16S rDNA ANALYSIS OF MICROBIAL COMMUNITIES IN ANOXIC MARINE SEDIMENTS OF THE MARMARA SEA

by

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ABSTRACT

The sediments of the Marmara Sea are of importance since they are believed to have been a rather sensitive recorder of climatic, biological and chemical changes and water-mass movements in the region. The Marmara Sea is now a critically polluted water body.

In this study, microbial diversity especially with a potential to degrade hydrocarbons and ability to remove carbon, sulphur and organic matter in anoxic sediments from two of the hydrocarbon polluted regions of the Marmara Sea were investigated using culture-independent techniques. Overall microbial community structures were characterized by cloning and sequencing of PCR-amplified taxonomic16S rRNA genes. Denaturing Gradient Gel Electrophoresis was used to investigate the seasonal distribution of the microbial communities in coastal sediments from Tuzla and Moda.

Globally important *Methanosaeta* species in respect to acetate metabolism and Methanosarcinales dominated the 16S rDNA archaeal clone library of Tuzla, whereas *Methanothermococcus sp.* dominated the archaeal clone library of Moda. A sulfur oxidizing bacterium, *Epsilon Proteobacterium Dex80-27* which is able to reduce nitrate dominanated the bacterial clone library of Tuzla sediments and a fermentative bacterium, *Catenibacterium mitsuokai* dominated the bacterial clone library of Moda. The sequencing of clone libraries revealed a higher microbial diversity in anoxic sediment samples of Tuzla than that of derived from Moda and served to understand the potential dominant metabolic processes prevailing under anoxic conditions. Based on the frequencies of archaeal and bacterial species in the clone libraries, methylotrophic methanogenesis and denitrification were found as the potential dominant metabolic processes in Tuzla sediments, whereas hydrogenotrophic methanogenesis and fermentation appeared to be the potential dominant metabolic processes in Moda sediments. The results provided a unique opportunity to compare the microbial composition of both sites which had not been investigated before. DGGE data revealed a more significant change in microbial community structure of Tuzla sediments.

ÖZET

Marmara Denizi sedimentleri iklimsel, biyolojik ve kimyasal değişimlerin büyük ölçüde hassas bir kaydını tuttuğuna inanıldığından önemlidirler. Marmara Denizi bugün ciddi olarak kirlenmiş bir su kütlesidir.

Bu çalışmada, Marmara Denizi'nin hidrokarbonlarla kirlenmiş iki bölgesinde özellikle hidrokarbonları degrede etme kapasitesi ve karbon, sulfur ile organik madde giderim yeteneği olan mikrobiyal çeşitliliğin varlığı kültürden-bağımsız tekniklerle incelenmiştir. Genel mikrobiyal komünite yapısı PCR ile çoğaltılmış 16S rRNA genlerinin klonlanması ve dizi analizi ile tanımlanmıştır. Denaturan Eğimli Jel Elektroforezi (DGGE) metodu Tuzla ve Moda kıyı sedimentlerinde mikrobiyal komünitelerin mevsimsel dağılımını incelemek için kullanılmıştır.

Asetat metabolizmasıyla ilişkili olan global öneme sahip *Methanosaeta* türleri ve Methanosarcinales Tuzla 16S rDNA arkeyal klon kütüphanelerinde baskın olarak görülmüşken, Moda arkeyal klon kütüphanerinde *Methanothermococcus sp*. baskın tür olarak bulunmuştur. Nitrat indirgeme kapasitesinde ki bir sulfur oksitleyici bakteri olan *Epsilon Proteobacterium Dex80-2* Tuzla sedimentlerinin bakteriyal klon kütüphanesinde baskın olarak görülmüştür ve bir fermentative bakteri olan *Catenibacterium mitsuokai* de Moda bakteriyal klon kütüphanesinde baskın olarak bulunmuştur. Klon kütüphanelerinin dizi analizi, Tuzla anoksik sediment numunelerinde Moda'dan elde edilenden daha yüksek bir mikrobiyal çeşitlilik olduğunu ortaya koymuştur ve anoksik koşullarda hüküm süren potansiyel metabolik süreçlerin anlaşılmasına hizmet etmiştir. Arkeyal ve bakteriyal türlerin klon kütüphanelerindeki görülme sıklığına dayanarak, metilotrofik methanogenesis ve denitrifikasyon Tuzla'da ki baskın prosesler olarak bulunmuşken, hidrogenotrofik metanogenesis ve fermentasyon da Moda'da baskın olan potansiyel prosesler olarak görülmüştür. Sonuçlar daha önce araştırılmamış olan bu iki alanın da mikrobiyal kompozisyonunun karşılaştırılmasına eşsiz bir fırsat sağlamıştır. DGGE datası, Tuzla sedimentlerinin mikrobiyal çeşitliliğinde daha kaydadeğer bir mevsimsel değişim ortaya koymuştur.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Units used
TOC	Total Organic Carbon	(mg g^{-1})
TS	Total Solid	(mg L^{-1})
TVS	Total Volatile Solid	(mg L^{-1})
TC	Total Carbon	(mg g^{-1})
TIC	Total Inorganic Carbon	(mg g^{-1})
Cr	Chromium	(mg kg^{-1})
Cd	Cadmium	(mg kg^{-1})
Cu	Copper	(mg kg^{-1})
Zn	Zinc	(mg kg^{-1})
Pb	Lead	(mg kg^{-1})
Ni	Nickel	(mg kg^{-1})
Mn	Manganese	(mg kg^{-1})
Fe	Iron	(mg kg^{-1})
Ag	Silver	(mg kg^{-1})
N	Nitrogen	(%)
C	Carbon	(%)
S	Sulfur	(%)
P	Phosphorus	(%)

AOM	Anaerobic Oxidation of Methane
DGGE	Denaturing Gradient Gel Electrophoresis
DSDP	Deep Sea Drilling Project
EDTA	Ethylene Diamine Tetra Acetic Acid
EF	Enrichment Factor
IODP	Integrated Ocean Drilling Program
LSU	Large Subunit
mbsf	Meters below sea floor
MK	Moda
MY	Tuzla

mV	Millivolt
ODP	The Ocean Drilling Program
PCR	Polymerase Chain Reaction
Pg	Petagram
Rpm	Revolutions per minute
SRB	Sulfate Reducing Bacteria
SSU	Small Subunit
TAE	Tris-Acetic Acid-EDTA
TN	Total Nitrogen
TP	Total Phopshorus
T-RFLP	Terminal-Restriction Length Polymorphism
TS*	Total Sulfur

1. INTRODUCTION

Despite the inhospitality of subsurface sites, it has been revealed that marine subsurface sediment constitutes one of the largest and most widespread reservoirs of biomass on Earth. Subsurface prokaryotic activities have profound effects on global biogeochemical cycles (Webster et al., 2004), particularly on global carbon cycling. The amount of buried carbon in marine sediments as biogenic gas hydrates seems to equal between four and eight times the amount of carbon in all the living organisms on Earth (Kvenvolden, 1993). Recent estimates of global biomass have indicated that the amount of living carbon in the deep biosphere may constitute between one-tenth and one-third of Earth's total biomass and a large fraction of the global prokaryotic biomass which makes up about 70% of the *Bacteria* and *Archaea* in sub-seafloor sediments (Parkes et al., 2000). Moreover, sediment bacteria constitute a huge reservoir of genetic variability with a local diversity equal to soil ecosystems which means that it is possible to find new life forms buried within the sediments (Torsvik et al., 2002). However, the deep subseafloor biosphere is among the least-understood habitats on Earth regarding the organisms, their physiologies and their influence on surface environments (Inagaki et al., 2006). This is mainly due to the difficulties involved in enriching and isolating the representative deep-sediment microorganisms (Toffin et al., 2004) and previous studies based on cultivation methods could not reveal the appropriate sedimentary microbial diversity. Molecular-based, culture independent techniques such as fluorescence in-situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), and 16S rDNA sequencing for investigating the prokaryotic diversity have given a more realistic picture of the community structure in marine sediments (Lysnes et al., 2004; Webster et al., 2004), and have been successfully employed to overcome the difficulties associated with culture dependent methods. Such studies have led not only to insights into the community diversity and structure of microbial systems, but have revealed new phylogenetic lineages of microorganisms, some of which serve as the dominant constituent in a given microbial community (Webster et al., 2004).

Depth-related gradient of physical and chemical properties in marine sediments provides niches for a wide variety of metabolically diverse microorganisms (Urakawa et

al., 2000). Microbial activities occurring in anoxic marine sediments include methanogenesis, fermentation and reduction of SO_4^{-2}, Fe (III), Mn (IV), NO_3^-, and O_2 (D'Hondt et al., 2003). Methanogenesis and sulfate reduction are found to be the most important terminal processes in the remineralization of organic compounds because of the rapid depletion of other electron acceptors and the overwhelming abundance of sulphate in seawater (D'Hondt et al., 2002). Sulfate reduction appears to be the most important microbial process, accounting for up to 50% of organic matter degradation in coastal marine sediments and generally, methanogenesis becomes the dominant terminal oxidation process when sulfate becomes depleted (Wilms et al., 2007). The dissimilatory sulfate reduction can be linked to the oxidation of substrates that are difficult to degrade under anoxic conditions, such as alkanes and aromatic compounds (Hansen, 1994), or even to the anaerobic oxidation of methane at sulfate-methane transition zones in marine sediments which is the major biological sink of the greenhouse methane, serving as an important control for emission of methane into hydrosphere (Knittel et al., 2005). Since diverse syntrophic and competitive interactions occur between different physiological types of microorganisms in subsurface marine environments, (Fenchel and Finlay, 1995), studying microbial diversity of anoxic marine sediments allows to investigating key players of nutrient recycling and organic pollutant degradation.

Subsurface sediment layers only recently became a focus of microbial investigations which mainly targets open-ocean and continental-margin sediments and has paid little attention to subsurface coastal sediments, but the number of publication is increasing (Wilms et al., 2006). Marine coastal sediments are known to contain a rich diversity of microorganisms from different physiological and phylogenetic groups. Therefore, previous molecular studies focused on the general microbial community structure and the abundance and depth distribution of specific functional groups, e.g., polymer degrading, sulfur-oxidizing or sulfate-reducing bacteria (SRB) (Webster et al., 2004). Although microbial communities in coastal sediments can also be influenced by the strong seasonality in temperature and organic matter availability, to our knowledge, this has not been examined yet and this study assessed seasonal change in microbial diversity of coastal sediments from Moda region within the Kadıköy district located in İstanbul, Turkey on the coast of Marmara Sea and from Tuzla Bay which is located in Turkey, on the Asian side, 60 km east of İstanbul, on the Marmara Sea. Other sampling point of the

study, namely Moda has been densely exposed to domestic wastewater discharges since the end of 1970s and has gone under amendment by İSKİ since the early 2000. Based on the water quality monitoring projects, it has been showed that anoxic conditions have been occurred within the marine sediment samples taken from Moda region which is at the junction of a small rivulet, namely Kurbağalıdere connecting to Marmara Sea. Nevertheless, hydrocarbon rich wastewater discharge still occurs in this region which is only exposed to pre-treatment. Tuzla Bay is an important area for local fisheries and recreation for İstanbul City. It also serves as a host for many dockyards. Although the bay used to be an important area regarding eukaryotic biodiversity (Dural et al., 2007), it has undergone heavy environmental stress due to expansion of the İstanbul Metropolitan City in terms of agricultural, industrial and municipal activities over the past 25 years. On February 13th, 1997, a tanker named TPAO exploded in Tuzla shipyards and an estimated amount of 215 tons of oil was spilled in to the bay (Kazezyılmaz et al., 1998; Ünlü et al., 2000). The dockyards have also been contributing the oil pollution in the bay. Earlier studies on the bay concerned heavy metal and hydrocarbon pollution (Ünlü et al., 2000; Dural et al., 2007) and no information is available on microbial populations inhabiting neither the sediments of the Tuzla bay or Moda region. Such information can be used to establish bioremediation strategies for this heavily polluted environment or to isolate biotechnologically important new organisms.

This study is one of the legs of a broad range project supported by TÜBİTAK, aiming to determine the microbial composition and anaerobic petroleum degradation capacity of anoxic marine sediments of the Marmara Sea, from different points two of which are Tuzla and Moda. In the scope of the mentioned project, the presence of microbial communities with the potential to carry out anaerobic hydrocarbon degradation of hydrocarbons and presence of biomarkers for *in situ* hydrocarbon degradation in anoxic marine sediments will be investigated to determine the distribution of anaerobic hydrocarbon degradation potential in the Marmara Sea, an important and heavily industrialized marine environment. Laboratory crude-oil degradation microcosm experiments will be used to determine under which terminal electron accepting conditions anaerobic processes will have a significant impact on the dissipation of crude oil contamination and to identify the systematic effects of nutrient amendment on oil degradation and bacterial community dynamics. The effect of different nutrient supply

regimes on biodegradation rates will be assessed in the context of resource ratio theory which has only previously been investigated for aerobic hydrocarbon-degrading systems. Thus, the data derived from this study of Moda and Tuzla along with the other important sampling points included in the project will provide the initial steps forward the above mentioned goals and the basic microbial ecological bases for this broad project.

2. POLLUTION OF THE MARMARA SEA

2.1. Description of the Marmara Sea

Being an intracontinental sea on a waterway between Mediterranean Sea and Black Sea, the sediments of the Sea of Marmara are believed to have been a rather sensitive recorder of climatic, biological and chemical changes and water-mass movements in the region (Çağatay et al., 1996).

The Sea of Marmara is a small (size ~70 x 250 km) intercontinental basin connecting the Black Sea and the Mediterranean Sea and has a volume of 3,380 km^3, and consists of a complex morphology including shelves, slopes, ridges and deep basins (Algan et al., 2004). It has a relatively broad shelf (40 km) in the South and a narrow one (10 km) in the North. The Marmara Sea is connected to Black Sea in the northeast via the İstanbul Strait (Bosphorus) and to the Aegean Sea in the southwest via the Çanakkale Strait (Dardanelles).

The oceanographic features (chemical, biological) of the basin are influenced by the Black Sea and the Aegean Sea via the Bosphorus Strait and the Dardanelles, respectively. The waters of the Bosphorus are strongly stratified, with the upper layer comprising low salinity outflow from the Black Sea and the bottom denser layer generated by northerly, highly saline flow from the Mediterranean. Mixing between the two layers along the Bosphorus and the Bosphorus / Marmara junction, is strongly affected by the main features related to the physical oceanography of the area, namely the respective salinity of the approaching currents, the topography of the strait and the prevailing meteorological conditions which results in a permanent two-layer flow system with halocline at a depth of 20-25 m (Orhon, 1995). The stratification of the water column, together with the topographic restriction of the two straits, prevents the efficient circulation of the sub-halocline layer. As a result, the dissolved oxygen content of the bottom waters decrease by microbial oxidation of organic matter from 7-10 mg/l near Çanakkale Strait towards east to about 1 mg/l above the deep basins, and 2.5 mg/l near the İstanbul Strait (Ünlüata and Özsoy, 1986). The water that came from the Mediterranean Sea is vitalizing to Marmara

Sea, because it has more oxygen and more salt (Algan et al., 2004). The significant feature of the hydrodynamics of the Bosphorus is the intense mixing of the deeper Marmara waters into the upper layer at the Bosphorus/Marmara junction (Orhon, 1995). The oxygen saturation of water below 25-30 meter depth from the surface varies between 20 % - 30 % which is problematic for the mineralization of organic matter and coastal discharges from the Black Sea.

In terms of primary production, the Sea of Marmara is intermediate between the Black Sea and Aegean Sea, with values of 60-160 g cm^2 year (Yılmaz, 1986; Ergin et al., 1993), the highest values being located in the inner southern shelf.

2.1.1. Pollution Sources at the Marmara Sea

The Marmara Sea is now a critically polluted water body and the recipient of a large number of wastewater discharges from landbased sources located along the coastal line, including the İstanbul metropolitan area (Orhon, 1995; Albayrak et al., 2006) and subject to several other anthropogenic activities that primarily cause severe hydrocarbon and heavy metal pollution. The pollution of Marmara Sea is based on sewages, industries and vessels. Sewage pollution is most important of them. The Marmara Sea turned into an open sewage, because there is not a purification system for sewages. Industrial pollution is mostly based on government-run factories (Algan et al., 2004). The water quality measurements indicate severe signs of present and future eutrophication problems (Orhon, 1995). There is dying species in the Marmara Sea over 50, such as monk seals, sturgeons, shrimps and crabs (Turkish Marine Research Foundation, 2004).

The contaminants are introduced through water ways by a surface current from Black Sea and a deep current from the Mediterranean, respectively (Ünlü et al., 2006).The Bosphorus, a strongly stratified natural channel between the Marmara Sea and Black Sea, with significant mixing at the entrance to the Marmara Sea is also a major polluter for the Marmara basin, since it carries the highly polluted waters of the Black Sea (Orhon, 1995). The Marmara Sea receives via the natural exchange from the Black Sea roughly 15 times more organic matter than what is contained in the sewage discharges from İstanbul (Orhon, 1995). The basin receives a total of 1.9 x 106 tons of TOC (total organic carbon) and 2.7 x

105 tons of TN (total nitrogen) per year from the Black Sea inflow (Albayrak et al., 2006). Nutrient input from the Black Sea, however, is much more significant then coastal wastewater discharges according to the experimental evidence on the basis of extensive observations (Orhon, 1995).

Furthermore, aside from coastal areas, the main pollution problem in the Marmara Sea is the nutrient accumulation which can not be remedied (Orhon, 1995). The Marmara Sea, being an internal water body with close interactions with the Black Sea and the Mediterranean, is permanently and strongly stratified with totally different characteristics between the euphotic layer in the upper 30 m and the lower layer showing typical properties of the Mediterranean. The primary productivity in the upper layer can also be considered as a significant index of pollution in the Marmara Sea (Orhon, 1995).

Increasing industrial and domestic activities in the Marmara Region mainly influence the coastal and shelf areas of the Marmara Sea (Algan et al., 2004). Meanwhile rapid urbanization on the coastal zone of the Marmara Sea has attracted congested population influx since the 1970's (Ünlü et al., 2006). Pollution loading from İstanbul alone, the biggest city of Turkey in population and industry, makes up the major portion (40–65 %) of the total anthropogenic discharges (Polat and Tuğrul, 1995). Anthropogenic activities in the coastal area of the North Marmara Sea include, urban effluent, summer resorts (untreated effluent discharged into the sea), agricultural run off, sunflower oil factories, a big cement factory, fishing and shipping (Öztürk et al., 2000). Aside from İstanbul, the İzmit Bay area, the Gemlik Bay area, which are also included within the scope of the Tabitha Project covering this present study, the Susurrus River and the adjacent residential areas, and the Tekirdağ area contribute different degrees to the pollution of the Marmara Sea (Orhon, 1995). There are major rivers in the South (the Biga, Gönen and Kocasu rivers) flowing into the Sea of Marmara that are responsible for high input of nutrients and allactonous organic matter to the southern shelf (Çağatay et al., 1996).

In addition, tanker traffic of several thousand oil carrying vessels per day, via the Bosphorus Strait is a constant threat to the marine ecosystem (Albayrak et al., 2006). There is a heavy traffic of shipping approximately 60.000 vessels per year involving tankers 10%. Tankers from oil exporting countries surrounding the Black Sea have only one exit to

the Mediterranean Sea: via the Bosphorus Strait, the Sea of Marmara and the Dardanelle Strait. The Bosphorus and the Dardanelle's are typical narrow water channels and navigation route through the Sea of Marmara. This route therefore increases the risk of collisions and running aground (Tan and Otay, 1999). Many accidents of merchant ships and tankers occurred in the strait. Nine tanker accidents, which resulted in almost 193 tons oil spill, occurred in Bosphorus and Sea of Marmara between 1964 -2002 (Güven et al., 2004). The major accidents happened by large tankers Independenta in 1979 and Nassia in 1994. In the Independenta accident at the exit of the Bosphorus to the Sea of Marmara in 1979, 95 000 tons of crude oil was spilt and burnt (Etkin, 1997). In the Nassia accident at the northern exit of the Bosphorus to the Black Sea in 1994, 13 500 tons of crude oil were spilt (Oğuzülgen, 1995). M/V GOTIA sank into Bosphorus and 25 tons fuel oil was spilt and pollution spread out into a large area by winds (Güven et al., 2004). Bilge water discharge is also a major problem for the Straits of İstanbul and Çanakkale, and the Sea of Marmara. Increase in petroleum hydrocarbon levels mainly from oil spills, sewage outfalls and ship bilge water, has been observed in the Sea of Marmara (Güven et al., 1997).

The levels of pollution, particularly the heavy metals, have increased dramatically due to large inputs from the Black Sea (Kut et al., 2000). At the same time, the Marmara Sea has been subject to very high levels of pollution due to industrial and municipal waste disposal. Recent study of Sayhan Topçuoğlu and friends (Topçuoğlu et al., 2004) on heavy metal levels in biota and sediments in the northern coast of the Marmara Sea revealed that the levels of Zn, Fe, Mn, Pb and Cu in the macroalgae are higher than previous studies in the Marmara Sea, however, studied sediments from the relevant sampling points showed lower heavy metal levels than other areas in the Marmara Sea.

Metal contents (Al, Fe, Mn, Cu, Pb, Zn, Ni, Cr, Co and Hg) of the surface sediments from the shelf areas of the Marmara Sea generally do not indicate shelf-wide pollution. The variability of the metal contents of the shelf sediments is mainly governed by the geochemical differences in the northern and southern hinterlands. Northern shelf sediments contain lower values compared to those of the southern shelf, where higher Ni, Cr, Pb, Cu and Zn are derived from the rock formations and mineralized zones. However, besides from the natural high background in the southern shelf, some anthropogenic influences are evident from EF values of Pb, Zn and Cu, and also from their high mobility in the semi-

isolated bay sediments (Algan et al., 2004). Anthropogenic influences are found to be limited at the confluence of İstanbul Strait in the northern shelf. However, Algan et al. (2004) found that suspended sediments along the shallow parts of the northern shelf were enriched in Pb and Hg and to a lesser degree in Zn, reflecting anthropogenic inputs from İstanbul Metropolitan and possibly from the Black Sea via the İstanbul Strait.

2.1.2. Pollution of Tuzla and Moda

Tuzla is located on the Asian side, 60 km east of İstanbul, on the Sea of Marmara coast. Along the coast of Tuzla, there are agricultural lands and industrial plants (iron–steel plants, LPG plants, oil transfer docks, and cargo ship's ballasts water).

Moda is located within the the Kadıköy district in İstanbul, Turkey on the Northern coast of Marmara Sea. Moda is at the junction of Kurbağalıdere which used to be an historical old rivulet surrounded by a recreational area connecting to Marmara Sea and a sanctuary for fisheries and boathouses.

Biogenic, diagenetic and anthropogenic components contribute to shelf sediments after their delivery to the marine environment. In coastal areas of densely populated large cities, the anthropogenic component of the sediments mostly exceeds the natural one. The surface sediments become a feeding source for biological life, a transporting agent for pollutants, and an ultimate sink for organic and inorganic settling matters (Algan et al., 2004). Marine sediments, particularly those in coastal areas, are commonly polluted with petroleum hydrocarbons (PHC) as a consequence of the extensive use of petroleum compounds by mankind (Miralles et al., 2007). In aquatic sediments, the depth of oxygen penetration through diffusion is controlled mainly by the consumption of degradable organic matter within the sediment and in coastal ecosystems rarely exceeds more than a few millimeters (Jorgensen, 1983). With the exception of the most superficial layer, the bulk of organic matter-rich marine sediments contaminated by PHC are assumed to be anoxic (Canfield et al., 1993b). Consequently, microbial processes depending on the availability of free dissolved oxygen are constrained to the uppermost surface or, in deeper sediment layers, are coupled to irrigation and bioturbation processes of burrowing microorganisms (Freitag and Prosser, 2003). During the last decade, studies have shown

the potential of coastal marine sediments for anaerobic hydrocarbon degradation under sulphate-reducing conditions (Coates et al., 1997; Townsend et al., 2003). In marine reduced sediments, hydrocarbon degradation coupled to sulphate-reduction seems to be the most relevant among the different anaerobic processes, because sulphate is abundant in coastal and estuarine seawater, whereas nitrate concentrations are typically low and Fe(III) is often only sparsely available, especially in heavily contaminated sediments (Rothermich et al., 2002).

Industrial activities, municipal wastewater, agricultural chemicals, oil pollution and airborne particles have been the main reasons for the pollution that has affected primarily the estuaries and bays of the Marmara Sea and has ultimately spread along the shoreline and continental shelf that constitutes 50% of its total area (Ünlü et al., 2006) Anthropic pollution trapped in bays, in particular, has created significant ecological damage resulting in the decrease or extinction of marine species (Ünlü et al., 2006). The northern shelf of the Marmara Sea is more subjected to increasing human interferences in the form of industrial (metal, food, chemistry, and textile) waste disposal, fisheries, dredging, recreation and dock activities, than to the southern shelf. It receives pollution not only from various local land-based sources, but also from the heavily populated and industrialized İstanbul Metropolitan and from maritime transportation (Algan et al., 2004). Because Marmara Region is an important coastal settlement in Turkey with rapidly increasing population and industrial activities, the Sea of Marmara and the Turkish Straits are subject to intensive navigation activity. With the recent increases in sea traffic, these waterways have become a prime site for oil spill pollution (Kazezyılmaz et al., 1998).

Tuzla has undergone heavy environmental stress due to expansion of the İstanbul Metropolitan City in terms of industrial and human settlement through this area over the past 25 years. Many buildings were built on the marshy rim of the Tuzla despite heavy criticism from environmentalists. Due to heavy industrial and agricultural activities in the region, the bay has the polluted coastal waters of Turkey. Therefore, mainly untreated agricultural municipal and industrial wastes affect the lagoon direct or indirectly.

Moreover, on February 13th, 1997, a tanker named TPAO exploded in Tuzla shipyards located on the northeastern coast of the Sea of Marmara. During the fire, an

estimated amount of 215 tons of oil was spilled in to the Aydınlık Bay and 250 ton oil burnt (Kazezyılmaz et al., 1998; Ünlü et al., 2000). The oil pollution was investigated and the pollution level was determined in seawater, sediments and mussels in Tuzla Bay after the TPAO tanker accident. The highest pollution was found as 33.2 mg/l in seawater and 423.0 µg/g in sediment on the first day after the accident (Ünlü et al., 2000).

Moda is relatively considered as a less polluted area in comparison to Tuzla. However, Moda has been densely exposed to domestic wastewater discharges since the end of 1970s and has gone under amendment by İSKİ since the early 2000. Based on the water quality monitoring projects, it has been showed that anoxic conditions have been occurred within the marine sediment samples taken from Moda region. Nevertheless, hydrocarbon rich wastewater discharge of cyanide containing wastewater has recently occurred in this region which was only exposed to pre-treatment.

2.2. Marine Sediments

Approximately 70% of the Earth's subsurface is marine and the underlying sediments, which can be more than a kilometer deep, cover 70 % of the total earth (Kormas et al., 2003). Deep sea sediments covering earth's surface may also be defined as "deep-sea floor" which are that portion of the ocean bottom overlaying by at least 1000 m of water column (Vetriani et al., 1999; Glover and Smith, 2003). The deep-sea floor is one of the vast regions with a number of distinct habitats (Glover and Smith, 2003). These cover sediment filled basins, continental slopes and abyssal plains, deep ocean trenches and the exposed pillow basalts of young mid-ocean ridges, seamount risings > 1000 m above the general seafloor and submarine canyons. The most extensive habitats constituting >90% of the deep-sea floor are the "mud" or "silt and clay" clad plains of the slope and abyss. Deep ocean trenches constitute 1-2% of the deep-sea floor, while the rocky substratum of mid-ocean ridges (~ 10 km wide and ~ 60 000 km long) , seamounts (perhaps 50.000-100.000 in number) and submarine canyons being the rare habitats of the deep sea occupy < 4% of the sea floor. (Glover and Smith, 2003)

2.2.1. Formation of Marine Sediments

Deep sea sediments are primarily formed through the deposition of particles from the productive ocean surface (Vetriani et al., 1999). The new ocean basin that forms at spreading ocean ridges due to plate tectonic forces migrates towards subduction zones where it moves under continental shelves are returned to the interior of our planet. During this tour of maximum 170 years more and more sediments build up on top of hard basement rock and ultimately thousands meters of thick layers can be formed (Glover and Smith, 2003). The various major sediment input sources into the ocean are rivers, glaciers and ice sheets, wind blown dust, coastal erosion, volcanic debris, ground water. Much of the organic input into the oceanic sediments is through the recycling by the benthic communities (Aller et al., 1998). Marine sediments, also known as pelagic sediments are those that accumulate in the abyssal plain of the deep ocean, far away from terrestrial sources which provide terrigenous sediments, one of the two main classifications for marine sediments. Terrigenous sediments are normally delivered by rivers and are primarily limited to the coastal shelf. There are many classification schemes such as size, deposition mode, source, locale and chemistry for deep sea sediments. Terrigenous sediments are normally classified according to their sediment grain size and named as boulder, cobble, pebble, gravel or granule, coarse sand, medium sand, fine sand, silt or clay. However, pelagic sediments are classified by their composition as follows: lithogenous, biogenous, hydrogenous, cosmogenous. Among pelagic sediments, biogenic sediments which are derived from living, mostly planktonic organisms in a variety of forms and species are the most important in marine sedimentological field since the most information can be derived from them. Those sediments have high sedimentation rates and contain information about water chemistry and climates.

2.2.2. General Characteristics of Marine Sediments

There are several characteristics distinguishing most deep-sea sediments from other Earth's ecosystems, perhaps the most important one of which is its low productivity (Glover and Smith, 2003). The detrital base of deep-sea food webs strongly differs from most epipelagic, shallow-water and terrestrial ecosystems, which are mostly maintained by locally produced organic matter, whereas detrital food particles for the deep-sea biota

ranges from fresh phytoplankton remains to the carcasses of whales (Glover and Smith, 2003). Therefore the biomass of the deep benthic communities is only 0.001-1% of that in shallow-water benthic or terrestrial communities due to the low flux of organic energy. Low food flux along with low temperatures (-1– 4 °C) results in relatively low rates of growth, respiration, reproduction, recruitment and bioturbation in the deep sea (Glover and Smith, 2003).

In the subsurface which is defined as terrestrial subsurface below 8 m and marine sediments below 10 cm, prokaryotic cellular carbon for the marine subsurface is estimated as 303 Pg of C, whereas the total prokaryotic cellular carbon value in soil yields an estimate of 26 Pg of C (Whitman et al., 1998). 5 to 10 billion tons of organic particulate matter is constantly sinking in the world's oceans and accumulating as sediment and only about 0.4 % of the carbon fixed by phytoplankton at the ocean surface is buried in the oceanic sediments which represents a net carbon dioxide removal from, and oxygen input into, the atmosphere (Middelburg and Meysman, 2007). About 95% of the organic matter produced photosynthetically appears to be recycled in the upper 100-300 m, whereas only about 1% of photosynthetically produced organic carbon reaches the deep-sea floor, and this remainder out of the vast majority of organic matter recycled by near-surface microbial activity accumulates and represents the largest global reservoir of organic carbon, approximately 15.000 x 10^{18} g carbon, including fossil fuels (Parkes et al., 2000). Therefore the major nutritional characteristics of the deep-sea environments are relatively low input of organic carbon and its consumption for living organisms and deep-sea sediments may be estimated as unique habitats for microbial communities where the availability of nutrients is geographically highly variable and pressures are highly elevated (Li et al., 1999b).

Other general characteristics for deep-sea floor are the low-physical energy, very slow sediment accumulation rates (0.1-10 cm/thousand years) and the absence of sunlight. Nonetheless, the studies showed that deep-sea soft-sediment communities often exhibit very high local species diversity, with 0.25 m^2 of deep-sea mud containing 21-250 macrofaunal species (Glover and Smith, 2003). Surprisingly, not all the deep-sea habitats are low in energy and productivity. Hydrothermal vents and some cold seeps are exceptional since energy for the deep-sea biota is derived from an attenuated 'rain' of

detritus from remote surface waters (1-10 g C_{org} / m^2 yr). In cold seeps biomass and productivity of the present communities, which are low in diversity, are high due to the chemoautotrophic production fuelled by reduced chemicals such as hydrogen sulphide .Besides seamounts, canyons and whale falls which also break the low-energy deep-sea 'rule' enhances the physical and /or biological energy yield resulting in high biomass communities (Glover and Smith, 2003).

