Sustainable treatment of oil contaminated waste: oil-based mud (OBM) drill cuttings and soil.

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SUSTAINABLE TREATMENT OF OIL CONTAMINATED WASTE: OIL-BASED MUD (OBM) DRILL CUTTINGS AND SOIL

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Sustainable Treatment of Oil Contaminated Waste: Oil-Based Mud (OBM) Drill Cuttings and Soil

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To my late Father, **Warisenibo, Roland Amiesima Pepple**, who passed away on the 2nd of January 2014, in a ghastly boat accident on Bonny River. May his peaceful soul Rest in Peace.

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Dedication

I dedicate my thesis to Almighty God, the lover of my soul for granting me the privilege to get an education, by providing full scholarships from my first degree to PhD level within and outside my home country. May His Holy name be praised.

Abstract

Environmental pollution from oilfield drilling waste poses potential hazards which can lead to ecological imbalance. The predominant pollutant from oilfield waste is petroleum hydrocarbons. Some effects of petroleum hydrocarbon contamination in soil include loss of nutrients, reduced fertility, foul odour, flora/fauna imbalance and potential for transport and distribution to other media. Several studies have been carried out to develop technologies for the reduction of petroleum hydrocarbons in oil based mud (OBM) drill cuttings and soil. Soil washing using biosurfactant is one of such technological developments. Biosurfactants are surface active compounds produced from biological origin. They are amphiphilic molecules, consisting of hydrophilic and hydrophobic moieties. The major advantage biosurfactants have over their synthetic counterpart is that they have low toxicity and are biodegradable. They can be produced from natural and renewable feedstock (agricultural and industrial waste).

This work focused on the production, purification and characterisation of rhamnolipid (RL) biosurfactant, produced from *Pseudomonas aeruginosa* ST5 and *Pseudomonas aeruginosa* PS1, and its consequent application for the removal of total petroleum hydrocarbon (TPH) in OBM drill cuttings and petroleum contaminated soil. First, the OBM drill cuttings and soil were characterised to investigate the following parameters; particle size analysis by laser diffraction and sieve, morphology and elemental content (qualitative) by Scanning Electron Microscope – Energy Dispersive X-ray Analysis (SEM-EDXA), elemental content by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) analysis (quantitative), hydrocarbon profile by Gas Chromatography–Mass Spectrometry (GC-MS) and TPH by Fourier-Transform Infrared Spectroscopy (FT-IR).

Second, the rhamnolipid was produced from both bacteria using mineral salts media with glycerol as carbon source in shake flask cultivation process. Approximately 3.5 g/L yield of crude ST5 rhamnolipid extract (ST5-RL) was determined from the culture broth from *Ps*. ST5 and PS1. Thin layer chromatography analysis carried out on the crude ST5 rhamnolipid

extract detected two fractions with retardation factors 0.76 and 0.39, which were purified by column chromatography and confirmed to be monorhamnolipid (R1) and dirhamnolipid (R2) respectively, consequent upon structural characterization using FTIR, NMR and LC-MS/MS. The surfactant potential of R1, R2 and ST5-RL were determined by investigating their surface active properties such as; critical micelle concentration (where R1 = 28 ppm, R2 = 24 ppm and ST5-RL = 48 ppm), surface tension and emulsification index after 24 hours (E_{24}).

The crude ST5 rhamnolipid, R1 and R2 were applied for the removal of total petroleum hydrocarbon (TPH) in diesel contaminated soil at 10, 100 and 1000 ppm concentration levels. R1 and R2 both showed TPH removals at approximately 77% at 10 ppm, approximately 87% at 100 ppm and approximately 91% at 1000 ppm. However, ST5-RL showed over 90% TPH reduction from the oil contaminated soil at 10, 100 and 1000 ppm, validating the potential of RL in the removal of TPH from soil without purification. Approximately 91% of TPH was removed at the optimum washing condition using ST5-RL. The rhamnolipids were able to remove TPH from the sample by the mechanism of solubilisation. Also, the biocidal effect of RL and RL-washings (from the soil treatment) at 10, 100 and 1000 ppm was studied by carrying out cytotoxicity test on breast cancer MDA-MB-231 cells using MTT assay. The unused RL showed significant anti-proliferative against the cancer cells at 100 and 1000 ppm, while RL-washings showed significant anti-proliferative against the cancer cells at only 1000ppm. The RL was seen to be safe at 10 and 100ppm where over 90% TPH was achieved. This result shows the crude ST5 rhamnolipid is safe to use at concentrations not exceeding 100ppm. The study shows that biosurfactants can be applied to remove TPH from the environment at room temperature.

Identifying Words

OBM drill cuttings, total petroleum hydrocarbon, biosurfactant, rhamnolipid, oil contaminated soil, heavy metals.

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Abbreviations

BEIS	-	Department for Business, Energy & Industrial Strategy
CAPP	-	Canadian Association of Petroleum Producers
CEFAS	-	Centre for Environment, Fisheries and Aquaculture Science
^{o}C	-	Degree Celsius
DCR	-	Dispersion-by-Chemical Reaction
DCS	-	Drill Cuttings Sample
DECC	-	Department of Energy and Climate Change
DMSO	-	Dimethyl sulfoxide (DMSO)
DPR	-	Department of Petroleum Resources
FTIR	-	Fourier transform infrared spectroscopy
GC-MS	-	Gas Chromatography with Mass Spectrometry
HQ	-	Hazard Quotient
HSE	-	Health Safety and Environment
ICP-OES	-	Inductively coupled plasma optical emission spectroscopy
Kg	-	Kilogram
kV	-	Kilovolts
MEF	-	Ministry of Environment, Finland
mg/L	-	Milligram per Liter
mL	-	Millilitre
OBM	-	Oil Based Mud
OBM –DCS	-	Oil Based Mud Drill Cuttings Sample
OCNS	-	Offshore Chemical Notification Scheme
OSPAR	-	Oslo and Paris Conventions

Pa	-	Pascal
ppb	-	Parts per Billion
ppm	-	Parts per Million
RL	-	Rhamnolipid
R1	-	Monorhamnolipid
R2	-	Dirhamnolipid
RSD	-	Relative Standard Deviation
SBF	-	Synthetic Based Fluid
SEM-EDXA	-	Scanning Electron Microscope – Energy Dispersive X-ray Analysis
S/S	-	Stabilization/Solidification
TPH	-	Total Petroleum Hydrocarbon
UKOOA	-	United Kingdom Offshore Operators Association
WBF	-	Water Based Fluid
WD	-	Working Distance
WOB	-	Weight on Bit

CHAPTER 1 - BACKGROUND AND LITERATURE REVIEW

1.1. Introduction

Prior to the early 1990s, waste management was not regarded as a single environmental issue within the oil and gas industry, because waste from production processes such as; produced water, drill cuttings and flares were regulated separately from the general waste streams (McFadden 1996). One of the major problems associated with offshore oil and gas exploration and production processes, is the generation of an enormous amount of drill waste and its consequent management (Ataya 2008). The waste generated during a drilling process is usually hazardous to the environment, especially when oil-based mud (OBM) is used as the drilling fluid. Thus it becomes expedient to treat the waste generated in the most sustainable manner.

The discharge of oilfield waste by some oil and gas companies in the early 90's led to the OSPARCOM Decisions 92/2 and 2000/3, which limits the disposal of oil on cuttings (OOC) offshore to a maximum of 1% i.e. 10,000mg total petroleum hydrocarbon/Kg cuttings (OSPAR 2000). This decision seeks to limit the disposal of organic phase spent drilling fluid and drill cuttings offshore. OSPAR considers an organic phase contaminant to be, "an emulsion of water and other additives in which the continuous phase is a water-immiscible organic fluid of animal, vegetable or mineral origin." The rule further requires the companies to transport the waste onshore for treatment before disposal

This stringent regulation as well as other regulations and waste directives, leaves the oil and gas companies with the challenge of exploring sustainable options for the management of oilfield waste. There are several challenges and logistics associated with shipping the waste onshore such as; storage space on the deck, cost and availability of shipping vessels. A number of treatment methods have been applied to treating drill cuttings in the past such as; thermal desorption technique (Melton et al. 2003), incineration (Aird 2008), dispersion by chemical reaction (Ifeadi 2007)., stabilization/solidification (Al-Ansary and Al-Tabbaa 2007) and chemical

washing using surfactants (Paria 2008). However, all of these treatment methods have their limitations and impacts to the environment.

The management of waste from industrial processes has been a challenge for decades. The major concern associated with irresponsible and unsustainable management of spent OBM drilling fluid and cuttings is the potential hazard caused by the toxic nature of the pollutants found in it such as; petroleum hydrocarbons, biocides, corrosion inhibitors, scale inhibitors, emulsifiers, oxygen adsorbents and heavy metals such as; mercury, cadmium, zinc, chromium and copper (Speight 2015).

1.2. Overview of the Oil and Gas Industry

Offshore drilling development started in 1896, off the coast of Summerfield, California, Santa Barbara, with gradual steps seaward into shallow waters, and by the mid-1960's, the offshore industry had acquired sufficient expertise and technology to handle the offshore basins with very minimal onshore production such as in the Cabinda, Gippsland, Gulf of Suez and Southern North Sea basins (Nehring 1985).

The oil and gas industry business involves the mining or extraction of natural resources, mainly petroleum and gas, which is processed and utilized to meet various aspects of human needs in areas such as; energy, agriculture and medicine. The oil and gas industry is made up of three major sectors; upstream, midstream and downstream sectors. Figure 1.1 below shows a general overview of the major operations in the oil and gas industry.

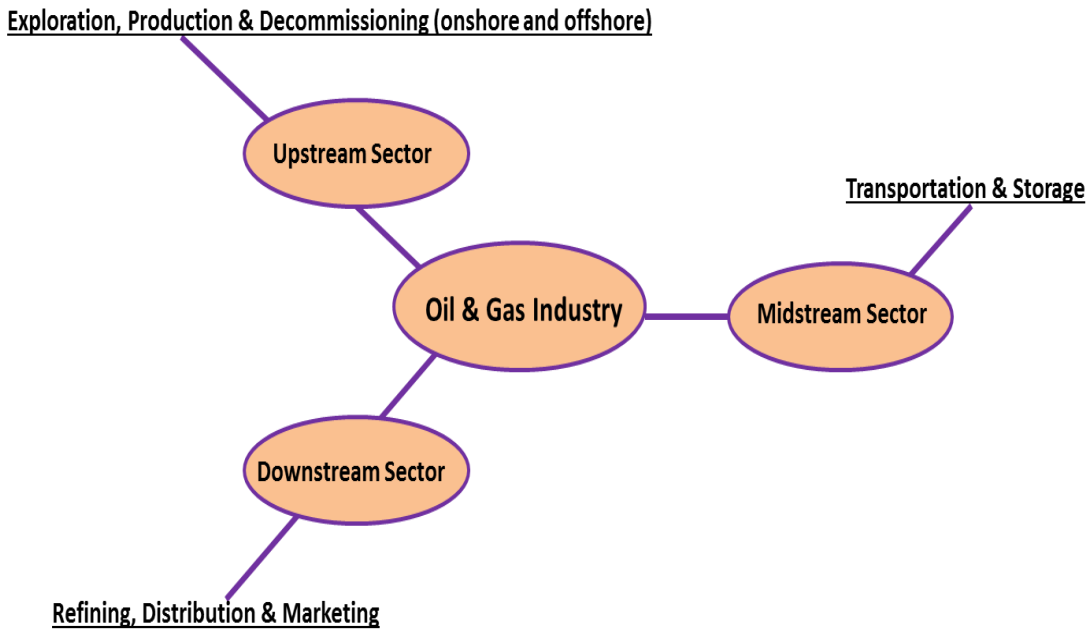


Figure 1.1: Major Operating Sectors of the Oil and Gas Industry

1.2.1. Waste Associated with the Oil and Gas Industry (Upstream).

The systematic record of advancement in the upstream section of the industry over the decades is not without its associated challenges. As with the creation of any product, oil and gas production generates a number of wastes during production. The European Waste Framework Directive (2008/98/EC) refers to waste as, "*any substance or object that the holder discards or intends or is required to discard*" (European Commission 2008). Figure 1.2 below shows some of the waste generated in the upstream sector of the oil and gas industry.

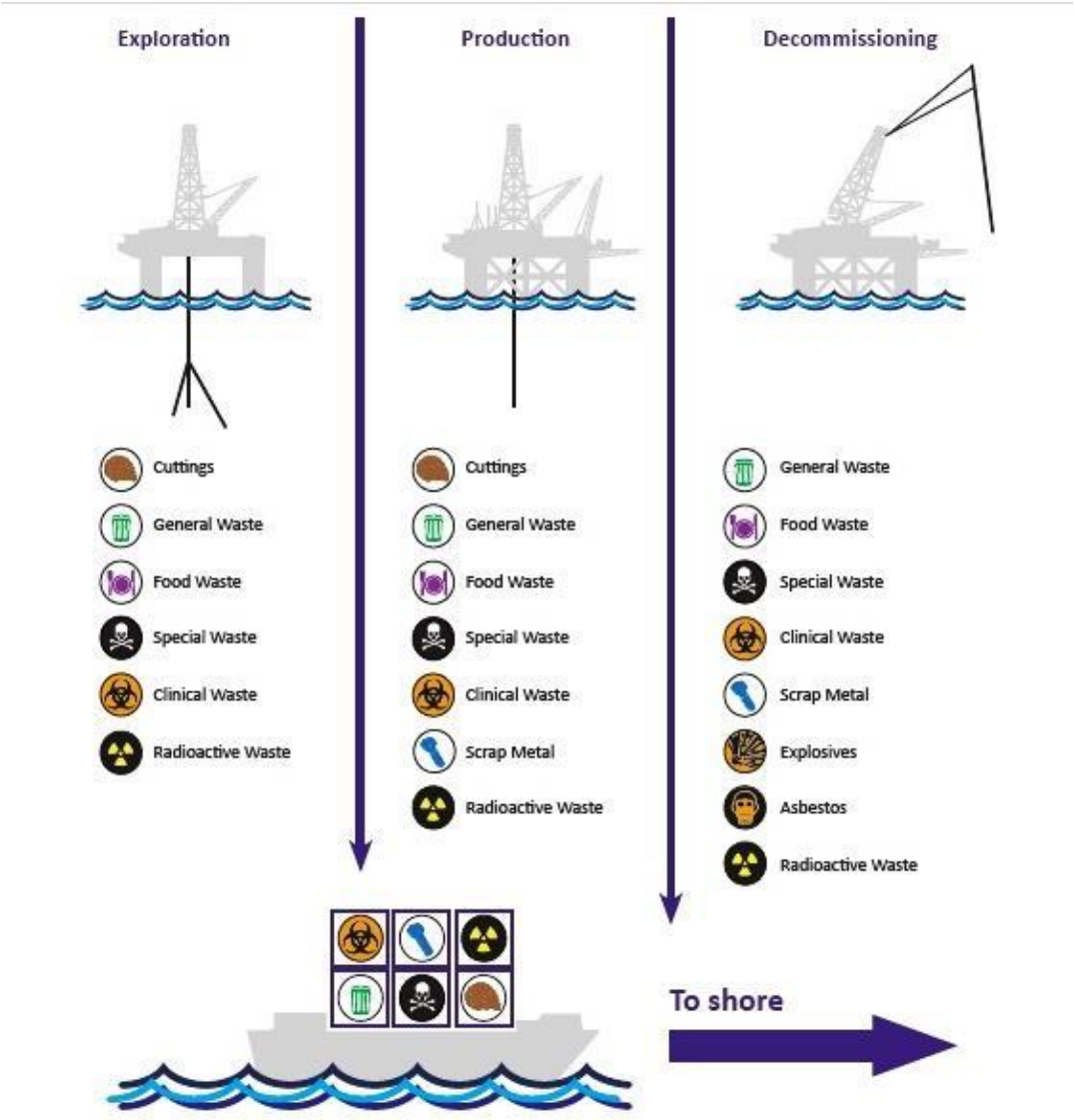


Figure 1.2: **Waste Generated Offshore by Source**

Available from: Oil and Gas UK (2016)

Taboas (1996), stated that "the ultimate objective of environmental management is the protection of human health." Table 1.1 below shows the effects of the waste generated in the upstream sector of the oil and gas industry on health, safety and environment.

Table 1.1: Summary of Waste Associated with Upstream Oil and Gas Industry

Available from: Ite et al. 2013

Operational Activity	Potential associated risks	Effects on Health, Safety and Environment
Exploration operations • Geological survey • Aerial survey • Seismic survey • Gravimetric and magnetic survey • Exploratory drilling • Appraisal	a. Noise pollution b. Habitat destruction and acoustic emission c. Drilling discharges e.g. drilling fluids (water based and oil based muds) and drill cuttings d. Atmospheric emission e. Accidental spills/ blowout f. Solid waste disposal	Ecosystem destruction and interference with land use to access onshore sites and marines resource areas; environmental pollution (air, soil and controlled water) and safety problems associated with the use of explosives; land pollution which affects plants and pose human health risks; groundwater contamination and adverse effects on ecological biodiversity.
Development and production • Development drilling • Processing: separation and treatment • Initial storage	a. Discharges of effluents (solids, liquids and gases) b. Operation discharges c. Atmospheric emission d. Accidental oil spills e. Deck drainage f. Sanitary waste disposal g. Noise pollution h. Transportation problems i. Socio–economic/ cultural issues	Ecosystem destruction and interference; Contamination of soils and sediments with petroleum–derived wastes; atmospheric emissions from fuel combustion and gas flaring/venting; environmental pollution (air, soil and sediments, controlled waters) and groundwater contamination; ecological problems in the host communities, adverse human health risks; safety related risks and interference with socio–cultural systems.
Decommissioning and rehabilitation • Well plugging • Removal of installations and equipment • Site restoration	a. Physical closure/removal b. Petroleum-contaminated waste disposal c. Leave in situ (partial or total) d. Dumping at sea	Environmental pollution and human safety; Pollution related to onshore and offshore operations; hazard to other human activities such as fishing and navigation; marine pollution, fishing and navigation hazards.

The potential associated risks emanating from the operational activities in the industry can cause detrimental effects on human health and the environment. It is therefore expedient to ensure that human health and safety is considered and the environment protected at every stage of the oil and gas operation. This can only be achieved when sustainable practices are followed because the quality of the human life depends on clean air, water and safe food free of pollution.

1.2.2. Sustainable Development in the Oil and Gas Industry

The concept of sustainability gained global prominence in 1987 from a United Nations World Commission on Environment and Development (WCED) report titled "*Our Common Future*," which defined sustainable development as, "*development that meets the needs of the present without compromising the ability of future generations to meet their own needs* (Brundtland 1987)." This report became necessary consequent upon health and safety concerns arising from environmental degradation associated with industrialisation and urban development. Most of the developments associated with modern human life have been enhanced by the supply of goods and services from the oil and gas industry.

The oil and gas industry is one of the most important industries in the world, adding value to the economic and social lives of people. It is common knowledge that, the operations of oil and gas industries has potential impacts on health, safety and environment requirements, which has led to the formulation of several regulations, standards and legislation to provide the essential guidance in a bid to control possible harmful impacts on HSE (Samarakoon and Gudmestad 2010).

The major challenge of every profit driven organisation is to carry out their operations under conditions that are environmentally and socially acceptable. Therefore, it is the responsibility of extractive industries to ensure that they strike a healthy balance and consider their impact on the critical sectors of the sustainability chart (Figure 1.3) which are: economic, social and environment. It is expected that whilst they seek to make profit in their business, they must adhere to best practices that are environmentally and socially responsible.

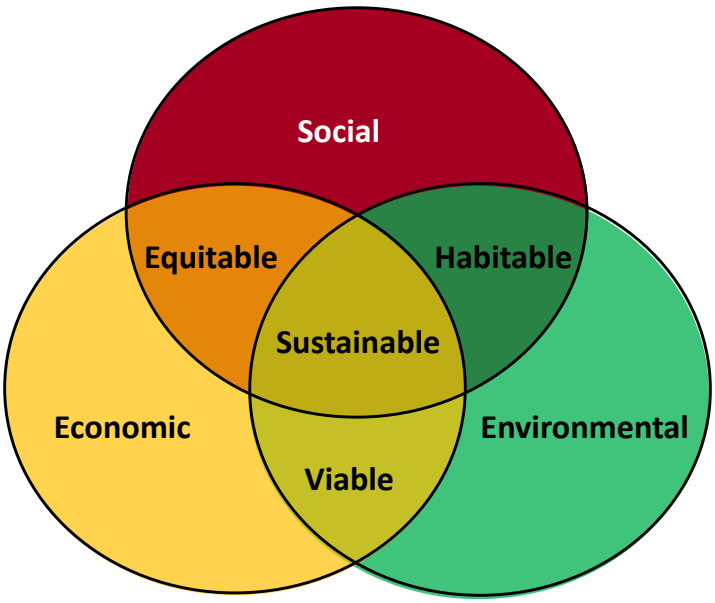


Figure 1.3: Sustainability Chart

Available from: Bevin and Steve (2012)

1.2.2.1. Economic Impact

The economic growth of a number of nations relies heavily on the oil and gas industry, generating income from exports and taxes as well as providing job opportunities (Turek 2013). The oil and gas industry has thrived over the years, boosting productivity with innovative technologies, birthed by continuous research and development. A number of processes in the industry has also been enhanced by automation and use of digital technologies. This technological advancement has increased the efficiency of the processes associated with the production of oil and gas, especially in the area of subsea infrastructure (Loffman 2015). Also, a significant decrease has been observed in; maintenance cost, fuel consumption and labour cost, whilst having an increase in productivity and value addition to their operation processes (Trent 2015).

1.2.2.2. Social Impact

Social impacts are effects that directly influence the human population, societies or people as a result of the operations of the company. The possible areas of impact includes; population, economic conditions, employment, religion, public health, education, culture, political institutions and processes, values, social wellbeing and quality of life. The operations and management of some oil and gas companies affect the way the people in the area live and relate with each other (Jones, Hartog and Sykes 1996). Although social impacts are not easily

quantifiable, most oil and gas companies have made significant contribution towards the development of their host communities. However, there is a need for improvement and consistency in the delivery of cooperate social responsibility by the oil and gas industries to their host communities (McHugh et al. 2006).

1.2.2.3. Environmental Impact

The operation of the oil and gas industry directly impacts the environment (air, land and water) in various ways such as; land clearing (removing vegetation and topsoil) during oil prospecting operation. This operation eliminates the forest canopy, exposing the environment to the risk of flooding and erosion. Also irresponsible handling of hazardous chemicals and disposal of drill cuttings and effluent water has potential impact on surface and ground water (Rosenfeld, Bowles and Thomsen 1998). Other impacts on the environment include; emission of toxic gases during gas flaring and refining processes causing respiratory diseases in humans and animals. It is the responsibility of the oil and gas industry to ensure that their operations do not impact negatively on the environment.

Furthermore, the industry must ensure that, until suitable alternative sources are available, the exploitation of the oil and gas resource is conducted with the least possible impact on the environment and within satisfactory financial and health and safety requirements for all stakeholders.

1.3. Understanding the Rotary Drilling System

In the oil and gas industry, the rotary drilling system is the major technique used to drill development wells. The Collins English Dictionary (2011) defines development wells as "wells drilled for the production of oil or gas from a field already proven by appraisal drilling to be suitable for exploitation." The rotary drilling system can be utilized for several purposes ranging from drilling for water, oil, gas, mineral assay coring, to geothermal and diverse construction projects (M-I Swaco 1998).

In 1844, Robert Beart received the first patent on the rotary drilling technique for boring holes in soil (Allen 1983). He devised a method of drilling with the aid of rotating hollow drills in a way that the cuttings may be removed by water (drilling fluid), although 2500 years before this time, the percussion drilling

technique was the technique applied in boring holes in China, Egypt and Europe (Darley and Gray 1988). These wells were drilled to obtain water, gas and brine. Water was the fluid used to soften the rock and enhance the elimination of drill cuttings (Darley and Gray 1988).

The rotary drilling system can be utilized for several purposes ranging from drilling for water, oil, gas, mineral assay coring, to geothermal and diverse construction projects (M-I Swaco 1998). The most relevant application of the rotary drilling system was introduced to oil and gas drilling in the early 1900's (Gray and Young 1973).

The rotary drilling technology used in the oil and gas industry has advanced over the years, and the drilling fluid industry has not been left out of this technological advancement. Researchers have developed more sophisticated drilling fluids with varying chemical compositions to meet the technical demands associated with drilling different reservoir formations and to enhance the production capabilities of the oil and gas industry.

For an effective understanding of offshore drilling waste, it is expedient to understand the fundamental aspects of the rotary drilling process utilised in drilling most oil and gas wells. The rotary drilling process is made up of three systems:

1. The drilling rig
2. The drill string, bit and casing and
3. The drilling fluid circulating system

These three systems have their technical limitations, which affects the type and quantity of waste generated during a drilling operation (CAPP 2001).

1.3.1. The Drilling Rig:

The basic components of a drilling rig are: the derrick (a four-legged steel structure), drill floor also known as the rig floor which is the working area surrounding the aperture through the platform from which tools are run down to the hole being drilled in the sea bed. It also functions as the central point for all activities on the drilling unit. Other components include; the drawworks and hoist system (a large winch mechanically or electrically driven), the swivel, kelly,

rotary hose and rotary table (Maclachlan 1987). Drilling rigs have six fundamental systems (Cunha and Ross, 2011), and these systems work in synergy during a drilling operation; they are:

1. The power system
2. The hoisting system
3. The circulating system
4. The rotary system
5. The well-control system
6. The well-monitoring system

The power system generates and transmits power on the drilling rig, the hoisting system provides a means of vertical movement of the pipe in the well, lowering and raising the drillstring, casing and other tools used in the rig. The fluid circulating system functions to provide hydraulic power to the drilling fluid to enable the pumpability of the fluid (with drilled cuttings) through the annulus.

The rotary system is used to achieve bit rotation downhole, the well-control system functions to prevent the unrestrained surge of formation fluids from the wellbore. The well-monitoring system is an inevitable part of the rig system, which ensures the tracking and monitoring drilling operation round the clock in order to aptly detect and amend any drilling operational problems (Cunha and Ross 2011). Figure 1.4 below is a schematic diagram showing a typical rotary drilling mud circulation system on a rotary drilling rig.

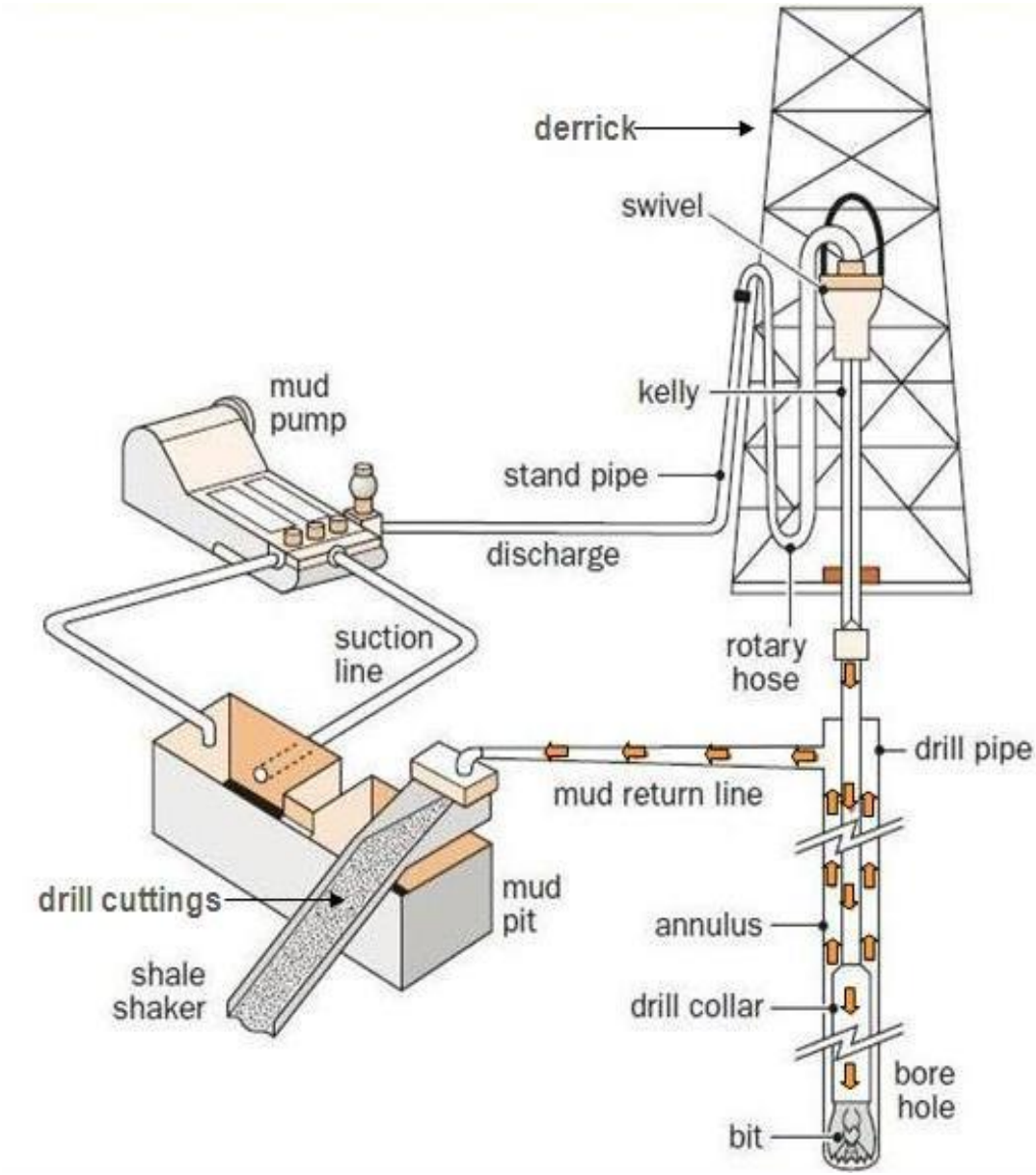


Figure 1.4: A Schematic Diagram of a Drilling Mud Circulation System

Adapted from: Visser and Larderel (1997)

1.3.2. The Drilling String, Bit and Casing.

The primary function of the drill string in a rotary drilling operation is to transmit rotary motion from the rotary table to the drill bit, and to transport drilling fluid to the surface of the drill bit, (Figure 1.5) whilst producing *weight on bit* (WOB) for efficient drilling action (Stefan 2011).

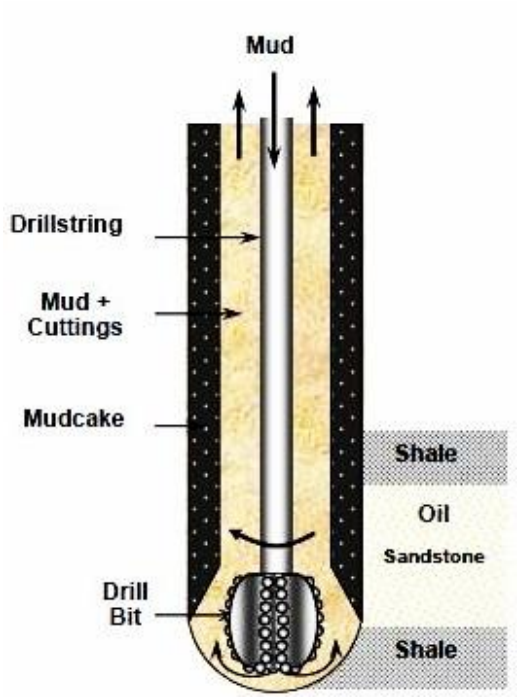


Figure 1.5: Schematic diagram showing a drill string and bit

(Available from: (Tehrani, 2007)

The bit is the cutting device used in drilling a well; it has nozzles via which the circulating drilling fluid is expelled at a high velocity. The bit is attached to the drill string and is rotated mechanically or electrically (Maclachlan 1987). The actual drilling operation takes place at the drill bit; as it rotates under the pressure of the drill-string, the bit shatters the rock under it. This activity generates drill cuttings (waste), which is removed from the well bore by the drilling fluid.

The size and morphology of the drill cuttings generated from a well bore during a drilling operation, is a function of the kind of drill bit utilized for the operation. The early drill bit used was the Drag bit or "Fish-tail" as it was called, and was only efficient in drilling soft formations because its blades could not drill hard formations. This led to the introduction of the Roller cone or rock bit (Figure 1.6) at the beginning of the 1900's (M-I Swaco 1998).

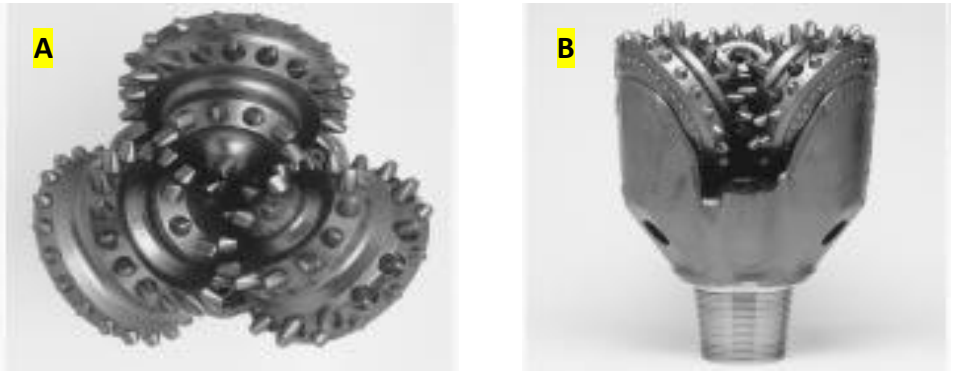


Figure 1.6: **Roller Cone Tungsten Carbide Insert (TCI) Drill Bit.**
Where A: Posterior view and B: Lateral view.
(Available from: M-I Swaco 1998)

The efficiency of the drill bit is a function of the speed of revolution, hardness of the rock or formation being drilled, pressure difference, the weight on it, and very importantly the drilling fluid viscosity and flow velocity (M-I Swaco 1998).

The drill casing serves several functions as the drilling operation progresses. It is a steel pipe placed in the bored hole to line its walls and prevents the caving-in or collapse of the wellbore during the process of drilling. It also reduces the damage caused by the drilling operation to the sub-surface environment (John, Jim and Mitchell 2011).

1.3.3. Drilling Fluid Circulating System.

Drilling mud (hereafter referred to as drilling fluid) mud is any fluid utilized during a drilling process in which fluid is pumped from the surface, down the drill string, out through the openings (nozzles) in the bit, and back to the surface, through the annulus (Growcock and Harvey 2005). The drilling fluid is basically added to the wellbore to enhance the efficiency and effectiveness of the drilling process. The drilling fluid is an overwhelming necessity for the promotion of drilling activities, both onshore and offshore (M-I Swaco 1998) and most challenges associated with drilling operations emanate directly or indirectly from the drilling fluid. However, the drilling fluid is often utilized as a tool in alleviating the challenges encountered during a drilling operation (Annis and Smith 1996).

1.3.3.1. Functions of Drilling Fluids

Basically, the fundamental purpose of the drilling fluid is to serve as a tool for the removal of cuttings from the bore hole, but until now the diverse applications of drilling fluids like control of sub-surface pressure and ensuring the formation is adequately evaluated, makes the task of specific functions difficult (Darley and Gray 1988). However, the overall function of all drilling fluids is achieved in the successful completion of a well. In rotary drilling, the principal functions performed by the drilling fluid are:

1. Cuttings suspension and transportation to the surface.
2. Control of sub-surface pressure.
3. Enhance well-bore stability, minimizing formation damage.
4. Cooling, lubricating and transmission of hydraulic power to the drill-string and drill-bit.
5. Cleaning the hole bottom.
6. Seal permeable formations thus reducing filtration rate.
7. Ensures adequate formation evaluation (data logging)

1.3.3.2. Classification of Drilling Fluids

The classification of drilling fluids depends on the base fluid being utilised to formulate the drilling fluid and other primary constituents (Growcock and Harvey 2005). There are three major types of drilling fluids (Figure 1.7) used in drilling oil and gas formations. They are; oil-based drilling fluids, water-based drilling fluids and pneumatic fluids.

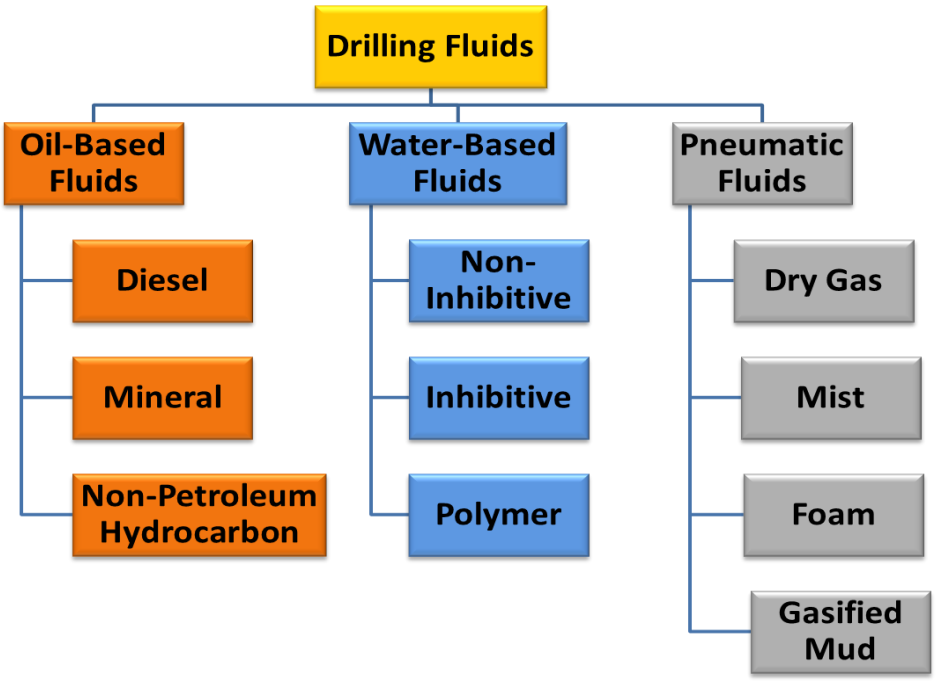


Figure 1.7: Drilling Fluid Classification

Available From: (Amoco Production Research 1994).

A. Oil Based Drilling Fluids

Traditionally, oil-based drilling fluids (OBF) are the ideal choice for drilling argillaceous formations (formations containing particles that are silt or clay-sized, approximately less than 0.625 µm in size), based on their high performance characteristics justified on the basis of borehole stability, penetration rate, filtration control, filter cake quality, lubricity, and temperature stability (Baker Hughes 2006) . Oil-based fluids are typically utilised for drilling difficult shales and to enhance bore hole stability (Amoco Production Research 1994). The major disadvantage associated with the use of oil-based fluids is the increasing HSE concerns based on the toxicity of the waste generated by the use of it, which is a function of the base fluid used in formulating it (Callaghan 1991). The base fluid used in formulating OBM drilling fluids could be either diesel or mineral oil, but mineral oil is mostly used (Fink 2012). The IARC (1984) states that, "mineral oil is a class of petroleum hydrocarbons from petroleum distillate streams such as light naphthenic or paraffinic distillates (containing C_{15} – C_{30} hydrocarbons), heavy naphthenic distillates (containing C_{20} – C_{50} hydrocarbons), white mineral oil (containing C_{15} – C_{50} hydrocarbons), and petrolatum and most residual oils (containing > C_{50} hydrocarbons).

The development of synthetic base fluids (SBF) resulted from the need to replace diesel and mineral OBF based on environmental restrictions as imposed by regulatory agencies. The SBFs offer better HSE characteristics than either diesel oil or mineral oil (M-I Swaco 1998), but still provide the same drilling performance characteristics of the conventional oil based drilling fluid (Eustes 2011). To produce an efficient synthetic base fluid, the diesel or mineral oil initially used in OBM drilling fluids is replaced with an organic fluid with reduced impact on the environment such as esters, polyolefins, acetal, ether, and linear alkyl benzenes. However, synthetic based muds are quite expensive to use, and the costs per barrel and mud losses are the two basic factors influencing the high cost associated with using synthetic based drilling fluids (Fink 2012).

There are vital characteristics required for the effectiveness and efficiency of a good drilling fluid such as; rheological properties (plastic viscosity, yield value, low-end rheology, and gel strengths), fluid loss prevention and stability against contaminating fluids from the formation (Fink 2012). The optimization of these characteristics can be achieved and manipulated during the formulation of the drilling fluid. The composition of a typical OBM drilling fluid is shown in Table 1.2 below.

Table 1.2: Composition of a Typical Oil Based Fluid (OBF)

(Available From: Health and Safety Executive 2000)

Component	Quantity	Mass (kg)	Volume (L)	% mass	% volume
Base fluid	0.52 bbl	63.64	83.31	30.37	52.40
Viscosifier	5.00 ppb	2.26	1.40	1.08	0.88
Emulsifier 1 (Primary emulsifier)	0.80 gpb	2.89	3.02	1.38	1.90
Emulsifier 2 (Secondary emulsifier)	0.40 gpb	1.49	1.51	0.71	0.95
Lime	5.00 ppb	2.26	1.00	1.08	0.63
Water	0.30 ppb	47.15	47.22	22.50	29.70
$CaCl_2$	30.20 ppb	13.70	3.35	6.54	2.11
Barite	167.9 ppb	76.15	18.16	36.34	11.42

**Characteristics of a typical OBM: Density 1318 kg/m^3, Salinity 22.5% and Oil to Water ratio (OWR) of 65:35. Components combined to give a total volume of One (1) barrel.*
Where bbl - barrel; ppb - pounds per barrel and gpb - gallons per barrel).

B. Water Based Drilling Fluids:

Water-based fluid can be formulated with fresh water, brine (commercial or natural) or seawater as the continuous phase depending on the formation to be drilled (M-I Swaco 1998). The majority of the wells drilled currently are drilled with water based drilling fluids based on its favourable HSE characteristics (Eustes 2011). Water based fluids are easy to produce, economical to maintain, environmentally friendly (green) and can be manipulated during formulation to surmount most drilling challenges (Amoco Production Research 1994).

C. Pneumatic Drilling Fluids (PDF):

Conventional fluids are inefficient when it comes to drilling formations with low reservoir pressures. Pneumatic drilling fluids are utilized in drilling formations with low and underbalanced reservoir pressures where loss of circulation occurs (Amoco Production Research 1994; Azar and Samuel 2007).

The major types of PDF are dry air, mist, foam and gasified mud. Pneumatic fluids have increased penetration rate when compared to liquid drilling fluids. The drill cuttings produced in this case are blown to the surface at a faster rate ahead of the drill bit, as a result of the pressure differential. At increased pressure differential, formation fluids from permeable zones flow into the wellbore. Pneumatic drilling fluids are useful in reducing formation damage which occurred as a result of (Amoco Production Research 1994):

1. Invasion of mud filtrate and solid particulates into reservoir pore spaces,
2. Flushing of hydrocarbons
3. Hydration of clays within the reservoir
4. Emulsion blocking
5. Formation of chemical precipitates within the reservoir.

The major advantages of using PDF are; longer drill bit life, better control of loss circulation zones, and less damage to formation. Some disadvantages include; possible down hole fire (in case of dry air/natural gas), hole deviation and hole erosion. Pneumatic drilling can also serve as a way of reducing the potential environmental effects associated with the use of oil based drilling fluid (Sharif et al. 2017). The major factors to consider before deciding to use PDF are: pore pressures, rock types, porosity and permeability, reservoir fluids, economics, and location (Donald 2018; Amoco Production Research 1994).

1.3.3.3. Other Additives in a Generic Drilling Fluid

One major challenge associated with the drilling operation is the management of the waste generated, such as produced water and drill cuttings. Generally, most challenges associated with drilling operations emanate directly or indirectly from the type of drilling fluid used in drilling the formation. The additives (Table 1.3) in the fluid, (added to improve the performance of the fluid), also have potential impact on the waste generated from the drilling process.

Table 1.3: Other Additives in a Generic Drilling Fluid

(Adapted from: Ball, Stewart and Schliephake (2012); Falk and Lawrence (1973); Fink (2012)

S/N	Major Components	Function	Examples
1.	Base fluid	The liquid phase of the fluid in which the solids are suspended.	Fresh water, salt water, paraffin, diesel, crude oil, mineral fluid.
2	Alkalinity and pH Control	Needed to control the degree of acidity or alkalinity of a drilling fluid.	Lime, caustic soda and bicarbonate of soda.
3	Biocides	Function is to reduce bacterial count	Carbamate, sodium, sulphide, aldehyde, chlorinated phenols, paraformaldehyde, caustic soda, lime
4.	Viscosifiers	Employed as viscosity builders for drilling fluids to ensure high viscosity solids relationship.	Bentonite, sodium carboxymethyl cellulose, attapulgite clays and sub-bentonites
5.	Calcium Removers	Designed to prevent and overcome the contaminating effects of gypsum. Forms of calcium sulphates can reduce the effectiveness of nearly any chemically treated mud not employing calcium removers.	Caustic soda, soda ash, bicarbonate of soda and certain polyphosphates
6.	Corrosion Inhibitors	A good mud, containing an adequate percentage of colloids, certain emulsion muds and oil muds exhibits excellent corrosion inhibiting properties.	Hydrated lime and amine salts are often added in mud systems to check corrosion.

7.	Defoamers	These are products designed to reduce foaming action, particularly that occurring in brackish water and saturated salt water muds.	Pure fluorosilicones and fatty acid esters of hydroxy alcohols, such as sorbitan monooleate can be used as defoamers.
8.	Emulsifiers	These function by creating a heterogeneous mixture of two liquids.	Included are modified lignosulfonates, certain surface active agents, anionic and non-ionic products.
9.	Filter Reducers	They serve to cut filter loss.	Included are bentonite clays, sodium carboxymethyl cellulose and pre-gelatinized starch.
10.	Flocculants	These are used to increase gel strength of the fluid. May be used to cause colloidal particles of a suspension to group, causing solids to settle out.	Salt, hydrated lime, gypsum and sodium tetraphosphates
11.	Lost Circulation Materials	These are used to plug the zone of fluid loss, back in the formation away from the face of the hole, so that subsequent drilling operations will not disturb the plug.	Water swellable polymers such as alkali metal polyacrylate or crosslinked polyacrylates can be used to control fluid loss.
12.	Lubricants	Extreme pressure lubricants are designed to reduce torque to increase horsepower at the bit by reducing the co-efficient of friction.	Certain oils, graphite powder and soaps are used for this purpose.
13.	Shale Control Inhibitors	These are products used to control caving by swelling or hydrous disintegration of shales.	Gypsum, sodium silicate, calcium lignosulfonates, as well as lime and salt
14.	Weighting Materials	Used to control formation pressures, check caving, facilitate pulling drill pipe on the round trip as well as combat circulation loss.	Barite, lead compounds, iron oxides

1.4. Legislative Overview on Offshore Discharge of Drill Cuttings

Pollution control legislation became necessary globally as a result of the negative impact of waste disposal from industrial and domestic activities on the environment and especially on human health (Andrew 1999). In a bid to ensure a steady flow of hydrocarbon energy source to the global world, the oil and gas industry engages in oil exploration and production activities onshore and offshore, and these activities directly or indirectly impacts negatively on the environment. The general public views the oil and gas industry as an industry whose activities deteriorate the environment (Apaleke, Al-Majed and Hossain 2012).

These environmental concerns have led to the enforcement of stringent regulations to ensure that the discharge limits of allowable oil on cuttings is adhered to by the oil and gas companies. Muherei and Junin (2007), claimed that, most regulatory bodies in Europe specifies that oily cuttings generated offshore have to be cleaned to a limit of 1% residual oil on cuttings (i.e. 10,000 mg oil per Kg dry cuttings). This specification is as a result of the pioneering embargo enforced in offshore North Sea countries by 1997 for diesel-based liquids (DBLs) and 2001 for synthetic-based liquids (SBLs). An example of such regulatory agency in the UK is the Department for Climate and Energy (DECC), (now changed to Business, Energy and Industrial Strategy (commonly abbreviated to BEIS) while that of Nigeria is the Department of Petroleum Resources (DPR) Nigeria.

These agencies regulate the activities of oil and gas companies in their areas of jurisdiction, to ensure that the environment is protected from potential degradation and consequent deterioration. However, the regulations on drill cuttings discharge vary from nation to nation since the discharge option or choice for the drill cuttings in most nations is a function of the type of drilling fluid utilized in drilling the formation, and the nation's commitment/political will to reduce environmental pollution (Heidi et al. 1999).

1.4.1. OSPAR Decision on Drill Cuttings Discharge Offshore

OSPAR (Oslo and Paris Commission) is an organisation through which fifteen nations of the western coasts, catchments of Europe, and the European Community, work in partnership to protect the marine environment of the North-

East Atlantic. It began in 1972 with the Oslo Convention against dumping and was further broadened to cover land-based sources and the offshore industry by the Paris Convention of 1974. These two conventions were put together, updated and extended by the 1992 OSPAR Convention (OSPAR 2012).

It is in a bid to protect the North-East Atlantic marine environment, that the Oslo and Paris Commission (OSPARCOM) Decisions 92/2 and 2000/3 prohibited the discharge into the sea of cuttings contaminated with oil based fluid at a concentration greater than 1% by weight on dry cuttings (OSPAR 2000). The OSPAR Quality Status Report of 2010, claimed that "discharges of contaminated drilled cuttings from offshore installations into the sea has largely stopped." This is due to the fact that in most OSPAR areas, cuttings from wells drilled with water-based drilling fluids are discharged into the sea, while the cuttings from wells drilled with organic-phase drilling fluids (still utilised in drilling lower sections of the well) are re-injected into sub-surface formations in line with OSPAR regulations or transported onshore for treatment and disposal (OSPAR 2010).

OSPAR decisions are intended to provide baseline requirements for discharge of chemicals in the North Sea. However, individual contracting parties are free to set their own requirements as long as they are at least as strict as OSPAR (Heidi et al. 1999). Table 1.4 shows the offshore discharge of drill cuttings standards in the United Kingdom.

Table 1.4: United Kingdom's Specific Requirements for Discharge of Drill Cuttings

Available from: Heidi et al. 1999

Type of Drill Cuttings	Standard Practice
From Water Based Drilling Fluids (WBF)	1. Discharge allowed subject to preapproval requirements for the drilling fluid chemicals. 2. Preapproval requirements include toxicity testing in line with OSPAR protocols
From Oil Based Drilling Fluids (OBF)	1. Effectively prohibits discharge. However, limit of 1% oil on cuttings (OOC). Practice is to inject cuttings or transport onshore for treatment and recover oil. 2. The UK government is also phasing out use of all but ester based synthetics. They have proposed that OSPAR adopt a decision to prohibit SBF discharges with allowances for some rare exceptions.
From Pneumatic Based Drilling Fluids (PDF)	Pneumatic drilling does not require treatment common to traditional drilling, which makes it the preferred drilling fluid for environmentally sensitive areas (Sharif et al. 2017)

However, before any discharge to the sea is carried out in the United Kingdom Continental Shelf (UKCS), operators are obligated to carry out an investigation to determine the potential environmental effects. The result of this assessment forms part of their permit application to the Department for Business, Energy & Industrial Strategy (BEIS) (Oil and Gas UK 2016).

