BIOREMEDIATION OF HYDROCARBON POLLUTED SOIL USING *PSEUDOMONAS AERUGINOSA*

BY

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Declaration

I declare that the work in this project dissertation titled "Bioremediation of Hydrocarbon Polluted Soil Using *Pseudomonas aeruginosa*" has been performed by me in the Department of Water Resources and Environmental Engineering. The information derived from the literature has been duly acknowledged in the test and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

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Certification

This dissertation titled "BIOREMEDIATION OF HYDROCARBON POLLUTED SOIL USING *PSEUDOMONAS AERUGINOSA*" by Isaac Abraham MHYA, meets the regulations governing the award of Master of Science degree in Water Resources and Environmental Engineering of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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Abstract

The bioremediation potential of *Pseudomonas aeruginosa* on hydrocarbon contaminated soil was studied. Twenty five kilograms of hydrocarbon polluted soil sample was collected at a depth of 20cm using an auger from an old mechanic workshop (Baba Kaduna Mechanic workshop) in Zaria city, Kaduna State Nigeria. The soil sample was room-dried for three days and sieved through a 2mm sieve. The soil physicochemical parameters, pH, Electrical conductivity, Oil and Grease content, % TOC, %Nitrogen, %Phosphorus, %Potassium, %Calcium and Magnesium were found to be 6.6, 0.2(µS/m), 580(Mg/L), 0.818, 0.175, 0.430, 0.682, 4.080 and 0.840 respectively and the soil was found to be a loamy soil. Hydrocarbon utilizing *Pseudomonas aeruginosa* were isolated and screened for their ability to utilize hydrocarbon as a sole source of carbon and energy. Four isolates of *Pseudomonas aeruginosa*were obtained (MWS1, MWS3, MWS4 and MWS6). Of the four isolates, MWS3 showed greater ability to utilize hydrocarbons as it had high growth across mineral salt medium containing different concentrations (0.5%, 1.0%, 1.5% and 2.0% v/v) of engine oil while isolate MWS1, MWS4 and MWS6 had only scanty growth on the medium. 25g of the polluted soil was treated with 10ml of standard suspension (3.0×10^{8}cfu/ml) of the selected isolate and incubated for a period of twenty eight days. The physicochemical properties of the treated soil was determined at intervals of seven days to assess the ability of the isolate to stabilize the pollutant. Recovery from the effect of the pollution was observed as the oil and grease content of the soil reduced drastically from 580Mg/L to 189Mg/L, the percentage of Nitrogen in the soil increased from 0.175 to 0.7 after the incubation period. It was therefore concluded that *Pseudomonas aeruginosa* is an active biological agent in the stabilization of hydrocarbon pollutants in the soil.

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CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Anthropogenic activities such as mining, construction, transportation and manufacturing not only deplete the natural resources but also produce large amount of wastes that leads to pollution of air, water, soil, and oceans; global warming and acid rains. Untreated or improperly treated waste is a major cause of pollution of soil and water environments consequently resulting in degradation of such environments with adverse public health significance (AbdelRahman, 2011).

Environmental Pollution is therefore the introduction of contaminants into the natural environment that causes adverse change. Pollution can take the form of chemical substances or energy, such as noise, heat or light. Pollution is often classed as point source or nonpoint source pollution (EPA, 2017).

Petroleum hydrocarbons are widespread in our environment as fuel and chemical compounds. The uncontrolled release of petroleum hydrocarbons negatively impacts many of our soi1 and water resources. The contamination can result from leaking Underground Storage Tanks (UST), petroleum refineries and bulk storage facilities, broken oil pipelines, spills of petroleum products in chemical plants and transportation processes. The risks of explosion and fire are also serious threats to the environment. It is estimated that 6,000,000tons petroleum waste enters the environment each year causing serious environmental problems (Banat*et al.*,2000).

With growing awareness in the negative impact of petroleum hydrocarbons to the environments, many guidelines have been set for the control of indiscriminate pollution of the

environment by hydrocarbon substances. However, even if the problems associated with fuel storage and distribution are solved; contamination incidental to production and commercial usage would continue to threaten surface and groundwater supplies. Many manufacturing processes necessarily produce water and sludge that are contaminated with hydrocarbons. At a typical oil refinery facility, more than 23 different waste streams have been identified, several of which have been classified as hazardous waste (Ma, 1998).

Since the contamination of soil and groundwater by uncontrolled releases of hydrocarbons (especially petroleum products) has become a significant problem, a number of technologies have been tested to remediate the polluted sites. Treatment processes have incorporated physical, chemical or biological methods, or a combination of them. Remedial action on a contaminated site can involve in situ or ex situ action. The conventional remediation methods include excavation and landfill disposal, use of chemical dispersants or incineration. However, these methods are expensive and only transfer the contamination from one place to another (Abha and Singh, 2012).

Other human activities in maintenance workshops, car repair workshops and tank farm sites also contribute to the pollution and ultimate damage of the immediate environment in which they are situated because such sites may never be usable for any other purpose.

Bioremediation has been claimed to be an inexpensive, natural method of cleanup of petroleum contaminated soil or water. Both in situ and ex situ treatment of bioremediation have been shown to be feasible. In situ biological treatment involves the stimulation of native microbial community to levels that effectively degrade contaminants. Treatment using in situ biological methods can prove to be efficient and cost effective for the cleanup of contaminated soils and groundwater (Ma, 1998).

1.2 Statement of Research Problem

1. Petroleum hydrocarbons occur in forms not readily usable by life forms. Most are recalcitrant and as such resist stabilization and constitute health hazard as they have been reported to be carcinogenic, mutagenic or growth inhibitory.
2. Many remediation technologies have been shown to be inefficient as they do not result in complete elimination of pollutants and may even leave the environment worse than it was.
3. Natural activities of oil degrading microorganisms in the soil have been found to be very slow as such complete elimination is not always feasible.
4. Reports have shown that hydrocarbon pollution adversely alter the physicochemical properties of soil resulting to increased soil infertility.

1.3 Justification

1. Since the physicochemical remediation technologies have been found to be inefficient and environmentally unfriendly, there is need to develop a more eco-friendly, efficient and cost effective means of hydrocarbon degradation.
2. Certain bacteria including members of the genus *Pseudomonas* have been used with relative success in the breakdown of hydrocarbons.
3. It is paramount to obtain bacteria isolates with high hydrocarbon degradation efficiency in order to increase the chances of containing the problem of environmental degradation by hydrocarbon pollutants.

1.4 Aim and Objectives

The aim of this research work is to remediate hydrocarbon polluted soil using *Pseudomonas aeruginosa*isolated from the soil.

The specific objectives of this research were to:

1. Determine the physicochemical properties of the hydrocarbon polluted soil.
2. Isolate and characterize *Pseudomonas aeruginosa* from the polluted soil.
3. Assess the capacity of *Pseudomonas aeruginosa* in remediation of the hydrocarbon polluted soil.

1.5 Scope and Limitations

This study will assess ability of *Pseudomonas aeruginosa* in bioremediation of hydrocarbon polluted soil. The *P. aeruginosa* was produced from the soil sample collected from a mechanic workshop in Zaria city (Baba Kaduna mechanic garage) located at 11°05'32.3"N 7°42'48.8"E. The soil sample was collected at plant root Zone (20cm) and will be the subject of the bioremediation. This work does not cover heavy metals present in the soil sample.

CHAPTER TWO

LITERATURE REVIEW

2.1 Hydrocarbons

Hydrocarbons are very simple organic compounds. They are composed only of two elements; carbon and hydrogen, hence their name (Moneke and Nwangwu, 2011). The majority of hydrocarbons found on Earth naturally occur in crude oil, where decomposed organic matter provides an abundance of carbon and hydrogen which, when bonded, can catenate to form seemingly limitless chains. (Mulligan, 2004)

Petroleum products (gotten from the fractional distillation of crude oil) are essential elements of life. Petroleum based products are the major source of energy for industry and daily life (Das and Chandran, 2010). The term petroleum (Latin for "rock oil") is referred to an extremely complex mixture of a wide variety of low and high molecular weight hydrocarbons. This complex mixture contains saturated alkanes, branched alkanes, alkenes, napthenes (homo-cyclics and hetero-cyclics), aromatics (including aromatics containing hetero atoms like sulfur, oxygen, nitrogen, and other heavy metal complexes), naptheno-aromatics, large aromatic molecules like resins, asphaltenes, and hydrocarbons containing different functional groups like carboxylic acids, ethers, etc (Abha and Singh, 2012). Petroleum is refined into various fractions such as light oil, naphtha, kerosene, diesel, lube oil waxes, and asphaltenes, etc. These hydrocarbon molecules are widespread in the environment due to the wide range of petroleum uses. Petroleum oils are used in large quantities as fuels (Hui, 2011).

2.1.1 Types of Hydrocarbons

Saturated hydrocarbons (alkanes) are the simplest of the hydrocarbon species. They are composed entirely of single bonds and are saturated with hydrogen. The general formula for saturated hydrocarbons is C_nH_{2n+2} (assuming non-cyclic structures) (IUPAC, 2011). Saturated hydrocarbons are the basis of petroleum fuels and are found as either linear or branched species. Substitution reaction is their characteristics property (like chlorination reaction to form chloroform). Hydrocarbons with the same molecular formula but different structural formulae are called structural isomers (IUPAC, 2011). Unsaturated hydrocarbons have one or more double or triple bonds between carbon atoms. Those with double bond are called alkenes. Those with one double bond have the formula C_nH_{2n} (assuming non-cyclic structures). Those containing triple bonds are called alkynes, with general formula C_nH_{2n-2}. Cycloalkanes are hydrocarbons containing one or more carbon rings to which hydrogen atoms are attached. The general formula for a saturated hydrocarbon containing one ring is C_nH_{2n}. Aromatic hydrocarbons, also known as arenes, are hydrocarbons that have at least one aromatic ring.Hydrocarbons can be gases (e.g. methane and propane), liquids (e.g. hexane and benzene), waxes or low melting solids (e.g. paraffin wax and naphthalene) or polymers (e.g. polyethylene, polypropylene and polystyrene) (Nunes *et al.*, 2009).

2.1.2 Hydrocarbon Pollution

Release of hydrocarbons into the environment whether accidentally or due to human activities is a main cause of water and soil pollution (Holliger *et al.*, 1997). Leaks and accidental spills occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products. The amount of natural crude oil seepage was estimated to be

600,000 metric tons per year with a range of uncertainty of 200,000 metric tons per year (Kvenvolden and Cooper, 2003). The contamination of the environment with petroleum hydrocarbons provides serious problems for many countries. (Mehrasbi and Hazhighi, 2012). Petroleum hydrocarbons are becoming a global problem for the environment. They are highly persistent in the environment, toxic and present significant health risks to human (Alrumman*et al.*, 2013).

Alvarez and Vogel, 1995 reported that hydrocarbon contamination in the environment is a very serious problem whether it comes from petroleum, pesticides or other toxic organic matter. Environmental pollution caused by petroleum is of great concern because petroleum hydrocarbons are toxic to all forms of life. Environmental contamination by crude oil is relatively common because of its widespread use and its associated disposal operations and accidental spills. Soil contamination with hydrocarbons causes extensive damage of local system since accumulation of pollutants in animals and plant tissue may cause death or mutations.

