BIOREMEDIATION OF CRUDE OIL-POLLUTED AGRICULTURAL SOIL USING BIOSURFACTANT-PRODUCING BACTERIAL ISOLATES

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MAY, 2021

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BY

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A DISSERTATION SUBMITTED TO THE DEPARTMENT OF APPLIED MICROBIOLOGY AND BREWING

IN PARTIAL FULFILLMENTOF THE REQUIREMENTS FOR THE AWARD OF DOCTOR OF PHILOSOPHY (PhD) DEGREE IN ENVIRONMENTAL MICROBIOLOGY OF NNAMDI AZIKIWE, UNIVERSITY, AWKA

DEPARTMENT OF APPLIED MICROBIOLOGY AND BREWING
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MARCH, 2021

CERTIFICATION

I Chizoba MaryannDidiugwu, with registration number NAU/PG/PhD/2014487015P hereby certify that I am responsible for the work submitted in this Dissertation and that this is an

original work which has not been submitted to this University or any other institution for the award of a degree or a diploma.

…………………… ………………………

Sign Date

APPROVAL

Didiugwu, Chizoba Maryann, a postgraduate student in the Department of Applied Microbiology and Brewing,majoring in Environmental Microbiology,has satisfactorily completed the requirements for course and research work, for the award of the Degree of Doctor of Philosophy (PhD) in Environmental Microbiology. The work embodied in this

dissertation is original and has not been submitted in part or full for any other diploma or degree of this or any other university.

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DEDICATION

This research work is dedicated to the Holy Trinity.

ACKNOWLEDGEMENTS

The Almighty God surely merits my hearty thanks and gratitude, for making it possible for me to start and accomplish this work in peace.

I fully appreciate the work of my supervisor and HOD, Prof. (Dame) E. I. Chukwura for her motherly care and direction during the course of this practical work.I also acknowledge the Dean of Faculty of Biosciences, Prof. S.C. Udedi, the Faculty PG Sub Dean, School of

Postgraduate Studies, Dr. S.C. Onuorah, the academic and non-academic staff of Department of Applied Microbiology and Brewing, for their help and support.

I appreciate Prof. Okpokwasili, Prof. L.O. Eneje, Mrs. Chukwujekwu, N., Mr. Onu and Ojuogu Doris for their help and advice.I also thank in a special way Dr. M.N. Unachukwu,Mrs. Udeh Chika, AkparantaIjeoma Maureen, Mrs. Ani-chuks Juliet, Mrs. Maria Ukwueze, Mr. Chosen Obihand Mrs. ChristabelEnehand other staff of Godfrey Okoye University where I carried out the research work on the role they played during the course of carrying out this research work.

I also thank the management and staff of Coal City University for granting me the opportunity to carry out this research work, especially the Vice Chancellor Prof. Okwueze, the Dean of Faculty of Natural and Applied Sciences, Prof. J.I. Umeh and the HOD of Microbiology Department, Rev.Sr. Dr. H. Uzoeto.

I appreciate the effort of my Lovely husband Mr. C. S. Ezema who gave me the emotional, moral and financial support to complete this research work. I thank my parents Mr. and Mrs. J.A. Didiugwu and my siblings with whom I started this journey of academic work; I fully appreciate your emotional, moral and financial support. I specially appreciate the effort of Mr. N. M. Ikegbunam for his moral and emotional support.

In addition, I am highly indebted to my friendsMrs. Ngwu Victoria, Mrs. Anaukwu Chika and Mrs. Chinenyeespecially those I met in the laboratory and all that contributed to the success of this work. I am also grateful to all project group members. I appreciate and value all your efforts.

ABSTRACT

Crude oil has remained one of the major pollutants in Nigeria. The use of biosurfactant producing bacteria Isolatesto remove this pollutant was assessed. The physicochemical properties and microbiological analysis of the polluted soil collected from Ibeno polluted site and unpolluted soils collected from Ibeno and Otuocha agricultural soils were carried out. Bacteria were isolated from the polluted soil while the unpolluted agricultural soils were artificially polluted with crude oil. The isolated organisms were characterized while bacterial metagenomics analysis and fungi characterization was done on the artificially polluted soil. The isolated bacterial were screened for their ability to degrade crude oil and produce

biosurfactant. Parameters like pH and optical density were used in determining the growth profile. Residual crude oil was analysed using gravimetric and gas chromatography method. Bioremediation exercise was then done on the artificially polluted agricultural soil. pH and total viable count was used in determining the progress of remediation in the soil. The success of the remediation was determined using gas chromatography profile, the soils physicochemical properties were also carried out. Bean seed was also planted to determine the ability of the remediated soil to support seed germination.The physicochemical properties of the polluted soil used for microbial isolation was 71.5 for electrical conductivity, 0.1987ppt for salinity, 24.4803cmol/kg for cation exchange capacity, 2.016% for nitrogen content, 2.55% for total organic carbon and 5.98% for phosphorus. The soil contained more of sand than clay and silt and other heavy metals were also determined.The physicochemical properties of the soil before crude oil pollution showed that Ibeno soil contained more sand and silt while Otuocha contained more clay. Some other physicochemical properties of Ibeno and Otuocha soil samples were pH 5.9 and 6.1, mercury 0.450 and 0.00, arsenic 0.184 and 0.083, nitrogen 0.336 and 0.672, carbon 0.0510 and 0.1275, phosphorus 7.82 and 8.79 respectively. The physicochemical properties of the soil after crude oil pollution for Ibeno and Otuocha soil samples were pH 6.19 and 5.97, mercury 1.039 and 0.216, arsenic 2.281 and 1.518, nitrogen 0.112 and 0.336, carbon 1.0772 and 0.3294, phosphorus 32.59 and 38.52 respectively.The microbial enumeration showed a decreased after crude oilpollution. The isolated organisms were identified as*Tsukamurellainochensis* and *Gordoniaalkanivorans*. The metagenomic analysis of the bacteria in the soil showed that some of the genera present in the soil after pollution were *Massilia, Phenylobacterium, Gordonia, Roseomonas, Microbacterium, Bacillus, Pseudomonas, Planococcus, Parviterribacter, Cellulomonas, Mycobacterium, Nocardioides, Tumebacillus*etc. The fungi isolates characterized were *AspergillusLentulus* and *Cylindrocarpon.*The mean percentage degradation of 100ul, 500ul 1000ul and 2000ul concentrations of crude oil by the isolate using gravimetric methods were 33.33%, 26.92%, 21.28% and 15.73% for *Gordoniaalkanivorans* and 50.00%, 42.31%, 25.53% and 17.98% for Tsukamurellainochensis. The pH of 28 days' degradation tends towards acidity for the medium containing the test isolates but no detectable change was noticed for that of control. The optical density increased gradually but started decreasing from the 21st day.The polycyclic aromatic hydrocarbon (PAH) content after 28 days of biodegradation were5510.4443 for the control, 869.8653 for*Gordonia*, 476.5867 for *Tsukamurella* and252.4649 for mixed culture while the total petroleum hydrocarbon (TPH) content were 10541.4180 for control, 1463.7610 for *Gordonia*, 619.0704 for *Tsukamurella*and269.8244 for mixed culture. The mean pH value of remediation exercise was between 5.61-6.87 before tending towards alkaline at the end of the remediation exercise. The total viable count increased gradually from the 4th week before decreasing on the 20th week. The PAH of Ibeno soil before remediation was 1191.1993 but treatment with *Gordonia* reduced it to 8.53390, 3.22127 by*Tsukamurella*, 1.04768 bytheir mixed cultureand the control was 11.00143. Otuocha soil PAH before remediation was 1879.1443but treatment with *Gordonia* reduced it to 516.9339, 539.6178 by *Tsukamurella*,340.6747 by their mixed culture and control value was 550.2319. TPH ofIbeno soil before remediation was 1975.2632 but treatment with *Gordonia*reduced it to 563.41279, 510.29552 by*Tsukamurella*,585.09108by

their mixed culture while control value was 778.76791. Otuocha soil before remediation was 3244.9021 but treatment with *Gordonia* reduced it to 559.55518, 526.17757 by*Tsukamurella*, 985.96555by their mixed culture while control value was 1326.70729. A significant difference ($p<0.05$) was observed for both TPH and PAH of the tested isolates and their control.The physicochemical parametresanalysedafter remediation showed that no crude oil was detected in the soil containing the tested isolates but was detected in the control; the water permeability of the control was negative while positive in the test samples. The nitrogen content, total organic carbon and phosphorus all decreased after remediation. The bean seed planted after remediation showed that the test samples and their positive control have a better plant growth than thenegative control. This research work showed that biosurfactantproducing bacterial isolates like *Tsukamurellainochensis* and *Gordoniaalkanivorans* can actually be used in cleaning up crude oil pollution from the environment.

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CHAPTER ONE

INTRODUCTION

1.1 Crude Oil Contaminated Site

Crude oil also known as petroleum is a naturally-occurring, unrefined petroleum product composed of hydrocarbon deposits and other organic materials. Petroleum hydrocarbons are major environmental pollutants generated by wide-scale production, transport, coastal oil refining, shipping activities, offshore oil production and accidental spilling (Arulazhagan *et al*., 2010). Human activities such as municipal run-offsandliquidrelease from industries,causecrude oilpollutionwhich impacts the environment and poses a direct or indirect health hazard to forms of life (Sajna *et al*., 2015). Petroleum hydrocarbon leakage due to frequent accidental and illegal disposal of oil waste at sea severely harms various ecosystems.

Crude oil drilling in Nigeria especially in the Niger Delta area of Nigeria has been the country's main source of income and foreign exchange. This Niger Delta area are then the main recipients of crude oil spillage, sometimes resulting in large-scale contamination of these environments (Chijioke-Osuji *et al*., 2014). Petroleum hydrocarbons are toxic compounds classified as priority pollutants (Costa *et al*., 2012). Crude oil is physically, chemically and biologically harmful to soil because it contains many toxic compounds in relatively high concentrations (e.g., polycyclic aromatic hydrocarbons, benzene and its substituted, cycloalkane rings).

Aliphatic and aromatic hydrocarbons are two major petroleum hydrocarbon components that have been reported because theyare recalcitrant and harmful to health. Aliphatic hydrocarbons are easily degraded by microorganisms, but large branched aliphatic chains

are not easily degraded; therefore, they persist in the environment. Likewise, aromatichydrocarbons are difficult to degrade because of their complex structures. In vitroandinvivoexperimentshaveshownthatpolycyclicaromatic hydrocarbons (PAHs) are carcinogenic, cytotoxic, genotoxic and environmentally toxic.

On land, crude oil spills have caused great negative impact on food productivity. Crude oil affects germination and growth of some plants. It also affects soil fertility but the scale of impact depends on the quantity and type of oil spilled. Severe crude oil spills in Cross-River State, Nigeria, have forced some farmers to migrate out of their traditional home, especially those that depend solely on agriculture. Soil fertility could be loss through loss of soil organic matter, leaching of nutrients, loss of the nutrients-laden top soil, changes in soil pH, reduction in cation exchange capacity, salinization, water logging and other forms of soil degradation. These are major problems associated with agricultural productivity in the oil producing and neighbouring communities in Nigeria (Okoye and Okunrobo, 2014).

Site highly contaminated with oil cannot be conducive for plant growth and those that are not highly contaminated could give room for growth but on the long run there is possibility of stagnant growth, once the soil composition and nitrifying bacteria have completely been altered and compounds have been absorbed by plants (Okoye and Okunrobo, 2014).A biological treatment is an alternative pollutant removal method because this technique does not elicit deleterious effects on the environment. This treatment may also be less expensive than other techniques. In soil microbial universe, certain microbes have a distinctive ability to degrade or to convert organic pollutants to harmless biological products. The fact of bioremediation mainly relies on the use of these talented microorganisms surviving in soil (Esin and Ayten, 2011). The ability to utilize hydrocarbons is widely distributed among diverse microbial populations. As no single microbial species is capable of

degrading all components of crude oil, complete oil degradation requires simultaneous action of different microbial populations (Esin and Ayten, 2011).

The success of bioremediation depends integrally on pollutant biodegradation, pollutant-degrading organisms, accessibility and biological activity optimization. Biodegradation by indigenous microorganisms is a major mechanism and a reliable method that operates by biologically removing foreign contaminants, such as crude oil. Bacteria and fungi can utilize petroleum hydrocarbons. Fungi such as *Aspergillus, Penicillium, Fusarium, Amorphotheca, Neosartorya, Paecilomyces, Talaromyces, Graphium Cunninghamella* while bacteria such as *Pseudomonas, Gordonia, Tsukamurella, Mycobacterium and Sphingomonas* are microorganisms which can degrade persistent pollutants.

1.2 Surfactants

The presence of high molecular weight compounds with very low solubility in water prevents natural biodegradation process from working efficiently in hydrocarbon contaminated soils. Generally, petroleum hydrocarbon compounds bind to soil components and are difficult to remove or degrade. Surfactants are used for bioremediation of the hydrocarbons and make the hydrocarbons available for the microorganisms to degrade. Hence the transfer of the hydrocarbons to the aqueous phase in bulk is the important process for its bioavailability (Adrion, *et al*., 2016). Among various methods, surfactants can be seen as the promising method for bioavailability related problems. The use of surfactants could increase the hydrocarbons mobility as well as the bioavailability which promotes the rate of biodegradation (Haftka*et al*., 2015). The diverse group of surfactants are divided on structural basis depending on the type of microorganisms that produced them (Cheng*et al*., 2016).

1.3 Biosurfactant

Biosurfactants produced mostly by microorganisms are the biological active surface molecules with vast applications in the field of industries, as they possess many of the versatile properties of specificity, minute toxicity and biological acceptability (Shivlata and Satyanarayana, 2015). They are used as an additive for the production of organic chemicals, petro-derivatives and petrochemicals. They possess several advantages over chemical surfactants. Bioremediation of waste water effluents can be done effectively by using biosurfactant producing microorganisms due to their specificity of utilizing the organic waste and hydrocarbon waste as raw materials. Biosurfactants bear surface activity, high tolerance to various environmental factors, withstand extreme conditions, such as acidity or basicity of an aqueous solution, temperature, salt concentration, ionic strength, biodegradable nature, demulsifying-emulsifying ability, anti-inflammatory potential and anti-microbial activity (Peele, 2017).

Biosurfactants consists of two different parts as they are amphiphilic compounds which possess hydrophilic polar moiety as well as a non polar group which is hydrophobic. The hydrophilic group has oligo or monosaccharide and proteins as well as polysaccharides or peptides and the hydrophobic moiety has unsaturated, saturated fatty alcohols or hydroxylated fatty acids (Rodrigues, 2015). One of the key features of biosurfactants is the hydrophilic- lipophilic balance which causes the hydrophobic as well as hydrophilic portions to be determined in substances that are surface active. Because of the amphiphilic structure, biosurfactants not only have the ability to increase the hydrophobic substance surface area but also have the ability to change the property of cell surface of the microorganisms along with the ability in increasing the bioavailability of substances. Because of the surface activity, surfactants behave as excellent foaming agents, emulsifiers and dispersing agents.

Naturally occurring surfactants are better and have many advantages when compared to their chemical versions. The naturally-derived surfactants are eco-friendly, low toxicity and biodegradable (De*et al*., 2015). They show good foaming capacity and selectivity of the substrate to degrade and are functionally active at extreme conditions of high temperatures, high salt concentrations, as well as pH which is caused by the by products and generated waste from industries. Due to the long lasting characteristics, the biosurfactants are cheap and reduces the cost and time of effect of biodegradation of the polluted soils and water bodies (Nercessian, *etal*., 2015). Due to their enormous advantages, they are widely used in many of the food production, pharmaceutical, agricultural and cosmetic industries. Different properties of surfactants are dispersion, emulsification or de-emulsification, wetting, foaming as well as coating and as a result, they are useful in bioremediation and physiochemical technologies of metal and organic contaminants (Wu & Lu, 2015). Biosurfactants show increase in the bioavailability of hydrocarbons which results in degradation of contaminants by the hydrocarbon degrading bacteria and enhanced growth of the bacteria in the polluted soil. In the soils with heavy-metal pollution, the biosurfactants form different complexes with metals and perform surface removal of heavy metals that cause increase of ion concentration of metals and the bioavailability (Sarma and Prasad, 2015). The pollutants which are hydrophobic that are present inside hydrocarbons, water and soil have the necessity to be solubilized before they will be degraded by the microorganisms.

Surfactants have the property of increasing hydrophobic particle surface area like pesticides applied in the soil and water, which in turn increases the solubility (Neitsch*et al*., 2016).
Increase in the microbial production of surfactants and the wide use of biosurfactants for the degradation of harmful compounds like chemicals that kill pest and insecticides in different kind of environment like soil, water has gained attention in the past few years (Shah *et al*.,

2016). The biosurfactants which are produced by various microorganisms are identified and characterized (Peele, 2017). Hence there are various types of biosurfactants based on the properties such as characterization, antimicrobial activity, production, and efficiency of hydrocarbon removal from environment and its ability of reducing the surface tension (Tabatabaei, 2015).

A wide range of compounds that are organic were used by microorganisms as the energy rich source and as the carbon source for their growth. But if carbon is an insoluble hydrocarbon, then microorganisms like yeast and bacteria will diffuse biosurfactants that can emulsify the insoluble hydrocarbons available in the medium (Leuchtle*et al*., 2015). Some examples of this type are different species of *Pseudomonas* producing rhamnolipids and sophorolipids which are produced by different species of *Torulopsis*. Most of the microorganisms could change the cell wall structure which was caused by the production of lipopolysaccharides in the cell wall (Saenz-Marta*et al*., 2015). *Candida lipolytica* produces lipopolysaccharides which are cell wall-bound when the medium contains n-alkanes. *Rhodococcus erythropolis* along with different *Mycobacterium* species and *Arthrobacter* species produce non-ionic trehalose corynomycolates. *Acinetobacter* species produce emulsan as well as lipoproteins like subtilisin which are produced by *Bacillus subtilis*. *Rhodococcus* sp. synthesises Mycolates, Corynomycolates synthesized by *Pseudomonas rubescens, Thiobacillus ferroxidans* and *Gluconobacter cerinus* synthesize ornithinlipids (Peele, 2017).

The diverse groups of surfactants are divided on structural basis depending on the type of microorganisms that produce those (Cheng *et al*., 2016). Biosurfactants produced by microorganisms are the biological active surface molecules with vast applications due to their specific versatile properties, minute toxicity and biological acceptability (Shivlata and

Satyanarayana, 2015). Microorganisms oxidize the organic hydrocarbon compounds by dissolving or emulsifying them while the major limiting factor of the biodegradation of the oil is its solubility rate. Biosurfactants increase the rate of biodegradation of the organic compounds by increasing their solubility by emulsification. Most of the crude oil degrading bacteria release extracellular biosurfactants to facilitate microbial oil uptake and facilitate degradation by emulsifying the hydrocarbon. Biosurfactants can increase the pseudo solubility due to their specificity and degradability.

1.4 Statement of the Problem

Crude oil contamination in the Niger Delta region of Nigeria is gaining more prominence as a result of increased upstream and downstream activities of the petroleum industry. The spillage caused by tankers as they convey petroleum products to other parts of Nigeria especially on farmland has brought increased deleterious effect on the ecology such as reduced crop yield, hence the need to use biological method in cleaning up this crude oil spills so as to restore the region and farmland back to a good shape for farming.

1.5 Significance of the Study

The problem of crude oil pollution as an oil producing country has drawn serious concern for a way out of it. Several methods have been employed to remove crude oil pollution from the soil and some of them can be detrimental to the environment. There are ongoing researches on the use of microorganisms and a non-chemical compound known as biosurfactant to remove this crude oil from the soil. This biosurfactant produced by microoragnisms are environmentally friendly and cheaper unlike the chemical surfactant, hence, were utilized in this research.

1.6 Aim

The aim of this study is to remediatecrude oil polluted agricultural soil using biosurfactant producing bacterial isolates.

Objectives of the Study

The specific objectives are to:

1. determine the physicochemical and microbiological properties of both the crude oil polluted and unpolluted soil samples.
2. characterize and identify the microbial isolates using morphological, biochemical and molecular tests.
3. screen the microbial isolates for crude oil degrading potentials.
4. study the growth profile of the crude oil degraders for 28 days in mineral salt medium.
5. screen the microbial isolates for ability to produce biosurfactants.
6. determine the gas chromatographic profile of crude oil and residual oil.
7. carry out hydrocarbon degradation study using the biosurfactant-producing bacterial isolates.
8. determine the potential of the remediated soil to support plant growth.

CHAPTER TWO

LITERATURE REVIEW

Petroleum is a naturally occurring, yellow-to-black liquid found in geological formations beneath the Earth's surface, which is commonly refined into various types of fuels. Components of petroleum are separated using a technique called fractional distillation. The name petroleum covers both naturally occurring unprocessed crude oil and petroleum products that are made up of refined crude oil. It consists of hydrocarbons of various molecular weights and other organic compounds.Crude oil is mainly composed of hundreds of different hydrocarbon molecules, which are mainly alkanes from C1 to C40 straight chain, C6–C8 branched-chain, cyclohexanes, aromatics and compounds containing sulphur, nitrogen and oxygen (Romanus *et al*., 2015).

A fossil fuel, petroleum is formed when large quantities of dead organisms, usually zooplankton and algae, are buried underneath sedimentary rock and subjected to both intense heat and pressure. Petroleum has mostly been recovered by oil drilling (natural petroleum springs are rare). Drilling is carried out after studies of structural geology (at the reservoir scale), sedimentary basin analysis, and reservoir characterization (mainly in terms of the porosity and permeability of geologic reservoir structures) have been completed (Guerriero *et al.*, 2013). The use of fossil fuels, such as petroleum will have a negative impact on Earth's biosphere, damaging ecosystems through events such as oil spills and releasing a range of pollutants into the air including ground-level ozone and sulfur dioxide from sulfur impurities in fossil fuels. The burning of fossil fuels plays the major role in the current episode of global warming.

2.1 Composition of Petroleum (Crude Oil)

Indeed, crude oil reservoirs have been found in vastly different parts of the world and their chemical composition varies greatly. Consequently, no single composition of crude oil can be defined. Thus crude oil-derived inputs to the environment vary considerably in composition and the complexity of crude oil composition is matched by the range of properties of the components and the physical, chemical, and biochemical processes that contribute to the distributive pathways and determine the fate of the inputs. Put simply, crude oil is a naturally occurring mixture of hydrocarbons, generally in a liquid state, which may also include compounds of sulfur, nitrogen, oxygen, metals and other elements (Speight, 2014). In terms of the elemental composition of crude oil, the carbon content is relatively constant, while the hydrogen and heteroatom contents are responsible for the major differences between crude oil from different sources. The nitrogen, oxygen, and sulfur can be present in only trace amounts in some crude oil, which as a result consists primarily of hydrocarbons. On the other hand, a crude oil containing 9.5% (w/w) heteroatoms may contain essentially no true hydrocarbon constituents insofar as the constituents contain at least one or more nitrogen, oxygen, and/or sulfur atoms within the molecular structures (Speight, 2017).

In terms of the composition of crude oil, it contains compounds that are composed of carbon and hydrogen only which do not contain any heteroatoms (nitrogen, oxygen, and sulfur as well as compounds containing metallic constituents, particularly vanadium, nickel, iron and copper). The hydrocarbons found in crude oil are classified into the following types: (1) paraffin derivatives, which are saturated hydrocarbons with straight or branched chains, but without any ring structure; (2) cycloparaffin derivatives also called naphthene derivatives but more correctly known as alicyclic hydrocarbons, which are saturated hydrocarbons containing one or more rings, each of which may have one or more paraffinic side-chains;

and (3) aromatic derivatives, which are hydrocarbons containing one or more aromatic nuclei such as the benzene ring system, the naphthalene ring system, and the phenanthrene ring system that may be linked up with (substituted) naphthalene rings and/or paraffinic side-chains (Speight, 2017).

2.2 Composition and Properties of Crude Oil in Nigeria

There are four main types of crude oil based on densities and toxicity levels as determined by their volatilities (Karras, 2010).

Light Distillates: These crude oils possess very high volatility and thus are capable of evaporating within a very short time (window of a few days). They diffuse at a very fast rate, thus decreasing toxicity levels. They include petroleum naphtha and ether, heavy and light virgin naphtha, kerosene, gasoline, and jet fuel.

Middle Distillates: These crude oils exude moderate volatility and are thus less evaporative and toxic. They are from a petroleum industry perspective referred to as grade 1 and grade 2 fuel and diesel fuel oils. Other examples include light crude marine gas oils and virtually all domestic fuels.

Medium Oils: These fall into the category of crude oils sold on local market floors nowadays. They are low-volatile oils that require very stringent cleanups, thus resulting in increased level of toxicity.

Heavy Fuel Oils: In terms of volatility and toxicity, heavy fuel oils are worse than medium oils. Examples include intermediate and heavy marine oils, grade 3, 4, 5, and 6 fuel oil (a strong equivalence of Bunker B and C) (Karras, 2010; Santos*et al.*, 2014).

Oils can also be categorized by virtue of sources and quality. One of them, called the OPEC Basket oil, is a combination of crude oils variants from seven countries (Nigeria, Venezuela, the Mexican Isthmus, Saudi Arabia, Algeria, Indonesia, and Dubai). The OPEC is a global organization created in 1960 to pass legal-binding policies that control and implement the importation and exportation of oil within its jurisdiction. Other oil types in this category that are somewhat foreign to Nigeria are the West Texas Intermediate and Brent blend (Bina and Vo, 2007).

2.2.1 Physical and Chemical Properties of Crude Oil in Nigeria

The physico-chemical properties of Nigeria's crude oil samples vary from one oil field to another. This is attributed to the fluctuating quantity of hydrocarbons (alkanes, alkenes, alkynes, cyclo compounds, and aromatics) and their derivatized forms (the presence of heteroatoms such as nitrogen, sulfur, and oxygen (Onyenekenwa, 2011) as well as organic compounds with carboxylic (-COOH) and alcohol (-OH) functionalities) (Onyema & Manilla, 2010). They also contain varying composition of heavy metals that have been confirmed as major pollutants in oil-producing regions (Isah *et al*., 2006; Madu*et al*., 2011). The variation in properties inevitably leads to carrying out a thorough analysis of the physico-chemical properties of crude oil variants. The following crude oil parameters are vital in the classification and specification of crude oil blends: pour point and kinematic viscosity as functions of temperature, density, metal contents, API gravity, water and salt contents (%), nitrogen and sulfur contents (%), and asphaltene (%) (Dickson and Udoessien, 2012; Riazi, 2005; Wilberforce, 2016).

2.2.1.1 Specific Gravity, Api Gravity and Sulfur Content

The classification of crude oil as heavy or light is determined by a standard scale called the American Petroleum Institute (API) gravity. It uses the index that is based on the relative density of oil as one of the criteria for oil classification. Depending on the nature of the oil, API gravity greater than 10 will float on water (immiscible liquids with oil being the upper organic layer and water the lower aqueous layer) whereas oil with API gravity less than 10 will form the lower aqueous layer. On the other hand, there have been reports about API gravity being used to classify crude oil as light (>31), medium (22-31) and heavy (<20). Specific gravity of crude oil is simply described as the ratio of the density or mass of a specific crude oil blend to the density or mass of a reference substance, which in most cases is water. It is also the ratio of the weight of a volume of the crude oil blend to the weight of an equal volume of water. Literature reports have affirmed that most of the crude oil blends obtained from Nigeria are light crude oils. Light crude oil samples are in high demand and are of higher market value in Nigeria than their heavy counterparts. On the contrary, heavy crude oils are characterized by low H/C ratios and very high levels of specific gravity, viscosity, asphaltene, sulfur, nitrogen, and heavy metals. This corroborates reports that API gravity of crude oil often increases as the specific gravity decreases (API, 2011; American Society for Testing and Materials (ASTM), 2011).

Sulfur content (expressed in percentage) in crude oil determines its crude sweetness or sourness. Sweet crude oil has sulfur content less than 0.5% whereas those with more than 0.5% are considered sour. However, Nigeria’s crude oil is sweet and one advantage this offers is the drastic reduction in its corrosion/pollution potentials, which leads to a reasonable cost of production. Moreover, this makes it more suited for the production of most valuable

refined products. Reports have also shown that API gravity has an inverse relationship with sulfur contents of crude oil blends (Al-Salem, 2015; Dickson and Udoessien, 2012).

2.2.1.2Pour Point, Viscosity, Water, Salt, Nitrogen, and Asphaltene Contents

Viscosity of crude oil is a measure of its ability to flow from one point to another. The majority of crude oil samples in Nigeria are light and have relatively low viscosity. This indicates that they are easily transported through pipes that connect oil wells to refineries. However, the implication of this property is that they have the intrinsic ability to flow rapidly during spillage, resulting in massive environmental pollution, which is often high temperaturedependent (Odilinye, 2012).

An absolute grip of nitrogen and water contents of any Nigerian crude oil sample is critical in the refining, procurement, and sales of crude oil. These parameters are also connected to the level of corrosion encountered in Nigeria's refineries. Nigeria's crude oils have appreciably low water and nitrogen contents, which expose refineries in Nigeria to mitigated risks associated with corrosion. Pour point is a measure of the low-temperature flow (viscosity) of crude oil blends (Salam*et al*., 2013). Pour points of heavy, viscous crude oil blends are above 5°C whereas those for light, less viscous crude oil blends can be as low as -15°C. These pour point values are indicative of crude oil blends' facile utilization in low-temperate operations (Stratiev*et al*., 2010). Asphaltene is one of the high molecular weight, high boiling point and C/H ratio involatile refinery products that are converted into a number of useful secondary products. It has been reported that its high concentration in crude oil blends results in heavy oil blends with high viscosity and pour points.

Salt content is an important index for refining operations. This is because salt content of crude oil blends is mainly sodium chloride dissolved in the aqueous phase of the oil or as a

suspension in the oil phase. Thus it is ideal to desalt crude oil blends before distillation to prevent salt particles from adsorbing on heat transfer surfaces. The adsorbed particles are capable of reducing the thermal efficiencies of the distillation procedure via buildup of deposits that will block refinery equipment. Therefore, high values of any of these parameters indicate high corrosion tendency of crude oil. However, Nigeria's crude oil samples have a very low content of salt, which make them favorable targets for both local and international marketers (Cani *et al.,* 2016).

2.2.2Heavy Metals

Levels of most of the trace metals found in crude oil in Nigeria are generally low except for nickel, iron, and vanadium. These validate reports that light crude oil samples in Nigeria usually contain relatively low trace metal contents compared to the heavy counterparts. The inference drawn from these reports is that crude oil samples from Nigeria, especially those with high concentrations of nickel and vanadium, exude very high tendencies to contaminate the environment. This contamination is on the threshold with nickel and vanadium such that a high vanadium-nickel heavy metal concentration ratio in soil and water bodies is strongly suggestive of the presence of crude oil contamination (Wilberforce, 2016). The crude oil blends in Nigeria can be categorized as light-sweet crude oil blends. They flow and spread out rapidly as well as possess low levels of water, salt, pour point, and trace metals. These topographical and developmental properties of Nigeria's crude oil blends account for their preference in indigenous and international oil markets as well as in refinery-based operations (Shotonwa*et al*., 2018).

2.3 Types of Crude Oil in Nigeria

It is interesting to note that Nigeria's crude oil, which is classified as light and sweet, has improved the economy tremendously. This is attributed to its paraffinic and low sulfur content, all of which are embraced by consumer refineries in the United States and Europe (Dickson and Udoessien, 2012).

The major classes of crude oil are so named in accordance with their export terminals. They are Bonny light (whose terminal is located in the city of Bonny in Rivers State, South-south Nigeria), Qua Iboe (Qua Iboe terminal is situated on the eastern side of the Qua Iboe River Estuary), Brass Blend (it is produced from a refinery located on the Brass River, which is a part of River Niger in the Niger Delta region of Nigeria), Escravos (which is located close to the Escravos site in Warri South Local Government Area of Delta State), and Forcados (whose terminal is located in a small town in the Burutu Local Government Area of Delta State) (Dickson and Udoessien, 2012).

The less important or minor crude oil types in Nigeria include Antan Blend, Bonny medium, Odudu Blend, Pennington light, Ukpokiti, Bonga, Yoho Blend, Agbami, Abo, Oyo, Okono Blend, Amenam Blend, Atam Blend, Okwori, Okoro, Ima, Obe, Okwuibome, Ebok, and Asaratoru. Bonny light has the highest demand of all the classes, and this is not unconnected to its highly desired grade, which is a function of its low sulfur content, low corrosive impact on infrastructural designs for refineries and the vehemently low impact of its refinery byproducts on the environment (Odularu and Okonkwo, 2009; Wilberforce, 2016). Therefore, it has received accolades as a major source of income for Nigeria as a country (Badmus *et al.,* 2012; Moscow 21st World Petroleum Congress, 2014).

2.4 Oil Spillage and Its Causes

Oil spills are discharges of oil (crude or refined) into the environment which normally occurs as a result of accident caused by the malfunctioning of equipment or through human error. According to U.S Environmental Protection agency (2004), oil spill can be defined as discreteevent in which oil is discharged through neglect, by accident or with intent over a relatively short time. It does not include operational spillages allowed or permitted by international or national regulations (such as MARPOL discharges from tankers) or that, which occur over a relatively long period of time (such as above >5 ppm oil discharge in refinery effluents) even if those discharges violate pollution regulations. According to Ifunanya (2010), oil spillages are forms of industrial pollution caused by the unwanted release of crude oil associated with exploration and transportation of petroleum. Considering oil spillage as oil pollution, the United Nations Convention defined pollution as the introduction by man, directly or indirectly of substances or energy into the environment which results or is likely to result in such deleterious effects as harm to living resources and marine life, hazards to human health, hindrance to marine activities and other impairment of quality for the use of sea water and reduction of amenities (Islam and Tanaka, 2004).

Since the discovery of oil in the 1950s in the Niger Delta region of Nigeria, there have beenvarying adverse environmental implications brought about by oil production activities in the region. The rapid development and production of its newly discovered resources in terms of crude oil coupled with an explosive increase in population have resulted in environmental degradation in oil producing states in Nigeria, particularly in the Niger Delta which comprises nine states and being the region with the most oil reserves (Badejo and Nwilo, 2004).

Earlier surveys in Nigeria have shown an increasing number of recorded oil spills leading to the damage of environment. The rate of spills has been rising with the increasing operations of petroleum production. In the Niger Delta region, due to the rise in energy consumption around the world, oil exploration in the region has seen a rise and in turn an increase in the number of oil spills. In 1970, only one spill of 150bbl was reported in the country, whereas a year later the number shot up to 15 incidents involving 15, 110bbl. In1974, there were 105 spills, another 154 in 1978, 241 in 1980 and 216 in 1982.

According to the Department of Petroleum Resources, from the period of 1976 to 1996 around 2.4million barrels of oil have been spilled in Nigeria in 4,835episodes. The period 1976-1996 witnessed a great number of oil spillage which (Nwilo and Badejo,2005) have suggested to be in the figure of 4647 and thus lead to an estimate of 2,369,470 barrels of oil liberated and polluting the resulting environment. A greater part of these oil spill episodes according to Twamasi and Merem(2006) and Uyigue and Agho (2007) transpired on land within the Niger Delta region and the prevailing offshore environment. In order words, the highest quantity of spilled oil was recorded between the year 1978 to1980 and of these spillages three were of major magnitude firstly in 1978, There was the GUCON'S Escravos spill resulting in a loss of approximately 300,000 barrels, the second in the same year which was of greater magnitude was as a result of a terminal tank failure at SPDC Forcados with a loss of about 580,000 barrels and lastly in 1980, a blow out from one of Texaco's unit specifically Texaco Funiwa – 5 resulted in an oil spillage of approximately 400,000 barrels of oil (Badejo and Nwilo, 2004).

Oil spills ravages the livelihood of many inhabitants in the oil producing areas in general and Niger Delta in particular. Most spillages occur as a result of corrosion in the pipelines used for oil production. Spillages could sometimes be quite devastating on people and

environment. Egbe and Thompson (2010) grouped the various causes of oil spills under eight headings as follow:

Blow Outs: Oil well blow out occurs when the well is not kept under control that the hydrostatic mud head counter balances the formation pressure and prevents the formation fluid from entering the well formation during drilling operations.

Sabotage: When the cause of spill is mischievously deliberate and not accidental.

Corrosion: When the cause of leakage is rusty equipment.

Equipment Malfunction: Breakdown and failure of equipment are often the most frequent causes of separator and tank over-flow.

Operations / Maintenance Error: Bad oil operation practices like untrained personnel and lack of maintenance of the equipment

Natural causes: Oil spillage: Oil spill could occur as a result of natural causes; theyare causes which are not manmade or induced thus, occurring without any fault of man (Examples motion of tectonic plates, rain, flood, etc.)

Accident from third party

Unknown Causes.

2.5 Oil spill incidents in Niger Delta

Different parts of the world have experienced oil spill incidents due to varying circumstances on different occasions and the Niger Delta region of Nigeria is no exception. The Department of Petroleum Resources (DPR) suggested that a total of 4647 oil spill incidents occurred between 1976 and 1996 in the Niger Delta region of Nigeria and these incidents contributed to oil spill in the amount of 2,369,470 barrels in which only about 23% was recovered.

Prominent oil spill incidents of note in the Niger Delta region include the GOCON"s Escravos spill in 1978 spilling out approximately 300,000 barrels of oil, the 580,000 barrels of oil spilled in 1978 as well from SPDC"s Forcados Terminal tank failure (Okoroji*et al.*, 2013), and the 1980 blowout from Texaco Funiwa-5 with an estimated 400,000 barrels spilled (Tolulope, 2004, Ukoli, 2005). A few other oil spill incidents of far less impact include the 1982 episode resulting in 18,818 barrels of oil spilled from the Abudu pipeline, the January 1998 Idoho oil Spill with a loss of approximately 40,000 barrels and the fire episode which lead to the death of over a thousand lives in Jesse. Unarguably, the year between1979 and 1980 recorded the most excessive oil spill having lost about 694,117.13 barrels and 600,511.02 barrels of oil apiece to the environment (Badejoand Nwilo, 2004).

According to International(2008) oil spill incidents in the Niger Delta region of Nigeriahave become a matter of regular occurrence with an appraisal from the United Kingdo m
WorldWildfire Federation in 2006 citing that the Niger Delta has experienced oil spills in the regionfor decades and within this period the quantity of oil spilled has been suggested to be roughly 50 times the quantity (10.7million gallons) spilled by Exxon Valdez in Alaska in1989. A huge amount, specifically 2405 oil spill episodes were recorded by the federal government between the year 2000 and 2006, bringing the average annual oil spill incidents to 600 per year. Another 2,405 spill was recorded by the National Oil Spill Detection Agency (NOSDRA) between 2006 and mid-2010, with an expanding pattern year-on-year: 252 in 2006, 598 in 2007, 927 in 2008 and 628 in 2009. According to Egwu (2012), it is only through government fulfilling its responsibilities of protecting life and creating gainful means of livelihood as being effected in other major oil producing countries that the amount of oil spill in the Niger-Delta will diminish.

2.6 Effect of Crude Oil Spill on Soil

Crude oil, a mixture of many thousands of organic compounds, can vary in composition from one source to another. This suggests that the effects of crude oil spill will vary from source to source. However, details of the potential biological damage will depend on the ecosystem where the spill occurred. Oil contamination can affect soil physical and chemical properties. The presence of oil in the soil lower soil fertility by reducing available Phosphorus and increasing soil pH, which potentially accelerated damage attributable to alkalescency in the wetlands in the semi-arid region (Wang *et al*., 2013). The daily maximum surface temperature of hydrocarbon-contaminated soils is often higher than that of adjacent control sites (Aislabie *et al*., 2004). Oil usually creates anaerobic environment in soil by smothering soil particles and blocking air diffusion in the soil pores and affects soil microbial communities (Townsend *et al*., 2003; Labud *et al*., 2007; Sutton *et al.,* 2013). Heavy crude oil pollution can cause complete mortality of marsh vegetation (Lin and Mendelssohn, 2012). In addition, crude oil-contaminated soils are hydrophobic compared with pristine sites (Quyum *et al.,* 2002; Aislabie *et al.,* 2004). Hydrocarbon contamination can also increase soil total organic carbon (Ekundayo and Obuekwe, 2000), and change soil pH values (Hu *et al*., 2006; Wang *et al.,* 2009; 2010) and other soil chemical properties (Arocena and Rutherford, 2005; Kisic *et al*., 2009).

2.6.1Effect of crude oil pollution on pH of soil

Crude oil pollution increased pH values in the marsh soil. The results of previous studies on oilfields in China showed that oil pollution raised soil pH (Jia *et al.,* 2009; Wang *et al*., 2010). The higher pH values in crude oil-polluted soil might be caused by two factors: first, the hydrophobic nature of crude oil might induce a potential drought in the surface and subsurface layers of polluted soil (Njoku *et al*., 2009), which could aggravate salinization and

thus raise the pH values compared with that in the control site; second, oil pollution in soil has been shown to be associated with the accumulation of exchangeable base (such as Ca2+, Na+) and a reduction in exchangeable acidity and effective cation exchange capacity (ECEC) (Osuji *et al*., 2006; Agbogidi *et al*., 2007). These mechanisms might also underpin the increase of pH values in the crude oil polluted soil. Okoye and Okunrobo(2014) reported that the pH of oil polluted soil is lower than the unpolluted soil; they further stated that the presence of oil may have had some direct impact in lowering the pH; it is also more likely that production of organic acids by microbial metabolism is responsible for the difference.

2.6.2Effect of crude oil pollution on available phosphorus of soil

Crude oil in the oilfield marshes reduces available phosphorus (AP) concentration in the soil. The results of previous studies showed that oil contamination decreased AP concentration by various degrees (Wang *et al*., 2009; 2010; Eneje *et al*., 2012). A field study on the Momoge wetlands showed that the concentration of AP decreased with increasing time of oil exploration and production (Wang *et al.,* 2010). In an experimental oil study, the concentration of AP in the crude oil-contaminated soil decreased as much as 66% compared with the control site when the content of crude oil reached 30 mg/kg (Eneje *et al*., 2012). However, Liu *et al*. (2007) reported that AP concentration was not significantly affected by oil contamination. However, lower AP concentrations in oil polluted site than in the unpolluted site, may be caused by two reasons. First, TPH in the soil could increase the carbon concentration, which might affect the equilibrium of nutrients in the soil. Microbes in soils, which utilize TPH as a carbon source, could utilize considerable amounts of AP when they degrade the hydrocarbons (Wang *et al*., 2009). Phosphorus is one of the most important macro-nutrients for plants and soil microorganisms. The decrease of AP concentrations in

oilfield marshes could change the structure of vegetation and soil microorganisms, and reduce marsh ecosystem services and values.

