ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

SULFATE REDUCERS AND METHANOGENS IN MARMARA SEA SEDIMENTS

M.Sc. Thesis by
Bahar ÖZTULUNÇ

Department : Environmental Engineering

Programme : Environmental Biotechnology

Thesis Supervisor: Prof. Dr. Orhan İNCE

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M.Sc. Thesis by
Bahar ÖZTULUNÇ
(501071803)

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Supervisor (Chairman) : Prof. Dr. Orhan İNCE (ITU)
Members of the Examining Committee : Prof. Dr. İzzet ÖZTÜRK (ITU)
Assoc. Prof. Dr. Didem A.GÜVEN (FU)

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MARMARA DENİZİ SEDİMENTLERİNDEKİ SÜLFAT INDİRGEYICILER VE METHANOJENLER

YÜKSEK LİSANS TEZİ
Bahar ÖZTULUNÇ
(501071803)

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Tez Danışmanı : Prof. Dr. Orhan İNCE(İTÜ)
Diğer Jüri Üyeleri : Prof. Dr. İzzet ÖZTÜRK (İTÜ)
Doç. Dr. Didem A.GÜVEN (FÜ)

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ABBREVIATIONS

TOC **:** Total Organic Carbon
EDTA : Ethylene diamine tetra acetic acid
TAE : Tris-Acetic Acid-EDTA
DGGE : Denaturing gradient gel Electrophoresis
EtBr : Ethidium Bromide
PCR : Polymerase Chain Reaction
SRB : Sulfate Reducing bacteria
MA : Methanogenic archaea
SO_4^{2-} : Sulfate
NO_3^- : Nitrate
Fe : Iron
Mn : Manganese
MOD : Moda Bay
TUZ : Tuzla Coast
KUC : Kucukcekmece coast
IZ : Izmit Bay
TPH : Total petroluem hydrocarbon
Cu : Copper
Cr : Chromium
Ni : Nickel
DNA : Deoxyribonucleicacid
RNA : Ribonucleicacid
PHC : Petroluem hydrocarbons
dsrB : Dissimilatory sulfite reductase gene
mcrA **:** Methyl coenzyme-M reductase
Ct : Threshold value
O_2 : Oxygen
Q-PCR : Quantitative PCR
MSS : Marmara sea sediment
Zn : Zinc
MGB : Miner groove binder
NTC : No template control
RT : Reverse transcriptase
Mmax : Specific growth rate
Ks : Half-saturation constant
r^2 : Regression coefficent
ITS : Internal transcribed spacer
N : Nitrogen
H_2S : Hydrogen sulfide
N_2O : Nitrous oxide
CH_4 : Methane
P : Phosphorus
GDNA : Genomic DNA

Mn : Manganes

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SULFATE REDUCERS AND METHANOGENS IN MARMARA SEA SEDIMENTS

SUMMARY

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

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

It has been estimated that less than 1% of the total microbial population in the land environment and even less in the marine environment have been successfully isolated in pure culture. Marmara Sea has great importance not only because of geological position but also its composition of microbial life which still remains in darkness. Sulfate reduction and methanogenesis are considered to be the most important microbial processes in marine sediments, and they consistently co-occur. Sulfate reduction and methanogenic community analyses together with chemical analyses of the sediments will undoubtly form a base to develop bioremediation strategies to overcome chronic pollution in the MSS.

In this study, abundance of sulfate reducing bacteria (SRB) and methanogenic archaea (MA) were monitored in sediments from 10 different locations in the Marmara Sea for 2 years to reveal how important these processes and what may control abundance of the responsible organisms. Microorganism quantifications were carried out using quantitative polymerase chain reaction (Q-PCR) and targeting functional genes (mcrA and dsrB). In order to mark suitable communities as a cornerstone for a bioremediation strategy, the results were evaluated along with other microbiological and chemical sediment characteristics which were determined by Kolukirik . (2009) during a TUBITAK project on bioremediation of petroleum hydrocarbons.

Q-PCR results indicated that Sulfate reducers and Methanogens cell contents of the sediments were high in the MSS ($1,46x10^{9}$- $1,56x10^{10}$and $1,45x10^{9}$-$3,82x10^{10}$cells/cm^{3} respectively).

TUBITAK project on bioremediation of petroleum hydrocarbons revealed that electron donors were not limited in the MSS. Scarcity of the electron acceptors determined dominancy of the organisms responsible for the relevant terminal e^{-}-accepting processes. Microorganisms, mainly sulfate reducers, and methanogens coexisted within a very short distance (15 cm) from the sediment surfaces. The sediment analyses targeting functional genes (mcrA and dsrB) also revealed that all of these metabolic groups were abundant in the sediments.

Sediment chacarteristics correlation analysis were done between heavy metal, elemental composition (C/N/P), anionic content (NO_3^-, SO_4^{2-}), petroleum hydrocarbon (TPH , aliphatics, aromatics, asphaleten, resene), total cell count (DAPI count, Q-PCR count), genes / transcrips responsible for Sulfate Reduction, Anoxic N cycle, BTEX degradation and Methanogenesis, total cell activity (rRNA level), physical characteristics (salinity, pH, temprature, sediment grain size) parameters (Kolukirik ,2009). Correlation results demonstrated that sediment variables were not related to Methanogens whereas Sulfate reducers were strongly related to sulfate concentration in the sediment. ($r= 0.98, p<0.05, n=47$).

Because the Marmara Sea Sediments (MSS) contains high amount of sulfate reducing and methanogenic microorganisms, a bioremediation strategy for the Marmara Sea based on stimulation of these microbes is possible. After this study, further laboratory hydrocarbon degradation microcosms were set up in the concenpt of TUBITAK 105Y307 project. The project overall results revealed that it is possible to increase hydrocarbon degrading activity of methanogenic-sulfate reducing microorganisms in the MSS for approximetly 10 by nutrient amendment. This will form a base for further filed scale bioremediation applications.

MARMARA DENİZİ SEDİMENTLERİNDE SÜLFAT İNDİRGEYİCİLER VE METHANOJENLER

OZET

Marmara denizi, Karadeniz ve Akdeniz arasındaki tek rotadır. Marmara bölgesinin nüfusu 25 milyona yaklaşmakta ve Marmara denizine çeşitli noktalardan büyük miktarda evsel ve endüstriyel atık boşaltılmaktadır. Ayrıca Marmara denizinde gemi ve tanker trafiği yoğundur. Kirlilik kaynaklarının toplam etkisi sonucu yoğun kirlenen bölgelerde anoksik sedimentler oluşmuştur. Bu bolgeler hem yerleşim hem de endüstriyel bazda yoğundur ve 3 milyondan fazla kişinin evsel ve endüstriyel atığına maruz kalır. Genelde, bölgelerde metal, tekstil ve deri, ilaç, kâğıt, kimya ve plastik endüstrileri gözlemlenir.

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

Tahmin edilmektedir ki karada yaşayan toplam mikrobiyal populasyonun %1'inden azı, deniz ortamlarında yaşayanların daha da azı saf kültüre alınmıştır. Marmara denizi sadece jeolojik pozisyonu sebebiyle değil hâlihazırda bilinmeyen mikrobiyal hayatın içeriği ile de büyük önem taşımaktadır. Sulfate indirgenmesi ve methanojenesis deniz sedimentlerindeki en onemli mikrobiyal proseslerdir.Sulfat indirgeyici ve methanojenik komünite analizleri, sediment kimyasal analizleri ile birlikte değerlendirilerek Marmara Denizindeki kronik kirlenmeyi gidermek için kullanılacak bir biyoıslah stratejisi oluşturabileceklerdir.

Bu çalışmanın esas amacı sulfat indirgeyici bakteriler ve methanojenik arkelerin Marmara denizinde ne derece önemli olduğu ve bu mikrobiyal kominıtelerin nasıl kontrol altına alınabileceğini belirlemektir bu amaçla Marmara denizinin 10 farklı bölgesi 2 yil boyunca gözlemlenmiştir. Mikrobiyal hücre sayısı gerçek zamanlı polimeraz zincir reaaksiyonu yöntemi ile belirlenmiş olup, işlevsel mcrA ve dsrB genleri hedeflenmistir. Uygun komüniteleri belirlemek için sonuçlar sediment kimyasal analizleri ve mikrobiyolojik sediment karakterizasyonu ile birlikte değerlendirilmiştir.

Gerçek zamanlı polimeraz zincir reaksiyonu sonuçları gostermiştir ki sülfat indirgeyici bakteriler ve methanojenik arkeler Marmara denizinde çok yüksek oranda bulunmaktadır (sırasıyla $1,46x10^{9}$- $1,56x10^{10}$ve $1,45x10^{9}$- $3,82x10^{10}$cells/cm^{3}).

Sediment karakterizasyonu korelasyon analizleri ağır metaller,elemental kompozisyon (C/N/P),Anyonik içerik (NO_3^-, SO_4^{2-}),petrol hidrokarbonu (TPH, alifatikler, aromatikler,asfaltan,rezen),toplam hücre miktari (DAPI yontemi ile sayim, Gerçek zamanli polimeraz zincir reaksiyonu ile sayim),Sülfat indirgenmesi,Methanojenesis,Anoksik azot döngüsü, BTEX degradasyonu ile ilgili

genlerin sayimi,Toplam hücre aktivitesi(RNA duzeyinde), fiziksel özellikler (tuzluluk, ph, sıcaklık, sediment tane büyüklüğü) parametreleri arasinda TUBITAK projesi kapsamında yapılmıştır. Korelasyon sonuçları göstermiştir ki; sediment karakterizasyon parametreleri ile methanojenler arasında bir bağlantı bulunamamaış bunun aksine, sülfat indirgeyici bakteriler ve sülfat konsantrasyonu arasında çok yüksek oranda bir korelasyon bulunmuştur.($r= 0.98, p<0.05, n=47$)

Marmara denizi sedimentlerinin (MSS) yüksek miktarlarda sülfat indirgeyen ve metanojen mikroorganizma içermesi nedeniyle, bu mikroorganizmaların stimülasyonuna dayanan biyoıslah stratejisi geliştirmek mümkündür. Bu çalışma sonrasında, 105Y307 No.'lu TÜBİTAK projesi kapsamında, laboratuvar ortamında daha ileri hidrokarbon degradasyonu mikrokozmosları kurulmuştur. Bu projenin sonuçları, MSS içerisindeki metanojen-sülfat indirgeyen mikroorganizmaların hidrokarbon degradasyonu etkinliklerinin, besin ıslahıyla yaklaşık olarak on kat arttırılabileceğini göstermiştir. Bu sonuçlar, daha büyük saha ölçekli biyoremediyasyon uygulamaları için bir temel oluşturacaktır.

1. INTRODUCTION

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

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

As a consequence of developments in molecular ecology, the application of molecular techniques such as quantitative polymerase chain reaction (Q-PCR), denaturing gradient gel electrophoresis (DGGE) (Muyzer , 1993) and cloning of 16s rDNA (Head and Rolling,2005) have led to new insights into microbial processes in different habitats. Q-PCR technique provides very accurate and reproducible quantitation of gene copies.unlike other quantitative PCR methods, real-time PCR

does not require post-PCR sample handling, preventing potential PCR product carry-over contamination and resulting in much faster and higher throughput assays (Williams, 2005).

The Marmara Sea is a small (size ≈ 70 x 250 km) intercontinental basin connecting and acting as the only route between Black Sea and Mediterranean Sea. The population of Marmara region reaches to 25 million and therefore there is large number of domestic wastewater discharge to the Marmara Sea from different points. Anthropogenic activities in the coastal area of the north Marmara Sea include,urban effluent, summer resorts (untreated effluent discharged into the sea), agricultural run off, sunflower oil factories, a big cement factory, fishing and shipping (Ozturk , 2000).Also large quantities of Central Asian oil and gas are transported to the west through the Marmara Sea. Combining effect of pollution sources create a chronic pollution at the Marmara Sea and formed several anoxic sediments in highly polluted sites The regions are populated by both residential and industrial sites and takes the domestic and industrial effluent of more than 3 million people. Industrial sites mainly composed of metal industry, textile and leather industry, medicine industry, paper industry, chemical industry, rubber and plastic industry. Also in 1999 due to tanker accident at Kucukcekmece beach the region was polluted with more than 3000 tones of petroleum (Otay and Yenigun, 2000)**.**Microbial community analyses together with chemical analyses of the sediments willundoubtly form a base to develop bioremediation strategies to overcome chronic pollution at MSS.

Usually oil spills are removed from the environment by mechanism of aerobic respiration to degrade petroleum hydrocarbons (Prince, 1997). Although the result may be beneficial, aerobic hydrocarbon degradation has a limiting parameter, which is presence of oxygen. Any treatment of contaminated sediments is not conventional since oxygen transfer to sediment by mechanical methods is laborious and expensive (Head and Swannell, 1999). On the other hand anaerobic biodegradationuses not dissolved oxygen but anaerobic electron acceptors that can be found abundantly in the sediment (Zwolinski, 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 , 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 , 2002). Sulfate reduction appears to be the most important microbial process, accounting forup to 50% of organic matter degradation in coastal marine sediments and generally,methanogenesis becomes the dominant terminal oxidation process when sulfate becomes depleted (Wilms , 2007). The dissimilatory sulfate reduction can be linked to the oxidation of substrates that are difficult to degradeunder 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, 2005).

Sulfate reduction and methanogenesis are considered to be the most important processes, and they consistently co-occur (Smith and D'Hondt, 2006). Sulfate reducing bacteria (SRB) rely on the availability of sulfate but do not obviously belong to the most abundant bacterial groups, even in those having high sulfate concentration (Schippers and Neretin 2006, Wilms, 2006). Distribution of methanogenic archaea (MA) correlates with sulfate and methane profiles and can be explained by electron donor competition with Sulfare reducing bacteria (Stams , 2006). In this study, abundance of sulfate reducing bacteria (SRB) and methanogenic archaea (MA) were monitored in sediments from 10 different locations in the Marmara Sea for 2 years to reveal how important these processes and what may control abundance of the responsible organisms. Microorganism quantifications were carried out using quantitative polymerase chain reaction (Q-PCR) and targeting functional genes (mcrA and dsrB). In order to mark suitable communities as a cornerstone for a bioremediation strategy, the results were evaluated along with other microbiological and chemical sediment characteristics which were determined by Kolukirik (2009) during a TUBITAK project on bioremediation of petroleum hydrocarbons.

2. POLLUTION OF MARMARA SEA

2.1 Description of Marmara Sea

The Marmara Sea is a small (size ≈ 70 x 250 km) intercontinental basin connecting the Black Sea and the Mediterranean Sea. Marmara Sea has its name from the region where it presents. The Marmara region is one of the important coastal settlements in Turkey. The region has evolved rapidly both in industrial activities and population. As being in the middle of the region, Marmara Sea becomes subject to a multitude of wastewater discharges from major land-based sources located along the coastline, including the Istanbul metropolitan area. The water quality measurements indicate severe signs of present and future eutrophication problems (Orhon, 1995). In addition to these, Marmara Sea and Turkish straits become a prime site for oil pollution because of inflow from Black Sea and increase in sea traffic mainly due to industrialization and dependence of petroleum. It has been reported approximately 450 sea accidents in 40 years between 1960 and 2000. Most of the accidents were not very important but there were some accidents which caused historic oil spills with major results on the environmental pollution (Kazezyilmaz, 1998).

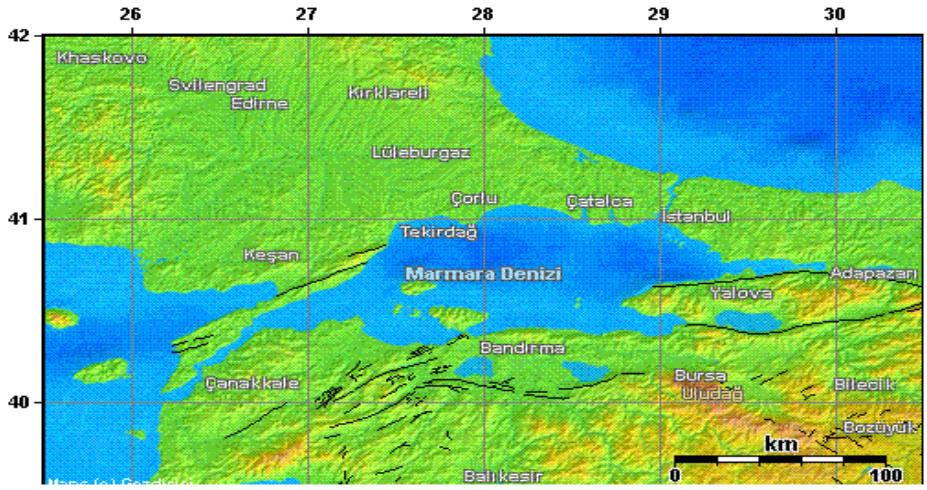

Figure 2.1: Location of Marmara Sea

2.1.1 Hydrography of Marmara Sea

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

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

2.1.2 Sources of Pollution in Marmara Sea

A large number of wastewater discharges to the Marmara Sea from different points. Anthropogenic activities in the coastal area of the north Marmara Sea include,urban effluent, summer resorts (untreated effluent discharged into the sea), agricultural run off, sunflower oil factories, a big cement factory, fishing and shipping (Ozturk, 2000).Industrial effluents with flushing of refinery plants can be considered also as sources of pollution too.

Benthic composition is one of the main elements of an aquatic system. Sediments are final destination of contaminants and other nonsoluble materials and due to accumulation of organic materials it becomes an oxygen trap for the bottom water (Venturini , 2004).It has been found that there is a positive correlation between

organic carbon contents and level of pollution in deep sediments. According to these arguments organic carbon level may beused as an indicator of pollution (Shine and Wallace, 2000, Hyland , 2005).The anthropogenic effect of pollution can be seen in the content of organic carbon. Total Organic Carbon (TOC) content of sediments varies from 2.1 mg/g to 22 mg/g with a highest average value of 12.5 mg/g at Buyukcekmece coast (Albayrak, 2006).

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

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

2.2 Region of Kucukcekmece

Kucukcekmece is on the Marmara coast, on the eastern shore of an inlet of the Marmara called Kucukcekmece Golu (Kucukcekmece Lagoon). The inlet is

connected to the Marmara Sea by a narrow channel, so the water is not salty.until the 1950's Kucukcekmece was a popular weekend excursion, people would come by train from Istanbul to swim or to fish. The streams running into the inlet now carry industrial waste and the inlet is highly polluted but efforts are being made to get it clean again. Thereused be wildlife and many kinds of birds and efforts to get the wildlife back are taking effect slowly.

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

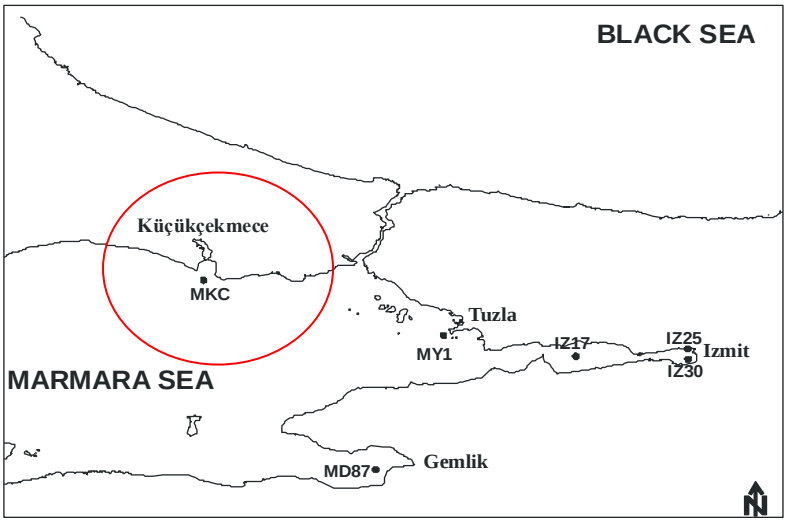

Figure 2.2: Location of Kucukcekmece region

2.2.1 Sources of Pollution at the Region

The region is polluted heavily due to awryurbanization and intensive industrialization. The Kucukcekmece lagoon is subjected to take effluent of 2 million people at the year of 2000. Industrial sites are mainly composed of metal industry, textile and leather industry, medicine industry, paper industry, chemical industry, rubber and plastic industry. The control of discharges are not controlled or regulated by the government. These problems coupled with incomplete sewage system create huge impact on the region. Therefore a recreation place once becomes now a place with lots of buildings and eutrophicated lagoon. The sources of pollution are

classified as point and nonpoint sources. Point sources composed of discharges from domestic and industrial sites. Waste loads of Nuclear Research Institution affect also rivers flowing to the lake. Nonpoint sources include drainage waters coming from runoff, groundwater including leachate and water coming from agricultural activities.

2.2.2 Petroleum Pollution due to Volganeft Accident

On December 29, 1999, the Volgoneft-248, a 25-year old Russian tanker, ran a ground and split in two in close proximity to the southwest shores of Istanbul at Kucukcekmece due to storm. More than 3000 tons of 4,300 tons of fuel oil on board spilled into the Marmara Sea. During the storm, spilled fuel oil spread to beach of Florya, about 5 square miles of the sea. According to the observations on the day of accident, spilled oil contaminated the shorelines between the grounded ship stern off the Menekşe Coast and the rock groin at Ciroz Park five kilometers to the East of the accident. Beaches, fishing ports, restaurants, recreation facilities, the Ataturk Pavillion, piers, groins and seawalls located in this area are directly affected. The concentration of oil was so high in some areas it reaches thickness of 5 cm on the surface of sea water. Fuel oil reached to the beach was then covered with sand creating a fuel oil saturated muddy layer along the beach. Heavy spill affected the aquatic life severely, killing many species of aquatic ecosystem including fishing birds (Dogan , 2005).

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

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

2.2.3 Pollution of Tuzla and Moda

Tuzla is located on the Asian side, 60 km east of Istanbul, 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 Kadıkoy district in Istanbul, Turkey on the Northern coast of Marmara Sea. Moda is at the junction of Kurbagalıdere whichused 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 anultimate sink for organic and inorganic settling matters (Algan,2004). Marine sediments, particularly those in coastal areas, are commonly polluted with petroleum hydrocarbons (PHC) as a consequence of the extensiveuse of petroleum compounds by mankind (Miralles, 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, 1993b).

Consequently, microbial processes depending on the availability of free dissolved oxygen are constrained to theuppermost 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 degradationunder sulphate-reducing conditions (Coates , 1997 ; Townsend, 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, 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 hasultimately spread along the shoreline and continental shelf that constitutes 50% of its total area (Unlu , 2006) Anthropic pollution trapped in bays, in particular, has created significant ecological damage resulting in the decrease or extinction of marine species (Unlu , 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 Istanbul metropolitan and from maritime transportation (Algan, 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, 1998).

Tuzla hasundergone heavy environmental stress due to expansion of the Istanbul 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, mainlyuntreated 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, 1998; Unlu , 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 (Unlu , 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 goneunder amendment by ISKI 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.

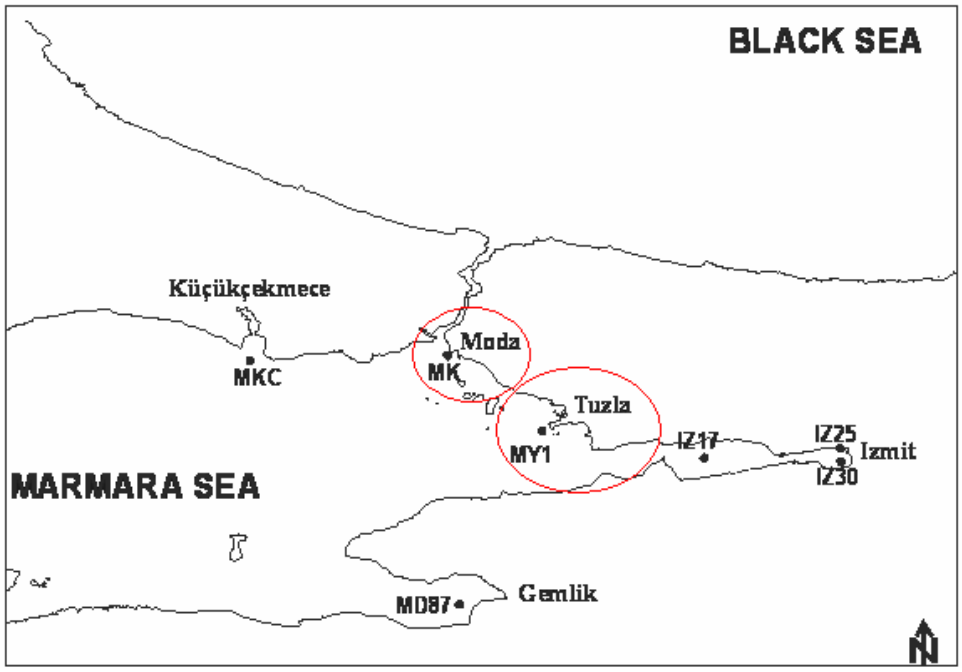

Figure 2.3: Location of Tuzla and Moda

2.2.4 Pollution of the Gemlik and Izmit Bays

Gemlik is a harbor town bordering the Sea of Marmara in Western Turkey, at approximately 29 kilometres from Bursa and not far from Istanbul. Gemlik was called Kiosuntil 1922 when its Greek inhabitants (around 80% of the population) left Asia Minor because of the population exchange. In 2004, Gemlik had approximately 70,000 inhabitants. The harbour is one of the most important in Turkey. Izmit Bay is one of the most polluted inner waters in the Marmara Sea and heavily impacted by petrogenic PAHs (Unlu and Alpar,2004). The Gemlik Bay is the second most polluted hot spot in this semi-enclosed sea connecting the Black Sea to the Aegean Sea via the Turkish straits (Bosphorus and Dardanelles). It is surrounded by areas of

high population growth and rapid economic developments in the Marmara Sea and receiving natural and anthropogenic discharges via rivers and atmosphere.

The bay, with a total surface area of 349 km^2, is most particularly subject to high anthropogenic pressure due to inputs from rivers, atmosphere, coastal shipping and industrial activities. The total of domestic wastewater discharge into the bay is as much as 7.5 million m^3/y (Solmaz, 2000). Only Gemlik town has their own deep sea outfall discharge system. Other coastal settlementsuse creeks or simple outfalls for their wastewater discharge.

Gemlik (GEM) Bay are the main industrial locations of the Marmara Region which receives various types of wastewaters. The easternmost part of the bay is subject to chronic severe contaminations, among which hydrocarbons play a major role. The main sources are ship traffic, fishery activities, domestic and industrial sewage waters and riverine inputs. The Karsak creek which discharges into the Gemlik port is the most important pollution source. Not only the discharges of a wide range of industrial plants in Gemlik town, but this creek also carries the waters of Lake Iznik, domestic and industrial wastewater discharges of Orhangazi town located 15 km in the west of the Gemlik Bay. The total load carried by Karsak River is therefore variable seasonally. The share of industrial waste water inputs is even higher, 13–20 million m^3/y (Solmaz , 2000). The total discharge of textile and chemistry plants is seemingly lower, but they introduce an important industrial pollution into the bay since they do notuse treatment systems. The impact of such an anthropogenic pressure can be observed often in summer with the phenomenon of red waters, resulting from eutrophication and disequilibrium processes for the exploitation of natural resources.

