ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

DEVELOPMENT OF EFFECTIVE MOLECULAR APPROACH FOR DETERMINING OF THE BIODEGRADATION POTENTIAL OF PETROLEUM CONTAMINATED SOIL

Ph.D. THESIS

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Department of Environmental Engineering

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

PETROLLE KİRLENMİŞ TOPRAĞIN BİYODEGRADASYON POTANSİYELİNİN BELİRLENMESİ İÇİN ETKİLİ MOLEKÜLER YAKLAŞIMININ GELİŞTİRİLMESİ

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To my family,

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ABBREVIATIONS

BTEX : Benzene, Toluene, Ethylbenzene, and Xylenes
CWAO : Catalytic Wet Air Oxidation
DRO : Diesel Range Organics
EPH : Extractable Petroleum Hydrocarbons
GRO : Gasoline Range Organics
HGT : Horizontal Gene Transfer
KOC : Organic Carbon Coefficient
OTU : Operational Taxonomic Unit
PAH : Polycyclic Aromatic Hydrocarbons
qPCR : Quantitative Polymerase Chain Reaction
TOC : Total Organic Carbon
TPH : Total Petroleum Hydrocarbon
VPA : Volatile petroleum hydrocarbons
WAO : Wet Air Oxidation
Nah : Naphthalene dioxygenase gene
alkB : Alkane monooxygenase gene
phnAc : Phenanthrene dioxygenase gene large subunit

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DEVELOPMENT OF EFFECTIVE MOLECULAR APPROACH FOR DETERMINING OF THE BIODEGRADATION POTENTIAL OF PETROLEUM CONTAMINATED SOIL

SUMMARY

Despite an increase in the production of renewable energy, petroleum products are still the main source of energy for industry and domestic consumption. World production of crude oil is more than three billion tons per year. Because of the fact that most of petroleum consumer counties are not major oil producers, huge amount of produced oil is transferred via pipeline and water ways. Vast amount of petroleum enters to the environment through exploration, extraction, production, transportation, refining and storage activities. Petroleum wastes involve toxic, mutagenic and carcinogenic compounds which cause health problems and disorders to human life and ecosystems. The release of these materials to the environment is restricted with regulations. Petroleum sludge has a complicated structure including water, oil emulsion, metals, tars and numerous petroleum hydrocarbon compounds. Improper disposal of oily sludge leads to serious soil, water and groundwater pollutions. Some physicochemical methods such as soil vapor extraction, soil washing and dispersion are common methods for treatment of petroleum polluted sites and remediation of oil sludge, however, these methods are not able to complete decompose petroleum pollution and may lead to increase the toxicity of contaminated sites. Bioremediation as a cost-effective and environmental friendly method overcomes these problems. During this process pollutant compounds are converted to nontoxic materials or completely degraded to CO_2 and H_2O. Indigenous microorganisms or inoculated ones in the bioremediation sites can be stimulated to metabolize organic pollutants using nutrients, oxygen, or other amendments. Bioaugmentation as an enhanced bioremediation type refers to improvement of the biodegradation potential of bioremediation site by adding of microbial strains. This process has remarkable effect on the degradation of various contaminants. However, concerns about using of genetically engineered microorganisms and their probable impacts on the environment are commonly raised. Biostimulation as an alternative for bioaugmentation and as a sustainable bioremediation method represents a more efficient method of soil bioremediation than bioaugmentation. Monitoring of the efficiency of this process and the evaluation of the biodegradation manner in the contaminated site is one of the important steps in biostimulation practice. An effective monitoring could reduce the bioremediation cost and treatment duration. Development of an effective molecular monitoring approach for evaluation of the biostimulation process and biodegradation potential of petroleum contaminated soil was the mission of this thesis.

The evaluation of the microbial community structure and functional gene dynamics during the biostimulation process was also the mission of this thesis. Setting up aerobic microcosms test, the biodegradation manner during biostimulation process,

microbial community structure and functional gene dynamics were evaluated. The occurrence of horizontal transfer of functional genes within microbial community was also analyzed. For this purpose, petroleum contaminated soil samples were collected from petroleum storage pit and characterized. Dominant bacterial species of collected samples were determined using Illumina sequencing. Obtained petroleum polluted samples were mixed with clean forest soil samples in different ratios and weighted in glass vessels. Obtained mixtures were amended with different N:P ratios and cultivated at 24 ºC for 90 days. Genomic DNA, total RNA, and plasmids were extracted. Quantitative real time PCR with TaqMan probe was used for the quantifying of alkane and poly aromatic hydrocarbons (PAHs) degrading bacteria and *alkB*, *phnAc* and *nah* functional genes. As a post-treatment method treated contaminated soil was bioaugmented with *Trametes versicolor* and *Bjerkandera adusta* fungi following the biostimulation practice. The effects of mentioned fungi on the indigenous microbial community and, *phnAc* and *nah* functional genes through 60 days bioaugmentation practice at 29 ºC were evaluated.

Throughout the biodegradation test, maximum biodegradation rate was obtained when C:N:P ratio was arranged to 100:15:1. The most total organic carbon (TOC) removal rate was 18% and obtained from setup with initial TOC amount of 15%. Microbial analysis of collected petroleum polluted soil samples showed that gram-negative bacteria were dominant bacterial group. γ-proteobacteria, Chloroflexi, Firmicutes and δ-proteobacteria were dominant bacterial phyla. These bacterial groups covered more than 65% of the determined bacterial strains. Biodegradation examination clearly revealed that biostimulation as a sustainable bioremediation approach enhances the biodegradation of petroleum contaminated soil. Quantitative analysis of indigenous microbial community in the biostimulation process showed that gram-negative bacteria have a competitive advantage over gram-positive bacteria in studied petroleum polluted soil. Dominance of this bacterial group in the petroleum contaminated soil samples and samples obtained from experimental phase demonstrates that due to a long term acclimation to the petroleum hydrocarbons in the polluted region these bacteria could overcome other indigenous bacteria of the biodegradation vessels which were originated from clean forest soil. Any failure in stability of these bacterial will lead to a decrease in alkanes and PAHs biodegradation process. A successful horizontal transfer of *alkB* and *phnAc* functional genes was detected. Detection of the occurrence of horizontal gene transfer (HGT) phenomenon during the biostimulation process shows that, HGT can be used a helpful biomarker for monitoring of bioremediation process. Furthermore, evaluation of HGT in a contaminated site can present the broader estimation of bioremediation potential of contaminated site and positive signs about the occurrence of HGT in a petroleum-contaminated site will indicate that biostimulation is a suitable choice for bioremediation of the contaminated site. Results obtained from the post-treatment phase showed that *T. versicolor* and *B.adusta* fungi cannot fully biodegrade the petroleum residues remained from biostimulation phase. However, based on the qPCR results *T. versicolor* is effective than *B. adusta* in biodegradation of the petroleum residues. During post-treatment phase, the expression level of *alkB*, *phnAc* and *nah* functional genes were increased synergistically. Any significant change in the amounts of active bacterial cells, gram-negative and gram-positive bacteria were observed. For further studies bioremediation studies using anaerobic microorganism because of their efficiency in the biodegradation of toxic compounds is recommended. Combination of different strains of anaerobic fungi and

combination of anaerobic fungi and bacteria for maximum biodegradation of petroleum contaminated soil are also suggested. Horizontal transfer of functional genes between inoculated microorganisms and indigenous ones during bioaugmentation process can also be tested as an efficient biomarker for monitoring of this enhanced bioremediation approach.

PETROLLE KİRLENMİŞ TOPRAĞIN BİYODEGRADASYON POTANSİYELİNİN BELİRLENMESİ İÇİN ETKİLİ MOLEKÜLER YAKLAŞIMININ GELİŞTİRİLMESİ

ÖZET

Yenilenebilir enerji üretimindeki artışa rağmen, petrol ürünleri hala sanayi ve yerel tüketim için enerjinin ana kaynağıdır. Dünyanın yıllık ham petrol üretim miktarı üç milyar tondan daha fazladır. En çok petrol tüketen ülkeler en büyük petrol üreticileri arasında olmadıklarından, üretilen petrolün büyük miktarı boru hattı ve su yolları vasıtasıyla aktarılmaktadır. Büyük miktarda petrolün arama, çıkarılma, üretim, taşıma, rafinaj ve depolama faaliyetleri esnasında çevreye girişim yapmaktadır. Petrol atıkları, sağlık sorunlarına ve insan hayatı ve ekosistemin düzenini bozmaya sebep olan toksik, mutajenik ve kanserojen bileşikler, içerir. Bu maddelerin çevreye bırakılması ilgili mevzuata göre kısıtlanmıştır. Petrol çamuru su, yağ emülsiyonu, metal, katran ve çok sayıda petrol hidrokarbon bileşiği içerir ve karmaşık bir yapıya sahiptir. Petrol çamurlarının yanlış bertarafı ciddi toprak, su ve yeraltı suyu kirliliğine neden olmaktadır. Toprak buhar ekstraksiyonu, toprak yıkama ve dispersiyon gibi bazı fizikokimyasal yöntemler yaygın olarak petrolle kirlenmiş ortamların ıslahı için kullanılmaktadır, fakat, bu yöntemler petrol kirliliğini tamamen bertaraf edemiyor aynı zamanda kirlenmiş bölgelerin toksikliğini artırabilir. Biyoremediasyon, ekonomik ve çevre dostu bir arıtma metodu olarak bu sorunları ortadan kaldırmaktadır. Bu işlem sırasında kirletici bileşikleri toksik olmayan materyallere dönüştürülür veya tamamen CO_2 ve H_2O'ya dönüşür. Biyoremediasyon uygulanan alanlarda yerli mikroorganizmalar veya aşılanmış olarak kullanılanları, oksijen veya başka bir ekleyici kullanılarak organik kirleticilerin metabolize olması için canlandırılabilir. Bioaugmentasyon, iyileştirilmiş biyoremediasyon tipi olarak, mikrobiyal suşların eklenmesiyle biyoremediasyon alanının biyolojik bozunma potansiyelinin gelişmesidir. Bu işlem çeşitli kirletici maddelerin indirgenmesinde önemli bir etkisi vardır. Ancak, genetik mühendisliği mikroorganizmalar kullanımı ile ilgili endişeler ve bu mikroorganizmaların çevre üzerindeki muhtemel etkileri yaygın olmaktadır. Biyostimülasyon, biyoaugmentasyon'a alternatif olarak ve sürdürülebilir bir biyoıslah yöntemi olarak biyoaugmentasyon'a göre toprak biyoremediasyonunda daha etkili bir yöntemdir. Kirlenmiş ortamda bu işlemin verimliliğin izlenmesi ve biyolojik bozunma durumunun değerlendirilmesi, biyostimülasyon uygulamasında önemli adımlardan biridir. Etkili bir izleme biyoremidasyon maliyetini ve ıslah süresini azaltabilir. Petrolle kirlenmiş toprakların biyoremediasyon işleminin değerlendirilmesi ve biyolojik bozunma potansiyelinin belirlenmesi için etkili bir moleküler izleme yaklaşımının geliştirilmesi bu tezin misyonudur.

Ayrıca, biyoıslah işlemi sırasında mikrobiyal topluluğun yapısının ve fonksiyonel gen dinamiğinin değerlendirilmesi de, bu tezin bir misyonudur. Aerobik mikrokozmaları testi kurularak, biyostimülasyon sürecinde biyolojik bozunma

durumu, mikrobiyal topluluğun yapısı, ve fonksiyonel gen dinamiği değerlendirilmiştir. Aynı zamanda mikrobiyal topluluğun içinde fonksiyonel genlerin horizontal geçiş oluşumu analiz edilmiştir. Bu amacı gerçekleştirmek için, petrolle kirlenmiş toprak örnekleri petrol depolama lagününden toplandı ve karakterize edildi. Alınan örneklerde dominant bakteri türleri Illumina Sekanslama yöntemini kullanılarak belirlenmiştir. Elde edilen petrolle kirlenmiş örnekler temiz orman toprağı örnekleri ile karıştırılıp farklı oranlarda ve ağırlıklarda cam kaplara konuldu. Elde edilen toprak karışımlarına farklı oranlarda N:P ekleyerek 90 gün boyunca 24 ° C'de yetiştirildi. Genomik DNA, toplam RNA, ve plazmidler çıkartıldı. Genomik DNA, toplam RNA, ve plazmidler ekstre edildi. TaqMan probu ile kantitatif gerçek zamanlı PCR alkan ve poli aromatik hidrokarbonlar (PAH) degrade eden bakteriler, *alkB*, *phnAc* ve *nah* fonksiyonel genlerin ölçülmesi için kullanılmıştır. Bir son-arıtma yöntemi olarak *Trametes versicolor* ve *Bjerkandera adusta* fungus türleri kullanarak biyostimülasyon uygulaması ardından biyoaugmentasyon uygulandı. Söz konusu fungusların yerli mikrobiyal topluluğuna ve *alkB*, *phnAc* ve *nah* fonksiyonel genlere etkisi, 29 ºC'de 60 günlük bir sürede biyoaugmentasyon uygulanarak değerlendirildi.

Biyodegradasyon testi boyunca, maksimum biyodegradasyon miktarı C:N:P oranı, 100:15:1'e ayarlanan mikrokozmoslardan elde edildi. En çok toplam organik karbon (TOK) giderim miktarı 18% ve başlangıç TOK miktarı 15% olan mikrokozmoslara ait idi. Petrolle kirlenmiş topraktan toplanan örneklerin mikrobiyal analizleri, gram-negatif bakterilerinin dominant bakteriyal grup olduklarını gösterdi: γ-proteobacteria, Chloroflexi, Firmicutes ve δ-proteobacteria dominant bakteri filumları oldukları belirlendi. Bu bakteri grupları, belirlenmiş bakteriyel suşların %65'inden fazlasını kapsamakta oldukları açıklandı. Biyostimülasyon bir sürdürülebilir biyoremediasyon yaklaşımı olarak, petrolle kirlenmiş toprağın biyodegradasyonun artırılabilmesi için yapılan biyodegradasyon testiyle açıkça ortaya koymuştur. Yapılan petrolle kirletilmiş toprağın biyostimülasyon sürecinde yerli mikrobiyal topluluğun miktari analizleri gram-negatif bakterilerin gram-pozitif bakteriler üzerinde bir rekabet avantajı olduğunu gösterdi. Petrolle kirlenmiş toprak örnekleri ve deneysel aşamada elde edilen örneklerde söz konusu bakteri grubunun dominant olması, kirlenmiş bölgedeki petrol hidrokarbonlarına uzun vadeli bir ortama alıştırma sürecinden dolayı bu bakteriler biyodegradasyon setlerindeki temiz orman toprağından alınan örneklerden kaynaklanan diğer yerli bakterilerin üstesinden gelebilmelerini gösteriyor. Bu bakterilerin istikrarında herhangi bir başarısızlık, alkan ve PAH biyodegradasyon işleminde bir düşüşe yol açacaktır. *alkB* ve *phnAc* genlerin başarılı horizontal geçişi tespit edildi. Biyostimülasyon işlemi sırasında horizontal gen geçişi (HGÇ) oluşumunun tespit edilmesi, HGÇ biyoremidasyon sürecinin izlenmesi için yararlı bir biyolojik gösterge olduğunu göstermektedir. Ayrıca, kirli alanlarda HGÇ değerlendirilmesi biyoremidasyon potansiyelinin daha geniş bir şekilde tahmin edilmesini sunabilir ve petrolle kirlenmiş bölgede HGÇ oluşumu hakkında olumlu işaretlerin elde edilmesi, biyostimülasyon işleminin kirlenmiş bölgenin biyoremediasyonu için uygun bir yaklaşım olduğunu teşhir etmektedir. Son-arıtım fazından elde edilen sonuçlar, *T. versicolor* and *B. adusta* fungusları biyostimülasyon fazdan kalan petrol kalıntılarını önemli miktarda indirgeyemediklerini gösterdi. Ancak qPCR sonuçlarına göre *T. versicolor,* petrol kalıntılarının biyodegradasyonunda *B. adusta*'dan daha etkilidir. Son-arıtım fazı aşamasında, *alkB, phnAc* ve *nah* fonksiyonel genlerin ekspresyon miktarı sinerjik olarak artırılmıştır. Aktif bakteriyel hücreler, negatif gram ve gram-pozitif bakter miktarında kayda değer bir değişiklik gözlemlenmemiştir. İlerideki çalışmalarda, anaerobik

mikroorganizmaların toksik bileşenlerin bozunması işleminde daha verimli olduklarından biyoremediasyon çalışmalarında kullanılması tavsiye edilir. Ayrıca, farklı anaerobik fungus türlerinin kombinasyonları ve anaerobik fungus ve bakteri türlerinin birleşimleri petrolle kirlenmiş toprakların maksimum biyodegradasyonu için önerilmektedir. Aynı zamanda, biyoaugmentasyon sürecinde inokulum mikroorganizmalar ve yerli olanlar arasındaki fonksiyonel genlerin horizontal geçişi, verimli bir biyomarker olarak biyoremediasyon yaklaşımının izlenmesi için test edilebilir.

1. INTRODUCTION

1.1 Importance of Thesis

Yearly production of crude oil is more than three billion tons. As the principal source of energy, oil has important role in human life and despite an increment in the utilization of renewable energy sources because the reliable alternative energy consumption has not yet been substituted petroleum will remain as a major source of energy in the next several decades. Utilizing of petroleum and its derivatives for many years as the main energy source caused the entrance of a significant amount of oil components to the environment in various ways and as a result, these substances formed one of the most important environmental pollutants. Leaks and accidental spills occur frequently during the exploration, production, refining, transport, and storage of petroleum and petroleum products. The worldwide amount of natural crude oil seepage only from storage tanks and during transferring from storage tanks is more than 600,000 metric tons per year. Petroleum wastes contain toxic, mutagenic and carcinogenic compounds which constitute a health hazard to human life and ecosystems. Therefore, the releases of these materials, which are classified as hazardous wastes, to the environment, are restricted with regulations.

One of the main parts of petroleum pollution is because of the fact that most of the oil consumer countries are not the major petroleum producers. Transportation of the massive amounts of petroleum via land pipeline or on water in tankers leads to huge amount of oil pollution. Refineries as the main petroleum processing units also produce vast amount of oily sludge during storage and refining process. Storing of crude oil in the storage tanks in refineries leads to deposition of the impurities in the bottom of the tanks and during the cleaning process oily sludge is recovered. In refineries, petroleum sludge also accumulates through seepages from faulty storage tank, pipeline ruptures, and pump failures and so on. Based on different estimations more than 28,000 tons of petroleum sludge are produced each year from each petroleum refinery.

Oily sludge is extremely complicated, including water, oil emulsion, and impurities of suspended solids and contains metals, tars, oils, and numerous petroleum hydrocarbon compounds. Petroleum hydrocarbons are made by multiple carbon bonds in which give intensity and raised complex structures as a result of bounding with other characteristic molecules. Petroleum hydrocarbons are shaped as various proportions of short, medium and long aliphatics (i.e. alkanes, alkenes), aromatics and polycyclic aromatic hydrocarbons (PAHs).

Inappropriate disposal of the petroleum sludge because of its mentioned composition causes environmental pollution, especially soil pollutions which in most cases lead to serious water and ground water contaminations. Soil pollution by petroleum hydrocarbons leads to some toxic effects on organisms that are exposed to such kind of chemicals damaging effects as accumulation into tissues and causing mutation and eventually occurring cancers in some significant organs. Unlike the physic-chemical methods, bioremediation with its different aspects, consists emerging technologies that are eco-friendly, feasible and cost-effective option. Bioremediation do not just transfer pollutants from one phase to another instead during this process, microorganisms convert such pollutants to nontoxic compounds or completely degrade to CO_2 and H_2O. Enhanced bioremediation as a sustainable remediation technology is a process in which indigenous microorganisms or inoculated ones such fungi and bacteria enhanced using nutrients, oxygen, or other amendments to metabolize organic pollutants found in the soil and ground water. There are two kinds of enhanced bioremediation: bioaugmentation and biostimulation. Bioaugmentation refers to improvement of the biodegradation capability of the natural existing microorganism population by adding microbial strains. This process has been reported that has a positively remarkable effect on the degradation of various contaminant hydrocarbons. However distinguishing of the exact role of new added microorganism on the biodegradation process and whether the introduced microorganisms or the endemic ones have the main role in the mentioned process is difficult. Some researchers have tried to introduce engineered microorganisms which are able to degrade various types of hydrocarbons. Even with successful realization of these studies because of negative concerns toward using of these strains the genetically modified microorganism could not be suitable choose for the sustainable

bioremediation. Biostimulation approach however is more effective than bioaugmentation and can be used a sustainable remediation method.

1.2 Mission and Scope

Nowadays environmental contamination by petroleum and its derivatives is a serious worldwide problem. Considerable amounts of petroleum hydrocarbons are released into water and soil because of pipeline leaks, transport accidents, and storage tank ruptures. It is well established that petroleum-derived wastes contains toxic, mutagenic and carcinogenic compounds that constitute health hazard to humans and other organisms in the ecosystem. Petroleum-derived wastes represent a large and diverse group of organic molecules having a broad range of properties, differing in molecular weight, structural configuration, water solubility, number of aromatic rings, volatility, sorption coefficients, etc. Bioremediation approach because of its numerous economic and environmental friendly advantages over other physicochemical methods is used world widely for sustainable treatment of the petroleum-contaminated soil. However, because of the lack of a precise and feasible monitoring system, successful evaluation of intrinsic biodegradation potential of contaminated site, optimizing of biodegradation process and evaluation of the bioremediation practice are main challenges of an effective practice. Advanced molecular biology techniques have been used for assessment of bioremediation efficiency however, a key point in monitoring and judgment about biodegradation potential which can be applied in in-situ and ex-situ bioremediation process, has not been reported yet. Development of an effective molecular approach for determining of the intrinsic potential of petroleum contaminated soil and evaluation of the biodegradation process during bioremediation practice is the main mission of this thesis.

2. LITERATURE REVIEW

2.1 Petroleum hydrocarbons and their transport in the environment

Petroleum hydrocarbons in general consist of two groups: aliphatics and aromatics. Aliphatic hydrocarbons include alkanes which contain only single bounds between carbon atoms, alkenes that contain at least one double bond between carbon atoms, and cycloalkanes which carbon atoms have been arranged in cyclic structures. Aromatic hydrocarbons contain benzene ring as part of their structure. Monoaromatics having single aromatic ring are among common aromatic hydrocarbons in petroleum. Benzene, toluene, ethylbenzene, and xylenes (BTEX) are the most prevalent monoaromatics which consist about two percent of crude oil by weight (Williams et al., 2006). These compounds are highly soluble in water therefore they are very mobile in water and soils and are available to microorganisms in soluble form (Prenafeta-Boldú et al., 2002). These compounds, however, because of their hydrophobic characteristics are very toxic for membrane of microorganisms (Dong et al., 2015). Monoaromatic hydrocarbons are also so toxic for eukaryotic cells and human organisms (Dean, 1985). Benzene as an important member of this group has carcinogenic effect (Kasai et al., 2007). Polycyclic aromatic hydrocarbons (PAHs) are aromatics that composed of multiple fused benzene rings sharing one or more side. These compounds which range from naphthalene (C10 H8, two rings) to coronene (C24 H12, seven rings) represent 0.2 to 7% crude oil. Low solubility in water high melting and boiling point are characteristics of these compounds (Almeda et al., 2013). Acenaphthene, acenaphtylene, anthracene, benzo(g,h,i)perylene, fluorene, phenanthrene, pyrene are examples of PAHs (Figure 2.1).

Crude oil contains usually high concentrations of aliphatic hydrocarbons than aromatics. Several aromatic hydrocarbons are known as human carcinogens (Potter and Simmons, 1998). BTEX and 16 PAH have been classified as priority pollutants regulated by the U.S. Environmental Protection Agency (USEPA) (Pampanin and Sydnes, 2013).

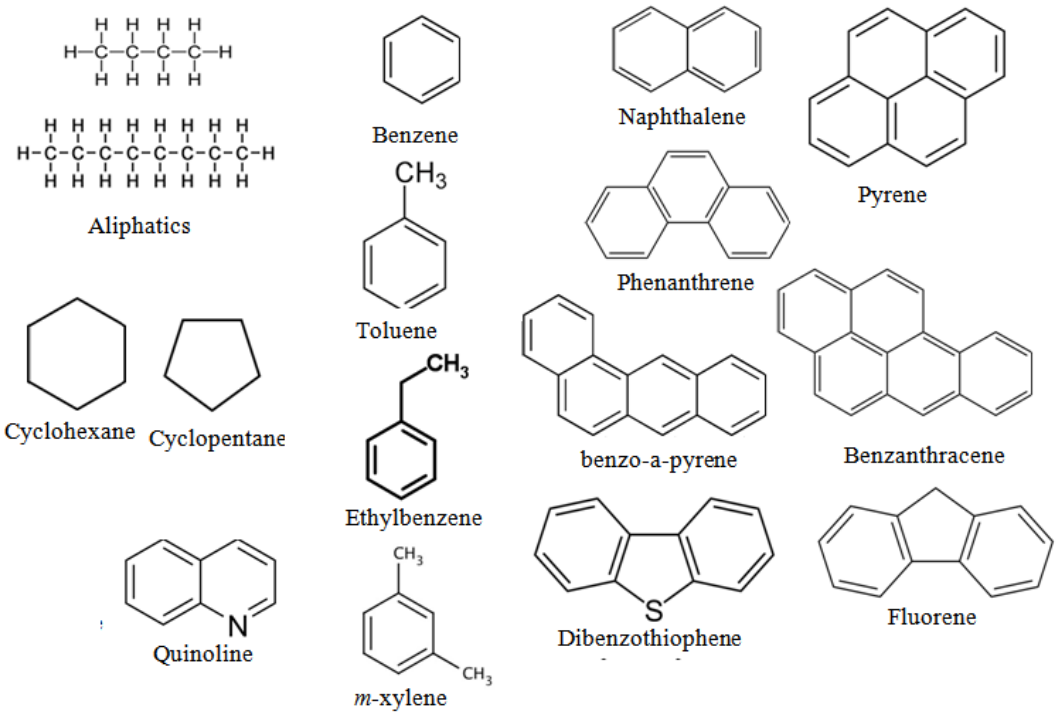

Figure 2.1 : Chemical structure of some hydrocarbons presented in the petroleum Sludge.

Mixture of petroleum hydrocarbons which compose of several hundred compounds commonly are described as total petroleum hydrocarbons (TPH). TPH is typically use as a common indicator of petroleum hydrocarbons in contaminated sites. When analyzing of TPH, two subset of gasoline range organic (GRO) which overlap hydrocarbons in the C5-C12 range and diesel range organic (DRO) which presents C9-C36 range hydrocarbons are measured by some of techniques mostly when GC is used (USEPA, 2007). Two additional subsets of petroleum hydrocarbons are Volatile petroleum hydrocarbons (VPH) which includes C5-C12 aliphatics, BTEX, methyl tertiary-butyl ether (MTBE), naphthalene, and C9-C10 aromatics and extractable petroleum hydrocarbons (EPH) which overlap C9-C36 aliphatics and C11-C22 aromatics, which can be detected by GC methods (Thompson and Nathanail, 2003). In the bioremediation of petroleum contaminated regions in some case site specific clean up level are used in many cases, however, standards generic clean up level which consist of clean up level for DRO or EPH are applied. Clean up levels are generally defined for each contaminant level in soil and groundwater however it can be defined based on TPH. The clean up levels for TPH which include DRO and EPH vary greatly for different regions. In generic numeric cleanup standards which established by Department of Environmental Quality in 2012 for soil and

groundwater, cleanup level for DRO vary between 50-500 mg/kg for soil whereas state of Maryland (report of June,2008) apply 200-600 mg/kg. This level based on the Tennessee Department of Environment and Conservation in the 1998 report has been determined from 10-1000 mg/kg (http://www.cleanuplevels.com). Cleanup levels for petroleum hydrocarbons are described mostly based on the human health risk standards and ecological risk-based approach (McMillen et al., 2001). Evaluating potential human health risks considers the compounds of risk-toxicity which defines as the potential of compound to cause undesirable health effects and the potential of exposure to such a compound (Dost, 2003). Two kinds of toxicity information for petroleum hydrocarbons are available in the literature; first sort are information about the toxicity of individual toxic petroleum hydrocarbon compounds such as benzene and benzo[a]pyrene and the other sort are information about the specific petroleum hydrocarbon mixtures such as gasoline (Mao et al., 2009). Regarding to the TPH mixture there is no available toxicity data (Michelsen and Boyce, 1993).

