ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

INVESTIGATION OF VARIOUS ANAEROBIC BIODEGRADATION STRATEGIES ON PETROLEUM CONTAMINATED SOIL

M.Sc. THESIS

Yıldız ERDENER

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ISTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

PETROLLE KİRLENMİŞ TOPRAKLARDA ÇEŞİTLİ ANAEROBİK BİYODEGRADASYON STRATEJİLERİNİN İNCELENMESİ

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Yıldız Erdener, a M.Sc. student of ITU Graduate School of Science Engineering and Technology student ID 501151808, successfully defended the thesis/dissertation entitled "INVESTIGATION OF VARIOUS ANAEROBIC BIODEGRADATION STRATEGIES ON PETROLEUM CONTAMINATED SOIL", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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Date of Submission : 12 September 2019
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To my family and friends,

FOREWORD

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ABBREVIATIONS

A : Aquifer
BTEX : Benzene, Toluene, Ethyl-benzene and Xylene
COD : Chemical Oxygen Demand
CTAB : Cetyl Trimethylammonium Bromide
E : Enrichment
EPA : Environmental Protection Agency
GC : Gas Chromatography
GITIP : Guadinium Isothiocyanate Isopropanol
HRM : High Resolution Melting Analysis
M : Manure
MGW : Molecular Grade Water
NGS : Next Generation Sequencing
PAH : Polycyclic Aromatic Hydrocarbons
PCA : Principal Component Analysis
PTSO : Pre-treated Soil
CSO : Contaminated Soil
qPCR : Quantitative Polymerase Chain Reaction
TOC : Total Organic Compound
TPH : Total Petroleum Hydrocarbons
TS : Total Solids
VS : Volatile Solids

SYMBOLS

CH_4	**:** Methane
CO_2	**:** Carbon dioxide
N_2	**:** Nitrogen
N_2O	**:** Nitrous oxide
NO	**:** Nitric oxide
NO_2^-	**:** Nitrite
NO_3^-	**:** Nitrate
H_2S	**:** Hydrogen sulfide
S	**:** Sulfide
SO_4^{2-}	**:** Sulfate

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INVESTIGATION OF VARIOUS ANAEROBIC BIODEGRADATION STRATEGIES ON PETROLEUM CONTAMINATED SOIL

SUMMARY

Nowadays, interest in renewable energy sources has increased, however petroleum is still important as an energy source. The demand for oil increases every day with the increase of the world's population and the need for energy. Crude oil and petroleum products are used in a wide range from electricity production to transportation. Oil spills that occur during the extraction, transportation and processing cause the contamination of the ground and surface water, seas and soil, briefly cause the entire ecosystem pollution. Hydrocarbons in the structure of oil have negative effects on human health and ecosystem. It is diffucult to remove these pollutants and they are persistent in nature for many years due to the complex structure of the compounds. Physical and chemical approaches were used for the removal of these pollutants in the past, however, nowadays it has been observed that biological methods are more useful in terms of cost and formation of secondary pollutants. Although many researches on this topic have focused on aerobic degredation, recent researches has shown that anaerobic degradation is less costly and leads to less sludge formation. The aim of this thesis to investigate the anaerobic biodegradation of petroleum-contaminated soil using nitrate and sulfate as electron acceptor in anaerobic conditions. For this purpose, four different sets of anaerobic bioreactors set up under mesophilic conditions. Aquifer, which was taken from the highly contaminated region of Leuna, Germany, rich in hydrocarbons and containing BTEX components mostly, was used as inoculum in the first two sets. In the other two sets, enrichment from samples of microcosmos, which were incubated under nitrate and sulfate reducing environments for 220 days in mezophilic condition, were used as inoculum. Also, chicken manure was added as co-substrate to assess its effect on biodegredation performance. In addition, a number of samples were pre-treated with persulfate oxidation method in order to increase the degradation efficiency, and these were used in some of the bioreactor sets. Total gas, CO_2 and CH_4 measurements were performed to monitor the activity of the bioreactors. The highest cumulative biogas and CO_2 production was observed in the manure and pre-treated soil (M+PTSO+E) in co-substrate applied bioreactors under sulfate reducing conditions. The highest production of CH_4 was observed at manure and contaminated soil (M+CSO+E) in co-substrate applied bioreactors under sulfate reducing conditions. The degradation efficiency was monitored by total organic carbon (TOC) measurements. The highest TOC removal was seen at manure and pre-treated soil (61%) in sulfate reducing condition with manure. Also, microbiological community analyses such as metagenomic, High Resolution Melting (HRM) and

Quantitative Polymerase Chain Reaction (qPCR) were carried out in order to determine the microorganisms involved in anaerobic degradation.

PETROLLE KİRLENMİŞ TOPRAKLARDA ÇEŞİTLİ ANAEROBİK BİYODEGRADASYON STRATEJİLERİNİN İNCELENMESİ

ÖZET

Günümüzde yenilenebilir enerji kaynaklarına olan ilgi artmasına rağmen, petrol halen enerji kaynağı olarak önemini sürdürmektedir. Dünya nüfusunun ve buna bağlı olarak enerji ihtiyacının her geçen gün artması ile birlikte petrole olan talep de artmakta, petrol ve petrol ürünleri elektrik üretiminden taşımacılığa kadar çok geniş bir alanda kullanılmaktadır. Petrolün çıkarılması, taşınması ve işlenmesi sırasında oluşan sızıntılar, yeraltı ve yerüstü sularına, denizlere, toprağa kısacası tüm ekosisteme karışmasına neden olur. Petrolle kontamine olan toprağın karakteristik yapısı kirlenmenin nerede olduğu, ne kadar zamandır devam ettiği vb. etkilere bağlı olarak değişkenlik göstermekte, petrolün yapısında bulunan hidrokarbonlar insan sağlığı ve ekosistem üzerinde olumsuz etkilere yol açmaktadır. İçeriğinde bulunan karmaşık yapılı bileşikler nedeniyle bu kirleticilerin giderimi zordur ve doğada uzun yıllar boyunca kalmaktadır. Daha önceleri bu kirleticilerin giderimi için fiziksel ve kimyasal yaklaşımlar kullanılırken günümüzde hem maliyetler açısından hemde ikincil kirleticilerin oluşumu açısından biyolojik yöntemlerin daha faydalı olduğu gözlenmiştir. Araştırmalar daha çok aerobik degredasyon üzerine yoğunlaşmış olsa da, son zamanlarda anaerobik degredasyon üzerine yapılan araştırmalar bu yöntemin daha düşük maliyetli olduğu ve daha az çamur oluşumuna neden olduğunu göstermiştir. Anaerobik ortamda mikroorganizmalar tarafından nitrat, sülfat ve demir gibi etkili elektron alıcılar kullanılmaktadır. Zenginleştirme, ön arıtma ve eş-substrat uygulamarı gibi stratejilerle bu kirleticilerin anaerobik koşullar alında arıtılabilirliği arttırılabilir. Bu tezin amacı anaerobik koşullar altında, farklı elektron alıcı ortamlarda, bu stratejilerin performanslarını incelemek ve en verimli stratejiyi bulmaktır. Bunun için mezofilik koşullarda dört ayrı anaerobik biyoreaktör seti kurulmuştur. Bu setlerden ilk ikisinde aşı numunesi olarak Almanya'nın Leuna şehrinin bir kirlilik bölgesinden alınan akifer ve daha önce yapılan mikrokozmoz çalışmalarından alınan numunelerde yapılan zenginleştirme, substrat olarak ise Adana'nın petrolle kirlenmiş bir bölgesinden temin edilen toprak örneği kullanılmıştır. Üçüncü ve dördüncü setlerde aşı kaynağı olarak zenginleştirme, substrat olarak kontamine toprak ve eş-substrat olarakta bir tavuk çiftliğinden gelen tavuk gübresi kullanılmıştır. İlk iki setin inkübasyon süresi 122 gün iken diğer setler için inkübasyon süresi 74 gündür. Tüm setler 37°C ve 100 rpm'de inkübe edilmişlerdir. Ayrıca biyolojik olarak parçalanmayı kolaylaştırmak amacıyla bir kısım toprak numunesine persülfat ile ön arıtma yapılmış, bu numunelerde biyoreaktörlerin bir kısmında kullanılmıştır. Biyoreaktörlerin

kurulumunda kullanılan kirlenmiş toprak, ön arıtma yapılmış toprak ve akiferin fiziksel ve kimyasal özelliklerinin tespiti için öncelikle karakterizasyon analizleri yapılmıştır. Bu kapsamında, pH, toplam katı, toplam uçucu katı, toplam fosfor, toplam azot, C/N oranı, sülfat, polisiklik aromatik hidrokarbonlar, poliklorobifeniller, toplam organik karbon, çözünmüş organik karbon ve toplam petrol hidrokarbonları analizleri yapılmıştır. Akiferin pH değerinin nötr olduğu görülürken, kirlenmiş toprakta 5 ve ön arıtma yapılmış toprakta 4 olduğu, koşulların asidik olduğu görülmektedir. Hidrokarbon parçalayıcı mikroorganizmalar genelde bazik ortamı tercih ederler ancak asidik koşullarda daha iyi degredasyon yapan mikroorganizmalar da mevcuttur. Toplam katı madde değeri akiferde %80, kirlenmiş toprakta %96, ön arıtma yapılmış toprakta %93 olarak tespit edilmiştir. Ön arıtmanın toplam katı yüzdesinin değişiminde belirgin bir etkisi olmadığı görülmüştür. Toplam uçucu katıya bakıldığında ise akiferde, kirlenmiş toprakta ve ön arıtma yapılmış toprakta sırasıyla %1, %42 ve %30 olduğu belirlenmiştir. Akiferdeki toplam uçucu katı yüzdesi çok düşüktür, diğer iki numunenin sonuçlarına bakıldığında ön arıtmanın burada nispeten daha etkili olduğu gözlenmiştir. C/N oranı akiferde (42) diğer iki numuneye göre oldukça düşüktür. Kirlenmiş toprak ve ön arıtma uygulanmış toprağın C/N oranlarına bakıldığında (sırasıyla 614, 638), ön arıtmanın bu oranda çok önemli bir değişiklik yaratmadığı, hem kirlenmiş hem ön arıtma uygulanmış toprakta bu oranın beklenen şekilde oldukça yüksek olduğu gözlenmiştir. Persülfat oksidasyonu sırasında sülfat radikalleri açığa çıkar. Bu durum analiz sonuçlarına da yansımış ön arıtma yapılmış topraktaki sülfat miktarının kirlenmiş toprağın yaklaşık 8 katı olduğu görülmüştür. Toplam petrol karbonlarının yüksek olduğu numunelerde bununla paralel olarak toplam organik karbonda yüksek oranda bulunmaktadır. Yapılan karakterizasyon analizleri sonucunda kirlenmiş toprak ve ön arıtma yapılmış toprakta iki değerinde oldukça yüksek olduğu görülmektedir. Ön arıtma toplam karbon değerlerinde çok büyük bir farklılık yaratmamıştır ancak alifatik ve aromatik fraksiyonların ayrı ayrı analiz sonuçlarına bakıldığında karbon zincirlerine göre oluşan farklılıklar görülmektedir. Ayrıntılı hidrokarbon analizleri incelendiğinde, her iki grupta da C5-C9 arası hidrokarbonların düşük miktarda olduğu ve tüm numunelerde alifatik fraksiyonların daha baskın olduğu anlaşılmaktadır. Ön arıtma hem aromatik hem de alifatik fraksiyonların C9-C16 ve C16-C35 arasındaki miktarlarını arttırmıştır. Bu durum ön arıtmanın C35'ten daha yüksek olan hidrokarbonları parçalayarak degredasyonu kolaylaştırdığının göstergesidir. Biyoreaktörlerin aktivitesini takip etmek için yaklaşık 2 haftada bir manometre ile toplam gaz analizleri ve GC ile N_2, CO_2, CH_4 gazları üretimlerini ayrıntılı olarak gösteren ayrıştırmalı analizler yapılmıştır. Gübre eklenmiş ve eklenmemiş setlerin inkübasyon süreleri farklı olduğu için değerlendirmeler ayrı ayrı yapılmış daha sonra genel profili görmek için tüm setlerin ilk iki aylık sonuçları alınarak genel bir değerlendirme yapılmıştır. Bu genel değerlendirme sonucunda toplam gaz ve CO_2 üretiminde en yüksek değerin, sülfat indirgeyici ortamda gübre ve ön arıtma yapılmış toprak ile hazırlanan biyoreaktörde (M+PTSO+E) olduğu gözlenmiştir. CH_4 üretim sonuçlarına bakıldığında ise gübre uygulaması olmayan setlerde herhangi bir üretim olmadığı, üretimin sadece gübre eklenen setlerin bir kısmında gerçekleştiği görülmektedir. Burada en yüksek üretim

nitrat indirgeyici ortamda sadece gübre ile hazırlanan reaktörde (M) gerçekleşmiş olup bunu sülfat indirgeyici ortamda gübre ile kirlenmiş toprak karışımı (M+CSO+E), gübre (M) ve gübre ile ön arıtma yapılmış toprak karışımı (M+PTSO+E) izlemektedir. Nitrat indirgemede denitrifikasyon sonucu ürün olarak N_2 üretilebileceği için, N_2 üretimi sadece nitrat indirgeyici ortamalarda incelenmiştir. Burada gübre eklenmiş sette ön arıtma yapılmış toprakla hazırlanan biyoreaktörde (M+PTSO+E) en yüksek üretim görülmektedir. Nitrat indirgeyici ortamlarda ilk ürün nitrittir, bu nedenle nitrat indirgeyici koşullarda hazırlanan biyoreaktörlerde nitrat giderimi ve nitrit üretimi de takip edilmiştir. Nitrat giderimine bakıldığında inkübasyon süresi daha kısa olmasına rağmen gübre eklenmiş setlerde giderimin daha yüksek olduğu görülmektedir. En yüksek giderim %97,3 ile sadece gübre ile hazırlanan reaktörde (M) görülmüş, bunu sırasıyla %96,7 ve %96,2 ile gübre, kirlenmiş toprak karışımı (M+CSO+E) ve gübre, ön arıtma yapılmış toprak karışımı (M+PTSO+E) izlemektedir. Nitrit üretimine bakıldığında ise gübre eklenmemiş sette üretimin daha yüksek olduğu görülmektedir. Sonuçlar N_2 analizlerini de içerecek şekilde değerlendirildiğinde gübre ile hazırlanan setlerde nitratın büyük oranda N_2'ye dönüştüğü görülmektedir. Biyoreaktörlerin degredasyon verimliliği inkübasyon süresi sonunda yapılan toplam organik karbon (TOK) ölçümleri ile izlenmiştir. Toplam organik karbon gideriminin %61 ile sülfat indirgeyici ortamda gübre ve ön arıtma yapılmış toprak ile hazırlanan biyoreaktörde (M+PSTO+E) gerçekleştiği görülmektedir. Gübresiz setlerde inkübasyon süresi daha uzun olmasına rağmen sülfat indirgeyici ortamda ön arıtılmış toprak ile hazırlanan biyoreaktörde (A+PSTO+E) ise bu değer %26,7'dir. Burada sülfat indirgeyici ortamda, ön arıtma yapılmış toprakta gübre ilavesinin degredasyon verimini yüksek oranda arttırdığı görülmektedir. Ayrıca anaerobik degredasyonda görev yapan mikroorganizmaların benzerliklerini, aktivitelerini, mikrobiyal popülasyondaki değişim vb. mikrobiyal profili belirlemek amacıyla mikrobiyal kominite analizleri yapılmıştır. Toprakta ve akiferde yapılan metagenomik analizler sonucu her iki numunede de alkanların, poliklorlubifenillerin ve aromatik hidrokarbonların degredasyonunda görev yaptığı bilinen *Actinomycetales* takımının yüzdesel olarak oldukça yüksek olduğu görülmektedir. Sülfat indirgeyici ve nitrat indirgeyici ortamlardaki mikrokozmozlardan alınan numunelerle yapılan zenginleştirmelerde ise *Bacillales'*in baskın duruma geçtiği gözlenmektedir. Sülfat indirgeyici ortamda *Actinomycetales* ikinci baskın takım iken, nitrat indirgeyici ortamda yapılan zenginleştirmelerde çok düşük oranda bulunduğu belirlenmiştir. Mikrobiyal benzerliğin değerlendirilmesini sağlayan yüksek çözünürlüklü erime (HRM) analizleri, biyoreaktörlerden iki haftada bir alınan örnekler üzerinde yapılmıştır. Bu analizin sonuçları incelendiğinde, gübre eklenmiş setlerde inkübasyon süresi sonlarında mikrobiyal komünitenin farklı gruba kaydığı, yani zamanla mikrobiyal komünitenin değiştiği gözlenmiştir.

1. INTRODUCTION

Petroleum is the most significant source of energy that meets major part of energy requests in the world. It is also raw material for many industries such as petrochemical, pharmaceutical, synthetic polymers, oil refineries, dye stuffs etc. (Zhang et al., 2011; Varjani and Upasani, 2017).

Petroleum consist of a mixture of liquid, solid and gases. Although the composition varies greatly depending on the properties of the source, it is composed of hydrocarbons in general (Eneh, 2011).

Oil spills cause serious pollution in both terrestrial and marine environments and are a threat to the diversity of marine ecosystem life forms and human health (Cheng et al., 2017). The oil spills, which have negative effects on the environment, economy and society, which stick to the soil with tidal and wave movements, making it difficult to clean the soil. The toxicity of the oil spill in the soil is a major concern for the environment and several researchers have focused on this issue (Qin et al., 2013; Lim et al., 2016). The vast application area of petroleum causes these pollutants to disrupt the ecological balance and for many years these pollutants can not be eliminated (Zhang et al., 2011). In addition, it can also damage vegetation that supplied food and shelter (Onuh et al., 2008).

Petroleum contamination affects ecosystem in a harmful way via adsorption of soil particles, it increases the rate of organic carbon as much as microorganisms cannot use it and also it decreases the nitrogen and phosphorus ratio in soil (Okolo et al., 2005; Onuh et al., 2008). These complicates the natural reclamation of oil-contaminated soils and various soil treatments such as biostimulation, phytoremediation, rhizoremediation can applied as bioremediation strategies to accelerate the process (Okolo et al., 2005).

1.1 Purpose of Thesis

The aim of this thesis is to observe the biodegradation efficiency using different electron acceptors under anaerobic conditions and minimize waste. For this purpose, four different environments were prepared using nitrate and sulfate as terminal electron acceptors under mesophilic conditions. In the first two sets, in nitrate and sulphate reducing media, the aquifer sample was used as inoculum and these sets were operated for 122 days. In the other two sets, enrichment culture from microcosm sets were used as an inoculum and chicken manure was used as co-substrate to increase the degradation efficiency. These sets operated for 74 days. In addition, pre-treatment was applied to the contaminated soil sample with persulfate oxidation method and some of bioreactors were also established with this sample. Thus, degradation efficiency in the case of pre-treatment and non-treatment was determined. In addition, the effect of chicken manure, which was used as co-substrate, on degradation was examined.

2. LITERATURE REVIEW

2.1 Petroleum Derivative Wastes

Petroleum consists of thermal decomposition of embedded organic matter for many years (Chandra et al., 2013; Varjani, 2017). It is a greasy, combustive liquid formed naturally under the soil surface (Chandra et al., 2013; Varjani and Upasani, 2017) It can have a wide variety of appearance which is depend on the chemical structure (Varjani and Upasani, 2017). The content of crude oil might vary depending on the location, age and depth of the well where it is located (Varjani, 2017).

Crude oil rarely used without any processing (Varjani and Upasani 2017), therefore it is sent to refineries and passed through different applications to produce several products (Varjani and Upasani, 2017; Varjani, 2017). Petroleum is processed in refineries to produce gasoline, heating oil, petrochemical raw materials, asphalt and various other products and by-products. Operations in the oil industry results hydrocarbon pollution in nature (Varjani and Upasani, 2017).

