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DEPARTMENT OF ENVIRONMENTAL SCIENCE

THE EFFECT OF BIOSURFACTANT (BILE) ON BIODEGRADATION OF PETROLEUM HYDROCARBON (USED MOTOR OIL) CONTAMINATED WATER

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BY

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DECLARATION

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

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DEDICATION

I dedicate this work to my little sister, Imoro Mariyam Tunteiya and to the memory of my friend and brother Mohammed Sani. May The Almighty Allah have mercy upon your soul.

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I wish to say thank you to The Almighty Allah for seeing me through this work. Also my profound gratitude goes to Dr. Bernard Fei-Baffoe, my project supervisor for his constructive criticisms, guidance and technical support throughout this work. Dr., thank you for your time, patience and expertise.

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I also say thank you to my family, you are the best!

ABSTRACT

This study was carried out to determine the effects of bile on biodegradation of a petroleum hydrocarbon contaminated water. Volumes of 1.2 ml, 1.4 ml, 1.6 ml and 1.8 ml bile were added to different preparations of contaminated water with and without nutrient supply in a fixed bed bioreactor. An increase in volume of bile did not directly correspond to an increase in percentage Total Petroleum Hydrocarbons (TPH) degraded. At an oxygen supply of 7.01 mg/l and average pH and temperature of 7.38±0.87 and 28.02±0.82 °C respectively, the highest percentage degradation was recorded in the treatment bile (1.6 ml) + nutrient supplements. Increments in volume of bile caused corresponding reductions in surface tension of contaminated water, thus demonstrating the ability of bile to emulsify and solubilise petroleum hydrocarbon contaminants. There was also a continuous reduction in surface tension in all treatment processes following the addition of bile except in the treatments, bile (1.6 ml) and nutrients + bile (1.6 ml) which showed a rather increasing trend in surface tension throughout their respective degradation periods. The Critical Micelle Concentration for bile was achieved at a concentration of 1.6 ml/l.

ACRONYMS

ATSDR - Agency for Toxic Substances and Disease Registry

CMC – Critical Micelle Concentration

DO – Dissolved Oxygen

DOE – Department of Energy

EC – Electrical Conductivity

IPCC - Intergovernmental Panel on Climate Change

MC – Microbial Counts

NAS – National Academy of Sciences

NM – Nutrient Medium

RTPH – Residual Total Petroleum Hydrocarbons

Sal – Salinity

Temp – Temperature

TPH – Total Petroleum Hydrocarbons

USEPA – USA Environmental Protection Agency

VMT – Vesicle – to – Micelle – Transition

APE – Alkyl Phenol Ethoxylate

PAH- Polycylic Aromatic Hydrocarbons

PHC – Polycyclic Hydrocarbon

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of study

Fossil fuel is the world's main energy source accounting for 80% of primary energy consumption (Friedleifsson, 2003). Current global crude oil utilization stands at around 80 million barrels per day (DOE, 2006). Today, one of the major environmental problems in the world is petroleum hydrocarbon contamination resulting from both natural and anthropogenic causes. The amount of natural crude oil seepage is estimated to be 600,000 metric tons per year with a range of uncertainty of 200,000 metric tons per year (Kvenvolden and Cooper, 2003). Also the annual global estimated input of petroleum into the environment due to anthropogenic sources is between 1.7 and 8.8 million metric tons (NAS, 1985). Faboya (1997) reports that in Nigeria alone, about 20 million gallons of waste motor oil are generated annually from mechanic workshops and discharged carelessly into the environment.

This situation is not different in Ghana as it is common to find people disposing of used motor oil on land and into drains. This is particularly evident in automobile workshops and garages throughout the country. A typical example of this practice is what pertains at Suame magazine in the Kumasi metropolis where land surfaces and a nearby stream can be described to contain oil based contaminants. The illegal dumping of used motor oil is an environmental concern with global ramification (Blodgett, 2001). Used motor oil renders the environment unsightly and constitutes a potential threat to humans, animals and vegetation (Edewor *et al.*, 2004). Hydrocarbon compounds have long been known to belong to the family of carcinogens and neurotoxic organic pollutants. Prolonged

exposure may cause the development of liver or kidney diseases, possible damage to the bone marrow and an increased risk of cancer (Mishra *et al.*, 2001). The presence of hydrocarbons in water and soil also constitutes a threat to aquatic and terrestrial flora and fauna (Edewor *et al.*, 2004).

The technologies commonly used in remediating water off petroleum hydrocarbon pollutants include evaporation, dispersion, photo oxidation, volatilization and adsorption. However, these technologies are expensive and can lead to incomplete decomposition of contaminants (Mandri and Lin, 2006). The process of bioremediation is considered a cheaper and more effective method. Bioremediation is non-destructive, cost and treatment effective and sometimes logistically favourable clean up technology that attempts to accelerate the naturally occurring biodegradation of contaminants through optimization of living conditions (Norris, 1994). Microorganisms known to be responsible for biodegradation include bacteria, fungi and yeast (Nilanjana and Preethy, 2011). Harder (2004) estimated that, bioremediation accounts for 5 to 10% of all pollution treatments and has been used successfully in cleaning up illegal dumping of used engine oil.

According to Nikolopoulou and Kalogerakis (2009), emulsification of hydrocarbons and making them bioavailable for degradation is made possible by the release of biosurfactants produced by bacteria consortium. These biosurfactants increases the oil surface area and that the right amount of oil is actually available for bacteria utilization. Research has shown that bile has this biosurfactant property due to its micelle-forming ability (Vethamuthu *et al.*, 1992c) and is responsible for solubilising dietary lipids, cholesterol and fat soluble vitamins in the gastrointestinal tract.

In this study, the biosurfactant property of bile was exploited to increase the rate of petroleum hydrocarbon (used motor oil) degradation in a fixed bed reactor.

1.2 Problem Statement

Indiscriminate dumping of used motor oil on land and into drainage systems is a common practice in Ghana especially by automobile garages. The release of oil into the environment is of environmental concern and attracts the public attention (Roling *et al.*, 2002). The presence of these pollutants in terrestrial and aquatic environments also constitutes public health and socio-economic hazards (Edewor *et al.*, 2004). It is documented that only one liter of waste engine oil is enough to contaminate one million gallons of fresh water (USEPA, 1996).

The predominant methods for degrading oil contaminants are the mechanical (separation, washing) and chemical (oxidation and photolysis) approaches. However, these methods are expensive, time consuming and do not completely degrade pollutants (Mandri and Lin, 2006). These methods are also not environmentally friendly since their applications mostly involve the transfer of pollutants from one environmental medium to the other. Environment and human health cannot be compromised, thus the motivation to join in the effort towards finding an environmentally and economically feasible solution to the treatment of hydrocarbon pollutants in water.

1.3 Objective of Study

The main objective of the study was to investigate the effect of bile on biodegradation of used motor oil contaminated water in a fixed bed bioreactor.

Specific Objectives

- To isolate hydrocarbon degrading microorganisms from oil contaminated soil
- To characterize hydrocarbon degrading microorganisms
- To investigate the effect of different volumes of bile on total petroleum hydrocarbon degradation.
- To monitor the biodegradation process by measuring some selected parameters: Dissolved Oxygen (DO), pH, Temperature, Surface tension, Microbial colony numbers, electrical conductivity and salinity.

1.4 Justification of the Study

Pollution of the environment due to indiscriminate disposal of used motor oil is a common practice in Ghana. It is done with impunity by individuals, road and bridge construction companies as well as automobile service stations. The most used methods in degrading this pollutant especially in industrial establishments are the physical/mechanical and chemical methods. However, these approaches have certain limitations. These include incomplete degradation of contaminants, cost, time, requirement of large space and environmental unfriendliness.

The environment and human health are definitely at risk if a more eco-friendly solution is not developed. Thus, this study is geared towards the development of an environmentally sound solution with economic benefits which will take care of the inefficiencies of the physical and chemical methods. Also in this era of converting wastes into resources, it is motivating to investigate the potential of bile (a slaughter house waste material), as a surfactant replacement for commercially available ones used in oil clean up technologies.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Used Motor Oil

According to Agency for Toxic Substances and Disease Registry (ATSDR) (1997), used motor oil is the brown-to-black, oily liquid removed from the engine of a motor vehicle when the oil is changed. The chemicals found in used motor oil vary depending on the brand and type of oil; whether gasoline or diesel fuel, the mechanical condition of the engine that the oil came from, and the amount of use between oil changes (ATSDR, 1997).

Substances usually present in used motor oil include metals such as zinc, magnesium, barium, and lead from engine parts and small amounts of gasoline, antifreeze, and chemicals that come from gasoline when it burns inside the engine (Roy *et al*., 1997). Also present in used oils are inorganic substances including sulphur, phosphorus, bromine and nitrogen (Roy *et al*., 1997). Used engine oil is a contaminant of concern, with large volumes entering aquatic ecosystems through water runoff.

2.2 Identified cases of used Motor Oil Pollution in the Environment

The pollution generated by discarding a ton of used oil per day is equivalent to the domestic sewer of 40,000 inhabitants, besides the fact that one liter of this substance is able to deplete the oxygen level of a million liters of water (Ambientebrasil, 2006). In Ghana, contamination of the environment due to indiscriminate release of used motor oil is quite common. This is particularly evident in automobile workshops and private garages throughout the country. For instance, at Suame magazine in the Kumasi

metropolis, it is a routine activity carried out at the full glare of city authorities. Though the exact amount of oil released annually, has not been cited in any literature, information gathered from the garages union revealed that an average of 240 liters of used oil is disposed off daily.

Similarly in Nigeria, about 20 million gallons of waste motor oil are generated annually from mechanic workshops and discharged carelessly into the environment (Adegoroye, 1997), out of which only one liter is enough to contaminate one million gallons of freshwater (USEPA, 1996). The presence of these pollutants in the terrestrial and aquatic environments constitutes public health and socio-economic hazards (Adelowo *et al.*, 2006). Also, used motor oil renders the environment unsightly and constitutes a potential threat to humans, animals and vegetation (Edewor *et al.*, 2004).

Hamblin (1995) reported that the percentage of used lubricating oil that was poured in ecosystems without any treatment were about 13% for European community and 32% for U.S.A. In Brazil, Lopes *et al.* (2008) found that a city with 200,000 inhabitants discarded daily about 47 liters of used lubricating oil into the environment and this volume was enough to pollute 47 million liters of water by causing the depletion of dissolved oxygen.

Between 1990 and 1991, sediment samples taken from water immediately downstream from a motorway in England revealed significantly elevated levels of inorganic substances (lead, zinc, chromium, copper, nickel, aluminum, calcium, and magnesium), total aromatic hydrocarbons, and PAHs, all of which had been detected in waste engine oils (Roy *et al.*, 1997). Thus this observation was attributable to indiscriminate dumping of waste engine oils around the site of contamination.

In a 1980 survey (Mahaney, 1994), it was revealed that residents living in Providence and Rhode Island generated an estimated amount of 44 metric tons of waste motor (crankcase) oil, which they dumped onto roads and into storm sewers without any prior treatment. In the United States also, a national survey of disposal methods indicated that 40% of used mineral-based crankcase (motor) oil was poured directly onto the ground without any treatment (Brinkman *et al.*, 1982).

In 2002 alone, Permitting, Environment and Regulatory Affairs in Miami-Dade County responded to over 300 incidents of careless disposal of motor oil, which varied from abandoned buckets and drums full of oil to contamination of storm drains and oil-soaked soils. This costs nearly $45,000 a year to remedy (www.miamedade.gov/derm/tips_oil_disposal.asp). Whatever the origin of contamination, some petroleum or decomposition products may reach groundwater reserves and surface waters providing water for domestic and industrial use (Adebusoye *et al.*, 2006).

2.3 Effects of Oil Pollution

Oil in water causes depletion of dissolved oxygen due to transformation of the organic components into inorganic compounds (Onwurah *et al.*, 2007). In mammals it possesses an anticoagulant potency (Onwurah, 2002a). Even species which are not directly in contact with oil can be harmed by a spill. Predators that consume contaminated prey can be exposed to oil through ingestion. Sometimes a local population of prey organisms is destroyed, leaving no food resources for predators (www.epa.gov/oem/docs/oil/edu/oilspill_book/chap1.pdf.).

Oil spill in the environment could also lead to increased exposure of by-products of Polycyclic Aromatic Hydrocarbons (PAHs) to a given human population and this may increase the risk of mortality from infectious diseases (Hall, *et al.*, 2006). Used motor oil contains more heavy metals and PAHs that can contribute to chronic hazards including carcinogenicity. Also, the indiscriminate burning of used lubricant oil, without prior treatment or de-metallization, generates significant metallic oxides emissions, besides other toxic gases, such as the dioxins and sulphur oxides (Ambientebrasil, 2006). All these emissions have possible harmful effects on the internal organs of man and other animals. Apart from the possible hazards to health such as liver damage and skin problems, such contamination is objectionable because of the very low concentration at which Polycyclic Hydrocarbons (PHC) and associated compounds can be detected by their smell and taste (Adebusoye *et al.*, 2006).

Oil is also associated with reproductive problems in birds as it smothers eggs by sealing pores in the eggs and preventing gas exchange (www.epa.gov/oem/docs/oil/edu/oilspill_book/chap1.pdf). Scientists have also observed some developmental defects in bird embryos that were exposed to oil. Long-term reproductive problems have also been shown in some studies with laboratory animals that were exposed to oil (www.epa.gov/oem/docs/oil/edu/oilspill_book/chap1.pdf).

2.4 Biological Approaches to the Cleanup of Hydrocarbon Contaminants

The technologies commonly used in remediating water off petroleum hydrocarbon pollutants include evaporation, chemical oxidative reduction, photo oxidation and volatilization. However, these technologies are expensive and can lead to incomplete decomposition of contaminants (Mandri and Lin, 2006). The processes of bioremediation

and natural attenuation are considered cheaper and more effective and are discussed below.

2.4.1 Bioremediation

Bioremediation is a technology that exploits the abilities of microorganisms and other natural habitats of the biosphere to improve environmental quality for all species, including man (Onwurah *et al*., 2007). This technology accelerates the naturally occurring biodegradation under optimized conditions such as oxygen supply, temperature, pH, the presence or addition of suitable microbial population and nutrients, water content and mixing (Trindade *et al*., 2005).

In oil contaminated sites, this technique makes use of indigenous oil – consuming microorganisms, called petrophiles, by enhancing and fertilizing them in their natural habitats. Petrophiles are very unique organisms that can naturally degrade large hydrocarbons and utilize them as a food source (Harder, 2004). It is estimated that bioremediation accounts for 5 to 10 percent of all pollution treatment and has been used successfully in cleaning up the illegal dumping of used motor oil (Harder, 2004).

Bioremediation systems have potentially broad-spectrum applications including ground water, soils, lagoons, sludge and process waste-streams, and it has been used in very large scale applications such as the shoreline cleanup efforts in Alaska, resulting from the oil tanker "Exxon Valdez" oil spill in 1989 (Caplan, 1993). Bioremediation technique can be as simple as applying a garden fertilizer to an oil-contaminated beach, or as complex as an engineered treatment "cell" where water or soils are manipulated, aerated, heated, or

treated with various chemical compounds to promote degradation (Hildebrandt and Wilson, 1991).

In the application of bioremediation technology four important aspects are necessary and these are microbial composition, contaminant type, geology of polluted medium and chemical conditions at the contaminated medium. Depending on these factors the choice of bioremediation approach may be decided upon, that is whether to apply biostimulation or bioaugmentation approaches (Aichberger *et al.*, 2005).

In biostimulation approach, the growth of indigenous oil degraders is stimulated by the addition of nutrients or other growth-limiting co-substrates. Biostimulation can also be achieved by enhancing oxygen concentration through the injection or infusion of air, or through the addition of slow oxygen releasing compounds (Nilanjana and Preethy, 2011).

Bioaugmentation, is the approach in which known oil degrading bacteria are added to supplement an existing microbial population (Nilanjana and Preethy, 2011). Usually, if the indigenous microbial population is low or inadequate (for example, due to toxicity) key microorganisms can be isolated from the site, grown up in large volumes, and used for inoculation.