Living matter is exposed to petroleum in many ways directly or indirectly. Some byproducts, formed during petroleum refining and processing which are used for the manufacturing of other products are highly toxic. Constantly, these toxic compounds are inadvertently released into the environment and if this effect is connected to the effect of accidental crude oil spills worldwide, then these combined sources of unrestricted hydrocarbons constitute the major cause of environmental pollution. Petroleum hydrocarbon molecules, which have a wide distribution of molecular weights and boiling points, cause diverse levels of toxicity to the environment. The toxicity of the hydrocarbon molecules and their availability for microbial metabolism depend on their chemical and physical nature. Petroleum is toxic and can

be lethal depending upon the nature of the petroleum fraction, the way of exposure to it, and the time of exposure (Madhavi, 2014).

2.1.3 Effects of Hydrocarbon Pollution on the Physicochemical Properties of Soil

Soil is a vital component, medium of unconsolidated nutrients and materials, forms the life layer of plants, it is a basic life support components of biosphere (Chaudhari, 2013). Soil biological activity, including soil microbial biomass and enzymatic activity, is influenced by a range of physicochemical, environmental parameters and perturbations (Labud *et al.*,, 2007).

Soil pollution comprises the pollution of soils with materials, mostly chemicals that are out of place or are present at concentrations higher than normal which may have adverse effects on humans or other organisms (Mittal and Singh, 2009). It is difficult to define soil pollution exactly because different opinions exist on how to characterize a pollutant; while some consider the use of pesticides acceptable if their effect does not exceed the intended result, others do not consider any use of pesticides or even chemical fertilizers acceptable (Khan *et al*., 2013). However, soil pollution is also caused by means other than the direct addition of xenobiotic (man-made) chemicals such as agricultural runoff waters, industrial waste materials, acidic precipitates, and radioactive fallout(Hui, 2011).

Heavy metals are naturally present in soils; however due to human activity, the concentration of heavy metals in soil is increasing. Some areas contain such a high concentrations of heavy metals and metalloids that they are affecting the natural ecosystem. It has been observed that hydrocarbon contaminated sites co-contaminated with heavy metals are difficult to bioremediate. The reason is that heavy metals and metalloids restrict microbe's

activity rendering it unable to degrade hydrocarbon or reducing its efficiency (Abha and Singh, 2012).

2.1.4 Public Health Significance of Hydrocarbon Pollution

At present, it is widely recognized that contaminated land or water systems are a potential threat to human health (Abha and Singh, 2012). The ever-increasing pollution of the environment has been one of the greatest concerns for science and the general public in the last fifty years (Peterson, 1994). The rapid industrialization of agriculture, expansion of the chemical industry, and the need to generate cheap forms of energy has caused the continuous release of man-made organic chemicals into natural ecosystems. Consequently, the atmosphere, bodies of water, and many soil environments have become polluted by a large variety of toxic compounds (Salanitro, 2001).Many of these compounds at high concentrations or following prolonged exposure have the potential to produce adverse effects in humans and other organisms: These include the danger of acute toxicity, mutagenesis (genetic changes), carcinogenesis, and teratogenesis (birth defects) for humans and other organisms. Some of these man-made toxic compounds are also resistant to physical, chemical, or biological degradation and thus represent an environmental burden of considerable magnitude ().

In 2010, the Deep water horizon oil rig owned by Transocean and leased to British Petroleum in the Gulf of Mexico had an “uncontrollable blowout”, killing about 11men, the oil spilled is about 4.9 million barrels causing the largest marine oil spill in the history of the petroleum industry. The damage to aquatic life continued for over four years (Viegas, 2013).

Chemicals and dispersants in crude oil can cause a wide range of health effects in people and wildlife, depending on the level of exposure and susceptibility. The highly toxic chemicals contained in crude oil can damage any organ system in the human body like the nervous system, respiratory system, circulatory system, immune system, reproductive system, sensory system, endocrine system, liver, kidney, etc. and consequently can cause a wide range of diseases and disorders (Costello, 1979).The damage caused by the toxicity of crude oil to organ systems may be immediate or it may take months or years (Singh *et al.*, 2004).

Awareness of this reality has led to international efforts to remediate many of these sites, either as a response to the health risks or to control the detrimental effects on the environment caused by contamination aiming the recovery of the contaminated sites (Abha and Singh, 2012).

2.2 Remediation

For decades efforts have been directed toward the evaluation of cost effective methods to cleanup oil contaminated soils (Abha and Singh, 2012). Over the years, many cleanup methods have been developed and applied. However, the remediation of oil contaminated environments is difficult because petroleum is a complex mixture of chemical compounds, and their degradation whether chemical or biological is not easy as different class of compounds needs different treatments (Hui, 2011). Numerous attempts are being made to decontaminate polluted soils, including an array of both *in situ* (on-site, in the soil) and *ex situ* (removal of contaminated soil for treatment) techniques. None of these is ideal for remediating contaminated soils, and often, more than one of the techniques may be necessary to optimize the cleanup effort (Harrison *et al.*, 2007). Furthermore, crude oil composition is reservoir dependent; therefore it is of great importance to know first the composition of the oil and the physicochemical nature of the

contaminated site before deciding the remediation strategies (Dhanjal and Cameotra, 2010). Crude oil degradation in the natural environment depends on several factors such as pH, chemical composition, and physical properties of the contaminated soil and/or water, among others (De *et al*., 2003). There exists some chemical, mechanical and biological methods to control oil spills, but most frequently applied are chemical methods because the chemical remediation of oil spills is faster ifcompared to mechanical and bioremediation. However, bioremediation is getting worldwide attention. In general the remediation of oil contaminated sites can be performed by two basic processes: in-situ and ex-situ treatment using different cleaning technologies, such as biological treatment, thermal treatment, chemical extraction and soil washing, and through aerated accumulation techniques (Haferburg and Kothe, 2007).

2.3 Bioremediation

Bioremediation is a "treatment that uses naturally occurring organisms to break down hazardous substances into less toxic or nontoxic substances". The use of indigenous microorganisms in bioremediation processes can reduce the risks associated with hydrocarbon contaminated soils (Suja *et al*.,, 2014).

Technologies can be generally classified as *in situ* or *ex situ*. *In situ* bioremediation involves treating the contaminated material at the site, while *ex situ* involves the removal of the contaminated material to be treated elsewhere. Some examples of bioremediation related technologies are phytoremediation, bioventing, bioleaching, landfarming, bioreactor, composting, bioaugmentation, rhizofiltration, and biostimulation. It may occur on its own (natural attenuation or intrinsic bioremediation) or may only effectively occur through the addition of fertilizers, oxygen, etc., that help encourage the growth of the pollution-eating

microbes within the medium (bio stimulation). In a non-polluted environment, bacteria, fungi, protists, and other microorganisms are constantly at work breaking down organic matter.

Nonetheless, bioremediation provides a technique for cleaning up pollution by enhancing the same biodegradation processes that occur in nature. Depending on the site and its contaminants, bioremediation may be safer and less expensive than alternative solutions such as incineration or landfilling of the contaminated materials. It also has the advantage of treating the contamination in place so that large quantities of soil, sediment or water do not have to be dug up or pumped out of the ground for treatment (Diaz, 2008). Microorganisms used to perform the function of bioremediation are known as bioremediators (biosurfactants fall under this group).

The efficient biodegradation of petroleum hydrocarbon often involves the manipulation of the environmental parameters to allow microbial growth and degradation to proceed at a faster rate, which include the availability of sufficient amount of oxygen or other electron acceptors, essential nutrients, the penetration depth of the hydrocarbon pollutants into the soil, and the nature of the soil (Ma, 1998).

According to Mohajeri*et al.*,(2010), bioremediation is not recommended for crude oil concentrations of 2000 mg/L or higher, lower concentration of crude oil demonstrated more efficient hydrocarbon removal. Besides, heavy crude oil is not dispersed as well as light and middle weight oils.

Whyte *et al.* (2001) assessed the bioremediation potential of three hydrocarbon-contaminated soil samples. Margesin and Schinner (2001) investigated the feasibility of bioremediation for a chronically diesel-oil-polluted soil in an alpine glacier area. They also examined the efficiencies of natural attenuation and biostimulation.

Van Hamme and Ward (2001) used mixed bacterial cultures to enhance crude oil biodegradation. They also studied physical and metabolic interactions of a *Pseudomonas sp.* and *a Rhodococcus sp*. strain during growth on crude oil and the effect of a chemical surfactant on them.

2.3.1 Biaugmentation

Bioaugmentation is the introduction of exogenous microorganisms to treatment facilities in order to enhance the degradation of organic pollutant (Watanabe, 2001). These pre-grown exogenous microorganisms have an affinity towards a specific contaminant. These microbes are suspended by a stabilizing agent and lie dormant in a spore until activated in solution and applied together with micronutrients and biostimulants. The purpose of seeding (introduction of exogenous microorganisms) is to increase the population of microorganisms that can biodegrade the spilled oil. Bacterial species that do not naturally exist in an area is added to the native population together with added nutrients (Hui 2011). Bioaugmentation can serve many functions in municipal waste-water treatment. This process improves treatment efficiency and reduces sludge production. A sufficient quality and diversify of microorganisms will allow a better processing of wastewater. The benefits of bioaugmentation are low operating and maintenance cost, production of a more thoroughly processed effluent containing fewer organic pollutants and less sludge to be treated and disposed of by the treatment plant (Ueno*et al.,* 2007). Bioaugmentation is particularly effective if indigenous microorganisms in a particular contaminated area are not capable of degrading the contaminants. However, the US EPA commented that bioaugmentation or seeding with native microorganisms do not result in faster biodegradation because hydrocarbon degrading bacteria exist almost everywhere and exogenous

species are often unable to compete successfully with native microorganisms (Norman*et al*, 2002).

In 1997, bioaugmentation using seed culture of petroleum-degrading bacteria, Terrazyme (Oppenheimer Biotechnology, Austin, Texas, USA) was attempted, on a trial basis on the coast of Japan facing the Japan Sea after a heavy oil spill from the Russian tanker Nakhodka. According to Tsutsumi *et al.,* (2000), Terrazyme was able to enhance the biodegradation of oil on the shore however such exogenous microbial products have never been widely used in Japan probably due to the unclear guidelines for bioremediation. On top of that, bioaugmentation using exogenous microorganisms were not enacted in Japan until 2004 (Hosokawa *et al.,* 2009). According to Hosokawa *et al.,* (2009), the efficiency of bioaugmentation is only during the early stages of the process. Hence, bioaugmentation treatment must be adjusted intermittently with the addition of more microorganisms. As an alternative to the survival and the xenobiotic degrading ability of introduced microorganisms, autochthonous bioaugmentation (ABA) is proposed by Hosokawa *et al.,* (2009) to overcome these difficulties.

