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COLLEGE OF ARTS AND SCIENCES

INVESTIGATION OF BACTERIAL DIVERSITY AND CHEMOMETRIC

ASSESSMENT FOR ENHANCING BIOREMEDIATION OF HYDROCARBONS IN

QATARI SOILS

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ABSTRACT

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Title: INVESTIGATION OF BACTERIAL DIVERSITY AND CHEMOMETRIC ASSESSMENT FOR

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Qatar is one of the leading countries in oil and gas industry worldwide. Qatar is located in the middle of the Arabian Gulf sea, a semi-closed sea surrounded by many petrochemical sites, besides the active offshore traffic of oil and gas transportation. Side-effects of production and transportation activities are expected and can affect the environment seriously. Bioremediation offers alternatives to alleviate such pollution caused by the thousands of oil components known by their high diversity, instability, toxicity and low bioavailability and biodegradability. Weathering, is an additional factor, making bioremediation of oil-polluted areas more difficult. Oil Weathering Processes results to changes of oil in terms of properties and composition. This is why Petroleum-derived contamination events constitute a unique environmental issue. In Qatar and the region in general, the weathering processes are accentuated due to harsh conditions, representing the main origin of failure of bioremediation applications. Some bacteria are able to adapt to such weathering conditions and absorb particles rich in pollutants through their hydrophobic wall to form biofilms. Others produce surfactants to improve accessibility.

In this study, a multidisciplinary approach was implemented to solve such an environmental issue, starting from the identification of the potential sources of the polluting

oil along AlZubara coast to Chemometric investigations allowing determination of weathered status. Following systematic sampling and chemical analyses of soils, biodiversity of hydrocarbon-degrading bacteria was studied. Through an integrated isolation and screening program, isolates were identified and differentiated through several molecular techniques and screened for their potential to remove classes of hydrocarbons with special focus on high efficiencies never reported with concentrations and weathered status. Biostimulation of indigenous bacteria showed low rates of removal. However, combining stimulation to augmentation improved the removal of weathered oil. Interestingly, by using ex-situ biopiling, augmentation using indigenous bacteria exhibited the best approach, but a high negative interaction was observed by using exogenous bacteria. These findings confirm the initial hypothesis of this work, that weathered hydrocarbons removal was enhanced by using adapted indigenous bacteria. It is then considered that bioremediation of a given area should be separately treated, using corresponding appropriate bioremediation strategy.

ACKNOWLEDGMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1 : **INTRODUCTION**

Qatar's National Vision 2030 is projected to propel Qatar towards an increased balance between its development projects and the protection of its air, sea, land, and natural environment. Qatar economic boom is based on the hydrocarbon industry. In addition, Qatar is in a unique position. Indeed, promoting environmental sustainability is a key component of Qatar's vision 2030. Qatar's National Development Strategy, 2011-2016, is based on the fact that Environmental degradation can be reduced by investing in advanced technologies to reduce the damage arising from economic activities in the country. Consequently, understanding and managing the impact of hazardous pollutants need new technologies which should be developed for remediation and recovering of resources while keeping concern on the Economy, Society and Environment.

Petroleum products composition can change through such processes as photodegradation, oxidation, partitioning, biodegradation, and volatilization, which makes it hard for experts to examine the extent of the risk of exposure (National Research Council, 2003). The more volatile or soluble compounds often migrate to different locations. In contrast, non-mobile petroleum components are often left behind and concentrate at the spillage site (National Research Council, 2003). This soil pollution is often the result of mining and oil production, or energy and fuel production (Abioye, 2011). Common contaminants include metals (e.g., copper, zinc, lead, cobalt, nickel, arsenic, and cadmium), light hydrocarbons (e.g., oil, gasoline, and diesel), heavy hydrocarbons (lubricants, heavy oil, and crude oil), and polycyclic aromatic hydrocarbons (PAHs) (e.g., phenanthrene and benzo[a]pyrene). (Castro-Jiménez, et al., 2012). Organic compounds are involved in nearly 90% of polluted sites with petroleum products and their derivatives

being the major source of water and soil pollution (Acher, et al., 1989).

Bioremediation is a common means to remediate hydrocarbon-polluted soils in temperate climates (Pelletier, et al., 2004). However, the climate of the Gulf region results in severe stress on the indigenous bacteria and little is known about the biodegradability of oil products (Hassanshahian & Cappello, 2013; Al-Thani & Potts, 2012; Radwan & Al-Hasan, 2000).

Numerous treatment technologies may be used to remediate soils and new technologies are always being developed. For example, since the 1980s biological techniques have been employed to decontaminate microorganism or plants grown in oil polluted soils (Perelo, 2010). Some of this technique have been perfected through research and necessary modification while others are still being subjected to research to assess their effectiveness (Leahy & Colwell, 1990; Atlas & Hazen, 2011). Researchers have taken a keen interest on these technologies as they are cheaper since there are no charges incurred in the name of excavation or transport, with exception. These technologies are classified into two broad categories (Meagher, 2000) first category: bioremediation techniques that use mainly bacteria and phytoremediation techniques that exploit the properties of plants (shrubs, trees). In the second category of bioremediation (Tyagi, et al., 2011), techniques which include biodegradation, bioaugmentation and biostimulation. Each technique has been designed to deal with a pollution situation.

Bioaugmentation of polluted soils has been considered a valid approach of bioremediation, where the biodegradation is stimulated through the addition of non-native microorganisms (Couto, et al., 2010). Many hydrocarbon-degrading bacteria have been studied and their genomes sequenced. As well as the completely sequenced organisms are

Pseudomonas putida (Lee, et al., 2003) and Acinetobacter sp. (Bento, et al., 2005).

Biodegradation process is usually affected abiotic factors such as temperature, moisture, oxygen, nutrient availability, pH and soil salinity (Leahy & Colwell, 1990; Mrozik & Piotrowska-Seget, 2010; Cébron, et al., 2013). The interaction of pollutants with soil components significantly affects bioremediation. When soils are exposed over a long period of time the hydrocarbons are strongly sorbed to soil particles, making them inaccessible to microorganisms (Ukiwe, et al., 2013). Bioavailability of hydrocarbons limits the effectiveness of bioremediation processes as they not only alter solubility but also resistance to degradation (Samanta, et al., 2002; Doyle, et al., 2008).

The qualitative hydrocarbon content of crude oil affects their ability to degrade (Nenningsland, et al., 2010). Susceptibility to degradation depends on the physical and chemical states of both the hydrocarbon and local environment (Haritash & Kaushik, 2009). For example, microbial populations ability to utilize hydrocarbons in crude oils depends on the components of unsaturated fraction and asphaltic fraction (Oudot & Chaillan, 2010). Moreover, alkanes and low-molecular-weight aromatics such as benzene, toluene, naphthalene, and methylnaphthalene can be degraded microorganisms to CO_2. However, higher-molecular-weight aromatics containing 4 or more fused benzene rings, such as benzo[a]pyrene, benz[a]anthracene and pyrene, were relatively resistant to microbial degradation (Kanaly & Harayama, 2000; Oudot & Chaillan, 2010). Studies have shown that PAHs can persist in the environment for long periods of time due to low biodegradation rates, with recalcitrance increasing with PAHs molecular weight. Additionally, some PAHs and other aromatic compounds may undergo only partial biodegradation (Zein, et al., 2006).

Several of bioremediation data of complex systems requires the use of several chemometric techniques to detect the optimal-most effective method for the bioremediation of petroleum (Kaczorek, et al., 2014). Moreover, these techniques are based on chemometric data analysis which is used to analyze correlations in large data sets to gather information from other analytical techniques such as Gas chromatography-mass spectrometry (GC–MS) and clustering a variety of unknown patterns in data associated with different chemical phenomena like in the case of crude oil biodegradation (Christensen & Tomasi, 2007; Al-Ghouti, et al., 2010; Christensen, et al., 2005 a). Nonetheless, these approaches have been limited in accuracy since they are curbed with errors as a result of the changing nature of spilled hydrocarbons when exposed to the environment (Schwartz, et al., 2011). One of the suitable approaches that have been used is risk assessment, which entails risk specific analysis on the environment and humans. On its part, total petroleum hydrocarbon (TPH) is not a reliable approach to risk assessment because the involved compounds have diverse toxicological and physicochemical properties (Schwartz, et al., 2011). Instead, products should be divided into fractions and in line with their toxicity and physicochemical properties for suitable risk analysis and soil quality to be performed. Such an approach caters for fractionation in terms of aromatic and aliphatic compounds in addition to their equivalent carbon number (ECN) (Christensen & Tomasi, 2007; Soleimani, et al., 2013).

Given some of the above potential limitations in the methods used to analyze petroleum components at polluted sites, the purpose of this research work is to:

- Determine the physical and chemical characteristics of the collected soil samples from the assigned polluted sites.

- Identify the main composition of the polluted soil matrix using various analytical techniques such as (GC-MS) (GC-FID), and FTIR.

- Isolate individual bacterial strains from collected soil samples from hydrocarbon-polluted sites in Qatar that can degrade hydrocarbons.

- Determine the nutritional requirements of the mixed cultures and individual bacterial strains for hydrocarbon degradation.

- Perform molecular identification and MALDI –TOF/MS profiling of the microbial communities to study the microbial diversity of the environments.

- Develop protocol(s) for enhancing in-situ bioremediation of hydrocarbons based on nutritional needs and examine the protocol(s).

The overall objective is to develop an analytical study for the characterization of soil hydrocarbon fractions in Qatar soil. The study will characterize the bacterial ecology of hydrocarbon-degrading communities in Qatar and contribute in the design of appropriate bioremediation approaches for each polluted site, to overcome the limitations often facing successful application of bioremediation processes. Chemometric techniques will be implemented, combining hydrocarbon occurrence and availability and potentialities of indigenous bacteria.

Chapter 2 : **LITERATURE REVIEW**

2.1 Pollution of The Environment with Crude Oil Components:

2.1.1 *Petroleum Components*

Natural gas and oil together make crude oil that contains hydrocarbons of diverse molecular weights in addition to numerous organic compounds (Kansha, *et al*., 2012). Crude oil is the source material for virtually all petroleum products (Guerriero, *et al*., 2012). Over 90% of petroleum is made up of hydrocarbons that are grouped according to their chemical structures such as aromatics, and straight, cyclic or branched alkanes. 10% of the petroleum component is made up of non-hydrocarbon elements such as sulfur, nitrogen and oxygen, in addition to trace amounts of metals such as vanadium and nickel (Likhatsky & Syunyaev, 2010; Hansen, 2007) .

Though oil and water are usually considered to be non-miscible, petroleum contains tiny quantities of soluble portions called the water soluble fraction (Simon, *et al*., 2010). The soluble portions consist of dissolved hydrocarbons, dispersed particulate oil and soluble contaminants like metallic ions. In addition to non-hydrocarbon components such as sulfur, oxygen, and nitrogen, the nitrogen-containing compounds includes quinoline and pyrimidine, while the oxygen-containing compounds includes ketones and esters (Waheed & Oni, 2014).

Therefore, it is possible that the concentration of non-hydrocarbon and hydrocarbon components in petroleum considerably differ from different sources. In most cases, petroleum components go up into solution and are absorbed by microorganisms and metabolized in living cells. This phenomenon is significant because in the event of an effluent discharge or oil spill from petroleum products or engine oil into the environment,

6

they cause serious adverse impacts on the ecosystem (Castro-Jiménez, et al., 2012) .

Some scientists have associated adverse ecosystem contamination from petroleum as result of dissolved low molecular weight hydrocarbons and mainly aromatic compounds such as toluene (Lee, et al., 2010). However, Zhao, et al. (2014); Castro-Jiménez, *et al.* (2012) have considered naphthalene as a more significant cause of petroleum toxicity compared to low molecular weight aromatic compounds. Another source argued that low boiling point unsaturated hydrocarbons like naphthalene, xylene, toluene, and benzene are the most toxic components of petroleum and its products (Kansha, et al., 2012). In all, toxicity of petroleum can be said to be a total function of the presence of all these mentioned substances. The next section elaborates about the major causes of petroleum pollution.

Complex mixtures of compounds are found in petroleum hydrocarbons. The rate of degradation differs from one component to another depending on their chemical structure and concentration. Four main fractions do characterize the petroleum hydrocarbons: Saturates, Aromatics, Resins and Asphaltenes (SARA), (Zeng H, 2012). The n-alkanes are the most willingly to be degraded and are average in length (C10-C25). The more difficult to degrade are the longer chain of alkanes consisting of (C25-C40) due to their water solubility scarcity and bioavailability. They are considered as hydrophobic solids (Zeng H, 2012). The branched chains of cycloalkanes and alkanes take more time in the degradation process compared to normal alkanes. Highly condensed cycloparaffinic structures, condensed aromatics, bitumen, tars and asphaltic materials are hardest to biodegrade and have a high boiling point. After oil degradation the residual product can be regarded as humic substance since it is similar in composition (Zeng H, 2012). Total petroleum

Hydrocarbons TPH include (C2-C40), it can be further separated to petrol range organics (C2-C5) and diesel range organics DRO (C6-C40) (Bishop, 1997).

The higher the volatilization, the lighter the components as these compounds tend to have a low boiling point that means that, during aeration process, these petroleum products can easily evaporate and less microbial activity is required, gasoline is an example of such products. In this case, Volatile organic compounds (VOCs) are bound to go to the air that is why treatment process to control VOCs emission is required before allowing the petroleum components to leave into the atmosphere (Jorgensen K.S, 2000). The hydrocarbon products, that are considered as mid-range, are less volatile compared to the lighter components. In this case, biodegradation is more important since evaporation requires a higher boiling point. Examples of such hydrocarbons are; diesel fuel and kerosene. For the heavier hydrocarbons almost, no evaporation happens and the biodegradation is the dominant mechanisms for such products. Examples are; lubricating oils and heating oil. However, the heavier the hydrocarbons, the higher the molecular weight and the longer the period of degradation. The more the number of carbon the higher the boiling point. The treatment of the contaminant may last from 6 months until 2 years under optimal conditions and depending on the target hydrocarbons that need to be treated (Jorgensen K.S, 2000).

Petroleum, benzene toluene ethylbenzene and xylene (BTEX), are mainly made by aliphatic hydrocarbons, whereas carcinogenic and toxic compounds are found in BTEX components. The pathways of most petroleum hydrocarbons are well established, in other words the main compounds should not be problematic during biodegradation if they were dealt with under natural environment (Lyon D, 2013). Natural attenuation or biostimulation

are biological treatments that can be incorporated due to lack of oxygen or/and nutrients, these are considered as limiting factors. Diesel pollution can be treated by bioaugmentation, as the site parameters are important to identify the most suitable treatment. The quicker the pollution is treated and the more efficient the treatment is cost and time wise the better outcomes will be attained (Lyon D, 2013). In terms of bioremediation applications and strategies, BTEX and petroleum hydrocarbons are included along the most studied pollutants. The Exxon Valdez spill helped in rising the public awareness about the significance of bioremediation activities, although bioaugmentation was not considered as a crucial step in treating the spill. Microorganisms are found in the environment and the ones able to degrade hydrocarbons are found in most sites, pristane areas hold these bacteria as well (Lyon D, 2013). Bioaugmentation is considered more successful when dealing with petroleum contaminants. Instead of petroleum itself, BTEX are rather more achievable in the treatment process. In the polluted site other waste products can be found such as heavy metals. In this scenario it is better to use metal resistant bacteria so that inhibition of bacterial growth does not occur, especially if the indigenous bacteria are already non -resistant to metals (Lyon D, 2013).

2.2 **Polycyclic Aromatic Hydrocarbons (PAHs)**

(PAHs) are chemicals with different arrangements, they can be angular, or cluster composed of two or more attached benzene rings. PAHs are toxic compounds in addition they are persistent. They are found in the environment in air, soil, and water. They are produced by large quantities into the environment and usually during fuel combustion, or from industries as byproducts of petroleum refinery or coal gasification. PAHs occur as

complex mixtures. The PAHs persistence in the environment can be explained due to the low water solubility as they are considered hydrophobic compounds which will lead to limit their availability to the microorganisms for biodegradation, as their sorption to soil particles increases due to the low solubility. Sometimes the oil droplets are trapped inside the soil particles making the process of degradation harder to be attained. Exposed over long periods in the soils, PAHs are strongly adsorbed to particles and/ or merged in very fined pores, which lead to be inaccessible to microorganisms (Doyle K, 2008). PAHs should be eliminated from the contaminated site as the treatment of the environment from PAHs is important because they are considered as toxic compounds and can cause mutagenic threats as well as carcinogenic effects (Sadrin T, 2007). Flora and fauna can both be provoked by PAHs and this may lead to detrimental effects on the living organisms. Toxic chemicals accumulate in food chains which may lead to genetic variation or/ and health issues in Humans (Sadrin T, 2007).

High temperatures cause PAHs formation, the rings are aromatic, and they are more stable than alkylated benzene rings that are their precursors (Sadrin T, 2007). The natural distribution, in addition to environmental stability, is affected by the arrangement of the aromatic rings. For instance, tetracene and anthracene are arranged linearly with benzene rings, they are considered as the least stable and usually are not found in natural environment as they can only survive when sequestered into inorganic or organic matrices. On the other hand, picene, phenanthrene, and chrysene have arrangements that are in the most stable form. The formation of these PAHs is through exposing organic compounds to high temperatures (Thapa B, 2012). Bioavailability of (PAHs) is an essential factor that usually limits the efficiency of bioremediation processes (Doyle K, 2008; Samanta K,

2002) and especially consortia of bacteria. Under specific circumstances, a process of bio-polymerization of compounds through a process of bio-autoxidation, generates intermediate degradation products, might occur, resulting in the production of non-biodegradable polymers. In the case of PAHs which are very recalcitrant to biodegradation, such as benzo [alfa] pyrene, cooperative processes can   be put in place: fungi known for their capacity to degrade high molecular weight PAHs (Mrozik A, 2003),initiate degradation. Somehow, the bacterial communities have an ability to degrade contaminants under the impact of environmental parameters, considering characteristics of contaminants and microorganism in the soil.

2.2.1 *Causes of Petroleum Pollution*

Petroleum pollution results when oil is directly or indirectly introduced into the environment through human activities. Oil discharge into the environment can lead to an unsuitable change in such a manner that the welfare and safety of any living microorganism become endangered (Li, *et al*., 2010). If spilled into the water, petroleum spreads over a wide area and immediately forms a slick and oil in water before undergoing various biological, chemical, and physical changes (Saeed, *et al*., 2011). The potential changes include sedimentation, microbial degradation, emulsification, drill, photochemical oxidation, dissolution of water soluble fractions, and evaporation of high volatile oil fractions (Lee, *et al*., 2010; Saeed, *et al*., 2011).

Some of the common causes of petroleum pollution results from oil spills that may come from oil wells, pipelines, and tankers (Farrington, 2013). These sources of pollution are called point sources of petroleum pollution because the origin of contaminants is traced from a single source (National Research Council, 2003). Some of the significant points

petroleum pollution in the past are Kuwait oil fires, deep-water Horizon oil spill, Gulf war oil spill, Lakeview Gusher, and Kuwait oil leaks. Therefore, point sources of pollution represents a catastrophic release of large oil volumes that can pollute enormous regions in a relatively short duration (Guerriero, *et al*., 2012). However, the majority of the causes of petroleum pollution come from nonpoint sources, where tiny amounts of spills from numerous and diverse places over time combine to cause large-scale pollution impacts. Nonpoint sources of petroleum pollution cause 70% of the oil released into the environment from human activities during inefficient consumption of oil and its products (Liu, 2013).

The minor diffuse pollution can include tiny, but the routine discharge of oil into the sea by commercial vessels or leakage from boats during recreational activities (Kansha *et al*., 2012). However, Freije (2014) has recently shown that, the 1991 Gulf war oil spill is considered one of the petroleum pollutions in Qatar. Another study that was done by Tolosa, et *al.* (2005) showed that the levels of TPH and PAHs in biota and sediments were relatively low compared to worldwide. Runoff from parking lots and asphalt covered roads enters streams, storm drains, and lakes and eventually washed downstream to reach oceans and results to negative impacts on the ecosystems through which it traverses (Liu, 2013). As the city population increases with time, there is an increased use of petroleum products such as gasoline, oil-based paint, solvents, and lubricants which are often disposed improperly into drainages and sewage pipes. Moreover, industrial plants use and produce small, but chronic spills that are hardly detected by individuals, but over time they build up and enter waterways (Li, et al., 2010).

Li and coworkers (2010) have shown that estuaries are particularly precarious habitats for diverse animals and plants, making them critical ecosystems more sensitive to

pollutants (Li, et al., 2010). Perhaps one of the most catastrophic oil tanker spills that caused widespread petroleum pollution is the 2013 Exxon Valdex spill that happened in Alaska, at Prince William Sound. It negatively affected the aquatic and the soil ecosystems, human health and natural resources (Maletić, *et al*., 2013).

2.2.2 Fate of Petroleum Pollution in the Environment

Crude oil discharged into the environment undergoes numerous chemical, physical, and biological changes (National Research Council, 2003). Rapid chemical and physical processes include movement and spreading by currents and wind, evaporation of volatile components, chemical oxidation and dissolution, injection into the air, and dispersion of small droplets into the water (Nwaichi, *et al*., 2011). Simultaneously with these, Lee and his coworkers (2010) reported that these are the appropriate with the biological procedure that may include the breakdown by microorganisms or absorption by larger animals, and the subsequent storage and metabolism or discharge (Lee, *et al*., 2010).

The fate of petroleum pollution in the environment became known in the early 2010s through semi-quantitative and qualitative studies as shown in Figure 2-2.
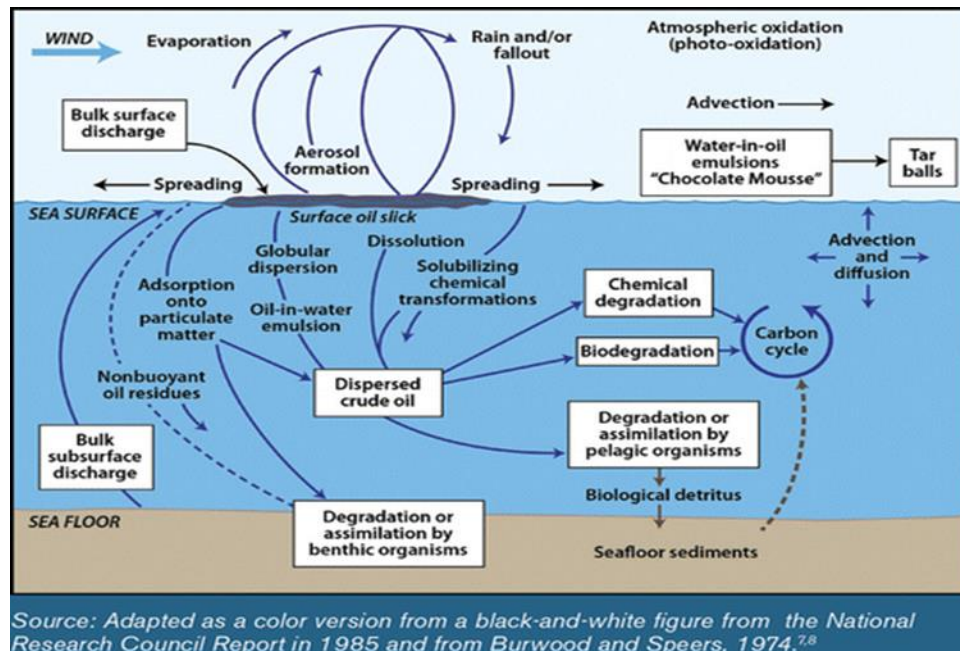
Figure 2-2: The primary fate of the oil spill processes involved in the marine ecosystem (Burwood & Speers, 1974; Farrington, Oil Pollution in the Marine Environment II: Fates and Effects of Oil Spills, 2014)

Figure 2-2 shows the primary fate of the oil spill processes involved that were hypothesized or identified over several decades. However, it showed that focus has shifted from a qualitative approach to quantitative and semi-quantitative comprehension of these processes for different types of petroleum and several ecosystems (Burwood & Speers, 1974). The biological, physical and chemical (photochemical) processes that act on petroleum spilled and its components operate at multiple benchmarks (Likhatsky & Syunyaev, 2010) From the small-scale turbulence to the size of molecules and large scale interactions if ocean, winds, currents and tide in hundreds to thousands of kilometers: from single organisms, small microbes to whales, and to the entire ecosystems.

Other processes like the spread of oil can act on the petroleum bulk. A number of

procedures can act differently in class of molecular and/or chemicals, weight ranges of the compounds in the petroleum spilled, and times specific single groups of substances (Castro-Jiménez, et al., 2012; Likhatsky & Syunyaev, 2010). Current studies have moved to expand the knowledge of the procedures in governing the outcome of petroleum spilled by researching various accidental petroleum leaks and a little induced spilled chemical undertaken for study purposes (National Research Council, 2003). The approach has provided the much-required understanding of the procedures involved in the fate of the petroleum pollution as displayed in Figure 1 and described as follows (Farrington, 2014).

First, the chemical and physical processes include horizontal deployed of spilled petroleum interacting with surface tension and viscosity phenomena between water and oil. Dispersion of spilled oil is spread on the ocean surface by the turbulence which is linked to eddies of ocean to different scales (Neff, 1990; National Research Council, 2003).

Second, petroleum can spread through Langmuir circulation where the interactive impact of the circulating wind blows over the ocean surface water in a way that generates divergence and convergence areas in surface water. This phenomenon frequently generates windrows of materials like natural organic material and flotsam to be released by microorganisms that forms slicks of these natural biological materials. Langmuir circulation results in the production of thicker slicks and long streamers during petroleum spills, and these streamers and slicks of oil, scatter to form thinner oil lusters in the marine ecosystems (Lehr & Debra, 2000).

Third, the fate of petroleum can also enter the evaporation chemical processes where the small molecular chemicals escape into the air. The process is more intensive in tropical areas, less fast in moderate regions, but least extensive and slow in colder polar

tropics (Fingas, 1995). The fate of spilled oil can also be estimated by identifying the air and water temperature and the vapor pressure of the petroleum chemicals.

Fourth, besides evaporation, petroleum can also dissolve through the water-soluble fraction consisting of soluble components such as a sugar glucose and salt (Mackay, *et al*., 1992). Nonetheless, low molecular weight compounds to medium molecular weight compounds of petroleum, such as chemicals, making kerosene and gasoline, can dissolve in seawater, through their solubility has been reported to be minimal compared to other elements such as salts (Schwarzenbach, *et al*., 2003).

Fifth, photo-oxidation of spilled oil can also undergo an important role to remove the aromatic and aliphatic fractions of oil spill oxidized photo-chemically, where the photo-oxidation reaction of chemicals, oxygen, and sunlight can act on the oil to produce oxygenated reaction products (Schwarzenbach, *et al*., 2003). In some cases, some petroleum chemicals are more reactive compared to other chemicals, making it difficult for photo-oxidation to be used to remove large amounts of oil during oil spills (Farrington, 2014). It was shown that the photo-oxidation of oils may contribute new compounds to high toxicity levels further, making spilled oil more dangerous to marine and land organisms (National Research Council, 2003). When the oil is not completely cleared from the sea environment, some may strand along the coastal areas where oil mousse and slicks can reach onshore and pollute the beaches, wetlands such as mangroves and marshes and rocky intertidal areas (Hayes & Michel, 2001; National Research Council, 2003).

Sixth, oil on the rocks is exposed to wave and tidal washing with continuous elimination of the highly dissolved chemicals in the oil, or the ultraviolet light may warm the oil causing additional evaporation of sunlight inducing photochemical reactions and

evaporation or volatile compounds (National Research Council, 2005). Oil bubbles and slicks of oil or mousse that reached the shore can also mix with the sand and soil, as the volatile components evaporate (National Research Council, 2003; Farrington, 2014). At this stage, the oil is also subjected to photochemical reactions where the more soluble and fragile components continue to be degraded into soluble petroleum chemicals (National Research Council, 2005). Petroleum buried in the soil can be re-exposed by wave actions and subsequent storms where it is broken down into fist size clumps and small thumbnail particles of oil and sand (National Research Council, 2003).

Seventh, the petroleum that reaches the shoreline wetlands such as mangroves and marshes has become a topic of concern since it can persist in this ecosystem for many years, and in some instances heavily polluted environments for decades. For instance, the existence of remaining crude oil causing from the Amoco Cadiz oil spill in France and Brittany lasted for decades where the total biodegrading was reported at 60% in 2011, 23 years later. The remaining oil chemicals were estimated to contain 49% asphaltenes resulting from photo-oxidation and microbial biodegrading, 29.5% resins, 34.7% higher molecular weight aromatic compounds, 6.8% saturated hydrocarbons such as cycloalkanes and alkanes (Oudot & Chaillan, 2010). Oils that do not pass through the discussed fate (photochemistry, dissolution, evaporation, and spreading processes) can sink in water including the Group V oils which are oils with a certain gravity of largest than (1.0). They have a negative buoyancy and sink in the water column. They include heavier fuel oils, bunker C oils, coal tar, and asphalt (National Research Council, 1999).

2.2.3 *Instability in The Environment*

Oil pollution impact on the environment vary based on the amount and type of petroleum involved. Environmental instability as a result of petroleum pollution depends on the time of the year, the age of the plant and plant species involved, and the degree of weathering. This contains the physical and chemical properties of the petroleum as well as the amount of water being polluted (Castro-Jiménez, *et al*., 2012; Aguilera, *et al*., 2010). The presence of macrophytes in the environment to be reduced during pollution depending on the concentration of water-soluble components and crude oil (Mendelssohn, et al., 2014).

The water-soluble fraction of petroleum depresses photosynthesis, phytoplankton, growth and respiration also kills or causes the developmental abnormalities of growing plants and animals (Nwaichi, *et al*., 2010). Low amounts of pollution can contribute to a high rate of rapid depression in the photosynthesis rate (Almeda, *et al*., 2014). The adverse environmental instability of petroleum and its products is important on plant and animal growth (Whitehead, 2013). Petroleum polluted environment with water soluble fraction are unstable for the growth and survival of organisms. This results from inhibition of metabolic processes as a result of the inhibition factor from the dissolved water soluble fraction due to increased ionic concentration and salts that negatively impact the stable environmental ecosystem. Continued spillage increases the buildup of ions in this aquatic environment. (Perhar & Arhonditsis, 2014; Mendelssohn, *et al*., 2014; Aguilera, *et al*., 2010).

2.2.4 *Environmental Issues*

The impact of petroleum pollution on the environment has been devastating. Petroleum pollution can take a number of forms resulting in various environmental issues. Pollution of the agricultural soils, the food grown, and the air all contributing to the health challenges and lowering the quality of life (Ogboghodo, *et al*., 2004; Perhar & Arhonditsis, 2014; Gros, *et al*., 2014). Petroleum contaminants are the most evident sources of environmental degradation today where their large concentrations contribute to high toxic emissions to the environment. Toxicity is one of the environmental concerns emerging from crude oil mixture and has been linked to be carcinogenic (Aniefiok, *et al*., 2013; Tobiszewski & Namiesnik, 2012).

Petroleum distillates and crude oil cause birth defects. Other petroleum components such as benzene have been known to cause low white cell count in humans, and this can cause people to be exposed to different diseases and susceptibility to infections (Kirkeleit, *et al*., 2005). Furthermore, benzene, toluene and naphthalene of exposure in the meager parts per billion that range from Hodgkins lymphoma, terminal leukemia, and other immune system and blood diseases (Solomon & Janssen, 2010).

Besides the toxic environmental problems, petroleum pollution has also raised a problem of exhaust emissions after oil distillates have been burned. Often, combustion is an incomplete problem where incompletely burned petroleum compounds emit toxic gases such as carbon dioxide (Tobiszewski & Namiesnik, 2012). Production of toxic fumes like carbon monoxide or even soot can negatively affect humans, animals, and other organisms resulting in respiratory problems, heart diseases and even death. Exhaust fumes have also been reported to be carcinogenic, implying that persons exposed to them are more prone to

the dangers of developing different types of cancer (Aguilera, *et al*., 2010; Whitehead, 2013; Garr, *et al*., 2014).

Petroleum spills also contributes to polluted soil as a result of oil-well accidents, pipeline accidents, leakage from underground tanks, and unregulated industrial waste can permanently contaminate vast areas of land contributing to less economic activities or production of polluted food products (Aguilera, *et al*., 2010; Bonvicini, *et al*., 2014). At times, waste oil used in the gearbox, motor oil, brake fluids, transmission oil, and hydraulic oil can further poison the soil. When washed in the rivers and streams, waste oil contributes to the poisoning of the surrounding ecosystems (Garr et al., 2014).

2.3 Interaction of Hydrocarbons with Microorganisms

2.3.1 Toxicity

Numerous classes of organic compounds from petroleum spills are toxic because they accumulate in cells after uptake resulting in the disruption of the cell membranes (Heipieper & Martı́nez, 2010). Under such circumstances, the toxicity of the dose-dependent compound relates to the partition logarithm coefficient LogP between water and octanol. Elements that have a logP that falls between 1 and 5 are considered to be toxic to the entire cells (Heipieper & Martı́nez, 2010). As such, the toxic impacts of petroleum contamination can result in a number of challenges in the bioremediation of highly polluted environments (Onwurah, *et al*., 2007). The toxic impacts of most petroleum spills are because of nonspecific impacts of the cell membrane fluidity because of their accumulation in the phospholipid bilayer (Zheng, *et al*., 2014). However, the only exception is the hydrocarbons that have precise and chemically active functional groups like epoxides and

aldehydes that indicate an additional organic toxicity (Cabral, *et al*., 2003).

The majority of the compounds that have hydrophobicity levels of LogP higher than four like biphenyls, polycyclic aromatic hydrocarbons, and alkanes have poor water solubility. This means that their bioavailability is very small to indicate the level of toxicity (Heipieper & Martı´nez, 2010). Prolonged exposure of petroleum pollutants in the most polluted environments like aquifers and soils may be as a result of two main physical and chemical properties of hydrocarbons; very high toxicity of numerous hydrocarbon classes and low bioavailability not reachable to microbial degradation. Specifically, the condition is true when considering monoaromatic compounds such as phenols, benzene, toluene, ethylbenzene, and xylenes, terpenoids and n-alkanols that are known to be highly toxic to plants, animals, humans, and microorganisms that are able to degrade them (Schmid, *et al*., 2001). Hence, environments that are polluted with such high levels of toxic compounds are difficult or impossible to be remediated because of the inhibitory impacts of the pollutants on the microbiota (Schmid, *et al*., 2001; Megharaja, *et al*., 2011).

Most microorganisms can tolerate water soluble solvents like acids and lower molecular alcohols. In contrast, it has been shown that highly lipophilic organic solvents and some hydrocarbons are not entirely toxic to cells (Heipieper, *et al*., 1991). Nonetheless, most of the natural solvents that are employed in petrochemical processes trigger a position among lipophilic compounds about water-soluble acids and alcohols. In elaboration, solvents from immediate hydrophobicity such as polycyclic solvents are highly toxic to microorganism cells (Sikkema, *et al*., 1992). Compounds like terpenoids, phenols, or aromatics have been extensively applied as antimicrobial materials (Heipieper, *et al*., 1991; Megharaja, *et al*., 2011). A number of them have been used as detergents, instruments for

permeabilization of cells, narcotic agents, and food preservatives. Notably, it should be noted that the toxic impacts of solvents, in addition to the compound's dose-response relation, have been found to be similar for various microorganisms. Therefore, hydrophobic organic hydrocarbon toxicity is caused by the non-specificity of membrane fluidity as a result of their concentration in the lipid bilayer. nevertheless, not all bacteria demonstrate similar sensitivity levels towards any organic toxins (Heipieper & Martı´nez, 2010; Heipieper, *et al*., 1991).

2.3.2 *Bioavailability of Hydrocarbons by Biosurfactants*

Biodegradation of hydrophobic natural compounds in polluted soil is a process that takes into account interactions between microorganisms, water, pollutants, and soil particles (Bustamante, *et al*., 2012). Surfactants or surface active agents, refer to compounds that can interrupt these interactions. Therefore, the use of surfactants in soil biodegradation can be a suitable approach to overcoming the problem of bioavailability of hydrophobic organic petroleum contaminants (Chrzanowski, *et al*., 2011). Surfactant effects on microorganism physiology can range from stimulation of growth as a result of the utilization of the surfactant as an eco-substrate, to inhibition of growth as a result of surfactant toxicity (Mohanty & Mukherji, 2011).

Importantly, surfactants are significant on the interactions between pollutants and soil in stimulating the mass transport of the pollutant from the polluted soils to the water bodies. The process is affected by three major mechanisms: facilitated transport, micellar solubilization, and liquid pollutant. With respect to the impacts that the three mechanisms have on surfactant bioavailability is the increased hydrophobic presence in different physical states. Biodegradation of hydrophobic organic compounds has shown that the

breakdown of such materials in the soil has been always a rate-limiting step process. The phenomenon is called limited bioavailability although the term is used differently by toxicologists (Wan, 2012).

Understanding of how various processes affect bioavailability depends on different microorganism factors stimulate the pollutant, microorganisms, and soil matrix. (Mulligan, 2005). Interactions rely on four processes; the state and type of a microorganism, physiochemical and state of the soil, external factors such as the presence of oxygen, nutrients, and/or temperatures. Factors determine the mode in which pollutants occur in the soil (Volkering, *et al*., 1998). Dissolution in the soil can occur through adsorption into soil particles, absorption into the soil, dissolved in the pore water, or be available in a separate phase of a solid or liquid. Since the bioavailability relies on mass transfer rates, the bioavailability of a contaminant is primarily governed by how the pollutant is transported into the aqueous bulk phase (Volkering, *et al*., 1998; Bailey, *et al*., 2012).

Some of the possible technological approaches to handle the problem of pollutant bioavailability is the use of these surface-active agents, surfactants, which will help to mobilize the hydrocarbon pollutants in the soil (Mulligan, 2005). In the case of *in situ* bioremediation, other approaches can be used like increasing the soil temperature, which is generally too expensive (Al-Awadhi, *et al*., 1993; Volkering, *et al*., 1998). Other processes such as the use of organic solvents, acoustic techniques, fungi or fungal enzymes, and chemical oxidants are still in the development phase (Muthusamy, *et al*., 2008). In the case of ex-situ remediation, raising the temperature and mechanically reducing the size of the soil aggregates and enhancing the mixing conditions appear to be highly realistic

approaches (Volkering, *et al*., 1998). At a pH of 7, cationic surfactants are highly toxic compounds while anionic surfactants indicate high toxicity at lower pH levels (Lang & F., 1993). Anionic surfactants are also less active against bacteria compared to ionic surfactants (Shekhar, *et al*., 2014).

2.3.3 *Biodegradability*

Biodegradation is one of the approaches of bioremediation that is used to treat water, soils, or sediments polluted with polycyclic aromatic hydrocarbons, polychlorinated biphenyls (PCBs), pesticides, fuels and dyes. In this process, microorganisms are used to detoxify or degrade environmental pollution (Joutey, *et al*., 2013). Biodegradation is a procedure that can be used as a cleanup technique that indicates the potentially to remove organic pollutants with the help of organic biological activity accessible in the substrate. Scholars agree that microorganisms employed in biodegrading polluted soils should be indigenous to the site or area of contamination (Marinescu, *et al*., 2009). Importantly, the bacteria should be in a position to multiply in number when degrading the polluted area and then reduce multiplication when the pollutant has finally been degraded (Mougin, 2002).