Izmit Bay, a semi-enclosed body of water located in the most industrialised area of the Marmara region, has been subjected to pollution by surrounding domestic and industrial discharges since the 1970s. Pollution prevention attempts resulted only to decrease the industrial organic carbon levels in the 1990s (Morkoc , 2001). However, previous studies show that many effluents discharging to the bay are toxic (Okay , 1996). Consistently the recent sediments were also found toxic throughout the bay (Tolun , 2001). The bay has a strong and permanent salinity stratification created by the low saline waters of the Black Sea overlaying high saline waters of the Mediterranean. Thus, there is an oxygen depletion in the bottom waters of the water

column which may stimulate organic carbon accumulation in the sediment (Morkoc , 2001). On August 17th, 1999, in the vicinity of Izmit, an earthquake of a moment magnitude Mw=7.4, a focal depth h=18 km and having approximately 120 km right lateral strike slip faulting was felt over the area. It caused great loss of life and extensive damage. It also generated a tsunami in the Izmit Bay (Yalcıner ,1999; Altınok and Ersoy, 2000; Altinok, 2001). The sea first receded then inundated both sides and ranup more than 2.5 m in some places of the Bay during the earthquake. Furthermore, the rise of the water was above 10 m in Degirmendere near Golcuk (a small town in the southern part of the Bay). There was a heavy concentration of petrochemical plants on the northeastern site of the Bay within about 10 km of the epicenter. This was the first time in about 35 years that large refineries and chemical plants have been so close to the epicenter of a major earthquake, and this may be the largest concentration ever of petrochemical facilities to experience such a shake. The most widely publicised and spectacular damage to any industrial facility occurred at the massive refinery near the town Korfez operated by the state-owned oil company, Tupra°. Following the earthquake the tank farm of the refinery burned out of control for several days. An oil spill occurred during the transfer operations the port when the earthquake began (Scawthron and Johnson, 2000). The oceanographic characteristics and the pollution levels of the bay before and after the earthquake have been investigated previously (Okay , 2001; Balkıs, 2003). These investigations showed that the subsequent fire after the earthquake caused an increase in the total PAH concentrations of the surface waters and local mussels (Okay , 2001, 2003) and the dissolved oxygen content of the lower layer was below the detection limit (Balkıs, 2003).

2.2.5 Pollution of the Horn Enstuary (Halic Bay)

Estuaries are special semi-enclosed systems displaying a wide range of physical and chemical properties. Like many of worlds natural resources, many estuaries have deteriorated due to waste disposal, recreation and power generation. The Golden Horn Estuary has been the favorite recreational area of Istanbuls cultures for centuries. It is 7.5 km long, 150–900 m wide, located southwest of the Strait of Istanbul (Bosphorus) (Figure.2.5).

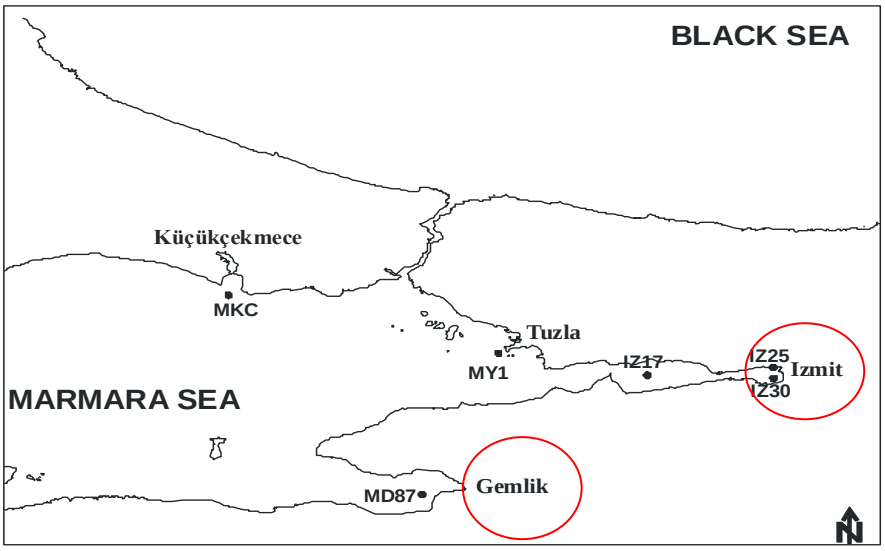

Figure 2.4: Location of the Gemlik and Izmit Bays

Maximum depth is 40 m at the entrance and decreases below 10 m at inner parts where a 3–4 km zone was completely filled with runoff carried by two small streamsuntil the early 1990s. These streams were described as the main sources of freshwater input (Kor, 1963). Following significant decreases in stream fluxes; rain and coastal inputs became the main sources of freshwater in the Golden Horn over time (Sur , 2002a). The estuary receives saline water from the highly stratified, two-layered Strait of Istanbul. Theupper layer with 25 m thickness has 20 psu salinity and lower layer has 38 psu salinity, which is separated by a transition zone. This stratified structure disappears in midestuary where maximum depth is 12–13 m. In addition to these layers, 2–3 m less saline permanent layer above the stratified waters of the estuary was reported due to the suspended sediment carried by local discharges and streams (Ozsoy , 1988). Such gradation in salinity should result in a system it high diversity in non-polluted waters.

However, the estuary has been polluted by wastewater of pharmaceutical, detergent, dye, leather industries and domestic discharges since the 1950s.(Tuncer , 2001) revealed that the metal pollution due to anthropogenic disturbance altered significantly within the second half of the century. In addition, the building of dam on the stream weakened freshwater renewal. Furthermore, bridges, floating on large buoys and shipyards with large buoyant dry docks blocked circulation ofupper layer and strengthen the pollution effect. Poor renewal of estuarine water and heavy

nutrient load including numerous types of organic and inorganic effluents resulted in low diversity, with some pollution resistant macroalgae species (e.g Enteromorpha intestinalis) (Aydın and Yuksek, 1990) and planktonic organisms such as Ceratium spp. and Dinophysis caudata (Tas and Okus , 2003) at the outer part of the estuary. The inner part, on the other hand, had only anaerobic life characterised by hydrogen sulfide formation (Dogan , 2001). The anthropogenic pollution at the estuary not only adversely affected the communities living in the estuary but also human life, giving a heavy odor of hydrogen sulfide and anunaesthetic appearance of this once recreational area. Therefore, a water rehabilitation plan was devised to improve water quality which focused on the inner estuary. First, 4.25 x 10^6 m^3 anoxic sediment filling the basin was removed and approximately 4–5 m depth was gained at the completely filled part. Afterwards, in May 2000, freshwater was released from the closest dam to the estuary for rapid oxygenation of the anoxic water body. Meanwhile, most of the domestic discharges were gradually connected to a collector system discharging deep into the lower layers of the strait, reaching deep water in the Black Sea (Aslan-Yılmaz, 2002). Finally, in May 2000, the floating bridge opened to ease water circulation. However, implementation of the plan and the provision of a better water quality in the estuary could not be successfully demonstratedunless continuous data on all aspects of ecosystem were collected.

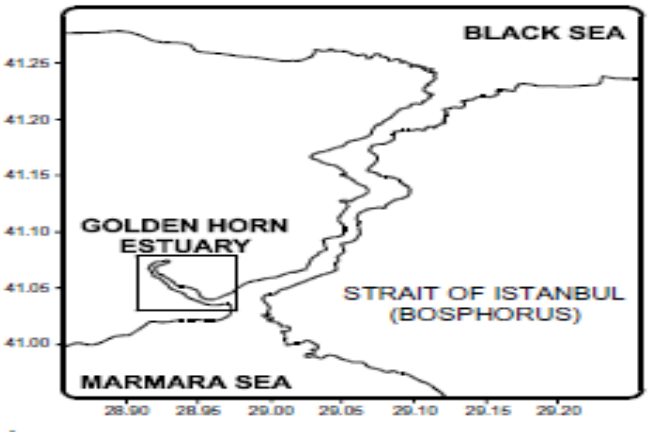

Figure 2.5: Location of the Horn Enstuary (Halic Bay)

3.ANOXIC MARINE SEDIMENTS AND ITS MICROBIOLOGY

3.1 Definition and Characteristics of Anoxic Marine Sediments

More than half of the earth's surface is covered by aquatic environments. Continual deposition of particles to oceans and seas forms hydrocarbon rich benthic environments, sea sediments (Vetriani , 1999). Sediments are a carbon and nutrient pool for aquatic environments. Processes for mineralization of organic matter mainly occur here by the benthic microbial communities (Aller ,1998). There are several studies about characterization of microbial communities involved carbon and sulfur cycling in the benthic environments (Devereux, 1994; Gray and Herwig, 1996; Llobet-Borassa , 1998; Munson, 1997; and Teske , 1996b), however the studies about microbial populations in deep sea sediments are very poor. Coastal and shelf sediments are especially important in the remineralization of organic matter. In those areas, an estimated 32 to 46% of the primary production settles to the sea floor. Prokaryotes reoxidize most part of the debris which is located in the sea sediments (Wollast, 1991).

A little knowledge about diversity and structures of indigenous microbial populations within the polluted costal and shelf areas is found in the literature. The few reports that are available for polluted marine sediments deal with main contaminants, such as polyaromatic hydrocarbons (Geiselbrecht , 1996; Gray and Herwig , 1996), heavy metals (Frischer ,2000; Gillan, 2004, Powell , 2003; Rasmussen and Sørenson, 1998), and organic matter (McCaig., 1999; Stephen , 1996), hydrocarbons (Macnaughton ,1999 ; Roling , 2004; and Roling , 2002). The presence of hydrocarbon compounds and low oxygen level creates a suitable environment for the growth of anaerobic bacteria. Although anaerobic biodegradation processes are slower than aerobic biodegradation, anaerobic processes can be a significant factor in removal of organic contaminants owing to the abundance of anaerobic electron acceptors relative to dissolved oxygen.

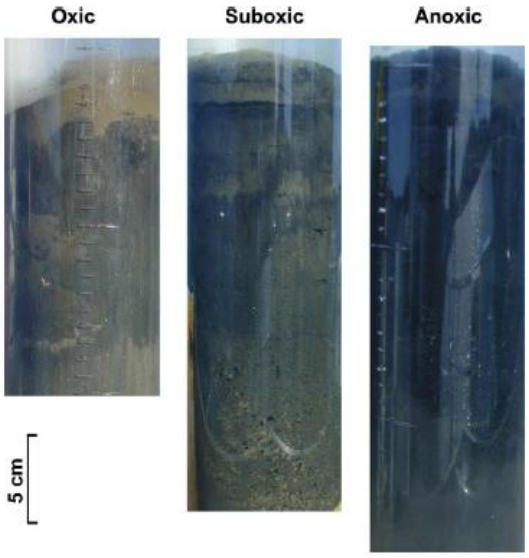

Figure 3.1: The oxic, suboxic and anoxic sediments (Virtasalo , 2005)

3.2 Microbial Life in the Anoxic Marine Sediments

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

3.2.1 Bacterial Communities in Anoxic Sediments

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

The first phylum of bacteria is proteobacteria. This is the widest phylum of the bacteria. As a group these organisms are all gram-negative, show extreme metabolic diversity, and represent the majority of known gram-negative bacteria of medical, industrial, and agricultural significance. Proteobacteria has five major subdivisions:

Alpha

Beta

Gamma

Delta

Epsilon

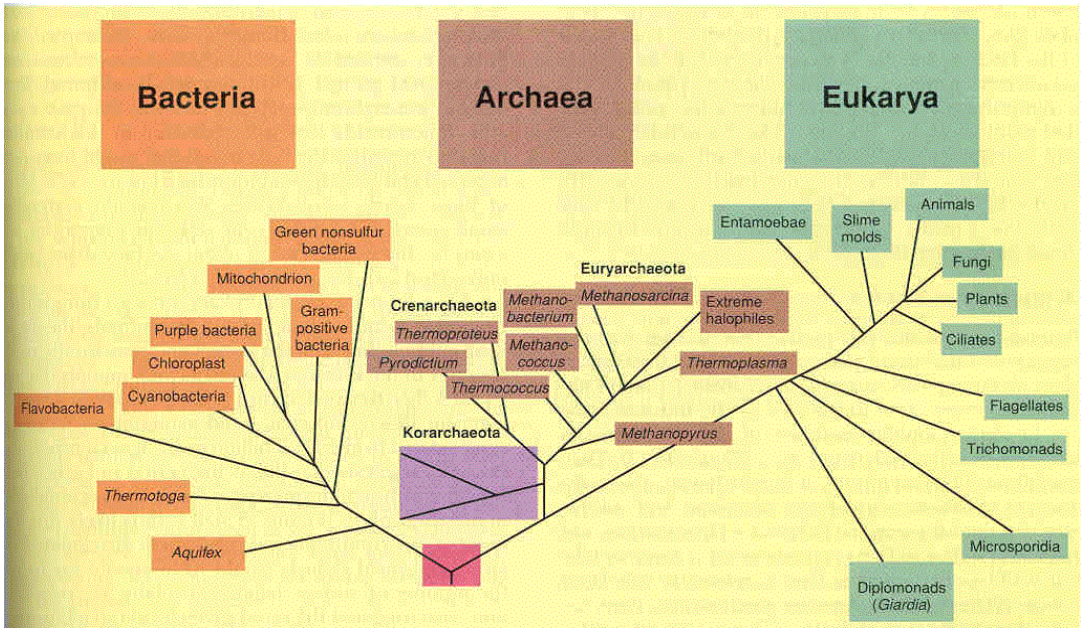

Figure 3.2:universal phylogenetic tree (Madigan , 2002)

One of the most important known groups of proteobacteria is purple phototrophic bacteria which carry out anoxygenic photosynthesis and contain chlorophyll pigments called *bacteriochlorophylls* with any variety of carotenoid pigments. The purple bacteria have different and spectacular colors,usually purple, red or brown.

The most known of purple bacteria are purple sulfur bacteria and purple nonsulfur bacteria (Madigan , 2002).

The other known groups of proteobacteria are the nitrifying bacteria which are chemolithotrophs as Nitrosifiers and Nitrifiers, sulfur- and iron-oxidizing bacteria, hydrogen-oxidizing bacteria, methanotrophs and methylotrophs, *Pseudomonas* and the *pseudomonads*, acetic acid bacteria, free-living aerobic nitrogen-fixing bacteria, *neisseria*, *chromobacterium* and relatives, enteric bacteria, *vibrio* and *photobacterium*, *rickettsia*, *spirilla*, sheathed proteobacteria as *sphaerotilus* and *leptothrix*, budding and prosthecate/stalked bacteria, gliding *myxobacteria*, and finally sulfate- and sulfur-reducing bacteria (Madigan , 2002).

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

3.2.2 Archaeal Communities in Anoxic Sediments

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

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

The first kingdom, Crenarchaeota derived from being phylogenetically close to ancestor or source of *Archaea* (Woese, 1990). It was believed to include only sulphur-dependent extreme thermophiles. Among cultured representatives, the Crenarchaeota contain mostly hyperthermophilic species including those able to grow at highest temperatures of all organisms

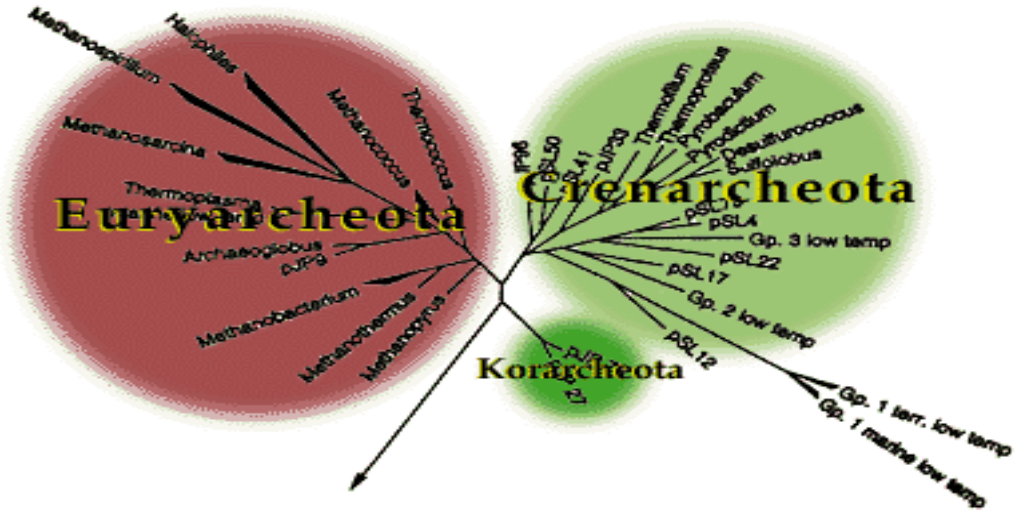

Figure 3. 3: Major lineages of *Archaea:* Crenarchaeota , Euryarchaeota Korarchaeota (http://www.ucmp.berkeley.edu)

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

Hyperthermophilic crenarchaeota tend to cluster closely together and occupy short branches on the 16S rRNA-based tree of life because these organisms have slow evolutionary clocks and have evolved the least away from the hypotheticaluniversal ancestor of life (Madigan, 2002).

The Euryarchaeota is a heterogeneous group compromising a broad spectrum of organisms with varied patterns of metabolism from different habitats. It includes extreme halophiles, methanogens, and some extreme thermophiles so far (Madigan , 2002). Moreover, a third archaeal kingdom has recently been discovered which is reported isolation of several archaeal sequences evolutionary distant from all *Archaea* known to date by Barns and coworkers in 1994 and then in 1996. The new group was placed on phylogenetic treeunder Crenarchaeota/Euryarchaeota and named as Korarchaeota (Madigan, 2002).

3.2.3 Microbial Ecology Studies in Marine Sediments

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 , 2007; Lovley and Klug, 1983), and the community structure of these groups in fresh water sediments has frequently been studied (Schwarz , 2007; Alm and Stahl, 2000; Glissmann, 2004; Go , 2000; Koizumi , 2003; Zepp-Falz , 1999). There are also a few studies that have analyzed sulfatereducing microbial community, and have used dsrB, genes encoding the dissimilatory (bi) sulfite reductase, as functional marker instead of 16S rRNA genes (Leloup , 2007; Baker , 2003; Dhillon , 2003; Nercessian , 2005). There are several studies on tidal flats that mostly focused on bacterial communities (Kim , 2004; Llobet-Brossa , 2002).

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

3.2.4 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 , 2004). Sulfate reduction, methanogenesis and other activities have been detected in cores from the subsurface (Whitman ,1998). Prokaryotic activity, in the form of sulphate reduction and/or methanogenesis, occurs in sediments throughout the world's oceans (D'Hondt , 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 , 2002): (i) Concentrations of dissolved SO4 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 , 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 , 2004). Previously mentioned metabolic activities such as carbon oxidation, Fe and Mn reductionultimately 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 transportupward from seawater flowing through theunderlying basalts. The oxidized Mn and Fe were originally introduced to the sediments by deposition of Mn and Fe at the seafloor (D'Hondt , 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 , (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 , 2004). Theultimate 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 , 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 , 2002 ; D'Hondt , 2004) and radiotracer experiments (Parkes , 2005) demonstrate that at least some of these reductive processes consistently co-occur in deep subseafloor sediments (e.g., sulfate reduction and methanogenesis). 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 , 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 , 2002; D'Hondt , 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 , 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)

4.METABOLIC INTERACTIONS BETWEEN METHANOGENIC CONSORTIA AND ANAEROBIC RESPIRING BACTERIA

4.1 Metabolic Interactions in Methanogenic Bioreactors

4.1.1 Competitive Interactions

Competition between two or more populations of microorganisms is a negative relationship in which the different populations often are adversely affected with respect to their survival and growth. Also competition is considered the most important interaction among organisms, and is one of the major responsible causes of the selection pressure leading to the evolution of species.

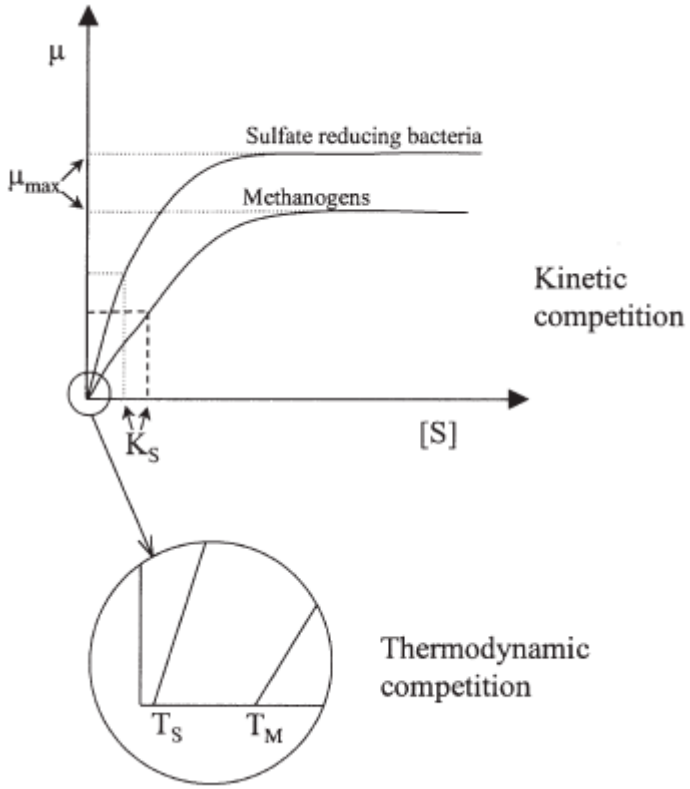

Figure 4.1: Model of kinetic and thermodynamic competition among sulfate Reducing bacteria and methanogenic Archaea.

The competitive interactions among anaerobic microorganisms can be roughly divided into kinetic competition and thermodynamic competition (Figure.4.1).

Kinetic competition refers to the determination of competitive capabilities by kinetic measurements of microbial growth,although theunderlying mechanism for the observed effects might be thermodynamic. Thermodynamic competition means that one organism is capable of growing at and maintaining a substrate concentration below the minimum concentration foruptake (threshold concentration) of other organisms due to a higher energy yield in the conversion of the compound.

In anaerobic fermentation of organic compounds, numerous pathways and combinations of pathways areused leading to different energy yields.However, since anaerobic fermentation is internally optimized in the cells to gain a maximum energy yield and an optimal redox balance (Thauer,1977) the energetic outcome is often the same. This has the consequence that fermentative competitive interactions are mainly of kinetic character.Most of the studies which have examined competition between anaerobic fermenting bacteria have focused on gastrointestinal systems (Coleman ME,1996) and very little is known on this type of competitive interaction in anaerobic digestion processes.

Table 4.1: The respiration hierarchy.

Acceptor	**Product**	**E'0(V)**
Oxygen 02	Water H_2O	+0.82
Manganic ion Mn^{4+}	Manganous ion Mn^{2+}	+0,80
Ferric ion Fe^{3+}	Ferrous ion Fe^{2+}	+0.77
Nitrate NO_3	Nitrogen N_2	+0.76
Selenate SeO_4	Selenite SeO_3^{2-}	+0.4S
Arsenate AsO_4^{3-}	Arsenite AsO_3^{3-}	+0.14
Sulfate SO_4^{2-}	Sulfide HS^-	-0.22
Carbon dioxide $C0_2$	MethaneCH_4	-0.24
Carbon dioxide $C0_2$	Acetate CH_3COO^-	-0.29

In contrast to aerobic conditions where most heterotrophic microorganismsutilize oxygen as a terminal electron acceptor and in most cases follow the same metabolic pathway ending in complete mineralization of the organic compounds into CO_2 and H_2O, the biochemical diversity of anaerobic microbial communities is huge.A large number of electron acceptors can beused by different anaerobic organisms in anaerobic respiration processes (Table 4. 1). The most important inorganic electron acceptors are Mn^{4+},Fe^{3+},NO^{3-},SO_4^{2-} and CO_2 . The respiration processes where

these acceptors areused are normally separated either in space or time. This is due to a different energy outcome of the processes according to the Gibbs equation:

$\Delta G_0' = -n \cdot F \cdot \Delta E_0'$ in which $\Delta G_0'$is the Gibbs free energy at pH = 7; n is the number of electrons transferred in the oxidation-reduction reaction; F is Faraday's constant (96.490 kJ/V) and $\Delta E_0'$is the redox potential (E_0') of the electron-accepting reaction minus the redox potential of the electron-donating reaction. From this equation it is obvious that the larger the difference is between the redox potentials of the half-reactions, the larger is the amount of energy available to the organism performing the reaction. The consequence is a hierarchy, which often resembles the order seen in Table 4. 1.

In most environments, some of the respiration processes do not occur,or only occur to a minor extent, due to the lack or exhaustion of available electron acceptors. The energy available to a respiring organism is not only dependentupon the difference in redox potential between electron donor and acceptor. Also concentrations of the reactants and temperatures deviating from Standard conditions affect the energy outcome according to the Nernst equation $\Delta G = \Delta G_0 + RT \ln [B]/[A]$ in which ΔG_0 is the change in Gibbs free energyunder standard conditions, R is the gas constant, T is temperature and [B] and [A] are the concentrations of the two components of the reaction A$\Longleftrightarrow$ B. According to the respiration hierarchy, sulfate reduction excludes methanogenicutilization of common substrates, which is verified in high-sulfate environments such as marine sediments (Abram JW,1978).However in, e.g., freshwater sediments, the two processes can coexist or even be dominated by methanogenesis due to equilibrium displacements caused by low sulfate concentrations making sulfate reduction thermodynamically less favorable than methane production (Lovley DR,1982).

4.1.2 Kinetic Competition

This is the classical competitive interaction, the theory of which has been established in studies of defined cultures in chemostats (Kuenen JG,1982). According to kinetically- based competition models, the outcome of interactions between two microorganisms competing for the same growth-limiting substrate can be predicted from the relationship between substrate concentration and the specific growth rate (μ) according to the Monod equation: $\mu = \mu max\ X\ S/Ks + S$. Two typical

relationships can be observed in studies of competitive interactions (Figure. 4.2: a, b).

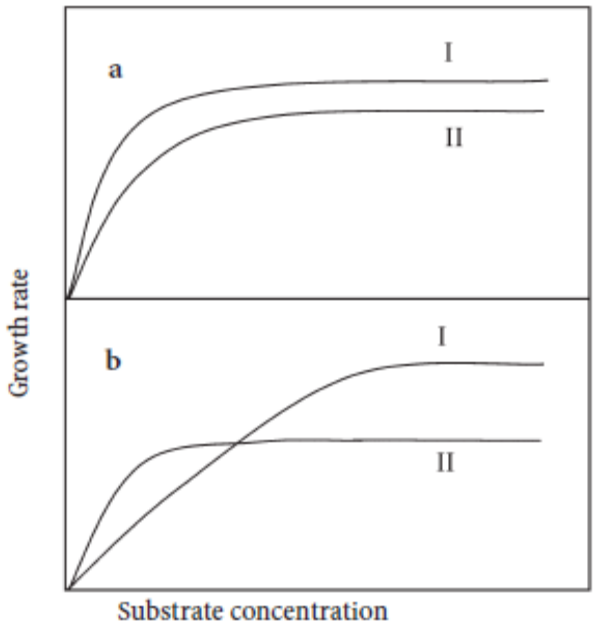

Figure 4.2: Growth rate as a function of substrate concentration in two differen scenarios (**a** and **b**). **A** represents two organisms with different energy metabolism , I having the highest energy yield. **b** represents two organisms with the same energy metabolism, but with different ecological strategies. I is assigned to *"r"* selection while II is assigned to "K"selection.

