BIODEGRADATION OF USED LUBRICATING ENGINE OIL CONTAMINATED WATER USING INDIGENOUS HYDROCARBON DEGRADING MICROBES IN A FIXED BED BIOREACTOR SYSTEM

By

Antwi-Akomeah Samuel, BSc. Laboratory Technology (Hons)

Dissertation Submitted To The Department Of Environmental Science, Kwame Nkrumah University Of Science And Technology In Partial Fulfillment Of The Requirements For The Award Of The Degree Of

MASTER OF SCIENCE IN ENVIRONMENTAL SCIENCE

College of Science

August 2011

DECLARATION

I, Antwi-Akomeah Samuel hereby declare that this thesis, "Biodegradation of hydrocarbon contaminated water using indigenous hydrocarbon degrading microbes in a fixed bed bioreactor system", consists entirely of my own work produced from research undertaken under supervision and that no part of it has been published or presented for another degree elsewhere, except for the permissible excepts/references from other sources, which have been duly acknowledged.

Antwi – Akomeah Samuel	……	……
(Candidate)	Signature	Date

Certified by:

Dr. Bernard Fei-Baffoe	……	……
(Supervisor)	Signature	Date

Rev. Stephen Acheampong	……	……
(Head of Department)	Signature	Date

DEDICATION

This work is whole heartedly dedicated to my parents in the persons of Mr. Henry Akomeah and Madam Comfort Tabi for their undying love and support towards my education up to this level. This thesis is also dedicated to my uncle, Mr. Samuel Kofi Bosompem and my siblings Miss Regina Akomeah and George Oteng Tabi Akomeah for being there for me.

ACKNOWLEDGEMENT

A challenging and thought provoking work of this nature would not have been possible if not for the assistance and guidance of certain individuals who willingly offered their enormous help towards the successful completion of this work.

My first and foremost gratitude goes to the Environmental Science department that granted me admission to pursue my graduate studies here at the Kwame Nkrumah University of Science and Technology (KNUST). My encounter with the board during the interview session was a real fun. Memories of that moment would remain fresh in my mind.

My profound gratitude also goes to my parents, Mr. Henry Akomeah and Madam Comfort Tabi for their financial support and encouragement throughout my study period. I say thank you mum and dad and may God richly bless you.

I am heartily thankful to my able supervisor, Dr. Bernard Fei-Baffoe, whose motivation, guidance and assistance from the beginning of the work to the end enabled me to develop a better understanding of the work.

My heartfelt gratitude also goes to the late Saani Mohammed Awudu who was more of a co-supervisor to me. His contributions in the form of suggestions and comments really did motivate me to develop interest in the work. May his soul find perfect eternal rest.

The following people also made their contribution to the success of this work by helping me in my laboratory work; Mr. Paul Osei and Mr. Samuel Frimpong all of Ghana Standards Authority; Mr. Jackson Napoleon, Mr. Douglas Amoah and Miss Ohenewa Agyare all of the Faculty of Renewable Natural Resources, KNUST not forgetting Mr. Eric Acheampong and Mr. Nii Odoi all of the microbiology lab, KNUST.

My thanks also goes to Mr. Eric Ayitey of the inorganic chemistry laboratory of KNUST who came in the dying minutes of the game to offer his support and expertise and Mr. Baah Philemon of the Mathematics department, KNUST who also assisted me in the analysis of my data.

I am also indebted to my colleagues particularly Mr. Kwadwo Yeboah Antwi a.k.a Senior who supported me by way of motivating me at all times.

Last but not the least, my thanks goes to my entire family, friends and the Almighty God, for answering my prayers and for giving me the strength to plod on despite the difficult times.

ABSTRACT

The performance of a mixed population of hydrocarbon-degrading microbes in degrading hydrocarbon contaminants in water was investigated using a fixed bed bioreactor system. Hydrocarbon-degrading microbes used for the study were isolated from oil-contaminated soil and further cultured in a nutrient medium. Sample concentrations of 500 mg/L, 1000 mg/L, 2000 mg/L and 6000 mg/L were studied. Each sample concentration was studied at loading rates of 0.5 L/min, 1.0 L/min, and 2.0 L/min for a week. Total petroleum hydrocarbon (TPH), pH, temperature, dissolved oxygen (DO), conductivity and the microbial population density were measured to ascertain the progress of microbial degradation of the oil contaminant in the water. A minimum degradation rate of 36.83±0.00 % was achieved at the least administered loading rate of 0.5 L/min at 1000 mg/L oil concentration. Maximum degradation rate of 93.85±0.00 % was also achieved at loading rate of 1.0 L/min at the highest oil concentration of 6000 mg/L. The minimum and maximum degradation rates were achieved by microbial populations of 1.53E+13 and 1.50E+13 respectively. The study revealed higher degradation rates occurring at higher oil concentrations and loading rates. The hydrocarbon degradation occurred in an optimum pH range of 6.63 and 7.32 and a temperature range of 27.3°C and 29.9°C.

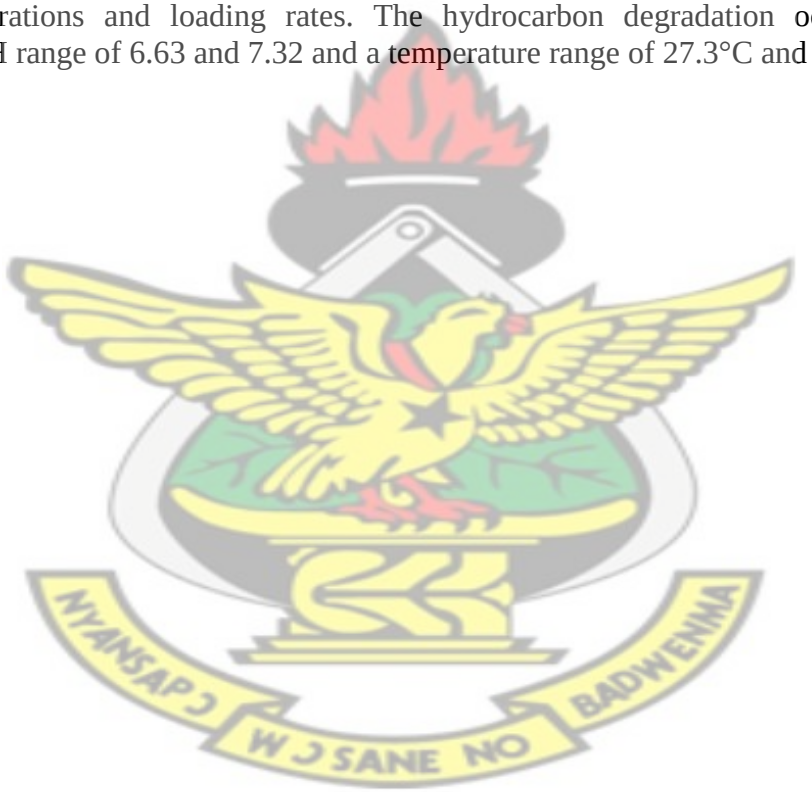


TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND ACRONYMS

APHA	American Public Health Association
ASTDR	Agency for Toxic Substances and Disease Registry
BTEX	Benzene, Toluene, Ethylbenzene and Xylene
CFC	Chlorofluorocarbons
DO	Dissolved Oxygen
FID	Flame Ionization Detector
GC	Gas Chromatography
HRT	Hydraulic Retention Time
PAH	Polyaromatic Hydrocarbon
PCB	Polychlorinated Biphenyl
PVC	Polyvinyl chloride
RNA	Ribonucleic acid
TPH	Total Petroleum Hydrocarbon
USEPA	United States Environmental Protection Agency

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Petroleum-based products are a major source of energy for industry and daily life (Kvenvolden & Cooper, 2003) and are primarily obtained from crude oil which is a naturally occurring liquid with a complex mixture of organic molecules, mostly hydrocarbons with varied chemical and physical properties. A precise description of the chemical composition of crude oil is not practicable because of its complexity (Amro, 2004). It has always entered the biosphere by natural seepage, but at rates much slower than the forced recovery by drilling (Balba *et al*., 1998a). The amount of natural crude oil seepage into the biosphere has been estimated to be 600,000 metric tons per year with a range of uncertainty of 200,000 metric tons per year. Leaks and accidental spills also occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products (Kvenvolden & Cooper, 2003; Nilanjana & Preethy, 2010). Manufactured from crude oil, petroleum hydrocarbons are found in gasoline, kerosene, fuel oil, lubricants, asphalt, and even in some chemicals used at home or at work. Thus petroleum-based products are by far the most commonly used chemicals in the industrial world now (Balba *et al*., 1998a).

Motor oil the subject of interest here is an oil used for lubrication of various internal combustion engines which include motor or road vehicles such as cars and motorcycles, heavier vehicles, etc. While the main function is to lubricate moving parts, motor oil also cleans, inhibits corrosion, improves sealing and cools the engine by carrying heat away from moving parts (Klamann, 1984). Most present day motor oils are derived from petroleum-based and non-petroleum synthesized chemical compounds. Motor oils are thus mainly blended by using base oils composed of hydrocarbons (organic compounds consisting entirely of carbon and hydrogen), e.g., mineral oil (Corsico *et al*., 1999).

Hydrocarbon or oil biodegradation as a process makes use of natural microbial biodegradative activities and this often employs the enzymatic capabilities of indigenous hydrocarbon-degrading microbial populations and modifying environmental factors (Atlas, 1981). That is, one major requirement for oil biodegradation is the presence of microorganisms with the appropriate metabolic capabilities. If these microorganisms are present, then optimal rates of growth and hydrocarbon biodegradation can be sustained by

ensuring that adequate concentrations of nutrients and oxygen are present and that the pH is ideal. The physical and chemical characteristics of the oil and oil surface area are also important determinants of bioremediation/biodegradation success. Two main approaches to oil bioremediation or biodegradation are (a) bioaugmentation, in which known oil-degrading microorganisms are added to supplement the existing microbial population, and (b) biostimulation, in which the growth of indigenous oil degraders is stimulated by the addition of nutrients or other growth-limiting co-substrates (Nilanjana & Preethy, 2010). Thus the ecology of hydrocarbon degradation by microbial populations in the natural environment is enhanced by physical, chemical, and biological factors that contribute to the biodegradation of petroleum and individual hydrocarbons. Rates of biodegradation depend greatly on the composition, state, and concentration of the oil or hydrocarbons, with dispersion and emulsification enhancing rates in aquatic systems and absorption by soil particulates being the key feature of terrestrial ecosystems. Temperature, oxygen and nutrient concentrations are important variables in both types of environments. Salinity and pressure may also affect biodegradation rates in some aquatic environments. Hydrocarbons are primarily biodegraded by bacteria and fungi (Leahy & Colwell, 1990).

Thus the success of oil biodegradation technology depends on the ability to establish and maintain conditions that favour enhanced oil biodegradation rates in the contaminated environment (Nilanjana & Preethy, 2010).

1.2 Problem Statement

The release of oil or hydrocarbons into the environment whether accidentally or due to human activities is a major cause of water (both surface and groundwater resources) and soil pollution (Holliger *et al*., 1997; Nilanjana & Preethy, 2010). The technologies commonly used for the remediation of oil spill and contamination include mechanical and physico-chemical processes such as burying, evaporation, dispersion, and washing. However, these technologies are expensive and can lead to incomplete decomposition or breakdown of contaminants (Nilanjana & Preethy, 2010).

The anthropogenic contamination of surface and ground water resources by oil products has thus become an object of intensive scientific research in the 21st century. Oil products are transported to places all over the world by ship, rail, truck, and pipelines.

Unfortunately, because of the large volumes of petroleum hydrocarbons produced and subsequent releases during transport, use and storage, such as in underground pipelines or storage tanks, petroleum hydrocarbons have become one of the most prevalent contaminants of water resources (Balba *et al*., 1998a, 1998b). The production, transportation, refining, and ultimate disposal of petroleum are introducing by conservative estimate, 3.2 million metric tons of petroleum annually into the oceans alone (National Research Council, 1985; Balba *et al*., 1998a).

Several gallons of waste engine oil are generated daily from mechanic workshops and automobiles and discharged carelessly into the environment (Faboya, 1997; Adegoroye, 1997; Adelowo *et al*., 2006). Out of this only one liter is enough to contaminate one million gallons of freshwater (USEPA, 1996; Adelowo *et al*., 2006). Apart from this, used engine oil constitutes a potential threat to humans, animals and vegetation. Thus the presence of these pollutants in the terrestrial and aquatic environments constitutes public health and socio-economic hazards (Edewor *et al*., 2004; Okerentugba & Ezeronye, 2003; Adelowo *et al*., 2006). For instance contamination of water or soil with hydrocarbons can cause extensive damage of local systems since accumulation of these pollutants in the tissues of animals and plants may cause mutations and/or death (Alvarez & Vogel, 1991; Nilanjana & Preethy, 2010). Several components of the oil, such as solvents and detergents added during the blending process, aliphatic hydrocarbon and polyaromatic hydrocarbons (PAHs) distilled from crude oil, and metals from engine wear are either toxic in themselves or can combine with products of combustion to generate carcinogens and endocrine disrupters, (USEPA, 1996; ATSDR, 1997; Adelowo *et al*., 2006).

1.3 Main Objective of the Study

The ultimate goal of the study is to investigate the microbial degradation of hydrocarbon (oil) in water in a fixed bed bioreactor system.

Specific objectives

1. To design and construct fixed bed bioreactor system.
2. To isolate indigenous hydrocarbon-degrading microorganisms from oil-contaminated soil sample.
3. To monitor the hydrocarbon (oil) degradation process by measuring parameters such as total petroleum hydrocarbon (TPH), pH, dissolved oxygen (DO), conductivity and microbial populations during the study period.

4. To determine the effect of oil concentration on the biodegradation rate.
5. To determine the effect of loading (flow) rate on the biodegradation rate.

1.4 Justification

In Ghana, several mechanic workshops, filling stations and washing bays are springing up with most of these workshops being sited close to water bodies and major drainage systems thereby making these water bodies and drainage systems vulnerable to petroleum contamination. Of particular concern is the indiscriminate and careless discharge of oil products particularly used motor oil from these workshops into the nearby water bodies and drainage systems. Also the constantly increasing number of automobiles, coupled with the increasing volume of transportation of oil products and the storage of these oil products pose a potential threat to the environment of Ghana in general (both terrestrial and aquatic).

Moreover, with the recent oil discovery in Ghana, the environment of Ghana is prone to contamination by hydrocarbons or oil products, which may find their way into the environment through several routes. Thus oil leakages from pipelines, garages, underground storage tanks, natural seepages and stranded oil spills (both off-shore and on-shore) during transportation, exploration, and production are likely problems to be encountered with the exploitation of the oil find in Ghana.

Petroleum hydrocarbons upon release through a spill, leak or careless disposal into the environment, have the potential of being washed or carried by run-offs into surface water bodies or migrating through soil particles until they reach groundwater. The study of microbial removal of hydrocarbon pollutants from hydrocarbon-contaminated water resources would therefore be an essential practice in mitigating some of the environmental and health concerns arising from hydrocarbon contamination of water resources in Ghana. This research therefore seeks to investigate the removal of hydrocarbon contaminants in water using indigenous hydrocarbon-degrading microorganisms.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Petroleum Formation

Petroleum or crude oil is a natural product, resulting from the anaerobic conversion of biological matter under high temperature and pressure (Balba *et al*., 1998a). Petroleum hydrocarbons refer to a mixture of compounds in petroleum products that are all made entirely from hydrogen and carbon, hence the name "hydrocarbon" (Kvenvolden, 2006).

According to generally accepted theory, petroleum is derived from ancient biomass. Formation of petroleum occurs from hydrocarbon pyrolysis, in a variety of mostly endothermic reactions at high temperature and/or pressure (Braun & Burnham, 1993). Present day crude oil is formed from the preserved remains of prehistoric zooplankton and algae, which has settled down a sea or lake bottom in large quantities under anoxic conditions (Kvenvolden, 2006). Over geological time the organic matter mixes with mud and is buried under heavy layers of sediment resulting in high heat and pressure. This process causes the organic matter to change, first into a waxy material known as kerogen (found in various oil shales around the world), and then with more heat into liquid and gaseous hydrocarbons via a process known as catagenesis (Braun & Burnham, 1993).

2.1.1 Classification of crude oil

Crude oil according to the United States Environmental Protection Agency (USEPA) (1996) can be classified into the following as discussed:

Class A: Light, volatile oils

These oils are often clear, spread rapidly on solid or water surfaces, have a strong odour, a high evaporation rate, and are usually flammable. They penetrate porous surfaces such as dirt and sand, and may be persistent in such a matrix. They do not tend to adhere to surfaces; flushing with water generally removes them. Class A oils may be highly toxic to humans, fish, and other biota. Most refined products and many of the highest quality light crudes can be included in this class.

Class B: Non-sticky oils

These oils have a waxy or oily feel. Class B oils are less toxic and adhere more firmly to surfaces than Class A oils, although they can be removed from surfaces by vigorous flushing. As temperature rises, their tendency to penetrate porous substrates increases and

they can be persistent. Evaporation of volatiles may lead to a Class C or D residue. Medium to heavy paraffin-based oils fall into this class.

Class C: Heavy, sticky oils

Class C oils are characteristically viscous, sticky or tarry, and brown or black in appearance. Flushing with water will not readily remove this material from surfaces, but the oil does not readily penetrate porous surfaces. The density of Class C oils may be near that of water. Weathering or evaporation of volatiles may produce solid or tarry Class D oil. Toxicity is low, but wildlife can be smothered or drowned when contaminated. This class includes residual fuel oils and medium to heavy crudes.

Class D: Non-fluid oils

Class D oils are relatively non-toxic, do not penetrate porous substrates, and are usually black or dark brown in colour. When heated, Class D oils may melt and coat surfaces making cleanup very difficult. Residual oils, heavy crude oils, some high paraffin oils, and some weathered oils fall into this class.

These classifications are dynamic for spilled oils; weather conditions and water temperature greatly influence the behaviour of oil and refined petroleum products in the environment. For example, as volatiles evaporate from Class B oil, it may become Class C oil. If a significant temperature drop occurs for instance at night, a Class C oil may solidify and resemble a Class D oil. Upon warming, the Class D oil may revert back to Class C oil (United States Environmental Protection Agency USEPA, 1996)

2.1.2 Crude oil composition and fractions

The hydrocarbons in crude oil or petroleum are mostly alkanes, cycloalkanes and various aromatic hydrocarbons while the other organic compounds contain nitrogen, oxygen and sulfur, and trace amounts of metals such as iron, nickel, copper and vanadium. The exact molecular composition varies widely from formation to formation but the proportions of chemical elements vary over fairly narrow limits. Carbon constitutes about 83 to 87%, hydrogen - 10 to 14%, nitrogen - 0.1 to 2%, oxygen-0.1 to 1.5%, sulphur-0.5 to 6% and metals < 0.1%. The various compounds in crude oil can be broadly categorized into four simple fractions:

- saturates (or alkanes);
- aromatics, including such compounds as benzene, toluene, ethylbenzene and xylenes (BTEX) and polyaromatic hydrocarbons (PAHs);

- resins, consisting of compounds containing nitrogen, sulphur, and oxygen, that are dissolved in oil; and
- asphaltenes, which are large and complex molecules that are colloidally dispersed in oil such as phenols, fatty acids, ketones, esters, and porphyrins (Shell International Ltd., 1983; Balba *et al*., 1998a).

The relative proportions of these fractions are dependent on many factors, including source, age, migration, etc. Of these fractions, the shorter alkane chain compounds and the lighter aromatics (such as BTEX) tend to be more readily biodegradable (Balba *et al*., 1998a, 1998b).

2.1.3 Motor oil composition

Most motor oils are made from a heavier, thicker petroleum hydrocarbon base stock derived from crude oil with additives to improve certain properties. The bulk of typical motor oil consists of hydrocarbons with between 18 and 34 carbon atoms per molecule. Prior to its use, motor oil consists of a complex mixture of hydrocarbons that make up 80 to 90 percent of its volume and performance-enhancing additives that make up 10 to 20 percent of its volume (Chris, 2007).

