KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

COLLEGE OF SCIENCE

DEPARTMENT OF ENVIRONMENTAL SCIENCE

BIOREMEDIATION OF HYDROCARBON CONTAMINATED SOIL USING COMPOST,NPK FERTILIZER AND CATTLE BILE AS AMENDMENT MATERIALS

BY

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Dissertation Submitted to the Department of Environmental Science, Kwame Nkrumah University of Science and Technology in Partial Fulfilment of the Requirements for the Degree of
Master of Science in Environmental Science

College of Science

May, 2013

DECLARATION

I hereby declare that this submission is my own work towards the MSc. Degree in Environmental Science and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

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DEDICATION

This work is dedicated to the Almighty for His boundless grace and limitless mercy that brought me to the point of pursuing my Masters degree and for seeing me through the course. It is also dedicated to my uncles; Dr. Emmanuel Annan and Mr. Daniel Annan whose generous financial support made all this possible to start with. Additionally, I dedicate this work to my beloved deary Stella Y. Doku and to my sister Ethel O. Annan.

ACKNOWLEDGEMENT

"No man is an island" Indeed, I could not have completed this work without the grace of the Almighty and the assistance of several people. I therefore acknowledge all those who in diverse ways helped me to complete this work. In particular I wish to acknowledge my supervisor Dr. Fei Baffoe for his guidance during this work. I would also like to acknowledge my uncles for their financial support. I wish to express my appreciation to Dr. Sarkodie (Head of Department Agric), for assisting me in the data processing and analysis as well as Jacob Ulzen. I further express my gratitude to Napoleon Jackson for his cooperation and support during the laboratory work.

ABSTRACT

The study seeks to investigate the extent of degradation of petroleum hydrocarbon contaminated soil by harnessing the remedial potential of compost, NPK fertilizer and cattle bile as amendment additives. A baseline study was carried out to determine the physicochemical properties (TPH, OM, TOC, pH, TN, P, K, and Moisture content) and the microbial load (THBC) of the contaminated soil and compost using appropriate methods. Homogenized soil (1000g) each was amended with 10.53 g of NPK fertilizer, 107.48 g of compost to achieve 0.2%, whereas to establish 0.4% nitrogen 23.87 g of NPK fertilizer and 243.54 g of compost as well as 2 ml of cattle bile were employed to establish different microcosm experiments such as; A, B, C, D, A2, B2, C2 and D2 including a control (devoid of afore-listed additives). The microcosm experiments were subjected to aeration thrice a week over six weeks of incubation, during which individual microcosm experiment was periodically sampled at two weeks intervals for analysis. The data of results revealed a sharp decrease in TPH concentration after two weeks and progressively decreased further over the subsequent sampling week periods during incubation; with a corresponding increase in the rate of hydrocarbon compounds removal. Microcosm B2 and the control (Ct1) emerged as the maximum and minimum rate of TPH components removal at the end of the bioremediation process as illustrated in the order; B2(98.43%) > B(94.87%) > C(94.64%) > A(94.14%) > D2(93.65%) > A2(93.57%) > C2(92.44%) > D(91.43%) > Ct1(79.57%). Generally it was observed that microcosms established at 0.4% nitrogen concentration exhibited higher rate of nitrogen consumption as compared to those microcosms established at 0.2% nitrogen concentration. In conclusion, biostimulation through homogenization, addition of moisture, compost, NPK fertilizer and inoculation of cattle bile, is a plausible approach to effect TPH components removal from contaminated soil. Microcosm B2 (1000 g contaminated soil + 0.2% fertilizer + cattle bile) evidently emerged as the best treatment option among the other nine microcosm experiments. Cattle bile, indeed possess remedial potential and function as biosurfactant to augment the biosurfactant produced by the bacterial population to enhance hydrocarbon degradation.

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TPH	Total Petroleum Hydrocarbon
THBC	Total Heterotrophic Bacterial Counts
NPK	Nitrogen-Phosphorous-Potassium
CB	Cattle bile
TEA	Terminal Electron Acceptor
BTEX	Benzene, Toluene, Ethylene and Xylene
TCE	Trichloroethylene
PAH	Poly aromatic hydrocarbon
PCBs	Polychlorinated biphenyls
CFU	Coliform forming unit
TOC	Total organic carbon
CMC	Critical micelle concentration
HLB	Hydrophilic-lipophilic balance
CSH	Cell surface hydrophobicity
ETPH	Extracted Total Petroleum Hydrocarbon
HCL	Hydrochloric Acid
PCA	Plate count agar
CRB	Complete randomized block
ANOVA	Analysis of variance

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Global industrialization has been attributed to the pollution crisis all around the world. One of the major environmental problems today is hydrocarbon contamination resulting from industrial activities such as petrochemical industry (Das and Chandran, 2011). Hydrocarbon constituents are known to be toxic persistent organic compounds which can contaminate large areas of soil and water reserviors. Accurate detail regarding the extent of hydrocarbon contamination in the terrestrial environment has been difficult to quantify because of the unintentional nature of the contamination (largely through accidental spillage or around factories and petrol stations). Hydrocarbon pollution is ubiquitous in the environment, and for example, in the United Kingdom accounts for over 15% of all pollution incidents (Stroud *et al*., 2007). There has been increasing international efforts to remediate contaminated sites using "green" technologies, either as a response to the risk of adverse health or environmental effects or to enable site redevelopment (Vidali, 2001).

Remediation technology can be use as a clean up tool to clean different polluted matrixes such as soil, water, sediments and air. Oil is a complex mixture of hydrocarbons and other organic compounds, including some organometallic constituents. It contains hundreds or thousands of aliphatic, branched and aromatic hydrocarbons most of which are toxic to living organisms (Jain *et al*., 2011). The Release of persistent, bioaccumulative and toxic chemicals (hydrocarbons) has a detrimental impact on human health and the environment. These contaminants find their way into the tissues of plants, animals and human beings by the movement of hazardous constituents in the environment. Oil spill from the industries, filling stations, refineries, loading and pumping stations, petroleum products

depots, during transportation and at auto-mechanics workshops all contribute to soil contamination, and actually make up a larger percentage of polluted ground in the world (Abdulsalam *et al*., 2010). There are three main potential sources of environmental pollution with petroleum hydrocarbons: (i) continuous low-level inputs from road surfaces and domestic waste, (ii) major spillages from tankers, pipelines and storage tanks, and (iii) slow, natural seepage from natural oil reservoirs. Effluent treatment by refineries and petrochemical plants generates large amounts of oily sludge. Accidental contamination of soil with hydrocarbons occurs primarily through production, transportation and storage accidents such as rupture of pipelines or storage tanks, road and railroad accidents. Leaky underground fuel tanks are the leading cause of groundwater pollution (Margesin and Schinner, 2001).

Bioremediation is an option that offers the possibility to destroy the contaminant or at least transform them into innocuous substances using natural biological activity. Bioremediation is defined as the use of living organisms primarily microorganisms, to degrade the environmental contaminants into less toxic forms. It uses naturally occurring bacteria and fungi or plants to degrade or detoxify substances hazardous to human health and the environment. The microorganisms may be indigenous to a contaminated area or they may be isolated from elsewhere and brought to the contaminated site. Contaminant compounds are transformed by living organisms through reactions that take place as a part of their metabolic processes. Biodegradation of a compound is often a result of the actions of multiple organisms (Vidali, 2001). The ability of microorganisms to transform and degrade many types of pollutants in different matrixes such as soil, water, sediments and air has been widely recognized during the last decade (Ilyina *et al*., 2003). Hydrocarbon metabolism by indigenous microorganisms is influenced by a number of

factors which include nutrients, temperature, oxygen, and pH value, water content in soils, bioavailability, quality and quantity of contaminants. The prior pollution history of the ecosystem may also be involved since chronically polluted systems are generally enriched in hydrocarbon-utilizing organisms (Atlas and Bartha, 1992).

Bioremediation techniques accelerate the naturally occurring biodegradation of hydrocarbons by optimizing the conditions of this process through aeration, addition of nutrients, controlling pH, moisture content, and temperature (Molina-Barahona *et al*., 2005). Physical and chemical technologies, such as dispersion, dilution, sorption, volatilization, abiotic transformations though important, have their limitations. These limitations include; expensive to implement at full scale, they are not environmentally friendly, their technologies are complex and they lead to destruction of soil texture and characteristics: Furthermore, the physicochemical technologies do not always result in complete neutralization of pollutants. Due to limitations of the physicochemical technologies stated above. Several literatures have reported that bioremediation technologies are alternatives and/or supplements to these technologies (Abdulsalam *et al*., 2010). This is because bioremediation of hydrocarbon-contaminated soils has been established as an efficient, economic, versatile, and environmentally sound treatment. The most widely used bioremediation procedure is bio-stimulation of the indigenous microorganisms by addition of nutrients, as input of large quantities of carbon sources (i.e. contamination) tends to result in rapid depletion of the available pools of major inorganic nutrients, such as Nitrogen and Phosphorous (Margesin and Schinner, 2001). The use of compost in bioremediation treatment is a form of biostimulating indigenous microorganisms to carry out contaminants clean up in contaminated soil. Compost bioremediation has received little attention despite its application in the treatment of soils

contaminated with organic compounds for many years. It is an established fact that composts have been reported to have potential for remediation of heavily contaminated sites (Atagana, 2008). In an attempt to further enhance this remediation technology, cattle bile will be applied in combination with compost for the treatment process. Bile has been found to possess properties of surfactants. Biosurfactants are a structurally diverse group of surface-active substances produced by microorganisms. Bio-surfactants increase bioavailability of hydrocarbon resulting in enhanced growth and degradation of contaminants by hydrocarbon-degrading bacteria present in polluted soil (Pacwa-Płociniczak, 2011). Bile decreases the surface tension by means of its surfactant properties. Surface tension may in turn be important for the wetting and mobilization of contaminants from soil. Furthermore, bile has been found to form complexes with metals and may result in an apolar environment in the interior of bile salt micelles for hydrophobic contaminants (Oomen *et al*., 2003).

1.2 Problem Statement and Justification

According to Vidali (2001), contaminated lands generally result from the production, use, and disposal of hazardous substances. Soil and groundwater at many existing and former industrial areas and disposal sites are contaminated with organic compounds and inorganic compounds that were chiefly released by the activities of petrochemical industries, mining companies and the disposal of hazardous waste and spill into the environment (Mayo-López *et al*., 2010). The environmental impacts that accompany the inadequate management of these compounds include contamination of soil and aquifers due to vertical migration, degradation of the aesthetic value of the landscape, and horizontal migration due to the overspill of waste pits during heavy rains. These problems have led to social conflicts and complaints of possible impacts to agricultural land, as

well as demands on the environmental authorities to address these problems. This has resulted in the recognition of the importance of developing useful technologies for the treatment of contaminated sites to achieve permissible criteria, and that these criteria are appropriate so that the biota is not affected. Hydrocarbon contaminated soil results in extensive damage of local ecosystems. Pollutants accumulate in plant and animal tissues and this often ensue in mutation or progeny's death. It is therefore imperative for mining companies to adopt appropriate measures such as selection, transportation, transfer, distribution, storage, use, collection and disposal, to minimize the negative impact of their activities on the environment.

Bioremediation generally is enhanced when the indigenous microbial population is stimulated by aeration and the addition of nutrients. In addition, biosurfactant plays a crucial role in the process of hydrocarbon contaminant degradation by enhancing bioavailability of hydrocarbon contaminant. Therefore, this study is intended to exploit the high nutrient contents of compost and fertilizer, as well as utilizing cattle bile (biosurfactant) in enhancing the microbial removal of hydrocarbons in contaminated soil and subsequently to assess the efficiency of bioremediation technique of the Hydrocarbon contaminated soil as an alternative to the physicochemical treatment processes.

1.3 Objectives

The main objective of the study is to investigate the extent of degradation of hydrocarbon contaminated soil through the process of biostimulation using compost and NPK fertilizer and cattle bile as amendment additives.

Specific Objectives:

- To determine the levels of TPH, THBC, C, N, P, K, moisture, and pH of contaminated soil taken from site.
- To design a bioremediation microcosm experiments of NPK fertilizer; Compost and application of cattle bile in combination with the individual nutrient supplements.
- Stimulation of heterotrophic bacterial population by adjusting nitrogen levels of the contaminated soil.
- To monitor appropriate parameters of the degradation process of the different amended treatments.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Principles of Bioremediation

Remediation technologies offer developing countries (in tropical regions) a great advantage as far as biological methods are concerned; with high temperatures and humidity that favours biological reactions that can be used for bioremediation and phytoremediation (Mayo-López *et al*., 2010). Bioremediation is defined as the process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state, or to levels below concentration limits established by regulatory authorities (Vidali, 2001).

By definition, bioremediation is the use of living organisms, primarily microorganisms, to degrade the environmental contaminants into less toxic forms (Vidali, 2001). Additionally, it includes methods that reduce mobility and migration of the contaminants, preventing their spreading to uncontaminated areas (Jain *et al*., 2011). Biodegradation of a compound uses naturally occurring bacteria and fungi or plants to degrade or detoxify substances hazardous to human health and / or the environment. The microorganisms may be indigenous to a contaminated area or they may be isolated from elsewhere and brought to the contaminated site (Vidali, 2001). Hydrocarbon degrading bacteria and fungi are widely distributed in marine, and soil habitats. Among these organisms, bacteria are the most numerous and biochemically active group, particularly at low oxygen levels. The metabolic process used by bacteria to produce energy requires a terminal electron acceptor (TEA) to enzymatically oxidize the carbon source to carbon dioxide (US. EPA, 2004). Typical bacterial groups already known for their capacity to degrade hydrocarbons include *Marinobacter* sp., *Micrococcus* sp., *Alcanivorax* sp., *Microbulbifer* sp.,

Pseudomonas sp., *Sphingomonas* sp., *Cellulomonas* sp. (Brito *et al*., 2006). Molds belonging to the genera *Penicillium* sp., *Aspergillus* sp., *Fusarium* sp., and the yeasts *Candida* sp., *Yarrowia* sp., have been implicated in hydrocarbon degradation (Chaillan *et al*., 2004). Other organisms such as fungi are also capable of degrading the hydrocarbons of engine oil to a certain extent. However, they take longer periods of time to grow as compared to their bacterial counterparts (Prenafeta-Boldu *et al*., 2001). In nature, biodegradation of a crude oil typically involves a succession of species within the consortia of microbes present. Degradation of petroleum involves progressive or sequential reactions, in which certain organisms may start the initial attack on the petroleum constituent; this produces intermediate compounds that are subsequently utilized by a different group of organisms, in the process that results in further degradation. There are two main approaches to bioremediation namely bioaugmentation and biostimulation (Vidali, 2001).

2.1.1 Bioaugmentation

Bioaugmentation is the process by which oil-degrading microorganisms are added to supplement the existing microbial population. The rationale for adding oil-degrading microorganisms is that indigenous microbial populations may not be capable of degrading the wide range of potential substrates present in complex mixtures such as petroleum. Other conditions under which bioaugmentation may be considered are when the indigenous hydrocarbon-degrading population is low (Venosa and Zhu, 2003). To examine whether microbial products can compete with the indigenous populations, Venosa *et al*. (2003) tested ten different commercial microbial products using weathered Alaskan crude oil in shake flask microcosms. Seeded microorganisms seemed to compete poorly with the indigenous population.

