BIOREMEDIATION OF SPENT ENGINE OIL IN SOME SELECTED CONTAMINATED SOILS

BY

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Certification

This is to certify that this thesis was carried out by **Hikmat Omolara SULAYMAN** with Matriculation Number **17/27/MMI009** under my supervision and it's a fair reflection of the student's input.

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Dedication

This project work is dedicated to my ever-supportive husband; Dr. Olawale Abiodun Ogundeji and the first fruit of our marriage; Abdullah Fakhir for bringing so much joy and fulfilment into our home.

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Abstract

The aim of this research is to investigate bioremediation of spent engine oil on some selected contaminated soils. Soil samples were collected from five (5) mechanic workshops along Taiwo-Oke within Ilorin metropolis. They were subjected to bioremediation using the land-farming approach. Physicochemical parameters of the soil samples before and after bioremediation were analyzed using standard methods. Bacteria were isolated using standard procedures and identified using biochemical tests and molecular methods. Physicochemical parameters of soil samples before bioremediation included particle size (all sandy in nature); pH (5.95 ± 0.07 - 6.25 ± 0.07); Organic carbon (8.18 ± 0.31 - 17.54 ± 1.87), Organic matter (18.34 ± 2.90 - 43.45 ± 9.12) and heavy metals (ND - 11.74 ± 0.07), while values obtained after bioremediation for pH, organic carbon, organic matter and heavy metals were 8.25 ± 0.07 - 8.90 ± 0.14, 13.07 ± 0.05 - 13.25 ± 0.84, 37.25 ± 1.06 - 44.80 ± 1.13, ND - 9.40 ± 0.04 respectively. Bacterial counts before and after bioremediation of soil samples were 2.50 ± 0.71 - 67.50 ± 2.12 x 10^5 CFU/mL and 6.50 ± 2.12 - 164.00 ± 11.31 x 10^5 CFU/mL respectively. The bacterial isolates were identified as *Pseudomonas* sp., *Acinetobacter* sp., *Klebsiella* sp., *Bacillus* sp., *Enterobacter* sp. and *Aeromonas* sp. while the hydrocarbon-utilizing bacteria were identified as *Thalassospira mesophila* strain JCM 18969; *Pseudomonas fluorescens* F113; *Siccibacter turicensis* LMG 23730; *Pseudomonas Zeshuii* strain KACC 15471; *Pseudomonas stutzeri* strain CGMCC 1.1803 and *Marinobacter hydrocarbonoclasticus* strain ATCC 49840. Total petroleum hydrocarbon in the soils before and after bioremediation had retention time of 7.182 - 24.96 and 22.752 - 28.513 respectively. It is concluded that the bacteria isolated effectively bioremediated the spent engine oil contaminated soils with a reduction of hydrocarbon pollutants.

Keywords: Bioremediation, Spent engine oil, Bacteria isolates, Contaminated Soil, Mechanic workshops

CHAPTER ONE

INTRODUCTION

Soil is one of the most treasured natural resources available to man as it is very important for sustainance of life on the earth. It is a mixture of minerals, organic matter, gases, liquids, and myriad of organisms that together support plant life. In other words, soil is the unconsolidated mineral or organic matter on the surface of the earth that has been subjected to and shows effects of genetic and environmental factors of climate (including water and temperature effects), and macro- and microorganisms, conditioned by relief, acting on parent material over a period of time. It is considered as one of the three (3) major ecological environments on earth along with water and air; wherein the soil is the top layer of the earth's lithosphere, formed from weathered rock that has been transformed by living organisms (SSSA, 2010). A number of components influence soil characteristics which include amount and type of nutrients, availability of moisture, degree of aeration, temperature, pH, practices and occurrence which contributes large numbers of organisms to the soil; for instance e.g. floods or addition of manure (Pelczar, 2005).

The environment is a common heritage that should be properly nurtured. Man's residence on earth has resulted in tremendous environmental degradation due to inhabitancy and developmental efforts. This degradation in turn puts the goals of sustainable development at risks and as such should be abated as a matter of both necessity and urgency (Okerentugba and Ezeronye, 2003).

Soil contamination can simply be said to be the presence of unwanted impure materials from human activities which can also include the distortion of the soil environment by human activities. Soil is the habitat for variety of organisms, including fungi, bacteria, protozoa, insects, nematodes, worms and many other animals. Viruses are also present in soils. This complex biological community contributes to the formation, maintenance, and in some situations, the degradation and disappearance of soils (Prescott *et al.,* 2005). One of the most common contaminant of soil is hydrocarbons.

Hydrocarbons are the most important class of organic compounds comprising entirely of hydrogen and carbon which occur in petroleum and natural gas. Commercial petroleum products including kerosene, engine oil, diesel, petrol, paraffin etc. are all mixtures of hydrocarbons (Gadwood, 2008). Petroleum hydrocarbon is a major environmental pollutant throughout the world today because exploration and downstream utilization are associated with economic development (Okerentugba and Ezeronye, 2003).

The petroleum industry is responsible for the generation of enormous amount of organic residues, as well as the pollution of soils, rivers and seas (Bento *et al*., 2005). The toxicity of petroleum products varies widely depending on their composition, concentration, environmental factors and the biological state of the organisms at the time of the contamination (Cerniglia and Setherland, 2001; Obire and Ayanwu, 2009). The toxic and carcinogenic components of used engine oil may also be formed as a result of the thermal decomposition of lubricating materials upon microbiology processing, transportation, exploitation and storage (Ameh *et al.,* 2012).

Environmental pollution with petroleum and petroleum products like engine oil has been recognized as one of the most severe current problems especially when associated with accidental spills on large-scale. The presence of different substrates and metabolites in hydrocarbon contaminated soils has no doubt provided an environment for the development of a quite complicated microbial community (Butler and Mason, 1997; Udeani *et al*., 2008). Very typical cases are those of the Niger-Delta oil spillages such as Ikot Abasi Local Government Area of Akwa Ibom state, Ikarama and Kalaba communities in Yenegoa Local Government Area of Bayelsa state and Exxon Valdez spillage which led to a considerable loss of aquatic lives in 1989. Of particular interest is the case of people of Eluku Ojutaye village in Ilorin East Local Government Area of Kwara State, who are still counting their losses sequel to the oil spill that occurred few years ago from broken Nigerian National Petroleum Corporation (NNPC) tanks located within the community which infiltrates into the soil and led to loss of vegetable farms (Musa *et al*., 2009). Also, the discharge of used engine oil from automobiles, industrial machines, plants and electric power generators are the main sources of oil pollution in Ota, a Western Nigeria town (Igwo-Ezikpe *et al*., 2009).

Oil released into the environment affects a wide array of plants, animals, microorganisms and humans within the oil impacted environment. Additionally, prolonged exposure to oil as well as high concentration of oil could cause the development of liver or kidney disease, possible damage to the bone marrow and an increased risk of cancer (Deni and Penninck, 1999; Lloyd and Cackette, 2001; Mishra *et al*., 2001; Igwo-Ezikpe *et al*., 2009). Pollution due to petroleum hydrocarbons and its derivatives, including diesel fuel, gasoline, heavy oil, motor oil, fuel residues, and mineral oil, has an increasing influence on the environmental reconquest (Su *et al*., 2011). They have been recognized as one of the most hazardous wastes (Udeani *et al*., 2009).

Engine oil is a mixture of base lubricant oil and additives, and the base oil contains long-chain (C_{16}–C_{36}) saturated hydrocarbons and more than 75 % cyclic alkanes (Koma *et al*., 2003). One billion gallons of waste lubricating oil (also called spent oil) are generated during oil-changing processes from automobile and mechanical workshops, a few of this huge amount is recycled, and most is disposed-off by incineration or dumping. Therefore, there is a need for appropriate recycling to avoid their dangerous threat towards the environment (Kalyani and Pandey, 2014). Used motor oil contains more metals, toxic, and carcinogenic polycyclic aromatic hydrocarbons (PAHs). Thus, it constitutes a potential threat to humans, animals, and vegetation (Adelowo *et al*., 2006). Lubricating oil is a common contaminant in water and soils. Generally, lubricating oil comprises 80 % of hydrocarbon lubricant, with the remainder being additives, which consists partly of zinc diaryl, molybdenum disulfide, zinc dithiophosphate, metal soaps and other organometallic compounds (Lu and Kaplan, 2008). Large amounts of lubricating oil are liberated into the environment when the motor oil is changed and disposed into gutters, water drains, open vacant plots and farmlands, a common practice by motor mechanics and generator mechanics (Odjegba and Sadiq, 2002). Despite efforts in some countries to recover and recycle used motor oils, significant amount of lubricants are inputted into the environment, particularly in environmentally sensitive applications such as forestry and mining, or through engine losses (Battersby, 2000). The rise in consumption of automotive lubricating oil is a worldwide problem and has increased, ending in large used oil volume and its waste (Koma *et al*., 2003).

Engine oil could simply be defined as a thick mineral liquid applied to a machine or engine so as to reduce friction between the moving parts of the machine (Whitefield, 2002; Ahmad *et al*., 2015). The consumption of engine oil in Nigeria has been on the increase in recent years due to the upsurge in the number of vehicles, power plants, and generators that make use of these lubricants (Odjegba and Atebe, 2007). Anthropogenic activities have led to immense environmental degradation due to man’s residency and developmental efforts. Contaminated lands are found mostly in developing countries because of ineffective environmental laws (Adams *et al*.,

2014) which directly affects the rate at which spent engine oil enters and pollutes the environment. Also, the indiscriminate disposal of this waste oil increases pollution incidents in the environment (Odjegba and Atebe, 2007). Some of the pollution effects of used oil in the environment include reduction in oxygen supply to microorganisms, pollution of ground and surface waters and accumulation of metal ions which are toxic to plant. Indiscriminate disposal of engine oil into gutters, water drains, open vacant plots and farms is a common practice in Nigeria especially by motor mechanics (Okonokhua *et al*., 2007).

Engine oils are lubricants for various internal combustion engines. In addition to lubricating moving parts, it also cleans, inhibits corrosion, improves sealing and cools the engine by carrying heat away from moving parts (Nwoko *et al*., 2007). Notwithstanding these important functions, engine oils also impact our environment negatively. While new engine oils contain more volatile and water soluble hydrocarbons that would be more of acute toxicity to organisms, used engine oils contain metals and heavy polycyclic aromatic hydrocarbons (PAHs) that could contribute to chronic hazards such as mutagenicity and carcinogenicity (Boonchan *et al*., 2000; Clemente *et al*., 2001; Mandri and Lin, 2007).

Engine oil is a pollutant in the environment and causes damage to our ecosystem as well as health hazard to human beings (Fatuyi *et al.,* 2012). After any oil spillage, polycyclic aromatic hydrocarbons (PAHs) are important contaminant retained in the environment (Kathi and Anisa, 2012). Oil pollution has contaminated soils used for agricultural lands and has not spared the aquatic and marine plants and animals in Nigeria. Groundwater has also been contaminated hence polluting the crops and farm animals (Eneh, 2011). Huge increase of vehicles due to rapid increase of human population has led to presence of various kinds of informal and formal automobiles hence increased use of motor oil (Husaini *et al.*, 2008). Petroleum hydrocarbon compounds bind to soil components, and they are difficult to be removed or degraded (Barathi and Vasudevan, 2001). Some compounds, such as the high molecular weight polycyclic aromatic hydrocarbons (PAHs), may not be biodegraded at all (Atlas and Bragg, 2009).

Spent engine oils contain high percentage of aromatic and aliphatic hydrocarbons, nitrogen, sulphur compounds, and metals (Zn, Pb, Cr and Fe) than fresh oils. Some of these metals in used engine oil can dissolve in water and move through the soil easily and may be found in surface water and groundwater (Mohd *et al*., 2011; Abdulsalam *et al*., 2012). Spent engine oil causes great damage to soil and soil microflora. It creates an unsatisfactory condition for life in the soil due to poor aeration, immobilization of soil nutrients and lowering of soil pH (Ugoh and Moneke, 2011). Also, marked changes occur in soil contaminated with hydrocarbons and these changes affect the physical, chemical and microbiological properties of the soil (Okonokhua *et al*., 2007).

Contamination of soil by spent lubricating oil is prevalent in oil producing and industrialized countries of the world. The problem is more severe in the developing countries where there are no effective regulatory policies on the environment (Onuoha *et al*., 2011). The presence of different types of automobile and machinery has resulted in an increase in the use of lubricating oil. Also, oil spills from industries, filling stations, loading and pumping stations, petroleum product depots during transportation and at auto mechanic workshops, all combine to contribute to soil contamination (Onuoha *et al*., 2011).

Mechanic workshops within Nigeria are poorly managed and can be sources of constant release of used spent oil discharged from the crank cases of cars and motorcycles which can be aesthetically unsightly and cause serious environmental pollution. Clean-up of mechanic sites is still elusive as operators of such sites are usually ignorant of the deleterious effects on the environment. There is also a likelihood of percolation to groundwater and a pint of engine oil is capable of contaminating 100,000/liters of groundwater (Lee *et al.,* 2007). Increased proliferation of automobile workshops within Nigeria has contributed markedly to the problem of soil contamination and this has resulted in the concomitant exposure of the surrounding soil within the vicinity of these workshops to high levels of spent engine oil and lubricating oils (Edna *et al*., 2016).

Microorganisms have limits as regards tolerance for particular environmental conditions and optimal conditions for peak performance. Factors that affect success and rate of microbial biodegradation include nutrient availability, moisture content, pH as well as temperature of the soil matrix. Inorganic nutrients including, but not limited to, nitrogen, and phosphorus are also necessary for microbial activity and cell growth. It has been shown that treating petroleum-contaminated soil with moderate amount of nitrogen and phosphorus can increase cell growth rate, decrease the microbial lag phase, help maintain microbial populations at high activity levels and as well increase the rate of engine oil degradation (Walworth *et al*., 2005).

Soils contain very large numbers of microorganisms which can include a number of hydrocarbons utilizing bacteria and fungi (Namkoong *et al*., 2002). Microorganisms such as fungi are extremely diverse and can adapt to survive in inhospitable environments. Microbes are capable of breaking down many complex molecules by adaptation of their degradative enzyme system (Boonchan *et al*., 2000). The survival of these microbes in altered environment depends on their ability to produce endospore and vegetative cell, which can stand harsh and unfavorable environment. These changes affect plants adversely and the amount of damage depends on the size of the area involved and the degree of saturation by the pollutant (Isinguzo and Bello, 2005).

Treatment of contaminated soil and water resources takes place using mechanical methods, such as skimming and chemical methods, such as surfactants and dispersants; though these methods are costly and with limited efficiency (Huang *et al*., 2008). In contrast, the utilization of microorganisms to degrade such pollutant has been found to be a promising alternative (Akio *et al*., 2006) as microorganisms have potential to detoxify hazardous organic compounds by means of polymerization, mineralization, or transformation (Sarma and Sarma, 2010). Therefore, as compared to other technologies, microbial degradation (bioremediation) is the method of choice, because it is cost effective, safe, and environmentally accepted (Singh *et al*., 2009).

Pollution is an undesirable change in the physical, chemical and biological characteristics of all the components of an environment (Aboriba, 2001). Consequently, the need for remediation will depend on the degree of actual or potential environmental threat or the level of risk (Ukoli, 2003). Remediation of a contaminated site is achieved by one or more of the following objectives: Removal or destruction of the contaminants, modification of the contaminants to a less toxic form and isolation of the contaminant from the target by interrupting the pathway of exposure (Onifade *et al*., 2007). Bioremediation, therefore is the application of naturally occurring process by which microorganisms transform environmental contaminants into harmless end products (Abdulsalam *et al*., 2012).

Bioremediation process is a microorganism mediated transformation or degradation of contaminants into non-hazardous or less hazardous substances. It is an attractive approach for cleaning up of hydrocarbons from the environment being a simple technique, easy to maintain, applicable over large areas, cost effective and leads to complete destruction of the contaminants (Achal *et al*., 2011; Nwankwegu *et al*., 2016). The main reason for this concept is that majority of the molecules in the crude oil and refined product are biodegradable. Many physical, chemical and environmental factors like temperature, nutrients, oxygen, biodegradability, photooxidation, bioavailability, soil moisture, soil acidity and alkalinity etc. affect the process of biodegradation of hydrocarbons (Rahman *et al*., 2003).

Bioremediation is one of the forms of biodegradation which involves the use of microorganisms to detoxify or remove organic and inorganic xenobiotic compounds from the environment. The process relied upon microbial enzymatic activities to transform or degrade the contaminants from the environment (Philip *et al*., 2005). The ability to isolate high numbers of certain oil-degrading microorganisms from oil-polluted environment is commonly taken as evidence that these microorganisms are the active degraders of that environment (Okerentugba and Ezeronye, 2003). Bioremediation process depends on the ability of microbes to remove the hydrocarbon pollutants completely (Go´mez *et al*., 2007). Interestingly, numerous hydrocarbon-degrading microbes are capable of producing biosurfactants/emulsifiers agents that could increase the solubility of these sparingly or insoluble substrates and facilitate their utilization as sole carbon sources (Franzetti *et al*., 2010; Su *et al*., 2011). The economic viability and environmental soundness of bioremediation makes it an attractive method for the remediation of oil-contaminated environments. It is simply the improvement of live organisms such as fungi, bacteria and plant to detoxify hydrocarbon and organic contaminants. As a cheaper and effective method, the use of microbes in pollution abatement is now receiving an increasing attention (Deni, and Penninck, 1999; Daane *et al*., 2001; Dinkla *et al*., 2001; Lalithakumari, 2001; Clementina and Omoanghe, 2008).

Bioaugmentation is a process that involves addition of desired and capable microorganisms at the contaminated site as an essential option for the treatment of contaminated site. Zanaroli *et al*. (2010) proposed two (2) microbial consortia ENZ-G_1 and ENZ-G_2 for bioaugmentation operations on diesel fuel contaminated soil and consist of bacteria belonging to genera *Chryseobacterium, Acinetobacter, Pseudomonas, Stenotrophomonas, Alcaligenes* and *Gordonia* along with the fungus *Trametes gibbosa* capable of extensively degrading a broad range of hydrocarbons mainly composing diesel fuels and characterized them for hydrocarbon biodegradation capability and specificity.

According to Harder (2004), bioremediation accounts for 5 - 10 % of all pollution treatment and has been used successfully to clean up used engine oil illegally dumped. During bioremediation, microorganisms exploit chemical contaminants in the soil as energy source and through oxidation-reduction reactions, metabolize the target contaminant into useable energy for microbes. By-products (metabolites) released back into the environment are typically in a less toxic form than the parent contaminants. For instance, engine oil can be degraded by microbes in the presence of oxygen through aerobic respiration. Here, the engine oil loses electrons and is oxidized while oxygen gains electrons and is reduced which results in the formation of carbon dioxide (CO_2) and water (H_2O). When oxygen is limited in supply or absent, as in saturated or anaerobic soils or lake sediment, anaerobic (without oxygen) respiration prevails. Generally, inorganic compounds such as nitrate, sulfate, ferric iron, manganese, or carbon dioxide serve as terminal electron acceptors to facilitate biodegradation (Nester *et al*., 2001).

It is known that greater degradation of oil pollutants is carried out in-situ by a consortium of microorganisms (Okpokwasili, 1996; Okpokwasili, 2003) and more than 200 species of bacteria, fungi and even algae are capable of degrading hydrocarbons because of their ubiquitous nature. Bacteria and fungi are known to be the principal agents of biodegradation of hydrocarbons. Fungi have a higher tolerance to the toxicity of hydrocarbons as a result of their physiology and adaptation to such variations in the environment and the mechanism for the elimination of spilled oil from the environment. Fungi have been found to be better degraders of petroleum than traditional bioremediation techniques with bacteria (Ojo, 2005).

Fungi and bacteria have been used in the past for biodegradation (Snape *et al.*, 2001). The filamentous fungi possess some attributes that enable them to be good potential agents of degradation. A fungus attaches itself quickly on the substratum then digests the substratum through the secretions of extracellular enzymes (Okerentugba and Ezeronye, 2003). Fungi are capable of growing under environmental stress including, low pH, poor nutrients and low water activity. Fungal bioremediation is an attractive approach over other techniques like physical-chemical for it is simple, easy to maintain, cost effective and can be produced in mass (Achal *et al.*, 2011). Studies by Smita *et al.* (2012) shows that, *Aspergillus*, *Penicillium, Fusarium, Rhizopus, Alternaria* and *Cladosporium* species have been identified as hydrocarbon biodegraders. Fungi have also demonstrated the ability to degrade and mineralize phenols, halogenated phenolic compounds, petroleum hydrocarbons, polycyclic aromatic compounds and polychlorinated biphenyls (Singh, 2006).

Various genera that have been reported to contain hydrocarbon degrading species include *Pseudomonas, Vibrio, Corynebacterium, Arthrobacter, Brevibacterium, Staphylococcus, Bacillus,* *Thiobacillus*, *Penicillium*, *Candida*, *Fusarium*, *Aspergillus*, *Talaromyces* and *Articulosporium* (Snape *et al*., 2001). These organisms have been isolated in large numbers from many oil polluted waters and soils but are found in less numbers in uncontaminated environments (Okoh, 2003).

The advantages associated with fungal bioremediation are primarily in the versatility of the technology and its cost efficiency compared to other remediation technologies such as incineration, thermal desorption and extraction. The use of fungi is expected to be relatively economical as they can be grown on a number of inexpensive agricultural or forest wastes such as corncobs and sawdust. More so, their utilization is a gentle non-aggressive approach (Edna *et al*., 2016).

Increasing exploration and production activities coupled with improper waste disposal practices has led to widespread contamination of both the aquatic and terrestrial ecological systems (Odokuma and Ikpe, 2003). A wide variety of microorganisms have the ability to utilize hydrocarbons as sole sources of carbon (energy) for metabolic activities and these microorganisms are widely distributed in nature. The microbial utilization of hydrocarbons depends on the chemical nature of the compounds within the petroleum mixture and on environmental determinant **(**Adeline *et al*., 2009).

1.1 Statement of the Problem

The effects of pollutants on the environment are detrimental and it warrants immediate attention and action. Spent engine oil is released indiscriminately into free land space, and drainage systems that carry them about. An engine oil polluted land is unhealthy for plant proliferation, microbial survival and can be aesthetically unpleasant. When placed in landfills, it may seep through the bottom of such landfill and subsequently contaminate groundwater supplies. When handled improperly, people’s health can be affected when inhaled and ingested if they get into drinking water sources and are not detected and removed. Spillage of engine oil can also bring about changes in the biological cycles in the soil, which in turn inhibits plant development.

1.2 Justification of the Study

The frequent contamination of soils with petroleum products in particular engine oil at the point of sales, mechanic workshops, generator houses and the likes negatively affect the aesthetics sense of the environment; some degree of contamination of the environment particularly the soil with engine oil occurs at these points. People often do not pay attention to the remediation of these sites as more research on biodegradation has focused on crude oil, and diesel oil. Hence, it becomes necessary to investigate the presence of natural microbial populations capable of degrading these polluted environments as well as isolate and characterize the microorganisms that are capable of degrading engine oil contaminated soil environment.

It is also important to estimate the biodegradation potential of the most promising strains, both individually and in combination as well as the factors which influence the biodegradation potential of such pollutant.

1.3 Aim of the Study

The aim of the study is to investigate the bioremediation of the spent engine oil contaminated soils with a consortium of indigenous occurring bacteria.

