ANAEROBIC BIODEGRADATION OF PETROLEUM BASED WASTE

by

Aslınur Çalışıyor

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ABSTRACT

ANAEROBIC BIODEGRADATION OF PETROLEUM BASED WASTE

Petroleum is the most common raw energy source which causes soil and groundwater pollution during the exploration, refining, transport and storage. Since the physical and chemical treatment methods have some disadvantages as high cost and secondary waste production, biological treatment methods have become important in environmental studies. Besides aerobic degradation, anaerobic biodegradation is an alternative method that has specific properties such as less biomass production and production of biogas under different electron accepting conditions. In this study, the biodegradability of petroleum-contaminated soil was observed under methanogenic and sulphate-reducing conditions at different temperatures in microcosms were inoculated with an aquifer from Leuna, Germany. Biodegradation efficiency, microbial community profile and biogas production rates of microcosms were monitored under different conditions and run for approximately 200 days. Microbial community profiles were observed by using quantitative real time polymerase chain reaction (qRT-PCR), high resolution melting (HRM) and Next Generation Sequencing (NGS) based metagenomics analyses. The performance of biodegradation was determined via Total Organic Carbon (TOC) analysis which can be pre-study for total petroleum hydrocarbon (TPH) analysis. According to the results, degradation of organic matter was more efficient under sulphate-reducing conditions than methanogenic conditions. The TOC removal efficiency was as high as 70% under sulphate-reducing conditions while the TOC removal was ignorable under methanogenic conditions. Especially, microbial community profile under 16S rRNA gene expression is highly related with chemical analysis components as TOC removal, gas production and electron acceptor utilization. Molecular microbial analyses showed that aliphatic and aromatic hydrocarbon degradation occurred in the microcosms and the higher TOC removal efficiencies were related to the change in the microbial community profiles under sulphate-reducing conditions.

ÖZET

PETROL BAZLI ATIKLARIN ANAEROBİK BİYODEGRADASYONU

Ham enerji kaynağı olarak yaygın halde kullanılan petrol sondaj, rafinasyon, taşınma ve depolama sırasında toprak ve yeraltı suyu kirliliğine neden olmaktadır. Biyolojik arıtma yöntemleri, fiziksel ve kimyasal arıtma tekniklerinin yüksek maliyetli ve ikincil atık üretimi gibi dezavantajları nedeniyle çevresel çalışmalarda önemli hale gelmiştir. Aerobik degradasyonun yanında, anaerobik biyodegradasyon farklı elektron alıcı koşullarında daha az biyokütle üretimi ve biyogaz oluşumu gibi özgün özellikleri ile alternatif metot olarak kullanılmaktadır. Bu çalışmada, metanojenik ve sülfat indirgeyici koşullarda ve farklı sıcaklıklarda Almanya'nın Leuna kentinden alınmış akiferin aşı olarak kullanıldığı mikrokozmoz testi ile petrolle kirlenmiş toprağın biyodegradasyonu gözlemlenmiştir. Mikrokozmozlarda biyodegradasyon verimliliği, mikrobiyal komünite profili ve biyogaz üretim oranı farklı koşullarda izlenmiştir ve inkübasyon yaklaşık 200 gün sürmüştür. Mikrobiyal topluluk dağılımı, kantitatif gerçek zamanlı polimeraz zincir reaksiyonu (qRT-PCR), yüksek çözünürlüklü erime (HRM) analizi ve metagenomik analizi olarak Yeni Nesil Dizileme (NGS) uygulanarak gözlemlenmiştir. Biyodegradasyon performansı toplam petrol hidrokarbon (TPH) analizine ön çalışma olarak toplam organic karbon (TOK) analizi ile belirlenmiştir. Çalışma sonuçlarına göre, organik madde degradasyonu metanojenik koşullara göre sülfat indirgeyici koşullarda daha verimli olmuştur. Metanojenik koşullardaki toplam organic karbon (TOK) giderimi ihmal edilecek kadar az iken, sülfat indirgeyici koşullarda TOK gideriminde %70 kadar verimlilik sağlanmıştır. Özellikle, 16S rRNA gen ekspresyonu altında mikrobiyal topluluk dağılımı, TOK giderimi, gaz oluşumu ve elektron alıcısı kullanımı gibi kimyasal analiz bileşenleri ile oldukça ilişkilidir. Moleküler mikrobiyal analizler, mikrokozmozlarda alifatik ve aromatik hidrokarbon degradasyonunun olduğunu ve daha yüksek TOK giderim verimliliğinin sülfat indirgeyici koşullar altında mikrobiyal topluluk dağılımındaki değişime bağlı olduğunu göstermiştir.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Unit
Al	Aluminum	
B	Boron	
bp	Base Pair	
$CaCl_2$	Calcium Chloride	
CH_4	Methane	µmol
CO_2	Carbon Dioxide	µmol
Cu	Copper	
Fe	Iron	
$FeSO_4$	Iron (II) Sulphate	
g	Gram	
H_2	Hydrogen	
HCO_3^-	Bicarbonate	
H_2O	Dihydrogen Oxide	
H_2S	Hydrogen Sulfide	
I_2	Iodine	
KCl	Potassium Chloride	
kg	Kilogram	
KI	Potassium Iodide	
L	Liter	
M	Molar	
mbar	Millibar	
mg	Milligram	
min	Minute	
mL	Milliliter	
mmol	Millimole	
Mg	Magnesium	
Mn	Manganase	
µg	Microgram	
µL	Microliter	
µM	Micromolar	
µmol	Micromole	

N_2	Nitrogen
$Na_2S_2O_8$	Sodium Persulfate
Ni	Nickel
nm	Nanometer
nM	Nanomolar
OH^-	Hydroxide
P	Phosphorus
pmol	Picomole
ppm	Parts Per Million
rpm	Revolutions Per Minute
S^0	Elemental Sulfur
SO_4^{-2}	Sulphate
Sec	Second
V	Vanadium
Zn	Zinc

mmol

Abbreviation	**Explanation**
A	Aquifer
assA	Alkylsuccinate Synthase α-subunit
bcrA	Benzoyl-CoA Reductase α-subunit
BTEX	Benzene, Toluene, Ethyl-benzene and Xylene
Bss	Benzylsuccinate Synthase
CTAB	Cetyltrimethylammonium Bromide
Cq	Quantitation Cycle
DNA	Deoxyribonucleic Acid
DOC	Dissolved Organic Carbon
dsrB	Dissimilatory Sulfite Reductase β-subunit
FAE	Fumarate Adding Enzymes
FID	Flama Ionization Detector
GEM	Genetically Engineered Microorganism
GC	Gas Chromatography
HMW	High Molecular Weight
HRM	High Resolution Melting
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry
LMW	Low Molecular Weight

M	Methanogenic Environment
mcrA	Methyl Coenzyme M Reductase α-subunit
MGW	Molecular Grade Water
MTBE	Methyl Tertbutyl Ether
MS	Mass Spectrometry
NA	Nucleic Acid
NGS	Next Generation Sequencing
PAH	Polycyclic Aromatic Hydrocarbon
PCA	Principal Component Analysis
PCC	Pearson Correlation Coefficient
PCR	Polymerase Chain Reaction
rDNA	Ribosomal Deoxyribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
RNA	Ribonucleic Acid
S	Sulphate Reducing Environment
SO	Contaminated Soil
TOC	Total Organic Carbon
TPH	Total Petroleum Hydrocarbon
TSO	Treated Contaminated Soil

1. INTRODUCTION

Petroleum or crude oil is a complex, naturally occurring liquid mixture also called as “black gold” containing mostly hydrocarbons, but containing also oxygen, nitrogen and sulfur. Petroleum is processed for utilization as petroleum products such as gasoline, diesel fuel or fuel oil (Radovic, 1997). Hydrocarbons, one of the major petroleum constituents, mainly include saturated alkanes and cycloalkanes, unsaturated alkenes, alkynes and aromatic hydrocarbons. The light crude oil comprises of mainly 78% saturates, 18% aromatics, 4% resins and <2% asphaltenes (Olah and Molnar, 2003). Processing pathways of petroleum to products is illustrated in Figure 1.1. Economic and population growth, development of industrialization have resulted in high demand on utilization of petrochemical products. Petroleum products are essential raw materials used as energy source in various types of industries. However, natural oil reserves for energy source have not been enough according to rise in human civilization and industrialization in time (Maletić et al., 2013). As a result of insufficient natural energy source, petroleum production has increased gradually. The predicted petroleum production of the World in a year reached twelve million metric tons. 1.7 to 8.8 million metric tons of estimated production amount are released into the environment annually (Koshlaf and Ball, 2017).

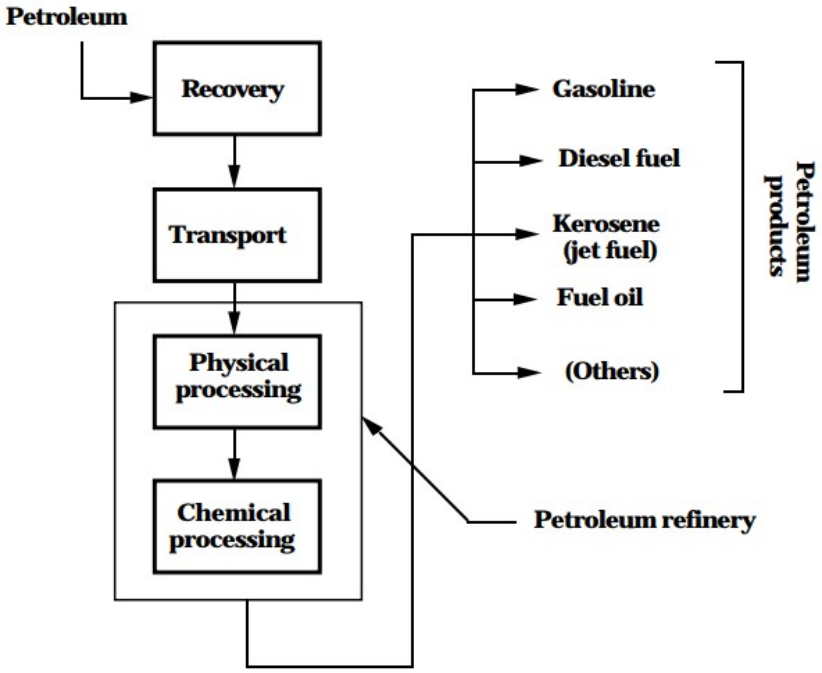

Figure 1.1. Pathways to petroleum utilization (Radovic, 1997).

Increment of petroleum production induces a major cause of environmental pollution. Petroleum hydrocarbon pollution has become one of the most remarkable global problems through its effects on environmental and human health due to its toxicity. The most noticeable activities in petroleum industries resulted in petroleum hydrocarbon pollution are associated with leaks and accidental spills during petroleum exploration, transport and processing. Besides oil waste leaks and accidental spills, the industrial companies are insufficient to manage waste oil recycling and disposal of hazardous oil wastes into landfills. Due to the inappropriate management of recycling and disposal, the contaminated sites and landfills have risen rapidly over the years (Brown et al., 2017)

1.1. Hazards of Petroleum Hydrocarbon Pollution

Based on the Toxic Release Inventory report from EPA in 2005, the oil refining and petroleum industry is one of ten main causes of releasing toxic and harmful chemicals into the environment. In recent years, the toxicity of petroleum hydrocarbons has resulted in serious problem to microorganisms and also in human life (Varjani and Upasani, 2017). From industrial zone, oil leakage or releases can also go through the subsurface resulting in natural oil and gas seeps. These leakages and releases through the subsurface of soil or sediment cause petroleum contaminated sites that pose many critical problems comprising of consequent risk to human health, ecology and especially water resources. Released petroleum hydrocarbons include soluble and volatile compounds such as benzene, toluene, ethyl-benzene and xylene (BTEX) and more stable compounds such as polycyclic aromatic hydrocarbons (PAHs) (Brown et al., 2017). Including petroleum hydrocarbon pollutants, 16 PAHs are called as priority pollutants because of their recalcitrance in the environment (Varjani, 2017). Presence of petroleum hydrocarbons in the environment such as soil surface, water resources and oceans leads to accumulation of hemotoxic, carcinogenic and teratogenic components (Meckenstock et al., 2016). Especially for human health, suffocation, anoxia, stunted growth, disturbance in metabolic reactions and hormone imbalance are reported as direct or indirect effects of toxicity of petroleum hydrocarbons. Some short-term impacts such as hypothermia, acute necrosis mortality, smothering and ingestion of toxic compounds and long-term impacts including developmental abnormalities in sea environment such as jaw reductions, lack of pigmentation and unfused skulls evolve out of hazards of petroleum hydrocarbon pollution. All these effects alter in a species population or community following with entire ecosystem (Varjani, 2014). Polycyclic aromatic hydrocarbons have potential to increase the malignant tumors that are prior to affecting skin and other epithelial tissue in order to their affinity for macromolecules like RNA, protein and DNA in the cell directly (Desforges et al., 2016)

1.2. Removal of Petroleum Hydrocarbon Contaminants

Due to its many hazards to the environment, the fate of petroleum hydrocarbons is one of the most considerable issues all over the world. In addition, it is vital to gain knowledge about oil remediation approaches for such contaminants. All treatment methods as physical, chemical and biological treatment have some benefits and limitations about cost, efficient, effectiveness or removal of specific level of contaminants. Especially, the physical or chemical remediation of petroleum products costs expensively, even it takes short time period. In the USA for example, the costs of polluted soil removal was predicted to be more than 1 trillion US dollars (Stroud et al., 2007). In addition, the basic costs for removal of contaminants from huge commercial costs as a minimum of 200,000 US dollars with an additional 40–70 US dollars for each cubic meter of contaminated soil (Arthur et al., 2005).

Petroleum and its products are put to physicochemical methods as soil washing, chemical inactivation and incineration. Some other physicochemical treatment methods are dispersion, dilution, sorption, volatilization and abiotic transformation. In last years, one of the innovative techniques is used for remediation of petroleum hydrocarbons called as bioremediation. Bioremediation is defined as treatment by living organisms for degradation of pollutants (Chandra et al., 2013). Through 20^{th} centuries, the use of microorganisms especially bacteria and fungi, or plants have been focused in remediation strategies of environmental pollutants such as petroleum. PAHs could be removed via volatilization, photo degradation, chemical oxidation, microbial degradation, etc. Previous studies have mainly focused on the aerobic degradation of low-molecular-weight (LMW) PAHs and the associated degradation pathways have been elucidated. The high-molecular-weight (HMW) PAHs were more recalcitrant and toxic to living organisms than those LMW ones. A close attention should be also paid considering the great public concerns caused by them. Meanwhile, most of the PAHs, contaminated sites, such as sediments and groundwater, were anaerobic and thus the anaerobes and anaerobic biodegradation of PAHs should play greater roles than the respective aerobic ones in natural environments. However, little attention has been paid to the anaerobic degradation of HMW PAHs, it was confined to specific pure strains if any. Besides, the anaerobes had the ability of destructing persistent organic pollutants and several studies have showed the potentially of organic pollutants via anaerobic treatment (Aydın et al., 2016). One the physicochemical treatment method is photo-oxidation affects in limited with only in sunlight-exposed oil. On the other hand, weathering processes such as spreading, evaporation, dispersion, sinking, and emulsification depend on environmental conditions such as temperature, weather factors and ocean currents (Souza et al., 2014). In addition, these conventional engineering

for physicochemical methods are not cost effective in order to the cost of excavation and transportation of huge quantities of contaminated sites. Instead of physicochemical applications, microbial degradation becomes an efficient, cost-effective, and applicable over large area and environmentally friendly treatment method especially in terrestrial environments (Chandra et al., 2013). However, like all techniques and strategies there are some disadvantages in biological treatment methods. These include the extended treatment time, low predictability and dependence on environmental factors.

The biodegradability of petroleum hydrocarbons depends on the concentration and bioavailability of the pollutants/contaminants. The bioavailability is affected by the fraction of pollutants, which can be substrate compounds for soil microbial community (Koshlaf and Ball, 2017). The differences between physical, chemical and biological treatment including advantages and disadvantages using one example of method are summarized in Table 1.1.

Table 1.1. Remediation techniques for petroleum hydrocarbons (Koshlaf and Ball, 2017).

Remediation Strategy	**Example of Method**	**Treating Site**	**Benefits**	**Limitations**
Physical	Vapour extraction	*Ex situ*	Fast Permanent removal High level of pollution	Costly Destructive Lead to secondary pollution
Chemical	Thermal desorption	*Ex situ*	Fast High level of pollution	Costly Destructive Lead to secondary pollution
Biological	Biostimulation	*In situ*	Environmentally friendly Cost effective Minimum site disruption Low level of pollutants	Long time processing Low predictability Based on environmental factors

1.3. Objective of the Thesis

The main aim of this study is to determine the biodegradability of petroleum hydrocarbon contaminated soil using mixed microbial population from a aquifer enriched petroleum hydrocarbon fractions under anaerobic condition with terminal electron acceptors at different temperature ranges. In this study, biodegradability test is carried with chemical pre-treatment for enhancement of bioavailability followed by anaerobic microcosm test under methanogenic and sulphate-reducing environment at psychrophilic, mesophilic and thermophilic ranges.

2. LITERATURE REVIEW

2.1. Chemical Composition of Petroleum Hydrocarbons

Over millions of years, petroleum is produced by thermal decay of buried organic material. Petroleum consists of nitrogen, sulfur and oxygen in some amount along with traces of metallic constituents, and mainly hydrocarbons in differentiated ratio of carbon and hydrogen. Crude oil which refers to naturally occurring raw oil is classified as light, medium or heavy oil based on the molecular weight of constituents. Petroleum hydrocarbons are classified in four different fractions: saturates (aliphatics), aromatics (ringed hydrocarbons), resins and asphaltenes as seen Figure 1.2. Oil compounds can be characterized and identified by using commonly High-resolution Gas Chromatography (GC) equipped with flame-ionization detection (FID) and capillary GC-Mass Spectrometry (MS) (Head et al., 2006).

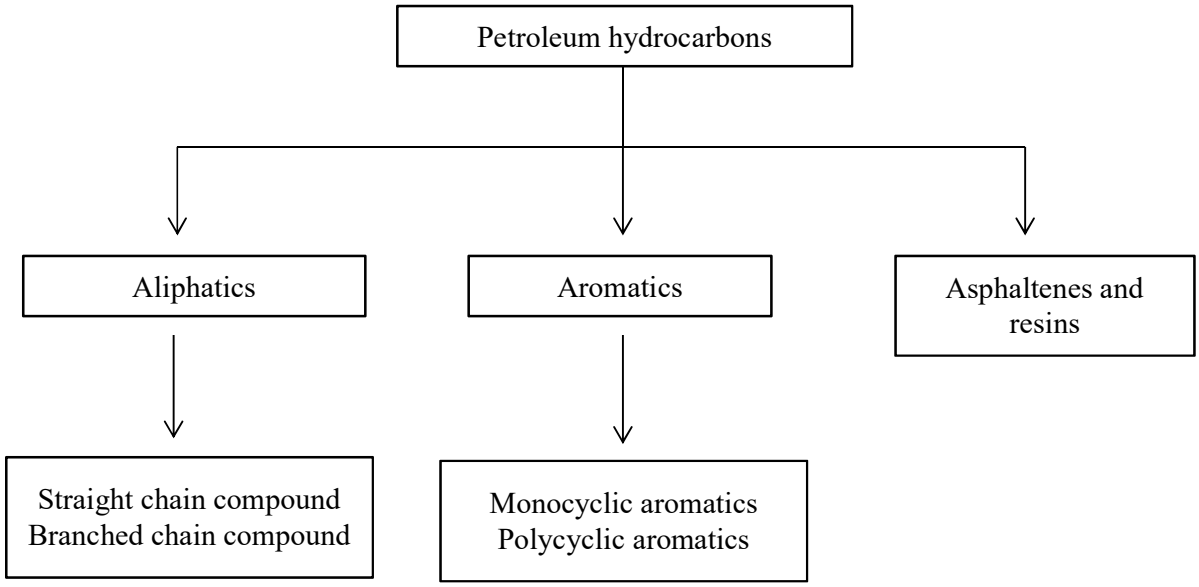

Figure 1.2. Classification of petroleum hydrocarbons.

