İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

DETERMINING IDENTITY OF ANAEROBIC/ANOXIC HYDROCARBON DEGRADING MICROBIAL SPECIES IN TUZLA AND HALIC BAY SEDIMENTS

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Programme : **Environmental Biotechnology**

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TUZLA VE HALİÇ KOYLARINDA BULUNAN ANAEROBİK/ANOKSİK HİDROKARBON YIKIMINDAN SORUMLU MİKROBİYAL TÜRLERİN BELİRLENMESİ

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ABBREVIATIONS

AODC : Acridine Orange Direct Count
AOM : Anaerobic Oxidation of Methane
APS : Amonium Per Sulphate
assA : Alkylsuccinate Synthase
bcrA : Benzoyl Coenzyme A Reductase
bssA : Benzylsuccinate Synthase
BTEX : Benzene, Toluene, Ethylbenzene and Xylene Isomers
CARD : Catalyzed Reporter Deposition-Fluorescence In Situ Hybridization
CH_4 : Methane
DAPI : 4',6-diamidino-2-phenylindole
DGGE : Denaturing Gradient Gel Electrophoresis
DNA : Deoxyribonucleic Acid
dsrA : Dissimilatory Sulphite Reductase
EDTA : Ethylenediaminetetraacetic Acid
EtOH : Ethyl Alcohol
FA : Fatty Acids
Fe : Iron
FISH : Fluorescent In Situ Hybridization
H2S : Hydrogen Sulfide
HC : Hydrocarbons
HC(-) : Without Hydrocarbon Addition
HC(+) : Hydrocarbon Addition
MCG : Miscellaneous Crenarchaeotic Group
Mn : Manganese
MnO_2 : Manganese Dioxide
NH_4 : Ammonia
NL : Nitrogen Limiting Nutrient Supply
NO_2^- : Nitrite
NO_3^- : Nitrate
ODP : Ocean Drilling Program
PAH : Polycyclic Aromatic Hydrocarbons
PCR : Polymerase Chain Reaction
PL : Phosphorus Limiting Nutrient Supply
Q-PCR : Quantitative Polymerase Chain Reaction
RNA : Ribonucleic Acid
rRNA : Ribosomal RNA
SO42- : Sulphate
TAE : Tris-acetate-EDTA
TEMED : N,N,N',N'-Tetramethylethylenediamine
TOC : Total Organic Carbon
TPH : Total Petroleum Hydrocarbon
UL : Unlimited Nutrient Supply

UPGMA : Unweighted Pair Group Method with Arithmetic Mean
WA : Without Electron Acceptor
WH : Without Hydrocarbon

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DETERMINING IDENTITY OF ANAEROBIC/ANOXIC HYDROCARBON DEGRADING MICROBIAL SPECIES IN TUZLA AND HALIC BAY SEDIMENTS

SUMMARY

Marmara sea is a small (size ≈ 70 x 250 km) intercontinental basin connecting Black Sea and Mediterranean Sea. Marmara Sea is one of the inland sea that has been extremely and chronically polluted via mainly oil transportation related accidents and discharges of hydrocarbons and hydrocarbon by products.

Sediments are the final destination of pollutants and organic matters. When the pollutants and/or organics enter aquatic environments, they can follow different biological, physical and chemical pathways but some portion of pollutants and/or organic matters eventually sink and they are accumulated within the sediments. Because of that reason sediments become carbon and nutrient pools for aquatic environments. Sediments of Marmara Sea are highly rich in organic content whereas bioavailable nitrogen and phosphorus are limited in the pore waters. Monitoring studies of Marmara Sea Sediments revealed that there is a diverse microbial life on the subsurface of the seafloor but vast majority of the microorganisms are not metabolically active due to limited nutrients. Thus, biostimulation of the sediments is one of the feasible option for bioremediation of Marmara Sea sediments.

In this study, microbial population dynamics in nutrient amended hydrocarbon degradation microcosms seeded with Halic and Tuzla Bay sediments under methanogenic and nitrate reducing conditions were investigated. The population dynamics was studied via Denaturing Gradient Gel Electrophoresis (DGGE) of microcosm samples and previous clone libraries from Halic and Tuzla Bay sediments. In this way, possible dominant anaerobic hydrocarbon degrading species were determined.

DGGE results revealed that microbial community within the nutrient amended hydrocarbon degradation microcosms changed over time. In nutrient amended hydrocarbon degradation microcosms seeded with Halic Bay sediments, 13 bacterial and 17 archaeal species were found to be correlated with hydrocarbon degradation ($p<0.05$ $r>0.95$, $n=3$). Also 6 bacterial and 4 archaeal species from nutrient amended hydrocarbon degradation microcosms seeded with Tuzla Bay sediments were found to be correlated with hydrocarbon degradation ($0,05<p<0,1, r>0.90$, $n=3$). These correlated microbial species are thought to be possible candidates for petroleum hydrocarbon degradation.

TUZLA VE HALİÇ KOYLARINDA BULUNAN ANAEROBİK/ANOKSİK HİDROKARBON YIKIMINDAN SORUMLU MİKROBİYAL TÜRLERİN BELİRLENMESİ

ÖZET

Marmara denizi, Karadeniz ve Akdenizi birbirine bağlayan, küçük (boyutu ≈ 70 x 250 km) bir denizdir. Marmara Denizi aşırı ve kronik bir şekilde, genellikle petrol taşımacılığına ilişkin tanker kazaları ile, hidrokarbonların ve hidrokarbon yan ürünlerinin deşarjları sonucunda kirletilmiş bir iç denizdir.

Sedimentler kirleticilerin ve organik maddelerin nihai durağıdır. Kirleticiler ve/veya organik maddeler sucul ortamlara girdiklerinde farklı biyolojik, fiziksel ve kimyasal yolakları izleyebilirler fakat bu kirleticilerin ve /veya organik maddelerin belirli bir kısmı mutlak suretle çökerek sedimentlerde birikmektedir. Bu nedenden dolayı sedimentler sucul ortamların karbon ve nutrient kaynaklarıdır. Marmara Denizi sedimentleri organik içerik açısından zengin olmasına rağmen sediment boşluk suyunda bulunan biyolojik olarak kullanılabilir azot ve fosfor miktarı kısıtlıdır. Marmara Denizi sedimentlerini izleme çalışmaları deniz tabanı yüzeyi altında geniş mikrobiyal bir yaşantının varlığını açığa çıkarmıştır ancak, mikroorganizmaların büyük çoğunluğu kısıtlı bulunan nütrientler nedeniyle metabolik olarak aktif değildir. Bu nedenle biyostimülasyon teknikleri Marmara Denizi sedimentlerinin biyoıslahı için elverişli bir seçenek olarak düşünülmektedir.

Yapılan bu çalışmada, metanojenik ve nitrat indirgeyici şartlarda, Haliç ve Tuzla körfezlerinden alınan sedimentler ile kurulan nutrient eklenmiş mikrokozmozlardaki mikrobiyal populasyon dinamikleri incelenmiştir. Populasyon dinamikleri, mikrokozmos örneklerinin Denatüre edici Gradiyent Jel Elektroforezi yöntemiyle ve daha önceden Tuzla ve Haliç Körfezlerinden alınan sedimentlerden oluşturulmuş klon kütüphaneleri ile çalışılmıştır. Bu yöntemler ile muhtemel baskın anaerobik hidrokarbon yıkımı yapan türler belirlenmiştir.DGGE sonuçları, nutrient eklenmiş hidrokarbon yıkım mikrokozmozlarında bulunan mikrobiyal komunitenin zamanla değiştiğini göstermiştir.Haliç körfezi sedimentleri ile kurulmuş hidrokarbon yıkım mikrokozmozlarında 13 bakteriyel ve 17 arkeyal tür hidrokarbon yıkımı ile anlamlı şekilde korele olduğu görülmüştür. ($p<0.05, r>0.95, n=5$). Ayrıca Tuzla körfezinden alınan sedimentler ile kurulan mikrokozmozlarda 4 arkeyal ve 6 bakteriyal türün anlamlı olarak hidrokarbon yıkımı ile korelasyon gösterdiği gözlemlenmiştir ($0.05<p<0.1$, $r>0.90$, $n=5$). Hidrokarbon yıkımıyla korele olan bu türlerin, petrol hidrokarbonlarının yıkımından sorumlu olduğu düşünülmektedir.

1. INTRODUCTION

Petroleum and petroleum by products have been utilized by humans throughout history. By the Industrial revolution, relationship between man and this valuable energy source became so irreplaceable that searching and the refining the crude oil have been a great competition all around the nations. The annual world production of crude oil is around 70 million barrels per day (Kilpatrick, 2007). Transportation of such quantities of crude oil has been aroused pollution risks to aquatic environments since nearly 50% of petroleum transported by sea. Majority of spilled petroleum hydrocarbons sink to bottom of the sea floor, called sediments through the water column. When the hydrocarbons reaches the sediments, they are absorbed and/or adsorbed within the particles of the sediments so these carbon rich organics began to accumulate on the subfloor of the marine environments. If renewal capacity of aquatic environment is lower than organic pollution rate, oxygen concentration of the sediments can reach critically low values. As a result of decreasing oxygen concentration values, marine sediments become anoxic (Kilpatrick, 2007).

Marmara sea is one of the unfortunate inland sea that has been extremely and chronically polluted via mainly oil transportation related accidents (these accidents includes the ones that happened in Black Sea because currents carry petroleum to the Marmara Sea), and discharges of petroleum hydrocarbons and related products without any treatment (Tolun, 2006; Alpar, 2004; Kucuksezgin , 2006). Pollution in the Marmara Sea has become chronic and renewal capacity is not enough to remedy the pollutants. As the pollution rate increases, oxic sediments are gradually become anoxic in the Marmara Sea so anaerobic and anoxic processes became dominant throughout the years (DSI, 2004).

Physical and chemical methods are capable of rapidly removing majority of the petroleum hydrocarbons from a contaminated area, but, in most cases, they are not feasible in terms of remediation cost and rarely completely successful. Bioremediation methodologies are promising technology for oil spillage. These technologies are mainly focused on stimulation of aerobic microorganisms that can be responsible of biodegradation of petroleum. Utilization of aerobic possesses is not

feasible due to physiochemical characteristics of the Marmara Sea sediments (Kolukırık, 2010). The most available bioremediation technique for Marmara Sea was revealed by Kolukırık, is the nutrient amendment (bioavailable nitrogen & phosphorus) to marine sediments that aiming the stimulation of anaerobic and/or anoxic microorganisms.

Since bioremediation methods are mainly focused on microorganisms, investigation of microbial communities plays crucial role in successful bioremediation. Although there are many studies about identification of microbial communities, majority of them are based on traditional microbiological methods that are insufficient to detect all microbial species due to cultivation technique's biases. **(**Teske, 2006a; Kormas, 2003; Delille, 1995). Since only 0.1-10 % of microscopically detected prokaryotic cells can be cultivated by using traditional microbiological techniques, DNA/RNA based analyses of environmental samples promises new microbial species as well as information about microbial processes (Moter, 2000; Cases, 2002)

Denaturing gradient gel electrophoresis (DGGE) is one of the widely used molecular tools for determination of population dynamics and diversity of microbial communities (Muyzer, 1993). Qualitative and semi quantitative estimations of diversity can be accomplished via DGGE. DGGE exploits the fact that DNA molecules that have the same length, but differ at least by one nucleotide, can be separated by electrophoresis through a linear gradient of increasing chemical denaturants of urea and formamide. As a result of DGGE experiment, band patterns are obtained.

The DGGE pattern provides a rapid identification of the dominant species. A big advantage of these techniques is that they make it possible to obtain taxonomic information by excising, re-amplifying and sequencing specific DNA fragments or by hybridization analysis with taxon specific oligonucleotides probes (Dorigo, 2005).

The purpose of this thesis is to investigate microbial population dynamics in nutrient amended hydrocarbon degradation microcosms seeded with Halic and Tuzla Bay sediments under methanogenic and nitrate reducing conditions (nitrate used as the final electron acceptor) respectively. The population dynamics was studied via DGGE of microcosm samples and the dominant anaerobic hydrocarbon degrading species were determined with the aid of previously constructed clone libraries from Marmara Sea Sediments by Kolukırık (2010) and statistical analysis.

2. MICROBIAL BIODIVERSITY AND ACTIVITY IN MARINE SEDIMENTS

Aquatic environments cover %70 of the earth surface and most marine bottom is covered with sediments which can consist of different particular size and type. Some sediment is composed of uniform particles and some of them are mixed particles ranging from fine clay to sand. Differentiation in the particle size makes marine sediments largest habitat of the entire planet that marine sediments can cover more than 2-3 times of the earth surface. Most of the sediments lay in the 1000 m depth (Snelgrove, 1997). Marine sediment habitats within these depths are confronted with extreme conditions such as lower temperatures at 2°C, very high pressure (can be up to 1100bar) and absence of appropriate light intensity which has crucial role for photosynthesis.

These extreme conditions may arouse the suspicion that no biological activity can take in place so no life form can exist at the bottom of the oceans however this is not reflecting the truth. Marine sediments provide good chemical components which supports diverse range of living conditions for their inhabitants.

First indication of metabolically active microorganisms leans upon 1980s. During pore water chemistry studies, samples that were obtained by core sampling from 167 meters below the sea floor indicated the potential prokaryotic sulphate reduction and methanogenic activity even though the results were inconclusive (Oremland, 1982; Whelan, 1986). First publications about comprehensive depth profiles of microbial activity, total and viable prokaryotic numbers and estimates of cultured biodiversity was started to submitted at early 90s. Several studies showed the certain connections between activity of the cells and bioavailability of organic carbons with electron acceptors (Cragg, 1990; Parkes, 1995). In 1994 mathematical model was constructed for the logarithmic decline of total prokaryotic cell numbers with sediment depth **(**Parkes, 1994). Afterwards the first culture-independent molecular study to reveal biodiversity in the deep marine subsurface was reported (Rochelle, 1994).

During the 1990s, the importance of the marine sediment habitat were began to realized when it was estimated that the deep subseafloor biosphere embraced one tenth to one- third of the Earth's total biomass and the majority (c. 65%) of the global prokaryotic biomass (Parkes, 1994; Whitman, 1998).

The aim of this chapter is to provide a summary of common properties of typical sediment and current knowledge of prokaryotic activity and biodiversity in subsurface marine sediments.

2.1 Common Characteristics of Marine Sediments

Sediments share some properties with soils and yet are distinct from soil environments for a variety of reasons, which carry great importance to the microbial communities that reside there. Because of sediments are, in general, overlain by a permanent water body. Thus sediments share common property of being continuously wet. Even though chemistry of the water may vary according to the level of primary production and inputs from intrinsic and/or extrinsic factors such as runoffs and anthropogenic activities, all sediments create moisturized habitat.

Depending on water temperature oxygen solubility in waters is limited around 300-400 μM so firstly organic matter is present in the water column should be aerobically degraded which is energetically favourable, when oxygen is depleted, residual organics must be degraded via various electron acceptors. Because of that reason undisturbed sediments almost universally become anoxic with depth. After depletion of oxygen, a series of almost stable horizontal gradients is formed within the sediments. Different electron acceptors, usually in the order of decreasing redox potentials, are dominant at relevant depth of the sediment. The stratification of marine sediments is a function of either anthropogenic or/and intrinsic organic inputs, microbial metabolic abilities, and the geochemistry of the environment (mineral content, salinity, currents etc.). Assuming that mixing is minimal, gradients will be formed whenever the production or consumption of any product or nutrient exceeds the diffusion rate of that product or reactant. Figure 2.1 describes vertical nutrient profiles of Lake Michigan and Black Sea that their profiles are accepted as guidelines for what might be expected while analysis of chemical component of marine or fresh pore water (Froelich, 1979; Reburgh, 1983).

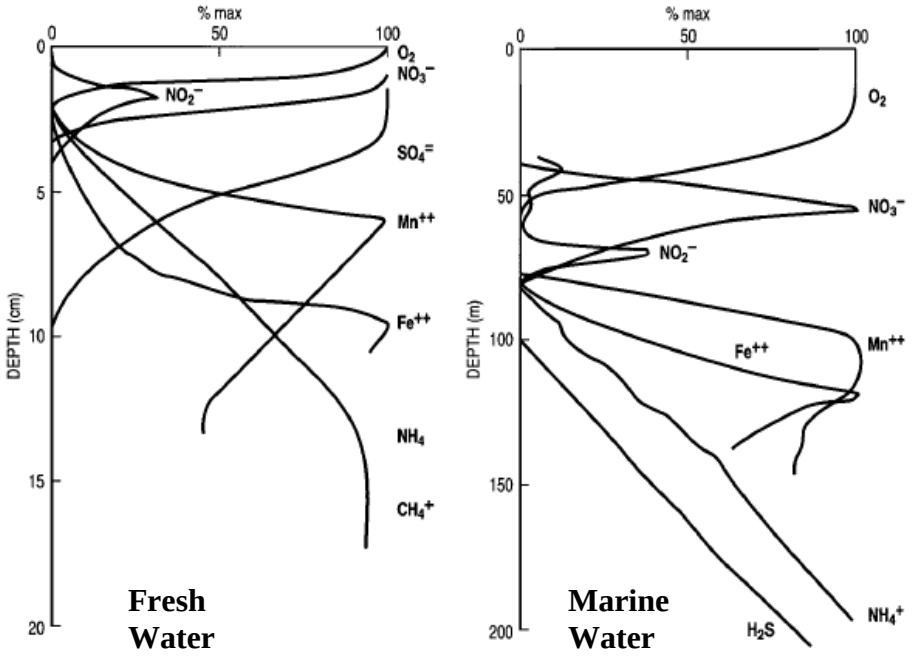


Figure 2.1: Vertical nutrient profiles of typical marine environment (Nealson, 1997).

In Figure 2.1 profiles of fresh water (Lake Michigan) and on the right marine (Blacksea) are shown. The upper regions in these freshwater and marine environments are oxic whereas the deeper parts are anoxic and anaerobic conditions become dominant where methane production is observed. The amount of organic carbon that reaches the sediment is the major function of the oxygen depletion. The primary difference between the freshwater and marine sediments relates to the amount of sulphate in the latter and the resulting dominance of the sulfur cycle, whereas in the freshwater sediments, methane formation is the terminal step, which dominates carbon metabolism at depth. The numbers presented here are percentages of maximum values that may be encountered in these environments: freshwater/marine: O_2, 300–400 µM for both; NO_3^- and NO_2^-, a few µM for both; SO_4^{2-}, 100–200 µM in freshwater; 25mM in marine systems (for this reason sulpfate depletion is often not seen until deep in profiles, and methane production often is minor in marine systems); Mn^{++}, 100µM/10µM; Fe^{++}, 10µM/25 nM; NH_4 , few micromoles in both; H_2S, usually not seen in freshwater, and rarely exceeds a few micromoles in marine systems. In this system, the H_2S is in the micromolar range and will not reach micrometer values until very deep parts (hundreds of meters). Thus, no significant sulphate depletion (sulphate profile not shown) will occur over this range (Nealson, 1997). CH_4 will range from a few nanomoles to saturation, forming

bubbles that are exported out of the system; because of the high sulphate in marine systems, methane is not usually a major component (Nealson, 1997).

The typically stratified sediments give historical information about geochemical and biological events that may reveal the chronological record of what had occurred above the sediments to the some degree because of the microbiological activities which includes competitions for the rich source of energy and minerals deposited in the sediments.

Another important common characteristic of marine sediments is the fact that they are major deposits of both organics and pollutants. Between 5 and 10 billion tons of particulate organics are abundant in oceans. These organics is continuously sinking to the subfloor of the oceans and accumulating as sediments. Even though vast majority of them is degraded by the aerobic community that located near surface, sediments are still the largest global reservoir ($15000x10^{18}$g C) due to accumulation that derived from geological times. Accumulation in the sediments is a function of organic inorganic sources and grain size of the sediments. Sediments which composed of fine-grained clays or silts are more prone to accumulate organics. For example Barent Sea is the first in terms of total annual sediment burial, with an average burial rate of 259×10^6 t/year is consist of fine-grained clays and silts interspersed with layers of sand, representing typical marine, hemipelagic sedimentation (Wellsbury, 2002).

A final important characteristic of sediments that the abundance of minerals (clays, carbonates, silicates, metal oxides, etc.). Minerals can be both reactants with land/or products of microbial metabolism, and they undoubtedly impact the microbial ecology and metabolism of the surrounding environments, both structurally and functionally.

Those are all the common characteristics of marine sediments that make microbial activity possible in the extreme conditions of deep subsea floor. Following section in this chapter will provide brief information about investigation methods for revealing prokaryotic diversity in the sediments.

2.2 Methods to Study Prokaryotic Diversity in Marine Sediments

Like all habitats, revealing the diversity of prokaryotes found in the sediment biosphere is a difficult mission to accomplish. Traditional methods in microbiology are not sufficient enough to identify the diversity of spatial microbial community in the marine sediments. Traditional methods are based on cultivation where solid or liquid media are used to stimulate the growth of the microorganisms. The taxa obtained by standard laboratory cultivation methods surely represent only a small fraction of the actual community due to low mimic ability to represent microbial interaction in the nature of commercially available media. Cultivation methods can generally represent lower than 0.1% of the entire microbial community (Wellsbury, 2002; Engelen, 2008). Thus most microbial ecology studies have used studies have used molecular methods, involving direct extraction of nucleic acids from sediments and PCR amplification and or quantification of 16S rRNA genes (Giovannoni, 1990) and/or functional genes indicative of key anaerobic sedimentary processes such as methanogenesis (*mcrA*) and sulphate reduction (*dsrp*). These amplified genes are then analysed for diversity by either the construction of gene libraries by cloning and sequencing or by more rapid and reliable profiling methods such as DGGE (Muyzer, 1993).

In spite of indispensible utilization of molecular methodologies, they are especially difficult to use in the studies of marine sediments because of high concentrations of inhibitory substances such as humic acids and fulvic acids that have potential to interfere the molecular methods. On the other hand, extraction of nucleic acids from sediments results with relatively lower DNA and/or RNA concentrations due to physicochemical properties of the sediment and low prokaryotic cell number (sediments typically have 10^6–10^7 cells cm^{-3}) (Parkes, 2000). Sampling such a low biomass may cause significant problems for the reliable Polymerase Chain Reaction (PCR) amplification. Low biomass concentrations are susceptible to PCR biases due to random amplification (Chandler, 1997) and also reagents used in PCR amplification and DNA extraction are often contaminated with exogenous nucleic acids. Even if small amounts of exogenous nucleic acids contaminate sediment samples, consequences of contamination cause over estimation of the microbial community (Kormas, 2003; Webster, 2003). Developments in biotechnology contribute to solve contamination problems. Carefully optimized DNA extraction

protocols, kits have been improved and also sensitivity of PCR have been improved via nested PCR (Rochelle, 1992; Reed, 2002; Webster, 2003; Sorensen, 2006) to ensure that sequences retrieved are representative of subsurface prokaryotes.

Subsampling of sediment cores is also important for both microbiological and molecular analyses to ensure that sediment samples are in good quality and uncontaminated with sea water, drilling fluids etc. which may affect subsequent DNA extraction. Hence, it has now become a routine procedure for deep subsurface drilling to use a combination of a water soluble chemical tracer and fluorescent microspheres to mimic penetration of bacterial sized particles to monitor possible contamination from seawater and drilling disturbance (Smith, 2000; House, 2003; Lever, 2006).

2.3 Microbial Diversity of Marine Sediments

2.3.1 Bacteria abundance in the marine sediments

Analysis of 16S rRNA gene libraries has shown that Diversity of bacteria in deep marine sediments is very versatile. In this section composition of the bacterial populations found in marine sediments are summarized briefly according to some previous studies.

16S rRNA gene libraries indicate broadly diverse bacterial population and composition of the population vary according to location. For example, while *Proteobacteria*, averaged 37.4% of clones, are dominated at the Cascadia Margin ODP site 889/890 (North end, off Vancouver Island) (Marchesi, 2001) and volcanic ash sediment layers of the Sea of Okhotsk (Inagaki, 2003) while they are not abundant at Cascadia Margin ODP sites 1244/5 and 1251 (South end, near Hydrate Ridge, off Oregon) and from the Peru Margin ODP site (Inagaki, 2006) *Gammaproteobacteria, Chloroflexi* which represented by the 62-70% of the clone libraries are the most dominant bacterial groups in the deep marine sediment biota (Webster, 2004).

The bacterial groups: *Alpha-*, *Beta-*, *Delta-* and *Epsilonproteobacteria* are also found but are not so common, averaging only 7.8%, 4.9%, 3.7% and 2.1% of the clones, respectively. 21.3% of the remaining clones, the *Planctomycetes* are significantly

found (up to 26%) at some Peru and Cascadia Margin sites and depths (Parkes, 2005; Inagaki, 2006) as are the novel groups NT-B2 and NT-B6, both originally found in the Nankai Forearc Basin (Reed, 2002).

Although co-existence of the *Chloroflexi* and the JS1 groups are often, they can also dominate sites with one group being much less abundant. Cascadia Margin sites 1224/5 and 1251, Peru Margin site 1230, Nankai Trough site 1173 and Sea of Okhotsk pelagic clay layers (below 22 mbsf) are dominated by JS1 (Inagaki, 2003; 2006; Newberry, 2004), while *Chloroflexi* are dominant at Peru Margin sites 1227 and 1229, and the upper clay layer (7.5 mbsf) from the Sea of Okhotsk (Parkes, 2005; Webster, 2006).

16S rRNA gene libraries showed that some Gram positive bacterial phylotypes and many of the *Proteobacteria* are related to cultured species. These genera include *Ralstonia*, *Comamonas*, *Halomonas*, *Pseudomonas*, *Acinetobacter*, *Pedomicrobium*, *Sphingomonas* and *Pelobacter* from the *Proteobacteria* and *Actinomyces*, *Bacillus* (*Firmicutes*), *Clavibacter* and *Arthrobacter* (*Acitinobacter*) from the Gram-positive Bacteria (Rochelle, 1994; Inagaki, 2006; Webster, 2006).