ABA method is a bioaugmentation technology that contains the data of microorganisms indigenous to the contaminated site or predicted contamination site. Indigenous microorganisms of that particular contaminated site will be enriched under conditions where bioaugmentations will be conducted. For these reasons information in advance on the chemical and physical characteristics of potential oil spill sites and type of oils that might be spilled is needed. Hosokawa *et al.,* (2009) also claimed that it is not impossible to predict location of oil spills. Areas close to oil fields are threatened with many types of oil spills, such as eruption of oil wells and spills from storage tanks and pipelines. The most recent oil wells leakage incident in 2010 was Deepwater Horizon Spill that contaminated the Gulf of Mexico. The advantages of ABA

method to decontaminate oil polluted lands, coasts and waters are shorter treatment time, greater potential efficiency, lower impact on the environment, and relative ease in obtaining public support.

Rahman *et al.* (2003) investigated possible methods to enhance the rate of biodegradation of oil sludge from crude oil tank bottom through bioaugmentation and biostimulation.

2.3.2 Biostimulation

Biostimulation involves aeration and the application of selected micronutrients and biostimulants such as phosphorus, nitrogen or biosurfactant to a contaminated environment to stimulate the growth of the microorganisms that break down the contaminants (Kim, *et al.,* 2005). According to Neralla *et al.*,(1995), inorganic nutrient supplementation may speed up the process, because the addition of large quantities of oil results in a high C: N ratio that is unfavourable for microbial activity. Biostimulation is effective if high indigenous microbial population is present in the contaminated site. When nutrients are added, the native microbial population can grow rapidly, potentially increasing the rate of biodegradation. The two nutrients most likely to limit microbial population in sea water are nitrogen and phosphorus (Capone & Bauer, 1992). The advantage of biostimulation is that bioremediation has been undertaken with the native microorganisms that are well suited to the subsurface environment, and are well distributed specially within the sub surface. The primary disadvantage is that the delivery allows the additives to be readily available to the subsurface microorganisms which are based on the local geology of the subsurface. Tight clays or other fine-grained materials make spreading additives throughout the hydrocarbon polluted area difficult. Fractures in the subsurface create preferential pathways in the subsurface where additives will flow through, hence preventing the even distribution of additives. In a nutshell, bioavailability of a compound to the microbes is

determined by the rate of mass transfer of the substrate to the microbial cells in relation to intrinsic catabolic activity (Johnsen *et al.*, 2005). Proof of the effectiveness of biostimulation as an oil spill cleanup technology was developed on the shoreline of Delaware Bay in 1994 (US EPA,1999). This EPA-funded study, which involved an intentional release of light crude oil into small plots, demonstrated a several-fold increase in the biodegradation rate due to the addition of fertilizer as compared to the unfertilized control plots (US EPA, 1999).

Another successful case was during the Exxon Valdez oil spill cleanup and restoration activities, the Alaska Regional Response Team authorised the use ofbioremediation products, including biostimulation and bioaugmentation (US EPA,1999). Addition of nutrients was approved for 100 miles of Prince William Soundshoreline. Data collected through a monitoring protocol required by the State ofAlaska indicated that nutrient addition accelerated the natural degradation of oilwithout observed eutrophication or toxicity (US EPA, 1999).

Effectiveness of biostimulation is also highly site-specific. When oxygen is not a limiting factor, one of the key factors for success is to maintain an optimal nutrient level in the interstitial pore water. In other words, background nutrient concentrations at the contaminated site should be a determining factor in the decision to apply bioremediation, and biostimulation might not always be necessary if sufficient nutrients are naturally present at a spill site in high enough concentrations to permit effective microbial treatment (Hui, 2011).

2.4 Micobial Genera Involved in Bioremediation

Microorganisms in the soil include bacteria fungi, algae and protozoa. The bacteria are most abundant in the soil and can be heterotrophic (requiring complex organic compounds of nitrogen and carbon (as that obtained from plant or animal matter) for metabolic synthesis) or autotrophic (requiring only carbon dioxide or carbonates as a source of carbon and a simple

inorganic nitrogen compound for metabolic synthesis of organic molecules (as glucose) in their metabolism. Heterotrophic bacteria are the most important organisms in the transformation of organic compounds and the purpose of engineered bioremediation is to enhance their activity.

In Nigeria, Ijah (1998) investigated the relative degradation ability of two bacterial species Serratia marcescens OCS-21 and Acinetobacter calcoaceticus COU-27 isolated from oil polluted soil of Rivers State and a yeast species Candida tropicalis PFS-95 isolated from unpolluted soil to degrade crude oil. Mcnally *et al.,* (1998) isolated two pseudomonad strains from PAHs- contaminated sites and a third one from uncontaminated site. They used the isolated pseudomonad strains for the biodegradation of three and four-ringed PAHs under anaerobic denitrifying conditions.

Bartha and Atlas (1977) listed 22 and 47 genera of oil degrading bacteria and fungi respectively. They reported that the most important hydrocarbon-degrading bacterial genera in aquatic environments were *Pseudomonas, Achromobacter, Arthrobacter, Micrococcus, Nocardia, Vibrio, Acinetobacter, Brevibacterium, Corynebacterium* and *Flavobacterium.*

Based on the number of published reports, the most important hydrocarbon-degrading bacteria in both marine and soil environments are *Achromobacter, Acinetobacter, Alcaligenes, Arthrobacter, Bacillus, Flavobacterium, Nocardia*, and *Pseudomonas*species (AbdelRahman, 2011).

Survery *et al.,* (2004) isolated and identified from Pakistani soil sixty degrader bacterial strains using four solid hydrocarbons and four liquid hydrocarbon (biphenyl, camphor, phenantheren and naphthalene benzene, toluene, octane and heptane) as source of carbon. The sixty isolates belong to different genera including *Staphylococcus, Corynebacterium, Bacillus, Pseudomonas, Proteus, Klebsiella* and *Escherichia.*

Sathishkumar *et al.,* (2008) isolated 57 oil-degrader bacteria and determined the optimal conditions for biodegradation of a crude oil for the best degrader strains. They named several genera including *Bacillus, Corynebacterium, Flavobacterium, Micrococcus, Moraxella,* and *Pseudomonas*isolated from hydrocarbon contaminated sites.

Eze and Eze (2010) carried out isolated and characterizated microorganisms involved in the degradation of refined petroleum products polluted sites in Elele.

Kaczorek and Olszanowski (2011) evaluated the effects of exogenous bacterial consortia surfactants on hydrocarbon biodegradation and on cell surface properties. The most effective amongst all, as regards biodegradation, were the consortia of *Pseudomonas*spp. and *Bacillus* spp. strains

Ibiene *et al.,* (2011) reported that 'The effect of organic fertilizers (spent mushroom, cow dung and poultry droppings) on the bioremediation of hydrocarbon contaminated soil was investigated on a 28 days study period. The hydrocarbon contaminated soil was supplemented with the different organic fertilizers and analyzed throughout the study period. Physicochemical and microbiological parameters like soil pH, moisture content, phosphate, nitrate, % total organic carbon (%TOC), total petroleum hydrocarbon (TPH) total heterotrophic counts, total hydrocarbon utilizing counts (bacteria and fungi) were studied from baseline to the 28th day. The concentrations of phosphate, nitrate and percentage TOC of the treatments decreased significantly during the study period whereas the controls slight decrease in the parameters. pH values of the treatments were within slight acidity to alkalinity as the control had acidic pH range during the study period. The percentages of TPH loss in the cow dung option poultrydroppings option spend mushroom option and control were 97.83%, 98.21%, 99.91% and 27.52%. The hydrocarbon utilizing bacterial isolates from the study include *Bacillus, Pseudomonas, Kebsiella,*

Proteus, Flavobacterium, Clostridium, Micrococcus, Acinetobacter. The hydrocarbon utilizing fungal isolates from the study were *Penicillium, Aspergillus,Sacharomyces, Rhizopus, Fusarium* and *Mucor*. This study showed that cow dung, spend mushroom and poultry droppings are effective nutrient sources for bioremediation'.

2.5 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a common Gram-negativerod-shapedbacterium that can cause disease in plants and animals, including humans.It is found in soil, water, skin flora, and most man-made environments throughout the world. It thrives not only in normal atmospheres, but also in hypoxic atmospheres, and has, thus, colonized many natural and artificial environments. It is also able to decompose hydrocarbons and has been used to break down tar balls and oil from oil spills. (Itah, *et al.*, 2005). It is aerobic (and can be facultative anaerobic), bacillus with unipolar motility (Ryan, *et al.*, 2004)

In certain conditions, *P. aeruginosa* can secrete a variety of pigments, including pyocyanin (blue-green), pyoverdine (yellow-green and fluorescent), and pyorubin (red-brown). These can be used to identify the organsim. (King, *et al.*, 1954). *P. aeruginosa* is often preliminarily identified by its pearlescent appearance and grape-like or tortilla-like odor *in vitro*. Definitive clinical identification of *P. aeruginosa* often includes identifying the production of both pyocyanin and fluorescein, as well as its ability to grow at 42 °C. *P. aeruginosa* is capable of growth in diesel and jet fuels, where it is known as a hydrocarbon-using microorganism, causing microbial corrosion. (Striebich, *et al.*, 2014). It creates dark, gellish mats sometimes improperly called "algae" because of their appearance (Brown, 1956).

P. aeruginosa is a facultative anaerobe, as it is well adapted to proliferate in conditions of partial or total oxygen depletion. This organism can achieve anaerobic growth with nitrate or nitrite as a terminal electron acceptor. When oxygen, nitrate, and nitrite are absent, it is able to ferment arginine and pyruvate by substrate-level phosphorylation(Schobert, *et al.,* 2010).

The outer membrane of Gram-negative bacteria is composed of lipopolysaccharides (LPS), phospholipids and lipoproteins, covalently linked to the peptidoglycan layer through hydrophobic interactions (Sikkema *et al.,*,1995) and also contains porins and efflux pump embedded in the LP Slayer. In *Pseudomonas aeruginosa*, four major (OprF, OprP, OprB, OprD) and two minor (OprC, OprE) outer membrane proteins (OMPs) were reported to act as porins (Denyer and Maillard, 2002). A number of studies suggest that it is possible to modify the outer membrane of Gram-negative bacteria by mutation (Yoneyma *et al.,* 1995; Nikaido, 2003; Ni and Chen, 2004) or by addition of chemical agents that can act as membrane permeabilizing agents (Daugelavicius *et al.,* 2000; Denyer and Maillard, 2002; Longbottom *et al.,*., 2004). These processes result in changes of the cell surface properties as well as in increased permeability and hydrophobicity of the bacterial membrane. Changes in cell surface properties were observed also in presence of surfactants and biosurfactants, surface active compounds with amphiphilic structure (Zhang and Miller, 1994; Al-Tahhan *et al.,*2000; Mobius *et al.,* 2001). The biosurfactants that were studied most extensively in terms of their industrial and environmental application are the rhamnolipids. Nevertheless little is known about their interaction with bacterial cells, although it is likely that their surface and membrane active properties play an important role (Lang and Wullbtandt, 1999; Singh and Cameotra, 2004). The rhamnolipid-biosurfactant was produced by bacterial strain *Pseudomonas* sp. PS-17 (Shulga *et al.,* 2000). The low parameters for surface and interfacial tensions and critical micelle concentration of the

rhamnolipid-biosurfactant indicate its high surface activity.*P. aeruginosa*produces two major types of rhamnolipid in liquid cultures: the monorhamnolipid, rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-C10-C10) and the dirhamnolipid, rhamnosyl-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-Rha-C10-C10). Besides these, twenty-five rhamnolipid congeners have been described in *P. aeruginosa*, varying in chain length and/or extent of saturation, showing that the addition of a hydrocarbon chain to dTDP-L-rhamnose is not specific to the carbon chains (Mulligan, 2004)

Norman *et al.,* (2002) studied variation of lipopolysaccharide expression in two *Pseudomonas aeruginosa* strains.