Oh and Kim (2000) reports that, in oil polluted fresh water medium biostimulation is often applied due to the limitation of nitrogen and phosphorus when oil is introduced into water. This is achieved by adding formulations containing oleophilic fertilizers. In marine environments however, a combination of biostimulation and bioaugmentation may be

required due to conditions, such as low water temperature, low concentration of oil degrading microorganisms and inorganic nutrients.

Bioremediation is considered a cheaper approach as compared to other treatment methods due to reduced time required for monitoring, reporting and management, as well as reduced need for maintenance, labour, and supplies (National Research Council, 1993). In addition, bioremediation technology is believed to be noninvasive and relatively cost-effective (April *et al.*, 2000). It eliminates waste effectively, eliminates long-term liability, and has greater public acceptance, with regulatory encouragement, and it can be coupled with other physical or chemical treatment methods (Boopathy, 2000).

The potential of bioremediation has successfully been demonstrated. Booyjzsen (2007) investigated the efficiency of bioremediation in remediating motor oil contaminated water. Results from her work showed a 98% degradation of the motor oil. Under normal operating conditions, Enid *et al.* (2006) observed a 97 to 99% of total hydrocarbons removal within only 14 min hydraulic retention time in a bioremediation application. Also, according to Anjana and Meenal (2009), laboratory investigation of diesel bioremediation in soil within 30 days showed evidence of the degradation of long chain hydrocarbons into short chain hydrocarbons.

2.4.2 Natural Attenuation

Another form of bioremediation is natural attenuation which is a passive process involving physical and chemical changes occurring over time, reducing the mass, toxicity and mobility of contaminant chemicals (EPA, 2006). Barker *et al.* (1987) used the term "natural attenuation" to describe the combined dilution, dispersion, sorption and

biodegradation processes that caused contaminant concentrations to decrease. It is one of the least expensive technologies where natural exposure of pollutants allows water/soil rehabilitation. It is extremely slow, but occurs ubiquitously, thus, indirectly protecting human health and environment.

Five processes dominate natural attenuation: biodegradation, sorption, dispersion/dilution, volatilization, and chemical reactions (EPA, 1999). Of these five processes, the most environmentally significant is biodegradation encompassing all of the changes in chemical contaminants performed by microorganisms, such as bacteria and fungi (Chapelle, 1999). This implies natural attenuation can be attributed mostly to biodegradation. Chiang *et al.* (1989) found out significant losses of benzene mass over a 3-year period in a contaminated aquifer underlying a gas plant, and attributed this to natural attenuation process as no efforts were made to clean up the contaminants prior to their investigation.

On a much larger scale, a study of 7167 municipal supply wells in California showed that, while leaking gasoline was by far the most common contaminant being released into ground water systems, benzene (component of gasoline) was found in only 10 wells (Hadley and Armstrong, 1991). This study concluded that natural attenuation processes were actively consuming Benzene, Toluene, Ethylbenzene and Xylene (BTEX) compounds, thereby protecting California ground water supplies from widespread petroleum hydrocarbon contamination. However, this kind of treatment is usually only allowed in situations where the spread of contamination and further harm to the environment are unlikely and usually requires careful and consistent monitoring (Booyjzsen, 2007).

2.5 Biodegradation of Petroleum Hydrocarbon Pollutants

Biodegradation is the transformation or breakdown of substances into simpler components through the biochemical reactions of microorganisms such as bacteria, yeasts and fungi (Hemond and Fechner, 2000). There are two types of biodegradation, primary biodegradation or biotransformation and ultimate biodegradation or mineralization.

The former is the destruction of the molecule by metabolic activity of microorganisms such that the chemical properties of the molecule are lost or altered. Ultimate biodegradation is the complete breakdown of the compound to carbon dioxide, methane, water, mineral salts and biomass (Scott and Jones, 2000). Susceptibility of a hydrocarbon to microbial degradation varies with type and size of hydrocarbon molecule (Ulrici, 2000). Alkanes of intermediate chain length (C_{10}-C_{24}) are often degraded rapidly, while very long chain alkanes are increasingly resistant to microbial degradation (Ijaha and Antaib, 2003). Long chain hydrocarbons and cyclic-alkanes are known to be recalcitrant to microbial degradation (Bagherzadeh *et al.*, 2008) and methyl branches on aliphatic hydrocarbons tend to increase resistance to microbial attack (Pirnik, 1977).

Some compounds such as the high molecular weight polycyclic aromatic hydrocarbons may not degrade at all (Atlas, 1992). The biodegradation of aromatic compounds was found to involve an oxidative attack upon the ring structure, the formation of a diol followed by ring cleavage, and the formation of organic acids (Gibson, 1971). For aromatic compounds, simple methyl substitutions on benzene ring such as in toluene or various xylene isomers were found to enhance biodegradability (Gibson and Subamanian, 1984).

2.5.1 The Role of Microbial Flora in Biodegradation

Microorganisms capable of degrading hydrocarbons are common and widely distributed in nature (Van Hamme *et al*., 2003) and they do so mainly in order to produce energy and biomass and also to reduce toxicity and to perform other functions. Zobell (1946) reports that, the degradation of petroleum hydrocarbons by microorganisms has been known since the 1940's. Typical bacterial groups already known for their capacity to degrade hydrocarbons include *Pseudomonas, Marinobacter, Alcanivorax, Microbulbifer, Sphingomonas, Micrococcus, Cellulomonas*, *Dietzia*, and *Gordonia* groups (Brito *et al*., 2006). Molds belonging to the genera *Aspergillus, Penicillium, Fusarium, Amorphoteca, Neosartorya, Paecilomyces, Talaromyces, Graphium* and the yeasts *Candida, Yarrowia* and *Pichia* have been implicated in hydrocarbon degradation (Chaillan *et al*., 2004).

Microbial degradation is the major and ultimate natural mechanism by which one can clean-up the petroleum hydrocarbon compounds pollutants from the environment (Lal and Khanna, 1996). However each individual strain is usually characterized by an ability to utilize only a few kinds of hydrocarbons. Yeasts, for example, can oxidize only the aliphatic hydrocarbons (Okpokwasili and Ibe, 1987), so mixed cultures with overall broad enzymatic capacities are required to increase the rate of petroleum biodegradation (Bagherzadeh *et al*., 2008). Such bacterial genera as *Acinetobacter, Arthrobacter, Bacillus, Corynebacterium*, *Flavobacterium, Vibrio* and *Pseudomonas* contain species that together can degrade most constituents of crude oil, including the aliphatic, alicyclic, aromatic, and polycyclic hydrocarbons (Ko and Lebeault, 1999).

Rambeloarisoa *et al*., (1984) reported that a mixed culture, containing 8 strains of 6 genera, could effectively degrade crude oil. Interestingly, only 5 strains between them

could grow as pure cultures on different hydrocarbons. However, when the other 3 strains were removed from the culture, the effectiveness of the mixed cultures was remarkably reduced. These results showed that, each member in the microbial community had significant role and depended on the presence of the other species or strains for survival. It is possible that one species can remove the toxic metabolites of the other species, or degrade some compounds better than others (Alexander, 1999).

Also, many organisms can metabolize aliphatic hydrocarbons that they cannot use directly as carbon sources for growth (Karpouzas *et al.*, 2000) but which others may require. This phenomenon is referred to as co-metabolism or co-oxidation. In demonstrating co-metabolism, a carbon source supporting growth is provided to the organisms together with the target substrate, and the latter is then oxidized concurrently with the former (Adebusoye *et al.*, 2006).

2.5.2 Factors Affecting Biodegradation

Hydrocarbon biodegradation is affected by a number of factors including oxygen and nutrient supply, temperature, pH, light (photo) and metabolic end products. According to Leila *et al.* (2006), aerobic conditions are generally considered necessary for extensive degradation of oil hydrocarbons in the environment since major degradative pathways for both saturates and aromatics involve oxygenases. Under aerobic conditions, microorganisms will convert many organic contaminants to carbon dioxide, water and other chemicals (nitrates, sulphates).

Invariably nutrients are also very essential for successful biodegradation of hydrocarbon pollutants especially nitrogen, phosphorus, and in some cases iron and when in limiting

supply affect biodegradation processes (Cooney, 1984). Atlas (1985) reported that, when a major oil spill occurred in marine and freshwater environments, the supply of carbon was significantly increased and the availability of nitrogen and phosphorus generally became the limiting factor for effective microbial action on oil contaminant.

The rate of biodegradation generally decreases with decreasing temperature. Highest degradation rates generally occur in the range of 30–40 °C in soil environments, 20–30 °C in some freshwater environments, and 15–20 °C in marine environments (Bossert and Bartha, 1984). Biodegradation can occur under a wide-range of pH; however, 5.5<pH<8.5 pH range have been determined as optimal for microbial activity in fresh water (Allia *et. al.*, 2006). Recent studies have also reported that photo-oxidation increases the biodegradability of petroleum hydrocarbon by increasing its bioavailability and thus enhancing microbial activities (Maki *et al.*, 2005) and as well could be a limiting factor in petroleum biodegradation.

Metabolic by-products also affect biodegradation as Margesin and Schinner (2001) reports that by-products of the biodegradation of aromatic hydrocarbons (saturate fraction) hinders microbial activities which could explain the persistence of aromatic petroleum hydrocarbons in the environment. Moreover, it was discovered in 1984 that the degradation products of Alky Phenol Ethoxylate (APE) are 10 times more toxic than the precursor surfactant (Booyjzsen, 2007). Thus it can be stated that the chemistry of biodegradation of hydrocarbons is of great importance when considering potentially detrimental environmental implication.

2.6 Pathways of Petroleum Biodegradation

2.6.1 Aerobic Biodegradation

The most rapid and complete degradation of the majority of organic pollutants is brought about under aerobic conditions (Nilanjan and Preethy). Aerobic biodegradation is the breakdown of organic contaminants by microorganisms when oxygen is present (Nilanjana and Preethy, 2011). Research has shown that degradative organisms need oxygen at two metabolic sites, at the initial attack of the substrate and at the end of the respiratory chain. Primary attack on intact hydrocarbons always requires the action of oxygenases and therefore requires the presence of free oxygen (Okoh, 2006). Enzymatic key reactions of aerobic biodegradation are oxidations catalyzed by oxygenases and peroxidases. Oxygenases are oxidoreductases that use molecular (O_2) to incorporate oxygen into the substrate.

Oxidation of alkanes is classified as being terminal (mono) or di-terminal (sub). The mono-terminal oxidation is the main pathway. It proceeds via the formation of the corresponding alcohol, aldehyde, and fatty acid. The sub terminal oxidation occurs with lower and longer alkanes with the formation of a secondary alcohol and subsequent ketone. Unsaturated 1- alkenes are oxidized at the saturated end of the chains. The first step of benzene oxidation is a hydroxylation catalyzed by a dioxygenase. The product, a diol, is then converted to catechol by a dehydrogenase. These initial reactions, hydroxylation and dehydrogenation, are also common to pathways of degradation of other aromatic hydrocarbons (www.wiley-vch.de/books/biotech/pdf).

Equations 1 and 2 below illustrate terminal and sub-terminal alkane degradation.

Equation 1. Terminal oxidation of alkanes.

$$R.CH_2.CH_2CH_2CH_3 \rightarrow R.CH_2CH_2CH_2CH_2\,OH \rightarrow R.CH_2CH_2CH_2CHO \rightarrow$$

$$R.CH_2CH_2CH_2CH_2\,OH \rightarrow R.CH_2.CO_2\,H + CH_3.CO_2\,H$$

Equation 2. Sub-terminal oxidation of alkanes

$$R.CH_2.CH_2CH_3 \rightarrow R.CH_2CH(OH)CH_3 \rightarrow R.CH_2COCH_3 \rightarrow R.CH_2(OH) \rightarrow CH_3.CO_2\,H$$

Adopted from (www.eolss.net/Sample-Chapters/C06/E6-13-01-02.pdf)

Theoretically, the oxygen demand to degrade oil contaminants is 3.5 g of oil oxidized per g of oxygen (Floodgate, 1979). In reference to marine environment, Zobell (1969) calculated that, dissolved oxygen in 3.2×10^5 liters of seawater is required for the complete oxidation of 1 liter of oil. In practice, to convert one pound of hydrocarbon material into carbon dioxide and water requires between 3 and 5 pounds of available oxygen (USEPA, 2004).

Many investigations have demonstrated the key role of oxygen for bioremediation of petroleum contaminants. Hambrick *et al.,* (1980) found out that, at pH values between 5 and 8, mineralization of hydrocarbons in estuarine sediments was highly dependent on oxygen availability and that rates of hydrocarbon degradation decreased with decreasing oxygen reduction potential. Ward and Brock (1978) similarly found that hexadecane was rapidly mineralized in freshwater lake sediments under aerobic conditions but that almost no hydrocarbon mineralization occurred under anaerobic conditions. Addition of nitrate and sulfate, in the study, failed to increase hydrocarbon mineralization under anaerobic conditions.

2.6.2 Anaerobic Biodegradation

Previously, anaerobic processes were thought to contribute negligibly to hydrocarbon biodegradation and were often discounted (Townsend *et al.*, 2003), but recent research has brought to light the role anaerobic processes play in natural attenuation, specifically in saturated and aquatic systems where oxygen supply is limited. Anaerobic digestion is a series of processes in which microorganisms break down biodegradable material in the absence of oxygen. It is widely used to treat wastewater sludge and biodegradable waste because it provides volume and mass reduction of the input material (Danny, 2010).

Numerous petroleum hydrocarbons are susceptible to biodegradation in anaerobic environments, including Benzene, Toluene, Ethyl benzene and Xylene (BTEX) (Kao and Wang, 2000), Methyl Tert-Butyl Ether (MTBE) (Bradley *et al.*, 2001), *n*-alkanes (Chayabutra and Lu, 2000), number 2 diesel fuel (Boopathy, 2004), polycyclic aromatic hydrocarbons (PAH) (Schmitt *et al.*, 1996), and crude oil (Bekins *et al.*, 2001).

Anaerobic biodegradation follows different biochemical pathways dependent on the electron acceptor utilized by the microorganism. Petroleum-based contaminants have been shown to degrade under various anaerobic conditions, including nitrate reduction, sulfate reduction and ferric iron reduction conditions. There are now several pure culture examples of nitrate-reducing, Fe (III)-reducing, and sulfate-reducing bacteria that are capable of completely oxidizing some of these hydrocarbon contaminants to carbon dioxide (Coates *et al.*, 1996).

Studies using nitrate as a terminal electron acceptor has been documented to be successful in aiding biodegradation of numerous petroleum-based contaminants.

Boopathy (2004) reported 47 % reduction in No. 2 diesel fuel from contaminated soils using nitrate amendment and native microorganisms. Also, Chayabutra and Lu (2000) demonstrated 40 % reduction of *n*-hexadecane by a pure culture of *Pseudomonas aeruginosa* under denitrifying conditions following an initial oxic period.

Sulfate is generally more abundant in groundwater systems than nitrate since sulfate is a micronutrient, required only in small amounts for cell biomass. Relatively high concentrations at typical field sites make sulfate a likely electron acceptor, as reported by Cho *et al.* (1997), who stoichiometrically linked it, to approximately 66 % of BTEX biodegradation in a jet fuel contaminated site. Moreover, Lovley *et al.*, (1994) documented the complete mineralization of benzene by un-amended sulfate reduction, the first report of a successful, un-amended anaerobic study at that time (Chapelle, 1999). Also a moderately thermophilic (optimum around 60 °C) sulfate-reducing bacterium isolated on *n*-decane consumed *n*-alkanes from crude oil especially in the range from C_8 to C_{11} (Rueter *et al.*, 1994).

Ferric iron reduction has been called "the most important chemical change that takes place in the development of anaerobic soils and sediments" (Ponnamperuma, 1972). This is because it is responsible for the regulation of many soil and groundwater systems, including the oxidation of organic matter and the distribution of phosphate and trace metals (Lovley, 1991). Ferric iron reducing microorganisms were the first anaerobic bacteria identified capable of degrading petroleum contaminants in laboratory studies providing the basis for future research focusing on anoxic systems (Chapelle, 1999).