Phylum *Chloroflexi* is a very common bacteria group that found not only in marine sediments (Webster, 2006) but also in hot springs, hydrothermal sediments, soils, wastewater and polluted sites (Sekiguchi, 1999; Teske, 2002). As far as the knowledge about phylum of *Chloroflexi* is that they are divided into five subphyla (Stackebrandt, 2004). Subphyla I, II and III are include small amount of cultured species while marine sediment clones mainly fall into subphyla II and IV.

Currently the *Chloroflexi* are divided into at least five subphyla (Hugenholtz, 1998; Hugenholtz & Stackebrandt, 2004) with a small number of representative cultured species belonging to subphyla I, II and III. Deep subsurface clones mainly fall into subphyla II and IV but this knowledge seems to change since one study showed that sequences that related with *Chloroflexi* belonged to three subphyla and one deep branching unclassified group, with individual clones showing up to about 30% sequence difference (Inagaki, 2006).

Distribution of another frequent group JS1 in the environment is not widespread as *Chloroflexi*. Habitat of JS1 phylotypes seem to be restricted to anoxic marine sediment (Webster, 2004; 2007).

Ecological and physiological role of the group JS1 in the deep marine sediment is difficult to reveal since there are no cultured members in this group and so does the *Chloroflexi* which has a few cultured members.

Ability of utilization and/or production of hydrogen are known phenomena (Sekiguchi, 2003; Martinko, 2006) for *Chloroflexi* because closest cultured relative (98% sequence similarity) of *Chloroflexi* is the H_2-dependent dehalorespiring bacterium *Dehalococcoides ethenogenes* (Maymó-Gatell, 1997).

Also it is thought that *Chloroflexi* may degrade slowly degradable carbon sources (Wilms, 2006). It has also been stated that JS1 dominate methane hydrate bearing sites and *Chloroflexi* dominate organic-rich subsea floor sediments (Inagaki, 2006).

Sulphate reduction is an important geochemical activity in the deep marine subsurface (D'Hondt, 2006) thus Sulphate-Reducing Bacteria (SRB) would be expected to be dominant physiological groups. However, only limited numbers of phylotypes belonging to *Deltaproteobacteria* have been isolated from this habitat (Kormas, 2003). Limitations about detection of SRB in marine sediments are thought to be related with PCR biases. (Parkes, 2005). It has been proposed the use of the specific functional gene *dsrA* (dissimilatory sulphite reductase) to target SRB in deep subsurface sediments of the Peru Margin ODP sites 1228 and 1229 (Webster, 2006c). Results from this work indicated that very low numbers of uncultured SRB must be present at these sites, as only one sediment depth at site 1228 showed the presence of detectable *dsrA* phylotypes (Webster, 2006a). Even though SRB population of marine sediment is very small, sulphate reduction have been carried over geological timescales so it is possible to think that sulphate reduction is carried out by unknown sulphate-reducing prokaryotes with divergent functional genes that are not detected using current PCR methods (Mauclaire, 2004). Same study showed that SRB population are dominant (6-22% of all prokaryotic cells) throughout sediments of Peru Margin site 1229 by using catalysed reporter deposition-fluorescence in-situ hybridisation (CARD-FISH) that aims to detect microorganisms have lower 16S rRNA (Mauclaire, 2004).

2.3.2 Abundance of archeae in the marine sediments

Accurate and more reliable identification of archaeal 16S rRNA gene clones is essential for understanding the distribution and ecological niches of these microorganisms in the deep marine sediment biosphere. Since archaeal populations include uncultured groups, there are many questions that cannot be revealed. Also there are many studies (Fang, 2005; Wang, 2005) that archaeal sequences are left ungrouped resulting in confusion. Most reliable source about Archaeal population has recently reviewed by (Sørensen, 2008) which includes 47 16S rRNA gene libraries from 11 published studies of the deep marine biosphere.

73.4% of the clones are belonged to The *Crenarchaeota* that dominates the archeal population, while only 24.5% of the clones belonged to the *Euryarchaeota* (eight groups). *Miscellaneous Crenarchaeotic Group* (MCG) (33% of the clones) and Marine Benthic Group B (MBG-B; synonymous with the Deep-Sea Archaeal Group, DSAG) (26.3% of the clones) are the most observed *crenarchaeotal* groups (Inagaki, 2003). Rest of the clones are belong to Marine Group 1 (8.4%; *Crenarchaeota*), the South African Gold Mine Groups (SAGMEG) 1 and 2 (7.6%; *Euryarchaeota*). There are another groups which include thermophilic *Euryarchaeota* (7.6%) and others that cannot account for about 4.5% overall. These two groups have also been found in other sedimentary, aquatic and terrestrial environments, and so are not confined to the deep marine biosphere (Teske, 2006a; b).

The only *Archaea* phylotypes closely related to cultured species were the *euryarchaeotal* methanogens, thermophiles and hyperthermophiles but amount of the cultured clones does not cover a vast majority (<8%) of the phylotype; thus, as with the *Bacteria*, most of the *Archaea* were from uncultured microorganisms.

Marine sediment biosphere archaeal clone libraries are relatively less diverse than observed in Bacteria with 24/47 Deep subsea floor biosphere archaeal clone libraries are seemingly less diverse than was seen in those for *Bacteria*, libraries containing only one or two of the 14 main archaeal taxa found in the deep marine biosphere. These results could be an artefact due to molecular methodology biases or it reveals real variation between locations (Webster, 2003). Nevertheless large phylogenetic diversity and widespread distribution of MCG *Archaea* supports the ideas about utilization of complex carbon substrates can be driven by the diverse group of anaerobes (Webster, 2006; Sørensen, 2008; Biddle, 2007).

Since the importance of biogenic methane production for geochemical processes and also contributions to methane hydrate reserves in the marine sediments, methanogenic community of the marine sediments are deliberately investigated (D'Hondt, 2006; Inagaki, 2006). However, only small numbers of methanogen clones have been detected directly in general archaeal 16S rRNA gene libraries. For example, 1 out of 103 clones was thought to be methanogen at Peru Margin ODP site 1229 (Parkes, 2005). Also methanogens that belong to *Methanosarcinales*, *Methanobacteriales* were found related with cultured methanogen species such as *Methanococcus aeolicus* and *Methanoculleus palmaeoli* at Peru and Cascadia Margins (Inagaki, 2006). Proportion of known methanogens is very small among the prokaryotes just like SRB in the deep marine sediments. By the aid of taxon spesific 16S rRNA and *mcrA* (α-methyl coenzyme-M reductase) gene primers, sequences of *Methanosarcina mazei* and *Methanosarcina barkeri* (*Methanosarcinales*), *Methanobrevibacter arboriphilus* (*Methanobacteriales*) are succesfully amplified (Newberry, 2004; Parkes RJ W. G., 2005). These results showed that diversity of methanogens is limited in the deep marine sediments.

2.3.3 Comparison of abundance of archaea vs. bacteria

Mainly vast majority of molecular studies of prokaryotic diversity in deep marine sediments are based on conventional or nested PCR with universal primers that specific for either Bacteria or Archaea on the extracted DNA site. This application makes comparision of magnitude of domains slightly impossible to accomplish. Utilization of quantitative molecular methods including Q-PCR and FISH, CARDFISH (These two also obtain visual data) has been aided to solve this problem. Schippers et al. (2005) used CARD-FISH to investigate bacterial and archeal total cell counts at two Peru Margin (sites 1227, 1230) and two equatorial Pacific sites (1225, 1226). Results of this study revealed the high abundance of the bacterial cells (according to Acridine Orange Direct Count, AODC) but amount of the archaeal cells could not be detected. These results were verified by the same authors by using Q-PCR. Quantification of 16S rRNA gene showed that number of archaeal cells was less abundant than bacterial cells in 10-1000 folds (Schippers, 2006). However another study by Biddle et al. (2006) reported that samples from ODP Leg 201 sites 1227, 1229 and 1230 contain 82% archaeal cells within the prokaryotic cells by using FISH.

Also these results were supported by high abundance of archaeal intact polar lipids at rate of 34% (Biddle, 2006). Divergent results suggest that more studies should be made on molecular methodologies before more accurate determination on abundance of Bacteria vs. Archaea could be made.

2.4 Microbial Activity of Marine Sediments

2.4.1 Processes that are driven by prokaryotes

Introduction of organic carbon to the marine sediments cause a series of redox processes that are driven by prokaryotes to get the most energy as possible (Reeburgh, 1983). In this section, sediments on which microbiological activities take place are assume to be in stable conditions with regard to temperature and carbon input, and a minimal amount of physical mixing occurs.

2.4.1.1 Aerobic respiration

Organic matter that reaches the sediments is aerobically converted to CO_2 and H_2O until the all oxygen that reached to the sediments by diffusion is depleted. Actually most of the organics are degraded before reaching the sediments, leaving very little to carbon to be further oxidized.

The result is that deep-sea surface sediments are usually oxidized, and oxygen can remain at high levels for many centimetres downward. However, in shallower, more carbon-rich sediments, it is usual to see oxygen depletion within millimetres or centimetres of the sediment surface (Hansen, 1991).

2.4.1.2 Nitrification and denitrification

As one proceeds downward across the oxygen depletion zone, it is common to see a zone in which nitrate concentration increases. This occurs at low concentrations of oxygen, where ammonia diffusing upward from below is converted into nitrate via a process called nitrification. Dissimilatory reduction of N_2 results in the decrease of nitrate with the concomitant oxidation of organic carbon CO_2. The magnitudes of nitrification and denitrification are not easy to measure because they occur in spatially adjacent samples and because for both, the product of one process is the reactant of the other. Thus, with small concentrations of nitrate, the effect on the nitrogen and carbon cycles can be substantial if the cycling rate is large (Kim, 1997).

2.4.1.3 Methane, manganese and iron oxidation

Major oxidant for methane is molecular oxygen, even some anaerobic methane oxidation occurs in nature (Reeburgh, 1983). The observed methane profiles in sediments often shows methane depletion, where the intersection of the oxygen is minimum. As Mn(II) and Fe(II) diffuse upward from reduced sediments, they are deposited as metal oxide layers or crusts in the presence of low levels of oxygen. The layer of oxidized manganese, commonly in the form of MnO_2 typically overlays a layer of oxidized iron, which is more rapidly oxidized in the presence of low levels of oxygen and precipitates just below the MnO_2. Such ferromanganese layers are common in sediments.

The oxidation of both Mn(II) and Fe(II) are thermodynamically favoured, but the kinetics of the two processes is substantially different at neutral pH values common to most sediments. Mn(II) is kinetically stable (Stumm, 1981) and usually requires biological catalysis, whereas Fe(II) oxidation is very rapid at neutral pH and biological catalysis is assumed to be unnecessary.

2.4.1.4 Sulfur oxidation

In deep sediments, when organic carbon oxidation occurs, reduced sulfur species such as sulphide, thiosulphate, or polysulfide are produced and as they diffuse upward. Sulphide is oxidized by Fe(III), Mn(IV), and oxygen while they diffuse upward, the latter two reactions are quite rapid. Each oxidant generates different sulfur intermediates that can interact with other compounds, which makes the system sufficiently complex to defy most efforts to quantify the separate parts. In most systems, the oxidizing potential of the sediment is such that sulphide is consumed within the sediment, either by oxygen itself or by other oxidants, such as nitrate or metals (Jørgensen, 1990; Thamdrup, 1994).

2.4.1.5 Manganese and iron reduction

Metal Reduction takes place in the zone below the nitrate reduction zone. Organics are oxidized via manganese and iron. Thus, levels of Mn(II) and Fe(II) are increased in porewaters. MnO_2 is reoxidized to Fe(III) by Fe(II) during this reaction (Myers, 1988). Mn(II) increases, followed by the appearance of Fe(II). In marine sediments, because of the sulfur cycle, monitoring of iron cycle is difficult. Diffusion of

sulphide to upper side of the sediment has a tendency to remove Fe (II) as iron sulphide (pyrite). Biological and abiological reduction of manganese and iron are possible but chemical reduction of iron is more difficult, even some reports discussed that all iron reduction in nature is due to biological catalysis (Lovley, 1991)

2.4.1.6 Sulfur reduction

Sulphate reduction is well characterized in sediments, whereas thiosulphate and sulfur reductions are much less well quantified. With the exception of reduction by very high temperatures, such as those found in hydrothermal waters, sulphate is stable unless reduced biologically—probably no chemical reduction of sulphate is known in sediment systems. The most reduced species below metal reduction zone in the marine sediments is sulphide which is reduced by SRB.

The production of sulphide (and the generation of other reduced sulfur species) as a result of sulphate reduction is one of the major biogeochemical differences between freshwater and marine systems. In freshwater systems, sulphate concentration is in the range of 100-250 mM, whereas in marine systems, it is approximately 25 mM. Sulphate is thus the dominant electron acceptor in the marine sediments (Jorgensen, 1977).

2.4.1.7 Methanogenesis

As in sulphate reduction, methanogenesis is a process that occurs only as a result of biological catalysis at temperatures and conditions common to most sediment. Methane appears in pore waters just below the oxic/anoxic interface, and it is the major indicator of organic carbon turnover in marine sediment systems (Oremland, 1978). Figure 2.2 summarize the general processes that take place in marine sediments.

2.4.2 Depth profiles of activity

Upper layers of marine sediments usually have metabolically active microorganisms while rates of activities decrease by down layers which have low oceanic carbon flux into the sediment. Activity rate of sulphate reduction decreases with depth.

Blake Ridge in the Western North Atlantic, in the Equatorial Pacific (site 1226) and Woodlark basin, near Papua New Guinea (ODP Leg 180, sites 1109 and 1115) are the proof for decreasing activity for sulphate reduction (Parkes, 2005; Wellsbury, 2002).

There is a significant competition between sulphate reducers and methanogens. Thus activity rates for methanogenesis normally increase below the regions where the highest sulphate reduction occurs. However, rates of sulphate reduction decreases much more rapidly more than methanogenesis because sulphate reduction depletes its own electron acceptors SO_4^{2-}, while electron acceptor of methanogenesis (CO_2) can be compensated by microbiological activity, especially by heterotrophic activity, at appropriate temperatures and environmental conditions (D'Hondt, 2006; Biddle, 2006). Methanogenesis in Equatorial Pacific sites 1225, the Peru Margin site 1229 (Parkes, 2005) and Woodlark Basin (ODP Leg 180, site 1109) (Wellsbury, 2002) supports the knowledge about rate for methanogenesis in the marine sediments.

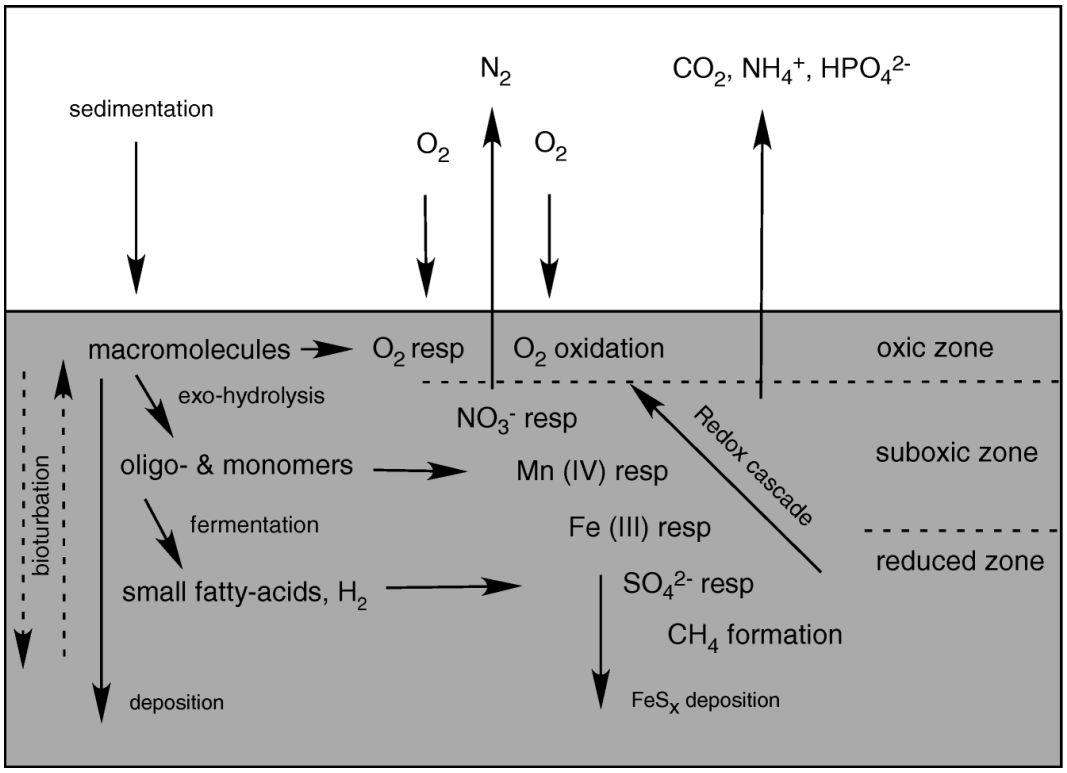


Figure 2.2: A schematic presentation of some important diagenetic processes in marine sediments (Glud, 2008).

In contrast to decreasing activity with depth, some spesific environmental conditions may stimulate activity in deeper layers of the sediments. Anaerobic oxidation of methane (AOM) is one of the unique processes that lead to enhanced activity. If the

methane accumulates in the presence of lower concentration of sulphate, AOM usually occurs (Hoehler, 1994).

Many studies showed that AOM takes place in above and also below of the top of the gas hydrate stability zones (Wellsbury, 2000; Cragg, 1996), upper and lower zones of the sulphate-methane transition zones of the marine sediments (Parkes, 2005).

Deep sulphate flux is also able to stimulate the activity of marine sediment microorganisms. This situation observed at number of other subsea floor sites (e.g. Nankai Trough and Eastern Flank of the Juan de Fuca Ridge) (Parkes, 2007b; Engelen, 2008; Mather, 2000).

3. ANAEROBIC HYDROCARBON DEGRADATION

3.1 Anaerobic Aliphatic Hydrocarbon Degradation

3.1.1 Anaerobic degradation of n- alkanes

There are two mechanisms of anaerobic *n*-alkane degradation that revealed recently. These mechanisms are different from aerobic n-alkane degradation and both processes can occur simultaneously in mixed sulphate reducing communities. The first mechanism involves activation at the subterminal carbon of the alkane and addition to a molecule of fumarate while the second one involves carboxylation of alkane at C-3. It should be noted that both two mechanisms could be observed as initial reactions during anaerobic digestion.

3.1.1.1 Addition of n-alkane to fumarate

Utilization of 13-C labelled alkanes revealed many questions about addition of n-alkane to fumarate during growth of a sulphate reducing bacterium (strain AK-01) (So and Young, 1999a). As a result of this reaction 2-Methyl (Me-), 4-Me- and 6-Me- branched fatty acids (FA) formation was observed correlating with length of the alkanes. That reaction is based on 2 main facts. i) Preservation of the original carbon chain of the alkane in these branched FAs and ii) that the methyl group was systematically the original terminal carbon of the alkane substrate. Rabus et al. (2001) reported that denitrifying bacterium (strain HxN1) stimulates homolytic C–H bond cleavage at C-2 and addition to fumarate by using labeled n-hexane. First stable product of these reactions is 1-methylpentyl succinate. Addition of n-alkane to fumarate shows analogy with anaerobic toluen oxidation, especially on the step of formation of benzyl succinate. This addition reaction is more likely radical reaction that is catalyzed by a glycyl radical enzyme, abstracting hydrogen from hydrocarbon substrate (Heider, 2007). Rearrangement of carbon skeleton results with alkyl succinate (CoA-thioester), followed by a decarboxylation step, and then yields 4-Me-branched Fatty acids (FA). 4-Me-branched FA can be degraded by β-oxidation via either by 2-Me-branched FA or a linear FA. The linear fatty acid possesses two

carbon atoms less than the original *n*-alkane. On the other hand, addition of fumarate at C-2 of *n*-alkanes with carbon chains C6 generally results in i) the preferential formation of cellular even and odd numbered FAs from even and odd numbered *n*-alkane substrates respectively and ii) the formation of some specific metabolites such as 1-methylalkyl succinates and 2-Me- and 4-Me-branched FAs. For such short chain *n*-alkanes, the relationship between the carbon chain length of the alkane substrate and that of the main cellular FAs is less obvious. Moreover, the addition of fumarate to an alkane can no longer be systematically envisaged at the sub-terminal C-2 carbon; this deserves further attention in future studies. Figure 3.1 shows the schematic illustration of this mechanism.

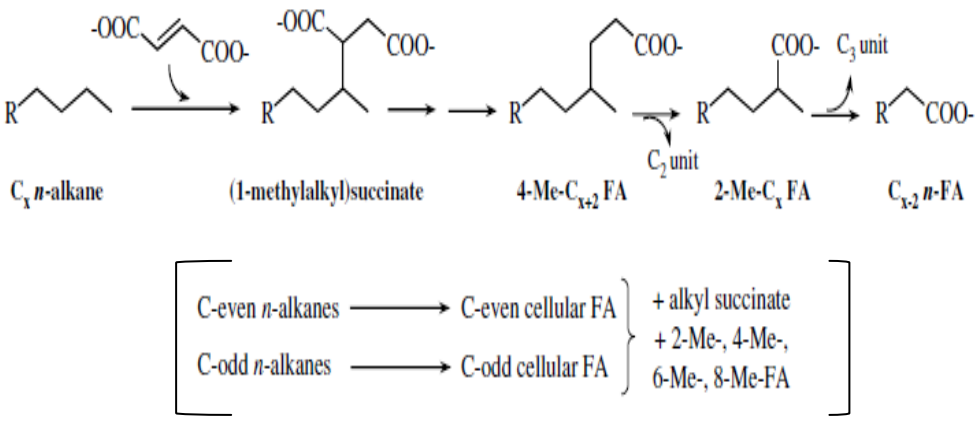


Figure 3.1: Pathway of addition n-alkane to fumarate in anaerobic bacteria (Grossi, 2008)

3.1.1.2 Carboxylation with inorganic carbon

Second pathway of anaerobic n-alkane oxidation was fully understood after first description of sulphate reducer strain Hxd3 by Aeckersberg et al. (1991) and experiments with labelled substrates (So, 2003). Mechanism of Strain Hxd3 is based on attacking the alkane by carboxylation with inorganic carbon (bicarbonate), most likely at C-3 and removal of two sub-terminal carbon atoms from the alkane chain terminus. This mechanism leads to even numbered and odd numbered FAs from odd and even numbered *n*-alkanes, respectively.

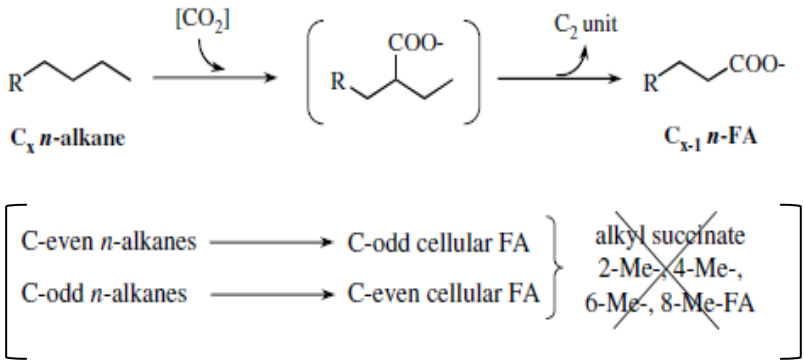


Figure 3.2: Pathway of carboxylation with inorganic carbon in anaerobic bacteria (Grossi, 2008)

between carbon chain length of *n*-alkane substrate and the main cellular FAs, there are Even though relationship still undiscovered parts about the subject such as inability of detection hypothetical 2-Et-branched FA intermediate, which should result from the addition of inorganic carbon at C-3 of the alkane. Figure 3.2 demonstrate the pathway.

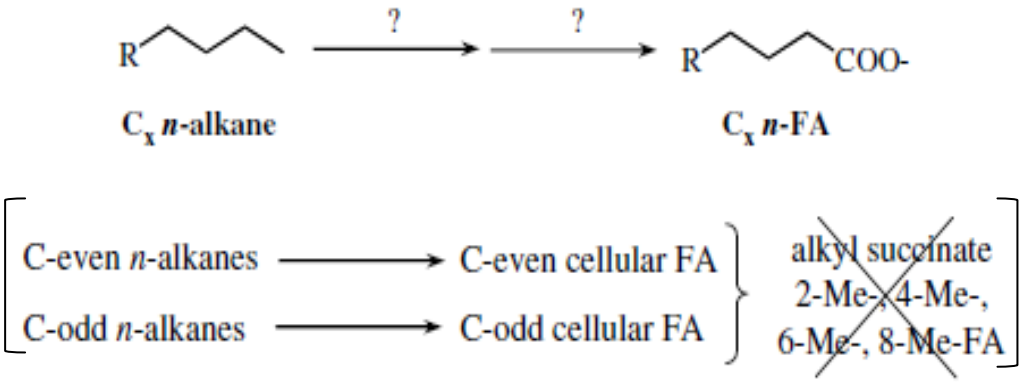
3.1.1.3 Possible alternative pathway for n-alkane degradation

A unique denitrifying microorganism *Pseudomonas balearica* strain BerOc6 can grow on C15–C18 *n*-alkanes. Total cellular FAs of strain BerOc6 are predominantly even carbon numbered when it is grown on an even carbon numbered alkane, and predominantly odd numbered when grown on an odd numbered alkane. Despite strain BerOc6 never showed any indication of using the addition to fumarate mechanism. Thus many questions about this mechanism have been aroused. Unfortunately no specific metabolite that might give a clue to such a third pathway has been identified. It is thought that the alkyl succinate synthase involved in the activation of some alkanes exhibits promiscuous activity (relaxed substrate specificity) towards longer saturated hydrocarbons that do not support bacterial growth (Wilkes, 2003). Degradation of long chain (>C-20) alkanes is still not fully understood and further studies are needed to reveal the possible the third pathway. Figure 3.3 shows the possible degradation mechanism.