Figure 1.8 shows the amount of cuttings from oil-based fluid and water-based fluid (in tonnes) discharged to sea in the UKCS from 2010 to 2015.

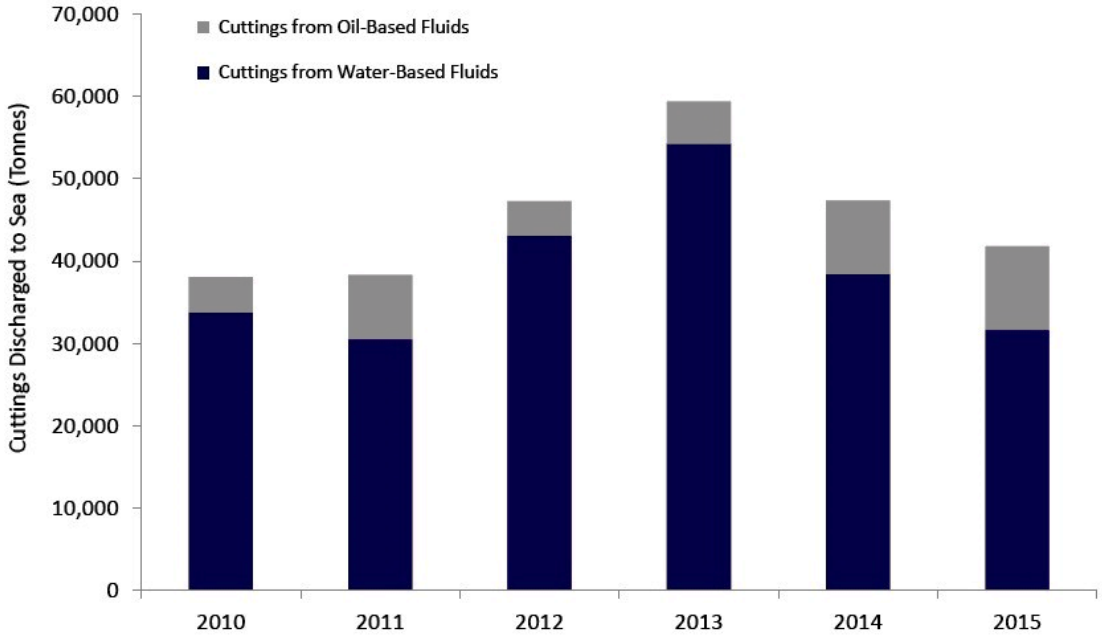


Figure 1.8: Drill Cuttings Discharged to Sea in the UKCS

Available from: Oil and Gas UK (2016)

The oil and gas UK 2016 report explains that, "the peak in cuttings discharged in 2013 (as shown in Figure 1.8), was due to more complex wells being drilled and is out of step with the general downward trend in drilling."

1.4.2. Department of Petroleum Resources (DPR) Nigeria Decision on the Discharge of Drill Cuttings

DPR Nigeria has a major statutory function to ensure that the petroleum industry operators in Nigeria do not pollute the environment in the course of their operational activities (EGASPIN 2002). DPR follows global HSE standards, and does well in adapting these standards to domestic circumstances (Clara 2011). The offshore discharge of drill cuttings standards in Nigeria as set by DPR are shown in Table 1.5.

Table 1.5: Specific Requirements for Discharge of Drill Cuttings in Nigeria

Available from: EGASPIN (2002)

Type of Drill Cuttings	Standard Practice
From Water Based Drilling Fluids (WBF)	Cuttings contaminated with WBF may be discharged offshore/deep water without treatment, provided the discharge does not contain free oil as determined by a visual sheen on the receiving water surface.
From Oil Based Drilling Fluids (OBF)	Cuttings contaminated with oil from Low Toxic Mineral Oil Based Mud (OBM) system shall not be discharged into offshore discharge zone unless treated to residual oil content less than 10,000 $mgkg^{-1}$ cuttings, i.e. 1% oil on cuttings.
From Pneumatic Based Drilling Fluids (PDF)	Pneumatic drilling does not require treatment common to traditional drilling, which makes it the preferred drilling fluid for environmentally sensitive areas (Sharif et al. 2017)

The oil and gas industry of the present day works hard to ensure that environmental and sustainable development concerns are thoroughly considered as they plan and execute projects at all stages of their drilling operations.

1.5. Overview of Waste Management Technologies for Drill Cuttings in the Oil and Gas Industry

Aloysius (2007) stated that, "*waste management is a system of practices and controls that is primarily designed to prevent the pollution of the environment.*" The ultimate aim of any waste management practice is to protect the environment from degradation and deterioration.

The type of contaminants present in drill cuttings is a function of the composition of the formation (rock) being drilled and the chemistry of the drilling fluid utilised during the drilling process (Leonard and Stegemann 2010). The waste management of drill cuttings is categorized into two options: the offshore option and the onshore option. The option chosen is basically hinged on the type of drilling fluid utilized in drilling the formation as discussed above. The drill cuttings are; either discharged directly to the seabed, re-injected into the well bore or transported onshore for treatment and disposal (UKOOA 2002).

Recent advances in treatment technologies, e.g. the TWMA's Rotomill technology has shown that drill cuttings can be treated offshore. The TWMA Company claims that, "the Rotomill technology processes and recycles drilling wastes by separating them into their constituent parts of oil, water and solids for recycling and reuse" (TWMA 2011). A major disadvantage of this process is that, solids and water recovered for the separation process are mostly discharged to sea, causing large footprint and potential environmental hazard in the future. However, there are stringent legislations prohibiting the disposal of drill cuttings from OBM offshore (OSPAR 2000). Figure 1.9 shows a summary of drill cuttings disposal options.

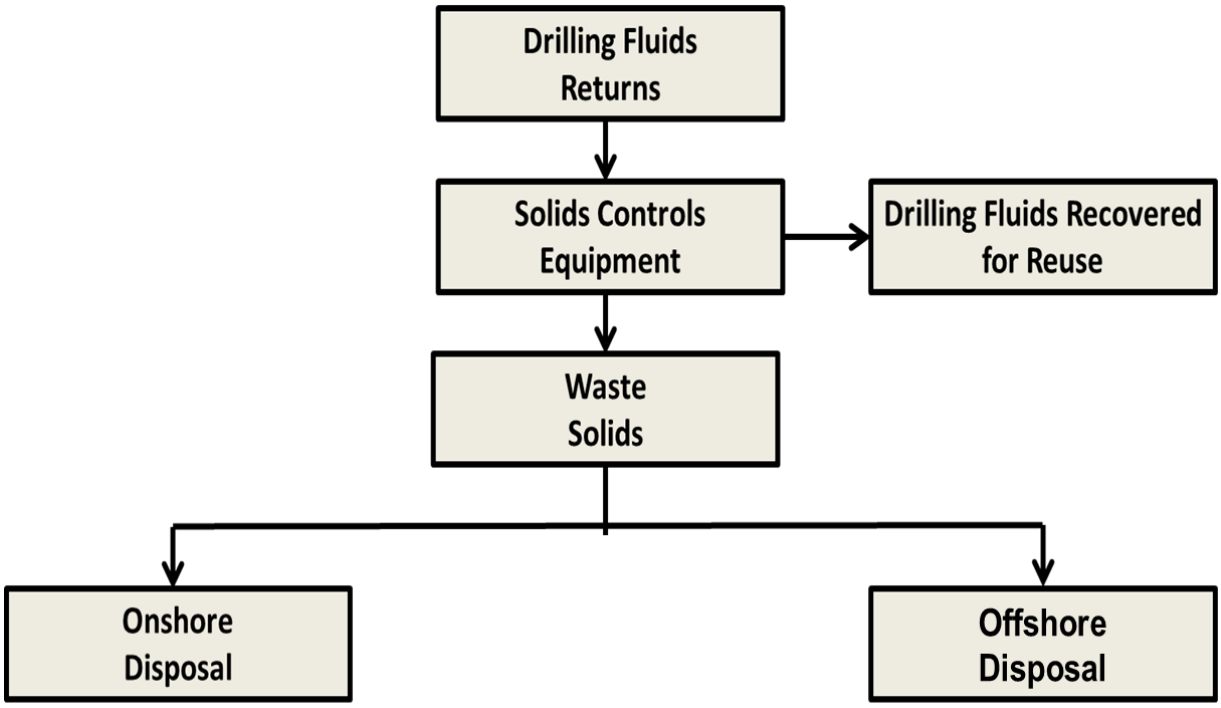


Figure 1.9: Schematic Flow Chart Showing Separation of Drill Cuttings from Drilling Fluids Solid Waste Disposal Options
(Available from: IOGP 2003)

1.5.1. The Offshore Discharge Option

In the past, drill cuttings were discharged indiscriminately to the seabed without treatment, and this impacted the surrounding environment to the platforms negatively (Al-Ansary and Al-Tabbaa 2007). This inappropriate discharge method raised some concerns on the consequent negative impact on the environment and particularly the long term effect on the seabed. Thus, the stringent operational conditions placed by regulatory and environmental agencies, prohibiting the discharge of drilling waste into the marine environment without prior treatment (Hinds et al. 1986) were enacted. There are three choices available to offshore oil and gas operators with regards to drill cuttings disposal offshore. They either grind the cuttings and discharge to seabed, inject to well bore or haul to shore (M-I Swaco 1998). Again these options depend largely on the type of drilling fluid utilized in drilling the formation and the regulatory framework operational in the region.

1.5.1.1. Discharge to Seabed

In this instance, drill cuttings from the shale shaker equipment (attached to the rig as shown in Figure 1.4 above) are flushed with water into a central discharge line which extends beneath the sea surface (CAPP 2001). However, current HSE regulations imposed by regulatory and environmental agencies only allow drill cuttings from WBF to be discharged to sea. Consequently, the drill cuttings from used WBF must be analysed to determine the concentration levels of contamination before discharged to seabed (Caenn, Darley and Gray 2011).

1.5.1.2. Cuttings Re-Injection (CRI) – Onsite Injection

Cuttings re-injection is basically a waste disposal procedure whereby drill cuttings and other oilfield waste are screened, ground to small particle size, mixed into slurry with the addition of water and pumped at a high pressure into an injection well (Ezell et al. 2011; Veil 2002). There are two forms of slurry injection: annular injection and injection into a disposal well. In annular injection, both solid and liquid waste are milled into slurry and pumped into the annulus between the casing strings down the subsurface fracture (M-I Swaco 1998). The injection into a disposal well involves; injecting the slurry either to a part of the drilled hole that is beneath all casing strings or to a part of the well bore that has been fractured with several holes at the depth of an injection formation (Ezell et al. 2011). This disposal method has its operational and environmental disadvantages as it is expensive to run with inconsistent efficiency and there may be possible breaches to the seafloor if incorrectly designed (Caenn, Darley and Gray 2011).

1.5.2. The Onshore Treatment and Disposal Option

The drill cuttings that do not meet the criteria for disposal offshore or re-injection into the well bore are typically transported to shore for treatment and disposal (CAPP 2001). Drilled cuttings processed from the shale shaker, are stored in steel boxes called skips and transported to shore for treatment and disposal (Caenn, Darley and Gray 2011), commonly referred to as "*skip and ship*" in the oil and gas industry (CAPP 2001). Different technologies have been developed and utilised in treating drill cuttings onshore. These technologies range from physical, chemical and thermal treatment and are not without some advantages and disadvantages (Mokhalalati, Al-Suwaidi and Hendi 2000).

1.5.2.1. Land Treatment

Land treatment of drill cuttings can be performed by land spreading, land farming and landfill. Land-spreading involves dispersing untreated cuttings uniformly over a piece of land and then tilling evenly with the addition of nutrients, water and air to initiate biodegradation by oil degrading bacteria (Melton et al. 2003). In land farming the background soil characteristics and biological population present in the soil is utilised in rendering the waste into by-products (e.g. carbon dioxide) of aerobes such as aerobic bacteria (Hinds et al. 1986). Landfill however, can be used to dispose inert, non-recyclable substances, and stabilized drilling waste (Visser and Larderel 1997). The major risk associated with land treatment of drill cuttings is pollution of groundwater if seepage and leaching are not controlled.

1.5.2.2. Thermal Treatment Technologies.

Thermal treatment technology utilises high temperatures to treat hydrocarbon polluted waste, and it is suggested to be the most efficient method for destroying organics present in waste (Aird 2008). There are two basic thermal treatment techniques for drill cuttings treatment;

A. Thermal Desorption Technique

This treatment technique involves placing the drill cuttings in a treatment unit, applying heat until the liquids (oil on cuttings and possibly water) are volatilised and re-condensed into water and non-aqueous based fluid (Melton et al. 2003). The resulting waste streams: oil, water and solids will then be separated for further treatment before disposal or reuse. The solid residue from the treated drill cuttings (usually contains heavy metals and salts) can be disposed by landfill or reused as construction material (Aird 2008). However, seepage of the metals to ground and leachate from the construction materials can be a potential source of pollution to the environment. Potentially, the recovered oil can be recycled and used to power the unit or utilities.

B. Incineration Technique:

This technique involves heating and oxidizing (hydrocarbons) the drill cuttings at high temperatures (between 1200 to 1500 ^{o}C), thus reducing the level of pollutants in the drill cuttings (Aird 2008). The residue may be

further treated by stabilization before disposal to stop the constituents (usually salts and heavy metals) from leaching into the environment (Melton et al. 2003). Incineration technology is used to destroy organic waste that are difficult to breakdown by biological means, pose high risk to human health and the environment, highly flammable and highly toxic. However the process of incineration is not environmentally friendly (Mokhalalati, Al-Suwaidi and Hendi 2000) due to the noxious emissions from the process. Also the extensive high energy requirement and cost of the thermal treatment technology has rendered it uneconomical and unsustainable for drill cuttings treatment (CAPP 2001).

1.5.2.3. Stabilization/Solidification Treatment Technology

Stabilization is a treatment technique whereby the hazard potential of a waste is reduced by converting the pollutants in the waste into a less soluble, immobile and less toxic form, while solidification involves the encapsulation of the waste in a monolithic solid matrix of high structural integrity (Conner and Hoeffner 1998). The stabilized/solidified treated waste must be in a form fit for storage and suitable for land filling or reuse as construction material (Al-Ansary and Al-Tabbaa 2007).

In the stabilization and solidification (S/S) treatment technique of drill cuttings, the cuttings are mixed with a binding agent (such as cement or lime) to stop the oily or organic component on the cuttings from seeping out, thus encapsulating the waste (Chen, Lin and Lin 2007). Cement however, is a common stabilization material based on the fact that its compression and compaction potential are steady over time thus reducing the surface area available for transferring the pollutants to the environment and also preventing fluid mobilization through the entire solid matrix (Razmgir, Afsari and Amani 2011). The major disadvantage of the S/S treatment of drill cuttings reported so far are; increase in waste volume (CAPP 2001), and the likelihood of leachability and interference of increased salt content (especially chlorides) with reinforced concrete (Al-Ansary and Al-Tabbaa 2007).

1.5.2.4. Dispersion-by-Chemical Reaction (DCR) Technology

Gurdarshan and Giles (1995) claimed that, "the Dispersion-by-Chemical-Reaction (DCR) technologies is a collection of patented waste treatment procedures

developed by Professor Friedrich Boelsing over 40 years ago in Europe for the stabilization of heavily oiled sludges, water-in-oil emulsions, oil-contaminated soil and industrial wastes such as acid-tars" (Gurdarshan and Giles 1995).

Similar to the S/S method, the DCR technique encapsulates waste as well and has been applied in a study in partnership with Tasmania Limited to treat drill cuttings in Nigeria (Ifeadi 2007). Basically, the DCR procedure involves treating the drill cuttings with hydrophobized calcium oxide to produce a dehydrated soil-like matter. The non-aqueous liquid phase of the drill cuttings are converted into solid phases, and it becomes insoluble via a non-reversible fixation of the water leachable components of drill cuttings waste.

The resulting immobile solid material can then be utilized as a construction material (Ifeadi 2007). However, the DCR technology is best when treating waste (organics) in the liquid phase (Gurdarshan and Giles 1995). Figure 1.10 below shows an interlocking block (construction material) produced using the DCR treated drill cuttings (Ifeadi 2007).

Figure 1.10: Interlocking Bricks Produced with DCR Treated Drill Cuttings.
(Available from: Ifeadi 2007).

1.5.2.5. Surfactant Enhanced Washing

Surfactants or surface active agents are compounds that lower or reduce the interfacial tension between two immiscible liquids or a liquid and a solid surface (Pereira et al. 2013). Surfactants are amphiphilic compounds, having both hydrophilic head group (water soluble) and hydrophobic tail group (water insoluble)

at either ends of the molecule chain (Dhanarajan and Sen 2014). Their amphiphilic structure enables the surfactant molecule to reduce interfacial tension at interfaces between fluids with different polarities such as; oil and water by aggregating at the fluid's interfaces (Soniyamby et al. 2011), thus increasing the solubility and movement of the oil within the water (Hogan et al. 2014).

Studies show that surfactants have been used to clean soils and sands contaminated with crude oil (Paria 2008), and surfactant based remediation methods for organic contaminated soil is gaining increasing attention (Rufino et al. 2013: Peng, Wu and Chen 2011). Muherei and Junin (2007) in their work on investigating the potential of surfactant washing to solve drilling waste environmental problems offshore, discovered that, "mixtures of anionic and nonionic surfactant were found to be excellent candidates for robust cleaners," for drill cuttings. The technique however, was considered as "*promising*" but will require more research and development to be utilized for offshore application.

However, most of the surfactants used in the industry are synthesized from chemicals of petroleum origin, and are mostly non-biodegradable (Makkar and Cameotra 2002). This research focuses on the utilization of biodegradable surfactant for the treatment of oil based mud drill cuttings.

1.6. Research Aim and Objectives

The aim of this research is *to develop an eco-friendly sustainable alternative for the removal of petroleum hydrocarbons in OBM drill cuttings and diesel contaminated soil, and explore the possibility of reusing same as a construction material* thus advancing the global quest for a clean and green environment. This will be achieved through the completion of the following objectives;

1. To carry out an extensive literature searching in order to understand the background on the subject area, critically reviewing the different treatment methods that have been applied in the past, finding out their advantages and disadvantages, in order to identify by research an environmentally sustainable treatment method.
2. To characterise the OBM drill cutting and soil samples utilising different analytical methods in order to investigate the properties of the sample, as well as determine the TPH concentration in them.
3. To identify a sustainable biological treatment method for oil contaminated solid (OBM drill cutting and soil samples), that is environmentally friendly, economically viable and technically practicable, with end products that will be innocuous to the environment when reused.
4. To identify and culture microorganisms that can be used for the biological treatment process.
5. Characterise the biological treatment compound, assessing the suitability and efficiency of the biosurfactant for the removal of TPH oil contaminated waste.
6. To carry out biosurfactant enhanced washing experiments to assess the efficiency and sustainability of the biological treatment compound for the removal of TPH in OBM drill cuttings and diesel contaminated soil. This would include optimisation of the cleaning or washing conditions with the biosurfactant on the oil contaminated waste.

1.6.1. Research Design

This research being a waste treatment project has been designed following the basic steps in a typical waste management plan. Figure 1.11 shows the key waste management decisions in a typical waste management plan.

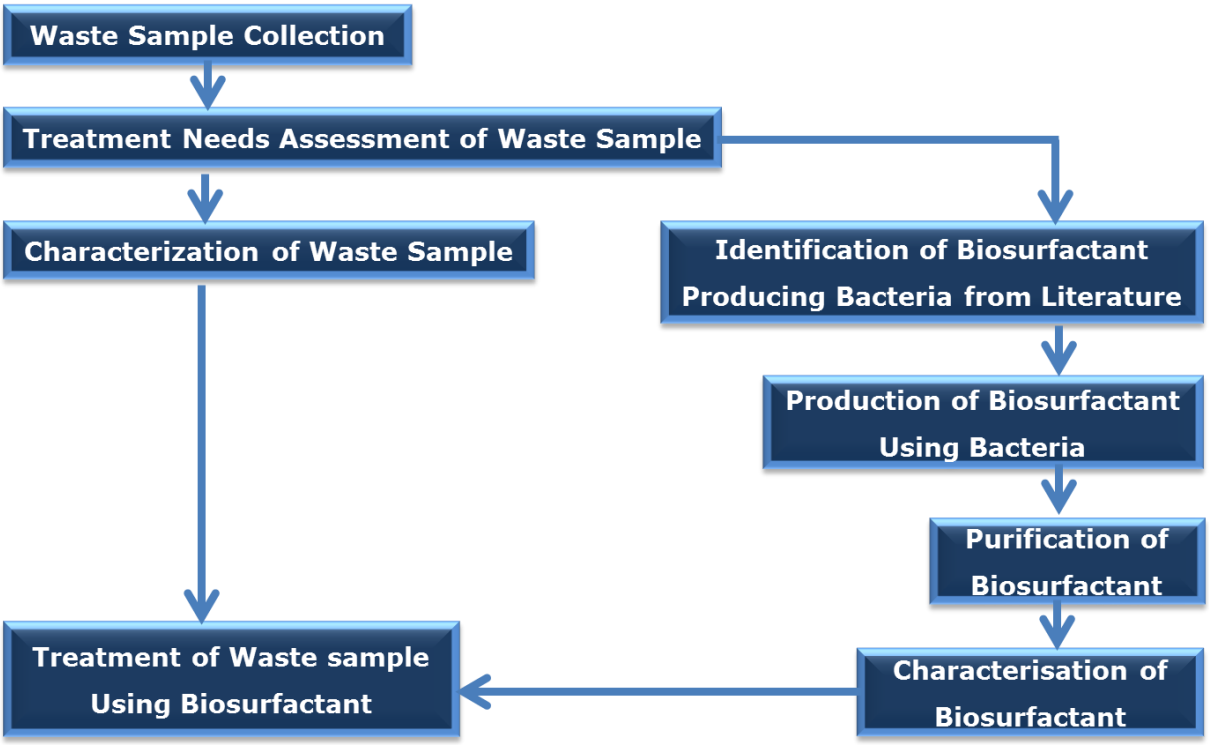


Figure 1.11: Schematic Diagram of Research Plan

CHAPTER 2 – CHARACTERISATION OF OIL-BASED MUD (OBM) DRILL CUTTINGS

2.1. Introduction

The production processes of the petroleum industry generates significant volumes of wastes, one such waste is drill cuttings (Janajreh, Arink and Shehhi 2014). Similar to the production of saw dust when a hole is drilled into a piece of wood with a domestic drill, so are pieces of rock or sand (called cuttings) produced when a formation is drilled for oil or gas using a drilling fluid (UKOOA 2002). Formations drilled with OBM drilling fluids, usually generate cuttings contaminated with petroleum hydrocarbons which cause potential human health hazards and thus cannot be disposed offshore.

Drill cutting discharge to seabed or indiscriminately to land has been an issue of concern based on its negative impact on the environment. When oil contaminated drill cuttings are discharged to the seabed, the benthic community found in the location of the discharge is threatened, and most times, all forms of aquatic life existing in the location are adversely affected, and this has negative impact on the food chain as well as the environment (Jonathan 2000; Kinigoma 2001).

The quantity of contaminated drill cuttings generated during a drilling operation requires proper handling and treatment, and as such cannot be disposed offshore due to stringent regulatory laws. Page et al. stated that "it is estimated that the United Kingdom Continental Shelf (UKCS), produces between 50,000 to 80,000 tonnes wet weight of oily drill cuttings annually" (Page et al. 2003).

2.1.1. Toxicity of OBM Drill Cuttings

The toxicity of drilling fluid emanates from some of the additives used in its formulation. Cranford et al (1999), investigated the toxicity of oil based mud on adult sea scallops, *Placopecten magellanicus* and recorded high mortalities at concentrations as low as 1.0ppm. Also, Sprague and Logan (1979), evaluated the toxicity of paraformaldehyde (a biocide), capryl alcohol, and 5 other surfactants found in drilling fluids on rainbow trout under controlled conditions, and results

showed lethal effects of the additives on the fish at concentrations less than 100 ppm.

The composition of the drill cuttings renders them heterogeneous and toxic, making them unsafe for disposal offshore or onshore without treatment (Abbe et al. 2009). In reality, the concentration levels of drilling fluids may be higher if the drill cuttings generated during a drilling operation (in tonnes) is disposed offshore. The toxicity of substances on living organisms vary and also, some benign substances can be toxic at high concentrations. Understanding the toxicity rating of chemicals used in the marine environment is important because it gives an understanding of the risk associated with the use of the chemicals at certain concentrations (IOGP 2016).

To this end, the Joint Group of Experts on Scientific Aspects of Marine Environmental Protection (GESAMP), established a globally harmonized system for rating (Table 2.1 below) the toxic effect of substances on aquatic life (GESAMP 2014).

Table 2.1: Revised Globally Harmonized Acute Aquatic Toxicity Rating System.
(Available from: GESAMP 2014)

Rating	Description	LC/LL50, EC/EL50, IC/IL50 (ppm)
0	Non-toxic	>1000
1	Practically non-toxic	>100 – ≤1000
2	Slightly toxic	>10 – ≤100
3	Moderately toxic	>1 – ≤10
4	Highly toxic	>0.1 – ≤1
5	Very highly toxic	>0.01 – ≤0.1
6	Extremely toxic	≤0.01

Key
LC/LL50: lethal concentration/lethal loading required to kill 50% of the population.
EC/EL50: effective concentration/effective loading of a drug that gives half-maximal response.
IC/IL50: inhibitory concentration/inhibitory loading where the response (or binding) is reduced by half.

The GESAMP rating was achieved using acute toxicity test data as it was considered the most practical test utilized for toxicity assessment with respect to the aquatic food chain. Microalgae, crustaceans and fish were used to generate the acute toxicity test data used for this rating. Also, all tests were carried on the same basis following international guidelines (GESAMP 2014).

Also the Centre for Environment, Fisheries and Aquaculture Science (CEFAS) United Kingdom ensures that, "chemicals are ranked according to their calculated hazard quotients (HQ) by the CHARM (Chemical Hazard Assessment and Risk Management) mathematical model, which uses toxicity, biodegradation and bioaccumulation data provided by suppliers on the Harmonised Offshore Chemical Notification Format (HOCNF) form" (CEFAS 2018). The Offshore Chemical Notification Scheme (OCNS) Team at CEFAS registers chemicals used in offshore oil and gas applications for use in the UK and Netherlands waters. The HQ is converted to a colour banding, as illustrated in Table 2.2.

Table 2.2: OCNS HQ and Colour Bands

Minimum HQ Value	Maximum HQ Value	Colour Banding	
>0	<1	Gold	
≥1	<30	Silver	Lowest Hazard
≥30	<100	White	
≥100	<300	Blue	
≥300	<1000	Orange	Highest Hazard
≥1000		Purple	

Some examples of contaminants (of concern) present in drill cuttings include; petroleum hydrocarbons which includes aliphatic hydrocarbons and aromatic hydrocarbons (including polycyclic aromatic hydrocarbons, (PAHs)), heavy metals such as lead, zinc, mercury, chromium, arsenic, nickel, cadmium and naturally occurring radioactive materials (Leonard and Stegemann 2010: Clark 2002). All these contaminants could cause potential hazard on aquatic life if disposed without treatment. The contaminants being investigated in this research are: heavy metals and petroleum hydrocarbons.

2.1.1.1. Heavy Metals

Heavy metals are naturally occurring elements that have a high atomic weight and a density at least 5 times greater than that of water i.e. 5 gmL^{-1} (Colin, Villegas and Abate 2012: Tchounwou 2012). Díaz, Martín-González and Gutiérrez (2006) classified heavy metals as persistent pollutants in the environment which are toxic and difficult to degrade, and as a result can be accumulated through the food chain in a process called bioaccumulation, causing potential health risk to living organisms in the environment.

The effect of heavy metals on human health has been studied and reviewed by numerous organisations such as the World Health Organisation (WHO) and the Agency for Toxic Substances and Disease Registry (ATSDR), (Järup 2003). Some toxic effects of heavy metals on humans include cancer which can be caused by arsenic (Saha, et al. 2016), kidney damage caused by cadmium and anaemia caused by lead (Pb) (Chowdhury et al. 2016). Typical values for heavy metals and barium content on OBM-DCS as found in literature is shown in Table 2.3 below.

Table 2.3: Typical Values for Heavy Metal Content in OBM-DCS

Heavy Metal	Heavy metal content in OBDC (mg/kg)		
	Schumacher et al. 1991	Kujawska and Cel 2017	XU et al. 2018
Copper	18.2	104.29	75.51
Zinc	79.21	62.1	642.84
Lead	30.16	41.92	345.13
Nickel	15.21	21.75	67.08
Chromium	8.42	65.76	65.52
Cadmium	0.3	ND	5.02
Mercury	0.29	NA	0.18
Manganese	148.77	469	25,229.92
Cobalt	N/A	0.2	N/A
Vanadium	23.05	N/A	N/A
Barium*	775.1	1911.33	N/A

Key: N/A: Not available

Studies show that, the accumulation of untreated mud and cuttings on the seabed (cutting pile) could potentially lead to increased concentrations of heavy metals in the environment when the piles are disturbed (Neff 2005; OSPAR 2009). Continual disposal of untreated drilling waste offshore potentially decreases the oxygen levels available for the life forms present in the environment. Another source of heavy metal introduction into the marine environment is via the irresponsible disposal of produced water containing heavy metals and other toxic compounds offshore (Clark 2002). Breuer et al. (2004) also suggested that, the cause of increased concentration of heavy metals detected drill cutting piles could emanate from two sources; the first being from the accumulation/dispersion from the natural sediment and the second from barite and chemicals present in the drilling mud.

Based on the human health risk associated with heavy metal contamination from untreated drill cuttings, it becomes necessary to treat the drill cuttings in a sustainable manner before disposal.

2.1.1.2. Petroleum Hydrocarbon Contamination

One major source of petroleum hydrocarbon contamination is from the reservoir formation being drilled (IPIECA 2009). The total petroleum hydrocarbon (TPH) is a parameter used in determining the gross level of contamination from petroleum hydrocarbon sources such as crude oil, lubricants, fuels etc (Schwartz, Ben-Dor and Eshel 2012). Basically, petroleum hydrocarbons contamination of the environment can occur in the 3 sectors of the oil and gas industry (upstream, midstream and downstream sectors). This usually happens through disposal of drilling waste, accidental spills and pipeline leakages. When this happens, the lighter fractions may evaporate or float (in case of surface water accidents), while the heavier fractions will accumulate in the sediments or seabed. The presence of petroleum hydrocarbons in the water has potential detrimental effect on the life forms found in the environment especially the bottom- feeding organisms such as benthic organisms (ATSDR 1999; Henry et al. 2017). Mostly due to the lipophilic and toxic nature of the aromatic components such as benzene and PAHs (Rocha et al. 2011).

Breuer et al. (2004) claimed that, "elevated hydrocarbon concentrations, up to 10,000 times background, have been found in the sediment and cuttings surrounding oil production platforms in the North Sea." This concentration is quite high and may cause potential harm to life forms if no remediation work is carried out on the sediments. It is expedient that drill cuttings be treated and disposed according to regulatory recommendations, because the occurrence of petroleum hydrocarbon in sea food is a potential human health hazard (Ansari, Desilva and Badesab 2012) such as acute central nervous system depression from the BTEXs (ATSDR 1999).

2.1.2. Aim

The aim of this chapter is to assess contaminant levels of the samples under study. This chapter reports the determination of total petroleum hydrocarbons (TPH) and heavy metals found in drill cuttings samples collected from a North Sea operation known to have been drilled with an oil based drilling mud and contaminated soil sample.

2.2. Materials and Methods

2.2.1. Samples

The oil based mud drill cuttings used for this work were obtained from an anonymous source. Freeze-dried sediment samples were used for the validation of the extraction method. Oil contaminated soil samples obtained from an undisclosed petroleum contaminated land were used as surrogate samples for the oil based mud drill cuttings. Figure 2.1 below shows the OBM drill cuttings as received.

Figure 2.1: Oil Based Mud (OBM) Drill Cuttings As Received

2.2.2. Chemicals and Reagents

Standard laboratory reagent (SLR) grade of *n*-pentane was used to wash the drill cuttings for particle size analysis. ICP grade standards of the following elements (individually at 10,000ppm): aluminium (Al), magnesium (Mg), barium (Ba), titanium (Ti), manganese (Mn), potassium (K), mercury (Hg), vanadium (V), cadmium (Cd), chromium (Cr), copper (Cu), cobalt (Co), iron (Fe), nickel (Ni), arsenic (As), lead (Pb), zinc (Zn) and gold (Au) were purchased from Fisher Scientific, UK. Analytical grade concentrated nitric acid (HNO_3, 70%) and concentrated hydrochloric acid (HCl) were used for the digestion of the drill cuttings. Anhydrous grade tetrachloroethylene (perklone) was used as extraction solvent for the TPH analysis and HPLC grade dichloromethane (DCM) was used for rinsing the glass ware used. All reagents were supplied by Fischer Scientific, UK, except for tetrachloroethylene, which was supplied by Sigma Aldrich, UK. Diesel

used for TPH analysis was procured from a BP filling station. Procedural blanks were run with individual analysis (where necessary).

2.2.3. Particle Size Distribution (PSD)

Particle size analysis is a descriptive analysis that classifies the size differences within the granular samples. It is important to measure the particle size of contaminated granular samples before treatment, due to potential correlation between pollutant levels and particle sizes as observed in a study carried out by Scott et al. (2009). Particle size analysis was carried out to determine the textural classes within the drill cuttings and soil samples. The PSD OBM-DCS was analysed using sieve analysis, while the soil sample was analysed by laser diffraction technique.

2.2.3.1. Particle Size Distribution Analysis of OBM-DCS by Sieve Analysis

The analysis was performed by washing approximately 30 g of the sample with *n*-pentane to remove the oil present in it. The washed drill cutting sample (DCS) was placed on a watch glass and allowed to air dry in the fume-hood for 24 hours. After which, the sample was stored in a desiccator for 48 hours to ensure that it was without any moisture. A Griffin sieve fitted with the following United States of America (U.S.A) mesh size; 10, 20, 40, 60, 80 and 100 was used for the sieve analysis. Approximately 20 g of the dried sample was transferred into the Griffin sieve analyser, and was screened thorough the following mesh sizes by agitating at 20rpm for 5 mins. The weight fraction in each mesh was classified according to its corresponding textural class, following the U.S.A. sieve series and Tyler mesh size equivalent (Kuo and Acharya 2012).

2.2.3.2. Particle Size Distribution Analysis of Soil Sample by Laser Diffractometry

Laser diffraction technology is useful for particle size measurement. The technique is simple, flexible and automated. Laser diffraction technique is well utilized in particulate processing industry for measuring particle size and particle size distribution (Levoguer 2013; Beuselinck et al. 1998). Levoguer (2013) explained that the laser diffraction technique functions by exploiting "the Mie theory of light,

which relates the scattering pattern produced as light passes through a sample to the size of any particles present. Large particles scatter light strongly at small angles to the incident ray while smaller particles scatter more weakly at wider angles. Through the analysis of detected angular scattering intensity data it is, therefore, possible to determine particle size and distribution." Some advantages of utilizing the laser diffraction technique for particle size distribution and analysis includes; speed, flexibility, high reproducibility, easy to use and it does not require calibration (Levoguer 2013; Xu 2002).

The analysis was carried out by wet dispersion method using a Malvern Mastersizer Basic, equipped with software version B.0, a Malvern QS small volume sample dispersion unit and Malvern in/out measuring cell. A beam width of 2.0 mm was used for the particle size distribution analysis of the soil sample. The analysis was performed by dispersing approximately 3 g of the samples in about 5 mL of water. The samples were added to the instrument until 10% obscuration is achieved.

2.2.4. Scanning Electron Microscope (SEM) – Energy Dispersive X-ray Analysis (EDXA).

SEM-EDXA was carried out to investigate the morphology and elemental composition of the samples (OBM-DCS and soil), before and after treatment. The SEM used for this analysis was a Zeiss EVO LS10 and the X-ray analyser was an Oxford Instruments INCA system. The system was set to chamber pressure of 100 Pa, magnification of 2000 times, working distance (WD) of 8.5 mm, and accelerating potential of 20 kV.

The procedure for the SEM-EDX analysis was carried out following a method obtained from the Zeiss EVO User Manual (Zeiss 2008). The samples were fixed to an aluminium SEM stub on which a double sided adhesive has been placed, and the stub was placed in the sample chamber for analysis. Control of the microscope was carried out using Zeiss Smart SEM software running on a Microsoft Windows XP operating system. All the microscope functions were executed using the main control console or by accessing the appropriate windows menu. The elemental content of the samples and photomicrographs were read and recorded on the instrument.

2.2.5. Microwave Assisted Digestion of Drill Cutting Samples

A microwave assisted acid digestion of the OBM drill cutting samples was carried out to extract the elements from the drilling cuttings into solution for Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) analysis. This analysis was carried out on the OBM-DCS alone (as received, washed with perklone and after treatment). A Milestone Ethos EZ Microwave Digestion instrument, (fitted with a touch-screen terminal, with an easy control software which runs the temperature control programme) was used for the digestion of the samples. The digestion was carried out following a modified procedure from the United States Environmental Protection Agency (EPA) Method 3051A (USEPA 2007a). Approximately 0.5 $\pm$ 0.1g of the sample was weighed into a digestion vessel in 3 replicates, to which 8 $\pm$ 0.1 mL of aqua regia (9 $\pm$ 0.1 mL concentrated nitric acid and 3 $\pm$ 0.1 mL concentrated hydrochloric acid) was added. A procedural blank sample (without the drill cuttings samples) was digested as well.

The samples were digested with the following temperature controlled programme: the temperature of the microwave digestion system was ramped to 200^{o}C over 15mins, and held constant at this temperature for another 15mins, and there after allowed to cool to room temperature for 30mins. The digested samples were quantitatively washed through a filter paper and transferred to a 50mL volumetric flask (cleaned previously with 10%v/v HNO_3) and analysed on the ICP-OES. The digests were diluted (50 times) prior to ICP-OES analysis.

2.2.6. ICP-OES Analysis of Digested Drill Cuttings Samples

ICP-OES analysis was carried out on the digested drill cuttings extract to determine the concentration of the following elements in the drill cuttings: Al, Mg, Ba, Ti, Mn, K, Hg, V, Cd, Cr, Cu, Co, Fe, Ni, As, Pb and Zn. The analysis was carried out using a Perkin Elmer Optical Emission Spectrometer Optima 7000 DV instrument, equipped with WinLab 32 version 4.0 software. The operating conditions for the ICP-OES used for this analysis is shown in Table 2.2 below.

Table 2.4: Operating Conditions for ICP OES with Axially Viewed Setting

Parameter	Setting/Value
Spectral purge gas flow	Normal
RF incident power (W)	1300
Spray chamber	Scott-Type (cyclonic)
Nebulizer	Gem-Cone
Plasma gas flow rate (L/min)	15
Plasma conditions	Vary by element
Auxiliary gas flow rate (L/min)	0.2
Replicate read time (s)	Auto
Nebulizer argon gas flow rate (L/min)	0.8
Instrument stabilization delay (s)	60
Pump rate (rpm)	15
Wash Frequency	Between Samples
Rate (mL/min)	1.5

2.2.6.1. *Calibration of ICP-OES Instrument*

The ICP was calibrated by preparing 100mL of 100ppm stock solutions of 2 mixed standards containing the following elements:

Mixed standard A: Cd, Cr, Cu, Co, Fe, Ni, As, Pb and Zn.

Mixed standard B*: Al, Mg, Ba, Ti, Mn, K, Hg, and V.

*Gold (Au) was added to mixed standard B to keep the mercury in solution.

The mixed standards A and B were used to prepare calibration standards with the following concentration levels: 0.1, 0.5, 1.0, 2.5, 5.0, 10.0ppm. A calibration blank of deionized water was also run with the calibration standards. All elements were run at specific wavelengths as shown in Table 2.3, and graphs plotted to assess linearity.

Table 2.5: Selected Wavelengths of Elements Analysed

S/N	Element	Wavelength (nm)
1.	Al	396.153
2.	Mg	285.213
3.	Ba	233.527
4.	Ti	334.940
5.	Mn	257.610
6.	K	766.490
7.	Hg	253.652
8.	V	270.093
9.	Cd	228.802
10.	Cr	267.716
11.	Cu	327.393
12.	Co	228.616
13.	Fe	238.204
14.	Ni	231.604
15.	As	188.980
16.	Pb	220.353
17.	Zn	206.200

2.2.7. Determination of the Hydrocarbon Profile of OBM Drill Cuttings by Gas Chromatography – Mass Spectrometry (GC-MS) using Head Space Solid Phase Micro Extraction (HS/SPME)

The HS-SPME, is a solvent-free sample preparation technique, in which a fused silica fibre coated with polymeric organic liquid is introduced into the headspace above the sample (whilst being heated to extract the volatile organic analytes in the sample). The volatilized analyte is adsorbed on the exposed fibre for a few minutes and then transferred to the GC for desorption and analysis (Zhang and Pawliszyn 1993).

The hydrocarbon profile of the OBM drill cuttings was determined by extracting the hydrocarbons using an HS/SPME method for analysis using the GC-MS. Approximately 1g of the drill cutting sample was weighed into a 20 mL glass vial (pre-washed with acetone and pre-dried in an oven at 100^{o}C). The vial was tightly capped with a Teflon septum. The glass vial was heated at 60^{o}C for 10 minutes on a heating block, after which a 100 µm CAR/PDMS SPME fibre (Carboxen/Polydimethylsiloxane Solid-phase micro extraction fibre), preconditioned following the manufacturer's instructions was inserted into the headspace above the sample being heated. Care was taken to avoid the needle/fibre from touching the sides of the vial and the sample. The fibre was exposed for 15 mins, retracted back into the needle and immediately desorbed for 30 mins in the GC injector port.

The hydrocarbon profile of the OBM drill cuttings extracts (extracted using HS/SPME) was determined using an Agilent HP 6890 gas chromatograph equipped with an Agilent 5971A mass selective detector. A non-polar Zebron ZB-5 column (30 m × 0.25 mm id, 0.25 µm film thickness; Supelco, UK; 5% phenyl, 95% dimethylpolysiloxane) was used for the analyses with helium as the carrier gas, controlled using the constant flow mode at 1.0 mL min^{-1}. Injections were made by placing the SPME fibre into the GC inlet in the splitless mode. The oven temperature programme of the GC was set as follows: initial oven temperature was set at 45.0^{o}C, and it was held at this temperature for 5 mins and then raised to 300^{o}C at 6°C/min and held for another 5 mins. The mass spectrometer was operated in the electron impact mode at 70eV and scanned in the range of m/z 40 – 450 in the full scan mode. The hydrocarbons found in the chromatogram/mass

spectra were confirmed using mass spectral NIST (National Institute of Standards and Technology) library software installed on the system. The HS-SPME technique minimises sample preparation steps and concentrates volatile analytes without the use of solvents. Figure 2.2 below shows 2 schematic diagrams showing;

A. Sample extraction using the SPME: analytes are adsorbed to the fibre-coating from the headspace of sample.
B. Sample desorption into the GC: analytes are desorbed from the fibre coating to the GC inlet

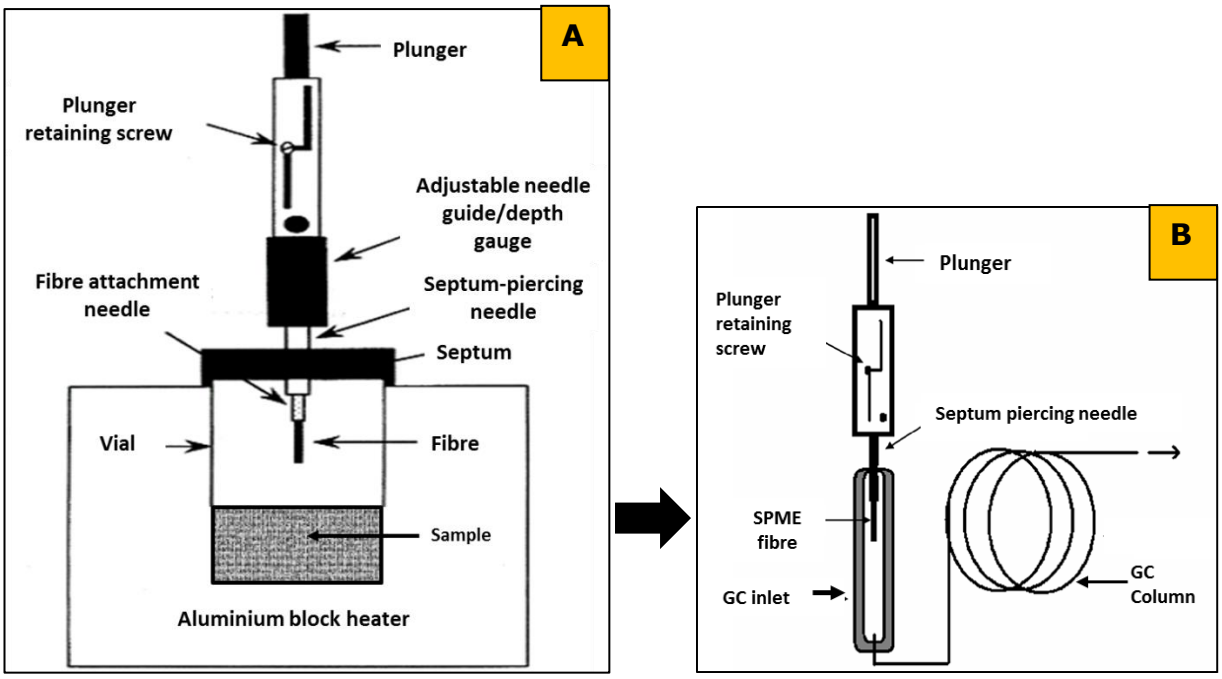


Figure 2.2: Schematic diagram of a HS-SPME system

(Available from Wang, McCaffery and Norwood 2008)

2.2.8. TPH Analysis of OBM-DCS and Soil using FTIR

The TPH content of the oil contaminated samples were determined using Fourier Transform Infrared (FT-IR) spectroscopy as described by Farmaki et al. (2007). A Perkin Elmer Spectrum BX FT-IR spectrophotometer was used for this analysis.

2.2.8.1. Calibration of FTIR Instrument for TPH Analysis

The FTIR instrument was calibrated following a modified DECC IR method (DECC 2013), using diesel in perklone standards. A 10,000ppm stock solution was prepared by adding 1.0 ± 0.1g of diesel in 100mL perklone, from which the following calibration standards were prepared: 20, 50, 100, 200, 300, 400 and 600ppm. Each standard was analysed by running 32 scans on the range of 4000 to 400cm^{-1}. The corrected area of the absorbance spectrum was measured between 3100 cm^{-1} and 2700 cm^{-1}, where hydrocarbons would normally absorb (C-H stretch). A calibration plot of the corrected area versus the calibration standard concentration was plotted to assess linearity of the graph.

2.2.8.2. Determination of TPH Extraction Method.

At the beginning of the experiment, preliminary extractions of TPH from the oil contaminated samples were carried out to determine the most appropriate method for extracting TPH from the oil contaminated sample (OBM drill cuttings). Three (3) extraction methods were applied, namely;

1. Extraction by sonication using a sonication bath.
2. Magnetic stirrer
3. Orbital shaker

The preliminary extractions were carried out using approximately 1 g of oil contaminated sample and 30mL of water as the extraction solvent. The extractions were carried out at room temperature for a duration of 30mins each. At the end of the extractions, the TPH content in the supernatant was discarded, and the residue extracted with 30 mL of perklone and ran on the FT-IR to determine the percentage TPH removal from the sample using the listed extraction methods with water as the extracting solvent. Figure 2.3 shows the extracted residue.

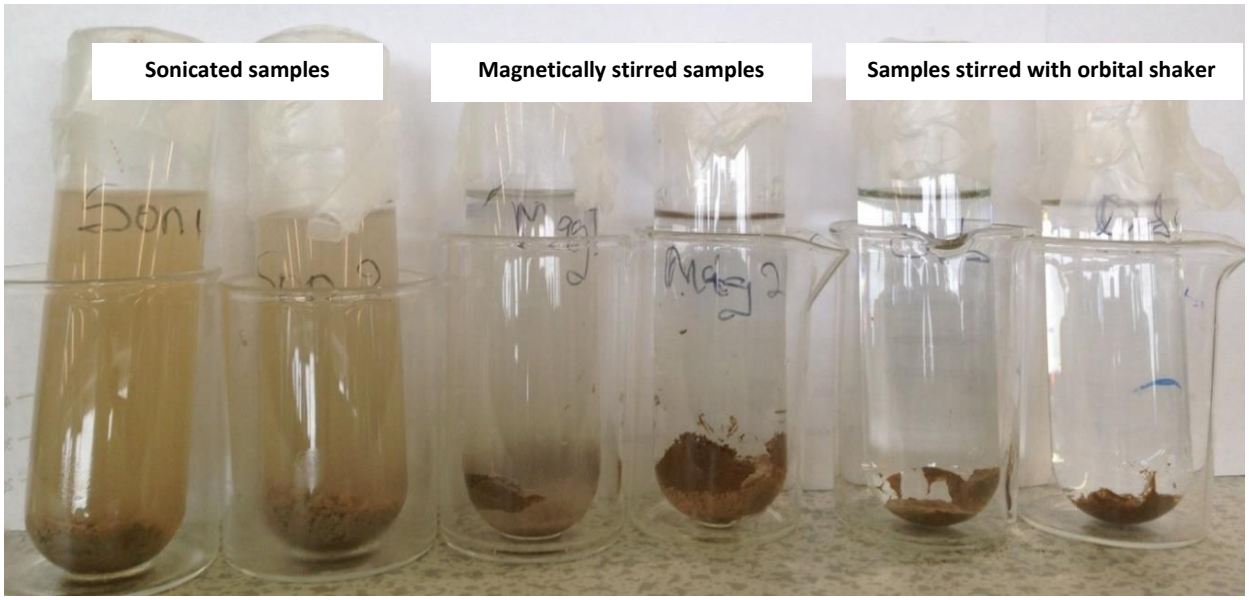


Figure 2.3: Determination of TPH Extraction Method from Contaminated Solids

2.2.8.3. Extraction and Determination of TPH from Samples

The TPH in the oil contaminated samples (OBM drill cuttings and soil) were extracted with perklone following a method adapted from the United States Environmental Protection Agency (EPA) Method 3550C Ultrasonic Extraction, (USEPA 2007b).

