İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

ANAEROBIC BTEX DEGRADING MICROORGANISM ABUNDANCES IN MARMARA SEA SEDIMENTS

M.Sc. Thesis by
Canan Zöhre KETRE

Department : Environmental Engineering

Programme : Environmental Biotechnology

Thesis Supervisor: Prof. Dr. Orhan İNCE

JUNE 2010

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M.Sc. Thesis by
Canan Zöhre KETRE
(501070812)

Date of submission : 07 May 2010
Date of defence examination: 11 June 2010

Supervisor (Chairman) : Prof. Dr. Orhan İNCE (ITU)
Members of the Examining Committee : Asst. Prof. Mahmut ALTINBAŞ (ITU)
Assoc. Prof. Bülent MERTOĞLU (MU)

JUNE 2010

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

MARMARA DENİZİ SEDİMENTLERİNDE ANAEROBİK BTEX AYRIŞTIRMASI YAPAN MİKROORGANİZMALARIN BULUNMASI

YÜKSEK LİSANS TEZİ
Canan Zöhre KETRE
(501070812)

Tezin Enstitüye Verildiği Tarih : 07 Mayıs 2010
Tezin Savunulduğu Tarih : 11 Haziran 2010

Tez Danışmanı : Prof. Dr. Orhan İNCE (İTÜ)
Diğer Jüri Üyeleri : Yard. Dr. Mahmut ALTINBAŞ (İTÜ)
Doç. Dr. Bülent MERTOĞLU (MÜ)

HAZİRAN 2010

FOREWORD

I would like to express my deep and sincere gratitude to my supervisor, Prof. Dr. Orhan İNCE who gave me the opportunity to work with them in the Microbial Ecology Group. His wide knowledge and his logical way of thinking have been of great value for me. His understanding, encouraging and personal guidance have provided a good basis for the present thesis.

I would like to express my gratitude to Prof. Dr. Bahar İnce for her valuable advice , kind support and guidance have been of great value in this study.

I owe my most sincere gratitude to my labratory supervisor, Res. Assist . Mustafa Kolukırık, for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my M.sc study.

During this work I have collaborated with many colleagues for whom I have great regard, and I wish to extend my warmest thanks to all those who have helped me with my work. In particular, I would like to thank Ezgi Demir , Bahar Öztulunç and Samet Azman, for their friendship and help, for the sleepless nights we were working together, for all the fun we have had in the last years.

My deepest gratitude goes to my family for their unflagging love and support throughout my life. Without their encouragement and understanding it would have been impossible for me to finish this work.

Last but not least, I would like to present special thanks to my friends for helping me get through the difficult times, and for all the emotional support and entertainment.

This work is supported by TUBITAK project no: 105Y307 “Anaerobic degradation of petroleum hydrocarbons in anoxic marine environments”.

June 2010

Canan Zöhre KETRE

Environmental Biotechnology

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ABBREVIATIONS

DNA :Deoxyribonucleic Acid
PCR **:**Polymerase Chain Reaction
FISH **:**Fluorescence in-situ Hybridization
DGGE **:**Denaturing Gradient Gel Electrophoresis
KUC **:**Küçükcekmece Coast
MOD **:**Moda Coast
HAL **:**Haliç Bay
TUZ **:**Tuzla Bay
GEM **:**Gemlik Bay
IZ **:**İzmit Bay
MSS **:**Marmara Sea Sediment
BTEX **:**Benzene, Toluene, Ethylbenzene, and the isomers of Xylene
rRNA **:**Ribosomal Ribonucleic Acid
Q-PCR **:**Quantitative Real-Time PCR
NA **:**Natural attenuation
BssA **:**Benzylsuccinate Synthase Gene
bss **:**Benzylsuccinate Synthase
SRB **:**Sulfate Reducing Bacteria
DB **:**Denitrifying Bacteria
NAFZ **:**North Anatolian Fault Zone
TOC **:**Total Organic Carbon
TN **:**Total Nitrogen
TPH **:**Total Petroleum Hydrocarbons
PLFAs **:**Phospholipid Ester-Linked Patty Acids
UM-BBD **:**University of Minnesota Biocatalysis/Biodegradation Databese
TEAP **:**Terminal Electron Accepting Processes
NTA **:**Nitrotriacetic Acid
EDTA **:**Ethylenediaminetetraacetic Acid
NTA **:**Nitrilotriacetic Acid
ATP **:**Adenosine Triphosphate
TGGE **:**Temperature Gradient Gel Electrophoresis
SSCP **:**Single-Strand Conformation Polymorphism
LH-PCR **:**Length Heterogeneity-PCR
tRFLP **:**Terminal-Restriction Fragment Length Polymorphism
SSU rRNA **:**Small Subunit Ribosomal Ribonucleic Acid
CLSM **:**Confocal Laser Scanning Microscopy
C_T :Threshold Cycle
NFQ **:**Non-Fluorescent Quencher
FRET :Fluorescence Resonance Energy Transfer
AnBTEXDeg : Anaerobic BTEX Degrader

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ANAEROBIC BTEX DEGRADING MICROORGANISM ABUNDANCES IN MARMARA SEA SEDIMENTS

SUMMARY

Microbial degradation is the only sustainable component of natural attenuation in contaminated marine environments which are still poorly understood. The monitoring of microbial capacities and their distribution at contaminated sites forms a basis for assessing natural attenuation. Although Marmara Sea has been extremely polluted with hydrocarbons, the level of benzene, toluene, ethylbenzene, and the isomers of xylene (BTEX) in this environment has not been determined yet. BTEX are priority environmental pollutants and can be removed by means of bioremediation as long as BTEX degrading microorganisms are abundant in the polluted environment. Benzylsuccinate synthase (BssA) is the key enzyme of anaerobic toluene and xylene oxidation and has repeatedly been proven to be a valuable functional marker gene for unknown anaerobic toluene and xylene degraders . BssA gene , codes for the R-subunit of benzylsuccinate synthase, can be targeted to quantify BTEX degraders in environmental samples. Quantitative real-time PCR (Q-PCR) have been widely used for the quantification of gene abundances. Q-PCR analysis was chosen for this study because it offers many advantages over traditional methods used to quantify AnBTEXDeg in environmental samples.We used the Q-PCR primers targeting the widest range of physiological and phylogenetical group of microorganisms carrying the bssA gene to monitor abundance of BTEX degraders in sediments from the most polluted regions of the Marmara Sea (10 different locations). The bssA gene abundance changed in a range of 2.4×10^{10} - 7×10^{12} during the 2 years monitoring period. The bssA was abundant where total petroleum hydrocarbon (TPH) levels are high, and BTEX % in TPH was negatively correlated to the relative abundance of the bssA in the total microbial community (3-40 %). The bssA gene abundance and activity were strongly related to the N/P ratios and the N-P levels. The results showed that the abundance of bssA gene was a good representetive to quantify the AnBTEXDeg. Overall results revealed that the Marmara Sea Sediments seems to be a promising candidate for further investigation of microbial BTEX degradation under anaerobic/anoxic conditions.

MARMARA DENİZİ SEDİMENTLERİNDE ANAEROBİK BTEX AYRIŞTIRMASI YAPAN MİKROORGANİZMALARIN BULUNMASI

ÖZET

Mikrobiyal degredasyon, kontamine olmuş deniz sedimentlerinde doğal atenüasyonunun tek sürdürülebilir bileşenidir ve halen tam anlamıyla açıklanamamıştır. Mikrobial kapasitelerin ve kontamine olmus bölgelere dağılımının gözlemlenmesi doğal atenuasyonun değerlendirilmesinin temelini oluşturur.. Marmara Denizi çok yüksek hidrokarbon kirliliği sahip olmasına rağmen , bu bölgedeki benzen, toluen , etilbenzen ve ksilen (BTEX) seviyeleri henüz belirlenmemiştir. BTEXler öncelikli çevre kirleticileridir ve BTEX degrede eden organizmaların çevrede bulunmasıyla bioıslah edilebilir. Benzilsüksinat sentaz (bssA) enzimi anaerobik toluen ve ksilen degradasyonunun anahtar enzimidir ayrıca Toluen ve ksilen degradasyonu için fonksiyonel bir indikatör olarak kullanıldığı pek çok kez ispatlanmıştır.

Benzilsüksinat sentazı kodlayan BssA geni, çevresel örneklerde BTEX ayrıştırıcılarının sayımı için hedeflenebilir. Gerçek zamanlı polimeraz zincir reaksiyonu (Q-PCR) genlerin bulunma çokluğunu belirlemek için çok kullanılan bir yöntemdir. Q-PCR, bu çalışmada geleneksel methodlara göre birçok avantaj sağladığı için seçilmiştir. Marmara Denizi'nin en kirli 10 bölgesinden alınan sediment örneklerinde, en geniş fizyolojik ve filogenetik mikroorganizma gruplarını hedeflemek için özel olarak dizayn edilen Q-PCR primerleri kullanarak analiz yapıldı. 2 yıllık gözlemlerin sonucunda, Marmara Denizi'nde BssA gen çokluğu 2.4×10^{10} - 7×10^{12} olarak bulundu. BssA geninin toplam petrol hidrokarbonlarının (TPH) çok olduğu bölgelerde daha fazla ve toplam mikrobiyal kominitedeki bssA gen çokluğu ile TPH içindeki BTEX yüzdesi ters orantılı olduğu belirlendi (%3-40). Yapılan korelasyon analizlerine göre bssA gen çokluğu ve aktivitesi azot fosfor oranı ve seviyesiyle yakından ilgili çıkmıştır. Sonuçlarımız anaerobik/ anoksik koşullarda mikrobiyal BTEX degradasyonun ileri incelemeleri için Marmara Denizi'nin ümit verici bir aday olduğunu göstermiştir.

1.INTRODUCTION

The ‘deep-sea floor’ is defined as that portion of the ocean bottom overlain by at least 1000 m of water column. The deep-sea floor is a vast region covering roughly 300 - 106 km2, or approximately 60% of the Earth’s solid surface.(Glover and Smiths 2003)**.**

The deep subseafloor biosphere is among the least-understood habitats on Earth regarding the organisms, their physiologies and their influence on surface environments (Inagaki, 2006). This is mainly due to the difficulties involved in enriching and isolating the representative deepsediment microorganisms (Toffin., 2004) and previous studies based on cultivation methods could not reveal the appropriate sedimentary microbial diversity.

However, marine subsurface sediment constitutes one of the largest and most widespread reservoirs of biomass on Earth, subsurface prokaryotic activities have profound effects on global biogeochemical cycles ,particularly on global carbon cycling (Webster, 2004). Most deep-sea floor habitats have several characteristics that distinguish them from other of Earth’s ecosystems and that influence their susceptibility to environmental change and human impacts. Perhaps the most important characteristic is low productivity. Except for hydrothermal vents and some cold seeps, the energy for the deep-sea biota is ultimately derived from an attenuated ‘rain’ of detritus from remote surface waters. Detrital food particles range from the fresh remains of phytoplankton (or ‘phytodetritus’) to the carcasses of whales. (Glover and Smiths 2003). In general, the deep-sea floor is also characterized by very low physical energy, including sluggish currents (_0.25 knots), very slow sediment accumulation rates (0.1–10 cm per thousand years), and an absence of sunlight (Gage & Tyler 1991; Smith & Demopoulos 2003).

Cultivation of organisms from this deep marine biosphere is difficult (Parkes ;1994), but it has been the focus of several molecular microbial ecology studies to investigate prokaryotic diversity.

Molecular based, culture independent techniques such as polymerase chain reaction (PCR), fluorescence in-situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), and 16S rDNA sequencing for investigating the prokaryotic diversity have given a more realistic picture of the community structure in marine sediments and have been successfully employed to overcome the difficulties associated with culture dependent methods (Lysnes, 2004; Webster, 2004).

The Marmara Sea is a small (size ≈ 70 x 250 km) intercontinental basin connecting and acting as the only route between Black Sea and Mediterranean Sea. The population of Marmara region reaches to 25 million and therefore there is large number of domestic wastewater discharge to the Marmara Sea from different points. Anthropogenic activities in the coastal area of the north Marmara Sea include, urban effluent, summer resorts (untreated effluent discharged into the sea), agricultural run off, sunflower oil factories, a big cement factory, fishing and shipping (Öztürk, 2000).

Marmara Sea has been extremely polluted with hydrocarbons (Kolukirik, 2009). The most polluted regions are Kucukcekmece Coast (KUC), Moda Coast (MOD), Halic Bay (HAL), Tuzla Bay (TUZ), Gemlik Bay (GEM) and Izmit Bay (IZ) (Sur, 2003). Subseafloor microbial and chemical diversities of these regions have just been explored (Kolukirik, 2009). Marmara Sea seems to be a perfect candidate for investigation of ecologically important microbial processes; since phylogenetically diverse communities exists within a very short distance from the sediment surfaces and chemical compositions of the sediments are unusual. The considerable amount of obtained phylotypes from the Marmara Sea Sediments (MSS) belongs to previously unknown an uncultivated groups; physiological properties of the MSS organisms remained largely unknown.

This study is one of the legs of a broad range project supported by TÜBİTAK, aiming to determine the microbial composition and anaerobic petroleum degradation potensial of anoxic marine sediments from the most polluted areas of the Marmara Sea, which are Tuzla, Moda, Gemlik, İzmit , Haliç and Küçükçekmece.

Benzene, toluene, ethylbenzene, and the isomers of xylene (BTEX), especially, are classified as priority environmental pollutants by the EPA (USEPA, 1986). This classification is based on their substantial toxicity and on the carcinogenic potential of the benzene component (Dean, 1985). High solubility in water relative to other

petroleum hydrocarbons cause BTEX compounds to be easily transported with the groundwater (Cozzarelli, 1990).

In the scope of the mentioned project, the presence of microbial communities with the potential to carry out anaerobic degradation of BTEX and presence of biomarkers for *in situ* hydrocarbon degradation in anoxic marine sediments will be investigated to determine the distribution of anaerobic hydrocarbon degradation potential in the Marmara Sea, an important and heavily industrialized marine environment. In this project monoaromatic hydrocarbons which are specified as BTEX have been investigated.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 methanogens. If the function is mediated by a diverse polyphyletic group of organisms, such as anaerobic BTEX degradation, rRNA-based approaches are of limited value. Instead, genes that encode key enzymes of important metabolic processes can be targeted (Smit, 2007).

DNA may persist in intact but inactive cells as well as in the environment as extracellular material after cell death (Naviaux, 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 genomic DNA rather than rRNA and mRNA in sediment samples, it is possible to count all the microorganisms even only one representative of the microbial group exist in the sediment.

Quantitative real-time PCR (Q-PCR) have been widely used for the quantification of gene abundances in environmental samples (Winderl ; 2008, Higashioka . 2009). There were a few studies that quantify functional gene transcripts in marine sediments (Smith, 2007, Chin, 2008). These studies targeted specific processes such as denitrification or DSR. Recently Kolukırık studied, for the first time, abundances and activities of organisms responsible for N cycling, AnHD, DSR and MG were assessed all together in marine sediments.

Natural attenuation (NA) is a bioremediation strategy based on natural processes. NA is of great interest for cost effective treatment of the chronic hydrocarbon pollution in the MSS. Aerobic respiration is much faster than anaerobic microbial processes for hydrocarbon degradation. Because the MSS were anoxic and oxygen mass transfer to the sediments is inappropriate, a bioremediation strategy based on aerobic respiration

is not feasible. Under such conditions anaerobic hydrocarbon degradation can be the alternative as long as oil-degrading microorganisms are abundant and active in the MSS. Monitoring techniques targeting catabolic genes essential to BTEX degradation which is bssA (Winderl, 2008) were established. The designed Q-PCR primers were able to amplify bssA of certain microbial groups such as sulfate reducing bacteria (SRB) (Beller, 2008), denitrifying bacteria (DB) (Beller ,2002) and a methanogenic consortium (Washer and Edwards 2007), or targeted certain clusters of previously identified bssA (Winderl ,2008)

1.1 Purpose of the Thesis

In this study, generic Q-PCR primers which has been designed to monitor abundance and activity of aromatic hydrocarbon degraders in the MSS was used for the first time. (Kolukırık, 2009.) .Advances in monitoring *in situ* BTEX biodegradation have been driven by dramatic improvements in understanding of the biochemistry and genetics underlying anaerobic alkylbenzene metabolism (Heider, 1998, Spormann, 2000) as well as advances in mass spectrometric and molecular techniques over the past 10 to 15 years. Some of these monitoring methods have focused on metabolites (benzylsuccinates) or genes (*bssA*) associated with benzylsuccinate synthase (BSS), an enzyme that catalyzes the first step of anaerobic toluene and xylene degradation (Heider ,1998,). Advantages of the BSS reaction with regard to *in situ* monitoring include the unique and diagnostic nature of its cognate metabolite (Beller , 2000;) as well as its relevance to physiologically and phylogenetically diverse bacteria. The presence of bss has been observed in cultures that degrade toluene under a wide range of environmentally relevant electron-accepting conditions, including denitrifying, sulfate-reducing, ferric iron-reducing, and methanogenic conditions (Heider,1998; Spormann, 2000; Kane, 2002, Edwards , 2000). BSS has also been shown to catalyze the first step in anaerobic xylene degradation (Krieger, 1999, Achong, 2001), and a homologous enzyme apparently catalyzes ethylbenzene degradation under sulfate-reducing conditions (Kniemeyer, 2003). To date, no enzyme other than BSS has been described that catalyzes the first step of anaerobic toluene or xylene degradation.

Microbial degradation is the only sustainable component of natural attenuation in contaminated groundwater environments, yet its controls, especially in anaerobic

aquifers, are still poorly understood. We conducted quantitative PCR of benzylsuccinate synthase genes (*bssA*) which is the key enzyme of anaerobic toluene degradation to quantify the distribution of specific anaerobic BTEX degraders.

2. POLLUTION OF THE MARMARA SEA

2.1.Characteristics of the Marmara Sea

The Sea of Marmara is a small (size 70 x 250 km) intercontinental basin connecting the Black Sea and the Mediterranean Sea. The oceanographic features (chemical, biological) of the basin are influenced by the Black Sea and the Aegean Sea via the Bosphorus Strait and the Dardanelles, respectively. Marmara Sea has an area of about 11,110km2 and a volume of 3380km3, 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 the 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 Istanbul Strait to Canakkale Strait, whereas saline Mediterranean waters (38%) flows as the lower layer in the opposite direction (Miller, 1983; Unlu , 1990).

The 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 Istanbul 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 tanker traffic of several thousand oil carrying vessels per day, via the Bosphorus Strait are a constant threat to the marine ecosystem.

The benthic environment is a fundamental compartment of any aquatic ecosystem. Bottom sediments are the final sink for many anthropogenic contaminants and they can accumulate great amounts of organic matter affecting the oxygen content of the bottom water (Venturini , 2004)

2.1.1. Pollution Sources of Marmara Sea

The Marmara Sea is now a critically polluted water body and the recipient of a large number of wastewater discharges from landbased sources located along the coastal line, including the İstanbul metropolitan area (Orhon, 1995; Albayrak., 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 (Ünlü, 2006).The Bosphorus, a strongly stratified natural channel between the Marmara Sea and Black Sea, with significant mixing at the entrance to the Marmara Sea is also a major polluter for the Marmara basin, since it carries the highly polluted waters of the Black Sea (Orhon, 1995).

The Marmara Sea receives via the natural exchange from the Black Sea roughly 15 times more organic matter than what is contained in the sewage discharges from İstanbul (Orhon, 1995). The basin receives a total of 1.9 x 106 tons of TOC (total organic carbon) and 2.7 x 105 tons of TN (total nitrogen) per year from the Black Sea inflow (Albayrak., 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 (Ünlü, 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 (Öztürk, 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 (total organic carbon) and $2.7x10^5$ tons of TN (total nitrogen) per year. Addition to oil pollution caused by inflow from Black Sea, heavy sea traffic and various refineries and facilities located around Marmara Sea increases the oil pollution dramatically (Fashchuk , 1991, Tuğrul and Polat, 1995). The oil concentration increased with years gradually as the sea traffic increases with years. The oil concentration at Bosphorus increased from 9.5 µg/L to 33.5 µg/L from 1995 to1996. The Dardanelles showed a higher increase in concentration from 5.25 µg/L to 42.5 µg/L in the same period. The concentration of the Marmara Sea increased from 36.9 µg/L to 103.7 µg/L at the same time (Guven , 1998).

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 (Güven, 2004). The major accidents happened by large tankers Independenta in 1979 and Nassia in 1994. In the Independenta accident at the exit of the Bosphorus to the Sea of Marmara in 1979, 95 000 tons of crude oil was spilt and burnt (Etkin, 1997).

In the Nassia accident at the northern exit of the Bosphorus to the Black Sea in 1994, 13 500 tons of crude oil were spilt (Oğuzülgen, 1995). M/V GOTIA sank into Bosphorus and 25 tons fuel oil was spilt and pollution spread out into a large area by winds (Güven , 2004). Bilge water discharge is also a major problem for the Straits of İstanbul and Çanakkale, and the Sea of Marmara. Increase in petroleum hydrocarbon levels mainly from oil spills, sewage outfalls and ship bilge water, has been observed in the Sea of Marmara (Güven , 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 Sayhan Topçuoglu and friends (Topçuoglu, 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 (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 (Ünlü , 2006) Anthropic pollution trapped in bays, in particular, has created significant ecological damage resulting in the decrease or extinction of marine species (Ünlü , 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 of Marmara Sea

2.2.1. İzmit Bay

Izmit Bay, the most important semi-enclosed body of water on the east side of the Sea of Marmara, about 50 km in length, 2–10 km in width and 310 km2 in surface area (Fig.2.1). 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 brakish (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 urbanisation of the coastal landscape have completely destroyed the previous serenity of Izmit Bay.

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

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 (Bauloubassi and Saliot, 1991), whereas the sediment may become sufficiently polluted to disrupt natural biological communities (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 (Chapman and Long, 1983; Long and Chapman, 1985).

İzmit Bay, located south of Istanbul 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 (Bauloubassi and Saliot, 1991), whereas the sediment may become

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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).

Figure 2.1: The location of the İzmit Bay

2.2.2.Küçükçekmece

Kücükcekmece Lagoon, located in the European part of Istanbul in Turkey, has typical spoon shaped topography (Figure 2.2). The surface area of the lake is approximately 17 km2, and the water volume is 145 million m3 at sea level. Untreated wastewaters, both domestic and industrial (metal, textile, plastic etc.), are routinely being discharged into the creeks of Kucukcekmece Lagoon (B. Ustun, N. Ince,2005)

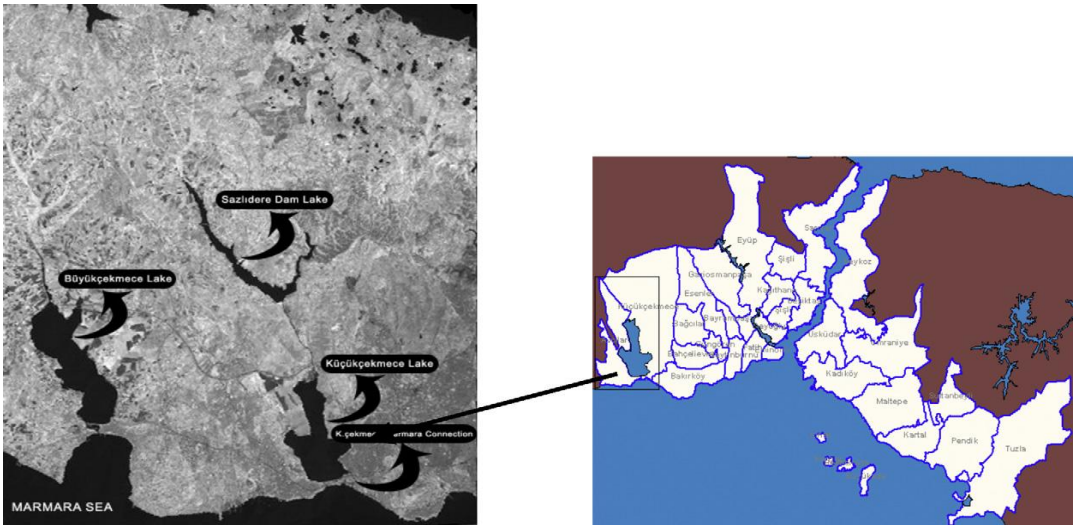

Figure 2.2: Topografical shape of Küçükçekmece Lagoon

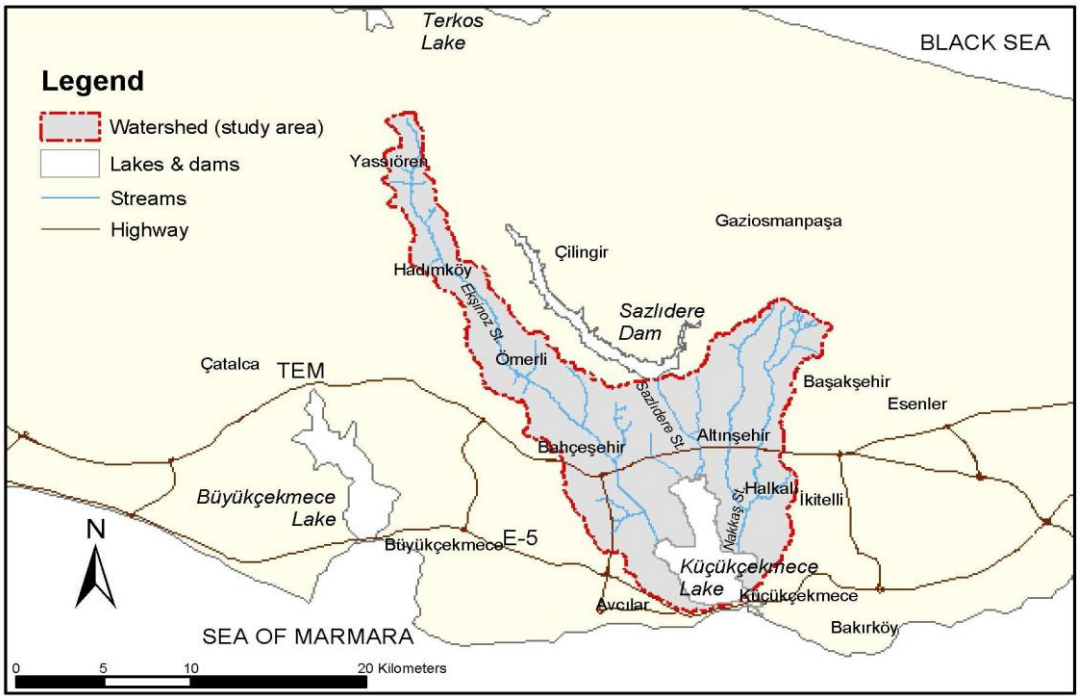

Figure 2.3: The location of Küçükçekmece

Three stream systems feed the lake: Nakkasdere, Sazlıdere and Ispartakule (Demirci, 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 (ĐSKĐ,2005a).

The degradation of a reservoir happened in the case of Kucukcemece reservoir in Istanbul, Turkey (Figure 2.3.). Kucukcemece reservoir was originally an estuary of the Marmara Sea. It was a clean natural habitat before the 1980's (Demirci, 2001). In the 1980s, Istanbul started to have a tremendous growth spurt. The development quickly transformed this previously sparsely populated semi-rural/exurban area of Kucukcemece 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 Istanbul 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; Ünlü, 2000). The oil pollution was investigated and the pollution level was determined in seawater, sediments and mussels in Tuzla Bay after the TPAO tanker accident. The highest pollution was found as 33.2 mg/L in seawater and 423.0 µg/g in sediment on the first day after the accident (Ünlü, 2000).

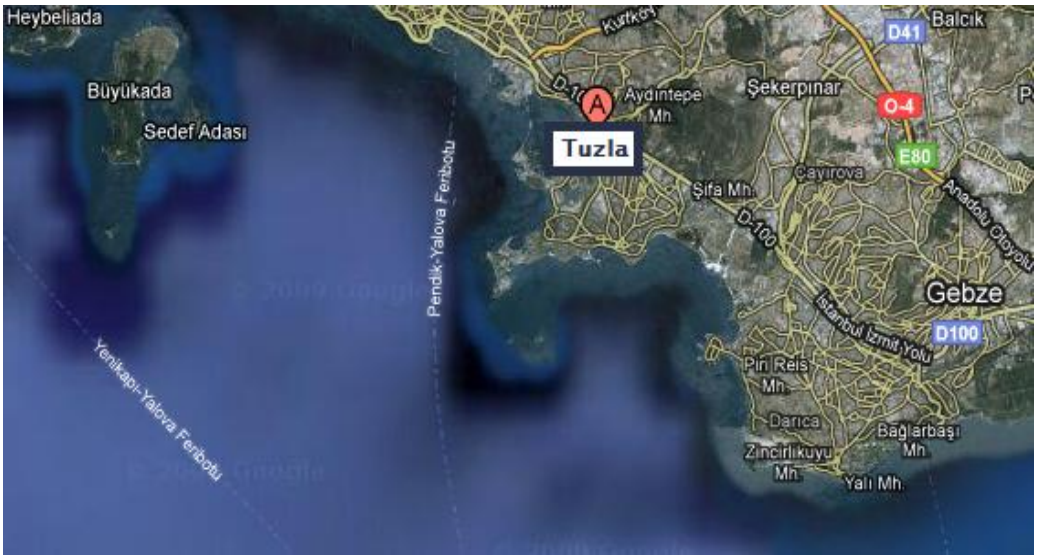

Figure 2.4: The location of Tuzla

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 , 2007). In aquatic sediments, the depth of oxygen penetration through diffusion is controlled mainly by the consumption of degradable organic matter within the sediment and in coastal ecosystems rarely exceeds more than a few millimeters (Jorgensen, 1983). With the exception of the most superficial layer, the bulk of organic matter-rich marine sediments contaminated by PHC are assumed to be anoxic (Canfield , 1993b).

Consequently, microbial processes depending on the availability of free dissolved oxygen are constrained to 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 , 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. This manuscript desribes how metal pollution evolved in Golden Horn between 1912 and 1987, by analysing Pb slices of a 3-m long core collected close to Galata bridge in 1989.

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 the 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 (Figure 2.5.), 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 Istanbul. 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 (w38 psu), Black Sea (w17 psu), and freshwater urban runoff, precipitation, and a small fluvial contribution. Freshwater remains on the surface due to a greater rate of input (300,000m3 of freshwater enter the estuary annually) than evaporative loss (Ozturk, 1998; Alpar , 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 (Aksit, 1977) (1963), Gunnerson (1974), Saydam (1986), Basturk (1988), and Ozsoy (1988). Low velocity surface winds and stable atmospheric conditions contribute to minimal air ventilation in the surrounding area (Incecik, 1986). 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).

Figure 2.5: Location of the Golden Horn and surrounding districts

2.2.6 Gemlik

The Gemlik Bay emerges as a 31-km-long tectonic trough between two topographic heights, with an increasing width westward (Figure 2.6.). 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 Samanlıdag˘ 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 (Figure 2.6.). With its 27600 km2 drainage area and 158 m3/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).

