations are the major contributors in the transformation of organic carbon, sulfur, nitrogenous compounds, and metals, with an important role in ecosystem food webs and nutrient cycling (Sjöling and Cowan, 2003).

Before the development of sequence-based methods, it was impossible to know the evolutionary relationships connecting all of life and thereby to draw a universal evolutionary tree (Pace, 1997). In the mid-1900s publications of R. H. MacArthur and G. E. Hatchinson spurred the field of ecology in the intense reserch and debate about significance of biodiversity. These and other workers claimed that biodiversity is a measure of important ecological processes such as resource partitioning, competition, succession and community productivity and is also an indicator of community stability. Bacteria and Archaea in sub-seafloor sediments make up about 70% of the global number of prokaryotes, but these have not been extensively studied. Prokaryote populations are highest in shallow water, high productivity sites where the organic carbon content of the sediment is high, and lowest in deep water, low productivity sites (Ravenschaleg et al., 2000).

Microbial diversity has also received particular attention in areas where industrial applications are evident, such as for marine, medical, and food biotechnology and search for new bioactive compounds in biochemical cycles and their influence on issues such as global climate change and for the bioremediation of polluted sites

(Dorigo et al., 2005; Morris et al., 2002). Unknown diversity hides novel metabolisms (such as the recently discovered photoheterotrophy in the sea) that force a re-evaluation of carbon and energy fluxes in the oceans. These processes must be understood so that, for example, precise models of global change can be constructed. Contribute to the unknown microorganisms are the largest potential reservoir of useful genes for medicine and biotechnology (Pedros-Alio et al., 2006). The estimation of bacterial diversity is required for understanding bacterial biogeography, community assembly, and ecological processes (Stach et al., 2003). In studies of the structure and function of aquatic ecosystems, reliable estimates of microbial numbers, diversity, and activity are critical (Sjöling and Cowan 2003).

2.4. Major Anaerobic Processes in Marine Sediments

2.4.1. Anaerobic Oxidation of Methane

Methane is a critical gas in the atmosphere; it currently accounts for >20% of the anthropogenically enhanced greenhouse effect, it is important in regulating the concentration of hydroxyl radicals (the atmospheres primary ‘’scrubbing’’ agent) and its oxidation is a major source of the water vapor in the stratosphere that is cirtical in forming polar stratospheric clouds, which play an important zone in ozone depletion (Schimel, 2005). In the methane budget proposed by Reeburgh (1996), more than 80% of the methane produced annually in marine sediments is consumed mostly in anoxic environments before it can reach the atmosphere (Hinrichs and Boetius, 2002).

More than 85% of the ocean’s organic carbon is deposited in shallow, anoxic marine sediments (Berner; 1982). Microbially mediated oxidation of methane in anoxic marine systems is a globally significant process, with up to 90% of the oceanic methane production recycled in anaerobic marine sediments (Holmer and Christensen, 1994). As the major biological sink of methane in marine sediments, the microbially mediated anaerobic oxidation of methane is crucial in its role of maintaining a sensitive balance of the atmosphere's greenhouse gas content (Hinrichs and Boetius, 2002). Methanogens play an important role in the degradation of this organic matter by converting low molecular weight compounds into methane (Kendall et al., 2007).

Molecular and isotopic data suggest that most of the methane produced in marine sediments is of biogenic origin; however, methanogens that inhabit permanently cold marine sediments have been poorly characterized and the contribution of marine systems to the global methane cycle is generally poorly understood (Kendall et al., 2007; Mc Donald et al., 2004).

Methane concentrations and oxidation rates have been determined from many oceanic sites, and these have shown the upper ocean is usually methane supersaturated with respect to the atmosphere (Mc Donald et al., 2004). In marine sediments, methane is present in dissolved form in the pore water, as free gas, or trapped within methane hydrates (Kendall et al., 2007). Both methane fluxes and oxidation rates in stratified water columns and at the air–sea interface suggest that biological oxidation is occurring in the sea although the methane turnover time may be slow (Mc Donald et al., 2004). Most of the methane encountered in the outer few kilometers of Earth's crust or on the surface is determined by isotopic analysis to be the product of methanogenic Archaea, past and present (Berner, 1982).

2.4.2. Sulfate Reduction

In marine sediments, sulfate reduction is the predominant terminal electron-accepting process in carbon metabolism quantitatively equivalent to or exceeding aerobic respiration (Smith and Klug, 1981; Tekse at al., 1991). Although sulfate reduction is of significance to carbon and electron flows in both of these ecosystems, the key electron donors utilized by natural populations of sulfate-reducing bacteria have not been definitively delineated (Smith and Klug, 1981).

The anaerobic mineralization of organic matter in marine sediments primarily occurs via sulfate reduction, and methanogenesis usually accounts for less than 5% (Holmer and Kristensen, 1994).

Sulfate-reducing bacteria are universally distributed in marine sediments and microbial mats (Smith and Klug, 1981). Sulfate-reducing prokaryotes constitute a diverse group of anaerobic microorganisms that share the exclusive ability to use sulfate as terminal electron acceptor while oxidizing various carbon sources. Dissimilatory sulfate reduction has a central role in the global sulfur cycle and represents one of the most important organic matter mineralization processes in marine and freshwater habitats (Bodelier and Miletto, 2007). Sulfate-reducing

prokaryotes presence was also demonstrated in oxic and anoxic biofilms, microbial mats, and wastewater treatment systems (Bodelier and Miletto, 2007). Sulfate-reducing bacteria can potentially compete with methanogenic bacteria for H_2 and acetate in both marine and freshwater sediments and have been demonstrated to oxidize the major portion of added hydrogen in marine sediments (Smith and Klug, 1981).

2.5. Molecular Approaches to Molecular Ecology

2.5.1. The Need for Molecular Techniques

Unfortunately, the study of microbial biodiversity is stil hampered by the difficulty of identifying and characterizing microorganisms prior to their isolation in pure culture (Giraffa and Neviani, 2001). For many decades it has been obvious to microbiologists that validly described prokaryotic species do not reflect the actual diversity of prokaryotic species in the environment (Wintzingerode et al., 1997). Uncultured organisms comprise the vast majority of the microbial world (Nocker et al., 2006).

Conventional culture dependent methods are inadequate for analysis of natural environments because of prokaryotic organisms small size, the absence of distinguishing phenotypic characters (Dorigo et al., 2005), lack of growth factors oxidative stress damage and, the formation of viable but ‘nonculturable’ cells unbalanced growth caused by a substrate shortage or excess, and the induction of lysogenic phages during starvation (Harayama et al., 2004) and the fact that most of these organsms can not be cultured and the existance of high numbers of unculturable bacteria (Kouzimi et al., 2004), are the most main factors that limit the evaluation of their biodiversity. A small fraction of microorganisms can currently be cultured from environmental samples, and even if a microorganism is cultured, its role in a community and contribution to ecosystem function are not necessarily revealed (Hamme et al., 2003). So far only 0.5% to 10% of the entire prokaryote biodiversity has actually been identified (Dorigo et al., 2005). Some of the traditional culture dependent microbiological methods are based on plate counts, isolation and biochemical identification (Rantsiou et al., 2005) and morphological criteria, such as optical, epifluorescence, or electron microscopy, can barely discriminate between these organisms, even at the class level (Diez et al., 2001).

Bacteria retrieved by culture-dependent methods are generally quite different from those identified by the culture-independent rRNA approaches. The rDNA sequences of bacteria that encode rRNA of the organism, obtained by culture-dependent methods rarely appear in the rDNA library. It has been concluded that the bacteria in seawater cannot be readily cultured and that bacteria isolated by culture-dependent methods represent a minor population in natural bacterial communities. Another hypothesis of 'uncultivability' is that microorganisms that cannot be cultivated might grow in a pure culture if the chemical components required for their growth are provided by other microorganisms. Lack of cell-to-cell communication may be one of the reasons why certain bacteria cannot be grown in axenic cultures. In gram-negative bacteria, *N*-acyl homoserine lactones are the signal molecules for cell-to-cell communication. The addition of *N*-acyl homoserine lactones was effective for increasing the cultivability of marine bacteria. A more striking increase in the cultivability was observed by the application of cyclic AMP (cAMP), which led to a cultivation efficiency of up to 100% of the total bacterial count. cAMP was found to be effective in the resuscitation (recovery of cultivability) of starved bacteria: when starved cells of a marine bacterial strain were cultivated in the presence of cAMP a higher growth rate was obtained than in the presence of AMP (Harayama et al., 2004).

The culture-dependent methods used to assess microbial diversity are inadequate as only liquid enrichment cultures were used that is not sufficient to study the microbial diversity in environmental samples (Smith et al., 1991). Selective enrichment cultures fail to mimic the conditions that particular microorganisms require for proliferation in their natural habitat. Furthermore many microorganisms are bound to sediment particles and are thus not detected by conventional microscopy (Muyzer at al., 1992). In fact only 0.1% to 10% of the total population present in environmental samples could be cultivated and different media have certain selective characteristics for different groups of organisms (Smith et al., 1991). Any deviation from the original environmental parameters during cultivation, such as the use of enrichment culture techniques, could alter the community structure through the imposition of new selective conditions. In most cases, therefore, it seems that we can not obtain a complete view of the microbial diversity of a given ecosystem because we are able to

characterise only a part of it. These limits restrict and bias in an unpredictable way our view of microbial diversity (Giraffa and Neviani, 2001).

The recent increase in awareness of our inability to cope with microbial diversity is due to a leap in methodologies (e.g. molecular cloning, polymerase chain reaction (PCR), DNA probing etc.). The development of concepts allowed biologists to come to a unified view of the genealogy of all living material (Muyzer, 1999).

This development can best be demonstrated by the revolutionary progress made in the use of ribosomal RNAs and their genes as reliable phylogenetic markers for the assessment of the natural relation between isolated and uncultured prokaryotes combined with improved PCR and sequencing technology (Muyzer, 1999). Consequently the limitations of current culture methods mean that molecular approaches must be used if the full diversity of prokaryotes in the deep biosphere is to be explored (Newberry et al., 2004).

2.5.2. Advantages of Molecular Techniques

For the past 20 years, the increased development and routine application of molecular-based techniques has made it possible to carry out detailed evaluations of the biodiversity of aquatic microbial communities (Dorigo et al., 2005). Molecular techniques provide an outstanding tool for the detection, identification, and characterisation of microorganisms involved in various environmental ecosystems (Newberry et al., 2004; Schmidt et al., 1991; Horikoski et al., 1991; Deverauxi snd Mundfrom, 1994; Lehours et al., 2007; Sjoling and Cowan, 2003). They have often focused on investigating the dynamics of the composition and structure of microbial populations and communities in defined environments, and the impact of specific factors, such as pollution by xenobiotics, on microbial diversity (Dorigo et al., 2005).