2.6.3Effect of crude oil pollution on total organic carbon and nitrogen of soil

Crude oil is mainly made up of carbon and hydrogen. Crude oil pollution tends to increase the total organic carbon in the soil because it's one of the components of crude oil. Wang *et al*. (2009; 2010) reported that oil contamination significantly increased the TOC contents in the soil. Uquetan *et al*. (2017) in their study reported that nitrogen and organic carbon increased markedly with an increase in treatment concentration with oil for all the crops. He stated that this can be attributed to slow decomposition by facultative and obligate anaerobes. Okoye and Okunrobo (2014) reported that there is rather a reduction in organic carbon and organic matter contents of the polluted soils than the unpolluted soil. This might be that spilled oil impaired the metabolic processes that would have facilitated the agronomic addition of organic carbon from the petroleum hydrocarbons by reducing the carbon-mineralizing capacity of the microflora.

2.6.4Effect of oil spill on other physicochemical properties of the soil

Exchangeable cations (Ca2+, K+, Mg+, Na+) were susceptible to reduction in the oil polluted soils. Uquetan *et al*.(2017) in their study on the effects of oil pollution on soil properties and growth of tree crops in Cross River State reported that exchangeable K+, Na+ and Mg+ decreased with increased treatment concentrations for all the crops. The plausible reasons for these decreasing trends may be due to the conversion of $H_2PO_4^-$ (most available form of phosphorus) to HPO_4^{2-} (less available form for plants uptake) and then to PO_4^{3-} as the soil pH increases (Asuquo *et al*., 2001; Kayode *et al*., 2009). Electrical conductivity in water (ECW) is a measure of salinity and the extent to which water is able to conduct an electric

current. It is expressed as micro Siemens per centimeter (us/cm). These salts typically include such cations as sodium, calcium, magnesium and potassium, and anions such as chloride, sulphate, and bicarbonate. The electrical conductivity of oil polluted area is usually higher than the unpolluted area (Okoye and Okunrobo, 2014).

2.7 Effect of Crude Oil Pollution on Microorganisms

The toxicity of petroleum hydrocarbons to microorganisms is well established. Crude oil pollution brings about a reduction in the population of microorganisms found in a soil sample. Okoye and Okunrobo (2014) reported the decrease experienced in the polluted area could be linked to the influence of oil spill in the soil. The oil alters the activities of many soil microbes and sometimes lead to it's eventual death due to high acidity/ alkalinity.

2.8 Effect of Crude Oil on Germination and Plant Growth

Crude oil has a negative effect on germination as well as plant growth. It causes a delay on the germination time of seeds. The reduced germination is adduced to the fact that volatile fractions of oil could enter the seed coat and induce unfavourable conditions for seeds germination. Uquetan *et al*. (2017) reported that the effect increased as concentration of treatment increases. Also soils polluted with crude oil and spent lubricating oil show poor wettability, reduced aeration and compaction and increased propensity to heavy metal accumulation. These observation is in tandem with Kayode *et al*. (2009) and Osuji and Nwonye (2007).

Site highly contaminated with oil cannot be conducive for plant growth and those that are not highly contaminated could give room for growth but on the long run there is possibility of stagnant growth, once the soil composition and nitrifying bacteria have completely been altered and compounds have been absorbed by plants. Soil fertility could be lost through loss

of soil organic matter, leaching of nutrients, loss of the nutrients-laden top soil, changes in soil pH, reduction in cation exchange capacity, salinization, water logging and other forms of soil degradation. These are major problems associated with agricultural productivity in the oil producing and neighbouring communities in Nigeria (Okoye and Okunrobo, 2014). Uquetan *et al.*(2017) in their research maintained that seedling growth rate is a function of the treatment concentration.

The toxic effects of hydrocarbons on terrestrial higher plants and their use as weed killers have been ascribed to the oil dissolving the lipid portion of the cytoplasmic membrane, thus allowing cell contents to escape (Currier & Peoples, 1954, as cited in Bijay *et al*., 2012). Soil contaminated with petroleum causes a decrease in the agricultural productivity of the soil (Wang *et al*., 2008), it also affects crop growth height (Uquetan *et al*., 2017).

2.9 Biosurfactant

Surfactants are used for bioremediation of the hydrocarbons and make them available for the microorganisms to degrade. Hence, the transfer of the hydrocarbons to the aqueous phase in bulk is an important process for its bioavailabity (Adrion *et al*., 2016). Among various methods, surfactants can be seen as the promising method for bioavailability related problems. The use of surfactants could increase the hydrocarbons mobility as well as bioavailability, which promotes the rate of biodegradation (Haftka *et al*., 2015). Biosurfactants have higher surface activity with high tolerance to various environmental factors and can withstand from mean to extreme conditions such as acidity or basicity of an aqueous solution, temperature, salt concentration, ionic strength, biodegradable nature, demulsifying-emulsifying ability, anti-inflammatory potential and anti-microbial activity (Karlapudi*et al*., 2018).

Biosurfactants are amphiphilic compounds that consist of hydrophilic polar moiety as oligo or monosaccharide and proteins as well as polysaccharides or peptides and the hydrophobic moiety has unsaturated, saturated fatty alcohols or hydroxylated fatty acids (Rodrigues, 2015). One of the key features of biosurfactant is the hydrophilic - lipophilic balance, which causes the hydrophobic as well as hydrophilic portions to be determined in substances that are surface active. Because of the amphiphilic structure, biosurfactants not only have the ability to increase the hydrophobic substance surface area, but also have the ability to change the property of cell surface of the microorganisms. Surfactants behave as an excellent foaming agent, emulsifiers and dispersing agents attributed to their surface activity (De *et al*., 2015). Biosurfactants show selectivity of the substrate to degrade and are functionally active at extreme conditions of high temperatures, high salt concentrations as well as pH that can be attributed to the products and generated waste from industries. Different properties of surfactants are dispersion, emulsification or de-emulsification, wetting, foaming as well as coating that make them effective in bioremediation and physiochemical technologies of metal and organic contaminants (Wu and Lu, 2015). Biosurfactants form different complexes with metals and perform surface removal of heavy metals that may cause the increase of ion concentration of metals and the bioavailability in the soils with heavy-metal pollution (Sarma and Prasad, 2015).

Biosurfactants play a role in bioremediation by increasing the surface area of substrates. Biosurfactant-producing microorganisms create their own microenvironment and promote emulsification by the release of certain compounds through various mechanisms such as quorum sensing. These biosurfactants are of different complex nature namely rhamnolipids, trehalolipids, sophorolipids, peptide-lipid complexes and carbohydrate-peptide-lipid complexes (Karlapudi *etal*., 2018). They may be located inside the cells (intracellular) or

secreted outside the cells (extracellular) (Antoniou *et al*., 2015). There are many reports available on bacterial biosurfactants, but the spectrum of activity depends on their chemical composition. A strain of *Pseudomonas aeruginosa* was reported to produce the rhamnolipid type biosurfactant which was mono as well as dirhamnolipid (Patel *et al*., 2015). It has been proved that the rhamnolipids and its producing microorganisms specifically degraded hexadecane, hence there is a clear correlation between the type of surfactant and the type of hydrocarbon/oil that gets degraded. It has been noted that several studies were done on phenanthrene degradation by various chemical surfactants. It was also indicated that there was increased phenanthrene degradation when it was associated with bacterial isolate that produced a non-ionic surfactant (Itrich*et al*., 2015). In another instance, oil degradation capacity of a chemical surfactant 'FinasolOSR-5' was multiplied when supplemented with a biosurfactant trehalose-5, 5'- dicorynomycolatesand reported to be the complete removal of aromatic hydrocarbons from the contaminated soil within a given period (Itrich *et al*., 2015).

2.9.1 Biosurfactant and Its Classification

Classification of the biosurfactants is mainly based on the origin of the microbes and their chemical composition. Biosurfactants are classified not like the artificial chemical surfactants which are categorized based on the polarity of the functional group (Sharma*et al.,* 2016). Biosurfactants are divided into two types based on the molecular weight, low molecular weight compounds which lower the interfacial surface tension, polymers of high molecular weight that are most of the efficient stabilizing agents. Glycolipids, lipopeptides and phospholipids constitute the majority of low mass biosurfactants, while particulate and polymeric surfactants come under the large mass biosurfactants (Saenz-Marta *et al*., 2015). Mostly are anionic biosurfactants and some are neutral, while hydrophobic moiety is based

on the derivatives of fatty acid long chains and have the hydrophilic moiety that could be an amino acid, phosphate group, carbohydrate part and a cyclic peptide (Harvey, 2015).

2.9.1.1 Glycolipids

Glycolipids are a group of carbohydrates which have a long-chain of aliphatic acids. They form a connection of either ester group or ether group. Some of the glycolipids are sophorolipds, rhamnolipids and trehalolipids (Rikalovic*et al*., 2015).

2.9.1.2 Rhamnolipids

These are the glycolipids in which any of the rhamnose sugar moieties linked to the myrmicacin, which is a derivative of β-hydroxycarboxylic acid hydroxyl group at the reducing end of rhamnose disaccharide, or present as one of the hydroxyl groups is occupied by ester formation (Nickzad*et al*., 2016).

2.9.1.3 Trehalolipids

Trehalolipids are present in most of the species such as *Corynebacterium* sp., *Mycobacterium* sp., and *Nocardia* sp. Trehalose is a disaccharide sugar which is linked at the 6th position of the carbon backbone to long chain fatty acids of mycolic acid. The structure and size of the mycolic acid vary from organism to organism by the different number in the presence of atoms of carbons and its unsaturation rate. Trehalose lipids obtained from *Arthrobacter* sp. and *Rhodococcus erythropolis* decreased the interfacial as well as surface tension in the growth medium (Sharma*et al.*, 2016).

2.9.1.4 Sophorolipids

Torulopsis bombicola synthesizes three types of glycolipids. *T. Petrophilum* as well as *T. apicola* contain a carbohydrate sophorose that is dimeric through the glycosidic linkage attached to the hydroxyl fatty acid. Generally, sophorolipids are heterogenous mixture of macrolactones and a free acidic group. Lactones, ester groups of hydroxycarboxylic acids extracted from sophorolipid molecules are required for various biomedical applications as polymers (Jimenez-Penalver*et al.*, 2016).

2.9.1.5 Lipoproteins and Lipopeptides

Cell walls of wide range of microorganisms have cyclic lipopeptides which trigger the responses of immune system. They include decapeptide-lipopeptide antibiotics. Lipopeptides and lipoproteins contain lipid as the functional group linked to the polypeptide chain. *Bacillussubtilis* synthesizes the cyclic lipopeptide surfactin which is the most effective biosurfactant. Surfactin is made of seven ring structure of amino acid which is joined to fatty acid chain with the help of a lactone linkage. Surfactin was reported to have reduced the surface tension below 28 mN/m (Nguyen and Gotz, 2016).

2.9.1.6 Lichenysin

Several of the biosurfactants synthesized by *Bacillus licheniformis* have exhibited great stability towards salt, temperature as well as pH and have same structural as well as physio-chemical properties as that of surfactin. Surfactant of *Bacillus licheniformis* is capable of lowering the surface tension of various liquids (Ronning *et al.*, 2015).

2.9.1.7 Phospholipids and Fatty acids

Yeast and bacteria when grown on n-alkane medium synthesize a large number of phospholipid and fatty acid molecules. *Acinetobacter* sp. produces rich vesicles of phosphatidylethanolamine which form microemulsions that are clear in water. *Rhodococcus erythropolis* produces phosphatidylethanolamine when grown on n- alkane and decreases the surface tension of water and hexadecane (Helfrich*et al*., 2015).

2.9.1.8 Polymeric biosurfactants

Liposan and Alasan are some of the most popular polysaccharide–protein complexes. Heteropolysaccharide biosurfactants show extracellular polyanionic activities that are synthesized by most of the *Acinetobacter* species. Emulsan is used to emulsify hydrocarbons present in water which is considered to be one of the effective emulsifying agents even if the concentration is lesser than 0.01%. Extracellular polymeric emulsifier, liposan is a water soluble emulsifier synthesized by *C. lipolytica* which consists more than 80% of carbohydrate and less than 20% of protein part (Wilton*et al*., 2016).

Biosurfactants derived from living organisms, mainly microorganisms have attracted much attention because of advantageous characteristics such as structural diversity, low toxicity, higher biodegradability, better environmental compatibility, higher substrate selectivity and lower critical micelle concentration (Saravanan and Vijayakumar, 2012).

2.9.2 Properties of biosurfactants

2.9.2.1 Surface and interface activity

An effective surfactant or a biosurfactant is the one that lowers the surface tension of water.

Bacillus subtilis produces surfactin that lowers surface tension of liquids most effectively even at adverse extreme conditions. *Pseudomonas aeruginosa* produces biosurfactant of

rhamnolipid nature that decreases the water surface tension effective than many other surfactants (Kim *et al*., 2015). Sophorolipids produced by *T. bombicola* reduces the surface tension of liquids. Biosurfactants are effective as well as efficient, Their Critical micelle concentration (CMC) is from 10 to 40 times lower than chemical surfactants and because of this reason, very less amount of biosurfactant is required to reduce the surface tension (Anjum *et al*., 2016).

2.9.2.2 Temperature, pH and ionic strength tolerance

Functions and parameters such as temperature and pH of most of the biosurfactants are not altered by the environmental conditions. Research studies suggest that lichenysin, which is produced by *B. licheniformis* was less affected by pH (4.5–9.0), temperature (up to 50°C) and by NaCl as well as Ca concentrations. At high temperatures beyond autoclavable temperature (121°C) and at low temperatures below minus 15°C, lipopeptides produced by *Bacillus subtilis* is found to be stable when stored for 180 days. At NaCl concentrations greater than 15% and pH range between 4 and 12, the activity was found to be stable (Cheng *et al*., 2016).

2.9.2.3 Biodegradability

Biosurfactants are regarded as non-toxic agents, as they are one of the best options to use in cosmetic, food and pharmaceutical fields. One of the recent studies suggest that the polyanionic

surfactant named emulsan has shown LC50 against *Photobacterium* phosphoreum, which is much lesser than *Pseudomonas* rhamnolipids. Biosurfactants produced by *Pseudomonas* species are widely in use in industries because of its wide applications and environmental toxic friendly nature compared with artificial surfactants. Studies indicated the range of

mutagenic and toxicity effects of biosurfactant when compared to that of chemical surfactant were less (Shah *et al*., 2016).

Formation and breaking of emulsion could be produced within a month. Emulsion may be stabilized or destabilized by the biosurfactants. Emulsifiers are generally a class of biosurfactants with high molecular weight compared with low mass biosurfactants. *T. bombicola* produces sophorolipid surfactant that can lower the surface tension and surface area. Stable emulsions were formed by the use of polymeric biosurfactants and have the additional advantage that they consist of oil coat droplets to form oil/water emulsions for cosmetics and food that are stable. Liposan produced by C. lipolyticacan emulsify edible oils but does not reduce surface tension effectively. Biosurfactants contain hydrophilic group which may be a sugar, or a protein, whereas hydrophobic group usually contains fatty acids or fatty alcohols (Karlapudi *et al*., 2018).

Bioemulsan is the best ever studied polymer produced by *Acinetobacter*. Most of the amphipathic polysaccharides were produced by *Acinetobacter* species. Rhamnolipids which are carbohydrate-lipid derivatives have been produced by *Pseudomonas* sp. and showed good emulsification ability, peptide linked bioemulsifiers produced by *Methyl bacterium* sp., *Methanobacterium* sp. and*A. calcoaceticus* has carbohydrate-protein derivative, Lipid-protein derivatives produced by *Bacillus velezensis* and *Streptococcus gordonii* and Lipid-fatty acid derivatives produced by *Myroides* species. Surface active agents show the surface property is made up of biological molecules such as carbohydrates, lipids and proteins in various combinations and compositions (Karlapudi *et al*., 2018). Microorganisms that produce bioemulsifiers have typical physiological behavior which was poorly understood by researchers as they perform definite functional roles in the microbes.

2.10Petroleum Hydrocarbon Degradation

Hydrocarbon biodegradation had immense ecological importance, because it incorporates the fundamental process for remediation of affected areas. Microorganisms capable of degrading a number of hydrocarbon chains were described and their mode of action had been studied. The soil affected with hydrophobic pollutants was restrained via non-availability of such contaminants to the microorganisms.

Surfactants ease the process of solubilization, stabilization and emulsification and deliver the processed hydrocarbons occluded to the soil's natural environment. Microorganisms such as yeasts, bacteria and filamentous fungi have been studied as remodeling representative way towards their capability to degrade a huge variety of pollutants. For this reason, micro-organisms were considered to be the most effective option for traditional techniques in solving environmental troubles. Oil contaminated sites that are affected with hydrocarbons could serve as the enrichment environments to the hydrocarbon degrading biosurfactant delivering microbial strains. Production and manufacturing of the biosurfactants with the aid of soil borne microorganisms isolated from the polluted sites was based on the fact that they can utilize hydrocarbons as a carbon source which were water insoluble. In fact, very little research has been carried out on marine bacteria that degrade hydrocarbons and PAHs, hence suggesting that bacteria belonging to genera *Cycloclasticus*, *Vibrio* and *Pseudalteromonas* had the ability of degrading hydrocarbons through biosurfactant production. Pereira and Mudge (2004) carried out experiments on microbial degradation of biodiesel and observed that biodiesel was completely degraded by a group of microorganisms.

2.11 Bioremediation

There are many technologies being used for the clean up of the contaminated sites. They include thermal evaporation, excavation and soil vapour extraction. Bioremediation is the most important method which has been the accepted treatment by using indigenous microbial flora. Certain biosurfactant producing bacteria can metabolize several classes of hydrocarbons (Karlapudi *et al*., 2018).

The microorganisms can be obtained originally by enrichment culture procedures, where maximum specific growth rate or maximum final cell concentration can be used as the selection criterion. Petroleum hydrocarbons can be degraded by microorganisms such as bacteria, mould, yeast and microalgae (Bundy *et al*., 2004). However, bacteria play the central role in hydrocarbon degradation (Geetha *et al*., 2013).

The constituents of oil differ distinctly in volatility and susceptibility to biodegradation. Some compounds are easily degraded, some resist degradation and some are non-biodegradable (Mukred *et al*., 2008). The biodegradation of different petroleum compounds occurs simultaneously but at different rates because different species of microbes preferentially attack different compounds. This leads to the successive disappearance of individual components of petroleum over time (Bijay *et al*.,2012).The effects of nutrients (i.e. NPK), aeration and biostimulation of indigenous soil microorganisms and inoculation of extraneous microbial consortia on the bioremediation of oil contaminated soil have been investigated (Vasudevan and Rajaram, 2001; Gogoi *et al*., 2003; Coulon *et al*., 2005; Ayotamuno *et al*., 2006; Sang-Hwan *et al*., 2007).

2.11.1 Biostimulation

Some microorganisms are present in the contaminated site, but for effective remediation, growth of microorganism should be stimulated. Biostimulation is the process of adding nutrient, electron acceptor, oxygen or any substance for example biosurfactant to stimulate existing bacteria involve in bioremediation. This is the process of optimizing the environment condition of the remediation site. Additives are usually added to the subsurface through injection wells. Subsurface characteristics such as groundwater velocity, hydraulic conductivity of the subsurface and lithology of the subsurface are important in developing a biostimulation system (Vidali, 2001). The indigenous microorganisms present in the soil are responsible for degradation of the pollutant, but biostimulation can be improved by bioaugmentation. Of the many remediation methods currently in use, biostimulation is viewed as one of the most promising technologies. Biostimulation involves the use of biological process to return a polluted environment to its original state by increasing the activity of micro-organisms that can degrade the contaminants through the addition of nutrients, oxygen or other electron donors and acceptors (Obire and Anyanwu, 2009; Blaise-Chikere, 2012).

2.11.2 Bioaugmentation

Bioaugmentation is the addition of a group of indigenous microbial strains or genetically engineered microbes to treat the contaminated soil. It is effective where native microorganisms are not identified in the soil or do not have the metabolic capability to perform the remediation process (Bijay *et al*., 2012).

The application of bacteria in bioremediation is termed Bioaugmentation; bacterial assemblies may provide a range of metabolic capabilities that cover the full spectrum of reactions required to completely degrade hydrocarbon mixtures and then utilize all of the breakdown products.

Therefore, the bacteria benefit from living in association due to synergistic and commensalistic relationships, thus faster and more complete biodegradation is possible than by individual species alone (Chijioke-Osuji *et al*., 2014).

2.11.3 Anaerobic Degradation

Most of bioremediation method aims in enhancing oxygen supply to contaminated sites assuming that the principal mechanism of hydrocarbon removal is aerobic respiration. But addition of urea and ammonia-based fertilizers sometimes used for oil-spill bioremediation can potentially exert an oxygen demand due to biological ammonia oxidation. On some sites, mass transfer of oxygen may not be sufficient to replenish oxygen consumed by microbial metabolism, though penetration of oil into deeper sediment layers is also likely to be reduced in fine sediments. Under such conditions anaerobic hydrocarbon degradation may be of relevance (Bijay *et al*., 2012).

2.11.4 Land Farming

Land farming is a method in which contaminated soil is spread over a prepared bed along with some fertilizers and occasionally rotated. It stimulates the activity of bacteria and enhances the degradation of oil. The criteria for determining proper site location includes: a minimum separation distance of 3feet between the ground surface and the seasonable high groundwater table and the slope of the land should not exceed 8% (Spormann and Widdel, 2000).

2.11.5 Composting

Composting is a process of piling contaminated soil organic substances such as manure or agricultural wastes. The added organic material supports the development of a rich microbial population and elevates temperature of the pile. Stimulation of microbial growth by added nutrients results in effective biodegradation in a relatively short period of time (Bijay *et al*., 2012).

2.12 Environmental Conditions Affecting Biodegradation

Microbial growth and activity are readily affected by pH, temperature, and moisture. Although microorganisms have been also isolated in extreme conditions, most of them grow optimally over a narrow range, so that it is important to achieve optimal conditions.

2.12.1 Nutrient

Although the microorganisms are present in contaminated soil, they cannot necessarily be there in the numbers required for bioremediation of the site. Their growth and activity must be stimulated. Carbon is the most basic form of nutrient required for living organisms. In addition to this, the bacteria also need macronutrients like nitrogen and phosphorous to ensure effective degradation of the oil. The optimum nutrient balance required for hydrocarbon remediation is Carbon: Nitrogen: Phosphorus equals 100:10:4. In general, at least 1 ppm of ammonium nitrogen and 0.4 ppm of orthophosphate needs to be present. Pathways can be influenced by further adjusting volumes of bio-nutrients (Bijay *et al*., 2012). Also, oil spills result in an imbalance in the carbon–nitrogen ratio at the spill site, because crude-oil is essentially a mixture of carbon and

hydrogen. This causes a nitrogen deficiency in an oil-soaked soil, which retards the growth of bacteria and the utilization of carbon source (Chorom *et al*., 2010).

2.12.2 Electron Acceptor / Oxygen

Although oxygen is not the rate limiting factor, it's one of the most essential elements of microbial degradation of hydrocarbons. Oxygen is necessary for the initial breakdown of hydrocarbons and succeeding reaction may require it. In the presence of oxygen complete degradation of oil takes place. The oxygen availability in the soil depends on microbial oxygen consumption rates and soil type, whether soil is water-logged, and the useable substrates presence which can drive the oxygen depletion (Haritash and Kaushik, 2009). If large quantities of oil are present, the oxygen in the soil will be depleted very fast causing anaerobic condition. Anaerobic bacteria will use other electron acceptors like nitrate, iron or sulphate, but the energy yield for the bacteria is less than oxygen used as the electron acceptor. Some studies have indicated that anaerobic degradation of petroleum hydrocarbons by microorganisms can happen at negligible rates (Haritash andKaushik, 2009).The lower energy yield by anaerobic bacteria results in lower degradation and hence a longer period of time is required for remediation. McNally *et al*.(1998) and Al-Hawash *et al*.(2018)reported that the aerobic biodegradation of petroleum hydrocarbons was higher compared with the anaerobic biodegradation. Biodegradation of petroleum hydrocarbons in anaerobic conditions was not as fast as in aerobic conditions (Grishchenkov *et al*., 2000). To increase the oxygen amount in the soil, it is possible to till or sparge air. In some cases, hydrogen peroxide or magnesium peroxide can be introduced to the environment (Vidali, 2001).Substrate oxidation by oxygenases in the catabolism of all

aliphatic, cyclic and aromatic compounds by microbes is considered a key step in the biodegradation process (Meng *et al*., 2017).

2.12.3 Temperature

Temperature is among the factors that influence petroleum hydrocarbon biodegradation by affecting the physical and the chemical compositions of petroleum hydrocarbons (Al-Hawash *et al*., 2018). At low temperatures, the degradation rate is generally observed to decrease, which is thought to be a result of reduced enzymatic activity rates (Bisht *et al*., 2015). Despite the fact that biodegradation of hydrocarbons can take place on a wide domain of temperatures, degradation rate decrease through declining temperature. The highest rates of degradation occur at the temperature range of 30–40 °C, 20–30 °C and 15–20 °C in soil, marine and freshwater environments, respectively (Al-Hawah *et al*., 2018)). Temperature affects biochemical reactions rates and the rates of many of them double for each 10 °C rise in temperature. Above a certain temperature, however, the cells die. Plastic covering can be used to enhance solar warming in late spring, summer and autumn (Vidali, 2001).

2.12.4 Moisture

Available water is essential for all the living organisms and irrigation is needed to achieve the optimal moisture level (Vidali, 2001). The biodegradation of hydrocarbons in terrestrial ecosystems may be restricted because of the water available for metabolism and growth of microbes. Al-Hawah *et al*.(2018) andDibble and Bartha (1979) showed that biodegradation was optimal with 30–90% water saturation in oil sludge. Availability of water directly impacts the movement and microorganism's growth.

2.12.5 pH

The pH can be highly variable and must be taken into consideration when improving biological treatment methods. The environmental pH affects processes such as cell membrane transport and catalytic reaction balance as well as enzyme activities (Bonomo*et al*., 2001). In general, heterotrophic fungi and bacteria prefer a nearly neutral pH, although fungi are tolerant to acidic conditions. Rates of octadecane mineralization increase remarkably when pH increases from 6.5 to 8.0, whereas the miner- alization rate of naphthalene remains unchanged. Thavasi *et al*.(2007) found that the maximum biodegradation of crude oil by *Pseudomonas aeruginosa* in water was at pH 8.0. Pawar (2015) observed that the soil pH 7.5 was most convenient for the degradation of all the petroleum hydrocarbons. The degradation of Phenanthrene in liquid media was favorable at a range of pH values (pH 6.5–7.0) by *Burkholderia cocovenenas*, isolated from a petroleum-polluted soil. Soil having too much acid is added lime to raise the pH (Bijay *et al*., 2012).

2.12.6 Type of Soil

Soil structure controls the effective delivery of air, water and nutrients. To improve soil structure, materials such as gypsum or organic matter can be applied. Low soil permeability can impede movement of water, nutrients and oxygen; hence, soils with low permeability may not be appropriate for *in situ* clean-up techniques (Bijay *et al*., 2012).

2.12.7 Bioavailability

Bioavailability refers to the portion of a chemical in soil, which can be taken up or transformed by living organisms. Bioavailability has also been defined as the influence of the physical, chemical and microbiological factors to the extent and rate of biodegradation. The pH, the

microbial community and the extent of deterioration of the hydrocarbon can be significantly affected by the restrictions in the bioavailability of hydrocarbons. The bioavailable part of the hydrocarbons is the area accessible to microorganisms. Petroleum hydrocarbons have low bioavailability and are classified as hydrophobic organic pollutants. Those chemicals have little water solubility, which makes them resistant to photolytic breakdown, chemical and biological (Semple *et al*., 2003).

2.13Adaptation Mechanism of Bacterial Strain

It has already been reported that some bacterial populations exhibited resistance to oiltransportation and also few bacterial populations efficiently degrade oils/hydrocarbons. Two different types of interactions are normally observed in the processes of oils/hydrocarbonbiodegradation. Oil adhesion, pseudo-solubilization and degradation of hydrocarbons to form small droplets of oils are the sequential steps involved in one of the mechanisms. Microbial cells adhere to the drops of hydrocarbons whose size was less than the cells and the substrate uptake takes place by active transport or by diffusion at the point of interference between cells and hydrocarbons (Palecek *et al*., 2015). Some types of microorganism are able to degrade petroleum hydrocarbons and use them as source of carbon and energy. The specificity of the degradation process is related to the genetic potential of the particular microorganism to introduce molecular oxygen into hydrocarbon and to generate the intermediates that subsequently enter the general energy- yielding metabolic pathway of the cell (Millioli *et al*., 2009). Some bacteria are mobile and exhibit a chemotactic response, sensing the contaminant and moving towards it, while other microbes like fungi grow in a filamentous form near the contaminant.Microorganisms produce enzymes in the presence of carbon sources which

are responsible for attacking the hydrocarbon molecules. Many different enzymes and metabolic pathways are involved to degrade hydrocarbons contained in petroleum. But lack of an appropriate enzyme will either prevents attack or will act as a barrier to complete hydrocarbon degradation (Bijay *et al*., 2012).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Description of the Sampling Areas

Ibeno Local government area has a coastal area of over 1,200 square kilometers. It is situated on the eastern bank of Niger Delta which in turn is part of the Gulf of Guinea. It is located at the

south end of Akwa Ibom State at latitude 4^o321 and 4^o341 North of Equator and longitude $7^0$541 and 8^o021 east of Greenwich Meridian.The area is characterized bya humid tropical climate with an annual rainfall of4021 mm andmean minimum and maximum temperatures of 22˚C and 30˚C. The people of those areas are mainly fishermen and farmers.

Otuocha land in Anambra East local government area is situated on the left bank of Anambra River which forms its north-western boundary. It is bounded on the north-east by the Emu stream, a tributary flowing into the Anambra river from the south-east, and on the south-west by the Akor river, another tributary of the Anambra, which joins it from the south-east at a point further downstream. It is located atLatitude 6°20'9.34"N and longitude 6°51'4.25"E.The climate is tropical with average annual rainfall of 2000 mm and mean temperature of 27℃. The main occupations of the people living around those areas are farming and fishing.

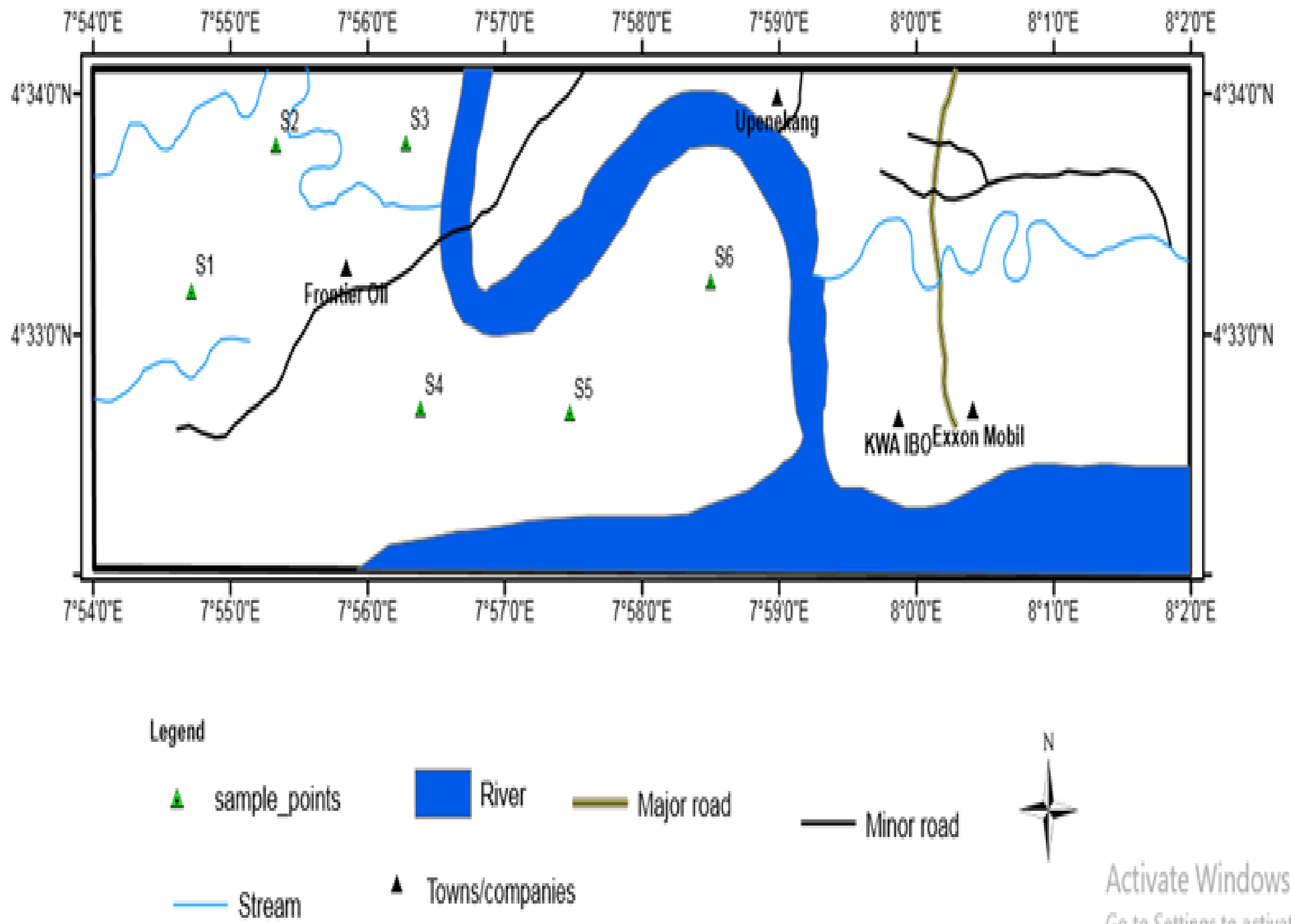


Figure 3.1: Map of Frontier Oil and Environs Showing Sampling Points

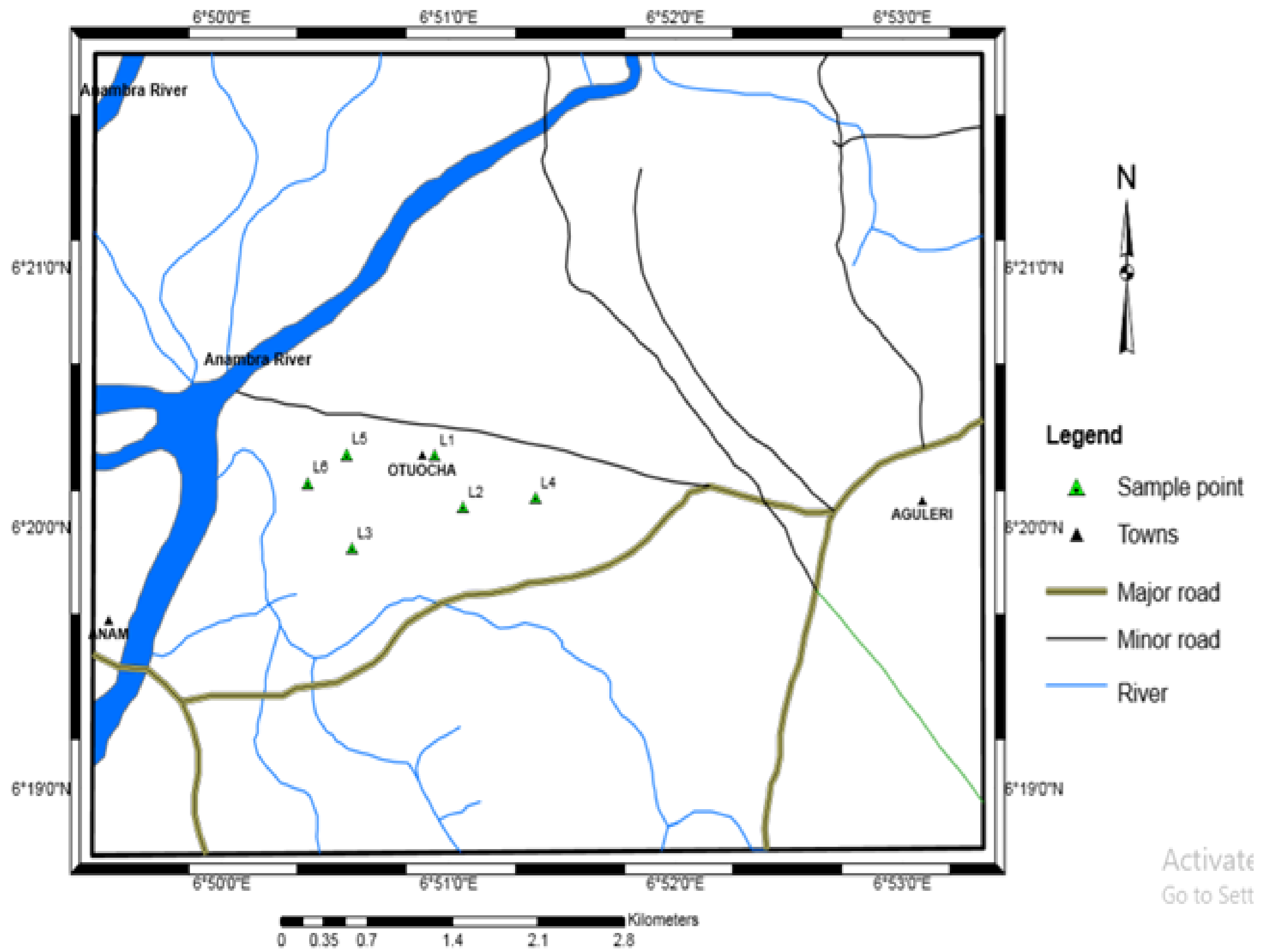


Figure 3.2: Map of Otuocha Showing Sampling Points

3.2 Sampling Site

Two different agricultural soil samples in crude oil producing area (i.e. Otuocha in Anambra East local government area in Anambra State and Qua Iboe in Ibeno local government area in Akwa Ibom State) were collected by random sampling at a depth of 5cm using a sterile trowel and zip lock bag. These were used in carrying out the remediation exercise. The soil for the isolation of biosurfactant-producing bacteria was collected from an oil drilling site in Ibeno, Akwa Ibom state. The soil samples were separatelybulked to form composite samples, labelled and immediately transported to Godfrey Okoye University Microbiology laboratory in an ice box for analysis as described by (Adieze, 2012).

3.3 Source of Crude Oil

Crude oil known as Qua Iboe Brent was collected from frontier Oil Company at Qua Iboe Terminal in Ibeno local government area of Akwa Ibom state. The Qua Iboe brent was used in carrying out all the research work.

3.4Determining the Sterility of Crude Oil

The sterility of Qua Iboe Brent was determined by inoculating one drop of it on Nutrient agar (TM media, India) and Sabourand dextrose agar (TM media, India). The plates were incubated at room temperature for 48 and 96 h for bacteria and fungi respectively. This was done to find out if the crude oil had some contaminants in it but none was observed. Crude oil sterilization was still carried out to ensure that the crude oil is free of contaminant.

3.4.1 Crude Oil sterilization

In order to sterilize the crude oil, micron Chromafil CA/S %45 syringe filters were used. Thus, at first the oil was pulled with a syringe and then the filter was connected to the syringe tip which was pushed slowly with a controlled pressure downward, to let the oil pass the filter and slowly enter the pipe in the device as described by Chorom *et al*.(2010). About half litre of oil was filtered and used in carrying out the analysis.

3.5 Soil Physicochemical Analysis

Physicochemical characteristics of the polluted and unpolluted soils were determined. Parameters such as Nitrogen determination, phosphorus, total organic carbon, particle size of the soil,pH, conductivity, salinity, cation exchange capacity (CEC) and water holding capacity were determined. Heavy metals such as Iron, copper, zinc, cadmium, nickel, lead, chromium, manganese, cobalt,vanadium, silver, mercury, arsenic, aluminium, selenium, molybdenum, and tin were also determined. The Nitrogen, total organic carbon, particulate size of the soil, mercury, arsenic, aluminium, selenium, molybdenium and tin were done at Springboard Research Laboratories, Awka. Water holding capacity was done at PRODA, Enugu. pH, conductivity, salinity, CEC, Iron, copper, zinc, cadmium, nickel, lead, chromium, manganese, cobalt, vanadium and silver was done at Halden Laboratories, Port-Harcourt. The physicochemical analysis was done before and after pollution inorder to ascertain the effect of crude oil pollution on soil’s physicochemical properties.

3.5.1 Nitrogen determination

This was determined as described by AOAC (1990).

Principle: The method is the digestion of sample with hot concentrated sulphuric acid in the presence of a metallic catalyst. Organic nitrogen in the sample is reduced to ammonia. This is

retained in the solution as ammonium sulphate. The solution is made alkaline and then distilled to release the ammonia. The ammonia is trapped in dilute acid and then titrated.

Procedure: 1g of sample was weighed into a 30ml kjehdal flask gently to prevent the sample from touching the walls of the side of each and then the flasks were stoppered and shaken. Then 1g of the kjedahl catalyst mixture was added. The mixture was heated cautiously in a digestion rack under fire until a clear solution appeared.

The clear solution was then allowed to stand for 30 minutes and allowed to cool. 100ml of distilled water was added after cooling to avoid caking and then transferred to the kjedahl digestion apparatus.

A 500ml receiver flask containing 5ml of boric acid indicator was placed under a condenser of the distillation apparatus so that the tap was 20cm inside the solution. The 10ml of 40% sodium hydroxide was added to the digested sample in the apparatus and distillation commenced immediately until distillation reached the 35ml mark of the receiver flask, after which it was titrated to pink colour using 0.01N hydrochloric acid.

Calculations:

% Nitrogen = Titre value x 0.01 x atomic mass of nitrogen x 4

Where 0.01 = normality of the acid, 4 = dilution factor

3.5.2 Phosphorous

Phosphate was measured using Standard Method 4500-P B.5 and 4500-PE as described by APHA(1998)

Procedure: 100ml of the homogenized and filtered sample was pipetted into a conical flask. The same volume of distilled water (serving as control) was also pipette into another conical flask.

1ml of 18M H_2S0_4 and 0.89g of ammonium persulphate were added to both conical flasks and gently boiled for 1 ½ hrs, keeping the volume of 25-50cm^3 with distilled water.

It was then cooled. One drop of phenolpthelein indicator was added and after neutralized to a faint pink colour with the 2M NaOH solution. The pink colour was discharged by drop wise addition of 2M HCl and the solution made up to 100ml with distilled water. For the colorimetric analysis, 20ml of the sample was pipetted into test tubes, 10ml of the combined reagent added, shaken and left to stand for 10mins before reading the absorbance at 690nm in a spectrophotometer, using 20ml of distilled water and 1ml of the reagent as reference.

Methods for Calibration

Standard phosphate solution: 219.5 mg of dried AR (analytical reagent) potassium hydrogen phosphate was dissolved in distilled water and made up to 1000ml, where 1ml = 50.0 µg. of phosphate. 10ml of the stock solution was made up to 1000ml to give 1ml = 0.05 mg. Standards of strength ranging from 0 (blank) to 0.05mg/lat intervals of 0.01mg was prepared by diluting the stock with distilled water.

$$\text{Conc of sample} = \frac{\text{Abs of sample}}{\text{Abs of std}} \times \text{conc of std}$$

3.5.3 Determination of Total Organic Carbon

This was determined as described in American Standard Test method (1995a).