3.1.2 Anaerobic n-alkane Degrading Microorganisms

Anaerobic n-alkane degradation microorganisms have been discussed since the 1940s but their demonstration lies back to late 1980s. (Widdel, 2000; Rabus, 2001). Since then, many microorganisms that can degrade *n*-alkanes and *n*-alk-1-enes under

different electron acceptor conditions such as denitrifying and sulphate reducing conditions have been isolated and identified (Grossi, 2007). Table 3.1 shows the identified microorganisms that can metabolize n-alkanes and/or n-alk-1-enes under anaerobic conditions.



? = conversion pathway is not clear but products are carboxylated

Figure 3.3: Pathway of possible alternative n-alkane degradation in anaerobic bacteria (Grossi, 2008)

3.2 Anaerobic Aromatic Hydrocarbon Degradation

3.2.1 Basic principles of aromatic hydrocarbon degradation

Four general enzymatic reactions are involved in anaerobic biodegradation of aromatic hydrocarbons. These are;

1. Fumarate addition yields aromatic-substituted succinates. This reaction is catalyzed by glycyl radical enzyme which shows analogy with n-alkane degradation.
2. Methylation of unsubstituted aromatics.
3. Dehydrogenase activity results with hydroxylation of an alkyl substituent.
4. Combination of two reaction(step 2 followed by 1) cause direct carboxylation (Foght, 2008).

These activation reactions feed into pathways that result in ring saturation, β-oxidation and/or ring cleavage reactions producing central metabolites such as benzoyl-coA that are eventually incorporated into biomass or completely oxidized.

3.2.2 Anaerobic Benzene, Toluene, Ethylbenzene and Xylenes (BTEX) Degradation

Benzene, Toluene, Ethylbenzene and Xylenes (BTEX) degradation has been debated up to date. There is a certain discussion about whether PAHs that contain three or more rings can stimulate or support microbial metabolism or they can support growth through cometabolism (Safinowski, 2006). Thus there is only limited number of BTEXs that are biodegraded anaerobically in situ or in microcosms under several electron acceptor conditions.

3.2.2.1 Anaerobic benzene degradation

Benzenes have relatively high water solubility and known toxicity combined with apparent chemical and biological stability in situ make them a priority pollutant even though their relatively low concentrations in many petroleum contaminants.

Although benzene can be degraded aerobically, anaerobic degradation of this structure is not usually thermodynamically favourable. Benzene degradation is usually subjected to inhibition so it occurs slowly and often results with incomplete degradation (Langenhoff, 1997).

There are five mechanisms have been reported about initiation of anaerobic attack on benzene. Two of them have little support in the scientific area. Although the most common mode of initial activation of methyl substituted aromatics is fumarate addition, it may be that the large activation energy required to remove hydrogen from the benzene ring precludes this mechanism for initiating benzene metabolism; and a proposed pathway involving initial attack by ring saturation has garnered little supporting evidence (Coates, 2002). Rest of the three pathways are:

a) **Hydroxylation:** producing phenol with subsequent carboxylation to the postulated intermediate *para*-hydroxybenzoate or ring reduction to yield cyclohexanone.

b) **Methylation:** producing toluene followed by fumarate addition to produce benzylsuccinate

c) **Carboxylation:** producing benzoate

Table 3.1: Bacteria that metabolize n-alkanes and/or n-alk-1-enes under anaerobic conditions (Grossi, 2008).

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

a- See Figure 2.1-2.3.
b- Not documented.
c- Three distinct strains were isolated.
d- DB, double bond.
e- Likely mechanism although not fully demonstrated.
f- This strain activates *n*-butane exclusively at C-2 but *n*-propane at both C-2 and C-1.
g- Bacteria from enrichment culture that were phylogenetically identified but not isolat

As a result of these 3 reaction, central intermediate on aromatic compound degradation (benzoyl-coA) is formed which will be oxidized to acetyl-CoA and carbon dioxide (Harwood, 1999). Figure 3.4 demonstrates these 3 pathways.

Even though these pathways are described, there are some unknown parts about intermediates. In the pathway a , the source of the hydroxyl group of the phenol intermediate has been debated: H_2O was proposed as the donor in methanogenic cultures but recent evidence points to a hydroxyl free radical as the donor used by *D. aromatica* RCB (Chakraborty, 2005). In the pathway b, the methyl group donor is not known but it is thought to be S-adenosylmethionine, methyltetrahydrofolate or cobalamin and also there is a strong analogy between pathway b and the methylation of naphthalene (Safinowski, 2006). The major challenge on revealing benzene degradation is the necessity of syntrophic interactions that is hard to mimic in the laboratory conditions. Instead of isolation of benzene degraders, application of molecular techniques is more feasible.

Ulrich and Edwards (2003) reported the indication of uncultured species involved in benzene degradation by using 16S rRNA gene sequencing for. This study indicates the syntrophic relationships between uncultivated bacteria (predominantly the sulpfate-reducing genus *Desulfosporosinus* and *delta-proteobacterial* sequence) and uncultivated archaea (predominantly acetoclastic methanogens) while anaerobic benzene degradation, whereas the nitrate-reducing consortium was dominated by bacterial sequences affiliated with denitrifying *beta-proteobacteria* similar to *Azoarcus* and *Dechloromonas*, with no archaea cloned. More studies should be carried to understand such a complex pathway.

3.2.2.2 Anaerobic toluene degradation

Previous toluene degradation studies reported that toluene is more readily biodegradable than other monoaromatic hydrocarbons. Toluene degradation was observed under several conditions such as manganese-, iron- and sulphate-reducing as well as methanogenic conditions. Toluene was readily degraded within 1–2 months under all redox conditions while benzene degradation can be continue up to 525days.

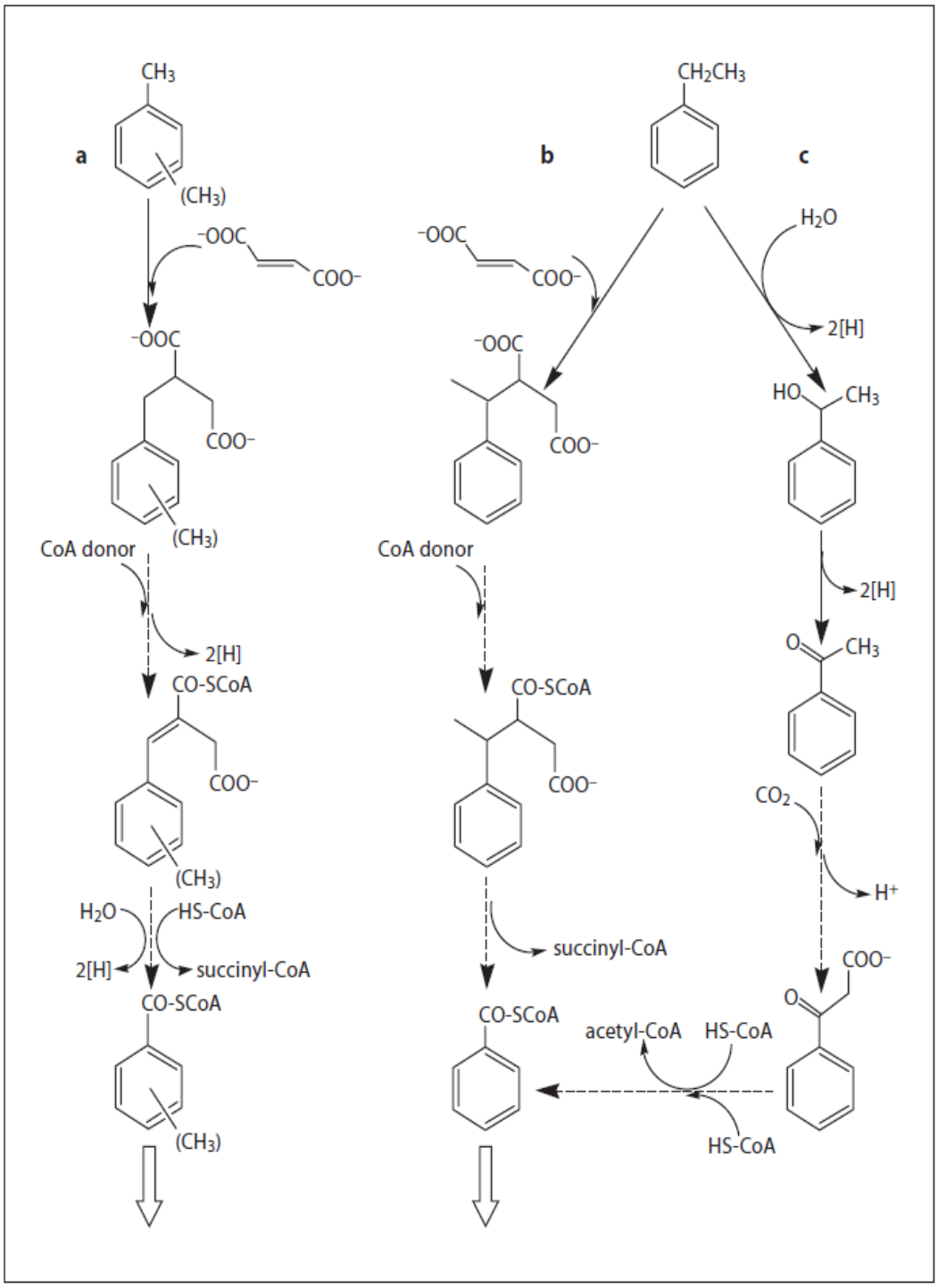


Figure3.4: Three of the anaerobic degradation pathways proposed for benzene. Square brackets indicate a postulated intermediate; broken arrows indicate multiple enzymatic steps; open arrows indicate further metabolism. a) Hydroxylation to form phenol, cyclohexanone, or *p*-hydroxybenzoate or benzoyl-CoA. b) Alkylation to form toluene, followed by fumarate addition to form benzylsuccinate and benzoyl- CoA c) Carboxylation to form benzoate (possibly through more than one enzymatic step) and benzoyl- CoA.

Since toluene degradation is driven by syntrophic interactions, Ficker et al. (1999) constructed a model to comprise physiological groups: a syntroph that initiates toluene oxidation, a homoacetogen that can reversibly oxidize acetate coupled to hydrogen generation, an acetoclastic methanogen and a hydrogenotrophic methanogen. This study identified two dominant archaeal and two bacterial representatives: a bacterial sequence with no significant homology to any known genus the presumptive (toluene-degrader), a *Desulfotomaculum* (like sulphate reducer a presumptive homoacetogen), a *Methanosaeta* sequence (type acetoclastic archaeon), and a *Methanospirillum* sequence (type presumed hydrogenotrophic methanogen). There is a significant similarity between benzene degradation and toluen degradation so it is thought that initial hydrocarbon attack generates partially oxidized products such as fatty acids or alcohols that become available for fermentative and syntrophic species, eventually being made available to methanogens as acetate and/or $H_2 + CO_2$.

Initiation of the attack on the toluene is mediated by addition of the double bond of fumarate to the methyl group of toluene, yielding benzylsuccinate (Leutwein, 1999). This intermediate is then further oxidized to *E*-phenylitaconate, and eventually benzoyl-coA, a central aromatic metabolite that subsequently undergoes ring reduction, cleavage and oxidation to CO_2 (Harwood, 1999).

3.2.2.3 Anaerobic ethylbenzene degradation

Ethylbenzene degradation under sulphate& nitrate reducing conditions has been previously reported (Reinhard, 1997). However ethylbenzene degradation under iron reducing condition was debated. Villatoro-Monzon (2003) reported that ethylbenzene was rapidly degraded under iron reducing conditions whereas Parsons et al. failed to observe ethylbenzene degradation even they used the same microcosms previously studied (Botton, 2006). Also Siddique (2007) showed toluene and xylene degradation under methanogenic conditions but in that study ethylbenzene was reported as a resistant molecule to biodegradation.

There are two described pathways for anaerobic biodegradation of ethylbenzene. First one involves the classic fumarate addition that produces the ethyl homolog of benzylsuccinate, observed under sulphate-reducing conditions (Kniemeyer, 2003).

Second pathway includes the hydroxylation of the benzylic carbon to form 1-phenylethanol (analogous to benzene attack) with water donating the oxygen atom of the hydroxyl group under denitrification conditions (Ball, 1996).

3.2.2.4 Anaerobic xylene degradation

Xylene has been reported that its biodegradation occurs via the fumarate addition pathway showing analogy with toluene but position of free radicals on xylene directly affect the anaerobic biodegradability of relevant molecule. Under nitrate reducing conditions, para- and meta- xylene (p-, m- xylene) are degraded while ortho- xylene (o-xylene) shows resistance to anaerobic degradation. However, after significantly long incubation times o-xylene degradation is observed under sulphate reducing conditions (Edwards, 1992). Under iron-reducing conditions, o-xylene (Jahn, 2005) and *p*-xylene (Botton, 2006) can be degraded by certain cultures. Although degradation rate of p-xylene is generally reported as a slow process, at least one sulphate-reducing enrichment culture recently was shown to degrade *p*-xylene via fumarate addition (Meckenstock, 2004).

It is noteworthy that the latter culture was enriched in the presence of Amberlite-XAD7 ion-exchange resin (Morasch, 2001), keeping the concentration of substrate and possibly inhibitory metabolites, low during the initial stages of enrichment. This method of acclimatizing the inoculum to a recalcitrant substrate may prove useful for isolation of additional cultures.

Homologs corresponding to toluene, metabolites (derived from fumarate addition) have been detected in cultures incubated with xylenes. For example, 4-methylbenzylsuccinate and 4-methylphenylitaconic acid were extracted from an enrichment culture incubated with *p*-xylene (Morasch, 2001).

3.2.2.5 Anaerobic degradation of unsubstituted Poly Aromatic Hydrocarbons (PAHs)

PAH biodegradation, under nitrate reducing conditions was confirmed by many authors. Mihelcic and Luthy (1988) were the first to report loss of naphthalene from enriched soil samples and Bregnard et al. (1996) showed naphtalene (14-C) degradation by measuring mineralization rates. Under sulphate reducing conditions, Coates et al. (1994) detected 14 C-naphthalene mineralization by sulphate-reducing marine harbour sediments. Langenhoff (1996) and Bedessem (1997) separately

reported partial degradation of naphthalene in sediment column under nitrate-(observed degradation 60%), manganese-(observed degradation50%) and sulphate-reducing as well as methanogenic conditions. There are also studies include PAH degradation via cometabolism which involves utilization of easily biodegradable substrate for growth while recalcitrant molecule is acting as a second source of energy (Langenhoff, 1996). Rockne and Strand (2001) found that naphthalene and phenanthrene could be degraded by a denitrifying enrichment culture originally derived from creosote-contaminated soil. However, the degree of mineralization varied considerably between substrates, with only partial mineralization of naphthalene versus 96% of phenanthrene; likewise, the proportion of PAH carbon incorporated into biomass varied between substrates, with naphthalene contributing the most to biomass- carbon.

There are two proposed pathways for the initiation of the anaerobic attack on naphthalene. First pathway involves carboxylation followed by fumarate addition and the second pathway uses methylation for the initiation of the attack on the carbon skeleton (Safinowski, 2006). Also there is a data proposed by Bedessem et al. (1997) that under sulfidogenic conditions this pathway initiated via hydroxylation of naphthalene to naphthol, but it is not verified. The two pathways converge at 2-naphthoic acid, and thereafter the aromatic rings are sequentially reduced, starting with the unsubstituted ring, to produce octahydronaphthoic acid.

There is a strong analogy between second pathway and methylation of benzene to toluene before further metabolism. These two reported modes of attack may actually represent a single pathway, since the methyl group may derive from bicarbonate via a reverse CO-dehydrogenase pathway (Safinowski, 2006). Despite the analogy to the benzene methylation pathway, experiments were carried by Coates et al. (2002) showed that sulfidogenic benzene-degrading sediments were unable to mineralize naphthalene. Results of this study highlighted the facts about certain microbial populations play crucial role on initiation of this pathway via relevant enzymes. Thus the study also suggests that initial enzymes for attack of these unsubstituted aromatics are substrate-specific. Figure 3.5 summarize the naphthalene degradation.

Phenanthrene degradation has been observed under nitrate- and sulphate-reducing conditions in marine sediments (Tang, 2005). By analogy to naphthalene the initial attack on phenanthrene may be carboxylation, or methylation as proposed by

Safinowski (2006) with subsequent fumarate addition and oxidation to phenanthroic acid. Mc Nally et al. (1999) found that concentration of phenanthrene has important effect on degradation rate. If the PAH concentrations are below their water solubility limit, degradation can be occurred without a lag phase under denitrifying conditions. The pathways for anaerobic phenanthrene degradation remain cryptic because the substrate concentrations used in this study were very low and the cell density high (10^8 cells/ml), possibility of the substrate sorption into the cell membranes may be a factor, since degradation products were not determined to verify anaerobic oxidation.

Other unsubstituted PAHs such as acenaphthene, fluorene and fluoranthene also have been shown to be removed by enrichments using nitrate or sulphate as electron acceptors by many authors (Foght, 2008). Rothermich et al. (2002) demonstrated that several indigenous as well as added PAHs were degraded in situ under sulphate reducing conditions in harbour sediments. The substrates monitored comprised a suite of 14 PAHs having 2–5 rings, including naphthalene, phenanthrene, and the high molecular weight PAHs chrysene, pyrene and benzo(a)pyrene, among others, including alkyl-substituted naphthalenes. All substrates monitored eventually showed at least some depletion 9% for benz(a)anthracene to 89% for acenaphthene, with the smaller PAHs generally degrading faster than the larger ones PAHs. This study demonstrated for the first time that high molecular weight unsubstituted PAHs could be degraded under sulphate- reducing conditions.

PAH degradation under methanogenic conditions has been approved thermodynamically feasible by applying molecular modelling that suggested by Christensen et al. (2004). After that, same group set up microcosms that include naphthalene or 1-methylnaphthalene at up to 65 °C. As a result of the experiment, the mass of both substrates decreased in all microcosms at a rate proportional to temperature.

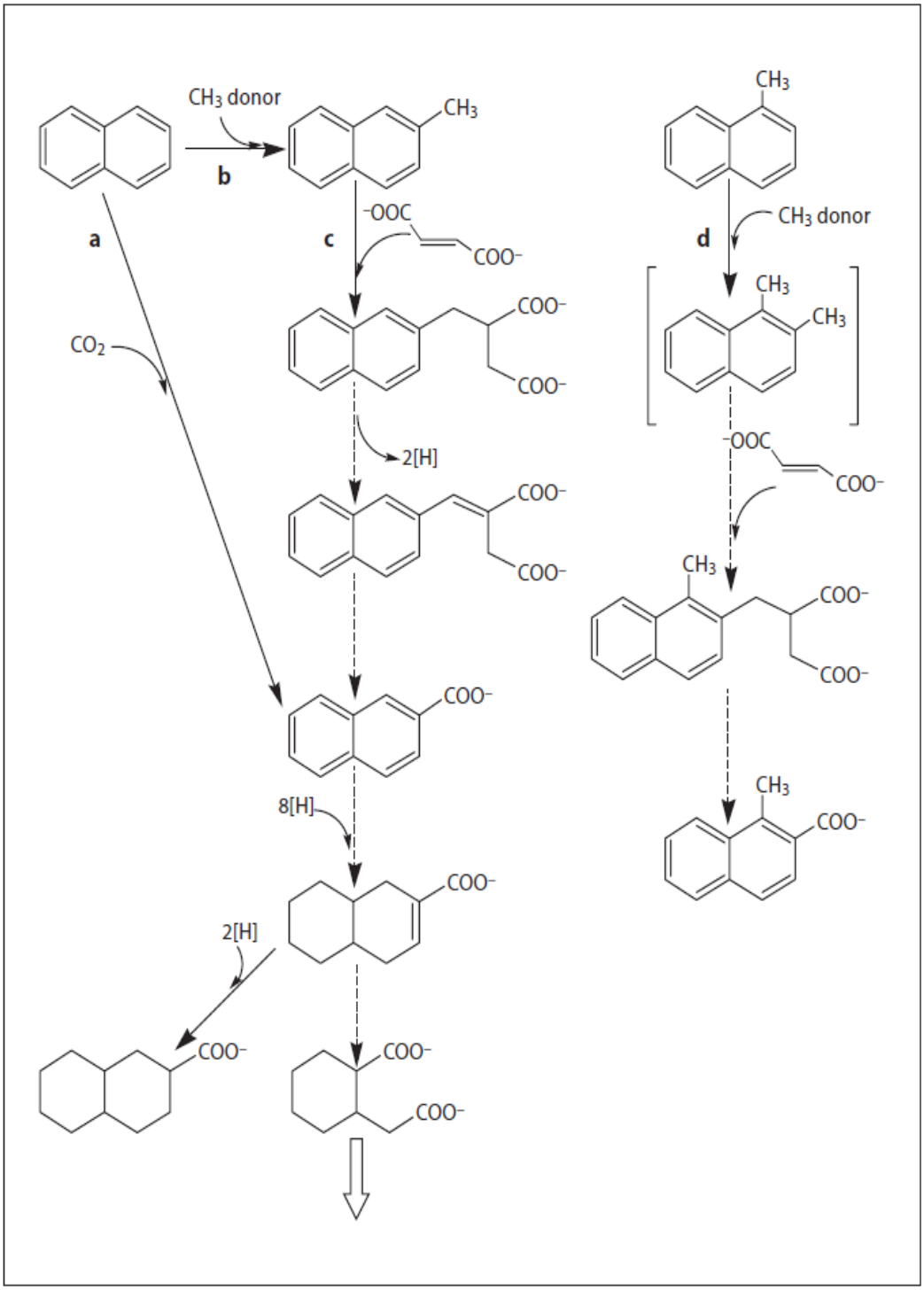


Figure 3.5: Anaerobic pathways for degradation of naphthalene and methylnaphthalenes. Square brackets indicate a postulated intermediate; broken arrows indicate multiple enzymatic steps; open arrows indicate further metabolism. **A** Carboxylation of naphthalene **b** Alkylation of naphthalene prior to oxidation via the 2- methylnaphthalene pathway. **c** Activation of 2-methylnaphthalene via fumarate addition **d** Alkylation of 1- methylnaphthalene to form the postulated intermediate 1,2-dimethylnaphthalene, followed by fumarate addition and eventual co-metabolic formation of the presumptive dead-end product 1-methyl-2-naphthoic acid (Foght, 2008)

3.2.3 Anaerobic aromatic hydrocarbon degrading microorganisms

Documentation of anaerobic degradation is a relatively recent within two decades. Several microorganisms are reported for anaerobic aromatic hydrocarbon degradation under different electron acceptor conditions. Amongst the identified microorganisms, there are many different groups which were limited to enrichment cultures and in situ sediments or ground waters or a few pure cultures. These identified microorganisms have initiated the elucidation of degradative pathways, intermediates, and genes encoding key enzymes. The list of organisms reported to degrade specific hydrocarbons was given in Table 3.2.

Degradation of unsubstituted PAHs requires more study using enrichment cultures, including metabolism under sulfidogenic and methanogenic conditions because much of the literature has been generated with denitrifiers. Also for complete understanding of the subject, mass balance of the systems should be determined whether they are completely mineralized or not.

Table 3.2: Identified microorganisms that are capable of degradation of aromatic hydrocarbons (Rabus, 2001).

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

4. POLLUTION PROFILE OF MARMARA SEA

4.1 Location and Properties of Marmara Sea

The Sea of Marmara is the inland sea that connects the Black Sea to the Aegean Sea. Thus, Marmara Sea separates Turkey's Asian and European parts. The Bosphorus strait connects it to the Black Sea and the Dardanelles strait connects it to the Aegean Sea. The former also separates Istanbul into its Asian and European sides. The Sea has an area of 11.350 km² (280 km x 80 km) with the greatest depth reaching 1.370 m. The salinity of the sea averages about 22 parts per thousand, which is slightly greater than that of the Black Sea but only about two-thirds that of most oceans. However, the water is much more saline at the sea-bottom, averaging salinities of around 38 parts per thousand similar to that of the Mediterranean Sea. This high-density saline water, like that of the Black Sea itself, does not migrate to the surface. Water from the Susurluk, Biga (Granicus) and Gonen Rivers also reduces the salinity of the sea.

There are two currents flowing from Black Sea to Marmara Sea upper water current has a speed of 0.5-4.8 knots sometimes reaching to 6.7 knots. Under current is slower and has a speed rate of 1.6 knots. The water circulation of the Marmara Sea mainly controlled by water entering the sea due to density differences, barometric pressure differences and sea level differences of connected seas. Local wind stress distribution also plays a role in circulation too. Water from Black Sea circulates mainly in clockwise. The denser water from Aegean Sea sinks deep after entering Marmara Sea and moves to shallower depths in warmer seasons due to density differences (Besiktepe, 1994). The south coast of the sea is heavily indented, and includes the Gulf of Izmit , the Gulf of Gemlik and the Gulf of Erdek. Figure 4.1 shows the location of Marmara Sea.

Figure 4.1: Location of Marmara Sea

4.2 Pollution Sources of Marmara Sea

Since Marmara Sea is in an important region because of its geopolitical position. Population has been dramatically increased over the years. Thus extensive anthropogenic activity, large number of wastewater discharges (both from domestic and industrial sources) pump out to the Marmara Sea (Öztürk, 2000). Also other anthropogenic activities that primarily cause severe hydrocarbon and heavy metal discharges generate the main problem in Marmara Sea. Pollution in the Marmara Sea has become chronic problem because of the absence in the number of wastewater treatment facilities. Water quality measurements also indicate the possible eutrophication potential due to uncontrolled discharges (Orhon, 1995).