Soil has an important place in the natural ecosystem as the environmental sustainability depends to a large extent on the sustainability of the soil ecosystem. Any interaction of soil with crude oil is harmful for this ecosystem (Onuh et al., 2008). Nonetheless, oil spills during refineries and other operations lead to significant environmental problems (Zhang et al., 2011; Varjani and Upasani, 2017). These organic pollutants cause serious environmental problems in numerous countries as a result of their resistance (Varjani and Upasani, 2017).

2.1.1 Chemical compound

Crude oil is a complicated mixture of various hydrocarbons and non-hydrocarbon compounds (Okafor et al., 2016). Petroleum hydrocarbons, largely containing of carbon and hydrogen, besides they involve a small amount of sulphur, oxygen, nitrogen, potassium and phosphorus. In addition, petroleum contains small quantities

of inorganic compounds such as metals (Adenipekun, 2008; Chandra et al., 2013; Koshlaf and Ball, 2017).

The petroleum hydrocarbons are composed of highly complex aromatic and aliphatic compounds, which may be branched or linear chain and may be saturated or unsaturated (Agarry et al., 2010;Varjani and Upasani, 2017). These compounds contain many components that are permanent in nature and cause toxic effects in the soil ecosystem (Onuh et al., 2008; Roy et al., 2014).

Crude oil content can be evaluated under four main titles as (a) Aliphatic hydrocarbons (saturates), (b) Aromatics (ringed) hydrocarbons, (c) Resins and (d) Asphaltenes (Figure 2.1) (Chandra et al., 2013; Koshlaf and Ball, 2017; Varjani and Upasani, 2017).

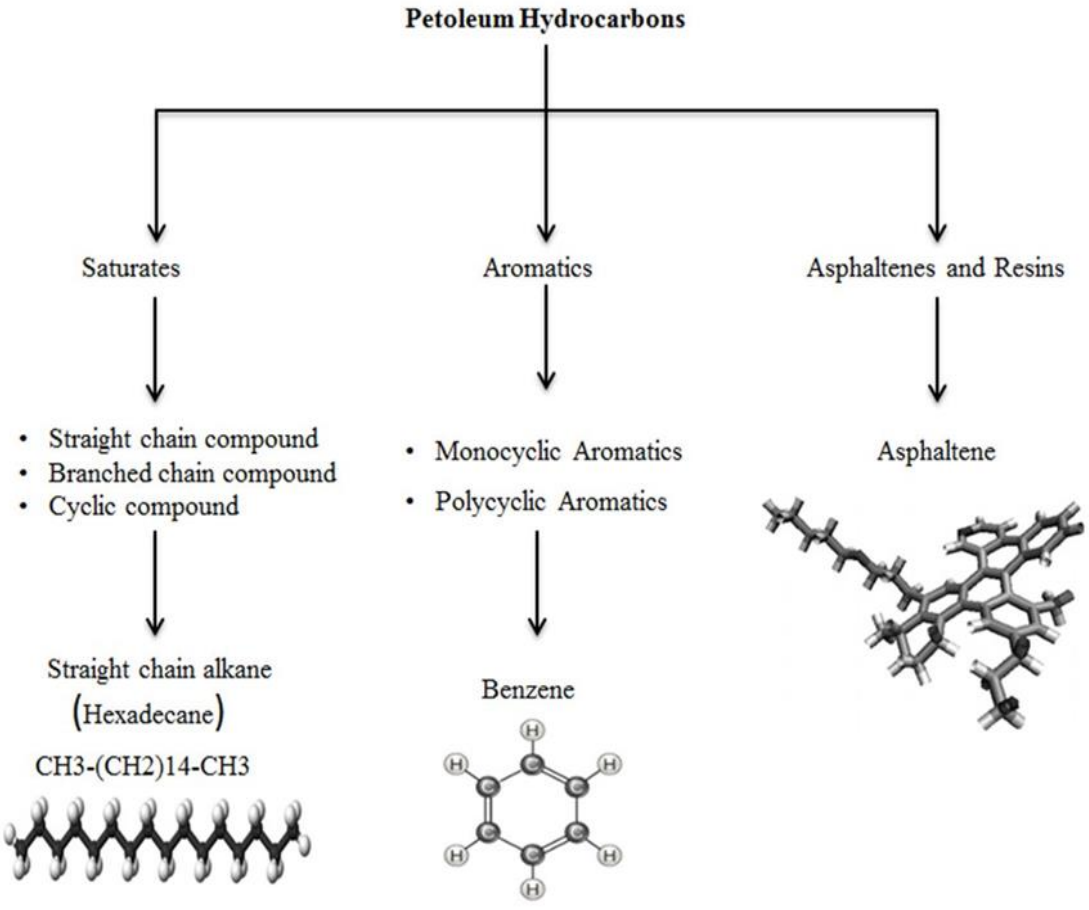


Figure 2.1 : Hydrocarbon fractions in crude oil (Koshlaf and Ball, 2017).

2.1.1.1 Aliphatic hydrocarbons

The major content of crude oil and its products consist of a mixture of various low- and high-molecular-weight aliphatic hydrocarbons (Abbasian et al., 2015; Koshlaf and Ball, 2017) They may be unsaturated or saturated and have branched or straight open chain structures such as n-alkanes, terpenes, isoalkanes and cycloalkanes (Koshlaf and Ball, 2017; Varjani, 2017).

Among aliphatic hydrocarbons n-Alkanes are the fastest decomposing components. They are examined in four groups due to their molecular weights: (a) the gaseous alkanes, (b) low-molecular weight aliphatic hydrocarbons, (C8–C16), (c) medium-molecular weight aliphatic hydrocarbons (C17–C28) and (d) high-molecular weight aliphatic hydrocarbons (>C28) (Abbasian et al., 2015).

Short-chain aliphatic hydrocarbons generally volatilise quickly, but sometimes they can spread on solid and liquid surfaces and have a toxic effect on the ecosystem. They are easily degraded by most hydrocarbon degrading microorganisms. Aliphatic hydrocarbons with larger chains (C20–C40) are more permanent, do not volatilise easily and are hard to degrade due to their low water solubility and chemical structure. Degradation of long chain (up to C44) aliphatic hydrocarbons also occurs, but they are enzymatically activated before degradation (Abbasian et al., 2015, Koshlaf and Ball, 2017).

2.1.1.2 Aromatic hydrocarbons

Aromatic hydrocarbons having ringed structure are mainly divided into two groups as mono aromatic (BTEX) and polycyclic aromatic hydrocarbons (PAHs) (Chandra et al., 2013; Varjani, 2017).

Monoaromatic fraction such as benzene, ethylbenzene, xylene and toluene are volatile organic compounds and constitute an important part of the petroleum hydrocarbon with a value of about 15%. Since these hydrocarbons have great toxicity and carcinogenic effect, they can cause adverse impact on human health, fishing, natural biota and ecosystem (Firmino et al., 2015; Daghio et al., 2016). Compared to other aromatic hydrocarbons, toluene permits rapid growth of microorganisms, while benzene is one of the most hardly degrading hydrocarbons by microorganisms (Widdel and Rabus, 2001; Zhang et al., 2010).

PAHs, which occur in the final stage of the crude oil process and comprise approximately 26-30% of the petroleum components (Koshlaf and Ball, 2017), are aromatic compounds having a fused-ring (2-6 fused rings) structure (Qin et al., 2018; Sun et al., 2018). They are another compound that is dispersed in oil (Zhang et al., 2011). PAHs, which are non-polar, hydrophobic and neutral molecules, have low volatility, low solubility in water and they tend to sorb onto the organic matter of the soil (Koshlaf and Ball, 2017; Peluffoa et al., 2018). They have common properties such as corrosion and heat resistance and photosensitivity (Koshlaf and Ball, 2017).

These compounds (PAHs) pose a threat to the environment and human health because of their carcinogenic, toxic, mutagenic and teratogenic potentials (Zhang et al., 2011; Su et al., 2012; Sun et al., 2014; Peluffoa et al., 2018; Qin et al., 2018). The physical-chemical properties of these compounds, which vary according to their molecular weight and the number of aromatic benzene rings, affect their distribution in water, soil and air and impacts on the environment (Koshlaf and Ball, 2017). PAHs, which typically have a larger number of aromatic rings, are considered to be more toxic and cause greater threats to the ecosystem (Qin et al., 2018).

Generally, low-molecular weight PAHs are easily degraded by fungi and bacteria, while high molecular weight PAHs with four or more rings are permanent pollutants due to their low bioavailability and hydrophobicity (Peluffoa et al., 2018). However, the 2 and 3-ring hydrocarbons have a particular importance because they dissolve in water and transport to long distances by underground and surface waters (Meckenstock et al., 2004; Su et al., 2012).

PAHs have been identified as priority pollutants by the EU and EPA due to their carcinogenic potential (Sun et al., 2018). Sixteen of these are listed as priority pollutants by EPA for their high stability (Figure 2.2) (Koshlaf and Ball, 2017; Varjani and Upasani, 2017).

The bioremediation of PAHs, which are more resistant to biological degradation because of their high hydrophobicity, generally requires more time than other technologies and may not be completed due to their recalcitrance and low availability (Zhang et al., 2011; Peluffoa et al., 2018). Anaerobic bioremediation effectiveness of soils contaminated with PAH may be limited to low bio accessibility due to lack of suitable electron acceptors and low water solubility (Sun et al., 2014).

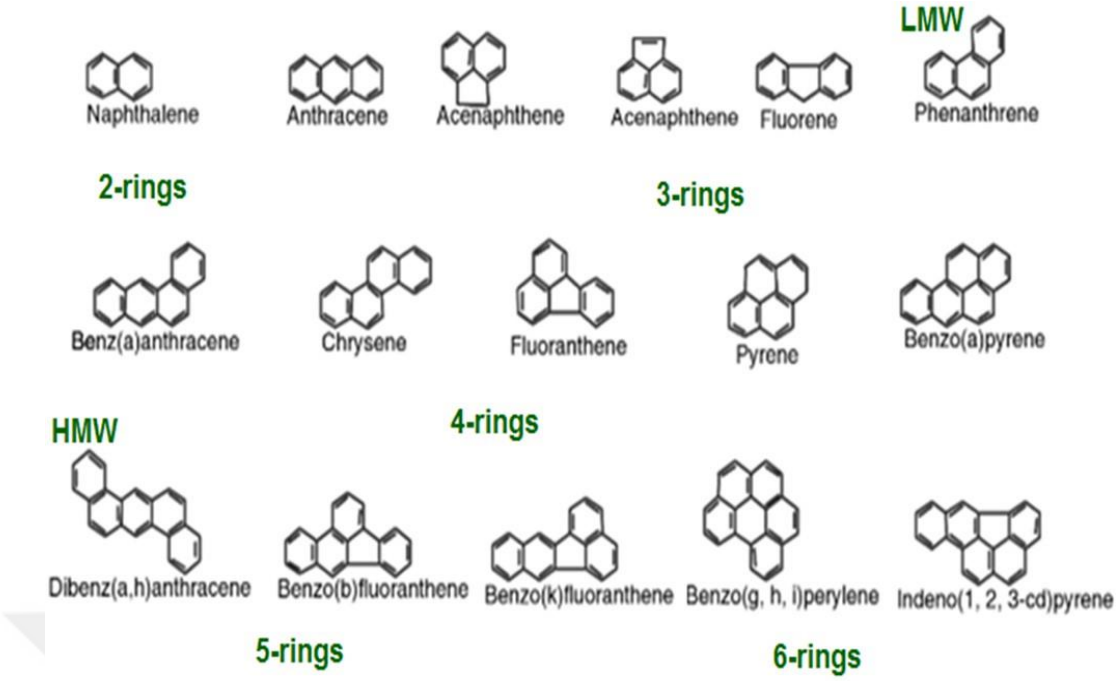


Figure 2.2 : Structure of PAH's in EPA priority list (Koshlaf and Ball, 2017).

2.1.1.3 Resins and asphalthenes

Resins and asphaltenes containing many nitrogen, oxygen and sulfur atoms have very complex and often unknown carbon structures (Figure 2.3) (Koshlaf and Ball, 2017; Varjani, 2017). Resins are oil-dissolved, amorphous solids containing nitrogen, oxygen, sulfur and some trace metals (Chandra et al., 2013; Varjani, 2017; Varjani and Upasani, 2017). They contain long alkyl chain aromatic compounds (Varjani, 2017). Asphaltenes contain a large number of polar functional groups, similar to resins. Colloidally dispersed asphaltenes in saturated and aromatics are dark brown colored, large and complex molecules (Chandra et al., 2013; Varjani, 2017).

The properties of resins and asphaltenes, which constitute for about 10% of the crude oil composition, affect the behaviour of crude oil during refining and production. Resins and asphaltenes are thought to have a strong affinity due to their composition and structural similarities. They are usually considered to be resistant to microbial degradation (Koshlaf and Ball, 2017).

Asphaltenes, which are high molecular weight, uncrystallisable, viscous and unstable compounds, consist of variable substituted with alkyd groups, polycyclic clusters and this contributes to their resistance to biodegradability (Chandra et al., 2013; Koshlaf and Ball, 2017; Varjani, 2017).

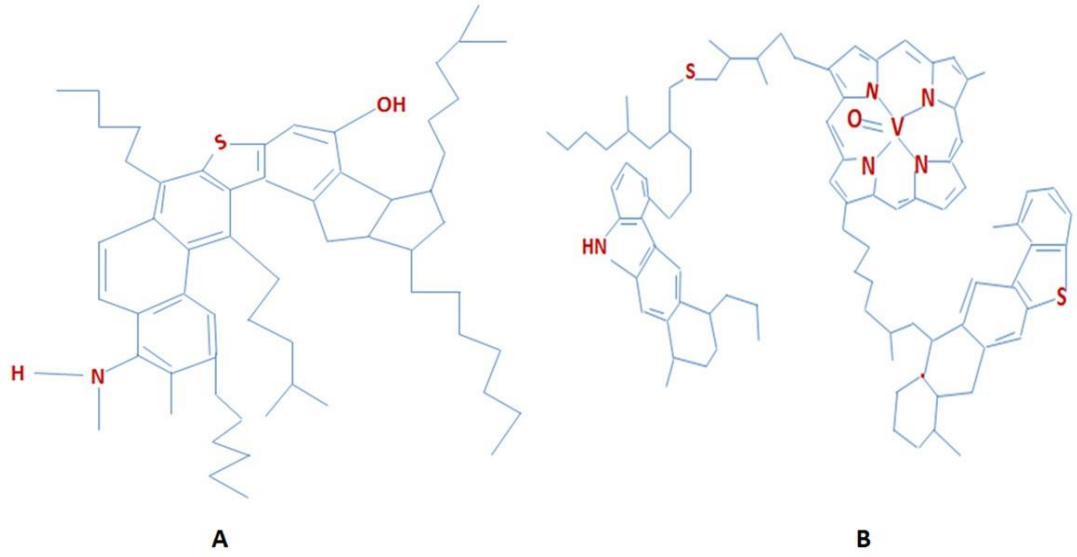


Figure 2.3 : Structures of (A) resins and (B) asphaltenes (Koshlaf and Ball, 2017).

2.1.2 Propagation and effects

The increase in global demand for energy and the excessive consumption of energy resources, especially for petroleum, cause many different types of pollution such as hydrocarbon contamination (Okafor et al., 2016; Cheng et al., 2017). There is a growing concern about the damage to the environment and human health from the accidental or deliberate release of petroleum hydrocarbons during the extraction, shipping and refining of crude oil (Scherr et al., 2012; Roy et al., 2014; Borah and Yadav, 2017; Varjani and Upasani, 2017).

The most common pollutants in the environment are petroleum hydrocarbons (Margesin et al., 2003). When petroleum hydrocarbons spread to the environment, their primary impact is the blocking of water, light, oxygen and nutrients in the environment (Koshlaf and Ball, 2017). Hydrocarbon pollution directly or indirectly poses a health risk for all living things in the world and effects the environment (Varjani, 2017). They cannot be wholly degraded by microorganisms and often leave more or less complex residues which are recalcitrant metabolites and compounds. Their bioavailability generally decreases over time due to sequestration by soils and low water solubility (Qin et al., 2013). The chemical and physical features of hydrocarbons identify potential risk to human health (Souza et al., 2014).

Exposure to aromatic hydrocarbons can cause many health problems like mucosal, eye and skin irritation, weakening of the nervous system, decreased bone marrow function

and cancer (Costa et al,. 2012). Among the mono-aromatic hydrocarbons, benzene is considered the most toxic, can damage the bone marrow, can cause liver cancer and leukemia, also affects blood cell production. Inhalation of toluene, which is another mono-aromatic hydrocarbon, can cause mucosal irritation, muscle weakness, kidney problems, and endangers the central nervous system. Ethylbenzene and xylene having low systemic toxicity have a depressant effect on the central nervous system (Souza et al., 2014) Exposure to PAHs, that is mostly located in soil and sediments at various concentrations, occurs through inhalation, ingestion and contact of contaminated soils or dust. These pollutants at low concentration can cause carcinogenic or mutagenic effects for humans and natural life (Koshlaf and Ball, 2017).

Aliphatic hydrocarbons, that may negatively impact soil microflora and limit nutrient and oxygen exchange in the soil, may also affect the nervous system, causing tremors, transient limb paralysis and loss of consciousness (Koshlaf and Ball, 2017).

Due to its resistant nature, petroleum contaminant remains in the soil extended period of time and affect the quality of soil and microbial diversity. The petroleum has a phytotoxic effect and blocks the presence of nutrients necessary for plants in the soil, for this reason plants are sensitive to the exposure of oil pollutants as well (Roy et al., 2014). Petroleum pollutants can accumulate in plant and animal tissues and be transported through the food chain, leading to genetic mutations and death (Koshlaf and Ball, 2017).

2.2 Remediation

Several studies have been conducted to minimize the effects of oil pollutants on the environment and human health (Zhang et al., 2011). Various methods may be applied to minimize environmental impact when oil contamination occurs (Okafor et al., 2016). Chemical, physical, biological and integrate degradation methods can be used to remediate petroleum contaminated soil (Ghoreishi et al., 2017; Galdamesa et al., 2017; Zhao et al., 2019). Previously, physical and chemical methods were preferred in the remediation of petroleum hydrocarbons, but nowadays, it is focused on bioremediation due to some advantages, (Covino et al., 2016). While chemical and physical methods have limitations such as changing ecosystem, inefficiency and high costs, biological methods are low cost and have low energy and chemical need (Patel et al., 2013; Borah and Yadav, 2017; Ghoreishi et al., 2017; Galdamesa et al., 2017).

Biological methods are also compatible with local conditions and can mineralize the pollutant into environmentally friendly carbon dioxide and water (Okafor et al., 2016).

Physical, chemical and biological methods such as chemical oxidation, combustion, composting, bioaugmentation, solvent extraction, thermal conduction, phytoremediation etc. can be used separately to remove PAH contaminated soils (Kuppusamy et al., 2017). On the other hand, physicochemical and biological methods can be used together for better remediation of PAH (Kuppusamy et al., 2017; Peluffoa et al., 2018).

Biological methods, which are an environmentally friendly approach instead of physical and chemical methods, have started to attract more attention in the remediation of petroleum contaminated soils (Roy et al., 2014).

2.2.1 Chemical/physical remediation

There is an immediate and obvious need to restore the oil-contaminated lands and a lot of physical and chemical methods are existing for clean-up this pollutants (Koshlaf and Ball, 2017). Physical and chemical methods used to remediate petroleum hydrocarbons include soil vapor extraction, pump and treat, thermal desorption, combustion, asphalt batching, soil washing, hydrolysis, excavation, solidification and photolysis. These methods are generally high cost, incomplete and their products can cause secondary contamination, which may lead to additional processing (Gkorezis et al., 2016; Okafor et al., 2016; Koshlaf and Ball, 2017).

