İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

NUTRIENT ENHANCED BIOREMEDIATION OF PETROLEUM HYDROCARBONS IN ANOXIC SEDIMENTS FROM MARMARA SEA

P.hD. Thesis by
Mustafa KOLUKIRIK

Department : Environmental Engineering

Programme : Environmental Biotechnology

Thesis Supervisor: Prof. Dr. Orhan İNCE

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Mustafa KOLUKIRIK
(501042801)

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Supervisor (Chairman) : **Prof. Dr. Orhan İNCE (ITU)**
Members of the Examining Committee : **Prof. Dr. Emine Ubay ÇOKGÖR (ITU)**
Prof. Dr. Candan TAMERLER (ITU)
Assoc. Dr. Bülent MERTOĞLU (MAUN
Assoc. Dr. Barış ÇALLIOĞLU (MAUN)

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

BESİ MADDESİ TAKVİYESİ İLE ANOKSİK MARMARA DENİZİ SEDİMENTLERİNDE PETROL HİDROKARBONLARININ BİYOISLAHININ İYİLESTİRİLMESİ

DOKTORA TEZİ
Mustafa KOLUKIRIK
(501042801)

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Tez Danışmanı : Prof. Dr. Orhan İNCE (İTÜ)
Diğer Jüri Üyeleri : Prof. Dr. Emine Ubay ÇOKGÖR (İTÜ)
Prof. Dr. Candan Tamerler (İTÜ)
Doç. Dr. Bülent MERTOĞLU (MÜ)
Doç. Dr. Barış ÇALLI (MÜ)

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FOREWORD

I would like to state my sincere gratitude to my supervisor, Prof. Dr. Orhan INCE whose experience and logical way of thinking have been the main guide for the present thesis. I believe that my Ph.D. studies were just the beginning of our lifelong collaboration.

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ABBREVIATIONS

AnAlHCD **:**Anaerobic aliphatic hydrocarbon degradation
AnAlHCDB **:**Anaerobic aliphatic hydrocarbon degrading Bacteria
AnArD **:**Anaerobic aromatic degradation
AnArDB **:**Anaerobic aromatic degrading Bacteria
AnArHCD **:**Anaerobic aromatic hydrocarbon degradation
AnArHCDB **:**Anaerobic aromatic hydrocarbon degrading Bacteria
AnHCD **:**Anaerobic hydrocarbon degradation
AnHCDB **:**Anaerobic hydrocarbon degrading Bacteria
assA **:**alkylsuccinate synthase
bcrA **:**benzoyl coenzyme A reductase
bssA **:**benzylsuccinate synthase
DGGE **:**Denaturant Gradient Gel Electrophoresis
DN **:**Denitirification
DNRA **:**Dissimilatory nitrate reduction to ammonia
DSR **:**Dissimilatory sulfate reduction
dsrB **:**dissimilatory sulfite reductase
FISH **:**Fluorescence in situ hybridization
HC **:**Hydrocarbon
HC(-) **:**Micrcosms without hydrocarbon addition
HC(+) **:**Hydrocarbon added microcosms
L **:**Limited nutrient supply
mcrA **:**Methyl-coenzyme M reductase
MG **:**Methanogenesis
MSS **:**Marmara Sea Sediments
N(-) **:**Micrcosms without nitrate addition
N(+) **:**Nitrate added microcosms
nasA **:**Assimilatory nitrate reductase
NL **:**N limited
nosZ **:**Nitrous oxide reductase
nrfA **:**Periplasmic nitrite reductase
PL **:**P limited
Q-PCR **:**Quntitative real time PCR
Q-RT-PCR **:**Reverse transcription combined with quntitative real time PCR
UL **:**Unlimited nutrient supply

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NUTRIENT ENHANCED BIOREMEDIATION OF PETROLEUM HYDROCARBONS IN ANOXIC SEDIMENTS FROM MARMARA SEA

SUMMARY

Marmara Sea has been extensively polluted with petroleum hydrocarbons. A cost effective remediation strategy which can be sustained for a long time with a minimal human intervention is needed to overcome the chronic hydrocarbon pollution in this environment. The best candidate for this purpose is bioremediation under anaerobic/anoxic conditions as long as oil-degrading anaerobes are abundant and active in the sediments, and there is a way to increase activity of this population. In order to assess this, physicochemical and microbiological characteristics of Marmara Sea sediments were monitored for two years. The total petroleum HC levels were similar to those from extremely polluted marine environments; the microbial cell contents were very high compared to the other marine environments; the sediments were dominated by dissimilatory nitrate reducers to ammonia, methanogens and anaerobic hydrocarbon degraders, and these microbes were active; and N and P were limited in the porewaters for biological activity. These indications raised the question that hydrocarbon degradation activity of Marmara Sea sediments can be increased by N-P amendment under methanogenic and nitrate reducing conditions. Natural TOC/N/P ratio of the sediment porewaters was gradually decreased which resulted in ~20× increase in hydrocarbon removal. Natural hydrocarbon content of the sediments were completely removed. The sediment microorganisms degraded wide range of aliphatic (n-C_{9-31} alkanes and acyclic isoprenoids) and aromatic (18 different 1-5 ring aromatics) hydrocarbons. Monitoring functional gene and transcript abundances revealed that methanogenesis, dissimilatory nitrate reduction to ammonia and denitrification processes were the three routes of hydrocarbon degradation in Marmara Sea sediments. Genes and transcripts related to initial activation of hydrocarbons were highly abundant throughout the incubation periods showing that fumarate addition was the main pathway of anaerobic hydrocarbon degradation. In conclusion, biostimulation of highly polluted anoxic Marmara Sea Sediments via nutrient amendment is possible which will lead to less human intervened and more economical field-scale bioremediation applications worldwide.

BESİ MADDESİ TAKVİYESİ İLE ANOKSİK MARMARA DENİZİ SEDİMENTLERİNDE PETROL HİDROKARBONLARI BİYOISLAHININ İYİLEŞTİRİLMESİ

ÖZET

Marmara Denizi petrol hidrokarbonlarıyla yoğun bir şeklide kirletilmektedir. Bu kronik hidrokarbon kirliliğinin üstesinden gelebilmek için sürdürülebilir, az insan müdahelesi gerektiren ve ekonomik bir ıslah stratejisi geliştirilmesi şarttır. Eğer Marmara Denizi sedimentlerinde anaerobik koşullarda hidrokarbon ayrıştırabilen mikroorganizmalar aktif bir şekilde bulunuyorsa ve bu organizmaların aktivitelerini arttırmanın bir yolu bulunabilirse, kirliliğin giderilmesi için en umut vaad eden yöntem anaerobik ya da anoksik koşullarda biyoıslah uygulanmasıdır. Bu ön koşulu sınamak için Marmara Denizi sedimentlerinin fiziksel, kimyasal ve mikrobiyolojik karakteristikleri 2 yıl boyunca izlenmiştir. Marmara Denizi sedimentlerindeki toplam petrol hidrokarbon seviyeleri, aşırı şekilde kirlenmiş deniz ortamlarındaki seviyelerle aynı düzeydedir. Toplam mikrobiyal hücre içerikleri ise deniz ortamları için belirtilen seviyelerin çok üstündedir. Sedimentlerde yürüyen başlıca metabolik prosesler nitratın amonyağa dissimilatif indirgenmesi, sülfat indirgenmesi, denitrifikasyon, metan üretimi ve anaerobik hidrokarbon ayrıştırılmasıdır. Sediment süzüntü sularında mevcut N ve P seviyeleri mikrobiyal çoğalmayı destekleyecek seviyelerin çok altındadır. Bu göstergeler nutrient takviyesi ile sedimentlerin aktivitelerinin arttırılabileceğine dair ipuçları vermiştir. Bunu sınamak için metanojenik ve nitrat indirgeyici koşullar altında hidrokarbon ayrışım mikrokozmosları kurulmuştur. Mikrokosmaslardaki nutrient seviyelerinin doğal seviyelerden başlanarak giderek arttırılması, hidrokarbon ayrıştırma aktivitesinde ~20× artışla sonuçlanmışıtr. Sedimentlerin doğal hidrokarbon içeriklerinin tümü bu şekilde giderilebilmiştir. Sedimentler bir çok farklı aromatik (18 farklı 1-5 halka aromatikler) ve alifatik (n-C_{9-31} alkanlar ve asiklik isoprenoidler) hidrokarbonları ayrıştırabilmiştir. Mikrokozmoslarda hidrokarbonların ayrıştırılması için 3 farklı mikrobiyal metabolik yol izlenmiştir. Bunlar metanojenesis, nitratın amonyağa dissimilatif indirgenmesi ve denitrifikasyon prosesleridir. Anaerobik ve anoksik koşullarda hidrokarbon ayrıştırılmasından sorumlu fonksiyonel genlerin miktarı ve bu genlerden üretilen transkriptlerin miktarı inkübasyon süresi boyunca yüksek seviyelerde seyretmiştir. Bu durum fümarat eklenmesi ile hidrokarbon aktivasyonu ve daha sonra anaerobik hidrakarbon ayrıştırılması prosesinin hidrokarbon ayrıştırılmasında en önemli rolu oynadığını göstermiştir. Sonuç olarak, bu çalışma Marmara Denizindeki yoğun ve kronik kirliliğin, sediment organizmalarının aktivitelerinin nutrient takviyesi ile arttırılması yoluyla giderilebilmesinin mümkün olduğunu göstermiştir. Bu çalışmanın çıktıları, daha az maliyet ve insan müdahalesi gerektiren biyoıslah uygulamalarının dünya çapında uygulanmaya başlanmasına öncülük edecek niteliktedir.

1. INTRODUCTION

1.1 Purpose of the Thesis

1.1.1 Investigating the microbial ecology of Marmara Sea Sediments

Sub-seafloor prokaryotic activities have profound effects on global biogeochemical cycles. This is because marine sediments cover more than two-thirds of Earth's surface and contain the largest reservoir of microbial biomass (Parkes, 2000). Moreover, sediment microorganisms constitute a huge reservoir of genetic variability which means that it is possible to find new life forms by studying different marine ecosystems (Torsvik, 2002). Despite its importance, fundamental aspects of marine benthic microbial life are poorly known (Smith and D'Hondt, 2006). What is the phylogenetic composition of sub-seafloor prokaryotic communities? What are their functional genes and metabolic activities that allow these prokaryotes to grow and survive in the subsurface?

Microbial investigations on subsurface sediment layers had mainly targeted open-ocean and continental-margin sediments (Smith and D'Hondt, 2006). However, as the investigations started to pay more attention to subsurface coastal sediments, the number of publications is currently increasing (Wilms et al., 2006). The previous microbiological studies on coastal marine sediments focused on vertical distribution of microbial communities (Bühring, 2005; Wilms et al., 2006). On the other hand, microbial communities can also be influenced by seasonality in the sediment's physical and chemical characteristics. This has not been studied yet, as far as we know.

Marmara Sea is a semi-enclosed water body connecting Black Sea to Aegean Sea. Inspite of its potential, the microbial ecology of Marmara Sea sub-seafloor has not been studied yet. In this study, 16S rRNA gene diversity (microbial composition), abundance and transcription levels of functional genes responsible for the key metabolic processes (microbial abundance and activity) as well as physiochemical characteristics of the Marmara Sea sediments (MSS) were monitored for two years.

The major outcomes of these efforts guided the feasibility studies on anaerobic bioremediation of petroleum hydrocarbons in MSS.

1.1.2 Assessing the bioremediation potential of Marmara Sea Sediments

The annual world production of crude oil is around 70 million barrels per day, with approximately 50% of this being transported by sea (Kilpatrick et al., 2007; McKew, 2007). Accidents occasionally result in large-scale marine pollution such as the recent explosion on an off-shore drilling platform in the Gulf of Mexico. Main sources of marine oil pollution are uncontrolled releases during crude oil production and refining, discharges during transportation, natural seepage from reservoirs, disposal by end users, and freshwater and terrestrial run-off (Diez et al., 2007; Lara and Martin, 2007; Short et al., 2007). It has been estimated that 1.7-8.8 x 10^6 tons of petroleum hydrocarbons impact marine waters and estuaries annually (McKew et. al., 2007). With world oil demand expected to grow by 50% by 2025 (US Department of Energy, 2006), oil pollution is likely to remain a significant threat to marine ecosystems.

Marmara Sea has extensively been polluted with petroleum hydrocarbons. The main sources of pollution are: highly polluted Black Sea, spills from oil tankers (≈35 accidents/year), discharges during marine transportation (≈50000 tankers/year), municipal wastes (≈20 million people), industrial wastes 40% of the Turkish Industry), atmospheric deposition, and urban surface and river runoff (Bebek, 2000; Pekey, 2007; Unlu and Alpar, 2006). Renewal capacity of Marmara Sea is much lower than the pollution rate which has led to increases in anoxic areas and 40% reduction in amount of viable marine species during the last 30 years (DSI, 2004).

Physical and chemical methods are capable of rapidly removing majority of petroleum hydrocarbons from a contaminated area, but, in most cases, they are not feasible in terms of remediation cost and rarely completely successful (Short et al., 2007; Head et al., 2006). Bioremediation is a promising technology for responding to the chronic hydrocarbon pollution in Marmara Sea (Van Hamme, 2003; Head and Swannell, 1999).

Anaerobic biodegradation processes are a significant component of natural attenuation owing to the abundance of anoxic electron acceptors relative to dissolved oxygen (Safinowski, 2006; Zwolinski, 2000). Furthermore, clean-up systems based

on anaerobic biodegradation require less human intervention. Despite its economical advantages, bioremediation strategies based on anaerobic microbial processes are very limited because they proceed at much lower rates than the aerobic ones (Prince, 2007).

An aerobic bioremediation strategy is unfeasible for Marmara Sea since oxygen penetration into anoxic MSS is poor and oxygen mass transfer enhancement by mechanical means is inappropriate for the inaccessible sediments. Moreover, economical long term solution for the chronic hydrocarbon pollution by continuous aeration of the huge anoxic areas in Marmara Sea is out of the question. Under these conditions anaerobic hydrocarbon degradation (AnHD) is the only alternative as long as oil-degrading anaerobes are abundant and active in MSS. Outcomes of the monitoring studies on MSS revealed that the genes related to AnHD were highly abundant and transcribed in the sediments; N and P was limited for the microbial activity. These indications raised the question that AnHD activity of the sediments can be increased via N-P amendment to bioremediate the chronic hydrocarbon pollution of MSS. In this study, this theory was assesses through AnHD microcosms.

2. POLLUTION PROFILE OF MARMARA SEA

Marmara Sea is a small size (70 x 250 km) intercontinental basin connecting Black Sea to Agean Sea via Bosphorus and Dardanelles straits. It has an area of about 11,110 km^2, a volume of 3380km^3, and consists of a complex morphology which is mainly controlled by the active tectonic regime of the North Anatolian Fault Zone (NAFZ). The present oceanography of Marmara Sea is controlled by a permanent two layer water stratification with a halocline at a depth of 20–25 m. Less saline Black Sea water (18%) flows as the upper layer from the İstanbul Strait to Canakkale Strait, whereas saline Mediterranean waters (38%) flows as the lower layer in the opposite direction (Miller, 1983; Unlu, 1990).

Marmara Sea is now the recipient of a large number of wastewater discharges from landbased sources. The basin receives a total of 1.9 x 106 tons of TOC total organic carbon) and 2.7 x 105 tons of TN (total nitrogen) per year from the Black Sea inflow. Pollution loading from İstanbul alone, the biggest city of Turkey in population and industry, makes up the major portion (40–65%) of the total anthropogenic discharges (Tugrul and Polat, 1995).

Anthropogenic activities in the coastal area of the North Marmara Sea include urban effluent, summer resorts (untreated effluent discharged into the sea), agricultural run off, sunflower oil factories, a big cement factory, fishing and shipping (Ozturk, 2000). In addition, the tanker traffic of several thousand oil carrying vessels per day via the Bosphorus Strait is a constant threat to the marine ecosystem.

The benthic environment of Marmara Sea is the fundamental compartment of this ecosystem since it is the final sink for many anthropogenic contaminants. Marmara Sea sediments have been accumulating great amounts of organic matter which has the major impact on oxygen content of the water column.

2.1 Pollution Sources

The Marmara Sea is now a critically polluted water body and the recipient of a large number of wastewater discharges from land-based sources located along the coastal line, including the İstanbul metropolitan area (Orhon, 1995; Albayrak., 2006) and subject to several other anthropogenic activities that primarily cause severe hydrocarbon and heavy metal pollution.

The pollution of Marmara Sea is based on sewages, industries and vessels. Sewage pollution is most important of them. The Marmara Sea turned into an open sewage, because there is not a purification system for sewages. Industrial pollution is mostly based on government-run factories (Algan, 2004). The water quality measurements indicate severe signs of present and future eutrophication problems (Orhon, 1995). There is dying species in the Marmara Sea over 50, such as monk seals, sturgeons, shrimps and crabs (Turkish Marine Research Foundation, 2004).

The contaminants are introduced through water ways by a surface current from Black Sea and a deep current from the Mediterranean, respectively (Unlu, 2006). The Bosphorus, a strongly stratified natural channel between the Marmara Sea and Black Sea, with significant mixing at the entrance to the Marmara Sea is also a major polluter for the Marmara basin, since it carries the highly polluted waters of the Black Sea (Orhon, 1995).

The Marmara Sea receives via the natural exchange from the Black Sea roughly 15 times more organic matter than what is contained in the sewage discharges from İstanbul (Orhon, 1995). The basin receives a total of 1.9 x 106 tons of TOC and 2.7 x 105 tons of TN per year from the Black Sea inflow (Albayrak, 2006). Nutrient input from the Black Sea, however, is much more significant then coastal wastewater discharges according to the experimental evidence on the basis of extensive observations (Orhon, 1995).

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

the upper layer can also be considered as a significant index of pollution in the Marmara Sea (Orhon, 1995).

Increasing industrial and domestic activities in the Marmara Region mainly influence the coastal and shelf areas of the Marmara Sea (Algan, 2004). Meanwhile rapid urbanization on the coastal zone of the Marmara Sea has attracted congested population influx since the 1970's (Unlu, 2006). Pollution loading from İstanbul alone, the biggest city of Turkey in population and industry, makes up the major portion (40–65%) of the total anthropogenic discharges (Polat and Tugrul, 1995). Anthropogenic activities in the coastal area of the North Marmara Sea include, urban effluent, summer resorts (untreated effluent discharged into the sea), agricultural run off, sunflower oil factories, a big cement factory, fishing and shipping (Ozturk, 2000).

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 and $2.7x10^5$ tons of TN 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; Tugrul 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 to 1996. Dardanelles showed a higher increase in concentration from 5.25 μg/L to 42.5 μg/L in the same period. The concentration of Marmara Sea increased from 36.9 μg/L to 103.7 μg/L at the same time (Guven, 1998).

In addition, tanker traffic of several thousand oil carrying vessels per day, via the Bosphorus Strait is a constant threat to the marine ecosystem (Albayrak, 2006). There is a heavy traffic of shipping approximately 60 000 vessels per year involving tankers 10%. Tankers from oil exporting countries surrounding the Black Sea have only one exit to the Mediterranean Sea: via the Bosphorus Strait, the Sea of Marmara and the Dardanelle Strait. The Bosphorus and the Dardanelle's are typical narrow water channels and navigation route through the Sea of Marmara. This route therefore increases the risk of collisions and running aground (Tan and Otay, 1999). Many accidents of merchant ships and tankers occurred in the strait. Nine tanker

accidents, which resulted in almost 193 tons oil spill, occurred in Bosphorus and Sea of Marmara between 1964-2002 (Guven, 2004). The major accidents happened by large tankers Independenta in 1979 and Nassia in 1994. In the Independenta accident at the exit of the Bosphorus to the Sea of Marmara in 1979, 95 000 tons of crude oil was spilt and burnt (Etkin, 1997).

In the Nassia accident at the northern exit of the Bosphorus to the Black Sea in 1994, 13500 tons of crude oil were spilt (Oguzulgen, 1995). M/V GOTIA sank into Bosphorus and 25 tons fuel oil was spilt and pollution spread out into a large area by winds (Guven, 2004). Bilge water discharge is also a major problem for the Straits of İstanbul and Çanakkale, and the Sea of Marmara. Increase in petroleum hydrocarbon levels mainly from oil spills, sewage outfalls and ship bilge water, has been observed in the Sea of Marmara (Guven, 1997).

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

Metal contents (Al, Fe, Mn, Cu, Pb, Zn, Ni, Cr, Co and Hg) of the surface sediments from the shelf areas of the Marmara Sea generally do not indicate shelf-wide pollution. The variability of the metal contents of the shelf sediments is mainly governed by the geochemical differences in the northern and southern hinterlands. Northern shelf sediments contain lower values compared to those of the southern shelf, where higher Ni, Cr, Pb, Cu and Zn are derived from the rock formations and mineralized zones. However, besides from the natural high background in the southern shelf, some anthropogenic influences are evident from EF values of Pb, Zn and Cu, and also from their high mobility in the semiisolated bay sediments Algan , 2004). Anthropogenic influences are found to be limited at the confluence of İstanbul Strait in the northern shelf. However, Algan et al. (2004) found that suspended sediments along the shallow parts of the northern shelf were enriched in Pb and Hg

and to a lesser degree in Zn, reflecting anthropogenic inputs from İstanbul Metropolitan and possibly from the Black Sea via the İstanbul Strait.

Industrial activities, municipal wastewater, agricultural chemicals, oil pollution and airborne particles have been the main reasons for the pollution that has affected primarily the estuaries and bays of the Marmara Sea and has ultimately spread along the shoreline and continental shelf that constitutes 50% of its total area (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 İstanbul 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).

2.2 The Most Polluted Areas in Marmara Sea

2.2.1 İzmit Bay

İzmit Bay, the most important semi-enclosed body of water on the east side of the Sea of Marmara, is about 50 km in length, 2–10 km in width and 310 km^2 in surface area. It consists of three district regions western, central and eastern) connected through narrow openings. The Bay is stratified throughout the year (Morkoc, 1996) having a lower layer swept by highly saline (37–38.5 ppt) water from the Mediterranean and a brackish (20–22 ppt) upper layer originating from the Black Sea.

The commissioning of more than 140 large industrial plants since 1965 and, in particular, the consequent urbanization of the coastal landscape have completely destroyed the previous serenity of İzmit Bay. Initially all solid and liquid wastes

were discharged directly into the Bay. Though major industrial effluents are now treated, there has yet to be treatment of domestic waste (Okay, 1996). The renewal capacity and water Exchange within İzmit Bay is insufficient for compensation and equilibration (Morkoc, 1996 and 2000). Eutrophication and deterioration of water quality have become serious problems.

Toxicity studies of the dominant wastes have led most factories contributing to the wastes to construct biological treatment plants during the past 10 years (Okay, 1996). Nevertheless, the treatments are still insufficient to eliminate toxicity (Okay, 1998). Sediments frequently contain higher concentrations of pollutants than are found in the water column and it is realised that, especially in turbulent waters, adsorption of pollutants by sediments scrubs the water column so that it appears relatively uncontaminated, whereas the sediment may become sufficiently polluted to disrupt natural biological communities (Bauloubassi and Saliot, 1991; Adams, 1992). Sediment bioassays that measure the toxic effects of contaminated sediments on the test organisms have been recently developed and a large variety of bioassays is becoming available. They provide information on the toxicity of contaminated sediments that can be neither derived from chemical analysis nor from ecological surveys (Long and Chapman, 1985).

İzmit Bay, located south of İstanbul on the southeast of the Marmara Sea, is the centre of burgeoning industrial development accompanied naturally by a rapid growth of population. The consequent, continuing danger of pollution has been minimised by ongoing monitoring and subsequent mitigation of the ecotoxicology of the bay, contributing to the wastes to construct biological treatment plants during the past 10 years. Nevertheless, the treatments are still insufficient to eliminate toxicity (Okay, 1998). Sediments frequently contain higher concentrations of pollutants than are found in the water column and it is realised that, especially in turbulent waters, adsorption of pollutants by sediments scrubs the water column so that it appears relatively uncontaminated , whereas the sediment may become sufficiently polluted to disrupt natural biological communities (Adam, 1992). Sediment bioassays that measure the toxic effects of contaminated sediments on the test organisms have been recently developed and a large variety of bioassays is becoming available. They provide information on the toxicity of contaminated sediments that can be neither derived from chemical analysis nor from ecological surveys (Long and Chapman,

1985). Testing procedures have included numerous techniques such as static tests, flow-through tests and elutriate tests (Burton and Scott, 1992; Schuytema, 1996; Mac, 1990; Malueg, 1984).

2.2.2 Kucukcekmece

Kucukcekmece Lagoon, located in the European part of İstanbul in Turkey, has typical spoon shaped topography. The surface area of the lake is approximately 17 km^2, and the water volume is 145 million m^3 at sea level. Untreated wastewaters, both domestic and industrial (metal, textile, plastic etc.), are routinely being discharged into the creeks of Kucukcekmece Lagoon (Ince et al., 2005)

Three stream systems feed the lake: Nakkasdere, Sazlıdere and Ispartakule (Demirci et al., 2001). The Sazlıdere stream output into the lake is much less due to the damming of this stream in 1995, which formed Sazlıdere Lake. The construction of a dam on this stream caused Kucukcekmece Lake to lose almost half of its watershed area. The lack of fresh water which was coming from the Sazlıdere stream did not affect Kucukcekmece's water level due to its connection with the Marmara Sea. However, its salinity has increased dramatically. Since the discharge of Nakkasdere stream was stopped and diverted offshore to the Marmara Sea by a new pipeline system in 2005, the lake has been fed by the Ispartakule stream from the northwest, surface runoff from the surrounding areas and by the sea water from the south.

The degradation of a reservoir happened in the case of Küçükçekmece reservoir in İstanbul, Turkey. Küçükçekmece reservoir was originally an estuary of the Marmara Sea. It was a clean natural habitat before the 1980's (Demirci et al., 2001). In the 1980s, İstanbul started to have a tremendous growth spurt. The development quickly transformed this previously sparsely populated semi-rural/exurban area of Küçükçekmece to an urbanized one. There were no regulations governing the protection of the watershed.

All types of development were allowed to build in this sensitive area. Industries directly dumped into streams their waste. High density residential development occurred on the edges of the reservoir. Raw sewage from these developments was allowed to be put into the streams and directly into the lake. The result was two fold: first, the greater İstanbul area lost a valuable reservoir; and second, the watershed

continued to be degraded by unregulated development so that it became an ecological disaster.

2.2.3 Tuzla

Tuzla is located on the Asian side, 60 km east of İstanbul, on the Sea of Marmara coast. Along the coast of Tuzla, there are agricultural lands and industrial plants (iron–steel plants, LPG plants, oil transfer docks, and cargo ship's ballasts water). Tuzla has undergone heavy environmental stress due to expansion of the İstanbul Metropolitan City in terms of industrial and human settlement through this area over the past 25 years. Many buildings were built on the marshy rim of the Tuzla despite heavy criticism from environmentalists. Due to heavy industrial and agricultural activities in the region, the bay has the polluted coastal waters of Turkey. Therefore, mainly untreated agricultural municipal and industrial wastes affect the lagoon direct or indirectly.

Moreover, on February 13th, 1997, a tanker named TPAO exploded in Tuzla shipyards located on the northeastern coast of the Sea of Marmara. During the fire, an estimated amount of 215 tons of oil was spilled in to the Aydınlık Bay and 250 ton oil burnt (Kazezyılmaz, 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).

2.2.4 Moda

Moda is located within the the Kadıköy district in İstanbul, Turkey on the Northerncoast of Marmara Sea. Moda is at the junction of Kurbağalıdere which used to be anhistorical old rivulet surrounded by a recreational area connecting to Marmara Sea and a sanctuary for fisheries and boathouses. Biogenic, diagenetic and anthropogenic components contribute to shelf sediments after their delivery to the marine environment. In coastal areas of densely populated large cities, the anthropogenic component of the sediments mostly exceeds the natural one. The surface sediments become a feeding source for biological life, a transporting agent for pollutants, and an ultimate sink for organic and inorganic settling matters (Algan, 2004).

Moda is relatively considered as a less polluted area in comparison to Tuzla. However, Moda has been densely exposed to domestic wastewater discharges since the end of 1970s and has gone under amendment 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.

Marine sediments, particularly those in coastal areas, are commonly polluted withpetroleum hydrocarbons (PHC) as a consequence of the extensive use of petroleumcompounds by mankind (Miralles et al., 2007). In aquatic sediments, the depth of oxygen penetration through diffusion is controlled mainly by the consumption of degradable organic matter within the sediment and in coastal ecosystems rarely exceeds more than a few millimeters (Jorgensen, 1983). With the exception of the most superficial layer, the bulk of organic matter-rich marine sediments contaminated by PHC are assumed to be anoxic (Canfield et al., 1993).

Consequently, microbial processes depending on the availability of free dissolved oxygen are constrained to the uppermost surface or, in deeper sediment layers, are coupled to irrigation and bioturbation processes of burrowing microorganisms (Freitag and Prosser, 2003). During the last decade, studies have shown the potential of coastal marine sediments for anaerobic hydrocarbon degradation under sulphate-reducing conditions (Coates, 1997; Townsend et al., 2003).

2.2.5 Haliç

The Golden Horn is a highly used water body in Turkey. It was a famous recreational area at the time of Ottomans, when it also served as the most important port of the region. The Golden Horn suffered fro heavy pollution due to extensive industrialization and rapid population growth in İstanbul in the twentieth century.

Estuaries are transitional zones between rivers and seas, and are important ecosystems typically rich in biodiversity. The Golden Horn Estuary supported thriving fisheries until the latter 20th century. It is a 7.5 km long, 200–900m wide hornshaped body of water that connects Alibeykoy and Kagithane Rivers to the Bosphorus Strait. Estuarine surface area covers 2.6 km2 and maximum depth is 36m

at the mouth, sloping to <1 m near tributary inflow. The shallow iner estuary, defined as the area north of the Valide Sultan/Old Galata Bridge, is more prone to anoxic conditions given that its depth abruptly slopes to <5m near the bridge.

The estuary receives saline water from the highly stratified, two-layered Strait of İstanbul. The upper 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 mid-estuary where maximum depth is 12–13 m. In addition to these layers, a 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).

The water column is a highly stratified product of the Mediterranean Sea (38 psu), Black Sea (17 psu), and freshwater urban runoff, precipitation, and a small fluvial contribution. Freshwater remains on the surface due to a greater rate of input (300000 m^3 of freshwater enter the estuary annually) than evaporative loss (Ozturk, 1998; Alpar et al., 2005). These three layers, separated by strong density gradients, effectively resist mixing of estuarine waters, and movement via tidal mixing or currents is negligible at <10 cm/s (Saydam, 1986; Basturk, 1988,). Low velocity surface winds and stable atmospheric conditions contribute to minimal air ventilation in the surrounding area. Thus, bothwater and air circulation are severely hampered in and around the Golden Horn, which has led to a local environment extremely prone to lasting pollution problems. These conditions are compounded by steep hills lacking foliage, the presence of stone quarries, and the absence of drainage systems, all encouraging substantial erosion and estuarine sedimentation (Aksit, 1977).

2.2.6 Gemlik

The Gemlik Bay emerges as a 31-km-long tectonic trough between two topographic heights, with an increasing width westward. It is 2–6 km wide in front of the Gemlik Town in the east of Tuzla point and 12–24 km in the west between Trilye and Bozburun (Armutlu Town). The length of its coasts along the step Samanlıdağ Mountains in the north, alluvial plains and deltas in the east and small hills along the southern coasts is about 76 km.

The regional winds, mainly controlled by the surrounding mountains, blow from northwest in winter and mainly northeast for the rest of the year. They play a

dominant role in the dynamics of this semi-enclosed sea. Gemlik Bay is open to the waves coming from the band between northwest and southwest (Ozhan and Abdalla, 1999). In winter, the dominant wave direction is from northwest with the significant wave heights less than 3 m. The dominant wave direction is from southwest in spring months with the significant wave height less than 2 m. The maximum hindcasted significant wave height for Gemlik wave is 3 m for the duration of wind data 1994–1998.

The maximum depth is 107 m in the middle of a small northwest-trending elliptical central trough which is a fault-controlled depositional area (Yaltırak and Alpar, 2002). The southern coasts of the Gemlik pull-apart basin are controlled by the central strand of the North Anatolian fault. Holocene alluvial fans in the east disturb the symmetry of this marine depression which is separated from the Marmara Sea by a sill with an average depth of 50 m in the west . With its 27600 km^2 drainage area and 158 m^3/s average water flows, the Karasu River is the most important geographic element in the region. It carries 0.5– 5.5 tons of suspended solids daily into the sea depending on the climatic conditions (Yıldız, 2003).

3. MICROBIAL DIVERSITY AND ACTIVITY IN MARINE SEDIMENTS

Oceans cover 70% of the Earth's surface, and between 5 and 10 billion tons of particulate organic matter is constantly sinking within them and accumulating as marine sediments. This then results in particulate organic matter being concentrated 10000–100000 fold higher in sediments, than in overlying seawaters. However, the vast majority of this organic matter is removed by near-surface microbial activity, but over geological time the remainder accumulates and results in the largest global reservoir of organic carbon ($15\,000 \times 10^{18}$ g C). Marine sediments have an average depth of around 500 m, but can be up to 10 km in depth with an average overlying water depth of 3800 m. Such water depths produce an average hydrostatic pressure of 380 bar and at maximum water depths 11 km, can be up to 1100 bar, which is increased further by the lithostatic pressure of the subsurface sediments. This high pressure combined with the very low near surface sediment temperatures (2 °C), decreasing porosity with increasing depth, and the fact that a large part of the seabed (95%) is at water depths where light intensity is too low to sustain photosynthesis led to the belief that the seabed was flat, biologically inactive and that seafloor sediments were insignificant in terms of their microbial activity (Jannasch et al., 1971; Jannasch and Wirsen, 1973). However, it has since been discovered that the seafloor environment is a dynamic geo- and biosphere that provides a diverse range of living conditions that are host to rich microbial communities (Jørgensen and Boetius, 2007).

In the 1980s, the first recognition of metabolically active microorganisms in deeply buried sediments arose from studies of pore-water chemistry and the use of radiotracers in deep sediment cores obtained by drilling down to 167 metres below seafloor (mbsf). Potential prokaryotic sulphate reduction and methanogenic activity were detected in buried sediments from three Deep Sea Drilling Project cruises sampling coastal North American sediments (Tarafa, 1987). However, positive results were patchy and data was inconclusive. It was early in the next decade that the first comprehensive depth profiles of microbial activity, total and viable

prokaryotic numbers and estimates of cultured biodiversity were published ,showing clear links between activity and the availability of organic carbon and terminal electron acceptors (Cragg et al., 1992). In 1994, a model was formulated for the logarithmic decline of total prokaryotic cell numbers with sediment depth, which has now become well accepted, and the first culture-independent molecular study to describe biodiversity in the deep marine subsurface was reported (Jørgensen et al., 2006).

During the 1990s geologists, geochemists and microbiologists began to realize the importance of the deep marine subsurface habitat, when it was estimated that the deep subseafloor biosphere comprised one-tenth to one-third of the Earth's total biomass and the majority (*c*. 65%) of the global prokaryotic biomass (Parkes et al., 1994; Whitman et al., 1998). Since then intact cells and intact membrane lipids have been consistently found in sediments <800 mbsf and recently in sediments down to 1626 mbsf. It was on this foundation that the first microbiology focused Ocean Drilling Program (ODP) cruise (Leg 201 in 2002) was planned with the aim to sample both highly productive sites in the Peru Margin and low activity sediments of the Equatorial Pacific and Peru Basin. ODP Leg 201 has now contributed more to our understanding of prokaryotes in the deep marine subsurface than any other ocean drilling expedition to date (Jørgensen et al., 2006).

The aim of this chapter is to provide a summary of current knowledge of prokaryotic activity and biodiversity in subsurface marine sediments. This habitat will be called as the deep subseafloor biosphere throughout the chapter.

3.1 Microbial Diversity

3.1.1 Methods to study prokaryotic diversity in subseafloor

As with other habitats, the diversity of prokaryotes found in the subseafloor biosphere by cultivation-independent molecular methods is much greater than obtained by standard laboratory culture. Furthermore, the taxa obtained by cultivation methods so far represent only a very small and unrepresentative subset of those revealed by molecular methodologies (D'Hondt et al., 2004). This is thought to be largely as a result of the observed low cultivability generally (<0.1%) of deep biosphere bacteria (Engelen et al., 2008). Thus, most biodiversity studies have used

molecular methods, involving direct extraction of nucleic acids from sediments and PCR amplification of 16S rRNA genes and/or functional genes indicative of key anaerobic sedimentary processes (e.g. methanogenesis mcrA) and sulphate reduction dsrA). These amplified genes are then analysed for diversity by either the construction of gene libraries or by more rapid profiling methods such as denaturing gradient gel electrophoresis (Muyzer, 1993).

However, molecular methodologies have been especially difficult to use in the study of deep marine sediments because of coextractable interfering substances like humic and fulvic acids, as well as the problem of low prokaryotic cell numbers and consequently low concentrations of extractable nucleic acids. Especially if we consider that the average prokaryotic cell contains <4 fg DNA per cell and subsurface sediments typically have 10^6–10^7 cells cm^{-3} (Parkes et al., 2000). Such low biomass samples have posed considerable problems for the reliable amplification of 16S rRNA genes from subsurfaceBacteria. Because low biomass samples are susceptible to PCR bias by random amplification and in addition most commercial thermostable DNA polymerases and other reagents used in PCR amplification and DNA extraction are often contaminated with small amounts of exogenous bacterial DNA (Webster et al., 2003). This has resulted in the development of a wide variety of specialist methods which include carefully optimized DNA extraction protocols, improved sensitivity using nested PCR and the use of very stringent anticontamination controls to ensure that sequences retrieved are representative of subsurface prokaryotes.

It is also important, before subsampling of sediment cores for microbiological analyses, to ensure that sediment samples are of sufficient quality and uncontaminated with drilling fluids, such as seawater, to warrant further time and effort for subsequent DNA extraction. Hence, it has now become a routine procedure for deep subsurface drilling to use a combination of a water soluble chemical tracer and fluorescent microspheres to mimic penetration of bacterial sized particles to monitor possible contamination from seawater and drilling disturbance (Lever et al., 2006).

3.1.2 Community composition of *Bacteria*

Analysis of 16S rRNA gene libraries has shown that there is substantial diversity of Bacteria in deep marine subsurface sediments, both within and between the phyla. The compositions of the bacterial populations found in a number of studies on the deep subseafloor biosphere are summarized in section. These data comprise 1909 clones from 34 16S rRNA gene libraries described in eight different studies, covering a wide range of sediment depths (1–503 mbsf). The 16S rRNA gene libraries are mainly from deep continental margin sites bordering the Pacific Ocean; unfortunately no comparable bacterial 16S rRNA gene libraries from other oceans have yet been obtained. It should also be noted that these libraries were constructed using different molecular approaches e.g. from different nucleic acid extraction protocols and/or using different domain-specific 16S rRNA gene PCR primers. Therefore, direct quantitative comparison of different clone libraries cannot be made due to the different methodological biases in each case, although qualitative assessments of prokaryotic diversity are possible.

It is clear that although the bacterial 16S rRNA gene libraries indicate a broadly diverse bacterial population, there is great variability in its composition. For example, although Proteobacteria averaged 37.4% of clones, and they almost completely dominated the Cascadia Margin ODP site 889/890 and volcanic ash sediment layers of the Sea of Okhotsk, they were almost absent from Cascadia Margin ODP sites 1244/5 and 1251 and from the Peru Margin ODP site 1230 (Inagaki et al., 2006). Overall, the most abundant subseafloor bacterial groups are *Gammaproteobacteria*, *Chloroflexi* and members of the candidate division *JS1* (Webster et al., 2004), which make up 18.9%, 17.3% and 26.1% of the clones, respectively, and so are the dominating groups of Bacteria in this deep sediment habitat. However, the *Alpha*-, *Beta*-, *Delta*- and *Epsilonproteobacteria* are also found in 3–50% of the libraries), but are not so common, averaging only 7.8%, 4.9%, 3.7% and 2.1% of the clones, respectively. Of the remaining 21.3% of clones the Planctomycetes are notably abundant (2–26%) at some Peru and Cascadia Margin sites and depths ,as are the novel groups NT-B2 and NT-B6, both originally found in the Nankai Forearc Basin. Although the Chloroflexi and the JS1 groups often co-occur, they can also dominate sites with one group being much less abundant. For example, Cascadia Margin sites 1224/5 and 1251, Peru Margin site

1230, Nankai Trough site 1173 and Sea of Okhotsk pelagic clay layers below 22 mbsf) are dominated by JS1, but Peru Margin sites 1227 and 1229, and the upper clay layer (7.5 mbsf) from the Sea of Okhotsk are dominated by Chloroflexi (Webster et al., 2006).

The exact taxonomic associations of the phylotypes in the 16S rRNA gene libraries show that many of the *Proteobacteria* and some of the Gram positive bacterial phylotypes are related to cultured species. Examples of their genera include *Ralstonia*, *Comamonas*, *Halomonas*, *Pseudomonas*, *Acinetobacter*, *Pedomicrobium* and *Pelobacter* from *Proteobacteria* and *Actinomces* and *Firmicutes* from the Gram-positive *Bacteria* (Webster et al., 2006). However, the phylotypes from other subsurface bacterial phyla are not closely related to known cultured species.

The extremely diverse but poorly characterized phylum *Chloroflexi* is a widespread group of bacteria found in a range of microbial communities, not only subsurface sediments but also hot springs, hydrothermal sediments, soils, wastewater and polluted sites (Teske et al., 2002). Currently *Chloroflexi* are divided into at least five subphyla with a small number of representative cultured species belonging to subphyla I, II and III. Deep subsurface clones mainly fall into subphyla II and IV, although this is likely to change as new sequences are retrieved, since subsurface sediments often contain a number of *Chloroflexi* with large sequence diversity belonging to different subphyla. For example, in one study (Inagaki et al., 2006). *Chloroflexi*-related sequences belonged to three subphyla and one deep branching unclassified group, with individual clones showing up to about 30% sequence difference. Conversely, candidate division *JS1* clones at present all belong to one monophyletic group with limited sequence variation, although greater sequence variation is suggested as a number of different *JS1* phylotypes can be recognized by *JS1*-targeted small 16S rRNA gene fragment PCR-DGGE. The environmental distribution of *JS1* does not seem to be as widespread as Chloroflexi with *JS1* phylotypes seemingly being restricted to anoxic sedimentary habitats (Webster et al., 2007). Within these two subsurface bacterial phyla there are no cultured members of *JS1* and few cultured members of the *Chloroflexi,* and so the physiology and ecological role of these bacteria in the deep subseafloor biosphere is difficult to predict. The closest matching cultured representative of the *Chloroflexi* to

many deep marine biosphere sequences is the H_2-dependent dehalorespiring bacterium *Dehalococcoides* ethenogenes with a sequence similarity of about 89%. For this reason and the ability of other cultured *Chloroflexi* to utilize/produce hydrogen (Madigan, 2009). It has been speculated that subsurface *Chloroflexi* may also consume hydrogen and anaerobically degrade recalcitrant carbon sources (Wilms et al., 2006).

Recently, investigations have considered the lithological and/or geochemical conditions that have led to the abundance of *Chloroflexi* and *JS1* in subsurface sediments. One study on the sediments from the Sea of Okhotsk found profound differences in the bacterial composition between different sediment lithologies. In this study pelagic clay layers were dominated by *Chloroflexi* and *JS1*, and volcanic ash layers were dominated by *Gammaproteobacteria*. However, clear indications of similar differences in community composition associated with sediment lithology are not apparent from other studies. It has also been stated that *JS1* dominate methane hydrate bearing sites and *Chloroflexi* dominate organic-rich subseafloor sediments (Inagaki et al., 2006). Although, we cannot substantiate either this or other overall differences in community composition due to lithology or geochemical composition with the data. In addition, *JS1* and *Chloroflexi* have also been detected in shallow subsurface sediments (Webster et al., 2007). In this environment it has been suggested that *JS1* dominate strictly anoxic organic-rich, but poor quality, recalcitrant carbon muddy sediments with low sulphate concentrations and small pore size, and are outcompeted by *Chloroflexi* in subsurface sandy sediments.

Sulphate reduction is an important geochemical activity in the deep marine subsurface (Parkes et al., 2000; D'Hondt et al., 2004) and therefore, sulphate-reducing bacteria (SRB) would be expected to be among the dominant physiological groups. However, only limited numbers of phylotypes belonging to *Deltaproteobacteria* have been isolated from this habitat, and most are only distantly related to known deltaproteobacterial SRB (Inagaki et al., 2006; Webster et al., 2006). It has been proposed by Parkes et al. (2005) from calculations of SRB numbers using specific sulphate reduction rates at ODP site 1229, that SRB are below detection in subsurface sediments by PCR using general bacterial 16S rRNA gene primers and are, therefore, absent from gene libraries.

One study reported the use of the specific functional gene dsrA (dissimilatory sulphite reductase) to target SRB in deep subsurface sediments of the Peru Margin ODP sites 1228 and 1229 (Webster et al., 2006). From this work it was suggested that very low numbers of uncultured SRB must be present at these sites, as only one sediment depth at site 1228 showed the presence of detectable dsrA phylotypes. Similarly, quantitative real-time PCR (Q-PCR) on deep sediments from the Peru Margin ODP site 1227 to determine the relative abundance of dsrAgenes (Schippers and Neretin, 2006), showed that DNA copy numbers of dsrA were about 10–3000-fold less than for the 16S rRNA genes ofBacteria or prokaryotes and had an irregular depth distribution.

The above studies may indicate that the subsurface SRB population is very small but active over geological timescales or, alternatively, that sulphate reduction is carried out by unknown sulphate-reducing prokaryotes with divergent functional genes that are not detected using current PCR methods. The data of (Mauclaire et al., 2004), in marked contrast to the above, demonstrated that SRB were a major population throughout sediments of Peru Margin site 1229 constituting 6–22% of all prokaryotic cells using catalysed reporter deposition-fluorescence in situ hybridisation (CARD-FISH) and at some depths 1, 3.5 and (110 mbsf) represented all detected bacterial cells. It should be noted that the SRB probes used in this study also target other *Bacteria* including *Chloroflexi*, which are a major component of the bacterial community within Peru Margin sediments.

3.1.3 Community composition of *Archaea*

Reliable identification of new subsurface archaeal 16S rRNA gene clones is essential for understanding the distribution of these microorganisms in the deep subseafloor biosphere, especially because subsurface archaeal populations comprise almost exclusively of uncultured lineages. Unfortunately, many archaeal sequences in molecular diversity studies of marine sediments are often left unidentified or assigned to groupings used in the specific publication, resulting in some confusion (Heijs et al., 2007). This section describes the composition of the archaeal populations from 47 16S rRNA gene libraries from 11 published studies of the deep marine biosphere from the Pacific Ocean and one recent study of deep subsurface sediments from the Atlantic Ocean. The *Crenarchaeota* (six groups) dominated the Archaea, with 73.4% of the clones, while only 24.5% of the clones belonged to

the Euryarchaeota (eight groups). The most abundant archaeal groupings overall were the crenarchaeotal groups, Miscellaneous Crenarchaeotic Group (MCG) and Marine Benthic Group B, comprising 33% and 26.3% of clones, respectively. The next most abundant groups were the Marine Group 1 (8.4% *Crenarchaeota*), the South African Gold Mine Groups (SAGMEG) 1 and 2 (7.6% *Euryarchaeota*) and the thermophilic *Euryarchaeota* (7.6%); none of the other groupings accounted for more than about 4.5% overall. These groups have also been found in other sedimentary, aquatic and terrestrial environments, and so are not confined to the deep marine biosphere. The only *Archaea* phylotypes closely related to cultured species were the euryarchaeotal methanogens, thermophiles and hyperthermophiles. However, these only accounted for <8% of the clones and were not a major component of the phylotypes; thus, as with the *Bacteria*, most of the *Archaea* were from uncultured lineages.

Most investigations of archaeal diversity in the marine subsurface have used PCR amplification of extracted sediment DNA, although (Sørensen and Teske, 2006) used extracted RNA and reverse transcription (RT)-PCR to make clone libraries. These authors found mainly MBG-B Archaea in the sulphate–methane transition zone (SMTZ) of ODP Peru Margin site 1227 and mainly MCG elsewhere in the core, but no methanogens despite high methane in the sediment at depth. However, it is unlikely that the MBG-B alone are indicative of the SMTZ, as another Peru Margin study (Biddle et al., 2006) found that four of these zones ODP sites (1227, 1229 and 1230) were dominated by mixtures of MBG-B and MCG Archaea. Because both of these studies used RT-PCR it is likely that these two groups are the most activeArchaea in deep subsurface sediment SMTZs. Another example of MBG-B archaeal signatures in different geochemical zones comes from Inagaki et al. (2006) who proposed that methane hydrate containing sediments are dominated by DSAG (MBG-B).

Deep subseafloor biosphere archaeal clone libraries are seemingly less diverse than was seen in those for Bacteria, with 24/47 libraries containing only one or two of the 14 main archaeal taxa found in the deep marine biosphere. This could be either an artefact, perhaps due to preferential PCR amplification of specific groups of related genes, or it may reflect real variation between sites and depths. Nevertheless, there is still a substantial diversity of *Archaea* phylotypes in the samples investigated. For

example, the MCG sequences alone show very large sequence variation up to (20%) in most of the 27 studies that found this group, and similar results have been reported for other major groups of subsurface *Archaea*. Such large phylogenetic diversity and widespread distribution of MCG Archaea supports the theory that these anaerobes are a metabolically diverse group of microorganisms that utilize complex carbon substrates (Teske and Sørensen, 2008).

The community composition of methanogens has been specifically investigated because of their inferred importance in biogeochemical processes and in the production of biogenic methane, which might contribute to the methane hydrate reserves in the deep marine biosphere (D'Hond, 2004; Inagaki et al., 2006). However, only small numbers of methanogen clones have been detected directly in general archaeal 16S rRNA gene libraries. For example, at Peru Margin ODP site 1229 only 1/103 clones was thought to be from a methanogen. This accounts for the very low average methanogen content. A small number (6/348 prokaryotic phylotypes investigated) of the *Thermococcales* and hyperthemophiles from the Peru and Cascadia Margins were also methanogens belonging to the *Methanosarcinales*, *Methanobacteriales* and *Methanococcales*, some of which were closely related to cultured methanogens (e.g. *Methanococcus aeolicus*, *Methanoculleus palmaeoli*). These results suggest that, like SRB, known methanogens constitute a very small proportion of the prokaryotic community in the deep sub-seafloor biosphere. Targeted studies using taxon-specific 16S rRNA and mcrA (α-methyl coenzyme-M reductase) gene primers have successfully amplified related sequences to *Methanosarcinamazei* and *Methanosarcinabarkeri* and *Methanobacteraboriphilus* (Parkes et al., 2005). Overall, these results suggest limited diversity of methanogens in the deep marine biosphere.

3.1.4 Abundance of *Bacteria* vs. *Archaea*

The majority of molecular studies of prokaryotic diversity in the deep subseafloor biosphere have tended to use conventional or nested PCR with primers specific for either *Bacteria* or *Archaea* on extracted DNA, making it difficult to compare directly the magnitude of these two domains within subsurface sediments. Recently, quantitative molecular techniques have been used to address this question, including FISH, CARD-FISH, and Q-PCR. Bacteria were enumerated by CARD-FISH in depth profiles at two Peru Margin sites (1227, 1230) and two equatorial Pacific sites

(1225, 1226) and were found to comprise a high proportion of the total cell count acridine orange direct count,(AODC), but *Archaea* could not be detected (Schippers et al., 2005). These authors also used Q-PCR on extracted DNA to estimate the 16S rRNA gene copy numbers of total prokaryotes, *Bacteria* and *Archaea,* and found that *Archaea* were 10–1000-fold less abundant than *Bacteria,* and that prokaryotic and bacterial 16S rRNA gene copy numbers were indistinguishable (Schippers and Neretin, 2006). Similar results were also found using Q-PCR. These results suggested that Bacteria dominated the prokaryotes in subsurface sediments, with Archaea contributing only a small proportion. In addition, a high proportion of the cells detected by AODC were also shown to be viable. Conversely, one study of samples from ODP Leg 201 sites 1227, 1229 and 1230 using FISH, showed that Archaea were, on average, 82% of the total prokaryotic community. Evidence for these high subsurface archaeal numbers was further supported by high abundance of archaeal intact polar lipids (Biddle et al., 2006). These conflicting results show that more work on the refinement of molecular methodologies is needed before the true relative abundance of *Bacteria* and *Archaea* can be determined in the deep marine biosphere.

3.1.5 Cultured biodiversity

Studies involving laboratory cultivation have been used to examine the diversity of culturable prokaryotes in the deep marine biosphere since the late 1980s, and many of these have been aimed at isolating pure cultures of typical deep sediment organisms. Some of the earliest studies used most probable number (MPN) techniques to enrich and count viable anaerobic heterotrophs, ammonifiers, acetogens, sulphate reducers, methanogens and aerobic ammonifiers in the Peru Margin (ODP sites 680, 681) and Japan Sea (ODP site 798) (Cragg et al., 1992). Generally the profiles of anaerobes followed those of the relevant geochemical and activity profiles, with highest counts near the surface and elevated counts coinciding with deeper peaks of activity. However, the aerobic ammonifier counts were 100–106-fold higher than anaerobic counts with little decrease with depth, suggesting that these heterotrophs were probably facultative anaerobes surviving in this anoxic habitat.