Rahman *et al.,* (2002a) determined optimal conditions for biodegradation of Bombay High crude by 130 oil degrading bacterial cultures isolated from soils contaminated with gasoline and diesel spilled hydrocarbons. The crude oil degraders they isolated belonged to the genera *Micrococcus, Corynebacterium, Bacillus, Enterobacteriaceae, Pseudomonas, Alcaligenes, Flavobacterium, Moraxella, Aeromonas, Acinetobacter* and *Vibrio.*

Okoh (2003) determined biodegradation of Bonny light crude oil in soil microcosm by four *Pseudomonas aeruginosa* hydrocarbon degrading strains isolated from crude oil pits in Nigeria.

2.6 Factors Affecting Bioremediation

Bioremediation generally occurs when the microorganisms use the pollutant as a carbon source. Hence degradation is accompanied by microorganism growth. An efficient degradation is dependent on the presence of other required nutrients, including nitrogen, phosphorus and so on.

Suitable environmental conditions, with respect to pH, temperature, moisture content and redox potential are also required (AbdelRahman, 2011).

2.6.1 Hydrocarbon Variety and Concentrations

Hydrocarbon variety and concentrations are factors that affect biodegradation. Those with a low molecular weight are relatively easy to biodegrade. Branched hydrocarbons degrade more slowly than the corresponding straight-chain hydrocarbons. Generally, when molecule size increases, the rate of biodegradation decreases, and mono aromatic compounds are more rapidly degraded than the two, three, four and five ring compounds. Comparatively lighter mixtures such as gasoline can be readily biodegraded to low levels. Heavier products or coal tar which contains many heavy molecular compounds, degrade much more slowly than gasoline. The concentration of hydrocarbon can affect the bioactivity and be toxic to the microorganisms. High concentration of hydrocarbons can be inhibitory to microorganisms, thus slowing down the remediation rate. At highly elevated concentration, contaminant can become toxic for microorganisms (Ma, 1998)

2.6.2 Microbial Factors

Many microorganisms are able to degrade petroleum hydrocarbons. They are present in contaminated soil and water. Most of them are aerobic organisms and can make use of organic contaminants for their growth. Since individual organisms can metabolize only a limited range of hydrocarbon substrate. It is necessary to assemble several bacteria with a broad catabolic potential which has the ability to dissimilate or break down complex organic molecules and release energy, in order to degrade the complex mixture of hydrocarbons that may affect a contaminated site. Banat (2000) used pure and mixed *Pseudomonas* cultures to degrade chlorobenzene, toluene, xylene, and ethanol. The results showed that a mixed culture made of

three strains demonstrated more stable growth behavior and degraded contaminants to much lower concentrations than pure cultures. Natural soil microorganisms may not have the metabolic capability to readily degrade certain compounds, and seeding of microorganisms into the soil has been performed to enhance the process of bioremediation (bioaugmentation). Generally, natural soil microorganisms have been previously isolated and enriched as a "seed". They are added during in-situ treatment, thus increasing the biomass and reducing the time necessary for remediation (Abha and Singh, 2012) .

2.6.3 Soil Structure

Soil structure controls the transmission of water, oxygen, and nutrients to the area of bioactivity. Generally, fine particles such as clay and silt transmit these substances slowly. Permeable soils, such as sands and gravels, are more favorable to nutrient transport and relatively rapid clean up can be achieved. Characteristics of the soils, such as composition, particle size distribution, percent moisture content, percent organic and cation exchange capacity may also be important for the remediation of contaminants (Ma, 1998).

2.6.4 Soi1 Moisture

According to Ma, 1998, microorganisms require water for microbial growth and for diffusion of nutrients and by products during the degradation process. If the soil is too dry, many microorganisms will die. If water content of the soil is too high oxygen transfer to microorganisms was resisted by the flooded soil and the rate of the hydrocarbon degradation was reduced. The optimum soil water content for bioremediation is dependent on the soil type. Generally, the optimum activity occurs when the soil moisture is 50-80% of the field capacity, also termed the water holding capacity which is defined as "the amount of the water remaining

within the soil after gravitational water has drained away" or the percentage of water in a soil when it was saturated (Hui, 2011). When moisture content is lower than 10% of the holding capacity, the bioactivity becomes marginal.

2.6.5 Oxygen

Bacteria activity proceeds more rapidly if sufficient oxygen is provided. During aerobic biodegradation, molecular oxygen is reduced to water while petroleum hydrocarbon is oxidized to create energy, cell mass, and carbon dioxide. The supply of oxygen to the scene of microbial activity is controlled by soil saturation and conduction. Ma, (1998) reported that the requirement of oxygen to degrade hydrocarbon is 3.1g of oxygen for 1.0g of hydrocarbon. The largest amount of oxygen required is approximately 200,000ppm in a well aerated soil and 8ppm in a saturated soil. Brown *et al.*,(1984) developed several projects where oxygen supply was identified as a critical point if the processes are to be generally applicable. This demonstration led to use hydrogen peroxide as an oxygen carrier (Brown *et al.,* 1984). Increasing oxygen availability by treating the soil with dilute hydrogen peroxide H_2O_2, at a concentration up to 1000 mg/l has been successfully tried.

Dua *et al.*,(2000) evaluated the utilization of hydrogen peroxide for enhanced biological treatment of petroleum hydrocarbon contaminated soil in laboratory. JP-5, Diesel fuel and lubricating oil were used as model petroleum hydrocarbons. The concentration of the H_2O_2 was approximately 500ma. Biotreatment was monitored by bacteria population density and concentration of petroleum hydrocarbons. Results showed enhanced removal of the petroleum hydrocarbon after comparing control sample and test sample. Soil venting is a method that provides oxygen to the contaminated area by introducing air into the vadose zone in order to increase the activity of native bacteria and allow them to degrade the contaminants (Ma, 1998).

2.6.6 Temperature

Soil temperature is another factor which can affect microbiological activity and the rate of the contaminant decomposition. Generally, a high temperature induces a high rate of biological degradation processes in the soil.Very low rates of hydrocarbon utilization were found by Banat (2000) at low temperature because low temperature leads to a slow rate of microbial growth. The rate of degradation can double for every 10°C rise in temperature. Most soil microorganisms have an optimal growth for temperature in the range of 20 to 35. The majority of organisms that degrade petroleum products are active in this range. Enrichments of thermophilic microorganisms have an optimal temperature for degradative activity comprised between 50°and 60°C (Ma, 1998).

2.6.7 Nutrients

Most microorganisms existing in the subsurface are part of an ecosystem that has low organic carbon content. The heterotrophic microorganisms found in soils possess the ability to degrade petroleum products but they require nutrients to grow. Nitrogen and phosphorous are the most common nutrients for bacteria. Other nutrients required for bacteria metabolism are potassium, magnesium, calcium, sulphur, sodium, rnanganese, iron, and trace metals (Ma, 1998)
Soil is made up of three main things: clay, humus and sand. There are also many small organisms that live in the soil, and most of these are useful to the plants.Clay is made from the breakdown and recombination of silicate rocks. It is made up of alternating layers of silicon oxides then aluminum oxides, with various cations such as Ca^{2+} loosely bound in between the layers. Anions adsorb onto the oxide surfaces, with doubly and triply charged cations sticking better than singly charged ones. Clays are the main source of nutrients in the soil. Humus is any

organic matter in the soil - i.e. the products of the decay of plants and animals. It is mostly made up of aromatic compounds. Over time it breaks down to carbon dioxide and water so it needs to be continually replaced. Humus is important in regulating the amount of water in the soil. Sand is solid particles of ground up rock. A small amount of sand is necessary to ensure the correct water content in the soil. (Curtis and Childs 2014)

2.6.8 pH Value

Biological activity in the soil can be affected by the pH. Some microorganisms can survive in a wide range of pH, but others are sensitive to small variations. The bacteria grow better in pH values between 6.5 and 8.5 (Dibble and Bartha 1979). Bioremediation is therefore favored by near neutral pH values (6-8). Soil pH can be adjusted if necessary to enhance microbial activity (Ma, 1998)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

(a) Equipment

- Sand bath (Gallenkamp)
- Atomic Absorption spectrophotometer (Unicom)
- Magnetic Stirrer hot plate (Gallenkamp)
- PW 4030 X-ray spectrophotometer
- Thermometer (Pyrex, England)
- Funnel (Chemex, England)
- Tape rule (Yuebao, China)
- Autoclave (Gallenkamp, model 1880R)
- Incubator (Gallenkamp, model IH-150)
- Sieves (Endecotts, England)
- Weighing balance (Atco, India)
- Soil Auger (Yonkang, China)
- Desiccator(Gallenkamp)
- pH meter (model pH s-25)
- Filter paper (Watman, England)
- Micro-filters (ADSL, USA)
- Retort Stand (Esel, India)

(b) Glassware

- Glass rod (Pyrex, England)
- Kjeldahl Flask (Pyrex, England)
- Measuring cylinder (Kinax, USA)
- Glass Beakers (Pyrex, England)
- Slides (Pyrex, England)
- Conical Flask (Pyrex, England)
- Test tubes (Pyrex, England)
- Petri dishes (Pyrex, England)
- Plastic beakers (Labplex, England)
- Pipette (Pyrex, England)
- Round Bottom Flask (Pyrex, England)
- Volumetric Flask (Pyrex, England)

(c) Chemicals and Reagents

- Ascobic Acid (Bayer)
- Ammonium Molybdate (Brakem)
- Anhydrous Na_2S (J.J Chemicals)
- Boric Acid (Continvest)
- Cetrimide agar (Bayer)
- Conc. $NaNO_3$(Megmani)
- Conc. Sulphuric Acid (Nauli Sumberdaya)
- Conc. NaCl (Jayam International)

- Conc. KH2PO4 (Bayer)
- Conc. KCl (Jayam International)
- Conc. Na2HPO4.12H2O (Nauli Sumberdaya)
- Conc. MgSO4 (Megmani)
- Ferrous Sulphate (Bayer)
- FeSO4.7H2O (Jayam International)
- Oslens reagent (Standard Surfactants)
- Oxidase reagent (Standard Surfactants)
- Phenylanthranilic acid (Bayer)
- Potassium Chloride (Jayam international)
- Potassium Permanganate (Standard Surfactants)
- Potassium dichromate solution (Standard Surfactants)
- Simmons Citrate (Bayer)
- Sodium Hydroxide (Bayer)

3.2 Collection and Processing of Soil Samples

Soil samples were collected from Baba Kaduna mechanic workshops located in Sabon Gari Local Government, Kaduna State. The samples were collected from 20cm layer using soil auger (Okoh, 2003; Ojo, 2006). The samples were placed in new black polythene bags and transported to the Department of Water Resources and Environmental Engineering, Ahmadu Bello University, Zaria under ambient conditions.