Motor oils are altered during use because of the breakdown of additives, contamination with the products of combustion, and the addition of metals such as magnesium, copper, zinc, lead, cadmium, etc from the wear and tear of the engine during operation. This makes the composition of waste oil therefore difficult to generalize or characterize in exact chemical terms. According to Irwin *et al*. (1997), the major components consist of aliphatic and aromatic hydrocarbons such as phenol, naphthalene, benzo (a) anthracene, benzo (a) pyrene, and fluoranthene.

2.2 Environmental and Health Impacts of Used Motor Oil

Several works attest to the fact that used engine oil renders the environment unsightly and constitutes a potential threat to humans, animals and the vegetation at large. Thus the presence of these pollutants in the terrestrial and aquatic environments constitutes public health and socio-economic hazards (Adelowo *et al*., 2006; Okerentugba & Ezeronye, 2003).

Used engine oil is a contaminant of concern, with large volumes entering aquatic ecosystems through water runoff. The major source of petroleum contamination in urbanized estuaries comes from waste motor oil. Fresh motor oil on the other hand contains fresh and lighter hydrocarbons that would be more of a concern for short-term (acute) toxicity to living organisms, whereas used motor oil contains more metals and heavy polyaromatic hydrocarbons (PAHs) that would contribute to long-term (chronic) hazards including carcinogenicity. Aromatics are considered to be the most acutely toxic components of petroleum products, and are also associated with chronic and carcinogenic effects. For instance chronic effects of naphthalene, a constituent of used motor oil, include changes in the liver and harmful effects on the kidneys, heart, lungs, and nervous system. Like several individual PAHs, waste motor oil has been shown to be mutagenic and teratogenic. Some immunological, reproductive, fetotoxic, and genotoxic effects have been associated with a few of the compounds found in used motor oil (Irwin *et al*., 1997).

Not only is used motor oil dangerous to human health, it also kills an unbelievable amount of aquatic organisms such as fish upon ingestion when spilled used oil for instance finds its way into aquatic ecosystems. Some toxic components of used motor oil are persistent and can therefore accumulate in the terrestrial and aquatic ecosystems, finding their way into the food chain and exerting their deleterious effects higher up the food chain. Thus used oil can affect fish population by both direct toxicity and by a reduction in the benthic species on which they feed (National Research Council, 1985).

On land, the release of used motor oil into the environment can have great negative impact on food productivity by its effects on soil fertility. The scale of impact would however depend on the quantity of oil spilled. Substantial quantities of petroleum hydrocarbons can thus 'sterilize' the soil and prevent crop growth and yield for a long period of time (Onwurah *et al*., 2007).

2.3 Biodegradation of Contaminants

Biodegradation is the chemical breakdown of materials by the physiological environment. The term is often used in relation to ecology, waste management and environmental remediation (bioremediation). Organic materials can be degraded aerobically with oxygen, or anaerobically, without oxygen. A term related to biodegradation is biomineralisation, in which organic matter is converted into minerals. Biosurfactant, an

extracellular surfactant secreted by microorganisms, enhances the process of biodegradation (Diaz, 2008).

Biodegradable matter basically refers to organic material such as plant and animal matter and other substances originating from living organisms, or artificial materials that are similar enough to plant and animal matter to be put to use by microorganisms. Some microorganisms have the astonishing, naturally occurring, microbial catabolic diversity to degrade, transform or accumulate a huge range of compounds including hydrocarbons (oil), polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), pharmaceutical substances, radionuclides and metals. Major methodological breakthroughs in microbial biodegradation have enabled detailed genomic, metagenomic, proteomic, bioinformatic and other analyses of environmentally relevant microorganisms providing unprecedented insights into key biodegradative pathways and the ability of microorganisms to adapt to changing environmental conditions (Diaz, 2008).

Biodegradation in other terms is a treatment process whereby contaminants or pollutants are metabolized into non toxic or less toxic compounds by microorganisms naturally existing in a given environment. Microorganisms can utilize many of the petroleum hydrocarbon constituents as a source of carbon and energy producing carbon dioxide and water as by-products. Once all of the contaminants have been consumed by microorganisms, the microbial population becomes dormant or dies out. Biodegradation can take place under aerobic or anaerobic conditions in the presence of other suitable electron acceptors such as nitrate, sulfate, or carbonate (Balba *et al*., 1998a).

Current research reveals that in aquatic and terrestrial environments microorganisms are the chief agents for the biodegradation of molecules of environmental concern, including petroleum hydrocarbons (Swanell & Head, 1994; Balba *et al*., 1998a). Hydrocarbon-degrading bacteria, yeast and fungi are widely distributed in marine, fresh water and soil habitats. Bacteria and yeast appear to be the dominant degraders in aquatic ecosystems while fungi and bacteria are the main degraders in soil environments (Cooney & Summers, 1976; Hanson *et al*., 1997; Balba *et al*., 1998b).

2.3.1 Oil degrading microbes

It is a well established fact that no single species of microorganisms can completely degrade any particular oil and this idea has been widely accepted by the scientific community (Colwell & Walker, 1977; Balba *et al*., 1998a). The breakdown of both crude and refined oils seems to involve a consortium of microorganisms, including both eukaryotic and prokaryotic forms. The most common genera known to be responsible for oil degradation or breakdown comprise mainly *Nocardia, Pseudomonas, Acinetobacter, Flavobacterium, Micrococcus, Arthrobacter, Corynebacterium, Achromobacter, Rhodococcus, Alcaligenes, Mycobacterium, Bacillus, Aspergillus, Mucor, Fusarium, Penicillium, Rhodotorula, Candida and Sporobolomyces* spp. (Atlas, 1981; Bossert & Bartha, 1984; Atlas & Bartha, 1992; Sarkhoh *et al*., 1990; Balba *et al*., 1998b).

Singer & Finnerty (1984), found out that of the various petroleum fractions, shorter alkanes of intermediate length ($C_{10} - C_{20}$) are the preferred substrates and tend to be most readily degradable unlike branched chain alkanes which are degraded more slowly than the corresponding normal alkanes. Longer chain alkanes known as waxes ($C_{20} - C_{40}$) are hydrophobic solids and consequently are difficult to degrade due to their poor water solubility and bioavailability (Bartha, 1986).

The actual contribution of a given density of oil-degrading microorganisms to the elimination of oil depends upon their inherent metabolic capability: that is, the heterotrophic potential and the degree to which environmental conditions allow this potential to be expressed (Bartha & Atlas, 1977).

2.4 Microbial Growth

Growth is an orderly increase in the quantity of cellular constituents or the acquisition of biomass leading to cell division, or reproduction. It depends upon the ability of the cell to form new protoplasm from nutrients available in the cell's immediate environment. In most bacteria, growth involves increase in cell mass and number of ribosomes, duplication of the bacterial chromosome, synthesis of new cell wall and plasma membrane, partitioning of the two chromosomes, septum formation, and cell division. This asexual type of reproduction that microbes undergo is called binary fission (Todar, 2008).

Methods for measurement of the cell mass involve both direct and indirect techniques. These include indirect measurement of chemical activity such as rate of O_2 production or consumption, CO_2 production or consumption as well as turbidity (optical density) measurements which employ a variety of instruments to determine the amount of light scattered by a suspension of cells. Particulate objects such as bacteria scatter light in proportion to their numbers. The turbidity/optical density of a suspension of cells directly relates to the cell number, after construction and calibration of a standard curve (Todar, 2008).

Microbial (bacterial) growth in batch culture can be modeled into four different phases namely lag phase (A), exponential or log phase (B), stationary phase (C), and death phase (D) as shown below:

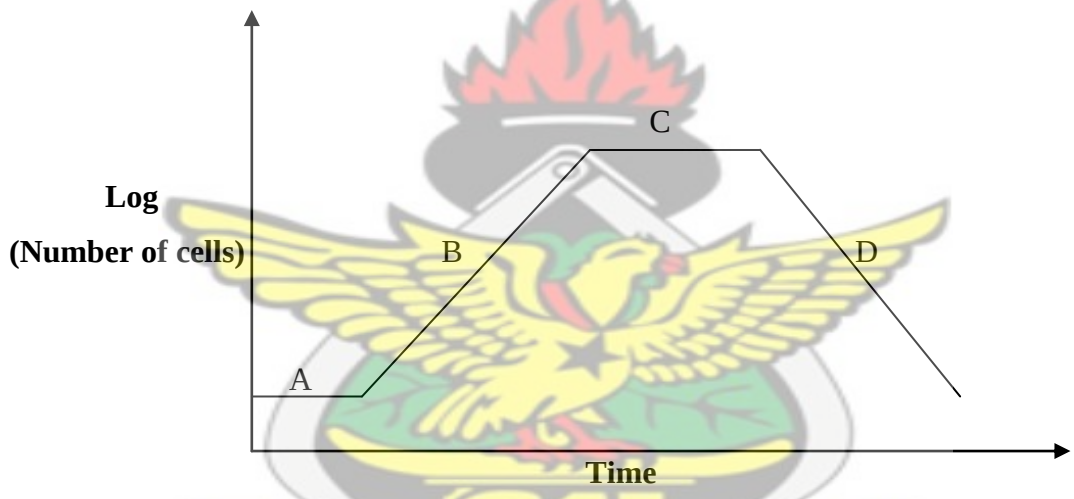


Figure 2.1 Microbial growth curve showing the various stages/phases of growth

During the ***lag phase***, bacteria adapt themselves to growth conditions. It is the period where the individual bacteria are maturing and not yet able to divide. During the lag phase of the bacterial growth cycle, synthesis of RNA, enzymes and other molecules occurs. In this phase the microorganisms are thus not dormant. ***Exponential phase*** (sometimes called the log phase or the logarithmic phase) is a period characterized by cell doubling. The number of new bacteria appearing per unit time is proportional to the initial population. If growth is not limited, doubling will continue at a constant rate such that both the number of cells and the rate of population increase doubles with each consecutive time period. For this type of exponential growth, plotting the natural logarithm of cell number against time produces a straight line. The slope of this line is the

specific growth rate of the organism, which is a measure of the number of divisions per cell per unit time. The actual rate of this growth depends upon the growth conditions, which affect the frequency of cell division events and the probability of both daughter cells surviving. Exponential growth however cannot continue indefinitely because the medium is soon depleted of nutrients and enriched with wastes. During the ***stationary phase,*** the growth rate slows as a result of nutrient depletion and accumulation of toxic products. This phase is reached as the bacteria begin to exhaust the resources that are available to them. This phase is a constant value as the rate of bacterial growth is equal to the rate of bacterial death. At the ***death phase,*** bacteria run out of nutrients, become dormant and eventually die. The build-up of toxic wastes in the medium also results in the death of microorganisms. The death of the microbes like their growth follows an exponential sequence (Zwietering *et al*., 1990; Novick, 1955).

2.5 Pathways of Petroleum Hydrocarbon Degradation

Regardless of whether microbes are native or newly introduced to a contaminated site, an understanding of how they destroy contaminants is critical to understanding the pathway or mechanism of biodegradation. The by-products of microbial processes can provide indicators that the biodegradation is successful. Microorganisms gain energy by catalyzing energy-producing chemical reactions. The type of chemical reaction is called oxidation-reduction reaction and involves the transfer of electrons away from the contaminant. In the process, the organic contaminant is oxidized, the technical term for losing electrons. Correspondingly, the chemical that gains the electrons is reduced. The contaminant is called the electron donor, while the electron recipient is called the electron acceptor. The energy gained from these electron transfers is then "invested", along with some electrons and carbon from the contaminant, to produce more cells. These two materials; the electron donor and acceptor are essential for cell growth and are called the primary substrates (Nyer, 1993).

There are three main energy-yielding oxidation-reduction processes by which non-photosynthetic microorganisms can break down hydrocarbons to obtain energy namely through; fermentation, aerobic respiration and anaerobic respiration. The amount of energy available depends on the metabolic pathway utilized and the available carbon source (Riser-Roberts, 1992).

In fermentation, the carbon and energy source (oil) is broken down by a series of enzyme-mediated reactions that do not involve an electron transport chain (Nyer, 1993).

The process of destroying organic compounds with the aid of O_2 is called aerobic respiration. In aerobic respiration, microbes use O_2 to oxidize part of the carbon in the contaminant (hydrocarbon) to carbon dioxide (CO_2), with the rest of the carbon used to produce new cell mass. In the process the O_2 gets reduced, producing water. Thus, the major by-products of aerobic respiration are carbon dioxide, water, and an increased population of microorganisms (Freeze & Cherry, 1979; Levin & Gealt, 1993).

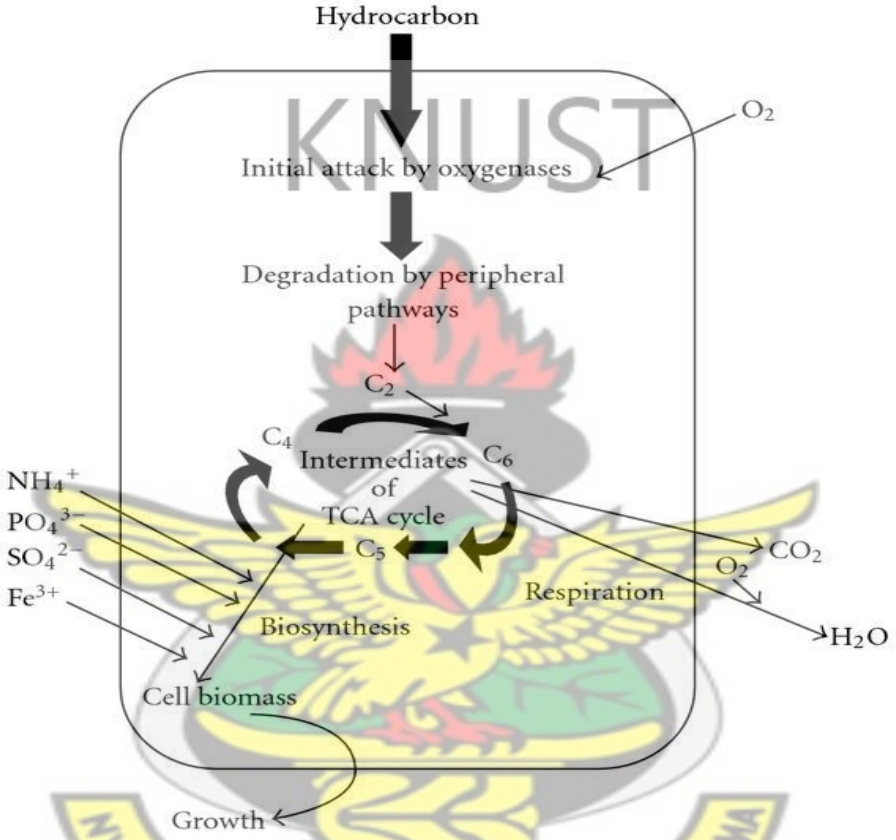
Many microorganisms can exist without oxygen, using a process called anaerobic respiration. In anaerobic respiration, nitrate (NO_3^-), sulfate (SO_4^{2-}), metals such as iron (Fe^{3+}) and manganese (Mn^{4+}), or even CO_2 can play the role of oxygen, accepting electrons from the contaminant. Thus, anaerobic respiration uses inorganic chemicals as electron acceptors. In addition to new cell matter, the by-products of anaerobic respiration may include nitrogen gas (N_2), hydrogen sulfide (H_2S), reduced forms of metals, and methane (CH_4), depending on the electron acceptor (Freeze & Cherry, 1979; Levin & Gealt, 1993).

Aerobic biodegradation of hydrocarbons compared to the other degradation pathways is thus fast enough to be observable on a short human timescale than fermentation and anaerobic biodegradation. Anaerobic biodegradation on the other hand could be much less expensive than the more commonly considered aerobic approach, which is costly and energy intensive due to the need for vigorous agitation and aeration in order to introduce sufficient quantities of oxygen (Aitken, 2004).

2.5.1 Principle and chemistry of aerobic degradation

The most rapid and complete degradation of majority of organic pollutants is brought about under aerobic conditions. The initial intracellular attack of organic pollutants is an oxidative process and the activation as well as incorporation of oxygen is the enzymatic key reaction which is catalyzed by oxygenases and peroxidases. According to Okoh, (2006) this attack normally results in the addition of hydroxide to the alkane (hydrocarbon) to form alcohol which is subsequently oxidized into aldehyde and finally into fatty acids. The addition of oxygen to hydrocarbon compounds makes them more

polar and thus more soluble. Thus 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, e.g. acetyl-CoA, succinate, and pyruvate. Sugars required for various biosyntheses and growth are synthesized through gluconeogenesis (Nilanjana & Preethy, 2010).



Source: (Nilanjana & Preethy, 2010).

Figure 2.2 Principle of aerobic degradation of hydrocarbons by microorganisms.

2.5.2 Strategies used by microbes in petroleum degradation

Several strategies used by microorganisms in the degradation of petroleum hydrocarbon contaminants include use of constitutive enzymes, enzyme induction, co-metabolism, transfer of plasmids coding for certain metabolic pathways; and production of biosurfactants to enhance bioavailability of hydrophobic compounds (Balba *et al*., 1998a).

Uptake of hydrocarbons by biosurfactants

Biosurfactants are heterogeneous group of surface active chemical compounds produced by a wide variety of microorganisms. Surfactants enhance solubilization and removal of contaminants. Biodegradation is also enhanced by surfactants due to increased bioavailability of pollutants. Biosurfactants increase the oil surface area and that amount of oil is actually available for bacteria to utilize it. Biosurfactants can also act as emulsifying agents by decreasing the surface tension and forming micelles thereby facilitating the degradation process (Nilanjana & Preethy, 2010).

Co-metabolism

Co-metabolism can be defined as the simultaneous degradation of two compounds, in which the degradation of the second compound (the secondary substrate) depends on the presence of the first compound (the primary substrate). For example, in the process of metabolizing methane, propane or simple sugars, some bacteria, such as *Pseudomonas stutzeri OX1*, can degrade hazardous chlorinated solvents, such as tetrachloroethylene and trichloroethylene, that they would otherwise be unable to attack. They do this by producing the methane monooxygenase, enzyme which is known to degrade some pollutants, such as chlorinated solvents, via co-metabolism. Co-metabolism is thus used as an approach to biological degradation of hazardous solvents (Ryoo *et al*., 2000).

Enzyme induction

Enzyme induction is the process whereby an (inducible) enzyme is synthesized in response to a specific molecule (inducer). The inducer molecule (often the substrate that needs the catalytic activity of the inducible enzyme for its metabolism) combines with a repressor and thereby prevents the blocking of an operator by the repressor (IUPAC Compendium of Chemical Terminology, 1997).

2.5.3 Enzymes involved in hydrocarbon degradation

The enzyme cytochrome P_{450} alkane hydroxylases constitute a super family of ubiquitous Heme-thiolate monooxygenases which play an important role in the microbial degradation of oil, chlorinated hydrocarbons, fuel additives, and several other compounds. Depending on the chain length, enzyme systems are required to introduce oxygen in the substrate to initiate biodegradation. Higher eukaryotes usually contain several different P_{450} families that comprise large number of individual P_{450} forms that

may contribute as an ensemble of isoforms to the metabolic conversion of a given substrate (Nilanjana & Preethy, 2010).

According to Nilanjana & Preethy, (2010), the capability of several yeast species to use n-alkanes and other aliphatic hydrocarbons as a sole source of carbon and energy is mediated by the existence of multiple microsomal cytochrome P_{450} forms. Dioxygenases are also another group of enzymes which play vital roles in organic matter degradation by microorganisms.

2.6 Monitoring of Oil Degradation

Degradation of oil or hydrocarbon contaminants in a bioreactor system using microorganisms is often directly or indirectly related to the metabolic activities of the degrading microbes and is usually associated with some changes which provide useful information on the degree of biodegradation. Biodegradation can thus be measured or monitored in a number of ways. The activity of aerobic microbes for instance can be measured by the amount of oxygen they consume, or the amount of carbon dioxide they produce. Biodegradation by anaerobic microbes can as well be measured or monitored by the amount of methane that they may be able to produce (Diaz, 2008). Also biodegradation can be measured or monitored in terms of nutrient (particularly nitrogen and phosphorus) uptake by the microorganisms (Kwaspisz *et al*., 2008). Other parameters commonly used in monitoring the progress of oil biodegradation include pH, temperature, total petroleum hydrocarbon (TPH), as well as the microbial population density at any point in time.

