Bioremediation of Libyan soil contaminated with crude oil tank bottom sludge

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis/project is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Abdulatif Mansur

28/11/2015
“Our progress as a nation can be no longer swifter than our progress in education... The human mind is our fundamental resource.”

John F. Kennedy
Acknowledgment

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Bioremediation of Libyan Soil Contaminated With Crude Oil Tank Bottom Sludge

Abdulatif Mansur

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Print name: Professor Andy S Ball

Signature: .................................................................

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CONFERENCE

- **WASTE CONVERSION TECHNOLOGY CONFERENCE & TRADE SHOW**; held on August 17-19th 2015 in San Diego, California, USA, “Recovery and Characterization of Oil from Waste Crude Oil Tank Bottom Sludge from Azzawiya Oil Refinery in Libya”.

POSTERS

- **ROYAL MELBOURNE INSTITUTE OF TECHNOLOGY** (RMIT Annual Research Day-2013), Screening of hydrocarbonoclastic activities of bacterial isolates on a range of petrogenic substrates, Abdulatif A. Mansur, Eric M. Adetutu, Krishna K. Kadali, Paul Morrison and Andrew S.Ball.
- **ROYAL MELBOURNE INSTITUTE OF TECHNOLOGY** (RMIT Annual Research Day-2014), Slurry Phase Bioremediation of Soil Contaminated With Crude Oil Tank Bottom Sludge (COTBS) Using Indigenous Bacterial Isolates, Abdulatif A. Mansur, Eric M. Adetutu and Andrew S.Ball.
STATEMENT OF AUTHORSHIP

Four peer-reviewed papers have been published from this project in international journals, and are presented as Chapters 3-6.

Abdulatif Mansur (PhD candidate) proposed the gap in knowledge, planned the experimental design, conducted the lab work, analysed and interpreted the results, wrote the manuscript draft and submitted the manuscripts for evaluation.

Signed Date 28/11/2015

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Mohamed Taha contributed to manuscript evaluation.
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Yuana Nurulita contributed to manuscript evaluation.
Tanvi Makadia contributed to manuscript evaluation.
Andrew S. Ball contributed in the planning of the experimental design, supervision of research, manuscript preparation and evaluation.

All authors have contributed to manuscript evaluation, read and approved the final manuscripts.
All co-authors give full consent to Abdulatif Mansur to present these 4 above papers for examination towards the Doctor of Philosophy.

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Bioremediation of Libyan Soil Contaminated With Crude Oil Tank Bottom Sludge

Abdulatif Mansur

Abstract

Petroleum crude oil represents about 50% of the world’s energy and heating services and secures raw materials for many industries. The oil industry generates massive amounts of crude oil tank bottom sludge (COTBS), a complex mixture of hydrocarbons. Disposal of untreated COTBS represents a significant economic loss and threatens human and environment health. This research project was designed to promote a safe and environmentally bioremediation technology which involved extraction of oil from COTBS followed by utilization of efficient bioremediation technologies including bioaugmentation (inoculating indigenous hydrocarbon degrading agents), biostimulation (supplying with the needed nutrients and air) and natural attenuation where the contaminants were left to be degraded naturally. In Libya, currently chemical and physical treatment methods are used to treat the contaminated soil, but both techniques are unsafe and expensive. Although bioremediation is a widely used methodology for petrogenic hydrocarbon contaminated soils, to date its application to the treatment of COTBS contaminated soils is limited especially in Mediterranean countries such as Libya. The aim of this project was to develop a cost effective, efficient, environment friendly and sustainable alternative treatment method that offers an alternative technology to current expensive, non-environmental friendly or sustainable physical and chemical approaches. In the first part of this study, the availability of oil within the COTBS for recycling purposes was investigated. Dichloromethane was used to extract the oil from the COTBS. The extracted oil was evaluated, characterised and compared to the parent oil (Hamada petroleum crude oil). The results indicated that COTBS contained a significant amount of recoverable oil (42.08 ± 1.1%). Gas Chromatography Mass Spectrometry detected 139 different hydrocarbon fractions within COTBS, composed of light hydrocarbons (30.7 ± 0.07%), heavy hydrocarbons (69.3 ± 0.4%), water (2.9 ± 0.2%) and solids (55.02 ± 0.6%). The API gravity of the extracted oil was 33.03 which classified the oil as Brent, similar to the parent oil. The benefits of oil reclamation is two-fold; firstly to improve oil utilization efficiency and secondly to reduce the environmental contamination from the oil industry. In the second part of the study, hydrocarbonclastic bacteria were isolated from COTBS, COTBS contaminated soil and treated COTBS contaminated soil and then characterized in terms of their hydrocarbonclastic potential. The results indicated the presence of 49 different bacterial phenotypes capable of growth on weathered Hamada crude oil. Evaluation of the substrate-
degrading abilities of the individual isolates confirmed the growth of the 49 isolates on at least one substrate from the six chosen. Seven organisms were able to degrade 5 out of the 6 substrates. Amongst the 6 substrates, phenanthrene was the most utilized and octadecane was the least utilized. Cluster analysis divided the hydrocarbon degraders into two separated clusters. Cluster 2 represented the highly hydrocarbon degrading group. They represented 7 bacterial isolates from four phylogenic groups (*Gammaproteobacteria*, *Firmicutes*, *Actinobacteria* and *Alphaproteobacteria*). These results confirmed that the isolation media was highly selective for hydrocarbon degrading organisms and the treated COTBS contaminated soil contained large numbers of hydrocarbonoclastic bacterial isolates. In the third part of the research, three bacterial isolates from cluster 2 (*Pseudomonas sp*, *Pseudomonas xanthomarina* and *Arthrobacter nitroguajacolicus*) were used in microcosm slurry phase bioremediation trials (through bioaugmentation, biostimulation and natural attenuation) of soil contaminated with COTBS. After 30 days, the degradation rate ranged from 97.8 to 99.4% where the total petroleum hydrocarbon (TPH) concentration was reduced from 30703 mg kg\(^{-1}\) to 170 to 664 mg kg\(^{-1}\), accompanied by a substantial reduction in PAH concentration, from 13816 mg kg\(^{-1}\) to below detection limit. In addition, the complete biodegradation of the carcinogenic and mutagenic fractions occurred. DNA-PCR-DGGE confirmed that no detectable changes in bacterial community between day 0 to day 30 were observed, with UPGMA analysis showing up to 100% similarity between day 0 and day 30 and between amended and control microcosms. In the final part of the research, larger scale slurry phase mesocosms (500 ml) were used to further assess the potential of the hydrocarbonoclastic isolates. The ability of two bacterial isolates (*Pseudomonas sp* and *Pseudomonas xanthomarina*) to bioremediate contaminated Libyan soils was investigated using three strategies; (i) (bioaugmentation) (BA), (ii) (biostimulation) (BS), (iii) both biostimulation-bioaugmentation (BA-BS). The results showed that BS-BA was the most efficient bioremediation option, with the greatest reduction in TPH for both isolates (97.19 and 96.67 %) observed (from 30703 mg kg\(^{-1}\) to 860 mg kg\(^{-1}\) and 1020 mg kg\(^{-1}\)). In contrast, the control showed only a 17.15% reduction in TPH concentration. After 90 days, BS-BA mesocosms also showed the highest rates of soil respiration (0.07 mg day\(^{-1}\) g soil\(^{-1}\)), some 2.5-fold higher than that observed in the control soils (0.021 mg day\(^{-1}\) g soil\(^{-1}\)), confirming that increased microbial activity correlated with degradation of the contaminant. Denaturing Gradient Gel Electrophoresis showed very little change in the bacterial community within the BS and BAS mesocosms while
analysis of the BS-BA mesocosms showed an increase in microbial diversity. This study provides further understanding and knowledge about the bioremediation of COTBS contaminated soil and confirms the potential and benefit of indigenous hydrocarbonoclastic bacteria and the potential of BS-BA remediation technology. The research suggests that this sustainable remediation technology can substitute for the currently used physical and chemical treatment methods applied in Libya.
Chapter 1

1 Introduction
1.1. Petroleum crude oil
Petroleum which in its raw phase is known as crude (petroleum) oil is a fossil mass that has accumulated below the surface of the earth millions of years ago (Chaudhuri 2010). Ancient petroleum crude oil was first utilised by the Chinese in pre-Christian times through surface leakage (Ayres & Alloway 1993). In 1859, in a well sunk in Pennsylvania the modern petroleum industry was started by Colonel E. A. Drake (Ayres & Alloway 1993). The first use of petroleum products was to replace expensive whale oil that has been used for lighting. Today, crude oil is used as a source of energy, lubricant and raw material for a range of diverse industries, making crude oil dominant in the world market.

1.2. Composition of crude oil
Crude oil is a complex heterogeneous liquid mixture of hundreds of hydrocarbon fractions and organic compounds (Zhang et al. 2010) in which carbon (85%-90%) and hydrogen (10%-14%) are the major elements, in addition to some non-hydrocarbon elements such as sulfur (0.2%-3%), nitrogen (≤ 0.1%-2%) and oxygen (1%-1.5%) and traces (parts per million) of metallic compounds including phosphorus, lead, nickel, arsenic and vanadium (Okoh 2006; Ray 2011). The organic compounds in petroleum crude oil are composed of linear and branched-chain volatile and non-volatile aromatic (26-30%) and aliphatic (up to 50%) fractions (Lal & Khanna 1996) ranging from light gases (C1-C4) to heavy residues (C35-C40) (Serrano et al. 2008), with resins constituting the remaining (Kadali 2012). However, the composition of crudes oil is not fixed and may vary depending on the age and location of an oil field, and upon the depth of each individual oil well.

1.3. Classification of crude oil
The characterization of oil is often referred to as a fingerprinting technique, requiring the integration of analytical methodologies with data processing. Each petroleum oil has an identifiable and often unique compositional profile. Gas chromatography (GC) and gas chromatography mass spectrometry (GC-MS) are techniques frequently used to analyse and determine the fractional composition of different oils (Pasadakis et al. 2008). In addition, physiochemical properties of oils such as American Petroleum Institute (API) gravity, density, viscosity and sulphur content are used to classify the oils.
Crude oils can be classified according to the relative proportions of the heavy molecular weight fraction present, with, crude oils classified as either light, medium or heavy crude. In addition the
constituents of the crude oil can also be broadly categorized based on their respective distillation residues into paraffins, iso-paraffins, naphthenes, olefins or aromatics (PIONA). Petroleum crude oils contain hundreds of individual compounds, these components are most commonly grouped into four categories (Fig. 1.1): (i): saturates (n- and branched-chain alkanes and cycloparaffins). (ii): aromatics (mono-, di- and polynuclear aromatic compounds connected to alkyl side chains and/or fused cycloparaffin rings). (iii): resins (aggregates with a multitude of building blocks such as thiophenes, sulfoxides, pyridines, carbazoles, quinolines and amides). (iv): asphaltenes (aggregates of extended naphthenic acids, polyaromatics, polyhydric phenols, fatty acids, sulfides and metalloporphyrins) (Sugiura et al. 1996). Further discussion of these 4 fractions is presented below.

Figure 1.1 The various fractions of hydrocarbons comprise the petroleum crude oil. Adopted from (Kadali 2012).

1.3.1. The saturated hydrocarbons
Saturated hydrocarbons are also known as aliphatic hydrocarbons and are the main components of petroleum crude oil (Figure 1.2). These fractions comprise about 50% of hydrocarbon products (Lal & Khanna 1996). Shorter chain aliphatic fractions are often toxic. In addition, these fractions readily penetrate into the soil and water surfaces, but they evaporate rapidly (Alvarez & Illman 2005). In regards to biodegradation, intermediate length (C10-C24) saturate hydrocarbons are easily degraded, but longer and branched alkanes with high molecular weights
are comparatively resistant to biological degradation (Kunihiro et al. 2005). Moreover, cyclic aliphatic hydrocarbons are less degradable compared to the linear and branched alkanes (Chaerun et al. 2004).

![Examples of saturated and branched hydrocarbons](http://chemwiki.ucdavis.edu)

**Figure 1.2.** Examples of saturated and branched hydrocarbons (http://chemwiki.ucdavis.edu).

### 1.3.2. The aromatic hydrocarbons

Petroleum crude oil consists of around 26-30% aromatic hydrocarbons which are naturally present in crude oil; more can be produced from oil processing (Lal & Khanna 1996) (Figure 1.3). These fractions are colourless, with low volatility and low solubility in water (Seo et al. 2009); these properties decrease with increasing molecular weight leading to resistance to biological degradation.

![Examples of aromatic hydrocarbons found in crude oil](http://chemwiki.ucdavis.edu)

**Figure 1.3.** Examples of aromatic hydrocarbons found in crude oil (http://chemwiki.ucdavis.edu)
1.3.3. The resin and asphaltene hydrocarbons
Crude oil consists of about 10% resins and asphaltenes. These compounds are polar, have high molecular weight and complex structure such as the oxygenated hydrocarbons (Vinas, M et al. 2002) (Figure 1.4). Although some studies have been conducted on the biodegradation of resins and asphaltenes, the biodegradation mechanism is still not identified (Morgado et al. 2009).

Figure 1.4. Two dimensional asphaltene molecule. (http://chemwiki.ucdavis.edu)

1.4. Properties of crude oil
There are a number of key parameters of oil that define the properties of the oil. Table 1.1 lists the main parameters that are measured, with values determined for Libyan Hamanda and Kuwait Light crude oils. One of the most important properties is the API gravity. API gravity is the relative density of the petroleum liquid compared with the density of water. API is the scale for denoting the 'lightness' or 'heaviness' of oil and oil products; the lighter the oil, the higher the API gravity. Lighter oils attract higher market values. Oils having an API more than 30º are known as light while oils with API between 22º and 30º are medium; an API less than 22º indicates a heavy oil and values below 10º are classified as very heavy oils. API values between 25º and 30º are preferred (Pusch & Gaida 1981). The viscosity, which is the fluid resistance to flow, is another important property. Viscosity affects the pumping and transportation abilities through the pipelines. Dealing with high viscosity oil is one of the main difficulties in transportation through the piping network (Centeno et al. 2011). Usually, the viscosity of petroleum oils ranges between100 mPa and $10^5$ mPa, with the maximum desired
viscosity being around 400 mPa (Hasan et al. 2010). Flash point is the minimum temperature at which the vapours of the material can ignite and is an indicator of the flammability of the hydrocarbon oils. Safe handling of oils including processing, storage and transportation requires estimation of accurate flash point values (Gharagheizi et al. 2008). Another key parameter is ash content; the ash content provides information on the metallic constituents remaining after the complete combustion of the fuel oils under specific conditions. High ash content lowers the heating values and is undesirable for direct combustion due to fouling and slagging of furnaces and boilers (Biller & Ross 2011). In addition, petroleum crude oil contains a small amount of salts, expressed as the amount of NaCl. If the salt content is higher than 30 ppm the salt needs to be minimized to reduce the fouling and corrosion and also to reduce the formation of acids by salts chlorides (Gary et al. 2010).

**Table 1.1.** Properties of Libyan Hamada petroleum crude oil and Kuwait light oil (Al-Besharah et al. 1987; Al-Sanad et al. 1995; Coker 2007; Pillon 2007; Riazi et al. 1999).

<table>
<thead>
<tr>
<th>DESCRIPTION</th>
<th>Libyan Hamada crude oil</th>
<th>Kuwait Light crude oil</th>
<th>Method of analysis</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>API gravity</td>
<td>38.8</td>
<td>29.65</td>
<td>Calculation</td>
<td></td>
</tr>
<tr>
<td>Density@ 15 ºC</td>
<td>0.8304</td>
<td>0.876</td>
<td>ASTM D-4052</td>
<td>g/ml</td>
</tr>
<tr>
<td>Specific gravity@60/60 ºF</td>
<td>0.8311</td>
<td>0.877</td>
<td>Calculation</td>
<td></td>
</tr>
<tr>
<td>Flash point ( PMCC )</td>
<td>23</td>
<td>41.5</td>
<td>ASTM D-93</td>
<td>ºC</td>
</tr>
<tr>
<td>Salt content (as NaCl)</td>
<td>2.14</td>
<td>2-3.5</td>
<td>IP 77</td>
<td>mg/l</td>
</tr>
<tr>
<td>Sulphur content</td>
<td>0.063</td>
<td>2.05</td>
<td>ASTM D-4294</td>
<td>wt.%</td>
</tr>
<tr>
<td>Kinematic viscosity @ 70 ºF</td>
<td>6.8431</td>
<td>9.15</td>
<td>ASTM D-445</td>
<td>cSt</td>
</tr>
<tr>
<td>Reid vapour pressure</td>
<td>4.2</td>
<td>7.39</td>
<td>ASTM D-323</td>
<td>psi</td>
</tr>
<tr>
<td>Hydrogen sulphide</td>
<td>17.04</td>
<td>IP 103</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water and sediment content</td>
<td>0.10</td>
<td>- 42</td>
<td>ASTM D-4007</td>
<td>vol.%</td>
</tr>
<tr>
<td>Pour point</td>
<td>-18</td>
<td>-</td>
<td>ASTM D-97</td>
<td>ºC</td>
</tr>
<tr>
<td>Kinematic viscosity @ 100 ºF</td>
<td>3.5742</td>
<td>5.5</td>
<td>ASTM D-445</td>
<td>cSt</td>
</tr>
<tr>
<td>Asphaltenes content</td>
<td>0.34</td>
<td>IP 143</td>
<td></td>
<td>wt.%</td>
</tr>
<tr>
<td>Conradson carbon residue</td>
<td>2.05</td>
<td>ASTM D-189</td>
<td></td>
<td>wt.%</td>
</tr>
<tr>
<td>Ash content</td>
<td>0.004</td>
<td>ASTM D-482</td>
<td></td>
<td>wt.%</td>
</tr>
<tr>
<td>Characterisation factor</td>
<td>12.0</td>
<td>UOP 375</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanadium</td>
<td>2.251</td>
<td>ASTM D 5708</td>
<td>ppm</td>
<td></td>
</tr>
<tr>
<td>Nickel</td>
<td>2.014</td>
<td>ASTM D 5708</td>
<td>ppm</td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>4.458</td>
<td>Dry ashing</td>
<td></td>
<td>ppm</td>
</tr>
<tr>
<td>Potassium</td>
<td>0.264</td>
<td>Dry ashing</td>
<td></td>
<td>ppm</td>
</tr>
</tbody>
</table>
Compared to other crude oils such as Kuwait light crude oil (Table 1.1), the Libyan Hamada crude oil is very light due to the presence of a high percentage of light fractions. The API value of Hamada oil is higher than 30º (38.8º) and the sulfur content is very low (0.063 % w/w), consequently this Libyan petroleum crude oil is classified as Brent and sweet oil.

1.5. Importance of crude oil and downstream products
In the first half of 2015, the annual daily global production of petroleum crude oil was about 87x10⁶ barrels per day (Benes et al. 2015). Crude oil continues to be the main source of energy for industry and daily life around the world (Rahman et al. 2003). It provides a wide range of raw materials for many industries such as plastics, fertilizers, pharmaceuticals and other products (Table 1.2) (Kadali et al. 2012). In addition, petroleum refined products including liquefied petroleum gas, liquid fuels, lubricating oils, asphalt, solvents, wax and coke are important and widely used substances consumed in modern society (Jha et al. 2008).

Table 1.2. The main products of petroleum crude oil refining.

<table>
<thead>
<tr>
<th>Petroleum refined product</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Refinery fuel gas</td>
<td></td>
</tr>
<tr>
<td>Liquefied Petroleum Gas (LPG)</td>
<td></td>
</tr>
<tr>
<td>Gasoline</td>
<td></td>
</tr>
<tr>
<td>Solvents</td>
<td></td>
</tr>
<tr>
<td>Aviation fuels</td>
<td></td>
</tr>
<tr>
<td>Diesel</td>
<td>(Gary et al. 2007; Kadali et al. 2012)</td>
</tr>
<tr>
<td>Heating oils</td>
<td></td>
</tr>
<tr>
<td>Lube oils</td>
<td></td>
</tr>
<tr>
<td>Grease</td>
<td></td>
</tr>
<tr>
<td>Asphalts</td>
<td></td>
</tr>
<tr>
<td>Industrial fuels</td>
<td></td>
</tr>
<tr>
<td>Refinery fuel oils</td>
<td></td>
</tr>
<tr>
<td>Plastics</td>
<td></td>
</tr>
<tr>
<td>Fertilizers</td>
<td></td>
</tr>
<tr>
<td>Pharmaceuticals</td>
<td></td>
</tr>
</tbody>
</table>
However while the petroleum industry brings numerous benefits to societies and is widely acknowledged as a key economic activity, especially among oil producing countries, globally this industry generates huge quantities with significant environmental impact (Cerqueira et al. 2011).

1.6. Problems associated with crude oil

1.6.1. Oil spills

There are many technical problems associated with the oil industry resulting in the large amounts of oil being released into the environment. Oil spills and discharges can occur at any stage in the life cycle of petrogenic hydrocarbons, during oil exploration and production, transportation (by vessels, through pipelines, by railroads or tanker trucks), storage facilities, consumption or usage of oil products as fuels or as raw materials for manufacturing or during waste disposal (Fingas, Mervin 2010). Oil spills are major economic and environmental issues because they lead to the release and loss of significant amounts of crude oils and refined products into the environment (Serrano et al. 2008; Sheppard et al. 2011). With approximately 50 % of transportation fuels being carried by sea, oil tanker accidents are inevitable, resulting in large-scale marine and coastal pollution (McKew et al. 2007). Yearly, about 20 accidents occur with thousands of tonnes of oil being spilled into seas and several weekly accidents with tens or hundreds of tonnes of hydrocarbons being spilled (Karpicza et al. 2005). Based on a comprehensive global database of major oil spills between 1967 and 2010, around 1200 oil spills occurred (Table 1.3) (Al-Majed et al. 2012; Eckle et al. 2012; Jernelöv 2010). The average estimated annual amounts of petrogenic hydrocarbons entering the marine and coastal environments from tankers and other sea-based activities is estimated to be 1,245,200 tonnes year$^{-1}$ (Betti et al. 2011). The USA national contingency plan (NCP) defines a minor oil spill as that less than 1000 gallons (3785 litres) to inland water or less than 10,000 gallons (37854 litres) to coastal water; a major discharge represents a spill of more than 10,000 gallons (37854 litres) to inland water or more than 100,000 gallons (378541 litres) to coastal water (Stalcup et al. 1997). Between 1988 and 2000, there were about 2,475 spills recording more than 800,000 litres of oil in Toronto and surrounding regions alone. In 1995, more than 5000 oil spills were reported by the US Emergency Response Notification System (ERNS) database (Stalcup et al. 1997). The sinking of the ship Prestige on 19th November 2002 contaminated the Galician coastline with 60,000 tonnes of oil resulting in ecological damage over 900 km of coastline (Vieites et al. 2004). In fact since
1960, a total of 410 large tanker oil spills of more than 700 tonnes have occurred, polluting the seas with over 5.5 million tonnes of oil (Vieites et al. 2004).

Although the number and size of oil spills have decreased significantly, blowouts (Table 1.4), spills from old, un-maintained or damaged pipelines and oil wells have increased dramatically (Figure 1.2), and regions like north-western Amazon, Niger Delta and Arctic Russia have become hot-spots of reoccurring hydrocarbon contamination (Jernelöv 2010).

1.6.1.1. Deepwater Horizon oil spill

The Deepwater Horizon oil spill (also known as the Gulf of Mexico Oil Spill or the BP Oil Spill) was the largest marine oil spill in history. On 20 April 2010, an explosion on the Deepwater Horizon offshore oil platform led to a massive spill and the platform sank in about 5,000 feet (1,500 m) of water (Bailus 2010). The spill was located about 50 miles southeast of the Mississippi River delta (28.74°N, 88.39°W. By July 15 2010, 86 days after the spill, British Petroleum (BP) finally successfully capped the well and stopped the flow of oil into the Gulf of Mexico. A total of approximately 4.9 million barrels (779 million liters, ±10%) of oil, consisting of a complex mixture of hydrocarbons including saturated hydrocarbons (74%), aromatic hydrocarbons (including polycyclic hydrocarbons (PAHs) which reached a maximal concentrations of 1200 mg l-1 at the surface was released affecting marine life and the activity and the diversity of the microbial community (Hazen, Dubinsky et al. 2010). In response to the deep-sea plume of hydrocarbons, documented shifts in the structure of the microbial community were examined by DNA based methods including cloning and sequencing of 16S rRNA genes and microarray analysis of functional genes (Hazen, Dubinsky et al. 2010). The analysis indicated that the oil affected microbial community was dominated by Oceanospirillales (Hazen, Dubinsky et al. 2010), Colwellia and Cycloclasticus together with methylotrophic bacteria (Valentine, Kessler et al. 2010).
Table 1.3. Examples of some of the largest oil spills from tankers occurring (Oil Tanker Spill Statistics 2009) (Al-Majed et al. 2012; Jernelöv 2010).

<table>
<thead>
<tr>
<th>Position</th>
<th>Ship name</th>
<th>Year</th>
<th>Location</th>
<th>Spill size (tonnes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Torrey Canyon</td>
<td>1967</td>
<td>Isles of Scilly, UK</td>
<td>119000</td>
<td></td>
</tr>
<tr>
<td>Sea Star</td>
<td>1972</td>
<td>Gulf of Oman</td>
<td>115000</td>
<td></td>
</tr>
<tr>
<td>Jakob Maersk</td>
<td>1975</td>
<td>Oporto, Portugal</td>
<td>88000</td>
<td></td>
</tr>
<tr>
<td>Urquiosa</td>
<td>1976</td>
<td>A Corunã, Spain</td>
<td>100000</td>
<td></td>
</tr>
<tr>
<td>Hawaiian Patriot</td>
<td>1977</td>
<td>300 nautical miles off Honolulu</td>
<td>95000</td>
<td></td>
</tr>
<tr>
<td>Amoco Cadiz</td>
<td>1978</td>
<td>Off Brittany, France</td>
<td>223000</td>
<td></td>
</tr>
<tr>
<td>Atlantic Empress</td>
<td>1979</td>
<td>Off Tobago, West Indies</td>
<td>287000</td>
<td></td>
</tr>
<tr>
<td>Independenta</td>
<td>1979</td>
<td>Bosphorus, Turkey</td>
<td>95000</td>
<td></td>
</tr>
<tr>
<td>Irenes Serenade</td>
<td>1980</td>
<td>Navarino Bay, Greece</td>
<td>100000</td>
<td></td>
</tr>
<tr>
<td>Castillo de Bellver</td>
<td>1983</td>
<td>Off Saldanha Bay, South Africa</td>
<td>252000</td>
<td></td>
</tr>
<tr>
<td>Khark 5</td>
<td>1983</td>
<td>120 nautical miles off Atlantic coast of Morocco</td>
<td>80000</td>
<td></td>
</tr>
<tr>
<td>Odyssey</td>
<td>1988</td>
<td>700 nautical miles off Nova Scotia, Canada</td>
<td>132000</td>
<td></td>
</tr>
<tr>
<td>ABT Summer</td>
<td>1991</td>
<td>700 nautical miles off Angola</td>
<td>260000</td>
<td></td>
</tr>
<tr>
<td>Haven</td>
<td>1991</td>
<td>Off Brittany, France</td>
<td>144000</td>
<td></td>
</tr>
<tr>
<td>Braer</td>
<td>1993</td>
<td>Shetland Islands, UK</td>
<td>85000</td>
<td></td>
</tr>
<tr>
<td>Sea Empress</td>
<td>1996</td>
<td>Milford Haven, Wales</td>
<td>70000</td>
<td></td>
</tr>
</tbody>
</table>
Table 1.4. The largest marine oil blowouts (Jernelöv 2010).

<table>
<thead>
<tr>
<th>Well</th>
<th>Country</th>
<th>Year</th>
<th>Spill (tonnes)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ixtoc I</td>
<td>Mexico</td>
<td>1979</td>
<td>475000</td>
<td>Spill figure from Mexican Petroleum</td>
</tr>
<tr>
<td>Nowruz</td>
<td>Iran</td>
<td>1983-1985</td>
<td>100000</td>
<td>After attack by Iraqi airplanes</td>
</tr>
<tr>
<td>Nowruz</td>
<td>Iran</td>
<td>1983</td>
<td>40000</td>
<td>After oil platform was hit by a tanker</td>
</tr>
<tr>
<td>Ecofisk</td>
<td>Norway</td>
<td>1977</td>
<td>27000</td>
<td>Bravo platform blowout</td>
</tr>
<tr>
<td>Funiwa 5</td>
<td>Nigeria</td>
<td>1980</td>
<td>26000</td>
<td>Chevron blowout and rig fire</td>
</tr>
<tr>
<td>Montara</td>
<td>Australia</td>
<td>2009</td>
<td>20000</td>
<td>Blowout of Montara oil wellhead</td>
</tr>
<tr>
<td>Deepwater Horizon</td>
<td>Gulf of Mexico</td>
<td>2010</td>
<td>680000</td>
<td>Explosion and sinking of the Deepwater Horizon oil rig</td>
</tr>
</tbody>
</table>

Figure 1.5. Water contamination with hydrocarbon. The Deep-water Horizon (Fingas, Merv & Brown 2014).
1.6.2. Environmental problems
Crude oil and downstream products have become a major environmental concern as many of the hydrocarbons present have a significant adverse effect on the environment including, water resources, marine life (Figure 1.5), soil surfaces (Figure 1.6) and air quality (Guo, W & Wang 2009; Molina-Barahona et al. 2004; Mukherjee, A.K. & Bordoloi 2011). Water contamination is a complex process and difficult to treat for many reasons including the low water solubility of oils and limited rate of mass transfer (Paria 2008). Hydrocarbon contamination of soils leads to a continuous and potentially long term source of contamination where the pollutants may migrate into the groundwater.

Figure 1.6. Soil contamination by hydrocarbon. (http://www.polyinform.com).

Petroleum hydrocarbons are the most widespread pollutants in the environment (Margesin et al. 2003; Zhang et al. 2012). Depending on toxicity, the spread of oil into the environment and flammability of spilled oil, the USEPA has categorized the petroleum crude oil into four categories, light, waxy, heavy and non-fluid oil in terms of their effect on the environment (Table. 1.5). Soil contamination with hydrocarbons is of concern in many countries and sites (Guo et al. 1997), as it can result in loss of soil fertility, destruction of plant communities, vegetation and the soil microbial community (Osuji et al. 2006). Even after remediation,
hydrocarbons may persist in surface and subsurface soils affecting the soil properties and microbial community (Osuji et al. 2006).

**Table 1.5.** EPA categorization of petroleum crude oil (Brody et al. 2012).

<table>
<thead>
<tr>
<th>Oil Class</th>
<th>Description</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Light and volatile oil</td>
<td>Highly toxic to human, marine creatures, spreads easily on liquid and solid surfaces, strong odour, evaporates easily and flammable.</td>
</tr>
<tr>
<td>B</td>
<td>Waxy oil</td>
<td>Less toxic, adheres more firmly to surfaces and has an oily feeling.</td>
</tr>
<tr>
<td>C</td>
<td>Heavy and Sticky oil</td>
<td>Low toxicity, viscous, sticky and sinks easily.</td>
</tr>
<tr>
<td>D</td>
<td>Non-fluid oil</td>
<td>Relatively non-toxic and does not penetrate porous materials easily.</td>
</tr>
</tbody>
</table>

1.6.3. **Polycyclic Aromatic Hydrocarbons**

The environmental risk of hydrocarbons comes largely from the presence of polycyclic aromatic hydrocarbons (PAHs). PAHs are chemical compounds consisting of two or more fused aromatic rings in a linear or clustered arrangement (Usman et al. 2012). Usually PAHs contain carbon and hydrogen atoms. Nitrogen, sulphur and oxygen atoms may substitute in the benzene ring to form heterocyclic aromatic compounds. In addition to their presence in oil, PAHs are formed from different sources including the incomplete combustion of fossil fuels, petroleum catalytic cracking, pyrolysis of organic matter, forest fires, volcanic eruptions, vehicles emissions and residual wood burning (Chen, B et al. 2012). PAHs are toxic organic contaminants to both environmental and human health. Due to their biological effects, toxicity, mutagenicity and carcinogenicity, PAHs are compounds of significant environmental concern (Gan, Lau et al. 2009, Haritash and Kaushik 2009). The USEPA and the European Union list 16 priority PAHs pollutants (Table 1.6). The list contains PAHs having severe toxic, mutagenic and mutagenic effects.
Bioremediation of Libyan Soil Contaminated With Crude Oil Tank Bottom Sludge

Abdulatif Mansur

Disposal of soil contaminated with petroleum hydrocarbons including PAHs is usually carried out with respect to the countrywide legislations which vary from country to country. For example, in Australia, the National Environmental Protection Agency regards soil contaminated with Total Petroleum Hydrocarbons (TPH) below 10 000 mg kg\(^{-1}\) as Low Level Contaminant Waste and safe for disposal in landfill sites (Makadia et al. 2011). At concentrations greater than 3% (w/w), hydrocarbons in soil are regarded as harmful to soil biota and to crop growth (Baker 1976).