In contrast to animals and plants, the morphology of microorganisms is generally too simple to serve as basis for a sound classification and to allow reliable identification. Thus, until very recently, microbial identification required the isolation of pure cultures (or defined cocultures) followed by testing for multiple physiological and biochemical traits. Culturability is determined as a percentage of culturable bacteria in comparison with total cell counts are 0,001-0,1% in seawater, 0,25% in freshwater, 0,1-1% in mesotrophic lakes, 0,1-3% in unpolluted estuarine waters, 1-15% in activated sludges, 0,25% in sediments, 0,3% in soil (Amann et al., 2005).

The advent of culture-independent methods, such as molecular biological techniques, has changed our view of microbial diversity (Dorigo et al., 2005). Molecular methods that based on 16S rRNA are highly sensitive and allow for a high degree of specificity, which, coupled with the ability to separate similar but distinct DNA molecules, means that a great deal of information can be gleaned from even very complex microbial communities (Spiegelman et al., 2005). The new discipline of molecular ecology offers the potential of determining the whole range of prokaryotic taxa without running into the problems of selective laboratory enrichment and growth media. Before the development of sequence-based methods, it was impossible to know the evolutionary relationships connecting all of life and thereby to draw a universal evolutionary tree. "Evolutionary distance" is the extent of sequence change, that the phylogenetic tree can be considered a rough map of the evolution of the genetic core of the cellular lineages that led to the modern organisms (sequences) included in the tree (Berner, 1982). Today, molecular genetic analysis of rDNA obtained from DNA extracted from natural habitats is routinely used in many laboratories world wide and a broad range of otherwise similar strategies are applied without prior cultivation and isolation of the organisms. Originally, emphasis was placed on targeting ribosomal RNAs from environmental specimens that were analyzed by hybridization of extracted rRNA or by direct rRNA sequencing (Muyzer, 1999). In recent years characterization of natural microbial communities by molecular methods based on 16S rRNA have been widely used to reveal intrinsic genetic diversity. The 16S ribosomal RNA (rRNA) gene was employed as a marker for diversity (Hejs et al., 2007).

Comparisons of nucleotide sequences of ribosomal genes from different organisms allowed inference of the evolutionary relationships between the organisms: the greater the similarity or difference between the rRNA sequences, the more or less closely related the organisms are (Robertson et al., 2005).

Culture-independent methods may detect species that are missed by plating, provided that the amplification efficiency is high enough (Kisand and Wikner, 2003). In particular fingerprinting methods such as denaturant gradient gel electrophoresis (DGGE), PCR amplified 16S rDNA fragments have been found suitable for monitoring the successive in microbial communities as their community structure can be disclosed by the motility of DGGE bands (Kouzimi et al., 2004; Mahmood et

al., 2006). Two major discoveries have revolutionized microbial ecology and have permitted culture-independent characterization of microbial communities

1. The recognition that phylogenetic relationships between microorganisms can be inferred from molecular sequences and

2. the ability to selectively amplify minute amounts of nucleic acids extracted from environmental samples by the polymerase chain reaction (PCR) (Roling and Head, 2005).

A significant number of studies dealing with microbial biodiversity involve the use of molecular tools (Dorigo et al., 2005; Dhung et al., 2004; Lehours et al., 2007; Scholten et al., 2005; Barns et al., 2005; Knittel et al., 2005; Heijs et al., 2007). The sequences of rRNAs from naturally occuring organisms are compared with known rRNA sequences by using techniques of molecular phylogeny. (Schmidth et al., 1991)

Several methods were developed for the assessment of genetic diversity which include (Muyzer, 1999) PCR amplification of a target, which is generally located within the ribosomal operon which is achieved by means of primers that are specific to the organisms of interest (Dorido et al., 2005).

Sequence analysis of randomly-picked clone inserts, hybridization with taxonspecific probes, restriction fragment length polymorphism (RFLP), and amplified ribosomal DNA restriction analysis (ARDRA) of clones or provides a visual separation of the mixture of fragments according to sequence polymorphism (Dorido et al., 2005) by amplified rDNA by gel electrophoresis (DGGE or Temperature Gradient Gel Electrophoresis (TGGE)). Also, the sample volume required for analysis is significantly reduced and micro-habitats are now open for investigation (Muyzer, 1999).

2.5.3. Advantages of DNA based molecular techniques

A survey of the available literature on methods most frequently used for the identification and characterization of microbial strains, communities, or consortia is presented. The advantages and disadvantages of the various methodologies were examined from several perspectives including technical, economical (time and cost), and regulatory. The methods fall into 3 broad categories: molecular biological, biochemical, and microbiological. Molecular biological methods comprise a broad

range of techniques that are based on the analysis and differentiation of microbial DNA. This class of methods possesses several distinct advantages. Unlike most other commonly used methods, which require the production of secondary materials via the manipulation of microbial growth, molecular biological methods recover and test their source materials (DNA) directly from the microbial cells themselves, without the requirement for culturing. This eliminates both the time required for growth and the biases associated with cultured growth, which is unavoidably and artificially selective. The recovered nucleic acid can be cloned and sequenced directly or subpopulations can be specifically amplified using polymerase chain reaction, and subsequently cloned and sequenced (Spiegelman et al., 2005). In the past few years, due to the use of molecular approaches, our knowledge on microbial diversity in complex, mixed microbial communities has dramatically increased (Giraffa and Neviani, 2001).

2.5.4. The Importance of 16S rRNA

The use of 16S rRNA has been an important step of microbial ecology and has led to a wealth of information concerning prokaryotic diversity (Huri and Chuni, 2004). Because of the properties of the gene that are; present in all prokaryotes, high information content, and good agreement with most physiological and genetic markers (Giovannoni et al., 2003), large amount of sequences available (Sjoling and Cowan, 2003) and its product shows functional constancy ribosomal RNA molecule is using, for constructing global phylogenies of all living things (Ward, 2002). 16SrRNA is part of the ribosomes that are required by all organisms to synthesize protein so that this gene is a very suitable molecular marker (Roling and Head, 2005), for giving an understanding of functional and biogeographical relationships, and such data is vital for an improved understanding of ecosystem processes and the role of bacteria play in various environments (Huri and Chuni, 2004). The gene is sufficiently long (1.5 kb) to be used as a document of evolutionary history and evidence for horizontal transfer of rRNA genes is limited. Due to the functional necessity of rRNAs, their primary and secondary structure is constrained. 16S rRNA consists of several sequence domains that have evolved at different rates some domains have remained almost universally conserved and are interspersed by more variable regions specific for phylum up to subspecies level. This permits unambigious alignment of homologus positions in a sequence and the identificaiton

of near universally conserved and taxon-specific ‘signature’ sequence motifs (Roling and Head, 2005).

2.5.5. Polymerase Chain Reaction (PCR)

PCR allows the selective amplification of small portions of DNA extracted from natural samples. It is generally used as the first step in phylogenetic analysis of microbial communities and for many other molecular ecological approaches (Roling and Head, 2005). The polymerase chain reaction is a powerful technique that has rapidly become one of the most widely used techniques in molecular biology because it is rapid, inexpensive and simple way of copying specific DNA fragments from minute quantities of source DNA material even when that source DNA is of relatively poor quality. The technique amplifies specific DNA fragments from minute quantities of source DNA material, even when that source DNA is of relatively poor quality (Polymenakou et al., 1998). Due to the power of the PCR to amplify small amounts of DNA, organisms occurring in small numbers in an environment are now detectable (Muyzer, 1999).

Primers annealing to conserved regions of the rDNA were used to amplify the variable V3 region flanked by the primers.(Novel techniques for analysing microbial diversity in natural and perturbed environments). It is nowadays generally used as the first step in phylogenetic analysis of microbial communities and for many other molecular ecological approaches (Roling and Head, 2005).

There are 3 steps in PCR reactions:

Denaturation:

During the denaturation (at 94^{o}C), the double strand melts open to single stranded DNA, all enzymatic reactions stop (for example: the extension from a previous cycle).

Annealing:

The reaction mixture is cooled down to 54°C. The primers jiggle around, caused by the Brownian motion. Ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), polymerase can attach and start copying the template. Primers anneal to the complementary regions in the DNA template strands, and double

strands are formed again between pimers and complementary sequences. Once there are a few bases built in, the ionic bond is so strong between the template and the primer, that it does not break anymore.

Extension:

The ideal working temperature for the polymerase is 72°C. The DNA polymerase synthesizes a complementary strand. Primers (where there are a few bases built in), already have a stronger ionic attraction to the template than the forces breaking these attractions. Positions of primers with no exact match get loosen again (because of the higher temperature) and (do not) give an extension of the fragment. The DNA polymerase synthesize a complementary strand
The bases (complementary to the template) are coupled to the primer on the 3' end (the polymerase adds dNTP's in 5' - 3' direction, reading the template from 3' - 5' direction, bases are added complementary to the template) The final PCR products obtained contain a mixture of multiple copies of the same fragment amplified at the chosen taxonomic level (strain, species, genus, etc.) (Dorigo et al., 2005).

PCR performance is influenced by the concentrations of the individual components including the magnesium ion concentration, the type of DNA polymerase, concentration and properties of primers and template in the PCR mixture, as well as the length of the product. Standard PCR conditions are generally adequate for the amplification of rRNA genes, though techniques such as hot start and touchdown PCR can reduce mispriming and increase specificity of amplification. Neverthless in some instances high specificity may not be desired (Roling and Head, 2005).

2.5.6. Denaturant Gradient Gel Electrophoresis (DGGE)

Denaturant Gradient Gel Electrophoresis (DGGE) was first introduced 10 years ago (Cody et al., 2000). Ward and coworkers were among the first who used DGGE of 16S rDNA fragments to study population changes in microbial communities.

It is now routinely used to assess the composition and diversity of microbial communities by analyzing PCR-amplified 16S rRNA fragments (Cody et al., 2000; Muyzer, 1999) and analysis of multiple samples which allows monitoring their dynamics that microbial communities may undergo by diel and seasonal fluctuations or after environmental perturbations and profile complex microbial communities (Dorigo et al., 2005; Fernis et al., 1996 and Muyzer, 1999). DGGE technique has

been used to characterize the microbial diversity in different environments such as activated sludge (Polymenakou et al., 2005), sediments (Kim et al., 2005), lake water (Lehours et al., 2007), hot springs (Ferris et al., 1996), soils (Torsvik and Ovreas, 2002), food associated ecosystems (Giraffa and Neviani, 2001), microbial fuel cell systems (Dehung et al., 2004).