2.1.2 Biostimulation

Biostimulation is the process by which the growth of indigenous oil degraders is stimulated by the addition of nutrients or other growth-limiting co-substrates and / or habitat alteration to accelerate the biodegradation process. Bench-scale treatability studies have been carried out to determine the type, concentration, and frequency of addition of amendments needed to achieve maximum stimulation in the field. The optimal nutrient types and concentrations vary widely depending on the oil properties and the environmental conditions (Venosa & Zhu, 2003). Decontamination of contaminated soil involves physicochemical and biological treatments (Jain *et al*., 2011).

2.2 Physicochemical treatment

This contributes to loss or alteration of some of the components. Volatile compounds are lost by evaporation. In aquatic environments and surface slicks photochemical reactions contribute to change; wind and wave action (in water) may cause formation of emulsions. Other processes are chemical dissolution; further, the oil may adsorb to detritus in water or to colloids such as humus particles in soil (Margesin and Schinner, 1999). Other physicochemical treatments are incineration, thermal desorption, solvent extraction and land filling etc. Incineration is a very effective treatment method, but it is costly and after burning, the soil loose most of its nutritional value and structure. Land filling does remove the contaminants but only relocates the problem: Hence, new innovative methods are needed to treat contaminated soils (Jain *et al*., 2011). Abiotic processes contribute, especially in recently contaminated soils, greatly to decontamination. After the Exxon Valdez oil spill, 30% of hydrocarbons were lost in a very short time by physical weathering such as vaporization and dissolution (Margesin and Schinner, 1999). Biodegradation is fundamentally an electron transfer process and involves biodegradation

of organic constituents by enzymes produced by microorganisms. Biological energy is obtained through the oxidation of reduced materials. Microbial enzymes catalyze the electron transfer. Electrons are removed from organic substrates to capture the energy that is available through the oxidation process. The electrons are moved through respiratory or electron transfer chains (metabolic pathways) composed of a series of compounds to terminal electron acceptors (WDNR, 1994).

For bioremediation to be effective, microorganisms must enzymatically attack the pollutants and convert them to harmless products (Vidali, 2001). Bacteria achieve contact with water-insoluble hydrocarbons by the following strategies: specific adhesion mechanisms and production of extracellular emulsifying agents. Many hydrocarbon-degrading microorganisms produce extracellular emulsifying agents (biosurfactant). In some cases, emulsifier production is induced by growth on hydrocarbons (Jain *et al*., 2011). Biodegradation of a compound is a stepwise process involving a variety of different enzymes and species of organisms: Therefore, in the natural environment, a constituent may not be completely degraded, but only transformed into intermediate product(s) that may be less, equally, or more hazardous than the original (parent) compound, as well as more or less mobile in the environment (WDNR, 1994). Petroleum oil degradation by bacteria can occur under both aerobic and anaerobic conditions. It has been established that the first step in the aerobic degradation of hydrocarbons by bacteria is usually the introduction of molecular oxygen into the hydrocarbon (Jaine *et al.*, 2011). Additionally, many hydrocarbon compounds that cannot be utilized as a carbon or energy source by microorganisms can be degraded by enzymes generated by microbes to metabolize other compounds. This process is referred to as co-metabolism. The non-growth substrate is typically only incompletely oxidized (transformed) by the microbe

involved, but other microbes may utilize by-products of the co-metabolic process (WDNR, 1994).

2.3 Biological Treatments / Pathways

2.3.1 Aerobic

A wide variety of organic materials are easily degraded under aerobic conditions. In aerobic metabolism, O_2 is the terminal electron acceptor. When biodegradation follows this pattern, microbial populations quickly adapt and reach high densities. As a result, the rate of biodegradation quickly becomes limited by rate of supply of oxygen or some nutrient, not the inherent microbial capacity to degrade the contaminant. The ultimate products of aerobic metabolism are carbon dioxide and water (WDNR, 1994). Under aerobic conditions the oil hydrocarbons are degraded according to the following reaction;

$$C_{16}H_{34} + 241/2\ O_2 \rightarrow 16CO_2 + 17\ H_2O$$

Hydrocarbons with less than 10 carbon atoms tend to be relatively easy to degrade as long as the concentration is not too high to be toxic to the organisms. Benzene, xylene and toluene are examples of gasoline components that are easily degraded. Complex molecular structures, such as branched paraffins, olefins, or cyclic alkanes, are much more resistant to biodegradation (Kosaric, 2001).

2.3.2 Anaerobic degradation

In the subsurface, oil biodegradation occurs primarily under anoxic conditions, mediated by sulfate reducing bacteria or other anaerobes using a variety of other electron acceptors as the oxidant. When oxygen is absent, nitrate, sulfate, ferric iron, manganese, and bicarbonate can serve as terminal electron acceptors, if the microbes have the appropriate

enzyme systems. Under anaerobic conditions, the rate of degradation is usually limited by the inherent reaction rate of the active microorganisms; adaptation is slow, requiring months or years, and metabolic activity results in the formation of incompletely oxidized, simple organic substances, such as organic acids, and by-products such as methane or hydrogen gas (WDNR, 1994). Hydrocarbon biodegradation under anaerobic, denitrifying conditions also follows an oxidative strategy. In the presence of nitrate hydrocarbon substrates e.g., toluene are metabolized to oxidized intermediates prior to further biodegradation. Anaerobic degradation of petroleum hydrocarbons in natural environments by microorganisms has been shown in some other studies to occur only at negligible rates and its ecological significance has been generally considered to be minor (Jain *et al*., 2011).

2.4 Mechanism of Petroleum Hydrocarbon Degradation

The most rapid and complete degradation of the majority of organic pollutants is brought about under aerobic conditions. The initial intracellular attack of organic pollutants is an oxidative process and the activationas well as incorporation of oxygen is the enzymatic key reaction catalyzed by oxygenases and peroxidases. Peripheral degradation pathways convert organic pollutants step by step into intermediates of the central intermediary metabolism, for example, the tricarboxylic acid cycle. Biosynthesis of cell biomass occurs from the central precursor metabolites, for example, succinate, acetyl-CoA, pyruvate. Sugars required for various biosyntheses and growth are synthesized by gluconeogenesis. The degradation of petroleum hydrocarbons can be mediated by specific enzyme system (Das and Chandran, 2011). Initial attack is achieved through the following mechanisms such as; attachment of microbial cells to the substrates and production of biosurfactants (Hommel, 1990).

2.4.1 Biodegradability

In general water soluble compounds are usually degraded faster than less soluble compounds; because bacteria in the unsaturated soil occur mainly in the interstitial water of soil; hence, hydrocarbons in soil correlate to their water solubility (Jaine *et al*., 2011). Biodegradability is inherently influenced by the composition of the oil pollutant. For example, kerosene (consists of almost exclusively medium chain alkanes) is totally biodegradable. Similarly, crude oil is also biodegradable quantitatively; the petroleum biodegradation has been reported to be mostly enhanced in the presence of a consortium of bacteria species compared to monospecies activities (Ghazali *et al*., 2004). The n-alkanes, n-alkylaromatics, and aromatic compounds in the C_5 to C_{22} range are usually readily biodegradable. These compounds comprise a major portion of gasoline, diesel, and fuel oil. The n-alkanes, n-alkylaromatics, and aromatic compounds above C_{22} have very low water solubilities which result in slow degradation rates. These compounds are common in heavier oils. Condensed or fused aromatic and cycloparaffinic compounds with four or more rings have very low biodegradation rates. These include most PAH compounds. The BTEX compounds are typically removed at about the same rate by aerobic metabolism. Under anaerobic metabolism, these aromatic compounds are first oxidized to phenols or organic acids, and then transformed to long-chain volatile fatty acids, which are finally metabolized to methane and carbon dioxide. The biodegradability and degradation rates for each of these compounds under anaerobic conditions can vary considerably. Many chlorinated hydrocarbons are also readily biodegradable through aerobic and/or anaerobic metabolism. However, when significant concentrations of these compounds are present, the application of naturally occurring biodegradation should be considered carefully due to the potential for production of metabolites having greater toxicity than the original contaminant. Knowledge of the applicable microbial metabolic

pathways is necessary. For example, the anaerobic metabolism of TCE produces vinyl chloride as a metabolite, which is significantly more toxic than the parent compound. In such situations, naturally occurring biodegradation would not be acceptable (WDNR, 1994).

2.4.2 Bioavailability

Bioavailability is the amount of a substance that is physiochemically accessible to microorganisms. It is a key factor in the efficient biodegradation of pollutants. Chemotaxis or the directed movement of motile organisms towards or away from chemicals in the environment is an important physiological response that may contribute to effective catabolism of molecules in the environment. In addition, mechanisms for the intracellular accumulation of aromatic molecules via various transport mechanisms are also important (Parales, 2008). Introduction of external nonionic surfactants, e.g., the main components of oil spill dispersants, influence the alkane degradation rate (Rahman *et al*., 2003). The use of surfactants in situations of oil contamination may have a stimulatory, inhibitory or neutral effect on the bacterial degradation of the oil components (Liu *et al*., 1995). In study using poultry manure as organic fertilizer in contaminated soil increased biodegradation was reported but the extent of biodegradation was influenced by the incorporation of alternate carbon substrates or surfactants (Okolo *et al*., 2005). Manilal and Alexander (1991), reported that mineralization rate of contaminants are lower in soils with a high organic matter content, which readily absorbs hydrophobic compounds. Soluble humic substances in particular humic and fulvic acids appear to be major binding sites. Their binding potential can be attenuated by mineral soil components, as well as pH and salt concentrations (Schlautman and Morgan, 1993). Weathering or the age of contamination may also affect bioavailability by physically

trapping, hindering and / or slowing desorption of contaminants from the soil (Jaine *et al*, 2011). Bioremediation can be effective only where environmental conditions permit microbial growth and activity, its application often involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate. Like other technologies, bioremediation has its limitations. Some contaminants, such as chlorinated organic or high aromatic hydrocarbons, are resistant to microbial attack. They are degraded either slowly or not at all (Vidali, 2001).

2.5 Petroleum Hydrocarbon

Petroleum hydrocarbons are a complex mixture of saturate aliphatic and aromatic organic compounds. They can be fractionated by distillation into saturates, aromatics, asphaltenes (phenols, fatty acids, ketones, esters and porphyrins) and resins (pyridines, quinolines, carbazoles, sulfoxides and arnides). Hydrocarbons differ in their susceptibility to microbial attack and ranked in the following order of decreasing susceptibility: n-alkanes > branched alkanes > low molecular weight aromatics > cyclic alkanes (Jaine *et al*., 2011).

Aliphatic hydrocarbons in soil

Aliphatic hydrocarbons constitute a large proportion of organic contamination in the terrestrial environment. Aliphatic hydrocarbons are saturated and unsaturated linear or branched open-chain structures (Stroud *et al.*, 2007). Stroud *et al.* in (2007) defined aliphatic hydrocarbons as open-chain methane derivatives, which are both non-aromatic and non-cyclic organic compounds, containing carbon and hydrogen. Generally, the saturated *n*-alkanes are the most readily degradable components in a petroleum mixture. Biodegradation of *n*-alkanes with molecular weights up to C_{44} has been demonstrated.

Alkanes in the C_{10} to C_{26} range are considered the most readily and frequently utilized hydrocarbons. The predominant mechanism of *n*-alkane degradation involves terminal oxidation to the corresponding alcohol, aldehydes, or fatty acid functional group. Branched alkanes are less readily degraded in comparison to *n*-alkanes. Highly branched isoprenoid alkanes, such as pristane and phytane can be readily biodegradable. Cycloalkanes, however, are particularly resistant to biodegradation. Complex alicyclic compounds such as hopanes and steranes are among the most persistent compounds of petroleum spills in the environment (Venosa and Zhu, 2003). Table 1 shows the members of aliphatic hydrocarbon groups and their properties. These physicochemical properties mean that mid-length aliphatic contaminants are not readily volatilised or leached from soil. Hydrophobicity has been determined as a critical property controlling hydrocarbon behaviour in soil, affecting sequestration and biological availability. For example, as shown in Table 1 the aliphatic hexadecane is a very hydrophobic hydrocarbon (high octanol-water partition coefficient), and several orders of magnitude more insoluble than the polycyclic aromatic hydrocarbon (PAH) phenanthrene (Stroud *et al*., 2007).

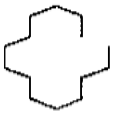
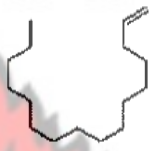
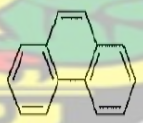
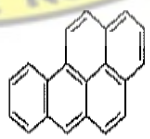
Diesel fuel is produced by refining crude oils and is a complex mixture of petroleum hydrocarbons with a carbon chain length of between C8 to C26. Engine oil is a petroleum distillate and highly refined mineral oil. Chemical constituents of engine oil include non-volatile mixture of long chain aliphatic, saturated and unsaturated hydrocarbons (C20-C50). Diesel fuel has a high content of normal, branched, cyclic and unsaturated alkanes. It also contains recalcitrant aromatic hydrocarbons and small amounts of. Benzene, toluene, ethylbenzene, xylenes and PAHs (especially naphthalene) may be present at levels of parts per million in diesel fuel. Owing to the complexity of the diesel fuel and

engine oil mixtures, analytical techniques used in most environmental assessments measure the total petroleum hydrocarbon mixture (Gaskin, 2008).

Aromatics

Although the aromatics are generally more resistant to biodegradation, some low-molecular-weight aromatics such as naphthalene may actually be oxidized before many saturates. Mono-aromatic hydrocarbons are toxic to some microorganisms due to their solvent action on cell membranes, but in low concentrations they are easily biodegradable under aerobic conditions. PAHs with 2–4 rings are less toxic and biodegradable at rates that decrease with the level of complexity. PAHs with five or more rings can only be degraded through co-metabolism, in which microorganisms fortuitously transform non-growth substrates while metabolizing simpler hydrocarbons or other primary substrates in the oil. Alkylated aromatics are degraded less rapidly than their parent compounds; the more highly alkylated groups are degraded less rapidly than less alkylated ones. The bacterial degradation of aromatics normally involves the formation of a diol, followed by ring cleavage and formation of a di-carboxylic acid. Fungi and other eukaryotes normally oxidize aromatics using mono-oxygenases, forming a trans-diol (Venosa and Zhu, 2003).

Table 1: Members of aliphatic and aromatic hydrocarbon groups and their physicochemical properties

Hydrocarbon group		Name	Formula	Molecular weight (g mol^{-1})	Structure	Melting point (°C)	Boiling point (°C)	Solubility (mg l^{-1})	log K_{ow}
Aliphatic	Alkane	Tetradecane	$C_{14}H_{30}$	198·38		5·5	253	0·000 282	7·2
	MODEL Alkane	Hexadecane	$C_{16}H_{34}$	226·44		18	287	0·0009	9·1
	Alkene	Hexadecene	$C_{16}H_{32}$	224·43		3–5	274	N/A	N/A
	Alkyne	Hexadecyne	$C_{16}H_{30}$	222·42		15	148	N/A	N/A
Aromatic	PAH	Naphthalene	$C_{10}H_8$	128·18		79–83	217·9	30	3·36
	MODEL PAH	Phenanthrene	$C_{14}H_{10}$	178·22		97–101	340	1·1	4·16
	PAH	Pyrene	$C_{16}H_{10}$	202·6		156	404	0·135	5·19
	PAH	Benzo[a]Pyrene	$C_{20}H_{12}$	252·31		175–179	495	0·0038	6·06

2.6 Factors Affecting Bioremediation

Bioremediation attempts to accelerate the biodegradation rates through the optimization of limiting environmental conditions (Margesin, 2007). These factors include: the existence of a microbial population capable of degrading the pollutants; the availability of contaminants to the microbial population and the environmental factors (type of soil, temperature, pH, the presence of oxygen or other electron acceptors, and nutrients) (Vidali, 2001).