Conventional physicochemical approaches are questioned due to their high cost and adverse effects on the ecosystem (Nwankwegu and Onwosi, 2017). Biological methods are considered as a good alternative to physical and chemical methods and researches focus on this method because they are effective, versatile, economic and environmentally friendly (Zhang et al., 2011; Qin et al., 2013).

2.2.2 Bioremediation

Bioremediation involves the transformation of organic pollutants into harmless substances, like water and carbon dioxide, by means of biological agents such as bacteria, archaea, fungi and plants (Adenipekun, 2008; Varjani and Upasani, 2017). The microorganisms, which act in this process, can be naturally occurring or

genetically modified (Okafor et al., 2016). Bioremediation also includes the implementation of nutrients such as nitrogen, phosphorus and phosphate to the polluted site (Adenipekun, 2008).

Bioremediation is an environmentally friendly method for remediating soils that contaminated with petroleum and it has been observed to be relatively effective in removing oil pollutants from the soil. Complex structures of oil pollutants make it difficult to reduce and monitor ecotoxicity (Shen et al., 2016).

Biodegradation efficiency depends on factors such as temperature, physicochemical composition, electron acceptors, nutrients, pH, pressure and salinity (Zhang et al., 2011; Chandra et al., 2013; ÁlvarezLM et al., 2017; Varjani and Upsani, 2017).

Temperature, which plays a major role in bioremediation, affects both the physicochemical state of hydrocarbons in the contaminated area and microorganisms (Varjani and Upsani, 2017). This parameter is effective on solubility, chemistry of the contaminant, viscosity, volatility, diversity and physiology of microorganisms (Chandra et al., 2013).

Oxygen enhance the bioremediation rate when used as an electron acceptor, but pollutants reduce air permeability of soil and restrict the growth of microorganisms (Chandra et al., 2013; Varjani and Upsani, 2017). Aerobic degradation activity limits oxygen when oxygen content is low and this leads to the sequencing of anaerobic microorganisms (Abbasian et al., 2015). In anaerobic condition, species use different electron acceptors such as iron, manganese, nitrate, sulphate, H_2 or carbon dioxide (Abbasian et al., 2015; Varjani and Upsani, 2017). They either present in the contaminated area or added externally. It is usually cheaper than the oxygen supplement even if it is added externally (Varjani and Upsani, 2017).

Nutrient is an important parameter for a successful biodegradation. When the oil contamination occurs, the amount of carbon considerably increases, while the amount of nitrogen and phosphorus decrease. This makes nitrogen and phosphorus a limiting factor (Chandra et al., 2013).

Pressure and salinity have an important effect on degradation in areas such as deep seas, salty lakes and degradation becomes more difficult as these parameters increase (Chandra et al., 2013; Varjani and Upsani, 2017).

The pH value varies according to the area where pollution occurs. Microorganisms can continue their activities in a certain range of pH, extremes in pH value can negatively affect degradation (Chandra et al., 2013).

The molecular structure and weight of the contaminant, concentration, solubility, volatility, hydrophobicity and bioavailability are also important effects on degradation (Chandra et al., 2013; Varjani and Upsani, 2017). High hydrocarbon concentration affects the growth and activity of microorganisms by making toxic effects (Koshlaf and Ball, 2017). Degradation of compounds with complex molecular structure is more difficult, and these contaminants are more permanent in nature (Chandra et al., 2013).

2.3 Anaerobic Degradation

Aerobic biodegradation has been well documented in the remediation of aliphatic and aromatic hydrocarbons, but studies on anaerobic biodegradation is relatively recent and new ideas are constantly generated. This degradation is reported under nitrate-, sulphate-, manganese-, iron-reducing and methanogenic conditions (Foght, 2008; Varjani and Upasani, 2017). The anaerobic biodegradation of petroleum hydrocarbons have been accepted as a naturally occurring process, and the recovery and utilization of methane produced during this degradation has been seen as an energy-efficient approach. Bacteria and archaea play a dominant role in anaerobic degradation while fungi are in third place (Scherr et al., 2012).

Besides the advantages of anaerobic degradation such as energy saving and low construction cost, there are some disadvantages such as anaerobic microorganisms being sensitive to inhibition and also it needs post-treatment (Oliveira et al., 2019).

2.3.1 Sulfate reduction

In anaerobic respiration, some of the inorganic sulfur compounds are important electron acceptors. The most important of these is sulfate, which is an easily oxidizable form. The use of sulfate as an electron acceptor is based on the reduction of sulfate, where sulfate reducing bacteria act (Madigan et al., 2012). Sulfate is reduced to elemental sulphur and to sulfide in the form H_2S, S^{2-}, HS^-. Some reactions of this are as follows (Equation 2.1 and 2.2) (Machel, 1989):

$$Crude\ oil \rightarrow Light\ crude\ oil + H_2S + CH_4 \quad (2.1)$$

$$Hydrocarbons + SO_4^{-2} \rightarrow altered\ hydrocarbons + bitumen + HCO_3^- + H_2S \quad (2.2)$$

Sulfate reducers are absolute anaerobic and use organic compounds as a source of energy and carbon. These bacteria can successfully break down aromatic compounds and convert them to CO_2 (Karthikeyan and Bhandari, 2001).

2.3.2 Nitrate reduction

One of the most important electron acceptors in anaerobic respiration is nitrate. It can be reduced to nitric oxide (NO), nitrous oxide (N_2O) and dinitrogen (N_2), which are in gas form, through denitrification. Nitrate reduction and denitrification steps are given in Figure 2.4. The first product in nitrate reduction is nitrite, and nitrite is then converted to nitric oxide. Nitrite can be converted into ammonium (NH_3) by some microorganisms but reduction to gas form is seen much more (Madigan, 2012)

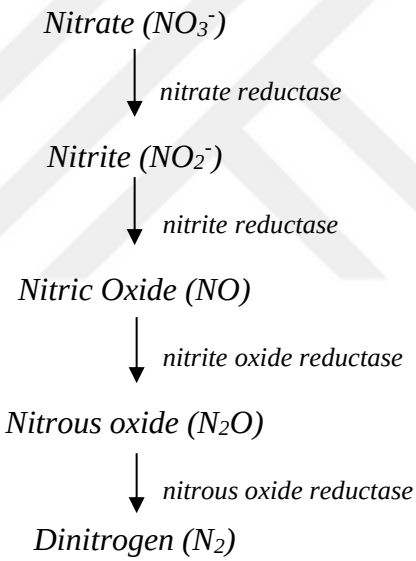


Figure 2. 4 : Nitrate reduction and denitrification.

2.4 Effect of Manure on Biodegradation of Petroleum Hydrocarbons

Petroleum contaminants increase the amount of organic carbon while they reduce the amount of nitrate and phosphorus in the soil and that makes the conditions difficult for degradation (Okolo et al., 2005). Economic and environmentally friendly applications for the remediation of oil hydrocarbons remain the focus of researches. One of these eco-friendly methods is the use of animal manure for remediation (Agarry et al., 2010). It has been shown that animal manure is a potential source of nutrient (Okafor et al., 2016) and microorganisms in manure have the ability to use petroleum wastes (Varjani

and Upasani, 2017). The application of organic fertilizers to petroleum-contaminated soil balances the amount of nitrogen but do not impact the amount of phosphorus (Onuh et al., 2008). Agarry et al. were reported that poultry manure is more effective in increasing the degradation efficiency.

Poultry manure, which has a rich organic matter content, is a potential nutrient source for microorganisms and contains microorganisms that use hydrocarbons as energy and carbon sources. For these reasons, they are useful in removing hydrocarbon pollution in soil (Okafor et al., 2016). Onuh et al., (2008) have reported that poultry manure provides an effective remediation by enhancement the physical and chemical properties of oil-contaminated soil.

3. MATERIALS AND METHODS

3.1 Aquifer, Petroleum Contaminated Soil and Chicken Manure Sampling

The polluted soil sample used in this study was obtained from BOTAŞ, Adana facilities waste pool, where petroleum derivative wastes were collected. The aquifer sample to be used as an inoculum was taken from Leuna, Germany. This aquifer is rich in fuel hydrocarbons generally containing BTEX components (Tischer et al., 2013) and includes fuel additives.

In order to increase biodegradation efficiency, chicken manure used in this study was obtained from a poultry farm in Sakarya.

All samples were stored in a cold room at 4°C until they were used.

3.2 Characterization

Total solids (TS), volatile solids (VS) and chemical oxygen demand (COD) analyses of chicken manure were performed by using Standard Methods (APHA, 2005). These analyses were carried out triplicate.

The analysis and methods applied to aquifer, contaminated soil and pre-treatment soil were as follows: The total solids (TS) and volatile solids (VS) contents were determined using the APHA (2005) standard methods. pH was determined by electrometric method in accordance with TS ISO 10390 standard. Total phosphorus analyses were carried out according to EPA 200.7 standard with inductively coupled plasma optical emission spectrometry (ICP-OES) method used for detection of chemical elements. The determination of total nitrogen was done with the modified kjeldahl method as specified in TS 8337 ISO 11261 standard. The C / N ratio was determined in accordance with TS 12089 EN 13137 and TS 8337 ISO 11261 standards with modified kjeldahl and high temperature combustion methods. Dissolved organic carbon (DOC) analysis were performed by high temperature burning method depending on the SM 5310 B standard. The determination of total petroleum

hydrocarbons and the aliphatic and aromatic fractions were made within the scope of the TS EN 14039 and S-TPHFID08 standards by Gas Chromatography with Flame Ionization Detector (GC-FID).

3.3 Pre-treatment Method

Oxidation is a method used to facilitate biodegradation. Although there are various chemical oxidation methods, there are some advantages of the persulfate oxidation. The persulfate can be activated by transition metals, ultraviolet light, heat and bases to form sulfate radicals, which provide high degradation efficiency of organic pollutants. Apart from that, their oxidation potential is high and they are not selective. (Peluffoa et al., 2018).

In this study, oxidation process with persulfate was done as follows (Wu et al., 2016): 150g of petroleum-contaminated soil, 50 g sodium persulfate ($Na_2S_2O_8$), 29.19 g iron (II) sulphate heptahydrate ($FeSO_4.7H_2O$), 20.17 g citric acid ($C_6H_8O_7$) were added into 500 mL of erlenmeyer flask. 300 mL of distilled water was added on it and closed with aluminium foil properly. Erlenmeyer flask, which was then placed in the shaker, was left to be mixed for two hours at the 200 rpm. After this process, 50 g sodium persulfate ($Na_2S_2O_8$), 29.19 g iron (II) sulphate heptahydrate ($FeSO_4.7H_2O$), 20.17 g citric acid ($C_6H_8O_7$) were added again, and re-mixed with shaker for 2 hours more at the 200 rpm. At the end of the four-hour period the erlenmeyer flask was taken from the shaker, after that the liquid part was split off from the sample and stored in a cold room at a temperature of 4°C until the sample was used. All scales performed during this oxidation process were made with a precision balance of four digits.

3.4 Preparation of Media

On the basis of the OECD 311 medium for the setup of anaerobic bioreactors, two different reducing conditions (nitrate and sulfate) that included trace elements and vitamin solutions were prepared as follows.

Preparation of reducing media: 0.27 g of anhydrous potassium dihydrogen phosphate (KH_2PO_4) and 1.12 g of disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$) were weighed and placed in a 2000 mL volumetric glass flask, then 500 mL of deionized water was added. Resazurine (oxygen indicator) stock solution

was prepared by adding 20 mg of resazurine to 20 mL of deionized water in a 50 mL glass bottle. 1 mL of this stock solution was taken and added to the 500 mL solution prepared with KH_2PO_4 and $Na_2HPO_4.12H_2O$. 0.53 g of ammonium chloride (NH_4Cl), 0.075 g of calcium chloride dihydrate ($CaCl_2.2H_2O$) and 0.10 g of magnesium chloride hexahydrate ($MgCl_2.6H_2O$) were placed in another 500 mL volumetric glass flask. Afterwards, two solutions were autoclaved at 121°C for 15 minutes. Iron (II) chloride tetrahydrate ($FeCl_2.4H_2O$) stock solution was prepared by placing 2 g $FeCl_2.4H_2O$ in 100 mL water and sodium sulphide nonahydrate ($Na_2S.9H_2O$) stock solution was prepared by adding 4 g of $Na_2S.9H_2O$ to 20 mL of deionized water.

Preparation of trace element solution: 100 mg cobalt chloride hexahydrate ($CoCl_2.6H_2O$), 50 mg manganese chloride tetrahydrate ($MnCl_2.4H_2O$), 10 mg nickel chloride hexahydrate ($NiCl_2.6H_2O$), 5 mg boric acid (H_3BO_3), 5 mg disodium selenite (Na_2SeO_3), 5 mg zinc chloride ($ZnCl_2$), 3 mg copper (II) chloride ($CuCl_2$), 1 mg disodium molybdate dihydrate (Na2MoO4.2H2O) was placed in a 2000 mL volumetric flaks and 1000 mL of autoclaved deionized water was added. The prepared solution was stirred for 20 minutes in a shaker and stored at room temperature until it was used.

Preparation of vitamin solution: First, 0.386 g of disodium phosphate (Na_2HPO_4), 0.315 g of monosodium phosphate (NaH_2PO_4) and 500 mL of autoclaved deionized water was added in 1000 mL volumetric flask to prepare the buffer (NaP buffer (10 Mm, pH 7.1)). 0.8 mg 4- Amino benzoic acid, 0.2 mg D (+)- biotin, 2.0 mg nicotinic acid, 1.0 mg calcium D (+) pentothenate, 3.0 mg pyridoxine dihydrochloride, 2.0 mg thiamine was placed in a 100 mL volumetric flask and 20 mL of buffer was added. This solution was mixed thoroughly and stored at 4°C until use.

After the two autoclaved solutions were cooled down, they were mixed in a 2000 mL volumetric glass flask. 1 mL from each iron (II) chloride tetrahydrate and sodium sulphide nonahydrate stock solutions were added to this mixture by 0.22 µm filter sterilization. 10 mL from each vitamin and trace element solutions were also added with sterile pipette.

Potassium nitrate (KNO_3) has been used as a source of nitrate in nitrate reducing medium. Potassium nitrate solution has been added to the mixture above by 0.22 µm filter sterilization to correspond to 1 gram per liter. Finally, this solution was completed

to 1 liter by adding autoclaved water. It was stored at 4°C to be used as a nitrate reduction medium in the installation of bioreactors.

Potassium sulfate (KSO_4) was used as a source of sulfate. Potassium sulfate solution was placed to the mixture above by 0.22 µm filter sterilization to correspond to 1.8 g per liter. This solution also was completed to 1 liter by adding autoclaved water and stored at 4°C as sulfate reducing media.

3.5 Bioreactors

Anaerobic bioreactors have been prepared in set of four including different media as nitrate reducing, nitrate reducing with chicken manure, sulfate reducing and sulfate reducing with chicken manure.

Nitrate and sulfate reducing anaerobic bioreactors were prepared by adding the amounts in Table 3.1 and Table 3.2 to 1000 mL volumetric glass reactor bottles.

Table 3.1 : Content of nitrate reducing anaerobic bioreactors.

Bioreactor	Nitrate Media (mL)	Aquifer (g)	Contaminated Soil (g)	Pre-treated Soil (g)	Enrichment (mL)
Control-aquifer (A)	700	50	-	-	-
Control-contaminated soil (CSO)	700	-	25	-	-
Control-pretreated soil (PTSO)	700	-	-	25	-
Control-enrichment (E)	700	-	-	-	2
Contaminated soil (A+CSO+E)	700	50	25	-	2
Pre-treated soil (A+PTSO+E)	700	50	-	25	2

Table 3.2 : Content of sulfate reducing anaerobic bioreactors.

Bioreactor	Sulfate Media (mL)	Aquifer (g)	Contaminated Soil (g)	Pre-treated Soil (g)	Enrichment (mL)
Control-aquifer (A)	700	50	-	-	-
Control-contaminated soil (CSO)	700	-	25	-	-
Control-pretreated soil (PTSO)	700	-	-	25	-
Control-enrichment (E)	700	-	-	-	2
Contaminated soil (A+CSO+E)	700	50	25	-	2
Pre-treated soil (A+PTSO+E)	700	50	-	25	2

Anaerobic bioreactors for nitrate reducing with chicken manure have been prepared by putting the following amounts in Table 3.3 into 500 mL volumetric glass reactor bottles.

Table 3.3 : Content of nitrate reducing bioreactors with manure.

Bioreactor	Nitrate Media (mL)	Manure (g)	Non-treated Soil (g)	Pre-treated Soil (g)	Enrichment (mL)
Control-manure (M)	250	4	-	-	-
Control-contaminated soil (CSO)	250	-	18	-	-
Control-pretreated soil (PTSO)	250	-	-	18	-
Control-enrichment (E)	250	-	-	-	2
Contaminated soil (M+CSO+E)	250	5	18	-	2
Pre-treated soil (M+PTSO+E)	250	3.6	-	18	2

Sulfate reducing with chicken manure bioreactor set was made with the addition of quantities given in Table 3.4 to glass reactor bottles with a volume of 2000 mL.

Table 3.4 : Content of sulfate reducing bioreactors with manure.

Bioreactor	Sulfate Media (mL)	Manure (g)	Non-treated Soil (g)	Pre-treated Soil (g)	Enrichment (mL)
Control-manure (M)	1000	8	-	-	-
Control-contaminated soil (CSO)	1000	-	36	-	-
Control-pretreated soil (PTSO)	1000	-	-	36	-
Control-enrichment (E)	1000	-	-	-	4
Contaminated soil (A+CSO+E)	1000	10.1	36	-	4
Pre-treated soil (A+PTSO+E)	1000	7.2	-	36	4

The reactors prepared as stated above were covered with a glass cover in the medium neck and the side necks were closed in silicone septa. The reactors were flushed with nitrogen gas for 20 minutes to create a completely oxygen-free environment. In order to prevent gas inlet and outlet, the caps were secured with parafilm. All the reactors were incubated under mesophilic conditions (37°C).

3.6 Gas Analysis

The gas measurements of bioreactors were performed once a week at the time of their first installation and then once every two weeks. Total gas measurements have been made with Lutron PM-9107 7000 mbar pressure and vacuum gauge (manometer). The biogas composition was determined by Agilent 6850 gas chromatography with thermal conductivity detector (GC-TCD), a technique used in inorganic gas and small hydrocarbon analysis. Accuracy and calibration curve studies for gas chromatography are carried out with reference standards.

3.7 Enrichment Method

The enrichment culture study was carried out separately for sulfate and nitrate reducing media at 37°C. Each study was performed duplicate. Bushnell Haas Broth, containing magnesium sulphate, calcium chloride, monopotassium phosphate, dipotassium phosphate, ammonium nitrate and ferric chloride, were used. Also, liquid samples were taken from the anaerobic microcosmos, which were operated in nitrate reducing and sulfate reducing media for approximately 200 days in mesophilic conditions (Seyis, 2019; Çalışıyor, 2018), were used for the enrichment.

Liquid medium was prepared by adding 3.2716 g Bushnell Haas Broth to 1 liter distilled water. 80 mL media, 1% crude oil (0.8 g) were placed in 120 mL volume serum bottle. Bottles sealed with aluminum foil and autoclave tape have been autoclaved at 121°C for 15 minutes. After cooling, 2 mL liquid sample from microcosmos were placed the bottles and bottle were closed with septa and then flushed with nitrogen gas for 10 minutes. These bottles were incubated at determined temperature and 100 rpm. The optical density measurements of these samples were performed every two days with the UV spectrophotometer set at 660 nm. After 1 week, 2 mL of this culture was transferred into 100 mL of liquid medium. This transfer was repeated 4 times. Afterwards the volume was scaled up to 1 liter to have enough amount of enrichment culture to inoculate bioreactors.

3.8 Ion Analysis

In order to perform ion analysis, a sample was taken from bioreactors every 2 weeks and stored at minus 20°C until the analysis was performed. Nitrite and nitrate analyzes were performed in samples taken from bioreactors set up with nitrate reducing media and sulfate analyzes were performed in bioreactors formed with sulfate reducing media.