Finneran and Lovely (2001) demonstrated that ferric iron utilizing microorganisms were capable of complete mineralization of MTBE, often thought to be recalcitrant in anaerobic systems. This mineralization was completed without the formation of Tert-Butyl Alcohol (TBA), a toxic byproduct of incomplete MTBE degradation. Kao and Wang (2000) also demonstrated 93.1 % reduction of BTEX within the iron-reducing zone of a gasoline-contaminated aquifer during an *in situ* bioremediation study.

2.7 Surfactants

The influence of surfactants on biodegradation of hydrocarbons has continued to be a stimulating topic of research and discussion (Boonchan *et al.*, 1998). They are amphipathic molecules consisting of a hydrophilic polar head and a hydrophobic non polar tail. Surfactants form a group of chemicals with a high overall environmental relevance, due to a combination of their inherent environmental properties and their very large production volume (Rouse *et al.*, 2001). Generally, surfactants are classified under the following groupings; anionic, having one or more negatively charged groupings and cationic, having one or more positively charged groupings. Non-ionic have no ionic constituents or groupings and amphoteric contain both anionic and cationic groupings (REMTECH, 2008).

REMTECH (2008) reported that surfactants are able to lower the surface tension of water from 72 dynes to <50 dynes and some are able to lower surface tension to <30 dynes. Surfactants tend to migrate to surfaces and alter free energy states or interfacial tensions, which can result in the formation of emulsions, greatly increasing the interfacial area between immiscible liquids (example, oil and water) (Rouse *et al.*, 2001). Both reduction

of free energy and increase in surface area can contribute to an enhanced rate of desorption or dissolution.

Another characteristic of surfactants is their tendency to form aggregates, or micelles, when the critical micelle concentration (CMC) is surpassed (Rosen, 1989). Partitioning of hydrocarbons into the micellar core, or solubilization, greatly increases the concentrations of poorly soluble compounds in solution. Solubilization potential can be characterized by the micelle-water partition coefficient, KM, and the Molar Solubilization Ratio, MSR (Edwards *et al.*, 1991).

Commercial and environmental uses of surfactants include: storage tank and parts cleaning, off-shore dispersants, and shoreline spill clean-up, In-situ Surfactant Enhanced Aquifer Remediation (SEAR), oil recovery from oil sand and oil-shale and improving oil recovery from production wells (REMTECH, 2008).

It is recommended that, in choosing a suitable surfactant for application in bioremediation, the following factors be considered; minimal tendency to form liquid crystals, gels, or macroemulsions. The surfactant should have high contaminant solubilization potential, environmentally acceptable and biodegradable. Low critical micelle concentration and amenable to recycling (Rouse *et al.*, 2001).

However some inhibitions to biodegradation of organic compounds have been observed upon addition of surfactants. This is demonstrated in the work done by Rouse *et al.* (2001) in which they used anionic diphenyloxide disulfonate (DPDS) surfactants (C_{12} and C_{16} alkyl moieties) with naphthalene in aqueous systems to investigate the influence of

these surfactants on biodegradation of hydrocarbons. The overall trend for both C_{12}- and C_{16}-DPDS assays indicated decreasing efficiency in degradation of naphthalene with increasing surfactant concentration.

2.8 Biosurfactants

Biosurfactants are microbially produced compounds which exhibit surface activity. All biosurfactants are amphiphiles, they consist of two parts, a polar (hydrophilic) moiety and non polar (hydrophobic) group (Pacwa-Płociniczak *et al.*, 2011). A hydrophilic group consists of mono-, oligo- or polysaccharides, peptides or proteins and a hydrophobic moiety usually contains saturated, unsaturated and hydroxylated fatty acids or fatty alcohols (Lang, 2002). Biosurfactants have applications in environmental protection, crude oil recovery, agriculture, mining, health care and food processing industries.

In comparison to their chemically synthesized (surfactants) equivalents, they have many advantages. According to Das *et al.* (2008) they are environmentally friendly, biodegradable, less toxic and non-hazardous. They have better foaming properties and higher selectivity. They are active at extreme temperatures, pH and salinity as well, and can be produced from industrial wastes and from by-products. This last feature makes cheap production of biosurfactants possible and allows utilizing waste substrates and reducing their polluting effect at the same time.

As environmental compatibility is becoming an increasingly important factor in the selection of industrial chemicals, the use of biosurfactants in environmental application, such as biodegradation and dispersion of oil spills is increasing. Due to their amphiphilic structure, biosurfactants increase the surface area of hydrophobic water-insoluble

substances, increase the water bioavailability of such substances and change the properties of the bacterial cell surface (Pacwa-Płociniczak *et al.*, 2011).

Table 2.0 Classification of biosurfactants and their uses in environmental biotechnology.

Biosurfactant		**Microorganism**	**Applications in Environmental Biotechnology**	**References**
Class	**Group**			
Glycolipids	Rhamnolipids	*Pseudomonas aeruginosa*	Enhancement of emulsification of hydrocarbons	Sifour *et al.*, 2007
	Trehalolipids	*Arthrobacter* sp.,	Enhancement of the bioavailability of hydrocarbons	Franzetti *et al.*, 2010
	Sophorolipids	*Torulopsis petrophilum*	Recovery of hydrocarbons from mud	Whang *et al.*, 2008
Fatty acids, phospholipids and neutral lipids	Corynomycolic acid	*Corynebacterium lepus*	Enhancement of bitumen recovery	Gerson *et al.*, 1978
	Spiculisporic acid	*Penicillium spiculisporum*	dispersion action for hydrophilic pigments;	Ishigami *et al.*, 1983
Lipopeptides	Surfactin	*Bacillus subtilis*	Enhancement of the biodegradation of hydrocarbons	Jennema *et al.*, 1983
	Lychenysin	*Bacillus licheniformis*	enhancement of oil recovery	Thomas *et al.*, 1993
Polymeric biosurfactants	Emulsan	*Acinetobacter calcoaceticus* RAG-1	Stabilization of the hydrocarbon-in-water emulsions	Zosim *et al.* (1982)
	Alasan	*Acinetobacter radioresistens* KA-53		Toren *et al.*, 2001
	Biodispersan	*Acinetobacter calcoaceticus* A2	Dispersion of limestone in water	Rosenberg *et al.*, 1988
	Liposan	*Candida lipolytica*	Stabilization of hydrocarbon-in-water emulsions	Cirigliano *et al.*, 1984
	Mannoprotein	*Saccharomyces cerevisiae*		Cameron *et al.*, 1988

Modified from Pacwa-Płociniczak *et al.* (2011)

2.8.1 Role of Biosurfactants in Biodegradation Processes

Biosurfactants enhance hydrocarbon bioremediation by two mechanisms. The first includes the increase of substrate bioavailability (by mobilization and solubilization/emulsification) for microorganisms, while the other involves interaction with the cell surface which increases the hydrophobicity of the surface allowing hydrophobic substrates to associate more easily with bacterial cells (Mulligan and Gibbs, 2004).

The mobilization mechanism occurs at concentrations below the biosurfactants critical micelle concentration (CMC). At such concentrations, biosurfactants reduce the surface and interfacial tension between air/water and soil/water systems. In turn, above the biosurfactants CMC, the solubilization process takes place. At these concentrations biosurfactants molecules associate to form micelles, which dramatically increase the solubility of oil (Pacwa-Płociniczak *et al*., 2011). The process of incorporation of these molecules into a micelle is known as solubilization (Urum and Pekdemir, 2004).

According to Franzetti *et al*. (2010) emulsification is a process that forms a liquid, known as an emulsion, containing very small droplets of fat or oil suspended in a fluid, usually water. The high molecular weight biosurfactants are efficient emulsifying agents. Inference to cell hydrophobicity, high cell-hydrophobicity allows microorganisms to directly contact oil drops and solid hydrocarbons while low cell-hydrophobicity permits their adhesion to micelles or emulsified oils (Franzetti *et al*., 2010).

Barkay *et al*. (1999) tested the effect of the bioemulsifier alasan produced by *Acinetobacter radioresistens* KA53 on the solubilization of polyaromatic hydrocarbons,

Barkay *et al.* (1999) tested the effect of the bioemulsifier alasan produced by *Acinetobacter radioresistens* KA53 on the solubilization of polyaromatic hydrocarbons, phenanthrene and fluoranthene and also, influence of alasan on mineralization of phenanthrene and fluoranthene by *Sphingomonas paucimobilis* EPA505. They concluded that aqueous solubility of phenanthrene and fluoranthene increased linearly in the presence of increasing concentrations of bioemulsifier (50 to 500 $\mu g \cdot mL^{-1}$) and that mineralization of PAHs by *S. paucimobilis* EPA505 was stimulated by the presence of alasan.

Also, Kang *et al.* (2010) used sophorolipid in studies on biodegradation of aliphatic and aromatic hydrocarbons and Iranian light crude oil under laboratory conditions. Addition of this biosurfactant increased biodegradation of tested hydrocarbons with the rate of degradation ranging from 85% to 97% of the total amount of hydrocarbons. Their results indicated that sophorolipid may have potential for facilitating the bioremediation of sites contaminated with hydrocarbons (with limited water solubility) by increasing the bioavailability of contaminants for biodegradation.

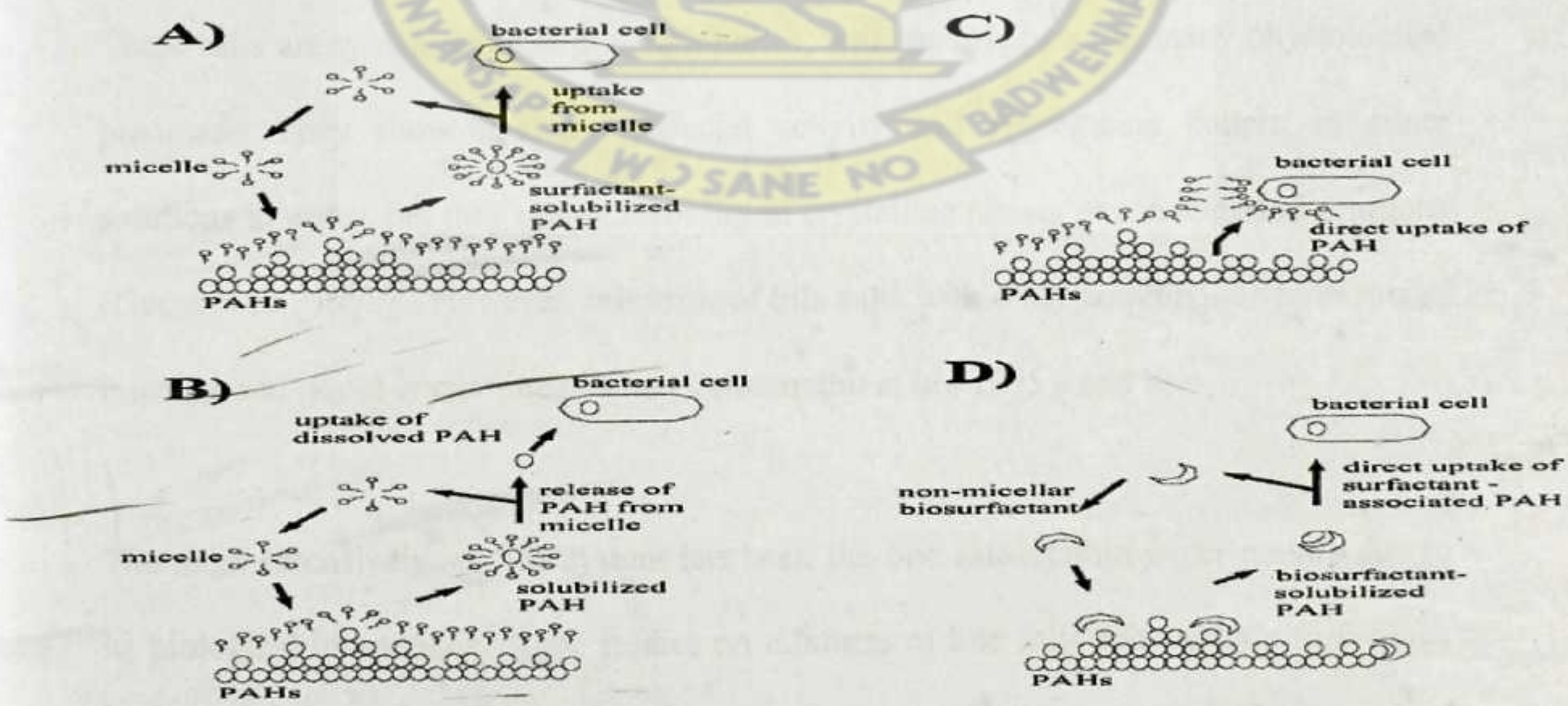

Fig 2.0 Surfactant micelle formation and uptake of PAHs by bacteria (REMTECH, 2008)

2.9 Bile Salts

Bile is a bitter-tasting, dark green to yellowish brown fluid, produced by the liver of most vertebrates that aids the process of digestion of lipids in the small intestine (en.wikipedia.org/wiki/Bile). In many species, bile is stored in the gallbladder and upon eating is discharged into the duodenum. Bile is a composition of the following materials: water (85%), bile salts (10%), mucus and pigments (3%), fats (1%), inorganic salts (0.7%) and cholesterol (0.3%).

Bile salts are synthesized from cholesterol in the liver. These are derivative of Cholanic acid that possesses a four-ring steroid nucleus and a five-carbon side chain terminating in a carboxylic acid. The arrangement of the rings is such that all the hydrophilic parts such as the hydroxyl groups and the carboxylic acid side arm positioned on one plane and the hydrophobic parts remain on the other, thus bile salts have a planar polarity. Because of the presence of amphiphilic nature and planar polarity, bile salts tend to aggregate in water (Swapnadip, 2010).

These salts are typical anionic bioamphiphiles, which participates in many physiological processes. They show unique interfacial activity and aggregation pattern in dilute solutions in water, but they do not form liquid crystalline phases in concentrated solutions (Gupta *et al.*, 1979). However, mixtures of bile salts with other amphiphiles form mixed micelles and liquid crystalline phases (Vethamuthu *et al.*, 1995 a and b).

The most extensively studied system has been the bile salt-lecithin-water mainly due to its biological importance. Some studies on mixtures of bile salts and cationic surfactants have also been reported. The role of bile salts as physiological surfactants is based on

their micelle-forming properties (Vethamuthu *et al*., 1992c). The addition of a bile salt to amphiphile aggregates usually increases the average head group area (referred to as the steric effect of a bile salt) and transforms these aggregates into highly curved ones, thus, increasing the surfactant ability of the combined product (Long *et al*., 1994).

2.9.1 Bile Salts as Biosurfactants

Bile acts as a surfactant, helpS to emulsify the fats in foods. Bile salts are regarded as the most important "bio-surfactants" because of their unusual solubilizing and emulsifying capacity (Swapnadip, 2010). The main function of bile salt is to solubilize dietary lipids, cholesterol and fat soluble vitamins in the gastrointestinal tract. The solubilization quality of bile salts is due to their micellization capacity (Vethamuthu *et al*., 1992c). In view of their unique molecular structure, they do not behave like conventional surfactants that contain a clear-cut polarity gradient between the hydrophilic and hydrophobic parts. They show distinct behavior with respect to self–association and molecular solubilization. They are also biocompatible and biodegradable (Hoffman *et al*., 1999).

Lingxiang *et al*. (2008) conducted an investigation and found out that, Vesicle-to-Micelle Transition (VMT) can be realized in catanionic surfactant systems by the addition of two kinds of bile salts, Sodium Cholate (SC) and Sodium DeoxyCholate (SDC). The facial amphiphilic structure and large occupied area of the bile salt played a crucial role in the enlargement of the average surfactant head group area that resulted in the VMT. Bile salts, thus, can react synergistically with other surfactants to increase solubilization of hydrocarbon compounds in aqueous systems.

CHAPTER THREE

3.0 METHODOLOGY

3.1 Materials and Chemicals

Used motor oil was obtained from Suame magazine industrial area. Soil samples were also obtained from three different sites at the magazine and fresh cattle bile from Kumasi abattoir. Chemicals for nutrient medium (2.0g of $Na_3HPO_4.2H_2O$, 0.8g of KH_2PO_4, 0.2g of $MgSO_4$ and 1.8g of $(NH_4)SO_4$) were obtained from the department of chemistry KNUST, Kumasi. Plate Count Agar (PCA) and Nutrient Agar (NA) were purchased from Amos chemical supplies.

