16S rDNA ANALYSIS OF MICROBIAL COMMUNITIES IN A HIGHLY POLLUTED REGION OF THE MARMARA SEA

by

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ABSTRACT

The Marmara Sea is a small (size ≈ 70 x 250 km) intercontinental basin connecting Black Sea and Mediterranean Sea. The population of Marmara region reaches to 25 million and therefore there is large number of domestic and industrial wastewater discharges to the Marmara Sea from different points. Also large quantities of Central Asian oil and gas are transported to the west through the Marmara Sea. Combining effect of pollution sources create a chronic pollution at the Marmara Sea and formed several anoxic sediments in highly polluted sites. One of the areas is Küçükçekmece region. The region is populated by both residential and industrial sites and takes domestic and industrial effluent of more than 3 million people. Industrial sites mainly composed of metal industry, textile and leather industry, medicine industry, paper industry, chemical industry, rubber and plastic industry. Also in 1999 due to tanker accident at Küçükçekmece beach, the region was polluted with more than 3000 tones of petroleum.

Sediment is a carbon and nutrient pool for aquatic environments. The presence of hydrocarbon compounds creates a suitable environment for the growth of anaerobic bacteria. Anaerobic biodegradation processes are slower than aerobic biodegradation. However, anaerobic processes can be a significant factor in removal of organic contaminants owing to the abundance of anaerobic electron acceptors relative to dissolved oxygen; therefore promising a stable and long term removal of contaminants.

It has been estimated that less than 1% of the total microbial population in the land environment and even less in the marine environment have been successfully isolated in pure culture. Marmara Sea has great importance not only because of geological position but also its composition of microbial life which still remains in darkness. The microbial diversity in this unique ecosystem has not been studied using culture-independent molecular techniques yet. Microbial community analyses together with chemical analyses of the sediments will undoubtly form a base to develop bioremediation strategies to overcome chronic pollution at the Küçükçekmece coast.

Main aim of this study is to find a suitable microbial community in anoxic sediments taken from Marmara Sea for a bioremediation strategy depending on anaerobic biodegradation. The microbial diversity and community structure were analyzed by Denaturing Gradient Gel Electrophoresis (DGGE) and 16S rDNA Clone Library method. The results were evaluated with sediment quality parameters along the sampling point. Established results will be used with other information from the literature to analyze the suitability of any of the community in the anoxic sediments from the Küçükçekmece coast for bioremediation purposes. Suitable communities will be marked and used as a cornerstone for a bioremediation strategy based on anaerobic biodegradation.

DGGE results indicate presence of 34 different bands for bacterial community and 15 different bands for archaeal community with each band representing a different organism. Clone library results are parallel to results of DGGE. In bacterial clone library there are 23 different clones and 26 different clones for September 05 and December 06 respectively. In archaeal 16S rRNA clone library, 19 different clones and 20 different clones were found in September 05 and December 06 respectively.

The result of sequencing of bacterial dominant clones indicate presence of *Trichococcus pasteurii, Clostridium glycolicum* in September 05 and Elbe River snow isolate Iso26, *Xanthomonas sp.* CC-FH5, and *Gallicola barnesae* in December 06. Archaeal dominant clones are *Methanoplanus petrolearius, Methanoplanus limicola*, *Methanogenium organophilum* in September 05 and *Methanogenium frigidum* and *Methanosaeta sp*. in December 06.

Results of clone library generation show that syntrophic relations are running in both times. In September 05, fermentation and hydrogenotrophic methanogenesis dominated the pathway. In December 06, anaerobic respiration and fermentation coupled with acetoclastic and hydrogenotrophic methanogenesis dominated the pathway.

ÖZET

Marmara denizi, Karadeniz ve Akdeniz arasındaki tek rotadır. Marmara bölgesinin nüfusu 25 milyona yaklaşmakta ve Marmara denizine çeşitli noktalardan büyük miktarda evsel ve endüstriyel atık boşaltılmaktadır. Ayrıca Marmara denizinde gemi ve tanker trafiği yoğundur. Kirlilik kaynaklarının toplam etkisi sonucu yoğun kirlenen bölgelerde anoksik sedimentler oluşmuştur. Bu bölgelerden biri Küçükçekmecedir. Bölge hem yerleşim hem de endüstriyel bazda yoğundur ve 3 milyondan fazla kişinin evsel ve endüstriyel atığına maruz kalır. Genelde bölgede metal, tekstil ve deri, ilaç, kâğıt, kimya ve plastik endüstrileri gözlemlenir. Ayrıca 1999'da meydana gelen bir tanker kazasında Küçükçekmece sahili 3000 tondan fazla petrol ile kirlenmiştir.

Sediment su ortamları için bir karbon ve besin havuzudur. Hidrokarbon bileşiklerinin varlığı anaerobik bakterilerin büyümesi için uygun bir ortam oluşturur. Anaerobik biyodegredasyon süreci aerobik biyodegredasyona göre yavaştır. Yine de anaerobik biyodegredasyon, anaerobik elektron alıcılarının çözülmüş oksijene kıyasla daha bol olması sebebiyle, organik kirleticilerin yok edilmesinde önemli bir faktör olup kirleticilerin devamlı ve uzun soluklu yok edilmesini vaat eder.

Tahmin edilmektedir ki karada yaşayan toplam mikrobiyal populasyonun %1'inden azı, deniz ortamlarında yaşayanların daha da azı saf kültüre alınmıştır. Marmara denizi sadece jeolojik pozisyonu sebebiyle değil hâlihazırda bilinmeyen mikrobiyal hayatın içeriği ile de büyük önem taşımaktadır. Bu ekosistemde ki mikrobiyel çeşitlilik henüz moleküler teknikler kullanılarak incelenmemiştir. Mikrobiyel komünite analizleri, sediment kimyasal analizleri ile birlikte değerlendirilerek Küçükçekmece sahilindeki kronik kirlenmeyi gidermek için kullanılacak bir biyoıslah stratejisi oluşturabileceklerdir.

Bu çalışmanın esas amacı anaerobik biyodegredasyon prensibine dayanan bir biyoıslah stratejisinde kullanılmak için Marmara denizinden alınan anoksik sedimentlerden uygun bir mikrobiyal komünite bulmaktır. Mikrobiyal çeşitlilik ve komünite yapısı Denaturan Eğimli Jel Elektroforezi(DGGE) ve 16S rDNA Klon Kütüphanesi metoduyla

analiz edilmiştir. Sonuçlar numune alma noktalarındaki sediment kalite parametreleri göz önünde bulundurularak değerlendirilmiştir. Marmara denizinden alınan anoksik sedimentlerde ki komünitelerin biyoıslah amacı için uygunluğu, elde edilen sonuçlar ve literatürdeki diğer bilgilerin yardımıyla incelenmiştir. Uygun komünite anaerobik biyodegredasyona dayalı bir biyoıslah stratejisinin temeli olarak işaretlenmiştir.

DGGE sonuçları göstermedir ki bakteriyel komünite jelinde her biri bir organizmayı temsil eden 34 farklı bant, arkeyel komünite de ise 15 farklı bant vardır. Klon kütüphanesi sonuçları DGGE sonuçlarına paralellik göstermiştir. Eylül 2005 ve Aralık 2006 için bakteriyel klon kütüphanesinde 23 ve 26 farklı klon bulunur iken Arkeyel klon kütüphanesinde is aynı aylar için 19 ve 20 farklı klon bulunmuştur.

Bakteriyel baskın klonların sekans sonuçları Eylül 2005 ayında *Trichococcus pasteurii ve Clostridium glycolicum* varlığını, Aralık 2006 ayında ise Elbe River snow isolate Iso26, *Xanthomonas sp.* CC-FH5, ve *Gallicola barnesae* varlığını göstermiştir. Arkeyel baskın klonlar ise Eylül 2005 ayı için *Methanoplanus petrolearius, Methanoplanus limicola* ve *Methanogenium organophilum* olarak; Aralık 2006 ayı içinse *Methanogenium frigidum* ve *Methanosaeta* sp. olarak belirlenmiştir.

Klon kütüphanesi sonuçları göstermektedir ki Eylül 2005 ve Aralık 2006 komüniteleri sintropik ilişki içindedirler. Eylül 2005'te fermantasyon ve hidrogenotrofik metanojenesis sediment örneğinde baskındır. Aralık 2006 da ise fermantasyon ve anaerobik solunum ile asetoklastik metanojenesis ve hidrogentrofik metanojenesis sediment örneğinde baskındır.

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Symbol	Explanation	Units used
COD	Chemical Oxygen Demand	($mgO_2\ L^{-1}$)
TS	Total Solid	($g\ L^{-1}$)
TVS	Total Volatile Solid	($g\ L^{-1}$)
TOC	Total Organic Carbon	($mg\ g^{-1}$)

EDTA	Ethylene diamine tetra acetic acid
TAE	Tris-Acetic Acid-EDTA
DGGE	Denaturing gradient gel Electrophoresis
PCR	Polymerase Chain Reaction
EtBr	Ethidium Bromide

1. INTRODUCTION

More than half of the earth's surface is covered by aquatic environments. Continual deposition of particles to oceans and seas forms hydrocarbon rich benthic environments, sea sediments (Vetriani et al., 1999). Sediments are a carbon and nutrient pool for aquatic environments. Processes for mineralization of organic matter mainly occur here by the benthic microbial communities (Aller et al., 1998). The presence of hydrocarbon compounds and absence of oxygen creates a suitable environment for the growth of anaerobic bacteria. Although anaerobic biodegradation processes are slower than the aerobic biodegradation, anaerobic processes can be a significant factor in removal of organic contaminants owing to the abundance of anaerobic electron acceptors relative to dissolved oxygen; therefore promising a stable and long term recycling and removal of organic matters (Zwolinski et al., 2000; Chan et al., 2002).

There are many studies focused on the characterization of microbial communities in coastal benthic environments (Devereux and Mundfrom, 1994; Gray and Herwig, 1996; Llobet-Brossa et al., 1998; Teske et al., 1996b). Although there are many attempts to identify microbial communities in marine sediments, most of them based on cultivation dependent techniques (Delille, 1995; Jørgenson and Bak, 1991; Parkes et al., 1995). Cultivation dependent techniques are laborious and contain many restrictions. Since only 0.1-10 % of microscopically detected prokaryotic cells can be cultivated by using traditional microbiological techniques, DNA/RNA based analyses of environmental samples promises new microbial species as well as information about microbial processes (Moter and Göbel, 2000; Sekiguchi et al., 1998; Cases and de Lorenzo, 2002; Amann et al., 1995a).

As a consequence of developments in molecular ecology, the application of molecular techniques such as polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993) and cloning of 16s rDNA (Head and Rölling,2005) have led to new insights into microbial processes in different habitats. DGGE technique provides valuable knowledge of dominant phylotypes within complex microbial communities. Thus, the microbial population dynamics and species responsible

for a specific degradation within the aquatic systems can be monitored and species can be identified by excising bands from the gel and sequencing their DNA. Cloning of 16S rDNA is another PCR dependent technique and reveals the composition of the community by amplifying each phylotype. Monitoring 16S rDNA libraries provides enough information to analyze and identify whole microbial community in the environmental ecosystem.

The Marmara Sea is a small (size ≈ 70 x 250 km) intercontinental basin connecting and acting as the only route between Black Sea and Mediterranean Sea. The population of Marmara region reaches to 25 million and therefore there is large number of domestic wastewater discharge to the Marmara Sea from different points. 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). Also large quantities of Central Asian oil and gas are transported to the west through the Marmara Sea. Combining effect of pollution sources create a chronic pollution at the Marmara Sea and formed several anoxic sediments in highly polluted sites. One of the areas is the Küçükçekmece region. The region is populated by both residential and industrial sites and takes the domestic and industrial effluent of more than 3 million people. Industrial sites mainly composed of metal industry, textile and leather industry, medicine industry, paper industry, chemical industry, rubber and plastic industry. Also in 1999 due to tanker accident at Küçükçekmece beach the region was polluted with more than 3000 tones of petroleum (Otay and Yenigün, 2000). The microbial diversity in this unique ecosystem has not been studied using culture-independent molecular techniques yet. Microbial community analyses together with chemical analyses of the sediments will undoubtly form a base to develop bioremediation strategies to overcome chronic pollution at the Küçükçekmece coast.

Usually oil spills are removed from the environment by mechanism of aerobic respiration to degrade petroleum hydrocarbons (Prince, 1997). Although the result may be beneficial, aerobic hydrocarbon degradation has a limiting parameter, which is presence of oxygen. Any treatment of contaminated sediments is not conventional since oxygen transfer to sediment by mechanical methods is laborious and expensive (Head and

Swannell, 1999). On the other hand anaerobic biodegradation uses not dissolved oxygen but anaerobic electron acceptors that can be found abundantly in the sediment (Zwolinski et al., 2000). Several studies clearly showed degradation of several petroleum hydrocarbons with nitrate or sulfate as electron acceptors, or by methanogenesis (Widdel and Rabus, 2001; Boll et al., 2002).

In this study, sediments taken from the coast of Küçükçekmece were analyzed in terms of microbial composition and chemical characteristics. Total solid/total volatile solid, heavy metal concentrations, total carbon, total organic carbon, total inorganic carbon and anions of the sediment were measured. Change in archaeal and bacteria diversity in the sediment monitored by DGGE throughout a year and microbial composition of the sediment was investigated by cloning and sequencing of archaeal and bacterial 16S rRNA genes.

2. POLLUTION OF MARMARA SEA

2.1. Description of Marmara Sea

The Marmara Sea is a small (size ≈ 70 x 250 km) intercontinental basin connecting the Black Sea and the Mediterranean Sea. Marmara Sea has its name from the region where it presents. The Marmara region is one of the important coastal settlements in Turkey. The region has evolved rapidly both in industrial activities and population. As being in the middle of the region, Marmara Sea becomes 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). In addition to these, Marmara Sea and Turkish straits become a prime site for oil pollution because of inflow from Black Sea and increase in sea traffic mainly due to industrialization and dependence of petroleum. It has been reported approximately 450 sea accidents in 40 years between 1960 and 2000. Most of the accidents were not very important but there were some accidents which caused historic oil spills with major results on the environmental pollution (Kazezyilmaz et al, 1998).

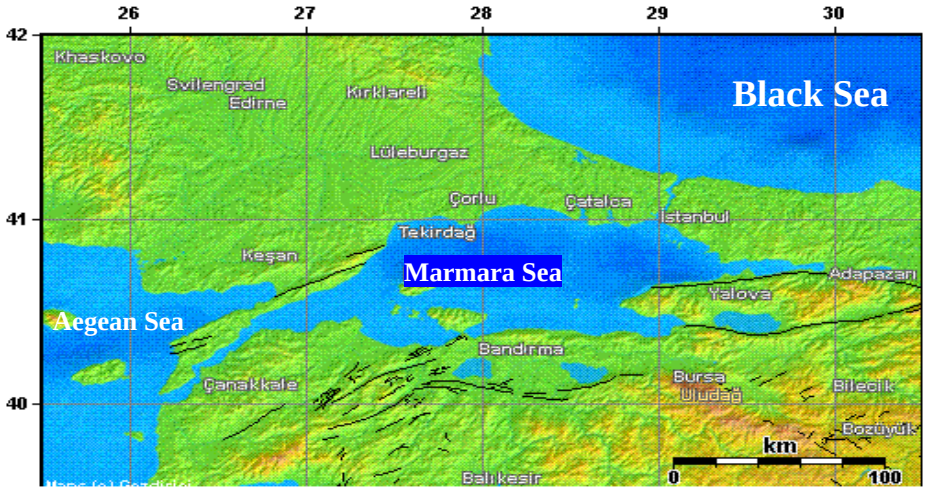

Figure 2.1. Location of Marmara Sea

2.1.1. Hydrography of Marmara Sea

Marmara Sea is one of the components of Turkish Strait which is also composed of Bosphorus and Dardanelles. Marmara Sea is connected to Black Sea via Bosphorus which is 31 km long and 1.6 km wide on the average. The maximum depth is 110 meters and the narrowest point is 70 meters. There are two currents flowing from Black Sea to Marmara Sea. Upper water current has a speed of 0.5-4.8 knots sometimes reaching to 6.7 knots. Undercurrent is slower and has a speed rate of 1.6 knots. Dardanelles connects Marmara Sea to Aegean Sea and it is 62 meters long and 6.5 km across at the widest point as 1.2 km at the narrowest point. The max depth is 105 meters. Upper current has a speed of 1.6 knots, as undercurrent has 0.4 knots. Due to density difference upper current carries water of Black Sea to Aegean Sea as the undercurrent do the opposite. Sea of Marmara has a surface area of 11.550 km^2 and maximum depth of 1268 m. Its upper current has speed of 0.4 knots and undercurrent has speed of 0.1 knots (Kocatas et al., 1993, Alpar and Yuce, 1998, Stashchuka and Hutter, 2001, Besiktepe et al., 1994).

The water circulation of the Marmara Sea mainly controlled by water entering the sea due to density differences, barometric pressure differences and sea level differences of connected seas. Local wind stress distribution also plays a role in circulation too. Water from Black Sea circulates mainly in clockwise. The denser water from Aegean Sea sinks deep after entering Marmara Sea and moves to shallower depths in warmer seasons due to density difference (Besiktepe et al., 2000).

2.1.2. Sources of Pollution in Marmara Sea

A large number of wastewater discharges to the Marmara Sea from different points. 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). Industrial effluents with flushing of refinery plants can be considered also as sources of pollution too.

Benthic composition is one of the main elements of an aquatic system. Sediments are final destination of contaminants and other nonsoluble materials and due to

accumulation of organic materials it becomes an oxygen trap for the bottom water (Venturini et al., 2004). It has been found that there is a positive correlation between organic carbon contents and level of pollution in deep sediments. According to these arguments organic carbon level may be used as an indicator of pollution (Shine and Wallace, 2000, Hyland et al., 2005).The anthropogenic effect of pollution can be seen in the content of organic carbon. Total Organic Carbon (TOC) content of sediments varies from 2.1 mg/g to 22 mg/g with a highest average value of 12.5 mg/g at Büyükçekmece coast (Albayrak et al., 2006).

Another important contaminant of Marmara Sea is petroleum hydrocarbons. Mainly oil pollution of Bosphorus occurred due to currents from the Black Sea. It has been estimated that 410.000 t of oil products are discharged into Black Sea each year. The estimated inflow from the Black Sea was calculated as total of $1.9x10^6$ tons of TOC (total organic carbon) and $2.7x10^5$ tons of TN (total nitrogen) per year. Addition to oil pollution caused by inflow from Black Sea, heavy sea traffic and various refineries and facilities located around Marmara Sea increases the oil pollution dramatically (Fashchuk et al., 1991, Tuğrul and Polat, 1995). The oil concentration increased with years gradually as the sea traffic increases with years. The oil concentration at Bosphorus increased from 9.5 µg/L to 33.5 µg/L from 1995 to1996. The Dardanelles showed a higher increase in concentration from 5.25 µg/L to 42.5 µg/L in the same period. The concentration of the Marmara Sea increased from 36.9 µg/L to 103.7 µg/L at the same time (Guven et al., 1998).

Large quantities of Central Asian oil and gas, which support a market worth billions of dollars, have passed through the Bosphorus Strait to reach the West and elsewhere. The pollution caused by sea traffic has two different sources, minor but continuous pollution due to ballast waters and major but seldom pollution due to ship accidents. High traffic in Bosphorus creates a great risk for the ships since strait has many narrow points and curves. In past years, two major and hundreds of minor tanker accidents resulted in great oil spills. In 1979 Independenta had caused an oil spill which was resulted with 95000 t crude oil at the southern part of Bosphorus. In 1994 another accident, Nassia, contaminated northern Bosphorus with 14000 t of crude oil (Dogan et al., 2005).