2.6.1 Microbial Population for Bioremediation Processes

Microorganisms can be isolated from almost any environmental conditions due to their ability to adapt to these environmental conditions. The main requirements are an energy source and a carbon source. Because of the adaptability of microbes and other biological systems, these can be used to remediate environmental hazards. Microorganisms are subdivided into the following groups:

Aerobic Microbes

In the presence of oxygen (which serves as TEA). The following bacteria are known for their degradative abilities namely, *Mycobacterium, Sphingomonas, Pseudomonas, Alcaligenes*, and *Rhodococcus*. These microbes have often been reported to degrade pesticides and hydrocarbons, both alkanes and polyaromatic compounds (Vidali, 2001).

Anaerobic Microbes

Unlike aerobic bacteria, anaerobic bacteria in the absence of oxygen utilize nitrate, sulphat, during their metabolic processes. There is an increasing interest in anaerobic

bacteria used for bioremediation of polychlorinated biphenyls (PCBs) in river sediments, dechlorination of the solvent trichloroethylene (Vidali, 2001).

Methylotrophs

Methylotrophs are aerobic bacteria that grow utilizing methane for carbon and energy. It is necessary that bacteria and the contaminants achieve contact for degradation to ensue. However, this is not readily achieved, as neither the microbes nor contaminants are uniformly distributed in the soil. Some bacteria are mobile and exhibit a chemotactic response, sensing the contaminant and moving toward it. It is possible to enhance the mobilization of the contaminant utilizing some surfactants such as sodium dodecyl sulphate (Vidali, 2001). Microbial population densities in typical soils range from 10^4 to 10^7cfu/gram of soil. For land farming (bioremediation technology) to be effective, the minimum heterotrophic plate count should be 10^3 cfu/gram or greater. Plate counts lower than 10^3 could indicate the presence of toxic concentrations of organic or inorganic (e.g., metals) compounds. In this situation, land farming may still be effective if the soil is amended to reduce the toxic concentrations and increase the microbial population density (US. EPA, 2004).

2.6.2 Concentration and Toxicity

The presence of very high concentrations of petroleum organics or heavy metals in site soils can be toxic or inhibit the growth and reproduction of bacteria responsible for biodegradation in land farms. In addition, very low concentrations of organic material will also result in diminished levels of bacteria activity. In general, soil concentrations of total petroleum hydrocarbons (TPH) in the range of 10,000 to 50,000 ppm, or heavy metals exceeding 2,500 ppm, are considered inhibitory and / or toxic to most

microorganisms. If TPH concentrations are greater than 10,000 ppm, or the concentration of heavy metals is greater than 2,500 ppm, then the contaminated soil should be thoroughly mixed with clean soil to dilute the contaminants so that the average concentrations are below toxic levels. Below a certain "threshold" constituent concentration, the bacteria cannot obtain sufficient carbon (from degradation of the constituents) to maintain adequate biological activity (US. EPA, 2004).

2.6.3 Environmental Factors

Soil Texture

Soil texture generally tends to affect the permeability, moisture content, and bulk density of the soil. To ensure that oxygen and moisture addition as well as nutrient distribution of the soils can be maintained within effective ranges, it is therefore imperative to consider the texture of the soils. Example, soils which tend to clump together are difficult to aerate and result in low oxygen concentrations. It is also difficult to uniformly distribute nutrients throughout these soils. They naturally retain water for extended periods (US. EPA, 2004).

Soil moisture

Soil moisture is essential to biodegradation since the majority of microorganisms live in the water film surrounding soil particles. Soil water serves as the medium through which many organic constituents and nutrients diffuse into the microbial cells by way of transportation, and through which metabolic waste products are removed. Soil moisture content also affects the nature of soil, soil aeration status, and amount of soluble materials, soil water osmotic pressure, and the pH of the soil. The extent to which the soil pore space is filled with water affects the exchange of gases through the soil. When soil

pores become filled with water, the diffusion of gases through the soil is severely restricted, oxygen is consumed faster than it is replenished in the soil vapor space, the soil becomes anaerobic, and major shifts in microbial metabolic activity occur. Soil moisture content should be in the range of 25% – 85% of the water holding capacity; a range of 50% – 80% is optimal for biodegradation. The soil water holding capacity is equivalent to the "field capacity," which is the percentage of water remaining in a soil after it has been saturated and gravitational drainage has ceased (WDNR, 1994).

Temperature

Temperature is essential to ensure biodegradation of petroleum hydrocarbons, as a result of its direct impact on the chemistry of the pollutants and on its effect on the physiology and diversity of the microorganisms. Ambient temperature of an environment affects both the property of spilled oil and the activity of microorganisms (Venosa & Zhu, 2003). At low temperatures, the viscosity of the oil increases, while the volatility of toxic low-molecular weight hydrocarbons is reduced, delaying the onset of biodegradation. Temperature affects the solubility of hydrocarbons; although, hydrocarbon biodegradation can occur over a wide ranges of temperature. The rate of biodegradation generally decreases with decreasing temperature. Highest degradation rates generally occur in the range of (30 - 40) °C in soil environments (Jaine *et al.*, 2011). Deeb and Alvarez-Cohen (1999) found that their consortium grew best at 35 °C. Temperature affects biochemical reactions rates, and the rates of many of them double for each 10 °C rise in temperature. Above a certain temperature, however, the cells die (Vidali, 2001).

Soil pH

Soil pH is an important soil parameter to be considered. Basically pH is an indicator of hydrogen ion activity in the soil. Soil pH in the range of 5 to 9 is generally acceptable for biodegradation; a pH of 6.5 to 8.5 is generally considered to be appropriate for optimal biodegradation efficiency. Organic soils in wetlands are often acidic, while mineral soils have more neutral and alkaline conditions. Most heterotrophic bacteria and fungi favor a neutral pH, with fungi being more tolerant of acidic conditions. Studies have shown that degradation of oil increases with increasing pH, and that optimum degradation occurs under slightly alkaline conditions (Venosa and Zhu, 2003). If the soil has too much acid it is possible to buffer the pH by adding crushed limestone or agricultural lime (Vidali, 2001). Most heterotrophic bacteria favour a pH 7.0. Extreme pH of soils would have a negative influence on the ability of microbial populations to degrade hydrocarbons (Jaine *et al*., 2011).

Oxygen Availability

Oxygen serves as a terminal electron acceptor (TEA) on the basis of which microbial population within soil depends on for their oxygen during metabolism. The rate of aerobic biodegradation is typically limited by the rate at which oxygen is supplied. The major source of oxygen in soil is diffusion from the atmosphere. When soil pores become filled with water, the diffusion of gases through the soil is restricted. Oxygen may be consumed faster than it can be replaced by diffusion from the atmosphere, and the soil may become anaerobic. Clayey soils tend to retain a higher moisture content, which restricts oxygen diffusion. Organic matter may increase microbial activity and deplete available oxygen. For aerobic metabolism, dissolved oxygen concentrations in the soil moisture of greater than 0.2 mg/l are necessary; oxygen becomes rate-limiting at

dissolved oxygen concentrations below about 1 mg/l. Oxygen levels in soil gas should be at least 2-5% in order to avoid oxygen limitation of aerobic microbial activity. A minimum air-filled porosity of 10% is necessary to allow for adequate oxygen diffusion in the soil gas (WDNR, 1994).

Presence of other electron acceptors

Oxygen is the primary electron acceptor for aerobic biodegradation processes. However, in situations where oxygen levels are low, other terminal electron acceptors may be utilized for microbial metabolism. Nitrate (NO_3^-), iron (Fe^{3+}), manganese (Mn^{3+}), and sulfate (SO_4^{2-}) can act as electron acceptors if the organisms have the appropriate enzyme systems. Sulfate is utilized by microbes when the supply of oxygen or nitrate is low. Microbes can reduce the sulfate molecule to provide energy for metabolism (WDNR, 1994).

Availability of Nutrients

Microbial metabolism and growth is dependent upon the availability of essential nutrients in a usable form, and appropriate concentrations, and proper ratios. Carbon (C), nitrogen (N), and phosphorous (P) are essential nutrients (WDNR, 1994). These nutrients serve as the basic building blocks of life and allow microbes to create the necessary enzymes to break down the contaminants. Carbon is the most basic element of living forms and is needed in greater quantities than other elements. The microbial cell is composed of Carbon 50%; Nitrogen 14%; Oxygen 20%; H 8%; Phosphorous 3%; Potassium1%. The nutritional requirement of carbon to nitrogen ratio is 10:1, and carbon to phosphorous is 30:1 (Vidali, 2001).

In theory, approximately 150 mg of nitrogen and 30 mg of phosphorus are utilized in the conversion of 1 g of hydrocarbon to cell materials (Rosenberg & Ron, 1996). Biodegradable organic compounds provide a carbon source, and total organic carbon (TOC) is a measure of the total carbon, or "food," that microbes may utilize for energy and growth. All organic chemicals present in the soil are included in the TOC measurement. Nitrogen in the form of organic nitrogen, ammonia (NH_3), nitrite (NO_2^-), and nitrate (NO_3^-), and phosphorous in the form of soluble or reactive phosphorous (*o*-PO_4, or orthophosphate) are suitable to microbes for growth. Nitrate can also provide energy to microbes when oxygen levels are low. The amount of nitrogen in decomposing organic matter is important in controlling the rate of contaminant degradation by microorganisms. Total organic nitrogen (TON) is a measure of this and total organic matter which contains greater than 1.5% to 1.7% nitrogen is probably adequate to meet the microbial nitrogen requirements during contaminant metabolism. TON is measured as kjeldahl N less inorganic N. A C:N ratio of less than 20-25 should lead to mineralization (excess N present); a C:N ratio of greater than 35-40 generally indicates inadequate nitrogen, which could limit biodegradation due to depletion of mineralized nitrogen resulting in nitrogen starvation. Similarly, immobilization of phosphorous can occur when the C:P ratio is greater than 120:1. A suggested C:N:P ratio of 100:10:1 is considered optimal. Ratios should be used with caution, however, since they do not indicate the availability of the carbon, nitrogen, or phosphorous to microorganisms. Other nutrients (K, Ca, Mg, S, etc.) are typically found in adequate supply for metabolic needs in most soils. However, high concentrations of calcium and magnesium may precipitate phosphates, and will reduce the amount available for microbial metabolism. High levels of chlorides may inhibit microbial activity (WDNR, 1994). Okolo *et al*. (2005) reported that the application of poultry manure as organic fertilizer in contaminated soil increased

biodegradation but the extent of biodegradation was influenced by the incorporation of alternate carbon substrates or surfactants. Chaillan *et al*. (2006), reported that excessive nutrient concentration can inhibit the biodegradation activity and several authors have also reported the negative effect of a high NPK levels on the hydrocarbons biodegradation and more especially on the aromatic (Jaine *et al*., 2011).

2.7 Advantages and Disadvantages of Bioremediation

2.7.1 Advantages of bioremediation

- Theoretically, bioremediation is a natural process useful for complete destruction of a broad spectrum of contaminants.Many compounds that are categorized as hazardous can be transformed to innocuous forms; hence eliminates the chance of future liability associated with treatment and disposal of contaminated substance.
- Bioremediation process avoids the transfer of contaminants from one environmental medium to another; example, from land to water.
- Bioremediation has gain public acceptance as a waste treatment process for contaminated reservoir such as soil. Microbes able to degrade the contaminant increase in numbers in the presence of the contaminant; when the contaminants undergo degradation the population declines. The resulting products are harmless and include carbon dioxide, water, and cell biomass.
- Bioremediation is considered to be less expensive compared to other clean-up technologies.
- Bioremediation can often be carried out on site, hence preventing the potential threats to human health as well as the environment that can arise during transportation.

2.7.2 Disadvantages of bioremediation

- Bioremediation is limited to those compounds that are biodegradable. However, there are some concerns that the products of biodegradation may turn out to be more persistent or toxic than the parent compound.
- Bioremediation often takes longer than other treatment options, such as excavation and incineration.
- Bioremediation is a biological process that requires metabolically capable microbial populations, suitable environmental growth conditions, and appropriate levels of nutrients and contaminants to ensure it success.
- The need to conduct research is imperative to develop and engineer bioremediation technologies that are appropriate for sites with complex mixtures of contaminants that are not evenly dispersed in the environment.

2.8 Biosurfactant

Biosurfactants are important biotechnological products that can efficiently enhance bioremediation of hydrocarbon contaminated environments. Biosurfactants or microbial surfactants are surface metabolites (surface-active substances) that are produced by microorganisms such as bacteria, yeast and fungi; that have very different chemical structures and properties. Biosurfactants are amphiphilic molecules consisting of hydrophobic and hydrophilic domains (Techaoei *et al*., 2007). Biosurfactant properties of interest are as follow: in changing surface active phenomena, such as lowering of surface and interfacial tensions, wetting and penetrating actions, spreading, hydrophylicity and hydrophobicity actions, microbial growth enhancement, metal sequestration and anti-microbial action (Kosaric, 2001). A characteristic feature of biosurfactants is a hydrophilic-lipophilic balance (HLB) which specifies the portion of hydrophilic and

hydrophobic constituents in surface-active substances. Due to their amphiphilic structure, biosurfactants increase the surface area of hydrophobic water-insoluble substances, increase the water bioavailability of such substances and change the properties of the bacterial cell surface. Surface activity makes surfactants excellent emulsifiers, foaming and dispersing agents (Pacwa-Płociniczak *et al*., 2011).

Biosurfactant, in comparison to their chemically synthesized equivalents possess many positive advantages. Some of these are: biodegradability, generally low toxicity, biocompatibility and digestibility (which allows their application in cosmetics, pharmaceuticals and as functional food additives); Availability of raw materials **(**biosurfactants can be produced from industrial wastes and from by-products which are available in large quantities), This feature makes cheap production of biosurfactants possible and allows utilizing waste substrates. Biosurfactants can also be produced from industrial wastes and by-products and this is of particular interest for bulk production (*e.g*. for use in petroleum-related technologies), Use in environmental control - biosurfactants can be efficiently used in handling industrial emulsions, control of oil spills, biodegradation and detoxification of industrial effluents and in bioremediation of contaminated soil.

Specificity – biosurfactants, being complex organic molecules with specific functional groups, are often specific in their action (this would be of particular interest in detoxification of specific pollutants), de-emulsification of industrial emulsions, specific cosmetic, pharmaceutical, and food applications, effectiveness at extreme temperatures, pH and salinity (Kosaric, 2001).

Table 2: Various biosurfactants produced by microorganisms

Microorganism	Type of surfactant
Torulopsis bombicola	Glycolipid (sophorose lipid)
Pseudomonas aeruginosa	Glycolipid (rhamnose lipid)
Bacillus subtilis	Lipoprotein (surfactin)
Corynebacterium lepus	Corynomycolic acids
Candida petrophilum	Peptidolipid
Rhodococcuseryithropolis	Trehalosedimycolates

Among the different classes of biosurfactants rhamnolipid and surfactin as shown in Table 2, are the best studied biosurfactants (Priya and Usharani, 2009). Biosurfactants increase the bioavailability of hydrocarbons resulting in enhanced biodegradation of hydrocarbon contaminants present in the polluted soil (Pacwa-Płociniczak *et al*., 2011).