The moisture content of the air – dry soil which had been grounded to pass a 0.42 sieve was determined. Soil which contained accurately between 10g and 20mg of carbon was weighed into a dry tared 20ml conical flask.

10ml .1N K_2Cr20_7 was added and the flask swirled gently to disperse the soil in the solution. 20ml concentration of H_2SO4 was added directing the stream into the suspension. The flask was immediately swirled until the soil and the reagent were mixed. A 200^0c thermometer was inserted and heated while swiring the flask on a hot plate or over a gas burner and guaze until the temperature reached 139^0C.

It was set aside to cool slowly on an asbestors sheet in a fume cupboard. Two blanks (without soil) was run in the same way to standardized $FeSO_4$ solution.

When cooled (20 – 30mins), it was diluted to 200ml with deionised water, and the $FeSO_4$ titration was proceeded using either the ferroin indicator or potentiometrically with an expending scale PH/MV meter or auto titrator.

Ferroin Titration

3 or 4 drops of ferroin indicator was added and titrated with 0.4N $FeSO_4$. As the end points was approached, the solution takes on a greenish colour and then changed to a dark green. At this point, the $FeSO_4$was added drop- by- drop until the colour changed from blue – green to reddish – grey. The end point was overshort, and so 1.0ml of 1N $K_2Cr_20_7$was added and the end points was reapproached drop by drop. The determination was repeated with a smaller soil sample.

3.5.4Determination of % Silt, Clay and Sand.

This was determined as described in American Standard Test method (1995b).

50g of the soil sample was dispensed into a 250ml beaker

The beaker was filled with distilled water to 200ml mark.

The sand was washed four times with distilled water. 25% sodium hexametaphosphate was prepared.

Then 20ml of the 25% sodium hexametaphosphate and 200ml of distilled water was added.

It was allowed to stand for 16hrs (ie overnight)

It was transfered into 0.2mm sieve and the liquid was collected in a pan. The sieve was the sand while the pan was the silt. The sand and silt was transferred in a preweighed container and dried. The container containing the residue was weighed again and calculated.

Residue = weight of container with residue - weight of empty container

$$\% \text{ residue} = \frac{\text{Residue}}{\text{sample}} \times 100$$

% Clay = 100 - % Silt + % Sand.

3.5.5 pH, Conductivity and Salinity

These were determined as described by APHA (1998).

pH, conductivity and salinity were measured using a digital Oakton multimeter (model PCD 650).

10g of the sample was weighed in a beaker and 10ml of distilled were added. The content was thoroughly mixed.

The pH and Conductivity/TDS meter (Oakton multimeter) was standardized with buffer solutions (4 & 7), and conductivity standard solutions respectively.

The tip of the probe in each case was rinsed with deionised water and cleaned with tissue paper. The probe was then immersed in the sample and the corresponding steady reading was taken in each case.

3.5.6Determination of CEC (Cation Exchange Capacity)

CEC can be described as the capacity of a soil to exchange cations for another.

The CEC is a part of the soil test which was calculated from the levels of k, Mg, Ca and Na which are extracted in the soil. The atomic weight of the cations was divided by the number of their valences to determine the equivalent weight. Hence CEC was reported as meq/100g.

For eg Ca: 40/2 = 20

Mg: 24/2 = 12

K: 39/1 = 39

Na: 23/1 = 23

The soil extract was analysed for Ca, Mg, K and Na using AAS. The value obtained was used in calculation. CEC was calculated thus:

$$Ca = \frac{\text{value obtained}}{200}$$

$$Mg = \frac{\text{value obtained}}{120}$$

$$K = \frac{\text{value obtained}}{390}$$

$$Na = \frac{\text{value obtained}}{230}$$

CEC was obtained by adding up the values

3.5.7Determination of water holding capacity of soils

30g of each of the soil was weighed out and transferred into a pre-weighed funnels lined with soaked filter paper. The soils were saturated with water. It was allowed to stand on a retort stand

for 48hours. The funnels were covered with aluminium foil to avoid loss of water through evaporation. After 48hours, the drained soils were weighed again (http://notesforfree.com).

$$\text{Water holding capacity} = \frac{(W_3 - W_2) - W_1 * 100}{W_1 * 1}$$

Where: W_1= weight of dry soil

W_2= weight of funnel with moist filter paper

W_3= weight of drained soil with filter paper and funnel

3.5.8 Heavy Metals

Sample Preparation:

This was done following the method adopted from ASTM (1999) and APHA (1998). 5g of the sample was weighed in a conical flask. 10ml and 1ml of HCl and HNO_3 respectively were added. Also 100ml of distilled water was added. The sample was heated uniformly on a heating mantle until the volume reduced to 15ml. Thereafter, the sample was filtered after cooling. The filtrate was made up to 50ml using distilled water.

Iron

The concentration of iron(Fe) in the sample was determined using Atomic Absorption Spectrophotometric (ASTM D1068) method after acid digestion of the sample. This was carried out using PG AA500 Atomic Absorption Spectrophotometer (AAS) with detection limit of 0.05mg/kg. Prior to the analysis, calibration was done with standard of known concentrations. Dissolved iron (Fe) was determined by aspirating a portion of the filtered sample (without

pretreatment) directly in AAS. Concentration of iron was determined from the data generated by the AAS and expressed in mg/kg.

Copper

The concentration of Copper (Cu) in the sample was determined using Atomic Absorption Spectrophotometric (ASTM D1688) methodafter acid digestion of the sample. This was carried out using PG AA500 Atomic Absorption Spectrophotometer (AAS) with detection limit of 0.05mg/kg. Prior to the analysis, calibration was done with standard of known concentrations. Dissolved copper (Cu) was determined by aspirating a portion of the filtered sample (without pretreatment) directly using AAS. Concentration of copper was ascertained from the data generated by the AAS and expressed in mg/kg.

Zinc

The concentration of zinc (Zn) in the sample was determined using Atomic Absorption Spectrophotometric (ASTM D1691) method after acid digestion of the sample. This was carried out using PG AA500 Atomic Absorption Spectrophotometer (AAS) with detection limit of 0.05mg/kg. Prior to the analysis, calibration was done with standard of known concentrations. Dissolved zinc (Zn) was determined by aspirating a portion of the filtered sample (without pretreatment) directly using AAS. Concentration of zinc was determined from the data generated by the AAS and expressed in mg/kg.

Cadmium

The concentration of cadmium (Cd) in the sample was determined using Atomic Absorption Spectrophotometric (ASTM D3557) method after acid digestion of the sample. This was carried

out using PG AA500 Atomic Absorption Spectrophotometer (AAS) with detection limit of 0.001mg/kg. Prior to the analysis, calibration was done with standard of known concentrations. Dissolved cadmium (Cd) was determined by aspirating a portion of the filtered sample (without pretreatment) directly using AAS. Concentration of cadmium was determined from the data generated by the AAS and expressed in mg/kg

Nickel

The concentration of nickel (Ni) in the sample was determined using Atomic Absorption Spectrophotometric (ASTM D1886) method after acid digestion of the sample. This was carried out using PG AA500 Atomic Absorption Spectrophotometer (AAS) with detection limit of 0.05mg/kg. Prior to the analysis, calibration was done with standard of known concentrations. Dissolved nickel (Ni) was determined by aspirating a portion of the filtered sample (without pretreatment) directly using AAS. Concentration of nickel was determined from the data generated by the AAS and expressed in mg/kg.

Lead

The concentration of lead (Pb) in the sample was determined using Atomic Absorption Spectrophotometric (ASTM D3559) methodafter acid digestion of the sample. This was carried out using PG AA500 Atomic Absorption Spectrophotometer (AAS) with detection limit of 0.05mg/kg. Prior to the analysis, calibration was done with standard of known concentrations. Dissolved lead (Pb) was determined by aspirating a portion of the filtered sample (without pretreatment) directly using AAS. Concentration of lead was determined from the data generated by the AAS and expressed in mg/kg.

Chromium

The concentration of Chromium (Cr) in the sample was determined using Atomic Absorption Spectrophotometric (ASTM D1687) method after acid digestion of the sample. This was carried out using PG AA500 Atomic Absorption Spectrophotometer (AAS) with detection limit of 0.001mg/kg. Prior to the analysis, calibration was done with standard of known concentrations. Dissolved chromium (Cr) was determined by aspirating a portion of the filtered sample (without pretreatment) directly using AAS. Concentration of chromium was determined from the data generated by the AAS and expressed in mg/kg.

Manganese

After acid digestion of the sample, the concentration of Manganese (Mn) in the samples were determined using Atomic Absorption Spectrophotometry (ASTM D858) method. This was carried out using PG AA500 Atomic Absorption Spectrophotometer (AAS) with detection limit of 0.001mg/kg. Prior to the analysis, calibration was done with standard of known concentrations. Dissolved Mn were determined by aspirating a portion of the filtered sample (without pretreatment) directly using AAS. Concentration of manganese was ascertained from the data generated by the AAS and expressed in mg/kg.

Cobalt

After acid digestion of the sample, the concentration of Cobalt (Co) in the samples were determined using Atomic Absorption Spectrophotometry (ASTM D3558) method. This was carried out using PG AA500 Atomic Absorption Spectrophotometer (AAS) with detection limit of 0.001mg/kg. Prior to the analysis, calibration was done with standard of known concentrations. Dissolved Co were determined by aspirating a portion of the filtered sample (without pretreatment) directly using AAS. Concentration of cobalt was ascertained from the data generated by the AAS and expressed in mg/kg.

Vanadium

After acid digestion of the sample, the concentration of Vanadium (V) in the samples were determined using Atomic Absorption Spectrophotometry (ASTM D3373) method. This was carried out using PG AA500 Atomic Absorption Spectrophotometer (AAS) with detection limit of 0.001mg/kg. Prior to the analysis, calibration was done with standard of known concentrations. Dissolved V were determined by aspirating a portion of the filtered sample (without pretreatment) directly using AAS. Concentration of vanadium was ascertained from the data generated by the AAS and expressed in mg/kg.

Silver

After acid digestion of the sample, the concentration of silver (Ag) in the samples were determined using Atomic Absorption Spectrophotometry (ASTM D3866) method. This was carried out using PG AA500 Atomic Absorption Spectrophotometer (AAS) with detection limit of 0.001mg/kg. Prior to the analysis, calibration was done with standard of known concentrations. Dissolved Ag were determined by aspirating a portion of the filtered sample (without pretreatment) directly using AAS. Concentration of silver was ascertained from the data generated by the AAS and expressed in mg/kg.

Mercury, Arsenic, Aluminium, Selenium, Molybdenum and Tin

Heavy metal analysis was conducted using Varian AA240 Atomic Absorption Spectrophometer according to the method of APHA (1995).

Working principle: Atomic absorption spectrometer's working principle is based on the sample being aspirated into the flame and atomized when the AAS's light beam is directed through the flame into the monochromator, and onto the detector that measures the amount of light absorbed by the atomized element in the flame. Since metals have their own characteristic absorption

wavelength, a source lamp composed of that element is used, making the method relatively free from spectral or radiational interferences. The amount of energy of
the characteristic wavelength absorbed in the flame is proportional to the concentration of the element in the sample.

Digestion

2g of the sample was weighed into a crucible and put into a muffle furnance for ashing at a temperature of 450^0C for 2hours. The sample was removed from the furnance and allowed to cool. The dry ash was emptied into a 250ml beaker 20ml of 20% H_2SO_4 was added, heated in a water bath for 20mins, filtered and made up to 50ml with distilled water and stored in a sample bottle for AAS macro and micro nutrient analysis.

Preparation of Reference Solution:

A series of standard metal solutions in the optimum concentration range was prepared, the reference solutions were prepared daily by diluting the single stock element solutions with water containing 1.5ml concentrated nitric acid/litre. A caliberation blank was prepared using all the reagents except for the metal stock solutions.

Caliberation curve for each metal was prepared by plotting the absorbance of standards versus their concentrations**.**

3.6 Soil Microbiological Enumeration

The soil microbiological enumeration was done on the unpolluted soil (agricultural soil) by carrying out a ten-fold serial dilution using normal saline on the soil samples. 1 g of each soil samples were added in a test tube containing 10 ml of normal saline (NaCl, 0.8%) and agitated well using vortex mixer. These test tubes were considered as stock culture for the different soil

samples. 1ml volume of the stock culture collected and used in carrying out a ten-fold serial dilution. The total heterotrophic bacterial and fungal counts were enumerated by plating aliquots (0.1 ml) of appropriate diluted soil samples on nutrient agar and Sabouraud dextrose agar containing chloramphenicol (1 mg 100^{-1} ml), respectively. They were incubated aerobically at 30°C and counted after 48 and 96 h for bacteria and fungi (Omotayo *et al*., 2012).

Actinomycetes were enumerated following the method described by Bizuye *et al.* (2013) and Salim *et al*. (2017). 0.1ml of the ten-fold serial dilution was taken and spread evenly with sterile L-shaped glass rod over the surface of sterile starch casein plates aseptically using spread plating technique. Amoxicillin (20 µg/mL) and nystatin75µg/mlwere added to the media to inhibit bacterial and fungal contamination, respectively. The plates were incubated aerobically at 30°C for 5 d. Actinomycetes on the plates were counted and its colony forming unit determined after incubation.

The nitrogen fixing bacterial counts were estimated using the method described by Bhavna *etal*. (2019) and Omotayo *et al*. (2012). 0.1ml of the ten-fold serial dilution was taken and inoculated on to Ashby's mannitol agar. The plates were incubated aerobically at 30°C for 7 d. Nitrogen fixers were counted after incubation and its colony forming unit determined.

3.7Isolation of Bacteria

Bacteria were isolated from the polluted soil using an enrichment culture technique described by Liu *et al*., (2010). Two hundred and fifty millilitre Erlenmeyer flasks containing 100 ml of sterile mineral salts medium, composed of NaCl (30 g /1), $K_2HPO_4.3H_2O$ (1.0 g /L), KH_2PO_4 (1.0 g /L), NH_4NO_3 (1.0 g /L), $MgSO_4.7H_2O$ (0.2 g L/1), $CaCl_2.2H_2O$ (0.02 g L/1) and $FeCl_3$ (0.05 g L/1), 0.2ml of crude oil and 1g of contaminated soil (its gas chromatography and physicochemical

parameters were analysed prior to isolation) were incubated in a rotary incubator at 130 revolutions per minute (rpm) at 30°C for 10 days. Later, 5 ml inoculum was transferred to a fresh MSM and incubated for another cycle. 1 ml of the culture was serially diluted in sterile saline solution (0.85% NaCl) after six successive transfers. A 100 µL of the appropriate dilutions were plated onto sterile MSM agar plates. The MSM was added with sterile crude oil and agitated before dispensing into plates. These were then incubated at room temperature for 7 days. Pure colonies were obtained and stored in nutrient agar slants at 4°C for characterization and various analysis.

3.8Artificial Contamination of Soil with Crude Oil

The two agricultural soil samples were thoroughly mixed with sterile crude oil to achieve 5% artificial contamination in a plastic bowl. 5% spiking was adopted to achieve severe contamination because beyond 3% concentration, oil has been reported to be increasingly deleterious to soil biota and crop growth (Osuji *et al*., 2005; Chukwu and Udoh, 2014). The crude oil was sprayed in such a way that the whole soil would be polluted homogeneously. The soils water holding capacity was at 30% during pollution so as to stimulate thorough mixing of the crude oil with the soil (Akpoveta *et al*., 2011). Following a modified method of Chorom *et al*. (2010) who left the soil undisturbed for 3 days, the soils were left undisturbed for 30 days. At the end of the 30 days the physicochemical analysis was repeated and microbiological analysis done. The bacteria isolate in the soil werecharacterized using metagenomics while the fungi were identified using atlas. The gas chromatography profile was analysed before embarking on remediating the soils with microorganisms.

3.9 Characterization and Identification of the Isolates

The isolated organismswere characterized using morphological, biochemical and molecular methods.

3.9.1 Morphological Characterization

3.9.1.1 Colonial characteristics

Pigmentation and morphological properties such as colour, elevation, edge, surface and optic characteristics were observed after incubation.

3.9.1.2 Gram staining

This was carried out as done by Rollins and Joseph (2000). This technique divides bacterial species into Gram positive and Gram negative groups. A smear of the culture was made on clean dry grease-free glass slide, using a sterile wire loop. The smear was air-dried and heat-fixed by passing over Bunsen burner flame three times. After cooling, the slide was flooded with 0.5% crystal violet solution and left for a minute. It was washed off with water, and then flooded with lugol iodine (which served as a mordant that fixes the dye inside the cell). The iodine was washed off after one minute and 95% ethanol was used to decolourize the smear for 10seconds. The smear was counter-stained with dilute safranin (0.25%) dye for 30 seconds. It was then washed off and the slide air-dried, a drop of immersion oil was placed on it and observed under the microscope using oil immersion objective lens. Gram positive and negative reactions were indicated by blue and red colours respectively.

3.9.2 Biochemical Characterization

Pure colonies of the bacterial isolates were characterized and identified using Bergey's manual of Determinative Bacteriology authored by Holt *et al*. (1994). Some biochemical tests were carried out to aid in the identification. They were motility test, catalase test, citrate test, Indole test, methyl red, urease test, hydrogen sulphide production test and sugar fermentation test.

3.9.2.1 Motility test

The method of Cheesbrough (2000) was used. A directional and purposeful movement of the organisms demonstrate motility. Nutrient broth was supplemented with 0.2% agar (HKM, Guangdong), dispensed into test tubes and sterilized by autoclaving at 121^0C and 15psi for 15 minutes. The inoculated test tubes were incubated for 24 hours. Diffused growth, which spreads throughout the medium, indicated motility. Non-motile organisms grew along the line of incubation.

3.9.2.2 Catalase test

The method of Cheesbrough (2000) was used. The test identifies organisms that produce the enzyme catalase from those that do not possess the enzyme. A drop of 30% freshly prepared hydrogen peroxide (3ml H_20_2 in 7ml H_20) was placed on a clean slide. A loopful of isolate was transferred into it and emulsified. The appearance of gas bubbles (effervescence) indicated the presence of catalase which was a positive test. Non release of bubbles indicated a negative reaction.

3.9.2.3 Citrate test

The method of Aryal (2019) was used. The test was used to determine the organisms that could utilize citrate as a sole-carbon source for metabolism. Slants of Simmon's Citrate Agar (Accumix, India) were prepared according to the manufacturer's instructions. The slants were inoculated by streaking over the surface with a loopful of an 18 hours old culture and incubated at 37^0C for 48 hours. Positive results were indicated by a change in colour from green to blue while the absence of colour change indicated a negative result.

3.9.2.4 Indole test

The method of Cheesbrough (2000) was used. The tryptone-broth was prepared and 5ml was dispensed into each tube and sterilized. Isolates were inoculated into the tube and incubated at 28^0C for 48 hours. Five drops of Kovac's reagent (4 p-dimethy-amino benzaldhyde) were added to the tubes, gently shaken and allowed to settle. A red colouration in the alcohol dye indicated a positive result.

3.9.2.5 Methyl red

The method of Mcdevitt (2009) was used. Sterile tubes of buffered glucose peptone broth (1.5% peptone water, 0.5% glucose and 0.5% dipotassium phosphate) were inoculated and incubated at 37^oC for 48 hours. Then 5 drops of methyl red reagent were added to 5ml of each of the culture. The production of bright red colour immediately on the addition of the reagent showed a positive result.

3.9.2.6 Urease test

The method of Tankeshwar (2012) was used. The medium for carrying out the urease test was prepared following the manufacturer's instruction (Himedia). 24g of the urease agar was

dissolved in 950ml of distilled water in a conical flask. It was heated and autoclaved at 115°C for 10 minutes. It was allowed to cool to 45°C, then 50ml of 40% filter sterilized urea was added to it, shaken and dispensed into test tubes. The tubes were slanted and allowed to cool. The slants were inoculated by streaking over the surface with a loopful of a 48-hour old culture and incubated at 30^{0}C for 48 hours. Positive results were indicated by a change in colour from yellow to pink while the absence of colour change indicated a negative result.

3.9.2.7 Hydrogen sulphide production test

The method of Aryal (2019) was used. Hydrogen sulphide (H_2S) production test is used for the detection of hydrogen sulphide gas produced by an organism. Peptone water (Accumix, India) was manufactured according to manufacturer's instruction. It was inoculated with the organism. Lead acetate paper strip was inserted in the neck of the test tube above the medium and stopped well. It was then incubated at 30°C and examined daily for blackening of the lower part of the strip which indicated a positive test for gas production.

3.9.2.8 Sugar fermentation test

The method of Aryal (2020) was used. The test determined the ability of isolates to ferment glucose, sucrose, lactose, sorbitol, fructose, sucrose, raffinose and durcitol with the production of acid and gas. The fermentation medium was 1% peptone water, 5 drops of 0.2% bromothymol blue indicator solution and 1% of the sugar granule. 9ml of medium was dispensed into clean dry test tubes in which Durham tubes have been dropped (inverted and without air space) and sterilized by autoclaving at 115^{0}C for 15 minutes. It was allowed to cool and inoculated with a loopful of the test organisms and incubated at 30^{0}C for 24hrs. A change in colour of the medium

from blue to yellow was recorded as a positive reaction, while the appearance of an empty space at the end of the Durham tubes indicated gas production.

3.9.3 Molecular Characterisation

Molecular characterization of the bacterial isolates and the metagenomics analysis of bacteria in the soil was done at the laboratory of the University of Illinois at Chicago Sequencing Core (UICSCQ). The molecular characterization involved; DNA extraction for bacterial isolates stored in DNA/RNA shield, Polymerase Chain Reaction (PCR) amplification and Sanger Sequencing.

3.9.3.1 Sanger sequencing of bacterial isolates

Genomic DNA from bacterial cells was extracted using an automated DNA extraction device, the Maxwell16 instrument (Promega), implementing the Maxwell® 16 Tissue DNA Purification Kit according to the manufacturer's protocol. Genomic DNA was subjected to PCR amplification with the primer set 27F (AGAGTTTGATCMTGGCTCAG) / 1492R (GGTTACCTTGTTACGACTT) using DreamTaq Green PCR Master Mix (2X) (ThermoFisher). PCR-amplified DNA was purified withAMPure XP beads (0.6X) to remove unused primers and dNTPs. Amplified genomic DNA was sequenced on an ABI 3730xl capillary sequencer using the 27F primer to initiate the sequencing reaction. Sequence data was trimmed to remove poor quality bases and sequences analyses were performed using the NCBI BLAST software, the SILVA on-line aligner, and the software package MEGA.

3.9.3.2 Microbial genomics analysis of bacteria in the artificially polluted soils

The workflow for this involves: DNA extraction from soil, PCR amplification and next generation sequencing (NGS) using an Illumina MiniSeq sequencer.

NGS amplicon sequencing of the soil samples

Genomic DNA was extracted from soil using the DNeasy PowerSoil Kit (Qiagen), implemented on a QIAcube automated extraction device. Genomic DNA was prepared for next-generation amplicon sequencing using a two-stage PCR protocol to generate amplicons with Illumina sequencing adapters and a sample-specific barcode.

Specifically, genomic DNA was PCR amplified with primers CS1_515F and CS2_806R (Walters *et al*., 2016; Apprill *et al*., 2015; Parada *et al*., 2016) targeting the V4 regions of microbial small subunit ribosomal RNA genes using a two-stage "targeted amplicon sequencing (TAS)" protocol (Naqib *et al*., 2018 and Bybee *et al*., 2011). The primers contained 5' common sequence tags (known as common sequence 1 and 2, CS1 and CS2) as described previously (Moonsamy *et al*., 2013). First stage PCR amplifications were performed in 10 microliter reactions in 96-well plates, using the MyTaq HS 2X mastermix. PCR conditions were 95°C for 5 minutes, followed by 28 cycles of 95°C for 30", 55°C for 45" and 72°C for 30".

Subsequently, a second PCR amplification was performed in 10 microliter reactions in 96-well plates. A mastermix for the entire plate was made using the MyTaq HS 2X mastermix. Each well received a separate primer pair with a unique 10-base barcode, obtained from the Access Array Barcode Library for Illumina (Fluidigm, USA). These AccessArray primers contained the CS1 and CS2 linkers at the 3' ends of the oligonucleotides. Cycling conditions were as follows: 95°C for 5 minutes, followed by 8 cycles of 95°C for 30", 60°C for 30" and 72°C for 30". A final, 7-minute elongation step was performed at 72°C. Samples were pooled in equal volume using an EpMotion5075 liquid handling robot (Eppendorf, Hamburg, Germany). The pooled library was

purified using an AMPure XP (Agencourt, Beckmann-Coulter) to remove fragments smaller than 300 bp. The pooled libraries, with a 20% phiX spike-in, were loaded onto an Illumina MiniSeq mid-output flow cell (paired-end reads). Based on the distribution of reads per barcode, the amplicons (before purification) were pooled to generate a more balanced distribution of reads. The pooled library was purified using AMPure XP, as described above. The pooled libraries, with a 15% phiX spike-in, were loaded onto a second MiniSeq mid-output flow cell and sequenced to generate additional data. Fluidigm sequencing primers, targeting the CS1 and CS2 linker regions, were used to initiate sequencing. De-multiplexing of reads was performed on instrument. Library preparation, pooling, and MiniSeq sequencing were performed at the University of Illinois at Chicago Sequencing Core (UICSQC).

3.9.4Characterisation of Fungi

3.9.4.1 Slide culture preparation

This was done following a method described in Devi (2011). An already prepared and autoclaved Sabourand Dextrose Agar (SDA) was pipetted with a sterile string and transferred acceptically to a sterile slide in drops. A pure culture of the fungal isolate was inoculated to each slide and covered with a cover slip. The slide was laid on a Petridish supported by a sterile folded u-shaped filter paper. It was incubated without inverting the position at a temperature of 30^oC for 5 days to allow the organism to grow very well.

3.9.4.2 Microscopic examination of the slide culture

A fresh slide was flooded with few drops of lactophenol cotton blue, the cover slip was removed from the slide culture and laid on the lactophenol cotton blue slide (Devi, 2011). It was thereafter

viewed under x40 objective lens. Fungi Atlas by Kidd *et al*. (2016) was then used in the identification.

3.10Screening Tests for the Ability of the Isolates to Utilize Crude Oil

3.10.1 Analysis of the ability of the isolates to degrade different concentrations of crude oil using gravimetric method

A method of Palanisamy *et al*. (2014) and Latha and Kalaivani (2012) was used. The isolates that showed ability to produce biosurfactants were inoculated into 100ml of conical flask containing 20ml of sterile mineral salt medium supplemented with different concentrations of crude oil i.e. 100ul, 500ul, 1000ul and 2000ul. Control flask contained no organism. Incubation was done at 130rpm in a rotary shaker at 30°C. The experimental set up was allowed to last for 14 days. At the end of the incubation period, the residual crude oil was extracted in a separating funnel using n-Hexane as the solvent. The supernatant which contained the n-Hexane and the residual hydrocarbon was transferred to a glass Petridish. The Petridish were lined up in an oven set at 50°C to allow the solvent to evaporate, after which the weight of the Petridish was taken.

The % of degradation was calculated as follows;

Weight of Residual crude oil= Weight of Petridish containing extracted crude oil – Weight of empty Petridish.

Residual oil = oil applied – oil recovered

$$\% \text{ oil degradation} = \frac{\text{oil applied} - \text{oil recovered}}{\text{Oil applied}} \times 100$$

3.10.2 The use of the isolates growth profile to study its ability to degrade crude oil

A method of Al-wasify and Hamed (2014) was used to determine the growth profile of *Gordonia alkanivorans* and *Tsukamurella inochensis*. The two isolates that showed an ability to produce biosurfactants were inoculated into 250ml conical flask containing 100ml of sterile mineral salt medium supplemented with 2mls of crude oil. There was also a flask for the mixed culture of the two isolates while control flask had no organism. Incubation was done at 130rpm in a shaker at 30^oC. The pH and optical density were checked at 7 days' interval for 28 days. The set up were analysed for Total Petroleum Hydrocarbon (TPH) and Polycyclic Aromatic Hydrocarbon (PAH) using gas chromatography after 28 days.

3.11Biosurfactant Screening

The resultant colonies were screened for its ability to produce biosurfactants using different methods, namely the oil displacement, drop collapse, haemolysis and emulsification test.

3.11.1 Oil displacement assay: The oil displacement assay developed by Morikawa *et al*. (2000) was used. 10ul of crude oil were added to the surface of 40 ml of distilled water in a Petri dish to form a thin oil layer. Then, 10ul of culture supernatant were gently placed on the centre of the oil layer. The presence of biosurfactant in the supernatant displaced the oil and a clearing zone was formed.

3.11.2 Drop collapse test: Crude oil was used in this test. Two microlitres of oil was applied to the well regions delimited on the covers of 96-well micro plates and these were left to equilibrate for 24 h. Five micro liters of the 72 h culture after centrifugation at 4000rev for 15 min to remove cells, were transferred to the oil-coated well regions and drop size was observed after 1 min with the aid of a magnifying glass as done by Saravanan and Vijayakumar(2012). The result

was considered positive for biosurfactant production when the drop was flat and those cultures that gave rounded drops were scored as negative, indicative of the lack of biosurfactant production as described by Youssef *et al*. (2004).

3.11.3 Emulsification test (E24): This was carried out as done by Bodour *et al*. (2004). Several colonies of pure culture were suspended in test tubes containing 2 ml of mineral salt medium and after 48 h of incubation, 2 ml hydrocarbon (oil) were added to each tube. Then, the mixtures were vortexed at 3200rpm for 1 min and allowed to stand for 24 h. The emulsion index (E24) is the height of the emulsion layer (cm) divided by total height (cm), multiplied by 100.

Emulsification index (E24) = Height of the emulsion layerX100

Total height

3.11.4 Haemolysis activity: Pure culture of bacterial isolates were streaked on the freshly prepared blood agar and incubated at 37°C for 48h as described by Saravanan and Vijayakumar(2012). Positive strains caused lysis of the blood cells and exhibited a colorless, transparent ring around the colonies.

3.12 Total Petroleum hydrocarbon and Polycyclic Aromatic Hydrocarbon analysis

3.12.1 Total Petroleum Hydrocarbon (TPH) and Polycyclic Aromatic Hydrocarbon analysis (PAH) of crude oil and residual oil after 28 days' degradation

TPH and PAH analysis was done at Halden Laboratories, Port-Harcourt.

20ml of each sample was extracted twice with 2ml dichloromethane. The extracts were then fractionated into aliphatic and aromatic components by column chromatography.

Total petroleum hydrocarbon (TPH) was analyzed using Agilent 6890 GC-FID as described by USEPA(2003).

Polycyclic Aromatic hydrocarbon (PAH) was analyzed using Agilent 7890 GC-MS as described by USEPA(2018).

3.12.2 Total Petroleum Hydrocarbon and Polycyclic Aromatic Hydrocarbon analysis of the soil samples

10g of soil sample was extracted by the ultrasonic extraction method using 20ml dichloromethane according to the USEPA 3550C (USEPA, 2007). The extract was then cleaned and concentrated.

Total petroleum hydrocarbon (TPH) was analyzedby injecting 1µl of the extract into the Agilent 6890GC-FID for analysis according to USEPA 8015 method (USEPA, 2003). The concentration in mg/kg was deduced from the calibration graph.

Polycyclic Aromatic hydrocarbon (PAH) was analyzed by injecting 1µl of the sample extract into the Agilent 7890 GC-MS for analysis according to USEPA 8270 (USEPA, 2018).

3.13 Inoculum Development

Following A modified method of Patowary *et al*., (2016) inoculum development was done with mineral salt medium. One loopful of the biosurfactant producing isolate was inoculated into a 250ml flask containing 100ml of sterile mineral salt medium supplemented with 1ml of crude oil. Incubation was done at 130rpm at 30°C for 7days. The medium after incubation contained the organism and its biosurfactant which were inoculated into the soil. A tenfold serial dilution

was carried out using normal saline (0.85%), it was plaited out on nutrient agar and incubated at 30°C for 48 hrs. The plates were then counted and the colony forming unit was determined.

3.14Bioremediation of Soil

The crude oil polluted soil samples were divided into four parts each prior to inoculation. Two parts each were inoculated with the two bacterial isolates; one part for their mixed culture while the remaining part without the tested isolates stood as control. Each soil application was sprayed with the liquid culture of the inoculum development using a syringe (20mls/500g). The control was not sprayed (Steven *et al*., 2000). The initial concentration of total petroleum hydrocarbon and polycyclic aromatic hydrocarbon in the soil was determined using gas chromatography. The polluted soil in the various treatment containers (i.e. plastic bowl) were turned twice a week to provide the necessary aeration and to facilitate mixing of nutrients and microbes with the contaminated soil as described by Ayotamuno *et al*. (2006). The water content was adjusted with distilled water by adding 45% of each soil's water holding capacity at three days' interval as described by Baldrian *et al*. (2000). The pH and soil microbial count were determined at 4 weeks' intervals. The remediation exercise lasted for five (5) months as described by mehrasbi *et al*. (2003). The extent of crude oil degradation was analysed at the end of bioremediation using gas chromatography to check for total petroleum hydrocarbon and polycyclic aromatic hydrocarbon. The soil physicochemical analysis (Electrical conductivity, salinity, CEC, heavy metals, nitrogen, carbon, phosphorus, water permeability, odour and texture) was also done. The odour was determined by perceiving the soil sample while the texture was determined by rubbing it between the palms.

3.15Planting on Remediated Soil

At the end of the degradation activity, the soils were planted with bean seed to know if they can support seed germination while the unpolluted soil served as control. The time of germination, the number of leaves, stem and root length were determined using a centimeter metre ruler for 14 days as described by Okoye and Okunrobo (2014). The root length was determined by uprooting the plant after 14 days.

3.16Statistical Analysis

The statistical analysis was carried out to determine the significance difference of TPH and PAH degradation using one way Anova.

CHAPTER FOUR

RESULTS

4.1 Physicochemical Properties of the Polluted and Unpolluted Soil Samples

4.1.1Physicochemical Properties of the Polluted Soil Sample for Microbial Isolation

The pH reading of the soil from Ibeno polluted with crude oil for microbial isolation was 6.9. Nitrogen, carbon and phosphorus values were 2.016%, 2.55% and 5.98% respectively. The electrical conductivity was 71.5, salinity was 0.1987ppt, CEC was 24.4803cmol/kg, vanadium was 0.385mg/kg, lead was 0.712mg/kg, chromium was 0.536mg/kg, zinc was 13.533mg/kg, cadmium was 0.002mg/kg, iron was 208.38mg/kg, manganese was 5.738mg/kg, copper was 0.590mg/kg, cobalt was 0.00mg/kg, silver was 0.008mg/kg, selenium was 0.523mg/kg,

molybdenium was 0.098mg/kg, aluminium was 0.244mg/kg, tin was 0.00mg/kg, mercury was 0.039ppm, arsenic was 0.284ppm. The soil sample contained more of sand (88.26%) than clay (6.34%) and silt (5.4%). These are presented in Table 4.1.

Table 4.1: Physicochemical properties of the polluted soil sample for microbial Isolation

Parameters	Value
pH	6.9
Electrical conductivity,us/cm	71.5
Salinity, ppt	0.1987
Cation Exchange Capacity ,cmol/kg	24.4803
Vanadium, mg/kg	0.385
Lead, mg/kg	0.712
Chromium, mg/kg	0.536
Zinc, mg/kg	13.533
Cadmium, mg/kg	0.002

Iron, mg/kg	208.38
Manganese, mg/kg	5.738
Copper, mg/kg	0.590
Cobalt, mg/kg	0.00
Silver, mg/kg	0.008
Selenium, mg/kg	0.523
Molybdenium, mg/kg	0.098
Aluminium, mg/kg	0.244
Tin, mg/kg	0.00
Mercury, ppm	0.039
Arsenic, ppm	0.284
Nitrogen, %	2.016
Carbon,%	2.55
Phosphorus, %	5.98
Sand,%	88.26
Silt, %	5.4
Clay,%	6.34

4.1.2Result of Physicochemical Properties of Ibeno soil before and after Crude Oil Pollution

The results of the physicochemical properties of Ibeno before and after crude oil pollution showed that the pH was 5.900 before pollution and 6.190 after pollution. Particulate size showed that Ibeno soil contained more sand and silt. Other physicochemical parameters result of Ibeno soil before and after pollution are95.400and 5.410us/cm for Electrical conductivity (EC), 160

and 4.100mg/kg for salinity, 0.883 and 3.745cmeq/100g for Cation Exchange Capacity (CEC), 0.336 and 0.112% for nitrogen, 0.051 and 1.077% for carbon and 7.820 and 32.590mg/kg for phosphorus. The heavy metal values before and after crude oil pollution measured in mg/kg were <0.050and <0.001 for vanadium, 0.413 and 0.343 for lead, 0.730 and 0.160 for chromium, 12.100 and 1.332 for zinc, <0.050 and 0.020 for cadmium. 139 and 23.170 for iron, 15.700 and 1.674 for manganese, 0.400and 0.060 for copper, 4.790 and <0.050 for cobalt, 1.080 and 0.107 for silver, 0.272 and 0.273 for selenium. 0.590 and 0.075 for nickel, 0.183 and 0.176 for aluminium, 0.000was the result gotten for tin and molybdenium before and after pollution. The heavy metals measured in ppm before and after pollution were 0.450 and 1.039 for mercury, 0.184 and 2.281 for arsenic. The percentage water holding capacity was 4.130%. After pollution, the soil sample had a crude oil smell; the soil texture was loose while the water permeability was negative. These are presented in Table 4.2.

Table 4.2: Result of physicochemical properties of Ibeno soil before and after crude oil pollution

Parameters	Before pollution	After pollution
pH	5.900	6.190
Electrical conductivity,us/cm	95.400	5.410
Salinity, mg/kg	160	4.100
Cation Exchange Capacity, meq/100g	0.883	3.745
Vanadium, mg/kg	<0.050	<0.001
Lead, mg/kg	0.413	0.343
Chromium, mg/kg	0.730	0.160
Zinc, mg/kg	12.100	1.332
Cadmium, mg/kg	<0.050	0.020

Iron, mg/kg	139	23.170
Manganese, mg/kg	15.700	1.674
Copper, mg/kg	0.400	0.060
Cobalt, mg/kg	4.790	<0.050
Silver, mg/kg	1.080	0.107
Selenium, mg/kg	0.272	0.273
Molybdenium, mg/kg	0.000	0.000
Nickel mg/kg	0.590	0.075
Aluminium, mg/kg	0.183	0.176
Tin, mg/kg	0.000	0.000
Mercury, ppm	0.450	1.039
Arsenic, ppm	0.184	2.281
Nitrogen, %	0.336	0.112
Carbon, %	0.051	1.077
Phosphorus, mg/kg	7.820	32.590
Sand, %	86.968	-
Silt, %	40.384	-
Clay, %	8.648	-
Water holding capacity, %	4.130	-
Water permeability	Positive	negative
Odour	no crude oil	crude oil
Texture	bound	loose

4.1.3 Result of physicochemical properties of Otuocha soil before and after crude oil pollution

The results of the physicochemical properties of Otuocha soils before and after crude oil pollution showed that pH was 6.100 and 5.970 respectively. Particulate size showed that Otuocha soil contained more clay than silt and sand.Other physicochemical parameters resultof Otuochabefore and after pollution were 74.500 and 19.130us/cm for EC, 42.300 and 12.600mg/kg for salinity, 0.494 and 2.561 meq/100g for CEC, 0.672 and 0.336% for nitrogen, 0.126 and 0.329% for carbon and 8.790 and 38.520mg/kg for phosphorus. The heavy metal

values before and after pollution measured in mg/kg were <0.050 and <0.001 for vanadium, 0.576 and 0.700 for lead, 0.670 and 1.607 for chromium, 6.40 and 0.689 for zinc, 0.050 and 0.031 for cadmium. 148 and 25.590 for iron, 118 and 12.080 for manganese, 1.61 and 0.209 for copper, 35.5 and <0.711 for cobalt, 0.690 and 1.618 for silver, 0.102 and 0.373 for selenium. 0.022 and 0.00 for molybdenium, 1.630 and 0.267 for nickel, 0.018 and 1.406 for aluminium, 0.000 for tin before and after pollution. The heavy metals measured in ppm before and after pollution were 0.000 and 0.216 for mercury, 0.083 and 1.518 for arsenic. The percentage water holding capacity was 13.66%. After pollution, the soil sample had a crude oil smell; the soil texture was loose while the water permeability was negative. These are presented in Table 4.3.

Table 4.3: Result of physicochemical properties of Otuocha soilsbefore and after crude oil pollution

Parameters	Before pollution	After pollution
pH	6.100	5.970
Electrical conductivity,us/cm	74.500	19.130
Salinity, mg/kg	42.300	12.600
Cation Exchange Capacity, meq/100g	0.494	2.561
Vanadium, mg/kg	<0.050	<0.001
Lead, mg/kg	0.576	0.700
Chromium, mg/kg	0.670	1.607
Zinc, mg/kg	6.400	0.689
Cadmium, mg/kg	0.050	0.031
Iron, mg/kg	148	25.590

Manganese, mg/kg	118	12.080
Copper, mg/kg	1.610	0.209
Cobalt, mg/kg	35.500	<0.711
Silver, mg/kg	0.690	1.618
Selenium, mg/kg	0.102	0.373
Molybdenium, mg/kg	0.022	0.000
Nickel mg/kg	1.630	0.267
Aluminium, mg/kg	0.018	1.406
Tin, mg/kg	0.000	0.000
Mercury, ppm	0.000	0.216
Arsenic, ppm	0.083	1.518
Nitrogen, %	0.672	0.336
Carbon, %	0.128	0.329
Phosphorus, mg/kg	8.790	38.520
Sand, %	84.345	-
Silt, %	4.600	-
Clay, %	11.260	-
Water holding capacity, %	13.660	-
Water permeability	positive	negative
Odour	not distinct	crude oil
Texture	bound	loose

4.2 Microbiological Analysis of the Soil Samples

4.2.1Microbiological Enumeration of Ibeno Soil Samples before and after Crude Oil Pollution (cfu/g)

The microbiological enumerations of Ibeno soil samples before and after crude oil pollution are presented in table 4.4. Bacteria, fungi, nitrogen fixers and actinomycetes were enumerated. Bacteria values were$3.00x10^{7} \pm 0.02$before pollution and $3.70x10^{5} \pm 0.02$after pollution. Nitrogen

fixers were $0.85x10^7 \pm 0.02$before pollution and $0.63x10^5 \pm 0.02$after pollution. Fungi values were $0.03x10^7 \pm 0.02$before pollution and $0.32x10^5 \pm 0.02$after pollution. Actinomycetes were $0.60x10^7 \pm 0.02$before pollution and $0.38x10^5 \pm 0.02$after pollution.

