EFFECTS OF TEMPERATURE ON THE BIODEGRADATION OF USED LUBRICATION OIL CONTAMINATED WATER

By

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DECLARATION

I hereby declare that this submission consists of my own work and that, to the best of my knowledge, it contains no material previously published or presented by another person or material which has been accepted for the award of any other degree elsewhere, except where due acknowledgement/references has been indicated in the text.

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DEDICATION

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ABSTRACT

Biodegradation of hydrocarbon contaminants in water by mixed culture of hydrocarbon degrading microorganisms was investigated using a fixed bed bioreactor system. The study was performed with special emphasis on the effects of temperature on the biodegradation process. Microorganism capable of degrading hydrocarbons were isolated from oil – contaminated water and then cultured in a nutrient (mineral salt) medium. Temperature levels of 27^{o}C, 37^{o}C, 47^{o}C and 57^{o}C within the bioreactors were studied. At each temperature, sample concentration of 500 mg/L and 1000 mg/L were studied respectively for five days. Microbial population density, pH, conductivity dissolved oxygen (DO), and total petroleum hydrocarbon (TPH) were monitored to ascertain the progress of biodegradation within the bioreactors. Maximum degradation rate of 98.29±0.0 % was obtained at temperature of 47^{o}C at oil concentration of 1000 mg/L. Minimum degradation rate of 55.55±0.0 % was also achieved at the temperature of 57^{o}C at oil concentration of 500 mg/L. The study revealed that degradation rates

generally increased as oil concentration was increased. Degradation rates also increased as temperature was increased from 27^{o}C to 47^{o}C but reduced when increased to 57^{o}C.

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CHAPTER ONE

1.0 INTRODUCTION

Petroleum hydrocarbons in crude oils are natural products derived from aquatic algae laid down between 180 and 85 million years ago. Crude oils, composed mostly of diverse aliphatic and aromatic hydrocarbons, regularly escape into the environment from underground reservoirs (Atlas, 2011). According to Thapy *et al.* (2012), crude oil is a composite mixture of thousands of different chemical compounds.

Currently, petroleum hydrocarbons are the most abundantly used chemicals in the world. Manufactured from crude oil, petroleum hydrocarbons are found in gasoline, kerosene, fuel oil, asphalt, and even some chemicals used in home or at work (Chandra *et al.,* 2012).

There are a number of incidents in which substantial quantities of oil were accidently released into the environment instigating environmental disasters of epic proportions.

Petroleum hydrocarbon pollution of the environment may arise from oil well drilling production operations, transportation and storage in the upstream industry, refining, transportation and marketing in the downstream industry (Margesin *et al*., 1999). Xu (2012), is of the view that areas of contaminated soil have increased rapidly in recent years due to the continuous growth and development of the oil industry. Meanwhile, the level of contamination becomes severe as time elapses. These contaminants cannot be easily eliminated and eventually these contaminants will leach into the groundwater systems. Consequently, oil contamination is a serious environmental problem to our living ecosystem (Xu, 2012).

Thapa *et al*. (2012), suggested that remediation of the contaminated soil can be done in many ways which include both physic-chemical and biological methods.

A range of techniques have been successfully used to cleanup soil and groundwater contaminated with petroleum hydrocarbons, comprising pump and treat of groundwater, excavation of shallow contaminated soils, and vapour extraction. Many of these approaches, are however very costly or not fully efficient and are unable to entirely remove the contaminants. Chandra *et al*. (2012), argues that biological treatment however has emerged as one of the most promising methods of removal of petroleum hydrocarbon contaminants. It was first used to counter the Exxon Valdez oil spills and showed remarkable results and has since been developed as a major tool to remove the contaminants ever since. Biological methods are more economical and efficient than chemical and physical ones (Thapy *et al.*, 2012).

Rahman *et al.* (2002), described bioremediation as the conversion of chemical compounds by living organisms, especially microorganisms, into energy, cell mass and biological waste products. The rates of uptake and mineralization of many organic compounds by a microbial population depends on the concentration of the compound. Biodegradation is the metabolic ability of microorganisms to transmogrify or mineralize organic contaminants into less harmful, non-hazardous substances, which are then assimilated into natural biogeochemical cycles. Boopathy (2000), iterates that primary metabolism of an organic compound has been defined as the use of the substrate as a source of carbon and energy. This substrate serves as an electron donor resulting in microbial growth.

Thapa *et al*. (2012), gave examples of bacteria that can degrade petroleum products as- *Pseudomonas, Aeromonas, Moraxella, Beijerinckia, Flavobacteria, chrobacteria, Nocardia, Corynebacteria, Atinetobacter, Mycobactena, Modococci, Streptomyces, Bacilli, Arthrobacter, Cyanobacteria,* etc.

Microbial biodegradation of petroleum hydrocarbon in the environment is said to be comparatively slow because it is influenced by a number of factors which include the population of hydrocarbon (Ekpo and Udofia, 2008).

Atlas (1981), suggested that factors which influence rates of microbial growth and enzymatic activities affect the rates of petroleum hydrocarbon biodegradation. The intensity of biodegradation is influenced by several factors, such as nutrients, oxygen, pH, composition, concentration and bioavailability of the contaminants, chemical and physical characteristics and the pollution history of the contaminated environment (Margesin *et al*., 1999).

Temperature plays very important roles in biodegradation of petroleum hydrocarbons, firstly by its direct effect on the chemistry of the pollutants, and secondly on its effect on the physiology and diversity of the microbial milieu (Chukwuma *et al.*, 2012).

Bossert and Bartha (1984), in Chukwuma *et al*. (2012), observed that the highest degradation rates generally occur in the range of 30 ^{o}C– 40 ^{o}C in soil environments, 20 ^{o}C– 30 ^{o}C in some freshwater environments, and 15 ^{o}C– 20 ^{o}C in marine environments.

According to Atlas (1981), at low temperatures, the viscosity of the oil increases, while the volatility of toxic low-molecular-weight hydrocarbons is reduced, delaying the onset of biodegradation. A temperature increase affects a decrease in viscosity and vice versa, thereby affecting the degree of distribution, and an increase or decrease in

diffusion rates of organic compounds. Therefore, higher reaction rates due to smaller boundary layers are expected at elevated temperatures.

Bioavailability and solubility of less soluble hydrophobic substances, such as aliphatic and polyaromatic hydrocarbons, are temperature dependent (Margesin *et al.,* 1999). Although hydrocarbon biodegradation can occur over a wide range of temperatures, the rate of biodegradation commonly decreases with decreasing temperature (Atlas, 1981).

1.1 PROBLEM STATEMENT AND JUSTIFICATION

Petroleum hydrocarbon continues to be used as the principal source of energy. Wide scale production, transport, use and disposal of petroleum globally have made it a major contaminant in both prevalence and quantity in the environment (Rahman *et al.*, 2002).

The offshore extraction of crude oil from the oceanic zone within the territorial boundaries poses serious risk of crude oil contamination of Ghana seas. A single major accidental spill or ruptured oil pipelines can be very disastrous. Also, the influx of motor vehicles into the country has undoubtedly increased consumption of crude oil refined products in the country. This has increased the sprain up of several fuel service station in the country, subsequently there is an increase in the rate of its distribution to various locations within the country. Spillages caused by accidents that involve these fuel distribution tankers releases large quantities of fuel which are either absorbed into the soil or washed by runoffs into nearby water bodies (Allen *et al.,* 2010). Also, leakages

may occur during storage at these fuel stations which can percolate and contaminate immediate phreatic waters.

Moreover, all these vehicles are expected to regularly change their engine oils for efficiency and this normally is done at the fitting shops and sometimes at the fuel service stations. The replaced oils are improperly disposed and thereby end up polluting the soil and water bodies. Oils are also spilled in small quantities during operations, servicing, washing especially the engine parts of the vehicles. These eventually add up to the polluting effects of crude oil and its products (Dvirka and Bartilucci, 2007).

Apart from vehicles, other heavy duty equipment also uses lubricants and other hydraulic oil in their operations. Most of these oils end up contaminating the soil and water bodies when changed or through spillages in small quantities.

This study seeks to investigate how different temperature ranges affect biodegradation on bioremediation processes of crude oil in water.

1.2 OBJECTIVES

The main objective of the study is to investigate the effect of temperature on the rate of biological degradation of lubricating oil.

1.3 SPECIFIC OBJECTIVES

- To construct a bioreactor, isolate and culture microorganisms for the biodegradation.

- To determine the effects of temperature (27^{o}C, 37^{o}C, 47^{o}C, 57^{o}C) on biodegradation of used lubricating oil contaminated water at different concentrations.
- To monitor the biodegradation process by measuring some selected physicochemical and biological parameters.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 HYDROCARBON CONTAMINATION AND ENVIRONMENTAL

EFFECTS

Contamination is the introduction of unwanted or undesirable substances into the environment. Materials responsible for contamination are known as contaminants or xenobiotics. A broader definition of xenobiotics includes 'all compounds that are released in any compartment of the environment by the action of man and thereby occur in a concentration that is higher than natural (Top and Springael, 2003).

This larger group also includes heavy metals, polycyclic aromatic hydrocarbons (PAHs), and oil derivatives such as toluene. Strictly speaking, mineral oil hydrocarbons are not xenobiotic, but their large scale use and various applications lead in many cases to environmental contamination. The behavior of pollutants in the environment is influenced primarily by the nature and amount of the contaminant present and the interplay among chemical, geochemical, and biological factors

(Bordenave *et al.,* 2007).

Petroleum hydrocarbon continues to be used as the principal source of energy. Wide scale production, transport, use and disposal of petroleum globally have made it a major contaminant in both prevalence and quantity in the environment (Rahman *et al.,* 2002). Margesin *et al.* (2001) iterates that mineral oil hydrocarbons are the most frequently occurring environmental contaminants. The most noticeable sources of contamination are releases from manufacturing and refining installations, oil-tanker spills and accidents during transportation of the oil. Crude oils are transported long distance either on land pipeline or on water in tankers and both of which are prone to oil spill and accidents (Thapa *et al.,* 2012).

Dibble and Bartha (1979), reported that accidental releases of petroleum products from pipelines and fuel-oil storage tanks are among the most common causes of groundwater contamination. Petroleum hydrocarbons contain benzene, toluene, ethyl benzene, and xylene isomers (BTEX), the major components of fuel oils (especially gasoline), which are hazardous substances regulated by many nations. Das and Mukherjee (2006), is of the view that apart from accidental contamination of ecosystem, the vast amounts of oil sludge's generated in refineries from water oil separation systems and accumulation of waste oily materials in crude oil storage tank's bottoms pose severe problem because many of the standard treatment processes used to decontaminate soil and groundwater have been limited in their application, are prohibitively expensive, or may be only partially effective.

Petroleum refining unavoidably generates considerable volumes of oil sludge. Common sources of this sludge are storage tank bottoms, oil-water separators, flotation and biological wastewater treatment units, cleaning of processing equipment, and soil from occasional minor spills on refinery grounds (Dibble and Bartha, 1979).

Kostka *et al.* (2011), reported that the blowout of the Deep-water Horizon (DH) drilling rig resulted in the world's largest accidental release of oil into the ocean in recorded history. The equivalent volume of approximately 4.9 million barrels of light crude oil were discharge into the Gulf of Mexico from April to July 2010 (OSAT/
NOAA report and oil budget calculator (2010)), and the total hydrocarbon discharge was 40% higher if gaseous hydrocarbons are included. Petroleum contamination of the subsurface from accidental oil spills or leaking underground storage tanks remains a

significant environmental problem. The U.S. Environmental Protection Agency reports a backlog of about 117,000 leaking underground storage tanks (Allen *et al.*, 2007).

Oil pollution accidents are nowadays become a common phenomenon and have caused ecological and social catastrophes (Ghazali *et al.*, 2004). Analysis of the crude oil waste revealed at least two important types of environmental contamination, hydrocarbons and heavy metals and that they should be taken into account in the design of an appropriate treatment sequence before the final disposal of the waste (Capelli, 2001).

Thapy *et al.* (2012), reported that soil contaminated with petroleum has a serious hazard to human health, causes organic pollution of ground water which limits its use, causes economic loss, environmental problems, and decreases the agricultural productivity of the soil. Many pollution problems resulting from releasing aromatic chemicals occur in rivers, lakes, ground waters, and process effluents of the industrialized world the bioremediation of various contaminated areas (Shourian *et al.*, 2009).

Hohener *et al.* (2003), argues that due to the widespread use of fuels, fuel components such as petroleum hydrocarbons and methyl tert-butyl ether (MTBE) are among the most frequent groundwater contaminants. Accidental release of fuel to the subsurface results in residual pools retained in the unsaturated zone. Gaseous transport of volatile organic compounds (VOCs) through the unsaturated zone has been identified as a serious threat for groundwater quality. VOC vapors may also volatilize into the

atmosphere, thereby creating a potential health threat to individuals living in the vicinity of emission sources (Hohener *et al.,* 2003).

2.2 HYDROCARBONS AND THEIR CONSTITUENTS

Complex petroleum hydrocarbon mixtures, including crude oil, diesel fuel, and creosote, consist of various concentrations of n- and branched alkanes, cycloalkanes, phenolics, aromatics, and polycyclic aromatic hydrocarbons (Hamamura *et al.*,2006). Diesel oil contains 2000 to 4000 hydrocarbons, a complex mixture of normal, branched and cyclic alkanes, and aromatic compounds obtained from the middledistillate fraction during petroleum separation (Mariano *et al.*, 2008).

Several reporters have defined petroleum in different sense but generally concluding with four main constituents of petroleum. Andrade *et al.* (2012), defines petroleum as a complex mixture of saturated and aromatic hydrocarbons, polar compounds, resins and asphaltenes whiles Atlas (1981), explained petroleum as a complex mixture of hydrocarbons and other organic compounds, including some organometallic constituents, most notably complex vanadium.

Sugiura *et al.* (1997) in Capelli (2001), and Keijisugiura *et al.* (1997), reported that petroleum contains hundreds of individual compounds, and its components are generally grouped into four classes according to their differential solubility in organic solvents: the saturates (n- and branched-chain alkanes and cycloparaffins), the aromatics (mono-, di and polynuclear aromatic compounds containing alkyl side chains and/or fused cycloparaffin rings), the resins (aggregates with a multitude of building blocks such as pyridines, quinolines, carbazoles, thiophenes, sulfoxides, and amides),

and the asphaltenes (aggregates of extended polyaromatics, naphthenic acids, sulfides, polyhydric phenols, fatty acids, and metalloporphyrins).

The composition of the crude oil can differ depending on its geological origin, and it can even vary within different zones of the same geological formation (OTA, 1991). This implies petroleum recovered from different reservoirs varies widely in compositional and physical properties. According to Capelli (2001), most of the crude oil range is in its relative composition from 33 % to 50 % of saturated hydrocarbons, 27 % to 39 % of aromatics, 2 % to 19 % of asphaltenes and $<$2 % of resins. Saturates are proportionally the most significant fraction by mass while the most toxic and persistent compounds are the polar and aromatic hydrocarbons (Andrade *et al.*, 2012). Aromatics are the second most abundant hydrocarbons in crude oil. Benzene, naphthalene, and phenanthrene and their alkyl substituted derivatives represent typical aromatics (Capelli, 2001).

2.3 REMEDIATION PROCESSES

Remediation can be considered to be the reclamation or detoxification of areas polluted/ contaminated with undesirable substances. Remediation of areas contaminated by crude petroleum oil is difficult because of the complex nature of constituent hydrocarbons. There are numerous challenges to determine the ecological risk of hydrocarbon contamination at waste sites with cleanup decisions averaging

$25 million for each site (Dorn and Salanitro, 2000). Given that it is often not possible to locate and remove the residual BTEX, remediation must focus on preventing further migration of the dissolved contamination (Kao *et al.*, 2008).

Hydrocarbons released into the environment are subject to biotic and abiotic weathering reactions in the soil and water media. These processes act together, with the rate of transformation being related to the chemical composition of the fuel and local environmental factors, including temperature, soil moisture and nutrient and oxygen contents (Mariano *et al.,* 2008). Abiotic weathering or remediation method comprises of both physical and chemical technologies to clean the contaminated environment. According to Mariano *et al.* (2008), major abiotic reactions include hydrolysis, dehydrogenation, oxidation and polymerization and Margesin and Schinner (2001), also reported that physical and chemical processes, such as dispersion, dilution, sorption, volatilization and abiotic transformations are also important in remediation. Recently, Zekri and Chaalal (2005) suggested utilizing insitu burning as the primary means of response in the event of a major oil spill but Weidemeier *et al.* (1996), argues that of all these processes, biodegradation is the only mechanism working to transform contaminants into innocuous byproducts.