2.2 Region of Küçükçekmece

Küçükçekmece is on the Marmara coast, on the eastern shore of an inlet of the Marmara called Küçükçekmece Gölü (Küçükçekmece Lagoon). The inlet is connected to the Marmara Sea by a narrow channel, so the water is not salty. Until the 1950's Küçükçekmece was a popular weekend excursion, people would come by train from Istanbul to swim or to fish. The streams running into the inlet now carry industrial waste and the inlet is highly polluted but efforts are being made to get it clean again. There used be wildlife and many kinds of birds and efforts to get the wildlife back are taking effect slowly.

Due to geographical easiness to build any installation, the area has become an industrial region and crowded with huge housing projects. This development is still going on and is indeed accelerated as the TEM motorway to Europe passes through here now. The Ikitelli region in particular is very industrial and still more factories are being built. The Nuclear Energy Research center is located on the lake side.

2.2.1. Sources of Pollution at the Region

The region is polluted heavily due to awry urbanization and intensive industrialization. The Küçükçekmece lagoon is subjected to take effluent of 2 million people at the year of 2000. Industrial sites are mainly composed of metal industry, textile and leather industry, medicine industry, paper industry, chemical industry, rubber and plastic industry. The control of discharges are not controlled or regulated by the government. These problems coupled with incomplete sewage system create huge impact on the region. Therefore a recreation place once becomes now a place with lots of buildings and eutrophicated lagoon. The sources of pollution are classified as point and nonpoint sources. Point sources composed of discharges from domestic and industrial sites. Waste loads of Nuclear Research Institution affect also rivers flowing to the lake. Nonpoint sources include drainage waters coming from runoff, groundwater including leachate and water coming from agricultural activities.

2.2.2. Petroleum Pollution due to Volganeft Accident

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 due to storm. More than 3000 tons of 4,300 tons of fuel oil on board spilled into the Marmara Sea. During the storm, spilled fuel oil spread to beach of Florya, about 5 square miles of the sea. According to the observations on the day of accident, 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 Pavillion, piers, groins and seawalls located in this area are directly affected. The concentration of oil was so high in some areas it reaches thickness of 5 cm on the surface of sea water. Fuel oil reached to the beach was then covered with sand creating a fuel oil saturated muddy layer along the beach. Heavy spill affected the aquatic life severely, killing many species of aquatic ecosystem including fishing birds (Dogan et al., 2005).

On the day of accident the measured oil contamination was 14.05 g/L. The same sampling point showed 450 µg/L of oil contamination after 4 days. This value was still approximately 35 times higher than the standard value of sea water which was 13µg/L according to WHO-1989. Even after one year, contamination in the sea water varied 5-20 folds of the standard. The severity of the spill can only be understood when a comparison was made with spills occurred in the past. In Rhode Island, USA, 2700 t of fuel oil was spilled and the oil present in sea water was 4-115 µg/L. In 1978, during Amoca Cadiz accident 221000 t of fuel oil was spilled and the amount of oil present in sea water was 10µg/L. The oil present in sea water in the day of Volganeft accident was 1.5 million fold of the standard value and the day after the accident it was 4000 fold of the standard. Even after more than one year, oil present in the sediments was also 10-44 folds of the standard value which is 10 µg/g (Dogan et al., 2005).

Although the oil spill caused a major impact on the aquatic ecosystem of the region, ecosystem is recovering with the time. After two years the number of diatoms in the total phytoplankton increased from 8% to 65% (Dogan et al., 2005).

3. ANOXIC MARINE SEDIMENTS AND ITS MICROBIOLOGY

3.1. Definition and Characteristics of Anoxic Marine Sediments

More than half of the earth's surface is covered by aquatic environments. Continual deposition of particles to oceans and seas forms hydrocarbon rich benthic environments, sea sediments (Vetriani et al., 1999). Sediments are a carbon and nutrient pool for aquatic environments. Processes for mineralization of organic matter mainly occur here by the benthic microbial communities (Aller et al., 1998). 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., 1996b), however the studies about microbial populations in deep sea sediments are very poor. Coastal and shelf sediments are especially important in the remineralization of organic matter. In those 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).

A little knowledge about diversity and structures of indigenous microbial populations within the polluted costal 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), heavy metals (Frischer et al., 2000; Gillan, 2004, Powell et al., 2003; Rasmussen and Sørenson, 1998), and organic matter (McCaig et al., 1999; Stephen et al., 1996), hydrocarbons (Macnaughton et al., 1999; Röling et al., 2004; and Röling et al., 2002). The presence of hydrocarbon compounds and low oxygen level creates a suitable environment for the growth of anaerobic bacteria. Although anaerobic biodegradation processes are slower than aerobic biodegradation, anaerobic processes can be a significant factor in removal of organic contaminants owing to the abundance of anaerobic electron acceptors relative to dissolved oxygen.

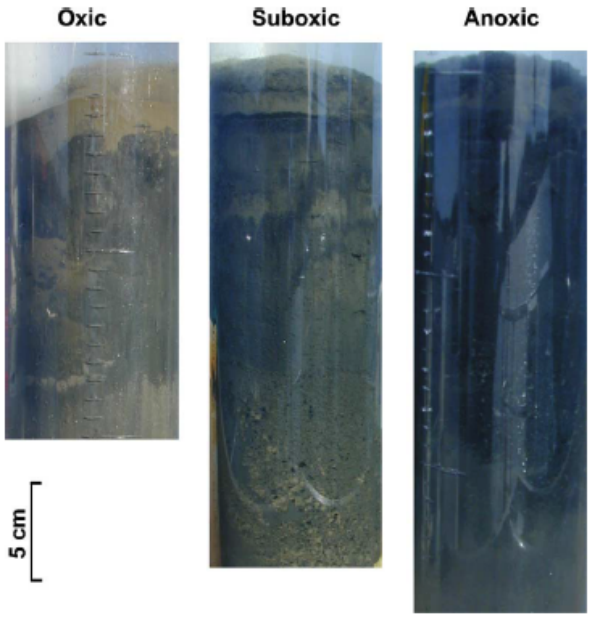

Figure 3.1. The oxic, suboxic and anoxic sediments (Virtasalo et al., 2005)

3.2. Microbial Life in the Anoxic Marine Sediments

In estimation of diversity of microbial life in aquatic communities, there are several difficulties in estimation of diversity of prokaryotes. Prokaryotic microorganisms are harder to identify at species level by their phenotypic character than eukaryotic ones. Their small size, the absence of distinguishing phenotypic characters, and the fact that nearly all of these organisms cannot be cultured are 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., 1995a; 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 Communities in Anoxic Sediments

According to laboratory studies including both culture dependent and independent techniques, there are at least 17 major phyla of bacteria. Figure 3.1 gives a phylogenetic overview of Bacteria.

The first phylum of bacteria is proteobacteria. 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:

- Alpha
- Beta
- Gamma
- Delta
- Epsilon

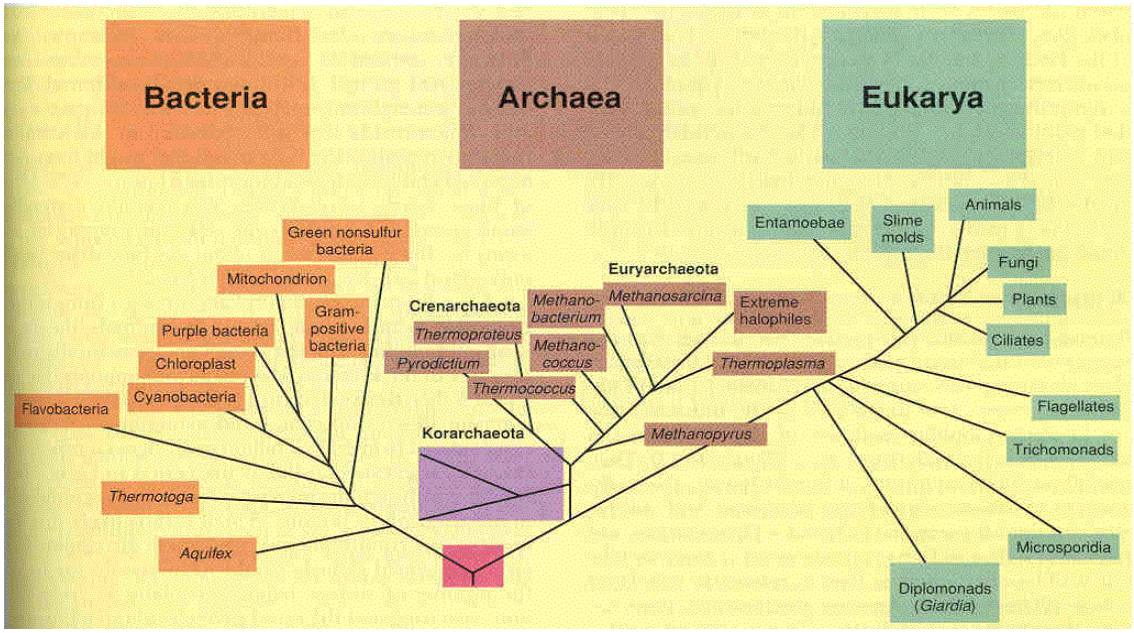

Figure 3.2. Universal phylogenetic tree (Madigan et al., 2002)

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., 2002).

The other known groups of proteobacteria are the nitrifying bacteria which are chemolithotrophs as Nitrosifiers and Nitrifiers, 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*, *rickettsia*, *spirilla*, sheathed proteobacteria as *sphaerotilus* and *leptothrix*, budding and prosthecate/stalked bacteria, gliding *myxobacteria*, and finally sulfate- and sulfur-reducing bacteria (Madigan et al., 2002).

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., 2002).

The other known phyla of the bacteria are *cynabacteria* and *prochlorophtes*, Chlamydia, *planctomyces/pirellula*, *verrucomicrobia*, *flavobacteria*, *cytophaga* group, green sulfur bacteria, *spirochetes*, *deinococci*, green nonsulfur bacteria, deeply branching hyperthermophilic bacteria and finally *nitrospira* and *defferibacter* (Madigan et al., 2002).

3.2.2. Archaeal Communities in Anoxic Sediments

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 *Archaea* are below:

- absence of peptidoglycan in cell walls
- presence of ether-linked lipids in membrane
- presence of the complex RNA polymerases

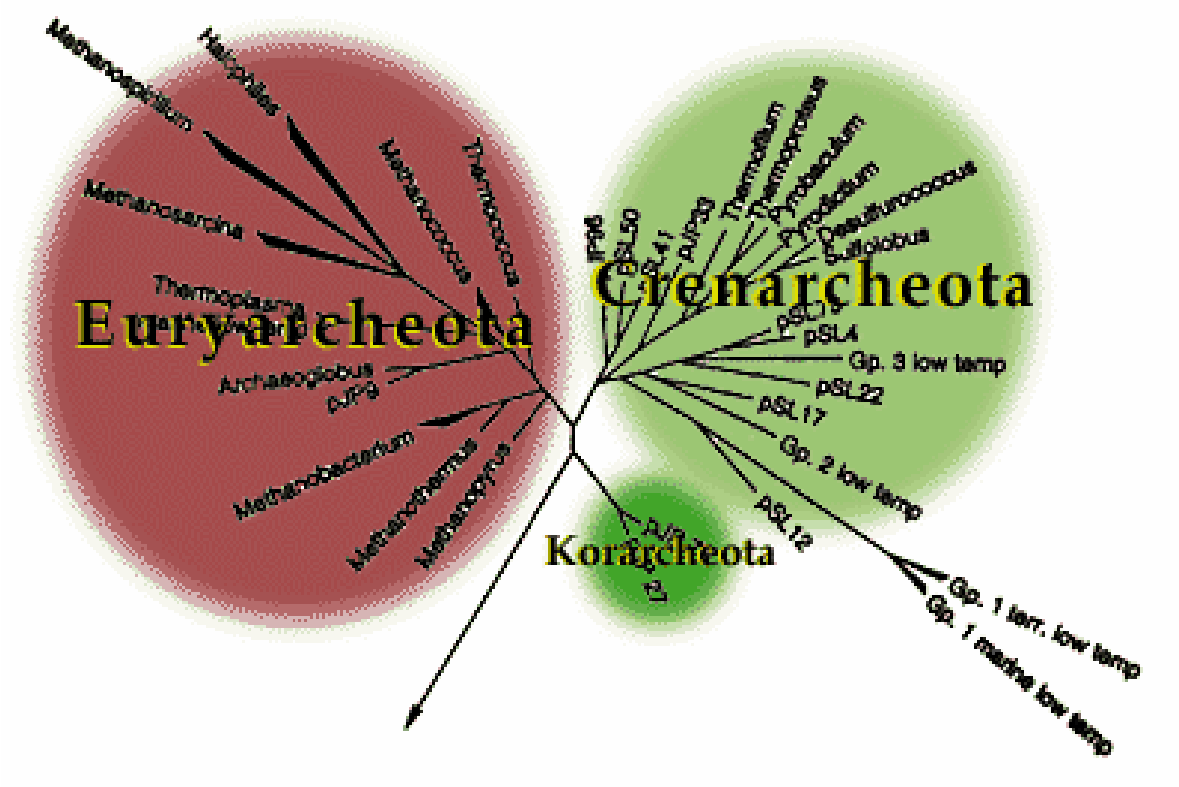

Figure 3.3. Major lineages of *Archaea:* Crenarchaeota, Euryarchaeota and Korarchaeota (http://www.ucmp.berkeley.edu)

A phylogenetic tree of *Archaea* 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., 2002).

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

chemolithotropic autotrophs and primary producers in the harsh environments because of their habitats and devoid of photosynthetic life.

Hyperthermophilic crenarchaeota 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., 2002).

The Euryarchaeota is a heterogeneous group compromising 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., 2002). Moreover, a third archaeal kingdom has recently been discovered which is reported isolation of several archaeal sequences evolutionary distant from all *Archaea* known to date by Barns and coworkers in 1994 and then in 1996. The new group was placed on phylogenetic tree under Crenarchaeota/Euryarchaeota and named as Korarchaeota (Madigan et al., 2002).

3.3. Petroleum Hydrocarbon Degradation by Microorganisms

With developments in industry, petroleum became a daily element of our lives. As the dependence of petroleum increases, the pollution caused by the petroleum hydrocarbon emission increased continuously. The input of petroleum hydrocarbons is high enough to cover the whole earth with a layer of oil. Although the scene is so bad, earth didn't covered with an oil layer only because of degradation of this petroleum by the activity of microorganisms. Individually or working in network, microorganisms are able to degrade hydrocarbons efficiently. Mostly marine environments are natural habitat of hydrocarbon degrading microorganisms.

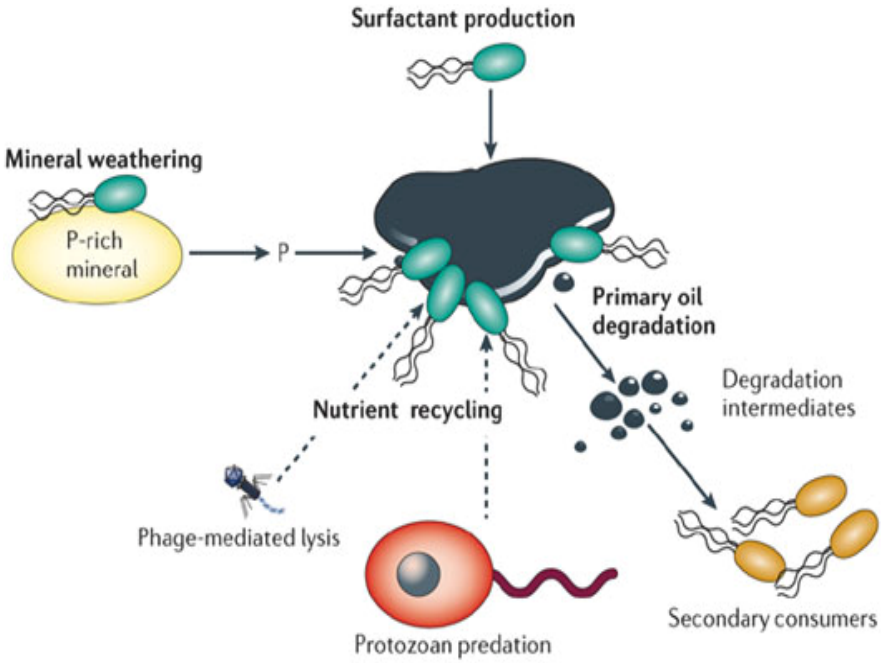

Figure 3.4. Biodegradation of petroleum hydrocarbon by microbial consortium (Head and Rolling, 2006)

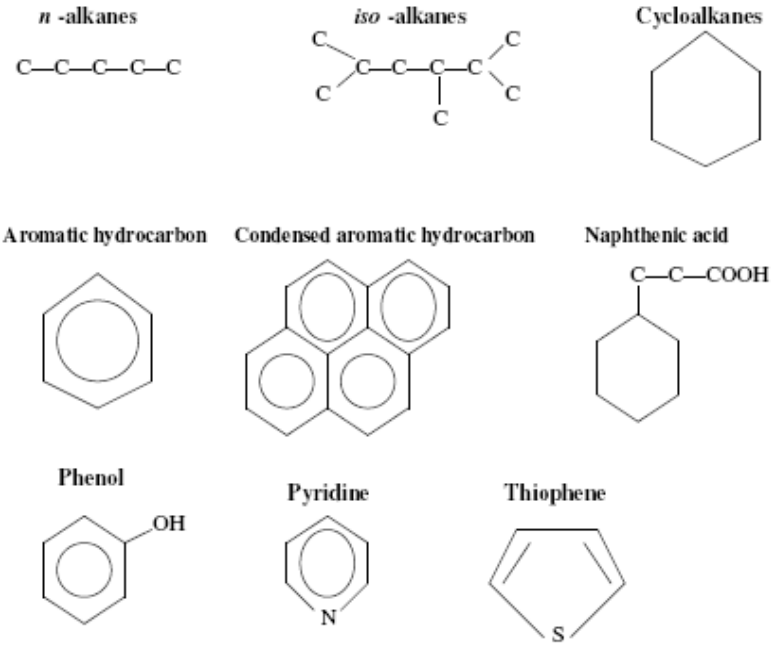

Figure 3.5. Structural classification of some crude oil components (Alloway and Ayres, 1993)

The biodegradation of petroleum hydrocarbons is not a new concept. The isolation of first hydrocarbon degrading bacteria spans to 1900s (Söhngen, 1913). Currently it has been found that there are 79 bacterial genera, 9 cyanobacterial genera, 103 fungal genera and 14 algal genera using, degrading and transforming hydrocarbons. With the new achievements in biochemistry of hydrocarbon degrading bacteria, degradation of hydrocarbons are well understood. The breakdown of hydrocarbons is mainly limited by the presence of nutrients like nitrogen and phosphorus (Atlas and Bartha, 1972); iron was also reported as a limiting factor in clean offshore seawater (Swannell et al., 1996). Sulphur is a well abundant in seawater as sulphate ion but can be a limiting factoring a freshwater system. Slightly alkaline pH of seawater is a favorable environment for the degradation. When these nutrients are abundant, bioavailability of hydrocarbons increases in importance and becomes the limiting factor.

