INVESTIGATION OF ARCHEAL AND BACTERIAL
DIVERSITY IN ANOXIC MARINE SEDIMENTS
FROM THE MARMARA SEA

M.Sc. Thesis by
Zeynep ÇETECİOĞLU, B.Sc.

Department: Environmental Engineering
Programme: Environmental Biotechnology

Supervisor: Prof. Dr. Orhan İNCE

JUNE 2006
INVESTIGATION OF ARCHEAL AND BACTERIAL DIVERSITY IN ANOXIC MARINE SEDIMENTS FROM THE MARMARA SEA

M.Sc. Thesis by
Zeynep ÇETECİOĞLU, B.Sc.
(501041807)

Date of submission : 8 May 2006
Date of defence examination: 13 June 2006
Supervisor (Chairman): Prof. Dr. Orhan İNCE
Members of the Examining Committee Prof.Dr. Rüya TAŞLI TORAMAN (İ.T.Ü.)
Prof.Dr. Betül KIRDAR (BÜ.)

JUNE 2006
MARMARA DENİZ İNDEE ALINAN ANOKSİK
SEDİMENTTEKİ ARKEAL VE BAKTERİYEL
ÇEŞİTLİLİĞİN İNCELENMESİ

YÜKSEK LİSANS TEZİ
Zeynep ÇETECİOĞLU
(501041807)

Tezin Enstitüye Verildiği Tarih : 8 Mayıs 2006
Tezin Savunulduğu Tarih : 13 Haziran 2006

Tez Danışmanı : Prof. Dr. Orhan İNCE
Diğer Jüri Üyeleri Prof. Dr. Rüya TAŞLI TORAMAN (İ.T.Ü.)
Prof. Dr. Betül KIRDAR (B.Ü.)

HAZİRAN 2006
ACKNOWLEDGEMENTS

I would like to thank to my supervisor Prof. Dr. Orhan İnce for his valuable advices, confidence and guidance.

I would like to thank Prof. Dr. Bahar Kasapgil İnce, the chair of the molecular ecology group at Bogazici University, for her support.

I want to present my special appreciation to Prof. Dr. Erdoğan Okuş, who lost his life by an accident at the time of this study, for his support.

I am very grateful to Res. Assis. Mustafa Kolukırık who shared his all knowledge about molecular biology and laboratory experience for his support and tolerance.

I would like to thank Res. Assis. Özge Eyice for her support and friendship.

I want to thank to may lab mate Gökhan Türker for his support in the laboratory at the last part of my study.

I would like to thank to my father for always supporting and being with me.

Last but not least, I would like to special thank to Mehdi Seyedzadeh for his endless supporting and patience.

June 2006

Zeynep Çetecioğlu
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABBREVIATIONS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>viii</td>
</tr>
<tr>
<td>ÖZET</td>
<td>x</td>
</tr>
<tr>
<td><strong>1. INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td><strong>2. AIM OF THE RESEARCH</strong></td>
<td>3</td>
</tr>
<tr>
<td><strong>3. MICROBIOLOGY OF ANOXIC MARINE SEDIMENTS</strong></td>
<td>4</td>
</tr>
<tr>
<td>3.1. Anoxic Marine Sediments</td>
<td>4</td>
</tr>
<tr>
<td>3.2. Microbial Diversity in the Anoxic Marine Sediments</td>
<td>5</td>
</tr>
<tr>
<td>3.2.1. Bacterial diversity</td>
<td>5</td>
</tr>
<tr>
<td>3.2.2. Archaeal diversity</td>
<td>7</td>
</tr>
<tr>
<td>3.3. Molecular Tools for Identification of Microbial Diversity</td>
<td>9</td>
</tr>
<tr>
<td>3.3.1. PCR-based molecular tools for the assessment of aquatic microbial diversity</td>
<td>11</td>
</tr>
<tr>
<td>3.3.1.1. Random sequencing in clone libraries</td>
<td>11</td>
</tr>
<tr>
<td>3.3.1.2. Denaturing gradient gel electrophoresis (DGGE)/Temperature gradient gel electrophoresis (TGGE)</td>
<td>12</td>
</tr>
<tr>
<td>3.3.1.3. Single-strand conformation polymorphism (SSCP)</td>
<td>14</td>
</tr>
<tr>
<td>3.3.1.4. Terminal-restriction fragment length polymorphism (T-RFLP)</td>
<td>15</td>
</tr>
<tr>
<td>3.3.1.5. Ribosomal intergenic spacer analysis (RISA) and automated ribosomal intergenic spacer analysis (ARISA)</td>
<td>16</td>
</tr>
<tr>
<td>3.3.2. Molecular tools for assessing aquatic microbial diversity that do not involve PCR</td>
<td>17</td>
</tr>
<tr>
<td>3.3.2.1. Fluorescence in-situ hybridization (FISH)</td>
<td>17</td>
</tr>
<tr>
<td>3.3.2.2. DNA re-association analysis</td>
<td>18</td>
</tr>
</tbody>
</table>
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>DGGE</td>
<td>Denaturing gradient gel Electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>TS</td>
<td>Total Solid</td>
</tr>
<tr>
<td>TVS</td>
<td>Total Volatile Solid</td>
</tr>
<tr>
<td>TC</td>
<td>Total Carbon</td>
</tr>
<tr>
<td>TOC</td>
<td>Total Organic Carbon</td>
</tr>
<tr>
<td>TIC</td>
<td>Total Inorganic Carbon</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-Acetic Acid-EDTA</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 5.1. Depths of the sampling points……………………………………………… 31
Table 5.2. Bacterial and archaeal oligonucleotide primers used for PCR amplification………………………………………………………………… 32
Table 6.1. DGGE band patterns of the bacterial community from the Marmara Sea……………………………………………………………… 44
Table 6.2. DGGE band patterns of the archaeal community from the Marmara Sea………………………………………………………………… 45
Table 6.3. TS and TVS concentrations of the anoxic sediment samples from the Marmara Sea…………………………………………………………… 46
Table 6.4. Heavy metal concentrations of the anoxic marine sediment samples from the Marmara Sea……………………………………………………… 47
Table 6.5. Carbon contents of the anoxic marine sediment samples from the Marmara Sea………………………………………………………………… 47
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 3.1.</td>
<td>Universal phylogenetic tree</td>
<td>6</td>
</tr>
<tr>
<td>Figure 3.2.</td>
<td>Major lineages of Archaea: Crenarchaeota, Euryarchaeota and Korarchaeota</td>
<td>8</td>
</tr>
<tr>
<td>Figure 3.3.</td>
<td>Diagram of the different molecular approaches for assessing the genetic diversity of microbial communities</td>
<td>10</td>
</tr>
<tr>
<td>Figure 5.1.</td>
<td>The research ship of İstanbul University-ARAR</td>
<td>29</td>
</tr>
<tr>
<td>Figure 5.2.</td>
<td>Van ween grab sampler</td>
<td>30</td>
</tr>
<tr>
<td>Figure 5.3.</td>
<td>Sampling Locations on the Marmara Sea</td>
<td>30</td>
</tr>
<tr>
<td>Figure 5.4.</td>
<td>Assembling the perpendicular gradient gel sandwich</td>
<td>34</td>
</tr>
<tr>
<td>Figure 5.5.</td>
<td>Casting the gel</td>
<td>35</td>
</tr>
<tr>
<td>Figure 5.6.</td>
<td>Loading the samples</td>
<td>36</td>
</tr>
<tr>
<td>Figure 5.7.</td>
<td>Bio-Rad DCode&lt;sup&gt;TM&lt;/sup&gt; system</td>
<td>36</td>
</tr>
<tr>
<td>Figure 6.1.</td>
<td>Extracted Genomic DNA</td>
<td>37</td>
</tr>
<tr>
<td>Figure 6.2.</td>
<td>Agarose Gel Electrophoresis Photograph of the Amplification of Bacterial 16S rDNA Gene (using pA-pHr primers)</td>
<td>38</td>
</tr>
<tr>
<td>Figure 6.3.</td>
<td>Agarose Gel Electrophoresis Photograph of the Amplification of Bacterial 16S rDNA Gene (using Vf-Vr primers)</td>
<td>39</td>
</tr>
<tr>
<td>Figure 6.4.</td>
<td>Agarose Gel Electrophoresis Photograph of the Amplification of Archaeal 16S rDNA Gene (using Arch46f-Arch1017r primers)</td>
<td>39</td>
</tr>
<tr>
<td>Figure 6.5.</td>
<td>Agarose Gel Electrophoresis Photograph of the Amplification of Archaeal 16S rDNA Gene (using Arch344fcla-Univ522r primers)</td>
<td>40</td>
</tr>
<tr>
<td>Figure 6.6.</td>
<td>DGGE Result of Anoxic Sediments from the Marmara Sea with Bacterial Primers</td>
<td>41</td>
</tr>
<tr>
<td>Figure 6.7.</td>
<td>DGGE Result of Anoxic Sediments from the Marmara Sea with Archaeal Primers</td>
<td>42</td>
</tr>
<tr>
<td>Figure 6.8.</td>
<td>Results of the phylogenetic analysis using Quantity One</td>
<td>46</td>
</tr>
</tbody>
</table>
INVESTIGATION OF ARCHEAL AND BACTERIAL DIVERSITY IN ANOXIC MARINE SEDIMENTS FROM THE MARMARA SEA

SUMMARY

Deep-sea sediments cover more than 50% of the earth’s surface. Investigations into these sediments have demonstrated the presence of a significant microbial biomass on a global scale. It is now believed that this deep biosphere plays a major role in the global cycling of elements and contains a large reservoir of organic carbons. Additionally, microorganisms are carrying out most of the biodegradation processes of pollutants. Therefore, the identification of the microbial community in the deep marine sediments is important.

The Marmara Sea is a small intercontinental basin connecting the Black Sea and the Aegean Sea via the Bosporus Strait and Dardanelles, respectively. The Marmara Sea has a great importance not only because of geological position but also its composition of microbial life which has not been studied using culture-independent methods yet. Unfortunately, the area is polluted by domestical and industrial wastewater discharges, and by petroleum hydrocarbons originated from oil tankers. As a result of the heavy pollution, deep sediments from some areas have become anoxic. Studying microbial diversity in these sediments will allow to make a bioremediation strategy to overcome the chronic pollution in the area by revealing microbial processes occurring in there.

In this study, bacterial and archaeal diversity in anoxic marine sediments from different regions of the Marmara Sea were investigated using cultivation-independent molecular methods. Combination of physical and chemical methods was used to extract genomic DNA from the sediment samples. The genes code for 16S rRNA were amplified by polymerase chain reaction (PCR) using archaeal and bacterial oligonucleotide primers. Amplified 16S rDNA fragments were analyzed using denaturing gradient gel electrophoresis (DGGE). DGGE gels were analyzed using a software-Quantity One and making a phylogenetic tree to explain relations between microbial communities from different points of the Marmara Sea. Microbial community fingerprints of each sampling location were evaluated with not only organic and inorganic contents of the sediments, but also heavy metal contents of the sediments.