This has been supported by numerous researchers in this field and most scientists have opted for this method of remediation because of its several advantages over both the physical and chemical processes. Rahman *et al.* (2002) reported that biological methods can have an edge over the physico-chemical treatment regimes in removing spills as they offer in situ biodegradation of oil fractions by the microorganisms.

Hamme *et al.* (2003), iterate that compared to physico-chemical methods, bioremediation offers an effective technology for the treatment of oil pollution because the majority of molecules in the crude oil and refined products are biodegradable and oil-degrading microorganisms are ubiquitous. Dojka *et al.* (1998) also examined that

the biological breakdown of hydrocarbons is particularly beneficial, because it ultimately converts hydrocarbons to carbon dioxide, water, and methane, rather than simply repartitioning the hydrocarbons.

Biodegradation is most often the primary mechanism for contaminant destruction. The most widely used bioremediation procedure is biostimulation of the indigenous microorganisms by addition of nutrients (Margesin and Schinner, 2001). "Intrinsic remediation" refers to a management strategy that relies on natural attenuation mechanisms to remediate contaminants and this occurs when indigenous microorganisms work to bring about a reduction in the total mass of contamination in the subsurface without the addition of nutrients (Weidemeier *et al.,* 1996).

2.4 BIOREMEDIATION

Bioremediation as defined by Thapa *et al.* (2012), is the productive use of biodegradative processes to remove or detoxify pollutants that have found their way into the environment and threaten public health, usually as contaminants of soil, water, or sediments. Rahman *et al.* (2002) also described it as the conversion of chemical compounds by living organisms, especially microorganisms, into energy, cell mass and biological waste products.

Bioremediation treatments are aimed at stimulating pollutant-degrading microorganisms to speed the recovery of contaminated ecosystem to a pre pollution state in terms of biodiversity and ecosystem function (Roling *et al.,* 2002). These techniques accelerate the naturally occurring biodegradation by optimizing conditions for biodegradation through aeration, addition of nutrients and control of pH and

temperature (Margesin *et al.,* 2001). The intrinsic susceptibility of the waste to a bioremediation process became an important factor for the selection of the treatment technology (Capelli, 2001).

Bioremediation is suggested for treating contaminated soil sites because of its low cost and ability to convert contaminants to harmless products (Rahman *et al.,* 2002). Both in situ and on-site treatment processes by involving the use of microorganisms to break down hazardous organic environmental contaminants will help to avoid the economic and technical disadvantages (Das and Mukherjee, 2006). It is an alternative technology capable of achieving permanent remediation at waste sites without such associated problems, as recognized by the US EPA for implementation under the Superfund Amendments and Reauthorization Act (SARA) of 1986 (Gogoia *et al.,* 2003). Acceptance by the general public is another major advantage of this technology.

Hamme *et al.* (2003), reported that heavy crude oil recovery, facilitated by microorganisms, was suggested in the 1920s and received growing interest in the 1980s as microbial enhanced oil recovery. As of 1998, only one productive microbial enhanced oil recovery project was being carried out in the United States.

According to Gogoia *et al.* (2003), the success of bioremediation (i.e. TPH removal) depends largely on the contaminant characteristics. In biological treatments it is always necessary to perform laboratory feasibility tests to determine the microbial potential to degrade the pollutants and to evaluate strategies to optimize the degradation rates before the design of real scale in-situ or ex-situ (bioreactors, land farming and others) treatments (Mariano *et al.*,2008).

Röling *et al.* (2004), suggested that restoration of the bacterial community structure to a state similar to that present prior to pollution could be used as a parameter for determination of the ecological end point of bioremediation. Kaplan and Kitts (2004) suggested that three types of bioremediation are predominant in the industry today: natural attenuation, biostimulation, and bioaugmentation.

2.4.1 TYPES OF BIOREMEDIATION

Natural attenuation

The simplest method of bioremediation to implement is natural attenuation, where contaminated sites are only monitored for contaminant concentration to assure regulators that natural processes of contaminant degradation are active (Kaplan and Kitts, 2004). Weidemeier *et al.* (1996), describe natural attenuation as the actual physical, chemical, and biological processes that facilitate intrinsic remediation. Mechanisms for natural attenuation of fuel hydrocarbons include advection, dispersion, dilution from recharge, sorption, volatilization and biodegradation.

Dojka *et al.* (1998), reported that since 1995, natural attenuation has been the most common treatment for contaminated groundwater and the second most common treatment for contaminated soil at these sites. About 17,000 contaminated groundwater sites (47 % of active sites) and 29,000 contaminated soil sites (28 % of active sites) are being remediated through natural attenuation.

Biostimulation

Biostimulation is the process of providing bacterial communities with a favorable environment in which they can effectively degrade contaminants (Kaplan and Kitts 2004). It consists of adding nutrients and other substances to soil to catalyze natural attenuation processes. Several studies of the effects of biostimulation with mainly NP-K or oleophilic fertilizers have reported positive effects on oil decontamination. According to Kasai *et al.* (2002), application of fertilizers promotes the growth of oildegrading bacteria and hence the rate of biodegradation of crude oil. Biostimulation uses indigenous microbial populations to remediate contaminated soils (Rikea *et al.*, 2003).

Bioaugmentation

In cases where natural communities of degrading bacteria are at low levels or not present, the addition of contaminant-degrading organisms, known as bioaugmentation, can speed up the process. Although significant research is being performed in this area, bioaugmentation is generally not practiced, since introduced bacteria usually cannot compete with well-adapted autochthonous bacterial communities (Kaplan and Kitts, 2004) but Whyte *et al.*(1998), argued that bioaugmentation of contaminated sites at cold temperatures are becoming a particularly viable in situ bioremediation strategy for such sites because the short summer seasons do not permit long acclimatization periods for hydrocarbondegradative populations.

Hamme *et al.* (2003), reported that the Exxon Valdez bioremediation experience, in particular, has been viewed by many as a general rule that bioaugmentation is ineffective in petroleum and other biodegradation processes and Macnaughton *et al.*

(1999), suggested that for bioaugmentation to be a viable bioremediation technology, the inoculum size should be at least equal to if not greater than the indigenous population after inoculation.

2.5 BIODEGRADATION OF HYDROCARBONS

Accidental spillage of petroleum often provokes serious damage to the natural environment, and the microbial degradation of spilled oil is one major route in the natural decontamination process (Keijisugiura *et al.,* 1997). Studies on the ability of microorganisms to degrade hydrocarbons of various structures which exist in crude oil started in the mid 1960's (Zekri and Chaalal, 2005).

Among biological factors, the diversity of microbial species and their metabolic capabilities constitute an important source of biocatalysis (Bordenave *et al.*, 2007). The biodegradation of hydrocarbons results in the production of bacterial metabolites, including organic acids and CO_2 (Allen *et al.*, 2007).

According to Walker and Colwell (1974), the success of biodegradation depends on the predominant environmental conditions, on the chemical structure of the pollutants, on the bioavailability of the contaminating compounds, and thus on the interaction between pollutant, soil matrix and microorganisms. Ramos *et al.* (1991), in Das and Mukherjee, (2006), reported that survival of microorganisms in petroleum hydrocarbons medium after spillage or contamination is a key deciding factor in the rate of biodegradation of hydrocarbons either in soil or in liquid phase. Rarely, contamination occurs suddenly (e.g. by tanker accidents or explosions), but more often creeping contamination occurs (pipeline or storage tank leakages) (Walker and

Colwell, 1974).

Some types of microorganism are able to degrade petroleum hydrocarbons and use them as source of carbon and energy. The specificity of the degradation process is related to the genetic potential of the particular microorganism to introduce molecular oxygen into hydrocarbon and to generate the intermediates that subsequently enter the general energy- yielding metabolic pathway of the cell. Some bacteria are mobile and exhibit a chemotactic response, sensing the contaminant and moving toward it, while other microbes like fungi grow in a filamentous form near the contaminant (Thapa *et al.*, 2012).

Das and Mukherjee (2006) observed that the bioavailability of a particular compound in a crude oil sample and not its chemical structure may be a sole determining factor for effective biodegradation of the compound. As hydrocarbons are mostly insoluble in water, bacterial cultures producing biosurfactant will be useful in solubilizing or emulsifying hydrocarbons leading to desorption and thereby enhancing biodegradation rate (Gogoia *et al.*, 2003).

Biotic weathering of a hydrocarbon fuel consists of two interdependent mechanisms: microbial uptake and metabolic degradation. These transformations are likely to occur stepwise, producing alcohols, phenols, aldehydes and carboxylic acids in sequence (Mariano *et al.*, 2008). Gogoia *et al.* (2003) observed that microorganisms are selective and attack specific hydrocarbons rather than all the components of the oily waste. It has been observed that the same compounds in different crude oil samples were degraded to different extents by the same organisms (Das and Mukherjee, 2006). Leahy and Colwell (1990), in Thapa *et al.* (2012), stated that the saturated hydrocarbons are in

general the most readily biodegraded component of a hydrocarbon mixture and that more than 60 % of the hydrocarbons present in the waste belong to this type. Rahman *et al.* (2002) argued that saturated compounds having a molecular weight larger than 500 may not be degraded by the organisms, since this size corresponds to the exclusion size for passage through the outer membrane of Gram-negative bacteria.

Ghazali *et al.* (2004) reported in their study that higher levels of hydrocarbon removal were seen with the medium chain alkanes compared to the longer chain alkanes. This is in agreement since short- and medium-chain alkanes are generally more easily degraded due to their lower hydrophobicity similarly the mineralization data of Whyte *et al.* (1998), suggested that microbes degrade shorter- chain alkanes (C12 and C16) more readily than the longer chain alkanes (C28 and C32), which is a common feature of many other alkane-degradative microorganisms.

2.6. MICROBIAL ECOLOGY OF HYDROCARBON BIODEGRADATION

Relatively little is known regarding the environmental determinants of microbial population selection in environments contaminated with complex hydrocarbon mixtures. The predominant factors influencing microbial community structure after contamination likely include; contaminant mixture type, physical, chemical, and biological conditions, time (Hamamura *et al.,* 2006). In general, the quantity and diversity of hydrocarbon-degrading microorganisms depends on the level and persistence of the hydrocarbons in the ecosystem (OTA, 1991).

Micro-organisms utilizing hydrocarbons as a source of energy and carbon are ubiquitous in nature (Lee *et al.,* 2005). According to Macnaughton *et al.* (1999), microbial communities within contaminated ecosystems tend to be dominated by those

organisms capable of utilizing and/or surviving toxic contamination. As a result, these communities are typically less diverse than those in non-stressed systems, although the diversity may be influenced by the complexity of chemical mixtures present and the length of time the populations have been exposed. The relationship between community structure and degradation appears to be complex since communities with similar structures showed different rates of degradation, while communities with different structures showed similar degrees of degradation (Röling *et al.,* 2004).

Top and Springael (2003), reported that there are several mechanisms or combinations which microbial communities can adapt to the presence of xenobiotics petroleum hydrocarbons in their environment. Firstly, there can be an increase in population size of those organisms that tolerate or even degrade the compound by induction of appropriate genes. Secondly, the cells can adapt through mutations of various kinds, such as single nucleotide changes or DNA rearrangements that result in resistance to or degradation of the compound. Thirdly, they may acquire genetic information from either related or phylogenetically distinct populations in the community by horizontal gene transfer (HGT), also called 'lateral gene transfer'. Eventually, the individual cells best suited to resist or degrade the xenobiotic will be selected and sweep through the population until they constitute a larger fraction of the total microbial community than before the presence of the xenobiotic.

Biodegradation of complex hydrocarbon usually requires the cooperation of more than a single species. This is particularly true in pollutants that are made up of many different compounds such as crude oil or petroleum and complete mineralization to

CO_2 and H_2O is desired (Ghazali *et al.*,2004).

Ghazali *et al.* (2004), reported that the advantages of employing mixed cultures as opposed to pure cultures in bioremediation have been widely demonstrated. It could be attributed to the effects of synergistic interactions among members of the association.

Walker and Colwell (1974), iterated that, a single bacterium usually has only a relatively small degradation range and thus not all fractions of the mineral oil hydrocarbon can be degraded by a single species. Microbial populations that consist of strains that belong to various genera have been detected in petroleum-contaminated soil or water. This strongly suggests that each strain or genera have their roles in the hydrocarbon transformation processes (Ghazali *et al.*, 2004). According to Rahman *et al.* (2002), their experimental results clearly showed that the mixed bacterial consortium could carry out a maximum of 78% of degradation after 20 days

incubation.

Several researchers/workers have reported on the different species of microorganisms capable of degrading hydrocarbon compounds. In their review, Bartha and Atlas (1977), in Atlas (1981), listed 22 genera of bacteria, 1 algal genus, and 14 genera of fungi which had been demonstrated to contain members which utilize petroleum hydrocarbons. ZoBell (1946), in Atlas (1981), noted that more than 100 species representing 30 microbial genera had been shown to be capable of utilizing hydrocarbons.

Atlas (1981), also reported that the most important (based on frequency of isolation) genera of hydrocarbon utilizers in aquatic environments were *Pseudomonas, Achromobacter, Arthrobacter, Micrococcus, Nocardia, Vibrio, Acinetobacter, Brevibacterium, Corynebacterium, Flavobacterium, Candida, Rhodotorula, and*

Sporobolomyces. Bacteria and yeasts appear to be the prevalent hydrocarbon degraders in aquatic ecosystems. In polluted freshwater ecosystems, bacteria, yeasts, and filamentous fungi and algae all appear to be important hydrocarbon degraders (Hamme *et al.*, 2003).

Kasai *et al.* (2002), noted that one group of bacteria capable of degrading aromatics in a marine environment is the members of the genus *Cycloclasticus*. Alonso-Gutierrez *et al.* (2009), analysis showed that *Actinobacteria*, mainly *Rhodococcus*species, was the key alkane-degrading group of bacteria in fresh waters and Kasai *et al.* (2002), identified *Alcanivorax*as the bacterium that was mainly responsible for the degradation of alkanes in an oil-contaminated marine environment.

Strains of microorganisms are selective in their degradation of hydrocarbon compounds or show preference or partiality in their consumption of petroleum hydrocarbon constituents.

2.7 LIMITING FACTORS TO BIODEGRADATION OF HYDROCARBONS

Factors which influence rates of microbial growth and enzymatic activities affect the rates of petroleum hydrocarbon biodegradation (Atlas, 1981). Taking into account the fact that each contaminated site can respond in a different way to distinct parameters that affect microbial biodegradation, laboratory-scale bioremediation protocols have been developed in order to determine the effects of different conditions (Vin˜as *et al.*, 2005).

Hamme *et al.* (2003), found that the rate of microbial degradation of crude oil or oil waste depends on a variety of factors, including the physical conditions and the nature, concentration, and ratios of various structural classes of hydrocarbons present, the bioavailability of the substrate, and the properties of the biological system involved. Kostka *et al.* (2011), reported that the in situ metabolism of oil-degrading bacteria is likely to be limited by a number of environmental parameters, including temperature, the availability of oxygen and major nutrients, oil hydrocarbon content, and weathering or dispersal of the oil. Various researchers have examined different parameters during hydrocarbon biodegradation experiments at different conditions.

These factors or parameters are generally grouped into three; physical, chemical and biological factors.

2.7.1 PHYSICAL FACTORS

Area: Availability of increased surface area should accelerate biodegradation. Not only is the oil made more readily available to microorganisms, but movement of emulsion droplets through a water column makes oxygen and nutrients more readily available to microorganisms (Atlas, 1981).

Atlas (1981), found that 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. The degree of spreading is reduced at low temperatures because of the viscosity of the oil and Shourian *et al.* (2009), showed that the biodegradation rate of phenol can be improved by immobilizing the cells and entrapping them on the solid-based biomaterial such as alginate, polyacrylamide and so on to obtain the maximum degradation capability.

Immobilization is considered to promote better survival and activity of the introduced organisms (Kastner *et al.*, 1998).

Lee *et al.* (2005) also reported that when the surface area becomes limiting, biomass increases arithmetically rather than exponentially.

Solubility: Solubility may be another factor to influence biodegradability. Its known that the solubility, and hence the accessibility to catabolic enzymes, of a hydrocarbon molecule decreases as the number of its carbon atoms increases (Keijisugiura *et al.*, 1997).

Lee *et al.* (2005) reported that low solubility of hydrocarbons and adsorption of high molecular weight hydrocarbons limits their availability to microorganisms.

The low water solubility of the majority of petroleum hydrocarbon compounds have the potential to limit the capacity of microbes, which generally exist in aqueous phases, to access and degrade these substrates (Hamme *et al.*, 2003).

Physical state of hydrocarbons: Some components in crude oils spilled are without difficulty degraded; others are more gradually and/or less entirely degraded; and some compounds are totally non-biodegradable (recalcitrant) (Lee and Merlin, 1999). The greater the intricacy of the hydrocarbon formation (i.e. the higher number of alkylbranched substituents or condensed aromatic rings), the slower the rates of degradation and the greater the likelihood of accumulating partially oxidized intermediary metabolites.