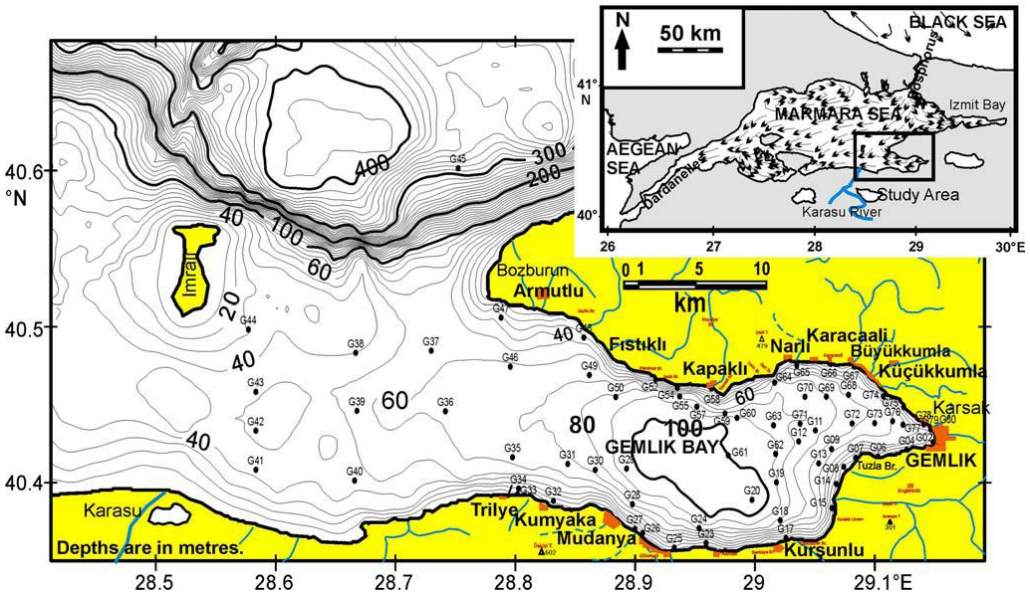

Figure 2.6 Location of the Gemlik Bay

3. MARINE SEDIMENTS

Approximately 70% of the Earth's subsurface is marine and the underlying sediments, which can be more than a kilometer deep, cover 70% of the total earth (Kormas , 2003). Deep sea sediments covering earth's surface may also be defined as "deep-sea floor" which are that portion of the ocean bottom overlaying by at least 1000 m of water column (Vetriani, 1999; Glover and Smith, 2003). The deep-sea floor is one of the vast regions with a number of distinct habitats (Glover and Smith, 2003). These cover sediment filled basins, continental slopes and abyssal plains, deep ocean trenches and the exposed pillow basalts of young mid-ocean ridges, seamount risings > 1000 m above the general seafloor and submarine canyons. The most extensive habitats constituting >90% of the deep-sea floor are the "mud" or "silt and clay" clad plains of the slope and abyss. Deep ocean trenches constitute 1-2% of the deep-sea floor, while the rocky substratum of midocean ridges (~ 10 km wide and ~ 60 000 km long) , seamounts (perhaps 50 000-100 000 in number) and submarine canyons being the rare habitats of the deep sea occupy < 4% of the sea floor. (Glover and Smith, 2003)

3.1 Properties of Marine Sediments

Deep sea sediments are primarily formed through the deposition of particles from the productive ocean surface (Vetriani , 1999). The new ocean basin that forms at spreading ocean ridges due to plate tectonic forces migrates towards subduction zones where it moves under continental shelves are returned to the interior of our planet. During this tour of maximum 170 years more and more sediments build up on top of hard basement rock and ultimately thousands meters of thick layers can be formed (Glover and Smith, 2003). The various major sediment input sources into the ocean are rivers, glaciers and ice sheets, wind blown dust, coastal erosion, volcanic debris, ground water. Much of the organic input into the oceanic sediments is through the recycling by the benthic communities (Aller , 1998). Marine sediments, also known as pelagic sediments are those that accumulate in the abyssal plain of the deep ocean, far away from terrestrial sources which provide terrigenous sediments,

one of the two main classifications for marine sediments. Terrigenous sediments are normally delivered by rivers and are primarily limited to the coastal shelf. There are many classification schemes such as size, deposition mode, source, locale and chemistry for deep sea sediments. Terrigenous sediments are normally classified according to their sediment grain size and named as boulder, cobble, pebble, gravel or granule, coarse sand, medium sand, fine sand, silt or clay.

However, pelagic sediments are classified by their composition as follows: lit hogenous, biogenous, hydrogenous, cosmogenous. Among pelagic sediments, biogenic sediments which are derived from living, mostly planktonic organisms in a variety of forms and species are the most important in marine sedimentological field since the most information can be derived from them. Those sediments have high sedimentation rates and contain information about water chemistry and climates.There are several characteristics distinguishing most deep-sea sediments from other

Earth's ecosystems, perhaps the most important one of which is its low productivity (Glover and Smith, 2003). The detrital base of deep-sea food webs strongly differs from most epipelagic, shallow-water and terrestrial ecosystems, which are mostly maintained by locally produced organic matter, whereas detrital food particles for the deep-sea biota ranges from fresh phytoplankton remains to the carcasses of whales (Glover and Smith, 2003). Therefore the biomass of the deep benthic communities is only 0.001-1% of that in shallow-water benthic or terrestrial communities due to the low flux of organic energy.

Low food flux along with low temperatures (-1– 4 °C) results in relatively low rates of growth, respiration, reproduction, recruitment and bioturbation in the deep sea (Glover and Smith, 2003). In the subsurface which is defined as terrestrial subsurface below 8 m and marine sediments below 10 cm, prokaryotic cellular carbon for the marine subsurface is estimated as 303 Pg of C, whereas the total prokaryotic cellular carbon value in soil yields an estimate of 26 Pg of C (Whitman , 1998). 5 to 10 billion tons of organic particulate matter is constantly sinking in the world's oceans and accumulating as sediment and only about 0.4 % of the carbon fixed by phytoplankton at the ocean surface is buried in the oceanic sediments which represents a net carbon dioxide removal from, and oxygen input into, the atmosphere (Middelburg and Meysman, 2007). About 95% of the organic matter produced

photosynthetically appears to be recycled in the upper 100-300 m, whereas only about 1% of photosynthetically produced organic carbon reaches the deep-sea floor, and this remainder out of the vast majority of organic matter recycled by near-surface microbial activity accumulates and represents the largest global reservoir of organic carbon, approximately 15.000 x 1018 g C, including fossil fuels (Parkes , 2000). Therefore the major nutritional characteristics of the deep-sea environments are relatively low input of organic carbon and its consumption for living organisms and deep-sea sediments may be estimated as unique habitats for microbial communities where the availability of nutrients is geographically highly variable and pressures are highly elevated (Li , 1999b).

Other general characteristics for deep-sea floor are the low-physical energy, very slow sediment accumulation rates (0.1-10 cm/thousand years) and the absence of sunlight. Nonetheless, the studies showed that deep-sea soft-sediment communities often exhibit very high local species diversity, with 0.25 m2 of deep-sea mud containing 21-250 macrofaunal species (Glover and Smith, 2003). Surprisingly, not all the deep-sea habitats are low in energy and productivity. Hydrothermal vents and some cold seeps are exceptional since energy for the deep-sea biota is derived from an attenuated ‘rain’ of detritus from remote surface waters (1-10 g Corg m-2 yr-1). In cold seeps biomass and productivity of the present communities, which are low in diversity, are high due to the chemoautotrophic production fuelled by reduced chemicals such as hydrogen sulphide. Besides seamounts, canyons and whale falls which also break the low-energy deep-sea ‘rule’ enhances the physical and /or biological energy yield resulting in high biomass communities (Glover and Smith, 2003).

3.1.1 Main Consideration on Marine Sediments

Sediments on the seafloor are a rich source of information on the history of the oceans (e.g., changes in ocean temperature, circulation patterns, and chemistry), on former climates, sea levels and pollution. They are very useful at providing information on changing global climates during the past few million years. Sediments play an important role in the remineralization of deposited matter in highly productive continental shelf areas (Mumann , 2005). Sediments have proved to be excellent indicators of environmental pollution, as they accumulate pollutants to the levels that can be measured reliably by a variety of analytical techniques and

they also store records about pollution history of a given water body due to sedimentation being a continuous process (Tuncer , 2001). Sedimentation with faster sedimentation rates in bays and estuaries are more suitable for investigating pollution history in the twentieth century as they provide higher resolution (Tuncer , 2001). Sediments are undoubtedly essential to the functioning of aquatic ecosystems, since they may act as sinks but also as sources of contaminants in aquatic systems (Mucha , 2003).

Besides all the geochemical importance of marine sediments, deep subsurface has been under the exploration of scientists for its biodiversity and the microbial processes occurring within, that are of importance as a result of general, social, professional and industrial motives (Pedersen, 2000), and mostly due to the environmental concerns.

However, the deep sea biosphere is among the least-understood habitats on Earth, eventhough the huge microbial biomass therein plays an important role for potential longterm controls on global biogeochemical cycles (Inagaki , 2006). Marine sediments are of significance since they play an important role in the global cycling of carbon and nutrients.(Rochelle , 1994). Chemical composition of the ocean and the atmosphere is profoundly effected by selective degradation of organic matter (Holland, 1984). The ocean sediments are a significant reservoir of carbon burial without which O2 would not have accumulated in the atmosphere (Middelburg and Meysman, 2007). Moreover, the small quantity of carbon transfer from surface to subsurface sediment supports prokaryotes that live deep in the Earth's crust and that make up about 30% of the total living biomass on Earth (Whitman , 1998).

Therefore, the subsurface is a major habitat for prokaryotes and the number of subsurface prokaryotes is expected to go beyond the numbers of the other components of the biosphere (Whitman , 1998). The studies have shown that the subsurface contains a variety of types of microbial ecosystems that are much more densely populated than expected (Krumholz , 2000). Thus deep subsurface environments harbor a vast diversity of communities that are responsible for various microbial processes which have a fundamental role in surface sediments and when microorganisms are mixed with the sediment, they catalyze the early diagenetic processes and thus appear to be important factors in the diagenesis of the sediments during the sedimentation process (Wellsbury , 1997).

3.1.2 Environmental Effects on the Ecosystem

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

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

The major natural environmental forcing factors on the deep-sea floor include food input such as organic carbon flux which has a major impact on the abundance and diversity of benthos on a seasonal or interannual regional scale, whale-falls with the latter mentioned have an impact between 1-100 years on local scale (Smith and

Baco, 2003) and the changes in the surface water has an interannual or decadal regional impact on the diversity and abundance of benthos (Smith , 1997). Other natural environmental forcing factors are biogenic disturbance and hydrodynamics and chemical emissions. Benthic storms with a temporal time scale of days and turbidity currents with a time scale between 1000-100 000 years both have a major impact on benthos' smothering and diversity and turbidity currents inhibit the settlement as well. Methane hydrate release is one of the chemical emissions whose major impacts on benthos are unknown (Glover and Smith, 2003), but has also a localized emission effect in terms of spatial scale, whereas another chemical

emission CO2 release lowers the pH and causes toxicity on a temporal scale of decades (Sakai , 1990). Hydrogen sulphide and trace metals from vents have an impact of toxicity on benthos and are an energy source for microbes on a temporal scale of decades (Van Dover, 2000).

3.1.3 Characterization of Anoxic Marine Sediments

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

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

(Macnaughton , 1999; Röling., 2004; and Röling , 2002). The presence of hydrocarbon compounds and low oxygen level creates a suitable environment for the growth of anaerobic bacteria. Although anaerobic biodegradation processes are slower than aerobic biodegradation, anaerobic processes can be a significant factor in removal of organic contaminants owing to the abundance of anaerobic electron acceptors relative to dissolved oxygen.

3.2 Microbial Ecology Of Anoxic Marine Sediments

3.2.1 Prokaryotes in Anoxic Marine Sediments

Although invisible to the naked eye, prokaryotes are anessential component of the earth's biota. They catalyze unique and indispensable transformations in the biogeochemical cycles of the biosphere, produce important components of the earth's atmosphere, and represent a large portion of life's genetic diversity.

Although the abundance of prokaryotes has been estimated indirectly the actual number of prokaryotes and the total amount of their cellular carbon on earth have never been directly assessed (Romankevich ,1988). Presumably, prokaryotes' very ubiquity has discouraged investigators, because an estimation of the number of prokaryotes would seem to require endless cataloging of numerous habitats.

To estimate the number and total carbon of prokaryotes on earth, several representative habitats were first examined. This analysis indicated that most of the prokaryotes reside in three large habitats: seawater, soil, and the sedimentysoil subsurface. Although many other habitats contain dense populations, their numerical contribution to the total number of prokaryotes is small. Thus, evaluating the total number and total carbon of prokaryotes on earth becomes a solvable problem. Results obtained through the Deep Sea Drilling Project and Ocean Drilling Program (ODP) have revealed that the activities of subsurface prokaryotes have profoundimplications for the global carbon cycle (Sorensen and Teske, 2006).

Microorganisms living in anoxic marine sediments consume more than 80% of the methane produced in the worlds oceans. In addition to single-species aggregates, consortia of metabolically interdependent bacteria and archaea are found in methane-rich sediments. A combination of florescence in situ hybridization and secondary ion mass spectrometry shows that cells belonging to one specific archaeal group

associated with the *Methanosarcinales* were all highly depleted in 13C (to values of Ð96ä). This depletion indicates assimilation of isotopically light methane into specifc archaeal cells. Additional microbial species apparently use other carbon sources, as indicated by significantly higher 13C/12C ratios in their cell carbon. Our results demonstrate the feasibility of simultaneous determination of the identity and the metabolic activity of naturally occurring microorganisms. (Victoria J. Orphan 2001)

Degradation and transformation of organic matter is the most important function of the microbial community in freshwater sediments. Therefore, information on the composition of the microbial community is of key importance for a better understanding of the metabolic processes in these aquatic ecosystems [S. Spring, 2000) Sediment bacteria also play a significant ecological and biogeochemical role in marine ecosystems (Polymenakou ,2005)

3.2.2 Subsurface Microbiota Characterization

The evidence of microbial life in the deep subsurface hasbeen postulated for a very long time, but the microbes thatwere recovered were suspected to be contaminants from the conventional biosphere. Interdisciplinary action by ateam from the Department of Energy Deep Subsurface Science Program developed a set of criteria and methods by which sampling could becarefully controlled; tracers of various sorts, such as bromine ions, perfluorohydrocarbons,microbe-sized fluorescent beads, community biologTM and signature lipid biomarkers, could be used to monitor contamination during the sample recovery process [FredricksonJ K, Phelps 1998]. One of the most powerful tests for contamination is community biology and phospholipid fatty acid signatureanalysis [Lehman RM, Colwell FS, 1995]. This shows distinct differences betweenmicrobial communities recovered from drilling muds,cuttings, and cores.

3.2.3 Subsurface Community Characterization

Phospholipid ester-linked fatty acids (PLFAs) are an excellent measure of the viable or potentially viable biomass in a wide range of environments. Viable microbes have intact membranes, which contain phospholipids (and PLFAs). Total PLFA often differs by three to five orders of magnitude between the drilling fluids and cores [Lehman RM, Colwell FS, 1995)

Cellular enzymes hydrolyze and release the phosphate group of phospholipids within minutes to hours following cell death (White DC, Davis WM 1979). The composition of the PLFAs and other lipids provides an insight into the community composition of the *in situ* microbiota.(White DC, Stair JO, 1996) and, because the lipids are modified in specific ways by shifts in the local environment, they reflect the physiological/ nutritional status of the communities (White De, 1995, White DC, Ringelberg DB 1997). The ratio of diglyceride fatty acids to PLFAs from lysed bacterial cells increases markedly with depth and the PLFA profiles can be significantly different at different subsurface horizons (Ringelberg DB, Sutton S 1997). Subsurface sediment samples collected in Western Washington, South Carolina, Northern New Mexico, and Central Idaho from a depth greater than 30m were analyzed for PLFAs (White DC, 1998). Comparison of the PLFA profiles shows that the distribution of the microbial community is influenced by the geology of the subsurface: 78% of the variance in the community PLFA profiles is influenced by the lithology (sand/sandstone to clay/basalt) and an additional 7% by the permeability of the subsurface sedimentary element (White DC 1998). The structure and chemistry of the subsurface clearly affect the microbial community composition and activit'> but do the microbes affect the rocks? Rates of mineral weathering in aquifers have been related to bacterial colonization (Bennet PC, 1996). Minerals that are not colonized by microbes are not weathered. Does soil mineral formation require a microbial biosphere?

3.2.4 Bacterial Communities in Anoxic Sediments

According to laboratory studies including both culture dependent and independent techniques, there are at least 17 major phyla of bacteria. Figure gives a phylogenetic overview of Bacteria. The first phylum of bacteria is proteobacteria. This is the widest phylum of the bacteria. As a group these organisms are all gram-negative, show extreme metabolic diversity, and represent the majority of known gram-negative bacteria of medical, industrial, and agricultural significance. Proteobacteria has five major subdivisions:

- Alpha
- Beta
- Gamma
- Delta
- Epsilon

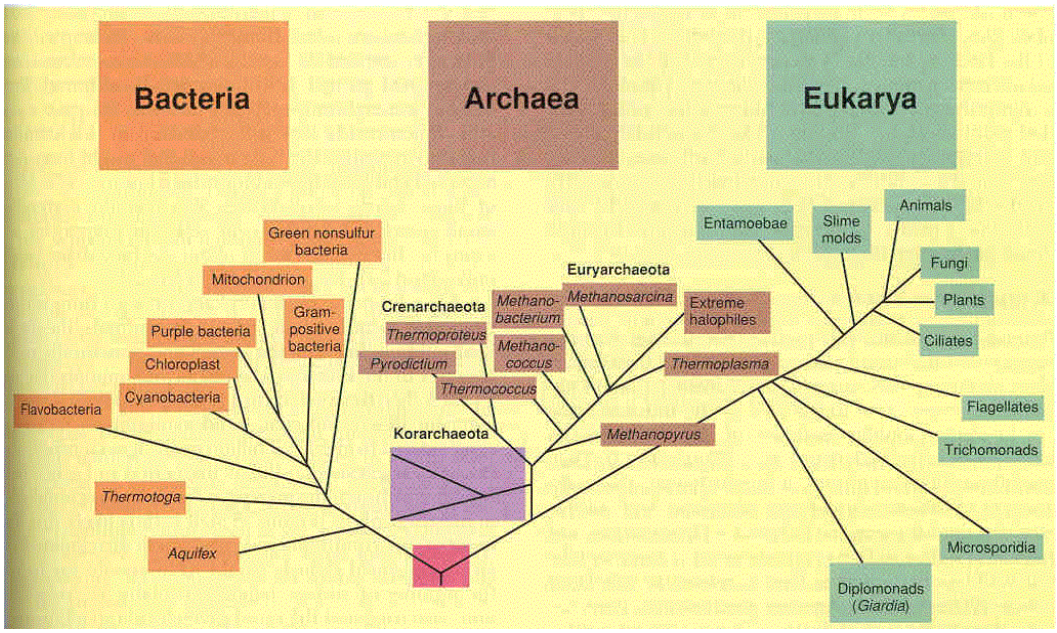

Figure 3.1: Universal phylogenetic tree , adapted from Madigan, 2002

One of the most important known groups of proteobacteria is purple phototrophic bacteria which carry out anoxygenic photosynthesis and contain chlorophyll pigments called *bacteriochlorophylls* with any variety of carotenoid pigments. The purple bacteria have different and spectacular colors, usually purple, red or brown. The most known of purple bacteria are purple sulfur bacteria and purple nonsulfur bacteria (Madigan , 2002).

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

The second phylum of bacteria is gram-positive bacteria which contain nonsporulating, low GC, gram-positive bacteria as lactic acid bacteria and relatives; endospore forming, low GC, gram-positive bacteria as *Bacillus*, *Clostridium* and relatives; cell wall-less, low GC, gram-positive bacteria as the Mycoplasmas; high GC, gram-positive bacteria as coryneform and propionic acid bacteria; high GC,

gram-positive bacteria: *Mycobacterium*; and lastly filamentous, high GC, gram-positive bacteria as *Streptomyces* and other *Actinomycetes* (Madigan , 2002).

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

3.2.5 Archaeal Communities in Anoxic Sediments

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

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

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

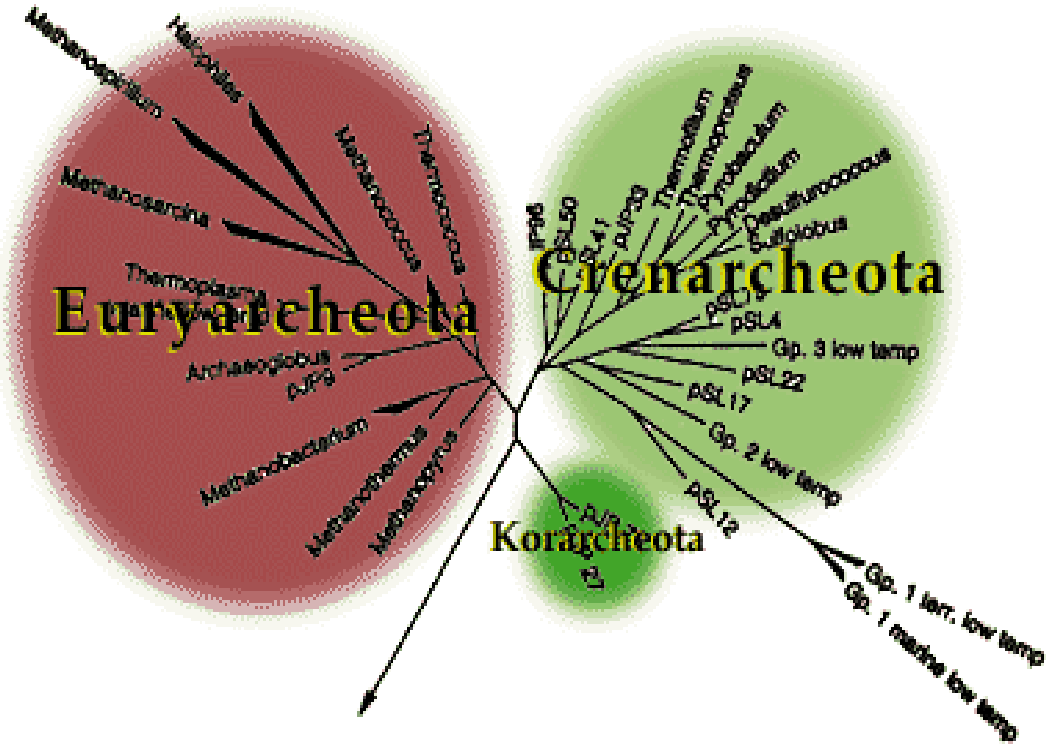

Figure 3.2: Phylogenetic Tree of Archaea

The tree is separated into two major phyla called the Crenarchaeota and Euryarchaeota. A third phylum is Korarchaeota which branches off close to the root (Madigan , 2002).

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

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

3.2.6 Petroleum Hydrocarbon Degradation by Microorganisms

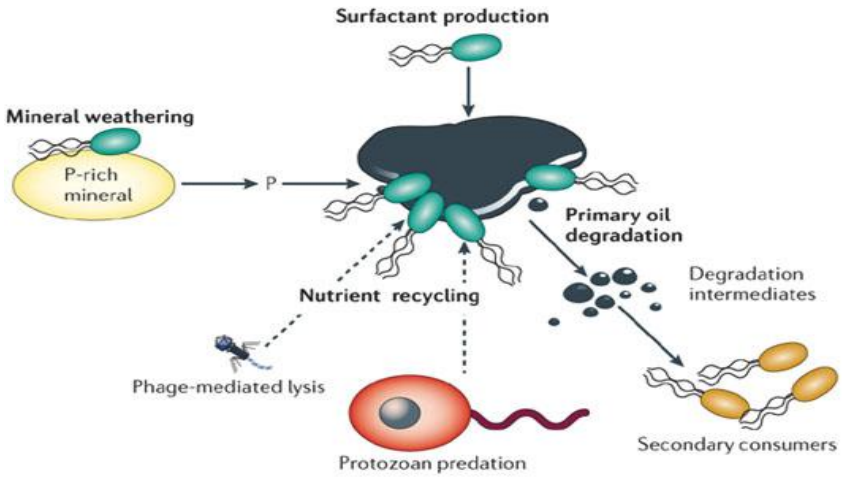

Figure 3.3: Biodegradation of petroleum hydrocarbon by microbial consortium adapted by Head and Rolling, 2006

With developments in industry, petroleum became a daily element of our lives. As the dependence of petroleum increases, the pollution caused by the petroleum hydrocarbon emission increased continuously. The input of petroleum hydrocarbons is high enough to cover the whole earth with a layer of oil. Although the scene is so bad, earth didn't covered with an oil layer only because of degradation of this petroleum by the activity of microorganisms. Individually or working in network, microorganisms are able to degrade hydrocarbons efficiently. Mostly marine environments are natural habitat of hydrocarbon degrading microorganism The biodegradation of petroleum hydrocarbons is not a new concept. The isolation of first hydrocarbon degrading bacteria spans to 1900s (Söhngen, 1913). Currently it has been found that there are 79 bacterial genera, 9 cyanobacterial genera, 103 fungal genera and 14 algal genera using, degrading and transforming hydrocarbons. With the new achievements in biochemistry of hydrocarbon degrading bacteria, degradation of hydrocarbons are well understood. The breakdown of hydrocarbons is mainly limited by the presence of nutrients like nitrogen and phosphorus (Atlas and Bartha, 1972); iron was also reported as a limiting factor in clean offshore seawater (Swannell , 1996). Sulphur is a well abundant in seawater as sulphate ion but can be a limiting factoring a freshwater system. Slightly alkaline pH of seawater is a favorable environment for the degradation. When these nutrients are abundant, bioavailability of hydrocarbons increases in importance and becomes the limiting factor.

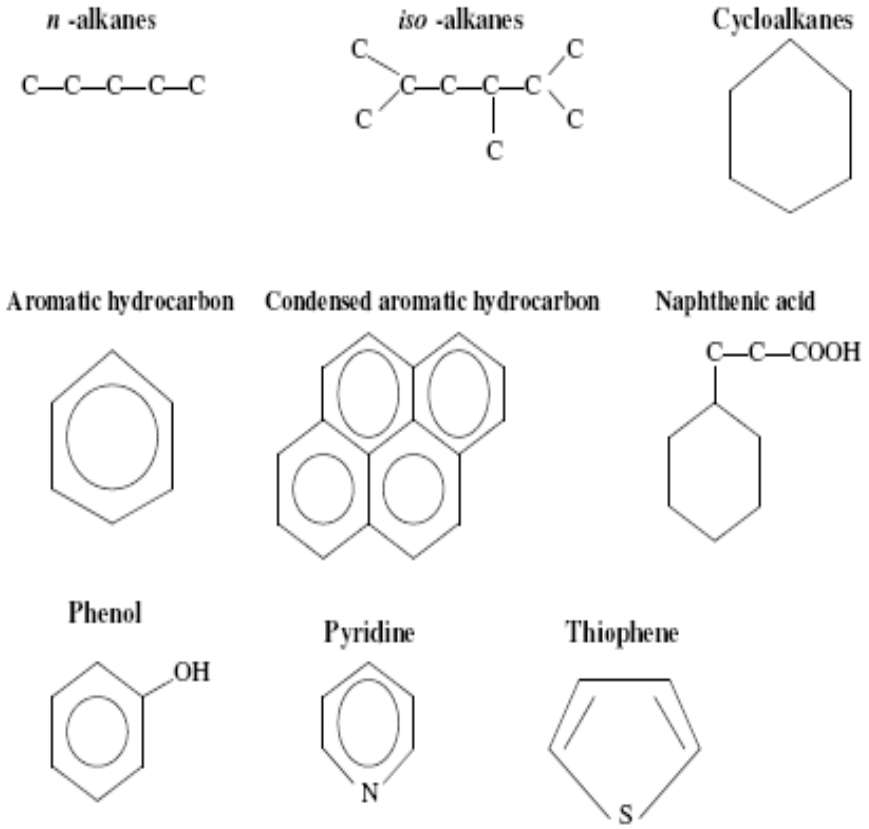

Figure 3.4: Structural classification of some crude oil components adapted by Alloway and Ayres, 1993

There were many studies about microbial hydrocarbon degradation in controlled conditions (Sugiura , 1997; Chaillan , 2004) and in open field experiments (Gogoi , 2003) but the knowledge about organisms play important role in biodegradation was rather limited. But there are some recent reviews about the degradation of hydrocarbons in anoxic conditions enlighten scientist in understanding these microorganisms. Studies have shown that the important players in hydrocarbon degradation come from marine environments. Those microorganisms are specialized in biodegradation of hydrocarbons by using them as a carbon source. *Alcanivorax* spp. (Yakimov , 1998), *Cycloclasticus* spp. (Dyksterhouse , 1995), *Oleiphilus* spp. (Golyshin , 2002), *Oleispira* spp.(Yakimov , 2003) and *Thalassolituus* spp. can be counted as important hydrocarbon biodegraders

Table 3.1: Some of common factors affecting petroleum hydrocarbon degradation adapted by Bartha, 1986

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

Bacterial groups of *Pseudomonas, Marinobacter, Microbulfier, Sphingomonas, Micrococcus, Cellulomonas, Dietzia and Gordonia* are also known as their capacity to degrade hydrocarbons (Brito , 2006). Some of these microorganisms specialized to degrade branched and straight chain hydrocarbons, as some others interest in polycyclic aromatic hydrocarbons. Although methanogens can not be classified petroleum hydrocarbon degraders, they are in the microbial network. Their possible role is to use acetate coming from reactions of anaerobic degraders as metabolite and produce methane and carbon dioxide. There are several studies showing hydrocarbon degradation in deep subsurface and petroleum reservoirs linked to methanogenesis (Nilsen and Torsvik, 1996, Zengler ,1999; Widdel and Rabus, 2001; Anderson and Lovley, 2000; Nazina , 1995; Ng , 1989).

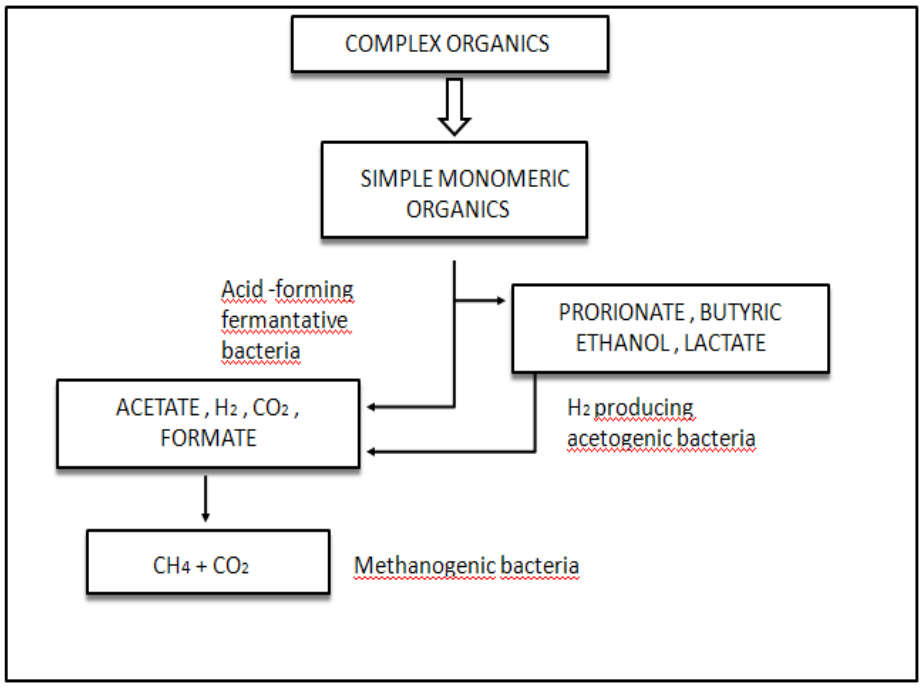

Figure 3.5: Anaerobic degradation of hydrocarbons

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

4.BTEX

BTEX is an acronym for benzene, toluene, ethylbenzene, and xylene. This group of volatile organic compounds (VOCs) is found in petroleum hydrocarbons, such as gasoline, and other common environmental contaminants.

4.1.Chemical Properties of BTEX

Benzene, toluene, ethylbenzene and xylenes isomers (BTEX) are important monoaromatic hydrocarbons that have been found in sites polluted by oil production facilities and industries (Kao, 2006) These hydrocarbons have higher water solubility than other organic compounds that are present in gasoline such as aliphatics. Generally, solubility of benzene, toluene, ethyl benzene, xylenes and gasoline in water are respectively 18, 25, 3, 20, 50–100 ppm when gasoline is introduced into water (Kermanshahi , 2005). Percent volume of benzene, toluene, ethylbenzene and xylenes in gasoline, are 1, 1.5, <1–1.5 and 8–10, respectively (An, 2004) They are also produced on the scale of megatons per year as bulk chemicals for industrial use as solvents and starting materials for the manufacture of pesticides, plastics, and synthetic fibers (Harwood ,1997). BTEX compounds under aerobic conditions are widely distributed, and researchers have isolated many of these strains by enrichment broth culturing with BTEX compounds as the sole carbon and energy sources, under aerobic and anaerobic conditions (Jahn, M. K. 2005)

Table 4.1: Physio-chemical properties of BTEX

Compound	Mole weight g $mole^{-1}$	Density g ml^{-1}	Boiling point °C	Water solubility mg l^{-1}	Vapor pressure mmHg	Log K_{ow}
Benzene	78	0.88	80.1	1780	76	2.13
Toluene	92	0.87	110.8	535	22	2.69
o-Xylene	106	0.88	144.4	175	5	2.77
m-Xylene	106	0.86	139	135	6	3.20
p-Xylene	106	0.86	138.4	198	6.5	3.15
Ethylbenzene	106	0.87	136.2	152	7	3.15

Table 4.2: Biodegradation of BTEX under different redox conditions

Compound	Aerobic conditions	Denitrifying conditions	Sulfate-reducing conditions	Iron-reducing conditions	Methano-genic conditions
Benzene	++	-	+	-	+
Toluene	++	++	+	+	+
m-Xylene	++	++	+	+	+
p-Xylene	++	+	+		+
o-Xylene	++	+/-	-	-	+/-
Ethylbenzene	++	+/-		-	+/-
1,2,4-trimethyl-benzene	++				+/-

4.2 Environmental Effects of BTEX

BTEX are considered one of the major causes of environmental pollution because of widespread occurrences of leakage from underground petroleum storage tanks and spills at petroleum production wells, refineries, pipelines, and distribution terminals

(Fries., 1994). Some estimate that 35% of the 1.4 million gasoline storage tanks in the United States are leaking (Harwood., 1997).