These studies have led to isolations of a few well-described novel prokaryotes with some physiological characteristics that suit the isolates for growth in the deep

biosphere. These pure cultures include the barophilic SRB *Desulfovibrio* profundus from the Japan Sea, the methanogen Methanoculleus submarinus from the Nankai Trough and thermophilic Firmicutes in the genus *Thermosediminibacter* (Lee , 2005) from Peru Margin sediments (sites 1227, 1228, 1230). Despite these prokaryotes having similar physiologies to *Bacteria* and *Archaea* represented in deep sediment clone libraries, only *M. submarinus* has any close sequence similarity (97%) to a clone from the subsurface (Inagaki et al., 2006).

Other studies have also isolated pure cultures from the marine subsurface that are less well described. Toffin et al. (2004) screened anaerobic enrichments designed to grow heterotrophs, acetogens and SRB from the Nankai Trough (ODP site 1173) and found mainly *Firmicutes, Gamma-* and *Deltaproteobacteria* and *Spirochaetes,* but only two were isolated as pure cultures and these were closely related to existing *Marinilactibacillus* and *Acetobacterium* species. Another study obtained 168 isolates from three Equatorial Pacific sites and four Peru Margin sites from Leg 201 belonging to six distinct lineages. These isolates included *Alpha-*, *Gamma-*, *Delta-Proteobacteria, Firmicutes, Actinobacteria* and *Bacteroidetes,* with the most abundant being close relatives of the genera *Rhizobium, Bacillus* and *Vibrio*. Another investigation of Leg 201 samples used aerobic heterotroph and anaerobic methanogen enrichments (Biddle et al., 2006). The methanogen enrichments failed to yield any methanogens, even after using the same techniques used previously to isolate *M. submarinus,* but the aerobic heterotroph enrichments gave six pure cultures of *Gammaproteobacteria* closely related (98–99% 16S rRNA gene sequence similarity) to the genera *Photobacterium, Halomonas, Shewanella* and *Vibrio*. As described earlier, *Gammaproteobacteria* are common in 16S rRNA gene libraries from deep subsurface sediments, and the genera isolated are often closely related facultative anaerobes with physiologies that suit them to life in the deep subseafloor biosphere. *Gammaproteobacteria* belonging to *Halomonas* and *Marinobacter* have also been isolated from the Sea of Okhotsk (Inagaki et al., 2006) and Peru Margin ODP sites 1228 and 1229 (Biddle et al., 2006). Interestingly, the isolation of facultative anaerobic genera, both aerobically and anaerobically, supports the earlier findings that many of the heterotrophic *Bacteria* in the deep subsurface may be facultative anaerobes.

However, despite a large number of the cultured prokaryotes isolated from deep subsurface sediments falling within higher taxa commonly obtained by cultivation-independent approaches (e.g. *Gammaproteobacteria*), they are very rarely representative of the abundant largely uncultivated phylotypes. This low culturability of subsurface prokaryotes clearly indicates that a lot more work is needed to develop new strategies for the isolation of deep biosphere prokaryotes, including at elevated pressure, which will undoubtedly reveal novel modes of metabolism.

3.1.6 Comparison of deep and coastal sediment communities

Recently, several studies have examined prokaryotic biodiversity in both marine surface sediments and the shallow subsurface, mainly by molecular methods, at depths down to around 6 mbsf, allowing comparison of these two adjacent habitats. Bacteria in cores from the Skagerrak, German Wadden Sea tidal-flats and the Benguela Upwelling System show that, overall, the upper layers are dominated byGamma- and Deltaproteobacteria, and the deeper layers by Chloroflexi and candidate division JS1 (Webster et al., 2007). It is also striking that in these sediments more SRB phylotypes were identified by 16S rRNA and dsrA genes, with similar results being found for methanogens using 16S rRNA and mcrA genes (Wilms et al., 2006; Parkes et al., 2007). In another study on the South China Sea the *Bacteria* were dominated by *Gammaproteobacteria* and the *Archaea*, similar to the deep subsurface, were dominated by MBG-B, MCG and uncultured *Euryarchaeota*. Overall, these studies show a transition from the near surface layers, where methanogens and SRB are easily detectable, to a deeper subsurface population below where sulphate reduction and methanogenesis still occurs, but the responsible prokaryotes are difficult to detect.

3.2 Prokaryotic Activity

3.2.1 Methods to study prokaryotic activity in subseafloor

Activity estimates have predominantly been undertaken anaerobically using replicate syringe mini-cores subsampled from intact whole round cores and injected evenly with radiotracer substrates (Parkes et al., 1995). Radiolabelled reaction products are then assessed after incubation at in situ temperature for varying incubation times, designed to give measurable product yields. For example, estimates of sulphate

reduction use $35SO_4^{2-}$ as a radiotracer and measure 35S in the sulphide produced, methanogenesis measures the transformation of 14C-labelled substrates, such as 14C-acetate or CO_2 to CH_4, and heterotrophic growth is estimated by the incorporation of 3H-thymidine into DNA. It should be noted that these activities are always referred to as potential rates because it is impossible to measure activity without disturbance of the deep biosphere. However, these potential rates are believed to be reliable (Parkes et al., 2000), because they: 1) correlate with geochemical and sedimentological changes; 2) correspond with stable isotopic values of reactants/products; 3) compare well with long-term sulphate removal rates in laboratory experiments incubated anaerobically at 4 °C. The details of these methods have been described and discussed extensively (Kallmeyer e al., 2004).

3.2.2 Depth profiles of activity

Rates of many activities in the deep biosphere are usually highest in the upper layers and decrease thereafter, especially at low activity sites which have low oceanic carbon flux into the sediment, and so AODC counts are low (D'Hondt et al., 2004; Parkes et al., 2005). Decreasing activity with depth occurs for sulphate reduction at Blake Ridge in the Western North Atlantic and in the Equatorial Pacific (Parkes et al., 2005). Similar profiles are seen for thymidine incorporation in the Equatorial Pacific ODP Leg 201 sites 1225 and the Japan Sea ODP Leg 128 site 798 (Cragg et al., 1992). Rates for methanogenesis normally peak just below the region of highest sulphate reduction activity due to reduced competition from SRB, this can be seen for $H_2 : CO_2$ methanogenesis in Equatorial Pacific sites 1225, the Peru Margin site 1229 (Parkes et al., 2005).

Rates of sulphate reduction and methanogenesis in upper layers of the subsurface marine biosphere are comparable to or below those generally found in anaerobic near surface coastal sediments (Parkes et al., 2000). However, sulphate reduction rates usually decrease to zero much more rapidly than methanogenesis because sulphate reduction consumes its own electron acceptor SO_4^{2-}, while CO_2, H_2 and acetate, common substrates for methanogenesis, can be replenished by heterotrophic activity, generation at elevated temperatures, and/or other processes occurring in deeper layers (D'Hondt et al., 2004; Biddle et al., 2006).

Against this background of decreasing activity with depth there are many cases when specific environmental conditions lead to enhanced activity in deeper layers and some examples of this are outlined below. Firstly, anaerobic oxidation of methane (AOM) often results when methane accumulates in the presence of low concentrations of sulphate. For example, AOM occurs in Blake Ridge subsurface sediments, in the region where methane accumulates above the top of the gas hydrate stability zone (GHSZ) and sulphate reduction is very low. Secondly, AOM is also stimulated deeper at the base of/and below the GHSZ, where both acetate and $H_2 : CO_2$ based methanogensis increase and methane accumulates. Similar changes have also been observed in the Cascadia Margin, again at the base of a methane hydrate zone. The accumulation of acetate at Blake Ridge also resulted in enhanced acetate turnover to CO_2 below the methane hydrate stability zone. Thirdly, stimulated AOM has also been inferred from geochemical and microbiological changes at the upper and lower SMTZ in the Peru Margin (Parkes et al., 2005). The lower SMTZ at site 1229 is due to the deep flow of brine bringing sulphate into the deep methane containing sediments, stimulating AOM linked with sulphate reduction (Parkes et al., 2005). Stimulation of activity by deep sulphate flux has also been observed at a number of other subseafloor sites (Engelen et al., 2008). Lastly, it is clear that thymidine incorporation is often maintained at significant rates deep into subsurface sediments, as observed in the equatorial Pacific, Peru Basin, Blake Ridge, and the Woodlark Basin (Wellsbury et al., 2002). It should be noted that these observed increases in subsurface activity are often reflected in local peaks of prokaryotic numbers estimated by AODC and MPN counts. For example, AODC values increased about 30-fold immediately below the GHSZ in Blake Ridge, and about 6- and 60-fold at the upper and lower SMTZs, respectively, in the Peru Margin site 1229.

3.3 Relating Biodiversity and Activity

It is important to take into account geochemical profiles when attempting to relate microbial biodiversity and activity. Before extensive molecular biodiversity data were available it was necessary to draw conclusions about prokaryotic composition from directly obtained activity and geochemical profiles (Wellsbury et al., 2000) or from activity estimates derived from geochemical data using geochemical flux

models (D'Hondt et al., 2004). For example, when SO_4^{2-} concentrations were decreasing rapidly with depth and sulphate reduction was high, SRB were thought to be dominant. Similarly, when methane accumulated and methanogenesis was high, methanogens were assumed to be dominant. Furthermore, when MnO_2 or NO_3^- reduction was predicted to be at its highest rate using flux models, manganese reducers and nitrate reducing heterotrophs would be expected. These views might not have always been clearly enunciated but the implications were often clear, especially when supported by evidence from MPN counts, cultured isolates and later from inferred physiologies of the phylotypes present in clone libraries.

However, recently many contradictions have become apparent and need to be explained. For example, methanogenesis, sulphate reduction and AOM are key processes when either measured directly or inferred from geochemical profiles. However, phylotypes with high sequence similarity to known SRB and methanogens are rare in 16S rRNA gene libraries, as are the archaeal members (ANME) of communities believed to be active in AOM (Orphan et al., 2001). Nevertheless, some methanogen and ANME phylotypes were enriched in a sealed ODP borehole at the Cascadia Margin site 892 and recently ANME sequences were found to be the dominant archaeal group in some deep sediments from the Newfoundland Margin. This apparent contradiction in the majority of studies presents a dilemma unless these groups are affecting the geochemical profiles by the activity of small-sized communities that do not dominate the total prokaryotic population assessed by AODC. In which case what type of physiologies do the majority of the population exhibit? Unfortunately, the clone libraries do not help to answer this question because nearly all of the archaeal and most of the bacterial phylotypes found are only distantly related to cultured species. Furthermore, it is well known from the 16S rRNA gene phylogenetic trees of cultured species that taxonomic closeness is not a reliable predictor of physiology (Gray and Head, 2001).

To resolve this dilemma scientists have used other approaches to link biodiversity with activity and associated geochemical profiles. One study concentrating on SMTZs at three ODP Leg 201 sites, used carbon flow reconstructions based on the δ13C content of individual cells identified using FISH, intact polar membrane lipids and sedimentary organic carbon. These authors concluded it was organic carbon compounds, other than methane, that provided the major carbon source for the

substantial populations of *Archaea* (MBG-B and MCG) in sediments at SMTZs. However, (Sørensen and Teske, 2006) suggested that as both MBG-B and MCG were most active in the SMTZ, based on RT-PCR amplified rRNA, then they must benefit directly or indirectly from AOM, although, as noted above, suggested that these *Archaea* do not incorporate methane-derived carbon. This idea is supported by the incorporation of 13C-acetate by stable isotope probing SIP; into the DNA of MCG *Archaea* from tidal sediment slurries incubated under anaerobic conditions. It is also possible that the abundant uncultivated groups of *Bacteria* are heterotrophic, as many isolates and phylotypes of *Gammaproteobacteria* from deep sediments are closely related to cultured heterotrophs. SIP has also shown that members of the abundant bacterial candidate division JS1 are able to utilize acetate and glucose or glucose metabolites (Webster, 2006). Furthermore, profiles of thymidine incorporation can show that heterotrophic prokaryotic growth is either maintained at significant rates (Wellsbury et al., 2000) or is maximal in subsurface sediments. Although, organic matter in deep sediments becomes recalcitrant during burial, this can be counteracted by thermal activation of organic matter at depth, providing deep substrates for prokaryotic growth (Parkes et al., 2007). Taken together these results strongly suggest that heterotrophy should be an important process in the deep marine biosphere.

Multivariate statistical methods have been used by small numbers of environmental microbiologists for many years to explore and simplify complex patterns in intricate datasets, and have mainly concentrated on cluster analysis and ordination techniques such as principal component analysis (PCA) and multidimensional scaling (MDS) (Ramette, 2007). Recently, cluster analysis has been used to investigate DGGE profiles of sediment communities down to about 5 mbsf (Wilms, 2006; Webster, 2007). These studies have effectively shown that community composition is different in distinct sediment depth horizons using 16S rRNA gene profiles and in different geochemical layers (e.g. at sulphate and methane peaks) with mcrA and dsrA genes (Wilms, 2006).

A more extensive approach was reported using cluster analysis, PCA, MDS, correlation and multiple regression of DGGE profiles of 16S rRNA genes, amplified from extracted DNA, to interrelate community composition with activity and geochemistry at two ODP Leg 201 Peru Margin sites (1228 and 1229). PCA was

shown to be the best method to explore the link between the diversity profiles, activity and geochemical variables. The first three PCA components accounted for more of the variability in the DGGE profiles of *Bacteria*, *Archaea*, *Euryarchaeota* and *JS1* 16S rRNA genes at site (1229 72–79%) than for site (1228 54–72%). Furthermore, multiple regression of the first three components with all activity and geochemical variables gave good explanations of the components at both sites 1229, 31–95% explanation with the best regression equations, two to five variables; 1228, 8–100%, (two to five variables). This clearly showed that community diversity, with all 16S rRNA gene primers used, was strongly related to both the geochemical environment and the prokaryotic activity in these deep subsurface sediments. Furthermore, it suggests that genomic DNA approaches, like directly extracted rRNA methods are also able to identify active and dynamic prokaryotic populations in the marine subsurface.

3.4 Challenges of Research on Microbial Ecology of Marine Sediments

Research over the last 20 years has increased understanding of the microbiology of the deep marine biosphere enormously. It is likely that the tools currently available and impending advances in molecular approaches will enable understanding of the way this complex habitat works to be increased even more over the next decade.

Direct quantitative evidence of the numbers and types of prokaryotes carrying out different functions need to be addressed urgently. Techniques such as CARD-FISH and Q-PCR need to be extended and applied to a range of functional genes and group specific bacterial and archaeal 16S rRNA genes and importantly ground truthed by comparison with geochemical and activity data. However, research over several years has shown that currently used PCR primers are biased against many prokaryotes present in the environment, so design of new more effective PCR primers is going to be important (Teske and Sørensen, 2008). Recent evidence from the deep sea, using 16S rRNA gene tag 454 pyrosequencing, points to a much greater diversity than demonstrated previously using PCR and cloning (Sogin et al., 2006). Thus, the application of high throughput, cloning-independent sequencing techniques that can produce very large numbers of ≥400-bp sequence reads will be especially important, and will enable large metagenomic libraries to be analysed, avoiding limitations associated with PCR (Biddle et al., 2007; Wommack et al., 2008). In

addition, inherent problems of extracting low concentrations of nucleic acids from low biomass deep subseafloor samples will also be enhanced using methods such as whole genome amplification (Abulencia et al., 2006) to improve DNA yields. Sequencing of metagenomic libraries from the deep subsurface will produce very large amounts of information so enhancements in the software used to analyse this data and computer storage to allow its distribution will also be vital (Hugenholtz, 2007). Such techniques are difficult to apply equally to all groups of prokaryotes and so initially concentrating studies on organic-rich sites with high AODC may give the best chance of success. To allow a full understanding of the deep subseafloor biosphere more multidisciplinary studies will be needed. International cooperation will be essential so that data from biodiversity, activity and geochemistry studies can be combined and the complex datasets analysed by appropriate multivariate analyses. Once all these techniques are working well together it will be essential to concentrate studies with a finer spatial resolution on two or three contrasting deep subsurface sites >1000 mbsf (Roussel et al., 2008) in another microbiology focused deep marine biosphere expedition.

4. ANAEROBIC DEGRADATION OF PETROLEUM HYDROCARBONS

4.1 Anaerobic Aliphatic Hydrocarbon Degradation

4.1.1 Aliphatic hydrocarbon degrading microorganisms

Although reports on the possible microbial utilisation of hydrocarbons in the absence of oxygen have regularly appeared since the 1940s, the biodegradation of such compounds under strictly anaerobic conditions was only clearly demonstrated in the late 1980s (Spormann and Widdel, 2000; Widdel and Rabus, 2001). Since then, numerous studies of the anaerobic degradation of saturated and unsaturated (aliphatic and also aromatic) hydrocarbons linked to the consumption of different electron acceptors have appeared and several bacterial strains capable of degrading *n*-alkanes and *n*-alkenes under denitrifying or sulfate reducing conditions have been isolated (Grossi et al., 2008) (Table 4.1). In a pioneering study involving two of these isolates sulfate reducing (strains Hxd3 and Pnd3) grown on C_{16}–C_{17} *n*-alkanes, observed a direct influence of the carbon chain length of the alkane substrate on the main cellular fatty acids of the strains. The relationship was, however, distinct for each strain, which led the authors to hypothesize that alkanes were activated differently in each strain. Later, by combining a study of the fatty acid composition of pure strains with the use of stable isotope-labelled hydrocarbon substrates, two distinct mechanisms for anaerobic *n*-alkane oxidation were delineated. Recently, labelling experiments have also allowed the anaerobic metabolism of alk-1-enes to be clarified, as this had remained speculative for a long time.

4.1.2 Anaerobic *n*-alkane metabolism

The two mechanisms of anaerobic *n*-alkane degradation described to date involve unprecedented biochemical reactions that differ completely from those employed in aerobic hydrocarbon metabolism. The first involves activation at the subterminal carbon of the alkane and addition to a molecule of fumarate Figure 4.1A). In the second, the alkane is carboxylated at C-3 (Figure 4.1A). Both pathways, which can occur simultaneously in mixed sulfate reducing communities, contradict a

hypothetical mechanism presented in previous literature involving the dehydrogenation of alkanes to the corresponding alk-1-enes (Aeckersberg et al., 1998). It is noteworthy that both addition to fumarate and carboxylation were also observed previously as initial reactions during the anaerobic degradation of some aromatic hydrocarbons (Spormann and Widdel, 2001).

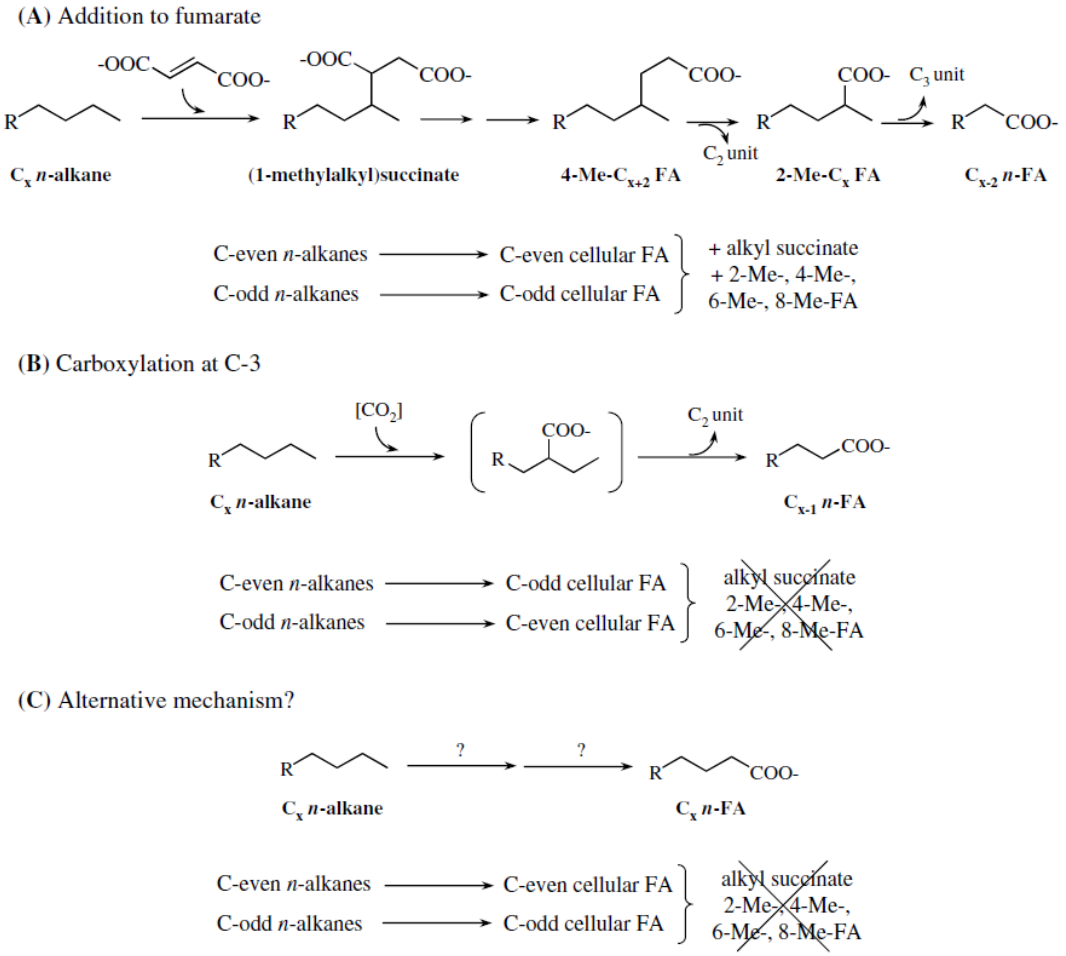


Figure 4.1: Mechanisms of *n*-alkane activation (Grossi et al., 2008)

4.1.2.1 Addition of *n*-alkane to fumarate

During growth of a sulfate reducing bacterium (strain AK-01) on deuteriated and ^{13}C-labelled alkanes, So and Young (1999) observed formation of labelled 2-Me-, 4-Me- and 6-Me-branched fatty acids FAs with chain lengths correlating with those of the alkanes. Careful mass spectrometric examination indicated i) preservation of the original carbon chain of the alkane in these branched FAs and ii) that the methyl group was systematically the original terminal carbon of the alkane substrate. This provided the first evidence for a possible addition of exogenous carbon at the subterminal position C-2 of alkanes in a pure anaerobic bacterium. Subsequently,

(Rabus et al., 2001) demonstrated that a denitrifying bacterium (strain HxN1) activates (labelled) *n*-hexane through homolytic C–H bond cleavage at C-2 and addition to fumarate, affording (1-methylpentyl) succinate as the first stable product. Based on the identification of several other (labelled) *n*-hexane metabolites, such as 4-Me-C_8 and 2-Me-C_6 FAs, the same authors subsequently proposed the first pathway for anaerobic bacterial degradation of an *n*-alkane (Wilkes et al., 2002). By analogy with the formation of benzyl succinate during anaerobic toluene oxidation e.g. (Heider, 2007), the addition of the alkane to fumarate is most likely a radical reaction (catalyzed by a glycyl radical enzyme) in which the abstracted hydrogen from the hydrocarbon substrate is retained. A carbon skeleton rearrangement of the resulting alkyl succinate as the (CoA-thioester), followed by a decarboxylation step, then yields a 4-Me-branched FA. This can be further degraded by β-oxidation via a 2-Me-branched FA and a linear FA. The linear fatty acid possesses two carbon atoms fewer then the original *n*-alkane (Figure 4.1A). Evidence for a similar degradation pathway was described later for the marine sulfate reducing bacteria *Desulfatibacillum aliphaticivorans* (Cravo-Laureau, 2005) and strain AK-01 (Callaghan et al., 2006) grown on longer chain *n*-alkanes (Table 4.1). The study with *D. aliphaticivorans* also allowed completion of the aforementioned degradation scheme, with some anabolic reactions explaining the formation of unsaturated 6-Me- and 8-Me-branched FAs from the alkane substrates.

Thus, addition of fumarate at C-2 of *n*-alkanes with carbon chains C_6 generally results in i) the preferential formation of cellular even and odd numbered FAs from even and odd numbered *n*-alkane substrates respectively (Figure 4.1A) and ii) the formation of some specific metabolites such as 1-methylalkyl succinates and 2-Me- and 4-Me-branched FAs. However, a mesophilic sulfate reducing bacterium (strain BuS5), able to grow only on propane and *n*-butane, was recently demonstrated to activate propane at both the secondary (C-2) and primary (C-1) carbon atoms, whereas activation of *n*-butane proceeded exclusively at C-2 (Kniemeyer et al., 2007). For such short chain *n*-alkanes, the relationship between the carbon chain length of the alkane substrate and that of the main cellular FAs (Figure 4.1A) is less obvious. Moreover, the addition of fumarate to an alkane can no longer be systematically envisaged at the subterminal C-2 carbon; this deserves further attention in future studies.

Table 4.1: Anaerobic alkane degrading Bacteria (Grossi et al., 2008)

Species/strain	*n*-Alkanes metabolized	Mechanism involved[a]	*n*-Alk-1-enes metabolized	Mechanism involved
Denitrifying bacteria				
Pseudomonas sp. strain All	None	–	C_{17}	n.d[b]
Azoarcus sp. strain HxN1	C_6–C_8	Fumarate	n.d[b]	–
Marinobacter sp. strains BC[c]	C_{18}	n.d[b]	n.d[b]	–
Pseudomonas balearica strain BerOc6	C_{15}–C_{18}	Unknown	n.d[b]	–
Strain OcN1	C_8–C_{12}	n.d[b]	n.d[b]	–
Strain HdN1	C_{14}–C_{20}	n.d[b]	n.d[b]	–
Sulfate-reducing bacteria				
Desulfatibacillum aliphaticivorans CV2803	C_{13}–C_{18}	Fumarate	C_7–C_{23}	DB oxidation + carbon addition[d]
Desulfatibacillum alkenivorans PF2803	None	–	C_8–C_{23}	n.d[b]
Desulfoglaeba alkanexedens ALDC	C_6–C_{12}	n.d[b]	n.d[b]	–
Desulfatiferula olefinivorans LM2801	None	–	C_{14}–C_{23}	n.d[b]
Strain Hxd3 Aeckersberg et al. (1991), So et al. (2003)	C_{12}–C_{20}		Carboxylation	C_{14}, C_{16}, C_{17}
Strain TD3	C_6–C_{16}	n.d[b]	n.d[b]	–
Strain Pnd3	C_{14}–C_{17}	Fumarate[e]	C_{16}	n.d[b]
Strain AK-01	C_{13}–C_{18}	Fumarate	C_{15}, C_{16}	n.d[b]
Strain Lake	C_6–C_{10}	n.d[b]	n.d[b]	–
Strain BuS5	C_3–C_4	Fumarate[f]	n.d[b]	–
Syntrophic bacteria				
Clones B1-B3[g]	C_{16}	n.d[b]	n.d[b]	–

[a] See Figure 4.1.
[b] Not documented.
[c] Three distinct strains were isolated.
[d] DB, double bond.
[e] Likely mechanism although not fully demonstrated.
[f] This strain activates *n*-butane exclusively at C-2 but *n*-propane at both C-2 and C-1.
[g] Bacteria from enrichment culture that were phylogenetically identified but not isolated.

4.1.2.2 Carboxylation with inorganic carbon

Based on experiments with the first well described, alkane-degrading, sulfate reducer strain Hxd3; (Aeckersberg et al., 1991) and labelled substrates, (So et al., 2003) proposed the existence of a second pathway for anaerobic *n*-alkane oxidation. Strain

Hxd3 was shown to attack the alkane by i) carboxylation with inorganic carbon (bicarbonate), most likely at C-3, and ii) removal of two subterminal carbon atoms from the alkane chain terminus. This mechanism leads to even numbered and odd numbered FAs from odd and even numbered *n*-alkanes, respectively (Figure 4.1B). Although this relationship between the carbon chain length of *n*-alkane substrate and the main cellular FAs of strain Hxd3 was unambiguously demonstrated, the hypothetical 2-Et-branched FA intermediate, which should result from the addition of inorganic carbon at C-3 of the alkane, has never been detected. More studies are therefore necessary to confirm the proposed reaction.

4.1.2.3 Indication for a possible alternative pathway

A denitrifying bacterium *Pseudomonas balearica* strain BerOc6, isolated recently from a brackish lagoon ,is able to grow on C_{15}–C_{18} *n*-alkanes. As for addition to fumarate in other strains (Figure 4.1A), the total cellular FAs of strain BerOc6 are predominantly even carbon numbered when it is grown on an even carbon numbered alkane, and predominantly odd numbered when grown on an odd numbered alkane. However, metabolites characteristic of activation of the alkane via addition to fumarate have never been observed with strain BerOc6. Thus, it may be that this strain activates *n*-alkanes by a new yet to be determined) mechanism, although no specific metabolite that might give a clue to such a third pathway has been identified.

It should be emphasized that the individual anaerobic bacterial strains currently available utilize only a distinct, narrow range of *n*-alkanes (Table 4.1). Unfortunately, their alkane metabolism was not systematically studied, although phylogenetic differences exist between some of the strains. It may be that the alkyl succinate synthase involved in the activation of some alkanes exhibits promiscuous activity (relaxed substrate specificity) towards longer saturated hydrocarbons that do not support bacterial growth (Wilkes et al., 2003). This highlights the need for further investigation of the (co-metabolic) reactions involved in the degradation of long chain (> C_{20}) alkanes by pure and mixed cultures. The isolation of anaerobic bacteria (eventually) able to use long chain (> C_{20}) alkanes as growth substrates also requires additional study.

4.1.3 Metabolites as biomarkers for aliphatic hydrocarbon degradation

Cultures grown on pure substrates have allowed the identification of a series of metabolites unique to the anaerobic degradation of hydrocarbons. Even if most of these transformation products are supposed to be transient metabolites, their high specificity may allow them to be used for assessing the biodegradation of hydrocarbons in anoxic environments. The potential usefulness of anaerobic metabolites of *n*-alkanes to trace anaerobic petroleum oxidation has been demonstrated both under laboratory and natural conditions (Gieg and Suflita, 2002). However, the use of metabolic biomarkers as a means of monitoring the in situ anaerobic oxidation of alkanes and/or alkenes is still rather limited.

The 1-methylalkyl succinates constitute biomarkers that are highly specific, albeit short-lived, in the anaerobic oxidation of *n*-alkanes. Monomethyl-branched FAs are more common and are found in plant, animal and microbial lipids. Bacterial monomethyl-branched FAs have substitution generally at the *iso*- (*n*−1), *anteiso*- (*n*−2) and C-10 positions, although branching at C-9, C-11, C-12 and C-13 is occasionally reported (Bergé and Barnathan, 2005). To our knowledge, unsaturated 2-Me- and/or 4-Me-branched FAs have only been detected in a marine sponge and in a few disparate animal tissues such as the uropygia (preen) gland of birds and rumen fats (Christie, 2007). Some animals also produce among many other branched (FAs) saturated 6-Me- and 2-Et- and 4-Et-branched acids, but such compounds have apparently never been reported in any aquatic organism. Monomethyl-branched FAs with branching at C-2, C-4 and C-6 and their eventual (unsaturated homologues) as well as 2-Et- and 4-Et-branched FAs, formed during the anaerobic oxidation of *n*-alkanes and/or *n*-alk-1-enes, can thus reasonably be envisaged as potential markers of this degradation process. The search for such structures in the lipid record of appropriate sedimentary settings may be worthwhile.

4.2 Anaerobic Aromatic Hydrocarbon Degradation

4.2.1 Aromatic hydrocarbon degrading microorganisms

Aromatic hydrocarbons enter the global environment through human activities such as crude oil spillage, fossil fuel combustion and gasoline leakage as well as natural inputs like forest fire smoke and natural petroleum seepage. These hydrocarbons

comprise simple aromatics like benzene and toluene as well as polycyclic aromatic hydrocarbons (PAHs) from naphthalene to pyrenes, as well as myriad alkyl-substituted isomers. Annually, large inputs of such compounds impact both aerobic and anaerobic environments such as aquifers, surface freshwater bodies, soils, and terrestrial and marine sediments. Whereas aerobic biodegradation of both aromatic and saturated hydrocarbons has been well known and studied for many years, the documentation of anaerobic degradation is relatively recent within the last two decades) and is constantly generating new insights. This activity is widespread, having been reported under nitrate-, iron-, manganese- and sulfate-reducing conditions as well as methanogenic conditions. Initial observations were limited to enrichment cultures and in situ sediments or ground waters, but a few pure cultures capable of anaerobic degradation of aromatic hydrocarbons have now been isolated and characterized. Such cultures have initiated the elucidation of degradative pathways, intermediates, and genes encoding key enzymes. At the same time, pathways and metabolites have been inferred from complementary studies of mixed populations using analytical chemical methods to demonstrate that laboratory results are relevant in situ. It is now clear that some key enzymatic steps in anaerobic hydrocarbon biodegradation involve novel biochemistry and versatile microbiota. The list of organisms reported to degrade specific hydrocarbons was given in Table 4.2 and can be found in Rabus (2005) and Bonin (2004).

4.2.2 Substrates, pathways and terminal electron acceptors

Initial activation of hydrocarbons is crucial for anaerobic biodegradation, and four general enzymatic reactions are recognized: 1) addition of fumarate, catalyzed by a glycyl radical enzyme to yield aromatic-substituted succinates; 2) methylation of unsubstituted aromatics; 3) hydroxylation of an alkyl substituent via a dehydrogenase, and 4) direct carboxylation, which may actually represent a combination of reaction 2) followed by reaction 1) (Foght, 2008). These activation reactions feed into pathways that result in ring saturation, oxidation and/or ring cleavage reactions, producing central metabolites such as benzoyl-coA that are eventually incorporated into biomass or completely oxidized.

4.2.2.1 Monoaromatics

BTEX components benzene, toluene, ethylbenzene and xylene isomers) are the best-studied substrates of anaerobic biodegradation. Interest in these compounds results from the fact that they comprise a significant proportion of conventional fuels such as gasoline, are the most water-soluble of the aromatic hydrocarbons and (therefore most likely to be mobile in situ and biodegradable) and also are reported to have acute toxicity as well as long-term potential carcinogenic effects (Snyder, 2000). The major pathways for anaerobic-BTEX degradation are presented in abbreviated form in Figures 4.2.

Benzene

Although benzene can be biodegraded aerobically, its thermodynamic stability dictates that enzymatic attacks very difficult to initiate under anaerobic conditions, commonly resulting in its persistence in anaerobic cultures (Langenhoff et al., 1996). Its relatively high water solubility and known toxicity combined with apparent chemical and biological stability in situ make it a priority pollutant despite its relatively low proportion in many petroleum contaminants. The occurrence and rate of biodegradation appears to be more site-specific for benzene than other BTEX components and is subject to inhibition by those co-contaminants; degradation is usually slow, incomplete and subject to long lag times. Anaerobic benzene-degrading enrichment cultures are not very common and pure isolates even more rare (Kasai , 2006). It is ironic, therefore, that the first anaerobic cultures unequivocally demonstrating aromatic hydrocarbon degradation were benzene degraders; methanogenic cultures from Dunja laboratory in the late 1980s led the way in documenting anaerobic BTEX degradation and in proposing pathway intermediates. In subsequent studies with enrichments, Edwards (1992) measured 90% mineralization of 14 Cbenzene to 14 CO_2, and although the actual TEA was not established in that study, sulfate was a likely candidate.

Similar levels of mineralization were later documented under methanogenic, iron-, sulfate-, and manganese-reducing conditions (Foght, 2008). Lovley (1994) found that benzene degradation in sediments incubated in microcosms under iron-reducing conditions was enhanced in the presence of iron chelators such as nitrilotriacetic acid, humic acids, EDTA, etc. that increase bioavailability of that electron acceptor. However, no pure cultures of iron-reducing, benzene-degrading bacteria have yet

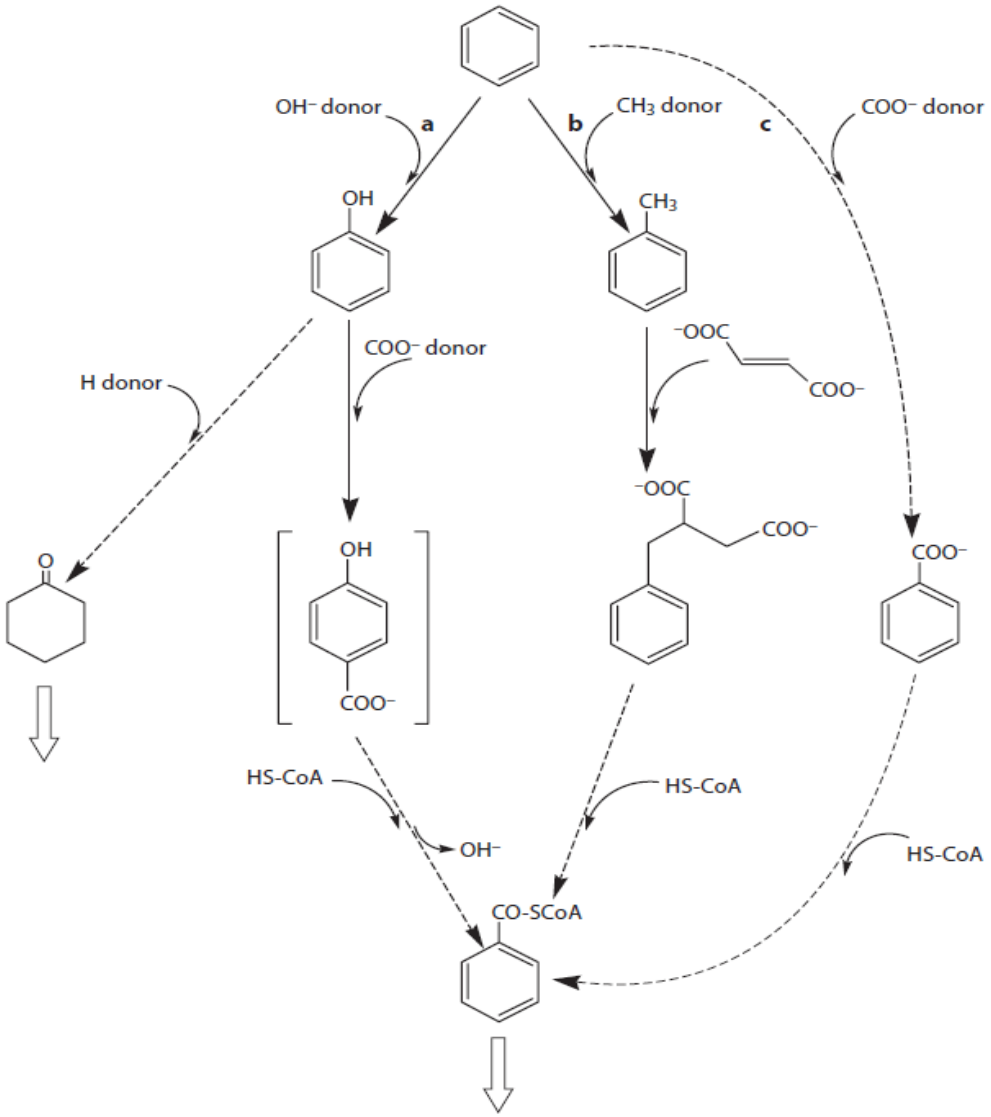
been isolated. Phelps et al. (1998) noted that, despite long-term (3- year) enrichment of a sulfate-reducing culture with benzene as the sole carbon source, pure benzene-degrading strains resisted isolation and the consortium remained quite complex, comprising at least 12 distinct phylotypes.

Table 4.2: Anaerobic aromatic hydrocarbon degrading bacteria (Cao, 2009)

Pollutants	Bacteria	Comments
Benzene	*Geobacter* spp.	Oxidize benzene in Fe(II)-reducing conditions
	Desulfobacterium spp.	Mineralize benzene into CO_2 in 5 days
Toluene	*G. metallireducens*	First pure culture for toluene oxidation
	Azoarcus spp.	Facultative toluene-oxidizing nitrate-reducers
	Thauera spp.	
Ethylbenzene	*Thauera*-related	Denitrifying bacteria completely mineralize methylbenzene
Xylene	*D. acetonicum*-related	Mineralizes *o*- and *m*-xylene
	Desulfosarcina variabilis-related	
PAHs	*Acidovorax*	Complete degradation for naphthalene and partial for three to five ring PAHs
	Bordetella	
	Pseudomonas	
	Sphingomonas	
	Variovorax	
	P. stutzeri	Mineralizes 7–20% naphthalene
	Vibrio pelagius-related	
PCBs	*Desulfitobacterium dehalogenans*	Dehalogenates flanking Cl of OH-PCBs
PCP	*Desulfitobacterium frappieri*	90–99% PCP removal forming 3-CP
	Desulfitobacterium halogenans	Dechlorinates at *o*- and *m*-position
	Desulfitobacterium chlororespirans	
	Desulfomnile tiedje	
Chlorinated pesticides	*Clostridium* sp.	Degrades DDT as the sole C source; degrades other chlorinated pesticides
	Aerobacter aerogenes	Degrades DDT
	Klebsiella pneumoniae	
	Nocardia vulgaris	
	Dehalospirilum multivorans	Preferentially dechlorinates technical toxaphene

Early studies reported that benzene was not degraded under denitrifying conditions. Anderson and Lovley (2000) found that addition of nitrate to a benzenedegrading consortium completely inhibited benzene degradation. However, Burland and

Edwards (1999) conclusively linked benzene biodegradation to nitrate reduction in enrichment cultures, with 92–95% of 14 C-benzene recovered as 14 CO_2 and the remainder presumably incorporated into biomass.



a: Hydroxylation to form phenol, cyclohexanone, or hydroxybenzoate and benzoyl-CoA.
b: Alkylation to form toluene and fumarate addition to form benzylsuccinate and benzoyl- CoA
c: Carboxylation to form benzoate and benzoyl- CoA.

Figure 4.2: Anaerobic degradation pathways proposed for benzene

Benzene oxidation in that study also seemed to be more dependent on nitrate reduction to nitrite than complete reduction to dinitrogen gas, and benzene utilization corresponded to a lower cell yield than predicted by the free energy of the reaction. Similarly, Ulrich and Edwards (2003) found that benzene degradation under nitrate-reducing conditions was less efficient than predicted by the theoretical free energy available. The intermittent reports of benzene oxidation under denitrifying conditions

appear to be site-specific and unpredictable; its degradation under these conditions may rely on the presence of specific consortium members capable of initiating attack on benzene rather than those metabolizing the oxidized intermedi-ates, since pathway intermediates like benzoate are readily degraded with all TEAs. Caldwell et al. (1999) suggested that these sporadic observations are due to an indirect effect of nitrate-dependent oxidation of Fe(II) to Fe(III) in some anaerobic environments containing both nitrate and Fe(II), generating a more suitable TEA for benzene degradation. Recently, two pure cultures identified as *Dechloromonas* spp. strains RCB and JJ were isolated through their ability to mineralize benzene concomitant with nitrate reduction (Coates et al., 2001). Strain RCB, now identified as *Dechloromonas aromatica* RCB, can also oxidize benzene using chlorate or oxygen as TEAs. Although its genome has now been fully sequenced, no genes or enzymes have yet been assigned to initiating benzene-degrading activity in this or any other organism.

The pathway for anaerobic benzene degradation is the subject of considerable debate, with five mechanisms having been proposed for initiating anaerobic attack on benzene. Two of these pathways have little support in the literature: although the most common mode of initial activation of methylsubstituted aromatics is fumarate addition, it may be that the large activation energy required to remove hydrogen from the benzene ring precludes this mechanism for initiating benzene metabolism; and a proposed pathway involving initial attack by ring saturation has garnered little supporting evidence (Coates et al., 2002). Three plausible pathways are presented in Figure 4.2: pathway A – hydroxylation, producing phenol with subsequent carboxylation to the postulated intermediate *para* –hydroxybenzoate or ring reduction to yield cyclohexanone; pathway B – methylation, producing toluene followed by fumarate addition to produce benzylsuccinate; and pathway C – carboxylation, producing benzoate ,although this pathway may represent the sum of several enzymatic steps carried out by a consortium. All three pathways converge on benzoyl-coA, a central intermediate in anaerobic oxidation of aromatic compounds, which is eventually oxidized to acetyl-CoA and carbon dioxide (Harwood et al., 1998). Evidence has been reported for all three modes of attack, with phenol, benzoate and cyclohexanone consistently detected in cultures incubated under various anaerobic conditions (Ulrich et al., 2005). The source of the hydroxyl group

of the phenol intermediate has been debated: H_2O was proposed as the donor in methanogenic cultures but recent evidence points to a hydroxyl free radical as the donor used by *D. aromatica* RCB (Chakraborty and Coates, 2005).

Likewise, the methyl group donor in pathway B (Figure 4.2) is unknown but may be S-adenosylmethionine, methyltetrahydrofolate or cobalamin. Activation of benzene by methylation may be analogous to the methylation of naphthalene proposed by Safinowski and Meckenstock (2006). Progress towards resolving the biochemistry of benzene degradation using pure cultures has been hampered in part by the fact that it is difficult to isolate hydrocarbon- degraders from methanogenic consortia because of the essential syntrophic interactions in such microbial communities. Whereas pure strains growing under nitrate- or sulfate-reducing conditions can in theory and in practice be enriched through utilization of benzene as the sole carbon source, in methanogenic consortia the organism initiating attack may not derive sufficient energy from benzene without a syntrophic partner to make the reaction thermodynamically favorable. That is, it may be energetically difficult to purify the benzene-oxidizing strain from a consortium using benzene itself. Instead of isolation, Ulrich and Edwards (2003) used physiological and nucleic acid techniques to characterize benzene-degrading methanogenic and denitrifying consortia. In the methanogenic culture they detected 16S rRNA gene sequences affiliated with uncultivated bacteria (predominantly the sulfate-reducing genus *Desulfosporosinus* and delta-proteobacterial sequence) and uncultivated archaea (predominantly acetoclastic methanogens), whereas the nitrate-reducing consortium was dominated by bacterial sequences affiliated with denitrifying beta-proteobacteria similar to *Azoarcus* and *Dechloromonas* , with no archaea cloned. Certainly, more research is required to establish the prevalence of pathways and intermediates involved in anaerobic benzene biodegradation, and to identify the genes and enzymes responsible for this environmentally important activity. Another factor that has not been adequately addressed in the literature is the toxicity of benzene (or other BTEX components) to particular species, and how solvent sensitivity or tolerance could shift the species composition of mixed populations, thus influencing net biodegradative activity.

Toluene

In sharp contrast to the recalcitrance of benzene, toluene is the most readily degraded of the BTEX compounds under all TEA conditions (Foght, 2008; Cao, 2009). Its relative susceptibility to anaerobic degradation has yielded more pure isolates than any other aromatic substrate, and concomitantly the best resolution of catabolic pathways, enzymes and genes. Early studies demonstrated toluene degradation under denitrifying conditions using inocula from a variety of sources. Subsequently, toluene degradation was documented under manganese-, iron- and sulfate-reducing as well as methanogenic conditions. Toluene was readily degraded within 1–2 months under all redox conditions in these cultures; conversely, benzene was recalcitrant over the duration of the experiment (up to 525 days). Similarly, toluene was preferentially degraded among BTEX components by oil sands enrichment cultures incubated under methanogenic conditions. There also have been intermittent reports of BTEX degradation under iron-reducing conditions in sediments incubated in the laboratory, as well as respiration with humic acids and the model quinone anthraquinone-2,6-disulfonate (AQDS) (Cervante, 2001). Addition of sub-stoichiometric amounts of humic acids enhanced toluene degradation linked to iron reduction in those studies, indicating that humus may be an adjunct electron shuttle and/or acceptor in situ facilitating aromatic hydrocarbon biodegradation.

Elucidating the pathways used by toluene-degrading consortia is complicated by the syntrophic interactions assumed to be integral to anaerobic degradation, particularly methanogenesis. For example, a model toluene-degrading methanogenic consortium is postulated to comprise four physiological groups: a syntroph that initiates toluene oxidation, a homoacetogen that can reversibly oxidize acetate coupled to hydrogen generation, an acetoclastic methanogen and a hydrogenotrophic methanogen. Ficker et al., (1999) used molecular biological tools to characterize a methanogenic consortium maintained by transfer with toluene for 10 years. They detected and phylogenetically identified two dominant archaeal and two bacterial representatives: a bacterial sequence with no significant homology to any known genus the presumptive toluene-degrader, *Desulfotomaculum*-like sulfate-reducer, presumptive homoacetogen, *Methanosaeta* type acetoclastic archaeon, and *Methanospirillum* type hydrogenotrophic methanogen. Interestingly, there is general metabolic similarity between these results and those reported by Ulrich and Edwards (2003) from a

benzene-degrading methanogenic consortium. Presumably, initial hydrocarbon attack generates partially oxidized products such as fatty acids or alcohols that become available for fermentative and syntrophic species, eventually being made available to methanogens as acetate and/or H_2 + CO_2. As metagenomic analysis techniques become more accessible, biochemical modeling of consortia operating under different TEA conditions should enable discernment of catabolic pathways and interactions between metabolic types. In the meantime, the published catabolic pathways described below have been based on a relatively limited suite of pure isolates representing a few genera, and a handful of characterized enzymes.

Several pure denitrifying, toluene-degrading cultures have been isolated, including *Thauera aromatica* strains T1 and K172, *Thauera* sp. DNT-1, *Azoarcus tolulyticus* strains Tol4 and ToN1, *Azoarcus* spp. from diverse environmental sources, and *Dechloromonas aromatica* strains RCB and JJ (Coates, 2001). A few pure strains that couple iron reduction to toluene degradation have been isolated, such as *Geobacter metallireducens* GS-15 and *Geobacter grbiciae* strains TACP-2T and TACP-5 (Coates, 2001). Sulfate-reducing toluenedegrading isolates include *Desulfobacula toluolica* Tol2 and oXyS1 as well as delta-proteobacterial strains TRM1 (Beller et al., 1996).

Toluene is the model for anaerobic biodegradation via the fumarate addition reaction mediated by benzylsuccinate synthase, a novel reaction elucidated for alkyl aromatics and also saturated hydrocarbons (Widdel and Rabus, 2001). Attack appears to be universally initiated by addition of the double bond of fumarate to the methyl group of toluene, yielding benzylsuccinate (Leutwein and Heider, 1999). This intermediate is then further oxidized to *E*-phenylitaconate, and eventually benzoyl-coA, a central aromatic metabolite that subsequently undergoes ring reduction, cleavage and oxidation to CO_2 (Harwood et al., 1998).

Xylenes

Although all three xylene isomers appear to be biodegraded via the fumarate addition pathway analogous to toluene, they have different susceptibilities to anaerobic biodegradation. Early conflicting reports of anaerobic xylene isomer degradation under nitrate-reducing conditions found that both (*p* – and *m* - but not *o*-) xylene were degraded, whereas Edwards (1992) observed degradation of *p*- and *o*-xylene

under sulfate-reducing conditions after significant lag times. Early work by Zeyer , (1986) reported 80% mineralization of ring-labelled 14 C- *m* -xylene added to denitrifying river sediments. Several subsequent studies found that *m*–xylene is the most readily degraded isomer in mixed cultures and in some cases its presence inhibits concomitant *o*- and *p*-xylene degradation (Meckenstock et al., 2004c; Morasch, 2004). *o*- Xylene can be degraded by certain cultures, and recently the first iron-reducing enrichment cultures were shown to oxidize *o*-xylene (Jahn et al., 2005) and *p*-xylene (Botton and Parsons, 2006). Although *p* -xylene is often reported to be recalcitrant (Rabus and Widdel, 1995), it was degraded by mixed denitrifying enrichment cultures that were very selective, being unable to grow on benzene, ethylbenzene or *o*-xylene (Haner et al., 1995), and at least one sulfate-reducing enrichment culture recently was shown to degrade *p* -xylene via fumarate addition (Morasch and Meckenstock, 2005). It is noteworthy that the latter culture was enriched in the presence of Amberlite-XAD7 ion-exchange resin (Morasch et al., 2001), keeping the concentration of substrate and possibly inhibitory metabolites low during the initial stages of enrichment. This method of acclimatizing the inoculum to a recalcitrant substrate may prove useful for isolation of additional cultures.

Several pure cultures have been shown to utilize *m*-xylene for growth under nitrate- (Morasch et al., 2004; Rabus and Widdel, 1995) and sulfate-reducing conditions, whereas only two pure cultures have been reported to mineralize *o*–xylene anaerobically, both under sulfate-reducing conditions: strain oXyS1 (Harms et al., 1999) and *Desulfotomaculum* sp. strain OX39 (Morasch et al., 2004). Such isolates often show narrow substrate specificity, with mXyS1 degrading only *meta* - and oXyS1 preferring *ortho* -substituted aromatics (Harms et al., 1999). No pure cultures utilizing *p*-xylene for growth have yet been reported. Such selectivity could account for the variable and site-specific patterns of xylene isomer degradation observed in situ and in enrichment cultures. It is also possible that *o*- and *p*-xylenes are being co-metabolized (via toluene) to deadend products in some enrichment cultures rather than supporting growth (Beller, 2000).

Homologs corresponding to toluene fumarate addition metabolites have been detected in cultures incubated with xylenes. For example, 4-methylbenzylsuccinate and 4-methylphenylitaconic acid were extracted from an enrichment culture incubated with *p* –xylene (Morasch and Meckenstock, 2005), and the expected 2-

methylbenzylsuccinate homolog was detected in cultures co-metabolizing *o* -xylene (Beller and Spormann, 1997).