These factors, as well as others such as volatility, set the practical operational limits for the appliance of bioremediation strategies. There is benefit to bioremediate a spill of light hydrocarbons such as gasoline, since it would evaporate rapidly (Lee and

Merlin, 1999).

2.7.2 CHEMICAL FACTORS

Concentration: Crude oil degradation is inversely proportional to the concentration of oil (Rahman *et al.,* 2002). Pfaender and Bartholomew (1982), showed the rates of uptake and mineralization of many organic compounds by microbial populations in the aquatic environment are proportional to the concentration of the compound, generally conforming to Michaelis-Menten kinetics.

Rahman *et al.* (2002) reported that 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. Fusey *et al.* (1984) reported that contamination of seashore sediments with crude oil above a threshold concentration prevented biodegradation of the oil because of oxygen and/or nutrient limitation.

The effects of crude oil concentrations on the growth of individual bacterial cultures and the mixed bacterial consortium, and crude oil degradation, were tested by Rahman *et al.*(2002) and they found that at 2.5 % BH crude oil, the mixed bacterial consortium showed 70 % degradation followed by 67 % at 5 %, 63 % at 7.5 % and 52 % at 10 %.

Oxygen: Aerobic situations are essential for microbial oxidation of hydrocarbons in petroleum contaminated or polluted environment. Conditions of oxygen constraints normally do not subsist in the upper levels of the water column in marine and freshwater environments (Cooney *et al.,* 1985).

In oil-contaminated soils it is well known that O_2 supply often limits biodegradation, although it is known that even low O_2 levels are able to sustain aerobic respiration (Rikea*et al.,* 2003). Kao *et al.* (2008), reported that BTEX are biodegradable under both aerobic and anaerobic conditions. Nevertheless, rates of BTEX biodegradation under aerobic conditions are higher than those under anaerobic conditions.

Hydrogen ion concentration (pH): Biodegradation of petroleum hydrocarbons is most favorable at a pH 7 (neutral), the suitable range is pH 6 – 8 (US EPA, 2006).

Low and high pH levels create acidic and alkaline conditions respectively in the aqueous medium and thereby impacts negatively on microbial populations. Rate of biodegradation is reduced at these extreme conditions.

Shourian *et al.* (2009), reported that the rate of phenol degradation was affected by initial

pH of culture medium. At pH 5 and 8.5 phenol degradation was inhibited and the cell growth was significantly retarded. These pH values created unfavorable growth conditions for the bacterial population.

2.7.3 BIOLOGICAL FACTORS

Biosurfactants: An important factor that affects microbial degradation is the hydrophobicity of diesel oil which limits its transfer to the cell surfaces of microorganisms. This limitation may be overcome by growing surfactant-producing microorganisms. This in turn results in the increased bioavailability of diesel oil to microorganisms (Lee *et al.,* 2005). Micro-organisms growing on petroleum usually produce potent emulsifiers and these surfactants help to degrade petroleum (Rosenberg, 1993).

Ron and Rosenberg (2002), in Basu (2005), found that biosurfactants are more effective than chemical surfactants in increasing the bioavailability of hydrophobic compounds and they are selective, environmentally friendly and generally less stable than most synthetic surfactants so easily degradable after bioremediation.

Hamme *et al.* (2003), reported that the low-molecular-weight biosurfactants (glycolipids, lipopeptides) are more effective in lowering the interfacial and surface tensions, whereas the high-molecular -weight biosurfactants (amphipathic polysaccharides, proteins, lipopolysaccharides and lipoproteins) are effective stabilizers of oil-in-water emulsions.

There are at least two ways in which biosurfactants are involved in bioremediation: increasing the surface area of hydrophobic water-insoluble substrates and increasing the bioavailability of hydrophobic compounds (Lee *et al.*, 2005).

Bioattachment: Microorganisms which are vigorous bio-oxidizers are normally attached to some substrate rather than in free aqueous phase. They are usually attached to sludge and soil particles where they form colonies. In some cases they simply attach to each other establishing biological floc. Soluble contaminants which persist in the aqueous phase are in contact with the microbes for instant destruction. At the same time, less soluble organics are adsorbed to soil particles. Adsorbed organics are accessible for intimate contact when colonizing microbes settle into form surface films over the solid particle.

Nutrient: The principal nutrients essential for biosynthesis and cell development of microbes concerned with bioremediation processes are carbon, nitrogen and phosphorus (C, N, and P). Hutchinson *et al.* (1994), reported that contaminant

degradation have shown that usual indigenous microbes have the potential to degrade HC contaminants more speedily when supplemented with nutrients in the form of fertilizers.

Roling *et al.* (2002), reported that because of the high carbon content of oil and the low level of other nutrients essential for microbial growth, the rate and extent of degradation are, in general, limited by the low availability of nitrogen and phosphorus and Hamme *et al.* (2003), demonstrated that nitrogen and phosphorus contents to a great extent affect the microbial degradation of hydrocarbons.

2.8 EFFECT OF TEMPERATURE ON BIODEGRADATION

The biodegradability of a variety of crude oils is highly dependent on crude oil composition and temperature (Atlas, 1975). Degrading micro-organisms are particularly active in phases of temperature increase in the system (Popp *et al.*, 2006). Therefore, increasing temperature increases and accelerates the growth of bacteria that resulted in increasing the degradation process of the crude oil (Zekri and Chaalal, 2005). Many studies have been done to investigate the influence of temperature on biodegradation of petroleum hydrocarbons and similar results were found.

Venosa and Zhu (2003), also reported that temperature plays very vital roles in biodegradation of petroleum hydrocarbons, initially by its direct effect on the chemistry of the pollutants, and secondly on its effect on the composition and diversity of the microbial milieu. Ambient temperature of an environment affects both the properties of spilled oil and the enzymatic activity or population of microorganisms. Rahman *et al.* (2002), found that temperature influences petroleum biodegradation by its effect on the

physico-chemical properties of the oil, rate of hydrocarbon metabolism by microorganisms and composition of the microbial community.

In another study Margesin and Schinner (2001), stated that field temperatures play a significant role in controlling the nature and extent of hydrocarbon metabolism. During winter, spring, and fall, temperature was a major limiting factor. Dibble and Bartha (1979), found that the rates of disappearance of hydrocarbons from an oilcontaminated field in New Jersey showed a definite correlation with mean monthly temperature.
Atlas and Bartha (1972), in Atlas (1981), suggested that the effects of temperature differ, depending on the hydrocarbon composition of a petroleum mixture. The effects of temperature are interactive with other factors, such as the quality of the hydrocarbon mixture and the composition of the microbial community. Hydrocarbon biodegradation can occur at the low temperatures (< 5ºC) that characterize most of the ecosystems which are likely to be contaminated by oil spills (Atlas, 1981).

Hydrocarbon biodegradation can occur over a wide range of temperatures, and psychrotrophic, mesophilic and thermophilic hydrocarbon-utilizing microorganisms have been isolated. In (Atlas, 1981), ZoBell (1973) and Traxler (1973) reported on hydrocarbon degradation at below 0°C and Mateles *et al*. (1967), reported on hydrocarbon degradation at 70°C.

Low Temperatures: Biodegradation of petroleum hydrocarbons is supposed to be reduced in cold region soils, since degradation is thought to obey the Arrhenius relationship and decreases as the temperature decreases (Rikea *et al.,* 2003). In many Arctic sites, the rates of biodegradation are thought to be too low to rapidly remove

hydrocarbon contaminants, and consequently, the contaminants remain in cold arctic ecosystems for long periods following contamination.

Whyte *et al.* (1998), reported that at low temperatures, the viscosity of oil increases, reducing the degree of spreading of the oil in soil and aquatic matrices. Conversely, low temperatures retard the volatilization of short-chain alkanes, which can increase their solubility in the aqueous phase and consequently, increase their microbial toxicity, which may delay the onset of biodegradation.

Atlas (1981), indicated that low temperatures retard the rates of volatilization of lowmolecular-weight hydrocarbons, some of which are toxic to microorganisms, he postulated that at low temperatures co-metabolism play an important role in determining the rates of disappearance of hydrocarbons in the mixture but Walker and Colwell (1974), argued that low temperatures clearly do not block or completely inhibit the autochthonous microbial degradation of oil. However, a selection for specific members of the indigenous microbial population capable of carrying out microbial degradation at low temperatures does occur.

High Temperatures: Thermophilic microorganisms are capable to survive and increase in population in conditions of extremely high temperatures. These microbes undergo metabolism and biodegradation within the environment they exist. Little work has been done concerning biodegradation of petroleum hydrocarbon within the thermophilic environment although Mateles *et al.* (1967) worked and reported on hydrocarbon degradation at 70°C.

Optimum Temperatures: Psychrotrophic microorganisms may be better suited for in situ bioremediation of contaminated sites from these environments than either

mesophiles, which have very low activities at $\leq$ 10°C, or psychrophiles, whose growth is inhibited at temperatures of $\geq$ 15ºC to 20°C (although in permanently cold habitats, psychrophiles may possess greater activities than psychrotrophs (Whyte *et al.*,1998). Psychrotrophic microorganisms able to degrade a wide range of hydrocarbons, including aliphatic, aromatic, and polycyclic aromatic hydrocarbons (Margesin and Schinner, 2001). This implies that an optimum temperature for hydrocarbon biodegradation ranges between 20ºC – 30ºC.

In their study Rahman *et al.* (2002), found out that all their five individual isolates and mixed bacterial consortium showed maximum crude oil degradation at 30ºC and the population also corresponded and Shourian *et al.* (2009), also reported that the bacterial growth and phenol consumption was very slow at 37ºC. In another study, Atlas (1975), also have reported that both low and high grade crude oils are subject to microbial degradation at 30ºC and Atlas and Bartha, (1993), concluded that within the range of 10ºC to 45ºC, the rate of microbial activity typically doubles for every 10ºC increase in temperature. Temperature also variously affects the solubility of hydrocarbons

These studies report that certain hydrocarbons are more readily degraded than others and that environmental factors such as temperature can influence the ability of microorganisms to degrade petroleum.

2.9 OPERATIONAL PARAMETERS

Biodegradation of petroleum hydrocarbon is linked with number of changes in bioreactor system. Examined parameters offer data on the progress of hydrocarbon degradation by microbes. These monitored parameters describe directly and indirectly the metabolic activities of degrading microorganisms. Kwapisz *et al.* (2008), suggested

some parameters which include, respiratory activities of cells, dissolved nutrient uptake, oxygen concentration and growth rate. In addition to the above, additional observed parameters include Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD).

Common parameters monitored in this work were temperature, pH, microbial population density, conductivity, dissolved oxygen and total petroleum hydrocarbon.

2.9.1 pH

The pH profile directly relates to the metabolic activities with the system. Microbes often alter the pH of their environment by generating acidic or basic metabolic waste products (Prescott *et al.*, 2002). Production of waste metabolic products such as organic acids acidifies the growth medium. Suspension of carbon dioxide in water also decreases the pH of growth media as hydrogen cations are released. The pH usually falls as microbes transform hydrocarbons into energy and waste products. Anaerobic reactor system usually indicates lesser pH values when compared to aerobic system owing to fermentative actives in the anaerobic systems. As pH values falls or rise with time, it is implied that microorganisms are vigorously feeding on the food source and are discharging waste products into the system (Prescott *et al.*, 2002).

2.9.2 DISSOLVED OXYGEN

Measuring dissolve oxygen relates directly to microbial activity. In aerobic biodegradation processes, microbial activity is strictly related to oxygen consumption. Oxygen usage follows microbial growth curve resulting in an activity profile whereby the microorganisms are relatively active originally, increasing their oxygen requirement up to highest values and thereafter declining. Hamme *et al.* (2003), reported that the presence and concentration of oxygen becomes limiting in aerobic biodegradation

process. The amount of dissolve oxygen in the system can also determine the rate of oxygen uptake. Oxygen uptake increases with increasing

microbial activity.

2.9.3 TOTAL PETROLEUM HYDROCARBON

Total Petroleum Hydrocarbon (TPH) is sometimes referred to as mineral oil, hydrocarbon oil, extractable hydrocarbons, or oil and grease. Gas Chromatography Mass Spectrometer (GC-MS) and Flame Ionization Detector (FID) are mostly used to assess the constituent fractions of hydrocarbons in petroleum oils. According to Basu (2005), this method can be applied to determine the amount of total organics at any particular period. Residuals from degradation processes can be assessed to determine the magnitude of total hydrocarbon remaining in the system. This technique of defining microbial activity suffers essential inconsistency because of the fact that losses that may occur as a consequence of seepages which may comprise of metabolic daughter compounds predominantly from aromatic compounds (Butler *et al.,* 1991).

2.9.4 CONDUCTIVITY

Conductivity is related to levels of contaminations with heavy metals. Heavy metals such as lead, zinc, magnesium, cadmium and copper exert their deleterious effects on microorganisms present (Fei-Baffoe *et al.,* 2012). Hydrocarbon biodegradation processes are limited as degrading microbes are either poisoned to death or forced to enter state of dormancy because of the harsh environmental conditions caused by presences of these heavy metals.

2.9.5 MICROBIAL POPULATION

Colony Forming Units per milliliter (CFU/mL) can be used as an indirect method to evaluate microbial quantities in bioreactor systems. Different methodologies are used among various researchers. Sondarkamp *et al*. (2001), described enumeration of CFU after incubating on Standard I nutrient media (St. I) whiles Soriano and Pereira (1998), described similar process after incubating on Tryptic Soy Agar (TSA) medium. Most Probable Number (MPN) method is a more flexible alternative which allows better quantitative value to be obtained. In all the methods for enumeration, microbes are grown in a media containing hydrocarbons as the only carbon and energy source.

Notwithstanding the general acceptability of this method of measuring microbial activity, Torstensson (1997), argues that there are large uncertainties about the true reflection of microbial population due to the inability to enumerate CFU of viable cells adhered to substrate matrix.

2.10 HYDRAULIC RETENTION TIME (HRT)

Hydraulic retention time is a measure of the average length of time that a soluble compound remains in a constructed reactor. Hydraulic retention is usually expressed in hours or days. Flow rate corresponds to hydraulic retention time. Higher rates of flow produce shorter HRTs. Low degradations are recorded at shorter HRT and vice versa. The longer microbes interact with the substrate, the better the degradation of substrate.

CHAPTER THREE

3.0 MATERIALS AND METHODOLOGY

This project is an experimental study to determine the effect of temperature on biodegradation of used lubricating oil contaminated water. The investigation was carried out at the department of theoretical and applied biology, KNUST.

3.1 DESIGN AND CONSTRUCTION OF BIOREACTORS

The experimental setup consisted of four (4) bioreactors made of 6 inch Polyvinyl chloride (PVC) pipe. Each bioreactor is approximately 0.5 m in height, 0.15 m in diameter and a volume of about 0.009 m^3. The bioreactors were sealed at the bottom with plastic material. Inlet holes were created on each of the bioreactor lids to allow influent while outlet holes were also created on the sealed bottom to allow effluents (Figure 3.1).