Figure 4.2a, organism I will grow faster than organism II at any substrate concentration, while the outcome in Figure.4.2b is dependentupon the substrate concentration. The pattern seen in Figure. 4.2a is typical of organismsutilizing different electron acceptors with different energy yields for the oxidation of a common substrate, since the energy yield is higher for the electron acceptor with the highest redox potential at all electron donor concentrations.The pattern seen in Figure 4.2b is typical for organismsutilizing the same metabolism but having different ecological strategies. In natural ecosystems, such as sediments, the concentration of nutrients needed to support growth is often very low. Among the organismsusing the same type of metabolismunder these conditions, type II in Figure 4.2b having a high substrate affinity (low Ks) and a relatively low maximal growth rate (μmax) will normally dominate. This group is assigned to "*K* selection" which refers to organisms that can most effectivelyutilize the resources available (MacArthur RH,1967). In gastrointestinal environments and anaerobic bioreactors,

opportunistic types of organisms (type I) will normally dominate, since type II has a longer doubling time than the retention time of the system. This group is assigned to "*r* selection" referring to a high potential *r* value (rate of population growth/individual) (MacArthur RH , 1967).

4.1.3 Thermodynamic Competition

In natural environments, the substrate concentration for most organisms is normally well below Ks. For all organisms, there is a specific minimum concentration of substrate necessary to gain conservable energy. This minimal "quantum" of energy, which can be conserved, corresponds to the energy needed for translocation of 1 proton. The phosphorylation of ATP to ADP has a ΔG_0 of +49 kJ/mol corresponding to 60–70 kJ/mol when compensating for energy conservation efficiency (Schink B,1994). Since 3 protons are needed in the phosphorylation of ADP to ATP, we can assume that the smallest amount of energy which can be conserved is 1/3 of the phosphorylation energy, corresponding to a minimum ΔG_0 of –20 kJ/mol. Inserting this value and ΔG_0 for different respiration processes in the Nernst equation, the substrate concentration yielding the minimum amount of energy (the threshold concentration) can be calculated for each processunder the prevailing conditions of the specific ecosystem. Several authors have shown that organismsutilizing electron acceptors with higher redox potentials can maintain electron donor concentrations below the threshold foruptake of organismsutilizing electron acceptors with lower redox potentials (Lovley DR 1985). Other studies have shown that significant differences in threshold values for common substrates also can be found among speciesutilizing the same type of metabolism (Westermann P,1989).

4.2. Inhibitory Interactions

Several compounds,which serve as electron donors to respiring bacteria, might inhibit members of the methanogenic consortia. Also some products from anaerobic respiration might affect the activity of these consortia. The modes of action can be indirect by increasing the redox potential to levels that interfere with the biochemistry of the anaerobic microorganisms, or direct by chemical reaction with proteins or other cell constituents.

It has been assumed that many anaerobic microorganisms have specific demands for low redox potentials in their environment to make their energy metabolism thermodynamically possible (Oremland R S 1988). This conception has since been moderated and several reports have shown that the parameters controlling growth of most anaerobes is the oxygen concentration and only to a lesser degree the redox potential of the environment. This has been demonstrated in studies of fermentative rumen bacteria, but also in studies of microorganisms considered extremely sensitive to aerobic conditions (Marounek M,1984). Fetzer and Conrad (Fetzer S,1993) have, for instance, demonstrated that methane production in axenic cultures of *Methanosarcina barkeri* proceeded at normal rates in oxygen-free media where the redox potential was elevated to +420 mV.

The direct inhibition of methanogenic consortia by electron acceptors is mediated by several mechanisms. Oxygen is toxic to all obligatory anaerobic microorganisms.Many anaerobes are rich in flavine enzymes,and may also contain quinones and iron-sulfur proteins,which can react spontaneously with oxygen to yield hydrogen peroxide, superoxide and hydroxyl radicals. Since most anaerobes lack peroxidase, catalase and superoxide dismutases, which destroy the reactive oxygen species,damage of essential cell components can occurupon oxygen exposure. Superoxide dismutase has, however, been demonstrated in some anaerobic microorganisms. Kirby (Kirby T,1981) have, for instance, characterized a superoxide dismutase from the obligatory anaerobe *Methanobacterium bryantii*. Other electron acceptors, such as oxidized nitrogen and sulfur species, have also been shown inhibitory to anaerobic microorganisms. Although the metabolism of these electron acceptors is competitive to anaerobesutilizing electron acceptors with a more negative redox potential, the reduction of the inhibitory compounds might lead to the production of less inhibitory compounds and, hence, relieve the inhibition. In some cases, however, the products of anaerobic respiration are more toxic than the parent compounds.

4.3 Competition

4.3.1 Competition Between Sulfate-Reducing and Acetogenic Bacteria and Methanogenic Consortia

In environments where sulfate is present, sulfate-reducing bacteria will compete with methanogenic consortia for common substrates. Direct competition will occur for substrates like hydrogen, acetate and methanol.Compared with methanogens, sulfate-reducing bacteria are much more versatile than methanogens. Compounds like propionate and butyrate,which require syntrophic consortia in methanogenic environments, are degraded directly by single species of sulfatereducing bacteria.

Kinetic properties of sulfate-reducers, methanogens, and acetogens can beused to predict the outcome of the competition for these common substrates (Verstraete W,1996). For bacteria growing in suspension,Monod kinetic parameters such as the half-saturation constant (Ks) and the specific growth rate (μmax) can beused. When bacterial growth is negligible, as is often the case in reactors with a dense biomass concentration,Michaelis-Menten kinetics may beused to predict which type of organism has the most appropriate enzyme systems to degrade substrates. Therefore, both the Vmax/Km and the μmax/Ks ratio gives an indication of the outcome of competition at low substrate concentrations (Robinson JA,1984).

4.3.2 Competition for Hydrogen

In anaerobic environments methanogens, homoacetogens and sulfate-reducers will compete for hydrogen.Thermodynamically,homoacetogenesis is less favorable than methanogenesis and sulfate reduction.Homoacetogens are very poor hydrogen-utilizing organisms (Cord-Ruwisch R,1988). When grown on organic substrates like ethanol and lactate in the presence of hydrogenotrophic methanogens, they even produce hydrogen. In the absence of methanogens 1.5 acetate is produced per lactate or ethanol that is degraded.However, in the presence of methanogens only 1 acetate per lactate or ethanol is produced,while reducing equivalents are disposed of as hydrogen.

Table 4.2: Acetogenic and methanogenic reactions, and sulfate-reducing reactions involved in the degradation of organic matter in methanogenic bioreactors, and sulfate-reducing bioreactors, respectively.

Reaction			**ΔG_0'[a]**
Syntrophic Acetogenic reactions			**[kj/reaction]**
$Propionate^- + 3\ H_2O$	⟶	$Acetate^- + HCO_3^- + H^+ + 3H_2$	+76.1
$Butyrate^- + 2\ H_2O$	⟶	$2\ Acetate^- + H^+ + 2H_2$	+48.3
$Lactate^- + 2\ H_2O$	⟶	$Acetate^- + HCO_3^- + H^+ + 2H_2$	–4.2
$Ethanol + H_2O$	⟶	$Acetate^- + H^+ + 2H_2$	+9.6
$Methanol + 2\ H_2O$	⟶	$HCO_3^- + H^+ + 3H_2$	+23.5
Methanogenic reactions			
$4\ H_2 + HCO_3^- + H^+$	⟶	$CH_4 + 3\ H_2O$	–135.6
$Acetate^- + H_2O$	⟶	$CH_4 + HCO_3^-$	–31.0
Methanol	⟶	$3/4\ CH_4 + 1/4\ HCO_3^- + 1/4\ H^+ + 1/4\ H_2O$	–78.2
Sulfate-reducing reactions			
$4\ H_2 + SO_4^{2-} + H^+$	⟶	$HS^- + 4\ H_2O$	–151.9
$Acetate^- + SO_4^{2-}$	⟶	$2\ HCO_3^- + HS^-$	–47.6
$Lactate^- + 1/2\ SO_4^{2-}$	⟶	$Acetate^- + HCO^{3-} + 1/2\ HS^- + 1/2\ H^+$	–80.0
$Ethanol + 1/2\ SO_4^{2-}$	⟶	$Acetate^- + 1/2\ HS^- + 1/2\ H^+ + H_2O$	–66.4
$Methanol + 3/4\ SO_4^{2-} + 1/4\ H^+$	⟶	$HCO_3^- + 3/4\ HS^-$	–90.4
Homoacetogenic reactions			
$Lactate^-$	⟶	$1\ 1/2\ Acetate^- + 1/2\ H^+$	–56.6
$Ethanol + HCO_3^-$	⟶	$1\ 1/2\ Acetate^- + H_2O + 1/2\ H^+$	–42.6
$Methanol + 1/2\ HCO_3^-$	⟶	$3/4\ Acetate^- + H_2O$	–55.0
$4\ H_2 + 2\ HCO_3^- + H^+$	⟶	$Acetate^- + 4\ H_2O$	–104.6
[a] $\Delta G'_O$ values are taken from Thauer . (1977)			

Studies with sediments and sludge from bioreactors have indicated that at an excess of sulfate hydrogen is mainly consumed by sulfate reducers (Banat IM,1981). In reactors with immobilized biomass the activity of hydrogenotrophic methanogens is completely suppressed within a few weeks when sulfate is added (Visser A,1993).As hydrogenotrophic methanogens are still present in high numbers in such reactors, this effect cannot simply be explained by Michaelis-Menten or Monod kinetic data (Table 4.3). In methanogenic environments the hydrogen partial pressure is low. However, by addition of sulfate the hydrogen partial pressure may even become

lower. The hydrogen partial pressure becomes so low that thermodynamically hydrogenotrophic methanogenesis is not possible any more (Figure 4.1). In freshwater sediments a threshold hydrogen concentration of 1.1 Pa has been measured; this value was lowered to 0.2 Pa by the addition of sulfate .

An additional effect of the addition of sulfate is that hydrogen formation becomes less important. In the absence of sulfate, hydrogen has to be formed by acetogenic bacteria in the oxidation of compounds like lactate, alcohols, propionate and butyrate. However, in the presence of sulfate, all these compounds can be oxidized directly by sulfate-reducers without the intermediate formation of hydrogen. However, this explanation cannot be the only one because fermentative glucose- and amino acid-degrading bacteria will always form some hydrogen.

Methanogens,which grow on H_2/CO_2,are autotrophic (Whitman WB,1992).Among the hydrogen-utilizing sulfate-reducing bacteria both autotrophic and heterotrophic species have been isolated (Smith RL,1981). The classical *Desulfovibrio* species require acetate and carbon dioxide or another organic carbon source for growth whereas, e.g.,*Desulfobacterium*sp. canuse CO_2 as the sole source of carbon (Widdel F,1992). Enrichments in media with H_2 and sulfate as energy substrates and carbon dioxide as the sole carbon substrate resulted in stable cultures of *Desulfovibrio* and *Acetobacterium*, in a cell ratio of about 20 to 1. The *Desulfovibrio* species required acetate for growth, which was provided by the homoacetogenic *Acetobacterium* species. Sulfate-reducing bacteria have a higher affinity for hydrogen than homoacetogens, but apparently the sulfate-reducers are dependent on the homoacetogens for synthesis of their carbon source acetate. It can be speculated thatunder these conditions the kinetic properties of homoacetogens determine the kinetic properties of the sulfate-reducers. In that case, methanogens would win the competition for hydrogen from the sulfate-reducers even at an excess of sulfate.unfortunately, an experiment which could demonstrate this has never been performed. Van Houten (Van Houten RT,1995) startedup bioreactors at high hydrogen partial pressures with solely bicarbonate as carbon source. This led to the coexistence of sulfate-reducers and homoacetogens.

Table 4.3: Selected growth kinetic data of hydrogenotrophic sulfate-reducing bacteria and methanogens.

Microorganism	K_s (μM)	μ_{max} (1/day)	$Yield^a$ (g/mal H_2)	K_m (μM)	V_{max} (μmol/min . g)
Sulfate reducers					
Desulfovibrio					
desulfuricans[b]		1.6-4.3	1.9	1.8-4.0	88
vulgaris[b]		0.7-5.5	0.6-3.1	1.3-4.0	30
Desulfovibr	2.4-4.2	1.2-1.6	1.4-2.0	1.1	65
Desulfobacter hydrogenophilus	1.0				
Desulfobacterium autotrophicum			0.7-1.1		
Desulfobulbus propionicus[b]			0.2- 1.7		
Desufomicrobium escambium			1.4		
Methanogens					
Methonabacterium					
bryantii		0.3-1.9	0.6		
formicicum[b]		1.2-3.1	0.9	2	
Ivanovii			0.8-1.7	11	14
Methanobrevibacter					
arboriphilus[b]		0.7-3.4	0.6-1.3	6.6	
Smithii		4.1			
Methanococcus vannielii		4.1			
Methanospirillum hungatei		1.2-1.8	0.3-0.6	5.0	70
Strain BD		2.4-4.8			
Methanosarcina		1.4-1.8	1.6-2.2	13	110

4.3.3 Competition for Acetate

It has been shown that in marine and freshwater sediments acetate is mainly consumed by sulfate-reducers when sufficient sulfate is present (Winfrey MR,1977).However, for anaerobic digesters it is less clear how acetate is degraded.A complete conversion of acetate by methanogens, even at an excess of sulfate, has been reported (Ueki K,,1988)However, in some studies a predominance of acetate-degrading sulfate-reducers was found (Visser A ,1995).

The work of Schonhei (Schönheit P,1982) has indicated that the predominance of *Desulfobacter postgatei* in marine sediments could be explained by its higher affinity for acetate than *Methanosarcina barkeri*. The Km values were 0.2 and 3.0 mM, respectively (Table 4.4). However, in bioreactors *Methanosarcina* sp. Are only present in high numbers when the reactors are operated at a high acetate concentration or operated at a low pH (Grotenhuis JTC 1992). Generally,

Methanosaeta (former *Methanothrix*,) sp. are the most important aceticlastic methanogens in anaerobic bioreactors (Morvai L,1992). Also in freshwater sediments *Methanosaeta* seems to be the most numerous acetoclastic methanogen (Scholten JCM 1999).*Methanosaeta* sp. have a higher affinity for acetate than *Methanosarcina* sp.; their Ks is about 0.4 mM (Jetten MSM,1992). In addition, *D. postgatei* and other *Desulfobacter* species are typical marine bacteria, which have not yet been isolated in freshwater media (Widdel F ,1987).

The aceticlastic sulfate-reducers that prefer freshwater conditions, such as *Desulfoarculus baarsii* , *Desulfobacterium catecholicum*, and *Desulfococcus biacutus* , show very poor growth with acetate. Only *Desulfobacterium* strain AcKo and *Desulfotomaculum acetoxidans* show good growth with acetateunder mesophilic conditions (see Table 4.4) unfortunately no Ks or Km values are available for these bacteria.

Two abundant acetate-degrading sulfate-reducers, *Desulforhabdus amnigenus* and *Desulfobacca acetoxidans*, were isolated from sulfate-reducing bioreactors (Oude Elferink SJWH,1995). The Michaelis-Menten parameters for *D. amnigenus* (KM = 0.2–1 mM,Vmax = 21–35 μmol · min^{-1} · g $protein^{-1}$) and *D. acetoxidans* (KM = 0.1–1 mM,Vmax = 29–57 μmol · min^{-1} · g $protein^{-1}$) were in the same range as or slightly better than those of most *Methanosaeta* species (KM = 0.4–1.2 mM, Vmax =32–170 μmol · min^{-1} · g $protein^{-1}$). This was also the case for the specific growth rate and the threshold value for acetate,which were 0.14–0.20 day^{-1} and <15 mM for *D. amnigenus* and 0.31–041 day^{-1} and <15 μM for *D. acetoxidans*. Reported values for *Methanosaeta* species are 0.08–0.69 day^{-1} and 7–69 μM, respectively. Putting all kinetic information together, it seems that the growth kinetic properties of acetate-degrading sulfate-reducers are only slightly better than those of *Methanosaeta*.

When the growth kinetic properties of the sulfate-reducers are only slightly better than those of the methanogens it can be expected that the initial relative cell numbers affect the outcome of competition experiments. This is in particular the case for methanogenic sludge from bioreactors where a major part of the microbial biomass may consist of *Methanosaeta*.When methanogenic bioreactors are fed with sulfate, the few initial acetate-degrading sulfate-reducers have to compete with huge numbers of aceticlastic *Methanosaeta* species. InuASB reactors the sludge age can be as high as 0.5^{-1} year (Hulshoff Pol LW ,1989) Visser (Boone,1988) have

simulated the competition between sulfate-reducing bacteria and methanogensusing a biomass retention time in the reactor of 0.02 day^{-1}, a maximum specific growth rate of 0.055 and 0.07 day^{-1} for the methanogen and sulfate-reducing bacterium, respectively, a Ks value for acetate of 0.08 and 0.4 mM acetate, respectively, and different initial ratios of bacteria. Starting with a ratio of methanogens/sulfate reducers of 104, it will take already one year before the numbers of acetate-degrading sulfate-reducing bacteria and acetate-degrading methanogens are equal. Nevertheless, long-termuASB reactor experiments of Visser (Grotenhuis JTC 1992) showed that sulfate-reducers are able to outcompete methanogens for acetate, even if the seed sludge initially only contains low numbers of aceticlastic sulfate-reducers. In his acetate- and sulfate-feduASB reactor it took 50 days before acetate degradation via sulfate reduction was observed, and another 50 days to increase it to 10%. The shift from 50 to 90% of acetate degradation via sulfate reduction took approximately 400 days.

Methanosaeta can only grow on acetate, whereas *Methanosarcina* canuse a few other substrates besides acetate, like hydrogen, methanol and methylated amines (Widdel F,1981). Aceticlastic *Desulfobacter* sp. alsouse a limited range of substrates; solely hydrogen, acetate and ethanol provide good growth *Desulfobacca acetoxidans* is also a true specialist. It only showed growth on acetate.However, *Desulfotomaculum acetoxidans* and *Desulforhabdus amnigenus*use a wide range of the common substrates for sulfate-reducers for growth. It is not clear to which extent these bacteria can grow mixotrophically. During growth on, e.g., butyrate or ethanol acetate is even excreted (Oude Elferink SJWH,1998). However, if low concentrations of acetate and other substrates areused at the same time the outcome of the competition between *Methanosaeta* and these sulfate- reducers will be affected.

4.3.4 Competition for Methanol

Methanol is an excellent substrate for mesophilic methanogens and homoacetogens. *Methanosarcina* species, *Acetobacterium woodii,Eubacterium limosum*and *Butyribacterium methylotrophicum* show very fast growth on methanol (Table 4.5). The homoacetogens require externally supplied bicarbonate for growth,while the methanogens do not. Remarkably, only a very few mesophilic species of sulfate-reducing bacteria can grow on methanol (Braun M,1985). The maximum specific

growth rates of these sulfate-reducers are much lower than those of the methanogens and homoacetogens. This suggests that sulfate-reducers are poor competitors for methanol.

Table 4.4: Selected growth kinetic data of acetotrophic sulfate-reducing bacteria and methanogenic bacteria.

Microorganism	K_s (µM)	μ_{max} (1/day)	$Yield^a$ (g/mal H_2)	K_m (µM)	V_{max} (µmol/min . g)
Sulfate reducers					
Desulfobacter					
curvatus		0.79			
hydrogenophilus		0.92			
latus		0.79			
postgatei[b]		0.72-1.11	4.3-4.8	0.07-0.23	53
Desulfotomaculum acetoxidans		0.65-1.39	5.6		
Desulforhabdus amigenous		0.14-0.20	0.6		28
Desulfobacca acetoxidans		0.31-0.41	0.6		43
Methonagens					
Methanosarcina barkeri[b]		0.46-0.69	1.6-3.4	3.0	
mazei[b]		0.49-0.53	1.9		
Methanosaeta					
soehngenii[b]	0.5	0.08-0.29	1.1-1.4	0.39-0.7	38
Concilii		0.21-0.69	1.1-1.2	0.84-1.2	16

[a] The Yield is given in gram celldry weight per mol.
[b] Several Strains.

The competition between methanogens and homoacetogens in bioreactors has been studied.(Florence ., 1994)It appears that the Ks value of methanogens for methanol is 0.25 mM, while that of the homoacetogens is much higher (16 mM).This indicates that at a low methanol concentration methanol is mainlyused by methanogens. Only at a high methanol concentration, and additionally a high bicarbonate concentration, was a substantial part of the methanol consumed by homoacetogens.

During growth on methanol methanogens and homoacetogens produce some hydrogen. The amount of hydrogen which is produced is affected by the presence of sulfate-reducers. This results in the coexistence of methanol-utilizing and hydrogen-utilizing anaerobes (Phelps TJ,1985). Thus, it seems that in mixed communities growing on methanol there is an indirect competition between methanogens and sulfate-reducers as well.

At low temperature methanogenesis became the dominant process, indicating that methanol is mainly consumed by methanogens. However, at a high temperature (65 °C) sulfate reduction became the dominant process (Weijma J,2000). Some thermophilic *Desulfotomaculum* species show excellent growth with methanol.

Table 4.5: Specific growth rates and growth yields (g dry weight · mol–1) of methanol utilizing anaerobic bacteria.

Microorganism	μ_{max} (1/day)	Yield (g / mol.methanol)
Methonagens		
Methanosarcina barkeri		
strain MS	2.35	3.5
strain 227	1.85	3.8
Methanosarcina mazei	3.24	
Mehanosarcina acetivorans	3.20	
Homoacetogens		
Acetobacterium woodii	5.3-8.2	
Eubacterium limosum	2.38	7.1
Butyribacterium methylotrophicum	1.85	8.2
Sulfate reducers		
Desulfovibrio carbinolicum	0.22	

4.3.5 Competition for Organic Acids and Ethanol

In anaerobic environments with high sulfate concentrations, sulfate-reducing bacteria compete with acetogenic bacteria for substrates like lactate, ethanol,propionate and butyrate. Little is known about this competition.

The fate of ethanol and lactate in anaerobic environments is not completely clear. A few methanogens are able to oxidize ethanol and other alcohols [97, 98]. In the presence of sulfate they can be oxidized by, e.g., *Desulfovibrio* species. However, lactate and ethanol (+CO_2) can also be fermented by bacteria in a propionic acid or homoacetogenic fermentation. In addition, lactate (+acetate) and ethanol (+acetate) can be fermented in a butyric acid fermentation by *Clostridium kluyveri*. Chemostat experiments have indicated that at low concentrations lactate and probably also ethanol are mainly consumed by sulfate-reducers. *Desulfomicrobium* outcompeted *Veillonella* and *Acetobacterium* at low acetate concentration. However, it appeared that the *Veillonella* sp. had a much higher specific growth rate than the sulfate-reducer, 0.30 and 0.17 h^{-1}, respectively. Interestingly, sulfate-reducers are also able to ferment lactate and ethanol. Lactate and ethanol can be oxidized to acetate and hydrogen, provided that the hydrogen partial pressure is kept low by methanogens

[99],while *Desulfobulbus* species are able to ferment lactate and ethanol in a propionic acid fermentation (Laanbroek HJ,1982; Tasaki M,1992).

For wastewater with an excess of sulfate it is to be expected that sulfate-reducing bacteria become predominant over syntrophic fatty acid-degrading consortia, because of their better growth kinetic properties. It is obvious that at high sulfate concentrations, sulfate-reducing bacteria grow much faster than the syntrophic consortia.Almost no Ks and Km values for propionate and butyrate degradation have been reported. Therefore, a comparison of the growth of syntrophic cultures and sulfate-reducers at low substrate concentrations is not possible.The existence of two subpopulations of propionate-oxidizers in methanogenic sludge was reported (Heyes RH,1983) a fast-growing one with a μmax of 1.2 day^{-1} and a Ks of 4.5 mM, and a slow-growing one with a higher affinity (μmax of 0.13 day^{-1} and a Ks of 0.15 mM).

Several researchers investigated the competition for propionate and butyrate between sulfate-reducers and acetogens in anaerobic reactors and in sediment slurries. In most cases syntrophic consortia are easily outcompeted by sulfatereducers. However, in some of these studies no distinction can be made between a direct oxidation of propionate and butyrate by sulfatereducers and an indirect conversion whereby the fatty acids are oxidized to acetate and hydrogen by the acetogenic bacteria followed by hydrogen conversion via sulfate reduction. In this respect it is important to note that sulfatereducers keep the hydrogen partial pressure lower than methanogens, and that propionate- and butyrate-degrading acetogens grow much faster in coculture with hydrogen-consuming sulfate-reducers than with hydrogen-consuming methanogens (Laanbroek HJ,1982; Tasaki M,1992). Therefore, the reported critical role of sulfatereducers in mediating propionate and butyrate degradation (Harmsen HJM ,1996) may be that of a hydrogen-consumer or that of a direct propionate or butyrateoxidizer.

The population dynamics of propionate- oxidizing bacteria in two UASB reactors, one fed with propionate and sulfate and the other with only propionate were studied.(Harmsen ,1996) In the first reactor the number of *Desulfobulbus* sp. increased rapidly, and in the second reactor the number of syntrophic propionate oxidizers increased. It seemsunlikely that *Desulfobulbus* acted as a hydrogen scavenger in the first reactor, although *Desulfobulbus* sp. are able touse H_2 as well as propionate, because no syntrophic propionate- oxidizers were enriched in this

reactor, and all *Desulfobulbus* cells were localized on the outside of the granule, not intertwined with other bacteria.Remarkably, *Syntrophobacter* species are also able to grow on propionate and sulfate (Kuijk van BLM,1995; Zellner G,1996).

4.4 Competition Between Sulfate-Reducers and Acetogens in the Absence of Sulfate

The role of sulfate-reducing bacteria in the anaerobic digestion in the absence of sulfate has hardly been investigated. Yet, recent studies showed that sulfatereducing bacteria can be present in methanogenic sludge toupto 15% of the total biomass . It is known that several types of sulfate-reducing bacteria have fermentative or syntrophic capacities.

Growth of sulfate-reducers in the absence of sulfate could explain the fast response of methanogenic ecosystems to the addition of sulfate.Some substrates which can be fermented by sulfate-reducers are pyruvate, lactate, ethanol, fumarate and malate, fructose, serine, choline, acetoin and *S*-1,2-propanediol and propanol + acetate. Sulfate-reducers can also grow as acetogens in the absence of sulfate. *Desulfovibrio* sp. oxidize ethanol or lactate to acetate when co-cultured with methanogens (Yadav VK,1988). It has been reported that *Desulfovibrio* sp. were the main lactate- and ethanol-degrading bacteria in a reactor treating whey in the absence of sulfate (Chartrain M,1986; Zellner G,1987). However, others reported that only in the presence of sulfate were *Desulfovibrio* sp. the dominant lactate degraders, while in the absence of sulfate lactate was fermented according to theusual fermentation pattern of *Propionibacterium*. Syntrophic formate degradation has been reported for *Desulfovibrio vulgaris* in association with *Methanobacterium bryantii* (Guyot J-P1986), and a *Desulfovibrio*-like organism could syntrophically degrade alcohols like 1,3-butanediol, 1,4-butanediol, 1-butanol and 1-propanol in the presence of 10 mM acetate and *Methanospirillum hungatei* (Tanaka K ,1992).

The syntrophic conversion of propionate was mainly performed by sulfate reducing bacteria, and they were able to isolate such an organism.This indicates that in the absence of sulfate certain propionate-degrading sulfate-reducing bacteria able to oxidize propionate in syntrophic association with H_2-consuming anaerobes,while in the presence of sulfate they couple propionate oxidation to sulfate reduction. This represents a considerable ecological advantage of this type of sulfate-reducing

bacteria over obligate syntrophic propionate degraders in ecosystems where sulfate is continuously or intermittently available.(Zeikus .,1992)

Interestingly, as mentioned before, several *Syntrophobacter* species, including *S.wolinii* , *S. pfennigii* , *S. fumaroxidans* , strain HP1.1, were shown to grow on propionate with sulfate. For *S. wolinii* this finding was very remarkable because *S. wolinii* grows as an acetogen in the presence of *Desulfovibrio* G11. Phylogenetic research, based on 16S rRNA sequences, showed that *Syntrophobacter* species belong to the Gram-negative sulfate-reducers (Harmsen HJM,1993).