The final products of the complete biodegradation of pollutants and mineralization include cell biomass, water and carbon dioxide (Ukiwe, *et al*., 2013). Optimizing the degradation process entails a number of factors which include the presence of microbial conglomerates able to break down the pollutant, the bioavailability of the pollutant to microorganism invasion, and a number of environmental factors such as soil nutrient content, electron acceptor agents, oxygen levels in the soil, soil pH, temperature, and soil type that facilitates the growth of the microorganism (Fritsche & Hofrichter, 2002).

Most scholars are of the school of thought that some bacterial isolates can degrade PAHs. Some important studies include that by Farag & Soliman (2011) who have studied the use of *Candida tropicalis* strain, Mao *et al.* (2012) who have studied the biodegradation of soil using the bacterial co-nsortium that have been augmented from the soil. Other studies have investigated the utilization of laccase enzyme produced of white fungus isolates from rotten *Genoderma lucidum* (Zhai, 2011). It has been shown that *Genoderma lucidum* can completely break down anthracene with or without of the redox mediator addition. nevertheless, other scholars have reported that *Thiobacter subterraneus* (Viana, *et al.*, 2011), *Alcoligenes* species (Yousefi, *et al.*, 2014), and *Escherichia coli* (Ikuma & Gunsch, 2010) are more efficient microbes for degrading phenanthrene and anthracene. Other studies have extensively reported the use of different microorganisms in biodegradation process. The most common includes microorganisms in the genera *Pseudomonas* (Ikuma & Gunsch, 2010; Rajeswari, *et al.*, 2011; Darsa & Thatheyus, 2014), and *Mycobacterium* (Chen, *et al.*, 2011; Olajire, 2014) have been found able of degrading and transforming (PAHs) under aerobic environments. These microorganisms use hydrocarbons, as carbon source for their metabolizing, respiration and fermentation (Johnsen, *et al.*, 2005; Kumari, *et al.*, 2013). Assimilation of these compounds mostly requires microbial extra cellular enzymes to convert, breakdown or detoxify most of them in the microenvironment. Capability of each of these bacteria are variable even from one isolate to anther within the same species. These potentialities determine the level of tolerance of each isolate to hydrocarbons (Johnsen, *et al.*, 2005).

There is a consensus that anthracene can be entirely mineralized using *Rhodococcus*, *Paracoccus*, *Beijerinckia*, *Norcardia*, and *Sphingomonas* with dihydriol as

the precusor oxygenated intermediary (Moody, *et al*., 2001; Mrozik, *et al*., 2003). The ultimate objective of the bioremediation process is not only to remove pollutants from the polluted areas, but also to restore the full function of the soil to regenerate back and perform its potential function. In line with this perception, the polycyclic aromatic hydrocarbon bioavailability becomes a central factor to take into consideration. The capacity to biodegrade PAHs is facilitated by multiple complex and interrelated interactions between abiotic and biotic factors (Simarro, *et al*., 2011; Ukiwe, *et al*., 2013).

Biotic factors which impacts the polycyclic aromatic hydrocarbon availability include the metabolic capability of a microorganism to break down the polycyclic aromatic hydrocarbon in addition to the various approaches through which bacteria improves accessibility to this compound. Microorganisms can encourage the polycyclic aromatic hydrocarbon accessibility through bio-surfactant production as previously discussed (Riser-Roberts, 1998; Ukiwe, *et al*., 2013). The production of the bio-surfactants increases solubility and emulsification of insoluble petroleum hydrocarbon in the polluted soils. Some microorganisms such as *Torulopsis bombicola*, *Pseudomonas aeruginosa* and *Bacillus subtilis* have shown potential to produce bioremediation surfactants like sophorolipid, rhamolipid, and surfactin able to improve the process of bioremediation by making PAHs easily soluble into an aqueous phase, while enhancing the pollutant bioavailability to degrade (Riser-Roberts, 1998).

2.4 **Different Types of Hydrocarbons Bioremediation.**

2.4.1 Phytoremediation

Phytoremediation includes using plants in situ in addition to their correlated microorganisms to detoxify, sequester, and extract hydrocarbon pollutants from polluted soils (Afzal, *et al*., 2014). Phytotransformation also called phytodegradation is a critical phytoremediations process that shows the functions of internal plant processes and mechanisms in eliminating pollutants from substrates. (Kang, 2014) has reported that various leguminous plants and grass are prospective candidates for phytodegradation of hydrocarbon pollutants in soil. A number of tropical plants have also been reported to be highly effective in degrading organic contaminants as a result of their integral features such as high tolerance to low nutrient availability, deep fibrous root system, and nutrient availability. Recently, Kumar (2013) indicated that the switch grass (*Pannicum virgatum*) and long fescue grass (*Festuca arundinacea*) can degrade about 38% of pyrene in 190 days.

Additional researches that evaluated the process degradation of pyrene include that of (Cheema, *et al*., 2010) and (Chouychai, *et al*., 2009) who have documented findings demonstrating that plants were enhanced in pyrene and phenanthrene degradation in acidic soils. The authors agreed that the existence of vegetation like rape seed (*Brassica napus*), alfalfa (*Medicago sativa*), and corn (*Zea mays*) significantly improved the adsorption of PAHs from polluted soil. Rice (Oryza sativa) has also been shown to be reliable in degrading PAHs pollutants from polluted soil (Cheema, *et al*., 2009) .

When the phytodegradation process has been used in secondary treatment, the process has been shown to be effective as a secondary treatment of polluted substrates (Parrish, *et al*., 2004). Nonetheless, in comparison to studies by (Parrish, *et al*., 2004), other

scholars have revealed that phytodegradation may be useful when utilized as a finishing step and as a primary remediation technology for soil treatment with hydrocarbons (Ukiwe, *et al*., 2013) more references. After six months, the reduction of PAHs were evident when they were treated with little blustem grass (*Schizachyrium scoparium*) and *P. virgatum*. In a similar research, (Campbell, *et al*., 2002) reported that using *Cannabis sativa* (industrial hemp) in treating soils polluted with hydrocarbons resulted in low concentration of chrysene and benzo(a)pyrene.

Recent advances in phytodegradation research have reported the application of bermuda grass and rye grass in breaking down alkylated two ring naphthalenes (Khan, *et al*., 2013). Moreover, (Kang, *et al*., 2010) has shown that the distribution of hydrocarbons in the sub-cellular root tissues of *L, multiflorium* increased the adsorption of organic compounds. However, previous studies by (Simonich & Hites, 1994; Sun, *et al*., 2011) were of the opinion that the plant's efficient uptake and hydrocarbon metabolism relied largely on the morphology of the plant being used. (Xia, 2008) has also researched the significance of water hyacinth in degrading hydrocarbons and found that it can accumulate high amounts of five ring PAHs. For successful phytoremediation, both microbes and plants used in the process must grow and survive in crude oil polluted soil (Ukiwe, *et al*., 2013).

2.4.2 *Microbial Degradation*

Complex hydrocarbon breakdown usually requires deployment of numerous biodegradation species. This is particularly true in contaminants that are composed of numerous petroleum compounds and there is a need to perform a complete mineralization to water and carbon dioxide (Joutey, *et al*., 2013). Individual microorganisms are only able

28

to degrade some limited number of hydrocarbons, so mixed populations of a assemblages with entire enzymatic properties is necessary to bring the extent and time of petroleum concentrations low. Microbial species that contain strains of various genera *Mycobacterium* and *Pseudomonas* have been observed and presented as very effective in degrading petroleum pollutants in water and soil (Mrozik, *et al*., 2003). This observation gives a strong indication that each genera and strain has its specific objective in the process of hydrocarbon transformation.

The cooperation between mixed species in degradation has been shown effective by Sorkhoh *et al*. (2013). The researchers noted the sequential breakdown of oil degrading bacteria in sand samples polluted with hydrocarbons. Similar observations have been reported by Venkateswaran and Harayama (2013) where there were a sequential enrichment in medium containing organic pollutants. Other studies that deployed the use of cultures have shown that after exhaustive growth of a single bacterial strain inorganic contaminants, the residual oil continued to support the growth of the second and third bacteria strain. These findings showed that a number of studies recommend the importance of employing numerous microorganisms as the most appropriate degradation practice. Al Disi (2013) have showed the effectiveness of as many as eight strains in effectively degrading crude oil. However, only five strains were able to grow in pure cultures to break down the hydrocarbons. Interestingly, the removal of the other three microorganisms from the consortium resulted in a significantly reduced the effectiveness of the entire mixed culture of the other five microorganisms. This observation shows that it supports the theory that every member of the microbiological ecosystem has a significant role to play. In addition, the organisms depend on the role that each of them plays in the presence of each

species in supporting their survival when there is a limited energy source and restrained complex carbons.

2.4.3 *Physical and Chemical Parameters of Biodegradation*

The evaluation of hydrocarbon degradation in the environment is relatively a burdensome undertaking. A number of factors determine the occurrence of hydrocarbons in aerobic conditions, including redox conditions, pH, and substrate interaction (Chang, *et al*., 2002). Stimulation of hydrocarbon degradation under sulfate reducing conditions has been researched (Bach, *et al*., 2005). Petroleum pollutants in soil can be broken down through biotic mechanisms (Riser-Roberts, 1998). In this regard, oxidation reactions are perceived as the most effective, although some scholars have argued that the photochemical reactions can potentially aid the process of oxidative reactions (Kim & Lee, 2012).

Notably, the majority of the chemical oxidation reactions in the polluted soil is triggered by oxidants like ozone, peroxide, and hydroxyl radical which are generated from the photochemical reactions, which oxidize hydrocarbons like PAHs through addition to double bonds or through abstracting hydrogen atoms. Ozone attacks the double bonds in the hydrocarbons and directly or it could form a reactive hydroxyl radical. in addition to decomposing water (Haag & Yao, 1992; Chu, *et al*., 2010). Numerous intermediates are generated when the reaction proceeds through several pathways. Nonetheless, the final products from this reaction include carboxylic acids, phenols, aldehydes, quinones, and ketones (Lee, *et al*., 2001; Liao, *et al*., 2014). The chemical degradation of hydrocarbons by ultraviolet light often involves similar oxidative species which are produced during the process of pure chemical oxidation of hydrocarbons (Ukiwe, *et al*., 2013).

When (PAHs) regulated by a chemical breakdown, they are converted to form other

poly-aromatic hydrocarbons and neither do they lose their aromatic properties (David & Boule, 1993; Barbas, *et al.*, 1996; Schwarzenbach, *et al.*, 2003). Their aromatic property is conserved in view of the fact that their considerable quantities of energy are needed to make alterations on the aromatic compound to a non-aromatic one. One concept that has been shown to explain this phenomenon is the localization energy concept which determines the reaction molecule position with the hydrocarbons.

Localization energy can isolate the pi-electron at the hydrocarbon center for the remaining pi-electron systems. Either a radical or an electophile can be the attacking species. This chemical attack contributes to the process of degradation where complete hydrocarbon mineralization results in water molecules, carbon dioxide, and other organic and inorganic compounds (Barbas, *et al.*, 1996; David & Boule, 1993). The addition of a chemical oxidant in sufficient amounts results to the metabolic transfer of the hydrocarbons into dead-end products that are resistant to additional degradation (Dong, *et al.*, 2014). Additional studies have shown that hydrocarbon degradation under anaerobic conditions is usually a slow process compared to the degradation under aerobic conditions (McNally, *et al.*, 1999) references.

Fenton's reagent has been reported to have increased effectiveness in breaking down hydrocarbons (Fenton, 1894). Additional chemicals that are employed to degrade hydrocarbons includes a number of electron donors like pyruvate, lactate, and acetate (Bach, *et al.*, 2005). Under both anaerobic and aerobic conditions, hydrocarbon degradation has been found to increase by both chemical and physical pre-treatment of polluted soil using light oils and biosurfactant produced by bacteria (Haritash & Kaushik, 2009). The effects of nitrogen and phosphorous by adding into the polluted soil can degrade

the hydrocarbons (Leys, *et al*., 2005).

Reduction of nitrate has also studied by (Burland & Edwards, 1999). Furthermore, anthracene, fluorene, and acenaphthrene are able to be degraded when exposed to a denitrifying environment. However, use of ferric oxide reduction is limited when exposed to hydrocarbon breakdown. This limitation has been accredited to low ferric oxide solubility and as a result reduced bioavailability (Lovley, *et al*., 1994; Yua, *et al*., 2014).

As noted previously, the hydrocarbon efficiency during chemical degradation is restricted by vapor pressure and low aqueous solubility (Prak & Pritchard, 2002). However, surfactants have been shown to have characteristics that can overcome the bioavaialability issues linked to hydrocarbons' low aqueous solubility (Boonchan, *et al*., 1998). Surfactants can boost the hydrocarbon solubility. As discussed in section 2.2, a number of studies have emphasized on the significance of surfactants in increasing the solubility of hydrocarbons by decreasing the surface tension of the interface among the soil/water and hydrocarbons (Li & Chen, 2009). The surfactant efficiency in promoting the hydrocarbon desorption from the polluted soils relative to water has been studied (Zhou & Zhu, 2007).

It has been shown that the surfactant efficiency promotes the desorption of polluted soils depending on the component factors of soil-surfactant and hydrocarbon system and therefore the efficiency of desorption being strong (Zhou & Zhu, 2007). This depends on the hydrocarbon properties, surfactant structure, and soil composition. Adding non-ionic surfactants to polluted soil positively improves the degradation of hydrocarbons (Zhou & Zhu, 2007; Li & Chen, 2009). Nonetheless, some scholars have indicated negligible or negative effects that adding surfactants have on soil substrates. Potential reasons to explain this negativity is attributed to the substrate competition in the utilization of the surfactant

among the hydrocarbons degrading bacteria in the soil matrix (Liu, *et al.*, 2001). Therefore, the process of hydrocarbon degradation entails the use of surfactants that is concentrated and specific enough in breaking down the pollutant without affecting the presence of the host bacteria (Ukiwe, *et al.*, 2013).

2.5 Bioremediation Processes

2.5.1 Bioaugmentation

Bioaugmentation is a bioremediation process that involves the introduction of microorganisms with biodegradation ability into the polluted environment. The introduced microorganisms aid the indigenous microbes in breaking down hydrocarbon contaminants (Couto, *et al.*, 2010). At times, genetically engineered microbes are also used in the biodegradation process of polluted soils. (Fan, *et al.*, 2014) points out that bioaugmentation has grown to be a low-cost and a promising bioremediation strategy where effective microbial consortium or bacterial isolates capable of degrading xenobiotics are disseminated to polluted sites.

Fan et al., (2014) have used yeast to study how pollutants can be removed from the environment, while Graj *et al.,* (2013) have evaluated if the selected microorganisms are efficient in degrading pollutants. Bagherzadeh-Namazi *et al.,* (2008) Findings indicate that five mixed cultures and three single bacterial strains of *Mycobacterium* species, *Arthrobacter* species, and *Pseudomonas* species were effective in breaking down crude oil by using individual hydrocarbons as their source of carbon. The species selection was done on the basis of their capability to grow in a medium that has petroleum hydrocarbons, used engine oil, or crude oil. Oil samples that had used engine oil were used to identify their

capacity to break down hydrocarbon pollutants (Bagherzadeh-Namazi, *et al*., 2008).

In a mixed culture, the combined species degraded 66% of the engine oil's aliphatic compounds following a 60 days incubation period. Individual culture removed 47% of aromatic hydrocarbons during the 60 days duration (Bagherzadeh-Namazi, *et al*., 2008), while (Hua, *et al*., 2010) have reported that light crude oil fraction, when subjected to the same culture, breaks down about 72.7% of the petroleum compounds. A consortium of *Pseudomonas* species, *Acinetobacter junii*, *Bacillus pumilus*, *Bacillus sphaericus*, and *Bacillus cereus* have been reported to break 75.2% of heavy hydrocarbon diesel in polluted soils (Bento, *et al*., 2005). (Tahhan, *et al*., 2011) augmented a polycyclic aromatic hydrocarbon polluted soil with *Parococcus* species and reported a 23.2% decline in total hydrocarbon concentration in the soil 30 days after the experiment.

Despite the relative success of the bioagumentation process, some scholars have reported that the process is very complicated. Other scholars have reported that the degradation by various microbial consortium affects the exogenous habitat of other microorganisms that tend to be affected by biological, physical, and chemical features present in the polluted soil (Wu, *et al*., 2011). At times, the use of petroleum degrading microbes contributes to the failure of bioaugmentation (Tuo, *et al*., 2012). Other studies have shown that bioaugmentation is a less suitable approach for remediating polluted environments since some bacterial strains may fail to grow and breakdown the pollutants, when compared to indigenous species (Herrero & Stuckey, 2014). These findings show that bioaugmentation is yet to receive full public acceptance due to fears that the seeding of genetically engineered microbes into the soil may alter the environmental ecology. In addition, there are fears that the microbes pose an environmental health risk if they persist

in the seeded sites after the degradation of pollutants is completed (Yan, *et al*., 2014; Herrero & Stuckey, 2014).

2.5.2 *Biostimulation*

Biostimulation is a bioremediation strategy that is often used in stimulating indigenous microbes during the remediation of polluted soil. The process includes the use of nutrients, either inorganic or organic, to promote the activity of the indigenous microorganisms (Agarry & Qion, 2012). The presence of large quantities of pollutant in the soil, including diesel oil, lubricating oil and crude oil often deplete the available inorganic nutrients in the soil such as phosphorous and nitrogen (Sang-Hwan, *et al*., 2007; Ghaly, *et al*., 2013). The levels of phosphorous and nitrogen, when added into polluted soil, stimulates the activity of microorganisms and this contributes to enhanced biodegradation mechanisms (Couto, *et al*., 2010).

Biostimulation is a nutrient enrichment approach similar to the application of fertilizer, which is similar to nitrogen and phosphorous that implemented to plant by adding polluted environment to farms to stimulate the growth of native's microorganisms that be able to degrade pollutants (Thieman & Palladino, 2009). In the presence of the added nutrients, bacteria can grow, and indigenous microbes increase in numbers sufficient enough to initiate the degradation of pollutants (Thieman & Palladino, 2009; Dadrasnia & Agamuthu, 2014). Most microbes require an abundance of the main elements such as phosphorous, oxygen, nitrogen, hydrogen, and carbon for building molecules. Adding fertilizers biostimulates the microbes thrive and reproduces. In other cases, straw, wood chips, and manure may be suitable sources of carbon as a micronutrient for the microorganisms (Thieman & Palladino, 2009).

Biostimulation concept is perceived as an approach where the addition of more nutrients will stimulate an increased number of microorganisms through replication, and this replication increases microbial growth and as a result the rate of bioremediation (Thieman & Palladino, 2009). In some cases, addition of nutrients stimulates biodegradation through autochthonous microbes, while in other cases the resident xenobiotic degrading microbes are added through the use of electron donors, nutrient addition, water, and electron acceptors (Widada, *et al*., 2002). Gong (2012) has reported that the use of combined inorganic nutrients is highly effective compared to the use of single nutrients. Laboratory studies on respiration have shown that balance of micronutrients based on an appropriate C:N:P ratios which are needed to stimulate the biodegradation capacity of indigenous biodegrading microbes . It has been shown that maximum biostimulation is attained when a nutrient solution contains 3% nitrogen, 11% phosphorous, and 75% sulfur (Liebeg & Cutright, 1999; Teng, *et al*., 2010).

Lee *et al*., (2003); Teng *et al.,* (2010) and Ma *et al.,* (2012) Have suggested that adding carbon in the form of pyruvate can stimulate additional microbe growth in addition to enhancing the speed of polycyclic aromatic hydrocarbon degradation. The use of composting bioremediation techniques can also be used to achieve biostimulation (Semple, *et al*., 2001). Bioremediation composting relies on the strategy of mixing the polluted soil contents with the primary composite ingredients (Semple, *et al*., 2001; Couling, *et al*., 2010).

This approach reduces the soil toxicity and increase nutrients, microorganisms, and enzymes for the biodegradation in the polluted sites. Other organic wastes like brewery spent cereal, spent mushroom compost, and banana skin have also been shown to heighten

the biodegradation of lubricating oil by 90% efficiency within 3 months (Lau, *et al*., 2003; Abioye, *et al*., 2009a). In addition, the use of melon shells has been reported to stimulate 75% of oil degradation in the soil in a period of 28 days (Abioye, *et al*., 2009b). However, depending on the nature of the polluted soil, some nutrients have been found to be less effective. This prompts the use of fertilizer nutrients and especially in the pristane environments (Coulon, *et al*., 2012). For instance, addition of commercial oleophilic fertilizer with phosphorous and nitrogen to petroleum polluted environments facilitated the rate of hydrocarbon breakdown by between 77% and 95% in total alkanes, while the polycyclic aromatic hydrocarbons reduced by 80% in six months. Manure from poultry firms has also been used to biostimulate microorganisms and findings indicates that bioremediation is enhanced in their presence, but the extent of these bioremediation relies on the use of surfactants or alternative carbon substrates (Okolo, *et al*., 2005). Nonetheless, the excessive application of nutrients can prevent the degradation activity of the microbes if potassium, phosphorous, and nitrogen levels are very high and mainly on the breakdown of aromatic (Chaillan, *et al*., 2006; Nikolopoulou, *et al*., 2013).

2.5.3 *In Situ Bioremediation.*

In-situ bioremediation is a technology used in enhancing the various mechanisms that degrade the polycyclic aromatic hydrocarbons. In addition, the technology is used in different eminent polluting hydrocarbons in groundwater and polluted soil. Notably, in-situ bioremediation uses engineered technologies to enhance the effects of naturally befalling biodegradation mechanisms (EPA, 2013). The process of in situ bioremediation includes the provision of suitable physiological conditions and nutrients for the growth of indigenous microbial species (EPA, 2013; Hu & Chan, 2015).

During the bioremediation of contaminants and soil pollutants, metabolic activity increases. In situ bioremediation entails a number of intrinsic engineered and bioremediation in-situ bioremediation (Bhatt, 2014). To degrade contaminants from the polluted sites, the process of bioremediation depends on diverse intrinsic factors. By definition, intrinsic bioremediation is the breakdown of the pollutants without amendment or alteration, with the aim of achieving in situ bioremediation (Lyon, *et al*., 2010). In the polluted sites where there is a monitored natural attenuation, intrinsic bioremediation is important as it plays a central role that helps to broaden the terms defined by the US National Research Council and the Environmental Protection Agency as destruction, transformation, biological or chemical stabilization, dispersion or biodegradation if contaminants (Hu & Chan, 2015).

2.5.4 *Biopile System Technology*

This technology is used for reducing petroleum contamination in soil, usually established through introducing bioremediation activities using both biostimulation and bioaugmentation. The contaminated soil is placed inside the piles followed by the addition of nutrients (nitrogen and phosphorus), biosurfactants, microorganisms, moisture (water), and introducing an aeration system. The petroleum decomposition happens through microbial respiration activity. This system occurs above ground. The aeration system is placed through the pile by pipes injected to provide oxygen into the soil (Jorgensen K.S, 2000). To evaluate biopile effectiveness, first identification of soil characteristics is important including microorganism's density, pH, temperature, soil texture, moisture content, and nutrient constituents. Followed to that the identification of contamination characteristics including percent volatility, concentration, toxicity and chemical structure

(Jorgensen K.S, 2000). Climate condition on site such as temperature, wind, and rainfall should be also taken into consideration. The biopile system is an engineered system depending on the previous identifications. Establishing a biopile design becomes possible by controlling air emission, aeration equipment, providing construction requirements, water management for water addition, pH adjustment, nutrient supply, land requirements, and site security. In addition, leachate collection and treatment, and soil cover are optional options in the treatment process. Biopile operation and monitoring plans are necessary for controlling the additions needed for the operational procedure success. These conditions can be also established on laboratory scale where same requirements are established but on a smaller scale (Jorgensen K.S, 2000). It is an environmental friendly technology, cost beneficial and sustain the ecosystem for future generation. This technology, if successfully applied, can provide a healthy environment for all living organisms.

2.6 **Microbial Biodiversity of Hydrocarbon-Degrading Bacteria**

Enrichment cultures strategies, microbial ecology and applied microbiology are the basis of the development of the described strategies of bioremediation. Molecular and genetic advanced techniques are implemented to identify but also to predict the biological activities of the microbial communities in the polluted soils. Additional information through the application of new metagenomic technologies will enable molecular ecology studies of natural microbial communities. Indeed, the use of analytical, microbiological and advanced molecular tools allow to evaluate different treatment options, focusing on the in-situ bioremediation.

Studies intend to investigate the microbial ecology of polluted sites, focusing on

those sites that have shown significant recalcitrance to remediation. Using enrichment culture techniques, they intend to isolate, identify and characterize hydrocarbon-degrading bacterial strains, including those that produce biosurfactants, and investigate their potentialities to degrade petroleum hydrocarbons under environmental conditions. More emphasis is generally on the more recalcitrant compounds present in soils as this contributes in overcoming large literature gaps in microbial community characterization and remedial design.

Comparative metagenomic studies with oil- polluted soils reveals common features of the microbial communities. The physical and chemical characteristics of polluted soils in the Gulf region are unique, with extremes in temperature, salinity, and water content influencing microbial communities and functions. In addition, Qatari polluted areas are unique because it are under high temperature conditions, the organic matter is transformed to complex hydrocarbon products, such as aromatic and aliphatic hydrocarbons (Survery, *et al*., 2004). Polluted soils in Qatar are ideal for comparative metagenomic analysis with the published metagenomes from other regions of the world. The metabolic ability, in particular for hydrocarbon degradation, of the microbial community has not been investigated in detail. Comprehensive studies on biodegradation, in particular under aerobic and micro-aerobic conditions, are required to characterize the metabolic potential and ecology of microbial ecosystems having the potential of hydrocarbon biodegradation.

The metagenome of oil-polluted soils must be analyzed to investigate the metabolic potential and functions of the inhabited microbial population. Key data provided by the metagenomic analysis include the presence and identification of genes responsible for hydrocarbon degradation; mapping of these genes as a function of the soil contamination

and local environmental conditions for the different sampling sites; and mapping of genes against each other for the different sites, allowing examination of coupled processes.

2.7 **Chemical Assessment and Analyses**

2.7.1 *Gas Chromatography (GC)*

Gas chromatography–mass spectrometry (GC-MS) technique gives critical information on both the group type distribution and conversion yields of aromatic and saturates. Besides, there is a need to have a previous knowledge of the samples so as to avoid potential errors and interference.

Both GC-MS and gas chromatography-flame ionization detection (GC-FID) methods have become a common tool in the quantification and identification of petroleum pollution is soils. Previous sample treatment has often been costly as additional steps such as purge and trap or solid phase microextraction, is the most costly and time consuming phase of the procedure and as well as being the frequent source of miscalculations. In the analytical laboratory, the throughput can be significantly increased if a non-separative method is developed to resolve different analytical problems. The outcome of this approach is the reduction is the operating costs per each of the samples produced (Vas & Vekey, 2004).

GC has been coupled with mass spectrometry and served as the main technology in identifying individual petroleum components, and mostly in gasoline range products. A list of around 400 peaks identified peaks in whole gasoline. Today, the common retention indices have been utilized in recognizing components that have been separated through the capillary column GC-FID. For light crude oil samples, when identifying light petroleum

products, the most preferred approach is the use of Kovat's retention index of n-alkanes (Wang, *et al*., 1999; Barman, *et al*., 2000). A coupled of GC-MS and retention indices have been used in the identification of petroleum products in the complex polluted hydrocarbon mixture. GC and retention indices middle oil fraction components have also been identified through this process where the retention indices of products such as picene, chrysene, phenanthrene, and naphthalene have been identified (Barman, *et al*., 2000).

2.7.2 *Identification of Petroleum Hydrocarbons in Soil*

A number of analytical techniques and methods are currently used to characterize soil polluted with petroleum and other related products, which include GC, GC–MS, high performance liquid chromatography HPLC, Size-exclusion chromatography, Fourier infrared spectroscopy (FTIR), thin layer chromatography (TLC), ultraviolet (UV), fluorescence spectroscopy and supercritical fluid chromatography (SFC). The common approach has been the hydrocarbon type analysis through GC (Beškoski, *et al*., 2012). With hydrocarbon type analysis, it is possible to determine the oil or fuel quality, determine variables in its processes of conversion, and examine reaction kinetics and pathways in addition to getting more information about the ability to process petroleum products. Identification of petroleum products in the soil is commonly done through two techniques GC–MS and GC–FID (Wang, *et al*., 1999; Dugo, *et al*., 2012).

GC has been widely used in the quantitative analysis of both semi-volatile and volatile compounds found in various matrices (such as liquids, solids and gases) in environmental samples and polluted soils. Flame-ionization detectors (FID) are useful in determining the specific compound concentration (Gasson, *et al*., 2013). Today, GC-FID has become one of the most popular, powerful, unique and versatile analytical technique

applied in the identification, separation, and quantitative assays of compounds in vapor state (Dijkmans, *et al.*, 2015). GC-FID popularity is largely centered on its high sensitivity, selectivity, and resolution in addition to having good precision and accuracy in a wide dynamic range of concentration (Gasson, *et al.*, 2013).

The determination of GC is based on the sum of all components or selected components within a specified range. Often, the approach utilizes two methods; one for the semi-volatile range and the other for a volatile range. Volatile compounds in solid or liquid samples are determined by the use of purge-and-trap GC-FID. This analysis is widely referred to as gasoline range organics method (GRO). Determination of semi-volatile range is evaluated using a GC-FID extract and is called diesel range organics (DRO). Different scientists have employed diverse approaches in the DRO and GRO analysis of the petroleum polluted sites and polluted soils and water. The precise requirements and method details vary from one region to another (Saari, *et al.*, 2010).

Recently, a comprehensive 2-dimensional GC has also been used to assess oil polluted soils. In most studies, GC has been fitted with FID where the choice of GC-FID over photo ionization detector (PID) has been as a result of a number of factors. One, FID is able to cope with very wet samples and soils with high humidity compared to PID. Two, the use of FID is suitable because the flame is able to ionize diverse range of volatile petroleum hydrocarbons and other organic compounds than PID, and as a result it is able to detect a wide hydrocarbon range. Third, FID is largely useful in detecting low concentrations of volatile organic compounds as a result of its low detection limits (Kelly-Hooper, *et al.*, 2013). Despite the advantages of FID, it has a number of limitations which include the ability to denature the sample being analyzed, it can detect volatile

hydrocarbons from organic materials such as peat and methane and other non-petroleum matter, thereby giving false positive results (Dugo, *et al*., 2012).

Hydrocarbon compounds biomarkers can be analysis and used widely to identify petroleum components can be saturates asphaltenes, resins, aromatic, saturates, and isoparaffins, or according to the number of aromatic rings. into polycyclic, tricyclic, dicyclic, and monocyclic aromatics (Wang, *et al*., 1999; Barman, *et al*., 2000). To be a reliable chromatography method for identifying individual petroleum products, the method has to meet various requirements to be chemically characterized for crude oil source detection as the following:

(1)   Saturated hydrocarbons as indiviuals n-alkanes (C8 – C40)

(2)   The volatile BTEX (toluene, benzene, three xylene isomers and ethylbenzene) and alkylated benzenes (C 3 –C 5- benzenes)

(3)   The EPA priority parent (PAHs) and the crude oil specific alkylated (C1–C4) homologues of selected (PAHs) (fluorene, phenanthrene, dibenzothiophene naphthalene, chrysene series, naphthalene, and alkylated). These alkylated (PAHs) homologues (Table 2-1) are the foundation of identification and chemical characterization of crude oil spill evaluations.

(4)   Biomarker sterane and terpane compounds (

Table 2-2). Analysis of selected ion peaks produced by these characteristic, environmentally persistent compounds produces great importance information of in determining source (Wang, *et al*., 1999).

Table 2-1 shows source specific target alkylated homologous (PAHs) and (PAHs) for crude oil spill studies. It should be applicable, quantitative, quality control, rapid, reproducible and rugged without necessarily undergoing pre-fractionation. However, in samples that cover a broad range of boiling points and heavy materials and diverse polarity, the current chromatographic methods in use often display large limitations of accuracy and detection. Most of the problems originate from inadequate power resolution, low component solubility, low volatility, poor detector sensitivity, strong sample adsorption at stationary phase, or low component solubility in the mobile chromatography phase. In complex samples, the task is always more challenging as some products contain hundreds of individual components and functional groups (Barman, *et al*., 2000; Beškoski, *et al*., 2012).

Table 2-1: Source specific target alkylated homologous (PAHs) and (PAHs) for crude (Wang, et al., 1999)

Compound Oil-characteristic alkylated PAHs	Code	Ring No.	Target ions
Naphthalenes C -naphthalene$_0$	C N$_0$	2	128
C -naphthalenes$_1$	C N$_1$	2	142
C -naphthalenes$_2$	C N$_2$	2	156
C -naphthalenes$_3$	C N$_3$	2	170
C -naphthalenes$_4$	C N$_4$	2	184
Phenanthrenes C -phenanthrene$_0$	C P$_0$	3	178
C -phenanthrenes$_1$	C P$_1$	3	192
C -phenanthrenes$_2$	C P$_2$	3	206
C -phenanthrenes$_3$	C P$_3$	3	220
C -phenanthrenes$_4$	C P$_4$	3	234
Dibenzothiophenes C -dibenzothiophene$_0$	C D$_0$	3	184
C -dibenzothiophenes$_1$	C D$_1$	3	198
C -dibenzothiophenes$_2$	C D$_2$	3	212
C -dibenzothiophenes$_3$	C D$_3$	3	226
Fluorenes C -fluorene$_0$	C F$_0$	3	166
C -fluorenes$_1$	C F$_1$	3	180
C -fluorens$_2$	C F$_2$	3	194
C -fluorenes$_3$	C F$_3$	3	208
Chrysenes C -chrysene$_0$	C C$_0$	4	228
C -chrysenes$_1$	C C$_1$	4	242
C -chrysenes$_2$	C C$_2$	4	256
C -chrysenes$_3$	C C$_3$	4	270
Other EPA priority PAH po	Llutants	2	154

Biphenyl	Bph		
Acenaphthylene	Acl	3	152
Acenaphthene	Ace	3	153
Anthracene	An	3	178
Fluoranthene	Fl	4	202
Pyrene	Py	4	202
Benz[*a*]anthracene	BaA	4	228
Benzo[*b*]fluoranthene	BbF	5	252
Benzo[*k*]fluoranthene	BkF	5	252
Benzo[*e*]pyrene	BeP	5	252
Benzo[*a*]pyrene	BaP	5	252
Perylene	Pe	5	252
Indeno[1,2,3-*cd*]pyrene	IP	6	276
Dibenz[*a,h*]anthracene	DA	5	278
Benzo[*ghi*]perylene	BP	6	276
Surrogates and Internal Standard			164
[^{2}H$_{10}$]Acenaphthene			
[^{2}H$_{10}$]Phenanthrene			188
[^{2}H$_{12}$]Benz[*a*]anthracene			240
[^{2}H$_{12}$]Perylene			264
[^{2}H$_{14}$]Terphenyl			244

Table 2-2: Source-specific analyzed biomarker terpane and sterane compounds for crude oil (Wang, et al., 1999)

Peak	Compound		Empirical formula	Molecular mass
Terpenes **1**	C19	tricyclic terpane	C19H34	262
2	C20	tricyclic terpane	C20H36	276
3	C21	tricyclic terpane	C21H38	290
4	C22	tricyclic terpane	C22H40	304
5	C23	tricyclic terpane	C23H42	318
6	C24	tricyclic terpane	C24H44	332
7	C25	tricyclic terpane	C25H46	346
8	C24	tetracyclic terpane1,C26 tricyclic terpanes	C24H421,C26H48	
9	C28	tricyclic terpane	C28H52	388
10	C28	tricyclic terpane	C28H52	388
11	C29	tricyclic terpane	C29H54	402
12	C29	tricyclic terpane	C29H54	402
13	Ts: 18a(H),21b(H)-22,29,30-trisnorhopane		C27H46	370
14	17a(H),18a(H),21b(H)-25,28,30-trisnorhopane		C27H46	370
15	Tm: 17a(H),21b(H)-22,29,30-trisnorhopane		C27H46	370
16	17a(H),18a(H),21b(H)-28,30-bisnorhopane		C28H48	384
17	17a(H),21b(H)-30-norhopane		C29H50	398
18	18a(H),21b(H)-30-norneohopane		C29H50	398
19	17a(H),21b(H)-hopane		C30H52	412
20	17b(H),21a(H)-hopane		C30H52	412
21	22*S*-17a(H),21b(H)-30-homohopane		C31H54	426
22	22*R*-17a(H),21b(H)-30-homohopane		C31H54	426
23	17b(H),21b(H)-hopane		(Internal standard)	
24	22*S*-17a(H),21b(H)-30,31-bishomohopane		C32H56	440
25	22*R*-17a(H),21b(H)-30,31-bishomohopane		C32H56	440
26	22*S*-17a(H),21b(H)-30,31,32-trishomohopane		C33H58	454
27	22*R*-17a(H),21b(H)-30,31,32-trishomohopane		C33H58	454
28	22*S*-17a(H),21b(H)-30,31,32,33-tetrakishomohopane		C34H60	468
29	22*R*-17a(H),21b(H)-30,31,32,33-tetrakishomohopane		C34H60	468
30	22*S*-17a(H),21b(H)-30,31,32,33,34-pentakishomohopane		C35H62	482
31	22*R*-17a(H),21b(H)-30,31,32,33,34-pentakishomohopane		C35H62	482
Steranes **32**	C_{20}5a(H),14a(H),17a(H)-sterane		C20H34	274
33	C_{21}5a(H),14b(H),17b(H)-sterane		C21H36	288
34	C_{22}5a(H),14b(H),17b(H)-sterane		C22H38	302

35	$C_{27}20S$-13b(H),17a(H)-diasterane	C27H48	372
36	$C_{27}20R$-13b(H),17a(H)-diasterane	C27H48	372
37	$C_{27}20S$-13a(H),17b(H)-diasterane	C27H48	372
38	$C_{27}20R$-13a(H),17b(H)-diasterane	C27H48	372
39	$C_{28}20S$-13b(H),17a(H)-diasterane	C28H50	386
40	$C_{29}20S$-13b(H),17a(H)-diasterane	C29H52	400
41	$C_{29}20R$-13a(H),17b(H)-diasterane	C29H52	400
42	$C_{27}20S$-5a(H),14a(H),17a(H)-cholestane	C27H48	372
43	$C_{27}20R$-5a(H),14b(H),17b(H)-cholestane	C27H48	372
44	$C_{27}20S$-5a(H),14b(H),17b(H)-cholestane	C27H48	372
45	$C_{27}20R$-5a(H),14a(H),17a(H)-cholestane	C27H48	372
46	$C_{28}20S$-5a(H),14a(H),17a(H)-ergostane	C28H50	386
47	$C_{28}20R$-5a(H),14b(H),17b(H)-ergostane	C28H50	386
48	$C_{28}20S$-5a(H),14b(H),17b(H)-ergostane	C28H50	386
49	$C_{28}20R$-5a(H),14a(H),17a(H)-ergostane	C28H50	386
50	$C_{29}20S$-5a(H),14a(H),17a(H)-stigmastane	C29H52	400
51	$C_{29}20R$-5a(H),14b(H),17b(H)-stigmastane	C29H52	400
52	$C_{29}20S$-5a(H),14b(H),17b(H)-stigmastane	C29H52	400
53	$C_{29}20R$-5a(H),14a(H),17a(H)-stigmastane	C29H52	400

2.7.3 *Chemometric Analysis and Bioremediation*

Chemometrics is the science of extracting data from chemical systems by using an application of mathematical and statistical methods in order to address problems in chemistry, chemical engineering, medicine, biochemistry and biology (Al-Ghouti, *et al*., 2010). The main objectives of multivariate chemometric methods in analytical chemistry include data grouping, reduction and the classification of large data and the modeling of relationships between variables (Christensen & Tomasi, 2007).