Leaks and accidental release of petroleum hydrocarbons occur frequently during the exploration, production, refining, transportation and storage of petroleum and petroleum products (Das and Chandran, 2011). When petroleum hydrocarbons are entered to the water through spills or leaks from transportation pipeline and so on, some fractions will float in the water surface and less than five percent of the petroleum hydrocarbons will dissolve in the water (Williams et al., 2005). Aromatics especially BTEX are most water soluble than other fractions. Some fractions of the petroleum hydrocarbon may be degraded by some organisms found in the water. The heavier sections will precipitate and accumulate in the sediment (Boesch and Rabalais, 1978). In the case that petroleum hydrocarbons are release to the soil these compounds may pass across the soil to the groundwater and pose a serious pollution of underground aquifer (Bojes and Pope, 2007). Petroleum hydrocarbons in the soil are exposed to the weathering (Suja et al., 2014). Adsorption to the particles of soil and volatilization are the main weathering process (Lisiecki et al., 2014). Alkanes and alkenes are the most volatile part and when spill of oil, leakage to the soil surface and storage of oil waste in open pits, these fractions will be volatized and lower molecular weight aliphatics will be lost (Potter and Simmons, 1998). Further more in large seepage and spill events aliphatics will be the major air pollutants because of

their high volatilization characters (Brewer et al., 2013). Petroleum hydrocarbons when attach to the soil particles may stay for a long time or be degraded by the soil organisms. Decrease in activity of soil enzymes, toxic effects on soil organism, nutrient deficiency, inhibition of seed germination, growth restriction of plants are some adverse effects of petroleum hydrocarbons when release to the soil (Gerhardt et al., 2009). Soil organic carbon coefficient (K_{oc}) is a parameter to determine the tendency of organic carbon sorption to the soil particles or sediments (Wang et al., 2015). This coefficient can vary depending on the soil type, pH and the type of organic substances (Delle Site, 1999). The less amount of Koc will indicate the high mobility of the compound, in other words compounds with less amount of K_{oc} will have the most concentration in the groundwater because of the high mobility nature (Williams et al., 2006). For instance benzene with K_{oc} of 59 is highly mobile than toluene with Koc amount of 182 and its concentration in the groundwater is higher. Typically BTEX because of their low K_{oc} values can be detected in groundwater near petroleum contaminated sites (Lovanh et al., 2000; Koo 2014). Poly aromatic hydrocarbons are less volatile and will not easily dissolve in water (Lohmann et al., 2011). These compounds because of their low solubility mostly enter to the sediments and soils and do not enter to groundwater. Accumulation of PAHs in the sediments may be a potential risk source to the aquatic life (Charles et al., 2010). Reduction in diversity and abundance of aquatic organisms are some adverse consequences of PAHs migration to groundwater and aquatic systems (Obiakor et al., 2014).

Degradation by microorganisms is an important process of petroleum hydrocarbon attenuation in the soil and sediments (Fathepure, 2014). Biodegradation rates depend on the kinds of petroleum hydrocarbons, microbial population diversity and ecological condition of the contaminated region (Das and Chandran, 2011). Microbial strains capable to degrade petroleum hydrocarbons are almost present in all soil and sediments and their population density increases when contact with contaminant. Microbial population uses the petroleum hydrocarbons as carbon source (Martins and Peixoto, 2012; Dindar et al., 2013). They used different electron acceptors regarding to the ecological and geochemical conditions of the region (Nyer et al., 2003). Dissolved oxygen is the main electron acceptor in the ground water and nitrate, insoluble manganese, ferric iron, sulfate and carbon dioxide are other

electron acceptors (Sahrawat, 2004). Bacteria capable of biodegrading petroleum hydrocarbons are usually classified based on the final electron acceptor which they use. Aerobic bacteria which use oxygen as final electron acceptor, nitrate reducing, iron reducing, manganese reducing, sulfur reducing, and methanogenic bacteria are bacterial groups which can degrade petroleum hydrocarbons (Maloney et al., 2004). In petroleum contaminated plumes different biodegradation zones based on the prevalent electron acceptor can be detected. These zones can be determined using obtained data from geochemical and microbial analysis (Tiehm and Schulze 2003). Biodegradation zones also give useful information about degradation rates. Aliphatic, Cycloalkane and aromatic hydrocarbons with low to moderate molecular weight have high biodegradation rate in optimum conditions whereas hydrocarbons with high molecular weight show low biodegradation rates (Das, 2011). Asphaltenes are among the most recalcitrant for biodegradation compounds (Liao et al., 2009; Singh et al 2009). Refinery sludge because of having a large amount of asphaltenes and resins is difficult for degradation by bioremediation process on its own (Hu et al., 2013; Ubani et al., 2013). Some studies, however, have reported large amount of organic conversion in this kind of sludge using bioremediation approach.

2.2 Petroleum sludge and sludge treatment methods

Typically, petroleum sludge is recalcitrant residuals characterized as a stable water-in-oil emulsion of water, petroleum hydrocarbons, solids, and metals (Mazlova and Meshcheryakov 1999). Chemical composition of oil sludge is different regarding to the origin of crude oil, petroleum processing methods and utilized reagents and equipments in refining process (Hu et la., 2013). TPH amount of the sludge in most cases are between 15 and 50% (Tahhan et al., 2011). The water contents and solids amounts are in the range of 30-85% and 5-46% respectively (Hu et al., 2013; Mansur et al., 2015). Petroleum hydrocarbons present in the oil sludge consists aliphatic and aromatic hydrocarbons which presents about 75% of hydrocarbons, nitrogen sulphur and oxygen (NSO) (Ward et al., 2003; Mrayyan and Battikhi, 2005; Reddy et al 2011). Asphaltenes are other substances found in the petroleum sludge. These materials which are combination of hundreds or maybe thousands of individual compounds and present about 10 percent of oily sludge do not have a specific chemical formula (Shedid and Abbas, 2005). The stability of the petroleum sludge

emulsion is mostly comes from the cooperation of these compounds and resins which contain hydrophilic functional groups (John and Eric, 1958). Petroleum sludge from different origins because of different chemical compositions and diverse constituents show different physical characteristics such as viscosity and density (Hu et al., 2013). Chemical properties of present compounds in the petroleum sludge such as polarity and molecular weight affect the physical characteristics of oil sludge. Petroleum sludge consists also variety of heavy metals (e.g. Zn, 60200 mg/kg for iron (Fe, Cu, Cr, Ni, and Pb) which their kinds and concentrations like the hydrocarbon fractions are very different in oil sludge obtained from different sites (Wuana and Okieimen, 2011).

Petroleum excavation, producing, transformation, refining, and probable accidences lead to producing of huge amounts of oily sludge. This sludge because of their poorly biodegradable nature cause environmental problems and different toxic effects originated from PHCs and heavy metals (Maletić et al., 2013). PAHs because of being genotoxic to humans and other organism are of the major concerns (Wickliffe et al., 2014). Therefore, safe disposal of produced sludge in the environment is one of the major problems faced by petroleum sector. For solving of the oily sludge problems petroleum industries apply some methods for minimizing of the sludge production and disposal of produced sludge. The first and the most important step in petroleum sludge management is to reduce the amount of sludge production (Khojasteh et al., 2012). Using suitable technologies petroleum industries try to decrease the quantity of waste sludge. Refineries as one of the main points in oily sludge production with employing new processing especially deep conversion process, using of modern catalysts, installation of mixers on crude oil storage tanks, and so on were somehow successful in reduction of the amount of refinery sludge (Dando and Martin, 2003). Oil recovery from the produced sludge is another important step in sludge management (Joseph, 2007). Some refineries use advanced petroleum recovery system when tank cleaning process to reclaim the maximum amount of hydrocarbons from oily sludge. However oil recovery after sludge production can be realized using some prevalent methods. This step also called pre-treatment phase when petroleum sludge is treated by bioremediation methods. Researchers believe that to remove the harmful contaminants from the pollution before discharge to the receiving system will increase the treatment effectiveness. Oil

recovery does this process in essence. Oil recovery has been done through various methods and many studies have been done for finding out a suitable method in terms of economic and environmental view point and the quality of recovered oil as well. Solvent extraction, ultrasonication, microwave technology and centrifugal oil recovery are among the common methods for this purpose (Xu et al., 2009; Yan et al., 2012). Each method has its own advantages and limitations and the most suitable one can be selected based on the oil sludge characteristics, oil recovery potential of techniques, economical view point, cleanup standards, hydrocarbon quality of recovered oil and so on (Lima et al., 2011). In most cases combination of two or more methods has been studied for a successful oil recovery (Radhi et al., 2011). Most of these studies, however, were in the lab scale and further studies are needed for increasing of the feasibility of these methods.

Solvent extraction which is extraction of oil from impurities using low boiler solvents is one of the frequently used methods. In this technique, using a proper amount of solvent, oil-solvent mixture is separate from the water and solid particles and then is distillated for separate petroleum hydrocarbons from the applied solvent (Diphare and Muzenda, 2013). In field-scale applications solvent is liquefied using a compressor and cooling system is used again for repeating cycle (Hu et al., 2013). Waste residues which consists impurities may need extra treatment. Different kinds of solvents have been studies successfully for oil extraction that turpentine, methylethyl ketone (MEK), methylene dichloride, ethylene dichloride, diethyl ether, n-heptane, hexane, xylene and toluene are some examples (Taiwo and Otolorin, 2009). Being a simple but effective method, applicable in short period of time, its potential to treat large amount of oily sludge are some advantages of this method (Xu et al., 2015). For field-scale a large amount of solvent is needed which reduce the efficiency of this method (Hu et al., 2013).

Ultrasonic technology is an effective method for treatment on petroleum sludge (Zhang et al., 2013, 2014). This technology along with using in petroleum production process for enhancing of oil recovery especially in the cases such as petroleum recovery from failing oil wells has been used effectively in the recycling of petroleum content of oily sludge (Abramov et al., 2013). The efficiency of ultrasonication in the increasing of biodegradability has been proved through various studies. Increasing of biogas production, when pre-treating with ultrasonic radiation,

has been increased (Houtmeyers et al 2014). The effectiveness of this technique in the recovery of oil from oily sludge has been concluded through various studies. This method improves the degradability through disrupting of the physical and chemical characteristics of the sludge (Chen et al., 2012; Tuan et al., 2013). Ultrasonic wave trough compression and rarefaction phenomena increase the destabilization of water-oil emulsion (Issaka et al., 2013). Furthermore, this method can increase the coalescence of droplets which promotes the separation of water-oil phases (Eow et al., 2001). Sonication parameters such as sunication duration, power and frequency, and characteristics of treated sludge such as water content, size of solid impurities, initial concentration of petroleum hydrocarbons determine the degree of disintegration (Pham, 2014). Therefore, different studies have been fulfilled for distinguishing of the optimum parameters and sonication devices for different types of sludge.

Microwave irradiation also is used for hydrocarbon recovery from oily sludge. Rapid heating which leads to higher reaction rates, easy to control, low overall cost, and compactness are advantages of this method (Jones et al., 2002). Such a short heating time increases the demulsification of water-oil emulsion and reduction in viscosity which leads the settlement of water droplets (Undri et al., 2013). Rapid increasing of temperature can also break down heavy hydrocarbons to light hydrocarbons (Miadonye and MacDonald, 2014). Several studies have demonstrated the efficiency of microwave irradiation on the treatment of water-oil emulsion and separation of water from oil which was significantly higher than when utilizing conventional heating system (Saifuddin and Chua, 2006). Oil recovery process using microwave irradiation can be affected by parameters such as microwave power and duration, pH, water/oil ratio and salt content of treated sludge (Kumar and Mohan, 2013). pH with affecting of hydrophilicity and existent salt of the sludge with affecting the conductivity and heating rate can affect the microwave-assisted oil recovery process (Xia et al., 2004; Fortuny et al., 2007). Application of this method in full-scale treatment is required specific equipment and high operation cost.

Freeze-thaw system is another method which can be used for oil recovery from petroleum sludge especially in cold regions such as Canada and Northern America where the natural freezing condition is accessible (Filler et al., 2008). This system can separate oil and water contents of the sludge from each other. This method is an

effective demulsification technique (Rajaković and Skala, 2006). As a brief during the freezing process water content and through the thawing process oil content of the sludge separate from the sludge (Kwon and Lee, 2015). Various studies have been done for evaluation of the performance of this method using different freezing techniques. Nearly ninety percent water removal and more than sixty percent oil recovery have been reported (Hu et al., 2013). Parameters such as freeze and thaw temperature and duration, water content and salinity affect the performance of process (Chen and He, 2003).

Centrifugation technique has been applied for oil-water separation. This technology is common in the field-scale remediation of petroleum sludge; however there is little research about this method (Miller, 1990). This method does not need large space and is clean and somehow effective in oil recovery. Using this cost-effective technique, sludge constituents separate from each other based on their densities (da Silva et al., 2012). Reduction of sludge viscosity improves its performance. The addition of organic solvents, demulsifying agents and heating of sludge are some methods for decreasing of sludge viscosity and reduction of consumed energy but using these agents causes environmental concerns (Lima et al., 2011; Yan et al., 2012; Hu et al., 2014). Some researches could increase the treatment performance more than 90 percent with reducing of the sludge viscosity. Generation of noise and vibration, and high energy consumption are some disadvantages of this method (Cambiella et al., 2006; Nahmad, 2012).

After oil recovery remained sludge needs to be disposal. In spite of that, most of the refineries don't have an established plan to dispose of oily sludge (Kuriakose and Manjooran, 2001), various approaches are used to overcome oily sludge problem. Two kinds of techniques are used for this purpose: physicochemical remediation and bioremediation. Incineration and oxidative treatment technologies are some common physicochemical methods.

Through incineration which is widely used by refineries, oily sludge is completely combusted using incinerators and under a high temperature which in some case is up to 1200 ◦C (Hu et al., 2013). The positive effects of an auxiliary fuel such as coal–water slurry and petroleum coke–sludge slurry on the stability of combustion process have been reported (Liu et al., 2009). With using of these auxiliary fuels the performance have been increased more than 90 percents. For increasing of the

process performance oily sludge with high viscosity and sludge with high moisture content need to be pre-treated before incineration (Sankaran et al., 1998). Air pollution risk and producing of hazardous ash are two disadvantages of this technique (Ururahy et al 1999).

Oxidative treatments, such as ultrasonication, ferro-sonication, Fenton's oxidation, ozonation, and wet air oxidation which are used for degradation of organic compounds, have been used for treatment of oil sludge (Rivas, 2006; Ferrarese et al., 2008). Sludge hydrolysis is the aim of these methods (Houtmeyers et al., 2014). Oxidative degradation is realized by chemical oxidation or accelerated oxidation (Ferrarese et al., 2008). Through chemical oxidation chemical reagent are added to the petroleum sludge that cause oxidation of organic compounds into CO2 and water or some other non-hazardous compounds (Ferrarese et al., 2008). Through oxidative treatment TCOD amount of the sludge decrease because of the partial oxidation of organic compounds (Loures et al., 2013). When oxidation by ultrasonic method, ultrasonic waves generate compressions and rarefactions micro bubbles are generated (Zhang et al., 2014). The negative and positive pressures created by these phenomena cause to generate and grow of microbubbles (Pilli et al., 2011). These bubbles finally collapse and produce huge amount of heat (about 5000K) and several hundred atmospheres pressure (Merouani et al., 2014). Under these extreme conditions compounds nearby will be destructed and hydrogen peroxide and OH•, HO2•, H• radicals will be formed. These radicals are so oxidative and can oxide the compounds around (Mason, 2007). In Fenton oxidation method hydrogen peroxide hydroxyl radical (OH•) is reduced to hydroxyl radical (OH•) (Zhang et al., 2003). Hydroxyl radical has powerful oxidation ability and can react with all of the components presented in sludge which leads to the disruption of treated sludge (Mason, 2007). Ozonation as an oxidation treatment has some advantages over the other methods. Ozone has high oxidation potential and through this method any oxidant residuals remain (Weller, 2014). Through ozonation treatment hydroxyl radical (OH•) is produced through a chain reaction. Hydroxyl radical as a powerful oxidant mineralize the sludge solids (Weller, 2014). Wet air oxidation (WAO) and catalytic wet air oxidation (CWAO) are other effective technologies to degrade concentrated hazardous compounds. Through this technique highly concentrated sludge such as petroleum sludge will convert to degradable compounds. In this method sludge will

treated under high temperature and pressure and using oxygen as oxidant. Through this process organic materials convert to carbon dioxide, water and other harmless end products (Jing et al., 2012).

The common drawback in most of these physicochemical treatments is that they are costly, and need complicated devices (Couto et al., 2010; Das and Chandran, 2011). Using addition chemicals as catalysts or oxidants also cause additional environmental problems. Furthermore in most cases these methods are not permanent solution and cannot completely remove contaminants (Calvert and Suib, 2007).

Bioremediation techniques as environment friendly and cost-effective methods can overcome most of limitations of physicochemical treatments (Megharaj et al., 2011). This technique is the process of employing microorganisms to degrade contaminants. Bioremediation is a simple to operate, economic and effective method which has been used widely in treatment of oily sludge and petroleum contaminated soil (Battikhi, 2014). The residues remained from bioremediation are nonhazardous products such as carbon dioxide, water and different fatty acids (Das and Chandran, 2011; Ubani et al., 2013; Dindar, et al., 2013). In recent years, bioremediation had a rapid development and a variety of microorganisms have been developed for treatment of different kinds of contaminants. The biodegradation of petroleum hydrocarbons is a complicated process and the type of bioremediation approach and operational conditions depend on the amount and kinds of hydrocarbon compounds in the contaminated site and sludge (Jain et al., 2011). Bioremediation is capable of degrade completely all petroleum wastes apart from heavy polyaromatics presence in the asphaltenes and resins (Liu et al., 2010). Land farming, biopiles and bioreactors methods are common and the most studied bioremediation approaches for treatment of petroleum sludge (Powell et al., 2007).

In bioremediation process, parameters such as types of microorganisms, remediation duration, nutrients amounts, and hydrocarbon concentrations in oily sludge are important factors which affect the biodegradation rates (Jordening and Winter, 2004). Many bacteria and fungi can degrade petroleum hydrocarbons but any single strain can afford biodegradation of all compounds found in petroleum sludge (Fan and Krishnamurthy, 1995). Biodegradation of petroleum hydrocarbons includes sequential reactions where bacteria in a microbial consortium collaborate with each other for degradation of oily sludge. Bioremediation duration is an important factor

which affects the biodegradation process. As time goes by, biodegradation rate decrease because of decreasing of biodegradable hydrocarbons and accumulation of recalcitrant compounds (Sihag et al., 2014). As it is declared trough different studies, the initial concentration of the petroleum hydrocarbons in the polluted soil is also an important factor in biodegradation rate (Boodoosingh et al., 2010).

Land farming which also know as land treatment is a remediation technology in which oily sludge mix with soil for removal of oil content by biodegradation (Hejazi et al., 2003). In this method oil polluted soil or petroleum sludge is spread in a thin layer on the ground and then the activity of present microbial community is increased by aeration and addition of nutrients, necessary minerals and moisture (Ubani et al., 2013). Through this process besides biodegradation, physicochemical degradations such as photodegradation have also important role in oil removal (Sihag et al., 2014). The performance of the biodegradation can be enhanced with adding of the bacterial strains to the soil. Through this process which called bioaugmentation new microbial inoculums, which are able to degrade contaminant are added to the soil (McGenity, 2014). Through land farming lighter components of petroleum sludge such as gasoline remove via evaporation and the remained fractions can be degraded by microorganisms in the soil. The heavier fractions may need more time for degradation. In hot and arid regions most part of the sludge evaporates and a small fraction may undergo the biodegradation process (Hejazi and Husain, 2004). Through land farming the volatile compounds emission should be controlled. The vapor emissions should be collects and treated before release to the atmosphere (Thibodeaux and Hwang 1982). Land farming is simple to design and needs short period of time. Being a cost-effective method and being effective in remediation of slow biodegradable components are the other advantages of this method.

Biopiles or compost piles technology which are used for biodegradation of petroleum sludge and petroleum contaminated soil involve piling up the contaminated soil and enhancing of the aerobic microbial bidegradation through aeration, addition of water and necessary nutrients (Hu et al., 2013). This method is similar to land treatment system whit a difference in the aeration system. While in land farming technique aeration is provided by tilling in the biopiles system aeration is supplied by forcing air using piping system inside piles. Biopiles system is effective in degradation of almost all of the components of petroleum products (Hazen et al., 2000). During this

process light components mostly remove by evaporation and the remained fractions degrade by microorganisms. The evaporated section then can be treated before release to the atmosphere. For increasing of porosity in the piles bulking agents such as wood chips, bark and saw dust can be used (Marín et al., 2006).

Through land farming and biopiles techniques biodegradation process can be accelerated. Nutrient addition especially adjusting of C:N:P ratio for an optimum amount can enhance the biodegradation (Ball et al., 2012). This process which known as biostimulation, is prevalent in bioremediation of petroleum contaminated soil. When natural microbial communities are not in sufficient number for biodegradation of petroleum hydrocarbons or degrading bacteria are not present in the treating system suitable microbial strains capable of degrading of petroleum hydrocarbon can be added to the bioremediation systems. This techniquewhich known as bioaugmentation, can also enhance the degradation process (Hamdi et al., 2007).

Soil column bioreactors and soil slurry phase bioreactors are to configurations of bioreactors for treatment of the petroleum hydrocarbons contaminated soil. In the bioreactor technology for treatment polluted soil or oily sludge mixes with water for increasing of the solubilization of the hydrocarbons which leads to increase the bioavailability of the hydrocarbons to the microorganisms (Robles-González et al., 2008). Hydrocarbon removal in this technique, therefore, is faster than soil-phase bioremediation methods such as land farming and biopiles. Furthermore, liquid phase facilitates the control of nutrients addition and introducing of additional carbon source and oxygen and optimizing of the conditions (Frank and Castaldi, 2003). Intrinsic microbial community are responsible for degradation of petroleum hydrocarbons in this technique however for enhancing of the bioremediation process additional microbial strains also can be used (i.e. bioaugmentation) (Ward et al., 2003). During bioreactor remediation hydrocarbons contents can be mineralize to carbon dioxide and water or transfer to the less toxic metabolites. Oily sludge because of its nonhomogene contents needs to be pretreated before biodegradation which needs extra budget (Hu et al., 2013). Furthermore generating of volute compounds and slurry mixture after bioreactor operation which will need to be treated also makes this system less economic in comparison with solid-phase treatment methods (Frank and Castaldi, 2003).

2.3 Petroleum hydrocarbons biodegradation: hydrocarbons metabolism, microbiology of degradation

2.3.1 Alkanes biodegradation

As a major fraction of crude oil, alkanes because of their non-polar and saturated nature are very immobile and less reactive (Labinger and Bercaw, 2002). This group of hydrocarbons can be found in three phase of gaseous (C1–C4), liquid (C5–C16) and solid (>C17). These hydrocarbons because of their poor water solubility are less bioavailable for microorganisms. However, alkanes can be degraded and converted to the easily degradable metabolites by some microorganisms (Berthe-Corti and Fetzner 2002). Different groups of microorganism have been reported in scientific literature which can use aliphatic compounds as carbon and energy source and can help to degrade these kinds of hydrocarbons. *Acinetobacter* sp., *Candida* sp., *Pseudomonas* sp., *Streptomyces* sp. *Arthrobacter* sp., *Bacillus* sp., *Rhodococcus* sp. are among the bacteria which have been reported to degrade alkanes (Whyte et al., 2002; Yousaf et al., 2010). Some bacterial strains are specialized in aliphatic hydrocarbons degradation. These bacteria can have an important position in the bioremediation of petroleum polluted sites. *Alcanivorax borkumensis*, *Alcanivorax dieselolei*, *Oleispira* sp., *Geobacillus* sp., *Bacillus* sp., *Thalassolituus* sp., *Oleiphilus* sp., and *Thermus* sp. are some reported alkane degrader bacteria (Golyshin et al., 2002; Yakimov et al., 2003; Yakimov et al., 2004; Liu and Shao, 2005; Schneiker et al., 2006; Marchant et al., 2006; Meintanis et al., 2006). Some bacteria are capable to effectively degrade special groups of alkanes. For instance, *Acinetobacter* sp. is able to degrade long chain n-alkanes (Singh et al., 2012). Hexadecane (HXD) which found in crude oil and is one of the main constituents of diesel has been reported to be degraded efficiently by *Pseudomonas putida*, *Bacillus thermoleovorans*, *Rhodococcus erythroplotis*, *Aspergillus niger*, *Arthrobacter* sp. and *Flavobacterium* sp. ATCC39723 (Steiert et al., 1987; Volke-Sepulveda, et al., 2003; Abdel-Megeed et al., 2010). Some alkane degraders such as *Desulfatibacillum alkenivorans* are reported to degrade effectively in the mesophilic conditions whereas some species such as *Goebacillus thermoleovorans* can degrade alkanes efficiently in thermophilic conditions and some others such as *Rhodococcus* sp. which are adapted to cold

conditions can degrade aliphatic hydrocarbons effectively in cold environments (Kato et al., 2001; Sanscartier et al., 2009; Singh et al., 2012).

Many fungi species also have potential to grow on alkane containing media (van Beilen et al., 2003). *Neosartorya*, *Graphium*, *Cephalosporium*, *Pencillium*, *Amorphoteca*, *Talaromyces*, *Aspergillus* are among fungi which are capable to degrade petroleum hydrocarbons. Some yeast species like *Pichia*, *Candida*, *Geotrichum* sp., *Rhodotrula mucilaginosa*, *Trichosporam mucoides* and *Yarrowia* have also potential to degrade crude oil hydrocarbons (Singh et al., 2012).

The solubility of alkanes in water depends on their molecular weight. The solubility decreases with increasing of the molecular weight (Eastcott et al., 1988). Alkanes with longer than C12 are practically insoluble. The insolubility of alkanes makes them difficult to be available for microbial uptake. For increasing the emulsification of insoluble hydrocarbons, alkane degrader bacteria produce various surfactants (Ron and Rosenberg 2002). Produced biosurfactants also facilitate the mobility of the bacteria and protect them from toxic substances (Kang and Park, 2010).

The most studied alkane degradation metabolic pathway is encoded by OCT plasmid carried by Pseudomonas oleovorans (Vandecasteele, 2008). With collaboration of membrane-bound monooxygenase with rubredoxin and rubredoxin reductase electrons transfer from NADH to the hydroxylase and converts alkane to an alcohol. The alcohol then can be oxidized to an aldehyde and acid which can be processed by the β-oxidation and tricarboxylic acid cycles (van Hamme et al., 2003). In the model offered by van Beilen et al., (1994 and 2001), all enzymes involved in oxidizing alkane to corresponding acyl-CoA are encoded by the *alkBFGHJKL* operon and *alkST* locus which are located on two detached regions of OCT plasmid. Alkane hydroxylase (AlkB), rubredoxins (AlkF and AlkG), aldehyde dehydrogenase (AlkH), alcohol dehydrogenase (AlkJ), acyl-CoA synthetase (AlkJ) and an outer membrane protein which may have a role in substrate transport, all are encoded by *alkBFGHJKL* operon. The *alkST* locus encodes the regulation protein (AlkS) and rubredoxin reductase (AlkT). The AlkS positively regulates (i.e. activates) the expression of the *alkBFGHJKL* operon. The *alkBFGHJKL* operon and *alkST* locus are separated by a 9.7-kb DNA (van Hamme et al., 2003; Vandecasteele, 2008). The separator DNA segment encodes a methyl acceptor transduction protein (AlkN)

which most probably has a role in alkane chemotactism process. Studies of *alk* genes in different alkane degrader strains showed that the regulation of alkane degradation genes and their clustering are various among bacteria. For example in *Acinetobacter* sp. *alk* genes are neither grouped in operons nor carried by a plasmid (van Hamme et al., 2003; Vandecasteele, 2008). Organization of alkane degradation model offered by Beilen et al., (2001) has been shown in Figure 2.2.