2.8.1 Properties of Biosurfactants

Biosurfactants are categorized by their chemical composition, molecular weight, physico-chemical properties and mode of action and microbial origin. Based on molecular weight they are divided into low-molecular-mass biosurfactants and high-molecular-mass biosurfactants / bioemulsifiers. Low-molecular-mass biosurfactants are efficient in lowering surface and interfacial tensions. The biosurfactants accumulate at the interface between two immiscible fluids or between a fluid and a solid. By reducing surface (liquid-air) and interfacial (liquid-liquid) tension they reduce the repulsive forces between two dissimilar phases and allow these two phases to mix and interact more easily. Biosurfactant activities depend on the concentration of the surface-active compounds

until the critical micelle concentration (CMC) is obtained. At concentrations above the CMC, biosurfactant molecules associate to form micelles, bilayers and vesicles. Micelle formation enables biosurfactants to reduce the surface and interfacial tension and increase the solubility and bioavailability of hydrophobic organic compounds (Whang *et al*., 2008). Biosurfactants also influence the bacterial cell surface hydrophobicity (CSH) by causing structural changes in the bacterial cell surface. This increase accessibility of hydrocarbons to microbial cells (Pacwa-Płociniczak *et al*., 2011).

2.8.2 Role of Biosurfactants in Biodegradation Processes

Biosurfactants enhance hydrocarbon bioremediation by two mechanisms. The first includes the increase of substrate bioavailability for microorganisms, while the other involves interaction with the cell surface which increases the hydrophobicity of the surface allowing hydrophobic substrates to associate more easily with bacterial cells. By reducing surface and interfacial tensions, biosurfactants increase the surface areas of insoluble compounds leading to increased mobility and bioavailability of hydrocarbons. In consequence, biosurfactants enhance biodegradation and removal of hydrocarbons. Addition of biosurfactants can be expected to enhance hydrocarbon biodegradation by mobilization, solubilization or emulsification (Pacwa-Płociniczak *et al.*, 2011). The mobilization mechanism occurs at concentrations below the biosurfactant CMC. At such concentrations, biosurfactants reduce the surface and interfacial tension between air / water and soil / water systems. In turn, above the biosurfactant CMC the solubilization process takes place. At these concentrations biosurfactant molecules associate to form micelles, which dramatically increase the solubility of oil. The hydrophobic ends of biosurfactant molecules connect together inside the micelle while the hydrophilic ends are exposed to the aqueous phase on the exterior. Consequently, the interior of a micelle

creates an environment compatible for hydrophobic organic molecules. The process of incorporation of these molecules into a micelle is known as solubilization (Urum and Pekdemir, 2004). Emulsification is a process that forms a liquid, known as an emulsion, containing very small droplets of fat or oil suspended in a fluid, usually water. The high molecular weight biosurfactants are efficient emulsifying agents (Pacwa-Płociniczak *et al*., 2011).

2.9 Bile

Generally bile acids are steroid acids found predominantly in the bile of humans and mammals. Cattle bile like any other bile is a digestive secretion. Cattle bile is green turbid appearance of aqueous solution of organic compounds. Bile salts are bile acids compounded with a cation, usually sodium. The two major bile acids are cholic acid, and chenodeoxycholic acid. Cattle bile (CB) is constituted of bile acids with a relatively high hydrophobicity, such as taurine and glycine conjugates of cholic and deoxycholic acids (Ishikawa and Watanabe, 2011). Bile acid constitutes approximately 50% of the organic components of bile. Table 3 shows bile acid composition of cattle bile and their corresponding percentages. Bile acids are synthesized from cholesterol by multienzyme process. All bile acids are conjugated as peptide linkage with their glycine (glycoconjugated) or taurine (tauroconjugated). Bile acids are amphipathic and can self-associate in water to form polymolecular aggregates called miscelles (Maire *et al.*, 2005). Bile acid above a certain conncentration called the critical micellar concentration result in the formation of micelle (Cohen and Carey, 1990). These micelles can in turn solubilize other lipids in the form of mixed micelles. Bile acids below their CMC are surface active binding to air-water and lipid-water interfaces.

Table 3: Bile acid composition of cattle bile

Bile acids Proportion	(%, w/w)
Taurocholic acid	(TCA) 19.5
Glycocholic acid	(GCA) 14.0
Glycodeoxycholic acid	(GDCA) 1.7
Taurodeoxycholic acid	(TDCA) 4.9

(Ishikawa and Watanabe, 2011).

2.9.1 Functions and dysfunction of bile acid

The major function of bile in vivo is to act as a biological detergent which emulsifies and solubilize fats. Bile decreases the surface tension by means of its surfactant properties (Luner, 2000). Surface tension may in turn be important for the wetting and mobilization of contaminants from soil (Charman *et al.,* 1997). The amphipathic nature of bile acids allows them to possess detergent action by causing emulsification. Emulsification greatly increase the surface area of fat making it bioaccessible. The ability of bile acid to interact with bacterial membrane lipids in order to increase the hydrophobicity of the cell surface membrane can be attributed to its detergent properties on bile. Hence high concentration of the bile acid can confer antimicrobial properties on bile (Maire *et al.,* 2005). Research works in digestion models have revealed many differences in bioaccessibility of different bile, seeing as bile composition to be species dependent (Oomen *et al.,* 2003).

2.9.2 Antimicrobial action of bile

Bile generally has been found to exert its effect on cell membranes. The addition of bile resulted in haemolysis. Electron microscope has shown that cells subjected to bile shrink and empty cell content. Enzyme assays have confirmed leakages of intracellular material.

Hence both experimental outcomes imply that bile alters membrane integrity or permeability (Noh and Gilliland, 1993).

CHAPTER THREE

3.0 METHODOLOGY

3.1 Materials and methods

3.1.1 Samples collection

The contaminated soil as well as the compost material employed in this study was obtained from Newmont Mining Company Limited at Kenyasi in the Brong Ahafo Region of Ghana. The petroleum hydrocarbon contaminated soil was taken from a volatilization site (where contaminated soil has been deposited); at a surface soil of (0 to15 cm). The compost on the other hand used in this experiment was obtained from a waste processing plant (where compost is manufactured using sewage sludge / foodwaste). The soil and compost sampled were carried inside sterile labelled polyethylene plastic bags and transported to KNUST. Inorganic fertilizer with nutrients NPK 20:10:10 was obtained from a chemical shop in Kumasi whereas the cattle bile was obtained from the Kumasi abattoir and refrigerated 4 ^{o}C.

3.1.2 Sample preparation

Large debris was removed from the surface of the contaminated soil. The contaminated soil sample was air-dried for a period of 5-days in a clean well-ventilated laboratory. The soil sample was pulverized and passed through a (2 mm pore size) sieve. The soil sample was thoroughly mixed to ensure proper mixing of the contaminant thereby achieving homogeneity. The prepared soil sample was kept at room temperature for later use. The compost was sun-dried, and was sieved by passing it through a (2 mm pore size) to achieve uniform particle size and kept in a neat polythene bag at room temperature for use. However both soil and compost samples awaiting microbial analysis were placed in polyethylene bags tightly closed and stored inside a refrigerator at 4 ^{o}C. The granular

inorganic NPK fertilizer was grinded in a clean sterile crucible into a powdered form for further usage.

3.2 Baseline study

According to Vidali (2001), for bioremediation process to be effective, information regarding the physicochemical properties and the indigenous microbial community of the contaminated soil are essential. In view of this assertion, the present study was focused on the assessment of physicochemical properties of contaminated soil. It is equally essential to evaluate the physicochemical properties of the amendment additives such as compost, NPK fertilizer and cattle bile.

3.3 Physicochemical analysis of contaminated soil sample, compost and cattle bile

Portion of the prepared contaminated soil was obtained and analyzed to determine total petroleum hydrocarbon, pH, moisture content, organic matter, organic carbon, nitrogen, phosphorous, and potassium prior to amendment. The compost as an amendment material was also subjected to analysis of the following; pH, moisture content, organic matter, organic carbon, nitrogen, phosphorous, potassium. Additionally cattle bile was analyzed for pH, nitrogen and phosphorous. The methods described below were employed in the analysis of the afore-mentioned physicochemical parameters.

3.3.1 Determination of TPH of prepared contaminated soil sample

Extraction of oil from the prepared soil for TPH analysis is achieved using the Soxhlet Extraction method: 10 g of test soil sample is mixed with 10 g of anhydrous sodium sulfate and placed in an extraction thimble and extracted with 200 ml of dichloromethane (methylene chloride) for 2 hours at 4 cycles / hour using a Soxhlet extractor as shown in appendix 2. The dichloromethane fraction was collected in a pre-weighed 250 ml round bottomed flask and the dichloromethane evaporated using a rotary evaporator at 40 °C. The oil extract was subjected to analysis of TPH using gas chromatography machine (GC) coupled with a flame ionization detector (FID), (ERI, 1999).

3.3.2 Determination of pH of soil sample, compost and cattle bile

Procedure:

10.0 g of dried contaminated soil sample was measured and transferred into a 50 ml cleaned beaker; 20 ml of distilled water was added as a suspension medium. The suspension was thoroughly stirred for 30 minutes, after which the calibrated pH meter (pH testr 20) was dipped into the beaker containing the suspension and the pH value was recorded. This was triplicated and the average pH values recorded. The pH of compost as well as cattle bile juice were equally determined by the procedural method described above: However, in the case of cattle bile juice 20 ml of bile was measured into the 50 ml beaker and the pH meter was dipped into the beaker containing the cattle bile juice and the pH value was recorded.

3.3.3 Determination of moisture content of soil sample and compost

Procedure:

100 g of the soil was put in the aluminium moisture box and placed in the oven, after removing the lid of the box. The sample was kept at 105 ºC for 24 hours until it attained a constant weight. The sample was then allowed to cool, first in the switched-off oven and then in a desiccator. The cooled sample was then weighed and the value recorded. The weight of sample was determined using electronic weighing balance. The loss in weight becomes the moisture contained in 100 g of soil sample (Motsara and Roy, 2008). The percentage of moisture is calculated as:

$$\text{Moisture percent} = \frac{Loss\ in\ weight}{Oven-dry\ weight\ of\ soil}\ x\ 100$$

The above described method was also employed in determining the moisture content of compost and the percentage of moisture calculated using the formula stated above.

3.3.4 Determination of Phosphorous in contaminated soil, compost and cattle bile

Procedure:

The soil sample was subjected to heating at 550 ºC by placing a suitable weight 1.0 g of the soil sample in a silica crucible and heated in a muffle furnace for 5 hours. The ash residue was dissolved in dilute HNO_3, and filtered through acid-washed filter paper in a 100 ml volumetric flask and the volume was made up to the mark. Phosphorous was then determined using spectrophotometer (Motsara and Roy, 2008). A blank determination was carried out to account for any contamination through the acids used in the digestion. This method was also used to determine the phosphorous present in compost and cattle bile: But in the case of cattle bile (liquid state) the sample was foremost solidified by evaporating the bile in a clean crucible on a hot plate before it was subjected to the method described above.

3.3.5 Determination of Organic Matter in soil sample and compost

Procedure:

10 g of sieved soil was put into an ashing vessel. The ashing vessel with soil was placed in a drying oven set at 105 ºC for 4 hours. The ashing vessel was removed from the drying oven and placed in a dry atmosphere. The ashing vessel was then weighed (after it had cooled) by using electronic balance. The ashing vessel with soil was placed into a muffle furnace set at a temperature of 400 ºC and ashed for 4 hours. The ashing vessel was removed from the muffle furnace and cooled in a dry atmosphere and weighed as shown in appendix 3. The percent organic matter and organic carbon is calculated as shown below.

Calculation:

$$(\%\ OM) = \frac{(W1-W2)}{W1}\ x\ 100$$

Where:

W_1 is the weight of soil at 105 ºC

W_2 is the weight of soil at 400 ºC

The percent of organic carbon is given by: % OM X 0.58

The above method was also used in determining the organic carbon content of compost and the percentage of organic carbon calculated using the formula above.

3.3.6 Determination of Potassium in soil sample and compost

The soil sample was heated at 550 ºC by placing a suitable weight 1.0 g of the soil sample in a silica crucible and heated in a muffle furnace for 5 hours. The ash residue was dissolved in dilute HNO_3, and filtered through acid-washed filter paper in a 100 ml volumetric flask and the volume was made up to the mark. Potassium was then determined using flame photometer (Motsara and Roy, 2008). A blank determination was

carried out to account for any contamination through the acids used in the digestion. The procedural method described above was also used to determine the potassium present in compost.

3.3.7 Determination of nitrogen in contaminated soil sample and compost

Digestion:

10 g air dry soil sample was weighed into 500 ml long-necked kjeldahl flask and 10 ml distilled water was added to moisten the sample. 1 spatula full of kjeldahl catalyst (mixture of 1 part selenium + 10 parts $CUSO_4$ + 100 parts Na_2SO_4), followed by 20 ml concentration of H_2SO_4 was added to the mixture in the kjeldahl flask. The mixture was then left to digest until the solution appeared clear and colourless. The flask was allowed to cool, and the fluid decanted into a 100 ml volumetric flask and made up to the mark with distilled water.

Distillation:

By means of a pipette, an aliquot of 10 ml fluid from the digested sample was transferred into kjeldahl distillation flask. 90 ml of distilled water was added to make it up to 100 ml in the distillation flask. 20 ml of 40% NaOH was added to the content of the distillation flask. Distillate was collected over 10 ml of 4% boric acid and 3 drops of mixed indicator in a 200 ml conical flask was collected as shown in appendix 4. The presence of nitrogen gives a light blue colour.

Titration:

The collected distillate was then titrated (about 100 ml) with 0.1N HCL till the blue colour changes to grey and then suddenly flashes to pink (Horwitz and Latimer, 2005). The titration process is shown in appendix 5. A blank determination was carried out without a sample.

Calculation:

Weight of sample used, considering the dilution and the aliquot taken for distillation

$$\frac{10\ g\ x\ 10\ ml}{100\ ml} = 1\ g$$

$$\%N = \frac{14\ X\ (A-B)\ X\ N\ X\ 100}{1000\ X\ 1}$$

Where:

A= volume of standard HCL used in sample titration

B= volume of standard HCL used in blank titration

N= normality of standard HCL

Nitrogen determination in compost was also carried out using the method as described above.

3.3.8 Determination of total nitrogen in cattle bile

Digestion:

50 ml distil water and 10 ml concentration H_2SO_4 were added to 100 ml of cattle bile juice. One digestion tablet and boiling chips were added and fitted in a digestion unit to boil until the solution is clear (straw colour/yellow-like). A blank determination was performed.

Distillation

After distillation 300 ml distilled water and 50 ml NaOH – $Na_2S_2O_3$ were added and the mixture placed in the distillation unit. Distillates were collected in 50 ml boric acid in Erlenmeyer flask till content of Erlenmeyer flask is 200 ml. Titrate with 0.02N HCl. The blank was included (Sadsivam and Manickam, 1991)

Calculation:

$$\% \text{ N} = \frac{(\text{Volume of ml HCl Sample} - \text{Volume of ml HCl Blank})(\text{N HCl})\ 1.401}{\text{Volume of Sample}}$$

3.3.9 Determination of amount of compost and NPK fertilizer required for the amendment process

Table 4 indicates the amounts or masses of the different amendment materials viz; compost and NPK fertilizer required to treat (by blending) 1000 g of contaminated soil in order to achieve 0.2% and 0.4% levels of nitrogen: Giving the fact that the test soil is limited in nitrogen content as illustrated in Table 6. The determination of the amount of amendment materials (compost and NPK fertilizer) needed for the amendment is as shown in appendix 1.