The samples were thoroughly mixed and approximately, 1±0.1 g of each sample was weighed in 3 replicates into glass centrifuge tubes. 25mL of perklone was added to each tube and sonicated for 15mins. The mixture was filtered through an Ashless Whatman 125mm filter paper grade 40 supplied by Sigma Aldrich. The filtrate was further diluted and analysed by FTIR. Figure 2.4 shows the OBM-DCS before and after cleaning with perklone.

Figure 2.4: Oil Based Mud Drill Cuttings:
Where (a) As received (b) Cleaned With Perklone

Equation 2.1: Determination of TPH from Calibration Curve

Calibration Equation: $y = mx + c$

Where: y = the absorbance value obtained from the extract
x = concentration of TPH in the extract (mg/L)
m = Slope
c = Intercept

*The value of x (obtained from this equation), is then multiplied by the dilution factor (if the samples were diluted before the FTIR analysis.

The unit of the value obtained from this equation will be in mg/L. To get the TPH concentration (*x*) in mg/Kg, Equation 4 was applied as shown below;

Equation 2.2: Determination of TPH in mg/Kg

$$x\ (mg/Kg) = \frac{x\ (mg/L) * Volume\ of\ extracting\ solvent\ (L)}{Weight\ of\ sample\ (Kg)}$$

2.2.8.4. Recovery Check of extraction method

The recovery check of the adapted EPA extraction method 3550C was assessed for efficiency using freeze-dried sediment samples. 3 sets of the samples were analysed in triplicate as follows:

Set A: Unspiked Set.

Set B: spiked with 100µL of 10,000ppm diesel in perklone standard before extraction with perklone for 15mins

Set C: spiked with 100µL of 10,000ppm diesel in perklone standard before extraction with perklone for 15mins and cleaned with approximately 1 g of florisil.

The TPH in the 3 sets of freeze dried sediment samples were extracted and analysed as described in section 2.2.8.2 above.

2.3. Results and Discussion

A summary of the parameters applied for the characterization of the oil contaminated samples are shown in Table 2.4 below.

Table 2.6: Summary of Techniques Applied for the Characterisation of the Oil Contaminated Samples

S/NO.	Technique	OBM-DCS	Soil
1.	Particle Size Distribution	By Sieve	By Laser Diffraction
2.	SEM-EDXA	√	√
3.	ICP-OES	√	X
4.	GC-MS by HS/SPME	√	X
5.	TPH by FT-IR	√	√

*Where the symbols: √ shows the technique was applied in characterising the sample
X shows the technique was not applied in characterising the sample

A diesel contaminated soil was initially used as a surrogate for the treatment of OBM drill cutting samples. Thus the ICP-OES and the GC-MS by HS/SPME analysis were not carried out on the soil samples and also for time constraints. The experimental data obtained from these parameters are discussed below.

2.3.1. Particle Size Determination

Studies show that pollutants tend to bind more to the finer particles than on the coarse or gravel sized particles (Trzciński, Williams and Żbikb 2015). Also, investigating the particle size distribution of materials is vital when monitoring product performance and consistency which sometimes gives an indication of the purity and quality of the product (Wedd 2005).

2.3.1.1. Particle Size Distribution Analysis of OBM-DCS by Sieve Analysis

Although, laser diffraction technique is usually the technique applied for the determination of particle size in most industries, sieve analysis has been applied here due to the size range of the OBM drill cutting samples. Also, Allen (2003),

commented that, particle size by sieve analysis is uncomplicated, economical to use, and gives reproducible results. The particle size/ textural class of the OBM drill cuttings was determined by using the United States Department of Agriculture (USDA), soil classification system. The result of the particle size distribution carried out on the OBM-DCS is shown in Table 2.5.

Table 2.7: Textural Classification of OBM-DCS using USDA Textural Soil Classification

Available from: USDA 1987

Textural Class	Diameter (µm)	Volume (%)
Fine Clay	<0.2	0
Clay	<2	0
Fine Silt	2-20	0
Coarse Silt	20-50	0
Very fine sand	50-100	16.1
Fine sand	100-250	4.3
Medium sand	250-500	25.5
Coarse sand	500-1000	39.1
Very coarse sand	1000-2000	15.1

The result of this analysis shows that the predominant textural class found in the OBM drill cuttings was coarse sand, making up approximately 40% of the sample. Xu et al. (2014), showed that treating of contaminated soil by soil washing has a higher efficiency with larger particle size (ranging from 2 to 25 mm) than with the sandy samples (between 1 and 0.05 mm). This is because most contaminants such as heavy metals and TPH bind more on the fines (mostly clay and silt), than on the larger particles and the percentage of fines present in this sample is approximately 20%. The proposed sustainable method for treating the contaminated drill cutting samples is by washing with a biosurfactant and the result of this analysis shows the possibility of treating the samples using the proposed method.

2.3.1.2. Particle Size Distribution Analysis of Oil Contaminated Soil Sample by Laser Diffractometry

The result of the particle size distribution of the soil sample carried out using laser diffraction technique is shown in Figure 2.3 below.

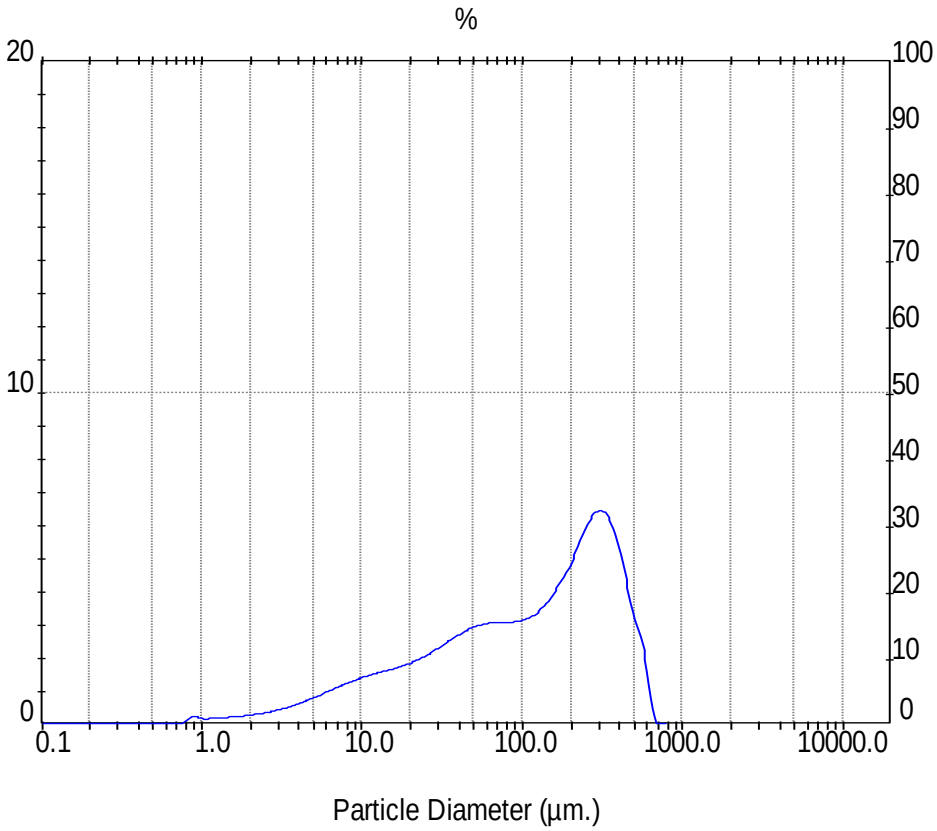


Figure 2.5: Particle Size Distribution (PSD) of Soil by Laser Diffraction Analysis

The data obtained from Figure 2.5 (as obtained from the instrument), was extracted and given as shown in Table 2.6 below.

Table 2.8: Textural Classification of Oil Contaminated Samples using USDA Textural Soil Classification

Textural Class	Diameter (μm)	Volume (%)	
		Soil	OBM-DCS
Fine Clay	<0.2	0	0
Clay	<2	1.5	0
Fine Silt	2-20	15.5	0
Coarse Silt	20-50	14.3	0
Very fine sand	50-100	12.3	16.1
Fine sand	100-250	23.1	4.3
Medium sand	250-500	28.7	25.5
Coarse sand	500-1000	4.7	39.1
Very coarse sand	1000-2000	0	15.1

The data extracted from the graph in Figure 2.5 shows that the soil has approximately 29% of medium sand in it, which potentially means that the intended soil washing to be applied for the cleaning of both samples will probably be more efficient with the cuttings than the soil. The Table from which the PSD data for the soil was extracted shall be seen in Appendix 1. Both results (OBM-DCS and soil) were then compared on a bar chart to show the difference in the results obtained. The bar chart is shown in Figure 2.6 below.

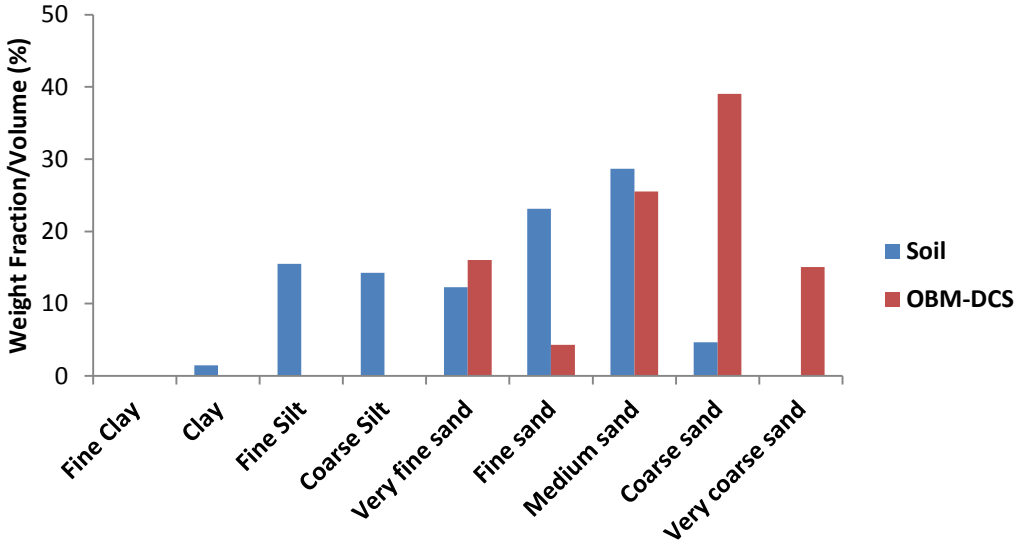


Figure 2.6: Comparing Textural Classes of Soil and OBM-DCS

Particle size distribution analysis is important because it gives useful information about any particulate matter being used in research and technology. Understanding the particle distribution of the OBM-DCS and soil is critical to the soil washing process. However, results from literature shows that contaminants bind more to finer particles because they have larger surface area (Xu 2014; Budianta et al. 2010). Budianta et al. (2010) in their work on the in-situ soil washing by sedimentation claimed that, the PAH contaminant found in the soil, was a function of the particle size of the soil. Their result showed that fine fraction of the soil had the highest PAH levels than the coarse fraction. However, this claim will be confirmed at the end of the washing process (see Chapter 4).

2.3.2. Scanning Electron Microscope (SEM) – Energy Dispersive X-ray Analysis (EDXA).

The SEM-EDXA was used to obtain information about the surface topography and elemental composition of the OBM drill cutting samples using back scattered electrons (BSE) process.

The SEM employs a high energy electron beam to illuminate a specimen for viewing on a monitor screen via a microscope. As the electron beam impinges on the sample a number of different interactions occur resulting in a variety of signals being emitted from the surface. The product of this process is the excitation of electrons in the gas molecules to higher energy states resulting in the emission of light photons upon relaxation of these electrons to the ground state. This process is termed gas luminescence. These light photons are then processed by the gas luminescence detector for viewing on the screen through the electron microscope. One advantage the SEM has over optical microscopy is the far greater depth of field when viewing a sample and it allows rough surfaces to be imaged in sharp focus even at high magnification (Zeiss 2008).

The EDX analysis functions via a BSE process. Upon the application of the high energy electron beam, backscattered electrons (primary beam electrons) escape the sample surface (elastically scattered) without losing much of their original energy, and these electrons are very directional (due to their high energy) as they emerge from the sample and therefore are not easily influenced by applied electrostatic fields. The backscattered electron yield is related to the atomic number of the sample atoms, thus providing an image which is said to have "atomic number contrast," as each element in the periodic table has a different backscattered electron co-efficient (i.e. the higher the atomic number, the greater the generation of backscattered electrons).

As a result of these properties BSE detectors are positioned in "line of sight" of the specimen, and typical images show areas with high atomic number as bright regions and areas with low atomic number as dark regions. Each element in the periodic table has a different backscattered electron co-efficient (Zeiss 2008), thus enabling the identification of the elements via the EDX analysis.

2.3.2.1. Microstructures of OBM Drill Cuttings Sample.

Figures 2.7 and 2.8 below shows the SEM images at 2000X magnification for OBM-DCS and soil (as received) respectively.

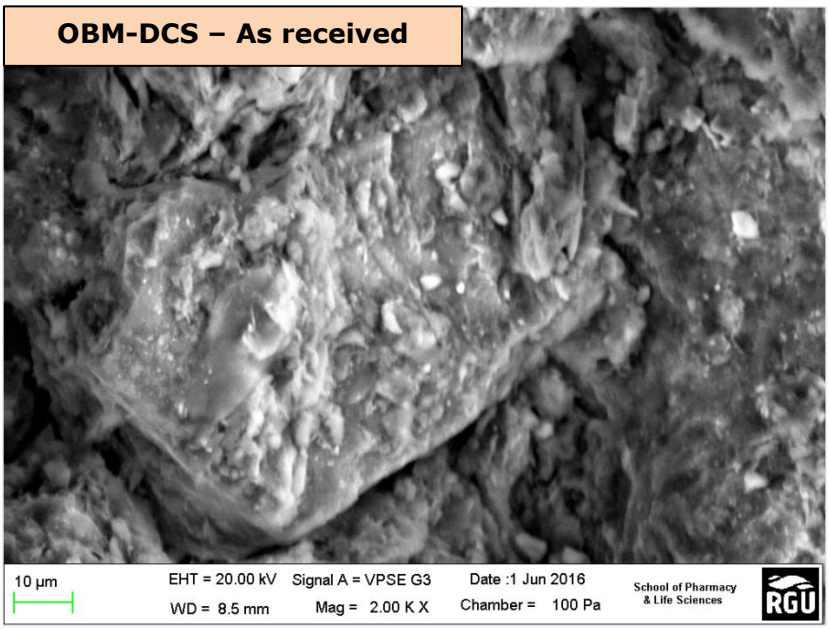


Figure 2.7: Microstructure of OBM Drill Cuttings at 2000X Magnification

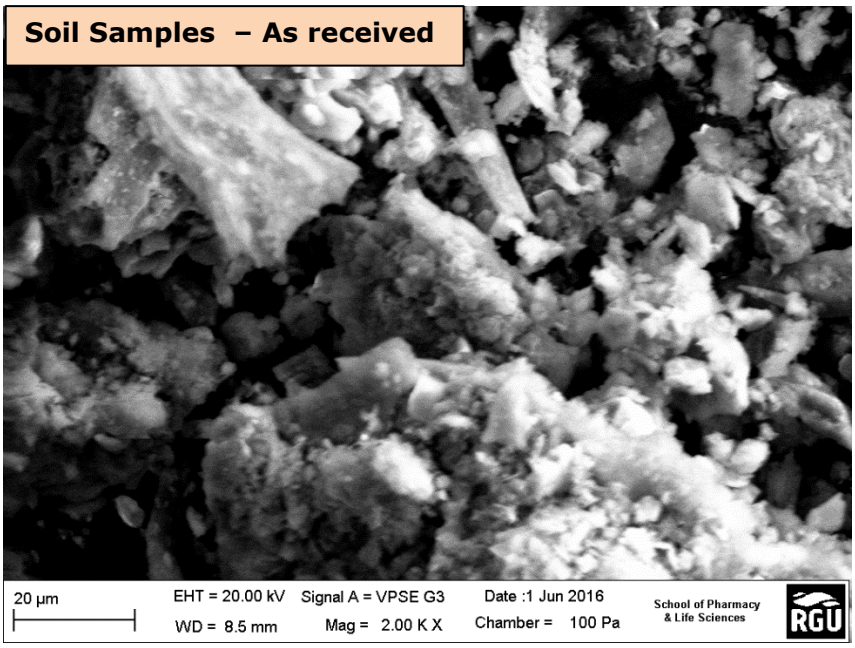
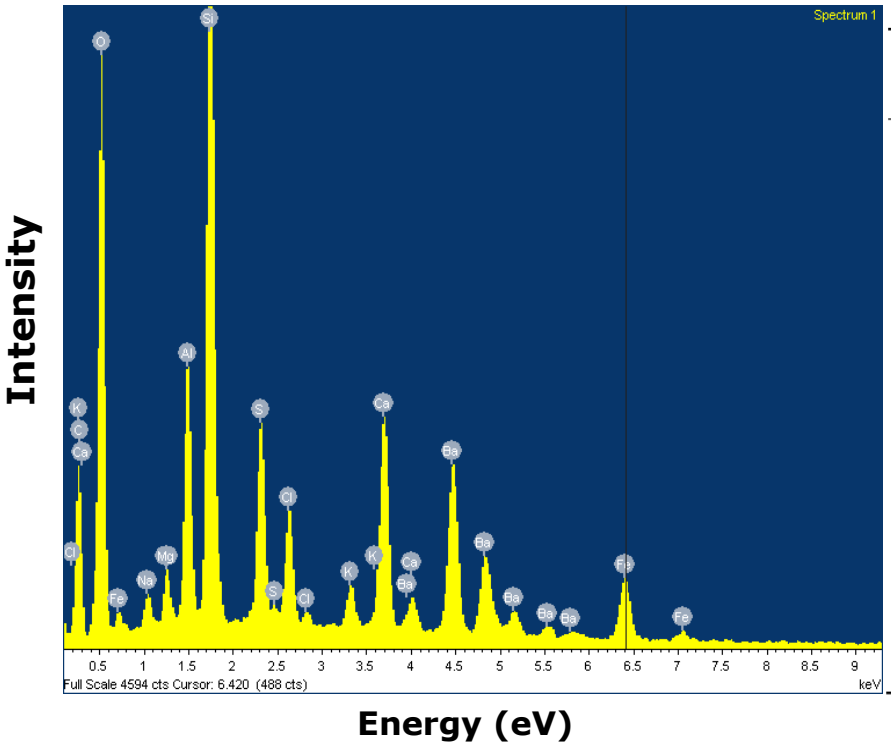


Figure 2.8: Microstructure of Soil Sample at 2000X Magnification

From the Figures 2.7 and 2.8, it can be seen that the grains in the soil samples are more loose (with finer particles in them) than that of the OBM-DCS, which is more compact. The SEM analysis shall be repeated and compared at the end of the washing, to study the level of cleaning/treatment received by both samples.

2.3.2.2. Determination of Elemental Composition of Drill Cuttings by EDXA

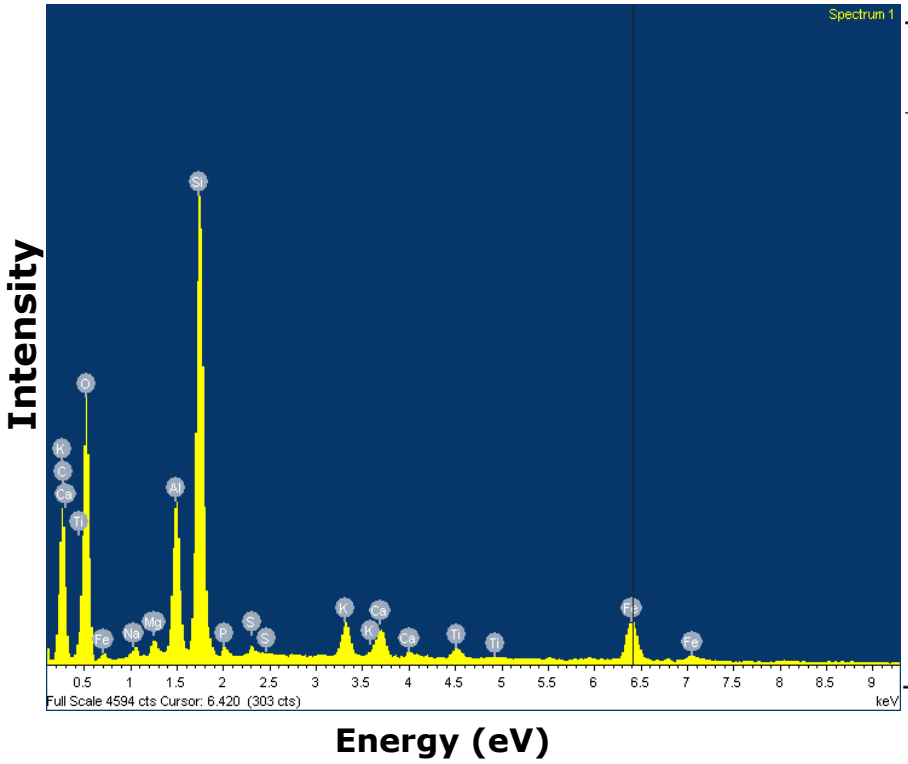
This is a qualitative analysis carried out to determine the elemental composition of the drill cuttings and the soil samples. The elements are identified from their characteristic X-ray peaks. However, the abundance of each element identified is not determined since the elements shall be quantified using ICP-OES. Based on the fact that this analysis was carried out qualitatively, random sites were analysed on the samples. Figures 2.9 and 2.10 shows the SEM-EDX spectras for OBM-DCS and soil respectively. The samples were analysed as received and reanalysed after treatment.



Composition Table

Element	Weight (%)	Atomic (%)
C	14.69	25.87
O	38.06	50.31
Na	0.77	0.71
Mg	0.94	0.82
Al	3.84	3.01
Si	11.91	8.97
S	3.48	2.30
Cl	2.29	1.36
K	0.85	0.46
Ca	4.83	2.55
Fe	3.68	1.39
Ba	14.66	2.26
Totals	**100.00**	

Figure 2.9: A SEM-EDX Spectrum of OBM-DCS – As received



Composition Table

Element	Weight (%)	Atomic (%)
C K	23.02	33.48
O K	44.75	48.86
Na K	0.53	0.40
Mg K	0.62	0.44
Al K	4.83	3.13
Si K	15.62	9.72
P K	0.44	0.25
S K	0.30	0.16
K K	1.67	0.75
Ca K	1.55	0.67
Ti K	0.76	0.28
Fe K	5.92	1.85
Totals	**100.00**	

Figure 2.10: An SEM-EDX Spectrum of Soil Samples – As Received

The elements identified from the OBM-DCS spectra above are; C, O, Na, Mg, Al, Si, S, Cl, K, Ca, Fe, and Ba. While the elements identified from the soil sample were; C, O, Na, Mg, Al, Si, P, S, K, Ca, Ti and Fe. Both samples have high amount of silicon in them, which is indicative of quartz based sample. Basically, the SEM-EDXA gives useful qualitative information on the elemental composition of the drill cutting samples, which serves as an appropriate guide to conducting further investigation on the elemental content of the samples. However, only the elements of interest in the OBM-DCS shall be quantified using ICP/OES. The seventh objective of this research was geared towards utilizing the treated OBM-DCS as construction material, consequent upon a successful treatment of the sample. It is expedient that the heavy metal content of the OBM-DCS be quantified before and after treatment to ensure that no heavy metal is leached into the environment when reused.

The SEM-EDXA is limited in monitoring the elemental content of samples for the following reasons;

1. The sample matrix must be solid and must fit into the sampling stubs for analysis in the chamber. This is a limiting factor when analysing

heterogeneous samples like drill cutting sample, and only a limited surface area of each particle is subjected to the analysis.

2. Although the drill cuttings sample was analysed as received, the presence of the mud on the sample can limit elemental investigation of the rock cuttings.

The SEM-EDXA is a recommended method for mineral identification and microstructural classification of samples (Haberlah et al. 2011)

2.3.3. Elemental Analysis of OBM DCS by Inductively Coupled Plasma - Optical Emission Spectroscopy (ICP-OES)

ICP-OES is a useful analytical technique for the determination and quantification of trace metals in a variety of diverse sample matrices (Ghosh et al. 2013). The ICP-OES is a fast and accurate technique best suited for multi-element analysis in different sample matrices (Froes et al., 2009). The concentration of the following elements were investigated in the drill cuttings samples, following a microwave assisted acid digestion; Al, Mg, Ba, Ti, Mn, K, Hg, V, Cd, Cr, Cu, Co, Fe, Ni, As, Pb and Zn. Also, gold (Au) was added to keep Hg in solution. The elements were selected based on; their toxic nature on the environment, data obtained from SEM-EDX analysis and references from relevant literature such as; Gbadebo, Taiwo and Eughele (2010) and Leonard and Stegemann (2010).

2.3.3.1. *Calibration for ICP-OES Analysis*

The calibration plot of emission intensity versus concentration of all 17 elements studied was linear. The calibration plot for three (3) elements; Al, Mg and Ba are shown in Figures 2.11, 2.12 and 2.13, while the remaining fifteen (14) elements; shall been seen in Appendix 2.

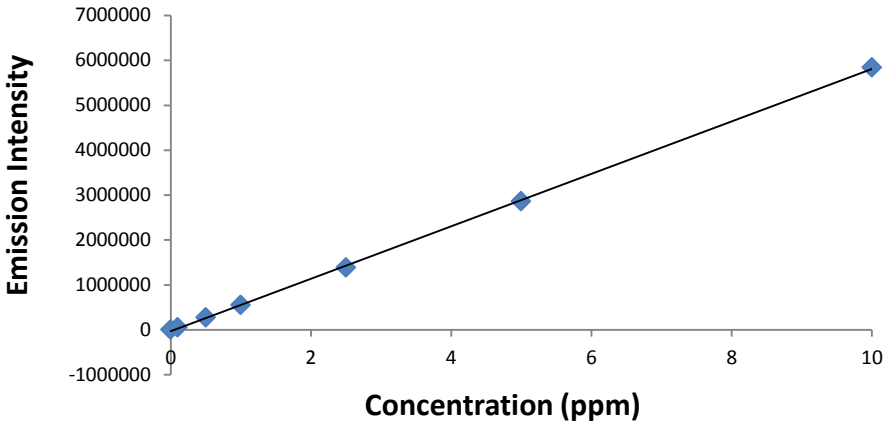


Figure 2.11: Calibration Plot for the Analysis of Al in DCS by ICP-OES

Calibration equation: y = 583591x – 24944 and R^2 = 0.9998

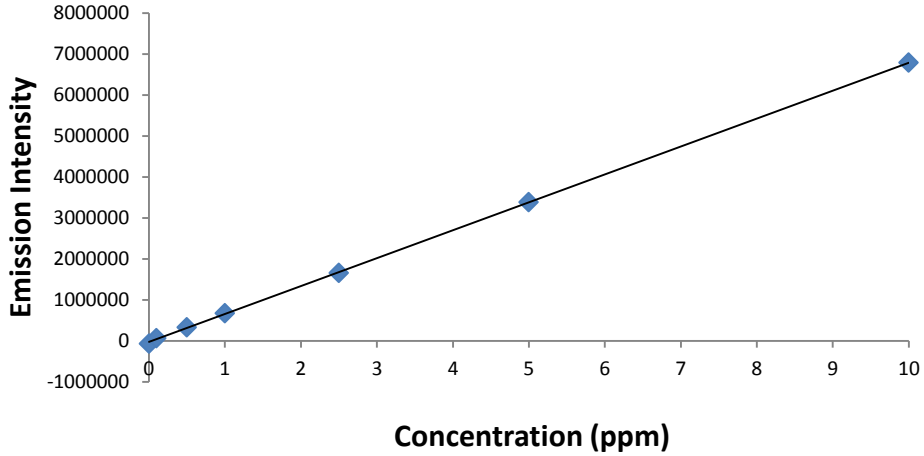


Figure 2.12: Calibration Curve for the Analysis of Mg in DCS by ICP-OES

Calibration equation: y = 681750x – 27626 and R^2 = 0.9999

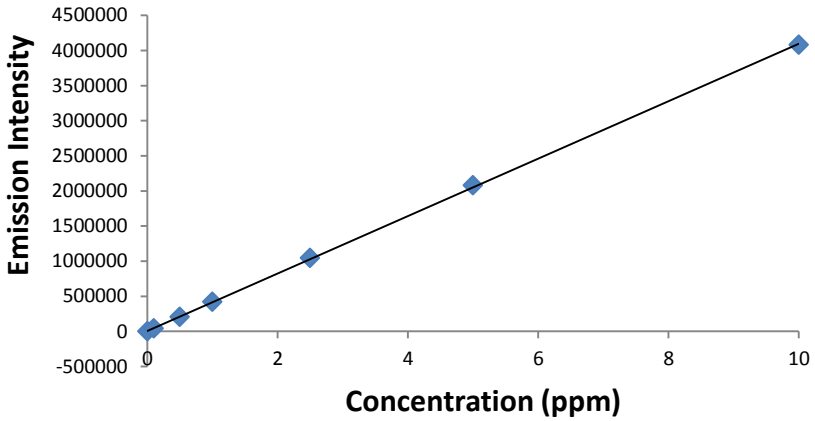


Figure 2.13: Calibration Curve for the Analysis of Ba in DCS by ICP-OES

Calibration equation: y = 408914x + 8415.7 and R^2 = 0.9999

The data obtained from the calibration curves of the 17 elements was used to calculate the concentration of the elements in the drill cuttings. The result of the analysis is shown in Table 2.9 below.

Table 2.9: Elemental Analysis of OBM Drill Cuttings by ICP-OES (n = 3)

S/NO	Elements	MEAN ± SD (mg/Kg)	RSD (%)	Calibration Equation	LOD (ppm)	LOQ (ppm)
1.	Al	47,436±2,380	5.0	y = 583591x - 24944	0.20	0.57
2.	Mg	11,384±131	1.2	y = 681750x - 27626	0.02	0.21
3.	K	8,324±562	6.8	y = 813152x - 98341	0.80	2.15
4.	Fe	51,068±3,212	6.3	y = 135,730x - 213	0.06	0.19
5.	Ba	6,177±1,520	24.6	y = 408914x + 8415	0.09	0.36
6.	Ti	19,993±1,958	9.8	y = 79500x + 1723	0.47	1.57
7.	Mn	255±37.8	14.8	y = 2,660,617x - 24,489	0.06	0.16
8.	Hg	Not detected	-	y = 14749x - 96	0.09	0.29
9.	V	178±4.3	2.4	y = 36,260x - 534	0.05	0.14
10.	Cd	Not detected	-	y = 116,677.7x + 353	0.04	0.15
11.	Cr	102±8.8	8.6	y = 75,732x + 799	0.05	0.13
12.	Cu	57±5.1	8.9	y = 184,271x - 1,141	0.08	0.26
13	Co	20±0.7	3.4	y = 59,532.2x - 747	0.06	0.21
14.	Ni	53±2.6	5.0	y = 37,478.8x - 354	0.11	0.31
15.	As	Not detected	-	y = 3,366.4x - 40.8	0.26	0.54
16.	Pb	Not detected	-	y = 3,778x + 337	0.53	1.07
17.	Zn	180±20.7	11.5	y = 22,861x - 902	0.20	0.57

Key

Detected from SEM-EDXA and ICP-OES

From Table 2.9 above, the calibration curve for all 17 elements showed strong linear relationships between the emission intensity and concentration with correlation coefficients (R^2) for all elements analysed ranged from 0.997 to 0.9998. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on three and ten times the standard error of the regression respectively and these were found to be in the ranging 0.02 – 0.8 ppm and 0.13 – 2.15 ppm respectively.

The result of this analysis is indicative and dependent on the mud composition and formation being drilled. Although 17 elements have been investigated in this study, only the heavy metals present in the sample and Barium (Ba) will be evaluated against set standards.

2.3.3.2. Evaluation of Heavy Metal Concentration in OBM-DCS against set Standards

The set standard or guidelines for the discharge of waste with heavy metals vary from region to region. The allowable discharge limit also varies for offshore and onshore (landfill) locations. The heavy metal content of the drill cutting sample was compared with the allowable limit of heavy metals in contaminated soil to determine the potential heavy metal toxicity of the waste sample if discharged onshore without treatment and also, to enable a comparison of the levels after treatment.

The standards used for this study were obtained from the Environmental Guidelines and Standards for the Petroleum Industry Nigeria (EGASPIN) authored by the Department of Petroleum Resources (DPR) Nigeria, the Ministry of Environment Finland (MEF), as well as soil guideline values (SGV) set by the Contaminated Land Exposure Assessment (CLEA) of the United Kingdom's Environment Agency. These standards were utilised to evaluate the result of elemental analysis carried out on the soil samples to see how they compare with the guideline values in the different regions.

The DPR target values indicates, "*the soil quality required for sustainability or expressed in terms of remedial policy, the soil quality required for the full restoration of the soil's functionality for human, animal and plant life*." While the intervention values indicate, "*the quality for which the functionality of soil for human, animal and plant life are, or threatened with being seriously impaired*," (EGASPIN 2002). Thus the elemental concentrations within the target values are values that express the soil quality being aimed for, while concentrations above the intervention values shows the evidence of serious contamination.

The MEF guideline was recommended by Tóth et al. (2016). They stated that the Finnish guideline for heavy metals in contaminated soil gives "a good approximation of the mean values of different national systems in Europe and India." Also the CLEA SGVs are trigger values for screening-out low risk areas of land contamination. They give an indication of representative average levels of chemicals in soil below which the long-term health risks are likely to be minimal. (ALS Environmental 2017)

The concentration of the heavy metals present in the drill cutting samples were evaluated against DPR, MEF and CLEA SGV as shown in Table 2.10 below;

Table 2.10: Heavy Metal Assessment Criteria for DPR (Nigeria) and MEF (Finland)
Available from: EGASPIN (2002), MEF (2007) and ALS Environmental (2017)

Heavy Metals & Ba*	Mean Concentration (mg/Kg)	DPR Nigeria Soil/Sediment (mg/Kg)		Ministry of Environment, Finland (mg/Kg)			Contaminated Land Exposure Assessment (CLEA), UK	
		Target Value	Intervention Value	Threshold Value	Lower guideline Value	Higher guideline Value	Function of Land Use	CLEA SGV mg/Kg
Cu	57 ± 5.1	36	190	100	150 (e)	200 (e)	NAAP	
Zn	179±20.7	140	720	200	250 (e)	400 (e)	NAAP	
Pb	Not detected	85	530	60	200 (t)	750 (e)	• Residential with home grown produce • Residential without home grown produce • Allotment • Commercial • Agricultural and after sewage sludge application	200 310 80 2300 -
Ni	52.8 ±2.6	35	210	50	100 (e)	150 (e)	• Residential • Allotment • Commercial • Agricultural and after sewage sludge application	130 230 1800 -
Cr	102.1 ± 8.8	100	380	100	200 (e)	300 (e)	• Residential with plant uptake • Residential without plant uptake • Commercial and Industrial • Agricultural and after sewage sludge application	130 200 5000 -
Cd	Not detected	0.8	12	1	10 (e)	20 (e)	• Residential with home grown produce • Residential without	22 150

							home grown produce • Allotment • Commercial • Agricultural and after sewage sludge application	 3.9 410 -
Hg	Not detected	0.3	10	0.5	2 (e)	5 (e)	• Residential • Allotment • Commercial • Agricultural and after sewage sludge application	10 26 26 -
As	Not detected	29	55	5	50 (e)	100 (e)	• Residential with home grown produce • Residential without home grown produce • Allotment • Commercial • Agricultural and after sewage sludge application	37 40 49 640 -
Co	19.5±0.7	20	240	20	100 (e)	250 (e)	NAAP	
V	178±4.3	NAAP	NAAP	100	150 (e)	250 (e)	NAAP	
Ba*	6,177±1,521	200	625	NAAP	NAAP	NAAP	NAAP	

Key: NAAP (Non available at present)

The Finnish government sets lower and higher concentration levels for each hazardous element in order to identify soil contamination and remediation needs. The MEF (2007) guideline states that, "the threshold value is applicable for all sites, and it indicates the need for further assessment of the area, while the guideline value is a value if exceeded indicates that the area has a contamination level which presents ecological or health risks." MEF sets a different guideline value for industrial and transport areas, (regarded as higher guideline value), and for all other land uses (lower guideline value).

As observed in Table 2.10, the following heavy metals were not detected in the drill cutting sample: Pb Cd, Hg and As. This is based on the fact that the levels of these metals in the sample were below the limit of detection of the instrument.

Evaluation of the metals under review against the CLEA SGV shows that the concentrations obtained were within the set guidelines for the functions of the land use at stated in the Table 2.10. The evaluation against the DPR standards shows that the concentration level of Co can be approximated to the target value set for soil and sediment, which indicates remediation due to contamination. Also, the concentration levels of Cu, Zn, Ni and Cr were above the target values for soil and sediment as set by DPR, except Barium which is 30 times greater than the target value and 10 times greater than the intervention value for DPR. The DPR has no set standard for vanadium. The results shows contamination of the OBM-DCS from: Co, Cu, Zn, Ni, Cr and Ba. Also, evaluating the result against the guidelines set by the Finnish government shows obvious contamination of the OBM-DCS from vanadium. The other metals slightly above/borderline to the threshold value includes: Ni, Cr and Co. Therefore the result of the evaluation of the metals against DPR and MEF guidelines shows the need for the sample to be treated before disposal or reuse.

2.3.4. Determination of the Hydrocarbon Profile of OBM-DCS by GC-MS

The determination of volatile contaminants in complex sample matrices is quite challenging without exhaustive sample preparation. The head space solid phase micro-extraction (HS-SPME) technique is useful for the extraction of volatile organic compounds (VOCs) and semi-volatile organic compounds (SVOCs) from complex sample matrices especially environmental waste samples such as soils, sediments and sludge (Ouyang 2012; Kotowska, Zalikowski and Isidorov 2012).

The hydrocarbons present in any OBM drill cuttings is a function of the base fluid used in formulating the drilling fluid and the nature of the formation being drilled. The hydrocarbon profile of the drill cuttings gives qualitative information on the range of hydrocarbons present in the oil on the cuttings. The total ion chromatogram of the OBM drill cutting samples showing the hydrocarbon profile is shown in Figure 2.14 below.

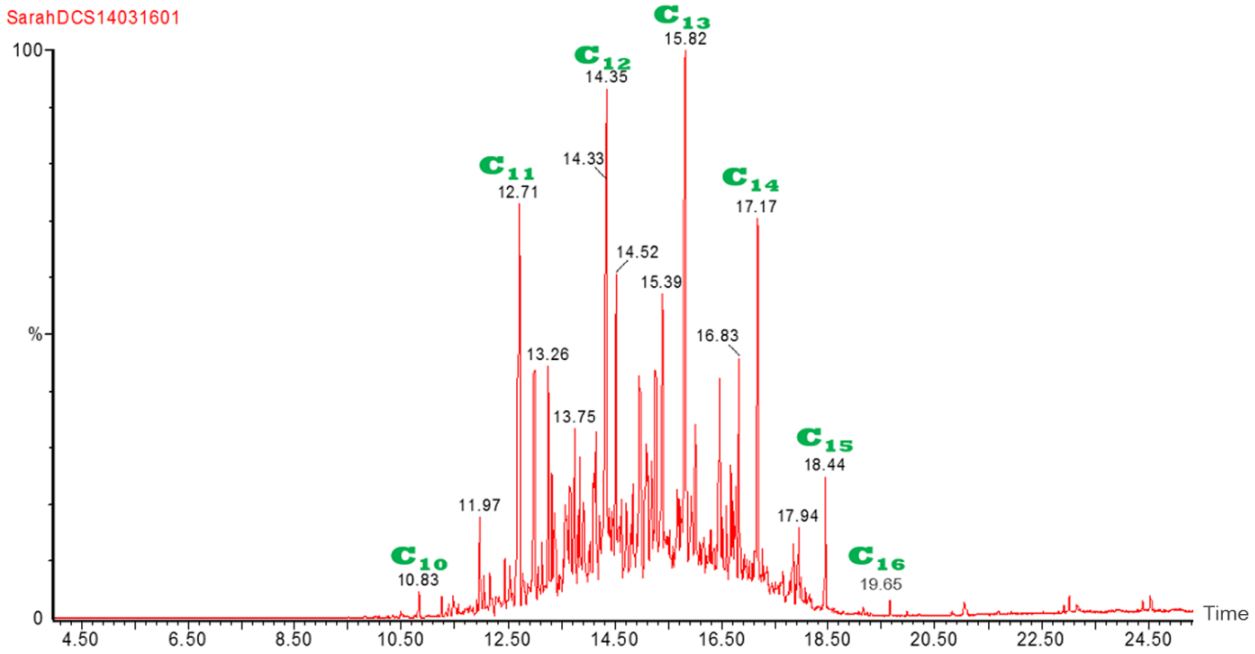


Figure 2.14: Total Ion Chromatogram of OBM Drill Cuttings

The hydrocarbons detected ranged between carbon numbers C_{10} to C_{16} (in green). The hydrocarbons in the sample were identified by checking the mass spectra of the individual peaks, which were confirmed using a mass spectral NIST library. Figure 2.15 and 2.16 below shows the mass spectras of the peaks at retention times, 10.83 and 12.71mins. The peaks were confirmed to be decane and undecane respectively.

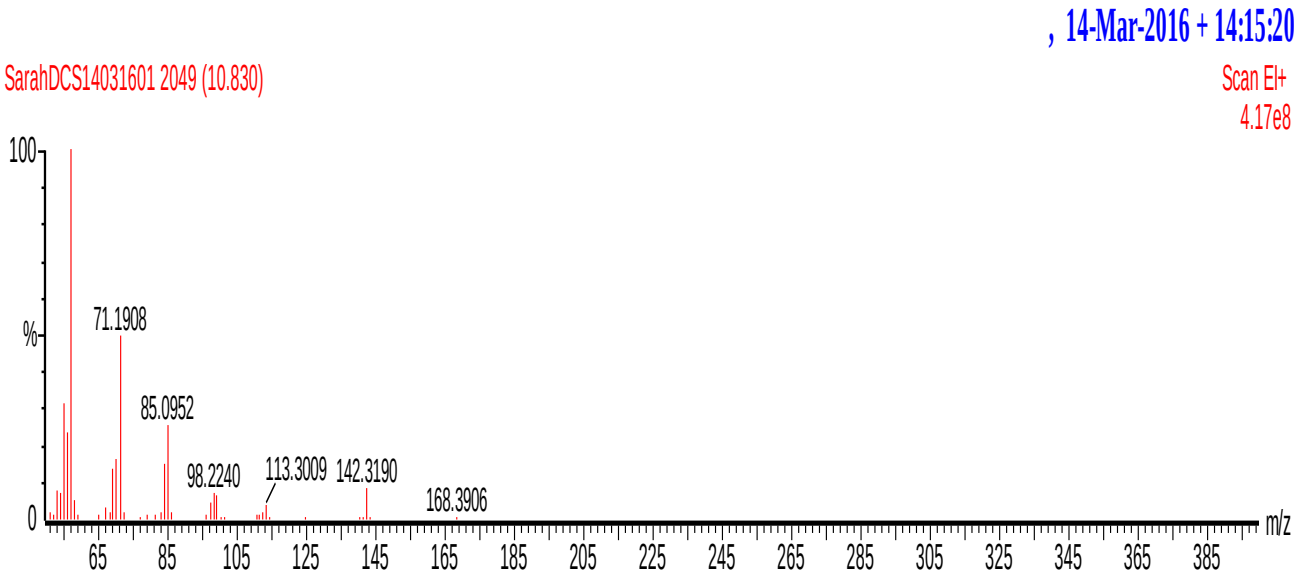


Figure 2.15: Mass Spectra of Decane ($C_{10}H_{22}$), @10.83 minutes

Identified as Decane using NIST Library

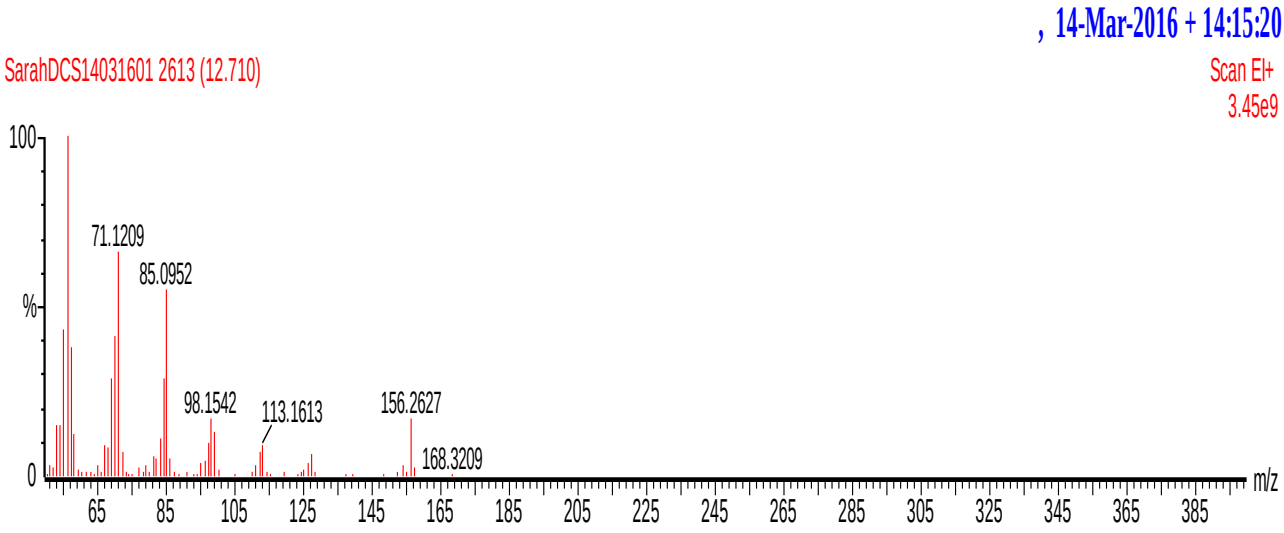


Figure 2.16: Mass Spectra of Undecane ($C_{11}H_{24}$), @12.71 minutes

Identified as Undecane using NIST Library

The rest of the peaks on the chromatogram were also confirmed using the same process. The result is shown in Table 2.11 below.

Table 2.11: Hydrocarbons Identified in OBM Drill Cuttings

S/N	Retention Time (mins)	Alkane	Formula
1	10.83	Decane	$C_{10}H_{22}$
2.	12.71	Undecane	$C_{11}H_{24}$
3.	14.35	Dodecane	$C_{12}H_{26}$
4.	15.82	Tridecane	$C_{13}H_{28}$
5.	17.17	Tetradecane	$C_{14}H_{30}$
6.	18.44	Pentadecane	$C_{15}H_{32}$
7.	19.65	Hexadecane	$C_{16}H_{34}$

This result shows that the base fluid used in formulating the drilling fluid is composed mainly of light hydrocarbons based on carbon range identified (C_{10} - C_{16}), which indicates hydrocarbons in the gasoline to light diesel range. This result is useful for the calibration of the FT-IR to be utilized for TPH analysis. The standard for the calibration will be within the diesel range.

2.3.5. Analysis of Total Petroleum Hydrocarbon (TPH) by FT-IR

The TPH level in the oil contaminated samples (OBM-DCS and soil) were determined using Fourier Transform Infrared (FT-IR) spectroscopy following extraction in perklone as described in section 2.2.8.

2.3.5.1. Calibration of FT-IR Instrument for TPH Analysis

The FTIR instrument was first calibrated using diesel in perklone standards ranging from 20, 50, 100, 200, 300, 400 and 600 ppm in order to assess the efficiency of the procedure. The calibration curve for the diesel in perklone standard, showing of the absorbance (corrected area) versus the concentration is shown in Figure 2.15 below.

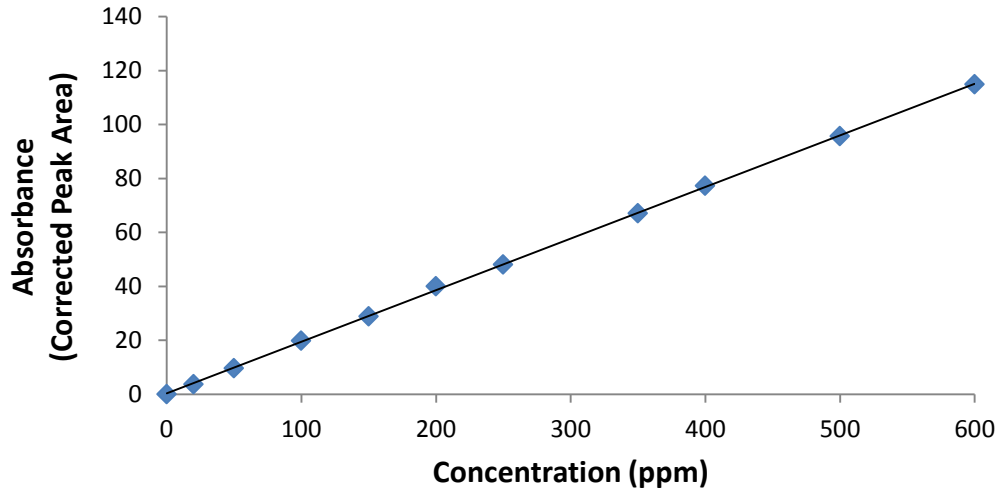


Figure 2.17: FT-IR Calibration Curve for Diesel in Perklone.

Calibration Equation y = 0.1913x + 0.3139 and R2 = 0.9998

A linear calibration plot was obtained from the plot of absorbance versus diesel in perklone standards with R^2 = 0.9986. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on three and ten times the standard error of the regression respectively and these were found to be in the ranges 7.1 ppm and 27.6 ppm respectively.

2.3.5.2. Determination of TPH Extraction Method

The result of the preliminary extractions carried out to determine the most appropriate TPH extraction method showed the following result (Figure 2.18).

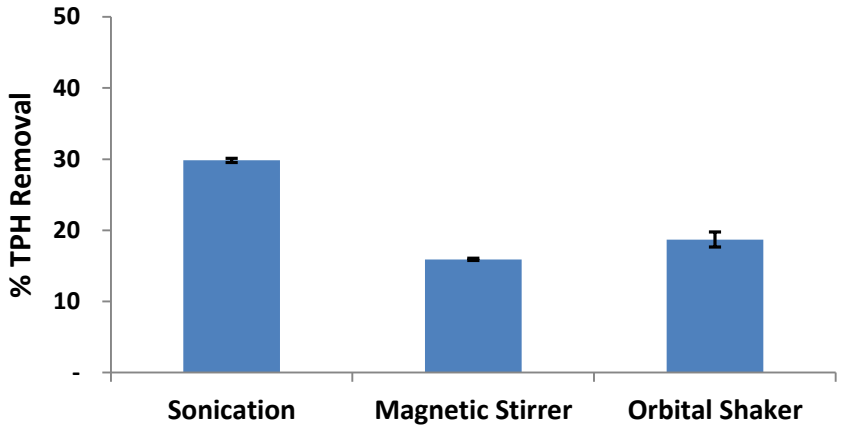


Figure 2.18: TPH Extraction Methods

As explained in section 2.2.8.2., the analysis was carried out using water. After which the percentage TPH removal from the samples were determined by extracting the residue with perklone and analysing on the FT-IR. The highest TPH removal was obtained from the sonication extraction method, and thus was chosen as the extraction method for this research.