DGGE results revealed a relationship between microbial communities and sampling depths. According to the DGGE band patterns, 26 different bacterial species and 27 different archaeal species were detected. The richest region about bacterial and archaeal diversity was found within Gemlik sediments, and the poorest diversity belongs to Iz 17.
According to phylogenetic analysis of DGGE bands, both bacterial and archaeal community of Tuzla and Küçükçekmece, Iz 17 and Gemlik, and Iz 25 and Iz 30 are similar. The furthest points within the phylogenetic tree are Iz 17 and Iz 25 for bacterial community and Gemlik and Iz 30 for archaeal community. The chemical analysis revealed that a relation between microbial community fingerprints and contents of total organic carbon (TOC), total carbon (TC) and heavy metals.
MARMARA DENİZİNDEN ALINAN ANOKSİK SEDIMENTTEKİ ARKEAL VE BAKTERİYEL ÇEŞİTLİLİĞİN İNCELENMESİ

ÖZET


Marmara Denizi Karadeniz’e İstanbul Boğazı ile, Ege Denizi’ne is Çanakkale Boğazı ile bağlanan küçük bir iç denizdir. Marmara Denizi’nin yalnız coğrafî olarak değil, literatürde kısıtlı bir bilgiye sahip olan mikrobiyal çeşitliliğinin açıktan da büyük bir öneme sahiptir. Ayrıca bu alan, hem evsel ve endüstriyel atıksuların deşarjı ile hem de petrol tankerleri kaynaklı hidrokarbonlarla kirletilmektedir. Büyük kirlenmenin sonucu olarak Marmara Denizi’nin bazı noktalarında anoksik sedimentler oluşmuştur. Bu alanlardaki mikrobiyal çeşitliliğinin çalışılması, buralarda oluşan kronik kirliliğe karşı biyolojik iyileştirme stratejileri geliştirmek için bir imkân sağlamaktadır.

Bu çalışmada, Marmara Denizi’nin farklı noktalarından alınan anoksik sedimentlerdeki bakteriyel ve arkeal çeşitlilik moleküler yöntemler kullanarak çalışılmıştır. Kimyasal ve fiziksel yöntemler kullanılarak sediment örneklerinden genomik DNA’lar çıkartılmıştır. 16S rRNA kodlayan genler, arkea ve bakterilere spesifik oligonükleotid primerler yardımıyla “polymerase chain reaction (PCR)” yardımıyla çoğaltılmıştır. Daha sonra çoğaltan bu DNA parçaları “denaturing gradient gel electrophoresis (DGGE)” jellere koşturulmuştur. Son adımda bu DNA parçaları bir bilgisayar programı (Quantity One) yardımıyla analiz edilmiş ve numune alınan noktalar arasındaki filogenetik ilişki ortaya koyan bir ağaç çizilmiştir.

Anoksik deniz sedimentlerine ayrıca toplam organik karbon (TOK), toplam karbon (TK) ve ağır metal analizleri uygulanmış ve her numunedeki mikrobiyal komünite bu analiz sonuçları ile birlikte değerlendirilmiştir.
1. INTRODUCTION

Deep-sea sediments cover more than %50 of the earth’s surface. They are originally formed through the incessant deposition of particles from the productive ocean surface (Aller et al., 1998). Investigation of the microbial community structure in the deep sea sediments is indispensable since benthic microbial communities recycle most of the organic compounds in the marine sediments. In addition, microorganisms are carrying out most of the biodegradation processes of pollutants (Calmona and Fürnster, 1996). There are several studies about characterization of microbial communities involved carbon and sulfur cycling in the benthic environments (Devereux et al., 1994, Gray and Herwig, 1996, Llobet-Borassa et al., 1998, Munson et al., 1997, and Teske et al., 1996), however the studies about microbial populations in deep sea sediments are very poor.

The most important factors that limit the evaluation of prokaryotic biodiversity are small size of the microorganisms, the absence of distinguishing phenotypic characters, and the fact that majority of these organisms can not be isolated in pure cultures mainly due to the ignorance of the culture conditions under which these microorganisms thrive in their natural environment (Bale et al., 1997; Kato et al., 1998; Pace, 1997; Torsvik and Øvreås, 2002; Torsvik et al., 2002). It would estimate that only between 0.5% and 10% of prokaryote biodiversity has actually been identified (Cases and de Lorenzo, 2002).

The use of macromolecular sequence comparisons to define phylogenetic relationships has revolutionised bacterial taxonomy. Methods based on analyses of nucleic acids allow to study a wide range of microorganisms as they occur in nature without cultivation. The refinement of sequencing techniques and perhaps more notably, the development of the highly versatile Polymerase Chain Reaction (PCR) has greatly facilitated the comparative analysis of large numbers of gene sequences (Saiki et al., 1985). Several fingerprinting techniques can be used for comparison of
microbial communities from different environments or to follow the behavior of one community over time. The general strategy for genetic fingerprinting of microbial communities consists of the extraction of nucleic acids (DNA and RNA), the amplification of genes encoding the 16S rRNA, and the analysis of PCR products by a genetic fingerprinting technique, such as denaturing gradient gel electrophoresis (DGGE) (Dorigo et al., 2005).

The Marmara Sea is a small intercontinental basin connecting the Black Sea and the Aegean Sea via the Bosporus Strait and Dardanelles, respectively. The Marmara Sea has a great importance not only because of geological position but also its composition of microbial life which has not been studied using culture-independent methods yet. Unfortunately, the area is polluted by domestical and industrial wastewater discharges, and by petroleum hydrocarbons originated from oil tankers (Tuğrul and Polat, 1995, Öztürk et al., 2000). As a result of the heavy pollution, deep sediments from some areas have become anoxic. Studying microbial diversity in these sediments will allow to make a bioremediation strategy to overcome the chronic pollution in the area by revealing microbial processes occurring in there.

Therefore, in this study, bacterial and archaeal diversity in anoxic marine sediments from different regions of the Marmara Sea were investigated using cultivation-independent molecular methods. Combination of physical and chemical methods was used to extract genomic DNA from the sediment samples. The genes code for 16S rRNA were amplified by polymerase chain reaction (PCR) using archaeal and bacterial oligonucleotide primers. Amplified 16S rDNA fragments were analysed using denaturing gradient gel electrophoresis (DGGE) (Vetriani et al., 1999). Microbial community fingerprints of each sampling location were evaluated with organic and inorganic contents of the sediments.
2. AIM OF THE RESEARCH

The Marmara Sea is a small intercontinental basin connecting the Black Sea and the Aegean Sea via the Bosphorus Strait and Dardanelles, respectively. The Marmara Sea has a great importance not only because of geological position but also its composition of microbial life which has not been studied using culture-independent methods yet. Unfortunately, the area is polluted by domestical and industrial wastewater discharges, and by petroleum hydrocarbons originated from oil tankers (Tuğrul and Polat, 1995, Öztürk et al., 2000). As a result of the heavy pollution, deep sediments from some areas have become anoxic. Studying microbial diversity in these sediments will allow to make a bioremediation strategy to overcome the chronic pollution in the area by revealing microbial processes occurring in there.

Therefore, in this study, bacterial and archaeal diversity in anoxic marine sediments from different regions of the Marmara Sea were investigated using 16s rRNA based molecular methods which do not include limitations of culture dependent microbiological techniques. Combination of physical and chemical methods was used to extract genomic DNA from the sediment samples. The genes code for 16S rRNA were amplified by polymerase chain reaction (PCR) using archaeal and bacterial oligonucleotide primers. Amplified 16S rDNA fragments were analysed using denaturing gradient gel electrophoresis (DGGE) (Vetriani et al., 1999). Microbial community fingerprints of each sampling location were compared and the results were discussed along with the organic and inorganic contents of the sediments.
3. MICROBIOLOGY OF ANOXIC MARINE SEDIMENTS

3.1. Anoxic Marine Sediments

Deep-sea sediments cover more than 50% of the earth’s surface. They are originally formed through the incessant deposition of particles from the productive ocean surface (Aller et al., 1998). Benthic microbial communities recycle most of the organic compounds in the marine sediments. There are several studies about characterization of microbial communities involved carbon and sulfur cycling in the benthic environments (Devereux et al., 1994; Gray and Herwig, 1996; Llobet-Borassa et al., 1998; Munson et al., 1997; and Teske et al., 1996), however the studies about microbial populations in deep sea sediments are very poor.

Coastal and shelf sediments have an important role in the remineralization of organic matter. In shelf areas, an estimated 32 to 46% of the primary production settles to the sea floor. Prokaryotes reoxidize most part of the debris which is located in the sea sediments (Wollast, 1991).

Investigation of the microbial community structure in the deep sea sediment, especially pollutant areas, is indispensable because of investigation of microbial processes underlying secondary pollution phenomena (Calmona and Fürnster, 1996).

A little knowledge about diversity and structures of indigenous microbial populations within the polluted coastal and shelf areas is found in the literature. The few reports that are available for polluted marine sediments deal with main contaminants, such as polyaromatic hydrocarbons (Geiselbrecht et al., 1996; Gray and Herwig, 1996), hydrocarbons (Macnaughton et al., 1999; Röling et al., 2004; and Röling et al., 2002), heavy metals (Frischer et al., 2000; Gillan, 2004, Powell et al., 2003; Rasmussen and Sørenson, 1998), and organic matter (OM) (McCaig et al., 1999; Stephen et al., 1996).
3.2. Microbial Diversity in the Anoxic Marine Sediments

The estimation of the diversity of the eukaryotic microorganisms in aquatic communities is easier than which of the diversity of the prokaryotic microorganisms because the eukaryotic microorganisms could be identified at the species level by their phenotypic characters. In contrast, knowledge of prokaryotic organisms is particularly limited. Their small size, the absence of distinguishing phenotypic characters, and the fact that nearly all of these organisms cannot be cultured are the most important factors that limit the evaluation of their biodiversity (Pace, 1997; Torsvik and Øvreås, 2002; Torsvik et al., 2002). It would estimate that only between 0.5% and 10% of prokaryote biodiversity has actually been identified (Cases and de Lorenzo, 2002). The advent of culture-independent methods, such as molecular tools, has changed visualization of microbial diversity (Hugenholtz et al., 1998; Vandamme et al., 1996; Giovannoni and Rappe´, 2000; Olsen et al., 1986; Amann et al., 1995; Rossello-Mora and Amann, 2001). Studies of Béjà et al. (2002) and Moon-van der Staay et al. (2001) identified unsuspected diversity among microbial marine communities of prokaryotes and eukaryotes, respectively.

3.2.1. Bacterial diversity

The laboratory studies including culturable techniques and 16S rRNA gene methods reveal at least 17 major phyla of Bacteria. The figure 3.1 gives a phylogenetic overview of Bacteria.