Sulfate analyzes were carried out under the scope of Method 9038 based on the principle of converting sulfate ion into barium sulfate suspension under controlled conditions and measuring the resulting turbidity with spectrophotometer. For these analyzes, the conditioning reagent was prepared first. 30 mL concentrated HCL was added slowly to 300 mL distilled water, then 100 mL ethanol and 75 g NaCl were placed, Finally 50 mL glycerol was added and mixed. After the control reagent was

prepared, the sample to be analyzed was diluted to 100 mL and placed in 250 mL Erlenmeyer flask. 5 mL control reagent was added to the solution and mixed. Barium chloride added while stirring continued. After stirring for one minute, the solution was placed on a UV spectrophotometer at 420 nm. The turbidity was measured for 4 minutes at 30 second intervals, the highest value recorded.

Nitrite analyzes were performed according to Method 8507. Hach DR / 2010 Portable Datalogging Spectrophotometer was used for these analyzes. Samples taken from the nitrate reducing medium were centrifuged and diluted at a rate of 1/100 in 10 mLvolumetrik flask. Program 371 was set on the spectrophotometer and the wavelength was adjusted to 507 nm. The diluted sample was put in the sample cell for use as a blank and placed into the cell holder. After zeroing with the blank sample, the sample cell was removed from the cell holder. Nitriver3 reagent was added to the sample cell and shaken until thoroughly dissolved. The sample cell was placed in the cell holder and a twenty-minute reaction time was initiated by pressing shift timer. After the twenty-minute reaction time was completed, press the read key to get the results in mg/L.

Method 8171 was used for nitrate analyzes. These analyzes were performed by Hach DR / 2010 Portable Datalogging Spectrophotometer. Program number 353 was entered and the wavelength was set to 400 nm. 1 / 100 diluted sample was put into 10 mL sample cell for use as blank and placed into the cell holder. After zeroing with the blank sample, Nitraver5 nitrate reagent was added to the sample cell. A one-minute mixing time is started by pressing the shift timer. Sample cell was placed in the cell holder after the end of one minute. A five-minute reaction time was initiated by shifting the shift timer. Results were taken by pressing read, after the reaction time was over.

3.9 Microbial Community Analysis

Total nucleic acid extraction, PCR Amplification of 16S rRNA genes, specific target genes, quantitative and real time PCR were carried out for aquifer, petroleum contaminated soil and samples taken from anaerobic bioreactors during the study. HRM analysis was conducted to determine the microbial community profile of bioreactor samples. Metagenomic analyses were performed to observe microbial community composition.

3.9.1 Total nucleic acid extraction

Total nucleic acid extraction was done by manual extraction using the CATB method for nucleic acid extraction. 50-100 mg sample was put into 2 mL screw-capped tube containing metal beads. 500µl of CATB solution was added to the tube and homogenized for 2 minutes at 7000 rpm. After two minutes of centrifugation at 14000g, the supernatant section was taken into a clean tube and vortexed with the addition of 500 µl Guanidium iythiocyanate (3M) and 20 µl Proteinase-K (20 mg/mL) on it. The sample solution was incubated at 55°C for one hour and vortexed every 10 minutes. After incubation at 55°C, it was incubated at 95°C for 15 minutes and vortexed every 5 minutes during incubation. Then the sample was cooled and centrifuged for 1 minute. The supernatant was taken into a new tube and vortexed with the addition of 500 µl of isopropanol (binding buffer) on it. Clean DNA column was put into new eppendorf tube and sample solution was placed into column. The sample was centrifuged for 1 minute at 14000 g and the filter was removed. This step was repeated until all sample finished. This stage was repeated until the sample was completely finished. Extracted nucleic acid was washed by using Guanidium isothiocynate isopropanol solution and washing buffer through the NA column. 500 µl washing buffer1 (GITIP) was added on the column, centrifuged for 1min and filtrate was removed. Then 500 µl washing buffer2 (WB2) was added on the column and centrifuged for 30secs and filtrate was removed. The washing 2 was repeated and the column was transferred to a new ependorf tube and centrifuged for one minute. Column transferred into the new micro centrifuge tube and 100 µl eluation buffer was added on it. Then the column was incubated at 50-60°C for 2-3 minutes and centrifuged for one minute at 14000 g. The column was removed the filtrate includes the isolated total nucleic acid. The sample was stored at -20°C for later analysis.

3.9.2 qPCR and specific targeted genes

Amplification of 16S rRNA and specific target genes was done using polymerase chain reaction. Universal primers pA (5’-GAGTTTGMTCCTGGCTCAG-3’) – pH (5’-ACGGYTACCTTGTTACGACTT-3’) was used for 16S rRNA amplification. The assA and BCR primers, which properties were given in Table 3.5, were used to observe hydrocarbon degrading genes. The extracted sample was diluted 1/10 with distilled

water. The reaction volume for qPCR was adjusted to 10 µl. Reaction volume was prepared by mixing 0.06 µl of 100xSybr Green dye, 5 µl of 2x Bioline mix, 2.84 µl of MGW, 0.05 µl of forward primer , 0.05 µl of reverse primer and 2 µl of template NA. After the mixture was centrifuged for 20 seconds, it was placed on well plate 96. Roche LightCycler 480 II was used for qPCR analysis. Program was set as:10 minutes pre-incubation at 95^0C, 45 cycles of amplification as 15 secs denaturation at 95°C, 20 secs annealing at 54°C and 30 secs extension at 72°C, following with melt curve analysis between 65°C – 95°C.

Table 3.5 : Sequences, target organism and annealing temperatures of target genes (Kolukırık, 2010).

Primer	Gene Sequence	Target Gene	Target Organism	Annealing Temp.
assA_1578f	5'-KGAYTTTGAGSASCTTTTCS-3'	assA	Aliphatic H.C. Degraders	56^0C
assA_1967r	5'-TCGTCCACRTARTCGTCGTC-3'			
BCR697f	5'-GTYGGMACCGGCTACGGCCG-3'	bcrA	Aromatic H.C. Degraders	55^0C
BCR1178r	5'-TTCTKVGCIACICCDCCGG-3'			

The reaction volume of RT-qPCR was set as 10 µl. This volume was composed of 0.06 µl of 100xSybr Green dye, 5 µl of 2xBioline RT mix, 0.05 µl of forward primer, 0.05µl of reverse primer, 2.54 µl of MGW, 0.3 µl of RT-RIN mix and 2 µl of template. Q-PCR analysis for 16S rRNA amplification was performed with Bio-Rad CFX Connect Instrument (Bio-Rad Inc., USA). Program was set as: pre-incubations for 30 minutes at 45°C and 3 minutes at 95°C, 45 cycles of amplification as 15 secs denaturation at 95°C, 25 secs annealing at 54°C and 30 secs extension at 72°C, following with melt curve analysis between 65°C – 95°C.

3.9.3 HRM analysis

The nested PCR approach was used to amplify microbial rDNAs (Kolukirik et al., 2011). The universal primaries, pA and pH, were used for the first cycle of qPCR. The vF (5'-CCTACGGGAGGCAGCAG-3') and vR (5'-ATTACCGCGGCTGCTGG-3') primers were used for the second cycle of PCR. The reactions involved 0.2 mM dNTP mix, 1.5 mM MgCl2, 0.5 mM of each primer, 1x Reaction Buffer, 0.1 U Fast Start

Proof Reading Recombinant Taq DNA Polymerase, 1x EvaGreen and 5 ng/mL DNA template. All reactions was performed with Bio-Rad CFX Connect Instrument and Biospeedy® HRM Master Mix (Bioeksen Ltd. Co., Turkey). Thermal cycling program was set as: 3 min pre-incubation at 95°C, 40 cycles of 20 secs at 95°C, 20 secs at 53°C and 30 secs at 72°C. To determine whether the expected product was amplified, melting curve analyses were performed at 60°C-95°C with a fluorescence reading ratio of 0.1 C/acquisition. HRM profiles were obtained as described by Reja et al. (2010). Microbial community profile dendrograms based on the similarity between HRM profiles and PCA ordinations were obtained with MINITAB 14 Software.

3.9.4 Metagenomic 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.

For the metagenomics analysis of raw data of sequencing, first the reverse and forward sequences are concatenated, than further cleaned, reduced and amazed via Mothur (ver 1.41.3) (Schloss et al., 2009). Raw sequence data is cleaned with the steps grooming, pairing, quality control and trimming via public server at usegalaxy.org. Cleaned data is analyzed using the Bayesian classifier within Mothur. After operational taxonomic unit (OTU) picking and taxonomic assignments using the SILVA rDNA database, the OTUs were binned in to phylotypes.

4. RESULTS AND DISCUSSIONS

4.1 Physical and Chemical Characterization

Chicken manure, aquifer, contaminated soil and pre-treated contaminated soil samples used in bioreactors were first subjected to characterization analysis. Total solids, volatile solids and COD analyses were performed in chicken manure. The results of these measurements are as follows: COD value is 31300 mg/L, TS is 23.2% and VS is 16.7%. In the other three samples pH, total kjeldahl nitrogen, total phosphorus, C/N ratio, sulfate, total solids, volatile solids, total organic carbon, dissolved organic carbon, polycyclic aromatic hydrocarbons, polychlorinatedbiphenyls, total petroleum haydrocarbons were measured. The results of these measurements are given in Table 4.1.

pH is one of the parameters affecting microbial diversity and activity. Soil degrading bacteria prefer alkaline environment rather than acidic environment. (Patel et al., 2013). It is seen from the table that pH is neutral in aquifer while contaminated soil and pre-treated soil have an acidic environment. Bamforth and Singleton (2005) reported that degradation can also occur in acidic environment. In some studies, it has been observed that some bacteria degrade PAH better in acidic environment (Moscoso et al., 2012).

The total solid (TS) is 80%, 96% and 93%, respectively, in aquifer, soil and pre-treated soil. Pre-treatment with persulfate (PS) shows a very small decrease in TS value. When the volatile solid (VS) values are examined, it is observed that VS is only 1% in the aquifer and 42% and 30% in the contaminated soil and pre-treated soil respectively. It is observed that PS oxidation is more effective in VS value.

The amount of nutrients such as phosphorus and nitrogen varies in different soils (ÁlvarezLM et al., 2017). These nutrients, essential for microbial growth and therefore biodegradation, are often found in small amounts in hydrocarbon-contaminated soil (Brown et al., 2017). An increase in the total nitrogen was observed with pretreatment, while an increase in C / N ratio was observed. It is understood that the amount of carbon is also increased with pre-treatment.

In pretreatment with persulfate, the sulfate radical is produced through the activation of persulfate (Achugasim et al., 2011).

$$S_2O_8^{-} + 2e^{-} \rightarrow 2\ SO_4^{2-} \quad (4.1)$$

It is seen from the table that the sulfate value is approximately the same in aquifer and contaminated soil. However, in accordance with previous studies, it appears that pre-treatment with persulfate increased the sulfate value in the soil by about 8 times.

Table 4.1 : Characterization of samples.

Parameter	Unit	Aquifer	Pretreated soil	Non-treated soil
pH		7	4	5
Total Solids (TS)	%	80	93	96
Total Volatile Solids (TVS)	%	1	30	42
Total Phosphor (P)	mg/kg	124	98	119
Total Kjeldahl Nitrogen (TKN)	mg/kg	340	320	280
C/N ratio	mg/kg	42	638	614
Sulfate (SO_4^{2-})	g/kg	37	1036	137
Polycyclic aromatic hydrocarbons (PAH_S)	mg/kg	<0.32	<0.32	<0.32
Polychlorinatedbiphenyls (PCBs)	mg/kg	<0.035	<0.035	<0.035
Total Organic Carbon (TOC)	g/kg	6	352	312
Dissolved Organic Carbon (DOC)	mg/kg	162	1422	53
Total Petroleum Hydrocarbons (TPHs)	mg/kg	1176	73743	68516

Polycyclic aromatic hydrocarbons (PAH_S) and polychlorinatedbiphenyls (PCBs) analysis results were below the detection limit in all three samples. The amount of dissolved organic matter in the aquifer is almost three times more than the contaminated soil. However, the DOC value of pre-treated soil is almost 27 times more than the value of contaminated soil. This situation leads to the expectation that the degradation of the pre-treated soil will be much easier.

The total organic carbon is 6 g / kg, 352 g/kg and 312 g / kg in aquifer, pre-treated soil and contaminated soil respectively . As seen in previous studies, there is not a very large change in TOC values before and after oxidation (Kakosová et al., 2016). In addition, previous studies have shown that the aquifer mostly has light hydrocarbons (Tischer et al., 2013). The TOC and TPH values of the aquifer were low conveniently.

The results of petroleum hydrocarbons aliphatic and aromatic fractions are detailed in Table 4.2. It can be seen that both contaminated soil and pretreated soil mostly contain complex structure of aromatic and aliphatic compounds.

Table 4.2 : Aliphatic and Aromatic Fractions.

Parameter	Unit	Aquifer	Pre-treated soil	Non-pretreated
C5-C8 Aliphatic Fraction	mg/kg	67.3	<60	34.1
C8-C16 Aliphatic Fraction	mg/kg	286	16100	10600
C16-C35 Aliphatic Fraction	mg/kg	<30	83200	49800
C5-C9 Aromatic Fraction	mg/kg	9.1	<80	<16.0
C9-C16 Aromatic Fraction	mg/kg	40	4290	999
C16-C35 Aromatic Fraction	mg/kg	<30	50100	10800

4.2 Gas Production of Bioreactors

Cumulative biogas production of bioreactors without manure is given in Figure 4.1 and Figure 4.2. The highest cumulative biogas production under sulfate reducing conditions is observed in control-contaminated soil (CSO) as seen in Figure 4.1. The lowest gas production occurs in control-enrichment (E). Figure 4.2 shows the production of cumulative biogas under nitrate reducing without manure. The highest gas production is observed here in control-pretreated soil (PTSO), while the lowest gas production is observed in E. When these two reducing media are evaluated together in terms of total gas production, it is observed that PTSO in the nitrate reducing medium has the highest gas production. The lowest gas production in both reducing conditions were observed in E with the same value. The cumulative biogas production is slowed after the 60^{th} day.

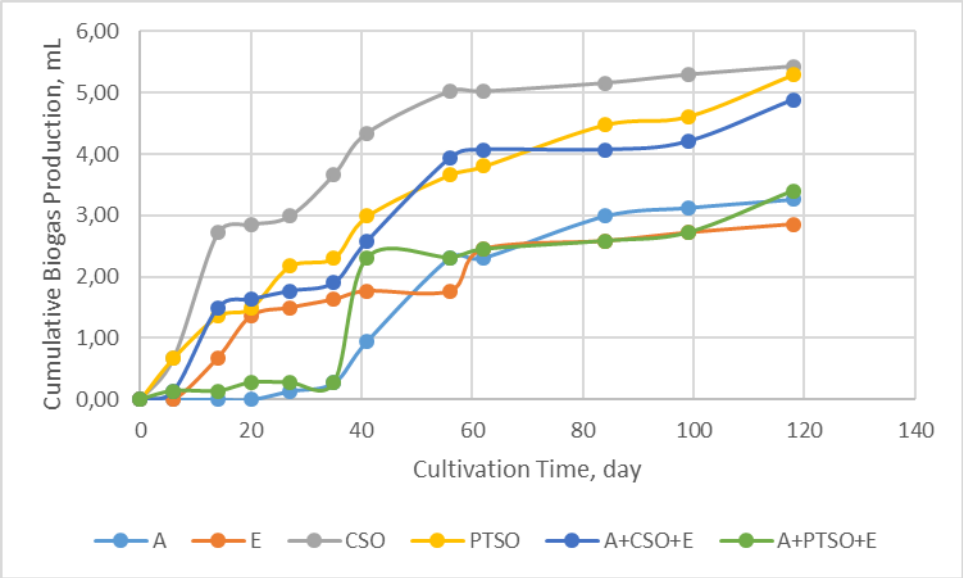


Figure 4.1 : Cumulative biogas production under sulfate reducing condition.

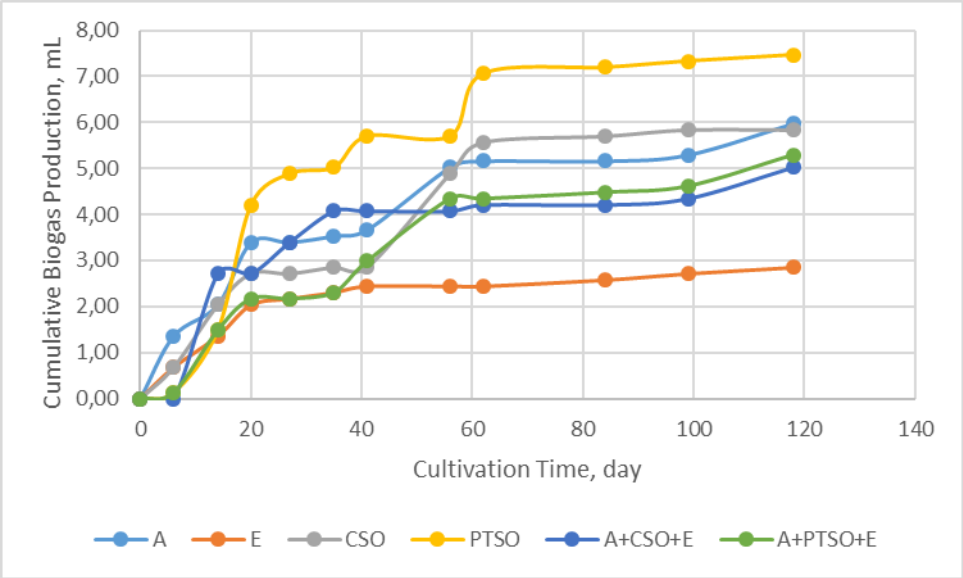


Figure 4.2 : Cumulative biogas production under nitrate reducing condition.

Cumulative biogas productions of bioreactors with manure are shown in Figure 4.3 and Figure 4.4. As it is stated in Figure 4.3, the highest gas production is observed in pre-treated soil (PTSO), this is followed by CSO and the lowest gas production is observed in E. The highest biogas production and subsequent value were also observed at M+PTSO+E and PTSO in the nitrate reducing, but the lowest gas production was observed at contaminated soil (M+CSO+E) (Figure 4.4). When two different reducing environments were examined together, the highest

gas production (M+PTSO+E) and the lowest gas production (E) were observed under sulfate reduction conditions.

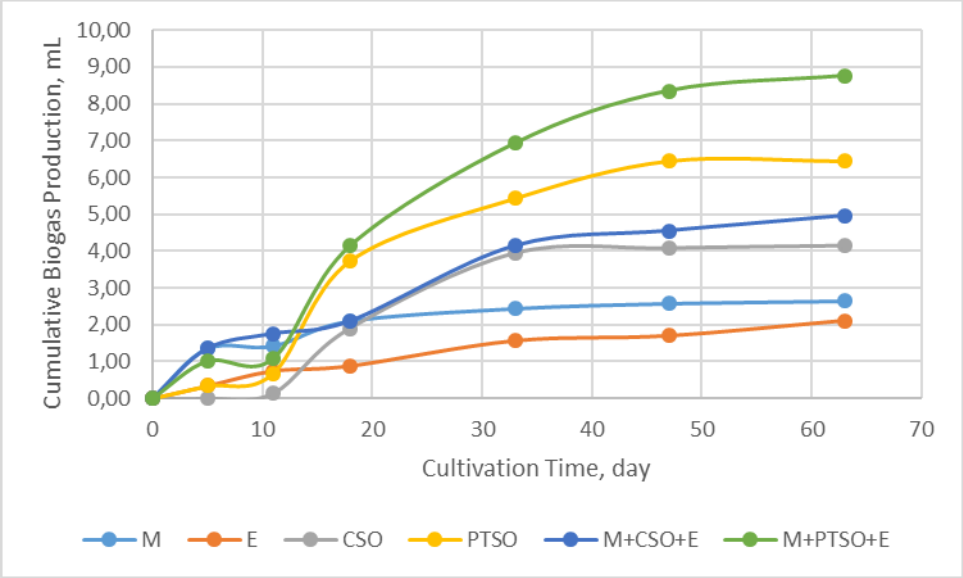


Figure 4.3 : Cumulative biogas productions in sulfate reducing with manure.