Black Sea and Mediterranean Sea are also pollutant source for Marmara Sea. Vast Majority of the pollutants that are carried by surface currents from Black Sea (Orhon, 1995) and carried by deep currents from and Mediterranean Sea (Unlu, 2006) sinks and accumulate in the Marmara Sea basin (Orhon, 1995). Nutrient input from the Black Sea is much more significant than coastal wastewater discharges. The basin receives a total of 1.9×10^6 tons of TOC (total organic carbon) and 2.7×10^5 tons of TN (total nitrogen) per year from the Black Sea inflow (Orhon, 1995). Thus, accumulation of nutrients and organic carbons brought main pollution problem in the Marmara Sea that cannot be remediated (Orhon, 1995).

Petroleum hydrocarbons are, may be the most considerable, another important contaminant of Marmara Sea. 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.

Heavy sea traffic and oil related facilities increase the oil pollution in the Marmara Sea significantly (Tuğrul, 1995). Between 1995 and 1996 oil concentration at Bosphorus increased from 9.5µg/L to 33.5µg/L while the oil concentration at the Dardanelles showed more significant increase on concentration from 5.25µg/L to 42.5µg/L in the same period. Increase on oil concentration at the overall Marmara Sea is more dramatically that in the same period concentration increased from 36.9µg/L to 103.7µg/L (Guven, 1998). There is a heavy traffic of shipping approximately 60000 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 (Otay, 2000). Thus many tanker accidents occurred in the strait between 1964 and 2002. As a result of these accidents, almost 193 tons of oil spilled into the Marmara Sea. Tankers Independenta accident in 1979 (95000 tons of crude oil was spilt) and Nassia accident in 1994 (13500 tons of crude oil was spilt) were the biggest tanker accident at the Bosphorus. There is also another accident which had happened more recently in 1999, the Russian oil tanker *Volgoneft* broke in two in the Marmara Sea, and more than 3000 tonnes of oil were spilled into the water (Otay, 2000).

Heavy metal pollution is another problem in the Marmara Sea. Industrial and municipal waste disposal increases the levels of this pollution. Metal contents (Al, Fe, Mn, Cu, Pb, Zn, Ni, Cr, Co and Hg) of the surface sediments from the shelf areas of the Marmara Sea generally do not indicate shelf-wide pollution. The variability of the metal contents of the shelf sediments is mainly governed by the geochemical differences in the northern and southern hinterlands. Northern shelf sediments contain lower values compared to those of the southern shelf, where higher Ni, Cr, Pb, Cu and Zn are derived from the rock formations and mineralized zones. However, besides from the natural high background in the southern shelf, some anthropogenic influences are evident from EF values of Pb, Zn and Cu, and also from their high mobility in the semi isolated bay sediments (Algan, 2001).

4.3 Polluted Areas of the Marmara Sea

4.3.1 Izmit Bay

Izmit Bay, a semi-enclosed body of water located in the most industrialised area of the Marmara region, has been subjected to pollution by surrounding domestic and industrial discharges since the 1970s (Morkoç, 2001). Unfortunately these effluents are also found toxic (Okay, 1996). Because of the accumulation ability of the sediments, they are also found toxic (Tolun, 2001).

Main oil pollution problem may be caused by earthquake of a moment magnitude Mw=7.4, a focal depth h=18 km and having approximately 120 km right lateral strike slip faulting was felt over the area on August 17th, 1999 in the Izmit Bay. There is heavy concentration of petrochemical plants on the northeaster site of the Bay. These facilities have been so close to epicentre of a major earthquake. The most damaged facility was Tupras, operated by the state-owned oil company. Leakage from the facility continued for a several days and spilled oils burned out of control even transfer procedures of the remained oil were applied after earthquake. Pollution level were measured by Okay, (2001) and Balkıs, (2003). Results from these studies showed the increase in the total PAH concentrations of the surface waters and local mussels. Also dissolved oxygen levels of the lower layers were found out of detection levels.

4.3.2 Gemlik Bay

The Gemlik Bay emerges as a 31-km-long tectonic trough between two topographic heights, with an increasing width west ward. 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.

Main pollution sources of the Gemlik Bay are ship traffic, fishery activities, domestic and industrial sewage waters and riverine inputs. The Karsak Creek is the most important riverine inputs because waters of this creek carries the domestic and industrial wastewater discharges of Orhangazi town located 15 km in the west of the Gemlik Bay (Solmaz, 2000). Even though the discharge of industrial plants are relatively lower than other Bays in Marmara Sea, effects are worrying and also can be observed visually in the summer with the phenomena of red waters.

4.3.3 Moda

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

Moda has been extensively exposed to wastewater discharges since the end of 1970s thus, in the early 2000; remediation techniques have been began to apply to save the coastal line. Because of the rich organic content of the domestic discharges, 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, 2001).

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. 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 millimetres.

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 bio-turbation processes of burrowing microorganisms.

4.3.4 Küçükçekmece

Kucukcekmece Lagoon, located in the European part of Istanbul in Turkey, has typical spoon shaped topography. The surface area of the lake is approximately 17

km^2, and the water volume is 145 million m3 at sea level. Untreated wastewaters, both domestic and industrial (metal, textile, plastic etc.), are routinely being discharged into the creeks of Kucukcekmece Lagoon (Gonenc, 1997). Three stream systems feed the lake: Nakkasdere, Sazlıdere and Ispartakule . 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 (Gonenc, 1997).

Küçükçekmece is a natural habitat before 1980's. After 1980, by the contribution of extensive immigration to the area and bad watershed management policies of the government, Küçükçekmece dramatically transformed to industrial area from rural area. The result was twofold: 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. Also watershed was polluted by petroleum hydrocarbons. The Russian oil tanker *Volgoneft* accident which is mentioned in section 4.2 happened near the region.

4.3.5 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). Industrial and human settlements along this area have been grown very rapidly over the past 25 years.

Main pollution problem in this site caused from untreated agricultural, municipal and industrial wastes. Moreover, on February 13th, 1997, a tanker named TPAO exploded in Tuzla shipyards located on the north eastern 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 (Ünlü, 2000). After the accident, oil concentrations were

measured on the water column and as well as in the sediments. 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).

4.3.6 Haliç Bay (Golden Horn)

Haliç bay is an estuarine that is located southwest of the Strait of Istanbul (Bosphorus). It is 7.5 km long, 150–900 m wide horn shaped body of water that connects the Alibeykoy and Kagithane Rivers to the Bosphorus strait. Estuarine surface area covers 2.6 km^2 and maximum depth is 36m at the mouth, sloping to <1 m near tributary inflow. The shallow inner estuary, defined as the area north of the Valide Sultan/Old Galata Bridge , is more prone to anoxic conditions given that its depth abruptly slopes to <5 m 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 (Ozsoy, 1989).

Such gradation in salinity should result in a system it high diversity in non-polluted waters. However, the estuary has been polluted by wastewater of pharmaceutical, detergent, dye, leather industries and domestic discharges since the 1950s. It is revealed that the metal pollution due to anthropogenic disturbance altered significantly within the second half of the century. Poor renewal capacity of Haliç causes the accumulation of the organic and inorganic matter within the water column and the sediments.

The anthropogenic activities adversely affect the communities living in the estuary and also aesthetic structure of Haliç was damaged due to odour problems caused by hydrogen sulfide formation (Narin, 2001). Therefore, a water rehabilitation plan was devised to improve water quality which focused on the inner estuary. First, 4.25 x 10^6 m^3 anoxic sediment filling the basin was removed and approximately 4–5 m depth was gained at the completely filled part. Afterwards, in May 2000, freshwater was released from the closest dam to the estuary for rapid oxygenation of the anoxic water body. Meanwhile, most of the domestic discharges were gradually connected to a collector system discharging deep into the lower layers of the strait, reaching deep water in the Black Sea. Finally, in May 2000, the floating bridge opened to ease

water circulation. However, implementation of the plan and the provision of a better water quality in the estuary could not be successfully demonstrated unless continuous data on all aspects of ecosystem were collected. Figure 4.2 demonstrates the location of the polluted sites that is mentioned above.

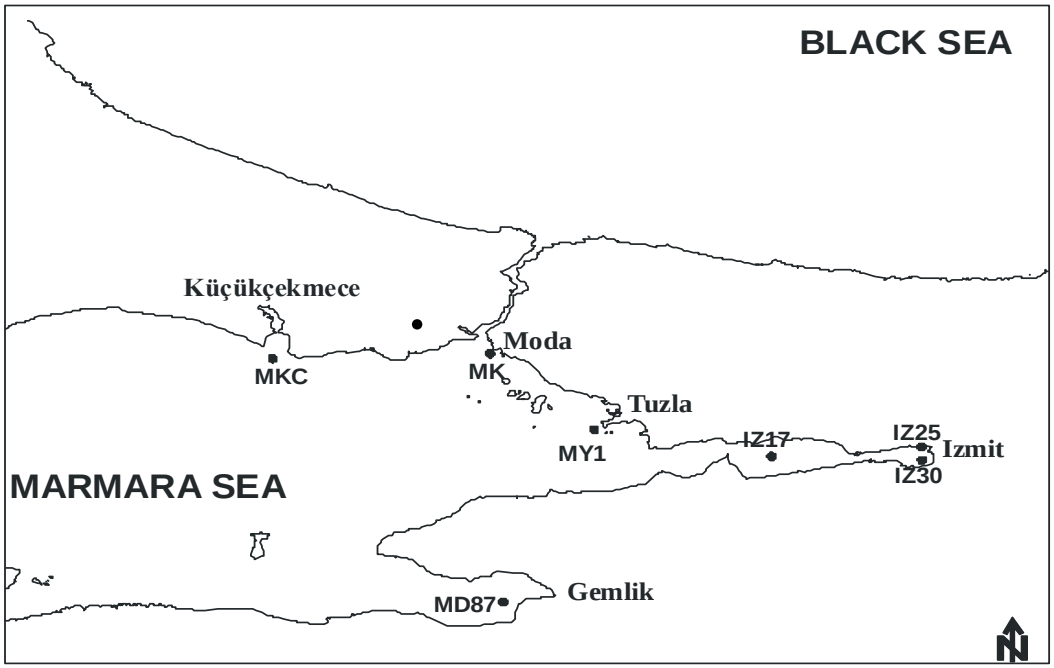


Figure 4.2: Location of polluted sites of Marmara Sea

4.4 Previous Studies on Physicochemical and Microbial Characteristics of Marmara Sea Sediments

4.4.1 Physicochemical characterization of Marmara Sea sediments

Recent study from Kolukırık (2010) revealed the physicochemical characteristics of Marmara Sea sediments. Investigation of the most polluted area in the Marmara Sea, mentioned in section 4.3, showed that all of the sediments that collected from these sites had fine-grained nature, being rich in mud (>90%) and poor in sand. Pore water of the sediments had the appropriate conditions (temperature varies between 14-21^{0}C and pH values changes between 7.5 and 8.3) to support wide variety of microbial processes such as methanogenesis, denitrification and sulphate reduction. Also redox potential of the sediments (-150 – -250 mV) were found to favour the iron reduction and sulphate reduction as well.

Surprisingly Marmara Sea Sediments have unusual sulphate and nitrate concentrations rather than generally observed. NO_3^- concentrations in porewaters

vary between 100-2200 μM (normal values; 10-50 μM) and SO_4^{2-} levels change between 0.5-17 mM (26-32 mM) (Jorgensen, 1977).

Also heavy metal measurements indicated the strong nickel pollution (50-100 mg/kg) and moderate zinc pollution (100-1000 mg/kg) compared with relevant studies (Morillo, 2008). Levels of TPH (1000-20000 ppm), aliphatic hydrocarbons (200-8000 ppm) and aromatic hydrocarbons (500-10000 ppm) in the sediments indicated the extreme and chronic pollution in Marmara Sea.

4.4.2 Microbial characteristics of Marmara Sea sediments

4.4.2.1 Amounts of microbial cells present in the Marmara Sea sediments

Studies about characterization of microbial communities via molecular tools in Marmara Sea sediments were carried by Kolukırık (2010).Studies of Kolukırık and Cetecioglu (2010) have brought the novel knowledge about microbial communities of Marmara Sea sediments to literature.

One of the main concerns about bioremediation is the abundance of local microbial communities. According to Kolukırık (2010), abundance of microbial species are mainly affected by bioavailable nitrogen and phosphorus. In this study quantification of microorganisms were done via Q-PCR and 4',6-diamidino-2-phenylindole (DAPI) staining of nucleic acid contents of the cells. Results obtained from this study showed that microbial cell contents of the sediments changed between 5×10^{9}-1.5×10^{11} cells/cm^3 (According to DAPI counts). However Q-PCR results were 6-12% higher than the DAPI cell counts. These results indicate that minority of microbial cells captured as detrital. In Marmara Sea sediments Bacteria population (1.6×10^{9}-9.4×10^{10} cells/cm^3) were dominated over Archeae (4.5×10^{8}-4.4×10^{10} cells/cm^3). Another important component of bioremediation is the activity of microbial cells, since microbial metabolisms are responsible for the degradation of deleterious compounds.

FISH results of the same study showed that 60%-85% of the total cells were active which were also correlated with bioavailable nitrogen and phosphorus in the porewater of the sediments.

4.4.2.2 Microbial composition of Marmara Sea Sediments

Cloning and Sequencing methods were used by Kolukırık (2010) to investigate the microbial composition of Marmara Sea sediments. 234 bacterial and 262 archaeal operational taxonomic units were detected by the authors.

The microbial communities were dominated by *Euryarchaeota* (43-55%) and *Proteobacteria* (32-48%) as well as MAC (24-44%) and MBC (13-23%) which were archaeal and bacterial phylogenetic clusters unique to Marmara Sea. *δ-proteobacteria* (13-37%) and *Methanomicrobia* (14-30%) were the most abundant classes.

4.4.2.3 Anaerobic hydrocarbon degrading bacteria abundance and activity

Anaerobic hydrocarbon degrading bacteria abundance and activity were investigated by Kolukırık and Cetecioglu (2010). They used Q-PCR and Q-RT PCR methods to quantify key enzymes and their transcripts of anaerobic aromatic hydrocarbon degradation such as benzoyl coenzyme A reductase (bcrA) and benzylsuccinate synthase (bssA) and alkylsuccinate synthase (assA). Bacteria which carried one of the functional genes for anaerobic hydrocarbon degradation were named as anaerobic Hydrocarbon Degrading Bacteria. This group comprised 3-40 % of the total prokaryotic cells in Marmara Sea sediments. bcrA, bssA and assA transcription levels showed that these bacteria were as active as other microbial groups that were responsible different metabolic processes such as sulphate and nitrate reduction. Figure 4.3 shows the relative abundance and transcription levels of ass, bcr and bss genes in MSS during the two years monitoring period.

4.5 Enrichment of Anaerobic Hydrocarbon Potential of Haliç Bay Sediments via Nutrient Amendment

Study about enrichment of anaerobic hydrocarbon potential was carried by Kolukırık (2010) via designated microcosms that were mentioned in section 4.2. In this study, sulphate reduction inhibitor was added to microcosms seeded with Halic Bay sediments sediments to stimulate methanogenesis in the serum bottle. Addition of dissimilatory sulphate reduction inhibitor and unobserved dissimilatory nitrate reduction provided direct correlation between gas production and syntrophic

consortium of fermentative Bacteria and methanogenic archaea. CO_2 and CH_4 production were given in the Figure 4.4 and Figure 4.5.

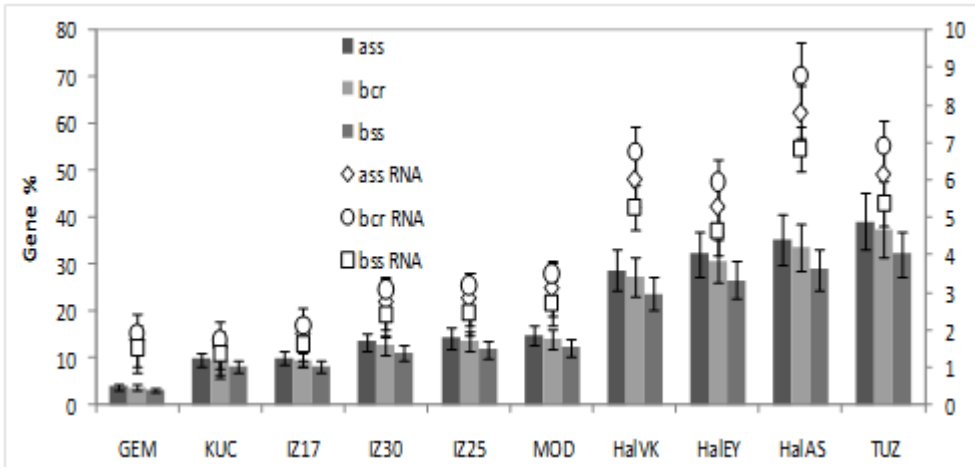


Figure 4.3: Relative abundance and transcription levels of ass, bcr and bss genes in MSS (Kolukırık, 2010).

Microcosms which have higher initial amount of N and P were resulted in higher gas production. These results indicated the possibility of bioremediation of Marmara Sea sediments via nutrient amendment. Also addition of external hydrocarbons to the microcosms was resulted in 2 times higher gas production for microcosms that are set up with unlimited nutrient supply (UL).

These results revealed that the added hydrocarbons (HCs) were biologically more available compared to the natural C sources in the sediments for the microbial growth.

4.5.1 N and P requirement

According to TOC:N:P ratios, N and P levels in the UL microcosms showed that HC added UL microcosms require more N and P levels (1000:78:12). Thus incomplete carbon removal was observed in these microcosms. WH-UL microcosms found out at the expected ratios (1000/47/7). Carbon removal stopped when N and P were depleted.

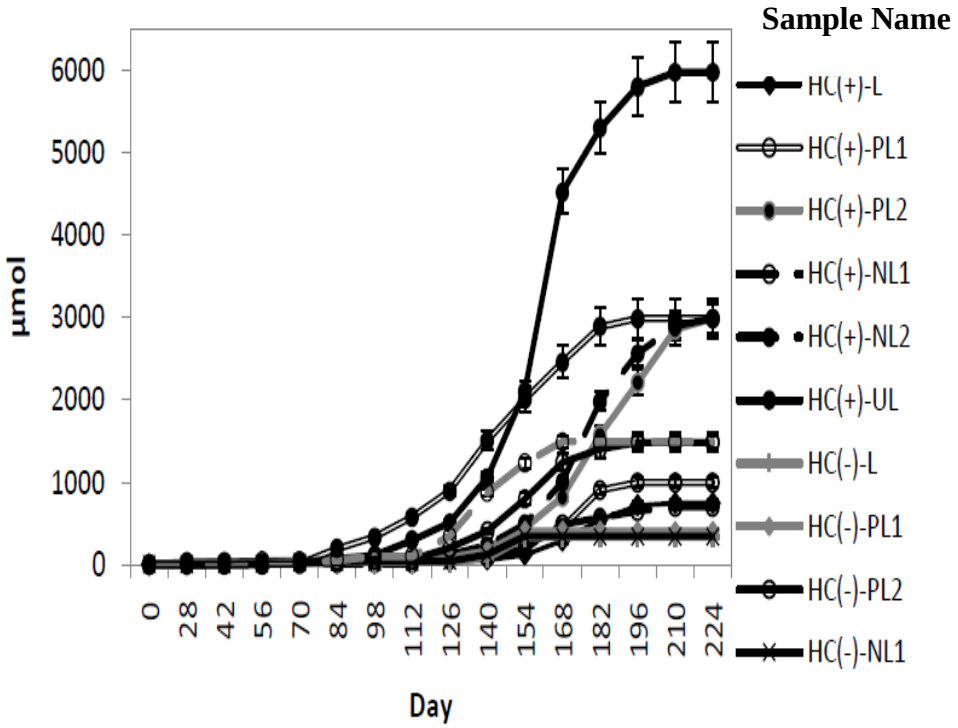


Figure 4.4: Cumulative CO_2 production in the microcosms (Kolukırık, 2010).

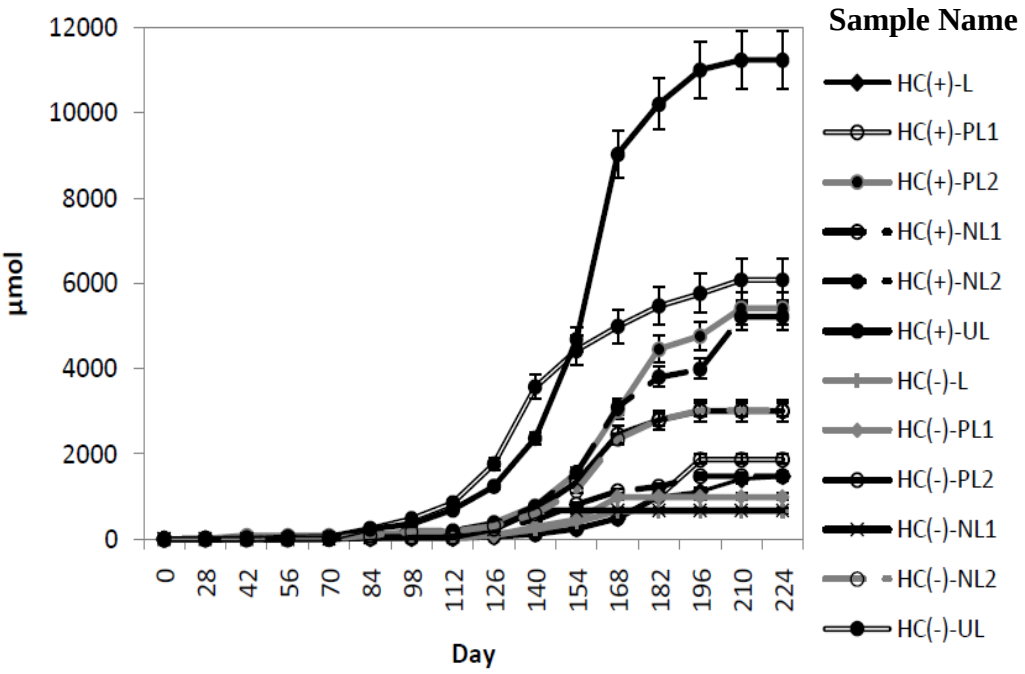


Figure 4.5: Cumulative CH_4 production in the microcosms (Kolukırık, 2010).

4.5.2 Changes in hydrocarbon composition

In Kolukırık's study (2010), removal percentage of aromatic and aliphatic HC of HC(+)-UL microcosms were observed 55 % and 57 % respectively. High proportion (92 %) of aromatic HCs and all aliphatic HCs were removed from HC(-)-UL microcosms.

4.5.2.1 Aromatic hydrocarbon degradation ratios

Aromatic hydrocarbons which have 1-3 rings have completely removed in both designated microcosms. However in HC(+)-UL microcosms, antracene was partially (40%) removed. 4-5 ring aromatic HCs were not degraded in HC(+)-UL microcosms whereas those in HC(-)-UL microcosms were completely consumed except benzo(g,h,i)perylene.

4.5.2.2 Aliphatic hydrocarbon degradation ratios

In HC(-)-UL microcosms n-C9-31 alkanes were depleted completely. In HC (+)-UL microcosms n-C9-20 alkanes were degraded, and n-C21-31 alkanes remained unchanged. n-C9-18 and n-C20 alkanes were also completely degraded in HC(+)-UL microcosms. Figure 4.6 shows the changes in aromatic and aliphatic hydrocarbon levels of HC(+)-UL and HC(-)-UL microcosms.

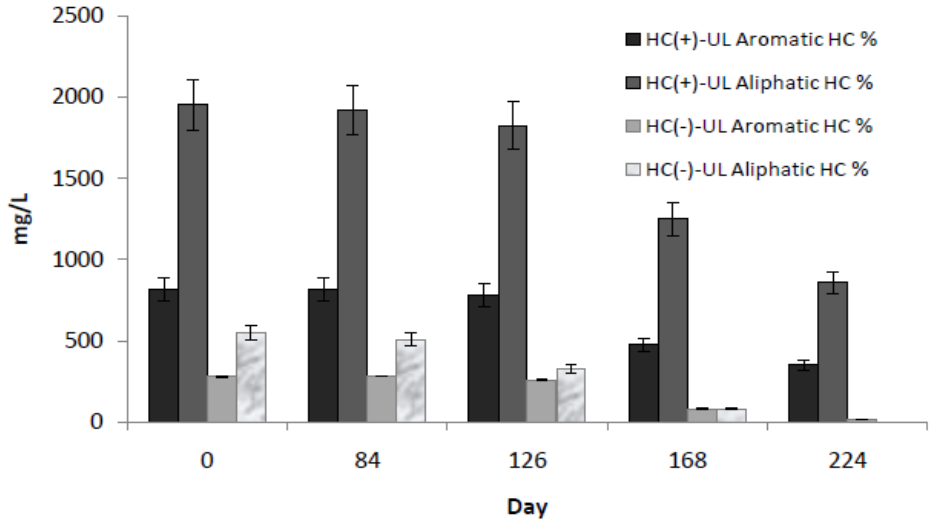


Figure 4.6: Changes in aromatic and aliphatic hydrocarbon levels of HC(+)-UL and HC(-)-UL microcosms seeded with Tuzla Bay sediments (Kolukırık,2010).

4.5.3 Recent findings about bioremediation potential of Haliç Bay sediments.

Throughout the literature, there is no study about increasing anaerobic hydrocarbon degradation activity of marine microbenthos through nutrient amendment. Novel study from Kolukırık (2010), proved the possibility of enhanced anaerobic hydrocarbon degradation activity of the Halic Bay sediments substantially (~9×) by supplying the limiting nutrients (N and P).

In anaerobic bioremediation of Haliç Bay sediments seems to be feasible due to high abundance of anaerobic hydrocarbon degrading microbiota, petroleum degradation ability of the Haliç Bay sediments and positive response of microbial communities to N and P amendment.