The soil samples thus collected were air dried, crushed and passed through 2mm sieve to remove pebbles and other large debris. The processed soil samples were placed in new black polythene bags and stored under ambient laboratory conditions (37^{o}C) for analysis (Okoh, 2003).

3.3 Preliminary Analysis of Soil Samples

Preliminary analysis was carried out to determine the physico-chemical properties of the soil samples that served as source of *Pseudomonas aeruginosa* that was used in the study.

3.3.1 Determination of Moisture Content

A container was dried and cooled in a desiccator and weighed. About 1g of the processed soil sample was then placed in the container and the weight of both was taken. The soil sample was dried in a hot air oven at 105^{o}C to constant weight (for at least three hours). The sample and container was cooled in desiccator and weighed again. The difference in weight after drying was used to calculate the moisture content by applying the formula below (Joel and Amajuoyi, 2009).

$$\text{Moisture (\%)} = \frac{\text{Loss in weight on drying (g)}}{\text{Initial sample weight (g)}} \times 100$$

3.3.2 Determination of Textural Class of the Soil

Fifty (50) gram of the soil sample was transferred into 500ml plastic bottle and 100ml of dispersion solution was added to the sample shaken on a mechanical shaker to obtain a homogenous solution. The soil solution was then transferred into a 1000ml glass measuring cylinder and the solution was made-up to 1000ml by adding distilled water and shaken, after which first pipetting was done using a 50ml pipette at 10cm depth and transferred into a 60ml petri dish. Second pipetting was done in same manner and transferred into another petri dish. The

remaining soil solution was transferred into a 1 litre measuring cylinder through a 0.02mm sieve and the material was washed through the sieve using jet water. Sand particles on the sieve were collected in a petri dish. The pipetted solutions in the dishes were dried overnight in an oven at 105^{o}C, cooled in a desiccator and weighed quickly. The weight of fine sand was determined by deducting the weight of clay, silt and sand particle from 100 (ISRIC, 2002).

3.3.3 Determination of pH

About 20g of the soil sample was weighed into a beaker and 50ml of distilled water was added. Thereafter, the mixture was vigorously stirred for a few minutes. The suspension was then allowed to stand for further 15 minutes, after which the electrode of a calibrated pH meter was immersed into the mixture and the reading was taken (Joel and Amajuoyi, 2009).

3.3.4 Determination of Electrical Conductivity

To determine the soil electrical conductivity, the conductivity cell method as described by Raymnent, 1992 where a 1:5 soil:water suspension was prepared by weighing 10 g air-dry soil (<2 mm) into a bottle. 50 mL of deionised water was added and was mechanically shaken at 15 rpm for 1 hour to dissolve soluble salts. The cell constant was obtained by calibrating the conductivity meter according to the manufacturer's instructions using the KCl reference solution. The cell was rinsed thoroughly and the electrical conductivity of the 0.01M KCl was measured at the same temperature as the soil suspensions. The conductivity cell was rinsed with the soil suspension and refilled without disturbing the settled soil. The value indicated by the conductivity meter was recorded as the electrical conductivity of the sample (APHA, 1995). The cell was constantly rinsed with deionised water between samples.

3.3.5 Determination Total Organic Content

One (1) gram of the air dried soil sample was weighed out in duplicate and transferred into a 250ml Erlenmeyer flask. Ten (10) ml of potassium dichromate solution and 20ml of concentrated sulphuric acid was added and the flasks swirled until the soil and reagents are mixed. The flasks were allowed to stand on a sheet of asbestos for about 30 minutes after which 100ml of distilled water was added. A blank was also prepared in the same manner without soil. Three drops of phenylanthranilic acid indicator was added and the preparation was titrated with 0.5N ferrous sulphate solution. The end point was observed when the colour changes rapidly from blue to red (maroon colour) in reflected light against a white background (ISRIC, 2002).

3.3.6 Determination of Total Nitrogen Content

Alkaline permanganate method was used to determine the total nitrogen content of the soil. Here, 20g of the soil sample was transferred into a round bottom flask, 3 glass beads and 1ml of liquid paraffin was also added. Then, 100ml of both potassium permanganate and sodium hydroxide was added to the flask. The mixture was distilled and the distillate was collected in a beaker containing 20ml of boric acid working solution. Approximately 150ml of distillate was collected and titrated with 0.02N standard H_2SO_4 till the colour changes from green to red (APHA, 1995). Then a blank was carried out without soil.

3.3.7 Determination of available Phosphorous

Oslen's method was used to determine the available phosporous. Here, 2.5g of the soil sample was transferred into a conical flask, and 0.3g of phosphate free activated charcoal grade was added. 50ml of Oslen reagent was added and the mixture was shaken for 20 minutes on a shaker at 180rpm. The contents were filtered immediately through filter paper and 5ml of aliquot

was transferred into a 25ml volumetric flask. Then 4ml of freshly prepared ascorbic and ammonium molybdate solution was added and the shaken for 30 minutes. A blank was prepared, and the absorbance measured at 882nm after half an hour (Oslen and Sommers 1982). The available phosphorus was obtained using the following formula

$$\text{Available phosphorus (ppm)} = \frac{GR \times 50 \times 5}{\text{Corrected Ht. of Soil}}$$

where,

GR = Concentration of Phosphorus in analysed sample (from standard curve).

3.3.8 Determination of available Calcium

A standard calcium solution is prepared by dissolving 2.4972 grams of oven-dried reagent-grade calcium carbonate in a solution containing approximately 500 milliliters of distilled water and 8 milliliters of concentrated hydrochloric acid. This solution is diluted to 1,000 milliliters with 0.10 hydrochloric acid and contains 1,000ppm of calcium. The 1,000ppm calcium solution serves as a stock solution from which other standard calcium reference solutions are prepared. Five standard reference solutions containing 0, 50, 100, 150, and 200ppm of calcium are prepared by measuring 0, 25, 50, 75, and 100 milliliters of the solution containing 1,000ppm of calcium and transferring these to 500-milliliter volumetric flasks. The calcium standards were diluted to a volume of 500 milliliters with 0.1% hydrochloric acid.

The concentration of calcium in the soil solution extract is determined by means of a Beckman Model DU Flame Spectrophotometer. The percent transmittance of each of the calcium standard reference solutions and the soil solution extract is measured according to the operating procedure described in Section II of the Instruction Manual for the Beckman Model DU Flame

Spectrophotometer (1957). The amount of calcium in the soil solution extract is determined by referring to a calibration curve obtained by plotting the percent transmittance readings against the calcium concentration of the five reference solutions (APHA, 19995).

3.3.9 Determination of available Magnesium

A standard magnesium solution is prepared by dissolving one gram of reagent-grade magnesium metal in dilute hydrochloric acid solution containing 400 milliliters of distilled water and 20 milliliters of concentrated hydrochloric acid. This solution, diluted to 1,000 milliliters with distilled water, contains 1,000 parts per million of magnesium. A solution containing 100ppm of magnesium is prepared by diluting exactly 100 milliliters of the 1,000-parts-permillion magnesium solution to 1,000 milliliters with distilled water. The solution containing 100ppm of magnesium serves as a stock solution from which other standard magnesium reference solutions are prepared. Five standard reference solutions containing 0, 5, 10, 15, and 20ppm are prepared by measuring 0, 25, 50, 75, and 100 milliliters of a solution containing 100ppm of magnesium and transferring these to 500-milliliter volumetric flasks. The magnesium standards are diluted to a volume of 500 milliliters with a 0.10 normal hydrochloric acid solution.

The concentration of magnesium in the soil solution extract is determined with the use of a Perkin-Elmer Model 303 Atomic Absorption Spectrophotometer. The percent absorption of each of the magnesium standard reference solutions and the soil solution extract is measured according to the operating procedures described in Section II, B, page 12 of the Instruction Manual for the operation of the Perkin-Elmer Model 303 Atomic Absorption Spectrophotometer (1964). The percent absorption of each of the magnesium standard solutions and the soil solution extract is converted to absorbance by using Table III on page 7 of the instruction manual. The

amount of magnesium present in the soil solution extract is determined by referring to a calibration curve obtained by plotting the absorbance readings against the magnesium concentration of the five reference solutions (APHA, 19995).

3.3.10 Determination of available Potassium

A standard potassium solution is prepared by dissolving exactly 0.9530 gram of oven-dried reagent-grade potassium chloride in 100 milliliters of distilled water. Dilute to 1,000 milliliters with a 0.10 normal hydrochloric acid solution. This solution contains 500ppm of potassium and serves as a stock solution from which other standard potassium reference solutions are prepared. Five standard reference solutions containing 0, 5, 10, 20, and 25ppm are prepared by measuring 0, 5, 10, 20, and 25 milliliters of the solution containing 500ppm of potassium and transferring them to 500-milliliter volumetric flasks. The potassium standards are diluted to a volume of 500 milliliters with a 0.10 normal hydrochloric acid solution.

The concentration of potassium in the soil solution extract is determined by means of a Beckman Model B Flame Spectrophotometer. The percent transmittance of each of the potassium standard reference solutions and the soil solution extract is measured according to the operating procedure described in Section D of the Instruction Manual for the Beckman Model B Flame Spectrophotometer (1957). The amount of potassium in the soil solution extract is determined by referring to a calibration curve obtained by plotting the per cent transmittance readings against the potassium concentration of the five reference solutions (APHA, 19995).

3.1.2.10*Determination of oil and grease content*

The Partition – Gravimetric method was used for the analysis of oil and grease. Fifty millilitres (50ml) of each sample was acidified to pH 2.0 using 5ml of hydrochloric acid, shaken

vigorously for three minutes. The sample was transferred into a separating funnel held by a retort stand, and then 15ml of hexane was used to carefully rinse the beaker before pouring the sample into the funnel which would separate the content into layers of oil, grease and water. The water being of higher density was collected below leaving oil and solvent at the surface. Twenty grams of anhydrous sodium sulphate was added to the filter cone and the emulsified solvent was collected. Extraction was done twice using 15ml of the organic solvent in each process. An additional 20ml of solvent was used to wash the filter paper and evaporation was carried out using water bath until all the solvent evaporate. The flask was transferred to desiccators before weighing (APHA, 19995). The oil and grease content was determined as follows:

$$\frac{A - B \times 1000\text{ml of oil and grease}}{\text{ml sample oil + grease (mg/l)}}$$

Where, A and B are the initial and final weight of the flask and its contents.

3.4 Isolation of *P. aeruginosa* from the Soil Sample.

3.4.1 PreparationMineral Salt Medium (MSM)

Mineral Salt Medium (MSM) with the following composition per litre: KH_2PO_4 (2.0g), $NaNO_3$ (2.0g), NaCl (0.8g), KCl (0.8g), $Na_2HPO_4.12H_2O$ (2.0g), $MgSO_4$ (0.2g), $FeSO_4.7H_2O$ (0.001g) was prepared in distilled water. The preparation was enriched with 1% (v/v) of engine oil and 20g of agar was added for solidification. It was then sterilized by autoclaving at 121^oC for 15 minutes. The preparation was allowed to cool to about 50^oC and dispensed into sterile petri dishes and allowed to solidify (Eniola *et al.,* 2014).