2.3.5.3. Recovery Check for TPH Extraction Method Applied: Sonication

The recovery of the sonication extraction method used was investigated using spiked freeze-dried sediment samples as a reference material. As explained in 2.2.8.3., 3 sets of the freeze-dried sediment samples were analysed to investigate the recoveries of the sonication extraction method. The result of the TPH analysis carried out on the freeze-dried sediment samples is shown in Table 2.12 below.

Table 2.12: TPH analysis on Freeze-Dried Sediment Samples

Sediment Samples	Mean Conc (mg/kg)	RSD (%)	Recovery (%)
SET A (not spiked)	Nil	Nil	Nil
SET B (spiked)	1009±45	4.5	100.9
SET C (spiked and cleaned with florisil)	926±76	8.4	92.6

As shown in Table 2.12 above, no petroleum hydrocarbon was detected in Set A samples, while that of Set B (spiked) had a 100±5% extraction recovery of TPH. The spiked Set C samples which were further cleaned with florisil, also yielded 92.6±7% extraction efficiency. The result of this recovery check validated the choice of the sonication method for the extraction of TPH from OBM drill cutting samples. Also the sonication method is ideal for the following reasons;

1. It is fast
2. It requires less solvent.
3. High (100%) extraction efficiency.

2.3.5.4. Determination of TPH in Oil Contaminated samples

The TPH in the oil contaminated samples were extracted and determined as described in section 2.2.8.3. Tables 2.13 shows the TPH content of the oil contaminated samples investigated in this work.

Table 2.13: TPH Content of OBM Drill Cutting Samples

Replicate Samples	TPH Content As Received (mg/Kg)	
	OBM-DCS	Soil
1	61,924.19	17,905.5
2	59,569.56	18,905.7
3	60,793.48	18,235.04
4	62,560.83	17,440.00
Mean	**61,212.01**	**18,121.57**
SD	1,316.48	616.17
RSD (%)	2.2	3.4

The TPH content of the oil contaminated samples were obtained as follows;

1. OBM drill cuttings: 61,212.01±2.2
2. Soil : 18,121.57±3.4

The result of the TPH values obtained from the analysis of the OBM drill cuttings is 6 times higher than the EGASPIN (2002) and OSPAR (2000) allowable limit of less than 1% (about 10,000 mg/kg) by dry weight on cuttings before disposal thus necessitating

the treatment of the waste DCS sample to reduce the levels of TPH before disposal. Following typical mechanical treatment offshore, cuttings are reported to retain 5-10% of oil by weight.

The drill cuttings sample under consideration falls within this range. The presence of hydrocarbons in the untreated drill cuttings is considered a potential risk to living organisms if disposed inappropriately to the environment. The TPH values obtained will provide the background data that will be used in assessing the effectiveness of the treatment that will be explored in subsequent chapters.

2.4. Conclusion

Treatment of oil contamination in the environment is important based on its potential detrimental effect on human health and the environment. The persistent nature of contaminants such as heavy metals poses potential detrimental risk when discharged in the environment. The result of the elemental characterization of the OBM drill cuttings gives an indication of the hazard potential of the OBM drill cuttings to the environment if untreated before disposal or reuse.

The particle size analysis showed that approximately 40% of the drill cuttings falls within the range of coarse sand (textural class). The sample is heterogeneous in nature, which may have contributed to the variability observed TPH and elemental analysis. Also the PSA carried out on the soil showed that approximately 30% of the soil consists of medium sand. This information is useful as it may give an indication to the efficiency of the treatment process when applied.

The SEM-EDXA carried out on both samples showed that the grains in the soil samples are more loose (with finer particles in them) than that of the OBM-DCS, which is more compact as shown on the microstructures obtained from the analysis. Also, the analysis gave a qualitative result of the elemental composition of the OBM-DCS and soil, indicating the presence of the following elements; Na, Mg, Al, K, Fe and Ti in both samples.

ICP-OES analysis carried out to determine the concentrations of Al, Mg, Ba, Ti, Mn, K, Hg, V, Cd, Cr, Cu, Co, Fe, Ni, As, Pb and Zn in the drill cuttings, showed that the sample had been contaminated with Co, Cu, Zn, Ni, Cr, V and Ba when evaluated with the DPR and MEF guidelines for heavy metals in contaminated soil. Also, Hg, Cd, As and Pb were not detected in the OBM drill cutting samples. This may be due to the fact that levels are probably lower than the limit of detection, which is also lower than the target values set by DPR.

The result of the hydrocarbon profiling carried out on the OBM-DCS gives the indication that the base fluid used in formulating the drilling fluid composed of light hydrocarbons based on the carbon range identified (C_{10} - C_{16}), which indicates hydrocarbons in the

gasoline to light diesel range. This result was useful for the calibration of the FT-IR to be utilized for TPH analysis.

The TPH result showed that the oil on cuttings (OOC) was more than 1%, with an average concentration of 61,212±1,316 mg/kg, over 6 times more than the allowable discharge limit (as stipulated by DPR and OSPAR). This value renders the OBM-DCS unsuitable for disposal offshore and must be treated before disposal or reuse.

The characterisation undertaken so far has provided benchmark values which can be compared after remedial treatment is carried out.

CHAPTER 3 - PRODUCTION AND CHARACTERIZATION OF RHAMNOLIPID BIOSURFACTANT

3.1. Introduction

The advancement of biotechnology in proffering solutions to environmental pollution has been on the increase in the last few decades. This advancement is driven by the quest for sustainable waste treatment technologies due to the increase in industrial activities and its consequent impact on the environment. With much waste being generated in the oil and gas industry it becomes expedient to source alternative sustainable technologies for waste treatment (Amulya, Dahiya and Mohan 2016). Shi (2010) believes that modern biotechnology has provided technologies and products that have helped to solve challenging issues in disease control, agriculture, reduction in environmental footprint by way of providing efficient industrial processes that requires less energy, as well as fostering the use of microbiology in the remediation of contaminated land (bioremediation).

The process of treating environmental contamination caused by oil and gas companies such as oil based mud drill cuttings is challenging and expensive. Some of the technologies available for treating drilling waste have negative impact on the environment, such as: potential pollution of groundwater, and as such are unsustainable in the long run. Research and development in biotechnology has led to the introduction of environmentally friendly solutions for treating contamination from oil and gas operations. One beneficial product from biotechnological research and innovation is the production of surfactants from biological origin typically referred to as biosurfactants.

3.1.1. Biosurfactants

Biosurfactants are a diverse group of surface active compounds produced from biological origin (Banat 1995a; Mulligan et al. 2014; Pacwa-Plociniczak et al. 2011). They share similar characteristics with synthetic surfactants; both being amphiphilic molecules, consisting of hydrophilic and hydrophobic moieties (Chakraborty and Das 2014). Figure 3.1 below shows the structure of a typical surfactant.

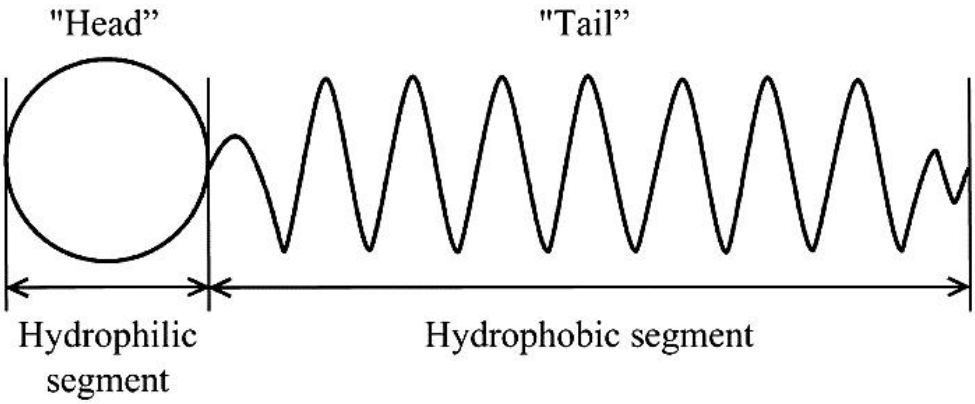


Figure 3.1: Structure of a Typical Surfactant
Available from Szymański (2008)

The hydrophilic head can be anionic, cationic, zwitterionic, or non-ionic, while the hydrophobic tail is usually a nonpolar hydrocarbon chain/segment. The presence of these moieties in the surfactant enables the biosurfactant to reduce surface tension and interfacial tension in non-aqueous and aqueous solutions, thus increasing the solubility of the non-aqueous solution in the mixture (Banat 1995b; Desai and Banat 1997).

3.1.2. Classification of Biosurfactant

Generally, biosurfactants can be classified into two broad groups: low and high molecular mass compounds. Biosurfactants, with low molecular mass are useful and efficient in lowering surface and interfacial tension, while the high molecular weight compounds are more efficient as emulsion stabilizing agents (Rikalović et al. 2012).
The major classes of biosurfactants are;

1. Glycolipids
2. Lipopeptides
3. Phospholipids & Fatty acids

Low molecular weight

4. Polymeric
5. Particulate

High molecular weight

Biosurfactants can also be classified according to their chemical composition, molecular weight, mode of action, physico-chemical properties and microbial source of origin (Dhanarajan and Sen 2014). Table 4.1 below shows the classification of biosurfactants and their applications in environmental solutions.

Table 3.1: Classification of Biosurfactants and their Applications in Environmental Solutions

(Available From: Chen, Juang and Wei (2015); Pacwa-Plociniczak, et al. *(2*011)

Biosurfactant		Producer organism	Applications in Environmental Biotechnology
Group	**Class**		
Glycolipids	Rhamnolipids	*Pseudomonas aeruginosa, Pseudomonas sp.*	Emulsification of hydrocarbons and vegetable oils, removal of metals from soil, Enhancement of the degradation and dispersion of different classes of hydrocarbons
	Trehalolipids	*Mycobacterium tuberculosis, Rhodococcus erythropolis, Arthrobacter sp., Nocardia sp., Corynebacterium sp.*	Enhancement of the bioavailability of hydrocarbons
	Sophorolipids	*Torulopsis bombicola, Torulopsis petrophilum, Torulopsis apicola*	Recovery of hydrocarbons from dregs and muds; removal of heavy metals from sediments; enhancement of oil recovery
Lipopeptides	Surfactin	*Bacillus subtilis*	Enhancement of the biodegradation of hydrocarbons and chlorinated pesticides; removal of heavy metals from a contaminated soil sediment and water; increasing the effectiveness of phytoextraction
	Lichenysin	*Bacillus licheniformis*	Enhancement of oil recovery
Phospholipids and Fatty acids	Corynomycolic acid	*Corynebacterium lepus*	Enhancement of bitumen recovery

	Spiculisporic acid	*Penicillium spiculisporum*	Removal of metal ions from aqueous solution; dispersion action for hydrophilic pigments; preparation of new emulsion-type organogels, superfine microcapsules (vesicles or liposomes), heavy metal sequestrants
	Phosphati-dylethanolamine	*Acinetobacter sp., Rhodococcus erythropolis*	Increasing the tolerance of bacteria to heavy metals
Polymeric Biosurfactants	Emulsan	*Acinetobacter calcoaceticus RAG-1*	Stabilization of hydrocarbon-in-water emulsions
	Alasan	*Acinetobacter radioresistens KA-53*	
	Biodispersan	*Acinetobacter calcoaceticus A2*	Dispersion of limestone in water
	Liposan	*Candida lipolytica*	Stabilization of hydrocarbon-in-water emulsions
	Mannoprotein	*Saccharomyces cerevisiae*	
	Mannoprotein	*Saccharomyces cerevisiae*	
Particulate Biosurfactants	Vesicles	*Acinetobacter Calcoaceticus*	Enhances hydrocarbon uptake, exhibits good emulsification activity
	Emulcyan	*Phormidium J-1*	

3.1.3. Properties of Biosurfactants

Biosurfactants have properties that make them useful in physico-chemical and biological treatment of organic and metal contaminants in diverse sample matrices. These include:

1. Surface and interface activity. Biosurfactants such as rhamnolipids, can reduce the surface tension of water from 72 mN/m to 30 mN/m (Raza, Khalid and Banat 2009).
2. Emulsion/de-emulsification, low toxicity and biodegradability (Dhanarajan and Sen 2014).
3. Wetting, penetrating actions and spreading (Mulligan et al. 2014)
4. Microbial and antimicrobial properties growth enhancements (Kuyukina et al. 2007)
5. Metal sequestration (Pacwa-Plociniczak et al. 2011; Açikel 2011),
6. Detergency and solubilisation (Hargreaves 2003; Childs et al. 2005).

3.1.3.1. Sustainable Environmental Considerations for the use of Biosurfactant

The major advantage biosurfactants have over their chemically synthesized counterparts is hinged on the fact that they are environmentally benign, having a low toxicity and are biodegradable (Makkar, Cameotra and Banat 2011).

Low Toxicity: Studies carried out by Lechuga et al. (2016) and Lémery et al. (2015), investigating the toxicity of chemically synthesized surfactants on aquatic organisms and human skin respectively, showed that synthetic surfactants can potentially cause harm to aquatic organisms and humans. A similar study carried out by Kuyukina et al., (2007), investigated the acute toxicity of a glycolipid on mice in doses of 1, 3, and 10 g/kg (fresh emulsion in 0.5% NaCl). The result of the analysis after a 14-day observation period showed no effect on the central nervous system (CNS) of the mice and caused no stimulation or inhibition of their behavioural activity at all the doses studied.

Biodegradability: It is ideal that the chemicals utilized in environmental solutions be biodegradable as the persistence of toxic chemicals in the environment can potentially be deleterious to the environment. A study carried out by Mohan, Nakhla and Yanful (2006), investigating the biodegradabilities of

triton X-100 and rhamnolipid under aerobic, nitrate reducing, sulphate reducing and anaerobic conditions, indicated that in terms of biodegradability, rhamnolipid is superior to triton X-100, since it was biodegradable under all the conditions studied. Other studies have also shown and confirmed the biodegradability of biosurfactants over their synthetic counterparts (Lima et al. 2011; Frank et al. 2010).

pH, Temperature, Salinity and Ionic Strength Tolerance: Biosurfactants are known to have better environmental compatibility and can function well at extreme temperatures, salinity and pH (Desai and Banat 1997; Amani et al. 2010). These properties give biosurfactants wide applicability in industrial and environmental applications. De Gusmão, Rufino and Sarubbo (2010) investigated the influence of pH, salt concentration and temperature on the surface tension reducing activity of cell-free broth of *Candida glabrata* strain UCP1002. Results of the chemical characterisation carried out on the surfactant extracted from the cell-free broth, confirmed the presence of a carbohydrate-protein-lipid complex. However, the cell-free broth was stable irrespective of the variations in pH (ranging from 2 to 12), NaCl concentration (ranging from 0 to 10%) and temperature (ranging from 4 to 120^{o}C). The stability of biosurfactants under these conditions makes them suitable and effective in environmental and pharmaceutical applications.

Production from low-cost renewable sources: One major challenge associated with the production of biosurfactants is the high cost of producing them on a commercial scale. The economics of large scale biosurfactant production has received increased attention in recent years (Açıkel 2011) and recent studies showed that, biosurfactants can be produced using a range of low cost renewable sources as substrate (energy source) for the cultivation of the microorganisms (Pereira et al. 2013; Bhardwaj, Cameotra and Chopra 2013). As an example, Soniyamby et al. (2011) utilized waste vegetable oil as a substrate to cultivate *Pseudomonas aeruginosa* for the production of rhamnolipid biosurfactant. Other low cost renewable substrates that have been used for the production of biosurfactants include:

a. Agricultural waste e.g. vine-trimming shoots using the halotolerant strain *Bacillus tequilensis* ZSB10 (Cortés-Camargo et al. 2016) and

wheat straw by enzymatic hydrolysis using cellulases (Prabu et al. 2015).

b. Dairy and sugar industry waste e.g. whey (Praveesh et al. 2011) and sugar molasses (Reis, Servulo and De Franca 2004) as carbon sources.
c. Industrial waste e.g. distillery waste such as spent wash (Sudhakar et al. 1996; Dubey and Juwarkar 2001).
d. By-products from oleo-chemical industry such as petrochemical waste water (Wei, Chou and Chang 2005) and soap-stock (Benincasa 2002).
e. Waste frying oils such as waste frying coconut oil (George and Jayachandran 2012; Raza et al. 2006; De Gusmão, Rufino and Sarubbo 2010)

The use of low cost renewable substrates for the production of biosurfactants is preferred because they are sustainable and less expensive. This research is focused on the production, characterisation and use of rhamnolipid, a glycolipid biosurfactant for the removal of TPH in oil contaminated drill cuttings and soil.

3.1.4. Rhamnolipids biosurfactant

Rhamnolipids (RL) are glycolipid biosurfactants consisting of a rhamnose sugar linked to one or two 3-hydroxydecanoic acid moieties, and are mainly produced by a strain of bacteria called *Pseudomonas aeruginosa*. This class of glycolipids were first studied by Jarvis and Johnson (1949). Rhamnolipids with one sugar molecule are known as monorhamnolipid (L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate), while the rhamnolipid with two sugar molecules is referred to as dirhamnolipid (L-rhamnosyl-β-l-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate) (Aşçi, Nurbaş and Açıkel 2008; Mulligan and Wang 2006). The typical structure of both rhamnolipids is shown in Figure 3.2.

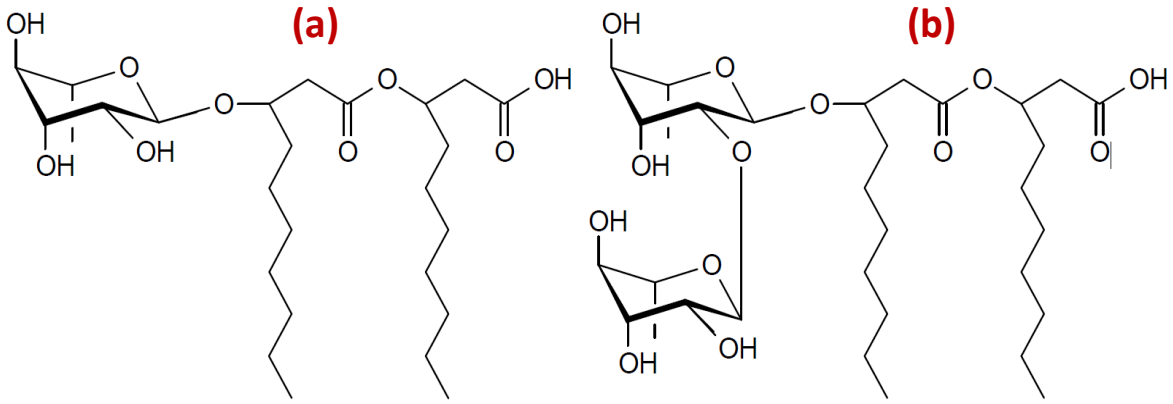


Figure 3.2: Structure of Rhamnolipids

(a) Monorhamnolipid and (b) Dirhamnolipid

Available from: Christie (2013)

Monorhamnolipids and dirhamnolipids have been reported (Behrens et al. 2016) to be formed to differing degrees along with their biosynthetic precursors 3-(3-hydroxyalkanoyloxy) alkanoic acids (HAAs). Similarly, Déziel et al. (1999) have suggested that differences observed in RL congener profiles could be due to the mode of RL isolation and analysis procedures, growth conditions, media used as well as strains or species utilised.

Irorere et al. (2017) recently recommended that detailed fermentation, isolation and purification steps be undertaken and reported including the use of analytical techniques such as liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) to identify the specific congeners and rhamnolipid composition. Other techniques that have been used for this include, infrared spectroscopy (IR), nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography mass spectrometry (LC-MS).

Furthermore Marchant and Banat (2017) have also recommended and outlined the use of gravimetric and analytical methods as mentioned above for the determination of RL production yield and characterisation. Based on their high surface activity and hydrocarbon solubilizing properties, the physical and chemical characteristics of rhamnolipids are continually being investigated to explore more areas of application. Some studies have shown the potential application of rhamnolipid in enhanced oil recovery and environmental remediation such as

removal of oils and heavy metals from polluted soils (Gudiña et al. (2015); Nikolopoulou et al. (2013); Abdel-Mawgoud et al. (2011)).

3.1.5. Potential application of rhamnolipid biosurfactant in waste treatment

In the past, synthetic surfactants such as sodium dodecyl sulphate (SDS) were applied for the clean-up of oil contaminated waste. Khalladi et al. (2009) achieved a 97% elimination of hydrocarbons from a polluted soil (after 10 days of washing) using sodium dodecyl sulphate (SDS), whilst Ceschia et al. (2014), achieved a 98% removal of oil from sand using SDS treated with NaCl (sodium chloride) at 50^{o}C. But the use of synthetic surfactants for environmental remediation is discouraged based on the fact that they were not biodegradable and were potentially harmful to the environment (Lechuga et al. 2016). This move by environmentalist and researchers led to a rise in the demand for biodegradable surfactants (Pacwa-Plociniczak et al. 2011). Rhamnolipids have been investigated for their suitability in:

1. **Treatment of Heavy Metals:** The detrimental health effects associated with the introduction of heavy metals in the environment have been reported worldwide (Järup 2003; Tchounwou, et al. 2012; WHO 2011). Studies show that rhamnolipids can be applied for the removal of heavy metals from solid and liquid waste (Mulligan and Wang 2006; Açıkel 2011; Dahrazma and Mulligan 2014; Elouzi et al. 2012). A review by Mao et al. (2015) shows that, rhamnolipids can be developed as washing agents capable of removing mixed heavy metals from soil by a mechanism of dissolution and complexation (with metal cations).

2. **Removal of TPH**: The result of a study carried out by Yan et al. (2011), using a commercial rhamnolipid to treat oil-based drill cuttings, achieved a reduction in the concentration of total extractable organics from 85,000 to 12,600 mg/kg (approximately 85%), after which a 120 day biodegradation process was further carried out to reduce the contaminants from 12,600 to 5470 mg/kg, thus making it a two-stage treatment process. The study carried out by Yan et al. (2011), seems to be the only study (to my knowledge) in which rhamnolipid have been applied for the treatment of drill cuttings; other studies have focused on utilizing rhamnolipid for the

treatment of hydrocarbon contaminated soil. Tahseen et al. (2016) achieved a 77.6% crude oil-degradation in polluted soil samples. However the rhamnolipid used in their study served as a supplement to the degradation process in which hydrocarbon degrading bacteria and nutrients were used.

3.1.6. Aim

This study however, aims to investigate the use of rhamnolipid biosurfactant for the removal of TPH and heavy metals from oil contaminated samples. Due to the prohibitive cost of utilising commercial rhamnolipids (10mg cost £158.00 from Sigma Aldrich), this chapter focuses on the production of rhamnolipids in the laboratory from two different bacterial strains of *Pseudomonas aeruginosa sp.,* using glycerol as carbon source. Subsequently the produced rhamnolipid (and its derivatives) were characterised using a range of analytical techniques such as Attenuated Total Reflectance - Fourier transform infrared (ATR-FTIR) spectroscopy, NMR and LC-MS/MS. Other techniques utilized in characterizing the products are surface tension (for the investigation of the critical micelle concentration), and emulsification activity.

3.2. Materials and Methods

3.2.1. Bacterial Stains

The microorganisms used in this study were *Pseudomonas aeruginosa* ST5 and *Pseudomonas aeruginosa* PS1. Both organisms were kindly provided by Professor Ibrahim Banat of School of Biomedical Sciences University of Ulster, Northern Ireland. The strains were maintained at 5^{o}C on nutrient agar slants and studied to investigate the growth rate and yield of biosurfactant.

3.2.2. Chemicals and Reagents

3.2.2.1. Media

Nutrient agar powder (containing Lab-Lemco powder, yeast extract, peptone, sodium chloride and agar) used for the preparation of nutrient broth and nutrient agar plates was produced by Oxoid Ltd., Hants, UK and supplied by Fischer Scientific, UK. Kay's minimal medium used for the cultivation of the inoculum was prepared using analar grade ammonium dihydrogen orthophosphate ($NH_4H_2PO_4$), dipotassium hydrogen orthophosphate (K_2HPO_4), magnesium sulphate ($MgSO_4$), iron sulphate ($FeSO_4$) and glucose all supplied by Fisher Scientific, UK. Mineral salts medium (MSM) used as the growth medium was prepared using laboratory reagent glycerol (as carbon substrate) supplied by Fischer Scientific, UK, sodium nitrate ($NaNO_3$), sodium hydrogen phosphate (Na_2HPO_4), dipotassium phosphate (K_2PO_4), magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), calcium chloride dihydrate ($CaCl_2.2H_2O$) and iron(II) sulphate heptahydrate ($FeSO_4.7H_2O$), all supplied by Fisher Scientific, UK. The MSM also contained the following elements in trace quantities; zinc sulphate heptahydrate ($ZnSO_4.7H_2O$), copper (II) sulphate pentahydrate $CuSO_4.5H_2O$, manganese (II) sulphate monohydrate ($MnSO_4.H_2O$), boric acid (H_3BO_3) and sodium molybdate dihydrate ($MoNa_2O_4.2H_2O$) also supplied by Fisher Scientific, UK. Sodium dodecyl sulphate (SDS) a synthetic surfactant used to compare the surfactant properties of the biosurfactant was supplied by Fisher Scientific, UK. Also approximately 10g of crude rhamnolipid sample was collected from Professor Ibrahim Banat as well to be utilised as a control for the thin layer chromatography analysis of the produced rhamnolipid.

3.2.2.2. Biosurfactant Extraction, Recovery, Identification and Purification

The biosurfactant was recovered from the culture by solvent extraction using HPLC grade ethyl acetate supplied by Fisher Scientific, UK. Magnesium sulphate used to remove traces of water in the ethyl acetate extract was from Sigma Aldrich. The mobile phase of the thin layer chromatography (TLC) analysis of the biosurfactant extract was made up with chloroform, methanol and acetic acid (in a ratio of 65:15:2) were all supplied by Fischer Scientific, UK. The sugar moiety of the biosurfactant was identified by staining with anisaldehyde reagent made up with anisaldehyde, sulphuric acid, glacial acetic acid and ethanol all supplied by Fischer Scientific, UK. The fatty acid moiety of the biosurfactant was identified by staining with ammonium molybdate/cerium sulphate reagent made up with ammonium molybdate supplied by Sigma Aldrich, cerium (IV) sulphate supplied by Sigma Aldrich and sulfuric acid supplied by Fischer Scientific, UK.

3.2.2.3. Biosurfactant Characterisation

Emulsification index of the biosurfactant was studied using the following hydrocarbon sources: Brent crude oil was obtained from an anonymous source from the North Sea, sunflower oil was obtained from a local shop, kerosene and diesel were from local Petrol station. Graduated cylindrical tubes and a vortex were used to study the emulsification index of the biosurfactant and purified fractions. Deuterated chloroform used to dissolve the biosurfactant for nuclear magnetic resonance (NMR), was supplied by Cambridge Isotope Laboratories Inc., U.S.A. The mobile phase for the liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis was constituted with deionised water as well as acetonitrile and formic acid supplied by Fischer Scientific, UK. The samples for LC-MS/MS analysis were dissolved in LC-MS grade methanol supplied by Arcos Organics, U.S.A.

3.2.3. Other Materials

Petri dishes, TLC tanks, watch glass and pre-coated TLC Silica 60-coated plates were from Machery-Nagel Co., Germany and were used as stationary phase for thin layer chromatography (TLC). A heat gun was used to dry the plates for the visual identification of the sugar and fatty acid moieties after staining. The chromatographic column for the purification of the identified fractions in the crude biosurfactant, was packed with silica gel 60, (particle size 0.060-0.2mm, with 70-

230 mesh), supplied by Alfa Aesar, Lancaster, UK. NMR tubes supplied by Wilmad LabGlass, UK were used to run the NMR analysis.

3.2.4. Growth Media Preparation

A. Nutrient Broth: The bacteria strains from the nutrient agar slants was revived in nutrient broth, which was aseptically prepared by weighing 6.5g of the nutrient agar powder into 500mL of deionised sterile water in a 1000mL conical flask. The mixture was autoclaved at 121^{o}C for 20mins and stored in the fridge.

B. Nutrient Agar Plates: The nutrient agar plates were aseptically prepared by adding 14g of the nutrient agar powder into 500mL of deionised sterile water, in a 1000mL conical flask. The mixture was sterilised in an autoclave at 121^{o}C for 20mins, and allowed to cool to 55^{o}C. Approximately 10mL of the sterilised agar was aliquoted into petri-dishes, and allowed to set at room temperature. The plates were then stored at 4^{o}C.

C. Pre-Culture Media: The pre-culture media is the media used in growing the colonies obtained from the plates (also known as inoculum). Kay's Minimal Medium (Gunther et al. 2005) was used as the inoculum for this work, and it was prepared using the chemicals listed in Table 3.2 below.

Table 3.2: Composition of Kay's Minimal Medium

Compound	Concentration (g/L)
$NH_4H_2PO_4$	3
K_2HPO_4	2
$MgSO_4$	1
$FeSO_4$	0.0005
Glucose	2

The pH was adjusted to 7 using dilute hydrochloric acid (0.1M) and 2M sodium hydroxide and stored in a cold room at 4^{o}C.

D. Growth Medium: The media used for the cultivation of the strains was mineral salts medium (MSM), with some trace elements added to it. The compounds used for the preparation of the MSM are found in Table 3.3.

Table 3.3: Composition of Mineral Salts Medium

Compound	Concentration (g/L)
Glycerol (Carbon Substrate)	2% (w/v)
$NaNO_3$	2
Na_2HPO_4	0.9
KH_2PO_4	0.7
$MgSO_4.7H_2O$	0.4
$CaCl_2.2H_2O$	0.1
$FeSO_4.7H_2O$	0.001
Trace Elements	**Concentration (g/L)**
$ZnSO_4.7H_2O$	0.7
$CuSO_4.5H_2O$	0.5
$MnSO_4.H_2O$	0.5
H_3BO_3	0.26
$MoNa_2O_4.2H_2O$	0.06

The pH was adjusted to 6.8 using sodium hydroxide and dilute hydrochloric acid, sterilised and stored at 4^{o}C.

3.2.5. Cultivation of Bacteria

A. Reviving the Bacteria: The bacteria on the nutrient agar slants were revived aseptically by transferring a sample of the bacteria into 10mL of sterilised nutrient broth using flamed loop. The bacteria were incubated at 37^{o}C for 24 hours.

B. Growing the Colonies: The revived bacteria in the nutrient broth were aseptically streaked on the nutrient agar plates using a flamed loop. The plates were incubated at 37^{o}C for 24 hours. Figure 3.3 shows the nutrient agar plates containing colonies of *P. aeruginosa PS1 and ST5* respectively.

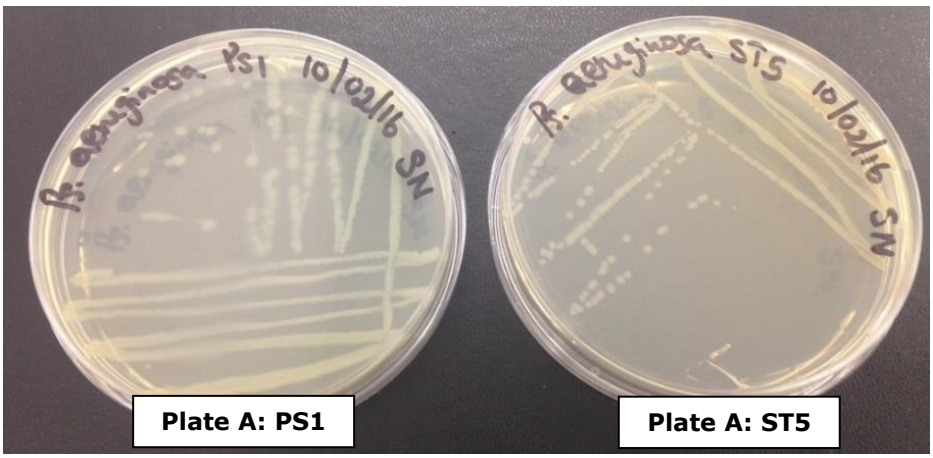


Figure 3.3: Nutrient Agar Plates Containing Colonies.

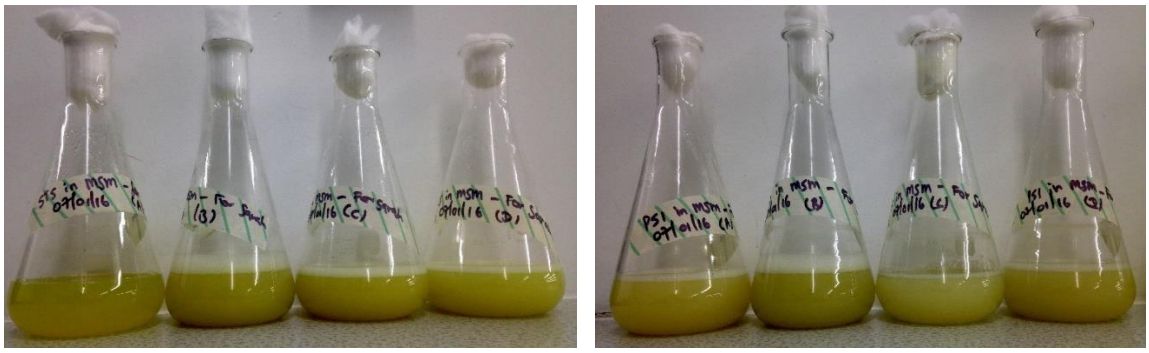
C. Cultivation of the Pre-culture: Distinct colonies of the grown bacteria (*Ps. aeruginosa PS1 and ST5* respectively) were transferred from the plate using a flamed loop into a 25mL screw-cap universal tube containing 10mL of sterilized Kay's minimal medium. The closed universal tubes were incubated at 37^{o}C for 24 hours. The cultivated pre-culture (Kay's minimal medium) containing *Pseudomonas aeruginosa* PS1 and ST5, is shown in Figure 3.4 below.

Figure 3.4: Pre-cultures of *Pseudomonas aeruginosa* PS1 and ST5

D. Cultivation of Growth Media by Shake Flask Method: The bacteria was cultivated by transferring 100 µL of the pre-culture (inoculum) into a sterile 500 mL conical flask containing 200 mL of sterile mineral salts media (MSM). The cultivation was carried out on a batch scale using an orbital shaker incubator at 37^{o}C, agitated at 200rpm (Figure 3.6) and the growth monitored (see section 3.2.6) to determine the growth curve.

Figure 3.5: Replicate flasks of culture broths containing Ps. Aeruginosa (ST5 & PS1)

Figure 3.7 below shows the culture broths at the end of the cultivation.



(a) **(b)**

Figure 3.6: Culture broths at the end of cultivation.

Where (a) Culture broth for Pseudomonas aeruginosa ST5
(b) Culture broth for Pseudomonas aeruginosa PS1

The flowchart for the cultivation of the bacteria from the slants is shown in Figure 3.8 below

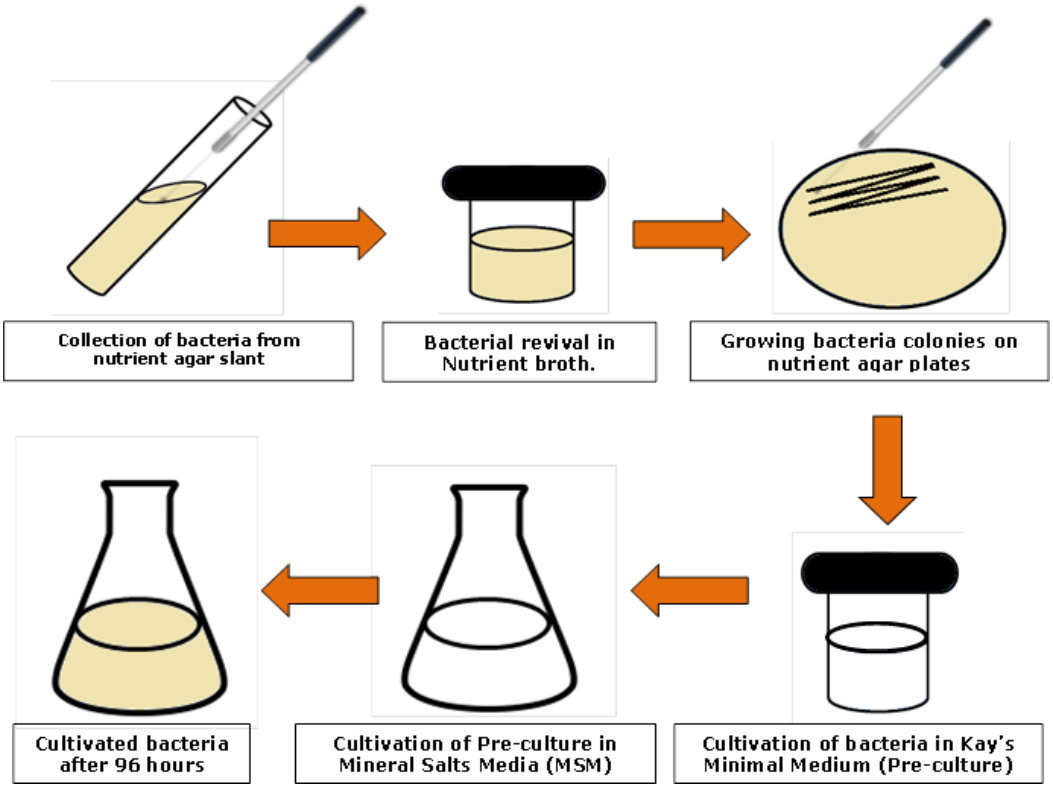


Figure 3.7: Flowchart of Bacteria Cultivation from Slants

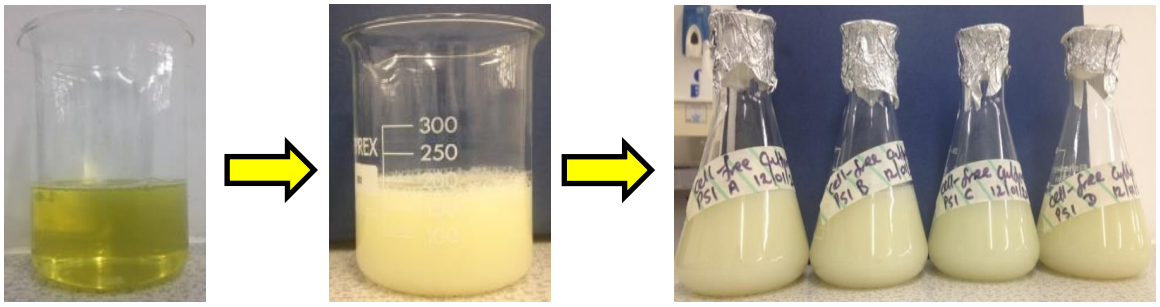
3.2.6. Cell Growth Determination

The growth of both strains was monitored by taking samples periodically from the culture broth and measuring optical density at 600 nm (OD_{600}) using a UV-visible spectrophotometer (Prabu et al. 2015). Optical density (OD) of a culture was measured to estimate the growth and metabolic activity of the cells. The growth curve of bacteria strains were obtained by plotting the absorbance of the culture broth (OD_{600}) against the cultivation time (hours). The growth of both strains was monitored for 160 hours at regular time intervals.

3.2.7. Extraction and Recovery of Biosurfactant

3.2.7.1. Removal of Bacteria Cells

At the end of the cultivation period, the cells in the broth were removed by centrifuging the broth at 13,000rpm for 15 min at 4°C. The cell-free broth was transferred to a 300 mL sterilised beaker (Figure 3.8a), and acidified to pH 2.5 using concentrated HCl (Figure 3.8b). The acidified cell-free broth was transferred to a sterile 500 mL conical flask, labelled and stored overnight at 4°C (Figure 3.8c).



(a) (b) (c)

Figure 3.8: Removal of Bacteria Cells for the Recovery of Biosurfactant

Where (a) Cell-free Culture after centrifugation and removal of cells
(b) Cell-free Culture after acidification
(c) Acidified cell-free culture left overnight at 4^{o}C

3.2.7.2. Recovery of Biosurfactant by Solvent Extraction

The biosurfactant in the acidified cell-free broth (left overnight) was recovered by solvent extraction following a method adapted from Smyth et al. (2010) via the following steps:

1. The biosurfactant in the broth was extracted by using equal volume of ethyl acetate (200mL) in a 500 mL separating funnel. The extraction was carried out by shaking the mixture vigorously, and allowing the two layers to separate in a separating funnel (Figure 3.9).
2. The aqueous layer made up of the acidified cell-free broth (below) and the organic layer made up of the extracted biosurfactant and ethyl acetate (top) were transferred into two separate flasks. The aqueous layer was re-extracted with equal volume of ethyl acetate until no further colour persists in the ethyl acetate layer (3 times).

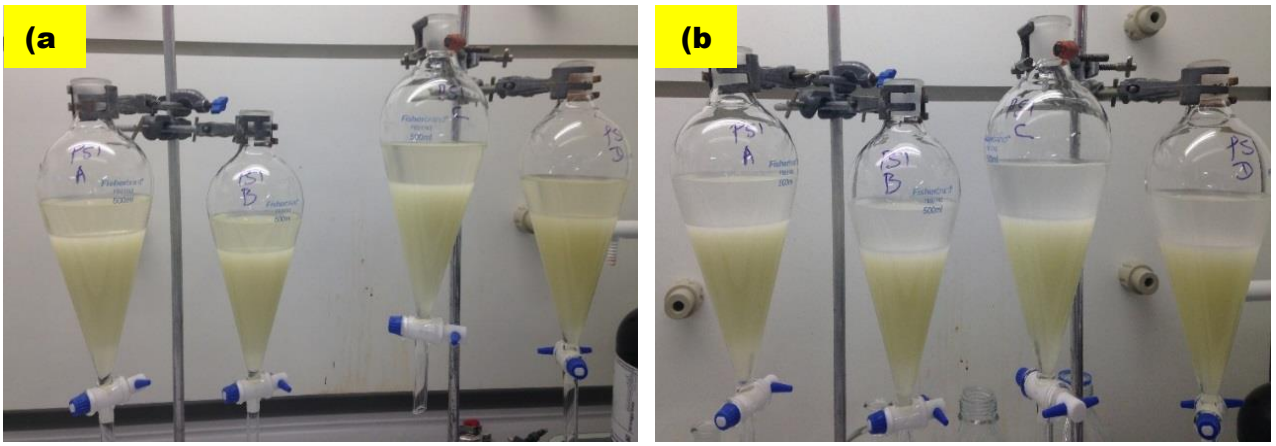


Figure 3.9: Solvent extraction of rhamnolipid using ethyl acetate.

Where: (a) First Extraction (b) Completed extraction (no further colour)

3. The ethyl acetate fractions were combined (per culture flask, approximately 600 mL), and the water content removed with approximately 3 g of anhydrous magnesium sulphate in a 1000 mL Duran glass bottle, and filtered to remove the magnesium sulphate.
4. The filtrate was rotary evaporated to remove the ethyl acetate to afford a yellowish-brown gum extract.
5. The extract was further dried in a vacuum oven at 30^{o}C overnight to remove any traces of ethyl acetate. Figure 3.10 shows the biosurfactant extract obtained at the end of the extraction and drying process.

Figure 3.10: Crude Biosurfactant Extract

3.2.8. Identification and Purification of Biosurfactant

The components in the crude biosurfactant extract were identified using thin layer chromatography (TLC) and further purified by column chromatography.

A. Identification by Thin layer chromatography (TLC)

Thin-layer chromatography is a technique applied for the separation of dissolved chemical substances, based on their differential migration over sheets coated with a thin layer of a finely ground adsorbent, such as silica gel or alumina. A TLC test was carried out on the crude extract using TLC Silica 60-coated plates and a solvent system (mobile phase) made up of chloroform, methanol and glacial acetic acid in a ratio of 65:15:2.

The crude extract was dissolved in chloroform and with a capillary glass pipette a spot of the dissolved sample was placed on the base line of the silica coated plates (1 plate each for sugar and lipid test). The silica plate was then placed in a TLC tank containing the mobile phase. The plate was removed when the solvent front reached near the top of the TLC plate. The plates were stained using the sugar and the lipid reagents:

Sugar Stain: Anisaldehyde reagent made up of; anisaldehyde, concentrated sulphuric acid, glacial acetic acid and ethanol in the following ratio: 1.2:4:1.2:80 respectively (personal communication from Professor Paul Kong Thoo Lin), was used to detect the sugar moiety in the crude extract.

Lipid Stain: Ceric Ammonium Molybdate reagent made up of 12.5 g ammonium molybdate, 0.5 g cerium (IV) sulphate, 25 mL sulphuric acid, in 225 mL water (Sulikowski 2015).

After each staining, the plates were visualized by heating the plates at about 100°C for 3 min.

B. Purification by Column Chromatography

In order to obtain pure samples of the fractions observed from the TLC analysis, the crude extract was purified using column chromatography, following a method adapted form Zhao et al. (2013) and Raza et al. (2009). About 1 g of the extract was dissolved in 4 mL of chloroform, and 3 mL loaded onto a 2 cm × 30 cm column packed with 10 g of Silica gel 60. The loaded column was eluted with 300 mL of chloroform to eliminate any phospholipids/lipids that may be present in the sample, and then with the following sequence of methanol:chloroform:

1. 5:95% (v/v); 100mL
2. 10:90% (v/v); 100mL
3. 20:80% (v/v); 200mL
4. 50:50% (v/v); 200mL

The elution flow rate was adjusted to 1 mL/min. The different fractions obtained from the elution were monitored and identified using TLC (as described in 3.2.8.A). The fractions with the same Rf value from the TLC analysis, were combined and rotary evaporated to remove the eluting solvent. The recovered extract was dried in a vacuum oven and weighed to determine the yield from the crude extract.

3.2.9. Physical Characterisation of Rhamnolipid

The physical characterisation of rhamnolipid was studied using the following analytical parameters;

1. Surface tension
2. Critical micelle concentration (CMC).
3. Emulsification index (E_{24})

3.2.9.1. Surface Tension

Surface tension measurements are used to study the surface activity of surfactants and their ability to reduce the surface tension of water. The surface tension of the crude rhamnolipid extracts, purified fractions and SDS were determined using a torsion balance (OS, White Electrical Instrument Co, London) and a Du Nouy Platinum ring.

The following concentrations were prepared for the analysis of the crude rhamnolipid and purified fractions: 2.5, 10, 20, 40, 50, 75, 100 ppm and a blank. The surface tension of the SDS was measured in millimoles (mM) to check similarity with already published work. The following concentrations were prepared for the surface tension analysis of SDS: 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 mM and a blank. The platinum ring and platform were cleaned with ethanol and twice with deionised water prior to the analysis of each sample. Approximately 1 mL of the standard solution was measured into a glass concave dish and the surface tension measured at temperatures ranging between 20 to 21 °C. The surface tension measurements were made in triplicates per concentration of sample. The surface tension of deionised water was determined intermittently between measurements as a quality control check of the instrument.

3.2.9.2. Critical Micelle Concentration (CMC)

The critical micelle concentration (CMC) is defined as the concentration of a surfactant above which micelles begin to form in the solution (Myers 1988). A micelle is an aggregate of surfactant molecules (with hydrophilic head and hydrophobic tail/chain) dispersed in the liquid. There are several techniques that can be used to determine the CMC of a surfactant and these include: tensiometry, conductometry, calorimetry, and viscometry (Fu et al. 2015). The CMC of the crude biosurfactant and purified fractions were determined by plotting the surface tension results against the concentrations studied. The value of the CMC was

determined at the point of inflection on the plot. Figure 3.11 shows a typical plot for the determination of CMC by tensiometry.

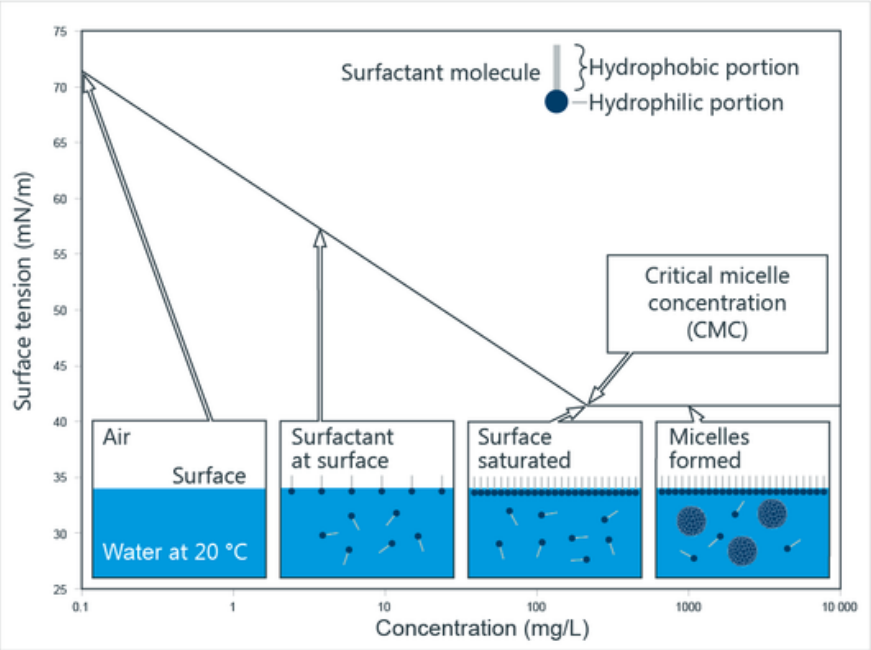


Figure 3.11: Typical Plot for the Determination of CMC by Tensiometry

Available from: KRÜSS GmbH (2017)

3.2.9.3. Emulsification Activity (E_{24})

The emulsification activity of the rhamnolipids and SDS were investigated by evaluating the emulsification index (E_{24}) after 24 hours. The E_{24} value is an index that gives an indication of the ability of the biosurfactants to solubilize hydrophobic molecules by trapping them in a pseudo-hydrophobic phase formed by micelles, thereby increasing their solubility in the hydrophilic phase (Bendaha et al. 2012).

Emulsification index (E_{24}) analysis was carried out following a method adapted from Abouseoud, Maachi and Amrane (2007). The following samples were analysed: kerosene, diesel, crude oil and sun-flower oil. E_{24} of the samples was determined by measuring 2 ml of the hydrocarbon source and 2 ml of surfactant into a 10 mL graduated test tube. The mixture was mixed on a vortex instrument for 2 minutes, placed on a test tube rack, and allowed to stand for 24 hours. The E_{24} was calculated as a percentage of height of emulsified layer (cm) divided by total height of the mixture (cm) as shown in equation 3.1 below;

Equation 3.1

$$E_{24} = \frac{\text{Height of emulsified oil}}{\text{Total height of the mixture}} \times 100$$

The emulsification analysis was carried out on SDS (Figure 3.12), crude ST5 rhamnolipid (Figure 3.13) and its corresponding purified fractions: ST5 monorhamnolipid (Figure 3.14) and ST5 dirhamnolipid (Figure 3.15). The emulsification study was not carried out on the crude PS1 rhamnolipid and its purified fractions as the research was later narrowed to focus on the ST5 rhamnolipid.