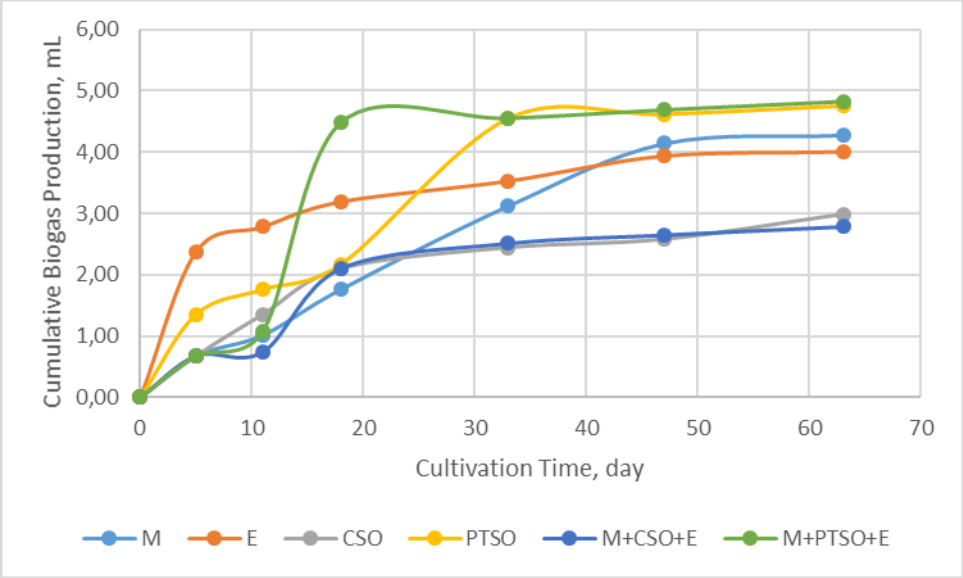


Figure 4.4 : Cumulative biogas productions in nitrate reducing with manure.

During the study, N_2 gas production for nitrate reducing conditions was monitored with GC instrument. Figure 4.5 demonstrates cumulative N_2 production for nitrate reducing without manure. The highest gas production is observed in the control-aquifer (A), while the lowest gas production is observed in E. The highest N_2 production in the nitrate reducing with manure is observed at M+PTSO+E and the lowest N_2 production is observed at E (Figure 4.6).

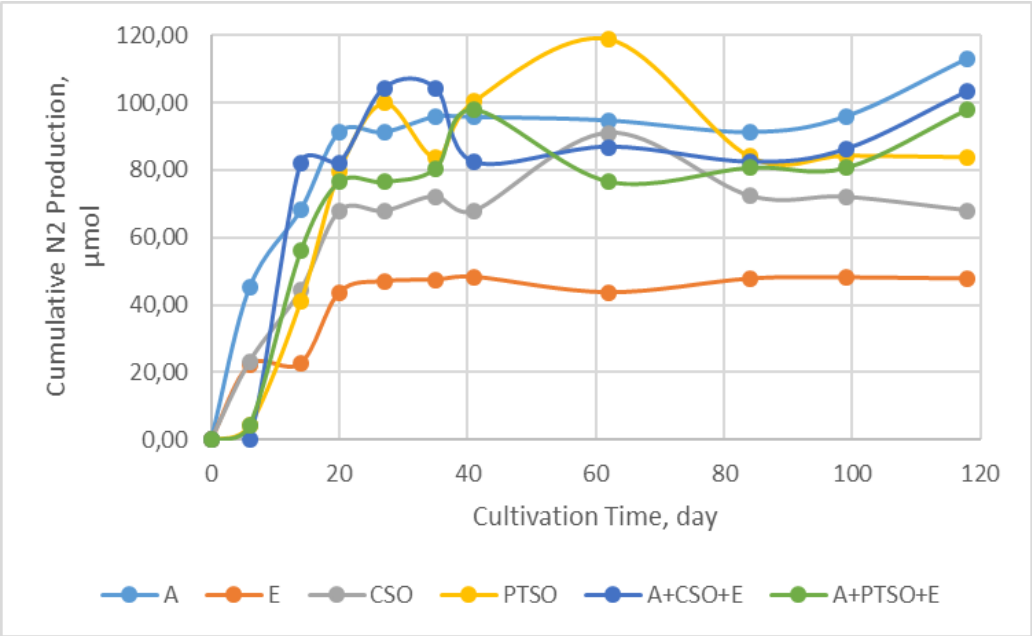


Figure 4.5 : Cumulative N_2 productions of nitrate reducing without manure.

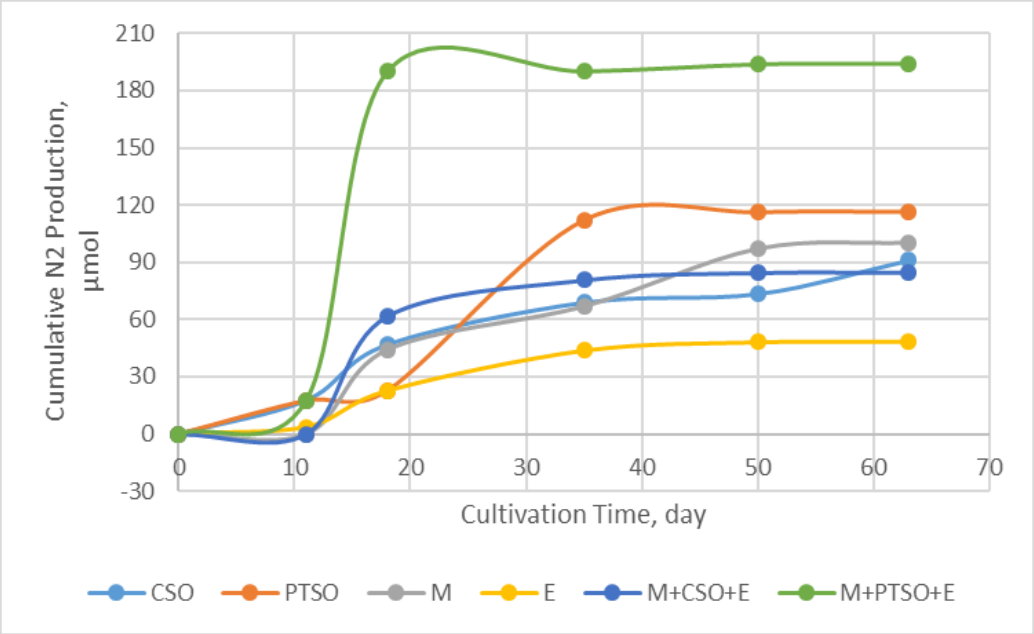


Figure 4.6 : Cumulative N_2 productions of nitrate reducing with manure.

The first two-month values were examined for comparing the N_2 production of these two sets. The highest N_2 production is observed here at M+PTSO+E in the fertilized set. This is followed by the PTSO of both sets with close values. In bioreactors prepared with treated soil, N_2 production is generally observed to be greater. The lowest gas production is seen in the E of the set without manure, followed by the E of the set with manure.

4.2.1 CO_2 production

Interest in bioremediation is high as it turns hydrocarbons in environmentally friendly CO_2. (Okafor et al., 2016). Some studies have shown that there are pure cultures examples of sulfate and nitrate reducing bacteria that can reduce monoaromatic hydrocarbons to CO_2 (Coates et al., 1997). CO_2 gas is produced during degradation of PAH, in compounds with low benzene rings, higher microbial degradation occurs and CO_2 minerilization increases (Brimo et al., 2018).

Cumulative CO_2 production for sulfate and nitrate reducing without manure are given in Figure 4.7 and Figure 4.8 respectively. For the sulfate reducing, the highest CO_2 production is observed in pre-treated soil (A+PTSO+E) and it is followed by contaminated soil (A+CSO+E) with a very close value. The highest CO_2 production in nitrate reducing medium is seen in control-pretreated soil (PTSO) and followed by pre-treated soil (A+PTSO+E) and contaminated soil (A+CSO+E) respectively. In the both reducing media, it is observed that CO_2 production was low in the other control sets except PTSO. CO_2 production at A in nitrate reducing medium is zero. The same is seen at E in the sulfate reducing medium.

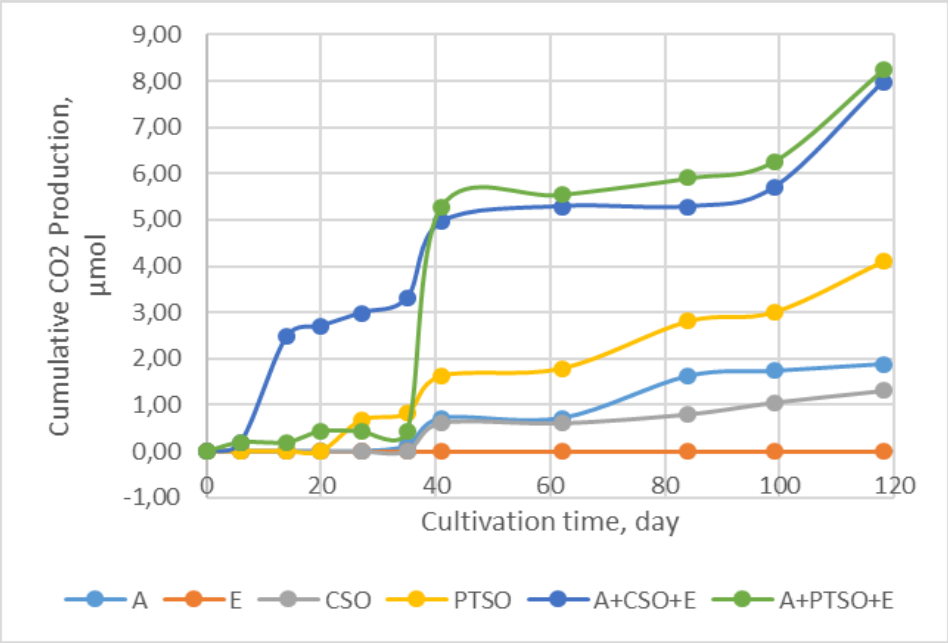


Figure 4.7 : Cumulative CO_2 productions of sulfate reducing conditions without manure.

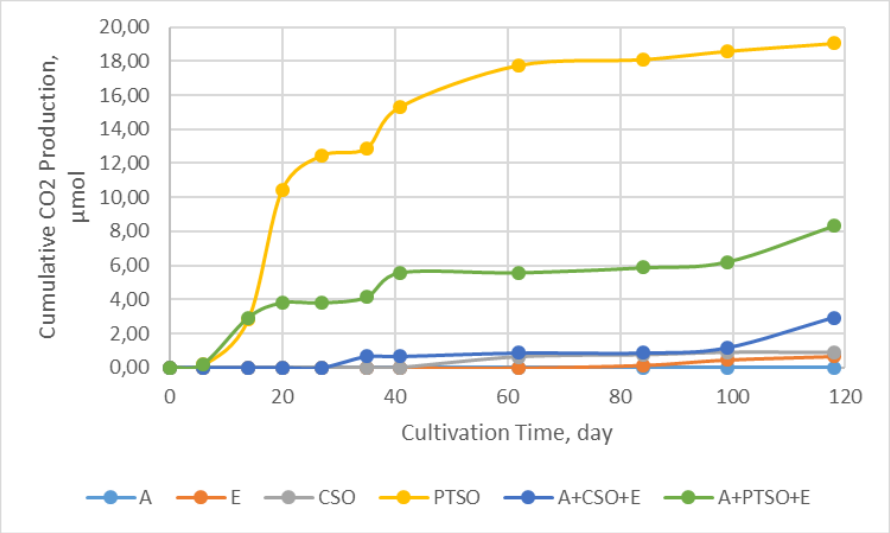


Figure 4. 8 : Cumulative CO_2 productions of nitrate reducing conditions without manure.

Figure 4.9 and Figure 4.10 shows CO_2 production in sulfate and nitrate reducing with manure. In the sulfate reducing with manure, production of CO_2 is started to increase at M+CSO+E and M+PTSO+E after 18th day and production slows after the 47th day. The highest CO_2 production is observed in M+PTSO+E and CO_2 production is not observed at E, CSO and PTSO. CO_2 production at M+CSO+E and M+PTSO+E has started to increase rapidly since the 11th day in nitrate reducing with manure and it become almost stable after 35th day. CO_2 production in control-manure (M) started to increase after the 18th day and the highest CO_2 production was seen here after the 50th day. Production of CO_2 is not observed at E while very low production is seen at CSO and PTSO on the 63rd day.

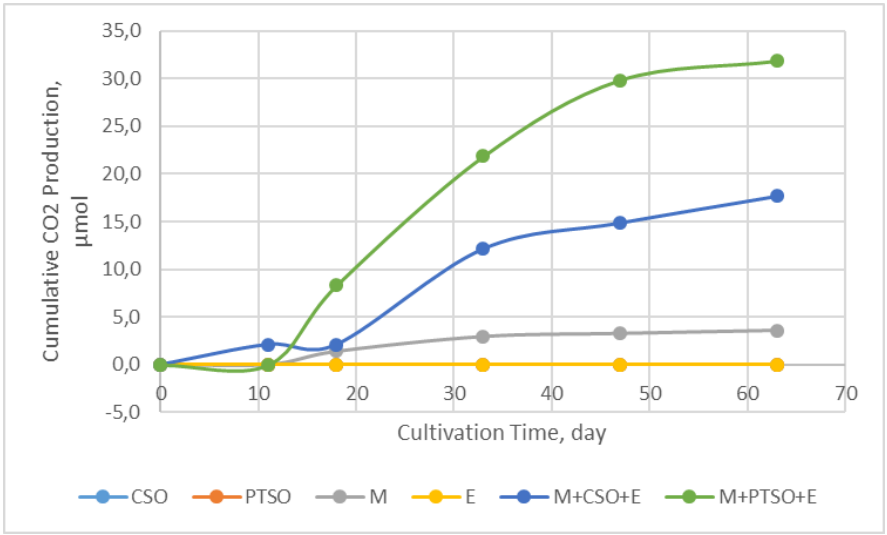


Figure 4.9 : Cumulative CO_2 productions of sulfate reducing with manure.

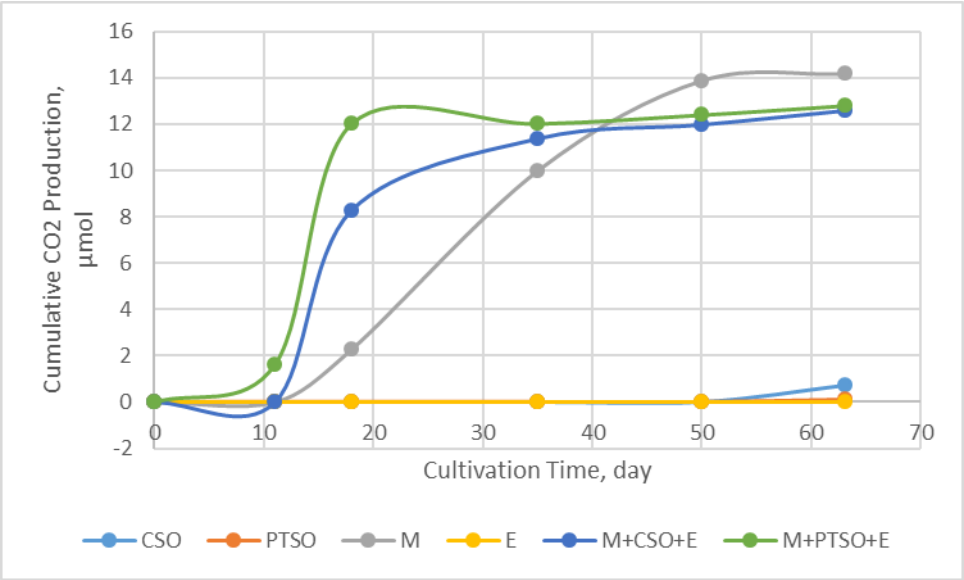


Figure 4. 10 : Cumulative CO_2 productions of nitrate reducing with manure.

The first two months results were evaluated in all sets to compare CO_2 production., the highest production was observed in the pre-treated soil (M+PTSO+E) under sulfate reduction conditions. Wloka et al. (2017) reported that the lower CO_2 production occured in sets with no added fertilizer. The results were found to be in line with previous studies.

4.2.2 CH_4 production

CH_4 production wasn't observed during the study in bioreactors without manure. The production of CH_4 in bioreactors with manure are seen in Figure 4.11 and Figure 4.12. Under the nitrate reducing conditions, CH_4 production was observed only in M. In here, CH_4 production, which began to increase rapidly after the 18th day, became almost stable after the 50th day. Production of CH_4 is observed at M, M+CSO+E and M+PTSO+E in sulfate reducing conditions. As seen from the figure, production in M and M+PTSO+E begins after the 11th day and proceeds in almost the same course and slowly. The highest CH_4 production is observed in M+CSO+E. Production began here after the 18th day and continued rapidly until the 63rd day. When this two different reducing media are evaluated together for CH_4 production, the highest production is seen to be in M under nitrat reducing conditions.

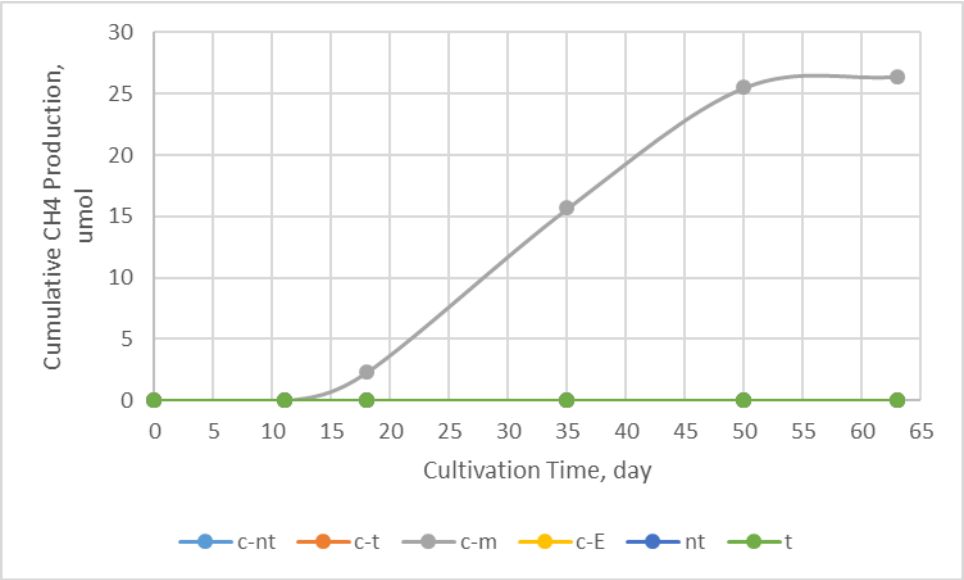


Figure 4.11 : Cumulative CH_4 productions in nitrate reducing with manure.