2.1.1. Aliphatic Hydrocarbons

Aliphatic hydrocarbons is the major constituent of crude oil and petroleum products. Based on their chemical structure, aliphatics include alkanes and cycloalkanes. Also, they are methane derivatives contains both saturated and unsaturated linear or branched open-chain structures (Stroud et al., 2007). According to their molecular weight, aliphatic hydrocarbons are classified into four

groups: gaseous alkanes, aliphatics with lower molecular weight (C_8-C_{16}), aliphatics with medium molecular weight (C_{17}-C_{28}) and aliphatics with high molecular weight (greater than C_{28}) (Abbasian et al., 2015). Aliphatic hydrocarbons with high molecular weight are more persistent in the soil and not easily volatilised and degradable due to their low solubility, bioavailability and stable structure (Stroud et al., 2007).

2.1.2. Aromatic Hydrocarbons

Aromatic hydrocarbons are ringed molecules classified as monocyclic aromatic hydrocarbons such as BTEX (benzene, toluene, ethylbenzene and xylenes) and polycyclic aromatic hydrocarbons (PAHs). Aromatics have two to six aromatic rings, with or without alkyl substituents aligned with linear, cluster or angular type. The major concern to environmental health is polycyclic aromatic hydrocarbons (PAHs) result in toxicity in order to their mutagenic and carcinogenic features (Mishra et al., 2001). PAHs have different structures than the other compounds with their physical-chemical properties such as molecular weight and number of benzene rings. They have low chemical reactivity and volatility and hydrophobic properties with high molecular weight. Due to their complexity and molecular weight, PAHs are highly toxic, carcinogenic and persistent compounds in the environment. According to US EPA, there are sixteen PAHs listed based on environmental significance as priority pollutants (Masih et al., 2012). These are seen in Figure 2.1 with their structures and nomenclatures.

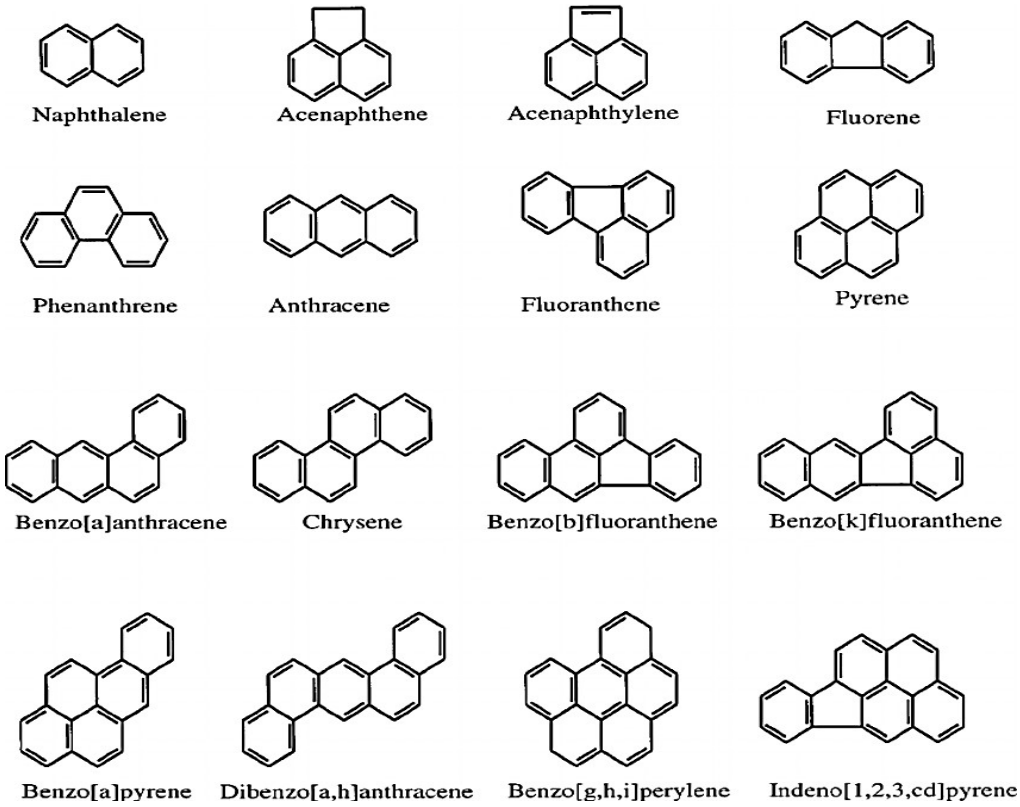

Figure 2.1. Structure of 16 US EPA priority pollutant PAH compounds.

2.1.3. Resins and Asphaltenes

Asphaltenes and resins have more complex structure with more polar constituents than aliphatic and aromatic hydrocarbons. Their properties have an effect on oil production and refining due to presence in crude oil compostion in 10% approximately. Phenols, ketones, esters and porphyrins belong to asphaltenes subgroup that do not have crystallization properties and unstability (Leyva et al., 2013). Asphaltenes are mostly polar compounds and composed of aromatic rings, aliphatic hydrocarbon chains and some heteroatoms. In comparison with asphaltenes, resins are more stable and have strong affinity. Both of them contain nitrogen, oxygen and sulphur with hydrocarbon chains. It is considered that resins and asphaltenes are mostly resistant to biodegradation by microorganisms (He et al., 2015).

2.2. Bioremediation Techniques for Petroleum Polluted Soils

Bioremediation methods are used for removal of toxic substances or pollutants by using microorganisms almost for 40 years (Ding et al., 2013). Bioremediation has eligible properties such as environmentally friendly, feasible and cost-effective features. According to many investigations

and studies, bioremediation process has high efficiency for removal of pollutants and clean up the contaminated sites than physicochemical treatment methods (Mrozik and Piotrowska-Seget, 2010). Environmental conditions such as microbial consortia, nutrient addition determined the application type of remediation due to its efficiency and feasibility. Since the presence of soil dwelling microbes which can degrade the wide range of hydrocarbon fractions, the petroleum contaminated sites may clean up by using some specified bioremediation treatment method including different conditions such as temperature, presence/absence of oxygen (Shahi et al., 2016). Different bioremediation methods being applied are natural attenuation, bioaugmentation, biostimulation and phytoremediation.

2.2.1. Natural Attenuation

Natural attenuation technique which is the simplest bioremediation method has been studied for many years due to removal of toxic substances and pollutants from contaminated sites. In this process, indigenous microbial population including bacteria and fungi which can degrade the hydrocarbon compounds are utilized for attenuation of petroleum polluted sites. These microbial communities use hydrocarbons as carbon source naturally in their metabolic pathways (McKew et al., 2007). According to bioremediation studies, natural attenuation is more efficient than biostimulation and bioaugmentation without any necessesity about enhancement of environment. In addition, this bioremediation technique is more cost-effective treatment method than the others. On the other hand, natural attenuation has some limitations such as unattainable nutrient source and absence of microbial populations which are able to degrade in high activity (Makadia et al., 2011).

2.2.2. Bioaugmentation

One of the *in-situ* bioremediation techniques is bioaugmentation which increase the biodegradability and bioavailability of hydrocarbon compound by utilization of single strains or consortia of microorganisms that have the petroleum hydrocarbon degradation capacity. In recent years, genetically engineered microorganisms (GEMs) are introduced as potential hydrocarbon degradative microbial populations for soil bioaugmentation as well (Mrozik and Piotrowska-Seget, 2010). Natural attenuation is an inapproprate technique in some cases depending on polluted site with insufficient or non-detectable number of microbial populations. Especially, bioaugmentation technique is applied after biostimulation and natural attenuation process for improvement of biodegradability. The hydrocarbon utilizers are isolated from hydrocarbon rich environments. On the other hand, bioaugmentation process requires extensive long-term monitoring and is not always

effective. The efficiency of bioremediation depends on adaptation of isolated strains and microbial consortia to the polluted environment. The introduced microorganisms can be inhibited by co-pollutants or native microorganisms El Fantroussi and Agathos, 2005).

2.2.3. Biostimulation

As bioattenuation, naturally occuring hydrocarbon degrader microorganisms are utilized for bioremediation process during biostimulation. In addition to excessive microbial population, the biodegradability enhances by nutrient addition such as nitrogen and phosphorus (Nikolopoulou and Kalogerakis, 2010). According to literature, petroleum hydrocarbon polluted environments containing 80% carbon can be clean up in a rapid reduction in the presence of inorganic nutrients (Koshlaf and Ball, 2017). These nutrients are essential for microbial cell growth, reducing the lag phase, improvent of log phase of microbial population. Hence, the microbial hydrocarbon degradation is affected positively. For the stimulation, fertilizers and biosurfactants can be used. However, nutrient balance and C:N:P ratio optimization are the most essential features of biostimulation process. Biostimulation technique is more efficient than natural attenuation due to nutrient enhancement as optimal C:N:P ratio for microbial growth (Li et al, 2007).

2.2.4. Phytoremediation

Phytoremediation technique is applied with plants and their associated microorganisms by supporting hydrocarbon degrading microbial populations within plant root. However, pollutants and toxic substances can be harmful for the plants in the contaminated sites (Koshlaf and Ball, 2017).

2.3. Anaerobic Degradation of Petroleum Hydrocarbons

Anaerobic hydrocarbon degradation metabolism has been studied in both highly enriched cultures or pure cultures isolated from hydrocarbon impact environments. Mostly, these cultures includes nitrate-reducing, sulphate-reducing and iron-reducing microorganisms, and methanogens as seen Figure 2.2. Aliphatic and aromatic hydrocarbon compounds are more degradable than asphaltenes and resins (Gieg and Toth, 2016).

Under anaerobic metabolism these aromatic compounds are first oxidized to phenols or organic acids, and then converted into long-chain volatile fatty acids, which result in the production of CH_4 and CO_2. Many types of terminal electron acceptors nitrate, ferrous iron, manganese or sulphate

ions are reported for anaerobic degradation of petroleum hydrocarbons (Varjani, 2017). Wilkes et al. (2016), observed two biochemical mechanisms that is described as initial activation of alkane degradation are addition of fumarate and carboxylation.

Benzoyl-CoA as common intermediate is present in anaerobic catabolism of many aromatic compounds. Toluene, alkyl benzene and ethylbenzene are oxidized to benzoyl-CoA. Benzene and naphthalene are initially activated by carboxylation. Followed by benzoyl-CoA or naphthalene-CoA pathways, hydrocarbon degradation pathway continues (Foght, 2008). Wilkes et al. (2016) have reported that central benzoyl-CoA pathways are different for many aspects in denitrifying, phototrophic and fermenting bacteria.

In brief, three major pathways for the anaerobic activation of petroleum hydrocarbons are: (i) for alkanes or alkylated aromatics the addition of a methyl or methylene group to fumarate known as fumarate addition; (ii) oxygen independent hydroxylation known to be involved in the degradation of ethylbenzene and related substituted benzenes and (iii) carboxylation as proposed for alkanes, benzene, and polycyclic aromatic compounds (von Netzer et al., 2018).

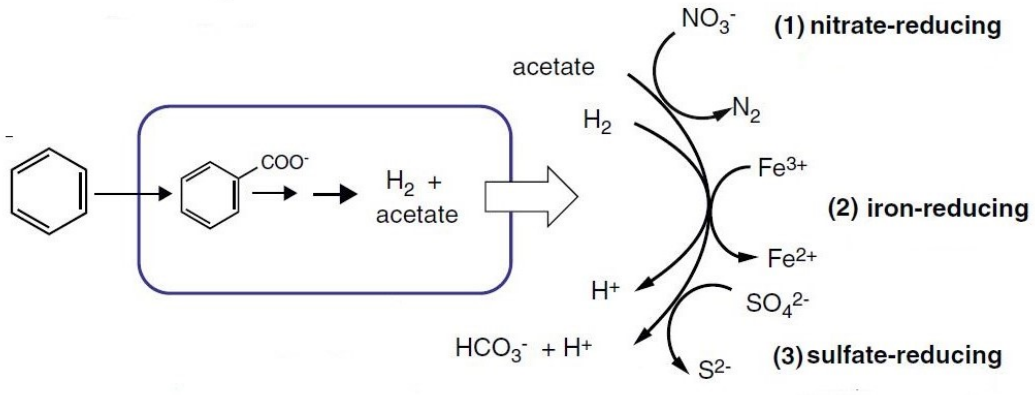

Figure 2.2. Anaerobic hydrocarbon degradation mechanism (Gieg et al., 2014).

2.3.1. Methanogenesis Pathways

Methanogenesis which is a biological process is performed by methanogenic archaeabacteria. Methanogenesis is carried out by high intracellular concentration of coenzyme F420, the enzyme methyl coenzyme M methylreductase (Mcr), and unique phospholipid membrane components. The two main pathways methane production are acetotrophic (acetate dependent) methanogenesis and hydrogenotrophic (H_2 dependent) methanogenesis as seen in Figure 2.3 (Gieg and Berdugo-Clavijo,

2014). In the acetotrophic step, acetate (CH_3COO) is converted into CH_4 and carbon dioxide (CO_2). During hydrogenotrophic methanogenesis, H_2 reacts with CO_2 forming CH_4 and H_2O. Other low molecular weight organic molecules, such as formate, methanol, dimethyl sulfide (DMS), tri-, di-, and monomethylamines, and ethylamine, can also be utilized for methanogenesis through intermediate steps (Bastviken, 2009). Microbial methanogenesis represents a terminal step of anaerobic organic matter degradation. Before the anaerobic degradation process, complex organic substrates have to undergo several degradation steps. On the other hand, in the presence of oxidized compounds as electron acceptors as nitrate, sulphate, iron resulting in terminal degradation steps like nitrification, iron reduction, sulphate reduction product more energy than methanogenesis does only (Jimenez et al., 2016).

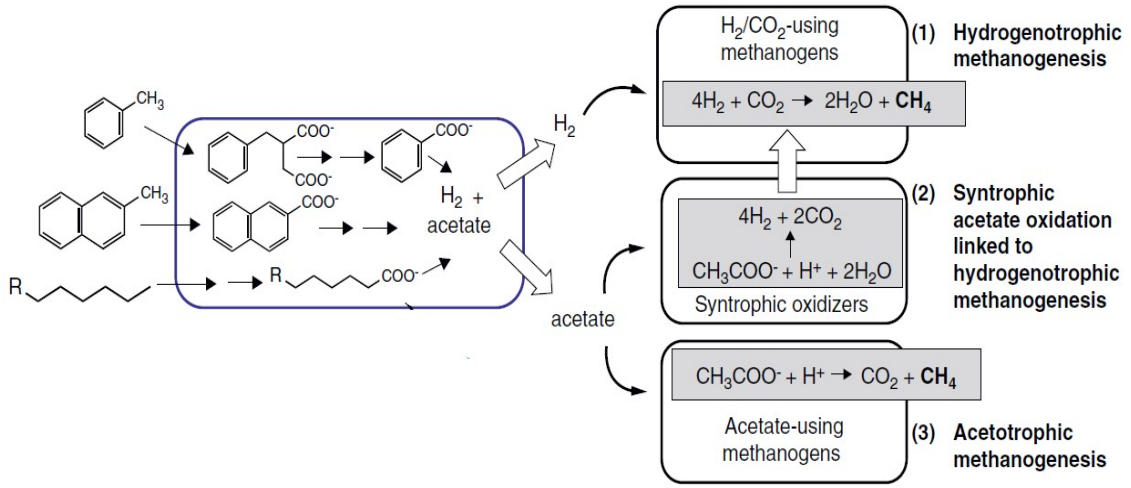

Figure 2.3. Methanogenesis pathways (Gieg et al., 2014).

2.3.2. Sulphate-reducing Pathways

Sulfur is the most abundant elements in ecosystem due to its reactivity in different reduction and oxidation states. The biological roles of inorganic sulfur compounds in anaerobic biodegradation system as acceptors or donors of electrons for dissimilatory energy-generating electron transport mainly among prokaryotes (Barton et al., 2014). Bioremediation using sulphate as the electron acceptor involves oxidation of aromatic hydrocarbons by sulfidogenic organisms coupled with reduction of sulphate to hydrogen sulfide.

The sulphate reduction pathway includes many different intermediate reactions as seen in Table 2.1. Reaction 1 shows that the end products of crude oil degradation are methane and

hydorgen sulfide. Reaction 2 involves the sulphate ion and reduction of S^{+6} to lower valance states. Essential reaction products are organic acids, bicarbonate ions and hydrogen sulphide. According to Reaction 4, sulphur is also utilized during degradation of hydrocarbon resulting in hydrogen sulfide and bicarbonate ion. Net reaction is given as overall results of these reactions, main net products of sulphate reuction pathway are bicarbonate and hydrogen sulfide. Carbon dioxide may be evolved during the intermediate steps (Machel, 1989). Also, sulphur, sulphite and thiosulphate are utilized via inorganic sulphur compoun fermantation to convert into sulphate ion (Barton et al., 2014).

Table 2.1. Different reactions through sulphate reduction pathway.

Reaction 1	Crude oil	$\longrightarrow$	Light crude oil + H_2S + CH_4
Reaction 2	$4R\text{-}CH_3 + 3SO_4^{-2} + 6H^+$	$\longrightarrow$	$4R\text{-}COOH + 4H_2O + 3H_2S$
	$R\text{-}CH_3 + 2R{=}CH_2 + CH_4 + 3SO_4^{-2} + 5H^+$	$\longrightarrow$	$3R\text{-}COOH + HCO_3^- + 3H_2O + 3H_2S$
	$2CH_2O + SO_4^{-2}$	$\longrightarrow$	$2HCO_3^- + H_2S$
Reaction 3	$3H_2S + SO_4^{-2} + 2H^+$	$\longrightarrow$	$4S^0 + 4H_2O$
	$H_2S + SO_4^{-2} + 2H^+$	$\longrightarrow$	$S^0 + 2H_2O + SO_2$
	H_2S + hydrocarbons	$\longrightarrow$	S^0 + altered hydrocarbons
	S^{-2}	$\longrightarrow$	S^0
Reaction 4	$4S^0 + 1.33(\text{-}CH_2\text{-}) + 2.66\ H_2O + 1.33\ OH^-$	$\longrightarrow$	$4H_2S + 1.33\ HCO_3^-$
Net Reaction	Hydrocarbons + SO_4^{-2}	$\longrightarrow$	altered hydrocarbons + HCO_3^- + H_2S ($+CO_2$?)

The presence of sulphate-reducing bacteria are detemined by using dsrB as a universal genetic marker. This gene encodes the β subunit of dissimilatory sulfite reductase, the key enzyme which catalyzes sulfite transformation into sulfide in all sulphate and sulphite reducing microorganisms as shown in Figure 2.4 (Korneeva et al., 2015).

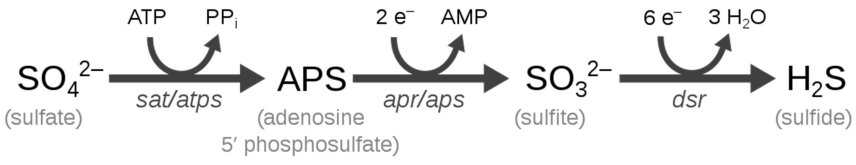

Figure 2.4. Transformation of sulphate ion in the presence of dsrB gene.

2.3.3. Anaerobic Degradation of Aliphatic and Aromatic Compounds

The initial activation reaction is often the crucial step in the degradation of aliphatic or aromatic hydrocarbons by anaerobic degraders. Similar to aerobic hydrocarbon catabolism, the anaerobic degradation of petroleum hydrocarbons also move through several initial activation and transformation mechanisms as intermediate degradation pathways as given in Figure 2.5.

Among the anaerobic biodegradation processes, fumarate addition is considered as initial of anaerobic hydrocarbon degradation mechanisms. It was first observed for the degradation of toluene by the denitrifying *Thauera aromatica* and is catalyzed by a glycyl radical enzyme named benzylsuccinate synthase (Bss). Meanwhile, a role of benzylsuccinate synthase and related fumarate adding enzymes (FAEs) has been reported for a wide phylogenetic diversity and respiratory variety of anaerobic degraders and also for the degradation of other alkylated mono- and polyaromatics, linear and cyclic alkanes. Depending on the nature of the substrate, the FAEs involved are also named as (1-methyl) alkylsuccinate synthases (Ass) (Kolukırık 2011a).