The first phylum of bacteria is protobacteria. This is the widest phylum of the bacteria. As a group these organisms are all gram-negative, show extreme metabolic diversity, and represent the majority of known gram-negative bacteria of medical, industrial, and agricultural significance. Proteobacteria has five major subdivisions:
One of the most important known groups of proteobacteria is purple phototrophic bacteria which carry out anoxygenic photosynthesis and contain chlorophyll pigments called *bacteriochlorophylls* with any variety of carotenoid pigments. The purple bacteria have different and spectacular colors, usually purple, red or brown. The most known of purple bacteria are purple sulfur bacteria and purple nonsulfur bacteria (Madigan *et al.*, 2000).
The other known groups of proteobacteria are the nitrifying bacteria which are chemolithotrophs as Nitrosifyers and Nitrifyers, sulfur- and iron-oxidizing bacteria, hydrogen-oxidizing bacteria, methanotrophs and methylotrophs, pseudomonas and the pseudomonads, acetic acid bacteria, free-living aerobic nitrogen-fixing bacteria, neisseria, chromobacterium and relatives, enteric bacteria, vibrio and photobacterium, rickettsias, spirilla, sheathed proteobacteria as sphaerotilus and leptothrix, budding and prosthicate/stalked bacteria, gliding myxobacteria, and finally sulfate- and sulfur-reducing bacteria (Madigan et al., 2000).

The second phylum of bacteria is gram-positive bacteria which contain nonsporulating, low GC, gram-positive bacteria as lactic acid bacteria and relatives; endospore forming, low GC, gram-positive bacteria as Bacillus, Clostridium and relatives; cell wall-less, low GC, gram-positive bacteria as the Mycoplasmas; high GC, gram-positive bacteria as coryneform and propionic acid bacteria; high GC, gram-positive bacteria: Mycobacterium; and lastly filamentous, high GC, gram-positive bacteria as Streptomyces and other Actinomycetes (Madigan et al., 2000).

The other known phyla of the bacteria are cyanobacteria and prochlorophites, Chlamydia, planctomyces/pirellula, the verrucomicrobia, the flavobacteria, the cytophaga group, green sulfur bacteria, the spirochetes, deinococci, the green nonsulfur bacteria, deeply branching hyperthermophilic bacteria and finally nitrospira and defferibacter (Madigan et al., 2000).

3.2.2. Archaeal diversity

Archaea is one of the major phylogenetic groups. Even though they have similar characteristics to the bacteria, not only their phenotypical characteristics but also their phylogenetic characteristics are different. Some of the major features of the Archeae are below:

- absence of peptidoglycan in cell walls
- presence of ether-linked lipids in membrane
- presence of the complex RNA polymerases
Figure 3.2. Major lineages of Archaea: Crenarchaeota, Euryarchaeota and Korarchaeota

A phylogenetic tree of Archeae is shown in Figure 3.2. The tree is separated into two major phyla called the Crenarchaeota and Euryarchaeota. A third phylum is Korarchaeota which branches off close to the root (Madigan et al., 2000).

The first kingdom, Crenarchaeota derived from being phylogenetically close to ancestor or source of Archaea (Woese et al. 1990). It was believed to include only sulphur-dependent extreme thermophiles. Among cultured representatives, the Crenarchaeota contain mostly hyperthermophilic species including those able to grow at highest temperatures of all organisms. Most hyperthermophiles of crenarchaeota are chemolithotrophic autotrophs and primary producers in the harsh environments because of their habitats and devoid of photosynthetic life.

Hyperthermophilic crenarchaeotes tend to cluster closely together and occupy short branches on the 16S rRNA-based tree of life because these organisms have slow evolutionary clocks and have evolved the least away from the hypothetical universal ancestor of life (Madigan et al., 2000).
The Euryarchaeota is a heterogeneous group comprising a broad spectrum of organisms with varied patterns of metabolism from different habitats. It includes extreme halophiles, methanogens, and some extreme thermophiles so far (Madigan et al., 2000).

Moreover, a third archaeal kingdom has recently been discovered which is reported isolation of several archaeal sequences evolutinary distant from all Archaea known to date by Barns et al. 1994 and then in 1996. The new group was placed on phylogenetic tree under Crenarchaeota/Euryarchaeota and named as Korarchaeota (Madigan et al., 2000).

3.3. Molecular Tools for Identification of Microbial Diversity

Traditional techniques are limited for identification microbial diversity. There is an increasing at studies dealing with biodiversity of microbial communities with using molecular tools last 15 years (Morris et al., 2002). Together using the molecular tools, the interest to the identification of microbial communities, new bioactive compounds with some peripheral interest in biogeochemical cycles, and their influence on issues such as global climate change has been driven (Chapin III et al., 2000). The diversity of eukaryotic microorganisms in aquatic microbial communities has now generally been estimated on the basis of phenotypic characters that make clear identification at the species level possible. On the other hand, the knowledge about prokaryotic organisms is particularly limited. 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 (Pace, 1997; Torsvik and Øvreås, 2002; Torsvik et al., 2002). A significant number of studies dealing with microbial biodiversity involve the use of molecular tools and 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 (Morris et al., 2002).
The molecular tools separate two main classes. Figure 3.3 shows these classes:

**Figure 3.3.** Diagram of the different molecular approaches for assessing the genetic diversity of microbial communities. DGGE: Denaturing gradient gel electrophoresis, TGGE: Temperature gradient gel electrophoresis, RISA: Ribosomal intergenic spacer analysis, ARISA: Automated ribosomal intergenic spacer analysis, SSCP: Single strand conformation polymorphism, RAPD: Random amplified polymorphic DNA, AFLP: Amplified fragment length polymorphism, (t)-RFLP: (terminal)-Restriction fragment length polymorphism, ARDRA: Amplified ribosomal DNA restriction analysis, (TSA)-FISH: (tyramide signal amplification)-Fluorescence in situ hybridization, FCM: Flow cytometry (Doriga et al., 2005).
3.3.1. PCR-based molecular tools for the assessment of aquatic microbial diversity

The first step of these tools is amplification of DNA via polymerase chain reaction (PCR). The choice of primers makes it possible to target the sequence at different taxonomic levels (strain, species, genus, etc.). 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.).

3.3.1.1. Random sequencing in clone libraries

Firstly PCR products are cloned at the random sequencing in clone library then these clones are carried out random sequencing within the clone library. Identification of the dominant copies present in the initial PCR products is possible using sequence analysis. Comparing these sequences with the available in sequence databases (GenebankTM or EMBL) gives information about the identity or relatedness of the new sequences to known species. Construction of phylogenetic trees to deduce phylogenetic relationship using molecular sequence data is the most incisive way (Olsen et al., 1986; Woese, 1987). The sequence information can be used to compare species richness or diversity in different samples, but can also be used as the basis for developing specific oligonucleotides required for other molecular methods. In this way probes are designed for use in, fluorescence in-situ hybridization (FISH), and primers for use in denaturing gradient gel electrophoresis (DGGE) analyses. 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 (e.g. Fuhrman et al., 1993; Mullins et al., 1995). Also, studies bacterial diversity in various lakes and revealed the predominance of some bacterial divisions, such as Proteobacteria (particularly in b-Proteobacteria), Bacteroides and Actinobacteria in freshwater, have used this approach (Eiler and Bertilsson, 2004; Glöckner et al., 2000; Hiorns et al., 1997). There have been fewer studies of eukaryotic diversity, but there are the papers of Díez et al. (2001) and Moon-van der Staay et al. (2001); the latter revealing unexpected diversity in oceanic eukaryotic picoplankton. Comparison of species composition in phytobenthic communities at different sampling sites in a small
French river containing both polluted and unpolluted areas has also made using cloning-sequencing strategy (Dorigo et al., 2002).

Apart from the skews linked to the PCR and cloning steps, the problem with this method is how to obtain a correct description of the diversity in a community. Parametric or non-parametric methods are both proposed by different authors as providing better estimates of the species richness from samples, and as ways to compare the diversity in different environments (Colwell and Coddington, 1994; Hughes et al. 2001; Bohannan and Hughes, 2003; Colwell et al., 2004; Singleton et al., 2001). Rarefaction curves or sample-based coverage estimators of species richness could be used to find out whether more sequences are required before diversity can be assessed correctly. In high diversified ecosystems, the PCR-cloning-sequencing approach can be very laborious, time-consuming and expensive. For the same reasons this technique is not well suited for studying seasonal or spatial successions of microbial communities.

3.3.1.2. Denaturing gradient gel electrophoresis (DGGE)/Temperature gradient gel electrophoresis (TGGE)

Denaturing gradient gel electrophoresis (DGGE) and thermal gradient gel electrophoresis (TGGE) have been studies for 10 years. They are now routinely used to assess the diversity of microbial communities, and to monitor their dynamics (Muyzer, 1999; Muyzer et al., 1996; Muyzer and Smalla, 1998). Qualitative and semi-quantitative estimations of biodiversity are obtained using these two methods. They typically involve amplifying the genes encoding the 16S rRNA and then, separating these fragments in a polyacrylamide gel. Studies of metabolically-active populations targeting 16S rRNA have also been reported (Felske et al., 1998). DGGE/TGGE exploits the fact that 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 (DGGE), or through a linear temperature gradient (TGGE). The two strands of a DNA molecule separate or melt at specific temperature, which depends on the hydrogen bonds formed between complementary base pairs (GC-rich domains melt at higher temperatures), and on the attraction between neighboring bases on the same strand. When a DNA fragment is run on a polyacrylamide gel, the mobility of the
molecule continues until 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 a GC clamp, which is added to one primer. The
DGGE/TGGE pattern obtained provides a rapid identification of the predominant
species. The most important advantage of these techniques is that they make it possible
to obtain taxonomic information by excising, re-amplifying and sequencing specific
DNA fragments or by hybridization analysis with taxon-specific oligonucleotide probes
(Heuer et al., 1999; Riemann and Winding, 2001). Most DGGE/TGGE studies focus on
the number of different bands in order to get an estimate of the community richness, and
there have been very few studies that also take into account the intensity of each band as
providing an estimate of the abundance of each band-population (Nübel et al., 1999).

DGGE/TGGE approaches have been used in a huge number of studies of eubacterial,
archaenal and eukaryotic communities in freshwater and coastal waters in relation to
herbicide exposure and/or spatio-temporal variations (Øvreås et al., 1997; van Hannen et
al., 1998; El Fantroussi et al., 1999; Casamayor et al., 2000, 2001, 2002; Lindström,
2000; Riemann and Middelboe, 2002; Zwart et al., 2002; Lyautey et al., 2003; Schauer
et al., 2003).

The choice of the primer set and the optimization of the gel running conditions before
the technique can be used to screen for sequence polymorphism of a particular gene are
the main limitations (Muyzer et al., 1993; Hayes et al., 1999), and the difficulty of
comparing patterns across gels, when these patterns include numerous bands. This
implies that multiple gels and different combinations of samples are required if
numerous samples are being investigated. Other limitations associated with this
technique are the fact that DNA fragments with differing sequences may migrate
together, and its limited sensitivity of detection of rare community members (Vallaeys et
al., 1997). There are several different ways to carry out the statistical analysis of data
obtained by DGGE/TGGE (Fromin et al., 2002).
3.3.1.3. Single-strand conformation polymorphism (SSCP)

Sequence variations among DNA fragments, which are usually PCR-amplified, 16S rRNA gene sequences are detected using single-strand conformation polymorphism analysis (SSCP). SSCP was originally described by Orita et al. (1989), and was first used to assess the diversity of natural microbial communities by Lee et al. (1996). A three-dimensional conformation determined by the intramolecular interactions that influence their electrophoretic mobility in a non-denaturing polyacrylamide gel will be adapted using single-stranded DNA at low temperatures. PCR fragments of the same size, but with differing nucleotide sequences will be separated due to their differing electrophoretic mobility. Differences in mobility are detected on autoradiograms (radioactive detection), by silver staining the bands or using fluorescently labeled primers that are subsequently detected by an automated DNA sequencer (non-radioactive detection).