Thus far, growth of sulfate-reducers on butyrate in the absence of sulfate but in the presence of methanogens was not yet demonstrated. However, *Desulfovibrio* sp. were detected in a fixed-bed reactor fed with butyrate without sulfate (Zellner G,1991).

4.5 Inhibition

Much of the decrease in methane production caused by intermediate nitrogenoxides of the denitrification process (NO_2^-, NO and N_2O) is due to toxicity of these compounds rather than competition andunfavorable redox conditions. The inhibition mechanism of nitrate and its denitrification products is stil largelyunknown. The reduction of oxidized nitrogen species for dissimilatory electron dissipation by fermentative bacteria yields ammonia which numerous authors have demonstrated to be toxic to methanogenic consortia. Ammonia is mainly toxic in itsun-ionized form (NH_3) while the ammonium ion (NH_4^+) is much less toxic, and toxicity is therefore dependentupon pH and temperature of the reactor. Figure 4.3 shows the effect of temperature and pH on the percentage of total ammonium (NH_4^++NH_3) which appears as NH_3 . It is obvious that increasing temperature and pH leads to increased NH_3 concentrations in a reactor.

If the sludge fed to the reactor simultaneously contains high amounts of proteinaceous material or/and pig manure, large amounts of ammonia are released from the fermentation of amino acids and other nitrogen-rich compounds (Angelidaki I,1993). Ammonia has been shown to mainly affect acetate-utilizing methanogenic Archaea, and to a lesser degree, hydrogen-utilizing methanogens and syntrophic bacteria (Heinrichs DM,1990). A decrease in pH and an increase in the

concentration of volatile fatty acids observed in ammonia-inhibited reactors, however, point towards an inhibition of all terminal microorganisms of the anaerobic degradation chain (Poggi-Varaldo HM,1997). In two studies on the effects of high ammonia concentrations (7 g NH_4^+– N/L) on methanogenesis from acetate, Blomgren (Blomgren A,1990) and Schnurer (Schnürer A,1994) demonstrated that aceticlastic methanogenesis was displaced in favor of syntrophic acetate oxidation in enriched and defined cultures growing with acetate as the only substrate.When the anaerobic processes are inhibited by ammonia, the decrease in pH will counteract the effect of ammonia due to a decrease in the free ammonia concentration.

Since anaerobic reactorsused in different ammonia toxicity studies have often been operated at different pH values, it is difficult to generalize about the inhibitory concentration as different concentrations of NH_3 ammonia are present. In most reactor studies,however, inhibitory concentrations are in the range 1.7–5 g total ammonia-N/L, corresponding to 0.4–1 g NH_3^- ammonia/L. Several authors have also shown that the biogas process can be adapted to ammonia concentrations above 4 g total ammonia/L without any reduction of the methane yield (van Velsen AFM ,1979).

Sulfide produced by sulfate-reducing bacteria and by fermentation of sulfurcontaining amino acids has been shown to be inhibitory to the biogas process by several authors . Similar to ammonia, it is generally assumed that the neutralundissociated sulfide is the agent of toxicity since it is only membrane permeable in this form (O'Flaherty,1998). The pH is therefore also an important determinant of the toxicity, but contrary to ammonia, low pH values and low temperatures favor theundissociated sulfide (Figure 4.3) .Much of the published literature on sulfide toxicity does not take pH into consideration, which makes general conclusions about toxicity levels difficult. Since sulfide readily reacts with most metals to form insoluble metal sulfides, the toxicity of sulfide is also related to metal concentrations in the sludge. However, several authors have found that sulfide inhibits the biogas process at concentrations around 50 mg/L (Parkin GF,1983). Sulfide and ammonia have been shown to inhibit methanogenesis in thermophilic anaerobic digesters synergistically. A sulfide concentration of only 23 mg/L led to an approximately 40% decrease of the methane production in a digester treating material with a high ammonium concentration (Hansen KH,1999). From Figure 4.3 it

is obvious that optimal conditions for maintaining a low concentration ofundissociated H_2S and NH_3 are occurring at lower pH values for thermophilic digesters than for mesophilic degistion.

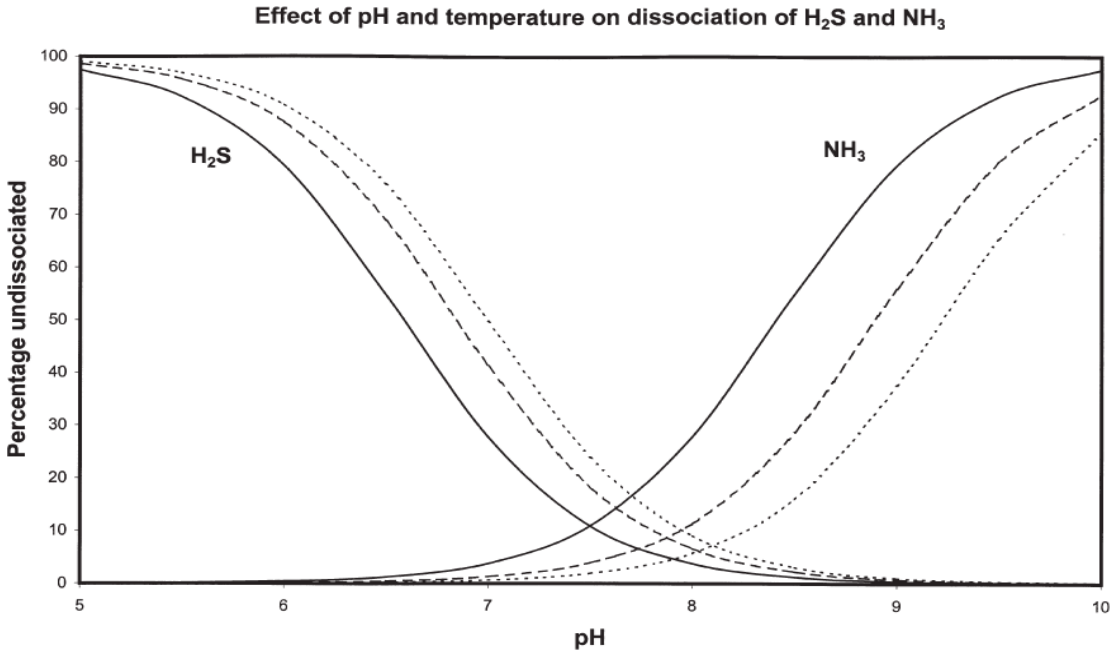

Figure 4.3: The effect of pH and temperature on the dissociation of H_2S and NH_3.(---)25 °C; (– – –) 37 °C; (——) 60 °C

5.MOLECULAR TECHNIQUES USED IN MOLECULAR ECOLOGY

5.1 The Need for Molecular Techniques

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

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

Despite the developments in the microscopy, direct microscopic analyses have many limitations in identifying microorganisms. The small size of prokaryotic organisms, the absence of distinguishing phenotypic characters, and the fact that most of these organisms cannot be cultured are the most important factors that limit the evaluation of the biodiversity (Pace, 1997; Torsvik and Øvreås, 2002; Torsvik ., 2002).In last 20 years, a significant number of studies dealing with microbial biodiversity involve theuse of molecular tools and have often focused on investigating the dynamics of the composition and structure of microbial populations and communities in defined environments, and the impact of specific factors, such as pollution by xenobiotics on microbial diversity (Morris ., 2002;Ranjard ., 2000).

5.1.1 The 16S rRNA and its Importance

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

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

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

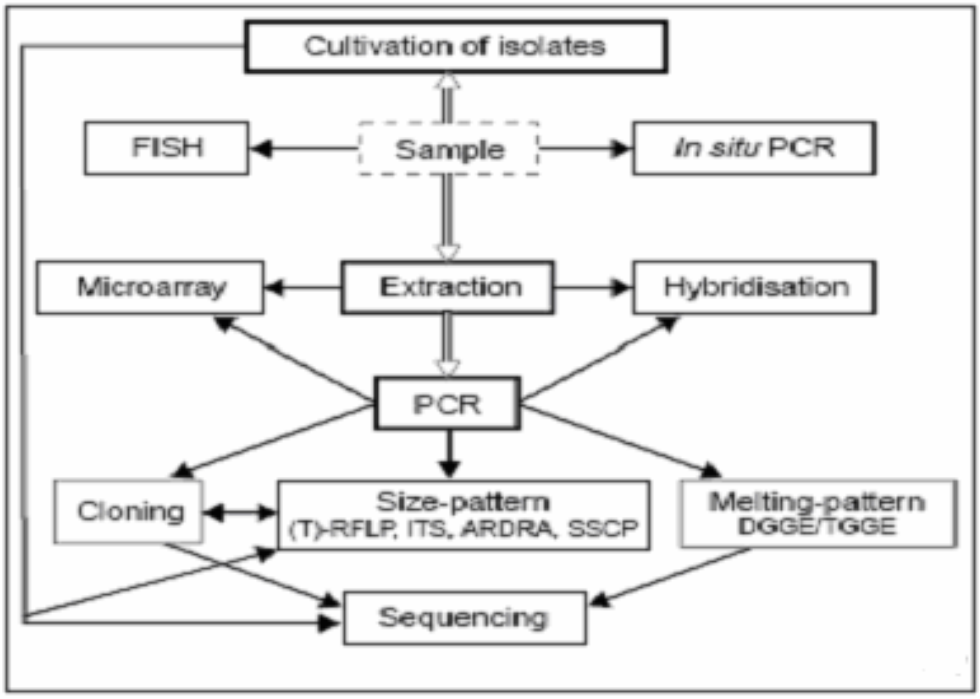

Figure 5.1: Common approaches to the analysis of microbial diversity (Dahllof,2002)

5.1.2 The Variable Regions in 16S rRNA and its Importance

The rRNA is highly conserved in nucleotide sequence as well as in secondary structure since its function remains same through years of evolution. It has many variable regions in which random changes occur time to time. These changes reflect evolutionary relationships of the organisms. Conserved regions functions as binding places for PCR primers or hybridization probes. Even data from this analysis is sufficient to compare statistically significant phylogenetic relations (Olsen ., 1986). Among the variable regions, V3 region is mostlyused in molecular analysis (Neefs, , 1990; Øvreas ., 1997).

5.2 Polymerase Chain Reaction (PCR)

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

PCR isused to amplify specific regions of a DNA strand. This can be a single gene, just a part of a gene, or a non-coding sequence. PCR process mainly based on three steps: Denaturation, Annealing, and Extension. In denaturation step double stranded

DNA templates melted and separated by high temperature. In annealing step the reaction temperature is lowered so that the primers can attach to the single-stranded DNA template. Then temperature is increased again to a level (72^0 C mostly) in which Taq polymerase can elongate the chain by adding nucleotides. (dNTPs) This cycle of binding of primer and elongation and then disassociation repeated 30-40 times to recover enough DNA segment of interest. The addressed sequence amplified in order of 2. (2^n where n is the cycle number) The resulted product will be run on an agarose gel to monitor efficiency of the PCR. Mostly Ethidium Bromide (EtBr) isused to stain DNA which renders DNA visibleunderuV light.

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

5.2.1 Limitations and Biases of PCR

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

Most common problem regarding PCR comes from its power to amplify DNA. Sensitivity of PCR is so high even a very small amount of DNA (a single copy in theory) out of the sample DNA can be detected and amplified by *Taq* polymerase. An extreme sterilization and care needed in performing PCR. A negative control

without a DNA template or DNaseI treatment of reagents can be done to prevent contamination caused by a foreign DNA (Schmidt ., 1991).

5.2.2 PCR Based Techniquesused in Molecular Ecology

5.2.2.1 Quantitative PCR

The application of PCR in combination with the extraction of nucleic acids (DNA and RNA) from environmental matrices has been central to the development of culture independent approaches in microbial ecology. These methods, which have been applied since the early 1990s (e.g. Giovannoni ., 1990), enabling the analysis of the total microbial communities present within environmental systems, have revolutionized ourunderstanding of microbial community structure and diversity within the environment. Coupling environmental nucleic acid isolation to subsequent PCR amplification of both taxonomic (i.e. rRNA) and functional gene markers and in combination with DNA fingerprinting- and sequencing-based analyses has enabled description of the hithertouncharacterized majority of environmental microorganisms (Head., 1998) driving the discovery of new microbial lineages and enabling the description of genetic diversity in a wealth of functional gene markers (Larkin ., 2005). Although recently developedultra-high-throughput sequencing technologies such as pyrosequencing (Margulies ., 2005; Edwards .,2006) now dwarf PCR-based sequence studies in terms of sequence coverage, the ability of the PCR to specifically target particular taxonomic or functional markers from domain – down to strain – or phylotype levels means that PCR will remain an invaluable method in the molecular microbial ecologist's toolbox. Nevertheless, PCR has inherent limitations (Von Wintzingerode., 1997), particularly those that result in biases in the template to product ratios of target sequences amplified during PCR from environmental DNA (Suzuki & Giovannoni, 1996; Polz & Cavanaugh, 1998), with such amplification biases found to increase with increasing numbers of PCR cycles. These limitations presented a significant challenge to microbial ecologists who were interested in determining the abundance of individual genes present in environmental samples. To circumvent such challenges, an adaptation of the PCR method developed by Holland .(1991)utilizing the so-called '50 nuclease assay' was applied to quantify target 16S rRNA genes amplified from environmental DNA by PCR (Becker., 2000; Suzuki ., 2000; Takai & Horikoshi, 2000).This development had been facilitated by the earlier

combination of the 50 nuclease assay developed by Holland (1991) with fluorescence detection following cleavage of an internal (TaqManTM) DNA probe (Livak, 1995), enabling the accumulation of amplicons to be monitored after each cycle (in real-time) and hence facilitating quantitative determination of the initial template gene (or transcript) numbers.

Quantitative-PCR or Q-PCR (often referred to as realtime PCR) is now widelyused in microbial ecology to determine gene and/or transcript numbers present within environmental samples. The target specificity of any Q-PCR assay is determined by the design of the primers (and in some cases an internal probe), allowing quantification of taxonomic or functional gene markers present within a mixed community from the domain level down to the quantification of individual species or phylotypes. Q-PCR has been shown to be a robust, highly reproducible and sensitive method to quantitatively track phylogenetic and functional gene changes across temporal and spatial scalesunder varying environmental or experimental conditions. Moreover, the quantitative data generated can beused to relate variation in gene abundances and/or levels of gene expression (in terms of transcript numbers) in comparison with variation in abiotic or biotic factors and/or biological activities and process rates. The provision of Q-PCR data sets that describe the abundance of specific bacteria or genes to complement other quantitative environmental data sets is of increasing importance in microbial ecology as it furthersunderstanding of the roles and contributions of particular microbial and functional groups within ecosystem functioning. Furthermore, reverse transcription (RT) analyses are now increasingly combined with Q-PCR methods in RT-Q-PCR assays, offering a powerful tool for quantifying gene expression (in terms of numbers of Rrna and mRNA transcripts) and relating biological activity to ecological function.

5.2.2.2 Advantages of Q-PCR over traditional endpoint PCR

Q-PCR approaches combine the detection of target template with quantification by recording the amplification of a PCR product via a corresponding increase in the fluorescent signal associated with product formation during each cycle in the PCR. Quantification of gene (or transcript) numbers is determined during the exponential phase of the PCR amplification when the numbers of amplicons detected are directly proportional to the initial numbers of target sequences present within the environment. Quantification of the target gene during exponential amplification

avoids problems that are associated with so-called ‘end-point’ PCR (in which amplicons are only analysed after completion of the final PCR cycle). In end-point PCR, the proportions of numerically dominant amplicons do not necessarily reflect the actual abundances of sequences present within the environment due to the inherent biases of PCR that are associated with amplification of targets from mixed template community DNA (Reysenbach, 1992; Suzuki & Giovannoni, 1996; Polz & Cavanaugh, 1998). Moreover, Q-PCR thatuses fluorescence-based detection offers greater sensitivity and enables discrimination of gene numbers across a wider dynamic range than is found with end-point PCR; for example twofold changes in target concentration can be discriminatedusing Q-PCR. Before the development of fluorescence-based Q-PCR-based methods, two alternative PCR-based methods for gene number quantification had been developed, namely competitive PCR (Diviacco , 1992) and limiting dilutions or most probable number (MPN)-PCR (Skyes , 1992). However, these methods are time- and resource-consuming, requiring post-PCR analysis, and have now largely been replaced by fluorescence-based Q-PCR methods.

5.2.2.3 Advantages ofusing Real-Time PCR

* Traditional PCR is measured at end-point (plateau), while real-time PCR collects data in the exponential growth phase

* An increase in reporter fluorescent signal is directly proportional to the number of amplicons generated

* The cleaved probe provides a permanent record amplification of an amplicon

* Increased dynamic range of detection

* Requirement of 1000-fold less RNA than conventional assays

* No-post PCR processing due to closed system (no electrophoretical separation of amplified DNA)

* Detection is capable down to a 2-fold change

* Small amplicon size results in increased amplification efficiency (Dorak .,2006)

5.2.2.4 Fluorescence detection chemistriesused to detect template amplification during Q-PCR

Quantitative real-time PCR works in essentially the same manner as end-point PCR, i.e. multiple amplification cycles in which template DNA is initially denatured, followed by annealing of oligonucleotide primers targeting specific sequences, followed by subsequent extension of a complementary strand from each annealed primer by a thermostable DNA polymerase, resulting in an exponential increase in amplicon numbers during the PCR. However, in contrast to end-point PCR, the increase in amplicon numbers is recorded in 'real-time' during the PCR via detection of a fluorescent reporter that indicates amplicon accumulation during every cycle. Two reporter systems are commonlyused, namely, the intercalating SYBR green assay (Wittwer ., 1997) and the TaqMan probe system (Holland., 1991; Livak ., 1995).

SYBR green binds to all double-stranded DNA via intercalation between adjacent base pairs. When bound to DNA, a fluorescent signal is emitted following light excitation (Fig. 1a). As amplicon numbers accumulate after each PCR cycle, there is a corresponding increase in fluorescence. Because SYBR green binds to all double-stranded DNA, it is essential touse primer pairs that are highly specific to their target

sequence to avoid generation of nonspecific products that would contribute to the fluorescent signal, resulting in an overestimation of the target. Extensive optimization of primer concentrationsused in SYBR green Q-PCR assays may be required to ensure that only the targeted product is formed. Primer pairs that exhibit self-complementarity should also be avoided to prevent primer–dimer formation. A post-PCR dissociation (melting) curve analysis should be carried out to confirm that the fluorescence signal is generated only from target templates and not from the formation of nonspecific PCR products. During a dissociation curve, the double-stranded template is heated over a temperature gradient and fluorescence levels are measured at each discrete temperature point. As the double-stranded template is heated, it denatures, resulting in a corresponding decline in fluorescence due to SYBR green dissociation from the double-stranded product (Giglio ., 2003; Gonzalez- Escalona ., 2006). The temperature at which 50% of the double-stranded template is denatured can beused to confirm that the template being targeted is present, along with the presence of other nonspecific template and primer dimers in

much the same way as agarose gel electrophoresis of an end-point PCR product isused.

The TaqMan probe methodutilizes a fluorescently labelled probe that hybridizes to an additional conserved region that lies within the target amplicon sequence. The TaqMan probe is fluorescently labelled at the 50 end and contains a quencher molecule at the 30 end (Livak.,1995). The close proximity on the probe of the quencher molecule to the fluorophore prevents it from fluorescing due to fluorescent resonance energy transfer. During the annealing step of each cycle of the PCR, primers and the intact probe bind to their target sequences. During subsequent template extension, the 50 exonuclease activity of the Taq polymerase enzyme cleaves the fluorophore from the TaqMan probe and a fluorescent signal is detected as the fluorophore is no longer in close proximity to the quencher (Figure 5.2b). Amplification of the template is thus measured by the release and accumulation of the fluorophore during the extension stage of each PCR cycle. The additional specificity afforded by the presence of the TaqMan probe ensures that the fluorescent signal generated during Q-PCR is derived only from amplification of the target sequence. Multiple TaqMan probes and primer sets can beused in different Q-PCR assays to differentiate between closely related sequences (Smith ., 2007), or alternatively, probes can be labelled with different fluorophores, facilitating the development of multiplex Q-PCR protocols whereby different targets can be coamplified and quantified within a single reaction (Neretin ., 2003;Baldwin ., 2003, 2008). For example, Baldwin (2003) developed a multiplex Q-PCR assay targeting a number of different aromatic oxygenase genesusing bacterial strains and then subsequently applied the assay to simultaneously quantify aromatic oxygenase genes in contaminated groundwater (Baldwin., 2008). TaqMan probes are, however, a more expensive option thanusing SYBR green chemistry and the former requires the presence of an additional conserved site within the short amplicon sequence to be present. Identification of three conserved regions within a short region (typically 100 bp) may not always be possible, especially when primer/probe combinations are being designed to target divergent gene sequences. More recent advances in TaqMan probe technology have involved the introduction of the minor groove binder (MGB) probe (Kutyavin ., 2000). The MGB molecule is attached to the 30 end of the probe and essentially folds back onto the probe. This not only increases the stability of the

probe, but allows the design of shorter probes (13–20 bp) than are required for traditional TaqMan probes (20–40 bp),while at the same time, maintaining the required hybridization annealing temperature.

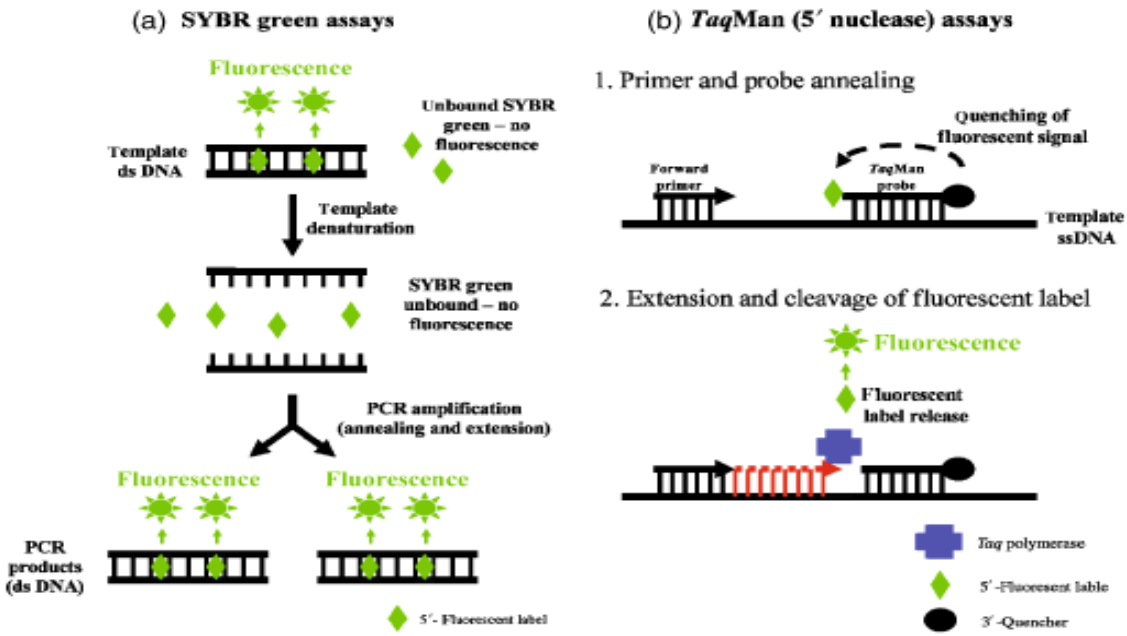

Figure5.2:. Real-time PCR chemistries: (a) SYBR green detection. SYBR greenbinds to all double-stranded DNA and emits a fluorescent signal. In itsunbound state,SYBR green does not fluoresce. Template amplification is therefore measured in each cycle by the corresponding increase in fluorescence. (b) TaqMan (50 nuclease) assayusing TaqMans probes. During annealing, the TaqMan probe and primers bind to the template. When the TaqMan probe is intact, energy is transferred between the quencher and the reporter; as a result, no fluorescent signal is detected. As the new strand is synthesized by Taqpolymerase, the 50 exonuclease activity of the enzyme cleaves the labelled 50 nucleotide of the probe, releasing the reporter from the probe. Once it is no longer in close proximity, the fluorescent signal from the probe is detected and template amplification is recorded by the corresponding increase influorescence.

5.2.2.5 Target quantification using the cycle threshold (Ct) method

Irrespective of the fluorescence chemistryused, quantification of the target template DNA is carried out in essentially the same manner. There are a number of different commercially available instruments to carry out Q-PCR, each with its own associated software. Currently, there is considerable debate as to which algorithms are the bestused to analyse Q-PCR data (reviewed in Rebrikov & Trofimov, 2006). All the Q-PCR platforms collect fluorescent data from every amplification cycle and the increase in fluorescence is plotted against the cycle number, resulting in the typical amplification curve shown in Figure 5.3. The Q-PCR amplification curve can be

subdivided into four stages, namely background noise, where the background fluorescence stil exceeds that derived from initial exponential template accumulation, exponential amplification, linear amplification and a plateau stage. During the exponential phase of the amplification, the amount of target amplified is proportional to the starting template and it is during these cycles that gene numbers are quantifiedusing the Ct method. The Ct is reached when the accumulation of fluorescence (template) is significantly greater than the background level (Heid ., 1996). During the initial cycles, the fluorescence signal due to background noise is greater than that derived from the amplification of the target template. Once the Ct value is exceeded, the exponential accumulation of product can be measured. When the initial concentration of the target template is higher, the Ct will be reached at an earlier amplification cycle.

Quantification of the initial target sequences of anunknown concentration is determined from the Ct values and can be described either in relative or in absolute terms. In relative quantification, changes in theunknown target are expressed relative to a coamplified steady state (typically a housekeeping) gene. Any variation in the presence (or expression) of the housekeeping gene can potentially mask real changes or indicate artificial changes in the abundance of the gene of interest. While this approach is commonly applied for studying eukaryotic gene expression (reviewed in Bustin, 2002), it is more difficult to apply this method for studying prokaryotic genes where the identification of a valid steady-state reference gene is problematic. Burgmann . (2007) nevertheless successfullyutilized such an approach when confirming microarray-based determination of the transcriptional responses of Silicibacter pomeroyi to dimethylsulphoniopropionate additions. From microarray experiments, they identified a gene whose expression was not altered by experimental conditions andused the expression of this gene to normalize levels of expression of the target genes of interest in RT-Q-PCR assays. In a number of other studies, gene and transcript numbers of the target gene of interest have been normalized to the numbers of 16S rRNA gene or transcripts (Neretin., 2003; Treusch .,2005; Kandeler ., 2006). For example, Treusch ,(2005) normalized the number of amoA transcripts to numbers of 16S rRNA gene transcripts in RNA extracted from ammonia-amended orunamended soils. They reported a statistically significant increase in amoA transcript numbers in the ammonia-amended soils. However,

although 16S rRNA genes and transcripts are now commonlyused in this manner, the application of such an approach is controversial, especially when studying genes/ transcripts amplified from nucleic acids extracted from complex environmental samples. This is, in particular, because 16S rRNA gene copy and transcript numbers are highly variable, with the number of 16S rRNA genes per operon varying dramatically between species (1–15 copies) while 16S rRNA gene transcription rates are regulated primarily by resource availability (Klappenbach, 2000). The 16S rRNA genes and transcripts cannot therefore be considered as a steady-state (housekeeping) gene, especially when studying genes/transcripts in environmental samples.