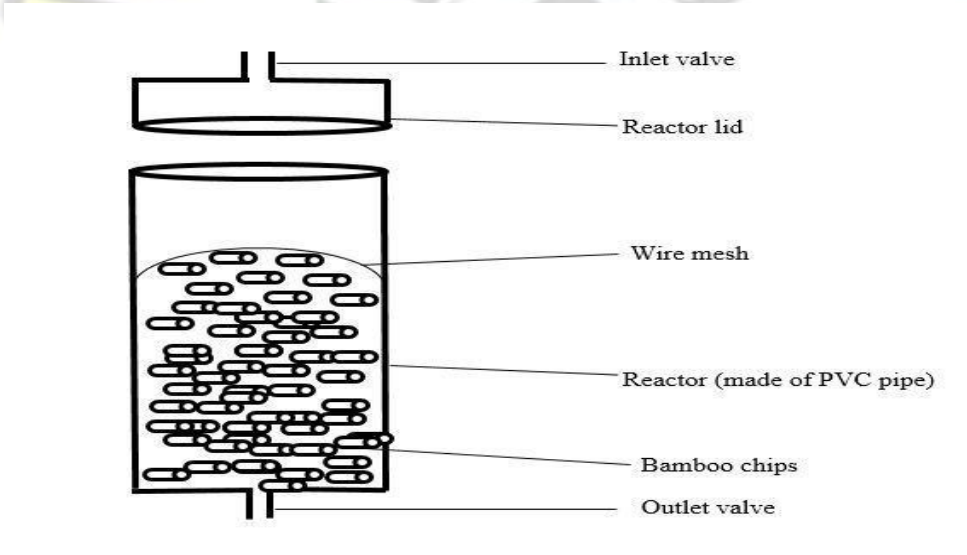


Figure 3.1: Diagram showing the bioreactor used for the study

3.2 BIOFILM SUPPORT MATERIAL

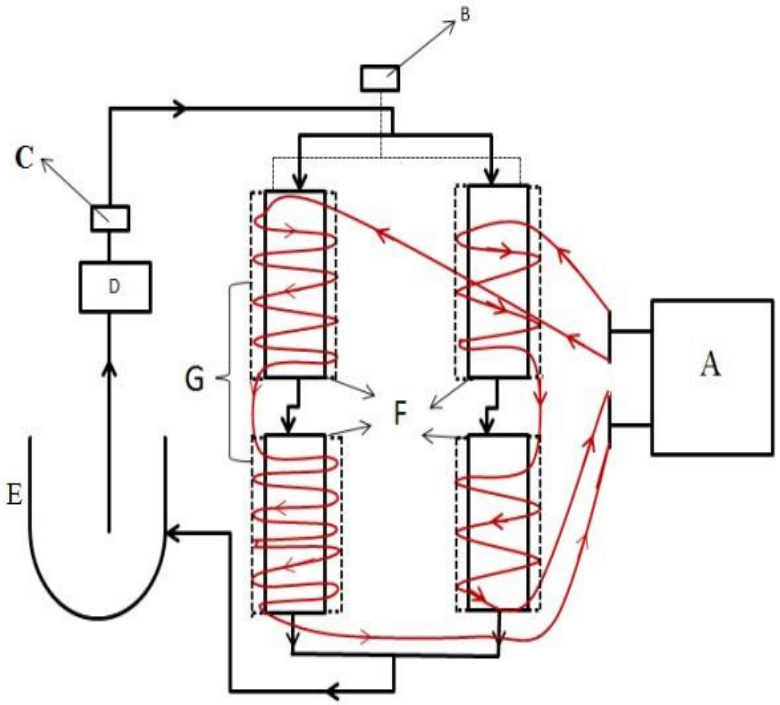
Hollow bamboo chips of about 1-2 cm in length were used as biofilm support materials. This is to serve as a supporting material for biofilm formation by preventing suspension

of microbes in the liquid medium but enable immobilization or fixation so as to achieve higher rates of degradation. The bamboo chips were dried for two weeks.

Plate 3.1: Bamboo Chips used as support materials

3.3 EXPERIMENTAL SETUP

Each bioreactor was filled with 1 kg of dried bamboo chips to about 2/3 of its volume (Figure 3.1). The bamboo chips were held together in the bioreactor by rubber mesh. Two set of the bioreactors were connected in series which was parallel to the other two sets (replicates) which were also in series (Figure 3.2). A low pressure tube of length 30 m was coiled around all four bioreactors and then connected to both the outlet and inlet valves of a circulating water bath (fisher scientific model 80). A quilt serving as an insulating material was then wrapped around the PVC pipes to reduce heat loss to the surroundings. All the bioreactors were connected to an aquarium air pump to create aerobic conditions within them.



A- Circulating Water bath

B- Air Pump

C- Timer

D- Water pump

E- Sample Container

F- Bioreactors

G-Insulating materials

Figure 3.2: Scheme showing the experimental setup.

3.4 ISOLATING HYDROCARBON DEGRADING MICROBES

Microorganisms capable of degrading hydrocarbons were used for the experiment. Hydrocarbon-degrading microbes were isolated from water contaminated with used lubricating oil obtained from Barima Oil Service Station at Kentikrono in Kumasi. Used lubricating oil used for the experiment was also obtained from the same place.

The microorganisms were cultured in a nutrient agar.

3.5 PREPARATION OF NUTRIENT MEDIUM

A nutrient agar containing all the essential nutrients (potassium, sodium, ammonium, magnesium and phosphorus) was prepared with a mixture of 0.2 g of Magnesium Sulphate ($MgSO_4$), 2.0 g of Anhydrous Sodium Phosphate ($Na_2HPO_4.2H_2O$), 1.8 g of

Ammonium Sulphate ($(NH_4)_2SO_4$) and 0.8 g of Potassium Hydrogen Phosphate (KH_2PO_4) in a 1 liter of distilled water (Fei-Baffoe, 2012).

3.6 CULTURING OF HYDROCARBON DEGRADING MICROBES

1000 ml of distilled water was inoculated with 40 ml of the oil contaminated water. 25 ml of prepared nutrient medium (mineral salt) was then added to the mixture followed by 5 drops of the engine oil. The mixture was then incubated at 37^{o}C for a week.

Monitoring cell growth of the culture

The degree of turbidity (cloudiness) exhibited by a broth culture is an indication of microbial growth. As cells propagated during the incubation period, broth culture turned cloudy or turbid, a signal that microorganisms were respiring, thus the usage of the oil as their carbon and energy source.

Turbidity of the prepared liquid culture was monitored every morning during the seven day incubation period using the Wagtech photometer WG 7100. The presence of these cells was confirmed by plate count at the end of the seven day incubation period.

3.7 MICROBIAL ENUMERATION

Preparation of agar plate

17.5 g of the plate count agar (PCA) was liquefied in 1 L of distilled water. The solution was sterilized by autoclaving at 121^{o}C for 15 minutes and afterwards cooled to about 40^{o}C before use. Petri-dishes used were also sterilized using a hot air oven at

180^{o}C for three hours 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^{-14}. Dilutions 10^{-10} through to dilution 10^{-14} were then plated. Microbial numbers were enumerated after 24 hours of incubation at 37ºC 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) = {Σ (number of colonies * dilution factor) / 5 ml}.

3.8 HYDROCARBON DEGRADATION

Aerobic microorganisms capable of utilizing the hydrocarbons in the used lubricating oil as their source of carbon and energy would be actively involved in the degradation process. The sample (oil contaminated water) was pumped from the storage tank and flowed through the bioreactors and back into the storage tank during each cycling regime.

3.8.1 Acclimatization of microbes in the bioreactor systems

1000 ml of liquid culture of hydrocarbon-degrading microbes was poured into each bioreactor (containing the bamboo chips). 50 ml of nutrient (mineral salt) medium was then added to each bioreactor. A sample (oil + water) concentration of 200 mg/L was pumped through the system at a flow rate of 0.5 L/minute for five days to allow for the microbes to acclimatize to conditions within the bioreactors.

3.8.2 Studied temperature

Temperatures (27ºC, 37ºC, 47ºC and 57ºC) within the bioreactors were studied to determine their effect on the rate of oil degradation by hydrocarbon – degrading

microbes. Temperature 27ºC was the ambient temperature within the bioreactors. To be able to create and maintain temperatures (37ºC, 47ºC and 57ºC) within the bioreactors for the required duration, low pressure tubes were connected to the inlet valve of a circulating water bath (fisher scientific model 80) and coiled around the bioreactors then back to the outlet valve of the same circulating water bath (Figure 3.2). The temperature regulator of the water bath and long thermometer were used to achieve the specific required temperature within the bioreactors at any particular experimental round.

3.8.3 Oil concentrations

Sample (water + oil) concentrations of 500 mg/L and 100 mg/L were used during the degradation processes. Both concentrations were prepared by mixing the required mass of oil with 1litre of distilled water. For each experimental round, 15 L of the sample concentration under study was used.

3.8.4 Flow rate

Both sample concentrations were studied at flow rates of 0.5 L/min. The flow rate was achieved by adjusting a regulating knob on the water pump until the required amount or volume of sample per minute was flowing.

3.8.5 Operation of the bioreactor set up

A water pump, 0.5 hp (horse power) was used to propel the contaminated water in the storage tank (15 L) through the bioreactors and eventually back into the storage tank. The cycle regimes through the bioreactors were controlled using an automatic timer to which the water pump was connected. A total of five (5) pumping regimes per day were

employed for each experimental round with each pumping regime lasting for 30 minutes.

Sampling for operational parameters

In order to monitor changes in operational parameters such as pH, 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 on the first, third and fifth days of each experimental round.

Sampling for microbial enumeration

For the purpose of determining the microbial density of the system, a sample volume of 20 ml was collected from the storage container. Samples were collected on the first, third and fifth days of each experimental round.

Sampling for extraction

At the end of the each experimental round, 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 five days degradation period was serially extracted from the collected sample for subsequent analysis using a gas chromatograph coupled with a flame ionization detector.

3.9 MEASURING OPERATIONAL PARAMETERS

Dissolve oxygen and Conductivity

Multiparameter probe from Hanna Instrument (HI 9828) was used to measure the dissolve oxygen and conductivity. The instrument was calibrated with 100 ml Hanna

Calibration Solution for dissolve oxygen and conductivity respectively. The probe was then inserted into 400 ml of sample and waited for the instrument to stabilize.

The value for dissolve oxygen and conductivity were recorded.

pH

The pH Test 20 produced by Aquatic Eco-Systems Inc. (S/N 1528630) was calibrated with buffer 4, 7 and 10. The probe of the instrument was inserted into 300 ml of sample and waited for the instrument to stabilize. The value was recorded.

Microbial population density

Microbial population density was obtained using the same procedure as described under section 3.7.

Total petroleum hydrocarbon analysis

- **Extraction of petroleum hydrocarbons from sample after degradation**

A sample volume of 500 ml was consecutively extracted three times in two successions with methylene chloride using a separatory funnel. 150 ml of the methylene chloride was initially added to the sample and was well shaken for about 20 minutes to homogenize it using an electronic shaker. About 250 ml of the well shaken sample containing methylene chloride was then conveyed into a 500 ml separatory funnel. The separatory funnel with its content was shaken for about 40 seconds and allowed to stand for about 20 minutes to allow for separation of the organic segment from the aqueous segment. The organic segment (containing the oil) was then carefully drained into a 50 ml beaker. The extraction was repeated two more times with 25 ml of the methylene chloride (in each case) and the oil extracts combined afterwards. The remaining three

sets of 250 ml of the sample were subjected to the same treatment as above and the extracts added to that from the first three extractions. The combined extracts was then poured in to soxhlet and processed to recover some of the methylene chloride used. After this process, the beaker containing the extracts was then subjected to heating using a hot plate to get rid of traces of any water remaining in the extracted oil. The final sample extract (oil), which was about 1 ml was then forced through a string clotted with cotton wool into a 2 ml vial. This was to further remove any trace of water in the extract. In total, eight samples went through the same treatment as described above.

- **Gas chromatographic (GC) analysis**

GC analysis was performed for all the samples (oil/hydrocarbon extracts) after extraction under these conditions stated in table 3.1:

Table 3.1: Parameters and their corresponding conditions used during GC analysis.

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

The order of chromatographic analysis was initiated with a solvent blank followed by calibration verification standard; method blank and lastly the sample extract (oil) analyses. The calibration verification standard was an n-alkane mixture that contained

C_{10} to C_{35} 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 RESULTS AND ANALYSIS

The results generated from the experimental study are presented below.

4.1 EFFECT OF TEMPERATURE ON DEGRADATION AT VARIED OIL CONCENTRATIONS

4.1.1 Effect of temperature on degradation at oil concentration 500 mg/L. The figure 4.1 below represents the degradation rates achieved for the studied temperatures of 27ºC, 37ºC, 47ºC and 57ºC at oil concentrations 500 mg/L.

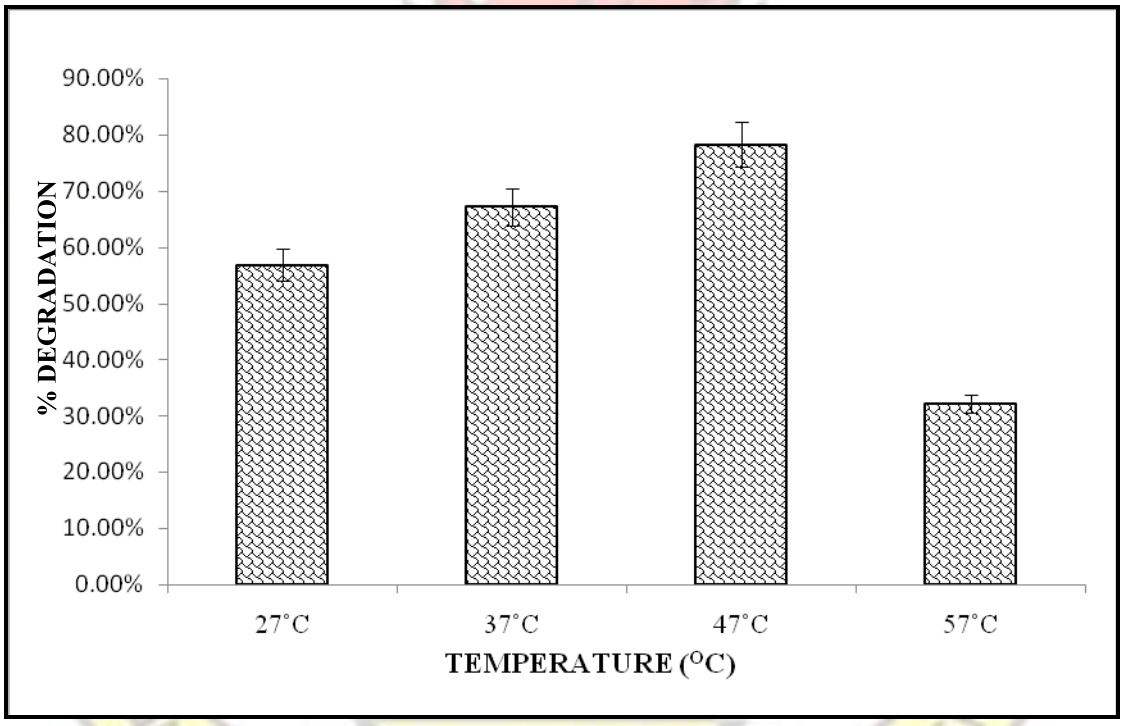


Figure 4.1 Degradation rates at different temperatures for oil concentration of 500 mg/L

At oil concentration 500 mg/L, degradation rate increased steadily as temperature was increased to 47ºC and declined sharply when temperature was increased to 57ºC.

Maximum and minimum degradation rates were 93.40 ±0.0 % and 55.55 ±0.0 % corresponding to temperatures 47ºC and 57ºC respectively (Figure 4.1).

4.1.1.1 Effect of temperature on microbial population at oil concentration 500 mg/L.

The figure below (4.2) represents the final microbial numbers obtained for the studied temperatures of 27ºC, 37ºC, 47ºC and 57ºC at oil concentrations 500 mg/L.

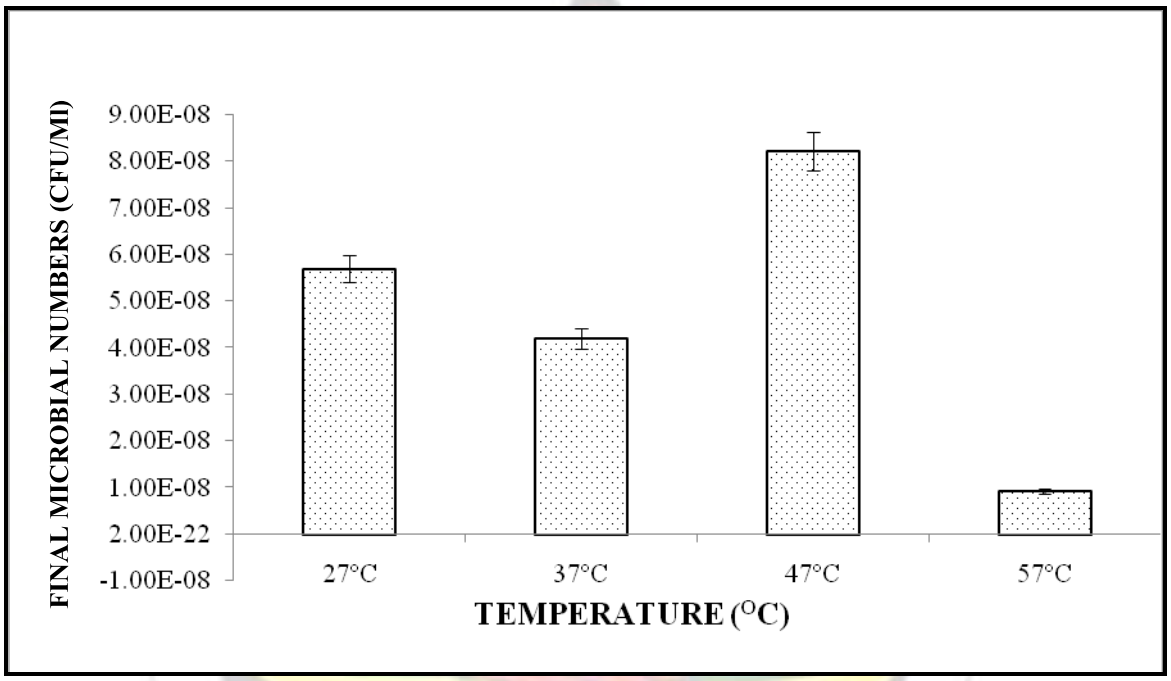


Figure 4.2 Microbial population obtained at varied temperatures for oil concentration 500 mg/L

At 500 mg/L of oil concentration, there was a fluctuation in the final microbial numbers as temperature increases from 27ºC to 57ºC. The maximum and minimum microbial numbers were 8.21E-08± 0.0 and 9.21E-09± 0.0 corresponding to temperatures 47ºC and 57ºC respectively (Figure 4.2).