There have been a few publications that report studies of microbial diversity in aquatic ecosystems using the SSCP method. Changes in groundwater microbacterial communities resulting from various strategies of bioremediation of polluted aquifers have been monitored by Ross et al. (2001) and Wenderoth et al. (2003). However, there have also been several papers reporting the use of this tool to assess the diversity of rhizosphere microbial communities (Schwieger and Tebbe, 1998; Schmalenberger and Tebbe, 2003), to study microbial succession during composting (Peters et al., 2000), and to characterize bacterial community dynamics in the Salers cheese (Duthoit et al., 2003).

A recent application of PCR–SSCP to functional genes has provided some very interesting information likely to be relevant to possible applications of this technique to investigate changes under various environmental conditions in gene families that play functional roles in the environment (Junca and Pieper, 2004). The detection of sequence variation using PCR–SSCP is generally good, but the detection sensitivity tends to decrease as the fragment length increases (Hayashi and Yandell, 1993). Phylogenetic assignation of the bands is possible if the fragment separation is carried out in gel. In the study of Schmalenberger and Tebbe (2003), sequencing was used to demonstrate that, as previously highlighted for DGGE analyses, a single band may consist of several
sequences, and that the electrophoretic conditions may therefore affect the resolution of genetic profiles and thus the estimated diversity. There is a suggestion that gene probes and Southern hybridization could provide valuable control instruments.

3.3.1.4. Terminal-restriction fragment length polymorphism (T-RFLP)

Terminal-restriction fragment length polymorphism (T-RFLP) analysis is a community fingerprinting technique that is based on the restriction digest of double-stranded fluorescently end-labeled PCR fragments (Liu et al., 1997; Marsh, 1999). One primer is labeled at the 50 terminus with a fluorescent dye. As a general rule, a single species will contribute a single terminal fragment of a given size, although several species may have terminal fragments of identical size. TRFLP is a high-throughput, reproducible method that can be used to carry out both qualitative and quantitative analyses of a particular gene in a community. Fragments of the 16S rRNA gene are usually targeted. The fragments are separated by gel electrophoresis in non-denaturing polyacrylamide gels or by capillary electrophoresis, and distinguished by laser induced fluorescent detection. These fluorescence data are converted into electrophoregrams, in which the peaks represent fragments differing in size, and the areas under the peaks indicate the relative proportions of the fragments. The advantage of this technique is its ability to detect even rarer members of a microbial community. In addition, phylogenetic assignments can be inferred from the sizes of the terminal restriction fragment (TRF) using web-based resources that predict T-RF sizes for known bacteria (Kent et al., 2003). Like SSCP, T-RFLP analysis has been used to compare the dynamics both between and within microbial populations in soils and activated sludge (e.g. Bruce, 1997; Liu et al., 1997; Marsh et al., 1998; Moeseneder et al., 1999; Osborn et al., 2000), but there are also some recent publications concerning aquatic ecosystems (Braker et al., 2001; Inagaki et al., 2002; Nusslein et al., 2002; Takai et al., 2002; Konstantinidis et al., 2003; Matz and Jurgens, 2003; Vetriani et al., 2003). The limitations and pitfalls that have been identified for this technique include the formation of pseudoterminal restriction fragments, which can result in the overestimation of microbial diversity, which was reported by Egert and Friedrich (2003). The choice of the primers and restriction enzymes also seems to be very important for obtaining an accurate evaluation of the
microbial diversity (Engebretson and Moyer, 2003; Lueders and Friedrich, 2003). In addition, Engebretson and Moyer (2003) have shown that T-RFLP seems to be very useful for estimating diversity in communities characterized by low-to-intermediate species richness, but is not suitable for complex microbial populations.

3.3.1.5. Ribosomal intergenic spacer analysis (RISA) and automated ribosomal intergenic spacer analysis (ARISA)

Ribosomal intergenic spacer analysis (RISA) was developed by Borneman and Triplett (1997) and was firstly applied to study the microbial diversity in soils. The method involves PCR amplification of the spacer region located between the small (16S) and large (23S) subunit rRNA genes in the rRNA operon. This region is extremely variable in size (ranging from 50 bp to more than 1.5 kb) and nucleotide sequence. Primers are thus defined to target to conserved regions in the 16S and 23S genes. In RISA, the polymorphism revealed is linked to the length heterogeneity. Amplification products differing in length are separated on polyacrylamide gels on the basis of their size and visualized by silver staining. This tool has been used successfully to assess community fingerprints, each band corresponding to at least one organism. Fisher and Triplett (1999) developed the automated version of RISA, which they named ARISA, in order to be able to assess community diversity more rapidly and more efficiently. PCR amplification of the 16S–23S region is performed using a fluorescently labeled, forward primer, which makes it possible to detect the amplicons by automated capillary electrophoresis. The total number of distinct fluorescent peaks in the ARISA data within a given sample is taken as an estimate of species diversity, and the sizes of the fragments can be compared to those in the GenBank database. In the study of Fisher et al. (2000), ARISA was successfully applied to a northern, temperate, oligotrophic lake to investigate the effects of resources and trophic interactions on freshwater bacterioplankton diversity (Fisher et al., 2000). Similarly, Graham et al. (2004) investigated the seasonal diversity pattern of bacterioplankton communities in a northern temperate humic lake, and tried to relate changes in ARISA fingerprints to changes in phytoplankton and protozoan diversity. In another study, Hewson and Fuhrman (2004) investigated richness and diversity of bacterioplankton species along an estuarine
gradient in an Australian Bay. Finally, in a recent and interesting study, Yannarell and Triplett (2005) assessed the influence of geographic and environmental variables on the diversity of lake bacterial assemblages. These authors also investigated the impact of various treatments and transformations applied to the bacterial data set.

Finally, it appears that all these PCR-based tools can provide a considerable amount of information about the species composition and diversity of aquatic microbial communities. However, they all suffer from the same sources of bias, linked for example to the DNA extraction and the generation of artifactual heteroduplex fragments during PCR amplification (Wang and Wang, 1996; von Wintzingerode et al., 1997; Muyzer, 1999; Giraffa and Neviani, 2001), plus the various specific biases inherent in each technique. In a few publications, different methods have been evaluated and compared using the same study models (Moeseneder et al., 1999; Casamayor et al., 2002; Nikolcheva et al., 2003; Dorigo et al., 2004). The results are generally similar, although some methods seem to perform less well in some specific situations.

3.3.2. Molecular tools for assessing aquatic microbial diversity that do not involve PCR

3.3.2.1. Fluorescence in-situ hybridization (FISH)

The fluorescence in-situ hybridization (FISH) technique using rRNA-targeted fluorescent probes is commonly used in microbiology to investigate the overall taxonomic composition of bacterial communities or assemblages. Probes can be designed to be complementary to species-, group-, or kingdom-specific target sites. Cells are fixed to make them permeable to the probe, which then hybridizes its specific target site. Generally, a probe targeting the Bacteria domain is used in combination with more specific probes. Various papers report use of this method to explore bacterial communities in activated sludge, marine and freshwater environments, and in both pristine and contaminated aquifers (Wagner et al., 1993; Borneman et al., 1996; Snaidr et al., 1997; Shi et al., 1999; Cottrell and Kirchman, 2000). Despite the numerous advantages of FISH, the application of the technique to investigate natural samples still produces some false positive and false negative results. Both methodological and environmental factors can influence FISH performance. The choice of the probe and of
the fluorochrome, the stringency conditions, the hybridization temperature, the fact that some microorganisms are autofluorescent, the ecosystem type, and the physiological state of the target cells can all significantly influence the efficiency of this tool (Ouverney and Fuhrman, 1997; Daims et al., 1999; Moter and Göbel, 2000; Fuchs et al., 2001; Bouvier and Del Giorgio, 2003). The main advantages of this combination are the multiparametric analysis of samples and its relatively fast and sensitive determination of even rare populations. However, very few publications report using this approach for ecological studies, although there are numerous papers in the domain of medical research (e.g. Baerlocher and Lansdorp, 2003). Recently, an improvement of the FISH technique has been proposed. The modified technique, known as TSAFISH (Tyramide Signal Amplification of FISH), boosts the fluorescent signal from hybridized cells 20–40 times fold versus background. It has been successfully applied, for example, to picoeukaryotes in Morlaix Bay, but this technique is time consuming, which limits the number of samples that can be studied (Biegala et al., 2003). Another improvement of the FISH method has been made by Pernthaler et al. (2002) who have introduced CARD-FISH (Catalyzed Reporter Deposition-FISH) in studies of natural microbial assemblages. Briefly, samples are concentrated on membrane filters, which are then embedded in low-gelling-point agarose, before permeabilizing the cells using lysozyme. In Pernthaler et al.’s study, up to 94% of bacterial plankton cells were labeled by CARD-FISH.

3.3.2.2. DNA re-association analysis

This technique is used for whole DNA comparisons between two communities, or for studying the sequence variety of a single community. In both cases, the total DNA is extracted and purified. When comparing two communities, the DNA of one community may be radioactively labeled and used as a template. Crosshybridization between the two DNA samples is then carried out, and the degree of similarity is monitored. To investigate the sequence complexity, the re-association kinetics of the denatured single-strand DNA with the homologous sequence is monitored, and this ultimately reflects the size of the genome or the complexity of the DNA. The higher the degree of similarity between two communities or the greater sequence homology of a single community
brings the faster re-association process and vice versa. The major limitation of this technique is the need to isolate the DNA of the community of interest from the complex matrix of DNA in the whole sample, and to produce very highly purified DNA. These shortcomings have so-far prevented routine use of this technique. For further details see the review of Øvreås (2000), the review of Rossello-Mora and Amann (2001) and finally the mini-review of culture-independent molecular techniques of Ranjard et al. (2000). This technique has been used by Torsvik et al. (1990) and Øvreås et al. (1998) to evaluate biodiversity in aquatic communities. A recent paper from this team (Øvreås et al., 2003) reports congruent results using T-RFLP and reassociation techniques for estimating species richness in hypersaline environments.
4. THE MARMARA SEA

The Marmara Sea is a small (size ≈ 70 x 250 km) intercontinental basin connecting the Black Sea and the Aegean Sea. The deepest water is in an underwater through that extends 1300 meters below the surface. This basin is a part of the North Anatolian fault, putting the area at risk for earthquakes. The Istanbul coast is relatively straight with few natural harbors. The sea products are very rich in their diversity due to currents and fish migrations that made Istanbul a fishing center. Fishing is now limited to the Black Sea for different varieties found there and lobsters and shellfish off the coast of the Marmara. Both sources of fish seem to be dwindling due to pollution of the water and over fishing through the years.

4.1. Hydrography of the Marmara Sea

The Black Sea is connected with the Mediterranean Sea by the Bosphorus Strait (length: ~30 km; width: ~0.7–3.5 km); the small basin of the Sea of Marmara (size: ~70 km x 250 km) and the Dardanelles (length: ~70 km; width: ~1.3–7.0 km).