Ethylbenzene

Ethylbenzene degradation has been reported in situ and in mixed cultures under sulfate-reducing and nitrate-reducing conditions (Reinhard et al., 1997). However, its recalcitrance varies with site and TEA: Villatoro-Monzon (2003) observed that ethylbenzene degraded most rapidly of all BTEX compounds under iron-reducing conditions (threefold more rapidly than benzene) whereas Botton and Parsons (2006) failed to detect oxidation of ethylbenzene under iron-reducing conditions even though the same microcosms were able to degrade benzene, toluene and xylenes. Jahn (2005) also reported ethylbenzene mineralization in two enrichment cultures incubated under iron-reducing conditions, but only after relatively long lag times, whereas Siddique (2007) observed recalcitrance of ethylbenzene under methanogenic conditions although toluene and xylenes were degraded. Only five pure cultures utilizing ethylbenzene have been reported to date, of which four are nitrate reducers and one is a sulfate reducer, and all of which appear to have fairly restricted hydrocarbon substrate ranges.

The first to be isolated were denitrifiers: strains EbN1 and PbN1 were originally affiliated with the genus *Thauera* (Rabus and Widdel, 1995) but strain EbN1 was recently fully sequenced and placed in the genus *Azoarcus* although referred to as *Aromatoleum aromaticum* by Kloer (2006). *Thauera* sp. PbN1 also has been renamed *Azoarcus* sp. strain PbN1 (Kniemeyer and Heider, 2001). *Azoarcus* sp. strain EB1 cannot grow on BTEX components other than ethylbenzene, whereas the denitrifying bacterium *D. Aromatica* RCB is capable of degrading toluene as well as ethylbenzene (Chakraborty, 2005). The ethylbenzenemineralizing marine delta-proteobacterium strain EbS7 is the only sulfate-reducing pure culture reported to date, and its aromatic hydrocarbon substrate range also appears to be limited to ethylbenzene (Kniemeyer et al., 2003).

Two distinct pathways for anaerobic biodegradation of ethylbenzene have been documented. One is the classic fumarate addition reaction described for toluene and xylenes that produces the ethyl homolog of benzylsuccinate, observed under sulfate-reducing conditions (Kniemeyer et al., 2003). An alternate pathway observed in

denitrifying organisms such as *Azoarcus* sp. strain EB1 is accomplished by hydroxylating the benzylic carbon to form 1-phenylethanol, analogous to benzene attack, with water donating the oxygen atom of the hydroxyl group (Ball et al., 1996). Subsequent dehydrogenation produces acetophenone and eventually the central aromatic metabolite benzoylcoA. The initial enzyme in this pathway, ethylbenzene dehydrogenase, has been characterized (Johnson et al., 2001; Kniemeyer and Heider, 2001). *Azoarcus* sp. EbN1, which degrades toluene by fumarate addition and ethylbenzene by hydroxylation, appears to use both pathways independently for initiating anaerobic oxidation of the two substrates (Heider et al., 1999; Rabus, 2005).

4.2.2.2 Polycyclic aromatic hydrocarbons (PAHs)

To date, only a limited number of PAHs definitively have been shown to biodegrade anaerobically in situ or in microcosms containing soil, river sediment, aquifer material or marine sediment using nitrate, iron, manganese, sulfate or carbon dioxide as TEAs (Foght, 2008). These include the unsubstituted PAHs naphthalene and phenanthrene as well as some alkyl-PAHs. However, as noted by Safinowski (2006), detailed information on anaerobic degradation of PAHs is scarce, and there is debate whether PAHs having three or more aromatic rings can support growth or whether they are only partially oxidized through co-metabolism with growth substrates. Cultivation of denitrifying or sulfate-reducing PAH-degrading pure cultures and enrichments has proven difficult, and only within the last decade have a few pure cultures (Galushko et al., 1999) and stable mixed cultures been obtained to enable the study of anaerobic degradation pathways. For most PAHs only circumstantial evidence for anaerobic degradation currently exists and it is not clear whether substrate losses represent partial oxidation by individual organisms to generate transient or dead-end metabolites, or complete oxidation by sequential activity of consortium members, or a combination of these processes.

Unsubstituted PAHs: naphthalene, phenanthrene and other PAHs

Mihelcic and Luthy (1988) were the first to report loss of naphthalene from soil enrichments incubated with nitrate as the electron acceptor. In later studies, Bregnard (1996) confirmed anaerobic naphthalene degradation by measuring mineralization of ^{14}C-naphthalene under nitrate-reducing conditions in microcosms containing

material from a chronically diesel-fuel contaminated aquifer. Coates et al., (1996) detected 14 C-naphthalene mineralization by sulfate-reducing marine harbour sediments and, interestingly, found that amendment of these sediments with insoluble iron oxides did not enhance contaminant degradation over the indigenous sulfate levels ,possibly due to the lower proportion of iron-reducers in the sediments compared with sulfate reducers. Subsequently, Bedessem (1997) observed mineralization of 14 C-naphthalene incubated with nine different enrichment cultures under sulfate-reducing conditions, although the lag times and degradation rates varied widely among enrichments. Nitrate-, manganese- and sulfate-reducing as well as methanogenic conditions also resulted in partial degradation of naphthalene in sediment columns (Langenhoff et al., 1996). In these studies using continuous upflow columns infused with a mixture of benzene, toluene and naphthalene, the naphthalene was not measurably degraded under methanogenic or iron-reducing conditions and was only partially degraded with manganese. Slightly more degradation was observed with nitrate or sulfate as TEA, with approximately 60% of 14 C-naphthalene mineralized under sulfate-reducing conditions. Addition of benzoate as a co-substrate accelerated naphthalene removal under nitrate-reducing conditions, either by providing an additional electron donor for naphthalene ring reduction, by inducing appropriate genes, or by providing an additional carbon source for growth (Langenhoff et al., 1996). Rockne et al. (2000) found that naphthalene and phenanthrene could be degraded by a denitrifying enrichment culture originally derived from creosote-contaminated soil. Radiolabel recoveries detected incorporation of carbon from 14 C-hydrocarbon into biomass and production of 14 CO_2 confirmed metabolism (Rockne and Strand, 2001). However, the degree of mineralization varied considerably between substrates, with only partial mineralization of naphthalene versus 96% of phenanthrene; likewise, the proportion of PAHcarbon incorporated into biomass varied between substrates, with naphthalene contributing the most to biomass- carbon. Anderson and Lovley (1999) were the first to demonstrate naphthalene degradation in aquifers dominated by iron reduction, but they found that this activity also was site-specific.

Only a few pure naphthalene-degrading cultures have been isolated, including the sulfate-reducing marine delta-proteobacterium strain NaphS2 (Galushko et al., 1999) and three nitrate-reducing, naphthalene-degrading strains isolated from a marine

sediment enrichment culture of which two were characterized: strain NAP-3-1 phylogenetically related to *Pseudomonas stutzeri* coupled partial naphthalene mineralization to complete denitrification whereas strain NAP-4-1 related to *Vibrio pelagius* reduced nitrate to nitrite. Both cultures produced a small amount of 14 CO_2 from labeled naphthalene initially (7–22%), with significant label (30–50%) incorporated into biomass.

Two pathways have been proposed for the initial anaerobic attack on naphthalene: carboxylation versus methylation followed by fumarate addition (Safinowski and Meckenstock, 2006). The two pathways converge at 2- naphthoic acid, and thereafter the aromatic rings are sequentially reduced, starting with the unsubstituted ring, to produce octahydronaphthoic acid (before ring cleavage or production of the dead-end product decahydronaphthoate. Under sulfidogenic conditions a third pathway not shown), initiated via hydroxylation of naphthalene to naphthol, has been proposed but not verified (Bedessem et al, 1997). Evidence for the carboxylation pathway comes from incubation of naphthalene-utilizing sediment cultures with 13 C-bicarbonate under sulfidogenic conditions. Zhang and Young (1997) observed that the label was incorporated into the carboxylic group of 2-naphthoic acid and inferred that the initial activation reactionwas direct carboxylation of naphthalene, a conclusion echoed by Meckenstock (2000).

However, because the 2-naphthoic acid was detected in a mixed culture it possibly represents the product of sequential enzymatic steps rather than primary attack. Subsequently, Safinowski and Meckenstock (2006) examined the sulfate-reducing culture N47, enriched from a tar-oil-contaminated aquifer. The culture was observed by phase microscopy to comprise a single cell morphology but was not considered to be axenic, and a demonstrably pure isolate could not be obtained by dilution. The culture could utilize naphthalene or 2-methylnaphthalene as the sole carbon and energy source individually but not simultaneously. When parallel cultures were incubated with deuterated naphthalene or 2-methylnaphthalene, the same fumarate addition pathway metabolites predicted by the 2-methylnaphthalene pathway were detected in both cultures, suggesting that naphthalene is first methylated to form 2-methylnaphthalene then undergoes fumarate addition. This is analogous to the proposed pathway for methylation of benzene to toluene before further metabolism. Interestingly, these two reported modes of attack may actually represent a single

pathway, since the methyl group may derive from bicarbonate via a reverse CO-dehydrogenase pathway (Safinowski and Meckenstock, 2006) eventually producing 2-naphthoic acid, consistent with Zhang and Young's observations but involving additional enzymatic steps.

Despite the possible analogy to the benzene methylation pathway, Coates (1997) found that sulfidogenic benzene-degrading sediments were unable to mineralize naphthalene, suggesting that the microbial populations were different and that initial enzymes for attack of these unsubstituted aromatics are substrate-specific. Phenanthrene degradation has been observed under nitrate- and sulfate-reducing conditions in marine sediments (Tang et al., 2005). By analogy to naphthalene the initial attack on phenanthrene may be carboxylation as proposed by Zhang and Young (1997), or methylation as proposed by Safinowski et al. (2006) with subsequent fumarate addition and oxidation to phenanthroic acid. Also, because some studies have documented concomitant naphthalene and phenanthrene degradation, it might be assumed that the same enzymes effecting 2-ring naphthalene attack could be recruited for 3-ring phenanthrene oxidation in parallel pathways. However, when Chang (2006) characterized the microbial communities in PAH-contaminated methanogenic marine sediments demonstrating naphthalene and phenanthrene degradation, they found that the two communities were distinct, suggesting that different species were responsible for naphthalene and phenanthrene degradation. Lack of pure cultures has hampered further elucidation of a phenanthrene pathway. McNally et al. (1998) used three *Pseudomonas* spp., isolated through their ability to degrade PAHs aerobically but also shown to reduce nitrate, to examine the degradation of phenanthrene, anthracene and pyrene under denitrifying conditions. They found that when the PAHs were provided at concentrations below their water solubility limit so as to eliminate the effects of dissolution and mass transport degradation occurred surprisingly quickly within hours and usually without a lag. However, because the substrate concentrations used in this study were very low and the cell density high (10^8 cells/ml), the possibility of substrate loss through partitioning into the cell membranes may be a factor, especially since degradation products were not determined to verify anaerobic oxidation. Thus, the pathways for anaerobic phenanthrene degradation remain cryptic.

Other unsubstituted PAHs also have been shown to be removed by enrichments using nitrate or sulfate, including acenaphthene (Yuan and Chang, 2007), fluorene and fluoranthene. Coates et al. (1997) determined that 14 C-labelled naphthalene, phenanthrene, fluorene and fluoranthene but not pyrene or benzo(a)pyrene) were mineralized to 14 CO_2 by sulfate-reducing sediments from San Diego Bay.

Rothermich et al. (2002) demonstrated that several indigenous as well as added PAHs were degraded in situ under sulfate-reducing conditions in harbor sediments. The substrates monitored comprised a suite of 14 PAHs having 2–5 rings, including naphthalene, phenanthrene, and the high molecular weight PAHs chrysene, pyrene and benzo(*a*)pyrene, among others, including alkyl-substituted naphthalenes. All substrates monitored eventually showed at least some depletion 9% for benz(a)anthracene to 89% for acenaphthene, with the smaller PAHs generally degrading faster than the larger PAHs. This study demonstrated for the first time that high molecular weight unsubstituted PAHs could be degraded under sulfate-reducing conditions (Rothermich et al., 2002). However, because signature metabolites were not assessed, and because the larger PAHs were not radiolabeled for measurement of 14 CO_2 evolution, it is not clear whether the PAH depletion was due to microbial growth resulting in complete mineralization, or to partial oxidation through co-metabolic processes, as has been suggested by Meckenstock et al. (2004) and Safinowski et al. (2006). More research involving mass balance of metabolites is required to resolve this question.

Although Meckenstock et al. (2004) concluded that unsubstituted PAHs are not attacked under methanogenic conditions, Christensen et al. (2004) assessed the potential for PAH removal under methanogenic conditions, applying molecular modelling to calculate the free energy of reaction for naphthalene and (1-methylnaphthalene) degradation using pathways proposed in the literature. Their calculations indicated that naphthalene oxidation should be thermodynamically feasible under methanogenic conditions. They then incubated naphthalene or 1-methylnaphthalene in microcosms inoculated with various anaerobic inocula. The mass of both substrates decreased in all microcosms at a rate proportional to temperature, up to 65 ° C for thermophilic enrichments. Similarly, Trably et al. (2003) measured removal of 13 unsubstituted PAHs from municipal sewage sludge in anaerobic stirred tank bioreactors under thermophilic methanogenic conditions and

found that removal was enhanced at 55 ° C compared with 35 ° C and 45 ° C, particularly for the larger PAHs.

However, in that study abiotic losses accounted for a significant portion of the total loss of the smaller PAHs at high temperatures, and no attempts were made to unequivocally demonstrate complete mineralization rather than partial cometabolic oxidation, so the results must be interpreted cautiously. Additional evidence was compiled by Chang et al. (2002) who followed the degradation of five 3-ring unsubstituted PAHs in soil under methanogenic, nitrate- or sulfate-reducing conditions. Over a period of 3 years, anaerobic cultures were established by periodic transfer with phenanthrene, and subsequently pure pyrene, anthracene, fluorene or acenaphthene were added individually or in combination for an additional year of enrichment with different TEAs. Significant removal of the PAHs from 100% to (approx. 60%) was observed for all compounds in the order phenanthrene 1 pyrene 1 anthracene 1 fluorene 1 acenaphthene, and PAH removal decreased in the order sulfate-reducing 1 methanogenic 1 nitrate-reducing conditions. Recently, Yuan and Chang (2007) documented removal of unsubstituted PAHs in anaerobic river sediment microcosms, again incubated under methanogenic, nitrate- or sulfate-reducing conditions.

The order of degradation differed from the previous study, with degradation rates decreasing from acenaphthene 1 fluorene 1 phenanthrene 1 anthracene 1 pyrene, but similar order of TEA efficacy sulfate-reducing 1 methanogenic 1 (nitrate-reducing conditions). From these microcosms, 12 strains were reported to degrade these PAHs anaerobically (although the TEA was not stated). The pure isolate with the best degradative ability, strain ER9, was identified morphologically and biochemically as a *Clostridium* sp. (Yuan and Chang, 2007). Notably, in both these studies (Chang, 2002; Yuan and Chang, 2007) neither mass balance using radiolabelled substrates nor detection of characteristic metabolites were attempted, nor was cell growth or mineralization determined; therefore, even though the initial inoculum had been enriched by repeated transfer on phenanthrene which was likely utilized as a carbon source, the measured decrease in the other PAH concentrations arguably could be ascribed to co-metabolic oxidation, as proposed by Meckenstock et al. (2004) and Safinowski et al. (2006).

Resolution of this controversy over utilization versus cometabolism of unsubstituted PAHs requires more study using enrichment cultures, including metabolism under sulfidogenic and methanogenic conditions because much of the literature has been generated with denitrifiers. It also demands studies in which mass balance is achieved, to determine whether the PAHs are being completely mineralized and serving as growth substrates, or are merely being co-metabolized. Finally, isolation of pure cultures that unequivocally oxidize these substrates anaerobically would allow pathways to be proposed and enzyme substrate ranges to be inferred.

4.2.3 Enzymes and genes

Enzymes

The fumarate addition pathway appears to be predominant in anaerobic transformation of alkyl-monoaromatics and -PAHs and may also be involved in catabolism of unsubstituted aromatics following methylation (Safinowski et al., 2006; Ulrich et al., 2005) (Figure 4.2). The enzymatic hydrocarbon activation reactions forming benzylsuccinates from monoaromatics are discussed briefly below, but for comprehensive coverage of the fumarate addition reactions and corresponding enzymes. No enzymes specific to attack on PAHs or alkyl-PAHs have been described, due at least in part to paucity of pure cultures to study. The initial enzyme in the toluene pathway, benzylsuccinate synthase Bss), belongs to a novel group of glycyl radical enzymes. Its activity, i.e. adding fumarate to the methyl group of toluene, has been demonstrated in enrichments incubated under nitrate- (Beller and Spormann, 1997), iron- (Kane et al., 2002), and sulfate-reducing (Beller and Spormann, 1998) as well as methanogenic conditions (Beller and Edwards, 2000; Washer and Edwards, 2007). The enzyme isolated from *T. aromatic* K172 has been characterized as has that from *Azoarcus* sp. strain T (Beller and Spormann, 1999). The gene encoding the alpha subunit of the enzyme, *bssA* , has been detected in all anaerobic toluenedegrading isolates surveyed to date, including *T. aromatica* K172, *Azoarcus* sp. strain T and *Geobacter metallireducens* and recently was used to develop a functional gene marker to screen for anaerobic toluene degraders (Winderl et al., 2007). Ethylbenzene dehydrogenase (EBD) catalyzes the initial attack in one pathway of ethylbenzene biodegradation, producing 1-phenylethanol. This was the first enzyme shown to hydroxylate an aromatic hydrocarbon in the absence of

molecular oxygen, deriving the hydroxyl group from water (Ball et al., 1996). The membrane- associated enzyme of the denitrifying organism *Azoarcus* sp. strain EB1, which grows only on ethylbenzene and no other BTEX compounds, oxidizes a limited number of fluorinated and nonaromatic homologs of ethylbenzene such as 4-fluoro-ethylbenzene and ethyldienecyclohexane, demonstrating a relatively broad substrate range, but does not transform either toluene or propylbenzene nor the saturated homolog ethylcyclohexane (Johnson et al., 2001). The enzyme is produced only during growth of strain EB1 on ethylbenzene or the direct pathway metabolites 1-phenylethanol and acetophenone. In contrast, the enzyme isolated from *Azoarcus* sp. EbN1 is reported to be periplasmic and can hydroxylate propylbenzene with low efficiency (Kniemeyer and Heider, 2001). The ethylbenzene-degrading *Azoarcus* sp. strain PbN1 also grows on propylbenzene with the intermediates postulated to be analogous to those of ethylbenzene i.e. phenylpropanol and propiophenone, its initial enzyme also can hydroxylate propylbenzene (Kniemeyer and Heider, 2001).

Enzymes responsible for subsequent catabolism of central metabolites of the BTEX pathways (i.e. benzylsuccinates and benzoyl-coA) have been studied in denitrifying bacteria such as *T. aromatica* K172 and the photosynthetic bacterium *Rhodopseudomonas palustris* (Egland et al, 1997). Many of these central steps require activation of the free acid by addition of co-enzyme A. Recent studies on the activity of a new type of benzoyl-coA reductase suggest that *G. Metallireducens* could be a model organism for coupling dearomatization to iron reduction (Carmona and Diaz, 2005).

The cellular location of pathway enzymes has been addressed by only a few researchers, even though this has implications for release of intermediates into the extracellular medium and therefore availability of metabolites or co-metabolites for further oxidation by other consortium members or for diffusion and mobility in the environment. Chakraborty and Coates (2005) proposed that hydroxylation of benzene occurs on the outer membrane or in the periplasm of *D. aromatica* RCB, allowing diffusion of phenol into the external medium during benzene degradation. Likewise, Rabus et al. (2002) have proposed a periplasmic location for ethylbenzene dehydrogenase in *Azoarcus* sp. EbN1. These locations would explain the appearance of pathway metabolites in culture supernatants and in the aqueous phase of contaminated environments, and limited uptake of the extracellular intermediates for

further metabolism would explain accumulation of such compounds in situ. However, neither a mechanism nor a biological rationale for excreting potential growth substrates has been examined; this aspect of anaerobic hydrocarbon biodegradation requires considerable experimental attention.

Co-Metabolism

Co-metabolism (i.e. gratuitous partial oxidation of a substrate by an organism during growth on a different substrate) appears to be an important fate for several aromatic hydrocarbons. For this to occur, the initial enzymes in the pathways must have a broad substrate range, initiating attack on a variety of aromatics. Partially purified benzylsuccinate synthase (Bss) from *Azoarcus* sp. Strain T was observed to catalyze fumarate addition to both toluene and *m*-xylene as well as several non-hydrocarbon aromatics (Beller and Spormann, 1999). In an analogous manner, Morasch et al. (2004) suggested that dimethylnaphthalenes are co-metabolized during growth of *Desulfotomaculum* sp. strain OX39 on *m*-xylene, producing the corresponding methylnaphthoic acids although the fumarate addition products were not detected and the initial enzymes are unknown. Ethylbenzene dehydrogenase also transforms other aromatic and non-aromatic substrates (Johnson et al., 2001). However, if the enzymes for the lower pathway reactions are more substrate-specific and unable to further transform those metabolites, it would account for the accumulation of metabolites characteristic of anaerobic biodegradation pathways (Beller, 2000). For example, *D. aromatica* RCB completely mineralized toluene and ethylbenzene to CO_2, but only transformed *p* -xylene to an unidentified metabolite detected in the culture supernatant (Chakraborty et al., 2005).

Likewise, denitrifying *T. aromatic* strain T1 did not grow on *o* -xylene and only transformed it in the presence of toluene (Evans et al., 1991). Sulfatereducing toluene-grown strain PRTOL1 co-metabolically transformed 90% of added *o*-xylene to the dead-end product 2-methylbenzylsuccinate, indicating the relative importance of co-metabolism in transformation of certain aromatics. Strain- and isomerspecific co-metabolism also seems to apply to PAHs: Safinowski et al. (2006) found evidence for co-metabolism of 1-methylnaphthalene by culture N47 grown on either naphthalene or 2-methylnaphthalene, generating the presumptive dead-end product 1-methyl-2-naphthoic acid. Interestingly, they also found that neither three-ring compounds i.e. fluorene, phenanthrene and anthracene nor biphenyl were co-

metabolic substrates for culture N47, although, because phenanthrene co-metabolism has been reported by others this observation may be specific to culture N47 (Safinowski, 2006). The question of toxicity of presumptive dead-end co-metabolites to the microbiota has not yet been addressed. Obviously, as more pure cultures are isolated and their enzymes purified and characterized, the picture of aromatic co-metabolism and substrate specificity will become clearer. It is hoped that such future studies will also address the question of gene regulation regarding alkyl isomers and heterocyclic homologs in particular.

Gene Regulation

Evidence exists for both repression and induction of genes involved in anaerobic aromatic hydrocarbon degradation; however, often the evidence is based on the sequence of attack on isomers or a mixture of substrates by an enrichment culture rather than a pure isolate, complicating interpretation of the data. Of the BTEX components, toluene is usually found to degrade most rapidly and with the shortest lag time (Meckenstock et al., 2004; Siddique et al., 2007). One explanation for this observation is repression by toluene of catabolic genes for other BTEX compounds. Meckenstock et al. (2004) observed that the presence of toluene at concentrations 1 20 _ M inhibited *o* -xylene degradation in sulfidogenic sediment columns, and that *o* -xylene degradation did not begin until toluene was depleted or was omitted from the culture). When tested with pure isolates, the phenomenon was found to be strain-specific: growth and *o* -xylene degradation by strain OX39 was sensitive to toluene, whereas toluene degradation by strain TRM1 which does not degrade xylenes was insensitive to the presence of *o* -xylene (Meckenstock et al., 2004). The recalcitrance of benzene can be similarly explained: results from early studies indicated that benzene degradation by a methanogenic consortium was impeded by the presence of more readily utilized substrates and benzene degradation in methanogenic aquifer columns was inhibited by the presence of toluene (Da Silva and Alvarez, 2004).

Likewise, a nitrate-reducing enrichment culture incubated with benzene plus toluene did not degrade benzene until toluene was depleted, whereas there was no inhibitory effect in a benzene-degrading methanogenic enrichment that was unable to degrade toluene (Ulrich et al., 2005). In addition, nonhydrocarbon substrates can repress hydrocarbon degradation: for example, toluene and *o* -xylene degradation by a methanogenic consortium was inhibited by the presence of preferred electron sources

(glucose, fatty acids, methanol, amino acids, etc.), implying catabolite repression. Considering the potential permutations of genes and regulatory systems and the complex mixtures of substrates and metabolites typically found in contaminated sites, prediction of inhibitory regulatory effects as opposed to general toxicity effects is difficult and in any case would likely be site and strain specific. Induction of monoaromatic catabolic genes also has been demonstrated. Prince and Suflita (2007) found that adding only 1 _ l of gasoline (representing about 5% of the indigenous hydrocarbon) to microcosms containing a natural gas condensate enhanced biodegradation of aromatic compounds under methanogenic and especially, sulfidogenic conditions. In particular, removal of *p*–xylene and ethyl-, propyl-, *iso* -propyl-, 1-ethyl-4-methyland two *iso* -butylbenzene isomers was enhanced when gasoline was added to cultures. In contrast, gasoline addition had little effect on biodegradation of alkanes or cycloalkanes, suggesting that the effect was a specific induction of aromatic catabolism genes. Similarly, overall PAH degradation was enhanced when a mixture of PAHs was incubated with anaerobic river sediment, versus individual substrates (Barbaro et al., 1992; Yuan and Chang, 2007). Induction of toluene catabolic genes in the denitrifying organism *T. aromatica* and *Thauera* sp. strain T1 involves multiple two-component regulatory systems (Coschigano and Young, 1997; Leuthner and Heider, 1998). The metabolite benzylsuccinate, which is often found in the supernatant of *T. aromatica* cultures grown on toluene, may be an inducer of the toluene pathway cited by Chakraborty and Coates (2004). Very recently, Washer and Edwards (2007) identified new putative benzylsuccinate synthase genes in a toluene-degrading methanogenic consortium. Their expression was upregulated in the presence of toluene but not the central metabolite benzoate, indicating specific induction by the primary substrate. The differential susceptibility of xylene isomers has been explained by Morasch (2004) as a consequence of having distinct enzymes for initial attack on *m* -, *o* - and *p* -xylenes, encoded by genes that are differentially induced in *Desulfotomaculum* sp. OX39. Similarly, the specificity of strain oXyS1 for *ortho* –alkylbenzenes versus strain mXyS1 for *meta* –alkylbenzenes could conceivably be due to specific gene induction and/or to substrate specificity of the Bss enzyme.

The denitrifying organism *Azoarcus* sp. EbN1 is unique because it can grow on toluene and ethylbenzene using two different pathways: toluene is degraded via

fumarate addition whereas ethylbenzene is hydroxylated. Early studies found that each pathway in EbN1 was separately and distinctly induced by its own substrate. The organization of toluene catabolic genes in EbN1 is similar to that of other denitrifying toluene degraders (Kube et al., 2004). Kuhner (2005) found that two toluene pathway operons were specifically induced by exposure to toluene, whereas genes involved in ethylbenzene degradation were induced by both ethylbenzene and toluene, suggesting that toluene is a fortuitous inducer of the second pathway.

The importance of such crossover induction is clear considering that environmental impact by a single contaminant is rare, and that most sites have multiple contaminant hydrocarbons. Full genome sequencing of EbN1 (GenBank accession number CR555306) ultimately revealed 10 presumptive pathways for anaerobic degradation (all but one converging at benzoyl-coA) and four pathways for aerobic biodegradation of aromatic compounds, plus paralogous genes as well as numerous transposable elements and multiple regulatory pathways, illustrating that regulation in this organism is probably more complex and versatile than can be discerned by simple incubation with individual substrates. PAH catabolic genes also appear to be induced. In studies with pure denitrifying isolates, McNally et al. (1999) found that the presence of naphthalene enhanced both phenanthrene and pyrene degradation, whereas phenanthrene apparently inhibited pyrene degradation, although these observations were not confirmed with metabolite analysis. Safinowski and Meckenstock (2006) noted that there was no lag phase when enrichment culture N47 was transferred from naphthalene as carbon source to 2-methylnaphthalene, indicating that induction of new genes was not required.

However, the converse was not true, and after growth on 2-methylnaphthalene the culture exhibited a lag phase of almost 100 days before growth resumed on naphthalene. This observation is consistent with the degradative pathways where an additional methylation step is required to activate naphthalene for fumarate addition induction of the requisite gene might account for the lag upon transfer from 2-methylnaphthalene to naphthalene. It is less apparent how this observation would support the existence of the pathway because induction of several upper pathway enzymes presumably would be required upon shift from naphthalene to 2-methylnaphthalene, yet the culture exhibited no lag phase. Notably, because culture N47 is not a confirmed pure culture these results are subject to alternative

interpretations including interplay between two or more microbial partners in a consortium. Other combinations of substrates also resulted in partial or complete inhibition of degradation by culture N47, showing yet again that strain- and substrate-specific effects may contribute to explaining site-specific field observations (Safinowski and Meckenstock, 2006).

Regulation of catabolic genes involved in central metabolism of aromatic intermediates such as benzoate has been investigated in *Azoarcus* spp., *Thauera* spp. and the anaerobic phototroph *Rhodopseudomonas palustris* (Egland and Harwood, 1999). Thus, from the relatively limited number of pure cultures available for study, a complex and strain-specific picture of enzyme substrate specificity, pathway redundancy, and gene regulation emerges. Most of these observations arose from toluene-degrading denitrifying isolates, and without more evidence from additional diverse organisms, particularly PAH-degraders, it will remain difficult to formulate general rules for aromatic hydrocarbon catabolic enzyme specificity or gene expression.

4.3 Applications of Anaerobic Hydrocarbon Degradation

4.3.1 Natural attenuation and bioremediation

Natural attenuation is a strategy for managing decontamination of soils, sediments and groundwaters, in which intrinsic physical and biological processes result in amelioration of the contamination. Biological processes typically comprise the major contributor to this treatment method. The term ‘bioremediation’ usually implies active intervention at the contaminated site to enhance biodegradation processes through, (for example, addition of limiting nutrients biostimulation) or of competent (microbes bioaugmentation). Because hydrocarbon-contaminated sites commonly include anaerobic sectors due to oxygen consumption during organic carbon metabolism, the importance of anaerobic biodegradation processes at such sites is obvious. Understanding the underlying principles discussed in previous sections of the Chapter 4 may enable prediction and manipulation of microbial processes to ensure or accelerate natural attenuation. Numerous reports have been published documenting natural attenuation of hydrocarbons in anaerobic aquifers, groundwater and sediments (Meckenstock et al., 2004).

Demonstration of natural attenuation requires at least three lines of evidence: documented loss of the contaminants from the site; laboratory data (e.g. microcosms or microbial enumeration methods) indicating the presence of competent microbiota in situ; and evidence of appropriate microbial activity in situ e.g. production of predicted metabolites, depletion of TEAs and/or accumulation of reduced TEA products. However, demonstrating anaerobic biodegradation in situ by measuring depletion of parent compounds can cause major technical problems because of difficulty distinguishing between losses due to biological transformation versus abiotic processes such as sorption, dilution or volatilization. To complement and augment depletion measurements, Beller (1995) proposed that specific anaerobic metabolites produced and excreted during degradation of the substrate be used as indicators of biodegradation. Ideally, anaerobic biomarkers are intermediates highly specific to the substrate's degradation pathway (i.e. are not products of metabolism of other compounds or abiotic processes); are produced only anaerobically; are not normally found in the environment unless the substrate is present; are water soluble so that they can be recovered from groundwater or saturated sediments; and are relatively stable chemically so that they can be detected, but are also relatively biologically labile so that their presence is an indicator of recent rather than relict metabolism (Phelps and Young, 2002). Through studies with pure cultures and substrates it has been possible to identify candidate biomarkers such as those shown in figures 1–3 , then correlate them with metabolites detected in situ (Beller, 1995).

The alkyl benzylsuccinates (i.e. the products of fumarate addition) fit these criteria and have been adopted as potentially universal signature metabolites for anaerobic biodegradation of BTEX compounds. In contrast, the alkylbenzoates (i.e. lower pathway intermediates) are not sufficiently specific because they can be formed aerobically or by metabolism of nonhydrocarbons like aromatic amino acids (Meckenstock et al., 2004). As new substrates and degradative pathways are identified, it is clear that we are still adding to the catalogue of potential biomarkers for anaerobic metabolism (Chakraborty and Coates, 2005). Technical difficulties with inconsistent detection and dentification of signature metabolites still hamper characterization of anaerobic biodegradation in situ, due to their low concentrations typically orders of magnitude lower than the parent hydrocarbons, heterogeneity and their transient nature due to biodegradation by other members of the consortium

(Beller, 2000). In addition, authentic standards of many hypothetical intermediates are not commercially available, preventing incontrovertible identification in culture extracts, groundwater, etc. Several current methods involve solvent extraction of relatively large volumes of groundwater with subsequent derivatization and GC-MS analysis (Gieg and Suflita, 2002).

Although well-established and definitive, these analyses can be costly and time-consuming. New sophisticated methods are being developed and combined to streamline analysis of signature metabolites, including liquid chromatography combined with stable carbon isotope or deuterated substrates, solid-phase extraction SPE and direct injection methods coupled with LC-MS to increase sensitivity of detection (Griebler et al., 2004).

The appearance of signature metabolites in the aqueous phase in situ or in culture medium is intriguing, because it is not clear why pathway intermediates that are potential growth substrates should be found outside the cell. Beller (2000) noted that benzylsuccinate and *E* -phenylitaconate, products of toluene metabolism, accumulate in growth medium and are more stable than expected in comparison with, (for example, benzoate), and speculated that after they leave the cell they are not taken up efficiently from the extracellular medium. For example, benzylsuccinate was not metabolized by toluene- grown cells but could be oxidized to phenylitaconate and benzoate by permeabilized cells (Beller and Spormann, 1998). Phelps et al. (2001) observed that exogenous benzoate was utilized less efficiently than benzene by a benzene- degrading enrichment culture, and suggested that it indicates inefficient uptake of benzoate by the cells. Similarly, Safinowski and Meckenstock (2006) observed that the fumarate addition pathway metabolites of 2-methylnaphthalene a carbon source for culture N47 accumulated in the medium with incubation. They proposed that the metabolites are continuously excreted from the cells and, once released, cannot be taken up again by culture N47 for metabolism. This raises the question of how efficiently the culture can grow on 2-methylnaphthalene if a proportion of the substrate is excreted as metabolites that cannot be re-acquired as carbon source. It also begs the question of the mechanism and biochemical rationale of export of catabolic pathway metabolites, although this is also observed during aerobic growth on aromatic hydrocarbons and heterocycles.

However, it is necessary to remember that the signature metabolites, at least, are typically found at low concentrations in the aqueous phase and probably represent only a small proportion of the total oxidized substrate. Obviously, further investigation of enzyme locations, metabolite export and uptake is warranted as more pure cultures are isolated and enzymes characterized. The implications for prediction, implementation and detection of natural attenuation processes are important for at least two reasons: excretion of polar metabolites without re-uptake will mobilize the aromatic skeleton because of increased water solubility compared with the parent hydrocarbon and may either enhance bioavailability in situ or increase mobility of the metabolite; conversely, excretion with re-uptake and further metabolism (by the same or different organisms in a consortium) may enhance biodegradation. Hydrocarbon-impacted environments usually experience complex mixtures of contaminants that can influence the overall activity of the microbial community (Zheng et al., 2001). In a survey of various aquatic sediments incubated under four different redox conditions, Phelps and Young (1999) found that degradation of a mixture of BTEX compounds was site and TEA specific: whereas toluene was degraded in all enrichment cultures under all conditions, benzene degraded only in sulfidogenic cultures from one site, and pristine sediments generally did not degrade BTEX whereas chronically contaminated sediments usually yielded active cultures. In cultures amended with gasoline which contains a large proportion of aliphatic hydrocarbons (in addition to aromatics) BTEX degradation was slower and incomplete, indicating that degradation of complex mixtures is influenced by site microbiota, redox conditions and the substrate itself. This observation could be explained by either inhibition of BTEX degradation by the other hydrocarbons in the gasoline through toxicity, gene expression effects, or shifts in the microbiota composition), or to their preferential degradation over BTEX compounds (Phelps and Young, 1999).

The negative effect of gasoline on BTEX degradation in the aquatic sediments contrasts sharply with the stimulatory effect of gasoline on aromatic hydrocarbon degradation noted by Prince and Suflita (2007) in microcosms under methanogenic and especially sulfidogenic conditions. These conflicting observations emphasize the unpredictability of site-specific responses to mixed substrates and the need for new, comprehensive approaches that do more than simply document substrate depletion.

Enhancing natural attenuation

The type and concentration of TEAs available in a contaminated environment will affect the outcome of natural attenuation or bioremediation. Common indigenous TEAs in impacted environments include nitrate, iron, sulfate and carbon dioxide, as discussed above, and in some cases also perchlorate and chlorate, quinones and humic acids although, to date, the latter TEAs have only been linked to biodegradation of certain aromatics (like toluene). Understanding the potential role of various TEAs in anaerobic biodegradation can inform remediation strategies such as amendment with TEAs to enhance natural attenuation. Sulfate, for example, has several potential advantages as a TEA amendment (Anderson and Lovley, 2000): unlike O_2, sulfate is not consumed by abiotic reactions with ferrous iron or sulfide; it does not form iron oxide precipitates in situ that can cause plugging; it is more soluble than O_2 and therefore can be added at higher concentrations, and furthermore accepts twice as many electrons as O_2; and it can be applied to groundwater at higher levels than nitrate, which is potentially toxic.

The disadvantage is that some contaminated sites may lack the microbes that initiate attack on benzene under sulfatereducing conditions (Weiner and Lovley, 1998). Notwithstanding that potential limitation, Weiner (1998) showed in laboratory microcosms. Then Anderson and Lovely (2000) demonstrated in situ that adding sulfate as a TEA to a petroleum-contaminated aquifer stimulated benzene oxidation whereas addition of nitrate completely inhibited benzene degradation in preliminary experiments. These effects were noted despite the fact that the sediments, contaminated with hydrocarbons for more than 50 years, were methanogenic when the TEAs were injected. This indicates that the potential for anaerobic degradation coupled to sulfate reduction persisted regardless of prevailing TEAs, and points to a possible role for sulfate-reducers activating aromatic hydrocarbons in methanogenic consortia.

Schreiber and Bahr (2002) added nitrate to a petroleum- contaminated aquifer and detected biodegradation of toluene, ethylbenzene and *m*- and *p*-xylenes but not benzene over a 60-day monitoring period. Interestingly, the stoichiometry of nitrate reduced to TEX oxidized was greater than predicted, and may have resulted from oxidation of other organics in the aquifer at the expense of the added nitrate. Ball and Reinhard (1996) also observed nonstoichiometric reduction of nitrate when

amending microcosms containing BTEX. This common phenomenon can complicate calculation of TEA demand for natural attenuation. Phenanthrene biodegradation in marine sediments was enhanced two- to three-fold by the addition of controlled-release TEAs, specifically nitrate as nitrocellulose and sulfate as gypsum (Tang et al., 2005). This approach may lead to refined 'capping' strategies in marine harbour sediments, where a slow-release TEA would be incorporated directly into otherwise undisturbed contaminated sediments, thus avoiding the issues associated with multiple applications of highly soluble, potentially inhibitory TEAs, particularly nitrate (Tang et al., 2005). Cunningham et al. (2001) enhanced in situ bioremediation of BTEX-contaminated groundwater by combining the advantages of nitrate and sulfate through amendment with both TEAs. The combination of TEAs accelerated the natural attenuation of the petroleum hydrocarbon contaminants; nitrate was used preferentially and so was rapidly consumed near the injection well, but sulfate had an effect outside the denitrifying zone.

Degradation of xylene isomers appeared to be linked specifically to sulfate reduction, validating the choice of amending with two TEAs rather than just nitrate. Benzene was the most recalcitrant contaminant in situ but eventually showed evidence of biodegradation after approximately 15 months. This study illustrates how understanding the potential diversity of in situ anaerobic processes and adjusting the remediation method to suit the contaminants and the indigenous microbial community can be used to relieve the limitations encountered by injection of a single TEA. The stimulatory effect of providing nutrients, such as fixed nitrogen and/or phosphate, has not been as thoroughly studied under anaerobic conditions as under aerobic conditions. However, at least two cases show the benefit of fertilizing nutrient-poor anaerobic environments contaminated with diesel fuel: Cross et al. (2006) observed enhanced anaerobic degradation when contaminated groundwater microcosms were amended with nutrients, specifically ammonium, nitrate and phosphate. Powell et al. (2006) noted the stimulatory effect of nutrients (nitrate, ammonium, calcium, sulfate and phosphate) on denitrifying hydrocarbon degraders in nutrient- poor Antarctic soils.

Whereas biostimulation through TEA addition has been studied, bioaugmentation with bacteria capable of anaerobic degradation is virtually untested. Da Silva and Alvarez (2004) inoculated flowthrough aquifer columns with enrichment cultures

and demonstrated increased benzene degradation under methanogenic conditions only in the bioaugmented columns, but this activity required a very long acclimation period and the observation was not verified in the field. It is possible that versatile degraders such as *D. aromatica* RCB, which can mineralize BTEX components under aerobic, nitrate-, perchlorate- and chlorate-reducing conditions (Chakraborty et al., 2005), may be valuable as bioaugmentation agents in certain applications. However, in general, added microbes are at a disadvantage in competition with the indigenous microbiota and, even under aerobic conditions, successful bioaugmentation trials are sparse.

4.3.2 Treatment of hydrocarbon-containing wastes

Processing of hydrocarbon-containing industrial wastewaters and municipal sewage sludge is another area that may benefit from increased understanding of anaerobic biodegradation. Soil-wash fluids from a wood preserving site containing both pentachlorophenol (PCP) and PAHs necessitated an integrated waste management system of soil washing and anaerobic bioremediation to deal with both classes of compounds (Miller et al., 1998). Removal of contaminants under anaerobic conditions was demonstrated with a simulated waste stream containing PCP 99.8% removal and four model PAHs.

Naphthalene and acenaphthene were removed efficiently 86% and 93% removal, respectively), although negligible removal of pyrene and benzofluoranthene was measured. In a recent study, Siddique et al. (2007) documented methanogenic removal of BTEX and other hydrocarbons (both aromatic and aliphatic) from naphtha in a slurry of oil sands tailings waste, without prior laboratory enrichment. Although high concentrations of an artificial mixture of BTEX or of naphtha inhibited methanogenesis in the microcosms, lower concentrations similar to those normally present in the tailings waste supported methane production and resulted in hydrocarbon depletion and methane production in the microcosms. Methanogenesis in the large volume tailings basins, sustained by anaerobic hydrocarbon biodegradation, apparently is responsible for daily emission of millions of liters of methane at this site. It is possible that anaerobic pretreatment of the tailings to remove hydrocarbons with capture of the produced CO_2 and methane could reduce greenhouse gas emissions from the current open system. Similarly, domestic sewage sludge might be pretreated to remove hydrocarbons before diversion to other

purposes such as application to agricultural soil (Trably et al., 2003). Chang et al. (2003) incubated samples from municipal and petrochemical sludge with a suite of five PAHs and found that degradation was slower in the municipal sludge, possibly due to the presence of more susceptible, competing organic compounds in the municipal sludge, or conversely the presence of a more competent microbiota in the petrochemical sludge. They also found that sulfate-reducing conditions were superior to methanogenic and denitrifying conditions for PAH removal. However, nitrate and sulfate are not usually practical or desirable TEAs for sewage sludge bioprocessing, and methanogenic conditions are considered more practical, even though evidence for PAH removal under these conditions is currently scarce. To test the ability of sludge to degrade PAHs under methanogenic conditions, enriched cultures from diverse sources: a wastewater treatment plant; digested manure and industrial food waste; leachate from a municipal landfill; or contaminated soils from gasoline stations. They found that each inoculum was able to degrade naphthalene and 1-methylnaphthalene, but the contaminated soil enrichments performed the best. Trably et al. (2003) also observed PAH losses from municipal sludge incubated as mesophilic and thermophilic enrichments and found that bioaugmentation with an adapted inoculum enhanced PAH degradation. This limited number of studies indicates the potential for waste stream processing although more research in this area is required, especially demonstrating mass balance to document complete oxidation of the hydrocarbons.

4.3.3 Petroleum reservoirs

Head et al. (2003) have reviewed the literature on deep subsurface oil reservoirs and support the proposition that heavy oils have arisen through anaerobic biodegradation of conventional oils over geologic time, occurring in reservoirs with a water interface and an in situ temperature 80 °C or perhaps higher (Spark et al., 2000). Deep subsurface biodegradation, involving aliphatic as well as aromatic hydrocarbons, is generally deleterious to the economic value of the oil, resulting in increased oil density and viscosity, sulfur content, acidity and metals, and decreased saturated and aromatic hydrocarbons corresponding to the extent of in situ biodegradation (Larter et al., 2006). Thus, archaic anaerobic biodegradation has had huge economic impacts on fossil fuel quality and crude oil recovery worldwide. Detection of anaerobic naphthalene signature metabolites, specifically 2-naphthoate and partially reduced 2-

naphthoates, during a screen of 77 degraded oil samples from around the world lends more specific support to the inference that anaerobic hydrocarbon biodegradation has occurred in a large proportion of oil reservoirs (Aitken et al., 2004; Magot et al., 2000). Despite this circumstantial evidence, no pure culture has yet been isolated that exhibits the ability to degrade hydrocarbons anaerobically under in situ reservoir conditions (Aitken et al., 2004; Roling et al., 2003). The succinate derivatives from fumarate addition pathways have not been confirmed yet in crude oils, possibly because these polar metabolites partition into the aqueous phase ,but they have been detected in production water from oil fields. Sulfidogenesis appears to dominate in sulfate-containing reservoirs, especially those undergoing waterflooding for secondary recovery. It is assumed that indigenous hydrocarbons support this detrimental sulfide generation (Rueter et al., 1994), perhaps by members of the family *Desulfobacteriaceae* (Rabus et al., 1996). Although pure aromatic hydrocarbon- degrading cultures demonstrating this activity have not yet been isolated. Chen and Taylor (1997) successfully enriched a thermophilic consortium from the produced water of an Alaskan oil field that could metabolize BTEX components to unknown water-soluble products concomitant with sulfide production, suggesting the potential for such activity *in situ*. Nitrate has been added to reservoirs as an alternate TEA for biological control of souring and has proved useful in some cases (Sunde and Torsvik, 2005); again, it is assumed that a portion of the oil in situ serves as carbon and energy source for the nitrate-reducers but this awaits proof. Rabus (1999) found that a succession of BTEX- and aliphatic-degrading bacteria grew on crude oil under nitrate-reducing conditions, and that degradation by the community exceeded that observed with individual strains and compounds.

In contrast, Kodama and Watanabe (2003) isolated sulfide-oxidizing, nitrate-reducing bacterial strains from underground oil storage facilities, but these strains apparently could not grow directly on crude oil as carbon source, so their importance in oil reservoirs is currently unknown. Nor has the possible role of biological iron reduction in reservoirs been well-addressed yet (Birkeland, 2004; Roling et al., 2003). In reservoirs low in available sulfate, methanogenesis appears to be the primary TEA process and is thought to have contributed over geological time to methane gas associated with heavily biodegraded petroleum such as the Canadian oil sands deposits (Head et al., 2003). It may be possible to exploit anaerobic activity (in

situ through biostimulation or bioaugmentation) to produce methane from reservoirs with otherwise economically unrecoverable oil, such as wells that have undergone extensive waterflooding and are marginal producers (Suflita et al., 2004). The contribution of aliphatic hydrocarbons to anaerobic degradation is likely to be more important in such environments because of the higher mass ratio of alkanes to aromatics in most crude oils. Thus, the potential exists to control reservoir souring (and associated metal corrosion in production facilities) or to enhance energy production via in situ methanogenesis through judicious manipulation of anaerobic hydrocarbon biodegradation in oil reservoirs. Alternatively, oil reservoirs may be sources of isolates capable of anaerobic biodesulfurization to improve crude oil quality through removal of organic sulfur from sulfur heterocycles (Marcelis et al., 2003).

5. MATERIALS AND METHODS

5.1 Sampling

Sampling locations were shown in Figure 5.1. Sampling depths and dates, and sample abbreviations were given in Table 5.1. The samples were taken using a Van Veen grab (volume of 3.5 L and penetration depth of 15 cm) on board of the RV Arar of İstanbul University, Institute of Marine Sciences during research cruises between the years 2005 and 2008. The samples were taken in three replicates and then subdivided for molecular analyses and sediment chemistry and stored at -20°C. Samples for FISH analyses were stored with the addition of absolute ethanol (1:1, v/v) on-site. All samples appeared visually similar possessing grayish-black color and had a noticeable odor of H2S. Sediment grain size analysis was performed using a method adapted from Folk (1974) as described by Unlu and Alpar (2006). All of the sediments have fine-grained nature, being rich in mud (>90%) and poor in sand.

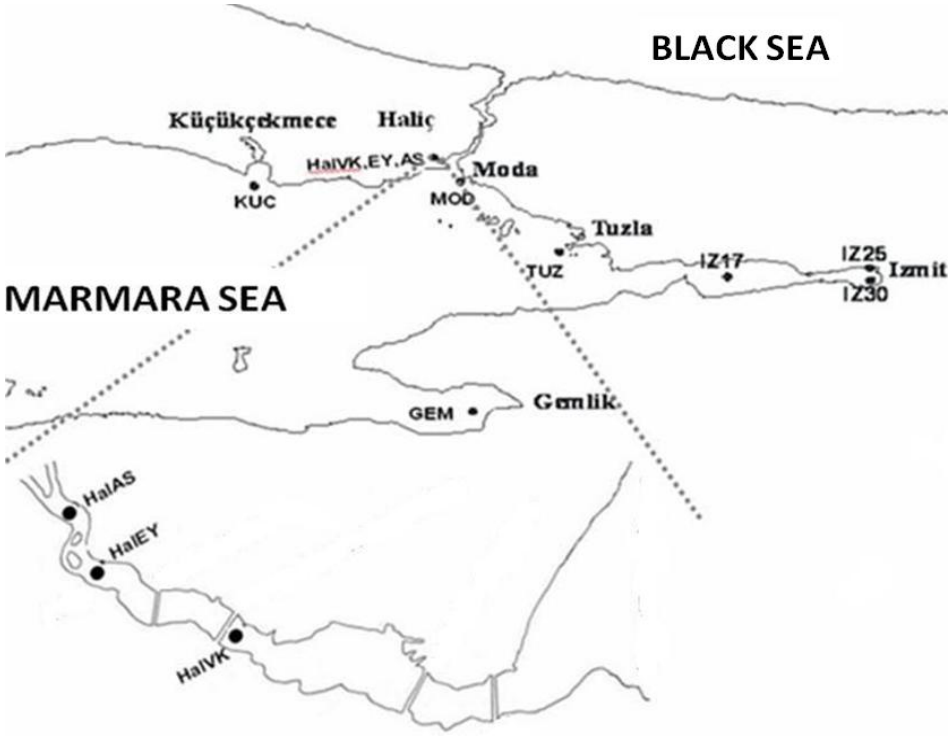


Figure 5.1: Sampling locations

Table 5.1: Sampling locations, depths and dates, and sample abbreviations

Location	**Coordinates**			**Sampling dates and sample abbreviations**					
	Latitude (N)	Longitude (E)	Depth (m)	August 2005	November 2005	February 2006	November 2006	February 2007	August 2007
İzmit	40˚43.30’	29˚37.00’	157	Iz17Aug05	IZ17Nov05	IZ17Feb06	Iz17Nov06		
İzmit	40˚44.00’	29˚47.00’	30	IZ25Aug05	IZ25Nov05	IZ25Feb06	IZ25Nov06		
İzmit	40˚44.20’	29˚53.50’	30	IZ30Aug05	IZ30Nov05	IZ30Feb06	IZ30Nov06		
Gemlik	40˚33.17’	27˚56.49’	87	GEMAug05	GEMNov05	GEMFeb06	GEMNov06		
Kucuk-cekmece	40˚58.24’	28˚45.44’	22	KUCAug05	KUCNov05	KUCFeb06	KUCNov06	KUCFeb07	KUCAug07
Moda	40˚58.62’	29˚01.49’	8			MODFeb06	MODNov06	MODFeb07	MODAug07
Tuzla	40˚50.60’	29˚13.60’	42	TUZAug05	TUZNov05	TUZFeb06	TUZNov06	TUZFeb07	TUZAug07
Haliç	41˚19.38’	28˚57.99’	6				HalVKNov06	HalVKFeb07	HalVKAug07
Haliç	41˚24.24’	28˚56.92’	6				HalEYNov06	HalEYFeb07	HalEYAug07
Haliç	41˚33.66’	28˚56.64’	2				HalASNov06	HalASFeb07	HalASAug07

5.2 Physicochemical Analyses

pH and conductivity were measured using HI 99121 Soil pH Test Kit and HI 993310 Water Conductivity and Soil Activity Meter respectively according to manufacturer's instructions (Hanna Instruments Ltd., UK). NH_4^+-N was measured using 1200-NH Colorimeter and the kit supplied by the manufacturer. (LaMotte Company, U.S.A.). Anion and heavy metal measurements were carried out according to Standard Methods (Clesceri et al., 1998). The anion analyses were performed using a Dionex Ion Chromatograph (Bannockburn, IL, USA). Atomspeck H 5150 Model atomic absorption spectrophotometer (Rank Hilger Ltd., U.K.) was used for heavy metal measurements. Organic carbon and nitrogen contents were analyzed by the dry combustion method (Polat & Tugrul, 1995), using a Carlo Erba Model 1108 CHN analyzer. Phosphorus content was determined colorimetrically by the routine orthophosphate method after exposure of the sediments to dry combustion and HCl treatment (Polat & Tuğrul, 1995). The analytical procedure for extraction of hydrocarbons derived from a modified UNEP’s (*1991*) protocol.