3.4.2 Inoculation

About 1g of the processed soil samples was serially diluted using sterilized distilled water. Then 0.1ml of the serially diluted sample was inoculated onto the prepared medium using spread plate method and incubated at ambient temperature for 48 hours (Naga *et al.*, 2016). The size, shape, margin consistency and pigmentation of the resulting colonies were examined (Ekhaise and Nkwelle, 2011).

3.5 Characterization of *Pseudomonas aeruginosa* from the Soil Sample

3.5.1 Gram Staining

The resulting distinctive colonies from the inoculation procedure as described in 3.4.1 were Gram stained and examined microscopically to determine their cellular morphology and Gram reaction. Briefly, a smear was prepared from the 18 - 24 hours culture. A loopful of well separated colony (pure culture) was placed directly into a drop of normal saline on a clean, dried, grease-free slide, smeared and then allowed to air-dry. The smear was then heat-fixed by passing the slide over a Bunsen flame for three quick successions.The slide was flooded with crystal violet solution and allowed to stand for one minute, then washed with slow-running tap water and then flooded with Gram's iodine (mordant) and allowed for one minute. The slide was washed with tap water and decolourize with 95% alcohol for 30seconds. The slide was rinsed with tap water and then counter-stained with safranin for another 30seconds. The slide was finally rinsed with slow-running tap water, allowed to air-dry and then examined microscopically under oil immersion objective lens after adding a drop of oil immersion (Cowan and Steel, 2003).

Then Gram negative rod isolates were inoculated onto nutrient agar slant and preserved in the refrigerator at 4-5^{o}C for further identification tests and analysis.

3.5.2 Biochemical Characterization of the Isolates

The isolates were biochemically characterized following the scheme described by Cowan and Steel (2003) which include:

3.5.2.1 *Motility Test*

This test was carried out by inoculating the motility medium with the 18 hours old culture of the isolates. A stab was made with a straight wire to a depth of about one third the total volume of the medium. The culture was then incubated at 35^{o}C for 24hrs. When the culture turns cloudy (turbid) after incubation, it means the organism was motile but when growth was restricted to the line of inoculation and the rest of the culture remained clear, then the organism was non-motile (Cowan and Steel, 2003).

3.5.2.2 *Citrate utilization test*

This test was carried out by inoculating the 18 hours old culture of the isolates on Simmons' citrate agar slant and then the inoculated slant was incubated at 35^{o}C for 48-72 hours and examined for development of a deep blue colour which indicates a positive reaction (Cowan and Steel, 2003).

3.5.2.3 *Urease test*

Urease test was carried out by inoculating urea agar slant with the colonies of the isolates. The slant was then incubated at 35^{o}C for 48-72 hours and examined for the development of bright pink or red colour indicates a positive reaction (Cowan and Steel, 2003).

3.5.2.4 *Nitrate reduction test*

This was carried out by inoculating Nitrate Broth lightly with colonies of the isolates. The inoculated broth was then incubated at 37^{o}C for 24-48hrs. One (1) ml of reagent A (sulphanilic acid) was added followed by 1 ml of reagent B (α-napthol amine) and examined. A deep red colour showed the presence of nitrite and thus, showed that nitrate has been reduced and indicates a positive reaction.

To tubes not showing a red colour within 5 min, powdered zinc (up to 5 mg/ml of culture) was added, allowed to stand and then examined for red colour which indicates nitrate present in the medium (i.e. not reduced by the organism) and absence of red colour indicates nitrate absent in the medium (i.e. reduced by the organism to nitrite, which in turn was itself reduced) (Cowan and Steel, 2003).

3.5.2.5 *O-nitro-phenyl-D-galactopyranoside (ONPG) test*

This was carried out by inoculating a tube of ONPG broth with the 18 hours old culture of the isolates. Then the inoculated tubes were incubated at 37^{o}C for 48 hours and examined for β-galactosidase activity which was indicated by the appearance of a yellow colour due to the production of o-nitrophenol (Cowan and Steel, 2003).

3.5.2.6 *Carbohydrate fermentation test*

Series of test tubes containing sterile nutrient broth with 1% each of membrane-filter-sterilized (0.45µm) single fermentable sugar were inoculated with the test organisms. The sugar fermentation tubes were incubated at 35^{o}C for 24 hours. At the end of the incubation period, all tubes were examined for acid production following the addition of methyl red indicator and then compared with the control for interpretation (Cowan and Steel, 2003).

3.5.2.7 *Oxidase test*

This was carried out by placing a piece of filter paper in a clean petri dish and then 2-3 drops of freshly prepared oxidase reagent was added. Using a piece of stick, a colony of the test organism was smeared on the filter paper and examined for the development of a blue-purple colour within a few seconds which indicates a positive test and absence of blue-purple colour within 10 seconds indicates a negative test (Cheesbrough, 2006).

3.6 Authentication of Isolates using MICROGEN Kit

Isolates suspected to be *Pseudomonas aeruginosa* on the basis of cultural, microscopic and biochemical characteristics were authenticated using the Microgen kits. Isolates thus obtained were stored in the refrigerator for further studies.

3.7 Screening of *P.aeruginosa* Isolates for the Ability to Utilize Hydrocarbon.

Mineral salt medium was prepared as earlier described and enriched with 1% (v/v) of engine oil. Each isolates were inoculated unto the medium in petri dishes by streaking. The ability of the isolates to utilize the hydrocarbon were determined by the amount of colonies emanating on each plate (Idise *et al*., 2010)

3.7.1 Standardization of Inoculum

Standardized Inoculum of the isolate of *Pseudomonas aeruginosa*was prepared by picking distinct colonies of the isolate and emulsifying them in normal saline and comparing the turbidity with that of McFarland standard 1 (3.0×10^{8}cfu/ml).

3.7.2 Determination of Hydrocarbon Degrading Capacity of the Selected *Pseudomonas aeruginosa*Isolate.

25g of the soil sample was weighed into 12 100ml flasks and autoclaved at 121^{o}C for 15minutes to eliminate all microorganisms present in the soil. Each flask was inoculated with 10ml of the standard suspension of the isolate and incubated at 37 ^{o}C for a period of 28days. After an interval of seven days (one week), a set of 3 flasks were assessed for the ability of the isolate to remediate the pollutants by determining the residual pH, EC, TOC, Total Nitrogen, Phosphorus, Potassium, Calcium, Magnesium and Oil & Grease (Anupama and Padma, 2009).

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Physicochemical Properties of the Soil Sample

Physical properties of soil like soil texture and bulk density have also 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 and several others are also under the direct or indirect influence of soil physical properties (Kumar *et al*., 2011) . In this present study, the soil was classified as sandy loam with 77% sand and 18% clay. The high percentage of sand indicates possibility of a high degree of hydrocarbon degradation as increased ventilation has a direct impact on microbial growth which can enhance the biodegradation of petroleum compounds (Hardik *et al*., 2011).

The physicochemical parameters of the soil are presented in Table 4.1. As shown in the Table, the soil is a loamy soil and contains high amounts of oil and grease (580mg/l). The pH is 5.5 and it contains significant amounts of carbon, nitrogen and phosphorus.

Table 4.1: Physicochemical Properties of Hydrocarbon Contaminated Mechanic Workshop Soil

Physicochemical Parameters	Values
Ph	6.6
Electrical Conductivity (EC) µS/m	0.20
Total Organic Carbon (TOC) (%)	0.818
Nitrogen Content (%)	0.175
Available Phosphorus (%)	0.430
Oil and Grease (Mg/L)	580
Potassium (%)	0.682
Calcium (%)	4.080
Magnesium (%)	0.840
Particle Size Distribution	
Silt	5
Clay	18
Sand	77
Textural Class	Loamy Soil

Key: µS/m; Micro-Siemens per meter, Mg/L; Milligram per litre, %; Percentage

4.2 Isolation and Characterization of *Pseudomonas aeruginosa* from Hydrocarbon Contaminated Soil.

The isolates had small green circular and convex (SGCC) colonies on centrimide agar on the basis of cultural characteristics and they were all gram negative with rod shaped morphology. They all showed positive reaction to motility, citrate, oxidase and arabinose utilization tests but negative reaction to the utilization of sucrose. Isolate WMS3, WMS4 and WMS6 showed positive reaction to fructose and Xylose utilization test while others were negative. With the exception of isolate WMS7, all the other isolates showed positive result to glucose utilization test as presented in Table 4.2

Four (4) isolates of *P. aeruginosa* were obtained in this study (Table 4.2). This is an indication that certain bacteria can adapt and survive in environment polluted with petroleum hydrocarbon as the mechanic workshop soil sample was found to have 580Mg/L of oil and grease. Hence, hydrocarbon polluted soil is a good source of bacteria with hydrocarbon utilization potential. This finding is in agreement with the work of researchers such asSalam and Obayori (2013); Stanley *et al*. (2015) and Liyange and Manage (2016) all who isolated bacterial species either or both of *Bacillus* and *Pseudomonas* from sources polluted by petroleum hydrocarbons.

Table 4.2: Cultural, Microscopic and Biochemical Characteristics of *P. aeruginosa*

Isolate Code	Growth on CA	GRM	BIOCHEMICAL CHARACTERISTICS										Tentative Identity
			M	C	U	OX	NR	Glu	Ara	Fruc	Xyl	Suc	
MWS1	SGCC	G-ve rods	+	+	-	+	+	+	+	-	+	-	*Pseudomonas*spp
MWS2	SGCC	G-ve rods	+	+	+	+	-	+	+	-	-	-	*Pseudomonas*spp
MWS3	SGCC	G-ve rods	+	+	+	+	+	+	+	+	+	-	*Pseudomonas*spp
MWS4	SGCC	G-ve rods	+	+	-	+	+	+	+	+	+	-	*Pseudomonas*spp
MWS5	SGCC	G-ve rods	+	+	-	+	-	+	+	-	-	-	*Pseudomonas*spp
MWS6	SGCC	G-ve rods	+	+	-	+	+	+	+	+	+	-	*Pseudomonas*spp
MSW7	SGCC	G-ve rods	+	+	+	+	+	-	+	-	-	-	*Pseudomonas*spp

KEY: MWS; Mechanic Workshop Soil, **SGCC**; Small Green Circular Convex, **CA**; Centrimide Agar, **GRM**; Gram Reaction and Morphology, **M**; Motility, **C**; Citrate, **U**; Urease, **NR**; Nitrate Reduction, **Glu**; Glucose **Ara**; Arabinose, **Fruc**; Fructose, **Xyl**; Xylose, **Suc**; Sucrose.

4.3 Identification of Isolates using MICROGEN® Enterobacteriaceae (GNA+GNB) Kits.

Table 4.3 depicts the identity of the isolates based on Microgen kit test. Of the isolates subjected to Microgen kit test, isolates WMS1, WMS3, WMS4 and WMS6 were *P. aeruginosa* with 87.95% probability and octal code of 760722001.