Benzene is of particular concern as a pollutant because of its carcinogenicity (Dean 1985). The others are toxic to varying degrees. BTEX also moves easily into sediments and other environments where oxygen is limited. In these anoxic environments, bacteria may use nitrate, iron, sulfate or carbonate as an electron acceptor while degrading organic compounds, but these ions cannot take the place of oxygen as a reactant in aromatic ring cleavage. Because of this, bacteria have had to evolve novel pathways for anaerobic ring cleavage (Harwood & Gibson 1997).

The BTEX compounds are especially difficult to degrade because they lack an activating oxygen or nitrogen substituent group which would make oxidation of the ring more energetically feasible. Although biodegradation of these hydrocarbons has been conclusively shown to occur under several anaerobic conditions, information regarding the extent of this activity, or the conditions that encourage it is limited.

4.2.1 Toxic Effects

There are not many data for the ecotoxicity of these compounds in the terrestrial environment, since they in most cases will not stay in this environment for very long time and thus are not considered toxic. For the same reason it is extremely difficult to perform toxicity tests in soil. This was also reported by Salanitro (1997), who observed losses of 40-95 % of BTEX applied to soil samples for preparation of toxicity testing. However, a number of studies have been performed estimating the toxicity towards marine or freshwater environments, since they might be exposed for longer periods of time in cases with large bulk spills of light fuel etc. Normally, there will be no additional problems with toxic metabolites, since the aerobic and anaerobic degradation of these compounds will lead to complete mineralization.

BTEX are commonly present as ground water contaminants, due to spillages or leakages from fuel tanks and effluents from petroleum refineries and manufacturing units. They are acutely toxic and produce noticeable health effects upon exposure to concentrations higher than the EPA maximum contaminant levels. For example, benzene with a concentration higher than 0.005 mg/L may cause anemia and increase cancer risk; toluene with concentrations of 1 mg/L and above may damage the nervous system, kidney, and liver.

4.2.2 Fate in the environment

Because of the relatively high water solubility and low K_{ow} values , these compounds will tend to be dissolved in the water phase or evaporated into the air spaces of the soil. Because of their relative hydrophilic nature, they are not attenuated very much by the soil particles or constituents and can be transported rather long distances if the right conditions are there. In some sites, some BTEX are found several kilometres downstream the source.

Mn(IV) reduction, Fe(III) reduction, and sulfate reduction are the primary terminal electron-accepting processes in most marine sediments, and nitrate is only a minor electron acceptor (Canfield ,1993). Thus, the microbial metabolism of hydrocarbon contaminants under anaerobic conditions can be effective for remediation of harbor sediments only if the hydrocarbon oxidizers are dissimilatory sulfate, Fe(III), or Mn(IV) reducers. Due to the abundance of sulfate in marine environments, in many instances bioremediation of hydrocarbon contaminants would be most effective under sulfate-reducing conditions. Contamination of groundwater with the BTEX compounds is difficult to remedy because these compounds are relatively soluble in water and can diffuse rapidly once introduced into an aquifer. Techniques for in situ bioremediation of the BTEX compounds are used to eliminate or reduce contamination levels in an aquifer. However, this would not occur, if it were not for the persistency of these compounds under certain redox conditions. All of the BTEX are highly biodegradable under aerobic conditions. However, in soil and groundwater oxygen is often depleted, and especially in groundwater, due to the low water solubility of oxygen, the flux of oxygen will in many cases not be enough to support aerobic degradation. Under anaerobic conditions, the biodegradation pattern for these compounds is rather complex, While most of them, including benzene, are shown to be degraded under strict anaerobic condition and sulfate reducing conditions (Edwards ,1992; Wilson, 1986), the biodegradation under denitrifying conditions are less favourable. Benzene cannot be degraded with nitrogen as terminal electron acceptor (Schreiber and Bahr, 2002), and o-xylene often depend on the existence of primary substrates, either toluene or phenol to be degraded (Flyvbjerg , 1993). In addition to this cometabolic behaviour, also prolonged lag periods for degradation of xylenes, ethylbenzene and 1,2,4-trimethylbenzene are often observed (Hutchins . 1991).

4.3 BTEX Metabolism Metapathway

Bacterial metabolic pathways of BTEX and other aromatic compounds can be divided into two main categories: peripheral pathways and trunk pathways. Peripheral pathways transform a unique compound into a compound common to many metabolic pathways. For example, toluene is anaerobically metabolized to benzoyl-CoA by a system of peripheral pathway enzymes. In turn, benzoyl-CoA is metabolized by trunk pathway enzymes and used as a growth substrate. Many anaerobic aromatic peripheral pathways end in the production of benzoyl-CoA. These pathways are said to "funnel" into the benzoyl-CoA trunk pathway. One of the reasons for the inclusion of metapathway maps in the UM-BBD is their ability to illustrate this funnelling phenomena.

In this metapathway map, *m*-xylene is used as an example of aerobic and anaerobic degradation of the xylenes. The UM-BBD also includes the aerobic *o*-xylene pathway and *p*-xylene pathway.

1)Aerobic BTEX Metabolism

2)Anaerobic BTEX Metabolism

4.3.1 Aerobic Metabolism

All of the BTEX compounds have at least one aerobic pathway which includes degradation to a substituted catechol. Benzene is degraded to catechol. Toluene has many separate biodegradative pathways, some of which include 3-methylcatechol as an intermediate product. Many separate pathways also exist for ethylbenzene, which can be degraded to 3-ethylcatechol. The xylenes are all metabolized to mono-methylated catechols; e.g., *m*-xylene goes to 3-methylcatechol. In each of these four cases, the aromatic ring of the substituted catechol is later cleaved by a dioxygenase. The asterisk indicates the pathway is currently included in the UM-BBD. Click on it for more information on the pathway or pathways of a particular compound.

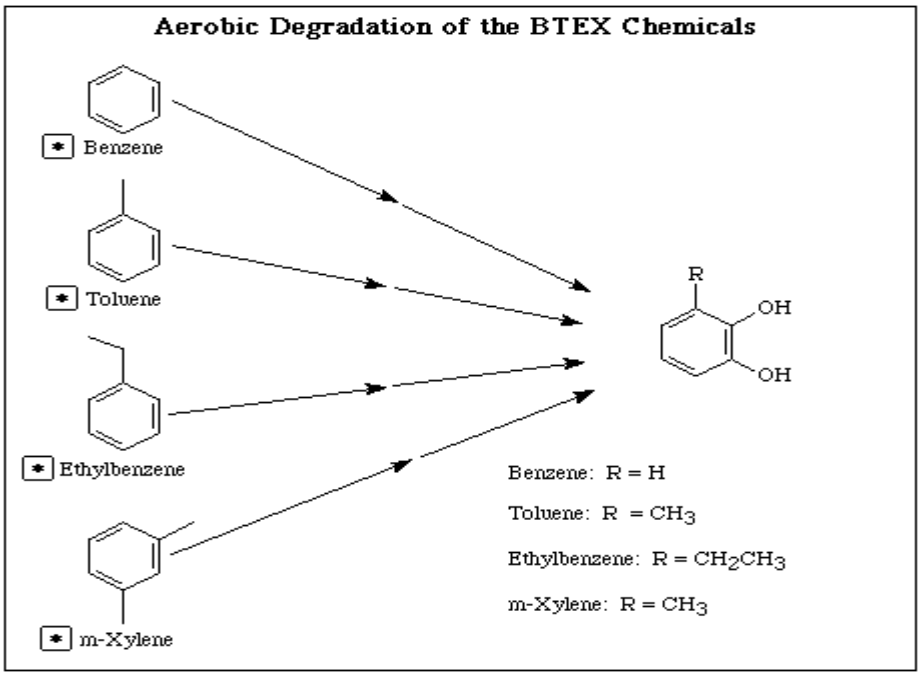

Figure 4.1: Aerobic Degradation of the BTEX Chemicals

4.3.2 Anaerobic Metabolism

Anaerobic pathways of BTEX biodegradation are important because these compounds are frequently found under conditions where the use of oxygen quickly exceeds the supply. These conditions are often found in such places as the sediments of all natural bodies of water, groundwater, and sometimes soil (Heider , 1997).

Dechloromonas spp. JJ and RCB completely mineralize benzene, through benzoyl-CoA, under anaerobic conditions (Chakraborty & Coates, 2005). Benzene, toluene and ethylbenzene have a common biodegradation intermediate: benzoyl-CoA. This compound is the most common central intermediate of anaerobic aromatic metabolism (Heider , 1997). The aromatic ring of benzoyl-CoA is reduced and eventually transformed to acetyl-CoA.

Few organisms are capable of anaerobically metabolizing xylene. They include strains of denitrifying bacteria capable of using m-xylene as a growth substrate (Harwood ,1997). Dechloromonas RCB can anerobically degrade all three xylene isomers (Chakraborty, 2005). The pathway(s) of anaerobic xylene biodegradation are not well-known.

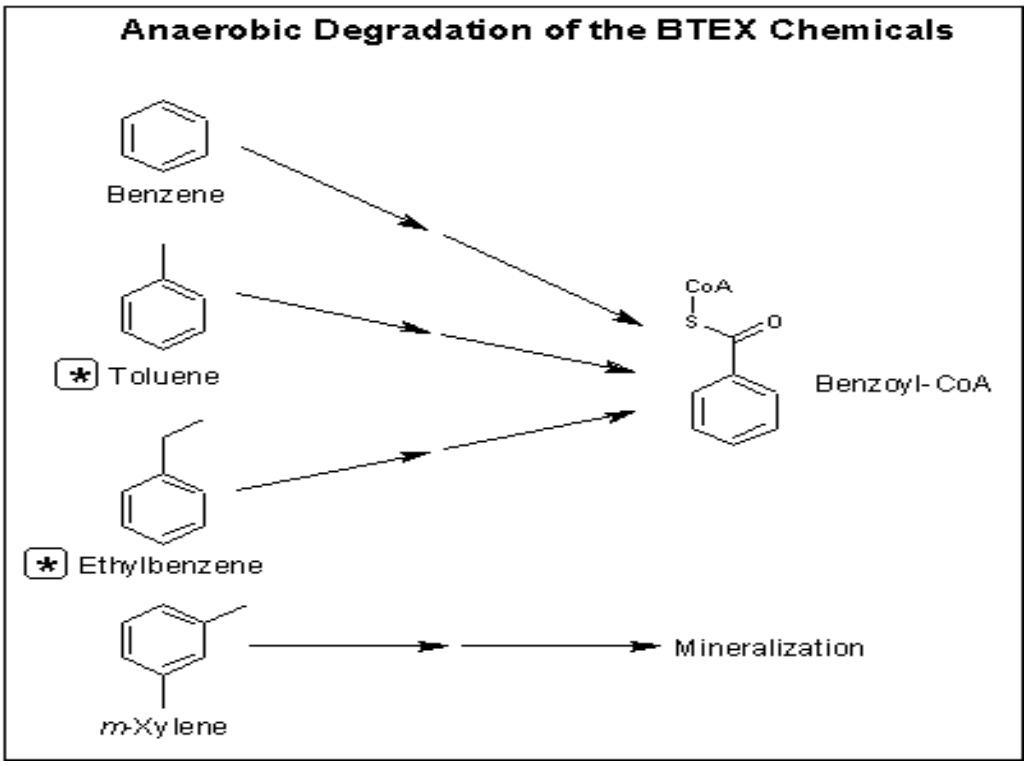

Figure 4.2: Anaerobic Degradation of the BTEX Chemicals

BTEX have in recent years attracted much attention, since they constitute one of the most common and serious threats to groundwater reservoirs and indoor climate deriving from contaminated sites. This is mainly due to the potential effects of benzene, which is considered a strong carcinogen, and which is highly mobile in the soil and groundwater environment, which is also the case for the other BTEX.

They are found in numerous sites, including areas used for fuel operations, refineries, gasoline stations, and gasification sites.

4.3.2.1 Benzene Biodegradation

Although isolates capable of the anaerobic oxidation of toluene and the metabolic pathways involved have been extensively documented in recent years, the anaerobic oxidation of benzene has been comparatively more difficult. Organisms capable of anaerobic benzene degradation have, until recently, been elusive and this metabolism has only been observed in sediment studies (Anderson and Lovley 1999; Anderson . 1998; Coates , 1996b, 1997; Lovley , 1994, 1995, 1996; Weiner and Lovley 1998b) or with microbial enrichments (Burland and Edwards 1999; Grbi'c-Gali'c and Vogel 1987; Vogel and Grbi'c-Gali'c 1986; Weiner and Lovley 1998a).

Although anaerobic benzene degradation has been demonstrated under nitrate-reducing (Burland and Edwards 1999), Fe(III)-reducing (Lovley, 1994; Lovley . 1996), sulfate-reducing (Coates, 1996b, 1997; Lovley, 1995), and methanogenic (Grbi'c-Gali'c and Vogel 1987; Weiner and Lovley 1998b) conditions, until recently, no specific organisms or genera were associated with this ability. Previous studies had demonstrated that Fe (III)-reducing sediments with which anaerobic benzene degradation was observed (Anderson and Lovley 1999; Anderson 1998) were enriched in organisms of the family Geobacteraceae (Rooney-Varga, 1999); however, there was no direct evidence to show that members of this family of organisms are capable of benzene degradation. Similarly, molecular community analysis of a sulfate-reducing enrichment that received benzene as the only carbon and energy source for a period of 3 years revealed a diverse collection of phylotypes (Phelp, 1998). 16S rRNA genes belonging to Proteobacteria, Cytophageles and Gram-positive phyla as well as one deeply branching clone not closely related to any known sequenced bacterium were detected. Four clones fell within the family Desulfobacteriaceae, one of which was closely related to a known aromatic hydrocarbon degrader Desulfobacula tolucia. The implication of a functional role of members of the Desulfobacteriaceae in anaerobic benzene degradation is further supported by the finding that a methanogenic benzene-degrading consortium was dominated (33% of the total population) by a phylogenetically similar clone within the family Desulfobacteriaceae (Ulrich and Edwards 2003). This enrichment culture was originally derived from a benzene-degrading sulfate-reducing enrichment (Ulrich and Edwards 2003).

Recently, the first two organisms capable of anaerobic benzene degradation were isolated and described (strains RCB and JJ; Coates,2001b). These organisms are closely related to each other and are members of the newly described Dechloromonas genus in the beta subclass of the Proteobacteria (Achenbach, 2001; Coates, 2001b). Both strains completely oxidized benzene in the absence of oxygen and both coupled benzene oxidation to the reduction of nitrate(Coates , 2001b) . The aromatic compounds were completely degraded to CO2 and concentrations of benzene as high as 160 μM were removed within 5 days (Coates ,2001b). Members of this genus are generally recognized for their ability to grow by dissimilatory perchlorate reduction (Achenbach and Coates 2000; Achenbach ,2001; Bruce ,1999; Coates 2003; Coates,1999) .Another common groundwater contaminant associated with the

activity of the munitions industry (Urbansky 1998). In support of this, one of the isolates, Dechloromonas strain RCB, could also couple growth and benzene oxidation to the reduction of perchlorate (Coates ,2001b). As such, this organism offers great potential for the bioremediation of environments co-contaminated with both BTEX and perchlorate in a single treatment strategy.

The Dechloromonas species, together with the closelyrelated Dechlorosoma species, are considered to represent the predominant perchlorate-reducing bacteria in the environment, and have been found ubiquitously regardless of whether or not there has been previous exposure of the environment to perchlorate (Coates and Lovley 2003; Coates, 1999). Because perchlorate-reducing bacteria are found in several pristine environments, the ubiquity of these organisms is unlikely to be related to their ability to grow by dissimilatory perchlorate reduction (Coates , 1999). Previous studies have demonstrated that these organisms are, in general, metabolically versatile and can use a broad range of alternative electrons donors (Coates and Lovley 2003). As such, the selective pressures for Dechloromonas species in the environment may be based on the diversity of their metabolic capabilities rather than any individual metabolism. In support of their potential importance in the nitrate-dependent anaerobic biodegradation of benzene, a recent molecular analysis of a benzene-degrading nitrate-reducing enrichment culture indicated that the microbial population was dominated (70% of the cloned 16S rRNA gene sequences) by an organism 93% identical to Dechloromonas strain JJ (Ulrich and Edwards 2003). Interestingly, this organism was equally related (93% identical based on 16S rDNA sequence homology) to Azoarcus species (Ulrich and Edwards 2003), which are well known for their ability to anaerobically degrade other BTEX components.

The biochemical pathway of anaerobic benzene degradation is currently unknown but several possibilities exist (Coates, 2002). These include initial carboxylation, hydroxylation, methylation, or reduction of the benzene ring with subsequent transformation to the central aromatic intermediate benzoyl-CoA and ring cleavage (Coates, 2002) (Fig. 4.3). Previous studies performed with various benzene-degrading enrichment cultures and sediment samples indicated that phenol and benzoate may be important intermediates of benzene degradation. Grbi'c-Gali'c and Vogel (1987) first detected phenol, cyclohexanone and propionate as putative intermediates in a methanogenic enrichment incubated with benzene, suggesting an

initial hydroxylation and subsequent ring reduction. Subsequent studies using isotopic trapping experiments or GC mass spectrometry analyses have similarly implicated phenol, benzoate, propionate and acetate as intermediates in benzene degradation by methanogenic Fe(III)-reducing, and sulfate-reducing enrichments (Caldwell and Suflita 2000; Phelps, 2001; Weiner and Lovley 1998b). Caldwell and Suflita (2000) demonstrated [13C-UL]-phenol and [13C-UL]-benzoate accumulation when [13C-UL]-benzene was fed to sulfatereducing enrichment culture. The mass spectrum of the benzoate produced suggested that the carboxyl carbon of the benzoate resulted from carboxylation of the benzene ring by a 13C-labeled fragment liberated from the metabolism of the starting compound. Phelps, (2001) detected deuterated benzoate in sulfate-reducing enrichmentsamended with deuterated benzene. The carboxyl group of benzoate was not labeled when 13C-bicarbonate, [1-13C]-acetate or [2-13C]-acetate were added. This is in contrast to the proposed pathway for anaerobic naphthalene and phenanthrene metabolism, where carboxylation of the aromatic ring by carbon dioxide is the initial activation step (Zhang and Young 1997).

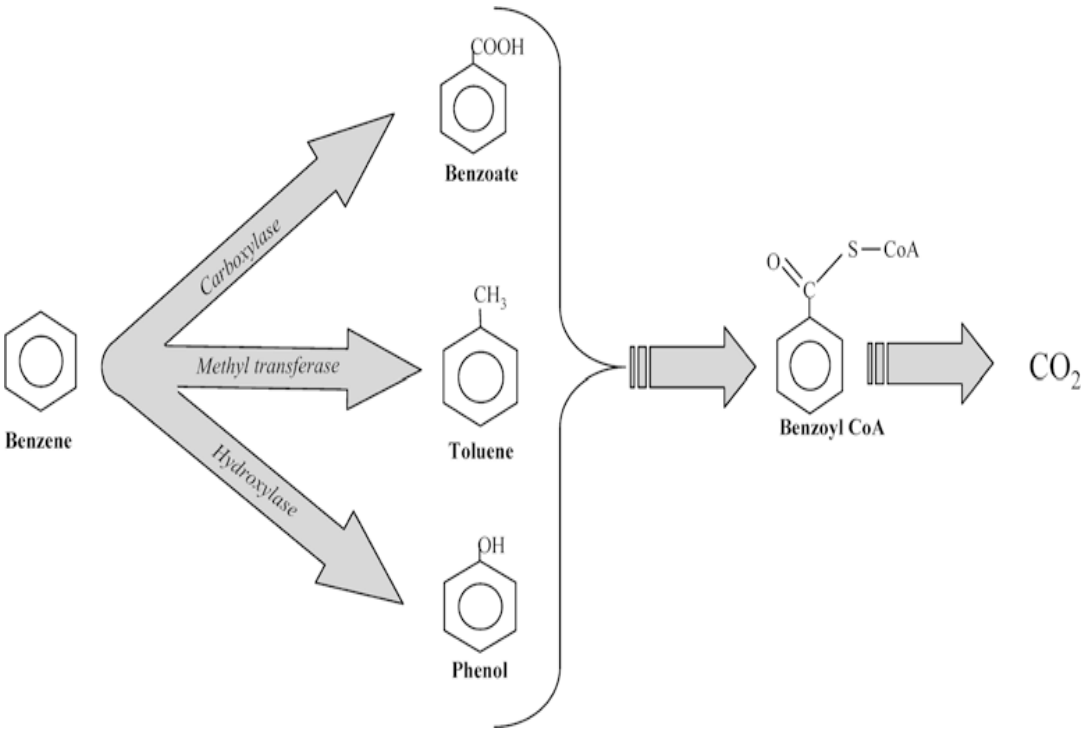

Figure 4.3: Anaerobic Degradation of Benzene

4.3.2.2 Toluene biodegradation

Among the BTEX components, the anaerobic biodegradation of toluene is probably the most comprehensively understood. Toluene is now known to be biodegradable with nitrate, Mn(IV), Fe(III), sulfate, or CO2 as terminal electron acceptors (Coates, 1996c; Dolfing,1990; Edwards , 1992; Evans, 1991a, 1991b; Fries , 1994; Grbi'c-Gali'c and Vogel 1987; Langenhoff, 1997; Lovley, 1989; Meckenstock 1999; Rabus . 1993; Vogel and Grbi'c-Gali'c 1986). More recently it has been demonstrated that anaerobic toluene degradation can also be coupled to the reduction of humic substances (Cervantes ,2001) or chlorine oxyanions such as chlorate or perchlorate (Coates, 2001b), or it can be assimilated as a carbon source by anoxygenic phototrophs (Zengler , 1999).

Geobacter metallireducens strain GS-15 was the first organism in pure culture demonstrated to be capable of the anaerobic oxidation of toluene (Lovley , 1989). G. metallireducens completely oxidized toluene to CO2 with the reduction of Fe(III) but was incapable of oxidizing other BTEX components (Lonergan and Lovley 1991; Lovley and Lonergan 1990; Lovley, 1989). Other Geobacter species, such as G. grbicium, that can alsooxidize toluene with Fe(III) have since been isolated and described (Coates and Lovley 2003; Coates ,1996c, 2001a). Geobacter species are considered the most prevalent of the known Fe(III)-reducing species in anoxic mesophilic environments and are often found to be dominant in the Fe(III)-reducing zone of environments contaminated with hydrocarbons (Rooney-Varga , 1999). In addition to Fe(III), both G. metallireducens and G. grbicium utilized nitrate, Mn(IV), or humic substances as alternative electron acceptors (Coates and Lovley 2003); however, it has not been demonstrated that toluene oxidation occurred under these conditions although there is no reason to suspect otherwise.

In addition to Fe(III) reduction, several organisms are now known to couple anaerobic toluene degradation to nitrate respiration [Thauera aromatica K172 (Anders, 1995), Thauera aromatica T1 (Evans , 1991a), Azoarcus sp. strain T (Dolfing, 1990), Azoarcus tolulyticus Tol4 (Zhou , 1995), Azoarcus tolulyticus Td15 (Fries , 1994), Strain ToN1 (Rabus and Widdel 1995), Dechloromonas strain RCB and Dechloromonas strain JJ (Coates,2001b)], perchlorate respiration [Dechloromonas strain RCB (Coates , 2001b)], and sulfate respiration [Desulfobacula toluolica (Rabus , 1993) and Desulfobacterium cetonicum (Harms

1999b)]. All of the toluene-oxidizing nitrate-reducers are facultative anaerobes and are members of the beta subclass of the Proteobacteria. Several of the Azoarcus and Thauera species were originally described as Pseudomonas species (Dolfing, 1990; Schocher, 1991) because of common physiological and morphological traits, but were subsequently reclassified into their current taxonomic positions (Anders, 1995; Zhou, 1995). These organisms are commonly isolated from anaerobic sludge or creek sediments with nitrate as the electron acceptor and various electron donors (Anders ,1995). Phenotypic characterization revealed that toluene oxidation may also be coupled to the reduction of nitrous oxide by some of these isolates, and cell yields for Azoarcus strain T with nitrous oxide were significantly higher than with nitrate as the sole electron acceptor (Dolfing, 1990). This was because of the severe growth inhibitory effect of the nitrite transiently produced during nitrate reduction by this organism (Dolfing , 1990). Use of nitrous oxide as an electron acceptor circumvented this effect (Dolfing, 1990). Further studies revealed that nitrous oxide could alternatively be used as an effective electron acceptor for the enrichment and isolation of organisms capable of anaerobic toluene degradation (Schocher, 1991). The relevance of this electron acceptor to the natural attenuation of hydrocarbons has yet to be determined and whether or not anaerobic oxidation of other hydrocarbons can similarly be coupled to nitrous oxide reduction is currently unknown.

The biochemistry and genetics of toluene degradation by the Azoarcus and Thauera species has been intensely investigated over the last decade (Beller and Spormann 1997a, 1999; Biegert, 1996; Coschigano 1999; Coschigano and Young 1997; Heider 1998; Krieger 1999) and is comprehensively reviewed elsewhere (Spormann and Widdel 2000; Widdel and Rabus 2001). Briefly, these studies revealed that the first step in the catabolism of toluene is the addition of fumarate onto the toluene methyl group to form benzylsuccinate (Fig. 4.4.). This reaction is mediated by a novel glycyl radical enzyme, benzylsuccinate synthase (BSS) (Leuthner, 1998). Free benzylsuccinate is often found in culture broths of T. aromatica as a transient intermediate when grown on toluene (Biegert , 1996), and is now believed to be an inducer of the genetic pathway for toluene catabolism (P. W. Coschigano, personal communication). Although the BSS-based toluene pathway was first identified in Thauera and Azoarcus species growing under nitrate-reducing conditions, this is now recognized as the common mechanism for the activation of toluene by phylogenetically diverse organisms growing under a rangeof alternative anaerobic

conditions. These include dissimilatory Fe(III)-reduction by Geobacter metallireducens (Kane ,2002) and dissimilatory sulfate reduction by Desulfobacula toluolica (Beller and Spormann 1997b), both of which are members of the delta subclass of the Proteobacteria. Similarly this mechanism is also utilized during the anoxic phototrophic assimilation of toluene by Blastochloris sulfoviridis of the alpha subclass of the Proteobacteria (Zengler , 1999).

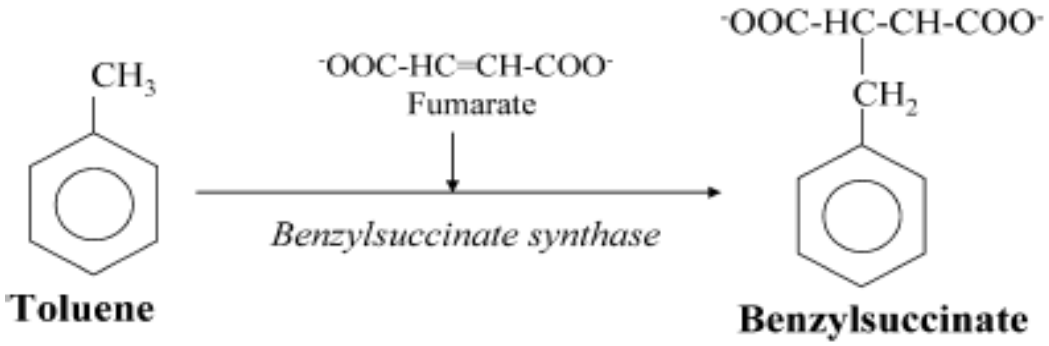

Figure 4.4: Anaerobic Degradation of Toluene

4.3.2.3 Ethylbenzene Biodegradation

In contrast to toluene and benzene, relatively little is known about the anaerobic biodegradation of ethylbenzene and, until recently, only three organisms had been

described that were capable of this metabolism. These organisms, strains EbN1, PbN1, (Rabus and Widdel 1995) and EB1 (Ball ,1996) are facultative anaerobes and couple ethylbenzene oxidation to the reduction of nitrate. The three isolates are closely related to each other and to the previously described Thauera species in the beta subclass of the Proteobacteria. Strains EbN1 and PbN1 were isolated with ethylbenzene and propylbenzene, respectively, from enrichments prepared with homogenized mixtures of river and ditch mud samples (Rabus and Widdel 1995) while strain EB1 was isolated from ethylbenzene-degrading enrichments prepared with sediment from an oil refinery treatment pond (Ball, 1996).

In general, ethylbenzene was completely mineralized to CO2 by these isolates with the reduction of nitrate, the transitory production of nitrite, and ultimate formation of N2 (Ball , 1996; Rabus and Widdel 1995). Phenotypic characterization revealed that these strains did not oxidize hydrocarbons aerobically, and were limited in their

ability to oxidize other aromatic hydrocarbons anaerobically. In addition to ethylbenzene, strain EbN1 could only utilize toluene while strain PbN1 could alternatively utilize propylbenzene (Rabus and Widdel 1995). In contrast, strain EB1 did not utilize any hydrocarbon other than ethylbenzene (Ball , 1996). Ethylbenzene is initially activated for degradation by these organisms through a

dehydrogenation reaction of the methylene group of the ethyl side chain to form 1-phenylethanol, which is further oxidized to form the aromatic ketone acetophenone (Ball, 1996; Rabus and Heider 1998) (Fig. 4.5.). Stable isotope labeling studies indicated that the hydroxyl group of the 1-phenylethanol formed in the initial dehydrogenation reaction is derived from water (Ball ,1996) and the reaction is mediated by ethylbenzene dehydrogenase, a novel member of the dimethyl sulfoxide reductase family of molybdopterin-containing enzymes (Johnson , 2001). In strain EbN1, complete ethylbenzene mineralization involves a complex genetic system with a twocomponent regulatory mechanism (Rabus , 2002). More recent studies on anaerobic ethylbenzene degradation under sulfate-reducing conditions resulted in the enrichment and isolation of novel organism, strain EbS7, from enrichments prepared with sediments collected from Guaymas Basin in the Gulf of California (Kniemeyer, 2003). Strain EbS7 is an obligate anaerobic marine sulfatereducing bacterium that couples the oxidation of ethylbenzene to the reduction of sulfate to sulfide (Kniemeyer, 2003). It is a member of the delta subclass of the Proteobacteria and is most closely related to strain NaphS2 and mXyS1, which can anaerobically oxidize naphthalene and m-xylene, respectively (Galushko, 1999; Harms, 1999b; Kniemeyer, 2003). In contrast to the initial dehydrogenation reaction used by denitrifying ethylbenzene degraders, activation of ethylbenzene for catabolism by strain EbS7 is achieved by a fumarate addition reaction at the secondary carbon atom of the ethyl group to form 1-phenylethyl-succinate (Kniemeyer 2003) (Fig. 4.6.). This alternative catabolic pathway for aromatic ethylbenzene degradation, which is similar to the activation step for toluene outlined above, is explained by the difference in the redox potential of the respective electron acceptors nitrate and sulfate (Kniemeyer, 2003). A similar type of activation reaction at the secondary carbon group has previously been observed in the anaerobic catabolism of n-alkanes under nitratereducing conditions (Rabus ,2001).