2.2.3. The Importance of Marine Sediments

Sediments on the seafloor are a rich source of information on the history of the oceans (e.g., changes in ocean temperature, circulation patterns, and chemistry), on former climates, sea levels and pollution. They are very useful at providing information on changing global climates during the past few million years. Sediments play an important role in the remineralization of deposited matter in highly productive continental shelf areas (Mußmann et al., 2005). Sediments have proved to be excellent indicators of environmental pollution, as they accumulate pollutants to the levels that can be measured reliably by a variety of analytical techniques and they also store records about pollution history of a given water body due to sedimentation being a continuous process (Tuncer et al., 2001). Sedimentation with faster sedimentation rates in bays and estuaries are more suitable for investigating pollution history in the twentieth century as they provide higher resolution (Tuncer et al., 2001). Sediments are undoubtedly essential to the functioning of aquatic ecosystems, since they may act as sinks but also as sources of contaminants in aquatic systems (Mucha et al., 2003).

Besides all the geochemical importance of marine sediments, deep subsurface has been under the exploration of scientists for its biodiversity and the microbial processes occurring within, that are of importance as a result of general, social, professional and industrial motives (Pedersen, 2000), and mostly due to the environmental concerns. However, the deep sea biosphere is among the least-understood habitats on Earth, eventhough the huge microbial biomass therein plays an important role for potential long-term controls on global biogeochemical cycles (Inagaki et al., 2006).

Marine sediments are of significance since they play an important role in the global cycling of carbon and nutrients (Rochelle et al., 1994). Chemical composition of the ocean and the atmosphere is profoundly effected by selective degradation of organic matter (Holland, 1984). The ocean sediments are a significant reservoir of carbon burial without which O_2 would not have accumulated in the atmosphere (Middelburg and Meysman, 2007). Moreover, the small quantity of carbon transfer from surface to subsurface sediment supports prokaryotes that live deep in the Earth's crust and that make up about 30% of the total living biomass on Earth (Whitman et al., 1998). Therefore, the subsurface is a major habitat for prokaryotes and the number of subsurface prokaryotes is expected to go beyond the numbers of the other components of the biosphere (Whitman et al., 1998). The studies have shown that the subsurface contains a variety of types of microbial ecosystems that are much more densely populated than expected (Krumholz et al., 2000). Thus deep subsurface environments harbor a vast diversity of communities that are responsible for various microbial processes which have a fundamental role in surface sediments and when microorganisms are mixed with the sediment, they catalyze the early diagenetic processes and thus appear to be important factors in the diagenesis of the sediments during the sedimentation process (Wellsbury et al., 1997).

2.2.4. Deep Subseafloor Studies

There have been several studies on marine sediments and deep intraterrestrial life. Scientists are exploring the subsurface and the questions of microbial diversity, the interactions among microorganisms and maintenance of subsurface microbial communities are being addressed (Krumholz et al., 2000). The microbiology of most intraterrestrial environments relates to the numbers of individual cells, the number of various physiological groups and the diversity of microbial populations which are mostly analyzed (Pedersen, 2000), leading to a successive inquiry of metabolic states of the communities, metabolically active communities and the favorable triggering conditions for such microbial communities.

Our understanding of the deep sea has changed during the 20^{th} century, the early expeditions of the British HMS Challenger and the Danish Galathea showed the presence of abundant life in all the area of deep ocean (Glover and Smith, 2003). One of the major

contribution in such studies is based on The Ocean Drilling Program (ODP) which is an international partnership of scientists and research institutions organized to explore the evolution and structure of the earth (Pedersen, 2000) and the international successor to the Deep Sea Drilling Project (DSDP) (Smith and D 'Hondt, 2006). The scientific ocean drilling community has been retrieving cores from hundreds of meters below the seafloor since the inception of DSDP in 1968 (Smith and D 'Hondt, 2006). The ODP, which can drill cores (long cylinders of sediment and rock), has recovered more than 160 000 cores since January 1985 and a high-priority research objective of the ODP is the exploration of the deep sub-sea floor (Pedersen, 2000).

Early studies of marine ecology were based on the paradigm of 'slow, steady pace of life' at the deep-sea floor (Smith, 1994) that agreed with the prevailing view of high species diversity in deep-sea sediments as a result of extreme resource partitioning under stable conditions during long time scales (Sanders, 1968). Data from deep sediment traps in the Sargasso Sea and North Atlantic showed dramatic variability in particulate organic flux and other evidences such as physical disturbance in the form of high energy benthic storms observed on Scotia Rise, pulsed biogenic disturbance and successional processes found in experimental studies in the Santa Catalina Basin countered the notion of a 'slow and stable' deep sea (Glover and Smith, 2003). Present view is of an ecosystem relatively homogeneous in space and time, interrupted by biogenic pulses and organic enrichment at scales ranging from centimeters to thousands of kilometers (Smith, 1994).

Study of subseafloor life has increased over the last 30 years (Smith and D 'Hondt, 2006). Over the past 20 years, ubiquity of the microscopic life beneath the seafloor has been revealed as a result of scientific drilling into the sediments and basaltic crust all over the world ocean (Jorgensen and D'Hondt, 2006). Results obtained so far reveals microbial life to be abundant both in deep sub-sea floor sediments and in the basement crust under the sediments (Wellsbury, et al., 1997). Initially microbiological research and such related studies were rare, however final DSDP expeditions of the Glomar Challenger (Legs 95 and 96) based on radiotracer experiments documented microbial activities in samples taken from depths as great as 167 meters below the seafloor (Tarafa et al., 1987). ODP which initally started with determination of cell counts and activity profiles in subseafloor sediments of Peru Margin (Cragg et al., 1990) brought a considerable momentum to the

exploration of subseafloor life and peaked with the first scientific drilling expedition of ODP Leg 201 (D'Hondt et al., 2003). The focus of the first drilling expedition launched by Ocean Drilling Program (ODP, Leg 201) was the exploration of deep sea (D'Hondt et al., 2004). Eastern tropical Pacific with sites ranging from the continental shelf to ocean depths of 5000 m was under investigation by drilling through the seafloor and down to the basaltic crust allowed the sampling of sediments with ages up to 35 million years (Jorgensen et al., 2006).

Recent advancement in the study of subseafloor is much faster during the successor of ODP, the Integrated Ocean Drilling Program (IODP), whose one of the three principal themes of the Initial Science Plan is the study of "Deep Biosphere and the Subseafloor Ocean" (Smith and D 'Hondt, 2006).

2.2.5. Environmental Impacts on the Deep-Sea Floor Ecosystems

Deep-sea floor ecosystem being one of the largest on the planet is under several human forcing and major natural environmental factors, some of which may be estimated as analogous to human forcing factors. Low productivity, low physical energy, low biomass and the vastness of the deep-sea increase the potential sensitivity to human impacts. Besides, high species diversity in the deep sea, in terms of number of species per sample, again makes the habitat more likely to be sensitive to human impacts (Glover and Smith, 2003). The large habitats of the deep sea may make the fauna more resistant to extinctions caused by local processes, with a potential for recolonization from widespread source populations whereas these large, continuous habitats may also allows the transportation of stressors, such as disease agents or radioactive contaminants over vast distances. Contaminants such as radioactive wastes could potentially move through deep-sea food web, through wide-ranging pelagic species and impact very large areas. Thus the unusual characteristics of the deep-sea ecosystems set forth conservation challenges different from shallow-water ecosystems (Glover and Smith, 2003).

The major human treats to the deep sea are the disposal of wastes (structures, radioactive wastes, munitions and carbon dioxide), deep-sea fishing, oil and gas extraction, marine mineral extraction and climate change. As represented in the study of Glover and

Smith, (2003) the past human forcing factors include dumping of oil/ gas structures, radioactive waste disposal, lost nuclear reactors and dumping of munitions in order of importance with a temporal scale of activity of minimum ~30 years and the present impacts include deep-sea fisheries, collateral damage by trawling, both of which have high regional effects, deep-sea oil and gas drilling, dumping of bycatch causing food falls, research and bioprospecting at vents and underwater noise. It is estimated for such examples of large ship wrecks or deep seabed mining that the impacts last > 100 years, consequently the time scale of deep-sea impact typically extends far beyond the time scales of activity due to low biological and chemical rates (Glover and Smith, 2003).

The major natural environmental forcing factors on the deep-sea floor include food input such as organic carbon flux which has a major impact on the abundance and diversity of benthos on a seasonal or interannual regional scale, whale-falls with the latter mentioned have an impact between 1-100 years on local scale (Smith and Baco, 2003) and the changes in the surface water has an interannual or decadal regional impact on the diversity and abundance of benthos (Smith et al., 1997). Other natural environmental forcing factors are biogenic disturbance and hydrodynamics and chemical emissions. Benthic storms with a temporal time scale of days and turbidity currents with a time scale between 1000-100 000 years both have a major impact on benthos' smothering and diversity and turbidity currents inhibit the settlement as well. Methane hydrate release is one of the chemical emissions whose major impacts on benthos are unknown (Glover and Smith, 2003), but has also a localized emission effect in terms of spatial scale, whereas another chemical emission CO_2 release lowers the pH and causes toxicity on a temporal scale of decades (Sakai et al., 1990). Hydrogen sulphide and trace metals from vents have an impact of toxicity on benthos and are an energy source for microbes on a temporal scale of decades (Van Dover, 2000).

2.3. Microbial Ecology of Marine Sediments

2.3.1. The Importance of Microorganisms

Prokaryotes are an essential part of the earth's biota for they catalyze unique and indispensable transformations in the biogeochemical cycles of the biosphere, produce important components of the atmosphere, and exhibit a large portion of life's genetic diversity (Whitman et al., 1998).

Results obtained through the Deep Sea Drilling Project and Ocean Drilling Program (ODP) have revealed that the activities of subsurface prokaryotes have profound implications for the global carbon cycle (Sorensen and Teske, 2006). One critical impact of microbes on the geochemical cycles is that microorganisms inhabiting anoxic marine sediments are significant in the consumption of more than 80% of the methane produced in the world's oceans (Orphan et al., 2001). Due to the amount of essential nutrients present in prokaryotes, they represent the largest living reservoir of C, N, and P on earth (Whitman et al., 1998). Microbial communities in marine sediments are responsible for various important biochemical functions, including the degradation of pollutants, transformation and mineralization of organic matter, one of which is the most important in freshwater sediments (Urakawa et al., 2000; Schwarz et al., 2007). Sediment bacteria also play a significant ecological and biogeochemical role in marine ecosystems (Polymenakou et al., 2005) for they are instrumental in the marine food web, where they are the key players for recycling of nutrients and degradation of pollutants. This is largely a result of their high abundance relative to the overlaying water column and their key function in mediating and regulating the transformation and speciation of major bioactive elements (e.g. carbon, nitrogen, phosphorus, oxygen, and sulphur) in these environments (Polymenakou et al., 2005). Sediment bacteria also represent a major genetic variability with a local diversity equal to soil systems (Torsvik et al., 2002).

2.3.2. Important Properties of Microbes

Since microorganisms have various important properties as mentioned above and may influence the maintenance of environment, amendment of polluted sites and have the potential to serve as cleaner energy resources, microbial and intraterrestrial life is the center of interest for general social, professional and industrial motives. First, the unknown diversity hides novel metabolisms (e.g. the recently discovered photoheterotrophy discovered in the sea) that force a re-evaluation of carbon and energy fluxes in the oceans (Fenchel, 2001; Karl, 2002), and it requires to be understood in order to be able to construct precise models of global change. Investigating the ecology of Archaea and bacteria is vital to understanding the functioning of global biogeochemical cycles. Thus the recognition of particular microbial groups that prevail under distinctive subseafloor environment is a significant step toward determining the role these communities play in Earth's essential biogeochemical processes (Inagaki et al., 2006). Secondly, the unknown microorganisms are the largest potential reservoir of useful genes for medicine and biotechnology and draw the attention of microbiologists for either their metabolisms or industrially interesting genes (Pedros-Alio et al., 2006). At last but not least, knowledge of microbial diversity will provide essential information to understand evolution and create a catalogue of microbial diversity. As it was estimated that up to 90% of all microbial cells on earth occurred in "deep biosphere" environments, interest in microbial communities inhabiting deep subseafloor sediments has increased rapidly (Leloup et al., 2007).

Recent observations have been made for a diversity of microorganisms which perform complete and unassisted biodegradation of certain anthropogenic contaminants in the subsurface for they are capable of carrying out almost any thermodynamically favorable reaction (Krumholz et al., 2000). There are also expectations to aid in predicting the fate of contaminants in different subsurface systems and to aid in developing procedures designed to stimulate the activity of endogenous microbiota for bioremediation purposes (Krumholz et al., 2000). Both the possible negative (e.g. through corrosion and well souring) and positive (e.g. through surfactant production) effects of microbial activity on oil extraction in oil wells, draws the attention of oil industry to deep oil reservoir microbiology (Pedersen, 2000). There is a widespread interest, that has been triggered as a result of contamination of groundwater from surface and underground disposal sites,

accidental spills, leakage and other human activities, in the possibilities of restoring contaminated underground sites with the help of autochthonous and/or allochthonous microorganisms (Heath, 1999). Disposal of radioactive wastes and heavy metals in deep geological formations requires in-depth knowledge about the host rock environment and the effects of microbes in future repositories (Pedersen, 1997). Moreover, microbes living deep below the deposits are supposed to produce most of the methane (Waseda, 1998) found in methane gas hydrates that are enormous reservoirs of energy, possibly twice the amount of energy contained in known oil and gas reservoirs (Kvenvolden, 1995). Another reason for the increased interest in subsurface life lies in the interest to find the origins of life. An increasing number of scientists propose an under-ground origin of life, possibly in the vicinity of hydrothermal-vents that suggests life on other planets should be searched for underground rather than on the surface (Pedersen, 2000). There is a publication of work proposing the hypothesis that extraterrestrial life existed within a Martian meteorite (Mckay et al., 1996).

2.3.3. Distribution and Abundance of Prokaryotes

Prokaryotes are an essential and abundant component of the earth's biota (Whitman et al., 1998). There were several studies on indirect estimates of prokaryote abundance. Since prokaryotes are highly ubiquitous, estimating the number of prokaryotes on earth requires analysis on numerous habitats. Thus figures for total number and total carbon of prokaryote estimates were based on the analysis from several representative habitats as follows; seawater, soil and sediment/soil subsurface that most of the prokaryotes reside in (Whitman et al., 1998), because the numerical contribution of prokaryotes in many other habitats to the total number of prokaryotes is still small, although such habitats contain dense populations (Whitman et al., 1998). Habitats other than subseafloor are of interest in their own and such habitats associated with prokaryotes include animals (birds, mammals, insects, gastrointestinal tracts of animals), leaves and air (Whitman et al., 1998). Diversity of microorganisms is highly controversial and even the right order of magnitude is unknown (Pedros-Alio, 2006). The known diversity of approximately 6000 species of prokaryotes and 100 000 species of protists have been previously described (Margulis et al., 1990).

A significant microbial biomass exists buried deep within the marine sediments that cover more than two thirds of Earth's surface where microbial life is widespread (Parkes et al., 1994; Whitman et al., 1998). The subsurface biomass of prokaryotes is proposed to be enormous based on circumstantial evidence (Gold, 1992). Studies of ODP cores have identified abundant prokaryotes in deeply buried oceanic sediments during the past 15 years (Parkes et al., 2000). Microorganisms have been proved to exist by intact cells and intact membrane lipids and have been recovered at depths as great as 800 m below the seafloor (D'Hondt et al., 2004). The number and mass of prokaryotes in subseafloor sediments have been estimated by extrapolation from direct counts of sedimentary organisms at a small number of ODP sites (Parkes et al., 1994; Whitman et al., 1998). ODP estimates of the biomass in subseafloor core sediments were more than 10^5 microbial cells/cm^3 even at a depth close to 1.000 m below seafloor (Parkes et al., 1994). Values ranging in between 10^3 to 10^8 per ml groundwater, formation water from petroleum deposits (Whitman et al., 1998), or g sediment are commonly reported for the total number of intraterrestrial microorganisms depending on the site studied (Pedersen, 1993). In summary, the number of prokaryotes is very large and on the basis of the exploration of prokaryotes, this "unseen majority" of microorganisms constitute one-tenth to one-third of Earth's biomass and accounts about 30% of the total living biomass (Parkes et al., 1994; Whitman et al., 1998).

2.3.4. Distribution of Archaea and Bacteria in Marine Sediments

Although around 70% of the Earth's surface is marine, little is known about the microbiology of underlying sediments (Parkes et al., 1994). During the past 15 years, studies using the small subunit rRNA (SSU rDNA) encoding gene sequences as a molecular tool have revealed a wealth of new marine microorganisms that belong to the three realms of life (Pedros-Alio et al., 2006): Bacteria, Archaea and Eukarya as shown in Figure 2.1.

Bacteria and Archaea in sub-seafloor sediments make up about 70% of the global number of prokaryotes (Whitman et al., 1998), however these haven't been extensively studied. Today, marine coastal sediments are known to contain a rich diversity of microorganisms from different physiological and phylogenetic groups (Musat et al., 2006).

Recent molecular analysis show that microbial communities of deep marine sediments harbor members of distinct, uncultured bacterial and archaeal lineages, in addition to Gram-positive bacteria and Proteobacteria that are detected by cultivation surveys (Teske, 2006). On the basis of 16S rRNA sequences, 52 phylum-level bacterial and 20 phylum level archaeal phylogenetic lineages, most of them with no or very few cultured representatives were listed up (Teske, 2006).

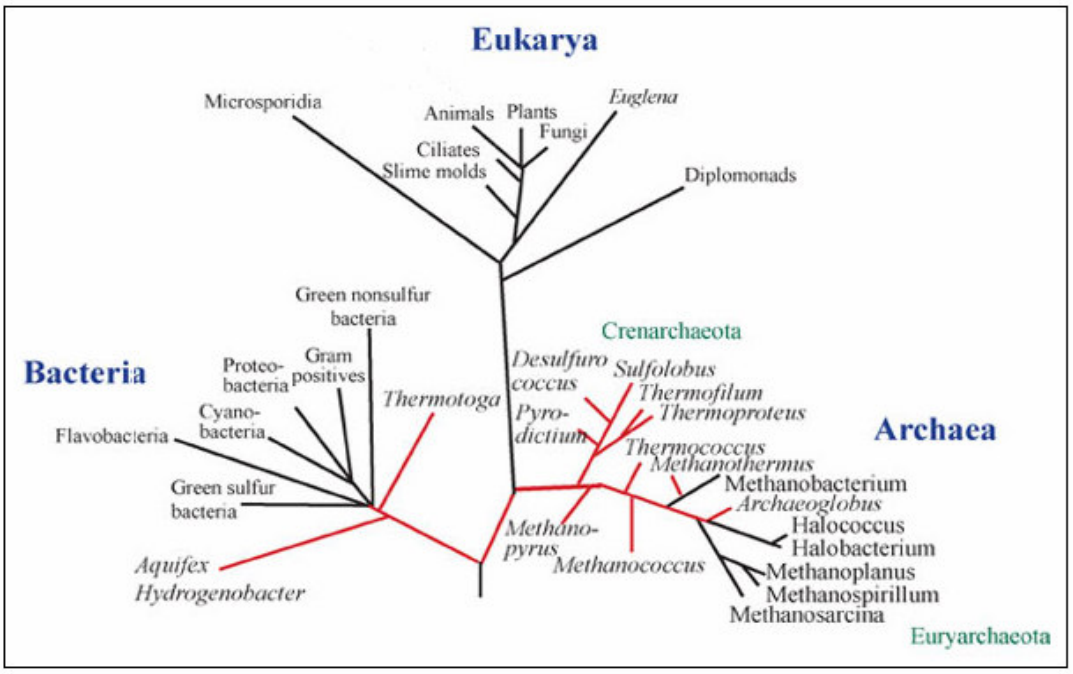

Figure 2.1. Universal phylogenetic tree (http://www.oceanexplorer.noaa.gov)

2.3.4.1. Bacteria. At least 17 major lineages of Bacteria are known from the study of laboratory cultures, any many others have been identified from retrieval and sequencing of ribosomal RNA genes from Bacteria in natural habitats (Madigan et al., 2003). Major phyla of Bacteria include gram positive bacteria, the cyanobacteria, and the Proteobacteria each of which is a large group containing many genera and are Bacteria about which much phenotypic information is known. However, the largest group of Bacteria being physiologically the most diverse of all is the phylum Proteobacteria. The Proteobacteria contains five clusters containing several genera each, designated as alpha, beta, gamma, delta, and epsilon (Madigan et al., 2003). Physiologically, Proteobacteria can be either phototrophic, chemolithotrophic, or chemoorganotrophic. The energy-generating

mechanisms of representatives of this group are greatly diverse. The newly discovered groups within the domain bacteria, most with no cultivated representatives, demonstrate that the microbial species in our culture collections provide only an incomplete picture of extant microbial diversity (Delong and Pace, 2001).

Recent estimates of global bacterial biomass indicate that a large fraction of bacterial biomass is present in the deep subsurface, most of which is in the marine deep subsurface (Whitman et al., 1998; Parkes et al., 2000). Cultivation surveys and most probable number quantifications have revealed the presence of fermentative, nitrate-reducing and sulphate reducing bacteria as well as methanogenic archaea (Cragg et al., 1996). Almost all cultured bacteria fall into well-known bacterial genera with many cultured species and strains from near-surface habitats. Many 16S rRNA gene sequences retrieved from subseafloor sediments belong to previously unidentified and uncultured groups of organisms, some of which have no clear phylogenetic affiliation (Kormas et al., 2003). Most of the genetic types are currently classified under provisional names such as "Japan Sea I Candidate Group" (bacteria) (Jorgensen and D'Hondt, 2006).

Recent observations report novel sequences representing similar taxa in near-surface, subsurface, and gas-hydrate-bearing sediments from deep marine sites such as the Guaymas Basin (Teske et al., 2002; Dhillon et al., 2003), the Japan Trench (Li et al., 1999a), the Nankai Through and forearc basin (Kormas, et al., 2003), and the Gulf of Mexico (Lanoil et al., 2001).

Most of the pure culture isolations and characterizations from deep marine sediments have focused on sulphate-reducing bacteria from Japan-Sea (Parkes et al., 1995) and the Cascadia Margin (Barnes et al., 1998). Moreover, diverse heterotrophic proteobacteria, Gram-positive bacteria and members of the Cytophaga-Flavobacterium-Bacteriodes (CFG) phylum have also been enriched from deep sediments of an ODP site, the Nankai Through (Toffin and Prieur, 2002). Some of the novel sequences fall into a single group which has been variously named, as flows: JAP504 cluster (Rochelle et al., 1994), OP9 associated (Teske et al., 2002), methane- and hydrocarbon-rich sediment group (Dhillon et al., 2003), hydrocarbon associated (Kormas et al., 2003), and the deep sediment group (Glover and Smith, 2003).

Among the bacteria, the recently identified candidate division JS-1 (Webster et al., 2004) and the Chloroflexi division (currently divided into four subphyla) (Hugenholtz et al., 1998) are well-represented in 16S rDNA clone libraries, at ODP Leg 201 sites and other subsurface locations (Teske, 2006).The divisions JS-1 and Chloroflexi are consistently found in diverse subsurface environments. The JS-1 candidate division was detected in marine sediments worldwide, in deep subsurface sediments as well as in coastal surficial sediments (Webster et al., 2004).

A study of microbial community composition of coastal marine sandy sediments over depth and over different seasons revealed high abundance of Planctomycetes, Bacteroidetes, Alphaproteobacteria (mainly in the upper layers of sediments), Gammaproteobacteria and of deltaproteobacterial sulphate reducers of the Desulfosarcina/ Desulfococcux group (Musat et al., 2006). Planctomycetes is known to be an abundant group in marine sediments, inhabiting the water column (Musat et al., 2006) and the sediment. The members of this group are present in the upper layers and in the anoxic regions (Ravenschlag et al., 2001). In other depth-related studies of microbial population changes in marine sediments, major phylogenetic groups of domain Bacteria: γ- and δ-Proteobacteria, high-GC Gram positive bacteria (Actinobacteria) and green non-sulphur bacteria and a candidate division of OP8, which was proposed by Hugenholtz et al. (1998), were recovered from sequenced clones (Musat et al., 2006). Moreover, α-subclass Proteobacteria in sediments is also predominant in marine sediments for they have been described as one of the predominating groups in marine plankton (Gonzalez and Moran, 1997). Cytophoga-Flavobacterium cluster was found to be the most predominant phylogenetic group in Wadden Sea sediments, followed by sulphate-reducing members of the δ subclass of Proteobacteria, although their members had not been found in marine sediments by either molecular methods (Gray and Herwig, 1996; Rochelle et al., 1994) or culture-based analysis (Delille, 1995) until then. Another analysis of 16S rDNA libraries for investigation of genetic diversity of microbial communities in marine sediments showed the presence of five major lineages of the Domain Bacteria: the gamma, delta and epsilon Protobacteria, high-GC Gram positive bacteria being the most dominant (Urakawa et al., 1999) and the division *Verrucomicrobia* (Hedlund et al., 1997) in Sagami Bay and Tokyo Bay, Japan. Gray and Herwig (1996) also reported the dominance of the gamma Proteobacteria and Gram-positive bacteria in a marine sediment sample, however alpha

Proteobacteria is also found to be dominant or nearly dominant in various soil (Zhou et al., 1997) and seawater samples (Fuhrman and Davis, 1997). Study of microbial communities associated with geological horizons of a sediment sample revealed the dominance of gamma subclass of the Proteobacteria, members of the candidate division OP9 and green nonsulfur bacteria. Research of D'Hondt and his colleagues (D'Hondt et al., 2004) reported the distribution of cultured members of γ-Proteobacteria to be consistently found at ocean-margin sites, whereas to be rare at open-ocean sites. However, Actinobacteria were most consistently found in sulphate-reducing sediments of the open-ocean sites and in the relevant ocean-margin site. One isolate from a site under investigation of D'Hondt and his colleagues (D'Hondt et al., 2004), along with most of the isolates related to known marine organisms and the recent discovery of deeply rooted but previously unknown archaeal 16S gene sequences in subseafloor sediments, demonstrated the existence of previously undiscovered prokaryotes in deep subseafloor sediments of the open ocean sites. Firmicutes that are most closely related to *Bacillus firmus* and α- Proteobacteria that are most closely related to *Rhizobium radiobacter*, are the most commonly cultured taxa (D'Hondt et al., 2004). Recent study of Schwarz and his colleagues showed that community structure of Archaea and Bacteria in an anoxic lake sediment was dominated by Deltaproteobacteria, sulphate-reducers, syntrophs in particular, and Bacteroidetes-Chlorobi group as the second most dominant in the relevant study site (Schwarz et al., 2007).

2.3.4.2. Archaea. The Archaea are divided into two kingdoms: the Euryarchaeota and the Crenarchaeota. A third phylum, the Korarchaeota branches of close to the root of archaeal phylogenetic tree and is a group not yet officially recognized in taxonomy. The Archaea were considered to be confined to specialized environments, including high temperature, high salinity, and extremes of pH and in strictly anaerobic niches that permit methanogenesis. However, several studies based on the comparison of 16S RRNA genes have radically changed our view of Archaea, revealing the ubiquitous character of these microorganisms, which also thrive in aquatic and terrestrial temperate environments (Vetriani et al., 1999). Culture independent surveys revealed widespread diversity of archaea in many other "non-extreme" habitats marine plankton (DeLong, 1998), forest and agricultural soils, deep subsurface environments, freshwater lake sediments, deep sea sediments, and in association with certain metazoan species (Delong, 1998).

The Euryarchaeota was traditionally considered the more physiologically diverse group, many of which like Crenarchaeotes, inhabit extreme environments of one sort or the other (Vetriani et al., 1999; Madigan et al., 2003). However, recent findings have revealed that this group is not just limited to extreme environments but also found associated with moderate environments such as the digestive tracts of marine fishes as well (van der Maarel et al., 1998). New lineages of the Euryarchaeota were also found among marine picoplankton (DeLong et al., 1994; Fuhrman and Davis, 1997), in salt marsh sediments (Munson et al., 1997), in continental shelf anoxic sediments (Vetriani et al., 1998) and in hydrothermal vent microbial mats (Moyer et al., 1998). Detailed studies on the distribution of planktonic Euryarchaeota illustrated that they were most abundant in surface waters (Massana et al., 1998). This kingdom includes the methanogens, extreme halophiles, some of the thermophiles, hyperthermophiles and finally a large group of yet uncultured marine euryarchaeotes. Methanogens within the Archaea are obligate anaerobes inhabiting strictly anaerobic niches, although they are related to several genera of extreme halophiles that are for the most part obligate aerobes. Their metabolism is linked to the production of methane (CH_4). Inspite of being a metabolically restricted group, methanogens are very cosmopolite exhibiting extreme habitat diversity. Species were isolated virtually from every habitat in which anaerobic biodegradation of organic compounds occurs, including fresh water sediments, digestive and intestinal tracts of animals, anaerobic waste digesters (Jones et al., 1983; Whitman, 1985), as well as extreme environments such as geothermal springs and both shallow and deep-sea hydrothermal vents (Iluber et al., 1982; Jones et al., 1983). Cultured methanogens are currently classified into five orders, 21 genera and more than 62 species (Aravalli et al., 1998). Extreme halophiles are limited to highly saline, land-locked water bodies and some of the thermophiles are usually found in close proximity to terrestrial and shallow-water hot springs and at deepsea hydrothermal vents.

Crenarchaeota were previously thought to include a group of organisms characterized by an extremely thermophilic, sulphur-metabolizing group and contain mostly hyperthermophilic species including those able to grow at the highest temperatures of all known organisms (Vetriani et al., 1999; Madigan et al., 2003). However even cold-dwelling relatives of hyperthermophilic crenarchaeotes have also been identified. A crenarchaeote inhabiting the tissues of a temperate water marine sponge was identified, demonstrating at temperatures of 10°C growth of this organism (Preston et al., 1996).

Moreover, Crenarchaeal phylotypes were found among marine picoplankton (Fuhrman et al., 1992; DeLong, 1994), in the gut of a deep-sea holothurian (McInerney et al., 1995), in fresh water sediments (Hershberger et al., 1996; MacGregor et al., 1997; Schleper et al., 1997), in soil (Bintrim et al., 1997; Jurgens et al., 1997; Buckley et al., 1998), in deep-subsurface sediments (Chandler et al., 1998), in continental shelf anoxic sediments (Vetriani et al., 1998), and in moderate temperature (15 to 30 °C) hydrothermal vent microbial mats. Planktonic Crenarchaeota were found to dominate at depth. Many hyperthermophiles are chemolithotrophic autotrophs; and because their habitats are devoid of photosynthetic life, these organisms are thus the only primary producers in these harsh organisms (Madigan et al., 2003)

The possible existence of a third kingdom, the *Korarchaeota*, was raised with the identification of two uncultured thermophilic organisms (Aravalli et al., 1998) whose 16S rRNA sequences were retrieved from an unusual Yellowstone hot spring (Vetriani et al., 1999; Madigan et al., 2003). The Korarchaeota was proposed to describe a group of as-yet-uncultivated organisms (Vetriani et al., 1999).