3.2 Equipment

The following equipments; Stuart's scientific automated colony counter, pH meter, YSI 550A temperature/DO and Krüss tensiometer used in this work were obtained from Department of Theoretical and Applied Biology, Fisheries Department and Department of Chemistry, KNUST respectively. Varian CP-3800 GC – FID was out sourced at Ghana Standards Authority, Accra.

3.3 Baseline Analysis of Used Motor Oil, Bile and Pipe Borne Water

The pH, surface tension, Total Petroleum Hydrocarbons (TPH), phosphorus and nitrogen contents, of used motor oil, bile and pipe borne water where determine to obtain information that will serve as a precedent for relevant comparisons and conclusions to be drawn at the end of the study. pH was determined with a pH meter, surface tension with a Krüss tensiometer, THP with a GC – FID after solvent extraction in methylene chloride (GC-grade), nitrogen content using the Kjeldhal's method and phosphorus using sprectrophotometric method .

3.4 Soil Sample Collection

Soil samples were collected from three different oil contaminated sites at Suame magazine. Soil samples were collected randomly 5-10 cm beneath the soil surface using a spatula and packed in sterile polythene bags (Ojo, 2006). Soil samples were immediately transferred to the laboratory for processing.

3.5 Designed Bioreactor used in the Study

The bioreactor used in this study is shown in Fig 3.0 below. The first three sets of reactors were supplied with oxygen to support aerobic degradation of contaminants, whiles the last set of reactors were made anoxic to make provision for anaerobic degradation of contaminants that otherwise might not be degraded by aerobes.

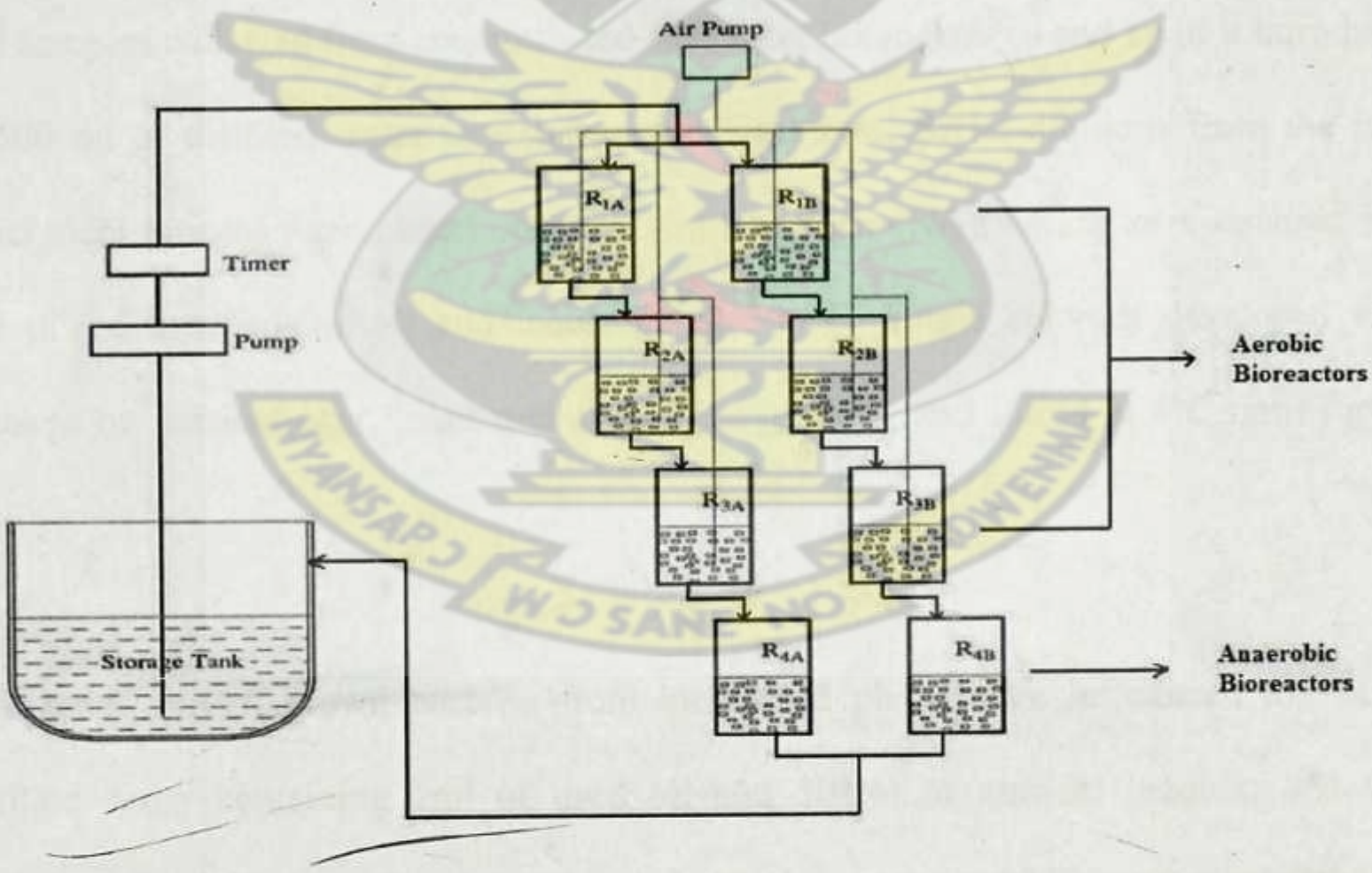

Fig 3.0 Bioreactor Set up

3.6 Microbial Support Material

Bamboo pieces of lengths 2-3 cm and 0.3-0.5 cm diameters were provided in the reactors as microbial support material. Bamboo has the advantages of absorbing very little

moisture and it provides a large surface area for microbial attachment (www.ehow.com). Its leathery surface reduces the tendency of absorption oil into it (www.guaduabamboo.com/bamboo-glossary.html). It is also biodegradable, thus allowing for microbial degradation under favourable conditions when disposed off after use.

3.7 Nutrient Medium

The nutrient medium used in the experiment comprised of; 2.0 g of $Na_2HPO_4.2H_2O$, 0.8 g of KH_2PO_4, 0.2 g of $MgSO_4$ and 1.8g of $(NH_4)SO_4$/L of distilled water (Fei-Baffoe, 2003).

3.8 Isolation of Heterogeneous Hydrocarbon Degrading Microorganisms

Soil samples collected from contaminated sites were homogenized and 1g of it introduced to 500 ml of distilled water and shaken for 5 minutes. Serial dilutions from the third enrichment process were plated out onto Nutrient Agar (NA), which were covered with 100 µL of used engine oil and incubated at 30 °C. Single colonies developed were streaked on nutrient agar, incubated at 37 °C overnight, and stored at 4°C until further use.

Two loops full of grown bacteria from inoculated plates were introduced to 1 L of distilled water containing 1ml of used oil and 50 ml of nutrient medium and then incubated for 72 hours. To determine microbial growth, turbidity measurements and heterotrophic plate counts were conducted. 1000 ml of the resulting liquid inoculum was dispensed aseptically into each reactor as microbial biomass.

3.9 Characterization of Isolates

The oil degrading isolates were characterized by gram stain and biochemical tests.

Gram Staining

Staining Protocol

A small drop of sterile distilled water was placed on a sterilized grease free slide. A small quantity of bacteria transferred from nutrient agar and placed on the slide with the aid of a sterilized loop. The preparation was allowed to dry and then fixed by passing 3 times through a Bunsen flame.

A 0.5% crystal violet stain was placed on the bacteria smear for 2 minutes. Then washed off with water and stained with dilute iodine for another 2 minutes. Absolute alcohol was carefully dripped on smear and allowed to run off. This process was repeated two more times and then washed off with water. A 1% safranin was used to counter-stain the smear for 2 minutes, and then smear washed, drained and blot dried (Standards Unit, Evaluations and Standards Laboratory, UK (2007)). This procedure was carried out on 2 more slides and the prepared smears presented for microscopy.

Microscopy

Stained smears were observed initially using low power objective lenses of X10 and X20. Afterwards immersion oil was carefully dripped on the smears and viewed under a high power lens (X100) to identify isolates.

Biochemical Test

Oxidase Test (Filter paper method)

Three pieces of filter papers were soaked in reagent solution (0.5ml of 1% N, N, N', N'-tetra-methyl-p-phenylenediamine dihydrochloride) after which a loop full each of freshly grown microbial colonies rubbed on them and the resultant reaction observed after 10 seconds for colour change (Standards Unit, Evaluations and Standards Laboratory, UK ,2007).

Catalase Test

Three millilitres (3 ml) of 3% H_2O_2 was poured in a test tube. Using a sterile glass rod, several colonies of isolates were removed and immersed in the hydrogen peroxide solution contained in a test tube. The test tube was immediately investigated for a bubbling reaction (Cheesbrough, 2006).

3.10 Simulation of Contaminated Water

6000 mg of used engine oil was added to 12 L of tap water as the simulated contaminated water. This mass of contaminant (6000 mg used oil) was used in all experiments in the study.

3.11 Experiments

3.11.1 Determining Hydrocarbon Loss due to Abiotic Factors (Control)

It was anticipated that, some of the oil contaminants might be adsorbed to the walls of bioreactor, container, delivery tubes and/ microbial support materials, therefore contributing to hydrocarbon loss in samples to be analyzed for residual TPH. It was also anticipated that some of the lighter chain hydrocarbon components of the contaminants

might be lost through volatilization. To account for this lost, contaminated water (6000mg used oil in 12 L water) without microbial inoculums was run for the same period as was done for all experiments in the study and samples collected for microbial biomass enumeration and TPH analysis. This served as control experiment.

3.11.2 Determining Critical Micelle Concentration of Bile

The Critical Micelle Concentration (CMC) for bile was determined with the aid of a micro syringe and Krüss tensiometer. This was achieved by dispensing aliquots of bile into oil contaminated water and immediately measuring the respective surface tension reductions over a period of one hour. CMC is the surfactant concentration at which an abrupt change in the rate of the surface tension reduction is observed with increasing surfactant concentration (Fox and Bala, 2000). Regardless of the surfactant concentration, a further reduction in the surface tension will not be observed with further addition of surfactant once the CMC has been reached, rather, more micelles are formed (Fox and Bala, 2000).

3.11.3 Biodegradation Treatments

These experiments were preceded with microbial acclimatization process. This was done by running a contaminated water containing 6000 mg of used engine oil for a week to expose the microbes to the reactor conditions. Subsequently, three sets of experiments were carried out as follows;

1. Effect of nutrients on biodegradation of used motor oil
2. Effects of bile on biodegradation of used motor oil
3. Effect of combinations of nutrient and bile on biodegradation of used motor oil

Effects of Nutrients on Biodegradation

This was determined by introducing 50 ml of nutrient medium into contaminated water. With a constant flow rate of 1 L/min and cycling period of 30 minutes for every 3 hours, biodegradation of contaminated water was carried out for a week. Samples were collected and processed for residual total petroleum hydrocarbon analysis.

Effects of Bile on Biodegradation

1.2, 1.4, 1.6 and 1.8 ml of bile were added to separately prepared contaminated water (6000 mg/L of oil in water). Each was run for a period of 7 days at a constant flow rate of 1 L/min and cycling period of 30 minutes for every 3 hours. At the end of each degradation period samples were collected and processed for residual Total Petroleum Hydrocarbon analysis.

Effect of Combination of Bile and Nutrients on Biodegradation

This was determined by combining 50 ml of nutrient medium and bile additions of 1.2, 1.4, 1.6 and 1.8 ml separately to four different preparations of contaminated water. Each combination of nutrient and bile was run for a week also at a constant flow rate of 1 L/min and cycling period of 30 minutes for every 3 hours. At the end of each experimental period, samples were collected for residual total petroleum hydrocarbon.

Biodegradation Monitoring Parameters

The following parameters were measured in the study. Dissolve oxygen (DO), temperature, pH, surface tension, electrical conductivity, salinity and microbial colonies. Measurement of DO and temperature was carried using YSI 550A DO/temperature meter. pH was monitored with a pH Testr 20 meter, surface tension with Krüss tensiometer,

conductivity and salinity with HANNA-HI9828 pH/ORP/EC/DO meter and microbial colony numbers determined by heterotrophic plate count.

Enumeration of Hydrocarbon Degrading Bacteria

Water samples were collected in tightly corked sterile bottles and immediately transported to the laboratory. Samples were serially diluted in sterile distilled water and the 10^{-7} to10^{-10} dilution plated on PCA. Inoculated agar plates were labeled and incubated at 37 °C for 24 hours. Microbial colony numbers on plates were obtained with the aid of Stuarts automated scientific colony counter.

3.12 TPH Analysis

Solvent Extraction

Extraction of petroleum hydrocarbons was carried out in methylene chloride (MeCl). Glassware was prepared by washing with soap, rinsing with tap water followed by three nominal rinses with deionized water, rinsing with methanol, and then rinsing with MeCl. After preparation, glassware was dried in the fume hood until used. Extractions were performed using 500 ml separatory funnels supported by ring stands in a chemical fume hood. Pyrex funnels were placed below the separatory funnels to collect extracts into receiving containers. 500 ml of each sample was presented for extraction. Samples were stored at 4°C and extracted within 7 days of sampling then analyzed within 40 days of the extraction (Environmental Research Institute, 1999).

100 ml of pure GC-grade MeCl was added to samples in separatory funnels. The separatory funnels were capped, removed from the ring-stand, and gently shaken several times to mix the water and MeCl. The funnels were inverted and the stopcock opened to

relieve accumulated gas pressure. This was repeated until gas pressure was equalized. At this point, the samples were vigorously shaken for one minute. The separatory funnels were returned to the ring stand, uncapped, and allowed to sit for ten minutes. During this time, the MeCl settled to the bottom of the separatory funnel, below the less dense aqueous layer. After 10 minutes, the MeCl extract was slowly drained from the separatory funnels through the Pyrex funnel into the collecting containers. The final extracts were prepared for GC-FID analyses.

GC-FID Analysis of Residual Oil

Each extracted sample was analyzed for residual Total Petroleum Hydrocarbons (TPH) using a Varian CP-3800 GC analyzer fitted with flame ionization detector. Standards were run periodically between sample analyses and with each sample run as a quality control measure to ensure consistency.

CHAPTER FOUR

4.0 RESULTS

Results obtained in the study are presented in this chapter.

4.1 Results on Baseline Analysis of Used Motor Oil, Bile and Pipe Borne Water

Used motor oil, bile and pipe borne water used in the study had pH values of 5.90 ±0.06, 7.15 ±0.03 and 7.00 ±0.02 respectively. Surface tension values determined for these materials were 38.00 ±0.09 mN/m for used motor oil, 36.50 ±0.05 mN/m for bile and 72.00 ±0.07 mN/m for pipe borne water. TPH in the used motor oil was 282.97 ±0.00 µg/µl. TPH was below detection limit in bile and pipe borne water. Used motor oil and bile contained 0.18 ± 0.01 mg/l and 0.58 ± 0.01 mg/l phosphorus, and 0.01 and 0.06 percent nitrogen, respectively. Both phosphorus and nitrogen were below detection limit in water (Table 4.0).

Table 4.0 Baseline Data of Used Motor Oil, Bile and Pipe Borne Water used in the Study

Parameter / Material	pH	Surface tension (mN/m)	TPH (µg/µl)	Phosphorus mg/l	Nitrogen (%)
Used Motor Oil	5.90 ±0.06	38.00 ±0.09	282.97 ±0.00	0.18 ± 0.01	0.01
Bile	7.15 ±0.03	36.50 ±0.05	0.00 ±0.00	0.58 ± 0.01	0.06
Pipe borne Water	7.00 ±0.02	72.00 ±0.07	0.00 ±0.00	0.00 ± 0.00	0.00

4.2 Microbial Isolates

Turbidity readings taken after the introduction of bacteria inoculums and used motor oil showed an increased from 180 ± 0.00 (NTU) to 265 ± 0.00 (NTU) within 24 hours incubation period. Heterotrophic plate counts also showed an increase in microbial colony

numbers from $3.0 \times 10^{10} \pm 0.01$ CFU/ml to $5.8 \times 10^{10} \pm 0.05$ CFU/ml within the same period of incubation.