4.6 Enrichment of Anaerobic Hydrocarbon Potential of Tuzla Bay Sediments via Nutrient Amendment

In the microcosms include 7.5x times more phosphorus, cumulative gas production resulted in 14× and 21× increase in total biogas amount. This finding indicated the possibility of stimulation of the microbial community within the Tuzla Bay sediments via nutrient amendment (Kolukırık, 2010).

4.6.1 Changes in hydrocarbon composition

Hydrocarbon removal stopped in all microcosms due to complete degradation of bioavailable phosphorus. 75% of hydrocarbon was removed in the hydrocarbon added microcosms. During exponential growth (at days 108 and 136) phase approximately 40 % of HC was removed. Also HCs were completely removed from HC(-) microcosms. Table C.1 and Table C.2. show the changes in HC composition.

Study revealed that short chain hydrocarbons were degraded faster than the long chain hydrocarbons. Aromatic hydrocarbons were only degraded after significant removal of n-alkanes and alteration of acyclic isoprenoids pristane and phytane (Kolukırık, 2010).

4.6.2 Anaerobic bioremediation feasibility of Tuzla Bay sediments

Kolukırık's study is the first report about 3-5 ring PAH degradation under methanogenic and nitrate reducing conditions. The study also indicate the degradation of acenaphtylene, benz(a)antracene, chrysene, benzo(k)flouranthene,

benzo(a)pyrene, dibenz(a,h)antracene and benzo(g,h,i)perylene biodegradation under nitrate reducing conditions.

The study showed that it is possible to increase anaerobic hydrocarbon degradation activity of Tuzla Bay sediments substantially 21 times by supplying the limiting nutrients (N and P).

5. METARIALS AND METHODS

5.1 Sampling

Sediment samples were collected by Institute of Marine Sciences and Management of Istanbul University in November, 2008. The samples were taken via a Van Veen grab (volume of 3.5 L and penetration depth of 15 cm) on board of the RV Arar of Istanbul University. Collected sediment samples were placed into 50 ml sterile Falcon tubes and transferred to the laboratory immediately in cool boxes (+4 °C) and stored at -20 °C. Sediment samples collected from Haliç and Tuzla Bay that having the following coordinates. Table 5.1 shows the sampling locations.

Table 5.1: Coordinates of sampling locations.

Location	Coordinates			
	Latitude (N)	**Longitude (E)**	**Depth (m)**	**Sampling Date**
Halic	**41˚24.24'**	**28˚56.92'**	**6**	**11.11.2008**
Tuzla	**40 ˚50.60'**	**29 ˚13.60'**	**42**	**12.11.2008**

5.2 Microcosm Setup

Sediment samples were collected from microcosms which were prepared with the Haliç and Tuzla Bay sediments under anaerobic and anoxic conditions respectively. Anaerobic microcosms were set up in glass 120-ml serum bottles sealed with butyl rubber stoppers and aluminium crimps (Aldrich). The total volume of liquid was 100 ml with 20 ml of headspace volume. An anaerobic cabinet (Coy Laboratory Products) fitted with an oxygen sensor and with a regulated atmosphere of nitrogen

(100%) was used in the preparation and incubation of the microcosms. Each microcosm comprised a carbonate-buffered nutrient medium containing sources of nitrogen (Nitrate) (Nitrate wasn't added to micrcosms that seeded with Haliç Bay sediments instead of ammonia was added) and phosphorus (Mono Potassium Phosphate), vitamins and trace minerals, mixed in deionized water, according to the brackish medium of Widdel and Bak (Widdel, 1992).

Microcosms were seeded with 10g of the sediments. Microcosms were fed with 200 mg of the hydrocarbon mixture (contains 20 aliphatic and 21 aromatics hydrocarbon). Composition of the hydrocarbon mix was defined based on the detected hydrocarbon types in the sediments during the two years monitoring study. Table C.1 shows the composition of HC mixture (Kolukırık, 2010).

The overall TOC/N/P ratio of Halic Bay sediments (~1000/5/1) was chosen as a nutrient limited condition. The unlimited nutrient condition was calculated as 1000/40/6 (C/N/P) based on the following assumptions: (1) molecular formula of the HC mix was C_5nH_8n (derived from the HC composition); (2) the maximum biomass yield was as high as 0.2 gcell/gHC mix and (3) C/N/P ratio of the marine microbes was 100/20/3 . Hence, the nutrient amendment was done by gradually decreasing TOC/N/P ratio from 1000/40/6 to 1000/5/1 for Halic Bay sedimets microcosms.

Microcosms were prepared in triplicates. 5 sets of each condition were prepared for destructive sampling. The destructive samplings were carried out based on the gas production data. Destructive samplings were done at the 0^{th}, 84^{th}, 126^{th}, 168^{th} and 224^{th} days of the experiment. The intended initial N and P concentrations, the experimental conditions and controls, and abbreviations of the sample names were summarized in Table 5.2 and Table 5.3.

Sediment samples for Denaturing Gradient Gel Electrophoresis (DGGE) were collected from these microcosms. These microsom xperiments were the part of the TUBITAK project no: 105Y307- Anaerobic degradation of petroleum hydrocarbons in anoxic marine environments.

Table 5.2: Summary of the experimental conditions and controls, and abbreviations of the sample names for Tuzla Bay sediment's microcosms (Kolukırık, 2010).

Condition	Abbreviation	C	N	P
Limiting	L	1000	5	1
N Limiting 1	NL 1	1000	5	6
N Limiting 2	NL 2	1000	20	6
Unlimited	UL	1000	40	6
P Limiting 1	PL 1	1000	40	1
P Limiting 2	PL 2	1000	40	3

Table 5.3: Summary of the experimental conditions and controls, and abbreviations of the sample names for Halic Bay sediment's microcosms (Kolukırık, 2010).

Condition	Abbreviation	C	N	P
Limiting	L	1000	5	1
P Limiting 1	PL 1	1000	100	1
P limiting 2	PL 2	1000	100	7.5
Unlimited	UL	1000	100	15

5.3 Genomic DNA Extraction

Genomic DNA extraction was performed by using Fast DNA Spin Kit for Soil (Q-Biogene, Bio 101 Thermo Electron Corporation, Belgium). Standard experimental protocol was applied according to manufacturer's protocol. Sediment samples were washed with 1X PBS solution (provided with the kit) twice to obtain the removal of humic substances which may cause inhibition of Polymerase chain reaction (PCR). Approximately 0.5 g sample 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 TM FP120 Bio 101 Thermo Electron Corporation) for 30 seconds at speed of 5.5 m/s. The tubes were then centrifuged at 14000 g 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 shaken by hands for 30 seconds. After mixing the tubes, centrifuged again at 14000 g for 5 minutes to pellet the precipitate completely. 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 14000 g 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 14000 g for 1 minute. Application-ready DNA was obtained in the tube. 1/100 diluted genomic DNA was run on the 1% (w/v) agarose gel, prestained with ethidium bromide (EtBr) in 1X Tris-acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; pH 8). Gel was visualized by using a gel documentation system, Mitsubishi 91. If the extracted DNA could be visualised via gel electrophoresis, genomic DNAs (gDNA), they were stored at -20 ^{0}C for further molecular analysis .

5.4 Polymerase Chain Reaction (PCR)

Amplification of 16S ribosomal DNA (rDNA) gene sequences was performed by PCR using archaeal and bacterial specific primers. Extracted gDNAs were used as a template for these primers. Bac341f-Bac534r and Arch344f-Univ522r primers were used to amplify V3 region of 16S rDNA (approximately 200 bp long) of Bacteria and Archaea, respectively. Primers used in the molecular analyses were shown in Table 5.4 and their sequences were given in Table 5.5. Table 5.6 shows the PCR conditions.

PCR reactions were performed in a 50 µl reaction volume containing 200 ng of DNA,1 µl of each primer that is diluted to 10 pmol, 10 mM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 5 µl of 10×*Taq* buffer and 4 unit of *Taq* DNA polymerase (Fermentas, Latvia). For the second-round nested amplification 1 µl of

the first-round product was used as template, with reaction composition being the same as previously**.**

Table 5.4: Bacterial and Archaeal oligonucleotide primers used for PCR amplification.

Primer	Experimental Stage	Annealing Temperature ^{0}C	Position	Reference
Bact341f-GC[2]	Second round for DGGE	55	341-357	Muyzer et al., 1993
Bact534r	Second round for DGGE	55	534-518	Muyzer et al., 1993
Bact8f	First round of nested PCR	55	8-27	Edwards et al., 1988
Bact1541r	First round of nested PCR	55	1541-1522	Edwards et al., 1988
Arch07f	First round of nested PCR	52	07-24	Lueders et al., 2004
Arch1384r	First round of nested PCR	52	1384-1368	Lueders et al., 2004
Arch344f-GC[2]	Second round for DGGE	53	344-358	Raskin et al., 1994
Univ522r	Second round for DGGE	53	522-504	Amann et al., 1995

Table 5.5: Bacterial and Archaeal oligonucleotide primer's sequence used for PCR amplification.

Primer	Sequence (5'-3')	Reference
Bact341f-GC[2]	GC* GCC TAC GGG AGG CAG CAG	Muyzer et al. 1993
Bact534r	ATT ACC GCG GCT GCT GG	Muyzer et al. 1993
Bact8f	AGA GTT TGA TCC TGG CTC AG	Edwards et al. 1988
Bact1541r	AAG GAG GTG ATC CAG CCG CA	Edwards et al. 1988
Arch07f	TTC YGG TTG ATC CYG CC	Lueders et al. 2004
Arch1384r	CGG TGT GTG CAA GGA GCA	Lueders et al. 2004
Arch344f-GC[2]	GC* GAC GGG GHG CAG CAG GCG CGA	Raskin et al. 1994
Univ522r	GWA TTA CCG CGG CKG CTG	Amann et al. 1995

Nested PCR was used for equaliztion of the DNA concentrtion of the samples. PCR amplifications were performed in a Techne TC-412 thermal cycler. Conditions are given in Table 4.5. Products of all reactions were screened for the amplification of correct band size. All PCR products were run on the %1 (w/v) agarose gel prestained with ethidium bromide (EtBr) in 1x Tris-acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; pH 8). Gels were visualized by using a gel documentation system, Mitsubishi 91(Bio-Rad Laboratories, Ltd., UK).

Table 5.6: PCR conditions for each relevant primer

Primers	Denaturation	Annealing	Elongation	# of Cycles
Bact8f-Bact1541r, pC	94 ^{0}C 45 sec.	55 ^{0}C 45 sec.	72 ^{0}C 60 sec.	30
Bact341f-Bact534r	94 ^{0}C 45 sec.	55 ^{0}C 45 sec.	72 ^{0}C 60 sec.	30
Arch07f-Arch1384r	94 ^{0}C 30 sec.	40 ^{0}C 30 sec.	72 ^{0}C 60 sec.	35
Arch344f-GC^2-Univ522r	94 ^{0}C 30 sec	53 ^{0}C 30 sec.	72 ^{0}C 60 sec.	35

5.5 Denaturing Gradient Gel Electrophoresis (DGGE)

Both samples were run on a gel electrophoresis using D-Code system (Bio-Rad Laboratories, Ltd., UK) .The first step was the assembly of gradient gel sandwich. Before assembly, glass plates were cleaned carefully with 70% EtOH to avoid any particle matter which may affect the gel and experiment procedure. The position of spacers were checked to avoid any leakage and glass plate sandwich then placed on the casting stand. The next step was preparation of the denaturing gradient gel. All samples were run on a 10% polyacrylamide gels (37.5:1 acrylamide:bisacrylamide) in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; pH 8.0) over a 40–70% denaturing gradient. 40% denaturant solution was prepared by mixing 33.3 ml of 30% acrylamide:bisacrylamide with 2 ml 50xTAE (2.0 M Tris, 50 mM EDTA, and 1.0 M acetic acid) and 12 ml formamide and 12.6 g urea. 70% denaturant solution was prepared by adding 24 ml formamide and 25.2 g urea to 33.3 ml of %30 acrylamide: bisacrylamide and 2 ml 50xTAE (2.0 M Tris, 50 mM EDTA, and 1.0 M acetic acid). Both solutions were added distilled water up to 100 ml. After solutions were prepared, they were filtered with 0.45 μm filter and sonicated to remove air bubles inside the solution for 15 minutes. The bottles were stored in

amber bottles to avoid sunlight and stored at 4^0C for further uses. Two syringe and wheel system were used to prepare the acrylamide gel. One of the syringe was filled, 18 mL of 10% (w/v) acrylamide:bisacrylamide solutions containing 40% denaturant and the other was filled 70% denaturant respectively. Each denaturant solutions previously were mixed with, 200 µl freshly prepared 10% ammonium per sulphate (APS) and 7.5 µl TEMED. Filled syringes were replaced to the wheel system and solutions were transferred to the assembled gel sandwich. After the transfer of solutions the comb was replaced in order to form appropriate well for loading samples. After polymerization (typically lasts in 2-3 hours), the comb was removed carefully to avoid collapsing of the wells. The wells are washed with distilled water to remove gel residues. After washing step, gel sandwich was replaced into the electrophoresis tank. Electrophoresis tank was filled with 1X TAE until marked level (approximately 7 liters of 1X TAE) and temperature was set to 60^0C.

Sample loading step was started with preparation of samples.4 µl of loading dye was mixed with 8 µl of PCR product to be run. The samples were carefully loaded into the wells. The DGGE was conducted at a constant voltage of 100 V, 63-68 mAmp at 60^0C for 17 hours in 1xTAE containing electrophoresis tank.

The last step was staining and visualizing gels. Gel sandwiches were taken from the tank and separated from it. Glass plates were disassembled and the direction of gel was marked with a cut on the upper left corner. 50 µl of 1:100000 diluted SYBR Gold DNA staining dye was added to 500 ml 1xTAE washing buffer and gels were incubated for 15 minutes. Gels were destained with distilled water to remove background impurity. Gels were visualized by using a gel documentation system, Mitsubishi 91 (Bio-Rad Laboratories, Ltd., UK).

5.5.1 Statistical analysis

For diversity analysis, DGGE images were converted, normalized and analyzed by using the Bionumerics 5.0 Software (Applied Maths, Kortrijk, Belgium). Similarities between tracks were calculated by using the Dice coefficient (S_D) (unweighted data based on band presence or absence) and UPGMA clustering. For analysis using Dice coefficient a band position tolerance of 0.7% was applied. This was the minimum tolerance at which all marker lanes clustered at 100%. For intensity analysis, samples

were clustered depending on band weights by using Pearson coefficient and UPGMA clustering. Further statistical analysis was carried out with MINITAB Release 14.

Relative abundance percentages of each relevant microorganisms were calculated according to numerical values of the band intensities at the exponential growth phase (at 108^{th} day) of the experimental groups. Numerical values of the band intensities were calculated via Bionumerics 5.0 Software (Applied Maths, Kortrijk, Belgium).

6. RESULTS AND DISCUSSION

6.1 Microbial Community Fingerprints of Nutrient Amended Microcosms Seeded with Haliç and Tuzla Bays

6.1.1 Community fingerprints of Haliç Bay microcosms

DGGE analysis of microcosms seeded with Haliç Bay sediments revealed that population dynamics of microbial species within the microcosms, changed. Change in the microbial community was showed by dendograms which are plotted via bionumerics software using Dice and Pearson coefficients. Dendograms are tree diagrams frequently used to illustrate the arrangement of the clusters that having similar characteristics. Dendograms plotted via Pearson correlation are related with band intensities of the samples while dendograms plotted via dice coefficient are related with abundance of the bands on the relevant position. In Figure 6.1, Figure 6.2, Figure A.3 and Figure A.4, dendograms of Haliç Bay microcosms were shown. Limited (in terms of nutrient) and sterile (treated with sodium azide) experimental groups were not given in the figures since there were no significant changes in the microbial communities ($p<0.05$).

As it can be seen in the dendogram, for archaeal communities within the nutrient amended microcosms seeded with Halic Bay Sediments, occasionally clustered according to time. Similarities between samples were found much higher in the dendograms plotted with Pearson coefficient. These results indicated the archaeal community components were changed by means of species type rather than relative quantity of each species.

Bacterial communities of the samples taken from nutrient amended microcosms seeded with Halic Bay sediments occasionally clustered according to time similar to archaeal community changes over time. Even though similarity of bacterial samples was higher than archaeal community samples, evaluation of dendograms gave the same results with archaeal community. Bacterial communities also changed by means of species type rather than relative quantity of each species.

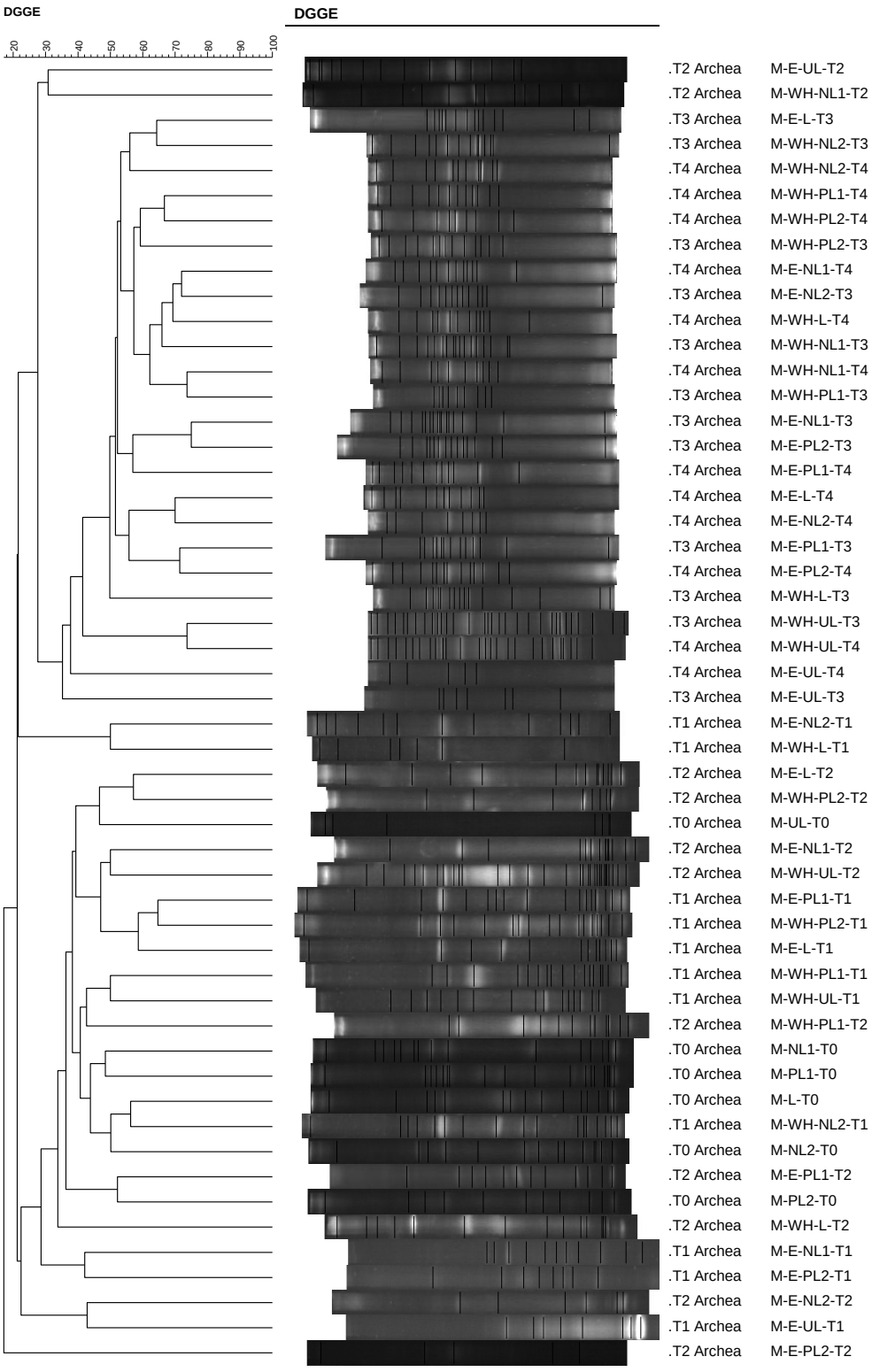


Figure 6.1: Archeal community dendogram plotted via dice coefficient from nutrient amended Halic Bay sediments.

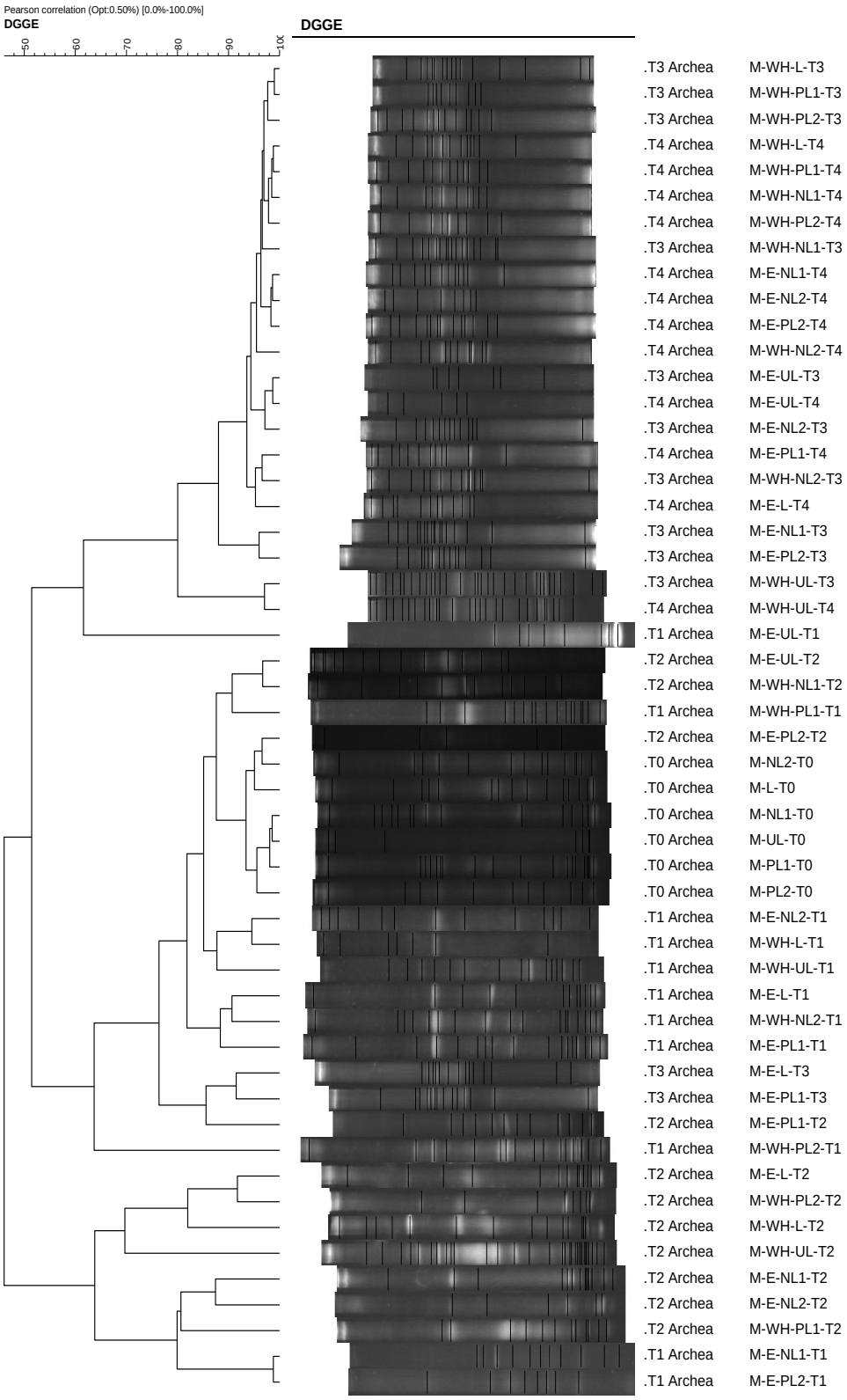


Figure 6.2: Archeal community dendogram plotted via Pearson correlation from nutrient amended microcosms seeded with Halic Bay Sediments

6.1.2 Community fingerprints of Tuzla Bay microcosms

Samples of nutrient amended microcosms seeded with Tuzla Bay sediments occasionally clustered according to time. Figure 6.3- Figure 6.4 show the relevant dendograms. Further dendograms can be found in appendix A and B.

Archaeal community of the sample of the nutrient amended microcosms seeded with Tuzla Bay sediments usually clustered according to sampling time. Similarity between samples was higher in the dendogram plotted with Pearson coefficient. This result indicated that archaeal communities changed by means of species type rather than relative quantity of each species.

Similarity of the bacterial samples showed congeneric results with archaeal samples. Bacterial communities were changed by means of species. Similar results with nutrient amended microcosms seeded with Halic Bay sediments intensify the differentiation of the community dynamics via biostimulation of the microorganisms in the presence of petroleum hydrocarbons.

6.1.3 Community fingerprints of unlimited experimental samples

Since samples taken from unlimited experimental serum bottles contain essential components for the petroleum biodegradation, evaluation of these samples plays a vital role in determination of the differentiation of possible candidates of petroleum biodegradation. Figures of these dendograms were shown in the appendix section.

Analysis of unlimited experimental samples from both experimental group indicate that some groups of microorganisms (can’t be revealed by using DGGE analysis solely) that can possibly degrade hydrocarbons became dominant over the time.