**Table 1.6.** US EPA’s 16 priority-pollutant PAHs and selected physical–chemical properties (Bojes & Pope 2007).

<table>
<thead>
<tr>
<th>Polycyclic aromatic hydrocarbons</th>
<th>Number of rings</th>
<th>Molecular weight (g/mole)</th>
<th>Solubility (mg/L)</th>
<th>Vapour pressure (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>2</td>
<td>128.17</td>
<td>31</td>
<td>8.89E-02</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>3</td>
<td>154.21</td>
<td>3.8</td>
<td>3.75E-03</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>3</td>
<td>152.20</td>
<td>16.1</td>
<td>2.90E-02</td>
</tr>
<tr>
<td>Anthracene</td>
<td>3</td>
<td>178.23</td>
<td>0.045</td>
<td>2.55E-05</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>3</td>
<td>178.23</td>
<td>1.1</td>
<td>6.80E-04</td>
</tr>
<tr>
<td>Fluorene</td>
<td>3</td>
<td>166.22</td>
<td>1.9</td>
<td>3.24E-03</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>4</td>
<td>202.26</td>
<td>0.26</td>
<td>8.13E-06</td>
</tr>
<tr>
<td>Benzo(a)anthracene</td>
<td>4</td>
<td>228.29</td>
<td>0.011</td>
<td>1.54E-07</td>
</tr>
<tr>
<td>Chrysene</td>
<td>4</td>
<td>228.29</td>
<td>0.0015</td>
<td>7.80E-09</td>
</tr>
<tr>
<td>pyrene</td>
<td>4</td>
<td>202.26</td>
<td>0.132</td>
<td>4.25E-06</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>5</td>
<td>252.32</td>
<td>0.0038</td>
<td>4.89E-09</td>
</tr>
<tr>
<td>Benzo(b)fluoranthene</td>
<td>5</td>
<td>252.32</td>
<td>0.0015</td>
<td>8.06E-08</td>
</tr>
<tr>
<td>Benzo(k)fluoranthene</td>
<td>5</td>
<td>252.32</td>
<td>0.0008</td>
<td>9.59E-11</td>
</tr>
<tr>
<td>Dibenzo(a,h)anthracene</td>
<td>6</td>
<td>278.35</td>
<td>0.0005</td>
<td>2.10E-11</td>
</tr>
<tr>
<td>Dibenzo(a,h)anthracene</td>
<td>6</td>
<td>276.34</td>
<td>0.00026</td>
<td>1.00E-10</td>
</tr>
<tr>
<td>Indeno[1,2,3-cd] pyrene</td>
<td>6</td>
<td>276.34</td>
<td>0.062</td>
<td>1.40E-10</td>
</tr>
</tbody>
</table>
1.7. Hydrocarbon oily sludge

Hydrocarbon oily sludge is a very complex oil/ water emulsion containing different petroleum hydrocarbons including petroleum crude oil, diesel, different concentrations of cycloalkanes, normal and branched alkanes, phenolic, aromatics, polycyclic aromatic hydrocarbons (PAH’s) and cycloalkanes in addition to suspended solids and mineral particles (Al-Futaisi et al. 2007; Jing et al. 2011; Makadia et al. 2011) (Table 1.7). Oily sludge normally contains 10–50 % (w/w) hydrocarbons in addition to 6–10 % (w/w) solids (Liu et al. 2012).

**Table 1.7.** The main components of oily sludge (Hu et al. 2013).

<table>
<thead>
<tr>
<th>Components</th>
<th>Value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocarbon</td>
<td>5-86.2 (more frequent 15-50)</td>
<td>% Wt.</td>
</tr>
<tr>
<td>Alkanes</td>
<td>40-52</td>
<td></td>
</tr>
<tr>
<td>Aromatic</td>
<td>28-31</td>
<td></td>
</tr>
<tr>
<td>Asphaltenes</td>
<td>8-10</td>
<td></td>
</tr>
<tr>
<td>Resins</td>
<td>7-22.4</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>30-85</td>
<td></td>
</tr>
<tr>
<td>Solids</td>
<td>5-46</td>
<td></td>
</tr>
<tr>
<td>Nitrogen</td>
<td>Less than 3</td>
<td></td>
</tr>
<tr>
<td>Sulphur</td>
<td>0.3-10</td>
<td></td>
</tr>
<tr>
<td>Oxygen</td>
<td>Less than 3</td>
<td></td>
</tr>
</tbody>
</table>

Oily sludges are mainly generated from water/oil separators and from the accumulation of crude oil tank bottom sludge’s (COTBS) (Ferrari et al. 1996). In petroleum oil refineries, COTBS is a major problem in terms of waste management because considerable amounts of oily COTBS is generated annually (Zubaidy & Abouelnasr 2010). The quantities of COTBS generated from petroleum oil refineries depends on many factors and conditions including the refinery capacity, oil storing methods, refinery processes in addition to the properties of the crude oil such as density and viscosity (Hu et al. 2013). However, generally for every 500 tonnes of crude oil processed, one tonne of oily sludge is generated (Hu et al. 2013). Oil refineries processing between 200 and 500 barrels of oil per day generate about 10,000 m³ of sludge annually (Cerqueira et al. 2011). In China it was estimated that the amount of oily sludge generated from
oil refineries and oil fields in 2006 was around 450 x10³ tonnes (Zilong & Shuixiang 2007). By 2011 this value had increased to 3 x 10⁶ tonnes. In one year, more than 10⁵ tonnes of sludge was generated by the Shengli oilfield alone (Wang et al. 2012). An investigation conducted by the US EPA indicated that in the USA, each oil refinery generates an average of 30 x10³ tonnes of oily sludge per year. Worldwide, in 2009 the amount of oil refined was 14.4 x 10⁶ m³ day⁻¹ with an estimated oily sludge generation of 19 x 10⁴ m³ day⁻¹ (da Silva et al. 2012). In Libya oil production is around 1.8 x10⁶ barrels per day of light crude oil. Concomitant with this oil production is the generation of oily sludge. Usually the accumulated sludge is treated physically, chemically or mechanically to remove hydrocarbons then dumped in landfills while still containing significant amounts of hydrocarbons that are potentially harmful to humans and the environment.

In Libya and worldwide, crude oil storage tanks at oil refineries are cleaned periodically and the generated oily COTBS is exposed to a routine cleaning and removal (Ferrari et al. 1996; Gallego et al. 2007). The removed COTBS poses difficulties for treatment and disposal because it contains many environmentally toxic compounds as well as carcinogenic hydrocarbon fractions. The USEPA lists many components of the COTBS as toxic, mutagenic and potentially carcinogenic (Cerqueira et al. 2011; Hamamura et al. 2006) (Table 1.8).

**Table 1.8.** EPA list of the main toxic, mutagenic and carcinogenic compounds found in sludge; adapted from (Keith & Telliard 1979).

<table>
<thead>
<tr>
<th>No</th>
<th>COTBS Component</th>
<th>No</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acrotein</td>
<td>41</td>
<td>Dichlorodifluoromethane</td>
</tr>
<tr>
<td>2</td>
<td>Acrylonitrile</td>
<td>42</td>
<td>Chlorodibromomethane</td>
</tr>
<tr>
<td>3</td>
<td>Benzene</td>
<td>43</td>
<td>Isophorne</td>
</tr>
<tr>
<td>4</td>
<td>Toluene</td>
<td>44</td>
<td>Nitrobenzene</td>
</tr>
<tr>
<td>5</td>
<td>Ethylbenzene</td>
<td>45</td>
<td>2,4-Dinitrotoluene</td>
</tr>
<tr>
<td>6</td>
<td>Carbon tetrachloride</td>
<td>46</td>
<td>2,6-Dinitrotoluene</td>
</tr>
<tr>
<td>7</td>
<td>Chloridebenzene</td>
<td>47</td>
<td>4-Bromophenyl phenyl ether</td>
</tr>
<tr>
<td>8</td>
<td>1,2-Dichloroethane</td>
<td>48</td>
<td>bis(2-Ethylhexyl) phthalate</td>
</tr>
<tr>
<td>9</td>
<td>1,1,1-Trichloroethane</td>
<td>49</td>
<td>Di-n-octyl phthalate</td>
</tr>
<tr>
<td>10</td>
<td>1,1-Dichloroethane</td>
<td>50</td>
<td>Dimethyl phthalate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>1,1-Dichloroethylene</td>
<td>51</td>
<td>Diethyl phthalate</td>
</tr>
<tr>
<td>12</td>
<td>1,1,2-Trichloroethane</td>
<td>52</td>
<td>Di-n-butyl phthalate</td>
</tr>
<tr>
<td>13</td>
<td>1,1,2,2-Tetrachloroethane</td>
<td>53</td>
<td>Fluorene</td>
</tr>
<tr>
<td>14</td>
<td>Chloroethane</td>
<td>54</td>
<td>Fluoranthene</td>
</tr>
<tr>
<td>15</td>
<td>2-Chloroethyle vinyl ether</td>
<td>55</td>
<td>Chrysene</td>
</tr>
<tr>
<td>16</td>
<td>Chloroform</td>
<td>56</td>
<td>Pyrene</td>
</tr>
<tr>
<td>17</td>
<td>1,2-Dichloropropane</td>
<td>57</td>
<td>Phenanthrene</td>
</tr>
<tr>
<td>18</td>
<td>Hexachlorocyclopentadiene</td>
<td>58</td>
<td>Anthracene</td>
</tr>
<tr>
<td>19</td>
<td>1,3-Dichloropropane</td>
<td>59</td>
<td>Benzo(a)anthracene</td>
</tr>
<tr>
<td>20</td>
<td>Methylene chloride</td>
<td>60</td>
<td>Benzo(b)fluoranthene</td>
</tr>
<tr>
<td>21</td>
<td>Methyl chloride</td>
<td>61</td>
<td>Benzo(k)fluoranthene</td>
</tr>
<tr>
<td>22</td>
<td>Tetrachloroethylene</td>
<td>62</td>
<td>Benzo(a)pyrene</td>
</tr>
<tr>
<td>23</td>
<td>Vinyl chloride</td>
<td>63</td>
<td>Indenol(1,2,3-c,d)pyrene</td>
</tr>
<tr>
<td>24</td>
<td>1,2-trans-Dichloroethylene</td>
<td>64</td>
<td>Dibeno(a,h)anthracene</td>
</tr>
<tr>
<td>25</td>
<td>bis (Chloromethyl) ether</td>
<td>65</td>
<td>Benzo(g,h,i)perylene</td>
</tr>
<tr>
<td>26</td>
<td>1,2-Dichlorobenzene</td>
<td>66</td>
<td>4-Chlorophenyl phenyl ether</td>
</tr>
<tr>
<td>27</td>
<td>1,3-Dichlorobenzene</td>
<td>67</td>
<td>3,3′-Dichlorobenzidine</td>
</tr>
<tr>
<td>28</td>
<td>1,4-Dichlorobenzene</td>
<td>68</td>
<td>Benzidine</td>
</tr>
<tr>
<td>29</td>
<td>Hexachloroethane</td>
<td>69</td>
<td>bis(2-Chloroethyl)ether</td>
</tr>
<tr>
<td>30</td>
<td>Hexachlorobutadiene</td>
<td>71</td>
<td>1,2-Diphenylhydrazine</td>
</tr>
<tr>
<td>31</td>
<td>Hexachlorobenzene</td>
<td>72</td>
<td>Copper and compounds</td>
</tr>
<tr>
<td>32</td>
<td>1,2,4-Trichlorobenzene</td>
<td>73</td>
<td>Chromium and compounds</td>
</tr>
<tr>
<td>33</td>
<td>bis(2-Chloroethoxy)methane</td>
<td>74</td>
<td>Lead and compounds</td>
</tr>
<tr>
<td>34</td>
<td>Naphthalene</td>
<td>75</td>
<td>Mercury and compounds</td>
</tr>
<tr>
<td>35</td>
<td>2-Chloronaphthalene</td>
<td>76</td>
<td>Nickel and compounds</td>
</tr>
<tr>
<td>36</td>
<td>N-Nitrosodiphenylamine</td>
<td>77</td>
<td>Selenium and compounds</td>
</tr>
<tr>
<td>37</td>
<td>Methyl bromide</td>
<td>78</td>
<td>Silver and compounds</td>
</tr>
<tr>
<td>38</td>
<td>Bromoform</td>
<td>79</td>
<td>Thallium and compounds</td>
</tr>
<tr>
<td>39</td>
<td>Dichlorobromomethane</td>
<td>80</td>
<td>Zink and compounds</td>
</tr>
<tr>
<td>40</td>
<td>Trichlorofluoromethane</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
COTBS is therefore considered a hazardous solid waste which is frequently reported as a soil contaminant (Jing et al. 2011). Once released into the environment COTBS affects the physiochemical properties of soils, leading to changes in soil morphology (Robertson et al. 2007). COTBS may also deplete soil nutrients (such as N and P), prevent seed germination, cause plant death or restrict plant growth (Al-Mutairi et al. 2008). Due to the high viscosity of COTBS its components may also penetrate and block soil pores, adsorbing to the mineral constituents of the soil. The overall result of these impacts are loss of hydraulic conductivity, hygroscopic moisture and water holding capacity of the soil (Trofimov & Rozanova 2003). In addition the high molecular weight components of COTBS may remain near the soil surface forming a hydrophobic layer which reduces moisture availability and prevents air/ water exchange (Tang et al. 2012). Specifically, the hydrocarbons present in COTBS have been shown to interact with the covalent bonds between humic polymers (such as humic acid and fulvic acid) forming fatty acids, long-chain alkanes and stable dialkylphthalates which are capable of restricting their microbial degradation (Alexander 2000; Certini 2005).

In addition, COTBS contain polycyclic aromatic hydrocarbons (PAHs) (Yu et al. 2005). PAHs are of major concern as they are toxic to the humans and to other ecological receptors (Robertson et al. 2007). PAH’s have been known to significantly affect soil enzyme activity and have toxic effects on the soil microbial community (Suleimanov et al. 2004). PAHs present in COTBS may migrate down through the soil and enter the groundwater and pass to the other aquatic systems leading to serious unwanted consequences including a reduction in fish density and diversity (Wake 2005).

As well as the presence of hydrocarbons, COTBS may also contain heavy metals which may also interfere with soil function. Many heavy metals are hazardous and have accumulative effects. In oil refineries COTBS are deposited in open lagoons exposed to open air, enabling the volatile organic compounds (VOCs) to evaporate polluting the surrounded atmosphere (Cheremisinoff & Rosenfeld 2009). Such air emissions can cause health risks to facility workers and surrounding communities (Epstein et al. 2002). As a consequence of the hazardous nature of the oily COTBS, many countries have established strict regulations for handling, storage and disposal. In the USA the Resource Conservation and Recovery Act (RCRA) was established and standards were applied for dealing with COTBS (EPA 1980).
1.8. Treatment of soil contaminated with COTBS
Being recognized as a hazardous waste in many countries, improper disposal or insufficient treatment of oily sludge can pose serious threats to the environment and human health (Adetutu et al. 2011; Hu et al. 2013; Kadali et al. 2012; Sheppard et al. 2011; Yuste et al. 2000; Zhang et al. 2010). Due to the carcinogenic, mutagenic and toxic potential of some of the hydrocarbons within the COTBS, the efficient remediation of the COTBS has become an urgent requirement (Jiang et al. 2013; Makadia et al. 2011; Molina-Barahona et al. 2004). In recent years a variety of treatment approaches have been developed including chemical and physical technologies together with biological treatments (Sarkar et al. 2005). Chemical and physical approaches have included solidification, stabilization with additives such as magnetite, solvent extraction, ultrasonic treatment, thermal desorption and incineration (Gallego et al. 2007; Li et al. 1995; Liu et al. 2009; Varanasi et al. 2007; Xu et al. 2009). However, all these approaches have environmental and/or economic disadvantages. For example, treating one cubic metre of COTBS contaminated soil by disposal into a landfill costs about $880 while incinerating one cubic meter costs about $700; thermal desorption costs $260 per cubic meter (Makadia et al. 2011; Rahman et al. 2003). In addition, some of the chemical treatment methods leave behind unwanted toxic chemicals such as solvents that themselves have potential deleterious impact on humans, soil and microbial communities. Overall these approaches offer only temporary solutions (Das et al. 2012). Further they are not safe and environmentally friendly. Consequently, there is a need to find fast, inexpensive, safe and environmental friendly approaches to the treatment of COBTS.

1.8.1. Bioremediation
Bioremediation is a safe, cost effective, efficient and environmental friendly approach compared to chemical and physical treatment approaches (Mansur et al. 2015; Zhang et al. 2010). Bioremediation of soil contaminated with petroleum hydrocarbons has been known for about 80 years but effective studies of the application of bioremediation began in 1967 when Davis summarized the early work and concluded that specific microorganisms showed the potential to degrade petroleum hydrocarbons and utilize them as a main carbon source for energy and growth (Kumar et al. 2011). Later studies showed that indigenous isolates from soil and water have the ability to degrade a wide range of contaminants in the environment including different hydrocarbons (Hazen et al. 2010; Jain et al. 2012; Mansur et al. 2014).
Bioremediation is the utilization of the contaminants by microorganisms such as bacteria, fungi and yeast, detoxifying or converting the pollutants to harmless products (mineralization) (Das et al. 2012; Kumar et al. 2011; Sarkar et al. 2005; Surridge et al. 2009). The removal of the pollutants depends on the mechanisms of enzymatic attack and the activity of the living organisms (Kumar et al. 2011). Studies on bioremediation indicated that this technology has the potential to clean up sites contaminated with hydrocarbon fractions and COTBS in particular (Mansur et al. 2015).

Effective bioremediation can be achieved only when optimized environmental conditions are allowed for the growth and activity of soil microorganisms and when potential degraders are present. In terms of bioremediation approaches, three main techniques can be used. First, enhancing bioremediation by supplying nutrients, biostimulation (BS) (Adetutu et al. 2011; El Fantroussi & Agathos 2005). BS can improve the degradation rate of the pollutants by optimizing important and effective conditions including addition of nutrients, aeration, controlling pH and temperature (Margesin et al. 2000). BS is a process in which nutrients are added and optimized to increase the population of indigenous contaminant degraders (Sarkar et al. 2005). In hydrocarbon degradation, traditionally nutrient supplementation focuses on the addition of N and P in various nutrient sources such as urea, inorganic fertilizers, compost, sawdust, biosolids and manure (Cho & Kende 1997; Namkoong et al. 2002; Walworth & Reynolds 1995). Many researchers have conducted successful BS studies on soils contaminated with different contaminants. One such study was conducted where BS was used as a strategy to enhance the bioremediation of soil contaminated with heavy hydrocarbons. After 140 days incubation, the degradation rate was increased significantly in the BS treatment, with a TPH removal efficiency of 30.80% (Yu et al. 2011) compared to control showing 9.2%. Under aerobic conditions (i.e. in the presence of oxygen), microorganisms utilize the hydrocarbon contaminants to generate carbon dioxide, water and microbial cell mass (biomass) which could be represented by the following equation (IMO 2001):

1 kg HC* + 2.6 kg O₂ + 0.07 kg N + 0.007 kg P → 1.6 kg CO₂ + 1 kg H₂O + 1 kg biomass

* Hydrocarbon contaminants

Another bioremediation approach is to enhance the degradation capacity of the soil microbial community by introducing specific microbial strains or consortia, bioaugmentation (BA) to the contaminated sites. Assessment of the success of the approach can be determined by examining
the changes occurring in the biotic factors following the addition of suitable microorganisms. For example increases in microbial biomass, degradative enzyme activity and survival (Mrozik & Piotrowska-Seget 2010; Thompson et al. 2005). Examination of the abiotic factors may also confirm the success of the approach, through assessment of the chemical structure and physicochemical properties of the contaminated soil and the concentration of pollutants (Tyagi et al. 2011). A microcosm study on the bioremediation of soil contaminated with PAHs was conducted where a bioaugmentation strategy was used. After 28 days incubation, the BA treatment showed a 23.2% decrease in PAH concentration (Teng et al. 2010) compared to 3.5 % in control (untreated) soil. Another study on the bioremediation of soil contaminated with hydrocarbon was conducted and BA and BS were applied to biopiles. After 140 days, bioaugmentation resulted in a reduction in contaminant concentration of between 64% and 68% compared to a 0.0 % reduction with biostimulation (Liu et al. 2011).

A third approach commonly used in bioremediation is natural attenuation (NA). NA is the reduction in toxicity of contaminants occurring naturally without physio-chemical or biological processes involvement (Scow & Hicks 2005). Because NA depends only on natural degrading processes, NA often requires a longer time to bring the contaminants to a lower concentration. This approach may raise concerns and objections from local communities as there are no outward signs of bioremediation (Bento et al. 2005). However, in spite of the longer time taken for NA to remediate the contaminated environment, NA has been used routinely at remediation sites of soil contaminated with petroleum hydrocarbon. In one study NA was conducted on a soil contaminated with diesel off Long Beach, California, USA. After 12 weeks incubation, the results indicated that NA was able to reduce the concentration of diesel by 36% compared to 59 % and 68 % reduction obtained by BA and BS respectively (Bento et al. 2005).

1.9. Factors influencing petroleum hydrocarbon biodegradation
As with the other remediation techniques, bioremediation has some limitations and barriers affecting the contaminant degradation rate (Table 1.9). One key factor influencing the rate of bioremediation is the concentration of contaminants. For example, aromatic hydrocarbons, residual oils and sludges and chlorinated organic compounds are all resistant to microbial attack (Kumar et al. 2011), especially at high concentrations because of their toxicity. In addition, the presence of additional inhibitory substances (e.g. heavy metals) may further limit the activities of the hydrocarbon degraders (Adetutu et al. 2011). Secondly a lack of an appropriate enzyme may
prevent the degradation of the pollutants e.g laccase an enzyme involved in PAH degradation (Peixoto et al. 2011; Thapa et al. 2012). This is often the case for xenobiotic compounds such as chlorinated hydrocarbons. Thirdly, a lack of nutrients such as nitrogen and phosphorus may significantly reduce biodegradation by preventing the native microbial communities achieving active growth and degradation of the contaminant (Röling & Van Verseveld 2002). Soil physiochemical characteristics, such pH and temperature also influence the activity and diversity of the soil microbial community (Hamamura et al. 2006; Molina-Barahona et al. 2004; Zhang et al. 2012). Oxygen limitation and a lack of moisture are also factors which may limit the degradative activities of the hydrocarbonoclastic microorganisms (Kabelitz et al. 2009). Studies on bioremediation have reported the rapid loss of between 50-80% of the soil moisture content (Calvo et al. 2009) during treatment. In summary to achieve a successful bioremediation outcome, all the above-mentioned factors are potentially important and need to be optimized before commencing any bioremediation strategy.

Table 1.9. Factors affecting the bioremediation potential of a contaminated site.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>High concentration of hydrocarbons</td>
<td>Resist microbial attack</td>
<td>(Kumar et al. 2011)</td>
</tr>
<tr>
<td>Presence of inhibitory substances</td>
<td>Inhibit the growth and activity</td>
<td>(Adetutu et al. 2011)</td>
</tr>
<tr>
<td>such as heavy metals</td>
<td>hydrocarbon degraders</td>
<td></td>
</tr>
<tr>
<td>Lack of appropriate enzymes</td>
<td>Prevents hydrocarbon degradation</td>
<td>(Thapa et al. 2012)</td>
</tr>
<tr>
<td>lack of nutrients</td>
<td>Prevents microbial growth and degradation</td>
<td>(Röling &amp; Van Verseveld 2002)</td>
</tr>
<tr>
<td>Soil pH and temperature</td>
<td>Influence the activity and diversity of</td>
<td>(Hamamura et al. 2006)</td>
</tr>
<tr>
<td></td>
<td>microbes</td>
<td></td>
</tr>
<tr>
<td>Lack of soil moisture and oxygen</td>
<td>Limits the degradative activity of the</td>
<td>(Calvo et al. 2009)</td>
</tr>
<tr>
<td></td>
<td>microorganisms</td>
<td></td>
</tr>
</tbody>
</table>
1.10. Hydrocarbon degrading bacteria

Bioremediation of soils contaminated with petroleum hydrocarbons has been investigated since the late 1940s. In 1946, the ability of microorganisms to utilize hydrocarbon pollutants was first reviewed and around 100 species were discovered (Atlas 1981). In 1967, the first major oil spill occurred through the SS Torrey Canyon running aground off the Western coast of Cornwall, UK. After this widely reported spill, more attention from scientists was focused on the environmental problems associated with oil spills and pollution and several studies were commenced (Atlas 1981). This interest was further fuelled following the 24th March 1989 oil spill when the Exxon Valdez released 41 million litres of crude oil into Prince William Sound, Alaska, US (Bence et al. 1996). To treat the spill, indigenous hydrocarbonoclastic microorganisms were supplied from Prince William Sound and bioremediation was conducted to treat the hydrocarbon contamination (Pritchard & Costa 1991). Although a very small percentage (less than 1%) of soil bacteria have been identified (Guo et al. 1997), recently more than 500 different strains and species have been listed as hydrocarbon degrading agents representing almost 200 bacterial, cyanobacterial, fungal and algal genera (Yakimov et al. 2007) (Table 1.10).

Table 1.10. Selected microorganisms used in bioremediation of soil contaminated with different hydrocarbons (Mrozik & Piotrowska-Seget 2010).

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Contaminants degraded</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Comamonas testosterone</em> BR60</td>
<td>Crude oil, PAHs</td>
<td>(Gentry et al. 2001)</td>
</tr>
<tr>
<td><em>Arthrobacter chlorophenolicus</em> A6L</td>
<td>4-Chlorophenol</td>
<td>(Jernberg &amp; Jansson 2002)</td>
</tr>
<tr>
<td><em>Absidia cylindrospora</em></td>
<td>Fluorene</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas sp.</em> ST41</td>
<td>Marine gas oil</td>
<td>(Garon et al. 2004)</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> WatG</td>
<td>Diesel oil</td>
<td>(Stallwood et al. 2005)</td>
</tr>
<tr>
<td><em>Sphingobium chlorophenolicum</em> ATCC 39723</td>
<td>Pentachlorophenol</td>
<td>(Ueno et al. 2006)</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em> ZWL73</td>
<td>4-Chloronitrobenzene</td>
<td>(Dams et al. 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Niu et al. 2009)</td>
</tr>
</tbody>
</table>
Bioremediation of Libyan Soil Contaminated With Crude Oil Tank Bottom Sludge

Abdulatif Mansur

<table>
<thead>
<tr>
<th>Consortia</th>
<th>PAHs (fluorine, phenanthrene, pyrene)</th>
<th>(Yu, S et al. 2005)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhodococcus sp., Acinetobacter sp., Pseudomonas sp.</td>
<td>Crude petroleum-oil hydrocarbons</td>
<td>(Das, K &amp; Mukherjee 2007)</td>
</tr>
<tr>
<td>Bacillus subtilis DM-04, Pseudomonas aeruginosa M and NM</td>
<td>Mixture of PAHs</td>
<td>(Jacques et al. 2008)</td>
</tr>
<tr>
<td>Mycobacterium fortuitum, Bacillus cereus, Microbacterium sp., Gordonia polyisoprenivorans, Microbacteriaceae bacterium, Fusarium oxysporum Bacillus strains B1F, B5A and B3G, Chromobacterium sp. 4015, Enterobacter agglomerans sp.</td>
<td>(naphthalene, phenanthrene, anthracene, pyrene, dibenzo[a]anthracene, benzo[a]pyrene)</td>
<td>(Silva et al. 2009)</td>
</tr>
</tbody>
</table>

Numerous studies investigating the potential of microorganisms for hydrocarbon utilization have been reported and not surprisingly have reported significant potential for the use of microorganisms for the commercial treatment and removal of hydrocarbons from contaminated environment sites (Cerniglia 1993; Huesemann & Moore 1993; Liebeg & Cutright 1999; Sarkar et al. 2005; Zhou & Crawford 1995). However, relatively few of these studies have focused on the bioremediation of soils contaminated with COTBS in natural environments. Of the reports on the use of bioremediation for the remediation of soil contaminated with COTBS, the potential of both single isolates and microbial consortia have been investigated (Bouchez, T et al. 2000; Gallego et al. 2007). The successful bioremediation of soil contaminated with COTBS has many benefits including; reduction of hydrocarbons resulting in lower toxicity, improving the soil properties and increasing the microbial population that can be used as a hydrocarbonoclastic stock to treat different contaminated sites. One in situ bioremediation study of oily sludge contaminated soil at the Shengli oilfield in northern China reported the use of an indigenous bacterial consortium of hydrocarbon degraders inoculated into the soil. After 360 days, the TPH concentration was reduced from 251 g kg\(^{-1}\) to 101 g kg\(^{-1}\) with a reduction rate of 58.2 \% compared to just 15.6\% degradation in the control (uninoculated) soil. In this study the number of TPH degraders increased during the bioremediation from 54.9 x10\(^4\) to 71.3 x10\(^4\) g\(^{-1}\). Interestingly, the water holding capacity (WHC) of the soil was also increased during the
remediation from 16.2 % to 86.6 % after 365 days (Liu et al. 2010). In the area of *ex situ* bioremediation of soil contaminated with oily hydrocarbons, a number of studies have been successfully conducted. In a trail of field bioremediation of soil (10 m$^3$) contaminated with oily hydrocarbons, a consortium of four indigenous bacterial hydrocarbon degraders were inoculated into the contaminated soil. After 100 days, the TPH was reduced from 25,000 g kg$^{-1}$ of dry soil to 2500 g kg$^{-1}$ of dry soil with 15 % degradation during the first 15 days. In this study the population of hydrocarbon degraders increased from $1.0 \times 10^8$ CFU to $3.16 \times 10^8$ CFU with an increase in respiration from 30 to 47 mg kg$^{-1}$ (Lin et al. 2011). In addition to using indigenous microorganisms, hydrocarbon utilizing microorganisms can be effectively isolates from hydrocarbon contaminated sites. Some studies have indicated the potential of oily sludge contaminated sites, including COTBS (Jasmine & Mukherji 2014a) as a good sources of hydrocarbonoclastic bacteria. In this instance the degraders are isolated and grown in nutrient rich environments then re-inoculated to treat the contaminated sites (Aislabie et al. 2006). However, due to the lack of nutrients, such as nitrogen and phosphorus and the potential depletion of moisture and air within the contaminated soils, these hydrocarbon degraders maybe far less effective *in situ* than in laboratory studies. In this case the addition of additional nutrients, water and air enhances the ability of the hydrocarbon degraders to utilize the contaminants and increases the biodegradation rate (Nanekar et al. 2015). In addition to using hydrocarbonoclastic bacteria in a consortium, some studies have reported the benefits of adding individual bacterial isolates to utilize the hydrocarbons within different contaminated environments. A recent study assessed the potential of individual bacterial isolates to degrade a complex hydrocarbon mixture of crude oil using bacterial isolates from petroleum crude oil contaminated environments, including *Alcanivorax borkumensis*, *Rhodococcus erythropolis* and *Pseudomonas stutzeri*. The initial concentration of TPH was 1000 ppm. After 15 day of biodegradation, the reduction in TPH ranged from 48 to 100 % while the population of the bacterial isolates increased from $1 \times 10^3$ to $1 \times 10^7$ cell ml$^{-1}$ (Santisi et al. 2015).

### 1.11. Techniques to study bioremediation

During the bioremediation process it is crucial to monitor the levels of the contaminant together with any intermediates. An array of different analytical techniques has been employed (Table 1.11) including gas chromatography (GC), gas chromatography mass spectrometry (GCMS), Fourier transform infrared spectroscopy (FTIR) and thermogravimetric infrared spectrometer.
(TGIR) or a combined TG-FTIR (Mittleman 1990; Poster et al. 2006; Yew et al. 2008; Zhu et al. 2008). These techniques can assess the concentration of total petroleum hydrocarbons (TPH) and polycyclic aromatic hydrocarbons (PAHs) (Sheppard et al. 2011). However, although critical measurements these techniques do not provide any information regarding the biological components of the bioremediation. Measuring and identifying the size, activity and diversity of the microbial community in the contaminated soil is essential for developing effective and robust bioremediation treatments. There are many methods used for assessing microbial activities in environments contaminated with hydrocarbons; both culture dependent and culture independent techniques have been used (Zhang et al. 2012).
Table 1.11. Analytical techniques used in bioremediation. Adapted from (Korda et al. 1997).

<table>
<thead>
<tr>
<th>Technique</th>
<th>Description</th>
<th>Parameter measured</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil respirometry</td>
<td>Hydrocarbon degradation is assessed by measuring the rate of CO₂ production and O₂ consumption</td>
<td>Microbial activity</td>
<td>(Montagnolli et al. 2015)</td>
</tr>
<tr>
<td>Gas chromatograph (GC)</td>
<td>Separating the complex mixture to components</td>
<td>Contaminant determination and identification</td>
<td>(O'Reilly et al. 2015)</td>
</tr>
<tr>
<td>Gas chromatograph mass spectroscopy (GCMS)</td>
<td>Separation and identification of compounds</td>
<td>Contaminant determination and identification</td>
<td>(Moore et al. 2014)</td>
</tr>
<tr>
<td>Gas chromatography – flame ionization detector (GC-FID)</td>
<td>Separation and identification of compounds</td>
<td>Contaminant determination and identification</td>
<td>(Weng et al. 2015)</td>
</tr>
<tr>
<td>Fluorescence analysis</td>
<td>During fluorescence analysis, excitation and emission spectra are used to identify compounds</td>
<td>Contaminant determination and identification</td>
<td>(Pena et al. 2015)</td>
</tr>
<tr>
<td>Internal petroleum biomarkers</td>
<td>Quantifying the extent of hydrocarbon on an oil weight basis. This method relies on GC/FID and GC/MS</td>
<td>Contaminant determination and identification</td>
<td>(Lin et al. 2014)</td>
</tr>
<tr>
<td>Fourier transform infrared spectroscopy (FTIR) and Thermogravimetric Infrared spectrometer (TGIR)</td>
<td>Identification of hydrocarbon compounds in oil samples depending on IR transmission and absorption by the sample resulting a spectrum representing a single molecule</td>
<td>Identify unknown materials, determine the quality or consistency of a sample and determine the amount of components in a mixture</td>
<td>(Djinović et al. 2015; Seitz et al. 2014)</td>
</tr>
</tbody>
</table>
1.11.1. Culture dependent techniques
To understand and improve the bioremediation process of hydrocarbons contaminants, it is essential to investigate the microbial communities involved in the biodegradation (Kadali 2012). To detect the presence of hydrocarbonoclastic microorganisms and investigate their ability to grow and utilize hydrocarbons as the sole source of carbon and energy as well as to assess the potential changes in the bacterial community composition, culture dependent methodologies have been widely used (Connon et al. 2005; Steven et al. 2007). Table 1.12 highlights some of the key culture dependent techniques used.