Table 4: Amount of amendment materials required to achieve 0.2% and 0.4% nitrogen level prior to incubation

Amendment Material	Nutrient supplement type	Mass of Amendment Material in (g) required for treatments	
		0.2% Nitrogen	0.4% Nitrogen
Compost	Nitrogen	107.48 gram	243.54 gram
NPK fertilizer	Nitrogen	10.53 gram	23.87 gram

3.4. Determination of total heterotrophic bacteria in the contaminated soil, compost and cattle bile

Microbial population of contaminated soil sample, compost, and cattle bile prior to the study was conducted, using the plate count method. Total viable count of heterotrophic bacterial population was determined by isolating using the pour plate technique and growth on plate count agar (PCA). Serial dilutions of 10^{-1} to 10^{-10} were prepared aseptically by diluting 1g of the contaminated soil sample into 10 ml of sterile distilled water and mixed using the pulsifier. 1 ml aliquots from each of the dilution were inoculated into or on sterile petri dishes with already prepared PCA at 40 ^{o}C. The plates were then incubated at a temperature of 35 ^{o}C for 24 hours. After incubation all white spots or spread were counted and recorded as total viable counts using the colony counter. Results were recorded as colony forming unit cfu per gram of soil.

3.5 Experimental setup

3.5.1 Basic treatment

1000 g of the prepared contaminated soil sample as mentioned earlier, was each measured using an electronic balance and transferred into nine sterile dry containers moistened without making the microcosms dripping wet with distilled water to ensure proper mixing with the contaminant. Adjusted nitrogen levels at 0.2% and 0.4% of the different nutrient sources were established. In order to establish microcosms at 0.2%, a calculated deficit mass of 107.43 g and 10.53 g of compost and 20:10:10 NPK fertilizer respectively were applied or added to 1000 g of the test contaminated soil sample contained in four sterile plastic containers; such that two containers would have compost blends at 0.2%, and the other two containers 0.2% fertilizer blends. The mixtures were thoroughly mixed up by using a sterile spatula: Furthermore 2 ml of cattle bile each was measured and transferred

into one of the containers with contaminated soil + compost (0.2% nitrogen concentration) matrix as well as another container with contaminated soil + fertilizer (0.2% nitrogen concentration) matrix. The mixtures were thoroughly mixed to achieve homogeneity. The act of stirring provides aeration and ensures mixing of nutrients and microbes with the contaminated soil. The other two containers containing mixtures of contaminated soil + compost at (0.2% nitrogen concentration) and contaminated soil + fertilizer at (0.2% nitrogen level) were without cattle bile. Four microcosms of exactly the same amendment blends as described above was established at 0.4% nitrogen level simultaneously, using a calculated deficit mass of 243.54 g and 23.87 g of compost and 20:10:10 NPK fertilizer respectively. The calculation of the mass deficit of nitrogen in contaminated soil to achieve 0.2% nitrogen level and 0.4% nitrogen level for the two nutrient sources can be seen in appendix 1. 2 ml of cattle bile was inoculated into two of the four microcosms maintained at 0.4% nitrogen level; whereas the other remaining two microcosms were devoid of cattle bile. Four experimental controls were established under intrinsic conditions (without amendments). The twelve experimental setups as shown in Table 5 were triplicated and incubated for six weeks by mounting them in a green house of temperature ranging from (25 – 45) ^{o}C. The microcosm experiments were left uncovered to allow diffusion of oxygen into the treated soil matrix. Complete randomized design was adopted as the experimental design for the microcosm experiments.

3.5.2 Experimental protocol

Table 5: Experimental design

Experimental set	Test experiment
Set A	1000g of contaminated soil + 0.2% compost
Set B	1000g of contaminated soil + 0.2% NPK fertilizer
Set C	1000g of contaminated soil + 0.4% compost
Set D	1000g of contaminated soil + 0.4% NPK fertilizer
Set A2	1000g of contaminated soil + 0.2% compost + CB
Set B2	1000g of contaminated soil + 0.2% NPK fertilizer + CB
Set C2	1000g of contaminated soil + 0.4% compost + CB
Set D2	1000g of contaminated soil + 0.4% NPK fertilizer + CB
Set Ct1	1000g of contaminated soil + no amendment

3.6 Monitoring and analysis of microcosm experiments

In order to remove the effect of the lack of oxygen and preparing aerobic soil conditions, all the triplicates of the microcosm experiments were subjected to stirring thrice each week during six weeks of incubation, to provide sufficient air and oxygen. Aeration was achieved by using sterile spatula to stir the microcosms under investigation. 50g each of the different microcosm experiments were sampled at two weeks interval during incubation. The samples were then subjected to physicochemical analyses viz, pH, temperature: Nutrient analyses such as; total nitrogen and organic carbon: and analyses of residual TPH as well as carrying out microbiological analyses. The various parameters listed were determined using the appropriate standard methods as illustrated above. Analysis of variance (ANOVA) was done to determine the level of significance at $p < 0.05$ between the results obtained at each period of the degradation process.

CHAPTER FOUR

4.0 RESULT

Physicochemical properties of the contaminated soil as well as the nutrient supplements used in the baseline study are shown in Table 6. The test soil under investigation can be described as been contaminated with hydrocarbon contaminants to some degree; with mean TPH concentration of 1009.12 mg/kg. The pH of the contaminated soil i.e. 7.95 falls within a suitable range (5 – 9) required for bioremediation processes to occur. However, the nitrogen content of the contaminated soil was found to be 0.042% which appeared to be lower as compared to the nitrogen content of compost (1.47%). The pH of compost and cattle bile was found to be 7.96 (slightly alkaline) and 7.05 (almost neutral) respectively.

Table 6: Baseline result of physicochemical parameters of the test soil and amendment additives

Physicochemical Parameter	Contaminated soil	Compost	Cattle bile
TPH (mg/kg)	1009.12 ± 0.0000	-	-
Total Nitrogen (%)	0.04 ± 0.0006	1.47 ± 0.0152	0.06 ± 0.0007
Organic matter (%)	2.76 ± 0.0000	10.19 ± 0.0004	-
Organic Carbon (%)	1.60 ± 0.0000	5.91 ± 0.0001	-
Potassium (mg/g)	0.82 ± 0.0142	1.31 ± 0.0226	-
Phosphorous (mg/g)	1.24 ± 0.0177	3.31 ± 0.0472	0.58 ± 0.0165
Moisture (%)	1.52 ± 0.0039	4.96 ± 0.0053	85 ± 0.0000
pH	7.95 ± 0.0153	7.76 ± 0.0151	7.05 ± 0.1440

The heterotrophic bacterial count of contaminated soil, and compost, and cattle bile is shown in Table 7. Compost was found to possess the highest number of heterotrophic bacteria of mean number of 5.00 x 10^5 cfu/g. Cattle bile has microbial load of mean

number of 7.37 x 10^4 cfu/g. The soil under study possesses the least mean number of heterotrophic bacteria i.e. 4.50 x 10^4 cfu/g.

Table 7: Baseline result of total heterotrophic bacteria in contaminated soil, compost, and cattle bile prior to incubation

Material	Total Heterotrophic Bacterial count (cfu/g)
Contaminated soil	4.50 x 10^4 ± 0.0 x 10^4
Compost	5.00 x 10^5 ± 7.0 x 10^4
Cattle bile	7.37 x 10^4 ± 5.8 x 10^2

4.1 Treatments with different nutrient sources only (devoid of cattle bile)

The data of result shows that TPH degradation indeed occurred in all five microcosm experiments as demonstrated in Table 8. After two weeks of incubation the rate of TPH component removal varied between a maximum value of approximately 93.15% and a minimum of 65.66% exhibited by microcosm A and Ct1 respectively from an initial TPH concentration of 1009.12 mg/kg. The other microcosm experiments follows the order; D(89.32%) > B(87.58%) > C(83.06%). It was observed that after week four of incubation, the following microcosm experiments i.e. A, C, D, B, and Ct1 exhibited 93.61%, 90.20%, 89.41%, 88.74% and 79.57% rate of TPH component removal from the soil respectively by an increased factor in the order; Ct1(13.91%) > C(7.14%) > B(1.16%) > A(0.46%) > D(0.09%). At the end of the sixth week it was observed that rate of TPH reduction generally increased. These rates of TPH reduction of the different microcosm experiments and their corresponding percentage factor of increased TPH reduction is in the order; B(94.87 – 6.13%) > C(94.64 - 4.44%) > A(4.14 – 0.53%) > D(91.43 – 2.02%) > Ct1(79.57 – 0.00%).

Table 8: Effect of different nutrient (compost and NPK fertilizer) only on the rate of TPH reduction during incubation

Treatment	Week 0	Week 2		Week 4		Week 6
	Initial TPH concentration (mg/kg)	Rate of TPH reduction (%)	Increased factor of rate of TPH reduction (%)	Rate of TPH reduction (%)	Increased factor of rate of TPH reduction (%)	Rate of TPH reduction (%)
Ct1 (0.0%)	1009.12	65.66 ± 3.00	13.91	79.57 ± 2.00	0.00	79.57 ± 2.00
A (0.2%)	1009.12	93.15 ± 2.00	0.46	93.61 ± 1.73	0.53	94.14 ± 1.73
C (0.4%)	1009.12	83.06 ± 2.00	7.14	90.20 ± 1.73	4.44	94.64 ± 7.23
B (0.2%)	1009.12	87.58 ± 2.08	1.16	88.74 ± 2.31	6.13	94.87 ± 2.00
D (0.4%)	1009.12	89.32 ± 1.53	0.09	89.41 ± 1.15	2.02	91.43 ± 2.00

Note: Each value is a mean of three replicates and ± indicates standard deviation among them.

4.2 Treatments with different nutrient sources in combination with cattle bile

Table 9 below indicates the rate of TPH degradation exhibited by the following microcosm experiments viz., B2, C2, A2, D2 and Ct1. The rate of TPH removal demonstrated by the aforementioned microcosm experiments are (95.12, 87.49, 81.89, 76.56, and 65.66%) respectively after two weeks of incubation. Furthermore, after four weeks of incubation, the rate of TPH components removal of each microcosm experiments was observed to have increased by a factor in the order; B2(2.10%), D2(10.14%), C2(2.27%), A2(3.21%), Ct1(13.91%), which eventually resulted in the following rate of TPH degradation in the order; B2(97.22%) > D2(90.00%) > C2(89.76%) > A2(85.10%) > Ct1(79.57%) respectively. Microcosms B2 and Ct1 emerged as the maximum and minimum rate of TPH residual reduction after the sixth week of incubation period according to the order; B2(98.43%) > D2(93.65%) > A2(93.57%) > C2(92.44%) > Ct1(79.57%). The percentage increase in the rate of TPH component reduction of the order above is as follows; B2(1.21%) > D2(3.65%) > A2(8.47%) > C2(2.68%) > Ct1(0.00%). The control after the forth and the sixth week remained constant whereas the four microcosm experiments showed a varing percentage increase of rate of TPH reduction.

Table 9: Effect of combination of different nutrient sources (compost and NPK fertilizer) and cattle bile on the rate of TPH reduction during incubation

Treatment	Week 0	Week 2		Week 4		Week 6
	Initial TPH concentration (mg/kg)	Rate of TPH reduction (%)	Increased factor of rate of TPH reduction (%)	Rate of TPH reduction (%)	Increased factor of rate of TPH reduction (%)	Rate of TPH reduction (%)
Ct1 (0.0%)	1009.12	65.66 ± 3.00	13.91	79.57 ± 2.00	0.00	79.57 ± 2.00
A2 (0.2%)	1009.12	81.89 ± 2.08	3.21	85.10 ± 1.73	8.47	93.57 ± 2.52
C2 (0.4%)	1009.12	87.49 ± 1.53	2.27	89.76 ± 1.15	2.68	92.44 ± 1.73
B2 (0.2%)	1009.12	95.12 ± 4.04	2.10	97.22 ± 6.93	1.21	98.43 ±1.53
D2 (0.4%)	1009.12	79.56 ± 2.52	10.14	90.00 ± 1.73	3.65	93.65 ± 2.52

4.3 Treatment with different nutrient sources only and combination of cattle bile with the different nutrient sources

According to the data of result, changes in TPH content of the different microcosms at each of the sampling period during inoculation were significantly different at $p < 0.05$ from the control experiment as shown in appendix 6.

Generally the microcosms treated with nutrients only and those amended with nutrients and cattle bile combination, showed a high rate of TPH reduction compared to the control (devoid of nutrient and cattle bile). Considering Table 10, the rate of TPH degradation after two weeks of incubation vary between a maximum of 95.12% and a minimum of 65.66% as exhibited by microcosm B2 and Ct1 respectively. The rate of TPH components removal of the rest of the microcosms within the same incubation period is in the order; A(93.15%) > D(89.32%) > B(87.58%) > C2(87.49%) > C(83.06%) > A2(81.89%) > D2(79.56%). The rate of TPH degradation of the microcosms after the fourth week increased according to the following order with their corresponding increased rate factor as shown; B2(97.22 – 2.10%) > A(93.61 - 0.46%) > C(90.20 – 7.14%) > D2(90.00 – 10.14%) > C2(89.76 – 2.27%) > D(89.41 – 0.09%) > B(88.74 – 1.16%) > A2(85.41 – 3.21%) > Ct1(79.57 – 13.91%).

The sixth week, which mark the end of the incubation period, further increased in the rate of TPH reduction for the following individual microcosms i.e. A, B, C, D, A2, B2, C2, and D2. The control however remained constant. Microcosm B2 recorded a maximum rate of TPH reduction of 98.43% having increased by a factor of 1.21%. Unlike microcosm B2, the control microcosm recorded 79.57% which represent the minimum rate of TPH degradation. The seven remaining treatments are in the order; B(94.87%) >

C(94.64%) > A(94.14%) > D2(93.65%) > A2(93.57%) > C2(92.44%) > D(91.43%) with their corresponding factor of increament of (6.13, 4.44, 0.53, 3.65, 8.47, 2.68 and 2.02%) respectively.

Table 10: Shows the rate of TPH reduction of different treatment options with nutrient only and cattle bile + nutrient

Treatment	Week 2		Week 4		Week 6
	Rate of TPH reduction (%)	Increased factor of rate of TPH reduction (%)	Rate of TPH reduction (%)	Increased factor of rate of TPH reduction (%)	Rate of TPH reduction (%)
Ct1 (0.0%)	65.66 ± 3.00	13.91	79.57 ± 2.00	0.00	79.57 ± 2.00
A (0.2%)	93.15 ± 2.00	0.46	93.61 ± 1.73	0.53	94.14 ± 1.73
C (0.4%)	83.06 ± 2.00	7.14	90.20 ± 1.73	4.44	94.64 ± 7.23
B (0.2%)	87.58 ± 2.08	1.16	88.74 ± 2.31	6.13	94.87 ± 2.00
D (0.4%)	89.32 ± 1.53	0.09	89.41 ± 1.15	2.02	91.43 ± 2.00
A2 (0.2%)	81.89 ± 2.08	3.21	85.10 ± 1.73	8.47	93.57 ± 2.52
C2 (0.4%)	87.49 ± 1.53	2.27	89.76 ± 1.15	2.68	92.44 ± 1.73
B2 (0.2%)	95.12 ± 4.04	2.10	97.22 ± 6.93	1.21	98.43 ±1.53
D2 (0.4%)	79.56 ± 2.52	10.14	90.00 ± 1.73	3.65	93.65 ± 2.52

Note: Each value is a mean of three replicates and ± indicates standard deviation among them.