In DGGE small PCR products (200-700 bp) are separated on an acrylamide gel having a low to high denaturant gradient (Nocker et al., 2006). The method is sufficiently sensitive to DNA duplexes that differ by only a single base pair and each band produced potentially represents a distinct bacterial population (Cody et al., 2000). DNA molecules that have the same length, but differ at least by one nucleotide, can be separated by electrophoresis through a linear gradient of increasing chemical denaturants of urea and formamide (Dorigo et al., 2005). Separation of DNA fragments is based on the decreased electrohoretic mobility of partial melted double-stranded DNA molecules (Muyzer, 1999). The two strands of a DNA molecule separate or melt at a specific temperature called melting temperature (Tm). The melting behavoir depends mainly on the length of the product, its GC (guanine and cytosine) content and the nucleotide sequence. And the physical shape of the molecules directly affects their mobility during electrophoresis (Nocker et al., 2006). If individual domains is sequence specific, which depends on hydrogen bonds formed between complementary base pairs (GC-rich domains melt at higher temperatures) and neighboring on the bases on the same strand. When run on a polyacrylamide gel, the mobility of the molecule is retarded when the first melting domain is reached, resulting in partial dissociation of the fragment. Complete strand separation is prevented by the presence of a high melting domain, known as s GC clamp, which is added to one primer (Dorigo et al., 2005). DGGE has heen used to determine the genetic diversity of total microbial communities or particular populations without further characterization of the individual inhabitants (Muyzer, 1999). The major advantage of DGGE is that it allows direct determination of bacterial genetic diversity, making it superior to cloning and subsequent sequencing (Cody et al., 2000).

Most DGGE/TGGE (temperature gradient gel electrophoresis) studies focussed on the number of bands as an estimate of community diversity, while little attention was given to quantification of band intensity. The number of bands in the gel was a

measure for ‘richness’ of community diversity, whereas the proportional abundance (‘evenness’) of the different sequence-defined populations was calculated from the intensity of the bands. Different diversity indices were found for different communities. These results were supported by similar diversity indices for the different mats obtained by two other cultivation-independent techniques (i.e. microscopical observation of morphotypes and HPLC (High Pressure Liquid Chromatograpy) analysis of carotenoids) (Muyzer, 1999).

The major advantages of DGGE are that it alows direct determination of microorganisms’ genetic diversity, making it superior to cloning and subsequent sequencing and there is no need for specific sequence information to create a probe (Cody et al., 2000; Muyzer, 1999). PCR-DGGE can also be used as a tool to follow the successful isolation of bacterial strains in pure cultures, such examples demonstrate how the combined use of PCR-DGGE/TGGE together with existing and new isolation strategies (e.g. the serial dilution technique can provide a new impulse in the isolation of ecologically relevant microorganisms (Muyzer, 1999).

DGGE also has some limitations, for instance, (the detection of heteroduplex molecules and molecules produced by different rRNA operons of the same organism.) Furthermore, separation of relatively small DNA fragments, co-migration of DNA fragments with different sequences, and limited sensitivity of detection of rare community members can cause problems. Some of these limitations such as the presence of heteroduplex molecules are not common found. However other limitations such as the limited sensitivity can be improved by hybridization analysis or by the application of a group-specific PCR (Muyzer, 1999).

2.5.7. Limitations of PCR and 16S rRNA based molecular methods

16S rRNA-based phylogenetic analysis allows the most detailed insight into microbial community composition. However, each physical, chemical and biological step involved in the molecular analysis of an environment is a source of bias which will lead to a distorted view of the ‘’real world" (Wintzingerode et al., 1997; Muyzer, 1999).

An exciting new direction in molecular microbial ecology is the analysis of enzyme encoding genes. These genes usually have more sequence variation than the relatively conserved 16s rRNA encoding genes, and might, therefore, be better

molecular markers to discriminate between closely related but ecologically different populations. Moreover, the use of functional genes makes it possible to study the specific activities of bacterial populations (Muyzer, 1999).

Some disadvantages of 16S rRNA gene are the heterogeneity of the gene between multiple copies within one species and hampers pattern analysis, and can confuse the interpretation of diversity from clone libraries and sequences retrieved from banding patterns (Sjoling and Cowan, 2003).

Although PCR is a routine method used for pure cultures, several problems arise when the methods are applied to mixed cultures. These problems are: (i) inhibition of PCR amplification by co-extracted contaminants, (ii) differential amplification, (iii) formation of artefactual PCR products, and one has also to consider that (iv) presence of contaminating DNA, and (v) 16S rRNA sequence variations due to *rrn* operon heterogeneity (that could unavoidably lead to a biased reflection of the microbial diversity. Humic acids or humic substances co-extracted with nucleic acids strongly inhibit DNA modifying enzymes (Wintzingerade et al., 1997). The PCR amplification step is known to introduce other biases and artifacts associated with cell lysis, mutations, deletions, chimeras nucleic acid extraction, choice of primer and amplification errors, irrespective of the gene targeted (Mahmood et al., 2006; Roling and Head, 2005) as it suggests the existence of organisms that do not actually exist in the sample investigated (Wintzingerade et al., 1997). Several types of PCR artefacts have been reported: (i) chimeras between two different homologous molecules, (ii) deletion mutants due to stable secondary structures, and (iii) point mutants due to misincorporation by DNA polymerases (Sjoling and Cowan, 2003).

Chimeras between two different DNA molecules with high sequence similarity (i.e. homologous genes) can be generated during PCR process as DNA strands compete with specific primers during the annealing step (Wintzingerade et al., 1997).

It is well known that PCR templates containing stable secondary structures often yield very low amplification efficiency or deletion mutagenesis in PCR products. As ribosomal RNAs usually exhibit intensive secondary structures (Wintzingerade et al., 1997). It was recently shown that the original sample template is amplified during the initial 5–6 cycles of the PCR reaction, and that in the following cycle's

amplification occur only on the PCR fragments produced earlier (Sjoling and Cowan, 2003).

Contaminating DNA, containing the specific target sequence of the PCR reaction, can lead to both amplification in negative controls without external DNA being added and co-amplification in experimental reactions (Wintzingerade et al., 1997).

Primer specificity is a major stumbling block, especially when attempting to quantify a mixture of homologous target sequences. Constructing truly general primers has proven difficult, and even single mismatches in the middle of the primer can cause a preferential selection. Lowering the annealing temperature allows for mismatches and increases diversity in the PCR product, but also increases the risk of unwanted by-products. Degenerate primers could be used, but these are not suitable for techniques like double-gradient gel electrophoresis as they produce multiple bands from one template, which gives the same problems as when using heterogeneous genes (Sjoling and Cowan, 2003). The method is time-consuming (Roling and Head, 2005), a number of laborous sequences are large enough to cover a whole community (Huri and Chuni, 2004). It is expensive, not reliably quantitative, is not necessarily informative regarding community function (Roling and Head, 2005) and generate only limited and may be problematic, especially when a sample with a highly diverse community structure is considered (Huri and Chuni, 2004). DNA polymerase enzymes are not 100% accurate and introduce point mutations due to intrinsic misincorporation of nucleotides during PCR (Roling and Head, 2005). Enzymes such as *Pyrococcus furiosus* (*Pfu*) DNA polymerase that have a 3' - 5' proofreading function have very low rates of nucleotide misincorporation. More commonly used enymes, such as *Taq* DNA polymerase from *Thermus aquaticus*, have a higher processivity but lack a proofreading exonuclease activity and hence have lower fidelity. The percentage of sequences with polymerase errors increases with the number of PCR cycles and the length of the amplified fragment. Due to stable secondary structures in rRNA, deletions can easily occur during PCR, resulting in smaller-sized PCR fragments (Roling and Head, 2005).

Some problems can occur in the sampling step. Extreme habitats, such as hot vents and deep marine sediments, as well as sampling sites which require extensive sampling effort, e.g. the marine environment, may require additional effort to collect, store or process samples at the sampling site. Special care must be taken during their

transport to the laboratory to avoid loss of nucleic acids due to lysis of specimens (Wintzingerade et al., 1997). However, this genetic diversity is not equivalent to species diversity as defined in ecology since quantification of the various types of bacteria is not possible because of the biases of PCR; the number of fragments detected is more comparable to species richness. Amplified 16S rDNA fragments are distinguishable using DGGE with as few as 1–10 nucleotide differences (Cody et al., 2000). All techniques that are based on PCR (cloning, pattern analysis and sequencing) will be affected by the biases introduced by PCR (Sjoling and Cowan, 2003).

2.5.8. Clone library or cloning

The cloning-sequencing approach was first used by Giovannoni et al. (1990) to target 16S rDNA, in order to estimate the diversity in the Sargasso Sea bacterioplankton, and later in numerous studies of the same topic (Dorigo et al., 2005). Cloning of PCR-amplified sequences into a cloning vector is the first step of molecular techniques for the unknown diversity subsequent species identification by sequencing (Roling and Head, 2005; Pedros-Alio, 2006; Nocker et al., 2006). The analysis of clone libraries created from 16S ribosomal DNA (rDNA) amplified from environmental DNA can be used to assess community structure and diversity at the highest possible resolution, with 16S rDNA gene data sets providing a qualitative guide to the microbial composition of a given sample (Bowman and Mc Cuaig, 2003). Several bacterial 16S rDNA clone libraries have been constructed from marine and marine-derived sediments (Heijs et al., 2007; Sjoling and Cowan, 2003; Kim et al., 2005; Schmidth et al., 1991).

Competent *Escherichia coli* cells are transformed with the recombinant plasmid DNA (vector and inserted PCR product) and the cells are plated on a medium that selects for a trait conferred on the recipient *E. coli* by the uptake of the vector. Usually an antibiotic resistance gene on the vector is used to select for maintenance of the recombinant plasmid in the host (Roling and Head, 2005). After cloning into the vectors, the inserts are randomly sequenced for the highest phylogenetic resolution, allowing either species identification or determination of similarity to already known species through the use of extensive and rapidly growing sequence databases. Sequence analysis makes it possible to identify the dominant copies present in the initial PCR products (Dorigo et al., 2005; Nocker et al., 2006).

Comparing these sequences with those available in sequence databases (Genebank or EMBL), yields information about the identity or relatedness of the new sequences to known species (Dorigo et al., 2005).

Sequencing of clone libraries from environmental samples has led to a wealth of information about prokaryotic diversity. The Ribosomal Database Project (RDP) on DNA currently has more than 253,000 entries (as of July 2006) and is frequently updated. Sequencing is the basis for construction of phylogenetic trees and for other comparative studies. Advances in sequencing technology and the cost-effectiveness of high-throughput systems in large genomic service facilities have increased the popularity of direct sequence analysis of clones as an alternative to laborious screening of clones by restriction analysis (Nocker et al., 2006).