Wide distribution of Archaea in oxic and anoxic marine sediments and in the water column was detected by using lipids as biological markers (Hahn and Haug, 1986; Hoefs et al., 1997; DeLong et al., 1998). Molecular surveys have shown that archaeal subsurface communities consist of novel, phylum-level lineages that are unrelated to cultured archaea, such as methanogens and are major components of deep subsurface microbial communities worldwide. Recent surveys of archaeal 16S genes in subseafloor sediments suggest that some archaeal lineages [the Deep-Sea Archaeal Group and the Benthic Marine Group A] are cosmopolitan members of subseafloor sedimentary communities (Lauer and Tekse, 2004). Members of the Marine Benthic Group B archaea (MBG-B) (Vetrani et al.,1999) are similarly well represented in clone libraries of archaeal 16S rRNA genes from diverse sampling sites and sediment types, thus show a cosmopolitan occurrence pattern in a wide spectrum of marine sediments, surficial as well as subsurface, and in hydrothermal vents (Teske, 2006). MBG-B archaea were originally found in surficial deep-sea sediments (Vetrani et al., 1999) and at hydrothermal vents (Takai and Horikoshi, 1999; Teske et al., 2002) before appearing as a dominant clone group in cold subsurface sediments and in surface sediments at methane seeps (Knittel et al., 2005). Archaeal groups other than

MBG-B, specifically members of the Miscellaneous Crenarchaeotal Group (MCG) and of the South African Goldmine Euryarchaeotal Group (SAGMEG) archaea appeared to be dominating some ODP sites on the Peru Margin (Takai et al., 2001; Inagaki et al., 2006). Members of these lineages have been found in Leg 201 sediments, but also in the deep terrestrial subsurface, such as South African Goldmines (Takai et al., 2001). The second major archaeal lineage that is frequently found in subsurface sediments are the Marine Group I (MG-I) archaea whose members were originally identified by sequencing of environmental reran genes from seawater (Fuhrman et al., 1992). MG-I archaea constitute the majority of prokaryotic picoplankton in the deep-sea water column below 3000 m depth (Karner et al., 2001). In contrast to the previously mentioned two groups of archaea, archaea of the Marine Benthic groups A and D have been detected in fewer samples and sites, and do not usually dominate deep subsurface clone libraries (Teske, 2006). Originally they have been found in 16S rDNA surveys of push cores retrieved from surficial sediments (upper 30 cm) of the Atlantic continental slope and abyssal plain of off-shore New England (Vetrani et al., 1999). Clones of these groups also occur in deep-subsurface sediments of ODP Leg 201 sites (Teske, 2006). Terrestrial Miscellaneous Euryarchaeotal group (TMEG) archaea also have a mixed habitat range; they occur in a wide range of terrestrial and freshwater environments, as well as marine subsurface sediments (Takai et al., 2001).

2.3.5. Microbial Ecology Studies in Marine Sediments

Identification of dominant members of microbial communities and determination of their different roles are the key questions in microbial ecology (Purdy et al., 2003). Studying the genetic diversity and analyzing the members of mixed microbial populations are two of the most important steps in microbial community studies (Urakawa et al., 1999). Fundamental aspects of the vast subseafloor ecosystem are poorly known. The phylogenetic composition of subseafloor prokaryotic communities, their functional genes and the metabolic activities that allow prokaryotes to grow and survive in the subsurface are the main questions to be answered (Teske, 2006).

Many studies were conducted to understand how microorganisms are distributed in the environment and how they influence geochemical processes (Leloup et al., 2007;

D'Hondt et al., 2004). Numerous studies in the recent past have focused on the identification and community diversity of microorganisms based on 16S rDNA analysis of naturally occurring microbial communities (Hugenholtz and Pace, 1996). Previous molecular studies focused on the general microbial community structure and the abundance and depth distribution of specific functional groups such as polymer degrading, sulphur-oxidizing or sulphate reducing bacteria (SRB), because the processes they catalyze can be measured directly (Sahm et al., 1999). Among these studies, ODP Leg 201 being the first ODP expedition dedicated to the study deep beneath the seafloor (D'Hondt et al., 2003) combined geochemical analyses, cell counts, cultivations and molecular screening of subsurface microbial communities with the objective of a census of subsurface microbial life (diversity, density, activity) in the context of geochemical controls that shape microbial community composition and activity (Teske, 2006).

The competition between specific groups of sulphate–reducing bacteria (SRB) and methane-producing archaea for common substrates such as acetate and hydrogen has been investigated repeatedly (Schwarz et al., 2007; Lovley and Klug, 1983), and the community structure of these groups in fresh water sediments has frequently been studied (Schwarz et al., 2007; Alm and Stahl, 2000; Glissmann et al., 2004; Go et al., 2000; Koizumi et al., 2003; Zepp-Falz et al., 1999). There are also a few studies that have analyzed sulfate-reducing microbial community, and have used dsrAB, genes encoding the dissimilatory (bi)sulfite reductase, as functional marker instead of 16S rRNA genes (Leloup et al., 2007; Baker et al., 2003; Dhillon et al., 2003; Nercessian et al., 2005). There are several studies on tidal flats that mostly focused on bacterial communities (Kim et al., 2004; Llobet-Brossa et al., 2002).

Limited information about the diversity of archaea and bacteria is also derived based on concentration profiles of biologically relevant porewater constituents (Parkes et al., 2000; D'Hondt et al., 2002), direct rate measurements of microbial processes (Cragg et al., 1992), and cultivations of subsurface bacteria and archaea (Parkes et al., 1995; Barnes et al., 1998) which have led to some insight into the metabolic activities and capabilities of deep marine subsurface microbial communities.

2.4. Major Anaerobic Processes in Marine Sediments

D'Hondt and colleagues (D'Hondt et al., 2004) now report evidence for metabolically diverse and active microbial communities buried deep within marine sediments nearly 0.5 km below seafloor (DeLong, 2004). Studies on samples collected during Deep Sea Drilling Project (DSDP) and Ocean Drilling Program (ODP) cruises have consistently demonstrated microbial activity in deep marine sediments millions of years after their initial deposition on the seafloor and several hundreds of meters below the sediment surface (Parkes et al., 1994, 2000; D'Hondt et al., 2002). In general , the depth-related gradient of physical and chemical properties provides niches for a wide variety of metabolically diverse microorganisms in marine sediments (Urakawa et al., 2000). In such environments, syntrophic and competitive interactions occur between different physiological types of microorganisms (Fenchel and Finlay, 1995).

2.4.1. Energy Sources of Marine Sediments

All actively growing organisms must keep their enzymatic machinery going above a critical level to maintain vital cell functions such as replacement of degraded enzymes, repair of DNA damaged by high-energy radiation from natural radionuclides and presumably, the maintenance of an electrochemical gradient across the cell membrane (Price and Sowers, 2004).

Although marine sediments harbor Earth's largest reactive carbon pool, the organic matter becomes increasingly unreactive with depth and age and would seem to be practically inaccessible for microorganisms several million years after its burial (Jorgensen and D'Hondt, 2006). However, circumstantial evidence suggests that this is not the case, and viability of subsurface prokaryotes is within the range observed for prokaryotes from surface sediments and soil (Hazen et al., 1991). Potential sources of food (electron donors) in deep subseafloor sediments include: (i) burial of organic matter from the surface photosynthetic world, (ii) cleaving of reductants (e.g., H_2) from water by radioactive bombardment from the surrounding mineral grains, (iii) burial of reduced minerals (e.g., minerals with reduced iron, manganese), and (iv) thermogenesis of reduced organic compounds (Smith and D'Hondt, 2006). In the anoxic subseafloor sediments that have

been studied to date, photosynthesized organic matter appears to be the principal food source (D'Hondt et al., 2004). Burial of reduced minerals is not likely to be a significant source of electron donors in anoxic deep marine sediments where SO_4^{2-} is the predominant electron acceptor, because SO_4^{2-} cannot be used to oxidize reduced metals. However, it could be a source of electron donors in very low-activity subseafloor environments where O_2 and NO_3^- diffuse down from the overlying ocean or up from the seawater that circulates through the underlying basement.

Extremely low energy flux per cell in the deep subsurface is a crucial problem, but molecular hydrogen (H_2) which is generated by chemical alterations in young basaltic crust along the mid-oceanic ridges is an additional energy source (Holm and Charlou, 2001). However, most of the seabed lies on old, crack-permeable crust, in which the potential oxidants for H_2 (such as oxygen and nitrate) seem to persist long enough to preclude a substantial H_2 supply (D'Hondt et al., 2004). Another possible source of H_2 may come from energy released by the decay of natural radionuclides of potassium, thorium, or uranium in the sediments dissociating the water molecules into free radicals and molecules such as H_2. Hence, such nuclear energy is not only destructive to microbial cells but may also support their metabolic activity. Water radiolysis is suggested to be the principal source of microbial energy in deep sea sediments that are much more depleted in organic matter (Jorgensen and D'Hondt, 2006) based on the estimates of Lin et al. (2005) on radiolytic H_2 production rates for a sedimentary basin. Smith and D'Hondt (2006) also suggest the radiolysis of (the breakup of chemicals into smaller components [e.g., water into H and O] by ionizing radiation) of water possibly to be a significant source of electron donors in the least active sub seafloor sediments, where very little organic carbon has been buried. This potential energy source is interesting since it does not require an external oxidant and water radiolysis produces not only H_2 but also oxidants such as H_2O_2 or O_2 which may be directly used for the energy-generating reoxidation of H_2 (Jorgensen and D'Hondt, 2006). There are examples of subsurface microbial communities that appear to be maintained by the action of lithotrophic bacteria growing on H_2 that is chemically generated within the subsurface (Krumholz, 2000). Lithoautotrophic processes may provide an additional source of energy for growth of subsurface prokaryotes (Whitman et al., 1998). Furthermore, there is evidence that most of the subsurface biomass is supported by organic matter deposited from the surface (Wellsbury et al., 1997). Energy sources for

the organisms living in the oceanic subsurface may originate as oceanic sedimentary deposits as well (Krumholz, 2000).

2.4.2. Diversity of Metabolic Activities in Deep Subsurface Sediments

Dissolved electron acceptors such as $SO_4^{2-}$and NO_3^- exhibit subsurface depletion, whereas dissolved metabolic products such as dissolved inorganic carbon, ammonia, sulphide, methane, manganese, and iron consistently exhibit concentration maxima deep in the drilled sediment columns, indicating the consumption and release of metabolites in the sediment column as a result of biologically catalyzed reactions (D'Hondt et al., 2004).

Sulfate reduction, methanogenesis and other activities have been detected in cores from the subsurface (Whitman et al., 1998). Prokaryotic activity, in the form of sulphate reduction and/or methanogenesis, occurs in sediments throughout the world's oceans (D'Hondt et al., 2002). SO_4^{2-} reduction, methanogenesis (CH_4 production), and fermentation are the principal degradative metabolic processes in subsurface (> 1.5 mbsf) marine sediments, for three reasons (D'Hondt et al., 2002): (i) Concentrations of dissolved SO_4^{2-} at the sediment-water interface are more than 50 times as great as concentrations of all electron acceptors with higher standard free energies combined (Pilson, 1998). (ii) External electron acceptors that yield more energy than SO_4^{2-} typically disappear within the first few centimeters to tens of meters sediment depth. (iii) Once all SO_4^{2-} has been reduced, methanogenesis and fermentation are the principal remaining avenues of metabolic activity (D'Hondt et al., 2002). Other microbial processes in deep subseafloor sediments include organic carbon oxidation, ammonification, methanotrophy and manganese reduction, iron reduction, and the production and consumption of formate, acetate, lactate, hydrogen, ethane, propane (D'Hondt et al., 2004)

Previously mentioned metabolic activities such as carbon oxidation, Fe and Mn reduction ultimately rely on electron acceptors from the photosynthetically oxidized surface world. O_2, NO_3^- and SO_4^{-2} ultimately enter sediments by diffusing down past the seafloor, and at the open ocean sites, by transport upward from seawater flowing through the underlying basalts. The oxidized Mn and Fe were originally introduced to the sediments by deposition of Mn and Fe at the seafloor (D'Hondt et al., 2004). Normally,

electron acceptors (oxidants such as oxygen, sulphate and nitrate) diffuse into the sediments from the overlying seawater and then consumed sequentially in a series of metabolic reactions which results in a predictable series of oxidant-depletion profile, with those yielding the greatest free energy being the first to be consumed, in which oxygen is reduced first, then nitrate, manganese, iron, sulphate and finally carbon dioxide (DeLong, 2004). However, D'Hondt et al. (2004) report that oxidants which normally diffuse downward from overlying seawater appear to have entered the sediments from subseafloor sources such as brines below sediment base generating sulfates and deep basaltic aquifers below the sediment base from where nitrate and oxygen enters as it's shown in Figure 2.2 (DeLong, 2004).

Those activities probably also rely on electron donors from the photosynthetically oxidized surface world (D'Hondt et al., 2004). The ultimate electron donors for subsurface ecosystems have been hypothesized to include buried organic matter from the surface world (Nealson, 1997), reduced minerals [such as Fe(II)-bearing silicates] (Bach and Edwards, 2003), and thermogenic CH_4 from deep within Earth (Gold, 1992). Thermogenesis may be a spectacular source of electron donors in some marine environments. However, it is not a significant source of electron donors in open-ocean sediments, where in situ temperatures are typically low (less than 30°C) and reduced compounds diffuse from the microbially active sediments into the basement below (D'Hondt et al., 2004).

Many of the reductive processes compete with each other for electron donors and have been assumed to competitively exclude each (Lovley and Chapelle, 1995). However, pore water chemical distributions (D'Hondt et al., 2002; D'Hondt et al., 2004) and radiotracer experiments (Parkes et al., 2005) demonstrate that at least some of these reductive processes consistently co-occur in deep subseafloor sediments (e.g., sulfate reduction and methanogenesis).

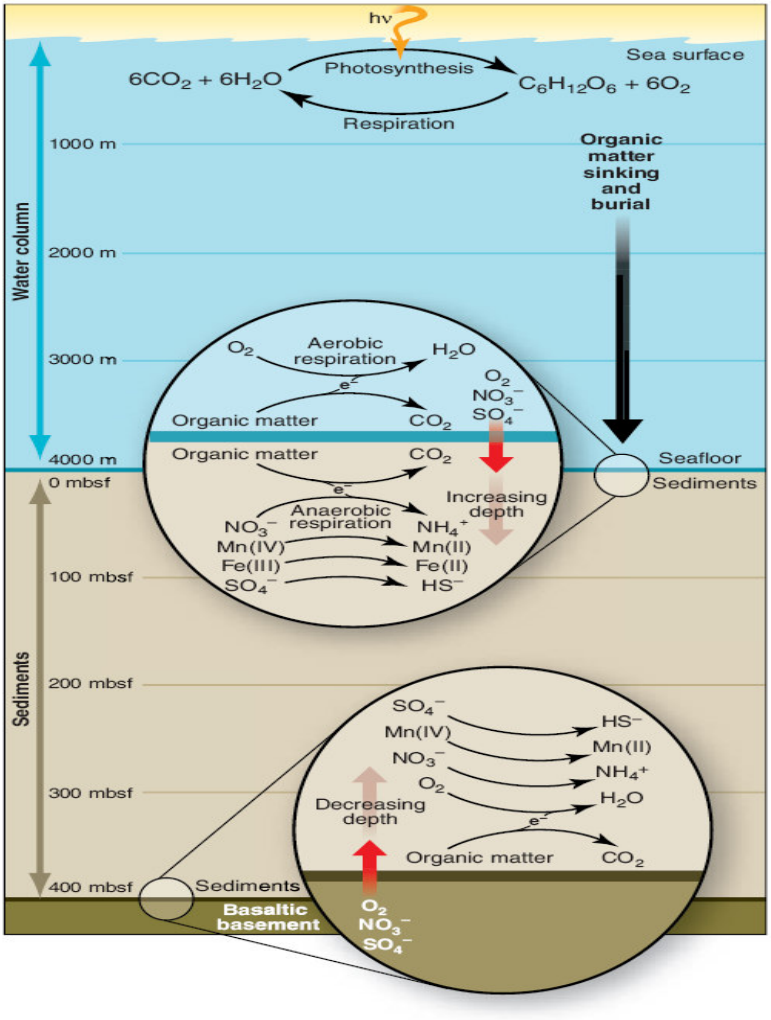

Figure 2.2. The ups and downs of organic matter (DeLong, 2004)

Radiotracer experiments demonstrate that potential rates of many microbial activities, such as sulfate reduction and methanogenesis, are often highest at very shallow depths in marine sediments (Parkes et al., 2000). However, rates of at least some activities, such as sulfate reduction, can exceed near-surface rates in deep subseafloor sediments where chemical transport brings electron donors and acceptors into contact at high rates (Smith and D'Hondt, 2006). Rates of activities over drilled sediment columns demonstrate that predominant activities and total rates of activities (as well as cell abundances) vary predictably from ocean margins to open-ocean anoxic sediments (D'Hondt et al., 2002; D'Hondt et al., 2004). Net redox activity is dominated by sulfate reduction in the anoxic sediments of ocean margins, where total activity and cell abundance are highest (D'Hondt

et al., 2004). In anoxic sediments of open-ocean sites, metal reduction and nitrate reduction become increasingly important as total activity and cell abundance decline. (Smith and D'Hondt, 2006).

2.4.2.1. Anaerobic Respiration. Aerobic oxidation of organic matter in marine sediments is generally accepted to be the most important form of respiration in most situations (Sorensen et al., 1979; Jorgensen and Revsbech, 1989). Aerobic respiration takes place in the oxic surface layer and is followed by nitrate reduction and sulfate reduction (Hansen and Blackburn, 1991). However, the oxic zone is only a few millimeters thick, and almost all very deep environments are anaerobic, with the exception of places where radioactivity may cause radiolysis of water, producing hydrogen and oxygen, thus anaerobic respiration becomes dominant. Furthermore, much of the oxygen uptake is used to reoxidate the products of anaerobic respiration (H_2S, NH_4^+, CH_4) at the oxic/anoxic interface (Jorgensen, 1983). The most common types of anaerobic respiration are given in the Figure 2.3.

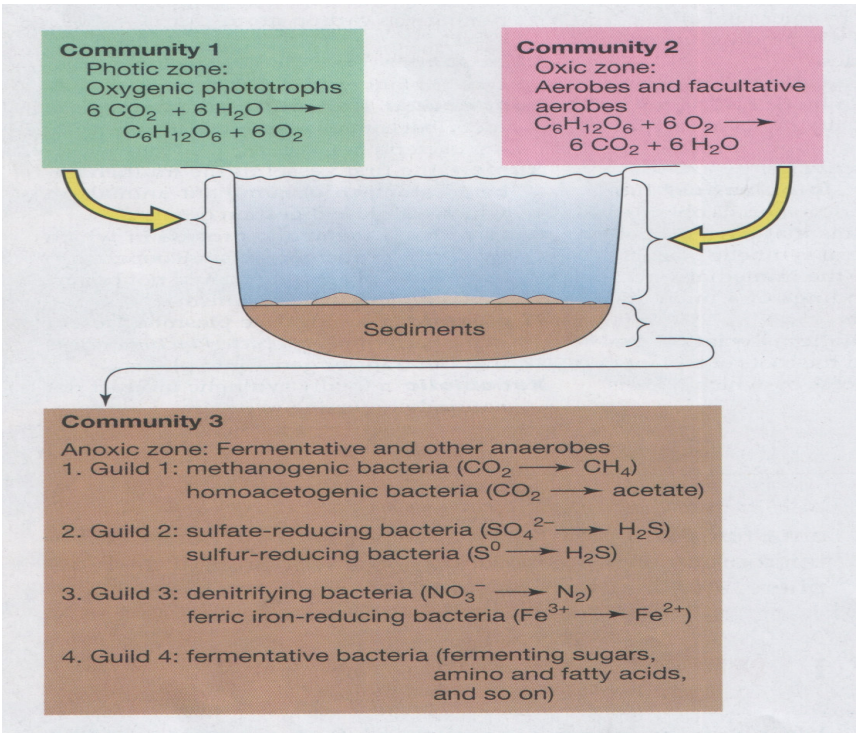

Figure 2.3. Populations, guilds and communities - an example of microbial community structure in a lake ecosystem (Madigan et al., 2003)

Anaerobic respiration is the process in which case a variety of other electron acceptors can be used instead of O_2. The energy released from the oxidation of an electron donor using O_2 as electron acceptor is higher than if some compound is oxidized with an alternate electron acceptor.

2.4.2.2. Anoxic Decomposition. Anaerobic digestion is a process of microbial decomposition in which, under anaerobic conditions, a community of microorganisms converts organic matter into methane, carbon dioxide, inorganic nutrients and humus. During this microbial process known as biomethnogenesis, microorganisms including protozoa, fungi and bacteria, decompose organic matter using carbondioxide and the methyl group of acetate as electron acceptors in the absence of dioxygen or other compounds. General scheme for anoxic decomposition is shown in Figure 2.4.

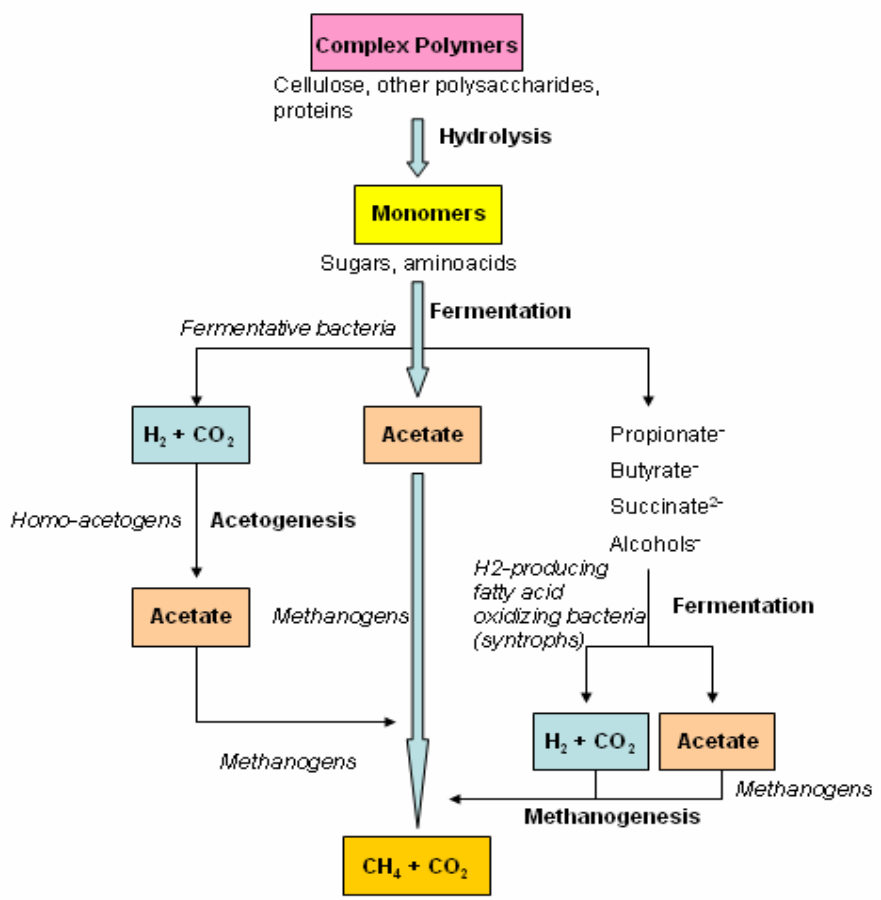

Figure 2.4. Overall process of anoxic decomposition (Madigan et al., 2003)

This microbial activity is responsible for carbon recycling in anaerobic environments, including wetlands, rice fields, intestines of animals, aquatic sediments and manure (Chouari et al., 2005). In the absence of methanogens to utilize these substrates, both hydrogen and acetic acid build up back-up for the reactions. Organic acids accumulate causing a decrease in pH, which ultimately inhibits and stops fermentation. The overall role of biomethanogenesis in the atmosphere is to complete the degradation process by removal of inhibitory fermentation products (Chouari et al., 2005). The process involves a consortium of different species of microorganisms, which decompose organic matter in a series of steps that ultimately produce methane and carbondioxide as terminal products (Chynoweth et al., 1991).

Hydrolysis: It's the decomposition of complex organic matter into simple soluble organic molecules using water and hydrolase enzymes (glucosidases, lipases, proteases, sulphatases, and phosphatases).

Fermentation: Because oxygen is not highly soluble (9.6 mg/l distilled water in equilibrium with air at 25 °C), many environments become anoxic. In such environments, decomposition of organic matter occurs anaerobically. If adequate supplies of electron acceptors like SO_4^{-2}, NO_3^-, Fe^{3+}, and the others are not available in such anoxic environments, much of the carbon will be catalyzed by fermentation. It's the anaerobic catabolism of an organic compound in which the compound serves as both an electron donor and an electron acceptor and in which ATP is produced by substrate-level phosphorlylation. The oxidation in fermentation is coupled to the reduction of a compound generated from the initial substrate; thus no externally supplied electron acceptor is involved. In terms of ATP synthesize mechanisms in fermentation, substrate-level phosphorlylation is the process in which ATP is synthesized during steps in the catabolism of an organic compound through the transfer of high energy phosphate bonds from organic intermediates of fermentation to ADP. (Madigan et al., 2003) Fermentations are either classified on the basis of products formed or substrate fermented.

Acetogenesis: CO_2 is common in nature and usually abundant in anoxic habitats because it's a major product of energy metabolism of chemoorganothrophs. There are two major groups of strictly anaerobic prokaryotes, homoacetogens and methanogens that can use

CO_2 as electron acceptor in energy metabolisms In addition to the major electron donor H_2, a variety of C1 compounds, alcohols, and certain nitrogen bases depending on the organism serve as electron donors for acetogenesis as well. Many homoacetogens can also reduce NO_3^- and $S_2O_3^{-2}$; however CO_2 reduction is probably the major reaction of ecological significance (Madigan et al., 2003).

Methanogenesis: It's the biological production of methane (CH_4) carried out by a group of strictly anaerobic Archaea called the methanogens. Research on methanogenesis has revealed that the biological production of methane occurs through a series of reactions involving novel coenzymes and amazing complexity. Methane is formed from two primary substrates, acetate and hydrogen/carbon dioxide (or formate).

Methane is produced both in sulfate-rich open-ocean province and in an ocean-margin province, but is abundant only in sulfate-depleted sediments and CH_4 concentrations below a few tens of mbsf along ocean margins are high because microbial activity is generally limited to fermentation and methanogenesis (D'Hondt et al., 2002). Generally, methanogenesis becomes the dominant terminal oxidation process when sulfate is depleted (Wilms et al., 2007). The methane flux to the atmosphere is influential in moderating the Earth's climate over a variety of timescales due to the strength of methane as a greenhouse gas (Coolen et al., 2004). Under anoxic conditions, methanogenesis is one of the most important processes in the mineralization of organic compounds and perhaps the most dominant microbial process in marine sediments, because of rapid depletion of other electron donors in seawater (D'Hondt et al., 2002). Global methane production in marine sediments is very significant at between 75 and 320 Tg / year (Valentine, 2002) and these sediments contain the largest global reservoir of methane. Methanogenesis in cold environments is an important source in the global methane budget (Franzmann et al., 1997). Low temperatures apparently inhibit hydrogenotrophic methanogenic archaea and shift sedimentary metabolism toward acetogenesis and acetoclastic methanogenesis (Schulz and Conrad, 1996; Nozhevnikova et al., 1997). Methanegesis accounts for almost up to 50% of organic matter degradation in fresh water sediments. Methane in cold seep fluids can have a biogenic origin resulting from the microbial degradation of organic matter as previously mentioned or a thermogenic origin resulting in the transformation of organic matter caused by high temperatures (Martens et al., 1991). Results from the study

of Inagaki et al. (2004b) indicate that methanogenesis occurs in close proximity with anaerobic methane oxidation and aerobic methane oxidation in the relevant sediments investigated.

2.4.2.3. Sulfate Reduction. Several inorganic sulfur compounds are important electron acceptors in anaerobic respiration. Sulfate, the most oxidized form of sulfur, is one of the major anions in seawater and is used by the sulfate-reducing bacteria. The end product of sulfate reduction is H_2S, an important natural product that participates in many biogeochemical processes. The ability to use sulfate as an electron acceptor for energy-generating processes, however involves a large scale reduction of $S0_4^{-2}$ and is restricted to sulfate-reducing bacteria. Nevertheless, sulfate is a much less favorable electron acceptor than either O_2 or NO_3^-. H_2, lactate and pyruvate are also some of the other electron donors used by a wide variety of sulfate-reducing bacteria. Sulfate-reducing bacteria are widespread in nature; however in many anoxic habitats, such as freshwaters and many soils, their activities are limited by the low levels of sulfate present. Populations of SRB are a major component of the microbial community of the upper layers of marine sediments. Sahm et al. (1999) found that 20% of the total prokaryotic rRNA in a coastal marine sediment originated from sulphate reducing bacteria. Because of the necessity for electron donors (or molecular hydrogen, which is a product of the fermentation of organic compounds) to derive sulfate reduction, sulfide production only occurs where significant amounts of organic material are presents. In many marine sediments, the rate of sulfate reduction is carbon limited, and the rate can be greatly increased by the addition of organic matter. This is important because disposal of sewage, sewage sludge, and garbage in the sea can lead to increases in marine sediments, leading to marine pollution. Since sulfide (HS^-) is a toxic substance to many organisms, formation of HS^- by sulfate reduction is potentially detrimental. Sulfide is commonly detoxified in the environment by combination with iron, forming the insoluble FeS which gives the black color of many sediments where sulfate reduction takes place (Madigan et al., 2003). Sulfate reducing prokaryotes (SRP) are metabolically highly diverse, and have been shown to be abundant in marine sediment and should dominate in the sulfate-containing upper sediment layers (Barnes et al., 1998). They share the capability to reduce sulfate, but may also reduce alternative electron acceptors such as nitrate, iron and manganese oxides, sulfur and even oxygen (Mußmann et al., 2005).

Under anoxic conditions, sulfate reduction is considered to be the most important process in organic matter mineralization (Lobet-Brossa et al., 2002). In particular, sulfate reduction are perhaps one of the two other dominant process, the other being methanogenesis, in marine sediments because of the overwhelming abundance of sulfate in seawater (D'Hondt et al., 2002). In the study of Reindard and his colleagues, tidal flats that are characterized by high production and sedimentation rates and intense reminalization, in contrast to open-ocean sites, step chemical gradients, with oxygen generally being depleted within a few millimeters below sediments surface, sulfate appears to be the most important one among a sequence of electron acceptors governing the microbial processes that are strictly anaerobic in layers beneath the oxic zones of 20 cm depth (Wilms et al., 2006). The dissimilatory sulfate reduction is regarded as the major anaerobic bacterial respiration process, accounting up to 50% of total carbon mineralization. The dissimilatory sulphate reduction was believed to be confined to anoxic environments (Postgate, 1984). However, repeated observations of high numbers of SRB and significant sulfate reduction rates in oxic zones are inconsistent with this assumption (Wieringa et al., 2000).The dissimilatory reduction of sulfate can be linked to the oxidation of substrates that are difficult to degrade under anoxic conditions, such as alkanes and aromatic compounds (Hansen, 1994), or even to the anaerobic oxidation of methane at sulfate-methane transition zones. In most sediments, the sulfate-depleted zone is located at a depth of tens of centimeters to several meters (Wilms et al., 2007).

2.4.2.4. Anaerobic Oxidation of Methane. The microbially mediated anaerobic oxidation of methane (AOM) is the major biological sink of the greenhouse gas methane in marine sediments and serves as an important control for emission of methane into the hydrosphere. The AOM metabolic process is assumed to be a reversal of methanogenesis coupled to the reduction of sulfate to sulfide (Knittel et al., 2005). In methane-rich environments a large part of the methane is oxidized aerobically by methanotrophic bacteria or anaerobically in marine sediments by a consortium of methane oxidizing archaea and sulfate reducing bacteria (Coolen et al., 2004). Microorganisms living in anoxic marine sediments consume more than 80% of the methane produced in the world's oceans (Orphan et al., 2001). Nearly all the methane moving upwards to the sediments surface is microbially oxidized, with anaerobic oxidation coupled to sulphate reduction (80%) playing the major role (Orphan et al., 2001).