2.6.1 Oxygen uptake

Oxygen uptake measurement is a simple method that provides a rapid estimate of microbial activity for samples containing large amounts of microorganisms and can be carried out by Winkler titration or with a suitable respirometer or oxygen electrode. Oxygen uptake increases with increasing microbial activity and vice versa (National Research Council, 1985).

2.6.2 Carbon dioxide evolution

Measurement of CO_2 evolution is also a simple method providing a rapid estimate of the activity of samples containing large numbers of microorganisms. CO_2 can be quantified

by titration of $BaCO_3$ or by infrared gas analysis (National Research Council, 1985). Carbon dioxide evolution measurement as a means of monitoring the degradation of hydrocarbon contaminants would as it stands not be far from precision as microbes in their quest to survive are able to use these hydrocarbon contaminants as their sole source of carbon and energy converting them into less harmful products mainly carbon dioxide and water.

Use of oxygen uptake and carbon dioxide evolution for estimating short term activity create the problem of determining effects of oil or oil degradation products on endogenous respiration. Each method of determining activity is very much dependent upon the experimental conditions employed including the type of oil and the physical state of the oil being degraded (National Research Council, 1985).

2.6.3 Temperature

Temperature as a parameter dictates the rate of degradation of a contaminant as well as the composition of the microbial community undertaking the degradation. In as much as no single species of microorganisms can completely degrade any particular oil, so it is that no single temperature can support the metabolism of all microorganisms. Optimum temperature ranges favouring metabolic activities of various species of microorganisms in a consortium would increase the degradation rate of contaminants. Low temperatures below the optimum range required for individual species of microorganisms in a consortium will adversely affect microbial growth and propagation by inhibiting enzymatic activities and thus decreasing the degradation rate. Also, at temperatures above the optimum range enzymatic activities are likely to be inhibited as proteins denature (Leahy & Colwell, 1990).

2.6.4 pH

Like temperature, pH also plays a role in determining the ability of microbes to grow or thrive in particular environments. Most commonly, microbes particularly bacteria grow optimally within a narrow pH range of between 6.7 and 7.5. Metabolic activities of microbes in a system can often be directly related to the pH (acidity or alkalinity) of the system under study. Several works have shown that microorganisms often change the pH of their habitat through the production of metabolic waste products that are either acidic or basic. Organic acids produced as a result of microbial activities function to lower the

pH of the system thereby making it more acidic unlike organic bases that would boost the pH of the system to make it alkaline. Anaerobic processes as a result of fermentative activities would usually lower the pH of the system. Depending on the nature of the metabolic waste product, the pH of the system would either increase or decline in correlation to microbial activity and numbers. A rise or fall in the pH of a system would therefore give an indication of the possible utilization of contaminants of concern by microbes (Prescott *et al*., 2002).

2.6.5 Microbial population density

The growth of microbes in a system is normally expected to follow the four main phases of the microbial growth curve namely the lag, exponential, stationary and death phases. Microbes upon the uptake of contaminants would flourish or increase in number; an indication that the microbes are successfully degrading or making use of the contaminant of concern. Reduced or stagnated microbial numbers generally give an indication of non utilization of the contaminant which possibly could be due to the presence of unfavourable factors in the system (Prescott *et al*., 2002).

2.6.6 Total petroleum hydrocarbon (TPH)

Total Petroleum Hydrocarbon (TPH) is sometimes referred to as mineral oil, hydrocarbon oil, extractable hydrocarbons, or oil and grease. There are many analytical techniques available that measure TPH concentrations in the environment. No single method measures or can measure the entire range of petroleum-derived hydrocarbons. Because the techniques vary in the way hydrocarbons are extracted, cleaned up, and detected, they each measure slightly different subsets of the petroleum-derived hydrocarbons present in a sample. Chemical composition of petroleum products is complex and change over time following their release into the environment. There is no single "best" method for measuring all types of petroleum contamination because of the complexity of petroleum and petroleum-derived products. These factors make it difficult in selecting the most appropriate analytical method for evaluating environmental samples containing hydrocarbons. Analytical methods for quantifying petroleum fractions include the following:

Gravimetric

With this method, the sample is extracted with a suitable organic solvent, filtered and the solvent evaporated to leave the oil/grease residue. This method does not give information

as to the exact carbon range and only measures oils and greases. An efficient extraction method such as soxhlet or microwave extraction is required to get a greater percentage of heavy hydrocarbons eluted for analysis.

Infrared

The sample is extracted using a suitable solvent, and the hydrocarbon concentration is measured by infrared spectrophotometry. With this method, the solvent is not evaporated. The light hydrocarbons are easily measured with this method. This method requires the use of solvents such as chlorofluorocarbons (CFC's) and as such is no longer routinely used due to the hazards associated with the use of CFC's.

Immunoassay

This is a relatively new method which is largely used for field measurements of indicative concentrations of the presence or absence of hydrocarbons. The method does not give information as to carbon range that can be eluted and is also prone to interferences from humic acids etc.

Gas Chromatography

This is the most commonly used method for the analysis of TPH. Samples are extracted with a suitable organic solvent using continuous liquid-liquid extraction, soxhlet or microwave extraction as well as separatory funnel extraction. The extract is analyzed by gas chromatography with the compounds present being detected in order of the boiling points of the compounds. The compounds are quantified by comparison to standards. The oil after extraction from the sample is injected onto a GC column. A flow of inert gas sweeps the sample through a narrow column, separating the components. The effluent from the GC column passes through the flame of the non-selective detector, which breaks down organic molecules and produces ions. The ions are collected and produce a measurable electrical signal used to confirm the identity and amount of the compounds of interest.

Some of the above mentioned methods measure more compounds than other methods because they probably employ more rigorous extraction techniques or employ the use of more efficient solvents for the extraction (http://www.mpl.com.au/total-petroleum-hydrocarbons-93.htm).

After biodegradation, TPH or oil samples would contain only some or a mixture, of the compounds present; an indication of the success of microbial activity and biodegradation of the oil sample (ATSDR, 1999).

2.6.7 Conductivity

Conductivity is a measure of the ability of water to pass an electrical current. Conductivity in water is affected by the presence of inorganic dissolved solids such as chloride, nitrate, sulfate, and phosphate anions or sodium, magnesium, calcium, iron, and aluminum cations. Organic compounds like oil, phenol, alcohol, and sugar have a low conductivity when in water. Conductivity is also affected by temperature: the warmer the water, the higher the conductivity. Significant changes in conductivity could thus be an indicator that a discharge or some other source of pollution has entered a system say a water body. Conductivity is also directly related to salinity in that conductivity increases with salinity. Aquatic organisms including microbes are adapted for a certain range of salinity. Outside of this range, they will be negatively affected and may die as some can handle high salinity, but not low salinity and others low salinity, but not high salinity (APHA, 1992; Hach Company, 1992).

2.7 Environmental Factors Influencing Biodegradation of Petroleum Hydrocarbons

The fate of petroleum hydrocarbons in the environment is largely determined by abiotic factors which influence the weathering or biodegradation of the oil. Factors which influence rates of microbial growth and enzymatic activities affect the rates of petroleum hydrocarbon biodegradation. The persistence of petroleum pollutants depend on the quantity and quality of the hydrocarbon mixture and on the properties of the affected ecosystem. In one environment petroleum hydrocarbons can persist indefinitely, whereas under another set of conditions the same hydrocarbons can be completely biodegraded within a relatively few hours or days (Leahy & Colwell, 1990). These factors include the following as discussed:

2.7.1 Physical state of the oil pollutant

The physical state of petroleum hydrocarbons has a considerable effect on their biodegradation. At very low concentrations, hydrocarbons are soluble in water, but most oil spill incidents release petroleum hydrocarbons in concentrations far in excess of the

solubility limits. The degree of spreading determines in part the surface area of oil available for microbial colonization by hydrocarbon-degrading microorganisms. In aquatic systems, the oil normally spreads, forming a thin slick (Atlas, 1981). As a result of wind and wave action, oil-in-water or water-in-oil ("mousse") emulsions may form. Dispersion of hydrocarbons in the water column in the form of oil-in-water emulsions increases the surface area of the oil and thus its availability for microbial attack. However, large masses of mousse establish unfavourably low surface-to-volume ratios, inhibiting biodegradation. The formation of emulsions through the microbial production and release of biosurfactants is an important process in the uptake of hydrocarbons by bacteria and fungi (Leahy & Colwell, 1990). The degree of spreading is reduced at low temperatures because of the viscosity of the oil (Atlas, 1981).

Artificial dispersants have been studied as a means of increasing the surface area and hence the biodegradability of oil slicks. The effectiveness of dispersants in enhancing the biodegradation of oil has been shown to be extremely variable and to be dependent on the chemical formulation of the dispersant, its concentration, and the dispersant/oil application ratio (Leahy & Colwell, 1990).

The key differences between petroleum biodegradation in soil and aquatic ecosystems following an oil spill are related to the movement and distribution of the oil and the presence of particulate matter, each of which affects the physical and chemical nature of the oil and hence its susceptibility to microbial degradation. Terrestrial oil spills are characterized primarily by vertical movement of the oil into the soil, rather than the horizontal spreading associated with slick formation (Bossert & Bartha., 1984; Leahy & Colwell, 1990).

2.7.2 Chemical composition of the oil pollutant

Hydrocarbons differ in their susceptibility to microbial attack and in the past, have generally been ranked in the following order of decreasing susceptibility: n-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes. Biodegradation rates have been demonstrated to be highest for saturates, followed by the light aromatics, with high-molecular weight aromatics and polar compounds exhibiting extremely low rates of degradation. These fractions have previously been considered relatively recalcitrant to biodegradation. Their microbial degradation however can be ascribed to

co-oxidation, in which non-growth hydrocarbons are oxidized in the presence of hydrocarbons which can serve as growth substrates. Compositional heterogeneity among different crude oils and refined products influences the overall rate of biodegradation both of the oil and of its component fractions (Leahy & Colwell, 1990).

2.7.3 Concentration of the oil pollutant

The rates of uptake and mineralization of many organic compounds by microbial populations in the aquatic environment are generally proportional to the concentration of the compound. High concentrations of hydrocarbons can be associated with heavy, undispersed oil slicks in water, causing inhibition of biodegradation by nutrient or oxygen limitation or through toxic effects exerted by volatile hydrocarbons. There is the likelihood that high concentrations of oil have similarly negative effects on biodegradation rates following oil spills in quiescent, low-energy environments such as beaches, harbours, and small lakes or ponds, in which the oil is relatively protected from dispersion by wind and wave action (Leahy & Colwell, 1990).

2.7.4 Temperature

Temperature influences petroleum biodegradation by its effect on the physical nature and chemical composition of the oil, rate of hydrocarbon metabolism by microorganisms, and composition of the microbial community (Leahy & Colwell, 1990; Atlas, 1981). At low temperatures, the viscosity of the oil increases, the volatilization of toxic short-chain alkanes is reduced, and their water solubility is decreased, delaying the onset of biodegradation. Rates of degradation are generally observed to decrease with decreasing temperature; this is believed to primarily be as a result of decreased rates of enzymatic activity. Higher temperatures increase the rates of hydrocarbon metabolism to a maximum, typically in the range of 30°C - 40°C in soil environments (Leahy & Colwell, 1990), 20°C - 30°C in some freshwater environments and 15°C - 20°C in marine environments (Bossert & Bartha, 1984) above which the membrane toxicity of hydrocarbons is increased with respect to the various environments.

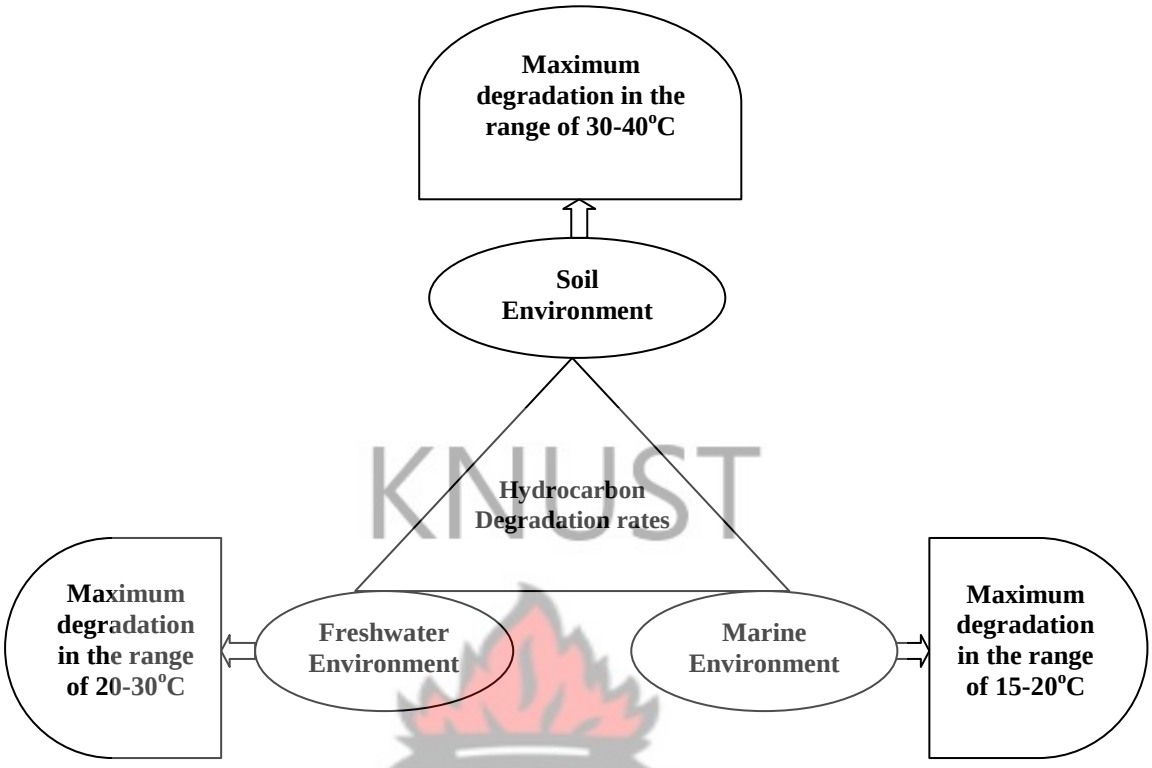


Figure 2.3 Hydrocarbon degradation rates in soil, fresh water, and marine environments

2.7.5 Oxygen

The initial steps in the catabolism of aliphatic, cyclic, and aromatic hydrocarbons by bacteria and fungi involve the oxidation of the substrate by oxygenases, for which molecular oxygen is required. Aerobic conditions are therefore necessary for this route of microbial oxidation of hydrocarbons in the environment. Conditions of oxygen limitation normally do not exist in the upper levels of the water column in aquatic environments. However, aquatic sediments are generally anoxic except for a thin layer at the surface of the sediment (Leahy & Colwell, 1990).

2.7.6 Nutrients

Microorganisms would only degrade oil to get energy and the building blocks for their biomass. However, the microorganisms would also require essential nutrients as the hydrocarbons contain hydrogen (H) and carbon (C) as the only elements. Microbes thus require nutrients for survival. Microorganisms might have varying nutritional requirements but basically all of them require nitrogen (N), phosphorus (P) and some trace elements in addition to the carbon source. The macronutrients nitrogen and

phosphorus are especially important to ensure fast and complete degradation of the oil (Braddock *et al*., 1997).

It is therefore important that the nutrients are supplied in the right ratio(s) in order to ensure optimal growth conditions for the microorganisms. Alexander *et al*. (1982), suggest that a C: N: P ratio of 100:10:2 is enough to ensure optimal growth of microorganisms. The nutrient concentration should be maintained at a level high enough to support maximum oil biodegradation based on the kinetics of nutrient consumption. Higher, concentrations will provide no added benefit but may lead to potentially detrimental ecological and toxicological impacts (Zhu *et al*., 2001). Thus nutrients especially nitrogen, phosphorus, and in some cases iron are very important ingredients for successful biodegradation of hydrocarbon pollutants. However some of these nutrients in excess or limited amounts could become limiting factors thus affecting the biodegradation process (Nilanjana & Preethy, 2010).

2.7.7 pH

Higher rates of degradation are often observed at neutral pH conditions. Most heterotrophic bacteria and fungi favour a pH near neutrality, with fungi being more capable of tolerating acidic conditions (Atlas, 1988). Extremes in pH, as can be observed in some environments, would therefore be expected to have a negative influence on the ability of microbial populations to degrade hydrocarbons (Leahy & Colwell, 1990). A study by Dibble and Bartha, (1979) suggests that biodegradation of oil as it stands increases with increasing pH with optimum degradation occurring under slightly alkaline conditions.

2.7.8 Water activity/moisture

Water constitutes a greater percentage of the cell's cytoplasm and it serves as a carrier for the transport of materials into and out of the cell. Most enzymatic reactions do take place in solution with water as the base solvent. The water activity or water potential (a_w) of soils for instance can range from 0.00 to 0.99, in contrast to aquatic environments, in which water activity is stable at a value near 0.98 (Bossert & Bartha., 1984; Leahy & Colwell, 1990). Hydrocarbon biodegradation in terrestrial ecosystems for instance may therefore be limited by the available water for microbial growth and metabolism (Leahy & Colwell, 1990).

2.7.9 Other environmental factors

Other variables such as pressure and salinity have also been reported to have significant effects on oil degradation rates by microorganisms. There are also reports that photo-oxidation increases the biodegradation of oil or petroleum hydrocarbons by increasing the bioavailability of the oil and thus enhancing microbial activities (Maki *et al*., 2005).

2.8 Bioreactor Defined

A bioreactor is essentially an engineered system in which biochemical transformation of materials is promoted by optimizing the activity of microorganisms, or by "in vitro" cellular components of the microbial cells/enzymes (Onwurah *et al*., 2007). A bioreactor thus may refer to any device or system that supports a biologically active environment. In one case, a bioreactor is a vessel in which a chemical process is carried out which involves organisms (suspended or immobilized) or biochemically active substances derived from such organisms. This process can either be aerobic or anaerobic. These bioreactors are commonly cylindrical, ranging in size from liters to cubic meters, and are often made of stainless steel or a suitable non corrosive material. A bioreactor may also refer to a device or system meant to grow cells or tissues in the context of cell culture (www.wikipedia.com/bioreactor).

2.8.1 Bioreactor design

Bioreactor design is a relatively complex engineering task, which is studied in the discipline of biochemical engineering. Under optimum conditions, the microorganisms or cells are able to perform their desired function with 100 percent rate of success. The bioreactor's environmental conditions like gas (i.e., air, oxygen, nitrogen, carbon dioxide) flow rates, temperature, pH and dissolved oxygen levels, and agitation speed or circulation rate need to be closely monitored and controlled to ensure optimum performance of the bioreactor. Aseptic conditions are also very critical to the performance of a bioreactor. Bioreactor vessels are usually made of nontoxic and noncorrosive materials that can easily be sterilized to avoid contamination. Most industrial bioreactor manufacturers use vessels, sensors and a control system networked together (www.wikipedia.com/bioreactor).

2.8.2 Classification of bioreactors

Key design considerations for bioreactor operations are the aeration and agitation schemes since these have greatest effect on the key process variables namely the bed temperature and water content as well as the void space oxygen concentration. It is useful to classify bioreactors in groups in terms of how they are aerated and agitated because of the many similarities in operating variables that can be manipulated to optimize bioreactor performance as well as the design strategies used. Four basic groups can be identified according to Mitchell *et al*. (2006):

Group I-Bioreactors that neither are agitated nor forcefully aerated

In Group I bioreactors, air is not blown forcefully through the bed but rather is circulated around the bed surfaces. The substrate bed either remains static during the whole process or is mixed only very infrequently of the order of once or twice per day.

Group II-Bioreactors that are not agitated but are forcefully aerated

The general feature of Group II bioreactors is that the substrate bed is forcefully aerated but remains static. In some processes it remains static for the whole process while in others it may be mixed infrequently on the order of once or twice a per day. The performance of this bioreactor is highly dependent on convective flow phenomena which require the establishment of axial gradients along the direction of air flow.

Group III-Bioreactors that are agitated but are not forcefully aerated

With Group III bioreactors, the beds are continuously or intermittently mixed or agitated but not forcefully aerated.

Group IV-Bioreactors that are both agitated and forcefully aerated

These bioreactors have their beds agitated or mixed continuously or intermittently with air forcefully blown through them.