Some cloning strategies make use of an overhanging 30-A added to PCR products by the Taq polymerase. The sticky end allows efficient ligation into vectors with an overhanging 30-T for more efficient cloning (Nocker et al., 2006).

Also there are some other methods like blunt-end ligation procedures but they are less efficient than sticky-end ligation, and PCR products generated with non-proofreading polymerases must be modified to produce blunt ends (Roling and Head, 2005).

3. MATERIALS AND METHODS

3.1. Sampling and preservation

Sediment samples were collected from Valide Sultan Köprüsü, Eyüp and Adalar Sonrası (Figure 3.1) in the northwest of the Marmara Sea at a water depths of 6, 6 and 2 m respectively, using a Van Veen grab (Figure 3.2) on board of the RV Arar of Istanbul University, Institute of Marine Sciences during research cruises, in February 2007. All samples appeared visually similar possessing grayish-black colour and had a noticeable odor of H_2S and densely packed clay-sized particles. Samples were taken in three replicates from the top 10 cm of sediment using 50 ml sterile syringes, with top end removed. Samples were then subdivided for molecular analyses (10 ml) and sediment chemistry (40ml). Samples were stored in sterile polypropylene tubes at -20°C.

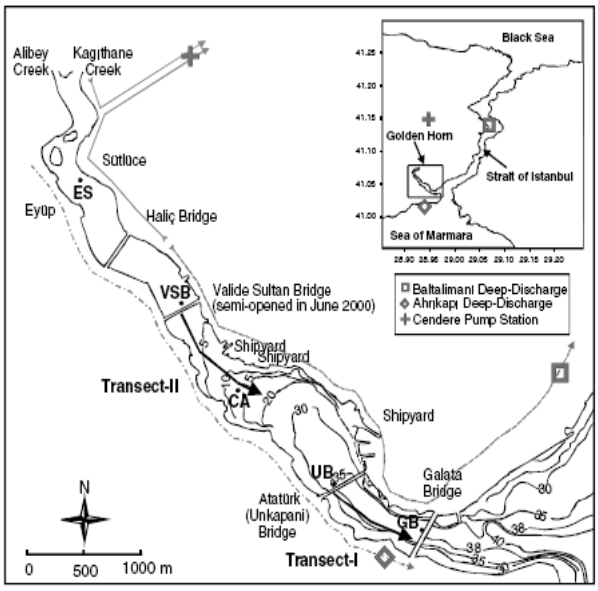


Figure 3.1: Locations of Adalar Sonrası, Eyüp, Valide Sultan Köprüsü sampling points



Figure 3.2: The research ship, ARAR, of İstanbul University and the Van Ween grab sampler

3.2. Chemical analysis

All the chemical analyses were conducted by another research group as part of this project.

pH and conductivity of the sediment samples were measured using HI 99121 Soil pH Test Kit and HI 993310 Water Conductivity and Soil Activity Meter, respectively, according to manufacturer's instructions (Hanna Instruments Ltd., UK). Total solids and total volatile solids (TS/TVS) measurements were carried out according to Standard Methods (Clesceri et al., 1998). For determination of the levels of PO_4^{3-}, NO_3^-, NO_2^-, SO_4^{2-}, F^-, Br^-, and Cl^-, sediment subsamples were centrifuged at 16.000×*g* (0°C) for 10 min. The resultant supernatants were filtered over a 0.2-µm membrane filter into fresh sterile tubes and stored at -20°C. Samples were diluted 1:100 (vol/vol) in deionized water prior to analysis. The anion analyses were performed using a Dionex Ion Chromatograph (Bannockburn, IL, USA). Atomspeck H 5150 Model atomic absorption spectrophotometer (Rank Hilger Ltd., U.K.) was used for Cr, Cu, Ag, Fe, Cd, Mn, Pb, Ni and Zn measurements. The both anion and heavy metal analyses were carried out according to Standard Methods (Clesceri et al., 1998).

Subsamples for elemental analyses were dried at 50–60 °C overnight and kept in a vacuum desiccator until analysis. Samples were further exposed to concentrated HCl fumes to remove inorganic carbonates for total organic carbon (TOC) measurements. Total carbon (TC), organic carbon, nitrogen (TN) and sulphur (TS) contents were analysed by the dry combustion method (Polat & Tugrul, 1995), using a Carlo Erba Model 1108 CHN analyzer. Total inorganic carbon (TIC) was calculated as the difference between TC and TOC. Samples for total phosphorus (TP) analyses were

first exposed to dry combustion at 500 °C for 2 h and then treated with 10 ml of 2N HCl for 10 h and filtered (Polat & Tuğrul, 1995). After the adjustment of pH to 8.0, the oxidized phosphorus in the solution was determined colorimetrically by the routine orthophosphate method. Statistical analysis of chemical data (Parametric two-way analysis of variance, Pearson correlation) was performed by using Systat 7.0 (SPSS Inc.)

3.3. Extraction of Genomic DNA from the Sediment Microbial Community

Genomic DNA was extracted from 0.5g sediments using the FastDNA Spin Kit for Soil (Qbiogene Inc., U.K.) following the manufacturer's instructions.

500 ml of sediment sample was added to lysing matrix tubes, which contains a mixture of silica and ceramic particles to lyse microorganisms' cell membrane, supplied by the kit. Then the lysing matrix tubes were spinned in the ribolyser for 30 seconds at the speed 5.5 m/s. The tubes were then centrifuged at 14000 rpm for 30 seconds. After centrifugation supernatants were transferred to a clean 1.5 ml eppendorf tubes and 250 µl PPS reagent was added. In order to mix the composition homology the tubes were hand-shaken by hands for 30 times then centrifuged at 14000 rpm for 5 minutes. Supernatants were transferred to 2 ml eppendorf tubes and 1 ml Binding Matrix Suspension was added. The tubes were inverted by hand for 2 minutes to allow binding of DNA to matrix. The tubes were placed in a rack for 3 minutes to allow settling of silica matrix. Then 500 µl of the supernatant was removed and resuspended with Binding Matrix and then transferred to a clean Spin Filter which was supplied in the kit.The filter was washed by 500 µl SEWS-M wash solution and centrifuged at 14000 rpm for 2 minutes. The filter was transferred to a clean catch tube (supplied in the kit) and 50 µl DES (DNase/Pyrogen free water) was added. The filter with DES was vortexed and followed by centrifugation at 14000 rpm for 1 minute to extract genomic DNA ready for application.

3.4. Polymerase Chain Reaction (PCR)

Three different PCR amplifications were used in this study: PCR for Eubacterial amplification, Nested PCR for Archaae and PCR for sequencing.

Amplification of 16S rDNA from the extracted DNA was performed with primers selective for the *Archaea* and *Bacteria (*Table1).

Table 3.1:. Primers and their annealing temperatures

Primer	Target	Experimental Stage	Annealing Temperature (°C)	Position[1]	Reference
Bact341f_GC[2]	Bacterial 16S rDNA	DGGE	55	341-357	Muyzer et al., 1993
Bact534r				534-518	
Bact8f		Cloning		8-27	Lane, 1991
Bact1541r				1541-1522	
Bact342f		Sequencing		342-361	Edwards et al., 1988
Arch46f	Archaeal 16S rDNA	First round of nested PCR	40	46-61	Ovreas et al., 1997
Arch1017r				1017-999	Barns et al., 1994
Arch344f		Cloning	53	344-358	Raskin et al., 1994
Arch855r				855-836	Shinzato et al., 1999
Arch344f_GC[2]		DGGE		344-358	Raskin et al., 1994
Univ522r				522-504	Amann et al., 1995
M13f	B-galactosidase	Clone screening	54	–	Schrenk et al., 2003
M13r					

[1]*Escherichia coli* numbering.

[2]5′-GC clamp on Arch344f and Bact341f (GCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACGGGG).

Amplification was done in a 50 µl reaction volume containing 200 ng of DNA, 10 pmol of each primer, 10 mM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 5 µl of 10×*Taq* buffer and 4 U of *Taq* DNA polymerase (Fermentas, Latvia). For the second-round nested amplification, 0.1 µl of the first-round product was used as template, with reaction composition being the same as described previously. PCR amplification was performed in a Techne TC-412 thermal cycler (Barloworld Scientific Ltd., U.K.) with an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing for 1 min and extension at 72°C for 2 min and a final extension at 72°C for 10 min. Mg^{+2} ions necessary for Taq polymerase activity was also present in the buffer solution. After the amplification completed, PCR products were visualized by electrophoresis to check for the specificity of amplification by (Thermo-Scientific Ltd., U.K.) on a 1% (w/v) agarose gel in 1× Tris–borate–EDTA buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) at 7 V cm^{-1} and gel images were recorded using a Chemi-Smart 3000 gel documentation system (Vilber Lourmat, France) following the staining with ethidium bromide.

The PCR process involved three stages: the DNA was denatured to convert double-stranded DNA into single-stranded DNA; oligonucleotide primers were annealed to complementary priming sites in the target DNA; finally the DNA was extended from the primers by the addition of nucleotides through DNA polymerase activity, resulting in double-stranded products (45).

To enhance sensitivity and specificity, a nested PCR approach was applied in all cases for Archaeal amplifications. For the first round of amplification of Archaeal genomic DNA, Arch46f and Arch1017r primers were used, and then these PCR products are used as a template for PCR using Arch 344f – Arch 855r. Bact8f and Bact1541r and Arch 344f – Arch 855r amplification products were used for cloning. For Eubacterial population Bact341f_GC[2] and Bact534r for archaeal Arch 344f – Arch 522r primers were used to amplify the region before loading on DGGE to analyse.

3.5. DGGE (Denaturant Gradient Gel Electrophoresis)

Community profiles of Archaea and Bacteria within the sediments were obtained using DGGE analysis of PCR amplification products from primers Arch344f_GC-

Arch855r and Bact341f_GC-Bact534r as described by Muyzer *et al.* (1993). For bacterial and archaeal population analyses in DGGE the same concentrations for gels were used.

3.5.1. Preparation and Electrophoresis of the Denaturant Gradient Gel

The gel sandwich was assembled by placing the small glass plate on top of the large plate. The thickness of the sandwich was established by using 1 mm spacers between two glass plates which are in size of 16x20 and 18x20 cm. The plate clamps attached (tight enough to hold everything together) and placed the entire assembly into the rear slot of the pouring stand. The clamps loosened slightly and the spacing card was used to assure the proper spacer alignment shown in figure 3.3.