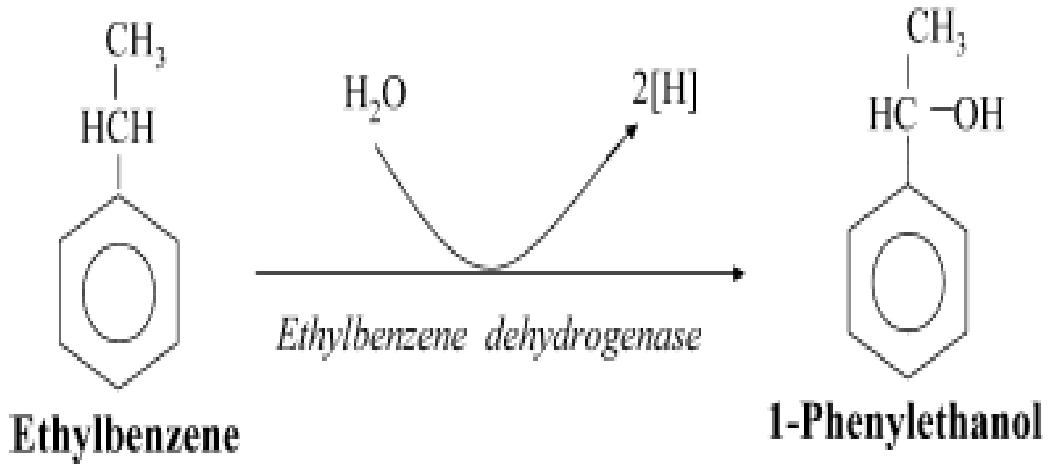

Figure 4.5: Activation of ethylbenzene with nitrate as the electron acceptor is effected through a dehydrogenation reaction at the methylene group of the ethyl side chain to form 1-phenylethanol

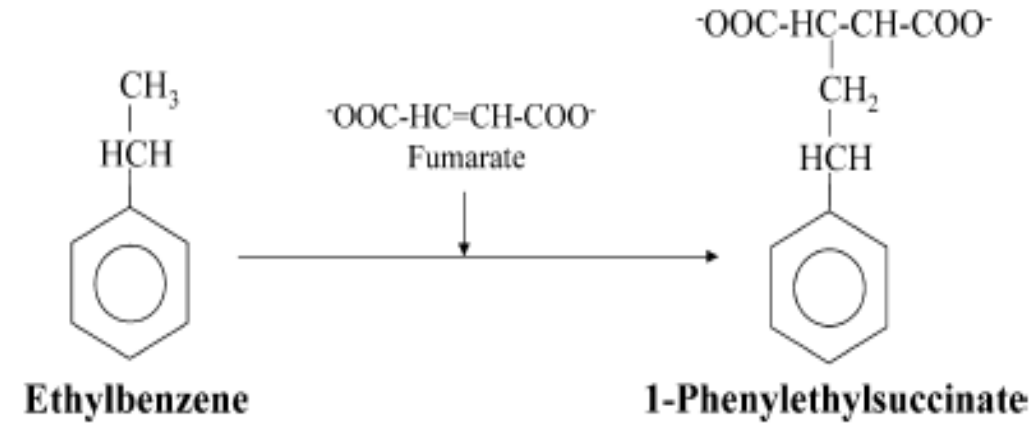

Figure 4.6: Ethylbenzene is activated with sulfate as the electron acceptor by a Fumarate addition reaction at the secondary carbon atom of the ethyl group to form 1-phenylethylsuccinate

4.3.2.4 Xylene Biodegradation

Anaerobic biodegradation of the three structural isomers of dimethylbenzene (meta, ortho, and para-xylene) has been predominantly studied under nitrate- and sulfatereducing conditions. Although studies based on sediments or enrichment cultures demonstrated biodegradation of para-xylene in the absence of oxygen (Haner, 1995; Kuhn ,1988), to date, no pure culture exists that can mineralize p-xylene completely to CO2. In contrast, several organisms have now been isolated that can completely mineralize meta- and ortho-xylene coupled to the reduction of nitrate (Hess , 1997; Rabus and Widdel 1995). Many of these organisms are closely related to each other and to the previously identified toluenedegrading denitrifiers

belonging to the beta-subclass of the Proteobacteria. In addition, several of the known toluenedegrading Azoarcus or Thauera species are alternatively capable of anaerobic xylene degradation, while several of the xylene-degrading isolates could also utilize toluene (Dolfing ,1990; Fries , 1994). Xylene-degrading denitrifyers have now been isolated from a broad range of environments, including aquifer material contaminated with diesel fuel (Hess , 1997), freshwater mud samples (Rabus and Widdel 1995), laboratory columns treating toluene (Dolfing, 1990), and compost (Fries , 1994).

The initial reactions involved in anaerobic m-xylene oxidation are thought to be similar to those of toluene oxidation under nitrate-reducing conditions and involve an initial addition of fumarate onto one of the methyl groups to form 3-methylbenzylsuccinate, which is subsequently oxidized to 3-methylbenzoate (Krieger 1999) (Fig.4.7.). The initial addition reaction is mediated by 3-methylbenzylsuccinate synthase, which, like BSS, retains the abstracted hydrogen atom from the methyl carbon during the reaction (Krieger 1999).

More recently, the marine dimethylbenzene-degrading, sulfate-reducing organisms strains oXyS1 and mXyS1, were isolated with ortho-xylene and meta-xylene as the sole carbon and energy sources, respectively (Harms, 1999b). Both strains are members of the Desulfobacteriaceae family in the delta subclass of the Proteobacteria, which is composed of a metabolically versatile group of organisms (Widdel and Bak 1992). While strain oXyS1 was closely related (greater than 98% identity based on 16S rDNA sequence analysis) to the Desulfobacterium cetonicum and Desulfosarcina variabilis species in this family, strain mXyS1 was only 86.9% similar to its closest relative, Desulfococcus multivorans, and represented the first example of a previously unrecognized line of descent within the delta subclass of the Proteobacteria (Harms, 1999b). The isolates were obtained from enrichment cultures growing anaerobically on crude oil and sulfate in seawater-based medium (Harms , 1999b). In addition to degrading dimethylbenzene, these isolates were able to grow on a range of alternative aromatic compounds, including toluene and higher homologs of dimethylbenzene such as ethyltoluene and isopropyltoluene (Harms, 1999b). In addition to pure substrates, the isolates were also able to grow with crude oil as the sole carbon and energy source (Harms , 1999b).

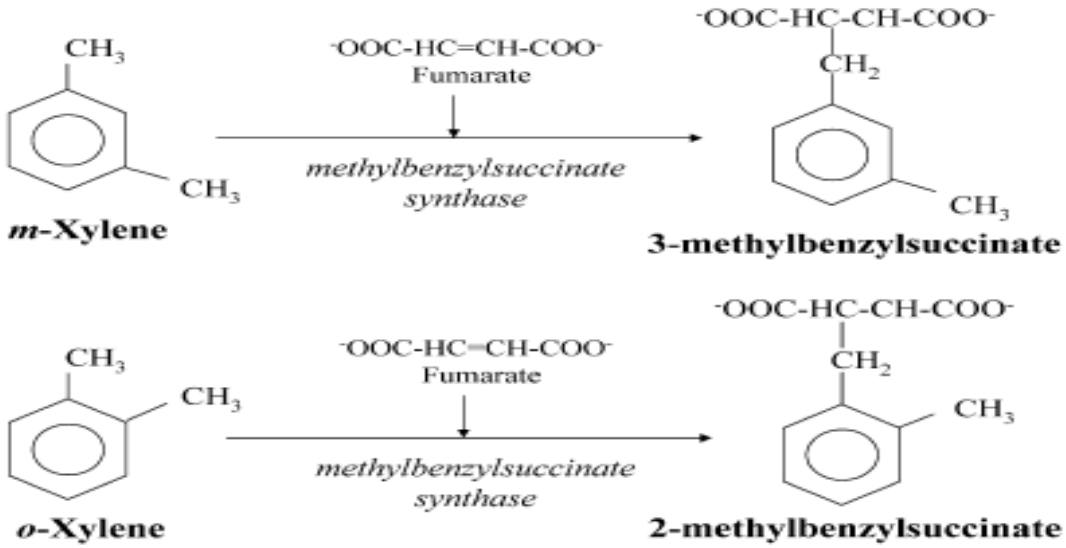

Figure 4.7: Anaerobic Degradation of Xylene

4.3.3 Biomarkers of Anaerobic BTEX Degradation

The practical application of various metabolic biomarkers to distinguish abiotic losses of hydrocarbons in contaminated sites from biologically mediated degradation processes has recently been proposed (Beller 2000; Elshahed , 2001; Mancini , 2003; Pelz , 2001). Specific intermediates of metabolic pathways can serve as biomarkers in the environment, where their detection is indicative of the presence of an active microbial population utilizing the metabolic substrate concerned. A compound can be used as a biomarker only if certain criteria are met. The compound must be readily detectable in soil, water, and sediment samples; it must be highly specific to a particular pathway (i.e., not a product of commonly utilized substrates by a host of diverse organisms), and it must not be found in pristine environments (Beller 2000). Ideally, the presence of the biomarker compound in a particular environment should signify its formation from a known substrate via a specific pathway by a particular group of microorganisms. If these criteria are met then monitoring the disappearance of the substrate concomitant with the appearance of the biomarker over time is a reliable indication of in situ biodegradation.

Benzylsuccinate and e-phenylitaconate have been commonly used as biomarkers for anaerobic toluene oxidation (Beller 2000). Similarly, methybenzylsuccinate, the first intermediate of the degradative pathway of dimethylbenzene (Krieger 1999), has also been identified as a suitable biomarker for xylene degradation. Benzylsuccinate, or

its methylated analogs are not anthropogenic compounds, and are highly specific to the anaerobic catabolic pathways of toluene, xylene and ethylbenzene, especially under nitrate-reducing conditions. Recent advances in the detection of such signature metabolites by LCMS techniques require minimal sample preparation and have detection limits as low as 0.3 mg l−1 (Beller 2002). As such, these compounds represent excellent candidates for use as biomarkers of aromatic hydrocarbon degradation in the environment. Detailed studies performed with Seal Beach groundwater in California indicated that the concentration of such biomarkers decreased over time simultaneously with a decrease in the concentration of the monoaromatic compounds (Beller, 1995). Because methylbenzylsuccinate was relatively more recalcitrant in the environment than benzylsuccinate, it was selected as a more effective biomarker for in-situ monitoring of intrinsic BTEX biodegradation (Beller, 1995). Transiently produced benzylsuccinate and methylbenzylsuccinate were similarly detected in sulfate-reducing and methanogenic sediments that actively degraded toluene and dimethylbenzene (Elshahed ,2001).

Furthermore, 3-phenyl-1,2-butanedicarboxylic acid was detected and identified as a possible metabolite of ethylbenzene oxidation under sulfate-reducing conditions in the same sediments (Elshahed, 2001). An alternative strategy for monitoring in situ biodegradation of a compound is to follow the changes in stable isotope composition of the molecule of interest. Variations in the stable isotope ratios of many elements have long been used to give valuable information about biogeochemical processes occurring in the environment (Bailey,1973; Coleman, 2003; Ku, 1999; Nissenbaum, 1972). Many atoms can exist in two or more forms, chemically identical but differing in mass.

The relative abundances of the stable (non-radioactive) isotopes are effectively constant for each element; however, microbial processes are known to make small but significant changes to isotopic compositions by preferentially utilizing the lighter isotopes (Ahad, 2000; Cloud, 1958; Coleman, 2003; Hall, 1999; Harrison and Thode 1957; Krichevsky, 1961; Meckenstock 1999). Isotopic fractionation studies have been successfully implemented to monitor in situ bioremediation of BTEX compounds (Mancini, 2003; Pelz , 2001).

When equal amounts of toluene-d8 (deuterated) and nonlabeled toluene were added as a mixture to sulfatereducing toluene-oxidizing organisms, hydrogen (D/H)

isotopic fractionation was nearly three times greater than the 13C/12C isotopic fractionation, thus suggesting that hydrogen isotopic fractionation of aromatic substrates could be used as an effective indicator of bacterial hydrocarbon degradation processes in situ (Morasch, 2001). The majority of the hydrogen isotopic fractionation occurred during the formation of benzylsuccinate by BSS and, in contrast to previous observations made with aerobic cultures, the extent of hydrogen isotopic fractionation in the anaerobic culture was unaffected by temperature (Morasch, 2001). This technique has also been recently extended to demonstrate anaerobic hydrocarbon degradation in BTEX-contaminated groundwater aquifers (Reusser, 2002). In these experiments, when deuterated toluene and deuterated ortho-xylene were added to the contaminated site along with nitrate as the electron acceptor, deuterated benzylsuccinic acid and deuterated o-methyl-benzylsuccinic acid were formed in situ as transformation products of anaerobic metabolism (Reusser, 2002). Similarly, isotope labeling studies were used to identify the specific microbial subpopulations involved in the anaerobic degradation of toluene in hydrocarbon-contaminated aquifer sediments by tracing the incorporation of [13C]-labeled toluene into the polar lipid derived fatty acids of a sulfate-reducing community (Pelz, 2001).In Table 4.3. the carcinogenity and the ecotoxicity towards different marine organisms were shown.

Table 4.3: Geno- and ecotoxicity (LC_{50} mg l^{-1})[1)] of BTEX

Compound	Carcinogen	LC_{50} Cancer magister	LC_{50} Paleo-monetes pugio	LC_{50} Marone saxitilis
Benzene	+	108	27	5.8
Toluene	-	28	9.5	7.3
o-Xylene	-	12	3.7	9.2
m-Xylene	-	6	1.3	11
p-Xylene	-	2	0.86	2
Ethylbenzene	-	13	0.49	4.3
1,2,4-Trimethylbenzene	-	5	5.4	

[1)] After Neff (1977)

4.3.4 Terminal electron Accepting Processes and The Development of Anaerobic Conditions

In pure cultures of chemotrophic organisms, reducing equivalents generated during transformation of hydrocarbons to metabolic intermediates, have to be transferred to an external electron acceptor with a more positive redox potential in order to conserve energy for growth. Growth without an external electron acceptor is not possible (Heider, 1998). In the absence of oxygen, energy conservation can be accomplished by anaerobic respiration with other inorganic chemicals as electron acceptor. These reduction processes are generally referred to as terminal electron accepting processes (TEAPs). Potential natural electron acceptors for anaerobic oxidation of organic matter to carbon dioxide in subsurface environments are nitrate, Mn(IV), Fe(III) and sulfate. Carbon dioxide can also function as an electronacceptor, yielding methane during methanogenesis. Along with these electron acceptors, BTEX components can be degraded anaerobically.

During contamination with organic compounds, such as petroleum or landfill leachate, in both time and space a sequence of redox zones develops downstream of the point of contamination (Christensen, 2000, Lovley, 1997). This sequential development relates to the energetics of TEAPs. Denitrification yields the highest amount of energy, while methanogenesis yields the lowest amount. The preference for electron acceptors decreases with nitrate > Mn(IV) > Fe(III) > sulfate > methanogenesis (Christensen, 2000, Lovley, 1997).Thus, most favorable electron acceptors will be depleted first.

TEAPs can be determined by measuring the changes in concentration of electron-acceptors and products of reduction along the groundwater flow path. However, the deduction of TEAPs can be hampered by precipitation reactions, such as Fe (II) with sulfide or carbonate, and does not always give information on the present on-going TEAPs, by transporting reduced and oxidized species in the groundwater from another place (Christensen, 2000) Over the recent years a more advanced technique based on hydrogen measurements in groundwater has been developed. Hydrogen is an intermediate in the degradation of organic compounds. Its turnover time is only a few seconds and its concentration indicates which TEAP is dominantly occurring(Christense, 2000). Hydrogen concentration decreases with methanogenesis > sulfate > Fe(III) > Mn(IV) > denitrification. By including the concentration of

redox species and performing simple thermodynamic calculations even a more precise delineation of TEAPs can be achieved. Thermodynamic calculations have shown that several redox processes can occur simultaneously (Jakobsen,1998). Analysis of microbial communities and measurement of redox activities in laboratory cosms have also shown that at one position in a contaminated aquifer microorganisms performing different TEAPs are present and active (Ludvigsen, 1998, Bekins, 1999). Such information provides more possibilities for specifically engineered bioremediation, such as addition of electron acceptors.

4.3.5 Microbial Comunities Involved In Anaerobic BTEX Degradation

Knowledge on TEAPs occurring at a contaminated site provides also a clue for which group of microorganisms to look for. Pollution leads to strong changes in microbial communities and research so far indicates that under each different redox condition other groups of microorganisms are involved in anaerobic BTEX degradation .

In present studies, molecular techniques play an important role in determining which microorganisms are involved in BTEX degradation (Rooney-Varga, 1999)and will also play a major role in future bioremediation practices (Brockman, 1995). The relatively fast cultivation-independent molecular methods[9], based on analysis of the generally occurring genes encoding 16S ribosomal DNA or genes involved anaerobic BTEX degradation, can theoretically address the whole microbial community present. Due to the limits of cultivation only a small fraction, 0.01 to 10%, will be addressed by using cultivation based studies (Brockman, 1995).

4.3.5.1 BTEX Degradation Coupled to Nitrate Reduction

Nitrate is not typically available in high concentrations in BTEX-contaminated aquifers. However, in some areas high concentrations of nitrate leach from agricultural fields receiving large amounts of fertilizers. All BTEX compounds can be degraded during denitrification (Table 4.4.). So far only beta-proteobacteria belonging to the Thauera/Azoarcus cluster have been found to play an important role in degradation under denitrifying conditions. This has been shown by culture-independent molecular analysis of BTEX degrading enrichments (Rabus 1999) and through-flow column experiments (Hess 1997) as well as by isolation of BTEX degrading bacteria. Up to now, at least 30 pure cultures of denitrifying microorganisms capable of oxidizing toluene, ethylbenzene, and m-, o- and p-xylene

have been obtained (Anders ,1995, Ball 1996, Dolfing 1990, Harms 1999) (Table 4.5.). All belong to the Thauera/Azoarcus cluster. For a long time it was not clear whether benzene could be degraded with nitrate as electron acceptor(Lovley 1997)Recently, evidence has been obtained that oxidation of benzene to carbon dioxide can also be linked to nitrate reduction(Burland 1999). In a benzene degrading enrichment culture, nitrite accumulates stoichiometrically as nitrate is reduced. When nitrate is depleted, only nitrite remains and the rate of benzene degradation decreases to almost zero. Benzene is mineralized to CO2 only in active cultures but not in sterile or uninoculated controls. Other electron acceptors (sulfate, iron, and CO2) are not involved in the degradation. No molecular studies have yet been performed to establish whether members of the Azoarcus/Thauera cluster are also involved in benzene degradation in this enrichment.

Table 4.4: Degradation of BTEX Compound During Denitrification.

Combinations of BTEX and TEAPs for Which Pure Cultures of Isolates Have Been Obtained

	Nitrate	Manganese	Iron	Sulphate
Benzene	-	-	-	-
Toluene	+	-	+	+
Xylene	+	-	-	+
Ethylbenzene	+	-	-	-

As nitrate dissolves well in water, it can easily be applied to contaminated groundwater to increase the concentration of electron acceptors for anaerobic degradation. Experiments performed by Hutchins .(Hutchins 1998, Bantle ,1998) showed that such an approach can be successful. In a fuelcontaminated aquifer, BTEX and JP-4 jet fuel were significantly removed after adding nitrate. A specific probe for Azoarcus has been described (Rabus 1999) (Table 4.5.).

Table 4.5: Pure Cultures of Denitrifiying Microorganisms.

Specific Probes for the Detection of Microorganisms Capable of BTEX Degradation

	TEAP	Sequence	Reference
Azoarcus/Thauera	nitrate	GAATCTCACCGTGGTAAGCGC (AT1458)	[10]
Geobacteraceae	Fe(III)	TACCCGCRACACCTAGT (Geo825R)	[28]
Desulfobacteriaceae	sulfate	CGGCGTTGCTGCGTCAGG (SRB385Db)[a]	[47]

[a] Reacts also with other delta-proteobacteria, like *Pelobacter* and *Desulfuromonas*.

4.3.5.2 BTEX Oxidation Coupled to Manganese Reduction

Manganese is a widespread transition metal, which occurs at about 5-10 times lowerconcentrations than iron. Mn(IV) reduction is energetically more favorable than Fe(III) reduction but the rate of degradation with this electron acceptor is low due to its limited availability and solubility. Little knowledge is available on BTEX degradation under Mn(IV) reducing conditions, and only toluene has been found to be degraded in column experiments (Langenhoff 1997) . No pure culture is available (Table 4.4.). However, as most Fe(III) reducting microorganisms can also reduce Mn(IV), results obtained under Fe(III) reduction likely also apply to Mn(IV) reducing conditions (Lovley 1997) The type of manganese oxide strongly affects the degradation rate of toluene. In a flow-through laboratory column filled with contaminated sediment and sludge, toluene was degraded in the presence of crystalline manganese oxide, amorphous manganese oxide and freeze-dried amorphous manganese oxide under anaerobic conditions. The degradation rate with crystalline manganese oxides was the lowest, with amorphous manganese oxides the highest(Langenhoff 1997). The larger specific surface area of amorphous manganese oxides provides better physical contact, which is required to transport the reducing power across the cell envelope and manganese oxide particleinterface (Langenhoff 1997, Lovley 1991, Nealson 1994). Increasing the solubility of manganese oxide by adding organic ligands such as nitrotriacetic acid (NTA) or EDTA further enhanced the toluene degradation rate Langenhoff 1997). The toluene degrading enrichment culture was identified by phylogenetic analysis of cloned rDNA sequences. At least two major groups of Gram-negative bacteria were present. One group showed a weak similarity with Bacteroides and Cytophaga, and

the other group consisted of members of the beta-proteobacteria, weakly related (91%) to Azoarcus (Langenhoff 1997).

4.3.5.3 BTEX Degradation Coupled to Fe (III) Reduction

Insoluble iron oxides typically provide the greatest electron-accepting capacity in shallow aquifers, because magnetite (Fe3O4) is ubiquitous in natural environments The zone of Fe (III) reduction can be quite extensive in contaminated aquifers Lovley 1997) Geobacters, belonging to the delta-proteobacteria appear to be the major iron reducers in iron reducing soils and sediments (Snoeyenbos-West 2000, Coates,1998). Cultivation-dependent and independent studies have showed that they are enriched in petroleum polluted aquifers (Rooney-Varga,1999, Snoeyenbos-West,1998, Anderson ,1998). Only members of the Geobacters are known to degrade BTEX compounds in pure culture (Coates, 1996 Lovley, 1989). The first evidence that BTEX can be degraded under anaerobic conditions was obtained by isolating the toluene degrading, Fe (III) reducer Geobacter metallireducens from a petroleum fuel contaminated aquifer (Lovley, 1989).

In this aquifer, Fe (II) accumulated in groundwater over time and sediment in contaminated portions of the contaminated aquifer contained much less Fe (III) than nearby uncontaminated parts, indicating the in situ degradation of toluene with associated Fe (III) reduction (Lovley, 1989). The most recalcitrant BTEX compound, benzene, can also be degraded under Fe (III) reducing conditions, although benzene degradation appears to be found in only a few aquifers as shown in experiments where sediments from anaerobic petroleum contaminated aquifers were anaerobically incubated without amendments (Anderson, 1998). Only in one case, added radiolabeled benzene was anaerobically oxidized to carbon dioxide without lag period, indicating the in situ degradation of benzene. Obvious differences in microbial composition were observed with nearby Fe (III) reducing locations where benzene was present but not degraded, with an uncontaminated part of the aquifer and a part of the aquifer which had turned methanogenic (Rooney-Varga ,1999)Both culturing and culture-independent methods (16S rDNA based denaturing gradient gel electrophoresis, most probable number PCR, 16S rDNA sequencing, phospholipid fatty acid analysis) demonstrated that in situ anaerobic benzene degradation was associated with much a larger number of Geobacteraceae (Rooney-Varga ,1999, Anderson, 1998).At Fe (III) reducing sites where no benzene degradation occurred,

Fe (III) reducing Geothrix fermentens dominated. Also benzene-oxidizing Fe (III) reducing enrichments were established (Anderson, 1998). After addition of radio-labeled benzene or toluene, monitoring the yield of 14C indicated both aromatic hydrocarbons were mineralized into CO2 without lag period.

Also in the enrichments Geobacters dominated, as revealed by culturing-independent methods. Bacteria closely related to Geothrix fermentens and Variovorax paradoxus were also encountered, but they are not known to be capable of anaerobic BTEX degradation(Rooney-Varga ,1999). These studies, indicate that organisms closely related to known Geobacter species are associated with anaerobic benzene degradation reduction(Rooney-Varga ,1999). Attempts to isolate the benzene oxidizer have not yet been successful. Sofar only two toluene degrading isolates have been obtained(Rooney-Varga ,1999,Coates,1996). Like manganese oxides, the quantity and form of iron oxides are major determinants in the rate and extent of organic contaminant degradation in Fe (III) reducing zones of polluted aquifers. Poorly crystalline Fe (III) oxides are the most readibly available for microbial reduction. The addition of synthetic Fe (III) chelators, such as NTA, can accelerate benzene degradation(Lovley, 1996). Chelators solubilize the highly insoluble Fe (III) oxides and this Fe (III) is much more accessible to Fe (III) reducers than natural Fe (III). Another limiting factor in degradation is the requirement for Fe (III) reducing microorganisms to establish direct physical contact with insoluble Fe (III) oxides in order to reduce them (Nevin, 2000). G. Metallireducens is not capable of reducing poorly crystalline Fe (III) oxide through the production of soluble reductants, electron-shuttling compounds or by solublilizing Fe (III) prior to reduction (Nevin,2000).Fe (III) reduction can be stimulated by the addition of humic acids which act as intermediates in iron reduction(Lovley, 1996).Electrons are donated to quinones in humic acids, then the humic acids non-enzymatically reduce Fe (III) or other metals like Mn (IV), regenerating an oxidized form of humic acid. Fe (III) reducers obtain energy to support cell growth from the process of electron transport to humics(Lovley,1996;2000). Even low concentration of humics can serve as electron-acceptor for significant amounts of organic-matter oxidation. Contact between humic acids and Fe (III) is much easier than between microorganisms and Fe (III) oxides, also humic acids can react with more types of Fe (III) oxides and reach Fe (III) oxides which can not be reached by microorganisms (Lovley,2000). As Fe (III) oxides are insoluble, they can not be added to aquifers. However, nitrate

addition might be capable of regenerating Fe (III). Anaerobic reoxidation of Fe (II) with associated reduction of nitrate has been observed in a variety of environments(Caldwell,1999). Addition of chelators or humic acids can help to enhance degradation under iron-reducing conditions. A recently developed 16S rDNA-based Geobacter primer set (Table 4.4.) allows the specific, culturing-independent monitoring of Geobacter populations in iron-reducing environments (Rooney-Varga ,1999, Anderson, 1998).

4.3.5.4 BTEX Degradation Linked to Sulfate Reduction

Some anaerobic environments are rich in sulfate, because rainwater provides a recharge of sulfate from fertilized soils and leaches sulfate from landfills and industrial waste. Furthermore, marine environments are by nature rich in sulfate.

Pure cultures of sulfate-reducing bacteria able to degrade toluene, o-xylene and mxylene have been isolated from anaerobic sulfate-reducing environments(Rabus,1998, Aeckersberg,1991, Wilkes,2000) (Table 4.4.). All isolates characterized in detail belong to the Desulfobacteriaceae cluster of delta-Proteobacteria (Desulfobacula toluolica, Desulfobacterium cetonicum, a close relative of Desulfobacterium cetonicum and Desulfosarcina variables and a close relative of Desulfococcus multivorans).

All BTEX compounds can be degraded with sulfate as electron acceptor . In two thermophilic consortia obtained from two different sulfate reducing environments, radio-labeled toluene, benzene, ethylbenzene and xylene were degraded to 14CO2, tightly coupled to H2S generation by sulfate reduction (Chen,1997). Degradation of benzene, the most recalcitrant BTEX compound, was observed with a lag period of 30-60 days in an enrichment established with aquifer sediment (Edwards,1992) and in sediments of a petroleumcontaminated aquifer under sulfate-reducing conditions (Chapelle,1996). The benzene was probably slowly degraded due to preferential utilization of easier consumable toluene and xylene and the requirement for sufficient bacterial biomass capable of benzene degradation.

Another enrichment of a benzene-degrading, sulfate-reducing consortium, obtained from Guaymas basin sediment and with benzene as the sole source of carbon and energy has been molecularly characterized (Phelps,1998).Phylogenetic analysis of 12 16S rRNA clones showed a broad diversity within the consortium. Clones were related to the gamma-, delta- and epsilon-proteobacteria, Cytophagales and low G+C

Gram-positives. Four clones fell within the Desulfobacteriaea cluster, one of them was closely related to aromatic degrader Desulfobacula toluolica and the remaining three were associated with Desulfosarcina variabilis. In addition, one clone was related to sulfide oxidizer Thiomicrospira. A bacteria related to Campylobacter/Wolinella (epsilon-proteobacteria) might be a member of a commensal relationship to scavenge hydrogen in the consortium. For the other members no possible role could be established. Desulfobacteriaceae were also dominant in another alkylbenzene degrading sulfate reducing enrichment, obtained from the water phase of North Sea oil tank. This group constituted 95% of the total bacterial population. When the same source of microorganisms was enriched with lactate as substrate, mainly Desulfovibrio were detected (Rabus,1996). In this study a probe specific for Desulfobacteriaceae was developed (Table 4.5.), which is of significance since both culturing and cultivation independent methods show a relation between the presence of Desulfobacteriaceae and BTEX degradation.

Stimulation of anaerobic in situ bioremediation under sulfate reducing conditions is an interesting option since sulfate can readily be added to a polluted aquifer due to its high solubility (Anerson,2000).However, this application should be done with care as during sulfate reduction toxic, volatile sulfide is formed. In environments with a high concentration of Fe (II) sulfate addition is a suitable option as the formed sulfide will precipitate with Fe (II), avoiding dangerous situations. Rates of benzene degradation could be stimulated in both batch and column experiments in the presence of added sulfate.These results led to the development of an anaerobic bioremediation field trail based on sulfate injection into the subsurface. Groundwater benzene concentrations as high as 0.1 mmol/L decreased substantially, and this coincided with decreases in supplied sulfate over 84 days of treatment(Anderson,2000)..

4.3.5.5 BTEX Degradation During Methanogenesis

Methanogenesis requires a syntrophic association between proton-reducing bacteria and hydrogen consuming methanogenic Archeae in order to obtain energy. Methanogens require hydrogen and acetate for their growth, which are provided by proton-reducing bacteria. The scavenging of hydrogen by methanogens keeps the hydrogen concentration sufficiently low for the proton-reducing or fermentative bacteria, so they can still gain energy from the degradation of BTEX (Heider,1998).BTEX degradation has been observed under methanogenic conditions

in enrichment cultures containing contaminated aquifers material (Acton,1992,; Edwards,1994; Kazumi,1997) as well as in petroleum contaminated aquifers (Dojka,1998, Weiner,1998). In enrichments, benzene degradation associated with methanogenesis normally takes a long lag period (140-400 days) (Kazumi,1997). It has been suggested that the microbial populations need to accumulate to sufficient density to degrade benzene.

However, recently the occurrence of benzene degradation without apparent lag period was demonstrated. This was probably due to the ability for the appropriate consortium to develop during long term in situ exposure to benzene (Weiner,1998). Benzene can not always be degraded under methanogenesis (Kazumi,1997). This possibly relates to high toxic benzene concentrations (1.4-4.3mmol/L), which affect the microorganisms adversely. On the other hand, no benzene metabolism is observed at very low concentrations (1.5μM) of benzene, because the concentration of benzene might too low to induce growth of benzene reducers(Hutchins,1998).

Two archaeal species related to the genera Methanosaeta and Methanospirillum, one eubacterium related to the genus Desulfotomaculum and a species not related to any previously described genus were responsible for toluene reduction in a methanogenic consortium(Ficker,1999). Methanosaeta species are aceticlastic methanogens that split acetate, oxidizing the carboxylic group to carbon dioxide and reducing the methyl group to methane. No other substrates support growth. Methanospirillum species use formate and hydrogen as electron donors. Thus these Archaea presumably utilize hydrogen or formate produced by other organisms in the culture. Desulfotomaculum is a sulfate reducing bacterium, but grows in the absence of sulfate acetogenically. Upon sulfate addition to the enrichment, toluene degradation was inhibited, indicating that the initiation of toluene degradation is not performed by this organism. Thus, by elimination it is likely that the unidentified species performs the first step in toluene degradation, resulting in the release of intermediates on which Desulfomaculum grows. Desulfomaculum in turn produces hydrogen and acetate which are utilized by the Archaea (Ficker,1999). Based on recent biochemical studies on this enrichment, interspecies transfer of the first metabolite in anaerobic toluene degradation, benzylsuccinate, has been proposed(Beller,2000).Samples collected from the methanogenic and combined methanogenic/sulfate reducing zone of a hydrocarbon and chlorinate contaminated aquifer undergoing intrinsic

bioremediation were analyzed by cloning PCR amplified 16S rDNA, screening, sequencing and phylogenetic analysis (Dojka,1998).. The microbial communities of the methanogenic and methanogenic-sulfate reducing samples had many clones in common, but were very different from the iron or sulfate reducing zone lower in the aquifer. Archaeal and bacterial diversity was high, bacterial clones were spread over 10 well-recognized divisions, as well as four candidate divisions with no cultivated representatives and six novel division level groups. In the methanogenic zones, one archeal clone sequence was highly abundant and comprised 81% of the archeal library, this clone was 97% identical to aceticlastic methanogen Methanosaeta spp. In the bacterial clone libraries of the methanogenic site, a sequence representing 7-16% of clones was 96% identical to Syntrophus gentianae.

Syntrophus spp. obtain energy from the anaerobic oxidation of organic acids to acetate and hydrogen. The authors proposed that the terminal step of hydrocarbon degradation in the methanogenic zone of the aquifer is aceticlastic methanogenesis and that Methanosaeta sp. and Synthrophus spp. occur in syntrophic association (Dojka,1998). More detailed information on the microbial participants in methanogenic BTEX degradation is so far not available.

4.3.5.5 BTEX Degradation in Synthrophic Associations

As described above, methanogenesis requires a synthrophic association of microorganisms. BTEX can not be degraded by disproportion to organic acids and hydrogen under standard conditions because the reaction is endogenic(Schink,1997).A negative Gibbs free energy can be obtained for this reaction only if a hydrogen-accepting partner organism reduces the hydrogen concentration. The fermentative oxidation of toluene in a defined synthrophic, non-methanogenic co-culture has recently been established (Schink ,1997). This syntrophic co-culture consisted of the sulfate reducing strain TRM1 or Fe (III) reducing Geobacter metallireducens in cooperation with Wolinella succinogenes as electron-accepting partner organism, with fumarate or nitrate as terminal electron acceptor(Meckenstock,1999).In pure culture none of these strains can degrade toluene under nitrate or fumarate reducing conditions, while TRM1 can degrade toluene under sulfate reducing conditions and G. metallireducens under Fe(III) reducing conditions. Only W. succinogenes can grow in pure culture with nitrate and fumarate as electron acceptors. It is proposed that hydrogen and acetate function as

electron shuttles between microorganisms in co-cultures. This result indicates that in nature BTEXfermenting microorganisms might cooperate with hydrogen-consuming organisms that use electron acceptors.