1.4 Specific Objectives of the Study

The objectives of the study are to:

i. carry out physicochemical analysis on the spent engine oil contaminated soil;

ii. isolate and identify naturally occurring bacteria in the engine oil contaminated soil;

iii. determine the total heterotrophic and hydrocarbon-utilizing bacteria in the engine oil contaminated soil;

iv. carry out biochemical and molecular characterization of the isolates;

v. carry out bioremediation of spent engine oil contaminated soil using bacterial isolates in soil supplemented with cow dung; and

vi. determine the physicochemical properties of the bioremediated soil.

CHAPTER TWO

LITERATURE REVIEW

2.1 Engine Oil Pollution

Petroleum products are widely widespread all over the world and their rigorous use is strongly connected to heavy discharge of hydrocarbons into the environment (Winkelmann *et al.*, 2009). A major concern for petroleum hydrocarbon bioremediation is the presence of heavy compounds such as polycyclic aromatic hydrocarbons (PAHs). These heavy hydrocarbon constituents are considered potential health risks due to their possible carcinogenic and mutagenic actions (Baheri and Meysami, 2002). Heavier compounds go further spreading in the layer above the groundwater, while the lighter parts continue their way downwards up to the water table. This is of interest for soil pollution, because it causes spreading of the pollution and creates the possibility of organic compound entering in the groundwater used for a variety of purposes (Qin and Huang, 2009).

Milic *et al*. (2009) performed an ex-situ experiment of bioremediation for 5.5 minutes for the treatment of soil heavily contaminated with crude oil and its products from the oil refinery Pancevo, Serbia. During this experiment the composition of the microbial consortium was examined. Ebrahimi *et al*. (2012) stated that bioremediation is the best way to treat pollutant by using microorganisms or other biological process that utilizes microorganisms or their enzymes to get original environment altered by contamination. They isolated some hydrocarbon degrading bacteria from oil contaminated site of Bushehr province. The hydrocarbon degrading efficiency of these isolates was tested in both solid and liquid medium enriched with gas oil (2 %), toluene (1 %) and phenanthrene (0.05 %). The utilization of hydrocarbon was analysed by measuring the growth diameter of colony in solid media and by observing growth turbidity at optical density 600 nm in liquid media.

2.2 Remediation Process

Physical, chemical and mechanical processes are traditional methods used in remediation of contaminated areas. Physical remediation method includes incineration, brick making and skimmers etc. which cannot biodegrade more than 10 - 15 % of spilled oil (Thavasi *et al.*, 2011). Use of chemical surfactants as remediating agent on the other hand is not favourable due to their toxic effects on flora and fauna (Thavasi *et al.*, 2011); however, this type of treatment system requires heavy machinery and the environmental consequences of this pollutant removal may result in massive air pollution (Bhupathiraju *et al.*, 2002). A lot of conventional methods including pump and treat systems, soil vapour extraction, incineration, digging up contaminated soil and moving it to landfill or to cap the contaminated area of soil land have been attempted for enhanced removal, reduction and mitigation of toxic materials including engine oil introduced into the soil by anthropogenic activities. These methods, however, suffered drawbacks and may even involve some level of risk (Perfumo *et al*., 2007).

Despite decades of research, successful bioremediation of oil contaminated environment stills remains a challenge (Perfumo *et al.,* 2010). Combining high efficiency and low costs, bioremediation processes represent an extremely important way of recovering oil contaminated areas among several other remediation processes (Bhupathiraju *et al.,* 2002). Bioremediation therefore, is the conversion of chemical compounds into energy, cell mass and biological waste products using living organisms, especially microorganisms (Rahman *et al.,* 2002). This bioremediation converts contaminants to harmless end products and the process is therefore economical and efficient (Das and Mukherjee, 2007). Several reports have been established on biodegradation of petroleum hydrocarbons of used engine oil contaminated soils. Koma *et al.* (2003) however submitted that microbial diversity in certain ecosystems even though rich in certain species might be deficient in certain proven strains of microorganism capable of degradation of petroleum hydrocarbons. They have shown that introduction of microorganism to polluted land area increased the rate of remediation by as much as 50 %.

Jorgensen *et al*. (2000) did the composting of lubricating oil contaminated soil in biopiles; an ex-situ bioremediation technology. It is performed in field scale where organic matter such as bark chips were utilized as the bulking agent. Inocula of two (2) commercially available mixed microbes were added and the effect of added nutrients (N, P, K) were tested at different level. In this treatment method, the general microbial activity was enhanced as measured by soil respiration with addition of large amount of organic matter and no particular effect of added inocula was found.

Marquez-Rocha *et al.* (2001) performed laboratory and pilot scale experiments for the biodegradation of diesel oil using bacterial consortium capable of degrading diesel oil. The bacterial consortium was prepared in two (2) steps; liquid and then in soil for laboratory tests and pilot scale experiment. Diesel contaminated soil after treatment with bacterial consortium showed reduced concentration of diesel in both laboratory and pilot scale experiment as compared with control experiment within five (5) weeks. Diesel biodegradation using bacterial consortium was enhanced by addition of NH_4NO_3. In the end, the combination of stepwise soil inoculation and nutrient additions in hydrocarbon contaminated soil enhanced the microbial activity in the contaminated soil.

Das and Mukherjee (2007) conducted a study in North-East India for evaluating the efficiency of bacterial isolates, *Bacillus subtilis* DM-04 and *Pseudomonas aeruginosa* M and NM strains from petroleum contaminated soil and compared their biodegradation potential for the degradation of crude petroleum-oil hydrocarbons in soil by utilizing them as a sole source of carbon and energy. As compared to control soil there was a significant decrease in the TPH contaminated soil treated with *Bacillus subtilis* DM-04 and *Pseudomonas aeruginosa* M and NM strains and was effective for in-situ bioremediation.

Mancera-López *et al*. (2007) performed a research to determine the capacity of the isolated bacteria and fungi for the degradation of total petroleum hydrocarbon specifically the aliphatic hydrocarbon (AH) and polycyclic aromatic hydrocarbon fractions (PAH). These fungi and bacteria were isolated from petroleum hydrocarbon contaminated soils having TPH consent rations of 60,600 and 500,000 mg Kg-. Media used for this experiment was modified Rennic medium consist of mineral liquid culture in addition with a complex mixture of TPH as a source of carbon. Only six (6) strains showed high efficiency to degrade PAHs, AHs and TPH which were identified as *Pseudomonas pseudoalcaligenes, Bacillus firmus, Bacillus alvei, Penicillium funiculosum, Aspergillus sydowii* and *Rhizopus* spp.

2.2.1 Bacteria as an Agent of Bioremediation

Bacteria are able to biodegrade petroleum compounds especially the PAHs as degrading bacteria are present in both the water and the soil environment (Prince *et al.,* 2003). Research on the isolation of bacteria associated with engine oil contaminated soil was carried out by Ugoh and Moneke (2011). They selected different mechanic sites in a town within the Northern part of

Nigeria. They suggested that *Bacillus sp.* is most adapted to conditions present in soils contaminated with used engine oil and hence could be exploited in bioremediation activities.

Su *et al*. (2011) demonstrated the motor-oil-degrading potential of an indigenous *Pseudomonas aeruginosa* SU-1 bacterial strain. This bacterium was found to have the ability to degrade motor oil efficiently upon growing in a medium containing such pollutant as the sole carbon source. Bhattacharya *et al*. (2015) reported that a newly isolated *Ochrobactrum* sp. C_1 could grow with waste lubricants, as the sole carbon and energy source and degrade a wide range of hydrocarbons present in this waste efficiently. Larik *et al*. (2016) also reported the biodegradation of used engine oil and diesel oil using an efficient bacterial consortium A2457: encompassing *Stenotrophomonas maltophilia*, *Bacillus pumilus* and *Bacillus cereus*.

Salam (2016) in his study established the extensive degradation ability of two *Pseudomonas aeruginosa* strains; RM1 and SK1 on waste engine oil. He reported the potentials of these strains in the degradation of aromatic, aliphatic, and branched alkane components of waste engine oils. Microbial consortiums have been usually suggested for complete biodegradation of petroleum pollutants (Farahat and El-Gendy, 2008), since the constituents of hydrocarbon mixtures differ in their solubility, volatility, and susceptibility to biodegradation, and in contrast, the required sets of enzymes cannot be present in a single microbial strain.

Ilyina *et al*. (2003) developed a product (COBE-10) for the bioremediation of oil contaminated from bacterial strains isolated from petroleum hydrocarbon contaminated sites. Thirty (30) bacterial strains were selected from total of eighty-two (82) strains by their isolation in selective agar (agar diesel, combustion oil and petroleum) and then on the basis of change in absorbance of mineral media (containing petroleum as a sole carbon source). The potential of these selected strains for the degradation of petroleum hydrocarbons in artificially contaminated soil under laboratory conditions were evaluated. For the final formulation of COBE-10, six (6) strains were selected on the bases of DTPH/day results and to select suitable vehicle seven (7) carrier materials were tested. The performance of the finally developed product COBE-10 was ex-situ evaluated in field with diesel and refinery wastes contaminated soils and results showed its high efficiency in bioremediation.

Nweke and Okpokwasili (2003) isolated *Staphylococcus* spp from oil contaminated soil. This was then grown in drilling fluid base oil (1 %) using HDF-2000 as a sole source of carbon and energy. For oil uptake the mechanisms of adherence and emulsification were used by the isolated strain. There was found no significant effect on the growth rates of *Staphylococcus* sp. in a nutrient-rich marine broth, base oil (up to 2 % V/V) and glucose (up to 1.6 % V/V). This investigation demonstrated that base oil was strongly utilized by the *Staphylococcus* spp. and hence has potential to be applied as a remedy for treatment of contamination with oil- based drilling fluids.

Makut and Ishaya (2010) studied the isolation of bacterial flora from petroleum products contaminated soils at ten (10) different mechanical workshops in Keffi town and was plated on plate count agar, brilliant green agar, mannitol salt agar, eosin methylene blue agar and desoxycholate citrate agar. The bacterial isolates were identified as *Pseudomonas* spp, *Staphylococcus* spp, *Escherichia coli*, *Streptococcus* spp, *Enterobacter aerogenes*, *Klebsiella* spp, *Micrococcus* spp, *Mycobacterium* spp, *Salmonella* spp and *Bacillus* spp. Four (4) petroleum hydrocarbon substrates i.e. petrol, kerosene, diesel and engine oil were used for the degradation by bacterial isolates. The limitation in the bioremediation of soils contaminated with diesel fuel is due to the absence of specified substrate and indigenous microflora at the site.

Hamza *et al*. (2010) determined the biodegradation of petroleum hydrocarbon by four (4) bacterial species namely *Acinetobacter lwoffii, Aeromonas hydrophila, Pseudomonas aeruginosa* and *Pseudomonas putida* isolated from hydrocarbon contaminated soil and their growth requirements. Mineral salt medium added with two (2) types of crude oil, sumandak and south angi at 1 % (V/V) concentration was used for the growth of the bacteria.

Onuoha *et al*. (2011) investigated the potential for the hydrocarbon degradation by bacterial isolates. There was a total of twenty-seven (27) bacterial isolates grown on mineral salt medium by enrichment method and using spent oil as a sole source of carbon. These bacterial isolates were identified and characterized by using standard methods. Hydrocarbon degradation by these bacterial isolates was checked by gas chromatography. Thenmozhi *et al*. (2011) studied the biodegradation of multi-components of used engine oil in both pure and consortium cultures. By using enrichment technique, three (3) bacterial strains were isolated from contaminated soil that were capable of utilizing used engine oil as a sole carbon source. Based on biochemical tests and 16S rRNA sequencing these bacteria were identified as *Serratia marcescens, Pseudomonas aeruginosa* and *Bacillus licheniformis*.

Kafilzadeh *et al*. (2011) conducted a research to isolate and identify hydrocarbons degrading bacteria from Shiraz Refinery soil using Nutrient agar (NA) and Mineral salt agar (MM2) as basic culture media. Isolated strains were identified as *Bacillus, Corynebacterium, Staphylococcus, Streptococcus, Klebsiella, Escherichia, Acinetobacter, Alcaligenes, Shigella and Enterobacter*. Naphthalene, Phenenthrene, Biphenyl and Camphor were discovered to be broken down by these bacteria. Jayashree *et al*. (2012) also studied the degradation of oil components by different bacterial species. The degradation efficiency of the isolated bacterial species were identified with solvent extraction method and confirmed by FT-IR analysis. In Iran, oil contamination is most dangerous pollution problem due to high consumption of oil products.

Luo *et al*. (2012) isolated some highly efficient diesel oil degrading bacteria from bilge water and identified one strain C7 as *Pseudomonas* spp. on the bases of various morphological, physiological, phylogenetic characteristics and 16S rRNA gene sequencing. Characteristics of C7 strain in various conditions and its biodegradation potential were analyzed. The optimal conditions used for the degradation was 30 ^{0}C temperature, 7.0 pH and 3 % initial diesel oil concentration and $4x10^7$ cells/mL initial bacterial concentration. The most components of diesel oil were shown to be degraded by this C7 strain (*Pseudomonas* spp.) as analyzed through GC-MS technique.

Umar *et al*. (2013) isolated waste lubricating oil utilizing bacteria from oil polluted soil samples collected from mechanical workshops and identified them as *Pseudomonas fluorescens, Bacillus mycoides* and *Acinetobacter* spp. Waste lubricating oil degrading potential of these isolates were tested in mineral salt medium and observed that the degradation of oil took place at varying rates. The ability to emulsify the waste lubricating oil was shown by these waste lubricating oil utilizing bacterial isolates which indicated the importance of production of biosurfactants by these isolates. Singh and Lin (2008) isolated ten (10) indigenous microorganisms from contaminated soils using the enrichment technique, out of which five (5) isolates were identified as *Acinetobacter calcoaceticus* (LT1 and ETS2), *Acinetobacter* spp (LT1A), *Citrobacter freundii* (MRC3) and *Bacillus pumilus* (JLB) under standard degradation conditions shows highest degradation potentials.

2.3 Environmental/Physicochemical Factors Affecting Biodegradation of Hydrocarbons

Biodegradation is an efficient, economic, versatile and environment friendly treatment for the hydrocarbon contaminated soils. Automobile workshop plays an important role in soil contamination through seepage of used engine oil and washed water into the soil. Sathiya-Moorthi *et al*. (2008) studied the degradation of oil from the automobile effluent by *Pseudomonas*. The *Pseudomonas* spp. responsible for the oil degradation was isolated from the automobile oil effluent polluted soil by enrichment technique. Various physicochemical properties such as pH, acidity, Total Dissolved Solids (TDS), Total suspended solids, hardness, Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD) etc. of the automobile effluent was analyzed. For their experiment, they used mini fermentor of two (2) liter capacity of batch type for the production of *Pseudomonas* sp. for the preparation of carrier based biodegradation and diatomaceous earth with lignite in the ratio 8:2 were used as a carrier.

In a research conducted by Osuji and Nwoye (2007), the authors examined the physicochemical conditions of soils contaminated with petroleum hydrocarbon, three (3) months after oil spillage at Owaza (Niger Delta), Southern Nigeria. Findings from the research revealed high temperature, high moisture content, high soil acidity, low electrical conductivity which imply low soil fertility.

Kayode *et al*. (2009) performed an experiment to investigate the impact of spent lubricating oil on the physicochemical properties of soil in Nigeria. It was concluded from their study that the spent lubricating oil altered the physicochemical properties of soil by decreasing soil capillarity, soil aeration, water holding capacity, nitrogen, phosphorous, sodium, potassium, magnesium and calcium contents and increased organic matter, organic carbon, bulk density and water porosity. The physicochemical properties of soil sample are improved during the experiment due to daily watering which cause reduction in the concentration of spent lubricating oil. Removal of the petroleum hydrocarbons contaminants is becoming a very important problem.

Akubugwo *et al*. (2009) studied the physicochemical properties of soil contaminated with refined oil three (3) years after spill in Isuikwuato, Abia state. Their result showed significant increase ($9 < 0.005$) in soil temperature, exchangeable acidity, effective cation exchangeable capacity, organic carbon, saturation base and organic matter in the polluted soil as compared to control. Soil nitrogen, magnesium, calcium and phosphorus were also estimated in contaminated soil and suggested the unsuitability of soil for agricultural activity.

Akoachere *et al*. (2008) isolated and characterized the waste engine oil degrading bacteria from auto mechanic workshops and petrol filling stations of the region Buea, Cameroon where the major source of oil pollution is due to indiscriminate disposal of crankcase oil. They also studied some environmental factors which influence the rate of degradation of oil by bacterial isolates. To check the degradation efficiency of the isolated bacterial culture at different temperature and nutrient concentrations, the test was set up individually and in mixed consortium. For the analysis of the results they used chi-square test. In Buea, their study was the first of its kind to indicate the presence of oil degrading bacteria in soils and also to found the environmental conditions for optimum degradation of oil by bacterial isolates.

To remediate oil contaminated site, Santhini *et al*. (2009) isolated seven (7) bacteria from the soil samples of three (3) different oil contaminated sites at Mayiladuthurai and Nagappattinam district in India. Out of all the seven (7) bacterial isolates, they selected *Micrococcus* spp for their study. The collected soil samples were tested for various physicochemical parameters such as temperature, electrical conductivity, nutrients including total nitrogen, phosphorous, potassium, iron, copper, magnesium and zinc. The biodegradation capability of *Micrococcus* spp was tested by liquid culture characteristics and emulsification activity in two (2) hydrocarbon substrates. The growth potential of hydrocarbon-utilizing bacterial was observed on petrol and diesel. The *Micrococcus* spp was tested for the biosurfactant activity and showed moderate results in petrol and diesel.

Pathak *et al*. (2011) carried out physicochemical analysis of petroleum contaminated soil using different methods. Physical properties analyzed include soil pH, soil moisture content, texture, organic matter, carbon/nitrogen ratio, bulk density, presence of heavy metal etc. Low water holding capacity and porosity as well as high Carbon-Nitrogen ratio (C:N) and bulk density in contaminated soil was observed.

Bahuguna *et al*. (2011) evaluated the physicochemical parameters of polycyclic aromatic hydrocarbons (PAHs) contaminated soil which involves temperature, pH, nutrient content, total organic carbon, inorganic phosphorous and inorganic nitrates. Results from the physicochemical parameters demonstrated a significant increase in total organic carbon, total PAHs content and temperature in the soil samples collected from automobile repair work stations having heavy transport activities but showing low moisture content and microbial counts.

Barua *et al*. (2011) assessed certain physicochemical changes in the soil due to contamination of crude oil in Rudrasagar oil field and Lakwa oil field of Sibsagar district of upper Assam, North-Eastern India, where oil exploration activities are conducted by Oil and Natural Gas Corporation limited (ONGCL). Findings from the study revealed an increase in total nitrogen, organic carbon, exchangeable potassium and decrease in soil moisture, pH, water holding capacity and extractable phosphorous.

Ujowundu *et al*. (2011) studied the biochemical and physical characteristics of diesel oil contaminated soil in South-Eastern Nigeria and concluded that the contamination by diesel resulted in decrease in pH and microbial mass and also adversely affect nutrient cycle, impede nutrient uptake by plants roots and decrease in crop yield.

In Niger Delta (Nigeria), the physicochemical properties of Ebocha - 8 soil was determined by Onojake and Osuji (2012) to estimate the extent of pollution after six (6) months of spill incident. The physicochemical properties of soil such as pH, moisture content, sulphate, conductivity, chloride, total acidity was determined by using standard analytical method. Ebocha - 8 soil was highly recommended for the liming, depollution measures and rehabilitation due to high hydrocarbon levels.

2.3.1 Soil Temperature

The most important factor affecting biodegradation of hydrocarbon is temperature, because the biological enzymes involved in the degradation pathway have an optimum temperature and will not have the same metabolic turnover for every temperature (Iranzo *et al.,* 2001). Temperature influences petroleum biodegradation by its effect on the physical nature and chemical composition of the oil, rate of hydrocarbon metabolism by microorganisms, and composition of the microbial community (Venosa and Zhu, 2003).

Hydrocarbon biodegradation can occur over a wide range of temperatures and the rate of biodegradation generally decreases with decreasing temperature. The highest degradation rates generally occur in the range of 30 - 40 ^{0}C in soil environments, 20 - 30 ^{0}C in some freshwater environments, and 15 - 20 ^{0}C in marine environments (Das and Chandran, 2011).

The effect of temperature is also complicated by other factors such as the composition of the microbial population (Zhu *et al.*, 2001). The small subunit (SSU) 18S rRNA gene is one of the most frequently used genes in phylogenetic studies and an important marker for random target polymerase chain reaction (PCR) in environmental biodiversity screening. In general, rRNA gene sequences are easy to access due to highly conserved flanking regions allowing for the use of universal primers (Perfumo *et al.*, 2010).

Temperature influences rate of biodegradation by controlling rate of enzymatic reactions within microorganisms. Generally, speed of enzymatic reactions in the cell approximately doubles for each 10 ^{0}C rise in temperature. There is an upper limit to the temperature that microorganisms can withstand. Most bacteria found in soil, including many bacteria that degrade engine oil, are mesophiles which have an optimum temperature ranging from 25 - 45 ^{0}C (Nester *et al*., 2001).

Bioremediation is an effective and efficient technology to accelerate the clean-up process for the remediation of soil contaminated with oil. Mandri and Lin (2007) isolated three (3) bacteria having capability of utilizing used engine oil as a source of carbon using enrichment technique from soil contaminated with oil. These bacterial isolates were identified on the bases of biochemical tests and 16S rRNA sequencing as *Flavobacterium* sp., *Acinetobacterium calcoaceticum* and *Pseudomonas aeruginosa*. Oil degradation by bacterial isolates was indicated by increase in cell number which cause increase in oil degradation. Biodegradation occurred at optimal temperature of 30 - 37 ^{0}C. The biodegradation test for the utilization of used engine oil by bacterial isolates was performed under laboratory condition at 30 ^{0}C and 160 rpm in Bushnell-

Haas media for four (4) weeks. These bacterial isolates showed high potential for oil bioremediation both in-situ and ex-situ.

2.3.2 Soil 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).

Soil pH is important because most microbial species can survive only within a certain pH range. Furthermore, soil pH can affect availability of nutrients. Biodegradation of engine oil is optimal at a pH 7 (neutral) and the acceptable range is pH 6 – 8 (US EPA, 2006). 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 cocovenena*, isolated from a petroleum-polluted soil.

Organic soils in wetlands are often acidic, while mineral soils have more neutral and alkaline conditions. Most heterotrophic bacteria and fungi favour a neutral pH, with fungi being more tolerant of acidic conditions (Margesin and Schinner, 2001). Studies have shown that degradation of oil increases with increasing pH, and that optimum degradation occurs under slightly alkaline conditions (Zhu *et al.*, 2001).

Okerentugba and Ezeronye (2003) studied the ability of three (3) bacterial isolates- *Bacillus* spp, *Micrococcus* spp and *Proteus* spp and three (3) fungal species- *Penicillium* spp, *Aspergillus* spp and *Rhizopus* spp isolated from two (2) rivers and refinery effluent to degrade two

(2) Nigerian crude oils. These isolates were observed to utilize and degrade crude oil components. Fungal isolates showed increase in biomass while the bacterial isolates showed increase in cell number and optical density as pH decreases.