4.3 Characterized Isolates

Gram stain protocols revealed that, the isolates belong to both gram positive and gram negative group of bacteria and were largely rod shaped. Moreover, oxidase, and catalase test performed on isolates showed positive reactions. Isolates that reacted positively to gram stain and catalase test (produced bubbling reactions in 3% H_2O_2) but negative to oxidase test where characterized as members of *Bacillus* sp., whiles those that reacted negatively to gram stain but positively to oxidase test (produced blue-purple colour within 10 seconds reaction with reagent) were associated to *Pseudomonas* sp.

4.4 Hydrocarbons Loss due to Abiotic Factors (Control)

GC-FID results from samples collected from this experiment showed a low percentage degradation of 2.59%. It was the lowest percentage TPH loss in the study. A total of 3×10^{10} CFU/ml microbial colony numbers were recorded from undiluted samples taken from this treatment.

4.5 Critical Micelle Concentration (CMC)

The Critical Micelle Concentration of bile was achieved at a concentration of 1.6 ml/l and corresponded to 1.6 ml volume of bile.

4. 6 Results from Treatments

4.6.1 Nutrient Medium Treatment

This treatment recorded a TPH degradation of 95.03% (Table 4.10). Microbial colony numbers increased from 45.00 ± 3.68 $x10^{10}$ CFU/ml on day 1 to 99.00 ± 3.42 $x10^{10}$ CFU/ml on day 3 but decreased to 86.00 ± 5.98 $x10^{10}$ CFU/ml on day 5 and then increased again to 94.00 ± 6.89 $x10^{10}$ CFU/ml on day 7 (Table 4.1). Temperature as well increased from 27.35 ± 0.02 °C on day 1 to 28.70 ± 0.04 °C on day3 and subsequently decreased to 28.0 ± 0.04 °C on day 5. It then increased to 28.04 ± 0.06 °C on day 7 (Table 4.1).

Electrical conductivity and salinity increased throughout the treatment period from 1825.00 ± 0.01 µS/cm to 1840.00 ± 0.06 µS/cm and 0.68 ± 0.01 PSU to 1.39 ± 0.03 PSU respectively (Table 4.1). However, DO and pH showed an initial decreasing trend from 6.03 ±0.08 mg/l and 7.07 ± 0.07 on day 1 to 5.01 ± 0.06 mg/l and 7.05 ±0.06 on day 3 respectively (Table 4.1). Subsequently DO increased to 5.09 ±0.08 mg/l on day 5 but decreased to 5.02 ± 0.07 on day 7, whereas pH continued to decrease until day 7 where it increased to 7.03 ±0.04 (Table 4.1). Surface tension however decreased from 64.00 ± 0.02 on day 1 to 63.10 ± 0.09 mN/m on day 7 (Table 4.1).

Table 4.1 Results of Nutrient Medium Treatment

Parameter / Day	pH	Temp (°C)	DO (mg/l)	EC (µS/cm)	Sal (PSU)	ST (mN/m)	MC $x10^{10}$ (CFU/ml)
1	7.07 ±0.07	27.35 ±0.02	6.03 ±0.08	1825.00 ±0.01	0.68 ±0.01	64.00 ±0.02	45.00 ±3.68
3	7.05 ±0.06	28.70 ±0.04	5.01 ±0.06	1829.00 ±0.02	0.93 ±0.02	63.60 ±0.04	99.00 ±3.42
5	7.01 ±0.07	28.02 ±0.04	5.09 ±0.08	1838.00 ±0.04	1.09 ±0.01	63.30 ±0.02	86.00 ± 5.98
7	7.03 ±0.04	28.04 ±0.06	5.02 ±0.07	1840.00 ±0.06	1.39 ±0.03	63.10 ±0.09	94.00 ±6.89

*Temp – Temperature, DO – Dissolve Oxygen, EC – Electrical Conductivity, Sal – Salinity, ST – Surface Tension, MC – Microbial Colony numbers.

4.6.2 Bile (1.2 ml) Treatment

Observations in this treatment showed temperature to have increased from 27.09 ± 0.03 °C on day 1 to 28.80 ± 0.04 °C on day 3 but decreased to 28.10 ± 0.05 °C on day 5 and 28.05 ± 0.08 °C on day 7 (Table 4.2). Microbial colonies too initially increased from 38.00 ±1.63 x 10^{10} CFU/ml on day 1 to 101.00 ± 5.82 x10^{10} CFU/ml on day 3 but decreased to 94.00 ± 5.27 x10^{10} CFU/ml on day 5 and 92.00 ± 6.66 CFU/ml on day 7 respectively (Table 4.2).

Electrical conductivity and salinity increased throughout the treatment period from 4029.00 ± 0.06 µS/cm to 4042.00 ± 0.01 µS/cm and 0.88 ± 0.09 PSU to 2.03 ±0.09 PSU consecutively (Table 4.2). Dissolved oxygen decreased from 6.09 ± 0.08 mg/l on day 1 to 3.94 ± 0.09 mg/l on day 3, then increased to 4.44 ± 0.04 mg/l on day 5 and 7 4.89 ± 0.06 mg/l on day 7 whiles pH decreased from 7.09 ± 0.09 on day 1 to 7.02 ± 0.06 on day 3 but remained unchanged to day 7 (6.54 ± 0.05) (Table 4.2). Surface tension however decreased throughout the treatment period from 62.00 ± 0.03 mN/m on day 1 to 53.50 ±0.09 mN/m on day 7 (Table 4.2). This treatment produced a 95.38% TPH degradation on completion (Table 4.10).

Table 4.2 Results of Bile (1.2 ml) Treatment

Parameter / Day	pH	Temp (°C)	DO (mg/l)	EC (µS/cm)	Sal (PSU)	ST (mN/m)	MC x10^{10} (CFU/ml)
1	7.09 ±0.09	27.09 ±0.03	6.09 ±0.08	4029.00 ±0.06	0.88 ±0.09	62.00 ±0.03	38.00 ±1.63
3	7.02 ±0.06	28.80 ±0.04	3.94 ±0.09	4033.00 ±0.01	0.93 ±0.08	58.70 ±0.07	101.00 ±5.82
5	6.54 ±0.05	28.10 ±0.05	4.44 ±0.04	4039.00 ±0.05	2.01 ±0.07	54.90 ±0.09	94.00 ±5.27
7	6.54 ±0.05	28.05 ±0.08	4.89 ±0.06	4042.00 ±0.01	2.03 ±0.09	53.50 ±0.09	92.00 ±6.66

4.6.3 Bile (1.4 ml) Treatment

This treatment produced a 94.52% TPH degradation at the end of its degradation period (Table 4.10). Microbial colony numbers, electrical conductivity and salinity increased in the order of 40.00 ± 2.64 x10^{10} CFU/ml on day 1 to 121.00 ± 6.49 x10^{10} CFU/ml on day 7, 3138.00 ± 0.01µS/cm on day 1 to 4109.00 ± 0.05 µS/cm on day 7 and 0.89 ± 0.02 PSU on day 1 to 1.10 ± 0.06 PSU on day 7 respectively (Table 4.3). Whereas surface tension, DO and pH, decreased in the order 61.90 ± 0.05 mN/m on day 1 to 52.70 ± 0.02 mN/m on day 7, 5.88 ± 0.07 mg/l on day 1 to 3.53 ± 0.03 mg/l on day 7 and 6.54 ± 0.05 on day 1 to 6.22 ± 0.06 on day 7 respectively (Table 4.3). Temperature interestingly showed the same readings on day 1 and 3 (27.29 ± 0.01 °C), then day 5 and 7 (29.08 ± 0.09 °C) (Table 4.3).

Table 4.3 Results of Bile (1.4 ml) Treatment

Parameter / Day	pH	Temp (°C)	DO (mg/l)	EC (µS/cm)	Sal (PSU)	ST (mN/m)	MC x10^{10} (CFU/ml)
1	6.54 ±0.05	27.29 ±0.01	5.88 ±0.07	3138.00 ±0.01	0.89 ±0.02	61.90 ±0.05	40.00 ±2.64
3	6.35 ±0.07	27.29 ±0.01	5.00 ±0.06	4040.00 ±0.03	0.97 ±0.09	58.40 ±0.07	43.00 ±2.04
5	6.27 ±0.05	29.08 ±0.09	3.57 ±0.04	4099.00 ±0.02	1.01 ±0.01	54.10 ±0.03	119.00 ±6.67
7	6.22 ±0.06	29.08 ±0.05	3.53 ±0.03	4109.00 ±0.05	1.10 ±0.06	52.70 ±0.02	121.00 ±6.49

4.6.4 Bile (1.6 ml) treatment

A 97.33% TPH degradation was recorded in this treatment (Table 4.10). Microbial colonies, temperature, electrical conductivity and salinity increased in the following order; 46.00 ± 2.45 x 10^{10} CFU/ml on day 1 to 118.00 x ± 5.27 x10^{10} CFU/ml on day 7, 27.35 ± 0.04 °C on day 1 to 29.06 ± 0.03 °C on day 7, 4139.00 ± 0.05 µS/cm on day 1 to 4978.00 ±0.02 µS/cm on day 7 and 0.86 PSU ± 0.05 on day 1 to 2.98 ± 0.02 PSU on day 7 respectively (Table 4.4). pH and DO on the other hand decreased throughout the treatment in the sequence of 7.50 ± 0.05 on day 1 to 6.07 ± 0.02 on day 7 and 5.53 ± 0.08 mg/l on day 1 to 3.89 ± 0.01 mg/l on day 7 respectively (Table 4.4). Surface tension showed an unusual trend by increasing from 60.00 ± 0.02 mN/m on day 1 to 66.48 ± 0.05 mN/m on day 7 (Table 4.4).

Table 4.4 Results of Bile (1.6 ml) Treatment

Parameter / Day	pH	Temp (°C)	DO (mg/l)	EC (µS/cm)	Sal (PSU)	ST (mN/m)	MC x10^{10} (CFU/ml)
1	7.50 ±0.05	27.35 ±0.04	5.53 ±0.08	4139.00 ±0.05	0.86 ±0.05	60.00 ±0.02	46.00 ±2.45
3	7.43 ±0.04	28.00 ±0.03	5.44 ±0.07	4240.00 ±0.09	1.66 ±0.01	66.90 ±0.07	61.00 ±3.51
5	6.50 ±0.01	29.01 ±0.02	3.93 ±0.07	4771.00 ±0.03	2.89 ±0.02	68.50 ±0.05	114.00 ±5.39
7	6.07 ±0.02	29.06 ±0.03	3.89 ±0.01	4978.00 ±0.02	2.98 ±0.02	70.50 ±0.07	118.00 ±5.27

4.6.5 Bile (1.8 ml) Treatment

This treatment recorded the lowest pH; 5.52 ± 0.14 and DO; 3.35 ± 0.70 mg/l in the study (Table 4.5). Temperature increased from 27.55 ± 0.04 on day 1 to 29.09 ± 0.05 °C on day 5 but decreased to 27.99 ± 0.08 on day 7. Also microbial colony numbers increased from 56.00 ± 2.65 CFU/ml on day 1 to 126.00 ± 3.44 CFU/ml on day 5 but decreased to 58.00 ± 2.76 CFU/ml on day 7 (Table 4.5). On the other hand, electrical conductivity and

salinity increased throughout the degradation period from 4019.00 ± 0.04 µS/cm on day 1 to 4636.00 ± 0.02 µS/cm on day 7 and 1.62 ± 0.01 PSU on day 1 to 1.88 ± 0.01 PSU on day 7 respectively (Table 4.5). DO and pH showed an initial decreasing trend from 4.89 ±0.07 mg/l and 7.47 ± 0.06 on day 1 to 3.35 ±0.07 mg/l and 5.52 ± 0.04 on day 5 but increased to 4.41 ± 0.05 mg/l and 6.53 ± 0.01 consecutively on day 7 (Table 4.5). Surface tension maintained a decreasing trend from 57.50 ±0.47 Nm/m on day 1 to 49.50 ±0.24 N/m on day 7 (Table 4.5). This treatment recorded a TPH degradation of 96.20% at the end of the seven days degradation period (Table 4.10).

Table 4.5 Results of Bile (1.8 ml) Treatment

Parameter / Day	pH	Temp (°C)	DO (mg/l)	EC (µS/cm)	Sal (PSU)	ST (mN/m)	MC x10^{10} (CFU/ml)
1	7.47 ±0.06	27.55 ±0.04	4.89 ±0.07	4019.00 ±0.04	1.62 ±0.01	57.50 ±0.07	56.00 ±2.65
3	7.45 ±0.03	28.09 ±0.01	4.53 ±0.01	4330.00 ±0.01	1.73 ±0.04	55.10 ±0.06	93.00 ±4.64
5	5.52 ±0.04	29.09 ±0.05	3.35 ±0.07	4428.00 ±0.04	1.84 ±0.02	51.34 ±0.07	126.00 ±3.44
7	6.53 ±0.01	27.99 ±0.08	4.41 ±0.05	4636.00 ±0.02	1.88 ±0.01	49.50 ±0.04	58.00 ±2.76

4.6.6 Nutrients + Bile (1.2 ml) Treatment

This treatment produced a percentage TPH degradation of 87.63% (Table 4.10). The highest pH (8.28 ±1.07) in the study was recorded in this treatment. Surface tension decreased throughout the treatment period from 55.00 ± 0.02 mN/m on day 1 to 45.50 ± 0.04 mN/m on day 7 (Table 4.6). pH decreased from 7.52 ± 0.03 on day 1 to 6.74 ± 0.07 on day 3 and subsequently increased to 6.99 ± 0.05 day 5 and 8.28 ±0.07 on day 7 (Table 4.6). DO initially decreased from 4.24 ± 0.02 mg/l on day 1 to 4.02 ±0.08 mg/l on day 3 but subsequently increased to 4.22 ± 0.02 mg/l on day 7 (Table 4.6). Microbial colony numbers and temperature showed an initial lower values but increased on day 3

from 57.00 ±2.22 CFU/ml and 27.89 ± 0.01 °C on day 1 to 98.00 ± 7.35 CFU/ml and 28.19 ± 0.02 °C respectively on day 3 and subsequently decreased to 76.00 ± 2.37 CFU/ml and 27.99 ± 0.08 °C respectively on day 7 (Table 4.6). Electrical conductivity increased from 2614.00 ± 0.09 µS/cm on day 1 to 2931.00 ± 0.09 µS/cm on day 7 (Table 4.6). Salinity also increased from 0.83 ± 0.01 PSU on day 1 to 0.89 ± 0.06 PSU on day 7 (Table 4.6)

Table 4.6 Results of Nutrients + Bile (1.2 ml) Treatment

Parameter / Day	pH	Temp (°C)	DO (mg/l)	EC (µS/cm)	Sal (PSU)	ST (mN/m)	MC x10^{10} (CFU/ml)
1	7.52 ±0.03	27.89 ±0.01	4.24 ±0.02	2614.00 ±0.09	0.83 ±0.01	55.00 ±0.02	57.00 ±2.22
3	6.74 ±0.07	28.19 ±0.02	4.02 ±0.08	2719.00 ±0.04	0.84 ±0.04	52.27 ±0.03	98.00 ±7.35
5	6.99 ±0.05	28.02 ±0.05	4.12 ±0.01	2924.00 ±0.06	0.86 ±0.02	50.07 ±0.08	82.00 ±4.24
7	8.28 ±0.07	27.99 ±0.08	4.22 ±0.02	2931.00 ±0.09	0.89 ±0.06	45.50 ±0.04	76.00 ±2.37