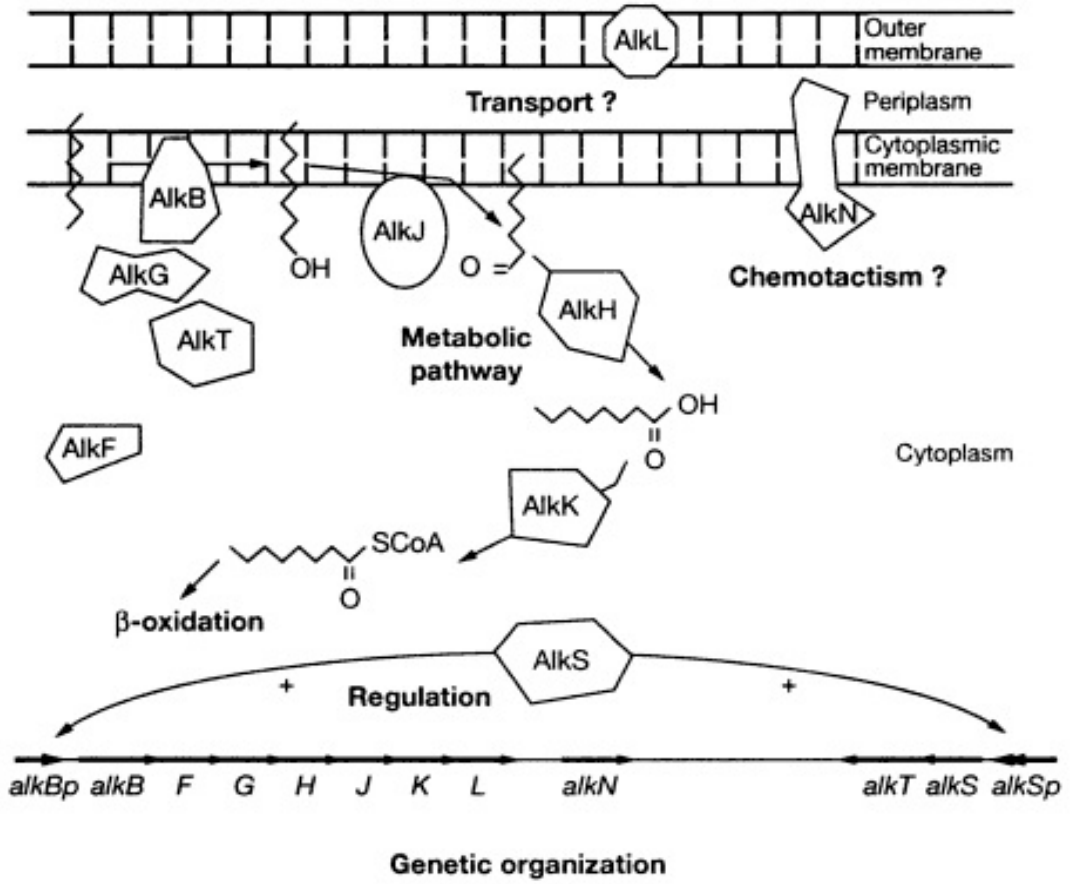

Figure 2.2 : Schematic of alkane degradation in *P. putida* GPo1, *alk* genes and intracellular enzymes locations (Vandecasteele 2008).

There is little information about the pathways other than monooxygenase-mediated model described for OCT plasmid. In Finnerty (1988) pathway which has been described for *Acinetobacter* sp. strain M1, by means of *n*-alkyl hydroperoxides a dioxygenase converts alkanes to aldehydes without an alcohol intermediate (Maeng et al., 1996; Sakai et al., 1996). Unlike the Beilen model, A flavin adenine dinucleotide chromophore has been detected in this model and rubredoxin and NAD(P)H are not required.

The majority of information about cyclohexane degradation has been obtained from the study of *Xanthobacter* sp. C20 (Trower et al., 1985). In the related pathway, the most important step is the initial hygroxylation and oxygen insertion on the cyclic ketone which leads to formation of lactone. Produced lactone then is metabolized or cleaves spontaneously.

n-alkyl cycloalkanes can be oxidized in both on the side chain and ring. Based on the studies by Beam and Beam and Perry (1974), *n*-alkyl cyclohexanes, these compounds after initial oxidation are degraded by β-oxidation on side chain. For odd chain (i.e. the number of carbon atoms are odd) cyclohexane carboxyl-CoA is formed and in the case of even chain cyclohexane acetyl-CoA will be produced. Cyclohexane carboxyl-CoA will then be further degraded by β-oxidation pathway but the metabolism of cyclohexane acetyl-CoA is not so easy and the involvement of a lyase will be required (Blakley 1978). Table 2.1 lists the bacteria and archaea which have the capacity to metabolize n-alkanes with varying chain lengths (Olajire and Essien 2015).

2.3.2 Monoaromatic hydrocarbons

Benzene, toluene, ethylbenzene and xylene, which collectively known as BTEX, are important monoaromatic hydrocarbons. These compounds because of being highly soluble in water are very mobile in water and soils and, therefore, are available to microorganisms in soluble form (Vandecasteele, 2008). The most privilege genera in degradation of monoaromatic hydrocarbon belong to *Pseudomonas* sp. Some other gram negative genus such as Flavobacterium can also degrade monoaromatic hydrocarbons. *Alcaligenes* sp., *Sphingomonas* sp., *Burkholderia*, and *Ralstonia* also contain some aromatic hydrocarbon degrading strains (Kaplan et al., 2004; Cébron et al., 2008). Although most of the studied aromatic degrading strains belong to gram-negative bacteria, bacteria belonging to gram-positive genera which have high C+G content such as *Mycobacterium, Nocardia* and *Rhodococcous* are excellent in degradation of monoaromatics. Gram-positive strains with low C+G content such as *Bacillus* can use some monoaromatic compounds as substrate (Vandecasteele, 2008). The first step in catabolism of monoaromatics is to hydroxylation of aromatic ring (Kim et al., 2007; Seo et al., 2012). This step which called upper pathway is

catalyzed by oxygenases. Regarding to BTEX as important monoaromatic hydrocarbons, oxygenases attack to the methyl group or aromatic ring.

Table 2.1 : Microorganisms having degradation potential for aliphatic hydrocarbons.

Hydrocarbon	Structure	Degrader
Octane	C_8H_{18}	*Arthrobacter* sp. *Bacillus* sp. *Rhodococcus* sp.
Decane	$C_{10}H_{22}$	*Bacillus* sp strain *DHT*
Tetradecane	$C_{14}H_{30}EH_4$	*Haloarcula vallismortis*
Pentadecane	$C_{15}H_{32}$	*Actinopolyspora* sp. *Marinobacter aquaeolei*
Hexadecane	$C_{16}H_{34}$	*Bacillus* sp strain DHT EH4 (*Haloarcula vallismortis*) *Halomonas* sp C2SS100 *Marinobacter aquaeolei* *Marinobacter hydrocarbonoclasticus* *Pseudomonas* sp. C450R
Octadecane	$C_{18}H_{38}$	*Halobacterium* sp. *Halococcus* sp. *Haloferax* sp.
Heptadecane	$C_{17}H_{36}$	*Haloarcula* sp. *Haloferax* sp.
Pristane	$C_{19}H_{40}$	*Alcanivorax* sp. Qtet3 EH4 (*Haloarcula vallismortis*) *Marinobacter hydrocarbonoclasticus*
Eicosane	$C_{20}H_{42}$	*Actinopolyspora* sp. DPD1 EH4 (*Haloarcula vallismortis*) *Haloarcula* sp. *Haloferax* sp. *Marinobacter hydrocarbonoclasticus*
Phytane	$C_{20}H_{42}$	*Alcanivorax* sp. strain Qtet3
Heneicosane	$C_{21}H_{44}$	EH4 (*Haloarcula vallismortis*) *Marinobacter hydrocarbonoclasticus*
Tetracosane	$C_{24}H_{50}$	*Alcanivorax* sp. strain Qtet3
Pentacosane	$C_{25}H_{52}$	*Actinopolyspora* sp. DPD1
n-Alkane	$C_{10}–C_{30}$	*Halobacterium* sp
	$C_{10}–C_{34}$	*Halobacterium* sp. *Halococcus* sp. *Haloferax* sp.
	$C_{10}–C_{34}$	*Alcanivorax* sp. strain Qtet3
	$C_9–C_{40}$	*Marinobacter falvimaris* *Marinobacter sedimentalis,*

There are a variety of metabolic pathways in degradation of monoaromatics but all leads to catechol or its derivatives which then undergo further degradation through

lower pathway (Shrivastava and Phale, 2011). Based on the studies of toluene degradation pathway in *Pseudomonas putida* mt-2, as one of the pioneer studies of monoaromatic degradation pathway, the TOL plasmid (known as pWW0) carried by this strain contains two *xyl* operons, one encodes the upper pathway which includes four enzymes and the second encodes the lower pathway (Fuentes et al., 2014). Some other strains carry similar TOL plasmids (Assinder and Williams, 1990). For instance, Pseudomonas putida MT53 contains pWW53 plasmid (Keil, et al., 1985). Chromosomal tod operon in Pseudomonas putida F1 encodes pathway for degradation of toluene. Toluene dioxygenase (TodC1C2BA) and dehydrogenase (TodD) are involved the upper section of related pathway which there catabolic activities leads to the producing of 3-methycatechol (Tan and Mason, 1990). The metabolic pathways of the aerobic biodegradation of toluene and benzene by *Pseudomonas putida* F1 has beem shown in Figure 2.3.

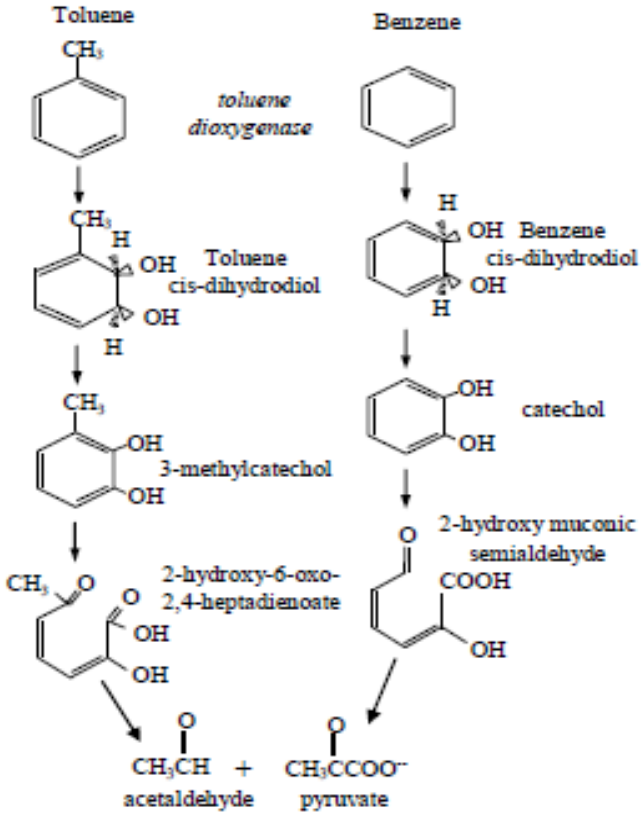

Figure 2.3 : Metabolic pathways of toluene and benzene in *Pseudomonas putida* F1. (Trigueros et al., 2010).

Similar to toluene, other BTEX members can also be used by a variety of strains. The degradation pathway for BTEX is essentially similar to the toluene pathway. For

example in *Pseudomonas putida* mt-2, m-xylene and p-xylene are degraded using *xyl* pathway (Ramos et al., 1997).

Other alkylbenzenes with short alkyl groups such as 1,2,3-trimethylbenzene, 1,3,5-trimethylbenzene, *n*-alkylbenzenes (C1-C7), isopropyl, isobutyl and etc can also be growth substrates for some strains (Lang, 1996). Initial attack positions are different in various strains. For example, in *Pseudomonas* strain initial attach by dioxygenase is carried out on the ring side whereas the initial attack site in the strains containing TOL plasmid when utilized 3-ethyltoluene was methyl group (Kunz and Chapman, 1981; Smith, 1990). Regarding to alkylbenzenes with long alkyl groups, attach by the dioxygenase occurs at the end of the alkyl chain (Smith, 1990). For example, in biodegradation of 1-phenyldodecane by *Acinetobacter lowoffii*, terminal hydroxylation of carbon chain took place and transformed to homogentisate. The homogentisate ring was then opened through lower pathway (Vandecasteele, 2008). In vinylbenzene which is a genotoxic alkylbenzene, initially attack by dioxygenase occur on the ring which results in production of 3-vinylcatechol (O'Leary et al., 2002). This compound then follows the lower metabolic pathway. Biphenyl as an important aromatic which has two unfused benzene rings takes the catabolic pathways of monoaromatic hydrocarbons. One of the benzene rings takes dihydroxylation by 2,3-dioxegenase and forms 2,3-dihydroxyphenyl which its ring then opens by meta fission and produces benzoate. The second ring then experiences further degradation (Smith, 1990). Intermediates produced in the upper pathway undergo lower pathway for degradation. Catechol and protocatechuate which are obligate intermediates of upper pathway can be metabolized according to two ring opening pathways. In one of them which called *meta*, dioxygenase bonds with carbon atom closed to the two hydroxyl groups and then starts the 2-oxiacid pathway. The other metabolic pathway which called *ortho*, happens between two hydroxyl groups and then starts the 3-oxoadipate pathway (Vandecasteele, 2008). Table 2.2 lists some microorganisms that degrade monoaromatics (Olajire and Essien 2015).

Table 2.2 : Microorganisms having degradation potential for some mono aromatic hydrocarbons.

Hydrocarbon	Degrader
Phenol	*Arhodomonas aquaeolei*, *Arthrobacter* sp. *Candida tropicals* *Halomonas campisalis* *Halomonas organivorans* *Thelassobacillusdevorans* *Modicisalibacter tunisiensis*
Benzoate	*Chromohalobacter* sp. strainHS-2 *Haloferax* sp. D1227 *Halomonas campisalis* *Halomonas elongate* *Halomonas eurihalina* *Halomonas halodurans* *Halomonas organivorans* *Marinobacter lipolyticus*
HydroxyBenzoate	*Arthrobacter* sp. *Bacillus* sp. *Bacillus* sp. strainDHT *Haloferax* sp. *Halomonas alimentaria* *Halomonas campisalis* *Halomonas organivorans* *Halomonas venusta* *Pseudomonas* sp. *Rhodococcus* sp. *Salinicoccus roseus*
o- Phthalate	*Arthrobacter* sp. *Bacillus* sp. *Rhodococcus* sp.
Gentisate	*Arthrobacter* sp. *Bacillus* sp. *Rhodococcus* sp.
4- Hydroxybenzoate	*Chromohalobacter* sp. strainHS-2 *Haloarcula* sp. strainD1, *Halobacterium* sp. *Haloferax* sp. *Halomonas elongate* *Halomonas organivorans* *Halorubrum* sp.

Table 2.2 (continued) : Microorganisms having degradation potential for some mono aromatic hydrocarbons.

Hydrocarbon	Degrader
Phenyl propionic acid	*Haloferax* sp. D1227 *Halomonas elongata* *Halomonas glaciei* *Halomonas organivorans*
Cinnamic acid	*Haloferax* sp. *Halomonas elongate* *Halomonas halophila* *Halomonas organivorans* *Halomonas salina* *Halomonas* strain IMPC
p- Coumaric acid	*Halomonas organivorans* *Chromohalobacter israelensis* *Halomonas organivorans* *Halomonas salina* *Halomonas* strain IMPC
Ferulic acid	*Halomonas elongate* *Halomonas organivorans* *Halomonas* strain IMPC
Benzene	*Alcanivorax* sp.HA03 *Arhodomonas* sp. strainRozel *Halobacterium* sp. *Halococcus* sp. *Haloferax* sp. *Marinobacter hydrocarbonoclasticus* *Marinobacter falvimaris* *Marinobacter sedimentalis*, *Marinobacter vinifirmus*, *Planococcus* sp. strainZD22
Toluene	*Alcanivorax* sp.HA03 3–15 *Arhodomonas* sp. strainRozel *Arhodomonas* sp. strainSeminole *Halobacterium* sp. *Halococcus sp.* *Haloferax* sp. *Marinobacter vinifirmus*, *Marinobacter hydrocarbonoclasticus* *Planococcus* sp. strainZD22
Ethylbenzene	*Marinobacter vinifirmus*, *M.hydrocarbonoclasticus* *Planococcus* sp. strainZD22
Xylene	*Marinobacter vinifirmus*, *M.hydrocarbonoclasticus* *Planococcus* sp. strainZD22

2.3.3 Poly aromatic hydrocarbons

Various studies have shown that a variety of microorganisms from bacteria, fungi, algae and cyanobacteria are capable to degrade low (three rings or fewer) and high molecular weight (four or more rings) PAHs. Regarding to the PAHs with more than four rings, no microorganism strain have been reported to use them as a sole carbon and energy source. Cometabolic transformation is the process for degradation of this group of hydrocarbons (Sutherland, 1992; Juhasz and Naidu, 2000; van Hamme et al., 2003). PAHs are characterized by their low water solubility and high sorption potential. There characters, which affect the biodegradation of PAHs, as well as some other factors such as producing of toxic metabolites, presence of other preferred substrate and lack of cosubstrates or inducer substrates should be considered while biodegradation of the PAHs (Molina et al., 1990; Juhasz et al., 2002). Understanding about the biodegradation process in the environment contaminated with mixture of PAHs and their various metabolites is more difficult. The majority of findings about the genetics of PAH metabolism have been obtained from the study of naphthalene catabolic plasmids such as NAH7 carried by *Pseudomonas putida* strain G7. All studied pathways are very similar. As a brief, catabolism starts with an initial attach by oxygenases and continue to conversion the naphthalene to salicylate (upper pathway) and then the conversion of salicylate to acetaldehyde and pyruvate (lower pathway) (Yen and Serdar, et al., 1988; Eaton and Chapman, et al., 1992; Platt et al., 1995) (Figure 2.4).

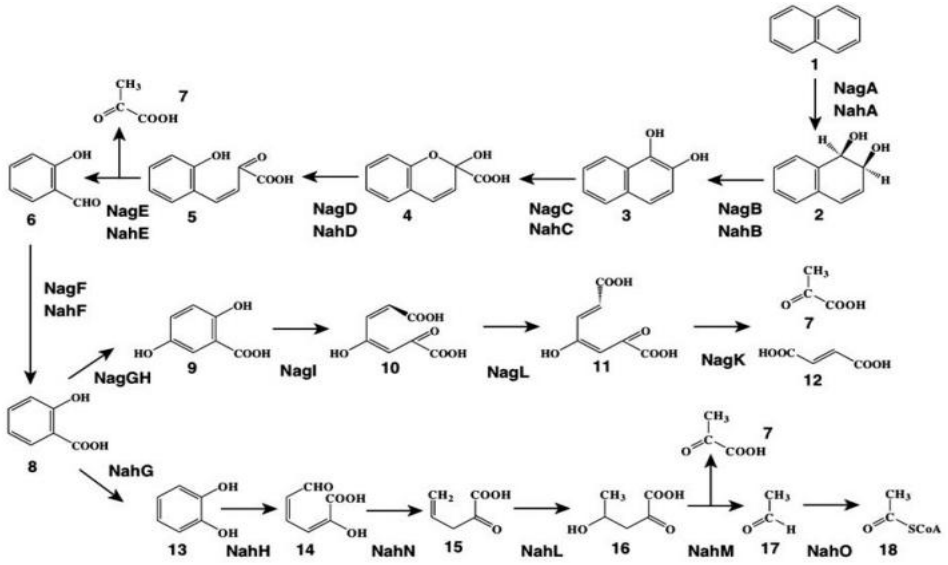

Figure 2.4 : Schematic of naphthalene degradation in *P. putida* G7 (Suenaga et al., 2009).

Enzymes involved in the upper pathway are encoded by *nahAaAbAcAdBFCED* operon whereas *nahGTHINLOMKJ* operon encodes the enzymes involved in lower pathway (Li et al., 2004). A third operon (*nahR*) encodes the regulator of both *nah* operons (Schell and Wender, 1986). It has been found that naphthalene dioxygenase is a flexible enzyme which can catalyze a variety of reactions (Parales et al., 200). Naphthalene degrading plasmid enzyme system can degrade some other PAHs such as phenanthrene and anthracene (Sanseverino et al., 1993; Mejáre and Bülow, 2001). With isolation and characterization of different PAH degrading bacteria a variety of PAH metabolic genes has been reported. Among these *ndo*, *pah*, and *dox* operons showed more than 90% homology to *nah* operon but it does not mean that they translate to enzymes specific to similar substrates (van Hamme et al., 2003). Different isofunctional gene sequences have been investigated in bacterial strains such as Rhodococcus, Nocardia, and Mycobacterium spp. which are able to degrade high molecular weight PAHs like pyrene. Regarding to the metabolism of PAH, other gene sequencing have been detected. *phnFECDAcAdB* (Laurie and Lloyd-Jones, 2000), *nagAaGHAbAcAdBF* (Hudlicky and Reed, 1995), *nidABCD* (Treadway et al., 1999), *nidDBA* (Khan et al., 2001), and *phdABCD* (Sato et al., 1997) are some examples which have been verified on *Burkholderia* sp. strain RP007, *Pseudomonas* sp. strain U2, *Rhodococcus* sp. strain I24, *Mycobacterium* sp. strain PYR1 and *Nocardiodes* sp. strain KP7 respectively. Naphthalene catabolic genes have been also found on the chromosomes indicate that genetic recombination and lateral gene transfer may have an important role in these metabolic pathways (Hudlicky and Reed, 1995; Sato et al., 1997; Laurie and Lloyd-Jones, 2000). Among PAH degrading bacteria, strains containing multiple gene operons for a similar enzyme has also been detected. For instance, Ferrero et al. (2002) reported two nahAc-like genes for upper pathway on *Pseudomonas* spp., and Bosch et al. (1999) detected two distinc genes (*nahG* and *nahW*) for lower pathway (i.e. for converting of salicylate to catechol) on *P. stutzeri* AN10 chromosome. *nahG* was detected in the *meta*-cleavage related operon unite while *nahW* was outside of this unit. Both of these genes are induced while exposure to the salicylate but *nahW* doesn't have the FAD-binding site (GxGxxG). *nahG* and *nahW* which encodes salicylate hydroxylases are examples of isofunctional genes in one strains (Bosch et al., 1999).

In degradation of phenanthrene as a three-ring PAH, upper pathway is similar to the naphthalene degradation pathway. A dioxygenase enzyme attack to the position of 3,4 and caused to formation of cis-3,4-phenanthrene dihydrodiol which is then converted to 1-hydroxy-2-naphthoate. This compound is converted into 1,2-dihydroxynaphthalene by Evans pathway (Evans et al., 1965). 1,2-dihydroxynaphthalene then can be degraded using the manner described to the naphthalene and finally catechol is formed. As an alternative for the Evans pathway, through a second pathway which called Kiyohara pathway, 1-hydroxy-2-naphthoate is oxidized to into protocatechuate (Kiyohara et al., 1994). Produced catechol and protocatechuate can then be degraded using the pathways of monoaromatic hydrocarbons.

Upper degradation pathway mostly has been declared through studies which have been done on *Pseudomonas putida* OUS82 and *Nocardioides* sp. strain KP7 (Kiyohara et al., 1994; Iwabuchi and Harayama, 1998). Utilizing phenanthrene by these bacteria as a carbon and energy source leads to produce 1-hydroxy-2-naphthoate. This metabolic pathway is encoded by *phdEFABGHCD* cluster which is a chromosomic gene. In most strains the upper phenanthrene degradation gene shows low level of homology with clasical *nah* genes. This case has been reported in *Comamonas testosterone* GZ39 when they grew on naphthalene, phenanthrene, and *Burkholderia* sp. strain RP007 when they grew on naphthalene, phenanthrene and anthracene (Goyal and Zylstra, 1997). On the other hand, plasmid pLP6a carried by *Pseudomonas Fluorescens* LP6a, has the genes fairly similar to naphthalene degradation genes of NAH7 and pWW60 plasmids when grows on naphthalene, phenanthrene and anthracene (Foght and Westlake, 1996).

Lower degradation pathways convert 1-hydroxy-2-naphthoate to salicylate. This reaction is take place similar to the upper pathway but for degradation of the second aromatic ring (Balashova et al., 2001). In Pseudomonas *putida* BS202-P1 for this purpose 1-hydroxy-2-naphthoate hydroxylates into 1,2-dihydroxynaphthalene. This reaction is catalyzed by salicylate 1-monooxygenenase. This path way is known as Evans pathway. In strain *P. putida* OUS82 almost a same pathway is used for degradation of naphthalene and phenanthrene into salicylate and 1-hydroxy-2-naphthoate respectively but for conversion of 1-hydroxy-2-naphthoate to salicylate a

pathway other than Evans pathway is used (Takizawa et al., 1994). This pathway which is called Kiyohara pathway in *Nocardioides* sp. strain KP7 has been studied (Saito et al., 2000). In this strain for conversion of 1-hydroxy-2-naphthoate is converted to *o*-phthalate, gene cluster *phdI*, *phdJ* and *phdK*. encodes this pathway. *phdIJK* is located following *phdEFABGHCD* cluster (upper pathway genes) (Iwabuchi and Harayama, 1998).

Anthracene as sole carbon and energy source is used by various strains. Very little information about the degradation pathway of anthracene is available. In the fulfilled studies the degradation pathway has been reported similar to the degradation of naphthalene (Vandecasteele, 2008). As in the *P. putida* OUS82 a dioxygenation attack is done in the position of 1,2 which cause a cleavage and producing of 2-hydroxy-3-naphthoate (kiyohara et al., 1994). Cernigilia and Heitkamp (1989) showed that in some strains further degradation of 2-hydroxy-3-naphthoate leads to 2,3-dihydroxynaphthalene production which degradation continues with salicylate and catechol.

Fluorene is the other three-ring PAH which possessing an alicyclic ring makes if different from the other PAHs. This hydrocarbon degrades using two different mechanisms. Based on the studies which have been done on *Arthrobacter* sp. strain F101, three catabolic pathways have been demonstrated. Dioxygenation of fluorene at the positions 1,2 and 3,4. is the initial step. Via the third pathway C-9 reacts with monooxygenation which leads to dead-end metabolites such as 9-fluorenol, 9-fluorenone, 4-hydroxy-9-fluorenone. Accumulated 9-fluorenone derivatives which are dead-end products can be degraded by mixed cultures. Casellas et al. (1998) could degrade fluorine without accumulation of 9-fluorenoneusing co-culture of *Pseudomonas mendocina* MC2 and *Arthrobacter* F101. The second type of mechanism for degradation of fluorine which reported in *Pseudomonas* sp. strain F274 and *Brevibacterium* sp. strain DPO 1361 takes place by dioxygenation of 9-fluorenone at C-1/C-1a which produces 1,1a-dihydroxy-1-hydro-9-fluorenone (Trenz et al., 1994).

In *Alcaligenes denitrificans* WW1, degradation of fluoranthene after the initial dioxygenase attack at position 9,10 the alicyclic ring degradation takes place by Baeyer-Villiger reaction. 3-substituted catechol is resulted which then further

progress by *meta* cleavage (Vandecasteele, 2008). 1-acenaphthenone is produce as intermediate. Studies of Mycobacterium PYR-1 showed another dioxygenase attach at position of 1,2 and producing of 9-fluorenone (Cerniglia, 2003). Existence of several pathways allow to broad mineralization of fluoranthene and producing of various intermediates.

Regarding to pyrene, similar to naphthalene and phenanthrene, biodegradation initiates with dioxygenase attack. This dioxygenation occurs at 4,5 position which then *cis*-4,5-pyrene dihydrodiol is produced. In this pathway which has been reported in some bacterial strains such as *Mycobacterium* sp. PYR-1 (Heitkamp et al., 1988), *Mycobacterium* sp. RFGII-145 (Schneider et al., 1996), *Pseudomonas* sp. XPW-2 (Zylstra et al., 1994), *P. yanoikuyae* R1, *Bacillus cereus* P21 (Kazunga and Aitken, 2000), and ets, mineralization process leads to generation of phthalate and in some case cinnamate. Some other attack positions have been reported for instance in *Mycobacterium* sp. PYR-1 dihydroxylation at 1,2 position leads to produce 4-hydroxyperinaphthenone.

Using of other four-ring PAHs as a carbon and energy sources reported very seldom but regarding to the degradation of chrysene has been shown through some studies. Strains such as *Rhodococcus* sp. UW1 (Walter et al., 1991), *Sphingomonas* sp. CHY-1 (Demaneche et al., 2004) and *Pseudomonas fluorescens* (Caldini et al., 1995) are some examples. *Pseudomonas fluorescens* can limitedly grow on benz(a)anthracene. For this compound as declared in *Beijerinckia* sp. B1, the initial attack by dioxygenase occurs at position 1,2 which leads to cleavage of one ring and prudusing of 1-hydoxy-1-anthranoate (Mahaffey et al., 1988). Benz(a)anthracene also can be attacked on several positions such as 1,2, 8,9 and 10,11 which has been reported in *Beijerinckia* sp. B1 and positions 5,6 and 10, 11 which has been reported in *Mycobacterium* sp. RJGII-135 (Schneider et al., 1996).