2.3.3 Soil Type

Soil type is an important parameter when determining the best suited bioremediation approach to a particular situation. Soil texture directly affect the soils porosity, just as permeability of soil to air and water is a function of soil texture. Fine-textured soils like clays have low permeability, which in turn prevents bio-vented oxygen and nutrients from dispersing throughout the soil. Also, it is difficult to control moisture content in fine textured soils because their smaller pores and high surface area allow it to retain water (Chaillana *et al.*, 2006). Fine textured soils are slow to drain from water-saturated soil conditions, thus preventing oxygen from reaching soil microbes throughout the contaminated area (US EPA, 2006).

2.3.4 Salinity

Changes in salinity may affect oil biodegradation through alteration of the microbial population. Many freshwater organisms can survive for long periods in seawater although few can reproduce (Iranzo *et al.,* 2001). In contrast, most marine species have an optimum salinity range of 2.5 - 3.5 % and grow poorly or not at all at salinity lower than 1.5 - 2 % (Venosa and Zhu, 2003). Qin *et al*. (2012) suggested that the salinity had a major influence on bioremediation and biodegradation process, and it also affects microbial growth and diversity. Salinity has an adverse influence on the activity of some key enzymes complicated in the process of hydrocarbon degradation (Ebadi *et al*., 2017).

Malkawi *et al*. (2009) isolated thirteen (13) bacteria and tested their salt tolerance ability for the remediation of soil contaminated with oil and in high saline environment by inducing random mutation on to hydrocarbon degrading key enzymes (Catechol 2,3 dioxygenase ($C_{23}O$) and Monooxygenase) created more effective oil-degrading microorganisms.

2.3.5 Oxygen

The oxygen availability in the soil depends on microbial oxygen consumption rates and soil type, whether soil is waterlogged, and the useable substrates presence which can drive to oxygen depletion. Some studies have indicated that anaerobic degradation of petroleum hydrocarbons by microorganisms can happen at negligible rates (Haritash and Kaushik, 2009).

Biodegradation of petroleum hydrocarbons (PHs) in anaerobic conditions was not as fast as in aerobic conditions (Grishchenkov *et al*., 2000). Substrate oxidation by oxygenases in the catabolism of all aliphatic, cyclic and aromatic compounds by microbial is considered a key step in the biodegradation process (Meng *et al*., 2017).

The enzymatic process of breaking down oil is usually most rapid in the presence of oxygen (Chaillana *et al*., 2006; Das and Chandan, 2011). Theoretically, given enough oil and other nutrients, microbial populations could grow so quickly that they exhaust the oxygen from the soil or water in the vicinity of an oil spill (Bamforth and Singleton, 2005). In practice, oxygen has not proven to be as important a limiting factor as a nutrient in restricting oil degradation in soils and oceans, although degradation rates could be slow if a spill occurred in a location where oxygen levels are low (Bamforth and Singleton, 2005; Chaillana *et al*., 2006).

2.3.6 Nutrients

Some nutrients can become a limiting factor thus impacting in the processes of biodegradation. Carbon comes from an organic source (PHs) while hydrogen and oxygen are supplied from the water (Kalantary *et al*., 2014). In marine and freshwater environments, oil spills cause a dramatic increase of carbon levels and a decrease of nitrogen and phosphorus levels which can affect the biodegradation process. In marine environments, nitrogen and phosphorus levels are low, and the wetlands are unable to provide the nutrients because of strong demands of nutrients by the plants. Thus, nutrients addition was necessary to promote the biodegradation of contaminants (Hesnawi and Adbeib, 2013).

Zafra *et al*. (2015) observed that the pollutants concentration had a selective pressure on petroleum-degrading organisms, the high polycyclic aromatic hydrocarbon (PAH) levels were limiting the growth of microorganisms that developed a response against PAHs, concerning the structure of cell membrane, alterations of sporulation and mycelia pigmentation. Balaji *et al*. (2014) also examined various sources of carbon for lipase production by *Penicillium chrysogenum*, *Lasiodiplodia theobromae* and *Mucor racemosus*, and found that sucrose and cellulose induced the highest activity in those species. Likewise, sources of nitrogen should be taken into consideration. Yeast extract was the better catalyst of high level production of lipase in the above-mentioned strains. Furthermore, Mineki *et al*. (2015) studied PAHs degradation by *Trichoderma/Hypocrea* using pyrene as a carbon source, after addition of 0.02 % yeast extract, 0.1 % lactose or 0.1 % sucrose, the strain growth and pyrene-degrading efficiency were enhanced compared with the control after 7 and 14 days of incubation.

2.3.7 Bioavailability

Bioavailability refers to the portion of a chemical in soil, which can be taken up or transformed by living organisms. It has also been defined as the influence of the physical, chemical and microbiological factors to the extent and rate of biodegradation. The pH, microbial community and 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 (PHs) have low bioavailability and are classified as hydrophobic organic pollutants. Those chemicals have little water solubility, which makes them resistant to photolytic breakdown and chemical biological (Semple *et al*., 2003).

Contaminants can adsorb to soil particles, rendering some contaminants unavailable to microorganisms for biodegradation (Chaillana *et al*., 2006). Thus in some circumstances, bioavailability of contaminants depends not only on the nature of the contaminant but also on soil type (State of Mississippi, Department of Environmental Quality, 1998; Chaillana *et al*., 2006). Hydrophobic contaminants, like petroleum hydrocarbons, have low solubility in water and tend to adsorb strongly in soil with high organic matter content (Chaillana *et al*., 2006). Additional research findings of the existence of thermophilic bacteria in cool soil also suggest that high temperatures enhance the rate of biodegradation by increasing the bioavailability of contaminants (Chaillana *et al*., 2006; Perfumo *et al*., 2007). It is suggested that contaminants adsorbed to soil particles are mobilized and their solubility increased by high temperatures (Perfumo *et al*., 2007).

2.3.8 Toxicity of End Product

The biological treatment principle is to put an end to toxins and pollutants from the restricted environment by using microorganisms. Recently, using a bioreactor for treating contaminated PAH gas-work soil evaluated both PAHs accumulation and removal of oxy-PAHs, such as coumarins, quinones and PAH-ketones (Lundstedt *et al*., 2003).

Most commercial bioremediation trials tend to monitor the success of the treatment by the degree of removal of the parent contaminant and do not consider the possibility of the biological production of more toxic breakdown metabolites. However, it is important to ensure that the contaminated material is suitably detoxified at the end of the treatment (Mendonca and Picado, 2002; Lundstedt *et al*., 2003).

2.3.9 Availability of Moisture

Soil moisture contents between 45 and 85 % of the water holding capacity (field capacity) of the soil or about 12 - 30 % by weight are optimal for petroleum hydrocarbon degradation (Chaillana *et al*., 2006; US EPA, 2006). Excess moisture, such as in saturated soil, is undesirable because it reduces the amount of available oxygen for aerobic respiration (Chaillana *et al*., 2006). Anaerobic respiration, which produces less energy for microorganisms (than aerobic respiration) and slows the rate of biodegradation, becomes the predominant process (Trindade *et al*., 2005).

2.3.10 Microorganisms

Natural microbial communities are diverse, with many different types of microorganisms that both compete and cooperate (Chaillana *et al*., 2006). The complex interactions that characterize healthy, natural microbial communities are only beginning to be understood, but interdependence is the norm (Perfumo *et al*., 2007). This is one reason why adding microbes to oil spills in the hope of speeding degradation is challenging; artificially introduced microbes have a hard time breaking into the existing community structure and competing with the local species that have evolved together over the millennia in a particular habitat (US EPA, 2006).

2.4 Soil Amendment with Biochar

Biochar is an extremely complex stable form of carbon produced by the controlled heating of plant and/or animal material (biomass feedstock) (Jenkins and Jenkinson, 2009). Biochar is generally obtained from the low-temperature (e.g., 400 - 700 ^{0}C) pyrolysis of various biomass which include manure (Cao *et al*., 2009), agricultural residues (Woolf *et al*., 2010), bamboos (Xu *et al*., 2012), and biosolids in the absence of oxygen and is often used as a soil amendment (Lehmann *et al*., 2011), which is therefore distinguished from fuel charcoal.

The complex chemical structure of biochar is defined by the feedstock it is made from and the temperature conditions used in its manufacture. Biochar is a form of charcoal but is different in that, it is produced in controlled conditions so that most of the carbon is converted to usable products. Charcoal usually has a total carbon content of over 75 % whilst biochar often has much less total carbon (often 40 -75 %) but it has a higher mineral content, containing minerals such as Calcium (Ca), Potassium (K), Phosphorus (P) and Nitrogen (N) (Jenkins and Jenkinson, 2009).

The characteristic of any biochar is a function of the material from which it is made and the temperature conditions used to make it. The range of biochar available could be considerable, representing a wide range of feedstock, temperature, residence times and heating rates used in their creation (Jenkins and Jenkinson, 2009). Incorporation of biochar into the soil is shown to affect the preexisting soil properties in ways attributed to the physical and chemical properties of biochar.

Biochar has high surface area, higher porosity, variable charge, and functional groups that can increase soil water holding capacity, pH, cation exchange capacity (CEC), surface sorption capacity, base saturation, and crop resistance to disease when added to soil (Glaser *et al*., 2002; Liang *et al*., 2006). These properties vary with the pyrolysis temperature and the properties of feedstocks (Gundale and DeLuca, 2006; Chan and Xu, 2009; Singh *et al*., 2010). An increased pyrolysis temperature could result in smaller biochar particles due to the decreased tensile strength of the feedstock at higher pyrolysis temperature (Hossain *et al*., 2011). The nutrient content and availability in biochar is influenced by the biomass type, processing conditions (Singh *et al*., 2010), and type of bonds associated with the elements involved (DeLuca *et al*., 2009).

Biochar application to soil is widely advocated for a variety of reasons related to sustainability (Jeffery *et al*., 2015).

2.4.1 Physical Properties of Biochar

Biochar is made of irregular arrangements of carbon (C) containing oxygen (O_2) and hydrogen (H) and, in some cases, minerals depending upon feedstock (Lehmann and Joseph, 2009). Charred biomass consists of recalcitrant aromatic rings as well as more easily degradable aliphatic and oxidized carbon structures (Lehmann, 2007). Key physical features of most biochar are their highly porous structure and large surface area which can provide refuge for beneficial soil microorganisms, like mycorrhizae and bacteria, and influences the binding of important nutritive cations and anions (Atkinson *et al*., 2010).

Biochar is often macroporous in nature which reflects cellular structures in the feedstock from which it is produced, that is potentially important for water holding and adsorption of soil (Sohi *et al*., 2010). When added to soil, biochar appears to divide rapidly into particles of silt size or less due to abrasion, shrink-swell, and other physical weathering processes (Brodowski *et al*., 2007). Low temperature biochar is stronger than high temperature products with regards to adsorptive properties, but it is more brittle and prone to abrading into finer fractions once incorporated into soil (Sohi *et al*., 2010).

2.4.2 Chemical Properties of Biochar

Much research has produced unequivocal proof that biochar is not only more stable than any other amendment to soil and increases nutrient availability beyond a fertilizer effect, but its stability and nutrient retention properties make it more effective than any other organic material in soil (Lehmann and Joseph, 2009).

Chemical and physical properties such as high charge density and its particulate nature along with specific chemical structure, and high microbial and chemical stability, all contribute to greater nutrient retention and resistance to microbial decay than other organic matter (Atkinson *et al*., 2010). Baldock and Smernik (2002) determined that thermal treatment of organic materials at temperatures > 200 ^{0}C induces significant variations in their chemical composition. Changes in chemical composition, as measured by 13C nuclear magnetic resonance (NMR) indicated that changes with increased pyrolysis temperature included a conversion of O-alkyl C to aryl and O-aryl furan-like structures, which are more chemically active oxygen-containing carbon ring.

Research suggests that biochar created at low temperatures may be suitable for controlling the release of fertilizer nutrients while high temperatures would lead to a material similar to activated carbon (Sohi *et al*., 2010).

2.4.3 Chemical Composition and Surface Chemistry of Biochar

Biochar is produced from biomass and is predominantly composed of recalcitrant organic carbon with contents of plant micro and macro-nutrients retained from the starting feedstock. It is known from research on wildfire occurrence and the development of Anthrosols (*e.g. Terra Preta* soils) in the Amazon that charcoal can remain in the soil for hundreds to thousands of years (Agee, 1996; Lehmann *et al.*, 2006). Consequently, biochar can rapidly increase the recalcitrant soil carbon fraction of soil. The carbon in biochar is held in aromatic form which is resistant to decomposition when added as a soil amendment (Amonette and Joseph, 2009), making it a carbon sequestration tool. However, composition varies by feedstock type and conditions of pyrolysis (Downie *et al*., 2009).

Actual carbon (C) contents can range between 172 g/kg and 905 g/kg. Nitrogen (N) content ranges from 1.8 g/kg - 56.4g/kg, total phosphorus (P) from 2.7g/kg - 480g/kg and total potassium (K) from 1.0g/kg - 58g/ kg (Lehmann *et al*., 2002; Lima and Marshall 2005; Chan *et al*., 2007). The outer surfaces contain various oxygen and hydrogen functional groups and the graphene sheets may contain oxygen groups and free radicals (Bourke *et al*., 2007). Additionally, biochar has been produced with a range of pH values between 4 and 12, dependent upon the starting feedstock and operating conditions (Lehmann, 2007). Generally, low pyrolysis temperatures (< 400 ^{0}C) yield acidic biochar, while increasing pyrolysis temperatures produce alkaline biochar. Once incorporated into the soil, surface oxidation occurs due to reactions of water (H_2O), oxygen (O_2) and various soil agents (Cheng *et al*., 2006; Lehmann, 2007). The cation exchange capacity (CEC) of fresh biochar is typically very low, but increases with time as the biochar ages in the presence of O_2 and water (Cheng *et al*., 2006; Liang *et al*., 2006; Cheng *et al*. 2008).

Biochar composition is highly heterogeneous, containing both stable and labile components (Sohi *et al*., 2009). Carbon, volatile matter, mineral matter (ash) and moisture are generally regarded as its major constituents (Antal and Grønli, 2003).

2.4.4 Pore Size Distribution and Connectivity of Biochar

Biomass feedstock and the processing conditions are the main factors determining pore size distribution in biochar, and therefore its total surface area. During thermal decomposition of biomass, mass loss occurs mostly in the form of organic volatiles, leaving behind voids, which form an extensive pore network. Biochar pores are classified in this review into three (3) categories, according to their internal diameters (ID): macropores (ID > 50 nm), mesopores (2 nm < ID < 50 nm) and micropores (ID < 2 nm). These categories are orders of magnitude different to the standard categories for pore sizes in soil science. The elementary porosity and structure of the biomass feedstock is retained in the biochar product formed (Downie *et al*., 2009). Micropores are mainly formed during processing of the parent material while macropores have been identified as a feeder to smaller pores (Martinez *et al*., 2006).

The development of microporosity in biochar, which is linked to an increase in structural and organizational order, has been shown to be favoured by higher pyrolysis temperature and retention times, as previously demonstrated for activated carbon (Lua *et al*., 2004). For instance, increasing pyrolysis temperature from 250 – 500 ^{0}C enhanced the development of micropores in chars derived from pistachio-nut shells, due to increased evolution of volatiles. Similarly, heating rate and pressure during processing have also been found to influence the mass transfer of volatiles produced at any given temperature range, and are therefore regarded as key contributing parameters influencing pore size distribution (Antal and Grønli, 2003).

2.4.5 Impacts of Biochar

The impact of biochar include but not limited to:

a) Alters soil microbial diversity and shift functional groups (Pietikäinen *et al*., 2000)

b) Reduce soil bulk density (Gundale and DeLuca, 2006)

c) Strongly sorbs salts and ameliorates salt stress effects on plants in agricultural, urban, and contaminated soils (Thomas *et al*., 2013).

d) Increases the availability of P and Zn, and the total N concentrations on soils (Glaser *et al.*, 2002; Lehmann *et al*., 2003).

e) Application of biochar with fertilizers helps to maximize the benefit on plant growth and nutrition as well as soil biology improvement (Solaiman *et al*., 2010).

f) Increase crop yields at lower rates of fertilizer use (Blackwell *et al*., 2010).

g) Banding of biochar in soil can minimize wind erosion risk and place biochar close to crop roots.

h) Improve nutrients availability, storage and cation exchange capacity (CEC)

i) Increases water holding capacity

j) Increases soil pH

k) Decreases aluminum toxicity

l) Decreases tensile strength

m) Changes the microbiology of the soil

n) Decrease greenhouse gases emissions from the soil (N_2O and CH_4)

o) Improve soil conditions for earthworm populations

p) Increase soil water infiltration and permeability

q) Carbon sequestration

r) Soil fertility improvement

s) Pollutant immobilization

t) Waste management (Hammond, 2014)

2.4.6 Effect of Biochar on Soil Biology

Soil is a highly complex and dynamic habitat for organisms, containing many different niches due to its incredibly high levels of heterogeneity at all scales. On the microscale, soil is often an aquatic habitat, as micro pores in soil are full of water at all times, apart from during very extreme drought, due to the high-water tension which exists there. The soil biota is vital to the functioning of soils and provides many essential ecosystem services. Understanding the interactions between biochar when it is used as a soil amendment, and the soil biota is therefore vital. It is largely through interactions with the soil biota, such as promoting arbuscular mychorrizal fungi (AMF) as well as influencing on water holding capacity, which lead to the reported effects of biochar on yields (Steiner *et al*., 2008a; Kolb *et al*., 2009).

Biochar, due to its highly porous nature, has been shown to provide increased levels of refugia where smaller organisms can live in small spaces which larger organisms cannot enter to prey on them. Microorganisms within these micropores are likely to be restricted in growth rate due to relying on diffusion to bring necessary nutrients and gases, but as this occurs in micropores within the soil, this demonstrates that microorganisms utilizing these refugia almost certainly would not be reliant of decomposition of the biochar for an energy source. This is likely to be one of the mechanisms for the demonstrated increases in microbial biomass and combined with the increased water holding potentials of soil is a possible mechanisms for the increased observed basal microbial activity (Steiner *et al.*, 2008a; Kolb *et al*., 2009). However, due to the complexities of the soil system and its biota, it is probable that many more mechanisms are at work. For instant, Kolb *et al*. (2009) demonstrated that while charcoal additions affected microbial biomass, microbial activity, as well as nutrient availability, differences in the magnitude of the microbial response was dependent on the differences in base nutrient availability in the soils studied. However, they noted that the influences of biochar on the soil microbiota acted in a relatively similar way in the soils they studied, albeit at different levels of magnitude, and so suggested that there is considerable predictability in the response of the soil biota to biochar application.

2.4.7 Biochar Effect on Sorption of Hydrophobic Organic Compounds (HOCS) and Others

The sorption of anthropogenic hydrophobic organic compounds (HOC) (e.g. polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyl (PCBs), pesticides and herbicides) in soils and sediments, is generally described based on two (2) coexisting simultaneous processes: absorption into natural (amorphous) organic matter (NOM) and adsorption onto occurring charcoal materials (Cornelissen *et al*., 2005; Koelmans *et al*., 2006). Comparatively to that of natural organic matter (NOM), charcoals (including soot) generally hold up to 10 - 1000 times higher sorption affinities towards such compounds (Chiou and Kile, 1998; Bucheli and Gustafsson, 2000; Bucheli and Gustafsson, 2003). It has been estimated that black carbon (BC) can account for as much as 80 – 90 % of total uptake of trace hydrophobic organic compounds (HOC) in soils and sediments (Cornelissen *et al*., 2005), and that it applies to a much broader range of chemical species than previously thought (Bucheli and Gustafsson, 2003).

Enhanced sorption capacity of a silt loam for diuron (Yang *et al*., 2006) has previously been reported following the incorporation of biochar ash from crop (wheat and rice) residues. Previous studies have convincingly demonstrated that adsorption to charcoals is mainly influenced by the structural and chemical properties of the contaminant (i.e. molecular weight, hydrophobicity, planarity) (Cornelissen *et al*., 2005; Zhu and Pignatello, 2005; Zhu *et al*., 2005).

For instance, sorption of tri- and tetra-substituted-benzenes (such as trichlorobenzene, trinitrotoluene and tetramethylbenzene) to maple wood charcoal (400 ^{0}C) was sterically restricted, when compared to that of the lower size benzene and toluene (Zhu and Pignatello, 2005).

Experimental evidence has recently demonstrated that organic structures in the form of black carbon (BC) (including biochar) or NOM, which are equipped with strong aromatic π-donor and -acceptor components, are capable of strongly adsorbing to other aromatic moieties through specific sorptive forces other than hydrophobic interactions (Keiluweit and Kleber, 2009). Although a large body of evidence is available on the way the characteristics of natural organic matter (HOC) influence sorption to biochar, the contribution of the char's properties to that process has been far less evaluated. It is generally accepted that mechanisms leading to an increase in surface area and/or hydrophobicity of the char, reflected in an enhanced sorption affinity and capacity towards trace contaminants, as demonstrated for other forms of black carbon (BC) (Jonker and Koelmans, 2002). The influence of pyrolysis temperatures mostly in the 340 - 400 ^{0}C range (James *et al*., 2005; Zhu *et al*., 2005) and feedstock type (Pastor-Villegas *et al*., 2006) on such phenomena has been recently evaluated for various wood chars by a number of authors. Interestingly, sorption to high-temperature chars appears to be exclusively by surface adsorption, while that to low-temperature chars derive from both surface adsorption and (at a smaller scale) absorption to residual organic matter (Chun *et al*., 2004).

The influence of micropore distribution on sorption to biochar has been clearly demonstrated by Wang *et al*. (2006). Diminished oxygen functionality on the edges of biochar‘s graphene sheets due to heat treatment (e.g. further charring), resulted in enhanced hydrophobicity and affinity for both polar and non-polar compounds, by reducing competitive adsorption by water molecules (Zhu et al., 2005; Wang et al., 2013). The treated char also revealed a consistent increase in micropore volume and pore surface area, resulting in better accessibility of solute molecules and an increase in sorption sites (Wang *et al*., 2013). The underlying sorption mechanism, including the way it is influenced by a wide range of factors inherent to the contaminant, to the char material and to the environment, remains far from being fully understood (Fernandes and Brooks, 2003). In this context, it is vital to comprehensively assess the environmental risk associated to these species in biochar-enriched soils, while re-evaluating both the use of generic OC-water distribution coefficients (Jonker *et al*., 2005) and of remediation endpoints (Cornelissen *et al*., 2005). For instance, remediation endpoints (undetectable, non-toxic or environmentally acceptable concentrations, as set by regulatory agencies) for common environmental contaminants in biochar-enriched soils would need to be assessed based on dissolved (bioavailable) concentrations rather than on total concentrations (Cornelissen *et al*., 2005).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection of Soil Samples

Soil samples were collected from five (5) mechanic workshops along Taiwo-Oke axis within Ilorin metropolis. The samples were aseptically collected randomly beneath the surface using sterile hand trowel, stored in sterile aluminum foils and transported to the laboratory immediately after collection for analysis. Unused soil samples were preserved in a refrigerator at 4 ^{0}C till further use.

3.2 Sterilization of the Materials

All glass wares were thoroughly washed with detergent, rinsed properly in several charges of tap water, drained and then wrapped in aluminum foil and sterilized in the hot-air oven at 170 ^{0}C for 60 minutes. The workbench was disinfected by swabbing with cotton wool soaked in 70 % ethanol before and after every experimental procedure in order to ensure an aseptic environment. Inoculating loop was flamed to redness in the Bunsen burner flame while spatula and pipettes were dipped in 70 % ethanol and flamed before and after use. All media and distilled water were sterilized in an autoclave at 121 ^{0}C for 15 minutes before use. All activities were carried out in a laminar flow chamber observing aseptic procedures as described by Fawole and Ọsọ (2007).