**Table 1.12. Culture dependent techniques.**

<table>
<thead>
<tr>
<th>Technique</th>
<th>Description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation</td>
<td>To identify and evaluate the capability of bacterial strains to degrade hydrocarbon contaminants.</td>
<td>(Adriaenssens et al. 2014; Mittal &amp; Singh 2009)</td>
</tr>
<tr>
<td>Analytical profile index</td>
<td>For classification and identification of microorganisms including bacteria.</td>
<td>(Sauer &amp; Kliem 2010; Venter et al. 1989)</td>
</tr>
<tr>
<td>Fatty acid profile</td>
<td>For evaluation, identification and identification of bacterial isolates</td>
<td>(Kalliomäki et al. 2001; Stead et al. 1992)</td>
</tr>
<tr>
<td>Respirometry measurements</td>
<td>Carbon dioxide production or oxygen consumption represents the metabolic potential of hydrocarbonoclastic bacteria and the mineralization of the contaminants.</td>
<td>(Arulazhagan et al. 2010; Mancera-López et al. 2008)</td>
</tr>
<tr>
<td>Biolog plates</td>
<td>To understand the functional diversity and metabolic potential of the individual isolates.</td>
<td>(Kadali et al. 2012; Manage et al. 2009)</td>
</tr>
</tbody>
</table>
1.11.1.1. **Culture isolation (plating isolation)**

Bacterial isolation is a technique used to culture, detect and isolate microorganisms from a polluted environment. Bacteria are cultured on a liquid or solid medium designed for their growth. There are many types of growth media used for bacterial growth (Table 1.13), but the most commonly used are nutrient broth (liquid medium) and nutrient agar (solid medium). This media contains a rich growth media designed to isolate a wide array of heterotrophic microorganisms, enabling an estimate of the culturable heterotrophic bacteria present in a sample (Schlegel & Zaborosch 1993).

**Table 1.13.** Different media used in growth, isolation and purification of environmental bacterial isolates.

<table>
<thead>
<tr>
<th>Media</th>
<th>Use</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bushnell Hass Mineral Salt</td>
<td>Used for studying microbial utilization of hydrocarbons</td>
<td>(Singh <em>et al.</em> 2015)</td>
</tr>
<tr>
<td>Medium (BHMSM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nutrient agar (NA)</td>
<td>Cultivation of less fastidious microorganisms (isolation and purification)</td>
<td>(Sreenivasulu <em>et al.</em> 2014)</td>
</tr>
<tr>
<td>Nutrient broth (NB)</td>
<td>Cultivation of a wide variety of microorganisms</td>
<td>(Vahabi <em>et al.</em> 2015)</td>
</tr>
<tr>
<td>Dextrose media</td>
<td>Growth media</td>
<td>(Gil <em>et al.</em> 2009)</td>
</tr>
<tr>
<td>Luria Bertani medium (LB)</td>
<td>Growth media</td>
<td>(Escobar-Niño <em>et al.</em> 2014)</td>
</tr>
<tr>
<td>Tryptan soya broth (TSB)</td>
<td>Culturing media</td>
<td>(Majtan <em>et al.</em> 2014)</td>
</tr>
<tr>
<td>Reasoner’s 2A agar (R2A)</td>
<td>Culturing media</td>
<td>(Hemala <em>et al.</em> 2014)</td>
</tr>
<tr>
<td>Marine agar</td>
<td>Isolation and enumeration of heterotrophic marine bacteria</td>
<td>(Lo <em>et al.</em> 2014)</td>
</tr>
<tr>
<td>Marine broth</td>
<td>Cultivation of marine heterotrophic bacteria</td>
<td>(Gutierrez <em>et al.</em> 2014)</td>
</tr>
<tr>
<td>Dextrose nitrate agar (DNA)</td>
<td>Growth and isolation of different microorganisms such as bacteria and fungi from different soil, water and air environments.</td>
<td>(Gil <em>et al.</em> 2009)</td>
</tr>
<tr>
<td>Glucose asparagine agar (GAA)</td>
<td></td>
<td>(Kokare <em>et al.</em> 2004)</td>
</tr>
<tr>
<td>Glycerol arginine agar (GliAA)</td>
<td></td>
<td>(Sipkema <em>et al.</em> 2011)</td>
</tr>
</tbody>
</table>
For the isolation and enumeration of culturable microorganisms capable of degrading the contaminant of interest such as hydrocarbon contaminants, selective enrichment using Bushnell Hass Mineral Salt Medium (BHMSM), enriched with either a single hydrocarbon fraction or a mixture of hydrocarbons such as petroleum crude oil, heavy oils or diesel as the only source of carbon and energy represents a successful isolation method (Bushnell & Haas 1941). Many studies report the use of BHMSM to isolate hydrocarbonoclastic bacterial isolates. For example, (Latha & Kalaivani 2012) reported the isolation of bacteria from a crude oil contaminated site using BHMSM. After culturing, a total viable count of between $248 \times 10^3$ CFU and $257 \times 10^6$ CFU were obtained. Another study used BHMSM amended with crude oil as a source of carbon to isolate hydrocarbonoclastic bacterial isolates from soil contaminated with hydrocarbons. After 7 days incubation of soil suspension at 27 ºC, $5 \times 10^2$ CFU g soil$^{-1}$ were enumerated (Kadali et al. 2012). In addition to the isolation potential of hydrocarbon degrading microorganisms, BHMSM has been used to increase the biodegradation rate of hydrocarbons. This study used autoclaved modified BHMSM to study the effect of nutrients on kerosene biodegradation. After 6 weeks, the amended experiments with BHMSM resulted in a 65 ± 7% reduction in hydrocarbon concentration compared with only a 27 ± 3% reduction in experiments without BHMSM (Shabir et al. 2008).

### 1.11.1.2. Biolog plates

Following the isolation and identification of the bacteria of interest, it is essential to understand the metabolic potential of the individual isolates in regard to environmental contaminants. In the late 1980s, the Biolog Corporation developed microplates enabling the metabolic fingerprinting of bacterial isolates (Garland & Heckbert 1997). The mechanism of the Biolog system depends on the bacterial utilization of different carbon sources where the biodegradation of the carbon source is confirmed by the generation of a violet colour due to the reduction of tetrazolium violet, quantified using a spectrophotometer (Hill et al. 2000). Currently there are a range of Biolog plates. For example Biolog MT2 consists of ninety six well microplates containing tetrazolium redox dye and a buffered nutrient medium without a carbon source. Simply by adding a carbon source this system allows for the rapid identification of bacterial isolates with a capability to degrade selected carbon sources (Bochner 1989). Recently, Biolog MT2 plates have been used successfully to identify and assist the hydrocarbon degrading potential of indigenous bacterial isolates (Kadali et al. 2012).
1.1.1.3. Soil respiration and respirometry measurements
Different studies report the use of a range of techniques to assess the biodegradation rate and the potential of the organisms to utilize the contaminants. During aerobic biodegradation of hydrocarbons, complete mineralization of the contaminants leads to the formation of H₂O, and CO₂ as end products (Fingerman 2005). Therefore, measuring the oxygen consumption or CO₂ generation in the gas phase during the bioremediation represent effective and reliable tools which provide information about the mineralization rate of the contaminants (Schoefs et al. 2004). Respiratory measurements during bioremediation directly represents bacterial and metabolic activity (Aspray et al. 2008). For example, a recent study was conducted to measure the potential of bacterial isolates to degrade polycyclic aromatic hydrocarbons (PAHs). CO₂ generation was used as an indication of the mineralization of PAHs. After four days of incubation, the results showed that the bacterial consortium with PAHs as sole carbon source produced higher level of CO₂ (1338 ppm) compared to the control (181 ppm), and was accompanied by high degradation (97-98 %) of PAHs (Arulazhagan et al. 2010).

1.1.2. Culture independent techniques
In addition to the culture dependent techniques there are a number of culture independent techniques used to assess the impact of hydrocarbon contamination on the microbial communities, such as molecular biological techniques and molecular genetic fingerprint techniques. Like culture dependent techniques, culture independent techniques have advantages and disadvantages (Table. 1.14) (Sheppard et al. 2011). Data obtained from culture independent methods helps to assess the diversity of the bacterial community together with assessment of the metabolic potential to degrade specific contaminants in addition to monitoring any changes in the bacterial community during the bioremediation process (Zhang et al. 2010). In addition, culture independent techniques can be used to compare the efficacy of different bioremediation strategies such as bioaugmentation, biostimulation and natural attenuation and to compare the changes on the microbial diversity (Vinas et al. 2005). Moreover, The ability through these techniques to examine and identify genes responsible for degrading specific contaminants has been of particular importance to the field of bioremediation (Matsuki et al. 2004).
Table 1.14. Advantages and disadvantages of culture-independent PCR-based microbial community fingerprinting methods. Obtained from (Garbeva et al. 2004) and adapted from (Hirsch et al. 2010).

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dependence on efficient cell lysis only and not on the physiological status of cells</td>
<td>Incomplete lysis of some species, notably Gram-positive spore-formers</td>
<td>(Garbeva et al. 2004; Temmerman et al. 2004)</td>
</tr>
<tr>
<td>Direct picture of the diversity of dominant microbial types, including the unculturables.</td>
<td>Possible biases in DNA extraction and PCR amplification, inhibition by soil compounds.</td>
<td></td>
</tr>
<tr>
<td>Direct assessment of shifts in microbial community structure.</td>
<td>Possible presence of one particular sequence or band in different organisms.</td>
<td></td>
</tr>
<tr>
<td>Ease in handling, simultaneous analysis of high sample numbers and Reproducible results.</td>
<td>Heterogeneous bands that may originate from one bacterial strain due to heterogeneity in the rDNA genes.</td>
<td></td>
</tr>
<tr>
<td>Generation of sequences resulting in identification and specific probes to track the specific organism in the ecosystem.</td>
<td>Phylogenetic information only is usually obtained and the link to functional information is difficult.</td>
<td></td>
</tr>
<tr>
<td>PCR exploits the semi conservative replication of DNA to enable exponential amplification of the target sequence and can provide &gt; $10^9$ copies after 30 cycles of DNA synthesis.</td>
<td>Does not provide information on abundance.</td>
<td>(Hirsch et al. 2010)</td>
</tr>
<tr>
<td>Ribosomal RNA (rRNA) is more stable and could survive for months even in dead cells and indicates the dominant active population.</td>
<td>mRNA is usually very short lived and indicates which genes are active at the time of extraction.</td>
<td></td>
</tr>
<tr>
<td>Provide identification of many soil bacteria, archaea and fungi.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precise information on which functional genes are active can be obtained from messenger RNA (mRNA).</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA can be extracted from nonviable microorganisms after many years of storage (up to 140 years).</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Molecular biological techniques have been used to detect and identify microorganisms through the application of some specific molecular markers including 16S rRNA or its encoding genes,
with results obtained following cloning and analysis. These results can indicate the presence of members of the microbial community but does not provide any data about the activity and microbial dynamics of the community (Muyzer & Smalla 1998). In contrast molecular genetic fingerprint techniques can provide information about the diversity of the microbial communities. These techniques begin with the extraction of DNA and RNA followed by the amplification of genes encoding 16S rRNA and finally, analysis of the PCR products by sequencing (Muyzer 1999).

A number of fingerprinting techniques have been developed and applied to investigations of microbial community changes in situations such as bioremediation. Among these techniques are Automated Ribosomal Intergenic Spacer Analysis (ARISA) (Fisher & Triplett 1999), Terminal Restriction Fragment Length Polymorphism (TRFLP) (Osborn et al. 2000), Denaturant Gradient Gel Electrophoresis (DGGE) (Muyzer et al. 1993) and temperature gradient gel electrophoresis (TGGE) (Fingerman 2005; Muyzer & Smalla 1998).

ARISA is a method used to analyse microbial communities that provides estimates of microbial richness at species level resolution (Kovacs et al. 2010). By using ARISA, DNA fragment found between the 16S and 23S genes in bacterial genomes are amplified and its natural variability in length is used for separation by capillary electrophoresis to indicate diversity. In a comparative study, ARISA was found to have performed better than other genetic fingerprinting techniques such as terminal restriction fragment length polymorphism (T-RFLP) (Danovaro et al. 2006). However, ARISA show some limitations in accurately describing the microbial diversity in samples. One study revealed that in spite of increasing species diversity in a microbial community, this technique tended to underestimate species richness (Kovacs et al. 2010). In addition, only limited fragment lengths could be detected by ARISA (between 200 and 1,150 bp) restricting the observation of different phylotypes within a sample. It can be concluded that ARISA might not be the most appropriate technique for comparing between samples with high taxon richness (Gobet et al. 2014; Koopman et al. 2010).

TRFLP is another genetic fingerprint technique for characterising microbial diversity and comparing bacterial community structure in environmental samples within a population of
amplified PCR products (Dickie & FitzJohn 2007; Garbeva et al. 2004). In TRFLP, fluorescently labelled primers combined with restriction digests are used to visualize sequence variation in either single or mixed species DNA samples. Dependent on the PCR amplified 16S rRNA genes, TRFLP is used to assess the structure, diversity and dynamics of microbial community (Liesack & Dunfield 2004). The obtained data represent the sizes of the fragments of PCR amplicons that contain the labelled primer (the terminal fragment lengths), observed as (electropherogram) peaks. Differences in the presence and location of cutting sites results in different species having terminal fragments of different lengths (Dickie & FitzJohn 2007). Although TRFLP offers some important advantages, it remains expensive and reliant on having specific primers for specific microbial groups of interest. TRFLP also has a number of important limitations in terms of peak profile, where only the terminal fragments are being read and any two distinct sequences sharing a terminal restriction site will result in one single peak (Röling & Van Verseveld 2002).

Single Strand Conformation Polymorphism (SSCP) also became an important and frequently used fingerprint tool in microbial ecology, providing information on the diversity and dynamics of microorganisms in an environmental sample. The strength and advantage of this fingerprinting technique is that large numbers of samples can be analysed and compared, making SSCP an ideal tool for ecological studies. Microorganisms can be characterised by the cloning and sequencing of differentiating bands or by probing (Smalla, Oros-Sichler et al. 2007). The choice to select SSCP is often be influenced by the expertise and equipment available in the laboratory. SSCP is based on the direct analysis of the 16S or 18S rRNA gene pool, after PCR amplification and separation in non-denaturing conditions. It provides a rapid fingerprint of a complex microbial community including dead and alive microbial cells (Callon, Delbès et al. 2006). SSCP separates PCR amplicons having the same length but different nucleotide sequences on the basis of the conformation of single-stranded DNA (Schwieger and Tebbe 1998).

DGGE and TGGE are two of the most commonly used techniques in the study of the microbial community associated with the bioremediation of hydrocarbon contaminated environments. DGGE and TGGE are forms of electrophoresis which use either a chemical (DGGE) or a temperature (TGGE) gradient to denature the polymerase chain reaction (PCR)-amplified fragments of nucleic acid as it passes through an acrylamide gel. DGGE and TGGE can be applied to both DNA and RNA nucleic acids (Muyzer & Smalla 1998) because they can separate
PCR amplified DNA fragments (200-700 bp) of the same length with different sequences (Muyzer et al. 1993). In DGGE, the separation of bands occurs in chemical denaturants and based on the differential denaturation (melting behaviour) profile of DNA fragments (Ercolini 2004), while TGGE uses a temperature gradient to denature the sample as it moves across the acrylamide gel. Both approaches (DGGE and TGGE) give similar results but not necessarily identical (Wartell et al. 1998).

DGGE is a powerful tool which overcomes this limitation and evaluates the diversity of the microbial community by separating amplified DNA fragments having the same length but with different sequences in differential denaturing characteristics (Muyzer 1999; Muyzer et al. 1993). Genetic fingerprinting techniques consist of (i), extracting the nucleic acids (DNA and RNA) from the environmental sample (ii), amplifying the genes encoding the 16S rRNA and (iii), analysing the PCR products by a genetic fingerprinting techniques (DGGE) (Muyzer 1999) (Muyzer 1999; Nicolaisen & Ramsing 2002). The separation process in DGGE is based on the decrease of electrophoretic mobility of partially melted double stranded molecules of DNA in polyacrylamide gel containing a linear gradient of DNA denaturants (Muyzer 1999). Briefly, during the DGGE process, as the amplified PCR products migrate through the polyacrylamide gel, they will pass through an increasingly higher concentration of chemical denaturant. When reaching the threshold denaturant concentration, the double stranded PCR product will start to denature and the migration begins to slow down dramatically (Muyzer 1999). A pattern of single bands will be obtained when different sequences of different bacterial isolates denature at different denaturant concentrations. Each separated band represents a different bacteria present in the community. The melting pattern depends on the product, its sequence of guanine (G) and cytosine (C) in addition to the nucleotide sequence. The two DNA strands are prevented from completely dissociating into single strands due to the GC rich sequence acts as a high melting domain (Sheffield et al. 1989).

In general, using universal 16S rRNA gene primers allow researchers to examine all the microbial communities involved in the remediation process (Borneman et al. 1996). In addition, specific primers can be used to detect genes responsible for degrading specific contaminants. Also, bands of specific genes can be excised from the gel and sequenced, providing important phylogenetic information (Kadali et al. 2012). Many studies reported the use of specific primers
to detect genes able to utilize hydrocarbon contaminants in different environments (Gargouri et al. 2014; Mao et al. 2012; Vinas et al. 2005). By using specific primers (23CAT-F, 23CAT-R, DEG-F, DEG-R and QUANT-F), the researchers were able to detect eight different bacterial genes capable of degrading the contaminants (Mesarch et al. 2000). DGGE has been found to be rapid profiling procedure allowing the analysis of multiple samples (Osborn et al. 2000), fast and comparatively cheap, providing precise results. However, as advanced molecular method s’ prices (such as Shotgun and MySeq) sequencing have currently dropped dramatically, it will be more ecologically advantageous to monitor the complete picture of microbial communities and their functional genes.

1.12. Aims
There are two main aims of this project:

The first aim was to recover and evaluate oil from waste oily crude oil tank bottom sludge and to compare it with parent oil (Hamada crude oil) in order to assess the commercial potential of recycling the oil.

The second aim was to develop a bioremediation technology as a safe, environmental friendly, cost effective and sustainable treatment approach to remediating Libyan soil contaminated with crude oil tank bottom sludge as a replacement of the currently used physical and chemical methods that are hazardous, costly and non-environmental friendly.

Completion of these aims will provide knowledge enabling greater understanding of the process of bioremediation as a tool for the removal of hydrocarbon contaminants from Libyan soils and to investigate the microbial community involved in the bioremediation treatment. The results obtained from this study will greatly assist the development of potential bioremediation technologies in the south Mediterranean countries including Libya.

The specific objectives of this study were:

1. Investigation of the availability of oil within the crude oil tank bottom sludge.
2. Qualitative assessment of the recovered oil in comparison with the parent oil.
3. Isolation of hydrocarbonoclastic bacterial isolates from three sources; crude oil tank bottom sludge, crude oil tank bottom sludge-contaminated soil and treated crude oil tank bottom sludge contaminated soil.

4. Screening of the hydrocarbonoclastic bacteria on five different aliphatic and aromatic hydrocarbon fractions in addition to Hamada petroleum crude oil.

5. Assessment of the hydrocarbon degrading ability of selected indigenous bacterial isolates in terms of hydrocarbon biodegradation in soil contaminated with crude oil tank bottom sludge through the application of replicated slurry phase microcosm based respiration bioreactors. Development of larger scale bioremediation protocols using low water content slurry phase bioreactors.

6. Examination of the changes in the soil microbial community through the bioremediation process by analysis of the 16S rRNA amplicons using DGGE from the mesocosms.

This thesis is structured into 7 main chapters. Chapter 1 provides a general literature review of the subject area together with the aims and objectives of the research. Chapter 2 provides details about the general materials and methods used throughout the research. Detailed methodology for specific chapters are presented in the relevant result chapter. Chapter 3 investigates the recovery of oil from the crude oil tank bottom sludge and assessment of the quality of the recovered oil. The recovered oil was compared to the parent oil. Chapter 4 describes the isolation of hydrocarbonoclastic bacteria from crude oil tank bottom sludge, crude oil tank bottom sludge contaminated soil and treated crude oil tank bottom sludge contaminated soil followed by screening of the isolates to identify the most promising hydrocarbon degraders. Chapter 5 examines the ability of the selected hydrocarbon degrading agents to degrade different hydrocarbon fractions within crude oil tank bottom sludge-contaminated soil in laboratory scale microcosm slurry phase bioreactors under controlled conditions. Chapter 6 assesses the degradative potential of the selected hydrocarbon degrading bacterial isolates from chapter 5 in larger scale mesocosms based on continuous aeration where the respiration of the microorganisms was measured and the changes in bacterial community examined using DGGE. Chapter 7 presents a general discussion of the research and a final conclusion. The result chapters (3-5) are presented as journal articles reproduced in their publication format. Chapter 6 has been submitted to an international peer reviewed journal and is reproduced in the submitted format.
Chapter 2

2 Materials and Methods
2.1. Chemicals and media
All the used chemical materials and media were obtained from Sigma-Aldrich (USA), Merck (Australia), Spectrum quality standards (USA), Difco laboratories (France) and Aax Finechem (Australia). Biolog (MT2) and Eco Plates were obtained from Biolog Inc. http://www.biolog.com/index.php (USA).

2.2. Sample collection
Crude oil tank bottom sludge (2 kg), soil contaminated with crude oil tank bottom sludge (5 kg) were sampled from collection lagoons together with Libyan Hamada petroleum crude oil (2 L) from Azzawiya oil refinery- Libya and stored in sealed plastic containers at room temperature in the Quarantine Facility at RMIT University.

![Figure 2.1](image.png)

**Figure 2.1.** Crude oil tank bottom sludge (A) and soil contaminated with crude oil tank bottom sludge (B).

2.3. Standards

2.3.1. PIONA standard
(Paraffines, Iso-paraffines, Olefines, Naphthalene and Aromatic) was obtained from Spectrum Quality Standards, Ltd. Sugarland, TX, USA.

2.4 Characterisation of COTBS
COTBS was characterised in terms of moisture content, solids content, organic matter content, concentration of volatile and non-volatile hydrocarbons and % recovered oil as described below.
2.4.1. Water content (%)
Following ASTM-D95, to triplicate samples of COTBS (25 g) dichloromethane (DCM) (79 ml solvent) were added (1:3 soil: solvent ratio). The oil, solvent and water were then distilled at 100 °C in a distillation apparatus and the condensate (water and solvent) continuously separated in a trap and transferred to a graduated cylinder and separated on the basis of density.

2.4.2. Volatile hydrocarbons (%)
Triplicate samples of COTBS (8 g) were placed in ceramic crucibles and heated to 105°C in a ventilated incubator for 24 h. The lost mass was attributed to light volatile hydrocarbons and moisture content. The light volatile hydrocarbons were calculated using the following equation:

\[
\text{Light hydrocarbons} = \frac{\text{reduced mass (g)}}{\text{mass of tested samples (g)}} \times 100\% - \text{water content } \%
\]

2.4.3. Solid content (%)
Triplicate samples of COTBS (8 g) were placed in ceramic crucibles and heated to 105°C in a ventilated incubator for 24 h then heated to 550°C for 30 min. The remaining samples were re-weighed. The solid (sediment and ash) content of the COTBS was calculated using the following equation:

\[
\text{Solid content} = \frac{\text{reduced mass (g)}}{\text{mass of tested sample (g)}} \times 100 \%
\]

2.4.4. Organic matter content (%)
The organic matter concentration was measured by loss on ignition of dry solid material in the muffle furnace (550°C for 30 min). The mass that was lost by the sample was attributed to organic material.

2.4.5. Non-volatile hydrocarbon content (NVH) (%)
The non-volatile hydrocarbon content was calculated according to the following equation:

\[
\text{NVH} = 100\%-\text{VH+SC+WC}
\]

Where: NVH is non-volatile hydrocarbons (wt %)

VH is volatile hydrocarbons (wt %)

SC is solid content (wt %)

WC is water content (wt %)
2.4.6. Oil recovery (%)
The oil was recovered from COTBS by solvent extraction using dichloromethane (DCM). Equal amounts of sludge and solvent (1:1 sludge: DCM ratio) were placed in Teflon coated centrifuge tubes and agitated (130 rpm min\(^{-1}\)) at room temperature for 30 min followed by centrifugation at 5000 rev min\(^{-1}\) for 5 min. The oil then was collected in separated glass bottles.

![Extracted oil from COTBS.](image)

2.4.7. Solvent recovery
The solvent/oil were separated using a rotary evaporator with a Buchi 461 water bath (Buchi RE 111 Rotavapot, Buchi, Switzerland) at 40 °C. The DCM was separated by evaporation, condensed and then collected prior to the collection of the remaining oil which was then measured.

2.5. Characterisation of oil

2.5.1. Quantitative and qualitative assessment of the hydrocarbon fraction present in the oil
A combined paraffins, isoparaffins, aromatic, naphthalenes and olefins (PIANO) PIANO-5-Piano (DHA) standard combined set (2 ml ampule) (Spectrum Quality Standards, Ltd. Sugarland, TX, USA) was used to analyse the hydrocarbon fractions within the recovered oil using the method described in ASTM-D513 using gas chromatograph mass spectrometer (GC-MS) equipped with autosampler (Aglient 6890 GC and Leco Pegasus III TOF-MS). Samples were injected and separated on a capillary column Agilent DB-5MS (60 m by 0.25 mm with 0.25 μm film thickness). The injection temperature and volume was 225°C and 0.2 μl respectively. Helium (1.8 ml min\(^{-1}\)) was used as a carrier gas at a constant flow rate. The concentration of each hydrocarbon fraction was analyzed and the total peak area of each fraction was compared to the peak area of each fraction in the PIANO standard curve.
2.5.2. Density (ρ)
Recovered oil (10 ml) was measured and weighed (Mettler AE 260, Mettler-Toledo, Switzerland). The density of the oil was derived from the following equation:

\[ \rho = \text{mass} / \text{volume} \]

2.5.3. Specific gravity (SG)
The specific gravity of the collected oil was calculated mathematically according to the specific gravity of water using the following equation:

\[ \text{SG} = (\rho \text{ sample}) / (\rho \text{ H}_2\text{O}) \]

2.5.4. API gravity
API gravity of the recovered oil was calculated using the following equation:

\[ \text{API gravity} = (141.5/\text{Specific Gravity}) - 131.5 \]

2.5.5. Viscosity (υ)
Kinematic viscosity of the oil was measured according to ASTM-D445. A Cannon-Fenske (Fisher Scientific, Pittsburgh, PA) glass capillary kinematic viscometer was used. Kinematic viscosity is determined by measuring the time (t) for a known volume of liquid flowing under gravity to pass through a calibrated glass capillary viscometer tube. The manufacturer of the Cannon-Fenske type viscometer tubes supplied calibration constants (c) at a range of temperature 40°F and 100°F. The kinematic viscosity was calculated using the following:

\[ \nu = c \cdot t \]

2.5.6. Ash content (%)
Triplicate samples (5 g) were heated overnight at 105°C and then transferred to a muffle furnace held at 550°C for 30 min to burn the organic matter. Ash content was calculated from the ratio of pre- and post-ignition sample mass.

2.5.7. Salt content
Salt content was measured according to ASTM-D3230 using a Pro 2030 multimeter (YSI Incorporation, Yellow Springs. OH 45387. USA. A known amount of oil (1 ml) was dissolved in hexane in a beaker. The test cell (probe) of two parallel stainless steel plates at constant alternative voltage was immersed in the mixture. The alternating voltage was passed through the
plates, and the salt content was obtained by reference to a calibration curve of the relationship of salt content of known mixtures to the current.

2.5.8. Thermogravimetric analysis
To measure the mass loss and to determine the functional group of the extracted oil at a given time and temperature, thermogravimetry-fourier transform infrared spectrometer (TGA-FTIR) was used. Thermogravimetric analysis was performed on a STA6000 thermal analyser operating under nitrogen through a furnace as following: 40 mg of oil sample was heated in a crucible with a heating rate of 20°C min\(^{-1}\) from 50 to 950°C in an inert atmosphere (nitrogen). The gas produced was transferred via a gas transfer line. The transfer line allows the transfer of the pyrolysis products from the thermal analyser to an FTIR 100 through a gas flow cell (20 ml min\(^{-1}\)). Spectrum time base was used to analyse the sp files collected during the testing to analyse the spectrum (Wilkie 1999).

2.5.9. Shear rheology
A Hybrid Discovery (HR3) rheometer was used to measure the rheological properties (viscosity) of oil samples. The HR3 rheometer was calibrated with viscosity standards including Polydimethyl Siloxane (PDMS). The calibration results of crossover frequency of G’ and G” were matched with the value recommended by instrument suppliers (TA Instruments). The shear rheology was conducted on the oil samples. Parallel plate geometry (40 mm smart swap, stainless steel) was used at constant temperature 50°C, constant strain (1 %) and angular frequency of 1 to 100 rad s\(^{-1}\) (Evdokimov et al. 2001).

2.6. Media

2.6.1. BH medium (Bushnell & Hass Medium)
BH medium contained per litre: Magnesium sulphate, 0.2 g, calcium chloride, 0.02 g, potassium dihydrogen phosphate, 1 g, dipotassium hydrogen phosphate, 1 g, ammonium sulphate, 1 g, ferric chloride, 0.05 g. The medium was sterilized by autoclaving for 15 min at 121 °C.

2.6.2. Nutrient agar
Nutrient agar contained per litre: bacteriological peptone, 5 g, sodium chloride, 5 g, yeast extract, 2 g, beef extract, 1 g and bacteriological agar, 13 g. pH was adjusted to 7.4 and the agar sterilized by autoclaving for 15 min at 121 °C.
2.6.3. Nutrient broth
Nutrient broth contained per litre: Lab-lemco powder, 1 g, yeast extract, 2 g, peptone, 5 g and sodium chloride, 5 g. pH was adjusted to 7.4 and the agar sterilized by autoclaving for 15 min at 121 °C.

2.7 Buffer Solutions

2.7.1. 50 X TAE buffer (Tris-acetic acid electrophoresis buffer)
TAE buffer (50 x) contained per litre: Tris-base, 242 g, glacial acetic acid, 57.1 mL, 0.5 M ethylenediaminetetra acetic acid (EDTA), 100 mL. pH was adjusted to 8.0 using NaOH.

2.8. Denaturing Gradient Gel Electrophoresis solutions

Fixing solution 1
Fixing Solution 1 comprised: ethanol (50 ml, 100%), glacial acetic acid, 2.5 ml in distilled water, 447.5 ml.

Fixing solution 2
Fixing Solution 2 contained sodium carbonate, 3.75 g dissolved in distilled water, 500 ml.

Silver nitrate solution
Silver nitrate solution contained silver nitrate, 0.2 g dissolved in distilled water, 200 ml.

Developing solution
Developing solution contained sodium borohydride, 0.02 g, formaldehyde, 0.80 ml and sodium hydroxide, 3 g in distilled water, 200 ml.

Preservation solution
Preservation solution contained ethanol (125 ml, 100%), glycerol, 50 ml in distilled water, 325 ml.

2.9. Bacterial Isolations
Bacteria were isolated in two batches by selective enrichment using Bushnell Hass (BH) mineral salts medium (Bushnell and Hass, 1941). One batch was amended with crude oil tank bottom sludge and the other was amended with Hamad petroleum crude oil from Azzawiya oil refinery.
Dilutions made with distilled water (pH 7.4) ($10^{-1}$ - $10^{-5}$ dilutions) were spread onto BH oil agar plates (Fig. 2.1) suspended with 0.5 % COTBS and Hamada crude oil as substrates and incubated for 5 days (d) at 30 °C. Colonies appearing on the plates were streaked onto BH agar supplemented with 0.5 % (w/v) COTBS and Hamada crude oil and incubated at 30 °C for 5 (d). Bacterial strains were then streaked on nutrient agar and incubated at 30 °C for 2 (d). Pure isolates were also cultured in nutrient broth and incubated at 30 °C for 2 (d) under constant shaking at 120 rpm.

**Figure 2.3.** Bacterial growth on BH oil agar supplemented with Hamada petroleum crude oil (A) and pure isolate growth on nutrient agar (B).
Figure 2.4. Bacterial growth in nutrient broth.