Benzoyl-CoA is the central metabolite of monoaromatic hydrocarbon degradation, and two enzyme systems are known to be involved: either the ATP-dependent class-I benzoyl-CoA reductase (Bcr/Bzd) in facultative anaerobes or the ATP-independent class-II benzoyl-CoA reductase (BamB) in strict anaerobes. After ring reduction, a ring-cleaving hydrolase (BamA) transforms the former aromatic ring into linear CoA-fatty acids, which are then subject to a beta oxidation-like degradation to acetyl-CoA or complete oxidation to CO_2. Such linear CoA-fatty acids are also products of the anaerobic degradation of n-alkanes (Varjani, 2017).

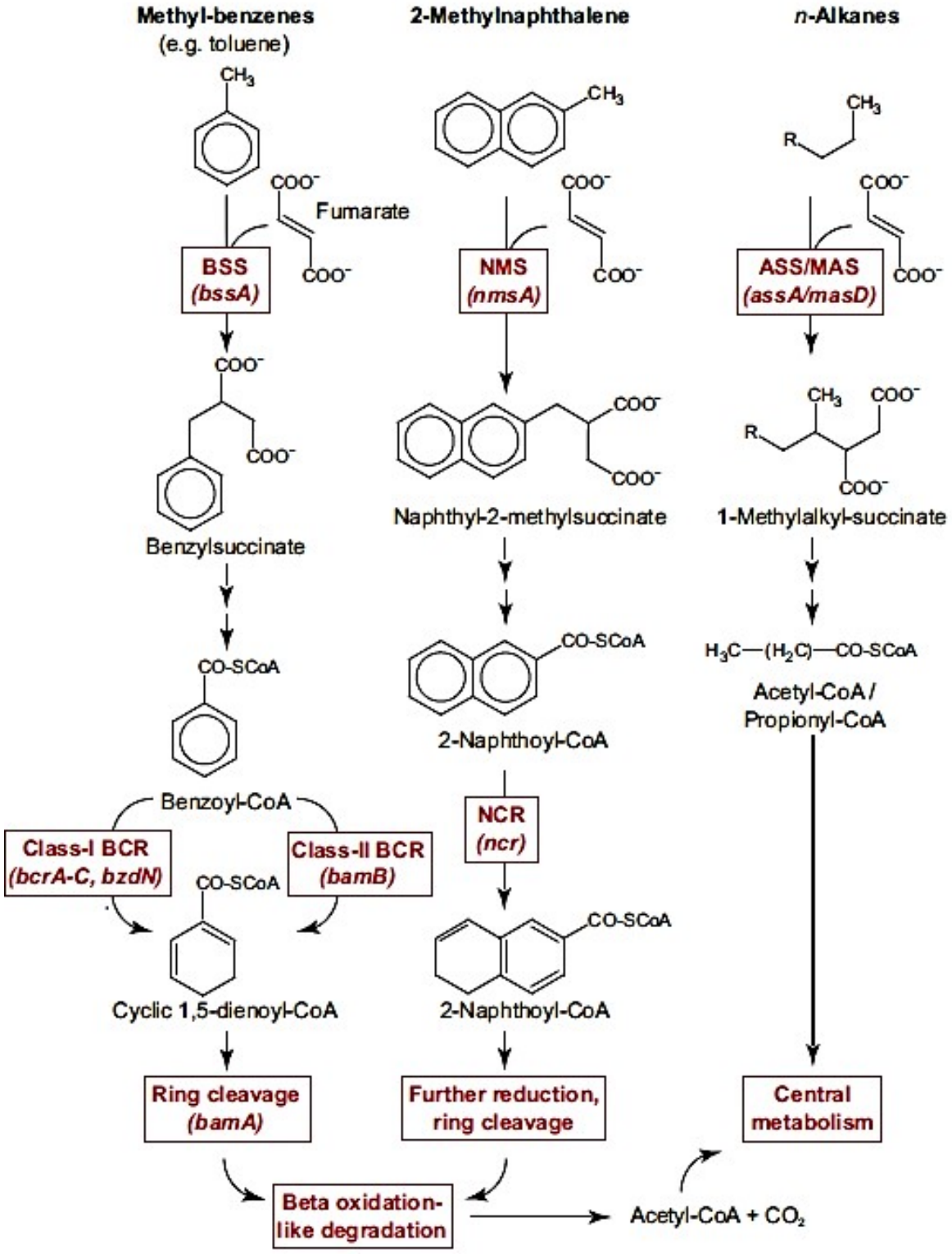

Figure 2.5. Anaerobic degradation of aliphatic and aromatic hydrocarbons.

2.4. Petroleum Utilizing Microorganisms

Some microorganisms have ability to degrade aliphatics; some can degrade monoaromatics or polyaromatics while others degrade resins. In general, Petroleum hydrocarbon pollutants degrading microorganisms and the type of hydrocarbon degraded by them are given in Table 2.2 and 2.3 (Koshlaf and Ball, 2017). Bacterial sp. of genera *Achromobacter*, *Acinetobacter*, *Arthrobacter*, *Azoarcus*, *Brevibacterium*, *Cellulomonas*, *Corynebacterium*, *Flavobacterium*, *Marinobacter*, *Micrococcus*, *Nocardia*, *Ochrobactrum*, *Pseudomonas*, *Stenotrophomaonas* and *Vibrio* are reported as hydrocarbon degraders Yakimov et al. (2007), and Brooijmans et al. (2009), have reported evolution of hydrocarbonoclastic bacterial genera *Oleispira*, *Marinobacter*, *Thalassolituus*, *Alcanivorax* and *Cycloclasticus* from petroleum hydrocarbon polluted sites. These indigenous bacteria were present at low or undetectable levels before pollution but were found to dominate in oil polluted sites. *Alcanivorax* strains grow on n-alkanes and branched alkanes, while they cannot grow on any sugars or amino acids as carbon sources. Cycloclasticus strains grow on aromatic hydrocarbons, naphthalene, phenanthrene and anthracene, whereas *Oleispira* strains grow on aliphatic hydrocarbons, alkanoles and alkanoates (Harayama et al., 2004). There are several reports available on isolation and identification of *P. aeruginosa*, *S. maltophilia*, *Rhodococcus sp.*, *Bacillus sp.*, *Acinetobacter sp*., *Ochrobactrum sp*., and *Exiguobacterium* for hydrocarbon utilization/degradation and biosurfactant production (Varjani and Upasani, 2017). In addition, it is determined that the dominant taxa were primarily known *Marinobacter*, *Vibrio, Pseudomonas*, *Acinetobacter* and *Arcobacter* being found in hydrocarbon enriched environments as halotolerant microorganisms (Gieg and Toth, 2016). Members of the genera *Pseudomonas*, *Bacillus*, *Burkholderia*, *Enterobacter* isolated from petoleum contaminated sites which are enriched with hydrocarbon degrading members (Sarkar, 2017).

Table 2.2. Petroleum utilizing microorganisms.

Species/Strain	Substrate	Species/Strain	Substrate
***Achromobacter sp.* NCW**	CBZ	*Geobacillus thermodenitrificans* NG80-2	C_{15}–C_{36}
***Acinetobacter baylyi* ADP1**	–C_{36}	*Gordonia sp.* TY-5	C_3, C_{13}–C_{22}
***A. calcoaceticus* 69-V**	C_{11}–C_{18}	*Janibacter sp.* YY-1	DBF, FLE, DBT, PHE, ANT, DD
***A. calcoaceticus* EB104**	C_6–C_{18}	*Marinobacter* NCE312	NAP
***A. calcoaceticus* NCIB 8250**	C_8–C_{16}	*Marinobacter sp.* BC36, BC38, & BC42	C_{18}
***Acinetobacter sp.* 2796A**	C_{10}–C_{16}	*Marinobacter hydrocarbonoclasticus* 617	C_{16}–C_{30}
***Acinetobacter sp.* M-1**	C_{13}–C_{44}	*Mycobacterium avium*	paraffins
***Acinetobacter calcoaceticus* RR8**	C_{10}–C_{34}	*M. avium subsp. paratuberculosis*	paraffins
Acinetobacter lwoffi	C_{12}–C_{28}	*M. bovis* BCG	C_{12}–C_{16}
***Acinetobacter sp.* ODDK71**	C_{12}–C_{30}	*M. smegmatis*	C_9–C_{16}
***Acinetobacter sp.* S30**	–C_{33}	*M. tuberculosis* H37Rv	C_{11}–C_{16}
***Acinetobacter sp.* DSM17874**	C_{10}–C_{40}	*Mycobacterium sp.* CH1	C_{12}–C_{28}
***Alcanivorax borkumensis* AP1**	C_{10}–C_{20}	*Mycobacterium sp.* HXN 600	C_6–C_{24}
***Alcanivorax borkumensis* SK2**	C_8–C_{32}	*Mycobacterium sp.* OFS	C_{11}–C_{28}
***Alcaligenes odorans* P20**	–C_{33}	*Mycobacterium sp.*	PYR, BaP
Alcaligenes denitrificans	FLA	*Mycobacterium sp.*JS14	FLA
***Arthrobacter nicotianae* KCC B35**	C_{10}–C_{40}	*Mycobacterium sp.* 6PY1, KR2, AP1	PYR
***Arthrobacter sp.*F101**	FLE	*Mycobacterium sp.* RJGII-135	PYR,BaA, BaP
***Arthrobacter sp.* P1-1**	DBT, CBZ, PHE	*Mycobacterium sp.* PYR-1, LB501T	FLA, PYR, PHE, ANT
***Arthrobacter sulphureus* RKJ4**	PHE	*Mycobacterium sp.* CH1, BG1, BB1, KR20	PHE, FLE, FLA, PYR
***Acidovorax delafieldii* P4-1**	PHE	*Mycobacterium flavescens*	PYR, FLA
***Bacillus cereus* P21**	PYR	*Mycobacterium vanbaalenii* PYR-1	PHE, PYR, dMBaA
***Bacillus thermoleovorans* B23 & H41**	C_9–C_{30}	*Mycobacterium sp. KMS*	PYR
***Bacillus thuringiensis/cereus* A2**	C_6–C_{28}	*Nocardioides aromaticivorans* IC177	CBZ
***Brevibacteriumsp.* HL4**	PHE	*Nocardioides sp.* CF8	C_7–C_{16}
***Burkholderia sp.*S3702, RP007, 2A 12TNFYE-5, BS3770**	PHE	*Paracoccus sereniphilus/marcusii* A7	C_6–C_{28}
***Burkholderia cepacia* ATCC 25416**	C_{10}–C_{16}	*Paracoccus sp. strains* Ophe1 & Sphe1	C_{10}–C_{28}
***Burkholderia cepacia* RR10**	C_{12}–C_{34}	*Pasteurella sp.* IFA	FLA
***Burkholderia sp.*C3**	PHE	*Planococcus alkanoclasticus* MAE2	C_{11}–C_{33}
***Burkholderia cepacia* BU-3**	NAP, PHE, PYR	*Polaromonas naphthalenivorans* CJ2	NAP
Burkholderia cocovenenans	PHE	*Prauserella rugosa* NRRL B-2295	C_8–C_{14}
***Burkholderia xenovorans* LB400**	BZ, BP	*Pseudomonas aureofaciens* RWTH 529	C_{10}
B. mallei	C_{10}–C_{16}	*Pseudomonas sp.* 7/156	n. d
B. pseudomallei	C_{10}–C_{16}	*Pseudomonas putida* GPo1	C_5–C_{12}
***Chryseobacterium sp.* NCY**	CBZ	*P. putida* P1	C_8

Table 2.3. Petroleum utilizing microorganisms.

***Cycloclasticus sp.*P1**	PYR	*Pseudomonas fluorescens* CHA0	C_{12}–C_{32}
Brachybacterium sp.	C_{10}–C_{20}	*Pseudomonas aeruginosa* PAO1	C_{12}–C_{24}
***Desulfatibacillum aliphaticivorans* CV2803**	C_{13}–C_{18}	*P. aeruginosa* PG201	C_{10}–C_{16}
***Dietzia cinnamea* P4**	C_{11}–C_{24}	*P. aeruginosa* KSLA473	C_5–C_{16}
Dietzia psychralcaliphila	C_{13}–C_{24}	*P. aeruginosa* NCIMB 8704 & 9571	C_8–C_{16}
***P. aeruginosa* ATCC 17423**	C_8–C_{16}	*R. erythropolis* Q15	C_8–C_{32}
***P. aeruginosa* RR1**	C_{12}–C_{34}	*R. erythropolis* 35-O	C_6–C_{16}
***P. aeruginosa strains* A1, A3, A4, A5, A6**	C_6–C_{28}	*R. erythropolis* 23-D	C_6–C_{36}
***Pseudomonas sp.* PUP6**	C_{12}–C_{28}	*R. erythropolis* NRRL B-16531	C_6–C_{36}
***Pseudomonas sp.*C18, PP2, DLC-P11**	NAP, PHE	*R. erythropolis* 42-O	C_6–C_{32}
***Pseudomonas sp.*BT1d**	HFBT	*R. erythropolis* 62-O	C_6–C_{16}
***Pseudomonas sp.*B4**	BP, CBP	*R. erythropolis* 23-D	C_6–C_{36}
***Pseudomonas sp.*HH69**	DBF	*R. erythropolis* 50-V	C_6–C_{32}
***Pseudomonas sp.*CA10**	CBZ, CDD	*R. erythropolis* NRRL B-16531	C_6–C_{36}
***Pseudomonas sp.* NCIB 9816-4**	FLE, DBF, DBT	*Rhodococcus fascians* 115-H	C_6–C_{32}
***Pseudomonas sp.* F274**	FLE	*R. fascians* 154-S	C_6–C_{24}
Pseudomonas paucimobilis	PHE	*Rhodococcus rhodochrous*	C_{12}–C_{20}
***Pseudomonas vesicularis* OUS82**	FLE	*Staphylococcus sp.* PN/Y	PHE
***Pseudomonas putida* P16, BS3701, BS3750, BS590-P, BS202-P1**	NAP, PHE	*Stenotrophomonas maltophilia* VUN 10,010	PYR, FLA, BaP
***Pseudomonas putida* CSV86**	MNAP	*S. maltophilia* VUN 10,003	PYR, FLA, BaA, BaP, DBA, COR
***Pseudomonas fluorescens* BS3760**	PHE, CHR, BaA	*Sphingomonas yanoikuyae* R1	PYR
***Pseudomonas stutzeri* P15**	PYR	*Sphingomonas yanoikuyae* JAR02	BaP
Pseudomonas saccharophilia	PYR	*Sphingomonas sp.*P2, LB126	FLE, PHE, FLA, ANT
Pseudomonas aeruginosa	PHE	*Sphingomonas sp.*	DBF, DBT, CBZ
***Ralstonia sp.* SBUG 290 U2**	DBF NAP	*Sphingomonas paucimobilis* EPA505	FLA, NAP, ANT, PHE
***Rhodanobacter sp.* BPC-1**	BaP	*Sphingomonas wittichii* RW1	CDD
Rhodococcus sp.	PYR, FLA	*strain* AK01	C_{13}–C_{18}
***Rhodococcus sp.* 1BN**	C_6–C_{28}	*strain* HdN1	C_{14}–C_{20}
***Rhodococcus sp.* RR12 & RR14**	C_{14}–C_{34}	*strain* Hxd3	C_{12}–C_{20}
***Rhodococcus sp. strains* T12 & TMP2**	C_9–C_{22}	*Terrabacter sp.*DBF63	DBF, CDBF, CDD, FLE
***Rhodococcus sp.* NCIM5126**	C_{13}–C_{20}	*Thalassolituus oleivorans*	C_7–C_{20}
***Rhodococcus sp.*WU-K2R**	NAT, BT	*Thermooleophilum album*	C_{13}–C_{29}
***Rhodococcus erythropolis* I-19**	ADBT	*Thermus sp.* C2	C_9–C_{39}
***R. erythropolis* D-1**	DBT	*Weeksella sp.* RR7	C_{12}–C_{34}
***P. aeruginosa* ATCC 17423**	C_8–C_{16}	*Xanthamonas sp.*	PYR, BaP, CBZ
***P. aeruginosa* RR1**	C_{12}–C_{34}	*Xylella fastidiosa* RR15	C_{14}–C_{34}

3. MATERIALS AND METHODS

3.1. Sampling of the Contaminated Soil and Aquifer

The aquifer that was used as a seed was collected from the capillary fringe and the subjacent region of an aquifer located in Leuna, Germany contaminated with several 10,000 tons of petroleum hydrocarbons including mainly benzene, toluene, ethyl benzene, and xylene (BTEX) and methyl tertbutyl ether (MTBE) (Tischer et al., 2013). The aquifer in this study was taken from below the water table from different cores in September 2017. The petroleum contaminated soil was collected from waste landfill in Adana, Turkey which was highly contaminated with oily wastes taken from petroleum pipeline for transportation and storage.

3.2. Pre-treatment of the Contaminated Soil

Persulfate treatment method is used for remediation of organic fuel-contaminated environment by chemical treatment. Besides some disadvantages such as non-selective for contaminants and harmful to soil organisms, there is a minimum concentration level for oxidants (Satapanajaru et al., 2017). Persulfate oxidation (Wu et al., 2016) was applied as pre-treatment method directly to petroleum contaminated soil. 0.4 g/g TS sodium persulfate ($Na_2S_2O_8$) was used as oxidizer and 0.5 M of iron (II) sulfate ($FeSO_4$) and citric acid was used as activator. The contaminated soil sample was placed into a 150 mL conical flask and persulfate, activator and distilled water was added into flask, respectively. In the batch experiment, the soil to liquid ratio was 1:5. After 2 hours incubation on orbital shaker at maximum rpm level, persulfate and activator was added into flask in the same amount. Sample was incubated 2 hours more incubated on the orbital shaker. The mixture of soil sample was centrifuged at 14,000 rpm and the pellet was stored at 4°C until physicochemical analyses and microcosms studies. The supernatant was analyzed for determination of consumption of persulfate by addition of potassium iodide (KI) and formation of iodine (I_2) via spectrophotometrically measurement at 352 nm (Wahba et al., 1959). In addition, dissolved organic carbon was determined for the contaminated soil before and after the pretreatment stage.

3.3. Physicochemical Characterization of the Contaminated Soil, Treated Soil and Aquifer

The pH was measured with an instrumental method for determination of pH using a glass electrode of soil in water, 1 M potassium chloride (KCl) or 0.01 M calcium chloride ($CaCl_2$) solution based on TS ISO 10390 standard. Total Organic Carbon (TOC) was measured based on SM 5310 standard by an accreditation certificated company. Total Petroleum Hydrocarbon (TPH), aliphatic fractions and aromatic fractions of petroleum hydrocarbons were measured according to TS EN 14039 and S-TPHFID08 standard method respectively by an other accreditation certificated company. Total phosphorus was measured using Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) according to EPA 200.7 standard. Total nitrogen was analyzed according to TS 8337 ISO 11261 standards include the modified method for determination of Total Kjeldahl Nitrogen. Dissolved Organic Carbon (DOC) was measured using high-temperature combustion method based on SM 5310 B standards. Preparation of sample for elemental analysis was applied through drying at 105°C overnight, digestion by nitric acid and sulfuric acid, and then microwave digestion. Elemental analyses to contaminated soil sample includes determination of magnesium, sulphur, iron, aluminum, manganese, copper, zinc, boron, nickel and vanadium were carried out by using inductively coupled plasma optical emission spectrometry (ICP-OES) through EPA 6010D standard. Total Solid and Volatile Solid were determined according to the Standard Methods (APHA, 2005).

3.4. Anaerobic Microcosm Cultivation

Anaerobic microcosms were set up in glass 120 mL serum bottles sealed with butyl rubber stoppers and aluminum crimps (Aldrich). The total volume of liquid was 80 mL with 40 mL of headspace volume. Microcosms were flushed with atmosphere of nitrogen (100%) and each microcosm had a growth medium with Resazurin for control of excess oxygen. Microcosms were incubated in cooled and heated incubator based on their temperature conditions.