3.3 Culture Media Preparation

Media used in the experimental procedure were all prepared according to the manufacturer’s instruction.

3.3.1 Preparation of Nutrient Agar (NA)

Nutrient agar was prepared by dissolving 14 g of the media in 500 mL distilled water in a conical flask. The conical flask was then plugged with cotton wool wrapped with aluminum foil and sealed properly with paper tape. The mixture obtained was boiled to ensure proper homogenization before sterilization (autoclaving) at 121 ^{0}C at 15 psi for 15 minutes.

3.3.2 Preparation of Mineral Salt Medium (MSM)

The mineral salt medium was prepared with the following composition for the bacterial isolates: 10 g/l of NaCl, 0.42 g/l of $MgSO_4.7H_2O$, 1.25 g/l of K_2HPO_4, 0.29 g/l of KCl, 0.83 g/l of KH_2PO_4, 0.42 g/l of $NaNO_3$, 1 % hydrocarbon source (engine oil) and 2.3 g of agar-agar powder for mineral salt medium (MSM) agar with the same composition for mineral salt medium (MSM) broth while omitting the solidifying agent (agar-agar). The mixture in the conical flask was then plugged with cotton wool wrapped with aluminum foil and sealed properly with paper tape. This was then boiled to ensure proper homogenization after which it was autoclaved (sterilized) at 121 ^{0}C at 15 psi for 15 minutes (Fagade *et al.*, 2009).

3.4 Determination of Soil Parameters of the Soil Samples

The soil samples collected from the five (5) mechanic workshops were analyzed individually (before bioremediation) and collectively (after bioremediation) to determine the following soil parameters:

3.4.1 Granulometric Determination (Particle Size Analysis/Distribution)

This was determined by weighing 50 g of air-dried soil and passed through 2 mm sieve after which stones and roots larger than 2 mm were separated out. Weight of the mass of sample

> 2 mm was then taken. Thereafter, 20 g of the soil that has passed through the 2 mm sieve was weighed and placed in a bottle together with 25 mL of sodium diphosphate ($Na_2P_2O_7$) solution. This was left for about 8 hours after which 200 mL of water was added and shaken. The suspension was passed through the sieve set arranged in order of decreasing pore size, and placed directly above a 1 litre measuring cylinder. Each sieve was washed with water until particles smaller than the nominal size of the sieve pores have passed through. The sieves were then dried in an oven at 105 ^{0}C until a constant weight was achieved with each sieve weighed before and after sieving the suspension. The cylinder containing the sieved suspension was made-up to 1 litre with water, stopper and shaken for 1 minute. The oven-dried sieves were then cooled in a desiccator and weighed. The mass of each particle fraction in the weighing bottles was then determined by difference. Twenty-five (25) mg was subtracted from each measurement in order to correct for the presence of sodium diphosphate ($Na_2P_2O_7$) reagent thereafter multiplied by 5 to give each fraction as % of the original weight. The percentage of the total mass that was present in each size fraction was then noted (Radojevic and Bashkin, 1999; Mbachu *et al.*, 2017).

3.4.2 Determination of Bulk Density

This was determined gravimetrically by drying the soil samples in an oven at 105 ^{0}C until a constant weight was achieved. Weight of the soil samples were then taken. Into a measuring cylinder, the soil samples were poured a little at a time while gently tapping the cylinder to compact it. There volume was then measured (1 mL = 1 cm^3) (Radojevic and Bashkin, 1999; Mbachu *et al.*, 2017). Bulk density was then determined as stated below:

$$\text{Bulk density (g/cm}^3\text{)} = \frac{\text{Weight (g)}}{\text{Volume (cm}^3\text{)}}$$

3.4.3 Determination of Soil pH

A 10% (w/v) suspension of air-dried soil was prepared in double distilled water, mixed thoroughly, allowed to settle for 1 hour and filtered through the Whatmann filter paper. The pH for all the soil filtrates was then checked using a calibrated pH meter (Bahuguna *et al*., 2011).

3.4.4 Determination of Total Organic Carbon

The soil samples were sieved through the 1 mm sieve and 1 g of the sieved soil sample was put in a 100 mL flask. Ten (10) mL potassium dichromate and 20 mL sulphuric acid was then added to the sample in the flask and shaken very well. The mixture was allowed to cool on asbestos sheet and the volume was made up of 100 mL with distilled water and kept overnight. The optical density was then measured at 660 nm wavelength using a spectrophotometer (Ayandele, 2018).

% Organic Carbon = Optical Density × Factor F

3.4.5 Determination of Total Organic Nitrogen

The nitrogen content of the oil contaminated soil was determined by Macro-kjeldahl method (Oyeleke and Manga, 2008). Percentage Nitrogen content in the soil was then calculated using the formula:

$$\%\ \text{Nitrogen} = \frac{N \text{ x } 0.014 \text{ x } Vd \text{ x } l0 \text{ x } 100}{A \text{ x Wt of sample}}$$

Where:

N = Normality of the acid

Vd = Volume of digest

A = Aliquot of digest

3.4.6 Determination of Available Phosphorus

This was determined using Vanado-molybdo- phosphoric acid colometric method by using ammonium molybdate which forms molybdo- phoshoric acid under acidic condition. The intensity of the yellow colour was measured using spectrophotometer at 490 nm (Rabah and Ibrahim, 2010).

3.4.7 Determination of Exchangeable Cations

This was determined by weighing 5 g of the soil and placed in a 100 mL polyethylene bottle. Twenty-five (25) mL of ammonium acetate solution was then added and the mixture shaken for 1 hour. The supernatant was filtered directly into a 100 mL volumetric flask through a filter paper held in a funnel inserted in the neck of the flask, leaving the soil in the bottle. Twenty (20) mL of 95 % ethanol was added to the bottle and shaken. This was allowed to settle and the supernatant filtered into the same 100 mL flask as before. The washing, shaking and filtering procedure was repeated, each time letting the soil remain in the bottle. The extract to 100 mL was made-up with distilled water and the concentrations of exchangeable cations (Ca^{2+}, Mg^{2+}, Na^{+}, Mn^{2+} and K^{+}) was determined by Atomic Absorption Spectrophotometer (AAS) (Model: BUCK Scientific ACCUSYS 211) (Radojevic and Bashkin, 1999; Mbachu *et al.*, 2017).

3.4.8 Determination of Electrical Conductivity

This was determined by measuring 10 g of the soil sample and dissolved in 100 mL of distilled water. The conductivity cell was rinsed with at least three (3) portions of the sample and then immersed in sufficient volume of the sample. The conductivity meter was then turned on and the conductivity of the sample was recorded (Aligwekwe, 2018).

3.4.9 Determination of Total Petroleum Hydrocarbons (Gravimetric Method)

The total petroleum hydrocarbon of the soil sample was determined spectrophotometrically based on the method of Darvishi *et al*. (2011). Each sample was mixed with an equal volume of hexane, shaken vigorously for 2 minutes on vortex, and then centrifuged at 10 x g at 4 ^{0}C for 15 minutes. The hexane phases were collected, and the total petroleum hydrocarbon contents determined by measuring the absorbance at 272 nm using spectrophotometer. The wavelength was chosen based on previous investigation performed using a mixture of used engine oil/hexane, which showed the highest absorbance at 272 nm. Accordingly, the relationship between the used engine oil (UEO) concentration and the absorbance was determined as follows:

$$Y = 1.0641X$$

Where:

Y = measured absorbance of sample (AB_{272nm})

X = concentration of UEO in the sample.

Degradation percentage was determined as the difference between the initial and final total petroleum hydrocarbon concentrations as follows:

Degradation (%) = $[(O_i - O_r)/O_i]$ x 100%

Where:

O_i = initial UEO concentration (mg/L)

O_r = residual concentration.

3.4.10 Determination of Heavy Metal Concentration

Heavy metals (Cd, Pb, Cu, Zn, Cr, and Fe) were estimated by weighing 0.5 g of dried soil and digested with conc. HNO_3, H_2SO_4 and H_2O_2 in the ratio 2:6:6 as prescribed by Nirmal Kumar *et al*. (2008). The blanks were run in a set, and the heavy metals present in the samples determined by using Atomic Absorption Spectrophotometer (AAS) (Model: BUCK Scientific ACCUSYS 211).

3.5 Microbiological Studies of the Soil Samples

The following microbiological studies were carried out:

3.5.1 Determination of Hydrocarbon-utilizing Bacterial Counts

Hydrocarbon-utilizing bacteria in the soil samples were enumerated using oil agar (1.8 g K_2HPO_4, 4.0 g NH_4Cl, 0.2 g $MgSO_4.7H_2O$, 1.2 g KH_2PO_4, 0.01 g $FeSO_4.7H_2O$, 0.1 g NaCl, 20 g agar and 1 mL of spent engine oil in 1000 mL distilled water). The oil agar plates were incubated at 30 ^{0}C for 7 days before visible growth was observed and the colonies were then counted. The bacterial colonies were randomly picked, and pure cultures were obtained by repeated sub-culturing on the oil agar (to ensure not to pick contaminants). This was subsequently sub-cultured on nutrient agar after which the isolates were then characterized using microscopic techniques (Gram staining), biochemical tests and molecular identification (Agamuthu *et al*., 2013).

3.5.2 Determination of Total Heterotrophic Bacterial Counts

This was determined by the pour plate method. Serial dilution was carried out on the soil sample collected from each site and 1 mL of the aliquot from each of the dilution was inoculated onto nutrient agar plates in duplicates. The plates were then incubated at 37 ^{0}C for 24 hours after which the total heterotrophic bacteria counts were determined (Ayandele, 2018).

3.6 Purification and Preservation of the Bacterial Isolates

Pure cultures of the bacteria isolates were isolated by using a sterile wire-loop to pick a representative colony from the parent plate. Sub-culturing was done on a solidified sterile nutrient agar plate by streaking under aseptic condition. The plates were then incubated upside-down at 37 ^{0}C for 24 hours. The incubated plates were examined for pure isolates which were thereafter transferred into separate sterile nutrient agar slant, incubated at 37 ^{0}C for 24 hours, labelled and stored in the refrigerator at 4 ^{0}C as stock cultures.

3.7 Characterization and Identification of the Bacterial Isolates

The bacteria isolates obtained were characterized and identified by their Colonial Morphology (macroscopically), Cellular Morphology (microscopically), Biochemical Test as well as by Molecular Characterization. The cellular morphology as well as biochemical test were carried out as described by Fawole and Ọsọ (2007).

3.7.1 Colonial Morphology

The colonial morphology used for the characterization and identification of the bacterial isolates include colony colour, colony shape, pigmentation of the colonies, optical characteristics of the colonies as well as the colonial edges which was determined macroscopically on the plates after incubation at 37 ^{0}C for 24 hours.

3.7.2 Cellular Morphology

The cellular characteristics of each bacterial isolate was observed under a compound microscope and in turn used for identification of the isolates. The cellular characteristics include:

3.7.2.1 Gram Staining

Gram staining was used to differentiate the bacteria isolates into either Gram positive or Gram negative. This was done by preparing thin smear of the bacteria isolates from the pure culture on clean, grease free-slides. The smears were air-dried and then heat fixed by passing the slide over flame a few times. The prepared smears were then flooded with crystal violet for 60 seconds, drained, flooded with Gram's iodine for 60 seconds and rinsed with distilled water. The smears were then decolourized using 95 % alcohol for 5 seconds and immediately rinsed with distilled water. The smears were thereafter counter-stained with safranin for 30 seconds, rinsed with distilled water and allowed to air-dry. The slide were then examined under oil immersion (X100) objective. Gram positive cells stained purple to blue while Gram negative cells stained pink to red. The cell arrangement as well as the cell shape was also observed in the course of viewing under the microscope (Fawole and Ọsọ, 2007).

3.7.2.2 Spore Staining

Thin smear of each isolate was prepared on clean, grease-free slides; air-dried and heat-fixed. The slides of the isolates were then arranged on staining racks over a pot of steaming water to stimulate spore production. On steaming, the smears were flooded continuously with malachite green for 10 minutes. The stain was then washed off with water and the smears further counter-stained with safranin for 20 seconds; which was in turn washed-off with distilled water. The slides were thereafter viewed under oil immersion objectives. Presence of spores was determined by the presence of green coloured spores inside pink coloured cells (Fawole and Ọsọ, 2007).

3.7.3 Biochemical Test

Biochemical test was carried out for each isolate to identify them. The tests include:

3.7.3.1 Catalase Test

A loop-full of each isolate was emulsified on different slides and a drop of 3 % hydrogen peroxide was added to it. Effervescence, caused by the liberation of oxygen as gas bubbles indicated the production of catalase, thus such organism was catalase positive, otherwise catalase negative (Fawole and Ọsọ, 2007).

3.7.3.2 Citrate Utilization Test

The test was based on the ability of an organism to utilize citrate as its only source of carbon. Simmon citrate agar was prepared in McCartney bottles and autoclaved at 121 ^{0}C for 15 minutes following manufacturer's instruction. Using a sterile wire-loop, each of the bacterial isolate was picked and inoculated into the slanted citrate agar. This was then incubated at 37 ^{0}C for 24 hours. The utilization of citrate was indicated by a colour change from dark green to blue (Fawole and Ọsọ, 2007).

3.7.3.3 Oxidase Test

This test was used for the identification of organisms that produces the enzyme oxidase. For each bacterial isolate, a colony from pure culture slant was rubbed on filter paper after which a drop of 1 % tetramethyl–p–phenylenediamine dihydrochloride solution was placed on the colony. Presence of the enzyme oxidase was determined by the formation of purple colour within 1 - 30 seconds of application (Fawole and Ọsọ, 2007).

3.7.3.4 Methyl Red and Voges Proskauer (MR-VP) Test

Methyl red (MR) test was used to determine whether or not glucose produced can be converted to acidic products like lactate, acetate and formate due to increase in pH while Vogas Proskauer (VP) test was used to identify those bacteria that ferments glucose leading to the formation of 2,3-butanediol accumulation in the medium. Methyl red and Voges Proskauer (MR-VP) broth was prepared according to the manufacturer's specification and dispensed into different McCartney bottles and autoclaved. Each bacterial isolate was then inoculated aseptically into the two (2) bottles. After incubation for 72 hours at 37 ^{0}C, reagents for methyl red test and Voges Proskauer test was added respectively to the two (2) bottles for each of the organisms.

For MR test, five (5) drops of methyl red indicator was added into the incubated isolates. Colour change from yellow to red indicated a positive result while no change in colouration indicated a negative result. For VP test; 1mL of 5 % α-naphthol, followed by 1 mL of 40 % NaOH containing 0.3 g of creatinine was added respectively. Development of red colour in the culture medium 1 hour following the addition of the reagent represented a positive VP test; while the appearance of dirty brown was indicative of a negative VP result (Fawole and Ọsọ, 2007).

3.7.3.5 Indole Test

This test was used to detect those bacteria that could breakdown the amino acid tryptophan to release indole, pyruvic acid and ammonia catalysed by tryptophanase. Tryptone soy broth was prepared in McCartney bottles and autoclaved according to the manufacturer's specification. The broth medium was then inoculated with the isolates at 37 ^{0}C for 48 hours. After incubation, 2 mL of chloroform and 2 mL Kovac's reagent was added respectively to each McCartney bottle. After 20 minutes, those bacteria that produces a red ring in the tube were considered indole positive while absence of red ring in the broth was indicative of a negative result for indole production (Fawole and Ọsọ, 2007).

3.7.3.6 Growth in 6.5% NaCl Broth

To 100 mL of nutrient broth, 6.5 g of NaCl was added to make 6.5 % NaCl-nutrient broth complex. Bacterial isolates were then inoculated into each of the broth and incubated at 37 ^{0}C for 24 hours. Ability of each isolate to tolerate the NaCl was determined by turbidity of the broth after incubation (Fawole and Ọsọ, 2007).

3.7.3.7 Growth in 7.5% NaCl Broth

To 100 mL of nutrient broth, 7.5 g of NaCl was added to make 7.5 % NaCl-nutrient broth complex. Bacterial isolates were then inoculated into each of the broth and incubated at 37 ^{0}C for 24 hours. Ability of each isolate to tolerate the NaCl was determined by turbidity of the broth after incubation (Fawole and Ọsọ, 2007).

3.7.3.8 Sugar Fermentation Test

This test was used to determine the ability of bacterial isolates to ferment certain carbohydrates and produce acidic products and gas. The sugars tested for included lactose, glucose, maltose and mannitol. To every 100 mL of nutrient broth in different conical flasks for each sugar, 0.5 g of each sugar was added respectively as well as phenol red indicator, and then the broths boiled. Ten (10) mL of the mixture was then dispensed into different McCartney bottles and Durham tubes inverted into the bottles, and the broths then autoclaved. Thereafter, bacterial isolates were inoculated into the different broth for all the sugars which was then incubated at 37 ^{0}C for 48 hours. Colour change from red to yellow after incubation was indicative of positive result; implying the sugar was fermented. Displacement of broth in Durham tubes indicated gas production (Fawole and Ọsọ, 2007).

3.7.3.9 Triple Sugar Iron Agar Test

This test is generally used for the identification of enteric bacteria and also used to distinguish the *Enterobacteriaceae* from other gram-negative intestinal bacilli by their ability to catabolize glucose, lactose, or sucrose, and to liberate sulphides from ferrous ammonium sulphate or sodium thiosulfate. For the test, triple sugar iron agar was prepared according to the manufacturer's specification and dispensed into test tubes, autoclaved and slanted slightly to form a slope after autoclaving. The agar was then inoculated by streaking the surface of each slant with each isolate, then stabbed deep down into butt. Tubes were thereafter incubated and observed for 24 hours after incubation in order to detect the presence of sugar fermentation, gas production, as well as H_2S production. Colour change from red to yellow indicated acid production, red indicated alkaline reaction and black colour indicated the production of H_2S while pushing up or splitting of the agar from the butt indicated gas production (Fawole and Ọsọ, 2007).

3.7.3.10 Coagulase test

This test was done using the slide coagulase test method to differentiate Gram positive cocci. A loop-full of bacterial culture was placed on a clean, grease-free slide. Thereafter, a loop-full of plasma was placed on the culture and stirred to emulsify the mixture. The suspension was then observed for clumping which indicates a positive result otherwise, it was regarded as negative (Fawole and Ọsọ, 2007).

3.7.4 Molecular Characterization

3.7.4.1 Genomic DNA Extraction

Fifty (50) mg (wet weight) of bacterial cells that has been re-suspended was taken and added in up to 200 mg of tissue in a ZR Bashing^{TM} Lysis Tube. Into the tube, 750 µl lysis solution was also added. A bead fitted with 2 mL tube holder assembly was secured and processed at maximum speed for ≥ 5 minutes. The ZR Bashing Bead^{TM} lysis tube in a micro-centrifuge was centrifuged at > 10,000 x g for 1 minute. Thereafter, 400 µl supernatant was transferred to a Zymo-Spin^{TM} IV spin filter (orange top) in a collection tube and centrifuged at 7,000 x g for 1 minute. The base of the Zymo-Spin^{TM} spin filter was snapped off prior to use.

To the above, 1,200 µl of bacterial DNA binding buffer was added to the filtrate in the collection tube. Thereafter, 800 µl of the mixture in the collection tube was transferred to Zymo-Spin^{TM} IIC column and centrifuged at 10,000 x g for 1 minute. The flow through was discarded from the collection tube and 800 µl of the mixture in the collection tube was transferred to Zymo-Spin^{TM} IIC column and centrifuged at 10,000 x g for 1 minute again. To it, 200 µl DNA pre-washed buffer was added to the Zymo-Spin^{TM} IIC column in new collection tube and centrifuged at 10,000 x g for 1 minute. In addition, 500 µl of bacterial DNA wash buffer was added to the Zymo-Spin^{TM} IIC column and centrifuged at 10,000 x g for 1 minute. The Zymo-Spin^{TM} IIC column was then transferred to a clean 1.5 mL micro-centrifuge tube and 100 µl (35 µl minimum) DNA elution buffer was added directly to the column matrix. This was then centrifuged at 10,000 x g for 30 seconds to elute the DNA (IITA, 2019).

3.7.4.2 PCR Amplification of 16S rDNA Gene

Aliquot of the genomic DNA extracted from each isolate was used as a template to amplify 16S rDNA gene. The gene was amplified using two (2) primers: 27F: AGAGTTTGATCMTGGCTCAG and 1525R: AAGGAGGTGWTCCARCCGCA. The PCR cycling parameters used was as follows: Initial denaturation was at 94 ^{0}C for 5 minutes, followed by 36 cycles of denaturation at 94 ^{0}C for 30 seconds, annealing at 56 ^{0}C for 30 seconds and elongation at 72 ^{0}C for 45 seconds. This was followed by a final elongation step at 72 ^{0}C for 7 minutes and holding temperature at 10 ^{0}C. Amplified fragments were visualized on safe view-stained 1.5 % agarose electrophoresis gels. The size of the amplicon was about 1500 bp and the DNA ladder used was Hyperladder from Bioline.

Sequencing was achieved with the machine 3130XL genetic analyzer from Applied Biosystems, while the PCR thermal cycler used was GeneAmp PCR system 9700 (IITA, 2019).

3.7.4.3 Phylogenetic Analysis of 16S rDNA Sequences

The obtained 16S rDNA sequences was compared with already known 16S rDNA sequences at National Centre for Biotechnology Information (NCBI) database using Basic Algorithm Search Tool (BLAST) algorithm (Mwaura, 2017).

3.8 Bioremediation of Spent Engine oil in Soil Microcosms

This was carried out by preparing 250 mL of stock each for the isolated organisms using Mineral Salt Medium (MSM) broth. The broth medium was prepared using the following composition: 10 g/l of NaCl, 0.42 g/l of $MgSO_4.7H_2O$, 1.25 g/l of K_2HPO_4, 0.29 g/l of KCl, 0.83 g/l of KH_2PO_4, 0.42 g/l of $NaNO_3$ and 1 % hydrocarbon source (engine oil). The mixture in the conical flask was then plugged with cotton wool wrapped with aluminum foil and sealed properly with paper tape. This was then boiled to ensure proper homogenization after which it was autoclaved (sterilized) at 121 ^{0}C at 15 psi for 15 minutes (Fagade *et al.,* 2009).

After sterilization, each of the hydrocarbon-utilizing bacteria kept as stock in the refrigerator was inoculated into the individual broth and incubated at 37 ^{0}C for 8 hours. On incubation, the broths were introduced collectively into the combined contaminated soil samples collected from the five (5) mechanic workshop and mixed thoroughly. A control sample was set aside containing only the contaminated soils. The soil samples were then monitored for parameters which included pH, total bacterial count, percentage moisture content and percentage organic matter for 15 days at 3 days interval.

3.8.1 Enhancing Treatment through the Addition of Organic Waste – Biochar

3.8.1.1 Biochar Production

Cow dung was collected from the Kwara State University (KWASU), Malete premises. The biochar was produced using a furnace at the University of Ilorin, Microbiology Laboratory at 600 ^{0}C for 3 hours pyrolysis temperature based on the recommendation of Lehmann *et al*. (2003).