4.6.7 Nutrients + Bile (1.4 ml) Treatment

A 51.11% TPH degradation was recorded at the end of this treatment (Table 4.10). Temperature and microbial colonies increased from 27.46 ± 0.09 °C and 47.00 ±1.74 CFU/ml on day 1 to 28.20 ±0.09 °C and 67.00 ± 6.27 CFU/ml on day 5 respectively but dropped to 28.01 ± 0.04 °C and 51.00 ± 3.08 CFU/ml respectively on day 7 (Table 4.7). Electrical conductivity and salinity on the other hand increased from 1029.00 ± 0.01 µS/cm and 0.35 ± 0.01 PSU on day 1 to 1057.00 ± 0.09 µS/cm and 0.53 ± 0.02 PSU on day 7 respectively (Table 4.7). DO showed a downward trend from 5.02 ±0.08 mg/l on day 1 to 3.79 ± 0.09 mg/l on day 7, whereas pH decreased from 6.49 ±0.07 on day 1 to 6.41 ± 0.02 on day 5 but increased to 6.98 ± 0.09 on day 7 (Table 4.7). Surface tension

decreased throughout the degradation period from 45.50 ±0.04 mN/m on day 1 to 40.00 ± 0.03 mN/m on day 7 (Table 4.7)

Table 4.7 Results of Nutrients + Bile (1.4 ml) Treatment

Parameter / Day	pH	Temp (°C)	DO (mg/l)	EC (µS/cm)	Sal (PSU)	ST (mN/m)	MC x10^{10} (CFU/ml)
1	6.49 ±0.07	27.46 ±0.09	5.02 ±0.08	1029.00 ±0.01	0.35 ±0.01	45.50 ±0.04	47.00 ±1.74
3	6.30 ±0.06	28.03 ±0.01	4.04 ±0.07	1031.00 ±0.01	0.31 ±0.09	44.30 ±0.03	61.00 ±7.15
5	6.41 ±0.02	28.20 ±0.09	3.80 ±0.03	1039.00 ±0.06	0.47 ±0.08	42.68 ±0.07	67.00 ±6.27
7	6.98 ±0.09	28.01 ±0.04	3.79 ±0.09	1057.00 ±0.09	0.53 ±0.02	40.00 ±0.03	51.00 ±3.08

4.6.8 Nutrients + Bile (1.6 ml) Treatment

Under this treatment the highest TPH degradation (97.34%, Table 4.10), microbial colony numbers (136.00 ±4.44 x10^{10} CFU/ml), electrical conductivity (5475.25 ± 0. 59 µS/cm), salinity (2.49 ± 0.36 PSU) and temperature (30.50 ± 0.35 °C) were recorded (Table 4.8). Microbial colony numbers increased from 31.00 ± 1.29 x 10^{10} CFU/ml on day 1 to 136.00 ± 4.44 x 10^{10} CFU/ml on day 7, temperature also increased from 27.0 ± 0.09 °C on day 1 to 30.50 ± 0.05 °C on day 7.

Electrical conductivity and salinity as well increased from 5401.00 ± 0.03 µS/cm and 2.41 ± 0.07 PSU on day 1 to 5475.00 ± 0.09 µS/cm and 2.49 ± 0.04 PSU on day 7 respectively. Surface tension in this treatment showed a rather increasing trend from day 1 to 7. It increased from 44.10 ± 0.03 mN/m on day 1 to 72.00 ± 0.05 mN/m on day 7. DO decreased from 4.97 ± 0.07 on day 1 to 3.37 ± 0.07 mg/l on day 7 whiles pH initially decreased from 7.57 ± 0.05 on day 1 to 6.97 ± 0.05 on day 5 but increased to 7.20 ± 0.09 on day 7 (Table 4.8).

Table 4.8 Results of Nutrients + Bile (1.6 ml) Treatment

Parameter / Day	pH	Temp (°C)	DO (mg/l)	EC (µS/cm)	Sal (PSU)	ST (mN/m)	MC x10^{10} (CFU/ml)
1	7.57 ±0.05	27.07 ±0.09	4.97 ±0.07	5401.00 ±0.03	2.41 ±0.07	44.10 ±0.03	31.00 ±1.29
3	7.54 ±0.07	28.01 ±0.03	4.80 ±0.01	5410.00 ±0.09	2.44 ±0.05	69.70 ±0.06	81.00 ±2.78
5	6.97 ±0.05	28.11 ±0.02	3.95 ±0.08	5471.00 ±0.07	2.46 ±0.06	70.90 ±0.08	96.00 ±3.45
7	7.20 ±0.09	30.50 ±0.05	3.37 ±0.07	5475.00 ±0.09	2.49 ±0.04	72.00 ±0.05	136.00 ±4.44

4.6.9 Nutrients + Bile (1.8 ml)

The lowest surface tension (36.90 mN/m ± 0.06) reading in the study was recorded in this treatment (Table 4.9). Temperature, electrical conductivity and salinity increased throughout the treatment period in the order; 27.54 ± 0.04 °C on day 1 to 28.58 ± 0.08 °C on day 7, 2475.00 ± 0.07 µS/cm on day 1 to 2600.00 ± 0.02 µS/cm on day 7 and 0.72 ± 0.06 PSU on day 1 to 0.96 ± 0.06 PSU on day 7 respectively (Table 4.9).

Microbial colonies increased from 55.00 ± 2.77 CFU/ml on day 1 to 79.00 ± 3.17 CFU/ml on day 3 and 97.00 ± 8.57 CFU/ml on day 5 but decreased to 77.00 ± 3.89 CFU/ml on day 7. On the other hand, surface tension decreased from 44.30 ± 0.03 mN/m on day 1 to 36.90 ±0.06 mN/m on day 7, pH from 7.70 ± 0.06 on day 1 to 6.23 ± 0.05 day 7and DO from 4.52 ± 0.07 mg/l on day 1 to 4.03 ± 0.05 mg/l on day 7 (Table 4.9). An 86.81% TPH degradation was produced in this treatment (Table 4.10).

Table 4.9 Results of Nutrients + Bile (1.8 ml) Treatment

Parameter / Day	pH	Temp (°C)	DO (mg/l)	EC (µS/cm)	Sal (PSU)	ST (mN/m)	MC x10^{10} (CFU/ml)
1	7.70 ±0.06	27.54 ±0.04	4.52 ±0.07	2475.00 ±0.07	0.72 ±0.06	44.30 ±0.03	55.00 ±2.77
3	7.40 ±0.08	28.00 ±0.02	4.24 ±0.05	2480.00 ±0.01	0.81 ±0.03	42.90 ±0.04	79.00 ±3.17
5	6.28 ±0.09	28.50 ±0.07	4.16 ±0.07	2591.00 ±0.04	0.90 ±0.04	40.20 ±0.08	97.00 ±8.57
7	6.23 ±0.05	28.58 ±0.08	4.03 ±0.05	2600.00 ±0.02	0.96 ±0.06	36.90 ±0.06	77.00 ±3.89

Percentage TPH Degraded by each Treatment

This study generally recorded high percentage TPH degradations. The highest percentage degradation (97.34%), was recorded in nutrient + bile (1.6ml) treatment. Also, a high percentage TPH degradation of 97.33 % was realized in the treatment Bile (1.6 ml). Control experiment had a percentage TPH degradation of 95.03%.

The treatments Bile 1.2 ml, 1.4 ml and 1.8 ml had percentage degradations of 95.38%, 94.52%, and 96.21 % respectively. Also treatments, Nutrients + Bile 1.2 ml, Nutrients + 1.4 ml, Nutrient + 1.8 ml recorded percentage degradations of 87.63%, 51.11% and 86.36% respectively. The lowest percentage (2.59) TPH loss was recorded in the treatment, Loss due to Abiotic Factor. Table 4.10 below shows the various percentage TPH degraded by each treatment in the study.

Table 4.10 Percentage TPH Degraded in each Treatment Process

Experiment	%TPH Biodegraded
Hydrocarbon Loss to Abiotic Factors (Control)	2.59
Nutrient Medium	95.03
Bile 1.2 ml	95.38
Bile 1.4 ml	94.52
Bile 1.6 ml	97.33
Bile 1.8 ml	96.21
Nutrient + Bile 1.2 ml	87.63
Nutrient + Bile 1.4 ml	51.11
Nutrient + Bile 1.6 ml	97.34
Nutrient + Bile 1.8 ml	86.36

4.7 Relationship between TPH Degraded and Volume of Bile Supplied

It was noted that an increase in volume of bile added did not necessarily result in an increase in percentage hydrocarbon degraded. This was evident in the treatments Bile (1.4 ml) and Bile (1.8 ml) and Nutrients + Bile (1.4 ml) and Nutrients + Bile (1.8 ml) which recorded lower percentage degradations relative to treatments with lower bile addition of 1.2 ml and 1.6 ml respectively. The extent of this disparity was illustrated by the medium relationship ($R^2 = 0.39$) shown between percentage TPH degraded and quantity of bile added (Figure 4.0). The relationship between percent TPH degraded and concentration of bile supplied was thus described as not strongly correlated. Also, it was realized that

treatments, Bile (1.2 ml), Nutrients + Bile (1.2 ml), Nutrients + Bile (1.4 ml) and Nutrients + Bile (1.8 ml) recorded lower percentage TPH degradations than nutrient medium treatment which received no bile supplements at all.

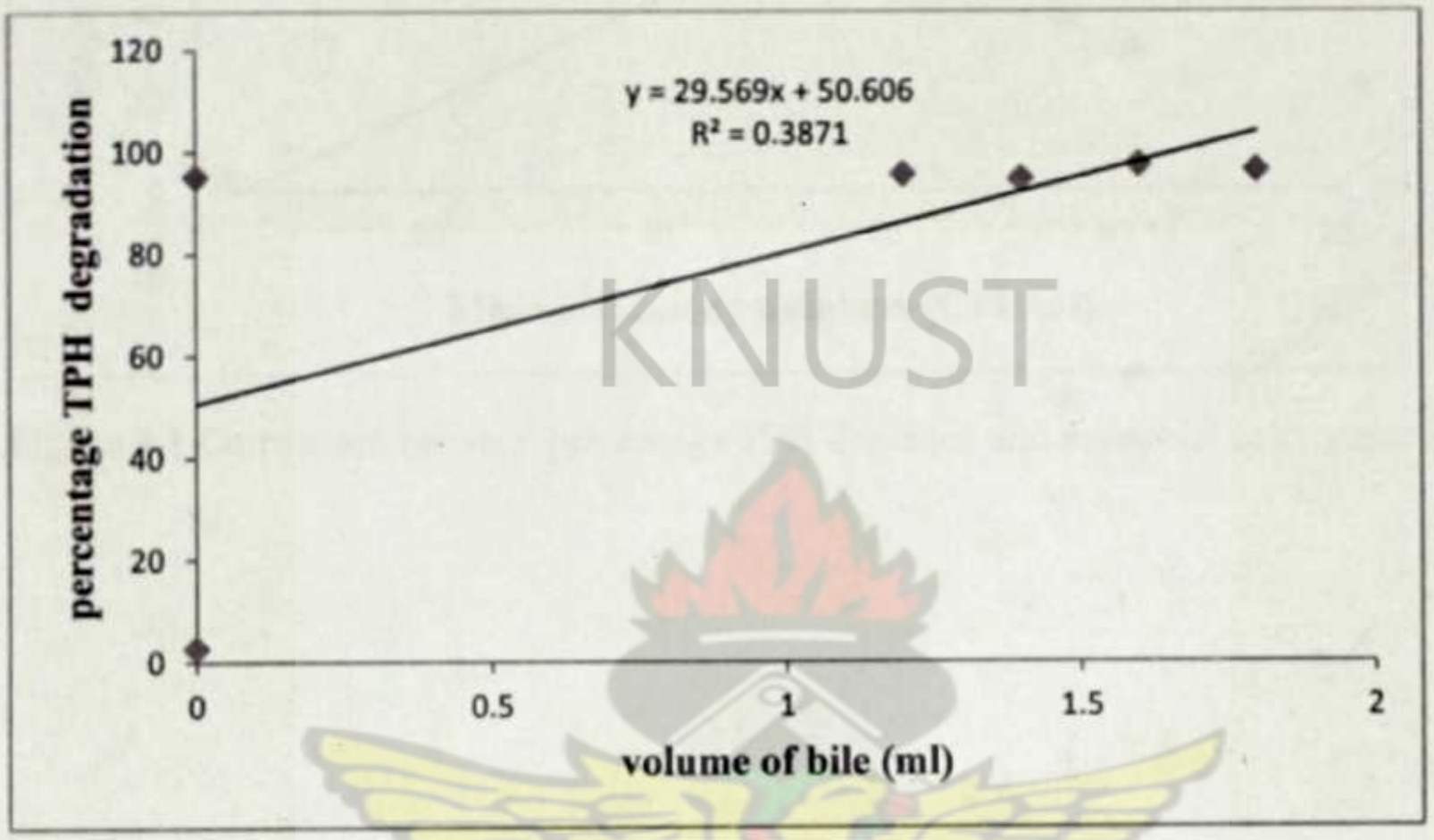

Figure 4.0 Correlation between quantity of bile supplied and percentage TPH degraded.

4.8 Relationship between TPH Degraded and Microbial Colony Numbers

An increase in microbial colony numbers corresponded to an increase in hydrocarbons degraded. This was evident in the observations that, increments in microbial colony numbers resulted in increments in percentage TPH biodegraded. A highly correlated relationship existed between microbial numbers and percentage TPH degraded (R^2 = 0.85, Figure 4.1).

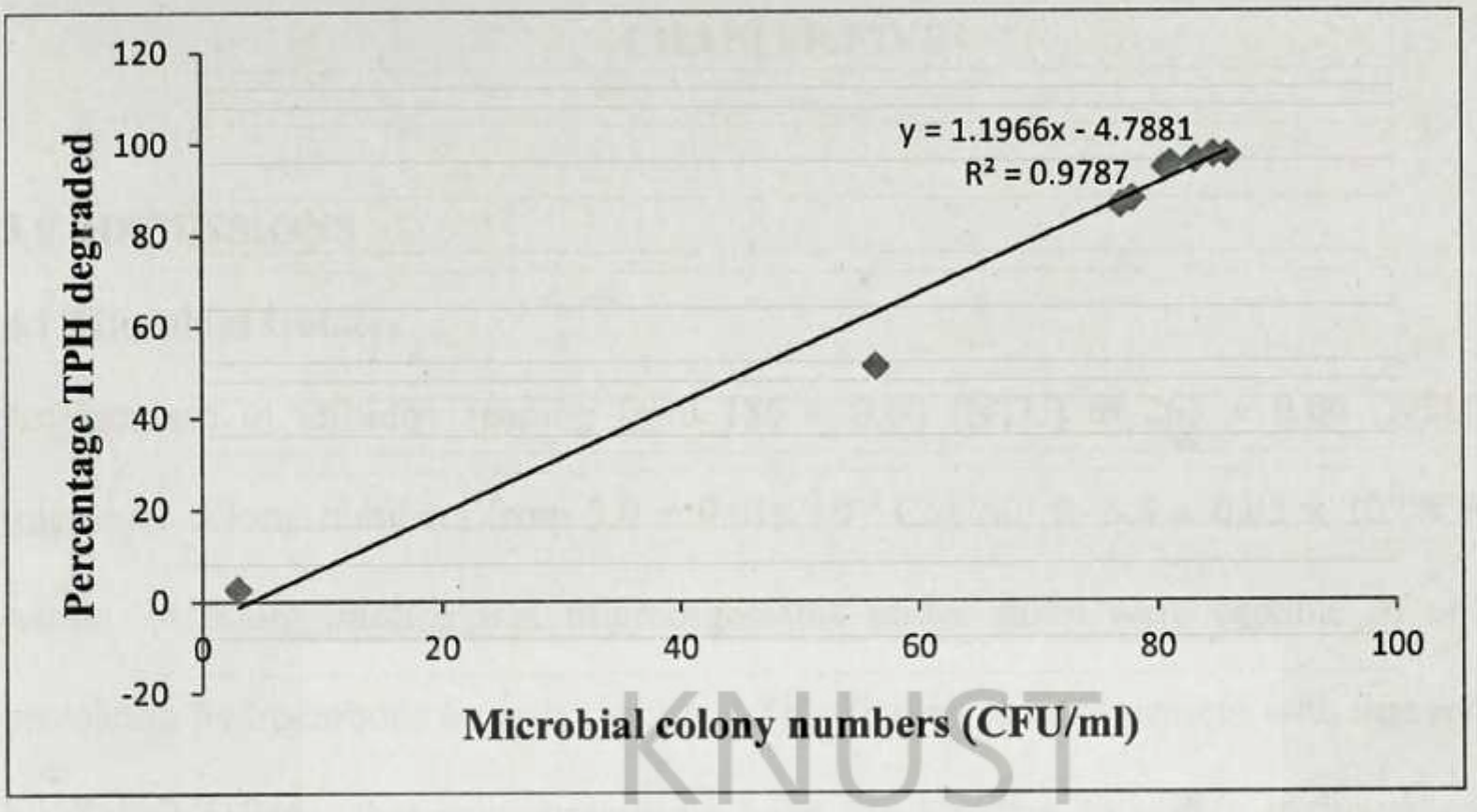

Figure 4.1 Correlation between percentage TPH degraded and microbial colony numbers

CHAPTER FIVE

5.0 DISCUSSIONS

5.1 Microbial Isolates

An increase in turbidity reading from 180 ± 0.00 (NTU) to 265 ± 0.00 (NTU) and microbial colony numbers from 3.0 ± 0.01x 10^{10} CFU/ml to 5.8 ± 0.05 x 10^{10} CFU/ml within 24 hours implied that microorganisms under study were capable of utilizing petroleum hydrocarbons as carbon source. This finding is in agreement with that reported by Zobell (1946), that microorganisms have the abilities to utilize hydrocarbons for growth.