The environmental medium conditions were modified with two different electron acceptor including trace elements and vitamin solution based on OECD 311 protocol “Anaerobic Biodegradability of Organic Compounds in Digested Sludge: by Measurement of Gas Production”. Both conditions comprised a medium with sources of nitrogen and phosphorus, vitamin and trace elements prepared in de-ionized water (OECD, 2006).

The base medium includes anhydrous potassium dihydrogen phosphate (KH_2PO_4) (0.27 g/L), disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$) (1.12 g/L), ammonium chloride (NH_4Cl) (0.53 g/L), calcium chloride dehydrate ($CaCl_2.2H_2O$) (0.075 g/L), magnesium chloride hexahydrate ($MgCl_2.6H_2O$) (0.1 g/L), iron (II) chloride tetrahydrate ($FeCl_2.4H_2O$) (0.02 g/L), resazurin as oxygen indicator (0.001 g/L), sodium sulphide nonahydrate ($Na_2S.9H_2O$) (0.1 g/L), 10 mL of trace element stock solution and 10 mL of vitamin stock solution. During preparation of medium, the bottle was flushed with atmosphere of nitrogen (100%). In the sulphate-reducing environment, potassium sulphate (K_2SO_4) (1.8 g/L) was used as source of sulphate. Trace element stock solution included manganese chloride tetrahydrate ($MnCl_2.4H_2O$) (50 mg/L), boric acid (H_3BO_3) (5 mg/L), zinc chloride ($ZnCl_2$) (5 mg/L), copper (II) chloride ($CuCl_2$) (3 mg/L), disodium molybdate dihydrate ($Na_2MoO_4.2H_2O$) (1 mg/L), cobalt chloride hexahydrate ($CoCl_2.6H_2O$) (0.1 g/L), nickel chloride hexahydrate ($NiCl_2.6H_2O$) (10 mg/L), disodium selenite (Na_2SeO_3) (6 mg/L) and sodium tungstate dehydrate ($Na_2WO_4 \cdot 2H_2O$) (8 mg/L). Vitamin stock solution comprised with 4-aminobenzoic acid (40 mg/L), D(+)-biotin (10 mg/L), nicotinic acid (0.1 g/L), calcium D(+)-pantothenate (0.05 g/L), pyroxidine dihydrochloride (0,15 g/L), thiamine (0.1 g/L) made up in NaP Buffer (10 mM, pH 7.1). All solutions were autoclaved at 121°C for 15 minutes.

Microcosms were inoculated with 10 g of the aquifer. Microcosms were fed with 5 g of the petroleum contaminated soil (Sherry et al., 2013).

Produced gas volume was determined by using 7,000 mbar manometer (Lutron PM-9107, China) manometer periodically (in each 30-40 days). Gas composition from headspace was analyzed for CH_4 and CO_2 using HP Agilent 6850 Gas Chromatograph (GC) with a thermal conductivity detector (HP Plot Q column 30 m × 0.53 mm). Peak areas were calibrated using the CH_4 and CO_2 gas standards and the reproducibility of replicate standard analyses were typically less than 1% relative standard deviation.

3.5. Microbial Community Analysis

Genomic DNA and Total RNA extraction, PCR Amplification of 16S rRNA genes and specific target genes, quantitative real time PCR was applied to aquifer and contaminated soil, and samples from microcosms at the beginning and end of cultivation. High resolution melting analysis was done for microbial community profile observation of samples from microcosms. Metagenomics analysis is applied to aquifer and contaminated soil due to determination of microbial population.

3.5.1. Genomic DNA and Total RNA Extraction

Total nucleic acid (Genomic DNA and Total RNA) was extracted by using manual extraction method for soil and aquifer samples by using CTAB Method for NA Extraction. 50-100 mg of soil sample is placed into screw-capped tube includes metal beads inside. After 500 µl of CTAB solution, the sample was vortexed strongly by homogenizator at 7,000 rpm for 2 minutes. After centrifugation at 14,000 g for 2 minutes, supernatant was used through the total nucleic acid extraction method. 500 µl Guanidium isothiocynate (3 M) and 20 µl of Proteinase-K (20 mg/mL) are placed onto the supernatant sample into the clean Eppendorf tube. The sample as incubated at 55°C for 1 hour and then at 95°C for 15 minutes. During incubation, the sample was vortexed in each 5-10 minutes. After cooling down, the sample is centrifuged and the supernatant was transferred into new Eppendorf tube for the binding step. 500 µl of isopropanol was added into the sample solution. Clean NA column was placed into new Eppendorf tube and the mixture of sample and binding buffer was put into the column. Until the end of the sample, the tube with NA column was centrifuged at 14,000 g for 1 minute and the filtrate was thrown away. Extracted nucleic acid was washed by using Guanidium isothiocynate isopropanol solution and washing buffer through the NA column. For the elution step, 100 µl of MGW was placed into the NA column and then the column was incubated at 50-60°C for 1 minute or at room temperature for 2-3 minutes. At last, the tube was centrifuged and the filtrate includes the extracted total nucleic acid. Total nucleic acid sample was stored at -40°C until polymerase chain reaction analyses and metagenomics analyses.

3.5.2. PCR Amplification of 16S rRNA Genes and Specific Target Genes

Amplification of 16S rRNA from the extracted nucleic acid was run with universal primers pA (5'-GAGTTTGMTCCTGGCTCAG-3') – pH (5'- ACGGYTACCTTGTTACGACTT-3'). The sequences of specific target genes are given in Table 2.1. PCR Amplification was performed with a 10 µl reaction volume containing 2 µl of template NA, 100 pmol/µl of each primer, 5 µl of 2×Bioline RT-qPCR mix, 0,5 µl of 10×LC green dye and completed with MGW. PCR amplification was done by Bio-Rad CFX Connect Instrument (Bio-Rad Inc., USA) with an initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 15 sec, annealing for 25 sec at 54°C and extension at 72°C for 45 sec, following with melt curve analysis between 65°C – 95°C. For target genes the annealing temperature was given in Table 3.1. The melt curve analysis of specific target genes was between 65°C – 98°C. The results of quantification and melt curve summary were observed from Bio-Rad CFX Manager 3.1.

Table 3.1. Primer sets and their targets (Kolukırık, 2010).

Primer	Target Gene	Target Organism
assA_1578f	assA	Aliphatic Hydrocarbon Degraders
assA_1967r	assA	Aliphatic Hydrocarbon Degraders
BCR697f	bcrA	Degraders of Aromatics
BCR1178r	bcrA	Degraders of Aromatics
mcrA1f	mcrA	Methanogens
mcrA1r	mcrA	Methanogens
DSRp2060F	dsrB	Sulphate Reducers
DSR4R	dsrB	Sulphate Reducers

3.5.3. Quantitative Real-Time PCR

Quantitative Real-Time PCR Amplification of 16S rRNA from the extracted nucleic acid was run with universal primers pA (5'-GAGTTTGMTCCTGGCTCAG-3') – pH (5'-ACGGYTACCTTGTTACGACTT-3'). The primer sets refer to specific target genes that are given in Table 2.1 used for the Q-PCR assays. Q-PCR Amplification was performed with a 10 µl reaction volume containing 2 µl of template NA, 100 pmol/µl of each primer, 5 µl of 2×Bioline RT-qPCR mix, 0,6 µl of RT-RIN mix, 0,5 µl of 10×LC green dye and completed with MGW. Q-PCR assay for 16S rRNA Amplification was done by Bio-Rad CFX Connect Instrument (Bio-Rad Inc., USA) with following thermocycling program as a reverse transcription step at 45°C for 30 min, following with an initial denaturation at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 15 sec, annealing for 50 secs at 54°C and extension at 72°C for 5 secs, and melt curve analysis was performed from 77°C to 95°C to determine if only one amplified product was generated during Q-PCR. For target genes, primer sequences and their annealing temperature is given in Table 3.2. The melt curve analysis for these target genes was performed between 65°C – 98°C. The results of quantification and melt curve summary were observed from Bio-Rad CFX Manager 3.1.

Table 3.2. Primer sequences and their annealing temperatures (Kolukırık, 2010).

Primer	Target Gene	Gene Sequence	Annealing (°C)
assA_1578f	assA	5'-KGAYTTTGAGSASCTTTTCS-3'	56
assA_1967r	assA	5'-TCGTCCACRTARTCGTCGTC-3'	
BCR697f	bcrA	5'-GTYGGMACCGGCTACGGCCG-3'	55
BCR1178r	bcrA	5'-TTCTKVGCIACICCDCCGG-3'	
mcrA1f	mcrA	5'-RTRYTMTWYGACCARATMTG-3'	58
mcrA1r	mcrA	5'-YTGDGAWCCWCCRAAGTG-3'	
DSRp2060F	dsrB	5'-CAACATCGTYCAYACCCAGGG-3'	60
DSR4R	dsrB	5'-GTGTAGCAGTTACCGCA-3'	

3.5.4. High Resolution Melting (HRM) Analysis

The nested PCR approach was used to amplify microbial rDNAs (Kolukirik et al., 2011b). The first round qPCRs were carried out using the pA (5'-GAGTTTGMTCCTGGCTCAG-3') – pH (5'-ACGGYTACCTTGTTACGACTT-3') primer set. The vF (5'-CCTACGGGAGGCAGCAG-3') and vR (5'-ATTACCGCGGCTGCTGG-3') primer set was used for the second round PCR. The following thermal cycling conditions were applied for all of the reactions: 3 min at 95°C, 40 cycles of 20 sec at 95°C, 20 sec at 53°C and 30 sec at 72°C. Biospeedy® HRM Master Mix (Bioeksen Ltd. Co., Turkey) and Bio-Rad CFX Connect Instrument (Bio-Rad Inc., USA) were used for all reactions. The reactions contained 1.5 mM MgCl2, 0.2 mM dNTP mix, 1x Reaction Buffer, 0.1 U Fast Start Proof Reading Recombinant Taq DNA Polymerase, 1x EvaGreen, 5 ng/mL DNA template and 0.5 mM of each primer. To ensure and detect whether the expected product was amplified during PCR and for HRM analysis, melting curve analyses were applied between 60 and 95°C at a fluorescence reading rate of 0.1 C/acquisition. HRM profiles were obtained as described by Reja et al. (2010). Microbial community profile dendrograms were obtained using Minitab 14 Software (Minitab Inc., England) based on the similarities between the HRM profiles. Bivariate correlation analyses between the bacterial and the other characteristics of the samples were performed using MINITAB 14. The correlations were evaluated using Pearson's method. Statistical significance was taken as $p < 0.05$. Principal component analysis (PCA) ordinations were calculated in MINITAB 14.

3.5.5. Metagenomics Analysis

The protocol included primer pair sequences for the V3 and V4 regions of the 16S rRNA that created a single amplicon of ~460 bp (Klindworth et al. 2013). The protocol also included overhang adapter sequences that must be appended to the primer pair sequences for compatibility with the Illumina index and sequencing adapters. Illumina adapter overhang nucleotide 16S rRNA-specific sequences were 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGG-CWGCAG-3' for the forward primer and 5'-GTCTCGTGGGCTCGGAGATGTGTATA-AGAGACAGGACTACHVGGGTATCTAATCC-3' for the reverse primer. The first PCR was performed using the Biospeedy Proof Reading DNA Polymerase 2× Reaction Mix (Bioeksen R&D Technologies) and 200 nm of each primer. The following program was performed on a Bio-Rad CFX Connect Instrument (Bio-Rad Laboratories, Hercules, CA, USA): 95°C for 3 min, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by 72°C for 5 min. The PCR products were separated on an agarose gel to verify the size (~550 bp) and purified using the Biospeedy PCR Product Purification Kit (Bioeksen R&D Technologies).

The dual indices and Illumina sequencing adapters were attached to the purified first PCR products via the second PCR, which was run using the Nextera XT Index Kit (Illumina, Inc., San Diego, CA, USA) and the following program: 95°C for 3 min, eight cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by 72°C for 5 min. The PCR products were purified using the Biospeedy PCR Product Purification Kit. The final library was assessed on a Bioanalyzer DNA 1000 chip to confirm the size (~630 bp). The final library was diluted using 10 mM Tris, pH 8.5, to 4 nM, and 5-µL aliquots were mixed for pooling the libraries. In preparation for cluster generation and sequencing, pooled libraries were denatured with NaOH, diluted with hybridization buffer (HT1), and then heat-denatured before the MiSeq sequencing. Illumina MiSeq (ver. 3) reagent kits were used. Each run included a minimum of 5% PhiX as an internal control.

The raw sequence data (concatenated forward and reverse sequence reads) were cleaned, reduced, and analyzed using Mothur (ver. 1.36.1) (Schloss et al. 2009). First, the barcode and the primer sequences were trimmed, and unique sequences were identified. The trimmed unique sequences were aligned with Greengenes rRNA database sequences using the BLASTN algorithm (DeSantis et al. 2006). Before this, the Greengenes database sequences were trimmed to include only the V3–V4 region. The overhangs at both ends were removed by filtering the sequences, and a redundancy check was performed. For further de-nosing, the sequences were pre-clustered. Chimeras were eliminated using the implanted code UCHIME (Edgar et al. 2011). The sequences

were classified using the Bayesian classifier within Mothur. The reference and taxonomy files were adopted from the Greengenes database (DeSantis et al. 2006). After operational taxonomic unit (OTU) picking and taxonomic assignments using the SILVA rDNA database, the OTUs were binned in to phylotypes.

3.6. Statistical Analysis

Statistical analyses were performed using the software MINITAB 14 (Minitab Ltd., England). Correlations were evaluated using Pearson's method. Statistical significance was taken as $p<0.05$.

4. RESULTS AND DISCUSSION

4.1. Results of Physical and Chemical Characterization of Contaminated Soil and Aquifer

The chemical and physical characterization of the aquifer (A), petroleum contaminated soil (SO) and pre-treated contaminated soil (TSO) was given in Table 4.1 including pH, total phosphorus, C/N ratio, total nitrogen, TS-TVS, TOC and DOC parameters. The results of TPH and, aliphatic and aromatic fractions of petroleum hydrocarbons for aquifer, contaminated soil and treated soil were given in Table 4.2. Tischer et al. (2013) indicated that the inoculum sampling from aquifer in Leuna, Germany consisted of sand and gravel with oxygen concentration in all cores ranged between 0.01 mg l^{-1} below the water table and almost 10 mg l^{-1} above the water table. According to Tischer and his colleagues, the aquifer which has 6-8 m depth where they measured MTBE concentrations are evenly distributed over dept and BTEX concentrations are peaked at the top.

The pH of aquifer was neutral, while contaminated soil slightly acidic condition (pH 5) was noted. Total phosphorus and nitrogen were present in relatively higher concentrations in aquifer than contaminated soil. C/N ratio was more in contaminated soil up to 15 times than aquifer. Sulphate concentration in aquifer was slightly same as contaminated soil.

Organic carbon is mostly derived from biota, microbial biomass and others. In general, crude oil contamination in the soil might contribute the TOC concentration due to petroleum hydrocarbon contents (Wang et al., 2013). According to many studies, crude oil or petroleum contaminated sites might have different TPH concentration in wide range such as from 10,000 ppm to 50,000 ppm. Wang and his colleagues reported that the higher TPH level refers to the higher TOC level, so TOC concentration might represent TPH concentation in the soil sample. TOC concentration was detected at considerably higher concentration in contaminated soil (312 g kg^{-1}), related to aquifer (6 g kg^{-1}). As mentioned before, the aquifer includes more low molecular weight hydrocarbons such as BTEX and MTBE than the contaminated soil which is polluted with high molecular weight petroleum hydrocarbons. Total petroleum hydrocarbons (TPHs) and the aliphatic and aromatic fractions of petroleum hydrocarbons were analyzed for the aquifer, contaminated soil and treated contaminated soil in the beginning of the study. As seen in Table 4.2, TPH concentration of both contaminated and treated contaminated soil (69 g kg^{-1}, 74 g kg^{-1}) is 70 times higher than the aquifer (1 g kg^{-1}).

Table 4.1. Physicochemical characterization of contaminated soil and aquifer.

Parameter	Unit	A	SO	TSO
pH	-	7	5	4
Total Phosphorus (P)	mg/kg	124	119	98
C/N ratio	-	42	614	638
Sulphate (SO_4^{-2})	mg/kg	800	790	11720
Total Kjeldahl Nitrogen (TKN)	mg/kg	340	280	320
Total Solids (TS)	%	80	96	93
Total Volatile Solids (TVS)	%	1	42	30
Total Organic Carbon (TOC)	g/kg	6	312	352
Dissolved Organic Carbon (DOC)	mg/kg	162	53	1422

Table 4.2. Results of TPH, aliphatic and aromatic fractions of petroleum hydrocarbons in contaminated soil and aquifer.

Parameter	Unit	A	SO	TSO
Total Petroleum Hydrocarbon (TPH)	g/kg	1	69	74
C5-C8 Aliphatic Fraction	mg/kg	67	<60	34
C8-C16 Aliphatic Fraction	mg/kg	286	16,100	10,600
C16-C35 Aliphatic Fraction	mg/kg	<30	83,200	49,800
C5-C9 Aromatic Fraction	mg/kg	9	<80	<16
C9-C16 Aromatic Fraction	mg/kg	40	4,290	999
C16-C35 Aromatic Fraction	mg/kg	<30	50100	10,800

The results of elemental analyses of contaminated soil (SO) were given in Table 4.3. Sulphur element was detected at considerably higher concentration (36,330 mg kg^{-1}) that could be utilized as electron acceptor. Iron and magnesium were present in relatively higher concentration (19,785 mg kg^{-1} and 16,830 mg kg^{-1}, respectively), while heavy metals like aluminum, manganase, zinc, copper, boron, nickel and vanadium were present at relatively lower concentrations.

Table 4.3. Elemental analyses of contaminated soil.

Parameter	Amount (mg/kg)
Magnesium (Mg)	16,830
Sulphur (S)	36,330
Iron (Fe)	19,785
Aluminum (Al)	28
Manganase (Mn)	3
Copper (Cu)	19
Zinc (Zn)	41
Boron (B)	18
Nickel (Ni)	51
Vanadium (V)	102

4.2. Results of Pre-treatment of the Contaminated Soil

Persulfate oxidation was used for enhancement of bioavailability of organic matter, especially petroleum hydrocarbons. Wu and his colleagues (2016) indicated that soil TPH was removed using a persulfate oxidation, mostly effective by the addition of Fe^{+2}. However, it was not completely removed in such cases due to complicated structure in the residual components of TPH and the existence of TPH components not oxidized by persulfate. As a result of chemical treatment of contaminated soil sample by persulfate oxidation, DOC and consumed persulfate amount was analyzed. Determination of iodine (I_2) formation 90% of persulfate was consumed during treatment process calculated by the calibration factor of 24.4 (MIT Open Course Ware, 2005). Zhao and his colleagues (2013) reported that the removal of PAHs enhanced by citrate-chelated ferrous ion activation. After persulfate oxidation, persulfate was converted into sulphate ion by oxidation reaction given as following sequential equations (Zhao et al., 2013):

$$S_2O_8^{-2} + Fe^{+2} \rightarrow SO_4^{-2} + SO_4^{\cdot -} + Fe^{+3} \ (Eq.\ 4.1)$$
$$2H_2O + S_2O_8^{-2} \rightarrow 2HSO_4^{-} + H_2O_2 \ (Eq.\ 4.2)$$
$$H_2O_2 + Fe^{+2} \rightarrow Fe^{+3} + OH^{\cdot} + OH^{-} \ (Eq.\ 4.3)$$
$$SO_4^{\cdot -} + Fe^{+2} \rightarrow SO_4^{-2} + Fe^{+3} \ (Eq.\ 4.4)$$

Thus, sulphate was present at relatively higher concentration (11,720 mg kg^{-1}) in chemically treated soil than the presence of sulphate in contaminated soil (790 mg kg^{-1}). As seen in Table 4.1,

dissolved carbon of pre-treated contaminated soil was detected greater than dissolved carbon of contaminated soil as 53 and 1,422 mg/kg, respectively. After chemical treatment as oxidation method, the amount of dissolved organic carbon increased (Sharp et al., 1993).

After persulfate oxidation, the removal efficiency is observed via TPH analysis. There is no significant difference in total petroleum hydrocarbon concentration for both contaminated and treated contaminated soil. Based on a study about persulfate oxidation of petroleum contaminated soil, it reported that persulfate reduced the C_{10}-C_{40} hydrocarbon concentrations in soil ecosystem effectively (Satapanajaru et al., 2017). Thus, according to the aliphatic and aromatic fractions of petroleum hydrocarbons results, the concentration of aliphatic and aromatic fractions decreased in all hydrocarbon chains after pre-treatment method (Table 4.2).