3.8.1.2 Biochar pH

A biochar to water ratio of 1:5 was prepared (appropriate for organic materials because of their ability to absorb more water) and pH determined using a standardized pH meter calibrated at pH 4, 7 and 9 (Hammond, 2014).

3.8.1.3 Calcium (Ca), Magnesium (Mg), Sodium (Na) and Potassium (K) Content of Biochar

A 0.5 g of the biochar sample was put into a crucible and then placed into a furnace for at least 1 hour at a temperature of 600 ^{0}C. The ash was later digested with 10 mL of nitric acid, accompanied by heating to speed up the rate of the reaction. This was then transferred into a 100 mL flask and topped up with distilled water to the 100mL mark. Calcium (Ca), Magnesium (Mg), Sodium (Na) and Potassium (K) Content of Biochar was determined on the Atomic Absorption Spectrophotometer (AAS) (Model: BUCK Scientific ACCUSYS 211) (Hammond, 2014).

3.8.1.4 Amendment of Soil with Biochar

The contaminated soil was amended with cow dung biochar at 0 (control) and 5 g/pot, and then replicated. The set up was left to stand under a shade at ambient temperature and samples were taken at an interval of 3 days for 15 days for the determination of total bacterial count, pH, percentage organic matter content as well as percentage moisture content. It was ensured that both the bioremediated and control samples was daily moistened in other to avoid the soil from drying-up.

3.8.2 Monitoring of Parameters

The following parameters was monitored at 3 days interval during the experimental period:

3.8.2.1 pH

pH changes in the amended soil containing biochar as well as the control soil was monitored over the course of the bioremediation period. This was done by taking known gram of the soil samples and distilled water introduced into them. The mixtures were then shaken vigorously to obtain homogenized solution. The pH electrode was standardized using buffer solutions of pH 4, 7 and 9 after which pH readings were taken on the pH meter scale by dipping the glass electrode into the soil solution (Ekundayo and Osunla, 2013).

3.8.2.2 Total Bacterial Count

The total bacterial count was determined during the course of the bioremediation study by quantitatively taking 1 g of each of the control and bioremediated soil samples and then making serial dilutions up to 10^{-5}. Dilutions 10^{-4} and 10^{-5} of each of the sample was then introduced into sterile Petri dishes for each of the samples. Sterile molten nutrient agar was then poured aseptically into each Petri dish and the plates swirled gently to mix the inoculum and agar properly. After the agar had solidified, the plates were incubated upside down at 37 ^{0}C for 24 hours. The numbers of colonies on each plate was then counted with the aid of colony counter and expressed as CFU/mL (Okerentugba and Ezeronye, 2003).

3.8.2.3 Percentage Moisture Content

The percentage moisture content of both the bioremediated and control soil sample was determined by making use of a machine referred to as Moisture Analyzer (RADWAG PCM 50/1 402496). Each of the soil sample was weighed into a pre-weighed aluminum dry-dish used for the analyzer. On completion, the percentage moisture content of the soil sample was displayed on the screen of the machine and the result recorded (AOAC, 2005).

3.8.2.4 Percentage Organic Matter

The percentage organic matter content of both the bioremediated and control soil sample was determined thus: The mass of an empty, clean and dry porcelain dish was determined and recorded. Thereafter, the entire oven-dried test specimen from the moisture content experiment was placed in the porcelain dish, and the mass of the dish and soil specimen was determined and recorded. The dish was then placed in a muffle furnace and the temperature in the furnace gradually increased to 440 ^{0}C and left overnight. The porcelain dish was then carefully removed using tongs and allowed to cool at room temperature after which the mass of the dish containing the ash was determined and recorded. The dish was then emptied and cleaned (Suryakanta, 2015).

The percentage organic matter content was determined using the formula:

$$\% \text{ Organic matter content} = \frac{\text{Mass of Organic matter}}{\text{Mass of dry soil}} \text{ X } 100$$

3.9 Statistical Analysis

Each set of data in the experiment was collected in replicates and the analytical results were taken as the mean of the replicated measurements. The standard deviations (error bars) and statistical significance (5% level of significance) was analyzed with IBM SPSS Statistic 20 using (analysis of variance) ANOVA (One-way ANOVA).

CHAPTER FOUR

RESULTS

Details of analysis carried out during the course of the research include physicochemical parameters of the soil samples before bioremediation; colonial and cellular morphology as well as biochemical characteristics of total heterotrophic bacteria; colonial and cellular morphology, biochemical characteristics as well as molecular identification of hydrocarbon-utilizing bacteria; pH, calcium (Ca), magnesium (Mg), sodium (Na), potassium (K) and percentage ash content of Biochar; total bacterial count, pH, percentage organic matter content as well as percentage moisture content during bioremediation and lastly physicochemical parameters of the soil samples after bioremediation. The results are documented as follows:

4.1 Physicochemical Parameters of the Soil Samples before Bioremediation

The physicochemical parameters studied in the soil samples collected from the five (5) mechanic workshop included particle size (percentage sand, silt and clay), bulk density, soil pH, total organic carbon, NO_3^{2-}, PO_3^{2-}, exchangeable cations (Ca^{2+}, Mn^{2+}, Mg^{2+}, Na^{2+}, K^{+}), water holding capacity, electrical conductivity, heavy metals (Cd, Pb, Cu, Zn, Cr, Fe), redox potential, percentage organic matter and total petroleum hydrocarbon. The results for the physicochemical parameters are as presented below:

From the result, the particle size (%) has values ranging between 91.96 ± 0.00 (sampling sites A, B and C) and 91.99 ± 0.00 (sampling site E) for sand while for silt, it ranged between 5.00 ± 0.00 (sampling site E) and 5.04 ± 0.00 (sampling sites A, B and C). The values for bulk density (g/cm^3) ranged between 1.03 ± 0.00 (sampling site E) and 1.68 ± 0.00 (sampling site B). For the soil pH, it ranged between 5.95 ± 0.07 (sampling site B) and 6.25 ± 0.07 (sampling site D). Total organic carbon ranged between 8.18 ± 0.31 (sampling site D) and 17.54 ± 1.87 (sampling site E). NO_3^{2-} (mg/kg) has values ranging between 17.85 ± 0.07 (sampling site A) and 27.80 ± 0.00 (sampling site C) while that of PO_3^{2-} (mg/kg) ranged between 23.70 ± 0.28 (sampling site D) and 39.05 ± 0.07 (sampling site C).

For the exchangeable cations (mg/kg), the values for Ca^{2+} ranged between 4.51 ± 0.00 (sampling site B) and 13.94 ± 0.72 (sampling site C), Mn^{2+} between 4.02 ± 0.16 (sampling site E) and 10.05 ± 0.00, Mg^{2+} between 5.95 ± 0.00 (sampling site B) and 6.92 ± 0.07 (sampling site C), Na^{2+} between 6.66 ± 0.00 (sampling site E) and 14.25 ± 0.00 (sampling site A) and K^{+} between 2.51 ± 0.07 (sampling sites B and E) and 3.59 ± 0.14 (sampling site D).The electrical conductivity (ms/cm) of the soil samples ranged between 0.19 ± 0.01 (sampling site B) and 0.58 ± 0.00 (sampling site A).

For the heavy metals (mg/kg) detected in the soil samples, the Cd content ranged between 0.01 ± 0.00 (sampling sites B and D) and 0.02 ± 0.00 (sampling sites A, C and E), Pb had values of 0.56 ± 0.07 (sampling site D) and 0.97 ± 0.22 (sampling site B), Cu ranged between 1.27 ± 0.04 (sampling site C) and 11.74 ± 0.07 (sampling site A), Zn between 2.23 ± 0.00 (sampling site B) and 2.67 ± 0.00 (sampling site C) and Fe between 12.04 ± 0.07 (sampling site E) and 12.86 ± 0.07 (sampling site C). The organic matter (%) mean and standard deviation ranged between 18.35 ± 2.90 (sampling site D) and 43.45 ± 9.12 (sampling site E). The results are presented in Table 1.

Table 1: Physicochemical Parameters of the Soil Samples before Bioremediation

Soil Parameters (Units)	Sampling Sites / Values of Physicochemical Parameters				
	A	**B**	**C**	**D**	**E**
Particle Size:					
Silt (%)	5.04 ± 0.00^a	5.04 ± 0.00^a	5.04 ± 0.00^a	5.02 ± 0.00^a	5.00 ± 0.00^a
Clay (%)	ND	ND	ND	ND	ND
Sand (%)	91.96 ± 0.00^a	91.96 ± 0.00^a	91.96 ± 0.00^a	91.98 ± 0.00^a	91.99 ± 0.00^a
Bulk Density (g/cm^3)	1.38 ± 0.00^d	1.68 ± 0.00^e	1.20 ± 0.00^c	1.14 ± 0.00^b	1.03 ± 0.00^a
Soil pH	6.20 ± 0.14^a	5.95 ± 0.07^a	6.15 ± 0.21^a	6.25 ± 0.07^a	6.00 ± 0.14^a
Total Organic Carbon (%)	14.65 ± 3.20^b	11.94 ± 0.74^a	16.87 ± 0.07^b	8.18 ± 0.31^a	17.54 ± 1.87^b
NO_3^{2-} (mg/kg)	17.85 ± 0.07^a	25.75 ± 0.07^c	27.80 ± 0.00^d	27.70 ± 0.00^d	24.90 ± 0.14^b
PO_3^{2-} (mg/kg)	23.80 ± 0.14^a	26.30 ± 0.00^c	39.05 ± 0.07^d	23.70 ± 0.28^a	24.60 ± 0.14^b
Exchangeable Cations:					
Ca^{2+} (mg/kg)	8.15 ± 0.94^b	4.51 ± 0.00^a	13.94 ± 0.72^c	10.20 ± 0.22^b	10.25 ± 0.58^b
Mn^{2+} (mg/kg)	8.25 ± 0.07^d	10.05 ± 0.00^e	7.44 ± 0.00^c	4.90 ± 0.01^b	4.02 ± 0.16^a
Mg^{2+} (mg/kg)	6.82 ± 0.07^c	5.95 ± 0.00^a	6.92 ± 0.07^c	6.87 ± 0.00^c	6.66 ± 0.00^b
Na^{2+} (mg/kg)	14.25 ± 0.00^d	8.41 ± 0.00^b	14.15 ± 0.00^d	9.53 ± 0.14^c	6.66 ± 0.43^a
K^+ (mg/kg)	3.49 ± 0.00^c	2.51 ± 0.07^a	3.08 ± 0.00^b	3.59 ± 0.14^c	2.51 ± 0.07^a

Key: Site A = Galadima 1 Site B = Galadima 2 Site C = Oko-Erin 1 Site D = Oko-Erin 2 Site E = Oko-Erin 3 ND = Not Detected

Values are means of duplicate reading and standard deviation of soil parameters of the soil samples from the five (5) sampling sites. Values in the same row with different superscript are significantly different at < 0.05 level of significance.

Table 1: Physicochemical Parameters of the Soil Samples before Bioremediation (Cont.)

Soil Parameters (Units)	**Sampling Sites / Values of Physicochemical Parameters**				
	Site A	**Site B**	**Site C**	**Site D**	**Site E**
Electrical Conductivity(ms/cm)	0.58 ± 0.00^{e}	0.19 ± 0.01^{a}	0.33 ± 0.00^{b}	0.40 ± 0.01^{c}	0.42 ± 0.01^{d}
Heavy Metals:					
Cd (mg/kg)	0.02 ± 0.00^{a}	0.01 ± 0.00^{a}	0.02 ± 0.00^{a}	0.01 ± 0.00^{a}	0.02 ± 0.00^{a}
Pb (mg/kg)	0.87 ± 0.07^{a}	0.97 ± 0.22^{a}	0.87 ± 0.07^{a}	0.56 ± 0.07^{a}	0.62 ± 0.00^{a}
Cu (mg/kg)	11.74 ± 0.07^{d}	1.69 ± 0.07^{c}	1.27 ± 0.04^{b}	1.88 ± 0.13^{c}	0.80 ± 0.01^{a}
Zn (mg/kg)	2.45 ± 0.00^{b}	2.23 ± 0.00^{a}	2.67 ± 0.00^{d}	2.49 ± 0.02^{c}	2.49 ± 0.00^{c}
Cr (mg/kg)	ND	ND	ND	ND	ND
Fe (mg/kg)	12.20 ± 0.00^{a}	12.81 ± 0.00^{c}	12.86 ± 0.07^{c}	12.56 ± 0.07^{b}	12.04 ± 0.07^{a}
Organic Matter (%)	38.30 ± 2.40^{b}	34.60 ± 1.27^{b}	33.50 ± 0.85^{b}	18.35 ± 2.90^{a}	43.45 ± 9.12^{b}

Key: Site A = Galadima 1 Site B = Galadima 2 Site C = Oko-Erin 1 Site D = Oko-Erin 2 Site E = Oko-Erin 3 ND = Not Detected

Values are means of duplicate reading and standard deviation of soil parameters of the soil samples from the five (5) sampling sites. Values in the same row with different superscript are significantly different at < 0.05 level of significance.

4.2 Total Heterotrophic Bacteria and Hydrocarbon-utilizing Bacteria Counts of the Soil Samples

Discrete colonies were counted where possible and expressed as the Log_{10} of colony forming unit per mL (CFU/mL) for both the total heterotrophic bacteria and hydrocarbon-utilizing bacteria. The total heterotrophic bacteria count of the soil samples ranged between the values of $2.50 \pm 0.71 \times 10^5$ CFU/mL (sampling site B) and $67.50 \pm 2.12 \times 10^5$ CFU/mL (sampling E). For the hydrocarbon-utilizing bacteria, it ranged between the values of $3.00 \pm 2.83 \times 10^5$ CFU/mL (sampling site B and D) and $18.50 \pm 0.71 \times 10^5$ CFU/mL (sampling site C). The result for the total heterotrophic bacteria and hydrocarbon-utilizing bacteria counts is presented in Table 2.

4.3 Distribution and Occurrence of the Bacteria in the Soil Samples

Eight (8) total heterotrophic bacteria and six (6) hydrocarbon-utilizing bacteria species were isolated from the soil samples collected from the five (5) mechanic workshops. The total heterotrophic bacterial isolates had majority of the isolates with 20 % frequency of occurrence (T_1, T_2, T_3, T_4, T_5, T_7 and T_8) with only T_6 having 40 %. The result is presented in Table 3a. For the hydrocarbon-utilizing bacterial isolates, H_1, H_2, H_3 and H_5 had 40 % frequency of occurrence while H_4 and H_6 had 60 %. The result is presented in Table 3b.

Table 2: Total Heterotrophic Bacteria and Hydrocarbon-utilizing Bacteria Counts of the Soil Samples

Sampling Sites	Total Heterotrophic Bacteria (10^5 CFU/mL)	Hydrocarbon-utilizing Bacteria (10^5 CFU/mL)
A	16.50 ± 0.71^{ab}	13.50 ± 7.78^{a}
B	2.50 ± 0.71^{a}	3.00 ± 2.83^{a}
C	23.50 ± 12.02^{ab}	18.50 ± 0.71^{a}
D	33.00 ± 4.24^{b}	3.00 ± 2.83^{a}
E	67.50 ± 2.12^{c}	8.00 ± 1.41^{a}

Key: Site A = Galadima 1 Site B = Galadima 2 Site C = Oko-Erin 1 Site D = Oko-Erin 2 Site E = Oko-Erin 3

Values are means of duplicate reading and standard deviation of bacteria counts from the soil samples collected from five (5) mechanic workshops. Values in the same column with different superscript are significantly different at < 0.05 level of significance.

Table 3a: Percentage Frequency of the Total Heterotrophic Bacteria

Bacterial Isolates	Sampling Site					Percentage Frequency (%)
	A	B	C	D	E	
T_1	+	-	-	-	-	20
T_2	-	+	-	-	-	20
T_3	-	-	+	-	-	20
T_4	-	-	+	-	-	20
T_5	-	-	-	+	-	20
T_6	+	-	-	+	-	40
T_7	-	-	-	+	-	20
T_8	-	-	-	-	+	20

Key: Site A = Galadima 1 Site B = Galadima 2 Site C = Oko-Erin 1 Site D = Oko-Erin 2 Site E = Oko-Erin 3 ND = Not Detected + = Present - = Absent

T_1 to T_8 represent the total heterotrophic bacterial isolates

Table 3b: Percentage Frequency of the Hydrocarbon-Utilizing Bacteria

Bacterial Isolates	Sampling Site					Percentage Frequency (%)
	A	B	C	D	E	
H_1	+	-	-	+	-	40
H_2	-	+	-	-	+	40
H_3	+	-	+	-	-	40
H_4	+	+	-	+	-	60
H_5	+	-	-	+	-	40
H_6	+	-	-	+	+	60

Key: Site A = Galadima 1 Site B = Galadima 2 Site C = Oko-Erin 1 Site D = Oko-Erin 2 Site E = Oko-Erin 3 ND = Not Detected + = Present - = Absent

H_1 to H_6 represent the hydrocarbon-utilizing bacterial isolates

4.4 Cellular Morphology and Biochemical Characteristics of the Bacterial Isolates

The total heterotrophic bacteria were gram negative (T_1, T_2, T_3, T_4, T_5, T_7 and T_8) with the exception of T_6 that was gram positive. The cell shape was mostly rod (T_1, T_2, T_4, T_5, T_6, T7 and T_8) with only T_3 being cocci-rod. The result is presented in Table 4a.

For the hydrocarbon-utilizing bacteria, isolates (H_1, H_2, H_3, H_4, H_5 and H_6) were all gram negative and non-spore formers. The result is presented in Table 4b.

4.5 Molecular Identification of Hydrocarbon-utilizing Bacteria

Molecular identification of the six (6) hydrocarbon-utilizing bacterial isolates isolated from the soil samples from the five (5) mechanic workshops under study gave the isolates as *Thalassospira mesophila* strain JCM 18969; *Pseudomonas rhizosphaerae* strain DSM 16299 and *Pseudomonas fluorescens* F113; *Siccibacter turicensis* LMG 23730; *Pseudomonas Zeshuii* strain KACC 15471; *Pseudomonas stutzeri* strain CGMCC 1.1803; *Marinobacter lutaoensis* strain T5054 DSM17466 and *Marinobacter hydrocarbonoclasticus* strain ATCC 49840 based on their percentage identity with the organisms in the GenBank.

Table 4a: Cellular Morphology and Biochemical Characteristics of the Total Heterotrophic Bacteria

Bacterial Isolates	Cellular Characteristics		Biochemical Characteristics											Tentative Identification
	Gram's Reaction	Cell Shape	Catalase Test	Citrate Test	Oxidase Test	Indole Test	Methyl Red (MR) Test	Voges Proskauer (VP) Test	Growth in 6.5% NaCl	Growth in 7.5% NaCl	Coagulase Test	Triple Sugar Iron (TSI) Test	Sugar Fermentation Test	
												H_2S	Mannitol	
T_1	-	Rod	+	+	+	-	-	+	-	-	-	-	+	*Enterobacter* sp.
T_2	-	Rod	+	+	+	+	ND	ND	-	-	-	+	+	*Pseudomonas* sp.
T_3	-	Cocci-Rod	+	+	+	+	-	+	+	+	-	+	+	*Acinetobacter* sp.
T_4	-	Rod	+	+	+	+	-	+	+	+	-	+	+	*Pseudomonas* sp.
T_5	-	Rod	+	+	-	+	+	+	-	-	-	-	+	*Klebsiella* sp.
T_6	+	Rod	+	+	+	+	+	+	-	-	-	+	+	*Bacillus* sp.
T_7	-	Rod	+	-	+	+	-	+	+	-	-	-	-	*Aeromonas* sp.
T_8	-	Rod	+	+	-	-	+	+	+	+	-	-	+	*Acinetobacter*

Key: + = Positive **- =** Negative ND = Not Determined

T_1 to T_8 represent the total heterotrophic bacterial isolates

Table 4b: Cellular Morphology, Biochemical Characteristics and Molecular Identification of the Hydrocarbon-utilizing Bacteria

	Cellular Characteristics				Biochemical Characteristics										
												Sugar Fermentation Test			
Bacterial Isolates	Gram's Reaction	Cell Shape	Arrangement	Spore Staining	Catallase Test	Citrate Test	Oxidase Test	Indole Test	Growth in 6.5% NaCl	Growth in 7.5% NaCl	Coagulase Test	Glucose	Lactose	Maltose	**Molecular Identification**
H_1	-	Rod	Chain	-	+	+	+	-	+	-	-	+ No Gas	+ No Gas	+ No Gas	*Thalassospira mesophila* strain JCM 18969
H_2	-	Rod	Chain	-	+	-	+	-	+	-	-	+ No Gas	+ No Gas	+ No Gas	*Pseudomonas fluorescens* F113
H_3	-	Cocci	Chain	-	+	+	+	+	+	+	-	+ Gas	+ No Gas	+ No Gas	*Siccibacter turicensis* LMG 23730
H_4	-	Rod	Cluster	-	+	-	+	-	+	+	-	+ No Gas	- No Gas	+ No Gas	*Pseudomonas Zeshuii* strain KACC 15471
H_5	-	Rod	Chain	-	+	-	+	-	+	+	-	- No Gas	- No Gas	- No Gas	*Pseudomonas stutzeri* strain CGMCC 1.1803
H_6	-	Rod	Singly	-	+	-	-	-	+	-	-	- No Gas	- No Gas	- No Gas	*Marinobacter hydrocarbonoclasticus* strain ATCC 49840

Key: + = Positive **-** = Negative

H_1 to H_6 represent the hydrocarbon-utilizing bacterial isolates

4.6 pH, Calcium (Ca), Magnesium (Mg), Sodium (Na), Potassium (K) Content and Percentage Ash Content of Biochar

The pH, calcium (Ca), magnesium (Mg), sodium (Na), potassium (K) content as well as the percentage ash content of the biochar supplemented into the bioremediated soil sample had values of 7.00 ± 0.28, 7.53 ± 1.66 mg/kg, 4.09 ± 0.01 mg/kg, 3.84 ± 0.07 mg/kg, 5.07 ± 0.21 mg/kg and 77.68 ± 1.07 % respectively. The result is presented in Table 5.

4.7 Total Bacterial Count during Bioremediation

Total bacteria count during the period of bioremediation for the control soil ranged between 6.50 ± 2.12 × 10^5 CFU/mL (day 6) and 60.00 ± 9.90 × 10^5 CFU/mL (day 9) while that of the bioremediated soil ranged between 7.00 ± 2.83 × 10^5 CFU/mL (day 0) and 164.00 ± 11.31 × 10^5 CFU/mL (day 9) (Table 6).

4.8 pH, Percentage Organic Matter Content and Percentage Moisture Content during Bioremediation

The pH of the control soil has values ranging between 6.10 ± 0.00 × 10^5 CFU/mL (day 9) and 8.80 ± 0.14 × 10^5 CFU/mL (day 12) while that of the bioremediated soil ranged between 6.30 ± 0.00 × 10^5 CFU/mL (day 6) and 8.80 ± 0.00 × 10^5 CFU/mL (day 12). For the percentage organic matter, the values ranged between 23.90 ± 0.14 % (day 12) and 91.30 ± 0.14 % (day 0) for the control soil while that of the bioremediated soil ranged between 24.65 ± 0.35 % (day 12) and 73.65 ± 0.07 % (day 9). The percentage moisture content had values ranging between 2.25 ± 0.07 % (day 3) and 5.69 ± 0.04 % (day 12) for the control soil while that of the bioremediated soil ranged between 1.75 ± 0.01 % (day 6) and 6.59 ± 0.29 % (day 0). The results are presented in Table 7.