Chemical fingerprinting is a collection of tools that trace the origin of a sample (e.g. pollutant mixture) based on its chemical composition. Oil spill fingerprinting, by matching of oil spilled to numerous candidate sources, is globally implemented to justifiably

determine oil source(s) (Christensen & Tomasi, 2007), to discriminate the contributions of background hydrocarbons (Christensen *et al*., 2010), and to observer the changes in spilled oil composition over time (weathering processes) (Christensen, *et al*., 2005 a; Barron & Holder, 2003). GC establishes a keystone of recent oil spill fingerprinting as mineral oil contains a complex matrix of individual compounds that can be separated and identified by this technique (Wang, *et al*., 2006). Specifically, GC-FID is the standard protocol for early screening of oil samples, and the GC combined with a mass spectrometric detector (GC–MS) is for detail fingerprints (Christensen & Tomasi, 2007).

Distributions of chromatograms of n-alkane by visual comparison, PAHs concentrations and univariate correlation analysis of source and spill samples are the standard data analysis in fingerprinting of oil hydrocarbon (see Christensen and Tomasi 2007 for more details). These methods establish the foundation of tiered methods for fingerprinting of oil hydrocarbon. However, complete quantitative analyses is costly and inefficient and consequently can deter the finding of innovative and more informative patterns in the data. Additionally, crude oil hydrocarbon with identical chemical composition may only have small variations that become indistinguishable even to the expert analyst and cannot be detected using normal statistical tests. Hence, these issues would be addressed using chemometry and multivariate statistical procedures which lead to easy characterization and more comprehensive of large data sets. Principal component analysis (PCA), as a one of the multivariate analytical tool, has broadly been used to categorize oil samples for oil–oil correlation and oil-source (Øygard, *et al*., 1984; Urdal, *et al*., 1986), and to provide information on the relations among the sampling sites, (PAHs) pollutant concentrations, diagnostic ratios of aliphatic and aromatic hydrocarbons, factors,

and patterns accountable in the environmental cases for the data-set structure (Aboul-Kassim & Simoneit, 1995).   In addition, various statistical methods such as linear discriminant and univariate methods analysis have been applied and the achieved results were compared with the ones obtained by PCA (Christensen & Tomasi, 2007; Al-Ghouti, *et al*., 2010; Yahya S. Al-Degs, *et al*., 2012).partial least squares regression (PLS), and Parallel factor analysis (PARAFAC) can also be used. However, applying (PCA) on sections of chromatograms to avoid peak identification and quantification are actually more efficient for data processing and for removing the variation unrelated of the largest part to the chemical composition (Al-Degsa, *et al*., 2009). Accordingly, Christensen *et al*. (2005b) demonstrated a PCA method on processed sections of GC–MS and GC-selected ion monitoring (SIM) chromatograms of petroleum biomarkers ('pixel-based approach'). Four meaningful PCs were identified for the analyzed refined petroleum and crude oils products: clay content, boiling point range, thermal maturity, and carbon number distribution of sterols in the source rock. (Christensen, *et al*., 2005 b; Gallotta & Christensen, 2012; Malmquist, *et al*., 2007; Soleimani, *et al*., 2013).

Although, state-of-the-art analytical and chemometric methods for fingerprinting of crude oil hydrocarbon have provided important information about the composition of oil there is a great need for development of next generation chemometric tools that can handle the extreme complexity of mineral oil with up to 1 million individual isomers of aliphatic, aromatic and more polar and larger resins and asphaltenes. One direction in research would be to develop integrated online sampling and sample preparation methods such as Solid Phase Extraction (SPE), dispersive liquid-liquid micro-extraction (DLLME) and pressurized liquid extraction (PLE) and combine these methods with cutting edge

analytical instrumentation and advanced signal processing and chemometric data analysis.

The cutting-edge analytical instrumentation for online screening purposes is Fourier Transform Infrared (FTIR) Spectroscopy in combination with multivariate regression techniques such as partial least squares (PLS) regression, and NPLS the latter that exploit the multiple data dimensions in excitation-emission landscapes. The use of comprehensive analytical techniques combined with chemometric data analysis has the potential for strongly improved source identification; study of weathering and biodegradation; and for apportionment of multiple oil sources.

Chemometric entails the application of statistical and mathematical methods to improve the comprehension of chemical information and to correlate the physical properties of quality parameters to analytical data instruments (Christensen & Tomasi, 2007; Mas, *et al*., 2012). The data patterns are modeled and the resultant models are then applied routinely in future data with an aim of predicting similar quality parameters (Christensen & Tomasi, 2007; Mas, *et al*., 2012). The results of chemometric approach is to gain efficiency in assessing the quality of a sample being analyzed. This analysis can be highly efficient in automated quality control and laboratory practice systems. The key requirement is suitable software and instrument to interpret the data patterns observed from the analyzed soil sample (Al-Ghouti, *et al*., 2010; Garcia-Perez, *et al*., 2010).

The chemometric science gives spectoscopists diverse and efficient approaches that are used to solve the problem of calibrating spectral data. The use of chemometrics can also be important in the routine and development of use of statistical model for analysis data. Analysts use different software packages such as "the Unscrambler" in chemometric data modeling, prediction, classification and analysis to meet the quality evaluation needs

and process monitoring (Christensen & Tomasi, 2007). Therefore, chemometrics is focused at researching how to extract chemically relevant data out of the measured pollutant sites and the hydrocarbons being analyzed (Mas, *et al*., 2012).

Studies have shown that the model concept gives a more realistic framework when it comes to chemical data evaluation compared to the often misleading exact looking associations obtained from the first principles. Notably, multivariate empirical modeling by principal component analysis (PCA), have often indicated unexpected patterns as a result of the joint effect of the entire variables taken into consideration, which give the most detailed description of biodegradation (Christensen, *et al*., 2005 a; Christensen & Tomasi, 2007).

Chemometric analysis can assess the quality of oil polluted soil after biodegradation processes such as biostimulation and bioaugmentation. (Soleimani, *et al*., 2013) has conducted a study using chemometric analysis for determining the type and level of hydrocarbon contamination soil. They also applied some methods to oil weathering of two to six ring (PAHs). They found that bioaugmentation and biostimulation were most efficient with (50%) to (62%) removal (TPH) and results confirmed that the bioaugmentation was more efficient in degradation of *n*-alkanes and low molecular weight (PAHs) in addition to alkylated (PAHs), for instance (C2) phenanthrenes, (C3–C4) naphthalenes and (C2–C3) dibenzothiophenes, while biostimulation cause a larger relative removal of isoprenoids (for instance pristane, norpristane, phytane). method is a helpful tool for evaluating bioremediation efficiency.

Chapter 3 :MATERIALS AND METHODS

3.1 Sampling

3.1.1 Selection of Sampling Sites

Three sites were selected to investigate the pollution issue, and the potential of microbial bioremediation approaches to alleviate the environmental issue, they included;

1. Alzubara, located northwestern coast of the Qatar peninsula. The soil contamination in this area is likely to be as a result of the oil spills during the 1991 Gulf War and from other potential contamination sources.

2. Dukhan, located west of the Qatar peninsula. The soil contamination is like a dumping area for the oil spill occurring on the Dukhan oil operation that transferred to this area, and the age of oil spill varied between aged and fresh oil spills.

3. A third set of samples were collected from three barrels of AlShaheen oil wastes and 2 others from local auto-shops were considered in the study

3.1.2 Sampling for Hydrocarbon Degrading Bacteria Isolation

From both Alzubara and Dukhan, sampling was carried out in a systematic way, 9 sampling points of a square area with 20 meters' distance between each 2 points (at a depth of 10 cm and 20 cm), from, soil samples were collected from surface and in depth bringing the total samples to 36. Soil samples from the sites selected was collected using a sterile spatula and stored in sterilized glass bottles. These bottles were sealed, labeled and wrapped with foil to protect them from the light to prevent any further reactions. The temperature of the samples of soil collected ranged from 25 to 26°C.

3.1.3 *Sampling for Identification of Polluting Oil Sources*

In Alzubara, two main areas were identified where sampling for hydrocarbon degrading bacteria was carried out. From the two areas, sediments including surface and subsurface sediments such as fresh and heavily weathered oil were collected. From the sample collected, 10 oil/sediment were isolated and analyzed (Samples were collected March 29th, 2014). 5 of the samples analyzed contained oil and thus used in the study (These samples were labelled X-01, X-02, X-03, X-04, X-05). 38 samples were collected using a shovel. Below are the maps of the sampling sites. Are shown in Figures Figure 3-1 and Figure 3-2.

Figure 3-1: AlZubara sampling area
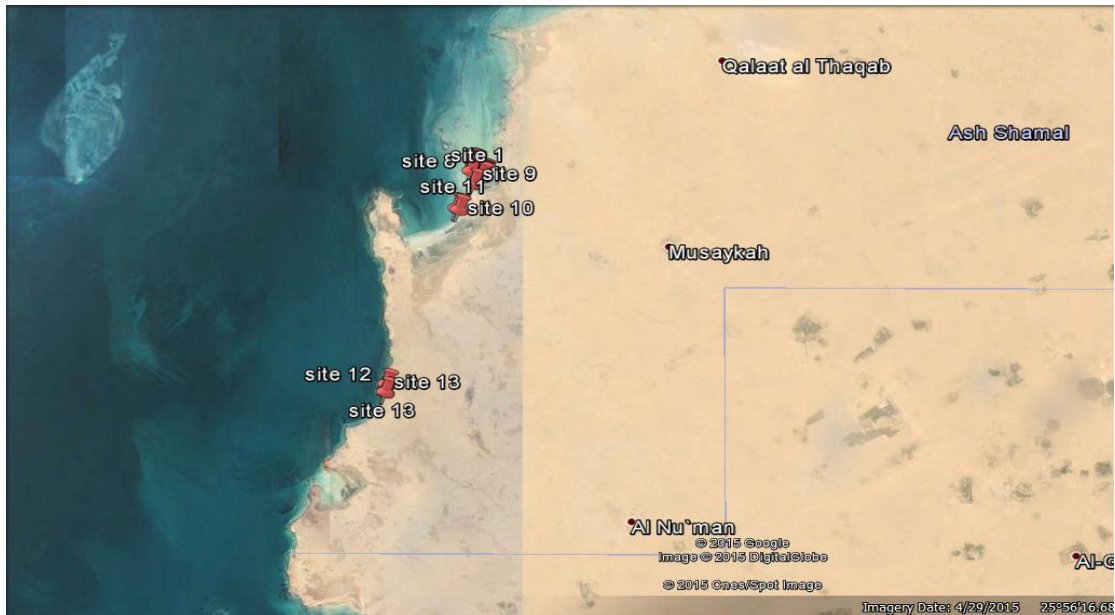
Figure 3-2: Alzubara sampling sites

- Area 1 consisted of 11 sampling sites (site 1-11) with a total of 26 collected samples of fresh and heavily weathered stranded oil as well as surface and subsurface sediments.

- Area 2 consisted of 2 sampling sites (site 12-13) with a total of 12 collected samples of fresh and heavily weathered crude oil.

3.1.4 *Soil Sampling for Bioremediation in Ex-situ Biopiles*

Three soil samples were collected from oil production and processing sites Qatar. The polluted soils are from a localized narrow spot with a long residence time under extreme climatic conditions. The existing indigenous bacteria were considered as highly adapted. The sampling protocol was described previously (Al-Kaabi, et al., 2017).

3.2 **Physical and Chemical Characterization of The Soil Samples**

3.2.1 Physical and Chemical Extraction

50ml of each soil sample was mixed with 50ml of distilled water in a 250 ml stopper conical flask and shaken for 30 minutes using a mechanical shaker. The samples were then let to settle for 30 minutes for equilibration after which filtration was carried out using Buchner funnels and Whatman filter papers no. 42. After shaking, the equilibration was taken place by leaving the samples for 30 minutes. On the other hand, turbid filtrates, were centrifuged for 5 mins using 3000 cycle/min centrifuge (Wilke, 2005).

3.2.2 Analysis of Major Elements

ICs 5000 ion chromatographic system (Dionex, Sunnyvale, CA, USA) was used to carry out Ion analyses which included anions (sulfate and chloride) and cations (sodium, potassium, calcium and magnesium). The ion chromatography system has a gradient, isocratic pump module, high-pressure injection valve with a 250 µl sample loop, a Dionex conductivity detector (CD), and automated sampler. Each of the ions use a specific gradient for computation. For example, anions line is: Dionex Ionpac AS4A-SC analytical column (250×4 mm I.D.) and AG4A- SC guard column (50×4 mm I.D.) together with an Anion Self-Regenerating Suppressor-1. The eluent was 1.8 mM Na_2CO_3 + 0.8 mM NaHCO3 with a flow rate of 0.25 ml/min. On the other hand, for the cations Dionex Ionpac CS12, analytical column (250 x 4 mm I.D.) and CG12 guard column (50 x 4 mm I.D.) with a Self Regenerating Suppressor for cations. The eluent was 0.020 M methanesulfonic acid and 0.25 ml/min eluent flow rate.

3.2.2.1 Analysis of Nutrient

Nutrient include NO_2, NO_3, and PO_4, has been performed using the following standard/non-standard methods:

- **Nitrite (NO_2)**

    Nitrite is usually determined using a method proposed by Strickland and Parson (1972), by diatozotizing it using sulfanilamide and coupling it with N-(1-Naphytyl)-ethylenediamine dihydrochloride to azo dye. The color of the dye is then easily measured spectrophotometrically at wave length 543 nm.

- **Nitrate (NO_3)**

    Nitrate determination is based on the ability of Nitrate to be reduced to nitrite by a cadmium metal in the form of Cadmium-copper column. The reaction of Nitrite with sulfanilamide in an acidic medium results to the formation of a diazonium salt. Which is then coupled with N-(1-Naphytyl)-ethylenediamine dihydrochloride to azo dye. The color of the dye is then easily measured spectrophotometrically at wave length 543 nm.

    $$2H^+ + NO_3^- \Leftrightarrow Cd^{2+} + NO_2^- + H_2 O$$

- **phosphate (PO_4)**

    The absorbance of reactive phosphorus is spectrophotometrically measured at 885nm wavelength after seawater is reacted with a composite reagent to form a complex that forms a blue solution when reduced in situ. The composite reagent utilized in this case is a mixture of ammonium molybdate, ascorbate acid and potassium antimony-tartrate.

3.3 Chemical Analysis (GC-MS) for Oil Source Identification of Beached Oily Polluted Soils at Al Zubarah

3.3.1 Chemicals Used for Source Identification Analysis

Different chemicals were used for the identification analysis process. Among the chemical that were used include, Dichloromethane and Anhydrous sodium sulphate. Anhydrous sodium sulphate was heated at 400 °C for 4 hours and cooled down in a desiccator. Moreover, GC–MS tune mixture was also used to assess the performance of the instrument used. GC–MS tune mixture is comprised of various chemicals which include benzidine (99.9%, Sigma), 4,4-DDT (99.7%, Sigma), pentachlorophenol (99.9%, Sigma, Supelco) and (decafluorotriphenyl-phosphine, dftpp) (99.3%, Sigma, Supelco) (50 µg/mL of each).

3.3.2 GC-MS for Source Identification

Chemical analysis was carried out by first dissolving the oil samples in dichloromethane until a concentration of 2500 µg/mL was obtained. Agilent 7890A/5975C GC–MS equipped with a 60 m ZB-5 capillary column was used to analyze the data. In this case, Helium with a flow rate of 1.1 mL/min was used as a GC for carrier purposes. Following which 1 µL of Helium was injected in a pulsated and split less mode at 315 °C. For effective results the column temperature of the program was 40 °C (2 min), 25 °C/min to 100 °C then an increase of 5 °C/min to 315 °C (13.4 min). 55 mass-to charge ratios was acquired using SIM mode is as shown in Table 3-1. (Gallotta & Christensen, 2012).

Table 3-1: List of compounds, mass-over-charge (m/z) and corresponding groups of GC/MS-SIM

Compounds	m/z	Group(s)	Compounds	m/z	Group(s)
n-alkyl cyclo hexanes	83	I to XII	C4-decalins C2-fluorenes	194	I + II + III + VI + VII
Alkanes	85	I to XII	C2-dibenzofurans	196	IV + V + VI + VII
alkyl toluenes	105	I to XII	C1-dibenzothiophenes	198	VI + VII
Sesquiterpanes	123	I to VI	C0-fluoranthene C0-pyrene	202	VII + VIII + IX
naphthalene	128	I	C2-phenanthrenes/anthracenes	206	VII + VIII + IX
benzo(b)thiophene	134	I	C3-fluorenes	208	VI + VII
d8-naphthalene	136	I	C2-dibenzothiophenes d10-fluoranthene d10-pyrene	212	VII + VIII
C0-decalin	138	I	C1-fluoranthenes/pyrenes	216	VIII + IX
C1-naphthalenes	142	II	Steranes	217	VIII to XII
C1-benzo(b)thiophenes	148	I + II	Steranes	218	X + XI + XII
C1-decalins acenaphthylene	152	I + II + III	C3-phenanthrenes/anthracenes	220	VII + VIII + IX
acenaphthene	154	II + III + IV	C3-dibenzothiophenes	226	VII + VIII + IX
C2-naphthalenes	156	III	C0-benzo(a)anthracene C0-chrysene	228	X
d8-acenaphthylene	160	III	C2-fluoranthenes/pyrenes	230	IX + X
C2-benzo(b)thiophenes	162	II + III	triaromatic steranes	231	X + XI + XII
d10-acenaphthene	164	III + IV	C4-phenanthrenes/anthracenes retene C0-benzonaphtothiophene	234	VIII + IX + X
C2-decalins C0-fluorene	166	I + II + V	C4-dibenzothiophenes d12-benzo(a)anthracene d12-chrysene	240	VIII + IX + X

C0-dibenzofuran	168	II + III + IV	C1-chrysenes	242	X + XI
C3-naphthalenes	170	IV + V	d14-p-terphenyl *	244	VIII
C3-benzo(b)thiophenes d10-fluorene	176	IV + V	C1-benzonaphtothiophenes	248	X + XI
C0-phenanthrene C0-anthracene	178	VI	5 Rings PAHs	252	XI + XII
C3-decalins C1-fluorenes	180	I + II + III + V	C2-chrysenes	256	XI
C1-dibenzofurans	182	IV + V + VI	d12-benzo(k)fluoranthene d12-benzo(a)pyrene d12-perylene	264	XI + XII
C4-naphthalenes C0-dibenzothiophene	184	IV + V + VI	C3-chrysenes	270	XI + XII
d10-phenanthrene d10-anthracene	188	VI	6 Rings PAHs	276	XII
C4-benzo(b)thiophenes	190	IV + V	6 Rings PAHs	278	XII
tricyclic terpanes hopanes	191	IX + X + XI + XII	d12-indeno(1,2,3-cd)pyrene d12-benzo(g,h,i)perylene	288	XII
C1-phenanthrenes/anthracenes d8-dibenzothiophene	192	V + VI + VII			

3.3.3 Data Set for Source Identification

The 267 samples analyzed were split into four training sets and three Validation sets which were used to construct models and predicted on the model respectively. The training sets consisted of 153 global samples, 18 heavy fuel oil, 35 Middle East samples and a subset of 11 of the Middle East samples labelled Gulf 2. Conversely, validation sets were made up of 27 quality control samples, 34 Qatar Spill samples and 13subsets of fresh Qatar Spill samples. Training and validation sets in the training and validation sets used for each model can be seen in Table 3-2.

Table 3-2: Training and validation sets in PCA model 1–4. Italic represent subsets.

Sets	No. of samples	Model 1	Model 2	Model 3	Model 4
Global	153	TrainSet		TrainSet	TrainSet
M. East	35	TrainSet		TrainSet	TrainSet
Gulf 2	11		TrainSet		
HFO's	18				
Qatar Spill	34	ValSet	ValSet	ValSet	
Qatar Spill	13				ValSet
UW					
QC	27	ValSet			ValSet

3.3.4 CHEMSIC

3.3.4.1 PCA modelling and data processing

Data processing was done using, CHEMSIC method which involves retention time alignment, baseline removal, and data normalisation (Christensen et al. (2005 b; Christensen & Tomasi, 2007) to get rid of samples with varying chemical composition. Consequentially, Four PCA models having different datasets were recorded as it is in Table 3-2. For all the four models recorded in the table, their baseline was removed using a function known as Savitzky–Golay (Savitzky and Golay, 1964). Correlation optimal warping (COW) was then used to align retention times of the sample chromatograms (Nielsen et al. 1998). The optimal warping parameters used in the process were determined by grid and discrete simplex-search method (Skov et al. 2007).

For the grid search the following information was collected 50 and 200 scan points, 1–3 slack parameter, and finally about 50 simplex iterations. Maximal deviation from the COW alignment was found to be 5%.

The PCA model was constructed on the basis of hopane using 1,153 Global and 35 Gulf sample. The other samples 191. 34 Qatar Spill and 27 quality control samples were predicted on the PCA model to show the differences in the oil hydrocarbon fingerprints which are not necessarily as a result of weathering of the samples. On the other side, a local model was constructed using 10 Middle East samples and 1 Gulf 2 sample to predict all the other spill samples onto the model.

The data obtained from the two models was normalized to unitary Euclidean norm to facilitate the analysis of variants within each SIC (Gallotta & Christensen, 2012). C1–C3 alkyl substituted polycyclic aromatic hydrocarbons model was used to define Qatar Spill samples (Christensen and Tomasi, 2007; Christensen et al., 2005). Several PAHs were utilized in the model which included phenanthrene and anthracene (m/z 178), C1-phenanthrenes and anthracenes (m/z 192), C2-phenanthrenes (m/z 206), C3-phrenanthrenes (m/z 220), C4phenanhthrenes (m/z 234), C1-pyrenes (m/z 216), C2-pyrenes (m/z 230), chrysene (m/z 228) and C1-chrysenes (m/z 242). This model constituted 188 and 34 samples from the Global & M. East and Qatar spill respectively.

Nevertheless, analysis of the variation between and within SICs was carried out combining and normalizing SICs to Euclidean norm (Gallotta & Christensen, 2012). All the 188 samples were then combined in model 4 where further analysis was done using hopanes and steranes. The relationship between methyl-anthracene (meAnt) and methyl-. phenanthrenes (meP), expressed by the diagnostic ratio meAnt /(1-meP+9/4-meP+meAnt), for the unweathered spill samples such as HFO's was calculated from the database.

3.4 **Isolation and Identification of Hydrocarbon Degrading Bacteria**

3.4.1 Culture Media

The isolation strategy employed in hydrocarbon-degrading bacteria shown in Figure 4-8 was based on enrichment cultures performed in MSM supplemented with 10% diesel. In fact, this culture medium should exhibit high toxicity to bacteria, provided by the 75 g/l hydrocarbons (diesel containing 750 total hydrocarbons, and representing 10% (v/v) of the culture media)Mineral salt medium (MSM) culture media composed of (g/l) : NH_4NO_3, 4.0 g; Na_2HPO_4, 2.0 g; KH_2PO_4, 0.53 g; K_2SO_4, 0.17 g, $MgSO_4.7H_2O$, 0.10 g, and trace element solution (per 100ml) ; EDTA 0.1g, $ZnSO_4$ 0.042, $MnSO_4$ 0.178g, H_3BO_3 0.05, $NiCl_2$ 0.1g. MSM solid media were obtained by adding (20 g/l) of agar.

Luria-Bertani (LB) broth and agar media from Sigma-Aldrich were used in this study as well. All the media was sterilized using an autoclave for 20 minutes at 121ºC.

3.4.2 Isolation of Hydrocarbon Degrading Bacteria

1g of each sample was weighed and suspended in 20 ml of Luria Broth (LB) used as enrichment medium. The liquid cultures were incubated for 72 hrs. at 30ºC in a shaker at 300 rpm. After the incubation period, 2 ml from each liquid culture were added to 20 ml MSM medium supplemented with 1ml diesel or crude oil used as the source for carbon. This adaptation step was repeated 3 times to ensure the media is rich with microorganisms capable of growing using crude oil and diesel as the main source of carbon. In addition, the spread plate technique (Survery et al. 2004) was also used to spread 100 µl of LB-liquid cultures on MSM agar medium that were subsequently sprayed with 100µl crude oil/Diesel.

Isolates demonstrating distinct morphologies were transferred to new LB agar plates, the purification steps were repeated until pure isolates were obtained. Similarly, colonies that formed clear zones on the solid MSM medium were selected.

3.4.3 Molecular Identification of the hydrocarbon degrading bacterial isolates

DNA from cells grown overnight in (LB) plates was removed using the PCR protocol. The cells were suspended distilled water (0.2ml), and placed at -80C for 20 min, then placed immediately in a water bath set at 100°C for 10 minutes. The cells were centrifuged for 10 minutes at a speed of 13,000 revolutions per minute (rpm). The supernatant obtained was transferred to a new tube for the next PCR protocol.

Amplification of the 16s rDNA was done using universal PCR primers; RibS73sp, AGAGTTTGATCCTGGCTCAG, and RibS74sp, AAGGAGGTGATCCAGCCGCA, (Weisburg et al. 1991).

The PCR protocol was carried out using 25µl PCR buffer containing 1.5µM MgCl2, 0.8µM dNTP, 1.35µM (forward and reverse primers), 10-20 ng genomic DNA isolated and 0.5IU Taq DNA. The first step of the PCR reaction was denaturation at 94 °C for 3 min then 35 cycles of 45 seconds denaturation step at 94 C, 45 seconds annealing step at 50°C, and 45 seconds elongation step at 72 °C, then a final 2 min extension step performed at 72°C. The DNA purification was carried out using QIAquick Gel Extraction Kit.

Sequencing of the bacterial 16S rRNA amplicons was done after purification of the amplicons. The sequencing data was generated from the sequencing unit. Then the 16S rDNA sequence obtained from each of the isolates was used to determine related sequences of DNA in the Gene Bank database using the Blast server at NCBI.

3.4.4 *Identification of Bacterial Isolates by Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI –TOF/MS)*

3.4.4.1 *Ethanol/Formic Acid Method for Protein Extraction*

The ethanol acid was used to extract proteins for the identification of bacteria. The bacterial cells were first thoroughly mixed with 300 µL of ultra-pure water to achieve a homogenous suspension, 900 µL of absolute ethanol was then added and vortex mixed to kill the cells. After which the mixture was centrifuged, and the supernatant discarded. The residue was dried at room temperature, then equal volumes (50 µL) of ethanol acid (70%) in water and HPLC grade acetonitrile were added and vortexed. Centrifugation was done at 13,000 revolutions per minute for 2 minutes. 1µL of supernatant was transferred to MALDI Biotarget plate; 2 spots were used for each sample. Once the sample spot was dried, it was then overlaid with 1µL matrix solution (α-cyano-4-hydroxycinnamic acid, HCCA, Bruker Daltonics). After drying, the Biotarget plate was then loaded into the MALDI-TOF Mass Spectrometer.

3.4.4.2 *Identification of Isolates by MALDI-TOF MS*

The procedure adopted for bacteria identification was similar to a previously described procedure Nacef et al. (2017). The Bruker Biotyper software was used on the MALDI-TOF spectra; in essence bacterial identification takes place through comparison of the present spectra those in the database. As such, in this case the matching profile was conveyed on a Log scale with a score 0 to 3.

Following the manufacturer's instruction, the value obtained was interpreted where a score of 2.3 – 3.000 describes a highly probable species identification, 2.00 - 2.299 was used for genus identification and probable species identification and 1.70 - 1.999 score was

used as a probable genus level identification. Calibration was carried out using Bacterial

Test Standards (Bruker Daltonics, Bremen, Germany (Item Catalogue #255343).

3.4.4.3 *Mass Spectra Acquisition*

The mass spectra acquisition was done by Bruker Flex Control using the default

settings. The acceleration and source voltages were set at 20 kV and 18.7 kV respectively.

The spectra from 240 laser shots in 40-shot bunches on different areas of the sample were

collected. Each sample was represented twice on the MALDI plate each of was run three

times thus a total of six spectra for each bacterium were obtained. For bacterial typing, the

mass spectra were recorded from 0 – 30,000 Da.

3.4.4.4 *MALDI-TOF MS Typing*

The Flex Analysis software was used to manually process the obtained raw spectra

of isolates obtained. The software was used for smoothing and baseline subtraction. The

visual inspection was done in order to find out potential biomarker peaks. The peaks were

then compared with other strains in order to find out the difference between the strains.

3.5 **Screening of Diesel Degrading Isolates Based on Biomass Production**

Isolates considered to be good in the degradation of petroleum components were

cultured in 10 ml LB broth for 48 hours at 30°C. Then, adaptation was performed by

pouring the selected isolates into sterilized 20 ml MSM-diesel (from Qatar Petroleum) or

MSM-petroleum (crude oil from Al-Shahen field) liquid media and incubated for up to 14

days at 30°C. Then the cells' biomasses were estimated by spreading 100 µl of serial

dilutions on the LB agar plates. Then Colony Forming Units (CFU) was used to count the

number of cells.

3.5.1 *Determination of The Total amount of Bacterial Cells*

The colony forming units (CFU) technique was applied to determine the total number of cells in the present liquid or soils cultures, by spreading 100 µl of serial dilutions on LB plates. Approximately 1 g was sampled after vigorous mixing and the corresponding cfu was reported to the exact weight of the sample.

3.5.2 *Screening of Diesel Degrading Isolates Based on Biodegradation Efficiency*

3.5.2.1 *Crude Oil Degradation on Solid MSM*

The selected isolates collected were inoculated on solid MSM agar plates sprayed by 100 µl fresh crude oil which is the source for carbon and energy. The plates were wrapped using foil then incubated in the dark at 30 ºC for 14 days. Biodegradation efficiency was visually evaluated on 1-3 scale based on the diameter of clear zones formed by bacteria coated solid MSM medium.

3.5.2.2 *Extraction of Hydrocarbons in Diesel*

After the incubation period, tubes containing MSM-5% diesel were centrifuged at 5000rpm for 10 min. 100 µl from the diesel layer was diluted with 1 ml of dichloromethane, then the mixture was placed in a sonicator for 10 min.

3.5.2.3 *Analysis of Hydrocarbons in Diesel by Gas Chromatography Flame Ionization Detector (GC-FID)*

A gas chromatograph (GC) equipped with a flame ionization detector (FID Agilent 6890N Network gas chromatography) was used to analyze the extraction process. This analysis is usually performed to investigate the degradation of diesel. using a column (Agilent HP-1 GC, 30 m, 0.25 mm, 0.10 µm, 7inch cage) with an oven temperature of 100 ºC to 280 ºC (hold 5 min) at 15ºC/min, nitrogen as vector gas, and a pressure of 7.85psi.

The total flow was 6.1 mL/min. A chromatograph is obtained, and the concentration was determined by the area under each peak (the U.S Environmental Protection Agency method # 8015).

The calculations were made using the following equations:

$$\text{RE (LMW)} = 100 - \left(\left(\frac{\Sigma\ n-alkaneLMW\ (Sample)}{total\ area\ (Sample)}\right) / \left(\frac{\Sigma\ n-alkane\ LMW\ (control)}{total\ area\ (control)}\right)\right) \times 100$$

$$\text{RE (MMW)} = 100 - \left(\left(\frac{\Sigma\ n-alkaneMMW\ (Sample)}{total\ area\ (Sample)}\right) / \left(\frac{\Sigma\ n-alkane\ MMW\ (control)}{total\ area\ (control)}\right)\right) \times 100$$

$$\text{RE (HMW)} = 100 - \left(\left(\frac{\Sigma\ n-alkaneHMW\ (Sample)}{total\ area\ (Sample)}\right) / \left(\frac{\Sigma\ n-alkaneHMW\ (control)}{total\ area\ (control)}\right)\right) \times 100$$

$$\text{RE (TPH)} = 100 - \left(\left(\frac{\Sigma\ n-alkaneTPH\ (Sample)}{total\ area\ (Sample)}\right) / \left(\frac{\Sigma\ n-alkane\ TPH\ (control)}{total\ area\ (control)}\right)\right) \times 100$$

$$\text{n-C17 /Pristane ratio:} = 100 - \left(\left(\frac{n-C17\ (Sample)}{Pr\ (Sample)}\right) / \left(\frac{n-C17\ (control)}{Pr\ (control)}\right)\right) \times 100$$

$$\text{n-C18 /Phytane ratio:} = 100 - \left(\left(\frac{n-C18\ (Sample)}{Ph\ (Sample)}\right) / \left(\frac{n-C18\ (control)}{Ph\ (control)}\right)\right) \times 100$$

Where RE is removal efficiency.

LMW: Low Molecular Weight Hydrocarbons = C10 – C15.

MMW: Medium Molecular Weight Hydrocarbons: C16 – C20.

HMW: High Molecular Weight Hydrocarbons= C21 – C 25.

3.5.3 **Determination of Diesel Solubilization Activity of Bacteria**

LB Cultures grown overnight were centrifuged at 10, 000 revolutions per minute (rpm) for 10 min. Then 0.9 ml was weighed and added to 10 ml of Tris HCl (pH 7.0) and 0.2 ml of diesel, control tubes were prepared by adding 0.2 ml Tris HCl instead of diesel (Ayed, et al., 2015). All tubes were placed in the shaker and centrifuged at 200 rpm for 1 day in the dark. After the incubation period, 4 mL of the solution were added to 4 mL of hexane in a 15 ml falcon tube, after vortex for2 min, the tubes were centrifuged at 4500

rpm for 15min, then 3 mL of hexane phase were used to measure optical density of hexane phase using spectrophotometer set at 295 nm, hexane was used as blank. Diesel solubilization was calculated used the following equation:

$$Solubilized\ diesel\ (\mu L/mL) = Assay\ concentration - Control\ concentration$$

$$\%\ Solubilization = \frac{Solubilized\ diesel}{Initial\ diesel\ concentration} \times 100$$

3.5.4 Determination of Diesel Emulsification Activity of Bacteria

In Eppendorf tube, 1 ml of culture broth was centrifuged at 10,000 revolutions per minute for 15 minutes then supernatant was transferred to a new Eppendorf tube, in which 150 µL of diesel were added, after vortex for 2 min, the tube was left for 1 hour to emulsify the diesel. The diesel layer on top were discarded and the optical density of solution measured using. spectrophotometer set at 400 nm. MSM media was used as blank (Hassan et al. 2014). The emulsification activity was calculated as following:

$$Emulsification\ activity\ (EU/mL) = \frac{Absorbance}{0.01}$$

3.5.5 Quantification of Carbon Dioxide Produced by Bacteria

CO_2 produced by the bacteria was measured by withdrawing 250 ml of the CO_2 at a set time interval using a glass syringe and then injecting it into Gas Chromatography (Agilent model 7890A) having a thermal detector and a 30 m by 250 mm capillary. The GC used Helium as the carrier gas. It was ensured that the temperatures of both the injector and detector was maintained throughout the quantification process at 200 ℃ and 210 ℃, respectively. Additionally, the oven temperature was also maintained at 50 ℃ to 80 ℃ with a hold time of 3 minutes increasing at a rate of 20 ℃ min1. The determine the extent of oil

mineralization as a result of CO_2 evolution the amount of CO_2 obtained experimentally was compared with the theoretical amount of CO_2 less the amount of CO_2 evolved from oil-free sediments (Abed et al. 2014).

3.5.6 *Biopiling System for Ex-Situ Bioremediation of Oil Contaminated Soils*

A sample of 685 g soil collected from Dukhan dumping area was placed in a biopile in the form of a cylindrical glass tank, tightly sealed with a rubber cap to avoid evaporation, covered with foil to prevent light oxidation reactions, and placed in an incubator set at 37°C during the periods indicated with the corresponding results. The soil was manually mixed twice a week to ensure homogenization. Two types of soils were used. A highly polluted soil with weathered hydrocarbons and a clean soil sample from the Qatar University biology field. A total 16 different biopiles were prepared in triplicates with different parameters; 11 biopiles by using highly weathered oil contaminated soil and 5 biopiles with clean soil. The latter was stained with 50 mL of fresh oil. The initial DRO contents in the biopiles were 584 + 7 ppm in fresh oil stained soil and 3067 + 67 ppm polluted weathered soil. PAHs concentrations were of 308 + 8 and 403+ 9 ppb (freshly stained vs. weathered). The C/N/P ratio was then adjusted to 100/10/1 by adding, to each biopile, 32.9 g CH_4N_2O as nitrogen source and 6.85 g KH_2PO_4 as phosphorous source. 70 mL of water was then added to achieve moisture of 10% in each biopile at the time zero of the incubation. A total of 0.8 mL Tween-80 was used as a surfactant in each biopile, corresponding to 1.1 mL/kg. The bacterial strains isolated from the two soil types that were introduced into the biopiles were added. The inoculation was performed with suspended pellets in MSM liquid medium from individual centrifuged cultures. The volume was calculated based on the CFU of the inoculum as determined by the CFU protocol described. The initial CFU in

each biopile was around 103 CFU/g soil. The produced biomass in the experiment was the difference between the counted CFU at the indicated incubation time and the initial CFU determined just after the inoculation. The incubation times are provided with the results.

3.5.7 *Polycyclic Aromatic Hydrocarbons (PAHs) Extraction and GC-MS Analysis.*

For the PAH extraction, dichloromethane was used, and filtration was performed through a washed silica column (SPE cartridge) using methanol once and dichloromethane three times. The sample was then injected onto the Agilent 6890N GC-FID equipped with a Restek Rxi®-5Sil MS, 30 m, 0.25 mm ID, 0.25 µm column. The oven temperature range was 50ºC (hold 0.5 min) to 290ºC with a temperature rise of 25ºC/min; subsequently the temperature was raised to 320ºC at 5oC/min increase rate. The inlet method was pulsed splitless using nitrogen flowing at a rate of 23.50ml/min and pressure of 11.747 psi. The detection method was mass spectrometry. The concentration was determined as described by the U.S Environmental Protection Agency method #8275, using a pre-established calibration curve (Worden, 1993).

3.5.8 *Fourier Transform Infrared (FTIR) analysis*

The soil samples collected were dried at 50ºC for 48 h, grinded and the placed directly onto the FTIR Perkin Elmer 400 FT-IR/FT-NIR spectrometer. The pressure exerted on the samples was ranging from 30-50 psi. The absorbance spectrum was recorded in the range of range of 400-4000 cm-1.

3.6 Biopiles of Dukhan Polluted Soil by Indigenous Bacteria

3.6.1 Experimental Design

The 8 biopiles prepared as in section 0 were performed at the same conditions to avoid any variations or difference in results that are not based on our research purposes. three indigenous bacteria named D1D2, D5D1 and D9S1 (Table 3-3) were selected for this study. In total 8 different biopiles were performed in triplicates, using single or mixed bacteria.

Table 3-3: Single and mixed indigenous bacteria used in bioaugmentation/biostimulation remediation of Dukhan dumping site.

Biopile code	indigenous isolate		
	D1D2	D5D1	D9S1
T1	X	-	-
T3	-	X	-
T5	-	-	X
T7	X	X	-
T9	X	-	X
T11	-	X	X
T13	X	X	X
T15 (control)	-	-	-

3.6.2 *Biopiles Diesel Range Organics (DRO) Extraction and Analysis*

The collected samples were dried at 50ºC for 48 h and grinded to insure homogeneity. 1.00 g of each sample was then used for DRO extraction. The samples were then placed into the extraction vials and 1 g of diatomaceous was added. Then, 20 g dried alumina were added to the vial, tightly sealed and placed into the ASE DINOX SE 500 evaporator for extraction. Hexane was the solvent used for DRO extraction. The extraction was performed by an accelerated solvent extractor machine through a programmed extraction cycle method; each sample took 30 min for complete extraction at 400 ºC. Once the extraction cycle was complete, the extracted solvent was concentrated to 1.0 mL using pure nitrogen and filtered through a washed silica column (SPE cartilage). The column was washed with methanol once and with hexane 3 times. Afterwards, 14 mL hexane were poured on the top of the column with the 1 mL extracted solvent. The liquid phase that was eluted from the column was evaporated to 1 mL by nitrogen gas was performed before injection to the Agilent 6890N Network gas chromatograph equipped with a flame ionization detector (GC-FID) (described in section 3.5.2.3).