4.3.1 Hydrocarbon Utilization Capacity of *Pseudomonas aeruginosa*

Isolating bacteria from hydrocarbon contaminated sample could be an indication of such bacteria to adapt to heavy hydrocarbon presence. However, their ability to utilize hydrocarbon as sole source of carbon and energy must be ascertained. All the isolates showed ability to utilize hydrocarbon as the only source of carbon (Table 4.3) but isolate WMS3 showed higher ability than others. This indicates that certain strains of organisms of same species could be more adapted, developed and boast a better developed enzyme system for hydrocarbon metabolism. Researchers such as Idise *et al*. (2010) also reported variations in the growth of different strains of bacteria on mineral salt medium containing petroleum hydrocarbons as the sole source of carbon and energy.

Table 4.3: Identity of *Pseudomonas aeruginosa*Isolates Based on Microgen Kit

Isolate's Code	Prophile Number	Probability (%)	Identity
MWS1	760722001	87.95	*P. aeruginosa*
MWS3	760722001	87.95	*P. aeruginosa*
MWS4	760722001	87.95	*P. aeruginosa*
MWS6	760722001	87.95	*P. aeruginosa*

KEY: MWS; Mechanic Workshop Soil

4.4 Engine Oil Utilization by the *Pseudomonas aeruginosa* Isolates

All the isolates had evidence of growth on mineral medium enriched with different concentration (0.5%, 1.0 %, 1.5% and 2.0%) of engine oil. While all the isolates showed high growth on medium containing 0.5% engine oil, only isolate MWS3 showed high growth on the medium containing 1.0% engine oil and moderate growth on that containing 1.5% and 2% engine oil. Isolate WMS1 had scanty growth on medium containing 2.0% engine oil while isolate MSW4 and MSW6 had no evidence of growth on the medium containing 2.0% engine oil as presented in table 4.4

Table 4.4: Growth of *P. aeruginosa* on Mineral Medium Containing Engine Oil

Isolate code	Crude Oil Concentration (%)			
	0.5	1.0	1.5	2.0
MSW1	+++	++	+	+
MSW3	+++	+++	++	++
MSW4	+++	+	+	-
MSW6	+++	++	+	-

KEY: MWS; Mechanic Workshop Soil, +++; High growth, ++; Moderate growth, +; Low growth, -; No growth

4.5 Remediation of the Soil by *P. aeruginosa*

Progressive decrease in concentration (580Mg/L to 189Mg/L) of the oil and grease of the soil was observed. This portrays that organisms were actively utilizing the petroleum hydrocarbons present in the soil as carbon and energy source, consequently degrading the pollutants and remediating the soil. The ability of the *P. aeruginosa* isolate to degrade hydrocarboncould be attributed to the presence and expression of certain catabolic genes that code for enzymes that are critical in activating and metabolizing hydrocarbons. The amount of oil and grease degraded by the organism was lowest in the first and last week if incubation. This could be attributed to a lag period in the first week when the organism may be synthesizing the enzymes that would enable the activation of the hydrocarbons. Also, the low amount degraded in the last week could be attributed to decrease in the amount of utilizable carbon which would result in competition between the cells and the accumulation of toxic waste and metabolic by products such as acids which could adversely affect the bacteria. This is in agreement with the work ofAjao *et al*. (2014) reported 90% oil and grease degradation after 20 days of incubation, Idise *et al*. (2010) reported 98.25 oil degradation and Rahman *et al*. (2002) who also reported the degradation of oil up to 78% after incubation of samples for 20 days inoculated with the bacterial consortium.

The remediation of the polluted soil also resulted in alteration or changes in the amounts of pH, organic carbon content, nitrogen, phosphorus, magnesium, calcium and potassium. This could suggest that certain metabolic by-products which interacted with these components either increasing or reducing the amounts were synthesized as the oil is degraded. Improvement in the amounts of nitrogen, phosphorus and organic carbon was observed as compared with the initial amounts indicating a recovery of the soil.

4.6 Changes in the Physicochemical Properties of the Soil during the Period Bioremediation

A consistent pattern was observed in the changes of the amount of the phosphorus and nitrogen in the soil sample during the incubation period. An increase was observed in the amount of phosphorus and nitrogen from the initial amounts and the amount after seven days with only a slight change between day 7 and 14 as shown in Fig. 4.6 while a sharp increase was observed between day 14 and 21 which was immediately followed by a decline.

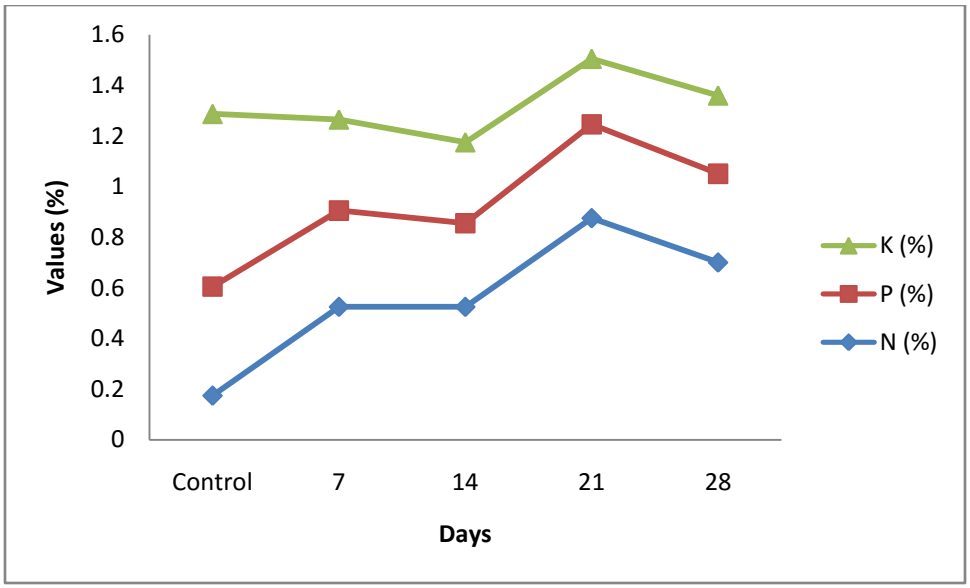

Fig. 4.6 Changes in the Amounts of N, P, K during bioremediation by *P. aeruginosa*

Key: N; Nitrogen, P; Phosphorus, K; Potassium.

4.7 Changes in Soil pH

The pH of the soil was in the neutral range. This pH condition have been shown to support the growth of most soil bacteria hence can be said to favour the growth of hydrocarbon degrading bacteria. Though, certain hydrocarbon degrading bacteria can thrive at a neutral or slightly acidic pH, research have shown that the activities of hydrocarbon degrading microorganisms is highest in an alkaline pH (Shamiyan *et al.,* 2013; Sharma and Pathak, 2014).

The pH increased, attaining highest value (7.6) at day 14 as shown in Fig. 4.7

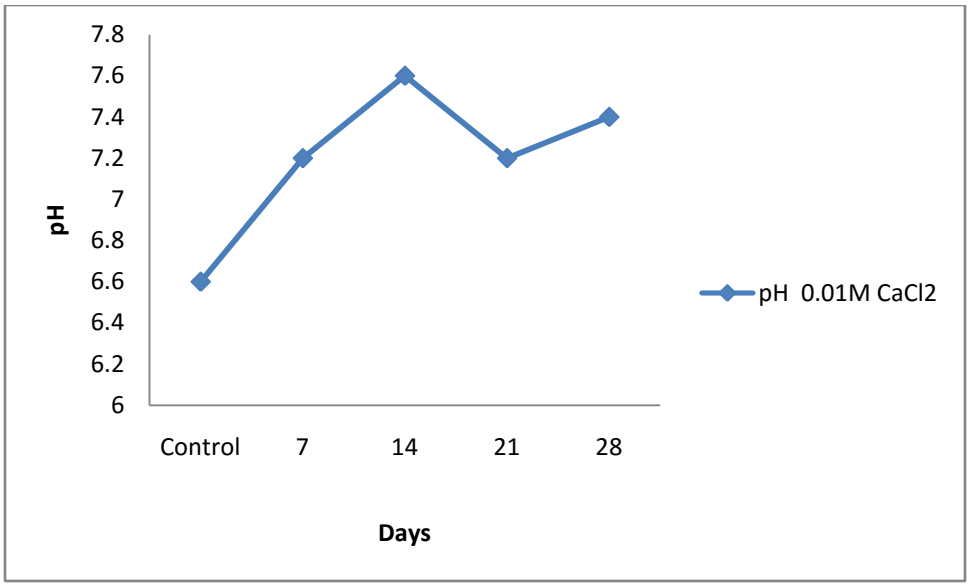

Fig. 4.7 Changes in pH during bioremediation by *P. aeruginosa*

4.8 Changes in Soil Electrical Conductivity

A sharp increase in the electrical conductivity of the sample was observed within the first 14 days of incubation which was immediately followed by a decline during the remaining period of incubation as shown in Fig. 4.8

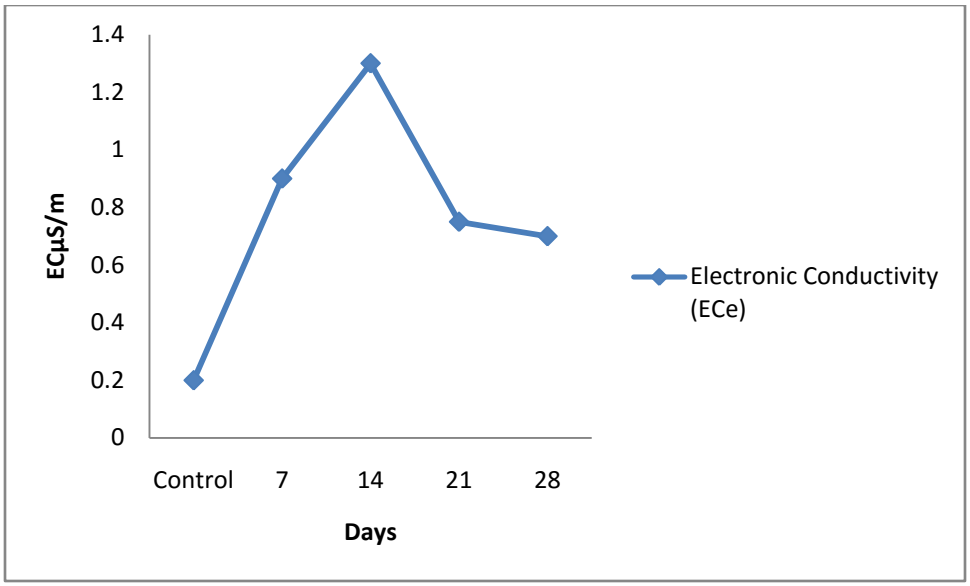

Fig. 4.8 Changes in Electrical Conductivity during Bioremediation by *P. aeruginosa*

4.9 Changes in Soil Total Organic Carbon

The Total organic carbon, nitrogen and phosphorus contents of the soil were very low when compared with the rating for soil data interpretation (compatible model) as suggested by Vidal (2001). The ability of microorganisms to degrade pollutants in soil greatly depends on the nutrient composition of the soil. Hence, the importance of the amounts of particular nutrients like Carbon, Nitrogen and Phosphorus cannot be overemphasized regarding success of a bioremediation process. The low organic carbon content, nitrogen and phosphorus could be attributed to the long exposure of the soil to petroleum products and heavy metal pollution leading to the alteration of the normal physicochemical properties of the soil which may impedes the activities of certain soil organisms like the nitrogen fixing bacteria. Also, the utilization of the available organic by indigenous hydrocarbon degrading microorganisms results in its depletion (Ajao *et al*., 2013). This is in agreement with the work of Ajao *et al.* (2013) who reported very low values of nitrogen and phosphorus in hydrocarbon polluted soil but disagrees with the findings of Salam and Abayori (2013) who reported high values of organic carbon in crude oil contaminated soil.