2.8.3 Immobilizing (support) material

An immobilizing material is a material used for the physical isolation of a given microorganism from the reaction medium in a bioreactor. Immobilization may be achieved by physical attachment, chemical attachment, and entrapment within a gel or within a membrane. Immobilized cells have been used and studied for the bioremediation of numerous toxic chemicals. Immobilization not only simplifies separation and recovery of immobilized cells but also make the application reusable which reduces the overall cost. Immobilization results in increased contact between cell and hydrocarbon droplets and has been proven to enhance the biodegradation rate of crude oil compared to free

living cells in a wide range of culture salinity. Materials commonly used as immobilizing materials include pebbles and plastic chips among others (Nilanjana & Preethy, 2010).

2.9 Hydraulic Retention Time (HRT)

The Hydraulic retention time (HRT) also known as hydraulic residence time is a measure of the average length of time that a soluble compound remains in a constructed bioreactor and is usually expressed in hours or sometimes days. Hydraulic retention time can also be defined as the ratio of the volume of the reaction vessel/tank to the influent flow rate:

$$\text{HRT} = \frac{\text{Volume of reaction vessel or tank}}{\text{influent flowrate}}$$

The longer microorganisms interact with the substrate, the better the degradation of the substrate (www.wikipedia.com/bioreactor).

CHAPTER THREE

3.0 METHODOLOGY

3.1 Introduction

The research basically involved laboratory experiments which were conducted in accordance with standard laboratory procedures. The experiments were conducted at the green house facility and the Biology laboratory of the Department of Environmental Science of Kwame Nkrumah University of Science and Technology, Kumasi.

3.2 Selection of Biofilm Support Material

Hollow bamboo chips of about 2-5 cm in length were used as biofilm support material. These were dried for a three week period. 0.95 kg of the bamboo chips was packed into each bioreactor to serve as the biofilm support material. The objective here was to ensure immobilization or fixation of the microbes in order to achieve desirable degradation rates.

Figure 3.2 Picture of bamboo chips used as support material

3.3 Design and Construction of Bioreactors

Eight bioreactors each with a volume of about 0.009 m^3were constructed using a six (6) inch polyvinyl chloride (PVC) pipe. Each bioreactor was approximately 50 cm in height and 15.24 cm in diameter. Each bioreactor was sealed at one end with a plastic material. Inlet and outlet holes were created on the lid and at the sealed bottom or end of each bioreactor for influent and effluent discharges respectively. 0.95 kg of bamboo chips was packed into each bioreactor occupying about 1/3rd the volume. Bamboo chips were held in place with a cylindrically molded wire mesh. The diagram below gives the details of each bioreactor;

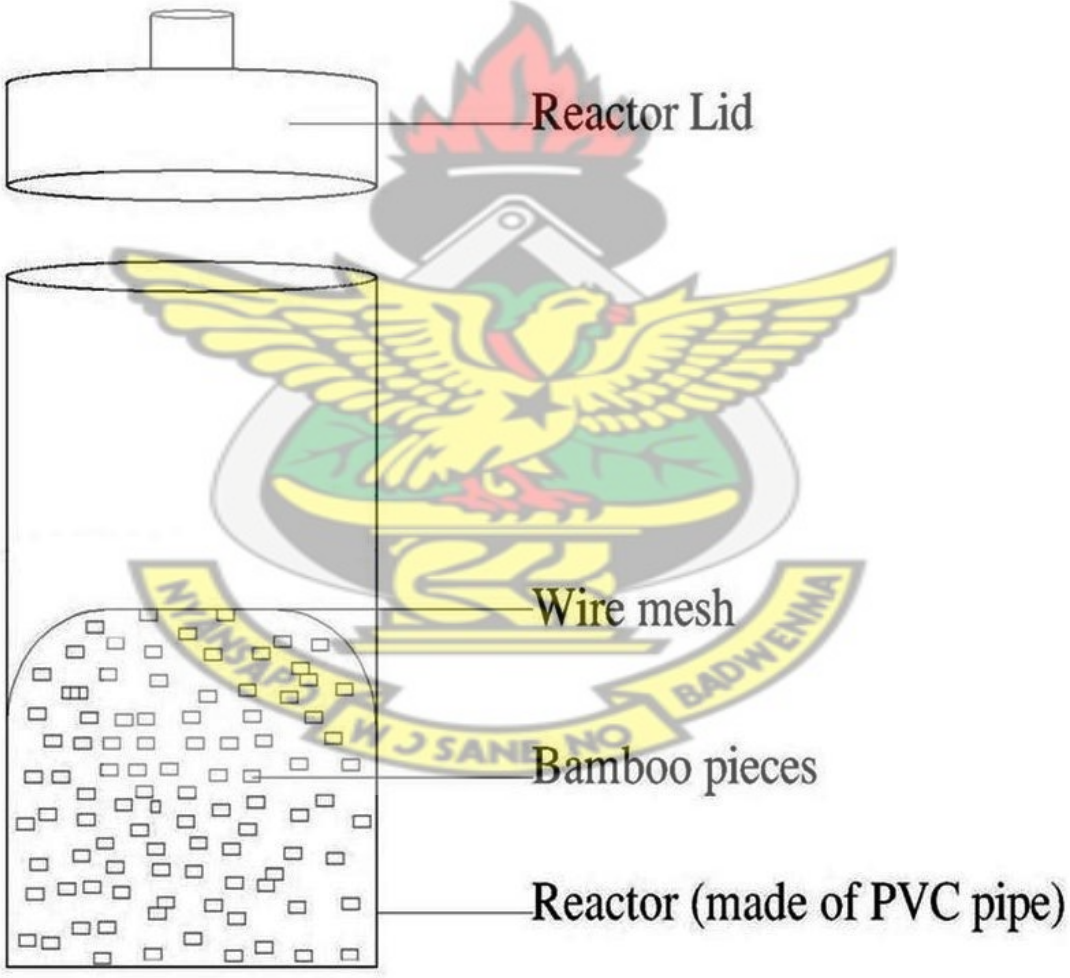


Figure 3.1 Details of each bioreactor

3.3.1 Arrangement of bioreactors

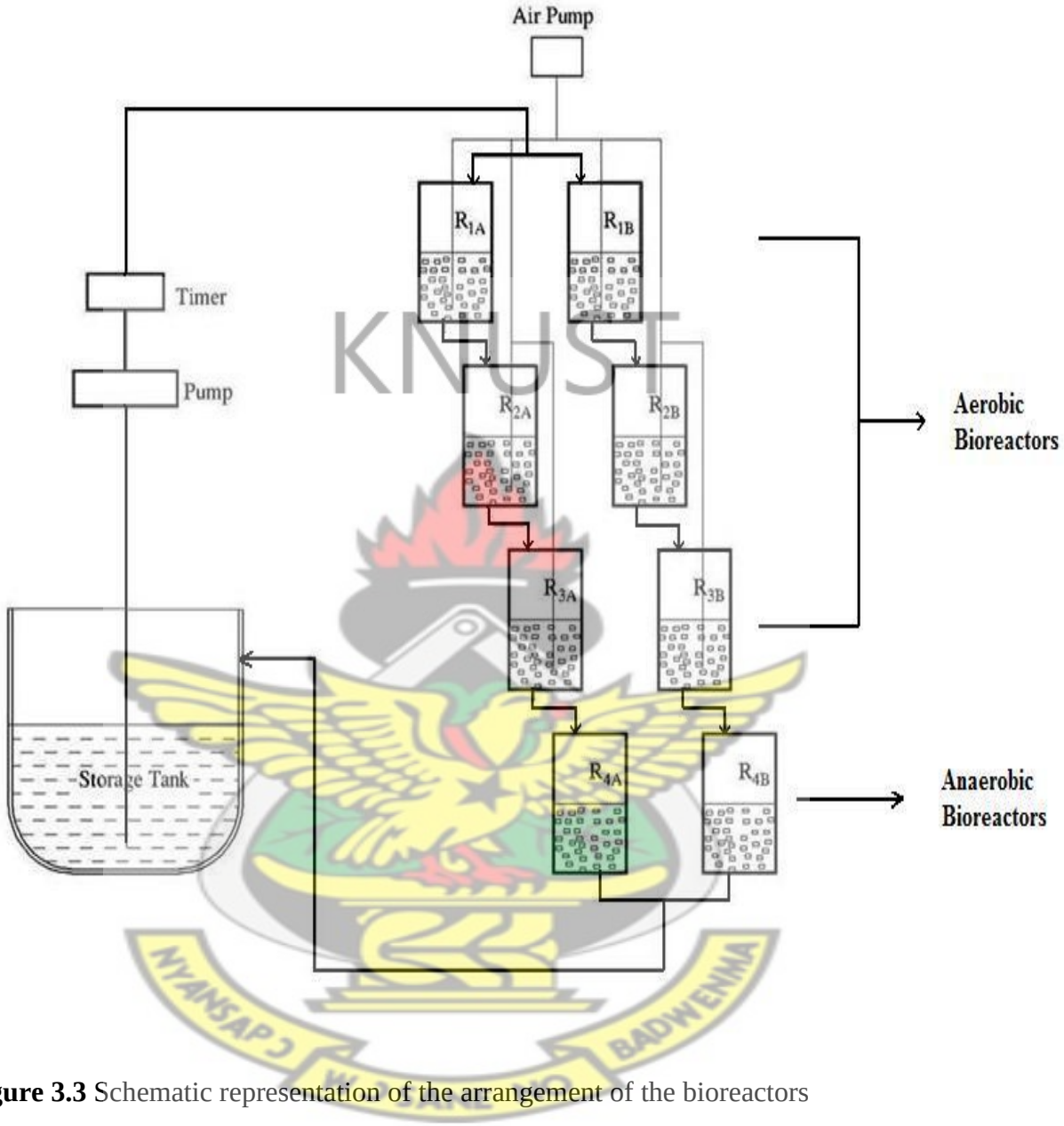


Figure 3.3 Schematic representation of the arrangement of the bioreactors

The eight bioreactors were sorted into two groups of four reactors each; namely R_{1A}, R_{2A}, R_{3A}, and R_{4A} as set A and R_{1B}, R_{2B}, R_{3B} and R_{4B} as set B. The four reactors in each set were connected in series to one another. The two sets were then arranged in parallel with bioreactor set B serving as a replicate of bioreactor set A (figure. 3.3). The first three bioreactors of each set (R_{1A}, R_{2A}, R_{3A} and R_{1B}, R_{2B}, R_{3B}) were aerated using an aquarium air pump making them aerobic. The last bioreactor of each set however was not aerated;

hence anaerobic. The idea was to make sure that substances that are not degraded aerobically could be removed in the anaerobic bioreactors.

3.4 Obtaining Motor Oil and Oil-Contaminated Soil Samples

Used motor oil sample (diesel) was obtained from a mechanic workshop at magazine in Kumasi for the study. Oil-contaminated soil samples were also fetched from the mechanic workshop at three different locations into sterile petri-dishes using a hand trowel. The used motor oil sample and oil-contaminated soil samples were then transported to the laboratory. Oil-contaminated soil samples were homogenized and a sub-sample taken to obtain a good representation of the hydrocarbon-degrading microbes required for the study.

3.5 Preparation of Nutrient (Mineral) Medium

A nutrient medium containing the essential nutrients (potassium, magnesium, sodium, phosphorus and ammonium salts) required by microorganisms for their growth was prepared by dissolving 2.0 g of $Na_2HPO_4.2H_20$, 0.8 g of KH_2PO_4, 0.2 g of $MgSO_4$ and 1.8 g of $(NH_4)_2SO_4$ in 1 L of distilled water (Soriano & Pereira, 1998; Fei-Baffoe, 2003).

3.6 Preparation of Culture of Hydrocarbon-Degrading Microbes

Hydrocarbon-degrading microorganisms used for the study were isolated from oil-contaminated soil sample (taken from the mechanic workshop) and cultured in a liquid medium (distilled water) containing mineral salts (nutrient medium) required for the growth of the microbes.

Procedure

5 g of the homogenized oil-contaminated soil sample was weighed and 50 ml of distilled water added after which the mixture was gently swirled. 1000 ml of distilled water was then inoculated with 40 ml of the supernatant resulting from the oil-contaminated soil and water mix. 25 ml of nutrient (mineral salt) medium was then added to the mixture followed by 5 drops of used motor oil. The mixture was then incubated at 37 ^oC for a week.

3.6.1 Monitoring cell growth of culture of hydrocarbon-degrading microbes

Turbidity of the prepared liquid culture was monitored every two days during the seven day incubation period using the Wagtech photometer WG 7100. As cells grew during the

incubation period, liquid culture turned cloudy or turbid an indication that microbes were respiring-thus making use of the oil as their carbon and energy source. The turbidity or optical density of the suspended cells was expected to be directly related to the cell mass. The presence of these cells was confirmed by plate count at the end of the seven day incubation period.

3.7 Microbial Enumeration by Plate Count

Microbes were enumerated by plate count using the pour plate technique.

Preparation of agar plate

17.5 g of the agar (PCA) was dissolved in 1 L of distilled water. The solution was sterilized by autoclaving at 121 ^{o}C for 15 minutes and subsequently cooled to about 47 ^{o}C before use. Petri-dishes used were also sterilized using a hot air oven at 180^{o}C for an hour and a half after which they were allowed to cool appreciably before use.

Serial dilution

1 ml of liquid culture (sample) was drawn and serially diluted through to 10^{-12}. Dilutions 10^{-7} through to dilution 10^{-12} were then plated. Microbial numbers were enumerated after 24 hours of incubation at 37 ^{o}C using a colony counter. The following relation was used in estimating the average microbial colonies per ml for the plated dilutions:

Colony Forming Unit (CFU/ml) = {$\sum$ (number of colonies * dilution factor) / 6 ml}

3.8 The Degradation Procedure

Aerobic and anaerobic hydrocarbon-degrading microbes capable of utilizing the oil as their source of carbon and energy would be actively involved in the oil degradation process as the sample (water + oil) continuously flows through the aerobic and anaerobic bioreactors and back into the storage tank during each cycling regime.

3.8.1 Acclimatization of Hydrocarbon-Degrading Microbes in the Bioreactors

The research basically employed the activities of hydrocarbon-degrading microbes. Before the start of the first experimental run, 1000 ml of liquid culture of hydrocarbon-degrading microbes was poured into each bioreactor (containing the bamboo chips). This was followed by the addition of 50 ml of nutrient (mineral salt) medium to each bioreactor. A sample (oil + water) concentration of 250 mg/L was pumped through the system at a flow rate of 0.5 L/minute for a week to allow for the microbes to acclimatize to conditions within the bioreactors. For subsequent reactions, acclimatization was done

for only twelve hours with the addition of only 50 ml of nutrient medium to each bioreactor to stimulate the growth of already existing microbes.

3.8.2 Testing oil concentrations

Four sample concentrations were studied to determine their effect on the rate of oil degradation by the hydrocarbon-degrading microbes. Sample (oil + water) concentrations of 500 mg/L, 1000 mg/L, 2000 mg/L and 6000 mg/L were prepared by doping the respective masses of oil in 1 L of distilled water. For each experimental run, 15 L of the sample concentration under study was used. Each sample concentration was studied for a week.

3.8.3 Testing loading rates

The above mentioned sample concentrations were studied at loading rates of 0.5 L/minute, 1.0 L/minute and 2.0 L/minute to determine the effect of loading rate on the degradation of the oil. Loading rates were achieved by adjusting a regulating knob on the water pump until the required amount or volume of sample per minute was delivered.

3.8.4 Operation of the bioreactor set up

A 0.5 hp (horse power) water pump was used in driving the sample (water + oil) in the storage tank (15 L) through the bioreactors and eventually back into the storage tank. The flow regimes through the bioreactors were regulated using an automatic timer to which the water pump was connected. A total of five (5) regimes per day were employed for each experimental run with each pumping regime lasting for 30 minutes. The set up was flushed continuously with water for about 10 minutes after each experimental run.

Sampling

At the end of the each experimental run, a sample volume of 1000 ml was collected (from the storage tank) using sterile sampling bottles. The amount of hydrocarbon (oil) remaining at the end of the one week degradation period was serially extracted from the collected sample for subsequent analysis using a gas chromatograph coupled with a flame ionization detector. For the purpose of monitoring the changes in pH, temperature, dissolved oxygen content, and conductivity level of the system, a sample volume of 500 ml was collected from the outlet of the last bioreactor of each bioreactor set. Samples were collected at two days intervals during each experimental run.

3.9 Monitoring the Degradation Process

Monitoring parameters measured as indicators of degradation during the study period included temperature, pH, dissolved oxygen, conductivity, microbial population density and total petroleum hydrocarbon (TPH).

3.9.1 Measuring of the monitoring parameters

Temperature, pH, dissolved oxygen, and conductivity, were measured using a multiparameter probe (YSI 600XL) and meter (YSI 650 MDS) by following manufacturer's instructions.

Procedure

A sample volume of about 500 ml was transferred into a clean 1000 ml measuring cylinder. The probe after being rinsed thoroughly with distilled water was then dipped into the sample and the corresponding readings recorded accordingly.

Microbial population density

Microbial populations were enumerated using same procedure as described under section 3.7.

Total petroleum hydrocarbon (TPH)

Gas chromatograph (GC) with a flame ionization detector was used (FID) for the analysis of the TPH. The method measured C_9 to C_{36} range of hydrocarbons. The method relies on the average response factor of alkanes to convert the total peak area of a sample chromatogram to a TPH concentration. The shortfall of this method is that organic compounds that are extracted, eluted through the column and detected by FID may be calculated as part of the TPH (Environmental Research Institute, 1999).

Procedure

- **Hydrocarbon extraction from samples after degradation**

A sample volume of 1 L was serially extracted three times with methylene chloride using a separatory funnel. 100 ml of the methylene chloride was first added to the sample and was well shaken for about 15 minutes to homogenize it using an electronic shaker. About 500 ml of the well shaken sample containing methylene chloride was then transferred into a 1000 ml separatory funnel. The separatory funnel with its content was shaken for a few seconds and allowed to stand for about 10 minutes to allow for separation of the organic phase from the aqueous phase. The organic phase (containing the oil) was then carefully drained into a 50 ml beaker. The extraction was repeated two more times with 40 ml of the methylene chloride (in each case) and the oil extracts combined afterwards. The

remaining 500 ml of the sample was subjected to the same treatment as above and the extracts added to that from the first three extractions. The beaker containing the combined extracts was then subjected to heating using a hot plate to get rid of traces of water. The final sample extract (oil), which was about 0.5 ml was then transferred into a 2 ml vial. A total of sixteen samples were worked on with each sample being subjected to same treatment as described above.

- **Gas chromatographic (GC) analysis**

Samples (oil/hydrocarbon extracts) after extraction were subjected to GC analysis under the following stated conditions in table 3.1:

Table 3.1 GC operating conditions

Parameter	**Condition**
Carrier gas flow rate	5 ml/minutes
Initial temperature	40 °C, hold for 0.5 minutes
Program	40 °C to 290 °C at 15 °C/min
Final temperature	290 °C, hold for 10 minutes
Injector Temperature	290 °C
Detector Temperature	300 °C
Make-up gas	25 ml/minutes

The sequence of chromatographic analysis begun with a solvent blank followed by calibration verification standard, then method blank and finally the sample extract (oil) analyses. The calibration verification standard was an n-alkane mixture that contained C_9 to C_{36} range of hydrocarbons. A 500 µg/ml working concentration was prepared for both the standard and sample extracts (Environmental Research Institute, 1999).