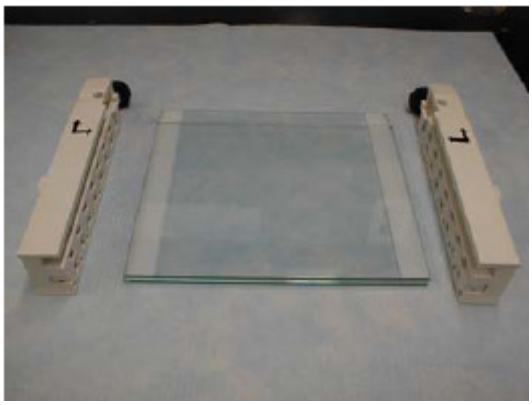


Figure 3.3: Components of the gel sandwich; gel clamps and spacers between two glass plates.

The gradient former was set up by inserting 30 ml syringes into a "closed" gradient wheel shown in figure 3.4. Two solutions of 16 ml volume prepared each; a "low" denaturant concentration solution, and a "high" denaturant concentration solution. 160 µl APS and 9 µl TEMED was added into each solution and swirl gently to mix. These reagents begin the polymerization of the acrylamide. At this point, a gradient wheel was used to set the gel.

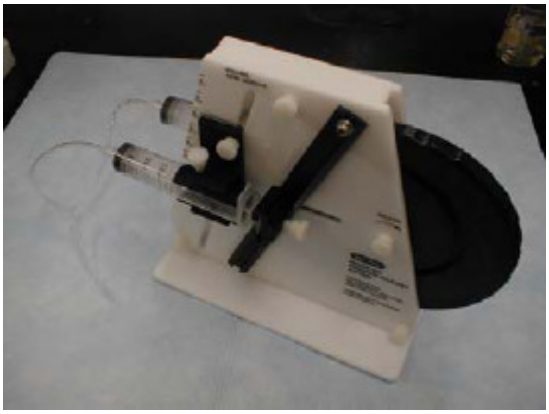


Figure 3.4: The gradient wheel

PCR product (10 µl) was mixed in equal volumes with loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll in water) and run on a 10% polyacrylamide gel (acrylamide–*N*,*N*'-methylenebisacrylamide ratio, 37.5:1) in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; pH 8.0) over a chemical denaturing gradient of urea and formamide equivalent to 30–60% denaturant (100% denaturant is 7 M urea and 40% (v/v) formamide). To aid the conversion and normalization of gels, a marker consisting of 16S rDNA mix from archaeal and bacterial clone libraries was added on the outside of the gel as well as after every four samples shown in figure 3.5.

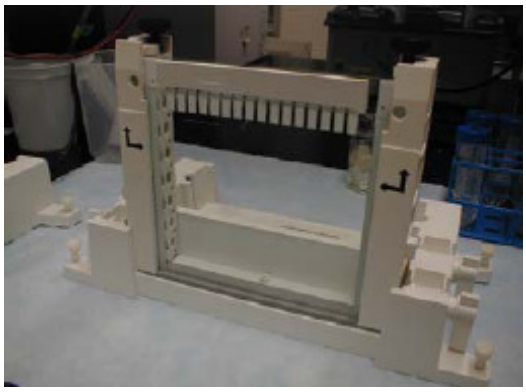


Figure 3.5: Gel assembly with comb inserted

3.5.2. Running the gel

Approximately 7 liters of 1X TAE was prepared and filled in the buffer chamber. The buffer in the DGGE apparatus was preheated to 65 °C; for 2 hours.

When the temperature reached 65 °C heating was interrupted and the gel plates attached to the core assembly shown in figure 3.6. The wells were washed for 3 times with 1X TAE buffer using a syringe to avoid denaturation of the sample because of

the denaturants that rises up to wells when the heat increases. Electrophoresis was performed using the D-Code system shown in figure 3.7. (Bio-Rad Laboratories, Ltd., UK) at 200 V constant current at 60 °C, for 4.5h.

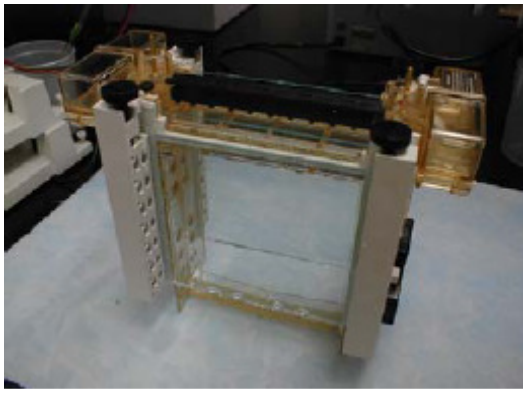


Figure 3.6: Gels attached to the core assembly



Figure 3.7: Bio-Rad DCodeTM system apparatus in action

The gel was stained for 15 minutes in 50 ml of GelStar nucleic acid stain (BioWhittaker) diluted 1:10,000 in 1X TAE. The gel was then viewed on a UV-Transilluminator. The core was taken from the tank and gel sandwiches were separated from it. Glass plates were disassembled and the direction of gel was marked with a cut on the upper left corner. 20 µl of 1:100000 diluted SYBR Gold DNA staining dye was added to 300 ml 1xTAE washing buffer and gels were incubated for 30 minutes. Gels were distained and washed three times with distilled water to remove background. Gel images were visualized and recorded using, Chemi-Smart 3000 gel documentation system (Vilber Lourmat, France). Images were converted, normalized and analyzed by using the Bionumerics 5.0 software (Applied Maths, Kortrijk, Belgium). Data were exported to Excel and used for numerical analysis with Systat 7.0 (SPSS Inc.)

3.6. Generation of 16S rRNA clone libraries

Bact8f-Bact1541r and Arch344f-Arch855r PCR products were purified with a High Pure PCR product purification kit (Roche Diagnostics GmbH, Mannheim, Germany) and cloned with a TOPO TA cloning kit (Invitrogen Ltd., Paisley, United Kingdom) according to manufacturer's instructions.

The first step of the procedure was preparation of 6 µl reaction mix by adding 4 µl PCR product, 1 µl salt solution (1.2 M NaCl, 0.06 M $MgCl_2$) and 1 µl TOPO vector in sterile conditions. The solution was mixed gently and incubated at room temperature for 30 minutes. Following the incubation, reaction mix was placed on ice before One Shot TOPO transformation step.

Prior to One Shot TOPO transformation, one tube of One Shot TOPO reaction was thawed on ice. After thawing, 3 µl of reaction mixture was added to One Shot vial. The solution was mixed gently without pipetting or shaking. The solution was incubated on ice for 30 minutes. After incubation, the tube was subjected to heat shock at 42 ºC for 30 seconds and transferred immediately to ice and 300 µl of SOC medium (2% Tyrptone, 0.5% Yeast Extract, 10 mM NacCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added then the solution was shaken horizontally for 60 minutes. Three LB plates containing 50 µg/ml Kanamycin were warmed to room temperature and then 100 µl of solution was spread on plates using glass spreader. The plates were incubated overnight and colonies were observed after incubation.

To test for correct insertation, randomly selected clonies on the plates were transferred to 200 µl PCR tubes containing 50 µl TE buffer (10mM Tris-HCI, 1mM EDTA pH 8.0) then tubes were boiled at 95 ºC for minutes then frozen at -20 ºC for 30 minutes. Thawed solution was used for PCR template for archaeal and bacterial populations with M13f – M13r primers. For bacterial inserted colonies, a 1100bp of DNA fragment, and for archaeal insert colonies, a 700 bp DNA fragment is expected to be seen in agarose gel electrophoresis.

3.7. Sequencing and Phylogenetic Analyses of 16S rRNA Gene Fragments

Sequencing PCR reaction was performed by mixing 4.7 µl molecular grade water, 1 µl template, and 0.3 µl of primers.

The PCR conditions were as follows: Denaturation at 95 ºC for 5 minutes, denaturation at 95 ºC for 30 seconds, annealing at 55 ºC or 53 ºC for 30 seconds, elongation at 60 ºC for 4 minutes. Run was continued for 30 cycles.

PCR products to be sequenced were purified by ethanol precipitation and sequenced using the ABI prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 377 DNA sequencer (Applied Biosystems, USA) using primers Arc344f and Bact342f generating 500bp and 800bp of archaeal and bacterial 16S rRNA gene fragment. Partial 16S rRNA gene sequences were analysed and manually edited in Chromas software package version 1.45 (http://www.technelysium.com/au/chromas.html). The sequences were scanned for reading errors using the alignment programs of the ARB package (Ludwig et al., 2004), which are based on secondary structures of rRNA. The 16S rDNA sequences were checked for chimerical constructs by using the CHECK-CHIMERA program of the Ribosomal Database Project (Cole et al., 2007) and the ARB software package. Homology searches of the EMBL and GenBank DNA databases for the 16S rRNA gene sequences were performed with FASTA (Pearson, 1988) provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/fasta33/nucleotide.html) to identify putative close phylogenetic relatives. Sequences representing distinct phylotypes (97% sequence similarity was required) and their closest relatives were aligned by using the ARB software fast aligner utility, followed by manual adjustments. Only unambiguously aligned base positions were used in the analysis. Distance analyses using the Jukes and Cantor (1969) correction and bootstrap resampling (1000 times) were done using the TREECON package (van De Peer and De Wachter, 1997) and trees were generated from distance matrices using the neighbour-joining method (Saitou and Nei, 1987).

16S rRNA gene sequences showing 97% similarity or higher was considered to belong to the same phylotype. Related 16S rRNA gene sequences were placed within tentative taxa (between Phylum and Order) by determining the taxonomic class (using the NCBI taxonomy database) of the closest relative in GenBank of sequences that formed a phylogenetic clade. Sequences that showed no or low (below 70%) relatedness with known bacterial or archaeal phylogenetic groups were listed as unclassified.

4. RESULTS

4.1. Chemical Analysis of the Sediment Samples

Chemical analyses provide information about sediment pollution, biological processes prevail in the sediments, presentation of microorganisms in the sediments and the metabolic processes.

It has been reported for anoxic marine sediments that pH is higher than 8 since sulfate and nitrate reduction processes consume H^+ (Radke et al., 2002). The pH values measured in Valide Sultan Köprüsü, Eyüp and Adalar Sonrası samples range between 8,6 – 8,7 that indicates anoxic conditions prevail at the sediment (Table 4.1.)

When electric potential is between +100 mv and -100 mv, iron reducing process occurs. However, if it's within the range of 100 mv and -200 mv, sulfate reduction process and below -200 mv methanogenesis occurs (Vorenhout et al., 2004). The redox potentials of three stations of Haliç sediments indicated that at Valide Sultan Köprüsü station iron reduction process was dominating, while at Eyüp and Adalar Sonrası stations methanogenesis was dominating.