4.3.5.6 Biochemistry and Molecular Genetics of BTEX degradation

Understanding the factors that control the rate and extent of anaerobic BTEX degradation requires information on the microorganisms responsible for the degradation and their metabolic capabilities, and thus requires their isolation. So far, only for some combinations of BTEX and TEAP (a quite limited number of) pure cultures of microorganisms have been obtained that are capable of complete oxidation of some of the BTEX compounds (Table 4.6.). No benzene degrading isolates are known.

Although it is well known that only a small part of the microbial communities can readibly be cultured and that culture-based studies can be biased, it is interesting to note that pure cultures of BTEX degraders are phylogenetically closely related to microorganisms that are dominantly detected using culture independent molecular analysis of enrichments or samples obtained from sites with on-going biodegradation Members of the *Thauera/Azoarcus* cluster, *Geobacteraceae* and *Desulfobacteriaceae* have been found to dominate in respectively denitrifying, Fe(III) reducing and sulfate

reducing environments as well as are the only groups of microorganism from which till present date BTEX degraders have been isolated in pure culture. Specific 16S rDNA probes to detect these microorganisms in the environment are available (Table 4.5.). It should also be noted that for all isolates close relatives are known which are not capable of BTEX degradation.

The isolates have allowed us to study in more detail the physiology, biochemistry and genetics of BTEX degradation. In the future, this might allow the development of more specific molecular probes, directed at the detection of (the expression of) genes involved in anaerobic degradation. So far the biochemical and especially the molecular studies have mainly be limited to denitrifying *Azoarcus* and *Thauera* species (Heider , 1998).

Anaerobic degradation has to differ considerably from aerobic degradation as under aerobic conditions the initial attack of hydrocarbons involves molecular oxygen as a cosubstrate for monoxygenases or dioxygenases (Heider , 1998). For anaerobic

degradation of aromatics several pathways have been described, sometimes occurring in the same strain (Philipp 2000). So far BTEX compounds appear to be degraded only via benzoyl-CoA in the benzyol-CoA pathway (Harwood, 1998). The individual BTEX compounds, as well as other aromatics, are funneled into this central pathway via different peripheral pathways. The enzymes of the peripheral and central pathways of anaerobic BTEX degradation are substrate-induced. The ethylbenzene degrading strain EbN1 is able to degrade both ethylbenzene and toluene. Cells grown on one of these substrates only exhibit the enzyme activities for metabolism of the respective substrate, but not for the other. This indicates differences in pathways and regulation of expression(Heider , 1998).

The Central Benzyol-CoA Pathway

The enzyme benzyol-CoA reductase plays a central role in the benzyol-CoA pathway. This enzyme performs the ATP-driven two-electron reductions of the benzene ring, this reaction uses a ferredoxin as electron. After nitrogenase this is the second enzyme known which overcomes the high activation energy required for reduction of a chemically stable bond by coupling electron transfer to the hydrolysis of ATP (Harwood, 1998). Benzyol-CoA reductase exhibit distinct similarities with 2-hydroxyglutaryl-CoA dehydratase and its ATPhydolysing activase protein of *Acidaminococcus fermentans*. The resulting cyclohex-1,5 diene-1-carboxyl CoA is oxidized to acetyl-CoA via a modified beta-oxidation pathway.

The genes for the enzymes of the central benzoyl-CoA pathway have been cloned and sequenced from denitrifying *Thauera aromatica* and *Azoarcus evansii*, as well as for the anoxygenic phototroph *Rhodopseudomonas palustris*.(Egland 1995 ; 1997). Sequence analyses of the genes support observations that phototrophic and denitrifying bacteria use two slightly different pathways to metabolize benzyol-CoA (Harwood, 1998). However, the benzoyl-CoA reductases show a high overall amino acid sequence identity of about 70%.

4.4 Management and Remediation

In most European countries, contaminated sites containing BTEX are on the clean up priority list, in particular because of the health threats posed by benzene. Since these compounds will be found both in the source zone and the plume area, they cannot be

remediated solely be excavation of the contamination soil. To contain the plume, pump-and-treat measures or barrier technology has to be applied. To remove the contamination, either natural attenuation can be relied on (MacDonald, 2000) or in situ remediation technologies such as bioremediation in the form of biostimulation with addition of nutrients and or electron acceptors, soil vapor extraction/bioventing, or reactive barriers can be applied. A number of field trials have shown successful removal of these compounds by in situ remediation (Cunnigham . 2001), but to achieve complete remediation, the geology and the biogeochemistry of the site must be accurately described, and a thorough monitoring scheme must be prepared in order to get exact information as to when the clean up criteria have been reached.

5.MOLECULAR TECHNIQUES USED IN MICROBIAL ECOLOGY

5.1 Tools to Study Microbial Ecology

Microbial ecology is a long-standing scientific discipline that is undergoing remarkable, even revolutionary, changes. The core of the field aims to understand microbial communities, which are selforganizing and self-sustaining assemblages of different microorganisms, and how these communities interact with their environment.(Alexander,1971)

The beginnings of microbial ecology can be traced back at least to the late 1940s and 1950s; great conceptual advancements were made in the 1960s and early 1970s (Alexander,1961,1971). The use of molecular biology tools, beginning around 1985, started to change the prospects for microbial ecologists. Selective and reliable amplification of defined DNA with the polymerase chain reaction (PCR) and hybridization with DNA oligonucleotides made it possible to interrogate directly the genetic information of individual microorganisms and entire communities. The small-subunit ribosomal RNA (SSU rRNA, also known as 16S rRNA for prokaryotes) was the first target for hybridization and is still the most widely used. (Pace ,1985)

5.1.1 Traditional Techniques

Knowledge of bacterial diversity obtained after more than 100 years of pure culture study is incomplete, and very few of the total number of microbial species are in culture (Torsvik ,1995). Until the middle of the last decade enumeration and identification of soil microorganisms hat to rely on phenotypic methods. However, phenotypic methods are restricted to only those bacteria that can be isolated and cultured. Other, maybe completely unsuspected groups, which may be abundant or very active, will not be considered, rendering the emerging picture of the soil microbial community false.

Only a small portion of all prokaryotes has so far been cultivated, and the majority of soil bacteria observed microscopically cannot be cultivated. In addition, the selective

enrichment culture has severe limitations as an approach to study the community composition of naturally occurring microorganisms (Ard, 1992; Amann , 1995). Results with pure culture isolates are not always reproducible due to the variability of phenotypic properties in relation to culture conditions. Furthermore, laboratory cultivation introduces serious bias to community analysis (Boivin-Jahns, 1995; Ferris ,1996), since the bacterial populations obtained through planting are mainly dependent on the isolation media used (Sorheim, 1989), as well as on purification and maintenance procedures. Since nutrient-rich media are used the selection might be biased towards copiotrophic bacteria rather than dominant community members. In addition, properties of microbes ex situ may differ, because they lack the interaction among populations or with the natural environment, or change their physiological state as a result of adaptation to the changed environment. Consequently, in situ diversity and community structure are unlikely to be represented by collections of natural isolates.

5.1.2 Molecular Approaches

Alternatively, molecular approaches base onphylogenetic analyses of biological markers amongst cellular components are capable of better encompassing the diversity of all well represented members of the entire community. Since the mid-1980s, the use of small-subunit ribosomal ribonucleic acid (SSU rRNA) based techniques has facilitated a cultureindependent approach of investigating microorganisms as they occur in nature (Olsen 1986; Ward, 1992; Amman , 1995). The comparison of these molecular "signature" sequences transformed microbial taxonomy from a pure identification system to an evolutionarily-based framework (Gray., 1984; Woese, 1987; Olsen., 1994). Based on these studies all forms of life are separated into three major evolutionary lines, the three so-called domains Bacteria, Archaea, and Eucarya (Fox , 1980; Woese , 1990).

5.1.3 Comparing Culture Independent and Culture Dependent Methods

In the past years, the use of molecular tools in microbial molecular ecology to study the diversity and dynamics of microbial communities increased steadily. The fast accumulation of molecular data necessitates new tools to provide interpretation of these data and to give them a predictive power that is of practical value (Prosser , 2007).

Culture-independent techniques can overcome problems associated with selective cultivation and isolation of bacteria from natural samples, by-passing the so-called 'great plate count anomaly' (Staley and Konopka, 1985; Amann , 1995). Several techniques have been used including denaturing and temperature gradient gel electrophoresis (DGGE and TGGE), single-strand conformation polymorphism (SSCP), length heterogeneity-PCR (LH-PCR), terminal-restriction fragment length polymorphism (tRFLP) and 16S rRNA gene clone libraries.

5.2 Molecular Biological Approach in the Field of Marine Biology

Divergence of the primary lines of microbial descent occurred early in biotic history so highly conserved molecular chronometers are best suited to the task of reconstructing microbial phylogeny (Woese, 1987). Ribosomal RNAs are integral elements of the protein synthesizing apparatus, the basic components of which are present in all primary kingdoms, and are among the most highly conserved cellular molecules. Yet, rRNAs also contain sufficient sequence variability so that relationships between closely related groups can be determined. Comparisons of rRNA sequences, pioneered by Woese and his colleagues, defined the main lineages in the evolution of microorganisms (Woese, 1987). An advantage of rRNA sequence comparisons is the generation of an increasingly expanding data base against which newly determined sequences may be compared (De Rijk , 1992;, Maidak , 2001). Nearly 60,000 16S rRNA sequences are currently available in the Ribosomal Database Project II (Maidak , 2001). Initially, sequences were obtained from well described pure cultures for phylogenetic research. Pace (1986) recognized that as the rRNA tree filled in, the data base would serve not only for continued comparison of sequences obtained from pure cultures, but also for comparison of sequences obtained directly from natural microbial communities without needing to grow the representative members in the laboratory. The concept of comparing gene sequences from microbial communities revolutionized microbial ecology. Subsequently, a suite of molecular methods was developed that employ rRNA sequences (Amann , 1995; Giovannoni, 1991; Stahl and Amann 1991).

Each methodology has specific advantages and disadvantages, or complications. However, molecular methods offer novel approaches to previously unresolved problems in microbial ecology. Microbial diversity in natural environments can be

assessed without the need for cultivation of isolates. Additionally, the role of the environment in regulating gene expression and growth in both prokaryotes and eukaryotes can be investigated utilizing these methods. By combining hybridization and cloning/sequencing techniques with the direct extraction of RNA from environmental samples, questions concerning the composition and activity of indigenous microbial communities may finally be answered.

5.3 Identification of Species Types

5.3.1 Ribosomal RNA and its Importance

rRNA is the most conserved (least variable) gene in all cells (Sit, 2007). For this reason, genes that encode the rRNA (rDNA) are sequenced to identify an organism's taxonomic group, calculate related groups, and estimate rates of species divergence. For this reason many thousands of rRNA sequences are known and stored in specialized databases such as RDP-II and the European SSU database (Cole, 2003;Wuyts, 2002).

Ribosomal RNA and the corresponding genes (rrn) are now widely used as powerful evolutionary and investigative biomarkers for the following reasons (Olsen , 1986): (i) Ribosomal RNAs are essential toprotein synthesis, and therefore are ubiquitous to all organisms, and structurally and functionally conserved; (ii) ribosomal RNAs are readily isolated and identified, (iii) they contain variable and highly conserved regions in both primary and secondary structure, (iv) and they appear to change in sequence very slowly, and they do not exhibit horizontal gene transfer found with many other prokaryotic genes; therefore relationships between rRNAs reflect evolutionary relationships. These traits make rRNAs not only the most widely used biomarker, but also a powerful tool for microbial ecology studies, particularly for complex terrestrial environments such as the soil with enormous and undiscovered diversity.

5.3.2 Ribosomal RNA Databases

Since the development and widespread application of the polymerase chain reaction (PCR; Saiki, 1988), rRNA sequences can directly be obtained from lysed cells, which have contributed to the exponential increase in known prokaryotic SSU rRNA sequences in recent years. Results from molecular ecological studies within the last

seven years frommarine (Giovannoni , 1990; Schmidt , 1991), thermophilic (Weller, 1991; Ferris , 1996), terrestrial (Liesack and Stackebrandt, 1992; Borneman , 1996) environments and on symbionts (Amann , 1991) documents the success of this strategy.

5.3.3 Nucleic Acid Extraction

Methodologies for DNA extraction yield information on the genetic composition of the microbial community. Methods for RNA extraction are used to determine the activity of the community. The extraction and determination of total RNA content has been used to detect total microbial activity, whereas techniques that determine the expression of specific target genes are used to measure the activity of specific components of the microbial community. In environmental molecular research RNA-based methods have been utilized to a lesser extent than DNA-based methods, possibly because of the difficulty in obtaining undegraded RNA. The ability to extract RNA in an undegraded form from cells is of fundamental importance in molecular and environmental microbiology.

5.4 Molecular Methods Used in Microbial Ecology

5.4.1 Polymerase chain reaction (PCR)

A major step forward in the study of microorganisms in the environment via their DNA and/or RNA has been the development of polymerase chain reaction (PCR) amplification (Erlich, 1989; Innis , 1990; Kolb , 2003; Smalla , 1993). PCR is based on the cyclic enzymatic extension from primers at two opposite ends of a DNA template, resulting in the generation of numerous copies of the template spanned by the two primers.

DNA extracted from the environment can be directly used as the template for PCR amplification, whereas RNA can be first reverse-transcribed into DNA using reverse transcriptase, after which the copy DNA (cDNA) is amplified. This process was coined reverse transcription (RT)-PCR. Analysis of the amplifed target sequences can then ensue, either via restriction analysis (RFLP-PCR), via hybridization to a specific probe, or via cloning and sequencing (Kolb , 2003; Smalla , 1993). The use of DNA microarrays has considerably expanded and this technology, which likely depends on PCR, will be increasingly applied, on a much wider scale than at present.

Finally, either one of the many 16S ribosomal RNA gene sequence based fingerprinting methods currently available, i.e. DGGE (Muyzer ,1993), TGGE (Heuer , 1997), T-RFLP (Buckley and Schmidt, 2001), SSCP (Lee, 1996), RISA (Selenska , 2001) and LH-PCR (Ritchie , 2000) can be used in the analyses to describe complex microbial communities.

Using PCR, DNA sequence information can be obtained from non-culturable organisms or dwarf forms which are known to abound in environment but traditionally form an enigmatic group there (Liesack and Stackebrandt, 1992). As PCR based on directly-extracted environmental DNA can detect organisms that are unculturable or non-viable, there is a need to complement the data obtained by PCR with those obtained by cultivation-based approaches.

The specificity of the PCR reaction is dictated by a variety of factors which affect the fidelity of the reaction. For instance, the specificity of the primers used, the cycling regime, the reaction components and their concentrations used, and the presence of PCR additives (adjuvants) are crucial in determining the success of amplification (Erlich , 1989; Innis , 1990). The difficulty when working with environmental, DNA, lies in the finetuning of the reaction conditions to the quality of the template DNA obtained, in terms of molecular integrity and level of purity.

It is often desirable, or necessary, to determine not only whether a specific DNA sequence is present, but at what concentration. Most investigators have relied on the use of DNA probes for quantitation of DNA in environmental samples by hybridization techniques. Although these methods are sensitive they have low detection limits. Quantitation of DNA by PCR has become fairly well established. There are currently four basic quantitative PCR approaches as follows:

1) Quantitation using an external standard

2) MPN-PCR

3) Co-amplification of an internal standard

4) Competitive PCR.

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

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

Although the general steps and ingredients are well defined, there will be small corrections or changes according the purpose of PCR or products planned to have. The changes can be made in enzyme conc., dNTP conc., magnesium conc., annealing and extension temperatures and times, cycle number and other reaction components. The steps were shown in the Figure 5.1.

5.4.2 PCR Based Molecular Methods

Molecular techniques provide an exciting opportunity to overcome the requirement for culturing, and have therefore greatly increased our understanding of microbial diversity and functioning of the marine environment (Thakur , 2008). In particular, PCR-based methods have wide application for the detection, enumeration, functional characterisation and biodiversity assessment of marine bacteria and marine microbial communities (Wilson, 1999). Optimal PCR performance using marine sediment is dependent upon the extraction and purification of nucleic acids (Luna 2006, Kormas , 2003).

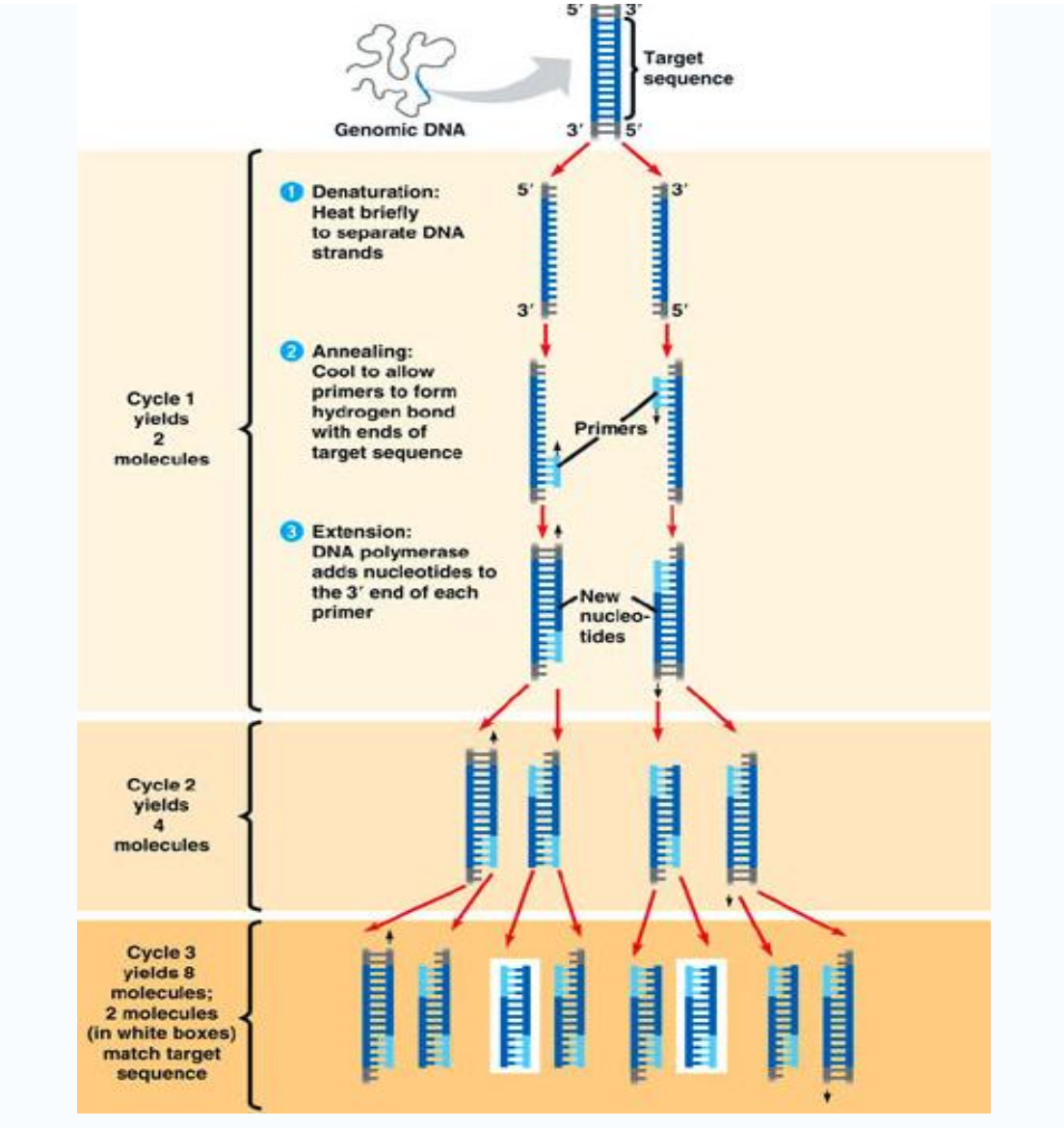

Figure 5.1: PCR Steps

In addition, PCR amplification can be severely hampered by the presence of inhibitory substances which are co-extracted with nucleic acids, such as humic acids, organic matter and clay particles (Kirk, 2004). While a number of studies have been conducted to assess and improve the methods used to extract and purify DNA from marine sediments (Luna ,2006; Zhou ,1996), little information is currently available on the efficiency of PCR reactions of the DNA from those samples. The efficiency of PCR is measured in terms of its specificity, yield and fidelity. More efficient amplification will generate more products using fewer cycles, thus improving sensitivity and accuracy of quantitative PCR. In this study, PCR efficiency was determined by the Real-Time PCR method using marine sediment samples of different origin and chemical composition, and in standard samples spiked with

known concentrations of target bacterial cells. In the analysis, a diagnostic species-specific primer set targeting the toxR gene of Vibrio parahaemolyticus and a universal primer set targeting bacterial 16S rRNA were tested. The comparison between reaction efficiency and sensitivity in the described experimental settings was carried out and discussed to improve the detection and quantification of microbial cells in marine sediments using Real-Time PCR.

Flow chart of a common nucleic-acid based approach in studying an environmental sample was shown in the figure 5.2. The resolution of the information obtained increase from gel electrophoresis to sequencing. Nucleic acids can be extracted directly from the environmental sample or cultured colony, followed by genetic profiling with or without polymerase chain reaction (PCR) amplification, including terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE). For a metagenomic approach, sequence information can be generated (aided by computer algorithm) from shotgun sequencing of a clone library or directly with pyrosequencing on a microtitre plate. The sequences can be stored in databases for future retrieval and feedback into the workflow in probe and primer design to allow further studies. Quantitative data on gene expressions can be gathered from dot blot, macroarray and microarray analysis, or more targeted and precisely with quantitative real time PCR (Q-RT-PCR). Nucleic acid probe, usually fluorescently labelled at 5' end can be used for *in situ* hybridisation study i.e. fluorescence *in situ* hybridisation (FISH). In addition, stable isotopes can also be used to investigate actively expressed genes and identify active organisms. Striped block arrow and dashed arrow indicate that the procedure is optional.

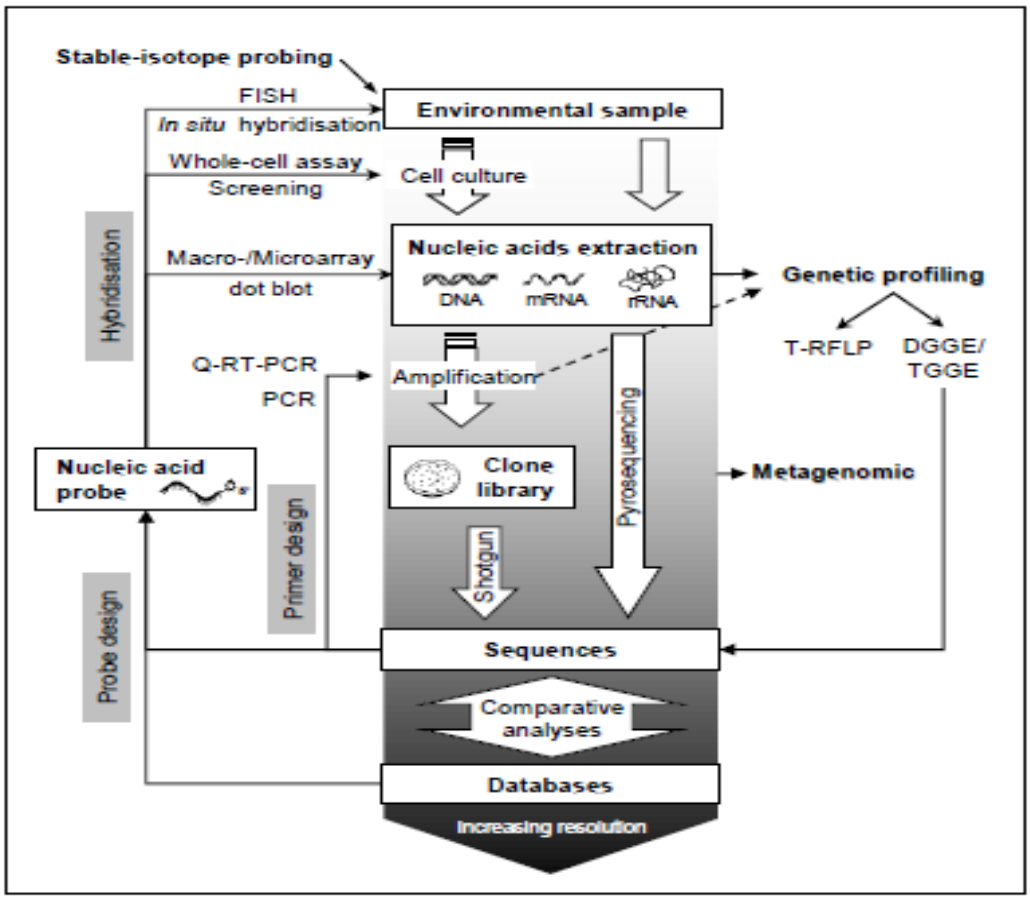

Figure 5.2: Most Commonly Used PCR-Based Molecular Techniques

5.4.3 Quantification of Certain Microbial Groups

In bioremediation studies, the quantitative analysis of functional genes has provided a valuable tool for studying the relationship between specific microbial populations and the performance of the degradation processes (Piskonen ,2005; Ringelberg , 2001). In the environment, however, a wide range of bacteria participate in the degradation of organic contaminants. Because diverse bacteria are associated with different phases of pollutant degradation (Katsivela, 2004; Ringelberg, 2001; Watanabe, 2002), better interpretation of the microbial community dynamics occurring during the progression of decontamination is important in the development of more efficient remediation processes. It is essential to not only obtain quantitative information about specific catabolic functions, but also to relate this information to the phylogeny and biology of the corresponding microbial populations. Instead of monitoring a single genotype, one should focus on the detection of a certain biological function performed by a specific group of organisms (Futamata , 2001).

5.4.3.1 Fluorescense In Situ Hybridization

Prokaryotic cells can be identified without cultivation by applying fluorescence in situ hybridisation (FISH) with ribosomal RNA (rRNA) targeted oligonucleotide probes. FISH can be used in combination with confocal laser scanning microscopy (CLSM) for accurate reconstruction of the spatial arrangement of microbial communities in their habitat (Daims , 2001). FISH combined with flowcytometry or CLSM and digital image analysis tools is also the method of choice for quantitative analyses of the composition and dynamics of microbial communities (Wagner, 2003).

The standard FISH technique (Figure 5.2.) (Amann , 1995) suffers from many limitations: 1) not all bacterial and archaeal cells can be permeabilised using standard fixation protocols, 2) the use of mono-labelled probes limits the sensitivity of the method and aggravates the use of FISH for identification of prokaryotes with low ribosome content per cell, 3) specific hybridisation and washing conditions cannot accurately be determined for probes that exclusively target rRNA sequences of uncultured prokaryotes, 4) the accuracy of quantification is relatively low in densely colonised biofilms, 5) Because of the relatively slow mutation rate of rRNA, this molecule generally possesses no target sites that differentiate between strains of a prokaryotic species, 6) FISH does not provide information on the ecophysiology of the identified microorganism and even the general physiological activity of a prokaryotic cell cannot always be inferred from the cellular rRNA content.

Fluorescence in situ hybridization (FISH) is a method that is used to detect specific RNA or DNA sequences in situ with fluorescently labeled oligonucleotide probes **(**Bauman ,1980**;** DeLong ,1989**).** This technique is used widely in environmental microbiology and clinical diagnostics (Budny, 2002**).** Although FISH has many advantages, automated analysis of FISH images remains challenging. The intensities of positive signals may be different in different experiments, even for the same sample. The differences in intensity are due to a number of factors, including the metabolic state of the cells, the hybridization conditions, and the image acquisition parameters. Many types of environmental samples have additional complications due to the presence of cell aggregates and nonuniform background fluorescence

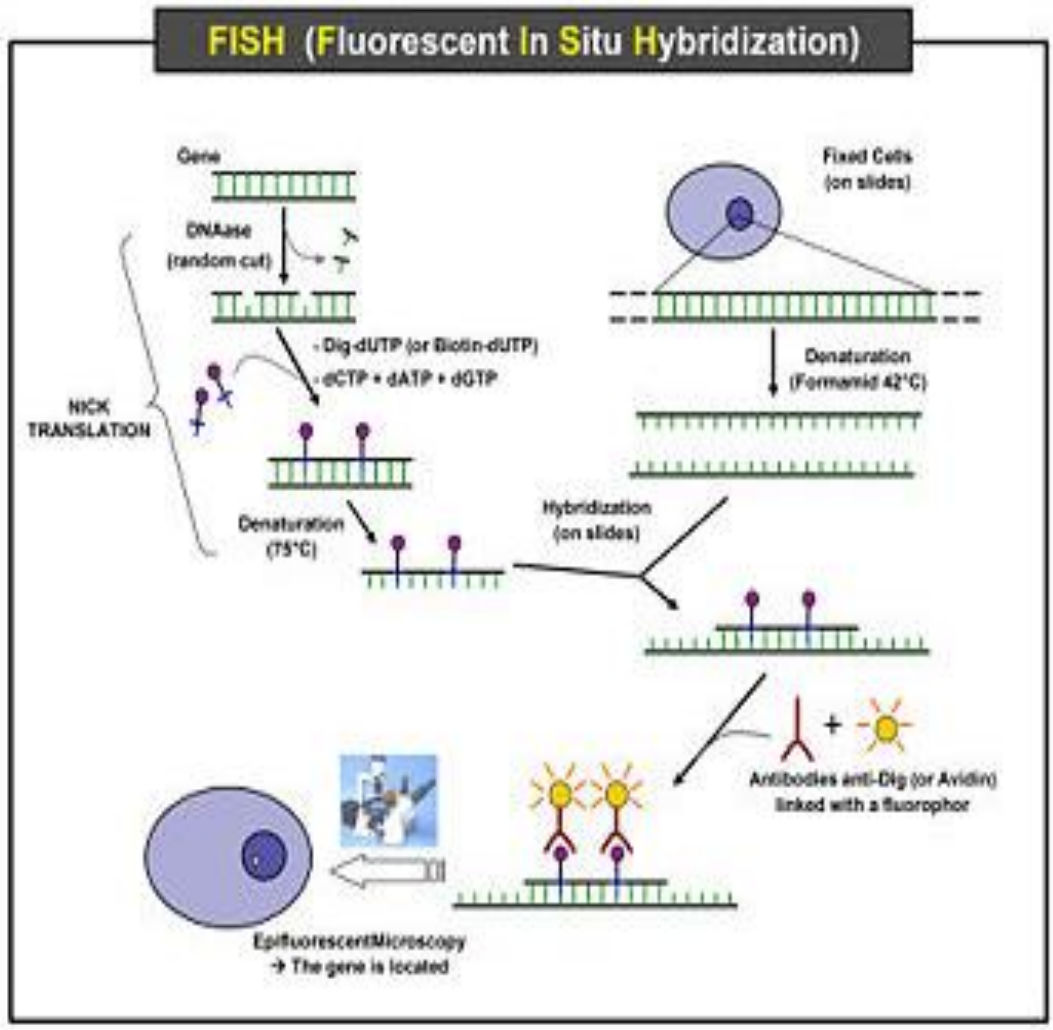

Figure 5.3: Fluorescence In Situ Hybridization Tecqnique

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The basic task in analysis of FISH images is to classify cells into two groups: target (positive) cells and nontarget (negative) cells. This classification is typically based on a threshold; i.e.,all cells with fluorescence intensity higher than a certain threshold are considered target cells, and other cells are considered nontarget cells. The simplest approach for setting a threshold is to choose a fixed value above the background level (Shopov,2000**).** As noted above, fluorescence intensity varies between experiments, so the threshold is often set manually for each experiment. Another common approach is to set a fixed signal-to-noise ratio (Garini, 1999**,** Andrews,1997**;** Pernthaler , 2003). Pernthaler . used a fixed signal-to-noise ratio and defined the threshold as the mean background gray value of a FISH gray image multiplied by a signal-to-noise factor (Pernthaler , 2003). The factor was empirically determined based on manual counting and varied from 110 to 200%, suggesting that

it may require adjustment for each experiment.Using an alternative approach, Langendijk ., set the threshold at the 95th percentile of the fluorescence intensity distribution of the negative control **(**Langendijk , 1995)**.**

However, as these authors noted, such a threshold is also not optimal, since the overlap of two fluorescence distributions in combination with a high fraction of potential target cells resulted in underestimation of the hybridization percentage. Furthermore, this method relies on comparable absolute intensities from two hybridizations (negative control and experiment), but as noted above, there is often substantial variability in intensities even in duplicate experiments. None of these methods is well suited for analysis of samples containing mixtures of cell aggregates and individual cells or samples with variable backgrounds.

5.4.3.2 Real Time PCR

The real-time PCR system is based on the detection and quantitation of a fluorescent reporter (Lee, 1993; Livak, 1995). This signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. A significant increase in fluorescence above the baseline value measured during the 3-15 cycles indicates the detection of accumulated PCR product.