4.9 Physicochemical Parameters of the Soil Samples after Bioremediation

The soil pH, total organic carbon, NO_3^{2-}, PO_3^{2-} and organic matter content of the control and bioremediated soil had values ranging between 8.25 ± 0.07 and 8.90 ± 0.14; 13.07 ± 0.05 % and 13.25 ± 0.84 %; 26.57 ± 0.12 (mg/kg) and 26. 61 ± 0.02 (mg/kg); 13.95 ± 0.07 (mg/kg) and 15.55 ± 0.07 (mg/kg); 37.25 ± 1.06 % and 44.80 ± 1.13 % respectively. The results are presented in Table 8.

Table 5: pH, Calcium (Ca), Magnesium (Mg), Sodium (Na), Potassium (K) Content and Percentage Ash Content of Biochar

Parameters (Unit)	Value
pH	7.00 ± 0.28
Calcium (Ca) (mg/kg)	7.53 ± 1.66
Magnesium (Mg) (mg/kg)	4.09 ± 0.01
Sodium (Na) (mg/kg)	3.84 ± 0.07
Potassium (K) (mg/kg)	5.07 ± 0.21
Ash Content (%)	77.68 ± 1.07

Source: Field Analysis, 2019

Values are means of duplicate reading and standard deviation of pH, Calcium (Ca), Magnesium (Mg), Sodium (Na), Potassium (K) Content and Percentage Ash Content of Biochar to be supplemented into the bioremediated soil.

Table 6: Total Bacterial Count of Soil Samples during Bioremediation

Soil Samples	Period of Bioremediation (Days) / Total Bacterial Count (10^5 CFU/mL)					
	0	**3**	**6**	**9**	**12**	**15**
Control	13.50 ± 6.36^a	11.00 ± 5.66^a	6.50 ± 2.12^a	60.00 ± 9.90^a	37.50 ± 10.61^{ab}	36.50 ± 23.33^a
Bioremediated	7.00 ± 2.83^a	42.50 ± 10.6^b	105.50 ± 65.76^a	164.00 ± 11.31^b	95.50 ± 34.65^b	36.50 ± 31.82^a

Values are means of duplicate reading and standard deviation of bacteria counts from the soil samples for the control and bioremediated soils. Values in the same column with different superscript are significantly different at < 0.05 level of significance.

Table 7: pH, Percentage Organic Matter Content and Percentage Moisture Content of Soil Samples during Bioremediation

Day of Bioremediation	pH		% Organic Matter		% Moisture Content	
	Control	Bioremediated	Control	Bioremediated	Control	Bioremediated
0	6.25 ± 0.07^{a}	7.15 ± 0.07^{b}	91.30 ± 0.14^{b}	58.48 ± 0.01^{a}	2.59 ± 0.28^{a}	6.59 ± 0.29^{b}
3	6.50 ± 0.28^{a}	6.35 ±0.21^{a}	28.80 ± 0.85^{a}	32.56 ± 1.63^{a}	2.25 ± 0.07^{a}	3.33 ± 0.19^{b}
6	6.20 ± 0.28^{a}	6.30 ± 0.00^{a}	32.25 ± 0.07^{a}	33.35 ± 0.35^{b}	3.71 ± 0.05^{b}	1.75 ± 0.01^{a}
9	6.10 ± 0.00^{b}	5.75 ± 0.07^{a}	72.60 ± 0.85^{a}	73.65 ± 0.07^{a}	4.69 ± 0.14^{b}	3.58 ± 0.02^{a}
12	8.80 ±0.14^{a}	8.80 ± 0.00^{a}	23.90 ± 0.14^{a}	24.65 ± 0.35^{a}	5.69 ± 0.04^{b}	4.52 ± 0.44^{a}
15	8.10 ± 0.14^{b}	7.75 ± 0.07^{a}	25.25 ± 0.35^{a}	31.65 ± 1.78^{b}	2.63 ± 0.05^{a}	6.44 ± 0.29^{b}

Values are means of duplicate reading and standard deviation of physicochemical parameters determined after bioremediation. Values along the same row for each parameter at different days with different superscript are significantly different at < 0.05 level of significance.

Table 8: Physicochemical Parameters of the Soil Samples after Bioremediation

Soil Parameters (Units)	**Soil Sample**	
	Control	**Bioremediated**
Soil pH	8.90 ± 0.14^{b}	8.25 ± 0.07^{a}
Total Organic Carbon (%)	13.25 ± 0.84^{a}	13.07 ± 0.05^{a}
NO_3^{2-} (mg/kg)	26.57 ± 0.12^{a}	$26.\ 61 \pm 0.02^{a}$
PO_3^{2-} (mg/kg)	13.95 ± 0.07^{a}	15.55 ± 0.07^{b}
Heavy Metals:		
Cd (mg/kg)	ND	ND
Pb (mg/kg)	0.308 ± 0.00	ND
Cu (mg/kg)	1.066 ± 0.00	ND
Zn (mg/kg)	0.103 ± 0.00^{a}	0.103 ± 0.00^{a}
Fe (mg/kg)	9.40 ± 0.04^{b}	0.021 ± 0.00^{a}
Organic Matter	44.80 ± 1.13^{b}	37.25 ± 1.06^{a}

Values are means of duplicate reading and standard deviation of physicochemical parameters after bioremediation. Values in the same row with different superscript are significantly different at < 0.05 level of significance.

CHAPTER FIVE

DISCUSSION

Microbial growth and metabolism in impacted areas can be mitigated by a number of factors such as pH, temperature, concentration of pollutant, moisture content, conductivity, oxygen content, nutrient availability and bioavailability and the property of the impacted soil medium (Rahman *et al*., 2001). According to Frey (2007), intact soil is a continuum of mineral particles, organic matter, pore spaces and organisms. Prevailing environmental conditions are among the most important limiting factors for optimum bioremediation. Factors that affects the success and rate of microbial bioremediation are nutrient availability, moisture content, soil pH, temperature, carbon/nitrogen ration, soil texture etc. (Pathak *et al*., 2011). Physicochemical properties of oil-spill contaminated sites are essential for successful bioremediation process. These factors have direct influence on the type, number and metabolic activities of the microflora of any ecosystem (Adebusoye *et al*., 2008). In addition, physical and chemical properties of soil have been shown to have a reflective influence on aeration, nutrient availability, water retention and consequently on microbial activity (Olaniran *et al*., 2006).

Soil pH is a critical factor for microbial growth and survival. Different microbial strains exhibit maximum growth potential in a limited pH range. A pH of near neutral is suitable for growth of diverse bacterial populations (U.S EPA, 2006). The pH influences a number of factors affecting microbial activity like solubility and ionization of inorganic and soil solution constituents, which will in turn affect soil enzyme activity (Voroney, 2007). The pH values for the five (5) soil samples before undergoing bioremediation indicates that all the samples were slightly acidic which could be due to the fact that hydrocarbons contain many free cations causing them to have properties of a weak acid. A reduction in pH implies 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 including Cd, Cu, Hg, Ni, Pb and Zn (Osuji and Nwoye, 2007). pH also influences the solubility and accessibility of soil components which indirectly influence biological activity in the soil (Onojake and Osuji, 2012).

The soil pH value obtained was similar to that detected by Ilembayo and Kolade (2008) who reported a range of pH values (5.96 – 8.88) for top soil samples. Low pH values usually enhance metal distribution and transport in soils (Ilembayo and Kolade, 2008). The pH values fall within the optimal soil pH range recommended to support bacterial growth which is from 6 to 8 (U.S EPA, 2006; Haritash and Kaushik, 2009; ASTS Incorporated, 2010) as well as the optimum soil pH for efficient bioremediation which is 5.5 to 8.8 as described by Vidali (2001). Boonchan (2000) reported that optimum pH for bioremediation is between 6.0 and 8.9 and opined that changes from initial levels of pH might be because of release of acidic and alkaline intermediates and final products during hydrocarbon degradation, which has an effect on pH. Soil pH is of importance because most microbial species can survive only within a pH range. In addition, soil pH can affect availability of nutrients (Agamuthu *et al*., 2013).

Physical properties of soil like soil texture and bulk density have been considered to be very important for bioremediation because several factors affecting the degradation process like soil aeration, movement of nutrients through soil pores, water holding capacity etc. are under the direct and indirect influence of soil physical properties (Luepromchai *et al*., 2007). Increased ventilation has a direct impact on microbial growth, which can enhance the biodegradation of petroleum compounds (Pathak *et al*., 2010).

The result of the particle size analysis for the five (5) sampling sites was indicative that all the soil was sandy in nature (Table 1). The particle size distribution is known to have an influence on the bacteria diversity of soils (Faoro *et al*., 2010).

The physicochemical property of spent engine oil contaminated soil collected from the mechanic workshops revealed that some heavy metal content of the soil was high and above the National Environmental Regulation of soil quality standard. According to European Committee for Standardization (ECS), most of these heavy metal content in used engine oil contaminated soil are high above the safe limit for lead, nickel, copper, zinc and cadmium respectively. Generally, all the heavy metals (Cd, Pb, Cu, Zn, Cr and Fe) concentrations recorded for the respective soil samples has values ranging from ND to 12.81 mg/kg. This report differs from the reports of Ipeaiyeda *et al*. (2007) and Ilembayo and Kolade (2008). Ipeaiyeda *et al*. (2007) reported values ranging from 0.05 mg/kg for zinc to 184 mg/kg for lead. Ilembayo and Kolade (2008) also reported values ranging from 730.97 mg/kg for zinc to 217 mg/kg for lead. The observed low levels of heavy metals especially lead in the soil samples could be suggestive of the amounts of leaded products disposed on the top soils within the vicinities of the workshops. The trend could also be reflective of increased mobility and infiltration of the heavy metals down the soil profile because of the sandy nature of the soils. Anikwe (2006) noted that heavy metals degrade soil quality and productivity.

The ability to isolate high numbers of certain oil degrading microorganisms from oil polluted environment is commonly taken as evidence that these microorganisms are the active degraders of the constituents of that environment (Okerentugba and Ezeronye, 2003). Although, hydrocarbon degraders may be expected to be readily isolated from an oil associated environment, the same degree can be isolated from a totally related environment such as soil (Santhini *et al*., 2009). *Pseudomonas* and *Acinetobacter* are the most common bacterial hydrocarbon degraders reported in literatures (Barathi and Vasudevan, 2001, Bhattacharya *et al*., 2002; Pokethitiyook *et al*., 2003; Van Hamme *et al*., 2003). The isolation of these organisms from the environment also shows that these organisms have evolved strategies of adapting to the environment and/or utilizing these substances as energy sources (Ghazali *et al*., 2004).

Other studies by Chikere *et al*. (2009); Ekhaise and Nkwelle (2011); Lawson *et al*. (2013) reported some bacteria species belonging to the genera in hydrocarbon degradation as a sole source of carbon and energy. It is well documented that bacteria genera like *Pseudomonas* and *Bacillus* are known hydrocarbon degraders which could be cosmopolitan in nature (Margesin *et al*., 2003; Hamamura *et al*., 2006; Van Beilen and Funhoff, 2007). These bacteria genera are of considerable environmental and biotechnological importance because of their wide catabolic abilities, resilience in harsh environmental conditions and ability to produce bio-surfactant (Arenskotter *et al*., 2004; Larkin *et al*., 2005; Van Beilen and Funhoff, 2007).

The results obtained for the hydrocarbon-utilizing bacteria is in correlation with the work reported by Mandri and Lin (2007), Khan and Rizvi (2011) and Abioye *et al*. (2012) who isolated *Pseudomonas, Bacillus, Micrococcus* and other bacterial strains from engine oil contaminated soil. Ogunbayo *et al*. (2012) also isolated *Pseudomonas*, *Bacillus* and *Rhodococcus* along with some other bacteria from an engine oil contaminated soil. The discovery of *Pseudomonas* is not surprising based on their frequency in the soil as well as their frequent biodegradability.

The presence of *Bacillus* sp. in engine oil contaminated sites was reported by Udeani *et al*. (2008). The presence of the bacteria could be attributed to their ability to produce spores which enable them to survive in a different environment including hydrocarbon polluted soils (Ghazali *et al*., 2004).

The average total heterotrophic bacterial count (Table 2) supports the statement that high polycyclic aromatic hydrocarbon (PAHs) concentration usually results in low moisture content. This in turn inhibits microbial growth, and hence low CFU count which correlates with previous findings with Yuan *et al*. (2001).

There was significant difference among the physicochemical parameters observed in the study and the activities of the microbes on the samples. The pH of the sample was significantly different as the day progressed between the control and bioremediated soil except for the pH of day 12 which showed no significant difference. Okonokhua *et al*. (2007) reported that there was no significant difference between the pH of the control and spent oil treated soil in their study while Shahida *et al*. (2015) reported pH of neutral from 7.02 - 7.5 in different points and slightly alkaline in one point of soil contaminated with hydrocarbons.

There was a gradual and significant reduction in the content of heavy metals present in the bioremediated soil as compared to the control soil. Adenipekun *et al*. (2013) also observed decrease in Mn, Pb, Ni and Cu in soil contaminated with fresh and spent cutting fluid after 2 months even in the control while Adams *et al*. (2014) reported a gradual decrease in the different metals analyzed in the spent oil contaminated site as the experimental days progressed. Asadu *et al*. (2008) and Nwite *et al*. (2008) reported that anthropogenic activities such as amendment of soil with agricultural wastes increased heavy metals concentration in the soil. Asadu *et al*. (2008) noted that there was significant increase in the amount of Zn, Cu, Pb and Cd of organic waste amended soil compared to the control. Asadu *et al*. (2008) pointed out that build-up of these metals (Zn, Cu, Pb and Cd) to critical levels could be phytotoxic and might result in reduced plant growth and increase within the food chain.

Significant difference at $P < 0.05$ in the statistical analysis carried out between the amended soil and the un-amended polluted soil (control) is an indication of the positive contribution of organic waste (biochar) to the biodegradation of spent engine oil in the soil. The higher counts of bacteria in the amended soil as compared to the control (Table 7) might also be due to the fact that the organic waste was able to neutralize the toxic effects of the oil on the microbial population by rapid improvement of the soil physicochemical properties (Basharudin, 2011; Floch *et al*., 2011). The organic waste might also help to improve the soil aeration thereby providing sufficient oxygen required by the microbial community which consequently favoured the growth of indigenous bacteria in the soil. The presence of macro and micro-elements in the biochar served as a nutrient source for the growth and maintenance of microbial community (Lee *et* a*l*., 2003). Odokuma and Dickson (2003) noted that bioremediation involved introduction of nutrients in form of organic matter to the contaminated soil. There was significant difference between the pH, PO_3^{2-} as well as the organic matter content between the control and bioremediated soil. There was no significant different between total organic carbon and NO_3^{2-} between the control and bioremediated soil.

Land-farming to biodegrade polycyclic aromatic hydrocarbons (PAHs) and organic contaminants in general requires a consistent number of aerobic and heterotrophic microorganisms (U.S EPA, 1994; Wick *et al*., 2011). Typical microbial population densities range from 104 to 107 CFU per gram of dry soil. For an effective land-farming process, total heterotrophic bacteria should have a presence of at least 1,000 CFU per gram of dry soil (U.S EPA, 1994).

Biodegradability potential exists among bacterial strains that inhabit PAHs polluted site (Yu *et al*., 2005; Igwo-Ezikpe *et al*., 2010).

5.1 Conclusion

The result obtained from the research revealed that soils within the premises of the mechanic workshops are good sources of hydrocarbon-utilizing bacteria namely: *Thalassospira mesophila* strain JCM 18969, *Pseudomonas fluorescens* F113, *Siccibacter turicensis* LMG 23730, *Pseudomonas Zeshuii* strain KACC 15471, *Pseudomonas stutzeri* strain CGMCC 1.1803 and *Marinobacter hydrocarbonoclasticus* strain ATCC 49840. These bacteria isolates can be effectively utilized in bioaugmentation aimed at the removal of hydrocarbon pollutants from contaminated environments especially those located within the residential vicinity.

5.2 Recommendation

More research could be done by introducing the hydrocarbon-utilizing bacterial isolates singly and then in combination randomly to treat the spent engine oil contaminated soil. Also, the introduction of the bacterial isolates on individual soils rather than on a combination of soil could be further researched on to ascertain their efficiency. Further studies need to also be done to confirm that these organisms can be used for bioremediation of engine oil contaminated soil.

Although many recommendations have been issued in various research papers on this subject, education, awareness and sensitization of people will help to reduce problems associated with engine oil contamination. Also, the development of effective regulatory policies on the environment in order to prevent contamination of soil by spent engine oil will go a long way in addressing this issue of public health concern.

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Appendix

Table 9a: Total Petroleum Hydrocarbon (TPH) of Galadima 1 Soil Before Bioremediation

S/N	Retention Time (RT)	Identification	Reference
1	7.182	2-Undecanone	38231
		(Z)-1-Phenylpropene	8954
		Benzene, 1-ethenyl-3-methyl-	8975
2	8.027	Acetic acid, octyl ester	39505
		N-[3-[N-Aziridyl]propylidene]tetrahydrofurfurylamine	46932
		1-Butanamine, N-pentylidene-	19119
3	8.233	1-Phenyl-1-pentyn-4-ol	30820
		1,4-Benzenediacetonitrile	28409
		4-(6,7-Dihydro-5H-benzo[1,2,5]oxadiazol-4 ylideneaminooxymethyl)-benzonitrile	116998
4	8.79	Benzenethiol, 2-methyl-	10473
		Benzenethiol, 4-methyl-	10476
		1,3,5-Tris[3-trichloromethyl-1,2,4-oxadiazoyl]benzene	241761
5	21.526	Cycloheptasiloxane, tetradecamethyl-	236968
		Cycloheptasiloxane, tetradecamethyl-	236969
		Pentasiloxane, dodecamethyl-	204093

Table 9b: Total Petroleum Hydrocarbon (TPH) of Galadima 2 Soil Before Bioremediation

S/N	Retention Time (RT)	Identification	Reference
1	7.201	Benzene, 1-ethenyl-2-methyl-	8974
		Benzene, 1-propenyl-	8962
		Benzene, 1-ethenyl-3-methyl-	8975
2	8.014	Methoxyacetic acid, tetradecyl ester	132959
		1-Hexanol, 2-ethyl-	13669
		2-tert-Butyl-3,4,5,6-tetrahydropyridine	17918
3	9.366	N.alpha.,N.alpha.-Dimethyllysine, methyl ester	52141
		2-Octanone	12438
		2-Octanone	12437
4	9.641	Benzenethiol, 2-methyl-	19953
		Benzenethiol, 4-methyl-	48738
		1,3,5-Tris[3-trichloromethyl-1,2,4-oxadiazoyl]benzene	12461
5	10.592	5-Aminoindole	14490
		1H-Indenol	14521
		5-Aminoindole	14491
6	13.025	4-Decenoic acid, ethyl ester, (Z)-	59568
		Octanoic acid, 6-hydroxy-8-methoxy-, .epsilon.-lactone	40396
		Fumaric acid, 2-hexyl pentadecyl ester	215723
7	13.55	Ethanol, 2-[2-(2-methoxyethoxy)ethoxy]-	34051
		Ethanol, 2-[2-(2-methoxyethoxy)ethoxy]-	34053
		Ethanol, 2-[2-(2-methoxyethoxy)ethoxy]-	34052
8	15.483	1H-Inden-1-one, 2,3-dihydro-	14545
		1H-Indazole, 3-methyl-	14499
		2-Methyl-5-phenyltetrazole	31388
9	15.834	2-[2-[2-(2-Ethoxyethoxy)ethoxy]ethoxy]ethyl 2,2,2-trifluoroacetate	159076
		2-(2-Ethoxyethoxy)ethyl nonanoate	122629

Table 9b: Total Petroleum Hydrocarbon of Galadima 2 Soil Before Bioremediation (Cont.)