5.3 Analysis of Petroleum Hydrocarbons

Dry sediment samples were Soxhlet-extracted with chloroform (1:2 m:v) for a period of 1 h at 50°C and concentrated on a rotary evaporator. The TPH content of extracts was quantified by infrared spectroscopy. The extracts were analysed using an Iatroscan MK-5 Thin Layer Chromatography-Flame Ionization Detector (TLC-FID) Analyzer (Iatron Laboratories, Tokyo) using the SARA (saturate, aromatic, resin,

asphaltene) method described by Karlsen and Larter (1991). Values (blank corrected) of the aliphatic, aromatic, resene and asphaltene fractions were also corrected for relative response factors using squalane, anthracene and undecanol standards, respectively. The results were recorded and processed using a LabSystems XChrom data system.

The extracts were fractionated into aliphatic and aromatic hydrocarbons by adsorption liquid chromatography using a column of alumina and silica-gel, and gradient solvents as eluent: *n*-hexane and 2:1 *n*-hexane/chloroform for aliphatic and aromatic fractions, respectively. The extracts for aromatic hydrocarbon analyses were evaporated under gentle steam of nitrogen after addition of dimethylformamide (DMF) as keeper and diluted with acetonitrile for HPLC analysis. The resulting solution were analysed by a Hewlett-Packard 1046A HPLC with a programmed fluorescence detector. The column (MZ-PAH 250 × 3 mm, 5 μm, from MZ-Analysentechnik, Mainz, Germany) was maintained at 35 °C and the flow of mobile phase was 0.5 ml/min. A linear gradient was applied from 52% acetonitrile in water to 100% acetonitrile and held constant for 10 min. Fourteen different polyaromatic hydrocarbons (PAHs) and benzene, toluene, ethylbenzene and m+p+o xylene (BTEX) were analysed in the samples. Certified Reference Materials (CRM 535) were used to assess the accuracy (> 85%) of the measurements. Another CRM (NIST-1647) was also used for recovery test and analysed three times.

Aliphatic hydrocarbon analyses were conducted on a Hewlett Packard (HP) 5972 II gas chromatograph-mass spectrometer (GC-MS). The samples were analyzed using a fused silica capillary column (25 m×0.32 mm, 0.52 μm) with nitrogen as carrier gas. The column temperature was programmed from 80°C to a final temperature of 280°C at a rate of 8°C/min. The MS operating conditions were: electron ionization of 50 eV and linear scanning over the mass range 35–500 Da were used. The samples were analyzed in the splitless mode. Compound identification was based on individual mass spectra and GC retention times in comparison to the literature, library data, and authentic standards. Standards were injected and analyzed under the same conditions as the samples. Quantification was made owing to internal standards such as *n*-C_{18}, *n*-C_{20}, *n*-C_{22} and *n*-C_{24}. Blank analyses were carried out, and all values were corrected for these blank concentrations.

5.4 Genomic DNA and Total RNA Extraction, and cDNA Synthesis

Genomic DNA (gDNA) was extracted using the FastDNA Spin Kit for Soil (Qbiogene, U.K.), and total RNA was extracted using the ChargeSwitch® Total RNA Cell Kit (Invitrogen, Germany) by following the manufacturer's instructions. To test for a DNA contamination, the RNA extracts were used in Q-RT-PCR as a negative control. The first-strand cDNA was synthesized from the total RNA using random hexamers and SuperScript® First-Strand Synthesis System for RT-PCR according to the kit's manual (Invitrogen, Germany).

5.5 PCR Amplification of 16S rRNA Genes

Amplification of 16S rDNA from the extracted DNA was performed with primers given in Table 5.2. 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 $MgCl_2$, 5 µl of 10×*Taq* buffer and 4 U of *Taq* DNA polymerase (Fermentas, Latvia). 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 staining with ethidium bromide after agarose gel electrophoresis (Thermo-Scientific Ltd., U.K.) and the images were recorded using a Chemi-Smart 3000 gel documentation system (Vilber Lourmat, France)

5.6 Primer Design for bssA, assA and hzoA

Equal amounts of gDNA extracts from the 47 different sediment samples were combined to create a gDNA mix for primer specificity assessment. Partial gene sequences were amplified from this template using the degenerate primer sets which were designed from alignments of known bssA, assA and hzoA gene sequences. Amplification was done under the conditions described above with the exception that Pfu DNA polymerase was used for the amplifications (Stratagene, Germany). To optimize PCR for newly designed primer sets, annealing temperature gradients were run between 50°C and 60°C. The amplicons were cloned using a TOPO TA cloning kit (Invitrogen, Germany) abd sequenced as described in the next session.

Table 5.2: Primers used in PCR amplifications

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

[a]*Escherichia coli* numbering.
[b]5´-GC clamp on Arch344f and Bact341f (GCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACGGGG).

5.7 Cloning and Sequencing of 16S rRNA Genes

PCR amplified 16S rRNA gene sequences from the samples taken in November 2006 were cloned. PCR products from the cloned sequences and the other samples were compared using DGGE to relate the bands in DGGE profiles with the cloned DNA.

PCR primers used for cloning, clone screening and sequencing were given in Table 5.2. PCR products were cloned with a TOPO TA cloning kit (Invitrogen Ltd., Paisley, United Kingdom) according to manufacturer's instructions. PCR amplified vector inserts of the correct size were purified by ethanol precipitation and sequenced using the ABI prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 377 DNA sequencer (Applied Biosystems, USA). 500bp and 800bp of archaeal and bacterial sequence data which can be sufficient for a phylogenetic assignment down to the genus level (Stackebrandt and Rainey, 1995) were generated respectively.The sequences were analyzed in Chromas software package version 1.45 (http://www.technelysium.com/au/chromas.html) and checked for reading errors with the alignment programs of the ARB package (Ludwig et al., 2004). Chimerical constructs were checked by using the CHECK-CHIMERA program of the Ribosomal Database Project (Cole et al., 2007). Homology searches of the sequences in DNA databases were performed with FASTA provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/fasta33/nucleotide.html). 16S rRNA gene sequences showing 97% similarity or higher were considered to belong to the same phylotype. Sequences that showed no or low (below 70%) relatedness with known bacterial or

archaeal phylogenetic groups were listed as unclassified. Sequences showing similarities between 70-97% were placed within tentative taxa according to their closest relative in DNA databases. The sequences showing less than 97% similarity were submitted to the EMBL database. Sequences representing distinct phylotypes and their closest relatives were aligned by using the ARB software fast aligner utility, followed by manual adjustments. Only unambiguously aligned base positions were used in the analysis. Distance analyses using the Jukes and Cantor (1969) correction and bootstrap resampling (1000 times) were done using the Treecon package (van De Peer and De Wachter, 1997) and trees were generated from distance matrices using the neighbour-joining method (Saitou and Nei, 1987). Rarefaction curves, coverage values, the Shannon-Weaver index of diversity (H?) and the Chao1 estimator of species richness (S_{chao1}) were obtained using the distribution of clone types present in the clone libraries (Röling and Head, 2005).

5.8 Denaturing Gradient Gel Electrophoresis (DGGE)

PCR primers for DGGE analysis were given in Table 2. PCR products were run on a 10% polyacrylamide gel (37.5:1 acrylamide:bisacrylamide) in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; pH 8.0) over a 30–60% denaturing gradient (100% denaturant is 7 M urea and 40% (v/v) formamide). To aid the conversion and normalization of gels, a marker was added on the outside of the gel as well as after every four samples. Electrophoresis was performed using the D-Code system (Bio-Rad Laboratories, Ltd., UK) at 200 V and 60°C for 4.5 h. Gel images were recorded using a Chemi-Smart 3000 gel documentation system (Vilber Lourmat, France) after stained with SybrGold (1:10000 diluted; Molecular Probes Inc., UK) according to the supplier's instructions.

Images were analyzed by using the Bionumerics 5.0 software (Applied Maths, Kortrijk, Belgium). Similarities between tracks were calculated by using the Dice coefficient (S_D) (unweighted data based on band presence or absence) and band-independent, whole-densitometric-curve-based Pearson product-moment correlation coefficients (r) and UPGMA clustering. For analysis using Dice coefficient a band position tolerance of 0.7% was applied. This was the minimum tolerance at which all marker lanes clustered at100%. DGGE data were analyzed by principal component

analysis (PCA) in order to correlate the first component (PC1) with the other variables.

5.9 Quantitative Real-Time PCR

PCR primer sets for the Q-PCR assays were given in Table 5.3. 10^{3-7} copies of the standard sequences were used to obtain the calibration curves. Roche LightCycler DNA Master SYBR Green I kit and Roche Light Cycler 2.0 (Roche Diagnostics GmbH, Mannheim, Germany) were utilized for all reactions. Reaction mixes contained 25 ng template DNA, 0.5 μM of each primer and 2.5 μM $MgCl_2$. Q-PCR conditions for the most of the primer sets were described previously (Table 5.3). 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. 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 analysed using 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.

5.10 Fluorescence *In Situ* Hybridization

The sediment samples were fixed in freshly prepared 4% paraformaldehyde (PFA) in PBS (130 mM NaCl, 10 mM sodium phosphate, pH 7.2) for at least 3 hours at 4°C. After fixation, cells were washed once with PBS, resuspended in PBS-absolute ethanol (1:1, v/v) and stored at -20°C (Harmsen et al., 1996).

16S rRNA-targeted oligonucleotide probes were obtained commercially and labeled at 5′ end with Cy3 (Qiagen, Germany). Arc915 (Stahl et al., 1988) and Univ1392 (Pace et al., 1986) probes were used to detect *Archaea* and whole microbial community respectively. Mix of EUB338 (Aman et al., 1990), EUB338 II and EUB338 III (Daims et al., 1999) probes were used to detect *Bacteria*. Two negative controls were used during the hybridization stage. One was to assess nonspecific binding (probe Non338), and the other was (lacking a probe) to monitor autofluorescence.

Table 5.3: Q-PCR primer sets

Primer	Target Gene	Target Organism	Reference
Bac519f	16S rRNA	*Bacteria*	Ruppel et al. 2006
Bac907r	16S rRNA	*Bacteria*	Ruppel et al. 2006
Arc349f	16S rRNA	*Archaea*	Takai and Horikoshi 2000
Arc806r	16S rRNA	*Archaea*	Takai and Horikoshi 2000
mcrA1f	mcrA	Methanogens	Colwell et al. 2008
mcrA1r	mcrA	Methanogens	Colwell et al. 2008
MSL812f	16S rRNA	*Methanosarcinales*	Yu et al. 2005
MSL1159r	16S rRNA	*Methanosarcinales*	Yu et al. 2005
Msn180f	16S rRNA	*Methanosarcina*	Duhamel and Edwards 2006
Msn511r	16S rRNA	*Methanosarcina*	Duhamel and Edwards 2006
Mst 170f	16S rRNA	*Methanosaeta*	Duhamel and Edwards 2006
Mst 390r	16S rRNA	*Methanosaeta*	Duhamel and Edwards 2006
DSRp2060F	dsrB	Sulfate Reducers	Geets et al. 2006
DSR4R	dsrB	Sulfate Reducers	Geets et al. 2006
BCR697f	bcrA	Degraders of Aromatics	Song and Ward 2005
BCR1178r	bcrA	Degraders of Aromatics	Song and Ward 2005
bssA_715f	bssA	Toluene-Xylene Degraders	This study
bssA_1107r	bssA	Toluene-Xylene Degraders	This study
assA_1578f	assA	Aliphatic Hydrocarbon Degraders	This study
assA_1967r	assA	Aliphatic Hydrocarbon Degraders	This study
hzoA_811f	hzoA	Anaerobic NH_4^+ oxidizers	This study
hzoA_1175r	hzoA	Anaerobic NH_4^+ oxidizers	This study
narGf	narG	Nitrate Reducers	Bru et al. 2007
narGr	narG	Nitrate Reducers	Bru et al. 2007
V17m	napA	Nitrate Reducers	Bru et al. 2007
napA4r	napA	Nitrate Reducers	Bru et al. 2007
nasAf	nasA	Nitrate assimilating *Bacteria*	Cai and Jiao 2008
nasAr	nasA	Nitrate assimilating *Bacteria*	Cai and Jiao 2008
nirK550r	nirK	Nitrite Reducers	Qiu et al. 2004
nirK 200f	nirK	Nitrite Reducers	Qiu et al. 2004
cd8F	nirS	Nitrite Reducers	Michotey et al. 2000
cd2R	nirS	Nitrite Reducers	Michotey et al. 2000
nosZf	nosZ	Denitrifiers	Henry et al. 2006
nosZr	nosZ	Denitrifiers	Henry et al. 2006
nrfAf	nrfA	Dissimilatory Nitrate Reducers to NH_4^+	Smith et al. 2007
nrfAr	nrfA	Dissimilatory Nitrate Reducers to NH_4^+	Smith et al. 2007

The fixed sample was spotted onto a gelatin-coated slide and air dried. Cells were dehydrated at room temperature in increasing concentrations of ethanol (50%, 80% and 98%). Dehydrated cells were prehybridized in hybridization buffer (0.9 mol l^{-1}

NaCl, 2 mg ml^{-1} Ficoll, 2 mg ml^{-1} Bovine Serum Albumin, 2 mg ml^{-1} polyvinyl pyrrolidone, 5 mmol l^{-1} EDTA, pH 8.0, 25 mmol l^{-1} NaH_2PO_4, pH 7.0, 0.1% SDS, 5-35% deionized formamide) at the intended hybridization temperature for 20 min (Manz *et al.*, 1992). After prehybridization, probe at a final concentration of 5 ng μl^{-1} was added into the hybridization buffer and incubated at the optimal hybridization temperature for 3 hours. Following hybridization, 2 μl of 4',6-diamidino-2-phenylindole (DAPI) DNA stain (final concentration of 3.3 μg/ml) was added into the hybridization buffer and incubated at room temperature for 10 minutes. The cells were washed twice in wash buffer containing 20 mmol l^{-1} Tris-HCl (pH 7.2), 0.01% SDS, 5 mmol l^{-1} EDTA and between 0.9 mol l^{-1} and 56 mmol l^{-1} NaCl according to the formula of Lathe (1985) for 15 min at the optimal washing temperature before a final wash with deionized water. Slides were air dried and one drop of Citifluor antifadent (Citifluor Ltd., U.K.) was added to the sample. Slides were examined under Olympus BX 50 Epifluorescence Microscope equipped with a 100 W high-pressure mercury lamp and U-MWIB/U-MWG filter cubes. Images were captured using a Spot RT charged coupled device (CCD) camera using the software supplied by the camera manufacturer (Diagnostic Instruments Ltd., UK). The images were processed and analyzed using Image-Pro Plus version 5.1 image analysis software (Media Cybernetics, U.S.A.). Counts for 10 random fields of view were obtained for each sample, and the average cell count was calculated.

5.11 Microcosm Setup

Anaerobic microcosms were set up in glass 120-ml serum bottles sealed with butyl rubber stoppers and aluminium crimps (Aldrich). The total volume of liquid was 100 ml with 20 ml of headspace volume. An anaerobic cabinet (Coy Laboratory Products) fitted with an oxygen sensor and with a regulated atmosphere of nitrogen (100%) was used in the preparation and incubation of the microcosms. Each microcosm comprised a carbonate-buffered nutrient medium containing sources of nitrogen and phosphorus, vitamins and trace minerals, made up in deionized water, according to the brackish medium of Widdel and Bak (1992).

Microcosms were seeded with 10g of the sediments. Microcosms were fed with 200 mg of the hydrocarbon mixture. Composition of the hydrocarbon mix was defined

based on the detected hydrocarbon types in the sediments during the two years monitoring study.

Nitrate reducing conditions were provided with NO_3^- addition; methanogenic conditions were established by the exclusion of the exogenous electron acceptors. Three control microcosms were included: (1) Na_2MoO_4 and 2-bromo-ethane sulfonate was added to inhibit DSR and MG respectively; (2) NaN_3 (1 g/L) treatment was applied to suppress microbial activity; and (3) external HCs were not added to assess degradation of natural TOC content of the sediments.

The overall TOC/N/P ratio of Haliç Bay sediments (~1000/5/1) was chosen as a nutrient limited condition. The unlimited nutrient condition was calculated as 1000/40/6 (C/N/P) based on the following assumptions: (1) molecular formula of the HC mix was $C_{5n}H_{8n}$ (derived from the HC composition); (2) the maximum biomass yield was as high as 0.2 gcell/gHC mix (Gavala et al., 2003); and (3) C/N/P ratio of the marine microbes was 100/20/3 (Vrede et al., 2002). Hence, the nutrient amendment was done by gradually decreasing TOC/N/P ratio from 1000/5/1 to 1000/40/6 for Haliç Bay sedimets' microcosms.

The Tuzla Bay sediments' microcosms without external N and P addition were regarded as nutrient limited. N and P which were enough to biologically remove 15 mmol TOC were added to the unlimited microcosms based on the following assumptions: (1) the maximum biomass yield was as high as 0.4 gcell/gHC mix; (2) C/N/P ratio of the marine microbes was 100/20/3; and (3) NO_3^- was used as both the e^--acceptor and the N source in nitrate reducing microcosms. NH_4^+ was used as a N source in methanogenic microcosms.

The intended initial N and P concentrations, the experimental conditions and controls, and abbreviations of the sample names were summarized in Table 5.4 and 5.5. Microcosms were prepared in triplicate. 5 sets of each condition were prepared for destructive sampling. The destructive samplings were carried out based on the gas production data.

Headspace gas (10 ml) was removed, periodically (2 weeks), throughout the course of the experiment from all microcosms and injected into evacuated gas tubes. The gas removed was replaced by 10 ml of 100% N_2. Gas samples were analysed for CH_4 using a GC-MS. Peak areas were calibrated using the CH_4 and CO_2 gas standards and

the reproducibility (n = 4) of replicate standard analyses were typically less than 1% relative standard deviation

Table 5.4: Experimental conditions and sample abbreviations for Tuzla

	150/0.9 (NO_3^-/PO_4^{3-} mM)	150/0.45 (NO_3^-/PO_4^{3-} mM)	150/0.12 (NO_3^-/PO_4^{3-} mM)	1/0.12 (NO_3^-/PO_4^{3-} mM)
	13/0.9 (NH_4^+/PO_4^{3-} mM)	13/0.45 (NH_4^+/PO_4^{3-} mM)	13/0.12 (NH_4^+/PO_4^{3-} mM)	1/0.12 (NH_4^+/PO_4^{3-} mM)
HC and NO_3^- added	HC(+)-N(+)-UL	HC(+)-N(+)-PL1	HC(+)-N(+)-PL2	HC(+)-N(+)-L
NO_3^- added	HC(-)-N(+)-UL	HC(-)-N(+)-PL1	HC(-)-N(+)-PL2	HC(-)-N(+)-L
HC and NH_4^+ added	HC(+)-N(-)-UL	HC(+)-N(-)-PL1	HC(+)-N(-)-PL2	HC(+)-N(-)-L
HC and NO_3^- added, inhibited	HC(+)-N(+)-I-UL	HC(+)-N(+)-I-PL1	HC(+)-N(+)-I-PL2	HC(+)-N(+)-I-L
HC and NO_3^- added, sterilized	HC(+)-N(+)-S-UL	HC(+)-N(+)-S-PL1	HC(+)-N(+)-S-PL2	HC(+)-N(+)-S-L

Table 5.5: Experimental conditions and sample abbreviations for Haliç

TOC/N/P→		**1000/40/6**	**1000/5/6**	**1000/20/6**	**1000/40/1**	**1000/40/3**	**1000/5/1**
External HC added→		HC(+)-UL	HC(+)-NL1	HC(+)-NL2	HC(+)-PL1	HC(+)-PL2	HC(+)-L
Control↓	**Without external HC**	HC(-)-UL	HC(-)-NL1	HC(-)-NL2	HC(-)-PL1	HC(-)-PL2	HC(-)-L
	Inhibited	HC(+)-I-UL	HC(+)-I-NL1	HC(+)-I-NL2	HC(+)-I-PL1	HC(+)-I-PL2	HC(+)-I-L
	Sterilized	HC(+)-S-UL	HC(+)-S-NL1	HC(+)-S-NL2	HC(+)-S-PL1	HC(+)-S-PL2	HC(+)-S-L

5.12 Statistical Analysis

Statistical analyses were performed using the softwares MINITAB 15 (Minitab Ltd., England) and SPSS 17.0 (SPSS Inc., U.S.A.).

6. RESULTS AND DISCUSSION

6.1 Changes in Microbial Diversity of Marmara Sea Sediments

This study was undertaken for microbiological characterization of coastal sediments from the most polluted regions in Marmara Sea. The microbial and chemical diversity were monitored for 2 years, and correlated to each other to determine how chemical composition may control the microbial diversity. Microbial diversity was determined using cloning, sequencing and denaturant gradient gel electrophoresis (DGGE) of PCR amplified 16S rRNA genes. Total cells, *Archaea* and *Bacteria* were also quantified using fluorescence *in-situ* hybridization (FISH). Total petroleum hydrocarbons (TPH), elemental composition, total and dissolved organic carbon (TOC and DOC), sediment grain size, anion and heavy metal content, salinity, redox potential and pH were measured for physicochemical characterization.

6.1.1 Chemical and physical characteristics

The results from the chemical analyses were given in Table 6.1 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.

Heavy metals

Comparison of heavy metal levels in the MSS with background metal concentration ranges in various uncontaminated marine sediments (Erydlmaz and Erydlmaz, 2002; MacDonald et al., 1996; Morillo et al., 2008) revealed that the MSS were moderately polluted with Zn and highly polluted with Ni. When this information was evaluated regarding ERL (effects range-low) and ERM (effects range-median) guideline values for metals and incidence of biological effects (Long et al., 1995), the heavy metals can be ranked for their potential adverse effects on benthic organisms in the MSS as Ni>Zn>Cu>Pb>Cr>Mn>Fe.

Table 6.1: Concentration ranges for chemical components

			IZ17	IZ25	IZ30	GEM	KUC	HalVK	HalEY	HalAS	TUZ	MOD
Sediment	Cr	mg/kg	22-32	26-38	48-67	45-66	40-60	190-270	100-140	80-100	240-360	32-40
	Cu	mg/kg	24-36	40-60	68-100	17-25	62-90	330-490	170-250	100-140	160-240	115-145
	Zn	mg/kg	105-155	190-280	670-960	130-195	145-210	440-600	290-420	220-335	400-580	450-650
	Pb	mg/kg	13-20	23-34	29-44	13-19	15-23	110-170	50-80	33-55	110-170	50-80
	Ni	mg/kg	65-100	60-95	60-85	70-105	60-90	55-75	55-80	55-85	60-85	60-90
	Mn	mg/kg	390-580	340-500	190-290	300-450	200-300	260-400	270-400	400-600	140-200	180-260
	Fe	mg/kg	15500-23200	20000-30000	20000-29900	23000-34000	9600-14400	25300-38000	24500-36800	23000-34500	14500-21700	16600-24900
	TPH	ppm	3300-4950	4700-7100	4400-6600	1300-1950	3200-4800	9500-1400	10500-16000	11500-17500	13000-19500	4900-7300
	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
Porewater	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
	NO_3^-	mM	0.6-0.9	1.3-1.9	1.1-1.6	0.4-0.5	0.5-0.7	1.2-1.8	0.1-0.2	0.2-0.3	1.2-1.9	1.5-2.2
	Salinity	psu	17.5-26	16-24	17-26	11-17	17-26	10-16	10-16	10-15	18-27	13-19.5

Horizantal "white → black" scale represents increasing level of the parameters

Elemental composition

TOC/N/P ratios of the MSS (≈100/50/10) were very low compared to the Redfield Ratio (106/16/1) (Redfield, 1958) indicating a perturbation of marine nutrient cycling by pollutants from anthropogenic sources. However, DOC/N/P ratios of the MSS porewaters (≈1000/5/1) were very high compared to C/N/P ratio (100/20/3) of the exponentially growing marine bacterioplankton (Vrede et al., 2002) indicating that N and P were limited in the porewaters for biological activity.

Petroleum hydrocarbons

Organic-rich marine sediments may naturally contain up to 100 ppm TPH, but concentrations much higher than this are usually associated with petroleum inputs (Readman et al., 2002). Total petroleum hydrocarbon (TPH) contents of the MSS were substantially higher than those from harbors, bays and other sediments

worldwide, and similar to those from extremely polluted marine environments (Ahmed et al., 2006; Guerra-García et al., 2003).

Sulfate, nitrate and salinity levels

Sulfate is abundant in coastal and estuarine seawater, whereas nitrate concentrations are typically low (Jørgensen, 1982). NO_3^- concentrations in the MSS porewaters were considerably higher than that of typical seawater (10-50 μM), while the SO_4^{2-} levels were lower than the typical levels (2600-3200 mg/l) (Millero, 1996). Moreover, NO_3^- was more abundant compared to SO_4^{2-} in the TUZ and MOD sediments. This is an unusual case for marine sediments.

The Marmara Sea is considered as metahaline (36-40 psu) (Lee et al., 2002). Salinity of typical sea water away from a freshwater source is more than 30 psu (Roy et al., 2001). Salinity measurements in this study showed that the MSS porewaters were brackish (0.5-29 psu) which may imply replacement of conductive porewater by a body of fresh groundwater in the studied sites.

Temperature, pH and redox potential

Deep water temperatures of HAL and MOD (17-21°C); KUC, TUZ and IZ25-30 (16-18°C); and GEM and IZ17 (14-15°C) were supporting psychrotolerant and/or mesophilic microbial activities (Arnosti et al., 1998). pH of the KUC, HAL, MOD and TUZ sediments changed between 7.5 and 8.3. This pH range is maintained by methanogenesis, denitrification and sulfate reduction in marine environments (Soetaert et al., 2007). pH of the GEM and IZ sediments fluctuated between 8-9. This pH range is sustained in seawater as a result of Fe and Mn reduction processes. Redox potential (Eh) ranges in the IZ, KUC and HalEY-AS sediments (-150 – -250 mV) favor sulfate and iron reduction, and methanogenesis while Eh ranges in the GEM, TUZ, MOD and HalVK sediments (-50 – -150 mV) favor only iron reduction (Schulz, 2000).

6.1.2 FISH results

Total, active, archaeal and bacterial cell counts were given in Figure 6.1. Total cell counts of the TUZ, MOD, HAL and KUC sediments 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). Although cell contents of the GEM and IZ sediments were considerably lower than the other sediments, their total cell counts were in the upper limit of the previously reported total cell counts.

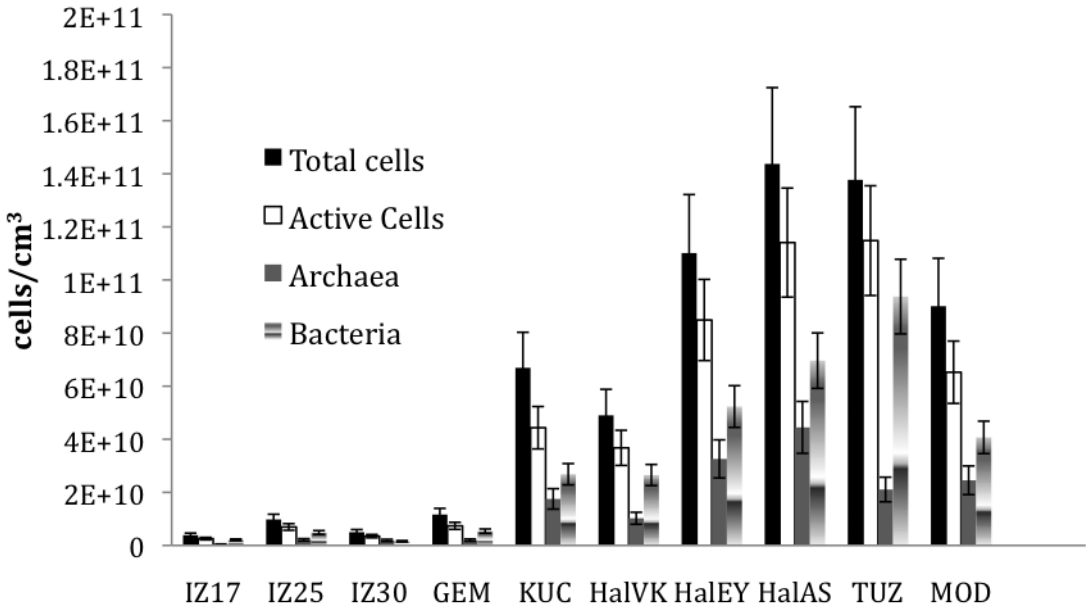


Figure 6.1: Cell counts for Marmara Sea sediments

Previous studies on marine sediments using FISH reported that 10-80% of the prokaryotic cells are alive and active (Biddle et al., 2006; Smith and D'Hondt, 2006). These studies disagreed considerably in relative proportion of *Archaea* and *Bacteria* which may point to the problems with application of *in situ* molecular techniques to complex sediment matrices (Smith and D'Hondt, 2006). Our FISH results showed that large fraction (60-85%) of subseafloor cells in the Marmara Sea were active.

Often, archaeal cells occur in higher numbers than bacterial cells, independently confirmed by FISH counts and lipid quantification (Biddle et al., 2006) and contesting earlier claims to the contrary (Schippers et al., 2005). *Bacteria* dominated over *Archaea* in the MSS except for the IZ30 sediments.

6.1.3 Cloning, sequencing and DGGE results

In this study, 2400 16S rRNA gene clones were screened; and 262 archaeal and 234 bacterial operational taxonomic units (OTUs) were identified. Identified OTUs in the clone libraries and the DGGE profiles, their classification, clone frequencies and DGGE band intensities, similarities between the OTUs and their cultured and uncultured closest relatives, and their respective nucleotide accession numbers were given in Appendice Tables A.1-A.18.

DGGE profiles

We were able to clone all the OTUs found in the DGGE profiles. Statistically significant high correlations ($r>0.85$, $p<0.05$) between clone frequencies and DGGE band intensities of the OTUs were obtained for all of the clone libraries. These correlations showed that DGGE patterns obtained in this represented the microbial composition of the sediments well.

Cluster analysis of bacterial and archaeal DGGE profiles using band position based Dice and band-independent, whole-densitometric-curve-based Pearson correlation coefficients were given in Appendice Figures B1-B4. The DGGE profiles perfectly clustered according to sampling location using Dice coefficient. Considerably higher similarities between the DGGE profiles also obtained using Dice coefficient compared to that obtained using Pearson correlation coefficient. These results showed that changes in microbial community structure of the MSS during the two years monitoring period occurred in terms of relative abundance of the community components (OTUs) rather than OTU types present.

Diversity Indices

Number of screened clones and identified OTUs, and coverage value, the Shannon-Weaver index of diversity (H?), evenness (J') and the Chao1 estimator of species richness (S_{chao1}) of the clone libraries were given in Table 6.2. The species richness was high (39-83), but the OTU frequencies were not evenly distributed ($J'<0.4$) in the clone libraries. This resulted in low diversity ($1.45< H?<1.71$) in the MSS. Rarefaction curves for all of the clone libraries were close to reach an asymptote predicting that most of the probable unique phylotypes were analyzed (Parkes et al., 2005).

Table 6.2: Diversity indices

		Screened clone[a]	OTU[a] (Clone)	OTU[a] (DGGE)	S_{chao1}[d]	H?[b]	J'[c]	Coverage
Bacterial	**Gemlik**	125	63	29	83	1,71	0,39	0,73
	Tuzla	125	59	30	73	1,66	0,39	0,77
	K.cekmece	125	57	30	69	1,64	0,39	0,78
	Moda	125	57	30	69	1,64	0,39	0,78
	Iz30	100	41	17	58	1,51	0,37	0,76
	Iz17	100	41	20	52	1,54	0,39	0,79
	HaliçAS	125	60	32	72	1,66	0,39	0,78
	HaliçVK	125	59	31	72	1,65	0,39	0,78
	HaliçEY	125	59	31	72	1,65	0,39	0,78
Archaeal	**Gemlik**	75	34	20	39	1,45	0,40	0,81
	Tuzla	175	61	41	66	1,58	0,38	0,89
	K.cekmece	175	63	42	68	1,64	0,39	0,88
	Moda	150	46	33	49	1,49	0,38	0,91
	Iz30	125	45	27	51	1,5	0,38	0,86
	Iz17	100	44	32	46	1,6	0,42	0,88
	HaliçAS	175	71	42	76	1,67	0,39	0,87
	HaliçVK	175	60	38	66	1,67	0,40	0,87
	HaliçEY	175	71	49	76	1,67	0,39	0,75

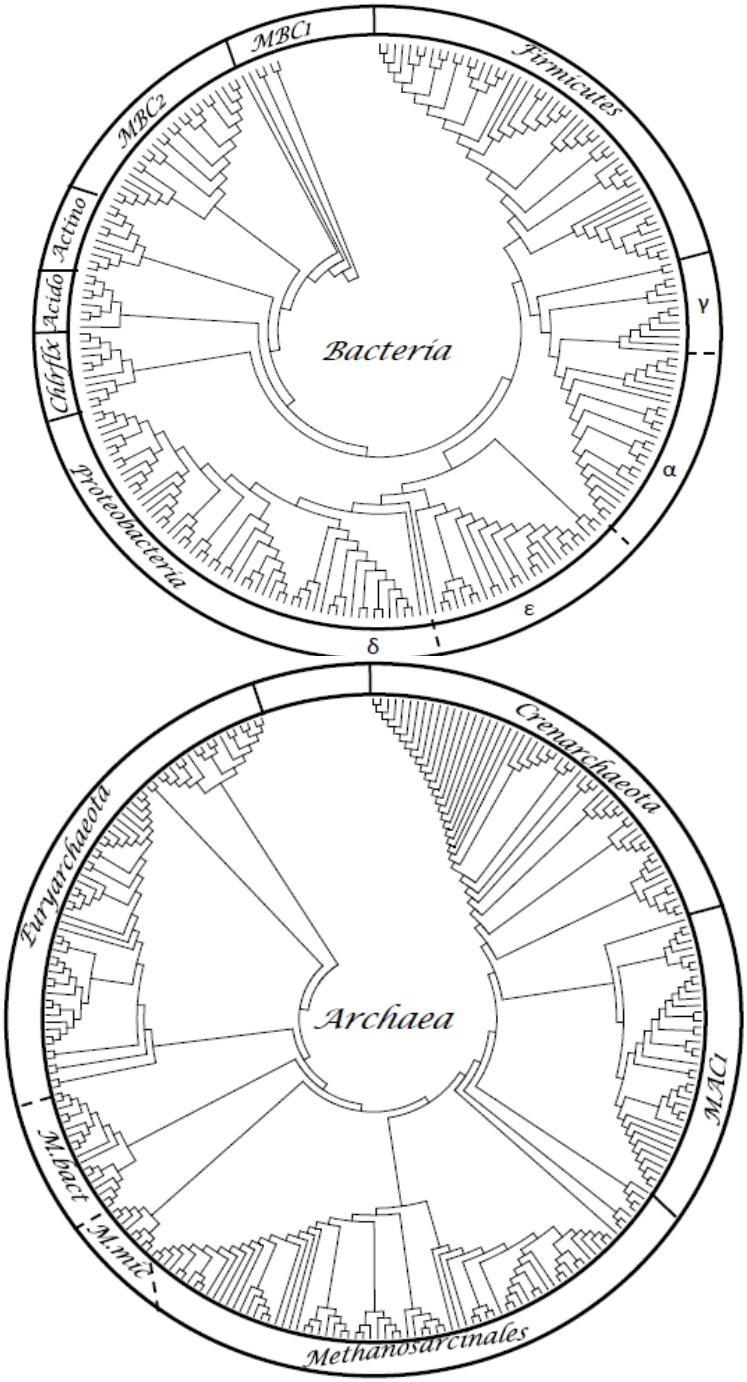
[a]Number of screened clones and identified OTUs
[b]Shannon-Weaver index of diversity (H?)
[c]Evenness (J')
[d]Chao1 estimator of species richness (Schao1)

Phylogenetic affiliation of dominant phylotypes

Bacterial and archaeal phylogenetic trees for 16S rRNA gene sequences were shown in Figure 6.2. Clone frequencies of the dominant bacterial and archaeal phylotypes were given in Figures 6.3 and 6.4. The phylum distributions were similar to those obtained from the previously studied coastal marine sediments (Wilms et al., 2006). The archaeal and bacterial clone libraries were dominated by euryarchaeotal and proteobacterial sequences respectively. *δ-proteobacteria* and *Methanomicrobia* were the most abundant classes.

The considerable portion of both archaeal and bacterial OTUs were unclassified. 24 of the bacterial unclassified OTUs formed a distinctive cluster, namely Marmara Bacterial Cluster 2 (MBC2) (Figure 2). MBC2 was found in all clone libraries (Supplementary Table 1-18). 5 bacterial unclassified sequences (MBC1) did not cluster with any known phyla or the other unclassified sequences. MBC1 was only found in the GEM sediments. The other bacterial unclassified sequences mainly clustered with *δ-proteobacteria*.

39 of the archaeal unclassified OTUs also formed a distinctive cluster namely Marmara Archaeal Cluster 1 (MAC1). MAC1 was found in all clone libraries. The other archaeal unclassified sequences mainly clustered with *Methanosarcinales.*



MAC1: Marmara Archaeal Cluster 1; MBC1-2: Marmara Bacterial Cluster12; M.bact: Methanobacteriales; M.mic: Methanomicrobiales; Chlrflx: Chloroflexi; Acido: Acidobacteria; Actino: Actinobacteria.

Figure 6.2: Phylogenetic trees for 16S rRNA gene sequences

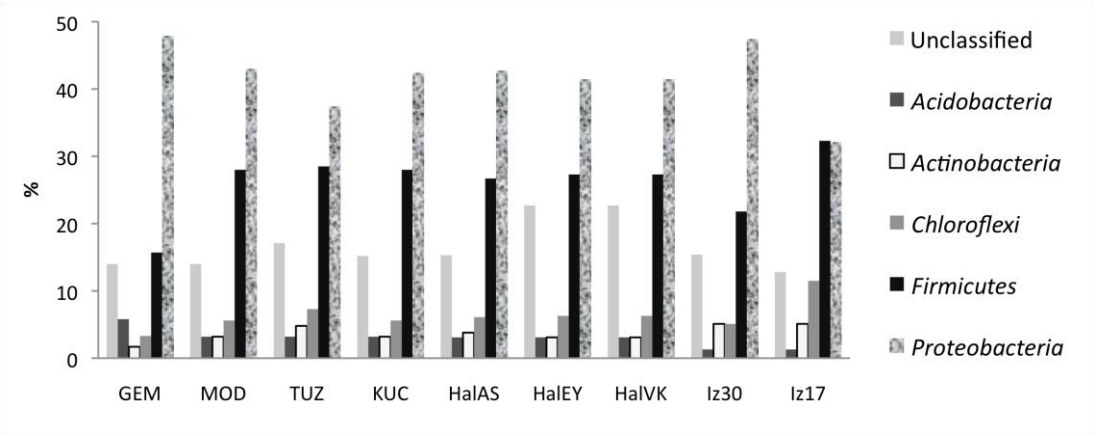


Figure 6.3: Clone frequencies of the bacterial phylotypes

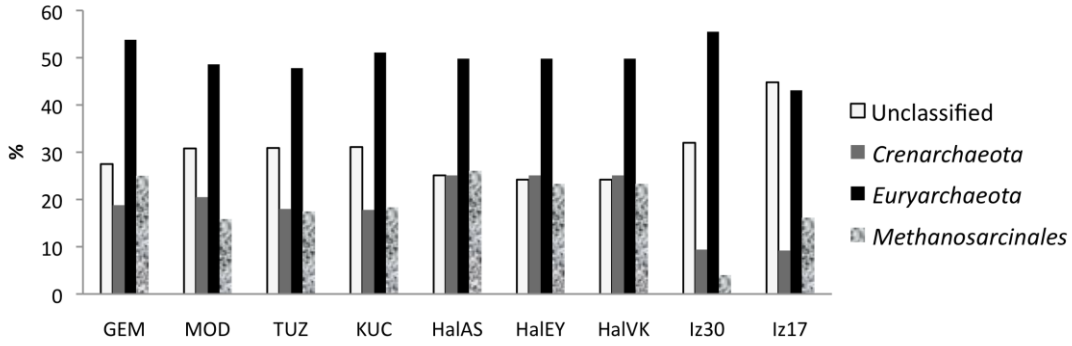
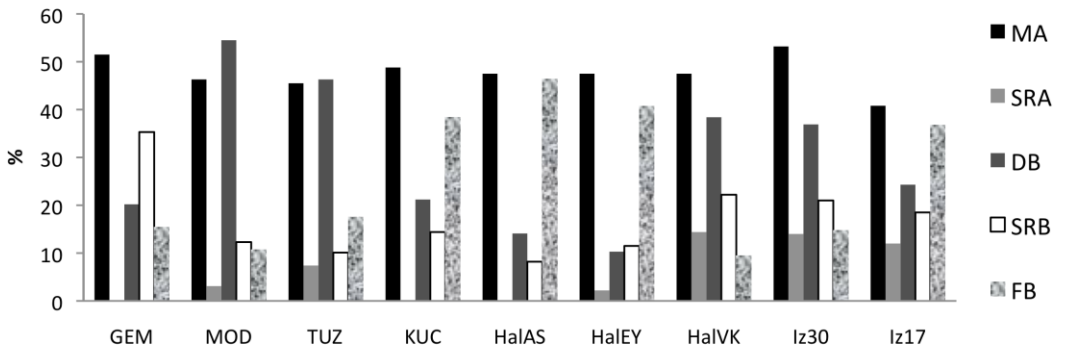


Figure 6.4: Clone frequencies of the archaeal phylotypes

Terminal e^--accepting processes

Since a considerable amount of obtained phylotypes belong to previously unknown and uncultivated groups, physiological properties of the MSS organisms remained largely unknown. We classified the clones according to their terminal electron accepting process by considering the physiologies of the closest cultured relatives as long as their similarity was over 80% (Figure 6.5). Methanogenic sequences over dominated the archaeal clone libraries. *Methanosarcinales* that utilize noncompetitive substrates were dominant in all sediments except Iz30. Typically hydrogenotrophic *Methanomicrobiales* and *Methanobacteriales* were dominant over *Methanosarcinales* in Iz30 sediments. Discernible amount of sulfate reducing archaeal sequences were also found in the IZ, HAL and TUZ archaeal clone libraries. The MOD, TUZ, HalVK and Iz30 sediments were predominated by denitrifying *Bacteria* whereas fermentative *Bacteria* predominated the KUC, HalAS-EY and Iz17 sediments. Sulfate reducing *Bacteria* were dominant in the GEM sediments.



MA: Archaea; SRA: Sulfate reducing Archaea; DB: Denitrifying Bacteria; SRB: Sulfate reducing Bacteria; FB: Fermentative Bacteria

Figure 6.5: Abundance of metabolic groups

Isolation/identification sources of the OTUs

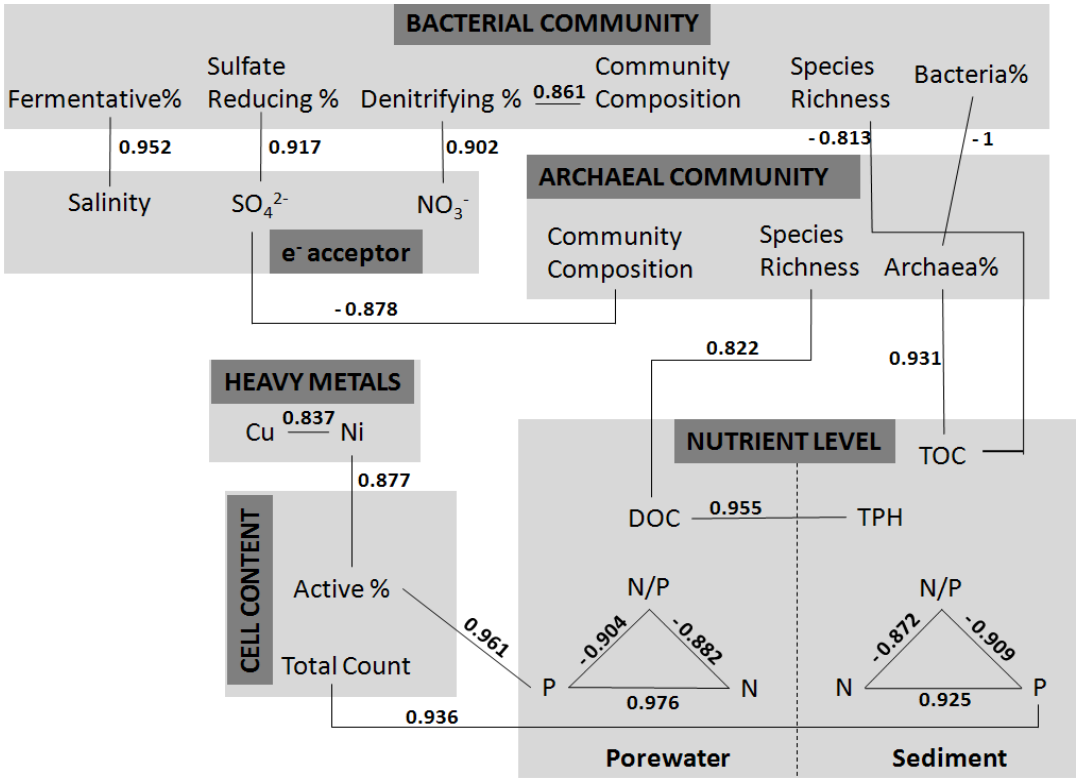
The clone libraries were dominated by the clones previously identified in marine sediments (42%), hydrothermal vents (15%) and anaerobic wastewater treatment systems (12%). The other isolation sources were animal feces (7%), soil (6%), saline lakes (6%), mud volcanos (4%), cold seeps (4%) and human (4%). 28% of the OTUs were hydrocarbon degraders or previously identified clones in oil rich environments. This coincided with the high hydrocarbon content of the MSS. Sequences affiliated with methanogens and denitrifying bacteria constituted the big majority of the clones identified in oil rich environments.

6.1.4 Correlation between microbial and chemical characteristics

It is well known that microbial diversity is related to the environmental chemistry in marine sediments (Gordon et al., 2006**;** Ley et al., 2006; Smith and D'Hondt, 2006; Sørensen and Teske, 2006; Tiquia et al., 2006; Wilms et al., 2006). The correlations leading to this statement have been obtained from the studies characterizing marine sediments along vertical profiles. In this study, we focused on seasonal change in microbial diversity of 10 horizontally distant (>5 km) sediments rather than depth-related gradient of physicochemical and microbiological sediment characteristics.

Statistically significant r values ($p<0.05$, $n=46$) between the microbiological and the chemical characteristics were shown in Figure 6.6. As seen, the microbial community structures were strongly related to the chemical compositions of the sediments, and/or the biological diversity drove the chemical diversity through niche creation. In other words, the chemical and biological diversity formed positive

feedback loops. As far as we know, this conclusion has not been derived from comparison of physically distant sediments.



p<0.05, n=46
Community composition: PC1 of the DGGE data

Figure 6.6: Correlations between microbial and chemical characteristics

Heavy metals-active cell abundance

The active cell percentage was higher where the Cu and Ni levels were higher. Both Cu and Ni are active part of many enzymes catalyzing anaerobic/anoxic reactions and transformations (Fermoso et al., 2008). Ni also stabilizes nucleic acid molecules which may be related to conservation of cellular rRNA content in the sediments.

N-P content-active and total cell abundance

The cell abundance and activity were strongly related to the N/P ratios and the N-P levels. This is an example of the resource ratio theory (Tilman, 1982) which postulates that different concentrations and ratios of N and P will select for the organisms most able to utilize the nutrients at the levels provided in the habitat.

The active part of the total cell content was related to the dissolved level rather than the total level of N-P. This is because the dissolved N-P levels were very low to

sustain exponential growth of marine bacterioplankton (Vrede et al., 2002). In other words, N and P were limited in the MSS porewaters for biological activity.

Species richness-Archaea/Bacteria-organic carbon

Organic carbon levels were related to dominancy of *Archaea* over *Bacteria* in terms of relative abundance and species richness. This is an example of the energy limitation hypothesis, which posits that the amount of energy available to the ecosystem limits species richness by limiting the density of its taxa (Kaspari et al., 2000).

Community Structure, e^- acceptor, terminal e^--accepting process

The interstitial water chemistry of coastal marine sediments generally exhibits a predictable zonation of different redox processes which has been ascribed to competition between metabolic pathways (D'Hondt et al., 2004). Vertical cascades of e^--accepting processes from the sediment surface are successively sustained as NO_3^-, Mn(IV), Fe(III), SO_4^{2-} reduction and methanogenesis. In organic-rich marine sediments, although heterotrophic microbial populations deplete electron acceptors quickly, formation of all the redox zones shallower than 100 cm below the seafloor (cmbsf) has not been reported yet. In this study, the results represented the overall microbial and chemical composition of the MSS in the first 15 cmbsf. The MSS were codominated by sequences belongs to denitrifying, sulfate reducing, fermentative and methanogenic microorganisms which is an indicator of formation of all the redox zones. The succession of these e^--accepting processes in such a short distance below the sediment surface has not been reported yet.

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. Hence, changes in the e^--acceptor levels were reflected in the microbial community compositions. As seen in Fig 6.6, the dominancy of sulfate reducing or denitrifying bacterial populations was related to the level of relevant e^--acceptor. The fermentative bacteria percentage increased as salinity (anoxic e^--acceptors) decreased.

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.

6.1.5 Concluding remarks on microbial diversity of Marmara Sea sediments

The Marmara Sea sediments were very rich in terms of hydrocarbon, nitrate, Ni and microbial cell content whereas N and P were limited in the porewaters. The microbial communities were dominated by *Euryarchaeota* and *Proteobacteria* as well as the MAC1 and the MBC2 which were archaeal and bacterial phylogenetic clusters unique to the Marmara Sea. Metabolically diverse organisms, mainly denitrifiers, sulfate reducers, fermenters and methanogens, coexisted within a very short distance from the sediment surfaces.

In the MSS, local and seasonal differences in the microbial community structure were strongly related to the changes in the chemical characteristics. The changes in the microbial community structure occurred in terms of relative abundances of the microbial species rather than the species types present. The large fraction of the cells was active; and the activity was strongly related to the nutrient and heavy metal contents. Electron donors were not limited in the MSS. Microbial community composition was related to the scarcity of electron acceptors.

Organic carbon levels were related to dominancy of *Archaea* over *Bacteria* in terms of relative abundance and species richness.

6.1.6 Future prospects on microbial diversity investigations

Existence of metabolically diverse communities within a very short distance from the sediment surfaces and uncommonly high concentrations of NO_3^- make the Marmara Sea Sediments perfect candidates for further investigation of ecologically important microbial processes. Recent observations indicated that present conceptual views of denitrification and pathways of nitrate reduction and N_2 formation are incomplete (Hulth et al., 2005). The Marmara Sea floor is a promising environment for further investigations on alternative N cycle pathways. Another example is sulfate-methane

transition zones (SMTZ) in which anaerobic oxidation of methane (AOM) occurs. SMTZs have not been reported to be formed shallower than 70 cmbsf (Leloup et al., 2007; Parkes et al., 2007; Wilms et al., 2007). Moreover, although nitrate is more energetically favorable than sulfate for AOM, AOM coupled to nitrate reduction in marine environments has not been reported yet. In the MSS, AOM coupled to both sulfate and nitrate reduction is possible since nitrate reducing, sulfate reducing and methanogenic organisms were coabundant along with their respective e^--acceptors in a very short distance (15 cm) below the sediment surface. We will direct our future research efforts on these speculations about the Marmara Sea Sediments.

6.2 Metabolic Activity Variations in Marmara Sea Sediments

In this chapter, the experimental results on physiological properties of Marmara Sea Sediments (MSS) organisms were discussed based on abundance and transcription level of functional genes responsible for the most important sub-seafloor microbial processes.

rRNA-based approaches can be used for metabolic function investigations as long as the target function is carried by a certain phylogenetic group of organisms such as methanogenic *Achaea* (MA). If the function is mediated by a diverse polyphyletic group of organisms, such as respiratory denitrification (RD), rRNA-based approaches are of limited value. Instead, genes that encode key enzymes of important metabolic processes can be targeted (Smith et al., 2007).

DNA may persist in intact but inactive cells as well as in the environment as extracellular material after cell death (Naviaux et al., 2005). In contrast to genomic DNA, ribosomes are being continuously turned over in cells and rRNA concentration is correlated to the growth rate (Kerkhof and Kemp, 1999). Thus, by analyzing rRNA and mRNA rather than genomic DNA in sediment samples, it is possible to specifically target the active part of the microbial community (Sørensen and Teske, 2006**).**

Quantitative real-time PCR (Q-PCR) have been widely used for quantification of gene abundances in environmental samples (Winderl et al., 2008; Higashioka et al., 2009). To investigate gene expression, Q-PCR can be combined with an initial reverse transcription (RT) reaction (Q-RT-PCR) to determine gene transcript

numbers in environmental systems. So far, such studies have been limited to the analysis of gene expression in aqueous systems or to quantify gene expression in individual species (Fey et al., 2004). There were a few studies that quantify functional gene transcripts in marine sediments (Smith et al., 2007; Chin et al., 2008). These studies targeted specific processes such as RD or dissimilatory sulfate reduction (DSR). This study reported the most comprehensive study of functional genes and their transcripts in sub-seafloor using Q-PCR. The target microbial processes and related functional genes were summarized in Figure 6.7. The Q-PCR primers for hzoA, assA and bssA genes were designed in this study.