4.3. Results of Performance of Anaerobic Microcosm Test

The analyses of anaerobic microcosm cultivation were monitored as cumulative gas production, CO_2 and CH_4 production, SO_4^{-2} removal, and TOC removal efficiency during test period. Cumulative gas production of methanogenic and sulphate-reducing environment under 4 different temperature were given in Figure 4.1 and 4.2, respectively. For all results, “M” refers to methanogenic conditions and “S” refers to sulphate-reducing conditions. As mentioned before, A, SO, and TSO meant the aquifer, contaminated soil and pre-treated contaminated soil sample, respectively. The numbers, which were written following environment conditions, were given the temperature conditions.

For both conditions, cumulative gas production of thermophilic conditions (55°C) was higher than the other temperature conditions. In methanogenic environment, the highest gas production was observed under aquifer and treated soil (M55-A+TSO) incubation with thermophilic conditions. In addition, except thermophilic conditions the other microcosms did not produce more than 40 mL gas over 214 days.

Under sulphate-reducing environment, the highest gas production was observed belongs to the incubation of both aquifer and soil (S55-A+SO) under thermophilic conditions. The incubation of both aquifer and treated soil (S55-A+TSO) continued as close to the highest gas produced microcosm over 205 days.

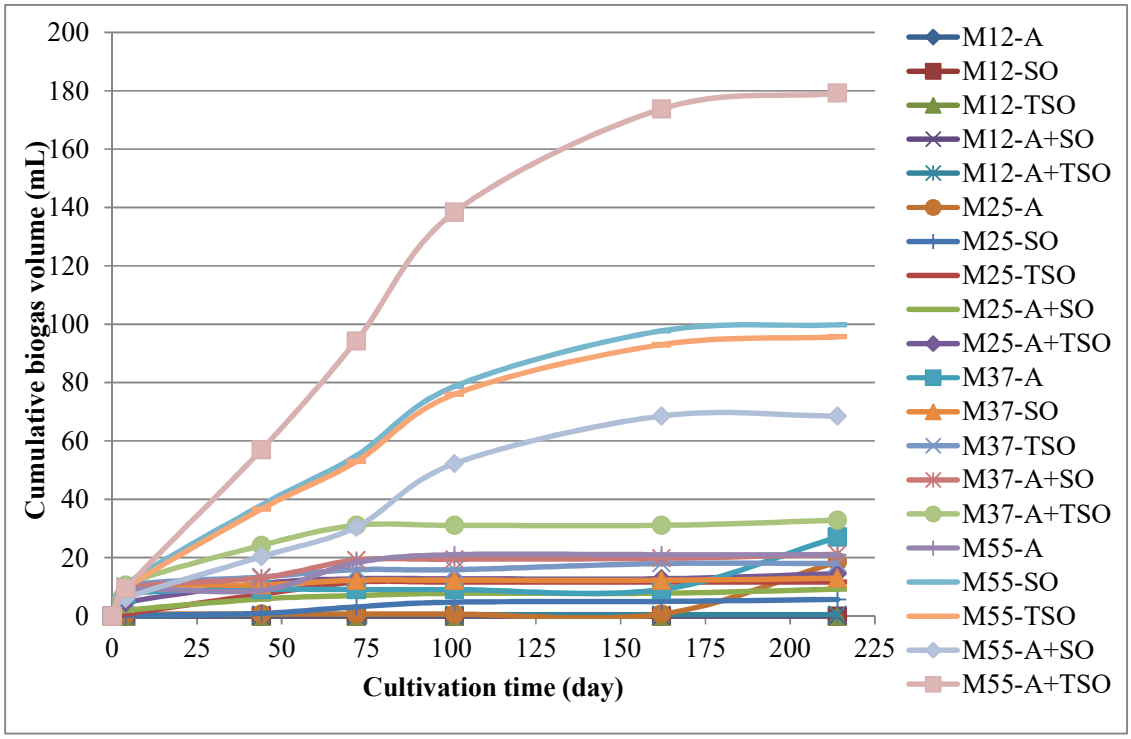

Figure 4.1. Cumulative gas production under methanogenic environment.

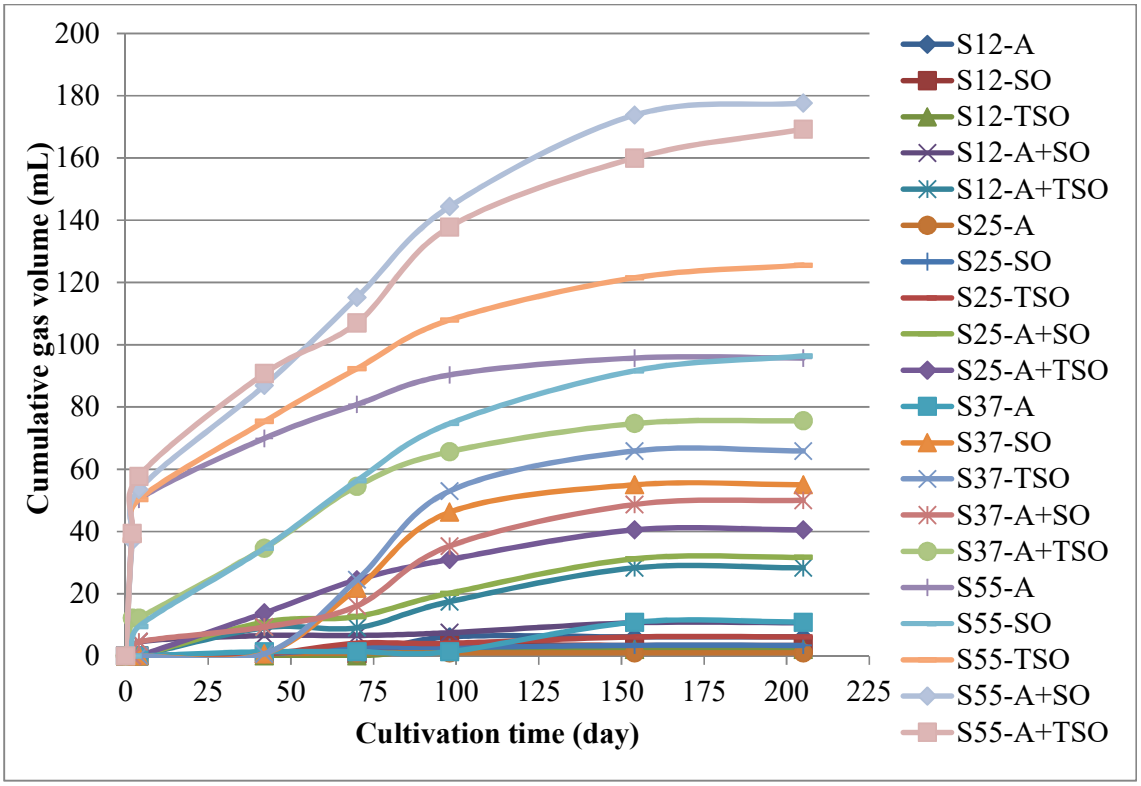

Figure 4.2. Cumulative gas production under sulphate-reducing environment.

4.3.1. CO_2 Production

Methanogenesis is performed by methanogenic archaebacteria under the presence of the enzyme methyl coenzyme M methylreductase (mcrA) resulting in CH_4 and CO_2 production. Before microbial methanogenesis, organic matter degradation process is carried out. Methanogenesis pathway is a strictly anaerobic process including acetrophic and hydrogenotrophic methanogenesis. Acetate (CH_3COO) iss converted into CH_4 and CO_2. Then, H_2 reacts with CO_2 through CH_4 and H_2O during hydrogentrophic process (Bastviken, 2009). According to many studies, degradation of organic matter may yield more energy in the presence of different electron acceptors as nitrate, sulphate, iron than methanogenesis. Anaerobic biodegradation of organic matter under sulphate-reducing conditions results in bicarbonate (HCO_3^-) and hydrogen sulfide (H_2S) formation in the net reaction. During intermediate steps of sulphate reduction pathway, CO_2 formation may occur depending on CO_2 evolution resulting from temperature and alkalinty (Jaekel et al., 2015; Machel, 1989).

Over incubation period, CO_2 formation was determined for both environment conditions. The results of CO_2 amount of all microcosms calculated by using produced gas volume from methanogenic and sulphate-reducing environment was given in Figure 4.3 and 4.4, respectively.

As seen in Figure 4.3, the highest CO_2 formation occurred in the microcosm including the aquifer and pre-treated contaminated soil (A+TSO) under mesophilic conditions in methanogenic environment. Over time period, CO_2 production increased rapidly for all different microcosms except aquifer incubation under psychrophilic and thermophilic conditions. In contrast to methanogenic environment, as temperature increased the CO_2 formation increased under sulphate-reducing environment. The highest CO_2 production occurred during incubation of aquifer and pre-treated contaminated soil (A+TSO) at thermophilic conditions under sulphate-reducing environment as approximately 1,600 μmol as seen in Figure 4.4.

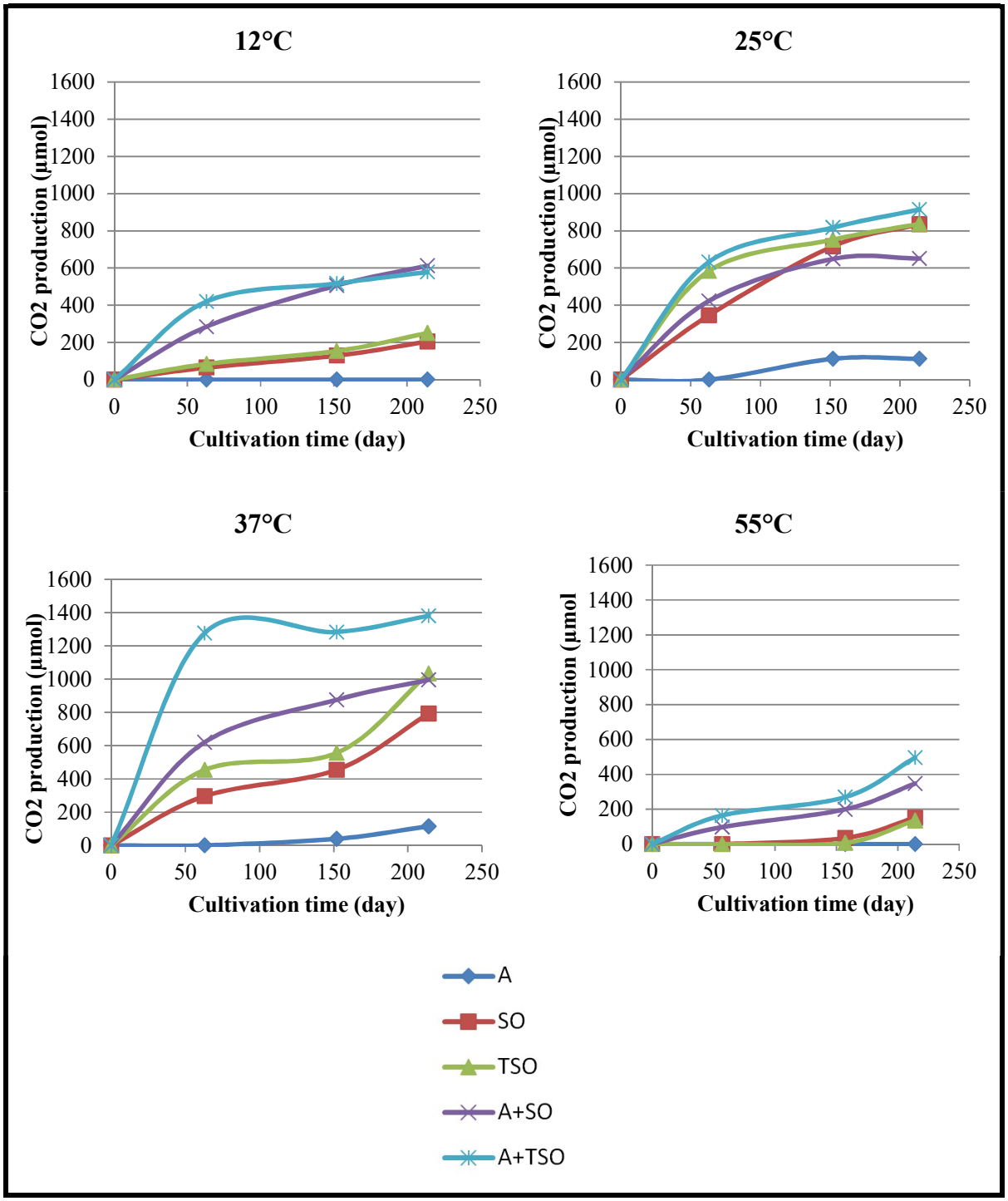

Figure 4.3. Carbon dioxide production under methanogenic environment.

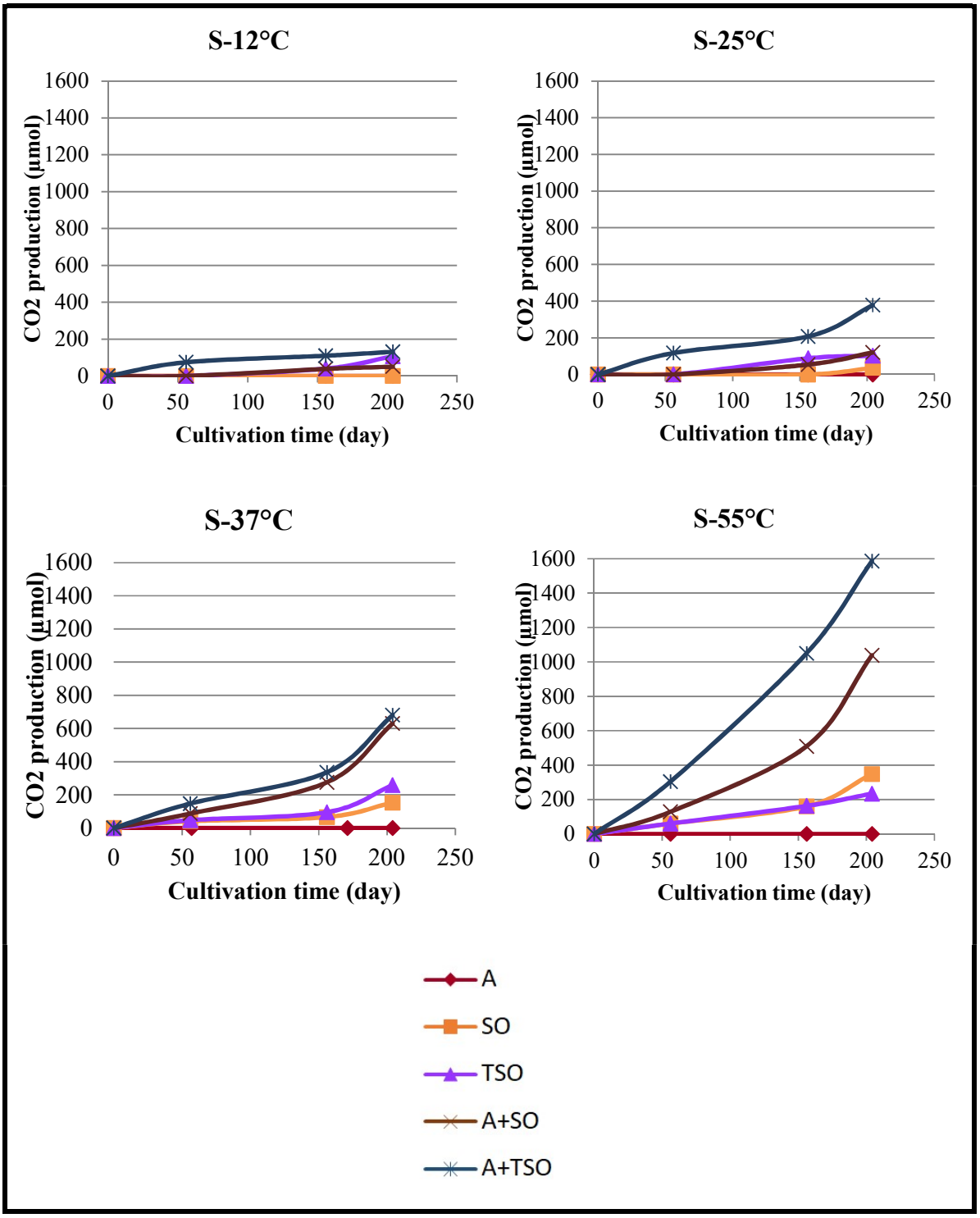

Figure 4.4. Carbon dioxide production under sulphate-reducing environment.

4.3.2. CH_4 production

During methanogenesis and sulphate-reducing environment, end production of net reaction may be methane gas (CH_4). Many hydrocarbon degradation pathways include methanogenesis and sulphate reduction processes, respectively. Thus, under net reaction equation of both reducing environment CH_4 formation was occurred as seen Table 2.1 (Jørgensen and Parkes, 2010). However, over time period only the incubation with aquifer at room temperature under methanogenic environment produced methane gas (CH_4) after 50 days as seen in Figure 4.5.

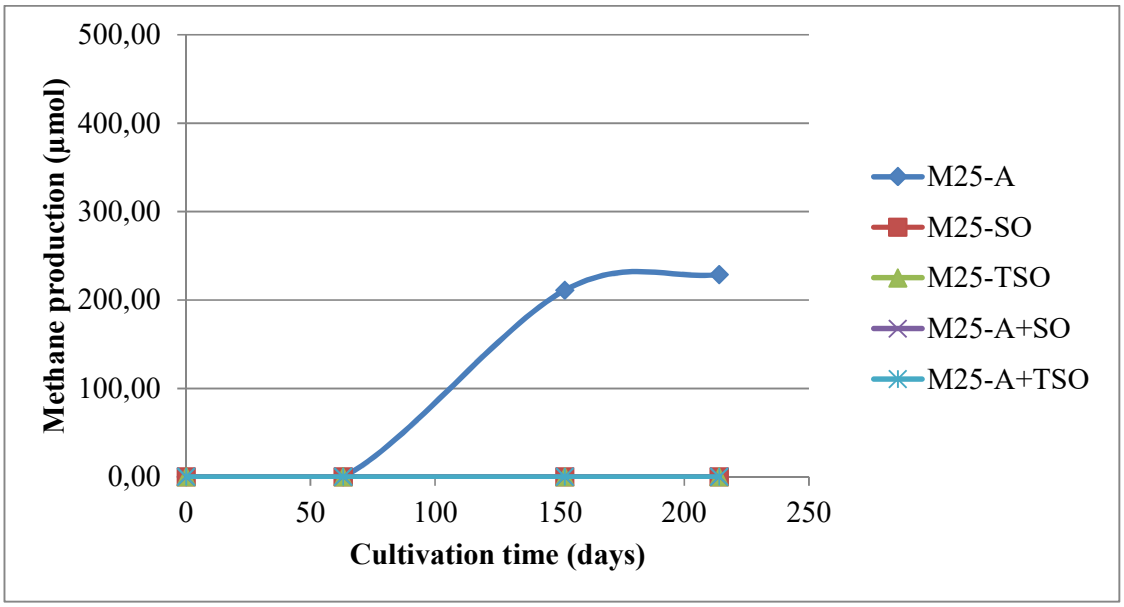

Figure 4.5. Methane production under methanogenic environment at mesophilic condition.

4.3.3. Sulphate Removal

Anaerobic biodegradation under sulphate-reducing environment is carried out by utilizing sulphate (SO_4^{-2}) as electron acceptor during bacterial sulphate reduction as mentioned before. According the net reaction of sulphate reduction pathway, degradation of organic matter results in SO_4^{-2} removal by reduction through the other states in the system. Besides the utilization of sulphate ion, sulphur and its variances may utilize for degradation of organic matter as seen based on Reaction 4 in Table 2.1 (Machel, 1989).