2.10. Biolog MT2 plates
Biology MT2 plates consists of a 96 wells microplate (Fig. 2.3), with each well containing a tetrazolium redox dye and a buffered nutrient medium optimised for a wide variety of bacteria. A re-suspended pellet (150 µl) was used to inoculate the Biolog MT2 plate wells. The hydrocarbon substrates (Table 2.1) were loaded (15 µl) into wells before inoculating with the normalised bacterial cultures (Fig 2.2). Control wells were inoculated with 150 µl cultures without any hydrocarbons. Plates were incubated at 30 ºC. Each bacterial isolate (n=3) was tested on the six different hydrocarbons. Readings were taken every hour over the first 12 h, then every 2 h from 12 to 36 h and every 12 h thereafter until day 7.
Figure 2.5. An example of the Biolog MT2 plates used in this study. The blue colour indicates oxidation of the substrates by the strains.
Figure 2.6. The principal technique of Biolog (MT2) microplate (metabolic fingerprint). (From http://www.biolog.com/).
Table 2.1. The six different hydrocarbons, found in Hamada crude oil and COTBS used in this study together with their chemical and structural formulas.

<table>
<thead>
<tr>
<th>Hydrocarbons</th>
<th>Category</th>
<th>Chemical formula</th>
<th>Structural formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexadecane</td>
<td>alkane</td>
<td>C_{16}H_{34}</td>
<td><img src="image" alt="Hexadecane Structure" /></td>
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<tr>
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<td>aromatic</td>
<td>C_{10}H_{8}</td>
<td><img src="image" alt="Naphthalene Structure" /></td>
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<tr>
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<td>aromatic</td>
<td>C_{14}H_{10}</td>
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<tr>
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<td>alkane</td>
<td>C_{20}H_{42}</td>
<td><img src="image" alt="Eicosane Structure" /></td>
</tr>
<tr>
<td>Octadecane</td>
<td>alkane</td>
<td>C_{18}H_{38}</td>
<td><img src="image" alt="Octadecane Structure" /></td>
</tr>
<tr>
<td>Hamada crude oil</td>
<td>Hydrocarbon mixture</td>
<td></td>
<td><img src="image" alt="Hamada Crude Oil Structure" /></td>
</tr>
</tbody>
</table>
2.11. Biolog EcoPlates

The Biolog EcoPlates consist of 96 wells containing 31 different carbon sources. These substrates consist of carbohydrates (n=10), carboxylic acids (n=7), amino acids (n=6), polymers (n=4), amines and phenols (n=2 for each) in addition to tetrazolium redox dye. Soil samples (1 g) were collected in triplicate from each flask before and after the bioremediation treatment. Deionized sterile water (10 ml) were added to the soil and shaken at 200 rpm for 20 min to dislodge bacteria before dilution. The resulting mixtures were diluted to (10^{-2}). Low speed centrifugation (1,500 g for 10 min) was used to remove the soil particles. Aliquots of bacterial suspension (150 µl) were then inoculated into each well of the Biolog EcoPlates. Control wells were inoculated with deionized sterile water only (150 µl). Plates were incubated at 30 °C in the dark. Readings were taken every 12 h for 7 days.

2.12. Analysis of colour formation

Colour formation in Biolog plate wells (absorbance at 595 nm) was analysed using a multiscan microplate reader equipped with an automated shaker-loader cassette (Labsystems, Finland, Multiskan EX Version 1.0).

2.13. Microcosm system

Slurry phase microcosms (250 ml) were prepared by adding COTBS contaminated soil (30 g) to autoclaved deionized water (150 ml) to obtain a 1: 5 soil: water solution ratio. Bacterial isolates (Pseudomonas sp (4M12), Pseudomonas xanthomarina (4M14) and Arthrobacter nitroguajacolicus (1B16A)) were inoculated (1 x 10^4 cell ml^{-1}) into the microcosms which contain nutrients ((NH4)_2SO_4 (0.43 g), K_2HPO_4 (0.05 g) and KH_2PO_4 (0.04 g)) to achieve a C:N:P molar ratio of around 100:10:1. Microcosms were incubated in the dark at 30 °C and 150 rpm for 30 d.

2.14. Mesocosm system

Slurry phase mesocosms (500 ml) were prepared by adding soil contaminated with COTBS (250 g) to autoclaved deionized water (250 ml) to obtain a 1:1 soil:solution ratio maintained during the whole experimental period. Nutrients ((NH_4)_2SO_4 (3.58 g), K_2HPO_4 (0.41 g) and KH_2PO_4 (0.33 g)) were added to achieve a C:N:P molar ratio of 100:10:1. An aliquot (5 ml) of each individual bacterial isolate ((Pseudomonas sp (4M12), Pseudomonas xanthomarina (4M14)) were inoculated into each experiment with an initial inoculum size of 1x10^4 cells / ml. Flasks
were then connected to a continuous flow of sterile air (0.2µm filter, 1.0 l/min) and maintained in the dark at 30°C and 150 rpm for 90 d.

2.15. Respiratory measurements in mesocosms
The system used is shown in Figure 2.3. Air was pumped at a constant flowrate (1.0 l m⁻¹) after sterilization using a 0.2 µm filter, and removal of CO₂ using a soda lime column. Carbon dioxide (CO₂) concentration in the effluent gas was captured in a NaOH trap (consisting of 150 ml of a 1 M solution). CO₂ production was determined every 4 d by titration with HCl (1M) using Ba(OH)₂ (25 ml) was added NaOH (25 ml) together with 2 drops of phenolphthalein. The NaOH trap was replaced every 4 d. To prevent photodegradation, experiments were carried out in the dark.

![Figure 2.7](image)

**Figure 2.7.** The respiratory measurements in mesocosms.

2.16. Determination of total petroleum hydrocarbon (TPH)
The TPH and PAH concentration of the COTBS in the microcosms and mesocosms were examined by weighing triplicate COTBS-contaminated soil samples (10 g each) into Teflon coated centrifuge tubes (25 ml) and adding hexane (15 ml). The oil in the soil samples was extracted by agitation (130 rpm) for 20 min, followed by centrifugation at 5,000 rpm for 5 min. The supernatants were removed into pre-weighed glass bottles and concentrated by solvent
evaporation overnight at room temperature. The reclaimed oil was then dissolved in hexane (1 ml) and transferred to GC-MS vials. Extracted oil was analysed on a gas chromatograph mass spectrometer (GC-MS) equipped with an auto-sampler (Agilent 6890 GC and Leco Pegasus III TOF-MS). Samples were injected and separated on a capillary column Agilent DB-5MS (60 m by 0.25 mm with 0.25 µm film thickness). The injection temperature and volume was 225 °C and 0.2 µl respectively. Helium (1.8 ml min⁻¹) was used as a carrier gas at a constant flow rate (Sabaté et al. 2004).

2.17. Nucleic acid extraction
DNA extraction was carried out using a MOBIO Power Soil DNA isolation kit (MO BIO Laboratories, Inc., USA) according to the manufacturer’s supplied protocol. Briefly, bacterial pellets were added to the supplied Power Bead tubes and gently vortexed. Solution C1 (60 µl) was added to the tube and the tube inverted several times and subject to bead beating for 30 s twice. The tubes were centrifuged at 10,000 g for 30 s at room temperature. The supernatants were then transferred to a clean collection tubes (2 ml) and the remaining steps performed as per the manufacturer’s protocol.

2.18. Bacterial 16S rDNA amplification
The PCR for the amplification was conducted in a 50-µl PCR master mix. The reactions were prepared using 2-µl bacterial primers 63f (10 µM) (5’-CAGG CCTAACACATGCAAGTC-3’) as forward primer and 1389r (10 µM) (5’-ACGGGCGGTGTGTACAAG-3’) as reverse primer (Osborn et al. 2000), MgCl₂ (5 µl, 25 mM), dNTP mixture (1 µl, 10 mM), GoTaq flexi buffer (10 µl, 5x), Taq polymerase enzyme (0.25 µl) and sterile nuclease-free water (27.75 µl) were added per PCR reaction. DNA extracts (2 µl) were added to 48 µl of master mix. The PCR thermocycling conditions were an initial denaturing step of 95 °C for 5 min and then 95 °C for 1 min, 65 °C for 1 min and 72 °C for 2 min (10 cycles in touchdown mode from 65 to 55 °C (1 °C decrease per cycle) and 20 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min followed by a final extension step at 72 °C for 10 min. Another PCR was also conducted with bacterial primers 341F-GC and 907R (Liang et al. 2011; Muyzer et al. 1993). The thermocycling conditions were; an initial denaturing step at 95 °C for 5 min, then 92 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min (25 cycles) and a final extension step at 72°C for 10 min.
2.19. Agarose gel electrophoresis
Agarose gels (1%) were run at 70 V for 50 min to detect the genomic DNA (using a 1 kb ladder to determine the DNA size). Other agarose gels (2%) were run for PCR products (using 100 bp ladder) to determine the size of PCR products.

2.20. Denaturing Gradient Gel Electrophoresis (DGGE)
PCR products were analysed by DGGE using Universal Mutation Detection System (BioRad) with a 9% acrylamide gel (Acrylamide: N,N'-Methylenebisacrylamide 37:1 solution). Polymerisation of gels were catalysed by addition of N,N,N’,N’-tetramethylethylenediamine (TEMED) (50 µl) and 10% ammonium persulfate solution (APS) (500 µl) added to the gel solution (50 ml). The denaturant gradient ranged between 45-65%. The gels were loaded with PCR products (8 µl) and dye solution (2µl) and run in 1x TAE buffer for 18 h at 60 °C and 60 V. The gels were then removed carefully and soaked in fixing solution (300 ml) (see section 2.6) for 10 min on a slow shaker. After 2 h, the gels were then silver stained for 20 min in silver nitrate solution (300 ml, see section 2.6) with gentle shaking. Silver nitrate was then discarded and developing solution was added (200 ml) to the gels with gentle shaking until bands appeared followed by placing the gels in fixing solution 2 for another 10 min. Finally, preservative solution (200 ml) was added to gels. The gels were then scanned with an Epson V700 scanner and the images were saved as a TIFF file. Images of DGGE gels were then analysed with Phoretix 1D software to generate a dendrogram using unweighted pair group method with mathematical averages (UPGMA).

2.21. Identification bacterial species
Amplified DNA was purified using a PCR clean up kit (Wizard SV PCR clean-up system, Promega, USA) and quantified using Nanodrop (Thermoscientific, USA). DNA samples were sent to the Australian Genome Research Facility (AGRF), Melbourne, for sequencing (www.agrf.org.au). Sequence chromatograms were then edited for quality using Sequencer (version 5) (Gene Codes Corporation, USA). Automated DNA sequencer generates a four colour chromatogram showing the results of sequences with each nucleotide representing a single peak (Fig. 2.4). The sample replicates were then aligned. The aligned sequence were analysed using the nucleotide BLAST program (www.ncbi.nlm.nih.gov). Species were matched with highest identity scoring.
Figure 2.8. Chromatogram of a bacterial sequence. (green peaks represent adenine, red peaks represent thymine, black peaks represent guanine and blue peaks represent cytosine).

2.22 Data analyses
In Biolog MT2 plates and Biolog Ecopeates, the absorbance values of individual and control wells were subtracted from the corresponding 0 hour value. At each other time point, the absorbance for substrates (raw difference) was calculated by subtracting the control wells’ colour formation values from the substrate wells’ colour formation values. For all test bacterial isolates, the average absorbance values were calculated at each time point up to day 7. For Biolog MT2 plates, the average absorbance values of the 49 bacterial isolates on the six hydrocarbon substrates were used for cluster analysis. Cluster analysis was conducted using the Biolog data to derive a hierarchical cluster analysis (HCA) dendrogram.

Digitized DGGE images were analysed TL120 software with the similarity relationship between the different communities analysed by the use of Unweighted Paired Group with Arithmetic Means (UPGMA). Statistical analyses were carried out using SPSS 20 using T Tests. Standard error bars were produced using the following formula in Microsoft® Office Excel 2010. Standard error = STDEV (range of values)/SQRT (number of replicates) Where: STDEV = standard deviation; SQRT = square root of the population size. The standard error (SE) was used where required.
Chapter 3

STATEMENT OF AUTHORSHIP (Chapter Three)
School of Applied Science

Chapter Declaration for Thesis with Publications

Chapter [3] is represented by the following paper: [Recovery and Characterization of Oil from Waste Crude Oil Tank Bottom Sludge from Azzawiya Oil Refinery in Libya]

Author List: Abdulatif A. Mansur, Muthu Pannirselvam, Khalid A Al-Hothaly, Eric M Adetutu1 & Andrew S Ball.

Journal name: Journal of Advanced Chemical Engineering

Year published: 2015

Declaration by candidate

I declare that I wrote the initial draft of this manuscript, and my overall contribution to this paper is detailed below:

<table>
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<tr>
<th>Nature of contribution</th>
<th>Extent of contribution (%)</th>
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<tr>
<td>wrote the first draft, collected all of the data, conducted most of the analysis</td>
<td>70</td>
</tr>
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</table>

The following co-authors contributed to the work. The undersigned declare that the contributions of the candidate and co-authors are correctly attributed below.

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<thead>
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<tr>
<td>Muthu Pannirselvam</td>
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<tr>
<td>Eric M. Adetutu</td>
<td>contributed to manuscript evaluation</td>
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<tr>
<td>Andrew S. Ball</td>
<td>contributed in the planning of the experimental design, supervision of research, manuscript preparation and evaluation</td>
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Candidate’s Signature

Date
28/11/2015

Primary Supervisor’s Signature

Date
28/11/2015
3. Abstract

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Bioremediation of Libyan Soil Contaminated With Crude Oil Tank Bottom Sludge

Abdulatif Mansur

Advanced Chemical Engineering

Research Article

Recovery and Characterization of Oil from Waste Crude Oil Tank Bottom Sludge from Azzawiya Oil Refinery in Libya

Abdulatif A Mansur1*, Muthu Paninselvam2, Khalid A Al-Hothaly1,3, Eric M Adetutu1 and Andrew S Ball1

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2Environmental and Natural Resources Engineering, Faculty of Engineering, Azzawiya University, Libya
3School of Civil, Environmental and Chemical Engineering, RMIT University, Melbourne 3000, Australia

Abstract

In this work we present the results of quantitative and qualitative analyses of oil obtained from crude oil tank bottom sludge (COTBS) generated from Azzawiya oil refinery in Libya. The aim of the study was to recover and evaluate oil from waste oily sludge and to compare it with parent oil (Hamada crude oil) in order to assess the commercial potential of recycling the oil. The benefits would be two-fold, firstly to improve oil utilisation efficiency and secondly in reducing the environmental contamination associated with the petrogenic hydrocarbon industry.

Oily COTBS and extracted oil were characterised and key properties were measured including water and oil content, light and heavy hydrocarbon content, solid content and organic matter content for COTBS and water content, density, specific gravity, API (American Petroleum Institute) gravity, viscosity, salt and ash content for the extracted oil. Solvent (hexane) extraction confirmed that the oily sludge contained 42.08% (± 1.1%) oil composed of light hydrocarbons (30.7 ± 0.07%) and heavy hydrocarbon (59.3 ± 0.4%) fractions. The water and solid contents were 2.9% (± 0.2%) and 55.02% (± 0.6%) respectively. The properties of the recovered oil were assessed; gas chromatograph spectrophotometer (GC-MS) results indicated that the oil contained 159 different hydrocarbon fractions with a total petroleum hydrocarbon (TPH) concentration of 29.367 mg/kg1 and a polycyclic aromatic hydrocarbons (PAH) concentration of 11.752 mg/kg. Several parameters of the oil were measured and compared to the parent oil (Hamada crude oil) including density, specific gravity, viscosity, salt and ash content. The API of the extracted oil (33.03) was lower than the parent oil (38.8) due to a reduced light hydrocarbon (LHC) content. TGA-TFTIR hyphenation shows both mass loss of hydrocarbons — low, medium and high molecular mass over a range of temperatures between 60°C and 450°C. crude oil extraction exhibited a non-Newtonian behaviour (shear thinning) for the shear rate sweep between 10 and 500/s. Dynamic shear rheology data showed that the extracted oil exhibit more like a solid than liquid. Overall the findings of the study confirmed that COTBS has a significant amount of oil similar in properties to Hamada crude oil. This large amount can be reclaimed and recycled. Depending on this essay, a commercial process could be performed which in parallel will reduce the environmental contamination with hydrocarbons.

Keywords: Solvent extraction; Oil recovery; Total petroleum hydrocarbon; Hyphenation; Dynamic shear rheology; Shear thinning; Storage modulus; Loss modulus; Thermogravimetry

Introduction

Petroleum crude oil represents one of the main current sources of energy. With the continuous increase in world population and industrialization, there is an increase in the global demand for petroleum crude oil and downstream products. In July 2012, Endurance International Group, Inc. (EIG) reported total global crude oil stocks of 7148 million barrels, with an estimation daily flow of oil production of around 75 million barrels [1]. In the processing of this crude oil the oil industry annually generates massive quantities of oily sludge during the different crude oil operations from exploration to refining [2]. The largest amount of the oily sludge is generated in oil refineries during oil storage operations. Most of the crude oil storage tanks contain bottom settling sediments accumulated over the time which are called crude oil tank bottom sludge (COTBS). During the cleaning processes, all the waste (COTBS) is removed and dumped in designated ponds. The continuous generation of COTBS during the bulk storage of crude oil is an unavoidable phenomenon [3]. COTBS usually contains a significant amount (30-50%) of oil (heavy hydrocarbons) [4], in addition to water (30-50%) and solids (10-12% w/w) [5]. However, the global composition of COTBS is highly variable varying from one facility to another and from tank to tank within the same facility. COTBS composition is dependent on the composition of the stored oil, storage conditions, storage period and the design and mechanical conditions of the storage tank [6].

Due to the accumulation of large quantities of COTBS together with its hazardous nature and associated waste management difficulties, COTBS has become a critical problem in most oil refineries [7]. In 2001, the USA petroleum industry was estimated to generate about 1.5 x 106 barrels of COTBS per annum [8] while large oil refineries (processing 2-5 x 106 barrels/day) were estimated to produce 10 x 106 m3 per year [9]. In India, petroleum oil refineries generate around 50 x 106 tonnes (t) per annum of oil rich COTBS (30-40% oil) [7]. In China, the petrochemical industry discharges nearly 3 x 106 t of COTBS per annum. One third of this amount (1 x 106 t) is derived from cleaning

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operations associated with crude oil storage tanks [10]. In parallel with oil refineries, oilfields also generate significant amounts of COTBS. In 2010, Shengli Oilfield alone discharged more than $10 \times 10^6$ t of COTBS [11].

The accumulation of COTBS inside the oil tanks reduces their oil storing capacities and inducing the oily sludge into the refinery can ultimately disturb the refining processes [12]. In contrast, spilling of COTBS in the environment without treatment poses a significant risk to the surrounding environment and population. Importantly, prolonged storage of COTBS in accumulation ponds leads to seepage and contamination of ground water as well as the reduction in the light (volatile) fractions. Many of these volatile compounds are known of suspected carcinogens and mutagens and their release into the air poses a significant threat to the ecosystem and human population. Moreover, if COTBS is disposed of inappropriately, the oily sludge will splash into the soil where hundreds of individual compounds will contaminate the soil [11,13,14]. As many of COTBS hydrocarbon components are considered as toxic, mutagenic or carcinogenic [15], in 1992 the United States Environmental Protection Agency (US EPA) announced a final rule (57 FR 37194, 37252) stating treatment regulations and standards under the land disposal restrictions program for several hazardous wastes including hydrocarbon materials (COTBS) [16].

COTBS is continuously generated and disposed of in large quantities [17]. Recently, development of treatment strategies for COTBS to reduce their environmental burden has received increased global attention [4] and different effective remediation techniques have been proposed [17] including physical, chemical and biological methods. Among the techniques described, landfiling, incineration, microwave liquefaction, centrifugation, encapsulation, biodegradation in landfarming, biopolys and bioreactors have all featured [18]. However, some methods (e.g. incineration) have become restricted in some countries through the implementation of rigorous environmental standards because of their potential environmental impact [19].

Given the high hydrocarbon content of the oily COTBS, the conventional treatment methods such as land farming, landfiling and incineration are time-consuming, ineffective, expensive and may potentially release more unwanted environmental pollutants [20,21]. Also as oily COTBS is recognized as a potentially valuable energy resource, decomposition (bioremediation) techniques using microorganisms are also inadvisable [21]. The current driving force for increased interest in studying and characterising the COTBS are to recover oil from waste oily sludge in order to assess the commercial potential of recycling the oil. The benefits would be two-fold, firstly to improve oil utilisation efficiency and secondly in reducing the environmental contamination associated with the petrogenic hydrocarbon industry [22,23].

To characterise and classify recovered oil for commercial use, its physicochemical properties should be known. To classify the oil, the API gravity is the most important property. It is the relative density of the petroleum liquids and the density of water, and used to compare the relative densities of petroleum products. API is a scale for denoting the 'lightness' or 'heaviness' of petroleum crude oils and products. The lighter hydrocarbon the higher API gravity and the lighter hydrocarbon the higher market value. Oils with API more than 30° are known as light while oils in the range between 22° and 30° are medium, but API less than 22° are heavy and below 10° are extra heavy. It is preferable to between 25° and 30° [24]. In addition to the density, the viscosity which is the resistance to flow is another important factor that affects the pumping and transportation abilities through the pipelines. Dealing with high viscosity oil is one of the main difficulties in transportation through the piping network [25]. Usually the viscosity of hydrocarbon oils ranges from 100 mPa to $10^5$ mPa and the maximum desired viscosity is 400 mPa [26], but the high viscosity can be reduced to the desired value by reducing the liquid temperature by adding gaseous or liquid diluents [25]. In addition, flash point which is the minimum temperature at which the vapours of the material can ignite is the indicator of the flammability of the hydrocarbon oils. Safe handling of oils including processing, storage and transportation needs knowing the accurate values of flash point [27]. Moreover, presence of ash in the oil can affect the quality of oil. The ash content provides knowledge of metallic constituent left after complete combustion of the oil under specific condition. High ash content lowers the heating values and it is undesirable for direct combustion due to fouling and sludging [28]. Usually the petroleum crude oil contains a small amount of salts expressed as the presence of NaCl. If the salt content is higher the 1000ppm, the salt need to be minimized to reduce the fouling and corrosion in addition to the formation of acids by salts chlorides [29].

Therefore this work aims to assess the quality of recovered oil from waste oily sludge and to compare it with parent oil (Hamada crude oil) for recycling purposes and (ii) to reduce the environmental impact of COTBS by reducing the oil content of the soil to the minimum possible levels.

Materials and Methods

Characterization of sludge

Petroleum-based COTBS samples used for this study were obtained from collection lagoons at Azzawiya oil refinery in Libya. After collection, the sludge samples were kept at room temperature for the duration of the study. The sludge was mixed well manually before each sample was taken.

Water content (wt%): The water content of the COTBS samples was measured as indicated by the American Society for Testing and materials (ASTM) standard method (D95). COTBS samples (25 g, in triplicate) were taken and placed in an extraction thimble and 75 ml of dichloromethane (DCM) (solvent) were added (1.3:1 solvent:sample ratio) [30]. The oil, solvent and water were then distilled and the condensate (water and solvent) continuously separated in a trap and transferred to a graduated cylinder. The triplicate condensates were pooled together [31]. It should be noted that due to the density of water (1g/cm³) being less than the density of solvent, the solvent layer settled at the bottom of the separation funnel and was measured.

Volatile hydrocarbons and moisture content (wt%): Volatile hydrocarbons (VH) and moisture content of the COTBS samples were determined in triplicate by weighing (8 g) in ceramic crucibles and heating to 105°C in a ventilated incubator for 24 h. The lost mass was attributed to light volatile hydrocarbons and moisture content [32]. The light volatile hydrocarbons were calculated using the following equation:

$$\text{Light hydrocarbons} = \frac{\text{reduced mass (g)}}{\text{mass of tested sample (g)}} \times 100\% - \text{water content (wt%)}. \quad (1)$$

Solid content (wt%): Solid materials (sediment, ash and organic) content were measured according to the method described by [2] with some adjustments. After measuring the light hydrocarbons and moisture content, the dried COTBS samples (at 105°C) were heated in a muffle furnace (LABEC, Laboratory Equipment, Pty Ltd, Australia).
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Specific gravity: Specific gravity ($SG_{20^\circ}$) is the ratio of the density of a liquid to the density of water ($g/cm^3$). The specific gravity of the claimed oil was measured according to [36] and can be expressed mathematically from the following equation:

\[ SG_{20^\circ} = (\text{density of sample}) / (\text{density of H}_2\text{O}) \]

API gravity: API gravity was calculated using the specific gravity of the oil extract, a unit-less property and determined at 60°F. API gravity was calculated according to [37] using the following equation:

\[ \text{API gravity} = (141.5 / \text{specific gravity}) - 131.5 \]

Viscosity: Viscosity ($\eta$) was measured using a Cannon-Fenske (Fisher Scientific, Pittsburgh, PA) glass capillary kinematic viscometer in a constant temperature bath in accordance with ASTM D445. Kinematic viscosity is determined by measuring the time (t) for a known volume of liquid flowing under gravity to pass through a calibrated glass capillary viscometer tube. The manufacturer of the Cannon- Fenske type viscometer tubes supplied calibration constants ($c$) at a range of temperature 40°F and 100°F [38,39].

Kinematic viscosity ($\eta$) in centistoke ($cSt$) was calculated from the following equation.

\[ \eta = ct \]

Ash content: Ash content of the extracted oil was determined using a loss-on-ignition procedure according to [40] with some adjustments. Triplicate samples (5 g) were heated overnight at 105°C and then transferred to a muffle furnace held at 550°C for 30 min to burn the organic matter. Ash content was calculated from the ratio of pre- and post-ignition sample mass.

Salt content: The salinity of the extracted oil was determined using the electrometric method according to ASTM D 3236 procedures using a Pro 2030 multimeter (YNI Incorporation, Yellow Springs, OH 45387, USA). In this method, the sample was dissolved in a mixed solvent and placed in a test cell consisting of a beaker and two parallel stainless steel plates. An alternating voltage was passed through the plates, and the salt content was obtained by reference to a calibration curve of the relationship of salt content of known mixtures to the current [41,42].

Thermogravimetric analysis (hyphenation with FTIR spectroscopy): The sample (COTBS extract) was used to measure mass loss and to determine functional group of the sample at a given time and temperature. Wilke previously described a TGA/FTIR hyphenation technique that could be applied to investigate the degradation of crude oil extract [43]. Thermogravimetric analysis was performed on STA6000 operating under nitrogen with a flow rate of 20 ml/min through the furnace in the following conditions. A sample mass of ~40 mg was heated in the crucible with heating rate of 20°C/min from 50 to 950°C in an inert atmosphere (nitrogen). The gas evolving from STA6000 was transferred via gas transfer line. This transfer line allows the transfer of combustion of pyrolys products from thermal analyser to FTIR 100 through the gas flow cell. Spectrum time base was used to analyse the sp files collected during the testing to analyse the spectrum (collected continuously for over 3600 s).

Shear rheology testing to measure viscosity: HR3 (Hybrid Discovery) rheometer was used to measure the rheological properties of sample (COTBS extract). The instrument (HR3 rheometer) was calibrated with viscosity standard including Polydimethyl Siloxane (PDMS). Crossover frequency of $G'$ and $G''$ of calibration results matched with the value recommended by instrument suppliers—TA instruments. Shear rheology was conducted on the sample at a constant
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Results

Composition of the oily sludge

Several key properties of the sludge were analysed and indicated in Table 1. The water content of the sludge was 2.9 (± 0.2%) and the solid content was 55.02 (± 0.6%). The amount of organic material in the solid content was found to be 70 (± 0.6%) of the original dry mass of the sludge. Solvent extraction of the hydrocarbon oil from COTBS using dichloromethane (DCM) showed that the sludge contained a significant amount of oil (oil content 42.08 ± 1.1%) composed of light (volatile) hydrocarbon (VH, 30.7 ± 0.07%) and non-volatile hydrocarbons (NVH, 69.3 ± 0.4%).

Composition of recovered oil

In addition to the amount of oil recovered, the quality of the oil has a major influence on determining the commercial viability of the recycling process. Selected physicochemical properties of the recovered oil from COTBS were tested including water content, organic material, density, specific gravity, API gravity, viscosity, salt content and ash content. The selected properties were compared with parent oil from Hamada crude oil. The properties are summarized in Tables 2 and 3. GC-MS based analysis of the recovered oil resulted in up to 136 different hydrocarbon fractions being detected including aromatic compounds (45.6%) and aliphatic compounds (34.6%) with some of these fractions (19.8%) being undefined (Figure 1). The concentrations of TPH and PAHs were 29,367 mg/kg and 11,752 mg/kg respectively. API gravity was calculated mathematically after determining the density of the oil (0.68 g/cm³) and was 33.03, confirming that this oil was as light as the parent oil. In addition, the kinematic viscosity of the recovered oil measured at 70°F and 100°F was 7.01 and 3.655 cSt respectively. According to the loss in ignition results, the ash content of this oil was 0.007 g/kg oil. Finally, the salinity test (as NaCl content) of the examined oil indicated that the salt content was 2.30 mg/L.

Thermogravimetric analysis

Mothe et al. [44] concluded that the study of thermogravimetry of crude oil based materials is very complicated due to the presence of many complex constituents [44]. In this research, we applied a hyphenation technique to analyse the gas evolving from the sample during thermogravimetric analysis (Table 4). The crude oil was exposed to a nitrogen atmosphere at heating rate of 20°C/min for 45 min (2700 s) from 50 to 930°C.

Rheology section

The International Energy Agency reported that heavy crude oil represents over 50% of the world’s recoverable oil resources. Crude oil is a composition of a large amount of hydrocarbons and varying amount of waxes. Ghannum stated the dynamic shear rheology test is a rheological investigation to study the viscoelastic behaviour of crude oil [45]. Rheological properties for petroleum oils are very useful for all processes in which fluids are transferred from one location to another. Evdokimov et al concluded that limited numbers of crude oil rheological properties are currently available, in particular for heavy crude oil [46].

The dynamic frequency sweep test shows the effect of oscillating stresses on this extracted crude oil. The storage modulus exhibits solid behaviour of sample and loss modulus exhibits liquid behaviour of samples. Storage modulus shows the contribution of stress energy that is stored during the test and can be recovered. As a standard procedure, linear viscoelastic region of sample was studied of the sample using strain sweep test (in dynamic mode). Three tests of the above mentioned methodology was conducted to check the reliability of the measured data. The rheograms matched well within the tolerance limits of about ± 3%.

Discussion

Due to the undesirable environmental impact of dumping COTBS with high hydrocarbon content and the economic benefits of the COTBS as a source of petroleum oil, there is current interest in studying the quantitative and qualitative characteristics of COTBS and the recovered oil. The management of oily wastes involves the analysis and characterization of both recovered oil and sludge. Knowing the
amounts produced, physical and chemical properties of sludge are important parameters in defining the applied conventional treatment strategies [18]. For the recovered oil, quantitative and qualitative assessment represents the most important parameters in determining the applicability of the oil for use as crude or fuel oil. In this research, the results of the studied COTBS showed that the average water content was low (2.9%). This low water content percentage was expected because the sludge studied in this research has been accumulated for several years and the water was separated into the bottom of the collection ponds due to the density difference between water and sludge. Comparing with other sludges, this percentage was very low. Heidarzadeh et al. [47] showed that the water content of a studied COTBS obtained from Iran refinery was 28.3%. Similarly, other research [48] determined the water content of 2 different oily sludges in China and found a significant amount of water (16.2 and 27.6%). On the basis of dry mass, the solid content of the oily sludge was 55.02%, of which 70% was organic matter. This relatively high solid content was expected since the stored COTBS was not exposed to a solid removal process. The presence of solid materials including sand and rust significantly increases the sludge viscosity [2,48]. Extraction of the oil from COTBS using a 1:1 ratio of COTBS: solvent revealed a high oil content (42.08%) of which with 29.7% were VH and 70.3% NVH. This oil could be potentially extracted and recycled. Recovering and recycling of valuable hydrocarbon oils aids the conservation of environment and energy resources [2,49] and decreases the consumption of non-renewable energy resources [50].

According to the American Petroleum Institute (USAPI), the primary environmental consideration in handling oily sludge is the maximum hydrocarbon recovery [51]. In the oil refining industry in the USA, more than 80% of the generated waste hydrocarbons were recycled while the remaining (20%) were disposal according to the (EPA) standards [52]. Generally, some studies suggested that high oil concentrations in COTBS (>50%) and a relatively low concentration of solids (<30%) are preferable for recycling [53]. Others suggested that even at low COTBS oil content (>10%), oil recovery is still accepted [7]. In this study, within the extracted oil (42.08%), the light hydrocarbon fractions (LHF) were not very high because of evaporation since the collection ponds were exposed to the environmental elements (wind, sun, etc) for many years. A similar study on exposed COTBS [47] indicated that the LHF was also very low (<10%). Storing COTBS in closed facilities results in increased volatile fractions [32] studied COTBS obtained from the Niger delta, Nigeria and determined the oil content to be 73.24%, of which 45.84% were light hydrocarbons. Light hydrocarbon fractions are important in qualifying the oil grade. The presence of (LHF) decreases the density and viscosity and increases the API of the oil. Hu et al. [4] reported that the oil content in COTBS range from 5% to 86.2% (w/w), although oil content in the range of 15-50% (w/w) was more frequent while the solids and water contents were in the range of 5-46% (w/w) and 30-85% (w/w) respectively. Increasing the solvent:sludge ratio could increase the amount of recovered oil. Zubaida and Abouelmor [2] studied various ratios and found that (4:1) solvent:sludge ratio facilitated the extraction.
of the highest amount of oil. Zabaide and Abouelnasr [2] studied the solvent extraction of hydrocarbon compounds and found that heavy molecular mass hydrocarbons were extracted more when less solvent was used. However, increasing the solvent-sludge ratio is not economical as most of the solvents used are expensive; in addition, in low condensation efficiencies, large amounts of solvent could not be reclaimed. Moreover, since the solid content was high (55.02%), a large amount of solvent will be adsorbed within the porous particles. The solvent path within a porous media depends largely on pore size; the more porous media the less solvent reclamation back [54]. Consequently, 1:1 solvent:sludge ratio is recommended for reclamation and economic purposes.