Anaerobic oxidation of methane is widespread and geochemically well documented process, yet very little is known about the physiology, biochemistry and identity of microbes involved (Orphan et al., 2001). It's hypothesized that the use of hydrogen as an electron donor by the SRB results in low partial pressure of H_2, creating thermodynamically favorable conditions for methanogenic archaea to act as methane-oxidizers (Hoehler et al., 1994; Hoehler and Alperin, 1996). Most of our knowledge about the microbiology and controls on anaerobic oxidation of methane (AOM) has come from studies and experiments on seep and gas hydrate sediments (e.g. Hinrichs et al., 1999; Knittel et al., 2005; Niemann et al., 2005). According to these studies, a unique group of Archaea, ANME, mainly within the methanogenic Methanosarcinales, is responsible for AOM usually in association with SRB, which are often members of *Desulfosarcina-Desulfococcus* (DSS) group (Orphan et al., 2001; Knittel et al., 2005). Syntrophic partnership between methanogens and SRB in anaerobic methane oxidation or 'reverse methanogenesis' have been demonstrated both by laboratory and field experiments. (Hinrichs et al., 1999; D'Hondt et al., 2002). These consortia of ANME and sulfate reducers oxidize methane with sulfate, yielding equimolar amounts of carbonate and sulfide (Nauhaus et al., 2002). Recently, different groups of ANME (1a, 1b, 2a, 2b, 2c, 3a) have been detected which seem to dominate AOM in various locations such as a mat and a field in Hydrate Ridge, a microbial mat in Black Sea (Knittel et al., 2005) and a gas seep in North Sea (Niemann et al., 2005). Nevertheless there are much larger areas of non-seep marine sediments with a discreet sulfate-methane-transition-zone (SMTZ) associated with AOM that have been much less studied (Parkes et al., 2007).

2.5. Molecular Techniques Used in Ecology

2.5.1. The Need for Molecular Techniques

The development of molecular techniques using nucleic acids has led to many new findings in studies of microbial ecology (Amann et al., 1995). Microbial diversity studies were limited in the past by the lack of methodological tools, but the availability of the new molecular methods has made it possible to investigate the dynamics of the composition and structure of microbial populations and communities in defined environments, the phylogenetic relationships, and the impact of environmental or specific factors such as

pollution by xenobiotics on microbial diversity (Morris et al., 2002), the origin and conservation of microbial biodiversity (Dorigo et al., 2005), allows enumeration, to study distribution and activity of microorganisms and detection of individual microbial taxa in natural habitats.

The classical approach for identification of viable microorganisms in environmental samples, including sediments, is plate counting on agar medium (Edlund and Jansson, 2006). It would appear that only between 0.5 % and 10 % prokaryote diversity has actually been identified due to the small size and the absence of distinguishing phenotypic characters of prokaryotic organisms, and the fact that most of these organisms cannot be cultured which are the most important factors that limit the evaluation of prokaryotic biodiversity (Torsvik et al., 2002). There are many other short-comings and disadvantages of the past traditional microbiological techniques. Microscopy based and culture dependent techniques have only a limited use for classification and identification of microorganisms (Muyzer, 1999). Because many microorganisms are bound to sediment particles, they can not be detected by conventional microscopy. Unrecognized nutrient and growth conditions, the failure of selective enrichment cultures to mimic the environmental conditions required by particular microorganisms for proliferation in their natural habitat, interruption of intrinsic interdependencies such as syntrophic reactions and the low growth rates, fastidious nutritional and environmental requirements of anaerobes are the limits of culture dependent methods (Hofman-Bang et al., 2003). Almost 99% of all microorganisms in nature can not be isolated and classified based on physiological and biochemical features mainly due to the previously mentioned limitations of cultivation (Muyzer, 1999). Recently more direct methods, such as immunology techniques have been developed for identification, quantification, and localization of microorganisms in environmental samples (Hofman-Bang et al., 2003), yet still with disadvantages of the need for axenic cultures to produce specific antibodies, the high specificity limiting the detection to the species or subspecies level, and the occurrence of cross-reactions (Kemp et al., 1988).

2.5.2. An Overall Look at Molecular Techniques

The development of molecular biological techniques has allowed us to study microbial diversity at a different level, the genetic level (Muyzer, 1999). Molecular phylogeny which employs nucleic acid sequences to document the history of evolution, has provided a new basis for the direct identification and quantification of microorganisms (Olsen and Woese, 1993). Nucleic-acid based methods allow microbial community characterization without cultivation (Hofman-Bang et al., 2003). Microbes are grouped according to similarities in their genes, which also reflect their evolutionary relationship (Woose, 1987).

The most powerful and basic approach to explore microbial diversity and to clarifying microbial communities is cloning and sequencing of 16S ribosomal rRNA encoding genes. 16S rDNAs are amplified by PCR from nucleic acids extracted from environmental samples, and then the PCR products are cloned and sequenced (Urakawa et al., 1999). This approach can avoid the limitation of traditional culturing techniques for assessing the microbial diversity in natural environments (Urakawa et al., 1999) and has been applied to water columns (DeLong et al., 1994; Fuhrman et al., 1992, Fuhrman and Davis, 1997; Giovanni et al., 1990), hot springs (Barns et al., 1994; Hugenholtz et al., 1998), soils (Kuske et al., 1997), deep subsurface environments (Boivin-Jahns et al., 1995), hydrothermal vents (Moyer et al., 1995) and the gut of animals (McInerney et al., 1995).

Molecular techniques have greatly increased our knowledge of marine microbial diversity, in contrast to the several attempts that have been made to describe marine sediment microbial communities based on cultivation (Parkes et al., 1994; Delille, 1995). Earlier studies based on traditional cultivation methods could not reveal the appropriate sedimentary microbial diversity due to the high selectivity of these methods. Such cultivation based approaches were subject to restrictions and biases leading to a distorted representation of the true community composition (Amann et al., 1995). Techniques such as reassociation analysis of DNA (Torsvik et al., 1996), denaturing gradient gel electrophoresis (DGGE) (Teske et al., 1996), and restriction fragment length polymorphism (Moyer et al., 1994) have yielded insight into bacterial diversity and community composition. However, phylogenetically based oligonucleotide hybridization

techniques permit not only the monitoring of individual phylogenetic groups but also a quantification of their abundance in natural habitats (Amann et al., 1995). Quantitative slot blot hybridization technique has been used to study marine sediment microbial diversity as well (Moran et al., 1995). In situ hybridization with rRNA-targeted fluorescent oligonucleotide probes not only permits the identification and quantification of individual cells, but also demonstrates great power in analysis of bacterial community composition in several environments (Llobet-Brossa et al., 1998). In recent years, there have been molecular approaches for linking activity and function or activity with identity (Edlung and Jansson; 2006). For example, Mar-FISH is based on combination of uptake of radioactive substrates with fluorescent in situ hybridization (FISH) (Kindaichi et al., 2004), and stable isotope probing can be combined with molecular fingerprinting approaches to link microbial identity (biomarker) and activity (Radajewski et al., 2003). Another promising molecular approach that has recently been used for identification of growing cells in environmental samples is based on incorporation of the thymidine analogue bromodeoxyuridine (BrdU) into the DNA of cells during DNA replication (Artursson and Jansson, 2003). The DNA with incorporated BrdU can be selectively extracted by immunocapture and analyzed by molecular fingerprinting techniques, such as T-RFLP, to determine the composition of the growing members of the community (Artursson and Jansson, 2003).

Culture independent approaches such as fluorescence in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), and 16S rDNA sequencing which has given a leapt forward since its advent to the study of microbial diversity and community analysis, give a more realistic approach of the community structure (Schwarz et al., 2007), but only few studies exist (Koizumi et al., 2003; Wobus et al., 2003). The ability to determine microbial diversity at a high-resolution level (groups, species and strains) without the need for cultivation will further our understanding of several issues; for example, it will help us to determine structure-function relationships and to analyze the interactions formed between microbes and the abiotic environment and other organisms.

2.5.3. The 16S rRNA and Its Importance

It has become common to investigate community diversity using the rRNA gene (rDNA) or the rRNA itself. The rapidly growing rDNA sequence data bank, accessible via the internet (www.ncbi.nlm.nih.gov/entrez/), now makes it possible to compare sequences from across the world (Dahllöf, 2002). The basic approach for molecular diversity analysis is shown in Figure 2.5. Briefly, isolates from a community can be subject to direct analysis, using in situ methods, or nucleic acid can be extracted for analysis using microarrays or dot-blot hybridization. A gene might also be amplified using the polymerase chain reaction (PCR) and evaluated using a range of techniques including pattern analysis, cloning and sequencing, probe hybridization and microarrays (Dahllöf, 2002).

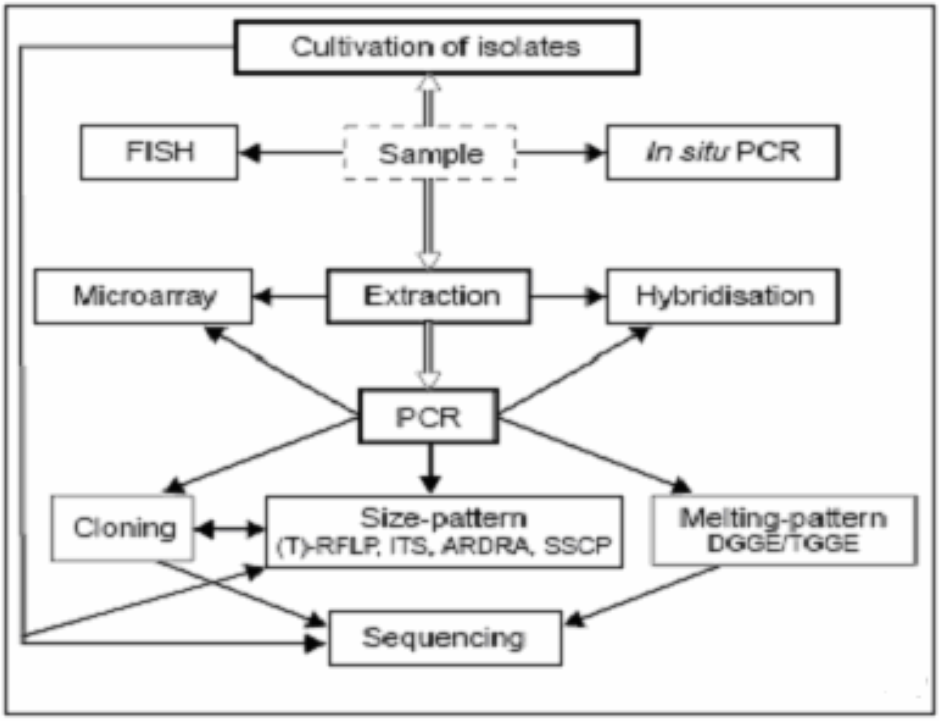

Figure 2.5. Common approaches to the analysis of microbial diversity (Dahllöf, 2002)

The use of 16s rDNA or rRNA is currently the most common approach for community analysis (Dahllöf, 2002). Studies of subsurface communities have focused on 16S rRNA gene analyses, which have provided information on the diversity of prokaryotes

in these environments (Parkes et al., 1994; D'Hondt et al., 2004). In the mid-1960s, Zuckerkandl and Pauling pointed out that molecular sequences could document evolutionary history (Zuckerkandl and Pauling, 1965).

The rRNAs have become the most commonly used molecules for phylogenetic analysis due to the pioneering work of Carl Woose. rRNA or the corresponding rDNA are particularly suitable as evolutionary chronometers (Stahl et al., 1988) since, (i) they are key elements of all cells and are functionally and evolutionarily homologous for all organisms, (ii) they are very conserved in overall structure, (iii)their regions of different conservation levels allow phylogenetic analysis and design of probes and primers, (iv) they are very abundant in most cells (10^3 to 10^5 copies) (Amann et al., 1995), and are easily recovered and detected, (v) the small subunit (SSU) rRNA (16S and 16S-like rRNA) and the large rRNA of the large subunit (LSU) of the ribosome (23S rRNA and 23S-like rRNA) are sufficiently long for statistically significant comparisons, and (vi) their genes have so far not been shown to be transferable among organisms (no lateral gene transfer) (Hofman-Bang et al., 2003).

Although it's obvious that the phylogenetic properties of 16S, as well as the large amount of sequences available offer a considerable advantage, there are also disadvantages (Dahllöf, 2002). For example, the heterogeneity of 16S between multiple copies within one species hampers pattern analysis, and confuses the interpretation of diversity from clone libraries and sequences retrieved from banding patterns (Dahllöf, 2002). The extent of 16S heterogeneity does vary between different regions, but so does resolution (Petri and Imhoff, 2001). It has also been shown that 16S lack resolution at the species level, most recently in Bacillus (Qi et al., 2001).

Caution and thoroughness are extremely important if 16S or any other heterogeneous gene is used to draw ecological conclusions concerning diversity and abundance (Dahllöf, 2002). The use of other genes, such as that for the σ factor rpoB (Qi et al., 2001), which appears to be present only in one copy ad has shown higher discrimination between species for some groups (Qi et al., 2001), can therefore be recommended both for pattern analysis and clone libraries. Moreover, functional genes – such as dsrAB genes for sulfate-reducing bacteria (Baker et al., 2003; Dhillon et al., 2003;

Nercessian et al., 2005), nasA gene for nitrate assimilation (Allen et al., 2001), nodD for rhizobia communities (Zeze et al., 2001), and pmoA and mxaF found in methanotrophic bacteria – are other alternatives used in diversity studies, especially when investigating structure – function relationships.

2.5.4. Most Commonly Used PCR-Based Molecular Techniques

2.5.4.1. Polymerase Chain Reaction. The polymerase chain reaction (PCR) can be used to amplify DNA sequences from environmental samples. The PCR products can be analyzed by techniques such as DGGE (denaturation gradient gel electrophoresis), TGGE (temperature gradient gel electrophoresis), T-RFLP (terminal restriction fragment length polymorphism), or SSCP (single stranded conformation polymorphism), which have the potential to separate the PCR products originating from different DNA sequences representing populations in the original samples. The PCR products can also be cloned and subsequently sequenced to allow identification of population (Hofman-Bang et al., 2003).

The development of synthetic DNA has spawned a new method for the rapid amplification of DNA in vitro, the polymerase chain reaction (PCR). In some molecular tools including the ones which will be discussed below, the initial step relies on the using PCR to amplify a target sequence. PCR can multiply DNA molecules up to a billion fold in the test tube, yielding large amounts of specific genes for cloning, sequencing or mutagenesis purposes.

PCR makes the use of the enzyme DNA polymerase, which copies DNA molecules (Madigan et al., 2003). The PCR technique requires that the nucleotide sequence of a portion of a desired gene to be known. This is necessary because short oligonucleotide primers complementary to sequences in the genes of interest must be available for PCR to work (Madigan et al., 2003). The choice of primers makes it possible to target the sequence at different taxonomic levels (strain, species, genus, etc.). The final PCR products obtained contain a mixture of the same fragment amplified at the chosen taxonomic level (strain, species, genus, etc.) (Dorigo et al., 2005). PCR technique is that each cycle literally doubles the content of the original target DNA. The extension products of one primer can

serve as a template for the other primer in the next cycle. In practice, 20-30 cycles are usually run, yielding a 10^6 to 10^9 fold increase in the target sequence

The PCR amplification step is known to introduce biases, with or sometimes without irrespective of the gene targeted. All techniques that are based on PCR (cloning, pattern analysis and sequencing) will be affected by the biases introduced by PCR (Dahllöf, 2002). Factors that cause bias of PCR are:

Primer specifity is a major stumbling block, especially when attempting to quantify a mixture of homologous target sequences (Becker et al., 2000). “Universal” primers or other specific primers are designed based on sequence information available in databases (obtained from cultured organisms and clones) (Pace, 1996). However, primers targeting multiple groups of organisms may not amplify all target genes since the primer sites are not completely conserved (Hofman-Bang et al., 2003). It was recently shown that the original sample template is amplified during the initial 5-6 cycles of the PCR reaction (Kurata et al., 2001), and that in the following cycles amplification occurs only on the PCR fragments produced earlier. This implies that sequences with a good primer match and high copy number will be selected for (Dahllöf, 2002). Even single mismatches in the middle of the primer can cause a preferential selection (Schafer et al., 2001). Degenerate primers could be used, but these are not suitable for techniques like DGGE as they produce multiple bands from one template, which gives the same problems when using heterogeneous genes (Dahllöf, 2002).

The common biases of PCR can be summarized as follows: (i) An inappropriate annealing stringency, which results in amplification of genes that are not intended to be amplified, can also cause a bias (Ward et al., 1992). Lowering the annealing temperature allows for mismatches and increases the diversity in PCR products (Ishii and Fukui, 2001), while increasing the risk of unwanted by-products. (ii) There is some evidence that PCR does not amplify all rRNA sequences in the sample to the same extent (preferential amplification) (Amann et al., 1995; Ward et al., 1992). (iii) Contaminating sequences from chemicals and enzymes can be erroneously included in the analysis (Hofman-Bang et al., 2003). (iv) Moreover, chimeric sequences are often produced (Ward et al., 1992) due to the presence of partial fragments of rDNA in DNA extracts, partially reverse transcribed DNA

when performing RT-PCR, or premature PCR products acting as primers in a subsequent PCR cycle (Amann et al., 1995).

2.5.4.2. Pattern Analysis and Denaturing Gradient Gel Electrophoresis. Pattern analysis or fingerprinting is often carried out by evaluating banding patterns of PCR products on gels (Dahllöf, 2002). Several fingerprinting techniques, such as DGGE, TGGE, RFLP, and SSCP, have been developed to screen clone libraries, to estimate the level of diversity in environmental samples, to follow changes in community structure (e.g., trace one or more populations over time), to compare diversity and community characteristics in various samples and simply to identify differences between communities (Hofman-Bang et al., 2003; Dahllöf, 2002). These techniques usually involve gel electrophoresis that can separate different DNA fragments of a community rDNA library (Dahllöf, 2002).

DGGE is now routinely used to asses the diversity of microbial communities, to monitor their dynamics (Muyzer, 1999; Muyzer and Smalla, 1998) and to screen clone libraries. This method can be used to obtain qualitative and semi-quantitative estimations of biodiversity. The DGGE pattern obtained provides a rapid identification of the predominant species. In a DGGE gel the number, precise position, and intensity of the bands in a gel track give an estimate of the number and relative abundance of numerically dominant ribotypes in the sample (Boon et al., 2002). The DGGE technique has been used to characterize the microbial diversity in different environments such as activated sludge (Curtis and Craine, 1998), sediments (Muyzer and De Wall, 1993), lake water (Ovreas et al., 1997), hot springs (Santegoeds et al., 1996), soils (Jensen et al., 1998), biofilm (Santegoeds et al., 1998). DGGE has been used to monitor changes in complex communities and to identify microorganisms present in wall painting (Santegoeds et al., 1996; Teske et al., 1996). It has recently been demonstrated that DGGE analysis of PCR products also works well in deep marine sediments and seafloor basalts (Lysnes et al., 2004). Denaturing gradient gel electrophoresis (DGGE) has been used extensively to profile prokaryotic community composition over both time and space in soils and aquatic environments (Schafer and Muyzer, 2001). It provides a quicker, less labor-intensive approach to comparing community composition in many different samples than sequencing of clone libraries. Although primarily used with bacterial communities by amplifying

fragments from 16S rRNA genes (Muyzer and Smalla, 1998), DGGE has also been used to explore the diversity of Archaea (Hoj et al., 2005)

DGGE is a gel electrophoresis method that separates genes/ DNA fragments of the same size (obtained after PCR of DNA extracted from an environmental sample) that differ in base sequence, at least by one nucleotide into distinct bands on a chemical denaturing gradient polyacrylamide gel. The technique employs a linear gradient of increasing chemical denaturant, such as a mixture of urea and formamide. When a double-stranded DNA fragment moving through the gel reaches a region containing sufficient denaturant, the strands begin to melt, at which point migration stops due to the larger volume of the denaturated molecule kept together by the GC clamp (Madigan et al., 2003, Dorigo et al., 2005). Separation or melting of the two strands of a DNA molecule depends on the hydrogen bonds formed between complementary base pairs (GC-rich domains melt at higher denaturant gradients), and on the attraction between neighboring bases on the same strand (Dorigo et al., 2005). When run on polyacrylamide gel, the mobility of the molecule is retarded when the first melting domain is reached resulting in partial dissociation of the fragment. Complete strand separation is prevented by the presence of a high melting domain, known as GC clamp, which is added to one primer (Dorigo et al., 2005). Differences in melting properties are to a large degree controlled by differences in base sequence. Thus, the different bands observed in a DGGE gel are different forms of a given gene that vary, sometimes only very slightly, in their sequences (Madigan et al., 2003). PCR amplification of the 16S rRNA gene utilizing conserved primers targeting either V3 or the V8 + V9 regions is normally used to produce a 300-500 bp fragment. Larger fragments are typically not used as the DGGE technique can not resolve these into distinct bands (Muyzer et al., 1993).

Fingerprinting provides a more rapid and less labor-intensive assessment of changing population structure and comparing community composition in many samples than sequencing of clone libraries, but little direct information on population identity (Muyzer and Smalla, 1998). A big advantage of the technique is that they make it possible to obtain taxonomic information by excising, re-amplifying and sequencing specific DNA fragments or by hybridization analysis with taxon-specific oligonucleotides probes (Riemann and Winding, 2001). Another advantage of the technique is that it can resolve

the microbial diversity of up to 15 different species by optimizing the DGGE in the gel. By using narrow gradients, rDNAs that differ in only one bp can be separated in DGGE (Muyzer et al., 1993). Bands from longer PCR products give better identification possibilities, although this must be weighed against separation efficiency. Creating a clone library which contains larger gene sequences is an alternative and thereby provides a more positive identification. However, a large number of clones have to be sequenced to get an appreciation of diversity, and clone libraries are victims of the same biases as other PCR methods (Hofman-Bang et al., 2003).

The main limitations are the choice of the primer set and the optimization of the gel running conditions before technique can be used to screen for sequence polymorphism of a particular gene (Muyzer et al., 1993) and the difficulty of comparing patterns across gels, when these patterns include numerous bands and its limited sensitivity of detection of rare community members (Vallaeys et al., 1997). The banding patterns of highly diverse microbial communities, present in soils, activated sludges and sediments, are usually very complex when bacterial primers are used. Since only the major populations of the analyzed community are represented on these DGGE patterns and thus relatively less abundant but potentially very important species may not be detected by this molecular method (Heuer et al., 1997). A drawback of the technique is that the reproducibility is not optimal; one DNA fragment may generate more than one band on the gel and a DNA sample analyzed on two different gels may not generate the same band pattern (Muyzer and Smalla, 1998). The possibility of a band in a DGGE gel to contain different sequences with similar denaturation characteristic may cause another bias to the technique (Hofman-Bang et al., 2003) because they may migrate together.

2.5.4.3. Molecular Cloning. Molecular cloning is at the base of most genetic engineering procedures and has greatly facilitated the analysis of any genome. The purpose of molecular cloning is to isolate large quantities of specific genes or chromosomal fragments in pure form (Madigan et al., 2003). It also allows the identification of the members of a community from environmental samples. Cloning can produce large amounts of DNA segments originally isolated from environmental samples. The DNA fragments can be produced after digestion with restriction enzymes of the DNA extracted from a sample (i.e., shotgun cloning), or after PCR or RT-PCR (if RNA is the template) (Hofman-Bang et

al., 2003). Analysis of 16S rRNA clone library to assess microbial diversity and populations in natural environments is an important approach (Giovanni et al., 1990). The unknown diversity is currently being explored with molecular techniques, particularly cloning and sequencing (Pedros-Alio, 1993). Molecular methods have mainly used cloning of PCR products amplified from deep subsurface sediment DNAs (Lysnes et al., 2004) and sequencing of clone libraries obtained after PCR amplification of extracted DNA with primers amplifying fragments of genes from Bacteria, Archaea and in some cases specific functional groups such as methanogens has been the predominant approach to studying prokaryotic diversity in deep subseafloor sediments.

A library is constructed with the DNA from a particular sample. Each clone has the SSU rDNA of one member of the original community. As more clones are sequenced, new taxa arise (Pedros-Alio, 2006). In general molecular cloning can be divided into several steps (Madigan et al., 2003): (i) Isolation and fragmentation of the source DNA. (ii) Joining the DNA fragments to a cloning vector with DNA ligase. The small, independently replicating genetic elements used to replicate genes are known as cloning vectors, and most are derived from plasmids or viruses. Cloning vectors are generally designed to allow recombination of foreign DNA at a restriction site that cuts the vector in a way that does not affect its replication. (iii) Introduction and maintenance in a host organism. The recombinant DNA molecule made in a test tube is introduced into a host organism, for example, by DNA transformation where it can replicate. Transfer of the DNA into the host usually yields a mixture of clones. Some cells contain the desired cloned gene, whereas other cells contain other clones generated by joining the source DNA to the vector. Such a mixture is known as a DNA library or gene library because many different clones can be purified from the mixture, each containing different cloned DNA segments from the source organism. Constructing a gene library by cloning random fragments of a genome is called shotgun cloning.

Cloning after PCR is rapid and convenient, but can be biased (Ward et al., 1992; Pace, 1996). The bias can be introduced during the PCR step or during cloning. For instance, the use of rare-cutting restriction enzymes during cloning might also cut amplified rDNA (Amann et al., 1995). Compared to cloning after PCR, shotgun cloning introduces less bias and produces clones of multiple genes at the same time (Pace, 1996).

In addition, different rRNA gene fragments may be cloned with different efficiencies. This technique is also time consuming and labor-intensive for the study of the vertical structure of communities in marine sediments.

3. MATERIALS AND METHODS

3.1. Sampling and Preservation

Sediment samples were taken from Tuzla and Moda (Figure 3.1.) in the northern east of the Marmara Sea, at a water depth of 42 m, using a Van Veen grab sampler (Figure 3.2) on board of the RV Arar of İstanbul University, Institute of Marine Sciences during research cruises in September 2005, December 2005, March 2006, November 2006 and February 2007. All samples appeared visually similar possessing grayish-black color as it can be seen in Figure 3.3 for anoxic sediments and had a noticeable odor of H_2S and densely packed clay-sized particles. Samples were taken in three replicates from the top 10 cm of sediment from the grab using 50 ml sterile syringes, with top end removed. Samples were then subdivided for molecular analyses (10 ml) and sediment chemistry (40ml). Samples were stored in sterile polypropylene tubes at -20°C.

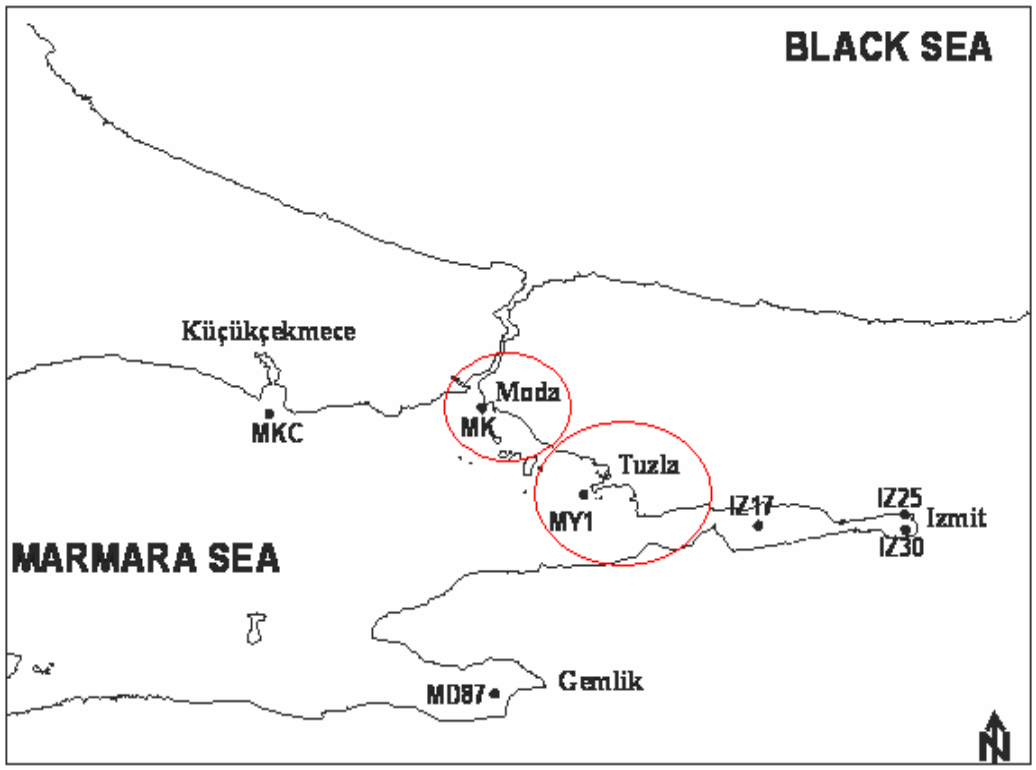

Figure 3. 1. Location of Tuzla & Moda and other sampling points

Figure 3. 2. The research ship, ARAR, of İstanbul University and Van Ween grab sampler

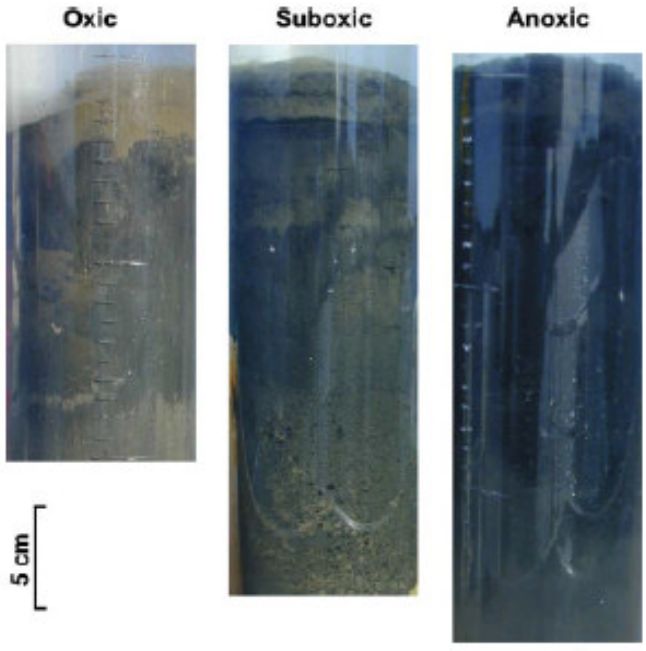

Figure 3. 3. The oxic, suboxic and anoxic sediment samples (Virtasalo et al., 2005)

3.2. Chemical Analysis

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

Subsamples for elemental analyses were dried at 50–60 °C overnight and kept in a vacuum desiccator until analysis. Samples were further exposed to concentrated HCl fumes to remove inorganic carbonates for total organic carbon (TOC) measurements. Total carbon (TC), organic carbon, nitrogen (TN) and sulphur (TS) contents were analyzed by the dry combustion method (Polat and Tuğrul, 1995), using a Carlo Erba Model 1108 CHN analyzer. Total inorganic carbon (TIC) was calculated as the difference between TC and TOC. Samples for total phosphorus (TP) analyses were first exposed to dry combustion at 500 °C for 2 h and then treated with 10 ml of 2N HCl for 10 h and filtered (Polat and Tuğrul, 1995). After the adjustment of pH to 8.0, the oxidized phosphorus in the solution was determined colorimetrically by the routine orthophosphate method. Statistical analysis of chemical data (Parametric two-way analysis of variance, Pearson correlation) was performed by using Systat 7.0 (SPSS Inc.).

3.3. Extraction of Sediment Microbial Community Genomic DNA and PCR Amplification of 16S rRNA Genes

Genomic DNA was extracted from 0.5 g sediments using the FastDNA Spin Kit for Soil (Qbiogene Inc., U.K.) following the manufacturer's instructions and a Ribolyser (Fast Prep™ FP120 Bio 101 Thermo Electron Corporation, Belgium), thus exposing the cells both to chemical and physical distruption, from which the genomic DNAs were extracted.