The density differences between the Black and the Aegean Seas drive the flows through the Turkish Straits (Stashchuka and Hutter, 2001). The Black Sea waters enter the Marmara Sea through the Bosphorus upper layer flow and exit the sea from the Dardanelles Strait. Likewise, the Aegean water enter the sea through the Dardanelles Strait lower layer flow and exit to the Black Sea with the Bosphorus underflow (Besiktepe et al., 1994).
In the mean, the Black Sea water with salinity ~17.8 ppt, flowing through the Bosphorus as a surface layer, enters the Sea of Marmara with ~19.4 ppt salinity. While crossing the Sea of Marmara, its salinity increases additionally by nearly 6 ppt. After increasing, it exits from the Dardanelles with a salinity of 29.6 ppt. On the other hand, the Aegean water with a salinity of 38.9 ppt when entering the Dardanelles, go acrosses the strait with little changes in its salt content. Approximately 2 ppt of salinity decreases within the Marmara basin. After a further dilution by another 2 ppt; while being transported through the Bosphorus, the Mediterranean waters enter the Black Sea with a salinity of nearly 35 ppt (Stashchuka, N., Hutter, K., 2001). The Marmara Sea is made up of two layers of water with either Black Sea or Mediterranean Sea origin, separated by a sharp interface. The upper layer has a volume of 230 km$^3$ and an average renewal time of 4-5 months. The lower layer has a volume of 3378 km$^3$ and an average renewal time of 6-7 years (Besiktepe et al., 2000).

The circulation of the Marmara Sea is controlled seasonally by the strength of the surface jet entering from the Bosphorus (a result of the differences of density and barometric pressure and sea level in the adjacent seas) and the local wind stress distribution. The surface circulation is mainly composed of a clockwise circulation. The dense water entering from the Dardanelles Strait sinks to the depths of the Marmara Sea, reaching the bottom in winter and possibly transiting through shallower depths in other seasons, as a function of the initial density difference (Besiktepe et al., 2000).

Mediterranean water, entering from the Dardanelles, supplies the suhalcline layer. The negatively buoyant plume of well-oxygenated water is the only means of renewal of the deep waters, partially compensating for the oxygen consumed by the degradation of organic matter sinking from the upper layer into the lower layer. Yet the subhalocline waters remain permanently deficient in oxygen, as a result of the internal balances of diffusion, advection and consumption. The depth to which the plume penetrates is a function of the seasonal characteristics of the inflow density (modified in the Strait) and the weak interior stratification (Besiktepe et al., 1994).
The moderate wind climate of the Marmara region is strongly influenced by land topography. The entire region of low lying topography surrounding the Turkish Strait Systems is a passageway for cold wind system from the north, and for cyclones moving from the Aegean into the Black Sea. The topographies of the valleys of the Bosphorus and Izmit Bay locally influence wind direction. The daily average wind speed is 4 ms\(^{-1}\). Strong wind events with typical ppeds of 8-25 ms\(^{-1}\) and durations of about 16 hours occur in winter. Moderate northeasterly and southeasterly winds are common during summer. The air temperature is coldest and precipitation highest during January. Warmest temperatures coincide with minimum precipitation during July. The sea surface temperature follows the air temperature with a lag of about one month reaching a minimum in February, and a maximum in August. (Besiktepe et al., 1994)

4.2. Pollution at the Marmara Sea

Black Sea and the Aegean Sea via the Bosphorus Strait and the Dardanelles influence on the oceanographic features (chemical, biological) of the basin. A large number of wastewater discharges to the Marmara Sea from different points. The basin receives a total of 1.9x10\(^6\) tons of TOC (total organic carbon) and 2.7x10\(^5\) tons of TN (total nitrogen) per year from the Black Sea inflow. Pollution loading from Istanbul alone, the biggest city of Turkey in population and industry, makes up the major portion (40–65%) of the total anthropogenic discharges (Tuğrul and Polat, 1995).

Anthropogenic activities in the coastal area of the north Marmara Sea include, urban effluent, summer resorts (untreated effluent discharged into the sea), agricultural run off, sunflower oil factories, a big cement factory, fishing and shipping (Öztürk et al., 2000). In addition of them, several thousand ton oil is carried and the result of this traffic there is an important risk as tanker accident. The Bosphorus Strait is a constant threat to the marine ecosystem. In the past, a lot of tanker accident occurred in not only the Bosphorus Strait, but also the Marmara Sea. Some examples of them are Volgoneft (1999), TPAO explosion (1997). With the tanker traffic of several thousand ton oil carrying vessel per day via the Bosphorus Straits the Marmara Sea environment is constantly threatened. Another negative impact of shipping on the coastal benthic
ecosystem seems to be the recent appearance of exotic species such as the manila clam Tapes philippinarum (Albayrak, 2005).

A fundamental compartment of any aquatic ecosystem is the benthic environment. Bottom sediments are the final sink for many anthropogenic contaminants and they can accumulate great amounts of organic matter affecting the oxygen content of the bottom water (Venturini et al., 2004). Frequently, there is a positive correlation between organic carbon and the contaminant level in coastal marine sediments. Content of organic carbon (TOC) in the sediment can be an indicator of pollution (Shine and Wallace, 2000; Hyland et al., 2005). There is a need for versatility in the use of indicators of biological change, in order to compensate for the effects of local variability in natural and anthropogenic sources of disturbance (Rees et al., 2005).

The Marmara Sea is a critically polluted waterbody, subject to a multitude of wastewater discharges from major land-based sources located along the coastline, including the Istanbul metropolitan area. The water quality measurements indicate severe signs of present and future eutrophication problems (Orhon, 1995) due mostly to the inputs from the Black Sea.

The identified species are typical for this location. That is the community composition has elements from the rich Aegean Sea and the poor Black Sea faunas. The diversity and number of species reflects not only the type of substratum, sandy (11–23 species per 0.1 m²) to muddy (4–24 species per 0.1 m²) but also the impact of anthropogenic stress the sites receive (Albayrak et al., 2006).

The impact of Istanbul’s urban and industrial effluent is also apparent in the TOC. TOC content of sediment varied from 2.1 mg/g to 22 mg/g. Highest average TOC content value (12.5 mg/g) was detected at Büyükçekmece transect (near Istanbul) by Albayrak et al. (2006).
4.2.1. Küçükçekmece

Küçükçekmece is a large, crowded suburb on the European side of Istanbul, Turkey near Atatürk Airport. Küçükçekmece is on the Marmara coast and is the eastern shore (nearest the city) of an inlet of the Marmara called Küçükçekmece Gölü. The inlet is highly polluted but there are works to get it clean again. There used being wild life and many kind of birds on the life. The inlet is connected to the Marmara Sea by a very narrow channel so the water is not salty.

On December 29, 1999, the Volgoneft-248, a 25-year old Russian tanker, ran a ground and split in two in close proximity to the southwest shores of Istanbul at Küçükçekmece. More than 800 tons of the 4,300 tons of fuel oil on board spilled into the Marmara Sea, covering the coast of Marmara with fuel-oil and affecting about 5 square miles of the sea. The amount of heavy fuel oil spilled from the Volgoneft-248 tanker to the Marmara Sea is estimated to be 1,290 tons. Approximately 1,000 tons of the remaining oil was discharged ashore, leaving another 2,000 tons in four tanks located in the sunken bow section. Field observations on the accident day evidenced that the spilled oil contaminated the shorelines between the grounded ship stern off the Menekşe Coast and the rock groin at Çiroz Park five kilometers to the East of the accident. Beaches, fishing ports, restaurants, recreation facilities, the Atatürk Pavilion, piers, groins and seawalls located in this area are directly affected.

Later field surveys associated with legal damage investigations were carried out to survey the state of pollution and the result of clean-up operations three to four months after the accident. It was found that the shorelines have been cleaned to a large extent except some minor leftover, and some fresh marks indicating a continuing contamination. The source for this recent pollution was found to be the oil leakage from the remaining oil in the sunken bow section of the tanker, which was later, recovered in summer, 2000 (Otay and Yenigün, 2000).
4.2.2. Tuzla

Aydınlık Bay, Tuzla located Northeast of the Marmara Sea accommodates nearly 40 shipyards. On February 13th 1997, the tanker named TPAO exploded at the Gemsan Shipyard where it was anchored for repair work. The explosion set the tanker on fire followed by oil spill. The fire extinguishing efforts continued for four days resulting with loss of human life and economic damages. A portion of the oil found on the ship was burned during this fire the rest was mixed to the sea water. The financial and ecological costs of the accident caused by the oil contamination are still under legal investigation.

According to the ship records the tanker was carrying 583 tons of fuel-oil, 29 tons of diesel oil and 9 tons of engine oil summing up to a total 621 tons of contaminant at the time of the accident. The spilled amount was estimated as 215 tons and rest was burned during the fire (Kazezyılmaz et al., 1998).

4.2.3. İzmit

İzmit Bay is an elongated semi-enclosed water body with a length of 50 km, width varying between 2 and 10 km and has an area of 310 km$^2$ (Balkıs, 2003).

Several industries have been developing rather rapidly around the bay. In addition to untreated or partly treated domestic wastewater originating from the increasing population, the substantial industrial development, the heavy maritime traffic and the agricultural activities in the surrounding areas have caused a considerable pollution burden. Furthermore, some factory and urban sewage systems were damaged by the earthquake of August, 1999. The bay ecosystem was strongly affected by the quake and subsequent refinery fire, as were the settlements and industrial regions (Aktan ve Aykulu, 2005).

The commissioning of more than 140 large industrial plants since 1965 and, in particular, the consequent urbanisation of the coastal landscape has completely destroyed the previous serenity of Izmit Bay. Initially all solid and liquid wastes were discharged directly into the Bay. Though major industrial effluents are now treated,
there has yet to be treatment of domestic waste. The renewal capacity and water exchange within Izmit Bay is insufficient for compensation and equilibration. Eutrophication and deterioration of water quality have become serious problems (Telli-Karakoc et al., 2002).

İzmit Bay and its surroundings are one of the most industrialized and populated area of Turkey, receiving more than 300 industrial and domestic effluents. Industrial wastewater discharges a total of 163,000 m$^3$/day wastewater, 24 tons/day BOD and 19.5 tons/day TSS to Izmit Bay. The dissolved oxygen content of İzmit Bay decreased dramatically from 1984 to 1999 and reached a minimum value at 20 m throughout the Bay (Balkis, 2003).

The inner part of the bay is the most polluted portion of the bay. Petroleum industries are situated in the central bay. The western portion (the outerpart), which is the entrance to the Marmara Sea, has a better water exchange capability; hence it is the less polluted part compared to the other parts of the bay. The Izmit Bay ecosystem has been monitored for more than 15 years; therefore, the oceanographic characteristics and pollution level in terms of conventional parameters in discharges such as total organic carbon, total nitrogen, total phosphorous, total suspended solids, etc., are easily available. Additionally, in the last 5–6 years, ecotoxicological studies in the Izmit Bay system have progressed to include finding out the fate and effects of pollutants—such as polycyclic aromatic hydrocarbon (PAHs), polychlorinated biphenyls (PCBs), heavy metals etc. All these previous data showed that the bay waters are eutrophic and the discharges that enter the bay are toxic. The last measurements and analysis, taken just after the earthquake, also showed that dissolved oxygen levels are very critical in the bottom waters and the sediment is polluted by PAHs. The highest concentrations of PAHs are known to be generally found around urban centers. They are aromatic substances, most of which are formed during incomplete combustion of organic material and are also components of crude oil and its refined products. Most of the PAHs are highly lipophilic and are therefore accumulated by mussels and tend to rapidly adsorb on
particles which are deposited in the sediments. Since it is found in the shallowest part of the bay, PAH-contaminated sediment is a big problem especially for the inner bay.