In addition to the amount of oil recovered, the quality of the oil is also a major concern. A set of oil grading physiochemical properties were conducted. Comparing with properties of Hamada crude oil, some properties of the recovered oil were higher including density, specific gravity (SG), viscosity, salt content and ash content; in contrast, water content and API gravity were less than those of Hamada crude oil (Table 2). Gas chromatographic mass spectrophotometer (GC-MS) analysis of the extracted oil showed the presence of a range of hydrocarbon fractions composed of 136 different compounds ranging from C_{24}-C_{32} (Figure 1) including both aromatic (45.6% w/w) and aliphatic compounds (34.6% w/w) with 19.8% being undefined. The total petroleum hydrocarbon (TPH) concentration of the recovered oil was 29,367 mgkg^{-1} and polycyclic aromatic hydrocarbon (PAH) concentration was 11,752 mgkg^{-1}. For comparison, [55] conducted a similar study on COTBS and found that the TPH of extracted oil was higher than 29,367 mgkg^{-1} (500,000 mgkg^{-1}), while another study found the TPH of an extracted oil to be very low (850 ± 150 mgkg^{-1}) [31]. Water content analysis indicated that the extracted oil was free of water. The presence of water lowers the heating value of the oil [56]; in addition, it generates steam and builds pressure in the refining processes [57]. The density of the oil was measured and found to be 0.86 which was higher than the density of the parent oil. Also, the SG has also been calculated at 60°F and found to be higher (0.86) than the original crude oil. Depending on the SG of the extracted oil, the mathematical calculation results show the API density to be 33.03. The API density is one of the main parameters used to grade the crude oil. Martinez-Palou et al. [58] indicated that oil with API<10 is classified as extra heavy while API=22.3 is heavy, API 22.3 to 31.1 is medium and API=31.1 is light. The lighter the oil is the higher content of light hydrocarbon compounds, and the less wax and asphaltenes are present. Viscosity is the resistance to flow and a measure of the internal molecular fraction of the fluid and is an important parameter affecting the pumping of oil and atomization of fuel [59]. The kinematic viscosity of the extracted oil was measured at 2 different temperatures and compared to that of Hamada crude oil. At 60°F viscosity was 7.01 cSt and at 100°F was 3.655 cSt while the viscosity of the Hamada oil was lower (6.8431 cSt and 3.5742 cSt) respectively. Lower LHF content in the oil increases the density and viscosity of the oil. Consequently more power is needed for pumping [59].

![Graph: Mass loss curve of the extracted oil](image_url)
In addition to magnesium chloride (MgCl₂), salts in crude oils consist of up to 75% sodium chloride (NaCl) [60]; typically the salinity of oil is measured as NaCl content. The salinity of the recovered oil was 2.30 mg/l. This value was slightly higher than the salt content of Hamada oil (2.14 mg/l). Comparing with other studies, this value was very low. Zubayid and Abouelnasr [2] investigated the properties of 3 extracted oils and found the salt content to be 5, 7 and 196.4 mg/l[1]. The presence of salts in oil is not favourable; even small concentrations of salt will accumulate in process equipment leading to fouling. In addition and more importantly, NaCl and MgCl₂ can be hydrolysed to hydrochloric acid as indicated in the following equations:

\[
2\text{NaCl} + \text{H}_2\text{O} \rightarrow 2\text{HCl} + \text{Na}_2\text{O} \tag{8}
\]

\[
\text{MgCl}_2 + \text{H}_2\text{O} \rightarrow 2\text{HCl} + \text{MgO} \tag{9}
\]

The produced hydrochloric acid is known to be extremely corrosive [42]; Ash content represents organic materials [61] and is another property used to assess the heating and calorific values of the oil [28,62]. The ash content in the recovered oil contains a higher ash (0.007 g/g oil) than Hamada oil (0.004 g/g oil). Previous studies confirm that lower ash content is indicative of high quality oil [63]; [28] studied the effects of ash content and found that presence of ash in oil reduces the heating value.

Thermogravimetric analysis

Thermogravimetric curve did not show decomposition stage at all temperatures as clearly shown in Differential Thermogravimetric (DTG curve) (Figure 2), however there was a sharp mass loss at the following temperatures: 66.82, 74.03, 89.40, 101.96, 114.73, 129.37, 146.07, 161.39, 209.28, 431.48, and 456.51°C. The decomposition of crude oil at various temperatures shows the mass loss of hydrocarbons (low, medium and high molecular weight) respectively at 161.39, 289.28, 431.48, 456.51°C. Figures 3 and 4 show the spectra obtained at various time intervals. EPA and NIST gas phase libraries were used to search/match the spectra obtained in this hyphenation experimentation and to compare the best possible match spectrum (Figures 5 and 6). The following are the gases evolved at various temperatures: methylene chloride, trans-1,4-dimethylcyclohexane, cyclohexane-1-hexyl-tertadecyl, isobutyl cyclo-hexane and 1-ethyl 2-methylcyclohexane (Table 4). The spectra were obtained via search/match technique using EPA and NIST gas phase library. These values are matching similar to results published in the article Mothe et al in the article titled “Thermal Evaluation of Heavy Oils by Simultaneous TG/DTG/FTIR” [44].

Dynamic shear rheology

It has been observed that 1% is within the linear viscoelastic region of crude oil (internal bonds of the sample tested are intact at that strain value). Figure 7 shows the storage and loss moduli versus frequency: storage modulus G’ values are higher than the values of loss modulus G”. It can be inferred that crude oil sludge exhibits solid like behaviour (sludge) rather than the viscous liquid behaviour (oil). This behaviour could be due to the large solid particles present in the sludge. In general, it has been stated that crude oil exhibits more viscous liquid like behaviour than solid-like material. This change in rheological behaviour could be due to the presence of solid waste present in this oil. The viscosity of the crude oil decreases with increasing temperature as expected. It can be inferred from Figure 8 that the crude oil clearly exhibited a non-Newtonian behaviour (shear thinning) for the shear rate sweep between 10 and 500/s.

Figure 7 shows the storage and loss moduli of crude oil for a frequency sweep (1 to 100 rad/s). The storage modulus and loss moduli increased with increase in angular frequency (1 to 100 rad/s). Viscosity and other rheological parameters are very important for any
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Figure 4: FTIR spectra collected at various time intervals.

Figure 5: FTIR spectra collected at 2322 seconds compared with search match NIST and EPA vapour phase database.

liquids including materials like crude oil that are often transported via pipelines [45,46,64]. The transportation of crude oil is very challenging due to the high viscosity (medium and high molecular weight and low fluidity). Figure 8 shows the viscosity over a temperature range (35 to 50°C). The viscosity decreases with increasing temperature. The low and medium molar mass compounds tend to affect the bonds between the solid particles, eventually reducing the oil viscosity. Ghannam et al concluded that the decrease in viscosity with increase in temperature could be due to the effect of temperature on the chemical structure of the components of the crude oil [45]. The change in viscosity over temperature could be attributed to changes in the chemical structure of high molecular mass components of the crude oil, in particular wax and asphalt [65].

For future work, the concentrations of heavy metals such as P, Gd,
Cr, Ca, Ni, Pb, Zn and Fe can be carried out. This can be determined using inductively coupled plasma optical emission spectrometry (ICP-OES) (Optima 2000DV, Perkin Elmer, Waltham, MA, USA) (detection limit 0.001-0.030 mg/l) and inductively coupled plasma mass spectrometry (ICP-MS) (7500a; Agilent Technologies, Santa Clara, CA, USA) (detection limit 0.015-0.120 mg/l) [66]. In addition, luminescent iridium (III) complex-base chemosensor and probes can be used to detect the heavy metals ions [67,68]. Moreover, logic gates technique is one of the useful tool that respond to the ions of metal [68,70].

Conclusion

The purpose of this study was (i) to extract oil from Libyan COTBS and present the most important properties through a qualitative and quantitative assessment and to compare it with the parent oil (Hamada crude oil), and (ii) to reduce the oil content of the sludge to the minimum level to reduce the environmental impact of COTBS. Some important properties of the COTBS were studied (Table 1). To determine the applicability of recovered oil for use as feedstock for recycling, the physiochemical properties were investigated (Table 2). The extraction yield confirmed that the oil content of COTBS was significant (42.08%). GC-MS analysis of the recovered oil indicated that the oil contained hydrocarbon fractions in the range of C_{10}-C_{32} with TPH and PAHs concentrations of 29.267 mg/kg and 11.75 mg/kg respectively. Comparing the properties of the recovered oil with the parent oil which was the original source of this sludge and classified as light oil, the reclaimed oil was heavier. Lower LHF content decreased the API gravity (33.03), but the API still higher than (31.1) meaning that the reclaimed oil is light oil. Consequently, the recovered oil can be classified as light oil and potentially used as good feedstock oil. The candidate (crude oil) exhibits non-Newtonian shear thinning behaviour over the shear rates sweep between 10 and 500/s. Viscosity of crude oil decreases significantly with increase in temperature due to the effect of temperature on chemical structure of the ingredient of the crude oil. This is the first report of the oil recovery from COTBS from Libya having significant potential of oil for use in the oil industry.
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References

Chapter 4

STATEMENT OF AUTHORSHIP (Chapter Four)
School of Applied Science

Chapter Declaration for Thesis with Publications

Chapter [4] is represented by the following paper: [Assessing the hydrocarbon degrading potential of indigenous bacteria isolated from crude oil tank bottom sludge and hydrocarbon-contaminated soil of Azzawiya oil refinery, Libya]

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Declaration by candidate

I declare that I wrote the initial draft of this manuscript, and my overall contribution to this paper is detailed below:

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<td>wrote the first draft, collected all of the data, conducted most of the analysis</td>
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The following co-authors contributed to the work. The undersigned declare that the contributions of the candidate and co-authors are correctly attributed below.

<table>
<thead>
<tr>
<th>Author</th>
<th>Nature of contribution</th>
<th>Extent of contribution (%)</th>
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<tbody>
<tr>
<td>Eric M. Adetutu</td>
<td>contributed to manuscript evaluation</td>
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<td>Krishna K. Kadali</td>
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<td>Andrew S. Ball</td>
<td>contributed in the planning of the experimental design, supervision of research, manuscript preparation and evaluation</td>
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Date
28/11/2015

Primary Supervisor’s Signature

Date
28/11/2015
4. Abstract

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Assessing the hydrocarbon degrading potential of indigenous bacteria isolated from crude oil tank bottom sludge and hydrocarbon-contaminated soil of Azzawiya oil refinery, Libya

Abdulatif A. Mansur · Eric M. Adetutu · Krishna K. Kadali · Paul D. Morrison · Yuana Nurulita · Andrew S. Ball

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Abstract The disposal of hazardous crude oil tank bottom sludge (COTBS) represents a significant waste management burden for South Mediterranean countries. Currently, the application of biological systems (bioremediation) for the treatment of COTBS is not widely practiced in these countries. Therefore, this study aims to develop the potential for bioremediation in this region through assessment of the abilities of indigenous hydrocarbonoclastic microorganisms from Libyan Hamada COTBS for the biotreatment of Libyan COTBS-contaminated environments. Bacteria were isolated from COTBS, COTBS-contaminated soil, treated COTBS-contaminated soil, and uncontaminated soil using Bushnell Hass medium amended with Hamada crude oil (1%) as the main carbon source. Overall, 49 bacterial phenotypes were detected, and their individual abilities to degrade Hamada crude and selected COBTS fractions (naphthalene, phenanthrene, eicosane, octadecane and hexane) were evaluated using MT2 Biolog plates. Analyses using average well colour development showed that ~90% of bacterial isolates were capable of utilizing representative aromatic fractions compared to 51% utilization of representative aliphatics. Interestingly, more hydrocarbonoclastic isolates were obtained from treated contaminated soils (42.9%) than from COTBS (26.5%) or COTBS-contaminated (30.6%) and control (0%) soils. Hierarchical cluster analysis (HCA) separated the isolates into two clusters with microorganisms in cluster 2 being 1.7- to 5-fold better at hydrocarbon degradation than those in cluster 1. Cluster 2 isolates belonged to the putative hydrocarbon-degrading genera; Pseudomonas, Bacillus, Arthrobacter and Brevundimonas with 57% of these isolates being obtained from treated COTBS-contaminated soil. Overall, this study demonstrates that the potential for PAH degradation exists for the bioremediation of Hamada COTBS-contaminated environments in Libya. This represents the first report on the isolation of hydrocarbonoclastic bacteria from Libyan COTBS and COTBS-contaminated soil.

Keywords Weathered Hamada crude oil · Crude oil tank bottom sludge (COTBS) · Contaminated soil · MT2 Biolog plates · Hydrocarbonoclastic bacteria · Bioremediation

Introduction

Petroleum crude oil is a complex mixture of hydrocarbons ranging from simple straight chain alkanes to complex polyaromatic hydrocarbons (PAH). As a result of this chemical complexity, the refinery of crude oil is known to yield a
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A diverse range of useful products such as liquefied petroleum gas, diesel, lubricating oils, bitumen and chemical solvents. However, there are also unwanted by-products such as crude oil tank bottom sludge (COTBS) associated with the refining process. COTBS is a mixture of high molecular weight paraffins, olefins and aromatic compounds, with compounds such as alkanes, cycloalkanes, benzene, toluene, xylenes, phenols and phenanthrene commonly detected (Kriipsalu et al. 2008). Oil-water emulsions and suspended solids such as sand, mud and metal scales are also found in COTBS.

COTBS is generated in significant quantities at crude oil refineries (Cerqueira et al. 2011). It is estimated that a refinery processing between 200 and 500 barrels per day will generate about 10,000 m³/year of sludge (Cerqueira et al. 2011). In addition to this, sludge and other oil impurities can accumulate over time at the bottom of crude oil storage or holding tanks. This accumulated sludge is removed periodically (usually once every 5 years) through a labour intensive cleaning process. The safe disposal of this removed sludge can be challenging and creates additional problems for hydrocarbon waste management. This is because the removed sludge or COTBS is difficult to degrade and toxic and because the associated costs of its treatment and disposal represent a significant additional expense (Ferrari et al. 1996; Gallego et al. 2007; Yuste et al. 2000). Improper handling, storage or disposal of COTBS can lead to soil and groundwater pollution (Bhattacharyya and Shekdar 2003), resulting in well-documented adverse effects on the environment (Bento et al. 2005).

Although a significant body of research has been carried out on the impact of hydrocarbon contamination on soils and water (Wang et al. 2008; Wang et al. 2011), only a few of these reports focus on soils contaminated with COTBS (Adetutu et al. 2014). This may partly be due to the fact that COTBS takes a long time (5–10 years) to accumulate in holding tanks prior to any removal and subsequent treatment. In addition, COTBS is usually stored on site (with restricted human access) for a number of years, thereby limiting their access for research-related activities.

The treatment of soils contaminated with COTBS can be carried out using either physical or chemical methods. Excavation, secured landfill disposal, thermal desorption, incineration, stabilization and solidification are some of the techniques that can be used to treat contaminants such as COTBS and PCBs (Varamasi et al. 2007). Some of these techniques can be expensive (Makadja et al. 2011), are not environmentally friendly and may not completely detoxify the waste material (Gallego et al. 2007). Biological treatment, based on the ability of naturally occurring microorganisms to degrade the pollutants, is efficient, economical and more environmentally friendly. This approach represents a versatile alternative to physical and chemical treatments (Zhang et al. 2010). Biotreatment can be carried out through nutrient addition (biostimulation), addition of hydrocarbon-degrading microorganisms (biotaugmentation) or a combined biostimulation-biotreatment approach. Previous research on bioremediation has shown that the natural microbial community present in the contaminated material will contain a range of organisms with the potential to degrade many of the oil fractions (Makadja et al. 2011). For COTBS containing light and heavy hydrocarbon fractions, the lighter fractions are readily degraded while the heavier fractions such as PAHs are more recalcitrant. The application of biostimulation to COTBS and contaminated soils may result in substantial hydrocarbon degradation (Gallego et al. 2007). Microorganisms isolated from COTBS are likely to have been well adapted to degrade a range of fractions including the more recalcitrant heavier fractions of the COTBS (Verna et al. 2006). Consequently, biological treatment of COTBS may require that isolates from the sludge be evaluated for their abilities to degrade the COTBS fractions before isolates from other sources are considered.

The study of the microbial resources naturally present in the contaminated and the contaminated environment can be achieved using a variety of culture-dependent and culture-independent tools. Culture-independent tools such as polymerase chain reaction (PCR)-DGGE (Vitte et al. 2011) and the use of next generation sequencing tools can play important roles in improving our understanding of the bioremediation process (Davey et al. 2011; Mardis 2008; Morozova and Murra 2008). However, if augmentation is to be used as a bioremediation technique alongside biostimulation, then, there is a requirement to isolate hydrocarbon-degrading organisms. The selection of an appropriate isolation protocol is important, as this is often a time-consuming process resulting in the screening of hundreds of isolates. The assessment of hydrocarbonoclastic activity of bacterial isolates can be carried out using microtitre plates such as Biolog MT2 plates containing a range of hydrocarbon substrates representative of the hydrocarbons found in the contaminating oil (Kadali et al. 2012). This assessment of the suitability of bacterial isolates step is crucial if the augmentation protocols are to be successful. Interestingly, to date, most research data examining the potential for bioremediation of COTBS have been carried out using microbial resources from other contaminated environments and not from COTBS (Gallego et al. 2007; Machín-Ramírez et al. 2008). Apart from this, limited information is available on the use of microbial resources in COTBS (for bioremediation) from south Mediterranean regions such as Libya. Therefore, in this study, the potential of indigenous bacterial isolates from Hamada COTBS, COTBS-contaminated soil and treated soil to degrade a range of hydrocarbon fractions was assessed. This represents one of the first reports on the assessment of the hydrocarbonoclastic potential of isolates from COBTS and soil contaminated with COBTS from Azzawiyah oil refinery, Libya. This represents a
significant study given the global problem associated with COBTS.

Materials and methods

Field sampling

The samples used in this study were obtained from four different sites. These were an aged collection lagoon of COTBS, stockpiles of soil with long-term contamination by COTBS, aged piles of historically treated soil at Azzawiyah oil refinery, Libya (latitude of 32° 97174813 and longitude of 12° 7089823), and uncontaminated control soils (similar soil type to treated and untreated contaminated soils; loamy sand). The aged piles of historically treated soils (also referred to as treated COTBS-contaminated soil) had been treated using thermal treatment with diesel followed by liquid/solid separation for oil recovery by refinery personnel prior to the start of this study. Samples were collected from the top 0–30 cm, sieved (2-mm mesh size) into ziplock plastic bags, transported to RMIT University, Melbourne, Australia, coded and stored in a quarantine facility at the university.

Oil extraction form COTBS-contaminated soil

Oil was extracted in triplicate by solvent extraction and air-drying using a previously described protocol (Tang et al. 2010) with some modifications. These modifications were carried out for ease of analysis and because they led to greater hydrocarbon yield from the contaminated soils used in our experiment compared to the original protocol. Briefly, COTBS-contaminated soil (10 g) was weighed into Teflon-coated centrifuge tubes (25 ml) and hexane (15 ml) added. The oil in the soil was extracted by agitation (130rpm) for 20 min, followed by centrifugation at 5,000 rpm for 5 min. The supernatants were removed into pre-heated glass bottles and concentrated by solvent evaporation overnight at room temperature.

Hydrocarbon profile of oil in COTBS-contaminated soil

A combined paraffin, isoparaffin, aromatic, naphthenes and olefin, PIANO-5-Piano (DHA) standard combined set (2-ml ampule), (Spectrum Quality Standards, Ltd. Sugarland, TX, USA) was used for hydrocarbon profiling. For the generation of the hydrocarbon profile, the extracted oil from soil contaminated with COTBS (untreated) was analyzed as described in ASTM D5134 with some modifications. These modifications which were carried out to enhance gas chromatograph (GC) separation and resolution were related to the injector temperature and the carrier gas flow rate. GC mass spectrometer (MS) equipped with autosampler (Agilent 6890 GC and Leco Pegasus III TOF-MS) was used for generating the hydrocarbon profile of extracted COTBS. All the samples were placed in a chromatographic vial (2 ml), injected and separated on a non-polar capillary column Supelco SPB (30 m by 0.25 mm with 0.25-μm film thickness). The injection temperature was 350 °C and injection volume was 1 μl. Helium (2 ml min⁻¹) was used as a carrier gas at a constant flow rate. The % weight of each hydrocarbon fraction was analyzed and compared to the weight of each fraction in the PIANO standard. Over 70% compatibility was obtained.

Isolations

Bacterial isolations were carried out on samples from four sources: (i) COTBS, (ii) soil contaminated with COTBS, (iii) treated COTBS-contaminated soil and (iv) clean agricultural (control) soil. One gram of each sample was added to sterile distilled water (9 ml) and serially diluted by a thousand fold using phosphate-buffered saline (PBS) solution. Bacterial species were isolated by selective enrichment using Bushnell Hass (BH) mineral salts medium (Bushnell and Haas 1941) enhanced with weathered Libyan Hamada petroleum crude oil. BH oil agar plates, supplemented with 1% (v/v) Hamada oil as a substrate, were inoculated with chosen diluents by a spread plate method and incubated in the dark at 30 °C for up to 5 days. For validation purposes, observed colonies were subcultured, re-streaked onto BH agar plates amended with 1% (v/v) Hamada crude oil and re-incubated at 30 °C. After validation, bacterial strains were purified on nutrient agar plates (Acumed, Lansing, MI, USA) (26 g l⁻¹ adjusted to pH 7.4) before being differentiated on the basis of their colonial morphologies. Purified isolates were inoculated into nutrient broth (10 ml; Acumed, Lansing, MI, USA) and incubated for 48 h at 120 rpm at 30 °C for the generation of cells for storage. Stocks of pure cultures were maintained at −80 °C in 50% glycerol.

Preparation of bacterial isolates for Biolog MT2 plates

Pure bacterial isolates were reactivated by inoculation into nutrient broth (15 ml) and incubated at 30 °C and 120 rpm for 2 days. Cultures between 0.980 and 1.702 (OD₆₀₀ values) were harvested by centrifugation at 4 °C at 16,000×g for 2 min, washed twice with sterile distilled water and standardized as previously described (Kadali et al. 2012). The obtained pellets of pure bacterial cultures were then resuspended in sterile water (1 ml) prior to use.

Biolog substrates

Based on the analysis of the components of the hydrocarbon profile of the extracted oil from COTBS-contaminated soil, five different hydrocarbon fractions (naphthalene, phenanthrene,
cicosane, octadecane and hexadecane) and Hamada crude oil were selected for use. Naphthalene and phenanthrene were used as representatives of aromatic fractions found in COTBS-contaminated soils while cicosane, octadecane and hexadecane (Sigma-Aldrich) were used as representatives of aliphatic contaminants. Hamada petroleum crude oil was also used for Biolog assay, as it was the source of the COTBS that contaminated the soils investigated in this study. The selected substrates were dissolved in hexane at a final concentration of 2% of (w/v).

Biolog MT2 plates

Ninety-six well microplates containing tetrazolium redox dye (Bochner 1989) and medium of buffered nutrients without a carbon source were used. The microplates were maintained on ice for 30 min before loading the substrates to prevent the microplates from cracking. Each of the hydrocarbon substrates (0.3 mg in 15 μl of 2% stock solution; 0.02 mg μl⁻¹) was loaded into the wells in a fume hood. Plates were then left on ice for up to 30 min to allow hexane to evaporate (Palmroth et al. 2006; Perfumo et al. 2006; Kadali et al. 2012) prior to inoculation of the plates. In the case of naphthalene (which can sublime at room temperature), visual confirmation of the formation of the naphthalene crystals was carried out prior to any inoculation. An aliquot of the re-suspended bacterial pellets (150 μl) was inoculated into the wells of Biolog MT2 plates. Control wells were inoculated with bacterial cultures (150 μl) without any substrates. Plates were incubated at 30 °C in the dark with the lids closed (only opened for taking readings). Each individual bacterial isolate (n = 3) was tested on the six different hydrocarbon compounds. A multispec microplate reader equipped with an automated shaker-loader cassette (Labsystems, Finland, Multiskan EX Version 1.0) was used to analyze the colour formation in the Biolog MT2 plate wells (absorption at 595 nm). Readings were taken every hour over the first 12 h, then every 2 h from 12 to 36 h and every 12 h thereafter for up to day 7.

Data analysis

The absorbance values of individual and control wells were subtracted from the corresponding 0 hour value. At each other time point, the absorbance for substrates (raw difference) was calculated by subtracting the control wells’ colour formation values from the substrate wells’ colour formation values. For all test bacterial isolates, the average absorbance values were calculated at each time point up to day 7. The average absorbance values of the 49 bacterial isolates on the six hydrocarbon substrates were used for cluster analysis.

Cluster analysis

Cluster analysis was carried out based on the method described by Kadali et al. (2012). This was used to classify bacterial isolates on a set of user-selected characteristics (day 7 absorption readings on six different hydrocarbons).

Nucleic acid extraction

Pure bacterial isolates were cultured in nutrient broth (10 ml) for 48 h and then harvested by centrifugation at 4 °C at 16,000×g for 2 min and washed twice with sterile distilled water. DNA extraction of the pellet was carried out using a MOBIO Power Soil DNA isolation kit (MO BIO Laboratories, Inc., USA) according to the manufacturer’s supplied protocol. Briefly, bacterial pellets were added to the supplied Power Bead tubes and gently vortexed. Solution C1 (60 μl) was added to the tube and the tube inverted several times and subject to bead beating for 30 s twice. The tubes were centrifuged at 10,000×g for 30 s at room temperature. The supernatants were then transferred to a clean collection tubes (2 ml) and the remaining steps performed as per the manufacturer’s protocol.

Bacterial identification

Bacterial isolates were identified based on the partial sequencing of the 16S rDNA gene. The PCR for the amplification was conducted in a 50-μl PCR master mix. The reactions were prepared using 2-μl bacterial primers 63f (10 μM) (5’CAG GCC TAA CCA CAT GCA GTC-3’) as forward primer and 1389r (10 μM) (5’-ACG GGCGGTGTTGACAG-3’) as reverse primer (Osborn et al. 2000), 5 μl MgCl₂ (25 mM), 1 μl dNTP mixture (10 mM), 10 μl of GoTaq flexi buffer (5×), 0.25 μl of Taq polymerase enzyme and 27.75 μl of sterile nuclease-free water per PCR reaction. DNA extracts (2 μl) were added to 48 μl of master mix. The PCR thermocycling conditions were an initial denaturing step of 95 °C for 5 min and then 95 °C for 1 min, 65 °C for 1 min and 72 °C for 2 min (10 cycles in touchdown mode from 65 to 55 °C (1 °C decrease per cycle) and 20 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min followed by a final extension step at 72 °C for 10 min. Amplified DNA were purified using a PCR clean up kit (Wizard SV PCR clean-up system, Promega, USA) and quantified using Nanodrop (Thermoscientific, USA). DNA samples were sent to Australian Genome Research Facility (AGRF), Melbourne, for sequencing (www.agrf.org.au). Sequence chromatograms were then edited for quality using Sequencher (version 5) (Gene Codes Corporation, USA).
Results

Analysis of the oil extracted from untreated COTBS-contaminated soil showed that it contained 30,703 mg kg\(^{-1}\) of total petroleum hydrocarbons. Hamada crude oil which is the original source of the COTBS used in this study is a Libyan light and sweet petroleum crude oil with a sulphur content of 0.063 % by wt and an API value of 38.8. GC-MS analysis of the extracted oil performed using PIANO standards (Fig. 1) confirmed the presence of a range of hydrocarbon compounds including aromatic compounds (46.3 %) and aliphatic compounds (33.8 %).

Using Hamada crude oil (1 % (v/v)) as the main source of carbon, selective plate isolations resulted in the recovery of \( \geq 8 \times 10^{6} \) CFU g\(^{-1}\) of hydrocarbonoclastic bacteria in contaminated soil (data not shown). From these isolations, 49 pure isolates or phenotypes were obtained from the contaminated soils (treated and untreated) and COTBS while no isolates were obtained from the clean soil. The highest number of bacterial isolates capable of growing on BH-Hamada crude oil agar plates was obtained in treated COTBS-contaminated soils (21) followed by COTBS-contaminated soils (15) and COTBS (13) (Table 1).

Assessment of the specific ability of each of these isolates to utilize or degrade five different hydrocarbon fractions and Hamada crude oil was carried out with Biolog M12 plates.

All the microorganisms were able to utilize Hamada crude oil on the Biolog plates after 3-day incubation (Table 1). Amongst the five hydrocarbon fractions, representative aromatic fractions (PAH) were utilized by a greater number of isolates than representative aliphatic fractions (Fig. 2). Overall, the ability to utilize representative aromatic substrates was the most common activity with 44 out of the 49 isolates capable of degrading naphthalene and phenanthrene (Table 1). For treated COTBS-contaminated soils, 16 out of the 21 morphological distinct isolates were able to utilize the representative aromatic and aliphatic fractions. All the 21 isolates utilized Hamada crude oil, suggesting the potential of this sample as a source of novel hydrocarbonoclastic bacteria (Table 1). All the six substrates were degraded over the first 3 days. In terms of the extent of substrate utilization, eicosane was the substrate with highest growth (greatest degradation) as measured by maximum average absorption (595 nm) while octadecane was the least degradable substrate (Fig. 3).

![GC-MS chromatogram of soil extracts obtained from COTBS-contaminated soil with some selected peaks identified. S1 2,6,10-trimethyldodecane, S2 2,6,10,14-tetramethylpentadecane, S3 2,6,10,14-tetramethylhexadecane, S4 cyclic octaatomic sulphur. GC-MS chromatograms of extracts from treated COTBS-contaminated soils and COTBS are presented in Supplementary Fig. 1a, b](image)

Fig. 1 GC-MS chromatogram of soil extracts obtained from COTBS-contaminated soil with some selected peaks identified. S1 2,6,10-trimethyldodecane, S2 2,6,10,14-tetramethylpentadecane, S3 2,6,10,14-tetramethylhexadecane, S4 cyclic octaatomic sulphur. GC-MS chromatograms of extracts from treated COTBS-contaminated soils and COTBS are presented in Supplementary Fig. 1a, b.
Bioremediation of Libyan Soil Contaminated With Crude Oil Tank Bottom Sludge

Abdulatif Mansur

Table 1 Sources of isolates and summary of their hydrocarbon-utilizing potentials

<table>
<thead>
<tr>
<th>Hydrocarbon</th>
<th>Long-term contaminated soil (&gt;3 years)</th>
<th>Treated contaminated soil (&gt;12 years)</th>
<th>COTBS</th>
<th>Clean (control) soil</th>
<th>Total</th>
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<td>Ability to utilize/grow</td>
<td>15</td>
<td>21</td>
<td>13</td>
<td>0</td>
<td>49</td>
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<tr>
<td>Aromatic&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15</td>
<td>16</td>
<td>13</td>
<td>0</td>
<td>44</td>
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<tr>
<td>Aliphatic&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4</td>
<td>16</td>
<td>5</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Hamada crude oil&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15</td>
<td>21</td>
<td>13</td>
<td>0</td>
<td>49</td>
</tr>
</tbody>
</table>

Aromatic substrates tested: naphthalene and phenanthrene, aliphatic substrates tested: eicosane, octadecane and hexadecane
<sup>a</sup> Number derived from growth on BH-Hamada crude oil agar plates
<sup>b</sup> Number derived from growth on selected substrates used in Biolog MT2 plates

Cluster analysis was conducted using the Biolog data to derive a hierarchical cluster analysis (HCA) dendrogram. Two separate clusters were observed (Fig. 4). Cluster 1 contains a large number of hydrocarbonoclastic bacteria while cluster 2 has fewer hydrocarbonoclastic bacteria (Fig. 4). However, the isolates in cluster 2 showed higher Biolog plate substrate utilization (1.7–5-folds higher) than cluster 1 isolates (Fig. 5). This was deduced from the analysis of the average absorbance of bacterial isolates of both clusters which was calculated for the six different hydrocarbons (Fig. 5). A significant increase in the average absorption of the six different hydrocarbon substrates was observed in cluster 2 microorganisms compared with cluster 1 microorganisms (P<0.05; Fig. 5).