Regarding to the studies on the degradation of benzo(a)pyrene and dibenz(a)anthracene as five-ring PAHs, these compounds are not used as sole carbon and energy sources. Co-metabolism of chrysene and benz(a)anthracene has been reported through different studies. Some findings about the cometabolism of these compounds with three or four-ring PAHs have been reported. Conversion of benzo(a)pyreneto cis-9,10- benzo(a)pyrene dihydrodiol and cis-7,8- benzo(a)pyrene

dihydrodiol by *Beijerinckia* sp. B8/36 has been reported (Kanaly and Harayama, 2000). Based on the studies of Schneider et al. (1996) on *Mycobacterium* sp. RJGII-135, benzo(a)pyrene, they found an initial attack on position 4,5 by dioxygenase which led to production of 4,5 chrysene dicarboxylate. They also have reported other initial attack sites at positions 7,8 and 9,10.

Obtained information about the biodegradation of individual PAH cannot be enough to describe the biodegradation of PAH mixture. PAH mixtures are not completely degraded by a single strain and because of limitations which mostly arise from the inhibitory effects of diverse metabolites and intermediates resulted from cometabolism in the biodegradation capacity of single strain, suitable consortia are needed. Table 2.3 lists some microorganisms that degrade polycyclic aromatic hydrocarbons (PAHs) (Olajire and Essien 2015).

2.3.4. Functional genes and horizontal gene transfer

The evaluation of hydrocarbon degrading functional gene gives valuable information about the degradation capability of the polluted sites. *alk* gene as functional gene in alkane-degrading bacteria are present in the genomes of different gram-negative and gram-positive bacteria. Many strains which can utilize alkane as carbon and energy source contain one or more *alk* genes. *alk* genes are generally chromosomal. Regarding to the PAH degrading functional genes, Foght and Westlake (1991) and Sayler and Layton (1990) were detected naphthalene degraders in aquatic and terrestrial regions using gene probes which have been prepared for NDO and NAH7 plasmids. Ahn et al., (1999) reported the ability of *nah*A-derived probe to detect a large proportion of the naphthalene and phenanthrene degrading strains. Using *phn*Ac gene Widada et al. (2002) could detect 19 strains of PAH-degrading bacteria from various environments. Hamann et al. (1999) using *ndoB* gene of the NDO plasmid from *Pseudomonas putida* NCIB 9816 could detect successfully a wide range of PAH-degrading bacterial strain from quit different genera. Bacterial strains carrying *pah* genotype are more numerous in the contaminated soils than those carrying nah genes (Laurie and Lloyd-Jones, 2000).

Table 2.3 : Some microbial strains having degradation potential for polycyclic aromatic hydrocarbons (PAHs).

Hydrocarbon	Degrader
Naphthalene	*Alcaligenes* sp. *Arthrobacter* sp. *Arthrobacter* spp. SN17 *Bacillus* sp strainDHT *Haloarcula hispanica* *Halobacterium piscisalsi*, *Halobacterium salinarium*, *Halobacterium* sp. *Haloferax* sp. *Halorubrum ezzemoulense*, *Marinobacter falvimaris* *Marinobacter nanhaiticus* *Marinobacter sedimentalis* *Micrococcus* sp. *Pseudomonas* sp. *Rhodococcus* sp.
Anthracene	*Alcaligenes* sp. EH4 (*Haloarcula vallismortis)* *Haloferax* spp *Marinobacter falvimaris* *Marinobacter nanhaiticus* *Marinobacter sedimentalis* *Micrococcus* sp. *Pseudomonas* sp.
Phenanthrene	*Alcaligenes* sp. *Arthrobacter* sp. EH4 *(Haloarcula vallismortis)* *Halobacterium* sp. *Haloferax* sp *Micrococcus* sp. *Pseudomonas* sp.
Acenaphthene	EH4 (*Haloarcula vallismortis)*
Fluorene	*Actinopolyspora* sp. DPD
Pyrene	*Bacillus* sp strainDHT *Haloarcula hispanica* *Halobacterium piscisalsi*, *Halobacterium salinarium*, *Haloferax* spp. *Halorubrum ezzemoulense*,
Biphenyl	*Arthrobacter* sp. *Halobacterium* sp. *Halococcus* *Haloferax* sp. *Marinobacter falvimaris* *Marinobacter sedimentalis* *Rhodococcus* sp.

In the bioremediation of petroleum polluted sites bioavailability is more important factor. Functional genes have also been used in the evaluation of the bioavailability of the petroleum hydrocarbons in contaminates sites. Sanseverino et al. (1993) have examined biosensor of lux casstte fused with the *nah*G gene for demonstration of naphthalene bioavailability.

Horizontal gene transfer (HGT) plays an important role in the efficiently adaptation of the bacteria to the contaminated environment (Boronin and Kosheleva 2014). In this process in which genetic material moves between bacterial strains plasmids play the main role. Trough HGT microbial community can adapt to the contaminated site and can degrade the contaminants via spreading of the catabolic pathways. Furthermore catabolic pathways can be evolved too due to HGT. HGT in microbial community in the environment have been previously demonstrated (Top and Springael, 2003). The presence of highly conserved *nah*AC gene in naphthalene catabolic plasmids (pDTG1-like) in diverse bacteria from different geographical regions (Herrick et al. 1997) and *dha*A gene in three different organisms *Rhodococcus rhodochrous* NCIMB13064, *Pseudomonas pavonaceae* 170, and *Mycobacterium* sp. strain GP1 are two examples. Horizontal gene transfer in naphthalene catabolic pathway has been also demonstrated due to experiments using labeled conjugative plasmids (Akhmetov, et al. 2008, Boronin and Kosheleva 2014). Horizontal transfer of naphthalene biodegradation genes in soil microbial community leads to enhance the biodegradation rate of petroleum hydrocarbons due to appearance of more effective degrader strains. By enhancing the development of an effective degrading microbial population, HGT can increase the biodegradation potential of contaminated soils. Therefore, determination and monitoring of suitable degrading genes on mobile genetic elements which can promote the HGT will be useful approach for ensuring successful bioremediation process. Based on the various related studies, *alk*B gene which encode alkane monooxygenase enzyme and *phn*Ac and *nah* genes are ideal markers for evaluation of the petroleum hydrocarbon degradation potential in the contaminated sites. Targeting and quantitive analysis of these genes have been examined successfully for characterizing the abundance and diversity petroleum degrading bacterial community. Monitoring is an essential necessity for assessment of the efficiency of biodegradation process throughout the

biostimulation. Analysis of HGT using quantitative techniques such as qPCR, as a feasible and effective monitoring approach needs to be investigated.

2.3.5. White rot fungi and there application in treatment of poly aromatic hydrocarbons

The capability of white rot fungi for biodegradation of recalcitrant pollutants and xenobiotics have been recently attracted research interests in the field of environmental biotechnology (Asgher et al., 2008). White rot fungi belong mostly to the basidiomycetes. Few members of ascomycetes also produce white rot. The mycelia of these fungi groups which cause white rot on tree can produce extracellular ligninolytic enzymes and decompose wood to white sponge-like mass (Gao et al., 2010). Recently, the capability of white rot fungi (WRF) for biodegradation of xenobiotics and recalcitrant pollutants has generated a considerable research interest in this area of industrial/environmental microbiology. These fungi play important decomposition role in the environment because of their ability to catabolism compounds such as cellulose, hemicelluloses and lignocelluloses.

White rot fungi produce three extracellular enzymes which are effective in lignin degradation. These enzyme systems when combining with other processes can mineralize lignin. This enzyme system comprises: lignin peroxidase, Mn dependent peroxidase and laccase (Pointing, 2001). While these fungi can effectively breakdown lignin however they cannot use it as energy source. Most probably they degrade the lignin just for access to the cellulose of cell wall (Gao et al., 2010). Some other accomplice enzymes such as H_2O_2-forming glyoxal oxidase, aryl alcohol oxidase oxalate producing oxalate decarboxylase (ODC), NAD-dependent formate dehydrogenase (FDH) and P450 monooxygenase have been isolated from some white rot strains too (Asgher et al., 2008).

The idea of using this organism in environmental technology was proposed in 1980s. Through last 20 years these fungi which can be applied in different environmental media have helped biotechnology for degradation various kinds of organic pollutants in the environment. Degradation of many pesticides, poly aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dyes, 2,4,6- trinitrotoluene (TNT), pentachlorophenol (PCP) and some other compounds using white rot fungi have been

reported in the literature (Cookson, 1995; Reddy and Mathew, 2001). *Phanerochaete chrysosporium*, *Trametes versicolor*, *Bjerkandere adusta* and *Pleurotus ostreatus*, have been mostly studied for the biodegradation of xenobiotic organic pollutants. Table 2.4 summarized some of the pollutants which can be degraded by these fungi.

Table 2.4 : Biodegradation of organic pollutants by white rot fungi (Gao et al., 2010).

Organism	Pollutant or waste
Bjerkandera adusta	PAHs (dibenzothiophene, fluoranthene, pyrene and chrysene) TNT Daunomycin producing wastes
Irpex lacteus	PAHs (phenanthrene, anthracene, fluoranthene, and pyrene) Synthetic dyes (Reactive Orange 16 and Remazol Brilliant Blue R.)
Lentinus tigrinus	Polycyclic aromatic hydrocarbons (PAHs)
Trametes versicolor	Trichloroethylene (TCE) Pentachlorophenol (PCP) Synthetic dyes (Amaranth) Polysaccharide
Phlebia radiata	TNT
Pleurotus ostreatus	PAHs
Phanerochaete chrysosporium	PAHs (anthracene) Molasses wastewater Textile wastewater (Victoria Blue)

The capability of white rot fungi in bioremediation of contaminated soil have been studied by some researchers. Degradation of Poly aromatic hydrocarbon using five strains of white rot fungi (*T. versicolor* PRL 572, *T. versicolor* MUCL 28407, *Pleurotus ostreatus* MUCL 29527, *P. sajorcaju* MUCL 29757 and *P. chrysosporium* DSM 1556) have been reported by Andersson and Henrysson (1996). Studied strains showed different degradation abilities. Yateem et al., (1998) have tested the ability of three strains of white rot fungi, namely Phanerochaete chrysosporium, *Pleurotus ostreatus*, and *Coriolus versicolor* to degrade oil in contaminated soil. Studied fungi could decrease the amount of TPHs from 32 g/kg to 7 g/kg within 12 months. Eggen and Sveum, (1999) studied the effects of *Pleurotus ostreatus* on bioremediation of PAHs in a creosote contaminated soil. *P. ostreatus* showed a positive effect on the biodegradation of PAHs. Marquez-Rocha et al., (2000) studied the degradation of PAHs using *Pleurotus ostreatus*. They reported 50% of pyrene, 68% of anthracene, and 63% of phenanthrene mineralization after 21 days of examination. They could

increase the biodegradation of mentioned compounds by adding of Tween40 (polyoxyethylene (20) sorbitan monolaurate) surfactant. Canet et al., (2001) have studied the effects of combination of white rot fungi and native strains on the biodegradation of PAHs. Acevedo et al., (2011) have studied the PAHs biodegradation capacities of Anthracophyllum discolor. High removal capacity for phenanthrene (62%), anthracene (73%), fluoranthene (54%), pyrene (60%) and benzo(a)pyrene (75%) were reported. Chen et al., (2005) with studying the degradation of PAHs by white rot fungi have demonstrated the effects of some ecological conditions such as temperature, medium composition, dissolved oxygen, and the moisture content on the biodegradation capability of these fungi.

Despite successes in using of white rot fungi in biodegradation of xenobiotic organic pollutants, there are some limitations and technical challenges for application of these organisms in bioremediation practice. The main limitation of the white rot fungi for bioremediation purpose is its sensitivity to biological process operational conditions. Suspended media is not suitable for growth of these organisms and mixing actions reduce the efficiency of these fungi furthermore, they have lower ability to attach to the fixed media (Cookson, 1995). Reddy and Mathew, (2001); Hestbjerg et al., (2003) and Gao et al (2010) have demonstrated, the biodegradation test using white rot fungi have been mostly conducted on autoclaved soil or synthetic media and under controlled conditions whereas, they may not be such effective in natural environmental conditions with changeable temperature, moisture, pH, native organisms and so on. The other challenge is the application of white rot fungi in nonsterile conditions. In in situ condition indigenous microbial strains can grow faster and compete for carbon and nutrient sources. Most of white rot fungi have strict growth conditions. For instance *Phanerochaete chrysosporium* needs high temperature (30–37°C) for growth and production of ligninolytic enzyme (Hestbjerg et al., 2003). Very few studies test biodegradation performance under field conditions. One problem with field application deals with the strict growth conditions required for most white rot fungi. For example, *P. chrysosporium* has a high temperature requirement (30–37°C) for growth and ligninolytic enzyme production (Hestbjerg et al., 2003). In the case of large-scale waste treatment, limitations are even more. *Phanerochaete chrysosporium* which is a widely studied white rot fungus has not shown effective results in any large-scale tests.

Trametes versicolor and *Bjerkandera adusta* as members of white-rot fungi (WRF) are good candidates for the degradation of diverse groups of xenobiotics (Rodarte-Morales et al., 2011; Rodríguez-Rodríguez et al., 2012 a; Jelic et al., 2012; Shahi et al., 2016a). *T. versicolor* is a filamentous WRF. This obligate aerobic fungus belongs to polyporaceae. As the most common shelf fungus in the northern hemisphere it plays an important role in degradation of wood wastes. In submerged culture it grows as dispersed or pelleted mycelium and neither fruiting body nor spore is formed. Its morphology is depends mostly on the medium composition and growth conditions such as C/N ratio, pH and ionic stress. The growth ability of *T. versicolor* some aromatic hydrocarbons such as phenol and methylated phenols and on petroleum contaminated soil have been reported (Yemendzhiev et al., 2008; Alexieva et al., 2010; Yanto et al., 2014). *B. adusta* as a member of Polyporales have been reported also for its capability in the biodegradation of PAHs, Polychlorinated biphenyls (PCBs) and Benzo(a)pyrene (Field et al., 1992; Kotterman et al., 1994; Beaudette et al., 1998). In this thesis *T. versicolor* and *B. adusta* were used in bioaugmentation of petroleum contaminated soil following a biostimulation practice.

2.3.6 Molecular methods and biodegradation monitoring

Understanding of the behavior of responsible degrader populations is a powerful approach for evaluation of the efficiency of different bioremediation approach. Physicochemical methods mostly explain the loss of contaminates and do not give enough information about the abiotic and biotic removal fractions. Furthermore methods for tracking and analyzing of the key metabolites, such as using stable isotope fractionations are very difficult for interpretation especially in the area with low concentration of contaminants. Analyses of microbial activities throughout the bioremediation practice gives direct information about the biodegradation process and will allow to intervention to the remediation procedure and enhancing the performance of the practice over needed.

Performance of bioremedation process depends above all on the activity of microbial community involved which required understanding of the dynamics of microbial community. In complex ecosystems such as bioremediation site, due to the complexity of microbial compositions and lack of suitable method for analysis of individual microbial population, evaluation of the treatment manner was very

difficult and deciphering of the biological events was almost impossible. Cultural based methods based on isolation and cultivation of strains in the laboratory, have several limitations. Based on the estimations 0.1 to 1% of the microbial strains can be can be cultured (Donachie et al., 2007). This method cannot give full picture of the microbial community involved in the bioremediation process and misses important populations. Isolation and growth of culturable species is time consuming process. Application of cultural-independent molecular techniques in microbial ecology studies has been common. Cultural-independent methods can provide a comprehensive image of the diversity of microbial community in the bioremediation site.

Molecular techniques which are used for explain microbial community can be divided into four groups: clone library, genetic fingerprinting, hybridization techniques, and quantitative real-time PCR (qPCR). Clone library methods have been widely used for microbial evaluation of bioremediation systems and still used when inclusive taxonomic information is needed (Sanz and Kochling, 2007). This method, however, because of being time-consuming technique is not suitable when working on large number of samples. In addition, large number of clones must be provided which leads to increase the cost and required time. Molecular finger printing techniques such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism analysis (T-RFLP) and temperature gradient gel electrophoresis (TGGE) are most popular today for analysis of microbial community in the environmental issues. In these methods PCR amplified fragments are separated by their differing mobility in a capillary or on a gel. The generated patterns generated based on the each band or peak reflects the diversity of microbial community. DNA fragments of each band can be separated and sequenced which will give the phylogenetic information of microbial strains. In nucleic acid hybridization techniques, short oligonucleotide probes which are specific to target microorganisms are used. Applied probe conducts with the specific extracting nucleic acids. Dot blotting technique frequently has been used in metabolic study of microbial community by investigation of the changes in the gene expression level. Fluorescence in situ hybridization (FISH), has been frequently employed in the bioremediation studies. This method which does not need nucleic acid extraction, using FISH microscopy system gives in-situ visual information about the abundance

of microorganisms (Amann and Fuchs, 2008; Wagner et al., 2003). Hybridization methods have some limitations mostly because of difficulties in some steps such as hybridization and washing steps. qPCR is currently the most effective and precise method for detecting and quantifying of nucleic acids. Despite other PCR-based methods such as DGGE which can just detect the presence of target sequences, qPCR can also quantify their numbers. This method has been frequently employed for evaluation of the bioremediation practice (Kim et al., 2013).

qPCR is highly sensitive technique to quantitatively follow changes of phylogenetic and functional genes during the bioremediation practice. Variations in the gene abundance and gene expression can be evaluated and compared throughout the process (Kim et al., 2013). The arrangement qPCR data sets describing the abundance of specific bacteria or genes, gives important information about the microbial community for interpretation of the role of each microbial group in the studied bioremediation site. Obtained quantitative information helps linking any changes in the structure of microbial community in the performance of bioremediation process because the functional features are deeply affected by the composition of microbial community (Akarsubasi et al., 2005; de los Reyes, 2010). Conventional PCR can only quantify the final concentration of amplified nucleic acids and the final concentration is not proportional to the initial concentrations therefore, is not recommended to use in quantitative analysis (Zhang and Fang, 2006). qPCR in comparison can follow real time progress of DNA amplification process (Heid et al., 1996). Real-time tracking of the amplification and absolute quantification of target sequences is obtained by the continuously quantifying of the fluorescence signs emitted through the accumulation of amplicons. Some detection approaches using various fluorescent molecules such as non-specific DNA-binding dyes, hybridization probes, light-up probes, molecular beacons are used in the qPCR assay (Lim et al., 2011). Detection in all kinds is based on the emitted signal level which reflects the cumulative amount of target amplicons. SYBR Green I (a non-specific fluorogenic molecule which binds to double stranded DNA) and a dual-labeled TaqMan probe (an oligonucleotide which binds to specific DNA target) are widely used methods in microbial ecology studies (Figure 2.5).

In SYBR green based qPCR, SYBR Green I day binds to the minor groove of doublestranded DNA and emits 1000-fold more fluorescence than when unbound (Wittwer et al., 1997). In this assay each amplification cycle is measured by the relative fluorescent intensity and the fluorescence signal increases with increasing of the amplicons accumulation. SYBR green I is non-specific binding dye and may cause false-positive results. The specificity of this assay determined just with forward and reverse oligonucleotides primers. Therefore, non-specific PCR amplification using non-specific primers should be avoided for a dependable analysis.

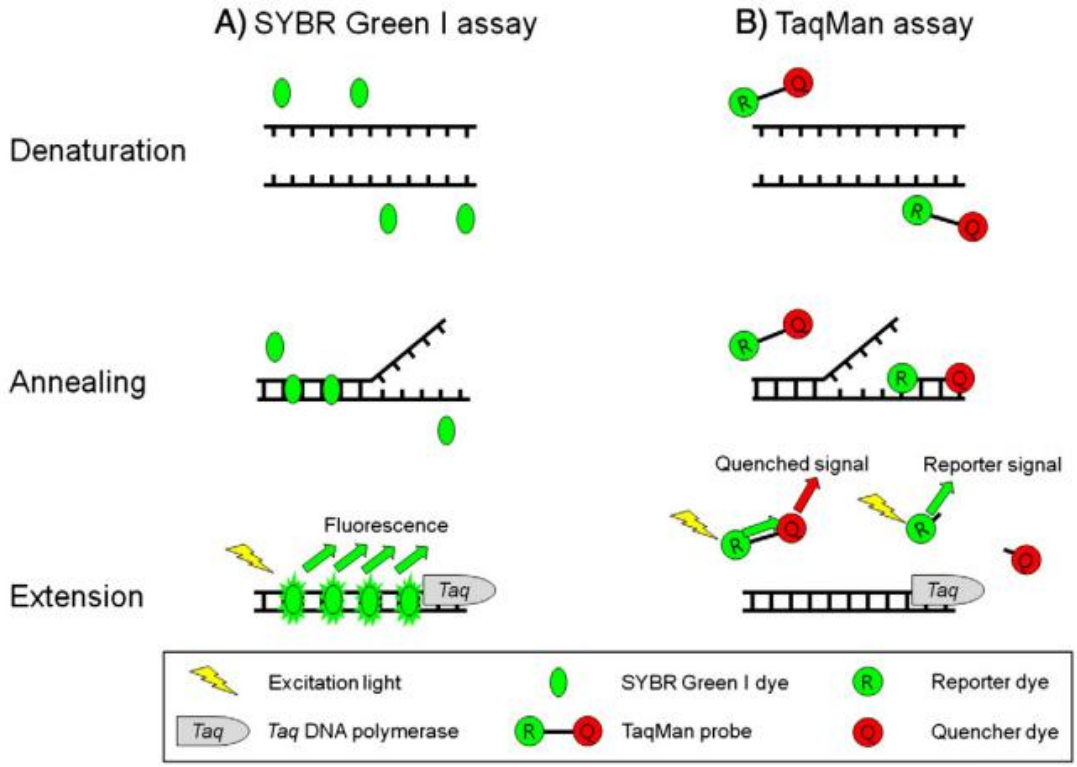

Figure 2.5 : Detection chemistries for qPCR: (A) SYBR Green I assay and (B) TaqMan assay (Kim et al., 2013).

As different DNA fragments have different melting temperatures (Tm) amplification specificity can be controlled and detected unwanted amplicone via melting point analysis (Ririe et al., 1997). However when using a group-specific primer set for targeting a taxonomic functional group where various microbial populations carry different target sequences, melting curve reading is hardly linked to amplification specificity. This limitation again noted the importance of primer specificity. In spite

of such limitations SYBR Green I because of its low cost and flexible applications is still broadly used (Kim et al 2013).

In TaqMan assay in addition to two primers one extra oligonucleotide, named TaqMan probe is used. In TaqMan probe an oligonucleotide is labeled with a reporter dye at the 5′ end and a quencher dye at the 3′ end. In TaqMan probe the reporter fluorescence is absorbed by the quencher end by fluorescence resonance energy transfer (Giulietti et al., 2001). TaqMan probe hybridize to the complementary sequences in template DNA and during PCR amplification when DNA extinction takes place, is degraded by DNA polymerase. One degradation reporter separate from the quencher and fluorescence emission signal can be detected. The fluorescence concentration indicates the amount of amplicons products and gives real-time information. Employing of probe in addition to the primers increase the specificity of TaqMan assay compared to the SYBR green (Yu et al., 2005). However, design of specific primer-probe sets is complicated. In design of primer-probe three specific oligonucleotides to different regions of target DNA with about 500 bases should be design. This process is difficult and may lead to produce wrong primer-probe sets which affect the result (Yu et al., 2005). Despite being difficult process, due to its unique specificity, TaqMan method is even more popular for evaluation of microbial community in mixed-culture environment. Lim et al., 2008; O'Reilly et al., 2009; Lee et al., 2010).

qPCR because of its wide quantification range, high reproducibility, high sensitivity and high specificity is currently an effective method for studying microbial ecology in complex bioremediation systems. Ever increasing publications number about the application of qPCR in the environmental researches proves this fact. However this method possesses some limitations especially when dealing with complex systems such as bioremediation site should be noticed before using this method. A critical factor which affects the qPCR results is nucleic acid extraction efficiency. Any deviations in the amount of extracted nucleic acids will affect the quantity of target sequence. Selected method and the nature of sample both affect the final nucleic acid yield. The purity of the template nucleic acids is another issue that affects the reproductively of qPCR assay. However inhibition caused by impurities can be mitigated using alternative DNA polymerase, chemical washing and so on (kim et

al., 2013). Group-specific primers and probes target different microbial groups which require designing primer-probe sets which are specific to the members target taxonomic or functional group. Design of such primer-probe sets is difficult and time-consuming although thanks to the huge amount of reported oligonucleotide sequences less necessary to design new primer and probes. For evaluation the performance of biological treatment process analysis of alive microbial cells is very important. qPCR like any other PCR based method cannot differentiate between viable and dead microbial cells. Employing RNA-based assay (i.e. targeting RNA instead of DNA), using DNA- intercalating dyes such as ethidium monoazide (EMA) propidium monoazide (PMA) which interact just with nonviable DNA can overcome this problem. Like the other nucleotide sequence based techniques an inherent limitation of PCR is that this technique cannot detect the unknown microbial diversity (Lim et al., 2011). This problem would be more obvious in the group-specific assays at higher taxonomic level. Designing of specific primers and probes is difficult task. However, the specificity of a probe or primer is ever improving with an increasing sequence database.

Precise and timely analysis of biodiversity is a continuing challenge for a successful biomonitoring process (Hajibabaei et al., 2011). Next-generation sequencing (NGS) approaches has recently introduced in biodiversity study. Next-generation sequencing (NGS) technologies have revolutionized molecular biology science 2005 (Shokralla et al., 2012). This technology with high-throughput capacity and low cost has become an important method for analysis of many genomic researches. The first DNA-sequencing approach known as Sanger method was introduced by Sanger et al. (1977) that is capable to give data of 1 kb of sequences from one sample at one time and up to 96 individual samples in most advanced version. Next generation technologies can potentially produce several hundred thousand to tens of millions sequence reads (Shokralla et al., 2012). Today, high-throughput next-generation sequencing enables the simultaneous barcoding of a mix of species so that, for example, all microbial species in a collected sample can be processed at once (Figure 2.6). This sequencing system can generate sequence reads from genomic sequencing, RNAseq or transcriptome sequencing, or from a PCR amplified fragments. In all case, there is no need for vector-based cloning procedure which is used to amplify separate DNA templates. Next generation sequencing technologies have faced some

challenges. Improvement in sequencing output in terms of read length and accuracy, cost and expenses, and challenges related to the amplification step prior to sequencing are important challenges in next generation sequencing methods. Despite these challenges this technology has potential to change the rout of genomic based studies. Application of next generation sequencing techniques in the study of complicated biological interactions in bioremediation studies has important ever increasing concerns. NGS can be divided into two main categories based on their dependence to the PCR amplification. First group which are PCR based technologies include four platform of Roche 454 Genome Sequencer (Roche Diagnostics Corp., Branford, CT, USA), HiSeq 2000 (Illumina Inc., San Diego, CA, USA), AB SOLiD System (Life Technologies Corp., Carlsbad, CA, USA) and Ion Personal Genome Machine (Life Technologies, South San Francisco, CA, USA) (Shokralla et al., 2012).

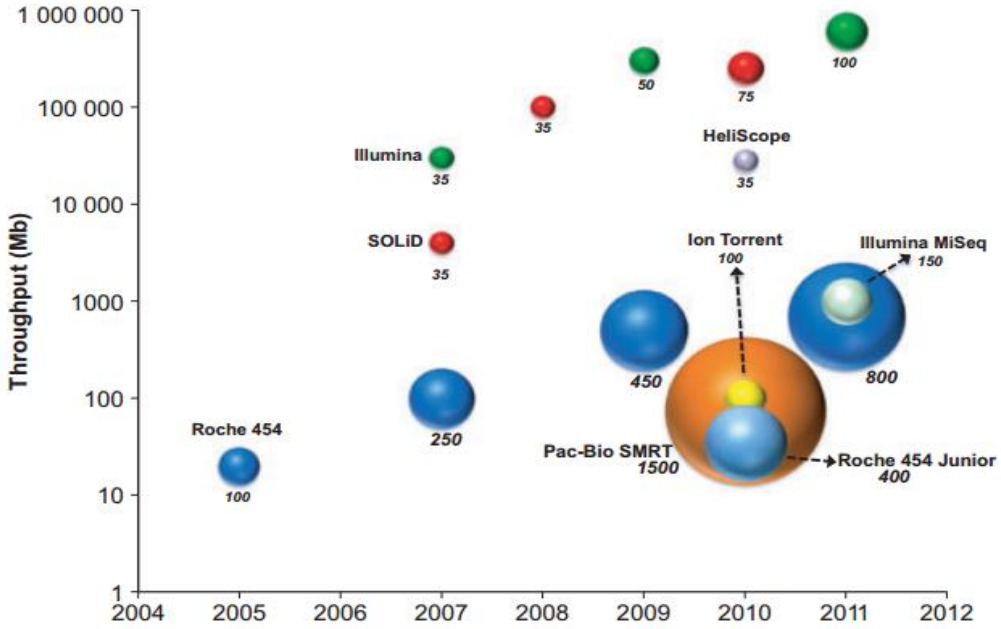

Figure 2.6 : Historical development of nextgeneration sequencing technologies. The diameter of each bubble represents the sequencing read length of the platform. Colours represent individual platforms (Shokralla et al., 2012).