Table 4.4: Microbiological enumeration of Ibeno soil samples before and after crude oil pollution (cfu/g)

Organism	before pollution	after pollution
Bacteria	$3.00x10^7 \pm 0.02$	$3.70x10^5 \pm 0.02$
Fungi	$0.03x10^7 \pm 0.02$	$0.32x10^5 \pm 0.02$

Nitrogen fixers	$0.85x10^7 \pm 0.02$	$0.63x10^5 \pm 0.02$
Actinomycetes	$0.60x10^7 \pm 0.02$	$0.38x10^5 \pm 0.02$

Values are mean $\pm$ S.D. of duplicate determination

4.2.2Microbiological Enumeration of Otuocha Soil Samples before and after Crude Oil Pollution (cfu/g)

Microbiological enumerations of the soil samples after crude oil pollution are presented in table 4.5. Bacteria count before pollution was $3.40x10^7 \pm 0.3$while after pollution was $8.60x10^5 \pm 0.4$. Fungi count was $0.03x10^7 \pm 0.2$ before pollution and $0.30x10^5 \pm 0.2$ after pollution. Nitrogen fixers were $2.70x10^7 \pm 0.3$before pollution and $0.96x10^5 \pm 0.2$ after pollution. Actinomycetes were $0.62x10^7 \pm 0.2$ before pollution and $0.49x10^5 \pm 0.2$after pollution.

Table 4.5: Microbiological enumeration of Otuocha soil samples before and after crude oil pollution (cfu/g)

Organism	before pollution	after pollution
Bacteria	$3.40 \times 10^7 \pm 0.3$	$8.60 \times 10^5 \pm 0.4$
Fungi	$0.03 \times 10^7 \pm 0.2$	$0.30 \times 10^5 \pm 0.2$
Nitrogen fixers	$2.70 \times 10^7 \pm 0.3$	$0.96 \times 10^5 \pm 0.2$
Actinomycetes	$0.62 \times 10^7 \pm 0.2$	$0.49 \times 10^5 \pm 0.2$

Values are mean $\pm$ S.D. of duplicate determination

4.2.3Morphological and Biochemical Characterisation of Isolated Bacteria

Morphological and biochemical characterisation of the isolates S2 and S13 are shown in Table 4.6. S2 appeared pink in colour, smooth and circular in texture and shape with a raised elevation. S13 appeared cream in colour with a dry/rough texture, irregular in shape and flat elevation. Both are gram positive. S2 were small cocci in chains while S13 were bacilli that appeared singly. They both gave a negative result to indole, methyl red, oxidase, mannose, galactose, sorbitol and both were non-motile. They both gave a positive result to catalase and they fermented fructose. They differed in their reaction to citrate, hydrogen sulphide, urease test, sucrose and glucose. S2 gave a negative result to citrate, hydrogen sulphide production test, urease test, sucrose and glucose fermentation test while S13 gave a positive result to the parameters.

Table 4.6: Morphological and biochemical characterization of isolated bacteria

	S2	S13
Colour	Pink	Cream
Texture	Smooth	Dry/rough
Shape	Circular	Irregular
Elevation	Raised	Flat
Gram reaction	+ve	+ve
Microscopic appearance	Small cocci in chains	Bacilli appearing singly
Citrate	-	+
Indole	-	-
Methyl red	-	-
Catalase	+	+
Oxidase	-	-
Hydrogen sulphide	-	+
Urease	-	+
Motility	-	-
Sucrose	-	+
Fructose	+	+
Glucose	-	+
Lactose	-	+
Mannose	-	-
Galactose	-	-
Sorbitol	-	-
	Gordonia sp	*Tsukamurella sp*

+ = positive, - = negative

4.2.4 Sequence Analysis

The sequence analysis of the two test bacterial isolates are presented in Figures 4.1 and 4.2. Figure 4.1 represented the sequence for *Gordonia alkanivorans* while Figure 2 represented the sequence for *Tsukamurella inochensis*.

CTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGG
AAAGGCCCAGCTTGCTGGGTACTCGAGTGGCGAACGGGTGAGTAACACGTG
GGTGATCTGCCCTGAACTTTGGGATAAGCCTGGGAAACTGGGTCTAATACCG
GATATGACCTTGGAGTGCATGCTCTGGGGTGGAAAGCTTTTGCGGTTCAGGA
TGGGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGAC
GACGGGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACG
GCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCA
AGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAAC
CTCTTTCACCAGGGACGAAGCGCAAGTGACGGTACCTGGAGAAGAAGCACC
GGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTC
CGGAATTACTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCGTCTGTGA
AATTCTGCAACTCAATTGTAGGCGTGCAGGCGATACGGGCAGACTTGAGTAC
TACAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAG
GAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGTAGTAACTGACGCTGAGG
AGCGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGGTGGGTACTAGGTGTGGGGCTCATTTCACGAGTTCCGTGCCGTAGC
TAACGCATTAAGTACCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGATTAATTCGA
TGCAACGCGAAGAACCTTACCTGGGTTTGACATACACCAGACGCATGTAGA
GATACATGTTCCCTTGTGGTTGGTGTACAGGTGGTGCATGGCTGTCGTCAGC
TCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCT
GTATTGCCAGCGGGTTATGCCGGGGACTTGCAGGAGACTGCCGGGGTCAAC
TCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGTCCAGGG
CTTCACACATGCTACAATGGCTGGTACAGAGGGCTGCGATACCGTGAGGTG
GAGCGAATCCCTTAAAGCCAGTCTCAGTTCGGATTGGGGTCTGCAACTCGAC
CCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAA
TACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAGTCGGTAAC
ACCCGAAGCCGGTGGCCTAACCCCTTGTGGGAGGGAGCTGTCGAAGGTGGG

ATCGGCGATTGGGACGAAGTCGTAACAAGGTAGCCGTACCGGAAGGTGCGG
CTG

Figure 4.1: The sequence analysis of isolate S2 (*Gordonia alkanivorans*)

CTATGGGTGCTTACACATGCAGTCGAACGGTAAGGCCCTTTCGGGGGTACAC
GAGTGGCGAACGGGTGAGTAACACGTGGGTGACCTGCCCTGTACTTCGGGA
TAAGCCTGGGAAACTGGGTCTAATACCGGATATGACCTTCTCCTGCATGGGG
GTTGGTGGAAAGCTTTTGCGGTACAGGATGGGCCCGCGGCCTATCAGCTTGT
TGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGG
CGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG
CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGT
GAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGACGAAGCGC
AAGTGACGGTACCTACAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCG
CGGTAATACGTAGGGTGCGAGCGTTGTCCGGATTTACTGGGCGTAAAGAGC
TCGTAGGCGGTTTGTCGCGTCGTCTGTGAAAACCCGAGGCTTAACCTCGGGC
CTGCAGGCGATACGGGCAGACTTGAGTACTGTAGGGGAGACTGGAATTCCT
GGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGC
GGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGTAGCGAAC
AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGTACTAGGTGTG
GGTTTCCTTCCACGGGATCCGTGCCGTAGCTAACGCATTAAGTACCCCGCCT
GGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGC
ACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACC
TGGGTTTGACATATAGAGGATCGCCGCAGAGATGTGGTTTGCCTTGTGCCTT
CTATACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTT
AAGTCCCGCAACGAGCGCAACCCTTGTCTCATGTTGCCAGCACGTTATGGTG
GGGACTCGTGAGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACG
TCAAGTCATCATGCCCCTTATGTCCAGGGCTTCACACATGCTACAATGGCGC
GTACAGAGGGCTGCGATACCGTGAGGTGGAGCGAATCCCTTAAAGCGCGTC
TCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGT
AATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACAC

CGCCCGTCACGTCATGAAAGTCGGTAACACCCGAAGCCGGTGGCCTAACCC
CTTGTGGGAGGAG CTGTCGAAGGTGGATGGCT

Figure 4.2: The sequence analysis of isolate S13 (*Tsukamurella inochensis* strain yaoman)

4.2.5 Metagenomic Analysis of the Soils Sample

The results of metagenomic analysis using Ibeno and Otuocha soil samples after crude oil pollution are presented in figures 4.3 and 4.4. From the genus-level analysis result, it could be seen that *Sphingomonas* was highest in both soil samples. *Nocardioides* was more in Otuocha soil than Ibeno soil sample. *Tetradesmus* was absent in Otuocha soil but present in Ibeno soil. Other organisms present in both soil samples were *Massilia, Phenylobacterium, Gordonia, Methylobacterium, Azospirillum, Singulisphaera, Roseomonas, Microbacterium, Bacillus, Pseudomonas, Paraburkholderia, Sphingomonas, Sphingobium, Methylorubrum, Candidatus solibacter, Aquabacterium, Tepidisphaera, Planococcus, Parviterribacter, Cellulomonas, Mycobacterium, Nocardioides, Tumebacillus* and *Hydrocarboniphaga*.

At the family-level the dominant taxa in both soil samples were Gammaproteobacteria, Oxalobacteraceae, Caulobacteraceae, Gordoniaceae, Isosphaeraceae, Rhodospirillaceae, Sphingomonadaceae, Burkholderiaceae, Acetobacteraceae, Methylobacteriaceae, Pseudomonadaceae, Microbacteriaceae, Bacteria, Acidobacteriaceae, Mycobacteriaceae,

Bacillaceae, Solibacteraceae, Tepidisphaeraceae, Planococcaceae, Cellulomonadaceae, Parviterribacteraceae, Comamonadaceae, Nocardioidaceae, Alicyclobacillaceae and Sinobacteraceae.

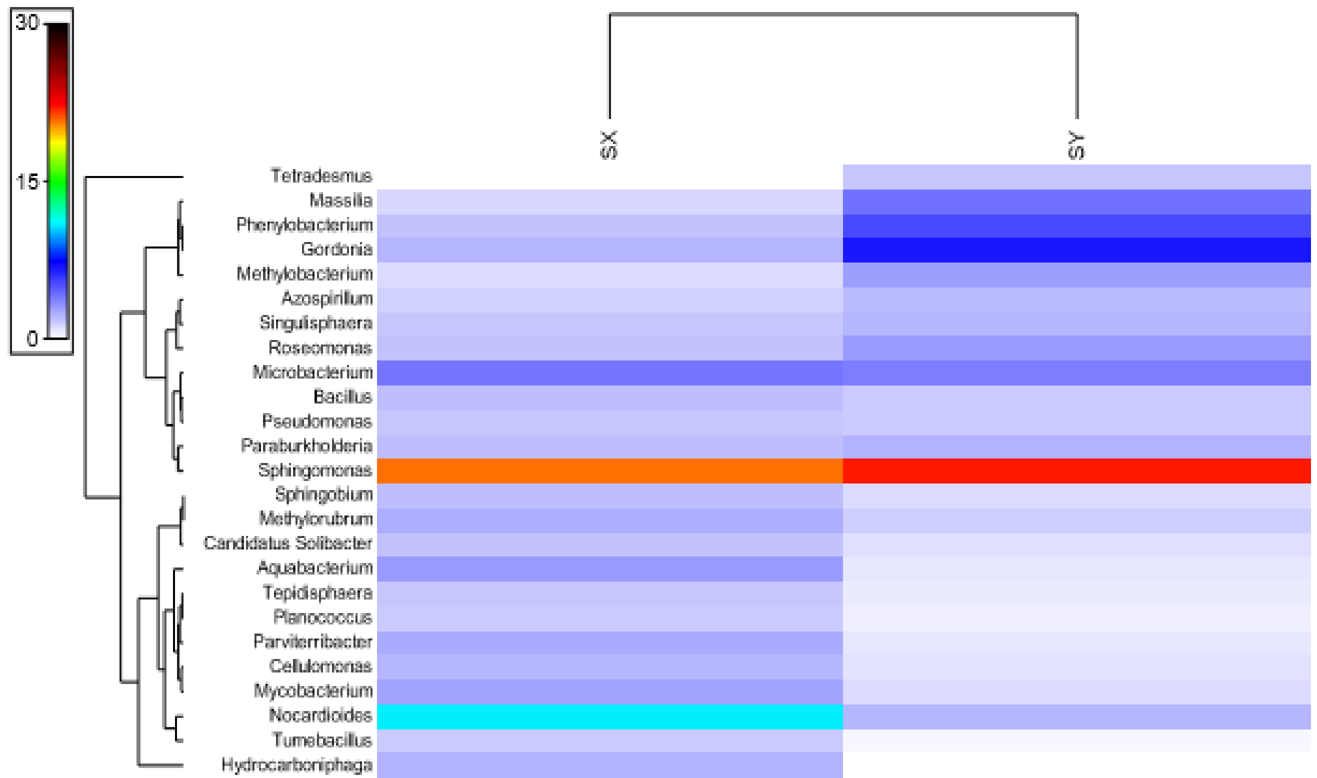


Figure 4.3: Genus-level analysis of dominant taxa in soil samples SX and SY.

SX= Otuocha soil sample

SY= Ibeno soil sample

Data are relative abundance of annotated reads in the OneCodex environment with the 'Targeted Loci' algorithm. Scale is percent relative abundance

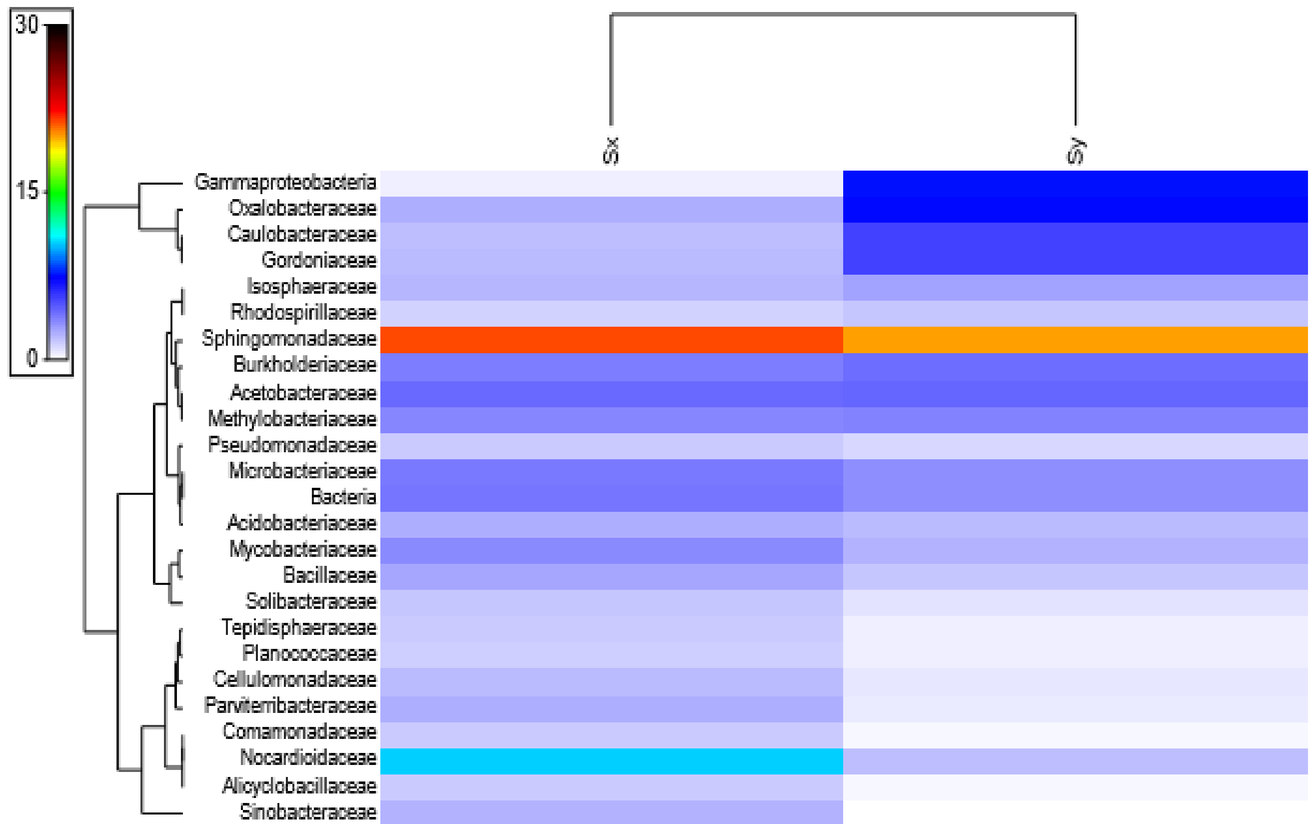


Figure 4.4: Family-level analysis of dominant taxa in soil samples SX and SY.

SX = Otuocha soil sample

SY = Ibeno soil sample

Data are relative abundance of annotated reads in the OneCodex environment with the ‘Targeted Loci’ algorithm. Scale is percent relative abundance.

4.2.6 Morphology and Microscopic Features of Fungi Isolated from the Artificially Polluted Soil

The fungi isolated from the artificial polluted soil were*Aspergillus lentulus* which was isolated from Ibeno soil sample and *Cylindrocarpon* isolated from Otuocha soil sample. They are presented in Table 4.7.

Table 4.7: Morphology and microscopic features of fungi isolated from the artificially polluted soil

organism	Morphology	Microscopic appearance	Suspected organism
IF	Whitish suede-like appearance with rumpled yellow reverse	Conidial heads are short and columnar. Conidiophore stipes are smooth-walled	*Aspergillus lentulus*
OF	White and fluffy that covers the plate within few days	Conidiophores consist of simple or repeatedly verticillate	*Cylindrocarpon*

phialides, arranged in

brush-like structures

OF=Organisms from Otuocha soil sample

IF =Organisms from Ibeno soil sample

4.3 Percentage Degradation of Crude Oil Using Gravimetric Method (%)

Figure 4.5 showed percentage degradation of different concentrations of crude oil. The percentage degradation of *Gordonia alkanivorans* at 100ul, 500ul, 1000ul and 2000ul were 33.33±0.03, 26.92±0.04, 21.28±0.03 and 15.73±0.23 respectively. *Tsukamurella inochensis* were 50.00±0.08, 42.31±0.49, 25.53±0.05 and 17.98±0.12 respectively.

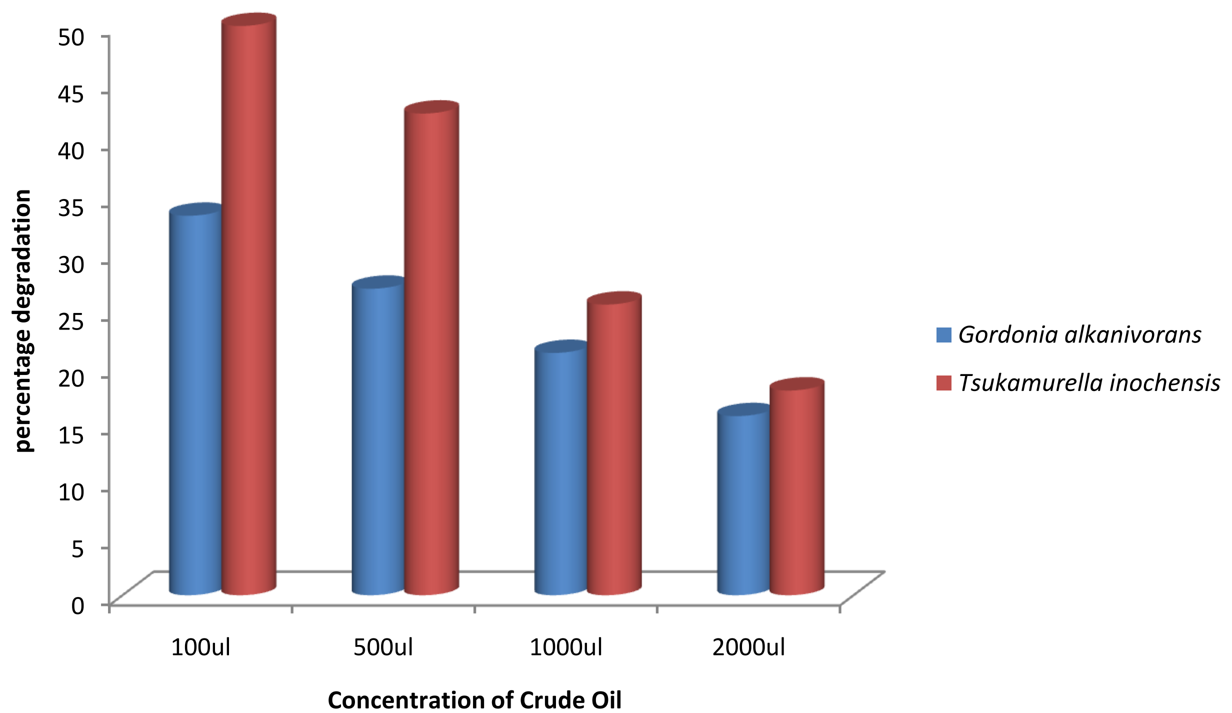


Figure 4.5: Percentage degradation of crude oil using gravimetric method (%)

4.4 pH Values During the 28 Days Degradation

The pH readings during the 28 days' degradation are presented in figure 4.6. The mean pH values of *Gordonia alkanivorans*during the 28 days' degradation was 4.8-5.0, *Tsukamurella inochensis* was 4.1-4.4 and Mixed culture was 4.4-4.7. There was no detectable change in the pH of the control.

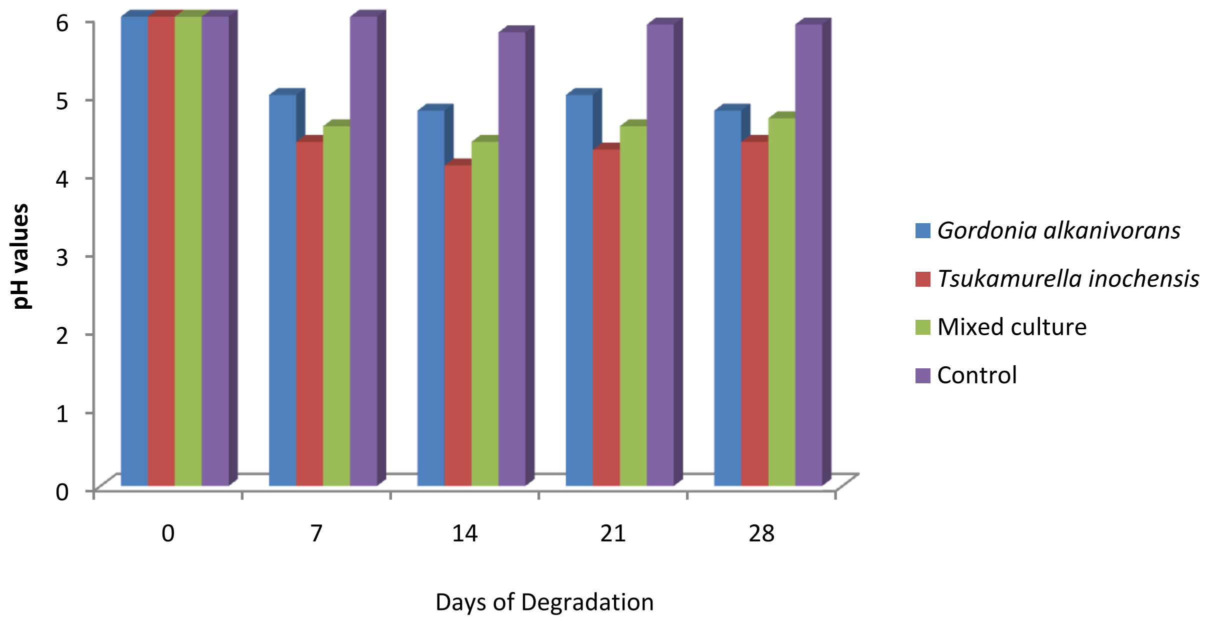


Figure 4.6: pH values during the 28 days' degradation

4.5OD Values During the 28 Days Degradation (600nm)

Optical Density values measured at 600nm for the 28 days' degradation showed that the reading increased on the 7thand 14thday for the test organisms and their mixed culture but no detectable increase for the control. Their mean values for 0, 7th, 14th, 21st and 28th days were 0.065, 0.358, 1.024, 0.670 and 0.370 for *Gordonia alkanivorans*. 0.059, 0.504, 1.558, 1.396 and 0.869 for *Tsukamurella inochensis*. 0.060, 0.408, 1.338, 1.026 and 0.694 for mixed culture. 0.047, 0.047, 0.334, 0.061 and 0.063 for control. These are presented in figure 4.7.

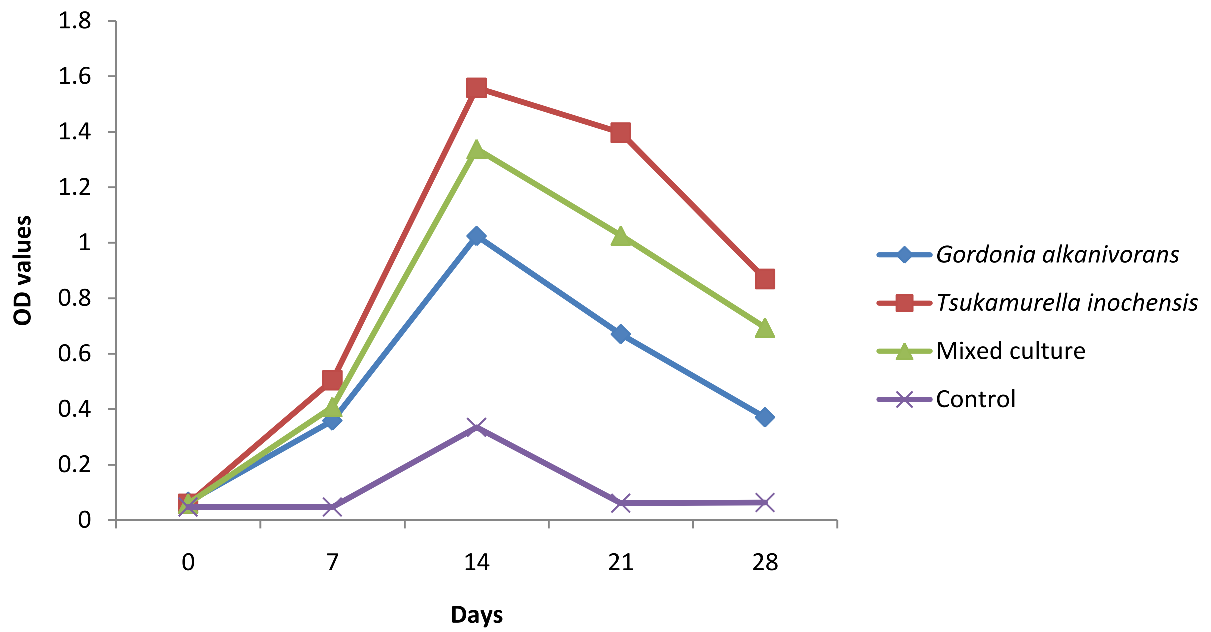


Figure 4.7: Optical Density values during the 28 days' degradation (600nm)

4.6The Values of Biosurfactant Screening Parameters

The isolated organisms were screened for its ability to produce biosurfactant. This was presented in Table 4.8. Only *Gordonia alkanivorans* and *Tsukamurella inochensis*were positive result to drop collapse and oil displacement test. Their emulsion index percentage 59.09% and 57.14% respectively was also higher than those of the other organisms and so were used in carrying out this research work.

Table 4.8: The values of biosurfactant screening parameters

Organism sample	Haemolysis test	Drop collapse	Oil displacement	Emulsion index (%)
S1	+	-	-	0
S2	+	+	+	59.09
S3	-	-	-	7.69
S4	-	-	-	0
S5	+	-	-	0
S6	+	-	-	7.41
S7	-	-	-	10
S8	-	-	-	7.14
S9	+	-	-	17.24
S10	+	-	-	3.57
S11	-	-	-	0
S12	+	-	-	0

S13	+	+	+	57.14

4.7Result of Total Petroleum Hydrocarbon and Polycyclic Aromatic Hydrocarbon Content After Crude Oil Degradation

4.7.1Total Petroleum Hydrocarbon Content after Crude Oil Degradation

The total petroleum hydrocarbons after 28 days' degradation are presented in Table 4.9-4.12.

The value of the total petroleum hydrocarbon of the test organisms and their mixed culture were lower when compared to control. This could be seen from the peaks in the figures. The control value was 10541.4180 but was degraded to1463.7610 by *Gordonia sp,*619.0704 by *Tsukamurella sp*and 269.8244 by their mixed culture.

Table 4.9: Total Petroleum Hydrocarbon content of crude oil (control)

Peak No	Peak Name	Ret Time (min)	Result (ppm)	Peak Area (counts)	Sep. Code
1	C8	2.073	9.5669	24900	VV
2	C9	2.762	155.9922	415302	VV
3	C10	3.676	440.0616	1223772	VV
4	C11	4.530	533.8961	1495275	VV
5	C12	5.302	514.9508	1486549	VV
6	C13	6.013	657.1946	1932603	VV
7	C14	6.676	506.0748	1483362	VV
8	C15	7.292	561.4563	1661840	VV
9	C16	7.872	466.7880	1412341	VV
10	C17	8.438	382.9052	1425247	VV
11	Pr	8.472	716.3463	1611369	VV
12	C18	8.952	343.7245	1072889	VV
13	Ph	8.994	157.9571	412896	VV
14	C19	9.453	281.2508	868949	VV
15	C20	9.932	450.9193	1315462	VV

16	C21	10.391	410.7138	1192467	VV
17	C22	10.832	418.1784	1223536	VV
18	C23	11.257	390.3202	1146701	VV
19	C24	11.665	409.2670	1196054	VV
20	C25	12.058	315.3884	921816	VV
21	C26	12.437	313.2524	917172	VV
22	C27	12.803	346.1620	992078	VV
23	C28	13.156	294.3683	875771	VV
24	C29	13.499	283.2456	827376	VV
25	C30	13.826	220.5703	666595	VV
26	C31	14.146	271.2165	843417	VV
27	C32	14.446	248.0091	759808	VV
28	C33	14.774	205.9713	624639	VV
29	C34	15.155	115.3227	346951	VV
30	C35	15.606	94.4871	267107	VP
31	C36	16.135	14.8352	42980	TS
32	C37	16.769	10.1814	25762	VB
33	C38	18.213	0.5294	1242	BB
34	C39	19.253	0.1181	225	BB
35	C40	20.733	0.1960	256	BV
Totals			**10541.4180**	**30714712**	

Table 4.10: Total Petroleum Hydrocarbon content after crude oil degradation with *Gordonia alkanivorans*

Peak No	Peak Name	Ret Time (min)	Result (ppm)	Peak Area (counts)	Sep. Code
1	C8	2.078	36.5234	95061	VB
2	C9	2.787	53.2324	141722	VV
3	C10	3.691	55.0673	153137	BV
4	C11	4.716	12.9041	36140	VV
5	C12	5.372	23.2210	67034	VV
6	C13	5.976	57.8815	170211	VV
7	C14	6.482	69.1358	202645	VV
8	C15	7.254	71.3337	211139	VV
9	C16	7.838	55.0146	166455	VV
10	C17	8.395	40.1939	149610	VV
11	Pr	8.430	180.9783	407098	VV

12	C18	8.919	41.9419	130916	VV
13	Ph	8.968	62.0597	162223	VV
14	C19	9.421	73.9909	228601	VV
15	C20	9.901	62.6770	182847	VV
16	C21	10.361	52. 8183	153353	VV
17	C22	10.802	43.6933	127841	VV
18	C23	11.226	36.3136	106684	VV
19	C24	11.634	37.5026	109599	VV
20	C25	12.026	35.1213	102653	VV
21	C26	12.405	35.6187	104288	VV
22	C27	12.770	44.2753	126890	VV
23	C28	13.123	55.1554	164092	VV
24	C29	13.464	64.1693	187442	VV
25	C30	13.795	37.6412	113757	VV
26	C31	14.112	40.1311	124798	VV
27	C32	14.421	41.3775	126765	VV
28	C33	14.746	30.0634	91172	VP
29	C34	15.131	7.4592	22441	TF
30	C35	15.583	5.4886	15516	VP
31	C36	16.215	0.0559	162	BV
32	C37	16.847	0.0429	109	BP
33	C38	18.299	0.2930	687	VV
34	C39	19.394	0.2432	463	VV
35	C40	20.652	0.1419	186	VV
Totals			**1463.7610**	**4183737**	

Table 4.11: Total Petroleum Hydrocarbon content after crude oil degradation with *Tsukamurella inochensis*

Peak No	Peak Name	Ret Time (min)	Result (ppm)	Peak Area (counts)	Sep. Code
1	C8	2.067	0.9362	2437	BP
2	C10	3.714	42.3577	117793	BB
3	C11	4.478	14.4100	40358	BB
4	C12	5.384	14.0248	40487	VV
5	C13	5.799	33.4824	98461	VV
6	C14	6.493	53.8927	157965	VV
7	C15	7.002	52.2318	154599	VV

8	C16	8.118	61.6454	186518	BV
9	C17	8.444	166.7417	620645	BB
10	Pr	8.471	0.2981	671	TS
11	C18	8.779	5.2152	16279	VB
12	Ph	8.979	76.8219	200811	VP
13	C19	9.392	16.8566	52080	BV
14	C20	9.909	7.2815	21242	BV
15	C21	10.370	5.0755	14736	BB
16	C22	10.810	3.1108	9102	BB
17	C23	11.238	2.4069	7071	BP
18	C24	11.643	3.4611	10115	BB
19	C25	12.033	5.2536	15355	VB
20	C26	12.413	3.4238	10025	BB
21	C27	12.776	2.2024	6312	BB
22	C28	13.132	1.8971	5644	BB
23	C29	13.468	2.8454	8312	BB
24	C30	13.808	4.1635	12583	BV
25	C31	14.046	17.3578	53979	VP
26	C32	14.287	19.9506	61121	BV
27	C33	14.691	1.7258	5234	PB
Totals			**619.0704**	**1929935**	

Table 4.12: Total Petroleum Hydrocarbon content after crude oil degradation with Mixed culture

Peak No	Peak Name	Ret Time (min)	Result (ppm)	Peak Area (counts)	Sep. Code
1	C8	2.076	6.1896	16110	TF
2	C9	2.796	7.7110	20529	TF
3	C10	3.693	3.0655	8525	TF
4	C11	4.535	2.1402	5994	TF
5	C12	5.371	5.6633	16349	TF
6	C13	5.786	21.5881	63484	TF

7	C14	6.479	30.1851	88476	TF
8	C15	7.019	28.7936	85225	TF
9	C16	8.107	17.9323	54257	TF
10	C17	8.422	40.4442	150541	TF
11	Pr	8.481	1.8929	4258	TF
12	C18	8.915	7.3641	22986	TF
13	Ph	8.963	19.0699	49848	TF
14	C19	9.417	6.8898	21287	TF
15	C20	9.899	6.7358	19650	TF
16	C21	10.359	5.9890	17388	TF
17	C22	10.800	5.4735	16015	TF
18	C23	11.224	5.4225	15930	TF
19	C24	11.632	5.2789	15427	TF
20	C25	12.024	5.4078	15806	TF
21	C26	12.403	4.0638	11898	TF
22	C27	12.767	4.0934	11731	TF
23	C28	13.120	4.7177	14035	TF
24	C29	13.460	5.7203	16709	TF
25	C30	13.792	4.8883	14773	TF
26	C31	14.032	5.6221	17483	TF
27	C32	14.270	5.6476	17302	TF
28	C33	14.748	0.6905	2094	TF
29	C34	15.130	0.4264	1283	BB
30	C35	15.599	0.2760	780	BB
31	C38	18.318	0.0131	31	BB
32	C39	19.381	0.0920	175	BV
33	C40	20.647	0.3361	440	VV
Totals			**269.8244**	**816819**	

4.7.2Polycyclic Aromatic Hydrocarbon Content after Crude Oil Degradation

The polycyclic aromatic hydrocarbon content after 28 days of degradation are presented in Table 4.13-4.16. The values of the aromatic hydrocarbons for that of the test organism and their mixed culture were lower when compared with the control. This could be seen from the peak presented in the figures.The control value was 5510.4443 but *Gordonia sp* degraded it to869.8653, *Tsukamurella sp* to476.5867, mixed culture to 252.4649.

Table 4.13: Polycyclic Aromatic Hydrocarbon content of crude oil (control)

Peak No	Peak Name	Ret Time (min)	Result (ppm)	Peak Area (counts)	Sep. Code

1	Naphthalene	5.307	568.9227	1348485	VP
2	Acenapthene	6.019	545.9747	1389842	BB
3	Acenaphthylene	6.683	817.1665	1223965	VV
4	Fluorene	8.479	547.8810	1149848	VB
5	Phenanthrene	8.958	399.7979	749889	BV
6	Anthracene	9.000	213.4037	279707	VB
7	Fluoranthene	9.460	403.0627	697321	VB
8	Pyrene	9.939	406.3939	671042	BB
9	Benzo[a]anthracene	14.058	58.5654	77170	BP
10	Chrysene	14.154	372.8059	528283	PB
11	Benzo[b]fluoranthene	14.308	171.2639	189292	BV
12	Benzo[k]fluoranthene	14.339	66.1258	68596	VP
13	Benzo[a]pyrene	14.453	362.4637	323835	PB
14	Indenol[1,2,3-cd]pyrene	14.748	363.5573	321213	BB
15	Dibenzo[a,h]anthracene	15.163	213.0591	162401	BB
Totals			**5510.4443**	**9180889**	

Table 4.14: Polycyclic Aromatic Hydrocarbon content after crude oil degradation with *Gordonia alkanivorans*

Peak	Peak Name	Ret Time	Result	Peak Area	Sep. Code

No		(min)	(ppm)	(counts)	
1	Naphthalene	5.374	23.5848	55902	VV
2	Acenapthene	5.977	62.8214	159919	VV
3	Acenaphthylene	6.637	115.1979	172545	VV
4	Fluorene	8.433	168.9350	354547	VV
5	Phenanthrene	8.922	62.2901	116836	BV
6	Anthracene	8.970	85.5258	112098	VB
7	Fluoranthene	9.425	61.8780	107052	VV
8	Pyrene	9.905	58.6210	96796	BB
9	Benzo[a]anthracene	14.115	59.4116	78285	PB
10	Benzo[b]fluoranthene	14.314	22.1096	24437	VP
11	Benzo[a]pyrene	14.423	50.6827	45281	PB
12	Indenol[1,2,3-cd]pyrene	14.748	46.0490	40686	BB
13	Dibenzo[a,h]anthracene	15.134	31.4211	23950	BB
14	Benzo[ghi]perylene	15.588	21.3373	18863	BB
Totals			**869.8653**	**1407197**	

Table 4.15: Polycyclic Aromatic Hydrocarbon content after crude oil degradation with*Tsukamurella Inochensis*

Peak No	Peak Name	Ret Time (min)	Result (ppm)	Peak Area (counts)	Sep. Code
1	Naphthalene	5.790	35.6123	84410	VV
2	Acenapthene	6.484	55.8964	142291	VV
3	Acenaphthylene	7.025	95.5052	143049	VV
4	Fluorene	8.431	146.7445	307976	VV
5	Phenanthrene	8.970	54.7068	102612	VV
6	Anthracene	9.066	17.3354	22721	VV
7	Fluoranthene	9.386	22.6392	39167	VV
8	Pyrene	9.826	16.1749	26708	VV
9	Benzo[a]anthracene	14.037	14.7599	19449	VP
10	Benzo[b]fluoranthene	14.314	17.2122	19024	VP
Totals			**476.5867**	**907407**	

Table 4.16: Polycyclic Aromatic Hydrocarbon content after crude oil degradation with Mixed culture

Peak No	Peak Name	Ret Time (min)	Result (ppm)	Peak Area (counts)	Sep. Code
1	Naphthalene	5.787	26.2399	62195	BB
2	Acenapthene	6.480	28.1437	71643	BB
3	Acenaphthylene	7.021	40.8767	61226	BB
4	Fluorene	8.424	74.2013	155728	VB
5	Phenanthrene	8.916	13.2543	24861	BV
6	Anthracene	8.965	42.4208	55601	VB
7	Fluoranthene	9.418	12.9373	22382	BB
8	Pyrene	9.899	14.3909	23762	BB
Totals			**252.4649**	**477398**	

4.8Total Petroleum Hydrocarbon (TPH) and Polycyclic Aromatic Hydrocarbon (PAH) Content after Artificially Polluting the Soils with Crude Oil

The TPH and PAH of Ibeno and Otuocha soil after artificially polluting the crude oil samples are presented in Table 4.17-4.20. The TPH value of Ibeno after pollution was 1975.2632 while the Otuocha value was 3244.9021. The PAH value of Ibeno after pollution was 1191.1993 while Otuocha value was 1879.1434.