5.2 Characterized Isolates

Pseudomonas sp. and *Bacillus* sp. are able to degrade hydrocarbons in order to produce energy and biomass (Van Hamme *et al*., 2003) and they do so because of their effective enzyme (oxygenases, dioxygenase, dehydrogenases) systems (www.wiley-vch.de/books/biotech/pdf). Therefore the identification of these bacteria in contaminated water implied that, they were able to utilize hydrocarbon contaminants as carbon and energy sources for growth.

The isolation of two different species of bacteria from contaminated water also implied that, these bacteria were possibly co-metabolizing hydrocarbon contaminants. In co-metabolism it is possible that one species can remove the toxic metabolites of the other species, or degrade some compounds better than others (Alexander, 1999). Thus it was possible that, one of the species was mineralizing the by-products of the other or was effectively degrading hydrocarbons more than the other.

5.3 Hydrocarbons Loss due to Abiotic Factors (Control)

The lowest percentage degradation (2.59%) was recorded in this experiment and was associated to adsorption of hydrocarbons to walls of bioreactors, delivery tubes and microbial support materials (bamboo). Volatilization of light chain hydrocarbons might have also contributed to this percentage loss of hydrocarbons. This conclusion was drawn because undiluted samples from this experiment when streaked on nutrient agar produced a total of only $3 \pm 0.00 \times 10^{10}$ CFU/ml microbial colony numbers therefore this few number of microbes could not effect a TPH degradation of 2.59%. If 2.59% of hydrocarbon lost in all experiments were attributable to adsorption to walls of bioreactor, delivery tubes, microbial support materials as well as to volatilization, then the larger percentage degradations was much due to microbial activity, thus it was concluded that, bioremediation is a feasible remediation technology.

5.4 Critical Micelle Concentration (CMC)

The achievement of Critical Micelle Concentration at a concentration of 1.6 ml/l meant that, it was the concentration at which an abrupt change in the rate of the surface tension reduction was observed with increasing surfactant concentration. Regardless of the surfactant concentration, a further reduction in surface tension was not observed with continuous addition of the biosurfactant. According to Fox and Bala, 2000, CMC is the surfactant concentration at which an abrupt change in the rate of the surface tension reduction is observed with increasing surfactant concentration.

5.5 Results from Treatments

5.5.1 Nutrient Medium Treatment

pH

The alkaline pH recorded in this treatment was associated to the possibility that, most of the ions released into solution reacted with each other to form alkaline products such as NaOH and KOH therefore increasing pH. Also, addition of nutrients possibly contributed to alkalinity recorded in the treatment. This is in line with findings that nutrient addition increases pH (Bagherzadeh *et al.*, 2008). According to Nilanjana and Preethy (2011), biodegradation of hydrocarbons produces acidic intermediates and by-products thus the decreases in pH also recorded were associated to the formation of acidic by-products.

Temperature

The increment in temperature recorded on day 3 and 7 of the experiment was attributed to bacteria respiration because cellular respiration is an exothermic redox reaction which releases energy in the form of heat (en.wikipedia.org/wiki/Cellular_respiration). On the other hand a decrease in temperature on day 5 was associated to a decrease in microbial activity. Rise in temperatures possibly was enhanced by the accumulation of CO_2. CO_2 has the ability to trap heat within a system (IPCC, 2007), thus capable of causing temperature increments.

DO

Major degradations of hydrocarbons are carried out by aerobes (Leila *et al.*, 2006). Therefore reduction in DO on day 3 and 7 of this treatment implied that, microorganisms (aerobes) were using more DO than was supplied. Temperature increments probably contributed to reductions in DO. This relation was made because increments in

temperature according to Biology with computers (2000), causes decrements in DO levels. The increase in DO on day 5 might however be due to low microbial activities in response to unfavourable experimental conditions.

Electrical Conductivity

The increasing trend in electrical conductivity from day 1 to 7 was associated to the increasing concentration of dissolved ions in solution due biodegradation. This linkage is justifiable by the findings of Moliterni *et al.* (2012), that biodegradation increases the presence of dissolved ions in solution.

Salinity

Biodegradation increases the presence of dissolved ions in solution (Moliterni *et al.*, 2012).Therefore increasing trend in salinity observed was attributed to the increasing concentration of dissolved ions in solution due to biodegradation. Observations of the trend of salinity showed that, it increased along with increments in electrical conductivity and the reason is that, they are both influenced by the amount of dissolved ions in solution. This finding is in line with that observed by McNeil and Cox, (2000) that salinity and conductivity are related and that one can be measured to give an indication of the other.

Surface Tension

The reason assigned to the continuous surface tension reduction from day 1 to 7 was that, bacteria are able to produce biosurfactants which have the abilities to reduce surface tensions of hydrocarbon contaminated waters. *Bacillus* sp and *Pseudomonas* sp isolated in the study are known biosurfactant producers and that *Pseudomonas* are especially capable

of producing biosurfactants when utilizing hydrocarbons as carbon and energy sources (Rahman *et al.*, 2003). Consequently biosurfactants produced by the bacteria were most responsible for surface tension reductions.

Microbial Colony Numbers

Microorganisms have the capacities to mineralized hydrocarbons for growth (Bagherzadeh, 2008). Therefore the increase in microbial colony numbers recorded on day 3 and 7 implied that, the microbes were effectively mineralizing hydrocarbon contaminants and that experimental conditions were favourable for multiplications. Decrease in microbial numbers on day 5 was however associated to bacteria encountering unfavourable conditions including toxic by-products of biodegradation. Biodegradation of certain hydrocarbons produces toxic products which hinders the abilities of certain bacteria (Alexander, 1999). Also increases and decreases observed in microbial colony numbers possibly were due to the normal bacterial growth curve. The normal bacteria growth curve follows the order lag, log, stationary and death phases (Friedrich, 2010).

Percent TPH Degraded

Percentage TPH degradation of 95.03% recorded in this treatment meant that nutrients supplied in the treatment were sufficient and appropriate for microbial utilization in degrading petroleum hydrocarbon contaminants. The presence of appropriate nutrients increases the capacities of microorganisms to degrade hydrocarbons (Atlas, 1985). Favourable experimental conditions possibly contributed to this high percentage degradation.

5.5.2 Bile (1.2 ml) Treatment

pH

The observation that, this treatment proceeded from an alkaline medium (7.09 and 7.02) to an acidic medium (6.54) was related to the following. Alkalinity was attributed to the possibility that, most of the ions released into solution reacted with each other to form products such as NaOH and KOH thus increasing pH. Whereas the acidity recorded on day 5 and 7 was most probably due to complete mineralization of hydrocarbons. Complete aerobic microbial mineralization of hydrocarbons releases CO_2 which reacts with water to form carbonic acids, thus reducing pH (Adebusoye *et. al.*, 2006). Biodegradation of hydrocarbons produces acidic intermediates and by-products (Nilanjana and Preethy, 2011). Consequently the formation of acidic intermediates/by-products (example, carboxylic acids) from biodegradation might have contributed to acidity observed on day 5 and 7.

Temperature

The initial increase in temperature on day 3 and subsequent decrease in the rest of the days was explained to be due bacteria respiration (activity). That is an increase in respiration caused an increase in temperature on day 3 and reductions in microbial activities (respiration) caused temperature reductions in the subsequent days. Bacteria respiration is an exothermic redox reaction which releases energy in the form of heat (en.wikipedia.org/wiki/Cellular_respiration). Also accumulation of CO_2 possibly contributed to rise in temperature on day 3. Carbon dioxide is able to cause rise in temperatures due to its heat trapping ability (IPCC, 2007).

DO

Major degradations of hydrocarbons are carried out by aerobes (Leila *et al.*, 2006). Thus reduction in DO on day 3 and subsequent increase on day 5 and 7 was associated to the following. Reduction in DO implied that aerobic microbes were using more DO than was supplied whereas the increments (day 5 and 7) were related to possible little oxygen utilizations or low microbial aerobic activity due to unfavourable experimental conditions. Increment in temperature might have also contributed to reductions in DO levels. This relation was made because an increase in temperature according to Biology with computers (2000) causes a decrease in DO levels.

Electrical Conductivity

Electrical conductivity is a measure of dissolved ions in solution (McNeil and Cox, 2000). Therefore an increase in conductivity implied an increase in dissolve ions in solution. Consequently, the increasing trend in electrical conductivity was related to an increasing dissolution of ions in solution due to microbial biodegradation of petroleum hydrocarbons. Biodegradation increases the presence of dissolved ions in solution (Moliterni *et al.*, 2012).

Salinity

The increasing trend in salinity was also associated to the increasing concentration of dissolved ions in solution due to biodegradation because according to Moliterni *et al.* (2012), biodegradation increases the presence of dissolved ions in solution. Observations of the trend of salinity showed that, it increased along with increments in electrical conductivity and the reason is that, they are both influenced by the amount of dissolved ions in solution. This finding is in agreement with that observed by McNeil and Cox,

(2000) that salinity and conductivity are related and that one can be measured to give an indication of the other.

Surface Tension

A continuous surface tension reduction from day 1 to 7 recorded in this treatment was attributed to the addition of bile to the treatment. Bile has the ability to reduce surface tension because of its micelle-forming properties (Vethamuthu *et al*., 1992c). Also, surface tension reduction might have been facilitated by biosurfactant produced by bacteria during the biodegradation process. *Bacilli* sp and *Pseudomonas* sp are known biosurfactant producers and that *Pseudomonas* especially are capable of producing biosurfactants when utilizing hydrocarbons as carbon and energy sources (Rahman *et al*., 2003). Moreover surface tension reductions possibly were enhanced by synergistic interactions between biosurfactants produced by bacteria and that present in bile.

Microbial Colony Numbers

Increase in microbial colony numbers recorded on day 3 implied that, the microbes were effectively utilizing the hydrocarbon contaminants and that experimental conditions were favourable for multiplications. Favourable environmental conditions are necessary for successful biodegradation (Nilanjana and Preethy). Decrease in bacteria numbers on day 5 and 7 were however associated to bacteria encountering unfavourable conditions including toxic by-products of biodegradation. Biodegradation of some class of hydrocarbons produces toxic products which hinders the abilities of most bacteria (Booyjzsen, 2007). According to Friedrich (2010), the normal bacteria growth curve follows the order lag, log, stationary and death phases. Therefore the increases and

decreases observed in microbial colony numbers were possibly due to the normal bacterial growth curve.

Percent TPH Degraded

The high percentage (95.38%) TPH degradation recorded in the study was associated to effective mineralization of hydrocarbons by microbes and that this was enhanced by the presence of bile in the treatment. Bile has a solubilising and emulsifying ability (Swapnadip, 2010). This implied that, bile increased the bioavailability of hydrocarbons for microbial degradation. A 95.38% degradation also implied that, bile at a volume of 1.2 ml is capable of supporting biodegradation of a hydrocarbon contaminated water of volume 12 liters.

5.5.3 Bile (1.4 ml) Treatment

pH

The acidic pH recorded throughout this treatment implied that, most of the by-products of degradation were largely acidic in nature (example, carboxylic acids). Biodegradation of hydrocarbons produces acidic intermediates and by-products (Nilanjana and Preethy, 2011). It was also possible that CO_2 from complete mineralization of hydrocarbons might have contributed to the acidity recorded. This is because complete mineralization of hydrocarbons according to Adebusoye *et al.* (2006) releases CO_2 which reacts with water to form carbonic acids, thus reducing pH.

Temperature

Microbial respiration is an exothermic redox reaction which releases energy in the form of heat (en.wikipedia.org/wiki/Cellular_respiration). Thus increments and decrements in temperature were attributed to microbial respiration. The recording of constant

temperatures on day 1 and 3 and then day 5 and 7 was possibly as a result of constant rate of microbial activities (respiration). That is the rate of respiration might have been constant from day 1 to 3, then increased on day 5 were it remain constant to day 7. Also accumulation of CO_2 possibly contributed to temperature increments. Accumulation of CO_2 increases temperature due to the heat trapping ability of CO_2 (IPCC, 2007).

DO

Major degradations of hydrocarbons are carried out by aerobes (Leila *et al.*, 2006). Consequently the reduction in DO throughout the experimental period meant that aerobic microorganisms were using more DO than was supplied. Temperature increments cause decreases in DO levels (Biology with computers, 2000).

Therefore, the increments in temperature possibly enhanced reductions in DO levels.

Electrical Conductivity

Biodegradation increases the presence of dissolved ions in solution (Moliterni *et al.*, 2012). Therefore the continuous increase in electrical conductivity from day 1 through 7 was associated to an increasing concentration of dissolve ions in solution due to microbial degradation of petroleum hydrocarbons. It was also found to increase with increasing temperature to some extent and the reason given to this observation was that, temperature increases the mobility of ions by reducing viscosity. This observation is in conformity with that of Barron and Ashton, (2007) that increment in temperature increase the mobility ions. Consequently increase in mobility of ions, increases the measurability of ions.

Salinity

The increasing trend in salinity was associated to the increasing presence of dissolved ions in solution due to biodegradation. That is biodegradation increases the presence of dissolved ions in solution (Moliterni *et al*., 2012). It was found to increase with increasing temperature to some extent and this was because temperature increases the mobility of ions by reducing viscosity. This observation is in conformity with that of Barron and Ashton, (2007) that increment in temperature increases the mobility of ions and thus increases the detection and measurability of these ions. Salinity evolution trend showed that, it increased along with increments in electrical conductivity and the reason is that, they are both influenced by the amount of dissolved ions in solution. This finding is in agreement with that observed by McNeil and Cox, (2000) that salinity and conductivity are related and that one could be measured to give an indication of the other.

Surface Tension

The decreasing trend in surface tension recorded in this treatment was attributed to bile added in the treatment. This because bile has the ability to reduce surface tension due to its micelle-forming properties (Vethamuthu *et al*., 1992c). Also, surface tension reduction might have been facilitated by biosurfactant produced by bacteria during the biodegradation process. *Bacilli* sp and *Pseudomonas* sp are known biosurfactant producers and that *Pseudomonas* especially is capable of producing biosurfactants when utilizing hydrocarbons as carbon and energy sources (Rahman *et al*., 2003). Moreover surface tension reductions possibly might have been enhanced by synergistic interactions between biosurfactants produced by bacteria and that present in bile.

Microbial Colony Numbers

Favourable environmental conditions are necessary for successful biodegradation (Nilanjana and Preethy). Therefore the increase in microbial numbers from day 1 to 7 implied that, microbes were effectively utilizing hydrocarbon contaminants under favourable experimental conditions for their growth.