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

0.5 g sediment was added up to lysing matrix tubes,which contains a mixture of ceramic and silica particles to lyse all microorganisms in the sample, supplied by the kit. The lysing matrix tubes were spinned in the ribolyser for 30 seconds at speed of 5.5 m/s. The tubes were then centrifuged at 14.000 rpm for 30 secons. After centrifugation, supernatants were transferred to clean 1.5 ml eppendorf tubesand 250 µl PPS reagent was added. In order to mix the composition, the tubes were shaked by hands for 30 seconds and then centrifuged again at 14.000 rpm for 5 minutes for the pellet to precipitate. Supernatants were transferred 2 ml eppendorf tubes and 1 ml of Binding Matrix Suspension was added to supernatant. Tubes were inverted upside down for 2 minutes to allow the binding of DNA to the maxtrix. The matrix tubes were incubated for 3 minutes at room temperature. 500 µl of the supernatant was removed carefully without disturbing the settked silica matrix. Then the binding matrix was resuspended in the remaining supernatant. All mixture was filtered and the filter was placed to a clean tube. The filter was washed by 500 µl SEWS-M wash solution and centrifuged at 14.000 for 2 minute. The filter was removed to a clean tube and 50 µl DES (DNase/Pyrogen free water) was added. The filter with DES was vortexed and followed by centrifugation at 14.000 rpm for 1 minute resulting in extracted genomic DNA yield ready for application.

Amplification of 16S rDNA from the extracted DNA was performed with primers selective for the Archaea and Bacteria. The primers and their annealing temperatures were given in Table 3.1.

Table 3. 1. Primers used in PCR amplifications

Primer	Target	Experimental Stage	Annealing (°C)	Reference
Bact341f_GC	Bacterial 16S rDNA	DGGE	55	Muyzer et al., 1993
Bact534r				
Bact8f		Cloning		Lane, 1991
Bact1541r				
Bact342f		Sequencing		Edwards et al., 1988
Arch46f	Archaeal 16S rDNA	First round of nested PCR	40	Ovreas et al., 1997
Arch1017r				Barns et al., 1994
Arch344f		Cloning	53	Raskin et al., 1994
Arch855r				Shinzato et al., 1999
Arch344f_GC		DGGE		Raskin et al., 1994
Univ522r				Amann et al., 1995
M13f	β-galactosidase	Clone screening	54	Schrenk et al., 2003
M13r				

To enhance sensitivity and specificity, a nested PCR approach was applied in all cases for Archaeal amplifications. The first round of archaeal 16S rDNA amplification employed primers Arch46f and Arch1017r.

Briefly, the major steps in PCR are as follows:

- A specific nucleic acid probe(primer) hybridizes to a complementary sequence in a target gene.
- DNA polymerase copies the target gene.
- Multiple copies of the target gene are made by repeated melting of complementary strands, binding of primers, and new synthesis.

Thus, each PCR cycle involved the following:

- Denaturation: heat denaturation of double stranded target DNA.
- Annealing: cooling to allow annealing of specific primers to target DNA.
- Extension: primer extension by the action of DNA polymerase.

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

3.4. Denaturing Gradient Gel Electrophoresis (DGGE)

Community profiles of *Archaea* and *Bacteria* within the sediments were obtained using DGGE analysis of PCR amplification products from primers Arch344f_GC-Arch855r and Bact341f_GC-Bact534r as described by Muyzer et al. (1993).

The first step of the DGGE protocol was to prepeare 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 16 x 20 and 18 x 20 cm. Before the assembly, attention to the cleanliness of the glass plates should be paid in order to avoid any particulate matter which may effect the separation of the gel. The position of the spacers were checked carefully to avoid any leakage of acrylamide, and then the prepeared gel sandwich was placed on the casting stand as shown in Figure 3.4.

Figure 3. 4. Assembling and loading of perpendicular gradient gel sandwich

PCR product (10 µl) was mixed in equal volumes with loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll in water) and run on a 10% polyacrylamide gel (acrylamide–N,N'-methylenebisacrylamide ratio, 37.5:1) in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; pH 8.0) over a chemical denaturing gradient of urea and formamide equivalent to 30–60% denaturant (100% denaturant is 7 M urea and 40% (v/v) formamide).

To aid the conversion and normalization of gels, a marker consisting of 16S rDNA mix from archaeal and bacterial clone libraries was added on the outside of the gel as well as after every four samples. Electrophoresis was performed using the D-Code system (Bio-Rad Laboratories, Ltd., UK) seen in Figure 3.5 at 200 V constant current at 60°C, for 4.5 h.

Figure 3. 5. Bio-Rad DCodeTM system

Prior to the visualization of the gels, the core carrying the gel sandwiches was taken from the electrophoresis tank and gel sandwiches were separated. Glass plates were disassembled from each other and the el images were recorded using a Chemi-Smart 3000 gel documentation system (Vilber Lourmat, France) after stained with SybrGold (1:10000 diluted; Molecular Probes Inc., UK) according to the supplier's instructions.

Images were converted, normalized and analyzed by using the Bionumerics 5.0 software (Applied Maths, Kortrijk, Belgium), and data were exported to Excel and used for numerical analysis with Systat 7.0 (SPSS Inc.). Similarities between tracks were calculated by using the Dice coefficient (S_D) (unweighted data based on band presence or absence) and band-independent, whole-densitometric-curve-based Pearson product-moment correlation coefficients (r) and UPGMA clustering. For analysis using Dice coefficient a band position tolerance of 0.7% was applied. This was the minimum tolerance at which all marker lanes clustered at 100%.

3.5. Cloning of 16S rRNA Gene Fragments

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

In general, molecular cloning can be divided in to several steps (Madigan et al., 2003):

- Isolation and fragmentation of the source DNA.
- Joining the DNA fragments to a cloning vector with DNA ligase. The small, independently replicating genetic elements used to replicate genes are known as cloning vectors, and most are derived from plasmids or viruses. Cloning vectors are generally designed to allow recombination of foreign DNA at a restriction site that cuts the vector in a way that does not affect its replication.
- Introduction and maintenance in a host organism. The recombinant DNA molecule made in a test tube is introduced into a host organism, for example, by DNA transformation where it can replicate. Transfer of the DNA into the host usually

yields a mixture of clones. Some cells contain the desired cloned gene, whereas other cells contain other clones generated by joining the source DNA to the vector. Such a mixture is known as a DNA library or gene library because many different clones can be purified from the mixture, each containing different cloned DNA segments from the source organism. Making a gene library by cloning random fragments of a genome is called shotgun cloning.

The initial step of the procedure was preparation of 6 µl 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 for 20 minutes. Following incubation, reaction mix was placed on ice before One Shot TOPO transformation step.

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

To test for the correct insert, colony PCRs were performed from 150-200 colonies using the vector-specific primers M13f and M13r. Randomly selected 60-65 vector inserts of the correct size were reamplified with primer pairs Arch344fgc-Univ522r and BactVf-BactVr and analyzed by DGGE to relate bands in DGGE profiles from the original samples with the cloned DNA. The conditions for DGGE analysis of cloned 16S rRNA gene fragments were as described above.

3.6. Sequencing, and Phylogenetic Analysis of 16S rRNA Gene Fragments

At least one representative of every clone type was sequenced, and for clone types that appeared more than once in the library at least two representatives were sequenced. PCR products to be sequenced were purified by ethanol precipitation and sequenced using the ABI prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 377 DNA sequencer (Applied Biosystems, USA) using primers Arc344f and Bact342f generating 500bp and 800bp of archaeal and bacterial sequence data respectively.

Partial 16S rRNA gene sequences were analyzed and manually edited in Chromas software package version 1.45 (http://www.technelysium.com/au/chromas.html). Homology searches of the EMBL and GenBank DNA databases for the 16S rRNA gene sequences were performed with FASTA (Pearson and Lipman, 1988) provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/fasta33/nucleotide.html) to identify putative close phylogenetic relatives. Sequences representing distinct phylotypes (as the criterion, 97% sequence similarity was used) and their closest relatives were aligned by using the ARB software fast aligner utility, followed by manual adjustments. Only unambiguously aligned base positions were used in the analysis. Distance analyses using the Jukes and Cantor (1969) correction and bootstrap resampling (1000 times) were done using the TREECON package (van De Peer and De Wachter, 1997) and trees were generated from distance matrices using the neighbor-joining method (Saitou and Nei, 1987).

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

4. RESULTS AND DISCUSSION

4.1. Chemical Analysis

The objective of the present study was to characterize the overall microbial community structure in anoxic sediment samples taken from the Tuzla Bay by cloning and sequencing of PCR-amplified taxonomic16S rRNA genes. Here we also report, for the first time, the seasonal distribution of archaeal and bacterial communities in a coastal sediment using DGGE of 16S rRNA genes. Results of this study provided a unique opportunity to compare the microbial ecology of the sediments taken from the same location (Moda and Tuzla, separately) at different time intervals.

Sediment samples taken from Tuzla (MY) and Moda (MK) were analyzed for physical and chemical characteristics to determine the pollution in those sampling points. pH, total solid (TS), total organic carbon (TOC), total volatile solid (TVS), heavy metal, anion analysis were carried out on the sediment samples, besides elemental carbon, nitrogen and sulfur contents were determined. All the results are given below.

It has been reported for anoxic sediments to have a pH higher than 8 since sulfate and nitrate reduction processes consume H^+ (Radke et al., 2002). The pH values measured for Tuzla and Moda samples range between 8.2-8.7 (Table 4.1) . When electric potential is within the range of +100 mV and -100 mV, iron reduction; when it's within the range of -100 mV and -200 mV, sulfate reduction and, below -200 mV methanogenesis occurs (Vorenhout et al., 2004). The electric potential for Tuzla and Moda sediment samples range within -100 mV and -150 mV which indicates the dominancy of sulfate reduction within these sediments. Sulfate reduction, iron and manganese reduction have previously found to be important microbial processes that occur within the marine sediments (Canfield et al., 1993a).

Tablo 4. 1. pH and electric potential of sediment samples

Sampling Point	Sampling Time	pH	Electric Potential (mV)
Moda	March 06	8.69	-146.25
	November 06	8.22	-101.25
	February 07	8.42	-121.50
Tuzla	September 05	8.71	-149.06
	December 05	8.58	-135.00
	March 06	8.55	-131.06
	November 06	8.36	-116.44
	February 07	8.61	-140.06

In the studies of Shine and Wallace (2000), and Hyland et al. (2005), it's mentioned that total organic carbon (TOC) value can be used as an indicator of pollution and the TOC values are found to be around 0.01-0.15 mg/mg within anoxic sediments depending on the pollutional load of the sediments. The TOC values for sediments from Tuzla and Moda vary in between 0.04-0.06 mg/mg (Table 4.2), showing that these sediments have been exposed to organic matter pollution to some tolerable degree. However, the data derived from TOC analyses is more meaningful when compared to the analysis from other sampling points of considerably polluted regions of Marmara Sea, such as Küçükçekmece. Küçükçekmece is a highly polluted region within Marmara Sea and TOC values are high when compared to other important points in the Marmara Sea (Türker, 2007). When TOC values are compared to the results from the study of Türker (2007), Moda and Tuzla are considered as polluted regions after Küçükçekmece.

The TOC/TS* ratios give an idea of the conditions prevailing within the sediments and about what biogeochemical processes might occur in the sediments. It has been previously estimated that TOC/TS* ratio is above 5 within sediments where aerobic conditions prevail, however if anoxic conditions prevail within the sediments while the water column is under aerobic conditions then this ratio is found to be between 1.5-5. When anoxic conditions prevail both within the sediments and water column, thus this ratio goes below 1.5. As the TOC/TS* ratio goes down the importance of sulfate reduction increases within such sediments (Craft et al., 1991). In this study, the TOC/TS ratios vary between 0.7-1. This shows that anoxic conditions prevail both within the sediments and in

the water column above the sediments. The TOC/TS* value is 0.7 in sediments from Tuzla, and reaches the value of 0.9 in sediments from Moda. These results indicate the dominancy of sulphate reduction within the deeper sampling point Tuzla over the sediments from shallow sampling point, Moda. Besides, SRBs could be detected in the bacterial clone library from Tuzla sediment samples, whereas no SRB was detected in the bacterial clone library constructed from Moda sediment samples correlates with this finding.

Tablo 4. 2. TC, TIC and TOC analysis

Sampling Point	Sampling Time	TC (mg/mg)	TOC (mg/mg)	TIC (mg/mg)	TOC/TC	TOC/TS*
Moda	March 06	0.055	0.052	0.003	0.939	0.93
	November 06	0.049	0.046	0.003	0.943	0.87
	February 07	0.051	0.047	0.003	0.932	0.88
Tuzla	September 05	0.045	0.039	0.006	0.871	0.76
	December 05	0.049	0.043	0.006	0.877	0.77
	March 06	0.049	0.043	0.006	0.8745	0.77
	November 06	0.052	0.045	0.007	0.868	0.77
	February 07	0.051	0.044	0.007	0.870	0.77

Moreover, TS concentrations and TS/TVS ratio can also be used as indicators of pollution as well, and the TS concentrations are within the range of 100-1000 mg/l (Spagnoli and Bergamini, 1997). However, the TS concentrations in this study vary between 200-700 mg/l (Table 4.3), also indicating that the sediments were exposed to pollution to some degree which correlates with results derived from TOC analysis. Moda appears to be densely polluted based on these results. The low TVS/TS ratios ranging between 2%-11% shows that Tuzla and Moda sediments are basically polluted with inorganic pollutants.

Tablo 4. 3. TS and TVS concentrations sediment samples

Sampling Point	Sampling Time	TS (mg/l)	TVS (mg/l)	TVS/TS (%)
Moda	March 06	611.0	46.5	7.6
	November 06	392.8	36.4	9.3
	February 07	685.6	19.5	2.8
Tuzla	September 05	429.9	20.3	4.7
	December 05	347.3	32.0	9.2
	March 06	377.6	40.6	10.8
	November 06	259.6	23.8	9.2
	February 07	692.1	15.3	2.2

The heavy metal concentrations within Tuzla and Moda sediments (Table 4.4) are extremely over the heavy metal levels found in unpolluted sediments and show close similarity values to the heavy metal levels detected in highly polluted sediments (Elder, 1988). These results show that Marmara Sea has been highly exposed to heavy metal pollution.

Tablo 4. 4. Heavy metal concentrations of sediment samples

Sampling Point	Sampling Time	Cr (mg/kg)	Cd (mg/kg)	Cu (mg/kg)	Zn (mg/kg)	Pb (mg/kg)	Ni (mg/kg)	Mn (mg/kg)	Fe (mg/kg)	Ag (mg/kg)
Tuzla	September 05	86	< 10	52	308	57	32	166	18060	< 25
	December 05	317	< 0.2	281	845	196	45	206	19900	< 0.25
	March 06	282	< 0.2	244	860	248	42	196	18450	< 0.25
	November 06	182	< 0.2	145	497	128	38	168	16500	< 0.5
	February 07	114	< 0.2	107	255	41	18	134	13993	< 0.5
Moda	March 06	34	< 10	160	634	84	48	227	22465	< 25
	November 06	36	< 0.2	118	488	70	42	250	20100	< 0.5
	February 07	38	< 0.2	107	485	62	34	213	20185	< 0.5

The C/N/P ratio is important in terms of understanding the potential for microbial decomposition of organic matter. In marine sediments, the most important limiting factor for biodegradation is the scarce amounts of nutrients such as N and P when compared to C (Roling et al., 2002). It has been reported in the previous studies that the rate of biodegradation increases in correlation with the N-P content (Goldman et al., 1987). In this

study, the C/N/P ratios for Tuzla and Moda sediment samples (Table 4.5) range between 20/1/1-20/8/1 which is within the range of C/N/P ratios that do not inhibit microbial proliferation under anoxic and anaerobic conditions (Lyons et al., 1996). The nutrient amounts are relatively high in Tuzla when it's compared to the results of analysis derived from Moda and other sampling points of the project. However, this situation does not show that biodegradation rate in Tuzla is relatively higher than the other sampling points, because nutrients neither in Moda nor in other sampling points are scarce.

Tablo 4. 5. Elemental analysis of samples

Sampling Point	Sampling Time	% N	% C	% S	% P	S / C / N / P
Moda	March 06	0.41	5.49	5.52	0.34	16 / 16 / 1.2 / 1
	November 06	0.35	4.87	5.23	0.24	22 / 20 / 1.5 / 1
	February 07	0.36	5.08	5.34	0.28	19 / 18 / 1.3 / 1
Tuzla	September 05	0.62	4.53	5.20	0.07	71 / 62 / 8.5 / 1
	December 05	1.70	4.90	5.54	0.24	23 / 21 / 7 / 1
	March 06	1.75	4.87	5.47	0.24	23 / 20 / 7.3 / 1
	November 06	1.83	5.23	5.9	0.26	23 / 20 / 7 / 1
	February 07	1.77	5.09	5.76	0.24	24 / 21 / 7.5 / 1

As it's common for sulfate concentrations to be abundant in marine sediments, the sulfate concentrations in Tuzla and Moda sediments were high as well (Table 4.6). However, nitrate concentrations were not within the detetable limits since it's common for nitrate to be quickly depleted within the marine sediments.

Tablo 4. 6. Anion concentrations within the sediments

Sampling Point	Sampling Time	Fluoride (mg/l)	Chloride (mg/l)	Nitrite (mg/l)	Bromide (mg/l)	Nitrate (mg/l)	Sulfate (mg/l)	Phosphate (mg/l)
Moda	March 06	3.5	22.9	26.8	20.1	13.0	10.4	14.6
	November 06	3.8	18563.1	00.0	44.3	00.0	3910.3	00.0
	February 07	2.9	3828.7	00.0	14.4	15.0	945.0	22.9
Tuzla	September 05	5.1	20969.7	00.0	79.0	6.0	1089.2	00.0
	March 06	5.1	16373.6	00.0	47.9	0.00	1287.3	00.0
	November 06	6.6	17608.8	15.8	51.2	0.00	2005.2	3.4
	February 07	7.8	14499.2	00.0	67.3	20.1	644.8	18.3

4.2. DNA Extraction, Visualization and PCR Amplification

The Fast DNA Spin Kit for Soil extracted amplifiable DNA from three seasonal intervals (March 2006, November 2006, February 2007) of Moda anoxic marine sediment samples, and from five seasonal intervals (September 2005, December 2005, March 2006, November 2006, February 2007) of Tuzla anoxic marine sediment samples. All extracted genomic DNAs were visible by agorose gel electrophoresis as in Figures 4.1 and 4.2. The relative concentration of the total extracted DNA from anoxic sediments was 200 µg/ml.

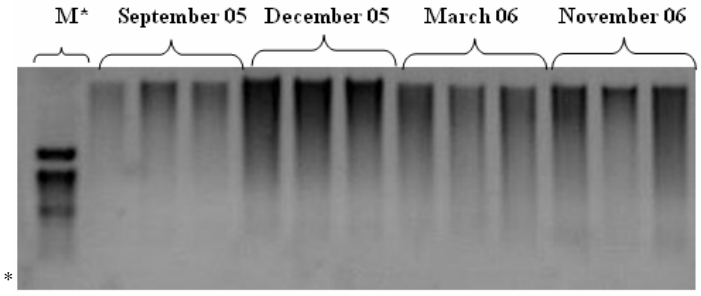

Figure 4.1. Agorose gel electrophoresis of gDNA extractions of Tuzla (MY) sediments

*M: Marker

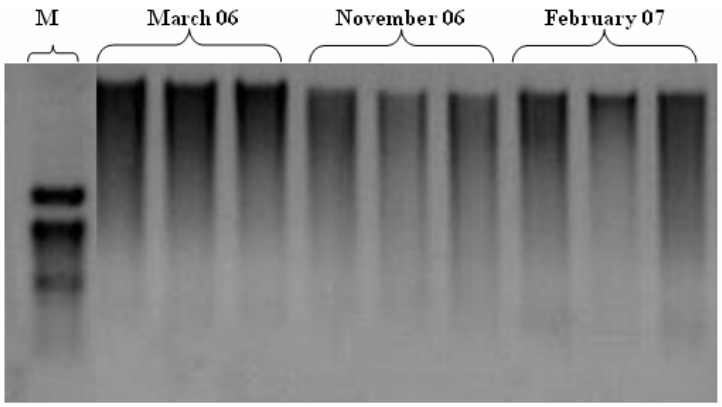

Figure 4.2. Agorose gel electrophoresis of gDNA extractions of Moda (MK) sediments

Variable (V3) regions of 16S rDNA from extracted genomic DNA of Moda and Tuzla Bay samples using bacteria specific primers pA-pH, could be amplified for cloning with a better PCR product yield after purification by High Pure PCR Product Purification Kit (Figure 4.3). No archaeal PCR product hat was visible by agarose gel electrophoresis could be amplified using archaea specific primers Arch46-Arch1017 from purified extracted genomic DNAs of Moda and Tuzla Bay samples, for cloning. However, smaller size of archaeal 16S rDNA fragment (~ 500 bp) sufficient to produce a satisfactory clone library could only be amplified after a nested PCR approach using archaea specific primers in the first (Arch46-Arch1017) and second (Arch344-Arch855) stage (Figure 4.4). ~ 200 bp bacterial and archaeal PCR products for DGGE analysis could also be amplified by nested PCR approach using bacterial primers Vf-Vr (Figures 4.5 and 4.6) and archaeal primers Arch344-Arch855 (Figures 4.7 and 4.8) in the second round from previously amplified bacterial (~1500 bp) and archaeal (~970 bp) PCR products by bacterial primers pA-pH and archaeal primers Arch46-Arch1017, respectively.

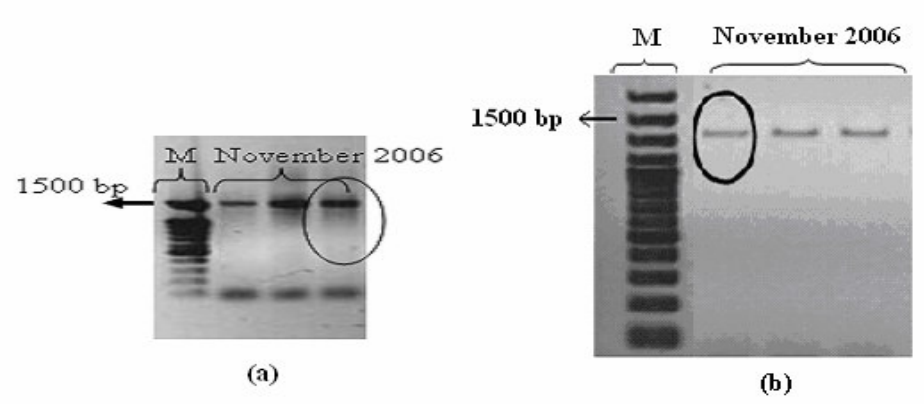

Figure 4.3. Agorose gel electrophoresis of the PCR products of 1500 bp bacterial 16S rDNA amplification from Tuzla (a) and Moda (b) samples

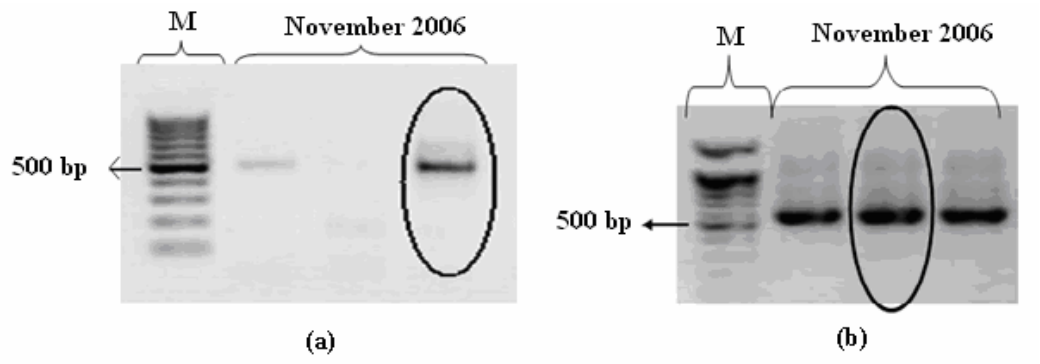

Figure 4.4. Agorose gel electrophoresis of the PCR products of 500 bp archeal 16S rDNA amplification from Tuzla (a) and Moda (b) samples

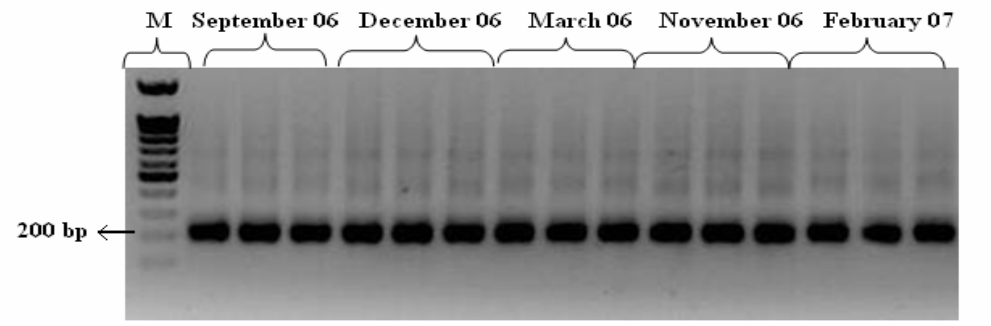

Figure 4.5. Agorose gel electrophoresis of 200 bp bacterial 16S rDNA amplification from Tuzla samples for 5 time intervals

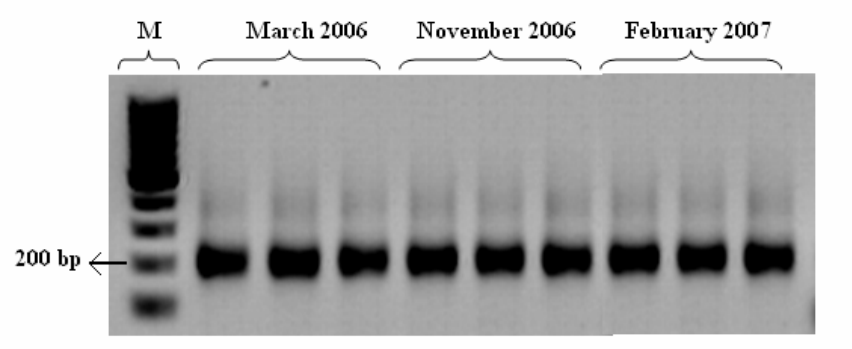

Figure 4.6. Agorose gel electrophoresis of the 200 bacterial 16S rDNA amplification from Moda samples for 3 time intervals

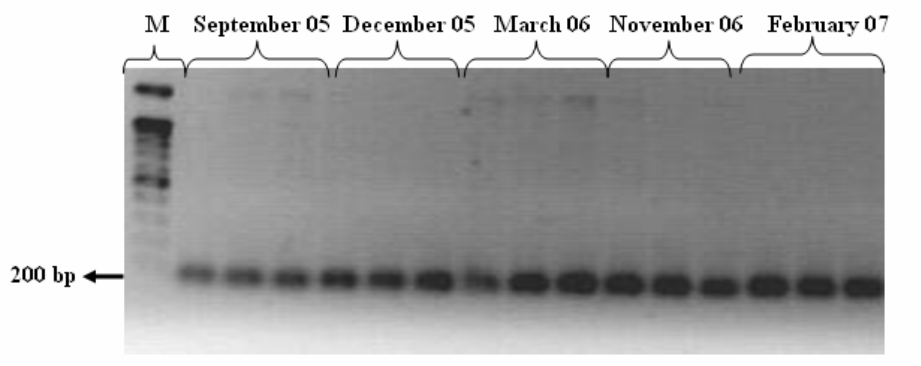

Figure 4.7. Agorose gel electrophoresis of 200 bp archeal 16S rDNA amplification from Tuzla samples for 5 time intervals

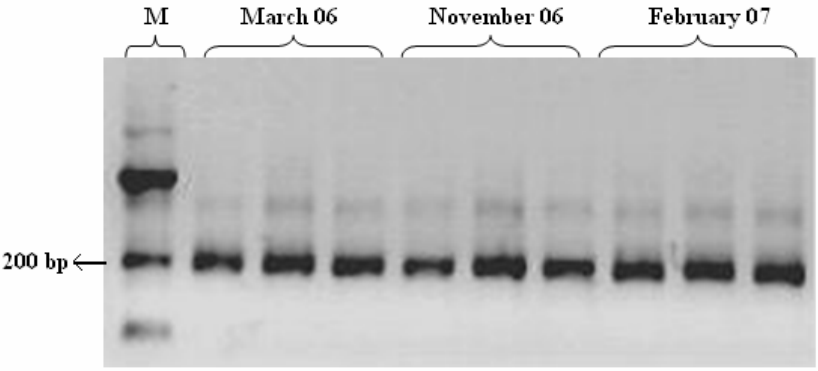

Figure 4.8. Agorose gel electrophoresis of 200 bp archeal 16S rDNA amplification from Moda samples for 3 time intervals

Recently, many investigators have raised questions about possible problems associated with PCR of the rRNA gene for the phylogenetic analysis of microbial communities. The increament of PCR cycles may bias the clone composition obtained (Sekiguchi et al., 1998, Suzuki & Giovannoni, 1996). A cautious selection of primers for PCR amplification may lessen a leveling effect of amplification (Suzuki & Giovannoni, 1996). Strong biases may be introduced by the copy number of 16S rRNA genes and the differential PCR amplification efficiency of DNA from heterogeneous templates (Chandler et al., 1997). These possible effects may mean that the proportions found in the clone libraries do not always represent the 16S rDNA proportions found in the original samples. However, the limitation of culture techniques (Amann et al., 1995) means that sequence-based phylogenetic techniques may still provide a less biased picture of community composition than would any single cultivation technique (Urakawa et al., 1999).

4.3. Archaeal Diversity of Tuzla Bay and Moda

To estimate the overall archaeal diversity of Moda and Tuzla anoxic marine sediments, two general archaeal 16S rRNA libraries were constructed from sediment samples taken in November 2006.

A total of 182 clones from Tuzla Bay November 2006 sediment samples were randomly picked and screened by agorose gel electrophoresis , of which 67 clones were positive and a total of 120 clones from Moda November 2006 sediment samples were randomly picked and screened, of which 88 clones were positive. All the positive clones from Tuzla and Moda sediment samples were screened by DGGE and grouped together based on their DGGE profiles. Representative clones were sequenced, and the 500 bp sequences were analyzed on 16S rDNA database of EBI (www.ebi.co.uk) as described in section 3.6 in order to determine their phylogenetic affiliations. Archaeal clone identification for both sites is given in Table 4.7 and the metabolic distributions of archaeal clones are shown in given in Tables 4.8 and 4.9.