CHAPTER FOUR

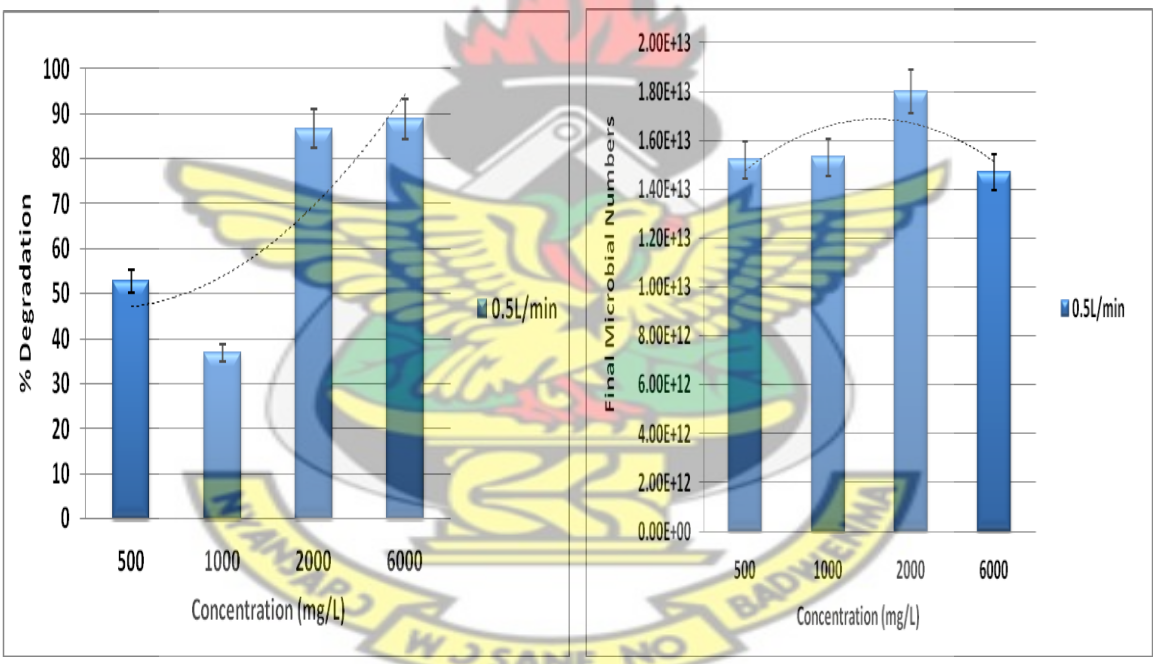
4.0 ANALYSIS OF RESULTS

4.1 Introduction

Presented below are the various profiles/results generated for the experimental study. Charts were plotted using Microsoft Office Excel (2007) software.

4.2 Effect of Oil Concentration on Degradation of Hydrocarbons

The figures-4.1a, 4.2a and 4.3a below respectively represent the degradation rates achieved at loading rates of 0.5 L/min, 1.0 L/min and 2.0 L/min for the various oil concentrations studied with figures-4.1b, 4.2b and 4.3b also respectively representing the corresponding microbial densities observed at the above mentioned loading rates for the various oil concentrations.

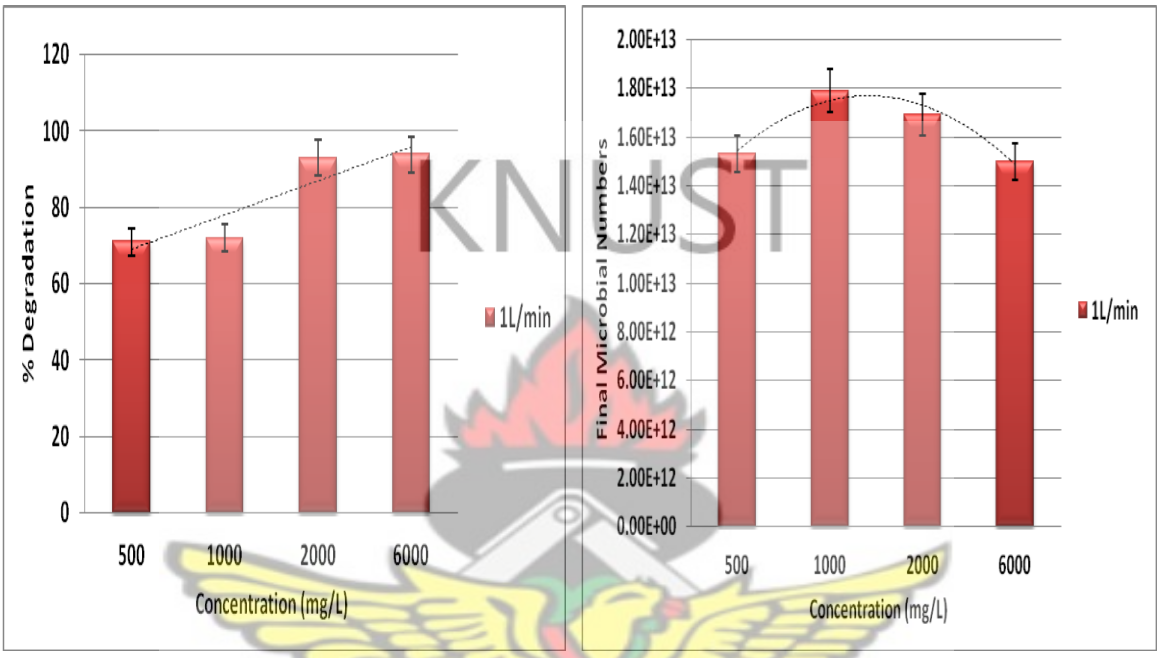


a. Degradation at 0.5 L/min b. Microbial variations at 0.5 L/min

Figure 4.1 Profile for degradation rates (%) obtained with the corresponding microbial population densities at 0.5 L/min loading rate

At the 0.5 L/min loading rate, a gradual increase in degradation was observed for the various oil concentrations studied. Minimum and maximum degradation rates of 36.83±0.00 % and 88.76±0.00 % corresponding to oil concentrations of 1000 mg/L and 6000 mg/L were achieved at the above mentioned loading rate (figure 4.1a). Similarly at

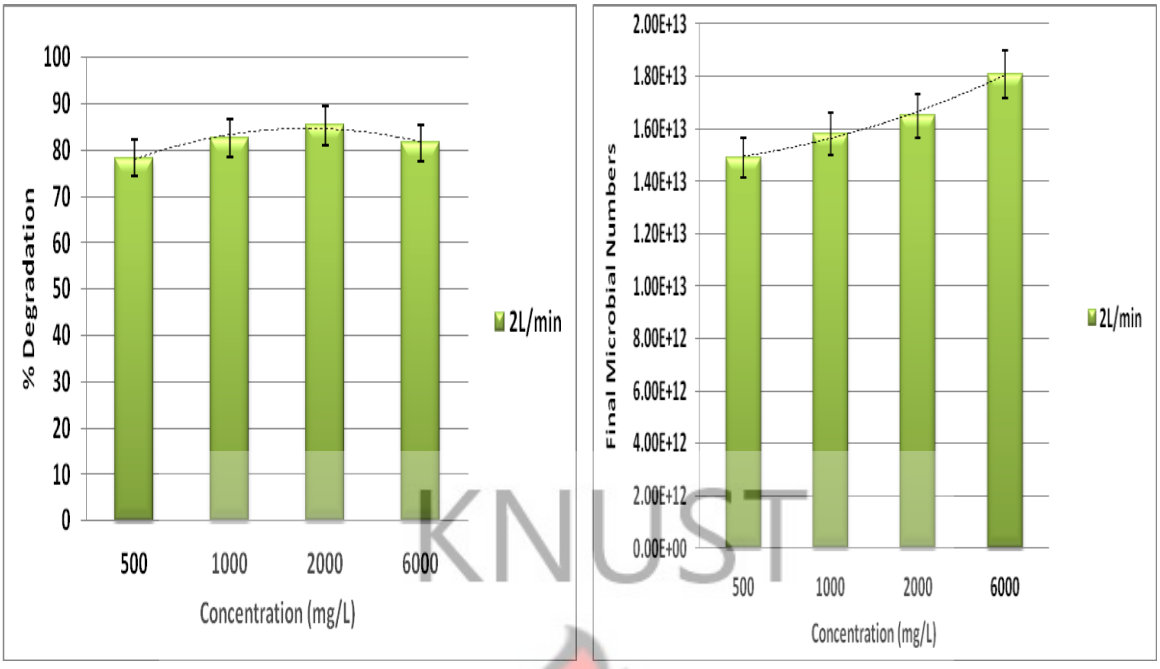
the same loading rate, minimum and maximum microbial populations of 1.47E+13±0.00 and 1.80E+13±0.00 occurred at oil concentrations of 6000 mg/L and 2000 mg/L respectively. An initial increase in microbial population density was observed until at a threshold concentration of 2000 mg/L microbial populations started declining (figure 4.1b).



a. Degradation at 1.0 L/min b. Microbial variations at 1.0 L/min

Figure 4.2 Profile for degradation rates (%) obtained with the corresponding microbial population densities at 1.0 L/min loading rate

With reference to figure 4.2a above, a general increase in degradation rate was attained for the various oil concentrations studied. Minimum and maximum degradation rates of 71.08±0.00 % and 93.85±0.00 % were achieved respectively for 500 mg/L and 6000 mg/L oil concentrations at the 1.0 L/min loading rate. A gradual increase in microbial population density was initially observed at this loading rate until at a threshold concentration of 1000 mg/L microbial populations began to decline gradually (figure 4.2b). Minimum and maximum microbial densities of 1.50E+13±0.00 and 1.79E+13±0.00 were obtained respectively at oil concentrations of 6000 mg/L and 1000 mg/L.



a. Degradation at 2.0 L/min b. Microbial variations at 2.0 L/min

Figure 4.3 Profile for degradation rates (%) obtained with the corresponding microbial population densities at 2.0 L/min loading rate

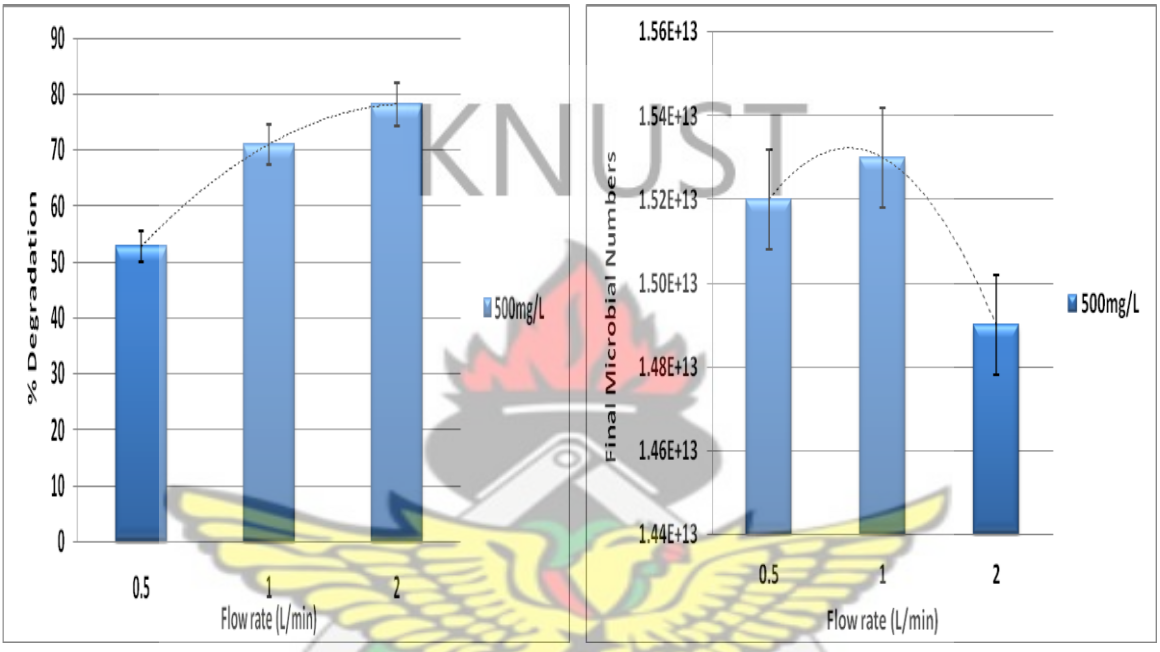
The 2.0 L/min loading rate saw a gradual increase in degradation rate and at a threshold concentration of 2000 mg/L, a gradual decrease was observed (figure 4.3a). A gradual increase in microbial population density was also observed (figure 4.3b). Minimum and maximum degradation rates of 78.26±0.00 % and 85.39±0.00 % corresponding to oil concentrations of 500 mg/L and 2000 mg/L were achieved at the above mentioned loading rate with minimum and maximum microbial population densities of 1.49E+13±0.00 and 1.81E+13±0.00 occurring when oil concentrations were 500 mg/L and 6000 mg/L respectively.

Table 4.1 Summarized results for degradation rates (%) obtained and the corresponding microbial population densities at the various oil concentrations

Loading rate (L/min)	Degradation (%)				Final Microbial Numbers			
	500 mg/L	1000 mg/L	2000 mg/L	6000 mg/L	500 mg/L	1000 mg/L	2000 mg/L	6000 mg/L
0.5	52.81	36.83	86.67	88.76	1.52E+13	1.53E+13	1.80E+13	1.47E+13
1.0	71.08	71.94	93.02	93.85	1.53E+13	1.79E+13	1.69E+13	1.50E+13
2.0	78.26	82.53	85.39	81.49	1.49E+13	1.58E+13	1.65E+13	1.81E+13

4.3 Effect of Loading Rate on Degradation of Hydrocarbons

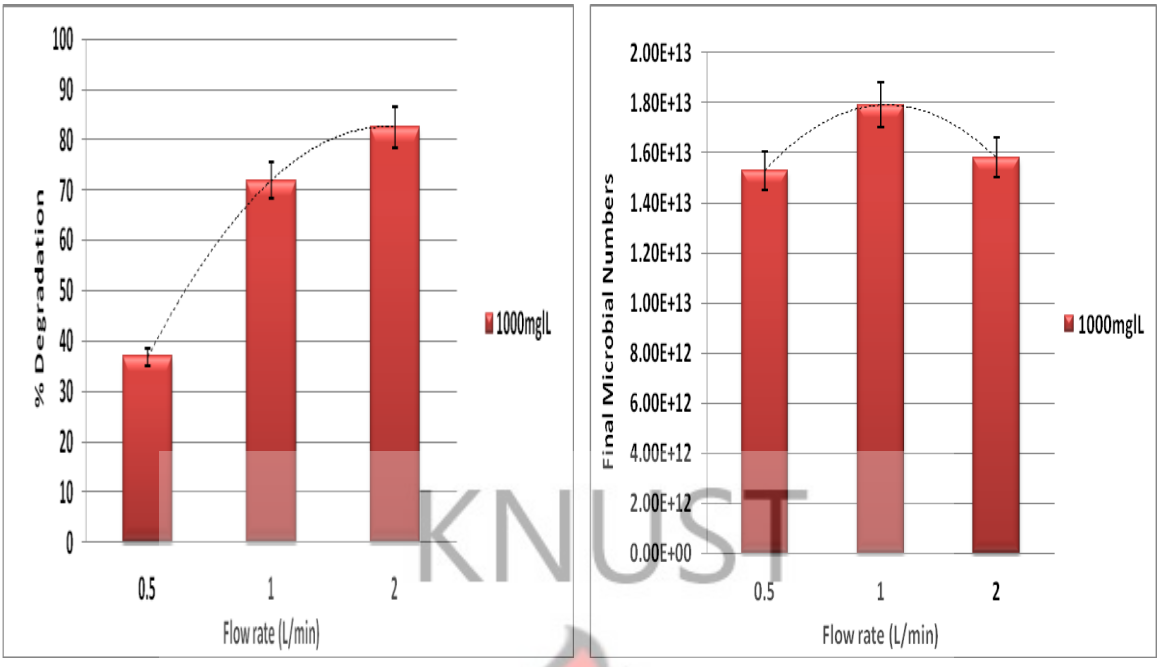
Profiles-4.4a, 4.5a, 4.6a and 4.7a below respectively represent the degradation rates attained at oil concentrations of 500 mg/L, 1000 mg/L, 2000 mg/L and 6000 mg/L for the various loading rates studied. Profiles-4.4b, 4.5b, 4.6b and 4.7b also respectively represent the corresponding microbial population densities at the above mentioned oil concentrations for the various loading rates.



a. Degradation at 500 mg/L concentration b. Microbial variations at 500 mg/L

Figure 4.4 Profile for degradation rates (%) achieved and the corresponding microbial population densities at 500 mg/L oil concentration

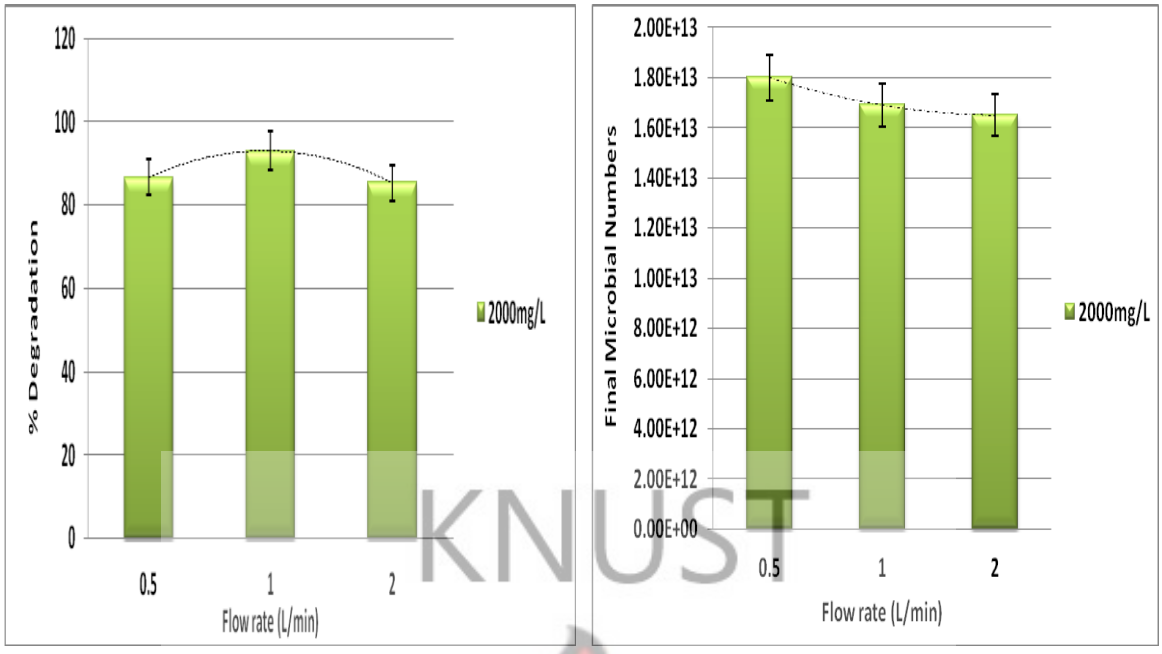
The 500 mg/L oil concentration study revealed a general increase in degradation rate at the various loading rates administered (figure 4.4a). However, the corresponding microbial population density profile (figure 4.4b) revealed an initial increase in microbial populations up to a threshold loading rate of 1.0 L/min beyond which there was a sharp decline in the microbial growth. Minimum and maximum degradation rates of 52.81±0.00 % and 78.26±0.00 % occurred respectively at loading rates of 0.5 L/min and 2.0 L/min with minimum and maximum microbial growths of 1.49E+13±0.00 and 1.53E+13±0.00 also occurring at 2.0 L/min and 1.0 L/min respectively.



a. Degradation at 1000 mg/L concentration b. Microbial variations at 1000 mg/L

Figure 4.5 Profile for degradation rates (%) achieved and the corresponding microbial population densities at 1000 mg/L oil concentration.