The T-PAH levels of Izmit Bay, in all matrices especially in sediments and in mussels, were found to be much higher than those found in other marine systems. The western portion of the bay is the less polluted part of the system in terms of PAH pollution. The central sector has a special importance because of the petroleum industries. Although the earthquake and the subsequent fire in the refinery increased the PAH level of the system, the discharges of these industries appear to be significant contributors to the PAH pollution in the bay. Due to several reasons—being the shallowest part of the bay, having limited water circulation, etc.—the eastern part is also a risky region. Additionally, unstable oceanographic characteristics resulting from sudden changes in meteorological conditions enhance the risk of pollution in the system, as was seen in case of the upwelling of anoxic bottom waters and resuspension of polluted sediment, which was caused by strong wind. Therefore, polluted sediment and untreated or ineffectively treated waste waters will always be problem for the system (Okay et al., 2003).

4.2.4. Gemlik

The Gemlik Bay is the second most polluted hot spot in the Marmara Sea. The organic carbon content is relatively high within the bay. Contrary to low values in the outer basin, the organic carbon content is relatively high within the bay. Highly-populated eastern and southern coasts are mainly influenced by rapid ecotourism development, direct discharges from rivers, surface run-off and drainage from port areas, domestic and industrial effluent discharges through outfalls and various contaminants from ships. Bathymetric features and the associated hydrodynamic processes seem to play an important role in the enrichment of organic carbon. Organic carbon contents show consistency with the sediment textural characteristics and the oxygen deficiency observed in Gemlik Bay (Alpar et al., 2006).
The bay, with a total surface area of 349 km$^2$, is most particularly subject to high anthropogenic pressure due to inputs from rivers, atmosphere, coastal shipping and industrial activities. The most important industrial towns along the bay are the Gemlik Town, Mudanya and Trilye. Mudanya port is the export gate of the second biggest industrial city of Turkey, Bursa. As opposed to the industrialization along the southern coasts, Armutlu, Fıstıklı, Kapaklı, Narlı, Karacaali, Buyukkumla and Kucukkumla villages are tourism centers along the northern coasts. Total population exceeds 129000 and doubles in summers. The most densely populated towns, however, are Gemlik (80 000) and Mudanya (24 000). The total of domestic wastewater discharge into the bay is as much as 7.5 million m$^3$. Only Gemlik town and Kuckkumla have their own deep sea outfall discharge system. Other coastal settlements use creeks or simple outfalls for their wastewater discharge. New systems are ready to operate for Mudanya and Armutlu (Alpar et al., article in press).

The easternmost part of the bay is subject to chronic severe contaminations, among which hydrocarbons play a major role. The main sources are ship traffic, fishery activities, domestic and industrial sewage waters and riverine inputs. The Karsak creek which discharges into the Gemlik port is the most important pollution source. Not only the discharges of a wide range of industrial plants in Gemlik town, but this creek also carries the waters of Lake Iznik, domestic and industrial wastewater discharges of Orhangazi town located 15 km in the west of the Gemlik Bay. The total load carried by Karsak River is therefore variable seasonally. The share of industrial waste water inputs is even higher, 13–20 million m$^3$/y. The total discharge of textile and chemistry plants is seemingly lower, but they introduce an important industrial pollution into the bay since they do not use treatment systems. The impact of such an anthropogenic pressure can be observed often in summer with the phenomenon of red waters, resulting from eutrophication and disequilibrium processes for the exploitation of natural resources (Alpar et al., article in press).
5. MATERIALS AND METHODS

5.1. Sampling

Samples were collected by a team from İstanbul University. Arar shown in figure 5.1 (the research ship of İstanbul University) was used for sampling. Samples were taken using Van Ween Grab sampler (Hydro-bios, Germany). Figure 5.2 gives Van Ween grab sampler. Triplicate samples were collected for DNA extraction from each point and these samples were stored at -20 °C until DNA extraction.
Samples collected from six different points of the Marmara Sea. Three of these points are at İzmit Bay. The locations are Küçükçekmece, Tuzla, İzmit Bay and Gemlik Bay. Figure 5.3 shows the sampling locations. Samples were collected from different depths. Depth of the each sampling is listed in Table 5.1.
Table 5.1. Depths of the sampling points

<table>
<thead>
<tr>
<th>SAMPLING POINT</th>
<th>CODE</th>
<th>DEPTH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tuzla</td>
<td>MY1</td>
<td>42 m</td>
</tr>
<tr>
<td>Küçükçekmece</td>
<td>MKC</td>
<td>22 m</td>
</tr>
<tr>
<td>İzmit I</td>
<td>IZ17</td>
<td>157 m</td>
</tr>
<tr>
<td>İzmit II</td>
<td>IZ25</td>
<td>30 m</td>
</tr>
<tr>
<td>İzmit III</td>
<td>IZ30</td>
<td>30 m</td>
</tr>
<tr>
<td>Gemlik</td>
<td>MD87</td>
<td>87 m</td>
</tr>
</tbody>
</table>

5.2. DNA Extraction

Physical and chemical disruption methods were used for DNA extraction. Genomic DNAs were extracted by using Fast DNA Spin Kit for Soil (Q-biogene). In addition to the commercial kit, physical disruption was occurred via ribolyser. The steps of the DNA extraction are given below:

Approximately 500 mg sample was added up to the lysing matrix tube which belonged to the commercial kit. The sample was processed for 30 second at speed 5.5 by ribolyser. After processing, the sample was centrifuged at 1400xg for 30 second. Supernatant was transferred to a clean tube. 250 µl PPS reagent was added to the supernatant and they were mixed by shaking the tube by hand. The sample was centrifuged again at 14000xg for 5 minutes to pellet participate. The supernatant was transferred to a new clean tube and 1 ml binding matrix suspension was added to the supernatant. The tube was inverted by hand for 2 minutes to allow binding of DNA to matrix. After inversion, the tube was placed in a rack for 3 minutes to allow settling of a silica matrix. Then, 500 µl of supernatant was removed. Binding matrix was resuspended in the remaining amount of supernatant. All mixture was filtered and filter was placed to a new tube. Filter was washed by 500 µl SEWS-M. After washing, filter was dried by centrifuge at 14000xg for 2 minutes. Filter was removed to a new tube and 50 µl DES (DNase/Pyrogen free water) was added. The filter with DES was vortexed and then centrifuged at 14000xg for 1 minute. Finally, there was a application-ready DNA in the tube.
5.3. Polymerase Chain Reaction (PCR)

In this study, 16S rDNAs of bacterial and archeael were amplified via PCR process. Nested PCR was chosen for amplification. Nested PCR is based on using the first PCR products as template in the second PCR. The primers for PCR are given table 5.2.

Table 5.2. Bacterial and archeael oligonucleotide primers used for PCR amplification

<table>
<thead>
<tr>
<th>Name of Primer</th>
<th>Sequence of Primer</th>
<th>Annealing Temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vf-clamp</td>
<td>5' -GC* GCC TAC GGG AGG CAG CAG-3'</td>
<td>55</td>
</tr>
<tr>
<td>Vr</td>
<td>5' - ATT ACC GCG GCT GCT GG-3'</td>
<td>55</td>
</tr>
<tr>
<td>Pa</td>
<td>5' - AGA GTT TGA TCC TGG CTC AG-3'</td>
<td>55</td>
</tr>
<tr>
<td>pHr</td>
<td>5' - AAG GAG GTG ATC CAG CCG CA-3'</td>
<td>55</td>
</tr>
<tr>
<td>Arch 46F</td>
<td>5' - YTA AGC CAT GCR AGT-3'</td>
<td>40</td>
</tr>
<tr>
<td>Arch1017R</td>
<td>5' - GGC CAT GCA CCW CCT CTC-3'</td>
<td>40</td>
</tr>
<tr>
<td>Arch344F</td>
<td>5' - GC* GAC GGG GHG CAG CAG GCG CGA -3'</td>
<td>53</td>
</tr>
<tr>
<td>Univ522R</td>
<td>5' - GWA TTA CCG CGG CKG CGT -3'</td>
<td>53</td>
</tr>
</tbody>
</table>

5.3.1. Bacterial 16S rDNA gene amplification

Firstly pA and pHr were used to amplify all 16S rDNA gene of bacteria as 1500 bp. The gene was amplified at thermal cycler (TECHNE-TC 412). The amplification conditions were below and total cycle number is 35:

Denaturation: 94 °C for 45 sec
Annealing: 55 °C for 45 sec
Extension: 72 °C for 1 minute

All PCR reactions occurred in a 50 µl reaction mixture containing 0.2 pmol each of primer, 0.2 µmol dNTP, 2.5 mM MgCl₂, 1U of taq polymerase enzyme and 1/10 volume of 10X PCR buffer. 1µl 1/100 diluted genomic DNAs were used as template.

After amplification of whole 16S rDNA gene, V3 region, a part is approximately 200 bp, of this gene was amplified by Vf-Vr primers for loading denaturing gradient gel electrophoresis (DGGE). The amplification process was occurred at the same thermal cycler under the same conditions and total cycle number is 35:

Denaturation: 94 °C for 45 sec
Annealing: 55 °C for 45 sec
Extension: 72 °C for 1 minute

All PCR reactions occurred in a 50 µl reaction mixture containing 0.2 pmol each of primer, 0.2 µmol dNTP, 2.5 mM MgCl₂, 1U of taq polymerase enzyme and 1/10 volume of 10X PCR buffer. 1µl products of PCR using pA-pHr were used as template.

Products of both amplification processes were run on the %1 (w/v) agarose gel, prestained with ethidium bromide in 1x Tris-Borate-EDTA buffer (89 mM Tris, 89 mM Boric acid, 2 mM EDTA; pH 8.3). Gels were visualized using a gel documentation system, Mitsubishi 91. PCR products were stored at 4°C prior to DGGE analysis.

5.3.2. Archeael 16S rDNA gene amplification

For first round of amplification, Arch46f and Arch1017f were used. These primers amplify a part of 16S rDNA gene of archaeae as approximately 1000 bp. The gene was amplified at thermal cycler (TECHNE-TC 412). The amplification conditions were below and total cycle number is 35:

Denaturation: 94 °C for 30 sec
Annealing: 40 °C for 30 sec
Extension: 72 °C for 1 minute

All PCR reactions occurred in a 50 µl reaction mixture containing 0,2 pmol each of primer, 0.2 µmol dNTP, 2.5 mM MgCl₂, 1U of taq polymerase enzyme and 1/10 volume of 10X PCR buffer. 1µl 1/100 diluted genomic DNAs were used as template.