Tablo 4. 7. Results of phylogenetic analysis of archaeal community

Closest identified relatives of Tuzla clones	**Similarity %**	**Clone name**	**Frequency %**
Methanosaeta concilii Opfikon	99	A	2
Methanolobus oregonensis WAL1	99	B	2
Methanosaeta concilii	98	C	10
Methanosarcina lacustris strain MS	98	D	10
Methanosarcina lacustris strain MM	97	E	5
Methanosarcina sp. AK-6	97	F	2
Methanosaeta concilii VeAC9	97	G	3
anaerobic methanogenic archaeon E15-2	97	H	2
Methanobrevibacter smithii B181	97	I	3
Methanosaeta sp. AMPB-Zg	97	J	3
Methanobrevibacter smithii DSM 2375	96	K	2
Methanobrevibacter smithii ALI	95	L	2
Methanobrevibacter smithii ALI-A	95	M	2
Methanobrevibacter smithii PS	95	N	3
Methanosarcina lacustris strain MS	94	O	11
Methanobrevibacter smithii ATCC 35061	93	P	2
uncultured archaeon PL-10A11	91	R	3
Methanosaeta concilii Opfikon	88	S	11
Uncultured archaeon clone RG-TJ31	84	T	3
Methanolobus oregonensis WAL1	82	U	13
uncultured archaeon 2-PML 8% pond	81	V	6
uncultured Methanolobus sp. SB03	71	Y	2
Methanolobus oregonensis	58	Z	2
Closest identified relative of Moda clones	**Similarity**	**Clone name**	**Frequency %**
Uncultured archaeon clone, MNTSA-B6 16S	95	a	2
Unidentified archaeon, clone 237	92	b	2
Uncultured archaeon, clone mrR1	83	c	1
Uncultured archaeon clone MKCSB-D5	98	d	10
Uncultured archaeon ZAR122	93	e	1
Uncultured archaeon clone met80	86	f	2
Uncultured archaeon, clone ODP1227A1.10	97	g	4
Uncultured archaeon clone 7F07	95	h	5
Uncultured archaeon clone 5H2_F22	96	i	2
Uncultured archaeon, clone ODP1251A1.1.	97	j	2
Uncultured archaeon clone 2H2_A14	97	k	5

Tablo 4.7. Results of phylogenetic analysis of archaeal community (continued)

Uncultured archaeon clone BCMS-6	98	m	2
Uncultured archaeon clone XMA37	96	n	1
Uncultured archaeon clone Ou2I-11	80	o	2
Uncultured crenarchaeote, clone BS1-1-87	95	ö	2
Uncultured crenarchaeote clone E_H10 16S	86	p	2
Uncultured crenarchaeote clone E_H04	91	r	6
Uncultured crenarchaeote clone EJ_B02	96	s	18
Uncultured euryarchaeote, clone ESYB34	90	t	5
Uncultured euryarchaeote EHB97 16	97	u	12
Uncultured euryarchaeote, clone:ESYB42.	94	v	2
Uncultured Methanosarcinaceae, HrhA66	91	w	2
Uncultured Methanosarcina sp. X4Ar38	94	x	2
Uncultured Methanosarcinaceae MRR25	94	y	2
Uncultured Methanosphaera sp., DI_C08	93	z	1

Tablo 4. 8. Dominant metabolic pathways, substrates and percentages of archaeal clones from Tuzla clone libraries

Microorganism and Its Frequency	**Metabolism**	**Substrates**
Methanosarcina lacustris (%26)	Methylotrophic Methanogenesis	H_2+CO_2, methanol, methylamines, methylsulfides
Methanosarcina sp. AK-6 (2%)		
Methanolobus oregonensis (%15)		Methylamines
Methanolobus sp. (2%)		Methanol, methylamines
Methanosaeta concilii (%40)	Acetoclastic Methanogenesis	Acetate
Methanosaeta sp. (3%)		
Methanobrevibacter smithii (%14)	Hydrogenotrophic Methanogenesis	H_2+CO_2 , formate

Tablo 4. 9. Dominant metabolic pathways, substrates and percentages of archaeal clones from Moda clone libraries

Microorganism and Its Frequency	Metabolism	Substrates
Methanolobus oregonensis (13%)	Methylotrophic Methanogenesis	Methanol, methylamines
Methanosarcina semesiae (8%)		Dimethylsulfide, methanethiol, methanol, methylated amines
Methanobrevibacter cuticularis (4%)	Hydrogenotrophic Methanogenesis	$H_2 + CO_2$, formate
Methanotorris formicicus (1%)		$H_2 + CO_2$, formate
Methanogenic endosymbiont of Nyctotherus ovalis (24%)		$H_2 + CO_2$, formate
Methanothermococcus okinawensis (2%)		$H_2 + CO_2$, formate
Methanospirillum hungatei JF-1 (%5)		$H_2 + CO_2$, formate
Methanothermococcus sp. (33%)		$H_2 + CO_2$
Methanosaeta sp. clone A1 (9%)	Acetoclastic Methanogenesis	Acetate, alkane

The archeal 16S rRNA clone library for Tuzla was dominated by Euryarchaeota constituting for 42 % of all the clones with more than 97% similarity to their closest cultured relatives, namely known methanogens on the databases. Phylogenetic analysis of 16S rRNA from diverse natural environments have identified a large number of both crenarchaeotal and euryarchaeotal sequences (Fuhrman et al., 1992; DeLong et al.; 1994, Bintrim et al.; 1997, Munson et al., 1997; Hinrichs et al., 1999). However, 60% of the archaeal clones from Tuzla clone library were putative organisms showing less than 97% similarity to their cultured and environmental relatives on databases. Only 2% of all the clones showing less than 70% similarity to any cultured or environmental clone on the database were thus grouped as unclassified.

Tuzla 16S rRNA library was dominated by *Methanosaeta* species covering 18% of all the clones, followed by *Methanosarcina* species by 17% and *Methanobrevibacter smithii* by 3% all of which showed higher than 97%, respectively. Clones related to *Methanolobus* with 99% similarity and *Methanobrevibacter smithii B181* with 97% similarity only accounted for 2% and %3 of all the clones, respectively. Archaea usually

represent no more than a few percent of the total prokaryotic community, with occasional reports of far higher proportions (up to 34%) in planktonic communities (DeLong et al., 1994; Massana et al., 1998), but high abundance and diversity were found in marine subsurface sediments (Inagaki et al., 2003). In this study, only the relative percentages are given since the percentages of the archaeal community members are evaluated within the Archaea, not in the whole prokaryotic community. In order to obtain exact figures of archaeal community, quantification of the archaea will further be accomplished by Real Time PCR.

Considering archaeal 16S rRNA clone library for Moda shows that all of the clones yielded reliable sequences that were affiliated with uncultured archaea, since none of the sequences belonged to any cultured organism on the databases showing more than 97% similarity, but were nevertheless phylogenetically related to previously cultivated methanogens.

Clones that could not fall into any subphylum among archaea represented 46% of the total archaeal community in Moda sediments, therefore all the archaeal sequences were analyzed in prokaryotic 16S rRNA databases to affiliate the sequences with known clones in order to find their physiologies and estimate the metabolic pathways dominant in the sediments. Cytryn and his colleagues (2000), also detected a cluster with sequences that did not fall into a cluster with any known methanogens and as he suggests, the inability to detect known methanogens could be because of a number of reasons, (i) Species related to known methanogens may exist in the sediments but represent a small fraction of the archaeal population, and thus the PCR analysis used was not sensitive enough to detect them. (ii) Sequences affiliated with uncultured archaea are phylogenetically distant from cultured archaea and thus nothing is known about their physiological characteristics. It is possible that members of these unidentified archaea represent novel methanogen types that have not yet been cultured. Methanogens are a polyphyletic group represented by a number of clusters throughout the euryarchaeotal kingdom. Therefore, additional methanogen clades may exist that contain species not yet cultured (Cytryn et al., 2000).

The rest of the uncultured archeal clones detected in the Moda clone library fell into major archaeal divisions of Crenarchaeota and Euryarchaeota, both of which are found to

inhabit a variety of environments. Phylogenetic analysis of 16S rRNA from diverse natural environments have identified a large number of both crenarchaeotal and euryarchaeotal sequences (Fuhrman et al. 1992; DeLong et al., 1994; Bintrim et al., 1997; Munson et al., 1997; Hinrichs et al., 1999). Many of these sequences form monophyletic clades unrelated to any known cultured organism (Cytryn et al., 2000).This correlates with the results of this study for archaeal sequences derived from Moda anoxic sediments. Methanogen *s*pecies were isolated virtually from every habitat in which anaerobic biodegradation of organic compounds occurs, including fresh water sediments, digestive and intestinal tracts of animals, anaerobic waste digesters (Jones et al., 1983; Whitman, 1985), as well as extreme environments such as geothermal springs and both shallow and deep-sea hydrothermal vents (Iluber et al., 1982; Jones et al., 1983). Crenarchaeal phylotypes were found among marine picoplankton (Fuhrman et al., 1992; DeLong, 1994), in the gut of a deep-sea holothurian (McInerney et al., 1995), in fresh water sediments (Hershberger et al., 1996; MacGregor et al., 1997; Schleper et al., 1997), in soil (Bintrim et al., 1997; Jurgens et al., 1997; Buckley et al., 1998), in deep-subsurface sediments (Chandler et al., 1998), in continental shelf anoxic sediments (Vetriani et al., 1998), and in moderate temperature (15 to 30 °C) hydrothermal vent microbial mats (Moyer et al., 1998). Since all the sequences derived were uncultured archaeal clones, in order to determine metabolic groups in the anoxic sediments of Moda, the closest cultured relatives of archaeal clones from Moda sediment samples were derived from the prokaryote 16S rDNA databases and they all fell into Euryarchaeota phylum only on an average of 81% covering major methanogenic groups, namely Methanosarcinales, Methanobacteriales and Methanococcales.

Phylogenetic analyses of ribosomal RNA gene sequences have revealed two distinct lineages among Euryarchaeota capable of anaerobic methanotrophy: the ANME-1 cluster, which does not contain any cultured relatives (Hinrichs et al., 1999), but form a distinct cluster within the Methanosarcinales, the only archaea that can utilize acetate, methylamines or methanol, and the ANME-2 cluster affiliated to the cultured members of the Methanosarcinales (Orphan et al. 2001). It has been showed that these archaea are capable of using methane as a carbon source. Their involvement in anaerobic oxidation of methane was showed (Stadnitskaia et al., 2005) and it's an important process in terms of controlling the global methane budget in the atmosphere. Sulfate reducing bacteria (SRBs) are also found to be present along with ANME groups of archaea and get involved in the

AOM process. Previous studies indicate a close metabolic association between SRB and methanotrophic archaea (Pancost et al., 2001). For example, sulfate-reducing bacteria affiliated to members of the genera *Desulfosarcina* and *Desulfococcus* have been found in association with ANME-1 and ANME-2 archaeal cells, representing in both cases putative methanotrophic consortia (Orphan et al., 2001, Michaelis et al., 2002). The uncultured archaeon species detected in Tuzla were affiliated with *Uncultured archaeon LFAc12* with a similarity of 94% which was previously found to have formed a distinct cluster with ANME groups within Methanosarcinales (Karr et al., 2006), was found to related to its closest cultured relative *Methanosarcina lacustris*. Thus analysis of functional genes can be aimed for future studies to detect the presence and abundance of SRBs in these sediments and support the findings of this study. The abundance of uncultured archaeal species in Moda and the presence of sequences affiliated with Methanosarcinales may be of importance both in Moda and Tuzla sediments in order to control methane emissions.

Moreover, the most abundant metabolic group, nitrate reducers in anoxic sediments of Tuzla along with the archaeal dominating members of methanogens suggests a novel process, anaerobic methane oxidation (AOM) coupled to denitrification which has recently been reported by Raghoebarsing et al. (2006). Methanotrophs' ability to oxidize methane aerobically to methanol or acetate at low oxygen concentrations, and that the methanol or acetate can subsequently be used to drive denitrification (Islas-Lima et al., 2004) was already established. But this recently defined new microbial guild of denitrifiers and bacteria related to ANME groups and cultivated methanogens (*Methanosarcina* and *Methanomethylovorans*) as also present in Tuzla sediments will support evidence for the syntrophic relationship among those groups. It will further explain their dominancy and the possible occurrence of this novel AOM process couple to denitrification in Tuzla sediments which may contribute significantly to methane oxidation, and could potentially counteract worldwide increases in methane production associated with intensive agriculture (Raghoebarsing et al., 2006).

Based on the results of the sequencing of Tuzla and Moda archaeal clone libraries, clones showing higher than 97% similarity are considered to be the same organism and less than 97% similarity are differentiated on strain level. Therefore, in overall 23 different

clones from Tuzla and 26 different clones from Moda were derived for November 2006 sediment samples as shown in the Figures 4.9 and 4.10.

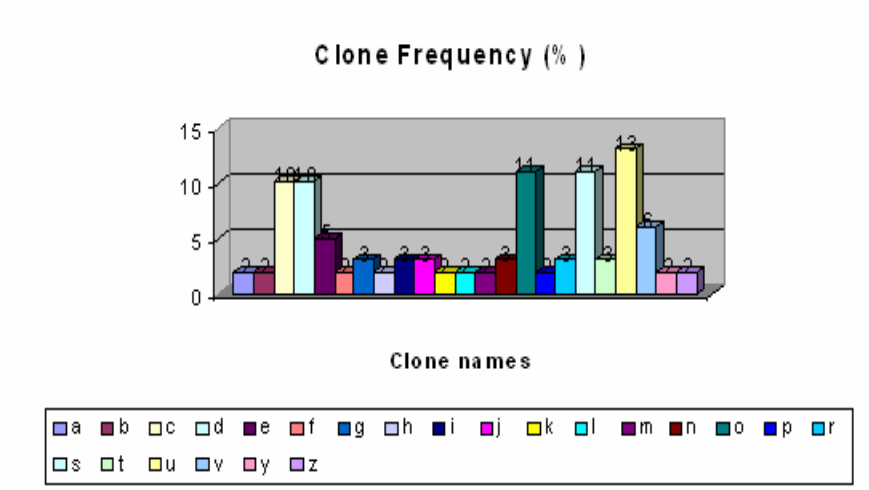

Figure 4.9. Percentages of archaeal clones in November 06 Tuzla sediments

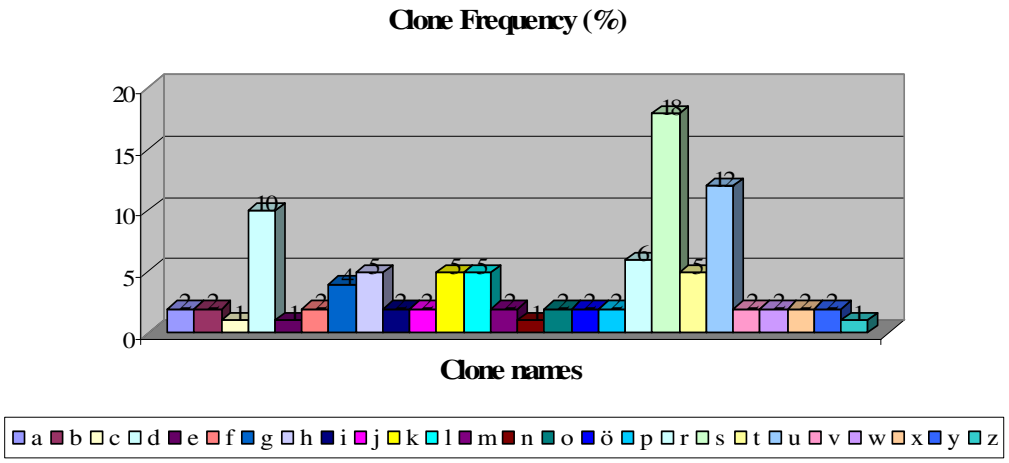

Figure 4.10. Percentages of archaeal clones in November 06 Moda sediments

The dominating sequence of the Tuzla 16S rRNA library is the putative clone (u) which constituted 13% of all the clones showing 82% similarity to his closest relative, *Methanolobus oregonensis WAL1,* followed by putative clone (o) showing 94 similarity to its closest relative *Methanosarcina lacustris strain MS* and the other one being putative

clone (s) showing 88% similarity to *Methanosaeta concilii Opfikon*, both of which alone cover 11% of all the clones. However, 12% of all the sequences showing less than 97% similarity were affiliated with uncultured archaeon clones whose closest cultured relative was *Methanosaeta concili.*

There are 3 dominant clones (s, u, d) in the archaeal clone library from Moda, *Uncultured crenarchaeote clone EJ-B02* constituting 18% of all the clones, *Uncultured euryarchaeote EHB97-16* constituting 12% and *Uncultured archaeon clone MKCSB-D5* constituting 10% of all the clones, respectively.

Methanogens dominated both the two 16S rDNA clone libraries. These methanogens could be assigned to subgroups Methanosarcinales, Methanobacteriales, Methanococcales and Methanomicrobiales. Methylotrophic methanogenesis, followed by acetoclastic methanogensis is assumed to be the dominating metabolic pathway in anoxic marine sediments from Tuzla, whereas hydrogenotrophic methanogensis seems to be highly favored in the anoxic marine sediments from Moda. Therefore, it is assumed that methanogenesis in Tuzla and Moda anoxic marine sediments represented a high proportion of the total carbon flow through the system, which was matched by the high proportion of archaeal sequences detected in these sediments, all of which are affiliated with methanogens. However, this assumption can only be proved after quantitative analysis of the whole microbial community together. The results derived in this study only give the relative figures since archaeal and bacterial communities are evaluated separately.

The dominancy of *Methanosaeta concilii* suggests that *Methanosaeta* organisms are important methanogens in anoxic marine sediments of Tuzla. It was suggested previously (Purdy et al., 2003) that the apparent ubiquity of clones related to *Methanosaeta* in freshwater sediments indicates that members of this group may be globally dominant acetoclastic methanogenic archaea which have a significant role in the degradation of acetate in freshwater systems (Go et al., 2000; Purdy et al., 2002), and the data presented here provide further support for the widespread importance of these organisms. Because of the abundancy of *Methanosaeta* species in Tuzla clone libraries, acetoclastic methanogenesis is one of the dominating terminal oxidation processes in the anoxic marine sediments of Tuzla.

Sequences detected in Tuzla clone library were all affiliated with isolates from anoxic habitats, mainly from cold terrestrial habitats (Simankova et al., 2003) and anoxic lake ecosystems (Cytryn et al., 2000), also from sewage digester (Eggen et al., 1990), gut ecosystem (Lin and Miller, 1998) and even from dental plaque (Kulik et al., 2001). Sequences retrieved from Moda clone library were more affiliated with isolates from marine environments, mainly from subsurface marine sediments (Inagaki et al., 2006), deep sea hydrothermal vents, tidal flat sediments (Kim et al., 2005), estuarine sediments (Kaku et al., 2005) as well as anaerobic sulfur spring (Elshahed et al., 2004), brackish freshwater sediments (Purdy et al., 2002), aquafarm sediments (Shao et al., 2004), mangrove sediment, anoxic rice fields and roots (Lu and Conrad, 2005) and even oil polluted saline soil which is of importance to indicate any microorganism present in our sediment samples with a potential to degrade petroleum hydrocarbons.

Acetoclastic methanogenesis is not the only pathway by which methane can be produced from acetate. It has been reported that syntrophic acetate degradation, in which acetate is oxidized with the production of H_2 which is then utilized by hydrogenotrophic methanogens, can be responsible for methane production in slurries amended with acetate (Nusslein et al., 2001). This correlated with the findings in this study that *Methanobrevibacter smithii* responsible from hydrogenotrophic methanogenesis constituted the rest of the archaeal clones by 14% and a sulphate reducer, *Desulfacinum subterraneum* although not constituting high proportions within the bacterial community is able to utilize acetate and produce H_2. Hydrogenotrophic methanogens are known to compete directly with sulfate reducers for hydrogen (Oremland and Taylor, 1977), thus this supports evidence for the syntrophic relationships between those groups and still relatively smaller proportion of the SRBs to hydrogenotrophs explain the dominancy of hydrogetrophic methanogens over SRBs.

However, in sediments where syntrophic acetate degradation occurs, no acetoclastic methanogens were detected in situ (Nusslein et al., 2001), in contrast to the results derived in this study. Although it seems likely that the methanogenic activity detected here is due to syntrophic acetate degradation, in overall the percentage of the methylotrophic methanogens dominate the sediments of Tuzla. The occurrence and dominancy of Methanosarcinales in these sediments can be explained by less competition with sulfate

reducers present as part of the bacterial community members. Members of the Methanosarcinales, detected in the anoxic sediments, are able to avoid competition by utilizing substrates like methylamines (Madigan et al., 2003) or dimethylsulfide (van der Maarel et al., 1997) that are generally neglected by most other physiological groups. These compounds are typically released during the decay of algae or other organisms and are mostly available near the sediment surface. In deeper layers, however, complex methylated aromatic compounds like humic acids or peat components (Killops and Killops, 2005) are a potential source of energy. *Methanosarcina* strains were shown to demethylate aromatic compounds like toluene sulfonate (Shcherbakova et al., 2003) or to demethoxylate lignin monomers (Phelps and Young**,** 1997). Thus, the ability of the methylotrophs to use non-competitive substrates might have favored them in the sediments of Tuzla and although *Methanosaeta* species are the dominant members of the community, the overall percentage of methylotrophs indicates the dominancy of the methylotrophic methanogesis as the dominating terminal oxidation process in these sediments.

4.4. Bacterial Diversity of Tuzla Bay and Moda

To estimate the overall bacterial diversity of Moda and Tuzla anoxic marine sediments, two general bacterial 16S rRNA libraries were constructed from sediment samples taken in November 2006.

For the identification of bacterial community, a total of 187 clones from Tuzla November 2006 sediment samples were randomly picked and screened, of which 79 clones were positive and a total of 279 clones from Moda November 2006 sediment samples were randomly picked and screened, of which 73 clones were positive. A total of clones, 79 from Tuzla sample and 73 from Moda sample, were screened by DGGE and grouped together based on their DGGE profiles. Representative clones were sequenced, and the 900 bp sequences were analyzed on 16s rRNA database of EBI to determine their phylogenetic affiliations. Bacterial clone identification for both sites is given in Table 4.10 and the metabolic distributions of archaeal clones are shown in given in Tables 4.11 and 4.12.

Tablo 4. 10. Results of phylogenetic analysis of bacterial community

Clostest identified relative of Tuzla clones	**Similarity %**	**Clone**	**Frequency %**
Uncultured bacterium clone RL183_aah27g12	100	a	2
Uncultured Acidobacteriales Belgica2005/10-140-14	100	b	2
Uncultured bacterium clone SZB8	100	c	2
Uncultured gamma proteobacterium Belgica2005/10-140-7	99	d	2
Uncultured actinobacterium clone Sylt 21	99	e	2
Uncultured bacterium clone SURF-GC205-Bac33	98	f	7
uncultured bacterium clone SZB76	98	g	7
Roseobacter sp. Strain UAzPsK-5	98	h	2
Uncultured Chloroflexi bacterium clone Y187	98	i	2
Uncultured epsilon proteobacterium BD2-5	98	j	4
Pelobacter sp. A3b3	98	k	2
Bacillus sp. Strain JH19	98	l	2
Uncultured bacterium clone KM87	98	m	2
Flavobacteriaceae bacterium T15	98	n	2
Uncultured bacterium clone 5-7	98	o	2
Proteobacterium Dex80-27	97	p	18
Epsilon proteobacterium 49MY	97	r	14
Sulfurovum lithotrophicum Strain 42BKT	97	s	2
Uncultured Desulfocapsa sp. clone SB1_88	97	t	2
Uncultured gamma proteobacterium VHS-B1-32	97	u	2
Alviniconcha aff. hessleri gill endosymbiont	97	v	4
Uncultured delta proteobacterium clone SM48	97	w	4
Uncultured bacterium clone GA456	96	x	2
Uncultured Holophaga sp. clone Hyd24-44	96	y	2
Uncultured bacterium clone DSBR-B064	96	z	2
Uncultured bacterium clone GN01-8.049	90	α	2
Uncultured candidate division GN10 bacterium	91	β	5
Uncultured bacterium clone MD2896-B28	89	γ	2
Halochromatium rosei	87	δ	2
Uncultured bacterium clone FCPP417	60	O	2
Gamma proteobacterium NEP68	98	σ	9
Closest identified relative of Moda clones	**Similarity**	**Clone**	**Frequency %**
Rhodobacteraceae bacterium CL-TA03	98	a	14
Unclassified/Paracoccus yeei	98	b	5
Catenibacterium mitsuokai	98	c	45

Tablo 4.10. Results of phylogenetic analysis of bacterial community (continued)

Finegoldia magna	90	d	8
Pseudoxanthomonas spadix	93	e	11
bacterium str. 37236	97	f	6

In contrast to the results derived from the archaeal community analysis for Tuzla and Moda sediment samples, bacterial sequences from Tuzla were relatively affiliated with more of uncultured clones on the 16S rRNA databases, whereas bacterial clones from Moda could be affiliated with cultured organisms on the 16S rRNA database. Of all the clones, only 7% of the sequences from Tuzla and 8% from Moda sediment samples showing less than 70% similarity could not be affiliated with any cultured or environmental clone on the databases.

Tablo 4. 11. Dominant metabolic pathways, substrates and percentages of bacterial clones from Moda clone libraries

Microorganism and Its Frequency	**Metabolism**	**Substrates**
Catenibacterium mitsuokai (45%)	Fermentation	Glucose, mannose, galactose, fructose, sucrose, maltose, cellulose, lactose, starch
Rhodobacteraceae bacterium CL-TA03 (14%)		Fatty acids, organic acids, amino acids, sugars, alcohols, aromatics
Pseudoxanthomonas spadix (11%)		Sugars, organic acids, aminoacids, H_2S
Finegoldia magna (8%)		Peptone
Clostridium sp. (8%)		Sugars, aminoacids
Clostridium saccharolyticum (3%)		Glucose, cellobiose, xylose
Paracoccus yeei (5%)	Nitrate Reduction	Glucose, lactose

Tablo 4. 12. Dominant metabolic pathways, substrates and percentages of bacterial clones from Tuzla clone libraries

Microorganism and Its Frequency	Metabolism	Substrates
Proteobacterium Dex80-27 (18%)	Nitrate Reduction	Sulfur, thiosulfate
Epsilon proteobacterium 49MY (14%)		Sulfur, thiosulfate
Sulfurovum lithotrophicum (4%)		Sulfur, thiosulfate
Endosymbiont of Ridgeia piscesae (4%)		Sulfur
Oligobrachia mashikoi (7%)		
Alviniconcha aff. hessleri gill endosymbiont (7%)		
Riftia pachyptila endosymbiont (2%)		carbon, hydrogen sulfide and nitrate
Endosymbiont of Lamellibrachia barhami (2%)		Sulfide
Candidatus Microthrix (2%)		Thiosulfate, bicarbonate
mixed culture isolate koll (4%)		Ammonia, nitrate, nitrite
Flavobacterium ferrugineum (2%)		Methanol
Gamma proteobacterium NEP68 (9%)	Fermentation	Nonylphenol polyethoxylate, acetate, pyruvate, propionate, s uccinate, lactate, tetraethylane glycol
Dehalococcoides sp. (5%)		Hexachlorobenzene, pentachlorobenzene
Caldilinea aerophila (2%)		Yeast extract, glucose, tryptone, sucrose, maltose, raffinose, starch, glycerol, acetate, pyruvate, lactate, succinate, fumarate, glutamate
marine gamma proteobacterium HTCC2246 (2%)		Formate
Roseobacter sp (2%)		Dimethylsulfoniopropionate, Mn(II)
Desulfoglaeba alkanexedens (2%)	Sulfate Reduction	n-alkanes
Desulfobulbus sp. BG25 (4%)		propionate, pyruvate, lactate, succinate, fumarate, malate, alanine, primary alcohols (C2–C5), mono- and disaccharides
Desulfacinum subterraneum (%2)		lactate, pyruvate, malate, fumarate, ethanol, formate, acetate, propionate, butyrate, yeast extract, H2+ CO2
Halochromatium rosei (2%)		Sulfur, sulfide

Tablo 4.12. Dominant metabolic pathways, substrates and percentages of bacterial clones from Tuzla clone libraries (continued)

Desulfotalea sp. (2%)	Sulfate Reduction	Lactate, alcohols, hydrogen
Acidimicrobium ferroxidans (2%)	Iron Reduction	Iron
Geobacter sp. CLFeRB (2%)		Mercury

Sequenced clones from Tuzla sediment samples fell into eight major lineages of Bacteria: the alpha, gamma, delta and epsilon Proteobacteria, Chloroflexi, Bacteroidetes, Actinobacteria, Acidobacteria and candidate division GN10. Gammaproteobacteria, Deltaproteobacteria, Bacteroidetes, and Planctomycetes have also been described from muddy sediments (Musat et al., 2006). Another study of microbial community composition of coastal marine sandy sediments over depth and over different seasons revealed high abundance of Planctomycetes, Bacteroidetes, Alphaproteobacteria (mainly in the upper layers of sediments), Gammaproteobacteria and of deltaproteobacterial sulphate reducers of the *Desulfosarcina/ Desulfococcux* group (Musat et al., 2006). Considering all the sequences, members of the Proteobacteria dominated Tuzla bacterial clone library, with 18% belonging to epsilonproteobacteria, %15 belonging to gammaproteobacteria, 7% belonging to deltaproteobacteria, and 2% belonging to alphaproteobacteria. In depth-related studies of microbial population changes in marine sediments, major phylogenetic groups of domain Bacteria: γ- and δ-Proteobacteria, high-GC Gram positive bacteria (Actinobacteria) and green non-sulphur bacteria and a candidate division of OP8, which was proposed by Hugenholtz et al. (1998), were recovered from sequenced clones (Musat et al., 2006). Another analysis of 16S rDNA libraries for investigation of genetic diversity of microbial communities in marine sediments also showed the presence of five major lineages of the Domain Bacteria: the gamma, delta and epsilon Protobacteria, high-GC Gram positive bacteria being the most dominant (Urakawa et al., 1999) in Sagami Bay and Tokya Bay, Japan. Sequences from Tuzla sediment samples related to Chloroflexi, Bacteriodes, Actinobacteria and Firmicutes accounted for 2% of all clones and these results from the study were in accordance with the findings in the literature as far as the common bacterial groups detected in other anoxic sediments are present in Tuzla sediments as well. Research of D'Hondt and his collegues (D'Hondt et al., 2004) reported the distribution of cultured members of γ-Proteobacteria to be consistently found at ocean-

margin sites, whereas to be rare at open-ocean sites. However, Actinobacteria were most consistently found in sulphate-reducing sediments of the open-ocean sites and in the relevant ocean-margin site. Clones from Tuzla sediment samples related to *Acidobacteria* accounted for 4% and sequences affiliated with candidate division GN10 accounted for 5% of all the clones. The kingdom Acidobacterium is a recently discovered bacterial lineage and contains only few cultured representatives (Ludwig et al., 1997). The Acidobacterium group seems to be present in many ecosystems, particularly in soils. The exact role and ecological significance of these bacteria is still unknown, however recent development indicate that members of the Acidobacterium are involved in methanol metabolism (Barns et al., 1999). Barns et al. (1999) have suggested that the members of the Acidobacterium kingdom could be genetically and metabolically diverse, as environmentally widespread and, perhaps, as ecologically important as important as the well-known Proteobacteria and Gram-positive bacterial kingdoms.

In overall 31 clones were derived from Tuzla sediment samples as shown in Figure 4.11. Only 21% of the sequences showed less than 97% (approximately 92%) similarity to their closest relatives on the database.

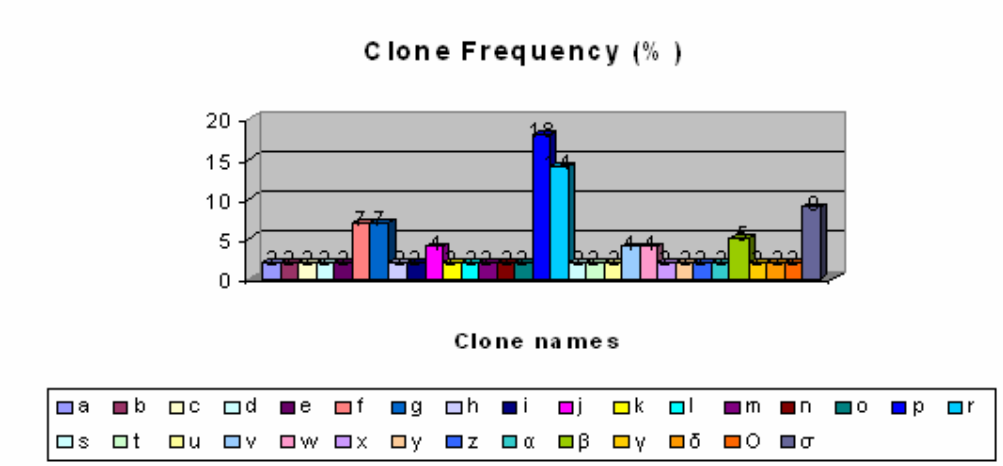

Figure 4.11. Percentages of bacterial clones in November 06 Tuzla sediments

Sequencing results of bacterial clones from Tuzla sediment samples shows that clone (p) showing 97% similarity to *Proteobacterium Dex80-27* dominates the archaeal clone library, followed by clone (r) affiliated with *Epsilon proteobacterium 49MY* by 97%

similarity constituting 13% of all the clones. The third dominant clone was (σ) showing 98% to *Gamma proteobacterium NEP68* which is able to utilize nonylphenols and various substrates acoounted for 9% of all the clones. The next dominant clone types, each accounting for 7% of all the clones were (f) and (g) with 98% similarity to their environmental relatives, *Uncultured bacterium clone SURF-GC205-Bac33* and *Uncultured bacterium clone SZB76*, respectively.