The second group, which is non-PCR, based and no need to amplification step is called single-molecule sequencing (SMS) technologies. These technologies are: HeliScope (Helicos BioSciences Corp. Cambridge, MA, USA) and PacBio RS

SMRT system (Pacific Biosciences, Menlo Park, CA, USA). Table 2.5 comprises the currently available next-generation sequencing technologies.

Table 2.5 : Comparison of currently available next-generation sequencing technologies (Shokralla et al., 2012).

Category	**Platform**	**Read length (bp)**	**Max. number of reads/run**	**Sequencing output/run**	**Run time**
PCR-based NGS technologies	Roche 454 GS	FLX 400–500	1×10^6	≤500 Mb	10 h
	Roche 454 GS	FLX+ 600–800	1×10^6	≤700 Mb	23 h
	Roche 454 GS Junior	400–450	1×10^5	35 Mb	10 h
	Illumina HiSeq 2000	100–200	6×10^9	≤540–600 Gb	11 d
	Illumina HiSeq 1000	100–200	3×10^9	≤270–300 Gb	8.5 d
	Illumina GAIIx	50–75	6.4×10^8	≤95 Gb	7.5–14.5 d
	Illumina MiSeq	100–150	7×10^6	≤1–2 Gb	19–27 h
	AB SOLiD 5500 system	35–75	2.4×10^9	100 Gb	4 d
	AB SOLiD 5500 xl system	35–75	6×10^9	250 Gb	7–8 d
	Ion Torrent - 314 chip	100–200	1×10^6	≥10 Mb	3.5 h
	Ion Torrent - 316 chip	100–200	6×10^6	≥100 Mb	4.7 h
	Ion Torrent - 318 chip	100–200	11×10^6	≥1 Gb	5.5 h
SMS technologies	Helicos HeliScope	30–35	1×10^9	20–28 Gb	≤1 d
	Pacific Biosciences system	≥1500	50×10^3	60–75 Mb	0.5 h

While different approaches are used to for next-generation sequencing, the Illumina recently has become the most effective method of deciphering DNA sequences (Hayes et al., 2013). Illumina firstly introduced as a genome analysis tool in 2007. Because of its high capability, Illumina soon became as the most active approach for whole-genome sequencing. Illumine platform employs a sequencing-by-synthesis approach with bridge amplification on the surface of a flow cell (Bentley et al., 2008). During each respective cycle of the sequencing process each of the four

nucleotides is labeled with an allocated dye and then simultaneously bounds to the flow cell. Each nucleotide incorporates a chemically blocked 3'-OH group, it means that only one nucleotide is incorporated per sequencing cycle; the unbound nucleotides are then washed away. Incorporated nucleotides can then be identified in an imaging step and the next round of sequencing can commence. The hybridization of DNA fragment-to-oligo on flow cell take place by heating and cooling steps followed by incubation with the amplification reactants and isothermal polymerase that generates millions of library fragments clusters. Through the sequencing process, four differentially labelled fluorescent nucleotides which their 3′- OH chemically inactivated are used. Inactivated 3′ end is for adding of only one base per flow cycle. Four commercial kinds of Illumine are currently available: HiSeq 2000, HiSeq 1000, Genome Analyzer IIx, and MiSeq platform which have sequencing outputs of 600 Gb, 300 Gb, 95 Gb, and 150-bp sequencing reads respectively. Illumine HiSeq 2500 which is upgraded from HiSeq 2000 can generate 120 Gb of data in 27 h and enable to prepare a information of a genome in a day. All of these advantages make Illumina sequencing as the better alternative for analysis of biodiversity and metagenomics analysis in the bioremediation site.

3. METHODS AND MATHERIALS

3.1 Petroleum Sludge and Soil Sampling and Characterization

Petroleum contaminated soil samples were collected form petroleum sludge storage pits located in terminal of Baku–Tbilisi–Ceyhan pipeline of Turkey Petroleum Pipeline Corporation (BOTAS). Sludge/soil samples were collected from four different points of storage pit after dredging process. Samples were sealed and transferred to the laboratory using polyethylene packets. Collected samples were homogenized, mixed thoroughly and stored at 4 ºC until further examinations. Clean soil samples were obtained from five different points of the forest near the Istanbul Technical University located at 41º6′9″ N and 29º1′58″ E. Physicochemical characteristics of petroleum polluted soil and forest soil samples were analyzed in the laboratory. Table 3.1 summarizes some of these physicochemical characteristics.

Table 3.1 : Physicochemical characteristics of soil samples.

	pH	**Moisture (%)**	**TOC (%)**	**N (mg/kg)**	**P (mg/kg)**	**C:N:P ratio**
Petroleum contaminated soil	6.45	15.91	41.33	540.60	31	100: 0.13: 0.007
Forest Soil	6.01	21.05	3.92	668.15	16.9	100: 1.7: 0.04

3.2 Microcosms operation and biostimulation experiments

Petroleum contaminated soil and clean forest soil samples were mixed in four different ratios to achieve four experimental groups based on the amounts of total organic carbon (TOC). Consequently, four experimental groups of T_1, T_2, T_3, and T_4 with their respective initial TOC amounts of 5, 10, 15, and 25% have been established. 300 g of final mixtures were weighed into 500 ml bechers. Each experimental group was amended with four different C:N:P ratios of 100:5:1, 100:10:1, 100:15:1 and 100:25:1. Therefore, sixteen microcosms setup were made using four different initial TOCs and amended C:N:P ratios. Table 3.2 summarizes the microcosms groups.

Table 3.2 : Microcosms setups in biostimulation process.

		Experimental groups (based on TOC amounts)			
		T1 (TOC=5%)	T2 (TOC=10%)	T3 (TOC=15%)	T4 (TOC=25%)
Nutrient ratio (C:N:P)	100:5:1	N_1T_1	N_1T_2	N_1T_3	N_1T_4
	100:10:1	N_2T_1	N_2T_2	N_2T_3	N_2T_4
	100:15:1	N_3T_1	N_3T_2	N_3T_3	N_3T_4
	100:25:1	N_4T_1	N_4T_2	N_4T_3	N_4T_4
	Control	C_1	C_2	C_3	C_4

Urea ($(NH_2)_2CO$) and KH_2PO_4 solutions were used as nitrogen and phosphorous sources respectively. Ghaly et al., (2013) and Dave et al., (2011) have previously reported the efficiency of urea as nitrogen source in microbial respiratory activity. On the other hand, Kauppi et al., (2011) have demonstrated the increase in the pH by using the urea. Concerning to the mentioned facts and in light of the fact that nutrient overdose is a potential problem in in-situ bioremediation process over biostimulation, the probable effect of high urea concentration on the microbial community and biodegradation process have also been tested using the C:N:P ratio of 100:25:1. Control microcosms (C sets) were not amended with nutrients .

Microcosms were prepared in triplicates and incubated at 24 ºC in dark condition for 90 days. Reactors were tilled manually for aeration purpose and their moisture were maintained at 70% of water holding capacity by adding distilled water (Gao et al., 2014). Periodic samples were done one in every 20 days.

3.3 Analytical methods

Total organic carbon (TOC) of collected samples was determined using dry combustion technique by TOC-5000A Total Organic Carbon Analyzer – Solid Sample Module (SSM -5000A) according to Turkish Standard Method TS 12089 EN 13137 (2003). Calibration curve were prepared using 20 to 100 mg CaCO3. Collected samples were dried and homogenized. 50 mg samples were weight into porselen boat and using HCl for removal of inorganic carbon sources. Samples were incubated for 1 day and then were combusted at 1300 ºC.

Total petroleum hydrocarbons (TPHs) were measured using Turkish Standard Method TS EN 14039 by GC-FID (2004). 20 g of dried samples were weight into glass flask. 20 ml of hexane and 40 of ml acetone were added. Mixture

was shaken for 2 hours. Any polar materials were removed using sodium sulfate and the mixture was clarified using Florisil column. Extract was then concentrated using evaporator and then injected to the CG device. Sample flow rate was arranged to 48.8 ml.min-1. Device was operated under temperature condition starting from 100 and elevated until 300 ºC. Helium gwas used as carrier gas. The DB-1 column was used for quantitation.

Phosphorous content of the samples was determined using Turkish Standard Method TS 8338 modified Bray and Kurt. No.1 (1990). Total nitrogen was analyzed using Turkish Standard Method TS 8337 ISO 111261 (1996). Moisture content was measured by the oven drying method described by Agarry et al., (2012). pH of collected samples were determined by ASTM D4972 method described by ASTM (1995).

3.4 Genomic DNA and total RNA extraction

Genomic DNA (gDNA) and total RNA were isolated from 500 g of collected samples using PowerSoil DNA and RNA isolation kit (Mo Bio Laboratories, USA) respectively, in accordance with recommended protocols. Concentration of isolated RNAs and DNAs were measured using NanoDrop UV-Vis Spectrophotometers (Thermo Scientific, USA). cDNA were synthesized from isolated RNAs immediately after sampling using Superscript Viloc DNA synthesis kit (Invitrogen, UK). Remained isolated RNAs were stored at -80 ºC. Isolated DNA and synthesized cDNA samples were stored at -20 ºC until further analysis. All gDNAs and cDNAs were further purified with Wizard DNA Clean-Up System (Promega).

3.5 Plasmid isolation for evaluation of horizontal gene transfer

Plasmids were isolated from 500 mg of soil samples using Plasmid isolation kits (Invitrogen, UK) in accordance with recommended procedures. Selected isolation kit allows to efficiently isolate the plasmid DNA from samples using anion-exchange columns, without the use of any organic solvents or cesium chloride (CsCl) and the isolated plasmid DNA is of high purity. In anion-exchange technology using a patented resin composed of small particles with a uniform pore size high yield and reproducible performance are provided. The unique patented ionexchange moiety

provides high effectiveness for separation of DNA from contaminants including RNA. With adding resuspension buffer using RNase A and lysing of cells with lysis buffer isolation protocol starts. Adding of precipitation buffer to the lysate clarifies the lysate. Clear lysate then is passed through the pre-packed anion exchange column. The phosphate groups of the backbone DNA interacts with the positive charges of the surface of the resin. Under moderate salt conditions, plasmid DNA bounds to the resin while using the wash buffer, RNA, proteins, carbohydrates and other impurities are washed away. Plasmid DNA then is eluted using elution buffer under high salt condition. Alcohol precipitation at the final step desalts and concentrates the plasmid DNA.

Concentration of isolated plasmid DNAs were measured using NanoDrop UV-Vis Spectrophotometers (Thermo scientific, USA). Isolated plasmid DNAs were stored at -20 ºC.

3.6 Real time quantitative PCR (qPCR)

In order to estimated the microbial community changes through the biostimulation process, the quantities of alkane and PAH-degrading bacterial communities and *alkB*, *nah* and *phnAc* functional genes were analyzed using quantitative real time PCR (qPCR). *alkB* gene encodes alkane monooxygenase which catalyzes the hydroxylation of n-alkanes in the presence of a NADH-rubredoxin reductase and rubredoxin. *nah* gene encodes naphthalene dioxygenase which catalyzes the oxidation of naphthalene to cis-1, 2-dihydroxy-1,2-dihydronaphthalene. *phnAc* gene encodes large subunit of phenanthrene dioxygenase which takes part in the degradation of phenanthrene via meta-cleavage by converting of phenanthrene to phenanthrene-cis-3,4-dihydrodiol. These genes have key roles in alkanes and polycyclic aromatic hydrocarbons and are commonly studied hydrocarbon-degrading genes. Their abundance and diversity have been investigated in a variety of hydrocarbon-contaminated aquatic and terrestrial ecosystems. However, the links between environmental parameters and the distribution of these genes are still remained unclear and the coexistence of these metabolic genes in the environment has been few studied this is why we have chosen these functional genes as indicator in this study. qPCR assay of samples obtained from all microcosms were done in triplicate using ABI 7500 SDS system (Applied Biosystems, Foster City, California)

by TaqMan probe and following related recommended procedure. Specific primer sets which were used in qPCR assay have been summarized in Table 3.3. Gradient PCR in the range of eight different temperatures, which was usually about 5 °C below the lowest Tm of the primer pairs, was used to optimize the annealing temperature.

Table 3.3 : Primers used for PCR amplification in this research.

Primer	**Sequence (5'→3')**	**Target gene**	**Ann-ealing (°C)**	**Reference**
alkB-F alkB-R	AAYACIGCICAYGARCTIGGICAYAA GCRTGRTGRTCIGARTGICGYTG	*alkB*	60	Yang et al., 2014
NAH-F NAH-R	CAAAA(A/G)CACCTGATT(C/T)ATGG A(C/T)(A/G)CG(A/G)G(C/G)GACTTCTT TCAA	*nah*	48	Yang et al., 2014
Primer P8073 Primer P9047	TTCGAGCTGGAATGTGAGC AATAACCGGCGATTCCAAAC	*phnAc*	55	Laurie and Lloyd-Jones, 2000
Gram-negative-F Gram-negative-R	GAGATGCATACCACGTKGGTTGGA AGCTGTTGTTCGGGAAGAYWGTGCM GTT	Gram negative PAH-RHD**α**	59	Cébron et al., 2008
Gram-positive-F Gram-positive-R	CGGCGCCGACAAYTTYGTNGG GGGGAACACGGTGCCRTGDATRAA	Gram positive PAH-RHD**α**	55	Cébron et al., 2008
Pse-F2 Pse-R	GGTCTTCGGATTGTAAAGCAC CCGGGGMTTTCACATCCAAC	16S rRNA	60	Juhanson et al., 2009
Bac519f Bac907r	CAGCMGCCGCGGTAANWC CCGTCAATTCMTTTRAGTT	16S rRNA	53	Aydin et al., 2015a

PCR products were analyzed using electrophoresis on a 3% agarose gel. The optimum annealing temperature was determined and then qPCR analyses were done. Standard curve was created based ob ten-fold dilution and calibration of standard curves for positive controls was done as described by Yang et al., (2014). Procedure recommended by Applied Biosystems was followed and TaqMan universal Master Mix (AppliedBiosystems, Foster City, California) was used in 25 µl reaction mixture. Applied Biosystems TaqMan Universal PCR Master Mix is optimized for TaqMan reactions contains AmpliTaq Gold DNA polymerase to provide a better yield and a more robust 5' nuclease assay than AmpliTaq DNA polymerase. The AmpliTaq Gold enzyme is a thermal stable DNA polymerase and has a 5´ to 3´ nuclease activity but lacks a 3´ to 5´ exonuclease activity. The mix contains also AmpErase UNG, dNTPs with dUTP, Passive Reference, and optimized buffer components. For amplification process, initial denaturation for 10 min at 95 °C followed by 30 cycles of 1 min at 95 °C, 1 min at specific annealing temperature as

given in Table 2, 2 min at 72 °C, and a final extension step of 10 min at 72 °C were applied. qPCR reactions were run in triplicates. qPCR efficiency ranges were 97.2 to 110% with R^2 values more than 0.99 for all calibration curves. Limit of detection (LOD) was 10 copies per 25 µl reaction for qPCR assays. To ensure the absence of primer dimers and any probable nonspecific products, melting curve analysis was done directly following each qPCR amplification assay for all reaction tubes.

3.7 Illumina sequencing and 16S rRNA data processing

Illumine MiSeq analysis was done for the sample obtained from petroleum contaminated soil, DNA of selected sample was extracted using PowerSoil DNA extraction kit (Mo Bio Laboratories, USA). Extracted DNA sample was quantified using Implen NanoPhotometer® P360. The quality of isolated DNA was verified on a 0.8 % agarose gel. 16S rRNA region of the isolated bacterial DNA was prepared for sequencing by IlluminaTruseq kit according to the manufacturer's instructions. Necessary adapter and index sequences have been integrated to the Bact339-F (5'-CTCCTACGGGAGGCAGCAG-3') and Bact815-R (5'-CTCCTACGGGAGGCAGCAG-3') primers based on the Illumina-Miseq platform and the necessary DNA library has been prepared. V3-V5 region was amplified. The validity of DNA library was verified using using Implen NanoPhotometer® P360 and real time PCR (ABI 7500 SDS system, Applied Biosystems, Foster City, California). DNA libraries then loaded on Illumina-Miseq device in accordance with manufacture's protocols. After completed of the sequencing process, information of 10,000 fragments of provided library was obtained. After sequencing process, adapter sequence which was previously added, was removed and sample was verified using index sequence. For elimination of the chimeras effects through the sequencing reading process, denoising was done (Edgar et al., 2011, Haas et al., 2011). Analyzing of the sequence data was done using Qiime platform. Reads containing ambiguous character (N) and average quality score <25, and reads which were not match to the used primers were filtered. Sequence reads with longer than 20 dp overlap and between 400 and 480 bp were assembled (You et al., 2015). Taxonomical analysis was done using Greengenes with PyNAST at 80% confidence threshold (You et al 2015) and operational taxonomic units (OTUs) were clustered

using BLASTN with 97% sequence identity threshold (DeSantis et al., 2006; Sun et al., 2011).

Illumine HiSeq analysis was done for samples optained from the operated microcosms. 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACVSGGGTATCTAAT-3′) primers specific for V4 region (length, ca. 250 bp) of the 16S rRNA gene were selected (Caporaso et al., 2010), required Illumina adapters and barcode sequence were added to the primers. Extracted DNAs were amplified using PCR with the amplification protocol which was as follow: initial denaturation for 3 minutes at 94 °C followed by 20 cycles of 45 seconds at 94 °C, 30 seconds at 53 °C, 90 seconds at 65 °C, and a final elongation step of 10 minutes at 65 °C (de Quadros et al., 2016). All DNAs were further purified using Wizard DNA Clean-Up System (Promega) in accordance with manufacture's protocol. The samples then quantified using Qubit 2.0 Fluorometer (Invitrogen, NY, USA). 16S rRNA genes were sequenced by the Illlumina GAIIx method (Illumina, Inc., CA, USA) with paired-end read cycles. Sequence analysis and the identification of operational taxonomic units (OTUs) were done using the methods of Giongo et al. (2010a, 2010b) and Fagen et al. (2012). After sequencing and for reading of the results, low quality bases and first 11 bases of the primer region were removed via a script based on Trim2 (Huang et al. 2003); Trim2_Illumina.pl. available online: https://gist.github.com/1006830). The reads were filtered with taking into account of 70% as the minimum percentage of bases using a minimum quality score of 20 in Phred + 33 encoding (de Quadros et al., 2016). Paired reads were mapped to the reference Ribosomal Database Project (RDP) using CLC Assembly Cell v3.0.2b (CLC Bio, QIAGEN). Taxonmic description was done based on the NCBI taxonomy database and entered in the RDP database using TaxCollector (Giongo et al., 2010a; Giongo et al., 2010b). 80% of fraction length matches were used for classification. At least 80% and 97% of sequence similarity was considered as phylum and species respectively. OUT abundance matrix for each taxonomic rank were created using total number of reads which showed 16S rRNA sequences matching with the database and matrices of each sample were divided by the total number of pairs for normalizing of varying sequencing depths (de Quadros et al., 2016).

3.8 Biaugmentation practice

3.8.1 Petroleum contaminated soil sample preparation

Soil samples included petroleum residuals were collected form previously operated microcosms of biostimulation practice phase. Microcosms had been operated for 90 days during biostimualtion process and soil samples for bioaugmentation practice collected from the end-point of biostimulation process.

3.8.2 Preparing fungi strains and sub culturing process

The biodegradation of petroleum residuals following biostimulation process was examined using white-rot fungi, *Trametes versicolor* ATCC 42530 and *Bjerkandera adusta* ATCC strains. *T. versicolor* ATCC 42530 and *B. adusta* ATCC 28314 fungus strains were obtained from American Type Culture Collection and maintained by sub culturing every 30 days on 2% malt agar on Petri dishes (pH 4.5) at 25 ^{o}C.

3.8.3 Mycelia suspension preparation

Agar plugs were cut from the fungi growing Petri dishes media and added to the flask containing 150 ml of sterilized malt extract medium. Flasks were incubated at 25 ^{o}C in a laboratory shaker (135 rmp) for 5 days. During this process mycelia was appeared in the flask. Produced mycelia were collected and suspended solution were prepared into a 8% NaCl and homogenized.

3.8.4 Fungi inocula preparation

Inocula were prepared by adding blended mycelium suspension to sterile WSP, 0.65 mL per gram of dry WSP and pre-growing for 7 d at 25 ºC. The Wheat straw pellets (WSP) were hydrated in a 1:2 ratio (w/v) prior mycelium inoculation.

3.8.5 Soil-phase treatment

Solid-phase treatment process was done in 24 x 150 mm tubes (Barloworld Scientific Ltd., Staffordshire, UK), containing of 7 g collected soil samples and 42% (w/w, dry basis) *T. versicolor* and *B. adusta* inoculum, separately. Wheat straw pellets (WSP)

were employed for two main purposes. First, they were used as a bulking material during the solid-treatment phase. Second, they were employed as a lignocellulosic substrate to increase the prevalence of *T. versicolor* and *B. adusta* in the sludge. Experimental setups were incubated at 30 ºC for 60 days. Setups were homogenized manually and the necessary moisture was provided by spiking of distilled water. Samples for DNA/ RNA isolation and functional gene expression quantification were collected from 60^{th} day of examination.

3.9 Statistical analysis

The normality of obtained data was assessed by examining histogram and q-p plots through the using of Shapiro-Wilk's test. Variance homogeneity was done by Leven's test. Differences in gene expression between control and experimental microcosms were compared using one-way analysis of variance (ANOVA) or in depended samples t test. Tukey's test was applied for multiple comparisons. Values presented as mean and standard deviation. Correlation tests were also assessed to reveal the HGT and functional genes (*alkB*, *phnAc* and *nah*) using Pearson's test. Analysis was contacted using R 3.1.1(www.r-project.org). P value less than 5% was considered to be statistically significant.

R program is a system for statistical analysis and graphs have been developed by R Development Core Team (2008). This extremely powerful system is a language and run-time environment for statistical computing. This program is a set of open source effective data handling and storage packages. When compared to most other stats packages used for statistical purpose, this program has at least three advantages: this program is free, open source and available through (Comprehensive R Archive Network). Users are free to modify the program. It runs on multiple platforms (Linux, Windows, and Macs). Furthermore it has combined many of the most useful statistical programs into one integrated environment. Statisticians and researchers which use statistic analysis around the world contribute packages to the R Group and have created a very active group to offer suggestions and help. The growing packages collection is maybe the greatest advantage of R.

4. RESULTS AND DISCOSSION

4.1 Biodegradation of petroleum contaminated soil

Maximum removal of petroleum hydrocarbons have been demonstrated in the T_3 microcosms (i.e. microcosms with initial TOC amount of 15%). Among all microcosms, maximum removal of 18% have been seen in the T_3N_3 microcosm which its C:N:P ration have been arranged to 100:15:1. Figures 4.1 and 4.2 show the biodegradation manner of petroleum-contaminated soil in all studied microcosms based of TOC and TPH removal, respectivally. When comparison the obtained results with other works, regarding to the amended nutrient ratios, obtained results were more or less the same with results reported by some other researchers such as Turgay et al., (2010), Qin et al., (2013) and Yerushalmi et al., (2003). The other microcosms demonstrated less TOC removal but significantly more than control microcosm ($p<0.05$). T_4N_4 microcosm (i.e. microcosm with 25% initial TOC when its C:N:P ratio has been arranged to 100:25:1). The amount of this parameter for N_4T_4 microcosm was non-significantly less than N_3T_3 but significantly higher than the others.

Figures 4.3 and 4.4 show the reducing manner of phosphorus and nitrogen amounts during the biostimulation practice. Phosphorus and nitrogen reducing manner in all microcosms showed positive correlation with the removal amounts of total organic carbon and total petroleum hydrocarbons ($p<0.05$).

Figure 4.5 shows the fluctuation of pH during the biostimulation practice. As it is clear form the graphs and as it is mentioned in the literature (Kauppi et al., 2011) at the beginning when adding of the nutrients because of the high amount of urea in the media pH is basic and more than 8. However, pH started to decrees because of consumption by bacteria and physicochemical disposal and the diminishing of its concentration in the microcosms.

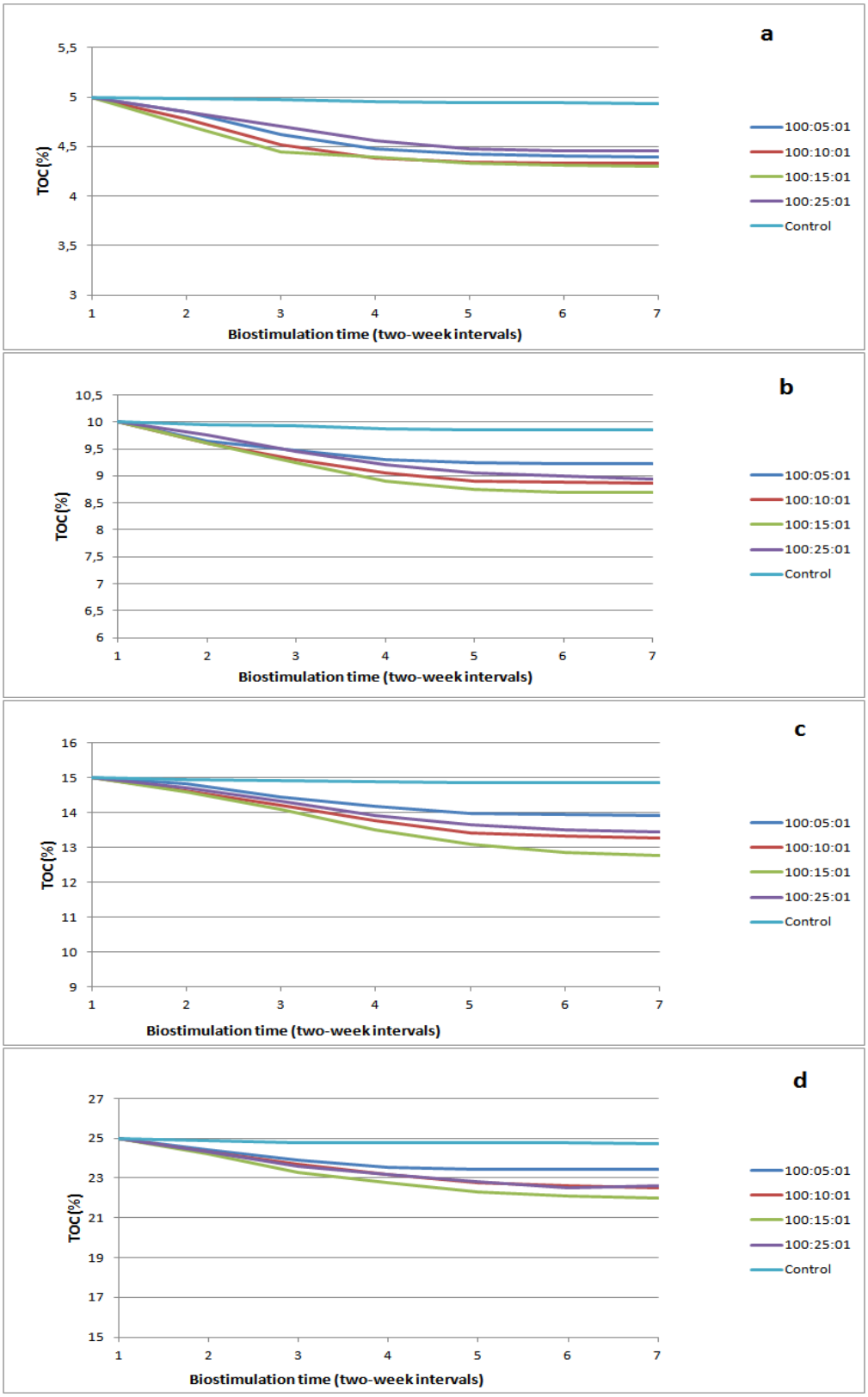

Figure 4.1 : Changes in Total Organic Carbon (TOC) in the microcosms with initial TOC amount of: (a) 5%, (b) 10%, (c) 15%, and (d) 25% through the ex-situ biostimulation of petroleum-contaminated soil.