3.6.3 *Soil Extraction and Gas Chromatography Mass Spectrometry (GC-MS)*

One gram of each soil sample was weighed and put in a vial tube. The hydrocarbons were extracted using 10 mL of dichloromethane mixed with internal standard (200 µl of 160 ppm), the mixture was then sonicated for 10 min. The dichloromethane extracts were analyzed with gas chromatography (GC) (Agilent 7890B) coupled to a quadrupole mass spectrometer (MS) (Agilent 5975C) which was measured using SIM mode that utilizes electron ionization process. The column was a 70 m GsBP-5MS capillary having 0.25 mm and 0.25 µm inner diameter and film thickness respectively. 1µl of the aliquots were

injected in splitless mode to the chromatographic column at an injection temperature of 315°C. Helium gas was used as the carrier gas that flows at a rate of 1.1 ml $\times$ s^{-1}. Initial temperature of the column was 40°C maintained for 2 min, ramped with 25°C $\times$ min^{-1} to 100°C, 5°C $\times$ min-1 to 315°C maintained for 13.4 min. The temperatures of the transfer line were 315°C, ion source temperature 230°C, and quadrupole temperatures of 150°C. The selected ions analyzed were divided into 12 groups where a total of 55 mass-to-charge ratios that were analyzed covered an *m/z* range from 83 to 288, see appendix I. Each *m/z* had a dwell time of 25 ms involving 2.81 scans $\times$ s^{-1}. The SIM method is further described in Gallotta & Christensen (2012). The selected saturated hydrocarbons that were investigated in this study are specified in Table 3-4 These compounds were chosen to represent the two groups; saturated hydrocarbons alkylated homologs. The saturated hydrocarbons were chosen along with branched alkanes to represent a range of different chain lengths (nC13-nC36) were chosen along with their alkylated homologs. See (Figure 3-3).
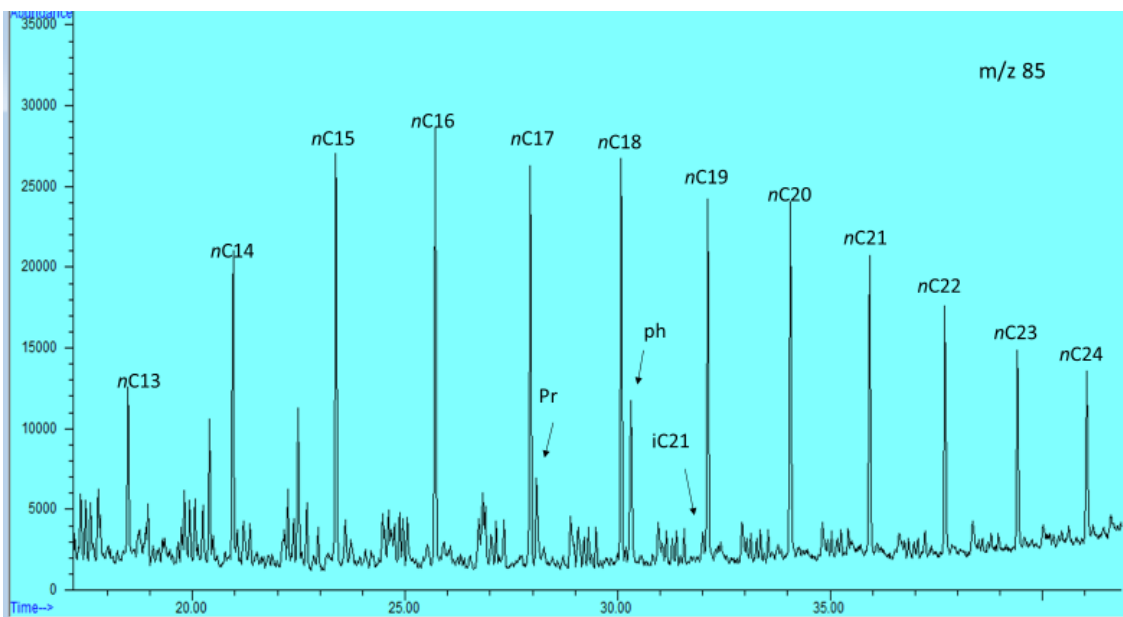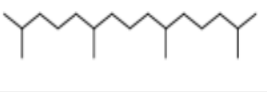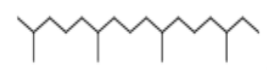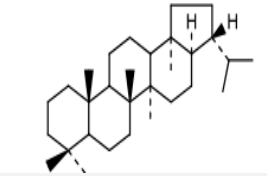
Figure 3-3:Selected ion chromatograms of n-alkanes, m/z 85.

Samples were divided into batches based on incubation weeks, the samples were analyzed in succession. Each batch contained solvent (dichloromethane) blanks, internal standard, and day zero as reference sample. For every 9-10 samples dichloromethane and instrument quality control samples were analyzed. Results of instrument quality control samples were evaluated daily to ensure that the instrument was properly tuned, and that the liner and the GC column were not contaminated. This was done by monitoring changes in relative abundance of components of the mixture, and changes in peak shapes. Furthermore, selected components of reference samples were compared on a daily basis to ensure stable instrument performance.

Table 3-4: Investigated oil components with selected molecular structures, molar weight $(g \times mol^{-1})$ and retention time (min).

Compound name	Chemical structure	Molar weight $(g \times mol)$	Retention time (min)
Saturated hydrocarbons			
n-tridecane, nC13		184.37	18.488
n-tetradecane, nC14		198.40	20.951
n-pentadecane, nC15		212.42	23.371
n-hexadecane, nC16		226.45	25.709
n-heptadecane, nC17		240.48	27.948
Pristane, iC19		268.53	28.091
n-octadecane, nC18		254.50	30.086
Phytane, iC20		282.55	30.314
Isoprenoid, iC21*		268.53	30.716
n-nonadecane, nC19		268.53	32,124
n-eicosane, nC20		282.56	34.070
n-heneicosane, nC21		296.58	35.928
n-docosane, nC22		310.61	37.705
n-tricosane, nC23		324.64	39.409
n-tetracosane, nC24		338.67	41.038
n-pentacosane, nC25		352.69	42.606
n-hexacosane, nC26		366.72	45.865
n-heptacosane, nC27		380.75	44.179
n-octacosane, nC28		394.77	46.932
n-nonacosane, nC29		408.80	48.299
n-triacontane, nC30		422.83	49.744
n-hentriacontane nC31		436.85	51.323

n-dotriacontane nC32	450.88	53.095
n-tritriacontane nC33	464.90	55.121
n-tetratriacontane nC34	478.93	57.473
n-pentatriacontane nC35	492.96	60.217
n-hexatriacontane nC36	506.98	63.462
Biomarker 17α,21β-hopane	412.7	53.670

3.6.4 GC-MS Data Analysis for Biopiles

Data obtained from GC-MS analyses was quantified with Enhanced Chemstation 2.0 (Agilent Technologies). To evaluate loss of *n*-alkanes due to biodegradation, *n*-C17 - *n*-C36 peak values were compared to the corresponding peak values in the original weathered samples (day zero). All data was normalized to 17α,21β-hopane and the averages and standard deviations for the triplicate samples were calculated. Data used to illustrate the disappearance of compounds over time was normalized to 17α,21β-hopane. Calculations of disappearance were performed by:

Compound remaining $100\% = \frac{Average_{day\,x}}{Average_{day\,0}} X\,100.$

3.7 Chemometric Analysis and Biopiles Bioremediation

3.7.1 Data Set for Biopiles

Each of the samples used in the data set consisted of retention time windows of 2 SICs (Table 3-5).

Table 3-5: List of compound group names, m/z's.

Compounds	m/z
Alkanes	85
tricyclic-pentacyclic terpanes	191

After analyzing 34 samples, 32 sample extracts were split into a 'training' set (8 samples from week two, 8 samples from week 4, 8 samples from week 8 and 8 samples from week 16) and two 'day zero' and one with 6 analytical replicates of the day zero sample 'Ref' in PCA plots).

3.7.2 Data Preprocessing for Biopiles

Data obtained from 2 GC–MS/SIM chromatograms from the samples was exported using a special commercial software known as ChemStation to an AIA file format. LatentiX v 2.12 (https://latentix.com) was used to analyze relevant data.

Sorting was done to eliminate target compounds which were not detected. Those compounds which had low signal to noise ratio and retention time were removed by visual inspection before data processing was carried out.

The CHEMSIC method was used in this case to reduce the variations that arise as a result of unrelated chemical composition (Christensen et al 2005; Christensen &Tomasi, 2007; Gallotta & Christensen, 2012). Calculation of the first derivatives obtained from SICs was carried out to remove the baseline. On the other side, retention time alignment procedure was carried out and it involved two critical steps i.e applying rigid shifts on the chromatograms and employing COW. In this case the function of the COW was to align the sample chromatogram to a target chromatogram. This process is usually achieved by stretching or compressing sample segments being used to tag along the retention time axis (Nielsen et. Al 1998). In optical warping parameters such as the slack parameter were determined using the grid search method using the parameter space and discrete simplex-search (Skov et. Al 2006). Training set samples with the highest sum of correlation coefficients was picked as the target for alignment. The highest sum of correlation co-efficient obtained from training set samples was used as the base for selecting the target for the alignment.

3.8 Statistical Analysis

Each experiment was carried-out in groups of three replicates. After which the mean values of the experiment was calculated and presented with standard deviation using M.s Excel 2013. Moreover, since there exists a huge difference between growth and removal of hydrocarbons, ANOVA was used to analyze the data at 95% confidence level.

Chapter 4 :POLLUTION BY OIL OF SELECTED COASTAL AND ACTIVITY SITES IN QATAR

4.1 Introduction

Bioremediation using microbial processes is an attractive approach to alleviate high pollution issues by its ability to remove complex mixtures of pollutants (Mittal & Singh, 2009). Some soil microorganisms, particularly bacteria, develop strategies to use organic molecules as the source of carbon and energy to transform them into substrates metabolized by other microorganisms (Johnsen et al. 2005; Mittal & Singh, 2009). For example, some hydrocarbon-degrading bacteria are powered with chemotaxis which is a signaling system, facilitating access to hydrocarbons via chemoreceptors and signaling pathways (Samanta et al., 2002). Mechanisms of degrading the organics include a bacterial accumulation between the pollutants based on hydrophobic-hydrophilic interactions (Kästner et al. 1994). Bacteria are also capable of transferring pollutants due the hydrophobicity of their walls (Lotfabad & Gray, 2002) to form biofilms (Johnsen et al. 2005). Finally, microorganisms produce surfactants which increase solubility of hydrocarbons, and consequently their accessibility (Masih & Taneja, 2006). Generally, hydrocarbons vary in terms of their susceptibility to microbial attack and depend on the hydrocarbon in question; cyclic alkanes, small aromatics, branched alkanes, and linear alkanes are the general subgroups (Das & Chandran, 2011). The complexity of biodegradation of such compounds is increased by the processes of weathering which characterize the arid areas such as the Arab Gulf region (AlDisi et al., 2017). In an extreme and harsh desert environment such as Qatar, microorganisms develop adaption routes leading to their selection and local

domination. They may acquire special abilities to remediate specific organic mixtures in weathered soils (AlDisi et al., 2017). The bioprocesses related to natural remediation in Qatar constitute a suitable general model of bacterial hydrocarbon degradation under the extreme environmental conditions. Such a model allows to address a highly variable organic composition in dry soils that are representative of arid regions. In a previous work, it was shown that the bioremediation strategy to alleviate pollution stress should be based on activity, adaptation and diversity of native bacteria and appropriate stimulation might be necessary (Attar et al. 2017; AlDisi et al., 2017). In fact, it is now well established that exogenous bacteria cannot be applied in bioaugmentation approaches to remediate oil-contaminated soils. The main source of failure is due to the lack of adaptation routes in such bacteria (Macaulay & Rees, 2014).

Three sites were selected to investigate the pollution issue, and the potential of microbial bioremediation approaches to alleviate the environmental issue. A coastal area, along AlZubara beach, the oily-wastes dumping site in the industrial area of Dukhan and oily-wastes from AhShaheen oil field and auto-workshops. AlZubara: a coastal area was of 12 Km located north-west of Qatar. 13 sampling points were identified along the beach, and 3 samples of each soil were collected from the 13 sampling point, giving a total of 39 samples. Then, the investigations were pointed out on one the site numbered (8) from which 9 specific points were identified for a Systematic Sampling from the surface soil area and 20 cm down soil area. Distances between the sampling points were of 20 m.

Dukhan oil field wastes dumping area was selected for a Systematic Sampling of soil samples from a site 3-year exposed to air and weathering processes (personal communication). Consequently, 9 points, each distanced 10 m, were identified for

82

sampling from the surface layer and 20 cm below the surface. Composite samples from three barrels of AlShaheen oil wastes and 2 others from local auto-shops were considered in the study.

Physical and chemical characterization of the soils and weathering degree of polluting oil was performed. In this study, we considered the fact that due to the warm weather in Qatar then oil weathering processes such activities as evaporation and biodegradation will be accelerated. The acceleration of the process resulted to complication in oil spill identification process since only recalcitrant compounds can be used to identify sources variation based on chemical differences.

4.2 **Investigation of Pollution and Bioremediation Potentialities in Alzubara Beach**

4.2.1 *Source Identification of Beached Oil at Al Zubarah, Northwestern Qatar*

To identify source of pollution and the weathering degree of the polluting oil, observed in the studied sites on AlZubara beach, samples were analysed for Total ion chromatograms, TIC and SICs (m/z 191 and m/z 217) for 5 samples having different weathering degree. Figure (4-1) shows weathering degree of the Qatar spill samples. According to the analysis sample 28 is seen to have several peaks in TIC and tin m/z 217 SIC which indicates that it is relatively unweathered. It is also evident that sample 2 had significantly weathered due to the pattern it dipplayed. On the other side, all the compounds found in TIC that had a retention time range of 0-40 mins were removed even though their sterane biomarker patterns were affected. From (Figure 4-1) steranes are significantly affected thus making hopanes the only recalcitrant that can be used to identify oil spills.
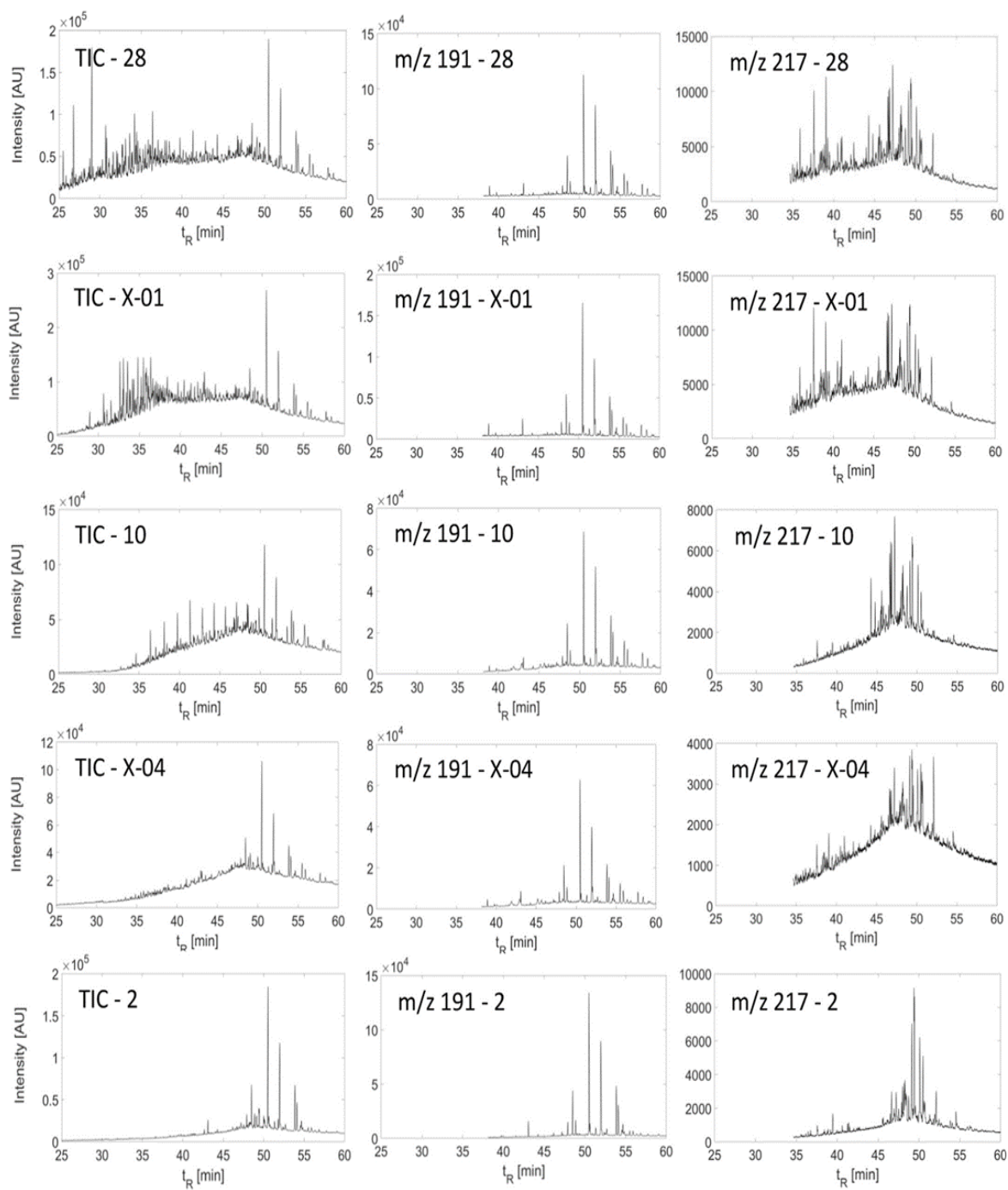
Figure 4-1: Total ion chromatogram, TIC and selected ion chromatograms, SICs of m/z 191 and m/z 217 for five samples of various weathering degree.

4.2.1.1 Global and Local Models for Oil Spill Identification

SICs of m/z 191 (hopanes) was analyzed using the CHEMSIC method. The first principal component (PC1) was used to explain the difference between samples collected based on their weight where m/z 191 crude oil heaviness was expressed with evaporative weathering. Consequently, PC1 cannot be used to identify where the oil was retrieve as it separates the oils partly based on their heaviness and evaporative weathering degree. PC2 and PC3 loadings for hopanes (m/z 191) and their score plots are as shown in (Figure 4-2 A) and (Figure 4-2 B), respectively. PC2 have high positive loadings for 17α(H),21β (H)−30-norhopane, whereas PC3 have high negative loadings for 17α(H), 21β(H)hopane, 17α(H),21β(H)−22S-homohopane, 17α(H),21β(H)−22Rhomohopane, Gammacerane, 17α(H), 21β(H)−22S-bishomohopane and 17α(H),21β(H)−22R-bishomohopane.

Clustering of 'QC' in the PCA score plot (Figure 4-2B) showed variations in the oil composition and not analytical variations. Model 1 was used to display Qatar oil spill samples and Gulf samples (Saudi Arabia, and Iraq) (Figure 4-3) cluster in areas of high PC2 scores (i.e 17α(H),21β (H)−30-norhopane). PCs can be used to mask the difference between oil sources that are related as they follow a possibly common trend. It is unfortunate that such PCs are ignored and are not included in the optimal PCA model. However, these variations in PCs were presented using a local PCA model which has a subset that is based on the source of oil (Christensen et al., 2004). It was found out that samples from Qatar oil spill and 10 crude oil obtained from Gulf and 1 sample from Syria cluster when the PC2 score were high and at PC3 score values of 0−1 as shown below. (Figure 4-3) shows Model 2.
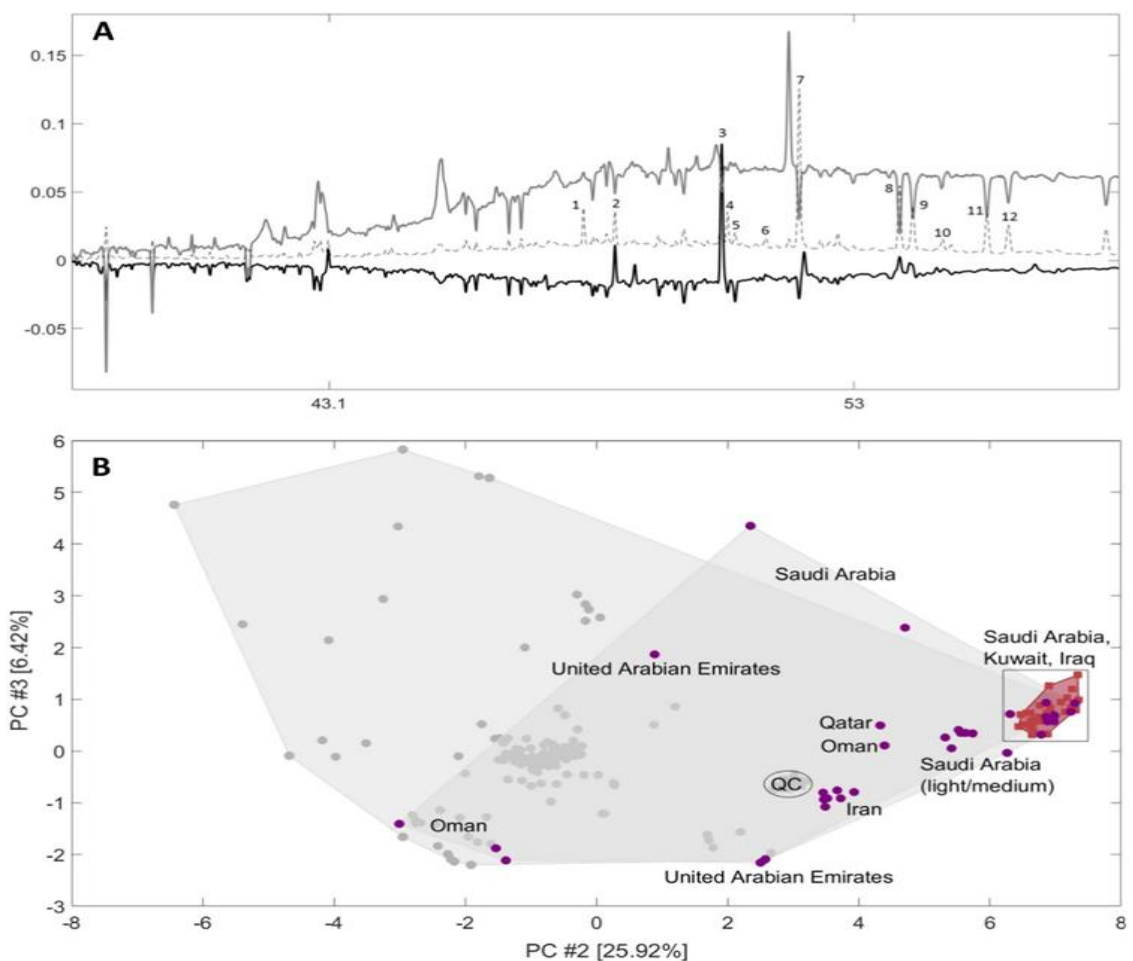
Figure 4-2: (A) PC2 (black solid line) and PC3 (grey solid line) loading plot for model 1 (m/z 191). The grey dotted line represents the average TIC of all samples in the training set. (1) 18α(H)−22,29,30-trisnorhopane, (2) 17α(H)−22,29,30-trisnorhopane, (3) 17α(H),21β (H)−30-norhopane, (4) 18α(H)−30-norneohopane, (5) 15α-methyl,17α(H)−27-norhopane, (6) 17β(H),21α(H)−30-norhopane, (7) 17α(H),21β(H)-hopane, (8) 17α(H),21β(H)−22S-homohopane, (9) 17α(H),21β(H)−22 R-homohopane, (10) Gammacerane, (11) 17α(H), 21β(H)−22S-bishomohopane, (12) 17α(H),21β(H)−22 R-bishomohopane. (B) PCA score plot of PC2 vs. PC3 of model 1 Samples from the Gulf (purple circles) and Qatar spill samples (red squares) are shown. Inside the grey square are all Qatar spill samples and 11 oils from the database. QC: quality control samples.
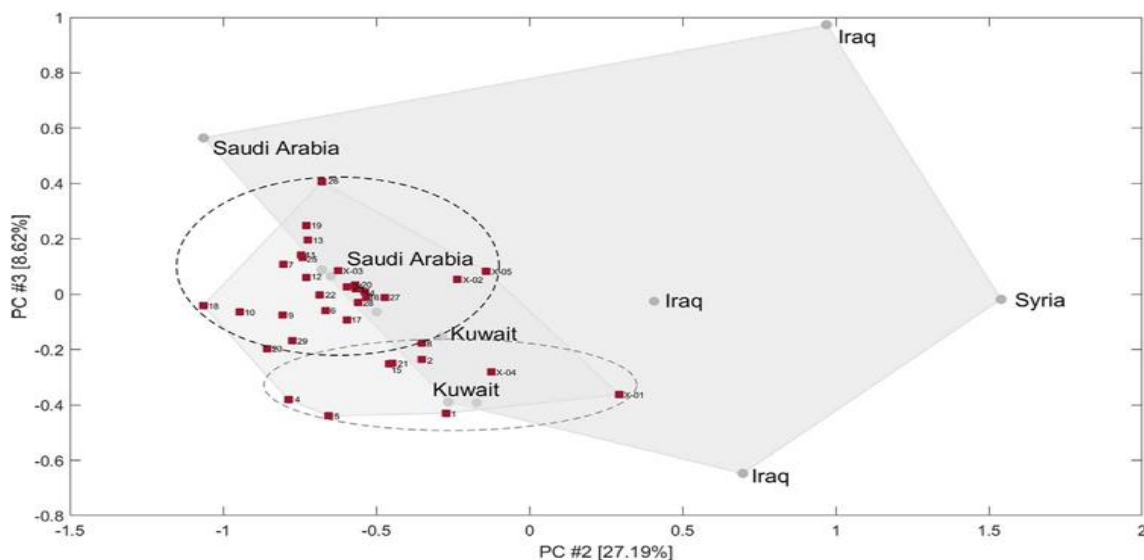
Figure 4-3:PCA score plot of PC2 vs. PC3 of the PCA model (m/z 191 SIC) of 34 Qatar spill samples (red squares) and 10 crude oil samples from the Gulf and 1 from Syria (data points inside the square in Fig. 2B). The grey dotted circle represents Qatar spill samples resembling Kuwait oils from the database, whereas the black dotted circle represents spill samples resembling Saudi Arabia oils.

4.2.1.2 *Selection of a Subset of Less Weathered Oil Spill Samples*

Calculation of PCA model of selected PAHs (m/z 178, 192, 206, 220, 234, 216, 230, 228 and 242) was carried out to determine the samples that are less weathered spill samples. On the other hand, for normalization purposes, SICs were combined and normalized using Euclidean norm. Model 3 as in (Figure 4-4). shows the loading and score plot for PC1 and PC2.
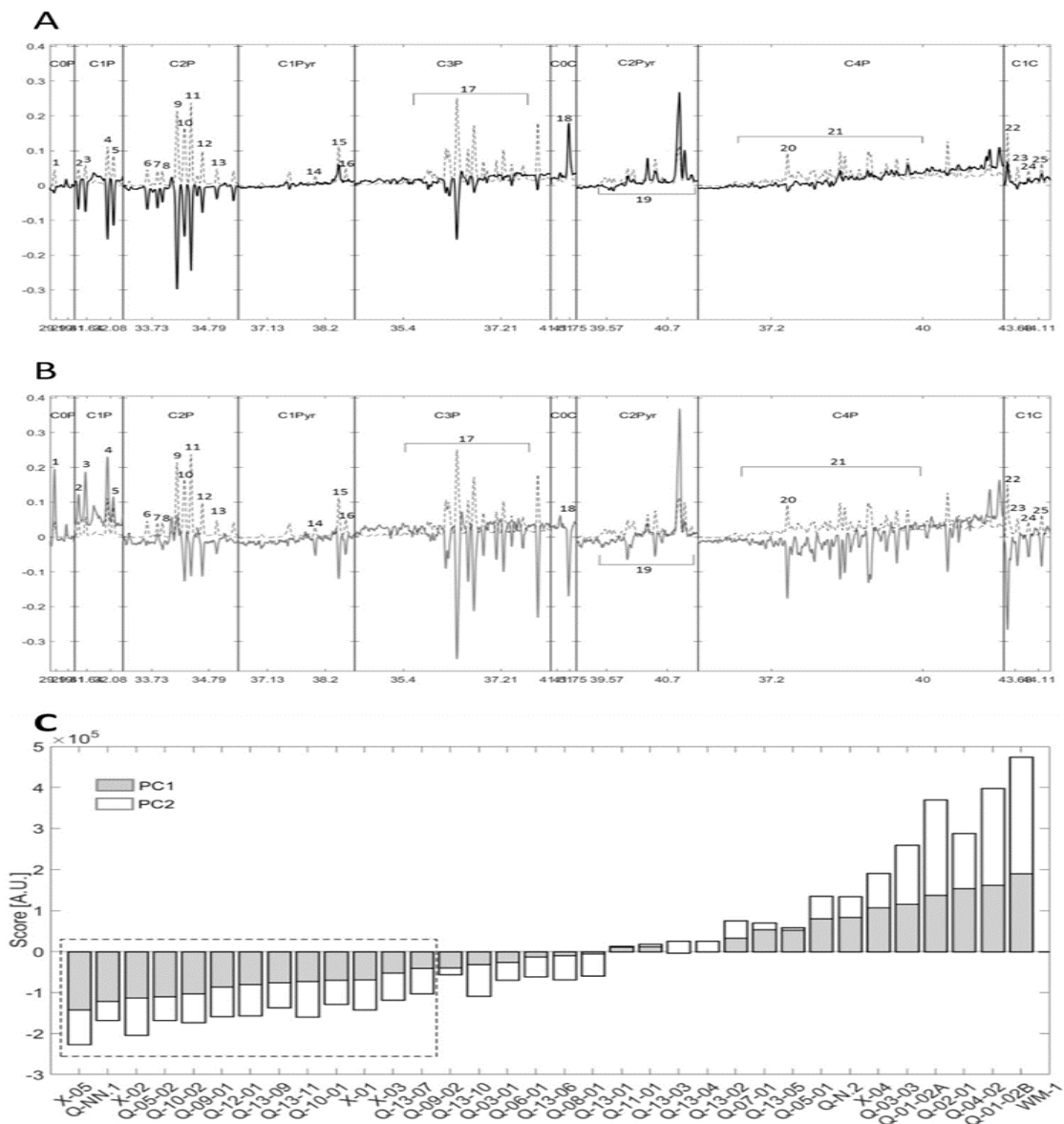
Figure 4-4:PCA loading plot of PC1 (A) and PC2 (B) for selected PAHs (m/z 178, 192, 206, 220, 234, 216, 230, 228 and 242) in Qatar spill samples. The grey dotted line represents the average TIC of all samples in the training set. (1) phenanthrene, (2) 3-methylphenanthrene, (3) 2-methylphenanthrene, (4) 9/4-methylphenanthrene, (5) 1-methylphenanthrne, (6) 2/9ethylphenanthrene and 3,6-dimethylphenanthrene, (7) 2,6-dimethylphenanthrene, (8) 2,7-dimethylphenanthrene, (9) 1,3/2,10/3,9/3,10-dimethylphenanthrene, (10) 1,6/2,9dimethylphenanthrene, (11) 1,7-dimethylphenanthrne, (12) 1,9-dimethylphenanthrene, (13) 1,8-dimethylphenanthrene, (14) 2-methylpyrene, (15) 4-methylpyrene, (16) 1methylpyrene, (17) C3-phenanthrenes, (18) chrysene, (19) C2-pyrenes, (20) Retene, (21) C4-phenanthrenes, (22) 3-methylchrysene, (23) 2-methylchrysene, (24) 6-methylchrysene.

According to the plot, samples that had high concentration of C1-C2- phenanthrene and low concentration of negative 4 ring PAHs chrysene and C2-pyrenes have negative PCI scores which is a characteristic for less weathered oils. Conversely, with high concentrations of C0-C1-phenanthrenes (Figure 4-4B) and low concentrations of the 3 and 4 ring PAHs, C2-C4-phenanthrenes and C0-C1-chrysenes have positive PC2 scores (Figure 4-4B). Notably, none of the samples used have both a positive and negative PC2 score. As such, this shows that all the samples used in the study were weathered. To promote the effectiveness of the method used to identify Qatar spill samples (model 4), m/z 191 (hopanes) were combined with m/ z 217 SIC (steranes) and tested to assess any changes in the identification process after which the 13 spill samples were predicted onto the model. Figure 4-5 below indicate that sample 15, X-05, X02 and X-01 had distinguishing features from the rest. Sample X-05, X-02 and X-01 resembled samples from Kuwait, Iraq, and a few from Saudi Arabia as they were hard, stony and dark, thus highly likely to be the remains of Gulf War oil spill. As for the other samples, they did not match with oils in the UCPH database, but they were found to somehow resemble crude oils from Saudi Arabia.
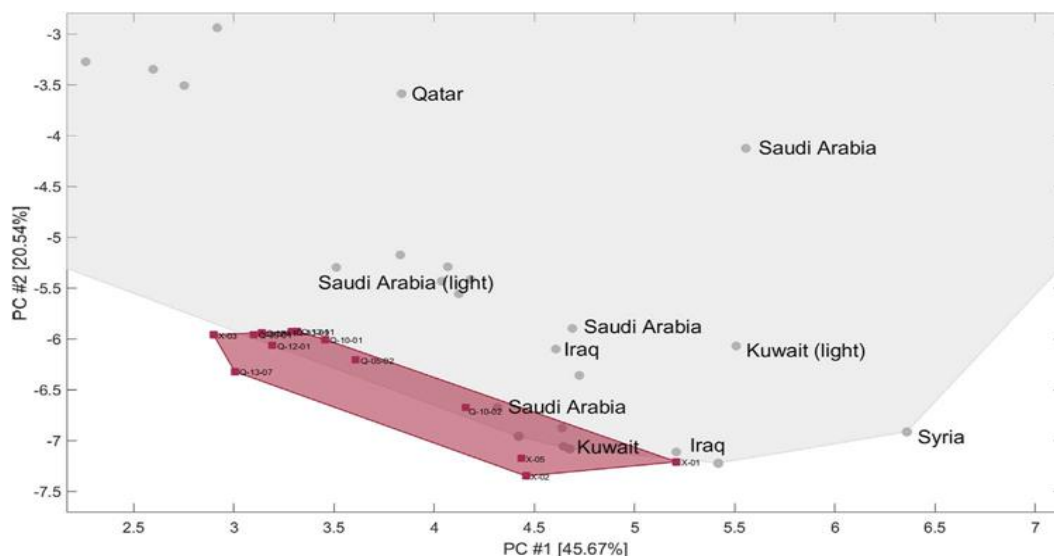
Figure 4-5:PC1 vs. PC2 score plot of hopanes (m/z 191) and steranes (m/z 217). 13 of the least weathered Qatar spill samples (red squares) were predicted onto the model.

4.2.1.3 Heavy Fuel Oil Characterization (m/z 192)

Heavy fuel oil and crude oils are easily distinguished due to their varying compositions of methyl-anthracene (meAnt) and methyl-phenanthrenes (meP) (Sun et al., 2015; Zhang et al., 2016) by calculating their diagnostic ratios (meAnt/($\sum$meP+meAnt) and meAnt/(1-meP+9/4-meP +meant).

Inspection of raw chromatograms showed that 2meP and 3-meP isomers were significantly affected by weathering and as result they were varied significantly from the other samples (data is not provided). According to the figures recorded in Figure 4-6 it is clearly evident that most of the samples are not HFO's as they have low diagnostic ratios. The results from the diagnostic ratio were significantly affected due to biological degradation as most of the samples were highly weathered. However, samples such as X-03, 7, 13 and 16 had high diagnostic ratio. Sample 7, 13 and 16, were found to be more

weathered through biological degradation resulting to confounded results. However, Sample X-03 was less weathered thus resembling oils from Saudi Arabia (Figure 4-4). As such, this shows that this spill sample is more likely to have originated from recent tank washings occurring in the Gulf.
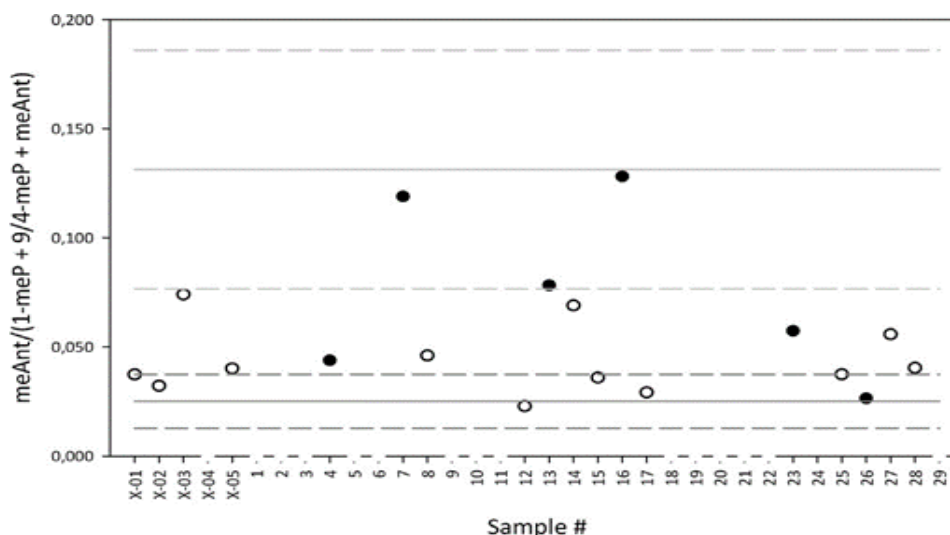
Figure 4-6:Diagnostic ratio of meAnt/(1-meP+9/4-meP+meAnt), for all spill samples (circles). Open circles are the 13 least weathered spill samples as defined from model 3 and black circles are the more weathered spill samples. The average diagnostic ratio for all M. East samples and all HFO's are shown as solid lines (dark and light grey for M. East and HFO's, respectively) with ± 2* stdev in dotted lines.

4.2.2 *Chemical and Physical Characterization of Alzubara Soil Samples (site 8).*

Following our observations, the widest area of oil pollution on AlZubara beach was in the site (8) (Figure 4-7). Moreover, the soil sample from site 8 was shown to be polluted with highly weathered oil, as explained above in the section (4.2.1).

Figure 4-7: sampling site 8 (AlZubarah)

In order to investigate the bioremediation potentialities to remediate weathered oil in the selected site 8, a full chemical and physical characterization of the corresponding soil was performed. A composite soil sample from 9 sampling points in a systematic sampling approach was used from the surface and another from 20 cm below the surface at each point. The results of major ions, nutrients concentrations, salinity and the pH in soil samples are shown in Table 4-1. Chemical and physical characteristics of the Alzubara oil spill was analyzed to assess the existing environmental conditions available to the indigenous microbial community.

Table 4-1: Major chemical and physical analyses of the soil sampled from the upper and down layers of soil from site 8 on AlZubara beach.