4.4 Variation in rate of TPH reduction, nitrogen utilization, and heterotrophic bacteria during bioremediation process

The data of result from Table 11 reveals that after two weeks of incubation the rate of TPH removal vary significantly between a maximum of 95.12% and a minimum of 65.66% as demonstrated by microcosm B2 and Ct1 (microcosm) respectively. In addition, microcosms B2 and Ct1 exhibited 51% and 47.62% rate of nitrogen utilization with a corresponding total heterotrophic bacterial count of 4.42 x 10^8 and 7.39 x 10^3 respectively. The rest of the treatments with their corresponding rate of nitrogen utilization and total heterotrophic bacterial count as well as the rate of TPH residual reduction are indicated on the table below. Generally, after four weeks of incubation all the microcosm experiments was observed to have exhibited an increase in the rate of TPH reduction, and percentage rate of nitrogen utilization as well as total heterotrophic bacterial count; with treatment B2 possessing a maximum rate of TPH reduction of 97.22% whereas the control exhibited 79.57% rate of TPH reduction; accompanied by 46.00% rate of nitrogen utilization which resulted in 4.70 10^7 cfu/g of total heterotrophic bacterial count and 35.71% rate of nitrogen utilization leading to the formation of 7.59 x 10^4 cfu/g heterotrophic bacterial count respectively. Furthermore, microcosms B2 and A2 were found to possess the maximal percentage rate of nitrogen utilization of 46.00% and a minimal of 23.50% respectively. Microcosms D2 possess 9.93 x 10^7 cfu/g heterotrophic bacterial count which represent the maximum and the control have a minimum of 7.59 x 10^4 cfu/g total heterotrophic bacterial count. The rest of the microcosms follow the order below with regards to the rate of TPH reduction: A > C > D2 > C2 > D > B > A2. The rate of TPH component removal of the different microcosm experiments further increased at the end (sixth week) of incubation period, hence the order; B2(98.43%) > B(94.87%) > C(94.64%) > A(94.14%) > D2(93.65%) > A2(93.57%) > C2(92.44%) > D(91.43%) >

Ct1(79.57%), with their corresponding rate of total nitrogen utilization given as (36.50, 21.50, 33.75, 27.50, 27.75, 18.50, 34.00, 33.25, and 23.81%) respectively as well as their respective total heterotrophic bacterial count (5.63 x 10^5, 4.56 x 10^6, 4.48 x 10^6, 2.88 x 10^6 , 8.38 x 10^6, 7.50 x 10^6, 4.67 x10^6 , 4.49 x10^6 , 5.80 x 10^4 cfu/g).

Table 11: Variation in rate of TPH reduction, nitrogen utilization and heterotrophic bacteria during bioremediation process

Treatment	Week 2			Week 4			Week 6		
	Rate of TPH reduction (%)	Rate of total nitrogen utilization (%)	Total heterotrophic bacterial count (cfu/g)	Rate of TPH reduction (%)	Rate of nitrogen utilization (%)	Total heterotrophic bacterial count (cfu/g)	Rate of TPH reduction (%)	Rate of nitrogen utilization (%)	Total heterotrophic bacterial count (cfu/g)
Ct1 (0.0%)	65.66±3.00	47.62±8.43	$7.39 \times 10^5 \pm 2.89 \times 10^3$	79.57±2.00	35.71±2.77	$7.59 \times 10^4 \pm 7.77 \times 10^2$	79.57±2.00	23.81±1.39	$5.80 \times 10^4 \pm 5.77 \times 10^2$
A (0.2%)	93.15±2.00	38.00±1.04	$4.51 \times 10^8 \pm 7.81 \times 10^6$	93.61±1.73	36.00±1.00	$5.45 \times 10^7 \pm 4.36 \times 10^5$	94.14±1.73	27.50±1.04	$2.88 \times 10^6 \pm 5.77 \times 10^3$
C (0.4%)	83.06±2.00	65.00±6.06	$2.75 \times 10^8 \pm 1.73 \times 10^6$	90.20±1.73	36.25±3.75	$7.50 \times 10^7 \pm 1.00 \times 10^6$	94.64±7.23	33.75±0.29	$4.48 \times 10^6 \pm 0.00 \times 10^0$
B (0.2%)	87.58 ± 2.08	42.00±0.00	$3.30 \times 10^8 \pm 3.46 \times 10^5$	88.7 ±2.31	31.50±1.04	$6.24 \times 10^7 \pm 1.00 \times 10^6$	94.87±2.00	21.50±0.58	$4.56 \times 10^6 \pm 1.73 \times 10^4$
D (0.4%)	89.32 ± 1.53	44.75±7.51	$3.41 \times 10^8 \pm 4.73 \times 10^6$	89.41±1.15	34.00±0.00	$5.24 \times 10^7 \pm 5.29 \times 0^5$	91.43±2.00	33.25±0.29	$4.49 \times 10^6 \pm 1.15 \times 10^4$
A2 (0.2%)	81.89 ± 2.08	34.00±0.87	$2.68 \times 10^8 \pm 1.15 \times 10^6$	85.10±1.73	23.50±0.58	$6.22 \times 10^7 \pm 6.66 \times 10^5$	93.57±2.52	18.50±4.62	$7.50 \times 10^6 \pm 0.00 \times 10^0$
C2 (0.4%)	87.49 ± 1.53	50.00±5.77	$3.25 \times 10^8 \pm 1.73 \times 10^6$	89.7 ±1.15	36.00±0.00	$3.39 \times 10^7 \pm 5.77 \times 10^5$	92.44±1.73	34.00±0.00	$4.67 \times 10^6 \pm 1.15 \times 10^4$
B2 (0.2%)	95.12 ± 4.04	51.00±0.00	$4.42 \times 10^8 \pm 1.73 \times 10^6$	97.22±6.93	46.00±1.15	$4.90 \times 10^7 \pm 5.77 \times 10^5$	98.43±1.53	36.50±1.15	$5.63 \times 10^5 \pm 5.77 \times 10^3$
D2 (0.4%)	79.56 ± 2.52	55.75±2.02	$2.12 \times 10^8 \pm 2.58 \times 10^7$	90.00±1.73	38.00±4.33	$9.93 \times 10^7 \pm 1.15 \times 10^6$	93.65±2.52	27.75±2.02	$8.38 \times 10^6 \pm 5.77 \times 10^4$

Note: Each value is a mean of three replicates and ± indicates standard deviation among them.

4.5 Effect of different nitrogen sources on rate of TPH removal, heterotrophic bacteria, nitrogen and organic matter utilization at the end of incubation period

Table 12 shows that microcosm B2 and Ct1 was observed to have exhibited 98.43% and 79.57% rate of TPH residual reduction which represent a maximum and a minimum hydrocarbon removal rate respectively: Whereas the remaining treatments follow the order; B(94.87%) > C(94.64%) > A(94.15%) > D2(93.65%) > A2(93.57%) > C2(92.44%) > D(91.43%) at the end of the incubation period. The corresponding rate of nitrogen and organic matter utilization of the aforementioned microcosms arranged above is as showm in the order; B2(36.50 – 31.05%) > B(21.50–28.31%) > C(33.75 – 31.82%) > A(27.50 – 29.83%) > D2(27.75 – 30.80%) > A2(18.50 – 34.44%) > C2(34.00 – 33.33%) > D(33.25 – 22.52%) > Ct1(23.81 - 13.33 %). Additionally, the corresponding heterotrophic bacterial count of the order stated above is as follows; (5.63 x 10^5, 5.63 x 10^5, 4.48 x 10^6, 2.88 x 10^6, 8.38 x 10^6, 7.50 x 10^6,4.67 x 10^6,4.49 x 10^6, 5.80 x 10^4cfu/g). Microcosm B2 appeared to have recorded the maximum rate of nitrogen utilization of 36.50% whereas microcosm A2 recorded the minimum rate of nitrogen utilization of 18.50% after six weeks of incubation. Furthermore, microcosm A2 appears to have utilized the highest rate of organic matter of 34.44% whereas the Ctl consumed 13.33% which represent the lowest rate of organic matter utilization. The data of results revealed that, generally at the end of incubation period, the microcosms established at a concentration of 0.4% nitrogen exhibited higher rate of nitrogen utilization as compared to those microcosms established at 0.2% nitrogen. However B2 microcosm (0.2% nitrogen level) is the only exception that exhibited higher rate of nitrogen utilization in comparison to the other microcosm experiments.

Table 12: Variation of the rate of TPH reduction, nitrogen and organic matter utilization and heterotrophic bacteria at the end of incubation period

Treatment	Week 6			
	Rate of TPH reduction (%)	Rate of total nitrogen utilization (%)	Rate of organic matter utilization (%)	Total heterotrophic bacterial count (cfu/g)
Ct1 (0.0%)	79.57 ± 2.00	23.81 ± 1.39	13.33 ± 0.23	$5.80 \times 10^4 \pm 5.77 \times 10^2$
A (0.2%)	94.14 ± 1.73	27.50 ±1.04	29.83 ± 0.09	$2.88 \times 10^6 \pm 5.77 \times 10^3$
C (0.4%)	94.64 ± 7.23	33.75 ± 0.29	31.82 ± 1.00	$4.48 \times 10^6 \pm 0.00 \times 10^0$
B (0.2%)	94.87 ± 2.00	21.50 ± 0.58	28.31 ± 0.00	$4.56 \times 10^6 \pm 1.73 \times 10^4$
D (0.4%)	91.43 ± 2.00	33.25 ± 0.29	22.52 ± 1.35	$4.49 \times 10^6 \pm 1.15 \times 10^4$
A2 (0.2%)	93.57 ± 2.52	18.50 ± 4.62	34.44 ± 0.07	$7.50 \times 10^6 \pm 0.00 \times 10^0$
C2 (0.4%)	92.44 ± 1.73	34.00 ± 0.00	33.33 ± 0.95	$4.67 \times 10^6 \pm 1.15 \times 10^4$
B2 (0.2%)	98.43 ± 1.53	36.50 ± 1.15	31.05 ± 0.00	$5.63 \times 10^5 \pm 5.77 \times 10^3$
D2 (0.4%)	93.65 ± 2.52	27.75 ± 2.02	30.80 ± 0.36	$8.38 \times 10^6 \pm 5.77 \times 10^4$

Note: Each value is a mean of three replicates and ± indicates standard deviation among them

4.6 Change in pH of the different microcosm experiments during bioremediation process

Figure 1 depicts variations in pH of different microcosm experiments under study over four sampling periods (Week 0, 2, 4 and 6) during bioremediation process. The variation in pH of the different microcosm experiments with amendments (compost only, fertilizer only, compost + bile and fertilizer + bile) over two sampling periods (week two and week four) were significantly different at $p < 0.05$ as shown in appendix 8. The graph generally

shows a decreasing trend of pH values of the different amendment treatments and the control microcosm over the four sampling periods. The initial pH of the amended microcosms at week zero after amendment apperared to have more or less the same pH as that of the test soil of pH value of 7.95 in its intrinsic state. Figure 1 clearly demonstrates an instantaneously high decrease in pH of the different microcosm experiments from an initial of A(7.98), B(7.97), C(7.96), D(7.97), A2(7.96), B2(7.97), C2(7.96), D2(7.98) and Ct1(7.95) after two weeks of incubation period. The high change in pH vary between a maximum of B2(6.48) and a minimum of Ct1(6.97) with the other treatment options in the order. A(6.66) > C(6.74) > D(6.74) > B(6.76) > C2(6.78) > D2(6.84) > A2(6.87). After the fourth week, all the microcosm experiments showed varing degrees of further decrease in pH value as demonstrated by the graph. Microcosms B, C, D, A2, C2, D2 and B2 exhibited a slight decrease and a sharp decrease in pH respectively considering the sixth period of incubation. The control and microcosm A however were the only exceptions i.e. the pH of the control microcosm remained constant from week 4 to week 6 whereas treatment A increased slightly. The order below depicts decrease in acidic strength of the different microcosm experiments; B2(5.59) > B(6.39) > C(6.45) > A(6.58) > D2(6.63) > A2(6.65) > C2(6.67) > D(6.69) > Ct1(6.96).

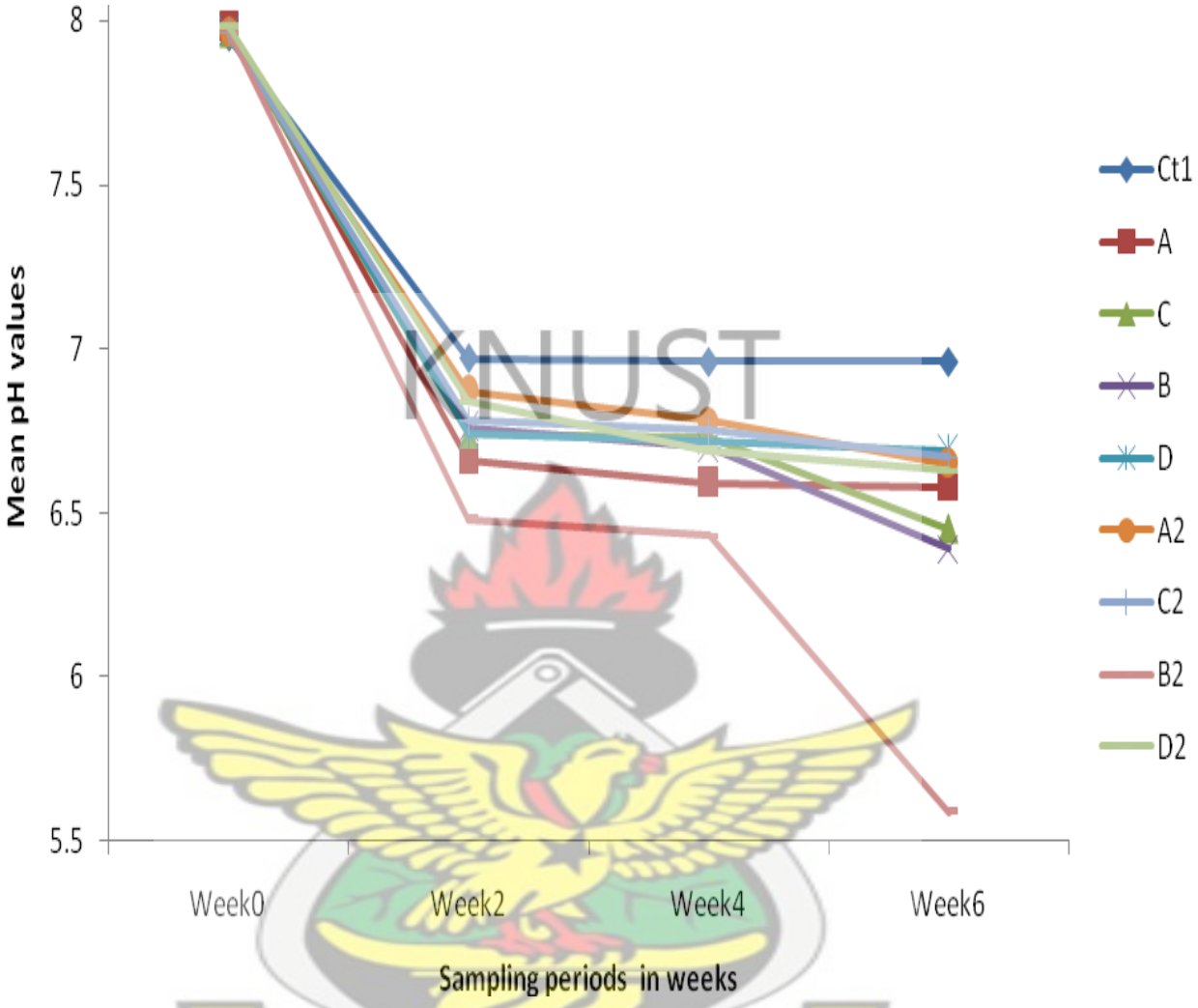


Figure 1: Variations in pH of the different microcosm experiments during bioremediation process.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Baseline information

This chapter is a discussion of the findings of the study. The main aim of the study was to investigate the extent of degradation of hydrocarbon contaminated soil through biostimulating the indeginous heterotrophic bacteria using compost and NPK fertilizer and cattle bile.