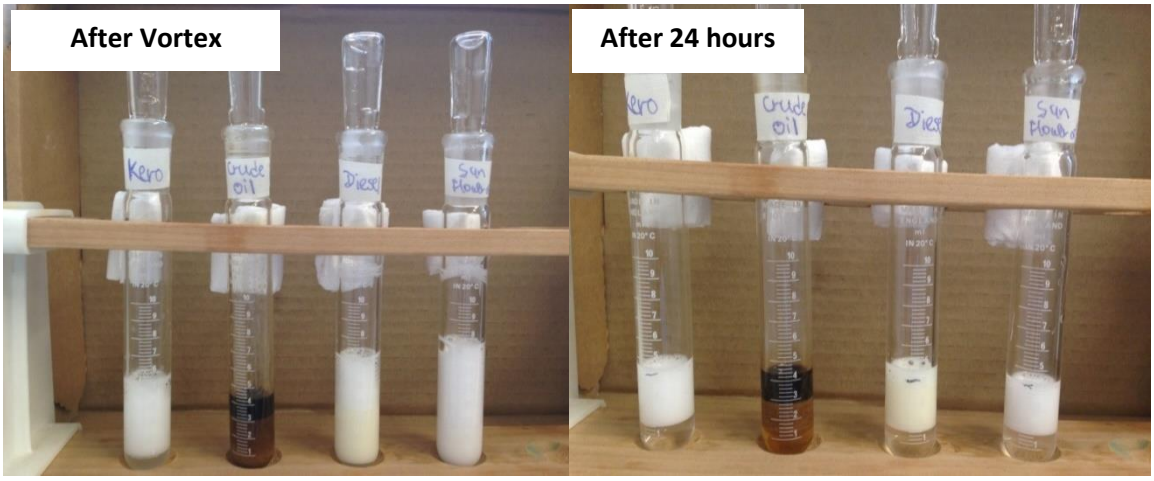


Figure 3.12: Emulsification Index Analysis on SDS

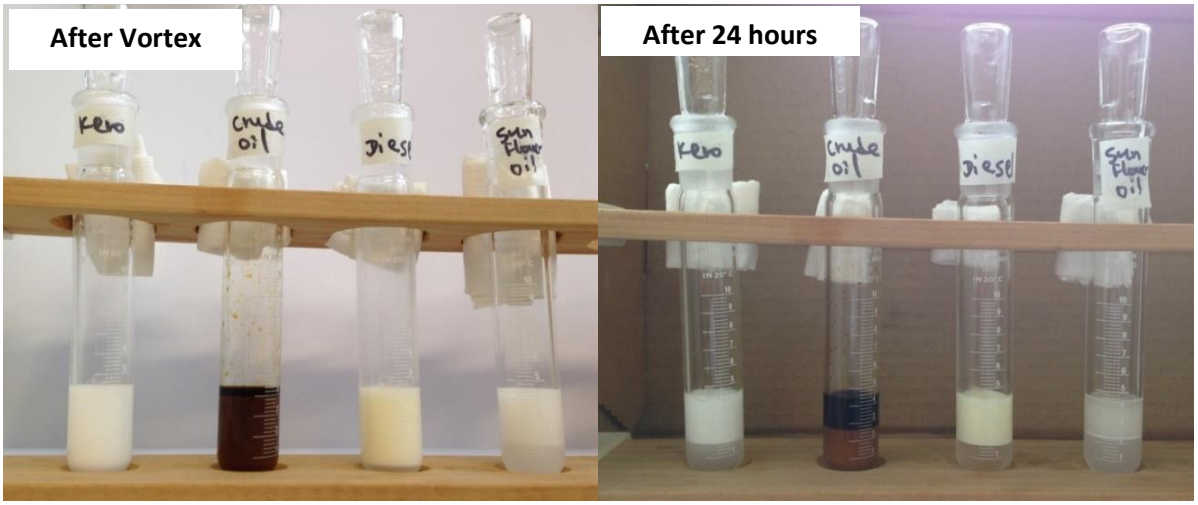


Figure 3.13: Emulsification Index Analysis on Crude ST5 Rhamnolipid

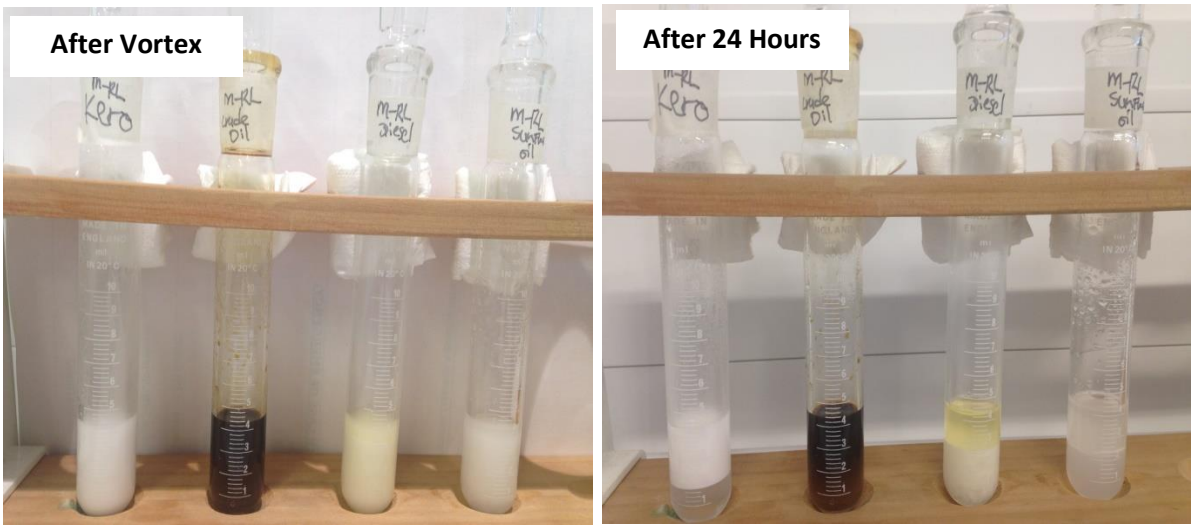


Figure 3.14: Emulsification Index Analysis on ST5 Monorhamnolipid

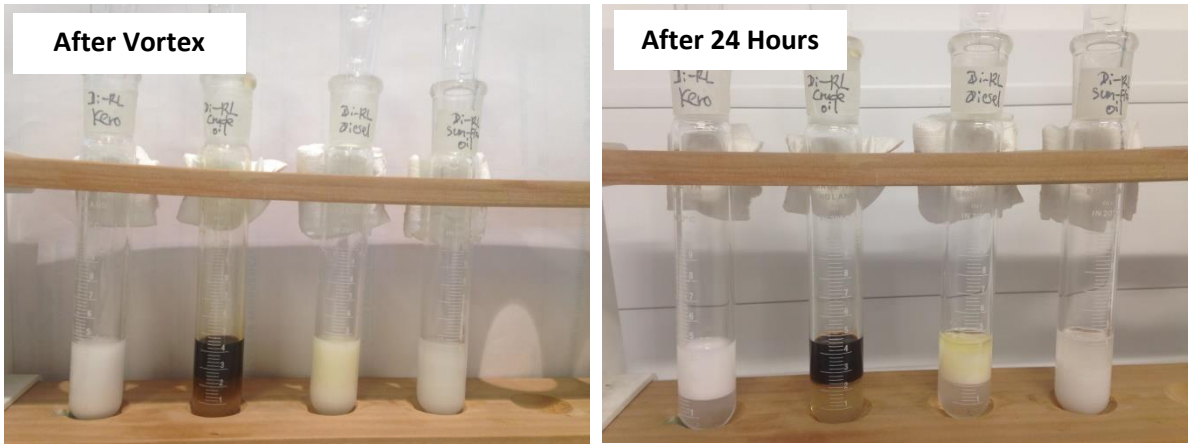


Figure 3.15: Emulsification Index Analysis on ST5 Dirhamnolipid

3.2.10. Chemical/Structural Characterisation of Rhamnolipid

The chemical structure of the produced biosurfactants was elucidated by characterising the samples with the following analytical methods;

1. Attenuated Total Reflectance (ATR) - Fourier transform infrared (FTIR) spectroscopy
2. Nuclear magnetic resonance (NMR) spectroscopy
3. Liquid chromatography – Mass spectrometry/Mass spectrometry (LC-MS/MS)

3.2.10.1. ATR-FTIR

ATR - FTIR technique gives information on the functional groups present in a sample. The information obtained from this analysis can be used in elucidating the chemical structure of the sample. The analysis involves the collection of radiation reflected from the interfacial surface between a sample and a reflection element (diamond crystal). Evanescent waves emanating from the crystal penetrates the sample, and is absorbed by the components inherent in the sample (Leitermann, Syldatk, and Hausmann 2008). The technique measures changes (in wavenumber) that take place in the sample as a result of this interaction (PerkinElmer 2005). This analysis was carried out using a Thermo Fisher Scientific Nicolet iS50 FTIR Spectrometer, fitted with an ATR accessory.

Approximately 5 mg was sampled from the biosurfactant on to the surface of the diamond crystal. The swivel pressure tower was then used to screw the sample tightly to the diamond crystal. The samples were run in the spectra region of 4000 – 400 cm^{-1} by averaging 30 scans at a resolution of 2 cm^{-1}.

3.2.10.2. Nuclear magnetic resonance (NMR)

NMR spectroscopy is an analytical technique that studies the transitions in atoms with a magnetic moment when in contact with an external magnetic field. NMR can be used to obtain structural information from a sample via the following parameters; the chemical shifts of the absorption frequency, the coupling constants (mutual influence of adjacent nuclei), and integral height (Heyd et al. 2008).

NMR spectroscopy is useful for the identification of some functional groups as well as the position of linkages within the sugar (rhamnose) and lipid molecules. The biosurfactants (crude extract and purified fractions) were dissolved in deuterated chloroform ($CDCl_3$) and analysed on an Avance 400MHz NMR spectrometer supplied by Bruker, Germany. The samples were analysed by one dimensional (1D) proton (^1H) and carbon 13 (^{13}C) NMR respectively.

3.2.10.3. Liquid chromatography – Mass spectrometry/Mass Spectrometry (LC-MS/MS)

LC-MS/MS is a hyphenated analytical technique that combines the separation technique of high performance liquid chromatography and the detection technique of mass spectrometry which involves the production, separation and identification of charged species in a sample. The experiment was carried out using an Agilent 6400 series Triple Quadrupole LC/MS system, fitted with the following; an Agilent 1260 Autosampler, an Agilent 1260 HPLC Quaternary Pump, an Agilent 1200 Variable Wavelength Detector and an Agilent 6420 Triple Quadrupole MS.

The system works on an Agilent Mass Hunter Workstation which was utilized for data acquisition and analysis. The samples were thoroughly oven dried at 50^oC, dissolved in methanol and placed on the autosampler crate for analysis. The analysis was carried out using the conditions itemised in Tables 3.4 (chromatographic conditions), 3.5 (Pump Gradient Time table) and 3.6 (Mass Spectrometer QQQ conditions).

Table 3.4: Chromatographic Conditions

Chromatographic Conditions	
Column	ZORBAX Eclipse Plus C18 2.1 x 50 mm, 1.8 µm
Column Temperature	40^{o}C
Mobile Phase	A: Deionised water + 0.1% formic acid B: Acetonitrile+ 0.1 % formic acid
Flow rate	0.25mL/min
Injection volume	20 µl
Pressure limit	High limit: 600.00 bar
Sample Temperature	5°C
Pump Gradient Time table	See Table 3.5
Sample Run Time	10 mins

Table 3.5: Pump Gradient Time Table

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0.00	40	60
4.00	5	95
8.50	5	95
9.50	40	60
10.00	40	60

Table 3.6: Mass Spectrometer QQQ Conditions

Mass Spectrometer QQQ conditions	
Ion source	Electrospray ionization
Nitrogen Gas Temperature	350°C
Gas Flow Rate	6 L/min
Nebulizer Pressure	15 psi
Capillary voltage	4000 V
Fragmentation voltage	25 V
mass range	100 – 1000 amu

3.3. Results and Discussion

3.3.1. Microorganism and Cultivation

The result of the growth curve study carried out on the two strains (*Pseudomonas aeruginosa* ST5 and PS1) is shown in Figure 3.16 below:

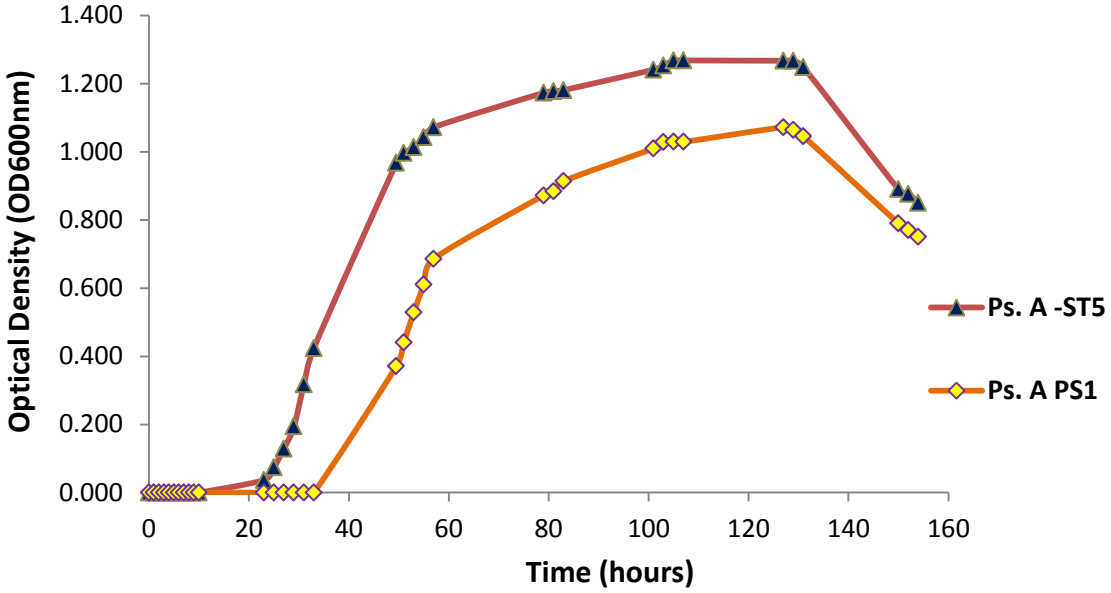


Figure 3.16: Growth Curve for *Ps. aeruginosa* ST5 and PS1

The growth curves for the culture broths cultivated with *Ps. aeruginosa* ST5 and *Ps. aeruginosa* PS1 showed similar trend, although the optical density (OD_{600}) of the *Ps. aeruginosa* ST5 medium was higher than that of *Ps. aeruginosa* PS1 by approximately 20% between 40 - 130hr, which could be as a result of the difference in the seeding density of the strains. The curves show that the PS1 strain has a slower growth rate than the ST5 strain. No growth was observed in PS1 until after 33 hours whilst the ST5 strain began to grow after 9 hours.

3.3.1.1. Product Yield

Although the start of the growth rates of both strains differs by 24 hours, the biosurfactant yields from the cultivation of both strains were not significantly different. The average yield of culturing four (4) replicate flasks for both strains gave an approximate yield of 3.31g/L and 3.44g/L (Table 3.7) from culture media cultivated using *Ps. aeruginosa* ST5 and PS1 respectively. This yield was obtained after 96 hours of incubation at 37°C.

Table 3.7: Product Yield for *Ps. Aeruginosa* ST5 and PS1

Strains	Replicates (g/200mL)				Mean (g/200mL)	Mean (g/L)	SD
ST5	0.57	0.61	0.73	0.74	0.6625	3.3125	0.07
PS1	0.70	0.78	0.62	0.65	0.6875	3.4375	0.06

The yield obtained in this study falls within the range reported by other researchers who utilised other strains of *Ps. aeruginosa* to produce rhamnolipids, using shake flask batch cultivation method, mineral salts media and glycerol as carbon source such as Rahman et al. (2002) obtained a yield of 1.77g/L, whilst Monteiro et al. (2007) reported a yield of 3.9 g/L. Although the latter used 3% (w/v) glycerol for the media formulation, and cultivated the culture for 9 days at 30^{o}C.

3.3.2. Identification and Purification of Rhamnolipid

3.3.2.1. Thin layer chromatography (TLC) Detection

TLC analysis was carried out on the following crude biosurfactant extract (Figure 3.17);

A. Crude rhamnolipid extract collected from Professor Ibrahim Banat of School of Biomedical Sciences University of Ulster, Northern Ireland. This sample served as a control check.
B. Crude rhamnolipid extract produced from *Ps. aeruginosa* ST5 using MSM
C. Crude rhamnolipid extract produced from *Ps. aeruginosa* PS1 using MSM

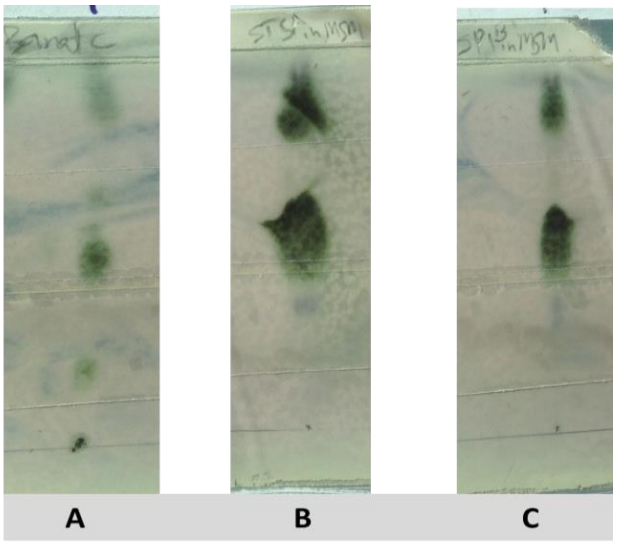


Figure 3.17: Thin Layer Chromatography of Rhamnolipids using Anisaldehyde Reagent (Sugar Test)
Mobile phase: Chloroform:Methanol:Glacial Acetic 65:15:2

Where A: Control check from Prof Banat's sample
B: Crude ST5 Rhamnolipid
C: Crude PS1 Rhamnolipid

The plates were stained with anisaldehyde reagent to confirm the presence of the sugar moiety, while the lipid moiety was confirmed by staining the plates with ceric ammonium molybdate reagent (as described in section 3.2.8). Figure 3.18 shows the TLC test result for the lipid moiety.

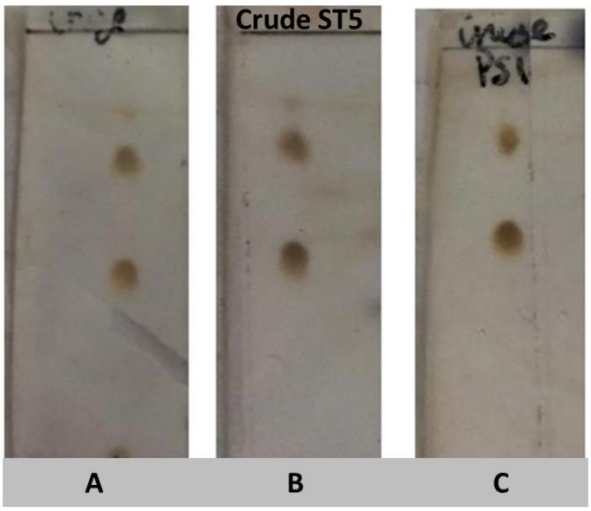


Figure 3.18: **Thin Layer Chromatography of Rhamnolipids using Ceric Ammonium Molybdate Reagent (Lipid Test)**
Mobile phase: Chloroform:Methanol:Glacial Acetic 65:15:2

Where A: Control check from Prof Banat's sample
B: Crude ST5 Rhamnolipid
C: Crude PS1 Rhamnolipid

The test carried out on the 3 samples, showed two distinct spots at approximately the same positions on the TLC plate for both the sugar and the lipid tests. Confirming the presence of sugar and lipid moieties in the samples.

A subsequent TLC analysis on a fresh sample (crude) of biosurfactant produced using *Ps. aeruginosa* ST5 (Figure 3.19), allowed for determination of the retardation factors of the 2 main spots or components from the sugar and lipid test were carried out.

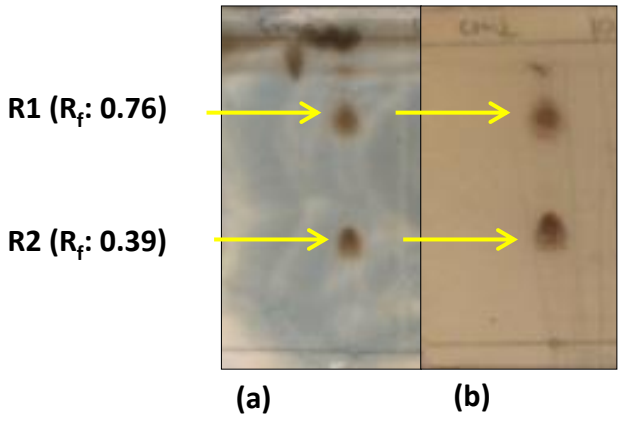


Figure 3.19: **Thin Layer Chromatography of Crude ST5 Rhamnolipid**
(a) With anisaldehyde reagent (sugar staining)
(b) With ceric ammonium molybdate reagent (lipid staining)

The retardation factors (R_f) values obtained for each spot was 0.76 and 0.39. Based on literature, these spots are taken to represent the monorhamnolipid (R1) and dirhamnolipid (R2) respectively (Wittgens et al., 2011). Lotfabad et al. (2010), for example found two main spots from a *P. aeruginosa* MR01 strain with Rf vales of 0.73 and 0.31 for R1 and R2 respectively. Arino, Marchal and Vandecasteele (1996) also reported similar retardation factors for R1 and R2 fractions (0.72 and 0.40 respectively). Further purification and characterisation of the identified sample was carried out to determine what the fractions were.

3.3.2.2. Purification by Column Chromatography

Column chromatography analysis was carried out on the crude extract produced from *Pseudomonas aeruginosa* ST5 and PS1 using silica as described in 3.2.8B. The analysis yielded 2 fractions from both strains, which confirms the result obtained from the TLC.

A. Fractionation of Biosurfactant Produced from *Pseudomonas aeruginosa* ST5

From the fractionation of 2,070 mg crude rhamnolipid produced using *Pseudomonas aeruginosa* ST5, the following yield was recovered for monorhamnolipid (R1) and dirhamnolipid (R2):

R1 = 650 mg

R2 = 270 mg

B. Fractionation of Biosurfactant Produced from *Pseudomonas aeruginosa* PS1

From the fractionation of 220 mg of crude rhamnolipid produced using *Pseudomonas aeruginosa* PS1, the following yield was recovered for R1 and R2:

R1 = 20 mg

R2 = 90 mg

The yields of both strains were calculated gravimetrically.

The differences observed in the yields obtained can be summarized as shown below;

- *Pseudomonas aeruginosa* ST5: R1 > R2 (2.4:1)
- *Pseudomonas aeruginosa* PS1: R1 < R2 (1:4.5)

3.3.3. Physical Characterisation of Rhamnolipid

In order to determine the surface active properties of crude rhamnolipid extracts (produced from *Pseudomonas aeruginosa* ST5 and PS1) and the recovered fractions (R1 and R2) from *Pseudomonas aeruginosa* ST5, physical characterization of the samples (4) was carried out using the following parameters: surface tension, CMC and emulsification activity.

3.3.3.1. Surface tension

Surface tension analysis carried out on the SDS showed a reduction of surface tension of deionised water from 72 mNm^{-1} to 30 mNm^{-1} at 20°C (Figure 3.21). The crude rhamnolipid recovered from *Pseudomonas aeruginosa* ST5 and PS1 strains showed both products reducing the surface tension of deionised water from 72 mNm^{-1} to 30 mNm^{-1} and 32 mNm^{-1} respectively at 21°C (Figure 3.22). Also, the purified fractions obtained from *Pseudomonas aeruginosa* ST5 reduced the surface tension of deionised water from 72 mNm^{-1} to 26 mNm^{-1} and 28 mNm^{-1} for R1 and R2 respectively at 21°C (Figure 3.23).

These results compare well with surface tension values reported in literature. Urum, Pekdemir and Gopur (2003) reported that SDS reduced the surface tension of water from 72 mNm^{-1} to 35 mNm^{-1}. Banat (1995a) also reported a surface tension value of 27.1 mNm^{-1} for SDS. A study carried out by Raza, Khalid and Banat (2009) using crude rhamnolipid obtained using canola waste frying oil (WFO) as carbon source, reduced the surface tension of water from 72 mNm^{-1} to 32 mNm^{-1}. Another study carried out by Raza et al. (2006), using crude rhamnolipid obtained with soybean using WFO as sole carbon source reduced the surface tension of water from 72 mNm^{-1} to 29.1 mNm^{-1}. The ability of the rhamnolipids to reduce the surface tension of water gives a good indication of the potential of the rhamnolipid to be utilised as a sustainable alternative for synthetic surfactants (Bai, Brusseau and Miller 1997).

3.3.3.2. Critical Micelle Concentration (CMC)

The CMC of is a useful parameter that gives a measure of the efficiency of a surfactant, especially when applied for cleaning purposes. It also guides the user by specifying the concentration the effective use. This is based on the fact that some surfactants are unable to reduce surface tension below CMC concentration. The CMC of SDS and the biosurfactants studied were obtained following the

method described in section 3.2.9.2. The plots of the surface tension against concentration for SDS (Figure 3.20), crude rhamnolipid produced using *Pseudomonas aeruginosa* ST5 and PS1 strains (Figure 3.21) and the purified rhamnolipid concentrations monorhamnolipid and dirhamnolipid produced from *Pseudomonas aeruginosa* ST5 (Figure 3.22) are shown below. The value of the CMC was determined at the point of inflection on the plot (as shown below).

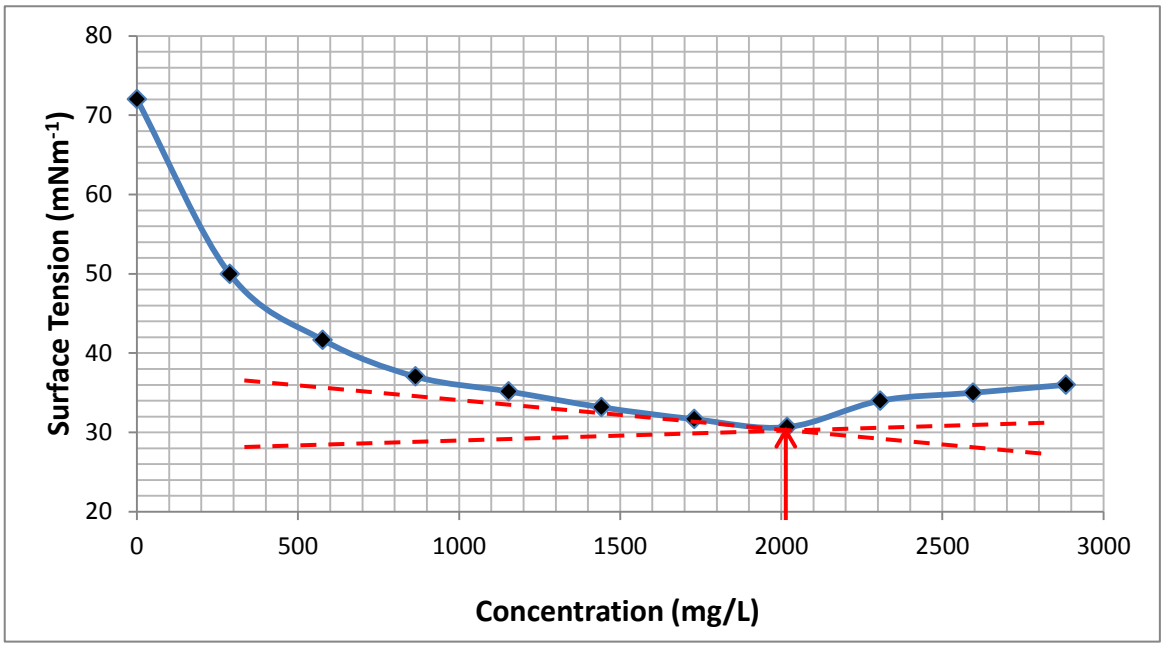


Figure 3.20: Determination of CMC of Sodium Dodecyl Sulphate (SDS)

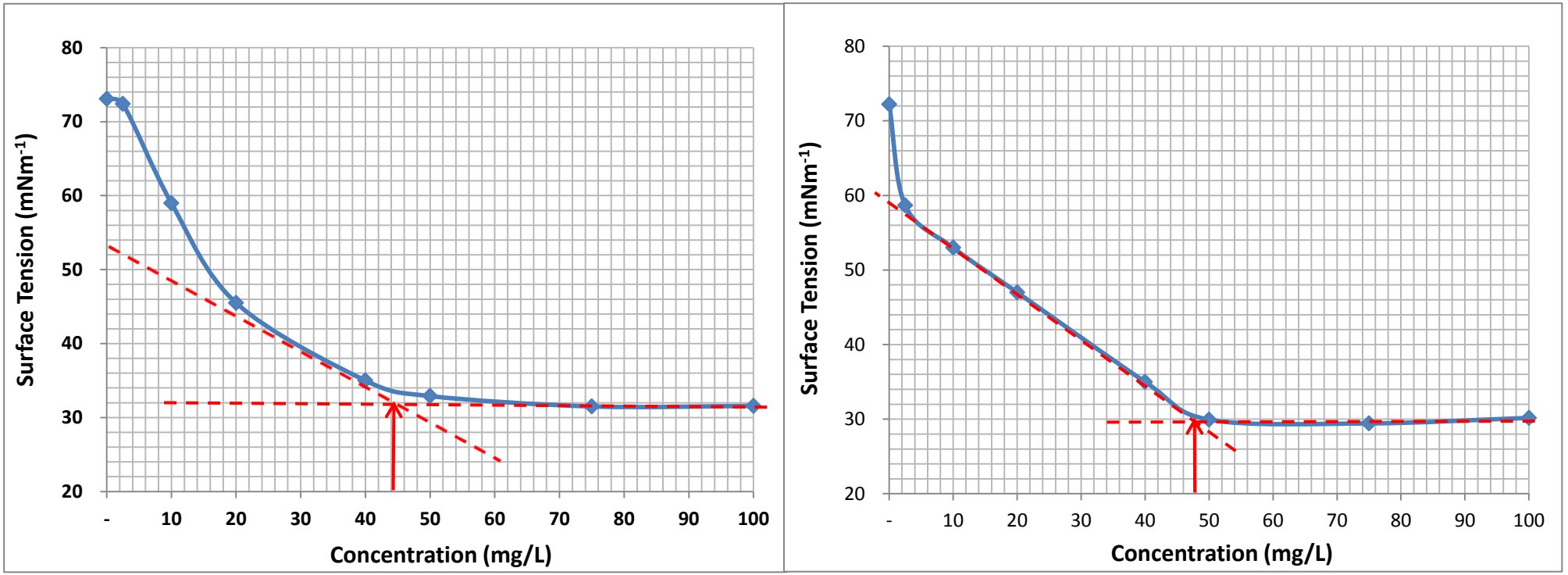


Figure 3.21: **Determination of CMC of Crude Rhamnolipid, Produced from *Ps. aeruginosa* sp.**
Where: *Ps. aeruginosa* PS1 (left) and *Ps. aeruginosa* ST5 (right)

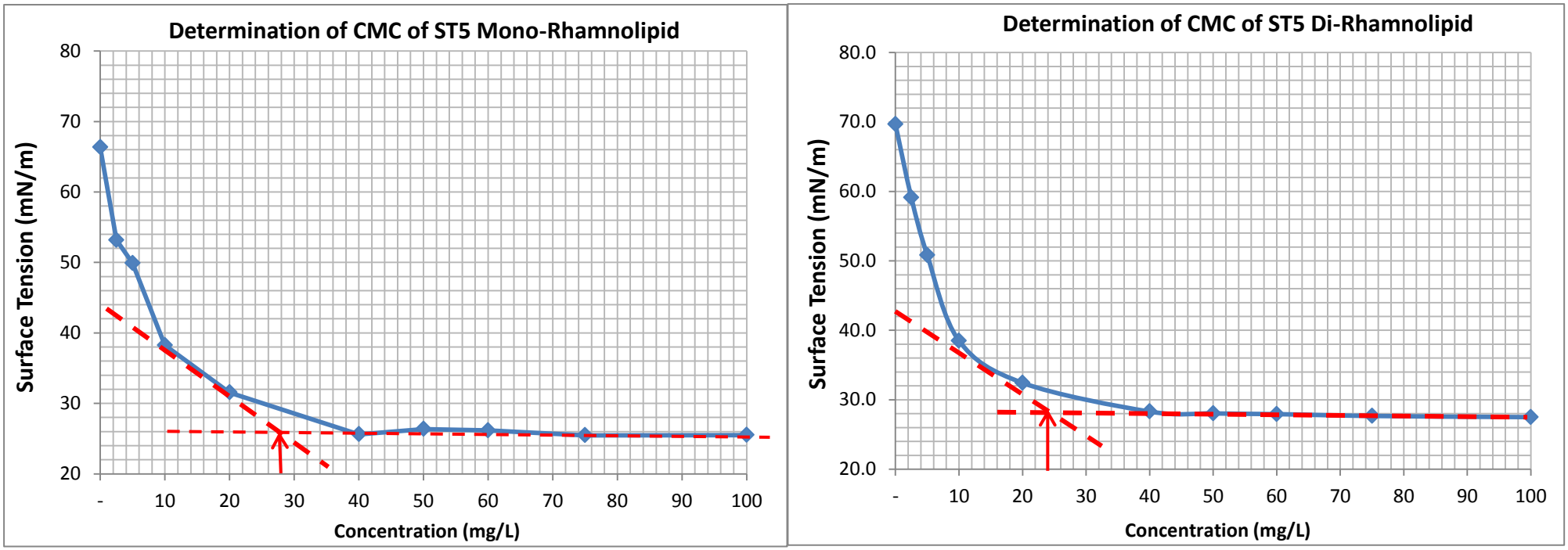


Figure 3.22: **Determination of CMC of Purified Rhamnolipid Produce from Ps. Aeruginosa ST5**
Monorhamnolipid (left) and Dirhamnolipid (right) Produced from *Ps. aeruginosa* ST5.

The CMCs and corresponding surface tension values of the rhamnolipids (Table 3.8) were determined at the point of inflection from the plots above. The point of inflection is the point on the plot where an increase in concentration of surfactant did not result in a significant reduction in surface tension (Mata-Sandoval, Karns and Torrents 1999).

Table 3.8: Critical Micelle Concentration (CMC) of Synthetic/Biological Surfactant

S/N	Surfactant	CMC (ppm)	At Surface Tension (mNm^{-1})	Temperature (^oC)
1.	SDS	2,018	30	20
2.	Crude ST5	48	30	21
3.	Crude PS1	46	32	21
4.	Monorhamnolipid - ST5	28	26	21
5.	Dirhamnolipid – ST5	24	28	21

The results obtained for the CMCs of the surfactants studied compared well with the result of similar studies found in literature. Noramiza et al. (2016), obtained a CMC of 2000 ppm for SDS, this value is comparable to the CMC value obtained in this work. Also, the CMC studies of rhamnolipid produced using different strains of *Ps. aeruginosa* falls in the range of 13 ppm and 200 ppm (Sathi et al. 2016; Wei, Chou and Chang 2005; Rahman et al. 2010 and Raza, Khalid and Banat 2009). As observed in Table 3.8, the CMC values obtained from the purified fractions were lower than that of the crude biosurfactant (ST5 and PS1). This is probably due to the fact that there may be mixtures of other congeners in the crude rhamnolipid. However, the CMC study for the fractions obtained from *Ps. aeruginosa* PS1 was not carried out due to time constraint

3.3.3.3. Emulsification Activity

The results of the emulsification activity (E_{24}) analysis carried out on SDS and crude ST5 rhamnolipid and the purified fractions using the kerosene, crude oil, diesel and sunflower oil is shown in Figure 3.23 below.

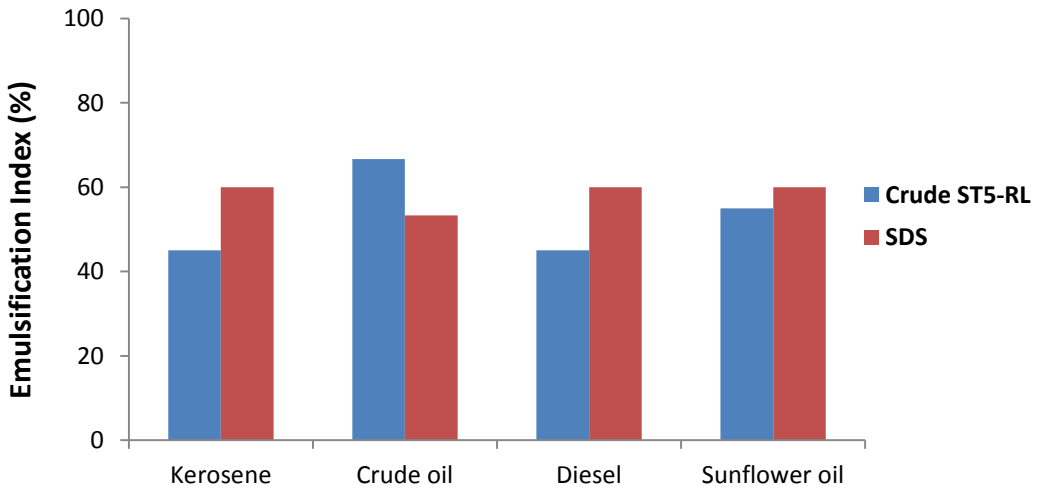


Figure 3.23: Emulsification Activity of SDS and Crude ST5 Rhamnolipid on Selected Oils

SDS compared with the crude ST5 to show difference between a synthetic surfactant and the biosurfactant. The crude ST5 rhamnolipid was also compared with its purified fractions (Figure 3.24). The measurements taken for the emulsification study were single measurements.

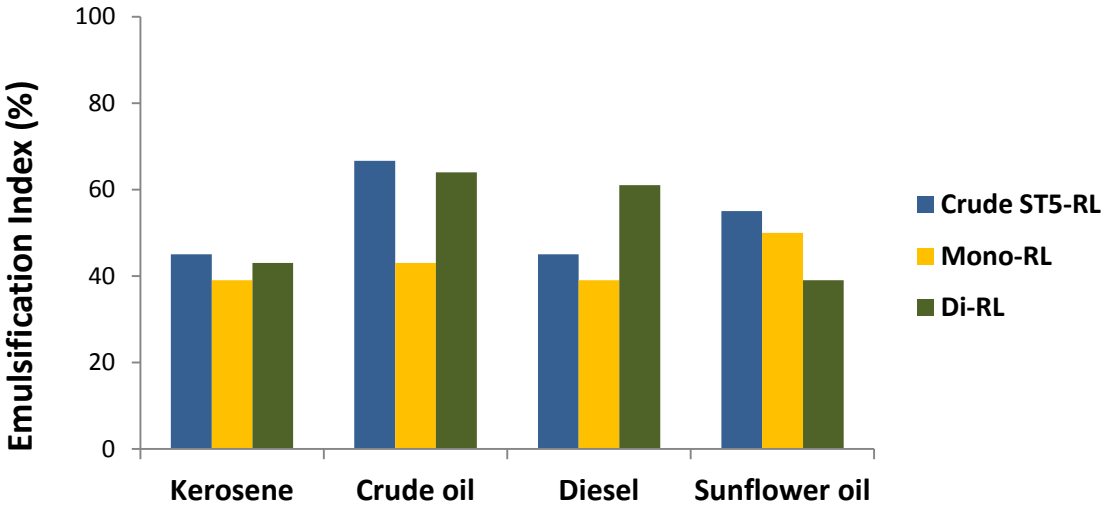


Figure 3.24: Emulsification Index of ST5 Rhamnolipids

The result from this test shows that dirhamnolipid (Di-RL) has higher emulsification index than that of monorhamnolipid (Mono-RL) on all the oils tested except sunflower oil. Also, the crude ST5-RL had a higher emulsification index than that of monorhamnolipid (Mono-RL) and dirhamnolipid (Di-RL) except diesel.

This may have implications in terms of the choice of biosurfactant for use in the treatment of hydrocarbon contaminated matrices.

3.3.3.4. Summary on Physical Characterisation of Rhamnolipids

The result of the physical characterisation of the rhamnolipids studied shows that, dirhamnolipid (Di-RL) had the lowest CMC value and higher emulsification index on the oils from petroleum origin than monorhamnolipid (Mono-RL). The chosen biosurfactant to be applied for this study is Crude ST5-RL. This choice is based primarily on the logistics (time and cost) associated with purifying the rhamnolipid to extract Di-RL especially when the biosurfactant is to be used for waste treament. Also the crude ST5-RL has a relatively low CMC of 48 ppm compared to CMC values of rhamnolipids reported in literature which ranges from 5 – 200 ppm.

3.3.4. Chemical/Structural Characterisation of Rhamnolipid

Although the physical characterisation confirms the potential of rhamnolipid as a viable biosurfactant, it is important that the chemical and structural characterisation be carried out to confirm the chemical structure of the biosurfactant (Irorere et al. 2017). The crude extract and fractionated rhamnolipid samples were investigated using ATR-FTIR, NMR and LC-MS/MS techniques. The procedures were adapted according to previous work reported in Smyth et al. (2010).

3.3.4.1. ATR - FTIR

The ATR - FTIR analysis carried out on the rhamnolipids can be seen in the spectra stacked in Figure 3.25 below.

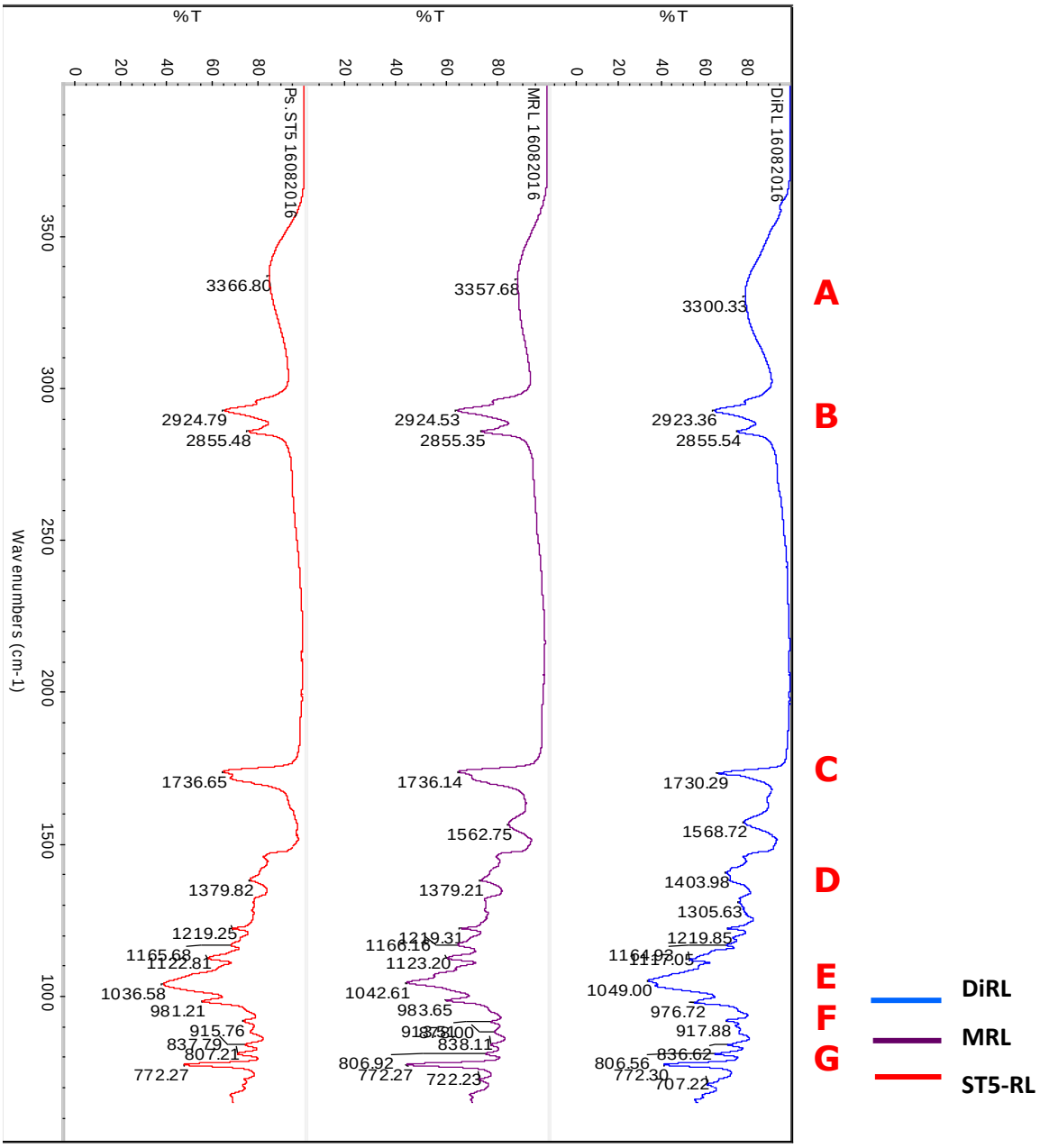


Figure 3.25: **FTIR Spectra for Rhamnolipids**
(For DiRL, MRL and ST5-RL).
Where A, B, C, D, E, F and G represent the regions

In order to elucidate the structure of the rhamnolipids, the functional groups identified with the characteristic peaks obtained from the spectra will be determined using a basic organic functional group reference chart from NIST

library. The FTIR – ATR analysis shows the presence of aliphatic hydrocarbons combined with a sugar moiety (Table 3.9) that is typical of a biosurfactant previously described in literature to be rhamnolipids (Moussai, Mohamed and Samak 2014; Leitermann, Syldatk and Hausmann 2008; Guo et al. 2009; Rahman et al. 2010 and Rikalovic et al. 2012).

Table 3.9: Assignment of FTIR Peaks

Region	Characteristic Peak Wave range (cm^{-1})	Assigned functional group
A	3366.80 - 3200.33	the –OH free stretch
B	2924.79 – 2855.48	aliphatic bond CH stretch
C	1736.65 - 1730.29	C=O stretching vibrations of the carbonyl groups
D	1455.69 – 1379.21	Bending of O–H bands in the carboxylic acid group)
E	1123.20 - 1100.29	C-O-C bond stretching that are characteristic of ether functional group found in the rhamnose.
F	983.65 – 913.51	pyranyl I sorption band
G	838.11 – 836.42	α-pyranyl II sorption band

From the spectra in Figure 3.19, the broad band at region **A** (3366.80 - 3200.33 cm^{-1}) indicates the presence of hydroxyl group (-OH stretching vibrations). The strong peaks observed at **B** (2921.96 cm^{-1}) indicates the presence of C-H stretch stretching of aliphatic chains, and a corresponding symmetric stretch is seen at 2855.98 cm^{-1}. The peaks observed **C** (1736.65 - 1730.29 cm^{-1}) shows the presence of C=O stretching vibrations of the carbonyl groups typical ofesters. The peaks at region **D** (1455.69 – 1379.21 cm^{-1}) indicates the bending of the hydroxyl (O-H) group confirms the presence of carboxylic acid functional groups in the molecule.

The absorption peak at region **E** (1123.20 - 1100.29 cm^{-1}) indicates the presence of C-O-C bond stretching that are characteristic of ether functional group found in the rhamnose (Moussa, Mohamed and Samak 2014; Rahman et al. 2010). The functional groups found on the peaks at regions **F** and **G** are associated with pyranyl I sorption band and α-pyranyl II sorption band, respectively. These peaks indicate the presence of dirhamnolipid in the spectrum (Moussa, Mohamed and Samak 2014).

3.3.4.2. Nuclear Magnetic Resonance (NMR)

NMR spectroscopy has been known as an excellent tool for elucidating the structure of organic compounds. It measures the absorption of radio frequencies by the atoms of the sample and, by interpreting the data, gives accurate information about the sample (Arab and Mulligan 2014).