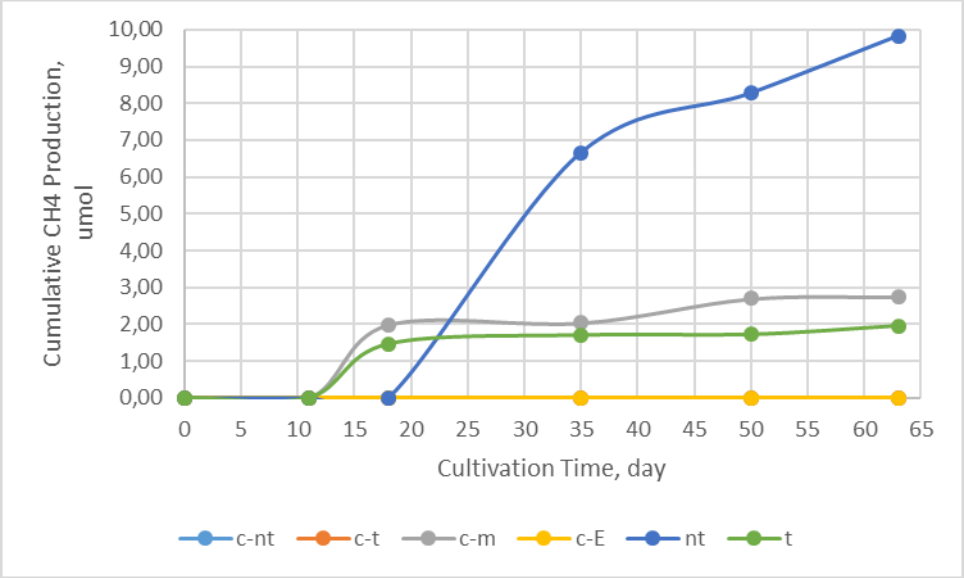


Figure 4.12 : Cumulative CH_4 productions in sulfate reducing with manure.

4.3 Ion Exchange in Bioreactors

4.3.1 Sulphate removal

In sulfate reduction conditions, sulfate is easily reduced to sulfite (SO_3^{2-}) and then to hydrogen sulfide (H_2S) (Madigan et al., 2012). Sulfate analyses in fertilized and non-fertilized bioreactors were conducted in sulfate reducing environments. Analysis of the fertilized bioreactor set was done in samples taken every two weeks, while samples taken once a month in non-fertilized

bioreactors were made. The results are shown in Table 4.3 and Figure 4.13. The control-pretreated soil (PTSO) and pretreated soil (t) of the bioreactors without manure have the highest removal with 94%. In the bioreactors with manure, the highest removal is observed in pre-treated soil (M+PTSO+E) with 91% and this is followed by control-pretreated soil (PTSO) with 89%. As can be seen from the table, the removal in both sets is very low in E's.

Table 4.3 : Sulfate removal rates in the bioreactors.

Without Manure (122 days)			With Manure (74 days)		
Sample	mmol	%	Sample	mmol	%
CSO	15.5	36	CSO	18.5	51
PTSO	260.2	94	PTSO	182.6	89
A	18.3	69	M	16.7	73
E	0.1	1	E	0.6	6
A+CSO+E	48.3	78	M+CSO+E	37.9	74
A+PTSO+E	277.6	94	M+PTSO+E	196.0	91

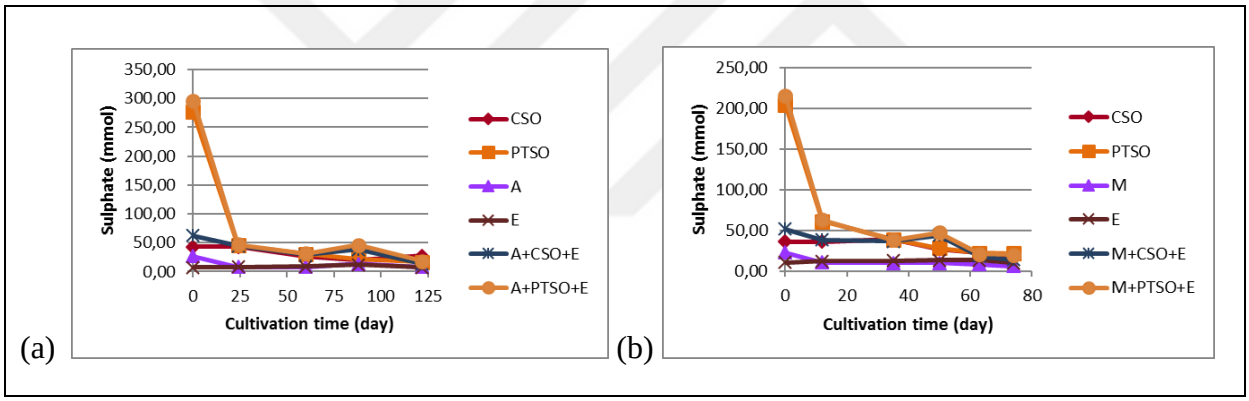


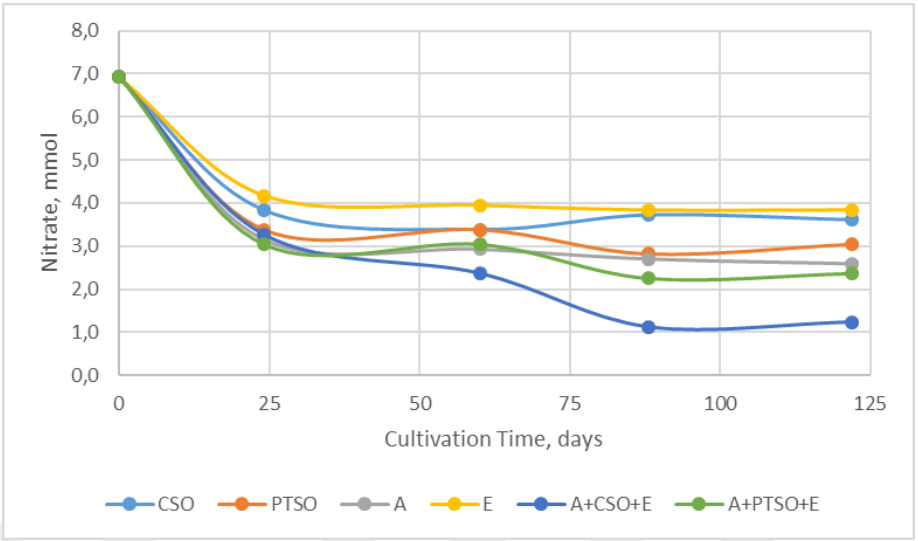
Figure 4.13 : Sulfate removal in bioreactors (a) without manure, (b) with manure.

4.3.2 Nitrate removal and nitrite production

The first product under nitrate reduction conditions is nitrite. Nitrate can also convert to N_2O, NO and N_2, which are in gas form as a result of denitrification (Madigan et al., 2012). Nitrate and nitrite analyses of bioreactors without manure were performed in samples taken monthly from bioreactors. They were conducted in nitrate reducing environments. The results of nitrate and nitrite analyses for non-fertilized bioreactors are seen in Figure 4.14. The highest nitrate removal is seen in A+CSO+E with 82.1%, followed by A+PTSO+E with 65.8%. The lowest nitrate removal was seen in E with 44.6%. On the other hand, when the nitrite results are

examined, it is observed that the highest production in here is at A+PTSO+E. This is followed by E, A, A+CSO+E, CSO, PTSO respectively.

(a)



(b)

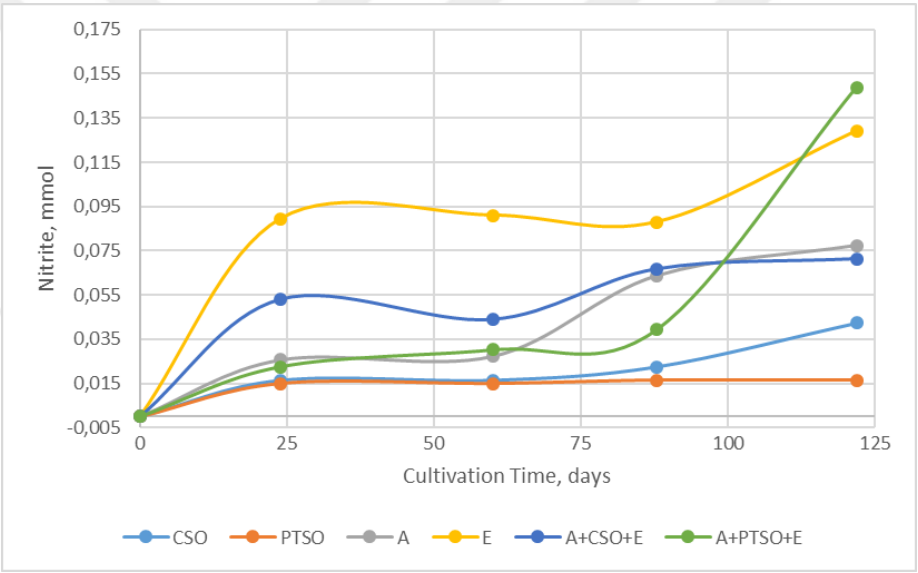
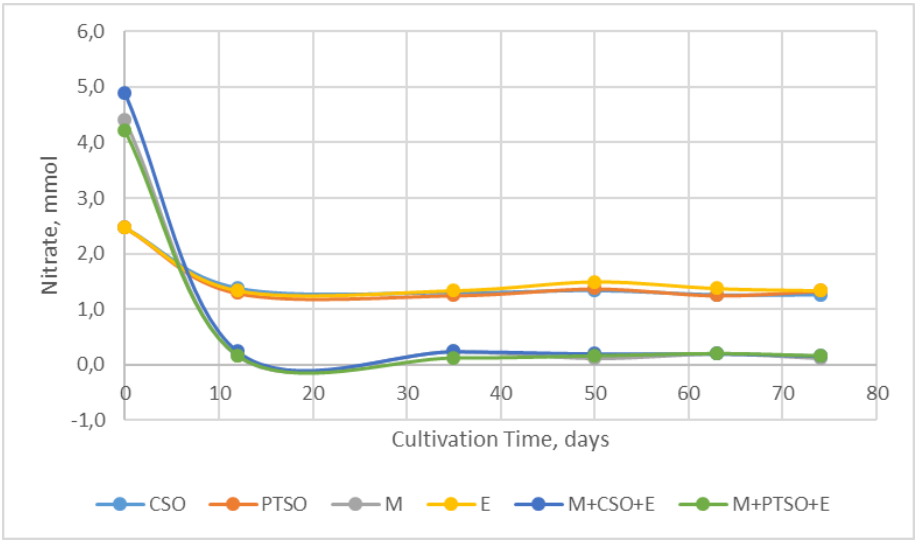


Figure 4.14 : (a) Nitrate removal, (b) nitrite production of bioreactors without manure.

Nitrate and nitrite analyses were performed in samples taken from fertilized bioreactors approximately every two weeks and the results were shown in Figure 4.15. The highest nitrate removal was observed at M with 97.3%. This is followed by M+CSO+E and M+PTSO+E with % 96.7 ve% 96.2 respectively. The lowest efficiency are seen in PTSO and E with 46.2%. As a result of nitrite analysis, it is observed from the graph that the highest production is at the E and this is followed by PTSO. The lowest production is seen in CSO and PTSO with the same value.

(a)



(b)

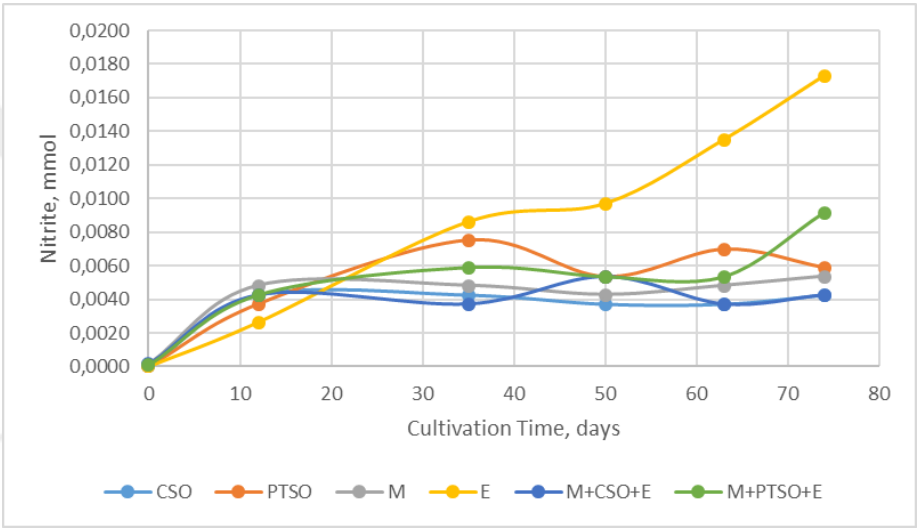


Figure 4.15 : (a) Nitrate removal, (b) nitrite production of bioreactors with manure.

4.4 TOC Removal

The use of different electron acceptors for hydrocarbon degradation results in different outcomes. It has been observed that low molecular weight alkanes in nitrate reducing conditions are more resistant than higher weight ones. Sulphate reducing conditions have been found to be effective on the longest chain alkanes (Hasinger et al., 2012).

TOC experiments to determine degredation in bioreactors were conducted at the beginning and at the end of cultivation. Table 4.4 and Figure 4.16 show TOC removal in non-fertilized bioreactor sets, which operated for 122 days. The highest efficiency is observed here in PTSO, which is pre-treated soil in nitrate reducing conditions with 51%. This is followed by

contaminated soil (A+CSO+E) in the sulfate reducing environment and control-contaminated soil (CSO) in the nitrate reducing environment with a 30% of removal efficiency. The lowest efficiency was observed in a mixture of contaminated soil (A+CSO+E) in nitrate reducing conditions.

Table 4.4 : TOC removal of bioreactors without manure.

	Nitrate Reducing		Sulfate Reducing	
	mmol	%	mmol	%
A	*	*	*	*
CSO	253	30	13	2
PTSO	483	51	250	27
A+CSO+E	3	0.3	258	30
A+PTSO+E	8	0.8	258	27

* TOC< 5000 mg/kg

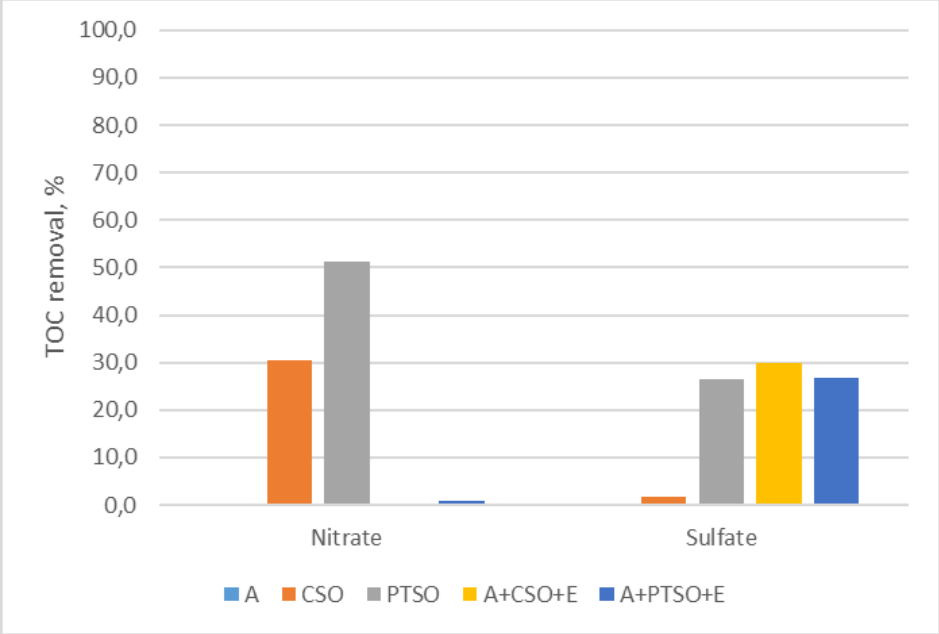


Figure 4.16 : TOC removal rates in the bioreactors without manure.

TOC removal in fertilized bioreactors, which operated for 74 days is shown in Table 4.5 and Figure 4.17. The highest degredation is observed in the sulphate reducing environment. 61% removal is seen in pre-treated soil (M+PTSO+E) and it is followed by control-manure(M) with 47%. Although the cultivation time was 200 days in previous microcosmos study where aquifer was used as a inoculum in sulfate reducing environment, 70% TOC removal was observed in the pre-treated soil (Çalışıyor, 2018). From this point of view, 61% TOC removal in 74 days in

manure added set shows that manure has a positive effect on removal. The lowest degredation is seen in control-pretreated soil (PTSO) in nitrate reducing environment with 1%.

Table 4.5 : TOC removal of bioreactors with manure.

	Nitrate Reducing		Sulfate Reducing	
	mmol	%	mmol	%
M	12	13	92	47
CSO	120	24	78	8
PTSO	7	1	120	11
M+CSO+E	41	7	68	6
M+PTSO+E	34	5	754	61

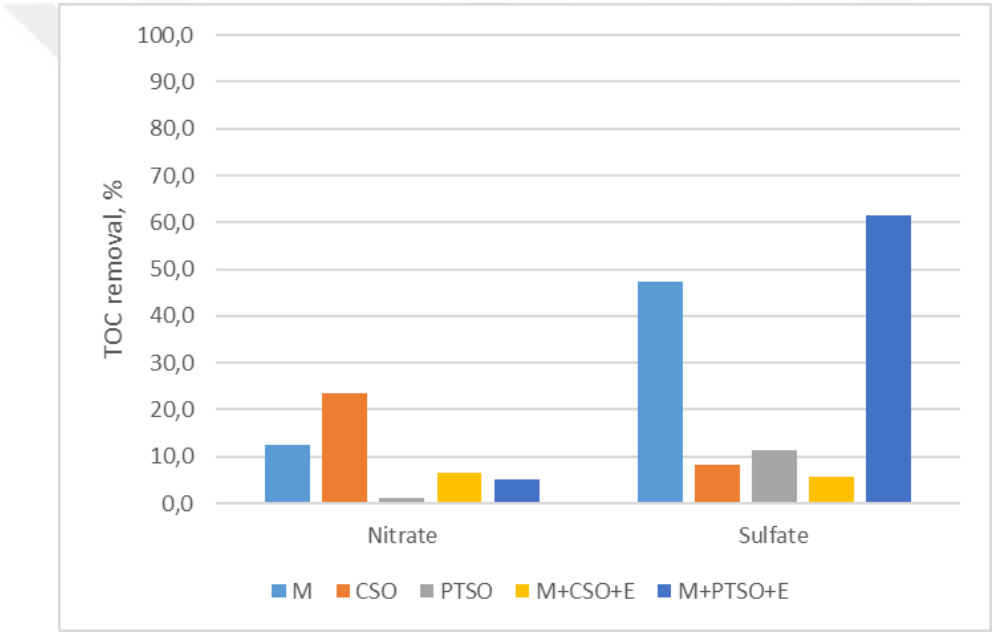


Figure 4.17 : TOC removal rates in the bioreactors with manure.

4.5 Microbial Community Analysis of Bioreactors

4.5.1 Results of metagonomic analysis

The order-based results of the aquifer from extracted nucleic acid, which expresses the activation of the microbial community, are given in Figure 4.18. As seen from the chart, *Actinomycetales* with 27.6%, *Pseudomanadales* with 10.8%, *Lactobacillales* with 10.6%, *Rhodobacterales* with 9.3% , *Rhizobiales* with 8.7% and *Burholderiales* with 6% were the

dominant orders. Gondek et al. (2016) have reported that several genera in the *Actinomycetales* order is functional in hydrocarbon degradation. Alkane sulfonate monooxygenase involvement in alkane degradation is mostly performed by members of *Actinomycetales, Pseudomonadales, Rhizobiales, Clostridiales* and *Burkholderiales* (Pal et al., 2017).