After the first round amplification, V3 region, a smaller part (approximately 200 bp), of this gene was amplified by Arch344f and Univ522r primers for denaturing gradient gel electrophoresis (DGGE). The amplification process was occurred at the same thermal cycler under the same conditions:

Denaturation: 94 °C for 30 sec
Annealing: 53 °C for 30 sec
Extension: 72 °C for 1 minute
All PCR reactions occurred in a 50 µl reaction mixture containing 0.2 pmol each of primer, 0.2 µmol dNTP, 2.5 mM MgCl$_2$, 1U of taq polymerase enzyme and 1/10 volume of 10X PCR buffer. 1µl products of PCR using Arch46-Arch1017 were used as template.

Products of both amplification processes were run on the %1 (w/v) agarose gel, prestained with ethidium bromide in 1x Tris-Borate-EDTA buffer (89 mM Tris, 89 mM Boric acid, 2 mM EDTA; pH 8.3). Gels were visualized using a gel documentation system, Mitsubishi 91. PCR products were stored at 4°C for DGGE analysis.

5.4. Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE has 8 main steps. The first step is assembling the perpendicular gradient gel sandwich. The thickness of desired gel should be 1 mm and this step is seen at figure 5.4.

Figure 5.4. Assembling the perpendicular gradient gel sandwich

After the assembling the system (16 and 18 cm glass plates, 1mm spacer and a 16 well comb), gels containing different concentration denaturant solutions were prepared. For %10 polyacrylamide gel containing %30 denaturant, 33.3 ml of %30
acrylamide:bisacrylamide, 2 ml of 50X TAE (2 M Tris, 50 mM EDTA, and 1 M Acetic acid), 12 ml formamide and 12.6 g urea were mixed and completed to 100 ml by dH₂O. For %10 polyacrylamide gel containing %60 denaturant, 33.3 ml of %30 acrylamide:bisacrylamide, 2 ml of 50X TAE, 24 ml formamide and 25.2 g urea were mixed and completed to 100 ml by dH₂O.

After adding 200 µl of %10 ammonium persulfate (APS) and 10 µl of TEMED to each gel, the gels were casted making a linear gradient by gradient maker. Figure 5.5 shows the gradient maker and casting step.

![Casting the gel](image)

Polymerization took approximately 1 hour and then the bacterial and archaeal PCR products were loaded to different two gels. This step is given at figure 5.6. DGGE was performed with Bio-Rad DCode™ system.
Figure 5.6. Loading the samples

The electrophoresis was occurred in 1X TAE buffer at 60 °C and 200 V for 4 hours. Figure 5.7 gives the Bio-Rad DCode™ system. After electrophoresis, the gels were stained by SYBR Gold and visualized using a gel documentation system, Mitsubishi 91.

Figure 5.7. Bio-Rad DCode™ system
5.5. Analysis of DGGE Gels Using a Software Program-Quantity One 4.4.1

Bacterial and archaeal DGGE gels were analyzed using Quantity One 4.4.1 (BioRad) for taking a phylogenetic relation between sediment collecting regions.

5.6. Chemical Analysis

Due to sediment characteristics of samples, total solids and total volatile solids (TS/TVS), heavy metal concentrations and total carbon, total inorganic carbon and total organic carbon were measured. All analyses were carried out according to Standard Methods (APHA, 1995).
6. RESULTS AND DISCUSSION

6.1. Genomic DNA Extraction and PCR Results

In this study, bacterial and archael community were investigated in the anoxic sediments from the Marmara Sea. Molecular tools were used which are based on 16S rDNA gene. Firstly, the genomic DNAs were extracted from the sediment samples and they were visualized via %1 agarose gel electrophoresis. Figure 6.1 gives the agarose gel photograph.

![Figure 6.1](image)

**Figure 6.1.** Extracted Genomic DNA (MD 87: Gemlik, MKC: Küçükçekmece, MY1: Tuzla, Iz17-25-30: İzmit)
As seen figure 6.1, the genomic DNAs were extracted from all triplicate samples. After extraction, the genomic DNAs were amplified using bacterial and archaeal primers. Amplified DNAs were run on the 1% agarose gel and controlled. Figure 6.2 gives agarose gel photo of the first amplification of the bacterial 16S rDNA gene using pA-pHr primers.

![Agarose Gel Electrophoresis Photograph of the Amplification of Bacterial 16S rDNA Gene (using pA-pHr primers) (MD 87: Gemlik, MKC: Küçükçekmece, MY1: Tuzla, Iz17-25-30: İzmit)](image)

**Figure 6.2.** Agarose Gel Electrophoresis Photograph of the Amplification of Bacterial 16S rDNA Gene (using pA-pHr primers) (MD 87: Gemlik, MKC: Küçükçekmece, MY1: Tuzla, Iz17-25-30: İzmit)

Bacterial 16S rDNA genes were amplified from all samples except three samples of Iz 17. After the second round of PCR amplification for bacterial 16S rDNA gene using Vf-Vr primers, the DNA fragments could be detected on the agarose gel. This result shows that whole 16S rDNA gene of the bacteria which were located at Iz 17 point could be amplified at the first round of amplification using pA-pHr primers, but the amount was not enough to detect them at the agarose gel electrophoresis. For the second round for the nested PCR, first round PCR products were used as template. Figure 6.3 gives the result of the second round PCR on the agarose gel.
As seen figure 6.3, PCR was worked for all samples from Iz17. 200 bp of 16S rDNA gene of bacteria were amplified to use at DGGE.

The same processes were applied the genomic DNA to detect archaeal community. The primers and PCR conditions were changed, but the procedure was same. Nested PCR was applied, too. Firstly, approximately 1000 bp fragments of genomic DNAs were amplified using Arch46f-Arch1017r primers. Figure 6.4 gives the results of first round of nested PCR to amplify archaeal 16S rDNA gene.
As seen in figure 6.4, the bands are faint on the gel. However, this problem is related to amount of PCR products. The first round PCR products were not enough to detect on the gel. After the second round of nested PCR, intensities of the bands increased.

The products of the first PCR to amplify archaeal 16S rDNA gene were used as templates of the second round which included Arch344fcla/Univ522r as primers. The aim of this second PCR is to gain 200 bp DNA fragments to use in DGGE process. After amplification of 200 bp DNA fragments, they were loaded %1 agarose gel to control. The agarose gel results were given in figure 6.5.

![Agarose Gel Electrophoresis Photograph of the Amplification of Archaeal 16S rDNA Gene (using Arch344fcla/Univ522r primers) (MD 87: Gemlik, MKC: Küçükçekmece, MY1: Tuzla, Iz17-25-30: İzmit)](image)

**Figure 6.5.** Agarose Gel Electrophoresis Photograph of the Amplification of Archaeal 16S rDNA Gene (using Arch344fcla/Univ522r primers) (MD 87: Gemlik, MKC: Küçükçekmece, MY1: Tuzla, Iz17-25-30: İzmit)

### 6.2. DGGE Results

The PCR products which were amplified using Vf-Vr primers for bacterial diversity and Arch344-Univ522 primers for archaeal diversity were analyzed by denaturing gradient gel electrophoresis (DGGE) gels to detect and compare community profiles of different points from the Marmara Sea. Figure 6.6 and 6.7 give the DGGE analysis from the
different anoxic sediment samples from the Marmara Sea amplified nested PCR with bacterial and archaeal primers, respectively.

According to the first DGGE analysis which reflected to the bacterial community (figure 6.6), 26 bands, each representing putative organism, were detected at anoxic sediments from the Marmara Sea.

![Figure 6.6. DGGE Result of Anoxic Sediments from the Marmara Sea with Bacterial Primers (MY1: Tuzla, MKC: Küçükçekmece, MD 87: Gemlik, IZ17, 25 and 30: Izmit Bay)](image)

At the other DGGE analysis which reflected to the archaeal community (figure 6.7), 27 bands, each representing putative organism, were detected at anoxic sediments from the Marmara Sea. Comparative DGGE analysis of the archaeal 16S rDNAs in different samples from the Marmara Sea revealed a relatively higher complexity in the community structure of the anoxic sediments than other studies in the literature. Vetriani et al. detected only 16 dominant bands (1999).
Figure 6.7. DGGE Result of Anoxic Sediments from the Marmara Sea with Archaeal Primers (MY1: Tuzla, MKC: Küçükçekmece, MD 87: Gemlik, IZ17, 25 and 30: Izmit Bay)

Table 6.1 and 6.2 show the band patterns of bacterial and archaeal community, respectively.
Table 6.1. DGGE band patterns of the bacterial community from the Marmara Sea

<table>
<thead>
<tr>
<th>Band</th>
<th>Tuzla (42 m)</th>
<th>MKC (22 m)</th>
<th>Gemlik (87 m)</th>
<th>Iz17 (157 m)</th>
<th>Iz25 (30 m)</th>
<th>Iz30 (30 m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>6</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>7</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>8</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>9</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>17</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>
Table 6.2. DGGE band patterns of the archaeal community from the Marmara Sea

<table>
<thead>
<tr>
<th>Band</th>
<th>Tuzla (42 m)</th>
<th>MKC (22 m)</th>
<th>Gemlik 1 (87 m)</th>
<th>Iz17 (157 m)</th>
<th>Iz25 (30 m)</th>
<th>Iz30 (30 m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
</tbody>
</table>
6.3. Phylogenetic Analysis

Using Quantity One 4.4.1, a phylogenetic tree was made to investigate relations between sampling point. Figure 6.8 gives these phylogenetic trees.

![Bacterial Diversity Diagram](image1)

![Archaeal Diversity Diagram](image2)

**Figure 6.8.** Results of the phylogenetic analysis using Quantity One

6.4. Results of Chemical Analysis

Results of total solid (TS) and total volatile solid (TVS) analysis are given table 6.3.