From the above data, identification of the most active isolates from cluster 2 was carried out. Seven bacterial strains were identified. Four of these isolates were isolated from treated COTBS-contaminated soils (57 %), two from COTBS (29 %) and one from COTBS-contaminated soil (14 %). These were classified into four groups: (i) three bacterial species belonging to the genus *Pseudomonas*, two were identified as *Pseudomonas* sp. (AB733546.1) and *Pseudomonas* sp (FJ662885.1) (98 and 96 % similarity, respectively) while the third was identified as *Pseudomonas xanthomarina* (HG008727.1) (96 % similarity); (ii) two isolates were identified as *Bacillus* strains, *Bacillus nocardiae* (KF015515.1, 88 % similarity) and *Bacillus aericus* (KC443115.1, 90 % similarity); (iii) one bacterial isolate was identified as *Arthrobacter nitroguajacolicus* (JX483740.1, 98 % similarity) and (iv) the final isolate, identified as showing high hydrocarbonoclastic activity, was identified as *Brevundimonas nasdae* (EU740900.1, 96 % similarity) (Table 2).

**Discussion**

GC-MS analysis of the oil extracted (4.5–5.5 % w/w) from COTBS-contaminated soils confirmed that the COTBS is a

![Graph](image)

**Fig. 2** The percentage of test bacterial species capable of utilizing selected representatives of aromatic and aliphatic compounds and Hamada crude oil

**Fig. 3** Growth (measured by absorption at 595 nm) of bacteria on six different hydrocarbons over 3 days. Values (average well colour development) represent the means of growth of 25 bacterial species on representative aliphatic substrates, 44 bacterial species on representative aromatic substrates and 49 bacterial species in Hamada crude oil. Results for growth over 72 h (3 days) are presented, as there was no further increase in average absorption after this time.
Fig. 4 Classification of 49 hydrocarbonoclastic bacteria based on their growth on the six different hydrocarbons. The vertical lines represent the bacteria, and the horizontal lines represent distance.

rich mixture of different hydrocarbons containing both aromatic compounds (46.3%) and aliphatic compounds (33.8%). This hydrocarbon mixture potentially represents a good source of carbon and energy to the bacterial community. Isolation by selective enrichment (Bushnell and Haas 1941) using weathered Libyan Hamada petroleum crude oil as the main carbon source resulted in the isolation of high numbers of hydrocarbonoclastic bacteria ($\geq 8 \times 10^6$ CFU g$^{-1}$) in all samples. All the three COBTS-containing samples used in this study (COTBS, treated COTBS-contaminated soil and COTBS-contaminated soil) were found to contain significant bacterial populations (Table 1). These populations represent a range of microorganisms adapted to the presence of COTBS. This is not surprising as previous research had shown that when weathered petroleum crude oil and engine oil were used as the main carbon sources, a high number of hydrocarbonoclastic bacteria were isolated ($4.8 - 5.2 \times 10^6$ CFU g$^{-1}$) from contaminated soil (Jain et al. 2010). However, this may not always be the case, as some other investigations have reported the recovery of far lower numbers of hydrocarbonoclastic bacteria ($10^5 - 10^6$ CFU g$^{-1}$ and $5 \times 10^5$ CFU g$^{-1}$) from hydrocarbon-contaminated soil (Roy et al. 2002; Kadali et al. 2012). In this case, the reduction in bacterial population (pristine or contaminated) could have been due
to the depletion of nutrients (nitrogen) and the presence of other contaminants such as heavy metals (Vézie et al. 2002; Nwuche and Ugoji 2008).

The selective isolation strategy used in this study resulted in the isolation of 49 different phenotypes of bacterial species from COTBS and COTBS-contaminated soils (treated and untreated). The use of indigenous strains of hydrocarbonoclastic organisms from COTBS contaminants for treating COTBS-contaminated environments is important, as these isolates are likely to be more efficient degraders of the hydrocarbons present than exogenous strains. This improved efficiency is probably due to their prior adaptation to the presence of contaminants (Li et al. 2002). This is especially important in environments such as those found in Libya which tend to have environmental characteristics that lie beyond those in which commercially available strains are adapted to. Such characteristics include a wide temperature variability (10–35 °C) and low moisture content in summer (<6 %). Research works based on the application of exogenous non-indigenous microbes to contaminated soils have shown variable results regarding the effectiveness of the treatment. These results ranged from having no observed additional beneficial effect on hydrocarbon removal (Makadia et al. 2011; Sheppard et al. 2011) to small increases in remediation efficiency (Xu et al. 2013; Alcer et al. 2011).

Interestingly in this study, historically treated COTBS-contaminated soil was the dominant source of most isolates (21 isolates (42.9 %)), followed by 15 isolates from COTBS-contaminated soils (30.6 %) and 13 isolates from COTBS (26.5 %) (Table 1). To the authors’ knowledge, this is the first report of the successful isolation of a greater number of hydrocarbonoclastic bacteria from treated COTBS-contaminated soils compared with COTBS and untreated COTBS-contaminated environment. The differences in the type and number of isolates obtained from COTBS and test soil samples may be related to the hydrocarbon profiles of the sludge (Fig. 1; Supplementary Fig. 1), history of soil use and environmental factors. In contrast, culture-based assay of the clean Libyan agriculture (control) soil did not result in the isolation of any hydrocarbonoclastic strain. This may not always be the case as previous work has shown that hydrocarbonoclastic isolates can be detected in pristine soil samples (Margesin et al. 2003). Some scientific investigations have suggested that the availability of readily degradable carbon sources other than petroleum hydrocarbon in the soil could prevent the growth of hydrocarbonoclastic bacteria (Brito et al. 2009). In addition, the specific growth media used may significantly affect the recovery of hydrocarbonoclastic bacteria (Haack et al. 1995; Jones 1970). These factors could have been responsible for the lack of culturable hydrocarbonoclastic isolates in the control soils.

In order to assess the degradative potential of hydrocarbonoclastic bacterial isolates, five different hydrocarbons present in the extracted oil from COTBS-contaminated soil and Hamada crude oil were selected for use on Biolog plates. Analysis of the obtained Biolog data of the growth of the individual bacterial isolates confirmed the relevance of a selected isolation technique. All 49 isolates were able to grow on weathered Hamada crude oil. The increased substrate degradability shown by bacterial isolates, as assessed by the rate of colour formation, may have occurred as a result of enzymatic activities, unique cell wall structure or biological properties (Martinková et al. 2009). Although no flask-based hydrocarbon degradation assays were carried out in this study,

Table 2 Identities of selected bacteria species isolated from COTBS, treated COTBS-contaminated soil and COTBS-contaminated soil

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Origin</th>
<th>Phylum/class</th>
<th>Identification</th>
<th>% Similarity</th>
<th>Accession no.</th>
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<td>4 M14</td>
<td>Treated soil</td>
<td>Gammaproteobacteria</td>
<td>Pseudomonas xanithorina</td>
<td>96</td>
<td>HG008728.1</td>
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<tr>
<td>4 T17</td>
<td>Treated soil</td>
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<td>Pseudomonas sp.</td>
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<td>AB733546.1</td>
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<tr>
<td>1 T5</td>
<td>COTBS</td>
<td>Firmicutes</td>
<td>Bacillus nasae strain</td>
<td>88</td>
<td>KP015515.1</td>
</tr>
<tr>
<td>4 M12</td>
<td>COTBS</td>
<td>Gammaproteobacteria</td>
<td>Pseudomonas sp.</td>
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<td>FJ662885.1</td>
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<td>1B16A</td>
<td>Treated soil</td>
<td>Actinobacteria</td>
<td>Arthrobacter nitrogojacilicus</td>
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<td>JX483740.1</td>
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<tr>
<td>1B25</td>
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<td>Bevrandimonas nasae strain</td>
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<td>EU741090.1</td>
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<tr>
<td>1B14</td>
<td>Contaminated soil</td>
<td>Firmicutes</td>
<td>Bacillus aerias strain</td>
<td>90</td>
<td>KC443115.1</td>
</tr>
</tbody>
</table>
the growth of bacterial cultures in microplate wells supplemented with desired hydrocarbon (as the sole carbon source) was indicative of the abilities of these cultures to degrade the supplied hydrocarbon.

The use of historically treated COTBS-contaminated soil as a source of hydrocarbonoclastic organisms resulted in the isolation of 21 indigenous bacterial isolates (Table 1). Sixteen of these isolates were able to degrade representative substrates of aromatic and aliphatic compounds along with the petroleum mixture (crude oil). In contrast, isolates from long-term (>3 years) contaminated soil and COTBS showed less activity, with only four and fifteen isolates capable of utilizing representative aliphatic and aromatic substrates, respectively. A number of studies have reported the successful isolation of highly degradative bacteria (with between 64.3 and 100 % contaminant degradation) from COTBS-contaminated sites (Gallego et al. 2007; Hamamura et al. 2013; Jacques et al. 2008; Verma et al. 2006). In this study, the highest utilization rate occurred with Hamada crude oil (100 % growth; all isolates), followed by growth of 90 and 51 % of the isolates on representative aromatic fractions and aliphatic fractions, respectively (Fig. 2). However, although more isolates were able to utilize representative PAH substrates (phenanthrene and naphthalene) than representative aliphatics, the extent of their utilization was lower when compared to specific aliphatic compounds such as eicosane and hexadecane (Fig. 3). Kubota et al. (2008) and Chaerun et al. (2004) also observed that amongst the hydrocarbons, alkanes were the most degradable fractions while PAHs were the most recalcitrant fractions.

HCA represents a promising and widely used tool for differentiating individuals groups of data (Kaufman and Rousseuw 2009; Maioli et al. 2010; Kniggendorf et al. 2011). In this study, the 49 bacterial isolates were classified into two different clusters (Fig. 4) based on the utilization pattern of the six different hydrocarbon substrates. Cluster 2 isolates showed substantially higher utilization performance (45 to 84 % higher) (data not shown) than cluster 1 isolates. The seven bacterial isolates in cluster 2 were identified and classified into four groups. The dominant bacterial species were Pseudomonas sp. Pseudomonas sp. have been shown to be capable of degrading a wide range of hydrocarbon substrates including crude oil, refined fuels, alkanes and PAHs (Kumar et al. 2008; Arun et al. 2008; Sopeña et al. 2013). Two bacterial species represented in cluster 2 were identified as Bacillus nasudae and Bacillus aerius. The growth of Bacillus strains in soil contaminated with petroleum-derived compounds with high utilization activities has been reported in situ long-term bioremediation studies (Benedek et al. 2013). Another study found that two native Bacillus strains showed significant potential for the in situ remediation of soil contaminated with low concentrations (500 ppm) of diesel, whilst also degrading 87 % of diesel fuel in soil contaminated with higher concentration (10,000 ppm) (Kebrina et al. 2009).

Amongst the bacterial strains isolated in this study, A. nitrosoaceticus was isolated and identified. Arthrobacter are well known for their hydrocarbonoclastic activities on PAHs with some Arthrobacter strains reported showing a high degradation efficiency for phenanthrene (Peng et al. 2008). Another isolate was putatively identified as Brevundimonas nasudae; this species has not been previously isolated from oil-contaminated materials nor has been reported as hydrocarbonoclastic, although members of the genus are known to be hydrocarbonoclastic. However, Brevundimonas nasudae has previously been isolated from soil contaminated with malathion (MLT) (C10H10O6S2P), an organophosphorus insecticide, where it exhibited very high degradative capabilities (87.32 %) in medium containing 20 mg l⁻¹ MLT after 48 h (Zhao et al. 2011).

Conclusion

The purpose of this study was to investigate the availability of hydrocarbonoclastic bacteria in Libyan COTBS and soils contaminated with COTBS and to test the degradability of selected hydrocarbon substrates by these isolates. A rich indigenous hydrocarbonoclastic community from treated COTBS-contaminated soil, COTBS and soil contaminated with COTBS was consequently identified using BH-Hamada crude oil agar plates. MT2 Biolog plates were successfully used to assess the abilities of these isolates to utilize selected substrates with this method representing a rapid and economic way of further screening these hydrocarbonoclastic bacteria. Cluster analysis was also successfully used to differentiate the most active degraders or utilizing of selected hydrocarbon substrates. This is the first report of the isolation of hydrocarbonoclastic bacteria from Libyan soils contaminated with COTBS with these bacteria having significant potential for use in the bioremediation of soil contaminated with COTBS.

Acknowledgments This work was supported by the Libyan Ministry of Higher Education and Science Research. The authors thank the Environmental and Natural Resources Engineering, Azzawiya University, Libya, the management of Azzawiya oil refinery, Libya, and brothers Ali Mansour, Mohammad Ahfeda and Nassraden Souf for supplying soil and COTBS samples.

References


Aksel S, Adetunnu EM, Makadia TH, Patil S, Ball AS (2011) Harnessing the hydrocarbon-degrading potential of contaminated soils for the
Bioremediation of Libyan Soil Contaminated With Crude Oil Tank Bottom Sludge

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Environ Sci Pollut Res

bioremediation of waste engine oil. Water Air Soil Pollut 218:121–130

© Springer
Pseudomonas xanthomarina isolated from an aged contaminated soil. CLEAN-Soil Air Water. doi:10.1002/clen.201300247
Chapter 5

STATEMENT OF AUTHORSHIP (Chapter Five)
School of Applied Science

Chapter Declaration for Thesis with Publications

Chapter [5] is represented by the following paper: [Assessment of the Hydrocarbon Degrading Abilities of Three Bioaugmentation Agents for the Bioremediation of Crude Oil Tank Bottom Sludge Contaminated Libyan Soil]

Author List: Abdulatif A. Mansur, Eric M. Adetutu1, Tanvi Makadia1, Paul D. Morrison1, Andrew S. Ball.

Journal name: International Journal of Environmental Bioremediation & Biodegradation

Year published: 2015

Declaration by candidate

I declare that I wrote the initial draft of this manuscript, and my overall contribution to this paper is detailed below:

<table>
<thead>
<tr>
<th>Nature of contribution</th>
<th>Extent of contribution (%)</th>
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</thead>
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<tr>
<td>wrote the first draft, collected all of the data, conducted most of the analysis</td>
<td>70</td>
</tr>
</tbody>
</table>

The following co-authors contributed to the work. The undersigned declare that the contributions of the candidate and co-authors are correctly attributed below.

<table>
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<tr>
<td>Eric M. Adetutu</td>
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<td>5</td>
<td></td>
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<tr>
<td>Tanvi Makadia1</td>
<td>contributed to manuscript evaluation</td>
<td>5</td>
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<tr>
<td>Paul D. Morrison</td>
<td>contributed to manuscript evaluation</td>
<td>5</td>
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<tr>
<td>Andrew S. Ball</td>
<td>contributed in the planning of the experimental design, supervision of research, manuscript preparation and evaluation</td>
<td>10</td>
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Candidate’s Signature                                      Date
                                                        28/11/2015

Primary Supervisor’s Signature                             Date
                                                        28/11/2015
5. Abstract

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Assessment of the Hydrocarbon Degrading Abilities of Three Bioaugmentation Agents for the Bioremediation of Crude Oil Tank Bottom Sludge Contaminated Libyan Soil

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Abstract Bioremediation is a widely used environmental friendly treatment method for petrogenic hydrocarbon contaminated soils but its application to the treatment of crude oil tank bottom sludge (COTBS) contaminated soil is limited especially in Mediterranean countries such as Libya. Therefore in this study, the hydrocarbon degrading abilities of three bioaugmentation agents Pseudomonas sp (4M12), Pseudomonas xanthomarina (4M14) and Arthrobacter nitroguajacolicus (1B16A) (isolated from COTBS polluted soils) applied as part of a biostimulation-bioaugmentation (BS/BA) strategy were assessed in COTBS contaminated Libyan soils. Biostimulated (BS) and natural attenuation (NA) microcosms were also set up for comparative purposes. Gas chromatograph mass spectrometer (GC-MS) analysis revealed a total soil petroleum hydrocarbon (TPH) and polycyclic aromatic hydrocarbon (PAHs) content of 30,703 mg kg⁻¹ and 13,816 mg kg⁻¹ respectively. Two carcinogenic fractions (naphthalene and benzenamine, 4, 4 methylenbis [2-methyl-]) and 4 mutagenic fractions (pyrene, phenanthrene, fluorene and anthracene) were detected. Substantial PAH degradation occurred in 4M14 and 4M12 samples within 15 days in contrast to up to 23 days in 1B16A, NA. However, substantial reduction in TPH (> 97%) was only observed in 4M12 and 4M14 inoculated microcosms within 15 days compared to 25-30 days in 1B16A inoculated, BS and NA microcosms. 4M14 inoculated microcosms were most efficient at complete removal (D100) of all carcinogenic and mutagenic fractions; 4M14 (9-10 days), 4M12 (9-15 days), 1B16A (15-23 days), BS (18-21 days) and NA (18-22 days). Pseudomonas xanthomarina was therefore shown as the best candidate for use in a BS/BA approach for treating COTBS contaminated Libyan soils. This study shows the importance of pre-screening bioaugmentation agents for the removal of carcinogenic and mutagenic fractions prior to use; in order to carry out safe, efficient and sustainable COTBS bioremediation in Libya.

Keywords: Crude oil tank bottom sludge (COTBS), slurry phase bioremediation, biostimulation, bioaugmentation, gas chromatograph mass spectrometer (GC-MS)


1. Introduction

Global oil production in 2013 was estimated at 75.6 M bbl/day [1]. At oil refineries, crude oil is stored in large storage tanks pending processing. During this time, crude oil tank bottom sludge (COTBS) is generated as a result of the settlement of suspended solids; millions of tonnes of sludge (COTBS) are generated annually worldwide [2,3]. Apart from labour costs and waste management issues associated with COTBS removal, its presence in holding tanks reduces the oil storing capacity of tanks with the increasing possibility of tank corrosion. Periodical removal of sludge therefore becomes necessary [4].

COTBS contains a mixture of hydrocarbon fractions ranging from alkanes to polycyclic aromatic hydrocarbons (PAHs), [5] and heavy molecular weight hydrocarbons (HMWHCs) above C₂₀ [6]. Some fractions such as PAHs (a significant component of COTBS [7]) have carcinogenic and mutagenic characteristics to living organisms [8]. Detoxification of COTBS contaminated soil is therefore essential prior to disposal or re-use.

Biological treatment or bioremediation is a well-established and widely used treatment method of hydrocarbon and COTBS contaminated soils [9,10,11,12]. This is because of its efficient, cost effective and
environmental friendly nature compared to chemical and physical treatment approaches. The mechanisms and methods involved in the bioremediation of different hydrocarbon polluted environments are well reviewed [13,14]. One commonly used bioremediation method involves the use of a combination of biostimulation (nutrient addition) and bioaugmentation (addition of hydrocarbon degrading microorganisms) strategies. This approach has been successfully used to treat hydrocarbon contaminated soils in solid and slurry phases, with slurry phase bioremediation providing faster and greater hydrocarbon degradation [10,15,16].

The focus of most bioremediation studies is on how effective a chosen treatment strategy is in reducing contaminant concentration to legislated disposal thresholds within a stipulated time frame and at minimal costs. Although, the efficiency of this process is affected by a variety of well-studied soil and environmental factors such as temperature, moisture content, nutrients, pollutant type and bioavailability, the available microbial capacity is crucial to successful bioremediation [17-21]. However, there are limited reports on the comparative hydrocarbon removal efficiencies of hydrocarbonoclastic isolates in most studies involving the use of a biostimulation-bioaugmentation strategy.

Selection of bioaugmentation agents is based on (i) endpoint hydrocarbon degradation values, with microorganisms with lower endpoint values being selected and (ii) the ability of the selected strains for fast multiplication and growth rate within the treatment conditions [17]. While hydrocarbonoclastic microorganisms are able to degrade hydrocarbons, the types of hydrocarbon degraded and rate of removal may vary [22]. There is limited information available on when a particular fraction is removed by a microbial isolate and whether this knowledge could be important in the selection of organisms to be used for bioaugmentation. For example, given that COTBS contains carcinogenic and mutagenic fractions such as PAH [23], selecting bioaugmentation agents that are able to remove these fractions faster would be beneficial to the bioremediation process in terms of process efficiency and reduction in health risks to practitioners.

In most Mediterranean countries such as Libya, bioremediation is poorly practised despite the occurrence of significant amount of soil pollution by COTBS and petrogenic hydrocarbons. This study is part of a larger study designed to isolate, screen and utilize hydrocarbonoclastic isolates from COTBS for large scale ex situ remediation of COTBS contaminated soils. Successful bioremediation outcome is expected to accelerate the acceptance and utilization of this technology for the treatment of contaminated environments in Mediterranean countries. Earlier work on a Libyan soil contaminated with COTBS had led to the isolation of 49 hydrocarbonoclastic bacterial isolates of which seven isolates were selected for further evaluations based on their high hydrocarbon degrading properties [24]. Out of these seven isolates, 3 were selected as the best COTBS degraders for use in this study.

This study therefore aims to evaluate and compare the efficiencies of these isolates in degrading the Hamada crude oil contaminants in soil samples in order to select the best isolates for use in a biostimulation-bioremediation strategy. Using chemical assays, this study will determine (i) the carcinogenic and mutagenic fractions that were degraded in microcosms inoculated with selected isolates (ii) when these fractions were degraded and (iii) the endpoint values for TPH and PAH in microcosms set-up with selected isolates in laboratory based slurry-phase experiments. Changes in the microbial community are also assessed using a PCR-DGGE approach.

2. Materials and Methods

2.1. Soil Samples

COTBS contaminated soil samples were collected from Azzawiya oil refinery in Libya. Samples (5 kg) were collected from the top 0-30 cm of contaminated soil stockpiles, sieved and stored in zip lock plastic bags. The samples were transported to RMIT University in Melbourne, Australia, coded and stored in a Quarantine facility.

2.2. Physico-chemical Characterizations

The soil texture, moisture content and pH of the soil samples were determined using standard methods [25]. The water-holding capacity was determined as described by [26]. The concentrations of C and N were measured by a model LECO TruMac CNS analyser following the manufacturer’s instructions. This involved sample combustion at 1250°C with infrared detection being used to detect the total carbon and a thermal conductivity cell for total nitrogen detection [27]. Phosphorus concentration was analysed using a Perkin Elmer Optima 7000 DV ICP-OSE (SSEL lab Thornleigh, NSW, Australia) as per US EPA methods (3050B) after acid digestion of the contaminated soils at 60°C. Metals (potassium and Calcium) concentrations were analysed using flame atomic absorption spectrophotometer (Varian Model Spectra AA 220) as per the manufacturer’s protocol. Briefly, soil samples were digested in test tubes at 60°C in a hot block with 5 ml HCl (70-75% concentration) and 5 ml hydrogen peroxide (30% concentration). The samples were then kept at 120°C for 150 min before being analysed as per the manufacturer’s suggested conditions [28].

2.3. Preparing Bacterial Isolates for Microcosms

Three bacterial isolates previously isolated and identified in a previous study [24] (Table 1) were selected for use based on their hydrocarbon degrading properties. These isolates (designated as 4M14, 4M12 and IB16A) were obtained from stocks of pure cultures maintained at 80°C in 50% glycerol and streaked individually on nutrient agar plates (Acumedia, Lansing, Michigan 48912) (26 g / l adjusted to pH 7.4). The streaked plates were incubated at 30°C for up to 3 d after which they were inoculated into nutrient broth (30 ml; Acumedia, Lansing, Michigan 48912). Nutrient broth incubation was at 30°C for 48 h at 120 rev min⁻¹. Cultures of OD₆₀₀ 0.689-1.656 were harvested by centrifugation at 4°C at 16,000 × g for 2 min and washed twice with sterile distilled water [23]. The pellets were then re-suspended individually in
Bioremediation of Libyan Soil Contaminated With Crude Oil Tank Bottom Sludge

Abdulatif Mansur

Table 1. Carcinogenic and mutagenic fractions detected in COTBS contaminated soil

<table>
<thead>
<tr>
<th>Hydrocarbon fraction</th>
<th>Effect on living organisms</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene**</td>
<td>Possibly carcinogenic to humans</td>
<td>(Lerda, 2010) (IARS, EPA and EU classifications)</td>
</tr>
<tr>
<td>Benzenamine.4,4'-methylenbis[2-methyl-]*a</td>
<td>Carcinogenic in rats</td>
<td>(Gini et al., 1999, Haiman et al., 1987)</td>
</tr>
<tr>
<td>Pyrene*b</td>
<td>Mutagenic but in other forms such as Diphenyl[a]pyrene</td>
<td>(Oostingh et al., 2008, Lerda, 2010, Pereló, 2010)</td>
</tr>
<tr>
<td>Phenanthrene*b</td>
<td>Mutagenic to humans (could destroy the immune system)</td>
<td>(Oostingh et al., 2008, Pereló, 2010)</td>
</tr>
<tr>
<td>Fluoranthene*b</td>
<td>Mutagenic to humans (harmful effects of fluoride intoxication on the living tissues)</td>
<td>(Oostingh et al., 2008, Pereló, 2010, Nabavi et al., 2012)</td>
</tr>
<tr>
<td>Anthracene*b</td>
<td>Mutagenic but in forms of 7,12-Dimethylbenz [a] anthracene (DMBA) is carcinogenic to humans</td>
<td>(Yardum et al., 2010, Pereló, 2010)</td>
</tr>
</tbody>
</table>

2.4. Preparation of Slurries and Strain Inoculation

Slurry phase experiments for assessing the biodegradation of COTBS in contaminated soil were carried out in 250 ml flasks. The slurries were prepared in triplicate by adding 30 g of COTBS contaminated soil to 150 ml of autoclaved deionized water to obtain a 1:5 soil: solution ratio [29]. During slurry preparation, nutrients (NH₄)₂SO₄ (0.43 g), K₂HPO₄ (0.05 g) and KCl (0.04 g) were added to achieve a C:N:P molar ratio of around 100:10:1 [29] to enhance bacterial growth. An aliquot (5 ml) of each individual bacterial isolate was inoculated with an initial inoculum size of 1 x 10⁴ cell ml⁻¹ [30,31]. Inoculated flasks were maintained on shaker incubators [32] with continuous rotation of 150 rpm for 35 d at 30°C. To prevent photo-degradation, experiments were carried out in the dark [32]. Three types of microcosms were incubated; (i) COTBS contaminated soil plus water plus nutrients and individual isolates (BS/BA), (ii) COTBS contaminated soil plus water plus nutrients (BS), (iii) COTBS contaminated soil plus water (NA). Sampling was carried every 5 days from each flask in order to assess the changes in total petroleum hydrocarbon (TPH) concentration and soil bacterial community.

2.5. Hydrocarbon Extraction

Oil was extracted in triplicate samples by solvent extraction (dichloromethane (DCM)) and air-dried using a previously described protocol [33] with some modifications. Briefly, triplicate slurry samples were air dried overnight at room temperature [31]. Dried samples were placed in a Teflon coated centrifuge tubes (25 ml) and (DCM) (3 ml) was added (1:3 soil: solvent ratio). The oil in this mixture was extracted by agitation (130 rev min⁻¹) for 20 min, followed by centrifugation at 5000 rev min⁻¹ for 5 min. The supernatants were removed into glass bottles (15 ml) and concentrated by solvent evaporation overnight at room temperature [32,33]. The extracted oils were concentrated to 1 ml in DCM [34] and placed in chromatographic vial (2 ml) (Adel Lab Scientific, The Barton, SA, Australia) for further analysis.

2.6. Determination of Hydrocarbon Concentration

For the analysis of Total Petroleum Hydrocarbons including aromatic and aliphatic compounds concentrations, a combined paraffins, isoparaffins, aromatic, naphthalenes and olefins (PIANO) PIANO-5-Piano (DHA) standard combined set (2 ml ampule) (Spectrum Quality Standards, Ltd. Sugarland, TX, USA) was used. The hydrocarbon content of the extracted oil was analysed as described in ASTM D5154 using gas chromatograph mass spectrometer (GC-MS) equipped with an autosampler (Agilent 6890 GC and Leco Pegasus III TOF-MS). Samples were injected and separated on a capillary column Agilent DB-5MS (60 m by 0.25 mm with 0.25 µm film thickness). The injection temperature and volume was 225°C and 0.2 µl respectively. Helium (1.8 ml min⁻¹) was used as a carrier gas at a constant flow rate. The concentration of each hydrocarbon fraction was analyzed and the total peak area of each fraction was compared to the peak area of each fraction in the PIANO standard curve [31].

2.7. DNA Extraction and PCR

DNA extraction was carried out in triplicate slurry samples from days 0, 5, 10, 15, 20, 25, 30 and 35 using a MO BIO Power Soil DNA isolation kit (MO BIO Laboratories, Inc, USA). One ml from each slurry sample was added to the PowerBead tubes and gently vortexed to mix. Solution C1 (60 µl) was added and the tube inverted several times. The tubes were centrifuged at 10,000 x g for 30 s at room temperature after which the supernatants were transferred into clean collection tubes (2 ml). Further processing was carried out as per the manufacturer’s protocol. DNA was also extracted from the bacteria isolates as described in [35].

The polymerase chain reactions (PCR) of DNA extracts from slurry samples and isolates were conducted using a 50 µl PCR master mix. The reactions were prepared using 2 µl bacterial primers 341 FGC (10 µM) and 518R (10 µM) [36,37], 3 µl MgCl₂ (25 mM), 1 µl dNTP mixture (10 mM), 10 µl of GoTaq flexi buffer (5x), 0.25 µl of l'ap polymerase enzyme (5U/µl) and 29.75 µl of sterile nuclease-free water per reaction. DNA extract (2 µl) was added to 48 µl of master mix. The thermocycling conditions were; an initial denaturing step at 95°C for 5 min, then 92°C for 30 s, 55°C for 30 s and 72°C for 1 min (25 cycles) and a final extension step at 72°C for 10 min.

2.8. Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

DGGE was performed on PCR amplicons using a Universal Mutation Detection System D-code apparatus (Bio-Rad, CA, USA) as per the manufacturer’s protocol. The PCR products were loaded onto 9% (w/v) polyacrylamide gel with a denaturing gradient of 45% to 0%
65% and ran for 18 h at 60°C and 60 V. PCR amplicons of the three isolates were loaded individually on single lanes and also mixed together and loaded as DGGE ladders. The DGGE gel was silver stained [38]. Briefly, DGGE gel was then incubated for 2 h in 200 ml of fixing solution (40% ethanol (100%), 10% acetic acid, v/v) before being incubated in 200 ml of silver nitrate solution (0.2 g silver nitrate in 200 ml of H2O2, v/v) for 20 min. The gel was then held for 10-30 min in 200 ml of developing solution (0.02 g sodium borohydride, 0.8 ml formaldehyde, 3 g sodium hydroxide in 200 ml of H2O2, v/v) followed by incubation in 200 ml of fixing solution (10% ethanol, 5% acetic acid, v/v) for 10 min. Finally, the gel was soaked in 200 ml preservative solution (125 ml of absolute ethanol, 50 ml glycercol in 325 ml of H2O2, v/v) for 10 min. DGGE gels were scanned with an Epson Expression 1600 V.2.65 E software [39] and digital images saved as tiff files.

2.9. Statistical Analysis
Digitized DGGE image was analysed with the similarity relationship between the different communities analysed by the use of Unweighted Pair Group with Arithmetic Means (UPGMA). Statistical analyses were carried out using SPSS 20 using T Tests Significance was taken at (P ≤ 0.05).

3. Results and Discussion
3.1. Physico-chemical Analysis
The contaminated soil used in this study was sandy loam in nature with a pH value of 6.8 (Table 2). TPH and PAH concentrations in this contaminated soil were 30,703 mg kg-1 and 13,816 mg kg-1 respectively. The results of heavy metal and nutrient assays are presented in Table 2.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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<tbody>
<tr>
<td>Moisture content (%)</td>
<td>7.02 ± 0.2</td>
</tr>
<tr>
<td>Soil texture</td>
<td>sandy loam</td>
</tr>
<tr>
<td>TPH (mg kg)</td>
<td>30,703 ± 101</td>
</tr>
<tr>
<td>PAHs (mg kg)</td>
<td>13,816 ± 88</td>
</tr>
<tr>
<td>K (mg kg)</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td>Cu (mg kg)</td>
<td>4.45 ± 0.04</td>
</tr>
<tr>
<td>Total N (% in 100 g)</td>
<td>0.035 ± 0.0</td>
</tr>
<tr>
<td>Total P (mg kg)</td>
<td>167 ± 0.0</td>
</tr>
<tr>
<td>TOC (% in 100 g)</td>
<td>10.39 ± 0.3</td>
</tr>
</tbody>
</table>

3.2. Hydrocarbonoclastic Bacteria
The identities of the three hydrocarbonoclastic bacteria had been determined in earlier studies [24]. The isolates were selected depending on the highest hydrocarbon degradation potential. Sequence analysis was used to identify 4M14 as Pseudomonas xanthomarina, 4M12 as Pseudomonas sp and IB16A as Arthrobacter nitroguajaciolicus. The source, phyla, similarity and accession numbers of these isolates are presented in Table 3.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Origin</th>
<th>Phylum</th>
<th>Identification</th>
<th>% similarity</th>
<th>Accession no</th>
</tr>
</thead>
<tbody>
<tr>
<td>4M14</td>
<td>Treated</td>
<td>Gamma Proteobacteria</td>
<td>Pseudomonas xanthomarina</td>
<td>96</td>
<td>HG088728.1</td>
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<tr>
<td>4M12</td>
<td>COTBS</td>
<td>Gamma Proteobacteria</td>
<td>Pseudomonas sp</td>
<td>96</td>
<td>JF662885.1</td>
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<tr>
<td>IB16A</td>
<td>Treated</td>
<td>Actinobacteria</td>
<td>Arthrobacter nitroguajaciolicus</td>
<td>98</td>
<td>JX33740.1</td>
</tr>
</tbody>
</table>

3.3. Hydrocarbon Degradation (TPH and PAH)

![Figure 1](image)

**Figure 1:** Reduction of TPH concentration by 3 individual bacterial isolates and NA and BS over 30 d. Diamond (4M12), box (IB16A), triangle (4M14), plus (BS) and multiplication sign (NA)

Note: The values of the standard errors are very low. Therefore the error bars are short and some of them are invisible.