Table 4.1: pH and electric potential of sediment samples

Sampling Point	pH	Redox Potential (mV)
Haliç VSB Feb07	8,7	-63
Haliç EYUP Feb07	8,6	-222
Haliç AS Feb07	8,7	-241

Heavy metal concentrations found in Eyüp (H2), Valide Sultan Köprüsü (H3) and Adalar Sonrası (H1) sediments (Table 4.2) were significantly above the levels found in unpolluted sediments and showed close similarity values to the heavy metal levels detected in highly polluted sediments (Elder et al., 1988).

Table 4.2: Heavy metal concentrations of sediment samples

Sampling Point	Cr (mg/kg)	Cu (mg/kg)	Zn (mg/kg)	Pb (mg/kg)	Ni (mg/kg)	Mn (mg/kg)	Fe (mg/kg)
Haliç VSB Feb 2007	304	520	640	168	74	293	29775
Haliç EYUP Feb 2007	126	230	434	63	54	448	30450
Haliç AS Feb 2007	83	124	284	47	49	695	27525
Typical Marmara Sea Sediments	123	154	389	70	46	299	28715

Table 4.3: Anion concentrations within the sediment

Sampling Point	Florur (mg/l)	Chloride (mg/l)	Bromide (mg/l)	Nitrate (mg/l)	Sulfate (mg/l)	Phosphate (mg/l)
Haliç VSB Feb 2007	6,0	6290	17,8	13,6	1066	0,0
Haliç EYUP Feb 2007	6,6	6493	39,2	15,5	139	0,0
Haliç AS Feb 2007	0,0	5836	14,7	11,2	85	0,0

The anoxic marine sediments of Valide Sultan Köprüsü station were shown to be rich in sulfur compounds, containing 1066mg\l of sulfide when compared with other two stations. Bromide concentration in Eyüp station is nearly two times higher than the other regions. Nitrate concentrations were relative in each sediment sample. It is significant that florur was not observed in Adalar Sonrası sediment samples when observed in Eyüp and Valide Sultan Köprüsü stations 6,6 and 6 mg\l respectively (Table 4.3.).

Table 4.4: TS, TVS, TC, TOC and hydrocarbon analysis

Sampling Point	TS (mg/ml)	TVS (mg/ml)	TVS/TS (%)	TC (mg/mg)	TOC (mg/mg)	TOC/TC (%)	TOC/TS	Hydrocarbon Concentration (mg/kg)
Haliç VSB Feb07	746	17	2	0,034	0,031	91	1,566	12769
Haliç EYUP Feb07	660	15	2	0,043	0,041	96	2,803	12347
Haliç AS Feb07	640	16	2	0,057	0,051	90	5,400	15653

In the Adalar Sonrası sediment samples, total organic carbon was 0,051mg/mg and total carbon was 0,057 mg/mg as being the most polluted region in Haliç. Anoxic marine sediment samples contained high concentrations of total solid 640 mg\ml to 746 mg\ml respectively. Hydrocarbon concentration was at the highest concentration in Adalar Sonrası sediment samples supported that the mostly poluted environment in the eustary. The TOC/TS value was 5,400 and 2,803 in the sediments from Adalar Sonrası, Eyüp and reached the value of 1,566 in sediments from Valide Sultan Köprüsü (Table 4.4.)

4.2. Molecular Community Analyses Based on Clone Libraries

Sediment samples from anoxic marine sediments of the three stations of Haliç estuary were analyzed for bacterial and archaeal diversity. Genomic DNA was extracted from sediment samples and amplified using universal bacteria and archaea specific 16S rDNA PCR primers.

The 16S rRNA gene sequence data generated in this study provided an overall picture of clear heterogeneity between the communities in the different sediment layers.

Amplification of 16S rRNA genes of extracted genomic DNAs (positive controls) with the appropriate primers yielded PCR product in all cases as expected. Yet, negative controls (no DNA) and inappropriate primer template pairs did not yield a

PCR product. With DNA isolated from the anoxic sediments of Haliç used as a template, specific amplification products were obtained with PCR primers that targeted the V3 variable region of 16S rRNA genes. Clone libraries were always constructed where PCR amplification was successful, and the number of clones obtained in each clone library ranged in at least total 60 clones.

4.3. Genomic DNA Extraction and PCR Amplification

The sediment samples were taken in triplicates. Fast DNA Spin Kit was used for Soil extracted genomic DNA of Haliç station samples. The genomic DNAs extracted were visualized by agorose gel electrophoresis. Genomic DNA's were diluted to 1/100 using molecular grade water. To amplify bacterial genomic DNAs for cloning, PCR were performed using PAf- PHr primers that amplify 1500 bp portion of 16S rRNA and for archaeal gDNAs for cloning Arch 46f – 1017r primers were used that amplify 1000 bp portion of 16S rRNA. To create archaeal clone library, a second round of nested PCR were taken using Arch344f-Arch855r primers that amplifies 500 bp gene products. To load on DGGE, a 200 bp portion of 16S rRNA V3 region was amplified. Arch 344-522 primers were used for archael population, Vf – Vr primers were used for bacterial population amplification.

To estimate the overall archaeal diversity of Eyüp, Valide Sultan Köprüsü and Adalar Sonrası stations of Haliç anoxic marine sediments, three archaeal 16S rRNA libraries were constructed from sediment samples taken in February 2007.

4.4. Archaeal Diversity of Adalar Sonrası Station (H1)

A total of 94 clones were randomly screened by agarose gel electrophoresis expecting to see the 700 bp DNA fragments (500bp of PCR product + 200 bp cloning vector) on agarose gel electrophoresis. 61 randomly selected clones that have the positive insertion were called positive clones. All the positive clones from Adalar Sonrası (H1) sediment samples were screened by DGGE and grouped together based on their DGGE profiles. Representative clones were sequenced, and the 500 bp sequences were analyzed on 16S rDNA database of EBI (www.ebi.co.uk) in order to determine their phylogenetic affiliations.

The archaeal 16S rRNA clone library obtained from Adalar Sonrası station consists of 62 clones and groups belonged to the Euryarchaeota and Crenarchaeota in the Archae domain. Adalar Sonrası clone library was dominated by Euryarchaeota constituting for 79% of all the clones with more than 85% similarity to their closest cultured relatives namely known as methanogens on the databases. In the clone library 20,9% of the all clones belonged to Crenarchaeota group.

Adalar Sonrası archaeal clone library was dominated by uncultured archaeons (19,3% of all clones), Methanosaeta species (16%) and Methanosarcinales species (11,2%). Clones obtained were related to Methanospirillium hungatei (97% similarity), Methanobacterium curvum (96%), Methanospirillium sp (95%), uncultured Methanosaeta sp (98%), Methanosarcina barkeri and uncultured Methanosarcinaceae (98%) and anaerobic methanogenic archeaon, uncultured eucaryotha (95%). Each one of these groups accounted for 1,7% of all the clones respectively.

11,2% of the archaeal clones from Adalar Sonrası clone library were putative methanogens showing less than 85% similarity to their cultured and environmental relatives on databases. Only 1,6% of all the clones showing less than 70% similarity to any cultured or environmental clone on the database were thus grouped as unclassified.

Sequences detected in the clone library were very diverse and their closest relatives were sequences from low temperature oil reservoir (Grabowski et al., 2005), petroleum contaminated soil (Kasai et al., 2005), marine sediment (Kendall et al., 2007), oil contaminated groundwater (Watanabe et al., 2007).

4.5. Bacterial Diversity of Adalar Sonrası Station (H1)

For the identification of bacterial community, a total of 85 clones from Adalar Sonrası sediment samples were randomly selected and screened. 62 of the clones were positive for 1100 bp DNA fragments (900bp of PCR product + 200 bp cloning vector). All clones from Adalar Sonrası sediment samples were screened by DGGE and classified based on their DGGE profiles. Representative clones were sequenced, and the 1100 bp sequences were analyzed on 16S rRNA database of EBI (www.ebi.co.uk) to determine their phylogenetic affiliations.

The clones in bacterial library belonged to two major groups of the domain Archaea; Euryarchaeota and Crenarchaeota. The clone library dominated by Euryarchaeota constituted of 79% of all the clones and showed more than 85% similarity to their closest cultured relatives, known as methanogens on the databases. 20,9% of the clones belonged to Crenarchaeota group.

The archeal 16S rRNA clone library for Adalar Sonrası station was consisted of 61 clones that grouped into 13 phylotypes. The bacterial phylotypes could be divided into six phyla, as follows: Firmicutes (21% of the total clones), Bacteriodetes (18%), Proteobacteria (14%), Chloroflexi (9%) and Candidate division (3%). Seven phylotypes could not be classified into known phylogenetic groups by the EBI classifier. Firmicutes and Bacteriodetes phylotypes represented nearly half of the clone library. The Proteobacteria was represented by 3 phylotypes that were Gammaproteobacteria, Epsilonproteobacteria, Betaproteobacteria and Deltaproteobacteria (above 95% confidence threshold). One of the obtained clones was related to uncultured Gammaproteobacteria with a clone frequency of 3% and with a similarity of 95%. This bacterium was reported to be previously found in wastes of uranium mining (Geissler and Selenka, 2005).

Clones that could not classified into any subphylum exhibiting less than 85% similarity represented 11,4% of the total archaeal community in Adalar Sonrasi sediments. The clones were called putative sulfate reducing and fermentative organisms and could not be affiliated with any cultured or environmental clone on the databases respectively. Among all the isolates, only 4,9% showed similarity less than 70%. These isolates could not be classified as any of the cultured or environmental clones, thus, they were grouped as unclassified on the database.

In general the clones were most closely related to groups isolated from human fecal sample (unpublished result), oil sands tailing ponds (unpublished result), sulfidic caves (unpublished result), hydrocarbon contaminated aquifer (Dojka et al., 1998), uranium mining waste pile (Geissler and Selenska, 2005).

4.6. Archaeal Diversity of Eyüp Station (H2)

Archeal 16S rRNA clone library of Eyüp station consisted of 60 clones that were dominated by Euryarchaeota, and they formed 66% of all the clones isolated. Eyüp

clone library was dominated by uncultured archaeon (%30 of all clones), Methanosaeta species (13%) and Methanomicrobiales species (11,6%). Clones related to uncultured Methanomicrobiales sp (with 96% similarity) and Methanosaeta sp (with 95% similarity) only accounted for 5% of all the clones. However, 25% of the all archaeal clones from Eyüp clone library were putative methanogen organisms showing <85% similarity to their cultured and environmental relatives on databases. Only 5% of all the clones showing <70% similarity to any cultured or environmental clone on the database were thus grouped as unclassified.