S/N	Retention Time (RT)	Identification	Reference
		Cyclopropanecarboxylic acid, 2,2-dimethyl, 3-(2-trimethylsilylethynyl),methyl ester, cis-	80825
10	16.115	2-Undecanone	38224
		Diethylvinylsilane	7396
		2-Nonanone	19930
11	16.353	2,5-Difluorobenzaldehyde	19578
		1,4-Methanonaphthalene, 1,4-dihydro-	19243
		2,5-Difluorobenzaldehyde	19574
12	16.49	Dodecane	1
13	16.603	Phthalic anhydride	23154
		1,2-Benzenedicarboxylic acid	35572
		N-(5-Amino-4-cyano-1-pyrazolyl)phthalimide	104564
14	17.26	Cyclohexasiloxane, dodecamethyl-	225658
		Cyclohexasiloxane, dodecamethyl-	225656
		Cyclohexasiloxane, dodecamethyl-	225657
15	17.541	1-Propanone, 1-phenyl-	15287
		Butanamide, 3-oxo, N-phenyl-2,2-dichloro	97814
		1-Propanone, 1-phenyl-	15292
16	19.199	N-Allyl-N,N-dimethylamine	1583
		o-Ethyl S-3-(dimethylamino)propyl methylphosphonothiolate	82062
		Doxylamine	119329
17	20.7	Ethanol, 2-[2-(2-butoxyethoxy)ethoxy]-	65549
		Ethanol, 2-[2-(2-butoxyethoxy)ethoxy]-	65551
		Ethanol, 2-[2-(2-butoxyethoxy)ethoxy]-	65552
18	21.526	Cycloheptasiloxane, tetradecamethyl-	236969
		Cycloheptasiloxane, tetradecamethyl-	236968
		N-Benzyl-N-ethyl-p-isopropylbenzamide	128464

Table 9c: Total Petroleum Hydrocarbon (TPH) of Oko-Erin 1 Soil Before Bioremediation

S/N	Retention Time (RT)	Identification	Reference
1	8.008	1-Chloro-1-n-decyloxy-1-silacyclopentane	124152
		2(1H)-Naphthalenone, octahydro-1,1,4a-trimethyl-, trans-	56427
		1,1,1-Trifluoroheptadecen-2-one	151185
2	8.233	Indene	8408
		Indene	8407
		1-Propyne, 3-phenyl-	8410
3	8.784	Benzenethiol, 3-methyl-	10471
		Benzenethiol, 2-methyl-	10474
		Benzenethiol, 2-methyl-	10470
4	17.26	Cyclohexasiloxane, dodecamethyl-	225657
		Cyclohexasiloxane, dodecamethyl-	225656
		Cyclohexasiloxane, dodecamethyl-	225658
5	19.186	3-Hydroxychlorpromazine	172168
		1-Methyl-1-ethyl-1-silacyclobutane	7407
		1-(2-Dimethylamino-ethyl)-4-(furan-carbonyl)-3-hydroxy-5-pyridin-4-yl-1,5-dihydro-pyrrol-2-one	177526
6	21.52	Cycloheptasiloxane, tetradecamethyl-	236968
		Cycloheptasiloxane, tetradecamethyl-	236969
		Pentasiloxane, dodecamethyl-	204092
7	24.96	Cyclooctasiloxane, hexadecamethyl-	240804
		Cyclooctasiloxane, hexadecamethyl-	240805
		Cyclopentasiloxane, decamethyl-	196316

Table 9d: Total Petroleum Hydrocarbon (TPH) of Oko-Erin 2 Soil Before Bioremediation

S/N	Retention Time (RT)	Identification	Reference
1	8.79	Pyridine-2-carboxylic acid, tetradecyl ester	160584
		1-Methyl-16-methoxyaspidospermidin-4-one	177021
		Benzenethiol, 2-methyl-	10473
2	17.26	Cyclohexasiloxane, dodecamethyl-	225656
		1H-Indole, 1,3-dimethyl-5,6-dimethoxy-2-(3, 5-dimethoxyphenyl)-	177669
		5-Amino-7-(4-methoxy-phenyl)-2-phenyl-pyrazolo[1,5-a]pyrimidine-6-carbonitrile	177640
3	21.526	Cycloheptasiloxane, tetradecamethyl-	236969
		Cycloheptasiloxane, tetradecamethyl-	236968
		N-Benzyl-N-ethyl-p-isopropylbenzamide	128464

Table 9e: Total Petroleum Hydrocarbon (TPH) of Oko-Erin 3 Soil Before Bioremediation

S/N	Retention Time (RT)	Identification	Reference
1	7.401	6-(3-Oxo-3-piperidin-1-ylpropylamino)-2H-[1,2,4] triazine-3,5-dione	116110
		Carveol	25057
		Pyridin-2(1H)-one, 1-phenyl-4-(1-iperidylmethylenamino)-	128383
2	17.26	Cyclohexasiloxane, dodecamethyl-	225658
		Cyclohexasiloxane, dodecamethyl-	225657
		Cyclohexasiloxane, dodecamethyl-	225656
3	19.293	Pyrrolidine-5-one-2-propionic acid, ethyl (ester)	49767
		4-(2-Pyrrolidin-1-yl-ethyl)-4-azatricyclo- [5.2.1.-02,6]dec-8-ene-3,5-dione	110831
		Dimethyl bicyclo[2.2.1]-2,5-heptadiene-2,3-dicarboxylate	67351
4	21.52	Cycloheptasiloxane, tetradecamethyl-	236969
		Pentasiloxane, dodecamethyl-	204092
		Pentasiloxane, dodecamethyl-	204091
5	24.96	Cyclooctasiloxane, hexadecamethyl-	240805
		Cyclooctasiloxane, hexadecamethyl-	240804
		Benzeneacetic acid, .alpha.,3,4-tris[(trimethylsilyl)oxy]-, trimethylsilyl ester	231302

Table 9f: Total Petroleum Hydrocarbon (TPH) of Control Soil After Bioremediation

S/N	Retention Time (RT)	Identification	Reference
1	21.519	Cycloheptasiloxane, tetradecamethyl-	236968
		Cycloheptasiloxane, tetradecamethyl-	236969
		3-Isopropoxy-1,1,1,7,7,7-l-3,5,5-tris(trimethylsiloxy)tetrasiloxane	240263

Table 9g: Total Petroleum Hydrocarbon (TPH) of Bioremediated Soil After Bioremediation

S/N	Retention Time (RT)	Identification	Reference
1	22.752	Cyclohexasiloxane, dodecamethyl-	225657
		Cycloheptasiloxane, tetradecamethyl-	225658
		3-Isopropoxy-1,1,1,7,7,7-l-3,5,5-tris(trimethylsiloxy)tetrasiloxane	225656
2	23.271	Cyclohexasiloxane, dodecamethyl-	225656
		Cyclohexasiloxane, dodecamethyl-	225657
		Cyclohexasiloxane, dodecamethyl-	225658
3	24.597	Cycloheptasiloxane, tetradecamethyl-	236968
		Cycloheptasiloxane, tetradecamethyl-	236968
		Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-	222021
4	25.879	Cyclooctasiloxane, hexadecamethyl-	240805
		Cyclooctasiloxane, hexadecamethyl-	240804
		Benzeneacetic acid, .alpha.,3,4-tris[(trimethylsilyl)oxy]-, trimethylsilyl ester	231302
5	27.005	Cyclononasiloxane, octadecamethyl-	242430
		Cyclononasiloxane, octadecamethyl-	242432
		Cyclononasiloxane, octadecamethyl-	242431
6	28.513	Cyclodecasiloxane, eicosamethyl-	243183
		Cyclononasiloxane, octadecamethyl-	242430
		Cyclononasiloxane, octadecamethyl-	242431

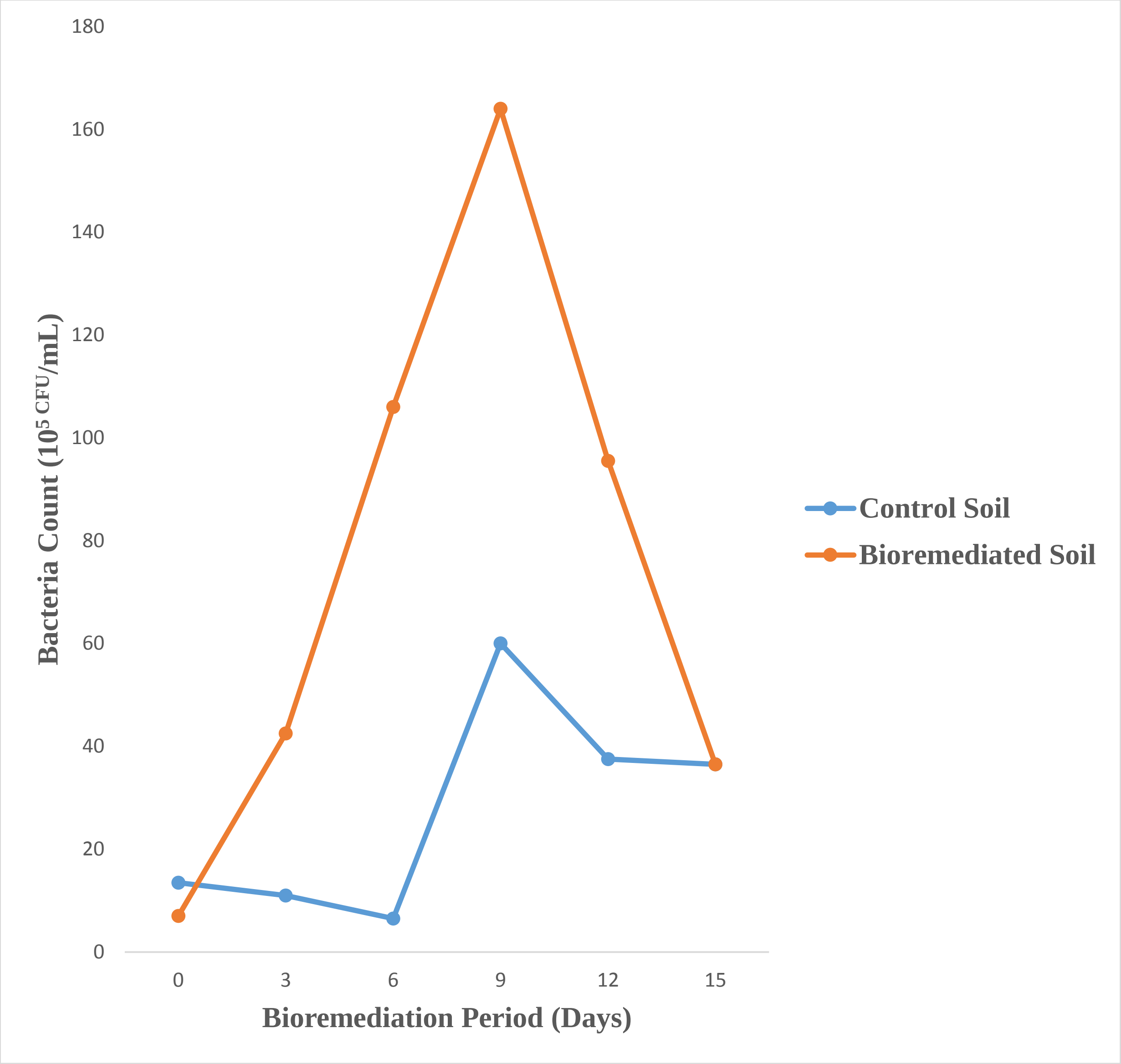

Figure 1: Total Bacteria Count of Soil Samples during Bioremediation

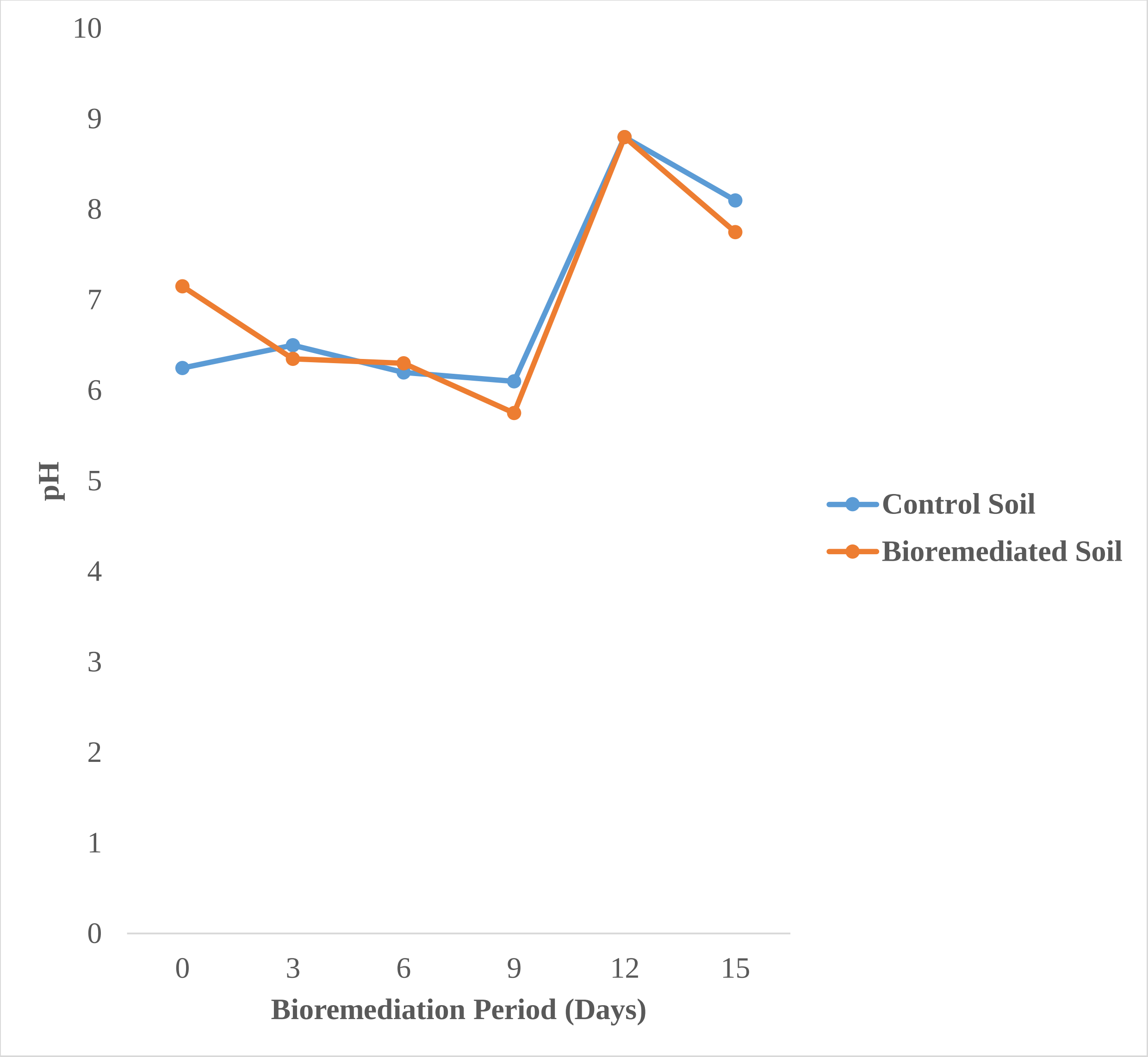

Figure 2: pH of Soil Samples during Bioremediation

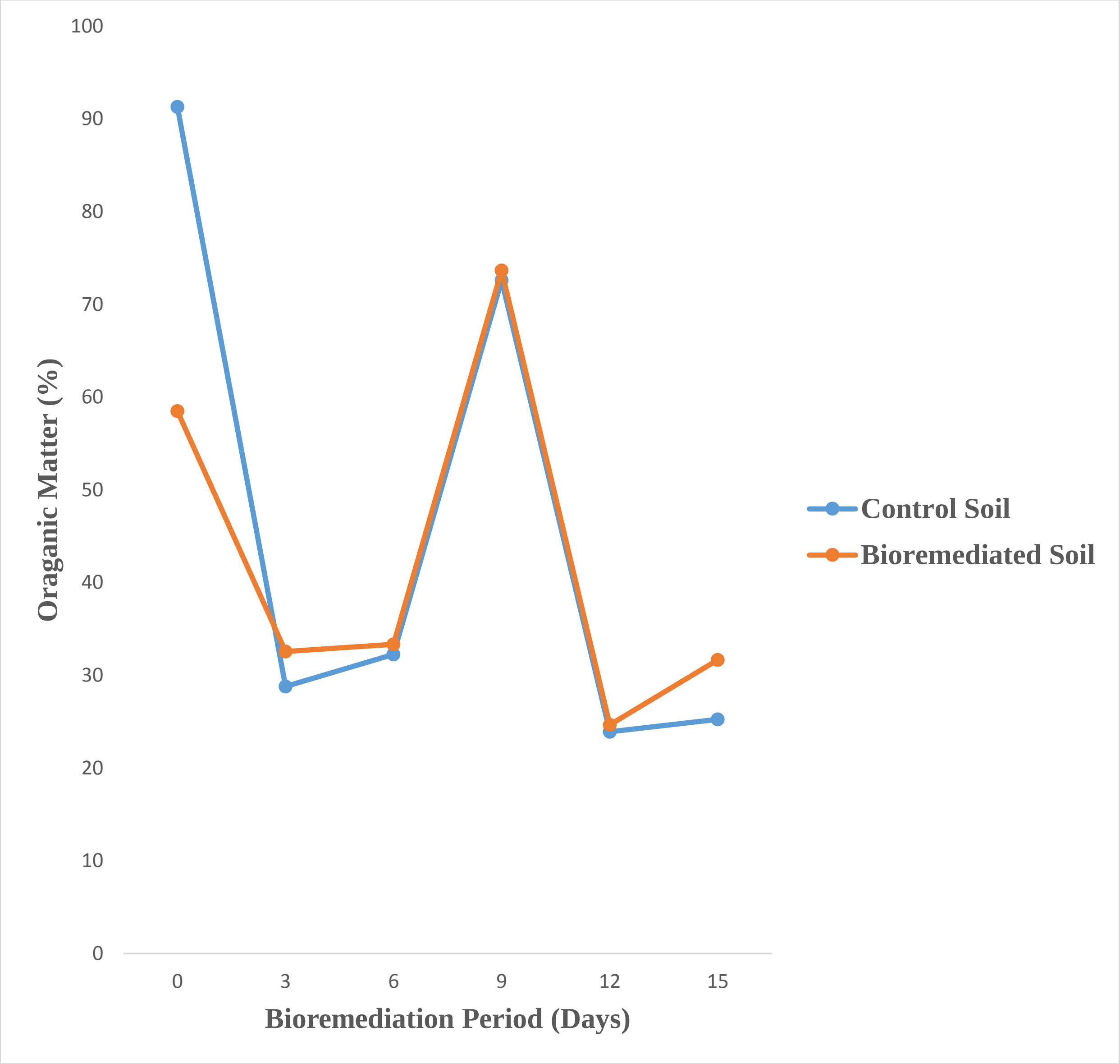

Figure 3: Percentage Organic Matter Content of Soil Samples during Bioremediation

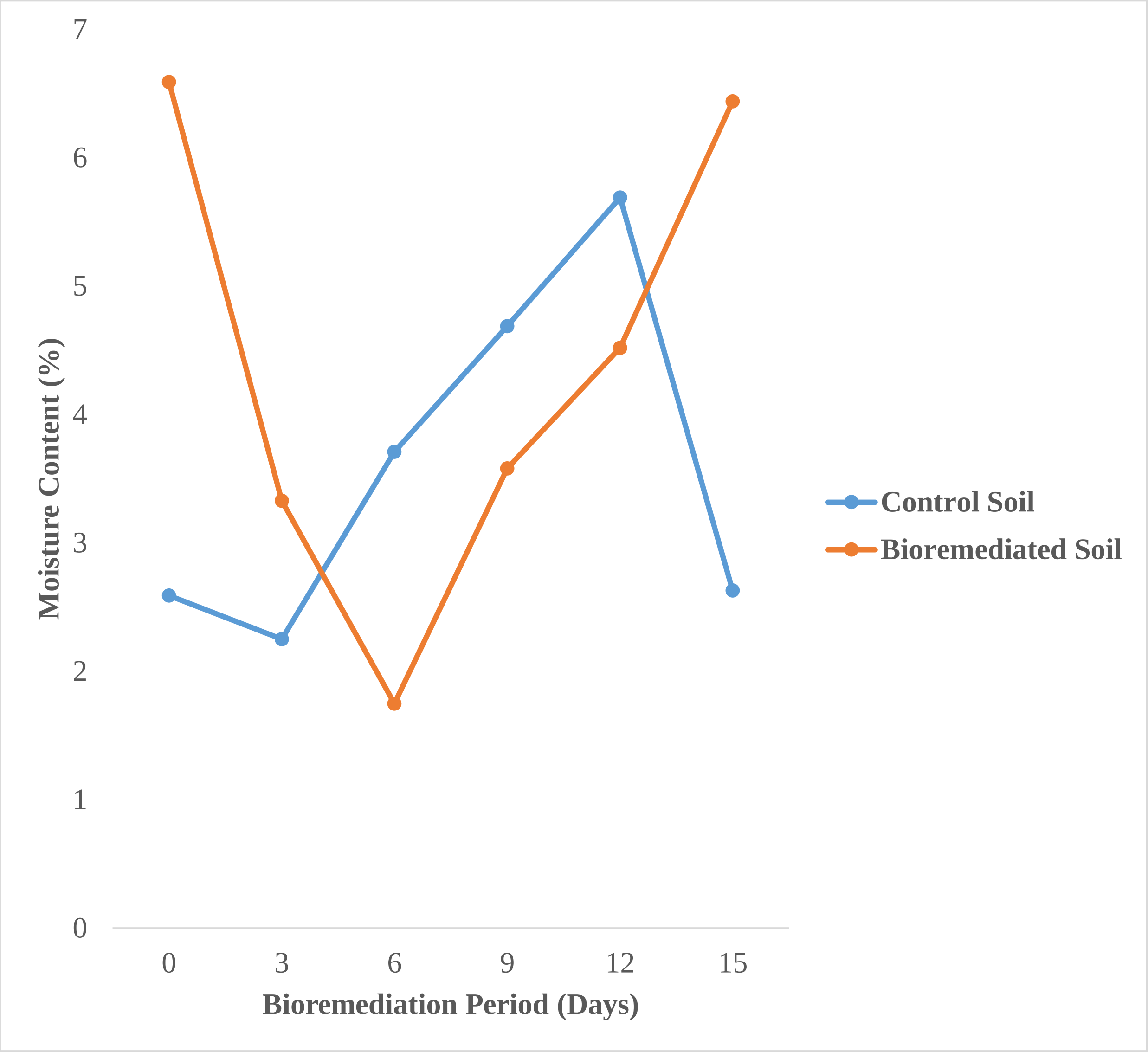

Figure 4: Percentage Moisture Content of Soil Samples during Bioremediation

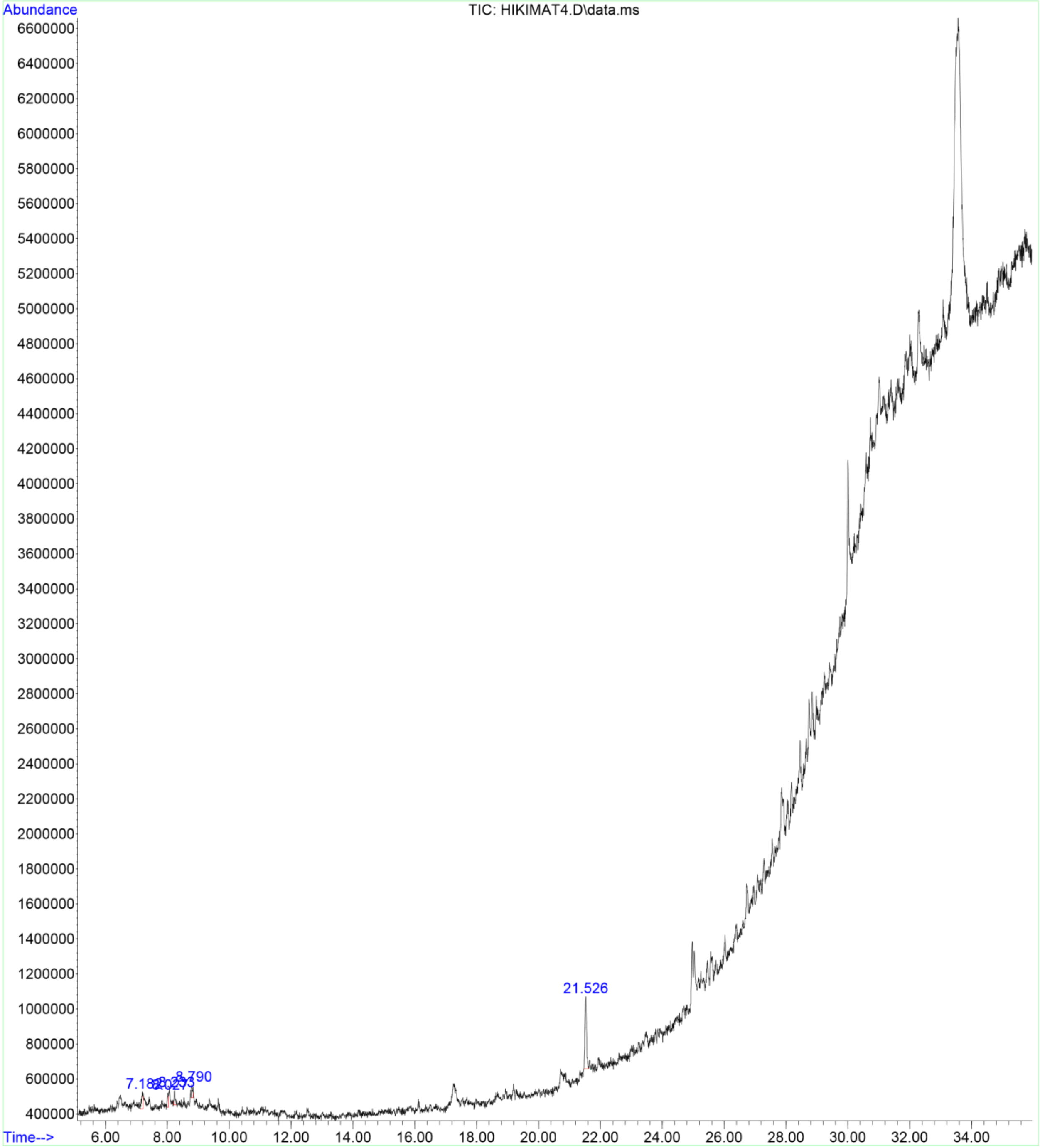

Figure 5: Total Petroleum Hydrocarbon (TPH) of Galadima 1 Soil Before Bioremediation