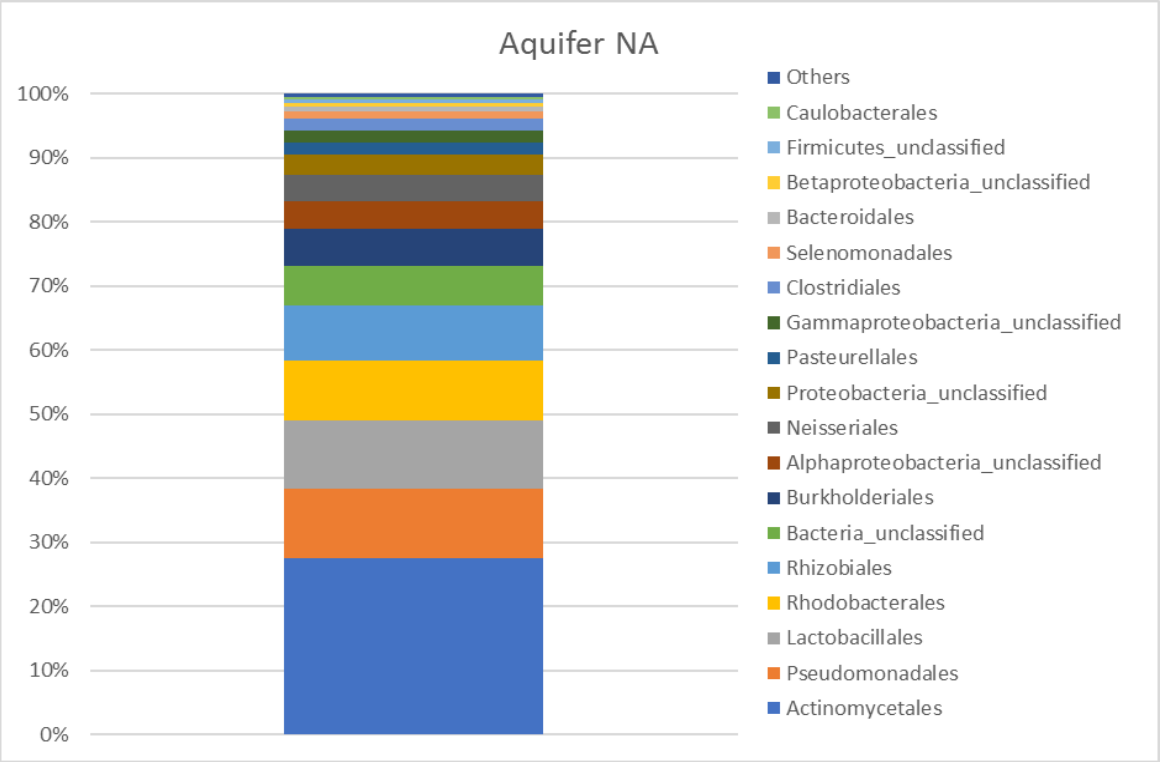


Figure 4.18 : Order-based results of aquifer.

The order-based results obtained from nucleic acid isolation of contaminated soil are given in Figure 4.19. Similar to the aquifer, the dominant order in contaminated soil was *Actinomycetales* (38.8%). It is followed by *Rhodobacterales*, *Rhizobiales* and *Burkholderiales* with 25%, 17.2% and 9.7% respectively. Abbasian et al. (2016) reported that petroleum-contaminated soils were places where *Actinomycetales, Rhizobiales*, and *Burkholderiales* were dominant, inhabited by a variety of microorganisms. They also stated that these microorganisms are beneficial for the degradation of aromatic and aliphatic hydrocarbons and for the treatment of oil-contaminated soil. In this study, it is observed that the actinomycetales were dominant order in both aquifer and contaminated soil samples and microbial community found in contaminated soil is comparable to previous studies.

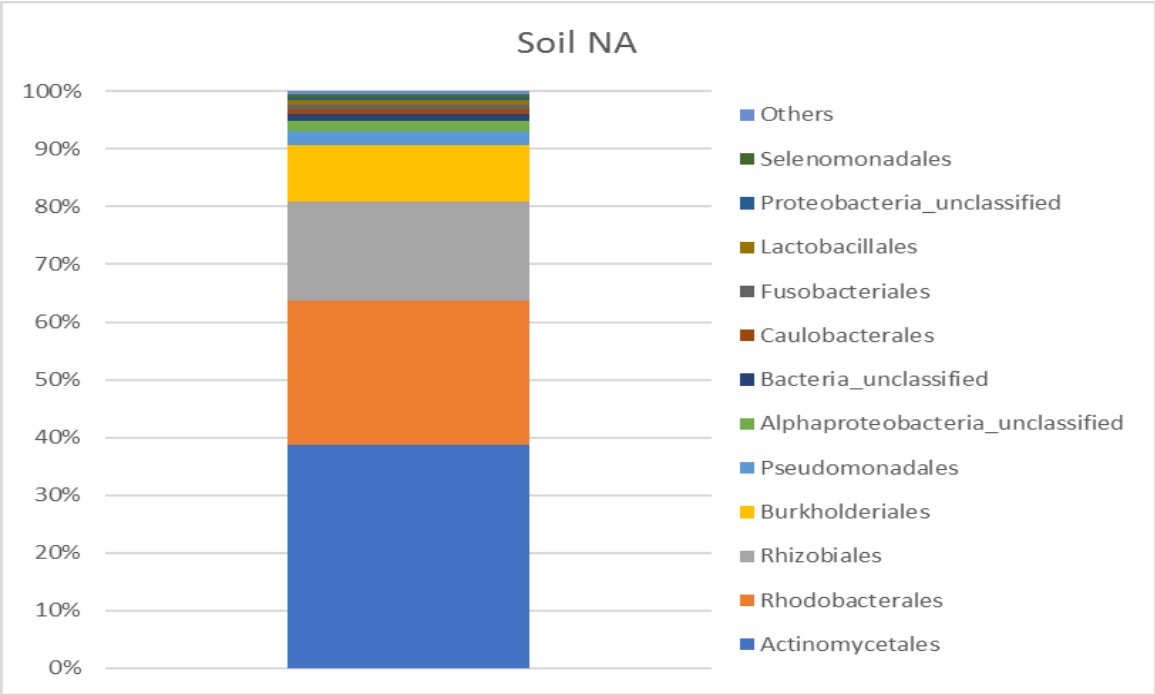


Figure 4.19 : Order-based results of metagenomic analyses in contaminated soil.

Enrichment N1 and enrichment N2 were performed with samples taken from aquifer and contaminated soil (nt) and aquifer and pre-treated soil (t) sets of microcosmoses in nitrate reducing media, respectively. These two enrichment were mixed and used as inoculum in bioreactors operated in nitrate reducing media. As can be seen from Figure 4.20, *Enterobacteriales* became the dominant order with 48.5% in the enrichment of aquifer and contaminated soil (Enrichment 1), while *Bacillales* became the dominant order with 45.2% in the enrichment of aquifer and pre-treatment soil (Enrichment 2). *Bacillales* with 32.6% in enrichment N1 and *Lactobacillus* with 34.5% in enrichment N2 were observed to be the second dominant species.

Enrichment S1 and enrichment S2 were carried out with samples taken from aquifer and contaminated soil (nt) and aquifer and pre-treated soil (t) sets of the microcosm under sulfate reducing conditions, respectively. These two enrichments were mixed and used as inoculum in bioreactors. The results of the next generation sequencing (NGS) analysis from nucleic acid isolation samples of these enrichments are given in Figure 4.21. It was observed that *Bacillales*, which has low abundance in aquifer and soil, became dominant with 19.75% in enrichment S1 and 37.2% in enrichment S2. *Bacillales* was followed by *Actinomycetales* and *Lactobacillales*, respectively, in both enrichment sample. Page et al. (2015) have reported that many members

of *Actinomycetales, Burkholderiales, Sphingomonadales, Bacillales* and *Pseudomonadales* were involved in the degradation of aromatic hydrocarbons, PCBs and alkanes. The genes for the desulfurization of hydrocarbons were carried on *Bacillales, Actinomycetales, Rhizobiales* and *Burkholderiales.* Furthermore, the genes that degrade long-chain alkanes (C20-C36) were found in *Actinomycetales, Pseudomonadales, Burkholderiales, Sphingobacteriales, Rhodobacterales, Oceanospirillales* and *Deinococcales* (Abbasian et al., 2016).

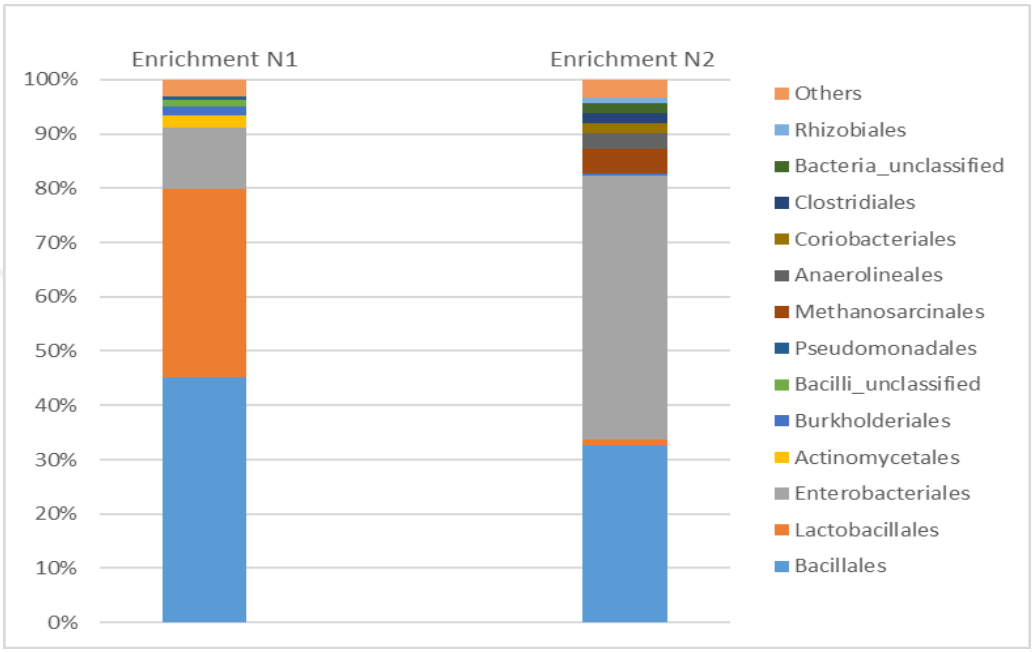


Figure 4.20 : Enrichment with samples from microcosmos in nitrate reducing conditions.

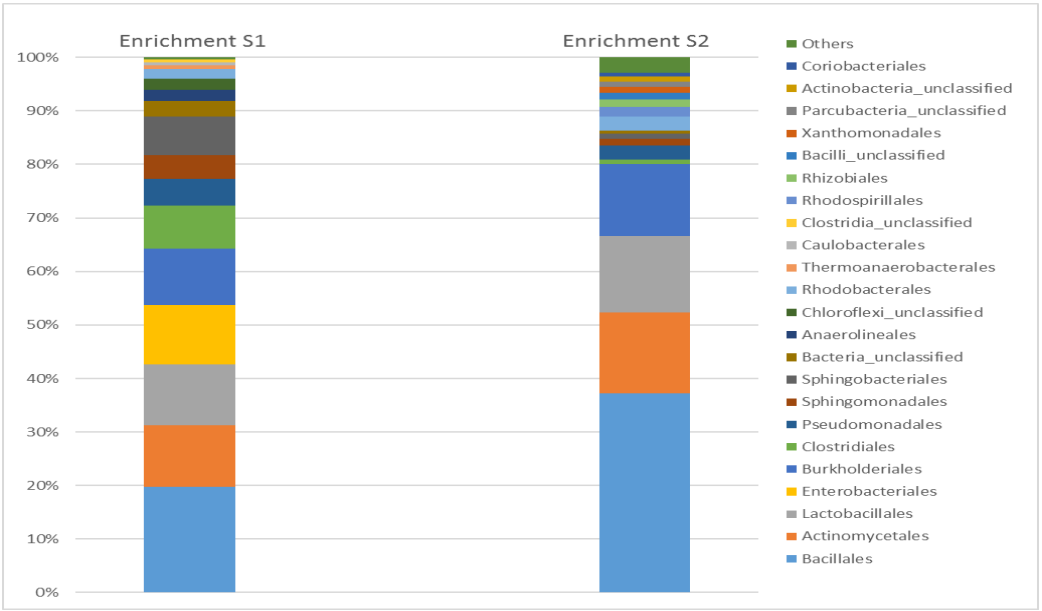


Figure 4.21 : Enrichment with samples from microcosmos in sulfate reducing conditions.

4.5.2 HRM analysis of bioreactors

In order to observe the change in microbial community profile during bioreactor operation, HRM analysis was performed using the data of qPCRs established with VFVR primer. Dendograms visualizing microbial abundance and microbial activity data were used in determining similarities between cultures. Figure 4.22 shows the similarity due to microbial abundance in bioreactors without manure. As seen from the figure, the overall similarity based on microbial abundance is 53.9% and three main groups are observed. In the first group, there are 38 samples with 97.7% similarity. There are 45 samples in the second group with 95% similarity and only one sample in the third group.

The overall similarity is 75.8% based on microbial activity in bioreactors without manure (Figure 4.23). It is observed that there are four main groups based on microbial activity. There are 46 samples with 97.4% similarity in the first group, 16 samples with 97.6% similarity in the second group, 21 samples with 98.8% similarity in the third group, and only one sample in the fourth group.

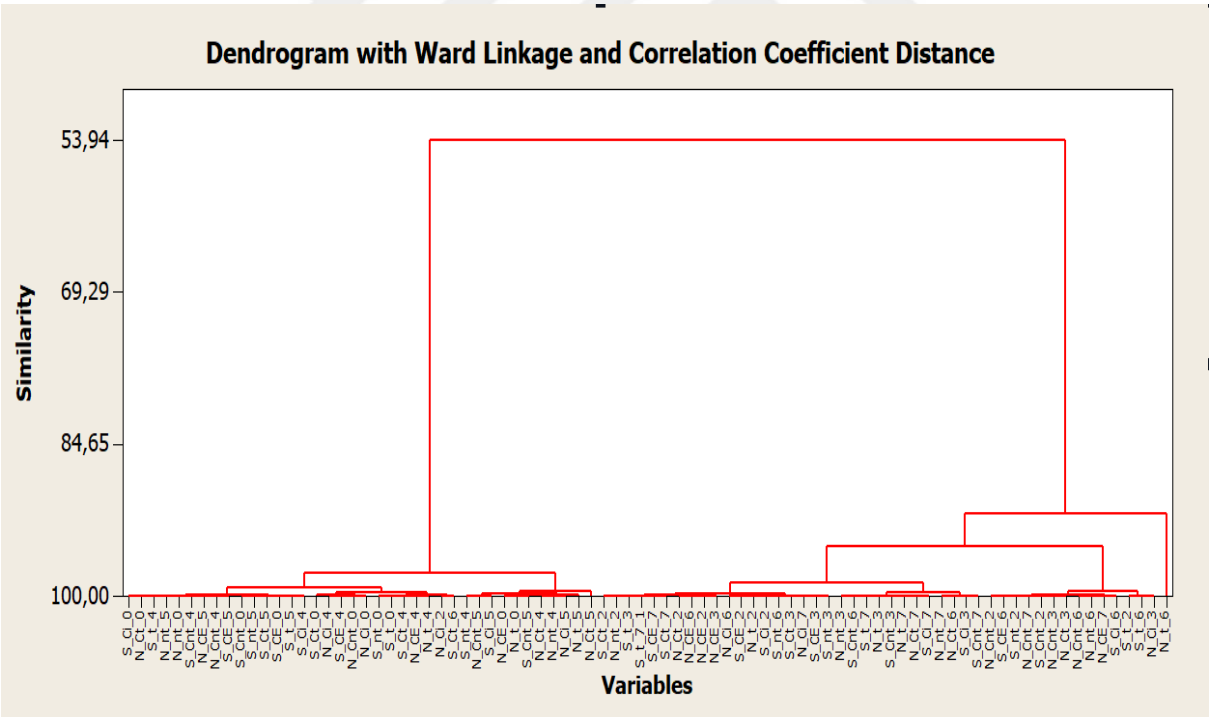


Figure 4.22 : Microbial community profile of bioreactors without manure based on microbial abundance.

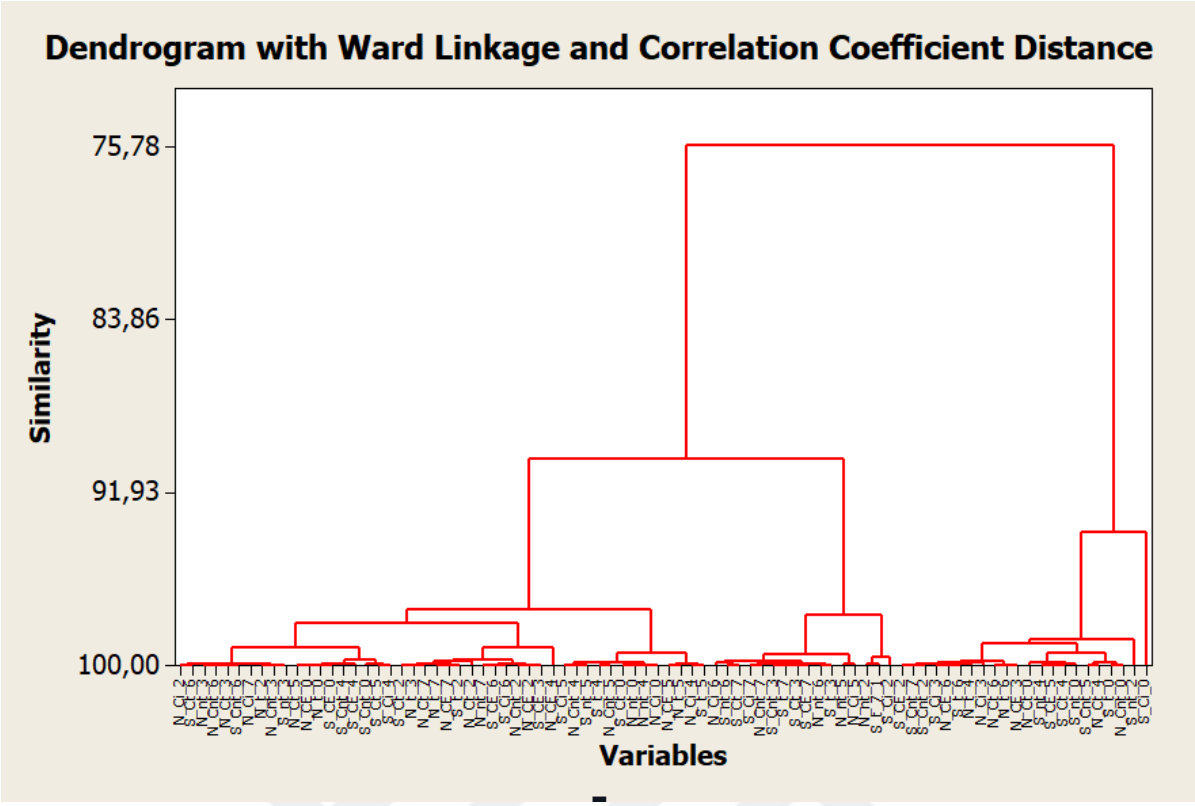


Figure 4.23 : Microbial community profile of bioreactors without manure based on microbial activity.

Figure 4.24 shows similarity based on microbial abundance of samples taken from bioreactors with manure throughout the study. The overall similarity based on microbial abundance is 82.2% and four main groups are observed. In the first group there were 37 samples with 99.2% similarity, in the second group there were 9 samples with 99.8% similarity, in the third group there were 13 samples with 99.5% similarity and in the fourth group there were 13 samples with 99.5% similarity. As seen from the figure, there has been a change in microbial community as time passes. Samples taken at the fourth and fifth time intervals are clustered in the third and fourth groups.

Microbial activity-based similarity for samples taken from bioreactors with manure are observed in Figure 4.25. The overall similarity here is 47.4% and there are four main groups. There were 31 samples with 97.3% similarity in the first group, 5 samples with 97% similarity in the second group, 22 samples with 97.8% similarity in the third group and 14 samples with 99.4% similarity in the fourth group.