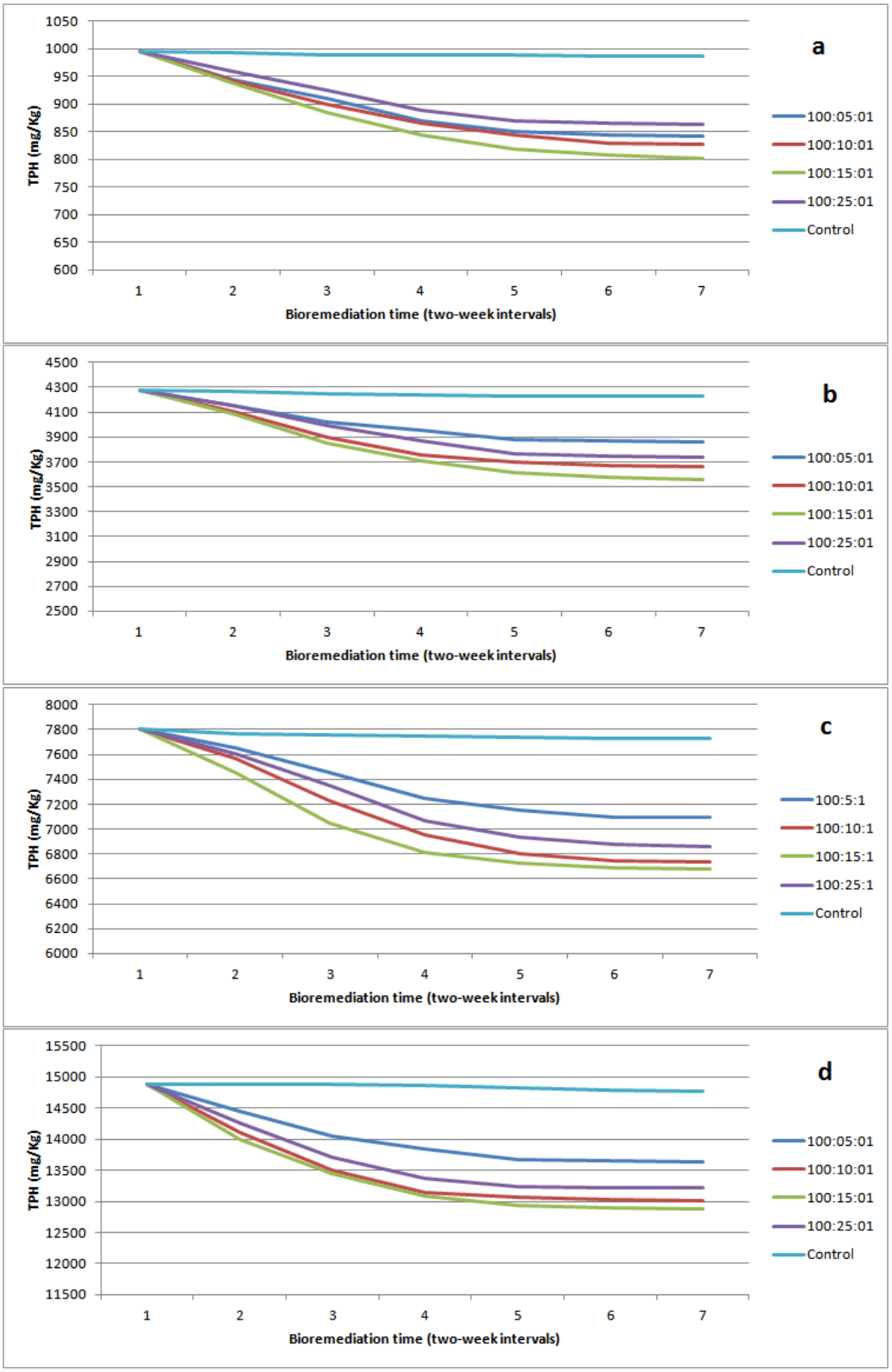

Figure 4.2 : Changes in Total Petroleum Hydrocarbons (TPHs) in the microcosms with initial TOC amount of: (a) 5%, (b) 10%, (c) 15%, and (d) 25% through the ex-situ biostimulation of petroleum-contaminated soil.

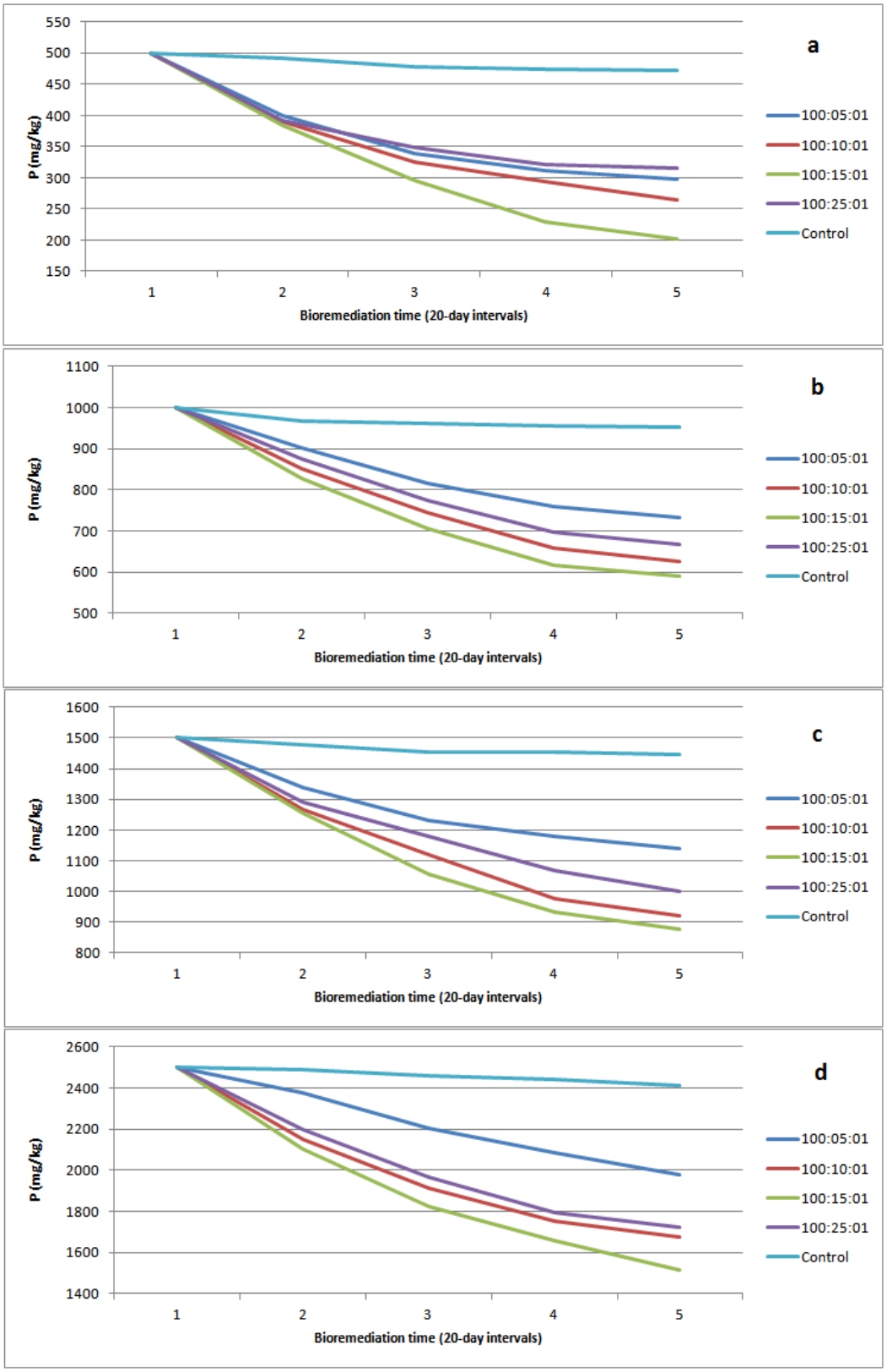

Figure 4.3 : Phosphorous removal in microcosms with initial TOC amount of: (a) 5%, (b) 10%, (c) 15%, and (d) 25% through the ex-situ biostimulation of petroleum-contaminated soil.

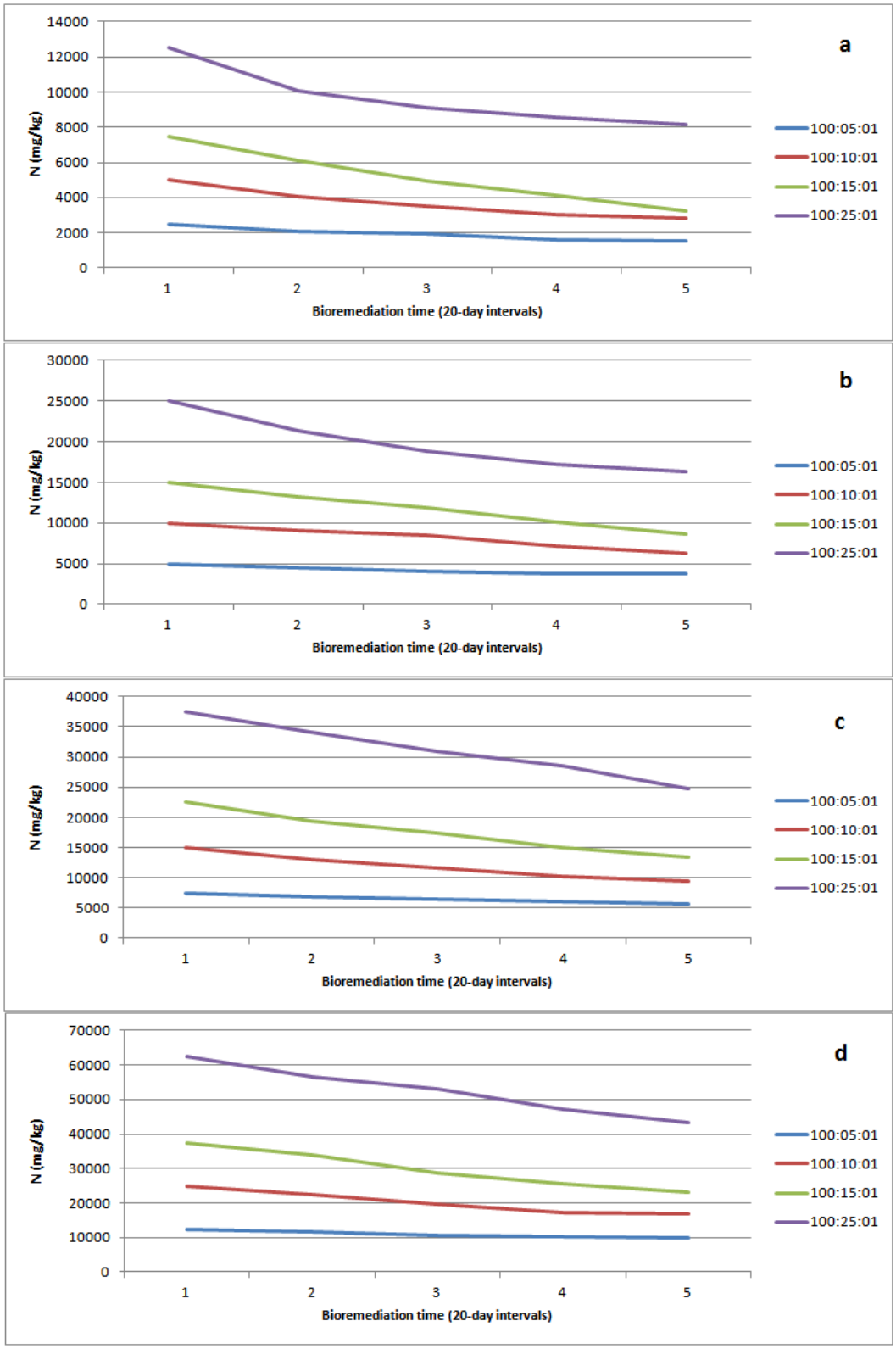

Figure 4.4 : Nitrogen removal in microcosms with initial TOC amount of: (a) 5%, (b) 10%, (c) 15%, and (d) 25% through the ex-situ biostimulation of petroleum-contaminated soil.

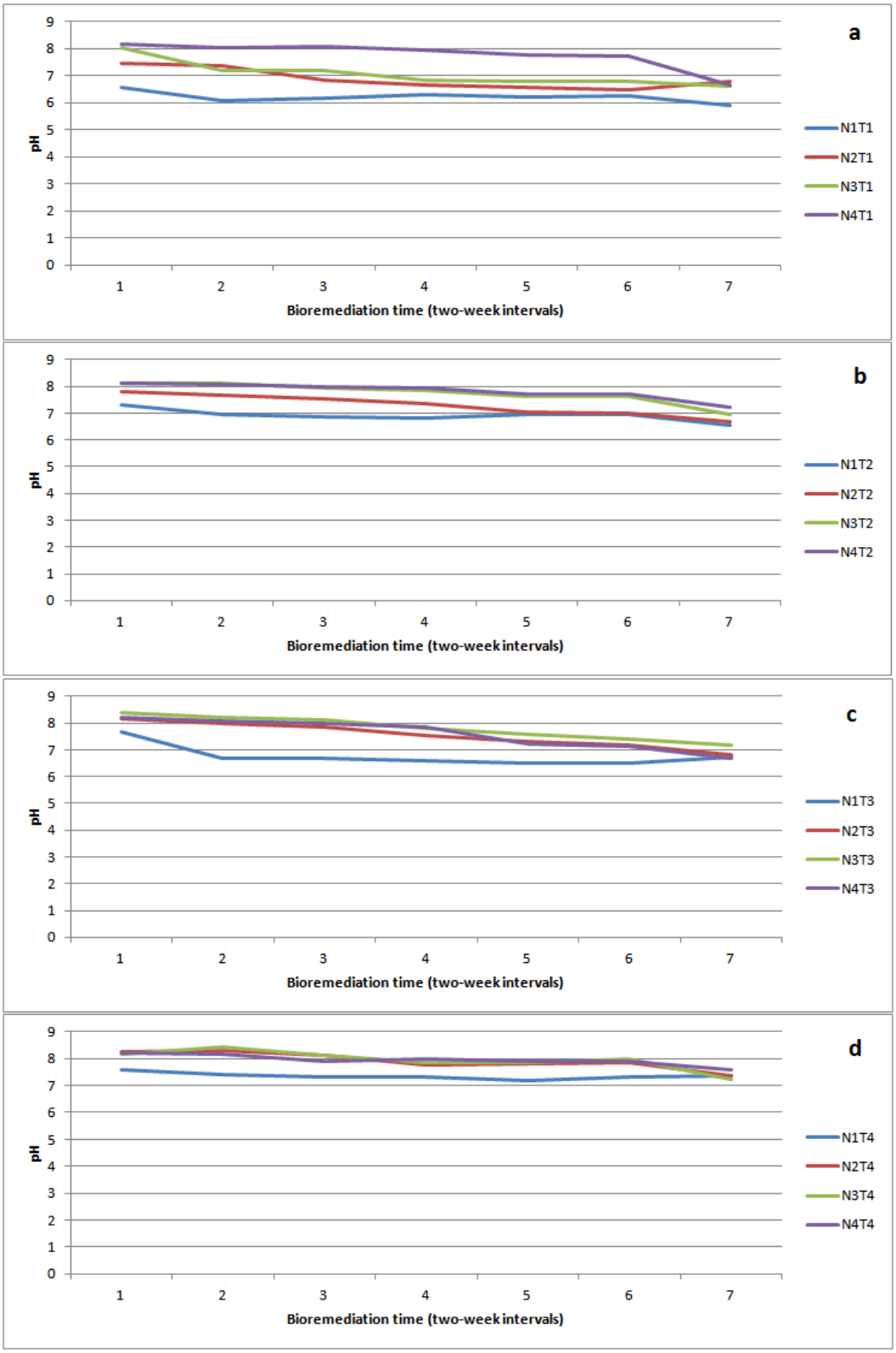

Figure 4.5 : pH flucuation in microcosms with initial TOC amount of: (a) 5%, (b) 10%, (c) 15%, and (d) 25% through the ex-situ biostimulation of petroleum-contaminated soil.

4.2 Evaluation of the diversity of microbial community and dominat bacterial species in the petroleum contamined soil using Illumia sequencing

Despite the fact that some bacteria can adapt to the high concentrations of nutrient and carbon sources, adding of nutrient to the oil contaminated make disorders in the bacterial communities (Adams et al., 2015). Consequently, assessment of indigenous bacteria in terms of their ability in degrading of petroleum hydrocarbons and the probable effects of petroleum hydrocarbons on the population dynamics of contaminated sites are necessary for a successful biostimulation practice. *Pseudomonas* spp consists more than 23% of total bacterial strains in the studies oil contaminated soil. The dominant bacterial phyla in the contaminated site were γ-proteobacteria, Chloroflexi, Firmicutes and δ-proteobacteria. These bacterial groups covered more than 65% of the determined bacterial strains. More than 85% of the determined bacterial strains belonged to the gram negative bacteria. Regarding to the dominant bacterial strains in the contaminated soil, *Anaerolinea* spp., *Longilinea* spp., *Desulfovibrio* spp. *Syntrophomonas palmitatica*, *Cupriavidus metallidurans*, *Syntrophobacter* spp., *Kosmotoga* spp., *Dermatophilus* spp., *Parabacteroides* spp., *Leptolinea* spp. and *Rhodopirellula* spp. were among important species. These species covered more than 40% of the dominant bacterial strains. Dominant phyla and bacterial strains in the studied petroleum contaminated soil have been shown in the Figures 4.6 and 4.7, respectively.

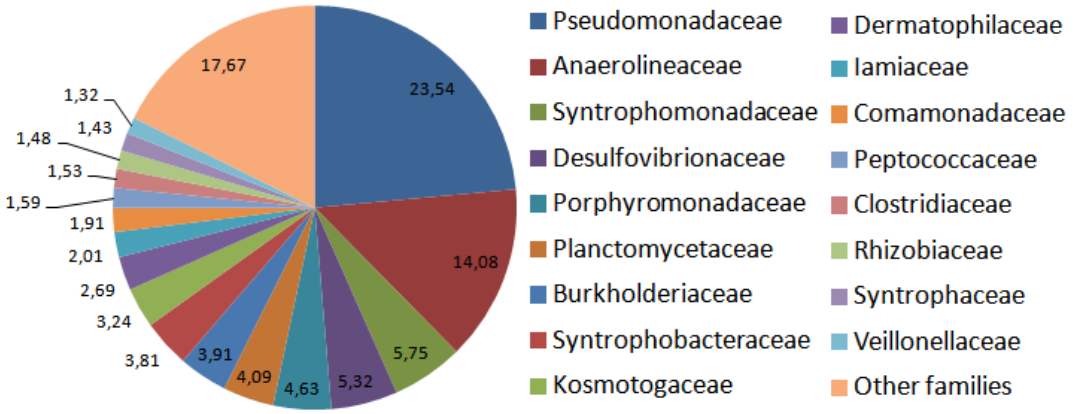

Figure 4.6 : Determined bacterial species in the studied petroleum contaminated soil.

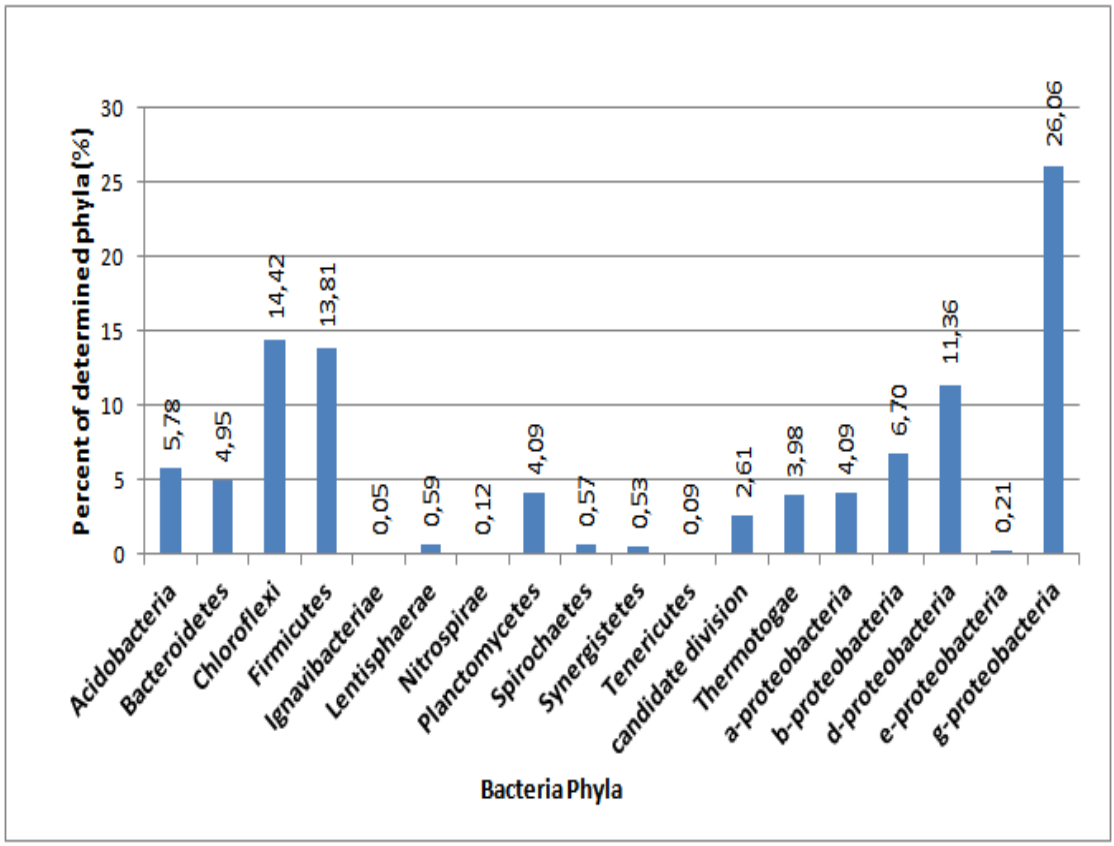

Figure 4.7 : The most abundant bacterial phyla in petroleum contaminated soil resulted from illumina sequencing.

4.3 Quantification of active and total Bacteria

Alkanes and polycyclic aromatic hydrocarbons (PAHs) are major hydrophobic organic pollutants and highly persistent pollutants in soil. These hydrocarbons constitute about 40-70 percent of crude oil (Jauhari et al., 2014). Biostimulation practice supports the suitable condition for growth of main hydrocarbon degrading bacteria (Chang et al., 2010). qPCR approach was used to estimate the abundance of total and active bacteria, *Pseudomonas* which is functionally important phylogenetic group, gram-positive and gram-negative bacteria to evaluate alkane and PAH degradation capacity during biostimulation process. Mentioned bacteria and functional gene quantitative analyses were done using samples collected from all microcosms. Figures 4.8 (a) and (b) Show the quanties of total and active bacteria in the studied microcosms.

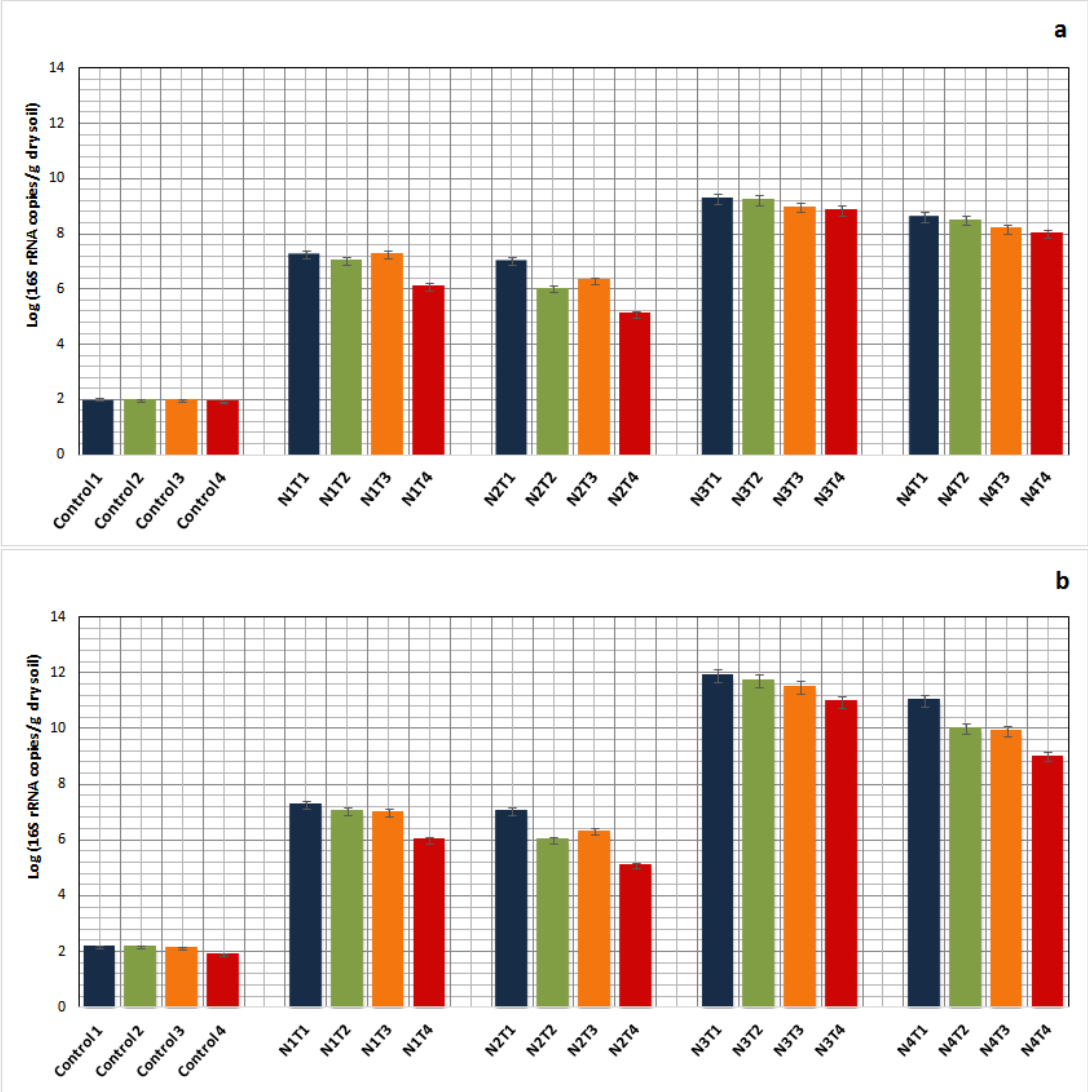

Figure 4.8 : Quantities of: (a) total and (b) active bacteria through the biostimulation of petroleum-contaminated soil.

Figure 4.9 (a), (b) and Figure 4.10 (a) and (b) show quantities of total and active gram-positive and total and active gram-negative bacteria in the studied microcosms.

Based on the results the quantities of total bacteria, gram-negative bacteria and *Pseudomonas* species showed positive correlation with the number of active bacteria. The obtained result because of introducing of sufficient nutrient per TOC amounts in studied microcosms was not surprising. The amount of active bacteria, gram-negative bacteria and *Pseudomonas* species showed significant positive correlations ($p<0.05$). Consequently, main section of the active bacterial community was belonged to the gram-negative groups.