The determination of sulphate (SO_4^{-2}) was applied through only the cultivations under sulphate-reducing environment in Figure 4.6. Removal of sulphate during the incubation with aquifer (A) as control is the highest ratio in each condition as temperature range. Sulphate was

removed from the system approximately 25-50% under the biodegradation test including aquifer and contaminated soil (A+SO), and aquifer and pre-treated contaminated soil (A+TSO). The highest removal of sulphate among them as A+SO occurred at room temperature incubation. In addition, the highest removal of sulphate occurred at psychrophilic conditions between both aquifer and pre-treated contaminated soil (A+TSO).

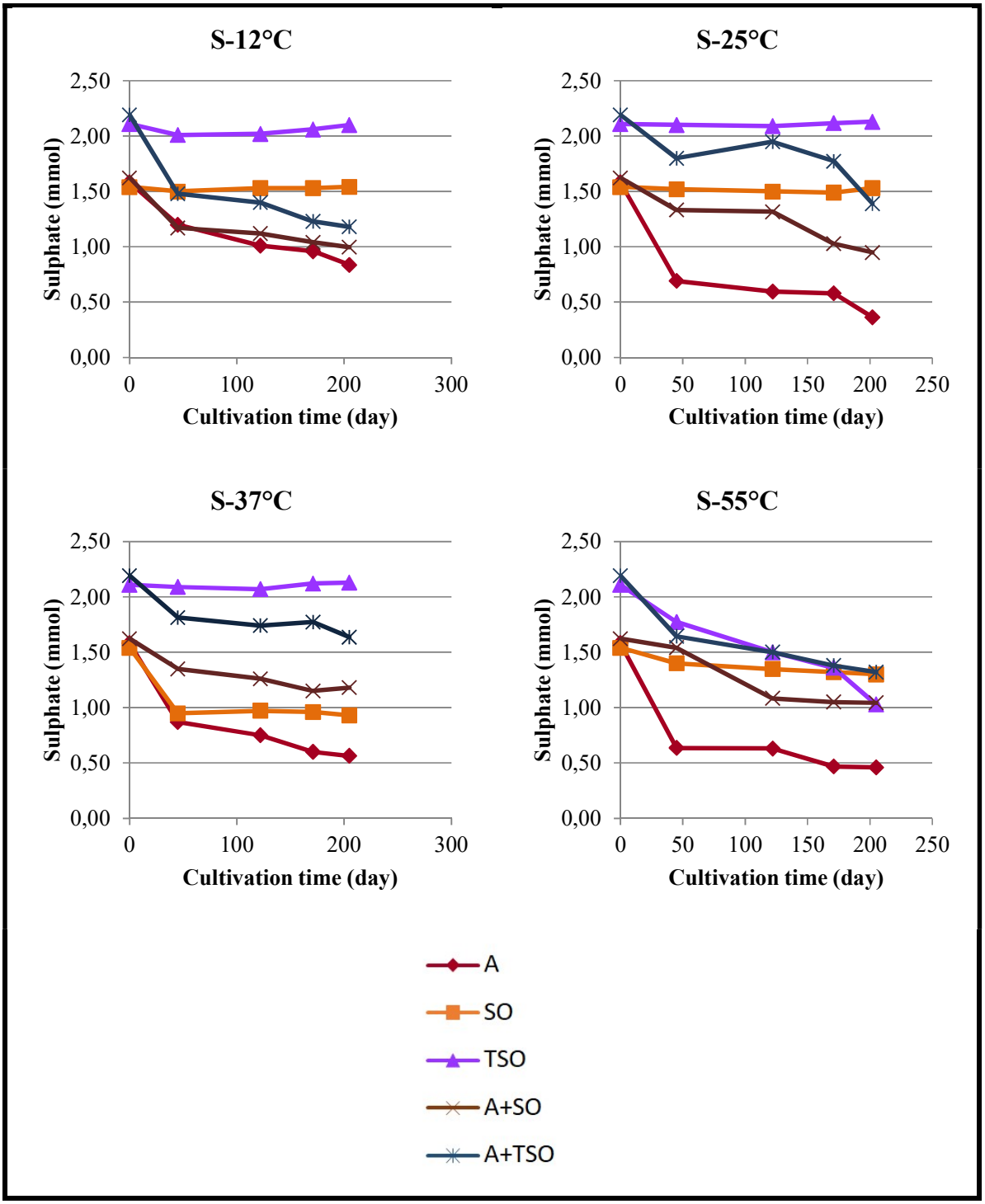

Figure 4.6. Sulphate removal under sulphate-reducing condition.

4.3.4. TOC Removal

TOC comprises all forms of organic carbon including petroleum hydrocarbons and natural organic matter. TOC is an useful quantitative method for determination of petroleum hydrocarbon presence because TOC includes all weight fractions of TPH (Wang et al.,2009). Besides TPH analysis, TOC concentration was monitored for all microcosms at last day of the incubation for determination of carbon removal efficiency via the difference between first and last samples.

Based on the results, organic carbon degradation under methanogenic condition with different temperature range is less than 3% for each microcosm. On the other hand, microcosm test under sulphate-reducing condition at different temperature ranges affected the efficiency of TOC degradation more than methanogenic environment. Especially, the incubation with aquifer and pre-treated contaminated soil (A+TSO) has the higher removal efficiency in psychrophilic, room temperature and mesophilic conditions. The highest efficiency as 70% is observed in sulphate-reducing environment under mesophilic conditions by running with aquifer and pre-treated contaminated soil (A+TSO). Incubation of aquifer (A) as control has effective TOC degradation between 20-30%. However, control microcosms including contaminated soil (SO) and pre-treated contaminated soil (TSO) had insufficient biodegradability of organic carbon, less than 1%.

The detailed results of TOC removal for methanogenic and sulphate-reducing environment is given in Table 4.4 and 4.5, respectively. In addition, percentage of removal efficiency over the incubation period for both reducing environment is given in Figure 4.7 and 4.8 as a graph.

Table 4.4. TOC removal under methanogenic environment.

Methanogenic Environment	TOC removal (mmol)	TOC removal (%)
M12-A	0.01	0.2
M12-SO	0.2	0.2
M12-TSO	0.3	0.2
M12-A+SO	0.6	0.4
M12-A+TSO	0.5	0.3
M25-A	0.1	2.1
M25-SO	0.8	0.6
M25-TSO	0.8	0.5
M25-A+SO	0.6	0.5
M25-A+TSO	0.9	0.6
M37-A	0.1	2.2
M37-SO	0.7	0.6
M37-TSO	1.0	0.7
M37-A+SO	1.0	0.7
M37-A+TSO	1.3	0.9
M55-A	0.01	0.3
M55-SO	0.1	0.1
M55-TSO	0.1	0.1
M55-A+SO	0.3	0.2
M55-A+TSO	0.5	0.3

Table 4.5. TOC removal under sulphate-reducing environment.

Sulphate-reducing Environment	TOC removal (mmol)	TOC removal (%)
S12-A	0.8	17
S12-SO	0.01	0.01
S12-TSO	0.1	0.1
S12-A+SO	65	48
S12-A+TSO	85	56
S25-A	1.4	28
S25-SO	0.04	0.03
S25-TSO	0.1	0.1
S25-A+SO	68	50
S25-A+TSO	79	52
S37-A	1.2	25
S37-SO	1.1	0.8
S37-TSO	0.3	0.2
S37-A+SO	58	43
S37-A+TSO	106	70
S55-A	1.1	22
S55-SO	0.4	0.3
S55-TSO	0.2	0.1
S55-A+SO	45	33
S55-A+TSO	24	16

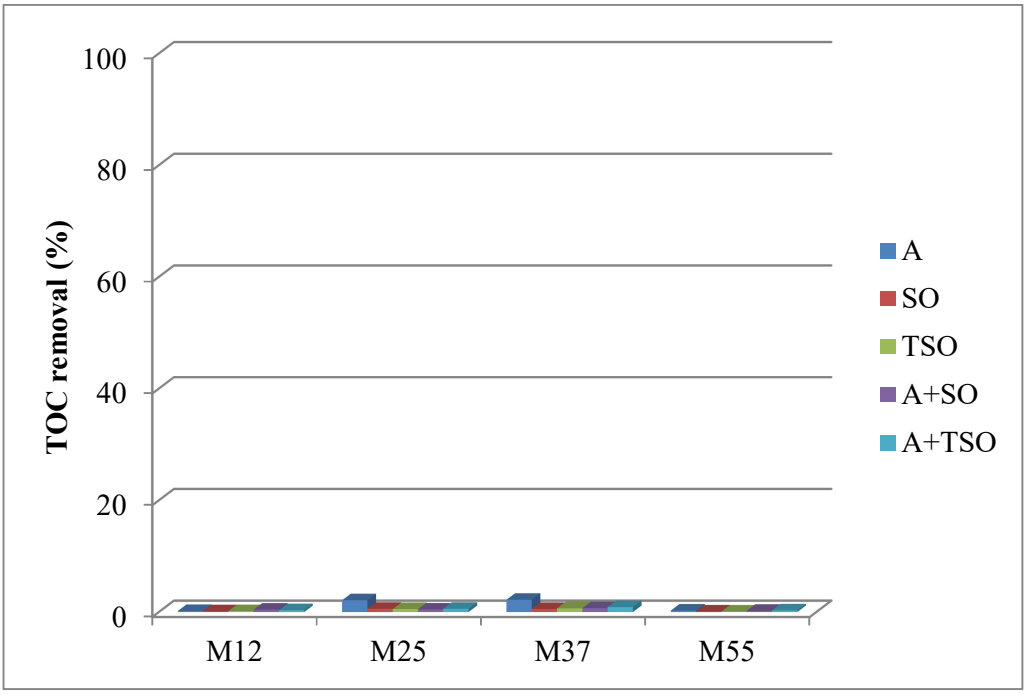

Figure 4.7. TOC removal efficiency under methanogenic conditions.

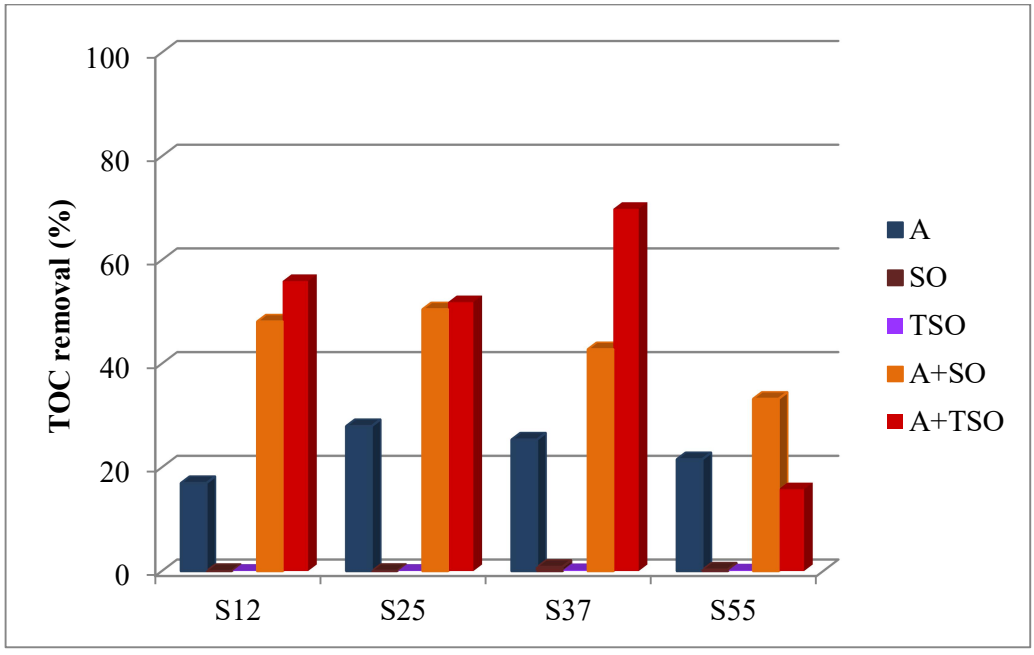

Figure 4.8. TOC removal efficiency under sulphate conditions.

4.4. Results of Microbial Community Analysis

16S rRNA based metagenomics analysis by Next Generation Sequencing was applied to contaminated soil and aquifer for determination of microbial population before microcosms set up. High Resolution Melting (HRM) analysis for microbial community profile was done for microcosms at last day of cultivation due to the determination of similarities between cultures used by dendrogram.

16S rRNA based metagenomics analysis is applied to contaminated soil and aquifer for determination of microbial communities before microcosm tests. In these results, metagenomics results are given for both contaminated soil and aquifer depending on bacterial abundance and microbial activity.

Based on the previous studies, the majority of anaerobic hydrocarbon-degrading bacteria have been indicated as *Proteobacteria* and *Firmicutes*. Some pilot studies show that the evolution of the indigenous bacterial community altered over time which is dominated by *Proteobacteria* and *Actinobacteria* (Greer and Juck, 2017). Yang et al. (2016) indicated that most abundant taxa belonged to known hydrocarbon-degrading species as *Sphingomonas*, *Novosphingobium*, *Burkholderia*, *Arthrobacter*, *Rhodococcus* and *Nocardia.* Furthermore, many nodes in the network of specialists for contaminated soil belonged to the *Rhizobiales* have been reported as capable to degrade PAHs.

4.4.1. Results of Metagenomics Analysis of Contaminated Soil

The results of 16S rRNA gene-based Illumina MiSeq high-throughput sequencing through isolated DNA of contaminated soil indicated that *Firmicutes* (30%), *Actinobacteria* (27%) and *Proteobacteria* (20%) were the most dominant phyla, and the most abundant orders were *Actinomycetales* (30%), *Lactobacillales* (30%) and *Burkholderiales* (14%) which can be classified into living microorganism in petroleum impact habitats in Figure 4.9. Based on the previous studies, *Actinobacteria*, *Firmicutes* and *Proteobacteria* were highly abundant in natural contaminated soil. Also, it is revealed that members of the genara *Bacillus* and *Burkholderia* including as order taxa *Lactobacillales* and *Burkholderiales* were present in the contaminated soil as petroleum utilizing microorganisms. Militon et al. (2010) reported that *Actinomycetales* under Actinobacteria phylum is observed to be able to play a role in a bioremediation of alkane-contaminated soils.

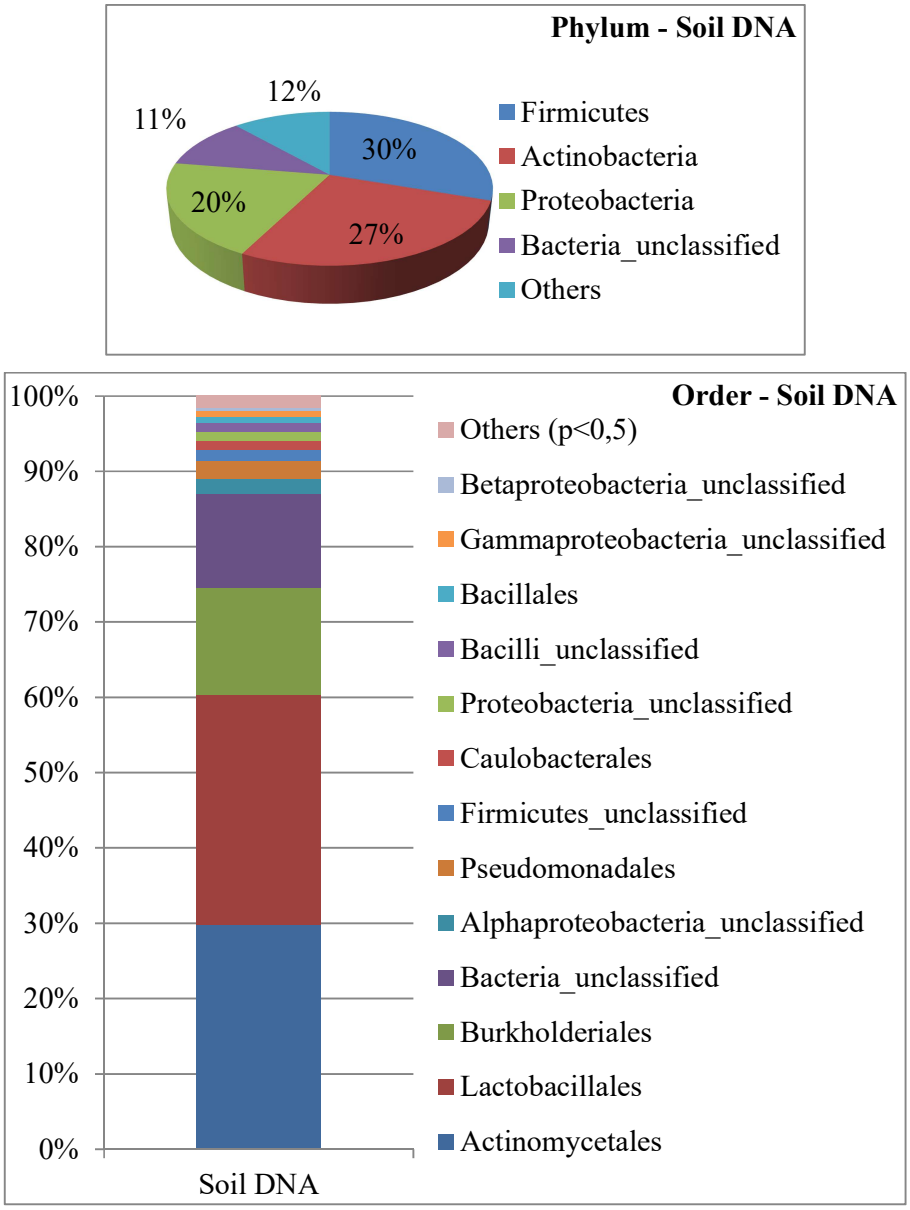

Figure 4.9. The phylogenetic results of DNA from contaminated soil.

Depending on Next Genome Sequencing method, from isolated total nucleic acid which refers to activation of microbial population the results show that the most abundant phyla were *Proteobacteria* (58%) and *Actinobacteria* (39%), and the most abundant orders were *Actinomycetales* (39%), *Rhodobacterales* (25%), *Rhizobiales* (17%) and *Burkholderiales* (10%) in Figure 4.10. Based on RNA-based microbial community analysis showed that the most abundant orders as *Actinomycetales*, *Rhodobacterales*, *Rhizobiales* and *Burkholderiales* has more microbial activity than the other taxa. It means that petroleum utilizing micoorganisms which is known as *Burkholderiales* and *Rhodobacterale*s were highly abundant as microbial activation in contaminated

soil. In addition, even they were present at low abundance *Pseudomonadales* and *Caulobacterales* are known as petroleum utilizing microorganisms.