A fixed fluorescence threshold is set significantly above the baseline that can be altered by the operator. The parameter C_T (threshold cycle) is defined as the cycle number at which the fluorescence emission exceeds the fixed threshold. There are three main fluorescence-monitoring systems for DNA amplification (Wittwer, 1997a): (1) hydrolysis probes; (2) hybridizing probes (see Hybridization Probe Chemistry); and (3) DNA-binding agents (Wittwer, 1997b; van der Velden, 2003). Hydrolysis probes include TaqMan probes (Heid, 1996), molecular beacons (Mhlanga, 2001; Vet, 2002; Abravaya, 2003; Tan, 2004; Vet & Marras, 2005) and scorpions (further details) (Saha, 2001; Solinas, 2001; Terry, 2002). They use the fluorogenic 5' exonuclease activity of Taq polymerase to measure the amount of target sequences in cDNA samples.

TaqMan probes are oligonucleotides longer than the primers (20-30 bases long with a Tm value of 10 ^{o}C higher) that contain a fluorescent dye usually on the 5' base, and a quenching dye (usually TAMRA or a non-fluorescent quencher (NFQ) typically on the 3' base (TaqMan MGB probes have a NFQ and minor groove binder at the 3' end). When irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing (this is called FRET = Förster or fluorescence resonance energy transfer) (Hiyoshi, 1994; Chen, 1997). Thus, the close proximity of the reporter and quencher prevents emission of any fluorescence while the probe is intact. TaqMan probes are designed to anneal to an internal region of a PCR product. When the polymerase replicates a template on which a TaqMan probe is bound, its 5' exonuclease activity cleaves the 5' end of probe which contains the reporter dye (Holland, 1991). This ends the activity of quencher (no FRET) and the reporter dye starts to emit fluorescence which increases in each cycle proportional to the rate of probe cleavage. Accumulation of PCR products is detected by monitoring the increase in fluorescence of the reporter dye (note that primers are not labeled). TaqMan assay uses universal thermal cycling parameters and PCR reaction conditions. Because the cleavage occurs only if the probe hybridizes to the target, the origin of the detected fluorescence is specific amplification. The process of hybridization and cleavage does not interfere with the exponential accumulation of the product. One specific requirement for fluorogenic probes is that there be no G at the 5' end. A 'G' adjacent to the reporter dye quenches reporter fluorescence even after cleavage. Well-designed TaqMan probes require very little optimization. To increase specificity of a TaqMan probe and have shorter probes, MGB or locked nucleic acid (LNA) probes can be used with equal efficiency (Kutyavin, 2000; Letertre, 2003; Johnson, 2004; Ugozzoli, 2004). See Glossary for LNA, MGB, NFQ; see also a list of SNP500 Cancer Validated TaqMan Allelic Discrimination Assays).

Real-time PCR chemistries: SYBR green detection (Fig 5.4). SYBR green binds to all double stranded DNA and emits a fluorescent signal. In its unbound state, SYBR green does not fluoresce. Template amplification is therefore measured in each cycle by the corresponding increase in fluorescence.

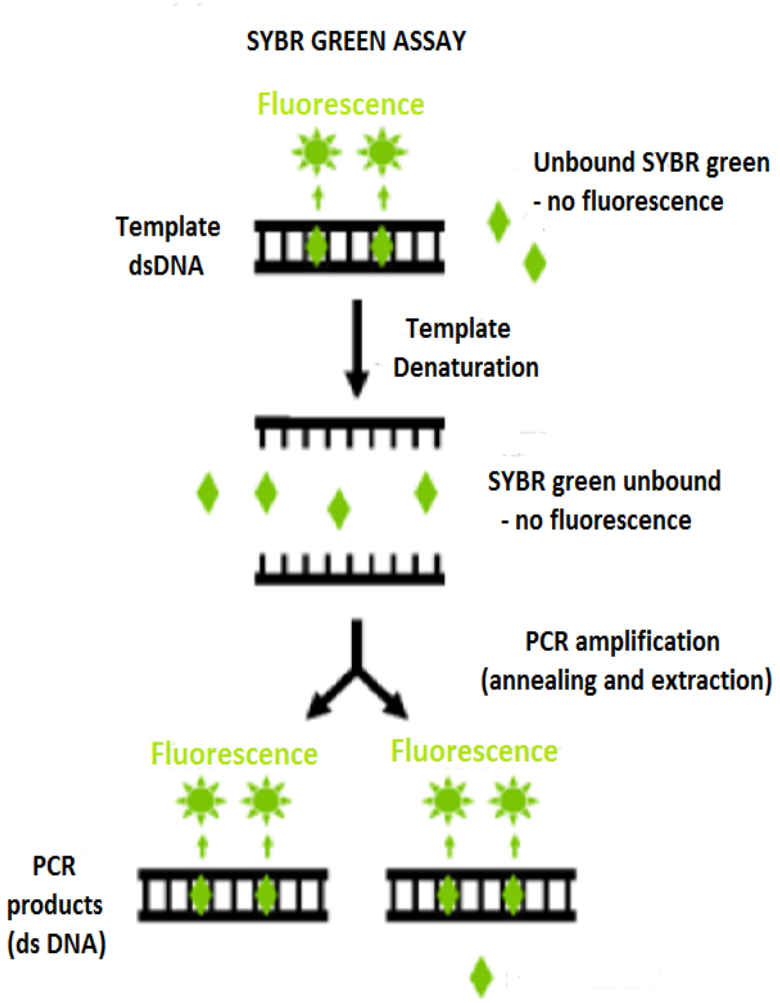

Figure 5.4: Syber Green Dye Method

TaqMan (Fig 5.5) (5 nuclease) assay using TaqMans probes. During annealing, the TaqMan probe and primers bind to the template. When the TaqMan probe is intact, energy is transferred between the quencher and the reporter; as a result, no fluorescent signal is detected. As the new strand is synthesized by Taq polymerase, the 50 exonuclease activity of the enzyme cleaves the labelled 50 nucleotide of the probe, releasing the reporter from the probe. Once it is no longer in close proximity, the fluorescent signal from the probe is detected an template amplification is recorded by the corresponding increase in fluorescence.

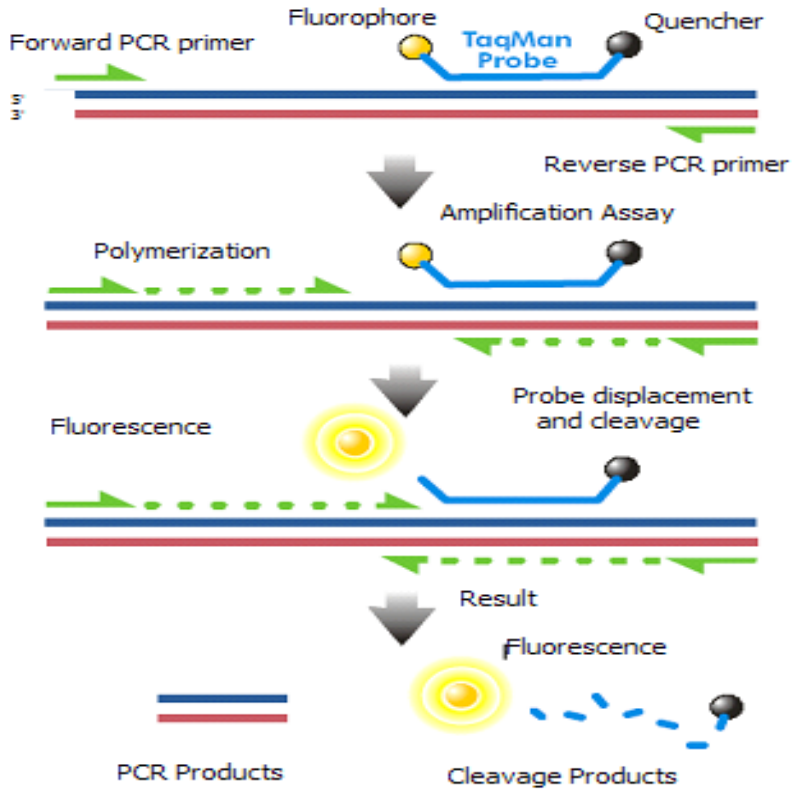

Figure 5.5: TaqMan Probe Method

Molecular beacons (Fig 5.6) also contain fluorescent (FAM, TAMRA, TET, ROX) and quenching dyes (typically DABCYL or BHQ) at either end but they are designed to adopt a hairpin structure while free in solution to bring the fluorescent dye and the quencher in close proximity for FRET to occur. They have two arms with complementary sequences that form a very stable hybrid or stem. The close proximity of the reporter and the quencher in this hairpin configuration suppresses reporter fluorescence. When the beacon hybridizes to the target during the annealing step, the reporter dye is separated from the quencher and the reporter fluoresces (FRET does not occur). Molecular beacons remain intact during PCR and must rebind to target every cycle for fluorescence emission. This will correlate to the amount of PCR product available. All real-time PCR chemistries allow detection of multiple DNA species (multiplexing) by designing each probe/beacon with a spectrally unique fluor/quench pair, or if SYBR green is used by melting curve analysis. By multiplexing, the target(s) and endogenous control can be amplified in single tube for qPCR purposes (Bernard, 1998; Vet, 1999; Lee, 1999; Donohoe, 2000; Read, 2001; Grace, 2003; Vrettou, 2004; Rickert, 2004; Persson, 2005.

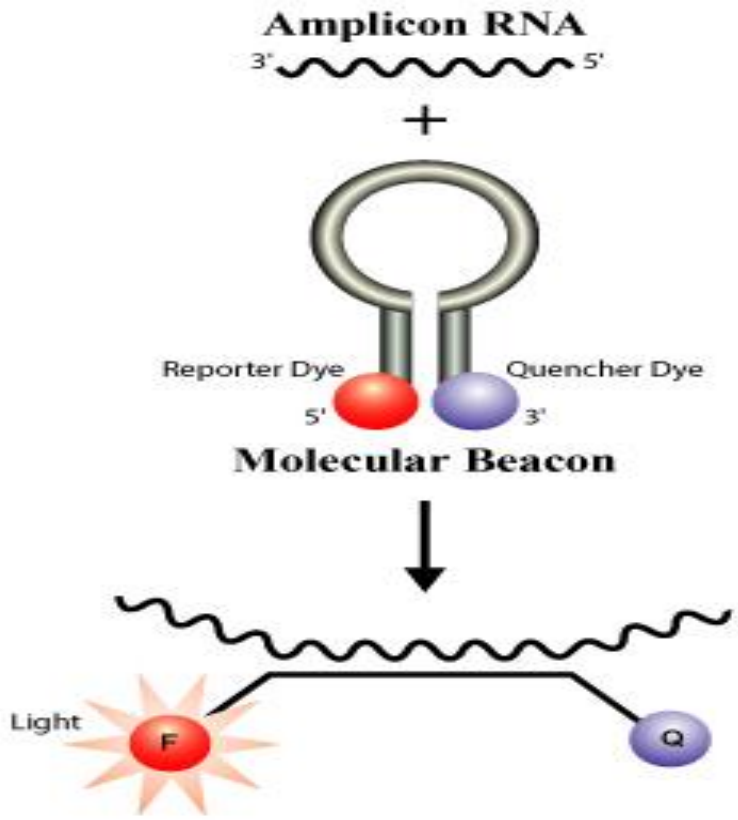

Figure 5.6: Molecular Beacon

With Scorpion primer/probes, sequence-specific priming and PCR product detection is achieved using a single oligonucleotide. The Scorpion probe maintains a stem-loop configuration in the unhybridized state. The fluorophore is attached to the 5' end and is quenched by a moiety coupled to the 3' end. The 3' portion of the stem also contains sequence that is complementary to the extension product of the primer. This sequence is linked to the 5' end of a specific primer via a non-amplifiable monomer. After extension of the Scorpion primer, the specific probe sequence is able to bind to its complement within the extended amplicon thus opening up the hairpin loop. This prevents the fluorescence from being quenched and a signal is observed .

The cheaper alternative is the double-stranded DNA binding dye chemistry, which quantitates the amplicon production (including non-specific amplification and primer-dimer complex) by the use of a non-sequence specific fluorescent intercalating agent (SYBR-green I or ethidium bromide). It does not bind to ssDNA. SYBR green is a fluorogenic minor groove binding dye that exhibits little fluorescence when in solution but emits a strong fluorescent signal upon binding to double-stranded DNA (Morrison, 1998). Disadvantages of SYBR green-based real-time PCR include the requirement for extensive optimization. Furthermore, non-

specific amplifications require follow-up assays (melting point or dissociation curve analysis) for amplicon identification (Ririe, 1997). The method has been used in HFE-C282Y genotyping (Donohoe, 2000). Another controllable problem is that longer amplicons create a stronger signal (if combined with other factors, this may cause CDC camera saturation, see below). Normally SYBR green is used in singleplex reactions, however when coupled with melting curve analysis, it can be used for multiplex reactions (Siraj, 2002).

The threshold cycle or the C_T value is the cycle at which a significant increase in DRn is first detected (for definition of DRn, see below and Glossary). The threshold cycle is when the system begins to detect the increase in the fluorescent signal associated with an exponential growth of PCR product during the log-linear phase. This phase provides the most useful information about the reaction (certainly more important than the end-point). The slope of the log-linear phase reflects the amplification efficiency (Eff). Eff can be calculated by the formula:

$$Eff = 10^{(-1/slope)} - 1$$

The efficiency of the PCR should be 90 - 100% ($-3.6 > slope > -3.1$) (Stratagene Slope to Efficiency Calculator). A number of variables can affect the efficiency of the PCR ¯Bustin, 2004; Wong, 2005; Yuan, 2006). These factors include length of the amplicon, secondary structure and primer quality. Although valid data can be obtained that fall outside of the efficiency range, the qRT-PCR should be further optimized or alternative amplicons designed (see Efficiency Determination Page by Pfaffl). For the slope to be an indicator of real amplification (rather than signal drift), there has to be an inflection point. This is the point on the growth curve when the log-linear phase begins. It also represents the greatest rate of change along the growth curve. (Signal drift is characterized by gradual increase or decrease in fluorescence without amplification of the product.) The important parameter for quantitation is the C_T. The higher the initial amount of genomic DNA, the sooner accumulated product is detected in the PCR process, and the lower the C_T value. The threshold should be placed above any baseline activity and within the exponential increase phase (which looks linear in the log transformation). Some software allows determination of the cycle threshold (C_T) by a mathematical analysis of the growth curve. This provides better run-to-run reproducibility. A C_T value of 40 or higher means no amplification and this value cannot be included in the calculations. Besides

being used for quantitation, the C_T value can be used for qualitative analysis as a pass/fail measure.

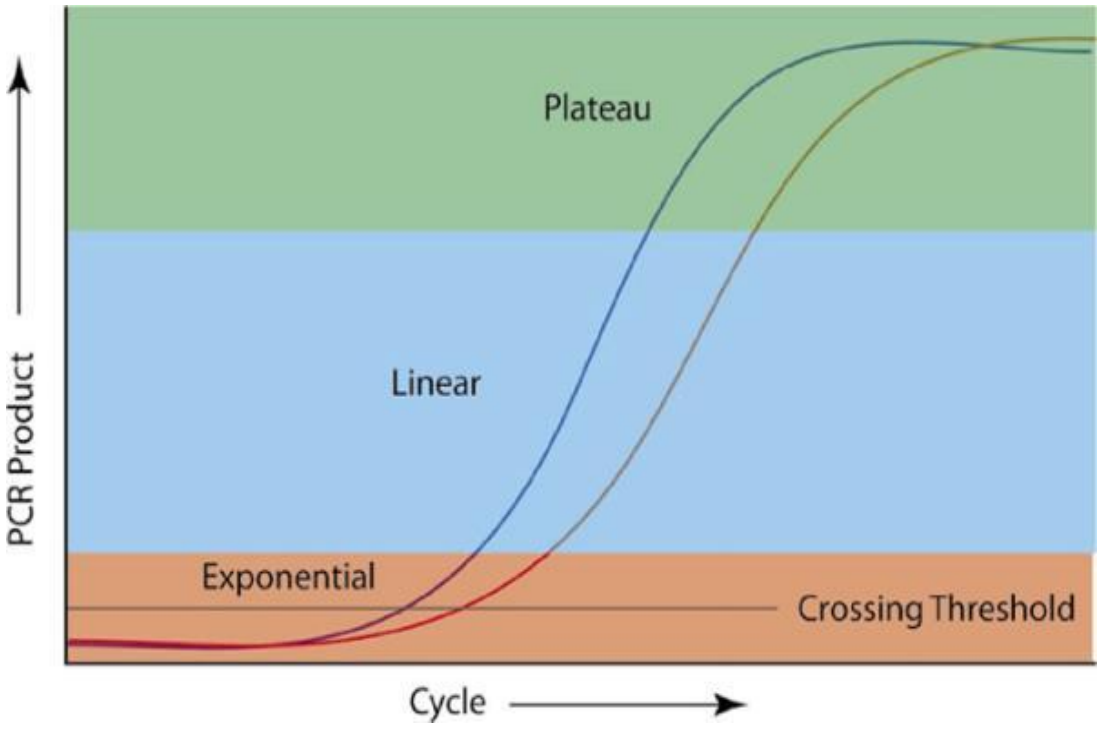

Figure 5.7: PCR Steps

The PCR process goes through three main phases as the number of cycles and the amount of product generated increase. Initially, when the amount of product is small and enzyme and reagents are not limiting, product generation is exponential and the reaction is closest to 100% efficiency. This exponential growth is hard to detect initially through real-time fluorescence because the amount of product is small. During the linear phase products continue to accumulate, but the reaction efficiency begins to fall and reagents become limiting. Finally, in the plateau phase of the reaction, accumulation of product ceases as the reaction is exhausted for a number of different reasons.

Q-PCR amplification from known concentrations of template DNA to construct standard curves for quantification of unknown environmental samples. Log plot of the increase in fluorescence vs. cycle number of DNA standards ranging from 1_104 to 1_108 16S rRNA gene amplicons *m*L_1.

Linear plot indicating the three phases of a PCR amplification, the corresponding Ct values for each of the amplified standards and for the NTC. (c)

Simple linear regression of the Ct values (from b) vs. log of the initial rRNA gene number. Q-PCR descriptors are shown (boxed).

CYCLE NUMBER	AMOUNT OF DNA
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1,024
11	2,048
12	4,096
13	8,192
14	16,384
15	32,768
16	65,536
17	131,072
18	262,144
19	524,288
20	1,048,576
21	2,097,152
22	4,194,304
23	8,388,608
24	16,777,216
25	33,554,432
26	67,108,864
27	134,217,728
28	268,435,456
29	536,870,912
30	1,073,741,824
31	1,400,000,000
32	1,500,000,000
33	1,550,000,000
34	1,580,000,000

Figure 5.8: Cycle numbers of PCR

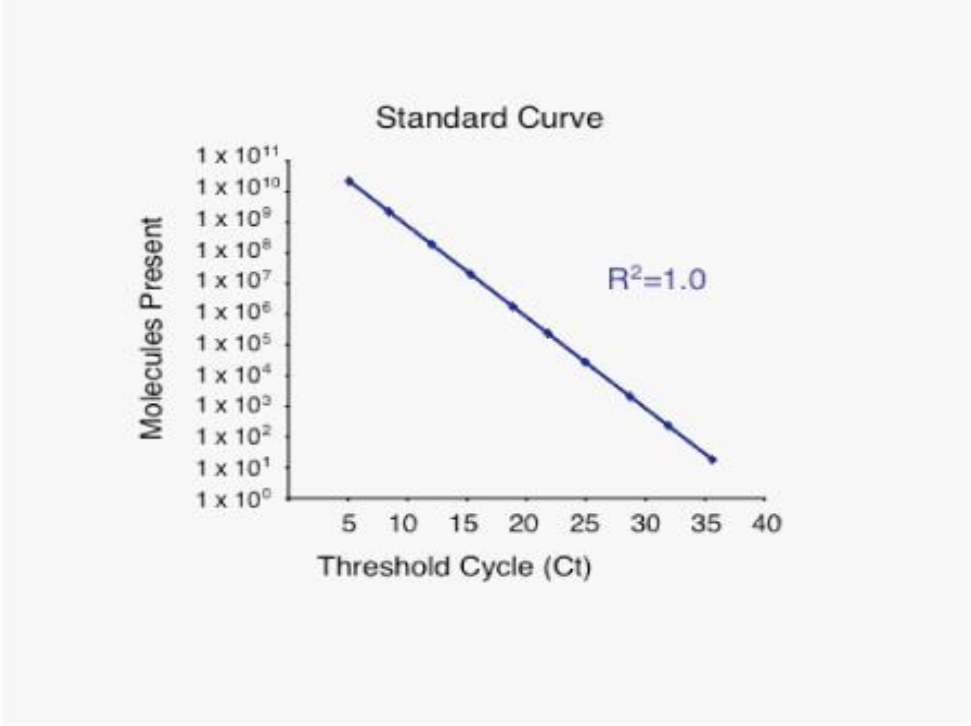

Figure 5.9: Standard Curve

6.MATERIALS AND METHODS

6.1 Sampling and Preservation

Sampling locations, depths and dates, and sample abbreviations were given in Table 6.1. The samples were taken using a Van Veen grab (Figure 6.1.) (volume of 3.5 L and penetration depth of 15 cm) on board of the RV Arar of Istanbul 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 RNA analyses were first treated with RNAprotect bacteria reagent (Qiagen, U.K.) following the manufacturer's instructions and stored at -80°C. All samples appeared visually similar possessing grayish-black color and had a noticeable odor of H_2S. Sediment grain size analysis was performed 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 6.1: The research ship, ARAR, of Istanbul University and Van Ween grab sampler

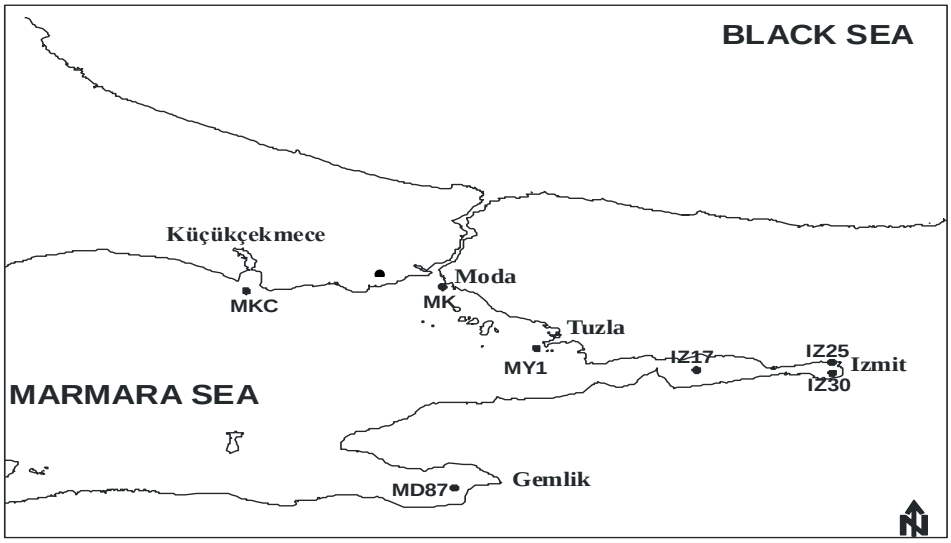

Figure 6.2: Sampling locations in the Marmara Sea

Table 6. 1: Sampling locations, depths and dates, and sample abbreviations.

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

6.2 Molecular Analysis

To analyze bssA gene abundance with culture independent techniques, different molecular methods were used. Molecular analysis of sediment samples began with extraction of Genomic DNA, followed by PCR to amplify bssA coding region with specific primers for preperation of Q-PCR standards. Flow chart of molecular analysis was given in Figure 6.3.

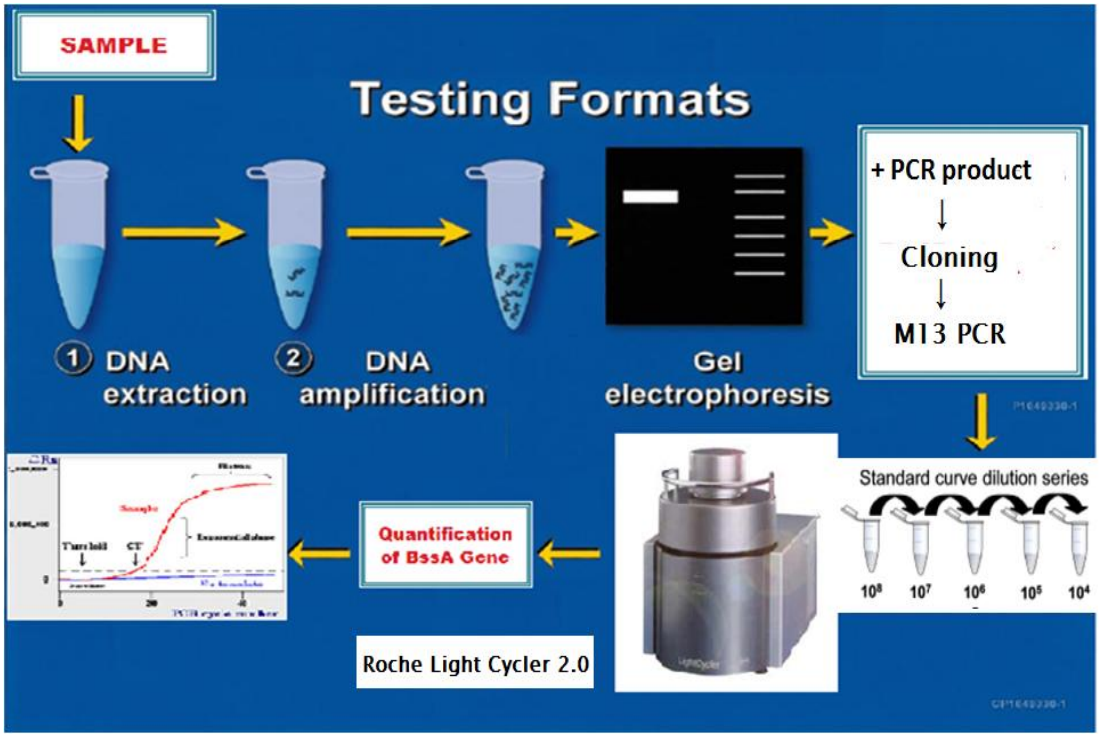

Figure 6.3: Flow Chart of Molecular Analysis

6.2.1 Genomic DNA Extraction

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

The methodology of Genomic DNA extraction of by Fast DNA Spin Kit for Soil was as follows: Approximately 0.5 g sediment was added up to lysing matrix tubes provided by the kit. The tube contains mixture of ceramic and silica particles to lyse all microorganisms in sample. Then lysing matrix tubes were spinned in Ribolyser (Fast Prep™ FP120 Bio 101 Thermo Electron Corporation) for 30 seconds at speed of 5.5 m/s. The tubes were then centrifuged at 14000xg for 30 seconds. After centrifugation supernatants were transferred to clean 1,5 ml eppendorf tubes and

added 250 µl PPS reagent. To mix the composition tubes were shaked by hands for 30 seconds. After mixing the tubes centrifuged again at 14000xg for 5 minutes to pellet the precipitate. Supernatants were transferred to 2 ml eppendorf tubes and 1 ml of Binding Matrix Suspension was added to supernatant. Tubes were inverted by hand for 2 minutes to allow binding of DNA to matrix. To settle the silica matrix tubes were incubated 3 minutes at room temperature. 500 µl of supernatant was removed carefully without disturbing settled silica matrix. Then the binding matrix was resuspended in the remaining supernatant. All mixture was filtered and filter was placed to a new tube. Filter was washed by 500 µl SEWS-M wash solution. After washing, filter was dried by centrifugation at 14000xg for 2 minutes. Filter was removed to a new tube and 50 µl DES (DNase/Pyrogen free water) was added. The filter with DES was vortexed and then centrifuged at 14000xg for 1 minute. Application-ready DNA was obtained in the tube.

To control the existance of extracted genomic DNA, 1/100 diluted genomic DNA was run on the %1 (w/v) agarose gel, prestained with Ethidium Bromide (EtBr) in 1x Tris-Acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM Acetic Acid, 1 mM EDTA; pH 8). Gel was visualized by using a gel documentation system, Mitsubishi 91.

The DNA concentration of each extract was determined using a fluorometer (Qubit, Invitrogen) according to the manufacturer's specifications. Quant-iT™ Working Solution was made by diluting the Quant-iT™ reagent 1:200 in Quant-iT™ buffer

All tubes was vortexed for 2–3 seconds and was incubated for 2 minutes at room temperature (15 minutes for the Quant-iT™ protein assay).Then, tubes were read in Qubit™ fluorometer. Qubit™ fluorometer performed the multiplication by the dilution factor to determine concentration of our original sample.

Purity of the genomic DNA samples were controlled by using Spectrophotometer. The ratio of the absorbance at 260 nm/ absorbance at 280 nm is a measure of the purity of a DNA sample. ddH_2O was used as a blank, values between 1.6 - 2.0 were used in the Q_PCR analysis.

6.2.2 Real Time PCR

6.2.2.1 Preparation of Standards:

Extracted GDNAs were used as templates, amplification of bssA gene sequences were performed by specific primers.

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) by using a a Techne TC-412 thermal cycler (Barloworld Scientific Ltd., U.K.) with an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing for 1 min and extension at 72°C for 2 min and a final extension at 72°C for 10 min. PCR products were visualized by electrophoresis (Thermo-Scientific Ltd., U.K.) on a 1% (w/v) agarose gel in 1× Tris–borate–EDTA buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) at 7 V cm^{-1} and gel images were recorded using a Chemi-Smart 3000 gel documentation system (Vilber Lourmat, France) after staining with ethidium bromide.

One of positive PCR product result was chosen for clonning.

The initial step of the clonning procedure was preparation of 6 µl reaction mix by adding 3 µl PCR product, 1 µl salt solution (1.2 M NaCl, 0.06 M $MgCl_2$), 1 µl TOPO vector and 1 µl Sterile Water. The solution was mixed gently and incubated at room temperature for 20 minutes. Following incubation, reaction mix was placed on ice before One Shot TOPO transformation step.

After incubation, the tube was subjected to heat shock at 42º C for 30 seconds and transferred immediately to ice and 300 µl of SOC medium (2% Tyrptone,0.5% Yeast Extract, 10 mM NacCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added.The solution was shaked horizontally for 60 minutes. Three LB plates containing 50 µg/ml kanamycin were warmed to room temperature and then 100 µl of solution was spread on plates using glass spreader. The plates were incubated overnight and white colonies were observed after incubation.

Colonies were picked from plate and transferred into 200 µl PCR tubes containing 50 µl TE buffer (10mM Tris-HCl, 1mM EDTA pH 8.0). Colonies were boiled at 95^0 C for 5 minutes then frozen at -20^0 C overnight. Thawed solution was used as templates for PCR. The DNA fragments were isolated from vector by PCR with primers M13f-

M13r (M13 Forward 5′-GTA AAA CGA CGG CCA G-3′/ M13 Reverse 5′-CAG GAA ACA GCT ATG AC-3′).

Other step of standart preparation is purification of M13 PCR products, which was done according to the Invitrogen PCR product purification Kit. according to the manufacturer's specifications. Purification with PureLink™ PCR Purification Kit, the yield of purified dsDNA has been estimated by agarose gel electrophoresis. To estimate the yield, agarose gel electrophoresis of the purified PCR product and known quantities of DNA fragment of the same size was performed . The band intensity of the purified PCR product with the standard DNA fragments was compared. So the purified PCR product was used as Q_PCR standards.The standartds concentration were determined using a fluorometer (Qubit, Invitrogen) according to the manufacturer's specifications.

Application ready standards were diluted in 1/100 ratio for Q_PCR experiments.

6.2.3 Q-PCR Experiment

PCR primer sets for the Q-PCR assays , 10^{3-7} copies of the standard sequences were used to obtain the calibration curves. Roche Light Cycler DNA Master SYBR Green I kit and Roche Light Cycler 2.0 (Figure 6.4.)(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 primer sets were described previously (Table 3). The following thermo cycling 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 analyzed using Roche Light Cycler 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

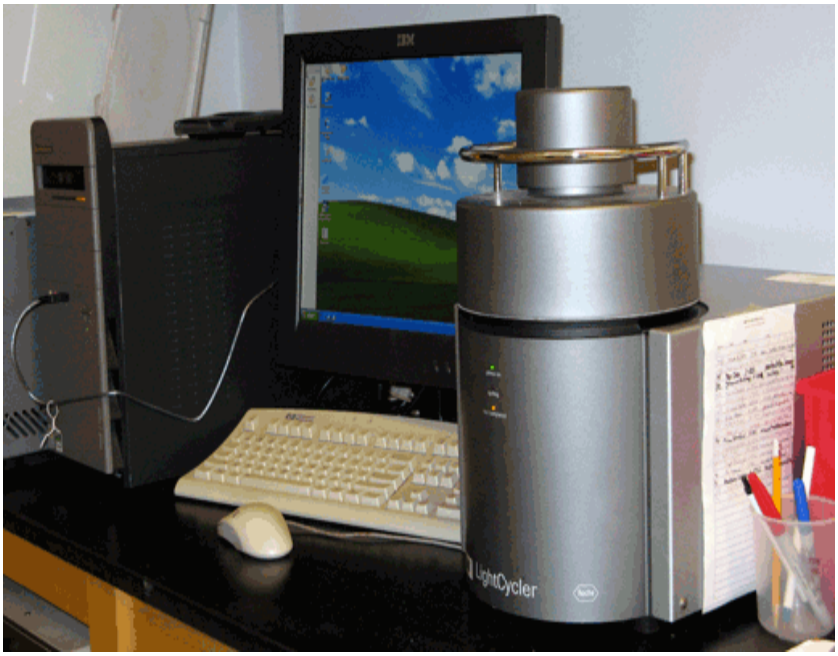

Figure 6.4: Roche Light Cycler 2.0.