The sequence similarities and the coverage of bacterial clones from Tuzla to the cultured relatives on the 16S rRNA database was relatively higher than the values derived for the archaeal sequences since 78% of all the bacterial clones were found to show an average of 98% similarity, whereas only 37% of all the archaeal sequences from the same site showing higher than 97% could be affiliated to their cultured relatives, as previously mentioned.

In general, our clones were most closely related to groups already identified from marine sediments, cold seep environments or deep-sea hydrothermal vent environments. Majority of the bacterial clones sequenced in this study were related to isolates from a wide variety of environments such as marine sediments (Knittel et al., 2003; Brinkhoff et al., 2004; Asami et al., 2005; Musat et al., 2006), deep-sea hydrothermal vent environments (Inagaki et al., 2004a), coastal estuary (Hansel and Francis, 2006; Milbrandt, unpublished), harbor sediments, hypersaline sediments (Lloyd et al., 2006), reservoir sediment, mangrove sediment (Liang et al., 2007), white tubes and gastropods (Suzuki et al., 2005), Antarctic sediments (Purdy et al., 2003), poultry farm, feces (Ley et al., 2006), sludge, saltern (Anil Kumar et al., 2007).

Comparison analysis of Moda bacterial clones on 16S rRNA prokaryote database showed that sequenced clones fell into three lineages of Bacteria: Firmicutes and Alfaproteobacteria and Gammaproteobacteria. Firmicutes that are most closely related to *Bacillus firmus* and α- Proteobacteria which are most closely related to *Rhizobium radiobacter*, are the most commonly cultured taxa (D'Hondt et al., 2004).

The bacterial community in Moda was less diverse than the community diversity seen in Tuzla sediment samples. As it is seen in Figure 4.12, clone (c) affiliated with

Catenibacterium mitsuoka belonging to Clostridia division of bacteria dominated the Tuzla clone library covering 45% of all the clones, followed by Rhodobacterales family member clone (a), *Rhodobacteraceae bacterium CL-TA03* from alpha subdivision of Proteobacteria constituting 14 % of all the clones. The next dominant clone type was affiliated to an oil polluted soil isolate clone (e), *Pseudoxanthomonas spadix* with 93% similarity accounted for 11% of all the clones.

The majority of the clones identified in Moda clone library were related to isolates mainly from feces (Kageyama and Benno, 2000), clinical specimen (Daneshvar et al., 2003; Todo et al., 2004), biofilm in coastal fish-farm and sewage sludge (Stackebrandt et al., 1999).

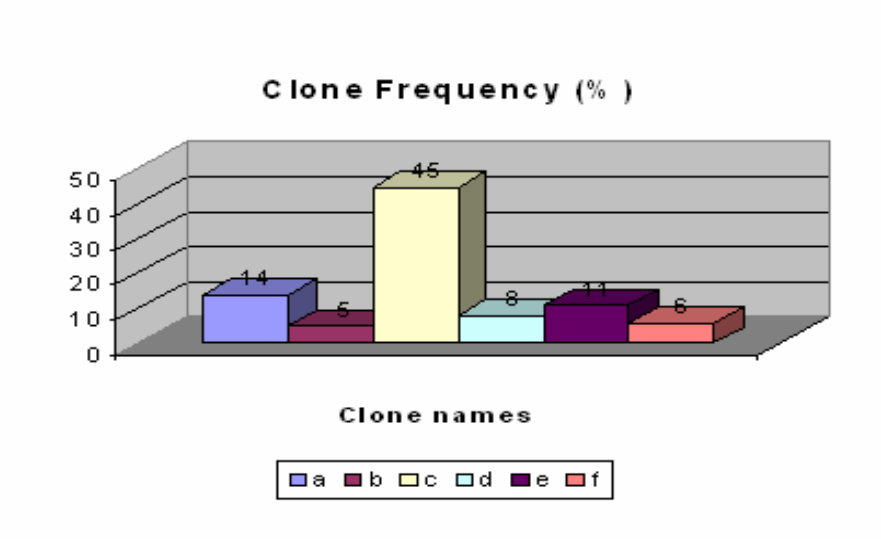

Figure 4.12. Percentages of bacterial clones in November 06 Moda sediments

Recent study of Schwarz and his collegues showed that community structure of Archaea and Bacteria in an anoxic lake sediment was dominated by Deltaproteobacteria, sulphate-reducers, syntrophs in particular, and Bacteroidetes-Chlorobi group as the second most dominant in the relevant study site (Schwarz et al., 2007). Clones belonging to sulphate reducing bacteria (δ-Proteobacteria) and sulfur oxidizing bacteria belonging to γ-Proteobacteria were also common by previous marine studies (Gray and Herwig, 1996; Urakawa et al., 1999). However, in contrast to the previous studies conducted in anoxic

sediments, our Tuzla sediments samples inhabited a small proportion of SRBs within the bacterial community, but were dominated by nitrate reducers (mainly sulfur oxidizers) as well. Purdy et al. (2003) also showed that sulfate reduction dominated the terminal oxidation processes in the Shallow Bay sediments and *Desulfotalea/Desulforhopalus* represented a substantial proportion of the prokaryotic community in the Shallow Bay sediments, and these organisms were the most commonly detected SRB in the Shallow Bay clone library along with *M. Concilii*. The 16S rRNA library from Tuzla sediment samples comprised sequences affiliated with a variety of metabolic groups: nitrate reducing bacteria that accounted for 51%, fermentative bacteria that accounted for 23%, sulphate reducing bacteria that accounted for 8% of all the clones and iron reducing bacteria that accounted for 2%. The closest environmental relative of *Uncultured Acidobacteriales bacterium clone Belgica2005/10-140-14* showing 85% similarity was found to be *Desulfoglaeba alkanexedens* isolate from oily sludge capable of degrading n-alkanes accounts for 4% of all the clones. Moreover, *Uncultured candidate division GN10 bacterium* showing 77% similarity to its closest environmental relative was found to be *Dehalococcoides sp. CBDB1* isolate from cholorobenzene degrading mixed culture (Jayachandran et al., 2003) which metabolizes hekzaklorobenzens and pentaklorobenzens is another clone of importance detected in the Tuzla sediment samples.

Moda sediment samples inhabited mainly two metabolic groups, one of which is fermentative bacteria dominating the samples by 89% and the other one *Paracoccus yeei*, being the only representative of nitrate reducers accounting for 5% of all the clones. Among the important metabolic groups, a fermentative bacterium *Pseudoxanthomonas spadix*, that is capable of of H_2S removal and may have the potential to degrade hydrocarbons since it's an oil contaminated soil isolate (Young et al., 2007), was related to clone (e) detected in our 16S rRNA library having relatively a high proportion within the bacterial clone library by 11%. In Moda sediment samples, the detected bacterial communities appear to be dominated by fermenters which are known to represent the majority of microorganisms in anoxic sediments (Schink, 2002).

In sediment samples from Tuzla, fermenters being the second dominant metabolic group also have relatively a high proportion within the whole bacterial community. Fermenting microorganisms are independent of sulfate, however, by releasing fermentation

products they provide substrates for terminal oxidizers like sulfate reducers and methanogens (Garcia et al., 2000). Sulfate reducing bacteria, in turn, rely on the availability of sulfate but do not obviously belong to the most abundant bacterial groups in sediments, even in those exhibiting intense sulfate reduction (Llobet-Brossa et al., 2002). This dovetails with the findings of the study, since SRBs are also found in the sediment samples from Tuzla but not dominate and probably they are present in the sediment samples from Moda, however being dependent on sulfate they can't compete with fermenters and only a small proportion may be maintained by the products of fermentation. The small percentage of SRBs found in Tuzla sediments and that SRBs were not present in Moda sediment samples, although they have been shown to be important community members in marine sediments (Sahm et al., 1999) could reflect the fact that their population sizes were under the limit of detection. Moreover, as previously mentioned, data derived from archaeal clone libraries showed the presence of uncultured archaea and *Methanosarcinales* that could be important in anaerobic oxidation of methane (AOM). Sulfate reducing bacteria (SRBs) are also found to be present along with ANME groups of archaea and get involved in the AOM process. Previous studies indicate a close metabolic association between SRB and methanotrophic archaea (Pancost et al., 2001). For example, sulfate-reducing bacteria affiliated to members of the genera *Desulfosarcina* and *Desulfococcus* have been found in association with ANME-1 and ANME-2 archaeal cells, representing in both cases putative methanotrophic consortia (Orphan et al., 2001; Michaelis et al., 2002). Thus analysis of functional genes can be aimed for future studies to detect the presence and abundance of SRBs in these sediments and support the findings of this study. Although nitrate reducers are the most dominant metabolic group in the bacterial clone library of Tuzla anoxic marine sediments, the nitrate concentration found in the anoxic sediment samples of Tuzla collected in November 2006, expected as the excess electron acceptor within the sediment was inconclusive. This may be because of nitrate reduction rates being quite high and the nitrate might have been consumed excessively thus the amount of NO_3^{-2} was not within the detectable limits of ion chromatograph or as it was put forth in the study of Nijburg et al. (1997), there was no correlation between NO_3^{-2} availability and the quantitative increase in the total number and activity of the potential nitrate-reducing bacteria. Since nitrate-reducing bacteria are common members of the total organotrophic bacterial community in sediments, their presence in the bulk sediment does not necessarily mean that their proliferation has been due to nitrate reduction. Other factors

might be responsible for their growth in the rhizosphere and bulk sediment (Nijburg et al., 1997). From another point of view, it should be considered that even the routine marine sampling, which generally involves sample sizes greater than 1 liter, homogenizes bacterial populations, destroying any spatial patchiness information the denitrification process itself exhibits temporal and spatial patchiness (Duarte and Vaque, 1992) and it seems likely that the microbes responsible for the process are also nonrandomly distributed (Scala and Kerkhof, 2000).

4.5. Archaeal Diversity and Distribution Patterns in Tuzla Bay and Moda

The community structure of the Archaea was investigated by DGGE analyses targeting the 16S rRNA gene using gDNA extracts from Moda and Tuzla sediment samples collected in November 2006. In order to identify the dominant representatives of DGGE banding patterns from November 2006 samples and then investigate the seasonal distribution of microbial groups, 16S rRNA gene fragments of PCR-amplified products were run with sequenced representative clones from Moda and Tuzla archaeal and bacterial16S rDNA clone libraries.

The archaeal banding pattern of Tuzla showed 5 bands from November 2006 sediment sample matching the dominant clones from the archaeal 16S rDNA library as shown in the Figure 4.13. Bands (i), (ii), (iv) and (v) showed high intensity which refers to the dominant types within the sample. Band (i) was an unclassified organism. Band (ii) was *Methanobrevibacter smithii B181*. Band (iii) was *Euryarchaeote J4.75-15*. Band (iv) *Methanosaeta concilii Opfikon*. Band (v) was *Methanosarcina lacustris strain MS*. Band (iii) which showed relatively low intensity compared to other four bands was related to *Euryarchaeote J4.75-15* from the clone library having relatively a smaller proportion among the other dominant clone types. DGGE is a technique which allows detecting the abundant types within a sample and the minor groups which are assumed to be quantitatively small are difficult to detect or show less band less intensity. Thus our results from the DGGE analysis correlates with the results from the analysis of clone libraries, since all of the four dominant bands (i, ii, iv, v) representing the major groups within Tuzla sediment samples matched with clones constituting high proportions in the overall 16s rDNA archaeal library of Tuzla sediments, whereas a less intense band (iii) matched a

clone showing relatively a smaller proportion in the overall archaeal community. The groups detected by DGEE correlates with the results from the cloning and sequencing of archaeal clones from Tuzla, since all the detected phylotypes are members of Euryarchaeota. As stated in many other previous studies, the presence of Euryarchaeota in marine sediments was not unexpected. The DGGE results in this study also showed the dominancy of Euryarchaeota in Tuzla samples as well.

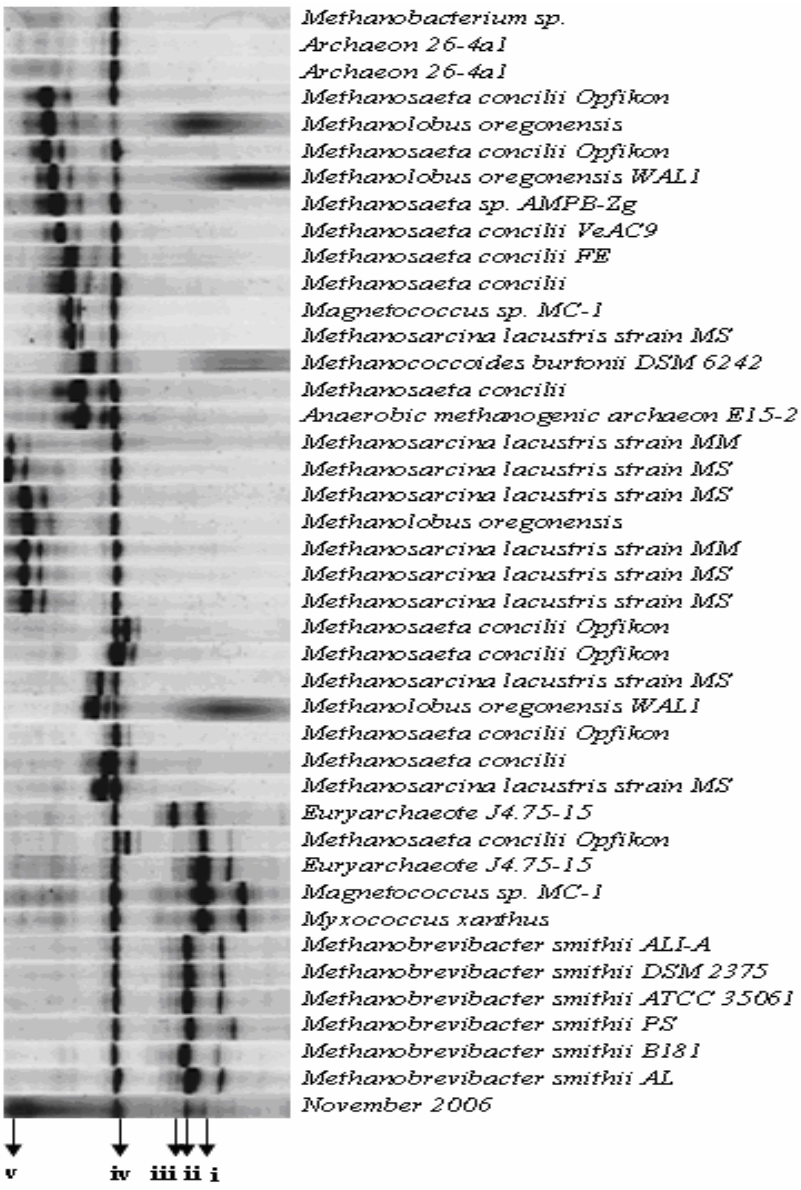

Figure 4.13. Archaeal clones and the PCR-amplified archaeal product (November 2006) from Tuzla sediment samples

The archaeal banding pattern of Moda showed 11 bands from November 2006 sediment samples matching the clones from the archaeal 16S rDNA library as in Figure 4.14.

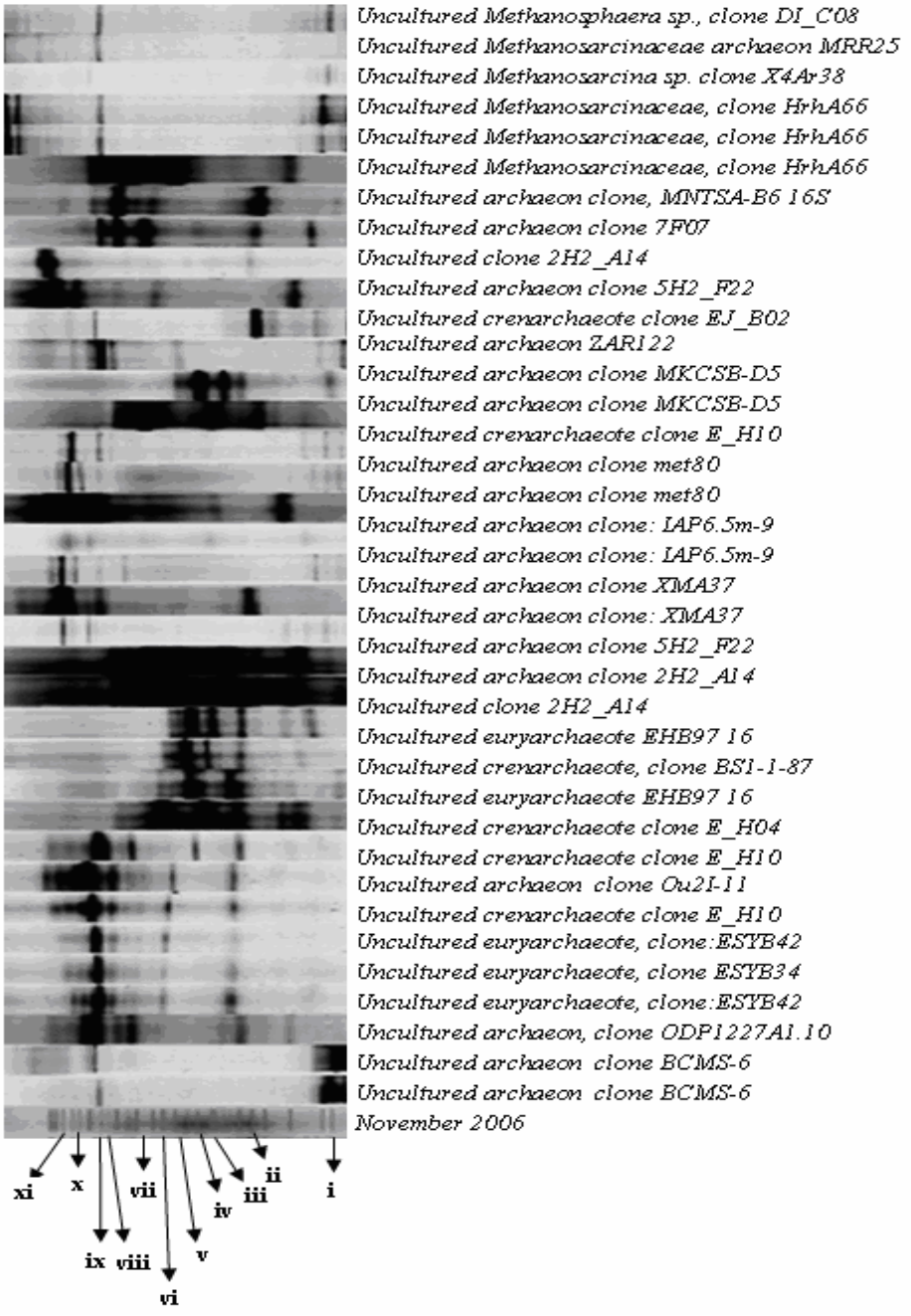

Figure 4.14. Archaeal clones and the PCR-amplified archaeal product (November 2006) from Moda sediment samples

Bands (ii) and (v), followed by band (vii) showed higher intensity compared to other bands out of eight and matched with *Uncultured crenarchaeote clone EJ_B02*, *Uncultured archaeon clone MKCSB-D5* and *Uncultured crenarchaeote clone E_H04*, respectively. Moreover, the second most dominant clone type *Uncultured euryarchaeote EHB9-16* matched band (iv) from November 2006 samples, as well. Band (i) was *Uncultured archaeon clone BCMS-6*. Band (iii) was *Uncultured crenarchaeote BS1-1-87*. Band (vi) was *Uncultured archaeon clone 7F*. Band (viii) was *Uncultured archaeon clone ODP1227A1.10*. Band (ix) was *Uncultured euryarchaeote clone ESYB34*. Band (x) was *Uncultured archaeon clone 2H2_A14* and band (xi) was *Uncultured archaeon clone 5H2_F22*. In overall the November 2006 sample gave 21 bands, most of which had high intensities and the rest of the visible but faint bands also matched clones from the library which had fewer proportions in the overall library. The bands related with the members of Euryarchaeote and Crenarchaeote, also correlates with the previous findings since those kingdoms represent the dominant groups in such environments.

In order to determine the seasonal changes on the archaeal communities present in Tuzla and Moda sediment samples taken at different time intervals, DGGE analysis was carried out on nested 200 bp 16S rDNA products, generated by using archaea specific primers Arch344f-Arch522r. These PCR products were shorter and their denaturing characteristics were more suitable for DGGE analysis**.**

In November 2006 and December 2005 Tuzla sediment samples 21 bands, in March 2006 sample 20 bands and in February 2007 sample 14 bands were detected as shown in Figure 4.15. Banding pattern of September 2005 Tuzla samples showed 23 bands indicating a higher diversity compared to other seasons. Based on the 16S rDNA cloning data, the dominant bands were compared to the banding patterns of other seasons through the analysis of DGGE gels on Bionumerics software. Since same sequence types are assumed to migrate to the same position on DGGE gels, the unclassified clone detected in November 2006 sediment samples were also seen in March 2006 and February 2007 sediment samples. *Methanobrevibacter smithii B181* and *Methanosarcina lacustris strain MS* were only seen in November 2006 and February 2007 samples. *Methanosaeta concilii Opfikon* showing a very high intensity was present at all the seasons. This may be because of changes in temperatures and relate to a change in microbial community over different

seasons, since it has been found that low temperatures apparently inhibit hydrogenotrophic methanogenic archaea and shift sedimentary metabolism toward acetogenesis and acetoclastic methanogenesis (Schulz and Conrad, 1996; Nozhevnikova et al., 1997). Thus the presence of *Methanosaeta conclii Opfikon* at all times may indicate acetoclastic methanogenesis becoming dominant by the changing temperatures as a result of seasonal change. There were two more bands detected at all times, however they couldn't be matched with any clones from Tuzla archaeal library. There were 4 bands unique for September 2005 samples and 2 bands February 2007 samples that couldn't be matched with any clones. Thus more number of clones should be screened or to avoid the time consuming process of clone screening, directly bands from the gels can be excised and reamplified and sequenced. However, creating a clone library which contains larger gene sequences provides a more positive identification but a large number of clones have to be sequenced to get an appreciation of diversity and clone libraries are victims of the same biases as other PCR methods (Hofman-Bang et al., 2003). The intensity of the bands matching *Methanosaeta concilii Opfikon* indicates its predominancy at all times which is relatively supported by its being a predominant clone in the archaeal clone library of Tuzla from November 2006 sediment samples. Another strain of *Methanobrevibacter smithii strain DSM* and *Methanosaeta concilii* were only detected in September 2005, December 2005 and March 2006 samples as shown bands (A) and (B) in the Figure 4.15. However, the low intensity of the bands (A) for *Methanobrevibacter smithii strain DSM* shows less abundancy, whereas the highly intense bands (B) for *Methanosaeta concilii* reveals this organism to be one of the predominant groups in September 2005, December 2005 and March 2006 samples.

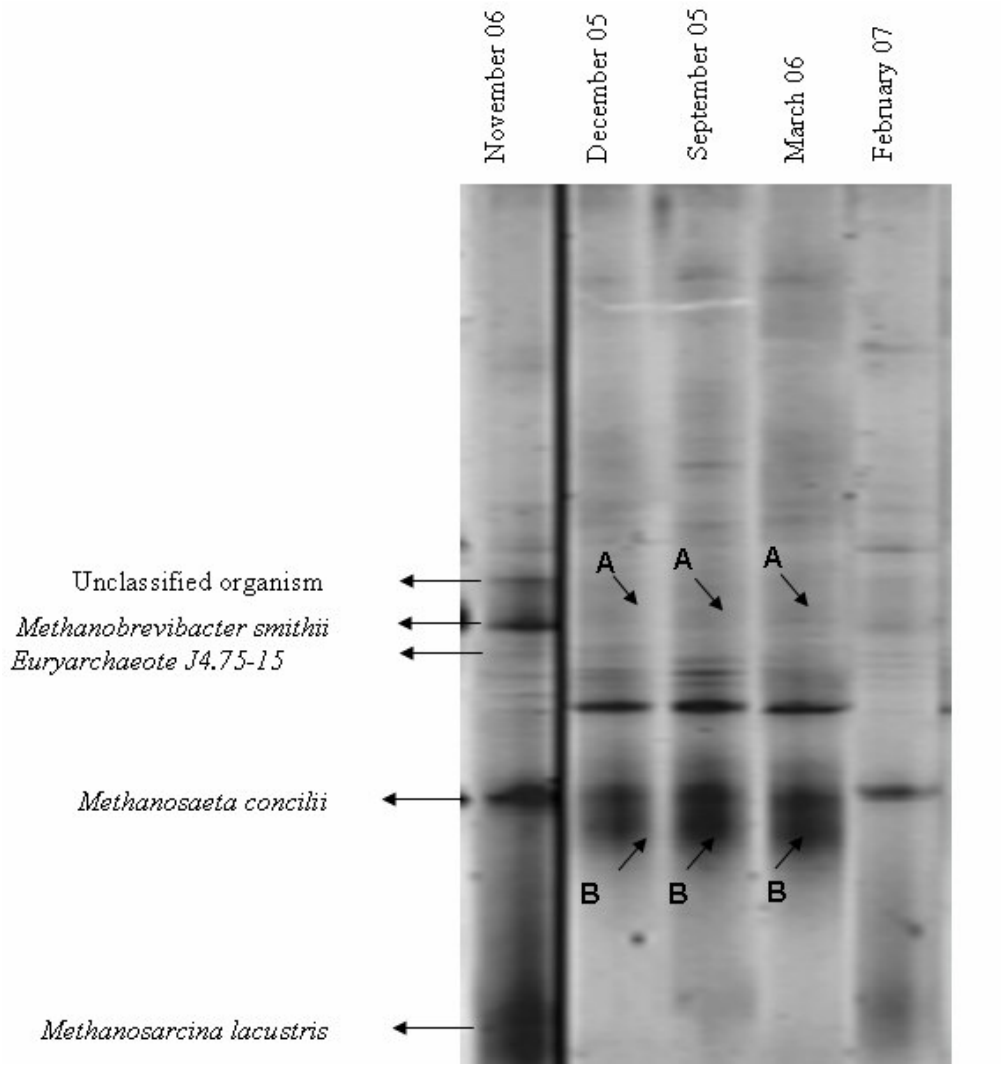

Figure 4.15. Analysis of archaeal community in sediment samples from Tuzla at different seasons

The phylogenetic analysis of the Tuzla sediment samples shows that December 2005 and March 2006 samples fell into same cluster and were related to September 2005 samples, whereas November 2006 and February 2006 sediment samples formed a cluster and distantly related to other samples as shown in the phylogenetic tree in the Figure 4.16.

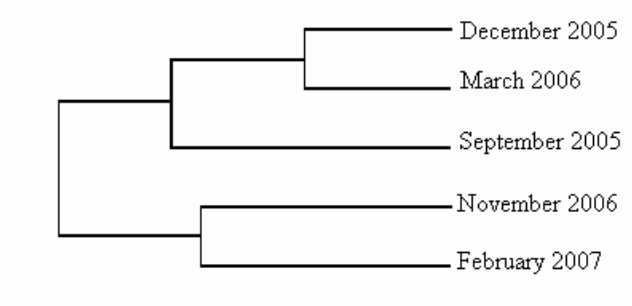

Figure 4.16. Phylogenetic analysis of Tuzla archaeal samples by Treecon

In November 2006 Moda sediment samples 28 bands were detected indicating the highest diversity of all other times as it can be seen in Figure 4.17. In March 2006 and February 2007 Moda sediment samples, 27 bands and 21 bands were detected, respectively. Based on the 16S rRNA cloning data, the dominant bands shown in the Figure 8.14 were compared to the banding patterns of other seasons through the analysis of DGGE gels on Bionumerics software. *Crenarchaeote clone EJ_B02* being the predominant clone of the Moda archaeal clone library also showed the highest intensity of bands on DGGE gel. *Uncultured archaeon clone BCMS-6, Crenarchaeote clone EJ_B02, Uncultured crenarchaeote clone BS1-1-87, Uncultured archaeon clone MKCSB-D5, Uncultured archaeon clone 7F, Uncultured archaeon, clone ODP1227A1.10, Uncultured euryarchaeote clone ESYB34, Uncultured archaeon clone 2H2_A14* and *Uncultured archaeon clone 5H2_F22* detected in November 2006 Moda sediment samples were also seen at all times based on the analysis of DGGE gels reflecting the seasonal community changes. The clone type *Uncultured crenarchaeote clone E_H04* detected in November 2006 was also seen in March 2006, but not in February 2007 sediment samples. There were seven more bands detected at all times through DGGE analysis but none of them matched any clones on the clone library which suggests cloning of more clones or due to same biases of PCR methods (Hofman-Bang et al., 2003).

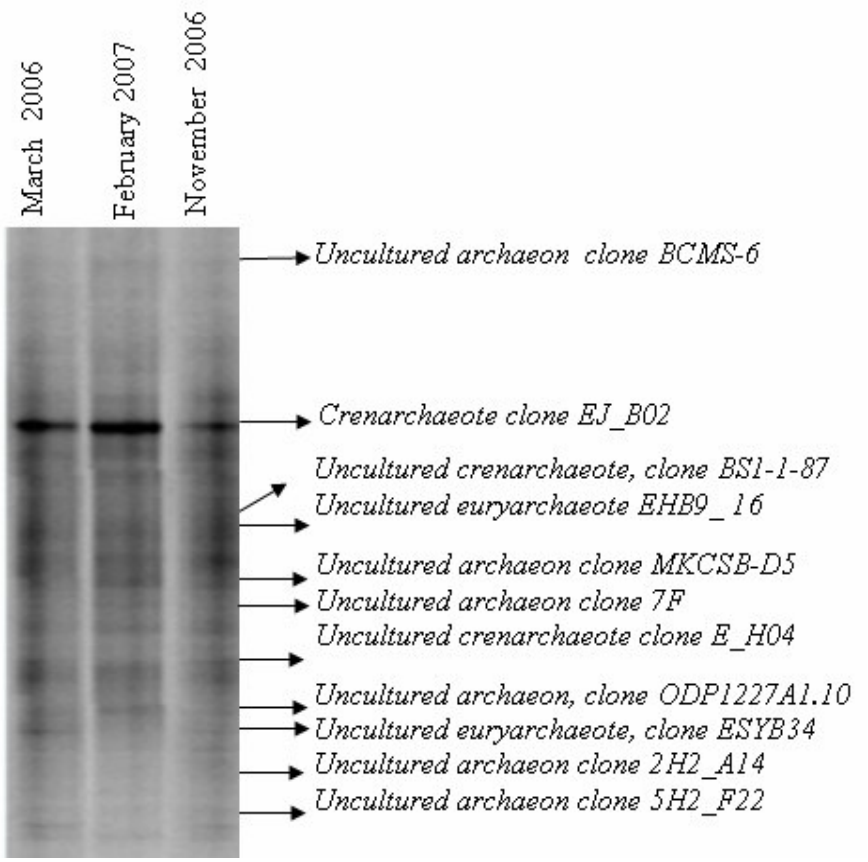

Figure 4.17. Analysis of archaeal community in sediment samples from Moda at different seasons

Based on the comparison analysis of seasonal community changes of Moda samples, March 2006 and November 2006 samples did not show a significant community shift and were closer to each other but community pattern of February 2007 sample was relatively distant from the other two sediment samples as shown in the tree in Figure 4.18.