4.1.2 Effect of temperature on degradation at oil concentration 1000 mg/L The figure 4.3 below represents the degradation rates achieved for the studied temperatures of 27ºC, 37ºC, 47ºC and 57ºC at oil concentrations 1000 mg/L.

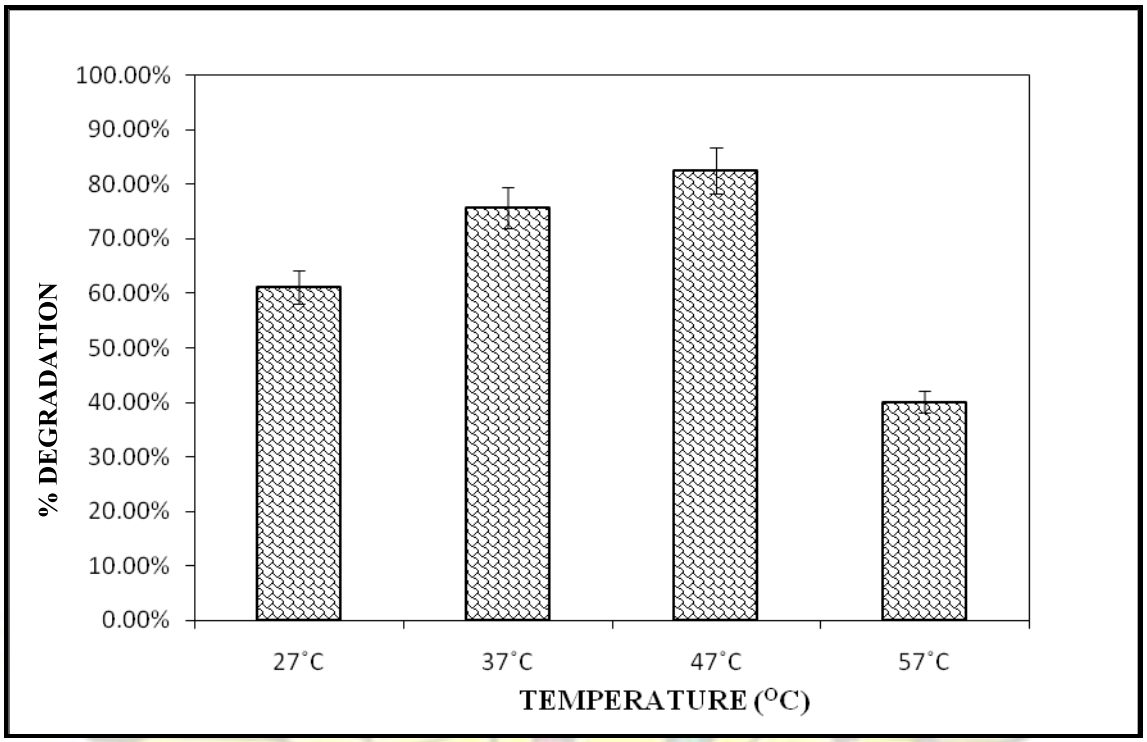


Figure 4.3 Degradation rates at different temperatures for oil concentration of 1000 mg/L

At oil concentration 1000 mg/L, there was a gradual increase in rate of degradation as temperature was increased 47ºC and declined sharply when temperature was increased to 57ºC. Maximum and minimum degradation rates were 98.29±0.0 % and 89.47±0.0 % corresponding to temperatures 47ºC and 57ºC respectively (Figure 4.3).

4.1.2.1 Effect of temperature on microbial population at oil concentration 1000 mg/L

The figure below (4.4) represents the final microbial numbers obtained for the studied temperatures of 27ºC, 37ºC, 47ºC and 57ºC at oil concentrations 1000 mg/L.

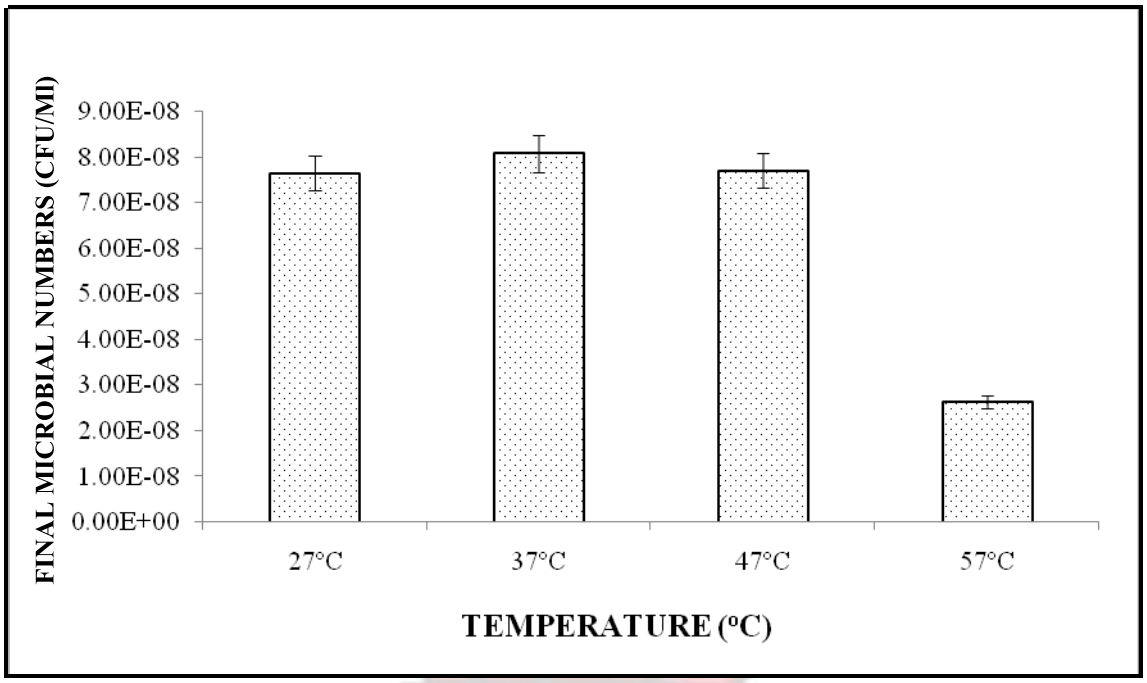


Figure 4.4 Microbial population obtained at varied temperatures for oil concentration 1000 mg/L

At 1000 mg/L of oil concentration, there was a gradual increase in microbial numbers from 7.65E-08±0.0 at 27°C to 8.09E-08±0.0 at 37°C, microbial numbers then decreased gradually to 7.71E-08±0.0 at 47°C and sharply to 2.63E-08±0.0 at 57°C. Maximum and minimum numbers obtained were 8.09E-08±0.0 and 2.63E-08±0.0 corresponding to temperatures of 37°C and 57°C respectively (Figure 4.4).

4.1.3 SUMMARY

4.1.3.1 Effect of temperature on degradation at different oil concentrations The figure 4.5 below represents the degradation rates achieved for the studied temperatures of 27°C, 37°C, 47°C and 57°C at different oil concentrations of 500 mg/L and 1000 mg/L respectively.

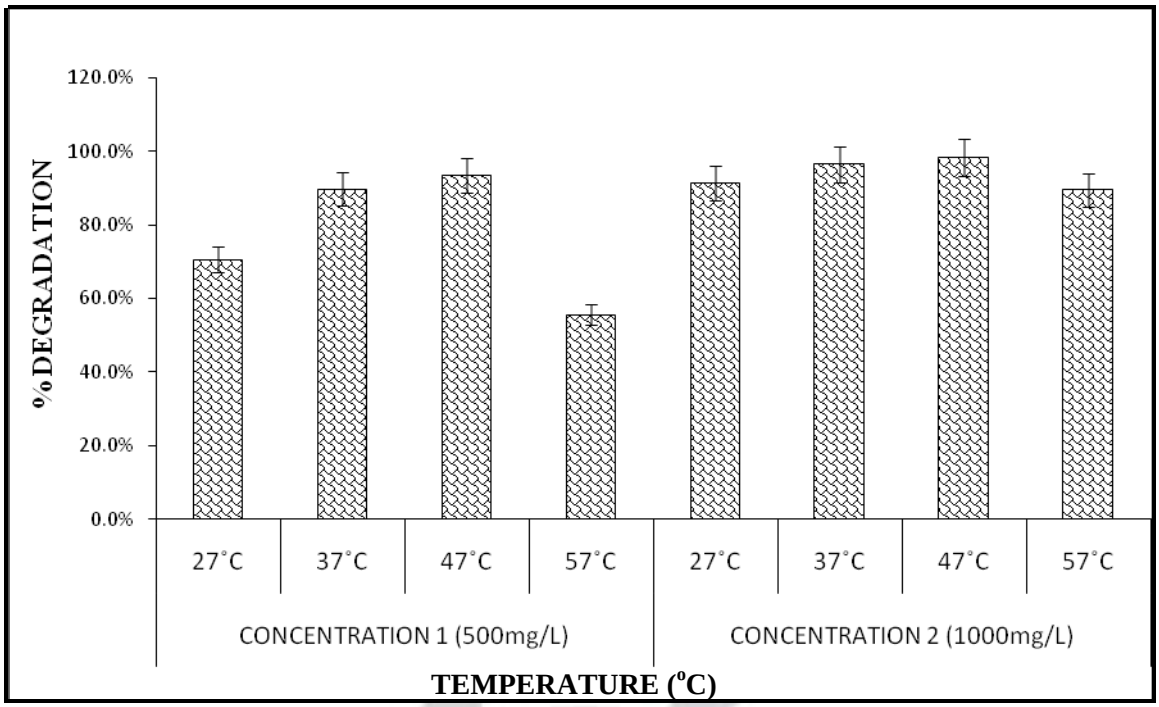


Figure 4.5 Degradation rates at different temperatures and oil concentrations

For both oil concentrations studied (500 mg/L and 1000 mg/L), gradual increase in degradation rates were observed as temperature increases from 27°C to 47°C but sharply decline with increase in temperature to 57°C (Figure 4.5). Maximum and minimum degradation rates across both oil concentrations were 98.29 ±0.0 % and 55.55±0.0 % corresponding to temperatures 47°C and 57°C respectively.

4.1.3.2 Effect of temperature on microbial population at different oil concentrations

The figure below (4.6) represents the final microbial numbers obtained for the studied temperatures of 27°C, 37°C, 47°C and 57°C at different oil concentrations (500 mg/L and 1000 mg/L respectively).

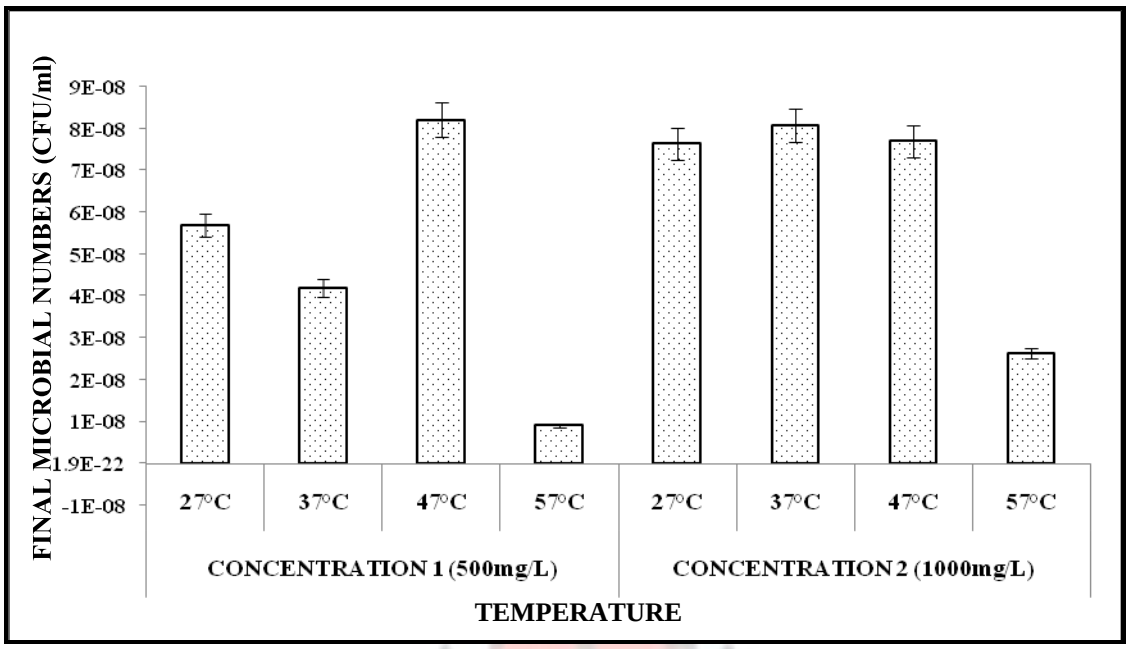


Figure 4.6 Microbial population at different temperatures and oil concentrations

For the both oil concentrations used (500 mg/L and 1000 mg/L), there was a general increase in microbial numbers as temperature increases from 27°C to 47°C but with some exceptions and sharply decline with increase in temperature to 57°C (Figure 4.6). Maximum and minimum microbial numbers obtained across both oil concentrations were 8.21E-08±0.0 and 9.21E-09±0.0 corresponding to temperatures 47°C and 57°C respectively.

4.4 ANALYSIS OF THE EFFECT OF pH, DISSOLVED OXYGEN (D.O) AND CONDUCTIVITY ON DEGRADATION AND MICROBIAL POPULATION

The table below (4.1) represents monitored parameters with their corresponding degradation rates, microbial numbers for the studied temperatures (27°C, 37°C, 47°C and 57°C) oil concentrations (500 mg/L and 1000 mg/L respectively).

Table 4.1 Summarized results showing the relationship between monitored parameters, degradation rates and microbial populations

STUDIED CONDITIONS	pH	D.O (mg/L)	CONDUCTIVITY (µS/cm)	DEGRADATION (%)	FINAL MICROBIAL NUMBERS (CFU/ml)
27˚C (500 mg/L)	6.48±0.11	1.05±0.46	1.16±0.20	70.56±0.0	5.69E-08±0.0
37˚C (500 mg/L)	6.66±0.3	0.83±0.11	1.05±0.22	89.66±0.0	4.20E-08±0.0
47˚C (500 mg/L)	6.79±0.22	1.04±0.34	0.81±0.03	93.40±0.0	8.21E-08±0.0
57˚C (500 mg/L)	6.65±0.17	1.07±0.18	0.67±0.02	55.55±0.0	9.21E-09±0.0
27˚C (1000 mg/L)	6.48±0.14	1.29±0.11	0.43±0.05	91.38±0.0	7.65E-08±0.0
37˚C (1000 mg/L)	6.74±0.05	2.77±0.68	0.33±0.01	96.52±0.0	8.09E-08±0.0
47˚C (1000 mg/L)	6.76±0.01	2.39±0.1	0.45±0.01	98.29±0.0	7.71E-08±0.0
57˚C (1000 mg/L)	6.67±0.11	3.26±0.13	0.45±0.01	89.47±0.0	2.63E-08±0.0

For both oil concentrations studied (500 mg/L and 1000 mg/L), it was observed that increase in pH was directly proportional to microbial numbers and degradation rates (Table 4.1). Maximum and minimum pH values recorded across both oil concentrations were 6.79±0.22 and 6.48±0.11 corresponding to microbial populations of 8.21E-08±0.0 CFU and 5.69E-08±0.0 CFU and degradation rates 93.40±0.0 % and 70.56±0.0 % respectively.

It was observed that increase in dissolved oxygen levels were directly proportional to microbial numbers. An exception to the above statement was observed at temperature 57^{o}C and for both oil concentration 500 mg/L and 1000 mg/L (Table 4.1). Maximum and

minimum dissolved oxygen levels obtained across both oil concentrations were 3.26±0.13 mg/L and 0.83±0.11 mg/L corresponding to microbial populations 2.63E08±0.0 CFU and 4.20E 08±0.0 CFU and degradation rates 89.47±0.0 % and 89.66±0.0 % respectively.