Table 4.17: Total Petroleum Hydrocarbon content of Ibeno soil after artificially polluting the soil (Before remediation)

Peak No	Peak Name	Ret Time(min)	Result(ppm)	Peak Area(counts)	Type
1	C8	2.053	0.3702	963	VV
2	C9	2.771	6.0062	15990	VV
3	C10	3.711	2.9060	8081	VV
4	C11	4.707	2.1146	5922	VP
5	C12	5.367	7.8661	22708	VV
6	C13	5.973	30.6571	90153	VV
7	C14	6.478	64.3512	188621	VV
8	C15	7.256	97.4961	288576	VV
9	C16	7.841	85.6910	259272	VV
10	C17	8.400	79.1606	294651	VV
11	Pr	8.441	325.3037	731747	VV
12	C18	8.926	87.6400	273556	VV
13	Ph	8.972	72.5205	189567	VV
14	C19	9.428	85.7455	264918	VV
15	C20	9.908	95.3417	278139	VV
16	C21	10.368	86.4709	251060	VV
17	C22	10.809	79.3478	232162	VV
18	C23	11.233	77.8823	228806	VV
19	C24	11.640	73.7280	215465	VV
20	C25	12.032	74.1124	216616	VV
21	C26	12.410	71.3106	208791	VV
22	C27	12.776	70.8181	202960	VV
23	C28	13.129	67.3372	200334	VV
24	C29	13.471	80.6708	235644	VV
25	C30	13.799	64.9138	196179	VV
26	C31	14.119	62.1358	193227	VV
27	C32	14.425	41.7269	127836	VV
28	C33	14.751	40.4339	122622	VV
29	C34	15.134	23.9325	72002	VV

30	C35	15.585	16.7203	47267	VV
31	C36	16.219	0.0008	2	TS
32	C37	16.851	0.0152	39	BB
33	C38	18.310	0.1062	249	PV
34	C39	19.359	0.1088	207	PB
35	C40	20.654	0.3202	419	VV
	Totals		**1975.2632**		

Table 4.18: Total Petroleum Hydrocarbon content of Otuocha soil after artificially polluting the soil (Before remediation)

Peak No	Peak Name	Ret Time(min)	Result(ppm)	Peak Area(counts)	Type
1	C8	2.053	0.1521	396	TF
2	C9	2.791	2.3124	6156	TF
3	C10	3.721	1.7975	4999	VV
4	C11	4.707	0.9587	2685	VV
5	C12	5.369	7.1214	20558	VV
6	C13	5.978	57.2456	168341	VV
7	C14	6.483	84.8300	248646	VV
8	C15	7.266	181.6299	537602	VV
9	C16	7.853	175.2086	530121	VV
10	C17	8.413	159.8770	595093	VV
11	Pr	8.453	378.1079	850526	VV
12	C18	8.937	171.0623	533947	VV
13	Ph	8.980	102.2273	267220	VV
14	C19	9.440	159.7405	493532	VV
15	C20	9.920	172.7950	504093	VV
16	C21	10.380	155.5024	451486	VV
17	C22	10.820	144.6347	423182	VV
18	C23	11.243	139.5842	410077	VV
19	C24	11.650	129.2782	377806	VV
20	C25	12.043	127.3697	372276	VV
21	C26	12.421	123.4682	361503	VV
22	C27	12.786	120.8479	346342	VV
23	C28	13.138	114.2596	339932	VV
24	C29	13.481	129.9333	379542	VV
25	C30	13.808	101.9200	308017	VV

26	C31	14.128	97.9912	304729	VV
27	C32	14.432	65.4178	200416	VV
28	C33	14.759	63.6537	193040	VV
29	C34	15.141	38.6707	116342	VV
30	C35	15.593	33.8242	95618	VV
31	C36	16.230	0.8970	2599	VV
32	C37	16.826	2.2551	5706	VV
33	C38	16.826	0.0957	224	VV
34	C39	19.394	0.0388	74	BV
35	C40	20.611	0.1937	253	VB
	Totals		**3244.9021**		

Table 4.19: Polycyclic Aromatic Hydrocarbon content of Ibeno soil after artificially polluting the soil (Before remediation)

Ret time (min)	type	Peak area	Amount (ppm)	Group name
5.366	BB	15727	6.6351	Naphthalene
5.973	BB	69296	27.2216	Acenaphthene
6.634	VB	145053	96.8432	Acenaphthylene
8.440	VB	509344	242.6928	Fluorene
8.925	BV	199996	106.6261	Phenanthrene
8.971	VB	122910	93.7747	Anthracene
9.428	VB	185420	107.1758	Fluoranthene
9.908	BB	179721	108.8418	Pyrene
14.120	PB	145478	110.4050	Benzo(a)anthracene
14.313	VP	30927	27.9811	Benzo(b)fluoranthene
14.427	PB	91005	101.8605	Benzo(a)pyrene
14.752	BB	90388	102.3039	Indeno(1,2,3-cd)pyrene

15.138	BB	44848	58.8377	Dibenzo(a,h)anthracene
Totals:			**1191.1993**	

Table 4.20: Polycyclic Aromatic Hydrocarbon content of Otuocha soil after artificially polluting the soil (Before remediation)

Ret time (min)	type	Peak area	Amount (ppm)	Group name
5.375	BB	15528	6.5511	Naphthalene
5.984	BB	134287	52.7523	Acenaphthene
6.648	VV	289995	193.6116	Acenaphthylene
8.458	VB	703528	335.2179	Fluorene
8.944	VV	417096	222.3714	Phenanthrene
8.986	VB	175463	133.8708	Anthracene
9.396	VV	55384	32.0128	Fluoranthene
9.926	BB	363552	220.1729	Pyrene
14.133	PB	215724	163.7162	Benzo(a)anthracene
14.291	BV	59008	53.3879	Benzo(b)fluoranthene
14.324	VP	38999	37.5944	Benzo(k)fluoranthene

14.437	PB	129865	145.3552	Benzo(a)pyrene
14.766	BB	127279	144.0581	Indeno(1,2,3-cd)pyrene
15.148	BB	63750	83.6354	Dibenzo(a,h)anthracene
15.600	BB	48477	54.8355	Benzo(g,h,i)perylene
Totals:			**1879.1434**	

4.9 TOTAL VIABLE COUNT OF THE INOCULUMS DEVELOPMENT

Total viable counts of the inoculum development needed for the remediation exercise are presented in figure 4.8. *Gordonia* sp was $22.7x10^5$cfu/ml on the 7th day while *Tsukamurella* was $11.0x10^5$cfu/ml.

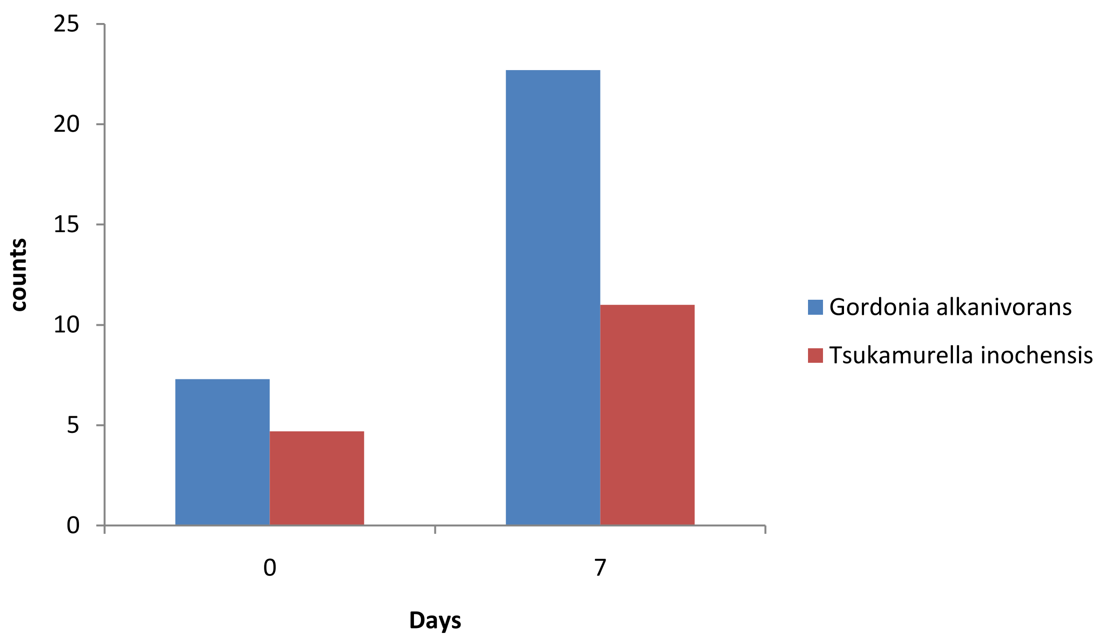
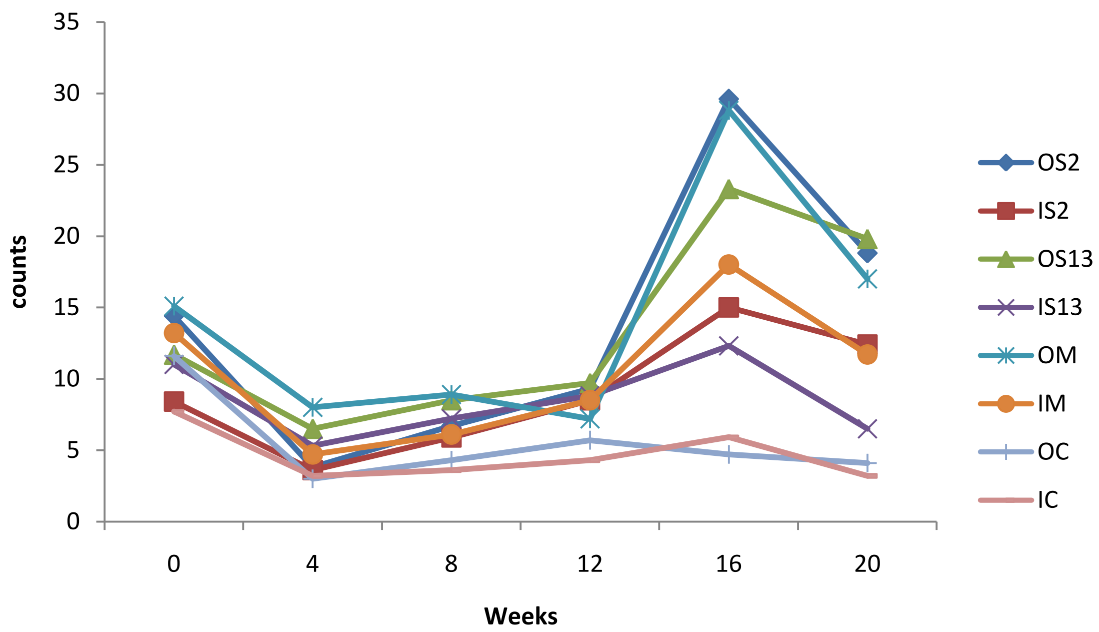


Figure 4.8: Total Viable Counts of the inoculums development (cfu/ml)

4.10Total Viable Counts During the 5 Months Remediation

Total viable counts during the 5 months' remediation are presented in figure 4.9. All the samples experienced a decrease in their total viable count on the fourth week which increased gradually on the eight and twelveth week. On the sixteenth week, they experienced a maximum increase except for the control. A decrease was again experienced on the twenthieth day.



35
30
25
20
15
10
5
0
counts
0
4
8
12
16
20
Weeks
OS2
IS2
OS13
IS13
OM
IM
OC
IC

Figure 4.9: Total viable count during the 5 months' remediation (cfu/g)

OS2= Otuocha soil with *Gordonia*

IS2= Ibeno soil with isolate *Gordonia*

OS13=Otuocha soil with isolate *Tsukamurella*

IS13= Ibeno soil with isolate *Tsukamurella*

OM= Otuocha soil with mixed culture

IM= Ibeno soil with mixed culture

OC= Otuocha soil without isolate (control)

IC= Ibeno soil without isolate (control)

4.11Dynamics of soil pH during bioremediation

During the five months of degradation of crude oil, The pH of the samples decreased on the fourth week except Otuocha soil containing *Gordonia sp* and Otuocha soil without any isolate. On the eight week, pH of all the soils decreased.However, on the twelveth week, pH increased in all the soils. Also, increase in soils' pH was observed on the twentieth week. Figure 4.22 presents the changes in pH during the 5 months of oil degradation in the soils.

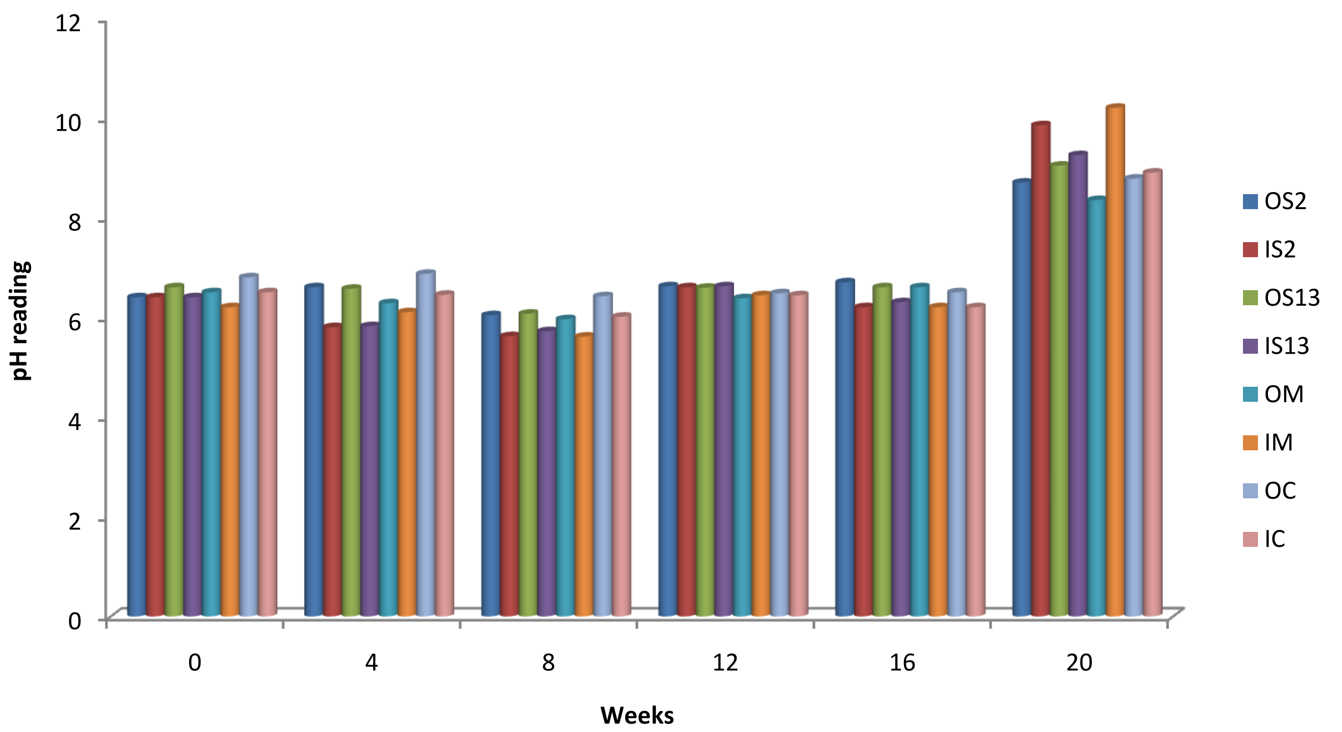


Figure 4.10: pH of the soil during the 5 months remediation

OS2= Otuocha soil with isolate *Gordonia*

IS2= Ibeno soil with isolate *Gordonia*

OS13=Otuocha soil with isolate *Tsukamurella*

IS13= Ibeno soil with isolate *Tsukamurella*

OM= Otuocha soil with mixed culture

IM= Ibeno soil with mixed culture

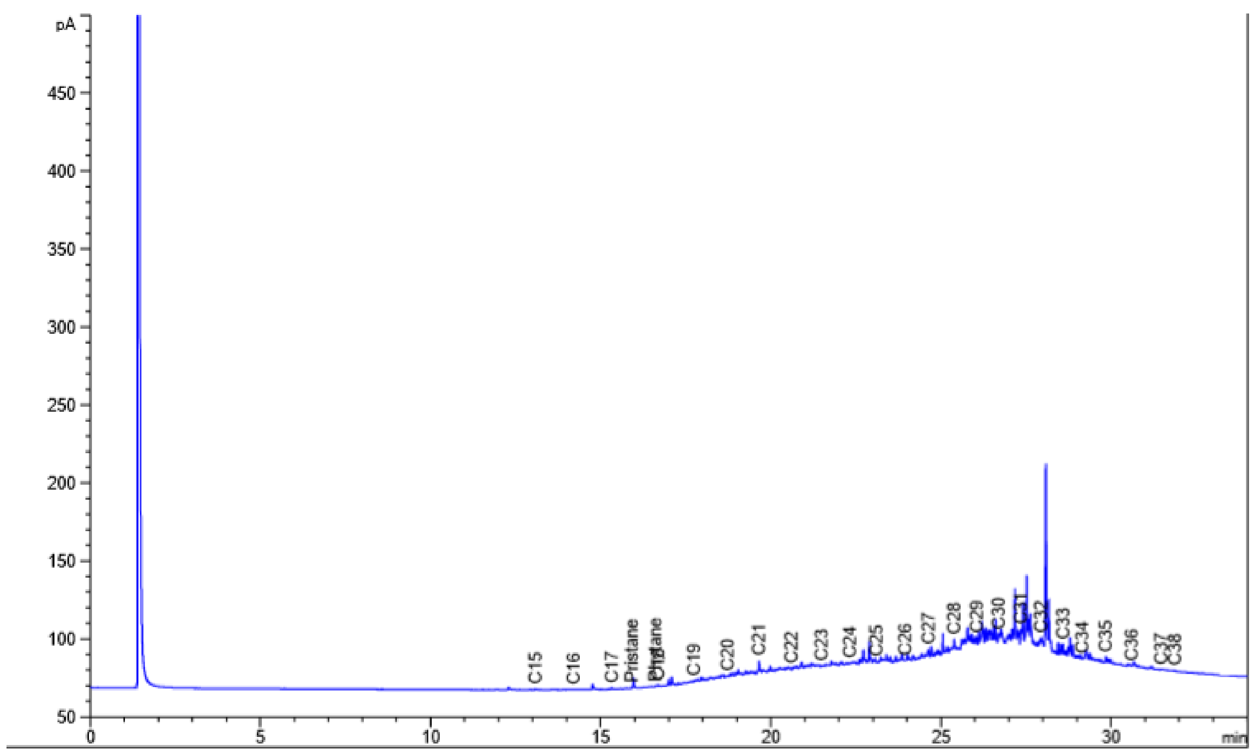
OC= Otuocha soil without isolate (control)

IC= Ibeno soil without isolate (control)

4.12 Total Petroleum Hydrocarbon and Polycyclic Aromatic Hydrocarbon Content after the Remediation

4.12.1Total Petroleum Hydrocarbon Content after the Remediation

The total petroleum hydrocarbon content before and after remediation of Ibeno and Otuocha soil samples are presented in figures 4.11-4.18. The values obtained from the soil amended with the test organisms were lower than those not amended but contained indigeneous microorganisms (control). Ibeno soil control value was 778.76791 but amendment with *Gordonia* reduced it to 563.41279, *Tsukamurella* to 510.29552 and their mixed culture to 585.09108. Otuocha soil control value was 1326.70729 but amendment with *Gordonia* reduced it to 559.55518, *Tsukamurella* to 526.17757 and their mixed culture to 985.96555.



pA
450
400
350
300
250
200
150
100
50
0
5
10
15
20
25
30
min
C15
C16
C17
Pristane
C18
C19
C20
C21
C22
C23
C24
C25
C26
C27
C28
C29
C30
C31
C32
C33
C34
C35
C36
C37
C38

Figure 4.11: Total petroleum hydrocarbon chromatography profile of Ibeno soil (Control)after the remediation

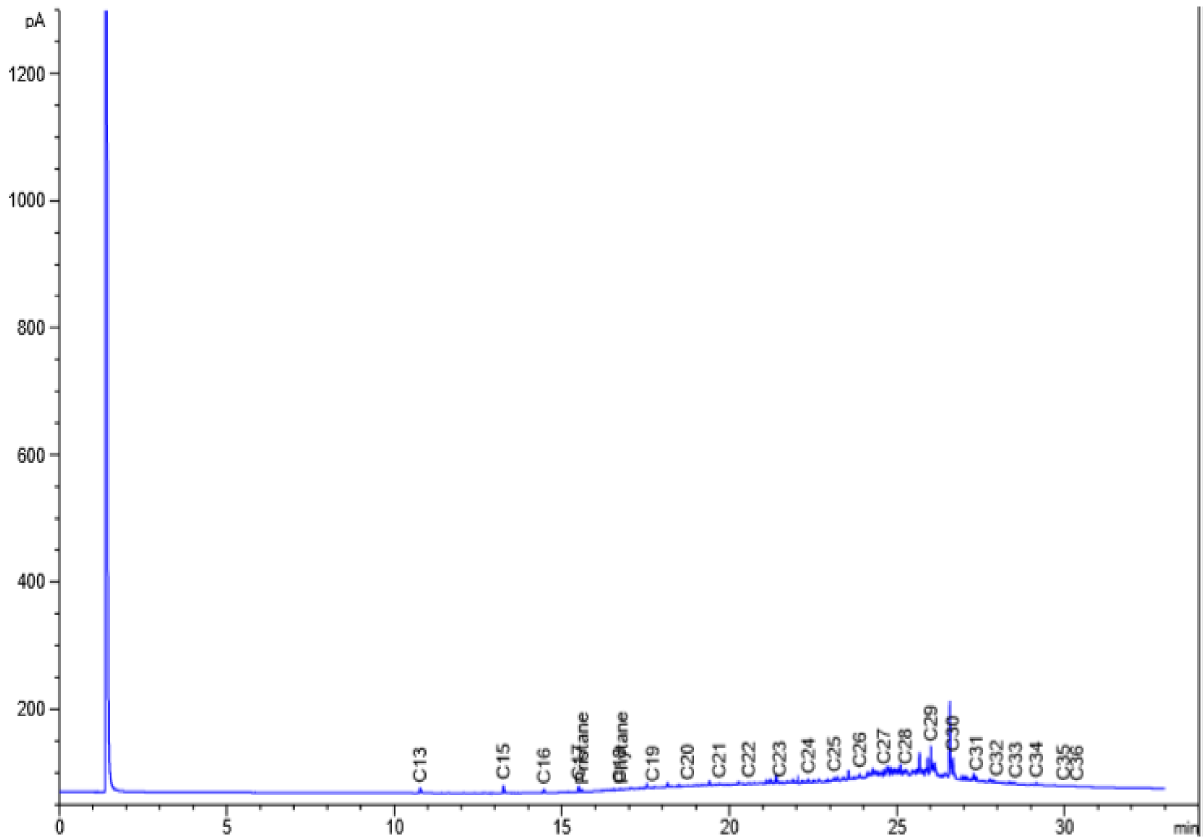


Figure 4.12: Total petroleum hydrocarbon chromatography profile after the remediation of Ibeno soil with *Gordonia alkanivorans*

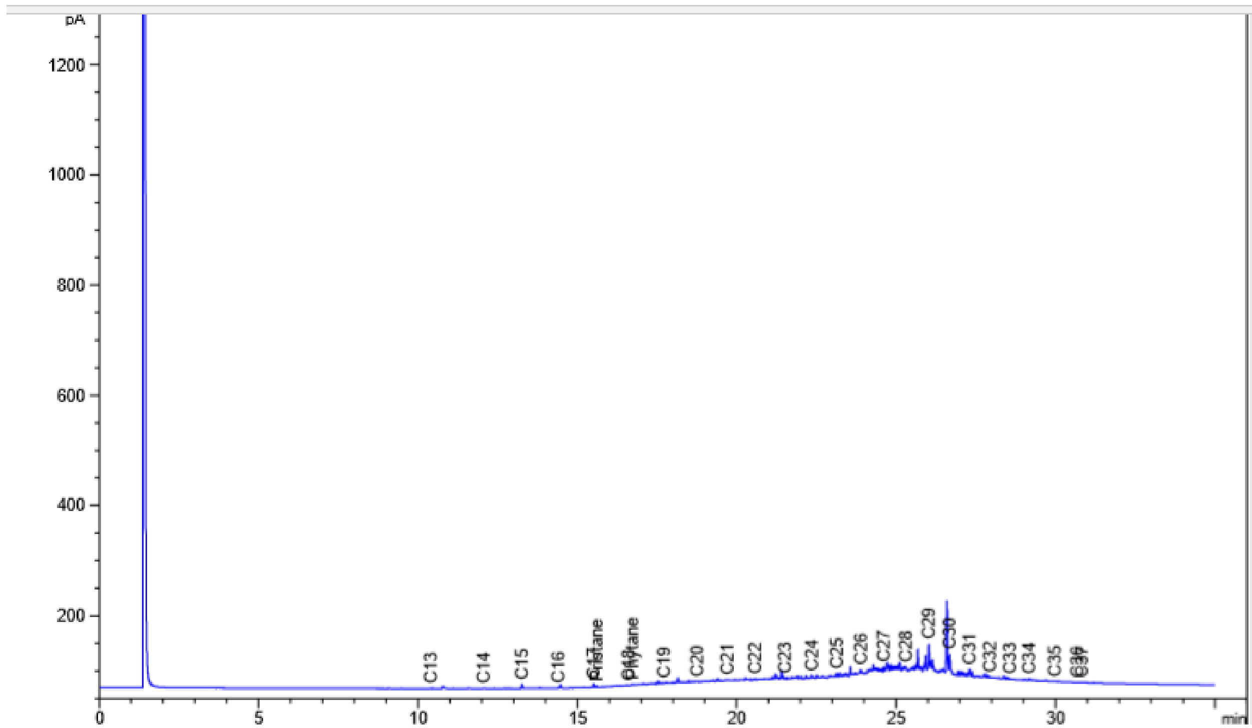


Figure 4.13: Total petroleum hydrocarbon chromatography profile after the remediation of Ibeno soil with *Tsukamurella inochensis*

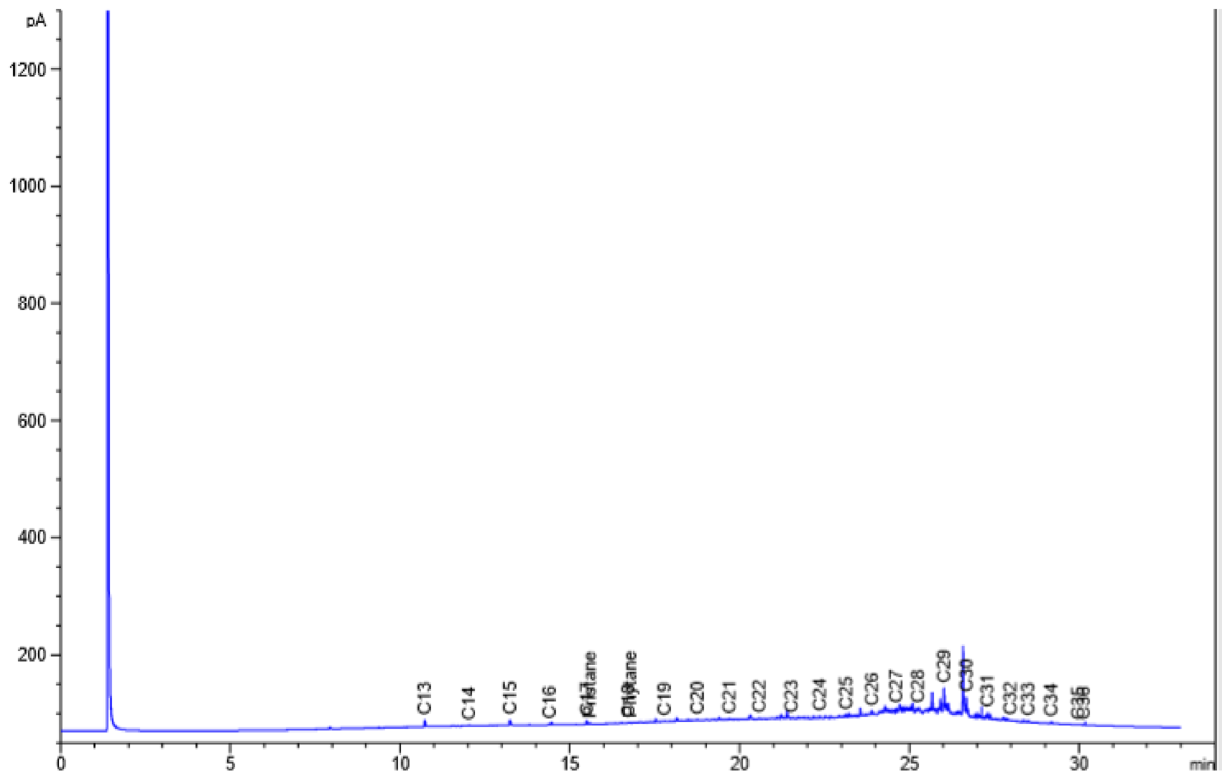


Figure 4.14: Total petroleum hydrocarbon chromatography profile after the remediation of Ibeno soil with mixed culture

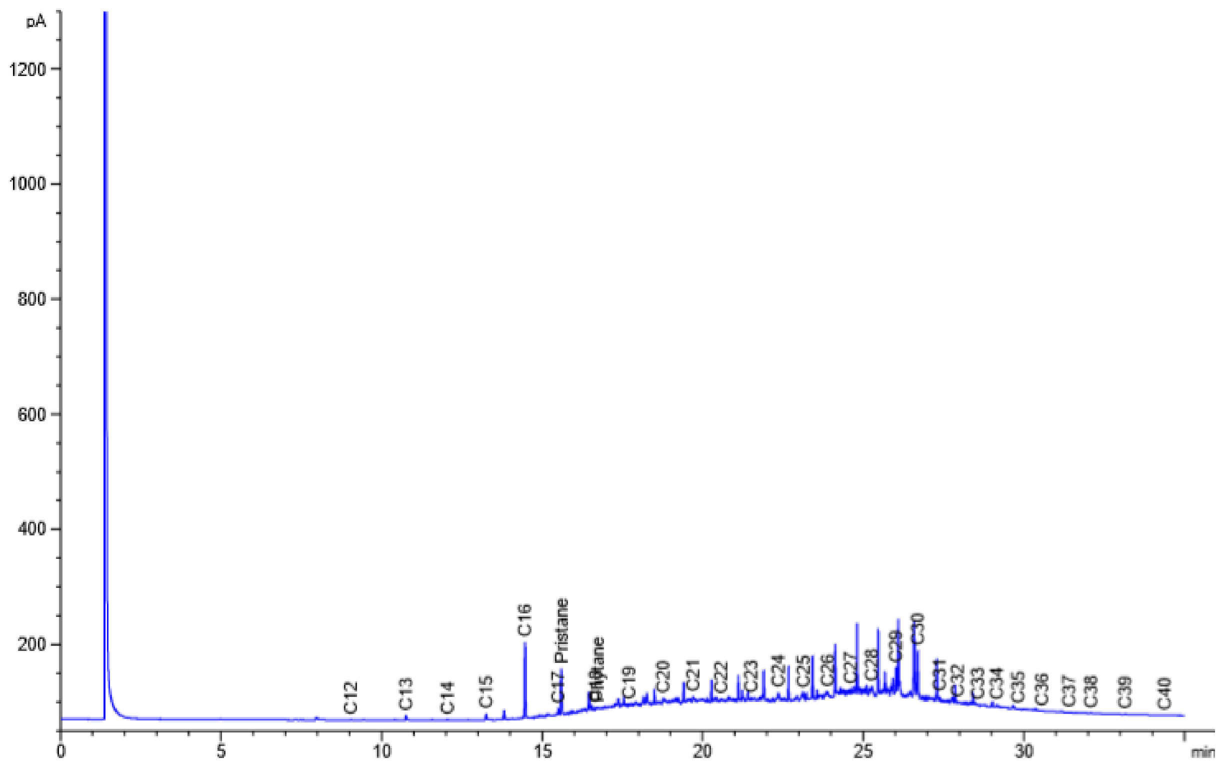


Figure 4.15: Total petroleum hydrocarbon chromatography profile of Otuocha soil (Control)after the remediation

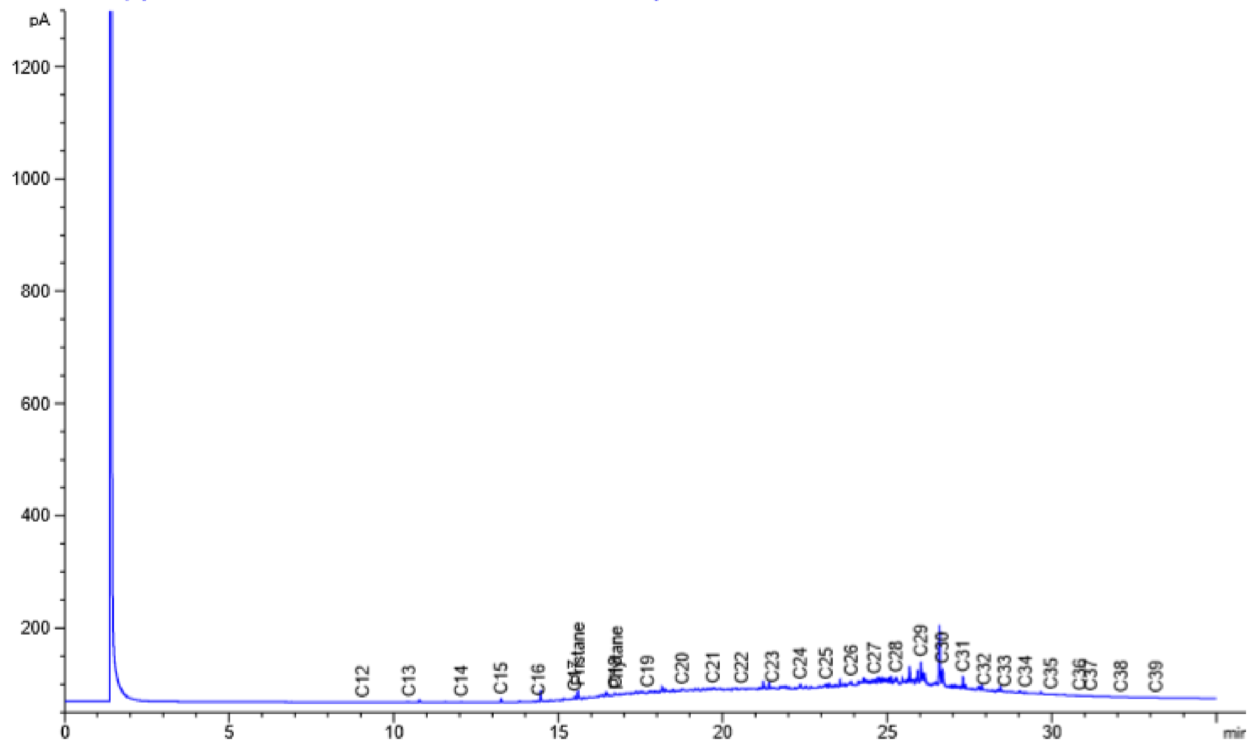


Figure 4.16: Total petroleum hydrocarbon chromatography profile after the remediation of Otuocha soil with *Gordonia alkanivorans*

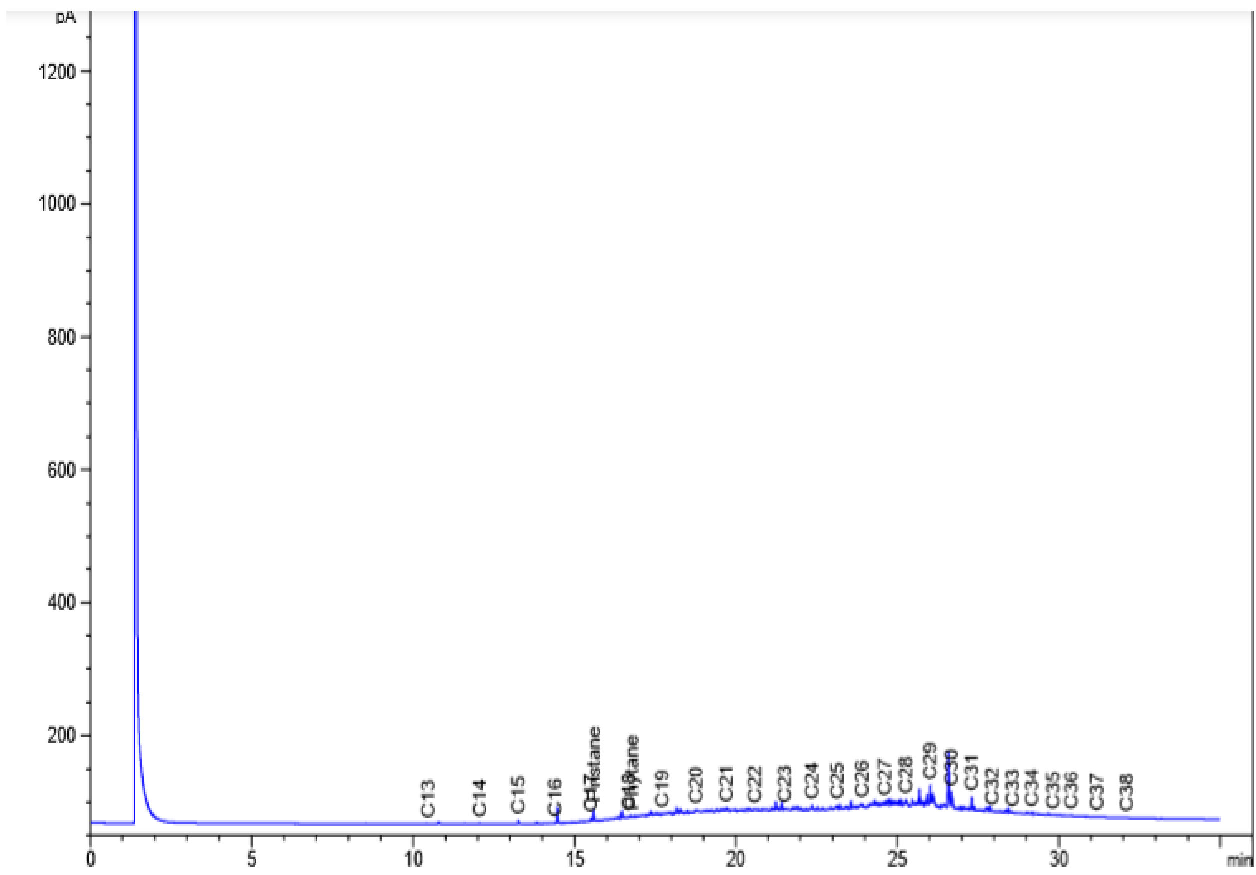


Figure 4.17: Total petroleum hydrocarbon chromatography profile after the remediation of Otuocha soil with *Tsukamurella inochensis*

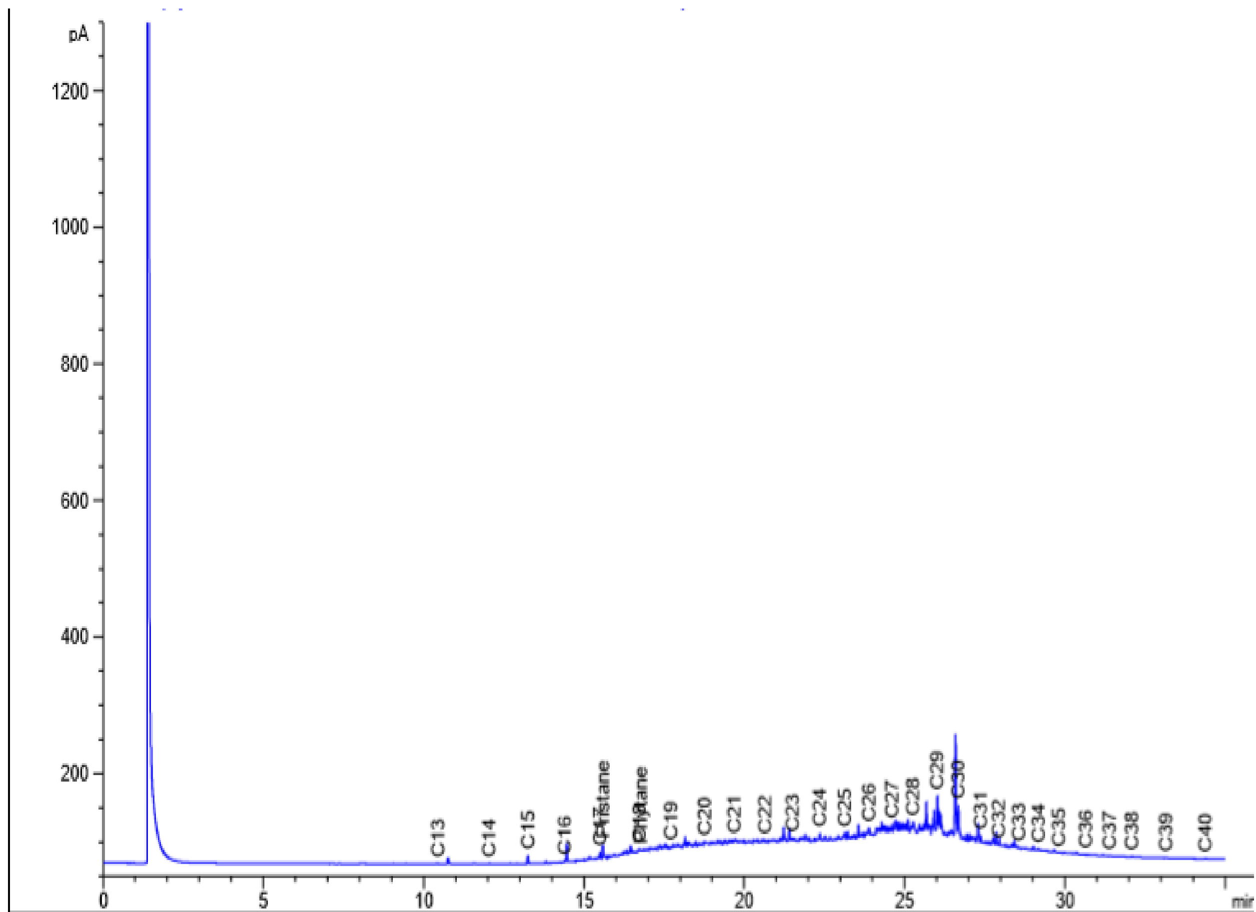


Figure 4.18: Total petroleum hydrocarbon chromatography profile after the remediation of Otuocha soil with mixed culture

4.12.2Polycyclic Aromatic Hydrocarbon Content after the Remediation

The polycyclic aromatic hydrocarbon content after remediation of Ibeno and Otuocha soil are presented in figures 4.19-4.26. The Ibeno soil control value was 11.00143 but amendment with *Gordonia* reduced it to 8.53390, *Tsukamurella* to 3.22127 and their mixed culture to 1.04768. Otuocha soil control value was 550.2319 but amendment with *Gordonia* reduced it to 516.9339, *Tsukamurella* to 539.6178 and their mixed culture was 340.6747. In Ibeno polluted soil, the soil amended with *Gordonia* sp and its biosurfactant completely removed 2 – Methylnaphalene, Acenaphthylene, Benzo (ghi) perylene and Indeno (1, 2, 3-cd) pyrene. The soil sample amended with *Tsukamurella* sp and its biosurfactant was able to completely remove 2 methylnaphalene, Acenaphthylene and acenaphthene. Their mixed culture and biosurfactant completely removed 2-methylnaphalene, Acenephthylene, Phenanthrene and Anthracene. The control was able to remove only acenaphthylene completely. In otuocha polluted soil, the test organism and its biosurfactant was able to reduce all the PAH but didn’t completely remove any.