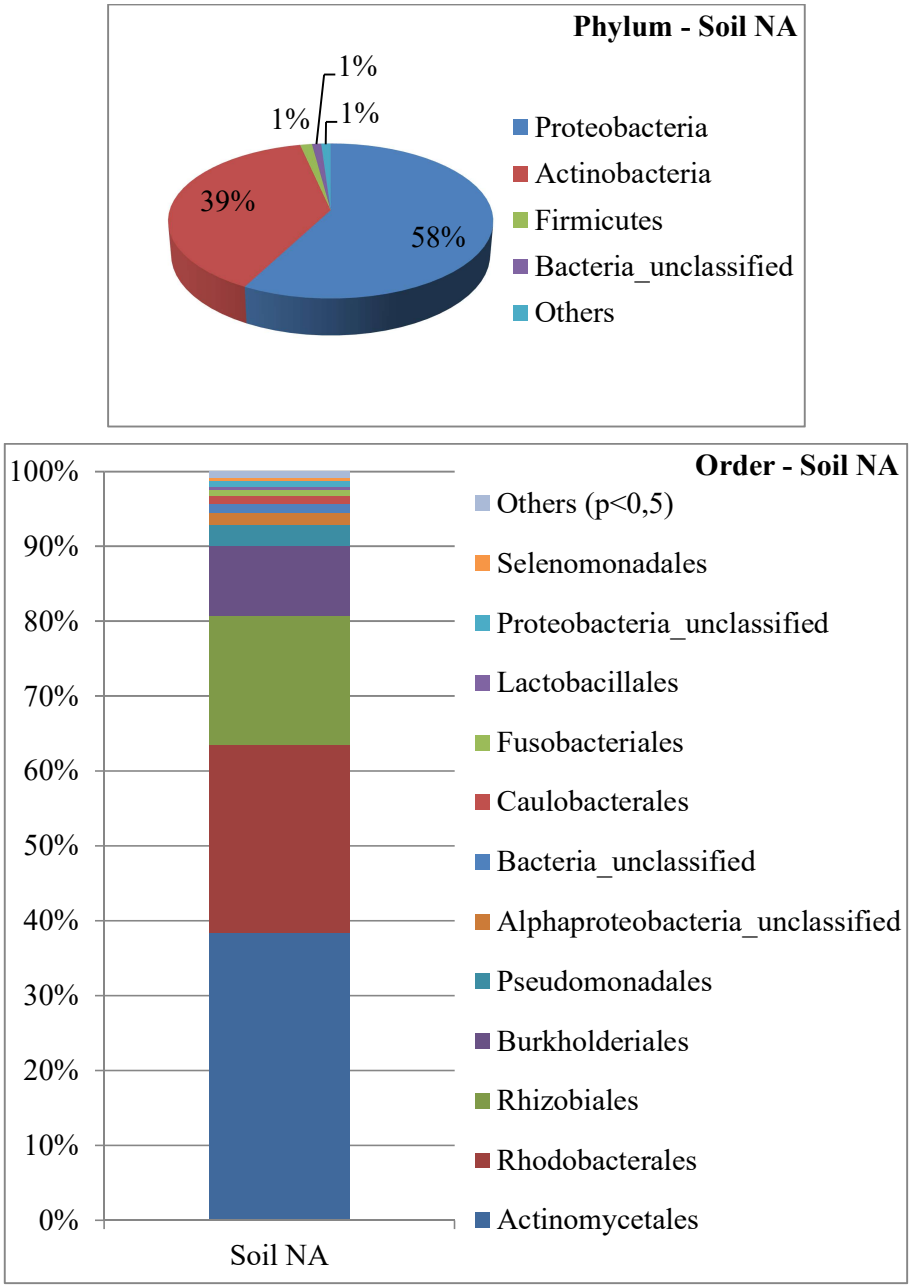

Figure 4.10. The phylogenetic results of NA from contaminated soil.

4.4.2. Results of Metagenomics Analysis of Aquifer

The results of 16S rRNA gene-based Next Genome Sequencing through isolated DNA of aquifer indicated that *Firmicutes* (30%), *Actinobacteria* (27%) and *Proteobacteria* (20%) were the most dominant phyla, and the most abundant orders were *Actinomycetales* (30%), *Lactobacillales*

(30%) and *Burkholderiales* (14%) which can be classified as living microorganism in petroleum impact habitats in Figure 4.11. The dominant taxa is as similar as microbial community profile of contaminated soil. Tischer et al. (2013) reported that the other important phylotypes which have a role in anaerobic biodegradation by terminal electron acceptors are *Dehalobacter*, *Desulfobulbaceae*, *Acidovorax*, *Bacilus*, *Clostridiales*, *Geobacter* and *Comamonadaceae*.

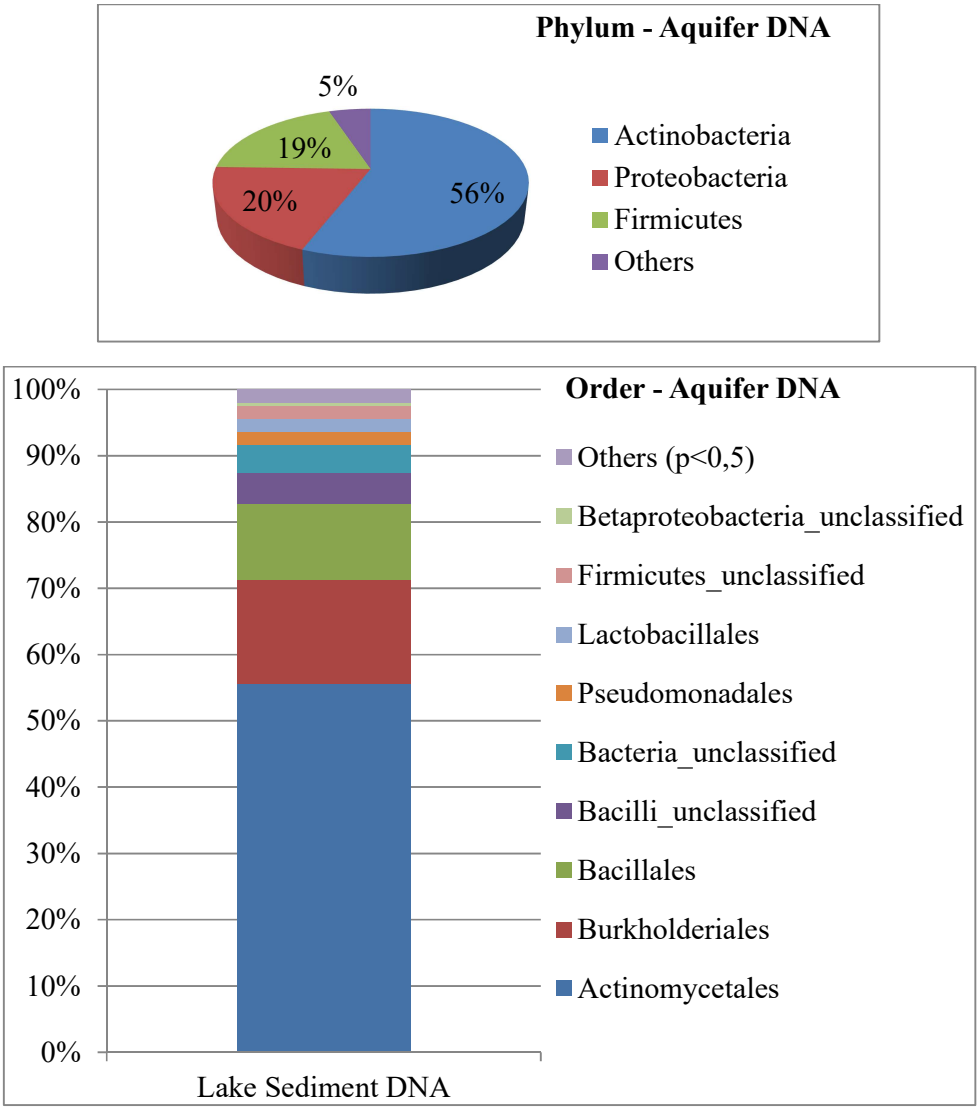

Figure 4.11. The phylogenetic results of DNA from aquifer.

Based on Next Genome Sequencing method, from isolated total nucleic acid which refers to activation of microbial population the results show that the most abundant phyla were *Proteobacteria* (51%) and *Actinobacteria* (28%), and the most abundant orders were *Actinomycetales* (28%), *Pseudomonadales* (11%), *Lactobacillales* (11%), *Rhodobacterales* (9%), *Rhizobiales* (9%) and *Burkholderiales* (6%) in Figure 4.12. The order of *Clostriadales* was present

into aquifer at lower abundance (2%) which microorganism plays an essential role in sulphate reduction pathway, especially in step including dissimilatory sulphite reductase gene (dsrB) (Gieg et al., 2014).

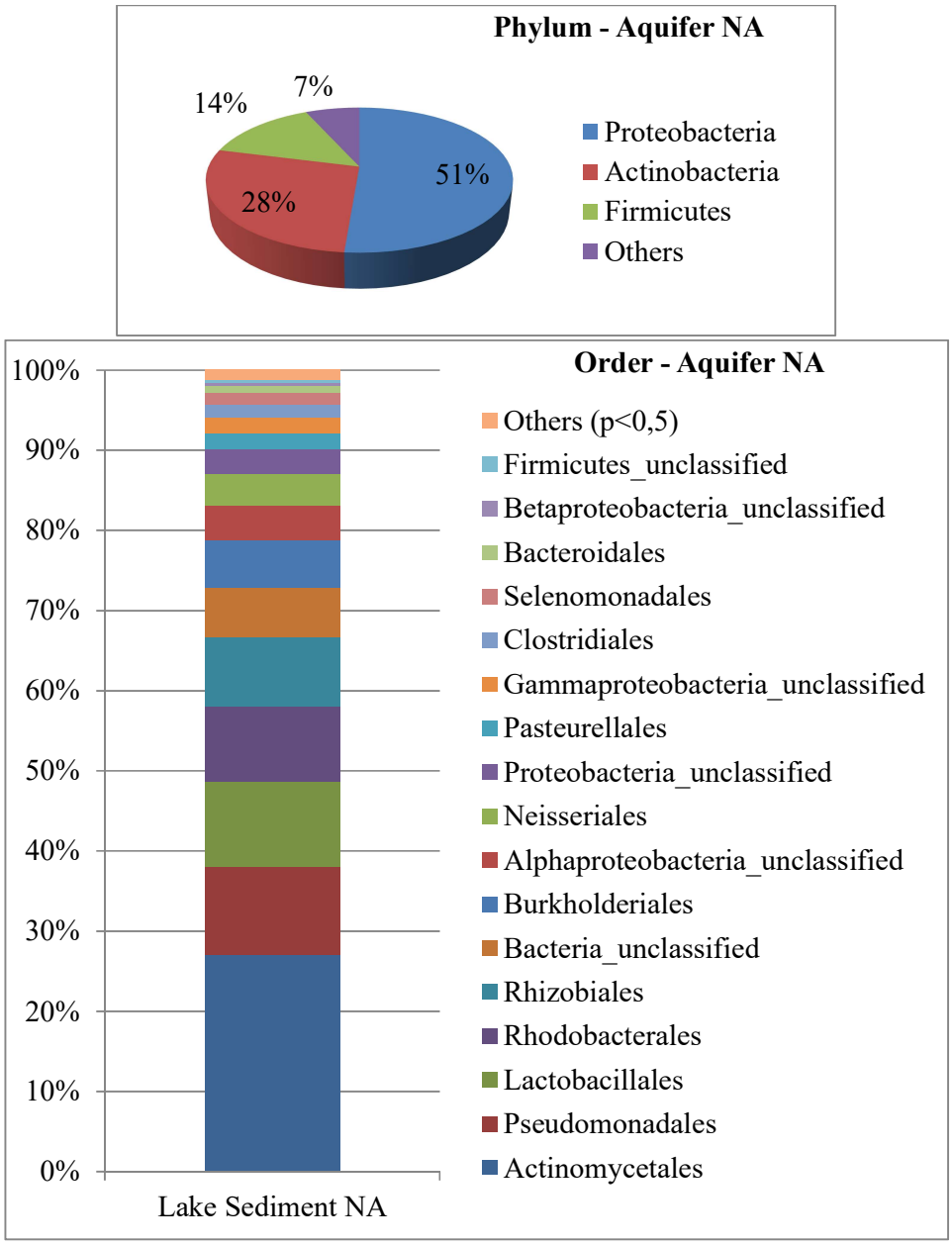

Figure 4.12. The phylogenetic results of NA from aquifer.

4.4.3. Results of High Resolution Melting (HRM) analysis

Microbial community profile dendrograms were obtained based on the similarities between the HRM profiles at last day of microcosm tests for all conditions. Based on HRM profiles principal component analysis (PCA) ordinations were calculated for correlation analysis due to the microbial community profile. Depending on the 16s rRNA based analysis for bacterial abundance and bacterial activity, microbial community profile dendrograms are given in Figure 4.13 and 4.14, respectively.

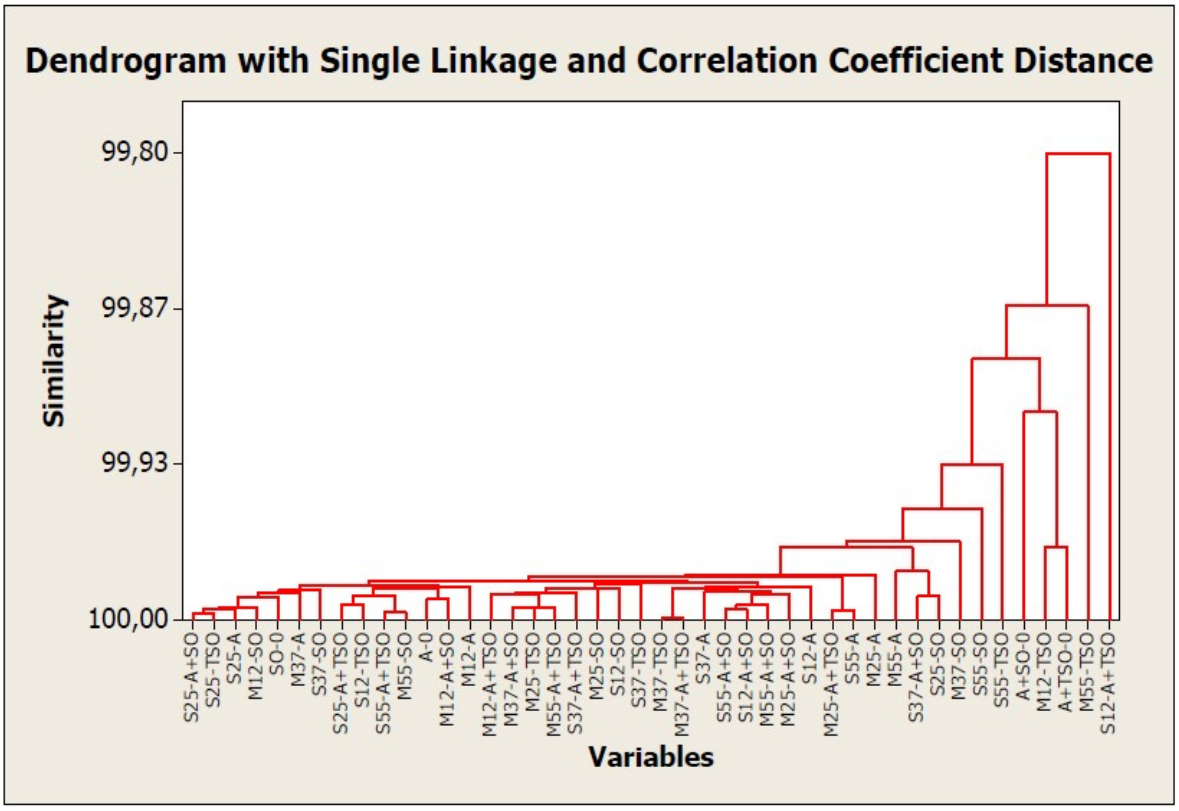

Figure 4.13. Microbial community profile dendrogram based on bacterial abundance.

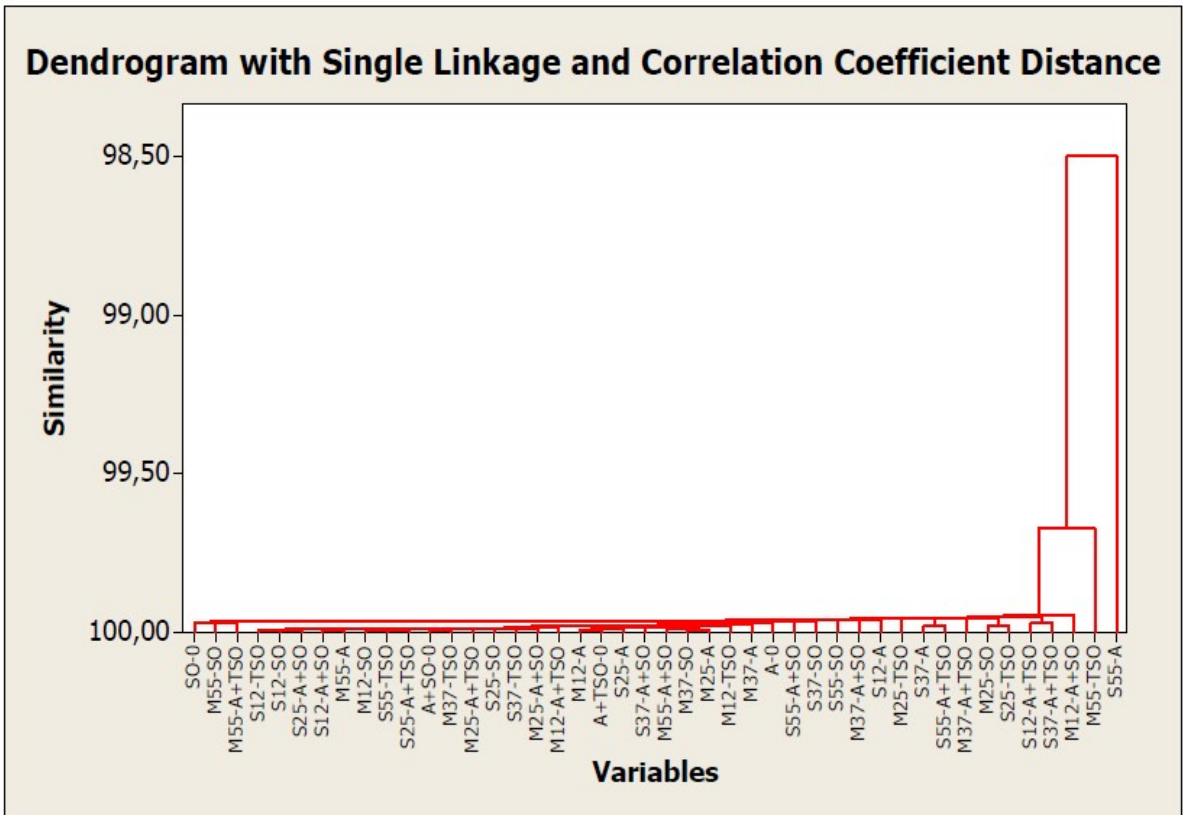

Figure 4.14. Microbial community profile dendrogram based on bacterial activity.

4.5. Results of Correlation between Microbial and Chemical Characteristics of Microcosm Tests

Correlation statistics are used for determination of relationship between two continuous variables. Pearson correlation coefficient (PCC) refers to the strength and direction of the linear relationship between two variables. The strength of linearity determines by PCC value from -1 to +1. The larger the absolute value of the coefficient makes stronger the relationship between the variables. For the Pearson correlation, an absolute value of 1 indicates a perfect linear relationship. A correlation close to 0 indicates no linear relationship between the variables.

The sign of the coefficient indicates the direction of the relationship. If both variables tend to increase or decrease together, the coefficient is positive, and the line that represents the correlation slopes upward. If one variable tends to increase as the other decreases, the coefficient is negative, and the line that represents the correlation slopes downward. The significance of correlation between variables, p-value is compared. Usually, the significance level in hypothesis is 0.05 works well. If the p-value is equal to or less than 0.05, the correlation is statistically significant. If it is greater than 0.05, the correlation is not statistically significant (MINITAB Inc., 2017).

In this study, microbial and chemical characterization results were correlated between them for statistical analyses. For correlation of the chemical characterization, CO_2 production as volume in µmol, CH_4 production as volume in µmol, SO_4^{-2} reduction as amount in mmol, TOC removal as amount in mmol and TOC removal as percentage were used. Relative bacteria quantity, relative bacterial activity, quantity of microbial activation per unit bacteria (Act/Bact), principal component analysis as community profile from high resolution melting analysis for both rDNAs and rRNAs were used for correlation to chemical characterization. Relative bacteria quantity and relative microbial activation quantity were determined by proportion to each other using ΔCq quantification method. Quantity of microbial activation per unit bacteria refers to ratio between relative microbial activation quantity and relative bacteria quantity. HRM results give the alteration of microbial community profile over the incubation period. The statistical analyses were calculated by the expression level of 16S rRNA genes, and expression level of specific target genes assA, bcrA, dsrB and mcrA seperately. In the correlation analyses, the grey highlighted results have PCC values that are close to absolute value of 1 and p-values that are equal or less than 0.05. Thus, results highlighted as grey refers to linearity and statistically significance test results.

Based on the amplification results of 16S rRNA genes using in the correlation analyses in Table 4.6 and 4.7, TOC removal is related to changing in bacterial community profile for M12, M55 and S25 conditions. CO_2 production and TOC removal under S37 condition are related to alteration in bacterial community profile. Specifically, the most related condition to microbial characterization is sulphate-reducing environment under thermophilic condition. In this condition, TOC removal is linearly related to relative bacteria quantity, quantity of microbial activation per unit bacteria and changing in bacterial community profile which is related to CO_2 production.