Component	Surface layer	20-cm down layer
Na (mg/g)	11.21 ± 1.02	11.220 ± 1.557
K (mg/g)	0.40 ± 0.011	0.405 ± 0.013
Mg (mg/g)	0.99 ± 0.05	0.994 ± 0.059
Ca (mg/g)	0.44 ± 0.02	0.435 ± 0.019
Cl (mg/g)	19.42 ± 1.18	19.376 ± 1.196
Br (mg/g)	0.082 ± 0.003	0.0817 ± 0.003
NO3 (µg/g)	0.007 ± 0.001	0.0078 ± 0.0009
SO4 (mg/g)	2.035 ± 0.064	2.037 ± 0.552
NO2 (µg/g)	0.136 ± 0.008	0.135 ± 0.007
PO4 (µg/g)	0.354 ± 0.031	0.359 ± 0.025
Salinity (ppt)	5.973 ± 0.122	5.983 ± 0.103
pH	7.20 ± 0.06	7.21 ± 0.08
TPH (DRO) (µg/kg)	<1.0	<1.0
TPH (ORO) (mg/kg)	280 ± 20	275 ± 16

In general, there is no significant difference in the overall chemical and physical characteristics between the surface and the down layers in AlZubara as revealed by Statistical analysis (one-way ANOVA), p-Values > 0.05. The TPH (ORO) content in upper and down layers were 280 ± 20, 275 ± 16 (mg/kg) of sediment, respectively (Table 1). The Neutral pH in upper and down layers was 7.20 ± 0.06, 7.21 ± 0.08 of sediment, respectively likely to be favorable for most of the microbes with degradation potential. Abundance of sulfate content in upper and down layers was 2.035 ± 0.064, 2.037 ± 0.552 (mg/g) of sediment, respectively, with low nitrate content in upper and down layers was 0.136 ± 0.008, 0.135 ± 0.007 (µg/g) of sediment, respectively, with low ammonia content in upper

and down layers was 0.007 ± 0.001, 0.0078 ± 0.0009 (µg/g) of sediment, respectively and phosphate content in upper and down layers was 0.354 ± 0.031, 0.359 ± 0.025 (µg/g) of sediment, respectively were noticed. The high salinity content in upper and down layers was 5.973 ± 0.122, 5.983 ± 0.103 ppt of sediment, respectively is an indicator of presence of halophilic and halotolerant microorganisms (Leahy and Colwell, 1990; Sarkar et al., 2016; Roy et al. 2018).

4.3 Investigation of Indigenous Hydrocarbon-Degrading Bacteria in Weathered Oily-Soil in Alzubara Beach

4.3.1 Isolation and Potential of Hydrocarbon-Degrading Bacteria from Site 8 At Alzubara

In order to, design an appropriate approach for bioremediation of AlZubara beach from the oil pollution, the indigenous microflora was important to study, to look at the possibility of in-situ bioremediation through biostimulation and or bioaugmentation strategies, using the indigenous bacteria. From the microbiological point of view, the study will also enrich our knowledge regarding diversity of hydrocarbon-degrading bacteria living in harsh conditions. The originality of our research is that it is related to highly adapted bacteria to weathered oil in harsh soils, which may lead to creation of new specific metabolic pathways by Qatari bacteria, due to the local harsh weather and soil conditions. The site 8 in AlZubara beach which was shown as polluted with highly weathered oil was selected to isolate hydrocarbon-degrading bacteria from the 9 points of the systematic sampling, each represented by 2 samples, one from the upper layer and one from the 20 cm-down layer.

The isolated strategy as in Figure 4-8) for isolation of the hydrocarbon-degrading bacteria was based on enrichment cultures performed in MSM supplemented with 10% diesel. In fact, this culture medium should exhibit high toxicity to bacteria, provided by the 75 g/l hydrocarbons (diesel containing 750 total hydrocarbons, and representing 10% (v/v) of the culture media). Highly resistant bacteria to fresh hydrocarbons were expected to be isolated. The isolation program based on enrichment cultures and screening was oriented to isolate and purify hydrocarbon-degrading bacteria with high degradation potential and tolerance to the toxicity of diesel and crude oil as performed by Al Disi et al. (2017) in other soil samples.
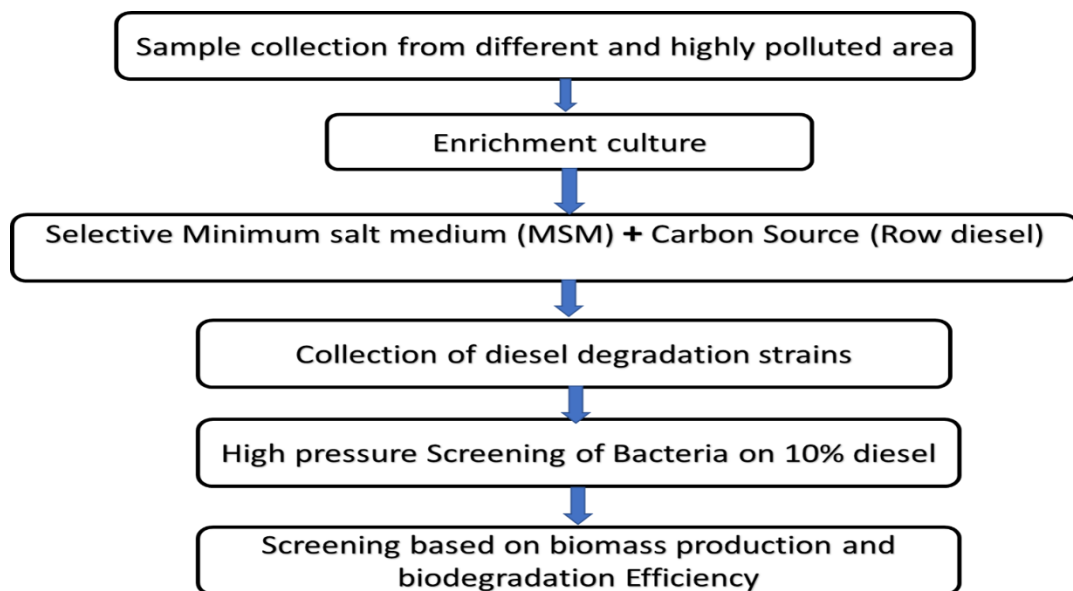
Figure 4-8: The isolated strategy for isolation of the hydrocarbon-degrading bacteria.

A total of 8 strains were isolated from both soil layers sampled systematically from the 9 points of site 8. The purified isolates were identified by ribotyping, based on sequencing the 16S rDNA, and comparison of sequences similarities to available sequences in databases (BLAST). (Table 4-2) shows the list of the isolated hydrocarbon-degrading.

Table 4-2 :isolated bacteria from the upper layer and down layer of soil samples systematically sampled in site 8 of AlZubara beach.

		Code	Identification	Similarity	Accession number
Point 3	Surface	Z3S1	*Bacillus subtilis*	100%	AF549498.1
	20 cm down	None			
Point 4	Surface				
	20 cm down	Z4D1	*Bacillus licheniformis*	100%	LN995452.1
Point 6	Surface	Z6S1	*Providencia rettgeri*	99%	CP027418.1
	20 cm down	Z6D1	*Virgibacillus marismortui*	99%	MF321845.1
Point 7	Surface	Z7S1	*Providencia rettgeri*	99%	CP027418.1
	20 cm down	Z7D1	*Virgibacillus halodenitrificans*	99%	KT945027.1
Point 8	Surface	None			
	20 cm down	Z8D1	*Morganella morganii*	98%	KU942503.1
Point 9	Surface	None			
	20 cm down	Z9D1	*Bacillus circulans*	100%	KY849415.1

In term of biodiversity, it is obvious that the genus *Bacillus* is represented by three isolates each belong to a different species. *Virgibacillus* genus is represented by two isolates belonging to two different species, both isolated from the down layer samples. Two isolates of *Providencia rettgeri* were isolated from surface layers samples of points 6 and

7. Table 4-3 provides reported works from literature, performed with bacteria of the genus and species of the isolated bacteria in this work.

Table 4-3: Some of the reported works performed with isolated hydrocarbon-degrading bacteria.

Strain Name	Reported REFERENCES
Bacillus licheniformis	(Al-Wahaibi, et al., 2016)
Bacillus subtilis	(Joo & Kim, 2013)
Pseudomonas aeruginosa	(Chen, et al., 2018)
Virgibacillus marismortui	Not reported as HDB
Providencia rettgeri	(WANJOHI, MWAMBURI, TOO, ALOO, & KOSGEI, 2015)
Morganella morganii	(DDT Pesticide) (Barragán-Huerta, et al., 2007)
Bacillus circulans	(Das, Mukherjee, & Sen, 2007)

The isolates strains through our program of isolation of hydrocarbon-degrading bacteria are expected to tolerate and degrade high concentrations of hydrocarbons. The high selection pressure would result in the isolation of bacterial strains that are most appropriate for the bioremediation of oil hydrocarbons. However, it seems that it inhibited the growth of other indigenous bacteria from soil samples, since, the corresponding enrichment cultures did not provide any bacterial isolate having the capacity to grow in solid diesel or crude oil through the spray plates technique. This result can also be explained

by the incapability of the lost bacteria to grow in a minimum medium, due to their auxotrophic nature. In both cases, sensitivity: toxicity in the enrichment media or auxotrophy, these bacteria would not be of interest for any further bioremediation strategy.

4.4 Investigation of The Diesel Hydrocarbon Degrading Activity of The Isolated Bacteria

The 8 bacterial isolates were studied for their ability to grow in the mineral salts medium (MSM) supplemented with 10% (v/v) diesel being the sole carbon source. The 10% diesel concentration is considered as a high concentration, in order to differentiate between the strains based on growth and tolerance to toxicity (translated into cell counts) and removal of main categories of diesel hydrocarbons, after two weeks of incubation. Results are presented in Table 4-4).

Table 4-4: Screening of the isolated bacteria from Site 8 in AlZubara beach in MSM medium with 10% diesel. Visual observation of growth of bacteria on crude oil-solid MSM on which 100 microliters fresh crude oil were spread: (1) :(low degradation); (2) :(medium degradation) ; (3):(high degradation) low molecular weight (LMW; nC$_{12}$-nC$_{16}$), medium molecular weight (MMW; nC$_{17}$-nC$_{20}$) and high molecular weight (HMW; nC$_{21}$-nC$_{25}$)

Strain	Solubilization (%)	Emulsification (EU/mL)	CFU (10^7/ml)	CO$_2$ ppm	TPH Ratios	C17/pristane Ratio	C18/phytane Ratio	LMW RE (%)	MMW RE (%)	HMW RE (%)	Crude oil Degradation on solid MSM
Z3S1	0.15	9.9	0.73 ± 0.14	122	38	4.31	8.0	14.74	35.2	33.94	1
Z4D1	0.09	0	2.33 ± 1.43	143.3	19	9.3	5.63	10.44	18.2	24.47	2
Z6D1	0.00	2	0.5 ± 0.01	101.5	22	6.57	2.44	18	26.9	22.27	2
Z6S1	0.05	6.4	1.10 ± 0.25	31.4	21.82	5.97	15.28	16.89	21.71	21.22	1
Z7D1	0.06	2.2	1.90 ± 0.25	198.1	23.45	7.23	4.72	27.0	18.2	28.7	1
Z7S1	0.16	0	1.00 ± 0.25	40	28.6	2.4	10.4	17 .63	22.84	24.48	1
Z8D1	0.00	0	1.03 ± 0.14	36.7	27	15	2.5	16.9	12.8	15.21	1
Z9D1	0.00	6	0.2 ± 0.01	38	23.06	9.0	23.02	13.23	24.01	26.42	2

*LMW: Low Molecular Weight Hydrocarbon, MMW Medium Molecular Weight Hydrocarbon, HMW: High Molecular Weight Hydrocarbon.

The results show that all the strains were able to grow and produce cell biomass at the experimental conditions. However, the produced biomass was clearly highly variable among the strains. The produced CO_2 as a metabolic product determined in the gas phase of the culture, was also variable, but not proportional to the produced biomass. It is also obvious that TPH removal efficiencies were not proportional to the produced biomass and CO_2. As an example, the strain Z3S1 produced 122 ppm CO_2 by a final cfu of 0.73 10^7/ ml ± 0.14 which removed 38% TPH, while Z4D1 produced 143 ppm CO_2 with a cfu of 2.33 10^7/ ml ± 1.43 which removed 19% TPH only (half of that of Z3S1). This result reflects that different metabolic pathways were employed by each bacterium, as metabolic diversity and adaptation processes. TPH removal was also considered a criterion of differentiation between the isolates. Five strains removed 19% to 23% as the largest category, while two removed 27% to 28% representing the second category. Interestingly, the isolate Z3S1 was the most performant isolate to remove 38% TPH. Most the isolates removed 13 to 18% of the low molecular weight hydrocarbons (LMW: nC_{12}-nC_{16}), while the strain Z7D1 removed 27%. The later, was also the efficient to remove 29% of high molecular weight (HMW; nC_{21}-nC_{25}). Many differences of removal of medium molecular weight hydrocarbons (MMW; nC_{17}-nC_{20}) was also clear. The isolate Z3S1exhibited the highest removal efficiencies on MMW and HMW hydrocarbons with almost 35 % removal. Biosurfactants determination was conducted to evaluate the capability of the strains to enhance biodegradation of diesel. Biosurfactants are crucial in bioremediation as they emulsify and solubilize hydrophobic compounds. In general, the measured biosurfactant activity in the culture broth of all the strains was very weak. Since such activity is compulsory for bacteria to interact with hydrocarbons, the obtained results may be

explained by full engagement of the produced biosurfactants with hydrocarbons and removed with the organic phase before analysis or attachment of the biosurfactants to cell walls. When biosurfactants are intracellular in nature they tend to either attach to the cell wall, and or can be excreted (Adamczak & Bednarski, 2000) Intracellular biosurfactants cell structure is made up of membrane lipids whose function is to facilitate e the transportation of insoluble substrates through the membrane. On the other hand, when biosurfactants are extracellular they consist of complex lipids, proteins and carbohydrates which allow them to facilitate substrate solubilization (Andrä, et al., 2006).

The main difference between the two types of biosurfactants is due to the chemical nature of the hydrophilic head varies significantly from one surfactants to another (20). However, the isolate Z3S1 produced the highest emulsification activity with 9.9 (EU/mL) followed by Z6S1 6.4 (EU/mL. For the solubilization activity, although poor, the isolates Z7S1 and Z3S1 exhibited 0.15-0.16 % solubilization. It is then clear that a high diversity of hydrocarbon-degradation activity is also detected among the 8 isolates. Complementary activities may be expected between some of them.

Ratios of n-heptadecane (n-C17) to pristane (Pr) and n-octadecane (n-C18) to phytane (Ph) were to indicate biodegradation assuming that the isoprenoid hydrocarbons pristane (C19) and phytane (C20) volatility is similar to n-C17 and n-C18 and that their disappearance at different rate is a result of a mechanism (i.e. biodegradation) rather than simple evaporation (Horel, Mortazavi, & Sobecky, 2015). Isoprenoids susceptibility to microbial degradation is less compared to n-alkanes of similar M.W. Also, their rate of evaporation and degradation tends to decrease depending on their degree of alkylation (Wang et al., 1998). for example, the isolate Z8D1 showed the highest C17 / pristane ratio

of 15% compared to the control, while each of Z4D1 and Z9D1 showed only 9%. The rest of the isolates showed C17 / pristane ratios from 2.3% to 7%. Moreover, the isolate Z9D1 showed the highest C18/ phytane ratio of (23%) compared to Z6S1 (15%) and Z7S1 (10%), while all other isolates showed C18/ phytane ratios from 2.5% to 8%.

4.5 Investigation of Pollution and Bioremediation Potentialities in The Controlled Dumping Area in Dukhan Oil Activity Site

4.5.1 Pollution Source and Characterization of The Polluted Soil

The site selected to sample oily-polluted soil was in the Dukhan oil industrial area which is a dumping site where solid and liquid oil wastes from oil extraction activity are collected in delimited areas. When the area is jugged full, it is kept in open air for years for self-remediation. The site is highly controlled to prevent any expansion of pollution, nor transfer to groundwater or watercourse. Since our approach is based on the impact of the weathered oil polluting of indigenous bacterial communities in soils at harsh conditions, an area left for three years without any oil wastes feeding was chosen. Systematic sampling was performed in such area, similarly as done at AlZubara sites. In total 9 sampling points were used, and two samples were sampled, one from the surface and one 20-cm down. The major chemical and physical characteristics of the samples were determined Table 4-5.

Table 4-5: Major chemical and physical analyses of the soils sampled from the upper and down layers of soil from Dukhan site.

Component	Surface layer	20-cm down layer
Na (mg/g)	1.890 ± 0.8465	1.884 ± 0.449
K (mg/g)	0.097 ± 0.052	0.096 ± 0.040
Mg (mg/g)	0.114 ± 0.0048	0.113 ± 0.0059
Ca (mg/g)	1.143 ± 0.007	1.148 ± 0.0076
Cl (mg/g)	2.426 ± 0.183	2.428 ± 0.143
Br (mg/g)	0.0572 ± 0.005	0.0564 ± 0.004
NO3 (µg/g)	0.0022 ± 0.0004	0.0023 ± 0.0005
SO4 (mg/g)	0.175 ± 0.011	0.174 ± 0.015
NO2 (µg/g)	0.025 ± 0.003	0.023 ± 0.002
PO4 (µg/g)	0.089 ± 0.005	0.0898 ± 0.0052
Salinity (ppt)	2.39 ± 0.07	2.38 ± 0.04
pH	6.73 ± 0.06	6.75 ± 0.06
TPH (DRO) (mg/kg)	6254.312 ± 285	6477.319 ± 412
TPH (ORO) (mg/kg)	39696.8 ± 1320	40595 ± 1435

In general, there is no significant difference in the overall chemical and physical characteristics between the surface and the down layers in Dukhan, as revealed by Statistical analysis (one-way ANOVA), p Values > 0.05. The TPH (DRO) content in upper

and down layers was 6254.312 ± 285, 6477.319 ± 412 (mg/kg) of sediment, respectively, The TPH (ORO) content in upper and down layers was 39696.8 ± 1320, 40595 ± 1435 (mg/kg) of sediment, respectively Table 4-5. The slightly acidic pH in upper and down layers was 6.73 ± 0.06, 6.75 ± 0.06 of sediment, respectively likely to be favorable for most of the microbes with degradation potential. A low of sulfate content in upper and down layers was 0.175 ± 0.011, 0.174 ± 0.015 (mg/g) of sediment, respectively, with low nitrate content in upper and down layers was 0.025 ± 0.003, 0.023 ± 0.002 (µg/g) of sediment, respectively, with low ammonia content in upper and down layers was 0.0022 ± 0.0004, 0.0023 ± 0.0005 (µg/g) of sediment, respectively and phosphate content in upper and down layers was 0.089 ± 0.005, 0.0898 ± 0.0052 (µg/g) of sediment, respectively were noticed. The high salinity content in upper and down layers was 2.39 ± 0.07, 2.38 ± 0.04ppt of sediment, respectively is an indicator of presence of halophilic and halotolerant microorganisms (Leahy and Colwell, 1990; Sarkar et al., 2016; Roy et al. 2018). A composite soil sample was prepared from the 18 soil samples, which was preserved at 22-25 C in the dark for further experiments along this study.

4.5.2 *Isolation and Potential of Hydrocarbon-Degrading Bacteria from Dukhan*

A total of 16 strains were isolated from both soil layers sampled systematically from the 9 points of Dukhan site. The isolation program based on enrichment cultures and high selection screening was oriented to isolate and purify hydrocarbon-degrading bacteria with high degradation potential and tolerance to the toxicity of diesel and crude oil (Al Disi et al., 2017). The isolation strategy is shown in Figure 4-9. The purified isolates were identified by ribotyping, based on sequencing of the 16S rDNA, and comparison of sequences similarities to available sequences in databases (BLAST).

Table 4-6 shows the list of the isolated hydrocarbon-degrading bacteria with an indication of their corresponding isolation sample and their molecular identification.

Table 4-6:isolated bacteria from the upper layer and down layer of soil samples systematically sampled from Dukhan dumping area.

		Code	Identification	Similarity	Access number
Point 1	Surface	D1S1	*Bacillus sp.*	100%	KY911251.1
	Down	D1D1	*Bacillus sp.*	100%	MG855692.1
	20cm	D1D2	*Bacillus licheniformis*	99%	KY962349.1
Point 2	Surface	D2S2	*Pseudomonas luteola*	100%	KC429633.1
	Down	D2D2	*Bacillus subtilis*	100%	MH071337.1
	20cm	D2D3	*Bacillus subtilis*	100%	MH040981.1
Point 4	Surface	D4S2	*Pseudomonas luteola*	100%	NR_114215.1
	Down 20cm	D4D3	*Pantoea calida*	99%	KX036541.1
Point 5	Surface	D5S1	*Pseudomonas aeruginosa*	100%	KF261029.1
	Down 20cm	D5D1	*Pseudomonas aeruginosa*	100%	KY040017.1
Point 6	Surface	D6S2	*Pseudomonas luteola*	100%	KX301304.1
	Down 20cm		None		
Point 7	Surface	D7S1	*Pseudomonas aeruginosa*	100%	KY040014.1
	Down 20cm	D7D1	*Bacillus sp. Strain*	100%	MG835309.1
Point 8	Surface		None		
		D8D2	*Pseudomonas stutzeri*	99%	MF421776.1
Point 9	Surface	D9S1	*Pseudomonas stutzeri*	99%	KX180912.1
		D9D1	*Pseudomonas aeruginosa*	100%	KF598858.1

In term of biodiversity, it is obvious that the genus *Pseudomonas* is represented by nine isolates each belong to three different species. *Bacillus* genus is represented by six isolates belonging to a different species, both isolated from the down layer samples. one isolates of *Pantoea calida* were isolated from down layers' samples of points 4.

Table 4-7 provides reported works from literature, performed with bacteria of the genus and species of the isolated bacteria in this work.

Table 4-7: Some of the reported works performed with isolated hydrocarbon-degrading bacteria.

Strain Name	Reported REFERENCES
Bacillus sp.	(Rahman, Thahira-Rahman, Lakshmanaperumalsamy, & Banat, 2002)
Bacillus licheniformis	(Al-Wahaibi, et al., 2016)
Bacillus subtilis	(Joo & Kim, 2013)
Pseudomonas luteola	(Atanasković, et al., 2016)
Pantoea calida	Not reported as HDB
Pseudomonas aeruginosa	(Chen, et al., 2018)
Pseudomonas stutzeri	(Lalucat, Bennasar, Bosch, Garcı´a-Valde´s, & Palleroni, 2006)

The hydrocarbon-degrading bacteria were isolated from the Dukhan site, *Bacillus* and *Pseudomonas* genera It may also be evident that some of them could be the same strain, which needs further investigations to differentiate between them. At this stage of the study,

such differentiation was based on the biological activity of each strain. Indeed, diesel and crude oil in MSM medium were used as the sole hydrocarbon sources, at 10% (v/v) corresponding to almost 75 g/l hydrocarbons, to investigate the potential of each strain to tolerate toxicity and grow by removing categories of hydrocarbons (Table 4-8).

Table 4-8 **:** Screening of the isolated bacteria from Dukhan dumping site in MSM medium with 10% diesel. Visual estimation of growth of bacteria on crude oil on solid MSM on which 100 microliters fresh crude oil were spread: (1) :(low degradation); (2):(medium degradation) ; (3):(high degradation)

Strain	Solubilization	Emulsification	CFU		Crude Diesel degradation in MSM liquid						Crude oil
	%	(EU/mL)	10^7	Co_2	TPH Ratios	C17 / pristane Ratio	C18/ phytane Ratio	LMW RE (%)	MMW RE (%)	HMW RE (%)	Degradation on solid MSM
				ppm							
D1D1	0.00	0	0.47 ± 0.14	47	31	9.5	4.4	9.28	19	44	1
D1D2	30.4	6.5	2.67 ± 1.43	33.5	48.05	54.54	65.28	72.60	60.27	80.32	3
D1S1	0.00	0	0.47 ± 0.14	66.1	8.7	2.97	1.99	4.53	0.55	19.2	1
D2D2	0.10	7.6	0.37 ± 0.14	63.3	17.6	18.4	6.5	9.6	24.4	20.2	2
D2D3	0.00	0	0.37 ± 0.14	44.9	16.5	19.4	4.3	2.59	23.2	21.1	2
D2S2	0.06	4.1	0.20 ± 0.25	81.8	21.3	17.4	8	7.5	31.8	33	2
D4D3	0.04	5.9	0.17 ± 0.14	60.6	17.5	7	15.5	15.58	16.3	19.0	1
D4S2	0.16	6.1	0.43 ± 0.14	127.5	24.5	6.2	8.5	19.63	33	17.3	1
D5D1	0.11	278	214.67 ± 13.68	1379.5	42.17	38.3	31.68	4.60	38.30	43.75	2

D5S1	0.00	113	13.67 ± 3.79	440.1	23	10.12	8.70	7.92	19.32	19.90	2
D6S2	0.08	5.2	0.10 ± 0.00	69.9	26.5	3.43	9.8	18.37	29	17.3	1
D7D1	0.08	36.4	1.43 ± 0.38	278.1	17.56	16.46	14.17	22.74	19.32	19	2
D7S1	0.18	116	65.33 ± 3.79	693.6	17.05	13.23	9.01	7.21	19.87	18.87	2
D8D2	0.08	2.1	0.60 ± 0.25	78.8	19.2	14.25	5.40	10.68	17.98	34.69	2
D9D1	0.01	178	27.33 ± 3.79	894.2	19.16	25.17	19.49	9.64	19.11	17.10	2
D9S1	0.05	12.1	4.67 ± 1.43	344	14.72	5.43	3.6	28.59	14.05	15.25	3

*Low molecular weight (LMW; $n\mathrm{C}_{12}$-$n\mathrm{C}_{16}$), medium molecular weight (MMW; $n\mathrm{C}_{17}$-$n\mathrm{C}_{20}$) and high molecular weight (HMW; $n\mathrm{C}_{21}$-$n\mathrm{C}_{25}$).

All the strains were able to grow at the experimental conditions and produce living cell biomass. But, the potential of the strains to produced new cell biomass was highly variable from one strain to another, even within the same genus-group of isolates. Similarly, the produced CO_2 determined as one metabolic product of the culture, was also variable, but not proportional to the produced biomass. Produced biomass and CO_2 were not proportional to removal efficiencies of TPH. As an example, the strain D1D1 produced 47 ppm CO_2 by a final cfu of 0.47 10^7/ ml ± 0.14 which removed 31% TPH, while D1D2 produced 33 ppm CO_2 with a cfu 2.67 ± 1.4 10^7/ ml which removed 48% TPH. This result reflects that different metabolic pathways were employed by each bacterium, as metabolic diversity and adaptation processes. TPH removal was also considered a criterion of differentiation between the isolates. Interestingly, D1D2 (*Bacillus licheniformis*) and D5D1 (*Pseudomonas aeruginosa*) were high to remove 48% and 42 % of TPH, respectively, at the experimental conditions. Removal efficiencies of TPH with most of the isolates were fluctuating between 16% and 26%. One isolate removed 8% TPH only. Most the isolates, including D5D1, removed less than 20% of the low molecular weight hydrocarbons (LMW: nC$_{12}$-nC$_{16}$), except D1D2 which was highly active with 72% removal. While all strains were shown moderately degraders of MMW and HMW hydrocarbons (less than 30% removal), D1D2 is also highly active on both hydrocarbons ranges (60% and 80%, respectively) and D5D1 removed 38%-43%. Biosurfactants production was also shown very poor in the supernatant of the cultures. Similar hypotheses than those formulated for bacteria isolated in AlZubara sites, may be drawn. The biosurfactants of the isolated bacteria should be intracellular thusa promote the transportation of insoluble substrates through the membrane the isolate D5D1 showed the highest emulsification activity with

278 (EU/mL) then D9D1, D7S1, and D5S1 with 178, 116 and 113 (EU/mL) respectively. For the solubilization activity, the isolate D1D2 produced a high activity with a 30.4% solubilization.

It is then clear that a high diversity of hydrocarbon-degradation activity is also detected among the 16 isolates. It was found out that the isolates purified through this program can tolerate and degrade high concentrations of hydrocarbons. The high selection pressure would result in the isolation of bacterial isolates that are most appropriate for the bioremediation of oil hydrocarbons. Complementary activities may be expected between some of them.

The isolate D1D2 showed the highest C17 / pristane ratio of (58%) compared to the control, while each of D5D1 (38.3%). The rest of the isolates showed C17 / pristane ratios from (2.4%) to (25%). Moreover, the isolate D1D2 showed the highest C17 / pristane ratio of (58%), while each of D5D1(31.68), while all other isolates showed C18/ phytane ratios from (2.5%) to (23%).

4.6 Extrapolation of Hydrocarbon-Degrading Bacterial Diversity and Adaptation to Weathered Oily-Soils and Harsh Conditions in, Qatar

In order to gather more evidences of metabolic adaptations and enrich our collection with interesting, highly adapted bacteria from Qatari weathered oily soils, the isolation of bacteria was extended to stored liquid and solid wastes in barrels at AlShaheen field, or local auto-workshops at not controlled weather condition. Sampling was done from seven highly oil-contaminated areas. Three sites (namely: D, B, and C) were selected in an industrial area in Qatar. To ensure that hydrocarbons are weathered and that the indigenous bacteria have

adapted to the conditions the Oily-polluted wastes were exposed to extreme weather for more than 5 years. These locations included auto-workshops contaminated with lubricants, diesel, and gasoil. Three other different barrels containing oil-wastes were also used for sampling a composite sample.

4.6.1 *Isolation of Hydrocarbon-Degrading Bacteria from Wastes Stored At Alshaheen Field and Auto-Shops*

As a first step, enrichment cultures were manufactured using 2.5 g sample in a Luria-Bertani (LB) liquid medium. Cultures were incubated during 3 days with shaking at 300 rpm and 30ºC. In a second step, a 25 mL minimum salt medium (MSM) liquid containing 2 mL of the first-step enrichment cultures (LB), were supplemented with 1 mL diesel as the unique carbon source and incubated for one week. Then, 2 mL of the obtained culture was used to inoculate 25 mL of fresh MSM medium as a new step of enrichment. The later step was repeated three times, and the obtained final culture was used to isolate and purify the enriched bacterial strains. The enrichment culture protocol allowed only isolation of hydrocarbon-degrading bacteria that are able to tolerate a high toxicity level. The 29 g/L added diesel hydrocarbon in the culture media is a rather high concentration. The results of Table 4-9 show that only 2 to 3 hydrocarbon-degrading bacterial isolates were isolated from each soil sample. This could be explained by the fact that oil weathering processes in soil such as those investigated previously by AlKaabi et al. (2017) strongly impact the abundance of bacteria. Purely based on Darwinistic expectations, these bacterial strains might be highly adapted to the existing weathered hydrocarbons. The adapted bacteria have the potential for application in bioremediation of soil to which they are adapted under the right circumstances. The stress conditions of warm weather for an

extended period of time, these bacteria will acquire and implement the ability to sustain in oil-polluted soils.

The identification of the isolates was performed by MALDI TOF technique, a rapid technique used for bacteria identification, recently introduced at Hamad Hospital (Qatar). MALDI-TOF MS was selected being a rapid and reliable technique to identify and differentiate the 7 bacterial isolates from the weathered soils Table 4-9. Preliminarily, microscopic observations and Gram staining showed high similarity among most of the isolates. The identification procedure showed that five isolates contain *Bacillus cereus*, and one isolate contains *Pseudomonas stutzeri*. Using "Probable Species-Level identification" procedure it was found out that six isolates were higher than 2. The isolate D1 was identified at the genus (*Bacillus*) level only, with a score corresponding to "Probable Genus Level". This may be due to the extensive exopolymeric substances (EPS) secretion by this isolate, disturbing protein profiling which represents the limitation of the MALDI TOF MS technique of identification of microorganisms. An additional attempt of identifying the bacterial strain D1 was made by ribotyping (16sRNA sequencing). This did indeed A showed that D1 consists of *Bacillus sonorensis* with a similarity (99%) to the reference BLAST database. Moreover, the proteins profile of each strain was determined showing high diversity among the *B. cereus* isolates. The isolates B2 and C2 (both are *Bacillus cereus*) showed highly matched profiles, meaning that they are highly similar, and could be identical. Moreover, proteins profiling of D1 was not successfully performed. MALDI-TOF MS was then concluded as not appropriate for identification of D1.

Table 4-9:Identification of the isolates and the corresponding produced biomass (10^7 cfu/ml) after 1 and 2 weeks incubation in MSM liquid medium with 5% or 10% supplemented diesel.

Isolate	Identification (Score of MALDI)	Cultures in 5 % diesel		Cultures in 10 % diesel	
		One week incubation	Two weeks incubation	One week incubation	Two weeks incubation
D1	*Bacillus sonorensis* (-Score: 1.87, PGI -Ribotyping: similarity 99%)	5.5± 0.2	5.8± 0.3	5.8± 0.2	5.6± 0.2
D2	*Bacillus cereus* (Score: 2.23, PSLI)	5.3± 0.2	5.4± 0.2	5.5± 0.2	5.7± 0.2
D3	*Pseudomonas Stutzeri* (Score: 2.09, PSLI)	5.2± 0.2	5.5± 0.3	5.7± 0.2	5.4± 0.2
B1	*Bacillus cereus* (Score: 2.11, PSLI)	6.2± 0.3	6.6± 0.3	6.6± 0.2	6.9± 0.2
B2	*Bacillus cereus* (Score: 1.91, PGI)	4.1± 0.2	4.3± 0.2	4.6± 0.1	4.8± 0.1
C1	*Bacillus cereus* (Score: 2.11, PSLI)	3.7± 0.2	3.9± 0.2	3.2± 0.1	4.1± 0.1
C2	*Bacillus cereus* (Score: 2.23, PSLI)	5.1± 0.2	5.2± 0.2	5.9± 0.2	6.2± 0.2

*PSLI: Probable Species-Level identification; PGI: Probable Genus identification.
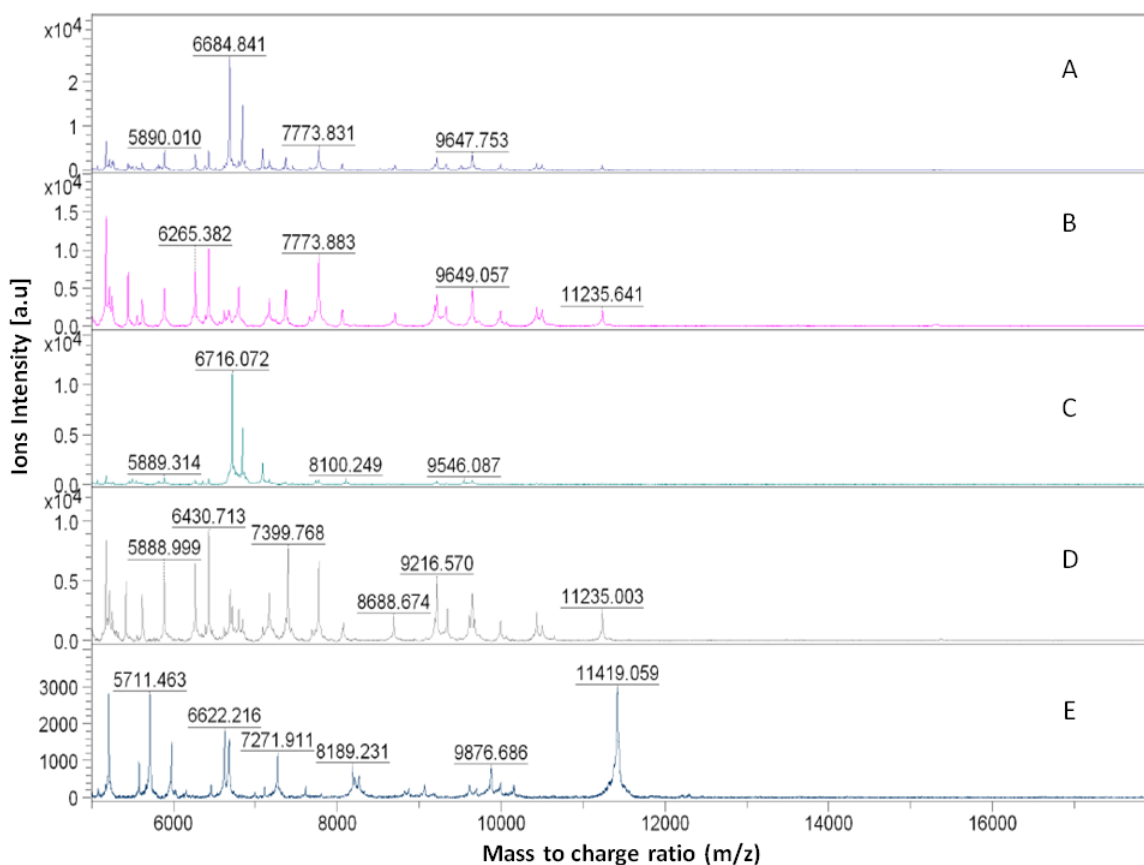
Figure 4-9: Mass spectra, profiling the isolated strains, using MALD-TOF-MS Typing. A – Isolate D2, B – Isolate B1,C – Isolates B2 and C2, D – Isolate C1, E – Isolate D3

4.6.2 Screening of Bacterial Isolates Based on Growth in 5% and 10% Diesel Media

In order to assess the diversity between the isolated strains and their growth potential using hydrocarbons as carbon source was cultured in MSM with 5% and 10% diesel. At such high diesel concentrations, a high level of toxicity was ensured. Bacterial growth was evaluated after 1 and 2 weeks and removal of the three categories of diesel hydrocarbons after 2 weeks (Table 4-9 and Table 4-10). Growth of the isolates may be based on partial or complete degradation of components of diesel. The results show that the four isolates produced 5.1×10^7 cfu/mL to 5.5×10^7 cfu/mL. Statistical analysis using

ANOVA at 95% confidence showed that the differences in this productivity are not significant. One *B. cereus* isolate (B1) gave 6.2×10^7 cfu/mL after one incubation, which is significantly higher than all the other isolates by the same incubation period, as per the ANOVA statistical analysis. Two other *B. cereus* isolates grew much less than all the others as concluded based on the statistical analysis. Moreover, the accumulated biomass after 1 or 2 weeks in 5% and 10% diesel were considered statistically insignificant for all the isolates; showing that some toxicity was exhibited at such concentrations, or a carbon catabolite repression may be exerted as a consequence of increasing substrate concentrations. This may also be a result of shifting the metabolism of the hydrocarbon-degrading bacteria as demonstrated for *Pseudomonas aeruginosa* by AlDisi et al. (2017).

Biodegradation and/or bioconversion of diesel-hydrocarbons was evaluated by GC-FID analysis of the three ranges of hydrocarbons based on their MW, in the remaining diesel fraction separated from the cultural media (MSM medium). The chromatogram of raw diesel was divided into three ranges of molecular weights of hydrocarbons, based on the retention time: low molecular weight (LMW; nC$_{10}$-nC$_{16}$), medium molecular weight (MMW; nC$_{17}$-nC$_{20}$) and high molecular weight (HMW; nC$_{21}$-nC$_{25}$). The removal efficiencies (RE) of each range of hydrocarbon was calculated based on separate integration of the corresponding area in the experiment and compared to the control (Table 4-10). According to the GC analysis results it is clear that biological activity of the isolated bacteria led to degradation of diesel hydrocarbons having a concentration of 5% and 10%. In addition, hydrocarbon removal efficiencies obtained at 5% and 10% diesel concentrations were statistically similar, explaining the similar produced biomasses (cfu) as shown in Table 4-10. Based on the statistical analysis by ANOVA (95% confidence

level), each isolate showed a different activity pattern. The two isolates B2 and C2 which showed similar MALDI TOF MS proteins profiles, exhibited some differences in their biological activities, although having the similar activity on MMW. While B2 showed 44% activity on LMW hydrocarbons and 34% on HMW ones, C2 exhibited the opposite. The isolate D1 (*B. sonorensis*) showed the highest activity on the three ranges of hydrocarbons. 85% to 89% of HMW hydrocarbons were removed or converted by *B. sonorensis* (D1) *and B. cereus* (D2). Moreover, most of the isolated bacteria were highly active on the HMW hydrocarbons, which may be related to their adaptation to the weathered oil from where they were isolated.