Table 3.1. Some of common factors affecting petroleum hydrocarbon degradation (Bartha, 1986)

Limiting Factor	Explanation and Examples
Petroleum Hydrocarbon Composition (PHC)	Structure, amount, toxicity
Physical state	Aggregation, spreading, adsorption
Weathering	Evaporation, photooxidation
Water potential	Osmotic and matrix forces
Temperature	Influence on evaporation and degradation rates
Oxidant	O_2 required to initiate oxidation, PHC biodegradation
Mineral Nutrients	N, P, Fe may be limiting
Reaction	Low pH may be limiting
Microorganisms	PHC degraders may be absent or low in numbers

There were many studies about microbial hydrocarbon degradation in controlled conditions (Sugiura et al., 1997; Chaillan et al., 2004) and in open field experiments (Gogoi et al., 2003) but the knowledge about organisms play important role in

biodegradation was rather limited. But there are some recent reviews about the degradation of hydrocarbons in anoxic conditions enlighten scientist in understanding these microorganisms. Studies have shown that the important players in hydrocarbon degradation come from marine environments. Those microorganisms are specialized in biodegradation of hydrocarbons by using them as a carbon source. *Alcanivorax* spp. (Yakimov et al., 1998), *Cycloclasticus* spp. (Dyksterhouse et al., 1995), *Oleiphilus* spp. (Golyshin et al., 2002), *Oleispira* spp.(Yakimov et al., 2003) and *Thalassolituus* spp. can be counted as important hydrocarbon biodegraders. Also bacterial groups of *Pseudomonas, Marinobacter, Microbulfier, Sphingomonas, Micrococcus, Cellulomonas, Dietzia and Gordonia* are also known as their capacity to degrade hydrocarbons (Brito et al., 2006). Some of these microorganisms specialized to degrade branched and straight chain hydrocarbons, as some others interest in polycyclic aromatic hydrocarbons. Although methanogens can not be classified petroleum hydrocarbon degraders, they are in the microbial network. Their possible role is to use acetate coming from reactions of anaerobic degraders as metabolite and produce methane and carbon dioxide. There are several studies showing hydrocarbon degradation in deep subsurface and petroleum reservoirs linked to methanogenesis (Nilsen and Torsvik, 1996, Zengler et al.,1999; Widdel and Rabus, 2001; Anderson and Lovley, 2000; Nazina et al., 1995; Ng et al., 1989).

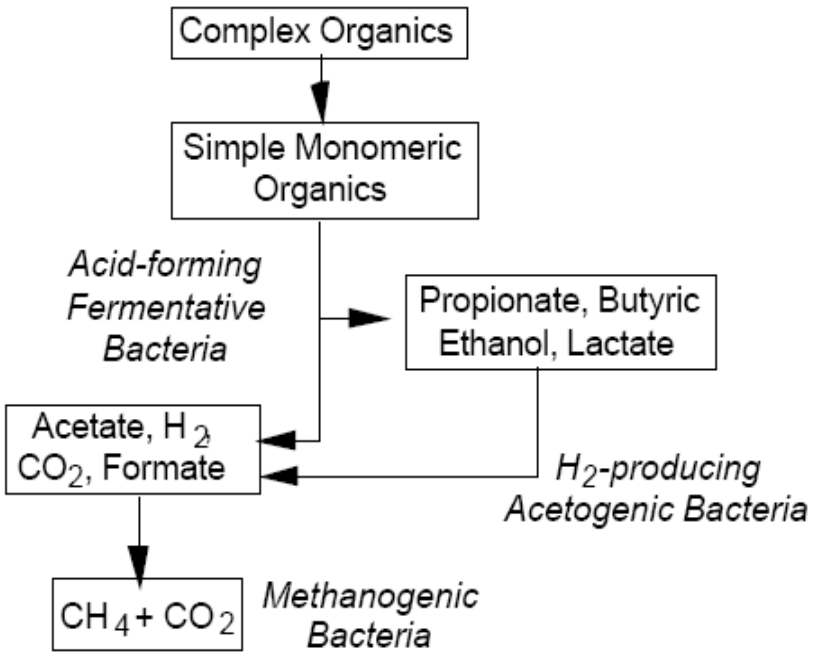

Figure 3.6. Anaerobic degradation of hydrocarbons

Many molecular studies haveshown that the number of hydrocarbon degraders can grow quite fast when nutrients are added to the hydrocarbon degradation. The general theory was that the number of these hydrocarbon degraders is in small amounts and they grow rapidly when they find suitable conditions like oil spill etc. Because all these microorganisms live in an ecological network, in which different microorganisms constantly interacting directly or indirectly with the environment and each other, a small change in conditions may be amplified by the network. So the increase in population of hydrocarbon degraders may be remarkable. This feature of biodegraders also shows why some bioremediation strategies fail. Since success of biodegradation do not solely depend on hydrocarbon degrading microorganisms, bioaugmentation strategies do not result with an increase in biodegradation. Also network of microorganisms and nature create various stresses and conditions those cannot be mimicked in laboratory environments. Addition of pollutant degrading microorganisms fails mainly at this point, since survival or activity of them is very poor in normal environment. The adaptability of introduced microorganisms is rather low because they did not encountered stresses under laboratory conditions (Head and Rolling, 2006).

4. MOLECULAR TECHNIQUES USED IN MOLECULAR ECOLOGY

4.1. The Need for Molecular Techniques

Classical microbiology techniques used in identification of environmental microorganisms are mostly based on cultivation dependent methods on selective growth media. These methods have certain limits which prevent an efficient identification of the community. Since there are many groups of microorganism difficult to grow, this technique is not able to address whole microorganisms.

In early years of modern microbiology, the most common method for identification of microorganisms is cultivation dependent method. The main limitation of this method is cultivability of a small fraction of all microorganisms. Microorganisms living in anaerobic environment are hard to grow because of low growth rates, syntrophic interactions and unknown growth requirements. Also cultivation dependent methods cause cultivation shift by favoring a normally not favorable microorganisms by changing competitions. Therefore a microbial community cannot be cultured as whole and cultured microorganisms do not reflect microbial community. The cultivable microorganisms make up 0.1%-10% of all microorganisms on earth (Amann et al., 1995a; Hugenholtz et al., 1998; Muyzer et al., 1993; Muyzer, 1999; Lim et al., 1999; Guillou et al., 1999).

Despite the developments in the microscopy, direct microscopic analyses have many limitations in identifying microorganisms. The small size of prokaryotic organisms, 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 the biodiversity (Pace, 1997; Torsvik and Øvreås, 2002; Torsvik et al., 2002).In last 20 years, 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;Ranjard et al., 2000).

4.1.1. The 16S rRNA and its Importance

Since a great percentage of microorganisms cannot be cultured on laboratory conditions, an alternative approach was created. In this approach, a unique and distinct characteristic of each microorganism was used. From the microorganism(s) DNA was extracted and a data bank of specific genes was created. With these genes, microorganisms can be identified without cultivation. Mostly ribosomal RNA (rRNA) molecules (16S and 23S) were used for phylogenetic marker. The molecule was selected for analysis since ribosome is a well abundant (10^3-10^5) and obligatory component of each cell. Because ribosomes are directly taking part in protein production, its number gives also clue about cell volume and growth rate (Amann, 1995b; Alcamo, 1996).

Both of the subunits of the ribosome are used for analyses. The extracted 16S and 23S rDNA are amplified by specific primers using polymerase chain reaction (PCR) (Saiki et al., 1988). Amplified subunit coding sequences then can be used in cloning or in other molecular methods for identification or monitoring of the microbial community. There are more than 15000 16S rRNA sequences uploaded to the public databases. 23S rRNA data base is smaller in size than the 16S rRNA database but it is growing rapidly with each day (Wilderer et al., 2002).

16S rRNA genes consist of highly conserved and highly variable regions (Lane et al., 1985). The amplification of this gene with suitable primers makes it possible to identify all microorganisms. The comparison of amplified genes with known sequences in database helps to build a phylogenetic classification system. With the developments in analysis of 16S rRNA, the detection and identification of microorganisms in nature enhances greatly. The 16S rRNA analysis also shows the truth of the suspicions about inefficiency of culture dependent techniques (Barns et al., 1994; Choi et al., 1994; DeLong, 1992; Liesack and Stackebrandt, 1992; Schmidt et al., 1991; Ward et al., 1990).

4.1.2. The Variable Regions in 16S rRNA and its Importance

The rRNA is highly conserved in nucleotide sequence as well as in secondary structure since its function remains same through years of evolution. It has many variable

regions in which random changes occur time to time. These changes reflect evolutionary relationships of the organisms. Conserved regions functions as binding places for PCR primers or hybridization probes. Even data from this analysis is sufficient to compare statistically significant phylogenetic relations (Olsen et al., 1986). Among the variable regions, V3 region is mostly used in molecular analysis (Neefs, et al., 1990; Øvreas et al., 1997).

4.2. Polymerase Chain Reaction (PCR)

Amplification of DNA segments via Polymerase Chain Reaction (PCR) using thermostable DNA polymerase was one of the most important advancement in molecular biology and opens wide range of alternatives of usage DNA in the field of environmental microbiology (Saiki et al., 1985).

PCR is used to amplify specific regions of a DNA strand. This can be a single gene, just a part of a gene, or a non-coding sequence. PCR process mainly based on three steps: Denaturation, Annealing, and Extension. In denaturation step double stranded DNA templates melted and separated by high temperature. In annealing step the reaction temperature is lowered so that the primers can attach to the single-stranded DNA template. Then temperature is increased again to a level (72^0 C mostly) in which Taq polymerase can elongate the chain by adding nucleotides. (dNTPs) This cycle of binding of primer and elongation and then disassociation repeated 30-40 times to recover enough DNA segment of interest. The addressed sequence amplified in order of 2. (2^n where n is the cycle number) The resulted product will be run on an agarose gel to monitor efficiency of the PCR. Mostly Ethidium Bromide (EtBr) is used to stain DNA which renders DNA visible under UV light.

Although the general steps and ingredients are well defined, there will be small corrections or changes according the purpose of PCR or products planned to have. The changes can be made in enzyme conc., dNTP conc., magnesium conc., annealing and extension temperatures and times, cycle number and other reaction components.

4.2.1. Limitations and Biases of PCR

PCR is one of the most important tools in molecular techniques. It is very powerful but without doubt it has also some limitations. First of all DNA polymerase is not 100% trustworthy in transcribing DNA. Approximately 0.02-0.3% incorrect nucleotides are incorporated during amplification (Bej et al., 1991). The contamination present in template like humic acids, phenolic compounds or chelating agents will decrease efficiency and fidelity of *Taq* polymerase. To overcome this problem the DNA purification methods were developed. Due to processive characteristics of *Taq* polymerase, the depletion of nucleotides may increase the error rate. Primer dimer formation is possible when primers compliment each other at 3' end (Bej et al., 1991). Creation of recombinant or chimeric products is another problem. This problem mostly arises when target sequence of primers was shared in other DNAs other than template. Mostly mixed culture DNA like environmental sample may create chimeric sequences of different species (Amann et al., 1995a).

Most common problem regarding PCR comes from its power to amplify DNA. Sensitivity of PCR is so high even a very small amount of DNA (a single copy in theory) out of the sample DNA can be detected and amplified by *Taq* polymerase. An extreme sterilization and care needed in performing PCR. A negative control without a DNA template or DNaseI treatment of reagents can be done to prevent contamination caused by a foreign DNA (Schmidt et al., 1991).

4.2.2. PCR Based Techniques Used in Molecular Ecology

Primer selection of PCR can produce DNA sequences at different taxonomic levels (strain, genus, species etc.). These sequences may belong to same organism or mixed culture of organism. With the help of some molecular techniques, these specific sequences reveal secrets of mixed cultures or relation of microorganisms. In some studies (Moeseneder et al., 1999; Casamayor et al., 2002; Nikolcheva et al., 2003; Dorigo et al., 2004) different techniques were used to analyze same data. Although results are generally similar, some methods are less efficient in specific situations.

Single-strand conformation polymorphism (SSCP) analysis is a mutation detection system. PCR Amplified DNA fragments mostly 16S rDNA sequences are used. SSCP described first in the study of Orita (Orita et al., 1989) and used firstly in the field of environmental microbiology with the study of Lee et al. (1996) on microbial diversity. The principle behind SSCP is nature of single stranded DNA in which it goes into a 3D conformation due to intramolecular interactions. The DNA fragments with the same length will have a unique conformation because of their unique nucleotide sequence. Different sequence will lead a different conformation which will have a distinct electromobility pattern in non-denaturing polyacrylamide gel. Differences in electromobility are detected then on autoradiogram, by silver staining or using fluorescent probes which are then detected by an automated DNA sequencer.

There are many areas of use of SSCP in the environmental microbiology. It can be used to monitor changes in an aquatic microbial community like in the study of Ross et al. (2001) or Wenderoth et al. (2003). It can also be used in different areas like monitoring diversity of rhizosphere (Schwieger and Tebbe, 1998; Schmalenberger and Tebbe, 2003), studying microbial succession during composting (Peters et al., 2000), and to characterize bacterial community dynamics in the Salers cheese (Duthoit et al., 2003).

The detection of sequence variation using PCR–SSCP is generally good, but the detection sensitivity decreases as the fragment length increases (Hayashi and Yandell, 1993). Phylogenetic analyses of the bands are possible if the fragment is recovered form the gel. In the study of Schmalenberger and Tebbe (2003), sequencing showed that a single band may consist of several sequences and electrophoretic conditions may affect the results.

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 5’ 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. T-RFLP is a high-throughput, reproducible

method that can be used to carry out both qualitative and quantitative analyses of a particular gene mostly 16S rRNA. The fragments are separated by gel electrophoresis in non-denaturing polyacrylamide gels or by capillary electrophoresis, and distinguished by laser induced fluorescent detection. Then data is converted into electrophoregram and different sizes are detected as different areas under peaks.

One of the advantages of this technique is its ability to detect rare members of a microbial community. By using web-based resources that predict T-RF sizes for known bacteria, phylogenetic assignments can be made from the sizes of the terminal restriction fragment (TRF) (Kent et al., 2003). T-RFLP analysis can be used in community dynamics (Bruce, 1997; Liu et al., 1997; Marsh et al., 1998; Moeseneder et al., 1999; Osborn et al., 2000). Recently it also begin to be used in 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 of the technique include formation of pseudoterminal restriction fragments, which affect the estimation of microbial diversity in positive way (Egert and Friedrich, 2003). In the study of Engebretson and Moyer (2003) it has been suggested that T-RFLP is very useful for estimating diversity in communities characterized by low-to-intermediate species richness, but is not suitable for complex microbial populations.

Ribosomal intergenic spacer analysis (RISA) and automated ribosomal intergenic spacer analysis (ARISA) is another community fingerprinting technique. RISA was introduced to environmental microbiology in the study of Borneman and Triplett (1997) about 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 targeting conserved regions of 16S and 23S genes produce sequences in different length. The PCR products separated in polyacrylamide gel and visualized by silver staining. Each band represents a different organism so used successfully in community fingerprinting. Later on in 1999, Fisher and Triplett (1999) developed an automated version of the technique. In this technique, 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 gives an estimate of diversity, and the sizes of the fragments can be compared to those in the GenBank database. ARISA was used successfully in finding diversity of bacterioplanktons in lakes, (Fisher et al. 2000, Graham et al. 2004) in marine samples. (Hewson and Fuhrman, 2004)

The limitations of technique based mostly on biases of PCR and DNA extraction (Wang and Wang, 1996; von Wintzingerode et al., 1997; Muyzer, 1999; Giraffa and Neviani, 2001).

4.3. Denaturing Gradient Gel Electrophoresis (DGGE)/Temperature Gradient Gel Electrophoresis (TGGE)

Denaturing gradient gel electrophoresis (DGGE) and thermal gradient gel electrophoresis (TGGE) have been studied for 10 years. In spite the principle is similar to SSCP and TGGE, DGGE becomes much effective, easy and fast in application. In DGGE, PCR amplified gene sequences with same length are run in denaturing gradient polyacrylamide gel and separated by its melting domain, literally according to sequence (Myers et al., 1987, Abrams and Stanton,1992). Double stranded DNA will melt in discrete segments called melting points due to increasing denaturant concentration. Each melting point is sequence specific therefore each melting and separation of double strand occurs in specific melting temperature (Tm). As the DNA partially melted at the melting point, branched molecule decreased in mobility and separated from other DNA molecules with different melting points. 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).

DGGE and TGGE were introduced to environmental sciences by Muyzer's studies (Muyzer et al., 1993). It becomes a routine technique to monitor microbial diversity and their dynamics (Muyzer et al., 1996; Muyzer and Smalla, 1998; Muyzer, 1999). 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).

The speed to monitor community and identify members increased greatly as individual bands begin to excise from the gel and sequenced. This feature is not possible in other fingerprinting techniques like T-RLFP (Liu, et al., 1997). With this feature DGGE/TGGE becomes an alternative to cloning which is rather difficult and laborious, in some situations (Heuer et al., 1999; Riemann and Winding, 2001).

The technique becomes a highly used technique in environmental microbiology after its introduction. Rapid and reliable results favor it and versatility of the technique makes it possible of its usage in a wide range area.

Analyzing community diversity. DGGE can be used to determine genetic diversity of a microbial community without identifying individuals. It can be used to compare different communities like two sludge plants (Curtis and Crane, 1996), soil samples (Heuer et al., 1997), bacterial and archaeal communities (Øvreås et al., 1997).

Studying community changes. In some cases microbial ecologist often require to have samples spanning long time periods. As cloning is not suitable to use in this kind of study, DGGE becomes a savior for the scientists. With DGGE different samples taken at different times can be analyzed and compared in one gel. The simultaneous analysis makes it a powerful tool to analyze microbial community changes over time (Donner et al., 1996; Santagoeds et al., 1997; Ferris et al., 1997).

Monitoring of enrichment and isolation of bacteria. As it is originally used in complex communities, DGGE can also be used in simple communities. Monitoring enrichment cultures make it possible to determine and analyze conditions of isolation and enrichment (Santagoeds et al., 1996; Ward et al., 1996; Teske et al., 1996; Muyzer, 1997; Bucholz-Cleven et al., 1997).

Comparison of different DNA extraction methods. DGGE can be used to compare efficiency of different DNA extraction protocols (Heuer and Smalla, 1997; Lieasack et al., 1997).

Screening of clone libraries. DGGE is one of the commonly used techniques to screen clone libraries. Rapid and reliable results of DGGE decreases the amount needed to perform clone libraries (Kowalchuk et al., 1997).

4.3.1. Problems and Biases of DGGE

DGGE is well known and abundantly used technique in environmental microbiology. It is very powerful and has usage in different areas. But like all techniques it is not without limitations. Many limitations may be avoided by careful planning and performing of experiments but some of them are inevitable.

The band excision is a powerful feature of DGGE but it has some difficulties since mostly a band will consist of 150-200bp DNA which is rather short for a phylogenetic analysis. Co-migration of bands also will give a poor identification in the sequencing (Felske et al., 1998; Vallaeys et al., 1997). This problem may be overcome by having a clone library screening. It is then combines both techniques' powerful aspects. (Giovanni et al, 1990, Ward et al, 1990) Another problem with excision is distance of two bands. In some cases bands are too close; a proper excision cannot be made. Also during excision step UV may affect DNA and in reamplification it may create ambiguous sequences.

The DGGE has biases also because it is a PCR dependent technique. DGGE is being negatively affected by biases of PCR like fidelity error of polymerase or chimeric products. Therefore it has been accepted that DGGE is a semi quantitative method since number of bands and intensities may be affected by PCR.

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.

4.4. 16S rDNA Clone Library

16S rDNA clone library generation is accepted as the last part of culture independent analysis of a natural microbial community. In this method, competent *Escherichia coli* cells are transformed with recombinant plasmid DNA (vector and inserted PCR product) and cells are plated on a selective medium. Mostly for the selection, antibiotic resistant gene on the vector is used to select for maintenance of the recombinant plasmid. *E.Coli* cells containing positive plasmid DNA may live in the antibiotic containing medium. There are several methods for inserting PCR product into vector. Mostly used method is TA-overhang cloning in which PCR products with 5' dA overhangs inserted into vectors with 3' dT overhangs (Clark, 1988, TOPO TA Manual). Other methods are blunt-end and sticky-end ligation (Weisburg et al., 1991, Rheims et al., 1996). Blunt end ligation is less efficient than sticky end ligation. PCR product must be modified for blunt end and have non directional insertion. Sticky end ligation has advantage of directional insertion due to presence of different restriction sites.