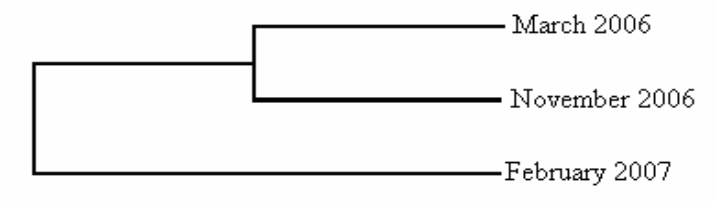

Figure 4.18. Phylogenetic analysis of Moda archaeal samples by Treecon

4.6. Bacterial Diversity and Distribution Patterns in Tuzla Bay and Moda

The community structure of the Bacteria was investigated by DGGE analyses targeting the 16S rRNA gene using gDNA extracts from Moda and Tuzla sediment samples collected in November 2006. In order to identify the dominant representatives of DGGE banding patterns from November 2006 samples and then investigate the seasonal distribution of microbial groups, 16S rRNA gene fragments of PCR-amplified products were run with sequenced representative clones from Moda and Tuzla archaeal and bacterial16S rDNA clone libraries

The bacterial banding pattern of Tuzla showed 11 bands from November 2006 sediment sample matching the dominant clones from the bacterial 16S rDNA library as it can be seen in Figure 4.19. Bands (i), (v) and (vi) matched the predominating members of the bacterial clone library for Tuzla, *Proteobacterium Dex80-27*, *Epsilon proteobacterium 49MY* ,*Uncultured bacterium clone SURF-GC205-Bac33*, respectively. The band (i) which was affiliated with *Proteobacterium Dex80-27* constituting 17% of the all the bacterial clones gave a distinct and but relatively a faint band compared to other bands derived on DGGE gel, and band (v) was affiliated with the second dominant clone type *Epsilon proteobacterium 49MY* in the bacterial clone library accounting for 13% of all the clones gave a thin but quite an intense band that comigrated with another band. Bands (ii), (iii) and (iv) also matched with clones from bacterial 16s rDNA library and gave distinct and visible bands on DGGE gels, band (ii) is one of the distinct bands on the gel that matched *Uncultured candidate division GN10 bacterium* which has a proportion of 5% in the clone library. Bands (vii) matching the *Uncultured bacterium clone SZB76* accounting for 7% of all the clones in bacterial 16S rRNA clone library gave a very distinct and a highly intense DGGE band. Band (iii) was *Uncultured candidate division GN10 bacterium*, band (iv) was *Uncultured gamma proteobacterium clone VHS-B3-62*, band (viii) was *Uncultured Chloroflexi bacterium clone Y187*, band (ix) was an unclassified clone, band (x) was *Uncultured Holophaga sp. clone Hyd24-44*, and band (xi) was *Uncultured bacterium clone RL183_aah27g12*.

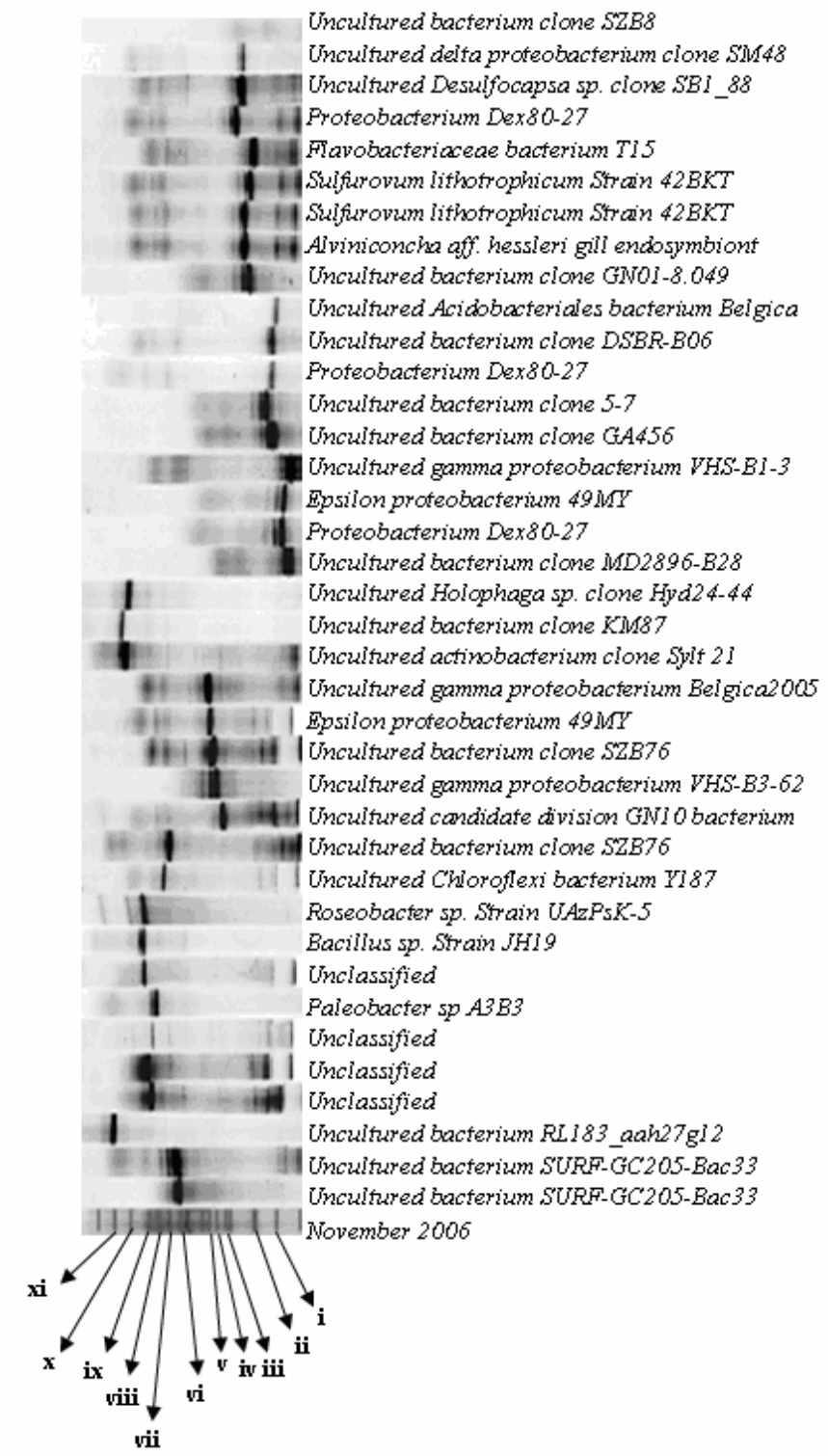

Figure 4.19. Bacterial clones and the PCR-amplified bacterial product (November 2006) from Tuzla sediment samples

The bacterial banding pattern of Moda showed 6 bands matching all the predominant and the rest of the clones detected within the bacterial clone library of Moda sediments, as it can be seen in Figure 4.20.

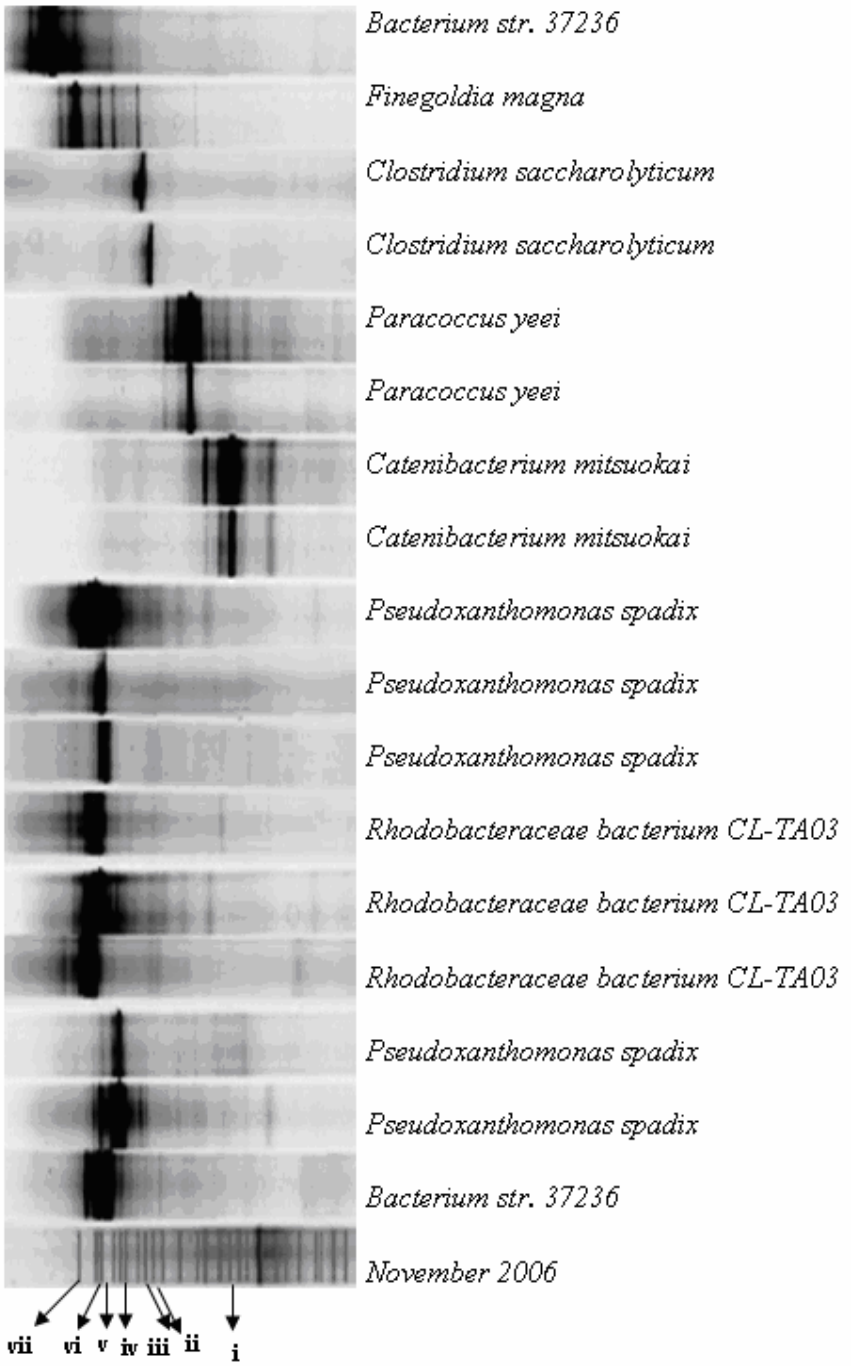

Figure 4.20. Bacterial clones and the PCR-amplified bacterial product (November 2006) from Moda sediment samples

Only *Bacterium str. 37236* and *Paracoccus yeei* which have relatively smaller proportions compared to the other clones detected by DGGE, could not match any of the visible bands. This may be due to some bias from the PCR reaction that might have caused the clones to migrate different positions on the DGGE gels or due to a drawback of DGGE since the reproducibility is not optimal, one DNA fragment may generate more than one band on the gel and a DNA sample analyzed on two different gels may not generate the same band pattern (Muyzer and Smalla, 1998). Bands (i), (v), (iv) matched *Catenibacterium mitsuokai, Rhodobacteraceae bacterium CL-TA03* and *Pseudoxanthomonas spadix* respectively, which are the most dominant clone types in the bacterial clone library for Tuzla. The bacterial banding pattern for Moda gave bands with have higher intensities compared to the bands matching with the clones but those couldn't match with any clones in the bacterial clone library. It is presumed that we couldn't detect all the representative clones which are dominant as well, since there are intense bands, semi-quantitatively indicating the abundancy of the species that it would match. Thus, it is necessary to increase the number of clones screened to increase the number of representative clones that would match the bands detected through DGGE. Band (ii) was *Clostridium saccharolyticum sp*. Band (iii) was *Clostridium saccharolyticum*. Band (iv) was *Rhodobacteraceae bacterium CL-TA03* and band (vii) was *Finegoldia magna*.

In order to determine the seasonal changes on the bacterial communities present in Tuzla and Moda sediment samples taken at different time intervals, DGGE analysis was carried out on nested 200 bp 16S rDNA products, generated by using bacteria specific primers Vf-Vf and spanning only the hypervariable V3 region These PCR products were shorter and their denaturing characteristics were more suitable for DGGE analysis.

DGGE is an advantageous technique in terms of its ability to resolve the microbial diversity of up to 15 different species by optimizing the DGGE in the gel. February 2007 from Tuzla sediment sample gave 26 bacterial bands most of which were clear, distinct bands and thus showed the highest diversity of all times under investigation throughout the study, followed by November 2006 sediment samples with 24 bands. In March 2006 samples 22 bands, in December 2005 samples 19 bands and in September 2005 sediment samples 18 bands were detected (Figure 4.21).

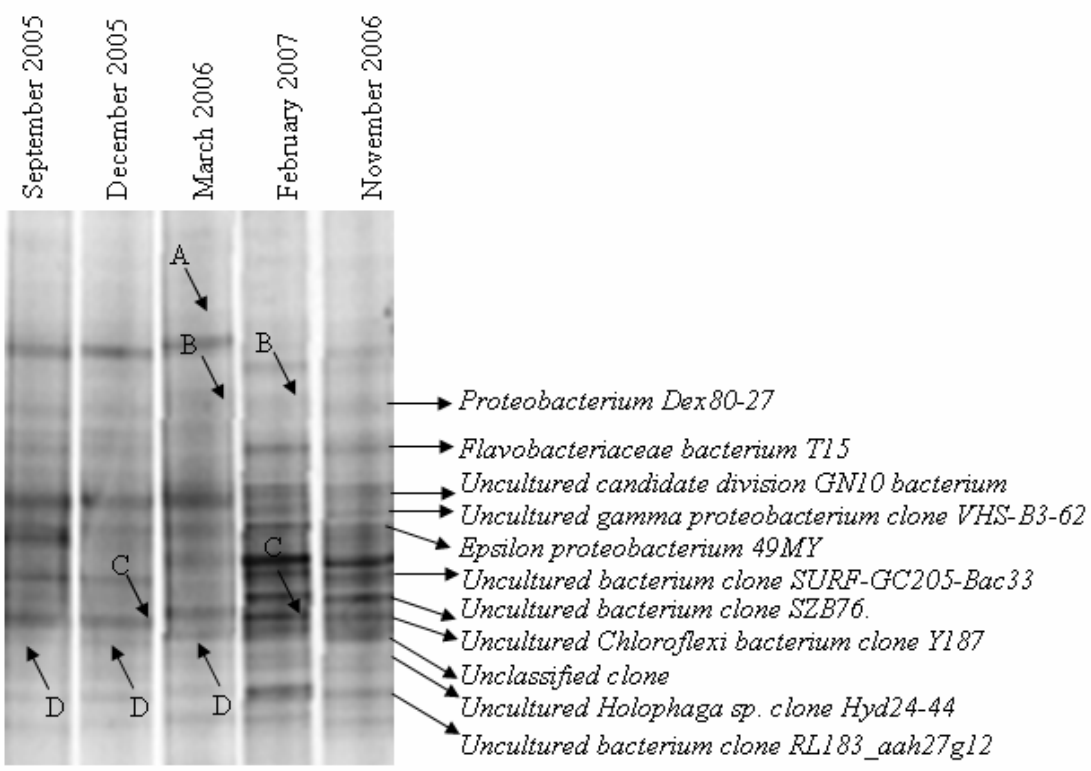

Figure 4.21. Analysis of bacterial community in sediment samples from Tuzla at different seasons

Some clones which were detected in 16S rDNA bacterial clone library but didn't show any match to the banding pattern of November 2006 samples, matched bands from other seasons. November 2006 sediment samples gave 4 unique bands not seen at other times, however they could not be affiliated with any clones on the bacterial clone library of Tuzla. February 2007 showed 3, and December 2005 showed 1 unique band. March 2006 gave 7 unique bands, one of which is band (A) as shown in the Figure 4.21 matching *Uncultured bacterium clone SZB8*. This clone is related to its environmental relative *Epsilon proteobacterium 49MY* which is a sulphur-oxidizer. Band B, which was also found to be related to another sulphur-oxidizer, *Proteobacterium Dex80-27* by 97% similarity, was seen both in March 2006 and February 2007 samples, although *Proteobacterium Dex80-27* migrated to a slightly different position in the November 2006 samples. One of the reasons for that when there are numerous bands as its seen for bacterial fingerprinting of Tuzla, there is difficulty to compare the patterns and there is especially little sensitivity of detection of rare community members. The oxidation of reduced sulfur compounds is a

major metabolic process in spring. Therefore the only detected bands to be affiliated with sulfur oxidizer related phylotypes confirms with previous studies (Perreault et al., 2007). Perreault et al. (2007) also found that sequences related to sulfur oxidizers were the most abundant and grouped into three subclasses of the Proteobacteria (Beta-, Epsilon-, and Gammaproteobacteria). Band (C) detected both in December 2005 and February 2007 samples matched *Paleobacter sp. A3B3* (Figure 4.21). Since the intensity of the bands give a semi-quantitative estimation of the numbers of a community, it's estimated according to the band intensities as seen on the DGGE gel for Paleobacter that number of the species increased in February 2007 sediment samples and even become one of the most dominant members of the whole community. Another clone type *Bacillus sp. Strain JH19* referring band (D) was seen in September 2005, December 2005 and March 2006 samples of Tuzla marine sediments. As it can be seen from the Figure 4.21, the intensity of the Band (D) increases throughout the time and number of *Bacillus sp.* species increases in March 2006 and it also becomes one of the dominant types in March 2006, since it's one of the six distinct and intense bands detected in the banding pattern for March 2006 sample.

The phylogenetic analysis of the Tuzla sediment samples shows that December 2005 and September 2005 samples fell into same cluster and were distantly related to March 2006 samples, whereas November 2006 and February 2006 sediment samples formed a cluster and distantly related to other samples as shown in the phylogenetic tree in the Figure 4.22. The bacterial phylogenetic relatedness was similar to that of archaeal relatedness. However, the seasonal community shift detected among bacteria in the samples were more than what was seen among archaea. The archaeal community showed a slight change, thus it's still possible to say that seasonal changes affect the community composition.

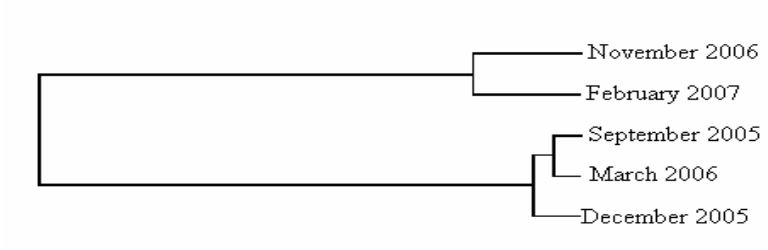

Figure 4.22. Phylogenetic analysis of Tuzla bacterial samples by Treecon

The bacterial banding pattern of Moda sediment samples gave 28 eight bands for November 2006 and February 2007 samples. March 2006 sediment samples gave 27 bands as shown in the Figure 4.23. Some clones which were detected in 16s rDNA bacterial clone library but didn't show any match to the banding pattern of November 2006 samples, matched bands from other seasons. This may be explained by a mismatch at the 3' end of the primer used. A single mismatch at or near the terminal 3' base of a primer affects PCR more dramatically than a single mismatch located internally or at the 5' end (Şimsek and Adnan, 2000). Bacterial clones, *Catenibacterium mitsuokai*, *Clostridium saccharolyticum sp*. and *Finegoldia magna* detected in November 2006 samples were also seen at all times from anoxic marine sediments from Moda. Based on the intensity of the bands relating to semi-quantitative estimation of abundancy of species in the community, it can be stated that the abundancy of *Catenibacterium mitsuokai* increased from March 2006 to November 2006, showing a clear and distinct band in February indicating a possible increase in the community members of the community. The intensity of the band relating *Clostridium saccharolyticum sp*. shows that the abundancy of the clone was highest in February 2007 and *Finegoldia magna* gave relatively the same intensity of band and its abundancy didn't change by time. The intensity of the band Bacterium str. 37236 decreased throughout the time and gave a fainter band in February 2007, whereas the band intensity seemed to be higher in March 2006, followed by November 2006. Another clone, *Pseudoxanthomonas spadix* detected in November 2006 samples was also seen in March 2006 sediment sample and the intensity of the band slight changed indicating a slight increase in the number of *Pseudoxanthomonas spadix* species within the community as well. *Rhodobacteraceae bacterium CL-TA03* was seen both in November 2006 and February 2007 sediment samples and the clear and distinctly thick band seen in the banding pattern for February 2007 reveals that its abundancy increased considerably. *Paracoccus yeei* detected in November 2006 bacterial clone library which couldn't match any bands from the same sample on DGGE analysis matched the band (A) from the banding pattern of February 2007 samples and was only unique to that sample.

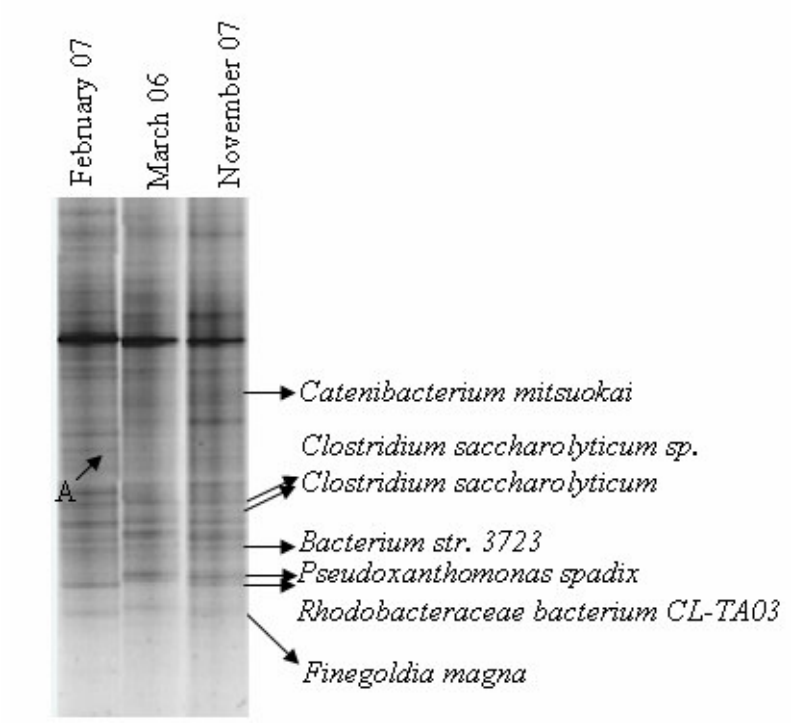

Figure 4. 23. Analysis of bacterial community in sediment samples from Moda at different seasons

The number of bands reflecting the bacterial diversity in the sediment samples from Moda and the phylogenetic tree seen in the Figure 4.24 indicates that the bacterial diversity did not change significantly but showed a slight change from March 2006 samples in February and November 2006.

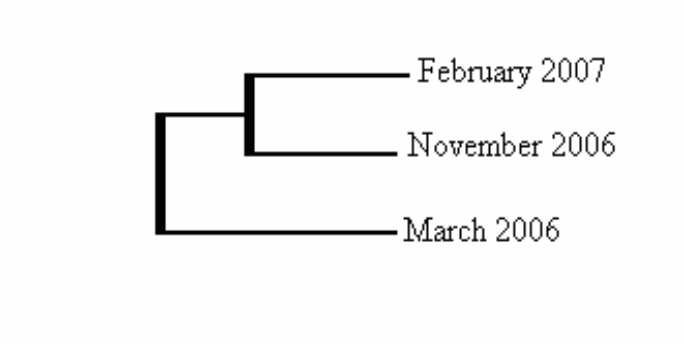

Figure 4. 24. Phylogenetic analysis of Moda bacterial samples by Treecon

Adopting a cautious approach to the molecular methodology to minimize the amplification of contaminating sequences is important because recent studies using 16S rRNA gene approaches with deep sediments have shown problems with PCR amplification of negative controls and probable contamination from γ-Proteobacteria (Kormas et al., 2003). In spite of DGGE being a useful method for providing a fingerprint of the composition of the dominant members of a community, the presence of 16S rRNA genes corresponding particular organisms does not necessarily imply that those organisms are active. Other molecular approaches for determining the activity of the dominant community members in important metabolic processes should be applied for further studies.

Based on the data derived from cloning which was complemented with DGGE method, all of the important clone types and the metabolic pathways, as previously mentioned, can be seen below in Figures 4.25 and 4.26. All the possible dominant pathways and processes that may be of environmental importance are shown by red arrows.

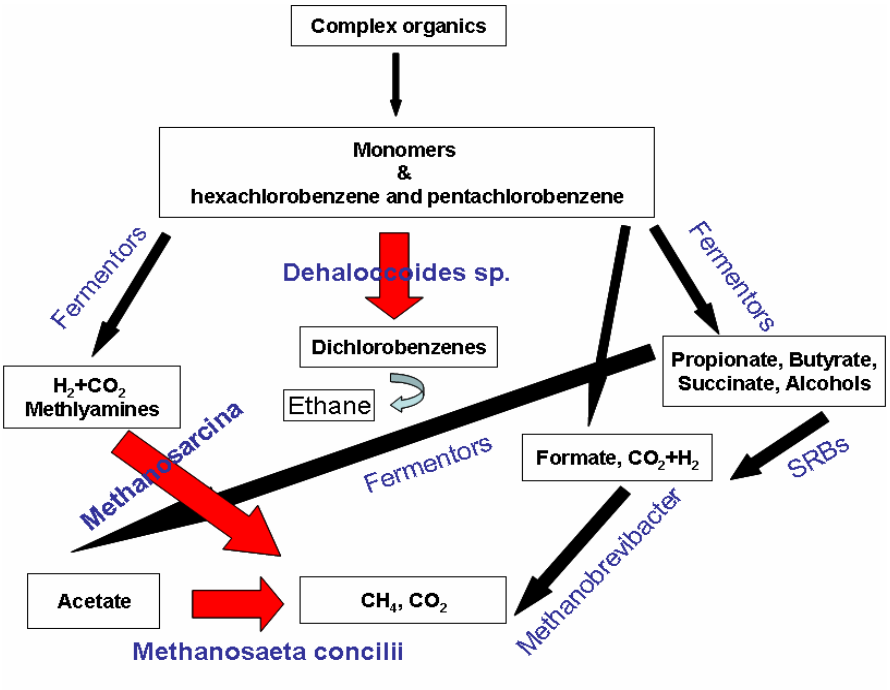

Figure 4. 25. Metabolic pathways in Tuzla anoxic sediments

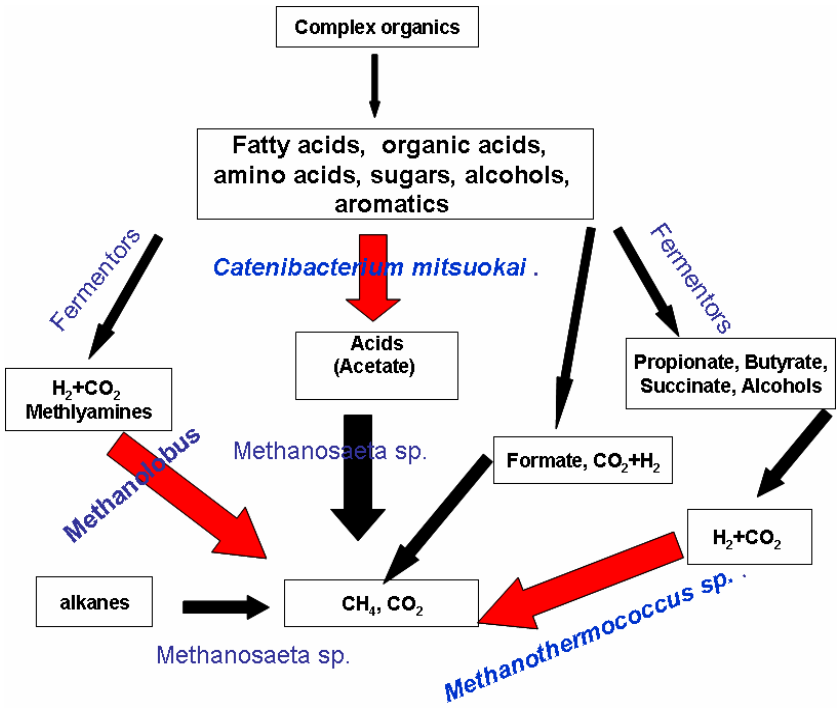

Figure 4. 26. Metabolic pathways in Moda anoxic sediments

5. CONCLUSIONS

This study investigated the archaeal and bacterial community composition in the heavily hydrocarbon polluted anoxic sediments from Tuzla and Moda of the Marmara Sea. Our data suggest that globally important *Methanosaeta* species in respect to acetate metabolism and Methanosarcinales predominantly occur in the anoxic sediments of Tuzla. Epsilonproteobacteria dominated the 16S rDNA bacterial clone libraries from Tuzla. The *Proteobacterium Dex80-27* found as the dominating bacterial clone in Tuzla sediments is a sulphur oxidizer which is capable of reducing nitrate. *Catenibacterium mitsuokai*, a fermentative bacteria dominated the sediment samples from Moda sediment samples. Clones belonging to sulphate reducing bacteria (δ-Proteobacteria) and sulfur oxidizing bacteria belonging to γ-Proteobacteria were also common by previous marine studies (Gray and Herwig, 1996; Urakawa et al., 1999), however, SRB constituted a small proportion of Tuzla sediments, and no SRBs could be detected in Moda sediment samples.

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

Cloning and sequencing approach of 16S rRNA genes showed the presence of methanotrophic archaea both in Moda and Tuzla sediment samples and refer to methanogenotrophic methanogenesis within anoxic sediment samples from Tuzla and hydrogenotrophic methanogensis within the anoxic sediment samples from Moda being the dominant processes. Nitrate reduction and fermentation appeared as the dominating metabolic processes in Tuzla and Moda sediments, respectively. Because fermenting microorganisms are independent of sulfate, however, by releasing fermentation products they provide substrates for terminal oxidizers like sulfate reducers and methanogens (Garcia et al., 2000) and previous studies indicates a close metabolic association between SRB and methanotrophic archaea, SRBs may still be present in Moda sediment samples as well. Therefore, future studies of quantification of the whole archaea and bacteria, methanogens and SRBs by group specific primers will reveal a better understanding of the syntrophic associations and metabolic processes prevailing in these sediments.

Furthermore, detection of methane and sulfate profiles in future studies are required to support the findings of the present study.

Although the involvement of SRBs and ANME groups of archaea are well accepted in AOM and reported by many studies, there is only recent information on AOM coupled to denitrification. The dominancy of denitrifiers and methanogens in anoxic sediments of Tuzla is of importance and supports evidence for the novel syntrophic relationship between those groups involved in AOM coupled with denitrification which has recently been reported. This finding will be base for the future studies of determining activity of microorganisms in such anoxic marine sediments.

Generally, all the sequences from the clone libraries, especially the archaeal phylotypes were related to isolates from a vast variety of environments which implies that these organisms have the capacity to thrive better under different environmental stresses such as heavy metal pollution and heavy organic loading as detected in Tuzla and Moda sediments. Besides, the archaeal sequences were related to isolates mostly from lake environments which may indicate a change in the microbial community of the coastal sediments of Tuzla and resemble more of a lake ecosystem due to pollution.

All existing fingerprinting methods currently in use to describe microbial communities have limitations and detect various components of the often very complex bacterial communities that exist in nature (Polymenakou et al., 2005), still the seasonal appearance and disappearance of some species were detected by DGGE as molecular method to monitor the community shifts. DGGE data in this study revealed that important community changes occurred and to our knowledge, although there are few studies for the analysis of seasonal community changes by DGGE, adopting this one molecular technique integrated with cloning and sequences was enough to observe the seasonal changes occurred in Tuzla sediment.

6. RECOMMENDATIONS

This was one of the first studies using DGGE integrated with cloning and sequencing to determine the seasonal changes of microbial communities the sediments of Marmara Sea The results are well enough to give an understanding of the potential dominant biochemical pathways, yet for a better understanding of the community structure, active microbial populations and their interactions with each other and activity tests should be accomplished in the future and this findings will be base for the future studies of determining activity of microorganisms in such anoxic marine sediments.

Microcosmos experiments will be conducted in the future for the investigation of biomarkers for in situ hydrocarbon degredation in anoxic marine sediments and intermediate products of anaerobic petroleum hydrocarbon degradation metabolism will be determined.

Determination of different nutrient suplly regimens on microbial communities should be investigated for a better understanding of the most efficient conditions under which on oil degredation occurs.

In order to minimize the experimental biases of molecular techniques such as DGGE, bigger clone libraries should be generated and more number of clones should be sequenced to cover all the members of the clone libraries.

In order to evaluate the importance of Archaea and Bacteria in natural ecosystems, vital information on the genotypes and phenotypes of these organisms is required and the molecular analysis of functional genes and the concomitant analysis of large genomic fragments from environmental DNA will lead to a better understanding of these complex microbial systems.

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