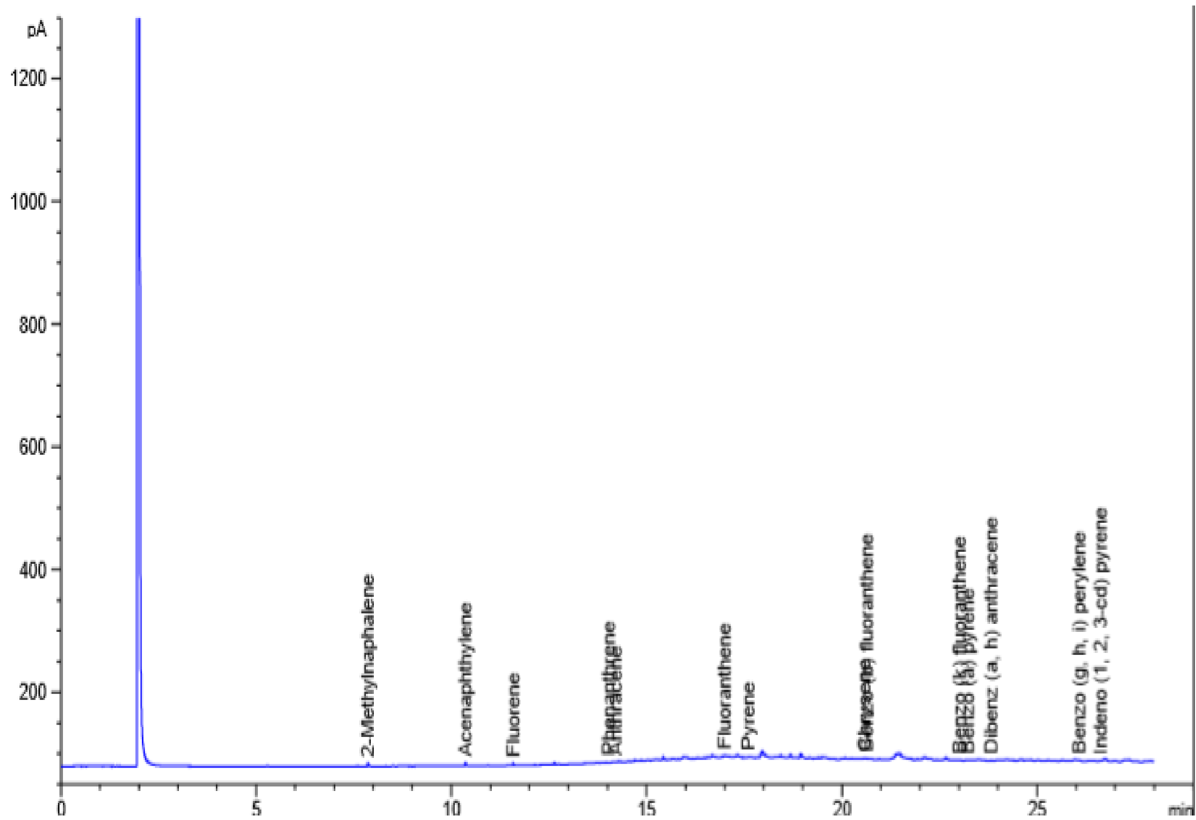


Figure 4.19: Polycyclic aromatic hydrocarbon chromatography profile of Ibeno soil (Control) after remediation

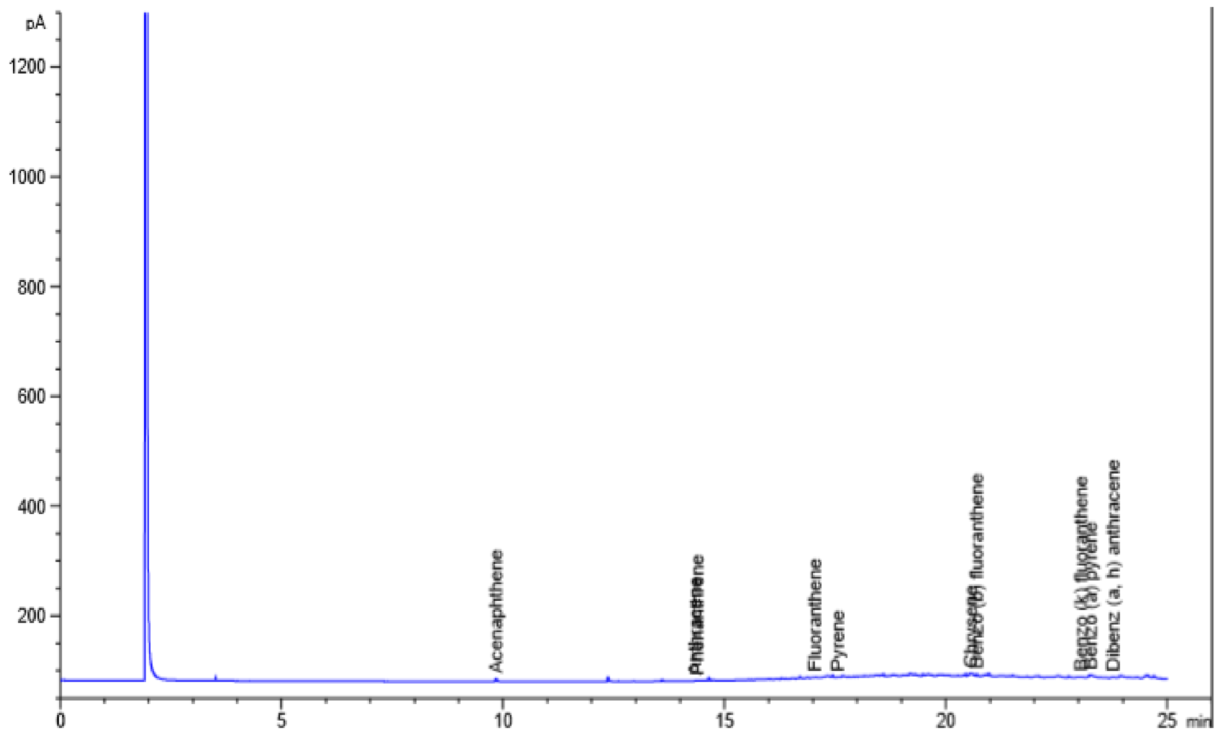


Figure 4.20: Polycyclic aromatic hydrocarbon chromatography profile after the remediation of Ibeno soil with *Gordonia alkanivorans*

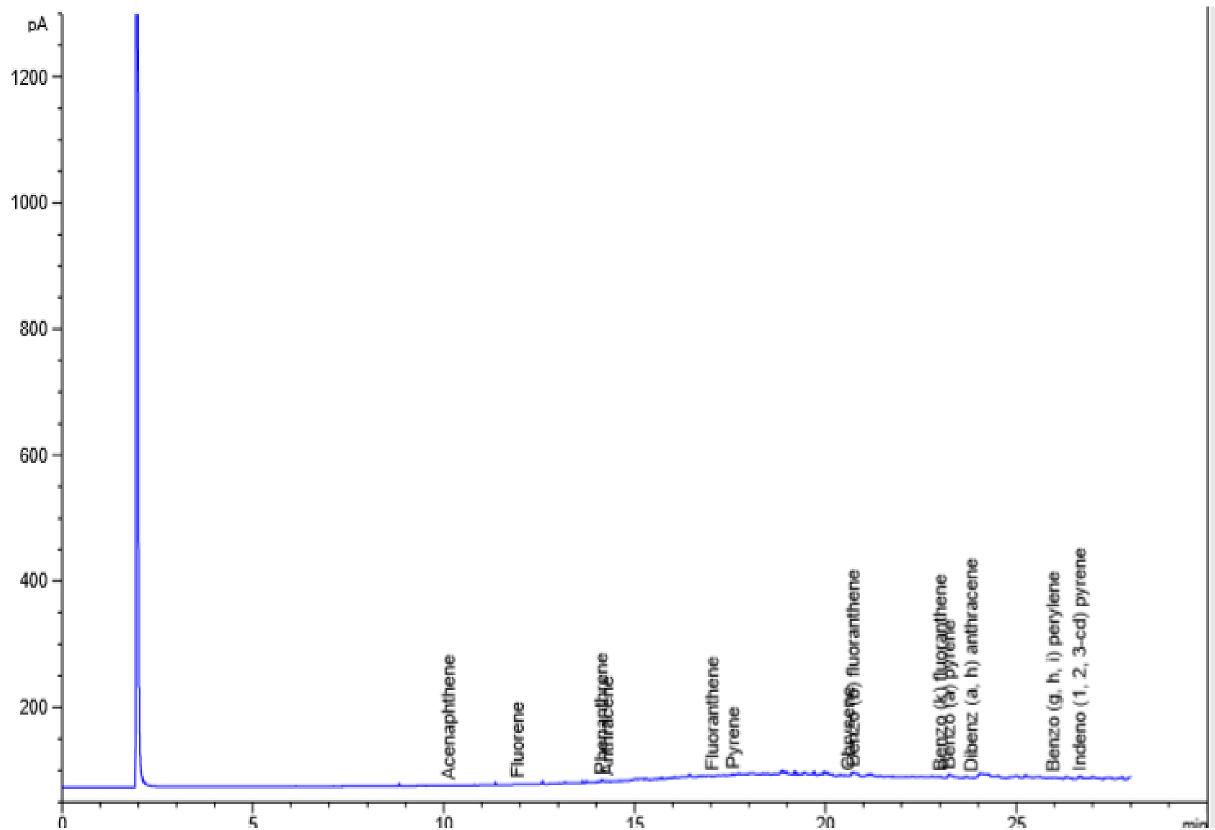


Figure 4.21: Polycyclic aromatic hydrocarbon chromatography profile after the remediation of Ibeno soil with *Tsukamurella inochensis*

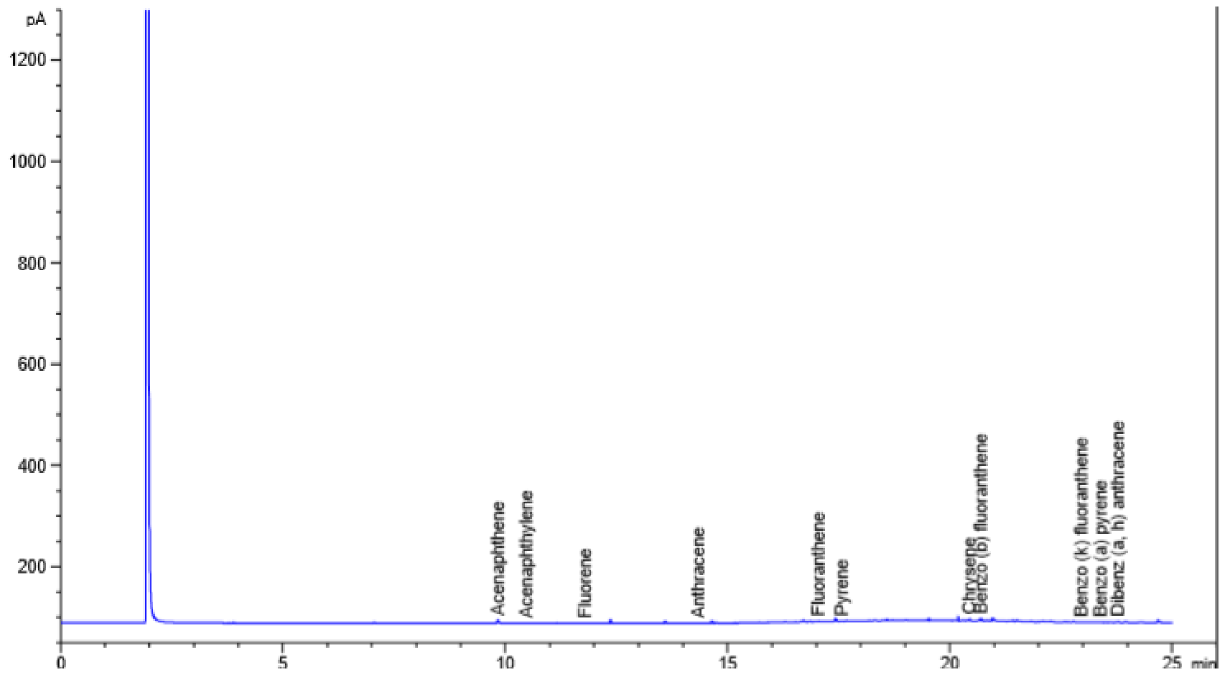


Figure 4.22: Polycyclic aromatic hydrocarbon chromatography profile after the remediation of Ibeno soil with mixed culture

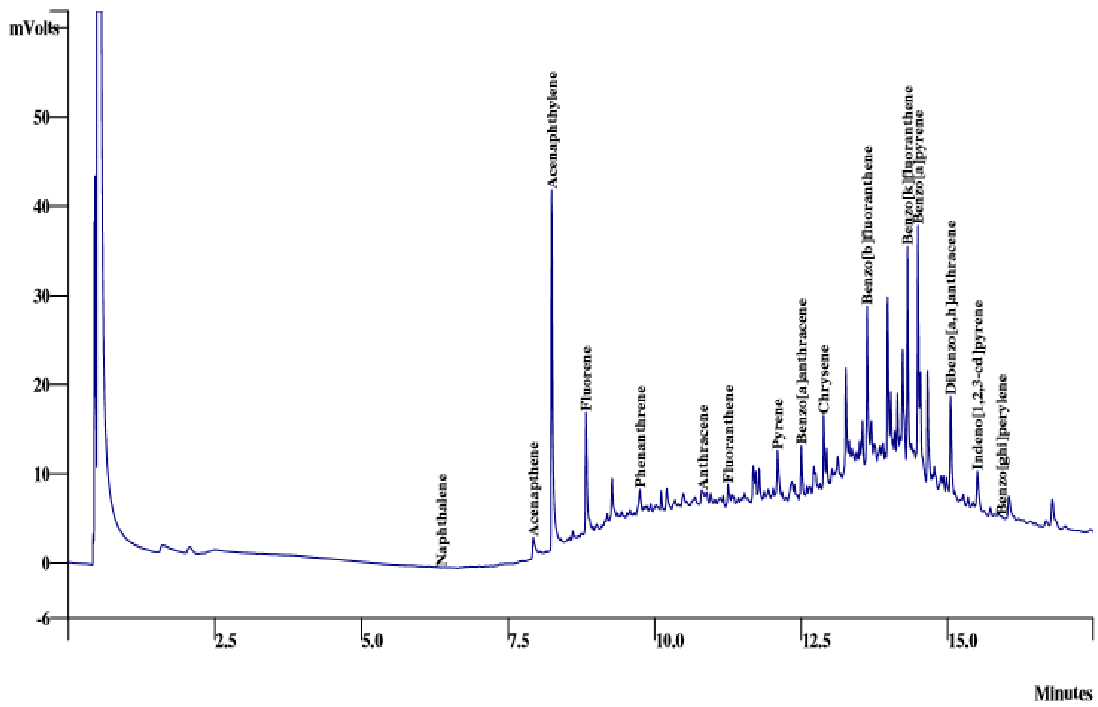


Figure 4.23: Polycyclic aromatic hydrocarbon chromatography profile of Otuocha soil (control) after remediation

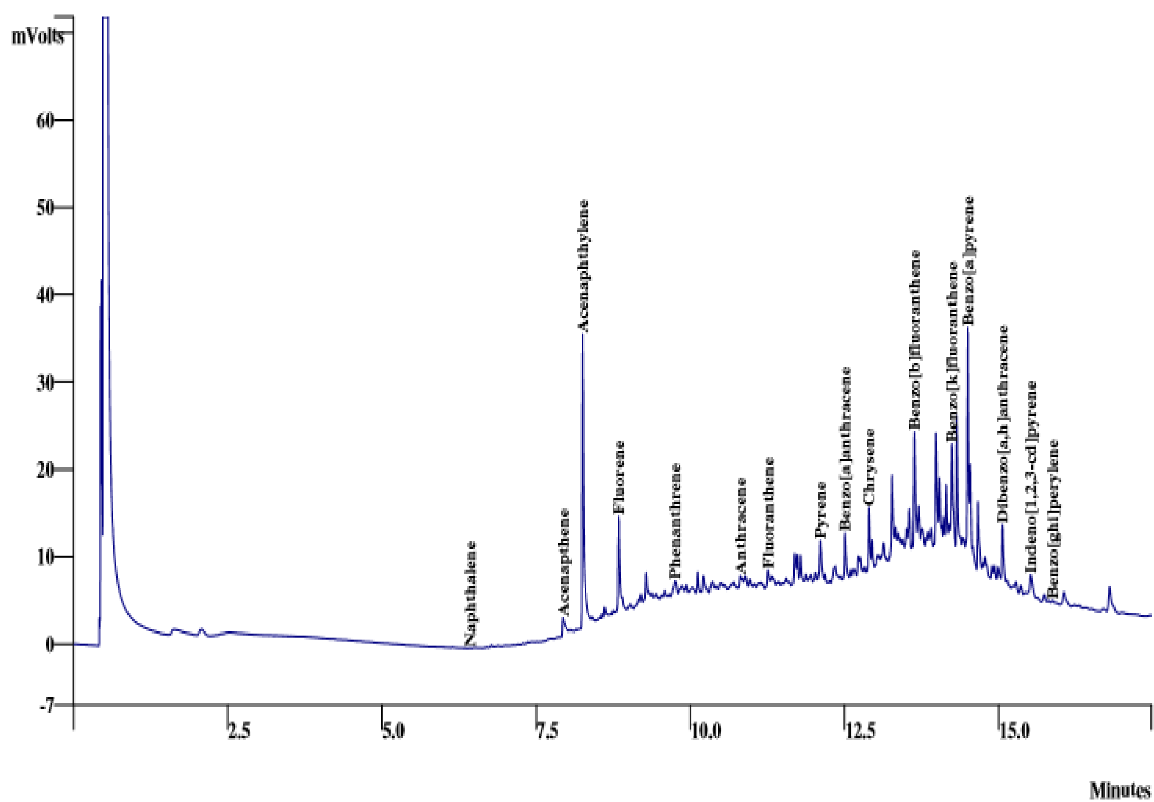


Figure 4.24: Polycyclic aromatic hydrocarbon chromatography profile after the remediation of Otuocha soil with *Gordonia alkanivorans*

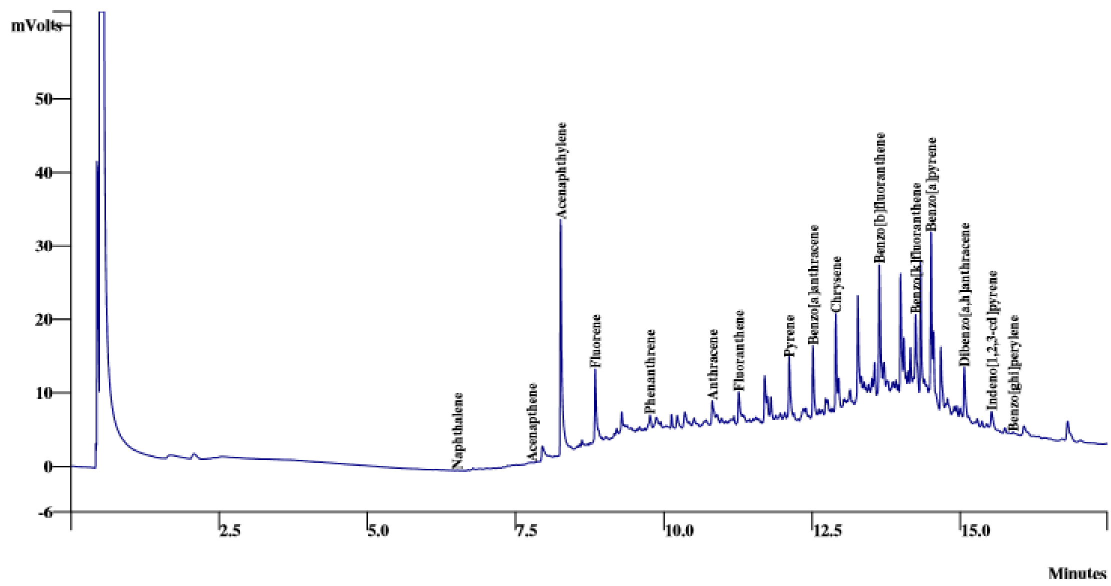


Figure 4.25: Polycyclic aromatic hydrocarbon chromatography profile after the remediation of Otuocha soil with *Tsukamurella inochensis*

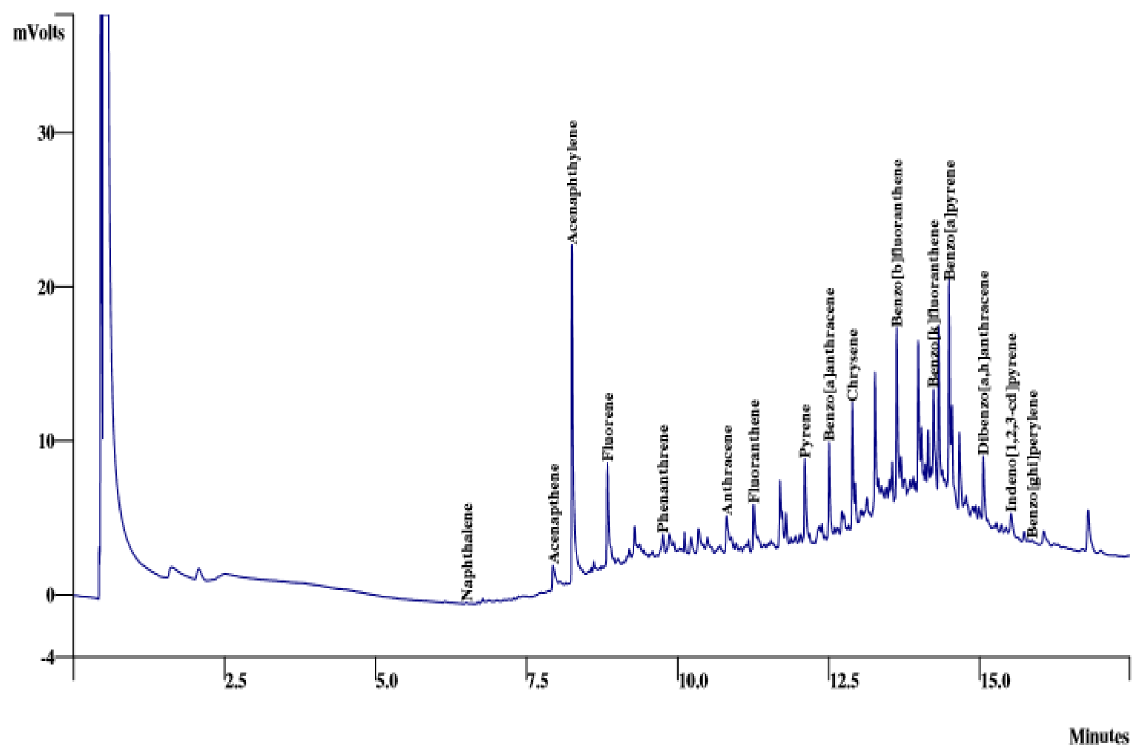


Figure 4.26: Polycyclic aromatic hydrocarbon chromatography profile after the remediation of Otuocha soil with mixed culture

4.13 Result of the Physicochemical Properties of Soils after Remediation

4.13.1 Result of the Physicochemical Properties of Ibeno Soil after Remediation

Result of the physicochemical properties of Ibeno soil samples after remediation are presented in Table 4.21. The electrical conductivity of the control was higher than the soil containing the test isolates likewise its salinity, lead, zinc and iron while cobalt, nickel and aluminium decreased. No crude oil odour was detected in the soil containing the test isolates while it was detected in the control. The water permeability of the control sample was negative while the test samples were positive. The soil texture of the control was loose while the test samples were bound. The value of chromium, manganese, copper, silver, selenium, molybdenium, tin and phosphorus for soil containing mixed culture was 2.14, 10.3, 0.56, 0.61, 0.371, 0.448, 0.100 and 5.738 all measured in mg/kg. The value of the mentioned parameters for soil containing *Gordonia alkanivorans*was 2.50, 9.91, 0.95, 1.10, 0.180, 0.181, 0.00 and 4.689. The value for soil containing *Tsukamurella inochensis* was 2.36, 8.68, 0.38, 1.07, 0.243, 0.132, 0.00 and 3.907. The value of the control was 0.605, 2.21, 9.53, 0.60, 0.98, 0.237, 0.257, 0.00 and 6.274. The value of vanadium and cadmium for all the soil samples was <0.05mg/kg. The value of mercury and arsenic measured in ppm for soil containing mixed culture was 0.031 and 0.788, *Gordonia alkanivorans* was 0.019 and 0.802, *Tsukamurella inochensis* was 0.00 and 0.922 while control was 0.00 and 0.870. The CEC measured in meq/100g was 0.645 for soil containing mixed culture, 0.646 for *Gordonia alkanivorans*, 0.594 for *Tsukamurella inochensis* and 0.605 for control. The percentage nitrogen and carbon was 0.324 and 0.459 for soil containing mixed

culture, 0.264 and 0.601 for *Gordonia alkanivorans*, 0.252 and 0.739 for *Tsukamurella inochensis* and 0.164 and 0.753 for control.

Table 4.21: Result of the physicochemical properties of Ibeno soil after remediation

Parameters	IM	IS2	IC	IS13
Electrical conductivity,us/cm	253	165	269	205
Salinity, mg/kg	120	90.0	122	100
Cation Exchange Capacity ,meq/100g	0.645	0.646	0.605	0.594
Vanadium, mg/kg	<0.05	<0.05	<0.05	<0.05
Lead, mg/kg	0.29	0.43	1.50	1.36
Chromium, mg/kg	2.14	2.50	2.21	2.36
Zinc, mg/kg	8.93	9.08	9.49	8.15
Cadmium, mg/kg	<0.05	<0.05	<0.05	<0.05
Iron, mg/kg	124	124	126	122
Manganese, mg/kg	10.3	9.91	9.53	8.68
Copper, mg/kg	0.56	0.95	0.60	0.38
Cobalt, mg/kg	0.36	0.21	0.08	0.23
Silver, mg/kg	0.61	1.10	0.98	1.07
Nickel	0.06	0.13	0.01	0.62
Selenium, mg/kg	0.371	0.180	0.237	0.243
Molybdenium, mg/kg	0.448	0.181	0.257	0.132
Aluminium, mg/kg	0.413	0.575	0.279	0.732
Tin, mg/kg	0.100	0.00	0.00	0.00
Mercury, ppm	0.031	0.019	0.00	0.00
Arsenic, ppm	0.788	0.802	0.870	0.922
Nitrogen, %	0.324	0.264	0.164	0.252
Carbon, %	0.459	0.601	0.753	0.739
Phosphorus, mg/kg	5.738	4.689	6.274	3.907
Water permeability	positive	positive	negative	positive
Odour	ND	ND	crude oil	ND
Texture	bound	bound	loose	bound

IS2= Ibeno soil with *Gordonia* IM=Ibeno with mixed culture ND=Not Distinct
IS13= Ibeno soil with *Tsukamurella* IC= Ibeno soil without Isolate

4.13.2 Result of the Physicochemical Properties of Otuocha Soil after Remediation

Result of the physicochemical properties of Otuocha soil after remediation is presented in Table 4.22. The electrical conductivity and salinity were lower likewise chromium and copper while selenium and mercury were higher in the control. No detectable crude oil odour in the test samples but was detected in the control. The water permeability of the test samples was fairly positive and negative in the control. The soil texture of the control was loose while bound in the test samples.

The value of lead, zinc, iron, manganese, cobalt, silver, nickel, molybdenium, aluminium, and phosphorus for soil containing mixed culture was 1.43, 5.59, 137, 94.1, 1.59, 0.67, 0.39, 0.00, 1.258 and 0.1896 all measured in mg/kg. The value of the mentioned parameters for soil containing *Gordonia alkanivorans* was 1.86, 5.35, 136, 92.6, 1.51, 0.94, 0.46, 0.015, 1.761 and 0.2987. The value for soil containing *Tsukamurella inochensis* was 0.14, 5.07, 136, 87.1, 1.37, 0.92, 0.65, 0.099, 1.452 and 0.1177. The value of the control was 0.86, 5.44, 136, 92.9, 1.56, 0.80, 0.43, 0.00, 1.268 and 0.267. The value of vanadium and tin for all the soil samples was <0.05mg/kg and 0.00mg/kg respectively. The value of cadmium for control and soil containing *Tsukamurella inochensis* was <0.05mg/kg while that of mixed culture and *Gordonia alkanivorans* was 0.05mg/kg. The value of arsenic measured in ppm for soil containing mixed

culture was 0.651, *Gordonia alkanivorans* was 0.824, *Tsukamurella inochensis* was 1.021 while control was 0.739. The CEC measured in meq/100g was 0.600 for soil containing mixed culture, 0.535 for *Gordonia alkanivorans*, 0.549 for *Tsukamurella inochensis* and 0.540 for control. The percentage nitrogen and carbon was 0.512 and 0.148 for soil containing mixed culture, 0.496 and 0.249 for *Gordonia alkanivorans*, 0.612 and 0.231 for *Tsukamurella inochensis* and 0.443 and 0.282 for control.

Table 4.22: Result of the physicochemical properties of Otuocha soil after remediation

Parameters	OS13	OS2	OM	OC
Electrical conductivity,ms/cm	292	291	271	28.8
Salinity, mg/kg	120	110	130	20.0
Cation Exchange Capacity ,meq/100g	0.549	0.535	0.600	0.540
Vanadium, mg/kg	<0.05	<0.05	<0.05	<0.05
Lead, mg/kg	0.14	1.86	1.43	0.86
Chromium, mg/kg	4.64	3.64	3.43	2.79
Zinc, mg/kg	5.07	5.35	5.59	5.44
Cadmium, mg/kg	<0.05	0.05	0.05	<0.05
Iron, mg/kg	136	136	137	136
Manganese, mg/kg	87.1	92.6	94.1	92.9
Copper, mg/kg	0.90	0.92	1.04	0.89
Cobalt, mg/kg	1.37	1.51	1.59	1.56
Silver, mg/kg	0.92	0.94	0.67	0.80
Nickel	0.65	0.46	0.39	0.43
Selenium, mg/kg	0.252	0.195	0.172	0.325
Molybdenium, mg/kg	0.099	0.015	0.00	0.00
Aluminium, mg/kg	1.452	1.761	1.258	1.268
Tin, mg/kg	0.00	0.00	0.00	0.00
Mercury	0.00	0.00	0.00	0.003
Arsenic	1.021	0.824	0.651	0.739
Nitrogen, %	0.612	0.496	0.512	0.443
Carbon, %	0.231	0.249	0.148	0.282
Phosphorus, mg/kg	0.1177	0.2987	0.1896	0.267
Water permeability	FPFPFP	negative		

Odour	NDND	ND	crude oil	
Texture	bound	bound	bound	loose

OS2= Otuocha soil with *Gordonia*OM=Otuocha soil with mixed culture ND= Not Distinct
OS13= Otuocha soil with *Tsukamurella* OC= Otuocha soil without Isolate FP= fairly positive

4.14 Germination of Bean Seed Monitored after Remediation of Polluted Soil

Table 4.23 showed the growth of bean seed monitored after remediation. The test sample including their positive and negative control seed germinated on the third day except the soils amended with *Tsukamurella* which germinated on the fourth and fifth day. At the end of fourteenth day of monitoring and uprooting the crop, the root and plant length of the test samples and the positive control were higher than those of the negative control.

Table 4.23: Germination of bean seed monitored after remediation of polluted soil

Soil	time of germination	after 14 days
IS2	germinated after 3 days	plant length=8.0, root length=25.5, no of leaf= 8
OS2	germinated after 3 days	plant length=11.3, root length=13.2, no of leaf=5
OS13	germinated after 4 days	plant length=11.0, root length=22.7, no of leaf=5
IS13	germinated after 5 days	plant length=8.0, root length=20.5, no of leaf=5
OM	germinated after 3 days	plant length=7.8, root length=12.3, no of leaf=5
IM	germinated after 3 days	plant length=7.5, root length=21.0, no of leaf=8
OC	germinated after 3 days	plant length=7.0, root length=6.5, no of leaf=5
IC	germinated after 3 days	plant length=7.0, root length=12.0, no of leaf =5

OUP	germinated after 3 days	plant length=12.5, root length=23.2, no of leaf=5
IUP	germinated after 3 days	plant length=12.0, root length=17.3, no of leaf=8

Length measured in centimeter (cm)
OS2= Otuocha soil with isolate *Gordonia alkanivorans*
IS2= Ibeno soil with isolate *Gordonia alkanivorans*
OS13=Otuocha soil with isolate *Tsukamurella inochensis*
IS13= Ibeno soil with isolate *Tsukamurella inochensis*
OM= Otuocha soil with mixed culture
IM= Ibeno soil with mixed culture
OC= Otuocha soil without isolate (control)
IC=Ibeno soil without isolate (control)
OUP= Otuocha unpolluted soil
IUP= Ibeno unpolluted soil

CHAPTER FIVE

DISCUSSION

The ability of biosurfactant producing bacterial isolates to degrade crude oil was assessed. This biosurfactant helps in emulsifying the crude oil so that it can easily be broken down by microbes.

The physicochemical properties of the Ibeno polluted soil sample such as pH, Electrical conductivity and some heavy metals indicated that the soil contains hydrocarbon components of crude oil (Table 4.1). The result correlates positively with the findings of Ogbonna and Amajuoyi (2009) who carried out the physicochemical properties of a crude oil polluted site and reported that the oil pollution made the values of the parametres to be above the specified limits.

The physicochemical properties of the soil before and after crude oil pollution showed that there was an increase in the pH of Ibeno soil after pollution but Otuocha soil recorded a decrease. Some of the metals analyzed showed some variability between the two soils as some were increasing in one soil and decreasing in the other. The observed reduction in pH and conductivity correlated positively with the findings of Osuji and Nwonye (2007). A reduction in pH for Otuocha soil implied increased acidity which is a problem for agricultural soils because many metal cations are more soluble and available in the soil solution at very low pH and can lead to an increase in the value of some of the metals that increased like mercury, arsenic, selenium etc. Akpoveta *et al.*(2011) also reported the same findings in their research work.

Reduced conductivity could be due to the non-polar nature of the crude oil bringing about reduced ionic movement in the soil. The increase in pH recorded for Ibeno soil may have been connected with the soil texture coupled with the fact that it was left undisturbed for 30 days and there are indigenous microbes inhabiting the soil. There is probability that the soil texture actually allowed degradation to be taking place which led to a slight increase in the pH. This may have also affected lead and cobalt which also experienced a decrease.

The decrease in some of the metals after crude oil pollution could be the trapping of the metals to the soil due to pollution. The reduction in nitrogen level after pollution correlated negatively with the findings by Akpe *et al*.(2015) and Akpoveta *et al*.(2011) who observed an increase following artificial pollution of the soil but it correlated positively with Agbogidi *et al.*(2007) who observed a decrease. The decrease experienced could be linked to the utilization of available nitrogen by indigenous microorganisms to carry out oil degradation when the polluted soil was left undisturbed for 30 days.

The microbiological enumeration of soil samples before and crude oil pollution showed that there was a decrease in the microbial count (cfu/g) of all the microorganisms after artificially polluting the soil with crude oil. The reduction was due to the toxicity of the crude oil to microorganism while leaving only the crude oil degraders that survived the pollution. This finding correlated positively with the findings of a research done by Akpoveta *et al*.(2011) who recorded a decrease in the colony forming unit of microorganism following crude oil pollution.

The two bacterial isolates that were identified and used in carrying out the research work were *Tsukamurella inochensis* strain Yaoman (JQ806393.1) and *Gordonia alkanivorans.*The morphological and biochemical characterization (Table 4.6) agreed with the named isolates which correlated positively with the molecular identification following sequence analysis (Figure 4.1 and 4.2).

The metagenomic analyses of the two soil samples showed the microbial population in the soils that survived the crude oil pollution. Most of the organisms have been researched on their ability to degrade petroleum hydrocarbon.

Microbacterium, Methylobacterium and *Pseudomonas*has been researched on by Godini *et al.* (2018). *Azospirillum, Bacillus, Gordonia, Mycobacterium, Nocardioides, Planococcus, Pseudomonas* and *Sphignomonas* have been published by Chikere *et al*. (2011) where he stated in a table that most were able to degrade petroleum by either carrying the enzyme alkane hydroxylase or plasmid-borne genes for dioxygenases.

*Paraburkholderia*has been published by Lee *et al*., 2019 as a petroleum degrader. *Sphingobium*has been researched on by Liu *et al.,* 2017. *Phenylobacterium, Aquabacterium, Massilia* and *Sphingomonas*has been researched on by Yang *et al.,* 2014. *Cellulomonas* has

been researched on by Fathepure, 2014. *Hydrocarboniphaga*has been researched on by Palleroni *et al.*, 2004. *Roseomonas*has been researched on by Jain *et al*., 2011. While *Candida solibacter* has been found through metagenomics in a soil polluted by oil (Patel *et al.,* 2016).

The fungi isolated from the polluted soil were *Cylindrocarpon* and *Aspergillus lentulus* as presented in Table 4.7. Only one fungal isolate was isolated from each soil. *Cylindrocarpon* has been observed by Beraldo de morais and Tauk-Tornisielo(2009) as a crude oil degrader. Aspergillus lentulus had been isolated from an oil polluted site by Ugboma *et al*., 2020.

The percentage degradation of crude oil using gravimetric method showed that 100ul gave higher percentage degradation than others. As the concentration of crude oil increased, the percentage degradation decreased. This correlated positively with the findings of Akpe *et al.*(2015) who observed a higher percentage of crude oil loss in the soil polluted with 5% crude oil than that of 10% and 15%. They stated that such high concentration could pose serious challenge to the metabolic activities of soil microorganisms.

The pH of the 28 days of degradation tended towards acidity during the course of the degradation except in the control. The increase towards acidity showed that organisms were actually growing but preferred an acidic medium; hence, it was actually utilizing the crude oil as a source of carbon. This correlated positively with the report of a research done by Sarma and Sarma (2010) who observed that bacterial strains were flourishing in a soil sample at a pH range of 4.98 – 5.5.

The optical density reading at 600nm during the 28 days of degradation showed an increase on the 7th day and highest value was observed on the 14th day. A decrease was observed on the 28th day (figure 4.7). The increase in the optical density at 600nm showed that the organisms were increasing in their number which means they are utilizing the crude oil as carbon and hence

degradation was taking place. The decrease observed on the 28th day showed that the organism has started reducing in their number because the carbon source has reduced thereby showing that the degradation is about to end. This increase in the optical density value by the day during spectrophotometry correlated positively with a similar work done by Vanishree *et al.* (2014) on petrol when he stated that the increase in the optical density during the treatment period indicated growth due to the utilization of petrol as a source of carbon.

In the biosurfactant screening test, more interest was laid on bacterial isolates that produced biosurfactant which could aid in biodegradation and remediation. Some of the isolates were positive for haemolysis test. Only *Gordonia alkanivorans* and *Tsukamurella inochensis*gave positive results to drop collapse and oil displacement tests. During incubation, emulsification of crude oil was evident in their culture media, suggesting the production of extracellular biosurfactant/bioemulsifier. Both isolates emulsified the crude oil within only 5 days of incubation; on standing the flask, a thin layer of oil was separated out which again became dispersed on gentle shaking. In contrast, the oil layer in the other flasks remained on the surface even after extra 5 days. The two isolates also gave a high emulsion index value. Five different methods were used as stipulated by Satpute *et al*. (2008) that more than one screening methods should be included in the primary screening for proper identification of potential biosurfactant producers. Some of the isolates which gave a positive result for hemolysis test but gave a negative result for other tests may not be biosurfactant producers. This is as a result of the fact that lytic enzymes can also give positive result for hemolysis test as reported by Jain *et al.*(1991). Plaza *et al*. (2006) and Youssef *et al.*(2004) also demonstrated that the oil spreading technique is a reliable method to detect biosurfactant production by diverse microorganisms. The result of the biosurfactant screening correlated positively with the findings of Kugler *et al.*(2014) which

stated that *Tsukamurella* sp was able to produce trehalose lipid biosurfactants. In addition, Nazina *et al*.(2003) and Ta-chen *et al*. (2008) also reported the production of exo-polysaccharides by *Gordonia* sp.

The total petroleum hydrocarbons (TPH) after 28 days of crude oil degradation for the test isolates and their mixed culture showed a less value of TPH to compare with the control, this was due to the utilization of the TPH component of the crude oil by the microorganisms.The result correlated positively with the research done by Ibrahim (2016) who observed a reduction in the level of TPH after degradation of used engine oil using bacteria.

The polycyclic aromatic hydrocarbon contents after the 28days degradation decreased in both the test organisms and their mixed culture to compare with the control. The PAH value during the 28 days' degradation showed that the test organism actually lowered the level of recalcitrance in the medium. The result correlated positively with the findings of Uba *et al.*(2016).

The values obtained for the TPH and PAH of Otuocha and Ibeno soil sample after artificial pollution indicates a strong pollution. It agrees with a publication by Osuji et al., 2005 who reported that beyond 3% concentration of crude oil pollution indicates severe pollution.

The total viable count of the inoculum development that was inoculated into the soil for the remediation exercise was 22.7×10^5cfu/mlfor *Gordonia alkanivorans* and 11.0×10^5cfu/mlfor *Tsukamurella inochensis*.The inoculum development was done to produce enough microbial seed and biosurfactant that will be used in the bioremediation exercise. This correlates with a publication done by Patoway *et al*., 2016 who prepared an inoculum development that he used in carrying out a degradation exercise.

The total viable counts of five months' soil remediation decreased on the first month, the decreasecould be as a resultof indigenous microbes and the added microbes trying to adapttotheir new environmentbecause the added biosurfactant reduced the pH of the environment. It increased on the second and third month after their adaptation. On the fourth month, there was a wide increase on the soil amended with the tested isolates to compare to the control and that was the peak of the microbes' growth and degradation. A reduction was observed on the fifth month because the source of carbon has reduced and hence a decline in the degradation rate. This result correlated positively with the findings of Akpe *et al*. (2015) who observed a decrease in the total viable count during the second week of their study, but it later increased again during the course of the experiment; they also observed that the total viable count was more in the amended soil samples than in the non-amended (control) samples.

The pH of the fivemonths' soil remediation decreased on the first day for soil containing the tested isolates because the added biosurfactant decreased the pH. On the second and third month, Ibeno soils containing tested isolates showed a slight increase in acidity compared to their control, on the third month, the pH value was almost the same. In Otuocha soil, the pH seems to be the same for the tested isolates and their control except on the third month where the tested isolates pH was slightly lower than the control's. The decrease in pH observed for the soils containing test isolates was because the added bacteria preferred an acidic environment and are more in number. But because indigenous hydrocarbon degraders were present in the soils, they might have influenced the pH of the environment to suite their growth condition, hence, the pH became almost the same with the control that also contained indigenous hydrocarbon degraders. The pH of the soil containing the test isolates and the control was at 5.61- 6.87 in the first four months to aid degradation. The pH values obtained during the course of soil remediation

supports the findings of a similar research work carried out by Sri and Lakshmi (2009) who reported that Pseudomonas Demolyticum degraded phenol more at pH 6 to compare to other pH values.

The values of TPH for Ibeno and Otuocha soilsafter the fivemonths' bioremediation for the tested isolates and their mixed culture were lower to compare with the control and also significantly different at 5% level of confidence, this was as a result of crude oil utilization by the isolates. The control showed a reduction in the value of TPH when compared with the artificially polluted soil because of the presence of indigeneous microbes that also degraded the crude oil in the soil. This finding correlated positively with a research done by Asadirad *et al*.(2016) who concluded on the use of microbes as a highly desirable and promising treatment option for crude oil polluted soil.

The PAH values of Ibenoand Otuocha soilsafter fivemonths'degradation of oil for the tested organisms and their mixed culture showed a lower value to compare to the control that has a higher value. In the experiment, the mixed culture showed a lower value than the individual tested isolates. The reduction observed in the value of the PAH showed that the tested isolates and their biosurfactant actually helped in removing more of the recalcitrant PAH in the soil to compare to control. The reduction in the value of PAH observed in the control when compared to the initial value after pollution was due to the presence of indigeneous microbes that helped in its degradation. The PAH value of Ibeno soil was lower than that of Otuocha soil because the soil texture of Ibeno allows for easy aeration and water movement which made the microorganisms to carry out metabolism at a higher rate in Ibeno soil and hence, the higher degradation rate. The decrease in the PAH for the samples treated with the test organism and their biosurfactant to compare to control correlated positively with a research work done by Belabbas *et al*.(2016) and

Bada *et al*.(2018) who reported the removal of polycyclic aromatic hydrocarbon in the soil using microorganisms.