**Table 6.3.** TS and TVS concentrations of the anoxic sediment samples from the Marmara Sea

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>TS(mg/ml)</th>
<th>TVS(mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MKC</td>
<td>450</td>
<td>31</td>
</tr>
<tr>
<td>MY 1</td>
<td>430</td>
<td>20</td>
</tr>
<tr>
<td>MD 87</td>
<td>444</td>
<td>32</td>
</tr>
<tr>
<td>IZ 17</td>
<td>569</td>
<td>29</td>
</tr>
<tr>
<td>IZ 25</td>
<td>302</td>
<td>7</td>
</tr>
<tr>
<td>IZ 30</td>
<td>235</td>
<td>24</td>
</tr>
</tbody>
</table>

Table 6.4. gives the results of heavy metal analysis of the marine sediments.
Table 6.4. Heavy metal concentrations of the anoxic marine sediment samples from the Marmara Sea

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>Cr (mg/kg)</th>
<th>Cu (mg/kg)</th>
<th>Ag (mg/kg)</th>
<th>Fe (mg/kg)</th>
<th>Cd (mg/kg)</th>
<th>Mn (mg/kg)</th>
<th>Pb (mg/kg)</th>
<th>Ni (mg/kg)</th>
<th>Zn (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MD 87</td>
<td>55.15</td>
<td>20.75</td>
<td>&lt; 25</td>
<td>28715</td>
<td>&lt; 10</td>
<td>376</td>
<td>16.3</td>
<td>103.75</td>
<td>164</td>
</tr>
<tr>
<td>MKC</td>
<td>12.9</td>
<td>37.85</td>
<td>&lt; 25</td>
<td>8725</td>
<td>&lt; 10</td>
<td>162</td>
<td>15.5</td>
<td>34.4</td>
<td>177.5</td>
</tr>
<tr>
<td>MODA</td>
<td>33.65</td>
<td>160.45</td>
<td>&lt; 25</td>
<td>22465</td>
<td>&lt; 10</td>
<td>227</td>
<td>84.3</td>
<td>48.45</td>
<td>634</td>
</tr>
<tr>
<td>MY 1</td>
<td>85.85</td>
<td>51.7</td>
<td>&lt; 25</td>
<td>18060</td>
<td>&lt; 10</td>
<td>165.5</td>
<td>56.7</td>
<td>31.85</td>
<td>307.5</td>
</tr>
<tr>
<td>IZ-17</td>
<td>20.9</td>
<td>18.95</td>
<td>&lt; 25</td>
<td>14990</td>
<td>&lt; 10</td>
<td>487.5</td>
<td>11.9</td>
<td>38.25</td>
<td>125</td>
</tr>
<tr>
<td>IZ-25</td>
<td>28.85</td>
<td>58.1</td>
<td>&lt; 25</td>
<td>25345</td>
<td>&lt; 10</td>
<td>243.5</td>
<td>32.15</td>
<td>45.55</td>
<td>255.5</td>
</tr>
<tr>
<td>IZ-30</td>
<td>43.6</td>
<td>103.75</td>
<td>&lt; 25</td>
<td>23515</td>
<td>&lt; 10</td>
<td>204</td>
<td>47.7</td>
<td>45.2</td>
<td>248.5</td>
</tr>
</tbody>
</table>

Table 6.5. gives the carbon contents of the sediment samples as total carbon (TC), total inorganic carbon (IC) and total organic carbon (OC).

Table 6.5. Carbon contents of the anoxic marine sediment samples from the Marmara Sea

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>TC (mg/l)</th>
<th>IC (mg/l)</th>
<th>TOC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MKC</td>
<td>0.283</td>
<td>0.01</td>
<td>0.273</td>
</tr>
<tr>
<td>MY 1</td>
<td>0.202</td>
<td>0.03</td>
<td>0.172</td>
</tr>
<tr>
<td>MD 87</td>
<td>0.199</td>
<td>0.028</td>
<td>0.171</td>
</tr>
<tr>
<td>IZ 17</td>
<td>0.239</td>
<td>0.046</td>
<td>0.193</td>
</tr>
<tr>
<td>IZ 25</td>
<td>0.461</td>
<td>0.044</td>
<td>0.417</td>
</tr>
<tr>
<td>IZ 30</td>
<td>0.48</td>
<td>0</td>
<td>0.48</td>
</tr>
</tbody>
</table>
7. DISCUSSION

Investigations of the compositions of microbial communities are important steps in understanding the role of bacterial and archaeal populations in biodegradation processes in the pollutant regions and biogeochemical processes. Due to poor culturability of natural bacteria, particularly anaerobic bacteria, molecular approaches based on rDNA sequences to investigate microbial community structure in the anoxic marine sediments of the Marmara Sea was used in this study. The aim of this study is to show the similarities and differences between microbial communities in the anoxic marine sediments samples which were collected different sites from the Marmara Sea.

Sampling points in this study are mostly polluted areas within the Marmara Sea and chemical analysis of the sediments (Table 6.2 and 6.3) show the pollution degree at the Marmara Sea.

For this study, highly variable V3 region of 16S rDNA of bacterial and archaeal genomic DNAs was amplified to run on DGGE gels. Figure 6.6 and 6.7 show the DGGE profiles of each duplicate sample taken from different anoxic sites of the Marmara Sea. Both bacterial and archaeal diversity profiles of samples within the different depth and different region have distinctly different banding patterns. However, some bands appear to be common to different sites (Table 6.1 and 6.2).

According to the first DGGE analysis which reflected to the bacterial community (figure 6.6), 26 bands, each representing putative organism, were detected at anoxic sediments from the Marmara Sea. Comparative DGGE analysis of the bacterial 16S rDNAs of different samples from the Marmara Sea revealed a relatively higher complexity in the community structure of the anoxic sediments. The similarity of the community profiles relates to the depth of the sampling. The DGGE analysis of the duplicate samples is similar. Furthermore, the Vf-Vr primers only amplified V3 region of the 16S rDNA, so DGGE analysis provided limited phylogenetic information. At a similar study (Webster et al., 2004), the number of detectable
bands is fewer than this study. This result shows that the Marmara Sea is an unknown environment and has wide range diversity. More detailed studies will help to investigate new indefinite species.

At the other DGGE analysis which reflected to the archaeal community (figure 6.7), 27 bands, each representing putative organism, were detected at anoxic sediments from the Marmara Sea. Comparative DGGE analysis of the archaeal 16S rDNAs in different samples from the Marmara Sea revealed a relatively higher complexity in the community structure of the anoxic sediments than other studies in the literature. Vetriani et al. detected only 16 dominant bands (1999). According to result of this study, the Marmara Sea should be studied completely. As seen figure 6.7, number of the detectable bands of the Iz17 was the lowest in the other samples and there is no important difference between the DGGE analyses of the duplicate samples. Furthermore, the Arch344-Univ522 primers only amplified V3 region of the 16S rDNA, so DGGE analysis provided limited phylogenetic information.

As given table 6.1, there are 16 different bacterial and 10 different archaeal species in the anoxic marine sediment sample from Tuzla. According to the phylogenetic tree drawn using Quantity One, bacterial community of Tuzla is similar to Küçükçekmece. 17 different bacterial and 11 different archaeal species were found in Küçükçekmece sample. Sampling depths of Küçükçekmece and Tuzla are alike. The sampling depth of Tuzla is 42 m. In addition of the similarity of bacterial diversity between Tuzla and Küçükçekmece, phylogenetic relationship is close. The contents of the total carbon and some of the heavy metals (Cu, Mn and Ni) are alike. However, there is no parallelism between results of other chemical analysis.

Tuzla has been mostly polluted with oil due to there are nearly 40 shipyards at Tuzla and in addition of that, an important tanker accident occurred here. The tanker named TPAO exploded in 1997 and the explosion set the tanker on fire followed by oil spill. According to the ship records the tanker was carrying 583 tons of fuel-oil, 29 tons of diesel oil and 9 tons of engine oil summing up to a total 621 tons of contaminant at the time of the accident. The spilled amount was estimated as 215 tons and the rest was burned during the fire (Aydın et al., 1998).

Similar to Tuzla, in Küçükçekmece, a tanker named Volganeflt broke into two pieces in 1999 and the important amount of oil spilled there. So, the similarity between
communities of Tuzla and Küçükçekmece has been expected because of oil pollution.

İzmit Bay is a zone, 50 km in length and consists of three basins connected by relatively narrow openings. The water permanently stratified, low salinity water from Black Sea overlaying Mediterranean water (Tuğrul et al., 1992), the oceanography well-understood (Tuğrul et al., 1992 and Legoviç et al., 1997). Domestic and industrial wastes along north costs of the bay are collected together into seven major discharges. Samples collected from three different points of İzmit Bay and two of them are near the shore, Iz 25 and Iz 30. Their sampling depths are same, 30 m, and their community profile is similar as seen phylogenetic tree. There are 11 different bacterial species in the sediment from Iz 25, furthermore there are 10 different bacterial species in the sediment from Iz 30 and 8 of them are same with Iz 25. Total carbon and total organic carbon contents of these sediments are similar. Additionally, Fe, Mn, Ni and Zn contents of samples are alike. Tolun et al. (2001) found similar heavy metal concentrations in the sediment to this study. Sampling depths, locations and some chemical analysis are similar between these two points, so the bacterial diversities are alike there.

The last similar areas are two points, one from İzmit Bay- Iz 17 and the other from Gemlik Bay- MD 87. There are the deepest points in the sample collected regions. According to the phylogenetic analysis, bacterial and archaeal diversities of Iz 17 and Gemlik (MD 87) are similar. Due to its sampling depth was reasonably different from them and this result was expected. 11 bacterial species, 5 archaeal species were found in Iz 17 samples and 18 bacterial, 12 archaeal species were detected in Gemlik samples. Total carbon and total inorganic carbon contents of Iz 17 and Gemlik are similar and some of the heavy metal concentrations (Cu, Pb and Zn) are alike. 8 same bacterial species were found within these two sediments; however there is no same archaeal species within these samples. Gemlik is an important region because of increasing industrialization and urbanization like İzmit.

The widest microbial diversity was found within Gemlik sediments as 18 different bacterial species and 12 different archaeal species. The poorest area is Iz17, only 11 bacterial and 5 archaeal species were detected.
8. CONCLUSION

This study has been based on to identify microbial ecology within the anoxic marine sediments from the Marmara Sea. The Marmara Sea has a great importance not only because of geological position but also its composition of microbial life which has not been studied using culture-independent methods yet. Unfortunately, the area is polluted by domestical and industrial wastewater discharges, and by petroleum hydrocarbons originated from oil tankers (Tuğrul and Polat, 1995, Öztürk et al., 2000).

There were six different points to collect anoxic marine sediment samples from the Marmara Sea. Depths and chemical characteristics of these sampling points were changed.

A PCR based method- denaturing gradient gel electrophoresis (DGGE) was applied to investigate bacterial and archaeal communities in anoxic sediments from the Marmara Sea.

To understand heavy pollution, some chemical characteristic of samples were examined as contents of carbon and heavy metals.

The results were compared with other anoxic marine sediment studies which include molecular tools. In addition of that, studies about water quality of the Marmara Sea, especially sampling point, were explored.

At the end of this study, 26 bacterial and 27 archaeal species were found within the anoxic marine sediments from the Marmara Sea. Tuzla-Küçükçekmece, Iz17-Gemlik and Iz25-Iz30 have similar microbial diversity.

The widest microbial diversity was found within Gemlik sediments as 18 different bacterial species and 12 different archaeal species. The poorest area is Iz17, only 11 bacterial and 5 archaeal species were detected.
A relation between depth of the sampling point and microbial community was found. Some chemical characteristics of the regions which have similar microbial communities were alike, however a direct relation could not be found.

In conclusion, this study showed molecular tool was applied successfully to identification microbial diversity in the anoxic sediments from the Marmara Sea.
REFERENCES


Alpar B., Unlu S., Kırbasoglu C. 2006. Records of anthropogenic pollution in sediment of Gemlik Bay (Marmara Sea, Turkey) during the last 15 years. Geophysical Research Abstracts. 8,392

Alpar B., Unlu S. Distribution and sources of hydrocarbons in surface of Gemlik Bay (Marmara Sea, Turkey). Chemosphere, Article in Press


CURRICULAM VITAE

Zeynep Çetecioğlu was born in İstanbul, 1981. She graduated from Kabataş High School and enrolled to the İstanbul Technical University, Environmental Engineering Department in 1999. After a year of English Preparation courses, she started the Molecular Biology and Genetic Department at the same university as double major student. She graduated from the Environmental Engineering Department in 2004 and started to MSc degree education in Environmental Biotechnology programme of the same department. She graduated from the Molecular Biology and Genetic department in 2006.