Table 4-10: Hydrocarbon Removal Efficiencies (% RE) of hydrocarbons grouped in the three ranges, by the isolated strains cultured in MSM medium with 5 % and 10% diesel and after two weeks incubation

Removal efficiency (%)	MSM-5% Diesel	MSM-10% Diesel
Isolate D1 (*Bacillus sorensis***)**		
LMW Hydrocarbons	78 ± 2.85	82 ± 3.20
MMW Hydrocarbons	61 ± 2.44	63 ± 2.78
HMW Hydrocarbons	84 ± 3.12	90 ± 3.32
Isolate D2 (*Bacillus cereus***)**		
LMW Hydrocarbons	46 ± 1.85	44 ± 2.55
MMW Hydrocarbons	59 ± 1.96	51 ± 2.75
HMW Hydrocarbons	83 ± 3.18	89 ± 2.92
Isolate D3 (*Pseudomonas Stutzeri***)**		
LMW Hydrocarbons	49 ± 2.72	50 ± 2.84
MMW Hydrocarbons	45± 2.54	42 ± 2.94
HMW Hydrocarbons	82 ± 3.05	84 ± 3.22
Isolate B1 (*Bacillus cereus***)**		
LMW Hydrocarbons	65 ± 2.62	64 ± 2.76
MMW Hydrocarbons	43 ± 1.65	41 ± 1.86
HMW Hydrocarbons	61 ± 2.58	64 ± 3.08
Isolate B2 (*Bacillus cereus***)**		
LMW Hydrocarbons	35 ± 2.05	34 ± 1.84
MMW Hydrocarbons	40 ± 1.92	41 ± 2.12
HMW Hydrocarbons	41 ± 1.76	44 + 1.54
Isolate C1 (*Bacillus cereus***)**		
LMW Hydrocarbons	32 ± 1.88	30 ± 1.94
MMW Hydrocarbons	34 ± 1.62	30 ± 1.58
HMW Hydrocarbons	40 + 2.10	34 ± 1.92
Isolate C2 (*Bacillus cereus***)**		
LMW Hydrocarbons	45 ± 2.54	44 ± 2.25
MMW Hydrocarbons	43 ± 2.66	41 ± 2.36
HMW Hydrocarbons	31 ± 1.95	34 ± 2.12

4.7 **Conclusion**

From this study, it is clear that Qatar can make great strides if it makes use of bacteria to clean up the environment. Qatari region is hot with acidic soil and an oily zone. These factors alone make the use of hydrocarbon-degrading bacteria in Qatar very promising method of bioremediation. If Qatar employs hydrocarbon-degrading bacteria, the ecosystem will remain balanced as well as the biodiversity. This study has performed deep analysis of Qatari environmental and ecological conditions to lay a foundation for any further research. The best of these studies was the investigation of microbial communities living in many polluted soils with hydrocarbons.

The oil spills from two locations in northwestern Qatar were analyzed by GC–MS and CHEMSIC method and it was concluded that the two originated from at least two different sources. Crude oils samples obtained from Saudi Arabia and Kuwait matched with oils in the University of Copenhagen oil database. Solidified heavily weathered oil was found to contain relatively unweathered oil as it prevented the interior oil from degrading any further. it was concluded that heavily weathered oil was from Gulf War proving that oil degradation is a very slow process. Moreover, from the analysis it was found that there were recent oil spills which indicate that Al Zubarah area is still experiencing oil spills.

The establishment of an isolation and screening of highly resistant hydrocarbon-degrading bacteria from Al-Zubara beach, Dukhan dumping site and many other representative sites showed limited biodiversity in term of Genus and Species. But, by studying the potential of each strain to degrade hydrocarbons, it was obvious that a high diversity was observed in the metabolic activity of the isolates even within the same group

of genus or species. These findings could allow to conclude that as initially postulated, the harsh conditions of soil and weather and the high weathered structure of the polluting hydrocarbons would lead to adaptation routes employed by each isolate to survive and overcome the high toxicity. The strategy of isolation we employed from the beginning of the study was highly helpful to achieve this task with microbial and metabolic evidences. The potential of local microbial strains to provide special and interesting biological activity due to their adaptation to extreme temperatures, desiccation and salinity are a big positive for Qatar. The most valuable thing about the current study is that it should be expected that each site polluted with oil components should be bioremediated by the intrinsic hydrocarbon-degrading bacteria, to overcome long adaptation periods and provide the appropriate metabolic activities to interact specifically with the existing pollutants. Here, the question which may asked is about the role of soil conditions and characteristics, the interaction soil-pollutants, the interaction bacterium-bacterium and the need for nutrients to succeed bioaugmentation-biostimulation approaches in Qatar. The soil sampled fronm Dukhan was selected to perform further studies to answer to such questions.

Chapter 5 **: APPLICATION OF BIOAUGMENTATION AND BIOSTIMULATION FOR THE REMEDIATION OF WEATHERED OILY-POLUTED SOILS FROM DUKHAN IN BIOPILE SYSTEM**

5.1 **Introduction**

Oil Weathering Processes affect biodegradation, by changing the properties and composition of the oil. As a result, oil contamination is classified as one of the most harmful and dominant sources of environmental deterioration in industrialized countries. The harsh conditions related to weather and soil characteristics accentuate the weathering processes leading to the failure of most of the bioremediation applications procedure to clean areas polluted with hydrocarbons. Moreover, weathering processes is more noticeable in arid areas such as in Gulf area. Polluted areas constitute a set of biotic and abiotic factors used a guide to select an effective bioremediation strategy of the corresponding pollutants. Pollution with hydrocarbons can easily destroy indigenous microorganisms, affect soil quality, production, and decline species richness. The higher the amount and concentration, and the longer the contamination is preserved into the environment, the harder the treatment mechanism is achieved, and the higher health risks on living creatures are anticipated. Contaminated soil treatment is very expensive. Cost effective mechanisms are being investigated to treat this threatening issue (Agamuthu et al. 2013). For this reason, bioremediation and biotechnology tools are introduced to accelerate and remove the pollution using *in-situ* or *ex-situ* process. The open biopile system technology is a cost-effective model to treat oil contaminated soils. Consequently, this study aimed to investigate the use of this technology in Qatar since oil and gas production are significant

in the country.

As such, there is need for further research to be conducted to characterize hydrocarbons-degrading bacteria and their metabolic activities, especially in harsh conditions. In defined media, selected bacteria can be shown highly efficient on the hydrocarbons provided as substrates. This is performed in the Chapter 4 of this work. It was then concluded about some selected bacteria, but not on their potentials to be implemented as unique or in mixed cultures. In this chapter, the polluted soil in the dumping site of Dukhan industrial area was used to develop approaches for removal of weathered hydrocarbons by bioremediation/biostimulation. The objectives of this work are to:

- Establish an open biopile system technology (*ex-situ*,) to study bioremediation of polluted Dukhan soils.

- Biostimulate growth of degrading-bacteria using nutrients, able to enhance the biodegradation process and degradation mechanism.

- Evaluate the performance of exogenous or indigenous bacteria able to biodegrade the hydrocarbons contaminating the soil as a bioaugmentation mechanism.

- Apply hydrocarbon-degrading bacterial consortia using selected bacteria from polluted soils in Qatar.

- Evaluate the efficiency of bacterial consortia in bioaugmentation/biostimulation approaches.

5.2 Physical and Chemical Characterization of The Soil Before Biopiling

5.2.1 Determination of The Physical Characteristics of The Soil

Biopiling requires adjustment of the chemical composition of the soils in order to provide hydrocarbon-degrading microorganisms with the optimal nutritional requirements (Benyahia & Embaby, 2016). Using multiple techniques, reported in the Material and Methods section, the composition of polluted soil was characterized, and the results reported in reported in Table (5-1).

Table 5-1: Chemical and physical characteristics of the oil-polluted soil used in biopiling.

Component	Amount
Nitrogen %	0.05±0.04
Carbon %	21.43±0.21
Hydrogen %	2.00±0.13
Carbon + Hydrogen %	23.43 ±0.34
Sulfur %	0.00± 0
Ash %	2.83±0.26
DM %	93.52±0.08
Moisture %	6.48±0.08
pH	7.52±0.02
TPH (DRO) (mg/kg)	3066.99 ± 198
TPH (ORO) (mg/kg)	402.51 ± 16

As expected, the carbon and hydrogen content of the soil is very high, representing almost 23%, of the dry matter, showing the high level of pollution. The soil does not contain nitrogen, which is also expected. The corresponding C/N ratio is 21.4/0.05 which

is not appropriate for microbial grow in bioremediation systems. The optimal C/N ratio should be fluctuating between 100/10 to 100/20. The sulfur content was not detectable. The pH was near neutrality. Using GC (FID) analysis techniques, the extracted compounds by hexane showed a Diesel Range Organics (DRO) content of 3066.99 ppm and using GC (MS) analysis technique, the extracted compounds by dichloromethane showed a total PAHs of 402.51 ppb, which are considered mostly as weathered hydrocarbons since the oil-polluted soil was exposed for more than three years at harsh conditions. Normally, all the volatile molecules were volatilized and/or degraded.

5.2.2 *GC-MS for Weathered Oil-Polluted Soils from Dukhan in Biopiles.*

GC-MS analysis revealed the absence of the peaks corresponding to nC13-nC15, which confirms that soil is weathered (Wang & Fingas, 1995). Figure 5-1 shows a GC-MS Chromatogram of soils used for biopiles.
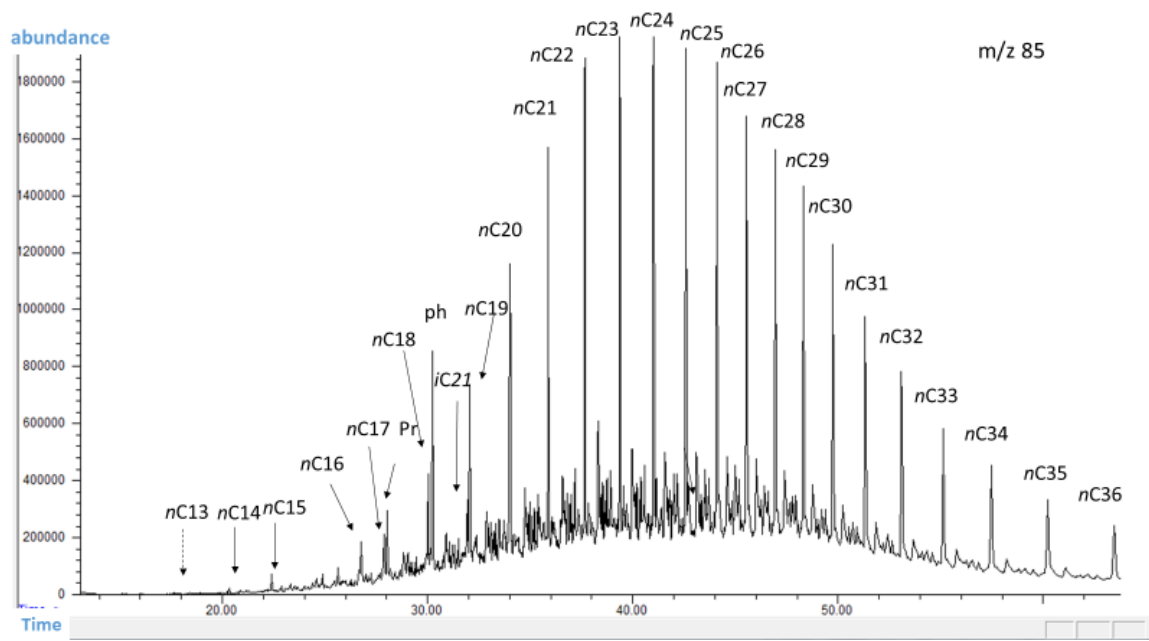
Figure 5-1: SIM of m/z 85 of soils used for biopiles.

5.2.3 *Bioremediation of the Weathered Oil-Polluted Soils from Dukhan in Biopiles*

In order to optimize the *ex-situ* technique in laboratory conditions, the biopiling system was used. The biopiles contained 685 g of homogenous soil, sieved with 2 mm pore size. Moisture in the samples was adjusted to 10 % and 13.5 % by adding 70 ml and 100 ml distilled water, respectively. The biopiles were incubated at room temperature, or at 37 C^o and growth of indigenous bacteria was periodically followed. GC analysis was taken

125

before and after treatment to determine DRO and PAHs content in the soil. The FTIR analysis was also carried out before and after analysis.

5.2.4 *Bioremediation of The Oil-Polluted Soils in Biopiles Using Urea as A Nitrogen Source for Biostimulation of Indigenous Bacteria*

The C/N/P ratios were adjusted at a first step by adding urea as nitrogen source containing 46.7 % Nitrogen and potassium phosphate containing 22 % Phosphorus at a C/N/P ratio of 100/10/1. The C/N/P ratios (100/10/1, 10/5/1, 100/5/0.5) and moistures were studied as indicated in Table 5-2. Urea was used as a nitrogen source due to the ability of all the indigenous bacteria isolated from Dukhan soils, to digest urea secreting urease activity. This was previously demonstrated in our laboratory by colleagues. All treatments at room temperature remained fairly constant until 90 days, an inhibition of growth using urea as a nitrogen source was clearly seen especially after the treatment reached 90 days where a drop of colonies formation units was at its minimum in the biopiles with C/N/P content of 100/10/1. For the C/N/P ratio 100/10/1 at 37 ℃, an inhibition of colonies formation was seen directly only after 10 days (not shown, performed in the Lab). The pH of all soils increased reaching a value 9.51.

DRO and PAH removal was evaluated at the beginning and after 90 days incubation. Results are shown in Table (5-2). DRO and PAH values did not see a dramatic decrease as the most biodegradation had been clearly shown in the control by the indigenous bacteria already inhibiting the soil. The biodegradation when comparing it to time zero until 160 days had 6.78 % DRO and 0.46 % PAHs removal. The pH was also optimal 7.28$\pm$0.5 after 90 days. On the other hand, it seemed that 10 % or 13.5 % humidity did not make much difference for biopiling of the polluted soil when comparing the

126

biopiles of C/N/P ratios 100/10/1. The DRO values were approximately the same around 1.4 % removal, and PAH values around 0 % meaning there was no degradation of PAHs compared to the control at time zero. Also, the temperature changes from room temperature to 37° C did not seem to enhance the biodegradation of the hydrocarbons, an aerator was introduced as well to the same biopile making no difference in the decrease of DRO and PAHs.

Table 5-2: DRO and PAHs content at the end of the treatment using urea as a nitrogen source with different C/N/P ratios.

C/N/P	Moisture (%)	Total DRO (%removal)	Total PAH (%removal)	pH
Control: 100/0.23/0(23 °C)	10	6.78	0.46	7.28±0.5
100/10/1 (23 °C)	10	1.44	0.00	9.35±0.32
100/5/1 (23 °C)	10	3.35	0.34	9.33±0.25
100/5/0.5 (23 °C)	10	0.14	0.21	9.36±0.21
100/10/1 (37 °C)	13.5	1.45	0.02	9.30±0.07

5.2.5 *Bioremediation of The Oil-Polluted Soils in Biopiles Using Ammonium Nitrate as A Nitrogen Source for Biostimulation of Indigenous Bacteria*

In the previous study, urea was used as nitrogen source, but it served also as carbon source when hydrolyzed. Potentialities of bacteria to hydrolyze urea was mainly dependent on their ability to secrete the enzyme named urease. Urea was normally hydrolyzed into ammonium and an organic acid. Ammonium was the responsible factor for pH increase if not totally used by bacteria as nitrogen source. To study the effect of the nitrogen source on biopiling, we compared urea to ammonium nitrate at the same C/N/P ratio of 100/10/1, temperature conditions 23°C and humidity 10%. The results are illustrated in Table 5-3. The findings clearly shown that the increase of pH in the soil was due to the use of urea. The growth obtained with ammonium nitrate was not inhibited and pH 7.12 ± 0.04 was not increased.

Table 5-3: Comparison of the effect of urea and ammonium nitrate as nitrogen sources with C/N/P ratio of 100/10/1.

Biopile	Moisture (%)	Treatment time (days)	Total DROs (%removal)	Total PAHs (%removal)	pH
Control 100/0.23/0 (23 ºC)	10	160	6.78	0.46	7.31+0.50
Urea 100/10/1 (23 ºC)	10	120	1.44	0.00	9.35+0.32
Ammonium Nitrate 100/10/1 (23 ºC)	10	42	7.38	7.99	7.12+0.04

Table 5-3 shows that the biodegradation was successful when using ammonium nitrate as a nitrogen source although the time of treatment was only 42 days with ammonium nitrate. It was clearly proved that the values of DRO and PAHs had decreased by 7.38 % and 7.99 % respectively compared to the control (6.78 % and 0.46 % only) after 90 days of treatment. This means that ammonium nitrate accelerated the process of degradation more than 4 times compared to the control. The nitrogen source helped in the acceleration of the biodegradation of the weathered hydrocarbons, making the difference between urea and ammonium nitrate as a nitrogen source very noticeable. The ammonium nitrate shown the advantageous effect in helping the indigenous bacteria in accelerating the biodegradation process in a short amount of time, with concerns of increasing the pH of the soil.

5.2.6 *Bioremediation of The Oil-Polluted Soils in Biopiles Using Tween-80 as Surfactant an Ammonium Nitrate as Nitrogen Source*

In the previous study it was clear that ammonium nitrate was successful in the biodegradation process and the number of hydrocarbons had been reduced but it remained high in the soil. However, further improvement is necessary to be implemented. Surfactants have proven to accelerate the biodegradation activities due to their hydrophobic and hydrophilic abilities, as they are able to reach the hydrocarbons inside the soil and help the indigenous bacteria found in the soil in the degradation of hydrocarbons process. Tween-80 is the surfactant that was used during this study with 0.4 ml. Table 5-4 shows the ability of surfactants in further decreasing the DRO content with the help of ammonium nitrate as a nitrogen source. The results of the treatment after 90 days showed an 8.27% removal compared to 7.38% removal without surfactant. On the other hand, the PAHs content

remained fairly constant with both treatments (7.99% and 7.64% without surfactants and with surfactants, respectively). The pH remained also in the acceptable range of approximately 7.1 for both treatments. The colony formation provides better understanding of the reason behind the drop of DRO values in the biopile containing surfactant (performed in our Lab). Indeed, it was shown that Tween-80 accelerated the process of biodegradation after 14 days whereas after 70 days both biopiles with and without Tween 80: 0.4 ml reached the same values of CFU (Oualha, M. 2017).

Table 5-4: Comparison of the effect of surfactant tween 80

Biopile	Moisture (%)	Treatment time (days)	Total DROs (% removal)	Total PAHs (%removal)	pH
Control (23 °C)	10	160	6.78	0.46	7.31+0.5
Ammonium Nitrate (23 °C)	10	42	7.38	7.99	7.12+0.04
Ammonium Nitrate with tween: 0.4 ml (23 °C)	10	42	8.27	7.64	7.14+0.09

When associating the results of DRO and PAH removal and pH in the biopiles, a trend can be identified and a confirmation of the success of combination of ammonium nitrate with surfactants is considered promising for the bioremediation of Dukhan soils.

5.3 Investigation of Dukhan Polluted Soil by Biostimulation/Bioaugmentation Using Exogenous Bacteria Isolated from Qatari Soils

Bioaugmentation/stimulation approaches were evaluated to assess the potential of exogenous selected bacteria to remediate the weathered hydrocarbons in Dukhan dumping site soil under study. The objective of the study was to determine the potentialities of local bacteria to remediate weathered oil at local conditions. Indeed, in the previous chapter, 7 isolates were isolated from highly polluted oil wastes from Dukhan solid wastes, auto-shops and AlShaheen residues and showed highly active on Diesel Hydrocarbons as illustrated on Table 4-10. The diversity of these bacteria and their corresponding isolation site was the basis of their use in a first step to investigate the bioremediation/biostimulation of Dukhan soils. Soils without prior contact with oil components, was used as control to assess the ability of the bacteria to remediate fresh oil at a DRO concentration of 584 ± 7 ppm and PAHs in concentrations of 308 ± 8 ppb. The weathered soil contained much more contaminant with DRO of 3067 ± 67 ppm DRO and 403 ± 9 ppb PAHs.

The results of Table (5-5) clearly show the high performance of the isolate D1 (*B. sorensis*) to remove 88% DRO from the freshly oil polluted clean soils within 90 days. This was translated into a biomass production of 7.2×10^7 cfu/g soil which means that the employed conditions were appropriate for growth by hydrocarbons degradation with a C/N/P ratio of 100/10/1, moisture of 10% and 1.1 mL/kg surfactant (Tween-80). The capabilities of D1 to grow in polluted soils with weathered oil hydrocarbons were shown to be much lower: produced biomass (8.2×10^6 cfu/g) and DRO removal of 25%, in the same period of 90 days. The other isolates were also effective on fresh oil with removal

efficiencies ranging from 80 % to 86% DRO. Their individual capabilities in weathered

soils were much lower than that in fresh oil and, statistically lower than those of D1.

Table 5-5: Efficiencies of the isolated bacteria in removal of DRO in weathered soils and
freshly oil contaminated soils.

	Oil-contaminated soil		Clean soil	
	DRO removal (%)	Final CFU (10^5/g soil)	DRO removal (%)	Final CFU (10^7/g soil)
Isolate D1 (*B. sorensis*)	25 ± 2.68	82 ± 4.58	88 ± 4.22	7.2 ± 0.26
Isolate D2 (*B. cereus*)	13 ± 1.72	17 ± 3.52	82 ± 3.96	3.3 ± 0.28
Isolate D3 (*P. stutzeri*)	9 ± 2. 55	55 ± 3.62	86 ± 4.12	1.9 ± 0.31
Isolate B1 (*B. cereus*)	11 ± 2.58	3 ± 0.88	83 ± 3.90	3.9 ± 0.29
Isolate B2 (*B. cereus*)	8 ± 1.74	2 ± 0.52	80 ± 4.47	1.8 ± 0.22
Isolate C1 (*B. cereus*)	12 ± 2.73	2 ± 0.17	81 ± 4.22	2.8 ± 0.28
Isolate C2 (*B. cereus*)	8 ± 2.25	1 ± 0.51	80 ± 4.45	1.9 ± 0.27

The FTIR analysis carried-out on soils samples from the biopiles performed with clean soil, supplemented with fresh oil, clearly confirmed that all the isolates strongly modify the organic composition, which is correlated to the high produced biomass and removal efficiencies of all oil components in liquid cultures. Figure 5-2 illustrates a representative result obtained with the strain D1 (*B. sorensis*). Figure 5-2 shows the FTIR spectra with soils from the control-biopile and D1-biopile using the weathered soil, indicating that D1 was responsible of inducing substantial changes in the hydrocarbon fingerprint of the polluted soil. The peaks at the wavenumbers 2921 cm^{-1}, and 2849 cm^{-1} completely vanished after the reaction. When associating the results of FTIR with DRO and CFU, a trend can be identified and a confirmation of the success of biodegradation using ammonium nitrate and Tween80 as surfactant with the isolate D1 in weathered soil.

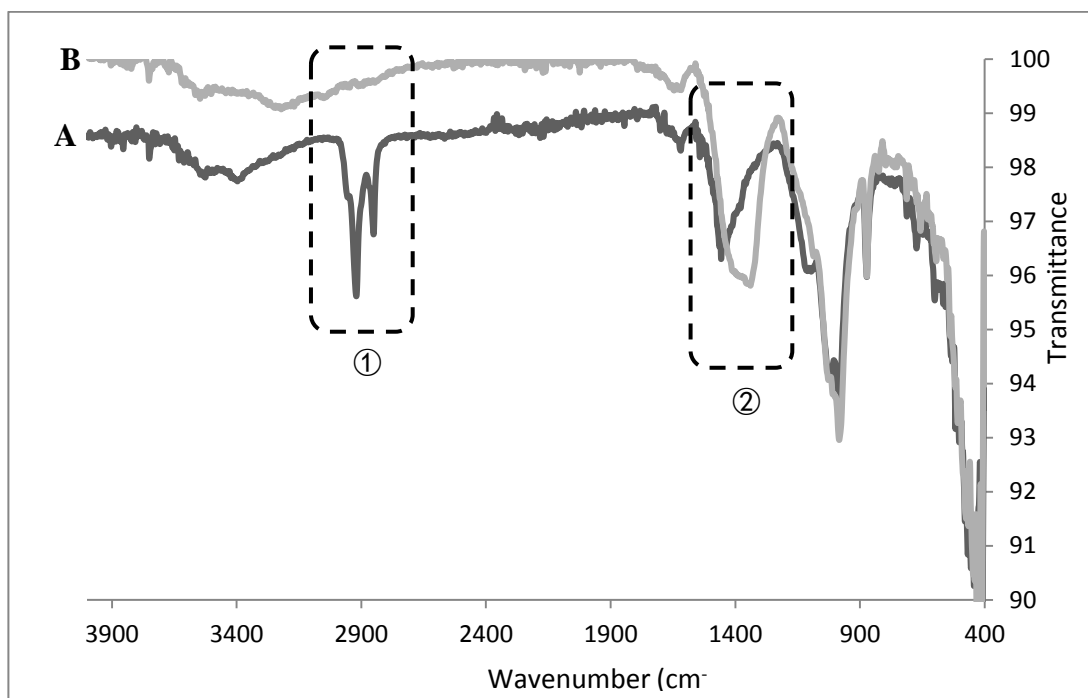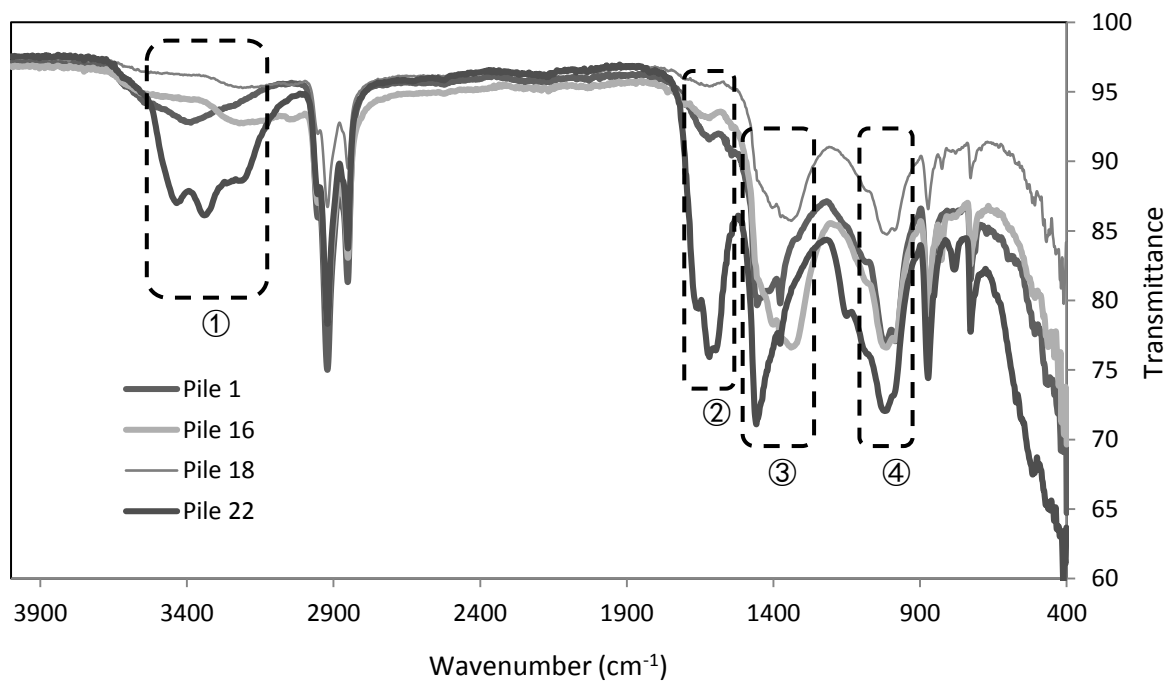Figure 5-2: FTIR spectra of soils with: (A) fresh oil and (B) treated with D1.

Figure 5-3:FTIR spectra of soils polluted with weathered soil (Control: pile 22), inoculated with Strain D1 (pile 18), inoculated with Strain D2 (pile 16), and inoculated with Strain D3 (pile 1).

The FTIR analysis Figure 5-3 of the soil inoculated with D3 (*B. stutzeri*) showed that the isolate was also able to exhibit some changes in the composition of the hydrocarbons of the weathered oil. When combining results of FTIR with DRO and CFU, it may be concluded that the isolate D3 was also able to grow and remove hydrocarbons although less than D1. Similar FTIR spectra as well as hydrocarbons removal efficiencies and produced biomass were obtained with all the *B. cereus* strains (D2, B1, B2, C1 and C2), with less performance than D1 and D3. On the other hand, it may be concluded that the isolate D1 (*B. sorensis*) may be considered highly interesting since the removal of weathered hydrocarbons was translated into cell growth producing 82×10^5 cfu/g soil compared to 55×10^5/g soil with D3 (*B. stutzeri*) and 17×10^5/g soil with D2 (*B. cereus*). With the other *B. cereus* strains, it seems that the hydrocarbons were more bioconverted than degraded, a process that generates less energy for the cell and thus leads to less biomass production.

The FTIR data obtained with fresh oil Figure 5-3 shows broad band at 3233, 3408 and 3525 cm^{-1}, respectively for the control (not inoculated) and D1 (*B. sonorensis*) pile. This could be allocated to the O–H stretching vibration in the chemical structures of the bio-surfactant. The strong peaks observed at 2921, 1426, and 2849 cm^{-1} in the un-inoculated fresh oil-contaminated soil matches the C–H stretching vibrations of the -CH_2 and -CH_3 hydrocarbon chains. These peaks vanished in the IR spectra after treatment with the D1 strain, while the peak at 1426 cm^{-1}, has been shifted to 1339 cm^{-1}. The peaks observed at 1108 cm^{-1}, could represent C-O stretching bands metabolic oxidation has taken place to form glycosidic bonds. As indicated in Figure 5-3 obtained with weathered oil-contaminated soils, peaks at 1459, 2852 and 2921 cm^{-1}, are allocated to the CH

bending and stretching of the aliphatic $-CH_3$, $-CH_2$, and $-CH$ bond.

In the pile serving as control (weathered soil, not inoculated with bacteria), the bands at 3050, 2940, 2840 and 1376 cm^{-1} reflect CH stretches and bends of aliphatic chains. The aliphatic C-H stretching with strong peaks at 2852 and about 2921 cm^{-1} observed in all piles is a result of their comparatively high hydrocarbon content. The relatively weaker aliphatic C-H stretching peaks in the soil incubated with D1 (*B. sorensis*) can be attributed to lower hydrocarbon content. In addition to this, the absence of aliphatic bending in all samples, 1446 confirms the likely removal of aliphatic compounds from the samples. Thus, the FTIR data corroborates the MS data and show what particular organic compounds that are removed in the biodegradation process.

5.3.1 *Discussion on Biostimulation/Bioaugmentation of Weathered Oily-Soils Using Selected Exogenous Bacteria from Local Harsh Weather and Soils*

This study confirms the presence of hydrocarbon-degrading bacteria in highly weathered oil-contaminated soils; that are adapted to the harsh Gulf environment. Only 2 to 3 bacterial strains were isolated from the cultures by enrichment. Identification and differentiation of bacteria by MALDI-TOF showed dominance of *B. cereus* bacteria categorized through protein profiling. *Pseudomonas stuzeri* was also present to a lesser extent. Ribotyping allowed identification of another strain as *B. sorensis*. All the bacterial strains were able to remove low categories of diesel hydrocarbons based on MW, with high efficiencies on HMW, which may be considered because of the occurrence and adaptation of the bacteria in the weathered oil-contaminated soil. Similarly, these bacteria were highly efficient in removing DRO from fresh oil within two weeks. However, their growth was much less pronounced in the weathered soils (compared to the fresh ones). The growth environment in the biopile was ensured by adjustment of C/N/P ratio to 100/10/1, moisture to 10% and surfactant at 1.1 g/Kg.

FTIR analyses showed that *B. sorensis* (strain D1) caused a significant change in the composition of the hydrocarbons in the soil. The D1 strain removed 20 to 25% of DRO. The stain D3 (*P. stuzeri*) was also effective although less than *B. sorensis* with almost 10% DRO removal. Small changes were observed in the FTIR spectrum after 90 days incubation. All *B. cereus* strains tolerated toxicity and grew slowly in the weathered oil-contaminated soil, with variability in growth, and DRO removal. This study clearly showed that bioremediation by bioaugmentation/stimulation of weathered oil-contaminated soils at harsh condition is possible but if suitably selected bacteria are used

with an appropriate screening program. However, the removal efficiencies of the hydrocarbons were not exceeding 25% after 90 days incubation. The reason for low removal efficiency by the selected bacteria may be associated to lack of metabolic activities or un-adapted structure-function characterizing the isolated.

5.4 Application of Indigenous Bacteria and Reconstituted Consortia for Bioremediation of Dukhan Weathered Oily-Soil

5.4.1 Introduction

Bacteria is the most commonly used in bioaugmentation. In commercial applications, either *in situ* or *ex situ*, single or mixed cultures of bacteria are added. They are usually naturally found in the environment and can establish biodegradation rapidly when dealing with pollutants. There is the possibility that the indigenous organisms found in the soil are not able to degrade the pollutants or the quantity of the organisms is not enough for a suitable treatment. This situation was shown applicable in the case of Dukhan soils from oil wastes dumping site, according to the section 5.3 of the current chapter 5 of this dissertation. In this scenario, bioaugmentation using indigenous bacteria can be convenient (Lyon & Vogel, 2013). Chemical and physical barriers to eliminate targeted contaminations by the degradation process using indigenous microorganisms was the main focus of bioaugmentation. Biostimulation alone was not successful until the introduction of bioaugmentation. Indeed, coupling bioaugmentation to biostimulation leads to improving environmental limitation by aeration in the case of aerobic microorganisms, adding nutrients if not sufficient, higher number and density of microorganisms is needed and this is established for better degradation of the target pollutants.

During the biodegradation of the hydrocarbons the main question that arises is how the microbe interacts with the substrate. Bioaugmentation (seeding) can be described as injection of the bacteria into the natural ecosystem that facilitate the rate of and/or extent of biodegradation of oil (Pathak H, 2009). Some microbes play the role of bio-degraders and are also the sources of biosurfactants facilitating the activity of indigenous ones (Lovley R, 2003; Kaczorek et al. 2011). Some studies also use indigenous species to treat the contaminated sites. The microorganisms of the soil do not necessarily possess the ability to metabolize the pollutants when they are not exposed to the contaminating compounds. However, once these microorganisms have been exposed to the pollutants, they can often be able to adapt by acquiring metabolic degradation of these pollutants recruitment vertical or horizontal gene specific (Hamme et al. 2003). Recruitment of metabolic pathways, present in the genome but not expressed, may be done by the events of mutation, genetic rearrangement or transposition referring to vertical recruitment (Weissenfels et al.1991).

Microorganisms can also acquire catabolic gene clusters via mobile elements transferred from a donor to a receiving host referring to horizontal transfer. In bacteria, transfer phenomena are best known through the combination of plasmids, transformation or transduction (Woo et al. 2004). These phenomena of horizontal transfers increase the metabolic capabilities of bacteria, including expanding their range of substrates, and allow adaptation of microbial populations to new contaminants (Xue & Warshawsky, 2005; Yu et al. 2005).

Biodegradation process of complex hydrocarbon mixture requires cooperation between more than one species together. This is true particularly in types of pollutions that

139

are made from various compounds such as petroleum or crude oil and desire for complete mineralization to H_2O and CO_2. Individual species of microorganisms metabolize a limited range of the hydrocarbon substrate, assembling mixed microbial populations with high range of enzymatic capacities to bring the extent and rate of hydrocarbon biodegradation further. Populations of microorganisms that consist of various strains that belong to different genera have been observed and detected in water or soils that are contaminated with petroleum (Sorkhoh et al., 1995)

Each genera or strain has a role in the transformation process of hydrocarbons. Each member in a microbial community plays a significant role and their presence and survival may be limited by the presence of other species or strains when the energy source is limited and being confined to the complex carbon (Ghazali et al., 2004). As such, metabolic diversity of the bacteria that degrade the hydrocarbon facilitate the process of degradation but also is the source of failure of some bioaugmentation applications, especially with weathered oil. The gap in the knowledge is about the behavior of reconstituted mixtures (consortia) of indigenous bacteria in a weathered oily-soil. Indeed, intermediates of metabolic pathways can play the role of substrates or inhibitors for other bacteria. The Dukhan soil represents a model of soil in which, it is to improve the understanding at the microbial behavior level not metabolic level.

*5.4.2 **Biopiling of Weathered Polluted Soils by Bioaugmentation Using Indigenous Bacteria***

All biopiles were performed at the same conditions to avoid any variations or difference in results that are not based on our research purposes. Soil was from the composite sample from Dukhan dumping site. Each biopile contained 685 g of soil that

140

was sieved with a sieve of 2 mm pore size and manually mixed rigorously to reach homogeneity. The average of the two soils moistures was similar of 6.5%, as measured in the soil stocks. This was not surprising since samples were from the upper layers. The added water content in the biopiles was adjusted to 18.8% or 20.9% moisture by adding 100 ml or 120 ml of water. All biopiles were incubated in an incubator set at 37°C. In this study, the focus was the removal efficiency of hydrocarbons. In fact, it was not so easy to compare the growth of each bacterial isolate in the mixed cultures. Gas chromatography was periodically performed for DRO'S determination to evaluate the efficiency of their removal by the bacterial isolates or consortia. pH in biopiles was measured continuously to observe if there is an oxidation or reduction processes or reactions.

The C/N/P ratio in all biopiles except the negative control was 100/10/1 as previously optimized in our laboratory, using ammonium nitrate as nitrogen source containing 46.7% Nitrogen, and potassium phosphate as phosphorus source, containing 22% Phosphorus. The soil contained 23% carbon of the total dry matter and negligible Nitrogen and Phosphorus contents (below detection level using CHNO analyzer).

The DRO and PAH in the two soils were measured using GC-FID and GC-MS and their contents in the control biopile were similar of 3066.99 ppm and 402.51 ppm respectively. This result shows that the hydrocarbons are highly adsorbed and stabilized in the soil particles. At the harsh weather, their adsorption might increase in Tarballs, which may only affect their structures. It is to mention that, hydrocarbons existing in the two soils are considered highly weathered hydrocarbons as exposed for harsh conditions for more than three years as shown by Alkaabi et al. (2017). In term of bacteria, three indigenous bacteria named D1D2, D5D1 and D9S1 hydrocarbon-degrading bacteria were isolated

from the Dukhan site, *Bacillus* and *Pseudomonas* genera It may also be evident that some of them could be the same strain, which needs further investigations to differentiate between them. At this stage of the study, such differentiation was based on the biological activity of each strain. Indeed, diesel and crude oil in MSM medium were used as the sole hydrocarbon sources, at 10% (v/v) corresponding to almost 75 g/l hydrocarbons, to investigate the potential of each strain to tolerate toxicity and grow by removing categories of hydrocarbons (Table 4-8).