A continuous increase in the amount of total organic carbon was observed throughout the period of incubation. Rapid increase was observed in within the first seven days which was then followed by a slight continuous increase as depicted by Fig. 4.9

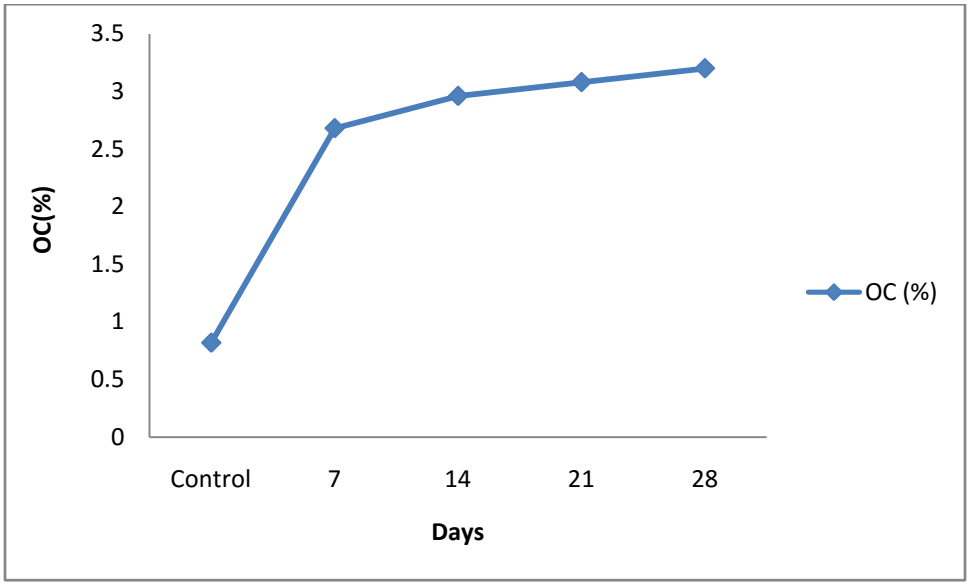

Fig. 4.9 Changes in Total Organic Carbon during Bioremediation by *P. aeruginosa*

4.10 Changes in Soil Calcium and Magnesium

Similar pattern was observed in the changes of the amounts of calcium and magnesium in the soil sample as depicted by Fig. 4.5

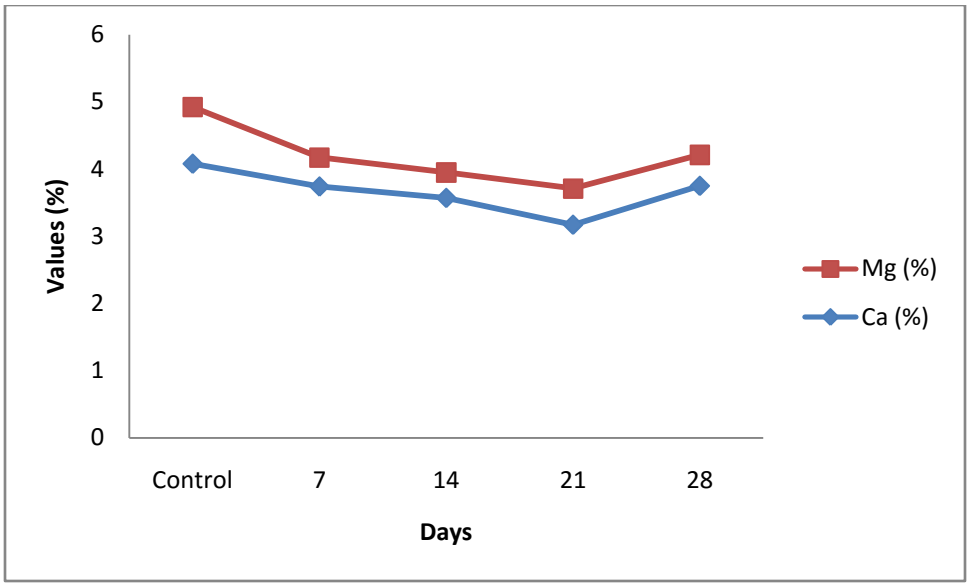

Fig. 4.10 Changes in the amount of Magnesium and Calcium during Bioremediation by *P. aeruginosa*

4.11 Changes in Soil Oil and Grease Content

The soil was found to contain high concentration of oil and grease which must have resulted from the consistent introduction of spent engine oil and other petroleum products into the soil of the mechanic workshop sampled. Large amount of hydrocarbon in soils discourages plant growth which in turn affects animals that depends on these plants for food and shelter (Ewetola, 2013). Crops like yams, cocoyam's, vegetables do not thrive well in hydrocarbon contaminated soils. This is due to insufficient aeration of the soil because of the displacement of air from the spaces between the soils particles by crude oil which have adverse effect on plant growth, this can cause seeds sown on contaminated soil not to germinate even after 30days (Hentati *et al*., 2013). Also, the diversity of microorganism may be drastically affected as only a certain groups are able to adapt in hydrocarbon polluted environment.

Fig. 4.11 shows the changes in the amount of oil and grease content of the soil. There was continuous decrease in the amount of the residual oil and grease.

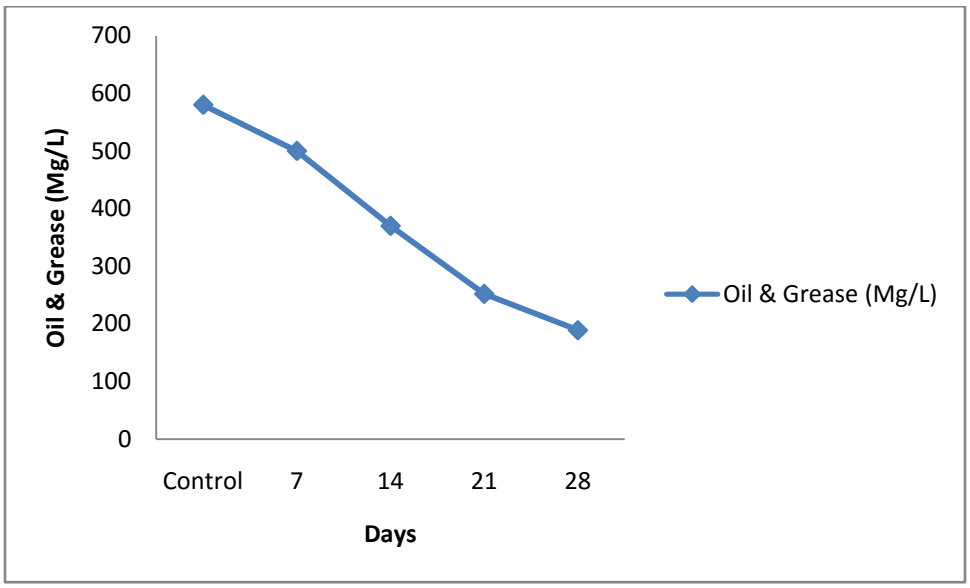

Fig. 4.11 Changes in the Oil and Grease Content of the Soil during Bioremediation by *P. aeruginosa*

CHAPTER FIVE

SUMMARY AND CONCLUSION

5.1 Summary

From the results obtained the pH of the soil was in the neutral range. This pH corresponds to conclusion made by Sharma and Pathak, 2014 where they concluded that the that the activities of hydrocarbon degrading microorganisms is highest in an alkaline pH .

The Total organic carbon, nitrogen and phosphorus contents of the soil were very low when compared with the rating for soil data interpretation (compatible model) as suggested by Vidal (2001). Also, the utilization of the available organic by indigenous hydrocarbon degrading microorganisms results in its depletion. This is in agreement with the work of Ajao *et al.* (2013) who reported very low values of nitrogen and phosphorus in hydrocarbon polluted soil but disagrees with the findings of Salam and Abayori (2013) who reported high values of organic carbon in crude oil contaminated soil.

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WMS3 showed higher ability than others. This indicates that certain strains of organisms of same species could be more adapted, developed and boast a better developed enzyme system for hydrocarbon metabolism. Researchers such as Idise *et al*. (2010) also reported variations in the growth of different strains of bacteria on mineral salt medium containing petroleum hydrocarbons as the sole source of carbon and energy.

The decrease in concentration of Oil and grease from (580Mg/L to 189Mg/L) shows that organisms were actively utilizing the petroleum hydrocarbons present in the soil as carbon and energy source, consequently degrading the pollutants and remediating the soil.This is in agreement with the work ofAjao *et al*. (2014) reported 90% oil and grease degradation after 20 days of incubation, Idise *et al*. (2010) reported 98.25 oil degradation and Rahman *et al*. (2002) who also reported the degradation of oil up to 78% after incubation of samples for 20 days inoculated with the bacterial consortium.

The remediation of the polluted soil also resulted in alteration or changes in the amounts of pH, organic carbon content, nitrogen, phosphorus, magnesium, calcium and potassium. This could suggest that certain metabolic by-products which interacted with these components either increasing or reducing the amounts were synthesized as the oil is degraded. Improvement in the amounts of nitrogen, phosphorus and organic carbon was observed as compared with the initial amounts indicating a recovery of the soil.

5.2 Conclusion

It can be concluded that bioremediation potential of*Pseudomonas aeruginosa* on hydrocarbon polluted soil was successful in that it reduced the Oil and grease content of the soil from 580Mg/l to 189Mg/l and the other elements studied indicated varying degree of remediation with some remaining in same state.

5.3 Recommendation

From the results of this work, each element showed the effect of *Pseudomonas aeruginosa* on their individual quantities in the soil and based on that, it will be recommended that further research should be carried out on these elements.

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Appendices

Appendix I

The physicochemical properties of the soil during treatment.

	pH 0.01M $CaCl^2$	Electronic Conductivity (ECe)	OC (%)	Oil & Grease (Mg/L)	N (%)	P (%)	K (%)	Ca (%)	Mg (%)
Control	6.6	0.2	0.818	580	0.175	0.43	0.682	4.08	0.84
7	7.2	0.9	2.68	500	0.525	0.38	0.36	3.74	0.43
14	7.6	1.3	2.96	370	0.525	0.33	0.32	3.57	0.38
21	7.2	0.75	3.08	252	0.875	0.37	0.26	3.17	0.54
28	7.4	0.7	3.2	189	0.7	0.35	0.31	3.75	0.46

Appendix II

Soil Sample collected from the Mechanic Workshop