In absolute quantification protocols, the numbers of a target gene or transcript are determined from a Standard curve generated from amplification of the target gene present at a range of initial template concentrations, and then the Ct values for each template concentration are determined. Subsequently, a simple linear regression of these Ct values is plotted against the log of the initial copy number (Figure 5.3). It should be ensured that the Ct value of the most diluted template DNAused to

construct the Standard curve is at least a log fold lower (3.3 cycles) than the Ct value of the no template control (NTC). Quantification of theunknown target template is determined by comparison of the Ct values of the target template against the Standard curve. However, in reality, this 'absolute' quantification of the target gene represents quantification of the target in comparison against a constructed standard curve, rather than as an absolute measurement of the number of target genes present within an environmental sample. Any number of factors involved in the construction of the standard curve including the initial quantification of the standard curve template, serial dilution of the template and the algorithmic determination of the Ct value (Love., 2006) contribute to the final quantification of the environmental sample. As a consequence, it is recommended that the following descriptors of the standard curve are reported for each Q-PCR amplification: amplification efficiency (E), the linear regression coefficient (r^2) and especially the y-intercept value, whichuniquely describes the standard curve and indicates the sensitivity of the reaction (Smith., 2006; Figure 5. 3). Furthermore, the Ct value of the NTC and its equivalent value in terms of gene numbers should be reported. Moreover, we have previously demonstrated that even highly reproducible standard curves may result in statistically significant differences in gene numbers for the same template (with

equivalent Ct values) when gene numbers are quantified within different Q-PCR assays (Smith .,2006) due to the log nature of the curve, where by minor differences in Ct values and standard curves result in large differences in gene copy numbers.

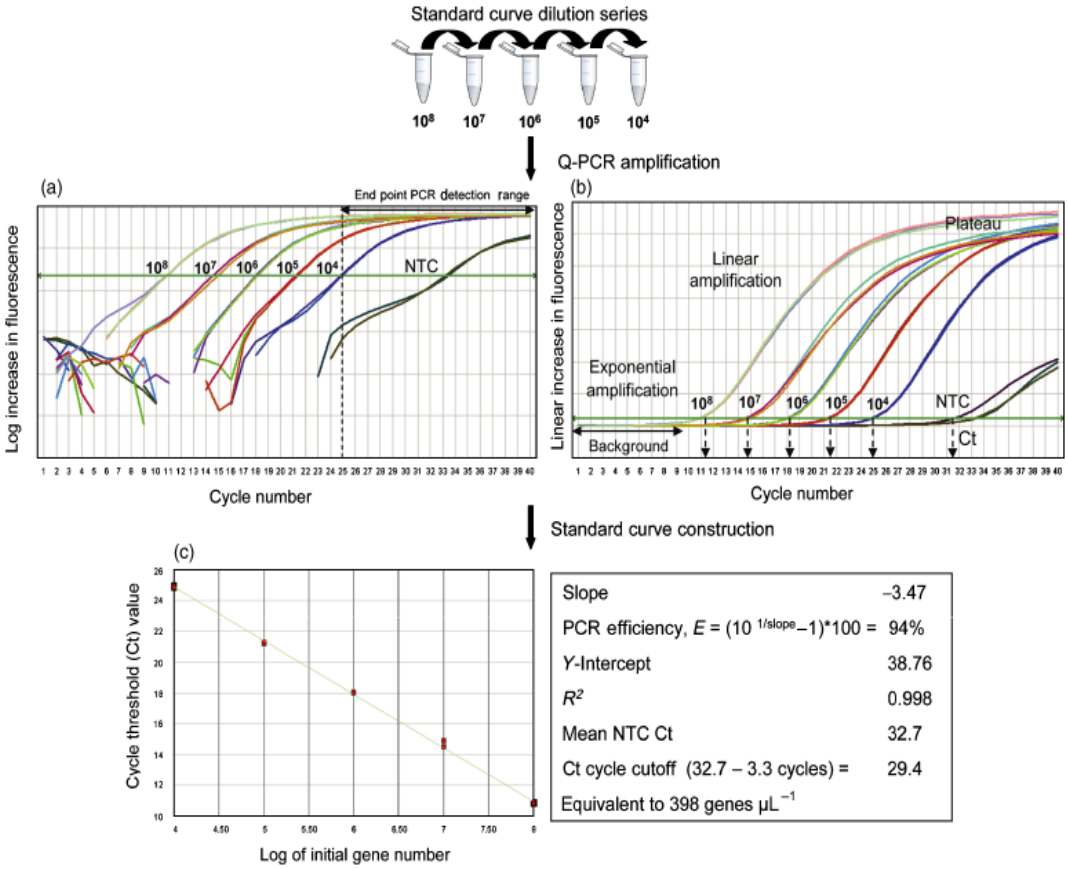

Figure 5.3:Q-PCR amplification from known concentrations of template DNA to construct standard curves for quantification ofunknown environmental samples. (a)Log plot of the increase in fluorescence vs. cycle number of DNA standards ranging from $1x10^4$ to $1x10^8$ 16S rRNA gene amplicons (b) Linear plot indicating the three phases of a PCR amplification, the corresponding Ct values for each of the amplified standards and for the NTC. (c) Simple linear regression of the Ct values (from b) vs. log of the initial rRNA gene number. Q-PCR descriptors are shown (boxed).

5.2.2.6 Application of Q-PCR for investigating the microbial genetic potential within the Environment

The first applications of Q-PCR in microbial ecology were reported in three papers published in November 2000, whichused TaqMan-based assays to target 16S rRNA genes(Becker, 2000; Suzuki, 2000; Takai & Horikoshi,2000). Becker (2000) demonstrated the ability of TaqMan probes to determine the abundance of a specific ecotype of Synechococcus sp. BO 8807 against a mixed background of phylogenetically related bacteriausing artificial mixed communities. Suzuki . (2000)

exploited the specificity and the sensitivity of TaqMan Q-PCR assays to determine spatial and temporal quantitative differences in the distributions of Synechococcus, Prochlorococcus and archaea in marine waters, while Takai & Horikoshi (2000) quantified archaeal 16S rRNA gene numbers within samples from a deep sea hydrothermal vent effluent, hot spring water and from hot spring and freshwater sediments. By targeting highly conserved regions of the 16S rRNA gene, Q-PCR assays have been designed to quantify 'total' bacterial (and or archaeal) numbers while targeting of taxa-specific sequences within hypervariable regions within the gene enables quantification of sequences from phylum to species levels, provided that there are sequence data available that enable the design of primers and probes. A caveat of this approach must be stressed; 16S rRNA gene numbers from environmental samples cannot be converted to cell numbers as the exact number of copies of the 16S rRNA gene in any given bacterial species varies (Klappenbach 2000).

Quantification of eukaryotes within environmental samples by Q-PCR can be carried out by targeting the 18S rRNA gene (Lueders, 2004; Zhu ., 2005) or the internal transcribed spacer (ITS) region (Landeweert ., 2003; Kennedy ., 2007). The ITS region is often targeted for the design of taxon-specific Q-PCR assays as it provides a greater degree of sequence differentiation between species and lower within-species variability (Kennedy , 2007) than is seen for the 18S rRNA gene. As with quantification of 16S rRNA gene numbers, Q-PCR-derived ITS region and 18S rRNA gene numbers cannot be directly equated to cell numbers. However, numbers of fungal rRNA gene or ITS numbers per volume of sample can beused to compare the relative numbers of fungi between different environmental samples (Guidot ., 2002).

In addition to quantitative data on taxonomic markers, Q-PCR has also been applied to quantify functional genes within the environment. By targeting functional genes that encode enzymes in key metabolic or catabolic pathways, the (genetic) potential for a particular microbial function within a particular environment can be assessed. To understand microbial functioning in the environment at a molecular level, it is essential not only to know what genes are present and the diversity of these genes but also to determine their abundance and distribution within the environment

Table 5.1: Quantitative PCR primer and probe sets targeting small subunit ribosomal RNA genes of bacteria, archaea

Target	Detection	Primer/probe	Sequence (5'-3')	Amplicon leight(bp)	Temperature (C^0)	References
Prokaryote	TM	Uni 340F	CCT ACG GGR BGC ASC AG	446	57	Takai & Horikoshi (2000)
16S rRNA gene	TM	Uni 806R	GGA CTA CNN GGG TAT CTA AT			
		TM 516F	TGY CAG CMG CCG CGG TAA HAC VNR S			
Bacterial	TM	BACT1369F	CGG TGA ATA CGT TCY CGG	123	56	Suzuki (2000)
16S rRNA gene		PROK1492R	GGW TAC CTT GTT ACG ACT T			
		Probe TM 1389F	CTT GTA CAC ACC GCC CG			
Bacterial	TM	331F	TTC TAC GGG AGG CAG CAG	466	60	Nadkarni (2002)
Archaeal	TM	Arch 349F	GYG CAS CAG KCG MGA A	457	59	Takai&Horikoshi (2000)

To this end, Q-PCR assays have been designed to target microbially mediated biogeochemical processes in the environment. Quantification of functional genes involved in ammonia oxidation (Hermansson & Lindgren, 2001;Okano., 2004; Treusch., 2005; Leininger, 2006; Mincer , 2007), nitrate reduction and denitrification (Lopez-Guti´errez ., 2004; Henry ., 2006; Smith ., 2007), sulphate reduction (Leloup ., 2007), methanogenesis (Denman ., 2007) and methane oxidation (Kolb, 2003) have been investigated . In a particularly striking example of the value of such functional gene Q-PCR assays,the relative contributions of ammonia-oxidizing archaea and bacteria to the first step of nitrification (ammonia oxidation) have been investigated both in soils (Leininger., 2006; He, 2007b) and in seawater (Mincer

.,2007) by determination of the abundance of archaeal- and bacterial-related amoA genes. These studies have suggested that archaea and not bacteria are the numerically dominant ammonia oxidizers in both environments. The results of such studies are therefore encouraging a re-evaluation of our basicunderstanding of nitrogen cycling and the relative importance of bacteria and archaea (or specific taxa or functional guilds within the domains) within key environmental processes. While these studies have greatly enhanced ourunderstanding of gene numbers in the environment, the next step to further ourunderstanding is to link variation in genetic potential (i.e. gene numbers) within a system in relation to variation in rates and activity of the biologically driven environmental processes in question, and hence enabling improvedunderstanding of theunderpinning factors that influence microbial functioning within the environment.

5.2.2.7 Pattern Analysis and Denaturing Gradient Gel Electrophoresis

Pattern analysis or fingerprinting is often carried out by evaluating banding patterns of PCR products on gels (Dahllof, 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 ., 2003; Dahllof, 2002). These techniquesusually involve gel electrophoresis that can separate different DNA fragments of a community Rona library (Dahllof, 2002).

DGGE is now routinelyused 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 beused 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 ., 2002). The DGGE technique has beenused 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 ., 1997), hot springs (Santegoeds., 1996), soils (Jensen., 1998), biofilm (Santegoeds, 1998). DGGE has beenused to

monitor changes in complex communities (Santegoeds ., 1996; Teske., 1996) and to identify microorganisms present in wall painting. It has recently been demonstrated that DGGE analysis of PCR products also works well in deep marine sediments and seafloor basalts (Lysnes., 2004). Denaturing gradient gel electrophoresis (DGGE) has beenused 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 primarilyused with bacterial communities by amplifying fragments from 16S rRNA genes (Muyzer and Smalla, 1998), DGGE has also beenused to explore the diversity of Archaea (Hoj., 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 ofurea 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, 2003, Dorigo ., 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., 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., 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, 2003). PCR amplification of the 16S rRNA geneutilizing conserved primers targeting either V3 or the V8 + V9 regions is normallyused to produce a 300-500 bp fragment. Larger fragments are typically

notused as the DGGE technique can not resolve these into distinct bands (Muyzer, 1993).

5.2.2.8 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., 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 ., 2003). Analysis of 16S rRNA clone library to assess microbial diversity and populations in natural environments is an important approach (Giovanni ., 1990). Theunknown diversity is currently being explored with molecular techniques, particularly cloning and sequencing (Pedros-Alio, 1993). Molecular methods have mainlyused cloning of PCR products amplified from deep subsurface sediment DNAs (Lysnes., 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.

In general molecular cloning can be divided into several steps (Madigan., 2003);

(1) Isolation and fragmentation of the source DNA.

(2) Joining the DNA fragments to a cloning vector with DNA ligase. The small, independently replicating genetic elementsused 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.

(3) 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 hostusually 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., 1992;Pace, 1996). The bias can be introduced during the PCR step or during cloning. For instance, theuse of rare-cutting restriction enzymes during cloning might also cut amplified rDNA (Amann, 1995). Compared to cloning after PCR, shotgun cloningintroduces 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.

6.MATERIALS AND METHODS

6.1 Sampling

Sediment samples were collected by Institute of Marine Sciences and Management of Istanbuluniversity (Figure 6.1.). The samples were takenusing a Van Veen grab (volume of 3.5 L and penetration depth of 15 cm) on board of the RV Arar of Istanbuluniversity, Institute of Marine Sciences during research cruises between the years 2005 and 2007.. Collected sediment samples were placed into 50 ml sterile Falcon tubes and transferred to the laboratory immediately in cool boxes (+4 ^{0}C or less) and stored at -20 ^{0}C. Sample collection was held in months of Augst 05, November 05, February 06, November 06, February 07, May 07, and Augst 07.

Figure 6.1: The research ship, ARAR, of Istanbuluniversity and Van Ween grab Sampler

Table 6.1: Sampling locations, depths and dates, and sample abbreviations.

Location	Coordinates			Sampling dates and sample abbreviations
	Latitude (N)	Longitude (E)	Depth (m)	
Tuzla	40˚50.60’	29˚13.60’	42	TUZAug05, TUZNov05, TUZFeb06, TUZNov06, TUZFeb07, TUZMay07, TUZAug07
Kucukcekmece	40˚58.24’	28˚45.44’	22	KUCAug05, KUCNov05, KUCFeb06, KUCNov06, KUCFeb07, KUCMay07, KUCAug07
Gemlik	40˚33.17’	27˚56.49’	87	GEMAug05, GEMNov05, GEMFeb06 , GEMNov06
Izmit	40˚43.30’	29˚37.00’	157	Iz17Aug05, IZ17Nov05, IZ17Feb06, IZ17Feb06
Izmit	40˚44.00’	29˚47.00’	30	IZ25Aug05, IZ25Nov05, IZ25Feb06, IZ25Nov06
Izmit	40˚44.20’	29˚53.50’	30	IZ30Aug05, IZ30Nov05, IZ30Feb06, IZ30Nov06
Moda	40˚58.62’	29˚01.49’	8	MODFeb06, MODNov06, MODFeb07, MODMay07, MODAug07
Halic	41˚19.38’	28˚57.99’	6	HalVKNov06, HalVKFeb07, HalVKMay07, HalVKAug07
Halic	41˚24.24’	28˚56.92’	6	HalEYNov06, HalEYFeb07, HalEYMay07, HalEYAug07
Halic	41˚33.66’	28˚56.64’	2	HalASNov06, HalASFeb07, HalASMay07, HalASAug07

6.2 Genomic DNA Extraction

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

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

Approximately 0.5 g sediment was addedup to lysing matrix tubes provided by the kit. The tube contains mixture of ceramic and silica particles to lyse all microorganisms in sample. Then lysing matrix tubes were spinned in Ribolyser (Fast Prep^{TM} FP120 Bio 101 Thermo Electron Corporation) for 30 seconds at speed of 5.5 m/s. The tubes were then centrifuged at 14000xg for 30 seconds. After centrifugation supernatants were transferred to clean 1,5 ml eppendorf tubes and added 250 µl PPS reagent. To mix the composition tubes were shaked by hands for 30 seconds. After mixing the tubes centrifuged again at 14000xg for 5 minutes to pellet the precipitate. Supernatants were transferred to 2 ml eppendorf tubes and 1 ml of Binding Matrix Suspension was added to supernatant. Tubes were inverted by hand for 2 minutes to allow binding of DNA to matrix. To settle the silica matrix tubes were incubated 3 minutes at room temperature. 500 µl of supernatant was removed carefully without disturbing settled silica matrix. Then the binding matrix was resuspended in the remaining supernatant. All mixture was filtered and filter was placed to a new tube. Filter was washed by 500 µl SEWS-M wash solution. After washing, filter was dried by centrifugation at 14000xg for 2 minutes. Filter was removed to a new tube and 50 µl DES (DNase/Pyrogen free water) was added. The filter with DES was vortexed and then centrifuged at 14000xg for 1 minute. Application-ready DNA was obtained in the tube. Extracted genomic DNA yield ready for application. 1/100 diluted genomic DNA was run on the %1 (w/v) agarose gel, prestained with Ethidium Bromide (EtBr) in 1x Tris-Acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM Acetic Acid, 1 mM EDTA; pH 8). Gel was visualized byusing a gel documentation system, Mitsubishi 91.

Briefly, the major steps in PCR are as follows:

(1) a specific nucleic acid probe(primer) hybridizes to a complementary sequence in a target gene

(2) DNA polymerase copies the target gene, and

(3) 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:

(1) Denaturation: heat denaturation of double stranded target DNA,

(2) Annealing: cooling to allow annealing of specific primers to target DNA, and

(3) 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 MgCl2, 5 µl of 10×*Taq* buffer and 4u of *Taq* DNA polymerase (Fermentas, Latvia). For the second-round nested amplification 0.1 µl of the first-round product wasused 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 min and extension at 72°C for 2 min and a final extension at 72°C for 10 min. 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 1 and gel images were recordedusing a Chemi-Smart 3000 gel documentation system (Vilber Lourmat, France) after staining with ethidium bromide.

6.3 Preparation of Q- PCR Standards

Extracted GDNAs wereused as templates, amplification of mcrA and dsrB gene sequences were performed by specific primers. The primers and their annealing temperatures were given in Table 6.2.

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 MgCl2, 5 µl of 10×*Taq* buffer and 4u of *Taq* DNA polymerase (Fermentas, Latvia) byusing a a Techne TC-412 thermal cycler (Barloworld Scientific Ltd.,u.K.) with an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing for 1 min and extension at 72°C for 2 min and a final extension at 72°C for 10 min.

Table 6.2: Bacterial and archaeal oligonucleotid primersused for PCR amplification

Name of Primer	Sequence of Primer	Target Gene	Annealing	References
mcrA1f	5'-GGTGGTGTMGGATTCACACARTAYGCWACAGC-3'	mcrA	**58°C**	Luton 2002
mcrA1r	5'-ACR TTC ATN GCR TAR TT-3'	mcrA	**58°C**	Luton 2002
DSRp2060F	5′-CAACATCGTYCAYACCCAGGG-3′	dsrB		Geets . 2006
DSR4R	5′-GTG TAG CAG TTA CCG CA-3′	dsrB	**58°C**	Wagner 1998

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 1 and gel images were recordedusing a Chemi-Smart 3000 gel documentation system (Vilber Lourmat, France) after staining with ethidium bromide.

One of positive PCR product result was chosen for clonning.

The initial step of the clonning 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 MgCl2), 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.

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 MgCl2, 10 mM MgSO4, 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 platesusing glass spreader. The plates were incubated overnight and white colonies were observed after incubation.

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

for 5 minutes then frozen at -20^0 C overnight. Thawed solution wasused as templates for PCR. The DNA fragments were isolated from vector by PCR with primers M13f-M13r (M13 Forward 5′-GTA AAA CGA CGG CCA G-3′/ M13 Reverse 5′-CAG GAA ACA GCT ATG AC-3′).

Other step of standart preparation is purification of M13 PCR products, which was done according to the Invitrogen PCR product purification Kit. According to the manufacturer's specifications. Purification with PureLink™ PCR Purification Kit, the yield of purified dsDNA has been estimated by agarose gel electrophoresis. To estimate the yield, agarose gel electrophoresis of the purified PCR product and known quantities of DNA fragment of the same size was performed . The band intensity of the purified PCR product with the standard DNA fragments was compared. So the purified PCR product wasused as Q-PCR standards.The standartds concentration were determinedusing a fluorometer (Qubit, Invitrogen) according to the manufacturer's specifications. Application ready standards were diluted in 1/100 ratio for Q-PCR experiments.

6.4 Q-PCR

PCR primer sets for the Q-PCR assays were given in Table 2. 10^{3-7} copies of the standard sequences wereused to obtain the calibration curves. Roche LightCycler DNA Master SYBR Green I kit and Roche Light Cycler 2.0 (Roche Diagnostics GmbH, Mannheim, Germany) wereutilized for all reactions. Reaction mixes contained 25 ng template DNA, 0.5 μM of each priMer and 2.5 μM $MgCl_2$. Simply add the master mix to PCR tubes along with template and primers. The chemically-modified and tightly controlled HotStart enzymeuniquely provides more accurate SYBR® Green results by preventing the amplification of primer dimers and other non-specific products. Q-PCR conditions for the most of the primer sets were described previously .The following thermocycling program was applied: 95°C, 10 min; 45 cycles of 10 s at 95°C, 5-10 s at primer dependent annealing temperature, 15 s at 72°C. Program the real-time thermal cycler to detect and record the SYBR Green signal from every reaction at the end of the 60ºC annealing / extension step of each cycle. A melt curve analysis was performed from 55°C to 95°C to determine if only one amplified product was generated during Q-PCR. Q-PCR runs were

analysedusing Roche LightCycler Software 4.05. The efficiencies were between 1.8 and 2.0, and the correlation factors (r^2) were not lower than 0.97 in all reactions.

To convert the detected gene targets into cell numbers, averages of 3.6 and 1.6 copies of the 16S rRNA gene were estimated for Bacteria and Archaea, respectively (Klappenbach,2001). Copy nembers of all other genes were directly correlated to cell numbers (Phillippot,2002;Da Silva and Alvarez,2002;Beller,2002).

Table 6.3: Primer sets specific for different phylogenetic domains and functional genes

Primer	Target Gene	Target Organism	Standard Gene Sequence	Reference
mcrA1f mcrA1r	Methyl-coenzyme M reductase alpha-subunit (mcrA)	Methanogenic *Archaea* (MA)	mcrA of *Methanococcus maripaludis S2* (NC_005791)	Luton ., 2002.
DSRp2060F	Dissimilatory sulfite reductase beta-subunit (dsrB)	Sulfate Reducing *Bacteria* (SRB)	dsrB of *Desulfitobacterium hafniense Y51 (*NC_007907)	Geets ., 2006

Figure 6.2: The Roche Lightcycle quantitative PCR instrument

Figure 6.3: System components

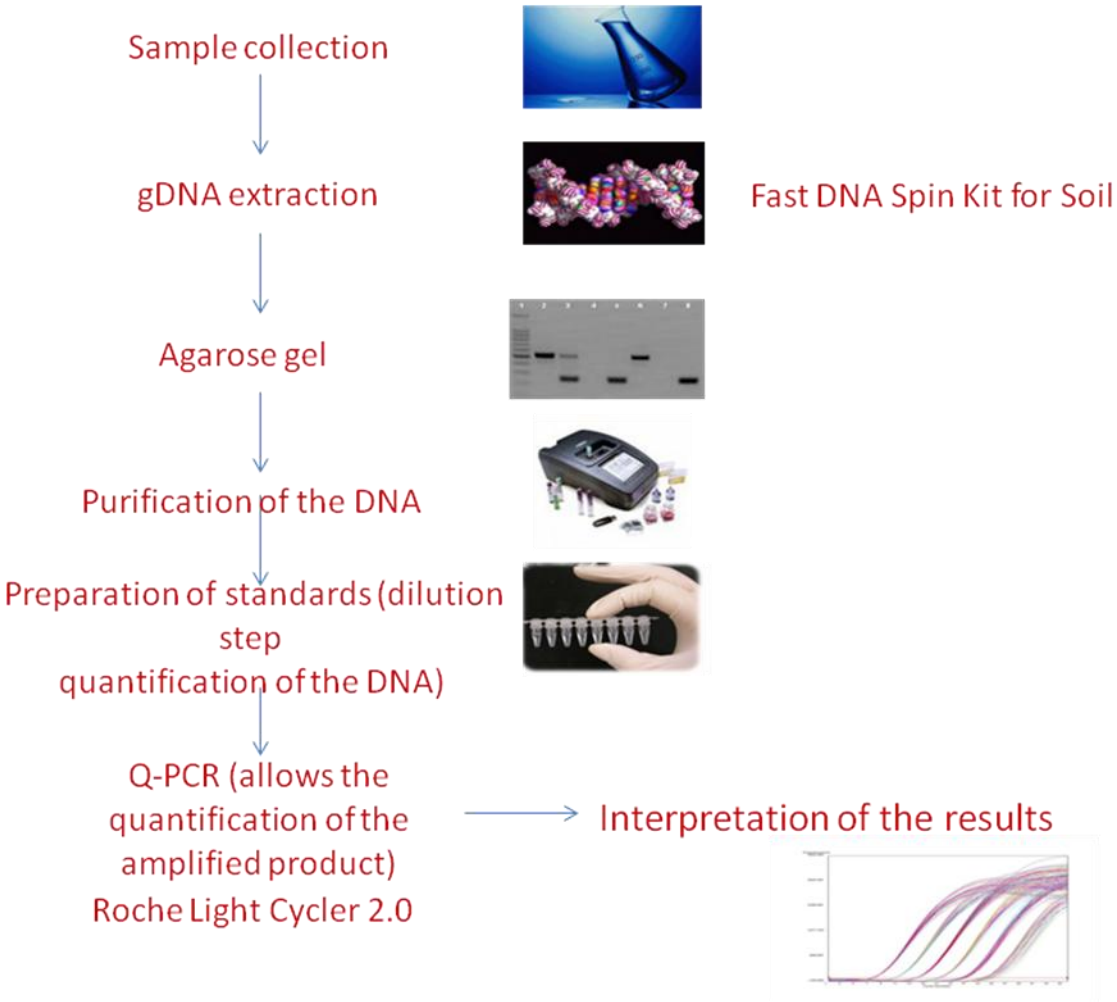

Figure 6.4: Flow-chart of experimental set-up

7.RESULTS

7.1 Microbial Abundance Analysis of Sediment Samples Using Q-PCR

In this study, the results represented the overall microbial and chemical composition of the MSS in the first 15 cmbsf. *Bacteria* and *Archaea* quantifications were carried out by targeting rRNA genes using Q-PCR and the total number of prokaryotic cells were calculated as the sum of *Archaea* and *Bacteria*.(Kolukirik, 2009). Quantitative real-time PCR (Q-PCR) have been widely used for the quantification of gene abundances in environmental samples (Winderl 2008, Higashioka 2009).We quantified Methanogens and SRB, by targeting functional genes (dsrB and mcrA) using Q-PCR (Figure7.1). The percentage of the functional genes quantification was calculated by total cell proportion to SRB and Methanogens quantity.(Figure 7.2)

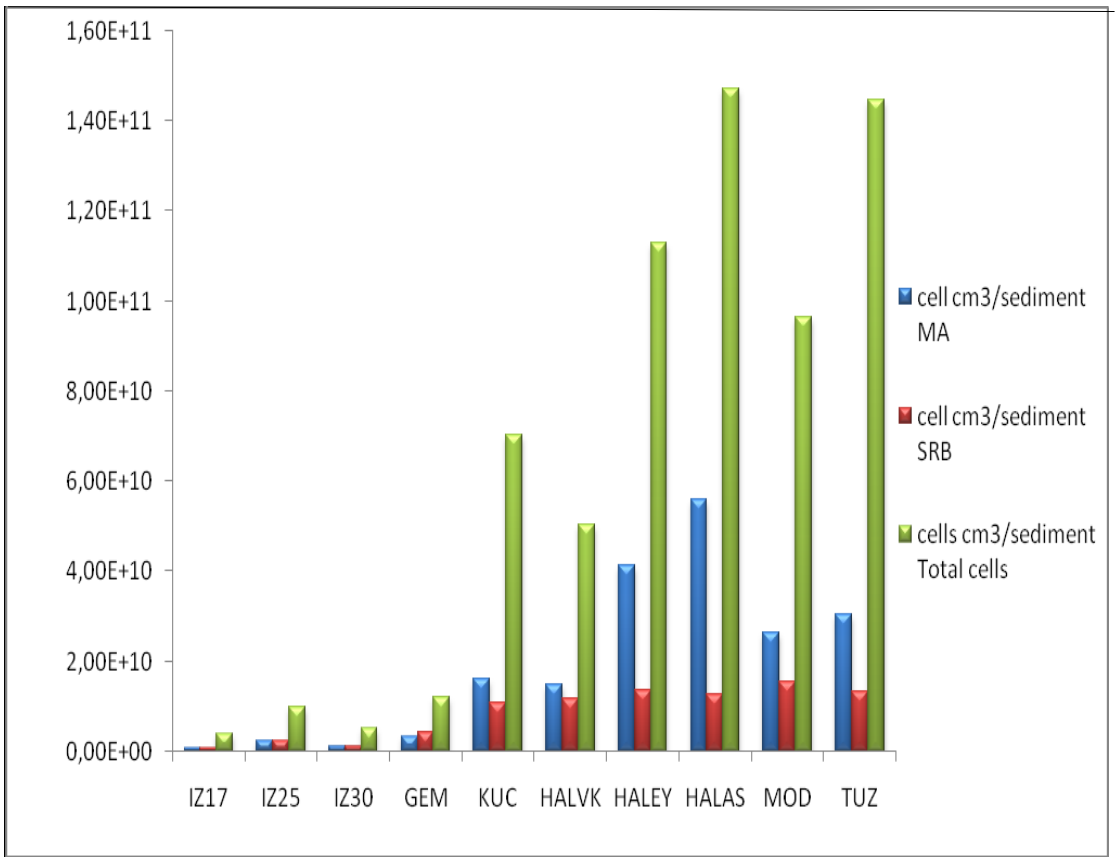

Figure 7.1: Cell concentration of Methanogens , SRB, and total cell concentarion grap

Table 7.1: Cell concentration of Methanogenic Archaea, Sulfate Reducing Bacteria, and total cell count(cell cm^3/sediment)and standard deviasons

	KUC	TUZ	MOD	IZ	GEM	HAL
Cell cont. Methanogens	$1,62X10^{10}$	$3,06X10^{10}$	$2,63X10^{10}$	$1,45X10^{9}$	$3,37X10^{9}$	$3,82X10^{10}$
cell cont.SRB	$1,08\ X10^{10}$	$1,33X10^{10}$	$1,56X10^{10}$	$1,46X10^{9}$	$4,40X10^{9}$	$1,30X10^{10}$
Total cell count	$7,026x10^{10}$	$1,45x10^{11}$	$9,64x10^{10}$	$6,37x10^{9}$	$1,19x10^{10}$	$5,99x10^{9}$
	KUC	TUZ	MOD	IZ	GEM	HAL
Standart deviasonsfor. Methanogens	1,80	1,4	3,9	2,29	2,5	2.52
Standart deviasons for SRB	1,44	0,8	1,7	2,18	0,8	1,99

The number of total cell changed in a range of $5,99x10^{9}$ - $1,45x10^{11}$ during the 2 years monitoring period (Kolukirik,2009). Total cell counts of the MSS were higher than the previously reported total cell count ranges (10^{8} – 10^{10} cells/cm^3) for marine sediments (Schippers and Neretin, 2006; Smith and D'Hondt, 2006). The results showed that Marmara sea sediment were very rich in terms of sulfate reducing bacterial and methanogenic cell contents which may imply that bioremediation is possible for the Marmara Sea as long as these organisms are stimulated for higher hydrocarbon degradation activity.