The physicochemical properties of the soil after remediation are presented in Table 4.13 for Ibeno soil and 4.14 for Otuocha soil. The treated soil samples for both Otuocha and Ibeno had positive water permeability while the control had negative. Water permeability being the capacity of a soil to allow water through, is a function of its texture, aggregation and swelling; the soil in polluted area has lost its colloid state and coagulating properties thus its lack of permeability. The Otuocha treated soil samples were fairly positive because of the composition of the soil. The crude oil odour was not distinct in the treated soils but noticeable in the control. The soil texture of the treated soils was bound while the untreated appears loose. This finding correlated positively with the research done by Chijioke-Osuji(2014) who stated that the polluted sample has fine grains while the treated samples have bound grains and that is as a result of degradation of the soil in the polluted area.

There was a general decrease in the total organic carbon; phosphorus and nitrogen contents in the samples after remediation but the value of the treated soil samples was lower than the control. This was because the bioaugmentation in addition to the biosurfactant added helped in solubilizing the crude oil and increasing the number of organisms needed to break down the crude oil, hence its utilization as a carbon source. Phosphorus and nitrogen were also utilized by the organisms during degradation. This result correlated positively with the findings of Chijioke-Osuji(2014) who also reported a decrease in the total organic carbon content and phosphorus in the treated soil after remediation. There was a general decrease in the level of arsenic, mercury and some other metals in the samples. This could be due to the bioremediation as there are possibilities of the test organisms removing the dangerous heavy metals as it is breaking down

the crude oil. Some of the physicochemical parameters that experienced some increase could be due to the solubilisation of the trapped metals in the soil due to bioremediation.

The germination and growth of bean after remediation showed that almost all the samples and their positive and negative controls germinated on the third day except for a few. The ones that delayed were the polluted soil treated with *Tsukamurella* and its biosurfactant. The root and plant length of all the test samples and their positive controls were more than that of the negative control. Ibeno test sample and their positive result had eight numbers of leaves each except for the soil sample treated with *Tsukamurella* sp and negative control which had 5 numbers of leaves. The reduction in the number of leaves and delayed germination for soils treated with *Tsukamurella* could be linked to its reduced cation exchange capacity which can come from the interaction between its surfactants and other microbes in the soil while that experienced in the negative control was because it wasn't treated with any organism. The result correlated positively with the findings of Okoye and Okunrobo (2014) and Uquetan *et al*.(2017) who maintained that crude oil affects soil fertility. This finding showed that removal of crude oil from the soil through biological means can help in improving soil fertility.

The statistical significant difference at the 5% level of confidence was observed from the PAH and TPH of the tested isolates and their control for both degradation and remediation. The result showed that the tested isolates and their biosurfactant actually helped in both degradation and bioremediation (see appendix XL).

CONCLUSION

The agricultural use and management of soil is largely dependent on the characteristics and qualities of the soil. Results from the study revealed that oil pollution had significant influence

on soil properties and crop growth which render such soils temporarily unsuitable for cropping for some time before being degraded.The use of biosurfactant producing bacterial isolates such as *Gordonia alkanivorans* and *Tsukamurella inochensis*have proved to be a biological tool that can be used in cleaning up this crude oil pollution from the environment.

RECOMMENDATION

The microbial biosurfactant synthesis has important applications in bioremediation for a range of hydrocarbon pollutants. It can be considered as a key strategy for bioremediation of agricultural soil due to their biodegradability and low toxicity. Necessary steps can be taken for the large scale production of biosurfactants using cheap raw materials such as organic wastes that is cost effective over chemically derived surfactants, with this much can be achieved interms of removing hydrocarbon or crude oil contaminationfromagricultural soil. In the present study, biosurfactant from soil bacterium was isolated and explored for biodegradation and bioremediation applications. Future research would be on complete structural characterization, genetic level studies and elucidation of the individual components of the biosurfactant produced by*Tsukamurella inochensis* and *Gordonia alkanivorans*. Secondly, the analysis of the crops from the bioremedated soil is essential to ascertain their safety for human consumption.

CONTRIBUTION TO SCIENCE

This research confirmed the use of *Tsukamurella inochensis* and *Gordonia alkanivorans* that can produce biosurfactant which is not toxic to the environment for the effective clean up of oil spills. These microbes provided an effective means of bioremediating crude-oil polluted soils

making them useful for farming again. This research also detected the effect of crude oil pollution on Otuocha soil, if oil drilling commence there and also clean up technique to adopt. The researchers used *Tsukamurella inochensis* and its biosurfactant to effectively carry out degradation and remediation which has not been published by any author.

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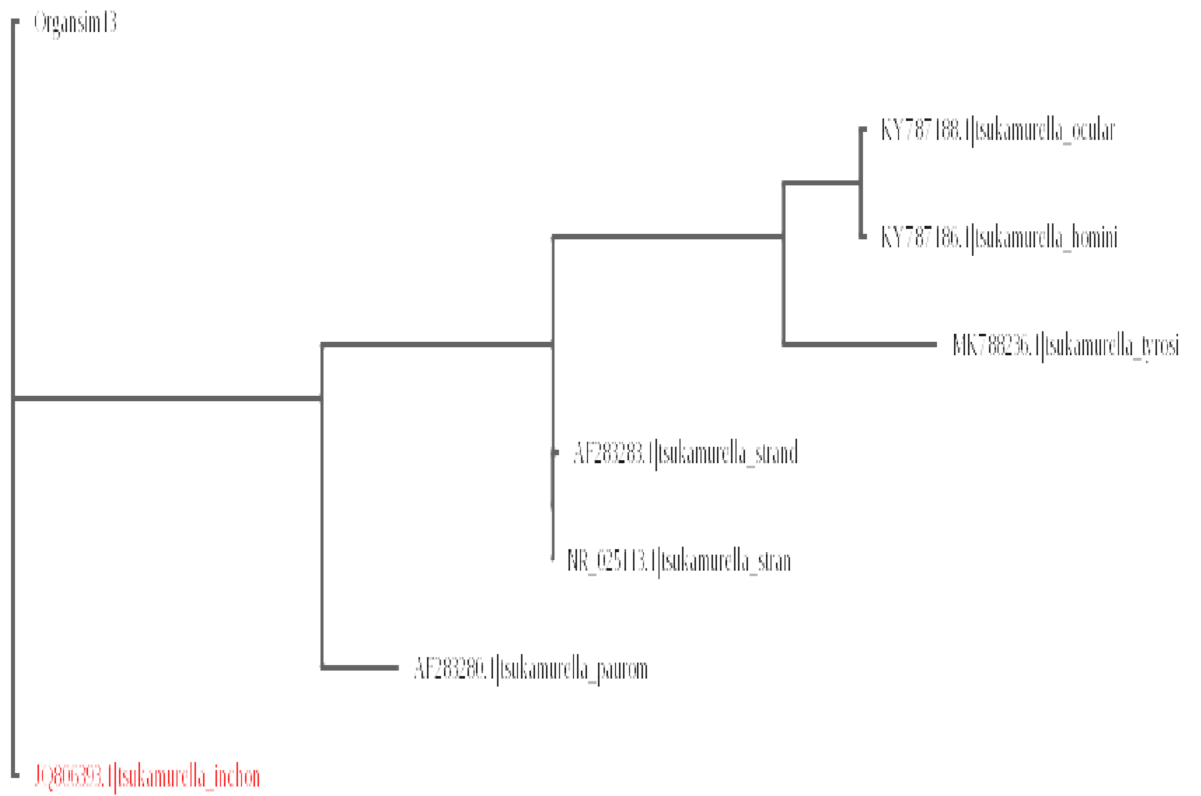
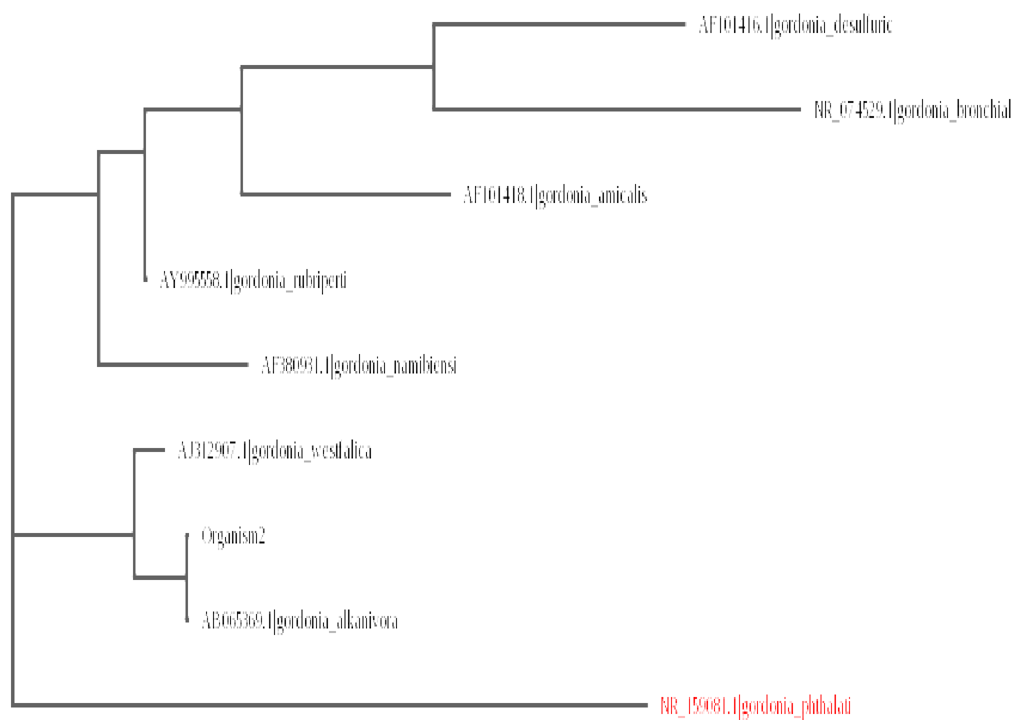
APPENDIX

Appendix I: PHYLOGENETIC TREE OF *Tsukamurella inochensis*



Appendix II: PHYLOGENETIC TREE OF *Gordonia alkanivorans*



Appendix III: Percentage degradation of crude oil by gravimetric method

Test organism	WP	WPECO	WRCO	WCOA	AD	% degradation
S2(100UL)	34.74±0.03	34.78±0.03	0.04±0.03	0.06±0.03	0.02±0.03	33.33±0.03
S2(500UL)	30.42±0.04	30.61±0.04	0.19±0.04	0.26±0.04	0.07±0.04	26.92±0.04
S2(1000UL)	36.53±0.03	36.90±0.03	0.37±0.03	0.47±0.03	0.10±0.03	21.28±0.03
S2(2000UL)	34.76±0.23	35.51±0.23	0.75±0.23	0.89±0.23	0.14±0.23	15.73±0.23
S13(100UL)	19.92±0.08	19.95±0.08	0.03±0.08	0.06±0.08	0.03±0.08	50.00±0.08
S13(500UL)	19.20±0.49	19.35±0.49	0.15±0.49	0.26±0.49	0.11±0.49	42.31±0.49
S13(1000UL)	29.08±0.05	29.43±0.05	0.35±0.05	0.47±0.05	0.12±0.05	25.53±0.05
S13(2000UL)	34.19±0.12	34.92±0.12	0.73±0.12	0.89±0.12	0.16±0.12	17.98±0.12

Values are mean ± S.D. of duplicate determination

S2= *Gordonia alkanivorans*

S13= *Tsukamurella inochensis*

WP= weight of petridish

WPECO= weight of petridish containing extracted crude oil

WRCO= weight of residual crude oil

WCOA= weight of crude oil added in the media

AD= amount degraded

Appendix IV: pH reading during the 28 days' degradation

Days	*Gordonia sp*	*Tsukamurella sp*	Mixed culture	Control
0	6.0±0.69	6.0±1.12	6.0±0.39	6.0±0.69
7	5.0±0.05	4.4±0.01	4.6±0.23	6.0±0.05
14	4.8±0.68	4.1±0.75	4.4±1.54	5.8±0.06
21	5.0±0.58	4.3±0.31	4.6±0.74	5.9±0.09
28	4.8±0.37	4.4 ±0.67	4.7±0.05	5.9±0.06

Values are mean ± S.D. of duplicate determination

Appendix V: OD reading during the 28 days' degradation (600nm)

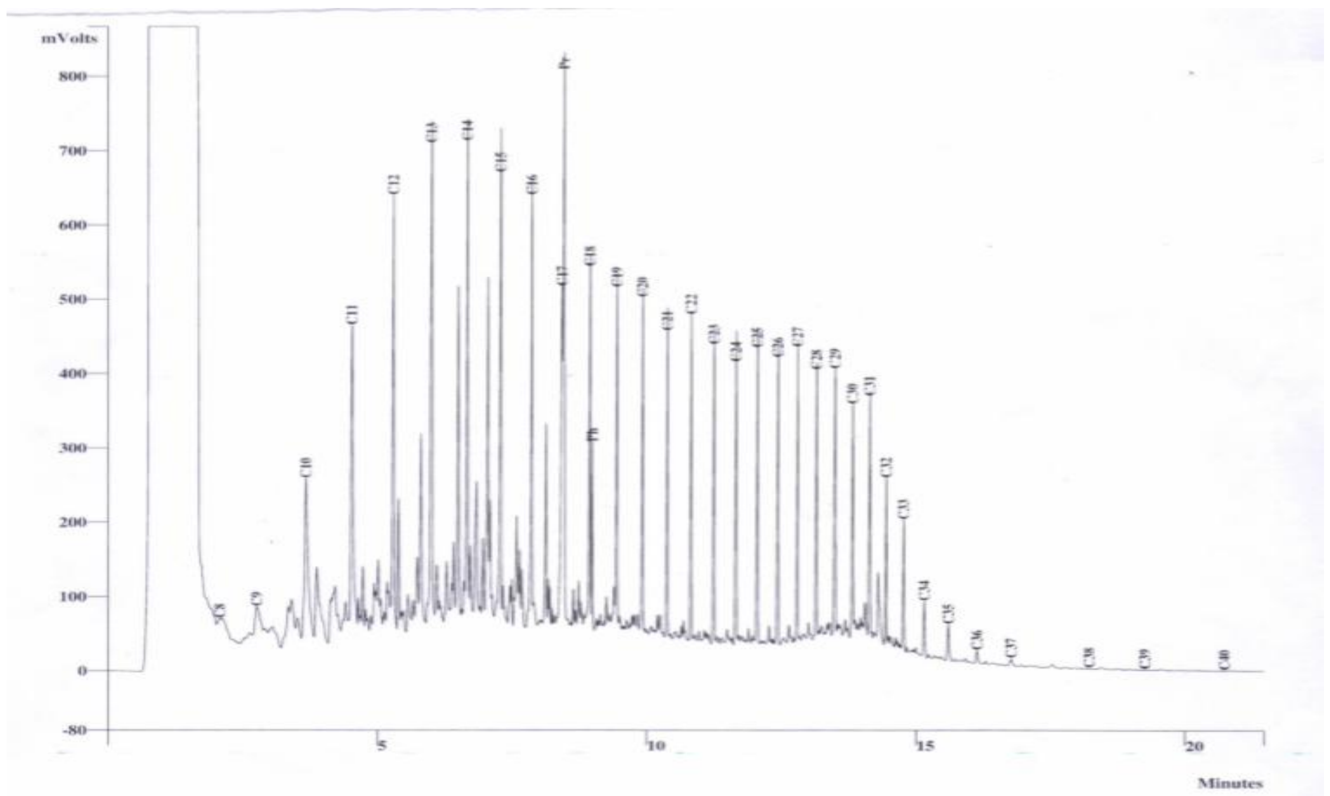
Days	*Gordonia sp*	*Tsukamurella sp*	Mixed culture	Control
0	0.065±0.16	0.059±0.29	0.060±0.18	0.047±0.08
7	0.358±0.11	0.504±0.29	0.408±0.02	0.047±0.16
14	1.024±0.23	1.558±0.28	1.338±0.31	0.334±0.17
21	0.670±0.31	1.396 ±0.12	1.026±0.30	0.061±0.02
28	0.370±0.09	0.869±0.49	0.694±0.39	0.063±0.08

Values are mean ± S.D. of duplicate determination

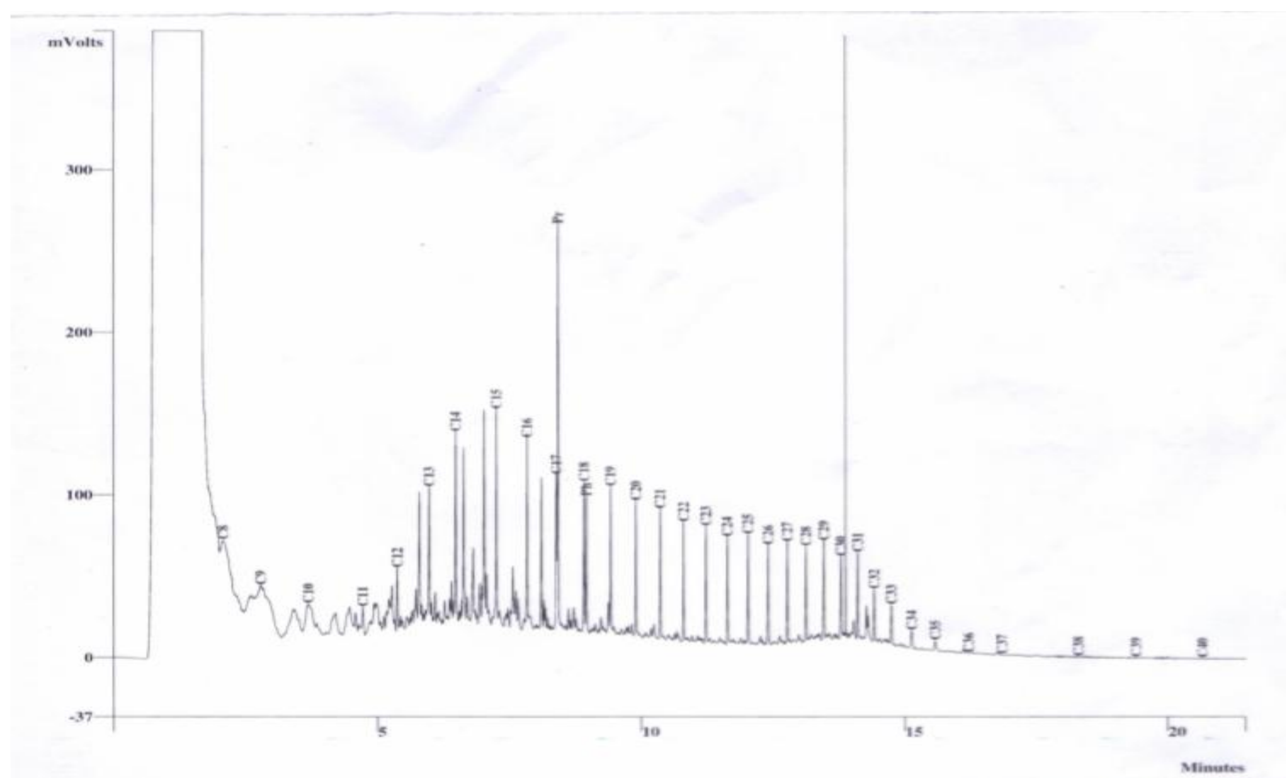
Appendix VI: Emulsion index

Test organism	emulsion layer	total height	emulsion index (%)
S1	0	2.1	0
S2	1.3	2.2	59.09
S3	0.2	2.6	7.69
S4	0	2.6	0
S5	0	2.7	0
S6	0.2	2.7	7.41
S7	0.3	3.0	10
S8	0.2	2.8	7.14
S9	0.5	2.9	17.24
S10	0.1	2.8	3.57
S11	0	2.7	0
S12	0	2.9	0
S13	1.6	2.8	57.14

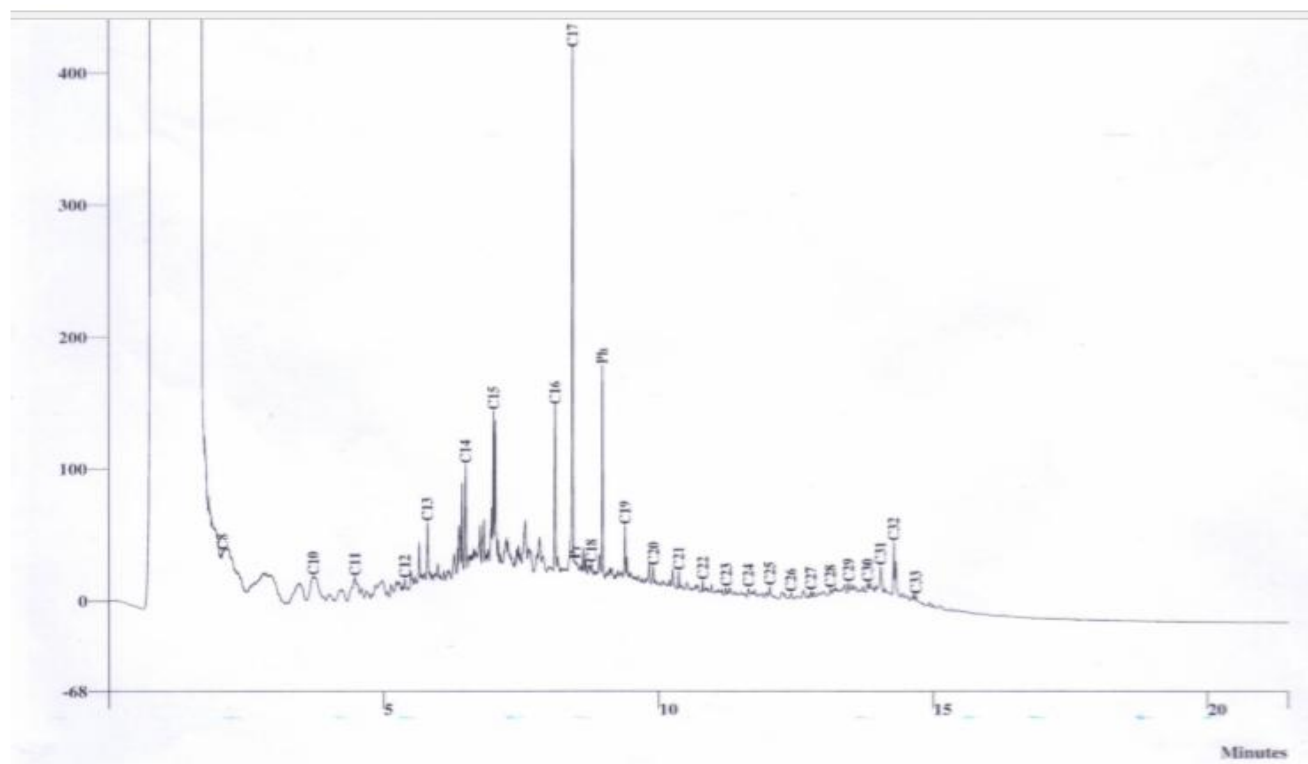
Appendix VII: Total petroleum Hydrocarbon Chromatography profile of crude oil (control)



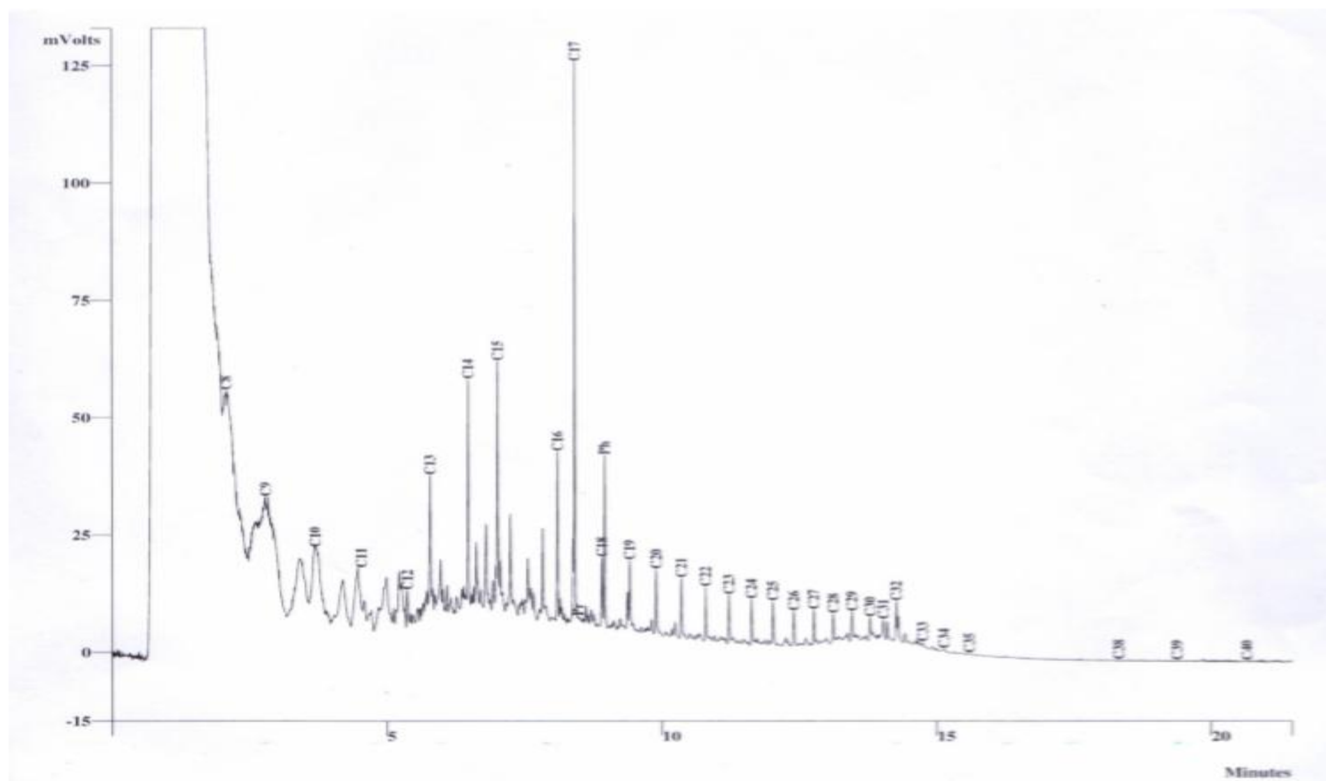
Appendix VIII: Total Petroleum Hydrocarbon chromatography profile after crude oil degradation with *Gordonia alkanivorans*



Appendix IX: Total Petroleum Hydrocarbon chromatography profile after crude oil degradation with *Tsukamurella inochensis*



Appendix X: Total Petroleum Hydrocarbon chromatography profile after crude oil degradation with mixed culture



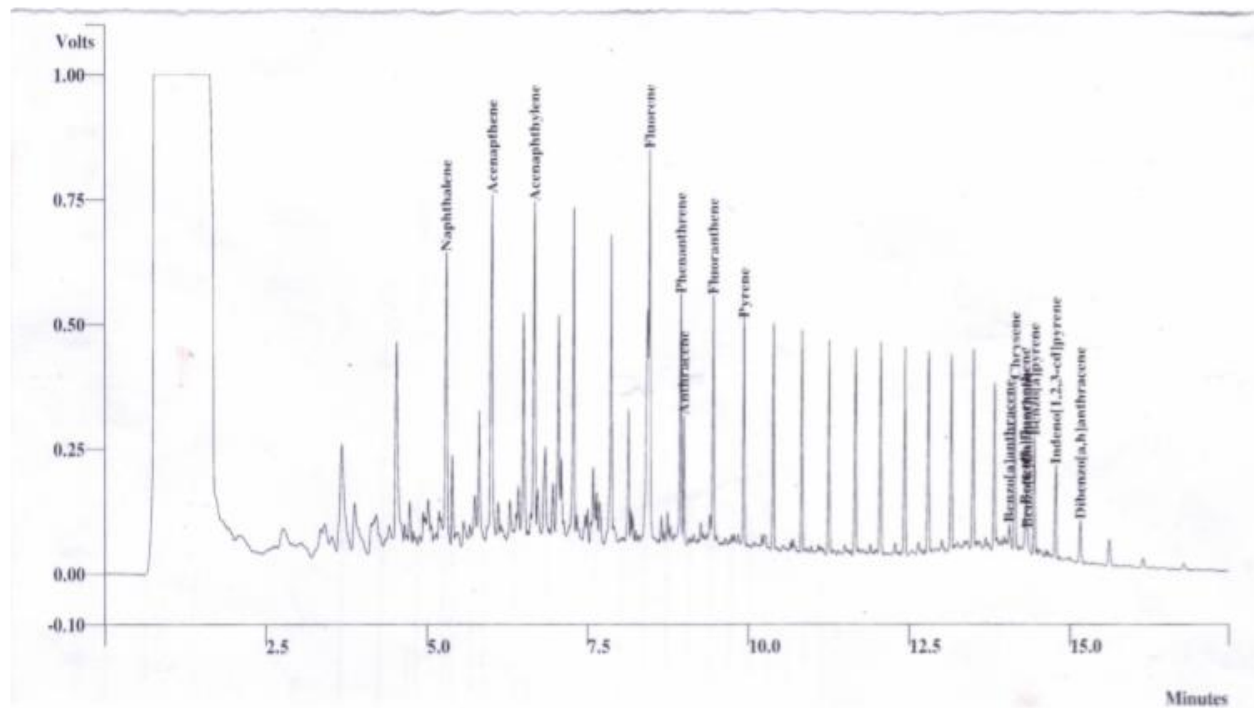
Appendix XI: Compound name of Total Petroleum Hydrocarbon

Group name	compound name

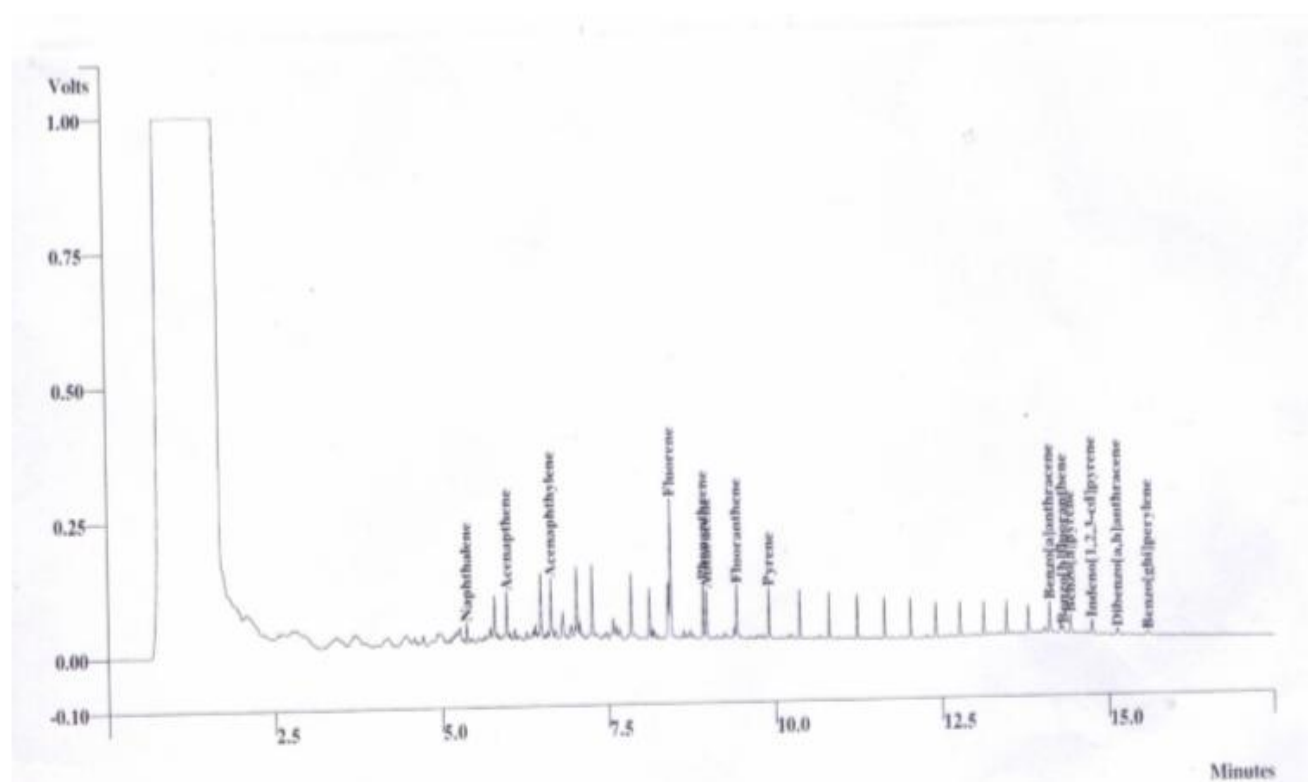
C8	n-cubane
C9	n-Nonane
C10	n-Decane
C11	n-Undecane
C12	n-Dodecane
C13	n-Tridecane
C14	n-Tetradecane
C15	n-Pentadecane
C16	n-Hexadecane
C17+	Pristane
C17	n-Heptadecane
C18	n-Octadecane
C18+	phytane
C19	n-Nonadecane
C20	n-Eicosane
C21	n-Henelcosane
C22	n-Docosane
C23	n-Tricosane
C24	n-Tetracosane
C25	n-Pentacosane
C26	n-Hexacosane
C27	n-Heptacosane
C28	n-Octocosane
C29	n-Nonacosane
C30	n-Tricotane

C31	n-Hentriacontane
C32	n-Dotriacotane
C33	n-Tritriacotane
C34	n-Tetratriacotane
C35	n-Pentatricontane
C36	n-Hexatriacontane
C37	n-Heptatriacontane
C38	n-Octatriacotane
C39	n-Nonatriacotane
C40	n-Tetracontane

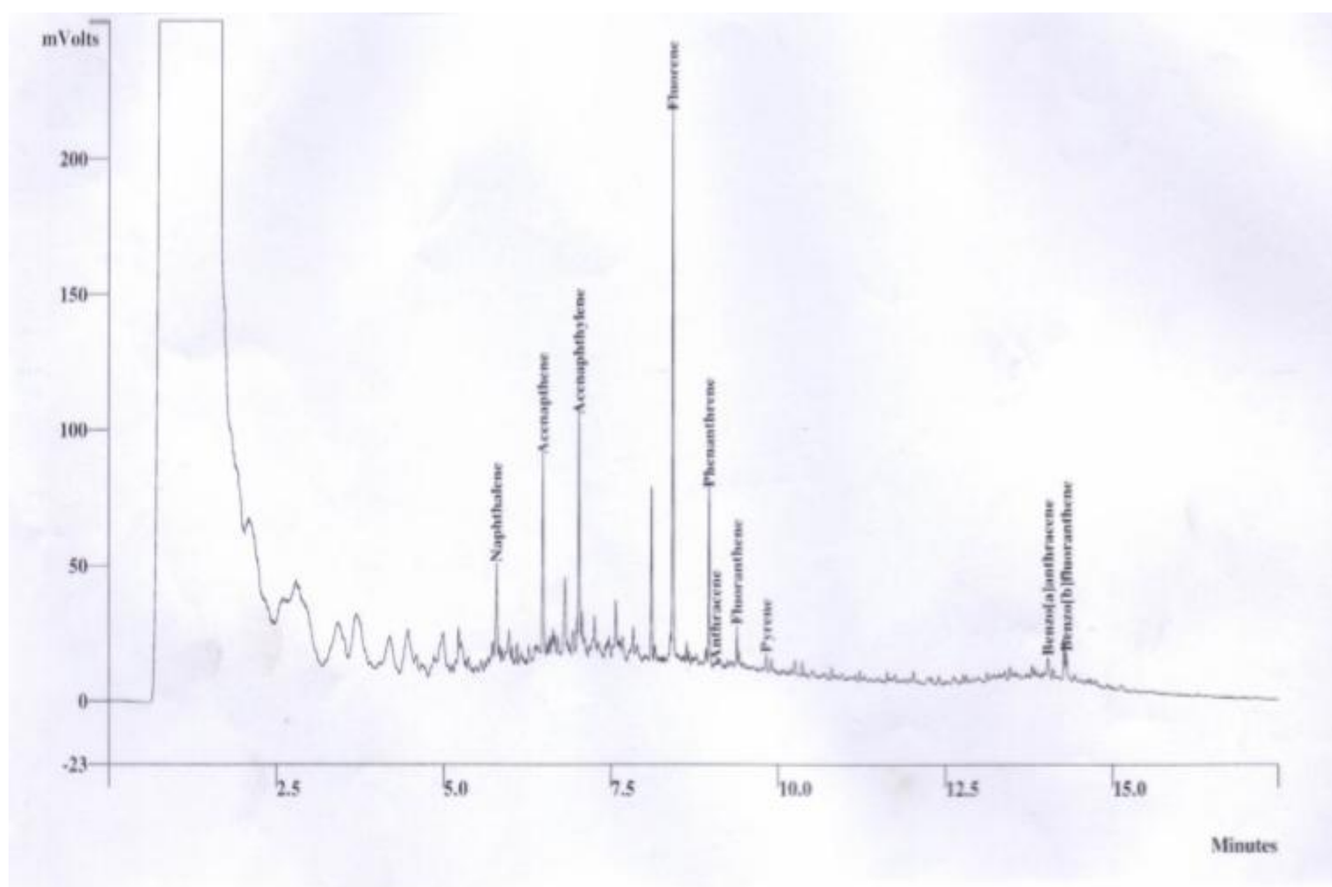
Appendix XII: Polycyclic Aromatic Hydrocarbon chromatography profile of crude oil (control)



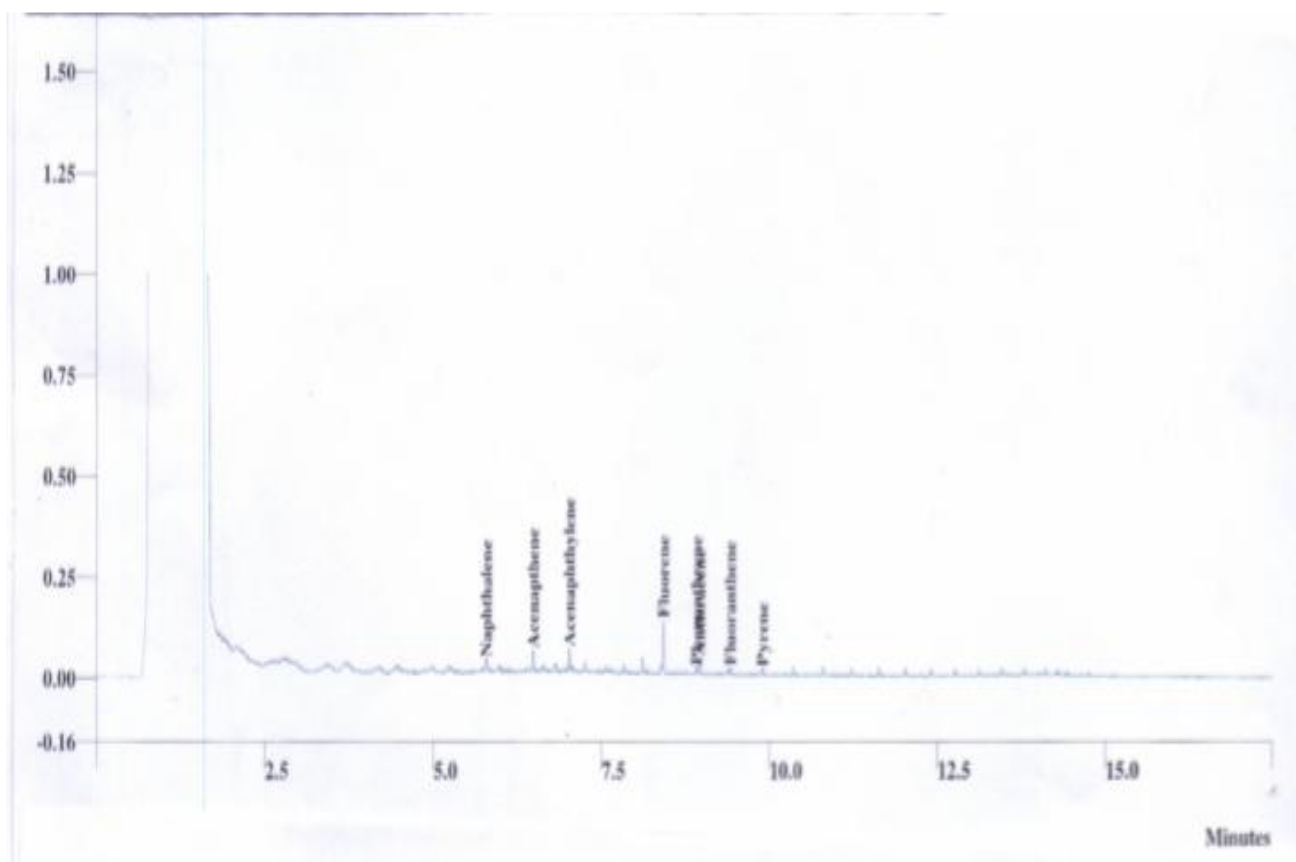
Appendix XIII: Polycyclic Aromatic Hydrocarbon chromatography profile after crude oil degradation with *Gordonia alkanivorans*