For both oil concentrations (500 mg/L and 1000 mg/L), it was observed that general decrease in conductivity levels translated into increase in microbial numbers and degradation rates (Table 4.1). Maximum and minimum conductivity levels obtained across both oil concentrations were 1.16±0.20 µS/cm and 0.33±0.01 µS/cm corresponding to microbial populations 5.69E-08±0.0 CFU and 8.09E-08±0.0 CFU and degradation rates 70.56±0.0 % and 96.52±0.0 % respectively.

CHAPTER FIVE

5.0 DISCUSSION

It is apparent that the microbial degradation of oil pollutants is a complex process and that environmental factors have a great influence on the fate of spilled oil. The biodegradability of a variety of crude oils is highly dependent on crude oil composition and temperature (Atlas, 1975).

5.1 EFFECTS OF TEMPERATURE ON THE DEGRADATION

Degrading micro-organisms are particularly active in phases of temperature increase in the system (Popp *et al.,* 2006). Therefore, increasing temperature increases and accelerates the growth of bacteria that resulted in increasing the degradation process of the crude oil at high temperature (Zekri and Chaalal, 2005).

This study revealed a general increase in degradation rates for the various studied temperatures except at 57ºC for the studied oil concentrations. The increase in degradation rates from temperature 27ºC to 47ºC possible suggests that microbial activities were enhanced as the temperature increases. This resulted in an increase in microbial numbers as observed in the study. Possibly, the increase in microbial numbers, which translated into an increase in their metabolic activities as temperature was increased gradually to 47ºC suggests that solubility of hydrocarbons regardless of the oil concentration also increased. Lee *et al*. (2005) reported that low solubility of hydrocarbons limits their availability to microorganisms. Solubility and hence accessibility of the petroleum hydrocarbons to microbes was enhanced as temperature was increased and thereby higher degradation rates.

Apart from microbial activities, the increase in degradation rates as the temperature increases from 27ºC to 47ºC as recorded in the study is also due to the fact temperature has influence on the physico-chemical properties of oil. Rahman *et al.* (2000), found that temperature influences petroleum biodegradation by its effect on the physico-chemical properties of the oil. The rate of physical weathering and breakages/disruption of chemical bonds within petroleum hydrocarbons increases as temperature increases. This resulted in breakdown of petroleum hydrocarbons and hence an increase in degradation rate.

Although the results recorded in this study did not have the maximum degradation occurring at 30ºC as observed by Atlas (1975) and also the degradation rate did not double for every 10ºC rise in temperature from 27ºC– 47ºC reported by Atlas and Bartha (1998), the study conforms to the general principle that petroleum hydrocarbon degradation increases as temperature increase from 20ºC– 45ºC. The differences observed could be attributed to the differences in the composition of substrate utilized by the hydrocarbon degrading microbes for the different studies.

Little work has been done on biodegradation of petroleum hydrocarbon within temperatures above 50^{o}C (Mateles *et al.*, 1967). The results of the study revealed a drastic drop/decrease of microbial numbers for both oil concentrations studied (500 mg/L and 1000 mg/L) at 57^{o}C. This possibly suggests that microorganisms used for the study barely survived at that temperature (57^{o}C) and had the duration been increased, all the microbes will be extinct. This indicates that the environmental conditions within the bioreactors were not favorable for the hydrocarbon degrading microbes at that temperature (57^{o}C) and hence the fall in their numbers.

The degradation rates (55.55±0.0% and 89.47±0.0%) recorded at temperature 57^{o}C for oil concentrations 500 mg/L and 1000 mg/L respectively could possibly be from two sources;

i) Partly due to the possible activation of dormant hydrocarbon degrading microbes within the bioreactors that thrive best at this temperature. Once favorable environmental conditions appeared, they revived from their state of dormancy and actively began to metabolize the petroleum hydrocarbons.

ii) Largely due to physico-chemical degradation of petroleum hydrocarbons. Venosa and Zhu (2003), reported that temperature plays very vital roles in biodegradation of petroleum hydrocarbons, initially by its direct effect on the chemistry of the pollutants. Temperature enhances the rate of physical weathering and also breaks down chemical bonds within hydrocarbon chains.

Statistical analysis (Table 4.16) revealed the differences in degradation rates for various temperatures studied to be statistically significant ($p < 0.05$).

5.2 MONITORED PARAMETERS ON DEGRADATION

5.2.1 pH

Low and high pH levels create acidic and alkaline conditions respectively in the aqueous medium and thereby influences negatively on microbial populations (Prescott *et al.,* 2002). Rate of biodegradation is reduced at these extreme conditions because of their effects on microbial growth. The inhibition of growth at a low pH could arise from insufficient energy to shift protons outwardly through the cell membrane to establish a proton motive gradient and from an additional expenditure of energy to maintain the membrane potential. The growth inhibitions that are seen at a low pH could be caused by a direct effect of the H ion on cellular components (Russell and

Dombrowski, 1980).

The study however revealed that the degradation process generally occurred in slightly acidic conditions (pH 6.48 – 6.79) for all the studied temperatures and oil concentrations (Table 4.2). This falls outside the inhibition range of low and high pH effects but lies within the assertion of US EPA, (2006) which states that biodegradation of petroleum hydrocarbons is most favorable at a pH 7 (neutral), the suitable range is pH 6 – 8. Statistical analysis (Table 4.18) revealed that pH had no significant influence on degradation ($p < 0.05$).

5.2.2 DISSOLVED OXYGEN

Aerobic situations are essential for microbial oxidation of hydrocarbons in petroleum contaminated or polluted environment. Kao *et al.* (2008), reported that BTEX are biodegradable under both aerobic and anaerobic conditions. Nevertheless, rates of BTEX biodegradation under aerobic conditions are higher than those under anaerobic

conditions. The solubility and availability of nutrients are affected by oxygen content of water and therefore the productivity of microbes (Ukpaka, 2013). Hydrocarbon degrading microbes also use dissolved oxygen to decompose organic materials within the oil contaminated water.

Chiang *et al.* (1989), stated that minimum dissolved oxygen level below which hydrocarbon biodegradation is either inhibited or slowed is 0.5 ppm (0.5 mg/L) and in order to prevent this, oxygen was supplied to all the bioreactors by an oxygen pump. The study revealed that there was oxygen present in the bioreactors at all times (0.83±0.11 mg/L - 3.26±0.13 mg/L) (Table 4.1).

The highest (3.26±0.13 mg/L) and lowest (0.83±0.11 mg/L) dissolved oxygen levels recorded had almost the same degradation rates (89.47±0.0 % and 89.66±0.0 % respectively). This is due to the difference in microbial numbers present at each particular period. Since both values exceed the minimum dissolved oxygen requirement for biodegradation, dissolved oxygen was not a limiting factor.

At the highest dissolved oxygen level which is expected to have higher degradation rate, microbial population (2.63E-08±0.0 CFU) was extremely low as compared to that of the lowest dissolved oxygen level (4.20E 08±0.0 CFU). This is because the temperature level (57^{o}C) at that period was very high and microbes barely survive at such temperatures. The little dissolved oxygen present at temperature 57^{o}C was in excess for the few microbes present and was then available in the water to be recorded.

The degradation rate achieved at this temperature may be attributed to second factor elaborated in section 5.1 above. Dissolved oxygen influence on the degradation was not statistically significant ($p < 0.05$) (Table 4.19).

5.2.3 CONDUCTIVITY

Conductivity is a measure of water's capability to pass electrical flow and this ability is directly related to the concentration of ions in the water. The ionic compositions of water sources are dependent on the surrounding environment (Kemker, 2013). This experimental study sampled used lubricating oil and because of the functions of this lubricating oil in the engine parts of automobiles, ions within the lubricating oil are likely to come from heavy metals. Conductivity is related to levels of contaminations with heavy metals. Heavy metals such as lead, zinc, magnesium, cadmium and copper exert their deleterious effects on microorganisms present (Fei-Baffoe *et al*., 2012).

Higher conductivity levels in the oil are a reflection of higher presence of heavy metals in the oil. The study reveals that higher conductivity translates into lower degradation rates and vice versa. The biodegradation processes are limited as degrading microbes are either poisoned to death or forced to enter state of dormancy because of the harsh environmental conditions caused by presences of these heavy metals. Higher conductivity also helps to inhibit protease activities in water. (Ukpaka, 2013). Statistical analysis (Table 4.20) revealed that conductivity had no significant influence on degradation ($p < 0.05$).

5. 3 Summary

The experiment provides a great lesson regarding the role of temperature in microbial degradation of petroleum hydrocarbons within the lubricating oil. Biodegradation of petroleum hydrocarbons occurred at ambient temperature but when temperatures within the bioreactors were increased, microbial numbers increased and consequently the rate of degradations also increased. This indicates that increasing temperature within the

bioreactors to a suitable level had positive impact on the growth and activities of microbes present. The efficiency of the biodegradation process of this study was optimized when temperature within the bioreactors were increased to 47^{o}C.

CHAPTER SIX 6.0 CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

In conclusion, the experimental study conducted proved to be successful. Generally, the study confirmed that petroleum hydrocarbon degradation rate increases as temperature is increased. Isolating petroleum degrading microbes for the study was successful and confirmed the efficiency of mixed consortium in degrading hydrocarbons. Culturing, inoculating and acclimatization of microbes within the bioreactors were also successful and this enabled the study to begin smoothly.

Degradation rates were greatly influenced by temperature. Increasing temperature generally caused an increase in degradation rates. At the highest temperature, there was a percentage degradation achieved even though microbial numbers decreased. Doubling the oil concentration did not have any adverse effects on microbial numbers as the oil concentrations were within solubility limits. Higher degradation rates were achieved at higher oil concentrations.

Microbial numbers generally increased as temperature was increased and also as the oil concentration was doubled. This translated into higher degradation rates. Monitored parameters – pH, Dissolved Oxygen (DO), conductivity, final microbial numbers and

Total Petroleum Hydrocarbon (TPH) measured to determine the progress of the biodegradation processes served as very good indicators to the study. Degradation processes occurred within slightly acidic conditions, lower conductivities translated into higher degradation rates.

6.2 RECOMMENDATIONS

Below are some recommendations being proposed for future consideration:

- Further research can be carried out to investigate why degradation rates are lowered at higher temperatures.

- The application of this technology in degrading other contaminates such as organochlorines can also be considered.

- Similar research can be carried out by adding fresh organic waste materials to the degradation process and the results compared with that of the used oil alone.

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APPENDICES

Presented below are the tables, charts for the various results generated and pictures of some prominent activities performed in the course of the experimental study.

APPENDIX 1.0 TABLE OF RESULTS FOR MONITORING PARAMETERS

Table 4.5: pH values for 500mg/L oil concentration

	SAMPLE1			SAMPLE 2			SAMPLE 3		
TEMPERATURE(˚C)	BLK A	BLK B	MEAN	BLK A	BLK B	MEAN	BLK A	BLK B	MEAN
27	6.59	6.34	6.47	6.51	6.69	6.60	6.56	6.20	6.38
37	6.29	6.34	6.32	6.83	6.68	6.76	6.92	6.88	6.90
47	7.03	6.97	7.00	6.82	6.79	6.81	6.53	6.60	6.57

57	6.82	6.77	6.80	6.75	6.63	6.69	6.25	6.67	6.46

i. **Table 4.6: Dissolved Oxygen (mg/L) values for 500mg/L oil concentration**

	SAMPLE 1			SAMPLE 2			SAMPLE 3		
TEMPERATURE(˚C)	BLKA	BLK B	MEAN	BLK A	BLK B	MEAN	BLK A	BLK B	MEAN
27	0.46	0.90	0.68	0.93	0.86	0.90	1.57	1.56	1.57
37	0.68	0.72	0.70	0.90	0.85	0.88	0.87	0.93	0.90
47	1.46	1.40	1.43	0.90	0.83	0.87	0.80	0.86	0.83
57	1.25	1.31	1.28	1.01	0.98	1.00	0.81	1.07	0.94

ii. **Table 4.7: Conductivity(µS/cm)values for 500mg/L oil concentration**

	SAMPLE 1	SAMPLE 2	SAMPLE 3

TEMPERATURE (˚C)	BLK A	BLK B	MEAN	BLK A	BLK B	MEAN	BLK A	BLK B	MEAN
27	1.12	0.82	0.97	1.27	1.04	1.16	1.43	1.30	1.37
37	0.81	0.81	0.81	1.31	1.15	1.23	1.12	1.12	1.12
47	0.85	0.73	0.79	0.80	0.82	0.81	0.87	0.82	0.84
57	0.65	0.66	0.66	0.66	0.68	0.67	0.69	0.69	0.69

iii. **Table 4.8: pH values for 1000mg/L oil concentration**

	SAMPLE 1			SAMPLE 2			SAMPLE 3		
TEMPERATURE (˚C)	BLK A	BLK B	MEAN ..	BLK A	BLK B	MEAN .	BLK A	BLK B	MEAN .
27	6.42	6.37	6.40	6.38	6.44	6.41	6.54	6.74	6.64
37	6.68	6.72	6.70	6.70	6.74	6.72	6.78	6.80	6.79
47	6.80	6.74	6.77	6.78	6.76	6.77	6.77	6.72	6.75
57	6.58	6.64	6.61	6.75	6.84	6.80	6.59	6.64	6.62

iv. **Table 4.9: Dissolved Oxygen (mg/L) values for 1000mg/L oil concentration**

	SAMPLE 1			SAMPLE 2			SAMPLE 3		
TEMPERATURE (˚C)	BLK A	BLK B	MEAN .	BLK A	BLK B	MEAN .	BLK A	BLK B	MEAN .

27	1.15	1.21	1.18	1.26	1.32	1.29	1.41	1.38	1.40
37	2.25	2.29	2.27	2.53	2.48	2.51	3.51	3.58	3.55
47	2.28	2.31	2.30	2.38	2.37	2.38	2.50	2.47	2.49
57	3.18	3.21	3.20	3.43	3.39	3.41	3.15	3.20	3.18

Table 4.10: Conductivity(µS/cm)values for 1000mg/L oil concentration

	SAMPLE 1			SAMPLE 2			SAMPLE 3		
TEMPERATURE (˚C)	BLK A	BLK B	MEAN.	BLK A	BLK B	MEAN.	BLK A	BLK B	MEAN.
27	0.45	0.46	0.46	0.47	0.46	0.46	0.38	0.36	0.37
37	0.33	0.34	0.34	0.33	0.46	0.39	0.34	0.32	0.33
47	0.43	0.44	0.44	0.45	0.46	0.46	0.45	0.45	0.45
57	0.44	0.45	0.45	0.45	0.46	0.46	0.46	0.46	0.46

APPENDIX 2.0 MICROBIAL EMUMERATION RESULTS

I. **Table 4.11: Microbial numbers for 500mg/L oil concentration** DILUTIONS

SAMPLES	TEMPERATURE (^{0}C)	10^{-10}	10^{-11}	10^{-12}	10^{-13}	10^{-14}	MICROBIAL NUMBERS (CFU/ml)
SAMPLE 1	27	145	99	68	53	23	3.11E-08
SAMPLE 2	27	172	111	84	79	38	3.68E-08
SAMPLE 3	27	260	228	168	116	120	5.69E-08
SAMPLE 1	37	97	82	63	49	22	2.12E-08
SAMPLE 2	37	124	102	88	61	35	2.70E-08
SAMPLE 3	37	191	172	166	146	92	4.20E-08
SAMPLE 1	47	204	92	56	26	11	4.28E-08

							4.72E-08
SAMPLE 2	47	216	192	76	19	13	
							8.21E-08
SAMPLE 3	47	372	356	292	140	79	
SAMPLE 1	57	192	132	68	83	52	4.12E-08
							2.59E-08
SAMPLE 2	57	120	89	61	52	28	
SAMPLE 3	57	42	37	33	21	15	9.21E-09

II. **Table 4.12: Microbial numbers for 1000mg/L oil concentration**

SAMPLES	TEMPERATURE(^{0}C)	10^{-10}	10^{-11}	10^{-12}	10^{-13}	10^{-14}	MICROBIAL NUMBERS (CFU/ml)
SAMPLE 1	27	109	93	79	61	48	2.38E-08
SAMPLE 2	27	248	192	96	78	71	5.36E-08
SAMPLE 3	27	356	248	144	192	80	7.65E-08
SAMPLE 1	37	126	102	89	63	48	2.74E-08

SAMPLE 2	37	202	187	145	104	91	4.45E-08
SAMPLE 3	37	368	332	284	260	172	8.09E-08
SAMPLE 1	47	120	98	85	71	43	2.61E-08
SAMPLE 2	47	252	216	196	144	85	5.51E-08
SAMPLE 3	47	356	272	212	103	91	7.71E-08
SAMPLE 1	57	248	200	128	97	81	5.39E-08
SAMPLE 2	57	197	176	153	109	91	4.32E-08
SAMPLE 3	57	120	107	91	78	59	2.63E-08