4.7. Bacterial Diversity of Eyüp Station (H2)

The bacterial clone library of Eyüp was composed of 65 clones that were grouped into eight phylotypes. The bacterial phylotypes could be divided into three phyla, as follows: Proteobacteria (57% of the total clones), Actinobacteria (%4,6) and Acidobacteria (3%). 3 phylotypes could not be classified as any of the known phylogenetic groups. Epsilonproteobacteria, Deltaproteobacteria and Gammaproteobacteria phylotypes represented more than half of the clone library. The Deltaproteobacteria phylotypes were classified into either Desulfobulbus rhabdoformis or Desulfumicrobium baculatum (98% and 99% confidence threshold, respectively). These organisms were previously isolated from water-oil separation systems and low temperature oil reservoir in the literature (Lien et al., 1998 and unpublished result)

From the Actinobacteria family, only *Corynebacteriacae sp* was isolated with the similarity of 99,8%.

One of the isolated clones was related to uncultured bacterium with a clone frequency of 3% and a similarity of 98% had also been previously reported among the isolates of a uranium mining depository sludge (Selenska, 2002).

18,46% of the bacterial clones in the library were organisms exhibiting less than 85% similarity to any cultured or environmental clones on the database, thus grouped as putative sulfur-metabolizing bacteria. 13,8% clones among the total clones with less than 70% similarity to any cultured or environmental clones on the database were grouped as unclassified.

4.8. Archaeal Diversity of Valide Sultan Köprüsü Station (H3)

Archeal 16S rRNA clone library of Valide Sultan Köprüsü consisted of 61 clones. Library was dominated by two major groups of the Archeae domain; Euryarchaeota and Crenarchaeota. Euryarchaeota group constituted of 9,8% of all the isolated clones (with more than 85% similarity to methanogens in the database) in the region while 1,6% of the all clones belonged to Crenarchaeota group. It appeared that 21,3% of the all clones from our library were putative methanogens showing less than 85% similarity to the relatives on databases. Meanwhile, 13,1% of all the clones (with <70% similarity to the clones on the database) were grouped as unclassified.

Valide Sultan Köprüsü archaeal clone library was dominated by uncultured archaeons, Methanosarcinales, uncultured eucaryotha, and Methanomicrobiales with clone frequencies of 29,5%, 16,3%, %9,8 and 8,1% respectively. Each one of these groups exhibited similarities above 85%.

4.9. Bacterial Diversity of Valide Sultan Köprüsü Station (H3)

Bacterial 16S rRNA clone library of Valide Sultan Köprüsü also consisted of 61 clones. Bacterial phylotypes could be divided into four phyla: Proteobacteria (52% of the total clones), Firmicutes (13%), Chloroflexi (8%), and Acidobacteria (5%). Six phylotypes could not be classified into known phylogenetic groups by the EBI Classifier. The isolated clones of Proteobacteria belonged to the following subclasses with the frequencies: Epsilon-Proteobacteria (19%), Delta-Proteobacteria (14%), Gamma-Proteobacteria (13%) and Alpha-Proteobacteria (4,8%).

Another group of clones appeared to be related to Clostridium species with the similarities as following: *Clostridium lituseburense* (98% similarity), *Clostridium glycolicum* (99% similarity) and *Clostridium sp* (99% similarity). These clones accounted for 3%, 3% and 5% of the all clones, respectively.

In the meantime, 3% of the bacterial clones in the library were organisms showing less than 85% similarity to any cultured or environmental clone on the database, thus, grouped as putative. Only 8% of all the clones showed less than 70% similarity to any cultured or environmental clone on the database were grouped as unclassified.

The clones in the 16S rRNA library were most closely related to groups isolated from human faeces (Ley ey al., 2006), oil polluted marine microbial mat (Delong et al., 2006), marine and deep sea sediments (unpublished result and Li et al., 1999), deep sea hydrothermal vents (unpublished result) and oil sand tailings (unpublished result). (unpublished result and Cho and Giovannoni, 2004)

5. DISCUSSION

This culture-independent survey of the sediments of Haliç has provided further insight into the microbial diversity of this special anoxic habitat. Three general 16S rRNA libraries were constructed from sediment samples of Adalar Sonrası, Eyüp and Valide Sultan Köprüsü. Since there are known biases associated with DNA extraction and PCR amplification (Dorigo et al., 2005; Cody et al., 2000), the abundance of a phylotype in a clone library does not necessarily reflect its abundance in the sample, and the corresponding ecological function cannot be inferred with certitude from the phylogenetic affiliation. Such assumptions should be made only when there is a high degree of sequence similarity between the phylotypes and known cultivated species.

In this study, a number of phylotypes had sequence homology with cultivated microorganisms at the species or genus levels, allowing some prediction of ecological function within the anoxic marine sediments of Haliç (Barns et al., 2005).

In order to obtain exact figures of archaeal community, quantification of the archaea and bacteria can further be accomplished by real time PCR. Clones showing higher than 97% similarity were considered to be the same organism and less than 97% similarity were differentiated on strain level; phylotypes with similarities less than 70% were accepted as unclassified while phylotypes with similarities less than 85% accepted as putative microorganisms.

Considering chemical analyses results, some predictions could be done. Due to the high levels of heavy metal concentrations at the sediment samples results showed that Haliç has been highly exposed to heavy metal pollution. Three of the sediments were shown to be rich in iron. Copper levels were relatively low in Adalar Sonrası in comparison to those in other two sediment samples. Among the samples of all three stations, hydrocarbon concentration was the highest in Adalar Sonrası station.

In anoxic sediments, TOC ranged between 0,01 and 0,15 mg/mg according to the level of pollution (Türker, 2007). In this study, total carbon content of the sediments

ranged between 0,03 and 0,05 mg/mg and this result indicated that sediments were fairly exposed to organic matter pollution.

In anoxic sediments acoording to the pollution level, TS concrentation ranged between 100-1000 mg/L (Spagnoli and Bergami, 1997). In our study, TS concentrations ranged between 640 and 746 mg/L indicating that the sediments exposed to high pollution. TVS/TS ratio of sediments was 2%. This result in the current study were lower, indicating that the sediments were polluted by inorganic pollutants.

The TOC/TS ratios give an idea about the conditions prevailing within the sediments and about what biogeochemical processes might occur.It has been previously estimated that TOC/TS ratio is above 5 within sediments where aerobic conditions dominates, however if anoxic conditions prevail within the sediments while the water column is under aerobic conditions then this ratio is found to be between 1,5-5. When anoxic conditions prevail both within the sediments and water column, thus this ratio goes below 1,5. As the TOC/TS ratio goes down the importance of sulfate reduction increases within such sediments (Craft, 1991).

In the current study, the TOC/TS ratios varied between 1,566 - 2,803 - 5,400 along Valide Sultan Köprüsü, Eyüp and Adalar Sonrası sediment samples. This shows that anoxic conditions prevail both within the sediments and in the water column above the sediments.

Additional to that from the results of phylogenetic analyses, sulfate concentration was at the highest concentration in Valide Sultan Köprüsü and sulfate reducing bacteria was dominating the bacterial clone library. The sediment samples correlates with this finding.

5.1. Evaluation of Microbial Community of Adalar Sonrası Station (H1)

The dominant phylum in the bacterial clone library of Adalar Sonrası station was Chloroflexi with a clone frequency of 9.8% and a similarity range between 92% and 96%. Sequences detected were all affiliated with isolates from oil contaminated environment (Dojka et al., 1998), anaerobic sludge and sulfidic caves (unpublished result)

From the literature various green non-sulfur bacterial phylotypes were identified both in the plankton and sediments (Briee et al., 2007). Chloroflexi are a class of eubacteria (formerly known as green non-sulfur bacteria) that produce energy through photosynthesis. They are filamentous anoxygenic phototrophs. They are facultatively anaerobic, but do not produce oxygen during photosynthesis, and have a different method of carbon fixation (photoheterotrophy) than other photosynthetic bacteria. They photosynthesize using H_2S as electron donor, although they can also grow photoheterotrophically, and some of them form symbiotic consortia with heterotrophic partners (Briee et al., 2007). In the anoxic subseafloor sediments that have been studied to date, photosynthesized organic matter appears to be the principal food source (D'Hondt et al., 2004). Chloroflexi species are frequent in anoxic layers of microbial mats and meromictic lakes, usually in deeper layers since they support higher H_2S concentrations and require less light intensity (Briee et al., 2007). In Adalar Sonrasi station, where the water depth is 2 m, Alibeyköy and Kağıthane streams carry high amounts of discharges. This creates a highly turbid and cloudy water profile, unable to allow light transmittion, providing a suitable environment for Chloroflexi survival. In comparison to other two stations of Haliç, sulfate levels in the sediment (85 mg/L), was low. From the phylogenetic results sulfate reducers were less abundant.

Anoxygenic phototrophs appear to contribute efficiently together with the anaerobic food chain to the conversion of organic carbon into easily degradable bacterial biomass by fueling the sulfur cycle, which, in turn, accelerates the carbon cycle in the system. Some types of symbiosis that could exist in the sediments, such as that between phototrophic bacteria and beta-Proteobacteria (Briee et al., 2007). In our total clone library the frequency of beta-Proteobacteria was 1,6%, therefore it could be speculated that there could be a symbiotic consortium between beta-Proteobacteria and anoxygenic green non sulfur bacteria.

A major metabolic process in the sediment appeared to be the oxidation of reduced sulfur compounds. Sequences related to sulfur oxidizers were the most abundant in our clone library of Adalar Sonrasi station, and belonged to the three subclasses of the Proteobacteria (Beta-, Epsilon-, and Gammaproteobacteria) (Barns et al., 2005).

In our study Bacteroidete phylotypes following Chloroflexi were very diverse that followed by uncultured compost bacterium, Actibacter sediminis,

Epsilonproteobacteria, Candidate division, Clostridiales, Sedimentibacter sp, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria and uncultured anaerobic bacterium respectively. Members of these phylotypes were dominated by sulfur reducers (24%) and fermentative bacteria (5%). Studies indicate that complex polymers such as cellulose, lignin or long-chain fatty acids are probably initially degraded by specialized microorganisms. Subsequently, fermenting bacteria degrade simpler organics to small molecules, such as acetate that can be utilized by acetogenic methanogens (Briee et al., 2007). Hydrogen, is a major end product of fermentation, and used as a substrate in various phylotypes. Sedimentibacter sp bacterium utilizes hydrogen in its metabolism which was isolated from the sediment samples in Adalar Sonrasi station.

In the bacterial clone library, Gamma-Proteobacterium was one of the identified bacteria, with a clone frequency of 3,2%, and with a 95% similarity. This organism has previously been reported to be isolated from a uranium mining waste pile.