After transformation of cells, plasmids need to be checked for the presence and size of the insert. Mostly, nowadays commercial cloning kits have plasmid with specific restriction sites or PCR primer binding sites. With help of these features insert can be screened by PCR or restriction digestion. Cloned and correct sized rRNA gene fragments can be sequenced to construct phylogenetic tree of species in the community. There are many screening techniques can be used prior to sequencing to avoid any sequencing of the same clone. Many of those techniques are described and discusses in Chapters 4.3. and 4.4. Sequences derived from the analysis of clone libraries can be used to identify species, to see the relation of clones to each other and other organisms, to create oligonucleotide probes like FISH probes or PCR primers.

The technique was used by Giovanni et al. (1990) to estimate diversity in Sargasso Sea bacterioplankton. Now it becomes a popular method to identify community structures

and dynamics of both natural and anthropologic communities (Eiler and Bertilsson, 2004; Glöckner et al., 2000; Hiorns et al., 1997; Dorigo et al., 2002).

There are several facts about cloning-sequencing method which eclipses its power as most detailed insight into microbial community composition. Method is time consuming and expensive and therefore not suitable for large number of samples. Again it is a PCR dependent technique and can be affected by PCR efficiency (Von Wintzingerode et al., 1997). It does not address community function. Although there are many drawbacks of the method, it can be overcome by using different methods together like screening colonies by DGGE or T-RFLP.

5. MATERIALS AND METHODS

5.1. Sampling and Preservation

Küçükçekmece sediment samples were collected by Institute of Marine Sciences and Management of Istanbul University (Figure 5.1.). Coordinates of sampling point are N 40˚5 8.24', E 28˚45.44' (Figure 5.2.). To perform molecular and chemical analysis, 50 ml of triplicate samples were collected using Van Ween Grab Sampler (Hydro-bios, Germany, Figure5.1.) from the depth of 22 meters. Collected sediment samples were placed into 50 ml sterile Falcon tubes and transferred to the laboratory immediately in cool boxes (+4 ^{0}C or less) and stored at -20 ^{0}C. Sample collection was held in months of September 05, December 05, March 06 and December 06.

Figure 5.1. The research ship, ARAR, of Istanbul University and Van Ween grab sampler

5.2. Chemical Analysis

To understand sediment characteristics of samples, total solids, total volatile solids (TS/TVS) were analyzed according to standard methods (APHA, 1995), heavy metal concentrations Cr^{+3}, CN^{-}, Cd^{+2}, Cu^{+2}, Mn^{+2}, Zn^{+2}, Hg^{+2} were analyzed by atomic adsorption spectrophotometry (ATIUNICAM 929, England), total phosphorus (TP), Total Carbon (TC), Total Organic Carbon (TOC),Total Inorganic Carbon (TIC) were analyzed by CHNP instrument (Carlo Erba, Italy).

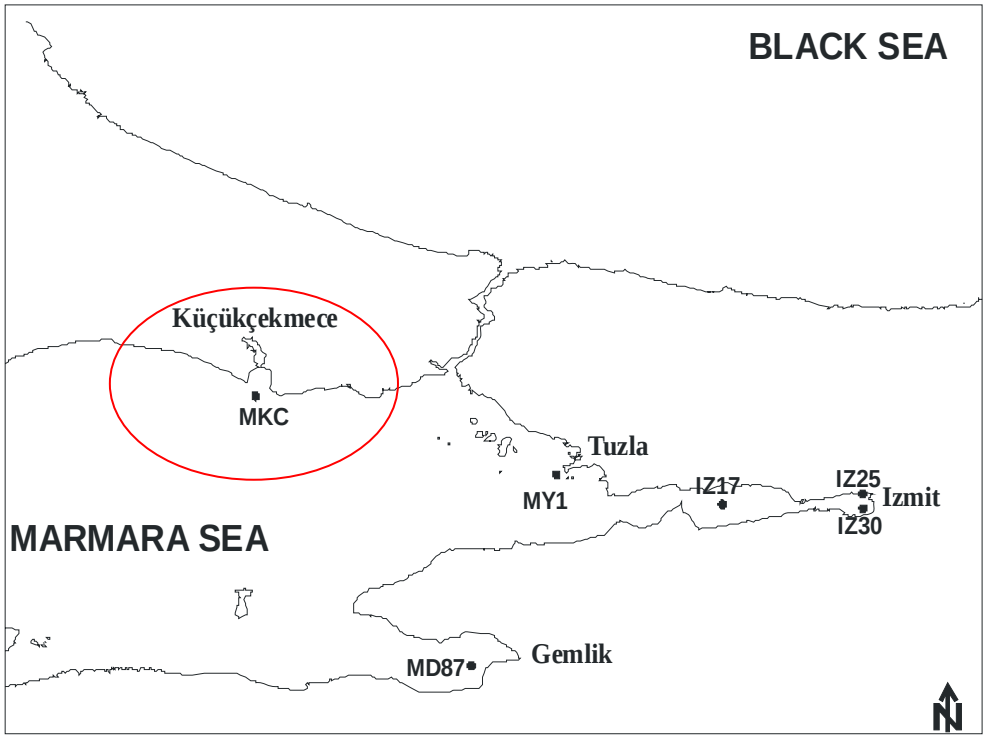

Figure 5.2. Location of Küçükçekmece and sampling point

5.3. Molecular Analysis

To analyze microbial community with culture independent techniques, different molecular methods were used. Molecular analysis of sediment samples began with extraction of Genomic DNA, followed by PCR to amplify 16S rDNA coding region and V3 region in the 16S rDNA coding region with specific primers. V3 region coding DNA bands were run in DGGE and 16S rDNA coding regions were used to generate a clone library. Clone library was screened again with DGGE technique and similarity analysis were done by Fasta in internet Genbank. Flow chart of molecular analysis was given in Figure 5.3.

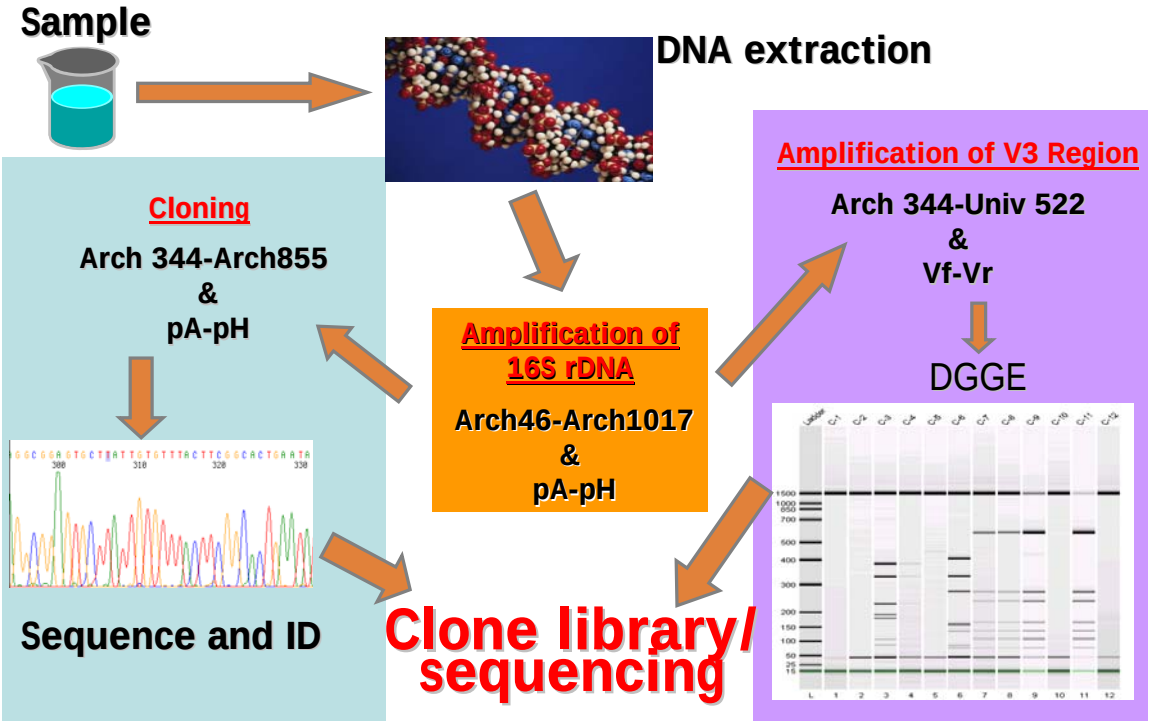

Figure 5.3. Flow chart of molecular analysis performed in this study.

5.3.1. Genomic DNA Extraction

DNA was extracted from 0.5 g sample by using Fast DNA Spin Kit for Soil (Q-Biogene, Bio 101 Thermo Electron Corporation, Belgium) and a Ribolyser (Fast Prep™ FP120 Bio 101 Thermo Electron Corporation, Belgium) according to the manufacturers' instructions.

The methodology of Genomic DNA extraction of by Fast DNA Spin Kit for Soil was as follows:

Approximately 0.5 g sediment was added up to lysing matrix tubes provided by the kit. The tube contains mixture of ceramic and silica particles to lyse all microorganisms in sample. Then lysing matrix tubes were spinned in Ribolyser (Fast Prep™ FP120 Bio 101 Thermo Electron Corporation) for 30 seconds at speed of 5.5 m/s. The tubes were then centrifuged at 14000xg for 30 seconds. After centrifugation supernatants were transferred to clean 1,5 ml eppendorf tubes and added 250 µl PPS reagent. To mix the composition tubes were shaked by hands for 30 seconds. After mixing the tubes centrifuged again at

14000xg for 5 minutes to pellet the precipitate. Supernatants were transferred to 2 ml eppendorf tubes and 1 ml of Binding Matrix Suspension was added to supernatant. Tubes were inverted by hand for 2 minutes to allow binding of DNA to matrix. To settle the silica matrix tubes were incubated 3 minutes at room temperature. 500 µl of supernatant was removed carefully without disturbing settled silica matrix. Then the binding matrix was resuspended in the remaining supernatant. All mixture was filtered and filter was placed to a new tube. Filter was washed by 500 µl SEWS-M wash solution. After washing, filter was dried by centrifugation 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. Application-ready DNA was obtained in the tube. 1/100 diluted genomic DNA was run on the %1 (w/v) agarose gel, prestained with Ethidium Bromide (EtBr) in 1x Tris-Acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM Acetic Acid, 1 mM EDTA; pH 8). Gel was visualized by using a gel documentation system, Mitsubishi 91.

5.3.2. Polymerase Chain Reaction for Cloning and DGGE

Amplification of 16S rDNA gene sequences was performed by PCR using archaeal and bacterial specific primers.

Primers used in this study are given in Table 5.1. pAf-pHr and Arch46f-Arch1017r primers were used for the amplification 16S rDNA of bacteria and archaea respectively. Extracted GDNAs were used as a template for these primers. Arch46f-Arch1017r PCR product was further used as a template for PCR using Arch344f-Arch855r. pAf-pHr and Arch344f-Arch855r amplification products were used for cloning and sequencing analysis.

Vf-Vr and Arch344f-Univ522r primers were used to amplify approximately 200 bp region of 16S rDNA of bacteria and archaea. Extracted GDNAs were used as a template for PCRs using Vf-Vr primers and Arch46f-Arch1017r PCR products were used as a template for amplification using Arch344f-Univ522r primers. The PCR products were used in DGGE analysis.

PCR reactions were performed in a 50 µl (total volume) mixture containing 0.2 µM forward primer, 0.2 µM reverse primer, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 1U of Taq polymerase enzyme and the buffer supplied with the enzyme (Fermentas Life Sciences), and 1 µl of template. Amplification was performed with a thermal cycler. (TECHNE-TC 412) PCR programs in thermal cycler were given in Table 5.2.

Products of all reactions were screened for the amplification of correct band size. All PCR products were run on the %1 (w/v) agarose gel prestained with Ethidium Bromide (EtBr) in 1x Tris-Acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM Acetic Acid, 1 mM EDTA; pH 8). Gels were visualized by using a gel documentation system, Mitsubishi 91.

Table 5.1. Bacterial and archaeal oligonucleotide primers used for PCR amplification

Name of Primer	Sequence of Primer	References
Vf	5'-GC* GCC TAC GGG AGG CAG CAG-3'	Muyzer et al.1993
Vr	5'-ATT ACC GCG GCT GCT GG-3'	Muyzer et al.1993
pA	5'-AGA GTT TGA TCC TGG CTC AG-3'	Edwards et al., 1988
pHr	5'-AAG GAG GTG ATC CAG CCG CA-3'	Edwards et al., 1988
Arch 46F	5'-YTA AGC CAT GCR AGT-3'	Ovreas et al., 1997
Arch1017R	5'-GGC CAT GCA CCW CCT CTC-3'	Barns et al., 1994
Arch344F	5'-GC* GAC GGG GHG CAG CAG GCG CGA -3'	Raskin et al 1994
Arch855F	5'-TCC CCC GCC AAT TCC TTT AA -3'	Shinzato et al., 1999
Univ522R	5'-GWA TTA CCG CGG CKG CTG -3'	Amann et al.1995a

*GC clamp: CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG

Table 5.2. PCR conditions used in the study

Primers	Denaturation	Time	Annealing	Time	Elongation	Time	#of cycles
pA-pH, pC Vf-Vr M13f-M13r	94^0 C	45 sec	55^0 C	45 sec	72^0 C	60 sec	30 cycles
A46-A1017	94^0 C	30 sec	40^0 C	30 sec	72^0 C	60 sec	35 cycles
A344-A855 A344-Univ 522	94^0 C	30 sec	53^0 C	30 sec	72^0 C	60 sec	35 cycles

5.3.3. Denaturing Gradient Gel Electrophoresis (DGGE)

The first step was the assembly of the perpendicular gradient gel sandwich. The thickness of the sandwich was established by using 1 mm spacers between two glass plates which are in size of 16x20 and 18x20 cm. Before assembly, glass plates were cleaned carefully to avoid any particle matter which may affect the gel. The position of spacers were checked to avoid any leakage and glass plate sandwich then placed on the casting stand. (Figure 5.4.)

The next step was preparation of the denaturing gradient gel. For bacterial DGGE, 10% (w/v) acrylamide:bisacrylamide 30% denaturant solution was prepared by mixing 33.3 ml of %30 acrylamide:bisacrylamide with 2 ml 50xTAE (2 M Tris, 50 mM EDTA, and 1 M Acetic acid) and 12 ml formamide and 12.6 g Urea. 60% of denaturant concentration was reached by adding 24 ml formamide and 25.2 g urea to 33.3 ml of %30 acrylamide: bisacrylamide and 2 ml 50xTAE (2 M Tris, 50 mM EDTA, and 1 M Acetic acid).Both solutions were added distilled water up to 100 ml.

For archaeal DGGE, 10% (w/v) acrylamide:bisacrylamide 40% denaturant solution was prepared by mixing 33.3 ml of %30 acrylamide:bisacrylamide with 2 ml 50xTAE (2 M Tris, 50 mM EDTA, and 1 M Acetic acid) and 16 ml formamide and 16.8 g urea. 70% of denaturant concentration was reached by adding 28 ml formamide and 29.4 g urea to 33.3 ml of %30 acrylamide:bisacrylamide and 2 ml 50xTAE (2 M Tris, 50 mM EDTA, and 1 M Acetic acid). 100% denaturant solution is defined as 40% (v/v) formamide and 7 M Urea. Both solutions were added distilled water up to final volume of 100 ml.

After solutions were prepared, they were filtered with 0.45 μm filter and sonicated for 10 minutes. The bottles were wrapped with foil paper to avoid sunlight and stored at 4^0 C for further uses.

Into two beakers, 20 ml of 10% (w/v) acrylamide:bisacrylamide solutions containing 30% and 60% (40% and 70% for archaeal samples) denaturants were poured. The lower denaturant containing solution was then stained with Bromophenol/Xylene loading dye. Gradient former was set to 16 ml. To both solutions, 200 ml freshly prepared

ammonium per sulfate (APS) and 10 ml TEMED was added and immediately 16 ml of it sucked with syringes of gradient former. The syringes were placed to their corresponding positions and the solutions were poured into the sandwich by turning the wheel of gradient former at a slow constant speed. When the gel sandwich was filled, 16 wells comb was placed carefully to avoid any bubble formation. The syringes were cleaned immediately with distilled water to prevent any polymerization in the syringes or capillaries. The polymerization was depending directly to the amount of APS and TEMED in solutions; usually took 60-90 minutes at room temp. During polymerization, electrophoresis tank was filled with 1xTAE until marked level and temperature was set to 65^0 C.

Figure 5.4. Assembling and loading of perpendicular gradient gel sandwich

Sample loading step was started with preparation of samples. 3 µl of loading dye was mixed with 7 µl of PCR product to be run. Polymerized gel sandwiches placed to the core and then the core was inserted into the preheated tank. The comb was removed and wells were washed with 1xTAE buffer to avoid any early denaturation due to presence of denaturants in wells. The samples were carefully loaded into the wells. The DGGE was conducted at a constant voltage of 200 V at 60^0 C for 300 minutes in 1xTAE containing electrophoresis tank.

The last step was staining and visualizing gels. The core was taken from the tank and gel sandwiches were separated from it. Glass plates were disassembled and the direction of gel was marked with a cut on the upper left corner.20 µl of 1:100000 diluted SYBR Gold DNA staining dye was added to 300 ml 1xTAE washing buffer and gels were incubated for 30 minutes. Gels were distained and washed three times with distilled water

to remove background. Again gels were visualized using a gel documentation system, Mitsubishi 91.

Figure 5.5. Bio-Rad DCode^{TM} system

5.3.4. Analysis of DGGE Gels Using Bionumerics & Treecon

DGGE gels were analyzed by using Bionumerics software (Applied Maths, Belgium) to determine the phylogenetic relationship between the samples. Presence-absence data of DGGE fingerprint were created based on similarity matrix obtained using Bionumerics software. Presence-absence data then was used to draw the phylogenetic tree using Treecon software.

5.3.5. Generation of 16S rDNA Clone Library

A clone library of 16S rDNA was generated to identify microorganisms present in the sample. Two clone libraries were generated to analyze bacterial community in sediment samples of September 05 and December 06, respectively. Two clone libraries

were generated to analyze archaeal community in sediment samples of September 05 and December 06, respectively.

Whole bacterial 16S rDNA (ca.1.5 kb) was amplified by using primers pA-pH, as described above. PCR products were cloned with a TOPO TA cloning kit (Invitrogen Ltd,) and gene libraries were screened by DGGE.

A part of archaeal 16S rDNA (ca.0.5 kb) was amplified by using primers Arch344f-Arch855r, as described above. PCR products were cloned with a TOPO TA Cloning Kit for Sequencing (Invitrogen Ltd,) and gene libraries were screened by DGGE.

The procedure of generating 16S rDNA Clone Library was as follows:

The process was started with preparing 6µl reaction mix by adding 3 µl PCR product, 1 µl salt solution (1.2 M NaCl, 0.06 M $MgCl_2$), 1 µl TOPO vector and 1 µl Sterile Water. The solution was mixed gently and incubated at room temperature (R.T.) for 20 minutes. After incubation reaction mix was placed on ice before its usage in One Shot TOPO transformation step.

One shot TOPO transformation was started by thawing one vial of One Shot TOPO reaction mix on ice. After thawing, 2 µl of reaction mix was added to One Shot vial. The solution was mixed gently without pipetting or shaking. The solution was incubated on ice for 30 minutes. After incubation, tube was subjected to a heat shock at 42^0C for 30 seconds and transferred immediately to ice and 300 µl of S.O.C. medium at R.T. (2% Tryptone,0.5% Yeast Extract,10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$,10 mM $MgSO_4$,20 mM glucose) The solution was shaked horizontally for 60 minutes. Three LB plates containing 50µg/ml Kanamycin were warmed to R.T. 100 µl of solution was spread on plates using glass spreader. The plates were incubated overnight and white colonies were observed after incubation.