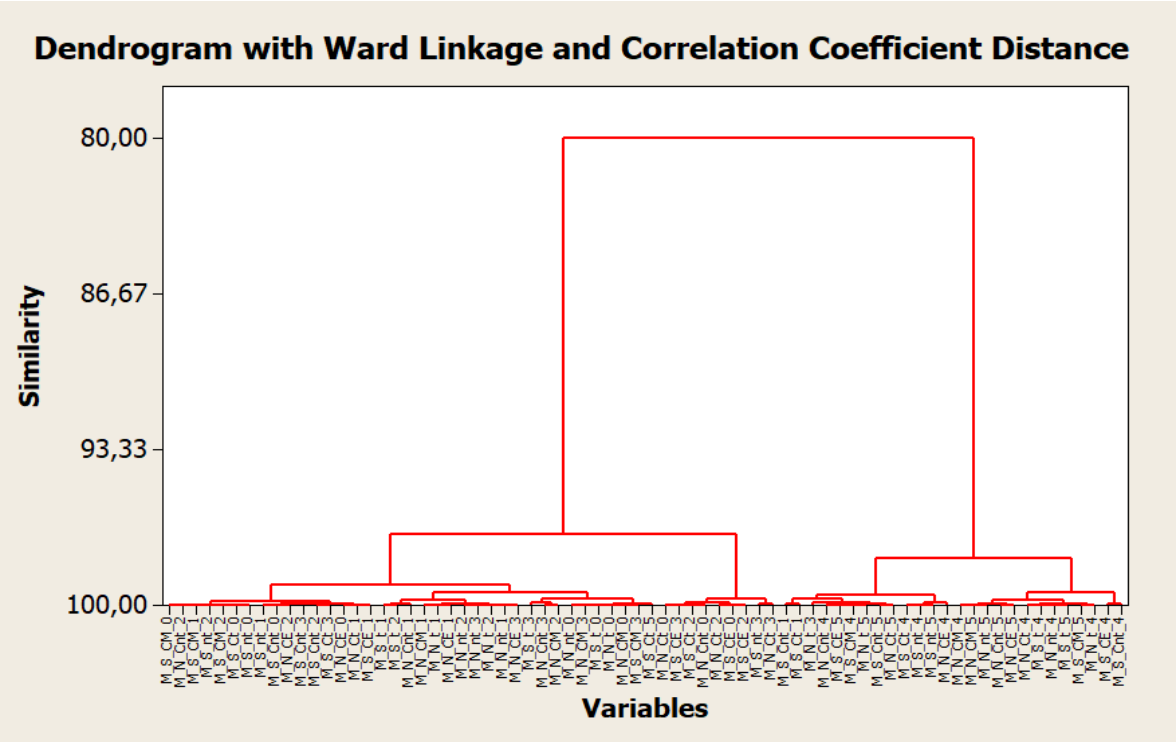


Figure 4.24 : Microbial community profile of bioreactors with manure based on microbial abundance.

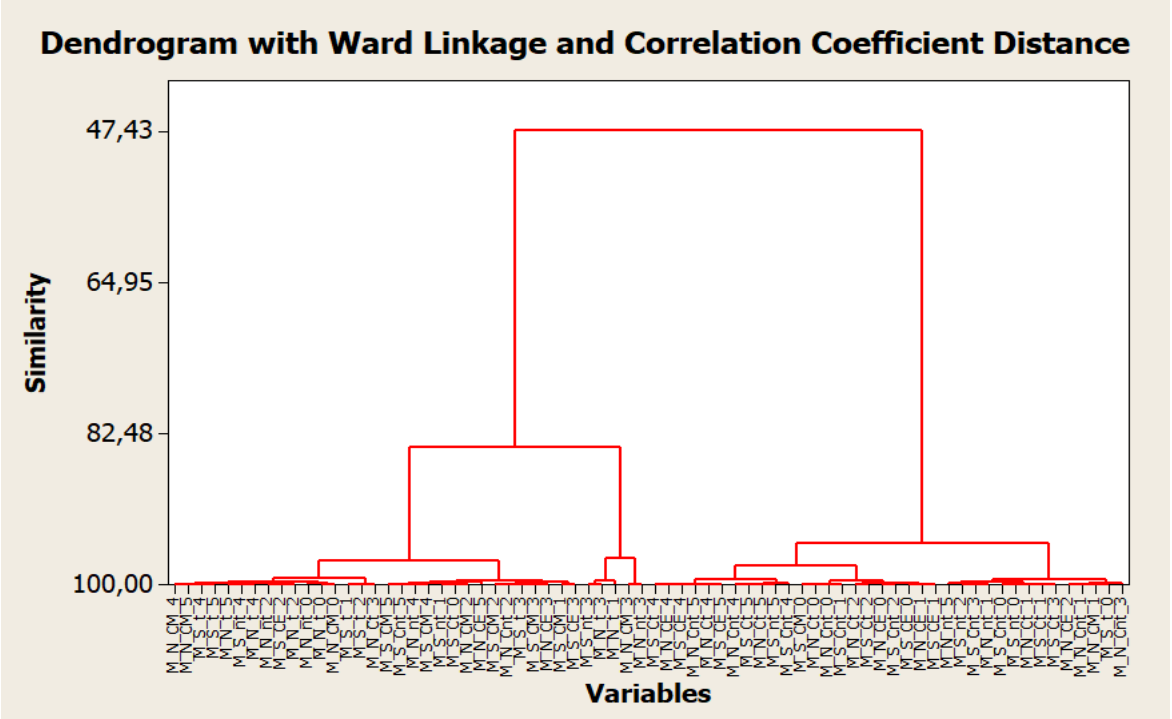


Figure 4.25 : Microbial community profile of bioreactors with manure based on microbial activity.

4.5.3 qPCR results of bioreactors

qPCR and reverse transcriptase qPCR analysis was performed using specific primers (pApH) to determine the relative variation of the 16S rRNA gene. The results were interpreted with graphs based on the percentage change compared to the beginning. In bioreactors with co-substrate, the results based on microbial abundance were given in Figure 4.26. In the control-manure group (M), there is a significant increase in the amount of bacteria on the 12th day compared to the beginning in both sulfate and nitrate reducing reactors. The predominance of the 16S rRNA gene has increased seventy-four times in the sulfate reducing condition and twelve times in the nitrate reducing condition. Since all of the groups observed a decrease in the amount of bacteria on the twelfth day were fertilizer-free mechanisms, it appears that the reason for the increase was the microbial population in the fertilizer. It was observed that none of the samples with fertilizer showed an increase after the high increase on the 12th day. After 35th day, microbial community was increased in the control soil (CSO) and control pre-treated soil (PTSO) groups of both sulfate reducing media and nitrate reducing media.

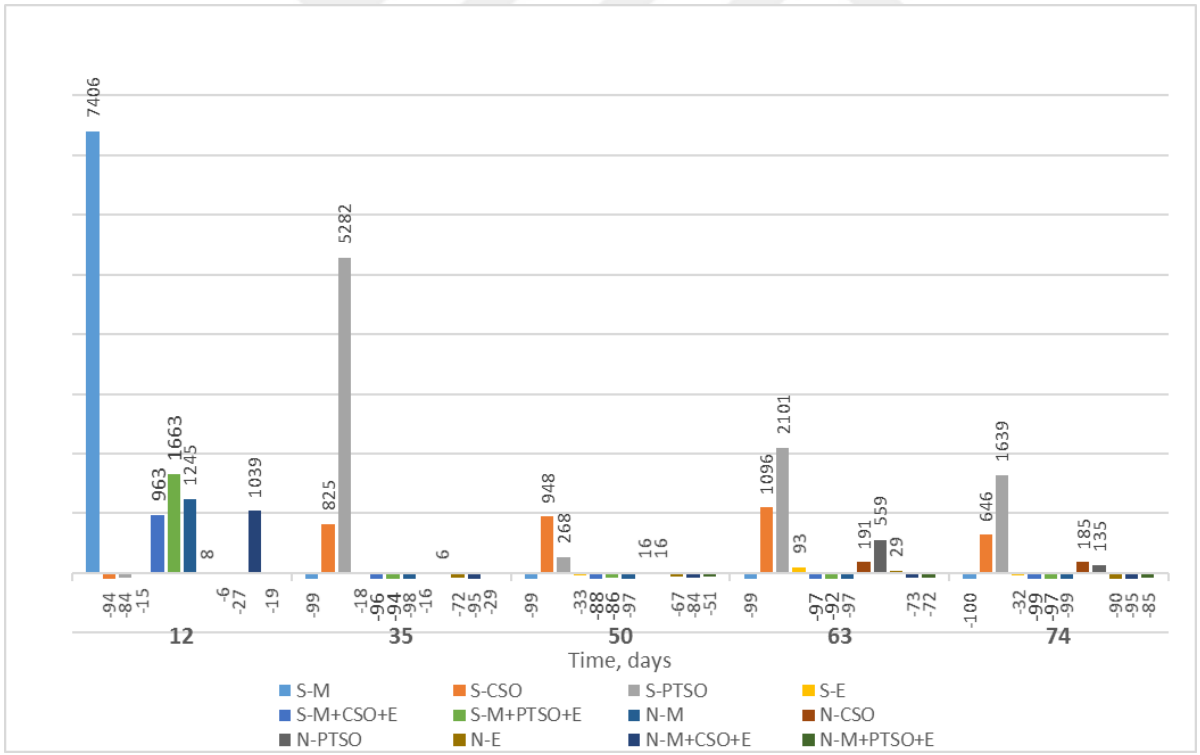


Figure 4.26 : Relative variation of the pApH gene in co-substrate applied bioreactors (based on microbial abundance).

The results based on microbial activity were given in Figure 4.27. As with the results due to microbial abundance, the results due to microbial activity also increase on 12th day in all samples with manure (M, M+CSO+E, M+PTSO+E). However, the effect on microbial activity appears to be lower than microbial abundance. It was observed that the activity in the other groups took longer time to increase. After 35th day, a decrease in the amount of bacteria was observed in all groups with co-substrate added. Except for the S-M group, the other manure groups have increased again after the 63rd day. From 63rd day, there was an increase in all groups compared to begining.

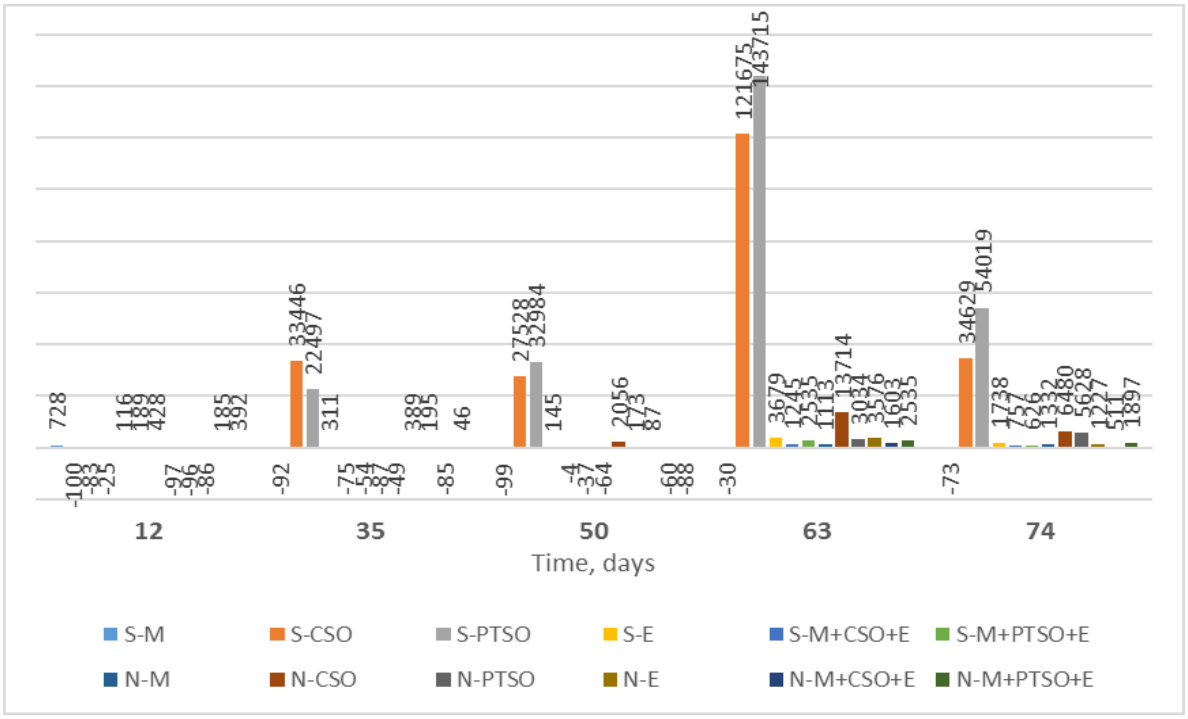


Figure 4.27 : Relative variation of the pApH gene in co-substrate applied bioreactors (based on microbial activity).

In bioreactors without co-substrate, the results based on microbial abundance were shown in Figure 4.28. As can be seen from the figure, in both reducing environments, on the 20th day only an increase was observed in control-pretreated soil groups. Microbial abundance-based analysis of the 16S rRNA gene for both reducing media showed that after 35 days of acclimatization, the amount increased in all samples compared to the beginning. On the following days, the highest increase was seen in S - A + CSO + E group. This group reached its highest value on 35th day. The relative variation of the paph gene based on microbial activity in bioreactors is given in Figure 4.29. In the first twenty days, microbial activity in all groups

decreased compared to beginning. It was observed that there was a general increase after the acclimation process. The S – A+CSO+E group showed the highest increase in activity-based analysis as well as microbial abundance-based analysis.

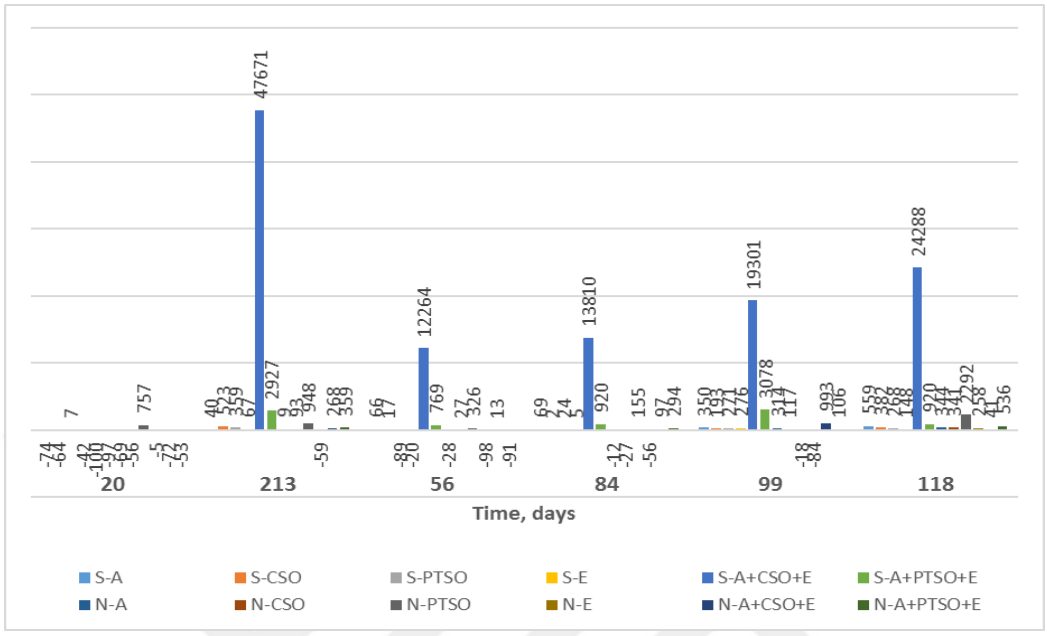


Figure 4.28 : Relative variation of the pApH gene in bioreactors (based on microbial abundance).

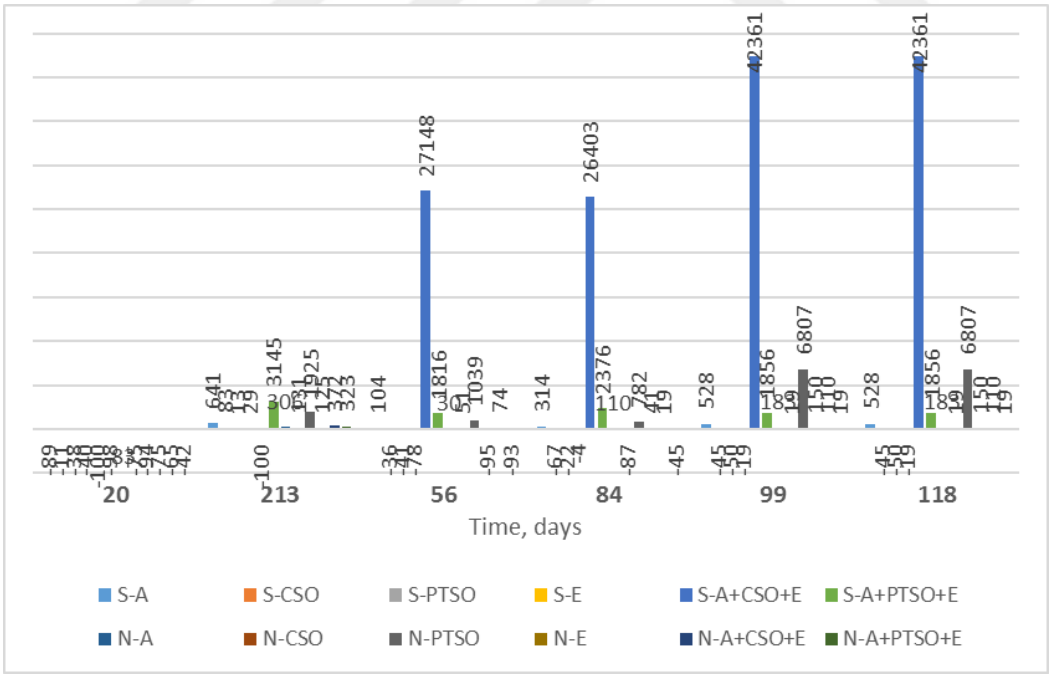


Figure 4.29 : Relative variation of the pApH gene in bioreactors (based on microbial activity).

5. CONCLUSION&FUTURE REMARKS

The results of this study can be outlined as follows:

It is observed that long-chain hydrocarbon structures in contaminated soil become short-chain as a result of pre-treatment with persulfate. In this case, biodegredation is expected to be easier.

The highest production of total biogas and CO2 was realized in the sulfate reducing environment at (M+PTSO+E), where treated soil and chicken manure were present.

CH4 production is observed in sets with manure while it is not observed in sets without manure. In nitrate reducing environment, production was observed in control group (M) where only chicken manure was present. CH4 production was monitored in control-manure (M), contaminated soil (M+CSO+E) and pre-treated soil (M+PTSO+E) in sulfate reducing media. The highest production was observed in M+CSO+E. It was understood that methane production was more supported in sulfate reducing conditions.

Ion removal was found to be high in co-substrate applied sets.

Although the incubation period (74 days) was shorter than the non-fertilizer sets , the highest TOC removal is observed at M+PTSO+E with 61% , where the pre-treated soil and manure were used together in sulfate reducing environment. In the sulfate-reducing environment without manure was observed 27% TOC removal at M+PTSO+E despite 122 days incubation period. It is showed that chicken manure increases degradation of pre-treated soil in sulfate reducing environment.

It is observed from the results that manure feeding is more effective in the pre-treated soil in sulfate reducing environment.

Analysis of aromatic and aliphatic fractions of total petroleum hydrocarbons is required to confirm the overall results.

For a detailed interpretation of the results, metagenomic analyses showing microbial community profile must be performed by NGS in the final samples taken from bioreactors at the end of the incubation period.

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