Analysis of the contaminant concentration throughout the experimental period showed that after 30 d, the TPH concentration in the slurry phase (BS, BA and BS) naturally attenuated microcosms had decreased significantly (Figure 1) (P < 0.05). However, highest TPH reduction occurred within the first 15 days in microcosms with hydrocarbonoclastic isolate 4M14 followed by 4M12 and IB16A than in the BS and NA, samples, with NA samples having the lowest reduction rate. For example in soil amended with 4M14 and 4M12, there was a substantial decrease in soil TPH concentration within 15 days from 30,703 mg kg-1 to 170 - 664 mg kg-1 (97.8 to 99.4%). The rate of TPH decrease was not significantly different in the Pseudomonas (4M14 & 4M12) inoculated samples (P > 0.05), with TPH degradation of up to 99.4% occurring by day 15. In contrast, and despite an initial rapid reduction (0-10 d) TPH reduction in samples inoculated with IB16A, BS and NA samples were slower, taking an extra 10-15 days (day 25-30) for the TPH concentration to be substantially reduced to 664 mg kg-1 (97.8%) and 936 mg kg-1 (96.9%) respectively (Figure 1).
Their TPH reduction rate after 10 days was significantly lower compared to TPH reduction with Pseudomonas (4M14 & 4M12). Overall, substantial hydrocarbon removal occurred in all microcosms by the end of the experimental period (Figure 2a and Figure 2b). Hydrocarbon degradation is thought to be enhanced in slurry phase bioremediation because the elevated moisture content used results in greater solubilisation and bio-availability of contaminants and nutrients to hydrocarbon degrading bacteria [10,16,41].

*Figure 2a.* Chromatogram of soil extract obtained from COTBS contaminated soil before treatment with some selected peaks identified. S1 2,6,10-trimethyldecane, S2 2,6,10,14-tetramethylpentadecane, S3 2,6,10,14-tetramethylhexadecane, S4 cyclic octa-atomic sulphur. GC-MS chromatograms of extracts from treated COTBS-contaminated soil

*Figure 2b.* Chromatogram of soil extract obtained from COTBS contaminated soil after treatment with some selected peaks identified. S1 2,6,10-trimethyldecane, S2 2,6,10,14-tetramethylpentadecane, S3 2,6,10,14-tetramethylhexadecane, S4 cyclic octa-atomic sulphur. GC-MS chromatograms of extracts from treated COTBS-contaminated soil
TPH assay results suggest that *Pseudomonas xanthomarina* (4M14) and *Pseudomonas* sp (4M12) were the best isolates for TPH removal. The substantial reduction observed was likely due to the presence of these isolates at high concentration (via bioaugmentation) given that it took an extra 15 days for the nutrients only microcosms to reach a similar level of TPH reduction. The genus pseudomonas is known for the presence of hydrocarbonoclastic capabilities. Pseudomonads have been widely studied and isolated from different environments worldwide and are known for their capabilities of utilizing hydrocarbons as source of carbon and energy [42,43,44]. *Pseudomonas* sp have been successfully used to treat hydrocarbon contaminated sites. [45] studied the efficiency of *Pseudomonas* for the biodegradation of hydrocarbon contaminated soil in north east of India and found *Pseudomonas* has the ability to reduce 75% of TPH. In addition, [46] used a *Pseudomonas* strain in the degradation of n-alkanes in diesel oil and concluded that this strain was able to reduce 51% of the TPH.

In addition, the accelerated removal of hydrocarbon in 4M14 and 4M12 inoculated microcosms shows the beneficial effects of bioaugmentation-biotostimulation over biostimulation alone or natural attenuation. Contaminated soil may sometimes lack a sufficient population of hydrocarbon degraders or be deficient in nutrients (N and P). Inoculating the hydrocarbon contaminated soil with indigenous hydrocarbon degrading isolates (BA) and providing the required nutrients (BS) can lead to substantial TPH reduction in contaminated soil. [47] reported that the adapted indigenous microbial isolates was responsible for better reduction of TPH (41.3% increase) and therefore increased biodegradation efficiency. According to [48], applying BS-BA together generally increases the degradation rate of the TPH by 63-84% compared to NA.

![Figure 3. Reduction of PAH concentration by 3 individual bacterial isolates and NA and BS over 50 d. Diamond (4M14), circle (IB16A), asterisk (NA), box (4M12) and triangle (BS).](image)

Note: The values of the standard errors are very low. Therefore the error bars are short and some of them are invisible.

Figure 3 shows the degradation of PAH in different slurry phase microcosms inoculated with *Pseudomonas xanthomarina* (4M14), *Pseudomonas* sp (4M12), *Arthrobacter nitroguajacolicus* (IB16A) and nutrients (BS/BA), nutrients only (BS) and controls (without any bioaugmentation or nutrients (NA)). All the microcosms inoculated with 4M14, 4M12 and nutrients only showed a substantial decrease in PAH concentration from 13,816 mg kg⁻¹ to 0 mg kg⁻¹ or below detection limit (98.8% - 100%) within 5-10 days. However, the PAH concentration in the IB16A and NA microcosms showed 100% reduction of PAH only after 15 days. This indicated that these isolates and other indigenous microbial communities in the slurry were better degraders of the PAH than TPH. PAH degradation depends on a number of environmental conditions, type of microorganisms and nature and chemical structure of the PAH fractions being degraded. PAH can be biodegraded and transformed into less toxic and complex fractions by mineralization into inorganic minerals and generating H₂O, CO₂ in aerobic conditions or generating CH₄ through anaerobic conditions [49].

Compared to other bacterial degraders, *Pseudomonas* is unique in its ability to metabolize hydrocarbons (single fractions and mixtures) rapidly and efficiently even at high hydrocarbon concentrations [50,51]. *Pseudomonas* was tested for its ability of growth on a different of hydrocarbons including various PAHs, n-alkanes and complex hydrocarbon mixture [52] reported that the *Pseudomonas* sp was able to remove 72% of PAH from PAH contaminated soil. [53] isolated different PAH bacterial degraders from sediments of the polluted Amlakadi canal, Gujarat, India and found that amongst the isolates, *Pseudomonas* sp showed the highest potential for PAH degradation being able to degrade 2000 ppm PAH within 24 h.

### 3.4. Degradation of Carcinogenic and Mutagenic Compounds

Given that the focus of this study on the removal of mutagenic and carcinogenic fractions of COTBS contaminated samples, the extent of removal of selected mutagenic and carcinogenic fractions was examined and qualified with D50 (Removal of target fraction by 50%) and D100 (Complete removal of target fraction). Microcosms inoculated with *Pseudomonas xanthomarina* (4M14) were most efficient at the complete degradation (D100) of pyrene (9 days), phenanthrene (10 days), naphthalene (9 days) and benzenamine, 4,4 methylbenz[2-methyl] (10 days). Even though microcosms inoculated with 4M12 were equally effective at TPH and PAH removal as those inoculated with 4M14, their rate of removing carcinogenic and mutagenic fractions was slower. For example, an additional 5 days were needed for complete removal (D100) of pyrene (day 14), additional 3 days for 100% phenanthrene degradation (day 13), additional 2 (day 11) and 5 (day 15) days for naphthalene and benzenamine, 4,4 methylbenz[2-methyl] removal respectively. Microcosms inoculated with *Arthrobacter nitroguajacolicus* (IB16A) were the slowest in removal of the 4 carcinogenic and mutagenic fractions taking between 15 to 23 days for complete removal. Overall, complete degradation of selected mutagenic and carcinogenic fractions was accomplished between 9-10 days in 4M12 and 9-15 days 4M14 while it took approximately twice this time-frame (18-22 days) to achieve the complete removal of these compounds in BS microcosms and (18-22 days) in NA (Table 4).

It is important to note that although soil amended with the three selected isolates were able to completely degrade the hydrocarbon contaminants, they did so at different rates. For example, 4M12 and 4M14 had similar and
better TPH removal profile than 1B16A, BS and NA samples. This ordinarily would have meant the selection of either of the candidates for use in a bioaugmentation-biotostimulation bioremediation strategy. However, consideration of the type of carcinogenic and mutagenic fractions removed and time of removal showed that 4M14 was a better (faster) removal of these toxic fractions than 4M12. This demonstrates the benefit of carrying out these kinds of assays to assess both the time and type of removal toxic hydrocarbon fractions in isolates selected for potential use as bioaugmentation agents prior to the initiation of bioremediation.

<table>
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<tr>
<th>Strategies</th>
<th>Degradation</th>
<th>Pyrene*</th>
<th>Phenanthrene*</th>
<th>Naphthalene**</th>
<th>Benzenamine, 4,4’-methylenebis[2-methyl]-**</th>
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<td>Microcosm + 4M12</td>
<td>D50</td>
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<td>6</td>
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<tr>
<td>Microcosm + 1B16A</td>
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<td>16</td>
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<tr>
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<td>BS</td>
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3.5. Bacterial Community Profile

Culture independent DGGE based bacterial community profiling was used to assess changes in the different microcosms during the experimental period in order to evaluate the effect of bioaugmentation-biotostimulation strategy on the natural microbial community. Figure 4a showed that there was no difference in the bacterial communities in inoculated and control microcosms at any time point. Figure 4b showed the bioaugmentation agents compared to the community profile. Two of the bacterial isolates (4M12 and 4M14) had melting domains that were similar to the melting domains of some bands found on the community profile. Given that these isolates were originally from the same COTBS contaminated samples used for this assay, it is possible that the sequences of these community profile bands would be highly similar to those of the bioaugmentation bacteria. However, this will have to be confirmed by band excision, cloning and sequence analyses (which was not the focus of this study and consequently not carried out). The non-detection of 1B16A is unusual but it might be that this isolate was not in the dominant groups that were observable through DGGE (DGGE only shows the top 1-2% population) despite its obvious importance in hydrocarbon degradation. Overall, there was no detectable change or shift in bacterial community from day 0 to day 30 with the UPGMA dendrogram showing up to 100% similarity between day 0 and day 30 and between amended and control (unamended) microcosms. This showed that despite the complete degradation of the carcinogenic and mutagenic fractions, application of hydrocarbonoclastic isolates in a nutrient solution did not change the natural community. This is important because it shows that complete detoxification of COTBS contaminated soils can be achieved without any adverse effect on the natural microbial community. This is also important for sustainable bioremediation; a concept that involves achieving detoxification targets with minimal impact on the natural community while providing long-term protection of human health and the environment (Ellis and Hadley, 2009). The accelerated removal of carcinogens and mutagens (which will result in the protection of health of personnel involved in bioremediation operations) using indigenous microbial resources without any harmful effect on the community as observed in this study suggests that the utilized biostimulation-bioaugmentation is a sustainable approach.
4. Conclusion

Selection of agents for use in bioaugmentation studies is based on their endpoint TPH or PAH values in degradation studies with the removal of carcinogens and mutagens (type and time of removal) being overlooked. However, carcinogens and mutagens pose the greatest risk to human and environmental health and the earlier removal of these compounds would be beneficial to the bioremediation process. This study has demonstrated the benefit of additionally screening bioaugmentation agents for carcinogen and mutagen removal, in order to carry out safe, efficient and sustainable bioremediation. Comparing three hydrocarbonoclastic bacteria, *Pseudomonas xanthomarina* (4M14) was shown to be the most effective candidate for carcinogens and mutagens removal in a sustainable manner (− twice as fast compared to NA and BS samples) with minimal effects on the natural community. The application of this isolate in BS/BA approach resulted in timely and efficient removal of these hydrocarbon fractions and also demonstrates the benefit of using isolates from COTBS for treating COTBS contaminated Libyan soils.

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Reference


Bioremediation of Libyan Soil Contaminated With Crude Oil Tank Bottom Sludge

Abdulatif Mansur


Chapter 6

STATEMENT OF AUTHORSHIP (Chapter Six)
School of Applied Science

Chapter Declaration for Thesis with Publications

Chapter [6] is represented by the following paper: [An effective soil slurry bioremediation protocol for the treatment of Libyan soil contaminated with crude oil tank bottom sludge]

Author List: Abdulatif Mansur; Mohamed Taha, Esmaeil Shahsavari, Nagalakshmi Haleyur, Eric Adetutu, Andrew Ball

Journal name: International Biodeterioration & Biodegradation.

Submitted Year: 2015

Declaration by candidate

I declare that I wrote the initial draft of this manuscript, and my overall contribution to this paper is detailed below:

<table>
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<tr>
<th>Nature of contribution</th>
<th>Extent of contribution (%)</th>
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<tr>
<td>wrote the first draft, collected all of the data, conducted most of the analysis</td>
<td>70</td>
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The following co-authors contributed to the work. The undersigned declare that the contributions of the candidate and co-authors are correctly attributed below.

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<tr>
<td>Mohamed Taha</td>
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<td>Eric M. Adetutu</td>
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<tr>
<td>Andrew S. Ball</td>
<td>contributed in the planning of the experimental design, supervision of research, manuscript preparation and evaluation</td>
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Candidate’s Signature

Date
28/11/2015

Primary Supervisor’s Signature

Date
28/11/2015
An effective soil slurry bioremediation protocol for the treatment of Libyan soil contaminated with crude oil tank bottom sludge

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An effective soil slurry bioremediation protocol for the treatment of Libyan soil contaminated with crude oil tank bottom sludge

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Abstract

Petroleum hydrocarbons are the most widespread contaminants in the environment. Soil contamination with crude oil tank bottom sludge (COTBS) represents a significant risk to both human and environmental health, and current traditional approaches only partially resolve the issue as they are inefficient, not environmentally friendly and left behind unwanted products and expensive; hence there is an urgent need, especially in developing countries to develop an environmentally friendly, cost effective solution to this issue. The aim of this study to develop a slurry phase bioremediation protocol for the treatment of Libyan soil contaminated with COTBS at a minimum soil: water ratio using indigenous bacterial isolates. In this study, two hydrocarbonoclastic bacterial isolates, *Pseudomonas sp* (4M12) and *Pseudomonas xanthomarina* (4M14), were used in three different strategies, bioaugmentation (BA), biostimulation (BS) and biostimulation-bioaugmentation (BS-BA) to assess their ability to reduce the total petroleum hydrocarbon concentration (TPH) in crude oil tank bottom sludge (COBTS) contaminated Libyan soil. The results indicated that a substantial reduction in TPH was observed in all three treatments, with the BS-BA treatments showing the highest reduction (96-97%, from 30703 to 860 and 1020 mg kg\(^{-1}\)), followed by the BS treatment (92-93% reduction). In contrast, control mesocosms showed only a 17.15 % reduction (30667 mg kg\(^{-1}\)) in TPH concentration. The results from CO\(_2\) respiration, community fingerprinting (DGGE) and metabolic profiling (Biology assay) confirmed increased activity in the BS/BA treatments. For example, BS-BA treatments accumulated produced the highest levels of CO\(_2\) after 90 d incubation (0.075 mg day\(^{-1}\) g soil\(^{-1}\) and 0.072 mg day\(^{-1}\) g soil\(^{-1}\)) respectively, some 244-258 % higher than the control (only 0.020 mg day\(^{-1}\) g soil\(^{-1}\)). This study confirms the beneficial of (BS-BA) approach and the ability of *Pseudomonas* isolates to reduce the TPH concentration dramatically in soil contaminated with COTBS. This approach can be utilized in commercial application at low water requirements.

Keywords: Mesocosms, Biostimulation/Bioaugmentation (BS/BA), Total Petroleum Hydrocarbon (TPH), Mineralisation, Respirometry.
1. Introduction

In the first quarter of 2015, the total world production of petroleum crude oil was $95.24 \times 10^6$ barrels per day while the total world consumption was $93.30 \times 10^6$ barrels per day (U.S. energy information administration 2015), with an expected 38% increase in the consumption of oil and other liquid fuels expected between 2010 and 2040. Fuels obtained from refined petroleum crude oil supply more than 50% of the world’s energy and heating services (Speight, 2014). Furthermore, in modern society petroleum crude oil is one of the most important materials, used as a source of raw materials for many industries (Speight, 2014). However, serious environmental problems are associated with the oil industry worldwide. Oil industry is a potential source of air, soil and water pollution and contamination (Costa et al., 2012). In addition to the accidental release of different hydrocarbon contaminants to the environment, significant quantities of oily sludges are generated from oil industry facilities. Of these the largest source of sludge is crude oil storage tanks where vast amounts of oily crude oil tank bottom sludge (COTBS) are generated every year causing an environmental threat to the environment (Liu et al., 2010). Improper handling or disposal of untreated COTBS represents a serious human and environmental threat (Hu et al., 2013). Remediation of soil contaminated with COTBS is therefore essential (Juan et al., 2008). Soil contaminated with COTBS is usually treated using one of three methods; physical, chemical or bioremediation treatments (Ting et al. 1999). In comparison to bioremediation, many of the physical and chemical technologies are either costly, do not remove contaminants completely or are not environmentally accepted (Liu et al., 2010). Moreover, unwanted chemical materials are generated or released during or after bioremediation. In this case, bioremediation, the utilization of the hydrocarbon contaminants by microorganisms converting the pollutants to harmless products (mineralization) can be considered as an effective and cost effective approach. However, effective bioremediation requires significant optimisation of key environmental parameters to permit the growth and activity of the microorganisms leading to faster degradation rate (Sharma, 2012). Failure of commercial bioremediation projects in the past has largely been due to poor preparation and pre-optimisation, resulting in inappropriate selection of micro-organisms for the pollutant or environmental conditions (Tyagi et al., 2011). While inoculation of soil with hydrocarbon degrading microorganisms (bioaugmentation) has resulted in increased rates of bioremediation in some cases (Ma et al., 2015) the use of indigenous bacterial isolates has also resulted in significant reductions in hydrocarbon concentration in contaminated soil
(Silva et al., 2009). For example, Karamalidis et al. (2010) conducted a study on the bioremediation of soil contaminated with hydrocarbon materials (Karamalidis et al., 2010). After 191 days, the selected indigenous isolates were able to reduce 94% of the hydrocarbon. However, poor selection of microorganism may significantly reduce the effectiveness of the approach. Teng et al. (2010) tested the biodegradation potential of Paracoccus strain HPD-2 on an aged hydrocarbon contaminated soil. After 28 days, the inoculated strain showed only a 23.2% decrease in soil total hydrocarbon concentrations (Teng et al., 2010). Other studies have also reported the ineffectiveness of a bioaugmentation strategy (Abdulsalam et al., 2011).

Optimizing key physical and chemical parameters such as temperature, aeration and supplying the required nitrogen and phosphorus (biostimulation) in some cases accelerates the bioremediation mechanism by enhancing the growth of the indigenous hydrocarbon degraders (Milić et al., 2009). In China, Dong et al. (2013) studied the effect of biostimulation on the bioremediation of soil contaminated with hydrocarbons and found that after 30 days the removal of TPH reached 88.3% (Dong et al., 2013). In this case the limiting factors preventing the bioremediation of the contaminant by the indigenous microbial population was a lack of nitrates (NO$_3^-$), phosphates (PO$_4^{3-}$), and iron (Fe) (Atlas and Hazen, 2011). However biostimulation has also been reported to be an ineffective treatment. Bento et al. (2005) studied the effect of biostimulation on the degradation rate of Hong Kong soil contaminated with diesel. The authors reported after 12 weeks the reduction of diesel concentration in the soil reached only 35.3%. Another study on the effects of biostimulation on the degradation rate was conducted by Couto et al. (2010) on a soil contaminated with petroleum hydrocarbon obtained from an oil refinery in Portugal. After 9 months, the results indicated that TPH reduction reached only between 10-35% in soil at a depth of 5-10 cm, while no TPH reduction was observed in contaminated soil obtained from depths below 20 cm (Couto et al., 2010).

The success of bioremediation of soil contaminated with hydrocarbons depends on the selection of the appropriate hydrocarbon degrading microorganisms and supplying the degraders with the required nutrients (bioaugmentation/biostimulation). For this reason, a number of researches have carried out the bioremediation of soils contaminated with petroleum hydrocarbon materials using a combined bioaugmentation/biostimulation strategy. For example, Xu (2010) carried out a bioremediation study on a soil contaminated with
petroleum crude oil using BS/BA as a remediation option. After 12 weeks the results indicated that 61% TPH reduction was achieved (Xu and Lu, 2010). In another study carried out on soil contaminated with hydrocarbon where BS/BA, BS and BA strategies were chosen, after 5 weeks, BS/BA showed the highest biodegradation (87.3%) compared to the other techniques (BS and BA). Moreover, highest bacterial growth was also observed with the BS/BA treatment (Agarry et al., 2010).

Many studies have also used slurry phase bioremediation of soil contaminated with hydrocarbons (Machin-Ramírez et al., 2008, Kriipsalu et al., 2007, De-qing et al., 2007). Soil slurry bioreactors offer maximum control and have shown a significant enhancement to both the initial rates and overall extent of hydrocarbon mineralization. Soil slurry phase bioreactors ensure an effective contact between contaminant and hydrocarbon degrading microorganisms obtaining a significant enhancement of hydrocarbon degradation in a shorter time (Mohan et al., 2009). Devi et al. (2001) conducted a bioremediation study using different techniques and found that by using BS/BA with indigenous hydrocarbonoclastic microorganisms in slurry phase bioreactors, the maximum TPH removal was observed by integrating biostimulation with bioaugmentation (BS/BA) (44.01%) (Devi et al., 2011). Another study carried on long term hydrocarbon contaminated soil used a BS/BA slurry phase with indigenous hydrocarbonoclastics bacteria (Aburto-Medina et al., 2012). After 42 days of incubation, removal of 51.6% of TPH was observed.

Libya is one of the oil producing countries in North Africa and generates considerable amounts of sludge every year. To date very few studies have been carried out on soil contaminated with COTBS and to the authors knowledge there have been no studies carried out on COTBS contaminated soils from the Middle East and North Africa. Early work investigated the potential of three bioaugmentation agents to degrade the TPH within COTBS contaminated Libyan soil in 5:1 water: soil ratio. Because Libya is among the most arid countries in the world, the purpose of the present study was to investigate and evaluate the effects of biostimulation/bioaugmentation, selecting the best two indigenous individual bacterial isolates in slurry phase bioreactors on the reduction of total petroleum hydrocarbon in Libyan soil contaminated with COTBS at lower water: soil ratios.
2. Materials and Methods

2.1. Contaminated Soil sampling
The COTBS contaminated soil samples used in this study were collected from Azzawiya oil refinery in Libya. Contaminated soil samples (~5 kg) were collected from the top 0-30 cm of historically stockpiled soil. Soils were homogenized, sieved to 2 mm mesh size in zip lock plastic bags, transported to RMIT University, Melbourne, Australia, coded and stored in a Quarantine facility. The sample was air-dried for 7 days at room temperature.

2.2. Preparing bacterial isolates for microcosms
Two bacterial isolates from these contaminated Libyan soils, previously isolated and identified (Mansur et al., 2014) were selected for use based on their hydrocarbon degrading properties. These isolates (designated as 4M14 and 4M12) were obtained from stocks of pure cultures maintained at -80°C in 50% glycerol and streaked individually on nutrient agar plates (26 g/ l adjusted to pH 7.4). The streaked plates were incubated at 30 °C for up to 3 d after which they were inoculated into nutrient broth (30 ml) at 30 °C for 48 h at 120 rev min⁻¹. Cultures of OD₆₀₀ 0.689-1.056 were harvested by centrifugation at 4°C at 16,000 × g for 2 min and washed twice with sterile distilled water (Cerqueira et al., 2011). The pellets were then re-suspended individually in distilled water (5 ml) for the bioremediation experiments. Triplicate samples were used for all experimental work.

2.3. Preparation of slurries and strains inoculation
In order to assess the actual and potential metabolic activity of indigenous hydrocarbonolytic bacterial isolates, slurry phase experiments for assessing the biodegradation of COTBS in contaminated soil were carried out in 500 ml flasks. The slurries were prepared in triplicate by adding 250 g of soil contaminated with COTBS to 250 ml of autoclaved deionized water to obtain (1: 1 soil : solution ratio) (Rutherford et al., 1998) and maintained during the whole experimental period. During slurry preparation, nutrients (NH₄)₂SO₄ (3.58 g), K₂HPO₄ (0.41 g) and KH₂PO₄ (0.33 g) were added to achieve a C:N:P molar ratio of 100:10:1 (Aburto-Medina et al., 2012) to enhance bacterial growth. Flasks were then sealed with cotton stoppers and autoclaved. After sterilization, an aliquot (5 ml) of each individual bacterial isolate were inoculated into each experiment with an initial inoculum size of 1x10⁴ cells / ml (Machin-Ramírez et al., 2008, Sabaté et al., 2004). Flasks where then
connected to a continuous flow of sterile air (0.2 μm filter, 1.0 l/min) then maintained in the
dark at 30 °C and 150 rpm (Oleszczuk and Baran, 2004) for 90 d.

The biodegradation experiments were performed in 4 different sets:

i. COTBS contaminated soil plus water plus air plus nutrients and individual isolates (BS/BA).
ii. COTBS contaminated soil plus water plus air plus nutrients mesocosms (BS).
iii. COTBS contaminated soil plus water plus air plus individual isolates mesocosms (BA).
iv. COTBS contaminated soil and air (control).

2.4 Respiratory Measurements in Mesocosms

Respirometry experiments were conducted in triplicate with the COTBS contaminated soil using a previously described protocol (Kadali et al., 2012) with some modifications. All the bioreactor flasks were maintained at 150 rpm at 30 °C and connected to a continuous flow of sterile air (0.2 μm filter, 1.0 l/min) which passed through a soda lime column to remove any CO₂ before entering the mesocosms. The CO₂ concentration in gas leaving each mesocosm was captured in 150 ml of 1 M NaOH. Carbon dioxide production was determined every 4 d by titrations with HCl (1M) where 25 ml of Ba(OH)₂ was added to 25 ml of NaOH together with 2 drops of phenolphthalein. The NaOH trap was replaced every 4 d. To prevent photodegradation, experiments were carried out in the dark (Oleszczuk and Baran, 2004) for 13 weeks. Every 2 weeks slurry samples (5 g) were taken in triplicate and analysed to assess changes in total petroleum hydrocarbon (TPH) concentration. In addition a further aliquot (1 g) was taken in triplicate every 2 weeks for DNA extraction.

2.5. DNA extraction and PCR

DNA extraction was carried out in triplicate slurry samples every 2 weeks from days (0) to week (13) using a MoBio Power Soil DNA isolation kit (MoBio Laboratories, Inc, USA) according to the manufacturer’s protocol.

The polymerase chain reaction (PCR) of obtained DNA from slurry samples was conducted using a 50 μl PCR master mix. The reactions were prepared using 2 μl bacterial primers 341F-GC (10 μM) and 907R (10 μM) (Liang et al., 2011, Muyzer et al., 1993), 3 μl MgCl₂
(25 mM), 1µl dNTP mixture (10 mM), 10 µl of GoTaq flexi buffer (5x), 0.25 µl of Taq polymerase enzyme (5U/µl) and 29.75 µl of sterile nuclease-free water per reaction. DNA extract (2 µl) was added to 48 µl of master mix. The thermocycling conditions were: an initial denaturing step at 95 °C for 5 min, then 92 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min (25 cycles) and a final extension step at 72°C for 10 min.

2.6. Denaturing gradient gel electrophoresis (DGGE) analysis

DGGE was performed on PCR amplicons using a Universal Mutation Detection System Decode apparatus (Bio-Rad, CA, USA) as per the manufacturer protocol. PCR products were loaded onto a 6 % (w/v) polyacrylamide gel with a denaturing gradient of 45% to 65% and run for 18 h at 60°C and 60 V. DGGE gel was silver stained (Girvan et al., 2003) and then scanned with an EPSON Expression 1600 V.2.65 E software (Patil et al., 2010) and digital images saved as tiff files.

2.7. Oil extraction form COTBS contaminated soil

Oil was extracted in triplicate by solvent extraction and air drying using a previously described protocol (Mansur et al., 2014, Mansur et al., 2015). Briefly, COTBS-contaminated soil (10 g) was weighed into Teflon coated centrifuge tubes (25 ml) and hexane (15 ml) added. The oil in the soil was extracted by agitation (130 rpm) for 20 min, followed by centrifugation at 5,000 rpm for 5 min. The supernatants were removed into pre-weighed glass bottles and concentrated by solvent evaporation overnight at room temperature. The reclaimed oil was then dissolved in (1 ml) hexane and transferred to GC-MS vile.

2.8 Determination of Total Petroleum Hydrocarbons.

The TPH concentration of the COTBS contaminated soil in the mesocosms was determined at the beginning and every 4 weeks throughout the experiment using a method previously described (Mansur et al., 2014). For the analysis of TPH, a standard (2 ml ampule) (SUPELCO, Bellefonte, PA, USA) was used. The hydrocarbon content of the extracted oil was analysed as described in ASTM D5134 using gas chromatograph mass spectrometer (GC-MS) equipped with an auto-sampler (Agilent 6890 GC and Leco Pegasus III TOF-MS). Samples were injected and separated on a capillary column Agilent DB-5MS (60 m by 0.25 mm with 0.25 µm film thickness). The injection temperature and volume was 225 °C and 0.2
μl respectively. Helium (1.8 ml min⁻¹) was used as a carrier gas at a constant flow rate. The concentration of each hydrocarbon fraction was analyzed and the total peak area of each fraction was compared to the peak area of each fraction in the TPH standard curve (Sabaté et al., 2004).

2.9 Biolog assay

Bacterial functional diversity was studied using Biolog EcoPlates which consist of 96 wells containing 31 different carbon sources. These substrates consist of carbohydrates (n=10), carboxylic acid (n=7), amino acid (n=6), polymer (n=4), amines and phenols (n=2 for each) (Christian and Lind, 2006) in addition to tetrazolium redox dye for soil community analysis. The ability of the microbial community to utilize different carbon substrates contained in Biolog EcoPlates was assessed colorimetrically using standardized inoculum densities. Briefly, soil samples (1 g) were collected in triplicate from each flask before and after the bioremediation treatment. Deionized sterile water (10 ml) were added to the soil and shaken at 200 rpm for 20 min (Floch et al., 2011) to dislodge bacteria before dilution. The resulting mixtures were diluted to (10⁻²) (Girvan et al., 2003). Low speed centrifugation (1,500 g, 10 min) was used to remove the soil particles. Aliquots of bacterial suspension (150 μl) were then inoculated into each well of the Biolog EcoPlates. Control wells were inoculated with deionized sterile water only (150 μl). Plates were incubated at 30 °C in the dark with the lids closed (only opened for taking readings). Each individual bacterial suspension (n=3) was tested. A multiscan microplate reader equipped with an automated shaker-loader cassette (Labsystems, Finland, Multiskan EX Version 1.0) was used to analyse the colour formation in the Biolog EcoPlate wells (absorption at 595 nm). Readings were taken every 12 h for 7 days (Mansur et al., 2014).

2.10 Statistical analysis for functional diversity

An average well-colour development (AWCD) that represents the potential utilization of different carbon sources by microbial communities was determined from BiologEco plates using the following equation:

\[ \text{AWCD} = \frac{\sum \text{OD}_i}{31} \]
Where OD<sub>i</sub> was the optical density (OD) value from each well (Ci), corrected by subtracting the blank well value (inoculated without a carbon source) from each plate well and the value from day 0 (R) (Garland, 1996, Garland and Mills, 1991). Substrate richness values were calculated as the number of oxidized carbon substrates that gave a positive response with an OD &gt; 0.10 (Garland, 1997, Gomez et al., 2000).

3. Results and Discussion

3.1. TPH degradation

The main goal of this study was to investigate the potential of two selected individual hydrocarbonoclastic bacterial isolates for use in the bioremediation of Libyan COBTS contaminated soil. These selected isolates, from Libyan COTBS and COTBS-contaminated soil identified, as *Pseudomonas sp* (4M12) and *Pseudomonas xanthomarina* (4M14) (Mansur et al., 2014) were assessed in terms of their potential to degrade COTBS as a carbon source were assessed in mesocosms in slurry phase bioreactors with a soil: water ratio of 1:1.

The intention here was to develop a method that can be translated to full scale in Libya. It was therefore important to minimise water usage even in a slurry type bioremediation process. Both isolates, irrespective of the bioremediation strategy (BS, BA or BS/BA) were able to survive in the bioreactor, utilizing the COTBS as a carbon source; after 90 d, the final concentration of TPH was significantly reduced (around 97%) in all treatments (Fig. 1), with significant TPH reduction occurring between 18 d and 54 d (from ~27500 to ~3500 mg kg<sup>-1</sup>). However, variations in patterns and levels of bioremediation were observed between strains and strategies. These findings support those reported by Liu et al. (2011) who studied the impact of different bioremediation strategies on the degradation of petrogenic hydrocarbons in contaminated soils. They reported that the combination of biostimulation/bioaugmentation achieved the highest TPH reduction (80%).