6.2.1 HzoA, bssA and assA clone libraries

Diversity indices and rarefaction curves for the clone libraries were shown in Table 6.3 and Figure 6.8. The detected clone types were given in Table 6.4. The rarefaction curves for all of the clone libraries reached an asymptote, and the all clone types were encountered more than once which predicting that all of the probable unique phylotypes were analyzed (Parkes et al., 2005). 14, 12 and 9 different sequence type of hzoA, bssA and assA genes were observed in the clone libraries. MSS can be assumed as very diverse in terms of hzoA, bssA and assA sequence types (Quan et al., 2008; Callaghan et al,. 2008; Winderl et al., 2007).

Table 6.3: Diversity indices for hzoA, assA and bssA clone libraries

Clone Library	Schao1[a]	H?[b]	J'[c]	Coverage
hzoA	14	1,1	0,42	1
assA	9	0,87	0,40	1
bssA	12	1,04	0,42	1

[a]Chao1 estimator of species richness
[b]Shannon-Weaver Index of Diversity
[c]Evenness

The bssA and hzoA primer sets did not amplify any non-target sequence. 4% of the sequences obtained using assA primers were bssA of *Magnetospirillum*. Since frequency of *Magnetospirillum*'s bssA was very low in both of bssA and assA clone libraries, the assA primer set was used in further Q-PCR assays.

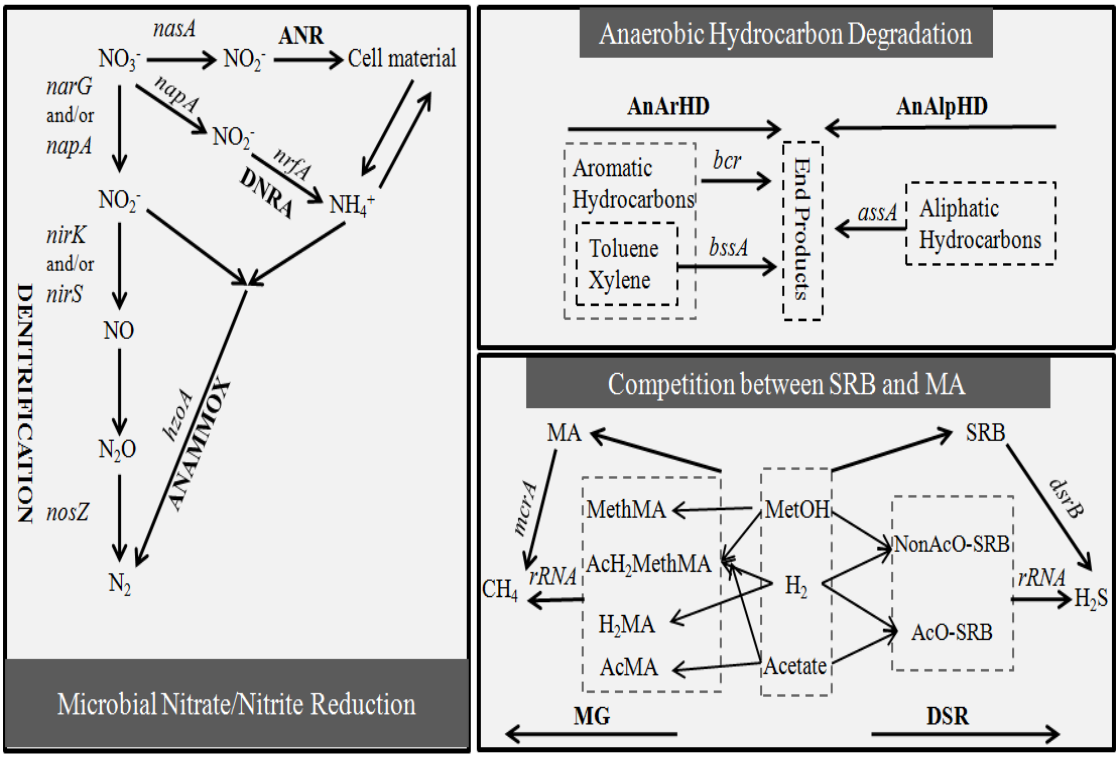


Figure 6.7: Target genes and the relevant metabolic processes

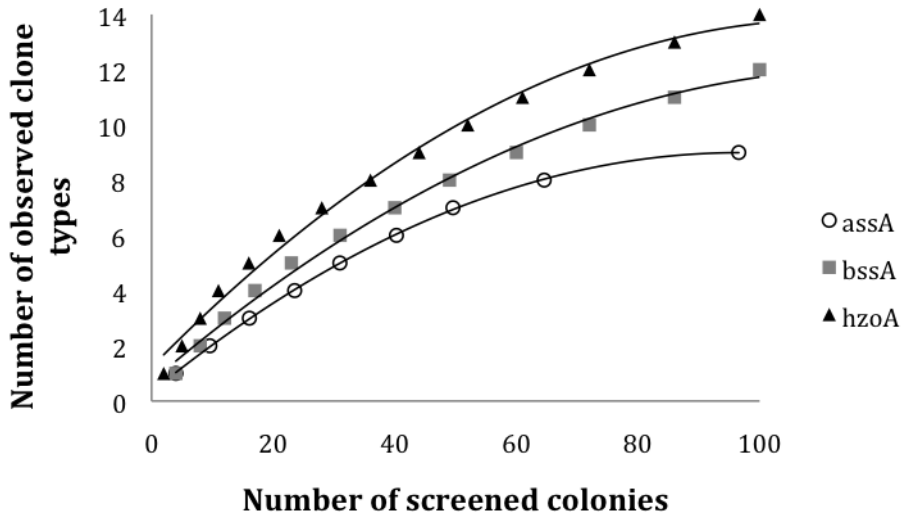


Figure 6.8: Rarefaction curves for hzoA, assA and bssA clone libraries

6.2.2 Correlating gene/transcript abundance with chemical diversity

In this study, we focused on local and seasonal changes in overall microbiological characteristics in the first 15 cm below the seafloor (cmbsf) rather than depth-related gradient of the sediment characteristics. Correlations between the quantitative results obtained in this study and in the accompanying study (Kolukirik et al., 2010) were shown in Figure 6.9. High correlations were obtained ($r>0.8$, $p<0.05$, $n=47$) despite

the considerably distant sampling dates (> 3 months) and locations (> 5 km). Microbial community structure changes in MSS can be explained based on chemical divergence of the sediments, and/or the biological diversity drove the chemical diversity through niche creation. In other words, chemical and biological diversity formed positive feedback loops in MSS.

Table 6.4: Clone types of partial hzoA, assA and bssA genes

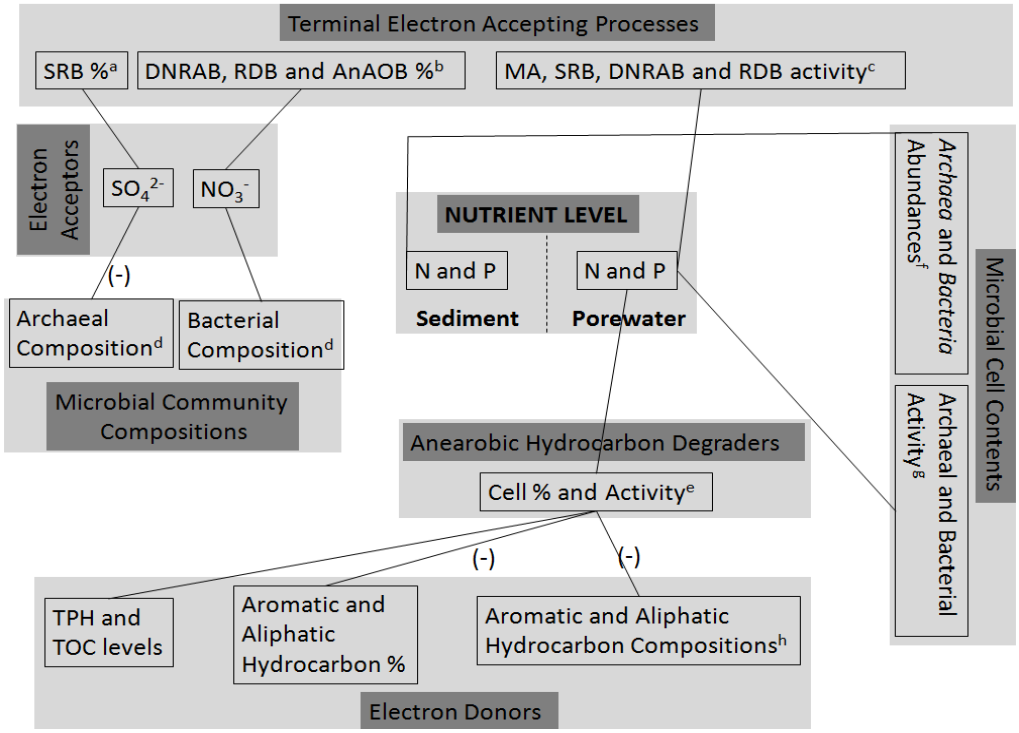
Clone Type	Gene	This Study[a]	F (%)[b]	Relative[c]	S[d] (%)	Organism
hzoA_MSS1	hzoA	GU477505	8	AB257585	93	*Planctomycete*
hzoA_MSS2	hzoA	GU477506	8	FJ901346	93	*Planctomycete*
hzoA_MSS3	hzoA	GU477507	11	FJ901345	93	*Planctomycete*
hzoA_MSS4	hzoA	GU477508	9	FJ901344	93	*Planctomycete*
hzoA_MSS5	hzoA	GU477509	5	EU294367	93	*Planctomycete*
hzoA_MSS6	hzoA	GU477510	7	EU294366	94	*Planctomycete*
hzoA_MSS7	hzoA	GU477511	3	EU294365	92	*Planctomycete*
hzoA_MSS8	hzoA	GU477512	14	ACF95877	95	*Planctomycete*
hzoA_MSS9	hzoA	GU477513	5	EU862268	93	*Planctomycete*
hzoA_MSS10	hzoA	GU477514	14	EU862267	94	*Planctomycete*
hzoA_MSS11	hzoA	GU477515	8	EU862266	92	*Planctomycete*
hzoA_MSS12	hzoA		3	AB257585	97	*Planctomycete*
hzoA_MSS13	hzoA		3	ACF95877	100	*Planctomycete*
hzoA_MSS14	hzoA		2	FJ901345	98	*Planctomycete*
assA_MSS1	assA1	GU477516	7	DQ826036	90	*δ-Proteobacteria*
assA_MSS2	assA1	GU477517	9	DQ826036	93	*δ-Proteobacteria*
assA_MSS3	assA1	GU477518	6	DQ826036	93	*δ-Proteobacteria*
assA_MSS4	assA1	GU477519	15	DQ826036	92	*δ-Proteobacteria*
assA_MSS5	assA2	GU477520	32	DQ826035	94	*δ-Proteobacteria*
assA_MSS6	assA2	GU477521	9	DQ826035	93	*δ-Proteobacteria*
assA_MSS7	assA2	GU477522	9	DQ826035	92	*δ-Proteobacteria*
assA_MSS8	assA2	GU477523	7	DQ826035	93	*δ-Proteobacteria*
assA_MSS9	assA2	GU477524	2	DQ826035	91	*δ-Proteobacteria*
bssA_MSS1	bssA	GU477525	11	AY032676	94	*Azoarcus*
bssA_MSS2	bssA	GU477526	8	CR555306	94	*Azoarcus*
bssA_MSS3	bssA	GU477527	14	AJ001848	94	*Thauera*
bssA_MSS6	bssA	GU477530	9	NC_006513	94	*Aromatoleum*
bssA_MSS7	bssA	GU477531	5	AB167725	93	*Magnetospirillum*
bssA_MSS12	bssA		4	NC_006513	98	*Aromatoleum*
bssA_MSS4	bssA	GU477528	12	CU466268	94	*Desulfobacula*
bssA_MSS8	bssA	GU477532	14	EF123663	92	*Desulfobacula*
bssA_MSS9	bssA	GU477533	9	EF123667	93	*δ-Proteobacteria*
bssA_MSS11	bssA		4	CU466268	100	*Desulfobacula*
bssA_MSS5	bssA	GU477529	4	EF123664	93	*Geobacter*
bssA_MSS10	bssA	GU477534	6	EF123666	93	*Geobacter*

[a]Accession number of the sequence obtained in this study

[b]Frequency of the observed clone type

[c]Accession number of the most similar sequence in the database

[d]Similarity to the most similar sequence in the database



$p<0.05$, n=46
Correlated variables were connected using the lines.
Only the variables between which pearson product-moment correlation coefficient (*r*) values were higher than 0.8 were connected.
Negative correlations were indicated as (-).
[a] Relative dsrB gene abundance. SRB % was also correlated (r>0.85) to relative abundances of acetate oxidizing SRB and non-acetate oxidizing SRB.
[b] Relative abundances of the genes (nirK, nirS, narG, napA, nosZ, nrfA, hzoA) related to respiratory denitrification *Bacteria* (RDB), dissimilatory nitrate reducing *Bacteria* (DNRAB), ANAMMOX *Bacteria* (AnAOB). *R* between relative abundance of these genes was between 0.85 and 0.92.
[c] mRNA levels of RDB (nosZ mRNA), DNRAB (nrfA mRNA), SRB (dsrB mRNA) and methanogenic *Archaea* (MA) (mcrA mRNA). *R* between relative abundance of these gene transcripts was between 0.9 and 0.95.
[d] Bacterial and archaeal community compositions denote the first principal component (PC1) of the denaturant gradient gel electrophoresis (DGGE) data (Kolukirik et al. 2010).
[e] Relative abundances and mRNA levels of the genes (bssA, assA, bcrA) related to anaerobic hydrocarbon degradation. *R* between these variables was between 0.85 and 0.94.
[f] Total cell counts for *Archaea* and *Bacteria* which were determined using both FISH and Q-PCR. *R* between the Q-PCR counts and the FISH counts was higher than 0.92. *R* between *Archaea* and *Bacteria* abundance was 0.8.
[g] rRNA levels of *Archaea* and *Bacteria*. *R* between *Archaea* and *Bacteria* rRNA level was 0.96.
[h] Aromatic and aliphatic hydrocarbon compositions denote PC1 of aromatic and aliphatic hydrocarbon components distribution data.

Figure 6.9: Correlations between microbial and chemical characteristics

6.2.3 Total cell activity and abundance

Comparison of Q-PCR counts with microscopic counts

The Q-PCR counts for *Archaea* and *Bacteria* were shown in Figure 6.10. The total cell abundances (*Archaea* Q-PCR count + *Bacteria* Q-PCR count) were 6-12%

higher than the DAPI cell counts (Kolukirik et al., 2010) which implies that a minor part of total DNA in MSS preserved as detrital, extracellular DNA (Dell'Anno and Corinaldesi, 2004).

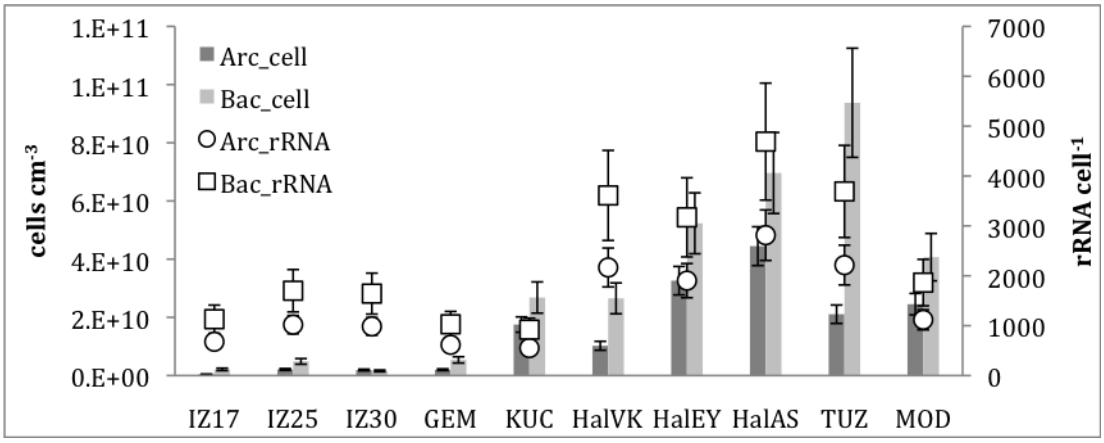


Figure 6.10: Cell and rRNA abundances

To convert gene abundances into cell numbers, averages of 3.6 and 1.6 copies of 16S rRNA gene were estimated for *Bacteria* and *Archaea*, respectively (Klappenbach et al., 2001). Series values show the average levels and error bars show the fluctuation ranges

The visual information from FISH (Kolukirik et al., 2010) verified accuracy of the quantitative estimates of Q-PCR. *R* values between the Q-PCR counts and the FISH counts for *Archaea* and *Bacteria* were 0.92 and 0.97 ($p < 0.05$, n= 47) respectively. The Q-PCR counts were 20-50 % higher than the FISH counts which implied that 50-80% of the Marmara sub-seafloor cells were active.

Archaeal and bacterial cell abundances

Total cell abundances in MSS were in the upper limit of 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). N-P availability was the main factor affecting cell enrichment in the sediments (Figure 6.9).

Archaeal and bacterial cell activities (rRNA contents)

Comparative evaluation of MSS with other marine sediments in terms of total rRNA level is not possible since there were a few studies that quantify gene transcripts in marine sediments (Smith et al., 2007; Chin et al., 2008). Extraction of RNA from

marine sediment samples is particularly difficult because of low metabolic activity of marine benthic prokaryotes and complex sediment matrices. rRNA numbers per cell in MSS were high when compared to the levels reported for marine oligotrophs (Fegatella et al., 1998). On the other hand, the rRNA levels were very low compared to that of exponentially growing *E. coli* (Bremer and Dennis, 1996). Total rRNA levels in MSS were positively correlated to the dissolved N-P contents (Figure 6.9).

6.2.4 Terminal electron accepting processes

Abundance and transcription levels of dsrB, mcrA, nosZ, nrfA and hzoA genes are indicators of abundance and activity of DSR bacteria (SRB), MA, RD *Bacteria* (RDB), dissimilatory nitrate reduction to ammonia (DNRA) *Bacteria* (DNRAB) and ANAMMOX *Bacteria* (AnAOB) respectively. SRB, MA, RDB, DNRAB and AnAOB were active and abundant in the first 15 cmbsf (Figure 6.11). As seen in Figure 6.9, their abundances were positively correlated to the relevant e^--acceptor levels (NO_3^- and SO_4^{2-}) while their activities were positively correlated to the dissolved N-P levels.

Cell abundances

Nitrate/nitrite reducing bacteria (NRB) (nrfA+hzoA+nosZ) dominated IZ25, TUZ and MOD sediments; MA dominated HalEY and HalAS sediments; and SRB dominated GEM sediments. IZ17, IZ30, KUC and HalVK sediments were co-dominated by SRB, NRB and MA. RDB, AnAOB, DNRAB, SRB and MA abundances in MSS were in ranges 2×10^8-1.5×10^{10}, 10^8-10^{10}, 3×10^8-3×10^{10}, 5×10^8-10^{10} and 4×10^8-4×10^{10} copies cm^{-3} sediment respectively. Previously reported abundances of these microbes in marine sediments were in ranges of 10^5-10^9, $<10^8$, 10^5-10^7, 10^6-10^9 and 10^2-10^7 copies cm^{-3} sediment respectively (Schmid et al., 2007; Smith et al., 2007; Wilms et al., 2007; Colwell et al., 2008). It can be concluded that all of these microbial groups were very abundant (at least an order of magnitude) compared to those in previously studied marine environments.

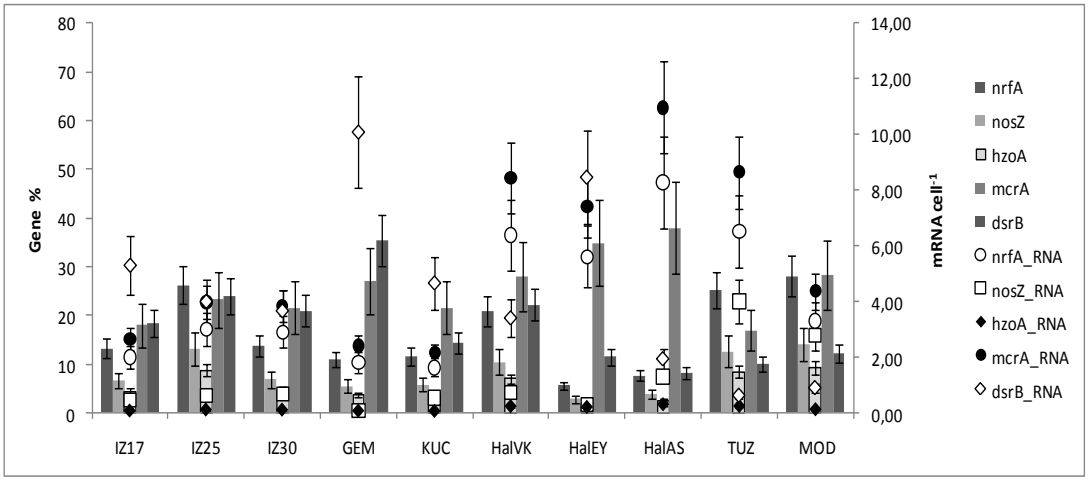


Figure 6.11: Functional gene and transcript abundances

Cell activities (mRNA contents)

Information on functional gene transcript numbers in marine sediments is very limited (Smith et al., 2007; Chin et al., 2008) since mRNAs in prokaryotes exhibit very short half-lives (Golding et al., 2005) and marine benthic prokaryotic activity levels are very low. Here we present the first report on mRNA copy numbers per cell for specific genes responsible for the key metabolic processes in marine sediments. MA, SRB and DNRAB were the most active prokaryotes in MSS. MA activity was high in all sampling locations. Substantial DSR activities were detected in IZ17, GEM and KUC sediments. DNRAB was considerably active in HalVK, HalAS and TUZ sediments. AnAOB and RDB were the least active microbes in MSS. Although AnAOB was abundant, their activity level was considerably low compared to the other microbial groups.

6.2.5 Activity and abundance of nitrate/nitrite reducing community

As seen in Figure 6.11 and 6.12, relative abundances of the N cycling genes were similarly distributed in all of the sediment sampling locations as ~1/1.5/2/2.5/3/3/6/7 for nasA/nirK/hzoA/nirS/narG/nosZ/nrfA/napA. Relative transcription levels of these genes can also be ranked as ~1/3/3/6/9/9/27/30 for hzoA/nasA/nirK/nirS/narG/nosZ/nrfA/napA. It can be concluded that DNRA was the most important pathway of nitrate reduction in MSS. AnAOB activity was substantially lower than that of the other microbial groups.

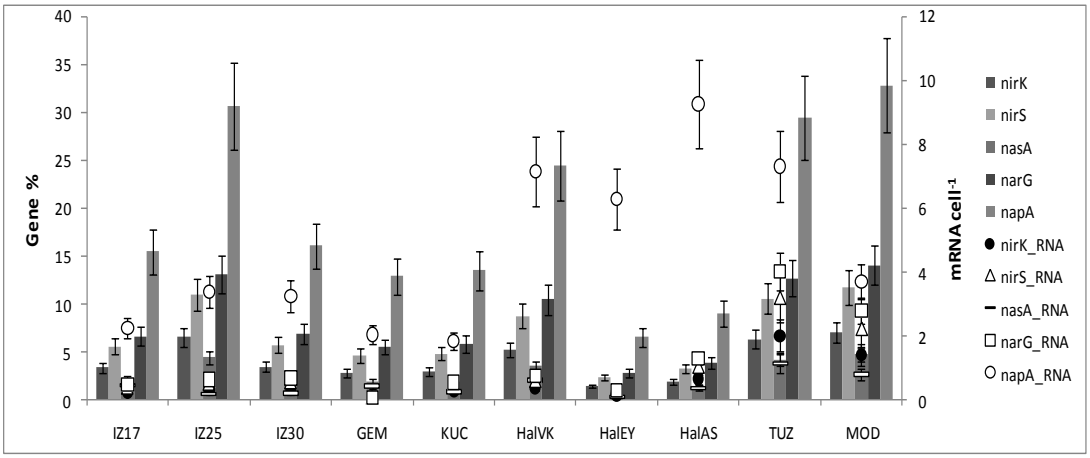


Figure 6.12: Cell and mRNA abundances of nitrate reducing microbial groups

6.2.6 Activities and abundances of methanogens and SRB

Description of the methanogenic and sulfate reducing microbial group names was given in Figure 1. AcH_2MethMA and NonAcO-SRB were the most dominant microbial groups (Figure 6.13). They were co-dominant in IZ, KUC and HalVK sediments. NonAcO-SRB dominated GEM sediments whereas AcH_2MethMA dominated HalEY, HalAS, TUZ and MOD sediments. NonAcO-SRB was the most active group in MSS. Activity levels of AcO-SRB and AcH_2MethMA were similar and considerably higher than the other methanogenic groups.

6.2.7 Petroleum hydrocarbons and the related microbes

Components of aliphatic hydrocarbons (AlpH) and aromatic hydrocarbons (ArH) in MSS were shown in Figure 6.14. AlpH and ArH indices for determination of contributions from biogenic, pyrolytic and petrogenic pollution sources can be find elsewhere (Ahmed et al., 2006; Baumard et al., 1998). The calculated index values (data were not shown) were suggested that both AlpH and ArH in MSS originated from petroleum and its products.

Genetic markers for anaerobic hydrocarbon degradation were shown in Figure 6.7. AnArHD was monitored by targeting genes and transcripts of bcrA (Higashioka et al., 2009) and bssA (Winderl et al., 2008) while assA gene (Callaghan et al., 2008) and its transcript were used as indicators of AnAlpHD. Bacteria which carry one of the functional genes for anaerobic hydrocarbon degradation were named as AnHDB. 3-40 % of the total prokaryotic cells in MSS were carrying the genes responsible for AnHD (Figure 6.16). BcrA, bssA and assA transcription levels were as high as those

of nrfA, mcrA and dsrB showing that AnHD process was as active as DNRA, MG and DSR.

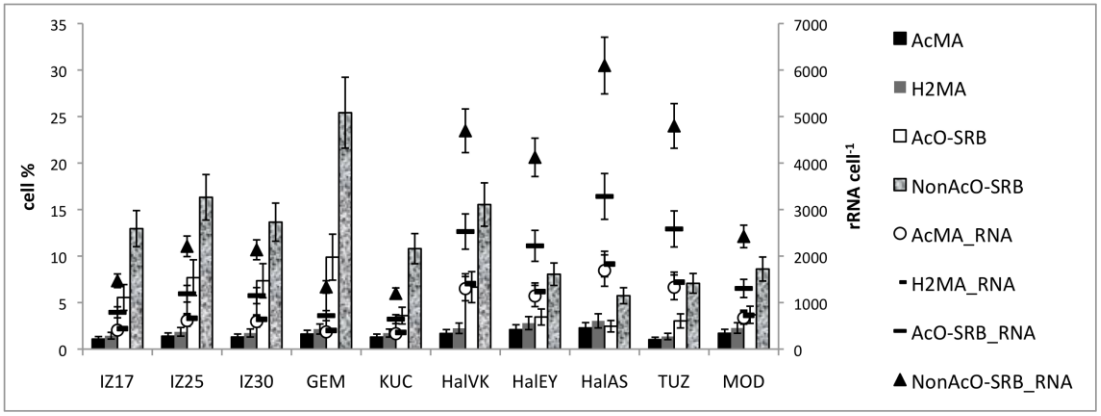


Figure 6.13: Cell and rRNA abundances of methanogens and SRB

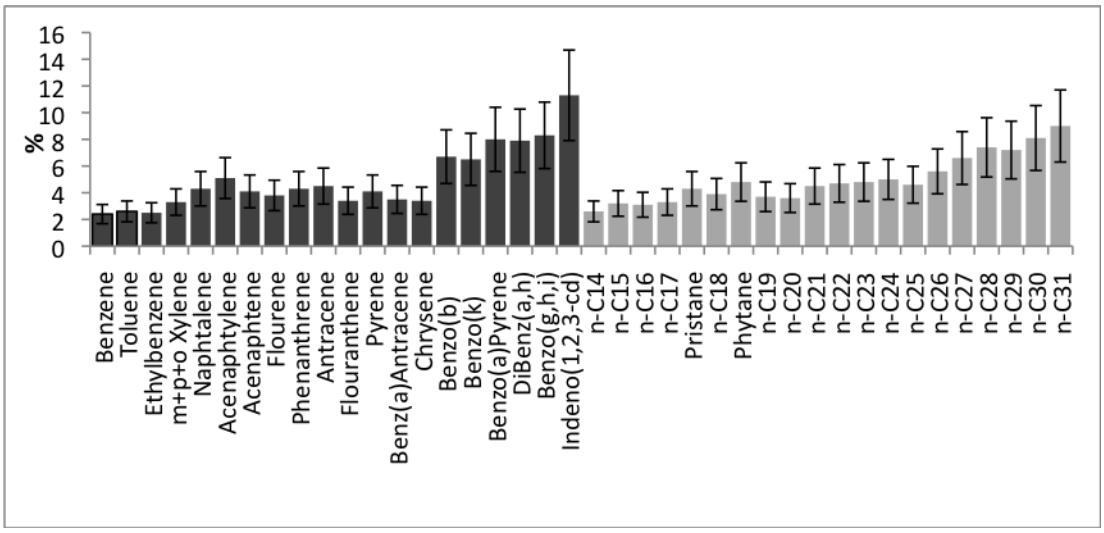


Figure 6.14: Levels of aliphatic and aromatic hydrocarbon fractions

Levels of TPH and its fractions in MSS (Figure 6.15) were similar to those from extremely polluted marine environments (Guerra-García et al., 2003; Ahmed et al., 2006; Tolun et al., 2006; Kolukirik et al., 2010). MSS can be considered as highly and chronically polluted with hydrocarbons. Natural attenuation (a bioremediation strategy) can be a cost effective solution for this pollution as long as oil-degrading microorganisms are abundant and active in the sediments. In this study, abundance and activity of anaerobic hydrocarbon degrading bacteria (AnHDB) were monitored in MSS for two years in order to assess feasibility of natural attenuation.

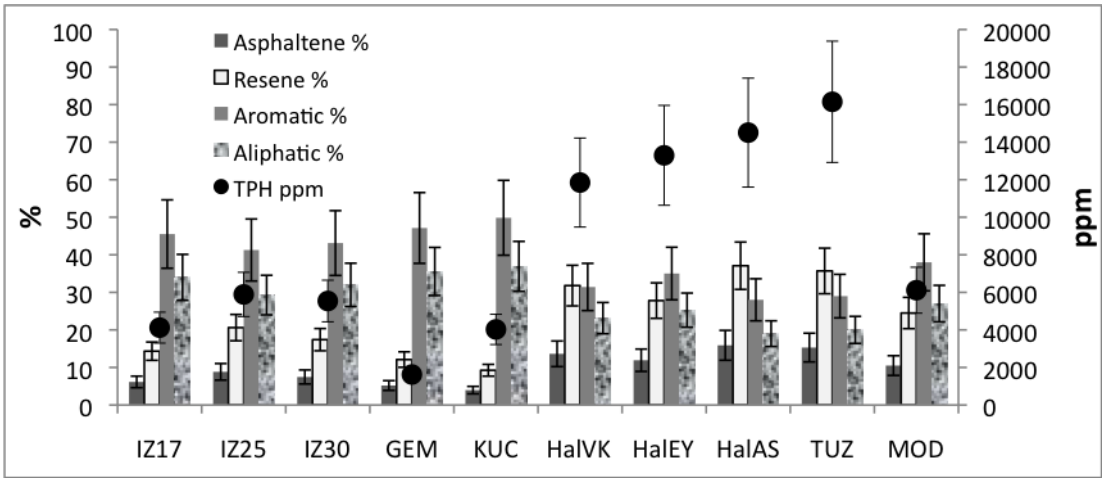


Figure 6.15: Levels of total petroleum hydrocarbons and their fractions

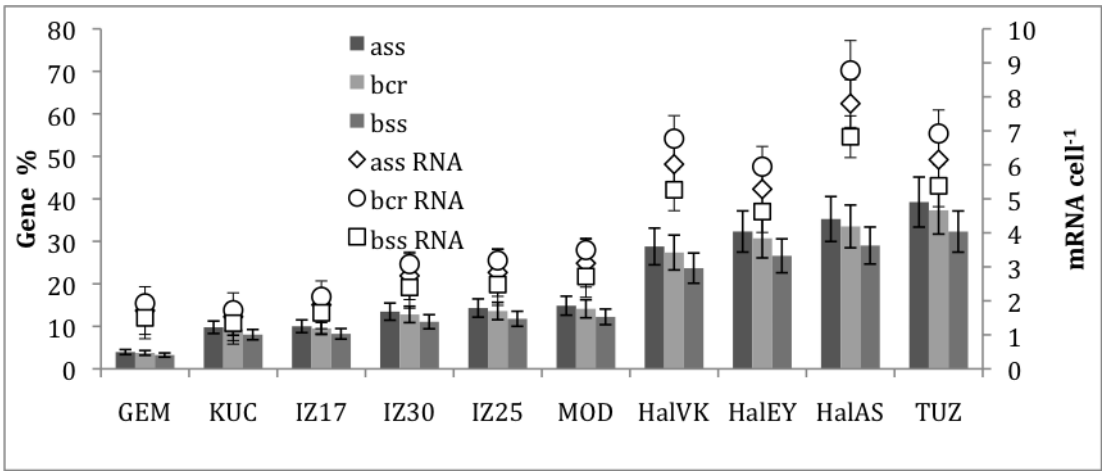


Figure 6.16: Abundances of ass, bcr and bss genes and transcripts

Statistically significant abundance and transcription level differences between bcrA, bssA and assA were not observed. They were positively correlated to each other. TPH levels, relative abundances of TPH components and aromatic and aliphatic hydrocarbon components' distributions were related to abundance and activity of AnHDB. It can be speculated based on the correlations that high sediment hydrocarbon content resulted in enrichment of AnHDB; higher abundance of AnHDB reduced the biodegradable fraction of TPH (aromatic and aliphatic hydrocarbons) and, thus, changed the distribution of hydrocarbon components.

6.2.8 Nutrient limited prokaryotic activity in MSS

Dissolved organic carbon (DOC)/N/P ratios of MSS porewaters (≈1000/5/1) were very high compared to C/N/P ratio (100/20/3) of the exponentially growing marine bacterioplankton (Vrede et al., 2002) indicating that N and P were limited in the

porewaters for biological activity. This conclusion was strongly supported by the high correlations between prokaryotic mRNA-rRNA levels and the dissolved N-P concentrations in MSS.

6.2.9 High abundance and activity of methanogens

Shallow marine sediments are characterized by intense and diverse microbial activities which generate steep chemical gradients (D'Hondt et al., 2004). As the products of O_2, NO_3^-, Mn(IV), Fe(III) and SO_4^{2-} reduction enter consecutively deeper zones of the sediments, vertical cascades of electron-accepting processes are sustained. Methanogenesis occurs after electron acceptors that yield higher standard free energies have been depleted. This is why high methanogenic activity has not been observed in the sediment zones shallower than 100 cmbsf. Methanogens in MSS were highly abundant and active along with NRB and SRB in 15 cmbsf. This was an expected result since electron acceptor (NO_3^- and SO_4^{2-}) levels in MSS were very low compared to the exceptionally high electron donor (TOC and TPH) contents. Scarcity of the electron acceptors was also evident from the positive correlations between the NRB-SRB abundances and the NO_3^--SO_4^{2-} levels. It can be speculated that limited amount of electron acceptors were quickly depleted in a very short distance below the MSS surfaces which resulted in succession of all the redox zones.

6.2.10 DNRA as the main pathway of nitrate reduction

Nitrate is either removed as N_2 gas by RD and ANAMMOX or converted via DNRA to biologically available NH_4^+ which is retained within the ecosystem. ANR also results in preservation of NO_3^--N in the system as a cell material. How important are these pathways in marine N cycling? This is a particularly difficult question to answer, because contribution of ANAMMOX, DNRA and ANR to marine N cycling is just beginning to be studied in detail (Burgin and Hamilton, 2007). We were able to answer this question for MSS via activity and abundance assessment of the functional genes related to N cycle. DNRA was the most important pathway of nitrate reduction in the sediments.

There are two recognized DNRA pathways: one involving fermentation and the other linked to sulfur oxidation. DNRA is mainly carried out by fermentative bacteria which can also carry out fermentation without using nitrate (Tiedje, 1988). Although

the conditions promoting fermentative DNRA and RD are similar, fermentative DNRA is thought to be favored in nitrate-limited environments rich in organic carbon, while RD would be favored under carbon-limited conditions (Burgin and Hamilton, 2007). Tiedje (1988) argued that high labile carbon availability would favor organisms that used electron acceptors most efficiently; DNRA transfers eight electrons per mole of nitrate reduced, whereas RD only transfers five. Dominancy of DNRA over RD was not surprising in organic carbon rich MSS.

6.2.11 High AnAOB abundance against low ANOMMOX activity

Although hzo enzyme which oxidizes the unique ANAMMOX intermediate is known, 16SrRNA gene of *Planctomycetes* has been targeted to quantify AnAOB (Li et al., 2009). Because the all *Planctomycetes* are not AnAOB, hzoA gene is a better target for quantitative assessment of AnAOB. The designed Q-PCR primer set in this study specifically amplified the hzo genes from 14 different *Planctomycetes*. Application of this primer set to quantify AnAOB in different environments will extend our knowledge beyond what is currently known about the microbial ecology of ANAMMOX.

High abundance of AnAOB in MSS (2-9 % of the total cells) compared to the other marine ecosystems can be attributable to the relatively high NO_3^- concentrations in MSS porewaters compared to the typically low nitrate levels of seawater. This was also evident from the high positive correlation between the AnAOB abundance and the nitrate content of the sediments.

ANAMMOX in marine ecosystems accounted for 0−80% of the total N_2 production (Burgin and Hamilton 2007). ANAMMOX activity in MSS was very low despite the high AnAOB abundance. This was probably due to inhibition of the process by simple organic compounds (Dalsgaard et al., 2005) which were originated from the high organic carbon content of the sediments. Within the various sedimentary studies, the importance of ANAMMOX relative to RD increased with increasing water depth because of the accompanying increase in C/N ratio (Brandes et al., 2007).

6.2.12 High abundance of non-acetate-oxidizing SRB

DSR and MG are considered to be the most important processes in marine sediments, and they consistently co-occur (Smith and D'Hondt, 2006). 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; Wilms et al., 2006). Distribution of MA correlates with sulfate and methane profiles and can be explained by electron donor competition with SRB (Stams et al., 2006). In this study, abundance and activity of SRB and MA, and their sub-trophic groups were monitored to evaluate the competition between SRB and MA in MSS.

Competition for acetate

Acetate can be consumed by AcH_2MethMA, AcMA and AcO-SRB. Acetate is mainly consumed by AcO-SRB when sufficient sulfate is present (Stams et al., 2006). Growth kinetic properties of AcO-SRB on acetate are only slightly better than those of AcMA at low acetate concentrations. AcMA and AcO-SRB have higher affinity for acetate than that of AcH_2MethMA. Putting all kinetic information together, it seems that AcH_2MethMA will dominate over AcO-SRB and AcMA at high acetate and low sulfate concentrations (Stams et al., 2006).

Acetate oxidation by AcO-SRB may be limited in sulfate rich zones of MSS because the big majority of the available sulfate seems to be depleted by overwhelmingly abundant and active NonAcO-SRB. In the sulfate depleted zones, it looked as if acetate concentration was high because AcH_2MethMA devastatingly dominated over both AcMA and AcO-SRB. High NonAcO-SRB activity in the upper layers of MSS could have contributed acetate input to lower zones since they can excrete acetate as an end product.

Competition for H_2

H_2 can be consumed by SRB, AcH_2MethMA and H_2MA. AcH_2MethMA and H_2MA have similar kinetic properties on H_2. SRB have higher growth rate and affinity on hydrogen compared to AcH_2MethMA and H_2MA (Stams et al., 2006). In the presence of sulfate, SRB will win the competition for H_2.

In methanogenic environments, H_2 partial pressure is low. If both SRB and MA actively consume H_2, the hydrogen partial pressure becomes so low that thermodynamically hydrogenotrophic methanogenesis is not possible any more. Both NonAcO-SRB and AcH_2MethMA were highly abundant and active in MSS. It can be speculated that H_2 was mainly consumed by NonAcO-SRB in MSS; the AcH_2MethMA dominance was due to their growth on acetate, MetOH and non-competitive substrates such as methylamines. AcH_2MethMA has much higher affinity and specific growth rate on MetOH compared to that of NonAcO-SRB. Regardless of sulfate level, AcH_2MethMA will win the competition for MetOH.

Messenger RNA is a better target than rRNA for comparative evaluation of DSR and MG

NonAcO-SRB rRNA contents were considerably higher than that of AcH_2MethMA. These results were not consistent with the activity results obtained by targeting functional genes which revealed that MG was taking place more actively than DSR in MSS. The rRNA-based approach is of limited value for investigation of DSR activity since many SRB can also carry out DNRA and/or fermentation (Madigan et al., 2009). Targeting dsrB gene transcripts for assessment of DSR activity certainly gave more specific results for MSS.

6.2.13 Evidences of anaerobic/anoxic hydrocarbon degradation

Bss enzyme catalyzes the first step of anaerobic toluene and xylene (TX) degradation. Advantage of bssA for *in situ* monitoring of TX degradation is its relevance to physiologically and phylogenetically diverse bacteria. The presence of bssA has been observed in cultures that degrade toluene under denitrifying (Beller et al. 2002), sulfate-reducing (Beller et al., 2008), ferric iron-reducing (Kane et al. 2002), and methanogenic conditions (Washer and Edwards, 2007). A general PCR primer pair which can amplify bssA from all of these microbial groups has not been designed yet. The previously designed PCR primers targeted certain microbial groups or certain clusters of previously identified bssA (Winderl et al., 2008). In this study, we reported the most generic Q-PCR primer set for bssA which specifically amplified bssA genes from various microbial groups in MSS.

Although the first description of a gene involved in anaerobic n-alkane metabolism (alkylsuccinate synthase) have recently been reported (Callaghan et al., 2008), none

has attempted to target this gene for monitoring AnAlpHD. In this study, we reported the first Q-PCR primer set targeting assA, and verified its applicability to marine sediments for tracking anaerobic aliphatic hydrocarbon degraders and their activities.

It is only in the last twenty years that the microorganisms and the mechanisms involved in anaerobic hydrocarbon biodegradation have been extensively studied (Fuchs, 2008; Grossi et al., 2008) and it has only recently been widely accepted that it takes place in marine environments (Harayama et al., 2004). As far as we know, our study is the first quantitative activity and abundance assessment of AnHDB in marine sediments. Application of the newly designed bssA and assA primer sets to various environments will expand our understanding of how widespread anaerobic/anoxic hydrocarbon degradation is in the environment.

High abundance of the genes related to AnHD, detection of their transcripts and the correlations between AnHD activity and TPH composition were the evidences of microbial anaerobic/anoxic hydrocarbon degradation in MSS. Dissolved N and P were limited in MSS for AnHD activity. These indications raised the question that AnHD activity in MSS can be increased by N-P amendment to bioremediate the chronic hydrocarbon pollution.

In order to assess nutrient amendment effect on the sediment activity, we set up oil degradation microcosms at different N-P supply conditions. Microcosms were inoculated using TUZ and HalAS sediments since AnHDB were the most abundant and active in these locations. TUZ and HalAS sediments were incubated under nitrate reducing and methanogenic conditions respectively because DNRA and MG were the dominant processes in these sediments. The results revealed that it was possible to increase AnHD potential of TUZ and HalAS sediments for ~18× and ~9× respectively by N-P amendment. AnHDB in TUZ and HalAS sediments were able to degrade wide range of aliphatic and aromatic hydrocarbons some of which were used to be known as recalcitrant to biodegradation. Our studies revealed that less human intervened and more economical field-scale bioremediation of petroleum hydrocarbons is possible by increasing AnHD activity of the natural benthic prokaryotic habitants of Marmara Sea.

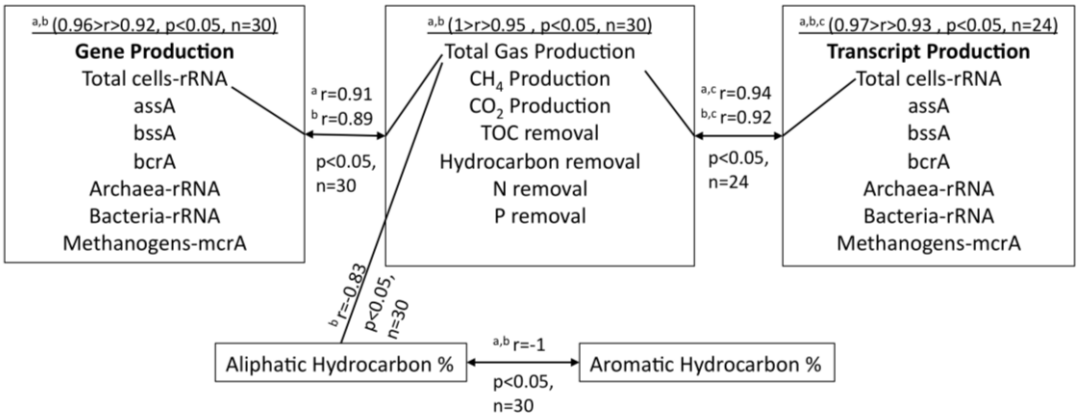
6.3 Increment in Activity of Haliç Bay Sediments via Nutrient Amendment

Haliç Bay is one of the extremely polluted anoxic area in Marmara Sea with a highly anaerobic deep sludge (Ince et al., 2006). It is an eight-kilometer long bay located in İstanbul, at a junction between Bospshorus Strait and Marmara Sea. It covers an area of 25 million m^2 and has a water surface area of 2.6 million m^2. The deepest region reaches 60 m, and the water depth decreases to 2-3 m in certain regions due to sedimentation of the wastes over the years. Efforts were made to improve the water quality of Haliç Bay by Water and Sewerage Administration of İstanbul (ISKI) and İstanbul Metropolitan Municipality (IBB). As a result, "Haliç Cleaning Project" started in the late 80's. $\sim 5\times10^6$ m^3 sludge was physically collected by screening which was followed by aeration of the water body. The water quality was improved significantly in a very short time period (15 months), on the other hand, the remediation cost was remarkably high. Haliç Bay ecosystem slowly returned its over polluted status after the completion of the project (during the last 20 years).

A cost effective remediation strategy which can be sustained for a long time with a minimal human intervention is needed to overcome the chronic hydrocarbon pollution in Haliç Bay. The best candidate for this purpose is bioremediation under anaerobic/anoxic conditions as long as oil-degrading anaerobes are abundant and active in the sediments, and there is a way to increase activity of this population (Head and Swannel, 1999). In order to assess the anaerobic bioremediation feasibility of Haliç Bay, a Turkish Academy of Sciences (TUBITAK) project was carried out. Monitoring the physicochemical and microbiological sediment characteristics revealed that (1) the total petroleum hydrocarbon levels (11000-18000 ppm) were similar to those from extremely polluted marine environments; (2) the microbial cell contents were very high compared to the other marine environments; (3) the sediments were dominated by methanogens and anaerobic hydrocarbon degraders, and these microbes were active; and (4) N and P were limited in the porewaters for biological activity. These indications raised the question that anaerobic hydrocarbon degradation activity of Haliç Bay sediments can be increased by N-P amendment under methanogenic conditions. This chapter describes assessment of this theory through methanogenic hydrocarbon degradation microcosms which were set up at different N-P supply conditions by using Haliç Bay sediments.

6.3.1 Correlation analysis

Statistically significant correlations were shown in Figure 6.17. All of the measured parameters except SRB abundance and activity were related to the gas production. The comparative evaluation of gas production data from the limiting and the unlimited nutrient microcosms reflected the relative changes in the other parameters. This is why only the results from unlimited nutrient conditions were given for the other parameters.



R values were separately calculated for HC(+) and HC(-) microcosms. A correlation range between the parameters in a same box was shown on top of the box.
[a]R values for the HC(+) microcosms.
[b]R values for the HC(-) microcosms.
[c]Since the microbial activity stopped at an uncertain time between the days 196 and 224, the data from the destructive sampling set on day 224 was omitted in the correlation analysis.

Figure 6.17: Statistically significant correlations

6.3.2 CO_2 and CH_4 production

Gas production was due to the microbial activity

The cumulative gas production data were shown in Figure 6.18 and 6.19. CO_2 and CH_4 production in sterilized and inhibited control microcosms were lower than 21 µmol; their gas production data were not shown. The much higher CO_2 and CH_4 production in HC(+) and HC(-) microcosms compared to the sterile controls indicated that the gas production activities were biological.

Addition of the dissimilatory sulfate reduction (DSR) inhibitor (Na_2MoO_4) inhibited the both DSR and methanogenic activity; the origin of CO_2 production (DSR or fermentation and methanogenesis) was discussed based on transcript abundance of the functional genes. The initial amount of SO_4 (120-127 µmol) was completely

removed before the day 126 (the data were not shown). DSR activity (dsrB transcript production) was not detected after the day 84 (Figure 6.34). Methanogenesis activity (mcrA transcript production) was very high during the all incubation period. Dissimilatory nitrate reduction (DNR) activity (nosZ and nrfA transcript production) was not observed in the microcosms. These results showed that gas production after the day 126 was originated from activities of syntrophic consortium of fermentative *Bacteria* and methanogenic *Archaea*.

Benthic microorganisms of the Haliç Bay can be activated via nutrient amendment

As seen in Figure 2 and 3, the higher initial amount of N and P was resulted in higher amount of total gas produced. Relative total gas production in HC(+) and HC(-) L/PL1/PL2/NL1/NL2/UL microcosms were ~ 1/1.3/3.7/1/3.5/7.6 and ~ 1/1.5/4.5/1/4.6/9.2 respectively. These results clearly indicated that it is possible to increase microbial activity of Haliç Bay sediments substantially by nutrient amendment.

Hydrocarbons as a favorable growth substrate for the micro-benthos of the Haliç Bay

Addition of external HCs to the microcosms was resulted in ~2× higher gas production for UL microcosms. This indicated that the added HCs were biologically more available compared to the natural C sources in the sediments for the microbial growth.

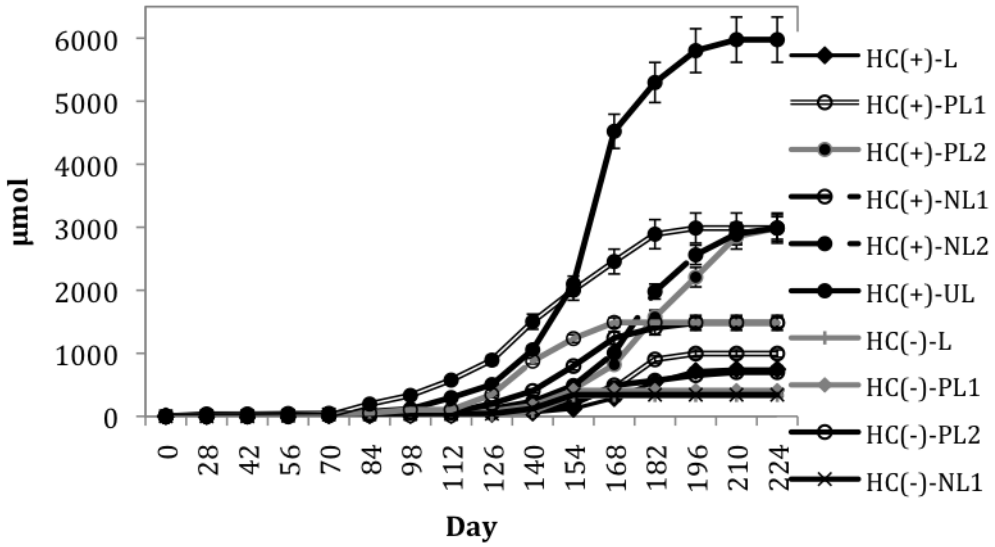


Figure 6.18: Cumulative CO_2 production in the microcosms

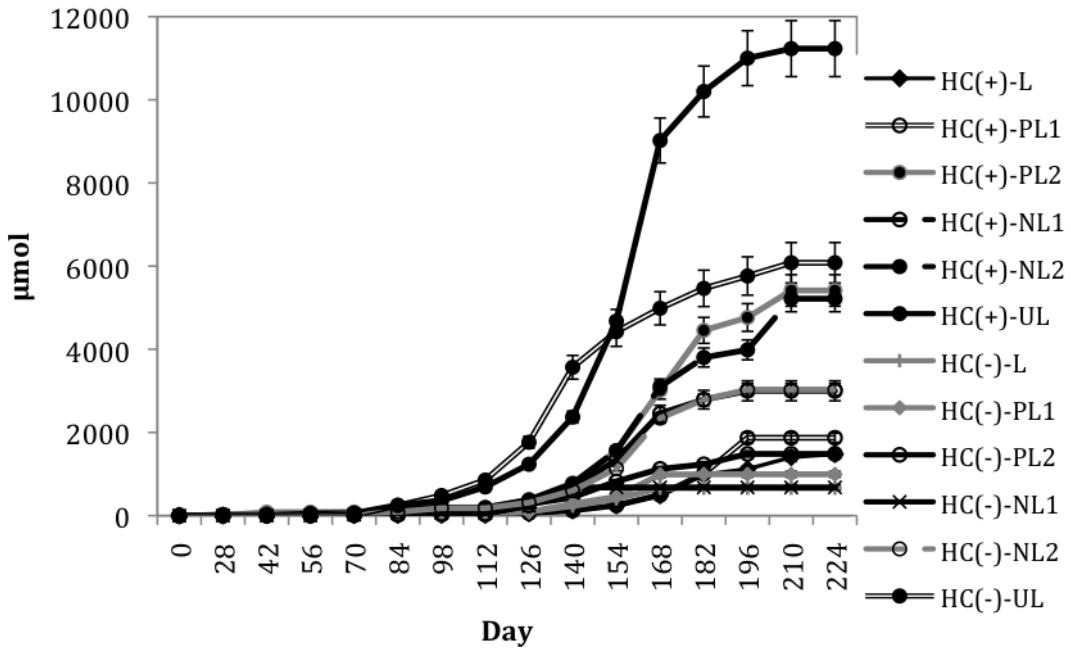


Figure 6.19: Cumulative CH_4 production in the microcosms

6.3.3 C balance

C balance in the microcosms was shown in Figure 6.20. In order to convert the removed mg HC into µmol C, molecular formula of the HC mix was calculated based on the composition of aliphatic and aromatic HCs (Figure 6.23-6.30). Molecular formulas of HC(+) microcosms on days 0, 84, 126, 168 and 224 were $C_{15}H_{28}$, $C_{15}H_{28}$, $C_{16}H_{29}$, $C_{18}H_{32}$ and $C_{20}H_{35}$; those of HC(-) microcosms were $C_{19}H_{32}$, $C_{20}H_{33}$, $C_{21}H_{34}$, $C_{24}H_{34}$ and $C_{22}H_{12}$ respectively. Cell numbers were coverted into µmol C based on the assumptions that molecular formula and average weight of a bacterial cell are $C_5H_7NO_2$ and 1 pg (Orhon and Artan 1994, Madigan 2009).