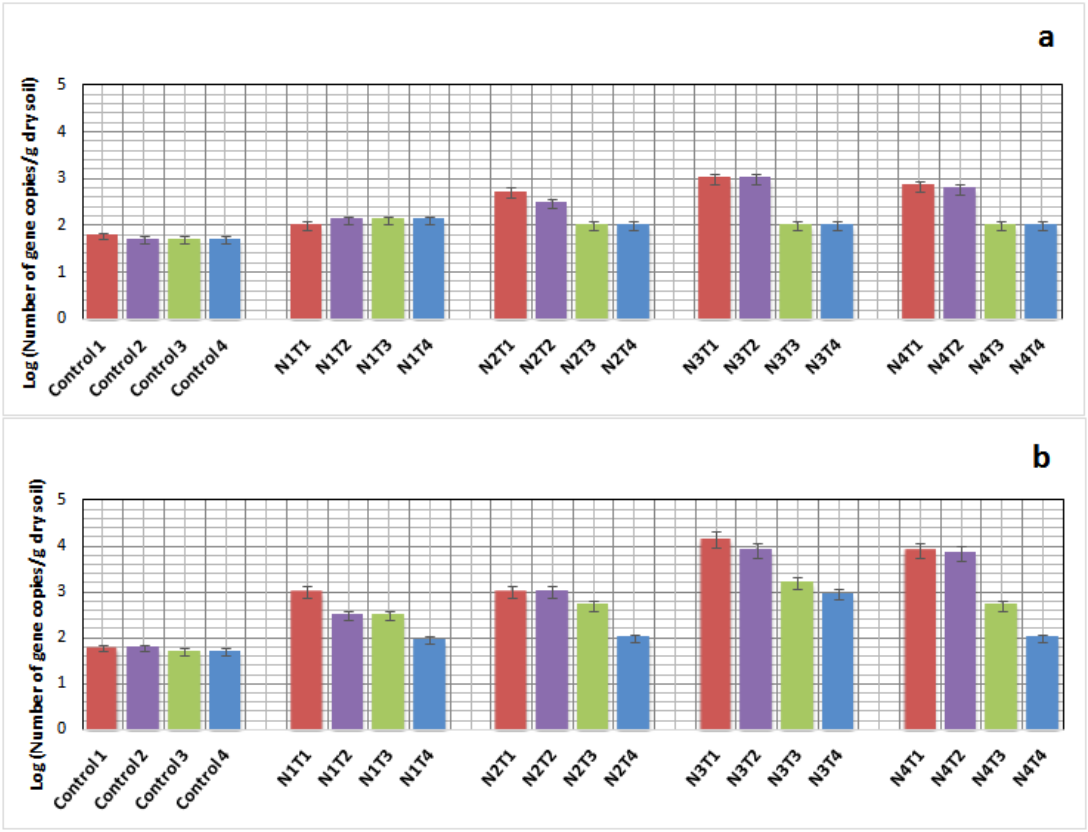

Figure 4.9 : Quantities of: (a) total geram-positive and (b) active gram-positive in the biostimulation of petroleum-contaminated soil.

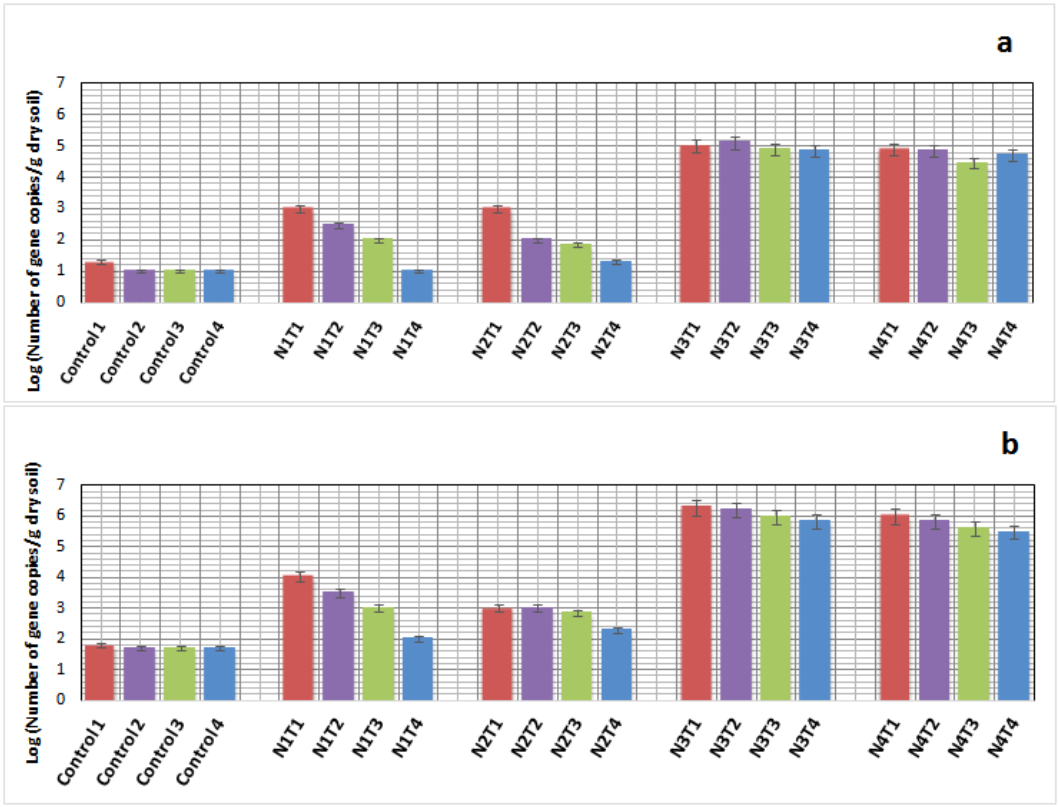

Figure 4.10 : Quantities of: (a) total gram-negative and (b) active gram-negative bacteria in the biostimulation of petroleum-contaminated soil.

Pseudomonas as a member of gram-negative bacteria had a major role in the biodegradation process. The quantity of active gram-positive bacteria was less than gram-negative bacteria. Regarding to the quantity of functional genes, qPCR assay declared a significant increase ($p<0.05$) in the active genes quantities in all microcosms with C:N:P ratio of 100:15:1. Figures 4.11 (a) and (b) show total and active *Pseudomonas* species in the studied microcosms.

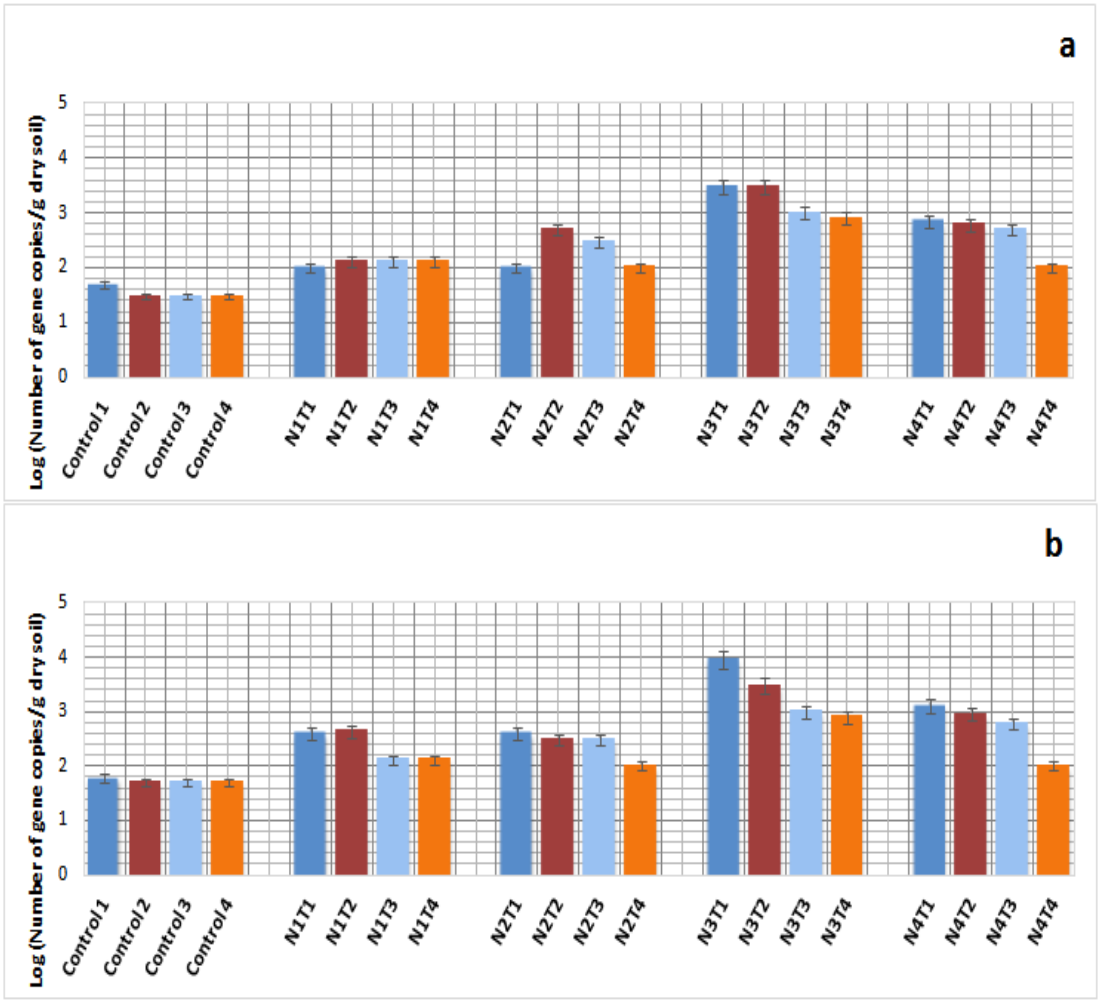

Figure 4.11 : Quantities of: (a) total and (b) active *Pseudomonas* sp. species in the biostimulation of petroleum-contaminated soil.

Results obtained from qPCR assay revealed that the quantitative study of active bacteria could be more reflective than quantitative analysis of total gene abundance through the bioremediation process. Therefore, analysis of 16S rRNA gene could be a good choice for assessment of petroleum hydrocarbon degrading bacteria in contaminated soil. Results of present research also highlighted the importance of gram-negative bacteria specially *Pseudomonas* species. This bacterial species had important role in the biostimulation of petroleum contaminated soil. Therefore the

effective control of these bacteria is necessary for a successful bioremediation process. The importance of *Pseudomonas* genus in biodegradation of petroleum contaminated soil has also been mentioned by some other works. Different strains of *Pseudomonas* have been isolated from oil-contaminated soil (Reddy et al., 2011; Masakorala et al., 2013). The petroleum hydrocarbon biodegradation ability of biosurfactant-producing *Pseudomonas* strains has been presented by Yan et al., (2012). The dominancy of gram-negative bacteria in the petroleum contaminated sites has been previously mentioned through some work. Kaplan and Kitts (2004) have reported a high amount of gram-negative bacteria in the petroleum contaminated soil. Fuentes et al., (2014) have found that gram-negative bacteria are likely more resistant to hydrocarbons in the petroleum contaminated soil.

4.4 Microbial composition and relative abundances in the biostimulation microcosms

Microbial composition of the studies setups were analyzed in the phylum level. Figure 4.12 shows the relative abundances in phyla in studied microcosmos. Proteobacteria (16.5%±2.9%), Firmicutes (9.9%±4.4%), Chloroflexi (5.2%±1.2%), Acidobacteria (4.6%±0.5%), Lentisphaerae (6.8%±3.1%), Planctomycetes (6.6%±2.6%), Thermotogae (6.1%±1.9%), Bacteroidetes (8.4%±5.3%), Synergistetes (5.2%±1.4%), and Verrucomicrobia (5.2%±1.5%) were dominant phyla in the studied microcosm setups. Importance of these phyla in the petroleum contaminated soil has been reported through various works (da Silva et al., 2012; Das and Kazy, 2014; Shahi et al., 2016). Most of the dominant phyla belonged to gram-negative bacteria which conform once again the importance of these bacterial groups in the biodegradation of petroleum contaminated soil (Shahi et al., 2016). As expected, the relative abundances of these phyla were different in studied microcosms regarding to the total petroleum hydrocarbon amounts and amended nutrient ratios. Relative abundances of Thermotogae, Synergistetes and Verrucomicrobia were affected significantly with the changes of TPHs concentrations whereas the other phyla were not sensitive significantly to TPHs concentrations between the studied ranges ($p<0.05$). On the other hand, the changes of C:N:P ratios affected significantly the relative abundances of Proteobacteria, Firmicutes, Chloroflexi, Lentisphaerae, and

Planctomycetes phyla. However, the relative abundances of other phyla were not affected by nutrient fluctuations in the studied concentration ranges.

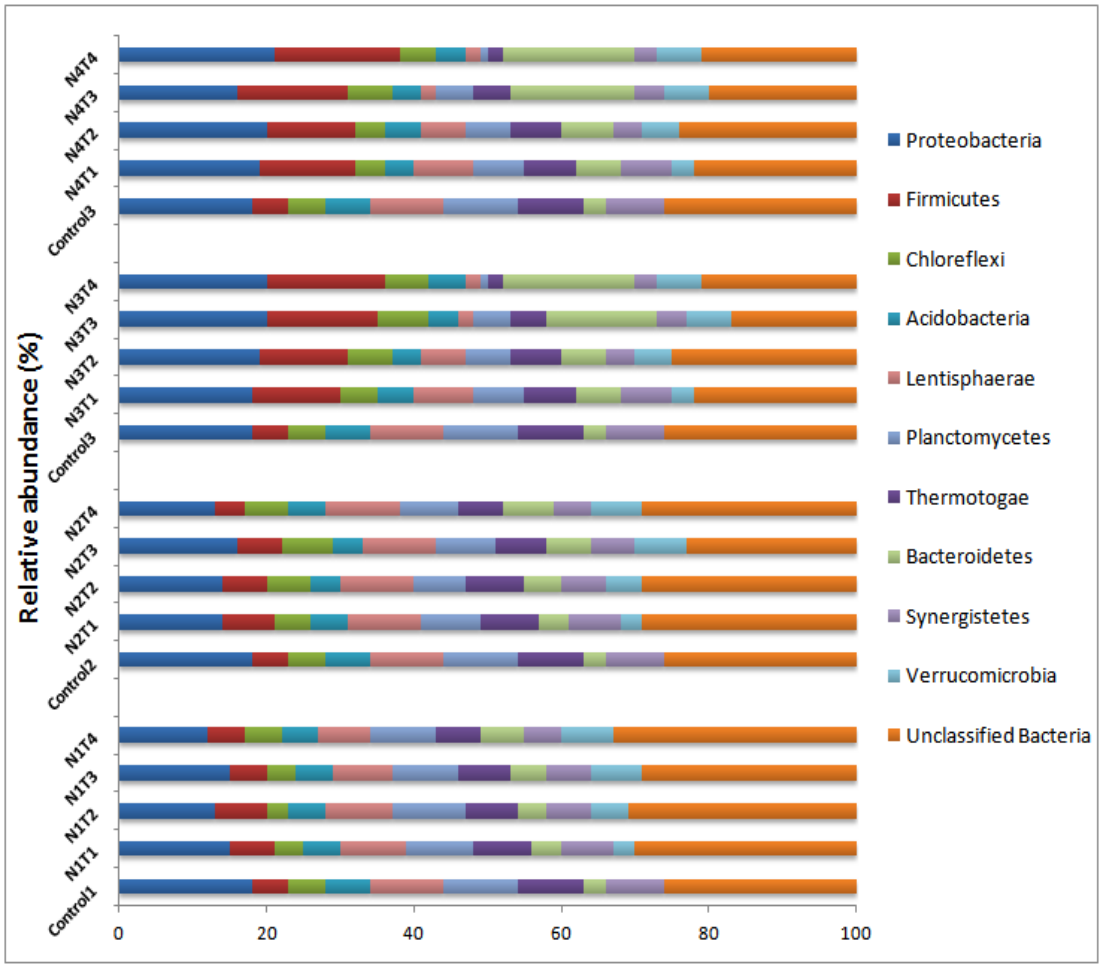

Figure 4.12 : Dominant bacterial phyla in the studied microcosms.

Proteobacteria was the most dominant phylum in all microcosms. This phylum have been reported as the most dominant phylum in the petroleum contaminated sites through other works (Viñas et al., 2005; Reddy et al., 2011; Shahi et al., 2016). Its abundance increased with increasing of the petroleum hydrocarbons concentrations. It had the highest relative abundance in N_4T_4 microcosm. Bacteroidetes and Verrucomicrobia were increased with the increasing of the TPHs concentrations in all microcosms groups (i.e. N_1, N_2, N_3, and N_4) which highlights the importance of these phyla in the biodegradation of petroleum contaminated soil. In N3 and N4 microcosms relative abundance of Firmicutes also was increase which indicates the importance of this phylum in soils with high concentration of petroleum hydrocarbons. Dominancy of this gram-positive phylum in the petroleum

contaminated soil has been previously reported (Reddy et al., 2011; Zhang et al., 2012; Shahi et al., 2016).

Relative abundance of Firmicutes, Bacteroidetes and Verrucomicrobia were increased with increasing of nutrient amounts between the studied concentration ranges. The increasing manner in the T_3 (i.e. N_1T_3, N_2T_3, N_3T_3, and N_4T_3) and T_4 (i.e. N_1T_4, N_2T_4, N_3T_4, and N_4T_4) microcosms were very notable. Relative abundance of Verrucomicrobia in the control microcosms was very low. Based on our previous work, it's abundance in the samples obtained from petroleum contaminated soil was lower than 1% (Shahi et al., 2016). But it started to increase with increasing of the nutrient concentration showing that it is a highly sensitive phylum to the available nutrient concentration. In microcosms with high concentration of petroleum hydrocarbons increasing of nutrient concentration lead to decrease in Planctomycetes, Synergistetes, Lentisphaerae phyla. In T_4 group the relative abundance of Thermotogae pylum was dramatically decreased with increasing of nutrient ratio most probably because of inhibition by high nitrogen concentration (Braddock et al., 1997; Walworth et al., 2007).

Since N_3T_3 microcosm had the most biodegradation amount among all studied microcosms, microbial diversity and their relative abundances in this microcosm give important information about the main phyla involved in the biodegradation process of petroleum contaminated soil. When comparing N_3T_3 with N_2T_3 setup which has the same amount of petroleum hydrocarbons but lower C:N:P ratio, increasing of N:P ratio was twin with the increasing of Proteobacteria, Firmicutes and Bacteroidetes and decrease of Lentisphaerae and Planctomycetes phyla. On the other hand, the comparison of N_3T_3 with the N_3T_2 microcosm which has the same N:P concentrations but lower hydrocarbon concentration shows that increasing petroleum hydrocarbon concentrations lead to increase the relative abundance of Proteobacteria, Firmicutes, and Bacteroidetes phyla and decrease in the abundance of Lentisphaerae, Thermotogae, and Planctomycetes phyla in N_3T_3 setup. Dramatic decrease in the amount of Lentisphaerae phylum which continues with increasing of the nutrient concentration in N_4T_3 and hydrocarbon concentration in the N_3T_4 can be because of, inhibitory effects of higher concentration of nitrogen (Braddock et al., 1997; Walworth et al., 2007), high pH value originates from high concentration of urea (Kauppi et al., 2011), inhibitory effects of high petroleum concentration (Margesin et

al., 2007), and/or its failure in competition with other phyla in achievement of the nutrient and carbon sources (Adams et al., 2014). Overall results highlighted the high relative abundances of the Proteobacteria, Firmicutes and Bacteroidetes phyla in the petroleum contaminated soil. These phyla covered 50% of the determined phyla in the N_3T_3 which had shown the most biodegradation amount. Chloroflexi which was one of the dominant phyla with relative abundance of 14.42% in the samples obtained from petroleum contaminated soil (Shahi et al., 2016) showed a decrease down to 5.1±1.2% in studied microcosms. Among all determined phyla Acidobacteria phylum with relative abundance of 4.6±0.51showed minimum fluctuation in studied microcosms.

Figure 4.13 shows the relative abundances of dominant species in the examined biostimulation microcosms. *Pseudomonas* sp. (8.3±2.1), *Streptococcus* sp. (4.8±1.3), *Desulfitobacterium* sp. (3.9±1.0), *Clostridium* sp. (3.6±0.9), *Kosmotoga* sp. (3.6±1.2), *Candida bombicola* (3.4±1.5), *Candida tropicalis* (3.3±1.8), *Syntrophobacter* sp. (3.1±0.8), Pseudomonas fluorescens (3.1±1.2), *Desulfovibrio* sp. (3.1±2.6), *Syntrophomonas* sp. (2.9±1.3), *Bacillus subtilis* (2.8±1.7), *Thermovirga* sp. (2.7±0.9), *Cupriavidus metallidurans* (2.4±1.0), *Aeromonas* sp. (2.4±1.5), *Rhodopirellula* sp. (2.1±1.1) , *Chthoniobacter* sp. (2.3±0.8), *Enterobacterium* sp. (1.9±0.9), *Tepidanaerobacter* sp. (1.8±0.9), and *Thermoanaerobacter* sp. (1.6±0.6) were the most dominant species. Dominant species are mostly belonged to the gram-negative bacteria. In N_3T_3 microcosm which showed the most carbon removal efficiency *Pseudomonas* sp., *Streptococcus* sp., *Candida tropicalis*, *Candida bombicola*, *Kosmotoga* sp. were five more dominant species. Relative abundance of *Desulfovibrio* sp. showed more fluctuation and *Thermoanaerobacter* sp. showed the less fluctuation in the studied microcosms.

4.5 Quantification of functional gene expression

Exact quantification of the functional genes responsible for the main enzymes in petroleum hydrocarbon biodegradation pathways is necessary for a successful biostimulation practice (LeFevre et al., 2012). *alkB, phnAc* and *nah* as functional genes of aerobic biodegradation of alkane and PAH were selected for quantitative studies using mRNA levels and plasmid DNA. The quantity and expression level of mentioned functional genes was evaluatzed in all experimental microcosms.

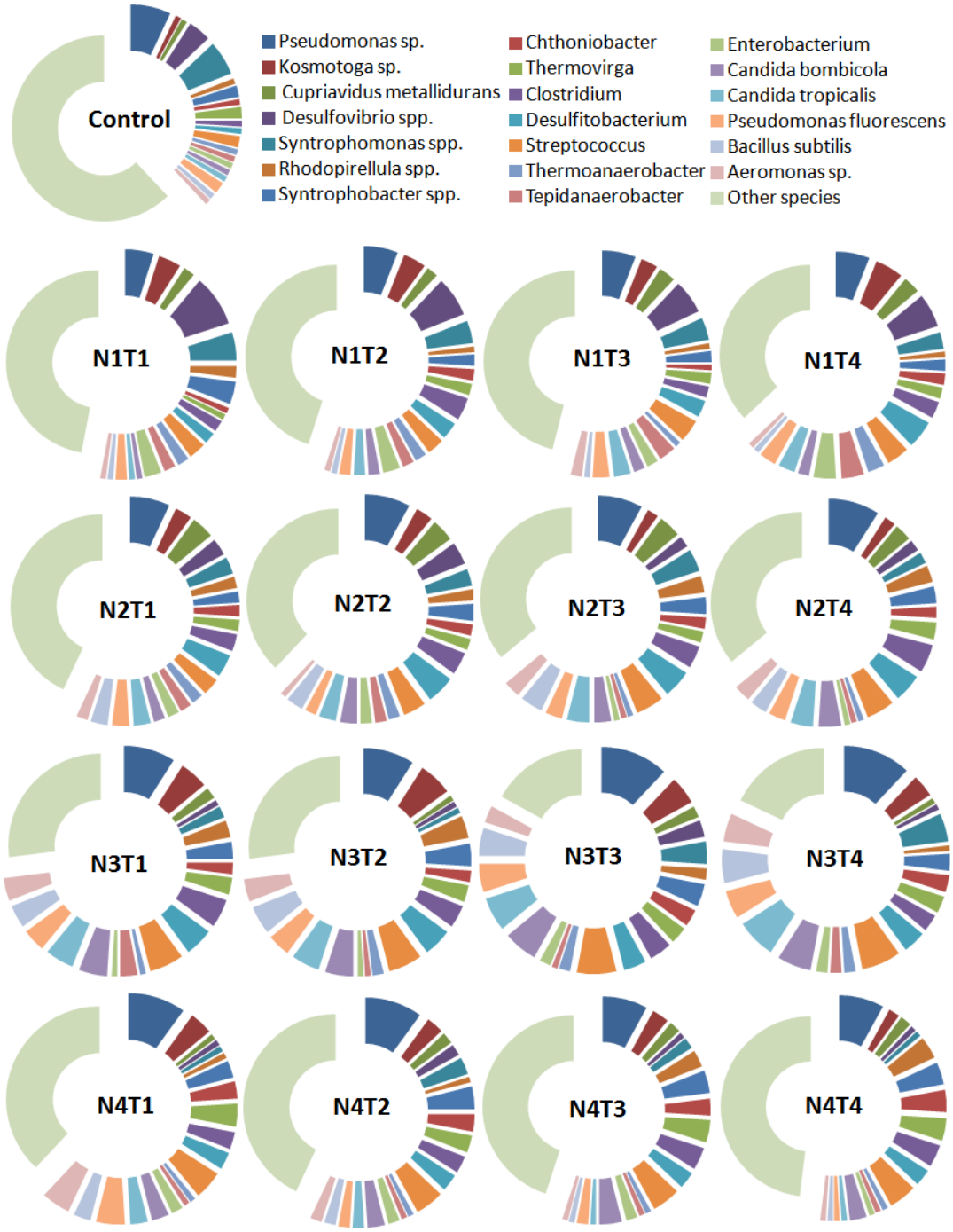

Figure 4.13 : Dominant bacterial species in the studied microcosms.

Figures 4.14 (a), (b) and (c) show the quantities of *nah*, *alkB* and *phnAc* functional genes in the studied microcosms, respectively. Studied functional genes were higher in the systems which were amended by 100:15:1 of C:N:P ratio (i.e. N_3 microcosms). These groups of microcosms showed significantly higher level of functional genes than N_1 and N_2 microcosms but non-significantly higher than N_4 microcosms.

Consequently, alkane and PAH degrading genes were high in the microcosms with C:N:P ratio of 100:15:1 and 100:25:1. Microbial population in these microcosms was also higher ($p < 0.05$).

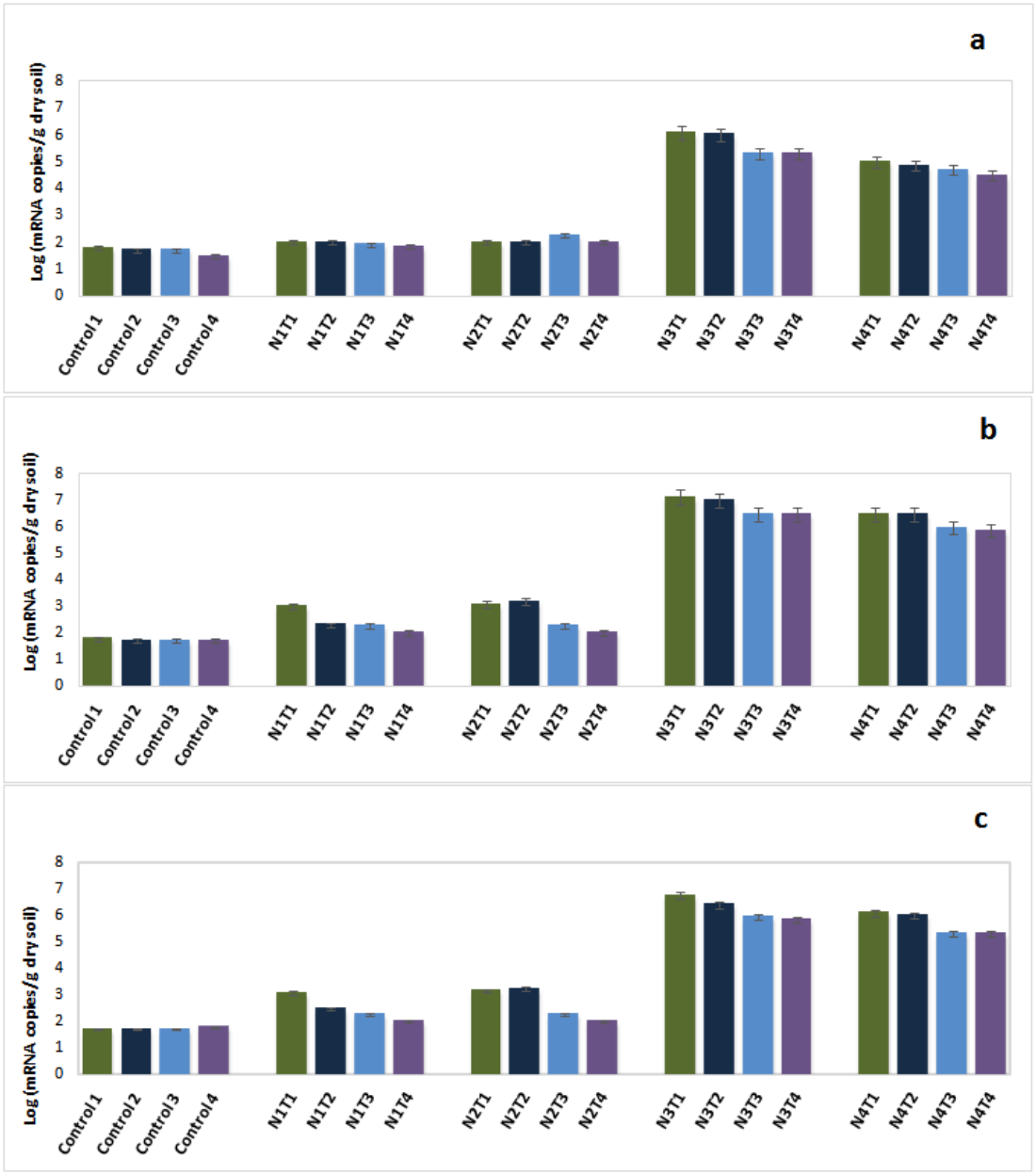

Figure 4.14 : Quantities of: (a) *nah* (b) *alkB* and (c) *phnAc* functional genes expression level through the ex-situ biostimulation of petroleum-contaminated soil.