Colonies were picked from plate and transferred into 200 µl PCR tubes containing 50 µl TE buffer (10mM Tris-HCl, 1mM EDTA pH 8.0). Colonies were boiled at 95^0 C for

5 minutes then frozen at -20^0 C overnight. Thawed solution was used as templates for PCR.

The 16S rDNA fragments were isolated from vector by PCR with primers M13f-M13r (M13 Forward 5′-GTA AAA CGA CGG CCA G-3′/ M13 Reverse 5′-CAG GAA ACA GCT ATG AC-3′). From PCR products of reaction with M13f-M13r primers nested PCRs were done to screen the clones in DGGE. Nested PCRs were performed for bacterial and archaeal separately as described above in section 5.5.

The screening of gene library was done with DGGE as described above in section 5.6 with a modification. The gels were stained with 25 µl EtBr (10 mg/ml) instead of SYBR Gold.

5.3.6. Phylogenetic Analysis of Microbial Community

Phylogenetic analysis of microbial community was done by sequencing clones and drawing phylogenetic tree by Treecon software (Treecon for windovs 1.3b. Univeristy of Konstanz, Germany). The whole process was done as described below:

Sequencing PCR was done using dominant clone DNA as template. Reaction was held by mixing 4.7 µl dIwater, 1 µl template, 0.3 µl primer, (10pM of pC primer [5'-CTA CGG GAG GCA GCA GTG GG-3'] was used for bacterial clones and Arch 344f primer was used for archaeal clones) 2µl Big Dye Reaction mix and 2 µl Big Dye Reaction buffer. PCR program was as follows:

denaturation at 95^0 C for 5 minutes,
denaturation at 95^0 C for 30 seconds
annealing at 55^0 C or 53^0 C for 30 seconds (according to primer, Table 5.2.) } 30 cycles
elongation at 60^0 C for 4 minutes

Products of PCR were then purified according to following protocol:

PCR products were mixed with 2 µl 3M Sodium Acetate (NaAc) and 50 µl of 95% ice cold ethanol. The mixture was incubated for 30 min on ice and centrifuged for 30 min at 14000 rpm. Supernatant was discarded and pellet was resuspended in 250 µl 70% ice cold ethanol and centrifuged for 30 min at 14000 rpm. Supernatant discarded and pellet was dried at 95^0 C for 5 minutes. Pellet was resuspended in 20 µl formamide and denatured at 95^0 C for 3 minutes. Then the mixture were loaded to sequencer (A 3130 Sequencer, Abi Prism, USA) and sequenced automatically.

The outcome of the sequencer then analyzed by Chromas software (Chromas Lite 2.01, Technelysium Pty Ltd.) and nucleotide sequence data was exported to Fasta format. Nucleotide sequence then analyzed with ClustalW software, available at the web site http://www.ebi.ac.uk/fasta33/nucleotide.html

6. RESULTS

6.1. Results of Chemical Analysis

Sediment samples taken from Küçükçekmece coast (MKC) were analyzed for physical and chemical characteristics to better understand the ecosystem where microbial life held place. Results of total solid (TS) and total volatile solid (TVS) analysis are given Table 6.1.

Table 6.1. TS and TVS concentrations sediment samples

Sample	TS(g/L)	TVS(g/L)	TVS/TS %
September 05	450	31	7
December 05	378	28	7
March 06	550	43	8
December 06	448	34	8

Heavy metal concentrations of sediment samples were analyzed and given in Table 6.2.

Table 6.2. Heavy metal concentrations of sediments

Sample	Mn (mg/kg)	Fe (mg/kg)	Ag (mg/kg)	Na (mg/kg)	K (mg/kg)	
September 05	162	8725	< 25			
December 05	254	11750	< 0,25	15814	5220	
March 06	228	9150	< 0,25	6022	3355	
December 06	236	10600	< 0,5	18570	3306	
Sample	Cr (mg/kg)	Cd (mg/kg)	Cu (mg/kg)	Zn (mg/kg)	Pb (mg/kg)	Ni (mg/kg)
September 05	13	< 10	38	178	16	34
December 05	56	< 0,2	135	557	11	46
March 06	64	< 0,2	81	109	5	42
December 06	39	< 0,2	73	120	31	45

Results of elemental analysis and carbon contents of the sediment samples as total organic carbon (TOC) and organic content were given in Table 6.3. along with the water quality above the sediment.

Table 6.3. Elemental analysis of samples

Sample	% N	% C	% S	% P
September 05	0.43	6.96	10.7	0.46
	TOC (mg/g)	OC % (TOC/TC)		COD (mgO_2/L)
September 05	6.8	91		603

pH and electric potential of the sample was also measured and given in Table 6.4.

Table 6.4. pH and electric potential of sediment samples

Sample	pH	electric potential (mV)
September 05	8.43	-99
December 05	8.25	-91
March 06	8.09	-82
December 06	7.53	-52

6.2. Genomic DNA Extraction and PCR Results

In this study molecular techniques are used to identify the microbial community in highly polluted marine sediment sample taken from the Küçükçekmece coast northwest of Marmara Sea. The usage of molecular techniques enlightens many points which cannot be found with culture-based methods.

The molecular techniques mainly deal with genetic material commonly DNA. To understand the microbial community in the sediment sample, first Genomic DNA of the microbial community should be extracted. Extracted GDNA was diluted to1/100 and run in agarose gel as mentioned above in Section 5.3.1. and can be seen in Figure 6.1.

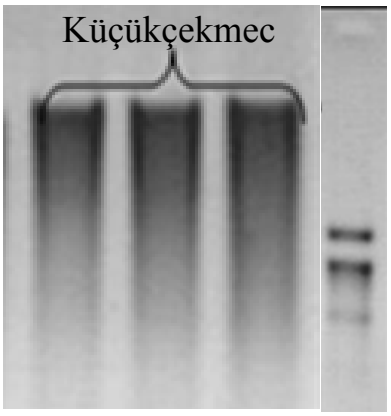

Figure 6.1 Electrophoretic analysis of extracted genomic DNA on agarose gel

As can be seen above in Figure 6.1. sediments samples prepared as triplicates. For amplifying bacterial and archaeal 16S rDNA, PCR was performed with primers pAf-pHr for bacterial 16S rDNA(1500bp); Arch46f-Arch1017r for archaeal 16S rDNA(1000 bp) (Figure 6.2.)

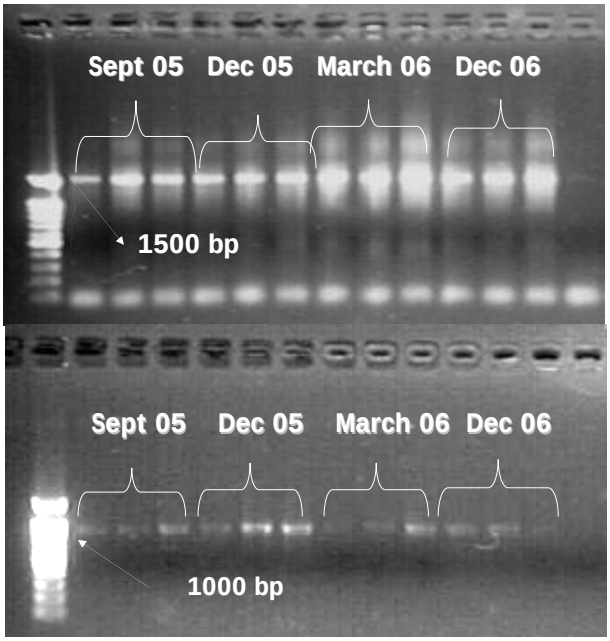

Figure 6.2. Electrophoretic analysis of 16S rDNA gene of bacterial and archaeal community on agarose gel

Products of PCR using primers pAf-pHr were used to generate clone library. For the DGGE analysis products of same reaction were used as templates to amplify V3 region by using primers Vf-Vr (Figure 6.3.).

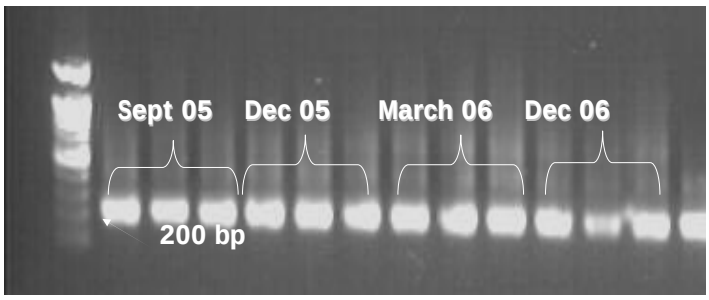

Figure 6.3. Electrophoretic analysis of V3 region of 16S rDNA gene of bacterial community on agarose gel

To create archaeal clone library, products of PCR using primer Arch46f-Arch1017r were used as template for PCR using Arch344f-Arch855r primers. Amplified 500 bp product was used to create archaeal clone library (Figure 6.4.).

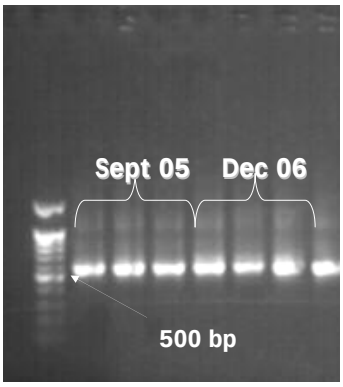

Figure 6.4. Electrophoretic analysis of 500 bp region on 16S rDNA of archaeal community on agarose gel

To analyze archaeal community in DGGE, primers Arch344f-Univ522 and product of PCR using Arch46f-Arch1017r primers, were used to amplify V3 region of Archaeal 16S rDNA (Figure 6.5.)

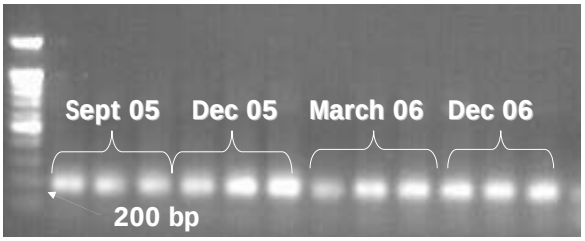

Figure 6.5. Electrophoretic analysis of V3 region of Archaeal 16S rDNA on agarose gel

6.3. Microbial Diversity Analysis of Sediment Samples with DGGE

Microbial diversity in the sediment samples was analyzed by DGGE. Both gels, Bacterial and archaeal, were analyzed by Bionumerics software for band visualization and intensity. Information of Bionumerics analysis was used in Treecon software to construct phylogenetic tree of samples. Bacterial and archaeal diversity of sediment samples can be seen in Figure 6.6. and 6.7. respectively.

According the DGGE band pattern table created with help of Bionumerics (Table 6.5.) samples of September 05 shows 27 different bands, samples of December 05 shows 26 different bands, samples of March 06 shows 31 different bands and samples of December 06 shows 32 different bands in bacterial DGGE gel. Among these bands 5 of them are dominant (I, II, III, IV, V) and 10 of them are intermediate bands. Band I gave an increasing intensity pattern throughout samples. Band II gave an increasing intensity pattern in first two samples but then diminished in intensity. Bands III and IV had intensity nearly same among samples. Band V is visible in September 05 and December 05 but show faint bands in other times.

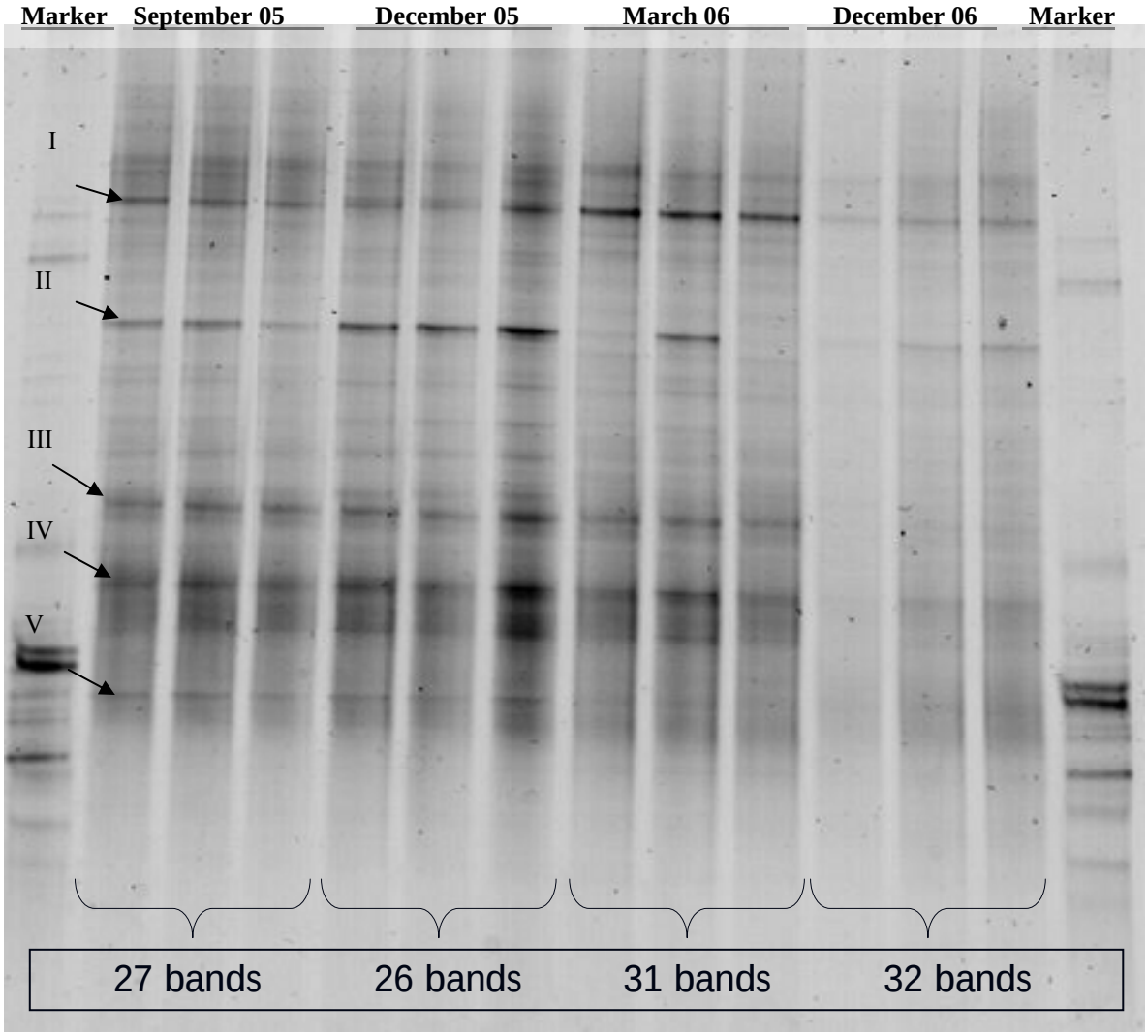

Figure 6.6. Analysis of bacterial community in the sediment samples by DGGE

In the archaeal DGGE gel 13 different band can be identified for samples of September 05 and samples of December 05, 15 different bands for samples of March 06 and again 13 different bands for samples of December 06 (Table 6.6.). Among them there are 3 dominant (I, II, III) and 3 intermediate bands in all samples and another dominant band appeared in samples of December 06 just below the band I. Band I showed same intensity among samples. The appeared band below band I was also present in sample September 05 as a faint band. Bands II and III showed same intensity. The bands down the middle of the gel and in the uppermost level of the gel appeared in all archaeal DGGE gels and should be considered as experimental error.

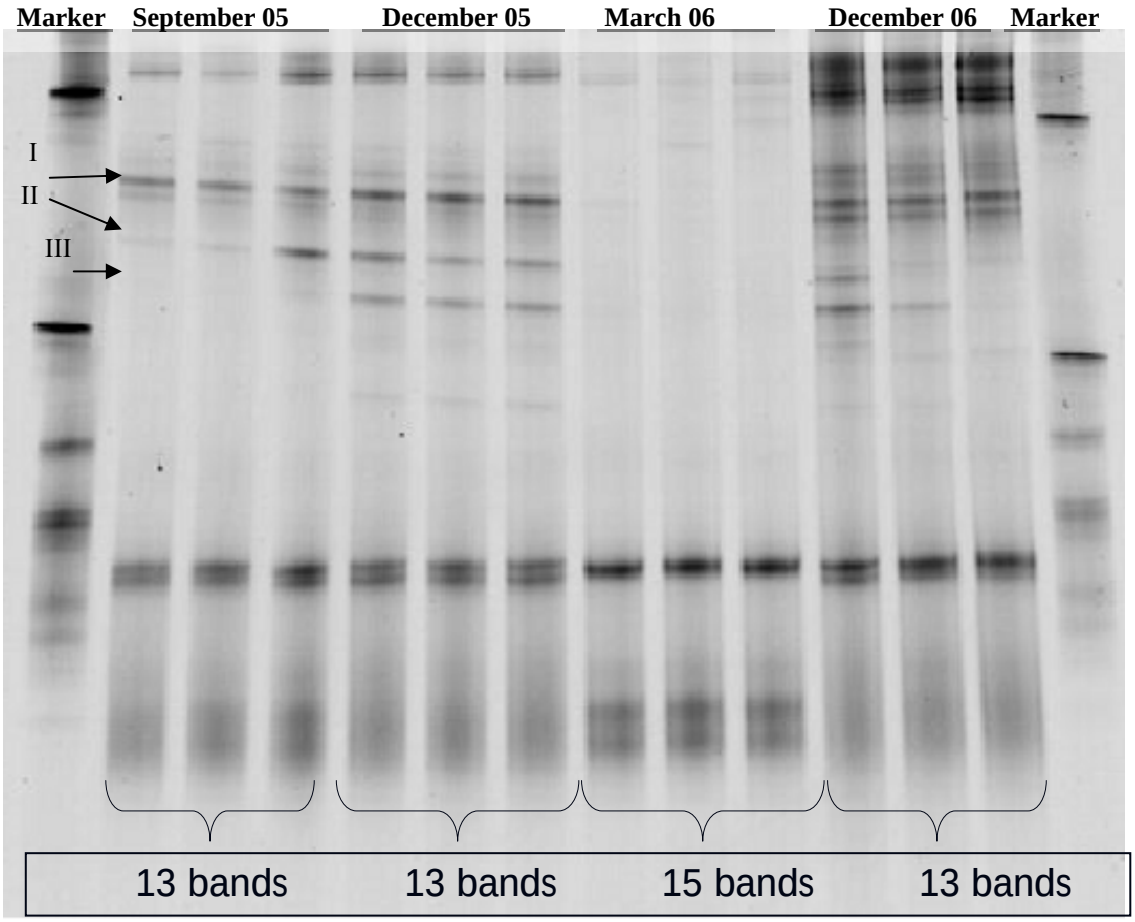

Figure 6.7. Analysis of archaeal community in the sediment samples by DGGE

Table 6.5. Presence (1)-absence (0) data of bacterial DGGE fingerprint

	Sept. 05 I	Sept. 05 II	Sept. 05 III	Dec. 05 I	Dec. 05 II	Dec. 05 III	March 06 I	March 06 II	March 06 III	Dec. 06 I	Dec. 06 II	Dec. 06 III
1	0	0	0	0	0	0	1	1	1	1	1	1
2	1	1	1	0	0	0	0	1	1	1	1	1
3	1	1	1	1	1	1	1	1	1	0	1	0
4	1	1	1	1	1	1	1	1	1	1	1	1
5	1	1	1	1	1	1	1	1	1	1	1	1
6	1	1	1	1	0	1	0	0	0	1	0	0
7	0	0	0	0	0	0	0	1	1	1	0	0
8	1	1	1	1	1	1	1	1	1	1	1	1
9	1	1	1	0	0	0	1	1	1	0	1	1
10	1	1	1	1	1	1	1	1	1	1	1	1
11	1	1	0	0	1	1	0	1	0	0	0	1
12	1	1	1	1	1	1	1	1	1	1	1	1
13	1	1	1	1	1	1	1	1	1	1	1	1
14	1	1	1	1	1	1	1	1	1	1	1	1
15	1	1	1	0	1	0	1	1	1	0	1	0
16	0	0	0	1	1	1	1	1	1	1	1	1
17	1	1	1	1	1	1	1	1	1	1	1	1
18	1	1	1	0	0	0	0	0	0	0	0	0
19	1	1	1	1	1	1	1	1	1	1	1	1
20	0	0	0	0	0	0	1	1	0	0	0	0
21	1	1	1	1	1	1	1	1	1	1	1	1
22	0	0	0	1	1	1	1	1	1	0	1	1
23	1	1	1	1	1	1	1	1	1	1	1	1
24	1	1	1	1	1	1	1	1	1	1	1	1
25	1	1	1	1	1	1	1	1	1	1	1	1
26	1	1	1	1	1	1	1	1	1	1	1	1
27	0	0	0	0	0	0	0	0	0	1	1	1
28	1	1	1	1	1	1	1	1	1	1	1	1
29	1	1	1	1	1	1	1	1	1	1	1	1
30	1	1	1	1	1	1	1	1	1	1	1	1
31	1	0	0	0	0	0	1	1	1	1	1	1
32	1	1	1	1	1	1	1	1	1	1	1	1
33	1	1	1	1	1	1	1	1	1	1	1	1
34	1	1	1	1	1	1	1	1	1	1	1	1

In the Bacterial DGGE fingerprint, band 1, 7, 16, 20, 22, 27 and 31 are not present in samples of September 05 but appeared in other samples. Bands 1, 2, 7, 9, 18, 20, 27 and 31 are not present in samples of December 05. Band 18 is not present in samples of March 06 and bands 18 and 20 are not present in samples of December 06.