DILUTIONS

APPENDIX 3.0 TABLE OF RESULTS FOR PERCENTAGE DIFEERENCE

INMICROBIAL NUMBERS

Table 4.13: results for percentage difference in microbial numbers

CONCENTRATION (mg/l)	TEMPERATURE (^{0}C)	INITIAL MICROBIAL COUNT (CFU/ml)	FINAL MICROBIAL COUNT (CFU/ml)	% DIFFERENCE IN MICROBIAL NUMBERS

500	27	3.11E-08	5.69E-08	45.32
500	37	2.12E-08	4.2E-08	49.58
500	47	4.28E-08	8.21E-08	47.94
500	57	4.12E-08	9.21E-09	-77.64
1000	27	2.38E-08	7.65E-08	68.84
1000	37	2.74E-08	8.09E-08	66.08
1000	47	2.16E-08	7.71E-08	71.98
1000	57	5.39E-08	2.63E-08	-51.11

APPENDIX 4.0TABLE OF RESULTS FOR THE TURBIDITY OF LIQUID CULTURES (INOCULUM)

Table 4.14: results for turbidity of liquid cultures

DATE	CONCENTRATION (mg/L)	TURBIDITY (FTU)
23/01/2014	500	97
24/01/2014	500	123
25/01/2014	500	198

26/01/2014	500	230
27/01/2014	500	287
28/01/2014	500	345
29/01/2014	500	394
16/02/2014	1000	74
17/02/2014	1000	108
18/02/2014	1000	186
19/02/2014	1000	253
20/02/2014	1000	291
21/02/2014	1000	348
22/02/2014	1000	407

APPENDIX 5.0TABLE OF RESULTS TOTAL PETROLEUM HYDROCARBON

Table 4.15: results for total petroleum hydrocarbon

TEMPERATURE (^{0}C)	CONCENTRATION (mg/L)	Total Petroleum Hydrocarbon (TPH) CONTROL	SAMPLE	DEGRADATION %
27	500	3159.26	930.241	70.56
37	500	3159.26	326.513	89.66
47	500	3159.26	208.514	93.40
57	500	3159.26	1404.19	55.55

27	1000	3522.358	303.627	91.38
37	1000	3522.358	122.444	96.52
47	1000	3522.358	60.098	98.29
57	1000	3522.358	371.054	89.47

APPENDIX 6.0 ANALYSIS OF VARIANCE

Table 4.16: Single Factor Anova for Degradation with respect to temperature at various concentrations

SUMMARY

	Groups	*Count*	*Sum*	*Average*	*Variance*
CONCENTRATION 1 (500mg/L)	27˚C	1	0.705551	0.705551	0.00 0.00
	37˚C	1	0.896649	0.896649	0.00
	47˚C	1	0.933999	0.933999	
	57˚C	1	0.555532	0.555532	0.00
CONCENTRATION 2 (1000mg/L)	27˚C	1	0.9138	0.9138	0.00 0.00
	37˚C	1	0.965238	0.965238	0.00
	47˚C	1	0.982938	0.982938	
	57˚C	1	0.894657	0.894657	0.00

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.153534	7	0.021933	65535	0.00	0.00
Within Groups	0	0	65535			
Total	0.153534	7				

Table 4.17: Single Factor Anova for Degradation with respect to concentrations at various temperatures

SUMMARY

	Groups	*Count*	*Sum*	*Average*	*Variance*	
CONCENTRATION 1 (500mg/L)	27˚C	1	0.705551	0.705551	0.00	0.00
	37˚C	1	0.896649	0.896649	0.00	
	47˚C	1	0.933999	0.933999		
	57˚C	1	0.555532	0.555532	0.00	
CONCENTRATION 2 (1000mg/L)	27˚C	1	0.9138	0.9138	0.00	0.00
	37˚C	1	0.965238	0.965238	0.00	
	47˚C	1	0.982938	0.982938		
	57˚C	1	0.894657	0.894657	0.00	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.153534	7	0.021933	65535	0.00	0.00
Within Groups	0	0	65535			
Total	0.153534	7				

Table 4.20: Two Factor Anova for conductivity with respect to Degradation

	SUMMARY	*Count*	*Sum*	*Average*	*Variance*	*sd*
CONCENTRATION (500mg/L)	27°C	3	3491	1163.667	39143.58	197.85
	37°C	3	3163	1054.333	47910.08	218.88
	47°C	3	2444	814.6667	761.5833	27.6
	57°C	3	2013.5	671.1667	293.5833	17.13

CONCENTRATION (1000mg/L)	27°C	3	1286.5	428.8333	2599.083	50.98
	37°C	3	991.5	330.5	33.25	5.77
	47°C	3	1343.5	447.8333	82.33333	9.07
	57°C	3	1360	453.3333	42.58333	6.53
	SAMPLE 1	8	4901	612.625	51175.2	226.22
	SAMPLE 2	8	5563	695.375	117669.4	343.03
	SAMPLE 3	8	5629	703.625	143643.8	379

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	2046211	7	292315.9	28.98155	2.73E-07	2.764199
Columns	40524.33	2	20262.17	2.008885	0.170997	3.738892
Error	141207.8	14	10086.27			
Total	2227943	23				

Table 4.18: Two Factor Anova for pH with respect to Degradation

	SUMMARY	*Count*	*Sum*	*Average*	*Variance*	*sd*
CONCENTRATION (500mg/L)	27°C	3	19.445	6.481667	0.012308	0.11
	37°C	3	19.97	6.656667	0.092808	0.3
	47°C	3	20.37	6.79	0.047475	0.22
	57°C	3	19.945	6.648333	0.029358	0.17
CONCENTRATION (1000mg/L)	27°C	3	19.445	6.481667	0.018858	0.14
	37°C	3	20.21	6.736667	0.002233	0.05
	47°C	3	20.285	6.761667	0.000208	0.01
	57°C	3	20.02	6.673333	0.011108	0.11
	SAMPLE 1	8	53.05	6.63125	0.052955	0.23
	SAMPLE2	8	53.545	6.693125	0.017457	0.13
	SAMPLE3	8	53.095	6.636875	0.029614	0.17

ANOVA

Source of	*df*	*MS*	*F*	*P-value*	*F crit*

Variation	*SS*				
Treatments	0.290196	7	0.041457	1.41564	0.274119 2.764199
Samples	0.018731	2	0.009366	0.319813	0.731453 3.738892
Error/Residuals	0.409985	14	0.029285		
Total	0.718913	23			

Table 4.19: Two Factor Anova for Dissolved Oxygen with respect to Degradation

	SUMMARY	*Count*	*Sum*	*Average*	*Variance*	*sd*
CONCENTRATION (500mg/L)	27°C 37°C 47°C	3 3 3	3.14	1.046667	0.213058	0.46
			2.475	0.825	0.011875	0.11
			3.125	1.041667	0.113408	0.34
	57°C	3	3.215	1.071667	0.033308	0.18
CONCENTRATION (1000mg/L)	27°C	3	3.865	1.288333	0.011558	0.11
	37°C	3	8.32	2.773333	0.460408	0.68
	47°C	3	7.155	2.385	0.0091	0.1
	57°C	3	9.78	3.26	0.016975	0.13

	SAMPLE 1	8	13.03	1.62875	0.776205	0.88
	SAMPLE 2	8	13.21	1.65125	0.957105	0.98
	SAMPLE 3	8	14.835	1.854375	1.155853	1.08

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatments	18.73189	7	2.675984	25.10546	6.82E-07	2.764199
Samples	0.247127	2	0.123564	1.159244	0.342087	3.738892
Error/Residuals	1.492256	14	0.10659			
Total	20.47127	23				

Table 4.21: Two Factor Anova for Microbial Density with respect to Degradation

SUMMARY	*Count*	*Sum*	*Average*	*Variance*	*sd*
27°C	2	8.8E-08	4.4E-08	3.33E-16	1.8E-08
37°C	2	5.53E-08	2.76E-08	8.33E-17	9E-09
47°C	2	1.25E-07	6.24E-08	7.75E-16	2.8E-08
57°C	2	5.04E-08	2.52E-08	5.11E-16	2.3E-08
27°C	2	1E-07	5.02E-08	1.39E-15	3.7E-08
37°C	2	1.08E-07	5.41E-08	1.43E-15	3.8E-08
47°C	2	9.87E-08	4.93E-08	1.54E-15	3.9E-08
57°C	2	8.02E-08	4.01E-08	3.79E-16	1.9E-08

INITIAL	8	2.63E-07	3.29E-08	1.41E-16	1.2E-08
FINAL	8	4.43E-07	5.54E-08	8.16E-16	2.9E-08

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatments	2.29E-15	7	3.27E-16	0.520089	0.796055	3.787044
Time	2.03E-15	1	2.03E-15	3.221112	0.115771	5.591448
Error/Residuals	4.41E-15	7	6.3E-16			
Total	8.73E-15	15				

APPENDIX 7.0 CHARTS SHOWING THE CORRELATION BETWEEN MONITORED PARAMETERS

Parameters	Concentration	Temperature	PH	Conductivity	DO	TPH	% Degradation	Change in Microbial Population	Final Microbial Population
Concentration	**1**								
Temperature	**0.9**	**1**							
pH	**0.62**	**0.66**	**1**						
Conductivity	**-0.86**	**-1**	**-0.73**	**1**					
DO	**0.64**	**0.91**	**0.67**	**-0.95**	**1**				
TPH	**0.27**	**0.61**	**-0.03**	**-0.59**	**0.73**	**1**			
% Degradation	**-0.16**	**-0.27**	**0.55**	**0.19**	**-0.2**	**-0.77**	**1**		
Final Microbial Population	**-0.3**	**-0.61**	**0.08**	**0.58**	**-0.7**	**-1**	**0.81**	**0.96**	**1**

Table 4.2: Correlation between the parameters under study

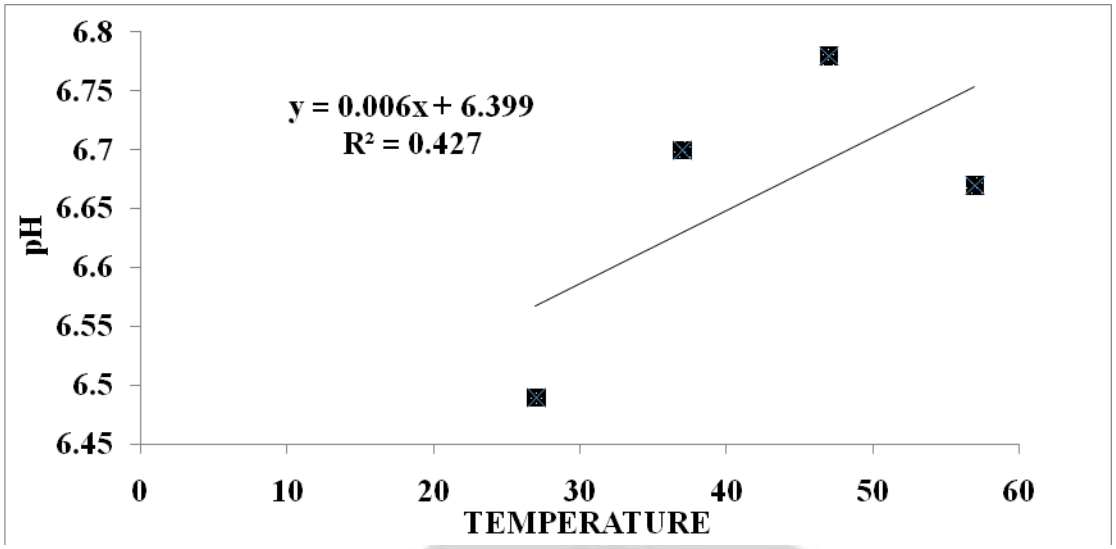

Figure 4.10: Correlation profile for temperature (˚C) and pH

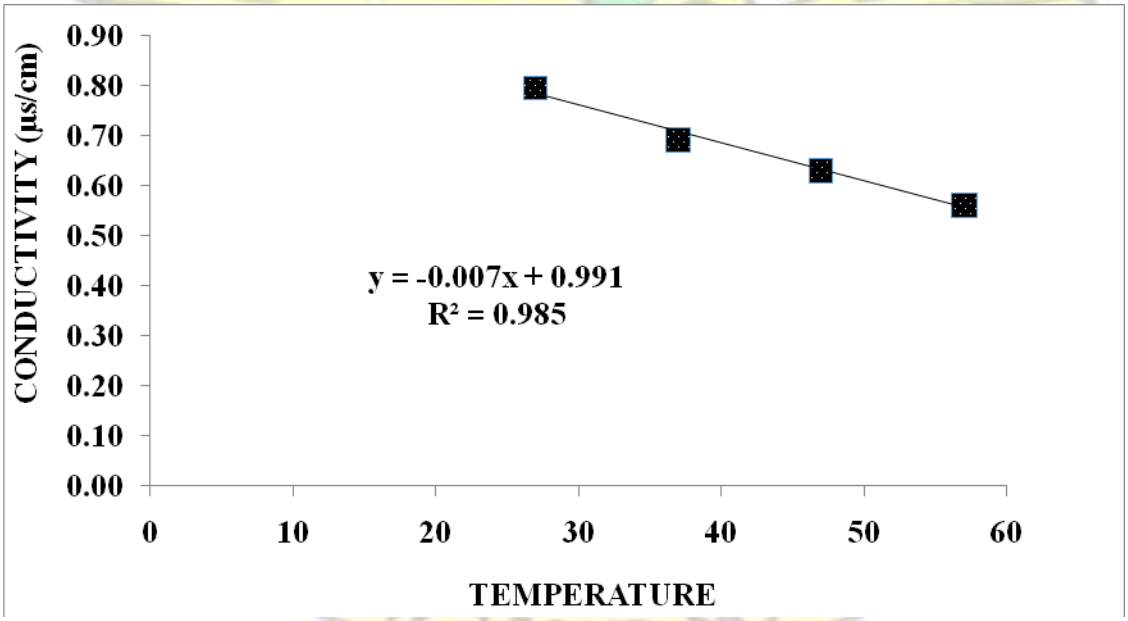

Figure 4.11: Correlation profile for temperature (˚C) and Conductivity (µs/cm)

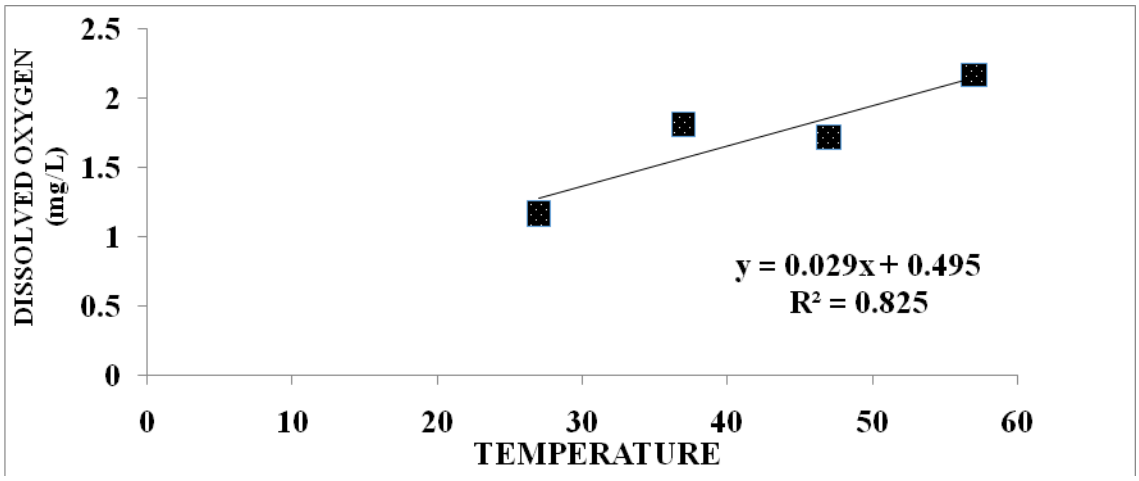

Figure 4.12: Correlation profile for temperature (˚C) and Dissolve Oxygen (mg/L)