The structures of the purified rhamnolipids obtained from ST5-RL were confirmed by using 1 D proton (^{1}H) and carbon 13 (^{13}C) NMR analyses with deuterated chloroform ($CDCl_3$) as solvent. The ^{1}H NMR spectra for monorhamnolipid and dirhamnolipids are shown on Figure 3.26 and 3.27 below;

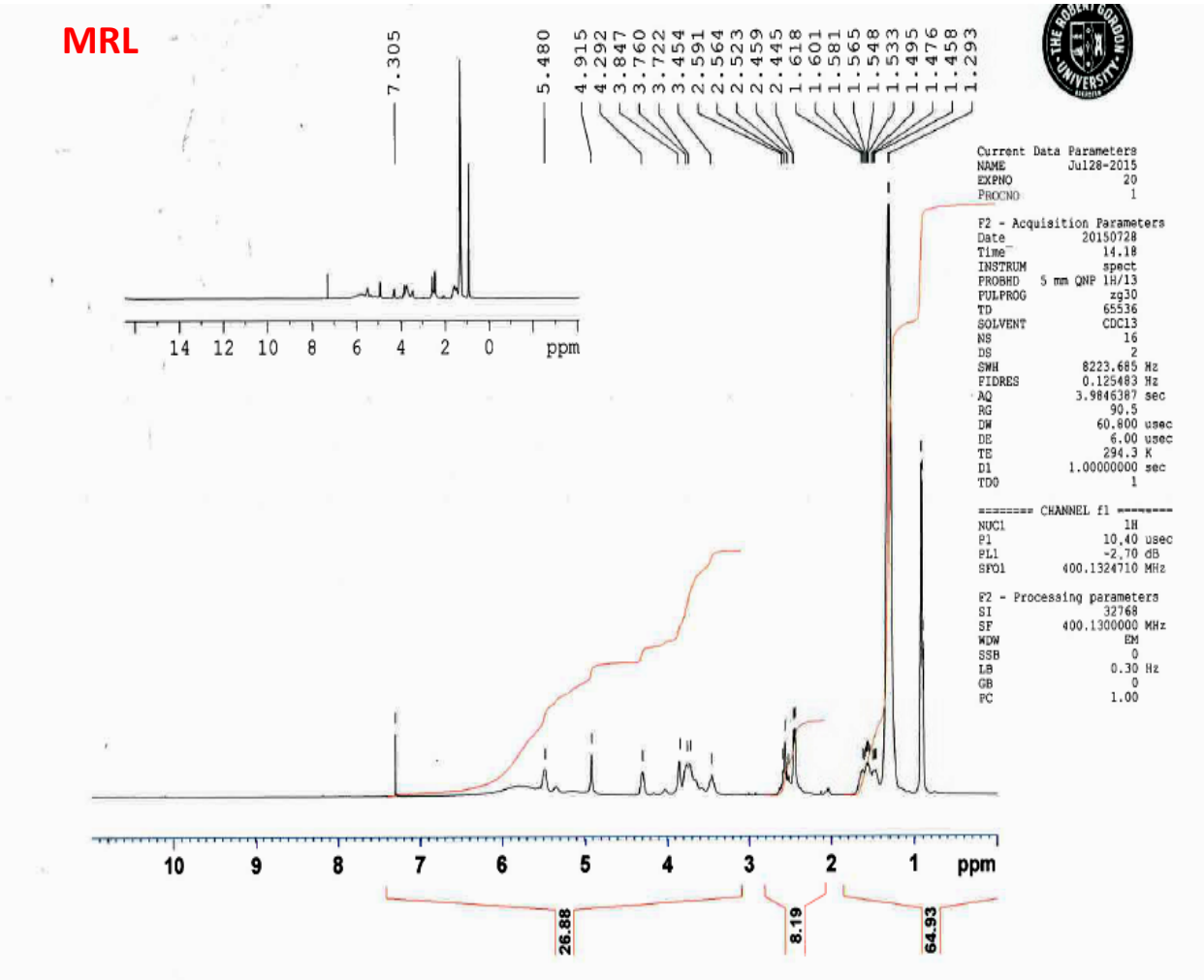


Figure 3.26: ^{1}H NMR spectra for ST5 Mono-RL ($CDCl_3$ solvent)

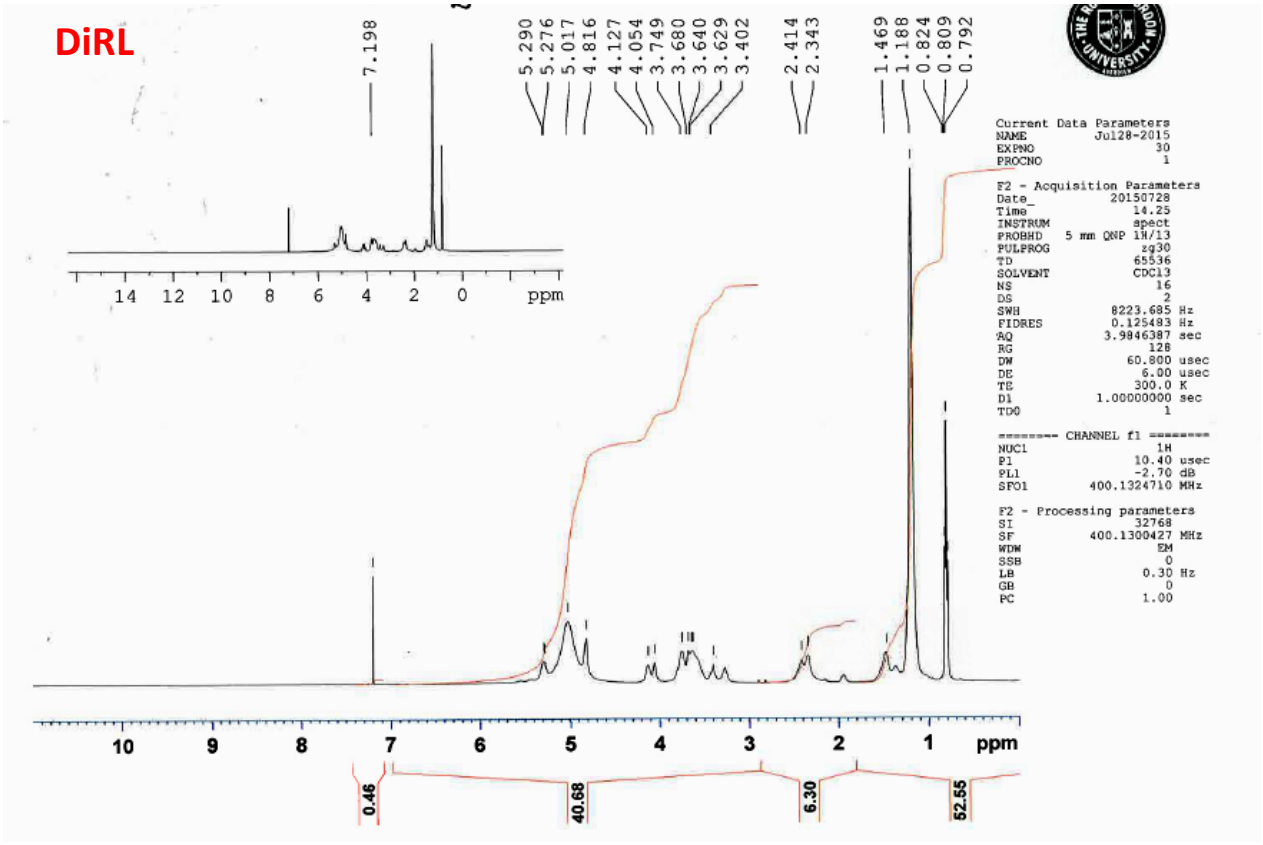


Figure 3.27: ^{1}H NMR spectra for ST5-Di-RL ($CDCl_3$ solvent)

Table 3.10 shows the results from ^{1}H NMR analysis of the fractions obtained from the purified rhamnolipid.

Table 3.10: Chemical Shift Assignment of Monorhamnolipid and Dirhamnolipid.

Assignment	Chemical Shift (ppm)	
	Monorhamnolipid	Dirhamnolipid
-CH_3	0.886	0.792 - 0.824
–$(CH_2)_n$–	1.293	1.188
–CH_2–COO–	2.445, 2.459	2.343, 2.414
–O–CH–	4.915	4.816
–COO–CH–	5.480	5.276, 5.290
CH (ring)	1.293	1.188
4'-H	3.454	3.25, 3.402
2',3'-,5'-H	3.722 - 3.847	3.629 - 3.749
1'-H	4.292	4.054, 4.127

Signal at 7.25ppm in 1H NMR spectrum due to solvent, $CDCl_3$. The ^{13}C NMR spectra for monorhamnolipid and dirhamnolipid are shown on Figure 3.28 and 3.29 below

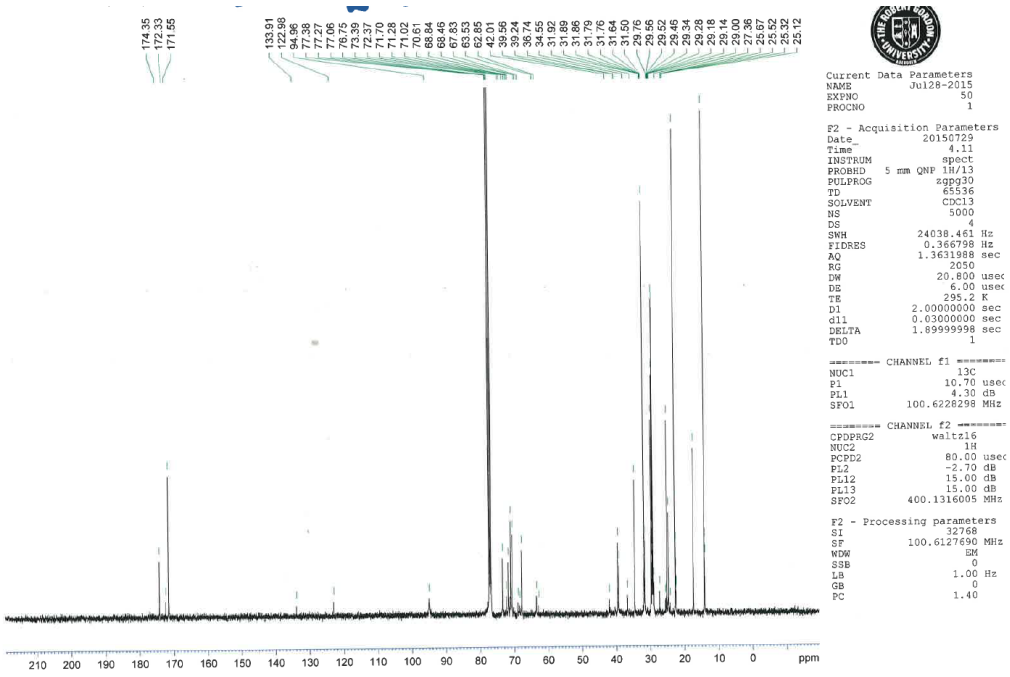


Figure 3.28: **^{13}C NMR spectra for ST5-Mono-RL**
(signals between 77.05 and 77.77ppm are due to solvent $CDCl_3$)

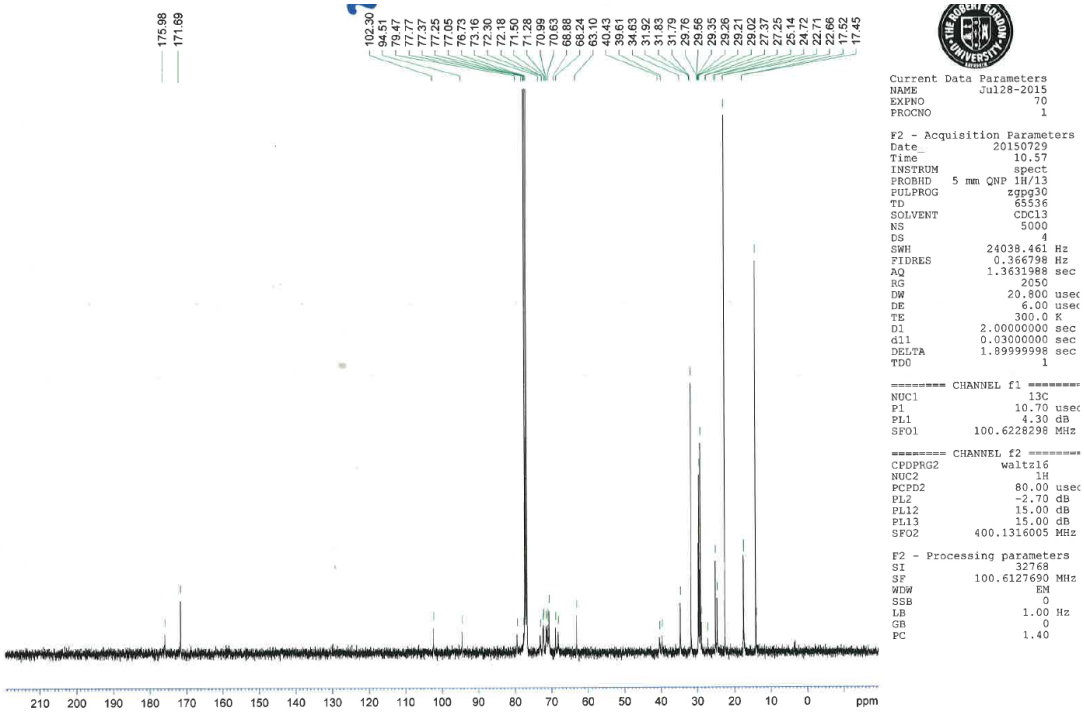


Figure 3.29: **^{13}C NMR spectra for Di-rhamnolipid**
(signals between 77.05 and 77.77ppm are due to solvent $CDCl_3$)

The ^{13}C NMR spectrum of monorhamnolipid (Figures 3.28) and dirhamnolipid (Figures 3.29) shows signals for carbonyl groups at δ 171.69 and δ 175.58ppm (dirhamnolipid) and δ 171.55 – 172.33 and δ 174.35ppm (monorhamnolipid). These could be due to the carboxyl and ester groups in both the monorhamnolipid

and dirhamnolipid). All lipid signals (alkyl chains) present in the samples showed resonances between 10.00-30ppm whereas the carbons due to the sugar moieties gave weaker signals from 22.00-100.00ppm) Those characteristic chemical shifts were comparable to previous reports found in literature (Lotfabad et al. 2010; Wei, Chou and Chang 2005).

The NMR results also indicate that the purified biosurfactant comprises two principal rhamnolipid homologs, i.e. monorhamnolipid and dirhamnolipid as confirmed in literature (Lotfabad et al. 2010; Wei, Chou and Chang 2005).

3.3.4.3. Liquid Chromatography-Mass Spectrometry/Mass Spectrometry LC-MS/MS

The rhamnolipids from the ST5 and PS1 strains were further characterised by an atypical negative mode electrospray ionisation LC-MS/MS analysis. Several authors (Deziel et al. 1999; Behrens et al. 2016; Lotfabad et al. 2010) have reported that RL biosurfactants are produced as mixtures of several RL congeners which can be linked to the cultivation method as well as strains involved. They elucidated the fragmentation mechanism and patterns for a number of these congeners. Hence characteristic pseudo-molecular ions were initially used to identify the presence of specific congeners in RLs obtained from both strains in this study.

Only the result of the purified fractions from rhamnolipid produced from *P. aeruginosa* ST5 (MRL and DiRL) are reported here, based on the similarities of the fractions produced by *P. aeruginosa* ST5 and *P. aeruginosa* PS1.

The total ion chromatogram (TIC) of Monorhamnolipid and Dirhamnolipid homologues produced by *P. aeruginosa* ST5 are shown in Figure 3.30 and 3.31 below.

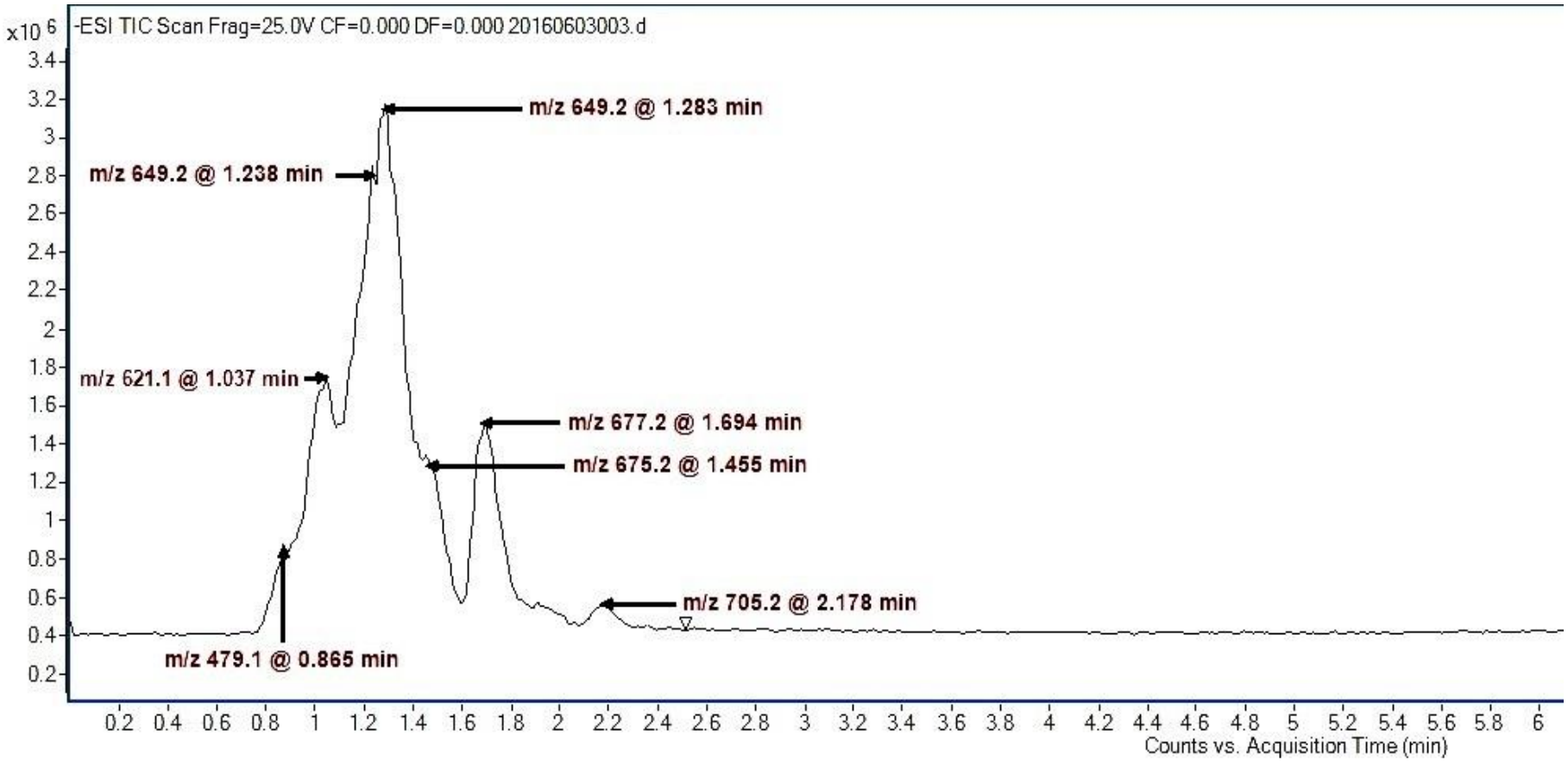


Figure 3.30: TIC of Di-RL Homologues Produced from Ps. Aeruginosa ST5

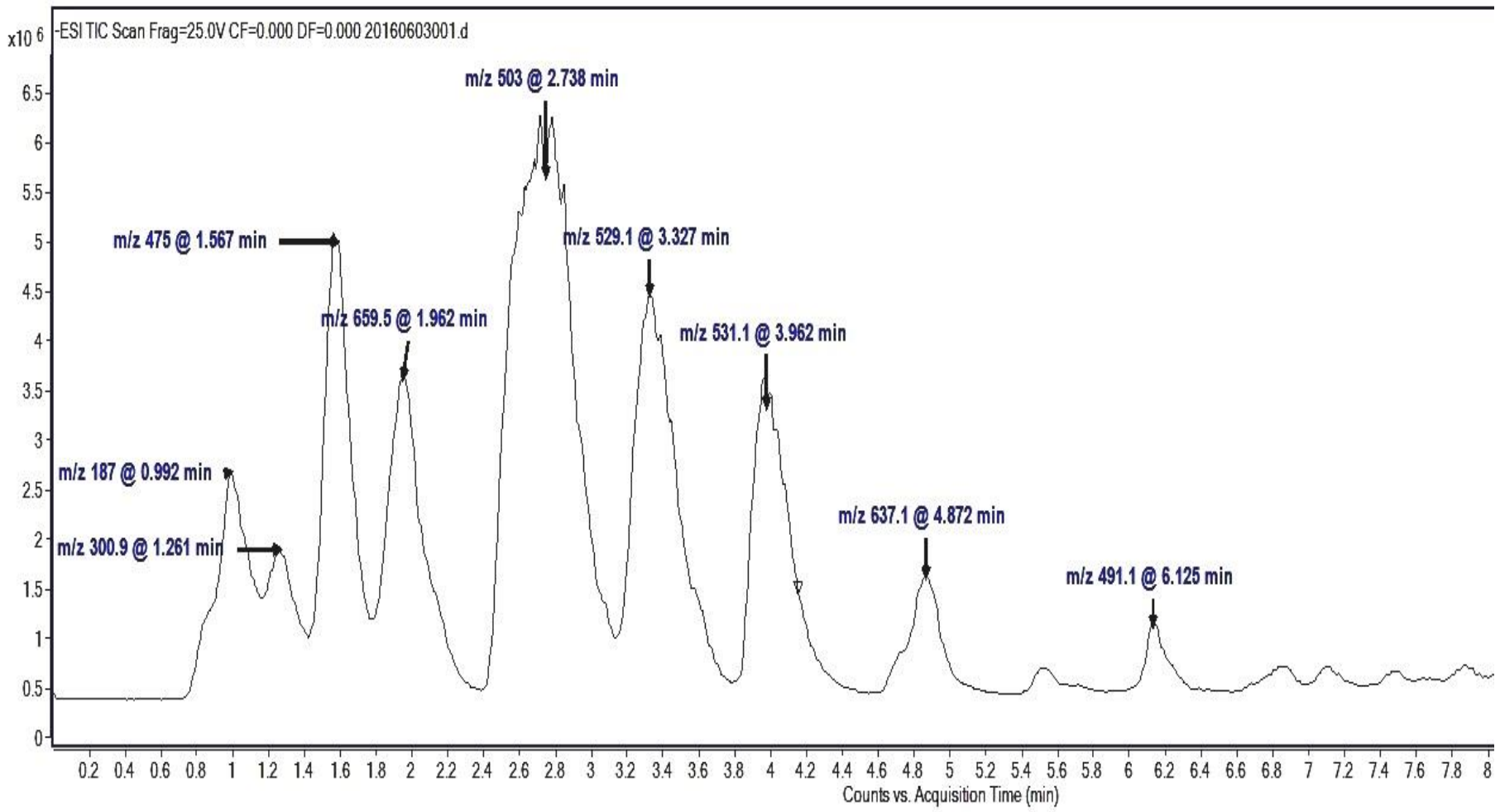


Figure 3.31: TIC of M-RL Homologues Produced from Ps. Aeruginosa ST5

From Figure 3.30 and 3.31 above, 6 major congeners were identified from the TIC of the dirhamnolipid and 5 major congeners were identified from the TIC of monorhamnolipid respectively (Table 3.11). However, four peaks (at 0.992, 1.962, 4.872 and 6.125 min) were unidentifiable on the TIC of MRL. The major m/z ions obtained for the 4 peaks were; 187, 660, 637 and 491. Further work will seek to establish the identity of the additional peaks seen within the TICs.

The fraction with the $[M - H]^-$ ion at m/z 503 ($RhC_{10}C_{10}$) predominated in the monorhamnolipid fraction whilst the $[M - H]^-$ ion at m/z 649 ($Rh_2C_{10}C_{10}$) was the most abundant in the dirhamnolipid fraction.

This dominance has been reported in several other studies such as Raza et al. 2009; Deziel et al, 1999 and specifically Rudden et al. (2015) who quantified both $RhC_{10}C_{10}$ and $Rh_2C_{10}C_{10}$ to be approximately 83.8% and 85.1% respectively of the total rhamnolipid congeners found in each fraction of the strain studied using Ultra-performance LC-MS/MS. These proportions were estimated in the monorhamnolipid and dirhamnolipid fractions of the ST5 extracts based on the relative intensities of the pseudo-molecular ions (without distinguishing any rhamnolipid isomers of the same molecular weight) to be 55.3 and 62.2% respectively.

Table 3.11: Composition of Rhamnolipid Congeners Found in the purified fractions ST5 Rhamnolipid

S/N	Pseudomolecular ion (*m/z*)	Congener	Retention time (min)	Reference
Dirhamnolipid				
1.	479	Rh_2C_{10}	0.865	Zhao et al. (2013);
2.	621	$Rh_2C_8C_{10}$ or $Rh_2C_{10}C_8$	1.037	Zhao et al. (2013);
3.	649	$Rh_2C_{10}C_{10}$	1.238 – 1.283	Lotfabad et al. (2010);
4.	675	$Rh_2C_{10}C_{12:1}$ or $Rh_2C_{12:1}C_{10}$	1.455	Abdel-Mawgoud et al. (2011); Rudden et al. (2015);
5.	677	$Rh_2C_{10}C_{12}$ or $Rh_2C_{12}C_{10}$	1.694	Samadi et al. (2012)
6.	705	$Rh_2C_{12}C_{12}$	2.178	Abdel-Mawgoud et al. (2011);
Monorhamnolipid				
1.	301	$RhC_{8.2}$	1.261	Zhao et al. (2013); Abdel-Mawgoud et al. (2011)
2.	475	RhC_8C_{10} or $RhC_{10}C_8$	1.567	Zhao et al. (2013);
3.	503	$RhC_{10}C_{10}$	2.738	Lotfabad et al. (2010);
4.	529	$RhC_{10}C_{12:1}$ or $RhC_{12:1}C_{10}$	3.327	Abdel-Mawgoud et al. (2011); Rudden et al. (2015); Samadi et al. (2012)
5.	531	$RhC_{10}C_{12}$ $RhC_{12}C_{10}$	3.962	Lotfabad et al. (2010); Abdel-Mawgoud et al. (2011); Rudden et al. (2015); Samadi et al. (2012)

Tandem mass spectrometry analysis was used to confirm the structures and identity of selected major pseudo-molecular ions identified earlier, i.e. m/z 503 and 649 as well as the di-rhamnolipid m/z 621 ($Rh_2C_8C_{10}$/ $Rh_2C_{10}C_8$) by fragmenting these ions to obtain product or daughter ions (Figure 3.32).

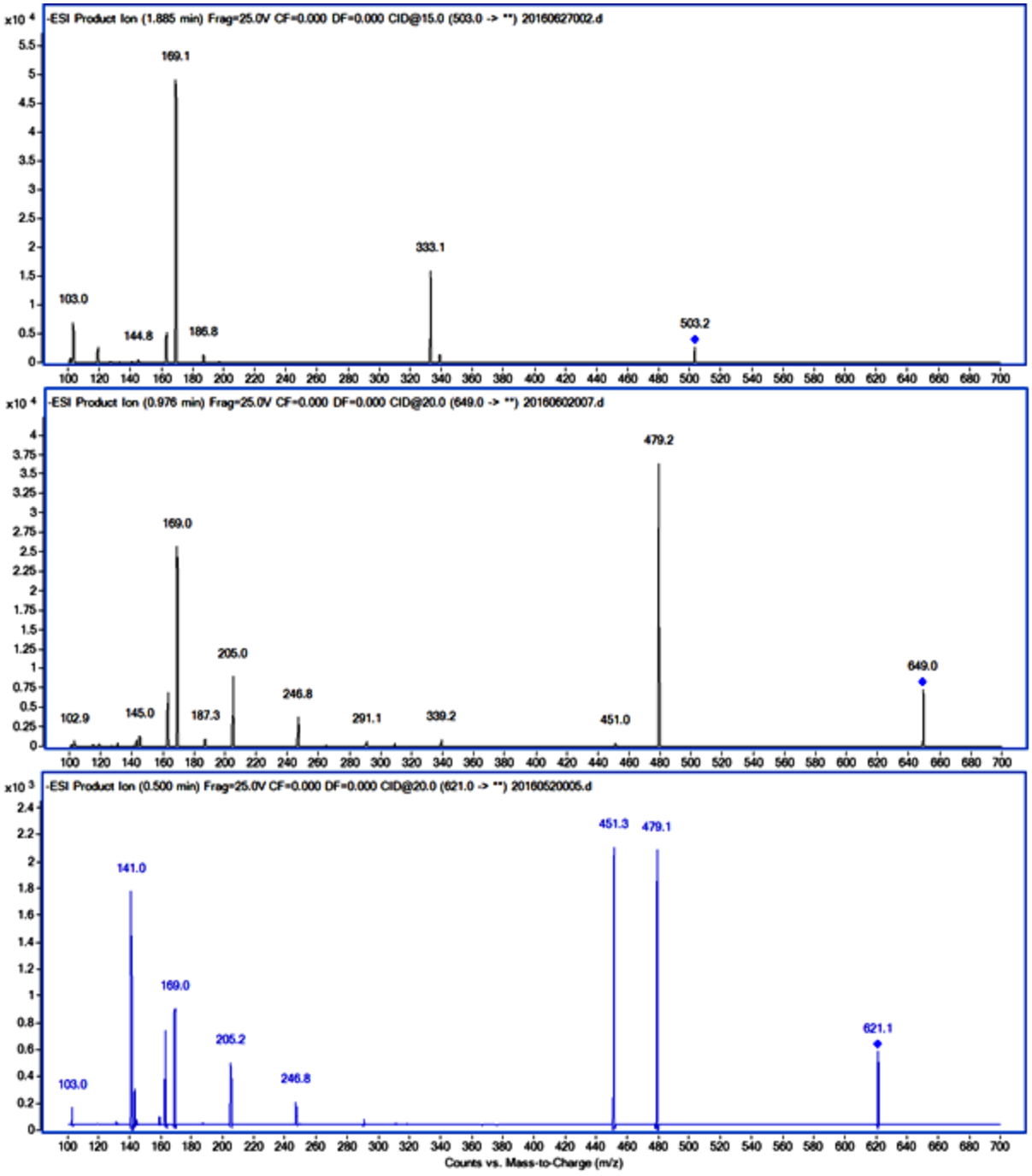


Figure 3.32: Product Ion Scan of Major Pseudo-molecular Ions

Where: Top: $RC_{10}C_{10}$ (m/z 503),
Middle: $Rh_2C_{10}C_{10}$ (m/z 649)
Bottom: $Rh_2C_8C_{10}$/ $Rh_2C_{10}C_8$ (m/z 621.1)

Rudden et al. (2015), Zhao et al. (2013), Samadi et al. (2012), Déziel et al. (1999) and Monteiro et al. (2007), have detailed similar fragmentation patterns as observed in this study. For example, m/z 621 ($Rh_2C_8C_{10}$/ $Rh_2C_{10}C_8$) showed product ions at m/z 451 and 479 corresponding to the cleavage of the ester bond in the rhamnolipid structure. The fragments m/z 141 and 169 represent the fatty acid moieties with a loss of a hydrogen ([C8]- and [C10]-) whilst the m/z 205 reflect the loss of the 2 rhamnose moieties in the rhamnolipid congener (Behrens et al. 2016).

The LC-MS/MS analysis of the purified fractions of the rhamnolipid produced from *P. aeruginosa* ST5, confirmed the presence of two predominant components, detected as the ions of *m/z* 503 and *m/z* 649, which corresponds to $RhC_{10}C_{10}$ (monorhamnolipid) and $Rh_2C_{10}C_{10}$ (dirhamnolipid) respectively.

3.3.4.4. Summary on Chemical Characterisation of Rhamnolipids

The results of the chemical characterization carried out on the biosurfactant produced by Pseudomonas *aeruginosa* ST5 and PS1 showed that both strains can produce rhamnolipids consisting mainly of monorhamnolipid and dirhamnolipids congeners. The ATR-FTIR analysis confirmed the presence of the functional groups present in the glycolipid, while the proton and carbon NMR of the samples showed characteristic chemical shifts comparable to previous reports found in literature on NMR analysis of rhamnolipid. The result of the LC-MS/MS confirmed the presence of the congeners (pseudomolecular ions) reported in literature for monorhamnolipid and dirhamnolipid.

The result of these analyses confirms the samples under review to be rhamnolipid biosurfactant. The results of the chemical analysis carried out on the biosurfactant produced by Pseudomonas *aeruginosa* PS1 shall be seen in appendix 3 to 4.

3.4. Conclusion

Biosurfactants are important because of their biodegradability and low toxicity. These properties make biosurfactants highly suitable in environmental applications. Studies have shown that biosurfactant has good surfactant properties when compared to their synthetic counterparts. The production and characterisation of the biosurfactant in this research shows that the biosurfactant is a suitable alternative for synthetic surfactants considering the effectiveness of their surfactants properties and sustainability. Although the cost of producing biosurfactants is considered expensive, more research should be focused on producing biosurfactants from cheap renewable resources such as agricultural waste, dairy and sugar industry waste and waste from food and beverage industries in order to reduce the cost of production of biosurfactant.

The effectiveness of the biosurfactants were determined using the following parameters; surface tension, critical micelle concentration and emulsification activity. All results obtained were comparable to previous studies carried out such as Raza et al. (2006);Bai, Brusseau and Miller (1997) and Christova et al. (2011). The structure of the biosurfactants were elucidated consequent upon identification using TLC and purification using column chromatography using the following structural characterization techniques; FTIR-ATR, NMR and LC-MS/MS. The purified fractions of the rhamnolipids were determined to be mainly $RhC_{10}C_{10}$ (monorhamnolipid) and $Rh_2C_{10}C_{10}$ (dirhamnolipid) respectively, although other congeners were identified. The biosurfactants were produced for the purpose of investigating their utility for the treatment of oil contaminated cuttings and soil.

CHAPTER 4 – TREATMENT OF OIL CONTAMINATED SOLIDS USING RHAMNOLIPID BIOSURFACTANT

4.1. Introduction

Oil contaminations can occur in any of the 3 sectors of the oil and gas industry: upstream, midstream and downstream. Oil contamination from petroleum hydrocarbons is an important human and environmental-health issue around the world (Sullivan 1991). Generally, an oil spill is considered to have occurred when liquid petroleum hydrocarbon is released into the environment via human activity.

Figure 4.1 below shows the global oil spill trend from 1970 to 2017 as reported by the International Tanker Owners Pollution Federation (ITOPF). Two categories of spills are reported in Figure 4.1, they are; large spills (> 700 tonnes) and medium spills (between 7 – 700 tonnes). The spills reported here are majorly from tanker accidents such as hull failure, equipment failure, fire, explosion etc.

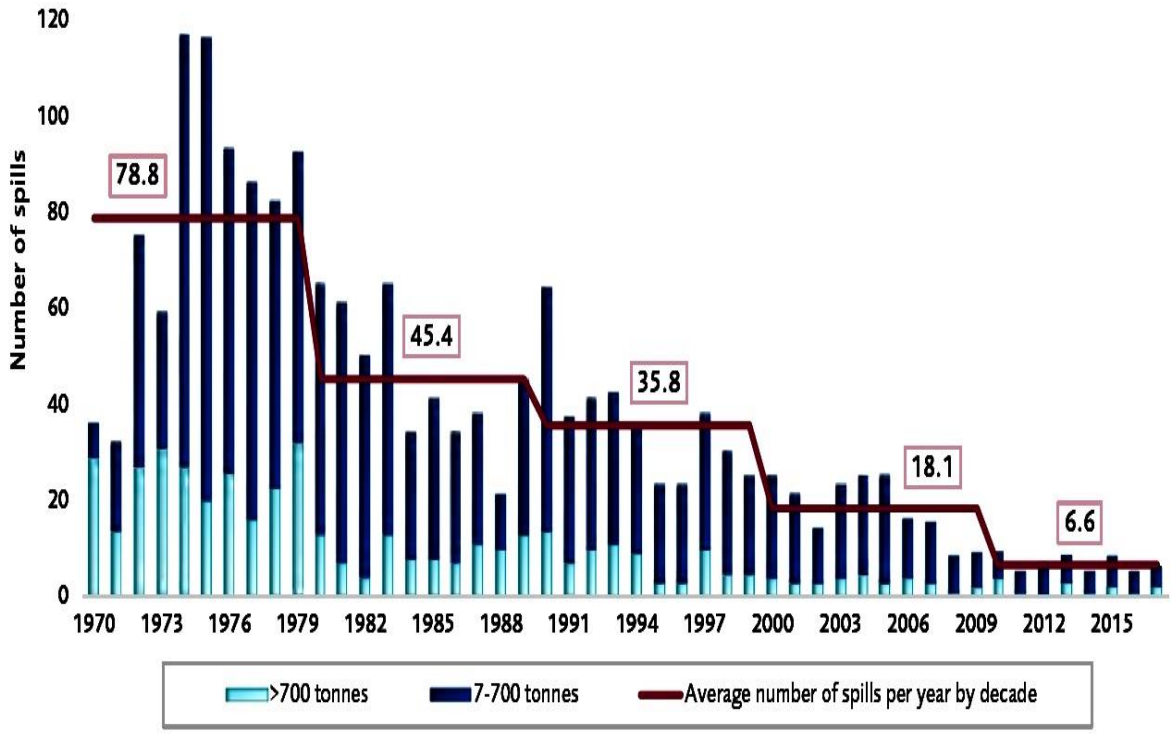


Figure 4.1: **Global Oil Spill Trend from 1970 to 2017**
Available from: ITOPF (2018)

Governments and industries are working in collaboration to reduce the occurrence and risk of oil contamination in the environment. They do this by making and enforcing regulations that guide the operational activities in the oil and gas industry.

The oil and gas sector is one of the most regulated sectors of the economy. Despite these regulations, oil contaminations still occur in the environment. This is partly due to the fact that systems operated by humans can be subject to errors and accidents do happen. Fingas (2011) commented that, "despite these measures, spill experts estimate that 30 to 50% of oil spills are either directly or indirectly caused by human error, with 20 to 40% of these incidents caused by equipment failure or malfunction." These estimates call for concerted effort in reducing the risk of oil contamination in the environment.

4.1.1. Treatment Technologies for Remediation of Oil Contamination in the Environment

Several technologies are available for the removal of oil contaminants in the environment. The technology available for remediation of oil contamination in the environment (such as soil and water) ranges from physical, chemical and biological treatment technologies. Table 4.1 shows some remediation technologies for soil and ground water, which can be applied for the removal of oil contaminated solid matrices such as soils and drill cuttings (waste from drilling operations).

Table 4.1: Common soil and groundwater remediation technologies

Available from: Bhandari (2007)

Technology	Types
Physical treatment technologies	• Free product recovery • Pump-and-treat • Soil vapour extraction • Air sparging • Groundwater circulation wells • Multiphase extraction • Induced fracturing • Soil heating
Chemical treatment technologies	• Precipitation • Chemical oxidation and reduction • Permeable reactive barriers • Stabilization/solidification • Adsorption and ion exchange • Electrochemical processes • Chemical leaching and solvent extraction • Soil flushing • Soil washing
Biological treatment technologies	• Biosparging • Bioventing • Biostimulation • Bioaugmentation • Anaerobic biotransformation • Aerobic biotransformation • Biological fixation • Enzyme- catalyzed treatment • Saprotrophic fungal processes • Mycorrhizal fungal processes • Phytoremediation • Monitored natural attenuation

4.1.1.1. Factors Affecting the Choice of Treatment Technology

The choice of treatment technology to be applied for the treatment of any contaminated soil or liquid is hinged on 3 major factors:

1. **Nature of contaminant:** The major categories of pollutants usually found in contaminated soil or water samples are grouped into organic and inorganic pollutants, and the extraction method applied for the extraction of these pollutants from contaminated samples can be different (Conti 2008).
   - **A. Organic pollutants:** The presence/level of organic pollutants in a sample is usually determined by extraction using solvents (in which the pollutant is soluble in) and also solvent-less techniques such as: SPME, headspace sampling, purge and trap approaches etc. Some examples of organic pollutants are:
     i. Volatile organic compounds (VOCs) such as benzene, toluene, ethylbenzene, xylene (BTEX).
     ii. Semi volatile organic compounds (SVOCs) such as petroleum hydrocarbons, oil and grease, and polycyclic aromatic hydrocarbons (PAHs), (Lopes and Dionne 1998).
   - **B. Inorganic pollutants:** The presence/level of inorganic pollutants in a sample is usually determined by digesting or extracting the sample in an appropriate solvent (typically acids/bases). The digestion can be wet or dry depending on the sample type. Some examples of inorganic pollutants are:
     i. Heavy metals (such as, Cd, Hg, As, Pb, Ni and Zn).
     ii. Non-metals (such as, nitrates, sulphates, cyanide and phosphates).
     iii. Naturally occurring radioactive materials (NORMs) such as uranium and thorium).

Before any treatment technology can be applied for the removal of these contaminants if found in soil or water samples, a key consideration will be the contaminant mobility which largely depends on the contaminant concentration in the sample, and the solubility of the contaminant in water and other solvents (Ong and Angela 2007).

2. **Nature/State of Contaminated Sample:** The nature and state of the contaminated sample is important for two (2) reasons:

A. **Surface area of sample:** Generally liquid samples have more surface area than solid samples. Contaminants tend to spread more in liquid sample by diffusion than in solid sample matrices and this will depend on solubility and matrix homogeneity.
B. **Sample preparation technique:** The nature and state of the contaminated sample determines the ease and duration of sample preparation for treatment. Some complex sample matrices such as solids (soils, sludges and sediments) require longer sample preparation time than liquid samples. Also, heterogeneous samples will require longer sample preparation time than homogeneous samples.

3. **Sustainability of the Treatment Technology**: As explained in Section 1.2.2, Brundtland (1987) described sustainable development is "*the development that meets the needs of the present without compromising the ability of future generations to meet their own needs*.". The sustainability of treatment technologies to be applied for the remediation of the environment should be assessed before application. The economic viability and social impact are important, but the paramount aspect of the sustainability to be considered before any treatment technology is applied for remediation is primarily hinged on the environmental friendliness of the treatment technology. Biosurfactants are reportedly to be environmentally benign, having low toxicity and are biodegradable. This makes biosurfactants a potential sustainable alternative for chemical surfactants in environmental solutions.

4.1.2. Soil Washing Treatment Technology

Soil washing technology is a water-based technique applied for the removal of contaminants from soil (Mulligan 2014). It is carried out ex-situ, and is usually applied for the removal of VOCs, SVOCs, metals and petroleum hydrocarbons from soil (Ong and Angela 2007). Soil washing involves mixing the soil and washing solvent (usually aqueous) in a vessel, in order to separate the contaminant from the soil by partitioning or complexation. The washings (with dissolved contaminant), is separated for further treatment and the treated soil is then tested and reused (Dadrasnia, Shahsavari and Emenike 2013). Figure 4.2 shows a schematic diagram of a typical soil washing process.

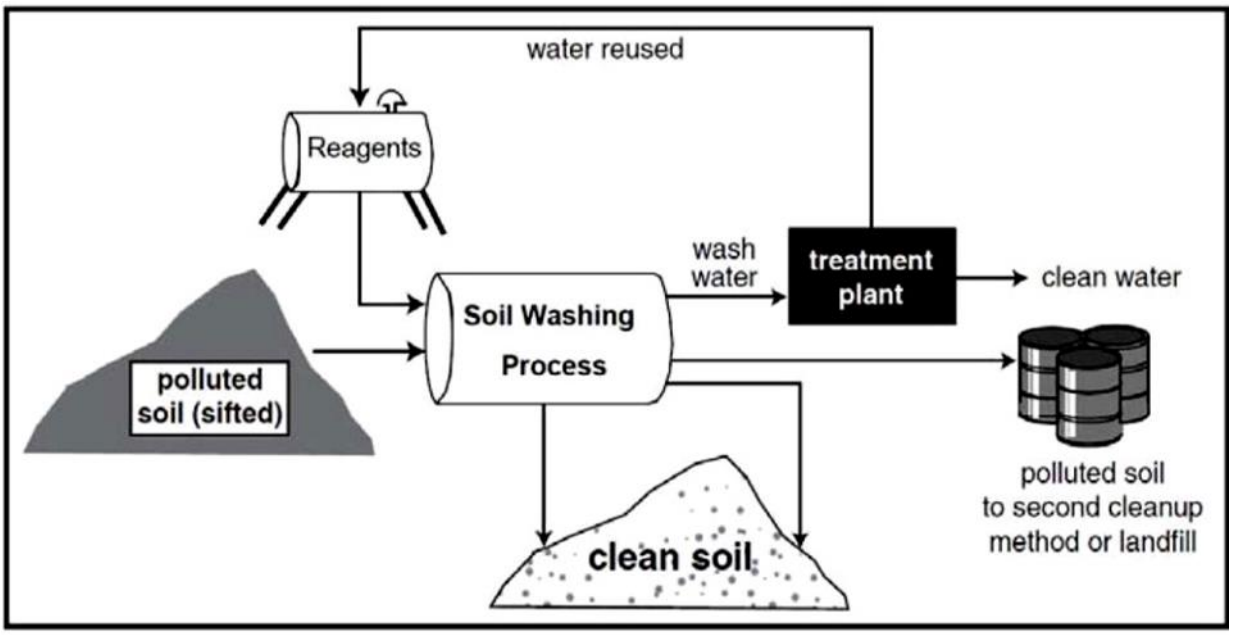


Figure 4.2: Schematic diagram of a Typical Soil Washing Process
Available from: Dadrasnia, Shahsavari and Emenike (2013)

As discussed in Section 1.5.2.5, surfactant enhanced washing has been applied for the removal of contaminants from soil (Paria 2008). The soil washing process usually generates large volume of contaminated wastewater (effluent), which must be treated before discharge or reuse, hence the need to utilize environmentally friendly surfactant in the clean-up process. Although chemical surfactants are usually applied for large scale clean ups, studies have shown that biosurfactants serves as a sustainable alternative for the use of chemical surfactants based on their properties such as; low toxicity, biodegradability, and the fact that they can be produced from renewable sources (Desai and Banat 1997; Marchant and Banat 2012; Soberón-Chávez and Maier 2011; Mulligan 2014).

4.1.2.1. Soil Washing with Biosurfactant for the Removal of Petroleum Hydrocarbons in OBM Drill Cuttings

One of the motivations behind this research is based on the fact that there has been a limited study carried out to remove petroleum hydrocarbons from oil based mud drill cuttings. The utilization of biosurfactant for the removal of petroleum contaminated soil and OBM drill cuttings has been studied by Urum, Pekdemir and Gopour (2003) and Yan et al. (2011) respectively. Both studies achieved 79.9 and

83% removal of organics from the soil and OBM drill cuttings respectively using a washing process. However, these studies were limited by the following;

1. The prohibitive cost of the commercial rhamnolipid biosurfactant (10mg cost £158.00 from Sigma Aldrich), which limits the economic viability of the study.
2. Also, the percentage removals of the organics in these studies were achieved at 50^{o}C and 60^{o}C respectively.

Also, studies carried out by Lai et al. (2009), to remove TPH from a contaminated soil (9000 mg TPH/kg) using two (2) biosurfactants: rhamnolipid and surfactin, and two synthetic surfactants (Tween 80 and Triton X-100). They showed a 63%, 62%, 40% and 35% removal efficiency for rhamnolipid, surfactin, Tween 80 and Triton X-100 respectively. This result shows that the biosurfactants were more effective in mobilizing the petroleum hydrocarbons from the soil than the synthetic surfactants.

4.1.3. Aim

The aim of this chapter is to investigate and discuss the removal of petroleum hydrocarbons from contaminated soil and OBM drill cuttings (at room temperature) using the rhamnolipid produced with *Pseudomonas aureginosa* ST5 via a soil washing process, and to investigate the potential toxicity of the rhamnolipid washings on the environment by carrying out a cytotoxicity analysis on cells. The potential reduction of heavy metals from the OBM drill cuttings was also studied.

4.2. Materials and Methods

The methodology in this chapter covers the removal of TPH and heavy metals from the contaminated solids (OBM-DCS and soil) using rhamnolipid biosurfactant, as well as the cytotoxicity investigation of the biosurfactant washings using a breast cancer cell line.

4.2.1. Samples for Analysis

Pure sand sample was obtained from Arcos organics. The oil contaminated samples; soil and OBM drill cutting samples used for this work were obtained from anonymous sources in Aberdeen. The oil contaminated samples were stored in air-tight amber coloured glass containers, to avoid loss of the volatiles and were kept away from light to minimise photo-degradation of the sample.

4.2.2. Chemicals and Reagents

Tetrachloroethylene (perklone) was purchased from Sigma Aldrich, UK. Sodium dodecyl sulphate (SDS) the synthetic surfactant used to compare the surfactant properties of the biosurfactant was supplied by Fisher Scientific, UK. Diesel used for the preparation of the diesel in perklone standards were obtained from a local gas station. The biosurfactant applied for the clean-ups (OBM-DCS and soil) were produced from *Pseudomonas aeruginosa* ST5 using glycerol as carbon source. The purified fractions; monorhamnolipid and dirhamnolipid, were also applied for the clean-up. The procedure for the production and characterization of the biosurfactants is described in chapter 3.

MTT assay {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide} was obtained from Sigma Aldrich. Breast cancer cells; MDA-MB-231 cell line was supplied by Sigma Aldrich, and maintained at 37°C in a humid atmosphere with 5% CO_2. Dimethyl sulfoxide (DMSO) solvent was supplied by Sigma Aldrich, UK.

The cells used for the cytotoxicity test were cancer cells (MDA-MB-231), based on availability and grown in Roswell Park Memorial Institute (RPMI) 1640 medium, which was supplied by Sigma Aldrich, UK. The RPMI 1640 media was supplemented with 10% heat-inactivated fetal bovine serum, 1% glutamine, 2 mM L-gluthamine, 100 U ml^{-1} penicillin, 100 µg mL^{-1} streptomycin and amphotericine B, all supplied by Sigma Aldrich. Phosphate-buffered saline (PBS) utilized for the washing of the cells and the trypsin used for the cell dissociation were also

supplied by Sigma Aldrich. Ethanol was used for aseptic clean-up for cell culture and assay apparatus was obtained from Acros Organics.

4.2.3. Other Materials

Syringes, 0.2 µm sterile filters, falcon tubes, T25 tissue culture flask, 96 well micro-titre tissue culture plate and Gilson 8 multichannel pipette were supplied by Fischer Scientific. 1 cm quartz curvette absorption cell used for FT-IR analysis and 50 mL screw-cap glass test tubes used for the washings were supplied by Sigma Aldrich. 125 mm Whatman filter paper used in filtering the washed and extracted samples were also supplied by Sigma Aldrich.

4.2.4. Washing Procedure

4.2.4.1. Washing Conditions

A. **Washing of Model Sand Samples:** A preliminary washing was carried out on pure sand samples (spiked with 10,000 ppm diesel in perklone standard), to investigate the efficiency of TPH removal using deionised water (control), crude ST5 rhamnolipid (biosurfactant) and SDS (synthetic surfactant). Approximately 3 g of the sand samples (weighed in triplicate) into a glass centrifuge tube. 300 µL of the diesel in perklone standard was used to spike the sand samples. Approximately 15 mL of the wash solutions (water, ST5 rhamnolipid and SDS) were added to the sand samples. The following concentrations ST5 rhamnolipid and SDS were used for the washing: 10, 100 and 1000 ppm. The tubes were stoppered and clamped on to a Stuart SF1 flask shaker for a 30 minutes agitation time at 700rpm. After the washing, the tubes were allowed to settle for 2 hours, after which the supernatant was decanted. The washed sand samples were rinsed twice with 15 mL each of deionised water to remove any residual wash solution left on the sample. The rinsing was carried out at 700rpm for 5 mins each. The TPH in the washed soil sample was subsequently extracted and determined following the FTIR procedure discussed in section 2.2.8.3. Figure 4.3 shows a schematic diagram of the spiked sand washing process.