Table 6.6. Presence(1)-absence (0) data of archaeal DGGE fingerprint

	Sept. 05 I	Sept. 05 II	Sept. 05 III	Dec. 05 I	Dec. 05 II	Dec. 05 III	March 06 I	March 06 II	March 06 III	Dec. 06 I	Dec. 06 II	Dec. 06 III
1	1	1	1	1	1	1	0	1	0	1	1	1
2	1	1	1	1	1	1	1	1	1	1	1	1
3	0	0	0	0	0	0	0	1	1	1	1	1
4	1	1	1	1	1	1	0	0	0	0	0	0
5	1	1	1	1	1	1	1	0	0	1	1	1
6	1	1	1	1	1	1	1	1	1	1	1	1
7	0	0	0	1	0	1	1	1	1	0	0	0
8	1	1	1	1	1	1	0	1	0	1	1	1
9	1	1	1	1	1	1	1	1	1	1	1	1
10	1	1	1	1	1	1	1	0	1	1	1	1
11	1	1	1	1	1	1	1	1	1	1	1	1
12	1	1	1	1	1	1	1	1	1	1	1	1
13	1	1	1	1	1	1	1	1	1	1	1	1
14	1	1	1	0	0	0	1	1	1	1	1	1
15	1	1	1	1	1	1	1	1	1	1	1	1

In archaeal DGGE fingerprint, band 3 and 7 are not present in samples of September 05. Bands 3 and 14 are missing in samples of December 05. Band 4 is not present in samples of March 06 and bands 4 and 6 are not present in samples of December 06.

The phylogenetic trees of samples can be seen in Figures 6.8. and 6.9. respectively.

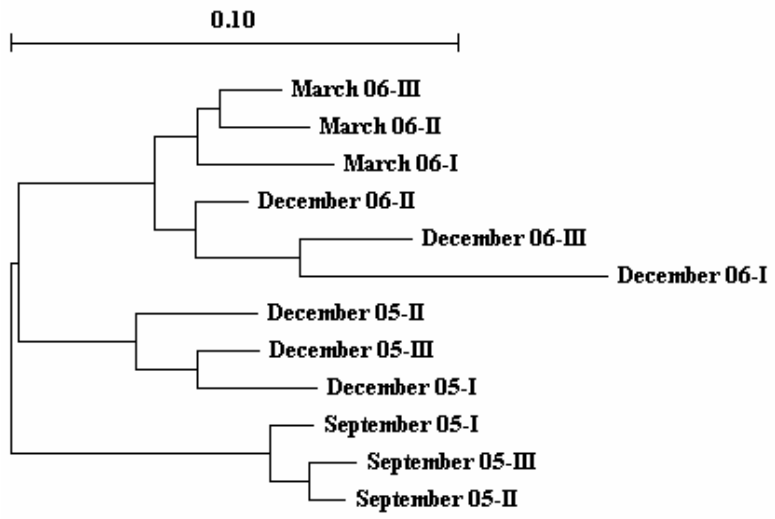

Figure 6.8. Phylogenetic analysis of bacterial samples by Treecon

Phylogenetic tree of bacterial samples shows all triplicates are in the same branch of the tree. The horizontal distance shows the distance of samples to each other. September 05, December 05 and March 06 samples are in the same distance from the root. Their difference from each other is minimal. Only samples of December 06 show a different pattern and have a distant relationship with other tree samples.

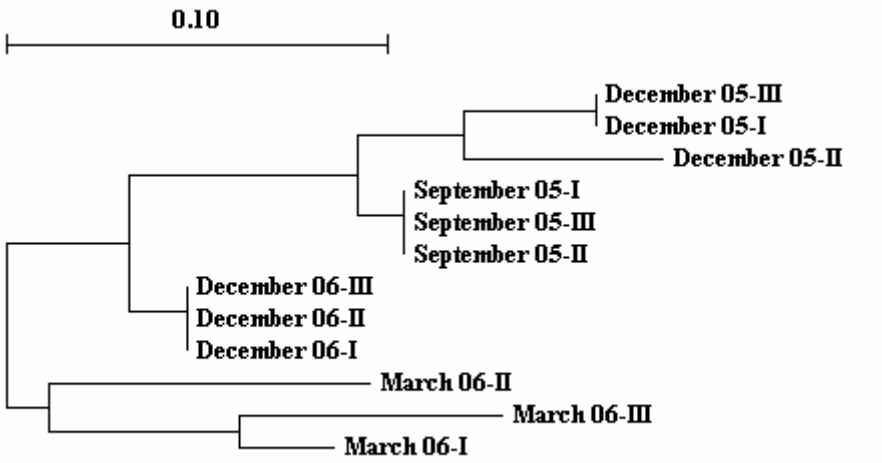

Figure 6.9. Phylogenetic analysis of archaeal samples by Treecon

Phylogenetic tree of archaeal samples shows all triplicates are in the same branch of the tree. The horizontal distance shows the distance of samples to each other. Different than bacterial tree, in archaeal phylogenetic tree samples are more distant from each other. March 06 samples are relatively close to December 06 and September 05 samples. December 05 samples are more distant to other samples. December 05 and December 06 samples are most distant in tree and can be considered as less related to each other. The different distance to the root of the tree shows that the samples are diversified during the time and become relatively different communities.

6.4. Identification of Archaeal and Bacterial Community

Two bacterial clone libraries were created from the sediments collected in months of September 05 and December 06; two archaeal clone libraries were also created for same sediments. Clone libraries were constructed as described in Section 5.3.5.

For the identification of bacterial community, 39 clones were screened for December 06 and 51 clones were screened for September 05. In the screened clones, 26 different clones were found for December 06 and 23 different clones were found for September 05. There are 3 dominant clones(c, n, and u) and 4 dominant clones (a, h, k m) for two sediments respectively. The results of screening can be seen in Figures 6.10. and 6.11.

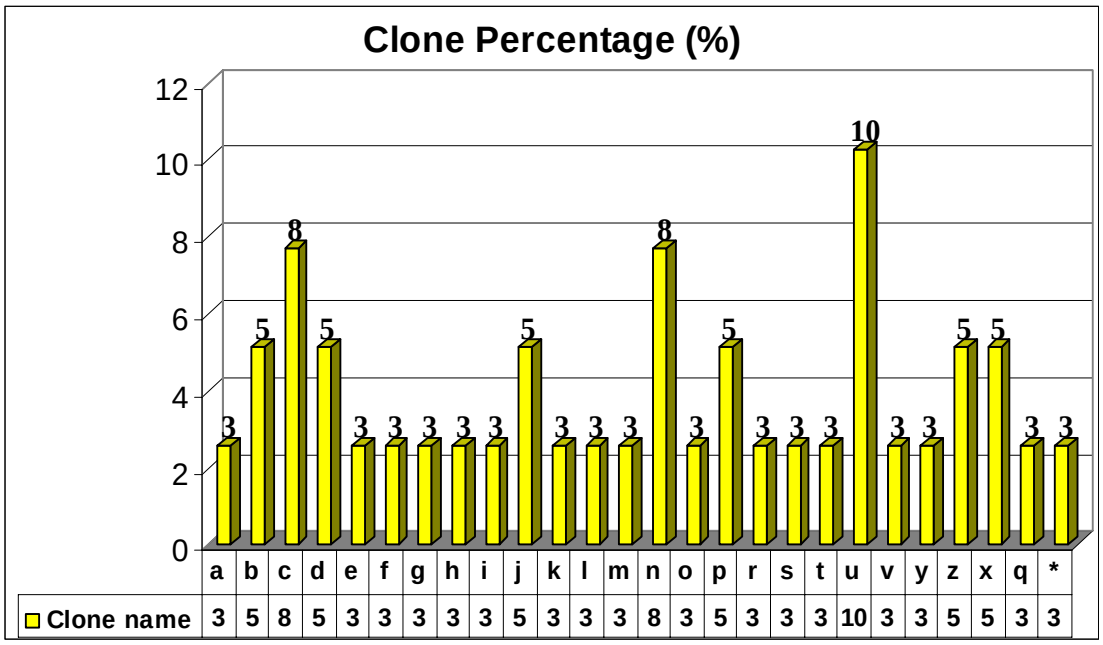

Figure 6.10. Percentages of bacterial clones in the sediment collected at December 06

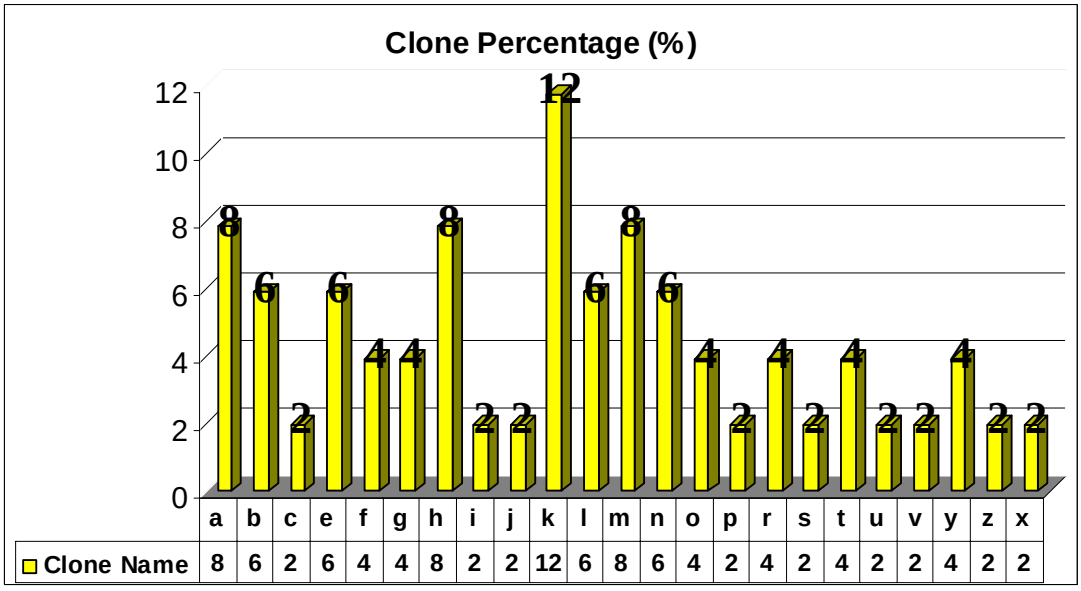

Figure 6.11. Percentages of bacterial clones in the sediment collected at September 05

The dominant clones were analyzed according to section 5.3.6. and sequence similarity data of bacterial community was given in Table 6.7. In September 05, mostly *Firmicutes* was dominant division in community. In Firmicutes division, Bacilli and Clostridia classes have seen as clones h and m. In December 06, Proteobacteria becomes dominant division in community. Firmicutes division was also present. In Firmicutes division, Clostridia class has seen as clone u. Among proteobacteria, Alpha and Gamma classes have been seen. Order of Rhizobiales of Alphaproteobacteria and order of Xanthomonadales of Gammaproteobacteria were present as clones c and n respectively.

Table 6.7. Results of phylogenetic analysis of bacterial community

Bacterial Clone	Species Name	Overlaps	Similarity
December 06 c	Elbe River snow isolate Iso26	1010/1010	99.0 %
December 06 n	*Xanthomonas* sp. CC-AFH5	1016/1017	97.5 %
December 06 u	*Gallicola barnesae*	851/971	93.3 %
September 05 h	*Trichococcus pasteurii*	726/725	92.4 %
September 05 m	*Clostridium glycolicum*	1013/1018	97.2 %

Metabolism and biochemical pathways of the bacterial dominant clones were given in Table 6.8. along with percentages in community and dominant metabolic products.

Table 6.8. Dominant metabolism, products and percentages of bacterial dominant clones

Elbe River snow isolate Iso26 (8%) (Anaerobic respiration)	- Chemoorganotrophs and able to utilize many carbohydrates
Xanthomonas sp. CC-FH5 (8%) (Anaerobic respiration)	- Respiratory metabolism no fermentative, - Use low molecular weight organic compounds - Uses H_2-CO_2 as sole electron donor
Gallicola barnesae (10%) (Fermentation)	- Carbohydrates =>acetate, formate, butyrate, ammonia and CO_2
Trichococcus pasteurii (8%) (Fermentation)	- Carbohydrates => lactate, acetate, formate, ethanol - Pyruvate, citrate => acetate, formate and CO_2
Clostridium glycolicum (8%) (Fermentation and homoacetogenesis)	- Fructose, glucose, maltose, xylose => acetate - Engagement of acetogenesis and fermentation was substrate dependent. - H_2-CO_2, formate, lactate, pyruvate => acetate

For the identification of archaeal community; 59 clones were screened for December 06 and 39 clones were screened for September 05. Among the screened clones, 20 different clones were found for December 06 and 19 different clones were found for September 05. There are 5 dominant clones (d, j, l, o, s) and 4 dominant clones (a, e, h, i) for archaeal community respectively. The results of screening can be seen in Figures 6.12. and 6.13.

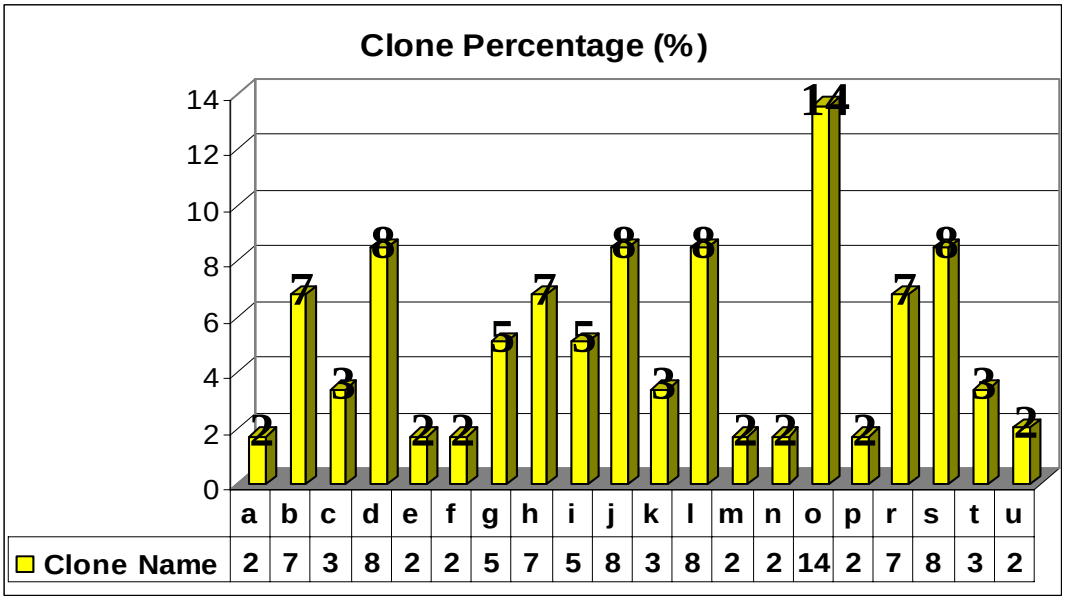

Figure 6.12. Percentages of archaeal clones in the sediment collected at December 06

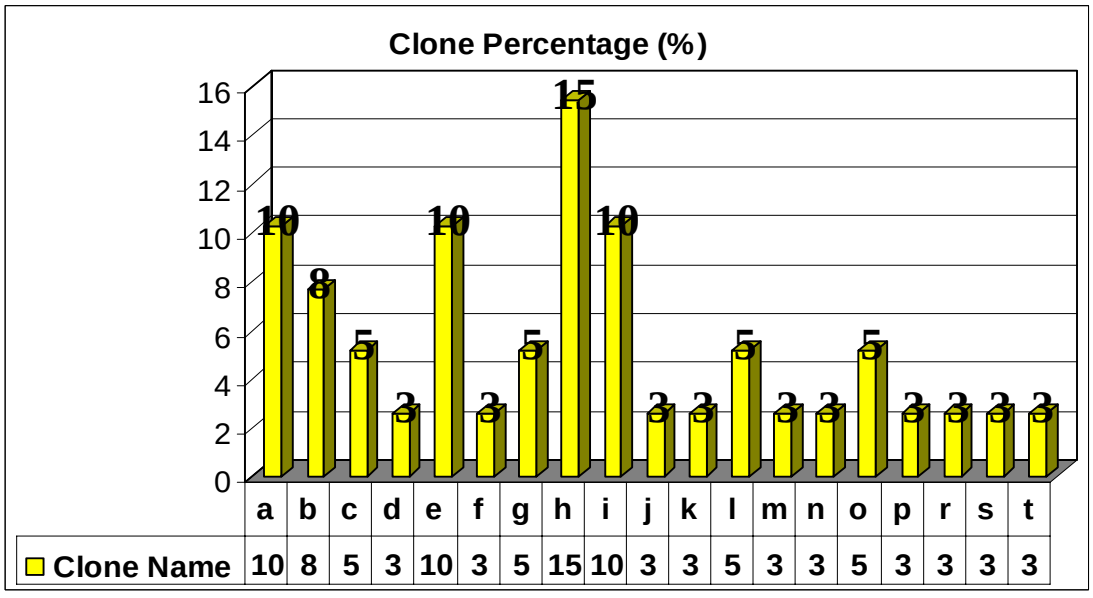

Figure 6.13. Percentages of archaeal clones in the sediment collected at September 05

Dominant clones of archaeal community were analyzed according to section 5.3.6.and sequence similarity data was given in Table 6.9. In September 05, the archaeal community was mainly dominated by class of Methanomicrobia. Under the methanomicrobia, order of methanomicrobiales was seen. Genus's *Methanogenium* and *Methanoplanus* were present as clones i and a, e respectively. In December 06, order methanosarcinale became dominant and *Methanosaeta* appeared as dominant genus. Presence of *Methanomicrobiales* as genus of *Methanogenium* continues in December 06 too. Clones d, j, o and s belong to *Methanosaeta* genus and clone l belongs to *Methanogenium* genus.