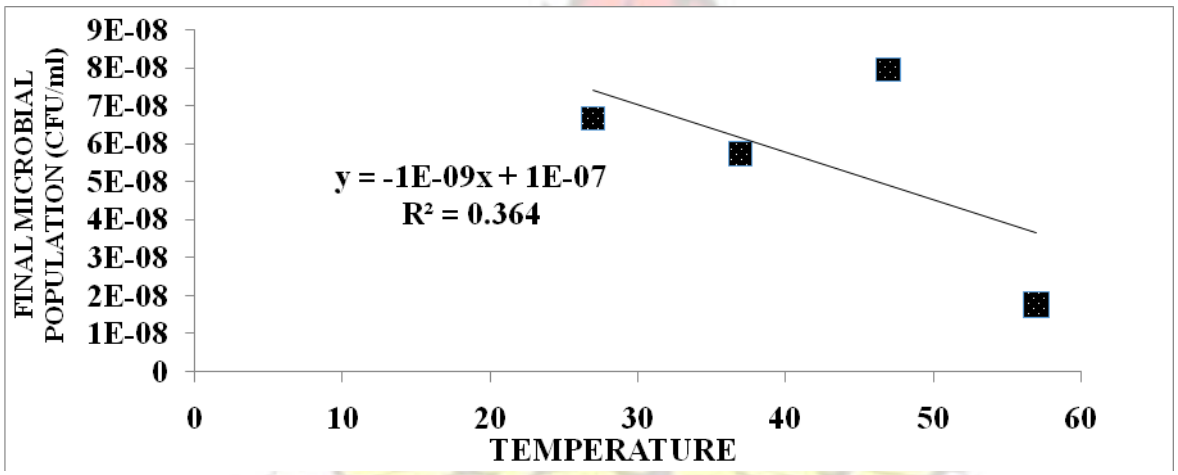

Figure 4.13: Correlation profile for temperature (˚C) and Final Microbial Numbers (CFU/ml)

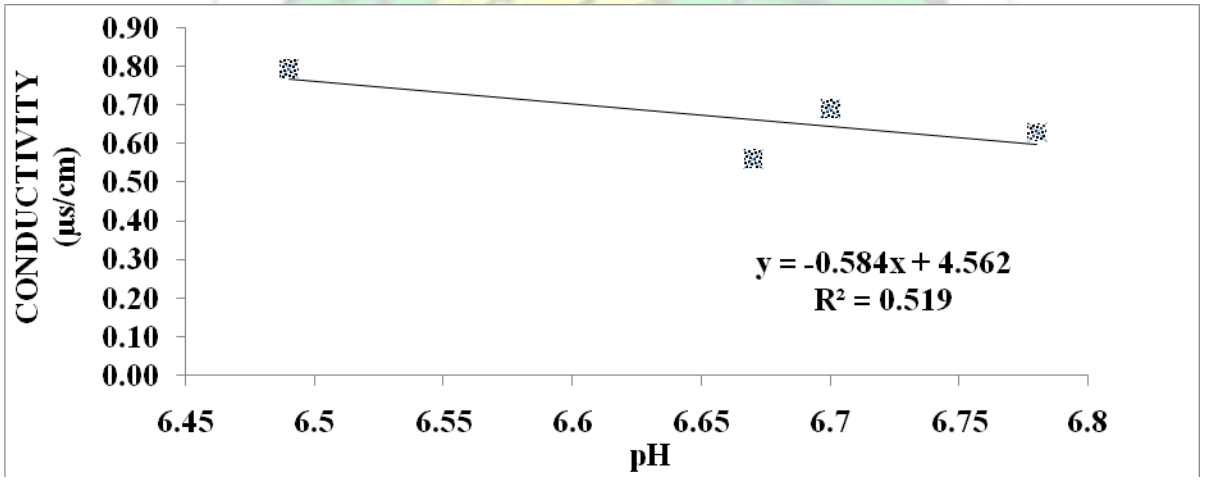

Figure 4.14: Correlation profile for pH and Conductivity (µs/cm)

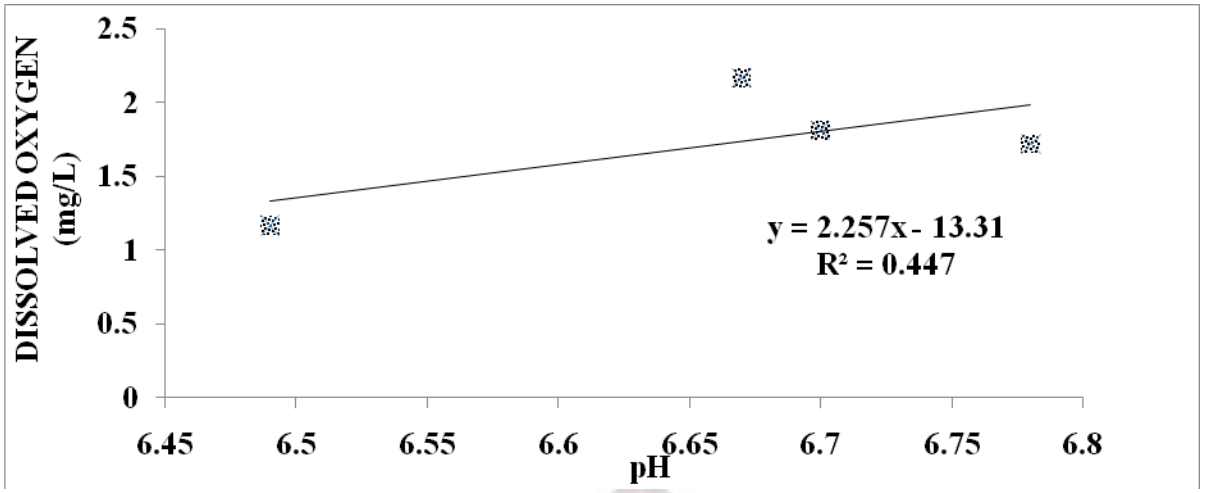

Figure 4.15: Correlation profile for pH and Dissolved Oxygen (mg/L)

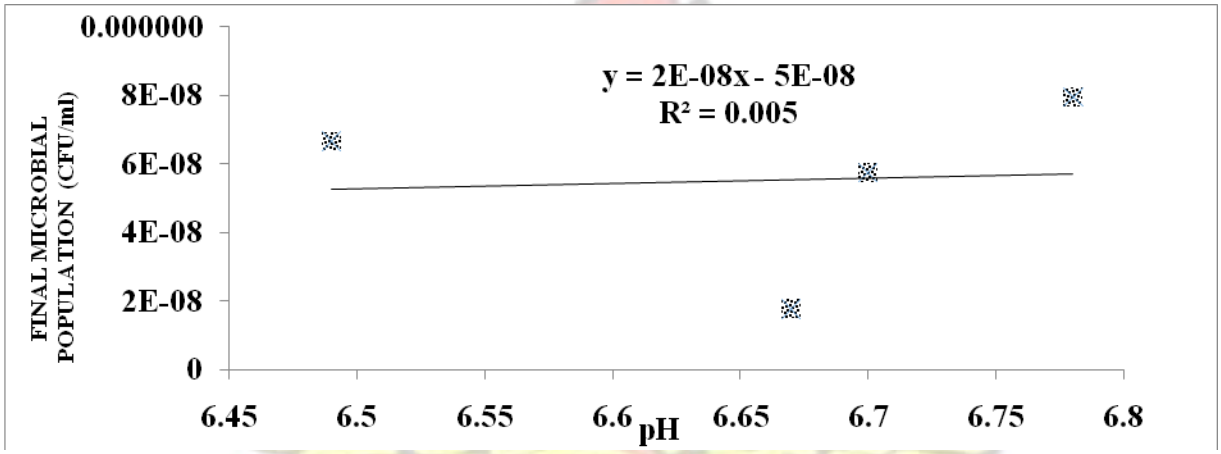

Figure 4.16: Correlation profile for pH and Final Microbial Numbers (CFU/ml)

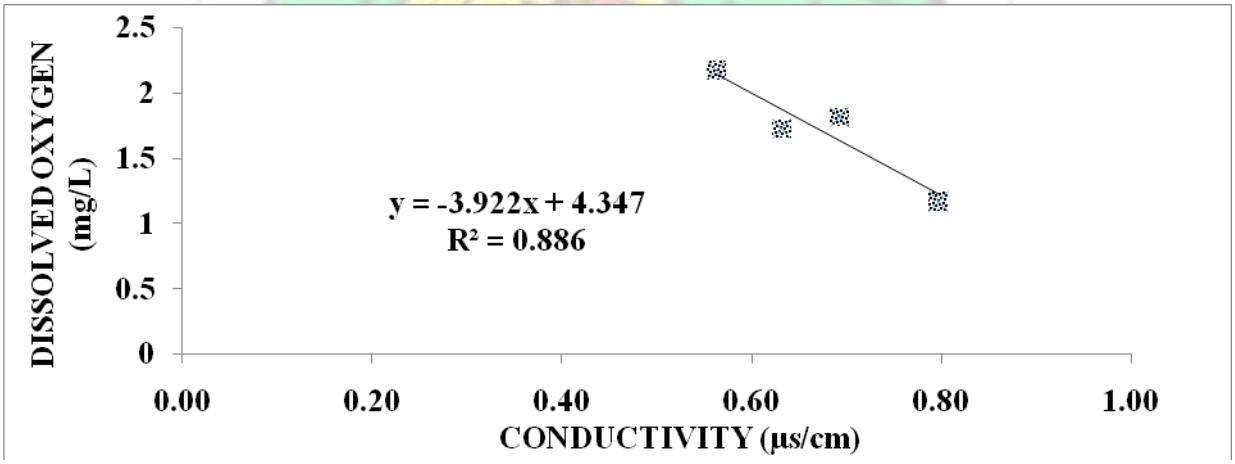

Figure 4.17: Correlation profile for Conductivity (µs/cm) and Dissolve Oxygen (mg/L)

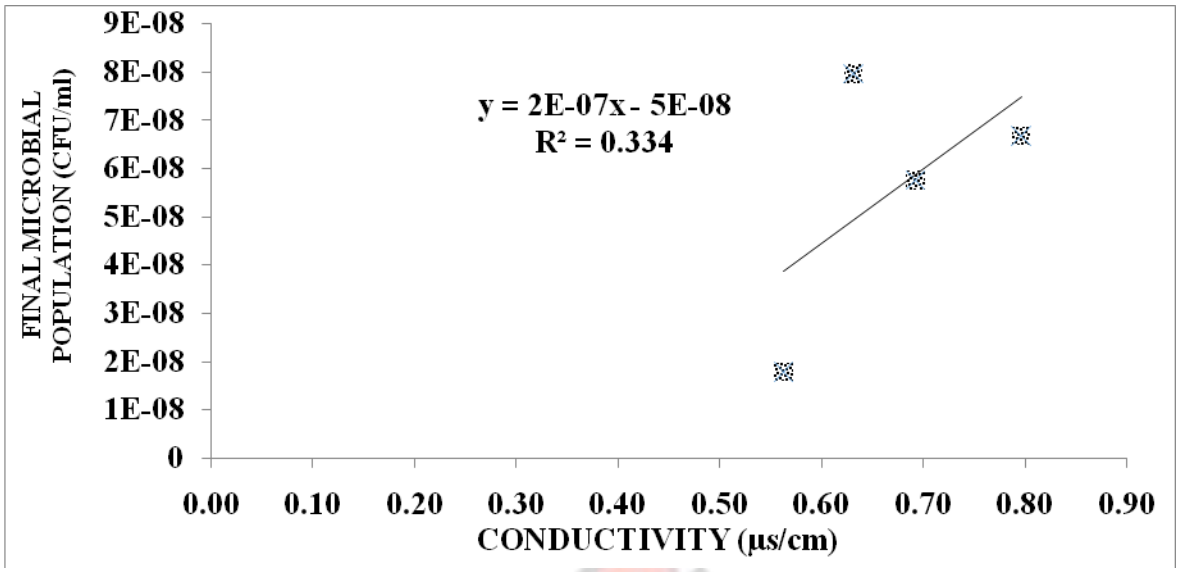

Figure 4.18: Correlation profile for Conductivity (µs/cm)and Final Microbial Numbers (CFU/ml)

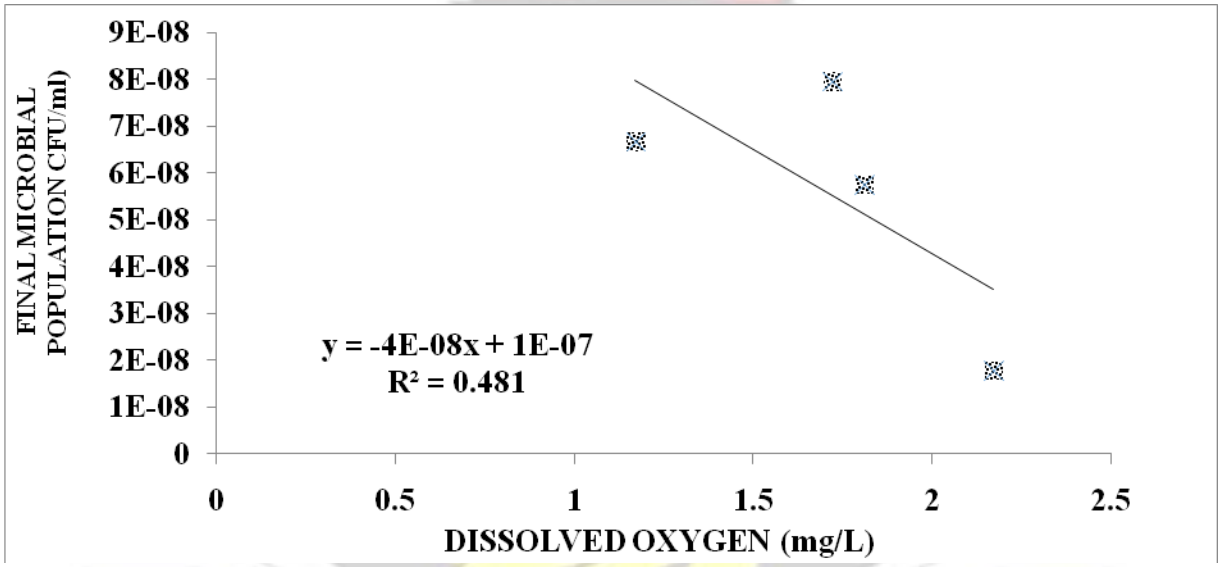

Figure 4.19: Correlation profile for Dissolved Oxygen (mg/L) and Final Microbial Numbers (CFU/ml)

APPENDIX 8.0 PICTURES OF THE EXPERIMENT

Plate 3.2: Bamboo chips used Plate 3.3: Packed Immobilizing Materials

Plate 3.4: Bioreactor Set Up

Plate 3.5: Bioreactor set up Plate 3.6:Coiled tubes around the pvc pipes

Plate 3.7: Prepared PCA for Microbial Enumeration

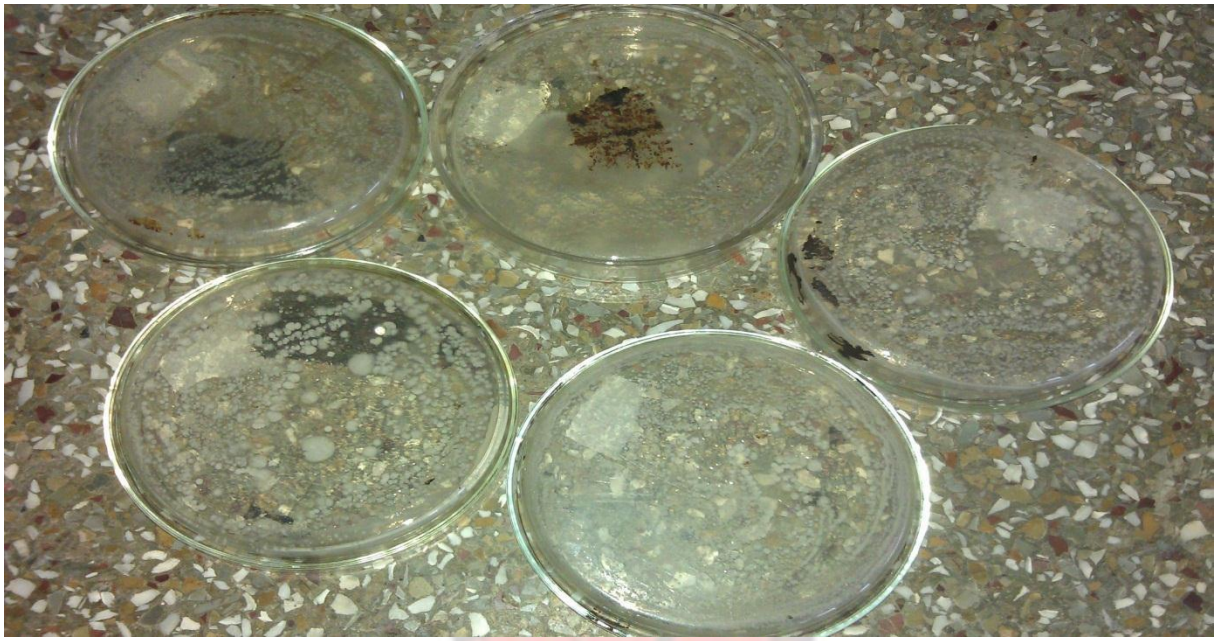

Plate 3.7: Coliforms on Petri Dishes after Incubation

Plate 3.9: Oil Extraction Set Up

APPENDIX 9.0 GAS CHROMATOGRAPHY (GC) RESULTS