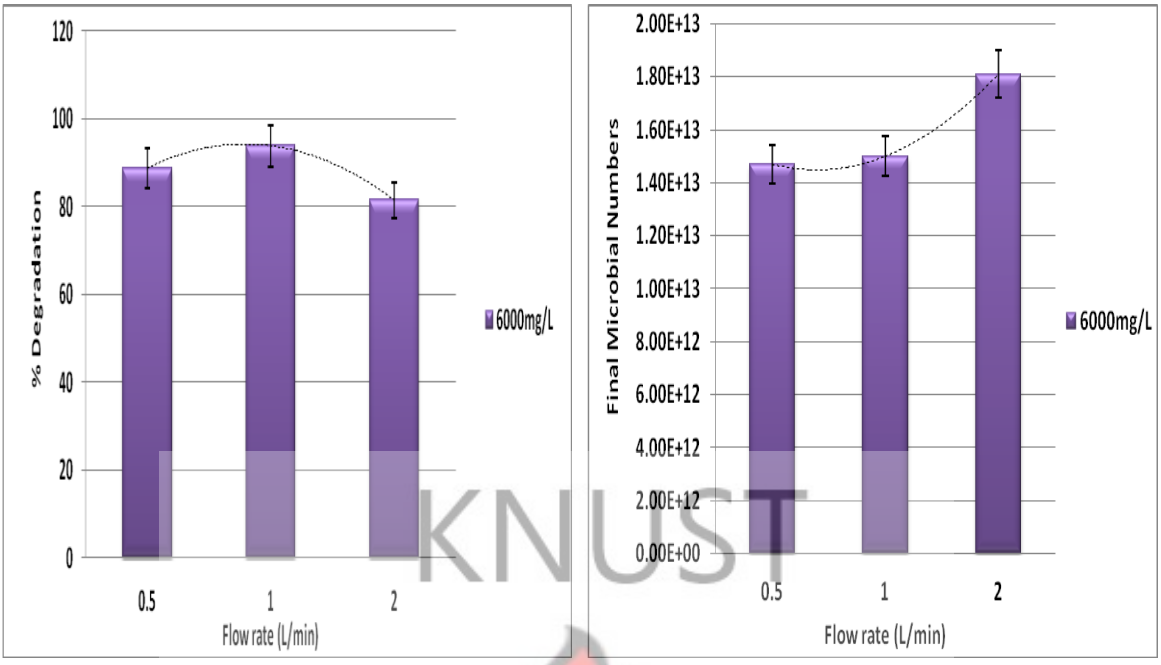
Similar to the 500 mg/L oil concentration study, the 1000 mg/L oil concentration study also saw a general increase in degradation rate at the various loading rates administered as can be inferred from figure 4.5a above. Minimum and maximum degradation rates of 36.83±0.00 % and 82.53±0.00 % occurred at loading rates of 0.5 L/min and 2.0 L/min respectively. The corresponding microbial population density profile (figure 4.5b) also revealed an initial increase in microbial population density up to a threshold loading rate of 1.0 L/min. Beyond this threshold, there was a decline in the microbial growth. A minimum microbial growth of 1.53E+13±0.00 occurred at a loading rate of 0.5 L/min with maximum microbial growth of 1.79E+13±0.00 also occurring at 1.0 L/min loading rate.



a. Degradation at 2000 mg/L concentration b. Microbial variations at 2000 mg/L

Figure 4.6 Profile for degradation rates (%) achieved and the corresponding microbial population densities at 2000 mg/L oil concentration

Minimum and and maximum degradation rates of 85.39±0.00 % and 93.02±0.00 % were observed respectively at loading rates of 2.0 L/min and 1.0 L/min during the 2000 mg/L oil concentration study (figure 4.6a). A gradual increase in degradation rate was observed initially. However, at a threshold loading rate of 1.0 L/min, a gradual decrease was observed. The corresponding microbial growth profile also revealed a gradual decrease in microbial density. Minimum and maximum microbial growths of 1.65E+13±0.00 and 1.80E+13±0.00 were recorded at loading rates of 0.5 L/min and 2.0 L/min respectively (figure 4.6b).



a. Degradation at 6000 mg/L concentration b. Microbial variations at 6000 mg/L

Figure 4.7 Profile for degradation rates (%) recorded and the corresponding microbial population densities at 6000 mg/L oil concentration

The 6000 mg/L oil concentration study also recorded minimum and and maximum degradation rates of 81.49±0.00 % and 93.85±0.00 % occurring respectively at loading rates of 2.0 L/min and 1.0 L/min (figure 4.7a). An initial gradual increase in degradation rate was observed. At a threshold loading rate of 1.0 L/min, a decline in degradation was recorded. The corresponding microbial growth profile recorded a gradual increase in microbial density with minimum and and maximum microbial growths of 1.47E+13±0.00 and 1.81E+13±0.00 occurring at loading rates of 0.5 L/min and 2.0 L/min respectively (figure 4.7b).

Table 4.2 Summarized results for degradation rates (%) achieved and the corresponding microbial population densities at the various loading rates

Concentration (mg/L)	Degradation (%)			Final Microbial Numbers		
	0.5 L/min	1.0 L/min	2.0 L/min	0.5 L/min	1.0 L/min	2.0 L/min
500	52.81	71.08	78.26	1.52E+13	1.53E+13	1.49E+13
1000	36.83	71.94	82.53	1.53E+13	1.79E+13	1.58E+13
2000	86.67	93.02	85.39	1.80E+13	1.69E+13	1.65E+13
6000	88.76	93.85	81.49	1.47E+13	1.50E+13	1.81E+13

4.4 Analysis of the Effect of Temperature, pH, Dissolved Oxygen (D.O) and Conductivity on Degradation

Table 4.3 Summarized results showing the relationship between monitoring parameters and degradation rates (%) achieved

Concentration (mg/L)	Temperature (°C)	pH	D.O (mg/L)	Conductivity (µS/cm)	Degradation (%)
500 (LR of 0.5 L/min)	28.06±0.67	7.32±0.11	2.80±0.70	0.29±0.02	52.81±0.00
500 (LR of 1.0 L/min)	29.90±0.41	7.15±0.10	2.30±0.06	0.23±0.01	71.08±0.00
500 (LR of 2.0 L/min)	28.73±0.56	7.11±0.37	3.44±1.21	0.23±0.01	78.26±0.00
1000 (LR of 0.5 L/min)	28.13±0.36	7.11±0.04	2.77±0.62	0.27±0.02	36.83±0.00
1000 (LR of 1.0 L/min)	29.74±0.47	7.08±0.18	2.29±0.47	0.23±0.02	71.94±0.00
1000 (LR of 2.0 L/min)	28.53±0.77	7.12±0.10	3.07±0.75	0.23±0.02	82.53±0.00
2000 (LR of 0.5 L/min)	27.26±0.76	6.97±0.22	2.76±0.69	0.21±0.01	86.67±0.00
2000 (LR of 1.0 L/min)	28.86±0.90	6.99±0.15	3.05±0.43	0.22±0.01	93.02±0.00
2000 (LR of 2.0 L/min)	28.28±0.19	6.76±0.08	3.61±1.33	0.22±0.02	85.39±0.00
6000 (LR of 0.5 L/min)	28.23±1.00	6.96±0.27	2.84±0.58	0.22±0.03	88.76±0.00
6000 (LR of 1.0 L/min)	28.94±0.78	6.75±0.45	3.55±1.14	0.21±0.04	93.85±0.00
6000 (LR of 2.0 L/min)	28.24±0.34	6.63±0.20	2.41±0.34	0.21±0.02	81.49±0.00

CHAPTER FIVE

5.0 DISCUSSION

5.1 Introduction

Petroleum contaminants in the environment are primarily biodegraded by bacteria and fungi, and these organisms appear to be ubiquitously distributed in aquatic and terrestrial ecosystems (Adebusoye *et al.*, 2006). Regarding aquatic ecosystems, several factors have been known to influence the microbial degradation of petroleum hydrocarbons (oil) and significant among these factors are the concentration of the oil contaminant in the water and the loading rate (flow rate) applied to the system under study.

5.2 Effect of Oil Concentration on Degradation of Hydrocarbons

The rate of uptake and mineralization of hydrocarbons by hydrocarbon-degrading microbial populations in aquatic environments is generally proportional to the concentration of the hydrocarbon contaminant present as suggested by Leahy & Colwell (1990). Thus petroleum hydrocarbon concentrations far in excess of the solubility limits would delay the onset of microbial degradation since the solubility determines in part the surface area of oil or hydrocarbon available for colonization by the hydrocarbon-degrading microbes (Atlas, 1981).

According to Leahy & Colwell (1990), high oil concentrations would be expected to inhibit the microbial degradation process in a number of ways namely through nutrient or oxygen limitation or through toxic effects exerted on hydrocarbon-degrading microbes by volatile hydrocarbon fractions.

The study revealed a general increase in degradation for the various oil concentrations studied at loading rates of 0.5 L/min, 1.0 L/min and 2.0 L/min (figures 4.1a, 4.2a, 4.3a). The general increase in degradation rate observed possibly suggests that oil concentrations in themselves were within solubility limits and hence a large surface area of the oil was available for colonization by the hydrocarbon-degrading microbes which perhaps were present in sufficient amounts to ensure degradation of the oil substrate. Hence the general increasing trends observed (as can be seen from the trendline patterns).

It was anticipated during the study that the multiple increases in oil concentration would significantly lower the rate of degradation of the oil substrate by way of its influence on

the hydrocarbon-degrading microbes. Despite the fact that oil concentrations were within solubility limits, this assertion was confirmed in a few instances.

The drastic decline in degradation rate when oil concentration was doubled from 500 to 1000 mg/L (figure 4.1a) could be explained in terms of the fact that hydrocarbon-degrading microbes in their quest to degrade the oil contaminant required some time to acclimatize or adapt their metabolism to be able to deal with the "shock" that resulted from the sudden doubling in oil concentration. Thus doubling of the oil concentration from 500 to 1000 mg/L possibly may have inhibited microbial activity either through nutrient or oxygen limitation or through toxic effects exerted by volatile hydrocarbon fractions. Subsequent higher degradation rates achieved suggest that hydrocarbon-degrading microbes probably became used to higher concentrations of the oil substrate or adjusted their metabolism to be able to deal with subsequent increases in oil concentration. The corresponding microbial population density when oil concentration was doubled from 500 to 1000 mg/L though high possibly did not reflect the actual amount of hydrocarbon-degrading microbes that were involved in the degradation process (figure 4.1b).

Similarly, the impact when oil concentration was tripled from 2000 to 6000 mg/L (figure 4.3a) could be explained in terms of its effects on the hydrocarbon-degrading microbes just as explained above. Statistical analysis revealed the differences in degradation rates for the various oil concentrations studied to be statistically not significant ($p>0.05$).

5.3 Effect of Loading Rate on Degradation of Hydrocarbons

The loading rate/flow rate of a system is another important parameter that exerts considerable influence on the microbial degradation of pollutants in water using a bioreactor. Microbial degradation of a soluble contaminant using a constructed bioreactor largely depends on the loading rate administered since the longer microorganisms interact with the substrate (pollutant), the better the degradation of the substrate and vice versa. Thus microbes can only interact with the pollutant/contaminant for a longer duration if and only if the loading rate administered is low enough (www.wikipedia.com/bioreactor). The loading rate in other words defines the contact time that microbes have at their disposal to interact with the substrate to be degraded.

Higher loading rates by way of their turbulent regimes were therefore expected to wash substantial amounts of the hydrocarbon-degrading microbes off the biofilm support material as well as reduce the contact time microbes have at their disposal to interact with the substrate to be degraded.

The study of the various loading rates of 0.5 L/min, 1.0 L/min and 2.0 L/min at concentrations of 500 and 1000 mg/L saw a general increase in the rate of uptake of hydrocarbons with maximum degradation rates occurring at the highest loading rate (figures 4.4a and 4.5a). The turbulent regime at the maximum loading rate of 2.0 L/min perhaps facilitated mixing of the oil, and water thereby enhancing the solubility and dispersion/emulsification of the oil in water. This obviously increased the surface area of oil available for colonization by the hydrocarbon-degrading microbes which were perhaps present in substantial amounts; hence the observed high rates of hydrocarbon uptake at the maximum loading rate. The microbial population density for the maximum loading rate though low possibly did not reflect the actual amount of hydrocarbon-degrading microbes that were involved in the degradation process (figures 4.4b and 4.5b)

The 2000 mg/L oil concentration study at the various loading rates also recorded an initial gradual increase in hydrocarbon uptake. At a threshold loading rate of 1.0L/min however, a decline in hydrocarbon uptake was observed (figure 4.6a). The highest microbial population density at this concentration was observed when loading rate was least (0.5 L/min). This possibly suggests that hydrocarbon-degrading microbes had ample time to interact with the oil substrate. This however did not translate into the respective degradation rate attained. It is therefore highly possible that the microbial population density as revealed by the microbial count results (figure 4.6b) did not truly reflect the actual amount of hydrocarbon-degrading microbes that were involved in the degradation process.

The 6000 mg/L oil concentration study just like the 2000 mg/L oil concentration study also saw an initial gradual increase in hydrocarbon uptake. Similarly at a threshold loading rate of 1.0L/min, a decline in hydrocarbon uptake was observed (figure 4.7a). However the highest microbial population density at this concentration occurred when loading rate was highest (2.0 L/min). This observation however did not correspond to the respective degradation rate achieved as the minimum degradation rate was attained at the

highest loading rate. Despite the fact that the turbulent regime at the maximum loading rate (2.0 L/min) may have facilitated mixing of the oil, and water, it is also possible that substantial amounts of the hydrocarbon-degrading microbes may have been washed off the biofilm support material. Thus the turbulent regime at the maximum loading rate (2.0 L/min) possibly reduced the contact time that microbes had at their disposal to interact with the oil substrate in the bioreactor (www.wikipedia.com/bioreactor). Differences in degradation rates for the various loading rates studied were not statistically significant ($p>0.05$).

5.4 Analysis of the Influence of Temperature, pH, Dissolved Oxygen and Conductivity on Degradation

The temperature readings recorded are attributable to the fluctuations in ambient temperature to which the set up was subjected (Table 4.3). As suggested by Leahy & Colwell, (1990), ambient temperature may have influenced the degradation of the oil by way of its influence on the physical nature of the oil and composition of the microbial community present. Temperature influence on the degradation and microbial community was not statistically significant ($p>0.05$).

Degradation in general occurred near neutral pH conditions. Slightly acidic conditions were observed at higher oil concentrations of 2000 and 6000 mg/L (Table 4.3). Slightly acidic conditions observed translated into higher degradation rates; an indication that microbes were more active at those concentrations making use of the oil as their sole carbon and energy source. Prescott *et al*. (2002), links slightly acidic conditions to the production of acidic intermediates. These acidic intermediates as it stands functioned to lower the pH of the system. Similarly, slightly basic conditions were observed at lower oil concentrations of 500 and 1000 mg/L. Statistical analysis however revealed that pH had no significant influence on degradation and the microbial population ($p>0.05$).

Although the amount of oxygen (mg/L) fed into the system from the source supply (air pump) was not known, dissolved oxygen levels recorded suggest that oxygen was present at all times to ensure the survival of aerobes in the aerobic bioreactors to undertake the degradation of the oil. Generally, higher dissolved oxygen levels translated into higher degradation rates at higher loading rates. Higher loading rates by way of their turbulent regimes or churning effect possibly enhanced the mixing and dissolution of supplied

oxygen in the oil contaminated water (Table 4.3). The influence of dissolved oxygen on degradation and the microbial population was not significant ($p>0.05$).

Higher degradation rates were achieved at low conductivities with lower degradation rates occurring at higher conductivities (Table 4.3). Waste engine oil by virtue of its use becomes contaminated with toxic metals such as magnesium, copper, zinc, lead, cadmium, in addition to by-products of combustion etc. from the wear and tear of the engine during operation (Irwin *et al*., 1997). These metals and combustion by-products possibly contributed to the conductivity readings recorded. Higher concentrations of these metals and combustion by-products possibly exerted their deleterious effects on hydrocarbon-degrading microbes; hence the lower degradation rates observed at higher conductivities. The influence of conductivity on degradation was found to be statistically significant ($p<0.05$). Conductivity however had no significant influence on the microbial population ($p>0.05$).

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

The experimental study conducted proved to be quite successful in confirming that mixed microbial consortium as a result of their diverse metabolic activities are potential degraders of hydrocarbon contaminants (used motor oil) in water. Employing the activities of these microbes in a constructed bioreactor to remove or degrade hydrocarbon contaminants (oil) thus proved to be an efficient technique in dealing with hydrocarbon contaminants in water.

Hydrocarbon-degrading microbes were successfully isolated for the study. Microbial count results generated showed an increase in microbial numbers throughout the study.

The parameters - total petroleum hydrocarbon (TPH), pH, temperature, dissolved oxygen (DO), conductivity and the final microbial populations measured to ascertain the progress of the microbial degradation of the oil contaminant proved to be good indicators for the investigation.

Hydrocarbon (oil) degradation actively occurred near neutral pH conditions, with slightly acidic conditions resulting in higher degradation rates. Higher dissolved oxygen levels and lower conductivities similarly translated into higher degradation rates.

Oil concentrations were found to be within solubility limits with higher degradation rates being achieved at higher oil concentrations. Loading rates also influenced degradation, with higher degradation rates occurring at higher loading rates and vice versa. The minimum degradation for the study occurred at the least loading rate with the maximum degradation also occurring at the highest oil concentration.

6.2 Recommendations

The following recommendations are being proposed for consideration:

- Further research could be conducted to compare the performance or efficiency of different biofilm supporting materials.
- Similar research or study could be carried out using fresh motor oil (diesel) and the results compared with that of the used oil.

- Feasibility studies on pilot application of this reactor model in dealing with issues of hydrocarbon contamination of water resources in parts of Ghana could be considered.
- The application of this technology in degrading pesticide contaminated water resources can also be looked at.

REFERENCES

Adebusoye S.A., Matthew O. I., Olukayode O.A., Olakunle D.T., Olatope S.O., (2006). "Microbial Degradation of Petroleum Hydrocarbons in Polluted Tropical Stream". The Journal of American Science, 2(3):48-57.

Adegoroye, G., (1997). "Environmental Considerations in Property Design, Urban Development and Renewal: In Akinjide O (Ed). Dimensions of Environmental Problems in Nigeria". Friedrich Ebert Foundation. pp: 12-25.

Adelowo, O.O., Alagbe, S. O., & Ayandele, A. A., (2006). "Time-dependent stability of used engine oil degradation by cultures of *Pseudomonas fragi* and *Achromobacter aerogenes*". African Journal of Biotechnology Vol. 5 (24), pp. 2476-2479.

Aitken, C. M., (2004). "Identification of Non-Hydrocarbon Metabolites of Deep Subsurface Anaerobic Petroleum Hydrocarbon Biodegradation". PhD thesis, Univ. Newcastle-upon-Tyne, UK.

Alexander, S.K., Schropp, S.J., & Schwarz, J.R., (1982). "Spatial and seasonal distribution of hydrocarbon-utilizing bacteria of sediment from the northwestern Gulf of Mexico". Contrib. Mar. Sci.25, 13–19.

Alvarez, P. J. J., & Vogel, T. M., (1991). "Substrate interactions of benzene, toluene, and para-xylene during microbial degradation by pure cultures and mixed culture aquifer slurries," *Applied and Environmental Microbiology*, vol. 57, no. 10, pp. 2981–2985.

Amro, M. M., (2004). "Treatment Techniques of Oil-Contaminated Soil and Water Aquifers", Presented at the International Conf. on Water Resources & Arid Environment, 2004.

American Public Health Association (APHA), (1992). "Standard methods for the examination of water and wastewater". 18th Edition. Washington, DC.

Atlas, R.M., (1981). "Biological Degradation of Petroleum Hydrocarbons: An Environmental Perspective". Microbiol. Rev. 45, pp.180-209.

Atlas, R. M., (1988). "Microbiology-fundamentals and applications", 2nd ed., p. 352-353. Macmillan Publishing Co., New York.

Atlas, R.M., & Bartha, R., (1992). "Hydrocarbon biodegradation and oil-spill bioremediation. In: Marshall, K.C. (Ed.), Advances in Microbial Ecology", Vol. 12, Plenum Press, New York, pp. 287–338.

Agency for Toxic Substances and Disease Registry (ATSDR), (1999). "Toxicological Profile for total petroleum hydrocarbons (TPH)." Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.

Agency for Toxic Substances and Disease Registry (ATSDR), (1997). Public Health Statements: Used Mineral Based Crankcase Oil.

Balba, M.T, Al-Daher, R., Al-Awadhi, N., Chino, H., & Tsuji, H., (1998a). "Bioremediation of Oil-Contaminated Desert Soil: The Kuwaiti Experience." Environment Institute, Vol. 24, No. 1/2, pp. 163-173.

Balba, M.T., Al-Awadhi, N., & Al-Daher, R., (1998b). "Bioremediation of oil-contaminated soil: microbiological methods for feasibility assessment and field evaluation". Journal of Microbiological Methods, Vol. 32, pp. 155-164.

Bartha, R. (1986). "Biotechnology of petroleum pollutant biodegradation." *Microbiol. Ecol*., 12, 155–172.

Bartha, R., & Atlas, R. M., (1977). "The microbiology of aquatic oil spills". Adv. Appl. Microbiol. 22:225-266.

Bossert, I., & Bartha, R., (1984). "The fate of petroleum in soil ecosystems, p. 434-476 .In R. M. Atlas (ed.), Petroleum microbiology". Macmillan Publishing Co., New York.