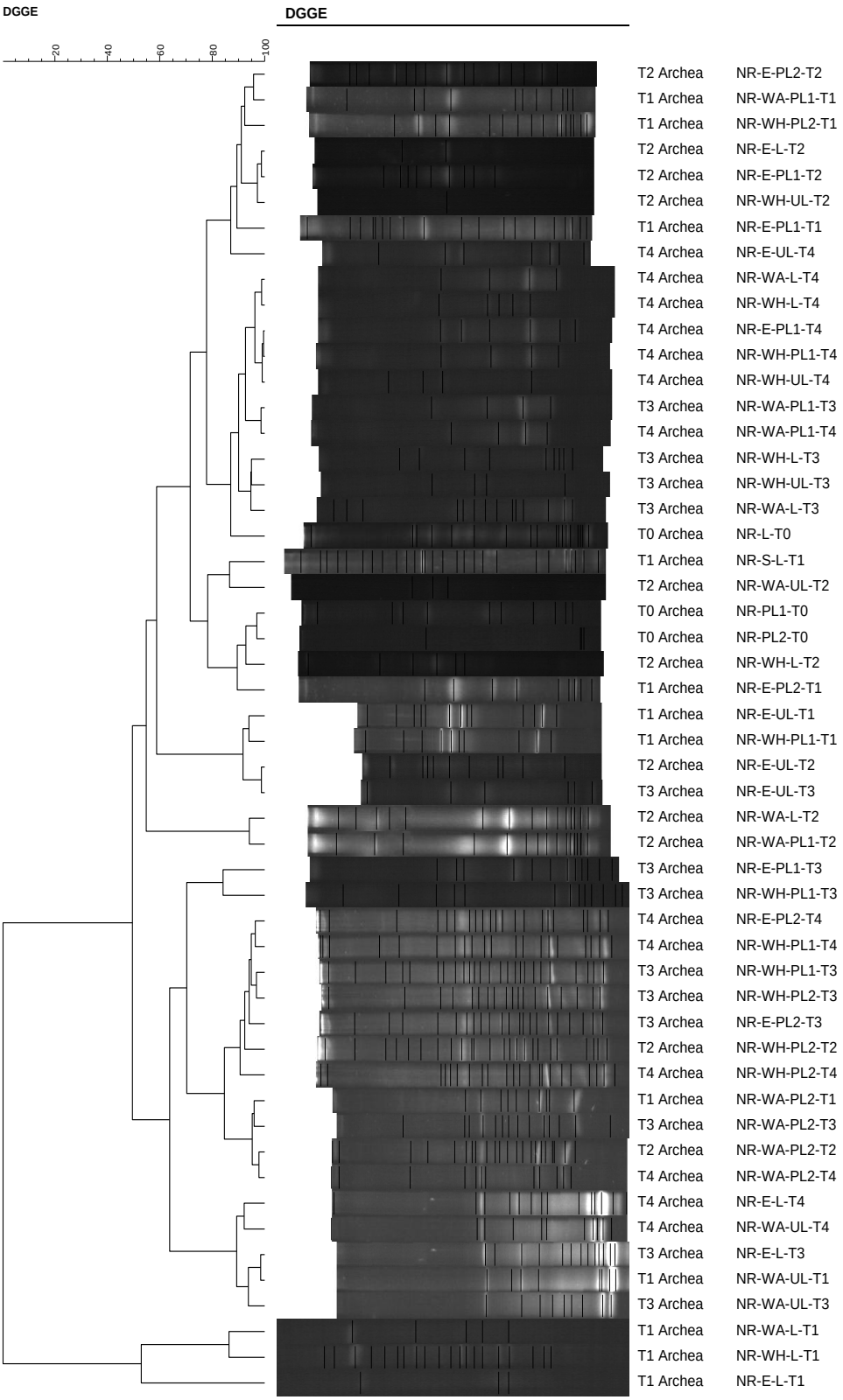


Figure 6.3: Archeal community dendogram plotted via Pearson coefficient from nutrient amended microcosms seeded with Tuzla Bay Sediments.

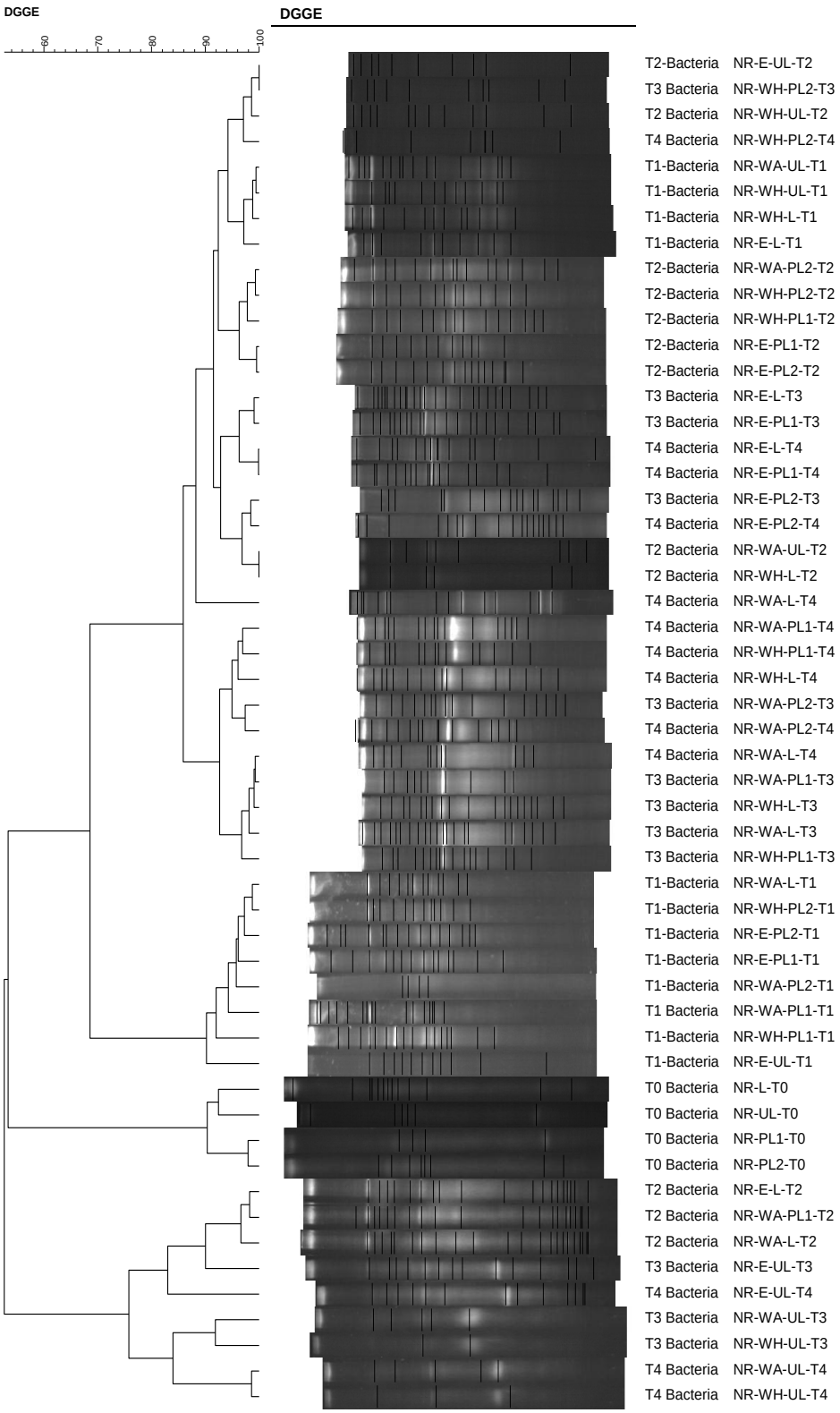


Figure 6.4: Bacterial community dendogram plotted via Pearson coefficient from nutrient amended microcosms seeded with Tuzla Bay sediments.

6.1.3.1 Unlimited microcosms seeded with Tuzla Bay sediments

Comparison of DGGE bands of unlimited experimental groups with the DGGE band data of the previously constructed clone libraries revealed that there were 75 different bacterial taxonomic units and 83 archaeal taxonomic units within the all microcosm samples. Abundance of these taxonomic units in each microcosm changed according to time and the type of the experimental groups.

In the E-UL experimental groups, microbial community changed over time while petroleum biodegradation taking place. Comparison of significant band intensity changes in the unlimited conditions with changes in limited conditions can indicate the dominant species that possibly degrade hydrocarbons.

Since hydrocarbon removal mostly occurred in the exponential phase of the experiments, differences between band's intensities were compared between three sampling time (0^{th}, 126^{th} and 168^{th} days). 6 bacterial species significantly changed over time when they were compared with limited experimental groups. Between bacterial species, two of the clones were firstly reported from Marmara Sea sediments. This finding strengthened the idea that they could be possible candidate for petroleum biodegradation since the habitat was chronically polluted. Correlation analysis showed that changes in the bacterial changes were significant ($0,1<p<0,05$, $r>0.90$ $n=3$). Correlated bacterial species includes sulphate reducing bacteria, nitrate reducing bacteria and chemolithotrophic bacteria. Information about these sepecies can be found in the Table D.1 and D.2 within the appendix section.

Correlation analysis between hydrocarbon removal and microbial population dynamics showed that 4 archaeal species changed significantly ($0,1<p<0,05$, $r>0.90$ $n=3$) over time when they were compared with limited experimental groups. Three of the correlated clones belonged to Marmara Sea sediments. All of the clones belonged to methanogenic Archaea. Three types of methanogens were detected; acetogenic, hydrotrophic and methylotrophic archaea. Uncultured archaeon clone Iz17_A10 was the dominant methanogen (represented 34% of the archaeal community) amongst the correlated species. Dominancy of methanogenic clones indicated the petroleum degradation coupled to methanogenesis.

Novel study from Chang et al. (2006) also supports the coupled hydrocarbon degradation to methanogenesis. *Methanococcus vannieli*, *Methanococcus*

maripaludis, Methanosarcina lacustris and *Methanosarcina mazei* were reported possible candidate for PAH degradation via syntrophic interactions.

In this study, no cultured species were identified but according to cultured similarity percentage, Methanobrevibacter sp. 1Y (98%) and Methanococcoides sp. NaT1 (97%) can be suggested as a possible candidate in the petroleum hydrocarbon degradation pathway. Information about archaeal species that were correlated with hydrocarbon degradation, was shown in the Table D.1 and Table D.2.

It can be speculated that methanogens can't compete with other species in nitrate or sulphate reducing conditions but this phenomena is not admissible to shallow marine sediments. Shallow marine sediments are characterized by intense and diverse microbial activities which generate steep chemical gradients. As the products of O_2, NO_3^-, Mn (IV), Fe (III) and SO_4^{2-} reduction enter consecutively deeper zones of the sediments, vertical cascades of electron-accepting processes are sustained. Methanogenesis occurs after electron acceptors that yield higher standard free energies have been depleted (D'Hondt, 2006). This is why high methanogenic activity has not been observed in the sediment zones shallower than 100 cmbsf. Methanogens in MSS were highly abundant and active along with NRB and SRB in 15 cmbsf. This was an expected result since electron acceptor (NO_3^- and SO_4^{2-}) levels in MSS were very low compared to the exceptionally high electron donor (TOC and TPH). Scarcity of the electron acceptors was also evident from the positive correlations between the NRB-SRB abundances and the NO_3^--SO_4^{2-} levels (Cetecioglu, 2009) (Kolukırık, 2010). It can be speculated that limited amount of electron acceptors were quickly depleted in a very short distance below the MSS surfaces which resulted in succession of all the redox zones. That finding supports the coupled hydrocarbon biodegradation to methanogenesis.

6.1.3.2 Unlimited microcosms seeded with Halic Bay sediments

a) <u>Hydrocarbon Added Microcosms</u>

In the E-UL experimental groups seeded with Halic bay sediments, microbial community changed over time while petroleum biodegradation taking place. Comparison between significant band intensity changes in the unlimited conditions could reveal the dominant species which can possibly degrade hydrocarbons. Since significant hydrocarbon removal mostly occurred between 84th and 126th days of the

experiment for microcosms seeded with Halic Bay sediments. Differences between band's intensities were compared between these three sampling time. 9 bacterial species were found highly correlated ($r>0.90$, $p<0.05$, $n=3$) with hydrocarbon removal rate when they were compared with limited experimental groups. Among bacterial species, 5 bacterial clones were not reported previously thus they belonged to Marmara Sea sediments. Significantly changed microbial species were highly varied and mostly belonged to phylum *Protobacteria* that overlapped with previously reported hydrocarbon degraders that given in the Table 3.1 and Table 3.2. Abundance of the gamma and *delta-Proteobacteria* subclass indicates the possible ability of hydrocarbon degradation since these 2 subphylum includes the major PAH-degrading genera, such as *Alcanivorax*, *Cycloclasticus*, *Pseudomonas*, *Oleiphilus*, *Oleispira,* and *Thalassolituus* (Watanabe, 2001; Head et al., 2006).

Metabolisms of identified bacteria were revealed by the aid of the knowledge about metabolisms of closest cultured relatives of relevant species. uncultured candidate division GN10 bacterium and Uncultured *Sinorhizobium sp.* clone HalAS_B10 were previously reported in PAH contaminated environments and their closest cultured relatives are also known as PAH degraders. Thus it can be speculated that these two species could be involved in initial attack on the hydrocarbon chains. Once larger hydrocarbons cleave into smaller hydrocarbon chains, they can be utilized by many microorganisms within the methanogenesis pathway. Only one species is found to be related with hydrolysis step of the anaerobic hydrocarbon degradation. Unidentified clone, showed 96% similarity with uncultured *Antarctobacter heliothermus strain EL-219*, was proposed as candidate hydrolytic bacteria of the system. 4 different bacterial species were found to be related with acidogenesis. These species includes uncultured bacterium clone HalAS_B9, delta proteobacterium LacK9, Uncultured *Longilinea* sp. clone HalAS_B4 and uncultured candidate division GN10 bacterium. Uncultured bacterium clone HalAS_B9 were the dominant acidogenic bacteria (represented 28% of the entire bacterial community). Acetogenic species that showed high correlation with hydrocarbon degradation were poorly varied. Only two predominant species could be associated with acetogenesis. *delta proteobacterium LacK9* (represents 1% of the bacterial community) and *Beta proteobacterium F06002* (represents 9% of the bacterial community) were the acetogenic species that involved in hydrocarbon degradation.

5 archaeal species changed significantly over time when they were compared with limited experimental groups. Correlation analysis ($r>0.90$, $p<0.05$, $n=3$) of archaeal species with hydrocarbon degradation rate showed that these species could be involved in the degradation process. Vast majority of the species were belonged to uncultured archaea. Uncultured archaeon HALEY_A4 and Uncultured archaeon clone HalVK_A6 were endemic to Marmara Sea sediments. All of the Archaea species were found to be methanogens. Methanogenic community embraced 3 different methanogens. These were acetoclastic methanogens, hydrogenotrophic methanogens and methonegens that can generate methane from methanol, methylamine or trimethylamine. Hydrogenotrophic methanogens were the dominant group within the microcosms seeded with Haliç Bay sediments. This finding is a strong proof that methanogenesis were carried via hydrogenotrophic methanogens while the hydrocarbons were used as a sole carbon source. Low abundance percentage of correlated acetoclastic methanogens supports the hypothesis that hydrogenotrophic methanogenesis was the main pathway for methane production.

Uncultured archeon V.8.ArD8 (29%), uncultured crenarchaeote E_F03 (56%) and uncultured archaeon CP-A21 (2,5%) were the abundant hydrogenotrophic methanogens in the relevant microcosms. *Methanosaeta concilii Opfikon* (2,5%) was the only correlated species within the acetoclastic methanogens. Also uncultured archaeon OHKA6.3 (2,4%) was identified in the the microcosms seeded with Haliç Bay sediments. This microorganism has a unique methabolism that can use methanol reduction with H_2 to methane.

Figure 6.5 shows the possible anaerobic hydrocarbon degradation pathway on the basis of anaerobic digestion and related microorganisms. Table D.3 and Table D.4 (can be found in appendix section) show the identified species and their uncultured and/or cultured closest relatives with possible metabolic ability and their abundance percentage within the archaeal or bacterial community.

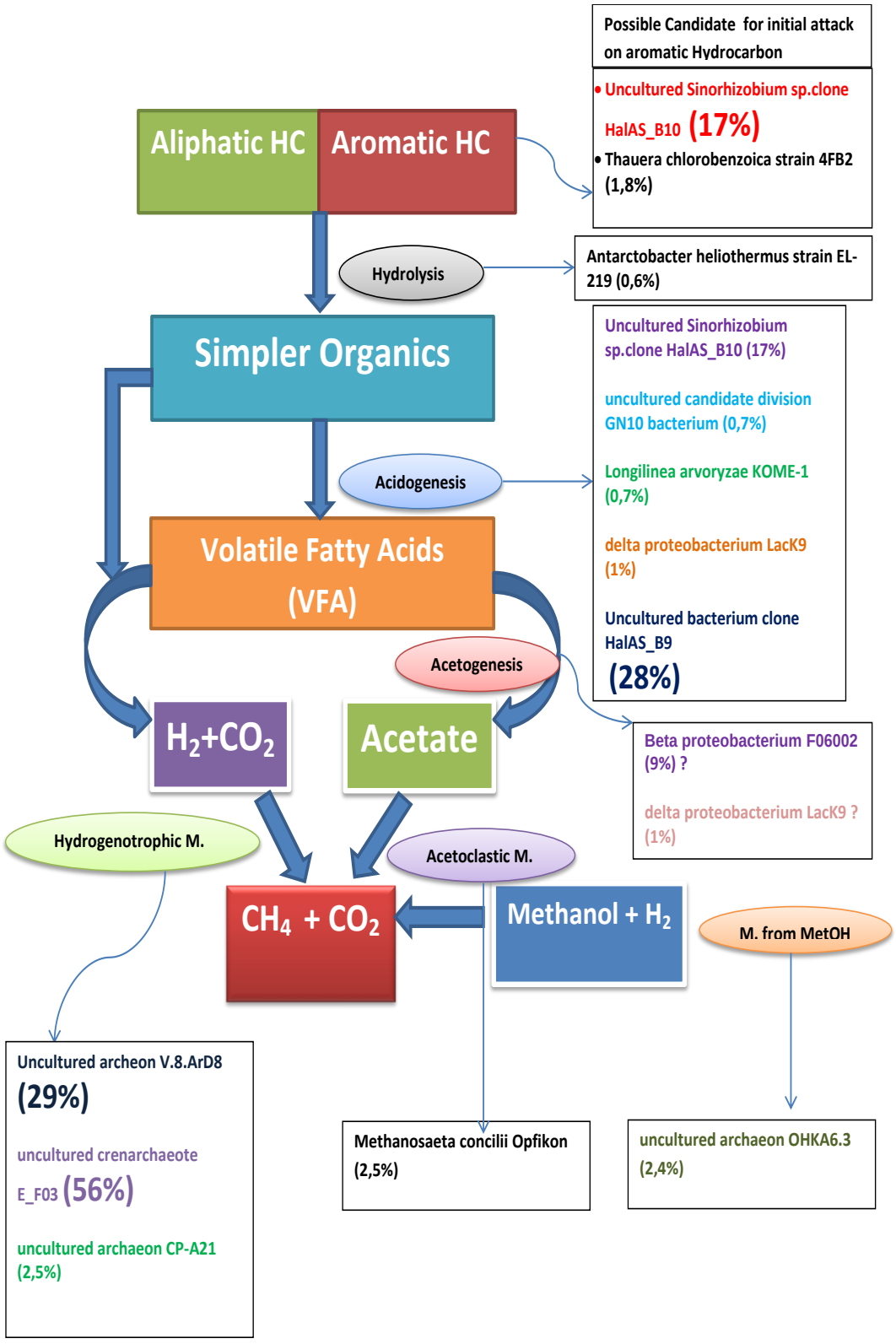


Figure 6.5: Possible anaerobic hydrocarbon degradation pathway on the basis of anaerobic digestion for UL experimental group seeded with Halic Bay sediments.

b) <u>Without Hydrocarbon Addition Microcosms</u>

Within the samples taken from WH microsms seeded with Haliç Bay sediments, 7 bacterial species were found highly correlated ($r>0.90$, $p<0.05$, $n=3$) with hydrocarbon removal rate when they were compared with limited experimental groups. Uncultured gamma proteobacterium clone HALEY_B5 was endemic to Marmara sea sediments. Bacterial community, correlated with hydrocarbon degradation rate, was relatively less diverse when it is compared to hydrocarbon added microcosms.

Two bacterial species were found related directly hydrocarbon degradation since their closest cultured relative can utilize aromatic and/or aliphatic hydrocarbons. Uncultured *Acidobacteriaceae* bacterium clone AT-s2 and Delta *proteobacterium Lac K9* were the proposed microorganisms for hydrocarbon degradation. *Delta proteobacterium Lac K9* was related with aromatic hydrocarbon degradation and was the dominant species amongst the correlated species (represented 30% of the bacterial community). Acidobacteriaceae bacterium clone AT-s2 represented 3% of the bacterial community and was mostly related with aliphatic hydrocarbon degradation. Unidentified clone that showed 96 % similarity with uncultured *Antarctobacter heliothermus strain EL-219*, was the only identified hydrolytic species in the pathway. Similar results observed in the UL experimental groups but abundance of unidentified clone within the WH experimental groups was more higher than UL groups (represented 7.4% of the bacterial community). Unidentified clone that showed 99 % similarity with uncultured *Succinivibrio dextrinosolvens* was found to be possible candidate for acidogenesis phase of the anaerobic hydrocarbon degradation. Correlated species for acetogenesis phase couldn't be detected for WH experimental groups.

14 archaeal species changed significantly over time when they were compared with limited experimental groups. Correlation analysis ($r>0.90$, $p<0.05$, $n=3$) of archaeal species with hydrocarbon degradation rate showed that these species could be involved in the degradation process. All of the archaeal were found to be methanogenic archaea. Hydrogenotrophic methanogens were the dominant species amongst the archaeal species that correlated with hydrocarbon degradation.

Uncultured archaeon clone HALEY_A6 was the dominant species (represented 14 % of the archaeal community) within the hydrogenotrophic methanogens and also within the archaeal species.

Figure 6.8 shows the possible anaerobic hydrocarbon degradation pathway on the basis of anaerobic digestion and related microorganisms. Table D.5 and Table D.6 shows the identified species and their uncultured and/or cultured closest relatives with possible metabolic ability and their abundance percentage within the archaeal or bacterial community.

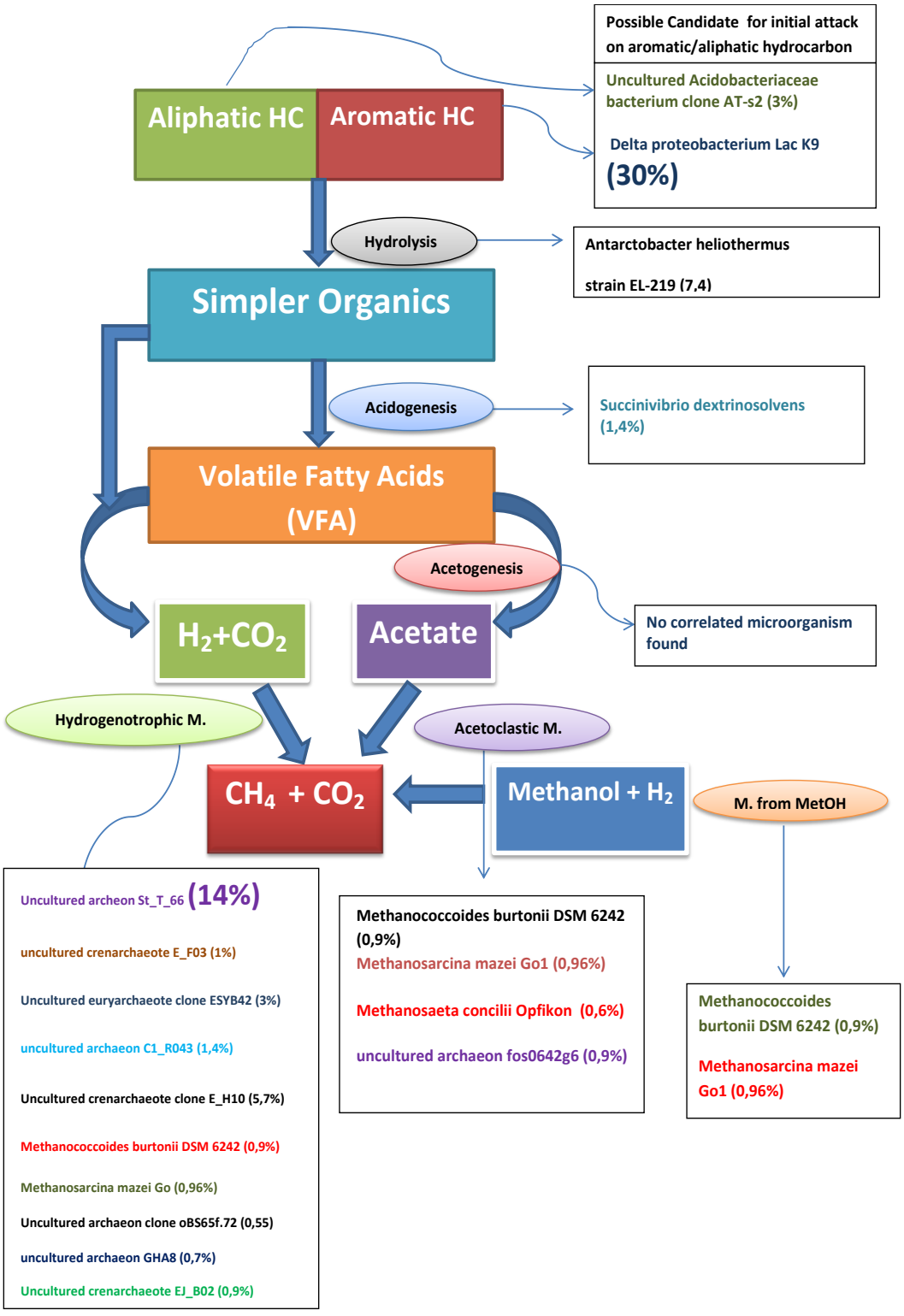


Figure 6.6: Possible anaerobic hydrocarbon degradation pathway on the basis of anaerobic digestion for WH-UL experimental group within UL experimental groups seeded with Tuzla sediments.