Figure 6.5: Roche Light Cycler carousel and capillaries

7.RESULTS AND DISCUSSION

Many various species of bacteria have now been described that can degrade one or more of the BTEX components in the absence of oxygen (Gibson and Harwood 2002)**.** Quantitative real-time PCR (Q-PCR) have been widely used for the quantification of gene abundances in environmental samples (Winderl, 2008, Higashioka, 2009). Q-PCR test had been carried out to assess the abundance of AnBTEXDeg. In order to determine metabolic potential of marine sediment organisms it is more advantageous to target functional genes that code for enzymes involved in metabolic activity than targeting the rRNA gene in which function is implied by virtue of belonging to a group where members are known to have the ability to perform a function (Kolukırık, 2009). BTEX degradation is mediated by a diverse polyphyletic group of *Bacteria* (Zumft, 1997) and rRNA-based approaches are of limited value for understanding the structure and diversity of these communities (Smith, 2007). For this reason, we quantified, AnBTEXDeg by targeting functional genes, bssA, using Q-PCR . The designed Q-PCR primers were able to amplify bssA of certain microbial groups such as sulfate reducing bacteria (SRB) (Beller, 2008), denitrifying bacteria (DB) (Beller , 2002) and a methanogenic consortium (Washer and Edwards 2007), or targeted certain clusters of previously identified bssA (Winderl . 2008). In this study, generic Q-PCR primers had been designed to monitor abundance and activity of BTEX degraders in the MSS were applied for time first time. Furthermore the *bssA* primer set appeared to function well when applied to microcosm samples containing indigenous (and uncharacterized) aquifer bacteria.

Copy numbers of bssA gene was directly correlated to cell numbers (Philippot, 2002 and 2005; Da Silva and Alvarez, 2002; Beller , 2002; Zang and Fang, 2006). Cell concentrations was calculated by the multiplication of the percentage of bssA gene to total cell per cm^3 of the sediment.

7.1.Chemical and Physical Characteristics of the Sediments

The results from the chemical analyses were given in Table 7.1. as ranges in which the concentrations fluctuate during the two years monitoring period according to the TUBITAK 105Y307 Project. The way sediments' chemical compositions changed along with the AnBTEXDeg abundancy will be discussed after presenting the correlation analysis results.

Table 7.1: Concentration ranges for chemical components of the Marmara Sea Sediment between the years 2005 and 2008. Horizantal "white → black" scale represents increasing level of the parameters. (Kolukırık , 2009).

			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

7.2 Petroleum Hydrocarbon Content of the Sediment

Marmara Sea has been extremely polluted with hydrocarbons. Total petroleum hydrocarbons (TPH) in the MSS and their fractions were given in Table 7.2. (Kolukirik; 2009) Organic-rich marine sediments may naturally contain up to 100 ppm TPH, but concentrations higher than this are usually associated with petroleum inputs (Readman , 2002). TPH levels implied that hydrocarbon pollution in the MSS were associated with petroleum inputs. Similar TPH values (1000-7000 ppm) to those from the MSS have been reported from extremely polluted environments (Guerra-García, 2003). Total Petroleum Hydrocarbon content in Tuzla sediments has the highest value with up to approx 17000 ppm TPH . Although Gemlik has the lowest value with approx 1600 ppm TPH but it is still very high for marine sediments.

Wang (2006) confirmed that the aliphatic hydrocarbons were mainly from petroleum contamination. Aliphatics levels were medium for Haliç sediments, high for Izmit sediments and very high for Gemlik and Küçükçekmece sediment compared to those from other areas worldwide (Ahmed, 2006; Guerra-García , 2003)

Hydrocarbons ranging from C10 to C26 and aromatics of low molecular weight are considered the most readily degraded (Atlas, 1995b), whereas more complex molecular structures are generally more resistant to biodegradation. The Aromatic levels showed the same characteristics as Aliphatics.

Asphaltene and Resene are considered to be recalcitrant to biodegradation (Oudot, 1998). However, Rontani (1985) have reported that about half of the Asphaltene components can be biodegraded in the presence of C12 to C18 n-alkanes which might be the reason for the low Asphaltene content of the TPH in the MSS. The level of Asphaltene was low in Küçükçekmce and Gemlik sediments and high in Haliç and Tuzla sediments.

Table 7.2: Total Petroleum Hydrocarbon and their fractions in the MSS (Kolukırık , 2009).

Sample	TPH (ppm)	Aliphatics (%)	Aromatics (%)	BTEX (%)	Asphaltene (%)	Resene (%)
GEM	1665±136	35,382	46,898	5,315±0,43	5,315	12,402
HALAS	14870±1516	18,464	27,228	3,160±0,32	16,291	38,014
HALEY	12630±577	24,846	34,438	2,871±0,25	12,214	28,5
HALVK	12145±1125	22,653	30,777	3,221±0,29	13,97	32,598
IZ17	4224±379	33,773	45,231	4,199±0,37	6,298	14,696
IZ25	6036±593	28,971	40,847	4,231±0,41	9,054	21,126
IZ30	5678±577	31,735	42,773	3,536±0,35	7,647	17,843
KUC	4237±412	36,72	49,585	4,722±0,46	4,172	9,735
MOD	6542±662	25,982	36,567	4,472±0,45	11,235	26,215
TUZ	16995±1443	18,997	27,546	2,441±0,20	16,101	37,569

The total petroleum hydrocarbon level was extremely high in all MMS sediments compared to those from other marine sediments. The ratios were given in the Table 7.7. TPH level in IZ17, IZ30 were lower than IZ25 but higher than Gemlik sediments. In all Haliç and Tuzla sediments the TPH level were very high in all MSS.

7.2.1 Locational BTEX Levels

BTEX compounds are monoaromatic hydrocarbons. The BTEX % was given in the fraction in total petroleum hydrocarbon. Although the TPH was very high in Tuzla sediments , BTEX % in TPH has the lowest value in all MSS. BTEX levels were medium for all Haliç and İzmit sediments, high for Moda sediment and very high for Küçükçekmece and Gemlik sediments compared to eachothers. The range of BTEX % was given in Figure 7.1 , 7.2. ,7.3. ,7.4. ,7.5., 7.6. , for each sampling points

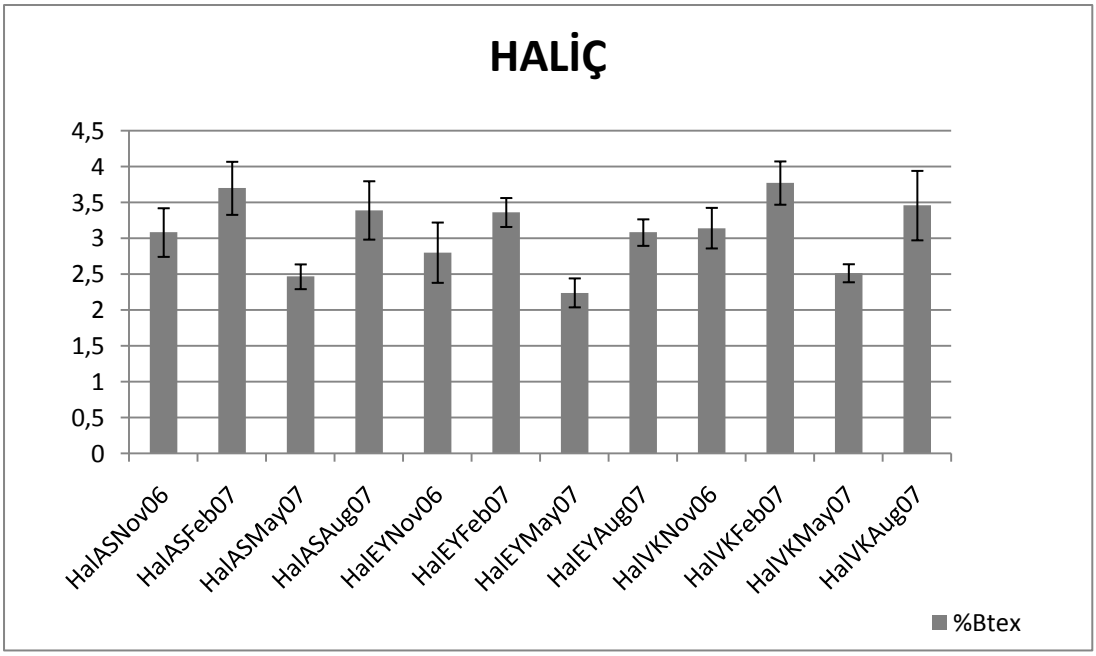

Figure 7.1: The BTEX content of Haliç sediments were taken at different times

The BTEX % in Haliç Bay sediments ranged from 2,24 – 3,77 % of the TPH amount. In all 3 sampling points the lowest abundance of BTEX were in May 2007 and the highest ranges were in February 2007.

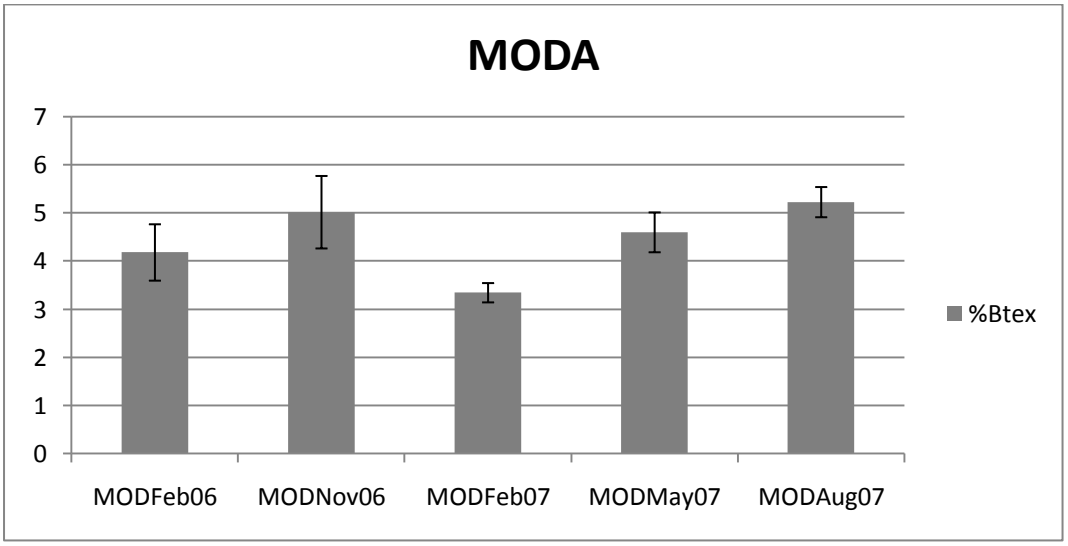

Figure 7.2: The BTEX content of Moda sediments were taken at different times

The BTEX % in Moda sediments ranged from 3.34-5.22 % of the TPH amount. The lowest BTEX % was in February 2007 and the highest was in August 2007.

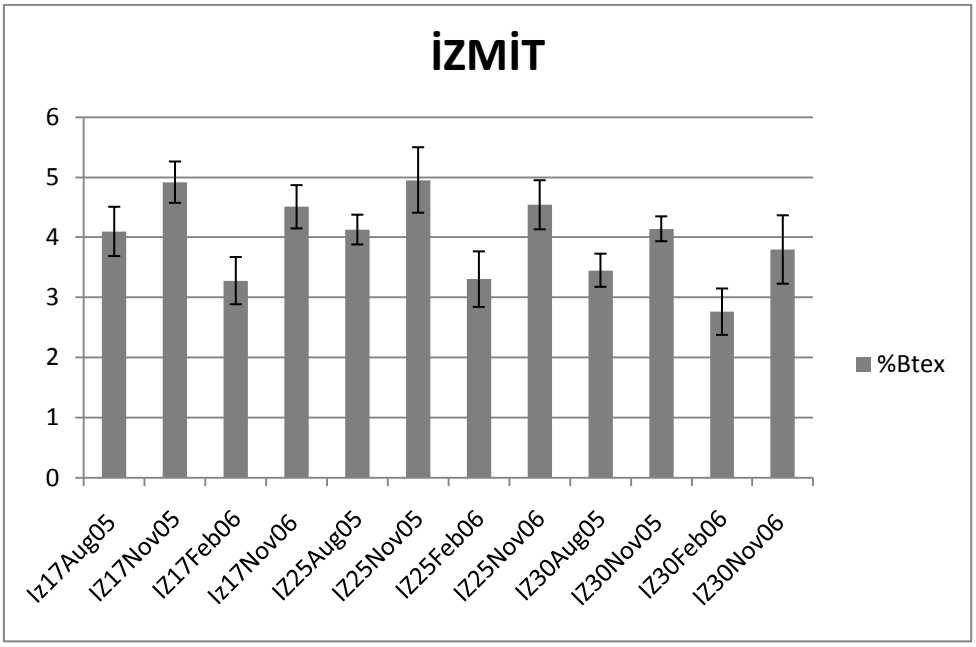

Figure 7.3: The BTEX content of İzmit sediments were taken at different times

The BTEX % in İzmit sediments ranged from 2,76 – 4,95 of TPH amount.In all 3 sampling locations in İzmit , the lowest BTEX % was in February 07 and the highest was in November 2005.

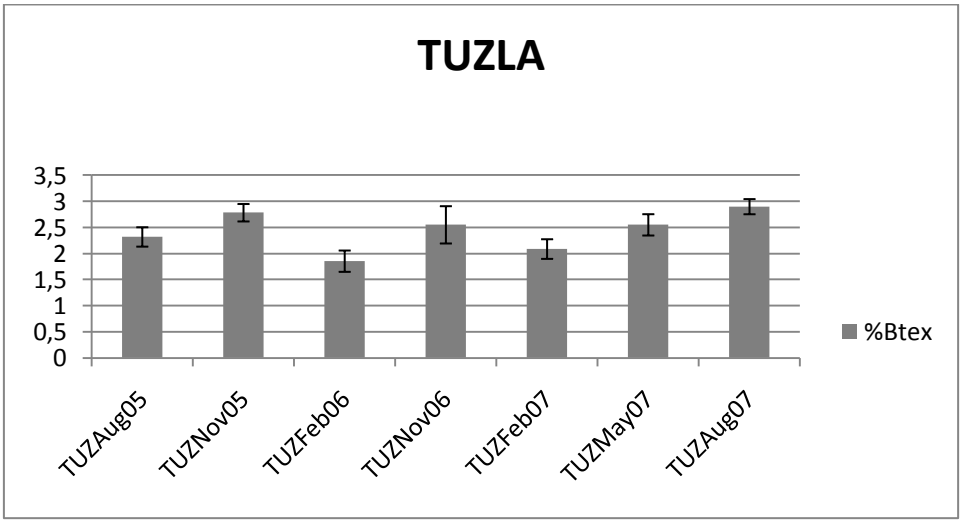

Figure 7.4. The BTEX content of Tuzla sediments were taken at different times

The BTEX % in Tuzla sediments ranged from 1,85-2,90 of TPH amount The lowest BTEX % was in February 2006 and the highest was in August 2007.

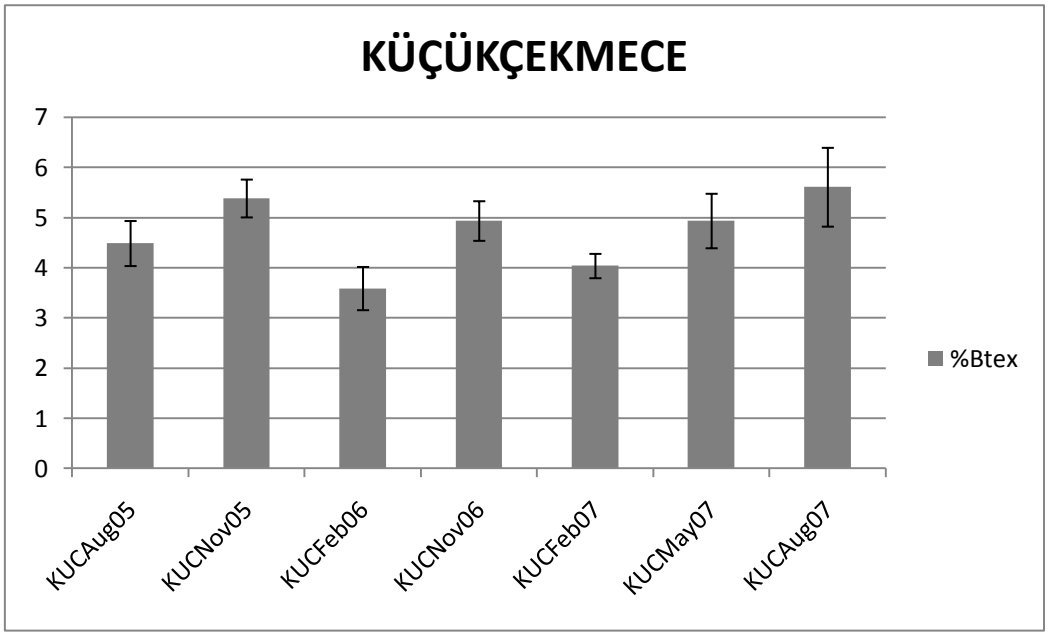

Figure 7.5: The BTEX content of Küçükçekmece sediments were taken at different times

The BTEX %in Küçükçekmece sediment ranged from 3,58-5,60 of TPH amount. The lowest BTEX % was in February 2006 and the highest was in August 2007.

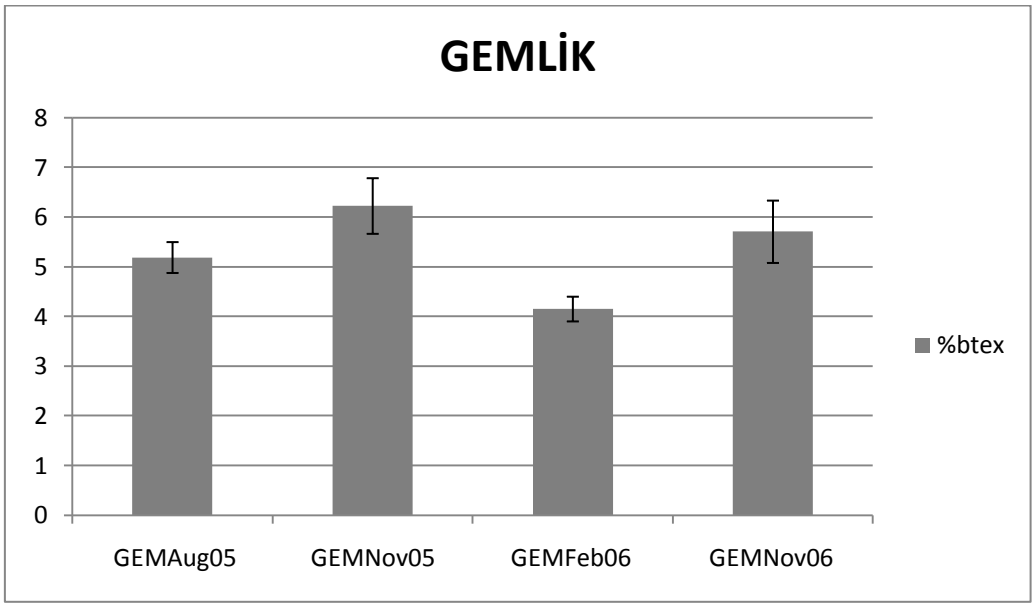

Figure 7.6: The BTEX content of Gemlik sediments were taken at different times

The BTEX % in Gemlik sediments ranged from 4,18-6,22 of TPH amount. The lowest BTEX % was in February 2006 and the highest was in November 2005.

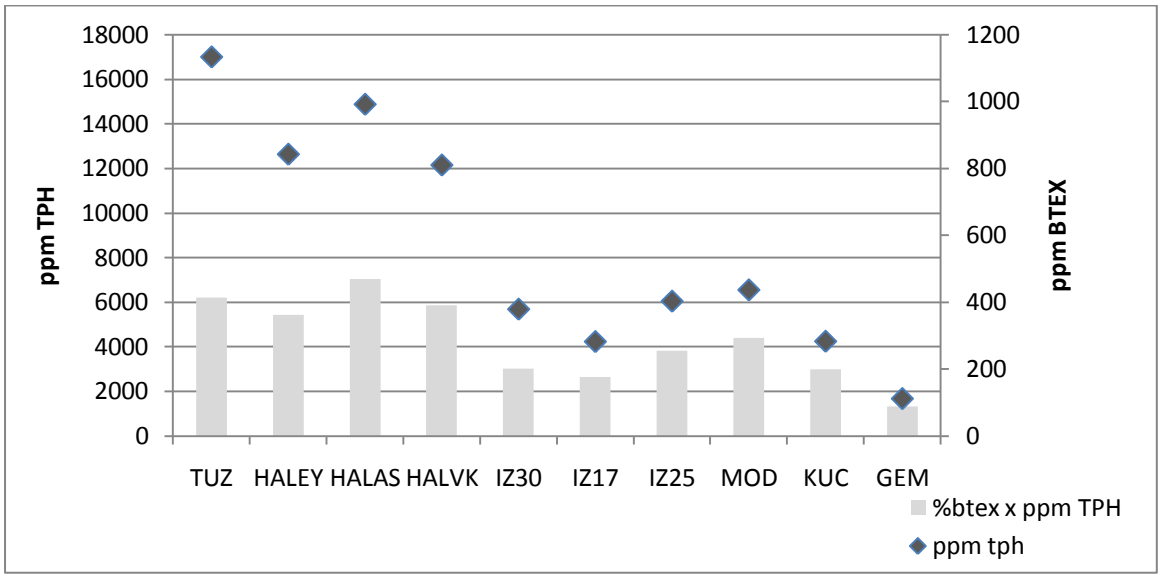

Figure 7.7: The Total Petroleum Hydrocarbon Pollution and BTEX pollution in MSS

The BTEXs amounts were measured by the multiplication of BTEX % to the total TPH amount. The BTEX ppm in the Gemlik sediment was the lowest in whole MSS. In IZ30, IZ17 and IZ25 sediments the BTEX levels were higher than Küçükçekmece and Gemlik sediment but considerably lower than Haliç and Tuzla sediments.

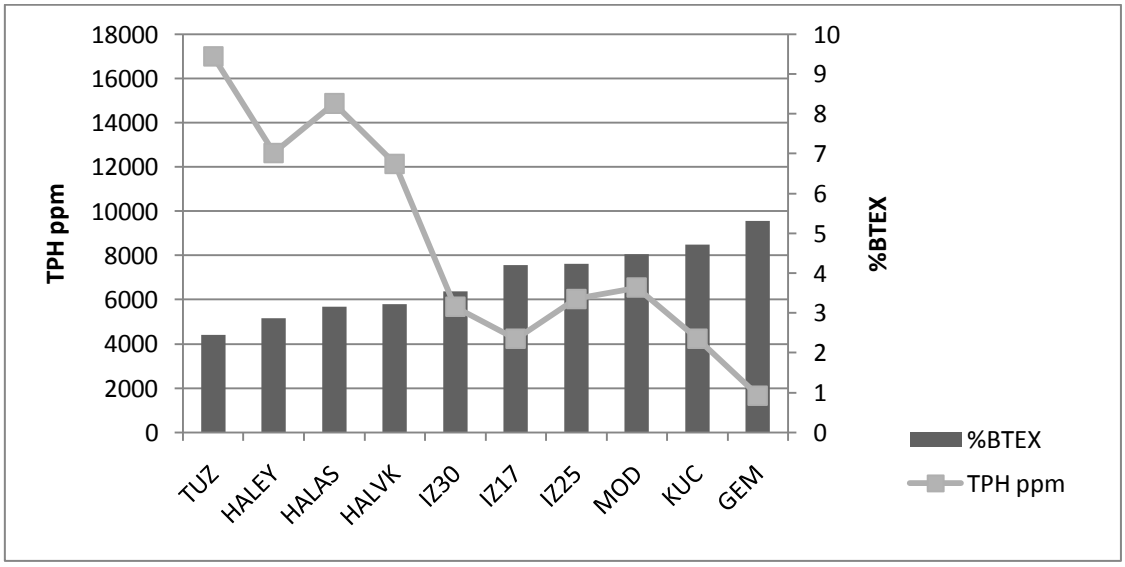

Figure 7.8: Locational BTEX % in TPH (Kolukırık , 2009)

As it seems on the graph, the two parameters TPH content of the sediment and BTEX % in TPH are inversely proportional. It can be interpreted that when the TPH level is high, it

increases the percentage of bssA and this causes an increase on the BTEX degregation so that the TPH percentage in BTEX decreases

7.2.2 Seasonal BTEX Levels

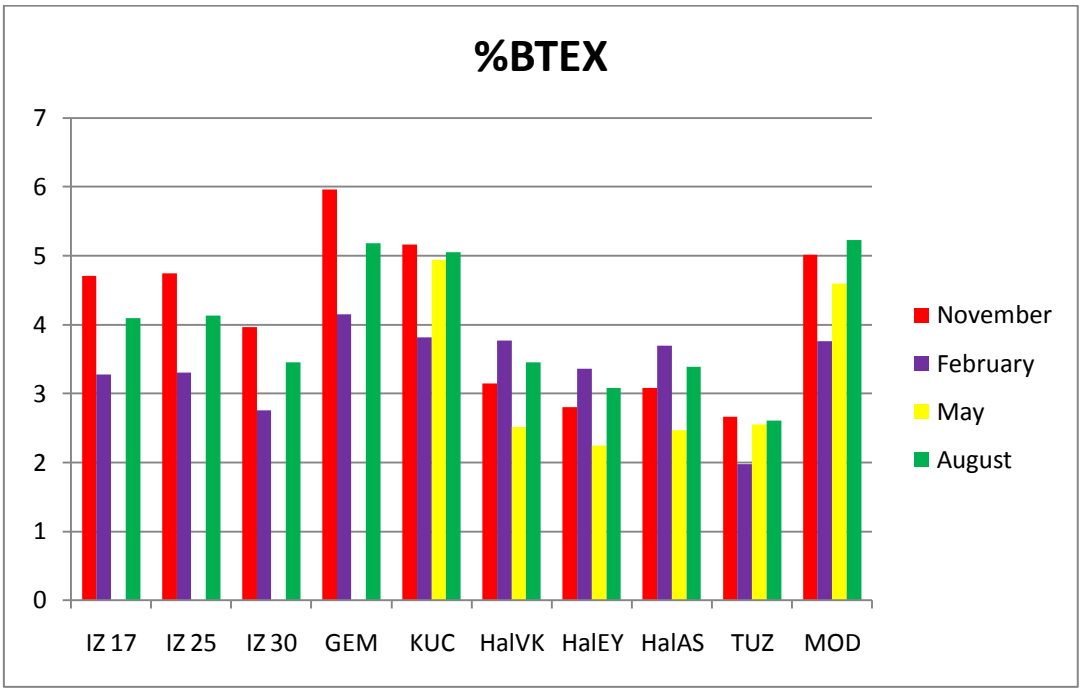

Figure 7.9. Seasonal analyses of % BTEX in MSS

The BTEX level in all sampling locations were considered by 4 months, which were Nowember , February ,May and August as representative for seasons. Generally, there wasn't any relation between the season and the BTEX level were determined. But except Haliç sediments the highest BTEX levels were in November and the lowest were in February. In Haliç sediments , the lowest levels of BTEX were obtained in May and the highest levels were in February.

7.3.Q-PCR Results -The bssA gene Abundance

Table 7.3: Quantitative estimates of Q-PCR for AnBTEXDeg. Number of cells were given as cells/mgSediment.

		%	% ±	cells cm-3	cells cm-3 ±	
Location	**Sample**	**bss**	**bss**	**Total cells**	**Total cells**	**# bss**
İZMİT 17	Iz17Aug05	8	0,8241	4,E+09	3,88E+08	2,63E+08
	IZ17Nov05	10	0,692244	5,E+09	3,26E+08	2,42E+08
	IZ17Feb06	7	0,791136	3,E+09	3,72E+08	3,78E+08
	Iz17Nov06	9	0,725208	4,E+09	3,41E+08	4,57E+08
İZMİT 25	IZ25Aug05	12	0,70668	1,E+10	5,88E+08	5,44E+08
	IZ25Nov05	14	1,554696	1,E+10	1,29E+09	3,45E+09
	IZ25Feb06	9	1,319136	8,E+09	1,10E+09	2,04E+08
	IZ25Nov06	13	1,166022	1,E+10	9,69E+08	4,36E+09
İZMİT 30	IZ30Aug05	11	0,88632	5,E+09	3,99E+08	5,39E+09
	IZ30Nov05	13	0,66474	6,E+09	2,99E+08	3,19E+08
	IZ30Feb06	9	1,240848	4,E+09	5,59E+08	6,52E+09
	IZ30Nov06	12	1,828035	5,E+09	8,23E+08	6,52E+09
GEMLİK	GEMAug05	3	0,19488	1,E+10	6,98E+08	3,54E+08
	GEMNov05	4	0,350784	1,E+10	1,26E+09	3,87E+08
	GEMFeb06	3	0,155904	9,E+09	5,58E+08	7,38E+08
	GEMNov06	4	0,393008	1,E+10	1,41E+09	7,76E+09
K.ÇEKMECE	KUCAug05	8	0,805171429	7,E+10	6,69E+09	7,05E+09
	KUCNov05	10	0,676344	8,E+10	5,62E+09	4,60E+08
	KUCFeb06	6	0,772964571	5,E+10	6,42E+09	8,42E+09
	KUCNov06	9	0,708550857	7,E+10	5,89E+09	5,53E+08
	KUCFeb07	7	0,434792571	6,E+10	3,61E+09	1,15E+09
	KUCMay07	9	0,974257429	7,E+10	8,10E+09	6,69E+08
	KUCAug07	10	1,40905	8,E+10	1,17E+10	1,10E+10
HALİÇ VK	HalVKNov06	24	2,13273	5,E+10	4,41E+09	1,40E+09
	HalVKFeb07	28	2,274912	6,E+10	4,71E+09	7,96E+08
	HalVKMay07	19	0,94788	4,E+10	1,96E+09	1,33E+10
	HalVKAug07	26	3,649338	5,E+10	7,55E+09	1,66E+09
HALİÇ EY	HalEYNov06	27	3,98925	1,E+11	1,65E+10	1,59E+10
	HalEYFeb07	32	1,91484	1,E+11	7,93E+09	1,72E+10
	HalEYMay07	0	1,91484	9,E+10	7,93E+09	7,43E+09
	HalEYAug07	29	1,75527	1,E+11	7,27E+09	1,87E+10

Table 7.3 (Contn'd): Quantitative estimates of Q-PCR for AnBTEXDeg. Number of cells were given as cells/mgSediment.

HALİÇ AS	HalASNov06	29	3,191595	1,E+11	1,58E+10	2,67E+10
	HalASFeb07	35	3,48174	2,E+11	1,72E+10	1,16E+10
	HalASMay07	23	1,624812	1,E+11	8,05E+09	2,85E+10
	HalASAug07	32	3,829914	2,E+11	1,90E+10	1,41E+10
TUZLA	TUZAug05	32	2,58384	1,E+11	1,10E+10	2,93E+10
	TUZNov05	39	2,325456	2,E+11	9,92E+09	1,67E+10
	TUZFeb06	26	2,842224	1,E+11	1,21E+10	4,17E+10
	TUZNov06	36	4,973892	2,E+11	2,12E+10	3,60E+10
	TUZFeb07	29	2,616138	1,E+11	1,12E+10	3,54E+10
	TUZMay07	36	2,842224	2,E+11	1,21E+10	4,22E+10
	TUZAug07	40	2,018625	2,E+11	8,61E+09	5,05E+10
MODA	MODFeb06	12	1,712032	9,E+10	1,26E+10	4,45E+10
	MODNov06	15	2,201184	1,E+11	1,62E+10	6,00E+10
	MODFeb07	10	0,5869824	7,E+10	4,33E+09	5,38E+10
	MODMay07	13	1,2106512	1,E+11	8,92E+09	5,38E+10
	MODAug07	15	0,91716	1,E+11	6,76E+09	6,41E+10

We quantified anaerobic BTEX degraders by targeting bssA gene using Q-PCR and calculated the bssA gene abundance as the multiplication of total cell count and bssA %. Copy numbers of bssA genes were directly correlated to cell numbers (Philippot, 2002 and 2005; Da Silva and Alvarez, 2002; Beller , 2002; Zang and Fang, 2006). The bssA gene abundance changed in a range of 2.4×10^{10} - 7×10^{12} during the 2 years monitoring period.