Braddock, J.F., Ruth, M.L., & Catterall, P.H., (1997). "Enhancement and inhibition of microbial activity in hydrocarbon-contaminated arctic soils: Implications for nutrient-amended bioremediation. Environmental Science and Technology", 31:2078-2084.

Braun, R.L. & Burnham, lan K., (1993). "Chemical Reaction Model for Oil and Gas Generation from Type I and Type II Kerogen". Lawrence Livermore National

Laboratory. (http://www.osti.gov/bridge/servlets/purl/10169154-cT5xip/10169154.PDF [Retrieved August, 29, 2010].

Chris C., (2007), "Implementing Phytoremediation of Petroleum Hydrocarbons, Methods in Biotechnology 23:99-108". Humana Press. ISBN 1588295419.

Colwell, R.E., & Walker, J.D., (1977). "Ecological aspects of microbial degradation of petroleum in the marine environment". Crit. Rev. Microbiol. 5, 423–445.

Cooney, J.J., & Summers, R.J., (1976). "Hydrocarbon-using microorganisms in three fresh water ecosystems. In: Sharpley, J.M. et al. (Eds.), Proceedings of the Third International Biodegradation Symposium, Applied Sciences", London, pp. 141–156.

Corsico, G., Mattei, L., Roselli, A. & Gommellini C., (1999), "Poly (internal olefins) – Synthetic Lubricants and high-performance functional fluids", Marcel Dekker, Chapter 2, p. 53-62, ISBN 0-8247-0194-1.

Diaz, E., (2008). "Microbial Biodegradation: Genomics and Molecular Biology", 1st Edition, Caister Academic Press. ISBN 978-1-904455-17-2 [http://www.horizonpress.com/biod].

Dibble, J. T., & Bartha. R., (1979). "Effect of environmental parameters on the biodegradation of oil sludge". Appl. Environ. Microbiol. 37:729-739.

Edewor, T.I., Adelowo, O.O., & Afolabi, T.J., (2004). "Preliminary Studies Into The Biological Activities Of A Broad Spectrum Disinfectant Formulated From Used Engine Oil". Poll. Res. 23(4): 581-586.

Environmental Research Institute, (1999). "Analysis of Extractable Total Petroleum Hydrocarbons (ETPH) Using Methylene Chloride: Gas Chromatograph/Flame Ionization Detection", University of Connecticut.

Faboya, O.O.P., (1997). "Industrial Pollution and Waste Management in Akinjide O (Ed). Dimensions of Environmental Problems in Nigeria", Friedrich Ebert Foundation. pp: 12-25.

Fei-Baffoe, B., (2003). "Biological degradation of fuel hydrocarbons in contaminated water". Unpublished M.Sc thesis, BTU-Cottbus.

Freeze, R. A., & Cherry, J. A., (1979), "Groundwater": Prentice-Hall, Inc.

Hach Company.,(1992). "Hach water analysis handbook". 2nd ed. Loveland, CO.

Hambrick, G. A., DeLaune R. D., and Patrick, W. H., Jr. (1980). "Effect of estuarine sediment pH and oxidation-reduction potential on microbial hydrocarbon degradation". Appl. Environ. Microbiol. 40:365-369.

Hanson, K.G., Nigam, A., Kapadia, M., & Desai, A., (1997). "Bioremediation of crude oil contamination with Acinetobacter sp." A3. Current Microbiol. 35, 191–193.

Holliger, C., Gaspard, S., Glod, G., Heijman, C., Schumacher, W., Schwarzenbach, R. P., & Vazquez, F., (1997). "Contaminated environments in the subsurface and bioremediation: organic contaminants," *FEMS Microbiology Reviews*, vol. 20, no. 3-4, pp. 517–523.

Irwin, R.J., VanMouwerik, M., Stevens, L., Seese, M.D., & Basham, W., (1997). "Environmental Contaminants Encyclopedia". National Park Service, Water Resources Division, Fort Collins, Colorado.

IUPAC Compendium of Chemical Terminology (1997). 2nd Edition, Volume: 2, Issue: Publisher: Blackwell Scientific Publications, Oxford.

Kvenvolden, K. A., & Cooper, C. K., (2003). "Natural seepage of crude oil into the marine environment," *Geo-Marine Letters*, vol. 23, no. 3-4, pp. 140–146.

Kvenvolden, K.A., (2006). "Organic geochemistry – A retrospective of its first 70 years". *Organic Geochemistry* **37**: 1. doi: 10.1016/j.orggeochem.2005.09.001.

Klamann, D., (1984) Lubricants and Related Products, Verlag Chemie, Weinheim, Deerfield Beach (Florida) and Basel, ISBN 0-89573- 177-0.

Kwaspisz, E., Wszelaka, J., Marchut, O., Bielecki, S., (2008). "The effect of nitrate and ammonium ions on kinetics of diesel oil degradation by *Gordonia alkanivorans S7*". International Biodeterioration & Biodegradation, Vol 61, Issue 3, 214-222.

Leahy, J.G., & Colwell, R.R., (1990). "Microbial degradation of hydrocarbons in the environment". Microbiol. Rev. 54, 305–315.

Levin, M. A. & Gealt, M. A., (1993). "Biotreatment of Industrial and Hazardous Waste": McGraw-Hill, Inc.

Maki, H., Sasaki, T., Haramaya, S., (2005). "Photo-oxidation of biodegradable crude oil and toxicity of the photo-oxidized products. Chemosph, 44, p. 1145-1151.

Mitchell, D.A., Krieger, N., & Berovic, M., (2006). "Solid-state fermentation bioreactors: fundamentals of design and operation. Springer, Germany.

MPL.com.au Available: <http://www.mpl.com.au/total-petroleum-hydrocarbons 93.htm> [Accessed June 10, 2011].

National Research Council, (1985). "Oil in the Sea: Inputs, Fates, and Effects." National Academy Press, Washington, D.C.

Nilanjana D., & Preethy C., (2010). "Microbial Degradation of Petroleum Hydrocarbon Contaminants: An Overview," Biotechnology Research International, vol. 2011, Article ID 941810, 13 pages, 2011. doi:10.4061/2011/941810.

Novick A., (1955). "Growth of Bacteria". Annual Review of Microbiology 9: 97–110. doi: 10.1146/annurev.mi.09.100155.000525. PMID 13259461.

Nyer, E.K., (1993). "Practical Techniques for Groundwater and Soil Remediation". CRC Press, Inc.

Okerentugba, P.O., & Ezeronye, O.U., (2003). "Petroleum Degrading Potentials of Single and Mixed Microbial Cultures Isolated From Rivers And Refinery Effluents In Nigeria". Afr. J. Biotechnol. Vol. 2(9): 293-295.

Okoh, A.I., (2006). "Biodegradation alternative in the cleanup of petroleum hydrocarbon pollutants". Biotechnology and Molecular Biology Review, Vol. 1 (2), 38-50.

Onwurah, I. N. E., Ogugua, V. N., Onyike, N. B. Ochonogor, A. E. & Otitoju, O. F., (2007). "Crude Oil Spills in the Environment, Effects and Some Innovative Clean-up

Biotechnologies." International Journal of Environmental Research, Vol. 1, No. 4, pp.307-320.

Prescott, L.M., Harley, J.P., Klein, D.E., (2002). "Microbiology." 5th Edition, McGraw Hill Inc., New York. p. 20-38, 96-132, 1012-1018.

Riser-Roberts, E., (1992). "Bioremediation of Petroleum Contaminated Sites": CRC Press, Inc.

Ryoo, D., Shim, H., Canada, K., Barbieri, P., Wood T. K., (2000). "Aerobic Degradation of Tetrachloroethylene by Toluene-o-xylene Monooxygenase of *Pseudomonas Stutzeri OX1"*. Nat Biotechnol, 18: 775-778.

Sarkhoh, N.A., Ghannoum, M.A., Ibrahim, A.S., Stretton, R.J., Radwan, S.S., (1990). "Crude oil and hydrocarbon degrading strains of Rhodococcus: Rhodococcus strains isolated from soil and marine environments in Kuwait". Environ. Pollut. 65, 1–18.

Shell International Ltd., (1983). "The chemistry of petroleum". In: The Petroleum Handbook", 6th ed., Elsevier, New York, pp.223–264.

Singer, M. E., & Finnerty, W. R., (1984). "Microbial metabolism of straight-chain and branched alkanes, p, 1-60. In R. M. Atlas (ed.), Petroleum microbiology". Macmillan Publishing Co., New York.

Soriano, A.U., & Pereira, N., (1998). "Oily sludge Biotreatment". Biochemistry Department of the Federal University of Rio de Janerio.

Swanell, R.P.J., & Head, I.M., (1994). "Bioremediation come of age". Nature 368, 396–397.

Todar K., (2008). "Online Textbook of Bacteriology" Available: <http://www.textbookofbacteriology.net>. [Accessed September 9, 2010].

United States Environmental Protection Agency (USEPA), (1996). "Recycling Used Oil: What Can You Do?" Cooperation Extension Services ENRI-317: 1-2.

Wikipedia.com/bioreactor. Available: <http://www.wikipedia.com/bioreactor>. [Accessed September 9, 2010].

Zhu, X., Venosa, A.D., Suidan, M.T., & Lee, K., (2001). "Guidelines for the Bioremediation of Marine Shorelines and Freshwater Wetlands", Report under a contract with Office of Research and Development, U.S. Environmental Protection Agency.

Zwietering M.H., Jongenburger I., Rombouts F.M., van 'T Riet K., (1990). "Modeling of the Bacterial Growth Curve". Applied and Environmental Microbiology 56 (6): 1875–1881. PMID 16348228.

APPENDICES

Introduction

Presented below are the tables for the various results generated in the course of the experimental study.

APPENDIX 1.0 TABLE OF RESULTS FOR MONITORING PARAMETERS (pH, Temperature, Dissolved Oxygen, Conductivity)

i) 500mg/L oil concentration @ 0.5L/minute flow rate

Date	pH			Temperature (^{o}C)		
	Point A	Point B	Average	Point A	Point B	Average
13/12/2010	7.30	7.34	7.32	28.40	28.60	28.50
15/12/2010	7.34	7.32	7.33	29.00	28.40	28.70
17/12/2010	7.46	7.44	7.45	27.80	27.80	27.80
19/12/2010	7.16	7.22	7.19	27.20	27.40	27.25

	Dissolved Oxygen (mg/L)			Conductivity (µS/cm)		
	Point A	Point B	Average	Point A	Point B	Average
13/12/2010	2.41	3.13	2.77	0.32	0.30	0.31
15/12/2010	2.47	2.43	2.45	0.29	0.29	0.29
17/12/2010	3.92	3.88	3.90	0.29	0.27	0.28
19/12/2010	2.03	2.09	2.06	0.26	0.28	0.27

ii) 1000mg/L oil concentration @ 0.5L/minute flow rate

Date	pH			Temperature (°C)		
	Point A	Point B	Average	Point A	Point B	Average
20/12/2010	7.08	7.10	7.09	28.60	28.50	28.55
22/12/2010	7.14	7.10	7.12	27.60	27.80	27.70
24/12/2010	7.15	7.17	7.16	28.30	28.20	28.25
26/12/2010	7.10	7.06	7.08	28.10	27.90	28.00

	Dissolved Oxygen (mg/L)			Conductivity (µS/cm)		
	Point A	Point B	Average	Point A	Point B	Average
20/12/2010	3.41	3.49	3.45	0.26	0.22	0.24
22/12/2010	1.85	2.25	2.05	0.30	0.28	0.29
24/12/2010	2.31	2.69	2.50	0.24	0.26	0.25
26/12/2010	3.25	2.89	3.07	0.29	0.27	0.28

iii) 2000mg/L oil concentration @ 0.5L/minute flow rate

Date	pH			Temperature (°C)		
	Point A	Point B	Average	Point A	Point B	Average
17/01/2011	6.50	6.80	6.65	27.50	27.20	27.35
19/01/2011	7.14	7.16	7.15	27.80	28.20	28.00
21/01/2011	7.04	6.98	7.01	27.60	27.40	27.50
23/01/2011	7.17	6.99	7.08	26.50	25.90	26.20

	Dissolved Oxygen (mg/L)			Conductivity (µS/cm)		
	Point A	Point B	Average	Point A	Point B	Average
17/01/2011	2.11	2.19	2.15	0.22	0.20	0.21
19/01/2011	2.79	2.77	2.78	0.21	0.19	0.20
21/01/2011	2.41	2.39	2.40	0.21	0.23	0.22
23/01/2011	3.78	3.66	3.72	0.19	0.21	0.20

iv) 6000mg/L oil concentration @ 0.5L/minute flow rate

Date	pH			Temperature (°C)		
	Point A	Point B	Average	Point A	Point B	Average
07/03/2011	6.65	6.53	6.59	29.40	29.60	29.50
09/03/2011	7.21	7.25	7.23	27.20	27.60	27.40
11/03/2011	7.05	7.11	7.08	27.50	27.40	27.45
13/03/2011	7.16	6.74	6.95	28.40	28.70	28.55
	Dissolved Oxygen (mg/L)			Conductivity (µS/cm)		
	Point A	Point B	Average	Point A	Point B	Average
07/03/2011	3.60	3.70	3.65	0.18	0.20	0.19
09/03/2011	2.63	2.61	2.62	0.25	0.21	0.23
11/03/2011	2.78	2.80	2.79	0.20	0.22	0.21
13/03/2011	2.26	2.34	2.30	0.24	0.26	0.25

v) 500mg/L oil concentration @ 1.0L/minute flow rate

Date	pH			Temperature (°C)		
	Point A	Point B	Average	Point A	Point B	Average
24/01/2011	7.19	7.31	7.25	30.80	29.90	30.35
26/01/2011	7.01	7.13	7.07	29.30	29.70	29.50
28/01/2011	7.03	7.09	7.06	29.40	29.80	29.60
30/01/2011	7.32	7.14	7.23	30.10	30.18	30.14
	Dissolved Oxygen (mg/L)			Conductivity (µS/cm)		
	Point A	Point B	Average	Point A	Point B	Average
24/01/2011	2.23	2.37	2.30	0.23	0.21	0.22
26/01/2011	2.37	2.41	2.39	0.26	0.24	0.25
28/01/2011	2.40	2.10	2.25	0.22	0.24	0.23
30/01/2011	2.40	2.14	2.27	0.21	0.25	0.23

vi) 1000mg/L oil concentration @ 1.0L/minute flow rate

Date	pH			Temperature (^{o}C)		
	Point A	Point B	Average	Point A	Point B	Average
31/01/2011	7.00	7.08	7.04	30.50	29.94	30.22
02/02/2011	7.01	6.95	6.98	30.10	30.00	30.05
04/02/2011	6.94	6.96	6.95	29.20	29.30	29.25
06/02/2011	7.37	7.33	7.35	29.60	29.30	29.45
	Dissolved Oxygen (mg/L)			Conductivity (µS/cm)		
	Point A	Point B	Average	Point A	Point B	Average
31/01/2011	3.01	2.97	2.99	0.21	0.19	0.20
02/02/2011	2.01	2.11	2.06	0.21	0.21	0.21
04/02/2011	2.00	2.06	2.03	0.21	0.27	0.24
06/02/2011	2.05	2.11	2.08	0.25	0.25	0.25

vii) 2000mg/L oil concentration @ 1.0L/minute

Date	pH			Temperature (^{o}C)		
	Point A	Point B	Average	Point A	Point B	Average
07/02/2011	7.27	7.03	7.15	29.0	28.60	28.80
09/02/2011	7.09	7.01	7.05	30.4	29.60	30.00
11/02/2011	7.00	6.62	6.81	28.7	26.90	27.80
13/02/2011	6.91	6.99	6.95	28.9	28.80	28.85
	Dissolved Oxygen (mg/L)			Conductivity (µS/cm)		
	Point A	Point B	Average	Point A	Point B	Average
07/02/2011	3.42	2.86	3.14	0.21	0.19	0.20
09/02/2011	2.50	2.38	2.44	0.22	0.24	0.23
11/02/2011	3.24	3.14	3.19	0.24	0.20	0.22
13/02/2011	3.40	3.46	3.43	0.21	0.25	0.23

viii) 6000mg/L oil concentration @ 1.0L/minute flow rate

Date	pH			Temperature (^oC)		
	Point A	Point B	Average	Point A	Point B	Average
14/03/2011	6.34	6.99	6.67	28.00	28.90	28.45
16/03/2011	7.28	7.32	7.30	30.20	30.00	30.10
18/03/2011	6.21	6.19	6.20	28.20	28.80	28.50
20/03/2011	6.98	6.70	6.84	28.70	28.70	28.70
	Dissolved Oxygen (mg/L)			Conductivity (µS/cm)		
	Point A	Point B	Average	Point A	Point B	Average
14/03/2011	3.22	3.38	3.30	0.17	0.17	0.17
16/03/2011	2.09	2.01	2.05	0.25	0.25	0.25
18/03/2011	4.02	4.48	4.25	0.22	0.26	0.24
20/03/2011	4.80	4.38	4.59	0.18	0.20	0.19

ix) 500mg/L oil concentration @ 2.0L/minute flow rate

Date	pH			Temperature (^oC)		
	Point A	Point B	Average	Point A	Point B	Average
21/03/2011	6.60	6.52	6.56	28.00	27.80	27.90
23/03/2011	7.24	7.28	7.26	28.50	29.50	29.00
25/03/2011	7.30	7.34	7.32	28.60	29.20	28.90
27/03/2011	7.26	7.34	7.30	29.00	29.22	29.11
	Dissolved Oxygen (mg/L)			Conductivity (µS/cm)		
	Point A	Point B	Average	Point A	Point B	Average
21/03/2011	2.60	3.00	2.80	0.23	0.21	0.22
23/03/2011	2.93	2.97	2.95	0.27	0.21	0.24
25/03/2011	2.61	2.89	2.75	0.22	0.22	0.22
27/03/2011	5.40	5.08	5.24	0.25	0.23	0.24

x) 1000mg/L oil concentration @ 2.0L/minute flow rate

Date	pH			Temperature (^oC)		
	Point A	Point B	Average	Point A	Point B	Average
21/02/2011	7.09	7.01	7.05	29.80	29.40	29.60
23/02/2011	7.13	7.17	7.15	28.40	28.60	28.50
25/02/2011	7.40	7.10	7.25	28.00	28.40	28.20
27/02/2011	6.94	7.10	7.02	27.60	28.00	27.80

	Dissolved Oxygen (mg/L)			Conductivity (µS/cm)		
	Point A	Point B	Average	Point A	Point B	Average
21/02/2011	2.11	2.49	2.30	0.21	0.21	0.21
23/02/2011	2.96	3.02	2.99	0.26	0.24	0.25
25/02/2011	2.87	2.89	2.88	0.23	0.21	0.22
27/02/2011	4.08	4.12	4.10	0.21	0.23	0.22

xi) 2000mg/L oil concentration @ 2.0L/minute flow rate

Date	pH			Temperature (^oC)		
	Point A	Point B	Average	Point A	Point B	Average
28/02/2011	6.78	6.90	6.84	28.20	28.60	28.40
02/03/2011	6.70	6.80	6.75	28.50	28.30	28.40
04/03/2011	6.45	6.85	6.65	27.60	28.40	28.00
06/03/2011	6.85	6.73	6.79	27.90	28.70	28.30

	Dissolved Oxygen (mg/L)			Conductivity (µS/cm)		
	Point A	Point B	Average	Point A	Point B	Average
28/02/2011	2.56	2.54	2.55	0.25	0.23	0.24
02/03/2011	2.40	2.48	2.44	0.20	0.20	0.20
04/03/2011	5.18	5.12	5.15	0.21	0.23	0.22
06/03/2011	4.15	4.45	4.30	0.22	0.22	0.22

xii) 6000mg/L oil concentration @ 2.0L/minute flow rate

Date	pH			Temperature (^{o}C)		
	Point A	Point B	Average	Point A	Point B	Average
21/03/2011	6.90	6.70	6.80	28.60	28.10	28.35
23/03/2011	6.78	6.78	6.78	27.60	28.00	27.80
25/03/2011	6.58	6.54	6.56	27.60	28.80	28.20
27/03/2011	6.38	6.36	6.37	28.50	28.74	28.62
	Dissolved Oxygen (mg/L)			Conductivity (µS/cm)		
	Point A	Point B	Average	Point A	Point B	Average
21/03/2011	2.50	2.90	2.70	0.20	0.22	0.21
23/03/2011	2.32	2.08	2.20	0.25	0.23	0.24
25/03/2011	1.97	2.13	2.05	0.20	0.18	0.19
27/03/2011	2.58	2.80	2.69	0.20	0.20	0.20