7. CONCLUSION

Sediment samples collected from nutrient amended hydrocarbon degradation microcosms that seeded with Tuzla and Halic Bay sediments from Kolukırık's study. This study showed that hydrocarbon degradation activity of Marmara Sea sediments can be increased by N-P amendment under methanogenic and nitrate reducing conditions. Biostimulation of the sediments microbial community resulted with ~20× and ~9× increase in hydrocarbon removal in the microcosms seeded with Tuzla and Haliç sediments respectively. The sediment microorganisms degraded wide range of aliphatic (n-C9-31 alkanes and acyclic isoprenoids) and aromatic (18 different 1-5 ring aromatics) hydrocarbons Apendix C shows the composition and changes in the composition.

In this study, investigation of microbial population dynamics in nutrient amended microcosms seeded with Tuzla and Halic Bay sediments under nitrate reducing and methanogenic conditions, was carried. The population dynamics was studied via DGGE of microcosm samples and species that are correlated with hydrocarbon removal, were identified via previous clone libraries from Marmara Sea sediments.

In Tuzla microcosms 14 bacterial and 14 archaeal species significantly changed ($0,15<p<0,05$) over time. This finding indicated that determined species can play a role in hydrocarbon degradation due to correlation analysis with hydrocarbon removal. Among the bacterial species, 3 of the clones belong to Marmara Sea sediments.

In Halic microcosms, significant changes ($0,15<p<0,05$) in 26 bacterial and 17 archaeal species were observed. Distribution of significantly changed bacterial species was so diverse but vast majority of the clones belonged to the phylum *Protobacteriacea.* Distribution of archaeal species belonged to uncultured archaeal species. Because of that reason successful comparison with literature about hydrocarbon degrading capacity can't be made. However correlation analysis with hydrocarbon removal indicates the possibility of hydrocarbon degradation ability of these taxanomic units.

In summary, in this study, possible candidates for petroleum hydrocarbon degradation were identified via sediment samples which taken from nutrient amended microcosms.

8. RECOMMENDATIONS

DGGE is a successful tool for investigation microbial community dynamics. However, It is foreseen that DGGE method can detect single base differences between taxonomic units theoretically but methods have some biases when studying spatial microbial communities such as marine sediments. Separation capacity of the DGGE instruments vary according to companies and single band may not represent one species all the time. In summary, DGGE method is not sufficient enough to identify species for a spesific tasks without statistical analysis.

Stable Isotope Probing (SIP) is an innovative method to track the environmental fate of a labelled contaminant of concern to unambiguously demonstrate biodegradation. The label serves as a tracer which can be detected in the end products of biodegradation (new biomass and CO_2 or dissolved inorganic carbon). SIP can be useful tool to confirm the detected species in this study whether they are actually responsible from biodegradation or not.

We hope that outputs from this study could be useful for studies that involve enrichment of hydrocarbon degrading mixed cultures since metabolisms of identified microorganisms from this study has been widely studied. Also Halic and Tuzla Bay sediments can be used as seeds for batch reactors to obtain metabolically more active mixed cultures for anaerobic/anoxic hydrocarbon biodegradation.

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APENDICES

APENDIX A

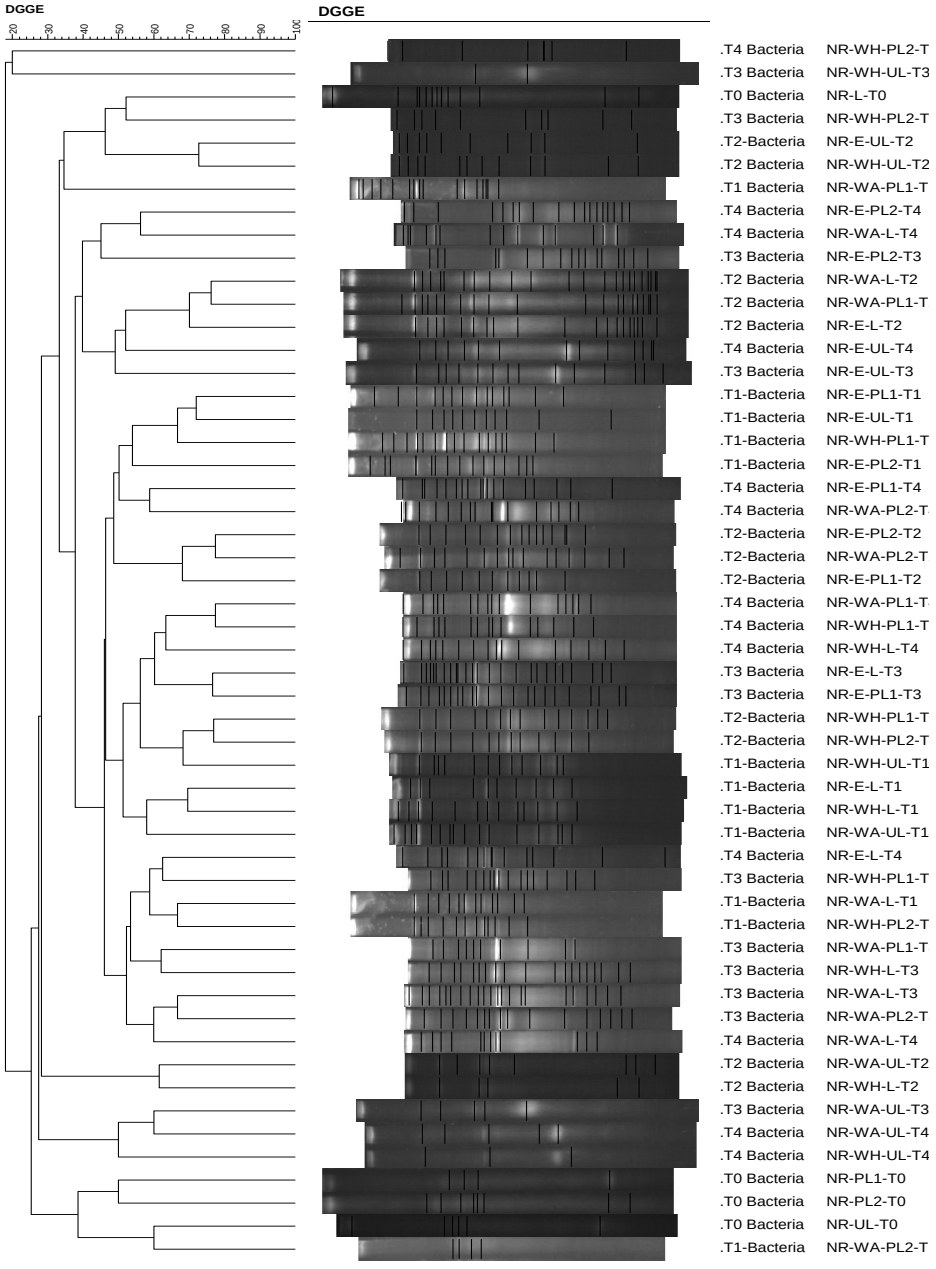


Figure A.1: Bacterial community dendogram plotted via Dice coefficient from nutrient amended microcosms seeded with Halic Bay sediments.

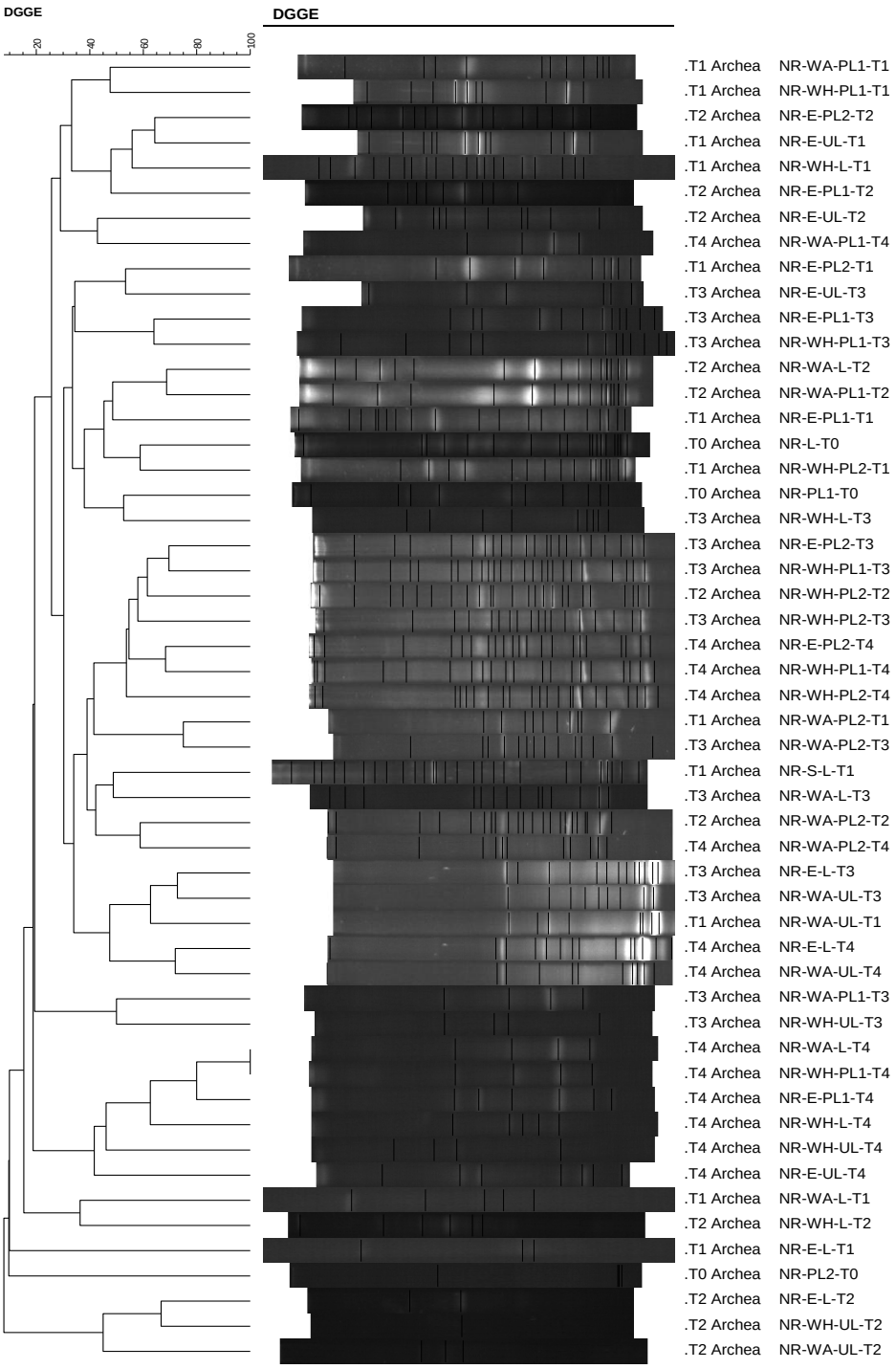


Figure A.2: Archeal community dendogram plotted via Dice coefficient from nutrient amended microcosms seeded with Tuzla Bay sediments.

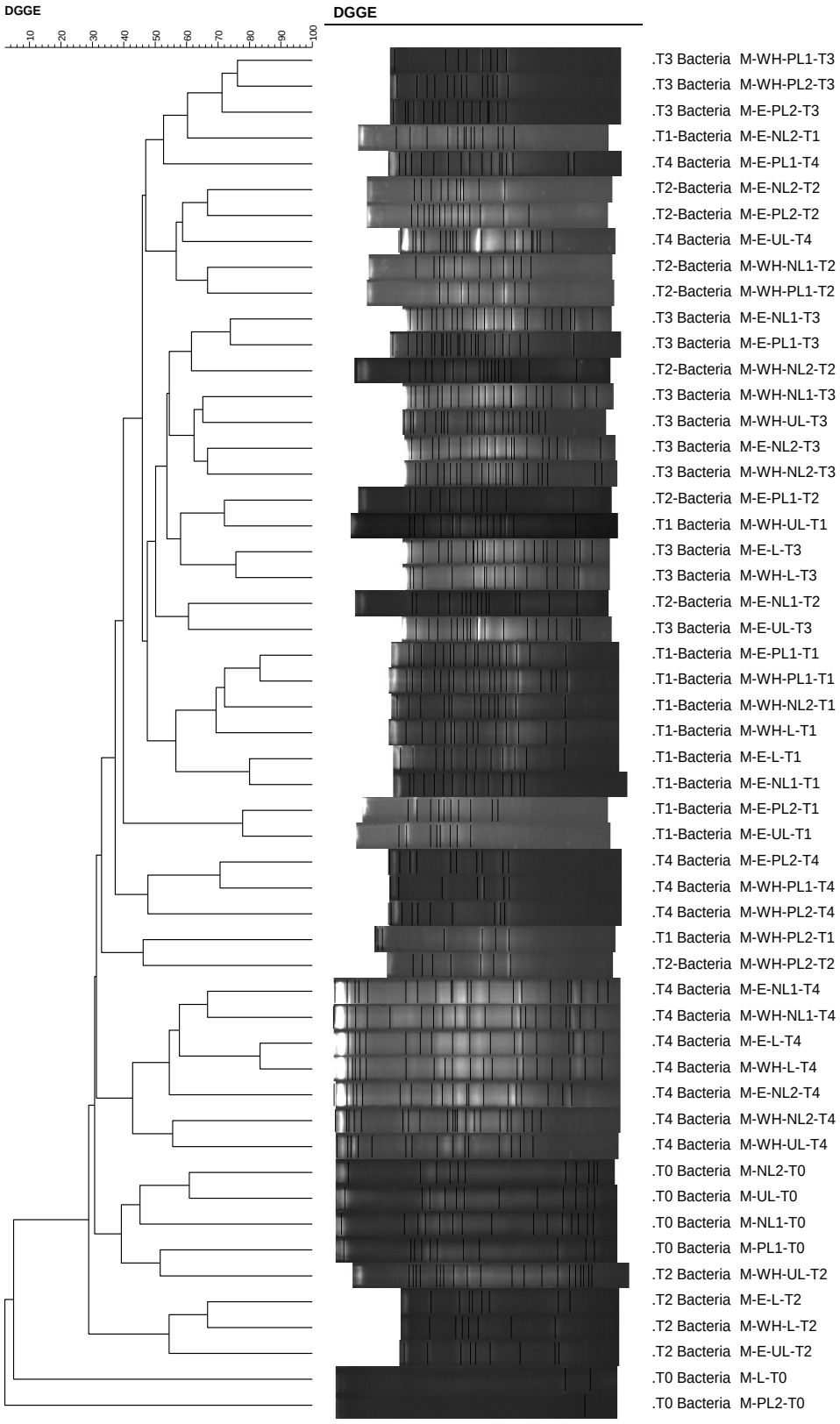


Figure A.3: Bacterial community dendogram plotted via Dice coefficient from nutrient amended microcosms seeded with Halic Bay Sediments.

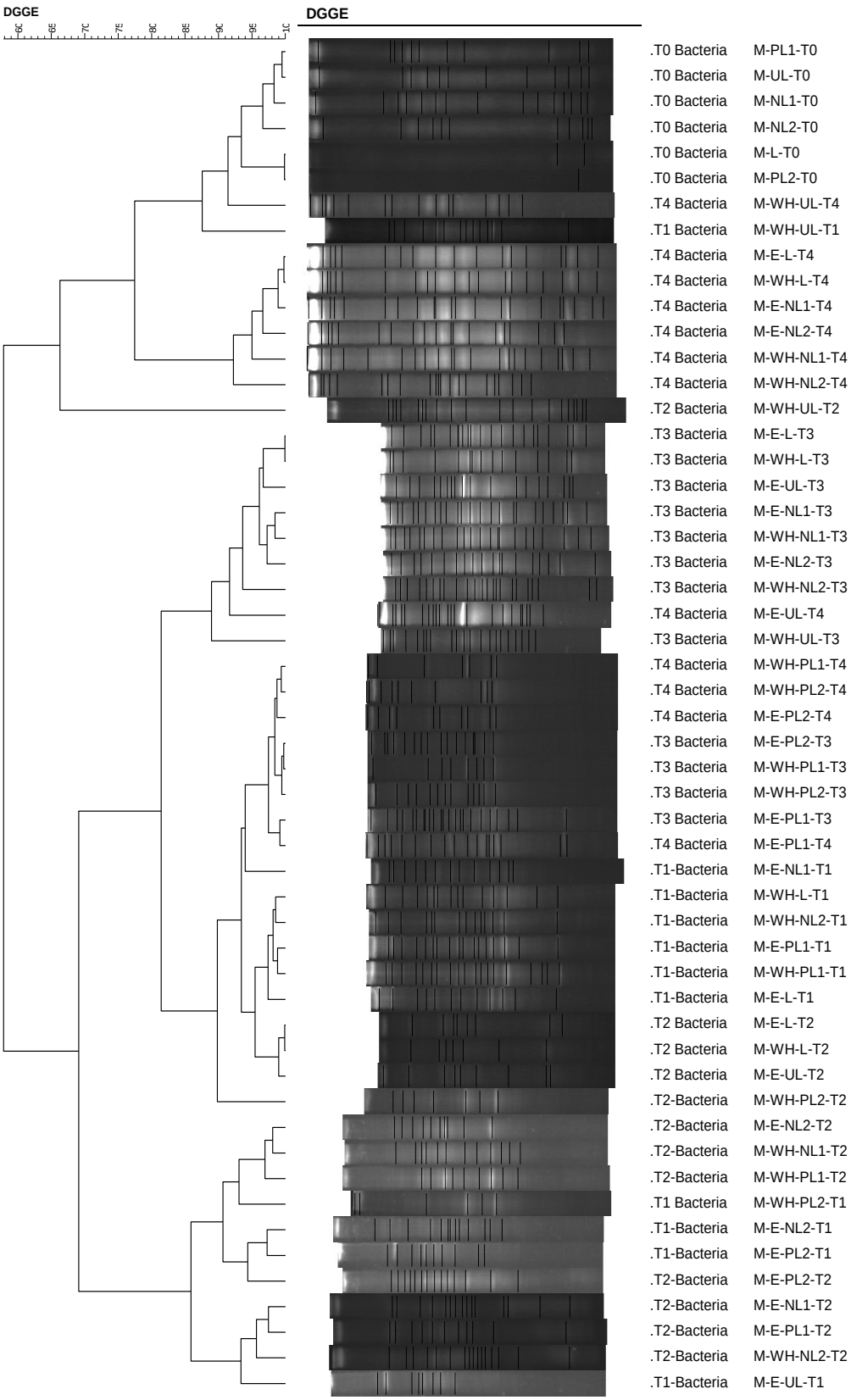


Figure A.4: Bacterial community dendogram plotted via Pearson coefficient from nutrient amended microcosms seeded with Halic Bay sediments

APENDIX B

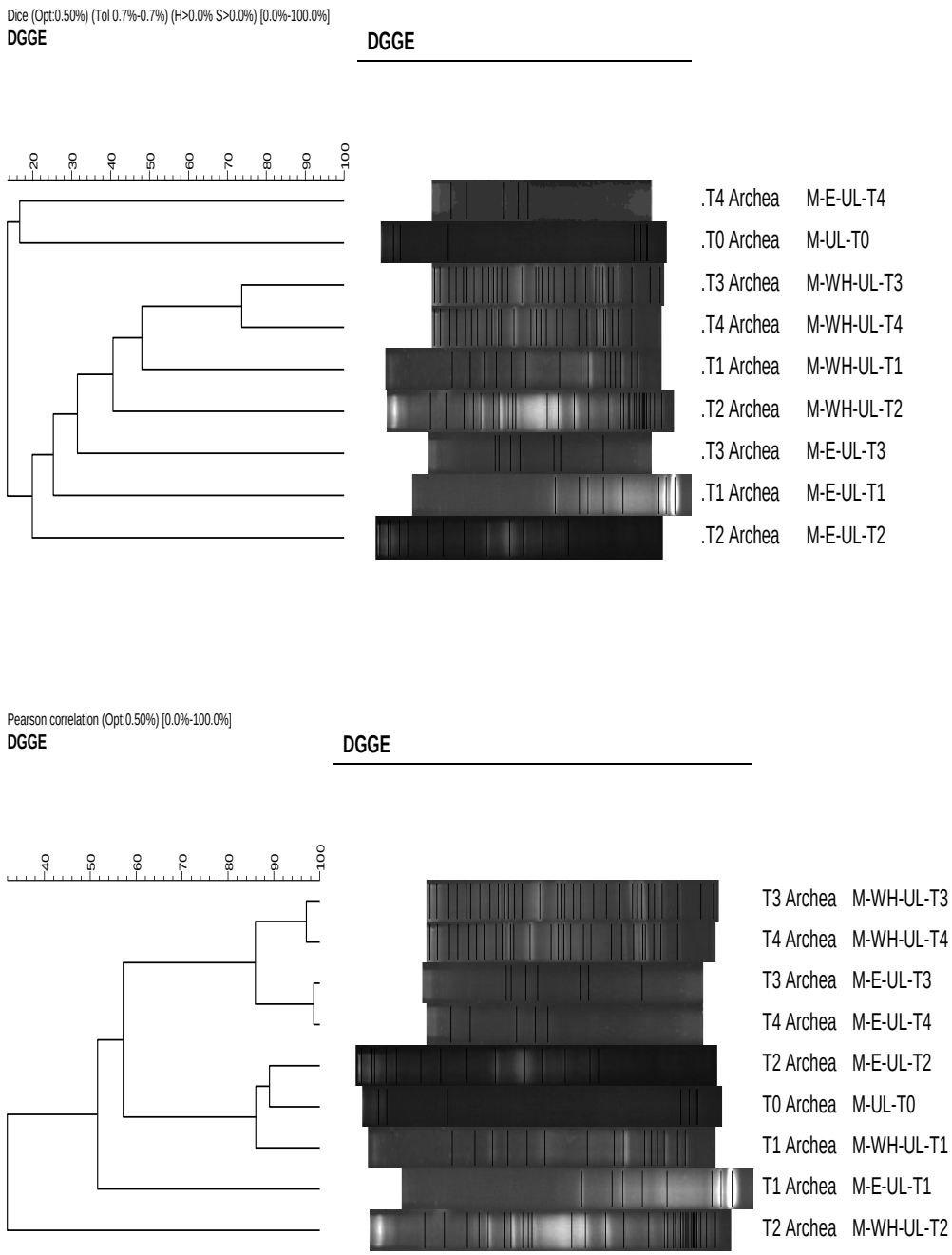


Figure B.1: Archaeal dendograms of UL microcosms seeded with Halic Bay sediments.

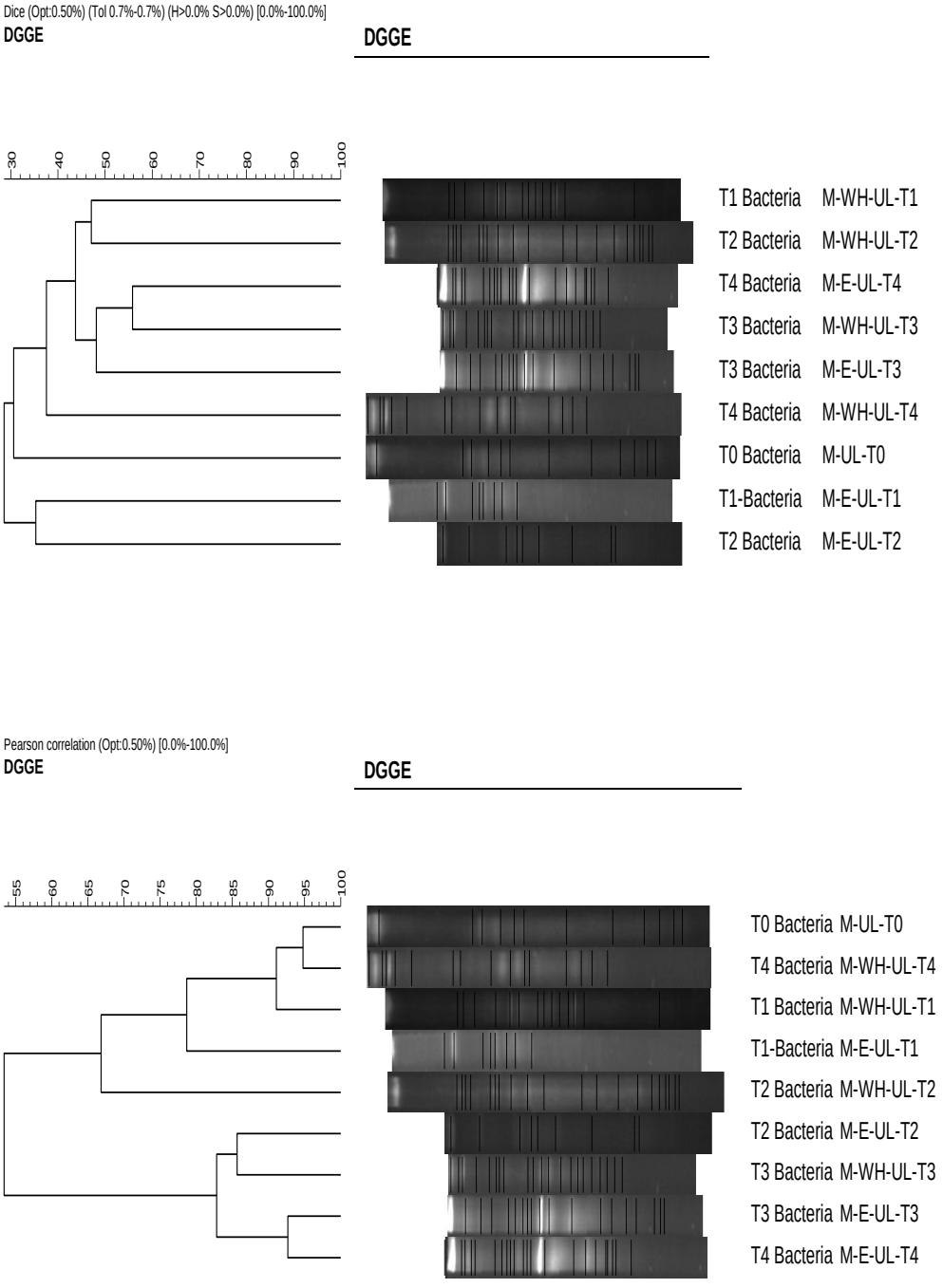


Figure B.2: Bacterial dendograms of UL microcosms seeded with Halic Bay sediments.