Table 4-8 were selected for this study. In total 8 different biopiles were performed in triplicates, using single or mixed bacteria as shown in (Table 3-3). Analysis of hydrocarbons by GC-MS was performed for 16 weeks (112 days). Similar results were obtained in the replicates. Changes over time in *n*-alkanes from *n*C17 to *n*C36 in the soil were monitored in each microcosm and presented in Figures. (5-3, 5-4 and 5-5). These graphs illustrate the total loss of n-alkanes over the incubation period.

5.4.3 *Efficiency of Biostimulation of Indigenous Bacteria in Oily-Soil of Dukhan Dumping Site*

The bioaugmentation strategy was valid only if biostimulation of indigenous bacteria shown slow or not promoting efficient removal of pollutants. Biostimulation is the addition of chemical and/ or physical components to the contaminated area for treatment to obtain better bioremediation technology that is time and cost effective. The addition of oxygen source can be an example of biostimulation when dealing with aerobic microorganisms. Nutrients composed of organic and inorganic compounds can also be added into a contaminated region to help enhance the degradation procedure by microorganisms found in the soil (Tabatabaeem et al 2005). Surfactants and fertilizers are some of the examples that can also be used in this treatment activity. The information on the polluted site is important to know for the strategy that will be followed in the treatment. Organisms are expected to exist in the soil, the addition of the stimulators should be well planned to not obtain an inhibition instead of a biodegradation (Lyon & Vogel, 2013). This will, therefore, rise biodegradation and bioavailability (Sandrin & Hoffman, 2007). Many of the hydrocarbon degrading bacteria have been isolated and studied, but those that degrade *in situ* are poorly understood (Mittal & Singh, 2009).In fact, less than 5% of the soil bacteria are cultured in the laboratory (Mittal & Singh, 2009). The abiotic factors that influence the rate of biodegradation include the salinity of the soil, level of pH, moisture,

oxygen, redox potential, and nutrient availability. The resistance to degradation and solubility is determined by the composition of the hydrocarbon mixture. Similarly, the effectiveness of bioremediation is determined by the interaction between the components of the soil and the pollutant to verify the impact of biostimulation on the degradation of hydrocarbon compounds in oily-soil from Dukhan dumping site, without augmenting the bacterial populations, the biopiles performed in triplicates were used to follow weekly, the fate of the hydrocarbons for 16 weeks Figure 5-4.
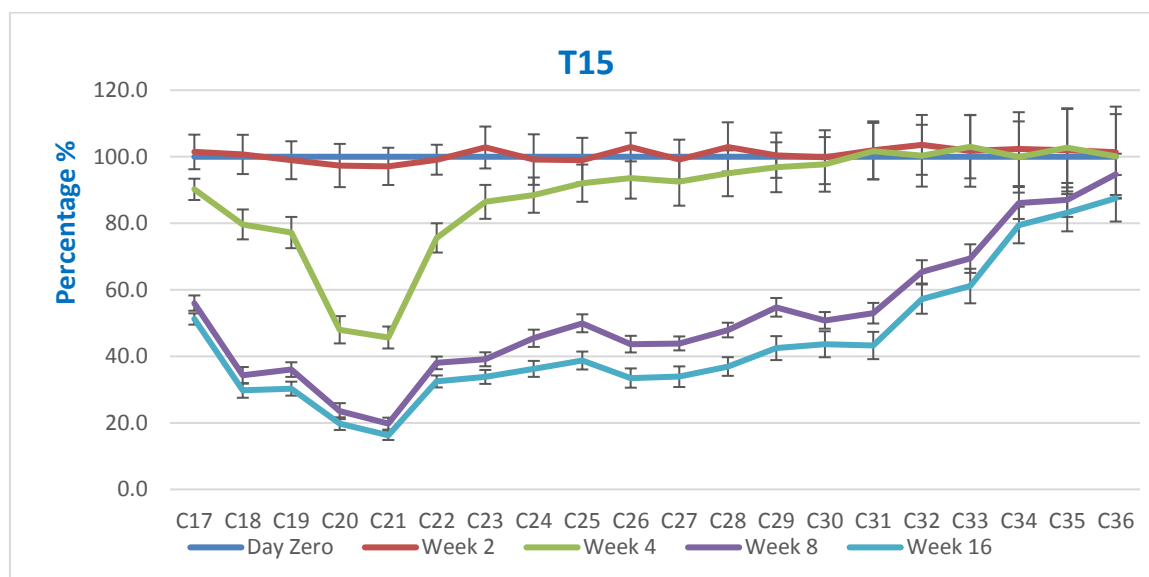
Figure 5-4: Hydrocarbons removal from oily-soil of Dukhan dumping area using biostimulation. The soil was stimulated with adjustment of C/N/P at 100/10/1 and 0.8 ml Twee-80/kg. The corresponding biopile is T15 from Table 3-3.

The largest removal of n-alkanes was seen in the 16th week with up to 85%. In fact, the biodegradation started effectively after 4 weeks to be almost highest at week 8. Interestingly noticed, degradation of hydrocarbons at week 4 started with low molecular weight (from C-17 to C-29) while on week 8 and week 16, most of the low molecular

weight hydrocarbons were degraded and bacteria developed a linear activity with increased molecular weight hydrocarbons. This result was expected, and a long lag phase was necessary for the indigenous bacterial communities to be performant, starting with the easiest to the difficult substrates.

5.4.4 Efficiency of Bioaugmentation Using Single Selected Indigenous Bacteria in Oily-Soil of Dukhan Dumping Site

To study the effect of bioaugmentation on the degradation of hydrocarbon compounds, isolates D1D2, D5D1 and D9S1 isolated from the same polluted soil (Chapter 4) were introduced in the soil separately in Biopiles T1, T3 and T5 respectively Figure 5-5. In T1 degradation started on week 2 whereas in T3 and T5 it started on week 4. The largest percentage removal of n-alkanes was seen in the week 16 to reach up to 95%, 92% and 85% on n-C21 in T5, T1 and T3 respectively. The hydrocarbons with lower molecular weight were pronounced to undergo a higher degradation as compared to the hydrocarbons with higher molecular weight. The removal efficiency of nC36 was 39%, 25% and 24% in T5, T3 and T1 respectively.
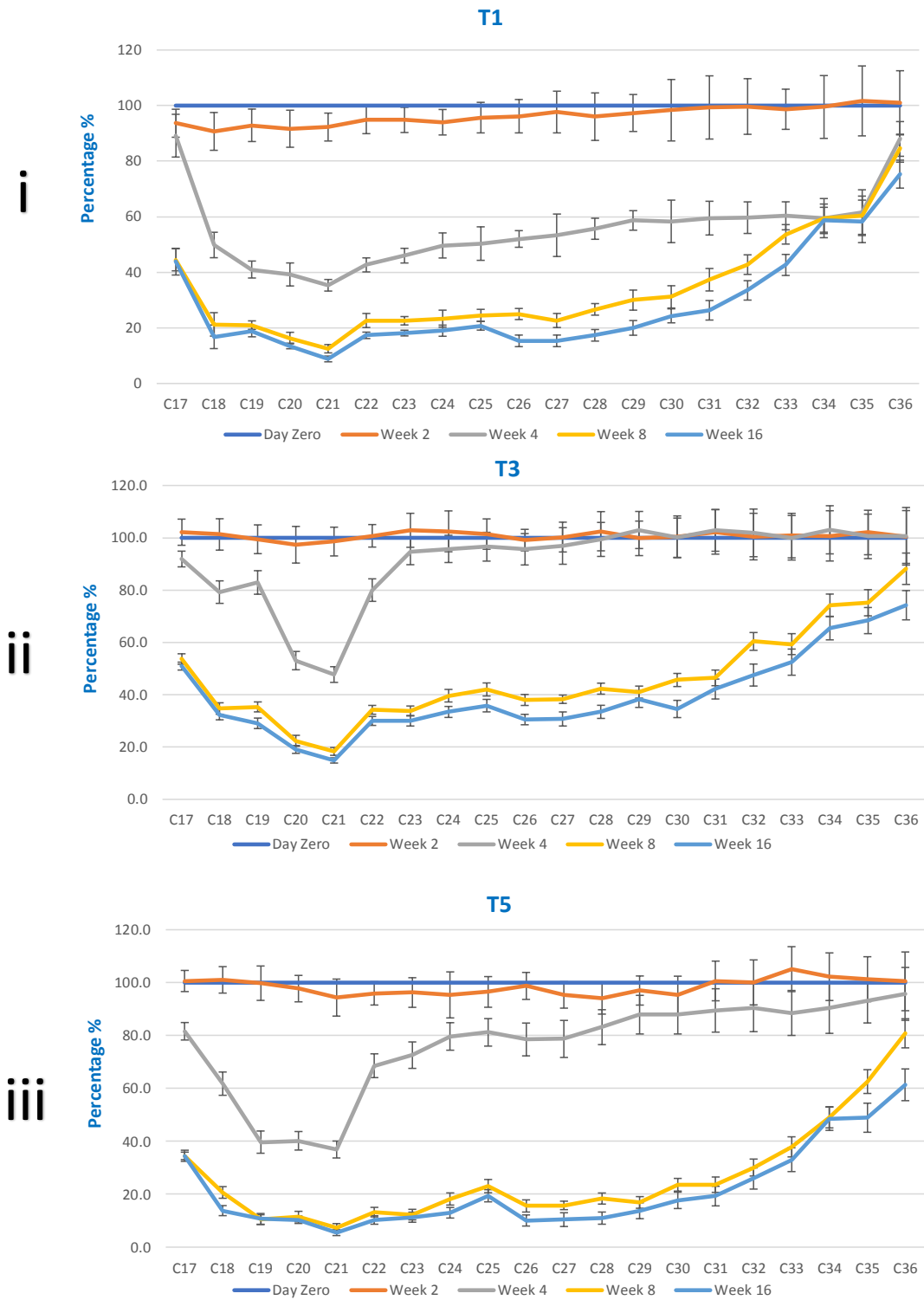
Figure 5-5: Effect of bioaugmentation on the degradation of hydrocarbon compounds, using isolates D1D2, D5D1 and D9S1 isolated from the same polluted soil and were in the soil separately in Biopiles T1, T3 and T5 respectively.

5.4.5 *Efficiency of Bioaugmentation Using Reconstituted Consortia Using Selected Indigenous Bacteria in Oily-Soil of Dukhan Dumping Site*

Reconstitution of consortia using the indigenous bacteria isolated from Dukhan soil, object of the current study, was investigated in Biopiles T7, T9 and T11 performed with combination of two isolates and T13 performed with the combination of all the three isolates together Figure 5-6. In all T7, T9, T11 and T13 biopiles, removal of hydrocarbons started on week 4 on low molecular weight hydrocarbons. The highest removal efficiencies of n-alkanes were in the week16 with up to 74%, 74%, 76% and 70% on n-C21 in T9, T11, T7 and T13 respectively. The degradation of low molecular weight hydrocarbons was much higher than that of the high molecular weight ones. The removal efficiency of nC36 was of 25%, 25%, 24% and 16% in T9, T11, T7 and T13 respectively.
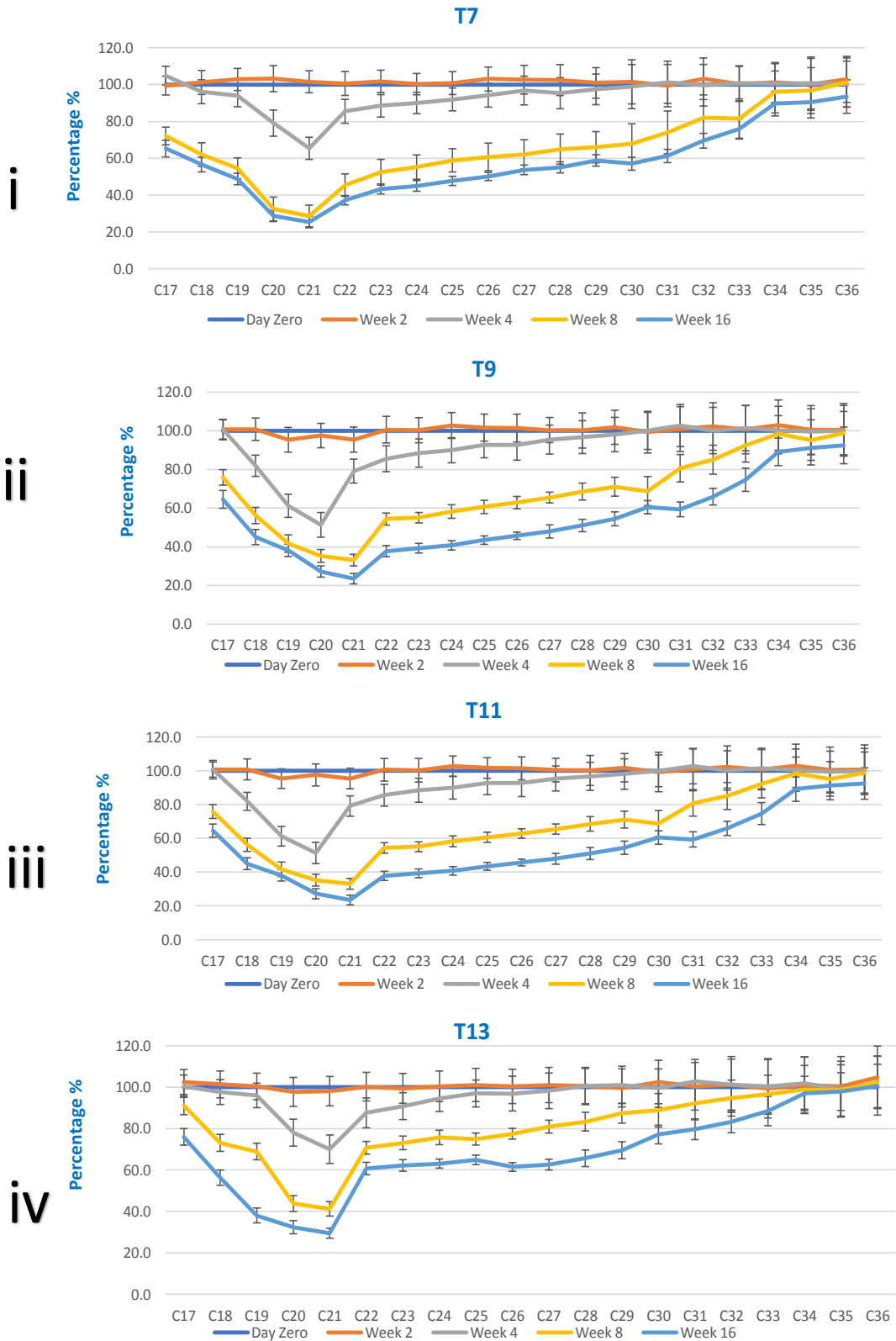
Figure 5-6: Effect of bioaugmentation on the degradation of hydrocarbon compounds, using combination of isolates D1D2, D5D1 and D9S1 isolated from the same polluted soil and were in the soil separately in Biopiles T7 (D1D2, D5D1), T9 (D1D2, D9S1) and T11 (D5D1,D9S1) and T13 (D1D2, D5D1 and D9S1).

5.4.6 *Comparison of The Efficiency of Bioaugmentation Using Single or Reconstituted Consortia Using Selected Indigenous Bacteria in Oily-Soil of Dukhan Dumping Site*

To compare the effect of biostimulation/bioaugmentation using single indigenous isolates or their mixtures (consortia) on the degradation of hydrocarbons, the corresponding removal profiles were compared after 16 weeks of treatment Figure 5-7. The best removal profile was obtained with bioaugmentation using the isolate D9S1 (biopile T5) with similar results obtained with the isolate D1D2 (Biopile T1). Biostimulated biopile (T15) showed similar results to that of the Biostimulated/ bioaugmented biopile (T3), using the isolate D9S1. The isolate D5D1 was not efficient to improve the overall performance of the existing bacteria in the soil. Surprisingly, bioaugmentation using the mixed bacterial isolates was less efficient that the biostimulation without bioaugmentation. Indeed, augmentation using D1D2 with D5D1 or D9S1 was less efficient that just biostimulation and much less than bioaugmentation with D1D2 alone. Similar conclusion can be drawn regarding the isolate D9S1 which was much more efficient than all the other combinations.

Combination of D5D1 with D9S1 or the reconstituted consortium with the three isolates together decreased a lot the performance of removal of hydrocarbons. These results clearly shown that mixing the isolated indigenous bacteria in bioaugmentation approaches, allowed the decrease of hydrocarbons removal and was not beneficial for the biostimulation/bioaugmentation approaches in the weathered oily-soils in Dukhan. It seems that the bacteria were inhibited through their respective activities. Compared to their respective performance to remove hydrocarbons obtained in separate cultures, the profile of removal was maintained but the removal efficiencies were much reduced. Thus, it can be concluded that the bacteria cannot mutually benefit from their metabolisms for growth but inhibition. This means that D9S1 isolate was able to perform better results alone than the other bacteria.
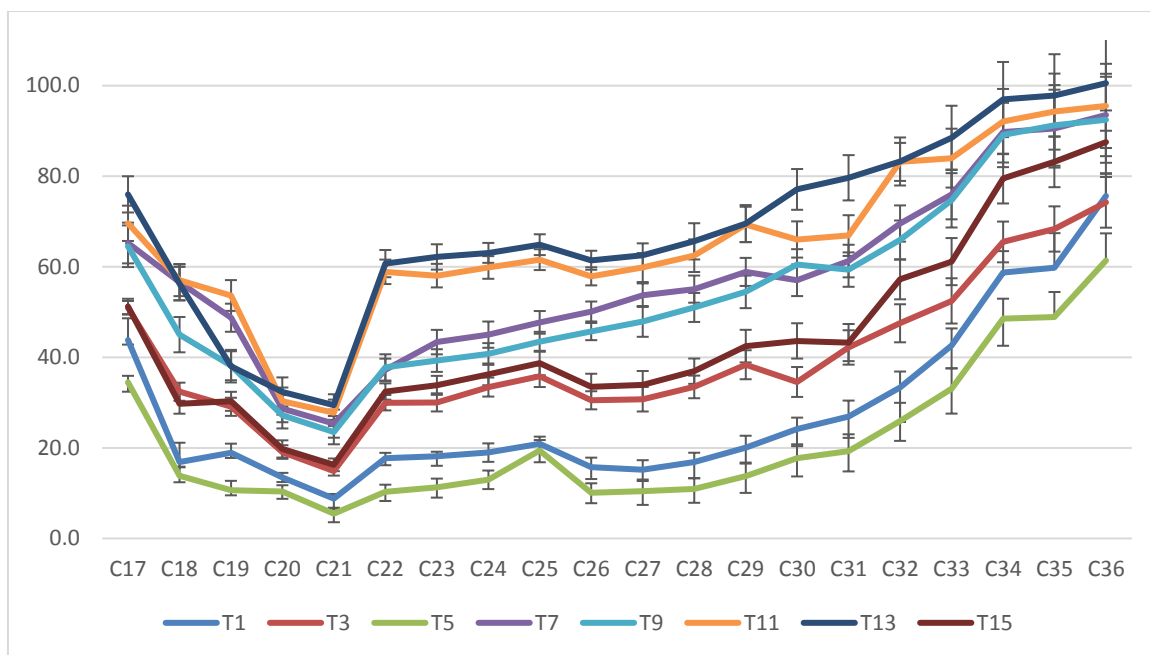
Figure 5-7: Comparison of the efficiency of bioaugmentation using single or reconstituted consortia using selected indigenous bacteria in oily-soil of Dukhan dumping site after 16 weeks incubation.

5.4.7 *Evaluation of The Performance of The Biostimulation/Bioaugmentation Treatments Using Indigenous Bacteria On n-Alkanes nC17-nC19 and Their Branched Homologs*

To further investigate the biodegradation effects, Figure 5-8 shows the removal of n-alkanes nC17-nC19 and their branched homologs that were evaluated during the incubation time in all the biopiles. Figure 5-8. depict the development over time because of the biological processes. The range of nC17-nC19 and branched homologs content in day zero samples is considered as the 100%. The bioaugmentation by single bacteria samples (T1, T3, T5) showed the largest reduction of 15%, at week 16 while remaining days were steady with 0-8% reduction. Depletion of nC17-nC19 as well as branched homologs was thus mainly attributed to biodegradation. At all biostimulation/bioaugmentation combinations, removal of iC21, Phytane and Pristane was

never exceeding 10% (residual contents were always near 90%).

For the branched alkanes in all microcosms, reductions were below 10% until week 8. By week 16, branched alkanes were further depleted, 10-13% for T1 microcosms, 15-18% for T3 microcosms; 11-15% for T5 microcosms; 5-8% for T7; 5-7% for T7; 4-9% for T9; 6-10% for T11; 5-7% for T13, and 8-11% for T15. As seen for nC17-nC36, both n-alkanes and branched alkanes were generally degraded at a higher rate with shorter chain length. Thus, the results are analogous to the other studies on n-alkanes as well as the branched alkanes (Wang, et al., 1998).
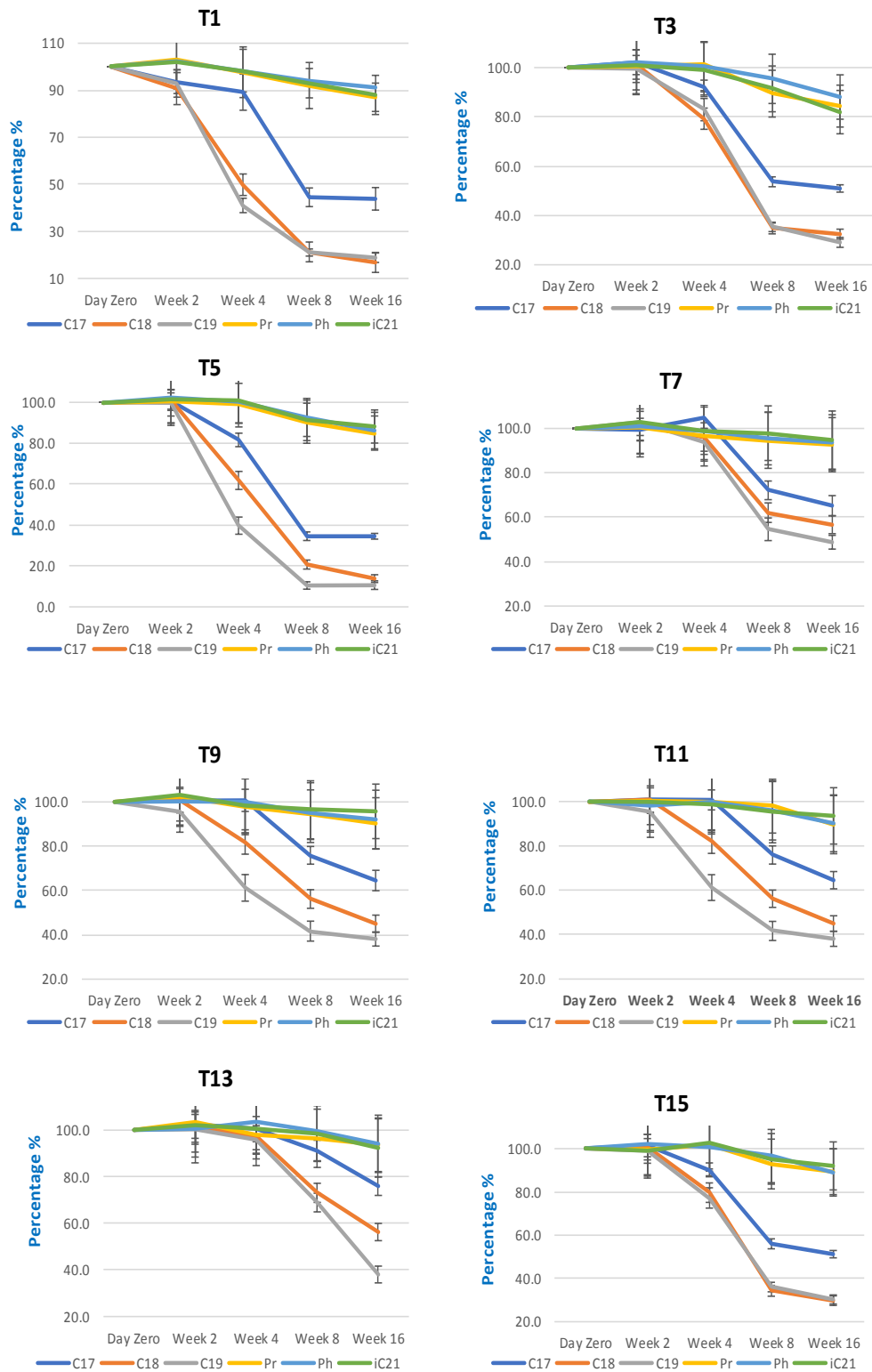
Figure 5-8: Evaluation of the performance of the biostimulation/bioaugmentation treatments using indigenous bacteria on n-alkanes nC17-nC19 and their branched homologs.

The intensity of the peaks is significantly decreased over time. Figure 5-9 shows an example of chromatogram illustrating the overall removal of hydrocarbons in soil after 16 weeks of incubation period using different treatments.
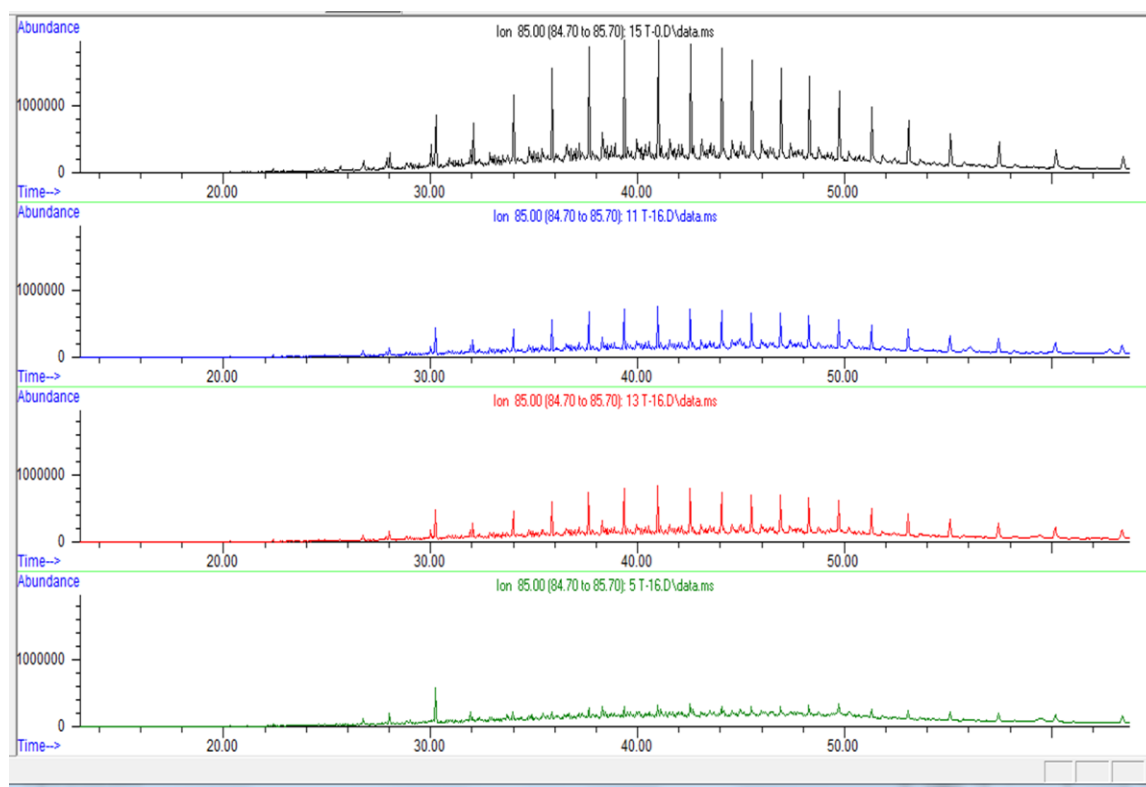
Figure 5-9; An example of overall removal of hydrocarbons through different treatments over time: (Black): Day zero; (Blue): T11 (D5D1 and D9S1) at week 16 ; (Red): T13 (D1D2, D5D1 and D9S1) at week 16; (Green): T5 (D5D1) at week 16.

5.4.8 *Investigation of Changes in Hydrocarbons Using FTIR Analyses*

The FTIR spectrum of Dukhan sandy oil spill (zero day) illustrate the aromatic C−H stretching bands ranging from 3000–3100 cm^{-1} and slight bands of aliphatic hydrocarbons (CH2 and CH3 stretching at 2987, cm^{-1}). The O-H stretching vibrations are responsible for the broad peaks estimated at 3200– 3550 cm^{-1}. The long straight-alkanes

illustrates an absorption band at 730 cm^{-1}. The changes in the structural properties of the Duckan sandy oil spill after addition of the non-ionic surfactant results to formation of the major peak illustrated between 1000– 1100 cm^{-1}. The major peak is associated with the S=O or the C-O stretching vibrations. As such, these findings can also be confirmed by the reports of (Feng, et al., 2006) showing that addition of Tween 80 in diesel oil enhances bio-desulfurization. The absorptions in the 680–730 cm^{-1} range represents the =C-H aromatic groups undergoing deformation stretching that is out-of-plane. A band at 1630 cm^{-1} is associated with carbonyl C=O.
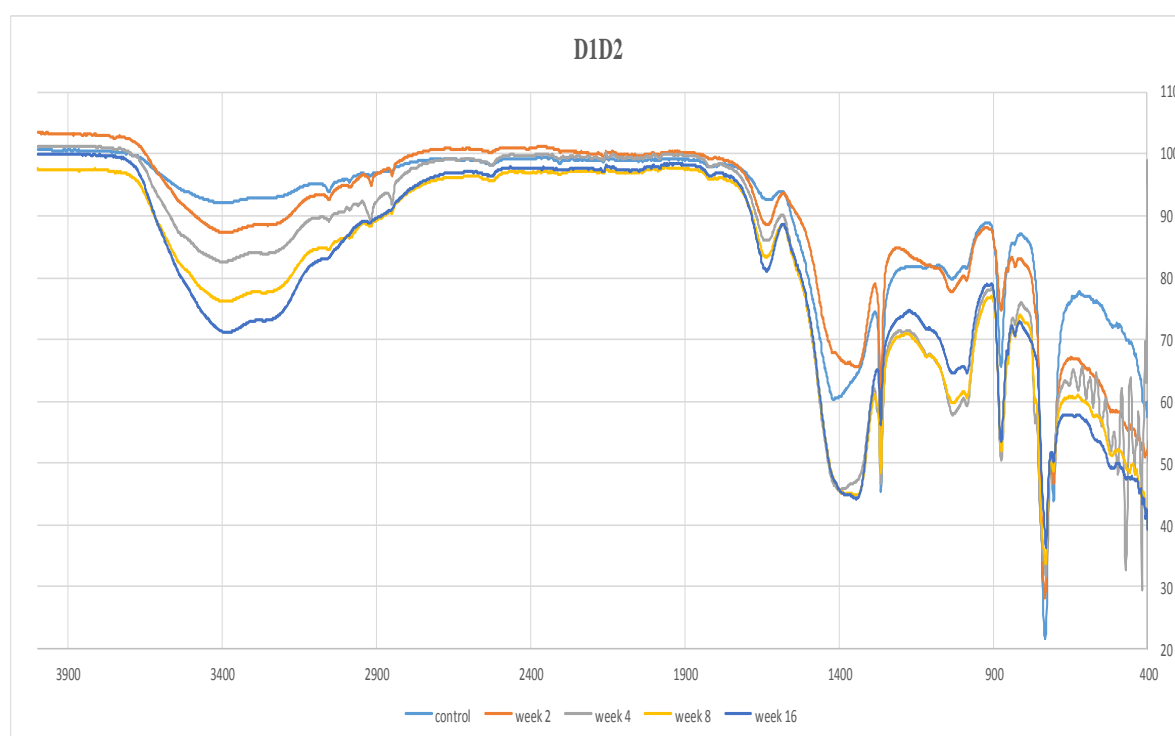

Figure 5-10: FTIR spectra of soils with (D1D2).

The spectra obtained after microbial treatment showed in Figure 3-10 by D1D2 as a single bacterium bioaugmentation on Duckan sandy oil spill with ionic surfactant

illustrates similar absorption bands. However, the spectra have varying relative densities. The microbial process induced extensive response on the carbonyl C=O at 1630 cm^{-1} through hydrocarbon oxidation during weeks 2, 4, 8 and 16. As such, the bacteria caused an oxidative alteration in the macromolecular structure resulting to formation of oxygenated functional groups after biodegradation. The peaks on week 2 and 4 present at 2834 and 2914 represents the CH2 and CH3 stretching but these bands do not appear on week 8 and 16. The decrease in the band 704 =C-H for alkene means that the bacteria start breaking down the alkenes that are transformed to alkanes and the band 2987 is eliminated. Also, for the band 730, alkanes start decreasing due to degradation (Muthukumar et al. 2007). IR spectra also showed on weeks 2, 4, 8 and 16 a decrease in the intensity of aromatic C-H stretching bands ranging between 3000 and 3100 cm^{-1}. Sequentially, there was disappearance of some aromatic nuclei peaks because of degradation. A band around 3200– 3550 cm^{-1} showed an increase in the intensity of O-H on week 2, 4, 8 and 16 generated by microbial oxidation process. The Alkene on band 2303 cm^{-1} do not appear after 2 weeks and alkene on band 703 cm^{-1} start to decrease in intensity after day Zero until 16 weeks which is an indication of biodegradation by the bacteria. Also, an increase in the intensity of the stretching vibrations of the C-O was illustrated by the IR spectra at a range of 1034 cm^{-1} indicating some kinds of biodegradation (Kumar, et al., 2014). Similarly, the FTIR analysis was carried out for all treated biopiles see Appendix A 1

5.4.9 *Investigation of Changes in Hydrocarbons Using Chemometery Analyses*

The SICs of *n*-alkane groups with *m/z* 85 of PACs and isoprenoids (*n*C17–*n*C19) were analyzed using CHEMSIC method. The first derivative was calculated to separate the baseline n-alkanes SICs and to facilitates separate alignment. A ±10 scan point was allowed for the maximum rigid shift while a search space of 25-175 was set for the COW with an increment segment length of 25-point and a slack parameter of 1-5 with a 1-point increment. 10 scan points was the maximum correction acceptable for COW. The combined SICs normalized to phytane and T2 (treatment 2, biopiles in week 2) norm was used to calculate the initial PC model. A projection of two validation sets and training sets (32 samples × 884 data points) was calculated on the PC model. The optimal numbers of the PCs were estimated using 10 random subsets of cross validation. The score plots for PC2 against PC1 using mean centering are shown in Figure 5-11 for all biopiles. Figure 5-12 illustrates the loading of PC2 and PC1 for n-alkanes. The information on biopiles, the type of weathering and the degree of weathering is illustrated on each PC.
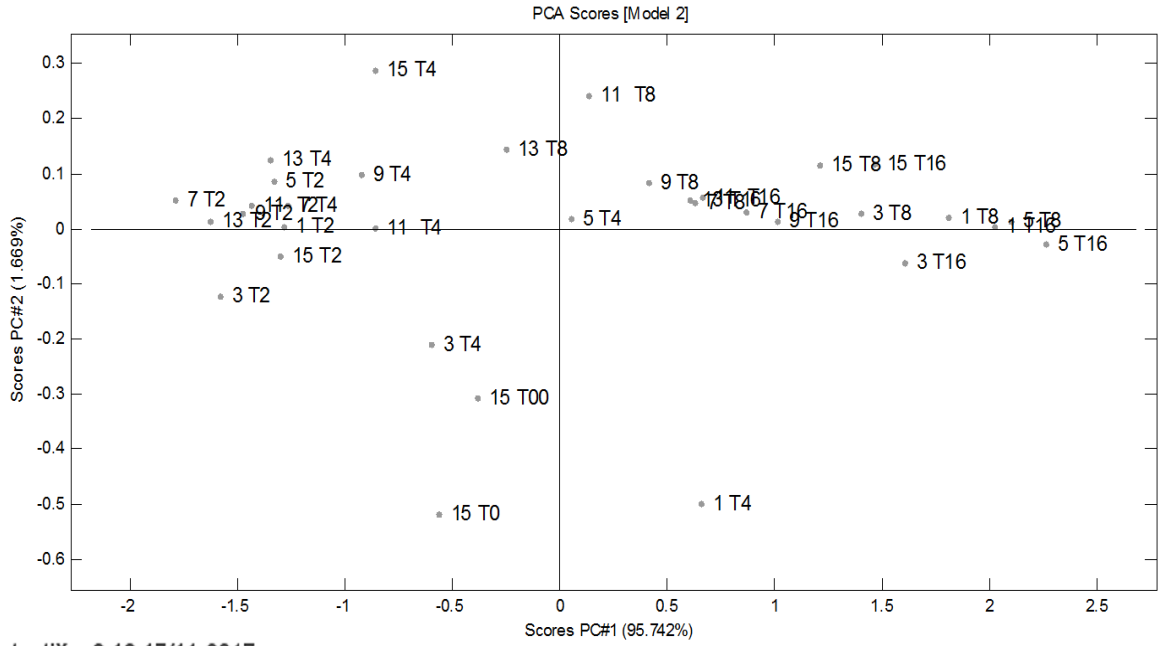
Figure 5-11: The score plots for PC1 vs. PC2 using mean centering for all biobiles.
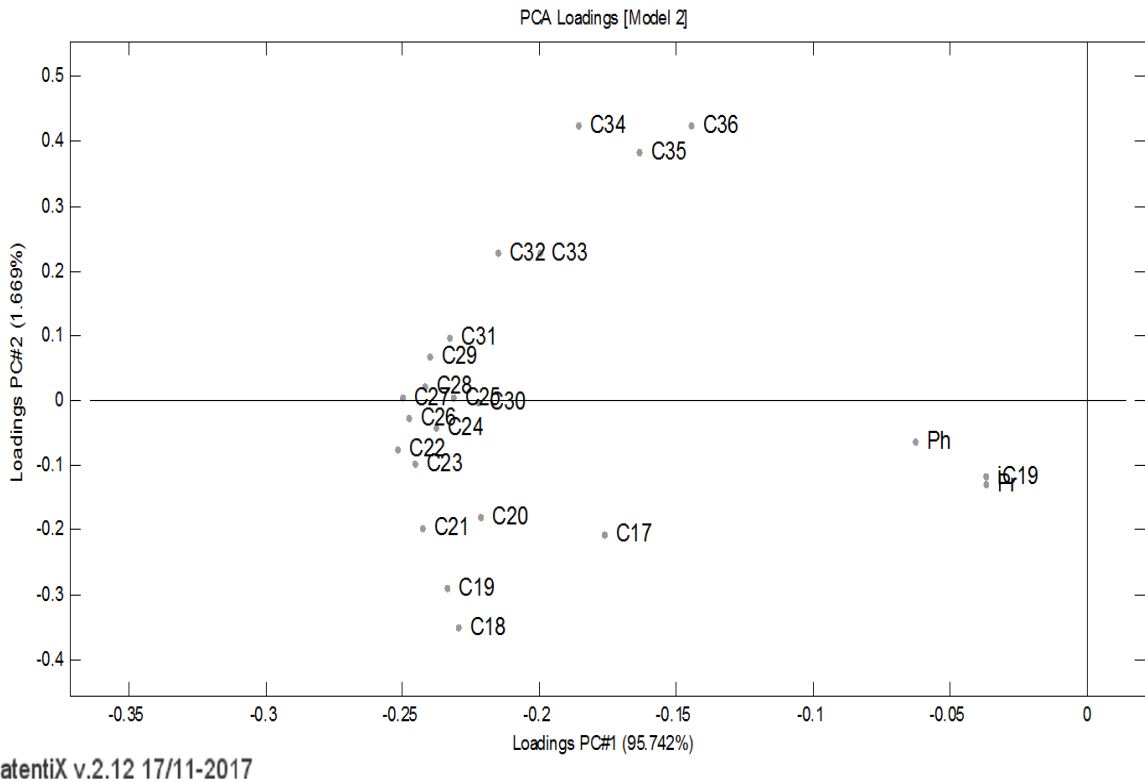
Figure 5-12: The PC1 and PC2 loadings for n-alkanes.