Table 6.9. Results of phylogenetic analysis of archaeal community

Archaeal Clone	Species Name	Overlap	Similarity
December 06 d	*Methanosaeta* sp.	493 /500	95.7 %
December 06 j	*Methanosaeta* sp.	498 /500	96.2 %
December 06 l	*Methanogenium frigidum*	268/291	89.6 %
December 06 o	*Methanosaeta* sp.	430/497	90.0 %
December 06 s	*Methanosaeta* sp.	395/403	92.4 %
September 05 a	*Methanoplanus petrolearius*	285/285	81.1 %
September 05 e	*Methanoplanus limicola*	477 /482	82.4 %
September 05 i	*Methanogenium organophilum*	495 /500	96.6 %

Metabolism and biochemical pathways of the archaeal dominant clones were given in Table 6.10. along with dominant metabolic products and percentages in community.

Table 6.10. Dominant metabolism, products and percentages of archaeal dominant clones

Methanosaeta (38%)	- Acetate + H => $CH_4 + CO_2$
Methanogenium Frigidium (8%)	- $H_2 + CO_2$, formate => $CH_4 + H_2O$
Methanoplanus petrolearius(10%) (Isolated from oil-producing well)	- $H_2 + CO_2$, formate => $CH_4 + H_2O$ - CO_2 + 2-Propanol => $CH_4 + H_2O$
Methanoplanus limicola (10%)	- $H_2 + CO_2$, formate => $CH_4 + H_2O$
Methanogenium organophilum (10%)	- $H_2 + CO_2$, formate => $CH_4 + H_2O$

In order to see the presence and position of dominant clones in microbial diversity, clones were also run in a DGGE gel together with PCR products showing microbial diversity of sediment samples collected from Küçükçekmece coast. Both DGGE gel can be seen in Figure 6.14 and Figure 6.15.

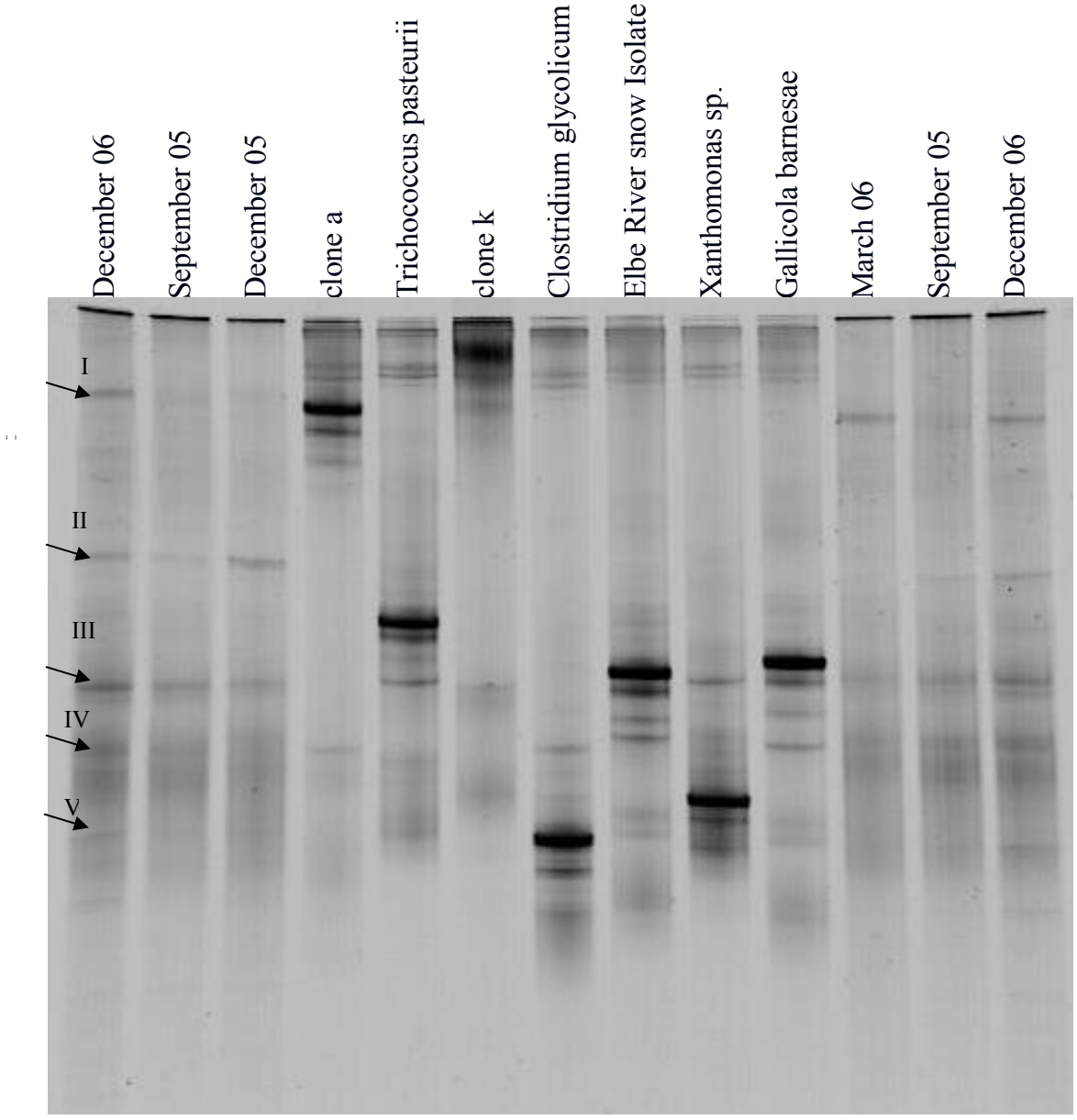

Figure 6.14. Analysis of bacterial clones and their position in bacterial diversity by DGGE (Dominant band I: Clone a of September 05, band III:Elbe River Snow Isolate Iso26, band V: *Clostridium glycolicum*, band II and band IV has no match.)

Bacterial dominant clones were analyzed with DGGE and their position in gel was monitored. The dominant band I in DGGE gel appeared as clone a of September 05. Band III was aligned with clone c of December 06(Elbe River Snow Isolate Iso26). Band V was aligned with clone m of September 05(*Clostridium glycolicum*). Clone h of September 05(*Trichococcus pasteurii*) and clone u of December 06(*Gallicola barnesae*) were aligned with intermediate bands between bands II and III. Clone n of December 06 (*Xanthomonas* sp. CC-FH5) was aligned with an intermediate band between bands IV and V. Clone k of September 05 was aligned with an intermediate band above the band I.

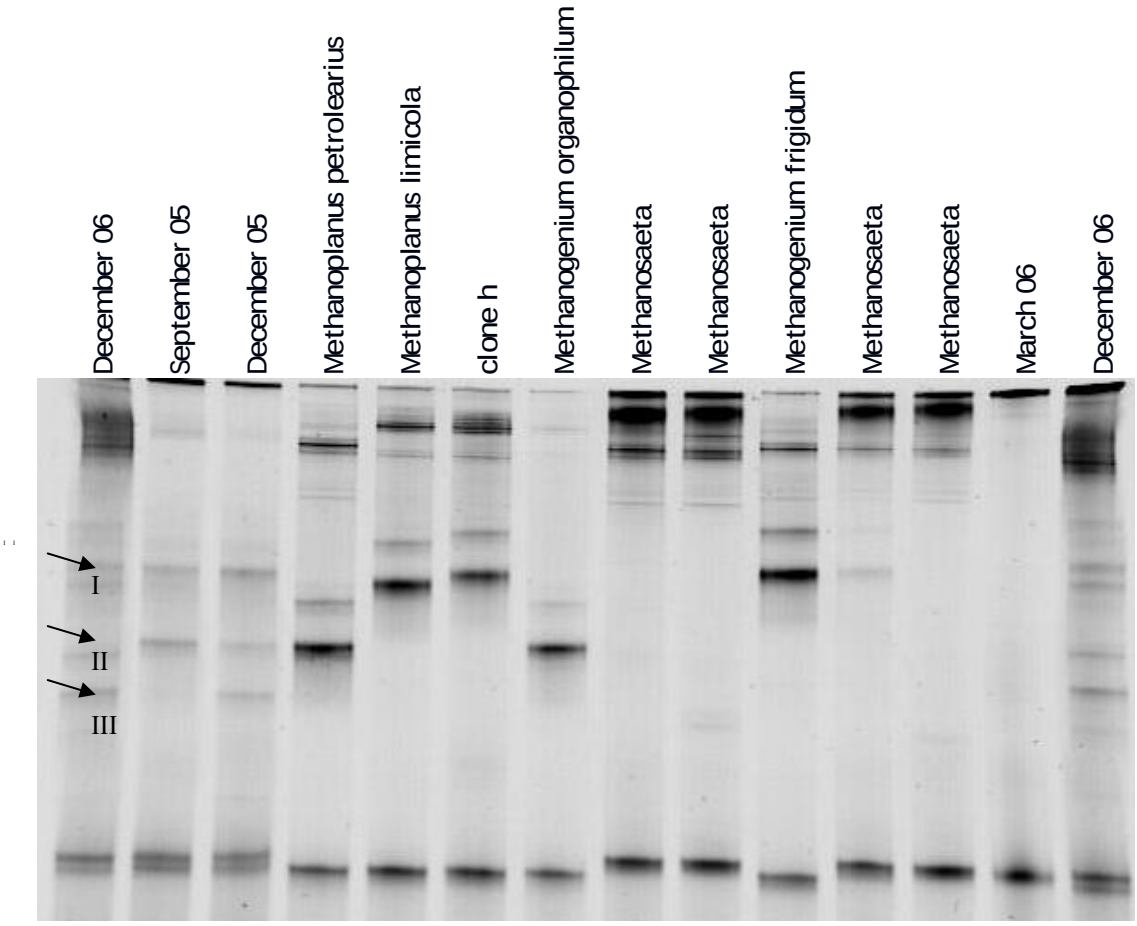

Figure 6.15. Archaeal clones and their position in archaeal diversity DGGE in sediment samples collected from Küçükçekmece coast (Dominant band I: Clone h of September 05 and *Methanogenium frigidum,* band II: *Methanoplanus petrolearius,* band III has no match.)

Archaeal clones were analyzed with DGGE and their position in gel was monitored. Band I was aligned with clone h of September 05 and clone l of December 06 (*Methanogenium frigidum*). Band II was aligned with clone a *(Methanoplanus petrolearius*) and clone i (*Methanogenium organophilum*) of September 05. None of clones aligned with band III. Clone e (*Methanoplanus limicola*) of September 05 was positioned at the new emerged dominant band of December 06. *Methanosaeta* species stacked at the top of the gel therefore no monitoring can be done with clones d, j, o, s of December 06.

According the data of similarity analysis, corresponding identification of dominant clones were made. Literature was reviewed for metabolism, biochemical pathway and final product of microorganisms. In the result of review, it has been found that bacterial and archaeal communities have containing microorganisms which are in syntrophic relationship with each other. In both times, September 05 and December 06, hydrocarbons degraded to same final product, methane. But the results of literature review and clone library indicate that the pathway and mechanism to final product was different in both times (Figure 6.16 and 6.17.).

In September 05, dominant clones are fermentative bacteria producing CO_2-H_2 gases and formate. Hydrogenotrophic methanogens are dominant in archaeal community and using these gases to produce methane (Figure 6.16.). But in December 06, anaerobic respiration became dominant and production of acetate is favored over production of CO_2-H_2 gases and formate. Therefore, acetoclastic methanogens dominated archaeal community and production of methane shifted from gases to acetate (Figrue 6.17.).

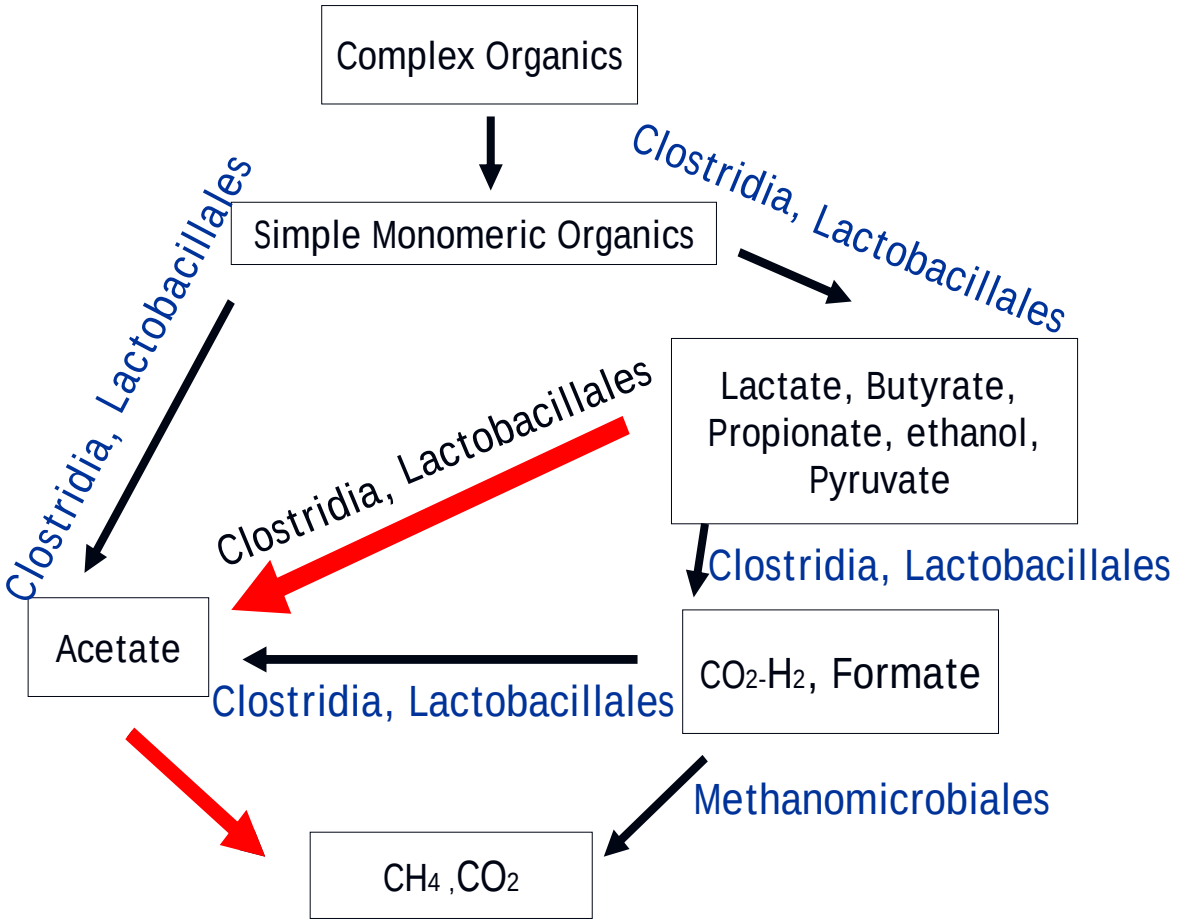

Figure 6.16. Anaerobic biodegradation pathway in September 05

In Figure 6.16. the anaerobic biodegradation pathway in September 05 can be seen. Red arrows show favored biodegradation pathway; formation of acetate from hydrocarbons and production of methane from acetate. Bacterial and archaeal groups responsible in the pathway were indicated with blue color and products were in black color. Font sizes of the microbial group also show the dominance of the group. Fermentation and hydrogenotrophic methanogenesis dominated the pathway.

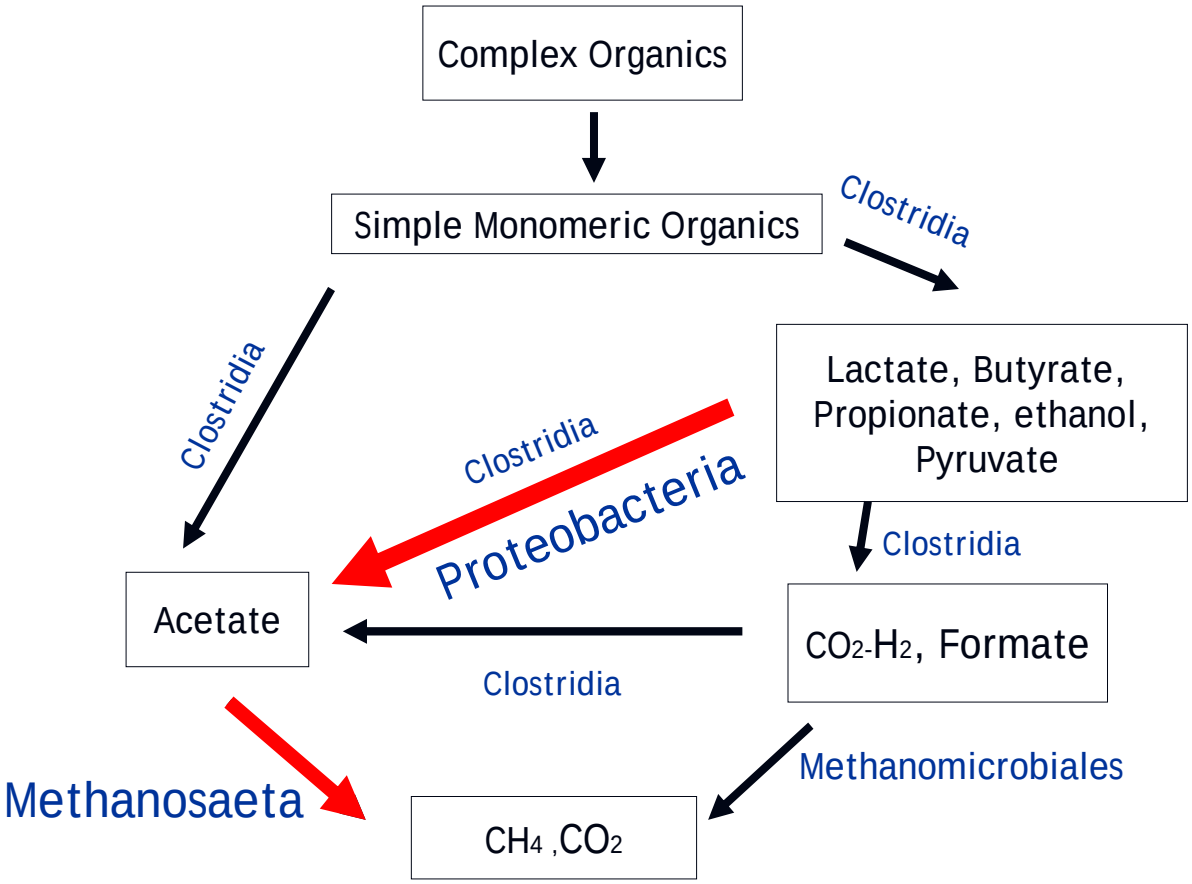

Figure 6.17. Anaerobic biodegradation pathway in December 06

In Figure 6.17. the anaerobic biodegradation pathway in December 06 can be seen. Red arrows show favored biodegradation pathway; formation of acetate from hydrocarbons and production of methane from acetate. Bacterial and archaeal groups responsible in the pathway were indicated with blue color and products were in black color. Font sizes of the microbial group also show the dominance of the group. Anaerobic respiration and fermentation coupled with acetoclastic and hydrogenotrophic methanogenesis dominated the pathway.

7. DISCUSSION

Analyzing sediment characteristics or microbial community alone does not give a satisfactory understandement of the problem of sediment sample. But chemical analysis coupled with biological analysis especially molecular ones can provide us enough information to understand and judge the situation in the sediment. Marmara Sea has not been studied in terms of molecular methods so far and as we think about the percentage of microorganisms that can be cultured, it can be said that Marmara Sea is still remains unexplored. Also anaerobic microorganisms are very hard to cultivate, therefore the benthic microbial life has not been studied even with culture dependent methods.