As given in the Table 7.3. , cell concentration in 3 sampling locations of İzmit were lower than all other locations and IZ17 was the lowest compare to IZ30 and IZ25. Cell concentration in the Gemlik sediment was very close to IZ25 , HALVK approx. 5 times higher than Gemlik sediment. Cell concentrations in HALEY, TUZLA and HALAS sediments were very close to each other and shared the highest concentrations.

As seen in the Table 7.3., although number of bss genes in the IZ25 sediment was higher than that of IZ17, Gemlik, and IZ30 sediments in order of magnitude, cell concentration in the Küçükçekmece sediment approx. 5 times higher than that of IZ25 sediment. The number of bss genes in HALVK and MODA sediment were very close and lower than HALEY sediment. HALAS and TUZLA sediments had the highest number of bssA genes in all MSS.

The relative abundance of the bssA in the total microbial community changed in ranges of 3-40 % sediment respectively during the 2 years monitoring period. (Figure 7.9.) Seasonal changes in sediments were shown in the Figure , 7.11, 7.12., 7.13. ,7.14. , 7.15. and 7.16. separately. Haliç and İzmit samples were shown together.

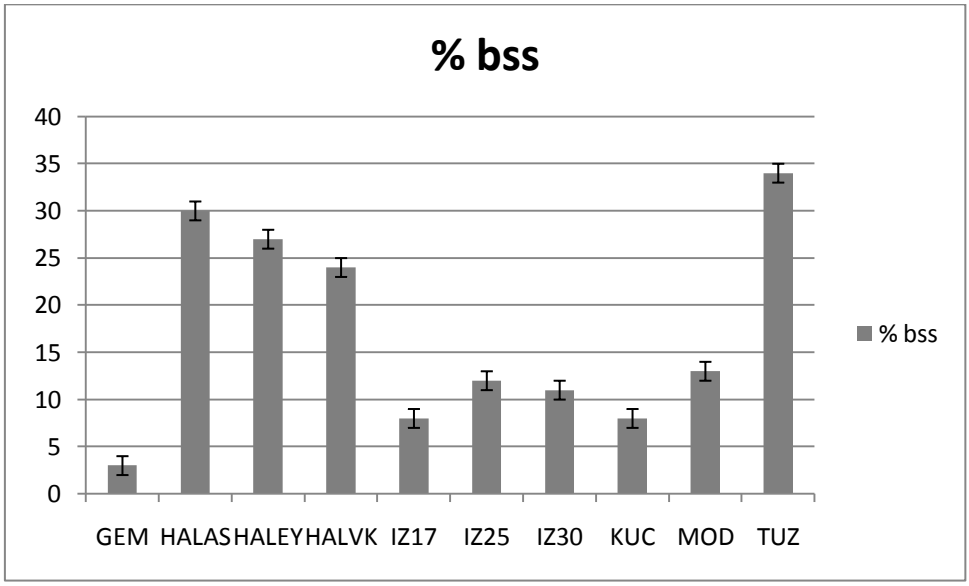

Figure 7.9: The relative abundance of the bssA in the total microbial community

The relative abundance of the bssA in the total microbial community changed in ranges of 3-40 % with an avarage of 17 %. Gemlik has the lowest percentage of all sampling points. Although the relative abundance of bssA gene in MOD sediment is higher than that of IZ17, KUC,IZ30 and IZ25 sediments in order of magnitude,the abundance of the HALVK sediment approx. 2 times higher than that of MOD sediment. The relative abundance of bssA gene in all Haliç sampling locations and Tuzla sediments are higher than the average of Marmara Sea Sediments.

Here we reported for the first time quantification anaerobic hydrocarbon degraders in marine sediments by targeting catabolic gene associated with anaerobic toluene and xylene degradation (bssA) (Da Silva and Alvarez, 2007) AnBTEXDeg constituted considerably high fraction (3-34%) of the MSS prokaryotic community.(Figure 7.10) Although total cell was most abundant in HALAs sediment (1,47 x 10^{11}) AnBTEXDeg was most abundant in the Tuzla Sediment (4,93x10^{10}).

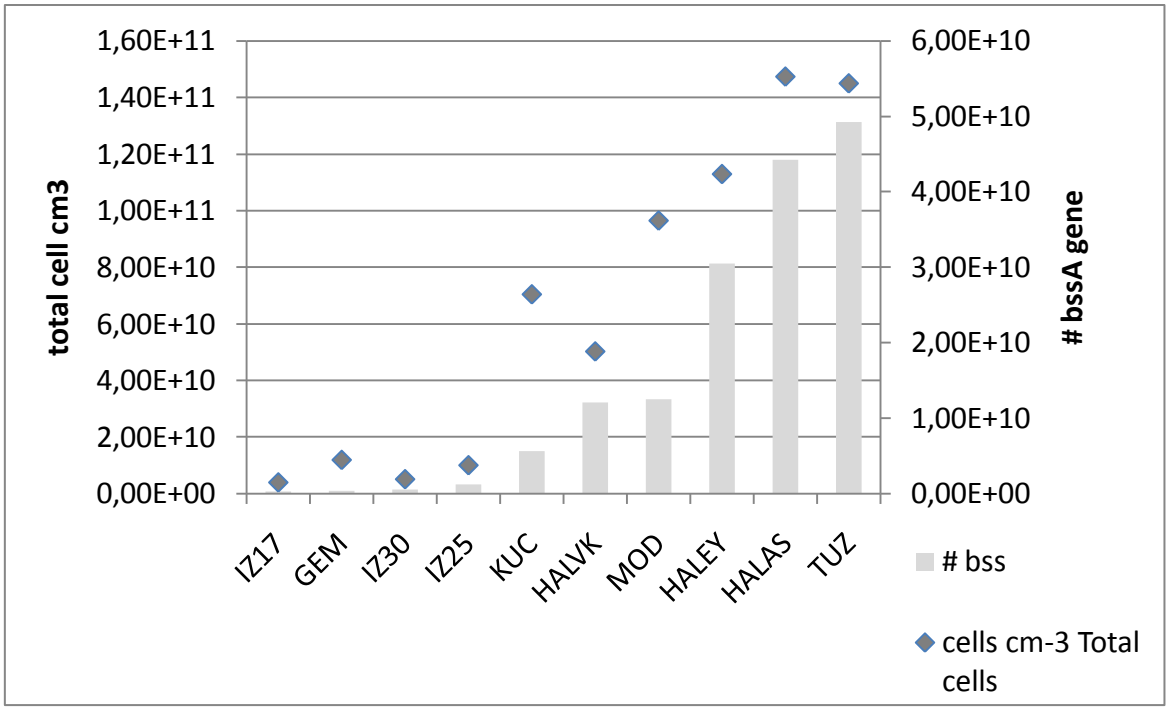

Figure 7.10: Comparison of the total cell count and the # bssA gene

The number of total cell changed in a range of $3{,}97 \times 10^{9}$ - $1{,}47 \times 10^{11}$ during the 2 years monitoring period. Total cell counts of the MSS were higher than the previously reported total cell count ranges (10^{8} – 10^{10} cells/cm^{3}) for marine sediments (Schippers and Neretin, 2006; Smith and D'Hondt, 2006) The bssA gene abundance changed in a range of 2.4×10^{10} - 7×10^{12} during the 2 years monitoring period.

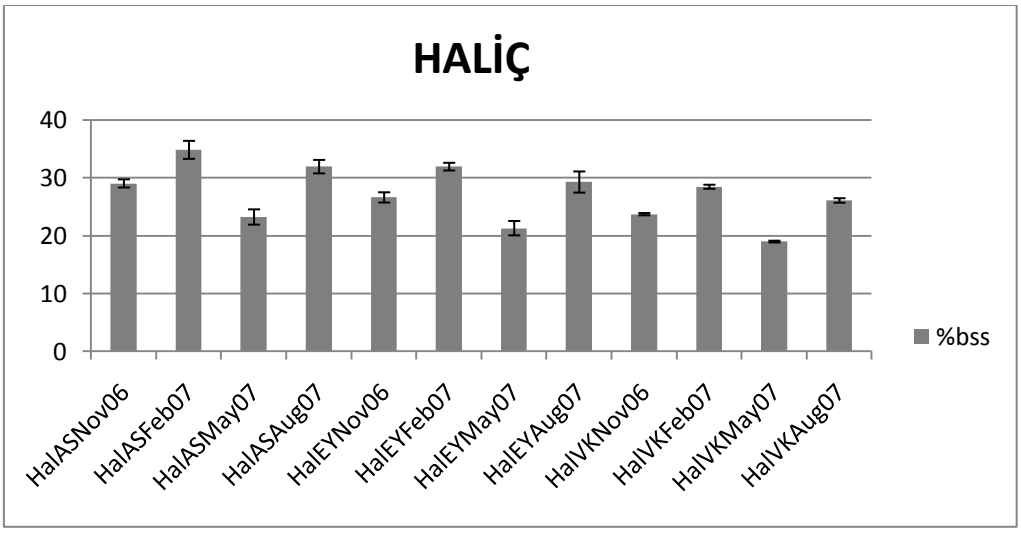

Figure 7.11: The relative abundance of bss genes in Haliç locations and HALAS, HALEY, HALVK sampling points.

In 3 differennt sampling points in Haliç , the relative abundance of the BssA gene was % 19-32 of the total community. Even the minimum BssA gene abundance in Haliç sediments ,were higher than the average of MSS (17 %).

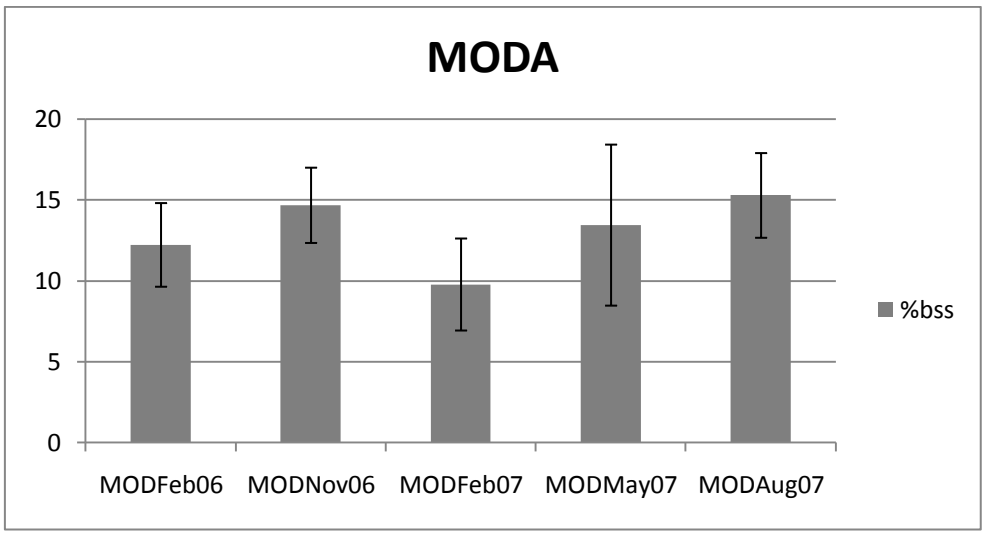

Figure 7.12: The relative abundance of bss genes in MODA sediment

In Moda sediment the relative abundance of the BssA gene was % 10-15 of the total community . Even the maximum BssA gene abundance in Moda sediment was lower than the average of MSS (17 %)

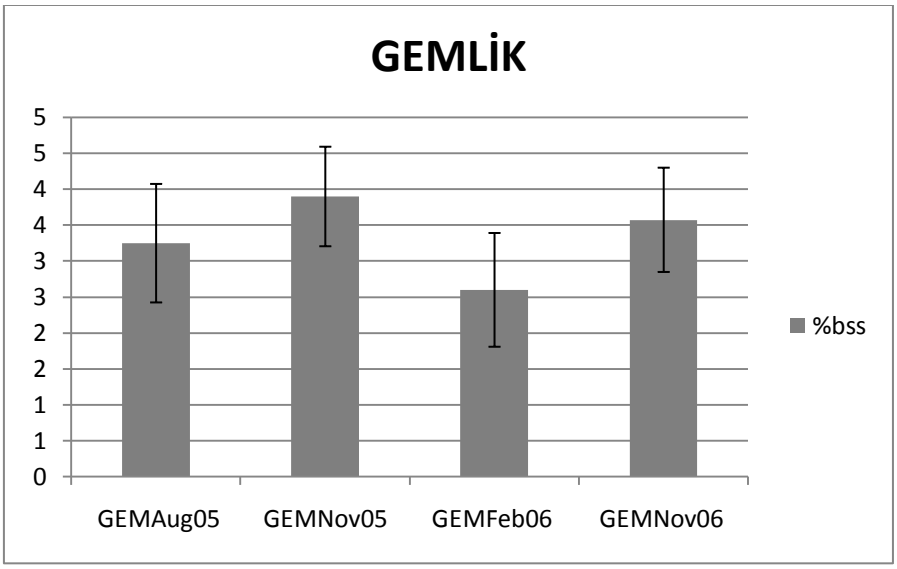

Figure 7.13: The relative abundance of bss genes in GEMLİK sediment

In Gemlik sediment the relative abundance of the BssA gene is % 3-4 of the total community. Although relative abundance of BssA gene in the Gemlik sediments was the low est compared to that of orher sampling locations, the bssA gene abundance in IZ17 sediment

was lower than in Gemlik sediment.The minimum BssA gene abundance in Gemlik sediment was much lower than the average of MSS (17 %).

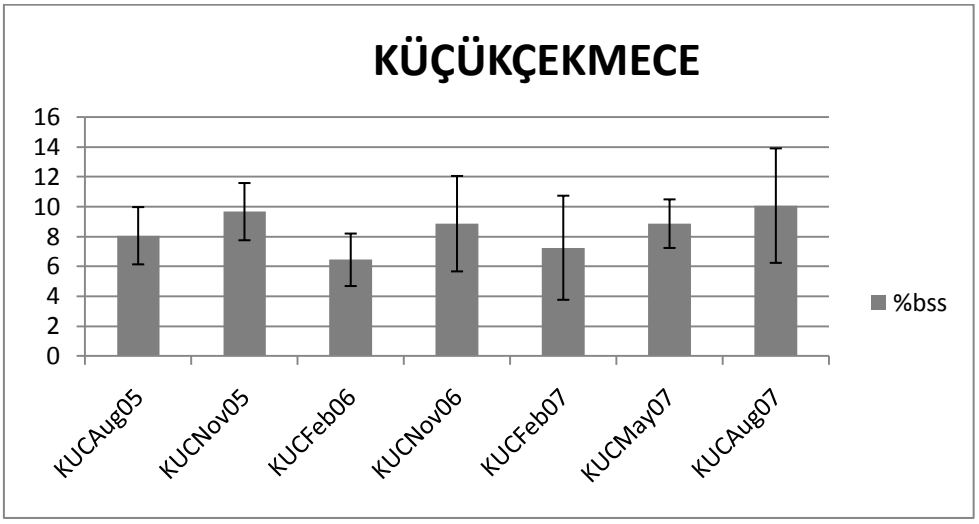

Figure 7.14: The relative abundance of bss genes in KÜÇÜKÇEKMECE sediment

In Küçükçekmece sediment, the relative abundance of the BssA gene is % 6-10 of the total community which was very low , but the BssA gene abundance was higher relatively. Even the maximum BssA gene abundance in Küçükçekmece sediment was lower than the average of MSS (17 %)

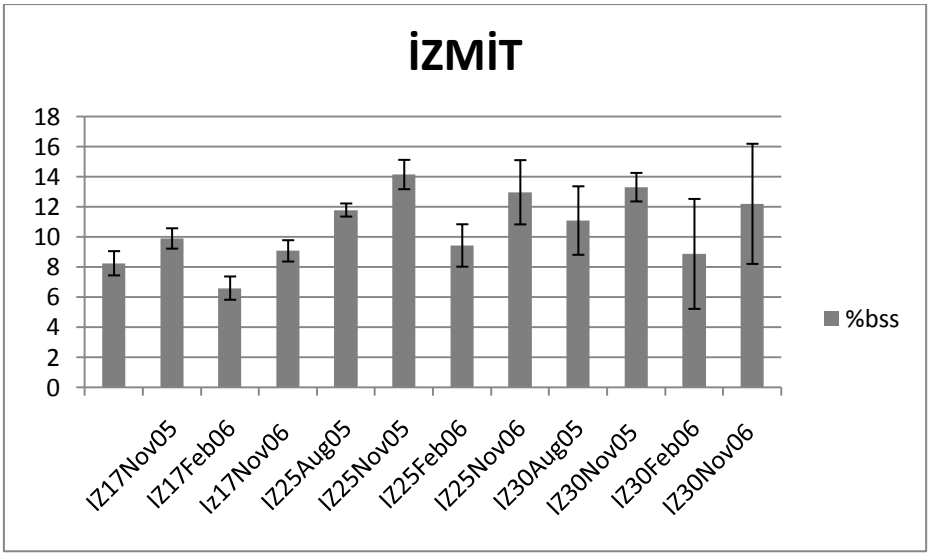

Figure 7.15: The relative abundance of bss genes in İZMİT locations and IZ17, IZ25 and IZ30 sampling point sediment.

In 3 different sampling points in İzmit ,the relative abundance of the BssA gene is % 7-14 of the total community. The maximum BssA gene abundance in İzmit sediments were much lower than the average of MSS (17 %)

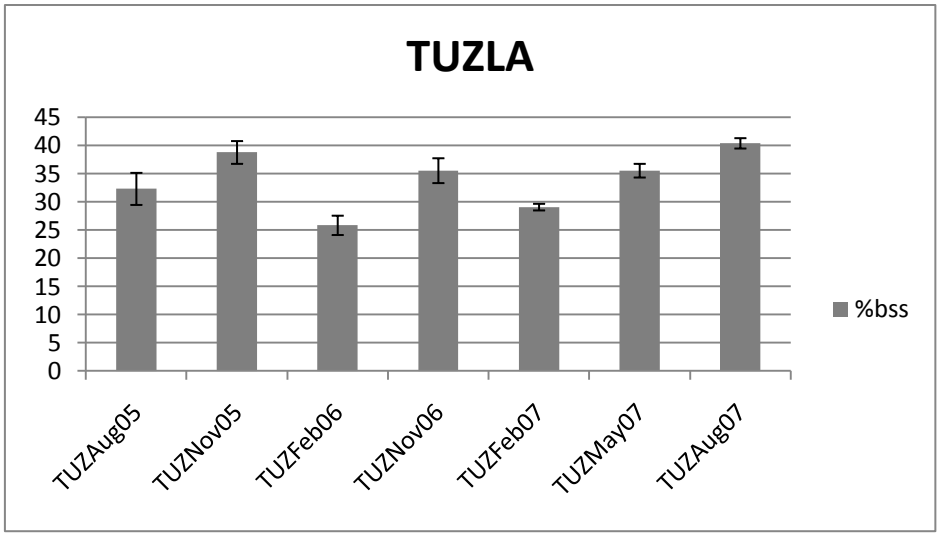

Figure 7.16: The relative abundance of bss genes in TUZLA sediment

In Tuzla sediment the relative abundance of the BssA gene is % 26-40 of the total community. Even the minimum BssA gene abundance in Tuzla sediment was much higher than the average of MSS (17 %) Although the total cell in the Tuzla sediments was lower compared to that of HALAs locations, the bssA gene abundance in Tuzla sediment was the highest in the whole MSS

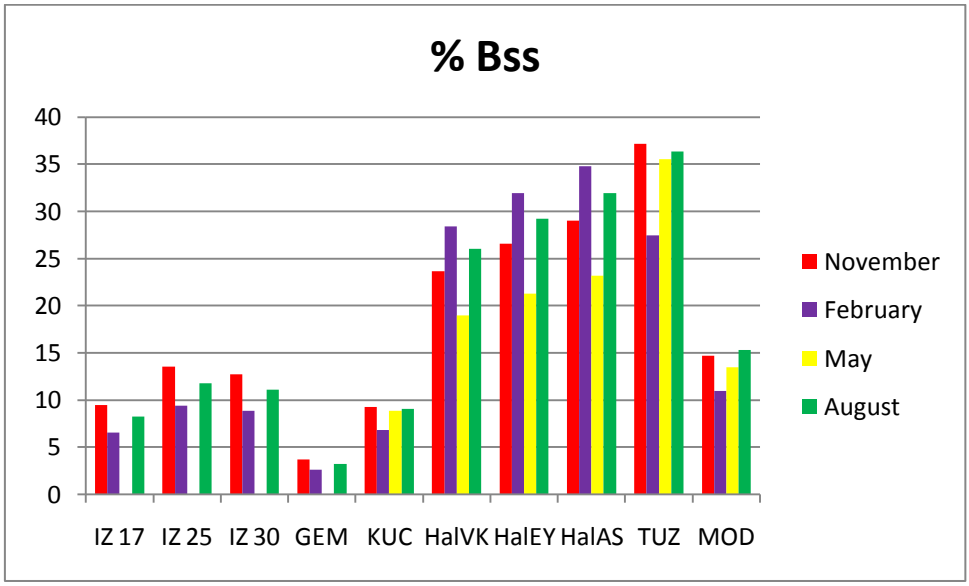

Figure 7.17: Seasonal changes in % BssA gene

The BssA gene abundance in all sampling locations were considered by 4 months , which were Nowember , February ,May and August as representative for seasons. Generally, there was no significant relation between the season and the bssA gene abundance were

determined. But except Haliç sediments the highest bssA percentages were in November and the lowest were in February. In Haliç sediments , the minimum bssA gene abundance were reached in May and the maximum levels were in February.

7.4 Correlating the bss gene abundance with the MSS Characteristics

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

The correlation analyses was made by MiniTab Programme. MSS physicochemical and microbiological characteristics from the Tubitak 105Y307 Project was correlated with the AnBTEXDeg abundancy. As seen, the bss gene abundance were strongly related to the elemental composition and petroleum hydrocarbon content of the sediments. The parameters for corelation analyses were shown in the Table 7.3.

For heavy metal characterization Cr, Zn, Pb, Mn, Fe, Cu, Ni was chosen. The physical characteristics of MSS which are salinity, pH , temperature and sediment grain size ;and the carbon ,nitrogen , phosphorus ratio (C/N/P) was evaluated as elemantal composition. The petroleum hydrocarbon and their fractions which are aromatics, aliphatics, asphaltene and resene was chosen for characteristics of MSS.

Total cell both with DAPI count and Q-PCR count , and their activity which was evaluated in RNA level was used. Genes responsible for Anoxic N Cycle, Sulfate Reduction, Methanogenesis, Hydrocarbon Degradation and their transcripsts were used in corelation analyses.

7.4.1 Petroleum Hydrocarbons

The total petroleum hydrocarbon (TPH ppm) was strongly correlated to the genes which are responsible for anaerobic hydrocarbon degradation. The biodegrable fractions of total petroleum hydrocarbons , aliphatics and aromatics were lower where the bss gene was abundant. This indicated that the bss gene was responsible for the anaerobic aromatics degradation. At the same time the nonbiodegradable part of the total petroleum hydrocarbons , asphaltene and resene were negatively correlated to the bss gene abundance. This is because when the percentage of biodegradables were low , nonbiodegradables started to accumulate in the sediment and the percentage of asphaltene and resene were getting to increase.

Table 7.4: Corelation Analyses Parameters (Kolukirik, 2009).

HEAVY METALS	Cr, Zn, Pb, Mn, Fe, Cu, Ni
PHYSICAL CHARACTERISTICS	Salinity, pH, Temperature, Sediment Grain Size
ELEMANTAL COMPOSITION	C/N/P
ANIONIC CONTENT	NO3- , SO4-
PETROLEUM HYDROCARBONS	TPH, Aliphatics, Aromatics, Asphaltene ,Resene
TOTAL CELL COUNT/ACTIVITY	Dapi Count, Q-PCR Count, RNA Level
GENES/TRANSCRIPTS	Responsible for Anoxic N Cycle, Sulfate Reduction, Methanogenesis, Anaerobic Hydrocarbon Degradation

7.4.2 C-N-P Content- The abundance of bss gene

The TOC content of the sediment was strongly correlated to the bss gene abundance . The bssA gene 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 claimed that different concentrations and ratios of N and P will decide for the organisms most able to utilize the nutrients at the levels provided in the habitat.

The dissolved level of N-P was directly related to the active part of the total cell content , instead of 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 , 2002). In other words, N and P were limited in the MSS pore waters for biological activity.

7.5 Correlation Analyses Results

According to the correlation analyses results, all correlation relations were shown in the Figure 7.17.

A very useful, often used, statistic that quantifies the link between two variables is the correlation coefficient, r, which has a value between +1 and -1. When r is close to +1, positive

corelation, an observation with a high value for one variable will likely have a high value for the other variable. The correlation coefficient, r, is close to zero if there is little association between the variables. When r is close to -1, negative corelation,an observation with a high value for one variable will likely have a low value for other variable.

The correlation coefficient ,r, higher than 0,9 and lower than – 0,9 were taken under consideration and the relations were shown in the Figure7.17. To get confidence intervals for the prediction, the p values under 0,005 were selected. ($p < 0.05$, $n = 47$)

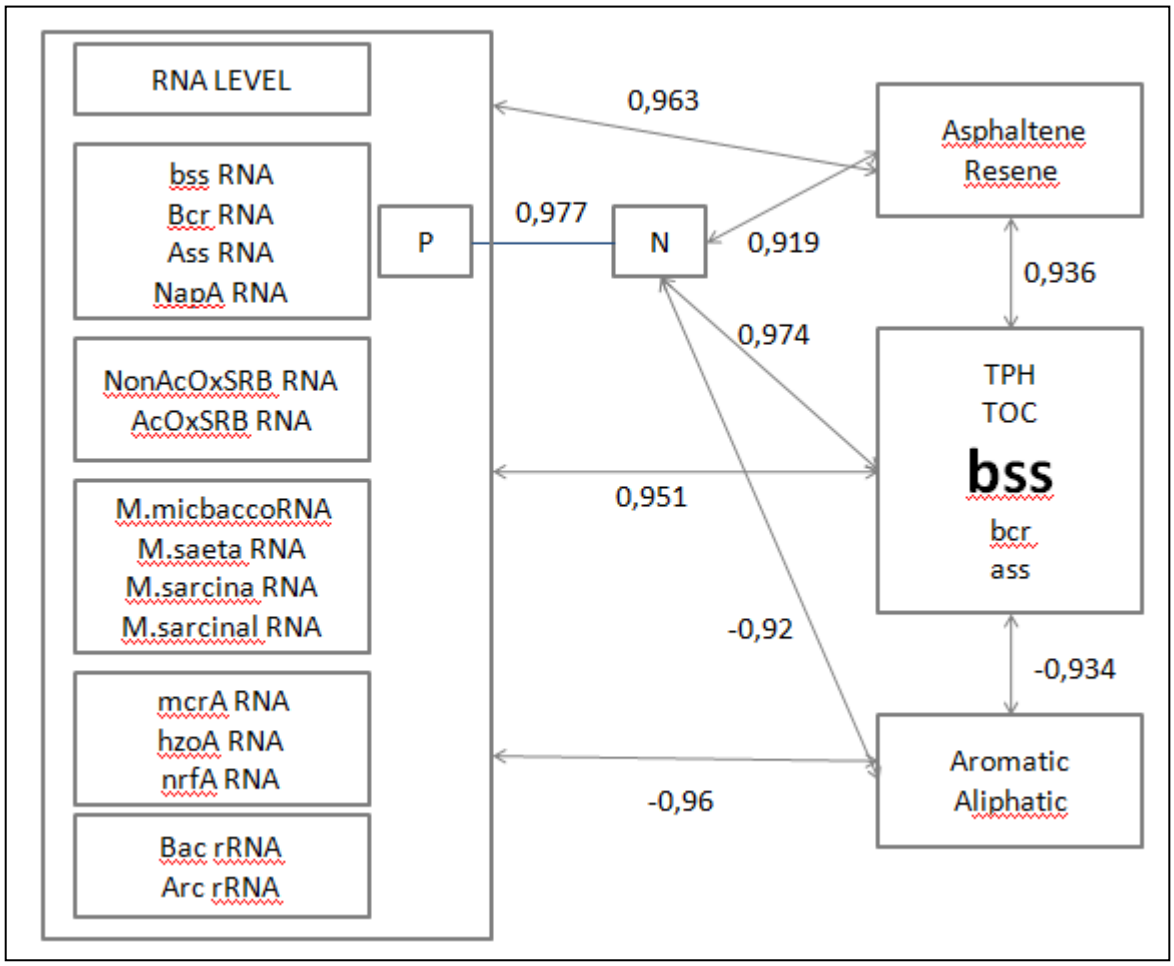

Figure 7.18: The relation with the bss gene abundance and the MSS characteristics.

The parameters were being in the same box , have a correlation more than 0,999 which means with a high value for bss gene will likely have a high value for bcr gene , ass gene, TPH ppm and TOC mg/L. This was meaningful because, the genes were abundant where the electron donors were high.

In fact that r is positive and near 1 in value indicates that the bss gene abundance tends to increase as the percentage of asphaltene and resene increase. When the r value is negative

and near 1 reveales that the bss gene abundance tends to increase as the percentage of aromatics and aliphatics decrease.

Statistically significant high correlations ($r>0.90$, $p<0.05$) between the P and N content of the sediment porewater and all of the gene tarnscript levels were obtained.

Also the percentage of asphaltene and resene were positively correlated to N and P content of the sediment porewater. Aromatic and aliphatic %s were also negatively correlated to the N and P content.

8.CONCLUSION

It has been known that the MSS were very rich in terms of hydrocarbon, nitrate, Ni and microbial cell content whereas N and P were limited in the porewaters. These characteristics indicated that MSS was a favorable candidate for investigating the anaerobic hydrocarbon degradation. BTEX, which are priority environmental pollutants, can be degraded in the absence of molecular oxygen as long as the other potential electron acceptors are present. We affirmed the possibility of anaerobic BTEX degradation by quantifying the bssA gene abundance in MSS which was affected by the level and type of nutrient sources and electron donors. In order to analyse the relationship among the prokaryotes we also correlated the bssA gene abundances and the other genes, responsible for the degradation of aromatic hydrocarbons (bcr) and aliphatic hydrocarbons (assA) presence.We detected strongly positive correlation among the bcr, assA and bssA genes, which may indicate that these genes were carried out by the same organisms. Here we also reported the first application of the generic bssA PCR primer in order to quantify the catabolic genes associated with anaerobic toluene and xylene degradation in marine sediments. Our results showed that the abundance of bssA gene was a good representative to analyse the quantification of the AnBTEXDeg .

In this study , 10 most polluted regions in Marmara Sea were selected for taking samples . The sediment samples were examined with the help of molecular techniques which we obtained faster and more precise results rather than traditional techniques. Q-PCR was an excellent application in order to reveal the quantification of AnBTEXDeg. Real Time PCR approach of bssA gene showed the presence of AnBTEXDeg in all MSS and refer to high potential of degradation in Haliç and Tuzla sediments .Correlation analyses was utilized to understand what may control the abundance of the bssA genes , thereby , the AnBTEXDeg.

Correlating abundance of the bssA genes with other geochemical and microbiological characteristics of the sediments revealed that there was a high potential in the Marmara Sea sediments for degradation of aromatic hydrocarbons under nitrate reducing conditions. Winderl . 2008, Higashioka . 2009).

The bssA gene abundance changed in a range of 2.4×1010 - 7×1012 during the 2 years monitoring period. The bssA was abundant where total petroleum hydrocarbon (TPH) levels are high, and BTEX % in TPH was negatively correlated to the relative abundance of the

bssA in the total microbial community (3-40 %). The changes in the microbial community structure occurred in terms of relative abundances of the microbial species rather than the species types present. Local and seasonal differences in the abundance of AnBTEXDeg were strongly related to the changes in the elemental compositions in the MSS. According to the TUBITAK-105Y307 project on bioremediation of petroleum hydrocarbons, 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.

Our results revealed that the Marmara Sea Sediments seems to be a promising candidate for further investigation of microbial BTEX degradation under anaerobic/anoxic conditions. For this reason with the help of the other studies , laboratory scale hydrocarbon degradation microcosms which were inoculated using the MSS under denitrifying and methanogenic conditions was the first step of further investigations. Microcosm studies verified that it was possible to degrade most of the aliphatic and aromatic hydrocarbons and also uncovered that the 10 fold increase of hydrocarbon degradation potential of the MSS by N-P amandment.

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CURRICULUM VITA

Candidate's full name:	**Canan Zöhre KETRE**
Place and date of birth:	**İstanbul , 01.04.1984**
Permanent Address:	**Marmara Sitesi E BLOK No:9 34347 Ortaköy İstanbul**
Universities and Colleges attended:	**İstanbul Technical University**