In another study, (Abdulsalam and Omale, 2009) studied the effect of biostimulation/bioaugmentation on TPH reduction in hydrocarbon contaminated soil at an initial TPH concentration of 40,000 ppm and found that the BS/BA strategy led to a reduction in soil TPH concentration of 65.2% (±0.25%). In the current experiment the biostimulation treatment followed a similar pattern, although by the end of the incubation (90 d) a lower level of TPH reduction was observed (93.45 %). Bioaugmentation treatments, using either
strain resulted in lower TPH degradation (around 92%). These degradation values obtained with bioaugmentation are significantly higher than those reported by (Xu and Lu, 2010) in a study on soil contaminated with petroleum crude oil in Liaohe Oilfield, Liaoning Province, China. They reported that after 12 weeks BA treated resulted in a TPH reduction of only 27%.

The results confirm the suitability of all 3 treatments for the bioremediation, although BS/BA was found to show the greatest efficacy in terms of TPH reduction. It is important to note that the final concentration of TPH remaining at the end of the remediation was only (860 and 1020 mg kg^{-1}) comparing to the control (30667 mg kg^{-1}). According to the Australian National Environmental Protection Measures (NEPM), the management limits for TPH concentration in residential, parkland and public space is 3500 mg kg^{-1} and in commercial and industrial sites is 5000 mg kg^{-1}. Consequently, the remediated soil was fit for reuse in residential and industrial facilities.

3.2 Soil respiration

Soil respiration results during the bioremediation for all treatments are presented in Fig 2. Overall respiration in the treatments was significantly increased and varying from an increase of 118.3% to 244.1% higher than the control. After 90 d incubation soil respiration was found to be up to 2.5 times greater in the treatments when compared to the control soils (Fig. 2). Highest soil respiration rates were observed in (0.07 mg CO_{2} day^{-1} g soil^{-1} respectively). Soil respiration has been proposed to be a quick and accurate assessment of bioremediation activity in a contaminated soil (Martinez-Carrera, 2010), with increased respiration indicative of active bioremediation on the basis that the hydrocarbon represents the main, readily available C and energy source in the soils. These values compare with soil respiration rates of 0.02 mg CO_{2} day^{-1} g soil^{-1} in control mesocosms. Kadali et al. (2012) reported increased respiration rates of 25% in a bioaugmentation using an indigenous bacterial isolate. The soil respiration results confirmed both the suitability of soil respiration measurements as an indicator of bioremediation activity and the efficacy of the BA/BS treatment.

3.4 Microbial community

To evaluate the effects of bioremediation strategy on the nature of bacterial community structure during the biodegradation of COTBS, culture independent DGGE bacterial profile
was used to evaluate the microbial changes in different mesocosms at the beginning \((t=0)\) and at the end \((t=90\text{ d})\) of the experiment (Fig. 3). Mesocosms inoculated with nutrients only (BS) and mesocosms inoculated with hydrocarbon degraders only (BA) show very little change apart from a general reduction in the total number of bands identified on the DGGE gel. This reduction in bacterial population may indicate that some bacteria cannot survive the toxicity of certain hydrocarbons or are not capable of competing for nutrients in the amended contaminated soils (Makadia et al., 2011). This reduction in bacterial diversity may, in part account for the reduction in TPH degradation in the BS and BA treatments. In contrast, detectable changes in the bacterial community in mesocosms amended with biostimulation-bioaugmentation were observed (Fig. 3).

DGGE analysis indicated that the number of bands representing the hydrocarbonoclastic bacterial community increased in the BS/BA mesocosms from 70 to 111 and from 84 to 111 for isolates 4M12 and 4M14 respectively. Clearly this combination of treatment stimulated the hydrocarbonoclastic community, suggesting the importance of the added bacterial strains, justifying the thorough assessment of their hydrocarbonoclastic potential reported earlier (Mansour et al. 2015).

This observation may also in part account for both increased soil respiration rates (Fig. 2) and increased TPH degradation (Fig. 1) and support the usefulness of such microbial fingerprinting technology in bioremediation studies. Finally, assessment of the soil functionality and the influence of the developed clean-up bioremediation strategy of soil contaminated with hydrocarbon on soil microbial community was further assessed using the Biolog Ecoplote system and compared with the pre-remediataed soils and a control (uncontaminated, agricultural) soil from the same location (Libya). Overall, all Biolog Ecoplates amended with soil taken from mesocosms following treatment (BS, BA, BS/ BA) demonstrated high metabolic capacity, as measured by AWCD values for all 31 substrates (Fig. 4).

Given the previous findings, these results were not surprising; through the addition of both bioaugmentation agents and nutrient, together with moisture and aeration an active microbial population was obtained (Agarry and Latinwo, 2015). In contrast, utilization of substrates in plates inoculated with either clean agricultural soils or contaminated soils was found to be significantly lower (Fig. 4). Both soils were nutritionally poor (Mansur et al., 2015). Further the presence of the contaminant did not affect the metabolic profile as both soils showed
similar activities (Fig. 4), suggesting that bioremediation through BS/BA treatment for this soil can lead to effective remediation resulting in a microbial community with strong resilience and metabolic capacity. Further these soils would appear to be of potential use as an agricultural supplement although further work in this area would be required.

4. Conclusion

This study has shown the benefits of using selected indigenous bacterial isolates to reduce the high concentration of TPH in COTBS-contaminated soil. Comparing the three bioremediation strategies, biostimulation-bioaugmentation using *Pseudomonas sp* (4M12) and *Pseudomonas xanthomarina* (4M12) were the most effective approach to remediate Libyan COTBS contaminated soil, reducing the TPH concentration to below Australian NEPM limits (3500 mg kg\(^{-1}\) for residential, parkland and public space and 3500 mg kg\(^{-1}\) for commercial and industrial purposes). Consequently, coupling biostimulation with bioaugmentation using indigenous hydrocarbonoclastic bacterial isolates can be scaled up for field trails and commercial bioremediation of environments contaminated with hydrocarbon contaminants. Selection of bioaugmentation agents that show high survival rates and significant hydrocarbonoclastic activities is important and will lead to enhanced bioremediation. Both soil respiration and community fingerprinting and metabolic profiling confirmed increased activity in the BS/BA soil and suggested that the remediated soil, may be of benefit for use as an agricultural amendment.

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Figure 1
Reduction in TPH in soil contaminated with COBTS over 90 d bioremediation course using different bioremediation strategies. Values are means of obtained results from BS (biostimulation), BA (bioaugmentation) and BS/BA (biostimulation/bioaugmentation) using *Pseudomonas sp.* (4M12) and *Pseudomonas xanthomarina* (4M14) treatment mesocosms compared to the control mesocosms.

Figure 2
Mean values of soil respiration rates in mesocosms containing COBTS contaminated soils over 90 d different bioremediation strategies. BS (biostimulation), BA (bioaugmentation) and BS/BA (biostimulation/bioaugmentation) using *Pseudomonas sp.* (4M12) and *Pseudomonas xanthomarina* (4M14) treatment mesocosms compared to the control mesocosms.

Figure 3
DGGE profile of bacterial community in microbial only (BA), nutrient amended microbial community (BS/BA), nutrient only (BS) before treatment (t=0) and after 90 days (t=90) incubation and the change in the number of bands before and after the treatment.
Note: BS (0) - Biostimulation mesocosms, BA 4M12 (0), BA 4M14(0) and BS/BA 4M12 (0), BS/BA 4M14(0) are biostimulated-bioaugmented microcosms before treatment. BS (90) - Biostimulation mesocosms, BA 4M12 (90), BA 4M14(90) and BS/BA 4M12 (90), BS/BA 4M14(90) are biostimulated-bioaugmented microcosms after treatment.

Figure 4
Growth of bacterial isolates (4M12 and 4M14) based on the utilization of 31 carbon sources in Biolog Ecoplates (measured by absorbance at 595 nm).
Figures for submission

Figure 1:

![Graph showing TPH reduction over time for different treatments.](image-url)
Figure 2

![Graph showing CO2 generation over time for different treatments.](image)
Figure 3
Figure 4
Chapter 7

General Discussion & Future Research
7.1. General Discussion
Due to the daily increased demand on oil and oil products, considerable amounts of crude oil tank bottom sludge (COTBS) are generated annually from different oil industry activities. COTBS is a very complex mixture of hydrocarbon and non-hydrocarbon materials (Jasmine & Mukherji 2015). Dumping untreated COTBS poses threats to both human and environmental components (Minai-Tehrani et al. 2015) and the conventional treatment methods are expensive, time consuming and not environmental friendly. But given the high hydrocarbon content within different COTBS (an average of 30-50%) (Hu et al. 2013), COTBS represents potentially valuable material and as such significant amounts of oil can be extracted and recycled.

In this study, COTBS was obtained from crude oil storage tanks used to store Hamada crude oil at Azzawiya oil refiner, Azawia, Libya. COTBS was characterised and the oil content was assessed. Oil was recovered from COTBS using dichloromethane (DCM) extraction methods. The results indicated that the studied COTBS contained 42.08 ± 1.1 % of oil. The recovered oil was characterised to investigate its composition and properties and compared to the parent oil (Hamada crude oil). Gas chromatograph mass spectrometer (GC-MS) analysis showed that this oil consists of about (29.7 %) light hydrocarbons and (70.3 %) non-volatile hydrocarbons, ranging from C14-C24 containing both aromatic (45.6% v/w) and aliphatic compounds (34.6% v/w), with 19.8% being undefined.

The water content of the COTBS was very low (2.9 ± 0.2 %) while the solid content was (55.02 ± 0.6 %). The extracted oil was qualified using different parameters. The results indicated that the density @ 15°C (0.86 g ml⁻¹), specific gravity@60/60°F (0.86), viscosity@70 °F and 100°F (7.03 and 3.655 cSt), salt content (as NaCl) (2.3 mg l⁻¹) and the ash content (0.007 g g⁻¹ oil) and API gravity (33.03). API gravity is the main parameter used for grading the oil. Due to the high percentage of light hydrocarbons within the extracted oil, this percentage has a potential to increase the value of API. Oil with API<10 is classified as extra heavy while API<22.3 is heavy, API 22.3 to 31.1 is medium and API>31.1 is light. Comparing the grading parameters of the extracted oil to those of the parent oil (API gravity of the extracted oil was 33.03 while that of the Hamada oil was 38.8) the obtained oil was very similar to Hamada oil. Consequently, the extracted oil can be graded as light oil where the lighter the oil is the higher content of light hydrocarbons with less wax and asphaltine compounds. The total petroleum hydrocarbon (TPH)
concentration of the recovered oil was 29,367 mg kg\(^{-1}\) and the polycyclic aromatic hydrocarbons (PAHs) concentration was 11,752 mg kg\(^{-1}\).

Solvent extraction has been used to extract oil from COTBS. Different studies investigated the potential of extracting oil from COTBS and varieties of oil percentages were obtained. The results showed a high percentage (67.5\%) of oil recovered (Hu et al. 2015). Zubaidy et al (2010) used two different solvents (methyl ethyl ketone (MEK) and LPG condensate (LPGC) at a ratio of 4: 1 (solvent: COTBS) to extract oil from COTBS and found that these solvent achieved 39 and 32\% oil recovery respectively (Zubaidy & Abouelnasr 2010). In contrast, some studies obtained only a very low oil recovery. Hu et al (2015) used 2-propane to extract oil from COTBS and only 8\% oil recovery was achieved (Hu et al. 2015). Other studies suggested that even at low (>10\%) COTBS oil content, it was still beneficial, both economically and environmentally to recover the oil (Ramaswamy et al. 2007).

After oil extraction, sludge usually contains a small percentage of oil that can be treated prior sending to final destination. To eliminate the human and environmental impact of COTBS, this soil must be treated. Physical and chemical methods are expensive, inefficient and release unwanted environmental pollutants (Shie et al. 2000). Bioremediation, the use of the microorganisms to utilize the hydrocarbons as the main source of carbon and energy represents safe and efficient alternative approach (Chen, M et al. 2015).

However, not all hydrocarbonoclastic microorganisms have the potential to utilize different hydrocarbon fractions and compounds (Kadali 2012). Currently, more than 500 different strains and species have been listed as hydrocarbon degrading, representing almost 200 bacterial, cyanobacterial, fungal and algal genera (Yakimov et al. 2007). In particular the use of indigenous hydrocarbonoclastic bacteria has been found to be a successful approach to developing a bioremediation strategy, resulting in significant hydrocarbon contaminant reduction (Dashti et al. 2015).

In the second part of this study, 49 hydrocarbonoclastic bacteria were successfully isolated from three different Libyan sources (15 from long-term contaminated soil, 21 from treated contaminated soil and 13 from COTBS) using an isolation enrichment strategy (BHMSM
amended with 1% Hamada crude oil as the core source of carbon and energy). However there were differences in isolate potential for hydrocarbon utilization.

It is important to differentiate between the activity of the isolates using a fast and efficient screening technology in order to assess the hydrocarbonoclastic potential of the isolates for bioremediation purposes. Biolog plate screening was performed for the first time with these isolates. Hierarchical cluster analysis (HCA) was used to separate the isolates into two clusters resulting in the identification of 7 isolates exhibiting relatively high hydrocarbonoclastic activity.

These 7 isolates were phylogenetically analysed and identified. Four of these were isolated from treated COTBS-contaminated soils, two from COTBS itself and one from COTBS contaminated soil. Three bacterial species were identified as belonging to the genus Pseudomonas, two were identified as Pseudomonas sp. while the third was identified as Pseudomonas xanthomarina; two isolates were identified as Bacillus strains, Bacillus nasdae and Bacillus aerius; one bacterial isolate was identified as Arthrobacter nitroguajacolicus and the final isolate was identified as Brevundimonas nasdae.

The Pseudomonas group has long been renowned for their ability to degrading a wide range of hydrocarbon compounds including crude oil, refined fuels, alkanes and PAHs and been reported as hydrocarbonoclastic (Kumar et al. 2008; Arun et al. 2008; Sopeña et al. 2013). Two bacterial species were identified as Bacillus nasdae and Bacillus aerius. In in situ long term bioremediation studies, Bacillus has shown to promote hydrocarbon degradation and the growth of Bacillus strains in soil contaminated with petroleum derived compounds with high utilization activities has previously been reported (Benedek et al. 2013).

In another study, Kebria et al. (2009) found that Bacillus strains showed significant potential for the bioremediation of soil contaminated with different hydrocarbon concentrations (500 – 10,000 ppm) with a degrading capacity of around 87 % (Kebria et al. 2009). Arthrobacter are well known for their hydrocarbonoclastic activities on PAHs with some Arthrobacter strains reportedly showing high degradation efficiency for specific pollutants such as phenanthrene (Peng et al. 2008). In this study, a novel isolate was isolated from soil contaminated with hydrocarbons and was identified as Brevundimonas nasdae. Interestingly, this species has not been previously isolated from hydrocarbon contaminated environments nor has been reported as
hydrocarbonoclastic, although members of the genus are known to be hydrocarbonoclastic (Brito et al. 2006).

*Brevundimonas nasdae* has previously been isolated from soil contaminated with malathion (MLT) (C10H10O6S2P), an organo-phosphorus insecticide, where it exhibited very high degradative capabilities (87.32 %) after 48 h (Zhao et al. 2011). In addition to the utilization of (MLT), in this study *Brevundimonas nasdae* showed a high potential to degrade hydrocarbon contaminants.

Although research in the field of bioremediation of soil contaminated with hydrocarbons has been intensively performed in last decade, only very few studies of bioremediation of soil contaminated with COTBS are available in the literature especially relating to work carried out in South Mediterranean counties such Libya. Despite the significant amount of soil contamination with petrogenic hydrocarbon including COTBS and the suggestions of the bioremediation potential, bioremediation is still completely neglected in Libya.

In the third part of this study, slurry phase (5:1 water: soil) microcosm experiments using three individual bioaugmentation agents (isolated in Chapter 2) *Pseudomonas sp*, *Pseudomonas xanthomarina* and *Arthrobacter nitroguajacolicus* in conjunction with biostimulation (BS) and natural attenuation (NA) microcosms studies were conducted to remediate Libyan soil contaminated with COTBS. The results showed that soils amended with all three isolates (and nutrients) were able to degrade the hydrocarbon fractions within COTBS; substantial hydrocarbon removal occurred in all microcosms by the end of the experimental period. Very high TPH reduction (97.8 to 99.4%, from 30,703 mg kg\(^{-1}\) to 170-664 mg kg\(^{-1}\)) was observed in BS/BA with *Pseudomonas sp* and *Pseudomonas xanthomarina* inoculated microcosms within 15 days. Although similar levels of degradation were achieved in *Arthrobacter nitroguajacolicus* inoculated microcosms, BS and NA microcosms, the time required to obtain these levels of degradation were significantly longer (25-30 d). Mesocosms inoculated with *Pseudomonas sp* and *Pseudomonas xanthomarina* also showed a remarkable reduction in PAHs concentration, from 13,816 mg kg\(^{-1}\) to 0 mg kg\(^{-1}\) or below detection limit (98.8% - 100%) within the first 5-10 days. In fact, all BS/BA mesocosms showed increased rates of TPH and PAH degradation compared with non-augmented mesocosms. This might suggest a lack of an active hydrocarbonoclastic population, although of course the origin of the isolates included the soil
used here. Agarry & Latinwo (2015) also investigated the potential of BS/BA in terms of their effect on the reduction of TPH in hydrocarbon contaminated soil and concluded that the BS/BA strategy led to a significant increase in TPH reduction (91.5%) compared to 40% TPH reduction achieved by natural bioattenuation (Agarry & Latinwo 2015). Another study investigated the effect of BS/BA using Pseudomonas sp as the main hydrocarbon degrader in soil contaminated with oily sludge obtained from a petroleum refinery sludge dam in Durban in South Africa. After 6 months of controlled incubation, the presence of the Pseudomonas sp led to a reduction in the TPH concentration of 91% compared to a TPH degradation of just 32% in the control. In the same study, Pseudomonas sp was able to degrade the low molecular weight PAHs completely. In contrast, in another study the addition of Pseudomonas sp did not result in any significant reduction in high molecular weight PAHs (Atagana 2014). Compared to other bacterial degraders, Pseudomonas is unique in its ability to metabolize hydrocarbons (single fractions and mixtures) rapidly and efficiently even at high hydrocarbon concentrations (Prabhu & Phale 2003).

Bioremediation studies on the degradation of different hydrocarbons have also been conducted in contaminated soil obtained from crude oil contaminated soil, North East of India where the potential of two individual Pseudomonas isolates (P. aeruginosa M and P. aeruginosa NM) to degrade three individual specific PAHs (Benzene, Toluene and m-Xylene) were assessed. After 90 days incubation, the results showed that P. aeruginosa M was able to reduce the concentration of Benzene, Toluene and m-Xylene by 79, 70 and 85% respectively comparing to 3.0% reduction by the control while P. aeruginosa NM was able to reduce the concentration of Benzene, Toluene and m-Xylene by 75, 68 and 88% compared to 3.2% PAH reduction achieved by the control (Mukherjee, Ashis K & Bordoloi 2012). In addition to the removal of TPH and PAH, Pseudomonas has been shown to specifically remove mutagenic and carcinogenic fractions from COTBS contaminated soil. In this present study, the addition of the Pseudomonas sp, Pseudomonas xanthomarina and Arthrobacter nitroguajacolicus were found to increase the rate of degradation of four mutagenic (pyrene, phenanthrene, flourene and anthracene) and two carcinogenic (naphthalene and benzenamine, 4,4`methylenbis[2-methyl-]) PAH fractions. In fact, complete degradation of selected mutagenic and carcinogenic fractions was accomplished between 9-10 days in mesocosms amended with Pseudomonas sp and 9-15 days in mesocosms amended with Pseudomonas xanthomarina. Microcosms amended with
Arthrobacter nitroguajacolicus were slower in terms of the removal of the 4 carcinogenic and mutagenic fractions, taking between 15 to 23 days for complete removal, similar with the time required to the complete removal of these compounds in BS and NA microcosms. In comparison to other studies, the potential of bacterial isolates used in this study showed remarkably high potential for removing hydrocarbon contaminants with COTBS contaminated soil. M’rassi et al. (2015) used Pseudomonas to degrade two mutagenic fractions in soil contaminated with hydrocarbons; after 30 days of treatment, Pseudomonas showed only 10 % degradation of pyrene and only 11 % degradation of anthracene (M’rassi et al. 2015). In the present study clearly the addition of the individual indigenous bacterial isolates in a slurry phase bioremediation treatment resulted in increased hydrocarbon degradation. The application of a slurry phase bioremediation approach ensures good contact between the hydrocarbonoclastic microbial community, the contaminants and nutrients, leading to rapid TPH and PAH reduction rates. Aburto et al. (2012) conducted three (BS-BA, BA and NA) slurry phase bioremediation strategies of long term hydrocarbon contaminated soil. After 42 days, the results indicated that BS-BA achieved the highest TPH reduction followed by BA (51.6 and 41.3 % respectively) compared to no significance hydrocarbon removal in NA (Aburto-Medina et al. 2012)

A number of studies already indicated the potential of hydrocarbon contaminated sites including contamination with oily sludge and COTBS as a good sources of hydrocarbonoclastic bacteria (Aislabie et al. 2006; Jasmine & Mukherji 2014b). Tam et al (2002) compared the potential of bacteria isolated from hydrocarbon contaminated and non-contaminated environments and found that bacteria isolated from contaminated sites showed higher hydrocarbon degrading abilities (Tam et al. 2002). (Bao et al. 2012) reported that single isolates could not metabolise all the fractions found within the hydrocarbon mixture. This is understandable give the array of contaminants present. The limitations of activity of any individual isolate include limited enzymatic activity and antagonistic interactions between the soil microbiota (Tam et al. 2002). In addition, prevention of bacterial coordination behaviors such as swarming mobility, antibiotic secretion and biofilm generation could interfere with the degradation abilities of bacterial communities (Teasdale et al. 2009). Kinetic studies of the biodegradation of hydrocarbon contaminants could provide a clearer description of these interactions (Bouchez, M et al. 1995). Jasmine & Mukherji (2015) investigated the biodegradation of oily sludge obtained from an oil refinery in Mumbai, India using 7 indigenous bacterial isolates in 3 sets of consortiums. After 30
days, the biodegradation rate of hydrocarbon fractions within the sludge in all consortia
ranged between 29 and 42 %. The authors concluded that antagonistic interactions among the
indigenous isolates may have lowered the hydrocarbon biodegradation (Jasmine & Mukherji
2015).

Slurry phase bioremediation of soil contaminated with hydrocarbon requires large amounts of
water, but Libya is facing a severe shortage of water with a very low rainfall in winter and
drought in the rest of the year. In an effort to reduce the volume of water required for the soil
slurry bioremediation treatment, the impact of a 5-fold reduction in water use during the
bioremediation treatment was assessed. In the final part of the study a low water content (1: 1
soil: solution ratio) soil slurry bioremediation was used. The two strains which when added
previously resulted in the highest rates of TPH and PAH degradation, *Pseudomonas sp* and
*Pseudomonas xanthomarina* were selected and their potential to reduce the TPH in COBTS
contaminated Libyan soil in a larger scale (meccocosms) bioremediation were evaluated. Three
different strategies, bioaugmentation (BA), biostimulation (BS) and biostimulation-
bioaugmentation (BS-BA) were again used to establish whether these isolates offered an effective
bioremediation strategy in the contaminated soil meccocosms during low water content slurry
phase bioremediation. The results showed a substantial reduction in TPH in all three experiments
in which the individual hydrocarbonoclastic isolates were added. BS-BA again showed the
highest reduction rates (96 and 97%), with TPH concentrations being reduced from 30,703 mg
kg\(^{-1}\) to 860 and 1020 mg kg\(^{-1}\) in mesocosms amended with for *Pseudomonas sp* and
*Pseudomonas xanthomarina* respectively. This compares to only 17.15% reduction in the TPH
concentration in the control mesocosms (from 30,703 mg kg\(^{-1}\) to 25,437 mg kg\(^{-1}\)). In a similar
study conducted on the bioremediation of tank bottom sludge and soil contaminated with tank oil
sludge obtained from the oil refinery Novi Sad, Serbia over 12 weeks, an 82-88% and 86-91 %
reduction in TPH were achieved in the BS/BA treatment of tank bottom sludge and soil
contaminated with tank oil sludge respectively compared to 6 % in the control (Gojgic-Cvijovic
*et al.* 2012). Mao *et al.* (2012) also conducted bioremediation study of an aged soil contaminated
with PAH’s using a bacterial consortium. After 56 days incubation, 35.8 % of PAH’s were
removed compared to 7.1 % was detected by control (Mao *et al.* 2012).
To monitor the bioremediation progress, carbon dioxide (CO$_2$) generation during the bioremediation was measured. CO$_2$ generation in the gas phase during the bioremediation represents an effective and reliable tool which provide information about the mineralization rate of the contaminants (Schoefs et al. 2004). Respiratory measurements during bioremediation directly represents bacterial and metabolic activity (Aspray et al. 2008). The results from CO$_2$ respiration obtained for each treatment showed that BS-BA treatments accumulated the highest levels of CO$_2$ (0.075 mg day$^{-1}$gs oil$^{-1}$ and 0.072 mg day$^{-1}$g soil$^{-1}$) for mesocosms amended with *Pseudomonas sp* and *Pseudomonas xanthomarina* respectively, some 2.5-fold higher than the soil respiration in the control mesocosm after 90 d incubation.

Overall this study has resulted in the isolation, screening and identification of the hydrocarbonoclastic bacterial isolates of potential commercial significance and demonstrated that this technology was capable, at least at a mesocosm scale of remediating Libyan oil waste or sludge contaminated soil even at low water content.

It is important to note that the addition of the bacterial isolates does not suggest that these organisms are capable of degrading all the hydrocarbons present. However these organisms may catalyze key reactions within the soil microbial community which enable, together with the additional nutrients the natural microbial community to more efficiently utilize the contaminants. Therefore in terms of hydrocarbon biodegradation, it is important to investigate the activity and diversity of the natural soil microbial communities involved in the bioremediation. In this present study, Denaturing Gradient Gel Electrophoresis (DGGE) was used to monitor microbial community members in different mesocosms and to assess the shift and diversity of bacterial communities in contaminated soil before ($t=0$) and after ($t=90$ d) treatments. In contrast, supplying nutrients led to an increase in the bacterial population (richness) and enhanced the survival of the isolates. In this study, higher species richness occurred in biostimulation/bioaugmentation mecososms (an increase in bands from 70 to 111 and from 84 to 111) accompanied by 97.19 and 96.67 % reduction in TPH for *Pseudomonas sp* and *Pseudomonas xanthomarina* strains respectively. A similar study was conducted by Taccari et al. (2012) on soil contaminated with hydrocarbon material where BS/ BA was carried out using individual bacterial isolates. DGGE was used to investigate the dynamics of the bacterial community. After 120 days of bioremediation, BS/ BA treatments augmented with *Pseudomonas* showed an
increase in both species diversity and biomass (Taccari et al. 2012). Another study conducted by Sprocati et al. (2012) involved the addition of bioaugmentation agents to soil contaminated with hydrocarbon. After incubation, they reported a large increase in the bacterial community and activity which was assessed using DGGE. DGGE profiles showed a very high Range-weighted richness (Rr) (250 < Rr < 350) in both the number of bands and distribution along the denaturing gradient (Sprocati et al. 2012).

In terms of the metabolic capacity of the soil microbial community, further assessment of bacterial community in contaminated soil before and after the treatment in addition to the clean (agricultural) soil was carried out using the Biolog Ecoplates which contain 31 different carbon substrates. In addition the metabolic capacity of clean (i.e. no petrogenic contamination) Libyan agricultural soil was also assessed. Interestingly, the functionality of clean soil was very low comparing to the contaminated and treated soils. This result highlights the importance of studying Libyan soils. Whilst Australian, European and American agricultural soils tend to show high metabolic activity due to nutrient amendments (O'Sullivan et al. 2013). Libyan agricultural soils are arid, nutrient poor and contain low organic matter, resulting in low substrate utilization. In contrast, the COTBS contaminated soil showed very high soil metabolic capacity perhaps as a result of the availability of carbon through the contaminating hydrocarbons in addition to nutrient and aeration; however greatest metabolic activity was found in the remediated soils. This could be due to a reduced hydrocarbon content and therefore greater microbial activity. In this case the use of the treated Libyan contaminated as an amendment to agricultural soils is worthy of further investigation. The addition of an active microbial together with additional carbon may enrich the natural soil.

7.2. Future Research

- Huge amounts of COTBS with high oil content are generated annually in Libya. Extracting the oil for recycling purposes and energy uses is economically beneficial. Future large scale oil extraction experiments would be useful in improving oil utilisation efficiency and in reducing the environmental contamination associated with the petrogenic hydrocarbon industry. Solvent extraction is one of the successfully applied technologies to reclaim oil from sludge. Because the oily sludge contains suspended solids, oil extraction would be followed by cyclone to separate the suspended solids from
the oil. Economically efficient solvents could be used such as warm diesel at 70°C. Low boiling point solvents could be used too and then separated from the oil by distillation or evaporation using large scale rotary evaporator. The solvent could be recycled. The technology has already been used successfully in Europe as a unit process on a larger scale to produce lubrication stock from used oil (AERCO 1995).

- Bioremediation of soil contaminated with petroleum hydrocarbons in Libya is still unpractised. This present study was the first study on bioremediation of soil contaminated with COTBS. The obtained results from this study were promising and great interest in field scale bioremediation would be conducted in future. Ex situ bioremediation has been increasingly viewed as an appropriate treating technology for soil contaminated with hydrocarbon. Instruction of biopiles of contaminated soil, inoculation with the appropriate bioaugmentation agents and supplying enough nutrient and aeration has been applied are required of bioremediation. Recent large scale bioremediation study was conducted in Canada on soil contaminated with hydrocarbon where 18 field-scale biopiles (16 m³ each) were constructed. Microbial isolates were inoculated in addition to mature compost. A response surface methodology (RSM) based on a factorial design was applied to investigate and optimize the effects of the microbial isolates application rate and amount of mature compost amendment on the TPH removal (964 µg g⁻¹ initial concentration). After 94 days, the final TPH removal achievement was in the range of 74-82% compared to 48% of TPH removal in control (Gomez & Sartaj 2014).

- The increased demand on fast, cheap and accurate DNA sequencing has led to an advancement of next generation sequencing (NGS). Since the introduction of (NGS) in 2005, a high impact on genome research was observed where NGS has been used for standard sequencing applications including genome sequencing and for novel applications which weren’t explored by Sanger sequencing. Although previous sequencing approaches have been widely adopted, some limitations in throughput, speed, cost and resolution may prevent researchers from obtaining essential genomic data. NGS technologies from 454/Roche, illumine/Solexa, ABI/SOLID and Helicos led to high throughput functional genomic research and applied in different contexts such as whole-genome sequencing and targeted genes. NGS has the ability to measure changes in the genome and can identify and qualify rare transcripts without previous knowledge (for un-
culturable organisms), it also provides information about how soil microbial community changes across time and space, requires very small amount (about 1µg) of template laying out millions of DNA fragments on a single chip and sequencing all the fragments in a parallel fashion. Consequently, NGS technology could be applied in future work to assess the microbial community and microbial community changes and diversity in Libyan soil contaminated with hydrocarbon. Moreover, NGS could be used to sequence the whole genes present in Libyan soil or to sequence specific genes responsible for degrading specific contaminants.

- As many aquatic systems in Libya are contaminated with hydrocarbon contaminants, bioremediation of water is another approach of interest that could be conducted in future studies. Previous studies indicated the beneficial of bioremediation of hydrocarbon contaminated water. Comparing to mechanical and physio-chemical treatment methods bioremediation was more efficient comparing. The use of bioremediation to treat the contaminated water offers some potential advantages including; lower cost, environmentally friendly, usually converts the contaminants to innocuous products (H₂O, CO₂ and methane). However, bioremediation has some disadvantages too where it can be effective only when environmental conditions permit microbial growth and activity. These conditions includes the presence of microbes capable to degrade the contaminants, type of soil, temperature, pH, nutrient and presence of oxygen and it is necessary that microbes and contaminants be in contact. Moreover, bioremediation requires more time and it is difficult to predict the future results and effectiveness.

7.3. Conclusions

In Libya, bioremediation technology should be considered as an alternative treatment method for cleaning soil contaminated with COTBS. For the treatment of polluted soil a safe, cheap, fast, efficient and environmental friendly method is required to be established. After extracting the highest possible amount of oil from COTBS, the hydrocarbon content in the soil has been reduced by 95%. This present research demonstrated a simple and rapid identification method of isolating and assessing hydrocarbonoclastic bacteria to identify those that can utilize the hydrocarbon contaminants within COTBS in soil.

Specifically, this study has shown:
• The benefit of oil recovery from COTBS for use as feedstock for recycling and energy purposes and to reduce the environmental impact of hydrocarbon contaminants by reducing the oil content in sludge to the minimum level.

• The benefit of selected medium (Bushnell Hass) enriched with crude oil to investigate the availability of hydrocarbonoclastic bacterial isolates in Libyan soil contaminated with COTBS and the benefit of Biolog MT2 plates-based assays as an alternative and successful method for screening the degradability of selected hydrocarbon substrates.

• The potential of additionally screened indigenous bioaugmentation agents (*Pseudomonas sp, Pseudomonas xanthomarina* and *Arthrobacter nitroguajacolicus*) to reduce 99.4% of the TPH concentration and 100% of PAHs concentration in addition to the 100% removal of two carcinogenic and four mutagenic contaminants within the COTBS in microcosms bioreactors.

• The commercial applicability of coupling biostimulation with bioaugmentation using indigenous *Pseudomonas sp* and *Pseudomonas xanthomarina* to reduce 97% of TPH concentration in contaminated soil in larger scale bioremediation at low water content bioreactors.
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