The baseline data of the physicochemical characteristics as well as the biological property of the test soil (obtained from Newmont) revealed that the contaminated soil possess an initial TPH concentration of 1009.12 mg/kg as well as 4.50×10^4 cfu/g total heterotrophic bacterial count (THBC), which is above the minimum value of 10^3 required for effective bioremediation (Abdulsalam *et al.*, 2010). Compost and cattle bile contain 5.00×10^5 cfu/g and 7.37×10^4 cfu/g THBC respectively. The pH value of the contaminated soil under study was found to be 7.95, which is slightly alkaline and adequate for effective bioremediation process according to Vidali (2001). Organic matter of 2.76% and 10.19% are present in the contaminated soil and compost respectively. Unlike the phosphorous with 1.24% in the test soil, the limiting nutrient i.e. nitrogen in the contaminated soil was found to be 0.04%. This percentage reflects low nitrogen content in the contaminated soil. 1000 grams of the contaminated soil was therefore amended with different nutrient sources such as compost and NPK fertilizer. The masses of the different nutrient sources needed to achieve 0.2% and 0.4% nitrogen levels are illustrated in Table 4.

5.2 The effect of compost and NPK fertilizer only, on the rate of TPH reduction of the different microcosms

Generally, considering Table 8 it was observed that the initial TPH concentration of 1009.12 mg/kg of the contaminated soil under investigation exhibited a high rate of TPH concentration reduction in all the different microcosm experiments such as A (1000 g of contaminated soil + 0.2% compost), B (1000 g of contaminated soil + 0.2% NPK fertilizer), C (1000 g of contaminated soil + 0.4% compost) and D (1000 g of contaminated soil + 0.4% NPK fertilizer); having subjected them to fertilization (once), moisture addition and homogenization over the four sampling period during incubation. The control appeared to be the only exception with minimal rate of TPH concentration reduction. This phemonenon is in consonance with the observation made by Hawrot and Nowak (2006). They reported that fertilization and stirring increased the efficiency of hydrocarbon biodegradation, as it resulted in the decrease of the amount of petroleum hydrocarbons: They attributed the fertilizer (nutrient source), and oxygen (generated by stirring) to have stimulated the growth of heterotrophic microorganisms and influenced their active participation in the biodegradation process repectively.

The data of results of the microcosm experiments subjected to only nutrient amendment treatment revealed that after the second week of incubation, all the microcosms exhibited instantaneously high but varing rate of TPH concentration reduction in the order; A(93.15%) > D(89.93%) > B(87.58%) > C(83.06%) > Ct1(65.66%) from an initial TPH concentration of 1009.12 mg/kg. The increasing factor in rate of hydrocarbon removal from the the second to fourth week and from the fourth to the sixth week of the different microcosm experiments are somewhat minimal: Additionally, comparing the rates of TPH concentration reduction of the different microcosms after the fourth week and sixth

week as well as their corresponding factors of increased rate of TPH reduction i.e. A(0.53%), B(6.13%), C(4.44%), and D(2.02%) as shown in Table 8. It can be suggested that perhaps by achieving or establishing an optimal condition the time period during incubation required to attain the aforelisted increased rate factors to ensure high TPH components removal from that of week four to week six might significantly be reduced. The following order; B(94.87%) > C(94.64%) > A(4.14%) > D(91.43%) > Ct1 (79.57%) ensued at the end (week six) of incubation. Microcosms B2 and Ct1 emerged as the maximum and minimum respectively. The control experiment did not show any increase in the rate of TPH concentration reduction from the fouth week to the sixth week; possibly because of insufficient nutrient source and probably because of the bacterial population lack the capacity to degrade the undegradable hydrocarbon constituents present within the control experiments.

5.3 The effect of combination of compost + cattle bile and NPK fertilizer + cattle bile on the rate of TPH reduction of the microcosms.

Microcosms B2 (1000 g of contaminated soil + 0.2% NPK fertilizer + cattle bile), C2 (1000 g of contaminated soil + 0.4% compost + cattle bile), A2 (1000 g of contaminated soil + 0.2% compost + cattle bile), D2 (1000 g of contaminated soil + 0.4% NPK fertilizer + cattle bile) and Ct1 (1000 g of contaminated soil + no amendment), after two weeks of incubation showed, (95.12, 87.49, 81.89, 76.56, and 65.66%) of rate of TPH removal respectively: However, the rate of TPH concentration reduction continued after the fourth week as shown in Table 9. The rate of TPH removal further increased according to the order; B2(98.43%) > D2(93.65%) > A2(93.57%) > C2(92.44%) > Ct1(79.57%) at the sixth week which marks the end of the incubation period. It was observed that the control setup did not show any increase in the rate of TPH removal

from week four to week six probably because microorganisms at the end of incubation lack the capability to degrade the remaining undegradable TPH compoments. It is imperative to note that, given the varing rates of TPH concentration reduction of the different microcosms as well as their corresponding increased factors of rate of TPH reduction; it can be inferred that under optimized conditions high hydrocarbon degradation rate can occure at a relatively shorter time than the six week duration of incubation during bioremediation.

5.4 Changes in the rate of TPH reduction, nitrogen utilization, pH and heterotrophic bacteria during bioremediation process

The anova analysis of the total petroleum hydrocarbon concentration reduction over four sampling period at $p < 0.05$ was found to be significant over the second week, fourth week and sixth week during the incubation period as demonstrated in appendix 6. Considering the microcosm experiments established by the application of treatment nutrients (compost and NPK fertilizer) only and nutrients + cattle bile matrix; the data obtained with regards to their individual rate of TPH components reduction indicate that biodegradation of hydrocarbons instantaneously occurred in all the microcosms including the control at varying degrees. This occurrence is attributable to the judicious utilization by the indigenous heterotrophic microbes of hydrocarbons as carbon source as well as compost and NPK fertilizer as nitrogen sources. Compost and NPK fertilizer are essential for microbial growth, multiplication and microbial activities. According to Ibiene *et al.* (2011) and Chikere *et al.* (2009), NPK fertilizer option effectively stimulates bacterial organisms into utilization of crude oil in a tropical crude oil polluted soil undergoing bioremediation. Compost on the other hand (made of sewage sludge / food waste) has

been established to enhance the degradation of hydrocarbons in soil mixtures (Atagana, 2008).

Initial TPH concentration of 1009.12 mg/kg of the test soil of the nine microcosm experiments over two weeks of incubation, varied between a maximum of 95.12% and a minimum of 65.66% rate of TPH reduction exhibited by microcosm B2 and Ct1 respectively. The order A > D > B > C2 > C > A2 > D2 with their respective rates of TPH reduction are as follows; 93.15%, 89.32%, 87.58%, 87.49%, 83.06%, 81.89%, and 79.56% respectively. The high initial heterotrophic bacterial load (as illustrated in appendix 10) present in the microcosm experiments at the onset of bioremediation process perhaps afforded the population the opportunity to remain high while adapting to and attacking the hydrocarbon substrate. Microcosm B2 appeared to have exhibited the maximum rate of TPH components degradation. This could possibly be due to the presence of more hydrocarbon degraders in B2 microcosm than the other microcosm experiments. The explanation stated above could as well be used to justify why the rate of hydrocarbon components were higher in microcosm B2 than in microcosm A even though the latter possesses higher total heterotrophic bacterial of 4.51 x 10^8 cfu/g than the former with 4.42 x 10^8 cfu/g. Nitrogen is an essential nutrient required by microbes to form cell protoplasm: Hence, the high rate of nitrogen utilization after two weeks of incubation as shown in Table 11. The afore-satated reason therefore substantiates the rapid growth of the heterotrophic bacterial population of the different microcosm experiments. Furthermore, from Table 11 the rate of organic matter utilization must have somewhat influenced the high numbers of heterotrophic bacterial load hence the rapidly high increase in TPH cocentration reduction. The initial level of organic matter in the test soil must have contributed to the high rate of mineralization of TPH contaminant since

according to Manilal and Alexander (1991), mineralization rate of contaminants are lower in soils with high organic matter content. The suitable pH range observed including the availability of moisture, and oxygen (stirring) may have significantly contributed to the observed rapid TPH component degradation.

Microcosm B2 evidently exhibited the highest rate of TPH residual reduction after the second week of sampling during incubation. This observation could probably be ascribed to the application of cattle bile and NPK fertilizer additives. The former acts as biosurfactant. Various literatures have reported the use of biosurfactant in bioremediation process. According to Luner (2000), bile decreases the surface tension by means of its surfactant properties. Surface tension may in turn be important for the wetting and mobilization of contaminants from soil (Charman *et al*., 1997). Biosurfactant enhanced biodegradation by mobilization, solubilization or emulsification (Pacwa-Płociniczak *et al*., 2011). Hence the subsequent removal of petroleum hydrocarbons by increasing the availability of petroleum hydrocarbon constituents for microbial utilization for energy and growth. Addition of biosurfactant agent augments the biosurfactant produced by the microorganisms (Jain *et al*., 2011). The latter i.e. NPK fertilizer which is an inorganic fertilizer unlike compost (organic fertilizer) dissolves readily hence, easily accessible by the heterotrophic microbes required to initiate and sustain hydrocarbon degradation process. The control exhibited the least rate of TPH concentration reduction possibly due to the fact that the control was devoid of essential nutrient (nitrogen) sources such as compost and NPK fertilizer as well as cattle bile. The control, however exhibited a somewhat appreciable level of percentage rate of TPH concentration reduction. This effect can be explained and justified by the fact that the control experiment was subjected to biostimulation by addition of moisture and aeration thrice each week during

incubation. It is imperative to mention that Schmitt and Rehn (2002), as well as Chorom, Sharifi and Motamedi (2010) reported that rapid degradation of hydrocarbons in soil + compost (sewage sludge) matrix and soil + NPK fertilizer was expected since sewage sludge as well as NPK fertilizer are rich in nutrients: Therefore the rapid degradation of hydrocarbons observed after two weeks. Generally, with regards to initial pH, the different microcosms fall within the range 7.96 – 7.98 as illustrated in Figure 1. A sharp decline in pH value was observed in all the different microcosm experiments. B2 and Ct1 microcosms have the highest and lowest decline in pH from (7.97 to 6.48) and (7.95 to 6.96) respectively, whereas the other microcosm experiments follow the order; A(6.66) > C(6.74) > D(6.74) > B(6.76) > C2(6.78) > D2(6.84) > A2(6.87) after week two of incubation. The variations observed in pH values of all the microcosms are indicative of the fact that mineralization (which results in elimination of hydrocarbon compounds) evidently occurred within the different microcosms including the control. Atagana (2008); Alexander (1999) and Eweis *et al*. (1999), reported that, the decreasing trend of pH indicate degradation of the compost and the hydrocarbons, which may have possibly resulted in the release of acidic intermediate and final products that probably lowered pH of the mixtures. As the rate of hydrocarbon degradation increased after the fourth week, the rate of TPH concentration reduction decreased, due to microbial activities: Hence the observed sharp decline in THBC population with microcosms D2 and Ct1 possessing a maximum microbial number of 9.93×10^7 cfu/g and a minimum number of 7.59×10^4 cfu/g respectively. The other treatment options and their corresponding microbial numbers are illustrated in Table 11. Aerobic hydrocarbon degradation involving oxygen has been established to result in quick reduction of microbial population. The decline in microbial population after the fourth week perhaps, can be attributed to the decrease in TPH concentration. Microcosm B2 obviously recorded the maximum nitrogen utilization of

46.00% whereas microcosm A2 recorded the minimum nitrogen utilization of 23.50% after the fourth week of incubation. Furthermore, microcosm A2 appears to have exhibitd the highest rate of organic matter consumption of 34.44% whereas the control possess 13.33% which represent the lowest organic matter utilization.

Microcosm B2 after the sixth week of incubation exhibited a maximum rate of TPH removal of 98.43% having increased by a factor of 1.21% after the fourth week of incubation. Unlike microcosm B2, the control microcosm recorded 79.57% which represent the minimum rate of TPH reduction with a corresponding increased rate factor of of 13.91%. The rate of TPH component reduction of the seven remaining treatment options are in the order; B(94.87%) > C(94.64%) > A(94.14%) > D2(93.65%) > A2(93.57%) > C2(92.44%) > D(91.43%) with their corresponding increased rate factor of (6.13, 4.44, 0.53, 3.65, 8.47, 2.68 and 2.02%) respectively. The length of time taken to achieve these varing but high levels of hydrocarbon degradation rates can possibly be reduced by optimizing bioremediation conditions necessary to ensure effective and effecient bioremediation process. This can lead to achieving almost the same or even higher rate levels of hydrocarbon compounds removal in the contaminated soil.

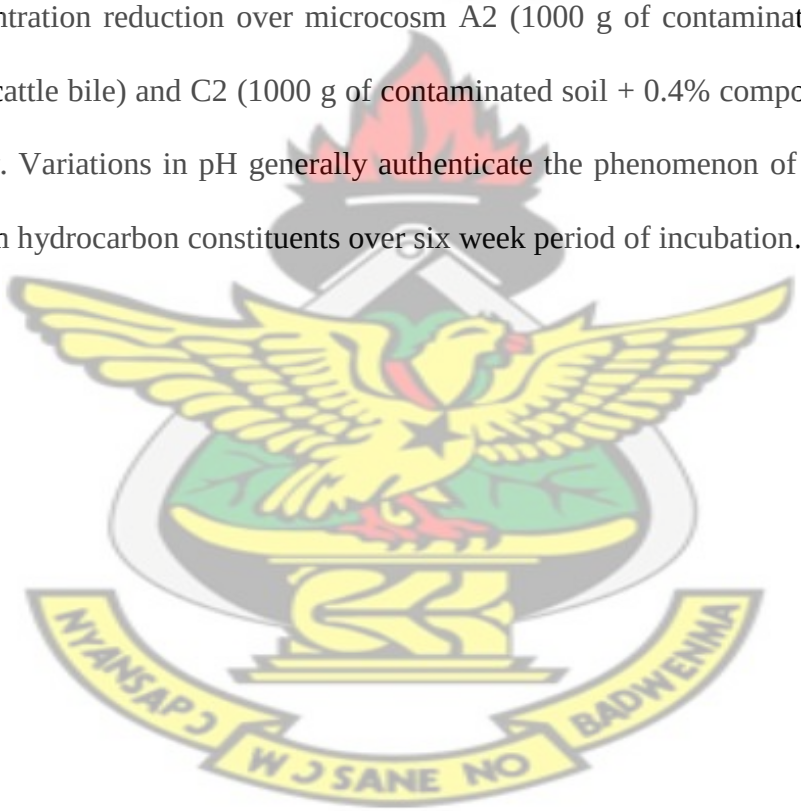
The progressive decline observed in THBC over the last sampling period i.e. week six, can be ascribed to unavailability of nutrients and reduced hydrocarbon concentration. The percentage rate of nitrogen utilization as demonstrated by the microcosm experiments generally decreased over the subsequent two sampling periods i.e. week four and six after the first two weeks interval of sub-sampling period during incubation. This effect could be attributed to the initialy high microbial activities. This is similar to that which was reported by Piccinini *et al*. (1996) and Atagana (2008). The pH of the control microcosm

remained constant after the fourth and the sixth week, whereas treatment B2 decreased sharply during that same period of incubation. The other microcosm experiments showed a slight decrease in pH value as demonstrated in the order below; B2(5.59) > B(6.39) > C(6.45) > A(6.58) > D2(6.63) A2(6.65) > C2(6.67) > D(6.69) > Ct1(6.96). The order depicts decrease in acidic strength of the different microcosms.