APPENDIX 2.0 TABLE OF RESULTS FOR MONITORING PARAMETERS

(Total Petroleum Hydrocarbon)

Concentration (mg/L)	Flow Rate (L/min)	Total Petroleum Hydrocarbon (TPH) SAMPLE (µg/ml)	CONTROL (µg/ml)	Degradation (%)
500	0.5	699.528	330.080	52.81
500	1.0	699.528	202.274	71.08
500	2.0	699.528	152.049	78.26
1000	0.5	924.400	583.965	36.83
1000	1.0	924.400	259.381	71.94
1000	2.0	924.400	161.480	82.53
2000	0.5	2284.079	304.417	86.67
2000	1.0	2284.079	159.407	93.02
2000	2.0	2284.079	333.669	85.39
6000	0.5	4840.571	543.971	88.76
6000	1.0	4840.571	297.899	93.85
6000	2.0	4840.571	896.108	81.49

APPENDIX 3.0 MICROBIAL COUNT RESULTS FOR MICROBIAL POPULATIONS PRESENT AT THE START OF EACH RUN

DILUTIONS

Date	Concentration (mg/L)	Flow Rate (L/min)	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}	Microbial Count (CFU/ml)
12/12/2010	500	0.5	84	92	52	36	32	60	1.06E+13
19/12/2010	1000	0.5	100	56	96	44	36	44	8.02E+12
16/01/2011	2000	0.5	96	116	100	46	80	60	1.21E+13
06/03/2011	6000	0.5	116	52	44	28	92	56	1.02E+13
23/01/2011	500	1.0	92	80	32	44	56	56	1.03E+13
30/01/2011	1000	1.0	104	112	72	120	96	60	1.18E+13
06/02/2011	2000	1.0	68	88	64	60	52	52	9.65E+12
13/03/2011	6000	1.0	72	132	192	40	44	60	1.08E+13
13/02/2011	500	2.0	76	72	44	52	40	56	1.01E+13
20/02/2011	1000	2.0	84	56	36	44	48	48	8.88E+12
27/02/2011	2000	2.0	92	52	124	92	64	44	8.58E+12
20/03/2011	6000	2.0	88	44	40	56	60	48	9.10E+12

APPENDIX 4.0 MICROBIAL COUNT RESULTS FOR SAMPLES TAKEN DURING THE DEGRADATION PROCESS

DILUTIONS

Date	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}	Microbial Count (CFU/ml)
13/12/2010	172	136	104	84	44	60	1.09E+13
15/12/2010	148	124	100	68	124	92	1.75E+13
17/12/2010	212	184	112	88	96	56	1.11E+13
19/12/2010	104	84	76	64	64	84	1.52E+13

20/12/2010	120	84	76	64	88	52	1.03E+13
22/12/2010	204	184	104	76	84	64	1.22E+13
24/12/2010	212	196	140	104	48	96	1.70E+13
26/12/2010	156	124	108	64	112	80	1.53E+13
17/01/2011	152	92	116	76	96	76	1.44E+13
19/01/2011	184	112	124	64	56	88	1.57E+13
21/01/2011	212	124	96	76	100	116	2.11E+13
23/01/2011	188	164	112	84	108	96	1.80E+13
07/03/2011	140	68	108	120	56	64	1.18E+13
09/03/2011	148	176	140	76	116	72	1.41E+13
11/03/2011	124	56	68	48	84	52	1.02E+13
13/03/2011	124	96	88	80	36	84	1.47E+13

DILUTIONS

Date	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}	Microbial Count (CFU/ml)
24/01/2011	104	84	132	112	104	92	1.73E+13
26/01/2011	116	80	116	104	100	88	1.65E+13
28/01/2011	92	68	120	128	96	76	1.52E+13
30/02/2011	112	180	136	88	108	80	1.53E+13
31/01/2011	184	208	108	236	76	120	2.17E+13
02/02/2011	216	144	136	164	80	148	2.63E+13
04/02/2011	144	104	72	168	84	104	1.90E+13
06/02/2011	168	112	132	68	108	96	1.79E+13
07/02/2011	88	140	60	48	104	48	9.83E+12
09/02/2011	92	76	80	52	132	68	1.36E+13
11/02/2011	96	88	72	44	124	64	1.28E+13
13/02/2011	76	80	68	48	48	96	1.69E+13
14/03/2011	68	84	116	68	56	80	1.44E+13
16/03/2011	96	56	76	56	72	68	1.26E+13
18/03/2011	124	44	56	68	44	76	1.35E+13
20/03/2011	224	216	52	44	52	84	1.50E+13

DILUTIONS

Date	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}	Microbial Count (CFU/ml)
14/02/2011	168	116	248	56	80	100	1.81E+13
16/02/2011	188	84	156	64	68	96	1.73E+13
18/02/2011	228	56	212	88	208	84	1.76E+13
20/02/2011	136	204	108	204	190	68	1.49E+13
21/02/2011	56	52	64	44	108	52	1.06E+13
23/02/2011	48	76	60	48	36	44	8.02E+12
25/02/2011	48	60	96	52	64	48	9.17E+12
27/02/2011	92	56	124	52	64	88	1.58E+13
28/02/2011	156	60	56	64	52	48	8.98E+12
02/03/2011	96	76	72	52	64	56	1.05E+13
04/03/2011	76	52	48	44	116	88	1.67E+13
06/03/2011	60	56	48	60	104	88	1.65E+13
21/03/2011	76	108	48	32	96	56	1.10E+13
23/03/2011	121	96	80	48	36	64	1.14E+13
25/03/2011	144	112	36	136	108	84	1.60E+13
27/03/2011	216	36	64	80	116	96	1.81E+13

APPENDIX 5.0 **TABLE OF RESULTS FOR PERCENTAGE INCREASE IN MICROBIAL NUMBERS**

Concentration (mg/L)	Flow Rate (L/min)	Initial Microbial Numbers (CFU/ml)	Final Microbial Numbers (CFU/ml)	% Increase in Microbial Numbers
500	0.5	1.06E+13	1.52E+13	43.40
1000	0.5	8.02E+12	1.53E+13	90.77
2000	0.5	1.21E+13	1.80E+13	48.76
6000	0.5	1.02E+13	1.47E+13	44.12
500	1.0	1.03E+13	1.53E+13	48.54
1000	1.0	1.18E+13	1.79E+13	51.69
2000	1.0	9.65E+12	1.69E+13	75.13
6000	1.0	1.08E+13	1.50E+13	38.89
500	2.0	1.01E+13	1.49E+13	47.52
1000	2.0	8.88E+12	1.58E+13	77.93
2000	2.0	8.58E+12	1.65E+13	92.31
6000	2.0	9.10E+12	1.81E+13	98.90

APPENDIX 6.0 **TABLE OF RESULTS FOR THE TURBIDITY OF LIQUID CULTURES (INOCULUM)**

Date	Concentration (mg/L)	Flow Rate (L/min)	Turbidity (FTU)
06/12/2010	500	0.5	135
08/12/2010	500	0.5	286
10/12/2010	500	0.5	330
12/12/2010	500	0.5	409
13/12/2010	1000	0.5	125
15/12/2010	1000	0.5	236
17/12/2010	1000	0.5	320
19/12/2010	1000	0.5	395
20/12/2010	2000	0.5	156
22/12/2010	2000	0.5	298
24/12/2010	2000	0.5	394
26/12/2010	2000	0.5	760
28/02/2011	6000	0.5	115
02/03/2011	6000	0.5	200
04/03/2011	6000	0.5	293
06/03/2011	6000	0.5	391

Date	Concentration (mg/L)	Flow Rate (L/min)	Turbidity (FTU)
17/01/2011	500	1.0	202
19/01/2011	500	1.0	297
21/01/2011	500	1.0	341
23/01/2011	500	1.0	395
24/01/2011	1000	1.0	220
26/01/2011	1000	1.0	250
28/01/2011	1000	1.0	265
30/01/2011	1000	1.0	280
31/01/2011	2000	1.0	66
02/02/2011	2000	1.0	151
04/02/2011	2000	1.0	250
06/02/2011	2000	1.0	309
07/03/2011	6000	1.0	71
09/03/2011	6000	1.0	96
11/03/2011	6000	1.0	166
13/03/2011	6000	1.0	210

Date	Concentration (mg/L)	Flow Rate (L/min)	Turbidity (FTU)
07/02/2011	500	2.0	105
09/02/2011	500	2.0	196
11/02/2011	500	2.0	270
13/02/2011	500	2.0	375
14/02/2011	1000	2.0	68
16/02/2011	1000	2.0	104
18/02/2011	1000	2.0	180
20/02/2011	1000	2.0	255
21/02/2011	2000	2.0	60
23/02/2011	2000	2.0	106
25/02/2011	2000	2.0	191
27/02/2011	2000	2.0	270
14/03/2011	6000	2.0	72
16/03/2011	6000	2.0	128
18/03/2011	6000	2.0	191
20/03/2011	6000	2.0	232

APPENDIX 7.0 STATISTICAL ANALYSIS RESULTS

Descriptive Statistics: pH, Temperature (^{o}C), Dissolved Oxygen (D.O) (mg/L), Conductivity (µS/cm)

Results for Oil concentration = 500 mg/L

Variable	Loading Rate	Mean	StDev
pH	0.5 L/minute	7.3225	0.1063
	1.0 L/minute	7.1525	0.1014
	2.0 L/minute	7.1100	0.3680
Temperature (^{o}C)	0.5 L/minute	28.063	0.665
	1.0 L/minute	29.898	0.412
	2.0 L/minute	28.727	0.558
Dissolved Oxygen (mg/L)	0.5 L/minute	2.7950	0.7920
	1.0 L/minute	2.3025	0.0618
	2.0 L/minute	3.4350	1.2060
Conductivity (µS/cm)	0.5 L/minute	0.28750	0.01708
	1.0 L/minute	0.23250	0.01258
	2.0 L/minute	0.23000	0.01155

Results for Oil concentration = 1000 mg/L

Variable	Loading Rate	Mean	StDev
pH	0.5 L/minute	7.1125	0.0359
	1.0 L/minute	7.0800	0.1838
	2.0 L/minute	7.1175	0.1044
Temperature (^{o}C)	0.5 L/minute	28.125	0.362
	1.0 L/minute	29.743	0.466
	2.0 L/minute	28.525	0.772
Dissolved Oxygen (mg/L)	0.5 L/minute	2.768	0.617
	1.0 L/minute	2.290	0.467
	2.0 L/minute	3.067	0.752
Conductivity (µS/cm)	0.5 L/minute	0.2650	0.02380
	1.0 L/minute	0.2250	0.02380
	2.0 L/minute	0.2250	0.01732

Results for Oil concentration = 2000 mg/L

Variable	Loading Rate	Mean	StDev
pH	0.5 L/minute	6.9730	0.2220
	1.0 L/minute	6.9900	0.1451
	2.0 L/minute	6.7575	0.0806
Temperature (^{o}C)	0.5 L/minute	27.262	0.761
	1.0 L/minute	28.862	0.899
	2.0 L/minute	28.275	0.189
Dissolved Oxygen (mg/L)	0.5 L/minute	2.763	0.689
	1.0 L/minute	3.050	0.426
	2.0 L/minute	3.610	1.334
Conductivity (µS/cm)	0.5 L/minute	0.20750	0.00957
	1.0 L/minute	0.22000	0.01414
	2.0 L/minute	0.22000	0.01633

Results for Oil concentration = 6000 mg/L

Variable	Loading Rate	Mean	StDev
pH	0.5 L/minute	6.962	0.273
	1.0 L/minute	6.752	0.454
	2.0 L/minute	6.628	0.203
Temperature (^{o}C)	0.5 L/minute	28.225	1.002
	1.0 L/minute	28.938	0.782
	2.0 L/minute	28.243	0.342

Dissolved Oxygen (mg/L)	0.5 L/minute	2.840	0.577
	1.0 L/minute	3.547	1.138
	2.0 L/minute	2.410	0.335
Conductivity (µS/cm)	0.5 L/minute	0.2200	0.0258
	1.0 L/minute	0.2125	0.0386
	2.0 L/minute	0.2100	0.0216

Regression Analysis: pH, Temperature (^{o}C), Dissolved Oxygen (mg/L), Conductivity (µS/cm), Microbial Population versus Degradation (%)

Summary Output: Degradation vs. pH

Regression Statistics	
Multiple R	0.535825876
R Square	0.28710937
Adjusted R Square	0.215820307
Standard Error	14.99573234
Observations	12

ANOVA

	df	SS	MS	F	Significance F
Regression	1	905.6488066	905.6488	4.02739715	0.072556332
Residual	10	2248.719885	224.872		
Total	11	3154.368692			

Summary Output: Degradation vs. Temperature

Regression Statistics	
Multiple R	0.04798344
R Square	0.00230241
Adjusted R Square	-0.0974673
Standard Error	17.7400847
Observations	12

ANOVA

	df	SS	MS	F	Significance F
Regression	1	7.262651636	7.262652	0.023077	0.882277507
Residual	10	3147.10604	314.7106		
Total	11	3154.368692			

Summary Output: Degradation vs. D.O

Regression Statistics	
Multiple R	0.3668619
R Square	0.1345876
Adjusted R Square	0.0480464
Standard Error	16.522196
Observations	12

ANOVA

	df	SS	MS	F	Significance F
Regression	1	424.5389987	424.539	1.555185	0.240788296
Residual	10	2729.829693	272.983		
Total	11	3154.368692			

Summary Output: Degradation vs. Conductivity

Regression Statistics	
Multiple R	0.861545
R Square	0.742259
Adjusted R Square	0.716485
Standard Error	9.016708
Observations	12

ANOVA

	df	SS	MS	F	Significance F
Regression	1	2341.358	2341.358	28.79863	0.000316124
Residual	10	813.0103	81.30103		
Total	11	3154.369			

Summary Output: Degradation vs. Microbial Numbers

Regression Statistics	
Multiple R	0.22786351
R Square	0.05192178
Adjusted R Square	-0.04288604
Standard Error	1.3217E+12
Observations	12

ANOVA

	df	SS	MS	F	Significance F
Regression	1	9.56745E+23	9.57E+23	0.5476529	0.476293194
Residual	10	1.74699E+25	1.75E+24		
Total	11	1.84267E+25			

Regression Analysis: pH, Temperature (^{o}C), Dissolved Oxygen (mg/L), Conductivity (µS/cm) versus Microbial Population

Summary Output: Microbial Numbers vs. pH

Regression Statistics	
Multiple R	0.358450177
R Square	0.12848653
Adjusted R Square	0.041335183
Standard Error	1.26724E+12
Observations	12

ANOVA

	df	SS	MS	F	Significance F
Regression	1	2.36758E+24	2.37E+24	1.474292	0.252564983
Residual	10	1.60591E+25	1.61E+24		
Total	11	1.84267E+25			

Summary Output: Microbial Numbers vs. Temperature

Regression Statistics	
Multiple R	0.12124788
R Square	0.01470105
Adjusted R Square	-0.0838288
Standard Error	1.3474E+12
Observations	12

ANOVA

	df	SS	MS	F	Significance F
Regression	1	2.70891E+23	2.70891E+23	0.149204	0.707388441
Residual	10	1.81558E+25	1.81558E+24		
Total	11	1.84267E+25			

Summary Output: Microbial Numbers vs. D.O

Regression Statistics	
Multiple R	0.3979241
R Square	0.1583436
Adjusted R Square	0.0741779
Standard Error	1.245E+12
Observations	12

ANOVA

	df	SS	MS	F	Significance F
Regression	1	2.91774E+24	2.92E+24	1.881333	0.200170857
Residual	10	1.55089E+25	1.55E+24		
Total	11	1.84267E+25			

Summary Output: Microbial Numbers vs. Conductivity

Regression Statistics	
Multiple R	0.4492238
R Square	0.201802
Adjusted R Square	0.1219822
Standard Error	1.213E+12
Observations	12

ANOVA

	df	SS	MS	F	Significance F
Regression	1	3.71854E+24	3.72E+24	2.52822	0.142910398
Residual	10	1.47081E+25	1.47E+24		
Total	11	1.84267E+25			

ANALYSIS OF VARIANCE

Anova: Single Factor for Degradation with respect to Oil Concentration at the various Loading Rates

SUMMARY

Groups	Count	Sum	Average	Variance
0.5 L/min	4	265.07	66.2675	656.6151
1.0 L/min	4	329.89	82.4725	160.4733
2.0 L/min	4	327.67	81.9175	8.664492

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	677.1101	2	338.555	1.229987	0.337105	4.256495
Within Groups	2477.259	9	275.251			
Total	3154.369	11				

Anova: Single Factor for Degradation with respect to Loading Rate at the various Oil Concentrations

SUMMARY

Groups	Count	Sum	Average	Variance
500 mg/L	3	202.15	67.38333	172.1746
1000 mg/L	3	191.3	63.76667	572.225
2000 mg/L	3	265.08	88.36	16.6963
6000 mg/L	3	264.1	88.03333	38.58843

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1555	3	518.3333	2.592689	0.125106	4.066181
Within Groups	1599.369	8	199.9211			
Total	3154.369	11				

Anova: Single Factor for Microbial Density with respect to Oil Concentration at the various Loading Rates

SUMMARY

Groups	Count	Sum	Average	Variance
0.5 L/min	4	6.32E+13	1.58E+13	2.22E+24
1.0 L/min	4	6.51E+13	1.6275E+13	1.87E+24
2.0 L/min	4	6.53E+13	1.6325E+13	1.83E+24

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	6.71667E+23	2	3.35833E+23	0.170234	0.846121	4.256495
Within Groups	1.7755E+25	9	1.97278E+24			
Total	1.84267E+25	11				

Anova: Single Factor for Microbial Density with respect to Loading rate at the various Oil Concentrations

SUMMARY

Groups	Count	Sum	Average	Variance
500 mg/L	3	4.54E+13	1.51E+13	4.33333E+22
1000 mg/L	3	4.9E+13	1.63E+13	1.90333E+24
2000 mg/L	3	5.14E+13	1.71E+13	6.03333E+23
6000 mg/L	3	4.78E+13	1.59E+13	3.54333E+24

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	6.24E+24	3	2.08E+24	1.36542669	0.321124	4.066181
Within Groups	1.21867E+25	8	1.52E+24			
Total	1.84267E+25	11				

APPENDIX 8.0 PICTURES OF SET UPS

Figure 8.1 Bioreactor set up

Figure 8.2 (a) Oil extraction set up

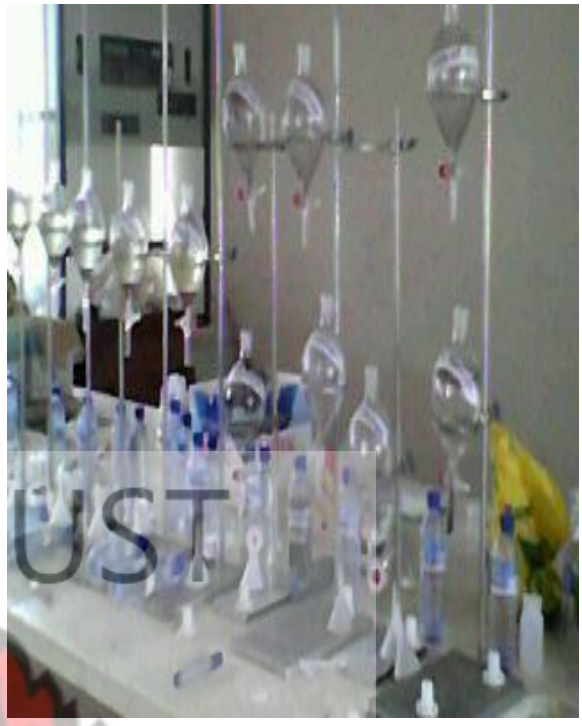


Figure 8.2 (b) Oil extraction set up

Figure 8.3 Turbid liquid culture

Figure 8.4 Cylindrically molded wire mesh

APPENDIX 9.0 GAS CHROMATOGRAPHY (GC) RESULTS