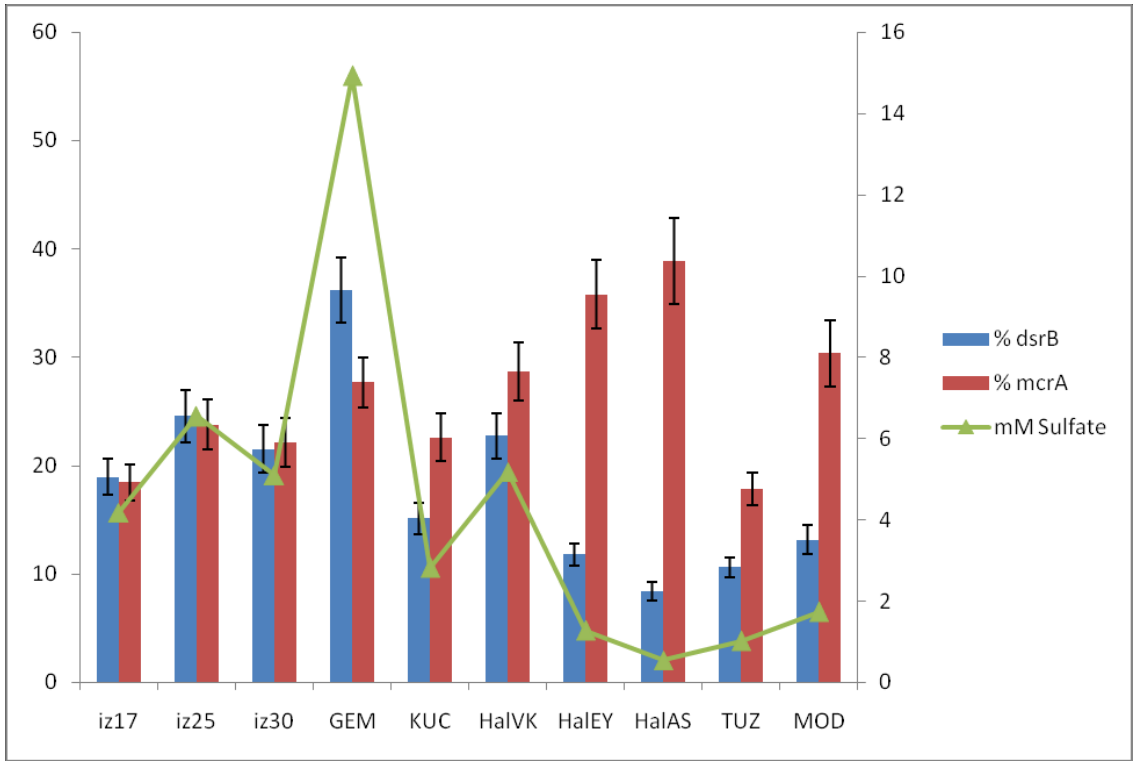

Figure 7.2: Percentage of the mcrA and dsrB genes abundance

7.2 Chemical and Physical Characteristics of the Sediments

Sediments characteristics results were taken from TUBITAK-105Y307 project on bioremediation of petroleum hydrocarbons as ranges in which the concentrations fluctuate during the two years monitoring period.

The way sediments' chemical compositions changed along with the microbial diversity will be discussed after presenting the correlation analysis results.

The correlations leading to this statement had been obtained from the studies characterizing marine sediments along vertical profiles.

Table 7.2: Concentration ranges for TOC, N, P and SO_4^{2-} of the Marmara Sea Sediments between the years 2005 and 2008(Sediment and Porewater respectively). Horizantal "white → black"scale represents increasing level (Kolukirik, 2009)

			IZ17	IZ25	IZ30	GEM	KUC	HalVK	HalEY	HalAS	TUZ	MOD
	TOC	**‰**	32-47	37-55	27-40	14-22	37-56	27-40	31-47	44-66	37-55	36-54
	N	**‰**	3-4	6-8	2-3	7-10	25-40	18-28	20-30	28-41	29-44	25-37
	P	**‰**	0.2-0.4	0.7-1	0.3-0.4	0.7-1.1	6-10	2-4	6-8	7-11	11-16	5-7
	TOC	**mg/L**	940-1400	1350-2000	1250-1900	750-1150	900-1350	2700-4000	3000-4550	3300-5000	3600-5500	1400-2000
	N	**mg/L**	5-7	7-10	6-9	4-6	5-8	14-21	15-23	18-27	16-24	6-9
	P	**mg/L**	0.9-1.4	1.4-2.1	1.4-2.1	0.9-1.3	0.8-1.2	3-4.5	2.6-4	4-6	3-4.5	1.6-2.3
	SO_4^{2-}	**mM**	3.3-4.9	5.1-7.7	4-6	11-17	2.2-3.2	4-6	1-1.5	0.4-0.6	0.8-1.2	1.3-2.0

7.3 Correlating the mcrA and dsrB genes abundance with the MSS Characteristics

In this study, we focused on the mcrA and dsrB genes abundance in total cell count of 10 horizontally distant (>5 km) sediments rather than depth-related gradient of physicochemical and microbiological sediment characteristics.

The correlation analyses was made by MiniTab Programme.Correlation analysis were done between Heavy Metal, Elemental Composition (C/N/P), Anionic Content (NO_3^-, SO_4^{-2}), Petroleum Hydrocarbon (TPH , Aliphatics, Aromatics, Asphaleten, Resene), Total Cell Count (DAPI count, Q-PCR count), Genes / Transcrips responsible for Anoxic N cycle,Sulfate Reduction and Methanogenesis,BTEX degradation Total Cell Activity (rRNA level), Physical Characteristics (Salinity, pH, Temprature, Sediment Grain Size) parameters (Kolukirik ,2009) (Table 7.3).

For heavy metal characterization Cr, Zn, Pb, Mn, Fe, Cu, Ni were measured. They can be rankend for their adverse effects on benthic organisms in the MSS as

Ni>Zn>Cu>Pb>Cr>Mn>Fe. The carbon ,nitrogen , phosphorus ratio (C/N/P) was evaluated as elemantal composition. The petroleum hydrocarbon and their fractions which are aromatics, aliphatics, asphaltene and resene were measured for petroleum hydrocarbon characterization. Total cell content using both DAPIcount and Q-PCR count , and their activity levels (RNA level) were determined. The measured physical characteristics of MSS are salinity, pH , temperature and sediment grain. Genes responsible for Anoxic N Cycle, Sulfate Reduction, Methanogenesis, BTEX degradation and their transcripsts were used in microbiological analyses.

Table 7.3: Sediment characteristics between Correlation variables (Kolukirik,2009)

Correlation Parameters

Heavy Metal	Ni>Zn>Cu>Pb>Cr>Mn>Fe
Elemental Composition	C/N/P
Anionic Content	NO_3^- SO_4^{2-}
Petroluem Hydrocarbon	TPH, Aliphatics,Aromatics Asphaltene, Resene
Total cell Count	DAPI, Q-PCR count
Genes/Transcripts responsible for	Sulfate Reduction, Methanogenesis Nitrate reduction BTEX degradation
Total cell activity	rRNA level
Physical Characteristics	pH, salinity,temprature, sediment grain size

Sediment characteristics results demonstrated that correlation parameters were not related to Methanogens whereas Sulfate Reducers abundance were strongly related to sulfate levels in the sediment. These correlation results imply that the main factor to govern the abundance of sulfate reducing community is the SO_4^{2-} level.These correlation results make sence because TUBITAK project determined that the MSS were organic rich. Their TOC content correlated to neither total cell content nor active cell abundance. This indicated that e^--donors were not limited in the MSS. Scarcity of the electron acceptors determined dominancy of the organisms

responsible for the relevant terminal e^--accepting processes. In marine sediments, e^--acceptors enter the sediment from the overlying water column. As the e^--acceptors are reduced; their reduced products enter successively deeper redox zones. Since e^--donors were not limited in the MSS, it is highly possible that heterotrophic microbial populations depleted electron acceptors quickly within a very short distance (15 cm) from the sediment surface which resulted in the succession of all the redox zones (Kolukirik,2009).Hence, changes in the e^--acceptor levels were reflected in the microbial community compositions. SRB rely on the availability of sulfate but do not obviously belong to the most abundant bacterial groups in marine sediments, even in those having high sulfate concentration (Schippers and Neretin, 2006; Parkes ., 2005; Inagaki, 2006**;** Wilms , 2006). The distribution of Methanogens in marine sediments correlated with the sulfate and methane profiles and could be explained by electron donor competition with SRB (Wilms , 2006)**.**

Table 7.4: Correlation of dsrB gene and Sulfate

Correlation parameters → Genes ↓	Sulfate
dsrB gene	O,963

TUBITAK project on bioremediation of petroleum hydrocarbons measurement determined that high amounts of sulfate concentration has been measured in the MSS (Table 7.3) and the highest sulfate concentration was measured in the Gemlik sediment consequently dsrB constituted important fraction of sulfate reducing population in the Gemlik Bay. dsrB was found to be the most dominant gene in Gemlik Bay,dsrB gene was in the range of 30- 40%.

Although SRB was the most dominated metabolic group in Gemlik sediment, mcrA percentage of the Methanogens in the Halic sediment was higher than other sampling locations. mcrA was found to be the most dominant gene in Halic Bay.mcrA gene was in the range was of 30-40%.,while dsrB gene was in the range of 10-20% .

There was no need to add e^-acceptor in Halic area because methanogens no need external electron acceptor they can use inorganic carbon (such as CO_2) as electron donor, but in order to enhance the carbon usage of Methanogens , N-P could be added. The cell abundance and activity were strongly related to the N/P ratios and the N-P levels. The active part of the total cell content was related to the dissolved level, rather than the total level of N-P. This arises from the fact that dissolved N-P levels were very low to sustain exponential growth of marine bacterioplankton (Vrede , 2002). In other words, N and P were limited in the MSS porewaters for biological activity. Chemical analysis results suggested that P release from the MSS occured at low rates and/or P removal from the pore water occurred at high rates (Kolukirik,2009).

SRB and Methanogens equally dominated in the Izmit Bay. We could supplement N and P in order to increase SRB population in Izmit Bay because metabolisms of Sulfate reducers were faster than those of Methanogenetic archaeal population.

Table 7.5: Correlation analysis table between correlation parameters with functional genes

Correlation parameters → Genes ↓	Sulfate	NonAcOxSRB	AcOxSRB	M.sarcinales	M.sarcina	M.saeta	M.micbacco
dsrB	0,963	0,996	0,979				
mcrA				1	1	1	1

Statistical analysis of Methanogenic Archaea results indicated that Methanogenic community structure was related to Methanosarcinales ,(Methanosarcina, Methanoseata), Methanomicrobiales, Methanobacteriales, Methanococcales groups. Methanosarcinales can growth on methanol except Methanoseata. Methanoseata can be used only acetate as electron donor.*Methanosarcinales* that utilize noncompetitive substrates were dominant in all sediments except Gemlik**,** which are are able to avoid competition by utilizing substrates like methylamines (Konneke, 2005) or dimethylsulfide (Takai , 2001). These compounds are mostly available near the sediment surface.In addition, *Methanosarcina* strains were shown to demethylate aromatic compounds (Parkes, 2000) which were very abundant in the Marmara Sea sediments and which were utilized H_2 , acetate, methanol, methylamines as electron

donor. Hydrogenotrophic methanogens *Methanobacteriales, Methanomicrobiales* which are known to compete directly with sulfate reducers for hydrogen. Coexistence of hydrogenotrophic methanogens with SRB in the anoxic marine surface layers were also previously reported. It was suggested that the coexistence is probably due to a substrate surplus that may be generated by exudates of benthic photosynthetic organisms (Wilms, 2006)**.**

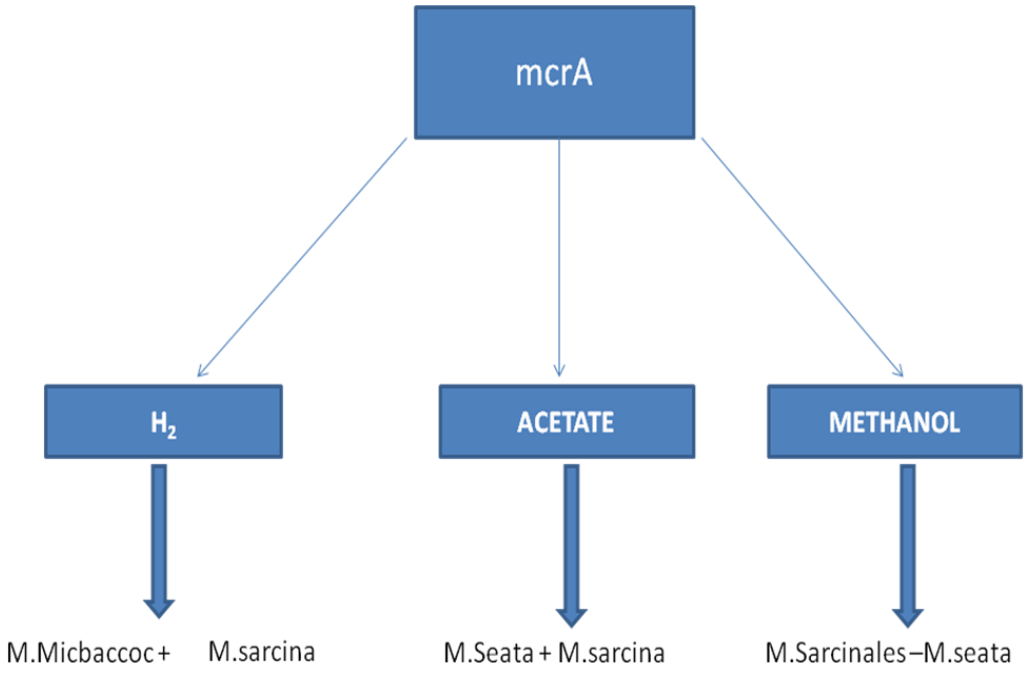

Figure 7.3: Flow chart of correlation analysis with mcrA gene

Furthermore, there was a strong correlation between acetate oxidizers (group I sulfate reducers) with non-acetate oxidizers (group II sulfate reducers). Group I sulfate reducers including Desulfovibrio, Desulfomonas, Desulfotomaculum and Desulfobulbus utilize lactate, pyruvate, malate,sulfonates, and certain primary alcohols (for example, ethanol, propanol, and butanol) or certain fatty acids as e^- donors, reducing sulfate to hydrogen sulfide; they are unable to catabolize acetate. The genera that group II, such as Desulfobacter, Desulfosarcina, and Desulfonema specialized in the oxidation of fatty acids, particulate acetate, lactate, succinate, and even benzoate in some species, reducing sulfate to sulfide. There was no negative correlation was observed between group I sulfate reducers with group II sulfate reducers because acetate oxidizers and non-acetate oxidizers don't compete each other for the same subsrate whereas sulfate concentration was positively related to group I sulfate recuders and group II sulfate reducers. Where sulfate concentration

was high, acetate-oxidizers and non-acetate oxidizers would be high because sulfate reducers use SO_4^{2-} as electron acceptor. Desulfosarcina, Desulfonema, Desulfococcus, Desulfobacterium, Desulfotomaculum, and certain species of Desulfovibria, are unique amount sulfate reducers in their ability to grow chemolithotrophically and autotrophically with H_2 as electron donor, SO_4^{2-} as electron acceptor, and CO_2 as sole carbon source. A few sulfate reducers can use hydrocarbons, even crude oil itself, as electron donors. This process is noteworthy because until such organisms were recognize, it was thought that hydrocarbons could only be oxidized under oxic conditions.

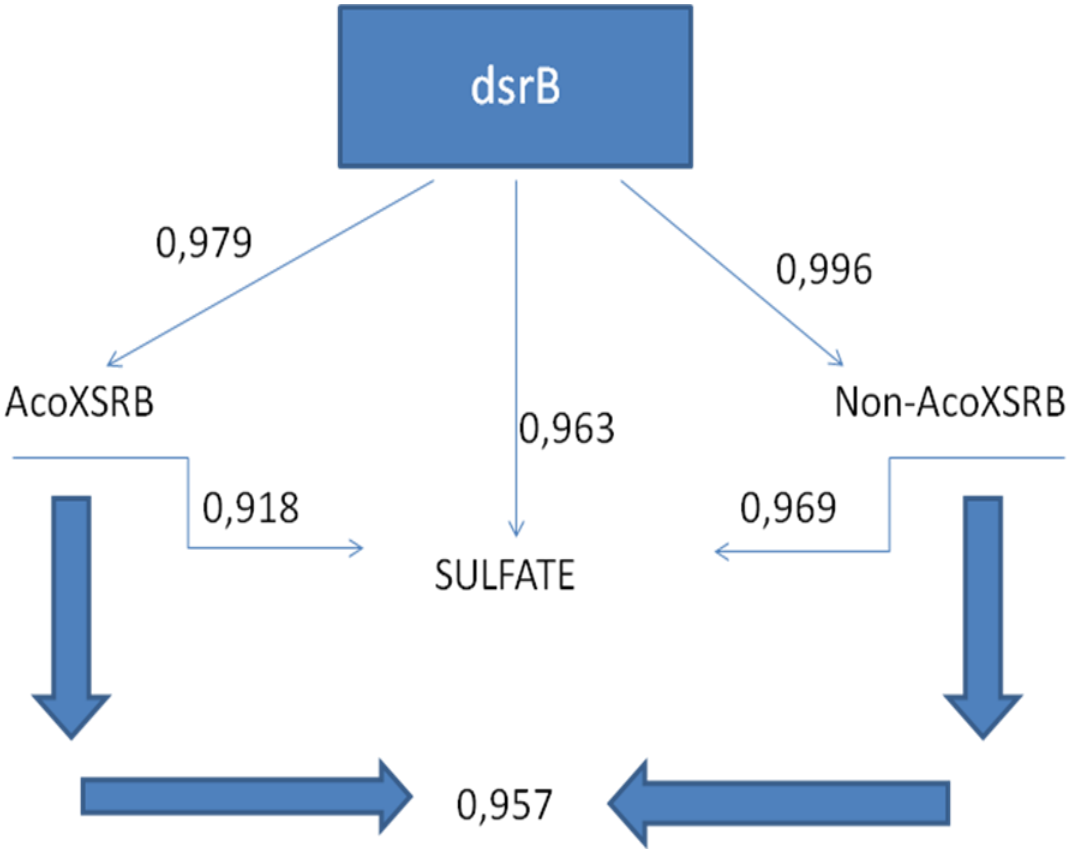

Figure 7.4: Flow chart of correlation analysis with dsrB gene

7.4 Seasonal SRB and Methanogens Abundance and Sulfate concentration

SRB and Methanogens abundance in all sampling locations were considered by 4 months , which were Nowember , February ,May and August as representative for seasons.

Seasonal differences in the microbial structure were not related to changes in the sediment's physical and chemical characteristics for 3 different sampling points in Izmit Bay. Deep water temperature were measured 16-18^{0}C, 16-18^{0}C , 14-15^{0}C for IZ25 , IZ30 and IZ17 locations (Kolukirik,2009).

The highest sulfate concentration was measured at nov05 (~7,5mM) in the IZ25 sampling location.

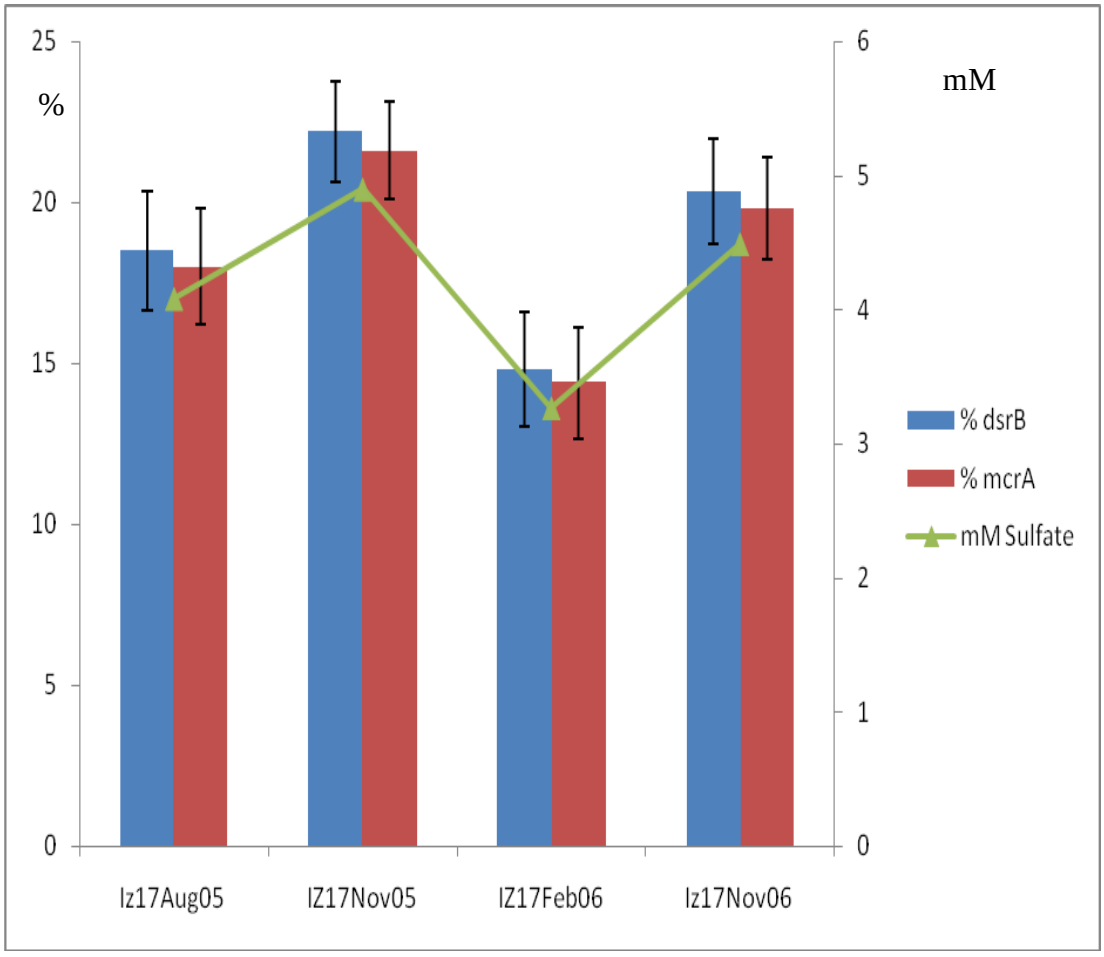

Figure 7.5: Seasonal changes in IZ17 for SRB and methanogens comminities and seasonal sulfate concentration.

Deep water temperature were measured 16-18^{0}C, 16-18^{0}C , 14-15^{0}C for IZ25 , IZ30 and IZ17 locations (Kolukirik,2009).