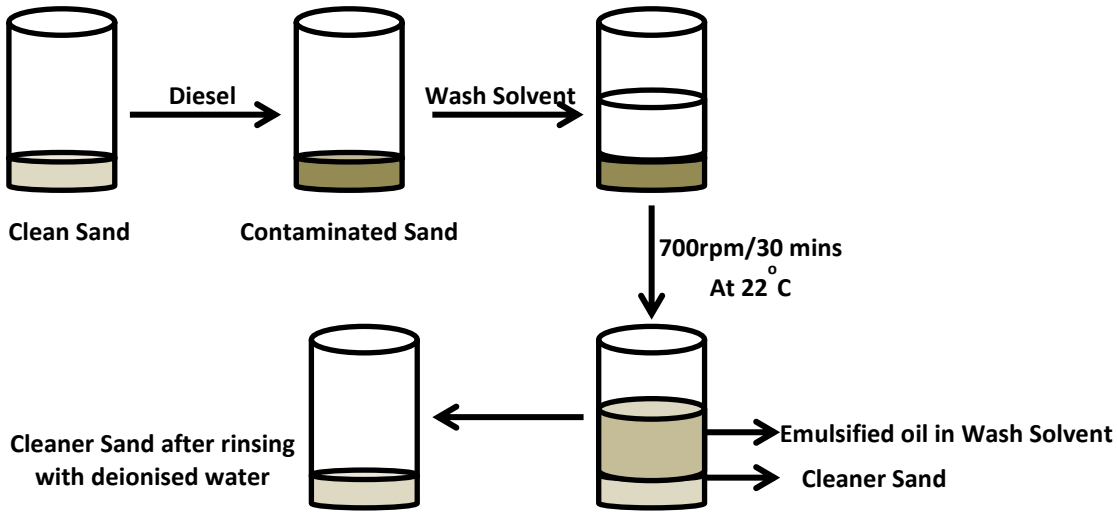


Figure 4.3: Schematic Diagram of the Sand Washing Process

B. **Washing of Oil Contaminated Samples:** The oil contaminated samples (OBM-DCS and soil) were washed using crude ST5 rhamnolipid and its purified fractions (where necessary) using the following concentrations 10, 100, 1000ppm and a blank. Approximately 3 g of oil contaminated soil and drill cutting samples were weighed (in triplicates each) into a glass centrifuge tube, to which 15 mL of the rhamnolipid solution was added. A 3:1 liquid to solid (L/S) ratio was used following a procedure obtained from Yan et al. (2011). The tubes were stoppered and clamped on to a Stuart SF1 flask shaker for a 30 minutes agitation time at 700rpm. After the washing, the tubes were allowed to settle for 2 hours. The supernatant was decanted into a container for a cytotoxicity investigation. The washed samples were rinsed twice with 15 mL each of deionised water to remove any residual biosurfactant left in the sample. The rinsing was carried out at 700rpm for 5 mins each. The TPH in the washed soil sample was subsequently extracted and determined following the FTIR procedure discussed in section 2.2.8.3.

4.2.4.2. Determination of Optimum Washing Conditions Using Taguchi Experimental Design

A Taguchi experimental design was applied to determine the optimum conditions using only the crude ST5 rhamnolipid. The Taguchi experimental design is a fractional factorial experimental design applied for experiments with multiple independent variables or parameters being investigated at different levels

(Cavazzuti 2013). The utilization of the Taguchi experimental design is important because, it is time saving and economically viable.

Similar studies carried out by Urum, Pekdemir and Gopour (2003) and Yan et al. (2011) investigated five (5) parameters namely;

1. Washing temperature,
2. Washing volume (liquid) to solid ratio (L:S),
3. Concentration of washing liquid
4. Washing speed
5. Washing time.

However, in determining the optimum washing conditions in this study, only three (3) parameters were varied, namely;

1. Concentration of washing liquid
2. Washing speed
3. Washing time.

Temperature and washing volume (liquid) to solid ratio (L:S) were not varied for the following reasons;

1. The washing was carried out at room temperature to reduce the energy consumption of the washing process. Thus enhancing the economic viability and sustainability of the process
2. The liquid to solid ratio (L:S) was not varied based on the result of the study carried out by Yan et al. (2011) which showed no significant increase in the removal of total extractable organics at L:S above 3:1. Yan et al. (2011) claimed that, "this may be due to more surfactant monomers available to mobilize the oil and more micelles available to stabilize the oil in the solution." A similar finding was reported previously by Urum and Pekedemir (2004). Thus the optimum L:S for this study was fixed at 3:1.

The experiment was carried out at three (3) levels (Table 4.2); $L_{27}(3^3)$, whilst keeping volume and temperature constant (Table 4.3).

Table 4.2: Experimental Parameter and Levels for Biosurfactant Washing

S/No.	Parameters	Levels		
		1	**2**	**3**
1.	Concentration of rhamnolipid (ppm)	1000	100	10
2.	Washing speed (rpm)	700	500	300
3.	Washing time (min)	15	30	45

Table 4.3: Taguchi Experimental Design L_{27} (3^3)

S/No.	Conc (ppm)	Speed (rpm)	Time (min)	No. of Replicates
1	10	300	15	3
2	10	500	30	3
3	10	700	45	3
4	100	300	30	3
5	100	500	45	3
6	100	700	15	3
7	1000	300	45	3
8	1000	500	15	3
9	1000	700	30	3
Total number of experiments				27

4.2.5. Total Petroleum Hydrocarbon Analysis

4.2.5.1. Calibration of FT-IR Instrument

The FTIR instrument was calibrated as described in section 2.2.8.1.

4.2.5.2. Extraction and Determination of TPH from Washed Samples

The TPH in the samples were extracted into perklone before and after the washing and determined using an FTIR following the procedure described in section 2.2.8.3.

4.2.5.3. Determination of Moisture Content of Washed Sample

However, to get the TPH concentration in the washed sample per dry weight (mg/Kg), the moisture content of the washed sample was determined by sub-sampling approximately 1 g of the washed sample, and drying to constant weight in a vacuum oven at 50^{o}C. The samples were dried for approximately 24 hours. The moisture content was obtained by calculating the percentage of the difference in sample weights, before and after drying as shown in Equation 4.1 below;

Equation 4.1: Moisture Content

$$\text{Moisture Content (\%)} = \frac{W1 - W2}{W1} \times 100$$

Where: W1 = Initial Weight of Sample (g)

W2 = Final Weight of Sample (g)

The value obtained was used for the calculation of the TPH content in the sample to obtain the result by dry weight (mg/Kg).

The samples were washed with the rhamnolipids following the washing procedure stated in section 4.2.4 B. The percentage TPH removal from the contaminated samples (after the washings), were obtained using equation 4.2 below;

Equation 4.2: TPH Reduction

$$\text{\% TPH Reduction} = \left\{ \frac{S_a - S_b}{S_a} \right\} \times 100\%$$

Where S_a = Initial TPH content (mg/kg) before treatment and

S_b = Final TPH content (mg/kg) after treatment

4.2.6. ICP-OES Analysis of Washed OBM-DCS

The washed OBM-DCS were air-dried overnight, weighed and digested as described in section 2.2.5. The elemental content of the samples were determined by ICP-OES as described in section 2.2.6

4.2.7. Cytotoxicity Analysis of Rhamnolipid Standards and Washings

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay also known as (MTT) assay is a colorimetric analysis used for the determination of cell viability. Succinate dehydrogenase is an enzyme found in cell mitochondria; where cellular respiration occurs (Septisetyani et al., 2014). When MTT solution is added to living cells, succinic dehydrogenase in the cells, coverts the MTT into insoluble formazan. Formazan, when dissolved in DMSO, produces a purple colour. The production of the purple colour after the addition of DMSO confirms the viability of the cells being tested. The cell viability is determined quantitatively by measuring the absorbance of the DMSO solution at 595nm using an ELISA plate reader (BioRad iMark^{TM}).

4.2.7.1. Preparation of MTT Solution

MTT solution was prepared by adding 5 mg MTT per 5 mL of RPMI-1640 media. The MTT solution was filtered using a 20 mL syringe through a 0.2 µm sterile filter into a sterile falcon tube. The falcon tube was covered with a tin foil and stored in the dark at 4^{o}C.

4.2.7.2. Preparation of Cancer Cell Solution

Breast cancer cells (MDA-MB-231) were maintained as described in section 4.2.2, and cultured in a T25 tissue culture flask using supplemented RPMI 1640 media. The cells were washed using PBS and dissociated from the flask using trypsin solution. The cells were counted using a hemocytometer and a light microscope. The cells were then seeded at density of 10,000 cells/well/100 µL in a 96 well micro titre plate for the MTT assay.

4.2.7.3. Test Solutions for Cytotoxicity Analysis

The cytotoxicity of the *Pseudomonas aureginosa* ST5 rhamnolipid was investigated before and after the washings of the contaminated soil sample. The cytotoxicity test was aimed at investigating the toxicological effect of the rhamnolipid and rhamnolipid washings on the environment. The protocol used for this test was

obtained from Plumb (2004). The test solutions used investigated in this analysis is listed in Table 4.4 below

Table 4.4: Test Solution Per Well Line for MTT Assay

Well Lines	Test Solution
1	Media (positive control)
2	Water (negative control)
3	10 ppm ST5 rhamnolipid standard
4	100 ppm ST5 rhamnolipid standard
5	1000 ppm ST5 rhamnolipid standard
6	10 ppm ST5 rhamnolipid washings
7	100 ppm ST5 rhamnolipid washings
8	1000 ppm ST5 rhamnolipid washings
9	No test solution added
10	No test solution added

**The test solutions per well line are as analysed.*

4.2.7.4. Cytotoxicity Test Using A 96 Well Micro-Titre Plate

A 96 well micro-titre plate was used for this analysis. 100 µL of the cancer cell solution was added to each well (under the blue line as shown in Figure 4.4). The wells on the border of the plate (with orange-coloured border) were left empty of cell solution, only media was added. The cells in the micro-titre plate were then incubated at 37^{o}C for 24 hours. After 24 hours the test solutions (Table 4.4) were added to the same wells (under the blue line within the orange border) and incubated at 37^{o}C for 48 hours.

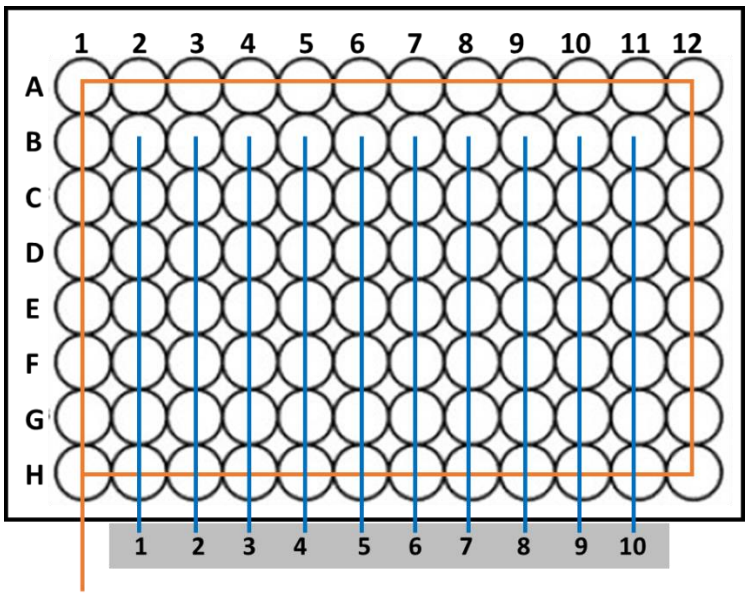


Figure 4.4: Schematic diagram of a 96 well micro-titre plate for MTT assay

4.2.7.5. *Cell Viability Analysis*

After 48 hours, the test solutions were removed (individually) from the plate using a Gilson pipette. 100 µL of the prepared MTT solution was added to all the wells in the plate, and incubated at 37°C for 4 hours. After 4 hours, the MTT solution was removed from the wells using a pipette. 200 µL DMSO was added to all the wells, after which the plate was covered with the lid and wrapped in a tin foil. The plate was then shaken for 20 minutes using the Thermo Scientific plate shaker.

The viability of the cells was confirmed qualitatively by purple colouration and quantitatively by reading the absorbance of the solutions at 595 nm using an ELISA plate reader. The viability of the cells was calculated in relation to the cells in the media (positive control) without the addition of the test solution using Equation 4.2

Equation 4.3: Cell Viability

$$\text{Cell viability (\%)} = \left\{ \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \right\} \times 100$$

The mean absorbance values (6 wells per test solution) were used for this calculation.

4.3. Results and Discussion

4.3.1. Washing of Contaminated Samples

4.3.1.1. Washing of Spiked Sand Samples

The result of the preliminary washings carried out on the spiked sand samples using deionised water (blank), biosurfactant (crude ST5 rhamnolipid) and synthetic surfactant (SDS) is shown in Figure 4.5.

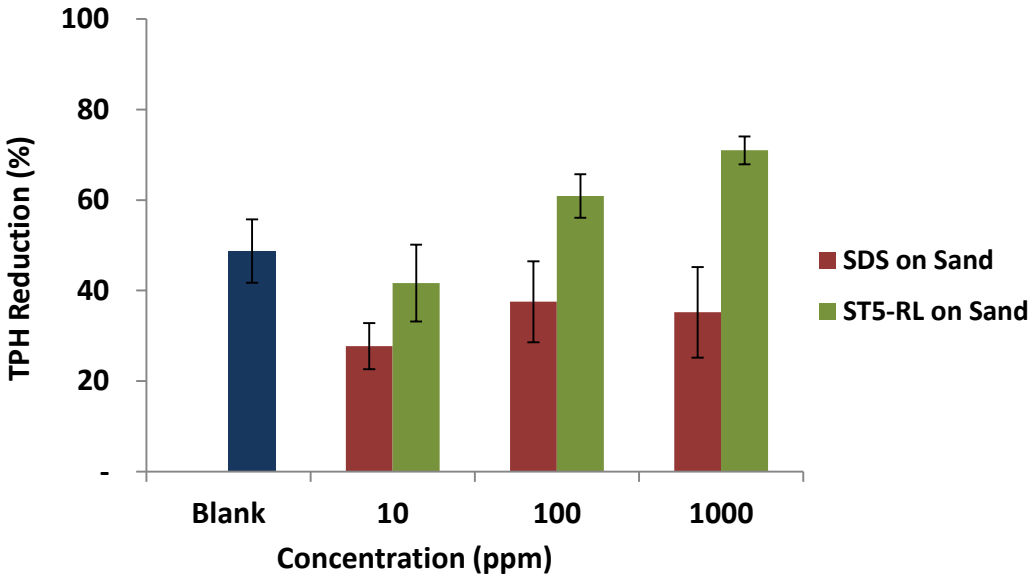


Figure 4.5: TPH Determination of Spiked Sand Samples (n=3)

The result of the washings carried out on the spiked sand samples showed that the produced crude ST5 rhamnolipid (CMC 48 ppm) was more effective in removing the oil than the SDS (CMC 2018 ppm) on two of the three concentrations investigated (100 and 1000 ppm). Also, approximately 50% of TPH was removed from the spiked sand sample using deionised water. As explained in Chapter 3, the CMC of surfactants gives a measure of the efficiency of a surfactant. The CMC of the surfactants would have contributed to the difference in the TPH reductions observed in this result.

This result compares well with a study carried out by Amani (2015) using biosurfactants (rhamnolipid and surfactin) and their synthetic counterparts (Triton X-100, and SDS) for the removal of crude oil from sand through a washing process, achieved the following results 80%, 77%, 65%, and 61% at room temperature for rhamnolipid, surfactin, Triton X-100, and SDS, respectively. The

result of this investigation also shows that the crude ST5 rhamnolipid can be applied for the remediation of coastal sands contaminated by oil (Arelli et al., 2018; Amani, 2015).

4.3.1.2. Determination of TPH Removal Efficiency of the Crude Rhamnolipid and Purified Fractions using Soil.

The effectiveness of the produced crude ST5 rhamnolipid and its purified fractions; monorhamnolipid (mono-RL) and dirhamnolipid (di-RL), in removing TPH from oil contaminated solids was investigated on the oil contaminated soil using the following conditions:

1. Rhamnolipid concentration: 10, 100 and 1000 ppm
2. Temperature: Room temperature (22^{o}C)
3. Liquid to solid ratio (L:S): 3:1
4. Washing time: 20 mins
5. Washing speed: 700rpm

The investigation was carried out for soil using all 3 rhamnolipids is shown in Figures 4.6.

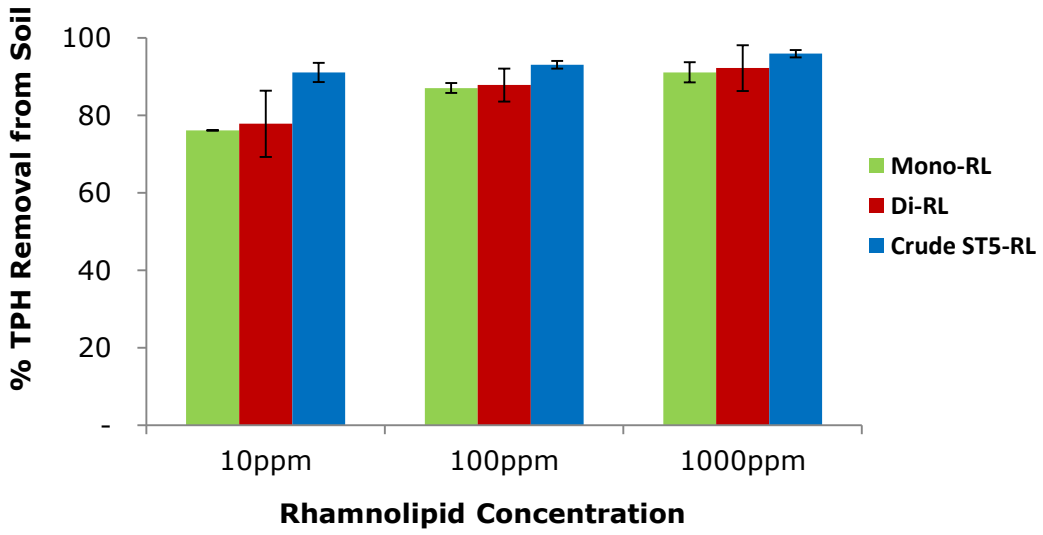


Figure 4.6: Perfomance of Biosurfactants in TPH Removal from Oil Contaminated Soil

As observed in Figure 4.6, over 80% TPH reduction was achieved using the crude ST5 rhamnolipid and its purified fractions, at all concentrations investigated. This result further shows the ability of the crude-RL to solubilise the oil (on both sand

and soil), lowering the interfacial tension between the oil and the biosurfactant at room temperature and at concentration below the CMC value. Childs et al. (2005), explained that the removal of oil from contaminated soil at low surfactant concentration occurs by a combination of two mechanisms called "roll-up" and "snap-off" mechanisms. They further explained that the roll-up and snap-off oil removal mechanisms are activated by reduced interfacial tension resulting from the surfactant concentration, which reduces the adhesion, cohesion and capillary forces holding the oil on to the surface of the soild. There was no significant difference between the concentrations analysed for crude ST5 rhamnolipid.

Also, there was no significant difference between the performance of the mono-RL from the di-RL at all the concentrations analysed. This was expected because of the closeness of their CMC values; 28 and 24 ppm respectively (as shown in Chapter 3). The efficiency of the crude ST5 rhamnolipid suggests that a purification step may not be required in treating the contaminated waste. Eliminating the purification of the crude ST5 rhamnolipid will reduce the cost of the cleaning process, thus making the process more economially viable and sustainable in the long run.

4.3.1.3. Determination of TPH Reduction in OBM-DCS and Oil Contaminated Soil samples

The washings carried out so far has shown that the crude ST5 rhamnolipid has the potential to efficiently clean oil contaminated waste on sand and soil. The crude ST5 rhamnolipid was then utilized to clean the OBM-DCS and soil in order to determine and compare the efficiency of the biosurfactant in cleaning oils on the two matrices.

The result of the washing carried out on the oil contaminated samples using crude ST5 rhamnolipid show that, the crude ST5 rhamnolipid was effective in removing over 60% of TPH from the OBM-DCS (approximately 36,600 mg/Kg), and over 90% of TPH from the soil (approximately 16,200 mg/Kg), with respect to the initial concentration of TPH in the OBM-DCS (61,212.01 mg/Kg) and the soil (18,121.57 mg/Kg) before the washings. The result is shown in Figure 4.7 below.

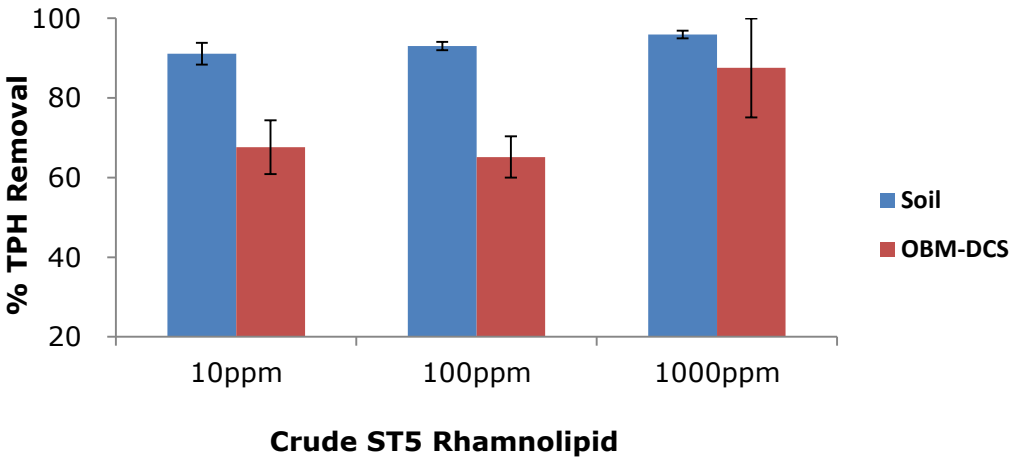


Figure 4.7: Treatment of Oil Contaminated Samples using Crude ST5 Rhamnolipid (n = 3)

This is interesting because the crude ST5 rhamnolipid is seen to be effective even at 10 ppm. The difference observed in the plot (with respect to precision and variability as shown by the error bars), could potentially be based on the following reasons:

a) **Textural/Particle Size Difference**: As shown in chapter 2, section 2.3.1, both samples are texturally different, with different particle sizes and thus, different surface areas. The OBM-DCS had more coarse sand in it (approximately 40%), while the soil sample had more medium sand in it (approximately 29%). Studies show that particle size could potentially limit or enhance the efficiency of the washing process. The United States Interstate Technology & Regulatory Council (ITRC), in their document on the technical and regulatory guideline for soil washing stated that, "soil washing is not cost effective for soils with silt/clay content in excess of 30 to 50%" (ITRC, 1997). This is interesting because the soil under study has a higher percentage of finer particles in it than the OBM-DCS. The biosurfactant was more effective in solubilizing the oil in the soil than in the OBM-DCS. Although the OBM -DCS is quite heterogeneous in appearance and very diverse in particle size when compared to the soil (see Table 2.5, Table 2.6 and Appendix 1 which shows a D90 of 398 µm for the soil). These characteristics could potentially affect the action of the biosurfactant on the sample which is evident in the larger variance (as shown by the error bars) of the OBM-DCS.

b) **Chemical Constituents/Additives**: As explained in chapter one, the oil based mud used in drilling is formulated with different types of base oil and

a range of additives depending on the technical demands of the formation being drilled. These oils/additives could possible limit the solubilisation of the oil on the drill cuttings, thus reducing the efficiency of the biosurfactant in removing the TPH found there on.

4.3.1.4. Determination of the Optimum Soil Washing Condition

The result of the analysis carried out so far shows the efficiency of the rhamnolipid in removing TPH from the sample matrices studied in this order soil>sand>OBM-DCS. Thus it will be best to study the optimum washing performance of the crude ST5 rhamnolipid using the oil contaminated soil. As discussed in section 4.2.5.2., Taguchi experimental design was applied for the determination of the optimum washing condition for the oil contaminated sample using soil as a case study. The effect of time and washing speed on the removal of TPH from the oil contaminated soil were varied along with the concentration (10, 100 and 1000ppm), and all samples were analyzed in 3 replicates.

A. Time Effect: In investigating the effect of time on the washing using 3 different concentrations of the crude ST5 rhamnolipid; the highest TPH removal (90.8±3.7%) occurred when the sample was washed with 1000ppm of the biosurfactant for 45 minutes, whilst the lowest TPH removal (56.7±1.04%) was observed at 45 minutes when the sample was washed with 10ppm of the biosurfactant (Figure 4.8). The increase in washing time from 30 to 45 minutes significantly increased the TPH removal from the soil at 100 and 1000 ppm rhamnolipid concentrations, whilst a decline in TPH removal was observed with 10 ppm concentration under the same condition.

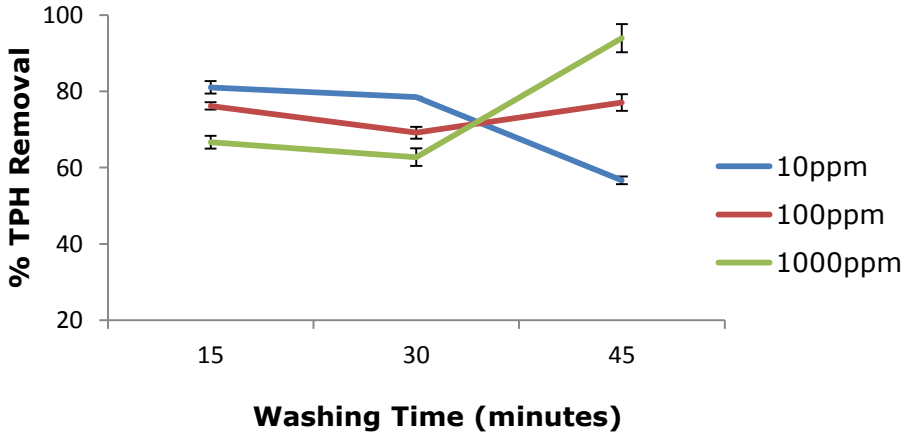


Figure 4.8: Effect of Washing Time on TPH Removal

B. **Washing Speed Effect:** Again, the highest TPH removal (90.8±3.7%) occurred when the sample was washed with 1000ppm of the biosurfactant at 300 rpm (Figure 4.9). Interestingly, the lowest TPH removal (56.7±1.04%) was observed at 45 minutes using 10ppm of the biosurfactant at a washing speed of 700 rpm. The decline in TPH removal at 700 rpm, may be due to possible breakage of the lipid due to vigorous shaking. This is based on the fact that the chain length of a lipid is a function of micelle formation, and the cleaning potential of the lipid is hinged on its ability to form micelles. This result shows that washing speed has significant impact on the ability of the biosurfactant to remove TPH from the soil. A significant decline in TPH removal was observed for the washing carried out using 1000 ppm at washing speed above 300 rpm, whilst an increase was observed for the washing carried out with 100 ppm rhamnolipid from 300 to 500 rpm and a decline after 500 rpm. For washings carried out at 10 ppm, no increase in TPH removal was observed at speeds above 300 rpm. As explained earlier, this decline may be as a result of possible breakage in the lipid chain length. However, the result shows that efficient cleaning can be achieved at low concentration with reduced speed, thus boosting the sustainability of the treatment process.

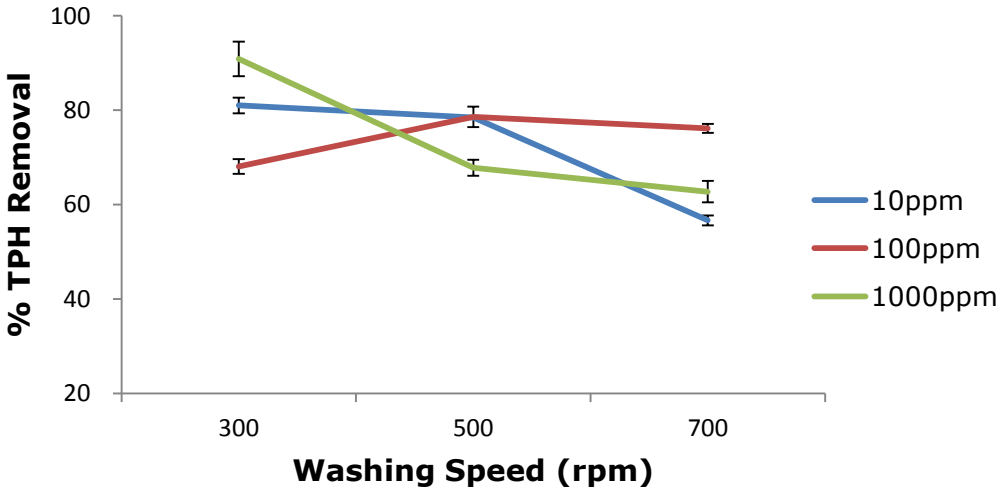


Figure 4.9: Effect of Washing Speed on TPH Removal

C. **Concentration Effect:** The CMC for the crude ST5 rhamnolipid is 48 ppm (as obtained in chapter 3, section 3.3.3.2). Although Urum and Pekdemir (2004) in their work showed that rhamnolipid solution may improve oil removal from oil contaminated soil at concentrations greater than its CMC

value up to a certain level, after which no significant oil removal can occur with increase in the rhamnolipid concentration. However, the result of this analysis shows that the crude rhamnolipid biosurfactant has the potential to remove TPH from soil at concentration below and above its CMC value. As observed in this study, higher TPH removal was achieved at 1000 ppm for the OBM-DCS, which is 20 times above the CMC. A number of factors may be responsible for the effectiveness of the rhamnolipid at concentrations below and above the CMC, such as washing speed and washing time.

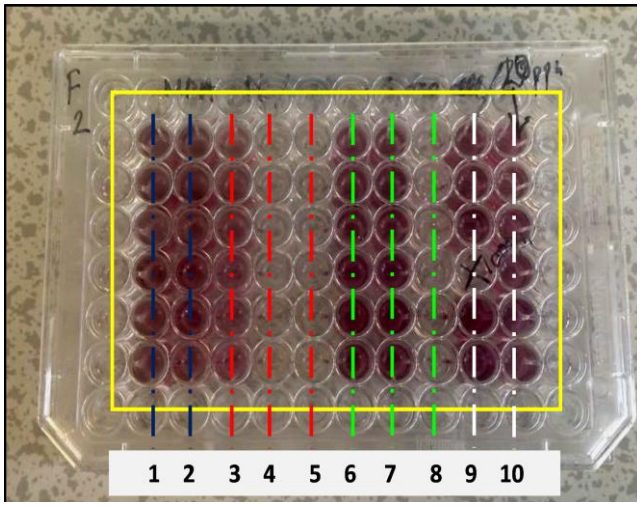
Table 4.5: Optimum Soil Washing Condition Obtained From Taguchi Experimental Design

TPH Removal (%)	Concentration (ppm)	Washing speed (rpm)	Washing time (mins)
90.8±3.7%	1000	300	45

It is interesting to see that over 90% TPH removal was achieved at room temperature and at concentration above the CMC. The result of this investigation shows that the cleansing action of the rhamnolipid can be significantly affected when the washing speed and time are varied.

4.3.2. Cytotoxicity of Rhamnolipid Standards and Washings

The result of the cytotoxicity test carried out on the rhamnolipid standards and washings to investigate the viability of the cells after 48 hours is shown in Figures 4.10 and 4.11 below.



Key

Lines	Test Solution
1	Media
2	Water
3	10ppm standard
4	100ppm standard
5	1000ppm standard
6	10ppm washing
7	100ppm washing
8	1000ppm washing
9	No test solution added
10	No test solution added

Figure 4.10: Cytotoxicity Test of ST5 Rhamnolipid Standards and Washings After DMSO Addition

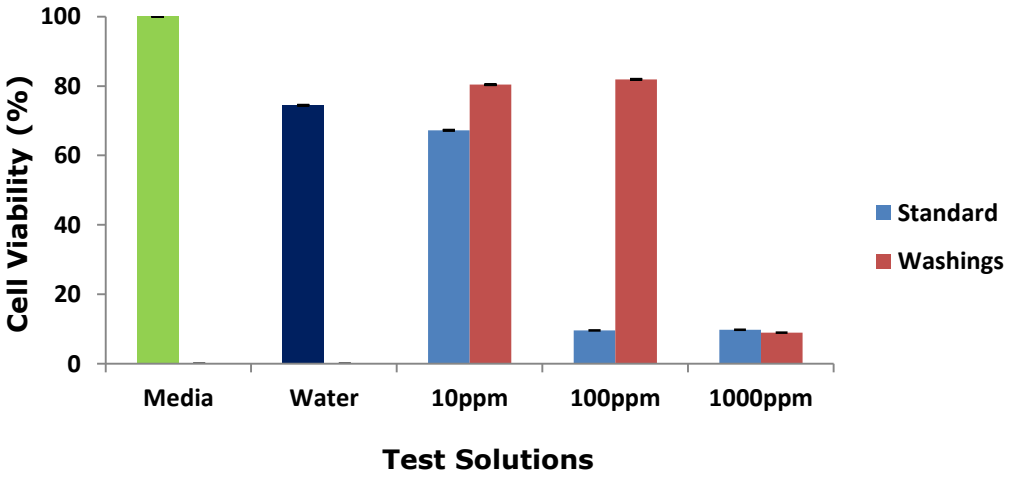


Figure 4.11: Effect of Rhamnolipid on Breast Cancer Cells Viability Measured by MTT Assay
(Viability test result after 48 hours (n=6)

As explained in section 4.2.7.4, the viability of the breast cancer cells were determined in relation to the viability of cells in the media, which served as the control for the experiment. The result of the cell viabilty test showed significant anti-proliferative properties against the breast cancer cells at 100 and 1000ppm for the crude ST5 rhamnolipid (standards), while rhamnolipid washings only showed anti-proliferation against the cancer cells at 1000ppm. A closer look at the test solutions (after the analysis) under the microscope at magnification x200 (Figures 4.12 – 4.15) further validated this result.

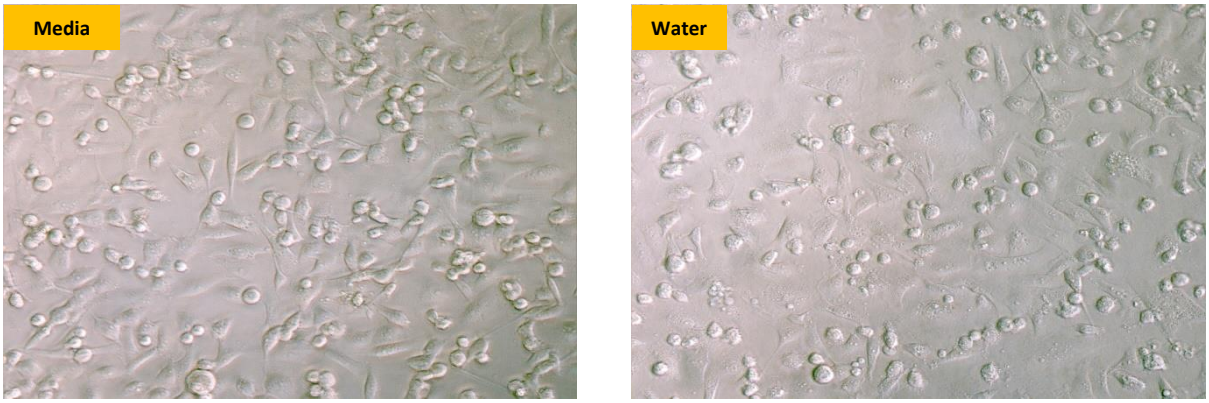


Figure 4.12: **Photomicrographs of Cytotoxicity Test Controls (Media & Water). Mag = 200x**

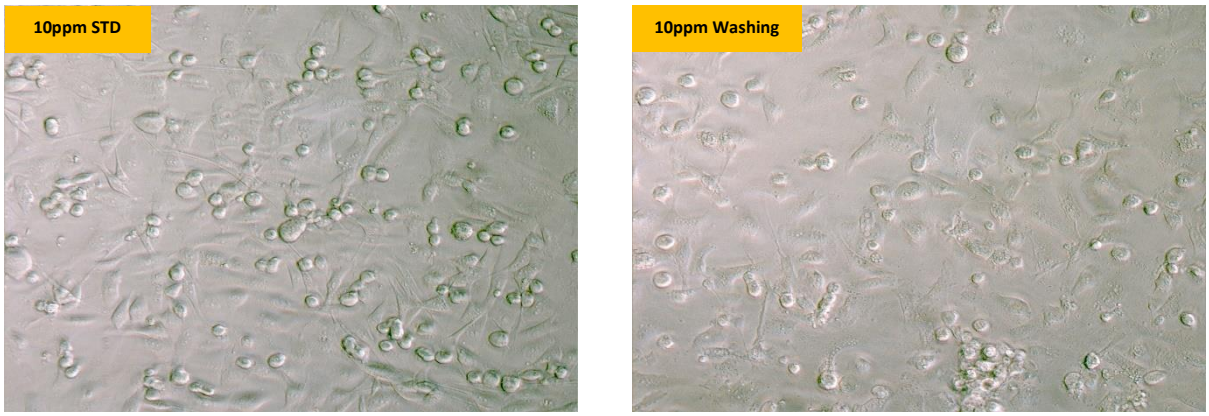


Figure 4.13: **Photomicrographs of Cytotoxicity Test on 10ppm Rhamnolipid Solution (Standard & Washings) Mag = 200x**

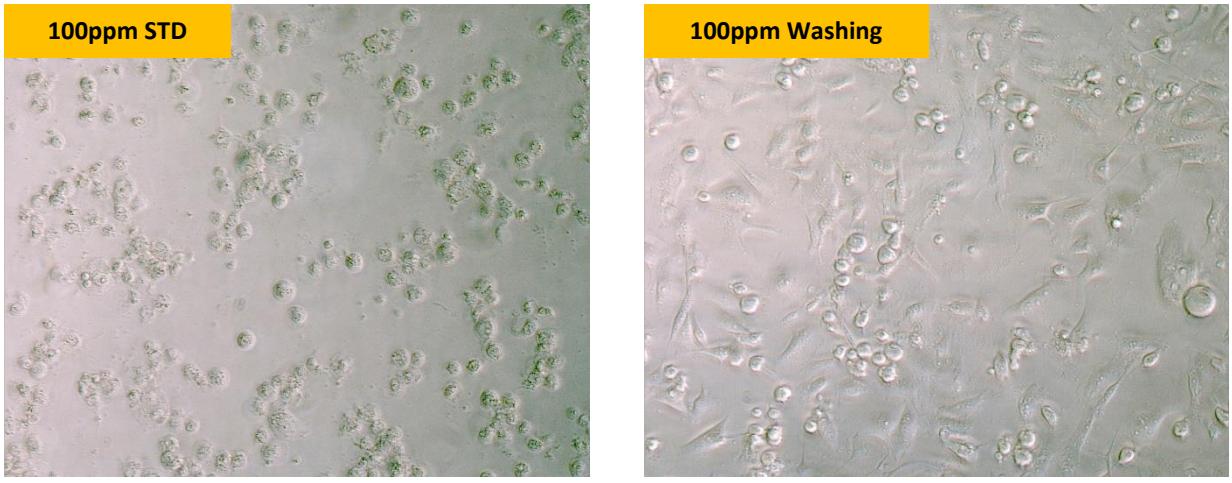


Figure 4.14: **Photomicrographs of Cytotoxicity Test on 100ppm Rhamnolipid Solution (Standard & Washings) Mag = 200x**

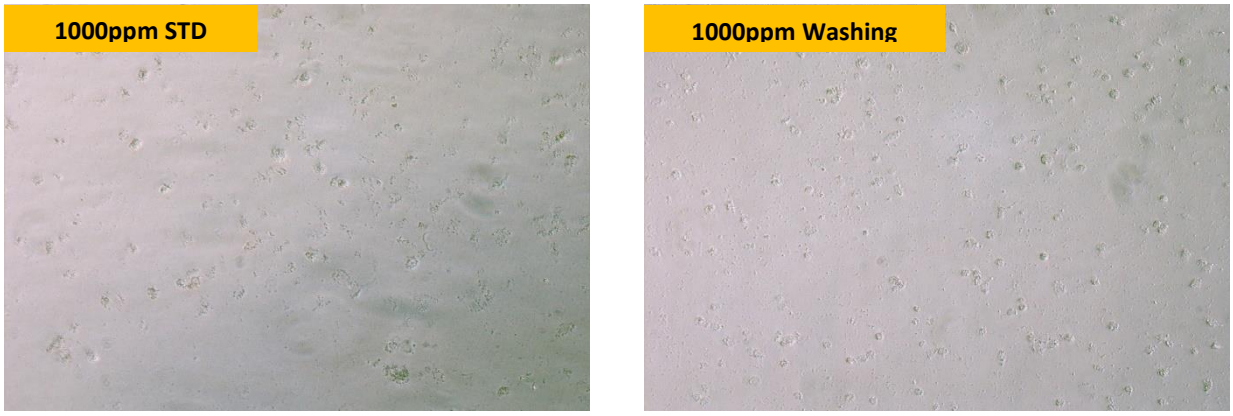


Figure 4.15: Photomicrograph of Cytotoxicity Test on 1000ppm Rhamnolipid Solution (Standard & Washings) Mag = 200x

The photomicrographs show healthy and confluent cells for the media, viable cells for water, 10ppm standard, 10ppm washings and 100ppm washings. The cells in the 100ppm standard are unhealthy since they appear shrunken and rounded, hallmark of dying cells, while 1000ppm standard and washings had dying cells in them.

However, the cells in the 100ppm washings appeared healthy with over 80% viability. But the cells tested with the 1000ppm standard appeared unhealthy with less than 10% viability (Figure 4.15).

Although the 1000ppm rhamnolipid was highly effective in removing TPH from the oil contaminated samples, the cytotoxicity analysis has shown that it is potentially unsafe to apply this concentration for TPH clean-up. This result clearly demonstrates the potential for safe use of rhamnolipid for the removal of TPH at 100ppm concentration where over 90% and 65% TPH removal was achieved from soil and OBM-DCS respectively (see Figure 4.7).

4.3.3. ICP-OES Analysis of Washed OBM-DCS.

The washed and air-dried OBM-DCS samples were digested as described in section 2.2.5, and ran on the ICP-OES as described in 2.2.6. The result of the analysis is compared with the as received OBM-DCS sample as shown in Figure 4.16 below.

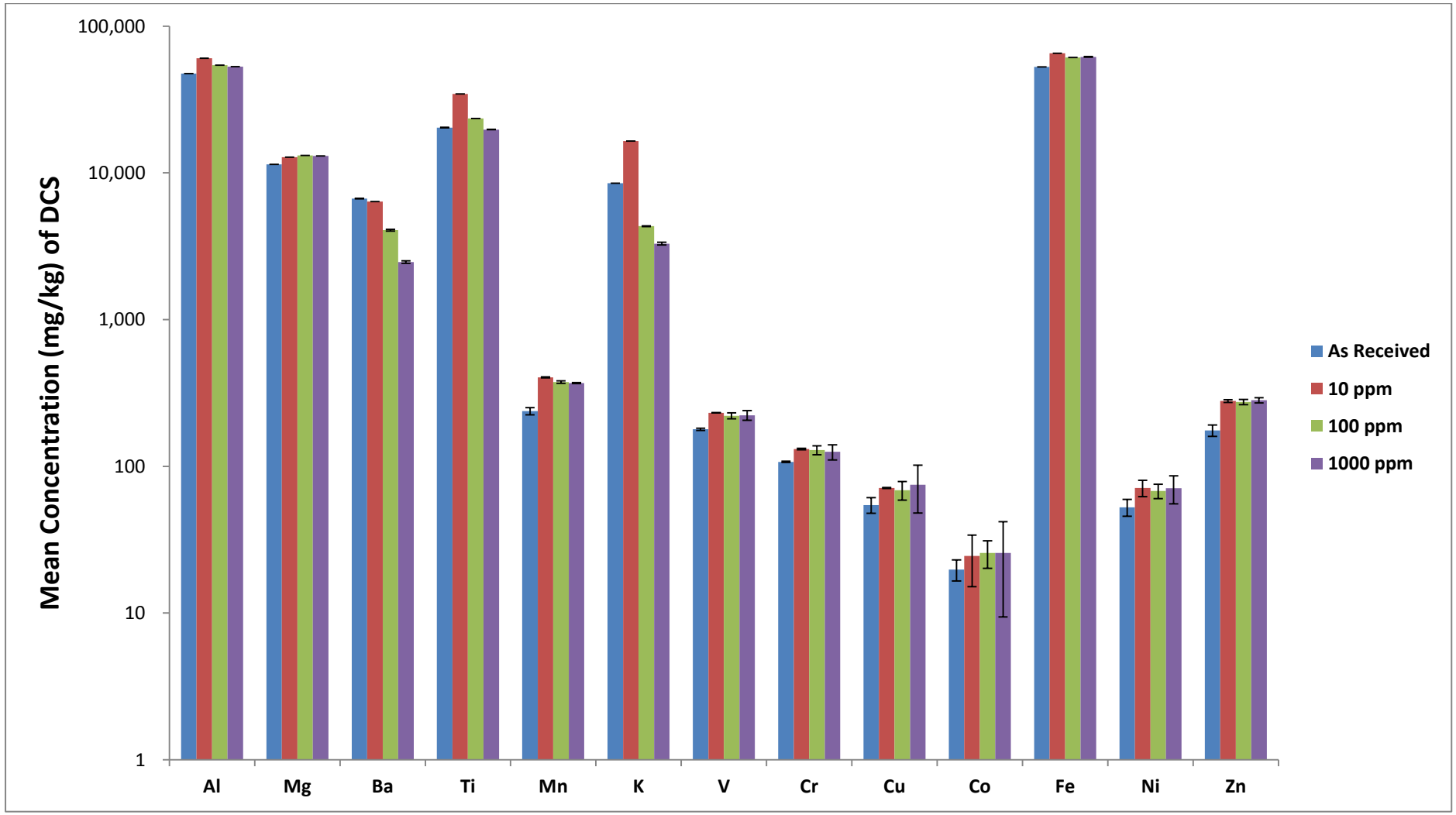


Figure 4.16: **Elemental Content by ICP-OES of OBM-DCS as received and following Crude ST5 Rhamnolipid Washing (n=2)**

As observed in section 2.3.3, Hg, Cd, As and Pb were not detected in the sample (as received). The result of the elemental analysis showed slight increases in the elemental content in the samples except for baruim in which an appreciable reduction in the sample after washing (Table 4.6).

Table 4.6: Barium (Ba) Content of OBM-DCS Before and After Washing

	As Received	Washed with 10 ppm	Washed with 100 ppm	Washed with 1000 ppm
Ba (mg/Kg)	6,668.27	6,381.78	4,070.49	2,466.15
% Reduction	-	4.3	36.2	61.4

The percentage reduction of Ba in the sample increased with increasing concentration of the biosurfactant. Barium is usually found in most drilling fluids where Barite is used as the weighting agent. It is possible that Ba detected in the analysis must have added (as an additive) to the fluid used in drilling the well, and as such could easily be removed under the washing conditions applied.

Generally, the result of the washing was not effective in reducing the elemental content of the sample. It is possible that this limitation could have been as a result of the washing conditions applied. The washing time, temperature and concentration of the washing solvent are variable factors that would have limited the biosurfactant from effectively reducing the elemental content of the samples (Mulligan 2014; Aşçi, Nurbaş and Açıkel 2008; Mulligan and Wang 2006).

Rhamnolipid have been succesfully applied for the removal of heavy metals from liquid and soild matrices. Elouzi et al. (2012), utilized 80ppm of rhamnolipid to remove Cd, Pb, Ni, Ba, Zn and Sr from contaminated water. They achieved a perentage reduction of (53%, 62%, 56%, 28%, 20% and 7%) for Cd, Pb, Ni, Ba, Zn and Sr respectively. Although the contaminated water samples was incubated at room temperature for one hour. It is possible that if the washing time is increased, the removal of the metal from the sample could potentailly increase.

4.4. Conclusion

Accidents, mistakes and errors are likely to occur during oil and gas operations, especially during transportation. It is imperative to ensure that oil contamnination is treated appropriately and sustainably. This chapter shows a successful removal of TPH from oil contaminated soil and OBM drill cuttings (at room temperature) using the rhamnolipid produced with *Pseudomonas aureginosa* ST5. A soil washing process was applied for the washing of the oil contaminated sample. The washing was achieved by the mechanism of solubilising the oil on the samples. The TPH was extracted by sonication to determine the percentage removal of the TPH from the contaminated samples. The optimum washing conditions were obtained following a Taguchi experimental design. The result of the optimum washing condition obtained from the Taguchi experimental design achieved a 90.8$\pm$3.7% TPH removal from oil contaminated soil when washed for 45 minutes, at 300 rpm washing speed using 1000 ppm crude ST5 rhamnolipid. The result from the cytotoxicity study showed that it was unsafe to use 1000 ppm crude ST5 rhamnolipid for the cleaning process as the cells tested using both the standard and washings from the 1000 ppm crude ST5 rhamnolipid were unviable at the end of the experiment.

However, it was interesting to note that the 10 and 100 ppm crude ST5 rhamnolipid was safe to be applied for the cleaning of the contaminated samples because the cells tested with the washings from both were viable at the end of the experiment. This is a positive observation because the 10 and 100 ppm crude ST5 rhamnolipid concentrations had over 90% removal of TPH from the oil contaminated soil. The effectiveness of the purified fractions (monorhamnolipid and dirhamnolipid) from the crude ST5 rhamnolipid in removing TPH from the oil contaminated samples were investigated. The results obtained showed that the crude ST5 rhamnolipid, was more effective in removing TPH from the oil contaminated sample than its purified fractions (over 90% removal of TPH achieved). This finding is crucial as it suggests the need to eliminate the purification step after production of the rhamnolipid, thus reducing the cost of the cleaning process, increasing the economic viability of the process and making the process sustainable in the long run. However, the washing did not reduce the elemental content of the OBM-DCS due to the washing conditions applied. Although there was a significant reduction of barium from the OBM-DCS (61.4%) using the 1000 ppm crude ST5 rhamnolipid.

CHAPTER 5 - CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

The treatment of oil contamination in the environment is important because such contamination can cause potential detrimental effects on the health and safety of humans and other living organisms. This research was carried out to develop an eco-friendly sustainable alternative for the removal of petroleum hydrocarbons from OBM drill cuttings and diesel contaminated soil, and explore the possibility of reusing the treated material as a construction material.

The summary of the specific objectives achieved in this research are;

(i) Critical review of existing treatment technologies applied in treating oil contaminated waste.
(ii) Characterisation of the oil contaminated waste using analytical parameters such as, particle size distribution, SEM-EDXA, ICP-OES, GC-MS by HS/SPME and FT-IR.
(iii) Production and characterisation of the rhamnolipid biosurfactant applied for the removal of TPH from the oil contaminated samples.
(iv) Treatment of the oil contaminated solids using rhamnolipid biosurfactant by a washing process.
(v) Accessing the safe use of the rhamnolipid biosurfactant by carrying out a cytotoxicity analysis using breast cancer cells.

5.1. Conclusions

Chapters one and two presented an overview of the oil and gas exploration and production industry, including an indepth review of existing literature discussing the waste associated with the upstream sector of the oil and gas industry, showing the potential risk associated at each level and the effects of these risks on health, safety and environment.

The second chapter showed the results of the characterisation of the oil contaminated samples (as received) to determine the treatment needs assessment of the samples. The results obtained from the characterisation of the OBM-DCS showed that the sample contained over 61,000 mg/Kg TPH (oil on

cutting). However, legislative requirement specifies that the oil on cuttings to be disposed offshore should have a maximum concentration of 10,000 mg/Kg. The obtained shows that the sample required treatment. The results also provided valuable data required to assess the efficiency of the treatment to be carried out on the contaminated samples.