The presence of this organism in the sampling stations could be explained by various possible scenarios. It is known that in certain industries like iron process, wastewaters might contain radioactive elements such as uranium. One of the largest iron-process plants of Turkiye; Demirdöküm used to be located nearby Haliç and its wastewater could have been discharged into the estuary. Other than that, boats carrying radioactive element-containing loads could have leaked material into the water. These examples could explain the enrichment of the organism mentioned above.

Methanogenic archea is mostly responsible of the hydrogen production. Indeed, two types of methanogens, whose relative dominance may vary seasonally as a function of temperature and population dynamics, may be operating in the system: acetogenic and hydrogenoclastic methanogens (Briee et al., 2007). In our study the dominancy of these methanogens were 25% in the total clones. These methanogens would take up acetate and hydrogen derived from fermentation while other methanogens would utilize methanol or methylamines derived from the hydrolysis of complex organic matter (Briee et al., 2007). From the results of chemical analyses and from previous studies on Haliç, due to a high level of organic matter deposition in the sediments it is predictable to encounter these types of methanogens.

Unclassified and putative clones, their similarities less than 85% and 70% are not negligible with the dominancy (11,4%) and (4,9%) respectively. From the phylogenetical results there is high level of microbial biodiversity in the station depending on classified, unclassified and putative organisms. This result warrants for further studies in identification of these organisms.

5.2. Evaluation of Microbial Community of Eyüp Station (H2)

In the phylogenetic study results of Eyüp station, the dominant bacterial group appeared to be sulfate reducing Proteobacteria, accounting for 52,3% of the total clones. Results of previous studies demonstrate that sulfate-reducing bacteria are universally distributed in marine sediments and microbial mats. Sulfate reduction is the dominant anaerobic biomineralization pathway (Verstraete and Grusenmeyer, 1986). Depending on chemical analyses of sediment samples, sulfate levels are in the range of 139 mg/L. This level was higher than that of Adalar Sonrası station. This can be explained by the fact that sulfate reducers are more dominant when compared with the other sampling stations.

In the current study, phylogenetic results of clone library demonstrate that in comparison to Epsilon-Proteobacteria (17% clone frequency), betaproteobacteria and gammaproteobacteria subclasses were less abundant in methane oxidizing sediments of Eyüp. This result was similar to archaeal phylotypes in the Adalar Sonrası sediments. Epsilon-Proteobacteria are likely involved in sulfur cycling by oxidizing sulfide or sulfur using small amounts of oxygen or nitrate. Some can also reduce elemental sulfur to sulfide. Most delta-Proteobacteria are sulfate reducers; they are abundant and play a cardinal role in anoxic settings, including meromictic and anoxic lakes (Briee et al., 2007). Sulfate reducers Desulfobulbus rhabdoformis 97%, (9% clone frequency) has previously been isolated from water-oil separation systems and Desulfomicrobium baculatum 98% (7,7% frequency) had previously been isolated from low temperature oil reservoirs. In marine sediments, sulfate reducing bacteria may play an important role in hydrocarbon degradation. Anaerobic hydrocarbon metabolism and the realization that it may be an important process in contaminated anoxic sediments where transfer of oxygen may not be sufficient to replenish oxygen consumed by microbial metabolism (Okus et al., 2004).

Corynebacterium sp (4,6% clone frequency) was identified with 99.8% similarity. This bacterium is known to ferment carbohydrates, organic acids, glucose and acetate. Fermenting bacteria produce H_2 and acetate (as well as other simple organic and alcohols) as waste products. These compounds can be used by methanogens, but can also be used by sulfate reducing bacteria (Schwarz et al., 2007).

The dominant archaeal strains in the sediments of Eyüp were Methanosaeta sp. and Methanosarcina sp. that metabolize only acetate. Because of the abundancy of *Methanosaeta* species in the clone library, acetoclastic methanogenesis can be considered as one of the dominating terminal oxidation processes in the anoxic marine sediments of Eyüp.

The previous studies show that the anoxic layers of marine sediments are dominated by sulfate reduction and by methanogenesis as the most important terminal oxidation processes in carbon metabolism. Previous studies with sediments demonstrated the presence of elevated numbers of sulfate reducers and methanogens within sulfate–methane transition zones, suggesting that anaerobic methane oxidation coupled to sulfate reduction. (Apaydın et al., 2006; Smith and Klug, 1981)

Acetate, H_2 and CO_2 are the major precursors of methanogenesis in a variety of natural environments. Hydrogen and acetate are used as an electron donor for sulfate reduction (Lane et al., 1985). Sulfate-reducing bacteria (SRB) can inhibit the activity of methanogenic bacteria by outcompeting them for hydrogen and acetate when millimolar quantities of sulfate are present. SRB apparently have a higher affinity for hydrogen and acetate, which are the major methane precursors, relative to the methane producing bacteria. Thus, when sulfate was not limiting, sulfate reducers enabled them to inhibit methane production by lowering the hydrogen partial pressure below levels that methanogens could effectively utilize. However, methanogens coexisted with sulfate reducers in the presence of sulfate, and the outcome of competition at any time was a function of the rate of hydrogen production, the relative population sizes, and sulfate availability (Lovley et al., 1982). Sulfate reducing bacteria are also found to be present along with archaea and get involved in the anaerobic oxidation of methane process. Previous studies indicate a close metabolic association between SRB and methanotrophic archaea (Pancost et al., 2001).

In estuarine sediments, up to 50% of organic carbon can be oxidized to CO_2 via pathways mediated by sulfate reducing bacteria (Devereuxi and Mund from, 1994). Therefore, from the phylogenetic results of the sediment samples isolation sources of the clones are generally contaminated environments with petroleum compounds rich in carbon nutrient.

In the sediment samples with the 98,6% similarity value and 3% clone frequency Acidobacterium group seems to be present in many ecosystems, particularly in soils. The exact role and ecological significance of these bacteria is still unknown, however recent development indicate that members of the Acidobacterium are involved in methanol metabolism (Tekse et al., 1998), suggesting that the members of the Acidobacterium kingdom could be genetically and metabolically diverse, as environmentally widespread and perhaps, ecologically important.

With high proportion of (18.46% and 13.8% of the total clones) were showing less than 85% and 70% similarity to any cultured or environmental clone on the database were thus grouped as putative and unclassified organisms. The station that sediment samples were taken could be a dynamic location in terms of the possibility of finding varieties of new organisms, and putative mutant unidentified microorganisms may be found.

Similar to the that in the sediment samples of Adalar Sonrası station, an uncultured bacterium type was detected with the same frequency occurance (3%) but higher in similarity (98%). This organism had been previously isolated from uranium mining depository sludge. However, it is interesting to identify the same microorganism in two separate locations along the Haliç.

5.3. Evaluation of Microbial Community of Valide Sultan Köprüsü (H3)

Proteobacteria was the most abundant phylum in sediment libraries of Valide Sultan Köprüsü station. However, important differences were observed in the relative distribution and abundance of the different proteobacterial subdivisions. Thus, epsilon-proteobacteria was much more abundant than delta- and gamma-proteobacterium.

The most abundant bacterial group following Proteobacteria (47%) was Clostridium with 97% to 99% similarity and 13% clone frequencies in all clones. Chloroflexi was

also commonly determined from the clone libraries from the study with 91%-98% similarity values and 8% frequency. The most seen archaeal subphylums were Methanosarcinales and Methanosaeta, which are acetate consumer (Scholten and Stams, 2000). This abundance can be correlated as fermenting bacteria degrade simpler organics to small molecules, such as acetate that can be utilized by acetogenic methanogens (Briee et al., 2007).

When looking to isolation source the organism is most likely anaerobic. The Acidobacteria were also identified in sediments 98% similarity and 4,8% frequency. However, Acidobacterial phylotypes were quantitatively much more frequent in sediment samples. The Acidobacteria is very diverse, thus far containing very few cultivated species that are chemoorganotrophic (Briee et al., 2007).

There are a large proportion of putative microorganisms with similarity less than 85% and unclassified with similarity less than 70%. Bacterial and archaeal groups in the sediments of Valide Sultan Köprüsü that indicates still there are microorganisms that have not identified yet. In archaeal clone library (21%) all the clones consisted of putative microorganisms and (13%) is unclassified, in bacterial clone library (8,1%) of the all clones are unclassified.

6. CONCLUSION

This study closely examined the microbial communities of three different sediment samples taken from a specific location on Haliç estuary. The region involves high heavy metal and hydrocarbon concentration, originated mostly from the shipyard. Haliç estuary is very important with regard to such studies because the region has been imposed to industrial and domestic wastewater for centuries; thus it has become a highly polluted region. We had expected to see different species of microorganisms mutating in order to adapt to the conditions of the region.

In Adalar Sonrası station we found that 11% of the all archaeal clones were isolated from low temperature oil reservoir, oil contaminated groundwater and oil sands tailing ponds, cited from the previous studies in the literature with similarities 92% between 98%.

(23%) of the all bacterial clones of Adalar Sonrası station isolated from oil sands tailing ponds, hydrocarbon contaminated aquifer and hydrocarbon degrader 92–98% similarity.

Eyüp archaeal clone library (10%) of potentially degrade long alkane chains and from oil sands tailing ponds 92–95% similarity. Eyüp bacterial clone library 20% oil polluted marine microbial mat, water-oil separation system and low temperature oil reservoir 96–99% similarity.

Valide S.B. bacterial (10%) of the clone library is isolated from hydrocarbon seep, oil polluted microbial mat and oil sands tailing ponds 96–98% similarity.

Additionally it is surprising concerning Adalar Sonrası anoxic marine sediment that we found uncultured Gammaproteobacterium which is with clone frequency of (3%) and the similarity of 95% similar to bacterium, isolated from waste pile of uranium mining.

When it comes to Eyüp station, we found an uncultured bacterium, that is isolated from uranium mining, as well with same clone frequency in Adalar Sonrasi station but higher similarity rate, 98%.

The resence of this organism in the sampling stations could be explained by various possible scenarios. It is known that in certain industries like iron process,wastewaters might contain radioactive elements such as uranium. One of the largest iron-process plants of Turkiye; Demirdöküm used to be located nearby Haliç and its wastewater could have been discharged into the estuary. Other than that, boats carrying radioactive element-containing loads could have leaked material into the water. These examples could explain the enrichment of the organism mentioned above.

Diverse bacterial and archaeal microbial communities of the anoxic sediments found in Haliç suggests a variety of physiologies, as well as a vast potential for the discovery of novel organisms which may be biotechnologically important as well.

As seen from above a bioremediation strategy can be constracted with using high clone percentage of isolated microorganisms from petroleum contaminated environments. To do so, the microorganisms should be adapted to the environment and should facilitate their metabolism to degrade hydrocarbons and petroleum related contaminants more efficiently.

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