The PC model confirmed the n-alkanes results in (Figure 5-7) (n-alkane) that the bacterial bioaugmentation arising from the use of a single bacteria was most effective for biopiles bioremediation. PC2 and PC1 illustrate the biodegradation effects of PC1 loading coefficients are positive for best biopiles in (Figure 5-13), T5 week16, T5 week16, T1 week16, T1 week16, T3 week16 and T3 week16 respectively, and negative for the lowest biopiles. On the other side, the loading coefficient for PC2 was negative for the lowest degradation on n-alkanes and positive for highest degradation on n-alkanes in Figure 5-14. The susceptibility to microbial degradation is lower in high molecular weight n-alkanes as compared to the lower molecular weight n-alkanes that illustrates high susceptibility (Wanga, et al., 1998). Hence, the main effects of degradation within a biopiles (T1, T3, T5, T7, T9, T11, T13 and T15) can be illustrated by their PC2 and PC1 scores. Samples with positive PC2 and PC1 scores are the best degradation of an-alkanes; and the least degradation to high negative PC1 and PC2.

Figure 5-13: PC1 loading coefficients for biobiles, the positive for best biopiles

Figure 5-14: PC2 loading coefficients for n-alkanes, High molecular weight n-alkanes are less susceptible to microbial degradation, low molecular weight n-alkanes are high susceptible to microbial degradation.
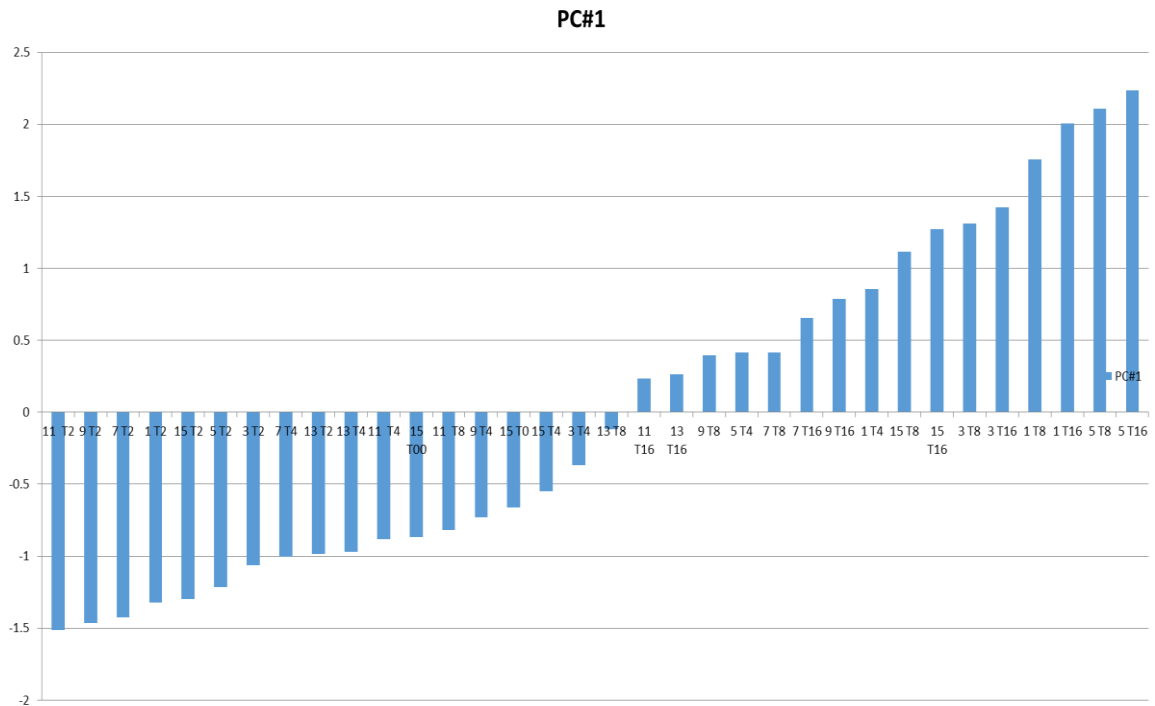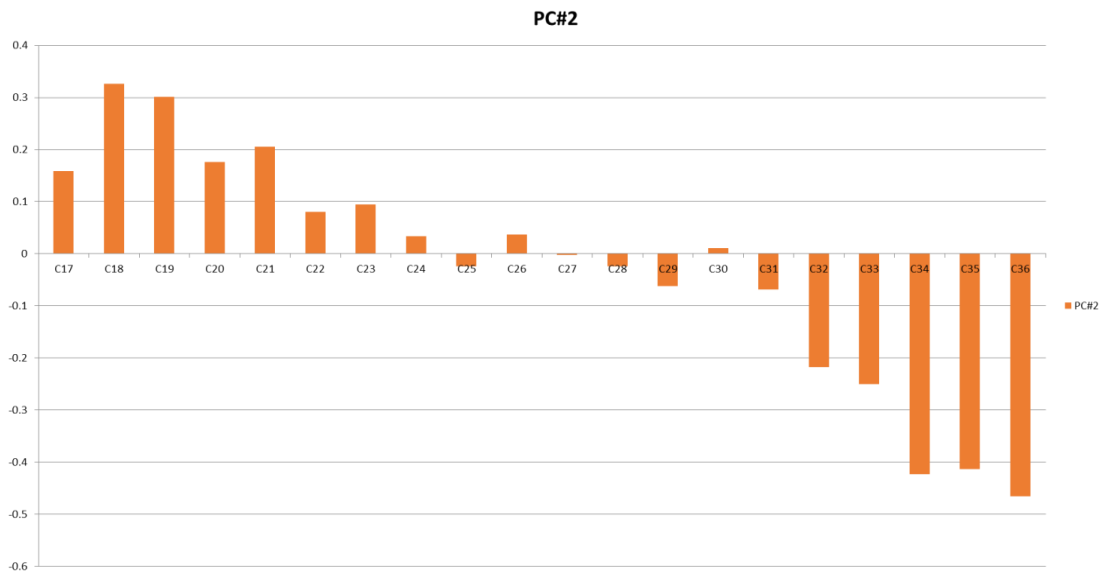
Chapter 6 : **CONCLUSION**

Oil and gas are two main cheap energy sources that are being used all over the world and transported for long distances, regardless attention drawn for running factories, cars, manufacturing, industries, etc. Oil and gas drilling, refinery process, storage, and transportation, are all possible sources of pollution. When the oil is being transported and exported from the producing countries to other countries or during the distributions within the country, via ships, pipes, vehicles (trucks), or rails, oil leakage or spills can occur no matter how cautious the transportation process is. Most cases of oil spills are accidental during the transportation on sea or land; during drilling process; or leaking from storage tank. In some cases, oil spills might occur in purposely as the situation in 1991 during the Persian Gulf War (Khamehchiyan et al., 2007). Oil sources determination is then an important parameter in limiting continuous pollution.

Also, the chemical stability and biodegradation of the pollutants is affected by the weather conditions and the chemical and physical features of the soil. The petroleum products undergo various changes through the process of biodegradation, oxidation, partitioning, photodegradation, and volatilization when they are exposed to the environment posing a greater challenge of evaluating the risk of exposure. An example of an area that is more prone to these weathering processes is the arid climate experienced in the Gulf area. As such, each polluted area illustrates unique features of abiotic and biotic factors that guide the selection of the best strategy of bioremediation to match the pollutant. Thus, additional studies are necessary to characterize the classes of hydrocarbon-degrading bacteria and the effects of the harsh conditions on the metabolic activities of these bacteria.

Bioremediation which is the best option from the environmental point of view to alleviating oil pollution; because it accelerates or imitates natural processes, may be performed in-situ or ex-situ, but within an overall strategy. As such, the strategies of bioremediation are centered around the main actors which are mostly hydrocarbon-degrading bacteria and their substrates, which are the pollutants. Here, biodiversity of such bacteria and their metabolic activities towards weathered hydrocarbons should be highly specific for the region and must be considered for any bioremediation strategy. The objectives of our work covered all the components of the environmental issue to contribute to filling the gap in knowledge regarding the specific case characterizing the area.

First, it was established that at least two different sources were responsible for the oil spills in the North-western Qatar. The solidified weathered oil covered the relatively weathered oil impeding further degradation. The source of the heavily weathered oil was found to be the Gulf War, thus illustrating that the process of degradation of oil is relatively slow. The existence of recent spill illustrated that AlZubara is constantly exposed to oil spills. Thus, this is demonstrated for the first time and may explain the low rates of bioremediation, compared to what is described in the literature. The establishment of appropriate isolation and screening strategy allowed to construct a local collection of highly resistant hydrocarbon-degrading bacteria from Al-Zubara beach, Dukhan dumping site, and many other representative sites in Qatar. This collection will be available for fundamental and applicable research.

The isolated bacteria were identified and differentiated through molecular techniques (16S rDNA sequencing and MALDI TOF). Our findings showed limited biodiversity in term of Genus and Species. However, by studying the potential of each strain to degrade hydrocarbons, it was obvious that a high diversity was observed in the

metabolic activity of the isolates even within the same group of genus or species. The finding of this research work confirms that the highly weathered soils because of harsh conditions would lead to adaptation routes employed by each isolate to survive and overcome the high toxicity. Our strategy of differentiation allowed identification of the bacteria but more interestingly demonstrated a diversity of the function related to the corresponding metabolism

In fact, this is the modern approach for function biodiversity studies useful to implement the microorganisms in applications, such as bioremediation. In our approach, hydrocarbon-degrading bacteria would work as a factory with high interaction with the microenvironment. This is the concept of cell factory. Our strategy of isolation was highly effective in providing evidence on the microbial and metabolic variabilities. Local microbial strains exhibited interesting biological activity due to their adaptation to prolonged extreme temperatures, desiccation and salinity are a big positive for Qatar. The most valuable thing about the current study is that it should be expected that each site polluted with oil components should be bioremediated by the intrinsic hydrocarbon-degrading bacteria, to overcome long adaptation periods and provide the appropriate metabolic activities to interact specifically with the existing pollutants.

The role of soil physiochemical characteristics and conditions and their interactions between soil-pollutants and bacterium were investigated to implement best bioremediation strategies regarding required nutrients to support bioaugmentation-biostimulation approaches in Qatar. As such, the success of the bacteria process originates from the metabolic variation that exist between the hydrocarbon-degrading bacteria. However, it is also the main source of failure of some bioaugmentation applications, especially with the weathered oil. The gap in the knowledge is about the behavior of reconstituted mixtures (consortia) of indigenous bacteria in a weathered oily-soil. Indeed, intermediates of

metabolic pathways can play the role of substrates or inhibitors for other bacteria. The Dukhan soil represented a model of soil in which, it is to improve the understanding at the microbial behavior level, not metabolic level.

Our results showed that a mixture of isolated indigenous bacteria caused a decrease in the removal efficiency of hydrocarbons, thus was not beneficial for the biostimulation/bioaugmentation approaches in the weathered oily-soils. It seemed that bacteria were inhibited through their respective activities. Thus, we can conclude that the bacteria may not be able to mutually benefit from their metabolisms for growth but rather inhibited. In this study we showed that bioremediation by bioaugmentation/stimulation of weathered oil contaminated soils at harsh condition is possible but if suitably selected indigenous bacteria are used with an appropriate screening program. Bioaugmentation must use native bacteria as they are, or at least can become, highly adapted particularly if the oil contaminants are weathered.

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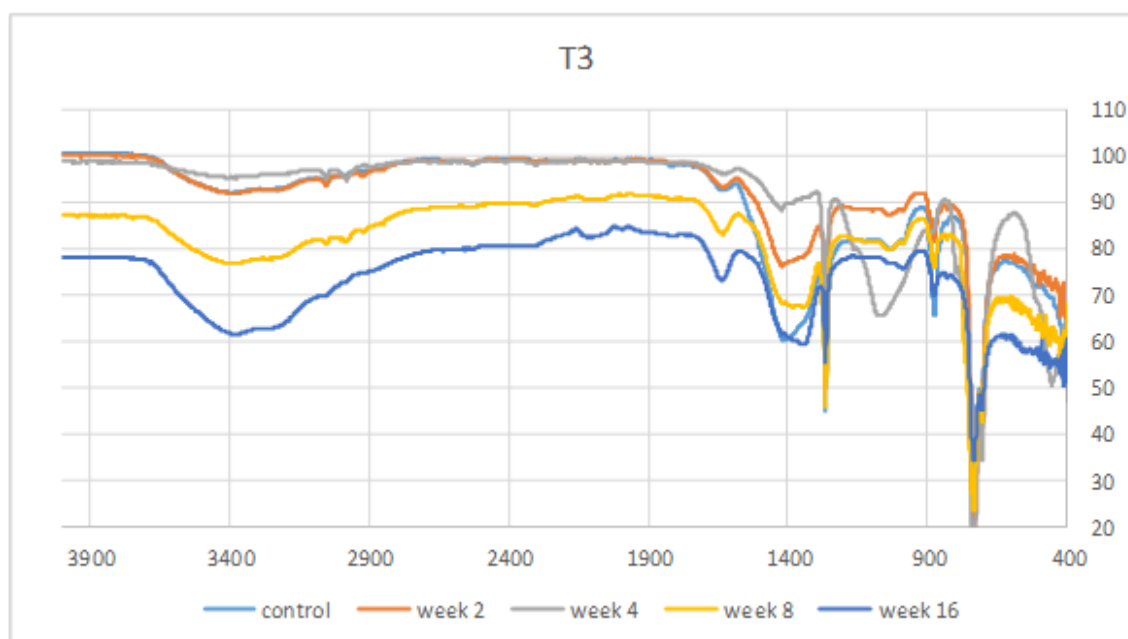
Figure 1: FTIR spectra of soils with T3 (D5D1)

The spectra obtained after microbial treatment showed in Figure 1 by T3 (D5D1) as a single bacterium as bioaugmentation on Duckan sandy oil spill with ionic surfactant exhibit the similar absorption bands. However, the spectra have varying relative densities. The microbial process induced extensive response on the carbonyl C=O at 1630 cm^{-1} through hydrocarbon oxidation during weeks 2, 4, 8 and 16. As such, the bacteria caused an oxidative alteration in the macromolecular structure resulting to formation of oxygenated functional groups after biodegradation. The peaks on weeks 2, 4 and 8 presents at 2834 and 2914 represents the CH2 and CH3 stretching but these bands do not appear on week 16. The decrease in the band 704 =C-H for alkene means that the bacteria start breaking down the alkenes that are transformed to alkanes and the band 2987 is eliminated. Also, for the band 730, alkanes start decreasing due to degradation. IR spectra also showed

on weeks 2, 4, 8 and 16 a decrease in the intensity of aromatic C-H stretching bands ranging between 3000 and 3100 cm^{-1}. Sequentially, there was disappearance of some aromatic nuclei peaks because of degradation. A band around 3200– 3550 cm^{-1} showed an increase in the intensity of O-H on week 2, 4, 8 and 16 generated by microbial oxidation process. The Alkene on band 2303 cm^{-1} do not appear after 2 weeks and alkene on band 703 cm^{-1} start to decrease in intensity after day Zero until 16 weeks which is an indication of biodegradation by the bacteria. Also, an increase in the intensity of the stretching vibrations of the C-O was illustrated by the IR spectra at a range of 1034 cm^{-1} indicating some kinds of biodegradation.
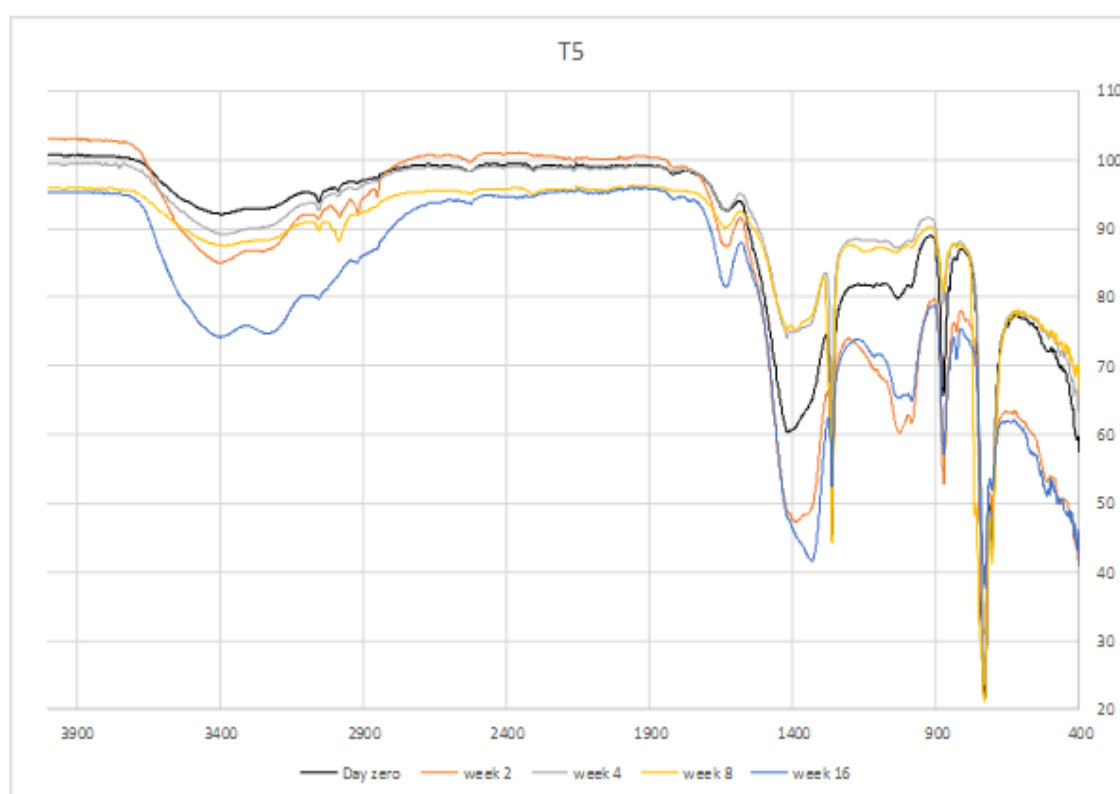

Figure 2: FTIR spectra of soils with T5 (D5D1)

The spectra obtained after microbial treatment showed in Figure 2 by T5 (D5D1) as a single bacterium as bioaugmentation on Duckan sandy oil spill with ionic surfactant illustrates similar absorption bands. However, the spectra have varying relative densities. The microbial process induced extensive response on the carbonyl C=O at 1630 cm^{-1} through hydrocarbon oxidation during weeks 2, 4, 8 and 16. As such, the bacteria caused an oxidative alteration in the macromolecular structure resulting to formation of oxygenated functional groups after biodegradation. The peaks on weeks 2, 4 and 8 presents at 2834 and 2914 represents the CH2 and CH3 stretching but these bands do not appear on week 16. The decrease in the band 704 =C-H for alkene means that the bacteria start breaking down the alkenes that are transformed to alkanes and the band 2987 is eliminated. Also, for the band 730, alkanes start decreasing due to degradation. IR spectra also showed on weeks 2, 4, 8 and 16 a decrease in the intensity of aromatic C-H stretching bands ranging between 3000 and 3100 cm^{-1}. Sequentially, there was disappearance of some aromatic nuclei peaks because of degradation. A band around 3200– 3550 cm^{-1} showed an increase in the intensity of O-H on week 2, 4, 8 and 16 generated by microbial oxidation process. The Alkene on band 2303 cm^{-1} do not appear after 2 weeks and alkene on band 703 cm^{-1} start to decrease in intensity after day Zero until 16 weeks which is an indication of biodegradation by the bacteria. Also, an increase in the intensity of the stretching vibrations of the C-O was illustrated by the IR spectra at a range of 1034 cm^{-1} indicating some kinds of biodegradation.
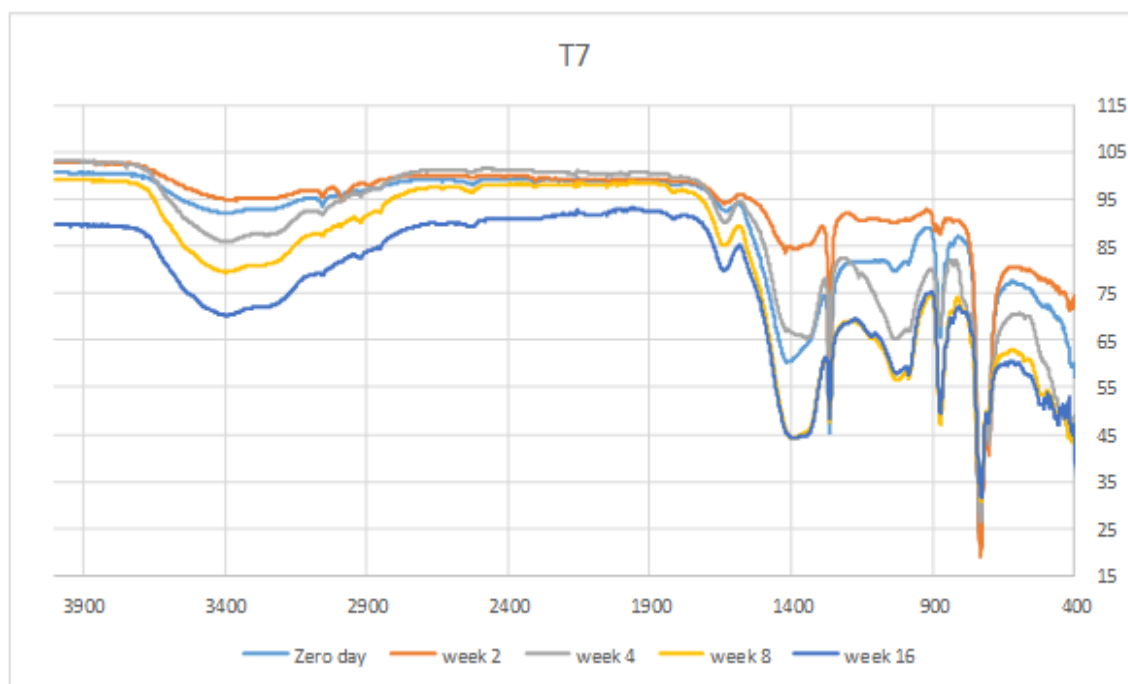
Figure 3: FTIR spectra of soils with T7 (D1D2 and D5D1).

The spectra obtained after microbial treatment showed in Figure 3 by T7 (D1D2 and D5D1). as consortia of bacteria as bioaugmentation on Duckan sandy oil spill with ionic surfactant illustrates similar absorption bands. However, the spectra have varying relative densities. The microbial process induced extensive response on the carbonyl C=O at 1630 cm^{-1} through hydrocarbon oxidation during weeks 2, 4, 8 and 16. As such, the bacteria caused an oxidative alteration in the macromolecular structure resulting to formation of oxygenated functional groups after biodegradation. The peak on weeks 4 present at 2914 represents the CH2 and CH3 stretching but these bands do not appear on weeks 2, 8 and 16. The decrease in the band 704 =C-H for alkene means that the bacteria start breaking down the alkenes that are transformed to alkanes and the band 2987 is eliminated. Also, for the band 730, alkanes start decreasing due to degradation. IR spectra also showed on weeks 2, 4, 8 and 16 a decrease in the intensity of aromatic C-H stretching

bands ranging between 3000 and 3100 cm^{-1}. Sequentially, there was disappearance of some aromatic nuclei peaks because of degradation. A band around 3200– 3550 cm^{-1} showed an increase in the intensity of O-H on week 2, 4, 8 and 16 generated by microbial oxidation process. The Alkene on band 2303 cm^{-1} do not appear after 2 weeks and alkene on band 703 cm^{-1} start to decrease in intensity after day Zero until 16 weeks which is an indication of biodegradation by the bacteria. Also, an increase in the intensity of the stretching vibrations of the C-O was illustrated by the IR spectra at a range of 1034 cm^{-1} indicating some kinds of biodegradation.
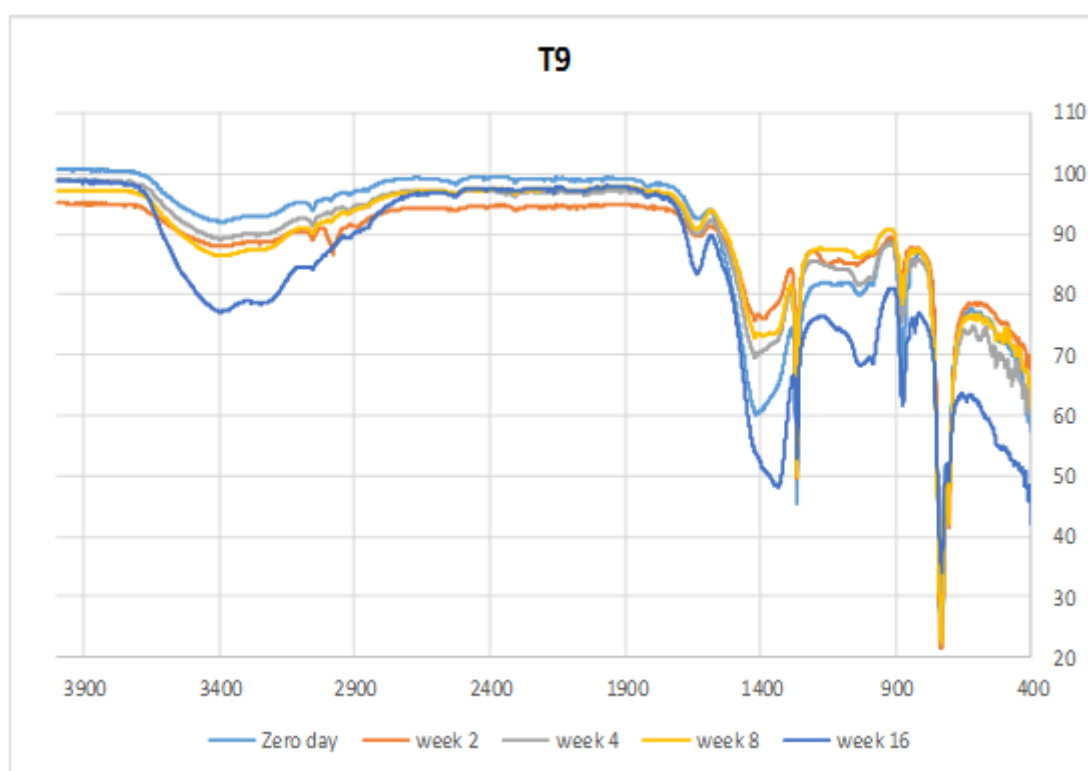

Figure 4: FTIR spectra of soils with T9 (D1D2 and D9S1)

The spectra obtained after microbial treatment showed in Figure 4 by T9 (D1D2 and D9S1) as consortia of bacteria as bioaugmentation on Duckan sandy oil spill with ionic surfactant illustrates similar absorption bands. However, the spectra have varying relative densities. The microbial process induced extensive response on the carbonyl C=O at 1630 cm^{-1} through hydrocarbon oxidation during week 16. As such, the bacteria caused an oxidative alteration in the macromolecular structure resulting to formation of oxygenated functional groups after biodegradation. The peak on weeks 2 present at 2914 represents the CH2 and CH3 stretching but these bands do not appear on weeks 4, 8 and 16. The decrease in the band 704 =C-H for alkene means that the bacteria start breaking down the alkenes that are transformed to alkanes and the band 2987 is eliminated. Also, for the band 730, alkanes start decreasing due to degradation. IR spectra also showed on weeks 2, 4, 8 and 16 a decrease in the intensity of aromatic C-H stretching bands ranging between 3000 and 3100 cm^{-1}. Sequentially, there was disappearance of some aromatic nuclei peaks because of degradation. A band around 3200– 3550 cm^{-1} showed an increase in the intensity of O-H on week 2, 4, 8 and 16 generated by microbial oxidation process. The Alkene on band 2303 cm^{-1} do not appear after 2 weeks and alkene on band 703 cm^{-1} start to decrease in intensity after day Zero until 16 weeks which is an indication of biodegradation by the bacteria. Also, an increase in the intensity of the stretching vibrations of the C-O was illustrated by the IR spectra at a range of 1034 cm^{-1} indicating some kinds of biodegradation.
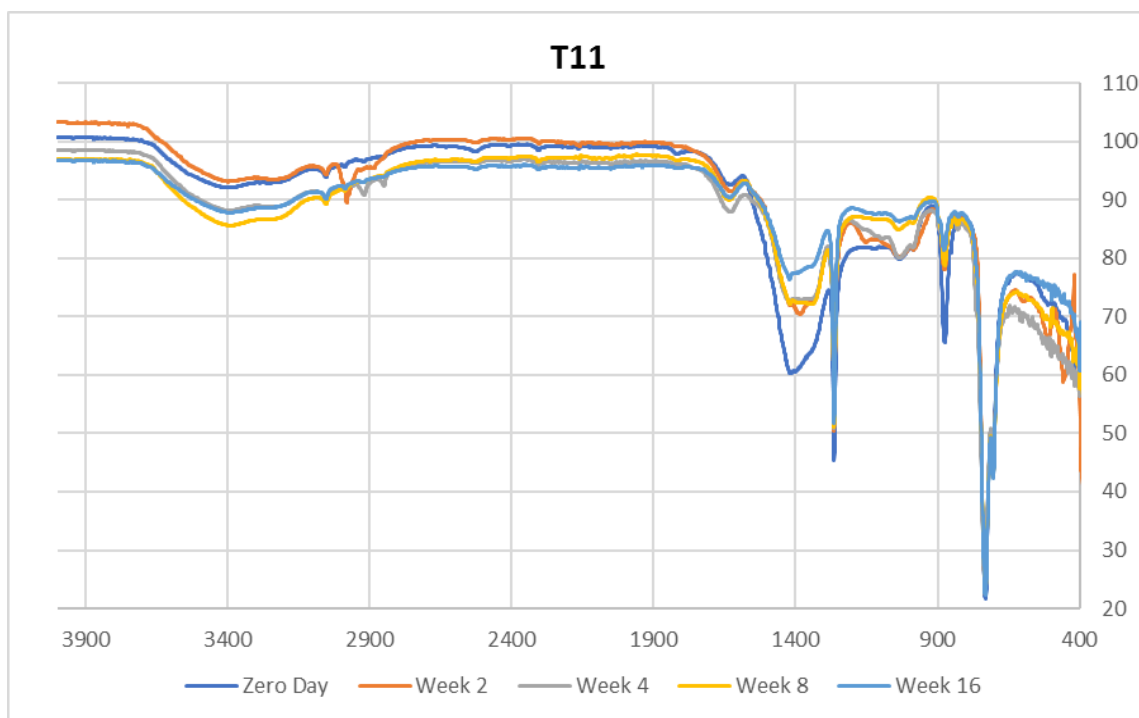
Figure 5: FTIR spectra of soils with T11 (D5D1 and D9S1)

The spectra obtained after microbial treatment showed in Figure 5 by T11 (D5D1 and D9S1) as consortia of bacteria as bioaugmentation on Duckan sandy oil spill with ionic surfactant illustrates similar absorption bands. However, the spectra have varying relative densities.

The microbial process induced extensive response on the carbonyl C=O at 1630 cm^{-1} through hydrocarbon oxidation during weeks 2, 4, 8 and 16. As such, the bacteria caused an oxidative alteration in the macromolecular structure resulting to formation of oxygenated functional groups after biodegradation. The peak on weeks 2 and 4 present at 2987, 2834 and 2914 represents the CH2 and CH3 stretching but these bands do not appear on weeks 8 and 16. The decrease in the band 704 =C-H for alkene means that the bacteria start breaking down the alkenes that are transformed to alkanes and the band 2987 is eliminated. Also, for the band 730, alkanes start decreasing due to degradation. IR spectra

207

also showed on weeks 2, 4, 8 and 16 a decrease in the intensity of aromatic C-H stretching bands ranging between 3000 and 3100 cm^{-1}. Sequentially, there was disappearance of some aromatic nuclei peaks because of degradation. A band around 3200– 3550 cm^{-1} showed an increase in the intensity of O-H on week 2, 4, 8 and 16 generated by microbial oxidation process. The Alkene on band 2303 cm^{-1} do not appear after 2 weeks and alkene on band 703 cm^{-1} start to decrease in intensity after day Zero until 16 weeks which is an indication of biodegradation by the bacteria. Also, an increase in the intensity of the stretching vibrations of the C-O was illustrated by the IR spectra at a range of 1034 cm^{-1} indicating some kinds of biodegradation.
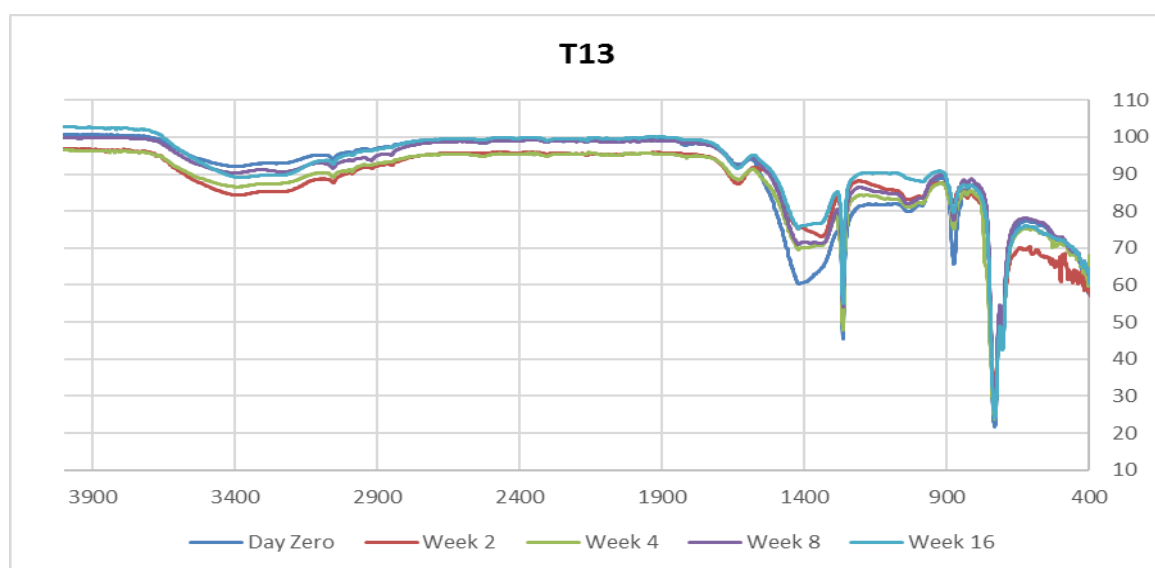

Figure 6: FTIR spectra of soils with T13 (D1D2, D5D1 and D9S1)

The spectra obtained after microbial treatment showed in Figure 6 by T13 (D1D2, D5D1 and D9S1) as consortia of bacteria as bioaugmentation **on** Duckan sandy oil spill with ionic surfactant exhibit the same absorption bands, which mean there are no degradation affecting the bands.
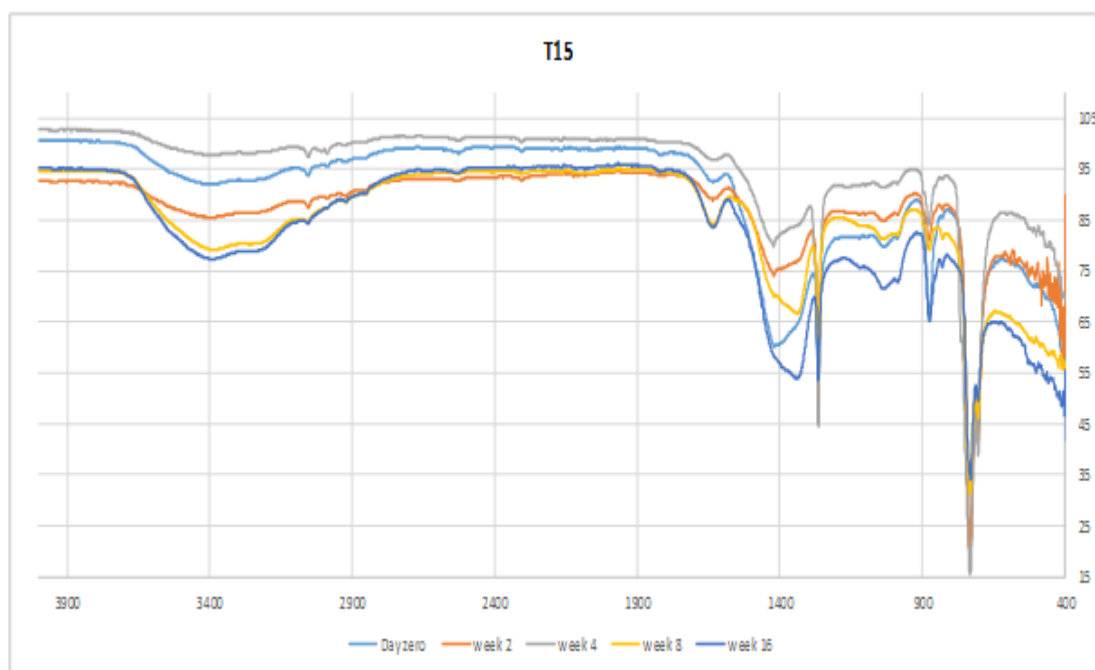
Figure 7: FTIR spectra of soils with T15 (without Bacteria)

The spectra obtained after microbial treatment showed in Figure 7 by T15 (without Bacteria) as biostimulation **on** Duckan sandy oil spill with ionic surfactant illustrates similar absorption bands. However, the spectra have varying relative densities. The microbial process induced extensive response on the carbonyl C=O at 1630 cm^{-1} through hydrocarbon oxidation during weeks 2, 4, 8 and 16. As such, the bacteria caused an oxidative alteration in the macromolecular structure resulting to formation of oxygenated functional groups after biodegradation. The peak on weeks 4 present at 2914 represents the CH2 and CH3 stretching but these bands do not appear on weeks 2, 8 and 16. The decrease in the band 704 =C-H for alkene means that the bacteria start breaking down the alkenes that are transformed to alkanes and the band 2987 is eliminated. Also, for the band 730, alkanes start decreasing due to degradation. IR spectra also showed on weeks 2, 4, 8 and 16 a decrease in the intensity of aromatic C-H stretching bands ranging between 3000 and 3100 cm^{-1}. Sequentially, there was disappearance of some aromatic nuclei peaks because

of degradation. A band around 3200– 3550 cm^{-1} showed an increase in the intensity of O-H on week 2, 4, 8 and 16 generated by microbial oxidation process. The Alkene on band 2303 cm^{-1} do not appear after 2 weeks and alkene on band 703 cm^{-1} start to decrease in intensity after day Zero until 16 weeks which is an indication of biodegradation by the bacteria. Also, an increase in the intensity of the stretching vibrations of the C-O was illustrated by the IR spectra at a range of 1034 cm^{-1} indicating some kinds of biodegradation.