At the end of bioremediation process, microcosm B2 (1000g of contaminated soil + 0.2% NPK fertilizer + cattle bile) emerged as the microcosm among the different treatment options with the highest rate of hydrocarbon removal. Therefore possessing the lowest TPH concentration as shown in appendix 6. The control however happens to have exhibited the least hydrocarbon compound removal. It was observed that amendment treatments involving the application of inorganic fertilizer i.e. NPK fertilizer appeared to have higher rate of remediation efficiency than those involving the use of organic fertilizer i.e. compost (sewage sludge / food waste). This phenomenon may be attributed to the fact that inorganic fertilizers are readily made available for microbial utilization than the organic fertilizers. Hence the NPK fertilizer becomes readily accessible to microbial population for microbial activities which result in biodegradation of petroleum hydrocarbon components. Generally, microcosms amended with compost and cattle bile, as well as those treatment options made of NPK fertilizer and cattle bile performed appreciably well with regards to their rate of TPH removal efficiency, which fall within the range of 92.00% - 94.00%. However, B2 microcosm was found to be the only exception with hydrocarbon degradation rates of 98.43% which is higher than A2 (1000 g of contaminated soil + 0.2% compost + cattle bile) and C2 (1000 g of contaminated soil + 0.4% compost + cattle bile) and D2 (1000 g of contaminated soil + 0.4% NPK fertilizer + cattle bile) microcosms. The following microcosm experiments; A2, C2 and D2 appeared

to have been out performed by microcosms A (1000 g of contaminated soil + 0.2% compost), B (1000g of contaminated soil + 0.2% NPK fertilizer) and C (1000g of contaminated soil + 0.4% compost) with TPH removal rates well within 94.00% - 95.00%. Liu *et al*. (1995) reported the use of surfactants in oil degradation may have a stimulatory, inhibitory or neutral effect on the bacterial degradation of the soil components. In addition bile, from literature has been reported to be injurious to microbes at high concentrations. The antibactericidal effect of bile is exerted on the bacterial cell membrane (Noh and Gilliland, 1993). Therefore the uneven distribution of bile in a mixture during homogenization may occur; inasmuch as cattle bile can concentrate in certain portions of the amended solid matrix. When this happens bacterial death may ensue, resulting in diminished TPH removal efficiency. This could possibly explain why microcosms inoculated with cattle bile were out-performed by microcosms without cattle bile. Microcosm experiments B2 (1000 g of contaminated soil + 0.2% NPK fertilizer + cattle bile) and D2 (1000 g of contaminated soil + 0.4% NPK fertilizer + cattle bile) with regards to the percentage rate of TPH removal efficiency, performed better than treatment B (1000 g of contaminated soil + 0.2% NPK fertilizer) and D (1000 g of contaminated soil + 0.4% NPK fertilizer) respectively. It was also observed from Table 11 that the corresponding heterotrophic bacterial count for treatments B2 (1000 g of contaminated soil + 0.2% NPK fertilizer + cattle bile) and B (1000 g of contaminated soil + 0.2% NPK fertilizer) after two weeks are 4.42 x 10^8 cfu/g and 3.30 x 10^8 cfu/g respectively, hence confirming the stimulatory effect of cattle bile on bacterial degradation of TPH components. Treatment option D2 (1000 g of contaminated soil + 0.4% NPK fertilizer + cattle bile) out performed D (1000 g of contaminated soil + 0.4% NPK fertilizer) microcosm in the depletion of hydrocarbon constituents having subjected all four microcosms to the same conditions over six week of incubation. The difference in

performance of depletion of TPH concentration; can be attributed to possibly the presence of inoculation of cattle bile, therefore, cattle bile can be said to have positively enhanced biodegradation of hydrocarbons. The expected enhancing effect of cattle bile clearly manifested in microcosms with NPK fertilizer as described above: Whereas the inhibitory impact of cattle bile to some extent, appeared to have been exerted on microcosms with compost amendment material. Considering the microcosm experiments involving the application of compost; microcosms A (1000 g of contaminated soil + 0.2% compost), and microcosms C (1000 g of contaminated soil + 0.4% compost) yielded higher rate of TPH concentration reduction over microcosm A2 (1000 g of contaminated soil + 0.2% compost + cattle bile) and C2 (1000 g of contaminated soil + 0.4% compost + cattle bile) respectively. Variations in pH generally authenticate the phenomenon of bioremediation of petroleum hydrocarbon constituents over six week period of incubation.



CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In this study, microcosm experiments were conducted to investigate the extent of degradation of hydrocarbon contaminants; using NPK fertilizer as well as compost (nutrient sources) and the application of cattle bile (as biosurfactant) to remediate petroleum hydrocarbon contaminated soil.

Microbial data as well as the rate of TPH residual reduction after six weeks of incubation, confirmed the presence and multiplication of indigenous heterotrophic bacterial population and the occurrence of biodegradation of petroleum hydrocarbon components in all the different microcosm experiments at varying degrees respectively. The results revealed that, application of different nutrient sources as a means of biostimulating indigenous microorganisms in a contaminated soil, resulted in a markedly enhanced rate of TPH component removal efficiency as exhibited by the microcosm experiments A (1000 g of contaminated soil + 0.2% compost), B (1000 g of contaminated soil + 0.2% NPK fertilizer), C (1000 g of contaminated soil + 0.4% compost) and D (1000 g of contaminated soil + 0.4% NPK fertilizer). NPK fertilizers and compost are therefore essential nutrient sources required to sustain a high rate of TPH constituents' removal in petroleum hydrocarbon contaminated soil.

The combined effect of cattle bile juice and the individual nutrient sources yielded the desired effect of enhancing remediation efficiency as compared to the control in it intrinsic state. B2 microcosm amended with NPK fertilizer at 0.2% and cattle bile out performed all other microcosm experiments: Therefore, cattle bile indeed possess

remedial potential as it function as a biosurfactant to augment the biosurfactant produced by the bacterial population to enhance hydrocarbon degradation. The treatment option B2 (contaminated soil + 0.2% fertilizer + cattle bile) can be adopted for decontamination of petroleum contaminated soil, giving it high rate of remediation efficiency.

6.2 Recommendations

The following recommendations were made on the basis of the findings.

- Research work must be conducted to further evaluate or investigate the remedial potentiality of cattle bile in isolated treatment and possibly in combination with other nutrient sources.

- Further investigation must be carried out, considering the frequency of inoculation of cattle bile and NPK fertilizer to establish if a maximum of 100% rate of TPH residual reduction can be achieved over six weeks of incubation period.

- Varying amount of cattle bile must be investigated in later research work in order to determine the maximum and minimum amount of cattle bile suitable for the survival of heterotrophic bacterial required to bring about enhanced remediation efficiency in contaminated soil.

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APPENDICES

Appendix 1: Calculating the amount of compost and NPK fertilizer required to achieve 0.2% and 0.4% nitrogen level

Calculating the amount of compost to achieve 0.2% nitrogen level

Contaminated soil = NC

Nitrogen present in compost is giving as 1.47%.

Initial level of nitrogen in contaminated soil = 0.042%

Let X represent amount of nitrogen present in 1000g of contaminated soil.

Therefore 100g = 0.042%

1000g = X

$$X = \frac{0.042 \; x \; 1000}{100}$$

X= 0.42g

Y = expected level of nitrogen to be achieved

0.2% of 1000g = Y

But $\frac{0.2}{100} \; x 1000$

Y = 2.0g

Nitrogen deficit (ND) in soil = (2.0 – 0.42) g

ND = 1.58g

But compost = 1.47%

Let Z represent the amount of compost to be added to achieve 0.2%

100g = 1.47g

Z = 1.58g

Therefore $Z = \frac{1.58g \; x \; 100g}{1.47g}$ = 107.48g

Calculating the amount of NPK fertilizer to achieve 0.2% nitrogen level

Let F represent the amount of NPK fertilizer required to be added to achieve 0.2%.

Fertilizer = 15%

100g = 15g nitrogen

F = 1.58g

$$F = \frac{1.58\ x\ 100g}{15}$$

F= 10.53g

Calculating the amount of compost to achieve 0.4% nitrogen level

α = expected level of nitrogen to be achieved

0.4% of 1000g =α

But $\frac{0.4}{100}\ x\ 1000$

α = 4.0g

Nitrogen deficit (ND) in soil = (4.0 – 0.42) g

ND = 3.58g

Nitrogen present in compost is giving as 1.47%.

Let β represent the amount of compost to be added to achieve 0.4%

100g = 1.47g

β = 3.58g

Therefore $\beta = \frac{3.58g\ x\ 100g}{1.47g}$

β= 243.54g

Calculating the amount of NPK fertilizer to achieve 0.4% nitrogen level

µ is mass of NPK fertilizer to be added to achieve 0.4% nitrogen.

Nitrogen present in NPK fertilizer is giving as 15%.

100g = 15g

µ = 3.58g

Therefore $\mu = \frac{3.58g \times 100g}{15g}$

µ = 23.87g

Appendix 2: Soxhlet Extractor used for the extraction of oil from petroleum comtaminated soil.

Plate 1.

Appendix 3: Ashing of sampled soil in a muffle furnace at 400 ^{o}C during organic matter determination.

Plate 2.

Appendix 4: Distillation unit and titration setup used in distillation and titration processes during nitrogen determination.

Distillation unit

Plate 3.

Titration Setup

Plate 4.

Appendix 5: Arrangement of the different microcosm experiments during incubation period.

Plate 5.

Appendix 6: A table showing the effect of different nitrogen sources on TPH concentration reduction during the bioremediation process

Treatment	Week 0	Week 2	Week 4	Week 6
	Initial TPH concentration (mg/kg)	TPH residual reduction (mg/kg)	TPH residual reduction (mg/kg)	TPH residual reduction (mg/kg)
Ct1(0.0%)	1009.12	346.49	206.16	206.15
A (0.2%)	1009.12	69.24	64.48	59.18
C (0.4%)	1009.12	170.92	98.91	54.08
B (0.2%)	1009.12	125.38	113.61	51.72
D (0.4%)	1009.12	107.76	106.87	86.50
A2 (0.2%)	1009.12	182.73	141.33	64.86
C2 (0.4%)	1009.12	126.23	103.32	76.27
B2 (0.2%)	1009.12	49.20	28.10	15.84
D2 (0.4%)	1009.12	206.28	100.91	64.42
F pr. < 5%	-	0.001	0.001	0.001
LSD (5%)	-	0.04	0.05	0.05
CV (%)	0.0	0.0	0.0	0.0

Appendix 7: A table showing variation in percentage total nitrogen during the bioremediation process

Treatment	Percentage total nitrogen over four sampling periods (%)			
	Week0	Week2	Week4	Week6
Ct1 (0.0%)	0.042	0.022	0.027	0.032
A (0.2%)	0.200	0.124	0.128	0.145
C (0.4%)	0.400	0.140	0.255	0.265
B (0.2%)	0.200	0.116	0.137	0.157
D (0.4%)	0.400	0.221	0.264	0.267
A2 (0.2%)	0.200	0.132	0.153	0.163
C2 (0.4%)	0.400	0.199	0.256	0.264
B2 (0.2%)	0.200	0.098	0.108	0.127
D2 (0.4%)	0.400	0.177	0.248	0.289
F pr. < 0.05	0.001	0.001	0.001	0.001
LSD (5%)	0.0003	0.026	0.013	0.007
CV (%)	0.1	11.2	4.4	2.3

Appendix 8: A table illustrating variation in percentage total organic matter during the bioremediation process

Treatment Identification	Percentage total organic carbon over four sampling period(%)			
	Week0	Week2	Week4	Week6
Ct1 (0.0%)	1.50	1.34	1.29	1.30
A (0.2%)	2.38	1.81	1.69	1.67
C (0.4%)	2.42	1.79	1.69	1.65
B (0.2%)	2.19	1.98	1.63	1.57
D (0.4%)	2.22	2.01	1.90	1.72
A2 (0.2%)	2.41	1.95	1.69	1.58
C2 (0.4%)	2.43	1.88	1.84	1.62
B2 (0.2%)	2.19	1.77	1.64	1.51
D2 (0.4%)	2.24	1.98	1.59	1.55
F pr. < 0.05	0.001	0.001	0.001	0.001
LSD (5%)	0.05	0.08	0.06	0.04
CV (%)	1.3	2.5	2.2	1.4

Appendix 9: A table showing variation in pH during the bioremediation process

Treatment Identification	Change in pH of microcosm experiment during incubation			
	Week0	Week2	Week4	Week6
Ct1 (0.0%)	7.95	6.97	6.96	6.96
A (0.2%)	7.98	6.66	6.59	6.58
C (0.4%)	7.96	6.74	6.73	6.45
B (0.2%)	7.97	6.76	6.70	6.39
D (0.4%)	7.97	6.74	6.72	6.69
A2 (0.2%)	7.96	6.87	6.78	6.65
C2 (0.4%)	7.96	6.78	6.75	6.67
B2 (0.2%)	7.97	6.48	6.43	5.59
D2 (0.4%)	7.98	6.84	6.69	6.63
F pr < (0.05)	-	0.00	0.001	-
LSD (5%)	-	0.01	0.05	-
CV (%)	0.0	0.1	0.5	0.0

Appendix 10: Illustrating change in the heterotrophic bacteria during the bioremediation process

Treatment Identification	Total heterotrophic viable count over four sampling periods (cfu/g)			
	Week0	Week2	Week4	Week6
Ct1 (0.0%)	4.50×10^4	7.39×10^5	7.59×10^4	5.80×10^4
A (0.2%)	3.56×10^6	4.51×10^8	5.45×10^7	2.88×10^6
C (0.4%)	3.78×10^6	2.75×10^8	7.50×10^7	4.48×10^6
B (0.2%)	7.58×10^5	3.30×10^8	6.24×10^7	4.56×10^6
D (0.4%)	8.77×10^5	3.41×10^8	5.24×10^7	4.49×10^6
A2 (0.2%)	3.95×10^6	2.68×10^8	6.22×10^7	7.50×10^6
C2 (0.4%)	4.03×10^6	3.25×10^8	3.39×10^7	4.67×10^6
B2 (0.2%)	9.10×10^5	4.42×10^8	4.90×10^7	5.63×10^5
D2 (0.4%)	9.44×10^5	2.12×10^8	9.93×10^7	8.38×10^6