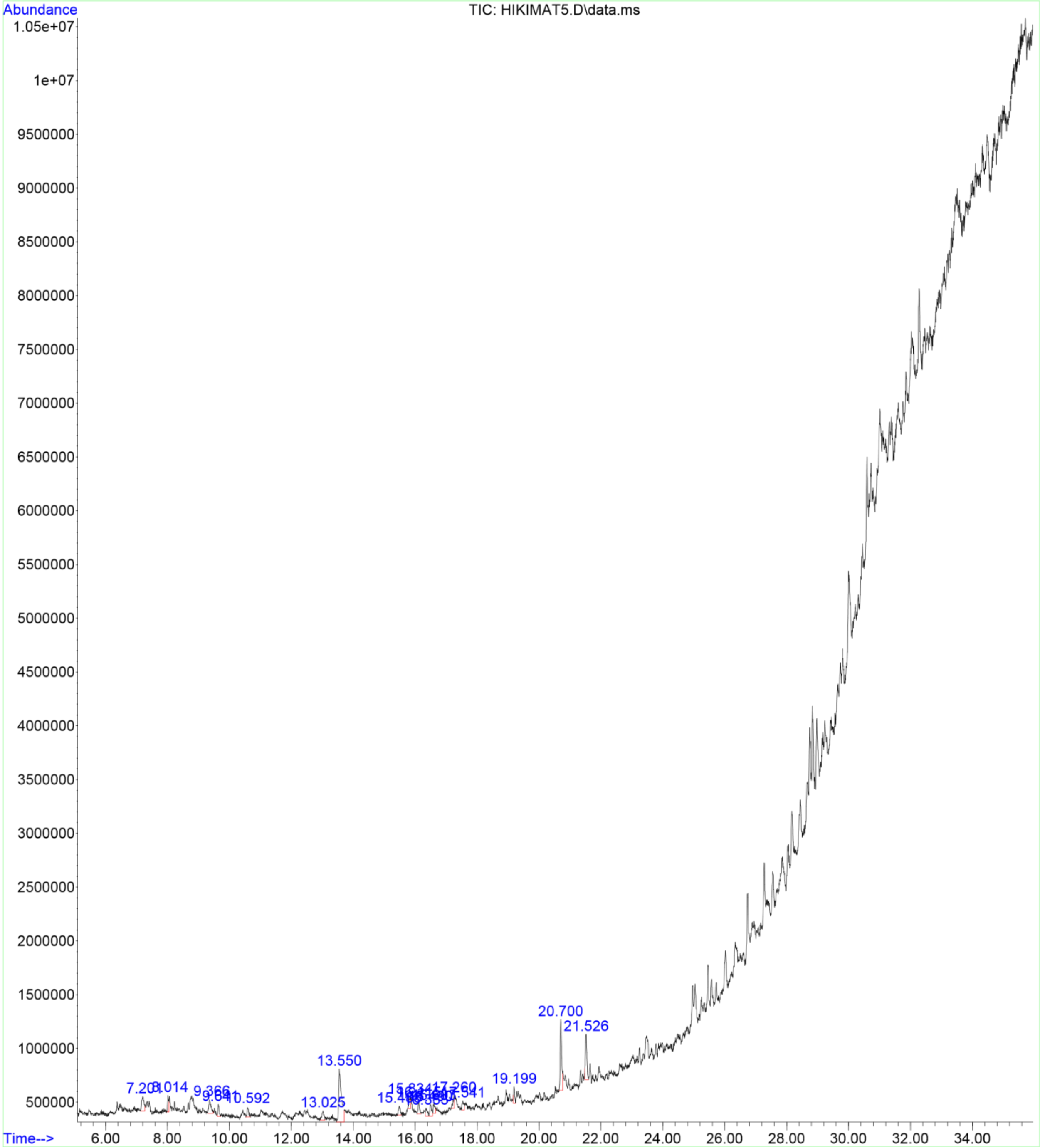

Figure 6: Total Petroleum Hydrocarbon (TPH) of Galadima 2 Soil Before Bioremediation

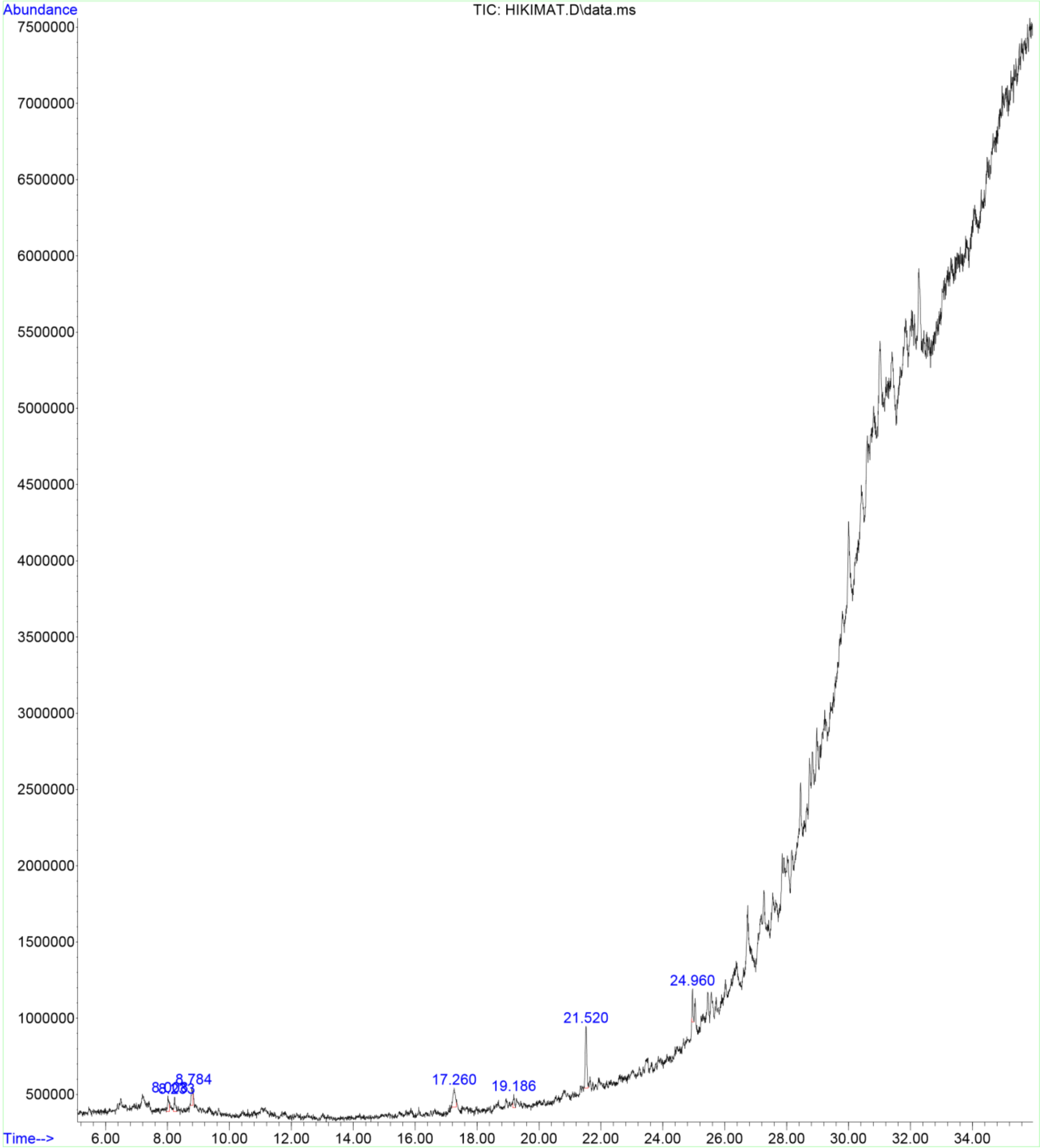

Figure 7: Total Petroleum Hydrocarbon (TPH) of Oko-Erin 1 Soil Before Bioremediation

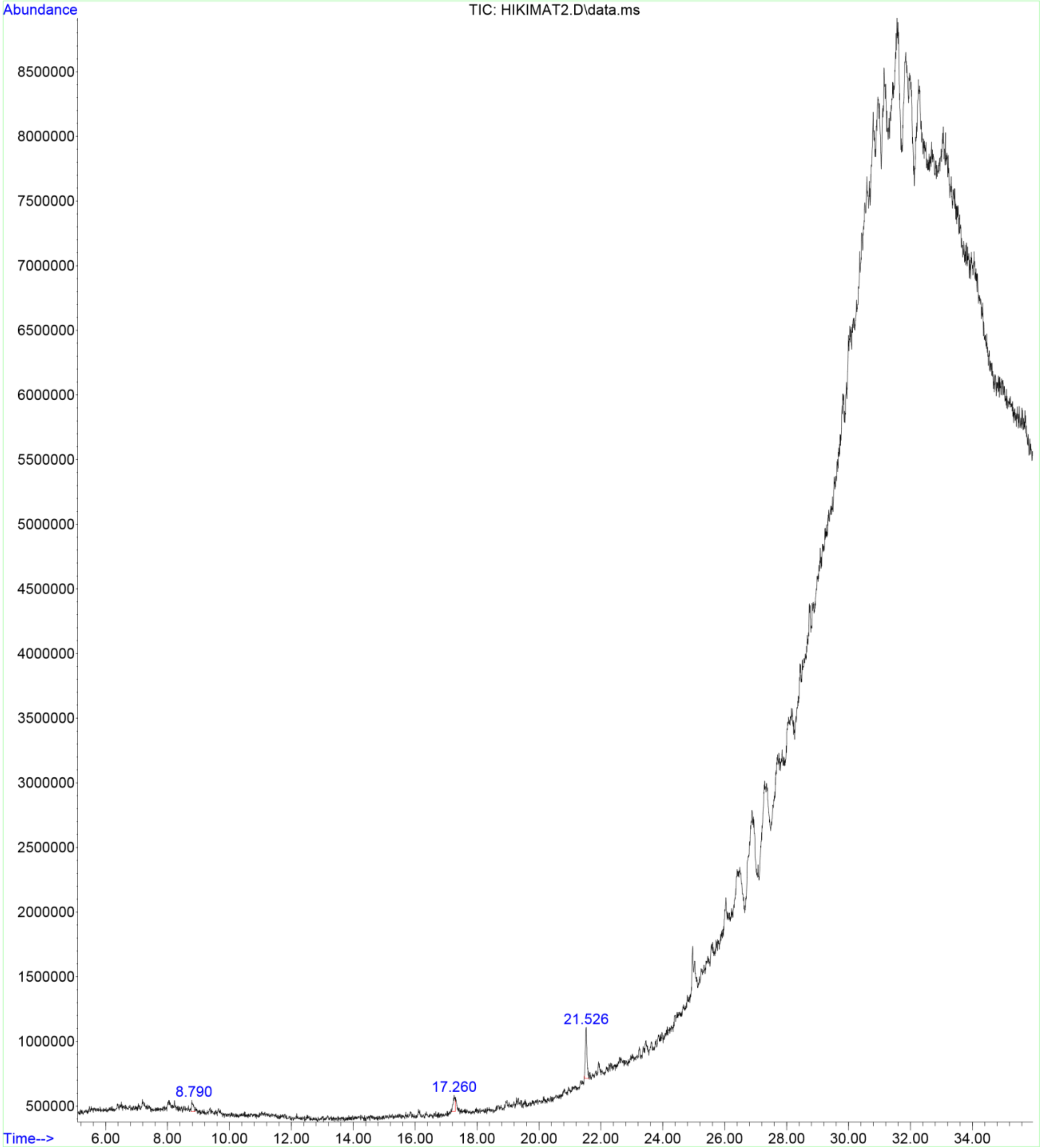

Figure 8: Total Petroleum Hydrocarbon (TPH) of Oko-Erin 2 Soil Before Bioremediation

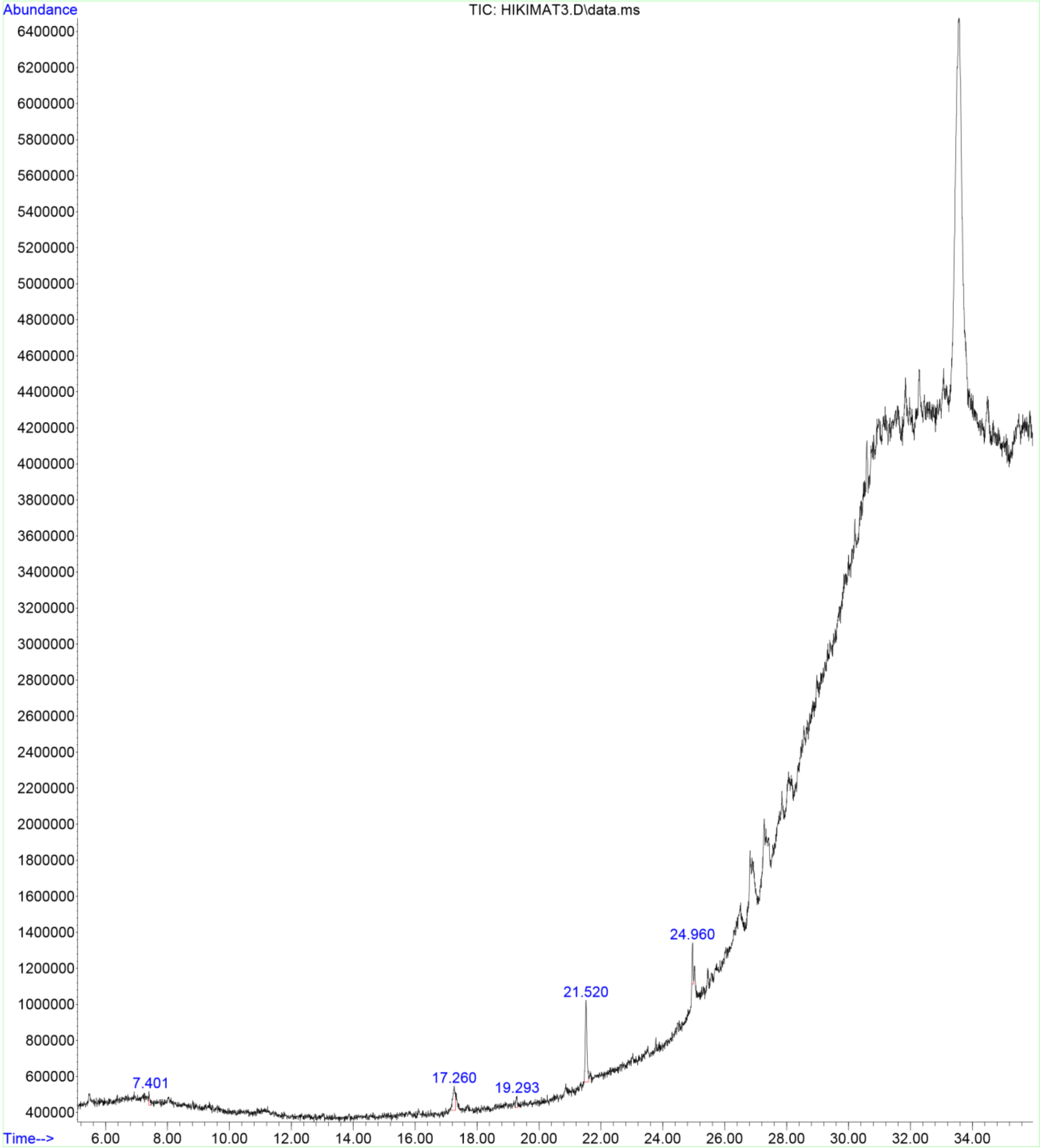

Figure 9: Total Petroleum Hydrocarbon (TPH) of Oko-Erin 3 Soil Before Bioremediation

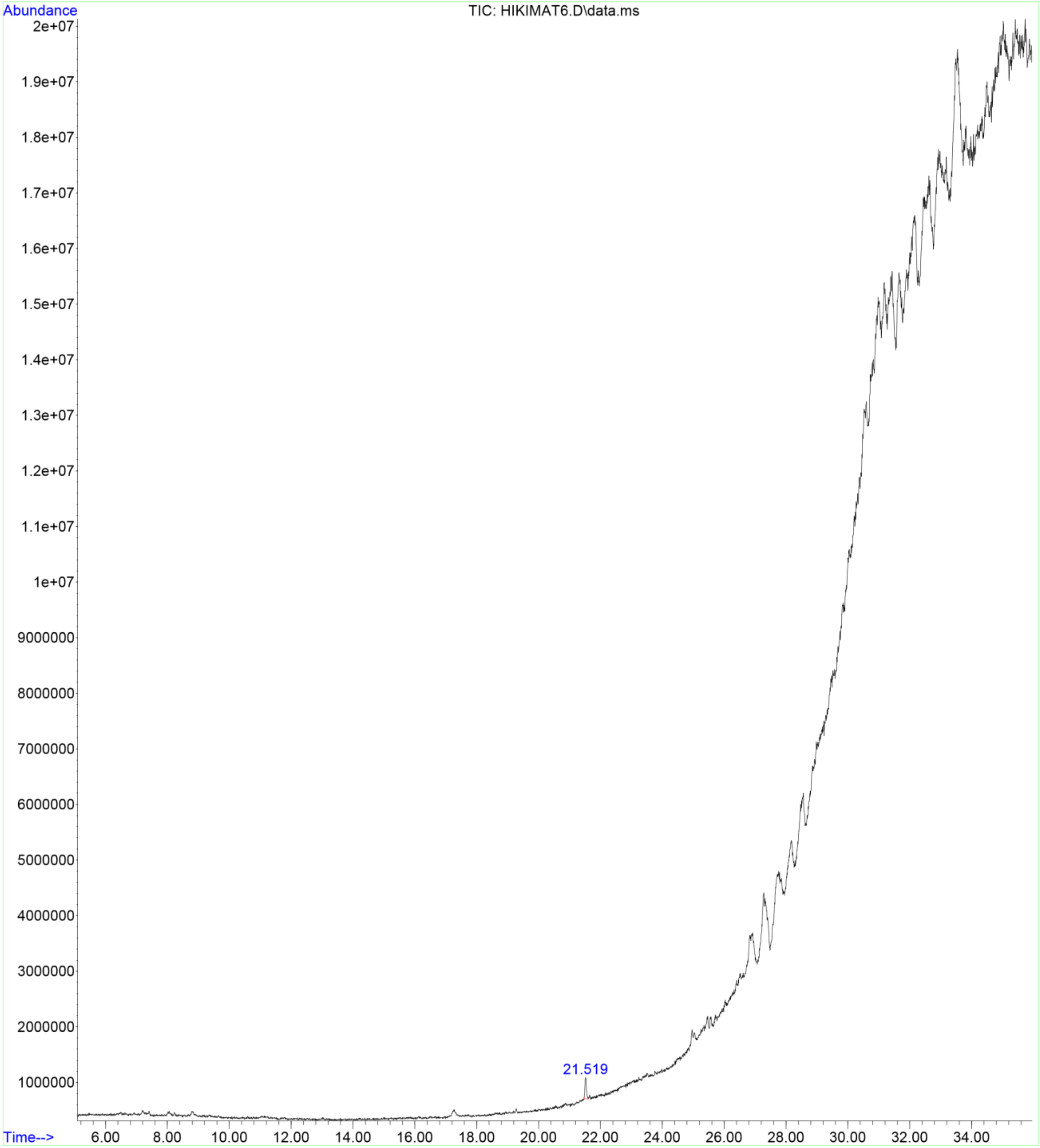

Figure 10: Total Petroleum Hydrocarbon (TPH) of Control Soil After Bioremediation

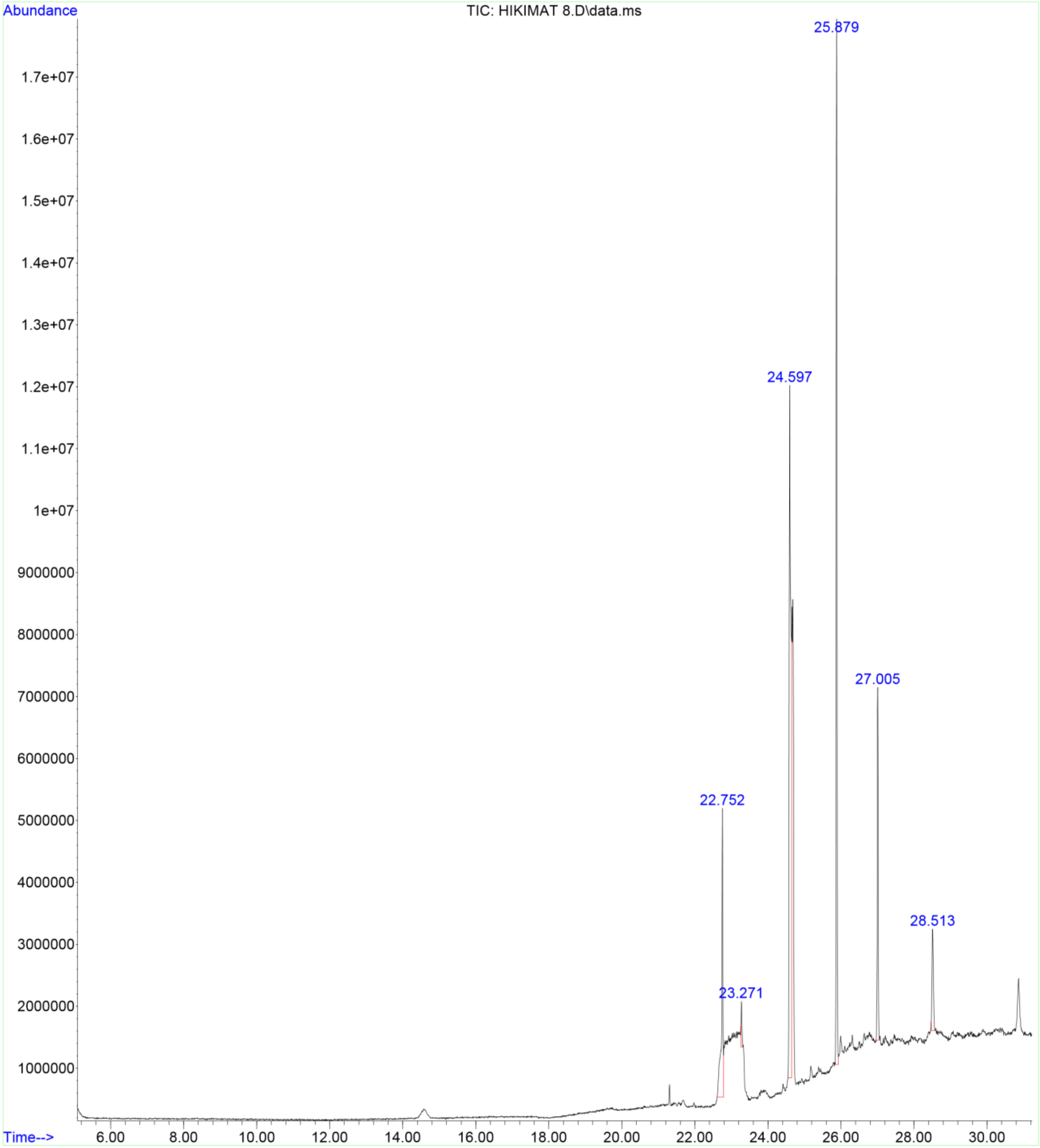

Figure 11: Total Petroleum Hydrocarbon (TPH) of Bioremediated Soil