51 % and 57 % of the initial TOC were removed from HC(+)-UL and HC(-)-UL microcosms respectively. These correspond to 56 % and 97 % HC removal in HC(+)-UL and HC(-)-UL microcosms respectively.

6.3.4 N and P removal

Changes in TOC, N and P content of HC(+)-UL and HC(-)-UL microcosms were shown in Figure 6.21 Initial TOC/N/P ratios of the both microcosms were adjusted to be 1000/40/6. Interestingly, addition of external HCs resulted in higher N and P consumption rate for HC(+)-UL microcosms. TOC/N/P removed from HC(+)-UL microcosms at 1000/78/12 ratio which resulted in incomplete C removal. Although TOC/N/P removed from HC(-)-UL microcosms at the expected ratio (1000/47/7), C

removal stopped before the all N and P depleted. This was probably due to depletion of the biodegradable fraction of TOC.

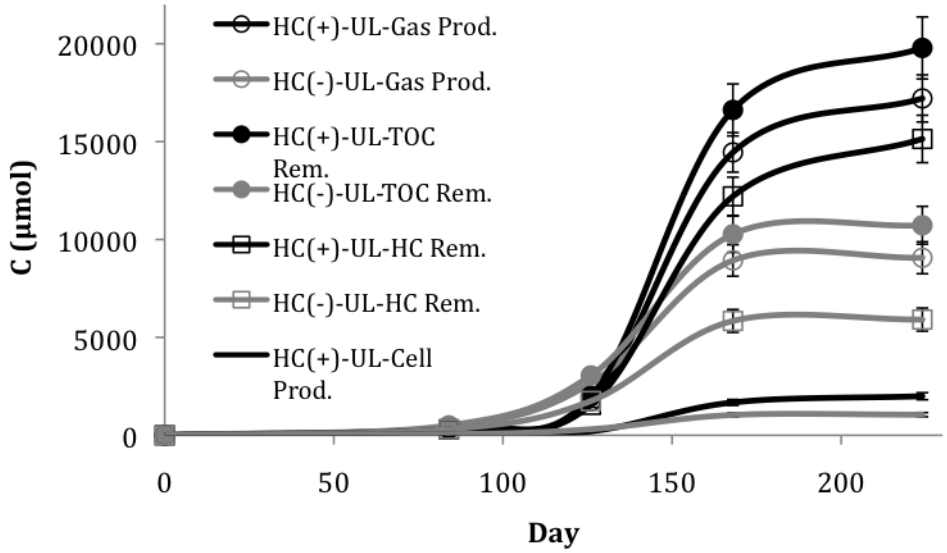


Figure 6.20: TOC removal, HC removal, gas production and cell production

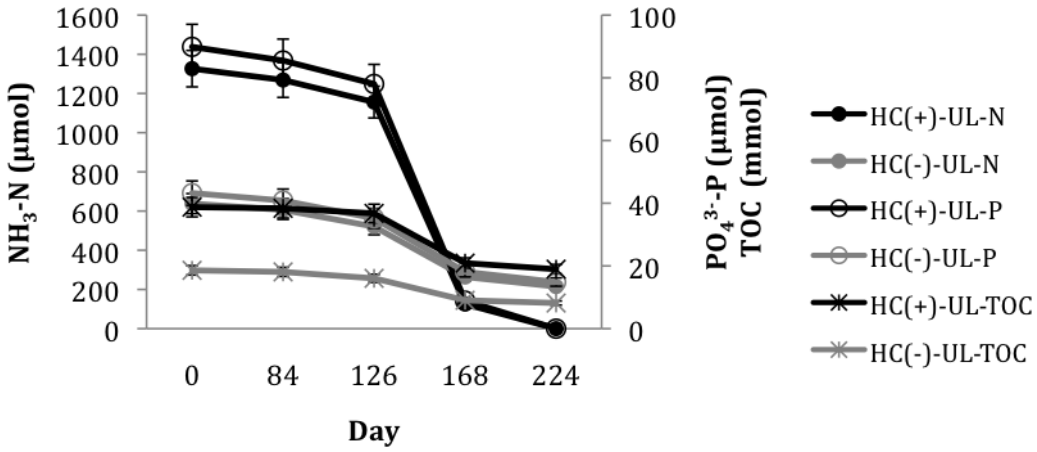


Figure 6.21: Changes in TOC, N and P contents

6.3.5 Changes in hydrocarbon composition

Changes in aromatic and aliphatic HC levels of HC(+)-UL and HC(-)-UL microcosms were shown in Figure 6.22. 55 % and 57 % of aromatic and aliphatic HCs in HC(+)-UL microcosms were removed respectively. High proportion (92 %) of aromatic HCs and all aliphatic HCs were removed from HC(-)-UL microcosms.

Changes in aromatic and aliphatic HC fractions were shown in Figures 6.23-6.30. As seen, the short chain hydrocarbons were degraded faster than the long chain

hydrocarbons. Aromatic hydrocarbons were only degraded after significant removal of n-alkanes and alteration of acyclic isoprenoids pristane and phytane.

Aromatic hydrocarbon fractions

Changes in aromatic HC fractions were shown in Figures 6.23-6.26. Complete removal of 1-3 ring aromatic HCs was achieved in both HC(+)-UL and HC(-)-UL microcosms. Only antracene was partially (40%) removed in HC(+)-UL microcosms. 4-5 ring aromatic HCs were not degraded in HC(+)-UL microcosms whereas those in HC(-)-UL microcosms were completely consumed except benzo(g,h,i)perylene.

Aliphatic hydrocarbon fractions

Changes in aliphatic HC fractions were shown in Figures 6.27-6.30. n-C9-31 alkanes were depleted completely in HC(-)-UL microcosms. n-C9-20 alkanes were degraded, and n-C21-31 alkanes remained unchanged in HC(+)-UL microcosms. n-C9-18 and n-C20 alkanes were also completely degraded in HC(+)-UL microcosms.

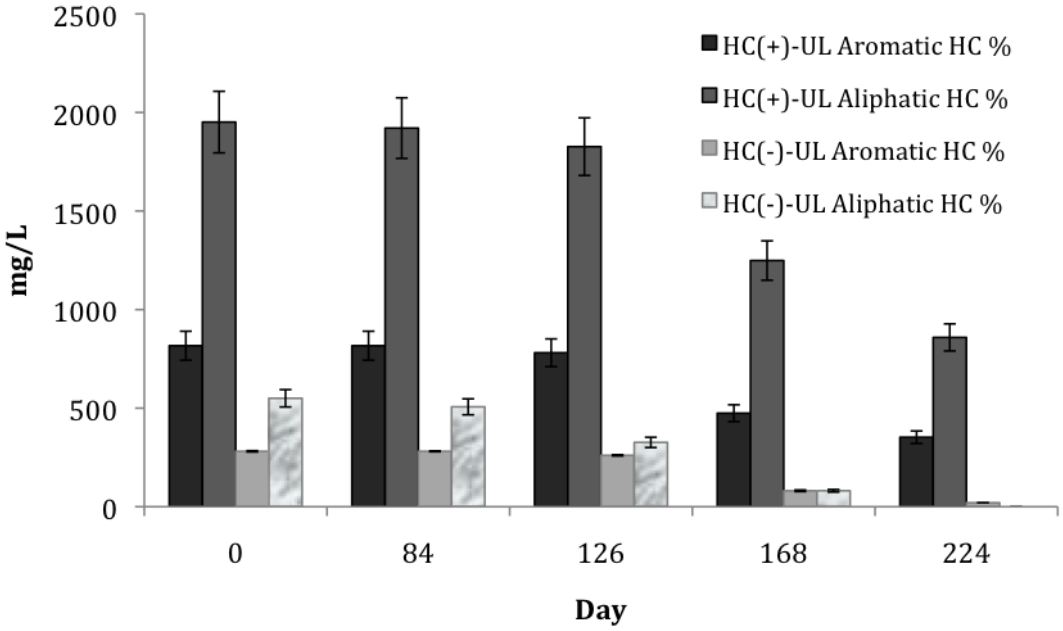


Figure 6.22: Changes in aromatic and aliphatic hydrocarbon levels.

6.3.6 Changes in microbial abundance and activity

Initial cell and transcript abundances in HC(+)-UL and HC(-)-UL microcosms were shown in Figure 6.31. The results were in accordance with the monitoring data on Haliç sediments between the years 2006 and 2008. Bacteria dominated over Archaea; archaeal community almost completely composed of methanogens. Methanogenic

archaea (MA) was highly dominant over sulfate reducing bacteria (SRB), denitrifying bacteria (DB) and dissimilatory nitrate reduction to ammonia bacteria (DNRB). Anaerobic aliphatic hydrocarbon degrading bacteria (AAHDB), anaerobic aromatic hydrocarbon degrading bacteria (AArHDB) and anaerobic aromatic degrading bacteria (AArDB) were as abundant as MA. All of the assessed microbial groups were active. The most active processes at the time 0 were methanogenesis and hydrocarbon degradation.

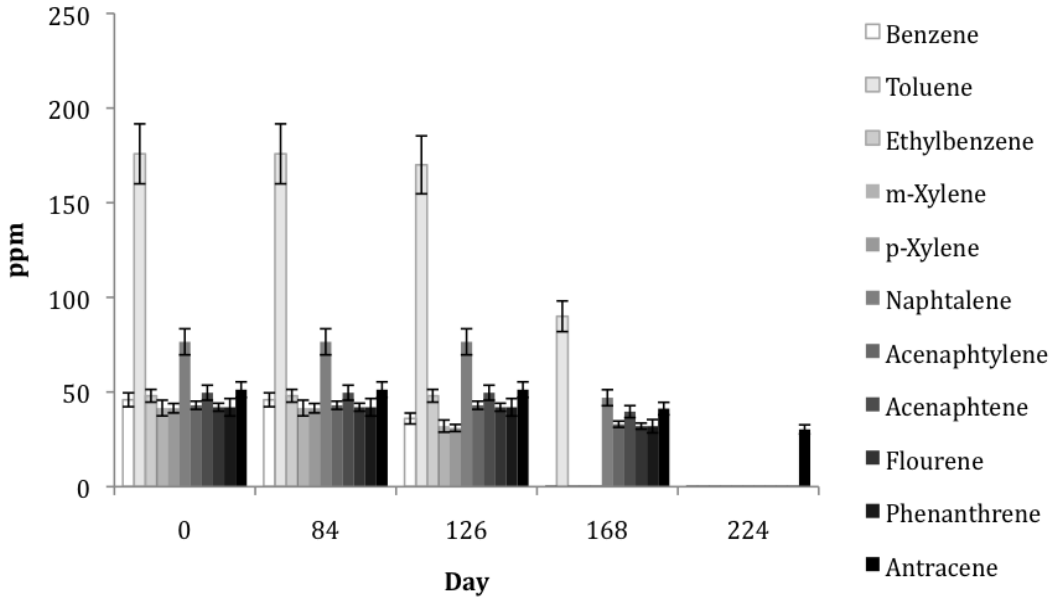


Figure 6.23: Changes in 1-3 ring aromatics in HC(+)-UL microcosms

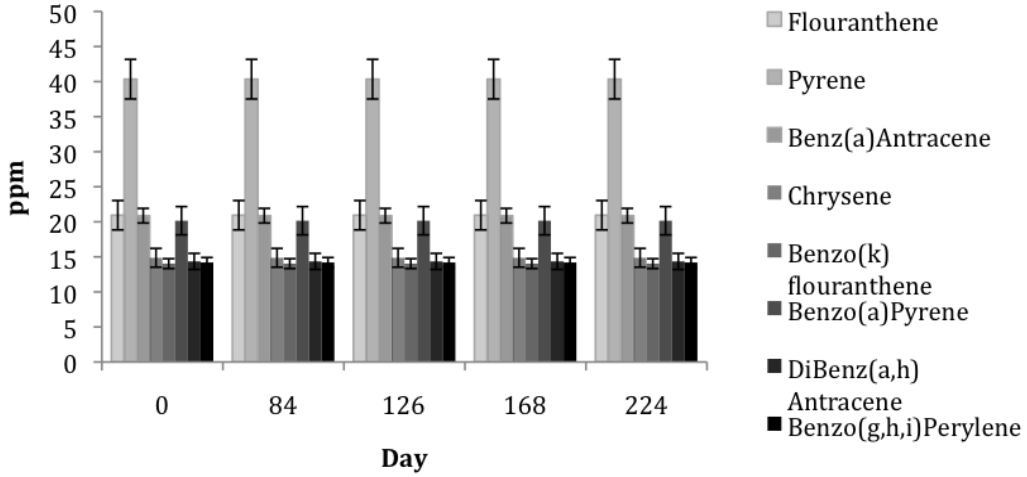


Figure 6.24: Changes in 4-5 ring aromatics in HC(+)-UL microcosms

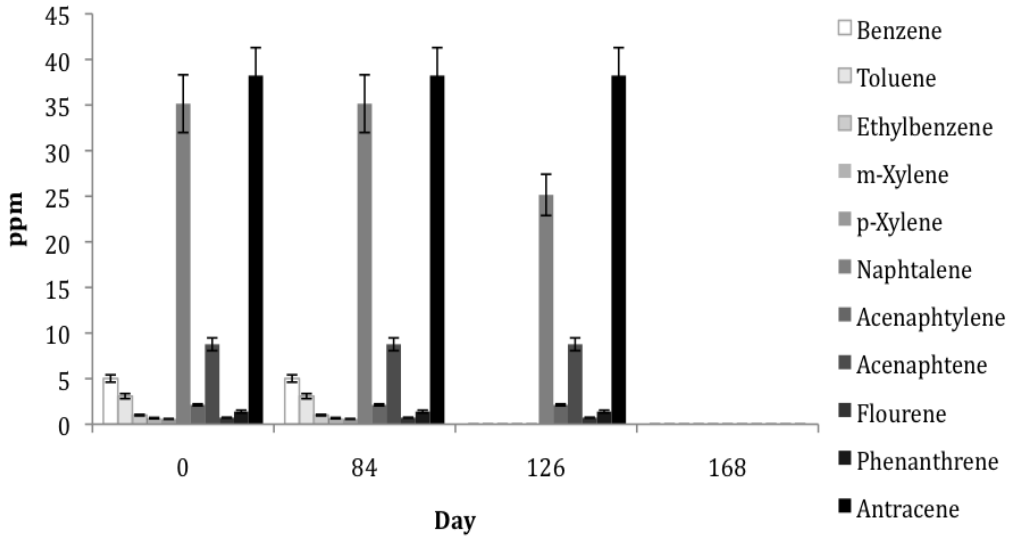


Figure 6.25: Changes in 1-3 ring aromatics in HC(-)-UL microcosms

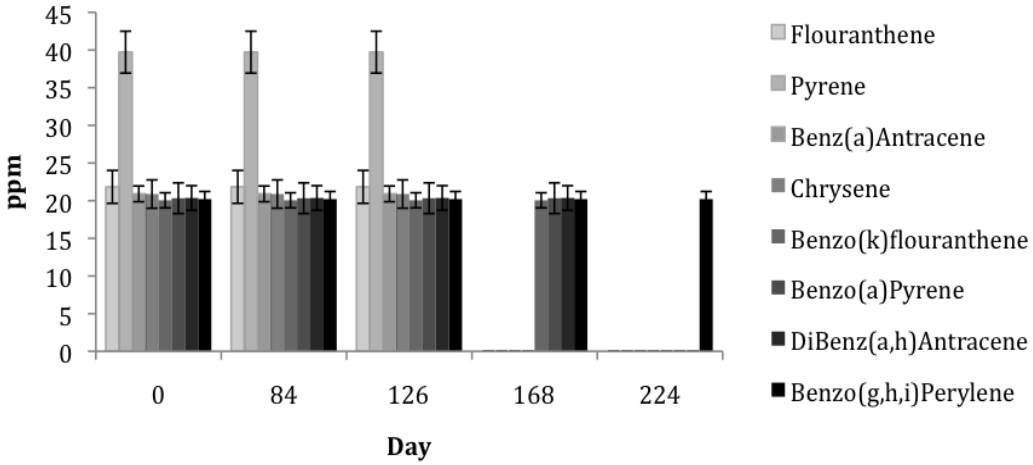


Figure 6.26: Changes in 4-5 ring aromatics in HC(-)-UL microcosms

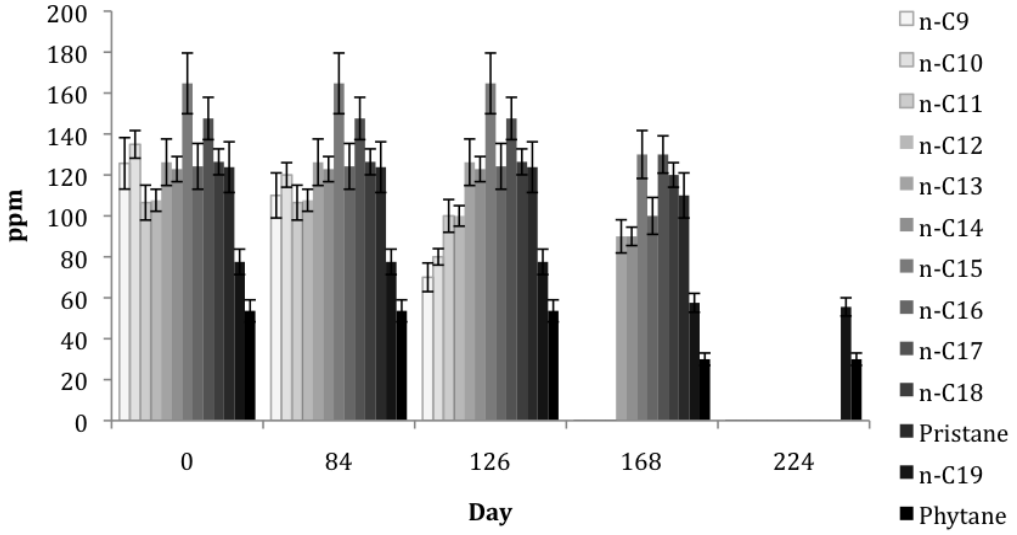


Figure 6.27: Changes in n-C9-19 alkanes in HC(+)-UL microcosms

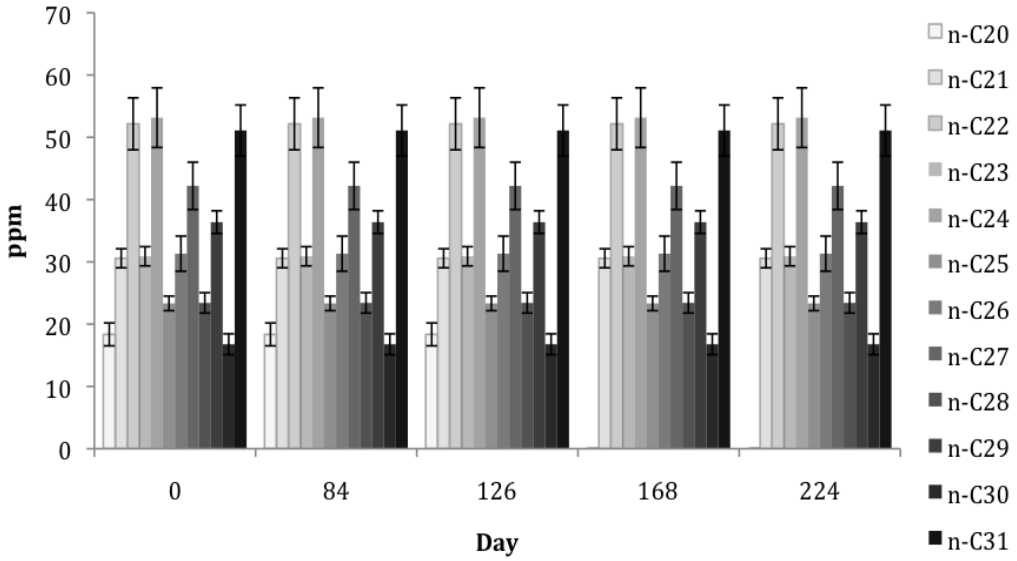


Figure 6.28: Changes in n-C20-31 alkanes in HC(+)-UL microcosms

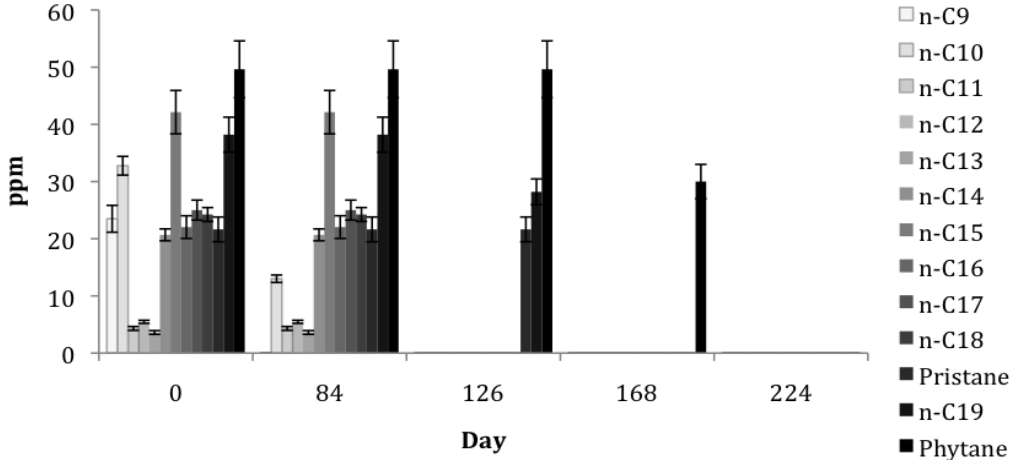


Figure 6.29: Changes in n-C9-19 alkanes in HC(-)-UL microcosms

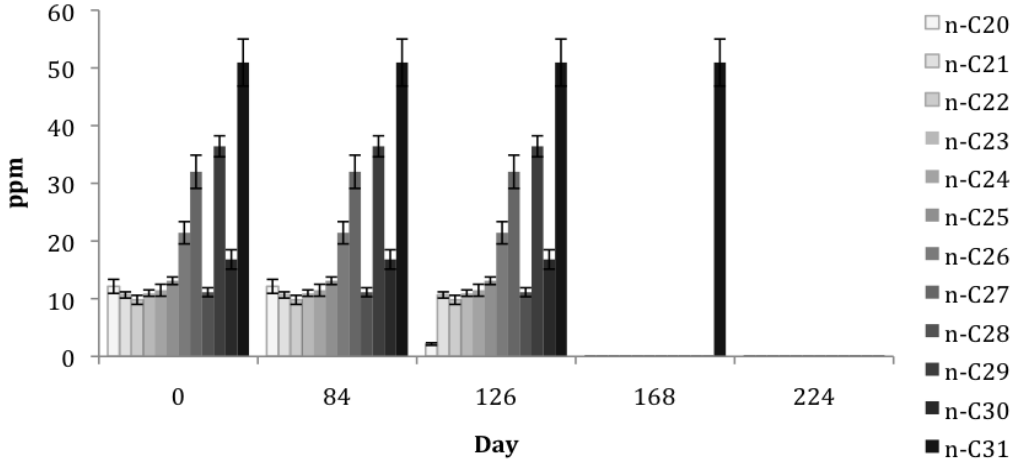


Figure 6.30: Changes in n-C20-31 alkanes in HC(-)-UL microcosms

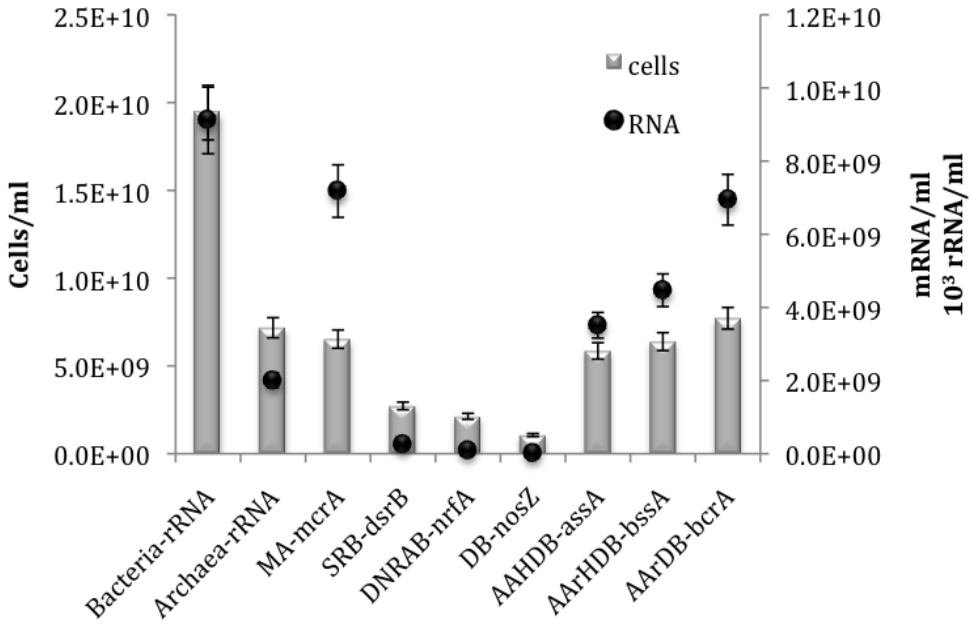


Figure 6.31: Initial microbial community structure

Bacteria, Archaea, MA and SRB abundance and activity changes

Differential changes in cell and RNA abundances compared to the levels on day 0 in HC(+)-UL and HC(-)-UL microcosms were shown in Figure 6.32 and 6.33. The most considerable increase was occurred in the abundance of SRB between the days 0 and 84 during which period sulfate concentration was decreased from 1310 μM to 660 and 170 μM in HC(+)-UL and HC(-)-UL microcosms respectively. Sulfate was completely depleted in the both microcosms before the day 126 after which SRB abundance decreased and no SRB activity was observed. Initial nitrate concentration in the microcosms was very low (~ 30 μM); DNRB and DB activities and nitrate were not detected throughout the incubation period.

Activity and abundance of *Archaea, Bacteria* and MA were very high and related to the C removal (Figure 1) all through the incubation period. *Archaea* abundance increases were ~1.5× higher than those of *Bacteria*. 74 and 67 % of the total C removal occurred between the days 126 and 168 during which period archaeal, bacterial and methanogenic activity levels increased 6-8×. Overall microbiological results implied that C removal in this period can be attributable to the activities of syntrophic consortium of fermentative bacteria and MA.

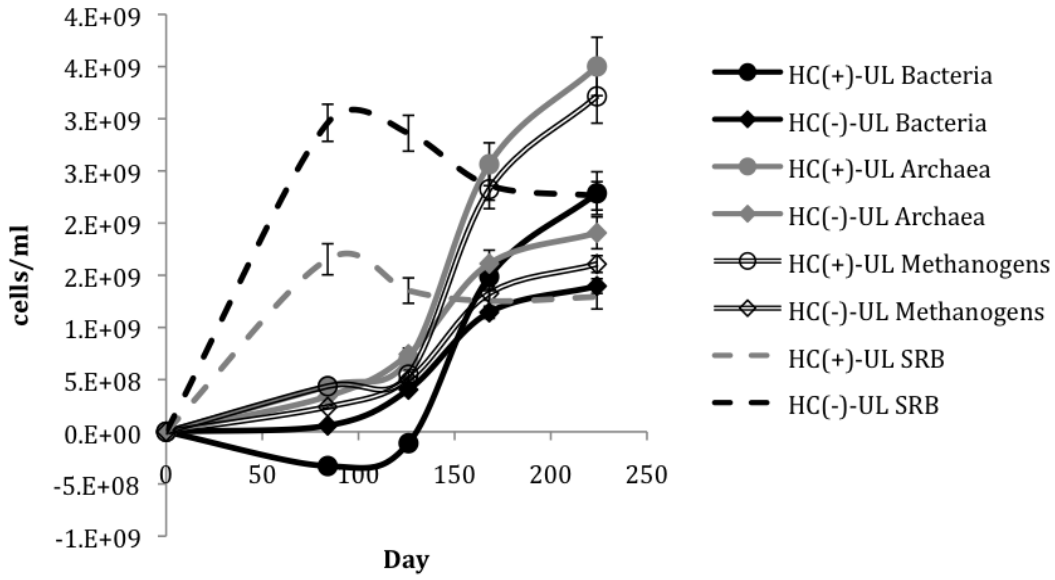


Figure 6.32: Differential changes in cell abundances

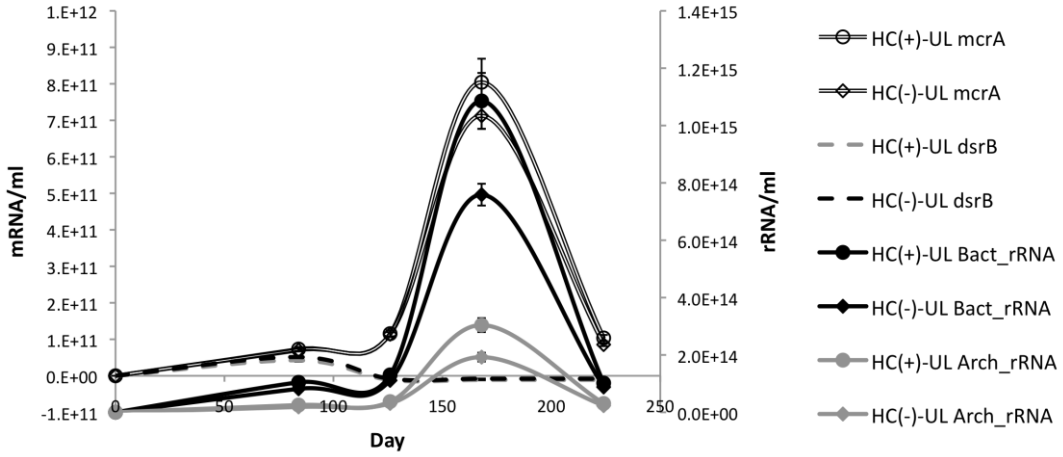


Figure 6.33: Differential changes in RNA abundances

Changes in activity and abundance of hydrocarbon degraders

Differential changes in gene and transcript abundance of enzymes related to anaerobic hydrocarbon degradation were shown in Figure 6.34. The changes were highly correlated to C and HC removal. Anaerobic hydrocarbon degrading bacteria (AHDB) were as abundant and active as MA in the whole incubation period. AHDB activity increased 9-12× and 4-5× in HC(+)-UL and HC(-)-UL microcosms respectively between the days 126 and 168 during which period ~70 % of the HC removal took place. Overall microbiological results implied that these genes were carried by syntrophic methanogenic consortium and the initial attack on

hydrocarbons was most probably carried out by fermentative bacteria after the day 126.

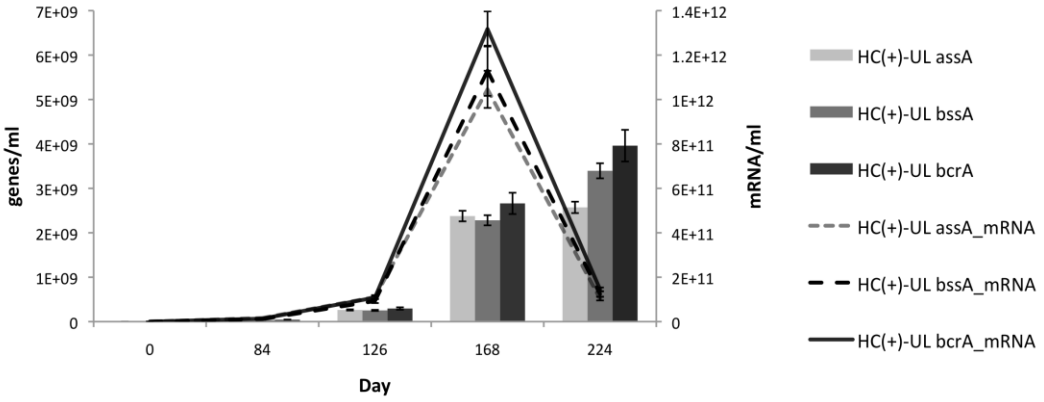


Figure 6.34: Differential changes in assA, bssA and bcrA abundances

6.3.7 Haliç Bay sediments degraded wide range of hydrocarbons

While aerobic hydrocarbon degradation has been well known and studied for many years, anaerobic degradation has been documented within the last two decades (Widdel and Rabus, 2001; Foght, 2008; Grossi et al., 2008). This activity is widespread and has been reported under nitrate-, iron-, manganese- and sulfate-reducing and methanogenic conditions.

The degraded hydrocarbons by Haliç Bay sediments were listed in Table 6.5. Only benzo(g,h,i)perylene was recalcitrant to biodegradation and remained intact during the incubation period. The much higher gas production in HC(+) and HC(-) microcosms compared to the sterile controls, increasing abundance and continous transcription of the genes related to anaerobic hydrocarbon degradation, and high correlations between hydrocarbon removal, gas production and microbial abundance-activity prooved that the hydrocarbon removal was biological, not due to the abiotic factors.

The previous literature on anaerobic metabolism of the degraded hydrocarbons was summarized in Table 6.5. $C_{9\text{-}31}$ n-alkanes have been reported to be biodegradable under nitrate- and sulfate-reducing and methanogenic conditions. The studies which used marine sediments as inoculum produced much of the biodegradability reports on $C_{20\text{-}31}$ n-alkanes (Jones et al. 2008, Miralles et al. 2007a and 2007b). We confirmed that marine benthic microbiota is able to degrade long chain n-alkanes.

Table 6.5: Degradability of hydrocarbons

Hydrocarbon	NR	SR	M	Hydrocarbon	NR	SR	M
n-C_{9-20} Alkanes	+	+	+Mar	Flourene	+Mar	+Mar	+
n-C_{20-31} Alkanes			+Mar	Phenanthrene	+Mar	+Mar	+Mar
Phytane and Pristane	+Mar	+Mar	+Mar	Anthracene	+	+Mar	+
Benzene	+Mar	+Mar	+Mar	Flouranthene	+Mar	+Mar	+
Toluene	+Mar	+Mar	+Mar	Pyrene	+	+Mar	+
Ethylbenzene	+Mar	+Mar	+Mar	Benz(a)Antracene		+Mar	+
m-Xylene	+Mar	+Mar	+Mar	Chrysene		+Mar	+
p-Xylene	+Mar	+Mar	+Mar	Benzo(k)flouranthene		+Mar	+
Naphtalene	+Mar	+Mar	+Mar	Benzo(a)Pyrene		+Mar	+
Acenaphtylene				DiBenz(a,h)Antracene			+
Acenaphtene	+Mar	+Mar	+	Benzo(g,h,i)Perylene			

SR: Dissimilatory sulfate reduction; NR: Dissimilatory nitrate reducing; M: Methanogenesis
+: Degradable hydrocarbons
+Mar: Degradability was shown for marine sediments

BTEX components are the best studied substrates of anaerobic biodegradation since they are the most water soluble of the aromatic hydrocarbons. As expected, Haliç Bay sediments degraded BTEX faster than the PAHs. A large amount of the literature on PAH degradation by anoxic marine sediments were obtained under sulfate reducing conditions because of overwhelming abundance of sulfate in seawater. Our study is the first that reports 3-5 ring PAHs (acenaphtylene, acenaphtene, flourene, anthracene, flouranthene, pyrene, benz(a)antracene, chrysene, benzo(k)flouranthene, benzo(a)pyrene and dibenz(a,h)antracene) were degraded by marine sediments under methanogenic conditions. Besides, this is the first indication of acenaphtylene biodegradation.

Our findings on the anaerobic degradation hierarchy of hydrocarbons were coincided with the previous reports: the most easily degradable compounds are n-alkanes, followed by more resistant branched acyclic and monocyclic hydrocarbons, the most resistant polycyclic steroidal and triterpenoidal hydrocarbons, and aromatic hydrocarbons (Foght, 2008; Grossi et al., 2008).

6.3.8 The pathway of anaerobic hydrocarbon degradation

Initial activation of hydrocarbons is crucial for anaerobic biodegradation, and four general enzymatic reactions are recognized: (1) addition of fumarate, catalyzed by a glycyl radical enzyme such as bss; (2) methylation of unsubstituted aromatics; and (3) hydroxylation of an alkyl substituent (Fuchs, 2008). The other proposed pathways

represent a combination of these reactions. These activation reactions feed into pathways that result in production of central metabolites such as benzoyl-coA which are eventually incorporated into biomass or completely oxidized.

Bss has been the only identified enzyme which specifically attacks on aromatic hydrocarbons (toluene and xylene) (Foght, 2008); bcr catalyzes dearomatization of the central metabolite benzoyl coenzyme A. This is why we chose bcr and bss genes and their transcripts as indicators of anaerobic aromatic hydrocarbon degradation. Abundance of these genes gradually increased during the incubation period and their transcription levels were very high even after depletion of toluene and xylene. These give an impression that fumarate addition played a major role in initial activation of the other aromatic hydrocarbons. Fumarate addition has previously been proposed to be included in degradation pathways of the other monoaromtic hydrocarbons (benzene, alkylbenzenes and ethylbenzene) and PAHs (naphtalene and phenanthrene) (Foght, 2008).

The two proposed mechanisms of anaerobic n-alkane degradation involves (1) activation at the subterminal carbon of the alkane and addition to a molecule of fumarate; and (2) the alkane carboxylation at C-3 (Grossi et al., 2008). Recently, Callaghan et al. (2008) reported a glycyl radical type enzyme (assA) which involve in alkane activation through fumarate addition in the SRB strain AK-01. This was the first description of a gene specifically involved in anaerobic n-alkane metabolism. Our results imply that fumarate addition was the main mechanism of initial activation of n-alkanes since both abundance and transcription level of the ass gene was very high throughout the incubation period.

6.3.9 Stimulation of Haliç Bay sediments' biological activity

Whereas biostimulation through TEA addition or supplementary carbonaceous substrate has been studied, the stimulatory effect of providing N and P has not been thoroughly studied under anaerobic conditions (Harayama et al., 2004; Andreoni and Gianfreda, 2007; Foght, 2008). Two cases showed the stimulatory effect of fertilizing nutrient-poor groundwater (Cross et al., 2006) and soil (Powell et al., 2006) on anaerobic hydrocarbon degraders. Although it is well known that N and P limit microbiological activity in marine environments, none has attempted to increase anaerobic hydrocarbon degradation activity of marine microbenthos through nutrient

amendment. We have shown that it is possible to increase anaerobic hydrocarbon degradation activity of the Haliç Bay sediments substantially (~9×) by supplying the limiting nutrients (N and P).

6.3.10 Feasibility of anaerobic bioremediation

Anaerobic biodegradation processes are a significant component of natural attenuation owing to the abundance of anoxic electron acceptors relative to dissolved oxygen. Furthermore, clean-up systems based on anaerobic biodegradation require less human intervention (Head and Swannel, 1999). Studies on the anaerobic/anoxic biodegradation of the hydrocarbons in natural habitats, microcosms and enrichment cultures were initiated to determine whether or not bioremediation processes are possible in petroleum/fuel-contaminated anoxic sediments, groundwaters and aquifers (Widdel and Rabus, 2001). Although numerous reports have been published documenting natural attenuation of hydrocarbons in these environments (Meckenstock et al., 2004; Andreoni and Gianfreda, 2007; Foght, 2008), bioremediation strategies based on anaerobic microbial processes are very limited because they proceed at much lower rates than the aerobic ones (Harayama et al., 2004; Miralles et al., 2007).

Recent reports on the anaerobic hydrocarbon degradation rates of marine sediments or marine enrichment cultures were summarized in Table 6.6. As seen, none has achieved anaerobic hydrocarbon degradation rates comparable to the aerobic ones. In this study, we obtained anaerobic hydrocarbon degradation rates as fast as SRB enrichment cultures' rates.

Although hydrocarbon degradation rates of Haliç Bay sediments were comparable to the aerobic ones, they are still much lower than the aerobic hydrocarbon degradation rates. Moreover, aerobic microorganisms degrade a wider range of hydrocarbon compounds than anaerobic microorganisms. Nevertheless, an aerobic bioremediation strategy is unfeasible for Haliç Bay since oxygen penetration into the anoxic sediments is poor and oxygen mass transfer enhancement by mechanical means is inappropriate for the inaccessible sediments. Moreover, economical long term solution for the chronic hydrocarbon pollution by continuous aeration of the huge anoxic area in Haliç Bay is out of the question. Under these conditions anaerobic hydrocarbon degradation is the only alternative.

In summary, we have obtained three lines of evidence for demonstrating anaerobic bioremediation feasibility of petroleum HC pollution in Haliç Bay sediments: (1) the anaerobic hydrocarbon degrading microbiota was highly abundant in the sediments; (2) anaerobic hydrocarbon degradation was taking place in the sediments; (3) the sediments were able to degrade wide range of hydrocarbons under anaerobic/anoxic conditions; (4) high anaerobic hydrocarbon degradation rates were achieved via biostimulation of the sediments through nutrient amendment.

As the oil spill catastrophe in gulf of Mexico highligted recently, anaerobic oil spill bioremediation has been a theoritical technology and must be applied in the field to verify its lab-scale advantages (Vrossi, 2010). We are now making the preliminary preparations in conjunction with Maritime Undersecretariat of Turkish Republic, ISKI and IBB to carry out a field-scale bioremediation trial to remove the accumulated hydrocarbons from the subsurface of Haliç Bay through biostimulation of the sediments. Success of this trial will certainly lead to less human intervened and more economical field-scale bioremediation applications for over polluted anoxic marine environments worldwide.

Table 6.6: Literature on anoxic/anaerobic hydrocarbon degradation rates

Inoculum	Substrate	e^--acceptor addition	Removal Rate - ug/gSediment.L.day	Reference
SRB Enrichment	Short-chain alkanes	Sulfate	0,6	Kniemeyer et al., 2007
SRB Enrichment	Phenanthrene	Sulfate	1,4	Davidova et al., 2007
Sediment	Crude oil	none	7	Miralles et al., 2007
Sediment	2-5 rings PAH	Sulfate	48	Rothermich et al., 2002
Sediment	n-alkanes	none	60	Jones et al., 2008
Sediment	PAH	none	228	Kim et al., 2008
SRB Enrichment	Naphthalene	Sulfate	880	Musat et al., 2009
Sediment	**n-alkanes, PAH and BTEX**	**none**	**700**	**This study**
Sediment	Crude oil	Oxygen	1970	Syakti et al., 2006
Sediment	Crude oil	Oxygen	4285	Roling et al., 2002

6.4 Nutrient Enhanced Bioremediation of Tuzla Bay Sediments

Bioremediation strategies based on anaerobic microbial processes are very limited because they proceed at much lower rates than the aerobic ones. Moreover, aerobic microorganisms degrade a wider range of HC compounds than anaerobic microorganisms. Nevertheless, an aerobic bioremediation strategy is unfeasible for marine sediments where (1) oxygen penetration is poor and oxygen mass transfer enhancement by mechanical means is inappropriate; and (2) the pollution is chronic, and affects a huge area which makes the aeration strategy economically unattainable. Tuzla Bay, an extremely polluted marine environment with hydrocarbons, meets these two criteria.

A bioremediation strategy under anaerobic/anoxic conditions is feasible for Tuzla Bay as long as oil-degrading anaerobes are abundant and active in the sediments, and there is a way to increase activity of this population. In order to assess this, physicochemical and microbiological characteristics of Tuzla Bay sediments were monitored for two years. The total petroleum HC levels (11000-18000 ppm) were similar to those from extremely polluted marine environments; the microbial cell contents were very high compared to the other marine environments; (3) the sediments were dominated by dissimilatory nitrate reducers to ammonia and anaerobic HC degraders, and these microbes were active; and (4) N and P were limited in the porewaters for biological activity. These indications raised the question that HC degradation activity of Tuzla Bay sediments can be increased by N-P amendment under nitrate reducing conditions. This chapter describes assessment of this theory through anaerobic/anoxic HC degradation microcosms.

6.4.1 Correlation analysis

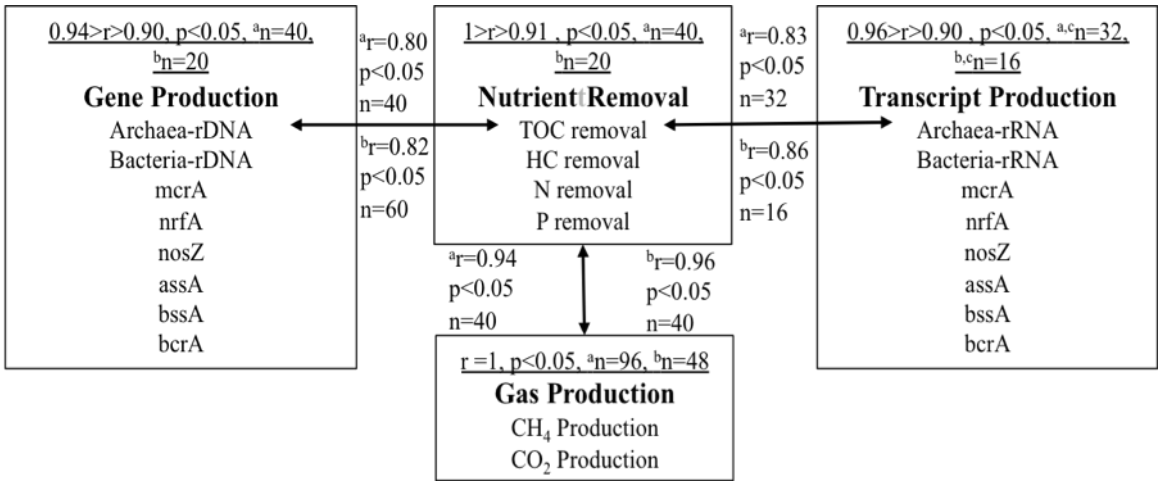
The reader should check the abbreviations part for the frequently used terms throughout the text. All of the measured parameters except SRB abundance and activity were highly related to the gas production (Figure 6.35); only the results from UL microcosms were given for the parameters other than gas production

6.4.2 CO_2 and CH_4 production

The much higher gas production compared to the sterile controls (total gas production<24 μmol, the data were not shown) indicated that the gas production

activities were biological. Addition of the DSR and MG inhibitors inhibited the all microbial groups (total gas production<267 μmol, the data were not shown); the metabolic origin of CO_2 production was discussed based on the transcript abundance of functional genes (Figure 6.37) and the stoichiometry of microbial growth (Table 6.11).

The cumulative gas production in the microcosms was shown in Figure 6.36. 7.5× increment in P concentration resulted in 14× and 21× increase in total gas production for N(-) and N(+) microcosms respectively. This indicated that it is possible to trigger the microbial activity, and to remove the accumulated TOC in Tuzla Bay sediments via nutrient amendment**.**



A correlation range between the parameters in a same box was shown on top of the box
a r values for the N(+) microcosms
b r values for the N(-) microcosms
c Since the microbial activity stopped at an uncertain time between the days 136 and 164, the data from the destructive sampling set on day 164 was omitted in the correlation analysis.

Figure 6.35: Pearson product-moment correlation coefficients (*r*)

6.4.3 TOC and HC removal

Nutrient removal data from UL microcosms were given in Table 6.7. C removal ceased in all microcosms due to the complete depletion of P. ~75% of the HCs was removed from the HC(+) microcosms where TOC removal was completely due to HC degradation. 1 unit of HC removal corresponded to ~0.7 unit of TOC removal which was mainly due to the contribution of produced cells to the TOC content (Table 6.11). ~40% of the HC removal took place during the exponential growth phase (between the days 108 and 136).

The HCs were completely removed from HC(-) microcosms. ~60% of the total TOC removal took place after the compete depletion of HCs. HCs were preferentially degraded compared to the other organic carbon sources in both HC(+) and HC(-) microcosms.

As seen in Table 6.8 and 6.9, the short chain hydrocarbons were degraded faster than the long chain hydrocarbons. Aromatic hydrocarbons were only degraded after significant removal of n-alkanes and alteration of acyclic isoprenoids pristane and phytane.

6.4.4 Microbial cell production

50-60% of the total cell production took place during the exponential growth phase (Table 6.10). ~7× more bacterial cells produced compared to the archaeal cells in N(+) microcosms which was mainly due to the proliferation of DNRAB. ~1.5× higher MA production compared to the bacterial cells in N(-) microcosms was due to the absence of exogenous e^--acceptors. ~90% of the bacterial population was carrying the genes related to anaerobic hydrocarbon degradation in HC(+) microcosms; addition of external HC sources resulted in ~20% higher relative abundance of the anaerobic HC degraders.

Even after cessation of sulfate depletion (Table 6.7) and dsrB-mRNA production, SRB was produced. Inferring DSR activity from production of dsrB gene is a misleading approach since some SRB can carry out DNRA or fermentation in addition to DSR (Madigan, 2009).

6.4.5 Microbial activity levels

The microbial activity level was strongly dependent on the nutrient removal rate (Figure 6.35). DNRA was the most dominant process in N(+) microcosms (Figure 6.37). Surprisingly, MG activity was higher than DN activity at very high nitrate concentrations in N(+) microcosms. MG was predominant over the all other terminal e^--accepting processes in N(-) microcosms. Genes related to the anaerobic hydrocarbon degradation were highly transcribed as long as the HC degradation took place.

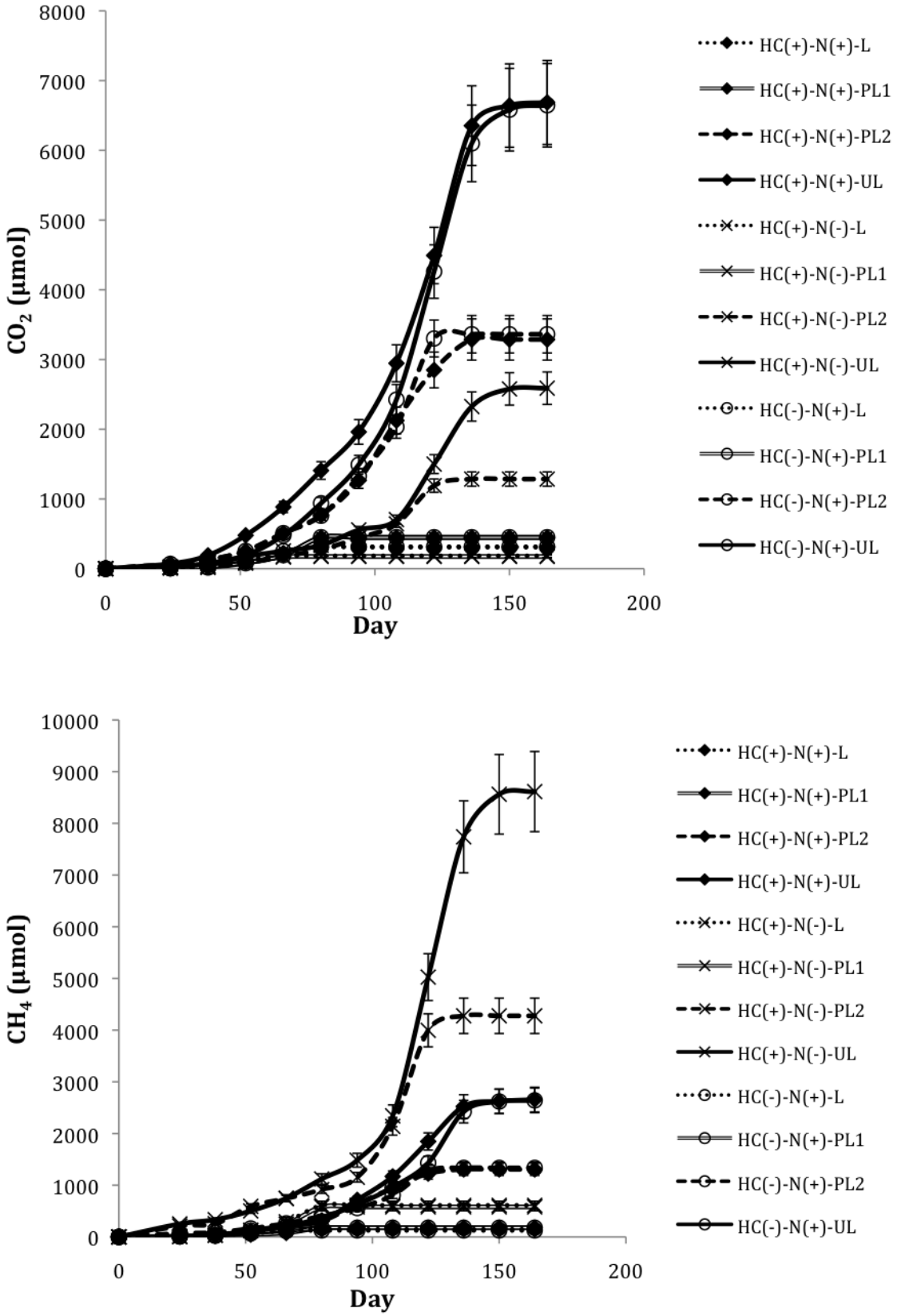


Figure 6.36: Cumulative gas production in the microcosms

As seen in Figure 6.37, TOC removal was as a result of MG in N(-) microcosms whereas DNRA, MG and DN processes were the concomitant routes of substrate degradation in N(+) microcosms during the exponential growth phase.