Table 4.6. Correlation analysis through the gene expression of 16S rRNA gene under methanogenic environment.

pA-pH	*Pearson correlation coefficient* *p value*	Relative Bacteria Quantity	Relative Bacterial Activity	Act/Bact	Active bacterial community profile	Bacterial community profile
M12	CO_2 production	-0.215 0.728	-0.153 0.806	-0.567 0.319	0.607 0.278	-0.8 0.104
	CH_4 production	* *	* *	* *	* *	* *
	TOC removal	-0.232 0.707	-0.159 0.798	-0.579 0.306	0.647 0.238	-0.797 0.106
	TOC removal (%)	-0.397 0.508	-0.189 0.761	-0.821 0.089	0.805 0.1	-0,961 0,009
M25	CO_2 production	0.434 0.466	0.304 0.62	-0.451 0.446	0.629 0.256	-0.084 0.894
	CH_4 production	-0.451 0.446	-0.265 0.666	0.771 0.127	-0.408 0.495	0.375 0.534
	TOC removal	0.393 0.513	0.242 0.695	-0.552 0.335	0.561 0.325	-0.19 0.759
	TOC removal (%)	-0.427 0.473	-0.234 0.705	0.818 0.091	-0.349 0.565	0.425 0.475
M37	CO_2 production	0.405 0.499	-0.272 0.658	0.043 0.946	0.616 0.268	-0.696 0.192
	CH_4 production	* *	* *	* *	* *	* *
	TOC removal	0.425 0.476	-0.294 0.631	0.02 0.975	0.65 0.235	-0.71 0.179
	TOC removal (%)	-0.192 0.758	-0.169 0.786	-0.199 0.748	-0.095 0.879	0.468 0.426
M55	CO_2 production	0.864 0.059	0.022 0.973	-0.724 0.167	-0.259 0.675	-0.898 0.038
	CH_4 production	* *	* *	* *	* *	* *
	TOC removal	0.863 0.059	0.009 0.989	-0.724 0.167	-0.258 0.675	-0,896 0,04
	TOC removal (%)	0.552 0.335	-0.152 0.807	-0.397 0.508	-0.648 0.237	-0.425 0.475

Table 4.7. Correlation analysis through the gene expression of 16S rRNA gene under sulphate-reducing environment.

pA-pH	*Pearson correlation coefficient*	Relative Bacteria Quantity	Relative Bacterial Activity	Act/Bact	Active bacterial community profile	Bacterial community profile
S12	*p value*	-0.463 0.432	-0.32 0.6	-0.314 0.607	* *	-0.106 0.865
	SO_4^{-2} reduction	-0.614 0.271	-0.662 0.223	0.015 0.98	* *	-0.359 0.553
	TOC removal	-0.296 0.629	-0.266 0.666	-0.597 0.287	* *	-0.676 0.21
	TOC removal (%)	-0.427 0.473	-0.432 0.467	-0.482 0.411	* *	-0.695 0.193
S25	CO_2 production	0.239 0.699	-0.469 0.426	0.284 0.643	-0.345 0.569	-0.741 0.152
	SO_4^{-2} reduction	-0.342 0.574	-0.877 0.051	-0.048 0.939	-0.566 0.32	-0.434 0.466
	TOC removal	0.241 0.696	-0.468 0.426	-0.127 0.839	-0.413 0.49	-0.973 0.005
	TOC removal (%)	0.045 0.943	-0.709 0.18	-0.188 0.762	-0.569 0.317	-0.922 0.026
S37	CO_2 production	0.873 0.053	-0.352 0.561	-0.498 0.393	-0.644 0.241	-0.95 0.013
	SO_4^{-2} reduction	-0.237 0.701	0.239 0.699	0.766 0.131	0.754 0.141	0.065 0.918
	TOC removal	0.692 0.195	-0.479 0.414	-0.095 0.879	-0.377 0.532	-0.934 0.02
	TOC removal (%)	0.613 0.271	-0.503 0.387	0.161 0.796	-0.046 0.941	-0.810 0.048
S55	CO_2 production	0.746 0.147	0.167 0.789	-0.502 0.388	-0.285 0.642	-0.892 0.042
	SO_4^{-2} reduction	0.474 0.42	-0.03 0.961	-0.602 0.282	0.751 0.144	-0.368 0.543
	TOC removal	1 0	0.593 0.292	-0.914 0.03	-0.088 0.888	-0.957 0.011
	TOC removal (%)	0.783 0.117	0.454 0.443	-0.926 0.024	0.535 0.352	-0.595 0.289

There is a statistical significant correlation between gene expression level of assA under S37 condition which means that assA gene was mainly carried and expressed by sulphate reducers which is the indicator of alkane degradation by sulphate reducers. Since, assA gene expression is highly related to sulphate reduction as seen in Table 4.8 and 4.9.

Table 4.8. Correlation analysis through the gene expression level of assA gene under methanogenic environment.

assA	*Pearson correlation coefficient* *p value*	Relative Bacteria Quantity	Relative Bacterial Activity	Act/Bact
M12	CO_2 production	-0.431 0.469	-0.494 0.398	-0.838 0.076
	CH_4 production	* *	* *	* *
	TOC removal	-0.386 0.521	-0.476 0.418	-0.849 0.069
	TOC removal (%)	-0.483 0.41	-0.729 0.163	-0.558 0.328
M25	CO_2 production	0.013 0.984	0.156 0.802	0.049 0.938
	CH_4 production	-0.13 0.834	-0.088 0.888	0.371 0.539
	TOC removal	0.006 0.992	0.142 0.82	-0.064 0.919
	TOC removal (%)	-0.142 0.819	-0.083 0.894	0.438 0.46
M37	CO_2 production	-0.457 0.439	-0.557 0.33	-0.6 0.285
	CH_4 production	* *	* *	* *
	TOC removal	-0.495 0.397	-0.568 0.318	-0.581 0.304
	TOC removal (%)	-0.102 0.871	0.192 0.757	0.727 0.164
M55	CO_2 production	-0.651 0.234	0.096 0.878	-0.239 0.699
	CH_4 production	* *	* *	* *
	TOC removal	-0.661 0.225	0.089 0.886	-0.244 0.692
	TOC removal (%)	-0.589 0.296	0.139 0.824	0.046 0.941

Table 4.9. Correlation analysis through the gene expression level of assA gene under sulphate-reducing environment.

assA	*Pearson correlation coefficient p value*	Relative Bacteria Quantity	Relative Bacterial Activity	Act/Bact
S12	CO_2 production	-0.492 0.4	-0.57 0.315	-0.242 0.695
	SO_4^{-2} reduction	-0.693 0.195	-0.634 0.251	0.195 0.753
	TOC removal	-0.446 0.452	-0.331 0.586	-0.332 0.585
	TOC removal (%)	-0.57 0.315	-0.435 0.465	-0.25 0.685
S25	CO_2 production	-0.14 0.823	-0.103 0.869	0.19 0.759
	SO_4^{-2} reduction	-0.564 0.322	-0.569 0.317	0.058 0.926
	TOC removal	-0.359 0.553	-0.422 0.48	-0.255 0.679
	TOC removal (%)	-0.514 0.376	-0.576 0.309	-0.235 0.703
S37	CO_2 production	-0.441 0.457	-0.315 0.606	-0.266 0.666
	SO_4^{-2} reduction	-0.608 0.277	-0.751 0.144	0.885 0.046
	TOC removal	-0.756 0.139	-0.564 0.322	0.157 0.801
	TOC removal (%)	-0.843 0.073	-0.69 0.197	0.365 0.546
S55	CO_2 production	-0.739 0.153	-0.144 0.817	0.058 0.926
	SO_4^{-2} reduction	-0.432 0.467	-0.345 0.569	-0.213 0.731
	TOC removal	-0.183 0.768	0.168 0.787	-0.425 0.475
	TOC removal (%)	-0.005 0.993	0.055 0.93	-0.527 0.361

For aromatic hydrocarbon degradation, the correlation test was applied by using the results of bcrA target gene amplification. As seen in Table 4.10 and 4.11, there is a statistical significance correlation between the gene expression level of bcrA and TOC removal under thermophilic methanogenic conditions. SO, bcrA gene expression which is indicator of aromatic hyrdrocarbon degrading pathway is highly related to sulphate reduction. For sulphate conditions, the gene expression level of bcrA is related to sulphate reduction and TOC removal.

Table 4.10. Correlation analysis through the gene expression level of bcrA gene under methanogenic environment.

bcrA	*Pearson correlation coefficient* *p value*	Relative Bacteria Quantity	Relative Bacterial Activity	Act/Bact
M12	CO_2 production	0.064 0.919	-0.091 0.885	0.336 0.581
	CH_4 production	* *	* *	* *
	TOC removal	0.037 0.953	-0.064 0.918	0.325 0.594
	TOC removal (%)	0.191 0.759	-0.242 0.695	0.682 0.204
M25	CO_2 production	-0.476 0.417	0.246 0.689	-0.249 0.687
	CH_4 production	0.408 0.495	-0.209 0.736	0.211 0.734
	TOC removal	-0.429 0.472	0.259 0.674	-0.261 0.672
	TOC removal (%)	0.38 0.528	-0.212 0.732	0.214 0.73
M37	CO_2 production	-0.091 0.884	0.075 0.905	0.003 0.997
	CH_4 production	* *	* *	* *
	TOC removal	-0.047 0.941	0.106 0.865	0.035 0.955
	TOC removal (%)	0.524 0.365	0.375 0.534	0.442 0.456
M55	CO_2 production	0.386 0.521	0.215 0.729	0.21 0.734
	CH_4 production	* *	* *	* *
	TOC removal	0.395 0.51	0.227 0.714	0.222 0.719
	TOC removal (%)	0.96 0.01	0.53 0.358	0.526 0.363

Table 4.11. Correlation analysis through the gene expression level of bcrA gene under sulphate-reducing environment.

bcrA	*Pearson correlation coefficient p value*	Relative Bacteria Quantity	Relative Bacterial Activity	Act/Bact
S12	CO_2 production	-0.057 0.927	-0.437 0.461	-0.524 0.365
	SO_4^{-2} reduction	0.908 0.033	0.399 0.505	0.34 0.575
	TOC removal	0.672 0.214	-0.322 0.598	-0.387 0.52
	TOC removal (%)	0.836 0.078	-0.076 0.904	-0.144 0.817
S25	CO_2 production	-0.018 0.976	0.341 0.574	0.345 0.57
	SO_4^{-2} reduction	-0.414 0.488	0.549 0.337	0.564 0.322
	TOC removal	-0.301 0.622	0.401 0.504	0.411 0.492
	TOC removal (%)	-0.415 0.487	0.552 0.335	0.566 0.32
S37	CO_2 production	0.217 0.68	0.216 0.681	0.207 0.694
	SO_4^{-2} reduction	0.565 0.242	0.032 0.951	0.017 0.974
	TOC removal	0.473 0.343	0.294 0.572	0.279 0.593
	TOC removal (%)	0.798 0.057	0.497 0.316	0.478 0.338
S55	CO_2 production	0.292 0.575	0.353 0.492	0.389 0.445
	SO_4^{-2} reduction	0.944 0.005	0.404 0.427	0.133 0.802
	TOC removal	0.486 0.329	0.787 0.063	0.876 0.022
	TOC removal (%)	0.906 0.013	0.748 0.087	0.63 0.18

The abundance of bacterial diversity having dissimilatory sulfate reductase gene is correlated to chemical characterization by using dsrB gene expression level in Table 4.12 and 4.13. Under M25 condition, the quantity of microbial activation per unit bacteria is related to all chemical characterization. As seen before, carbon dioxide production is more than methane gas production under methanogenic conditions. Beside methanogenesis depending on the constituents of the environment such as sulphur or sulphite, sulphate reduction pathway may take place, following with carbon dioxide gas production (Bastviken, 2009). Thus, the gene expression level of dsrB gene is highly related to the quantity of microbial activation per unit bacteria. For sulphate-reducing

environment, TOC removal and CO_2 production are related to relative bacterial acitivity for mesophilic and thermophilic conditions which means that the gene expression level of dsrB is highly related to degradation of hydrocarbons.

Table 4.12. Correlation analysis through the gene expression level of dsrB gene under methanogenic environment.

dsrB	*Pearson correlation coefficient* *p value*	Relative Bacteria Quantity	Relative Bacterial Activity	Act/Bact
M12	CO_2 production	-0.647 0.238	-0.699 0.189	-0.023 0.971
	CH_4 production	* *	* *	* *
	TOC removal	-0.61 0.274	-0.739 0.154	0.013 0.984
	TOC removal (%)	-0.318 0.602	-0.648 0.237	0.421 0.48
M25	CO_2 production	0.31 0.612	-0.218 0.725	-0.903 0.036
	CH_4 production	-0.271 0.66	0.319 0.601	1 0
	TOC removal	0.249 0.687	-0.253 0.682	-0.943 0.016
	TOC removal (%)	-0.239 0.699	0.325 0.594	0.998 0
M37	CO_2 production	-0.738 0.155	-0.305 0.618	0.276 0.653
	CH_4 production	* *	* *	* *
	TOC removal	-0.762 0.134	-0.335 0.582	0.243 0.694
	TOC removal (%)	0.253 0.681	-0.148 0.813	-0.465 0.43
M55	CO_2 production	0.788 0.114	0.677 0.209	-0.873 0.054
	CH_4 production	* *	* *	* *
	TOC removal	0.796 0.107	0.686 0.201	-0.865 0.059
	TOC removal (%)	0.62 0.265	0.402 0.502	-0.023 0.97

Table 4.13. Correlation analysis through the gene expression level of dsrB gene under sulphate-reducing environment.

dsrB	*Pearson correlation coefficient p value*	Relative Bacteria Quantity	Relative Bacterial Activity	Act/Bact
S12	CO_2 production	-0.513 0.377	-0.271 0.659	-0.215 0.729
	SO_4^{-2} reduction	-0.644 0.241	-0.784 0.116	0.083 0.895
	TOC removal	-0.433 0.466	-0.715 0.175	-0.356 0.556
	TOC removal (%)	-0.549 0.338	-0.839 0.076	-0.315 0.606
S25	CO_2 production	-0.127 0.838	-0.171 0.783	0.26 0.672
	SO_4^{-2} reduction	-0.566 0.32	-0.562 0.324	0.567 0.319
	TOC removal	-0.437 0.462	-0.53 0.358	-0.014 0.982
	TOC removal (%)	-0.585 0.301	-0.658 0.227	0.186 0.764
S37	CO_2 production	-0.385 0.522	-0.721 0.17	-0.063 0.92
	SO_4^{-2} reduction	-0.671 0.215	-0.186 0.765	0.137 0.825
	TOC removal	-0.603 0.282	-0.793 0.11	-0.164 0.792
	TOC removal (%)	-0.684 0.203	-0.901 0.037	-0.29 0.637
S55	CO_2 production	-0.804 0.101	-0.756 0.14	-0.663 0.223
	SO_4^{-2} reduction	-0.214 0.73	-0.565 0.321	-0.291 0.635
	TOC removal	-0.235 0.703	0.886 0.046	-0.789 0.113
	TOC removal (%)	0.116 0.852	-0.743 0.15	-0.547 0.34

As mentioned before, CH_4 production occurred under M25 condition. Depending on statistical analyses including the gene expression level of Methyl coenzyme M reductase (McrA) genes, all chemical characterization is related to relative bacteria quantity as given in Table 4.14 and 4.15. Following with sulphate-reducing environment, there is a statistical significance correlation between TOC removal and relative bacteria quantity under S12 and S55 conditions. Under sulphate-reducing environment, methanogenesis may carry out by methanogens resulting in degradation of hydrocarbons to altered hydrocarbons through production of methane and carbon

dioxide (Machel, 1989). Thus, the gene expression level of Mcr is highly related to TOC removal under sulphate condition.

Table 4.14. Correlation analysis through the gene expression level of mcrA gene under methanogenic conditions.

mcrA	*Pearson correlation coefficient* *p value*	Relative Bacteria Quantity	Relative Bacterial Activity	Act/Bact
M12	CO_2 production	-0.79 0.112	0.461 0.435	0.642 0.243
	CH_4 production	* *	* *	* *
	TOC removal	-0.813 0.094	0.491 0.401	0.68 0.206
	TOC removal (%)	-0.945 0.015	0.713 0.176	0.881 0.048
M25	CO_2 production	-0.905 0.035	0.158 0.8	0.822 0.087
	CH_4 production	1 0	-0.063 0.919	-0.844 0.072
	TOC removal	-0.945 0.015	0.078 0.9	0.817 0.091
	TOC removal (%)	0.997 0	-0.025 0.968	-0.826 0.085
M37	CO_2 production	-0.089 0.886	-0.062 0.921	0.086 0.89
	CH_4 production	* *	* *	* *
	TOC removal	-0.087 0.889	-0.039 0.951	0.118 0.851
	TOC removal (%)	0.207 0.739	0.467 0.428	0.343 0.572
M55	CO_2 production	-0.755 0.14	-0.117 0.851	0.337 0.579
	CH_4 production	* *	* *	* *
	TOC removal	-0.754 0.141	-0.102 0.87	0.35 0.564
	TOC removal (%)	-0.596 0.288	0.277 0.652	0.66 0.226

Table 4.15. Correlation analysis through the gene expression level of mcrA gene under sulphate-reducing environment.

mcrA	*Pearson correlation coefficient* *p value*	Relative Bacteria Quantity	Relative Bacterial Activity	Act/Bact
S12	CO_2 production	-0.213 0.731	-0.058 0.927	0.659 0.226
	SO_4^{-2} reduction	-0.73 0.161	-0.628 0.257	0.676 0.211
	TOC removal	-0.848 0.069	-0.658 0.227	0.508 0.382
	TOC removal (%)	-0.918 0.028	-0.772 0.126	0.587 0.298
S25	CO_2 production	-0.281 0.646	-0.067 0.915	0.219 0.723
	SO_4^{-2} reduction	-0.413 0.489	-0.52 0.369	-0.17 0.785
	TOC removal	-0.755 0.14	-0.404 0.5	0.011 0.986
	TOC removal (%)	-0.778 0.121	-0.545 0.342	-0.073 0.907
S37	CO_2 production	-0.787 0.114	-0.131 0.834	0.31 0.612
	SO_4^{-2} reduction	0.154 0.805	-0.253 0.681	-0.507 0.384
	TOC removal	-0.624 0.26	-0.008 0.989	0.246 0.69
	TOC removal (%)	-0.614 0.27	0.027 0.966	0.253 0.682
S55	CO_2 production	-0.465 0.431	-0.164 0.793	0.334 0.583
	SO_4^{-2} reduction	-0.293 0.632	0.176 0.777	0.608 0.276
	TOC removal	-0.922 0.026	-0.56 0.326	0.516 0.374
	TOC removal (%)	-0.763 0.133	-0.335 0.581	0.625 0.259

5. CONCLUSIONS AND RECOMMENDATIONS

The main findings of our study are summarized as follows:

The highest TOC removal of up to 70% was achieved in microcosms that were setup under sulphate-reducing conditions, operated at mesophilic temperature, seeded with microbial community from petroleum hydrocarbon rich habitat and fed with chemically treated soil.

Although the best results were obtained at mesophilic temperature range, the TOC removal efficiencies at the psychrophilic temperature were comparable.

TOC removal, gas production and the electron acceptor consumption were highly related to the microbial community structure, bacterial abundance and activity that were revealed by molecular analyses.

Measurement of gene expression of mcrA and dsrB gene was the indicator of the presence of methanogenic and sulphate-reducing biodegradation activity, respectively. There were available electron sources and acceptors for both methanogens and sulphate reducers in the microcosms. Our results showed that mcrA and dsrB genes were expressed under both methanogenic and sulphate-reducing conditions.

For the overall results, measurement of gene expression level of assA and bcrA gene was the indicator of degradation of alkanes and aromatics in the microcosm, respectively. However, that conclusion should be confirmed via further analysis of aliphatic and aromatic fraction of total petroleum hydrocarbons by GC–MS.

Considering the environmental impacts, biodegradation test under sulphate reducing conditions at mesophilic and psychrophilic ranges should be improved by nutrient optimization or enrichment; fed with chemically non-treated soil.

At last, the changes in microbial community profile should be confirmed via metagenomics analysis through the last samples of microcosm tests by Next Generation Sequencing (NGS).

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