Obtained results from qPCR analysis about the importance of functional genes evaluation in the assessment of the biodegradation efficiency, have also been confirm by some other works and suggests that the quantity of *alkB*, *phnAC* and *nah* genes are more susceptible to changes in hydrocarbon and nutrient concentrations in contaminated soil than the quantity of microorganisms. In addition, functional genes

were positively correlated to gram-negative bacteria in all collected samples. Therefore, an effective control of alkB, *phnAC* and *nah* functional genes is necessary for a successful biostimulation practice.

Evaluation of the role of horizontal gene transfer (HGT) in the appearance of pollutant resistance in bacterial community and the impact of changes in petroleum hydrocarbon concentration on the abundance of resistance genes was done by quantitative PCR assays of plasmid DNA. *alkB and phnAc* genes located on plasmid were quantified in the samples collected from N_3 and N_4 microcosms. Figures 4.15 (a) and (b) show the quantities of *alkB* and *phnAc* genes on the plasmids obtained from microcosms which C:N:P ratio was arranged to 100:15:1 and 100:25:1 (i.e. N_3 and N_4 microcosms), respectively.

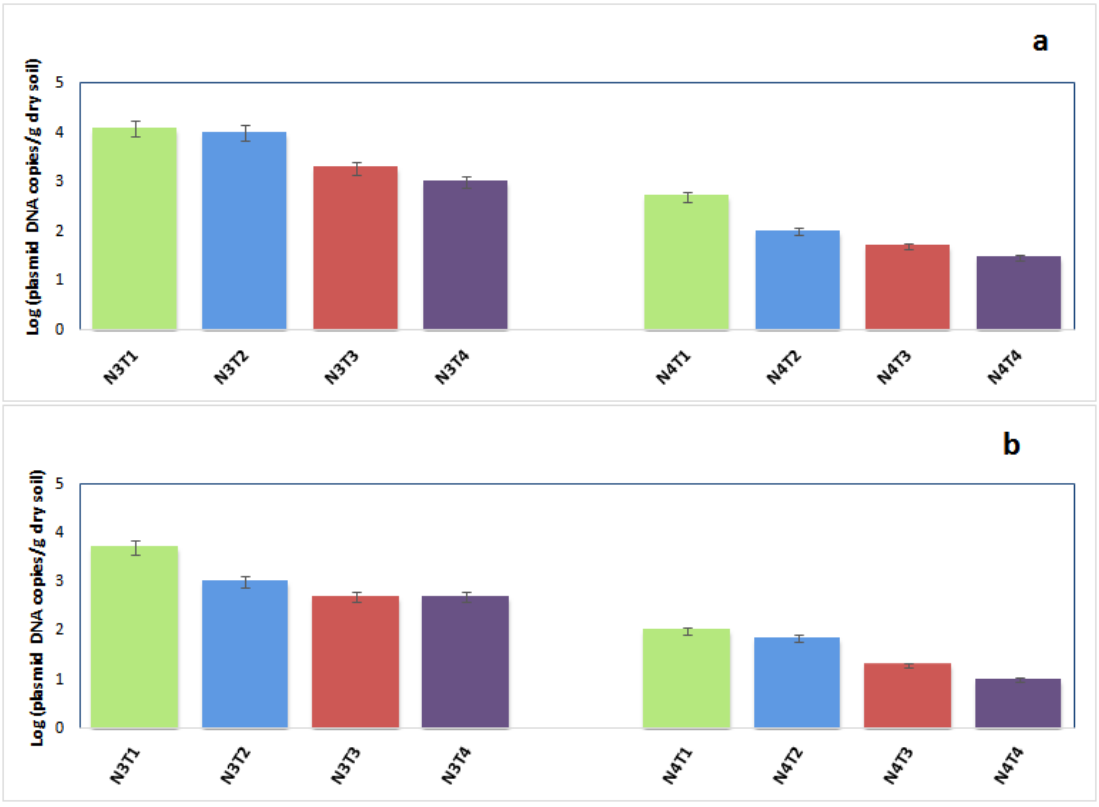

Figure 4.15 : Quantities of: (a) *alkB* and (b) *phnAc* expression level in plasmid DNA through the ex-situ biostimulation of petroleum contaminated soil.

Results suggested a relation between *alkB* and *phnAc* genes with HGT, which was supported by the by the finding that high amount of *alkB* and *phnAc* genes were located on plasmid during effective biostimulation practice. A strong relationship between these functional genes and HGT has been reported through other works. Wilson et al. (2003) have reported the horizontal transfer of *phnAc* gene by

naphthalene-degrading bacteria isolated from petroleum polluted soil. Andria (2010) has demonstrated the transporting of *alkB* gene through horizontal gene transfer between gram-negative and gram-positive bacteria in petroleum contaminated soil. Nie et al., (2014) have supported these findings and showed the horizontal transfer of alkB in oil-polluted environment.

4.6 The effects of petroleum hydrocarbon contaminants and biostimulation practice on soil bacterial population dynamics

Current work investigated the diversity and quantity of soil *n*-alkane and PAH-degrading bacterial communities with targeting of total and active bacteria, Gram-negative and Gram-positive bacteria, *Pseudomonas* species as well as *alkB*, *phnAc* and *nah* functional genes. Present work also highlighted the importance of nitrogen and phosphorous as very important elements for successful biodegradation of hydrocarbon pollutants. Adding of nutrients had significant effects and enhanced the biodegradation of petroleum contaminated soil. Active bacterial community during biostimulation practice in N3 and N4 microcosms when C:N:P ratio were arranged to 100:15:1 and 100:25:1 most probably were the most tolerated bacteria to high concentration of petroleum hydrocarbons and were able to metabolize the petroleum hydrocarbons as carbon sources for their growth using specific catabolic genes. Furthermore, current study emphasized biostimulation is a suitable choice for remediation of petroleum contaminated soil due to the low cost and engineering in comparison with bioaugmentation approach and physicochemical methods as well.

Obtained results also provide insight into the impact of abiotic factors like macronutrient availability on the HGT. Association between HGT and functional genes was assessed by qPCR analysis in which DNA sequences of given genes found in different bacterial genera are considered as horizontal transfer of such genes through bacterial community. However, to which degree these factors affect natural HGT still not known and need study more. Further studies are also necessary to reveal the relations between HGT and functional genes by nucleotide sequence analysis.

4.7 Quantification of functional gene expression through bioaugmentation phase

Based on the obtained results from the gene transfer by plasmids (horizontal gene transfer), a significant correlation between HGT and successful bioremediation of petroleum contaminated soil is expectable. Therefore, in this part of the work evaluation of the functional genes expression was used for understanding of the efficiency of the biodegradation practice. For assessment of the biodegradation status of the studied petroleum polluted soil in post-treatment phase using *T. versicolor* and *B. adusta* fungi, *alkB*, *phnAc*, and *nah* genes were quantified. The expression level of the studied functional genes in the bacterial community during treatment with *T. versicolor* than *B. adusta* fungi have been illustrated in Figures 4.16 and 4.17.

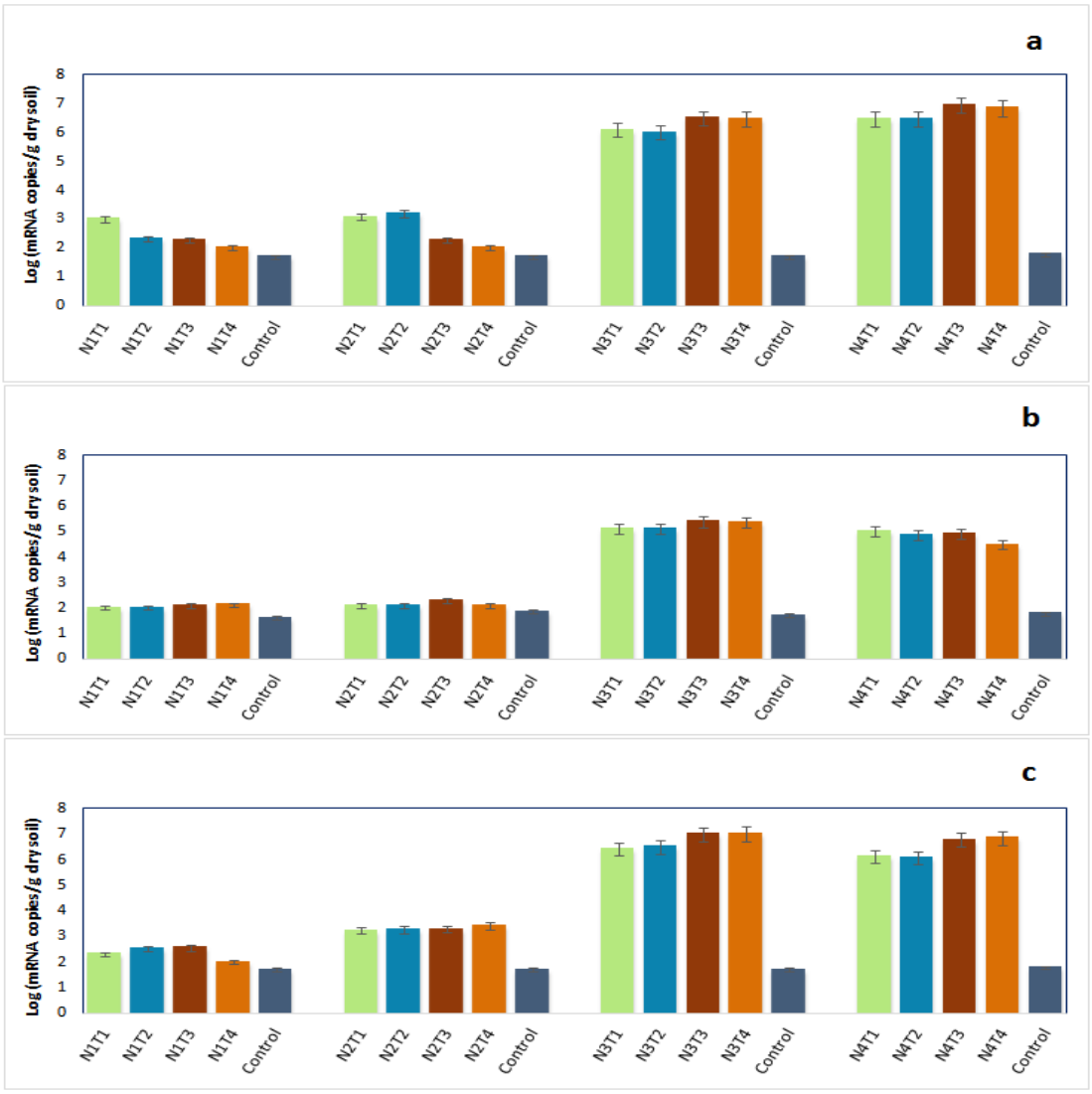

Figure 4.16 : The Expression of *alkB* (a), *nah* (b),and *phnAc* (c) genes in bacteria when petroleum contaminated soil post-treated by *T. versicolor*.

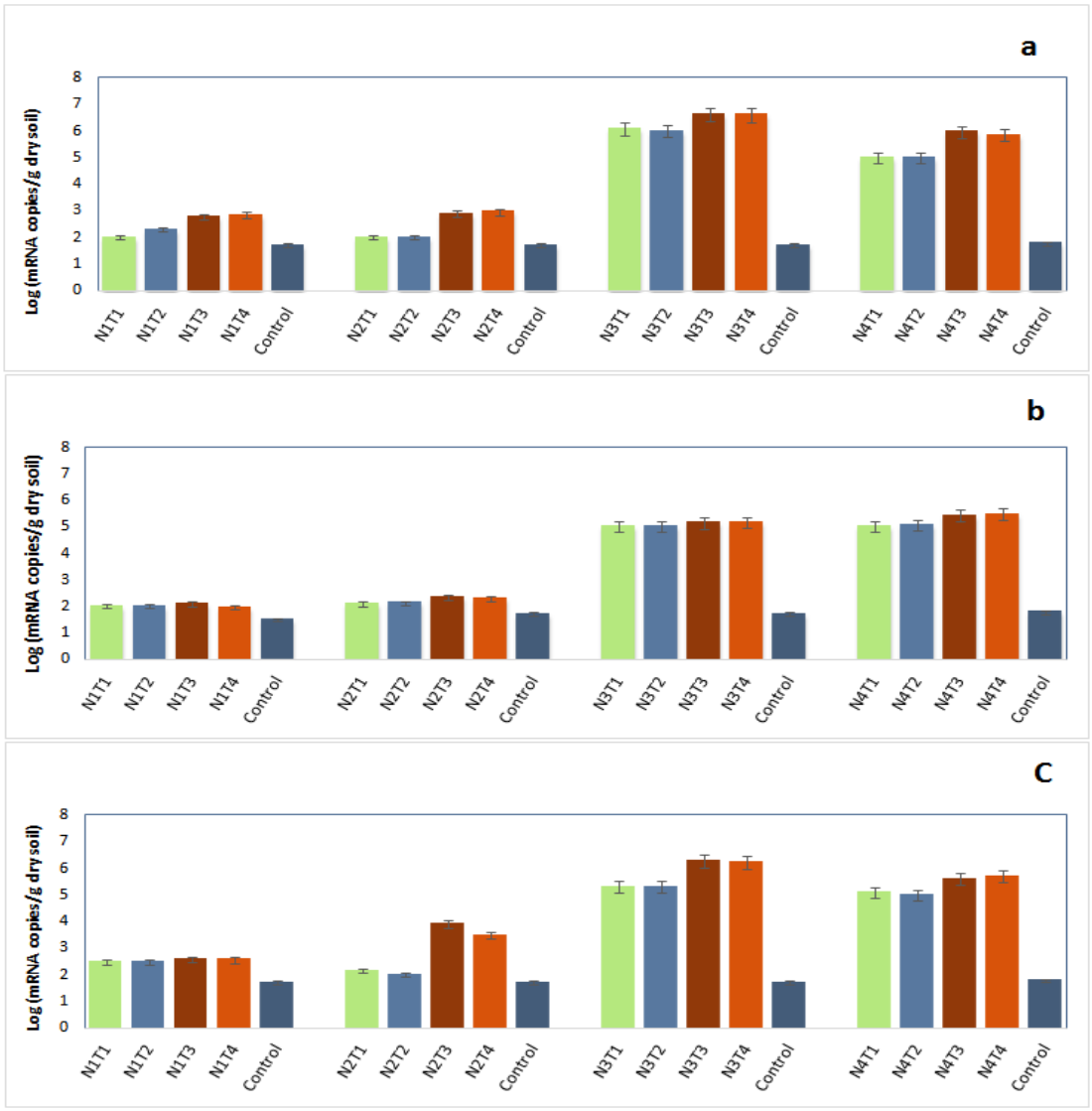

Figure 4.17 : The Expression of *alkB* (a), *nah* (b), and *phnAc* (c) functional genes in bacteria when petroleum contaminated soil post-treated by *B. adusta*.

These functional genes as previously mentioned, are essential genes in the aerobic biodegradation pathways of alkane and PAHs. Real time changes of these genes and bacterial community during post-treatment phase were evaluated using qPCR approach. As results show, the quantity of the studied functional genes where higher when combination of *T. versicolor* than *B. adusta* fungi were used. Such an increase in the biodegradation and a kind of synergistic biodegradation using combination of mentioned fungi have been previously reported by Sayara et al., (2011). A positive correlation ($p<0.05$) between C:N:P ratios and expression level of functional genes was demonstrated.

The quantities of the studied functional genes were increased when adding of fungi strains to the microcosms while the nutrients amount were low because most part of

amended nitrogen and phosphorous had been used during biostimulation process. However, increasing of the biodegradation through the adding of fungi most probably was because the fungi were able to survive in the environment with low nutrient availability as Tišma and Zeli (2010) have been previously reported. The highest amounts of functional genes were quantified in T_3 and T_4 microcosms which the initial TOC amounts were 15 and 25% respectively. *T. versicolor* was effective in increasing of the functional genes. Expression amount of studied genes in the bacterial community when bioaugmented by *T. versicolor* were higher than when were tested by *B. adusta*. Similarly, Beaudette et al., (2000) found *B. adusta* more effective than *T. versicolor* in biodegradation of polychlorinated biphenyl.

Figure 4.18 (a), (b) and (c) illustrates the quantity of active bacteria cells, active gram-negative and gram-positive bacteria, respectively.

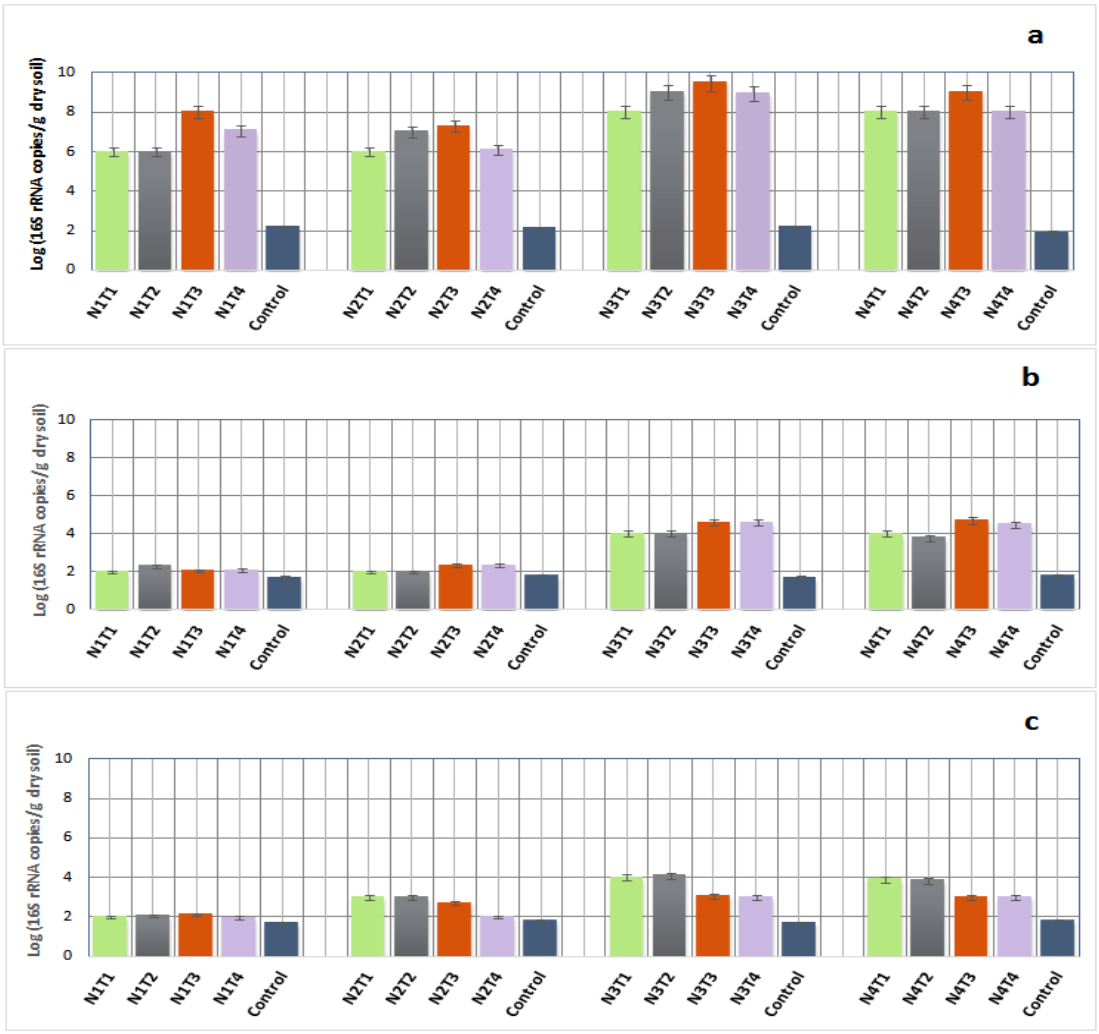

Figure 4.18 : Quantity of: (a) active, (b) active gram-negative, and (c) active gram-positive bacterial cells when petroleum contaminated soil post-treated by studied fungi.

Active bacterial cell numbers increased in N_1 and N_2 while their quantities decreased significantly in N_3 and N_4 microcosms ($p<0.05$). The number of active gram-negative bacteria reduced significantly in the end of bioaugmentation process compared to the biostimulation process. Gram-positive bacteria did not show significant reducing in bioaugmentation process ($p<0.05$). The quantities of active bacterial cell, gram-negative and gram-positive bacteria when bioaugmented with *T. versicolor* and *B. adusta* were not significantly different ($p<0.05$).

4.8 Evaluation of fungal bioaugmentation and the removal of petroleum hydrocarbons

Results obtained form the evaluation of the functional gene quantities, an increase in the biodegradation of the PAH in the examined microcosms can be implicity deducted. The higher amount of PAH removal are expected in the T_3 and T_4 microcosms. Accepting this assumption and taking into consideration that the biodegradation capability when using combination of *T. versicolor* and *B. adusta* fungi was higher than individual effects, each fungus species has different biodegradation capability petroleum hydrocarbon degradation capability and studied fungi likely use different transformation pathways. Therefore, combination of different fungi may increase the removal efficiency of petroleum and its derivatives. Successful biodegradation of various types of hydrocarbon pollutants using *T. versicolor* and *B.adusta* white-rot fungi strains have been reported through different studies. For example, Boonchan et al., (2000), Acevedo et al., (2011), Ding et al., (2013) have successful biodegradation results in treatment of PAHs. The efficiency of white-rot fungi in biodegradation of petroleum derivatives in the polluted sites have also been reported by through some other works (Ting et al., 2011; Yanto and Tachibana 2014; Khan et al., 2015). However, current study which examined the petroleum biodegradation efficiency of these fungi as a post-treatment phase showed that mentioned fungi could biodegrade the petroleum hydrocarbons but the biodegradation efficiency was not adequate. Most likely major part of the biodegradable hydrocarbons had been removed through the biostimulation practice by indigenous bacterial community and the remained part of hydrocarbons was recalcitrant and less biodegradable so studied fungi couldn’t completely remove the remained petroleum hydrocarbons. As a suggestion, because anaerobic

microorganisms show high performance in biodegradation of toxic substrates (Aydin et al., 2015b) they could be used effectively in post-treatment of the petroleum residuals after a bioremediation process.

4.9 Application in environmental biotechnology

Bioremediation as an effective approach is used for treatment of petroleum contaminated soil. When naturally existing bacteria are exposed to organic pollutants they start to develop, adapt and increase their ability to degrade the contaminant. Many groups of microorganisms can degrade the hydrocarbons including petroleum hydrocarbons. However, not all the contaminated sites have the potential for natural attenuation and the capability of such region for a successful bioremediation should be enhanced due adding of necessary nutrient, electron acceptors or some effective microorganisms. For an effective bioremediation practice the natural potential of the native microorganisms should be evaluated and the best alternative for bioremediation then can be chosen. After selecting of a suitable approach the bioremediation procedure should be observed during the process. Therefore, for a successful bioremediation practice an acquisitive monitoring before and during the bioremediation process is needed. Different monitoring approaches have been tested by researchers. Physicochemical methods have been used widely by different researchers. Geophysical analysis using electrical induced polarization (IP) measurement and finding correlation between changes in groundwater geochemistry accompanying stimulated iron and sulfate reduction and sulfide mineral precipitation when acetate injection to groundwater (Williams et al., 2009), employing of electrode-based approach with installing of borehole graphite anodes from a region of acetate injection for stimulation of bioreduction of U(VI) and reporting of a correlation between levels and availability of acetate and the removal of uranium from groundwater (Williams et al., 2010), using successfully remote sensing technique for the monitoring of benzene level throughout the pipeline (Noomen et al., 2015) and benefiting from the different level of deformation in microfossils under different concentrations of petroleum contamination for monitoring of bioremediation process in a crude oil polluted costal region (Sabean et al., 2009) are some examples from researches which have done for finding a suitable physical monitoring approach. Using stable isotope ratio of contaminant residuals (Stehmeier

et al., 1999; Meckenstock et al., 2004) and stable isotope probing method for analyzing of the microorganisms that metabolize and assimilate specific substrates in environmental samples (Andreoni and Gianfreda, 2007) are some chemical methods for monitoring of bioremediation practice. Almost all these methods rely on the monitoring of contaminant and detecting any diminishing in the contaminant concentration in the contaminated site during bioremediation process. However, they cannot give detailed information about the fate of the contaminant and cannot separate the biotic and abiotic removals. Monitoring approaches which are based on the microbial analysis give closer information about the biologic degradation of pollutants. Microbial community as the main players of bioremediations process gives us the exact information about what happened in the contaminated site. Evaluation of microbial population and functional genes are employed successfully as monitoring approaches for analysis of the efficiency of bioremediation system and were used (Shahi et al., 2015a, Shahi et al., 2016b). Horizontal transfer of functional gene which was tested successfully in this project in petroleum contaminated soil is an effective monitoring approach which can be used for evaluation of the bioremediation process. This developed monitoring approach not only is a method for checking out the bioremediation manner but also can be used for assessment of the biodegradation potential of the contaminated site (Shahi et al., 2015b).

5. CONCLUSION

Gram-negative bacteria play main role in the studied petroleum contaminated soil and show a competitive advantage over other bacterial community. In addition, this bacterial group was dominant in collected oily sludge samples and also in the end-point after biostimulation and bioaugmentation practices proves that Gram-negative bacteria due to a long term acclimation to the petroleum hydrocarbons in the contaminates site could overcome other bacteria in the operated microcosms. *Pseudomonas sp. PAMC 26824* species was the most dominat Gram-negative bacterium in the petroleum sludge and in the end-point of biostimulation and bioaugmentation processes. Regarding to the molecular tools, this study demonstrated that quantitative real time PCR (qPCR) is a precise and sensitive molecular approach for evaluating of 16S rRNA and functional genes and evaluation of the dynamics of petroleum hydrocarbon-degrading bacteria. Illumina Sequencing method because of its high-throughput capacity and low cost is the most suitable technique for analysis of microbial diversity in bioremediation site. Horizontal gene transfer is a developed monitoring approach not only for the assessment of biodegradation process but also for the estimating of bioremediation potential of contaminated sites. *T. versicolor* and *B.adusta* fungi cannot completely biodegrade the petroleum residues. However, *T. versicolor* is more effective than *B.adusta*. The expression level of *alkB*, *phnAc* and *nah* functional genes are synergistically increased in the presence of white rot fungi whereas the quantity of active bacterial cells, gram-negative and gram-positive bacteria do not change significantly. Overall conclusion can be summarized as below:

- Gram-negative bacteria have a competitive advantage over bacterial community on petroleum contaminated soil.
- Assesment of 16S rRNA and functional gene using qPCR is helpful approach for a real time monitoring of biostimulation process.

- Horizontal Gene Transfer is an effective molecular biomarker, which can be used for evaluation of bioremediation potential of petroleum-contaminated soil.
- In highly petroleum polluted soils Proteobacteria, Bacteroidetes, Firmicutes can successfully degrade petroleum hydrocarbons if necessary nutrients are provided.
- *Trametes versicolor* (a white rot fungus) increase the expression level of *alkB* and *phnAc* functional genes.

Isolation the most dominant species and detailed genomic analysis for HGT event is recommended for future studies. Furthermore, the examination of HGT as a developed monitoring approach in different bioremediation media especially in groundwater and anaerobic environments is alos recommended. Since anaerobic microorganism is more effective in the biotreatment of toxic compounds, the assessment of anaerobic fungi potential in the biodegradation of petroleum residues is recommended.

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