Due to excess pollution in Marmara Sea, many regions become anoxic and create suitable sources for anoxic marine sediment studies. In Figure 5.2. anoxic regions can be seen. The same characteristic of all points is there have been major oil or petroleum hydrocarbon contamination and a continuous anthropological pollution via domestic and industrial wastewater discharges. In the studies of Shine and Wallace (2000), and Hyland et al. (2005), it mentioned that TOC value can be used as an indicator of pollution. Deposition of hydrocarbons of various types will increase the organic carbon content in that area. The value of 6.8 mg/g can be classified as an intermediate value since the range of TOC in Marmara Sea has been found between 2.1 mg/g to 22 mg/g according to Albayrak et al. (2006). The other anoxic regions shown in Figure 5.2 have TOC values ranging from 4.3 mg/g to 13.2 mg/g in September of 2005. The organic content of the sediment sample of Küçükçekmece coast is 91% as another highly polluted region; Golden Horn (Halic) has an organic material content ranging 5% to 63 % (Akarsubasi et al., 2006). The high organic content of the sediment collected from Küçükçekmece coast indicate that hydrocarbon pollution in the region is higher than the Golden Horn.

In study of Virtasalo et al. (2005) it has been found that the water depth is directly related with anoxia. He and his coworkers have found that all of anoxic bottoms in their study have water depth less than 47 meters. Suboxic and oxic bottoms are dominated by water depth 20-60 meters and over 60 meters respectively. The sediments from

Küçükçekmece coast were collected from 22 meters deep bottom. This depth was in the range of anoxic bottom criteria found in the study of Virtasalo et al. (2005).

The pH and redox potential can also be accepted as an indicator of oxygen level. In the study of Virtasalo et al. (2005), the average redox potential of anoxic sediment has been measured as -102 mV. In sediments of Küçükçekmece coast, the electric potential was measured as -90 mV in average for first three samples; a main characteristic of anoxic sediment. The last sample shows a lower electric potential of -52 mV which is still in the range of anoxic sediment criteria but the decrease can also be interpreted as a recovery of the sediment. The reducing environment favors mineralization of hydrocarbon by exergonic processes, creating a suitable media for microbial energy conservation.

The percentages of nitrogen, phosphorus and sulfur are given in Table 6.3. along with percentage of carbon in the sediment sample of September 2005. The concentration of carbon is 15 times higher than those. In Ince et al. (2006), they studied another anoxic marine system, Black Sea. In the study of Ince et al. (2006), the percentage of nitrogen of different sampling points was ranging from 0.20% to 2.1% as C/N ratio ranging from 11.7 to 14.1. In our study, percentage of nitrogen is 0.43% which is in the range of results found in Black Sea. The C/N ratio is 16.2 in our study which is higher than the measurements in Black Sea. It has been already mentioned in section 3.3., mainly in the marine environments those two ions are main limiting factors in biodegradation since hydrocarbon sources are abundant. The high ratio of C/N in sediments collected from Küçükçekmece coast show that this region is more limited in biodegradation than the Black Sea.

A comparison of the concentration of heavy metals in the sediments of Küçükçekmece coast with another anoxic region can be made according Akarsubasi et al. (2006). He and his coworkers analyzed the sediment samples of another highly polluted marine system, Golden Horn (Halic). In their study, the concentration of Cu, Cr, Zn, Pb, Fe, Ni and Cd are 5-10 times higher than the concentration of sediments collected from Küçükçekmece coast. Also the COD level of water above the sediment ranged from 147-1940 mgO_2/L as the COD level of water above the Küçükçekmece coast sediment measured as 603 mgO_2/L. According this comparison it can be said that Golden Horn is more polluted than Küçükçekmece coast. The pollution history of Golden Horn is longer

than Küçükçekmece coast and the characteristic of wastewater discharge is also different. Golden Horn as a marine inlet is more isolated to environmental changes and become more polluted (Akarsubasi et al., 2006).

The molecular techniques open a new frontier in the environmental analysis and studies. Until then scientists use culture based or microscopic methods to identify microorganism, which is rather hard and laborious as well as not reliable. As mentioned in section 4.1. the culture based methods can only identify a limited amount of bacteria. Especially anaerobic ones are extremely hard to cultivate. But usage of molecular techniques in environmental samples enables to reach any kind of microorganism in the sample. Molecular techniques address genetic materials mainly DNA and RNA. The GDNA should be extracted first and the analysis will be carried out after that.

Molecular techniques have also some limitations. Mostly these limitations come from genetic material. 16S rDNA is the leading element in environmental molecular analysis since it is present in all living organisms and carries information of evolution. To reach 16S rDNA, the Genomic DNA should be extracted. There are many extraction methods used to obtain GDNA from the sediment. Although the result is same, efficiency varies greatly. To monitor efficiency of different DNA extraction protocols, Webster and his coworkers conduct some experiment in 2003 (Webster et al., 2003). In their study, they compare different DNA extraction procedures according to preparation time, efficiency of DNA yield, suitability for further analysis. Their study shows that the procedure used in this study (FAST DNA extraction kit for Soil by Q-Biogene) is very efficient than the other procedures. Also with slight modifications they are able to increase efficiency of DNA yield by 100%. They indicate the increase in DNA yield is achieved by adding Poly A to the lysis buffer since it will block the sites on sediments particles where DNA can bind after cell lysis. Obtaining a pure GDNA is the key to success in following steps.

PCR is a powerful method as well as very precise. Having a pure GDNA lowers risks of inefficient PCR products, in this case mainly 16S rDNA. It has been already mentioned above the importance of 16S rDNA. Because most of the molecular techniques are obligatory PCR dependent, the 16S rDNA amplification carries a great importance for the further experiments and efficiency of molecular techniques. One of the main inhibitors

of PCR is humic substances. The presence of humic substance in the GDNA will decrease the efficiency of PCR greatly. It will also affect further PCRs. The GDNA should be washed carefully to avoid presence of humic substances. In this study PCRs produce good amount of amplification products in desired length.

DGGE is a versatile fingerprinting method. The easy usage, adaptability to different conditions and band excision makes DGGE most used fingerprinting method. In this study four different triplicate samples were amplified by suitable primers (Section 5.3.2.) and monitored for the diversity in bacterial and archaeal community separately. The main points in having a good DGGE gel photo are creating a good gradient of denaturants and having a good sample. DGGE is a precise technique and can be used to separate even one nucleotide change if gel gradient and DNA sample is good enough. During this study it has been encountered many problems regarding DGGE. Most common problems are fuzzy bands and short run of DNA in gel. The draft procedure of DGGE is modified according these problems and mentioned in section 5.3.3. Fuzzy bands mainly created from uneven distributed gradient as well as not fully polymerized gel. Therefore several optimizations were made to obtain denaturant gradient of desire. APS and TEMED concentrations were optimized. Short run of DNA problem overcame by applying fresh running buffer in each electrophoresis and increasing run time.

DGGE gel is easy to visualize and bands are can be fairly enumerated by naked eye. But to see the minor bands software is needed. With the help of Bionumerics, the bands not visible to naked eye can be detected by software. In band pattern table, it can be seen some bands appear and disappear in different times. The microbial community representing these bands may decrease in number due to environmental factors. The reason of loss may be change in environmental conditions input of an inhibitor or decrease of the substrate. Unfortunately we have only data of total phosphorus which is one of the limiting factors in marine sediments. The phosphorus percentage decreases with time and a careful analysis of band pattern table will indicate mostly the appearance and disappearance of the bands happened in era between December of 2005 and March of 2006. Ion chromatography results also show an increase in sulfate level in samples of March 06 and December 06. This may be another factor affecting community. Sulfate was

increased more than 15 times in concentration. This elevated level of sulfate may affect microbial community since a community shift can be seen in DGGE gels.

Phylogenetic analysis of samples shows a clear branching with triplicates residing in the same branch. The phylogenetic distance of bacterial samples is pretty close. It can be said that the bacterial population do not show a community chance during the time. Archaeal population becomes more diverse during sampling times. Although the branching remains same, the distance of each branch to each other increased. March 06 and December 06 samples are closer to each other than other samples. Possibly competition among archaea is higher than bacteria and change in conditions create a bigger impact on archaeal community. Also it is well known that the microbial communities coexist together and are mostly in syntrophic relationship. As bacterial community was not changed as much as archaeal community, change in a key bacteria group may amplify the change in archaeal community. Phylogenetic tree of samples shows bacterial population can be assumed as one population throughout the year. Archaeal community changes remarkably therefore it should be treated individually according to each sample time.

It has been commonly known the presence of sulfate reducing bacteria (SRB) and methanogenic archaea are favored under anaerobic conditions (Santagoeds, et al., 1999). Previous study on this sediment by FISH technique clearly shows the presence of these microorganisms. Both microorganisms are in syntrophic interaction and used metabolites of each other. Literature concerning this interaction shows methanogenesis is inhibited by sulfate addition in sediments (Widdel, 1988). But also there are other reports that there is no effect on methanogenesis under sulfate-rich conditions in the same environment (Senior et al., 1982; Ueki et al., 1992). This two different results might be explained by substrate availability, adaptation times of SRB population, species type and substrate demand. The previous study on SRB and methanogens shows that the percentage of SRB community reaches to 25% of the total community in sediment samples of September 05. The same study estimated the percentage of *Methanogenium* species as 6% and *Methanosaeta* species as 7%. In this study according to data revealed from 16S rRNA clone library of archaeal community of September 05, estimated percentage of *Methanogenium* species is 10%. The dominant bacteria groups in September 05 are not sulfate reducers. The

complete sequencing of clone library will give necessary information about SRB community in sample.

Sequence similarity analysis of clones indicated that bacterial clones of September 05 are mainly aerotolerant, fermentative bacteria (Madigan et al., 2002). Bacilli and Clostridia classes are responsible of fermentation of lactic acid, butyric acid and acetic acid. *Trichococcus pasteurii* ferments and converts L isomers of organic and amino acids and D isomers of sugars. Pyruvate and citrate are fermented to acetate and formate (and CO_2). Glucose and other carbohydrates are fermented to L-lactate, acetate, formate, and ethanol (Janssen et al., 1995). *Clostridium glycolicum* produced acetate from H_2-CO_2, formate, pyruvate, and lactate but also responsible in formation of high amount of gases. The change in pathways fermentation-homoacetogenesis is rather substrate dependent (Kusel et al., 2001). Clones of December 06 are in classes of alpha-, gamma-proteobacteria and clostridia. Genus *Rhizobiaceae* are mainly plant pathogen and nitrogen fixers. *Gallicola barnesae* grew mainly on purine and uric acid. Its fermentation products are acetate, formate, butyrate, ammonia and CO_2 (Ezaki et al.,2001). *Xanthomonas* species are despite to other bacteria described above not fermentative. They have respiratory metabolism. They degrade low molecular weight organic compounds with H_2 or CO_2 sometimes nitrate as sole electron donor (Madigan et al., 2002). Elbe River snow isolate Iso26 is one of the many new isolates found in study of Boeckelmann (Boeckelman et al., 2000). In her study, she and her coworkers analyze bacterial community in Elbe River snow and found it is dominated by proteobacteria. Isolate Iso 26 was identified as a *Rhizobiaceae* species which are chemoorganotrophs and able to utilize many carbohydrates.

Clones of archaeal community are less diverse than bacterial ones. September 05 clones are dominated by *Methanogenium* and *Methanoplanus* species. Both genus are under the division of methanomicrobiales and mainly responsible in utilization of H_2-CO_2, and formate to methane. Founded species are *Methanoplanus petrolearius, Methanoplanus limicola* and *Methanogenium organophilum* (Ollivier et al., 1997; Rouviere et al., 1992). December 06 community is dominated by *Methanosaeta* species. Although *Methanogenium* species are present in the community (*Methanogenium frigidum,* Franzmann, et al., 1997), *Methanosaeta* species are numerous over *Methanogenium*

species. *Methanosaeta* species are well known for their strictly dependency to acetate and use only acetate to produce methane.

According the sequence similarity analysis and information of metabolism of the analyzed species, it can be said that; bacterial and archaeal community are in a syntrophic relationship. Archaeal species live on acetate and H_2-CO_2, formate produced by fermentative bacteria. Also according to information gathered from clone library, it can be also said that community in September 05 and community in December 06 are diverse but operate to same final product, methane. In September 05, fermentative bacteria dominated community and produce lactate, butyrate, propionate and ethanol. Acetate is both produced directly from monomeric organics and indirectly via lactate and pyruvate. Methane formation is achieved by methanomicrobiales division by utilizing H_2-CO_2, and formate. In December 06, fermentative bacteria were less dominant and anaerobic respiration metabolism became more active in which CO_2 is converted to acetate. Dominance over archaeal population is also shifted from methanomicrobiales division to methanosarcinales division. *Methanosaeta* species utilize solely acetate in methanogenesis. The shift in microbial community can be explained by production of acetate by proteobacteria in December 06. Decrease in number of H_2-CO_2 utilizing archaea may triggered by the decrease of fermentative bacteria.

In the study of Delbes et al. (2001), in an anaerobic digestor system *Methanobacterium*, *Methanosaeta*, *Clostridium*, *Bacteriodes* genera has been found. Also in this present study, the microbial community consists of genera mentioned above. Although *Methanobacterium* is not encountered in this study, the genera *Methanomicrobiales* do the same operation which is formation of methane from H_2-CO_2. Both of methanogenic groups work simultaneously in an ecosystem, natural or anthropological, maximizing methane formation (Madigan et al., 2002). Clostridia exhibit diverse metabolic capabilities which make it a versatile organism to cope with stresses of different environments. It can use classic fermentations, homoacetogenesis or syntrophic acetate oxidation. In her study, Delbes found a community shift in archaea during an acetate crisis in an anaerobic digester system. A sharp decrease in acetate level and an increase in *Methanosaeta* population were observed. The high population of methanomicrobiales community in September 05 shows that hydrogenotrophic

methanogens are dominated methane formation by route of hydrogen-dependent methanogenesis. Also presence of clostridia genera promoted this suggest by its gas producing activity. The decrease in methanomicrobiales and increase in *Methanosaeta* population during the time coupled with decrease of fermentative bacteria may mean hydrogen-dependent methanogenesis was overwhelmed by acetate dependent methanogenesis. Dominant bacterial genera became proteobacteria and acetate formation is accelerated both activity of proteobacteria and loss of fermentative bacteria.

Hydrocarbons can be metabolized under Fe(III)–reducing, denitrifying and sulfate-reducing conditions by anoxygenic photosynthetic bacteria or in syntrophic consortia of proton-reducing and methanogenic bacteria. There are several microorganisms used in enhanced oil recovery. *Xanthomonas campestris*, Methanogens and various clostridium species are among these microorganisms (Van hamme et al., 2003). The presence of these microorganisms in sediment samples collected from Küçükçekmece coast may indicate the presence of oil and petroleum hydrocarbons in the sediment.

8. CONCLUSIONS

Sediment samples collected from Küçükçekmece Coast at four different times were analyzed successfully with molecular techniques for the microbial community and its composition. Both bacterial and archaeal population were monitored for the microbial diversity using Denaturing Gradient Gel Electrophoresis (DGGE) and dominant species were identified by generating 16S rDNA clone library. The results showed that there was a community shift between first sample and last sample, September 05 and December 06 respectively.

In the studies of Shine and Wallace (2000), and Hyland et al. (2005), it mentioned that Total Organic Carbon (TOC) value can be used as an indicator of pollution. Study of Albayrak et al., 2006 showed that Marmara Sea has many anoxic regions ranging TOC values from 2.1 mg/g to 22 mg/g. In this study, the TOC value of Küçükçekmece sediment samples measured as 6.8 mg/g. This value is equal to an intermediate anoxic system.

There are several indicators of oxic-anoxic difference. TOC is one of them, and redox potential can also be accepted as an indicator of oxygen level. (Virtasalo et al, 2005). In this study, the potential was measured at -90 mV in average for first three sampling times and -52 mV for the last sampling time. This result may mean there is a recovery in sediment.

DGGE is a fast and versatile method for microbial diversity analysis. In this study both bacterial and archaeal diversity was monitored with DGGE technique. In bacterial DGGE gel it has been found that 27 different bands for September 05, 26 different bands for December 05, 31 different bands for March 06 and 32 different bands for December 06. Among these bands 5 of them are dominant and 10 of them are intermediate bands. In the archaeal DGGE gel, 13 different bands can be identified for samples of September 05 and samples of December 05, 15 different bands for samples of March 06 and again 13 different bands for samples of December 06. Among them there are 3 dominant and 3 intermediate bands. The diversity of both communities in DGGE is parallel to results of 16S rRNA clone library. In bacterial clone library there are 23 different clones and 26

different clones for September 05 and December 06 respectively. Also there are 4 dominant clones and 3 dominant clones for two sediments respectively. In Archaeal 16S rRNA clone library, 19 different clones and 20 different clones were found in September 05 and December 06 respectively. There are 4 dominant clones and 5 dominant clones for archaeal community respectively.

According to phylogenetic tree of samples derived from the data of DGGE, the bacterial community was not diverse as archaeal community. The triplicate samples resided in same branch. Bacterial community is more closely related to each other through the time as archaeal branches are more distant to each other and to the root. Therefore there is not a representative sample in among archaeal community in opposite to bacterial community in which one of the times can be used to represent bacterial community in sediments of Küçükçekmece coast.

The result of sequencing of bacterial dominant clones indicate presence of *Trichococcus pasteurii, Clostridium glycolicum* in September 05 and Elbe River snow isolate Iso26, *Xanthomonas sp.* CC-FH5, and *Gallicola barnesae* in December 06. Archaeal dominant clones are *Methanoplanus petrolearius, Methanoplanus limicola*, *Methanogenium organophilum* in September 05 and *Methanogenium frigidum* and *Methanosaeta* sp. in December 06.

According to results of sequence similarity analysis and information of metabolism of the analyzed species, bacterial and archaeal community are in a syntrophic relationship. Archaeal species live on acetate and H_2-CO_2, formate produced by fermentative bacteria. Results of clone library generation show that community in September 05 and community in December 06 are diverse but operate to same final product, methane. In September 05, fermentative bacteria and hydrogenotrophic methanogens are dominated sediment sample In December 06, fermentative bacteria were less dominant and chemoorganotrophs became more active. Acetoclastic methanogens are dominant in archaeal community. The shift in microbial community can be explained by production of acetate by proteobacteria in December 06. Decrease in the number of H_2-CO_2 utilizing archaea may be triggered by the decline in number of fermentative and of homoacetogenetic bacteria.

As a conclusion, this study demonstrates the dominant biochemical pathway in sediments samples collected from Küçükçekmece coast. Fermentative and respiratory metabolism present as bacterial biochemical pathway. Hydrogenotrophic and acetoclastic methanogenic pathway dominated archaeal life. Syntrophic relations were observed as fermentative bacteria-hydrogenotrophic methanogens and anaerobic respirators-acetoclastic methanogens.

9. RECOMMENDATIONS

This was the first study using molecular techniques on sediments of Marmara Sea. The results are sufficient for understanding dominant biochemical pathway. But to understand the community structure and all biochemical relations, a bigger clone library should be generated and all clones should be sequenced.

One disadvantage of using DNA as genetic material in molecular techniques is inefficiency in interpretation of activity of microorganisms. DNA bands will show presence and abundance of microorganism but high DNA content does not mean those microorganisms are active. In order to analyze activity of microorganisms RNA should be used. With molecular analysis using RNA as genetic material may inform us about the activity of microorganism. 16S rRNA analysis alone will not be enough to see the activity. Possible explanation of dominant pathway can be analyzed by mRNA micro arrays. So the results coming from 16S rRNA molecular analysis and mRNA analysis from micro arrays can be compared to interpret activity of microorganism and activity of biochemical pathway in the sediment samples.

To understand biochemical pathways chemical analysis are also needed, measurements about acetate, CO_2-H_2 and other substrates and products may be coupled with results of molecular analysis to better understand microbial ecology in the sediments.

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