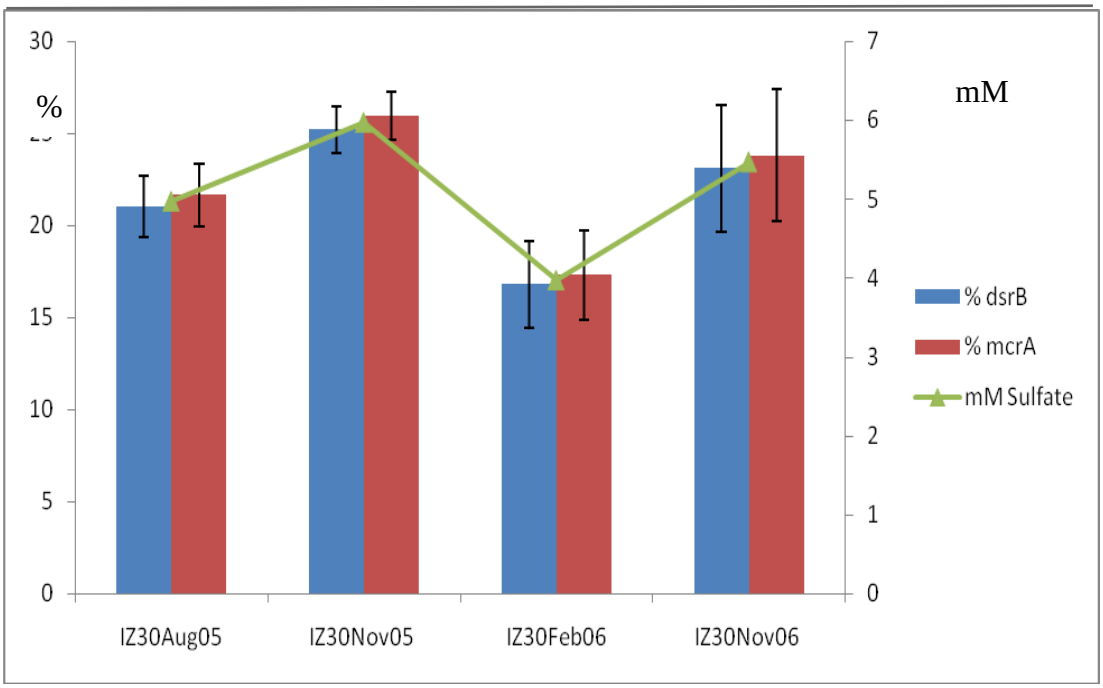

Figure 7.6: Seasonal changes in IZ30for SRB and methanogens comminities and seasonal sulfate concentration.

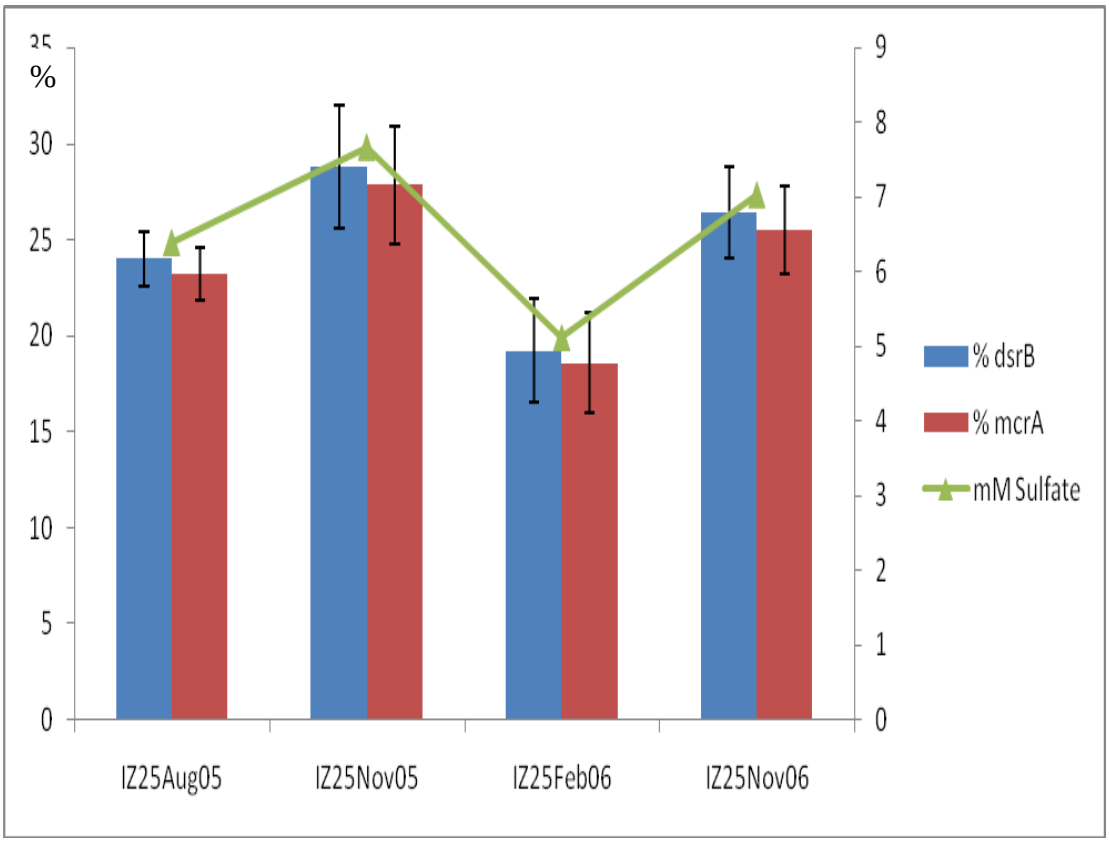

Figure 7.7: Seasonal changes in IZ25 for SRB and methanogens comminities and seasonal sulfate concentration.

Microbial communities couldn't be influenced by seasonality in the KUC sediment's physical and chemical characteristics. Deep water temperatures were measured 16-18^{0}C for Kucukcekmece coast. The highest sulfate concentration was measured at aug07 (~3,3mM) in the KUC sampling location.

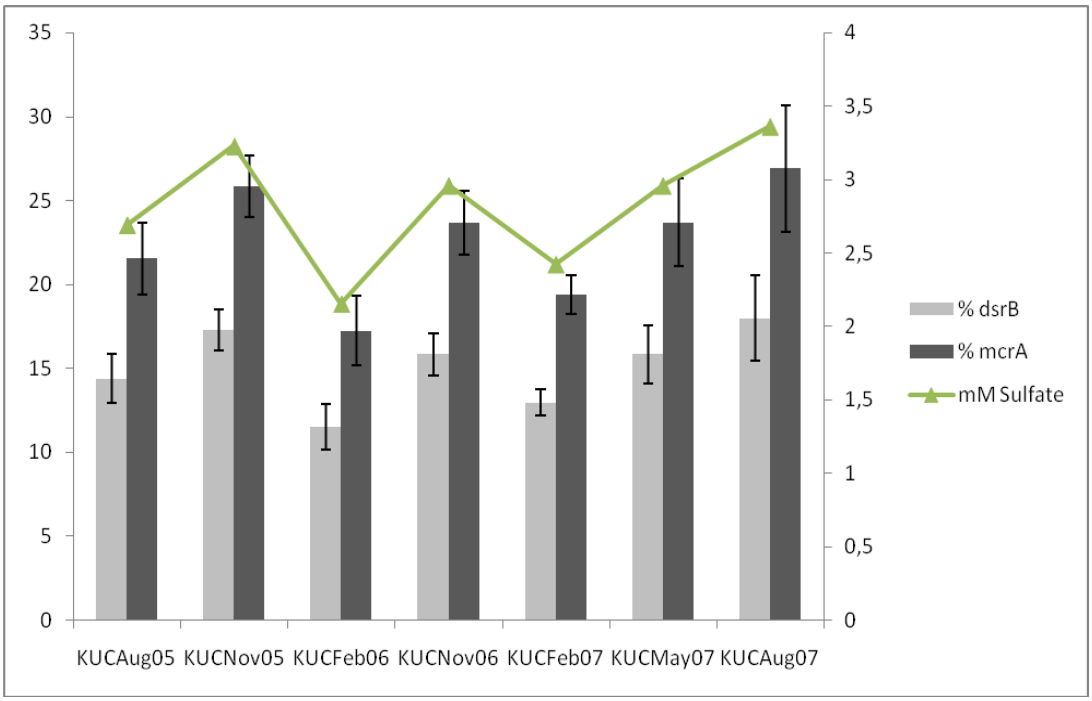

Figure 7.8: Seasonal changes in KUCUKCEKMECE coast for SRB and methanogens comminities and seasonal sulfate concentration.

Generally, there wasn't any relation between the season with SRB and Methanogens comminities were determined in 3 diffrent sampling points for Halic Bay. Deep water temperatures were measured 17-21^{0}C for Halic Bay.The excess sulfate concentration was measured at feb07 (~6 mM) in HalVK sampling point.

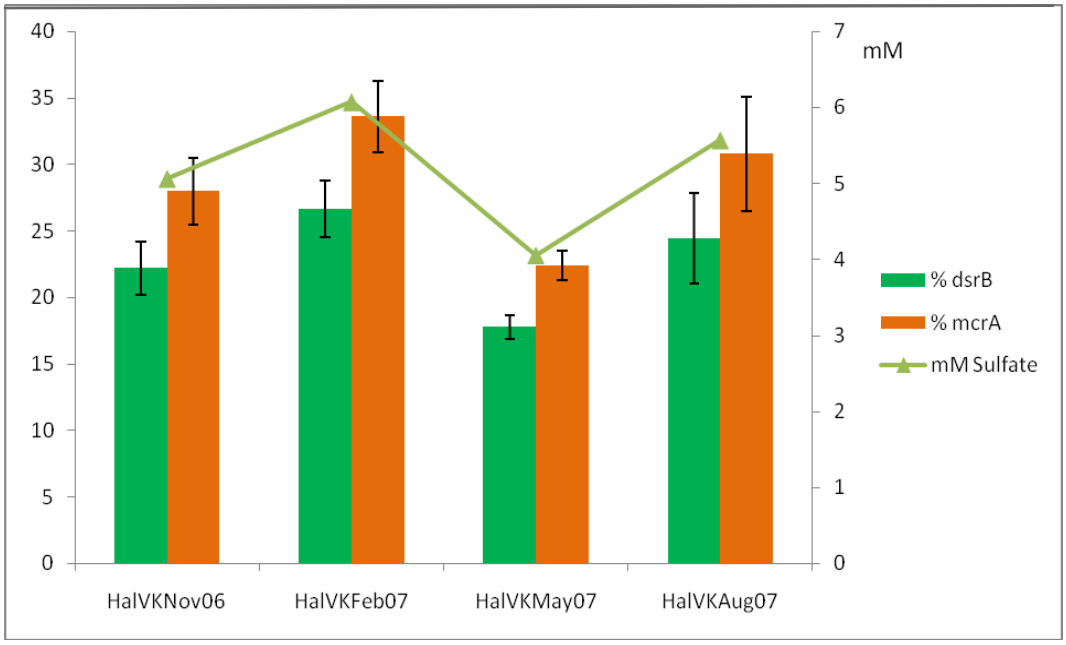

Figure 7.9: Seasonal changes in HalVK Bay for SRB and methanogens comminities and seasonal sulfate concentration.

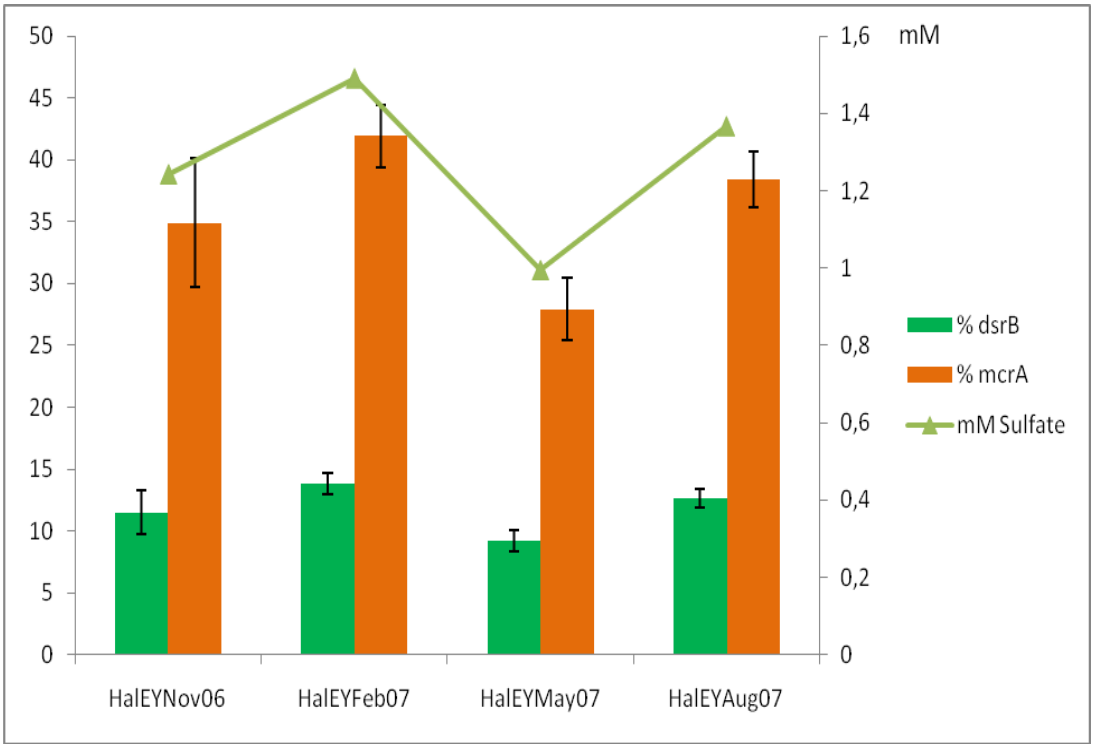

Figure 7.10: Seasonal changes in HalEY Bay for SRB and methanogens comminities and seasonal sulfate concentration

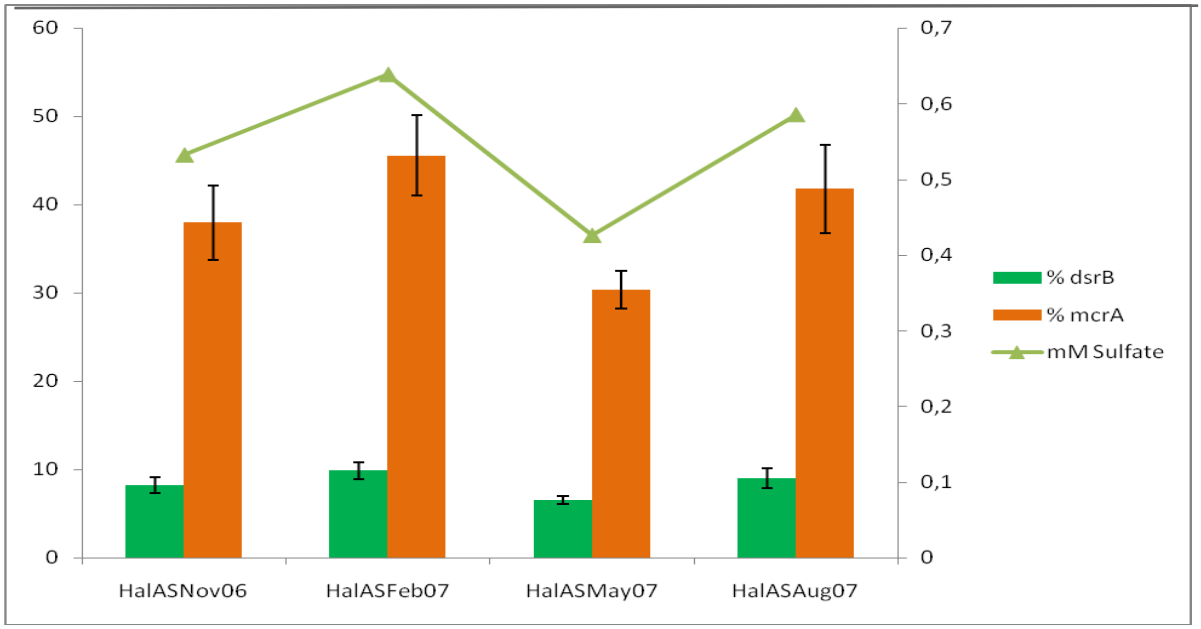

Figure 7.11: Seasonal changes in HalAS Bay for SRB and methanogens comminities and seasonal sulfate concentration.

Seasonal differences in the microbial structure were not related to changes in the sediment's physical and chemical characteristics for Tuzla Coast. Deep water temperature were measured 16-18^{0}C.(Kolukirik,2009).

The excess sulfate concentration was measured at aug07 (~1,2 mM) in Tuzla Coast.

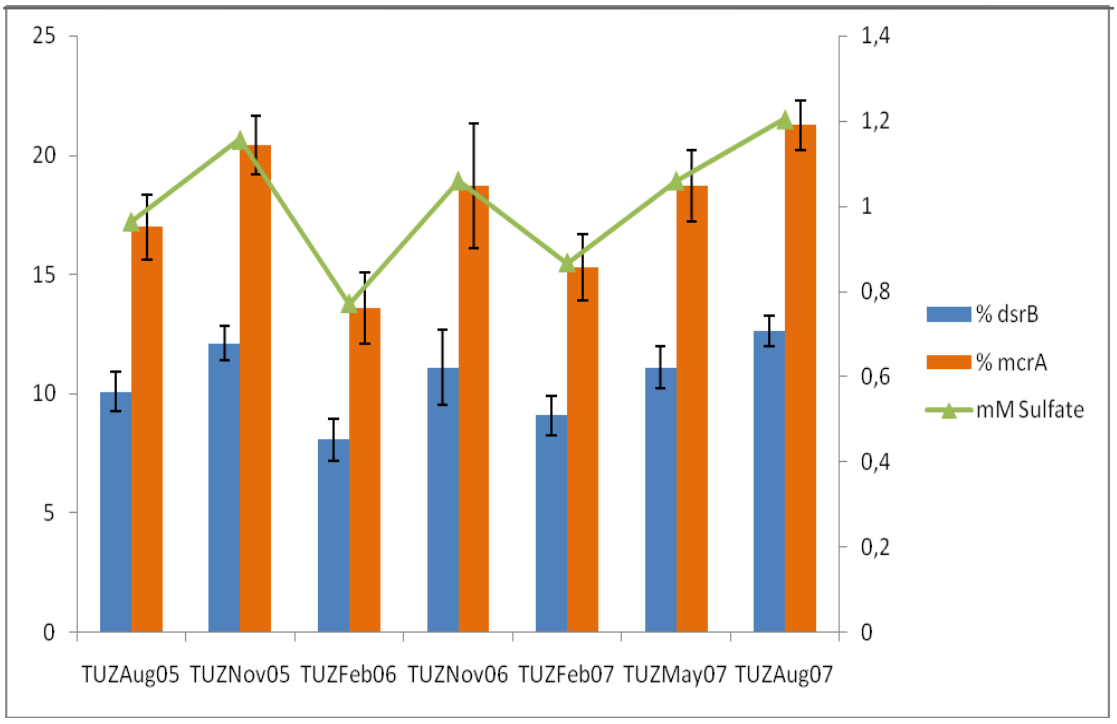

Figure 7.12: Seasonal changes in Tuzla Bay for SRB and methanogens comminities and seasonal sulfate concentration.

Seasonal changes were not observed between SRB and Methanogens in Moda Bay. Deep water temperature were measured 17-21^{0}C (Kolukirik,2009).The excess sulfate concentration was measured at aug07 (~2 mM) in Moda Bay.

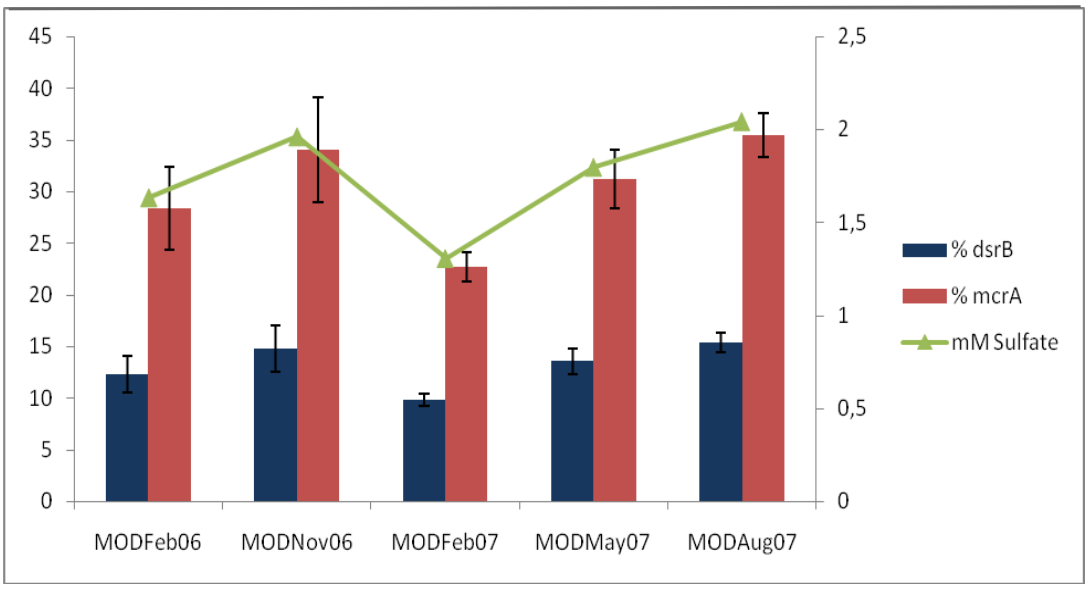

Figure 7.13: Seasonal changes in Moda Bay for SRB and methanogens comminities and seasonal sulfate concentration.

Seasonal changes were not observed between SRB and Methanogens in Gemlik bay.

Deep water temperature were measured 14-15^{0}C (Kolukirik,2009).The excess sulfate concentration was measured at nov05 (~18 mM) in Gemlik Bay.

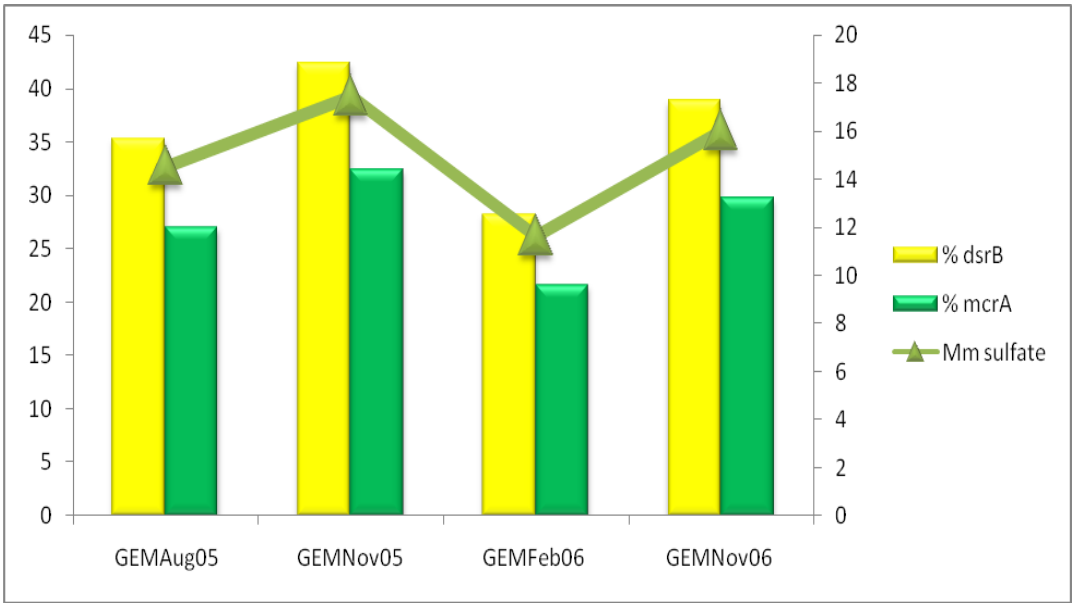

Figure 7.14: Seasonal changes in Gemlik Bay for SRB and methanogens comminities and seasonal sulfate concentration.

Generally there was not observed relationship between microbial comminities(SRB and Methanogens) and sulfate concentration in terms of seasonal changes in the MSS.

Temperature measurement showed that there was no correlation between temperature and seasonal changes in all sampling locations.

Deep water temperatures of all sampling locations were supporting psychrotolerant and/or mesophilic microbial activities (Arnosti,1998). pH of KUC, HAL, MOD, TUZ sediments changed between 7.5-8.3.This pH range is maintained by methanogenesis, and sulfate reduction in marine environments (Soetaert,2007).

8.CONCLUSIONS AND RECOMMENDATIONS

Sediment samples collected from the most polluted regions in the Marmara Sea were analyzed successfully with molecular techniques in order to reveal how important sulfate reduction and methanogenesis processes and what may control abundance of the responsible organisms. Abundance of sulfate reducing bacteria and methanogenic archaea were monitored in sediments from 10 different locations in the Marmara Sea for 2 years. Microorganism quantifications were carried out using quantitative polymerase chain reaction (Q-PCR) and targeting functional genes (mcrA and dsrB). The results showed that SRB and Methanogens microbial cell contents of the sediments were high. ($1,46x10^{9}$- $1,56x10^{10}$and $1,45x10^{9}$- $3,82x10^{10}$cells/cm^{3} respectively)

In this study the results represented the overall microbial and chemical composition of the MSS in the first 15 cmbsf. TUBITAK-105Y307 project on bioremediation of petroleum hydrocarbons revealed that electron donors were not limited in the MSS. Scarcity of the electron acceptors determined dominancy of the organisms responsible for the relevant terminal e^{-}-accepting processes. Microorganisms, mainly sulfate reducers, and methanogens coexisted within a very short distance (15 cm) from the sediment surfaces.

Q-PCR is a fast method in order to determine quantity of SRB and Methanogens. Microorganism quantifications were carried out using quantitative polymerase chain reaction (Q-PCR) and targeting functional genes (mcrA and dsrB). Sediment characteristics were taken from TUBITAK project, sediment characteristics results demonstrated that correlation variables were not related to Methanogens whereas Sulfate reducers were strongly related to sulfate concentration in the sediment.

High concentration of sulfate has been measured in the MSS for all sampling locations and the highest sulfate concentration was determined in the Gemlik sediment. Consequently dsrB constituted important fraction of sulfate reducing population in the Gemlik Bay.

Although SRB was the most dominated metabolic group in Gemlik sediment, mcrA percentage of the Methanogens in the Halic sediment was higher than other sampling locations. There was no need to add e^-acceptor in Halic area because Methanogens no need external electron acceptor they can use inorganic carbon (such as CO_2) as electron donor, but in order to enhance the carbon usage of Methanogens , N-P could be added. The cell abundance and activity were strongly related to the N/P ratios and the N-P levels. The active part of the total cell content was related to the dissolved level, rather than the total level of N-P (Kolukirik,2009). This arises from the fact that dissolved N-P levels were very low to sustain exponential growth of marine bacterioplankton (Vrede, 2002). In other words, N and P were limited in the MSS porewaters for biological activity**.**

It can be concluded that because the Marmara Sea Sediments (MSS) contains high amount of sulfate reducing and methanogenic microorganisms, a bioremediation strategy for the Marmara Sea based on stimulation of these microbes is possible. After this study, further laboratory hydrocarbon degradation microcosms were set up in the concenpt of TUBITAK 105Y307 project. The project overall results revealed that it is possible to increase hydrocarbon degrading activity of methanogenic-sulfate reducing microorganisms in the MSS for approx. 10 by nutrient amendment. This will form a base for further filed scale bioremediation applications.

REFERENCES

Alpar, B., Yuce, H., 1998. Sea-level variations and their interactions between the Black Sea and the Aegean Sea. Estuarine , Coast al and Shelf Science, Volume 46, Issue 5, 609-619.

Algan, O., Balkıs, N., Çagatay, M.N., Sarı, E. 2004. The sources of metal contents in the shelf sediments from the Marmara Sea,Turkey.Environmental Geology, 46, 932-950.

Altınok Y, Ersoy Ş. Tsunami observed on and near the Turkish coast. Nat Hazards 2000;21:185–205.

Amann, R.I., Ludwig, W., Schleifer, K.-H. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev., 59, 143-169

Aslan-Yılmaz, A., 2002. Monitoring discharges at Bosphorus, Black Sea exit of the Strait and the northeastern Marmara Sea by indicator microorganisms. M.Sc. Thesis, Department of Marine Biology, Institute of Marine Sciences and Management, Istanbul University.