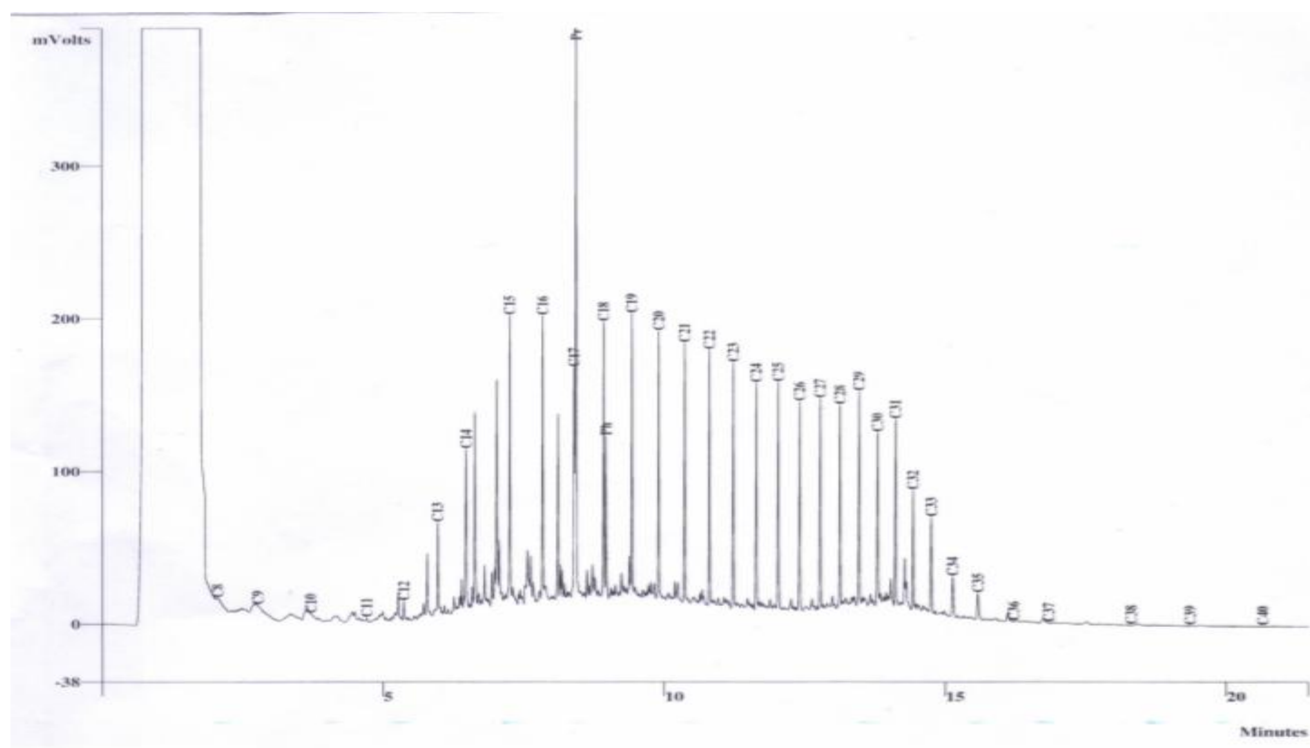
Appendix XIV: Polycyclic Aromatic Hydrocarbon chromatography profile after crude oil degradation with *Tsukamurella Inochensis*



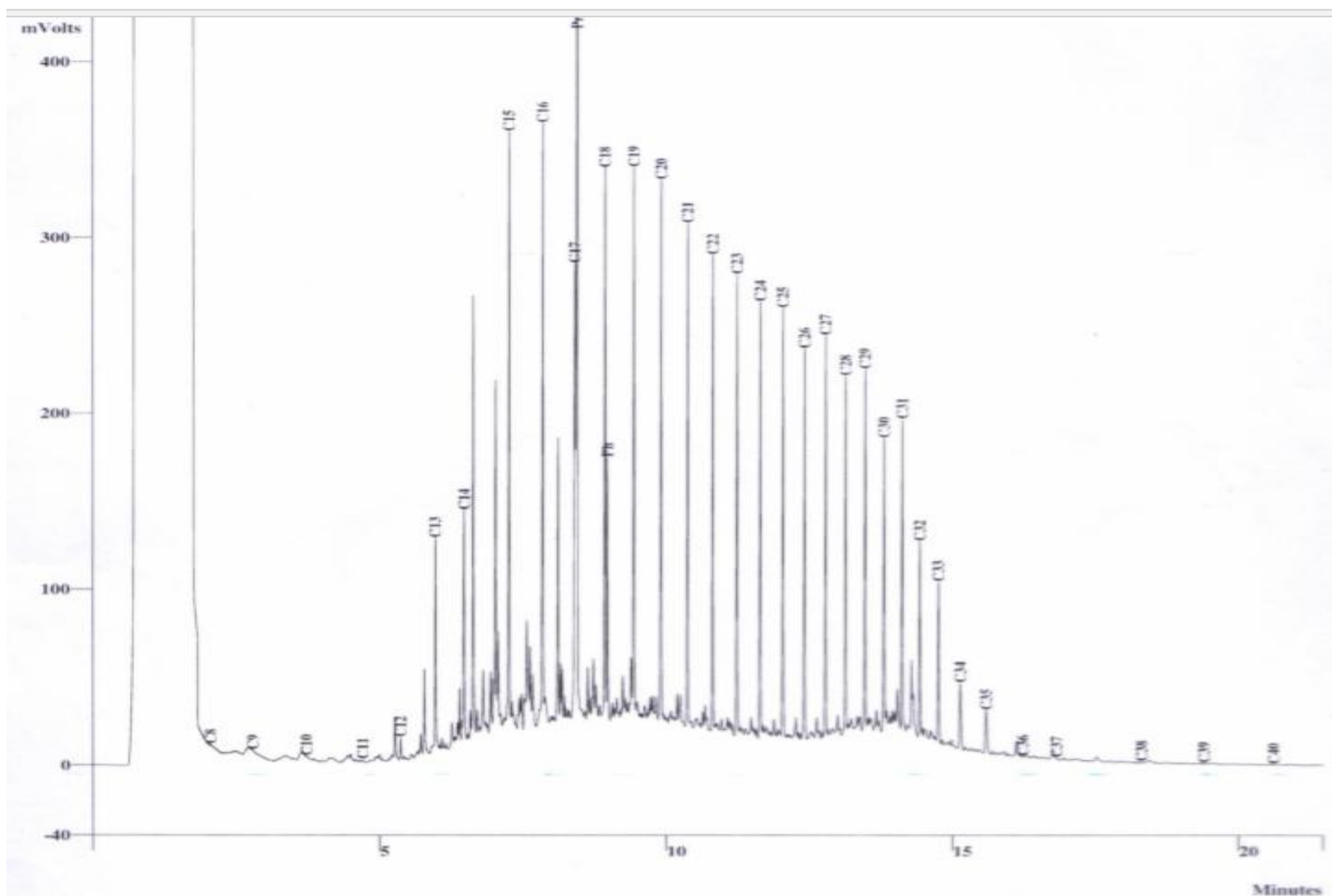
Appendix XV: Polycyclic Aromatic Hydrocarbon chromatography profile after crude oil degradation with mixed culture



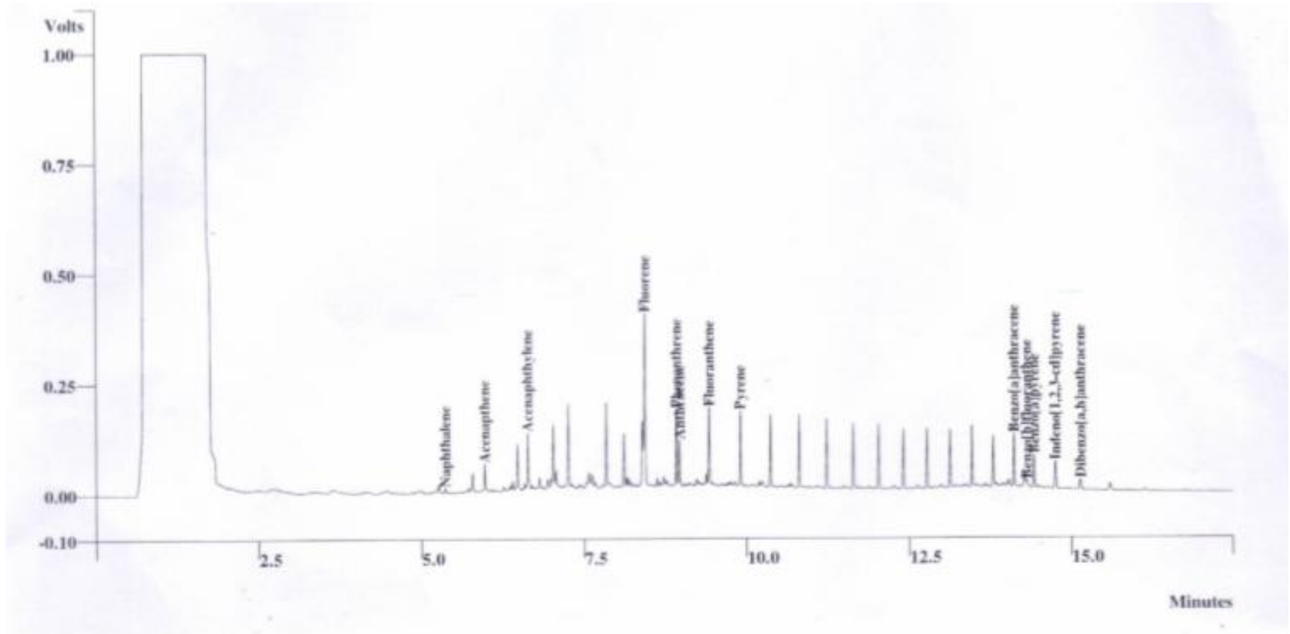
Appendix XVI: Total Petroleum Hydrocarbon chromatography profile of Ibeno soil after artificially polluting the soil (before remediation)



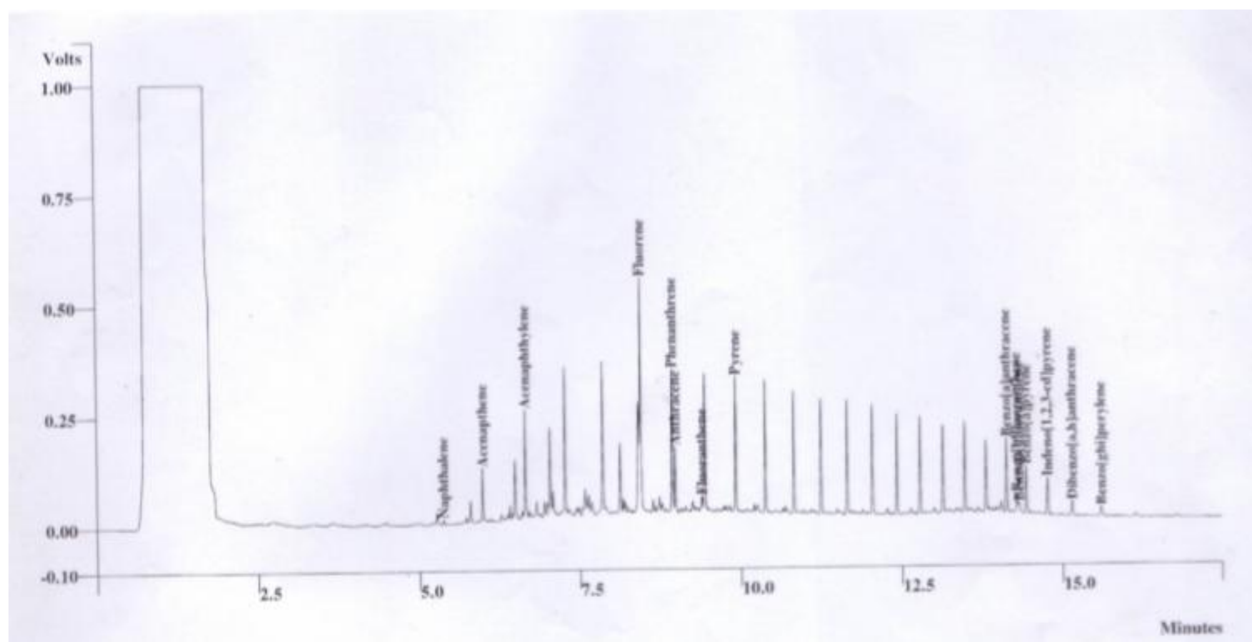
Appendix XVII: Total Petroleum Hydrocarbon chromatography profile of Otuocha soil after artificially polluting the soil (before remediation)



Appendix XVIII: Polycyclic Aromatic Hydrocarbon chromatography profile of Ibeno soil before remediation (after artificial pollution)


Volts
1.00
0.75
0.50
0.25
0.00
-0.10
2.5
5.0
7.5
10.0
12.5
15.0
Minutes
Naphthalene
Acenapthene
Acenaphthylene
Fluorene
Fluoranthene
Pyrene
Benzo[a]anthracene
Indeno[1,2,3-cd]pyrene
Dibenzo[a,h]anthracene

Appendix XIX: Polycyclic Aromatic Hydrocarbon chromatography profile of Otuocha soil after artificially polluting the soil (before remediation)



Appendix XX: Total Viable Counts of the inoculums development (cfu/ml)

Days	*Gordonia* sp	*Tsukamurella* sp
0	$7.3x10^5 \pm 0.15$	$4.7x10^5 \pm 0.35$
7	$22.7x10^5 \pm 0.78$	$11.0x10^5 \pm 1.27$

Values are mean $\pm$ S.D. of duplicate determination

Appendix XXI: Total Viable Count during the 5 months' remediation (cfu/g)

Sample	0	4	8	12	16	20
OS2	$14.4x10^{6}\pm2.0$	$3.8x10^{6}\pm0.8$	$6.7x10^{6}\pm1.0$	$9.3x10^{6}\pm1.0$	$29.6x10^{6}\pm3.0$	$18.8x10^{6}\pm2.0$
IS2	$8.4x10^{6}\pm1.0$	$3.6x10^{6}\pm0.6$	$5.9x10^{6}\pm0.8$	$8.5x10^{6}\pm3.6$	$15.0x10^{6}\pm4.0$	$12.4x10^{6}\pm2.0$
OS13	$11.7x10^{6}\pm2.0$	$6.5x10^{6}\pm0.5$	$8.5x10^{6}\pm1.0$	$9.7x10^{6}\pm1.0$	$23.3x10^{6}\pm2.0$	$19.8x10^{6}\pm2.0$
IS13	$11.0x10^{6}\pm2.0$	$5.3x10^{6}\pm0.3$	$7.2x10^{6}\pm1.0$	$8.8x10^{6}\pm2.0$	$12.3x10^{6}\pm2.0$	$6.5x10^{6}\pm1.0$
OM	$15.1x10^{6}\pm1.0$	$8.0x10^{6}\pm1.0$	$8.9x10^{6}\pm1.0$	$7.2x10^{6}\pm1.0$	$28.8x10^{6}\pm2.0$	$17.0x10^{6}\pm1.0$
IM	$13.2x10^{6}\pm3.0$	$4.7x10^{6}\pm0.6$	$6.1x10^{6}\pm1.0$	$8.5x10^{6}\pm2.0$	$18.0x10^{6}\pm5.0$	$11.7x10^{6}\pm2.0$
OC	$11.6x10^{6}\pm1.0$	$3.0x10^{6}\pm0.1$	$4.3x10^{6}\pm0.3$	$5.7x10^{6}\pm1.0$	$4.7x10^{6}\pm0.7$	$4.1x10^{6}\pm1.0$
IC	$7.7x10^{6}\pm2.0$	$3.2x10^{6}\pm0.1$	$3.6x10^{6}\pm0.3$	$4.3x10^{6}\pm0.3$	$5.9x10^{6}\pm1.4$	$3.2x10^{6}\pm0.1$

Values are mean ± S.D. of duplicate determination

OS2= Otuocha soil with *Gordonia sp*

IS2= Ibeno soil with isolate *Gordonia sp*

OS13=Otuocha soil with isolate *Tsukamurella sp*

IS13= Ibeno soil with isolate *Tsukamurella sp*

OM= Otuocha soil with Mixed culture

IM= Ibeno soil with Mixed culture

OC= Otuocha soil without isolate (control)

IC= Ibeno soil without isolate (control)

Appendix XXII: pH of the soil during the 5 months' remediation

Sample	0	4	8	12	16	20
OS2	6.40±1.0	6.60±1.0	6.04±1.0	6.62±1.0	6.70 ±1.0	8.70±1.0
IS2	6.40±0.5	5.80±0.4	5.62±0.3	6.60±0.8	6.20±0.1	9.85±1.3
OS13	6.60±1.0	6.57±1.0	6.07±1.0	6.59±1.0	6.60±1.0	9.04±1.0
IS13	6.40±0.4	5.82±0.4	5.72±0.4	6.62±0.6	6.30±0.5	9.25±0.2

OM	6.50±1.0	6.28±1.0	5.96±1.0	6.38±1.0	6.60±1.0	8.35±1.0
IM	6.20±1.0	6.10±0.2	5.61±0.5	6.44±1.2	6.20±0.2	10.2±0.9
OC	6.80±1.0	6.87±1.0	6.42 ±1.0	6.48±1.0	6.50±1.0	8.78±1.0
IC	6.50±0.1	6.45±0.2	6.01±0.8	6.44±0.5	6.20±0.3	8.90±0.2

Values are mean ± S.D. of duplicate determination

OS2= Otuocha soil with isolate *Gordonia sp*

IS2= Ibeno soil with isolate *Gordonia sp*

OS13=Otuocha soil with isolate *Tsukamurella sp*

IS13= Ibeno soil with isolate *Tsukamurella sp*

OM= Otuocha soil with Mixed culture

IM= Ibeno soil with Mixed culture

OC= Otuocha soil without isolate (control)

IC= Ibeno soil without isolate (control)

Appendix XXIII: Total Petroleum Hydrocarbon content after the remediation of Ibeno soil (Control)

Peak No	Peak Name	Ret Time (min)	Result (ppm)	Amt/Area	Type
1	C8	3.130	-	-	
2	C9	4.397	-	-	
3	C10	5.958	-	-	
4	C11	7.589	-	-	
5	C12	9.102	-	-	

6	C13	10.541	-	-	
7	C14	11.894	-	-	
8	C15	13.077	3.77401	6.83122	BB
9	C16	14.196	3.16796	5.83108	BB
10	C17	15.317	6.54866	8.36317	BB
11	Pr	15.906	0.00000	0.00000	BV
12	C18	16.677	7.54024	2.63931	VV
13	Ph	16.596	0.00000	0.00000	VV
14	C19	17.742	9.96334	1.74597	VV
15	C20	18.721	26.62242	1.48344	VV
16	C21	19.650	50.86474	1.40966	VV
17	C22	20.602	17.10370	1.54887	VV
18	C23	21.480	25.78818	1.47075	VV
19	C24	22.327	35.05770	1.47040	VV
20	C25	23.105	19.34509	1.54246	VV
21	C26	23.929	22.38504	1.51352	VV
22	C27	24.626	65.57405	1.51355	VV
23	C28	25.383	90.22895	1.44874	VV
24	C29	26.040	45.96180	1.52167	VV
25	C30	26.694	70.21637	1.52552	VV
26	C31	27.347	50.16802	1.60896	VV
27	C32	27.940	84.51756	1.66115	VV
28	C33	28.586	61.60850	1.74345	VV
29	C34	29.144	19.38816	1.86665	VV
30	C35	29.844	33.35050	1.92226	VV
31	C36	30.605	9.62047	2.16668	VV
32	C37	31.464	13.60287	2.38974	VV
33	C38	31.872	6.36959	3.77963	VB
34	C39	33.411	-	-	
35	C40	34.649	-	-	
Totals			**778.76791**		

Appendix XXIV: Total Petroleum Hydrocarbon content after the remediation of Ibeno soil with *Gordonia alkanivorans*

Peak No	Peak Name	Ret Time (min)	Result (ppm)	Amt/Area	Type
1	C8	3.130	-	-	
2	C9	4.397	-	-	

3	C10	5.958	-	-	
4	C11	7.589	-	-	
5	C12	9.102	-	-	
6	C13	10.766	8.64654	1.87167	BB
7	C14	11.894	-	-	
8	C15	13.251	9.34185	1.67723	VB
9	C16	14.464	4.66309	1.86535	VB
10	C17	15.496	11.82801	1.93472	VV
11	Pr	15.593	2.14370	6.59326e-1	VV
12	C18	16.718	9.47405	1.72097	VV
13	Ph	16.778	2.70118	8.47958e-1	VV
14	C19	17.701	7.85629	1.58198	VV
15	C20	18.749	12.29906	1.49439	VV
16	C21	19.689	26.90130	1.40634	VV
17	C22	20.573	26.62600	1.41745	VV
18	C23	21.509	10.11227	1.49832	VV
19	C24	22.348	34.67789	1.42514	VV
20	C25	23.124	28.54936	1.43753	VV
21	C26	23.882	49.51082	1.41914	VV
22	C27	24.602	29.66682	1.51658	VV
23	C28	25.256	62.92714	1.43956	VV
24	C29	26.011	71.67982	1.49135	VV
25	C30	26.667	75.26170	1.50953	VV
26	C31	27.366	26.20165	1.60701	VV
27	C32	27.960	17.05600	1.70386	VV
28	C33	28.541	10.70871	1.83082	VV
29	C34	29.156	16.19861	1.80137	VV
30	C35	29.961	5.72431	2.17378	VV
31	C36	30.368	2.65661	2.84438	VB
32	C37	31.430	-	-	
33	C38	32.351	-	-	
34	C39	33.411	-	-	
35	C40	34.649	-	-	
Totals			**563.41279**		

Appendix XXV: Total Petroleum Hydrocarbon content after the remediation of Ibeno soil with *Tsukamurella inochensis*

Peak No	Peak Name	Ret Time (min)	Result (ppm)	Amt/Area	Type
1	C8	3.130	-	-	
2	C9	4.397	-	-	
3	C10	5.958	-	-	
4	C11	7.589	-	-	
5	C12	9.102	-	-	
6	C13	10.397	2.20537	8.68209	BV
7	C14	12.054	2.25222	5.21691	BB
8	C15	13.252	5.47268	1.93904	VB
9	C16	14.405	1.84717	3.99731	BV
10	C17	15.498	9.74446	2.06462	VV
11	Pr	15.596	1.74366	6.08640e-1	VV
12	C18	16.603	5.08878	2.14635	VV
13	Ph	16.720	6.20708	1.03249	VV
14	C19	17.711	8.42684	1.56473	VV
15	C20	18.747	14.36800	1.47380	VV
16	C21	19.691	14.09315	1.46047	VV
17	C22	20.576	23.61027	1.42476	VV
18	C23	21.511	11.81224	1.47981	VV
19	C24	22.354	20.91705	1.45526	VV
20	C25	23.130	34.51291	1.42893	VV
21	C26	23.888	46.32510	1.42092	VV
22	C27	24.611	35.15778	1.51161	VV
23	C28	25.294	45.35498	1.44857	VV
24	C29	26.021	62.85476	1.49335	VV
25	C30	26.678	72.67519	1.51002	VV
26	C31	27.296	27.49552	1.60496	VV
27	C32	27.937	9.54864	1.76364	VV
28	C33	28.555	14.59736	1.79410	VV
29	C34	29.163	16.67978	1.79866	VV
30	C35	29.969	9.68691	2.00996	VV
31	C36	30.678	4.06528	2.29001	BV
32	C37	30.678	3.55234	2.99647	VB
33	C38	32.351	-	-	
34	C39	33.411	-	-	
35	C40	34.649	-	-	
Totals			**510.29552**		

Appendix XXVI: Total Petroleum Hydrocarbon content after the remediation of Ibeno soil with Mixed culture

Peak No	Peak Name	Ret Time (min)	Result (ppm)	Amt/Area	Type
1	C8	3.130	-	-	
2	C9	4.397	-	-	
3	C10	5.958	-	-	
4	C11	7.589	-	-	
5	C12	9.102	-	-	
6	C13	10.723	8.28610	1.89380	BB
7	C14	12.023	3.57419	2.64250	VV
8	C15	13.234	7.04632	1.78851	VB
9	C16	14.392	2.25137	2.98353	VV
10	C17	15.489	9.56002	2.07991	VV
11	Pr	15.587	1.78475	6.14468e-1	VV
12	C18	16.715	8.78849	1.75241	VV
13	Ph	16.761	1.94591	7.55209e-1	VV
14	C19	17.781	18.98024	1.44421	VV
15	C20	18.749	21.66751	1.43422	VV
16	C21	19.689	26.44475	1.40733	VV
17	C22	20.574	24.58278	1.42220	VV
18	C23	21.512	10.58744	1.49250	VV
19	C24	22.352	41.65362	1.41767	VV
20	C25	23.126	26.00029	1.44246	VV
21	C26	23.886	55.55838	1.41633	VV
22	C27	24.606	29.27982	1.51700	VV
23	C28	25.260	63.44720	1.43937	VV
24	C29	26.016	69.46367	1.49180	VV
25	C30	26.674	72.75310	1.51001	VV
26	C31	27.395	21.22427	1.61734	VV
27	C32	27.968	22.49535	1.68628	VV
28	C33	28.495	18.07612	1.77523	VV
29	C34	29.166	14.30609	1.81387	VV
30	C35	29.965	3.14074	2.59990	VV
31	C36	30.121	2.19257	3.33792	VB
32	C37	31.430	-	-	
33	C38	32.351	-	-	
34	C39	33.411	-	-	
35	C40	34.649	-	-	

Totals	**585.09108**

Appendix XXVII: Total Petroleum Hydrocarbon content after the remediation of Otuocha soil (Control)

Peak No	Peak Name	Ret Time (min)	Result (ppm)	Amt/Area	Type
1	C8	3.130	-	-	
2	C9	4.397	-	-	
3	C10	5.958	-	-	
4	C11	7.589	-	-	
5	C12	9.026	2.74578	7.08143	
6	C13	10.755	8.32458	1.89132	BB
7	C14	12.047	2.40153	4.48277	BB
8	C15	13.245	7.49828	1.75987	VB
9	C16	14.458	88.08601	1.40110	VV
10	C17	15.495	18.44212	1.75004	VV
11	Pr	15.591	48.52210	1.00959	VV
12	C18	16.668	20.99201	1.52814	VV
13	Ph	16.721	22.74243	1.17583	VV
14	C19	17.701	32.75979	1.40779	VV
15	C20	18.767	52.17180	1.39123	VV
16	C21	19.698	82.17299	1.36880	VV
17	C22	20.575	57.40746	1.38758	VV
18	C23	21.500	35.00042	1.41112	VV
19	C24	22.349	120.16141	1.39394	VV
20	C25	23.126	105.97213	1.40183	VV
21	C26	23.888	35.72360	1.42918	VV
22	C27	24.610	46.84984	1.50497	VV
23	C28	25.262	103.34959	1.43059	VV
24	C29	26.017	96.31947	1.48777	VV
25	C30	26.692	133.91905	1.50349	VV
26	C31	27.372	43.42993	1.58984	VV
27	C32	27.932	41.20894	1.66187	VV
28	C33	28.556	22.61414	1.75965	VV
29	C34	29.150	35.19284	1.75208	VV
30	C35	29.817	26.21494	1.88086	VV

31	C36	30.560	17.31236	1.78732	VV
32	C37	31.406	9.06087	2.26472	VV
33	C38	32.087	2.87754	4.21584	BB
34	C39	33.150	3.36950	5.67046	BB
35	C40	34.390	3.86385	7.08510	BB
Totals			**1326.70729**		

Appendix XXVIII: Total Petroleum Hydrocarbon content after the remediation of Otuocha soil with *Gordonia alkanivorans*

Peak No	Peak Name	Ret Time (min)	Result (ppm)	Amt/Area	Type
1	C8	3.130	-	-	
2	C9	4.397	-	-	
3	C10	5.958	-	-	
4	C11	7.589	-	-	
5	C12	9.046	2.48831	11.53453	
6	C13	10.453	2.32833	6.90180	BB
7	C14	12.055	2.20455	5.53196	VB
8	C15	13.251	4.33534	2.15180	BB
9	C16	14.402	2.28064	2.93980	VB
10	C17	15.498	9.59501	2.07695	VV
11	Pr	15.591	12.27510	9.41338e-1	VV
12	C18	16.723	20.18775	1.53377	VV
13	Ph	16.761	9.78912	1.09997	VV
14	C19	17.710	16.55273	1.45735	VV
15	C20	18.761	23.04420	1.42971	VV
16	C21	19.696	24.99008	1.41074	VV
17	C22	20.573	31.83687	1.40820	VV
18	C23	21.511	19.16299	1.43925	VV
19	C24	22.354	44.59548	1.41524	VV
20	C25	23.132	57.62426	1.41269	VV
21	C26	23.892	26.53829	1.44187	VV
22	C27	24.612	28.54979	1.51783	VV
23	C28	25.265	63.44154	1.43938	VV
24	C29	26.021	49.35528	1.49772	VV
25	C30	26.679	60.24920	1.51299	VV

26	C31	27.293	25.73011	1.60782	VV
27	C32	27.941	5.38469	1.87949	VV
28	C33	28.562	3.90147	2.11419	VV
29	C34	29.194	2.59506	2.47858	VV
30	C35	29.977	2.14969	3.25084	VV
31	C36	30.843	1.48686	6.31557	VB
32	C37	31.185	2.06174	4.86631	BB
33	C38	32.105	2.19944	6.67928	BB
34	C39	33.172	2.62127	10.54138	BB
35	C40	34.649	-	-	
Totals			**559.55518**		

Appendix XXIX: Total Petroleum Hydrocarbon content after the remediation of Otuocha soil with *Tsukamurella inochensis*

Peak No	Peak Name	Ret Time (min)	Result (ppm)	Amt/Area	Type
1	C8	3.130	-	-	
2	C9	4.397	-	-	
3	C10	5.958	-	-	
4	C11	7.589	-	-	
5	C12	9.102	-	-	
6	C13	10.462	2.20614	8.66726	VB
7	C14	12.060	2.24378	5.26911	BB
8	C15	13.255	4.55535	2.09829	VB
9	C16	14.405	2.40444	2.77851	VV
10	C17	15.499	8.62354	2.17223	VV
11	Pr	15.592	12.52985	9.43074e-1	VV
12	C18	16.682	9.54729	1.71794	VV
13	Ph	16.762	3.52087	9.15392e-1	VV
14	C19	17.709	15.51504	1.46432	VV
15	C20	18.765	27.91080	1.41747	VV
16	C21	19.698	24.62579	1.41166	VV
17	C22	20.583	31.91981	1.40808	VV
18	C23	21.504	19.92954	1.43681	VV
19	C24	22.354	44.64904	1.41519	VV
20	C25	23.129	49.44991	1.41666	VV

21	C26	23.891	24.09069	1.44695	VV
22	C27	24.609	23.80760	1.52447	VV
23	C28	25.262	57.12193	1.44191	VV
24	C29	26.018	41.54620	1.50159	VV
25	C30	26.674	56.06195	1.51428	VV
26	C31	27.291	27.24812	1.60533	VV
27	C32	27.939	7.59159	1.80064	VV
28	C33	28.559	9.57758	1.84758	VV
29	C34	29.163	10.31008	1.85664	VV
30	C35	29.825	3.74825	2.42892	VV
31	C36	30.380	1.69821	4.69582	VB
32	C37	31.183	1.68939	7.23917	BB
33	C38	32.110	2.05478	8.09190	BB
34	C39	33.411	-	-	
35	C40	34.649	-	-	
Totals			**526.17757**		

Appendix XXX: Total Petroleum Hydrocarbon content after the remediation of Otuocha soil with mixed culture

Peak No	Peak Name	Ret Time (min)	Result (ppm)	Amt/Area	Type
1	C8	3.130	-	-	
2	C9	4.397	-	-	
3	C10	5.958	-	-	
4	C11	7.589	-	-	
5	C12	9.102	-	-	
6	C13	10.440	2.32750	6.91078	VB
7	C14	12.042	2.48119	4.19679	VB
8	C15	13.242	8.30444	1.71823	VV
9	C16	14.397	4.12415	1.95472	VV
10	C17	15.494	17.32341	1.76955	VV
11	Pr	15.588	21.76326	9.80006e-1	VV
12	C18	16.720	34.40499	1.47513	VV
13	Ph	16.755	13.01049	1.13171	VV
14	C19	17.706	28.23600	1.41567	VV
15	C20	18.759	38.12285	1.40223	VV

16	C21	19.690	38.56486	1.38921	VV
17	C22	20.643	22.81962	1.42701	VV
18	C23	21.503	34.84613	1.41127	VV
19	C24	22.351	84.11707	1.39926	VV
20	C25	23.129	69.96061	1.40847	VV
21	C26	23.888	49.61675	1.41908	VV
22	C27	24.609	49.26366	1.50400	VV
23	C28	25.264	112.23126	1.42949	VV
24	C29	26.019	84.17561	1.48927	VV
25	C30	26.679	115.05305	1.50476	VV
26	C31	27.373	45.99704	1.58840	VV
27	C32	27.934	22.36681	1.68660	VV
28	C33	28.551	24.97916	1.75385	VV
29	C34	29.188	18.19568	1.79112	VV
30	C35	29.814	21.23724	1.89760	VV
31	C36	30.666	5.24225	2.11544	VV
32	C37	31.399	8.63962	2.28225	VB
33	C38	32.089	2.49338	5.16845	BB
34	C39	33.146	2.82541	8.23871	BB
35	C40	34.390	3.24207	10.93671	BB
Totals			**985.96555**		

Appendix XXXI: Polycyclic Aromatic Hydrocarbon Content after the remediation of Ibeno soil (Control)

Ret time (min)	type	Amt/area	Amount (ppm)	Group name
6.438		-	-	Naphthalene
7.861	BB	4.06407e-2	7.91588e-2	2-Methylnaphalene
10.067		-	-	Acenaphthene
10.357	BB	1.78523e-2	3.29082e-2	Acenaphthylene
11.583	VB	2.36133e-2	3.35131e-2	Fluorene
14.028	VV	3.65005e-2	1.05207e-1	Phenanthrene

14.203	VV	3.27739e-2	5.34347e-2	Anthracene
16.995	VV	4.49278e-2	2.33033	Fluoranthene
17.599	VV	4.18111e-2	3.64650e-1	Pyrene
20.568	VV	7.08832e-2	1.21724	Chrysene
20.638	VB	4.96510e-2	4.86874e-1	Benzo(b)fluoranthene
23.011	VV	1.07868e-1	1.36931	Benzo(k)fluoranthene
23.236	VV	6.30442e-2	8.95902e-1	Benzo(a)pyrene
23.819	VV	9.80690e-2	1.24522	Dibenz(a,h)anthracene
26.070		1.09930e-1	1.34415	Benzo(g,h,i)perylene
26.627		2.04044e-1	1.44353	Indeno(1,2,3-cd)pyrene
Totals:			**11.00143**	

Appendix XXXII: Polycyclic Aromatic Hydrocarbon content after the remediation of Ibeno soil with *Gordonia alkanivorans*

Ret time (min)	type	Amt/area	Amount (ppm)	Group name
6.438		-	-	Naphthalene
7.973		-	-	2-Methylnaphalene
9.836	BB	3.60127e-2	1.08753e-1	Acenaphthene
10.484		-	-	Acenaphthylene

11.761		-	-	Fluorene
14.334	BV	1.30704e-3	7.07344e-4	Anthracene
14.376	VV	3.56404e-3	1.88976e-3	Phenanthrene
17.038	VV	4.33842e-2	7.78039e-1	Fluoranthene
17.551	VV	4.15899e-2	3.39541e-1	Pyrene
20.570	VV	6.72915e-2	1.87363	Chrysene
20.700	VV	4.99357e-2	6.27257e-1	Benzo(b)fluoranthene
23.051	VV	1.06249e-1	1.59558	Benzo(k)fluoranthene
23.269	VV	6.39556e-2	1.86659	Benzo(a)pyrene
23.778	VV	9.75630e-2	1.34191	Dibenz(a,h)anthracene
26.040		-	-	Benzo(g,h,i)perylene
26.555		-	-	Indeno(1,2,3-cd)pyrene
Totals:			**8.53390**	

Appendix XXXIII: Polycyclic Aromatic Hydrocarbon content after the remediation of Ibeno soil with *Tsukamurella inochensis*

Ret time (min)	type	Amt/area	Amount (ppm)	Group name
6.438		-	-	Naphthalene
7.973		-	-	2-Methylnaphalene

10.124	BB	0.00000	0.00000	Acenaphthene
10.484		-	-	Acenaphthylene
11.924	BB	2.56379e-3	1.76128e-3	Fluorene
14.137	VV	3.50081e-2	8.40178e-2	Phenanthrene
14.278	VV	2.95215e-2	3.98434e-2	Anthracene
17.030	VV	0.00000	0.00000	Fluoranthene
17.579	VV	2.18300e-2	2.65606e-2	Pyrene
20.591	VV	3.99790e-1	1.90198e-1	Chrysene
20.716	VV	4.92860e-2	3.77189e-1	Benzo(b)fluoranthene
23.012	VV	1.92865e-1	2.68302e-1	Benzo(k)fluoranthene
23.228	VV	5.98259e-2	3.02357e-1	Benzo(a)pyrene
23.815	VV	2.79519e-1	1.24310e-1	Dibenz(a,h)anthracene
25.958	VV	1.39916e-1	5.64093e-1	Benzo(g,h,i)perylene
26.656	VV	2.21408e-1	1.24264	Indeno(1,2,3-cd)pyrene
Totals:			**3.22127**	

Appendix XXXIV: Polycyclic Aromatic Hydrocarbon Content after the remediation of Ibeno soil with Mixed culture

Ret time (min)	type	Amt/area	Amount (ppm)	Group name
6.438		-	-	Naphthalene

7.973		-	-	2-Methylnaphalene
9.832	BB	3.57198e-2	1.04058e-1	Acenaphthene
10.473	BV	0.00000	0.00000	Acenaphthylene
11.797	BB	4.89919e-3	3.57002e-3	Fluorene
14.091		-	-	Phenanthrene
14.337	VB	0.00000	0.00000	Anthracene
17.038	VV	1.57000e-2	2.21109e-2	Fluoranthene
17.565	VV	0.00000	0.00000	Pyrene
20.433	VV	1.38819e-1	2.88985e-1	Chrysene
20.698	VV	4.76889e-2	1.86154e-1	Benzo(b)fluoranthene
22.946	VB	3.68503e-1	1.80511e-1	Benzo(k)fluoranthene
23.386	BV	2.30063e-2	1.38886e-2	Benzo(a)pyrene
23.777	VV	1.37912e-1	2.48399e-1	Dibenz(a,h)anthracene
26.040		-	-	Benzo(g,h,i)perylene
26.555		-	-	Indeno(1,2,3-cd)pyrene
Totals:			**1.04768**	

Appendix XXXV: Polycyclic Aromatic Hydrocarbon content after the remediation of Otuocha soil (control)

Ret time (min)	type	Peak area	Amount (ppm)	Group name
6.356	BB	3	0.0014	Naphthalene
7.930	VV	13618	9.0919	Acenaphthene
8.241	VV	94541	37.1388	Acenaphthylene
8.825	VV	42423	20.2136	Fluorene
9.746	VV	40143	21.4018	Phenanthrene
10.814	VV	16541	12.6203	Anthracene
11.248	VV	33367	19.2866	Fluoranthene
12.094	VV	52743	31.9422	Pyrene
12.498	VV	47982	36.4141	Benzo(a)anthracene
12.883	VV	39441	27.8332	Chrysene
13.619	VV	74145	71.4750	Benzo(b)fluoranthene
14.310	VV	76275	69.0105	Benzo(k)fluoranthene
14.489	VV	74676	83.5834	Benzo(a)pyrene
15.047	VV	46989	61.6459	Dibenz(a,h)anthracene
15.502	VV	35793	40.5118	Indeno(1,2,3-cd)pyrene
15.914	VV	7127	8.0614	Benzo(g,h,i)perylene
Totals:			**550.2319**	

Appendix XXXVI: Polycyclic Aromatic Hydrocarbon Content after the remediation of Otuocha soil with *Gordonia alkanivorans*

Ret time (min)	type	Peak area	Amount (ppm)	Group name
6.426	BB	18	0.0074	Naphthalene
7.938	VV	16037	10.7069	Acenaphthene
8.250	VV	87639	34.4275	Acenaphthylene
8.835	VV	40645	19.3665	Fluorene
9.753	VV	40354	21.5146	Phenanthrene
10.809	VV	18290	13.9544	Anthracene
11.261	VV	27927	16.1423	Fluoranthene
12.106	VV	47566	28.8066	Pyrene
12.507	VV	47301	35.8974	Benzo(a)anthracene
12.894	VV	42037	29.6651	Chrysene
13.631	VV	63980	61.6758	Benzo(b)fluoranthene
14.237	VV	73240	66.2645	Benzo(k)fluoranthene
14.498	VV	73422	82.1796	Benzo(a)pyrene
15.061	VV	37059	48.6190	Dibenz(a,h)anthracene
15.519	VV	32562	36.8540	Indeno(1,2,3-cd)pyrene
15.872	VV	9599	10.8583	Benzo(g,h,i)perylene
Totals:			**516.9339**	

Appendix XXXVII: Polycyclic Aromatic Hydrocarbon Content after the remediation of Otuocha soil with *Tsukamurella inochensis*

Ret time (min)	type	Peak area	Amount (ppm)	Group name
6.511	BB	112	0.0471	Naphthalene
7.765	VV	2081	1.389	Acenaphthene
8.256	VV	84838	33.3272	Acenaphthylene
8.842	VV	52395	24.9650	Fluorene
9.761	VV	37117	19.7889	Phenanthrene
10.816	VV	36160	27.5883	Anthracene
11.264	VV	37848	21.8769	Fluoranthene
12.111	VV	52094	31.5487	Pyrene
12.512	VV	53291	40.4435	Benzo(a)anthracene
12.898	VV	43836	30.9346	Chrysene
13.635	VV	74799	72.1058	Benzo(b)fluoranthene
14.241	VV	66772	60.4129	Benzo(k)fluoranthene
14.504	VV	65607	73.4333	Benzo(a)pyrene
15.067	VV	37952	49.7911	Dibenz(a,h)anthracene
15.529	VV	28742	32.5315	Indeno(1,2,3-cd)pyrene
15.884	VV	17180	19.4334	Benzo(g,h,i)perylene
Totals:			**539.6178**	

Appendix XXXVIII: Polycyclic Aromatic Hydrocarbon content after the remediation of Otuocha soil with Mixed culture

Ret time (min)	type	Peak area	Amount (ppm)	Group name
6.502	BB	234	0.0987	Naphthalene
7.938	VV	10508	7.0157	Acenaphthene
8.248	VV	59726	23.4623	Acenaphthylene
8.833	VV	34245	16.3170	Fluorene
9.753	VV	20707	11.0399	Phenanthrene
10.809	VV	19339	14.7551	Anthracene
11.257	VV	18292	10.5733	Fluoranthene
12.102	VV	27953	16.9286	Pyrene
12.503	VV	30223	22.9364	Benzo(a)anthracene
12.889	VV	26149	18.4530	Chrysene
13.626	VV	46592	44.9139	Benzo(b)fluoranthene
14.233	VV	42135	38.1224	Benzo(k)fluoranthene
14.494	VV	42348	47.3996	Benzo(a)pyrene
15.057	VV	24878	32.6379	Dibenz(a,h)anthracene
15.519	VV	20051	22.6941	Indeno(1,2,3-cd)pyrene
15.865	VV	11781	13.3268	Benzo(g,h,i)perylene
Totals:			**340.6747**	

Appendix XXXIX: Picture of germinated seed planted after remediation

IS2

OS2

OS13

IS13

OM

IM

OS2= Otuocha soil with *Gordonia sp*
IS2= Ibeno soil with isolate *Gordonia sp*
OS13=Otuocha soil with isolate *Tsukamurella sp*
IS13= Ibeno soil with isolate *Tsukamurella sp*
OM= Otuocha soil with Mixed culture
IM= Ibeno soil with Mixed culture
OC= Otuocha soil without isolate (control)
IC= Ibeno soil without isolate (control)
OUP= Otuocha unpolluted soil
IUP = Ibeno unpolluted soil