Chapter three focused on the production and characterisation of the biosurfactant to be utilized for the treatment of the contaminated samples. The production was carried out using *Pseudomonas aeruginosa* ST5 and *Pseudomonas aeruginosa* PS1. The growth rate of both strains were studied, giving approximate yields of 3.3$\pm$0.1 g/L and 3.4$\pm$0.1 g/L of rhamnolipid respectively. Thin layer chromatography showed that there were two major fractions present in both rhamnolipids (monorhamnolipid and dirhamnolipid). The crude extracts were further purified and fractionated into the two major fractions by column chromatography with silica as the adsorbent. The surface activity of the crude biosurfactants, purified fractions and SDS (a synthetic surfactant used as a positive control) were investigated using the following parameters: surface tension by Du Nouy Platinum ring, critical micelle concentration (CMC) and emulsification activity on kerosene, crude oil, diesel and sun flower oil after 24 hours. The results obtained from the surface activity investigation showed that the crude biosurfactant, purified fractions and SDS had results that were comparable to previous studies carried out. Chemical and structural characterisation was also carried out on the crude ST5 rhamnolipid and purified fractions using FTIR-ATR, NMR, and LC-MS/MS. Specific rhamnolipid congeners were identified in each of the fractions with the $RhC_{10}C_{10}$ dominating in the monorhamnolipid fraction whilst $Rh_2C_{10}C_{10}$ was dominant in the di-rhamnolipids. The results obtained from the structural characterisation were also comparable to previous studies carried out as well confirming the production of the rhamnolipid biosurfactant.

Chapter four focused on assessing the efficiency and suitability of the produced rhamnolipid in removing TPH from OBM-DCS and oil contaminated soil. The efficiency was first assessed by using the crude ST5 rhamnolipid and SDS to remove TPH from sand spiked with diesel at room temperature. Results show that the crude ST5 rhamnolipid was more efficient in removing TPH from the spiked sand than the synthetic surfactant at all the concentrations investigated (10, 100 and 1000 ppm). This was expected as the CMC of the crude ST5 rhamnolipid (48

ppm) is much lower than the CMC of SDS (2,018 ppm) and the highest concentration applied for this investigation was lower than the CMC of SDS. The crude ST5-RL and purified fractions were applied for the removal of TPH from oil contaminated soil at 10, 100 and 1000ppm. Over 70% TPH reduction was achieved using the crude ST5 rhamnolipid and its purified fractions, at all concentrations investigated. The result of this investigation shows the ability of the crude-RL to solubilise the oil (on both sand and soil), lowering the interfacial tension between the oil and the biosurfactant at room temperature and at concentration below the CMC value.

The efficiency of the crude ST5 rhamnolipid on the OBM-DCS and oil contaminated soil was investigated using the following concentrations 10, 100 and 1000ppm. The result of this analysis showed over 90% TPH removal from the soil across the concentrations studied, while the 10, 100 and 1000ppm crude ST5 rhamnolipid removed 67.7%, 65% and 87.6% TPH repectively from the OBM-DCS. Based on the efficiency of the biosurfactant to remove TPH from oil contaminated soil, the optimum washing conditions were studied using only soil. Furthermore, only the crude surfactant was utilised as the additional purification step did not justify the use of the purified fractions. The result of the investigation of the optimum washing condition for the removal of TPH from soil achieved approximately 90.8+3.7% TPH removal using 1000ppm of crude ST5 rhamnolipid, washing at the washing speed of 300 rpm for 45 minutes using a liquid to washing ratio of 3:1.

A trial treatment was carried out on the washed OBM-DCS to see if the biosurfactant reduced the elemental heavy metal content in the sample. The results showed that the biosurfactant was able to reduce the concentration of only barium. Approximately 61.4% barium was removed from the washed sample using 1000ppm of the sample. Although studies show that rhamnolipid have been applied in removing heavy metals from liquid and solid matrices, this trial was not succesful in removing the metals from the sample. A better result can be achieved if the sample is digested with stronger acids and washing conditions altered.

Also the safe use of the crude ST5-RL was investigated by carrying out a cytotoxicity analysis using breast cancer cells via an MTT assay. The result of this analysis showed that the biosurfactant is safe to use from 10 to 100ppm

concentration where over 90% and 65% TPH removal was achieved for the oil contaminated soil and OBM-DCS respectively.

This work has been able to show that biosurfactant can be used as a sustainable alternative for the removal of TPH from oil contaminated soil and OBM-DCS at room temperature.

5.2. Contribution to Knowledge

The contributions to knowledge made from this research are as follows:

1. A comprehensive literature review and characterisation of OBM drill cuttings that serves as a benchmark for future studies.
2. Production and characterisation of an economically viable and environmentally friendly biosurfactant using strains of *Ps. Aeruginosa.*
3. This work has confirmed that biosurfactant can serve as a sustainable alternative or replacement for synthetic surfactants in the removal or treatment of oil contaminated drilling waste.
4. This work has obtained an optimum concentration at which rhamnolipid produced with *Ps. Aeruginosa* ST5 can effectively remove TPH from oil contaminated waste at room temperature.
5. The work has shown that the low toxicity of biosurfactants is limited to certain concentrations. Thus the safe use of the product can only be achieved at a certain concentration.

5.3. Recommendations for Future Work

The following future studies can be carried out:

1. The process of recovering the rhamnolipids from the bacteria culture is quite expensive. Further research should be carried out to finding less expensive options for the recovery of rhamnolipid from the bacteria culture, as well as utilize waste oils as carbon sources for the cultivation of the bacteria.
2. Investigation and identification of all the congeners present in the rhamnolipids produced from ST5 and PS1 rhamnolipid can be explored by utilizing high resolution mass spectrometry.

3. Investigation of the potential for the biosurfactants to remove heavy metals from OBM-drill cuttings should be carried out. Studies should be focused on biosurfactant complexation of metals using different ligands (Hogan et al. 2014).
4. The exploration of reuse options for treated drill cuttings as construction materials will be useful.

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Appendices

Appendix 1 – Particle Size Distribution

Table A.1: Particle Size Distribution of Soil by Laser Diffraction

Result: Analysis Table

ID: wm soil dispersion	Run No: 2	Measured: 29/4/2016 09:45PM
File: KYARI	Rec. No: 2	Analysed: 29/4/2016 09:45PM
Path: C:\SIZERS\DATA\		Source: Analysed

Range: 300RF mm	Beam: 14.30 mm	Sampler: MS1	Obs': 31.9 %
Presentation: 3OHD		Analysis: Polydisperse	Residual: 5.285 %
Modifications: None			

Conc. = 0.0194 %Vol	Density = 1.000 g/cm^3	S.S.A.= 0.2616 m^2/g
Distribution: Volume	D[4, 3] = 168.52 um	D[3, 2] = 22.93 um
D(v, 0.1) = 10.08 um	D(v, 0.5) = 121.18 um	D(v, 0.9) = 398.08 um
Span = 3.202E+00	Uniformity = 1.042E+00	

Size (um)	Volume In %	Size (um)	Volume In %	Size (um)	Volume In %	Size (um)	Volume In %
0.05	0.00	0.58	0.00	6.63	1.17	76.32	3.11
0.06	0.00	0.67	0.00	7.72	1.31	88.91	3.14
0.07	0.00	0.78	0.25	9.00	1.42	103.58	3.26
0.08	0.00	0.91	0.22	10.48	1.52	120.67	3.49
0.09	0.00	1.06	0.22	12.21	1.61	140.58	3.87
0.11	0.00	1.24	0.24	14.22	1.70	163.77	4.39
0.13	0.00	1.44	0.26	16.57	1.79	190.80	4.99
0.15	0.00	1.68	0.29	19.31	1.91	222.28	5.80
0.17	0.00	1.95	0.33	22.49	2.07	258.95	6.36
0.20	0.00	2.28	0.38	26.20	2.26	301.68	6.40
0.23	0.00	2.65	0.45	30.53	2.47	351.46	5.75
0.27	0.00	3.09	0.53	35.56	2.68	409.45	4.38
0.31	0.00	3.60	0.63	41.43	2.86	477.01	3.01
0.36	0.00	4.19	0.75	48.27	2.99	555.71	1.64
0.42	0.00	4.88	0.89	56.23	3.07	647.41	0.00
0.49	0.00	5.69	1.03	65.51	3.10	754.23	0.00
0.58		6.63		76.32		878.67	

Appendix 2 – Calibration Curves for Elemental Analysis

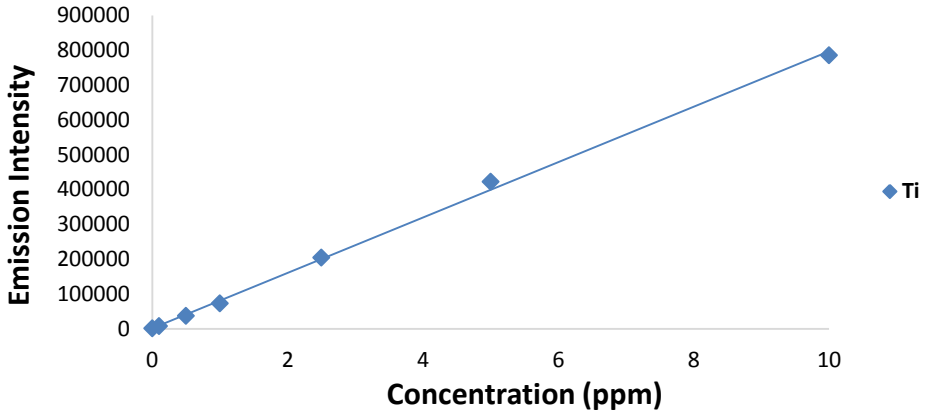


Figure A.1: Calibration Plot for the Analysis of Ti in DCS by ICP-OES

Calibration equation: y = 79500x + 1723.2 and R^2 = 0.9985

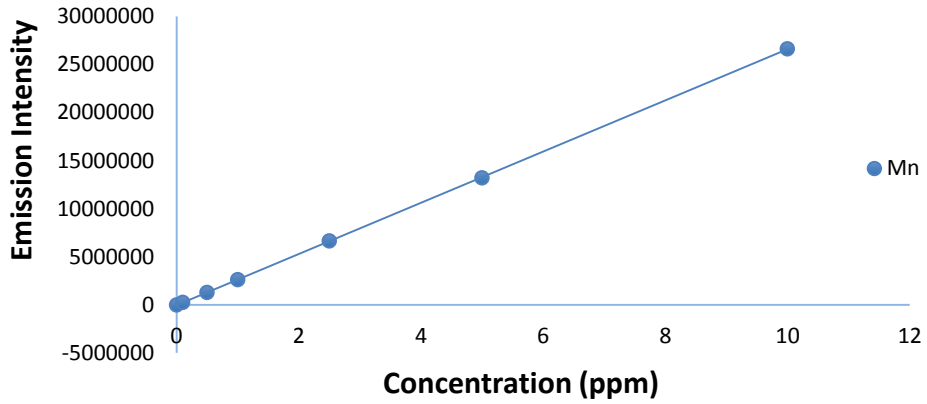


Figure A.2: Calibration Plot for the Analysis of Mn in DCS by ICP-OES

Calibration equation: y = 2,660,617.67x - 24,489.41 and R^2 = 1.00

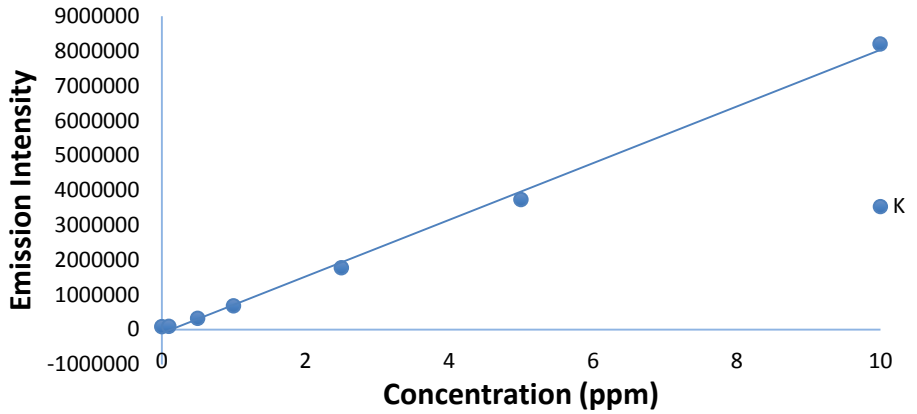


Figure A.3: Calibration Plot for the Analysis of K in DCS by ICP-OES

Calibration equation: y = 813152x – 98341 and R^2 = 0.9971

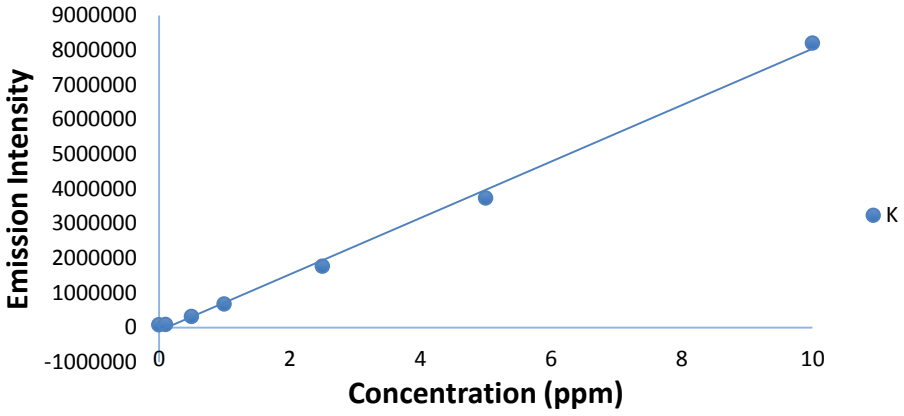


Figure A.4: Calibration Plot for the Analysis of K in DCS by ICP-OES

Calibration equation: y = 813152x – 98341 and R^2 = 0.9971

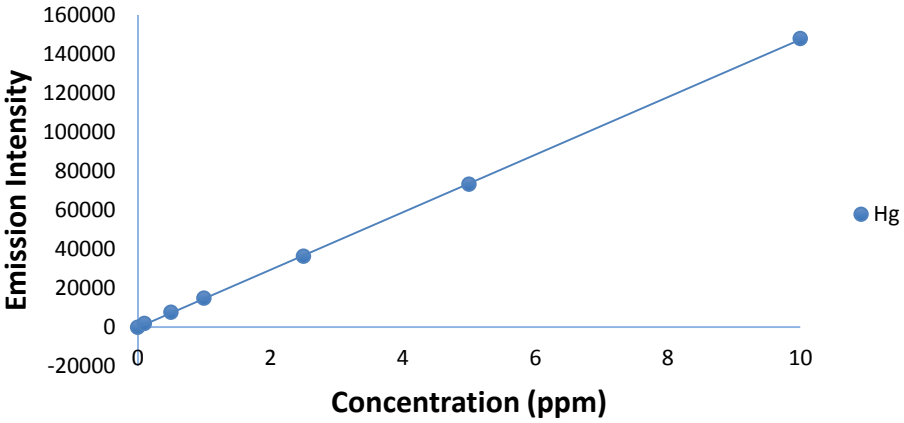


Figure A.5: Calibration Plot for the Analysis of Hg in DCS by ICP-OES

Calibration equation: y = 14749x - 95.999 and R^2 = 0.9999

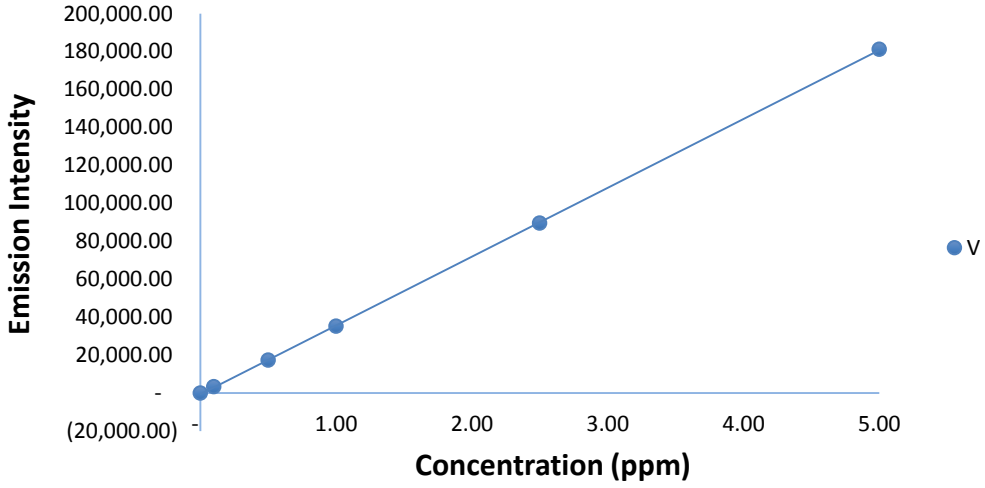


Figure A.6: Calibration Plot for the Analysis of V in DCS by ICP-OES

Calibration equation: y = 36,260.91x - 534.93 and R^2 = 1.0

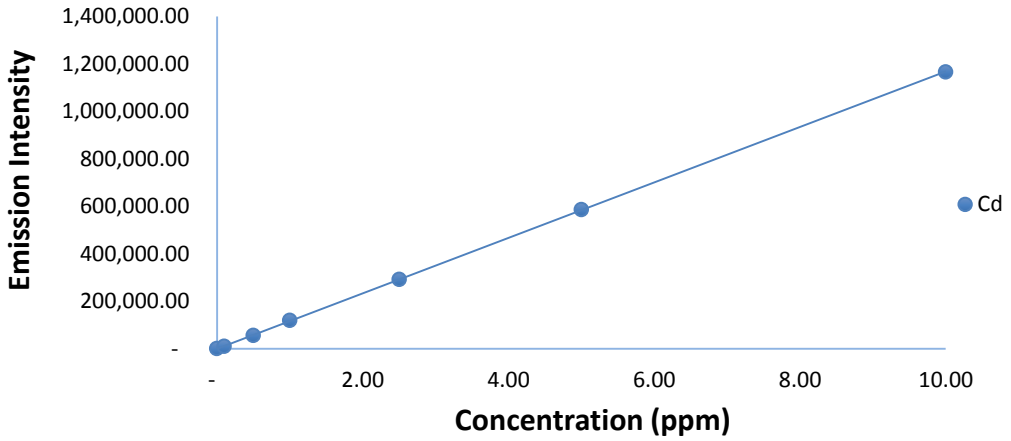


Figure A.7: Calibration Plot for the Analysis of Cd in DCS by ICP-OES

Calibration equation: y = 116,677.70x + 353.00 and R^2 = 1.00

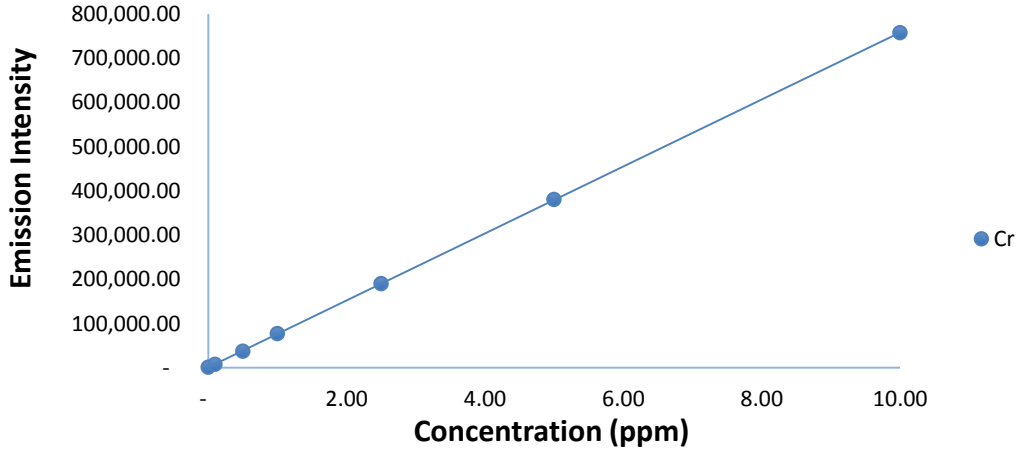


Figure A.8: Calibration Plot for the Analysis of Cr in DCS by ICP-OES

Calibration equation: y = 75,731.81x + 799.61 and R^2 = 1.00

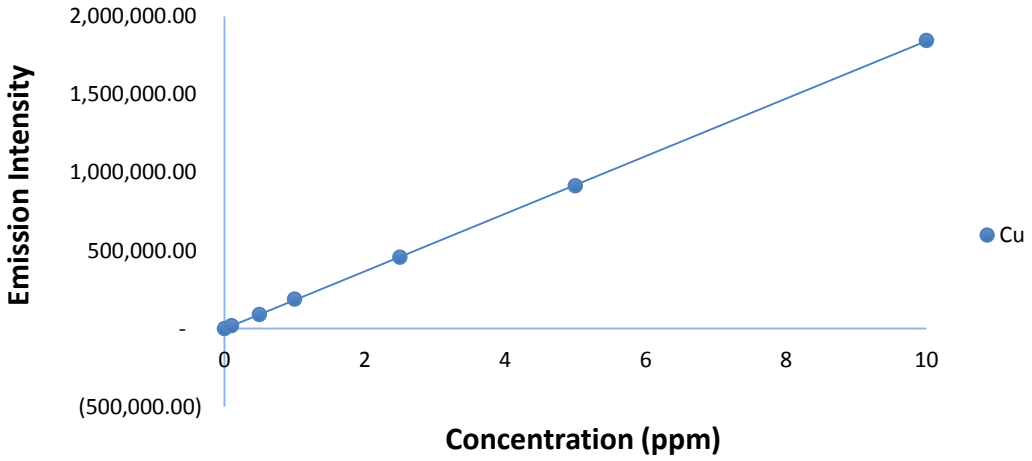


Figure A.9: Calibration Plot for the Analysis of Cu in DCS by ICP-OES

Calibration equation: y = 184,271.01x - 1,141.54 and R^2 = 1.00

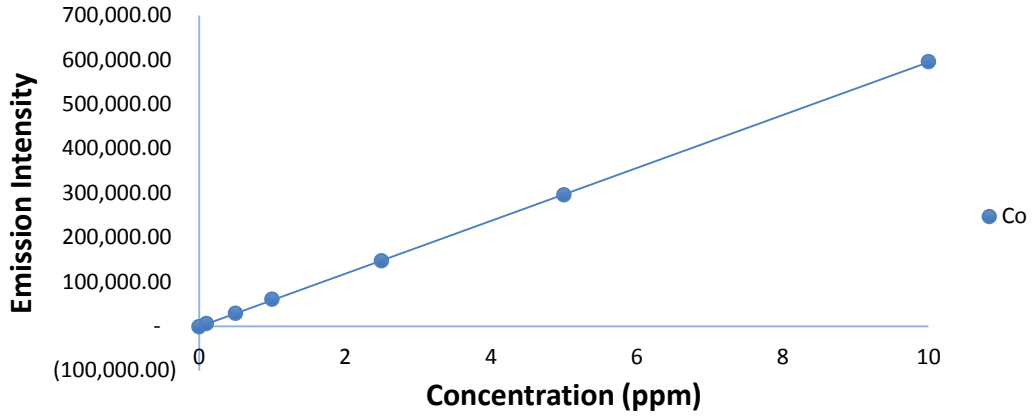


Figure A.10: Calibration Plot for the Analysis of Co in DCS by ICP-OES
Calibration equation: y = 59,532.19x - 747.83 and R^2 = 1.00

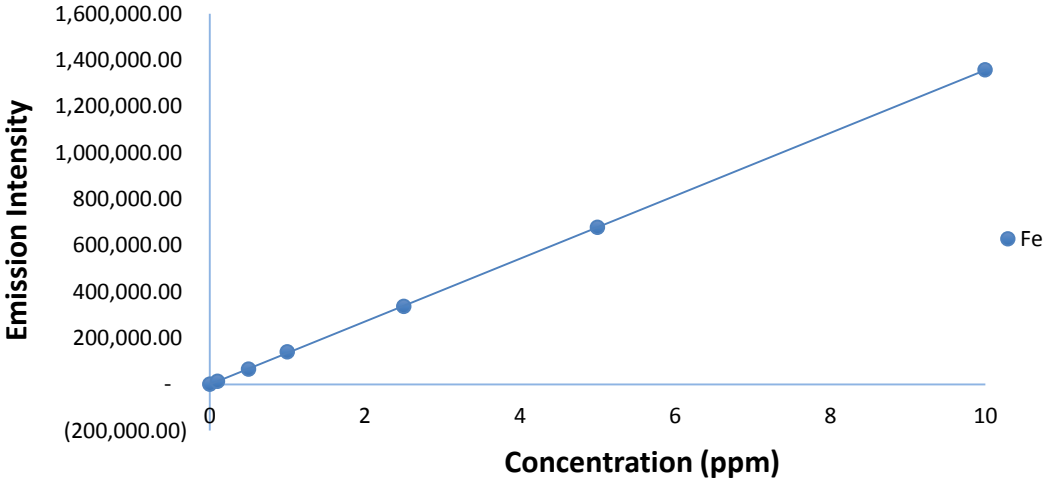


Figure A.11: Calibration Plot for the Analysis of Fe in DCS by ICP-OES
Calibration equation: y = 135,729.87x - 213.74 and R^2 = 1.00

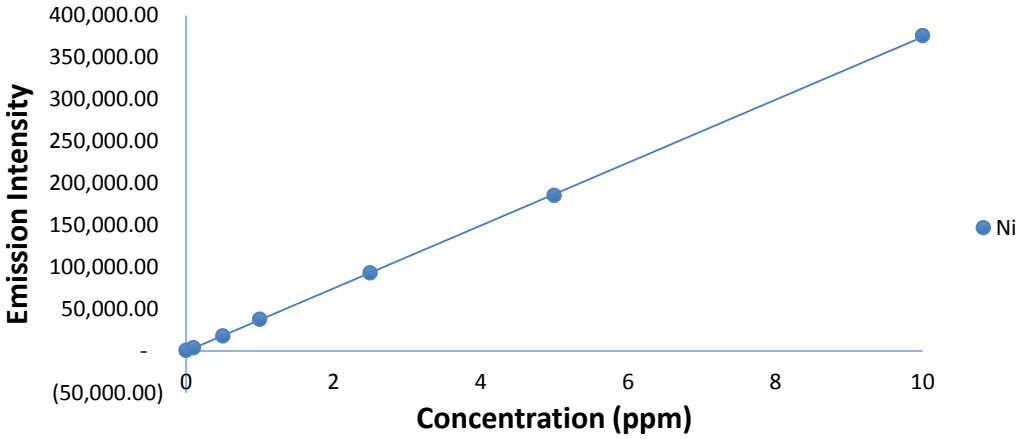


Figure A.12: Calibration Plot for the Analysis of Ni in DCS by ICP-OES
Calibration equation: y = 37,478.76x - 354.36 and R^2 = 1.00

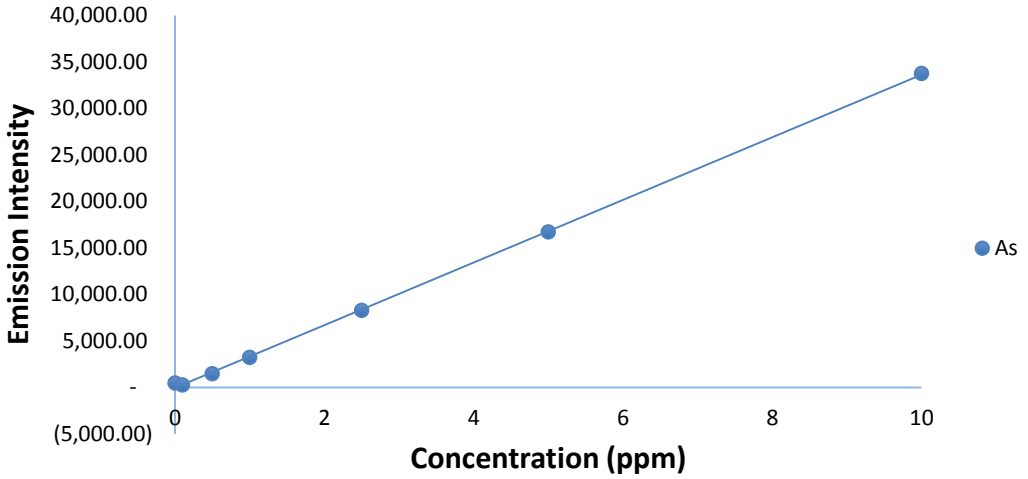


Figure A.13: Calibration Plot for the Analysis of As in DCS by ICP-OES
Calibration equation: y = 3,366.43x - 40.78 and R^2 = 1.00

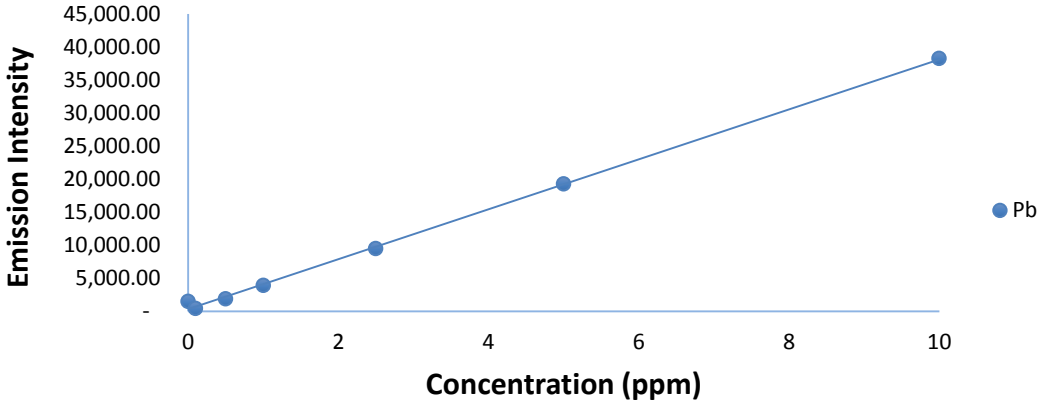


Figure A.14: Calibration Plot for the Analysis of Pb in DCS by ICP-OES
Calibration equation: y = 3,777.87x + 337.68 and R^2 = 1.00

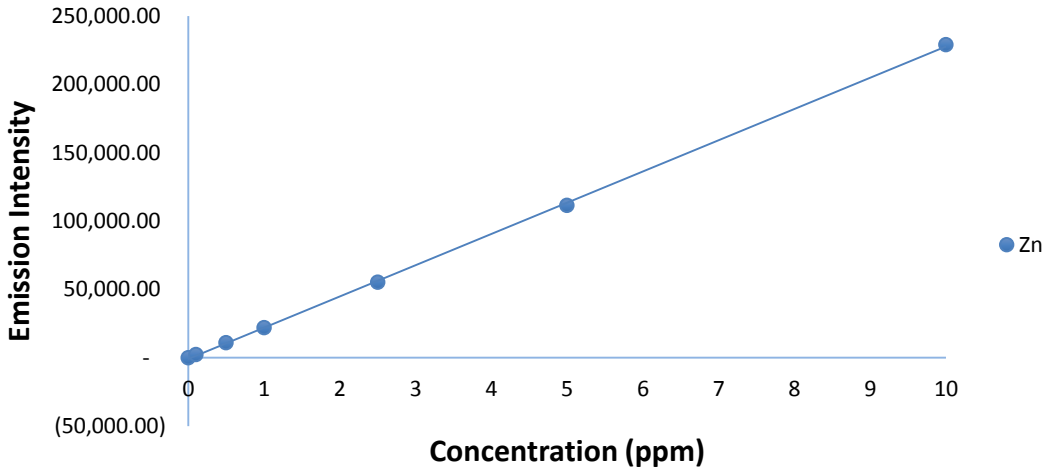


Figure A.15: Calibration Plot for the Analysis of Zn in DCS by ICP-OES
Calibration equation: y = 22,861.11x - 902.19 and R^2 = 1.00

Appendix 3 – FT-IR of Rhamnolipids

The result of the FTIR-ATR analysis carried out on all the rhamnolipids can be seen in the spectra stacked in Figure A.16 below.

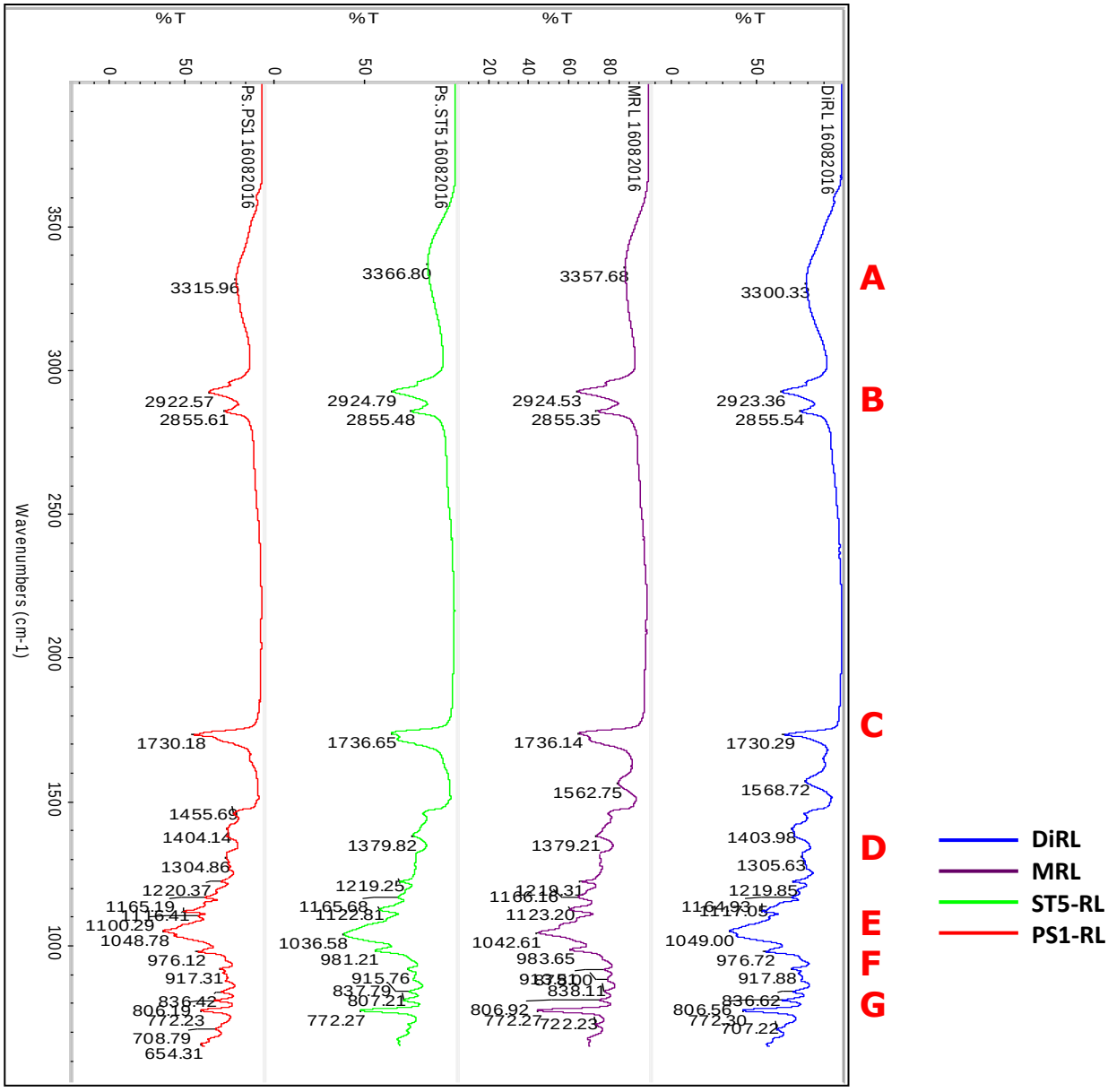


Figure A.16: **FTIR Spectra for Rhamnolipids**
(DiRL, MRL, ST5-RL and PS1-RL).
Where A, B, C, D, E, F and G represent the regions

Appendix 4 – LC-MS/MS of PS1 of Rhamnolipids

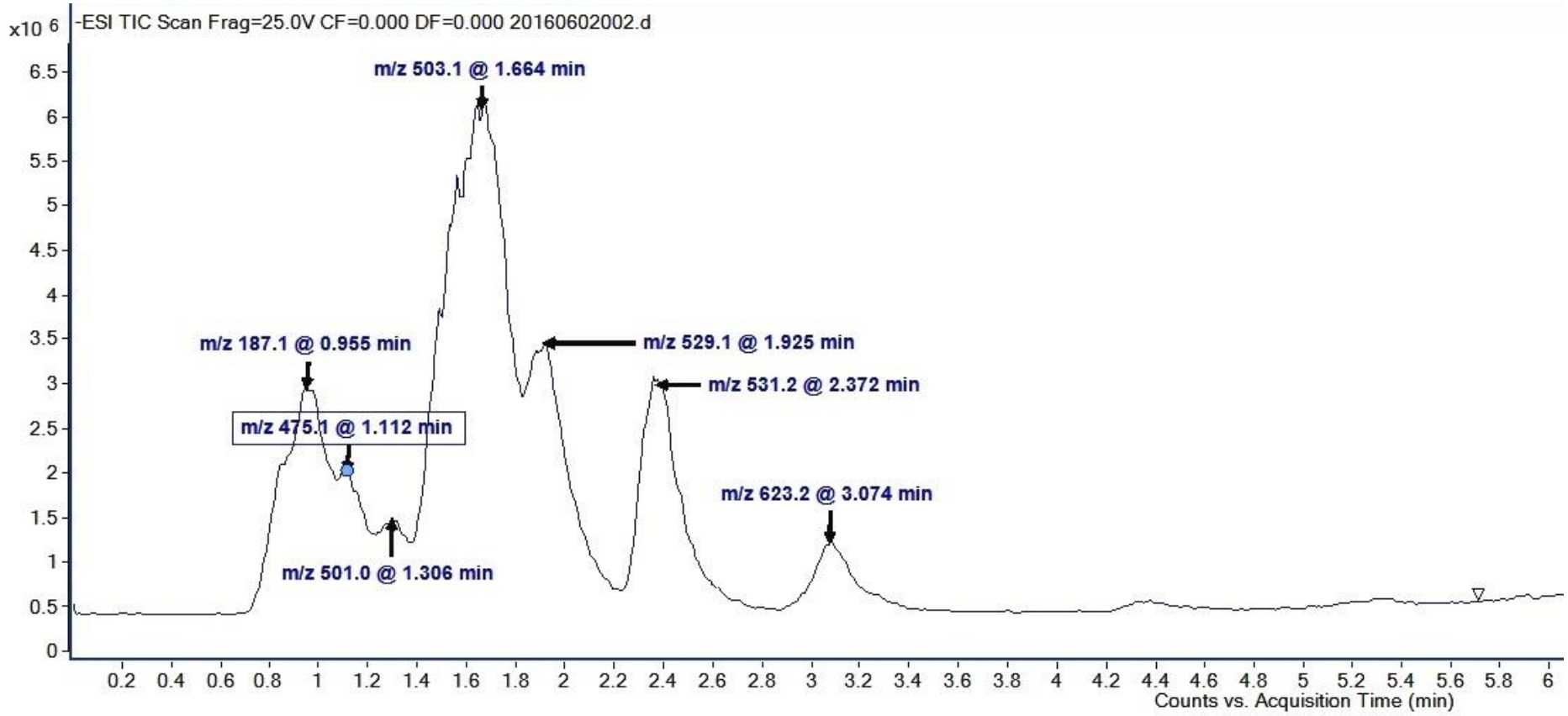


Figure A.17: TIC of M-RL Homologues Produced from Ps. Aeruginosa PS1

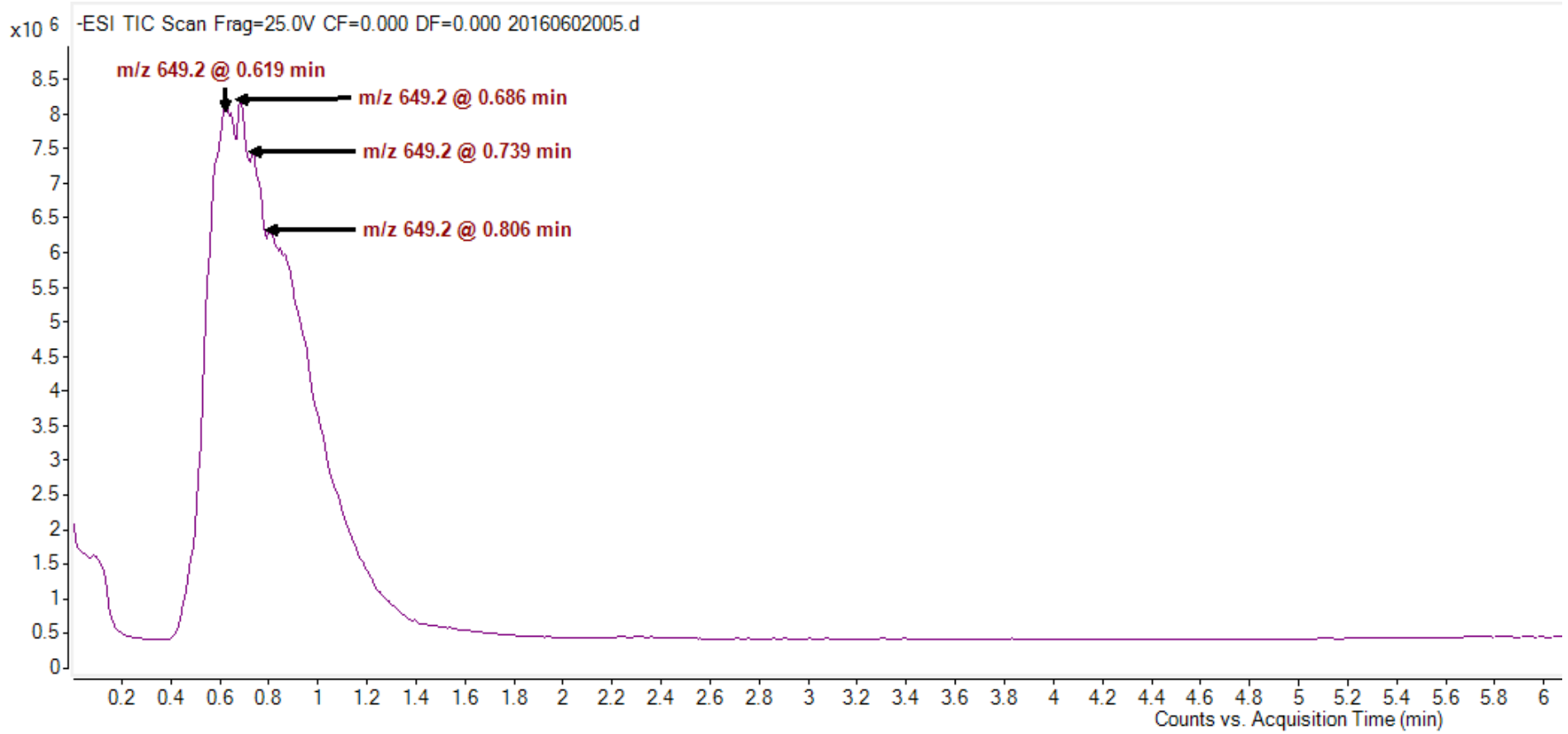


Figure A.18: TIC of Di-RL Homologues Produced from Ps. Aeruginosa PS1

Appendix 5 – Research Output

Conference papers

NWINEE, S.A., YATES K., LIN, P.K.T., COWIE, E. and BANAT, I.M., 2016. Biosurfactant: A Sustainable Alternative for the Treatment of Petroleum Contaminated Soils. A short oral paper presented at the *6th EuCheMS Chemistry Congress, organized by the European Association for Chemical and Molecular Sciences (EuCheMS).* The conference was held at Seville, Spain, from 11th -15th September 2016.

NWINEE, S.A., YATES K., and POLLARD, P., 2012. Sustainable Treatment and Disposal of Oilfield Waste (Drill Cuttings) Onshore. A paper presented at the 15th International HSE Biennial Conference on the Oil and Gas Industry, Abuja Nigeria, 5 – 7 November 2012.

Poster Presentations

NWINEE, S.A., YATES K., LIN, P.K.T., COWIE, E. and BANAT, I.M., 2015. Laboratory production and characterization of rhamnolipid biosurfactant, for the treatment of petroleum-contaminated soils. A poster p*resented at the Faculty of Design and Technology's Lunch/Poster Event in Robert Gordon University, Aberdeen, UK*, 10 December, 2015. (Award: 3rd Position School of Engineering)

NWINEE, S.A., YATES K., LIN, P.K.T. and COWIE, E., 2015. A Sustainable Alternative for the Treatment of Oil-Based Mud (OBM) Drill Cuttings. A poster presented at the *Chemistry in the Oil Industry (CITOI) XIV conference, a Royal Society of Chemistry International Symposium*, Manchester, UK, 2-4 November, 2015.

NWINEE, S.A., YATES K., and POLLARD, P., 2014. Treat Needs Assessment of Oil-Based Mud (OBM) Drill Cuttings. A poster presented at the *Royal Society of Chemistry Emerging Analytical Professionals (EAP) 2014 Conference* held at Penrith Cumbria, UK, from 4th - 6th April 2014.

Appendix 6 – Papers Being Worked on For Publication

The following papers are being worked on for publication in Marine Bulletin Journal:

1. Laboratory production and characterization of rhamnolipid biosurfactant from Pseudomonas species.
2. Removal of TPH from petroleum-contaminated soils using rhamnolipid biosurfactant.

Appendix 7 – Awards Received from Study Experience

1. Volunteering Award from RGU Union

Volunteering Award

presented to

Sarah Nwinee

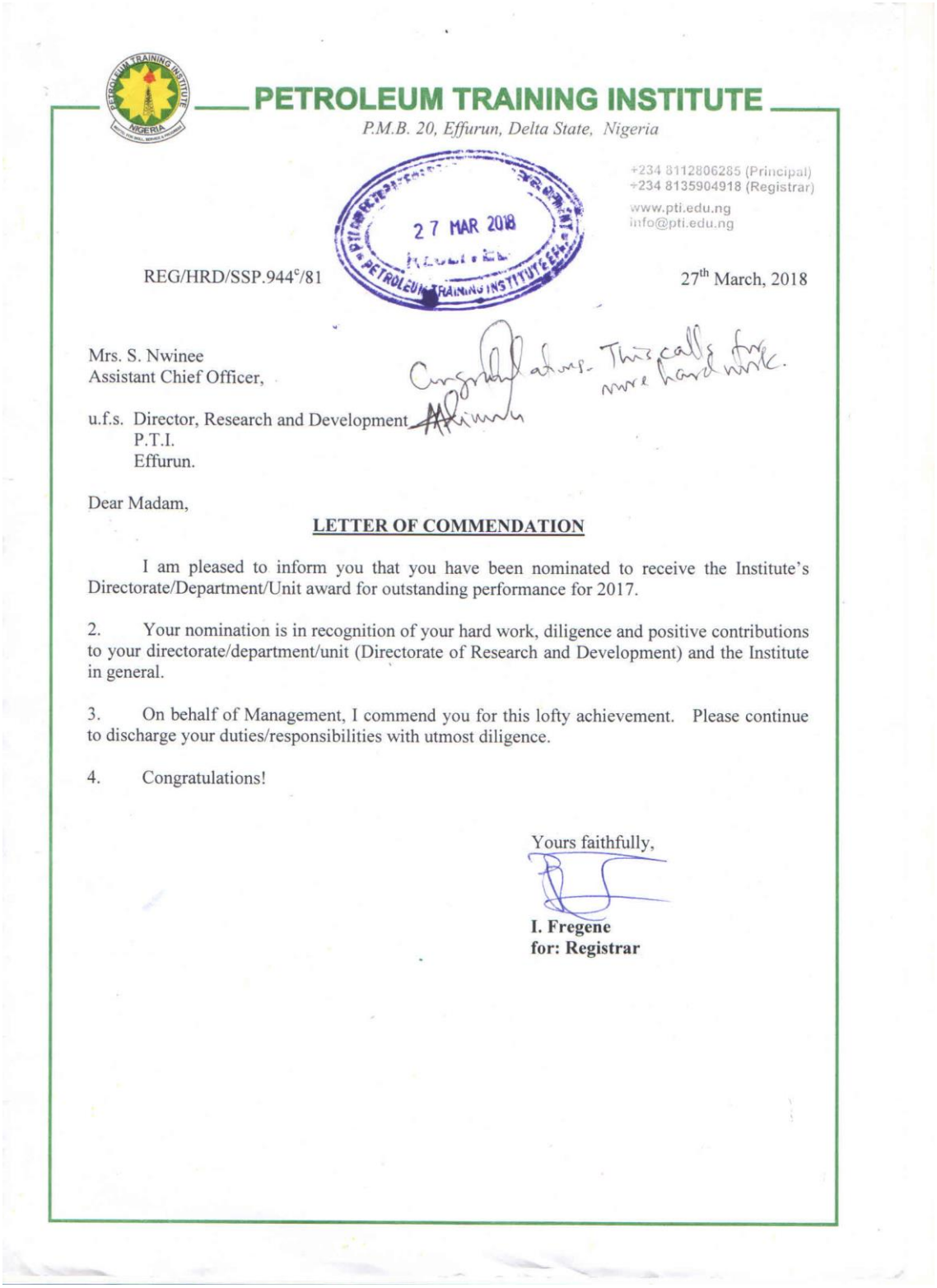
In recognition of 200 hours of dedicated
contribution to volunteering projects

Stevie Kearney, RGU:Union Student Development & Volunteering Co-ordinator — Date: 25/03/16

Ferdinand Von Prondzynski, Robert Gordon University Principal and Vice-Chancellor — Date: 25/03/16

Student ACHIEVEMENT Awards — rgu:union — ROBERT GORDON UNIVERSITY ABERDEEN

2. Letter of Commendation from Petroleum Training Institute, Nigeria (Employer).



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27 MAR 2018

REG/HRD/SSP.944^c/81

27th March, 2018

Mrs. S. Nwinee
Assistant Chief Officer,

Congratulations. This calls for more hard work.

u.f.s. Director, Research and Development
P.T.I.
Effurun.

Dear Madam,

LETTER OF COMMENDATION

I am pleased to inform you that you have been nominated to receive the Institute's Directorate/Department/Unit award for outstanding performance for 2017.

2. Your nomination is in recognition of your hard work, diligence and positive contributions to your directorate/department/unit (Directorate of Research and Development) and the Institute in general.

3. On behalf of Management, I commend you for this lofty achievement. Please continue to discharge your duties/responsibilities with utmost diligence.

4. Congratulations!

Yours faithfully,

I. Fregene
for: Registrar