Aydın, A., Yuksek, A., 1990. Investigations on the macroscopic and epiphytic algae of the Golden Horn. Istanbul University Journal of Biology 54, 15 20.

Baldwin BR, Nakatsu CH & Nies L (2003) Detection and enumeration of aromatic oxygenase genes by multiplex and real-time PCR. Appl Environ 69: 3350–3358.

Baldwin BR, Nakatsu CH & Nies L (2008) Enumeration of aromatic oxygenase genes to evaluate monitored natural attenuation at gasoline contaminated sites. Water Res 42: 723–731

Becker S, Boger P, Oehlmann R & Ernst A (2000) PCR bias in ecological analysis: a case study for quantitative Taq nuclease assays in analyses of microbial communities. Appl Environ Microb 66: 4945–4953.

Béjà, O., Suzuki, M..T., Heidelberg, J.F., Nelson, W.C., Preston, C.M., Hamada, T., Eisen, J.A., Fraser, C.M., DeLong, E.F. 2002. Unsuspected diversity among marine aerobic anoxygenic phototrophs. Nature. 415: 630–633.

Besiktepe, S., Sur H., I., Ozsoy, E., Latif, M., A., Oguz, T., Unluata, U. 1994. The circulation and hydrography of the Marmara Sea. Progressive Oceanography, 34, 285-234.

Besiktepe, S., Ozsoy, E., Latif, M., A., Oguz, T. 2000. Hydrography and Circulation of the Marmara Sea. Marmara Denizi 2000 Sempozyumu, Istanbul, 11-12 November 2000, 314-326.

Canfield D, Thamdrup B, Hansen JW. 1993a. The anaerobic degradation of organic matter in Danish coastal sediments : Fe reduction, Mn reduction and sulfate reduction. Geochim. Cosmochim. Acta., 57, 3867-3883

Canfield, D.E., Jorgensen, B.B., Fossing, H., Glud, R., Gundersen, J., Ramsing, N.B., Thamdrup, B. Hansen, J.W., Nielsen, L.P., Hall, P.O.J. 1993b. Pathways of organic carbon oxidation in three continental margin sediments. Mar. Geol., 113, 27-40.

Cases, I., de Lorenzo, V. 2002. The grammar of (micro)-biological diversity. Environmental Microbiology, 4, 623–627

Chan,O.C., Wolf, M., Hepperle, D., Casper, P. 2002. Methanogenic archaeal community in the sediment of an artificially partitioned acidic bog lake. FEMS Microbial Ecology, 42:119-129.

Chin KJ, Sharma ML, Russell LA, O'Neill KR , Lovley DR (2008) Quantifying Expression of a Dissimilatory (bi)Sulfite Reductase Gene in Petroleum-Contaminated Marine Harbor Sediments. Microb Ecol 55:489-499.

Coates, J.D., Woodward, J., Allen, J., Philip, P., Lovley, D.R. 1997. Anaerobic degradation of polycyclic aromatic hydrocarbons and alkanes in petroleum-contaminated marine harbor sediments. Appl. Environ. Microbiol., 63, 3589-3593

Coates, J.D., Woodward, J., Allen, J., Philip, P., Lovley, D.R. 1997. Anaerobic degradation of polycyclic aromatic hydrocarbons and alkanes in petroleum-contaminated marine harbor sediments. Appl. Environ. Microbiol., 63, 3589-3593

Coleman ME, Dreesen DW,Wiegert RG (1996) Appl Environ Microbiol 62:3632

Colleran E, Finnegan S, Lens P (1995) Antonie van Leeuwenhoek 67:29

Colwell FS, Boyd S, Delwiche ME, Reed DW, Phelps TJ , Newby DT (2008) Estimates of Biogenic Methane Production Rates in Deep Marine Sediments at Hydrate Ridge, Cascadia Margin. Appl Environ Microbiol 74:3444-3452

Delille, D., 1995. Seasonal Changes of Subantarctic Benthic Bacterial Communities, Hydrobiologia, 310,45-57

D'Hondt, S., Jorgensen, B. B., Miller, D. J., Batzke, A., Blake, R., Cragg, B. A., Cypionka, H., Dickens , G. R., Ferdelman, T., Hinrich , K .-U., Holm, N. G., Mitterer, R., Spivack , A., Wang, G., Bekins, B., Engelen, B., Ford, K., Gettemy, G., Rutherford, S. D., Sass, H., Skilbeck, C. G., Aiello, I. W.,Guerin, G.,House, C.H., Inagaki, F., Meister, P., Naehr, T., Niitsuma, S., Parkes, R. J., Schippers, A., Smith, D. C., Teske, A., Wiegel, J., Padilla, C. N., Acosta, J. L. S. 2004. Distributions of microbial activities in deep subseafloor sediments. Science, 306, 2216–2221.

D'Hondt, S. L., Rutherford, S., Spivack, A. J. 2002. Metabolic activity of subsurface life in deep-sea sediments. Science, 295, 2067–2070.

D'Hondt, S.L., BB. Jorgensen, D.J. Miller, and scientists from ODP Expedition 201. 2003.Proceedings of the Ocean Drilling Program, Initial Reports, 201, Ocean Drilling Program,Texas A&M University, College Station, TX.

Dardanelles. Continental Shelf Research, 21, 1361–1382.

Devereux, R., Mundform, G.W., 1994. A Phylogenetic Tree of 16S rRNA Sequences From Sulfate-Reducing Bacteria in a Sandy Marine Sediment, Applied Environmental Microbiology, 60, 3437-3439

Diviacco S, Norio P, Zentilin L, Menzo S, Clementi M, Biamonti G, Riva S, Falaschi A & Giacca M(1992) A novel procedure for quantitative polymerase chain reaction by co-amplification of competitive templates. Gene 122: 313–320.

Dogan, E., Guven, K.C., Sur, H.I., Okus, E., Yuksek, A., Unlu, S., Altiok, H., Tas, S., **Muftuoglu, E., Yilmaz,** N., Aslan-Yilmaz, A. and Karjan,U. 2005 Volganeft 248 Petrol Kirliligi Takibi Final Rapor.

Dogan, E., Sur, H._I., Guven, K.C., Okus, E., Polat C, , Yuksek, A., Uysal, A., Algan, O., Kıratlı, N., Balkıs, N., Unlu¨ , S., Altıok, H., Gazioglu, C., Tas, S., Aslan, A., Yılmaz, N., Cebeci, M., 2000.Water Quality Monitoring in Black Sea, Marmara Sea and the Strait of Istanbul, Final Report (1999). Submitted to: General Directorate of Istanbul Water and Sewerage Administration,Istanbul.

Dorigo, U., Volatier, L., Humbert, J-F. 2005. Molecular approaches to the assesment of biodiveristy in aquatic microbial communities. Water Research, 39, 2207-2218.

Edwards R, Rodriguez-Brito B,Wegley L, Haynes M, Breitbart M, Peterson D, Saar M, Alexander S, Alexander EC & Rohwer F (2006) Using pyrosequencing to shed light on deep mine microbial ecology. BMC Genomics 7: 57–70.

Fashchuk, D., Ya., Samyshev, E. Z., Sebakh, L. K., Shlyakhov, V. A., 1991. Forms of antropogenic impact on the Black Sea ecosystem and is modern state. Ekologia Morya, 19-27.

Florencio L (1994) The fate of methanol in anaerobic bioreactors. PhD thesis,Wageningen Agricultural University

Freitag, T.E. and Prosser, J.I. 2003. Community Structure of Ammonia-Oxidizing Bacteria within Anoxic Marine Sediments. Applied and Environmental Microbiology, 69, 1359-1371.

Frischer, M. E., J. M. Danforth, M. A. Newton Healy, F. M. Saunders. 2000. Whole-cell versus total RNA extraction for analysis of microbial community structure with 16S rRNA-targeted oligonucleotide probes in salt marsh sediments. Applied Environmental Microbiology, 66, 3037–3043.

Geets J, Borremans B, Diels L, Springael D, Vangronsveld J, van der Lelie D, Vanbroekhoven K (2006) dsrB gene-based DGGE for community and diversity surveys of sulfate-reducing *Bacteria*. J Microbiol Methods 66:194-205.

Giglio S, Monis PT & Saint CP (2003) Demonstration of preferential binding of SYBR green I to specific DNA fragments in real-time multiplex PCR. Nucliec Acids Res 31: e136.

Gray, J.P. and Herwig, R.P. 1996. Phylogenetic Analysis of the Bacterial Communities in Marine Sediments, 62: 4049-4059

Guidot A, Debaud JC & Marmeisse R (2002) Spatial distribution of the below ground mycelia of an ectomycorrhizal fungus inferred from specific quantification of its DNA in soil samples. FEMS Microbiol Ecol 42: 477–486.

Guven, K.C., Okus, E., Unlu, S. Dogan, E., 1998. The oil pollution of Turkish Straits in 1995-1996, Proceedings of the First Conference on Oil Spills in the Mediterranean and Black Sea Regions, Istanbul, 15-18 September 1998, 19-32.

Hansen, T.A. 1994. Metabolism of sulfate-reducing prokaryotes. Antonie Van Leeuwenhoek, 66, 165–185

Heijthuijsen JHFG,Hansen TA (1986) FEMS Microbiol Lett 38:57

Head, I.M., and Swannell, R.P., 1999. Bioremediation of petroleum hydrocarbon contaminants in marine habitats. Current Opinions in Biotechnology, 10, 234-239.

Head, I.M. and Roling, 2005. W.F. Prokaryotic systematics: PCR and sequence analysis of amplified 16S rRNA genes. In Osborn A.M.(Eds), 25-64, Molecular Microbial Ecology, Routledge Pubs

Head, I. M. 2004. Bacterial community dynamics and hydrocarbon degradation during a field-scale evaluation of bioremediation on a mudflat beach contaminated with buried oil. Applied Environmental Microbiology, 70, 2603–2613.

Head IM, **Saunders JR & Pickup RW**(1998) Microbial evolution, diversity and ecology: a decade of ribosomal RNA analysis of uncultured microorganisms. Microb Ecol 35: 1–28.

He JZ, Shen JP, Zhang LM, Zhu YG, Zheng YM, Xu MG & Di H (2007b) Quantitative analyses of the abundance and composition of ammonia oxidizing bacteria and ammoniaoxidizing archaea of a Chinese upland red soil under longterm fertilization practices. Environ Microbiol 9: 2364–2374.

Hofman-Bang, J., Zheng, D., Westermann, P., Ahring, B.K., Raskin, L. 2003. Molecular Ecology of Anaerobic Reactor Systems. Advances in Biochemical Engineering/Biotechnology, 81, 153-203.

Holland PM, Abramson RD, Watson R & Gelfand DH (1991) Detection of specific polymerase chain reaction product by utilizing the 50–30 exonuclease activity of Thermus aquaticus DNA polymerase.

Hoj, L., Olsen, R.A., Torsvik, V.L. 2005. Archaeal communities in high arctic wetlands at Spitsbergen, Norway (78 degrees N) as characterized by 16S rRNA gene fingerprinting. FEMS Microbiol. Ecol., 53, 89–101.

Hyland, J., Balthis, L., Karakassis, I., Magni, P., Petrov, A., Shine, J., Vestergaard, O., Warwick, R. 2005. Organic carbon content of sediments as an indicator of stress in the marine benthos. Marine Ecology Progress Series, 295, 91–103.

Jorgensen, B.B. 1983. Processes at the sediment-water interface. In: Bolin, B., Cook, R. B.(eds.) The major biochemical cycles and their interactions. Wiley, Chichester, 477-509.

Jørgensen, B. B. and Bak, F. 1991. Pathways and Microbiology of Thiosulfate Transformations and Sulfate Reduction in a Marine Sediment (Kattegat, Denmark) Applied Environmental Microbiology, 57, 847-856.

Kazezyilmaz, M.C., Gulec, S.B., and Otay, E.N. 1998. A Case Study of Contaminant Transport Modelling; Tuzla Oil Spill, Proceedings of the first Conference on Oil Spills in the Mediterranean and Black Sea Regions, Istanbul, 15-18 September 1998, 19-32.

Knittel, K., Lösekann, T., Boetius, A., Kort, R., Amann, R. 2005. Diversity and Distribution of Methanotrophic Archaea at Cold Seeps. Applied and Environmental Microbiology, 71, 467–479

Kocatas, A., Koray, T., Kaya, M., and Kara,O.F., 1993. A review of the fishery resources and their environment in the Sea of Marmara, F.A.O. studies and reviews. G.FC.FM

Kor, N., 1963. Preliminary studies on the pollution of the Golden Horn Estuary. Ph.D. Thesis. Istanbul Technical University, Istanbul.

Kolukirik M, Ince O, Ince BK (2009) Local and Seasonal Changes in Microbial Diversity of the Marmara Sea Sediments. Mar Pollut Bull MPB-D-09 00623 Under Review.

Kuenen JG, Harder W (1982) In Burns RG, Slater JH (eds), Experimental Microbial Ecology. Blackwell, London , p 342

Kuijk van BLM, Stams AJM (1995) Antonie van Leeuwenhoek 68:293

Klappenbach JA, Dunbar JM & Schmidt TM (2000) rRNA operon copy number reflects ecological strategies of bacteria.Appl Environ Microb 66: 1328 1333.

Kutyavin IV, Afonina IA, Mills A et al. (2000) 3'-Minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. Nucl Acids Res 28: 655–661.

Larkin MJ, Osborn AM & Fairley D (2005) A molecular toolbox for bacterial ecologists: PCR primers for functional gene analysis. Molecular Microbial Ecology (Osborn AM & Smith CJ,eds), pp. 281–301. Taylor & Francis, Abingdon, UK.

Livak KJ, Flood SJ, Marmaro J, Giusti W & Deetz K (1995) Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl 4:

Llobet-Brossa, E., Rosello-Mora, R., Amann, R. 1998. Microbial Community Composition of Wadden Sea Sediments as Revealed by Fluorescence in Situ Hybridization. Applied Environmental Microbiology, 64, 2691-2696.

Love JL, Scholes P, Gilpin B, Saville M, Lin S & Samuel L (2006) Evaluation of uncertainty in quantitative real-time PCR. J Microbiol Meth 67: 349 356.

Lovley DR, Dwyer DF,Klug MJ (1982) Appl Environ Microbiol 43:1373

Lovley, D.R., Klug, M.J. 1983. Sulfate reducers can outcompete methanogens at freshwater sulfate concentrations. Appl. Environ. Microbiol., 45, 187–192

Lyons, W.B., Lent, R.M., Burnett, W.C., Chin, P., Landing, W.M., Orem, W.H., McArthur, J.M. 1996. Jellyfish Lake, Palau: Regeneration of C, N, Si, and P in Anoxic Marine Lake Sediments. Limnology and Oceanography, 41, 1394-1403.

MacArthur RH, Wilson EO (1967) The theory of island biogeography. Princeton NJ, Princeton University Press

Macnaughton, S. J., Stephen,J. R., Venosa,A. D., Davis, ,G. A., Chang , Y. J., White, D. C. 1999 . Microbial population changes during bioremediation of an experimental oil spill. Applied Environmental Microbiology, 65, 3566–3574

Madigan, M.T., Martinko, J.M., Parker, J. 2003. Biology of microorganisms, 10th ed. Pearson Education, Inc. Upper Saddle River, N.J.

Manz W, Eisenbrecher M, Neu TR, Szewzyk U (1998) Abundance and spatial organization of Gram-negative sulfate-reducing bacteria in activated sludge investigated by in situ probing with specific 16S rRNA targeted oligonucleotides. FEMS Microbiol Ecol 25:43– 61.

Margulies M, Egholm M, Altman WE et al. (2005) Genome sequencing in microfabricated high-density picolitre reactions. Nature 437: 376-380.

Moon-van der Staay, S.Y., De Wachter, R., Vaulot, D. 2001. Oceanic 18S rRNA sequences from picoplankton reveal new eukaryotic lineages. Nature, 409, 607–610.

Moter A., Göbel, U.B. 2000. Fluorescent in situ hybridization (FISH) for direct Visualization of microorganisms, Journal of Microbiological Methods, 41, 85-112. Cases, I., de Lorenzo, V. 2002. The grammar of (micro)-biological diversity. Environmental Microbiology, 4, 623 627.

Morkoç E, Okay OS, Tolun L, Tufekçi V, Tufekçi H, Legoviç T. Towards a clean Izmit Bay. Environ Int 2001;26:157–61.

Muyzer, G. 1999. DGGE/TGGE a method for identifying genes from natural ecosystems. Current Opinion in Biotechnology, 2, 317-322.

Muyzer, G., Smalla, K. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and t emperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek, 73, 127–141.

Munson, M. A., Nedwell, D. B., Embley, T. M. 1997. Phylogenetic diversity of Archaea in sediment samples from a coastal salt marsh. Applied Environmental Microbiology, 63, 4729–4733.

Muyzer, G., Hottenträger, S., Teske, A., Wawer, C. 1996 . Denaturing gradient gel electrophoresis of PCR- amplified 16S rDNA–a new molecular approach to analyse the genetic diversity of mixed microbial communities. Molecular Microbial Ecology Manual 3.4.4.

Muyzer, G., De Waal, E. C., Uitterlinden, A, G. 1993. Profiling of Complex Microbial Populations by Denaturing Gradient Gel Electrophoresis Analysis of Polymerase Chain Reaction- Amplified Genes Coding for 16S rRNA. Applied Environmental Microbiology, 59, 695-700.

Muyzer, G., Hottenträger, S., Teske, A., Wawer, C. 1996. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA–a new molecular approach to analyse the genetic diversity of mixed microbial communities. Molecular Microbial Ecology Manual 3.4.4

Myers, R.M., Maniatis, T., Lerman, L.S. 1987. Detection and localization of single base changers by DGGE. Methods in Enzymology, 155,501-527.

Okano Y, Hristova KR, Leutenegger CM, Jackson LE, Denison RF, Gebreyesus B, Lebauer D & Scow KM (2004) Application of real-time PCR to study effects of ammonium on population size of ammonia-oxidizing bacteria in soil. Appl Environ Microb 70: 1008–1016.

Okay OS, Legoviç T, Tüfekci V, Egesel(Tolun) L, Morkoç E. Environmental impact of land based pollutants on Izmit Bay: short term algal bioassays and simulation of toxicity distributions in the marine environment. Arch Environ Contam Toxicol 1996;31:459–65.

Oremland R S (1988) In: Zehnder AJB (ed),Biology of anaerobic microorganisms.Wiley, New York, p 641

Orhon, D.1995. Evaluation of the impact from the Black Sea on the pollution of the Marmara Sea, Water Science and Technology, 32,(7),191–198.

Otay, E. N., Yenigün, O. 2000. Volgoneft-248 oil spill in the Marmara Sea. In: Proceedings of 2nd International Conference on Oil Spills in the Mediterranean and Black Sea Regions. 31 October–03 November 2000, Istanbul, pp. 13–23.

Øvreas, L., Forney, L., Daae, F. L., Torsvik, V. 1997. Distribution of bacterioplankton in meromictic Lake Saalenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Applied Environmental Microbiology, 63, 3367-3373.

Öztürk, B., Kadıoğlu, M., Öztürk, H. 2000. Marmara Sea 2000 symposium report. In: Öztürk, B., Kadıoglu, M., Öztürk, H. (Eds.), TÜDAV Yayın no. 5, Istanbul.

Pace, N.R. 1997. A molecular view of microbial diversity and the biosphere. Science, 276, 734–740.

Prince, R. C. 1997. Bioremediation of oil spills Trends Biotechnology, 15, 158–160.

Polz MF & Cavanaugh CM (1998) Bias in template -to -product ratios in multi template PCR. Appl Environ Microb 64: 3724–3730.

Rebrikov DV & Trofimov DY (2006) Real-time PCR: a review of approaches to data analysis. Appl Biochem Micro 42: 455–463.

Reysenbach AL, Giver LJ, Wickman GS & Pace NR (1992) Differential amplification of rRNA genes by polymerase chain reaction. Appl Environ Microb 58: 3417–3418.

Rothermich, M.M., Hayes, L.A., Lovley, D.R. 2002. Anaerobic, sulfatedependent degradation of polycyclic aromatic hydrocarbons in petroleum contaminated harbor sediment. Environ. Sci. Technol., 36, 4811– 4817.

Röling, W. F. M., Milner, M. G., Jones, D. M., Lee, K., Daniel, F., R., Swannell, J. P., Head, I. M. 2002. Robust hydrocarbon degradation and dynamics of bacterial communities during nutrient-enhanced oil spill bioremediation. Applied Environmental Microbiology, 68, 5537–5548.

Röling, W. F. M., Milner, M. G. , Jones, D. M., Fratepietro, F., Swannell, R. P., Daniel, J. F.,

Skyes PJ, Neoh SH, Brisco MJ, Hughes E, Condon J & Morley AA (1992) Quantitation of targets for PCR by use of limiting dilutions. BioTechniques 13: 444–449.

Schink B, Friedrich M (1994) FEMS Microbiol Rev 15:85

Schwarz, J.I.K., Eckert, W., Conrad, R. 2007. Community structure of Archaea and Bacteria in a profundal lake sediment Lake Kinneret (Israel). Systematic and Applied Microbiology, 30, 239–254.

Shine, J., Wallace, G. 2000. Chemical aspects of organic carbon and ecological stress in benthic ecosystems. Ad hoc Benthic Indicator Group-Results of Initial Planning Meeting. IOC Technical Series, UNESCO, 57, 40–44

Stams AJM, Oude Elferink SJWH, Westermann P (2003) Metabolic Interactions Between Methanogenic Consortia and Anaerobic Respiring Bacteria. Adv Biochem Eng Biotechnol 81:32-56.

Smith, D.C., D 'Hondt, S. 2006. Exploration of life in deep subseafloor sediments. Oceanography, 19, 58-70.

Stubner S (2004) Quantification of Gram-negative sulphate-reducing bacteria in rice field soil by 16S rRNA gene-targeted real-time PCR. J Microbiol Methods 57:219-230.

Sur, H.I., Okus, E., Sarıkaya, H.Z., Altıok, H., Eroglu, V., Ozturk, I., 2002a. Rehabilitation and water quality monitoring in the Golden Horn. Water Science and Technology 46 (8), 29–36.

Suzuki MT & Giovannoni SJ (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl Environ Microb 62: 625–630.

Suzuki MT, Taylor LT & DeLong EF (2000) Quantitative analysis of small subunit rRNA genes in mixed microbial populations via 50-nuclease assays. Appl Environ Microb 66: 4605–4614.

Takai K, Horikoshi K (2000) Rapid detection and quantification of members of the Archaeal community by quantitative PCR using fluorogenic probes. Appl Environ Microbiol 66:5066-5072.

Thauer RK, Jungermann K, Decker K (1977) Bacteriol Rev 41:100

Treusch AH, Leininger S, Kletzin A, Schuster SC, Klenk HP & Schleper C (2005) Novel genes for nitrite reductase and Amorelated proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. Environ Microbiol 7: 1985–1995.

Tuğrul, S., Polat, C. 1995. Quantitative comparison of the influxes of nutrients and organic carbon into the Sea of Marmara both from anthropogenic sources and from the Black Sea. Water Science and Technology, 32, 115–121.

Tuncer, G., Tuncel, G., Balkas, T.I., 2001. Evolution of metal pollution in the Golden Horn (Turkey) sediments between 1912–1987. Marine Pollution Bulletin 42 (5), 350–360.

Townsend, G.T., Prince, R.C., Suflita, J.M. 2003. Anaerobic oxidation of crude oil hydrocarbons by resistent microorganisms of a contaminated anoxic aquifer. Environ. Sci. Technol., 37, 5213–5218.

Townsend, G.T., Prince, R.C., Suflita, J.M. 2003. Anaerobic oxidation of crude oil hydrocarbons by resistent microorganisms of a contaminated anoxic aquifer. Environ. Sci.Technol., 37, 5213–5218.

Tolun, L.G., Okay, O.S., Grains, A.F., Tolay, M., Tufekci, H., Kiratli, N.,2001. The pollution status and the toxicity of surface sediments in Izmit Bay (Marmara Sea), Turkey. Environ. Int. 26, 163–168.

Unlu, S., Alpar, B., 2006. Distribution and sources of hydrocarbons in surface sediments of Gemlik Bay (Marmara Sea, Turkey) Chemosphere 64: 764 777.

Ünlü, S., Güven, K.C., Dogan, E., Okus, E. 2000. Oil pollution in Tuzla Bay after the TPAO accident. Turkish J. Marine Seciences, 6, 135-150.

Veldkamp H, Jannasch HW (1972) J Appl Chem Biotechnol 22:105

Vetriani, C., Jannasch, H.W., MacGregor , B, J., Stahl, D.A., Reysenbach, A.L. 1999. Population Structure and Phylogenetic Characterization of Marine Benthic Archaea in Deep-Sea Sediments. Applied Environmental Microbiology, 65, 4375-4384.

Venturini, N., Tommasi, L.R., Bicego, M.C., Martins, C.C. 2004. Characterization of the benthic environment of a coastal area adjacent to an oil refinery, Todos Os santos Bay (NE-Brazil). Brazilian Journal of Oceanography, 52, 123–134.

Von Wintzingerode F, G¨obel UB & Stackebrandt E (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol Rev 21: 213–229.

Vrede, K., Heldal, M., Norland, S., Bratbak, G., 2002. Elemental composition (C, N, P) and cell volume of exponentially growing and nutrient-limited bacterioplankton. Applied and Environmental Microbiology 68, pp. 2965–2971.

Ward, D.M., Bateson, M.M., Weller, R., Ruff-Roberts, A.L . 1992. Ribosomal RNA analysis of microorganisms as they occur in nature.Adv. Micro. Ecol.,12, 219-286.

Wilms, R., Sass, H., Köpke, B., Köster, J., Cypionka, H., Engelen, B., 2006. Specific Bacterial, Archaeal, and Eukaryotic Communities in Tidal-Flat Sediments along a Vertical Profile of Several Meters. Appl Environ Microbiol 72:2756-2764

Wittwer CT, Herrmann MG, Moss AA & Rasmussen RP (1997) Continuous fluorescence monitoring of rapid cycle DNA amplification. BioTechniques 22: 130–138

Wollast, R. 1991. The coastal organic carbon cycle: fluxes, sources, and sinks, p. 365–381. In R. F. C. Mantoura, J.-M. Martin, and R. Wollast (ed.), Ocean margin processes in global change. John Wiley & Sons, New York, N.Y.

Yalçıner AC, Synolakis CE, Borrero J, Altınok Y, Watts P, Imamura F, et al. Tsunami generation in Izmit Bay by the 1999 Izmit Earthquake. Proceedings of the International Conference on Kocaeli Earthquake, Kocaeli, Turkey; 1999. p. 217–21.

Yu Y, Lee C, Kim J, Hwang S (2005) Group-Specific Primer and Probe Sets to Detect Methanogenic Communities Using Quantitative Real-Time Polymerase Chain Reaction. Biotechnol Bioeng 89:670-679.

Zwolinski, M.D., Harris, R.F., Hickey, W.J., 2000. Microbial Consortia involved in the anaerobic degradation of hydrocarbons. Biodegradation, 11, 141-158

CURRICULUM VITA

Candidate's full name: **Bahar Öztulunç**

Place and date of birth: **Istanbul 20.03.1985**

Permanent Address: Tercüman Sitesi A3 blok Kat:6 Daire:27

Universities and

Colleges attended: **Istanbul University Biology 2007**

Istanbul Technical University Environmental Biotechnology 2010