Table 6.7: Nutrient concentration changes in the UL microcosms

			TOC µmol C	[a]HC µmol C	ArHC mg	AlpHC mg	PO_4^{3-}-P µmol	NO_3^--N µmol	NO_2^--N µmol	NH_4^+-N µmol	SO_4^{2-} µmol
HC(+)-N(+)	Day	0	30130	20103	76	203	88	15100	168	55	108
		80	28114	16899	63	172	70	13995	142	735	0
		108	25911	13398	54	132	50	12787	131	1479	0
		136	21031	5641	25	56	7	10112	116	3126	0
		164	20552	4881	21	50	1	9849	115	3288	0
HC(+)-N(-)	Day	0	30100	20039	76	202	85	103	11	1280	106
		80	28726	18156	70	182	74	0	0	1177	0
		108	27227	16101	63	160	62	0	0	1064	0
		136	20608	7028	31	66	9	0	0	568	0
		164	19533	5555	30	47	0	0	0	487	0
HC(-)-N(+)	Day	0	15986	5967	17	66	85	15051	160	52	105
		80	14642	3831	11	42	73	14314	157	506	0
		108	12514	449	0	7	54	13148	153	1224	0
		136	7250	0	0	0	7	10262	141	3000	0
		164	6466	0	0	0	0	9832	139	3265	0

The color scale in columns, ■→□, represents highest to lowest parameter value rank.
The standard deviations were between 7% and 11 % of the mean values
[a] The HC amount in mg was converted to µmol based on the molecular formula of the HC mix

6.4.6 Stoichiometry of the exponential microbial growth on HCs

Since the microcosms were fed with a defined mixture of hydrocarbons, we were able to calculate the stioichiometry of the growth on HCs (Table 6.11) based on the following assumptions: (1) NH_4^+ produced by DNRA was used in MG and DN; (2) NH_4^+ was used in the all cell synthesis reactions; (3) NO_3^- was converted to N_2 and NH_4^+ in e^--acceptor half reactions for DN and DNRA respectively; (4) CO_2 is the e^--acceptor for the overall reaction of fermentation and MG; (5) The weight of a microbial cell is 1 pg (6) Molecular formula of the cell is $C_5H_7NO_2$; (7) All of the removed TOC was HC; (8) Molecular formula of the HC mixture between the days 108 and 136 was $C_{17}H_{31}$ which was based on the determined HC composition; (9) The fraction of HC used in energy reactions (f_e) was 0.8 for N(-)-MG. The f_e was calculated from the measured CH_4 production between the days 108 and 136 in N(-) microcosms; (10) The stoichiometry of N(+)-MG and N(-)-MG is same; (11) 30% of the removed HC was used in MG for N(+) microcosms. This is calculated from the measured CH_4 production between the days 108 and 136 in N(+) microcosms; (12) 5% and 65 % of the removed carbon was used in DN and DNRA respectively. The

5/65 was calculated by dividing the produced nosZ-mRNA to nrfA-mRNA. This ratio was not calculated based on the gene production since a single bacterial cell can carry the genes for the both process.

As given in Table 6.11, differences between the measured and theoretical coefficients were less than 10%. This consistency was obtained due to the rational assumptions made based on the molecular microbiological data, and due to the accuracy of the measurements.

The information given in Table 6.11 implied that the major route of HC degradation was DNRA in N(+) microcosms. There are two recognized DNRA pathways: one involving fermentation and the other linked to sulfur oxidation. DNRA is mainly carried out by fermentative bacteria which can also carry out fermentation without using nitrate (Tiedje, 1988). Although the conditions promoting fermentative DNRA and DN are similar, fermentative DNRA is thought to be favored in environments rich in organic carbon, while DN would be favored under carbon-limited conditions (Burgin and Hamilton, 2007). Tiedje (1988) argued that high labile carbon availability would favor organisms that used electron acceptors most efficiently; DNRA transfers eight electrons per mole of nitrate reduced, whereas DN only transfers five. Pre-dominancy of DNRA over DN was not surprising in HC(+)-N(+) microcosms which contain extremely high levels of organic carbon.

~90% of NO_3^- was consumed via DNRA (Table 6.11) which: (1) prevented NO_3^--N loss as N_2 gas and kept it within the system as more biologically available NH_4^+-N (2) reduced the formation of toxic denitrification intermediates (NO_2^-, NO, N_2O) which would otherwise inhibit methanogenesis (Roy and Conrad, 1999; Kluber and Conrad, 1998). Three hypotheses have been proposed to explain the mechanism of methanogenesis suppression by nitrate: (1) redox shift; (2) competition for methanogenic substrates by denitrifying bacteria; or (3) inhibition by toxic denitrification intermediates (NO_2^-, NO, N_2O). Since the prevous studies showed that (1) methanogenesis is not necessarily prevented in being active under positive redox potential; (2) addition of electron donors could not prevent the inhibition of methanogenesis by nitrate, only the third hypothesis remain valid (Roy and Conrad, 1999). Roy and Conrad (1999) also reported that the extent of inhibiton by nitrous oxides was lower at the greater ratio of electron donor to electron acceptor where potentially toxic denitrification intermediates were faster reduced to non-toxic N_2.

This was also the case for HC(+) microcosms where unlimited amount of organic carbon is available for microbial growth. Although it was relatively much less important and omitted when calculating the stoichiometry, nitrate was also assimilated (evident from the nasA gene transcription) which would also resulted in reduction of toxic denitrification intermediates.

Table 6.8: Changes in aliphatic hydrocarbon composition

	mg/100mL													
	HC(+)-N(+)-UL					HC(+)-N(-)-UL					HC(-)-N(+)-UL			
	0	80	108	136	164	0	80	108	136	164	0	80	108	136
n-C9	13	4	0	0	0	13	2	0	0	0	2,6	0	0	0
n-C10	13	5	0	0	0	13	4	0	0	0	3,6	0	0	0
n-C11	11	4	0	0	0	10	10	0	0	0	0,5	0	0	0
n-C12	11	6	0	0	0	11	11	5	0	0	0,6	0	0	0
n-C13	13	11	0	0	0	14	13	14	0	0	0,4	0	0	0
n-C14	15	14	8	0	0	15	14	15	0	0	5,6	0	0	0
n-C15	16	15	15	0	0	16	17	16	0	0	4,7	0	0	0
n-C16	12	13	12	0	0	12	13	12	0	0	2,4	0	0	0
n-C17	17	17	16	0	0	17	18	17	0	0	5	1	0	0
Pristane	15	16	16	6	4	15	14	15	16	3	5,7	5,6	0	0
n-C18	13	12	13	0	0	13	14	13	0	0	2,7	2,5	0	0
Phytane	5,4	5,4	5,6	5,4	5,3	5,8	6,0	5,8	6,0	4,8	5,5	5,7	0	0
n-C19	7,8	8,2	4,2	4,0	0,0	7,1	7,0	7,1	3,8	0,0	4,2	4	0	0
n-C20	1,8	2,0	2,0	1,8	1,7	1,2	1,4	1,2	1,3	1,2	1,3	1,4	0	0
n-C21	3,1	3,2	3,4	3,1	3,0	3,1	3,0	3,1	3,3	3,1	1,2	1,2	0	0
n-C22	5,2	5,1	5,3	5,2	5,3	4,8	4,6	4,8	5,0	4,8	1,1	1	0	0
n-C23	3,1	3,0	3,2	3,1	3,0	2,8	3,0	2,8	3,0	2,8	1,2	1	0	0
n-C24	5,3	5,0	5,5	5,3	5,4	5,3	5,0	5,3	5,2	5,3	1,3	1,4	0	0
n-C25	2,3	2,4	2,3	2,4	2,3	2,4	2,3	2,2	2,3	2,3	1,5	1,4	0	0
n-C26	3,1	3,3	3,1	3,0	3,1	3,3	3,1	3,0	3,1	3,1	2,4	2,5	0	0
n-C27	4,2	4,0	4,2	4,0	4,2	4,0	4,2	4,0	4,2	4,2	3,5	3,6	0	0

The reducing color scale in rows represents decrease in the content level. The standard deviations were between 8% and 11 % of the mean values.

Table 6.9: Changes in aromatic hydrocarbon composition

	mg/100mL												
	HC(+)-N(+)-UL					HC(+)-N(-)-UL					HC(-)-N(+)-UL		
	0	80	108	136	164	0	80	108	136	164	0	80	108
Benzene	4,6	0	0	0	0	6,0	4,0	2,0	0	0	0,6	0	0
Toluene	18	9	0,2	0	0	16	13	7,0	0	0	0,3	0	0
m-Xylene	4,2	4,2	4,3	0	0	4,0	4,0	4,0	0	0	0,1	0	0
p-Xylene	4,1	4,2	4,4	0	0	4,1	4,1	4,1	0	0	0,1	0	0
o-Xylene	4,1	4,2	4,1	0	0	4,2	4,2	4,2	0	0	0,1	0	0
Naphtalene	7,6	7,0	7,3	0	0	7,0	7,3	7,3	0	0	3,9	0	0
Acenaphtylene	4,1	4,5	4,0	0	0	4,0	4,1	4,0	2	2	0,2	0	0
Acenaphtene	5,0	5,0	5,5	1,0	0	5,2	5,0	5,0	5,1	5,0	1	0	0
Flourene	4,2	4,6	4,4	4,0	1,0	3,9	3,8	4,0	4,0	3,9	0,1	0,1	0
Phenanthrene	4,2	4,3	4,3	4,1	4,2	3,9	3,9	3,9	3,9	3,9	0,2	0,2	0
Antracene	8,0	8,1	7,4	7,8	8,0	7,8	7,2	7,8	7,4	7,8	7,9	7,6	0
Flouranthene	1,1	1,3	1,1	1,2	1,1	1,3	1,3	1,1	1,0	1,0	0,2	0,2	0
Pyrene	3,0	2,8	3,1	3,1	3,0	3,2	3,2	3,1	3,2	3,2	2	2,2	0
Benz(a)Antracene	1,1	1,1	1,2	1,2	1,2	1,4	1,3	1,4	1,4	1,4	0,1	0,1	0
Chrysene	0,5	0,5	0,5	0,5	0,5	1,0	1,0	1,0	1,0	1,0	0,1	0,1	0
Benzo(k)flouranthene	0,4	0,4	0,4	0,4	0,4	0,6	0,7	0,6	0,6	0,6	0,1	0,1	0
Benzo(a)Pyrene	1,2	1,0	1,1	1,0	1,0	1,3	1,2	1,3	1,3	1,3	0,1	0,1	0
DiBenz(a,h)Antracene	0,5	0,5	0,4	0,5	0,4	0,6	0,6	0,6	0,6	0,6	0,1	0,1	0
Benzo(g,h,i)Perylene	0,5	0,4	0,4	0,4	0,4	0,5	0,5	0,5	0,5	0,5	0,1	0,1	0

The reducing color scale in rows, black→white, represents decrease in the content level.
The standard deviations were between 6% and 10 % of the mean values

Table 6.10: Differential microbial cell production

		Cells/100ml									
		rRNA	rRNA	ass	bcr	bss	nrfA	nosZ	mcrA	nasA	dsrB
	Day	Bac	Arc	AlHCDB	ArDB	ArHCDB	DNRAB	DB	MA	NAB	SRB
HC(+)-N(+)-UL	80	2,4E+10	3,3E+09	2,2E+10	1,7E+10	1,6E+10	1,9E+10	1,5E+09	3,2E+09	1,5E+08	4,6E+07
	108	5,0E+10	6,8E+09	4,6E+10	3,7E+10	3,3E+10	4,0E+10	3,2E+09	6,6E+09	3,2E+08	9,5E+07
	136	1,1E+11	1,5E+10	1,0E+11	7,6E+10	6,8E+10	8,6E+10	6,9E+09	1,4E+10	6,9E+08	2,1E+08
	164	1,1E+11	1,5E+10	1,1E+11	7,6E+10	6,8E+10	9,0E+10	7,2E+09	1,5E+10	7,2E+08	2,2E+08
HC(+)-N(-)-UL	80	4,5E+09	7,0E+09	4,3E+09	3,7E+09	2,6E+09	6,7E+08	1,3E+07	6,8E+09	1,1E+06	5,9E+08
	108	9,3E+09	1,5E+10	7,4E+09	7,0E+09	5,4E+09	6,5E+08	1,3E+07	1,4E+10	1,0E+06	5,7E+08
	136	3,1E+10	4,9E+10	2,8E+10	2,0E+10	1,4E+10	2,2E+09	4,3E+07	4,7E+10	3,4E+06	1,9E+09
	164	3,5E+10	5,4E+10	3,4E+10	2,3E+10	2,0E+09	2,4E+09	4,8E+07	5,3E+10	3,8E+06	2,1E+09
HC(-)-N(+)-UL	80	1,6E+10	2,2E+09	1,5E+10	1,2E+10	1,0E+10	1,3E+10	1,0E+09	2,1E+09	1,0E+08	3,0E+07
	108	4,1E+10	5,6E+09	3,8E+10	3,0E+10	2,7E+10	3,3E+10	2,6E+09	5,4E+09	2,6E+08	7,8E+07
	136	1,0E+11	1,4E+10	7,6E+10	7,6E+10	6,8E+10	8,2E+10	6,6E+09	1,4E+10	6,6E+08	2,0E+08
	164	1,1E+11	1,5E+10	8,1E+10	7,6E+10	6,8E+10	9,0E+10	7,2E+09	1,5E+10	7,2E+08	2,2E+08

The color scale in rows, black→white, represents highest to lowest parameter value rank.
The standard deviations were between 5% and 9 % of the mean values.

The overall results obtained in this study implied that methanogenesis can dominate over denitrification at high nitrate concentrations in environments where unlimited amount of organic carbon is available for microbial growth, and a bacterial population carrying out DNRA is predominant.

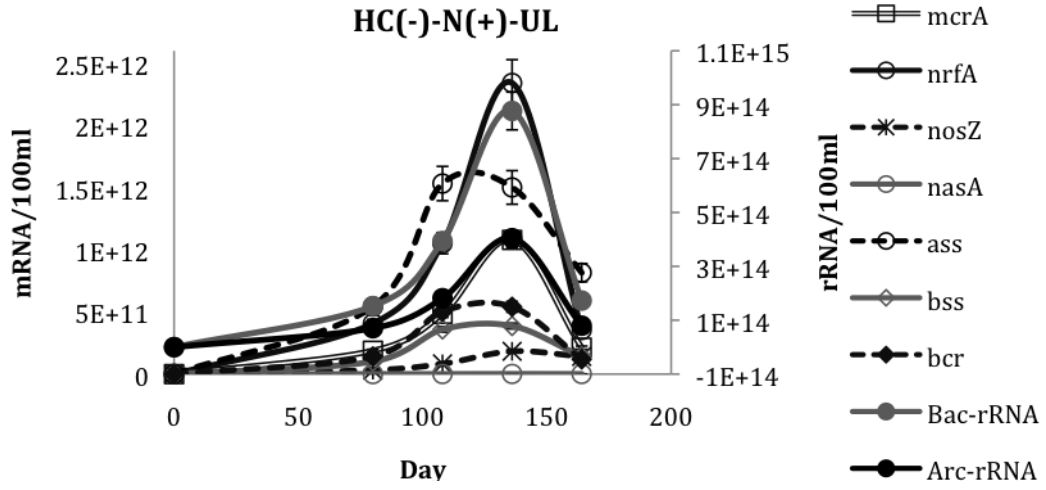


Figure 6.37: Differential RNA production compared to the level on day 0.

Table 6.11: Stoichiometry of exponential microbial growth

	Process	$C_{17}H_{31}$	H_2O	NH_4^+	NO_3^-	HCO_3^-	H^+	→	$C_5H_7NO_2$	CO_2	CH_4	H_2O	NH_4^+	N_2
N(-)	MG[a]	1	5,3	1		1		→	1	3,2	10			
	MG[b]	1	?	0,93		?		→	0,92	3,04	10			
N(+)	MG[a]	0,3	1,59	0,3		0,3		→	0,3	0,96	3			
	DNRA[a]	0,65		0,98	5,67	0,98	11	→	0,99	6,69		3,25	5,28	
	DN[a]	0,05		0,08	0,70	0,08	0,7	→	0,08	0,56		1,06		0,4
	Overall[a]	1			6,38	1,35	12	→	1,37	8,21	3	2,71	3,93	0,4
	Overall[b]	1			5,87	?	?	→	1,27	7,47	3	?	3,62	?

[a] The theoretical stoichiometry of methanogenesis (MG), dissimilatory nitrate reduction to ammonia (DNRA) and denitrification (DN).
[b] The measured stoichiometry of the exponential microbial growth.

6.4.7 Anaerobic hydrocarbon degradation pathway

Initial activation of hydrocarbons is crucial for anaerobic biodegradation, and four general enzymatic reactions are recognized: (1) addition of fumarate, catalyzed by a glycyl radical enzyme such as bss; (2) methylation of unsubstituted aromatics; and (3) hydroxylation of an alkyl substituent (Fuchs, 2008). The other proposed pathways represent a combination of these reactions. These activation reactions feed into pathways that result in production of central metabolites such as benzoyl-coA which are eventually incorporated into biomass or completely oxidized.

Bss has been the only identified enzyme which specifically attacks on aromatic hydrocarbons (toluene and xylene) (Foght, 2008); bcr catalyzes dearomatization of the central metabolite benzoyl coenzyme A. This is why we chose bcr and bss genes and their transcripts as indicators of anaerobic aromatic hydrocarbon degradation. Abundance of these genes gradually increased during the incubation period and their transcription levels were very high even after depletion of toluene and xylene. These give an impression that fumarate addition played a major role in initial activation of aromatic hydrocarbons other than toluene and xylene. Fumarate addition has previously been proposed to be included in degradation pathways of the other monoaromtic hydrocarbons (benzene, alkylbenzenes and ethylbenzene) and PAHs (naphtalene and phenanthrene) (Foght 2008 and references therein).

The two proposed mechanisms of anaerobic n-alkane degradation involves (1) activation at the subterminal carbon of the alkane and addition to a molecule of fumarate; and (2) the alkane carboxylation at C-3 (Grossi et al., 2008). Recently, Callaghan et al. (2008) reported a glycyl radical type enzyme (ass) which involve in

alkane activation through fumarate addition in the SRB strain AK-01. This was the first description of a gene specifically involved in anaerobic n-alkane metabolism. Our results imply that fumarate addition was the main mechanism of initial activation of n-alkanes since both abundance and transcription level of the ass gene was very high throughout the incubation period.

6.4.8 Anaerobic bioremediation feasibility of Tuzla Bay sediments

Our findings on the anaerobic degradation hierarchy of hydrocarbons were coincided with the previous reports: the most easily degradable compounds are n-alkanes, followed by more resistant branched acyclic and monocyclic hydrocarbons, the most resistant polycyclic steroidal and triterpenoidal hydrocarbons, and aromatic hydrocarbons (Foght, 2008; Grossi et al., 2008).

The previous literature on anaerobic metabolism of the HC mix components was summarized in Table 8. C_{9-31} n-alkanes have been reported to be biodegradable under nitrate- and sulfate-reducing and methanogenic conditions. The studies which used marine sediments as inoculum produced much of the biodegradability reports on C_{20-31} n-alkanes (Jones et al., 2008; Miralles et al., 2007). We showed for the first time that marine benthic microbiota is able to degrade long chain n-alkanes under nitrate reducing conditions.

Table 6.12: Degradability of hydrocarbons

Hydrocarbon	NR	SR	M	Hydrocarbon	NR	SR	M
n-C_{9-20} Alkanes	+	+	+Mar	Flourene	+Mar	+Mar	+
n-C_{20-31} Alkanes			+Mar	Phenanthrene	+Mar	+Mar	+Mar
Phytane and Pristane	+Mar	+Mar	+Mar	Anthracene	+	+Mar	+
Benzene	+Mar	+Mar	+Mar	Flouranthene	+Mar	+Mar	+
Toluene	+Mar	+Mar	+Mar	Pyrene	+	+Mar	+
Ethylbenzene	+Mar	+Mar	+Mar	Benz(a)Antracene		+Mar	+
m-Xylene	+Mar	+Mar	+Mar	Chrysene		+Mar	+
p-Xylene	+Mar	+Mar	+Mar	Benzo(k)flouranthene		+Mar	+
Naphtalene	+Mar	+Mar	+Mar	Benzo(a)Pyrene		+Mar	+
Acenaphtylene				DiBenz(a,h)Antracene			+
Acenaphtene	+Mar	+Mar	+	Benzo(g,h,i)Perylene			

SR: Dissimilatory sulfate reduction; NR: Dissimilatory nitrate reducing; M: Methanogenesis
+: Degradable hydrocarbons
+Mar: Degradability was shown for marine sediments

BTEX components are the best studied substrates of anaerobic biodegradation since they are the most water soluble of the aromatic hydrocarbons. As expected, Tuzla Bay sediments degraded BTEX faster than the PAHs. A large amount of the literature on PAH degradation by anoxic marine sediments were obtained under sulfate reducing conditions because of overwhelming abundance of sulfate in seawater. Our study is the first that reports wide range of 3-5 ring PAHs were degraded by marine sediments under methanogenic and nitrate reducing conditions. Besides, this is the first indication of acenaphtylene, benz(a)antracene, chrysene, benzo(k)flouranthene, benzo(a)pyrene, dibenz(a,h)antracene and benzo(g,h,i)perylene biodegradation under nitrate reducing conditions.

Whereas biostimulation through e^--acceptor or supplementary carbonaceous substrate addition has been studied, the stimulatory effect of providing N and P has not been thoroughly studied under anaerobic conditions (Harayama et al. 2004, Andreoni and Gianfreda 2007, Foght 2008). Two cases showed the stimulatory effect of fertilizing nutrient-poor groundwater (Cross et al. 2006) and soil (Powell et al. 2006) on anaerobic hydrocarbon degraders. Although it is well known that N and P limit microbiological activity in marine environments, none has attempted to increase anaerobic hydrocarbon degradation activity of marine microbenthos through nutrient amendment. We have shown that it is possible to increase anaerobic hydrocarbon degradation activity of Tuzla Bay sediments substantially (21×) by supplying the limiting nutrients (N and P).

Recent reports on the anaerobic hydrocarbon degradation rates of marine sediments or marine enrichment cultures were summarized in Table 9. As seen, none has achieved anaerobic hydrocarbon degradation rates comparable to the aerobic ones. In this study, we obtained anaerobic hydrocarbon degradation rates faster than SRB enrichment cultures' rates. Moreover, the obtained rates were comparable to the aerobic ones. In summary, we have obtained three lines of evidence for demonstrating anaerobic bioremediation feasibility of petroleum HC pollution in Tuzla Bay sediments: (1) the anaerobic hydrocarbon degrading microbiota was highly abundant in the sediments (Kolukirik et al., 2010); (2) anaerobic hydrocarbon degradation was taking place in the sediments (Kolukirik et al., 2010); (3) the sediments were able to degrade wide range of hydrocarbons under anaerobic/anoxic conditions (this study); (4) high anaerobic hydrocarbon degradation rates were

achieved via biostimulation of the sediments through nutrient amendment (this study).

Table 6.13: Literature on anaerobic hydrocarbon degradation

Inoculum	Substrate	e^--acceptor addition	Removal Rate - ug/gSediment.L.day	Reference
SRB Enrichment	Short-chain alkanes	Sulfate	0,6	Kniemeyer et al., 2007
SRB Enrichment	Phenanthrene	Sulfate	1,4	Davidova et al., 2007
Sediment	Crude oil	none	7	Miralles et al., 2007
Sediment	2-5 rings PAH	Sulfate	48	Rothermich et al., 2002
Sediment	n-alkanes	none	60	Jones et al., 2008
Sediment	PAH	none	228	Kim et al., 2008
SRB Enrichment	Naphthalene	Sulfate	880	Musat et al., 2009
Sediment	**n-alkanes, PAH and BTEX**	**none**	**1268**	**This study**
Sediment	Crude oil	Oxygen	1970	Syakti et al., 2006
Sediment	Crude oil	Oxygen	4285	Roling et al., 2002

7. CONCLUSION

A less human intervened, sustainable and cost effective remediation strategy is needed to overcome the chronic hydrocarbon pollution in Marmara Sea. The best candidate for this purpose is bioremediation under anaerobic/anoxic conditions as long as oil-degrading anaerobes are abundant and active in the sediments, and there is a way to increase activity of this population. In order to assess this, physicochemical and microbiological characteristics of Marmara Sea sediments were monitored for two years; the results of the monitoring study guided us for establishing a strategy to increase activity of anaerobic hydrocarbon degraders.

Monitoring the sediments from 10 different locations in the Marmara Sea for 2 years revealed that microbial composition, abundance and activity were strongly related to the level and type of nutrient sources. The sediments were extremely polluted with hydrocarbons (2-20 g/kg), and contained abnormally high concentrations of nitrate (0.1-2 mM) and Ni (55-105 mg/kg). The clone libraries were dominated by *Euryarchaeota* and *Proteobacteria*, as well as unclassified archaeal and bacterial phylogenetic clusters unique to the Marmara Sea. Microbial cell contents of the sediments were high (5×10^{9}-1.5×10^{11} cells/cm^{3}) and a considerable fraction of the cells was active (60-85%). The Marmara Sea seems to be a perfect candidate for further investigation of ecologically important microbial processes; since metabolically diverse communities existed within a very short distance from the sediment surfaces and chemical compositions of the sediments were unusual.

Seasonal and local changes in abundance and activity of organisms responsible for respiratory denitrification (RD), dissimilatory nitrate reduction to ammonia (DNRA), anaerobic ammonium oxidation (ANAMMOX), assimilatory nitrate reduction (ANR), dissimilatory sulfate reduction (DSR), methanogenesis (MG) and anaerobic hydrocarbon degradation (AnHD) in Marmara Sea Sediments were also monitored for 2 years. Functional genes and their transcripts were quantified using real-time PCR as the microbial process indicators. All of the target genes were abundant and transcribed in the first 15 cm below the sea floor. The most active processes were

DNRA, MG and AnHD. Microbial activity and abundance differences were related to chemical divergence of the sediments. Abundances of organisms responsible for DSR, RD, DNRA and ANAMMOX were positively correlated to the relevant e^--acceptor levels. Microbial activity levels (mRNA and rRNA levels) were strongly related to N and P contents of the porewaters. Abundance and activity of AnHD organisms were related to level and composition of petroleum hydrocarbons. This study reported the most comprehensive abundance and transcription level assessment of functional genes responsible for the key metabolic processes in marine sediments.

The most important outcomes of the monitoring study which guided the next bioremediation feasibility studies were: (1) the total petroleum HC levels were similar to those from extremely polluted marine environments; (2) the microbial cell contents were very high compared to the other marine environments; (3) the sediments were dominated by dissimilatory nitrate reducers to ammonia, methanogens and anaerobic hydrocarbon degraders, and these microbes were active; and (4) N and P were limited in the porewaters for biological activity. These indications raised the question that hydrocarbon degradation activity of Marmara Sea sediments can be increased by N-P amendment under methanogenic and nitrate reducing conditions. Natural TOC/N/P ratio of the sediment porewaters was gradually decreased which resulted in ~20× increase in hydrocarbon removal. Natural hydrocarbon content of the sediments were completely removed. The sediment microorganisms degraded wide range of aliphatic (n-C_{9-31} alkanes and acyclic isoprenoids) and aromatic (18 different 1-5 ring aromatics) hydrocarbons. Monitoring functional gene and transcript abundances revealed that methanogenesis, dissimilatory nitrate reduction to ammonia and denitrification processes were the three routes of hydrocarbon degradation in Marmara Sea sediments. Genes and transcripts related to initial activation of hydrocarbons were highly abundant throughout the incubation periods showing that fumarate addition was the main pathway of anaerobic hydrocarbon degradation.

In summary, we have obtained three lines of evidence for demonstrating anaerobic bioremediation feasibility of petroleum HC pollution in Tuzla Bay sediments: (1) the anaerobic hydrocarbon degrading microbiota was highly abundant in the sediments; (2) anaerobic hydrocarbon degradation was taking place in the sediments; (3) the sediments were able to degrade wide range of hydrocarbons under anaerobic/anoxic

conditions; (4) high anaerobic hydrocarbon degradation rates were achieved via biostimulation of the sediments through nutrient amendment. In conclusion, biostimulation of highly polluted anoxic Marmara Sea Sediments via nutrient amendment is possible which will lead to less human intervened and more economical field-scale bioremediation applications worldwide.

We are now making the preliminary preparations in conjunction with Maritime Undersecretariat of Turkish Republic, Water and Sewerage Administration of İstanbul (ISKI) and İstanbul Metropolitan Municipality (IBB) to carry out a field-scale bioremediation trial to remove the accumulated HCs from the subsurface of the Marmara Sea through biostimulation of the sediments. Success of this trial will certainly lead to less human intervened and more economical field-scale bioremediation applications for highly polluted anoxic marine environments worldwide.

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APPENDICES

APPENDIX A1: 16S rRNA gene accession numbers and frequencies

APPENDIX A.2: Cluster analysis of DGGE profiles

APPENDIX A1

Table A.1.1: Cloning results for Bacteria in Gemlik Bay sediments

Classification	Clone				DGGE	Accession Number			Similarity (%)	
						This Study	Closest Relative		Closest Relative	
	Name	Frequency (%)			Intensity (%)		Uncultured (UC)	Cultured (C)	UC	C
Marmara Bacterial Cluster2 (MBC2)	Mar_B11	14,0		0,8			AY592661	AB109438	99	90
	Mar_B8			0,8				AB188780		98
	Mar_B18			0,8			EU048671	DQ309326	97	85
	Mar_B29			0,8		AM980564	AF419690	AJ229236	94	84
	Mar_B34			0,8			AB294297	AY928240	96	91
	Mar_B36			0,8		AM980567		AJ536674		94
	Mar_B37			0,8			AY592648	AB109439	95	86
	Mar_B44			0,8			AM176853	AB286031	100	98
	Mar_B45			1,7	2,3	AM980569	AB267027	X85132	90	78
Marmara Bacterial Cluster1 (MBC1)	Mar_B52			0,8			AY592648	AB109439	95	86
	Mar_B56			0,8			DQ238608	Y18174	99	96
	Mar_B57			1,7	2,3		DQ302422	AJ224539	95	85
	Mar_B63			0,8		AM980577	AJ241004	AF357915	93	85
	Mar_B66			1,7	2,3	AM980578	DQ499326	CP000232	88	81
Acidobacteria	Mar_B4	5,8		1,7	2,3		AY225640	AM086646	96	82
	Mar_B48			0,8		AM980572	DQ811918	X91170	88	81
	Mar_B58			0,8			DQ351783	DQ303458	99	85
	Mar_B64			0,8			AJ241003	AJ300509	96	82
	Mar_B61			1,7	2,3	AM980575		CP000473		92
Actinobacteria	Mar_B53	1,7		0,8		AM980573	DQ811925	AF498707	94	83
	Mar_B7			0,8		AM980557		DQ076484		88
Bacteroidetes	Mar_B26	5,0		4,1	5,7			AM497875		96
	Mar_B9			0,8				DQ530470		95
Candidate division WS3	Mar_B43	1,7		1,7	2,3	AM980568	AM229499	U41049	85	72
Chlorobi	Mar_B5	2,5		2,5	3,4	AM980555	AJ428454	AJ290830	91	80
Chloroflexi	Mar_B2	3,3		0,8		AM980553	AB189336	AB109439	92	87
	Mar_B28			0,8			EF420218	AB109439	98	90
	Mar_B47			1,7	2,3	AM980571		AF524858		91
Firmicutes	Mar_B24	15,7		0,8		AM980563		AY169818		94
	Mar_B55			0,8				AM411529		96
	Mar_B12			0,8				DQ126679		98
	Mar_B21			0,8		AM980561		Y18185		93
	Mar_B50			1,7	2,3			AY438672		95
	Mar_B59			1,7	2,3		DQ325561	Y18176	99	95
	Mar_B20			0,8		AM980560		DQ117468		92
	Mar_B60			5,0	6,9	AM980574		AF542227		91
	Mar_B38			3,3	4,6			X87150		99
Planctomycetes	Mar_B46	2,5		2,5	3,4	AM980570	AF424486	AJ250882	93	80

Table A.1.2: Cloning results for Archaea in Gemlik Bay sediments

	Clone			DGGE	Accession Number			Similarity (%)	
					This Study	Closest Relative		Closest Relative	
	Name	Frequency (%)		Intensity (%)		Uncultured (UC)	Cultured (C)	UC	C
Marmara Archaeal Cluster1 (MAC1)	Mar_A6		3,8	4,5	AM980585	DQ082931	AF524848	87	79
	Mar_A16		3,8	2,5	AM980593		DQ058817		88
	Mar_A21		1,3		AM980598	DQ088719	AB026175	79	68
	Mar_A22		1,3			AJ969749	AF199378	96	90
Unclassified Archaea	Mar_A3		3,8	3,5	AM980582	DQ146745	U55239	84	75
	Mar_A5		1,3		AM980584	AY592249	DQ058822	74	62
	Mar_A10		3,8	4,5	AM980589	AY592500	AY196684	94	74
	Mar_A13		2,5	3,3	AM980590	DQ521768	CP000505	90	73
	Mar_A27		1,3			AY835408	M59124	95	78
	Mar_A28		1,3		AM980601	AF119137	BD445502	94	79
	Mar_A30		2,5	3,3	AM980603		DQ522944		83
	Mar_A33	17,5	1,3		AM980604	DQ521152	AF093061	89	72
Crenarchaeota	Mar_A2		1,3		AM980581	AY454668	AB197163	91	77
	Mar_A14		1,3		AM980591	AY396700	CP000505	86	75
	Mar_A23		11	10		AF004345	CP000505	97	80
	Mar_A7		1,3		AM980586	AY454668	U38486	92	75
	Mar_A24	18,8	3,8	4,5	AM980599		DQ085097		88
Euryarchaeota	Mar_A9		5,0	6,7	AM980588	AB301990	DQ445723	92	79
	Mar_A12		3,8	4,5			AF199378		95
	Mar_A15		1,3		AM980592		DQ445723		89
	Mar_A17		2,5	3,3	AM980594		AF199374		77
	Mar_A18		3,8	4,5	AM980595	AY380683	DQ445723	76	73
	Mar_A1		2,5	3,3	AM980580	AB119590	DQ451875	88	67
	Mar_A4		3,8	4,5	AM980583	AB119590	AB260046	93	67
	Mar_A20		2,5	3,3	AM980597		U55236		77
	Mar_A19		1,3		AM980596		AB057722		91
	Mar_A35		2,5	3,3			DQ177344		98
	Mar_A25		1,3			AJ133791	AJ133791	95	95
	Mar_A31		7,5	10,0			Y15402		97
	Mar_A32		3,8	4,5			M59146		95
	Mar_A38		5,0	6,7	AM980607		CP000300		94
	Mar_A37		3,8	5,5	AM980606		DQ522915		92
	Mar_A11		2,5	3,3			U20152		95
	Mar_A36	53,8	1,3				AB288264		97

Table A.1.3: Cloning results for Bacteria in Moda Coast sediments

							Accession number			Similarity	
		Clone				DGGE Intensity (%)		Closest Relative		Closest Relative	
		Name	Frequency (%)				This Study	Uncultured (UC)	Cultured (C)	UC	C
Marmara Bacterial Cluster2 (MBC2)		MAR_BAC_7	14		0,8				AB188780		98
		MAR_BAC_18			1,6	0,8		DQ809773	AJ012591	99	85
		MAR_BAC_32			0,8		AM990999	AY592788	AY165308	78	72
		MAR_BAC_80			0,8		AM980564	AF419690	AJ229236	94	84
		MAR_BAC_150			1,6	1,8	AM980569	AB267027	X85132	90	78
		MAR_BAC_169			0,8	2,0		AY592648	AB109439	95	86
		MAR_BAC_187			1,6	1,8	AM998360	AY214200	AJ431246	93	88
		MAR_BAC_201			1,6	1,8		DQ302422	AJ224539	95	85
		MAR_BAC_210			0,8			AY592152	AF295656	98	82
		MAR_BAC_226			0,8				AB205405		96
		MAR_BAC_143			1,6	0,8	AM980568	AM229499	U41049	85	71
		MAR_BAC_107			1,6	1,8		DQ325561	Y18176	99	95
Acidobacteria		MAR_BAC_206	3,2		0,8			DQ351783	DQ303457	99	85
		MAR_BAC_222			0,8	1,8		AJ241003	AJ300509	96	82
		MAR_BAC_213			1,6	1,8	AM980575		CP000473		92
eria		MAR_BAC_191			0,8		AM980573	DQ811925	AF498707	94	83
		MAR_BAC_135			2,4	2,7			DQ219354		100
Chloroflexi		MAR_BAC_70	5,6		0,8			AB116439	AB243672	97	86
		MAR_BAC_79			0,8			EF420218	AB109439	96	90
		MAR_BAC_109			1,6	1,8		EF420218	AB109439	98	90
		MAR_BAC_160			1,6	1,8	AM980571		AF524858		91
		MAR_BAC_223			0,8			AB288599	AB243672	95	86
Firmicutes		MAR_BAC_63	28		0,8		AM980563		AY169818		94
		MAR_BAC_167			2,4	2,7	AM998354		AF349724		65
	Clostridia	MAR_BAC_14		22,4	0,8				DQ126679		98
		MAR_BAC_58			0,8		AM980561		Y18185		93
		MAR_BAC_60			0,8				X76161		96
		MAR_BAC_106			1,6	1,8		DQ478749	AJ297449	97	87
		MAR_BAC_147			7,2	8,2			AB030224		99
		MAR_BAC_219			6,4	7,3			AB030221		98
		MAR_BAC_97			0,8				AF491333		95
		MAR_BAC_128			4	4,5	AM998349		AB038361		94
		MAR_BAC_145			2,4	2,7	AM998352		Y11466		80
		MAR_BAC_157			2,4	2,7	AM980570	AF424486	AJ250882	93	80

Table A.1.4: Cloning results for Archaea in Moda Coast sediments

							Accession number			Similarity	
		Clone				DGGE		Closest Relative		Closest Relative	
		Name	Frequency (%)			Intensity (%)	This Study	Uncultured (UC)	Cultured (C)	UC	C
Marmara Archaeal Cluster1 (MAC1)		MAR_ARC_6	30,8		2,1	3,6	AM980582	DQ146745	U55239	84	75
		MAR_ARC_11			1,4	2,7	AM992679	EF153105	DQ058818	60	55
		MAR_ARC_12			3,4	3,3	AM992680		L77117	80	69
		MAR_ARC_14			1,4	1,7	AM980584	AY592249	DQ058822	74	
		MAR_ARC_16			2,1	2,6	AM980585	DQ082931	AF524848	87	79
		MAR_ARC_22			0,7		AM992710	AJ305078	AF199376	79	79
		MAR_ARC_26			2,1	2,6	AM980589	AY592500	AY196684	94	74
		MAR_ARC_41			1,4	1,7	AM992692	DQ640163	CP000254	77	76
		MAR_ARC_50			1,4	2,8	AM992695	DQ640137	AB033290	91	77
		MAR_ARC_61			2,1	3,6	AM980593		DQ058817		88
		MAR_ARC_85			1,4	1,7	AM992707	AY592230	AY196658	94	75
		MAR_ARC_132			1,4	1,7		DQ103671	AF255608	96	81
		MAR_ARC_137			0,7			AY835408	M59124	95	78
		MAR_ARC_141			1,4	1,7	AM980601	AF119137	BD445502	94	79
		MAR_ARC_142			0,7		AM998424	AY835427	AF028689	89	75
		MAR_ARC_145			1,4	1,7	AM992716	DQ522944	AF255608	92	84
		MAR_ARC_152			1,4	2,5	AM992718	AY592258	AF255608	77	67
		MAR_ARC_154			1,4	1,7		AY341269	AE000666	96	80
		MAR_ARC_158			1,4	2,4	AM980603		AJ745145		83
		MAR_ARC_190			1,4	1,7	AM998446	EF444599	AB057722	86	76
		MAR_ARC_214			0,7		AM992723	AY592484	DQ445723	91	76
Crenarchaeota		MAR_ARC_15	20,5		0,7		AM992683	AY454678	AB269873	86	75
		MAR_ARC_17			1,4	2,1		AY454663	AB260046	96	76
		MAR_ARC_20			9,6	8,1	AM998384	AY454668	DQ451875	80	68
		MAR_ARC_119			6,2	5,2		AF004345	CP000505	97	80
		MAR_ARC_53			2,7	2,3	AM998395		DQ397841		67
Euryarchaeota		MAR_ARC_18	48,6		2,1	1,7		DQ417474	AB197163	90	77
		MAR_ARC_30			1,4	2,2	AM998386	AB119590	CP000743	93	67
		MAR_ARC_135			0,7		AM980600	AB119598	CP000102	94	69
		MAR_ARC_32			2,7	2,3	AM992691		AF199378		82
		MAR_ARC_1			2,7	2,3	AM998380	AB119590	CP000743	93	67
		MAR_ARC_4			9,6	8,1	AM980583	AB119590	AB260046	92	77
		MAR_ARC_5			9,6	8,1	AM980580	AB119590	DQ451875	88	74
		MAR_ARC_58			1,4	2,2	AM99270		AF509441		69
		MAR_ARC_55			1,4	2,2	AM998397		AE000666		80
		MAR_ARC_13			0,7		AM992681		DQ195164		75
		MAR_ARC_60			0,7		AM980596		AB057722		91
	Methanomicrobia	MAR_ARC_191		15,8	0,7			AY211687	AY970347	96	87
		MAR_ARC_175			0,7		AM998436		AJ133791		88
		MAR_ARC_184			0,7		AM998442	EF420177	AJ133791	84	84
		MAR_ARC_234			7,5	6,4			U20152		98
		MAR_ARC_95			1,4	2,2	AM992708		AJ012742		91
		MAR_ARC_165			1,4	2,2	AM992721		AE010299		86
		MAR_ARC_222			0,7				AY260431		95
		MAR_ARC_200			2,1	1,7	AM998453		CP000099		84

Table A.1.5: Cloning results for Bacteria in Tuzla Bay sediments

							Accession number			Similarity		
		Clone				DGGE		Closest Relative			Closest Relative	
		Name	Frequency (%)			Intensity (%)	This Study	Uncultured (UC)	Cultured (C)	UC	C	
Marmara Bacterial Cluster2 (MBC2)		MAR_BAC_4	17,1		0,8				AB188780		98	
		MAR_BAC_7			0,8				AB188780		98	
		MAR_BAC_18			1,6	2,1		DQ809773	AJ012591	99	85	
		MAR_BAC_32			0,8		AM990999	AY592788	AY165308	78	72	
		MAR_BAC_80			0,8		AM980564	AF419690	AJ229236	94	84	
		MAR_BAC_150			1,6	2,1	AM980569	AB267027	X85132	90	78	
		MAR_BAC_169			1,6	2,1		AY592648	AB109439	95	86	
		MAR_BAC_187			1,6	2,1	AM998360	AY214200	AJ431246	93	88	
		MAR_BAC_201			0,8			DQ302422	AJ224539	95	85	
		MAR_BAC_210			0,8			AY592152	AF295656	98	82	
		MAR_BAC_226			1,6	2,1			AB205405		96	
		MAR_BAC_228			0,8		AM980578	DQ499326	CP000232	88	81	
		MAR_BAC_143			0,8		AM980568	AM229499	U41049	85	71	
		MAR_BAC_107			2,4	3,2		DQ325561	Y18176	99	95	
Acidobacteria		MAR_BAC_206	8,1	3,3	0,8			DQ351783	DQ303457	99	85	
		MAR_BAC_222			1,6	2,1		AJ241003	AJ300509	96	82	
		MAR_BAC_213			0,8		AM980575		CP000473		92	
Actinobacteria		MAR_BAC_191		4,9	2,4	3,2	AM980573	DQ811925	AF498707	94	83	
		MAR_BAC_135			0,8				DQ219354		100	
		MAR_BAC_232			1,6	2,1	AM980557		DQ076484		88	
Chloroflexi		MAR_BAC_2	7,3		0,8		AM990998	AB189336	AB109439	92	87	
		MAR_BAC_70			1,6	2,1		AB116439	AB243672	97	86	
		MAR_BAC_79			1,6	2,1		EF420218	AB109439	96	90	
		MAR_BAC_109			0,8			EF420218	AB109439	98	90	
		MAR_BAC_160			1,6	2,1	AM980571		AF524858		91	
		MAR_BAC_223			0,8			AB288599	AB243672	95	86	
Firmicutes		MAR_BAC_63	28,5		0,8		AM980563		AY169818		94	
		MAR_BAC_167			0,8		AM998354		AF349724		65	
	Clostridiales	MAR_BAC_14			0,8				DQ126679		98	
		MAR_BAC_58			1,6	2,1	AM980561		Y18185		93	
		MAR_BAC_60			7,3	9,6			X76161		96	

Table A.1.6: Cloning results for Archaea in Tuzla Bay sediments

					Accession number			Similarity	
	Clone			DGGE		Closest Relative		Closest Relative	
	Name	Frequency (%)		Intensity (%)	This Study	Uncultured (UC)	Cultured (C)	UC	C
Marmara Archaeal Cluster1 (MAC1)	MAR_ARC_6	30,9	1,7	2,0	AM980582	DQ146745	U55239	84	75
	MAR_ARC_11		1,1	1,3	AM992679	EF153105	DQ058818	60	55
	MAR_ARC_12		2,8	3,3	AM992680		L77117	80	69
	MAR_ARC_14		1,1	1,3	AM980584	AY592249		74	
	MAR_ARC_16		1,7	2,0	AM980585	DQ082931	AF524848	87	79
	MAR_ARC_21		1,1	1,3	AM980587	EF104088	AF255608	72	59
	MAR_ARC_22		0,6		AM992710	AJ305078	AF199376	79	79
	MAR_ARC_26		1,7	2,0	AM980589	AY592500	AY196684	94	74
	MAR_ARC_41		1,1	1,3	AM992692	DQ640163	CP000254	77	76
	MAR_ARC_50		1,1	1,3	AM992695	DQ640137	AB033290	91	77
	MAR_ARC_61		1,7	2,0	AM980593		DQ058817		88
	MAR_ARC_80		0,6		AM992704	AY592484	AF255608	91	81
	MAR_ARC_85		1,1	1,3	AM992707	AY592230	AY196658	94	75
	MAR_ARC_114		1,1	1,3		AF119128	AE000666	95	76
	MAR_ARC_118		0,6			AJ969749	AF199378	96	90
	MAR_ARC_127		0,6			DQ363763	U41095	98	76
	MAR_ARC_132		1,1	1,3		DQ103671	AF255608	96	81
	MAR_ARC_137		0,6			AY835408	M59124	95	78
	MAR_ARC_141		1,1	1,3	AM980601	AF119137	BD445502	94	79
	MAR_ARC_145		1,1	1,3	AM992716	DQ522944	AF255608	92	84
	MAR_ARC_152		1,1	1,3	AM992718	AY592258	AF255608	77	67
	MAR_ARC_154		1,1	1,3		AY341269	AE000666	96	80
	MAR_ARC_158		1,1	1,3	AM980603		AJ745145		83
	MAR_ARC_190		1,1	1,3	AM998446	EF444599	AB057722	86	76
	MAR_ARC_214		0,6		AM992723	AY592484	DQ445723	91	76
	MAR_ARC_263		1,1	1,3	AM992725	AY367339	DQ058818	64	59
	MAR_ARC_245		1,1	1,3	AM992726	DQ522909	AF199378	86	81
Crenarchaeota	MAR_ARC_15	18,0	0,6		AM992683	AY454678	AB269873	86	75
	MAR_ARC_17		1,1	1,3		AY454663	AB260046	96	76
	MAR_ARC_20		7,9	9,2	AM998384	AY454668	DQ451875	80	68
	MAR_ARC_119		1,1	1,3		AF004345	CP000505	97	80
	MAR_ARC_53		5,1	5,9	AM998395		DQ397841		67
	MAR_ARC_247		2,2	2,6	AM998469	AF068822	CP000505	76	63
Euryarchaeota	MAR_ARC_18	47,8	1,1	1,3		DQ417474	AB197163	90	77
	MAR_ARC_30		1,7	2,0	AM998386	AB119590	CP000743	93	67
	MAR_ARC_69		1,1	1,3	AM980595	AY380683	DQ445723	76	73
	MAR_ARC_135		1,7	2,0	AM980600	AB119598	CP000102	94	69
	MAR_ARC_32		0,6		AM992691		AF199378		82
	MAR_ARC_1		2,2	2,6	AM998380	AB119590	CP000743	93	67
	MAR_ARC_4		2,2	2,6	AM980583	AB119590	AB260046	92	77
	MAR_ARC_5		7,9	9,2	AM980580	AB119590	DQ451875	88	74
	MAR_ARC_58		7,9	9,2	AM99270		AF509441		69
	MAR_ARC_230		1,1	1,3			CP000102		95
	MAR_ARC_55		0,6		AM998397		AE000666		80

Table A.1.7: Cloning results for Bacteria in K.cekmece sediments

		Clone				DGGE	Accession number			Similarity	
								Closest Relative		Closest Relative	
		Name	Frequency (%)			Intensity (%)	This Study	Uncultured (UC)	Cultured (C)	UC	C
Marmara Bacterial Cluster2 (MBC2)		MAR_BAC_4	15,2		0,8				AB188780		98
		MAR_BAC_7			0,8				AB188780		98
		MAR_BAC_32			0,8		AM990999	AY592788	AY165308	78	72
		MAR_BAC_80			0,8		AM980564	AF419690	AJ229236	94	84
		MAR_BAC_150			1,6	1,5	AM980569	AB267027	X85132	90	78
		MAR_BAC_169			0,8			AY592648	AB109439	95	86
		MAR_BAC_187			1,6	1,5	AM998360	AY214200	AJ431246	93	88
		MAR_BAC_201			1,6	2,5		DQ302422	AJ224539	95	85
		MAR_BAC_210			0,8			AY592152	AF295656	98	82
		MAR_BAC_226			0,8				AB205405		96
		MAR_BAC_228			1,6	2,5	AM980578	DQ499326	CP000232	88	81
		MAR_BAC_143			1,6	1,5	AM980568	AM229499	U41049	85	71
		MAR_BAC_107			1,6	2,5		DQ325561	Y18176	99	95
Acidobacteria		MAR_BAC_206	3,2		0,8			DQ351783	DQ303457	99	85
		MAR_BAC_222			0,8			AJ241003	AJ300509	96	82
		MAR_BAC_213			1,6	2,5	AM980575		CP000473		92
Actinobacteria		MAR_BAC_191	3,2		0,8		AM980573	DQ811925	AF498707	94	83
		MAR_BAC_135			2,4	3,6			DQ219354		100
Chloroflexi		MAR_BAC_70	5,6		0,8			AB116439	AB243672	97	86
		MAR_BAC_79			0,8			EF420218	AB109439	96	90
		MAR_BAC_109			1,6	2,5		EF420218	AB109439	98	90
		MAR_BAC_160			1,6	2,5	AM980571		AF524858		91
		MAR_BAC_223			0,8			AB288599	AB243672	95	86
Firmicutes		MAR_BAC_63	28		0,8		AM980563		AY169818		94
		MAR_BAC_167			2,4	3,6	AM998354		AF349724		65
	Clostridia	MAR_BAC_14		22,4	0,8				DQ126679		98
		MAR_BAC_58			0,8		AM980561		Y18185		93
		MAR_BAC_60			0,8				X76161		96
		MAR_BAC_106			1,6	2,5		DQ478749	AJ297449	97	87
		MAR_BAC_147			7,2	9,2			AB030224		99
		MAR_BAC_219			6,4	8,2			AB030221		98
		MAR_BAC_97			0,8				AF491333		95
		MAR_BAC_128			4	5,1	AM998349		AB038361		94
		MAR_BAC_145			2,4	3,6	AM998352		Y11466		80
		MAR_BAC_157			2,4	3,6	AM980570	AF424486	AJ250882	93	80
Proteobacteria		MAR_BAC_39	42,4		1,6	2,5	AM980559		AJ431222		94
		MAR_BAC_90		8,8	0,8				U78037		99
	α	MAR_BAC_138			8	1,2			AY962292		96
		MAR_BAC_220			2,4	3,6	AM980576		AF229868		60
	δ	MAR_BAC_59		19,2	0,8		AM991008		AJ132804		92
		MAR_BAC_129			3,2	4,8	AM998350	AJ704679	AJ012591	91	85