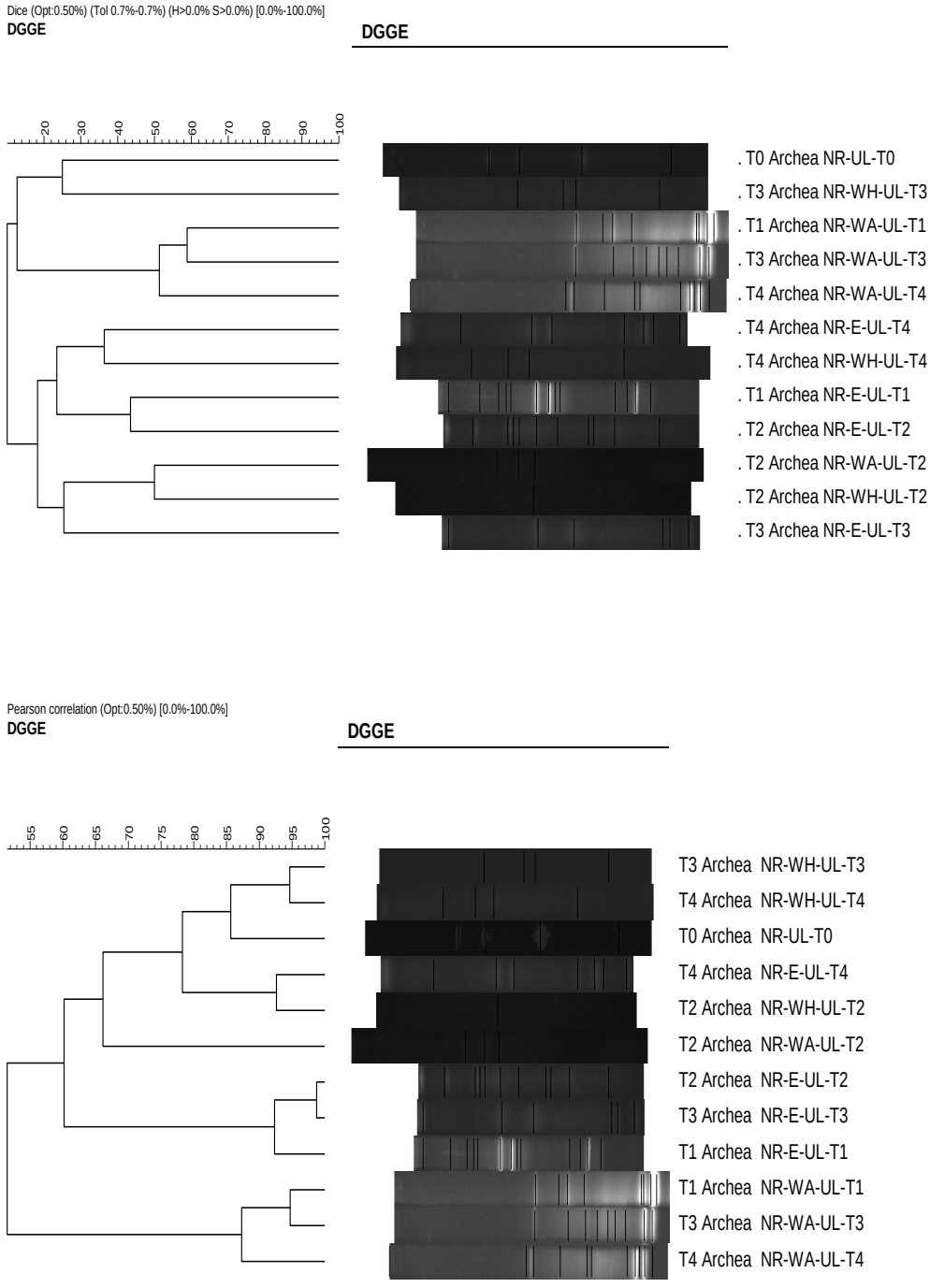
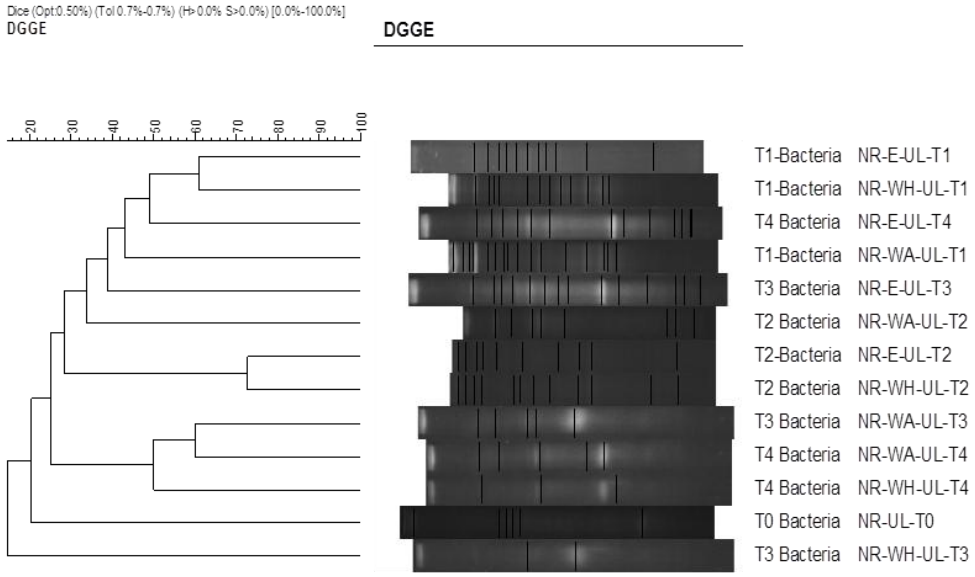


Figure B.3: Archaeal dendograms of UL microcosms seeded with Tuzla Bay sediments.



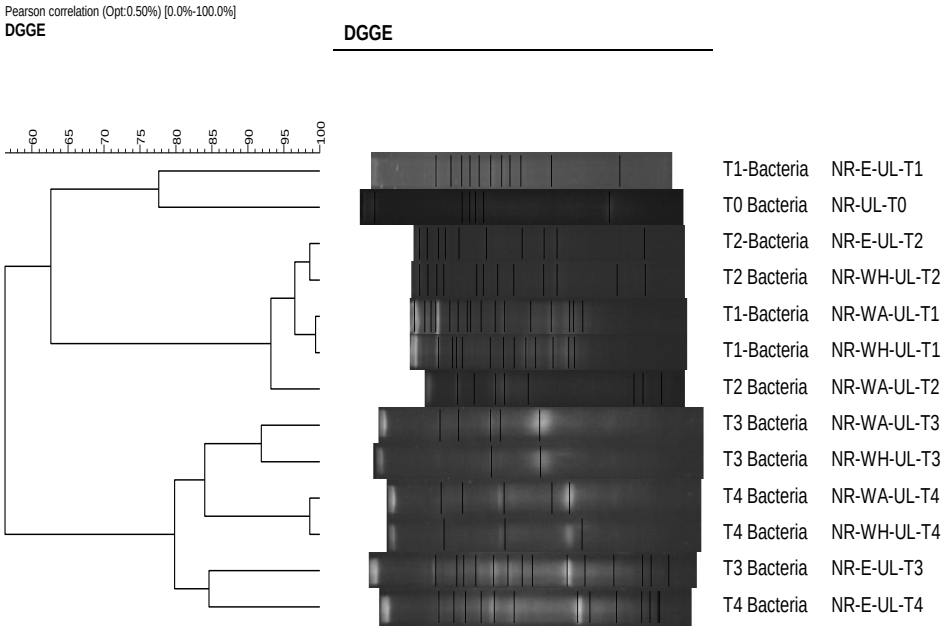


Figure B.4: Bacterial dendograms of UL microcosms seeded with Tuzla Bay sediments.

APENDIX C: Composition of the hydrocarbon mixture that was used in the set-up of microcosms and their removal in the microcosms which were seeded Tuzla Bay Sediments.

Table C.1: List of hydrocarbons that were used in the set-up of the microcosms (Kolukırık, 2010).

Aromatic Hydrocarbons	**Aliphatic Hydrocarbons**
Benzene	n-C14
Toluene	n-C15
Ethylbenzene	n-C16
m-p-o Xylene	n-C17
Naphtalene	Pristane
Acenaphtylene	n-C18
Acenaphtene	Phytane
Flourene	n-C19
Phenanthrene	n-C20
Antracene	n-C21
Flouranthene	n-C22
Pyrene	n-C23
Benz(a)Antracene	n-C24
Chrysene	n-C25
Benzo(b)Flouranthene	n-C26
Benzo(k)Flouranthene	n-C27
Benzo(a)Pyrene	n-C28
DiBenz(a,h)Antra	n-C29
Benzo(g,h,i)Perylene	n-C30
Indeno(1,2,3,c,d)Pyrene	n-C31

Table C.2: Changes in the aliphatic HC components of the microcosms seeded with Tuzla Bay sediments (Kolukırık, 2010).

Sample	**NR-E-UL**					**NR-WH-UL**			
Days	0	80	108	136	164	0	80	108	136
n-C9	13	4	0	0	0	2.6	0	0	0
n-C10	13	5	0	0	0	3.6	0	0	0
n-C11	11	4	0	0	0	0.5	0	0	0
n-C12	11	6	0	0	0	0.6	0	0	0
n-C13	13	11	0	0	0	0.4	0	0	0
n-C14	15	14	8	0	0	5.6	0	0	0
n-C15	16	15	15	0	0	4.7	0	0	0
n-C16	12	13	12	0	0	2.4	0	0	0
n-C17	17	17	16	0	0	5	1	0	0
Pristane	15	16	16	6	4	5.7	5.6	0	0
n-C18	13	12	13	0	0	2.7	2.5	0	0
Phytane	5.4	5.4	5.6	5.4	5.3	5.5	5.7	0	0
n-C19	7.8	8.2	4.2	4	0	4.2	4	0	0
n-C20	1.8	2	2	2	1.8	1.2	1.3	1.4	0
n-C21	3.1	3.2	3.4	3.1	3	1.2	0	0	0
n-C22	5.2	5.1	5.3	5.2	5.3	1.1	1	0	0
n-C23	3.1	3	3.2	3.1	3	1.2	1	0	0
n-C24	5.3	5	5.5	5.3	5.4	1.4	1.3	0	0
n-C25	2.3	2.4	2.3	2.4	2.3	1.5	1.4	0	0
n-C26	3.1	3.3	3.1	3	3.1	2.5	0	0	0
n-C27	4.2	4	4.2	4	4.2	3.5	3.6	0	0
n-C28	2.3	2	2.3	2	2.3	1.2	1	0	0
n-C29	3.6	3.8	3.6	3.9	3.6	2.9	3	2.9	0
n-C30	1.7	1.8	1.7	1.8	1.7	0.8	0.8	0	0
n-C31	5.1	5	5.1	4.9	4.9	4.5	4.6	4.4	0

Table C.3: Changes in the aromatic HC components of the microcosms seeded with Tuzla Bay sediments (Kolukırık, 2010).

Sample	**NR-E-UL(mg/100mL)**					**NR-WH-UL (mg/100mL)**		
Days	**0**	**80**	**108**	**136**	**164**	**0**	**80**	**108**
Benzene	4.6	0	0	0	0	0.6	0	0
Toluene	18	9	0.2	0	0	0.3	0	0
m-Xylene	4.2	4.2	4.3	0	0	0.1	0	0
p-Xylene	4.1	4.2	4.4	0	0	0.1	0	0
o-Xylene	4.1	4.2	4.1	0	0	0.1	0	0
Naphtalene	7.6	7	7.3	0	0	3.9	0	0
Acenaphtylene	4.1	4.5	4	0	0	0.2	0	0
Acenaphtene	5	5	5.5	1	0	1	0	0
Flourene	4.2	4.6	4.4	4	1	0.1	0	0
Phenanthrene	4.2	4.3	4.3	4.1	4.2	0.2	0.2	0
Antracene	8	8.1	7.4	7.8	8	7.9	7.6	0
Flouranthene	1.1	1.3	1.1	1.2	1.1	0.2	0.2	0
Pyrene	3	2.8	3.1	3.1	3	2	2.2	0
Benz(a)Antracene	1.1	1.1	1.2	1.2	1.4	0.1	0.1	0
Chrysene	0.5	0.6	0.7	0.8	0.9	0.1	0.1	1
Benzo(k)Flouranthene	0.4	0.4	0.4	0.4	0.4	0.1	0.1	0
Benzo(a)Pyrene	1.2	1	1.1	1	1	0.1	0.1	0
DiBenz(a,h)Antracene	0.5	0.5	0.4	0.5	0.4	0.1	0.1	0
Benzo(g,h,i)Perylene	0.5	0.4	0.4	0.4	0.4	0.1	0.1	0

APENDIX D: Species that are correlated with hydrocarbon degradation ($p<0.05$, $r>0.95$ $n=3$). Species was found via clone libraries previously constructed via cloning and sequencing analysis by Kolukırık (2010). All informations about microbial species obtained from www.ebi.ac.uk.

Table D.1: Species that are correlated with hydrocarbon degradation and their uncultured closest relatives in UL microcosms seeded with Tuzla Bay sediments.

Clone Code	Species	Accession Number	Uncultured Closeset Relative	Accession Number	Similarity %
Gemlik Bac 22	Unknown	-	*Acidobacteriacea* bacterium clone AT-s	**AY225640**	96
IZ 30 Bac 57	Unknown	-	-	-	-
MKC-Bac-E23	Unknown	-	*Deltaproteobacterium* clone Belgica 2005/10-130-7	**EF442982**	96
Gemlik Bac 2	Uncultured Microorganism Marmara 24	**AM980576**	-	-	-
H7 Bac 15	Unknown	-	*Chloroflexi* bacterium RPS-C9	**AB288599**	95
Gemlik Bac 12	Uncultured bacterium clone Marmara 26	**AM980578**	Bacterium clone CW106	**DQ499326**	88
IZ 30 Arc 2	Unknown	-	-	-	-
MK Arc 9	Unculturedeuyarchaeote clone MOD A21	**AM998435**	Euyarchaeote clone ESYB34	**AB119590**	90
IZ 17 Arc 1	Uncultured archaeone clone IZ 17 A10	**AM992704**	Archaeon clone Napoli 2A 20	**AY592484**	91
Gemlik Arc 19	Clone Marmara54	**AM980606**	-	-	-

Table D.2: Species that are correlated with hydrocarbon degradation and their cultured closest relatives in UL microcosms seeded with Tuzla Bay sediments.

Clone Code	Cultured Closeset Relative	Accession Number	Similarity %	Substrate	Metabolism	Abundance Percentage %
Gemlik Bac 22	*Desulfomonile tiedji*	**AM08664**	82	**H_2 or reduced elemental sulfur**	Sulfur Reduction	**0.5**
IZ 30 Bac 57	*Bacillus sp.* DTY1	**DQ363561**	97	**?**	Nitrate Reduction	**0.1**
MKC-Bac-E23	*Desulfobacterium catecholicum*	**EF442982**	92	**Catechol**	Sulphate Reduction	**7.4**
Gemlik Bac 2	*Thauera chlorobenzoicea* strain 4FB2	**AF229868**	98	**Aromatics: Halobenzoate**	Denitrification	**6.2**
H7 Bac 15	*Bellinea caldifistulae* GOMI-1	**AB243672**	86	**CO_2**	Chemo litoautotroph	**16**
Gemlik Bac 12	*Moorella thermoacetica* ATCC39073	**CP00032**	81	**CO, H_2**	Acetogenic	**4.1**
IZ 30 Arc 2	*Methanobrevibacter* sp. 1Y	**DQ135938**	98	**Formate, H_2**	Methanogenesis	**4.2**
MK Arc 9	*Methanothermococcus* sp. Ep70	**AB260046**	75	**Acetate, H_2, methylamines**	Methanogenesis	**4.8**
IZ 17 Arc 1	*Crenarchaeote* SRI-298	**AF255608**	81	**?**	Methanogenesis	**34**
Gemlik Arc 19	*Methanococcoides* sp. NaT1	**DQ522915**	92	**Methanol**	Methanogenesis	**4.9**

Table D.3: Species that are correlated with hydrocarbon degradation and their uncultured closest relatives in UL microcosms seeded with Halic Bay sediments.

Clone Code	Species	Accession Number	Uncultured Closeset Relative	Accession Number	Similarity %
My Bac 38	Candidate division GN10 bacterium Clone TUZ-B1	**AM998335**	Candidate division GN10 bacterium	**DQ329592**	91
H7 Bac 29	Bacterium clone HalAS-B9	**AM998370**	Bacterium C762	**AY985733**	98
H7 Bac 27	Unknown	-	-	-	-
H3 Bac 3	Unknown	-	-	-	-
MKC Bac E-10	Unknown	-	*Antarctobacter heliothermus strain EL-219*	**Y11552**	96
H7 Bac 30	Longilinea sp. clone	**AM998367**	-	-	-
MKC A6 Bac	Unknown	-	-	-	-
Gemlik Bac 2	Isolate 24	-	-	-	-
H4 Arc 1	Archaeon HALEY-A4	**AM998387**	Archeon V.8.Ard.8	**AY367345**	90
H3 Arc 6	Archaeon HALVK-A6	**AM998387**	Archeon OHKA63	**AB094550**	96
H3 Arc 20	Crenarchaeote E-F03	**AY454636**	Crenarchaeote E-F03	**AY454636**	99
H3 Arc 5	Archaeon CP-A21	**DQ521205**	-	-	-
MY Arc 31	*Methanosaeta concilii* Opfikon	**X51423**	*Methanosaeta concilii* Opfikon	**X51423**	99

Table D.4: Species that are correlated with hydrocarbon degradation and their uncultured closest relatives in UL microcosms seeded with Halic Bay sediments.

Clone Code	Cultured Closeset Relative	Accession Number	Similarity %	Substrate	Metabolism	Abundance Percentage %
My Bac 38	*Dehalococcoides sp. CBDB1*	**AF230461**	77	**Aromatic Hydrocarbons**	Fermentation	**0.66**
H7 Bac 29	*Clostridium sp. FG4*	**AB207248**	85	**Several organics**	Fermentation	**28**
H7 Bac 27	*Betaproteobacterium F06002*	**AF236014**	96	**?**	?	**9**
H3 Bac 3	*Delta-proteobacterium LacK9*	**AY771933**	97	**Catechol**	Fermentation	**1**
MKC Bac E-10	-	**-**	-	**Malate, citrate, succinate**	Hydrolysis	**0.59**
H7 Bac 30	*Longilinea arvoryzae KOME-1*	**AB243673**	90	**Xylose**	Acidogenesis	**0.68**
MKC A6 Bac	*Pseudoxanthomonas spadix*	**AM418384**	96	**Fatty Acids**	Fermentation	**1**
H7 Bac 5	*Sinorhizobium meliloti*	**DQ145546**	92	**Phenanthere ne**	Acidogenesis	**17**
Gemlik Bac 2	*Thauera chlorobenzoica strain 4FB2*	**AF229868**	90	**Aromatic Compounds**	Denitrification	**1.8**
H4 Arc 1	*Methanobacterium bryantii*	**MF59124**	78	**H_2+CO_2**	Methanogenesis	**29**
H3 Arc 6	*Methanosphera stadmanae*	**CP00102**	67	**Methanol**	Methanogenesis	**2.4**
H3 Arc 20	*Methanococcus maripaludis C5*	**CP00609**	77	**H_2+CO_2**	Methanogenesis	**56**
H3 Arc 5	*Methanobacterium palustre*	**-**	-	**Formate**	Methanogenesis	**2.5**
MY Arc 31	*Methanosaeta concilii* Opfikon	**X51423**	99	**Acetate**	Methanogenesis	**2.5**

Table D.5: Species that are correlated with hydrocarbon degradation and their uncultured closest relatives in WH microcosms seeded with Halic Bay sediments.

Clone Code	Species	Accession Number	Uncultured Closeset Relative	Accession Number	Similarity %
Gemlik Bac 22	Unknown	-	*Acidobacteriaceae clone AT-2*	**AY225640**	96
H3 Bac 3	Unknown	-	-	-	-
H4Bac 2	Unknown	-	-	-	-
MKC Bac-E 10	Unknown	-	-	-	-
MKC Bac A	Unknown	-	-	-	-
H7 Bac 13	Unknown	-	*Chloroflexi* clone KB40	**AB074960**	96
H4 Bac 7	*Gamma-proteobacterium* clone HALEY5	**AM998362**	-	-	-
Gemlik Arc 20	Archaeon clone Marmara 30	**AM980582**	Archaeon clone ObS65f72	**DQ146745**	84
H4 Arc 19	Archaeon clone Marmara 38	**AM980590**	Archaeon SURF-GC 205-Arc11	**DQ521768**	90
H3 Arc 18	Archaeon clone Marmara 52	**AM980604**	Archaeon GHA8	**DQ521152**	89
H4 Arc 38	*Crenarchaeote* clone HALEY A1	**AM992683**	*Crenarchaeote E-A11*	**AY454678**	86
MK Arc 40	Unknown	-	*Crenarchaeote clone EJ-B02*	**AY454663**	96
H4 Arc 5	Archaeon clone HALEY A6	**AM998398**	Archaeon St-T-66	**AY531726**	81
H3 Arc 20	Unknown	-	*Crenarchaeote E-F03*	**AY454636**	96
MK Arc 5	*Euryarchaeote* clone MODA14	**AM998406**	*Euryarchaeote* clone ESYB42	**AB119598**	94
H3 Arc 25	Archaeon clone HALVK_ A12	**AM998409**	Archaeon C1R043	**AF419642**	91
MK Arc 13	*Crenarchaeote* clone MODA17	**AM998425**	*Crenarchaeote* clone *E-H10*	**AY454641**	82
MY Arc 34	Methanococcoides clone TUZ A5	**AM998429**	-	-	-
H7 Arc 7	*Methanosarcina sp.* clone HALASA6	**AM998439**	-	-	-
MY Arc 19	Unknown	-	-	-	-
H3 Arc 10	Unknown	-	Archaeon fos0642g6	**CR937012**	95

Table D.6: Species that are correlated with hydrocarbon degradation and their cultured closest relatives in WH microcosms seeded with Halic Bay sediments.

Clone Code	Cultured Closeset Relative	Accession Number	Similarity %	Substrate	Metabolism	percentage %
Gemlik Bac 22	*Desulfomonile tiedji*	**AM086646**	82	**3-chlorobenzoate**	Sulphate Reduction	**2.9**
H3 Bac 3	*Delta-proteobacterium LacK9*	**AY771933**	97	**Catechol**	?	**30**
H4Bac 2	*Gamma-proteobacterium* RBE2cd-118	**EF111257**	98	**Unclassified**	Unclassified	**1.3**
MKC Bac-E 10	*Antarctobacter heliothermus EL-219*	**Y11552**	96	**Several Organics**	Hydrolysis	**7.4**
MKC Bac A	*Succinivibrio dextrinosolvens*	**Y17600**	99	**Carbohydrates**	Fermentation	**1.5**
H7 Bac 13	*Arenimonas sp YC06267*	**EU376961**	93	**CO_2**	Anoxic Photosynthesis	**1.5**
H4 Bac 7	Sulfur oxidizing bacterium ODIII6	**AF170422**	94	**Elemental Sulfur**	Sulfur Oxidation	**1.6**
Gemlik Arc 20	*Methanobrevibacter gottschlkii*	**U55239**	75	**H_2+CO_2**	Methanogenesis	**0.8**
H4 Arc 19	*Thermofilum pendens Hrk 5*	**CP000505**	73	**Acetate**	Methanogenesis	**0.9**
H3 Arc 18	*Methanobacterium palustre*	**AF093061**	72	**Formate**	Methanogenesis	**7.3**
H4 Arc 38	*Thermogymnomonas acidocola*	**AB269873**	75	**Unknown**	Unknown	**4.5**
MK Arc 40	*Methano thermococcus sp. Ep70*	**AB260046**	76	**H_2+CO_2**	Methanogenesis	**0.8**
H4 Arc 5	*Methanobrevibacter sp. LRsD4*	**AB033290**	70	**H_2+CO_2**	Methanogenesis	**14**
H3 Arc 20	*Methanococcus maripaludis C5*	**CP000609**	77	**H_2+CO_2**	Methanogenesis	**1.1**
MK Arc 5	*Methanococcus vannielii*	**CP000742**	78	**H_2+CO_2**	Methanogenesis	**3**
H3 Arc 25	*Methanobacterium sp. M2*	**DQ517520**	74	**H_2+CO_2**	Methanogenesis	**1.4**
MK Arc 13	*Methanococcus aeolicus Nankai-3*	**CP000300**	73	**H_2+CO_2**	Methanogenesis	**5.7**
MY Arc 34	*Methanococcoides burtonii DSM6242*	**AE008384**	69	**Acetate,Methanol,Fumarate**	Methanogenesis	**0.9**
H7 Arc 7	*Methanosarcina mazei Go1*	**X51423**	94	**Acetate,Methanol,Fumarate**	Methanogenesis	**0.96**
MY Arc 19	*Methanosaeta concilii*	**AJ133791**	95	**Acetate**	Methanogenesis	**0.6**

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