Table A.1.8: Cloning results for Archaea in K.cekmece sediments

					Accession number			Similarity	
	Clone			DGGE		Closest Relative		Closest Relative	
	Name	Frequency (%)		Intensity (%)	This Study	Uncultured (UC)	Cultured (C)	UC	C
Marmara Archaeal Cluster1 (MAC1)	MAR_ARC_6	31,1	1,7	1,9	AM980582	DQ146745	U55239	84	75
	MAR_ARC_11		1,1	1,3	AM992679	EF153105	DQ058818	60	55
	MAR_ARC_12		2,8	3,1	AM992680		L77117	80	69
	MAR_ARC_14		1,1	1,3	AM980584	AY592249	DQ058822	74	
	MAR_ARC_16		1,7	1,9	AM980585	DQ082931	AF524848	87	79
	MAR_ARC_21		1,1	1,3	AM980587	EF104088	AF255608	72	59
	MAR_ARC_22		0,6		AM992710	AJ305078	AF199376	79	79
	MAR_ARC_26		1,7	1,9	AM980589	AY592500	AY196684	94	74
	MAR_ARC_41		1,1	1,3	AM992692	DQ640163	CP000254	77	76
	MAR_ARC_50		1,1	1,3	AM992695	DQ640137	AB033290	91	77
	MAR_ARC_61		1,7	1,9	AM980593		DQ058817		88
	MAR_ARC_80		0,6		AM992704	AY592484	AF255608	91	81
	MAR_ARC_85		1,1	1,3	AM992707	AY592230	AY196658	94	75
	MAR_ARC_114		1,1	1,3		AF119128	AE000666	95	76
	MAR_ARC_118		0,6			AJ969749	AF199378	96	90
	MAR_ARC_127		0,6			DQ363763	U41095	98	76
	MAR_ARC_132		1,1	1,3		DQ103671	AF255608	96	81
	MAR_ARC_137		0,6			AY835408	M59124	95	78
	MAR_ARC_141		1,1	1,3	AM980601	AF119137	BD445502	94	79
	MAR_ARC_142		0,6		AM998424	AY835427	AF028689	89	75
	MAR_ARC_145		1,1	1,3	AM992716	DQ522944	AF255608	92	84
	MAR_ARC_152		1,1	1,3	AM992718	AY592258	AF255608	77	67
	MAR_ARC_154		1,1	1,3		AY341269	AE000666	96	80
	MAR_ARC_158		1,1	1,3	AM980603		AJ745145		83
	MAR_ARC_190		1,1	1,3	AM998446	EF444599	AB057722	86	76
	MAR_ARC_214		0,6		AM992723	AY592484	DQ445723	91	76
	MAR_ARC_263		1,1	1,3	AM992725	AY367339	DQ058818	64	59
	MAR_ARC_245		1,1	1,3	AM992726	DQ522909	AF199378	86	81
Crenarchaeota	MAR_ARC_15	17,8	0,6		AM992683	AY454678	AB269873	86	75
	MAR_ARC_17		1,1	1,3		AY454663	AB260046	96	76
	MAR_ARC_20		7,8	8,9	AM998384	AY454668	DQ451875	80	68
	MAR_ARC_119		5,0	5,7		AF004345	CP000505	97	80
	MAR_ARC_53		2,2	2,5	AM998395		DQ397841		67
	MAR_ARC_247		1,1	1,3	AM998469	AF068822	CP000505	76	63
Euryarchaeota	MAR_ARC_18	51,1	1,7	1,9		DQ417474	AB197163	90	77,00
	MAR_ARC_30		1,1	1,3	AM998386	AB119590	CP000743	93	67
	MAR_ARC_69		1,7	1,9	AM980595	AY380683	DQ445723	76	73
	MAR_ARC_135		0,6		AM980600	AB119598	CP000102	94	69
	MAR_ARC_32		2,2	2,5	AM992691		AF199378		82
	MAR_ARC_1		2,2	2,5	AM998380	AB119590	CP000743	93	67
	MAR_ARC_4		7,8	8,9	AM980583	AB119590	AB260046	92	77
	MAR_ARC_5		7,8	8,9	AM980580	AB119590	DQ451875	88	74
	MAR_ARC_58		1,1	1,3	AM99270		AF509441		69
	MAR_ARC_230		0,6				CP000102		95

Table A.1.9: Cloning results for Bacteria in HalAS sediments

							Accession number			Similarity	
		Clone			DGGE		Closest Relative			Closest Relative	
		Name	Frequency (%)		Intensity (%)	This Study	Uncultured (UC)	Cultured (C)		UC	C
Marmara Bacterial Cluster2 (MBC2)		MAR_BAC_4	15,3		0,8			AB188780			98
		MAR_BAC_7			0,8			AB188780			98
		MAR_BAC_18			1,5	2,8	DQ809773	AJ012591		99	85
		MAR_BAC_80			0,8	AM980564	AF419690	AJ229236		94	84
		MAR_BAC_150			1,5	1,8	AM980569	AB267027	X85132	90	78
		MAR_BAC_169			0,8		AY592648	AB109439		95	86
		MAR_BAC_187			1,5	1,4	AY214200	AJ431246		93	88
		MAR_BAC_201			1,5	1,8	DQ302422	AJ224539		95	85
		MAR_BAC_210			0,8		AY592152	AF295656		98	82
		MAR_BAC_226			0,8			AB205405			96
		MAR_BAC_228			1,5	1,8	AM980578	DQ499326	CP000232	88	81
		MAR_BAC_143			1,5		AM980568	AM229499	U41049	85	71
		MAR_BAC_107			1,5	1,8		DQ325561	Y18176	99	95
Acidobacteria		MAR_BAC_206	3,1		0,8	1,4		DQ351783	DQ303457	99	85
		MAR_BAC_222			0,8			AJ241003	AJ300509	96	82
		MAR_BAC_213			1,5		AM980575		CP000473		92
Actinobacteria		MAR_BAC_34	3,8		0,8			DQ811922	AJ224541	95	87
		MAR_BAC_191			0,8	1,4	AM980573	DQ811925	AF498707	94	83
		MAR_BAC_135			2,3	2,7			DQ219354		100
Chloroflexi		MAR_BAC_2	6,1		0,8		AM990998	AB189336	AB109439	92	87
		MAR_BAC_70			0,8			AB116439	AB243672	97	86
		MAR_BAC_79			0,8			EF420218	AB109439	96	90
		MAR_BAC_109			1,5	1,8		EF420218	AB109439	98	90
		MAR_BAC_160			1,5		AM980571		AF524858		91
		MAR_BAC_223			0,8			AB288599	AB243672	95	86
Firmicutes		MAR_BAC_63	26,7		0,8		AM980563		AY169818		94
		MAR_BAC_167			2,3	2,7			AF349724		65
	Clostridia	MAR_BAC_14		21,4	0,8				DQ126679		98
		MAR_BAC_58			0,8		AM980561		Y18185		93
		MAR_BAC_60			0,8				X76161		96
		MAR_BAC_106			1,5	1,8		DQ478749	AJ297449	97	87
		MAR_BAC_147			6,9	8,0			AB030224		99
		MAR_BAC_219			6,1	7,1			AB030221		98
		MAR_BAC_97			0,8				AF491333		95
		MAR_BAC_128			3,8	4,5			AB038361		94
		MAR_BAC_145			2,3	2,7			Y11466		80
		MAR_BAC_157			2,3	4,9	AM980570	AF424486	AJ250882	93	80
Proteobacteria		MAR_BAC_32	42,7		2,3	2,7		AM176844	AB271125	98	92
		MAR_BAC_39			1,5	1,8	AM980559		AJ431222		94
	α	MAR_BAC_90			0,8				U78037		99
		MAR_BAC_138			7,6	8,9			AY962292		96
	β	MAR_BAC_220			2,3	2,7	AM980576		AF229868		60
	δ	MAR_BAC_59		18,3	0,8	5,2	AM991008		AJ132804		92
		MAR_BAC_129			3,1	3,6		AJ704679	AJ012591	91	85

Table A.1.10: Cloning results for Achaea in HalAS sediments

							Accession number			Similarity	
		Clone				DGGE		Closest Relative		Closest Relative	
		Name	Frequency (%)			Intensity (%)	This Study	Uncultured (UC)	Cultured (C)	UC	C
Marmara Archaeal Cluster1 (MAC1)		MAR_ARC_6			1,3	2,6	DQ146745	DQ146745	U55239	84	75
		MAR_ARC_11			0,9	1,0	EF153105	EF153105	DQ058818	60	55
		MAR_ARC_12			2,2	2,6			L77117	80	69
		MAR_ARC_14			0,9		AY592249	AY592249	DQ058822	74	69
		MAR_ARC_16			1,3	1,6	DQ082931	DQ082931	AF524848	87	79
		MAR_ARC_21			0,9	1,0	EF104088	EF104088	AF255608	72	59
		MAR_ARC_22			0,4		AJ305078	AJ305078	AF199376	79	79
		MAR_ARC_26			1,3		AY592500	AY592500	AY196684	94	74
		MAR_ARC_41			0,9	2,0	DQ640163	DQ640163	CP000254	77	76
		MAR_ARC_50			0,9	1,6	DQ640137	DQ640137	AB033290	91	77
		MAR_ARC_61			1,3	1,6			DQ058817		88
		MAR_ARC_80			0,4		AY592484	AY592484	AF255608	91	81
		MAR_ARC_85			0,9	1,0	AY592230	AY592230	AY196658	94	75
		MAR_ARC_114			0,9	1,0	AF119128	AF119128	AE000666	95	76
		MAR_ARC_118			0,4		AJ969749	AJ969749	AF199378	96	90
		MAR_ARC_127			0,4		DQ363763	DQ363763	U41095	98	76
		MAR_ARC_132			0,9	1,0	DQ103671	DQ103671	AF255608	96	81
		MAR_ARC_137			0,4		AY835408	AY835408	M59124	95	78
		MAR_ARC_141			0,9	1,0	AF119137	AF119137	BD445502	94	79
		MAR_ARC_142			0,4		AY835427	AY835427	AF028689	89	75
		MAR_ARC_145			0,9	1,0	DQ522944	DQ522944	AF255608	92	84
		MAR_ARC_152			0,9		AY592258	AY592258	AF255608	77	67
		MAR_ARC_154			0,9		AY341269	AY341269	AE000666	96	80
		MAR_ARC_158			0,9				AJ745145		83
		MAR_ARC_190			0,9	1,0	EF444599	EF444599	AB057722	86	76
		MAR_ARC_214			0,4		AY592484	AY592484	DQ445723	91	76
		MAR_ARC_263			0,9		AY367339	AY367339	DQ058818	64	59
		MAR_ARC_245	25,1		0,9		DQ522909	DQ522909	AF199378	86	81
Crenarchaeota		MAR_ARC_15			0,4		AY454678	AY454678	AB269873	86	75
		MAR_ARC_17			0,9	1,0	AY454663	AY454663	AB260046	96	76
		MAR_ARC_20			6,3	7,3	AY454668	AY454668	DQ451875	80	68
		MAR_ARC_33			2,2	2,6	AY454663	AY454663	L77117	95	76
		MAR_ARC_119			4,0	4,7	AF004345	AF004345	CP000505	97	80
		MAR_ARC_34			2,2	2,6	AY454663	AY454663	CP000609	90	75
		MAR_ARC_36			6,3	7,3	AY454668	AY454668	CP000609	90	75
		MAR_ARC_53			1,8	2,1			DQ397841		67
		MAR_ARC_247	25,1		0,9	1,0	AF068822	AF068822	CP000505	76	63
Euryarchaeota		MAR_ARC_18			1,3	1,6	DQ417474	DQ417474	AB197163	90	77
		MAR_ARC_30			0,9	1,0	AB119590	AB119590	CP000743	93	67
		MAR_ARC_69			1,3	1,6	AY380683	AY380683	DQ445723	76	73
		MAR_ARC_135			0,4		AB119598	AB119598	CP000102	94	69
		MAR_ARC_32			1,8	2,1			AF199378		82
		MAR_ARC_1			1,8	2,1	AB119590	AB119590	CP000743	93	67
		MAR_ARC_4			6,3	7,3	AB119590	AB119590	AB260046	92	77
		MAR_ARC_5			6,3	7,3	AB119590	AB119590	DQ451875	88	74
		MAR_ARC_58	49,8		0,9	1,0			AF509441		69

Table A.1.11: Cloning results for Bacteria HalVK sediments

							Accession number			Similarity	
		Clone			DGGE		Closest Relative			Closest Relative	
		Name	Frequency (%)		Intensity %	This Study	Uncultured (UC)	Cultured (C)		UC	C
Marmara Bacterial Cluster2 (MBC2)		MAR_BAC_4	22,7	0,8				AB188780			98
		MAR_BAC_7		0,8				AB188780			98
		MAR_BAC_18		1,6	3,9		DQ809773	AJ012591		99	85
		MAR_BAC_32		0,8		AM990999	AY592788	AY165308		78	72
		MAR_BAC_80		0,8		AM980564	AF419690	AJ229236		94	84
		MAR_BAC_150		1,6	3,0	AM980569	AB267027	X85132		90	78
		MAR_BAC_169		0,8			AY592648	AB109439		95	86
		MAR_BAC_187		1,6	1,9	AM998360	AY214200	AJ431246		93	88
		MAR_BAC_201		1,6	2,4		DQ302422	AJ224539		95	85
		MAR_BAC_210		0,8			AY592152	AF295656		98	82
		MAR_BAC_226		0,8				AB205405			96
		MAR_BAC_228		1,6	2,3	AM980578	DQ499326	CP000232		88	81
		MAR_BAC_107		1,6	3,2		DQ325561	Y18176		99	95
		MAR_BAC_143		1,6	2,7	AM980568	AM229499	U41049		85	71
Acidobacteria		MAR_BAC_206	3,1	0,8			DQ351783	DQ303457		99	85
		MAR_BAC_222		0,8			AJ241003	AJ300509		96	82
		MAR_BAC_213		1,6	1,9	AM980575		CP000473			92
Actinobacteria		MAR_BAC_191	3,1	0,8		AM980573	DQ811925	AF498707		94	83
		MAR_BAC_135		2,3	2,8			DQ219354			100
Chloroflexi		MAR_BAC_2	6,3	0,8		AM990998	AB189336	AB109439		92	87
		MAR_BAC_70		0,8			AB116439	AB243672		97	86
		MAR_BAC_79		0,8			EF420218	AB109439		96	90
		MAR_BAC_109		1,6	1,9		EF420218	AB109439		98	90
		MAR_BAC_160		1,6	1,9	AM980571		AF524858			91
		MAR_BAC_223		0,8			AB288599	AB243672		95	86
Firmicutes		MAR_BAC_63	27,3	0,8	0,9	AM980563		AY169818			94
		MAR_BAC_167		2,3	2,8	AM998354		AF349724			65
	Clostridia	MAR_BAC_14	21,9	0,8				DQ126679			98
		MAR_BAC_58		0,8		AM980561		Y18185			93
		MAR_BAC_60		0,8				X76161			96
		MAR_BAC_106		1,6	1,9		DQ478749	AJ297449		97	87
		MAR_BAC_147		7,0	8,4			AB030224			99
		MAR_BAC_219		6,3	7,5			AB030221			98
		MAR_BAC_97		0,8				AF491333			95
		MAR_BAC_128		3,9	4,7	AM998349		AB038361			94
		MAR_BAC_145		2,3	2,8	AM998352		Y11466			80
		MAR_BAC_157		2,3	2,8	AM980570	AF424486	AJ250882		93	80

Table A.1.12: Cloning results for Archaea in HalVK sediments

							Accession number			Similarity	
		Clone			DGGE		Closest Relative			Closest Relative	
		Name	Frequency (%)		Intensity (%)	This Study	Uncultured (UC)	Cultured (C)	UC	C
Marmara Archaeal Cluster1 (MAC1)		MAR_ARC_6	24,2	1,3	1,5	DQ146745	DQ146745	U55239	84	75
		MAR_ARC_11		0,9	2,0	EF153105	EF153105	DQ058818	60	55
		MAR_ARC_12		2,2	3,4			L77117	80	69
		MAR_ARC_14		0,9		AY592249	AY592249	DQ058822	74	69
		MAR_ARC_16		1,3	2,5	DQ082931	DQ082931	AF524848	87	79
		MAR_ARC_21		0,9	1,0	EF104088	EF104088	AF255608	72	59
		MAR_ARC_22		0,4		AJ305078	AJ305078	AF199376	79	79
		MAR_ARC_26		1,3	1,5	AY592500	AY592500	AY196684	94	74
		MAR_ARC_41		0,9	1,2	DQ640163	DQ640163	CP000254	77	76
		MAR_ARC_50		0,9	1,0	DQ640137	DQ640137	AB033290	91	77
		MAR_ARC_61		1,3	1,5			DQ058817		88
		MAR_ARC_80		0,4		AY592484	AY592484	AF255608	91	81
		MAR_ARC_85		0,9	0,8	AY592230	AY592230	AY196658	94	75
		MAR_ARC_114		0,9	1,0	AF119128	AF119128	AE000666	95	76
		MAR_ARC_118		0,4		AJ969749	AJ969749	AF199378	96	90
		MAR_ARC_127		0,4		DQ363763	DQ363763	U41095	98	76
		MAR_ARC_132		0,9	1,0	DQ103671	DQ103671	AF255608	96	81
		MAR_ARC_137		0,4		AY835408	AY835408	M59124	95	78
		MAR_ARC_141		0,9	1,6	AF119137	AF119137	BD445502	94	79
		MAR_ARC_142		0,4		AY835427	AY835427	AF028689	89	75
		MAR_ARC_145		0,9	1,0	DQ522944	DQ522944	AF255608	92	84
		MAR_ARC_152		0,9	1,0	AY592258	AY592258	AF255608	77	67
		MAR_ARC_154		0,9	1,0	AY341269	AY341269	AE000666	96	80
		MAR_ARC_158		0,9	1,0			AJ745145		83
		MAR_ARC_190		0,9	1,0	EF444599	EF444599	AB057722	86	76
		MAR_ARC_214		0,4		AY592484	AY592484	DQ445723	91	76
		MAR_ARC_263		0,9	1,0	AY367339	AY367339	DQ058818	64	59
		MAR_ARC_245		0,9	1,0	DQ522909	DQ522909	AF199378	86	81
Crenarchaeota		MAR_ARC_15	25,1	0,4		AY454678	AY454678	AB269873	86	75
		MAR_ARC_17		0,9	1,0	AY454663	AY454663	AB260046	96	76
		MAR_ARC_20		6,3	6,9	AY454668	AY454668	DQ451875	80	68
		MAR_ARC_33		2,2	2,5	AY454663	AY454663	L77117	95	76
		MAR_ARC_119		4,0	4,4	AF004345	AF004345	CP000505	97	80
		MAR_ARC_34		2,2	2,5	AY454663	AY454663	CP000609	90	75
		MAR_ARC_36		6,3	6,0	AY454668	AY454668	CP000609	90	75
		MAR_ARC_53		1,8	2,0			DQ397841		67
		MAR_ARC_247		0,9	1,0	AF068822	AF068822	CP000505	76	63
Euryarchaeota		MAR_ARC_18	49,8	1,3	1,5	DQ417474	DQ417474	AB197163	90	77
		MAR_ARC_30		0,9	1,0	AB119590	AB119590	CP000743	93	67
		MAR_ARC_69		1,3	1,5	AY380683	AY380683	DQ445723	76	73
		MAR_ARC_135		0,4		AB119598	AB119598	CP000102	94	69
		MAR_ARC_32		1,8	2,0			AF199378		82
		MAR_ARC_1		1,8	2,0	AB119590	AB119590	CP000743	93	67
		MAR_ARC_4		6,3	6,9	AB119590	AB119590	AB260046	92	77

Table A.1.13: Cloning results for Bacteria in HalEY sediments

							Accession number			Similarity	
		Clone						Closest Relative		Closest Relative	
		Name	Frequency (%)			Int %	This Study	Uncultured (UC)	Cultured (C)	UC	C
Marmara Bacterial Cluster2 (MBC2)		MAR_BAC_4	22,7		0,8				AB188780		98
		MAR_BAC_7			0,8				AB188780		98
		MAR_BAC_18			1,6	1,9		DQ809773	AJ012591	99	85
		MAR_BAC_32			0,8		AM990999	AY592788	AY165308	78	72
		MAR_BAC_80			0,8		AM980564	AF419690	AJ229236	94	84
		MAR_BAC_150			1,6	2,8	AM980569	AB267027	X85132	90	78
		MAR_BAC_169			0,8			AY592648	AB109439	95	86
		MAR_BAC_187			1,6	1,9	AM998360	AY214200	AJ431246	93	88
		MAR_BAC_201			1,6	1,9		DQ302422	AJ224539	95	85
		MAR_BAC_210			0,8			AY592152	AF295656	98	82
		MAR_BAC_226			0,8				AB205405		96
		MAR_BAC_228			1,6	3,0	AM980578	DQ499326	CP000232	88	81
		MAR_BAC_143			1,6	2,4	AM980568	AM229499	U41049	85	71
		MAR_BAC_107			1,6	2,9		DQ325561	Y18176	99	95
Acidobacteria		MAR_BAC_206	3,1		0,8			DQ351783	DQ303457	99	85
		MAR_BAC_222			0,8			AJ241003	AJ300509	96	82
		MAR_BAC_213			1,6	1,9	AM980575		CP000473		92
Actinobacteria		MAR_BAC_191			0,8		AM980573	DQ811925	AF498707	94	83
		MAR_BAC_135			2,3	2,8			DQ219354		100
Chloroflexi		MAR_BAC_2	6,3		0,8		AM990998	AB189336	AB109439	92	87
		MAR_BAC_70			0,8			AB116439	AB243672	97	86
		MAR_BAC_79			0,8			EF420218	AB109439	96	90
		MAR_BAC_109			1,6	1,9		EF420218	AB109439	98	90
		MAR_BAC_160			1,6	1,9	AM980571		AF524858		91
		MAR_BAC_223			0,8			AB288599	AB243672	95	86
Firmicutes		MAR_BAC_63	27,3		0,8		AM980563		AY169818		94
		MAR_BAC_167			2,3	2,8	AM998354		AF349724		65
	Clostridiales	MAR_BAC_14		21,9	0,8				DQ126679		98
		MAR_BAC_58			0,8		AM980561		Y18185		93
		MAR_BAC_60			0,8				X76161		96
		MAR_BAC_106			1,6	1,9		DQ478749	AJ297449	97	87
		MAR_BAC_147			7,0	8,5			AB030224		99
		MAR_BAC_219			6,3	7,5			AB030221		98
		MAR_BAC_97			0,8				AF491333		95
		MAR_BAC_128			3,9	4,7	AM998349		AB038361		94

Table A.1.14: Cloning results for Archaea in HalEY sediments

							Accession number			Similarity	
		Clone				DGGE		Closest Relative		Closest Relative	
		Name	Frequency (%)			Intensity (%)	This Study	Uncultured (UC)	Cultured (C)	UC	C
Marmara Archaeal Cluster1 (MAC1)		MAR_ARC_6	24,2		1,3	1,4	DQ146745	DQ146745	U55239	84	75
		MAR_ARC_11			0,9		EF153105	EF153105	DQ058818	60	55
		MAR_ARC_12			2,2	2,4			L77117	80	69
		MAR_ARC_14			0,9	2,1	AY592249	AY592249	DQ058822	74	69
		MAR_ARC_16			1,3	2,4	DQ082931	DQ082931	AF524848	87	79
		MAR_ARC_21			0,9	2,0	EF104088	EF104088	AF255608	72	59
		MAR_ARC_22			0,4		AJ305078	AJ305078	AF199376	79	79
		MAR_ARC_26			1,3	2,3	AY592500	AY592500	AY196684	94	74
		MAR_ARC_41			0,9	1,0	DQ640163	DQ640163	CP000254	77	76
		MAR_ARC_50			0,9	1,5	DQ640137	DQ640137	AB033290	91	77
		MAR_ARC_61			1,3	1,0			DQ058817		88
		MAR_ARC_80			0,4		AY592484	AY592484	AF255608	91	81
		MAR_ARC_85			0,9	1,0	AY592230	AY592230	AY196658	94	75
		MAR_ARC_114			0,9	1,0	AF119128	AF119128	AE000666	95	76
		MAR_ARC_118			0,4		AJ969749	AJ969749	AF199378	96	90
		MAR_ARC_127			0,4		DQ363763	DQ363763	U41095	98	76
		MAR_ARC_132			0,9	1,0	DQ103671	DQ103671	AF255608	96	81
		MAR_ARC_137			0,4		AY835408	AY835408	M59124	95	78
		MAR_ARC_141			0,9	1,0	AF119137	AF119137	BD445502	94	79
		MAR_ARC_142			0,4		AY835427	AY835427	AF028689	89	75
		MAR_ARC_145			0,9	1,0	DQ522944	DQ522944	AF255608	92	84
		MAR_ARC_152			0,9	1,0	AY592258	AY592258	AF255608	77	67
		MAR_ARC_154			0,9	1,0	AY341269	AY341269	AE000666	96	80
		MAR_ARC_158			0,9	1,0			AJ745145		83
		MAR_ARC_190			0,9	1,0	EF444599	EF444599	AB057722	86	76
		MAR_ARC_214			0,4		AY592484	AY592484	DQ445723	91	76
		MAR_ARC_263			0,9	1,0	AY367339	AY367339	DQ058818	64	59
		MAR_ARC_245			0,9	1,0	DQ522909	DQ522909	AF199378	86	81
Crenarchaeota		MAR_ARC_15	25,1		0,4		AY454678	AY454678	AB269873	86	75
		MAR_ARC_17			0,9	1,0	AY454663	AY454663	AB260046	96	76
		MAR_ARC_20			6,3	6,7	AY454668	AY454668	DQ451875	80	68
		MAR_ARC_33			2,2	2,4	AY454663	AY454663	L77117	95	76
		MAR_ARC_119			4,0	4,3	AF004345	AF004345	CP000505	97	80
		MAR_ARC_34			2,2	2,4	AY454663	AY454663	CP000609	90	75

Table A.1.15: Cloning results for Bacteria in IZ17 sediments

							Accession number			Similarity	
		Clone				DGGE	This Study	Closest Relative		Closest Relative	
		Name	Frequency (%)			Intensity (%)		Uncultured (UC)	Cultured (C)	UC	C
Marmara Bacterial Cluster2 (MBC2)		MAR_BAC_7	12,8		1,3				AB188780		98
		MAR_BAC_18			2,6	3,4		DQ809773	AJ012591	99	85
		MAR_BAC_32			1,3		AM990999	AY592788	AY165308	78	72
		MAR_BAC_80			1,3		AM980564	AF419690	AJ229236	94	84
		MAR_BAC_150			2,6	3,4	AM980569	AB267027	X85132	90	78
		MAR_BAC_169			1,3			AY592648	AB109439	95	86
		MAR_BAC_187			1,3		AM998360	AY214200	AJ431246	93	88
		MAR_BAC_226			1,3				AB205405		96
		MAR_BAC_143			1,3		AM980568	AM229499	U41049	85	71
		MAR_BAC_107			1,3			DQ325561	Y18176	99	95
		MAR_BAC_206			1,3			DQ351783	DQ303457	99	85
		MAR_BAC_191			3,8	5,0	AM980573	DQ811925	AF498707	94	83
		MAR_BAC_135			1,3				DQ219354		100
Chloroflexi		MAR_BAC_2	11,5		2,6	3,4	AM990998	AB189336	AB109439	92	87
		MAR_BAC_70			2,6	4,4		AB116439	AB243672	97	86
		MAR_BAC_109			2,6	2,4		EF420218	AB109439	98	90
		MAR_BAC_160			3,8	5,0	AM980571		AF524858		91
Firmicutes		MAR_BAC_167	32,3		1,3		AM998354		AF349724		65
	Clostridia	MAR_BAC_14		19,2	2,6	3,4			DQ126679		98
		MAR_BAC_58			1,3		AM980561		Y18185		93
		MAR_BAC_106			6,4	8,4		DQ478749	AJ297449	97	87
		MAR_BAC_1			3,8	5,0			DQ882650		100
		MAR_BAC_97			3,8	5,0			AF491333		95
		MAR_BAC_128			1,3		AM998349		AB038361		94
		MAR_BAC_145			11,8	15,5	AM998352		Y11466		80
		MAR_BAC_157			4,1	5,4	AM980570	AF424486	AJ250882	93	80
Proteobacteria	α	MAR_BAC_90	32,1		2,6	3,4			U78037		99
		MAR_BAC_138			1,3				AY962292		96
	δ	MAR_BAC_129		12,8	2,6	3,4	AM998350	AJ704679	AJ012591	91	85
		MAR_BAC_194			1,3			AJ535245	X82874	97	90
		MAR_BAC_123			3,8	5,0	AM991010		AJ511275		90
		MAR_BAC_64			1,3				AF118453		96
		MAR_BAC_108			1,3				U96918		98
		MAR_BAC_98			2,6	3,4	AM980566	DQ267496	AF382399	92	87
	ε	MAR_BAC_27		11,5	3,8	5,0			AB091293		98
		MAR_BAC_49			2,6	3,4			AB091293		96
		MAR_BAC_72			1,3		AM998340		AB091293		80
		MAR_BAC_207			3,8	5,0			AB091293		96
	γ	MAR_BAC_119			1,3			AJ535246	AB271125	97	93
		MAR_BAC_35			1,3			DQ351781	U77482	98	91
		MAR_BAC_94			1,3				U62131		97

Table A.1.16: Cloning results for Archaea in IZ17 sediments

							Accession number			Similarity	
		Clone				DGGE		Closest Relative		Closest Relative	
		Name	Frequency (%)			Intensity (%)	This Study	Uncultured (UC)	Cultured (C)	UC	C
Marmara Archaeal Cluster1 (MAC1)		MAR_ARC_61			2,3	2,7	AM980593		DQ058817		88
		MAR_ARC_263			2,3	3,7	AM992725	AY367339	DQ058818	64	59
		MAR_ARC_80			1,1		AM992704	AY592484	AF255608	91	81
		MAR_ARC_11			2,3	2,7	AM992679	EF153105	DQ058818	60	55
		MAR_ARC_50			2,3	1,5	AM992695	DQ640137	AB033290	91	77
		MAR_ARC_51			1,1		AM992696	DQ640149	DQ445723	80	71
		MAR_ARC_85			2,3	2,7	AM992707	AY592230	AY196658	94	75
		MAR_ARC_152			2,3	2,9	AM992718	AY592258	AF255608	77	67
		MAR_ARC_52			2,3	2,7	AM992699	AY627488	DQ060323	67	63
		MAR_ARC_114			2,3	2,7		AF119128	AE000666	95	76
		MAR_ARC_14			2,3	2,7	AM980584	AY592249	DQ058822	74	69
		MAR_ARC_214			1,1		AM992723	AY592484	DQ445723	91	76
		MAR_ARC_132			2,3	2,7		DQ103671	AF255608	96	81
		MAR_ARC_141			2,3	2,7	AM980601	AF119137	BD445502	94	79
		MAR_ARC_96			1,1		AM992709	AY592522	DQ445723	94	80
		MAR_ARC_145			2,3	2,7	AM992716	DQ522944	AF255608	92	84
		MAR_ARC_83			1,1		AM992705	DQ522917	DQ445723	86	74
		MAR_ARC_21			2,3	2,7	AM980587	EF104088	AF255608	72	59
		MAR_ARC_41			2,3	2,7	AM992692	DQ640163	CP000254	77	76
		MAR_ARC_127			1,1			DQ363763	U41095	98	76
		MAR_ARC_12			1,1		AM992680		L77117	80	69
		MAR_ARC_118	44,8		4,6	5,3		AJ969749	AF199378	96	90
		MAR_ARC_245			2,3	2,7	AM992726	DQ522909	AF199378	86	81
Crenarchaeota		MAR_ARC_119			2,3	2,7		AF004345	CP000505	97	80
		MAR_ARC_86			2,3	2,7	AM992706		AF255608		80
		MAR_ARC_166			2,3	2,7	AM992719		AF255608		80
		MAR_ARC_53			1,1		AM998395		DQ397841		67
		MAR_ARC_164	9,2		1,1		AM992720		CP000493		70
Euryarchaeota		MAR_ARC_69			2,3	2,7	AM980595	AY380683	DQ445723	76	73
		MAR_ARC_135			2,3	2,7	AM980600	AB119598	CP000102	94	69
		MAR_ARC_133			2,3	2,7	AM992711		AF199376		85
		MAR_ARC_32	43,7		4,6	5,3	AM992691		AF199378		82

		MAR_ARC_5			2,3	2,7	AM980580	AB119590	DQ451875	88	74
		MAR_ARC_58			2,3	2,7	AM99270		AF509441		69
		MAR_ARC_55			2,3	2,7	AM998397		AE000666		80
		MAR_ARC_60			3,4	4,0	AM980596		AB057722		91
	Methanosarcinales	MAR_ARC_191			1,1			AY211687	AY970347	96	87
		MAR_ARC_25			1,1		AM992688		AF255608		71
		MAR_ARC_184			5,7	6,7	AM998442	EF420177	AJ133791	84	84
		MAR_ARC_95			2,3	2,7	AM992708		AJ012742		91
		MAR_ARC_165			2,3	2,7	AM992721		AE010299		86
		MAR_ARC_232			1,1				AB288264		97
		MAR_ARC_200		16,1	2,3	2,7	AM998453		CP000099		84
		MAR_ARC_66			5,7	6,7		AY133904	AJ419871	84	82

Table A.17: Cloning results for Bacteria in IZ30 sediments

							Accession number			Similarity	
		Clone				DGGE		Closest Relative		Closest Relative	
		Name	Frequency (%)			Intensity (%)	This Study	Uncultured (UC)	Cultured (C)	UC	C
Marmara Bacterial Cluster2 (MBC2)		MAR_BAC_7	15,4		1,3	0,0			AB188780		98
		MAR_BAC_18			2,6	2,6		DQ809773	AJ012591	99	85
		MAR_BAC_32			1,3	0,0	AM990999	AY592788	AY165308	78	72
		MAR_BAC_80			1,3	0,0	AM980564	AF419690	AJ229236	94	84
		MAR_BAC_150			1,3	0,0	AM980569	AB267027	X85132	90	78
		MAR_BAC_169			1,3	0,0		AY592648	AB109439	95	86
		MAR_BAC_187			1,3	0,0	AM998360	AY214200	AJ431246	93	88
		MAR_BAC_226			1,3	0,0			AB205405		96
		MAR_BAC_143			1,3	0,0	AM980568	AM229499	U41049	85	71
		MAR_BAC_107			2,6	2,6		DQ325561	Y18176	99	95
		MAR_BAC_206			1,3	0,0		DQ351783	DQ303457	99	85
		MAR_BAC_191	5,1		1,3	0,0	AM980573	DQ811925	AF498707	94	83
		MAR_BAC_135			3,8	3,8			DQ219354		100
Chloroflexi		MAR_BAC_2	5,1		1,3	0,0	AM990998	AB189336	AB109439	92	87
		MAR_BAC_70			1,3	0,0		AB116439	AB243672	97	86
		MAR_BAC_109			1,3	0,0		EF420218	AB109439	98	90
		MAR_BAC_160			1,3	0,0	AM980571		AF524858		91
Firmicutes		MAR_BAC_167	21,8		3,8	3,8	AM998354		AF349724		65
	Clostridia	MAR_BAC_14		14,1	1,3	0,0			DQ126679		98
		MAR_BAC_58			1,3	0,0	AM980561		Y18185		93
		MAR_BAC_106			2,6	2,6		DQ478749	AJ297449	97	87
		MAR_BAC_1			1,3	0,0			DQ882650		100
		MAR_BAC_97			1,3	0,0			AF491333		95
		MAR_BAC_128			6,4	6,4	AM998349		AB038361		94
		MAR_BAC_145			3,8	3,8	AM998352		Y11466		80
		MAR_BAC_157			3,8	3,8	AM980570	AF424486	AJ250882	93	80

Proteobacteria	α	MAR_BAC_90	47,4	14,1	1,3	0,0			U78037		99
		MAR_BAC_138			12,8	12,8			AY962292		96
	δ	MAR_BAC_129		16,7	5,1	5,1	AM998350	AJ704679	AJ012591	91	85
		MAR_BAC_194			2,6	2,6		AJ535245	X82874	97	90
		MAR_BAC_123			1,3	0,0	AM991010		AJ511275		90
		MAR_BAC_64			2,6	2,6			AF118453		96
		MAR_BAC_108			1,3	0,0			U96918		98
		MAR_BAC_98			3,8	3,8	AM980566	DQ267496	AF382399	92	87
	ε	MAR_BAC_27		9,0	1,3	0,0			AB091293		98
		MAR_BAC_49			1,3	0,0			AB091293		96
		MAR_BAC_72			2,6	2,6	AM998340		AB091293		80
		MAR_BAC_207			3,8	3,8			AB091293		96
	γ	MAR_BAC_119		7,7	2,6	2,6		AJ535246	AB271125	97	93
		MAR_BAC_35			1,3	0,0		DQ351781	U77482	98	91
		MAR_BAC_94			3,8	3,8			U62131		97

Table A.1.18: Cloning results for Archaea in IZ30 sediments

					Accession number			Similarity	
	Clone			DGGE		Closest Relative		Closest Relative	
	Name	Frequency (%)		Intensity (%)	This Study	Uncultured (UC)	Cultured (C)	UC	C
Marmara Archaeal Cluster1 (MAC1)	MAR_ARC_6	32	2,3	2,7	AM980582	DQ146745	U55239	84	75
	MAR_ARC_12		3,9	4,5	AM992680		L77117	80	69
	MAR_ARC_21		1,6	1,8	AM980587	EF104088	AF255608	72	59
	MAR_ARC_22		0,8		AM992710	AJ305078	AF199376	79	79
	MAR_ARC_26		2,3	2,7	AM980589	AY592500	AY196684	94	74
	MAR_ARC_40		0,8		AM992693	AJ969761	AJ132639	82	74
	MAR_ARC_42		0,8		AM992694	AY592232	DQ251043	90	74
	MAR_ARC_48		1,6	1,8	AM992698	AY592463	AJ132639	94	77
	MAR_ARC_49		0,8			AB177226	AJ132639	97	77
	MAR_ARC_57		0,8		AM992700	DQ522926	U41095	93	77
	MAR_ARC_61		2,3	2,7	AM980593		DQ058817		88
	MAR_ARC_72		0,8		AM992703	AY592232	U41095	90	78
	MAR_ARC_73		0,8			DQ640150	AF199376	96	88
	MAR_ARC_118		0,8			AJ969749	AF199378	96	90
	MAR_ARC_145		1,6	1,8	AM992716	DQ522944	AF255608	92	84
	MAR_ARC_127		0,8			DQ363763	U41095	98	76
	MAR_ARC_142		0,8		AM998424	AY835427	AF028689	89	75
	MAR_ARC_124		4,7	5,5	AM992689	AY436520	AF199378	80	73
	MAR_ARC_151		3,9	4,5	AM992717	DQ640156	AJ745133	83	73
Crenarchaeota	MAR_ARC_17	9,4	1,6	1,8		AY454663	AB260046	96	76
	MAR_ARC_81		1,6	1,8		AB239076	DQ445723	97	82
	MAR_ARC_82		1,6	1,8	AM992702	AY454637	AB269873	82	72
	MAR_ARC_131		0,8			AF004345	CP000505	97	81
	MAR_ARC_226		0,8			DQ278118	AJ132639	90	76
	MAR_ARC_53		3,1	3,6	AM998395		DQ397841		67
	MAR_ARC_155		3,1	3,6		DQ640153	AF199378	95	88
Euryarchaeota	MAR_ARC_18	55,5	2,3	2,7		DQ417474	AB197163	90	77
	MAR_ARC_23		3,1	3,6	AM980588	AB301990	DQ445723	92	79
	MAR_ARC_24		0,8		AM992687	AB235349	DQ445723	91	70
	MAR_ARC_30		1,6	1,8	AM998386	AB119590	CP000743	93	67
	MAR_ARC_69		2,3	2,7	AM980595	AY380683	DQ445723	76	73
	MAR_ARC_135		0,8		AM980600	AB119598	CP000102	94	69
	MAR_ARC_32		3,1	3,6	AM992691		AF199378		82
	MAR_ARC_1		3,1	3,6	AM998380	AB119590	CP000743	93	67

Table A.1.19: Clone library statistics

		Screened clone	OTU (Clone)	OTU (DGGE)	S_{chao1}	H?	J'	Coverage
Bacterial	**Gemlik**	125	63	29	83	1,71	0,39	0,73
	Tuzla	125	59	30	73	1,66	0,39	0,77
	K.cekmece	125	57	30	69	1,64	0,39	0,78
	Moda	125	57	30	69	1,64	0,39	0,78
	Iz30	100	41	17	58	1,51	0,37	0,76
	Iz17	100	41	20	52	1,54	0,39	0,79
	HaliçAS	125	60	32	72	1,66	0,39	0,78
	HaliçVK	125	59	31	72	1,65	0,39	0,78
	HaliçEY	125	59	31	72	1,65	0,39	0,78
Archaeal	**Gemlik**	75	34	20	39	1,45	0,40	0,81
	Tuzla	175	61	41	66	1,58	0,38	0,89
	K.cekmece	175	63	42	68	1,64	0,39	0,88
	Moda	150	46	33	49	1,49	0,38	0,91
	Iz30	125	45	27	51	1,5	0,38	0,86
	Iz17	100	44	32	46	1,6	0,42	0,88
	HaliçAS	175	71	42	76	1,67	0,39	0,87
	HaliçVK	175	60	38	66	1,67	0,40	0,87
	HaliçEY	175	71	49	76	1,67	0,39	0,75

H?: Shannon-Weaver index of diversity
J': Evenness
S_{chao1}: Chao1 estimator of species richness

APPENDIX A.2

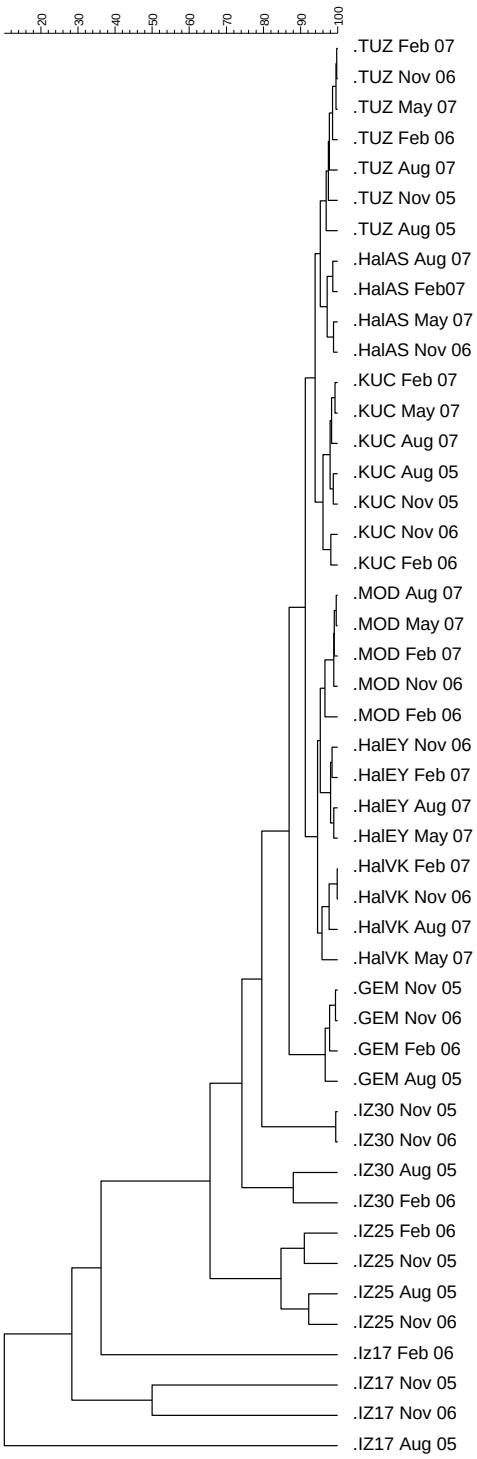

Figure A.2.1: Bacterial DGGE clusters using Dice coefficient.

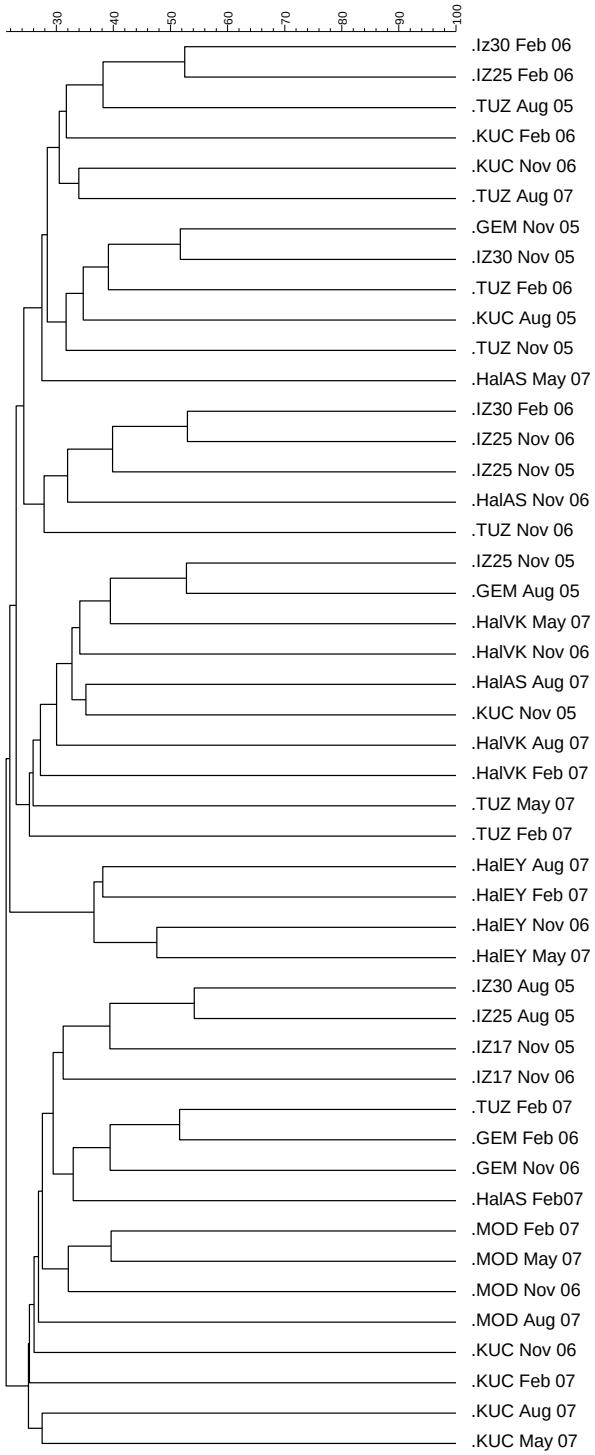

Figure A.2.2: Bacterial DGGE clusters using Pearson coefficient

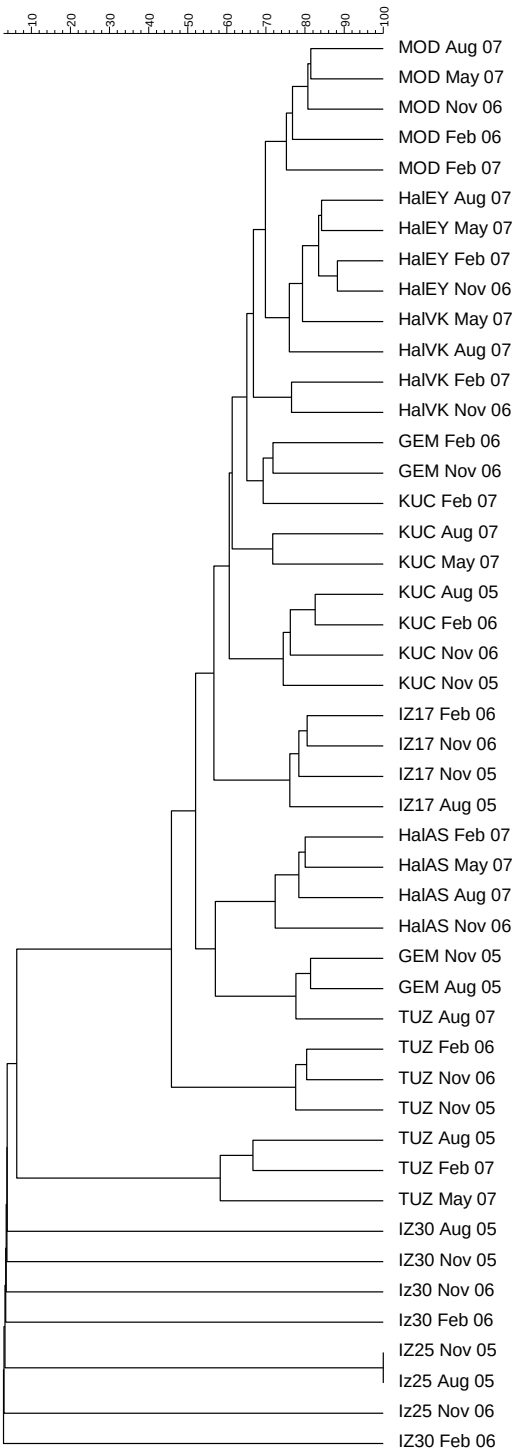

Figure A.2.3: Archaeal DGGE clusters using Dice coefficient

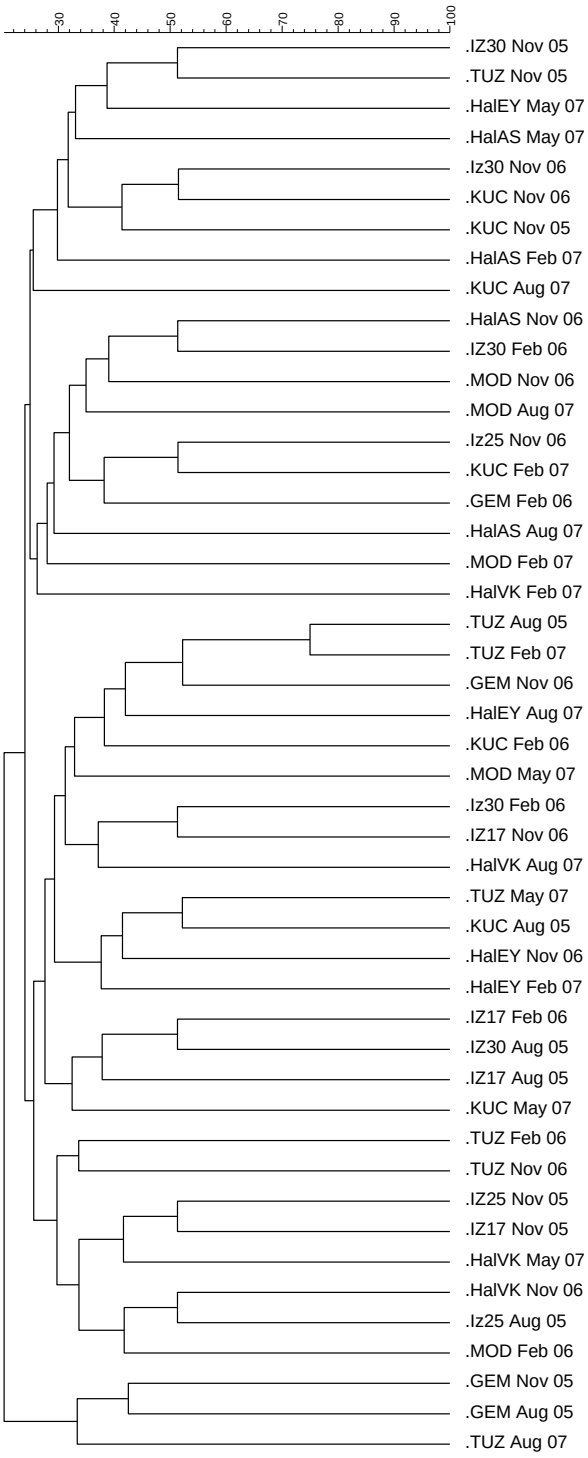

Figure A.2.4: Archaeal DGGE clusters using Pearson coefficient

CURRICULUM VITAE

Candidate's full name: Mustafa Kolukırık

Place and date of birth: Adana/Turkey, 28/04/1979

Permanent Address: Department of Molecular Biology and Genetics; İstanbul Technical University (ITU), 34469 Maslak-İstanbul.

Universities and Colleges attended:

2004 – PhD, Environmental Biotechnology Programme, ITU.

2004 –2005 ERASMUS exchange student, School of Civil Engineering and Geosciences, University of Newcastle Upon Tyne (NU).

2001 – 2004 MSc, Department of Environmental Engineering, ITU.

1997 – 2001 BSc, Department of Environmental Engineering, ITU.

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