Percent TPH Degraded

94.52% though a high percent degradation was less than that recorded in the treatment Bile (1.2 ml). This implied that increasing the volume of bile to 1.4 ml did not produce a corresponding increase in percentage TPH degradation. The reasons given to this relatively lower percentage degradation are; possibly this treatment produced biodegradation by-products that were largely toxic to bacteria involved in mineralization of hydrocarbons and therefore their capacities were limited. This linkage was made because according to Alexander (1999) biodegradation of certain hydrocarbons produces toxic products which hinders the abilities of certain bacteria. Additionally this outcome was related to possible biochemical interactions between bacteria cells and bile that might have triggered the bactericidal properties of bile by releasing sodium taurodeoxycholate and sodium deoxycholate which are known chemical substances present in bile that inhibit bacterial growth (Sung *et al.*, 1993).

5.5.4 Bile (1.6 ml) Treatment

pH

The change in pH from an initial alkaline medium to an acidic medium was related to an initial concentration of dissolved ions that reacted to form alkaline compounds (NaOH, KOH) on day 1 and 3 then followed by the formation of acidic by-products (carboxylic

acids) on day 5 and 7. Moreover the achievement of acidic pH on day 5 and 7 was possibly facilitated by some complete aerobic microbial mineralization of hydrocarbons in the treatment. Complete mineralization of hydrocarbons releases CO_2 which reacts with water to form carbonic acids, thus reducing pH (Adebusoye *et. al.*, 2006).

Temperature

Microbial respiration is an exothermic redox reaction which releases energy in the form of heat (en.wikipedia.org/wiki/Cellular_respiration). Thus the increasing trend in temperature recorded from day 1 to 7 in this experiment was related increments in microbial respiration (activities) in the treatment. The continuous rise in temperature observed was possibly facilitated by the accumulation of CO_2. Accumulation of carbon dioxide increases temperature due to its heat trapping ability (IPCC, 2007).

DO

Major degradations of hydrocarbons are carried out by aerobic microorganisms (Leila *et al.*, 2006). Thus the reduction in DO throughout this treatment meant that microorganisms were using more DO for degradation than was supplied. Increments in temperature were implicated to have contributed to the decreases in DO levels recorded. This observation is in line with that reported by Biology with computers (2000) that an increase in temperature causes a decrease in DO levels.

Electrical Conductivity

Biodegradation increases the presence of dissolved ions in solution (Moliterni *et al.*, 2012). As such, an increase in electrical conductivity implied an increase in dissolve ions in solution due to biodegradation of contaminants. Also electrical conductivity was found

to be influenced by increments in temperature and the reason is that, temperature increases the mobility of ions by reducing viscosity. This observation is in conformity with that of Barron and Ashton (2007) that, increasing temperature increases the detection and measurability of ions by increasing the mobility of the ions.

Salinity

Biodegradation increases the presence of dissolved ions in solution (Moliterni *et al.*, 2012). Consequently the increasing trend in salinity too was associated to the increasing concentration of dissolved ions in solution due to biodegradation. Observations of the trend of salinity in the treatment showed that, it increased along with increments in electrical conductivity and the reason is that, they both are influence by the amount of dissolved ions in solution. This finding is in agreement with that observed by McNeil and Cox, (2000) that salinity and conductivity are related and that one can be measured to give an indication of the other.

Surface Tension

The rather increasing trend in surface tension recorded in this treatment was explained to be due to high microbial degradation. According to Moliterni *et al.* (2012), high percentage hydrocarbon biodegradation results in surface tension increments. Also, BostrÖm *et al.* (2001) stated that, the increasing presence of dissolved ions increases the surface tension of air-water interface of a given solution as such the presence of large amount of dissolved ions in this treatment could have contributed to this increasing trend in surface tension.

Microbial Colony Numbers

Favourable environmental conditions are necessary for successful biodegradation (Nilanjana and Preethy, 2011). Therefore the increase in microbial colony numbers from day 1 to 7 implied that, microbes were effectively utilizing hydrocarbon contaminants under favourable experimental conditions for growth.

Percent TPH Degraded

A 97.33 % TPH degradation achieved in this treatment meant that hydrocarbon contaminants were effectively mineralized by microbes and that the mineralization process was enhanced by bile supplied in the treatment. Bile has a solubilizing and emulsifying ability (Swapnadip, 2010). This implied that, bile increased the bioavailability of hydrocarbons by increasing their solubilities. It was also deduced that, if 1.6 ml volume of bile produced a 97.33% degradation, then bile alone could be used to support biodegradation but only for a short period. This is because nutrient analysis of bile used in this study showed a nitrogen level of 0.06% and 0.58 ± 0.01 mg/l phosphorus which are rather too low to sustain biodegradation compared to the component (C: N: P) requirement of 120:10:1 needed for sustainable biodegradation (Salleh *et al.*, 2003).

5.5.5 Bile (1.8 ml) treatment

pH

The alkaline nature of this treatment on day 1 and 3 implied that, most of the ions released into solution reacted to form products such as NaOH and KOH therefore increasing pH. Whereas the acidity recorded on day 5 and 7 meant that most biodegradation products were mainly acidic, example carboxylic acids. Biodegradation of hydrocarbons produces acidic intermediates and by-products (Nilanjana and Preethy,

2011). Accumulation CO_2 possibly contributed to the acidity recorded. This conclusion was made because complete mineralization of hydrocarbons according to Adebusoye *et al.* (2006) produces carbon dioxide which reacts with water to form carbonic acids (Adebusoye *et. al.*, 2006).

Temperature

The initial increase in temperature from day 1 through 5 and subsequent decrease on day 7 was explained to be due to an initial increase in bacteria respiration followed by a reduction in respiration. This association was made because bacteria respiration is an exothermic redox reaction which releases energy in the form of heat (en.wikipedia.org/wiki/Cellular_respiration). Increments in temperature was possibly facilitated by the accumulation of CO_2. Accumulation of carbon dioxide according to IPCC (2007) increases temperature due to its heat trapping ability.

DO

The reduction in DO from day 1 through 5 and subsequent increase on day 7 was associated to the following. Major degradations of hydrocarbons are carried out by aerobic microorganisms (Leila *et al.*, 2006). Thus the reductions in DO observed meant that microorganisms were using more DO for degradation than was supplied. Increments in temperature were implicated to have also contributed to the decreases in DO levels recorded. This observation is in line with that reported by Biology with computers (2000) that an increase in temperature causes a decrease in DO levels.

Electrical Conductivity

Biodegradation increases the presence of dissolved ions in solution (Moliterni *et al.*, 2012). Thus the continuous increase in electrical conductivity from day 1 through 7 was associated to an increasing concentration in dissolve ions due to microbial degradation of petroleum hydrocarbons. It was also found to increase with increasing temperature to some extent and the reason given to this observation was that, temperature increases the mobility of ions by reducing viscosity. This observation is in conformity with that of Barron and Ashton, (2007) that, temperature increases the detection and measurability of dissolved ions.

Salinity

The increasing trend in salinity was associated to the increasing presence of dissolved ions in solution due to biodegradation. This conclusion was made in relation to Moliterni *et al.* (2012) that biodegradation increases the presence of dissolved ions in solution. It was also found to increase with increasing temperature to some extent and this is because temperature increases the mobility of ions by reducing viscosity.

This observation is in conformity with that of Barron and Ashton, (2007) that temperature increases the measurability of dissolved ions. Observations of the trend in salinity evolution showed that, it increased along with increments in electrical conductivity and the reason associated to this observation was that, they are both influenced by the amount of dissolved ion in solution. This finding is in agreement with that observed by McNeil and Cox, (2000) that salinity and conductivity are related and that one can be measured to give an indication of the other.

Surface Tension

The decreasing trend in surface tension was attributed to the addition of bile to the treatment process. Bile has the ability to reduce surface tension because of its micelle-forming properties (Vethamuthu *et al.*, 1992c). Also, surface tension reduction was possibly facilitated by biosurfactant produced by bacteria during the biodegradation process. *Bacilli* sp and *Pseudomonas* sp are known biosurfactant producers and that *Pseudomonas* especially is capable of producing biosurfactants when utilizing hydrocarbons as carbon and energy sources (Rahman *et al.*, 2003). Moreover surface tension reductions were possibly enhanced by synergistic interactions between biosurfactants produced by bacteria and that present in bile.

Microbial Colony Numbers

Increase in microbial colony numbers was interpreted to mean an effective utilization of hydrocarbon contaminants under favourable experimental conditions. Favourable environmental conditions are necessary for successful biodegradation (Nilanjana and Preethy, 2011). Decrease in microbial colony numbers on day 7 however was associated to microbial colony numbers encountering unfavourable conditions including toxic by-products of biodegradation. Biodegradation of some class of hydrocarbons produces toxic products which hinders the capabilities of most bacteria (Booyjzsen, 2007). According to Friedrich (2010), the normal bacteria growth curve follows the order lag, log, stationary and death phases. Therefore the increases and decreases observed in microbial colony numbers were possibly due to the normal bacterial growth curve.

The decrease in microbial numbers was also related to the possibility that microbes encountered recalcitrant hydrocarbon compounds. Some recalcitrant compounds, such as

the high molecular weight polycyclic aromatic hydrocarbons (PAHs), may not be degraded at all (Atlas and Bragg, 2009). According to Friedrich (2010), the normal bacteria growth curve follows the order lag, log, stationary and death phases. Therefore the increases and decreases observed in microbial colony numbers were possibly due to the normal bacterial growth curve.

Percent TPH Degraded

Although it received 1.8 ml bile, it produced a lower percentage TPH degradation of 96.21% compared to the 97.33% produced by the 1.6 ml bile treatment. The reasons assigned to this relatively lower percentage degradation are that; possibly this treatment produced biodegradation by-products that were to some extent toxic to bacteria involved in mineralization of hydrocarbons and therefore their capacities were limited. This association was made because biodegradation of certain hydrocarbons produces toxic products which hinder the abilities of certain bacteria (Alexander, 1999). Additionally this outcome was related to possible biochemical interactions between bacteria cells and bile that might have triggered the bactericidal properties of bile by releasing sodium taurodeoxycholate and sodium deoxycholate which are known chemical substances present in bile that inhibit bacterial growth (Sung *et al.*, 1993).

5.5.6 Nutrients + Bile (1.2 ml) Treatment

pH

The observation that, pH initially reduced and subsequently increased on day 7 was associated to the following. Reduction in pH implied that acidic by-products were mainly formed. This is in conformity with Nilanjana and Preethy (2011) that biodegradation of hydrocarbons produces acidic intermediates and by-products. Increment in pH was

associated to the possibility that, most ions released into solution reacted to form alkaline products such as NaOH and KOH thereby increasing pH. According to Bagherzadeh *et al.* (2008) nutrient addition increases the pH of a given solution (Bagherzadeh *et al.*, 2008). Therefore, the addition of nutrients also possibly contributed to the increment in pH observed.

Temperature

Bacteria respiration is an exothermic redox reaction which releases energy in the form of heat (en.wikipedia.org/wiki/Cellular_respiration). Thus the initial increase in temperature and the subsequent decrease on day 7 was explained to be due bacteria activities (respiration). Hence an increase in temperature was related to an increase in respiration and a decrease in temperature associate with a decrease in respiration. Also accumulation of CO_2 possibly contributed to temperature increments. This relation was made because IPCC (2007) reported that accumulation of CO_2 increases temperature due to the heat trapping ability of CO_2.

DO

Major degradations of hydrocarbons are carried out by aerobic microorganisms (Leila *et al.*, 2006). Therefore increments and decrements in DO were related to aerobic microbial activities. Consequently reduction in DO on day 3 and subsequent increase on day 5 and 7 was explained as follows. Reduction in DO implied microorganisms were using more DO than was supplied whereas the increments (on day 5 and 7) were related to possible little oxygen utilizations due low microbial activities. Increments in temperature possibly enhanced reductions in DO levels. According to Biology with computers (2000),

temperature increments cause decreases in DO levels, thus the implication of temperature in the reduction of DO levels.

Electrical Conductivity

The continuous increase in electrical conductivity from day 1 through 7 was associated to an increasing concentration in dissolve ions in solution due to microbial degradation of petroleum hydrocarbons. This conclusion was drawn with reference to Moliterni *et al.* (2012), that biodegradation increases the presence of dissolved ions in solution.

Salinity

Biodegradation increases the presence of dissolved ions in solution (Moliterni *et al.*, 2012). Hence the increasing trend in salinity was associated to the increasing concentration of dissolved ions in solution due to biodegradation. Salinity evolutions showed that, it increased along with increments in electrical conductivity. This finding is in agreement with that observed by McNeil and Cox, (2000) that salinity and conductivity are related and that one could be measured to give an indication of the other because their measurements both depend on the amount of dissolve ions in solution.

Surface Tension

The decreasing trend in surface tension in this treatment was attributed to bile added to the treatment process. Bile has the ability to reduce surface tension due to its micelle-forming properties (Vethamuthu *et al.*, 1992c). *Bacilli* sp and *Pseudomonas* sp are known biosurfactant producers and that *Pseudomonas* especially is capable of producing biosurfactants when utilizing hydrocarbons as carbon and energy sources (Rahman *et al.*, 2003). Therefore surface tension reductions were possibly facilitated by biosurfactant

produced by bacteria during the biodegradation process. Moreover surface tension reductions possibly were enhanced by synergistic interactions between biosurfactants produced by bacteria and that present in bile.

Microbial Colony Numbers

Favourable environmental conditions are necessary for successful biodegradation (Nilanjana and Preethy, 2011). Consequently increase in microbial colony numbers recorded on day 3 implied that, the microbes were effectively utilizing the hydrocarbon contaminants and that experimental conditions were favourable for growth. However the decrease in microbial colony numbers on day 5 and 7 were associated to bacteria encountering unfavourable conditions including toxic by-products of their biodegradation process. Biodegradation of certain hydrocarbons produces toxic products which hinders the abilities of certain bacteria (Alexander, 1999).

The decrease in microbial colony numbers on day 5 and 7 was also related to the possibility that microbes encountered recalcitrant hydrocarbon compound. Some recalcitrant compounds, such as the high molecular weight Polycyclic Aromatic Hydrocarbons (PAHs), may not be degraded at all (Atlas and Bragg, 2009). According to Friedrich (2010), bacteria growth follows a particular pattern and that is lag, log, stationary and death phases. Therefore the increases and decreases observed in microbial colony numbers were possibly due to the normal bacterial growth pattern.

Percent TPH Degraded

Biodegradation of some class of hydrocarbons produces toxic products which hinders the abilities of most bacteria (Booyjzsen, 2007). Therefore the rather relatively lower

percentage TPH degradation of 87.63% produced in this treatment was attributed to the possibility that this treatment produced biodegradation by-products that were largely toxic to bacteria involved in the mineralization of hydrocarbons. Additionally this outcome was related to possible biochemical interactions between bacteria cells and bile that might have triggered the bactericidal properties of bile by releasing sodium taurodeoxycholate and sodium deoxycholate which are known chemical substances present in bile that inhibit bacterial growth (Sung *et al.*, 1993).

5.5.7 Nutrients + Bile (1.4 ml) Treatment

pH

Biodegradation of hydrocarbons produces acidic by-products (Nilanjana and Preethy, 2011). Therefore the acidic pH recorded throughout this treatment implied that, most of the by-products of degradation were largely acidic in nature (example, carboxylic acids). Accumulation CO_2 possibly contributed to the acidity recorded. This linkage was made because complete mineralization of hydrocarbons according to Adebusoye *et al.* (2006) produces carbon dioxide which reacts with water to form carbonic acids (Adebusoye *et. al.*, 2006).

Temperature

The initial increase in temperature from day 1 through 5 and subsequent decrease on day 7 was explained to be due to an initial increase in bacteria respiration follow by a reduction in respiration. This relation was made because bacteria respiration is an exothermic redox reaction which releases energy in the form of heat (en.wikipedia.org/wiki/Cellular_respiration). Also accumulation of CO_2 possibly

contributed to temperature increments. Accumulation of CO_2 increases temperature due to the heat trapping ability of CO_2 (IPCC, 2007).

DO

Major degradations of hydrocarbons are carried out by aerobic microorganisms (Leila *et al.,* 2006). Thus the reduction in DO throughout this treatment implied that microorganisms were using more DO for degradation than was supplied. Increments in temperature were implicated to have also contributed to the decreases in DO levels recorded. This observation is in line with that reported by Biology with computers (2000) that an increase in temperature causes a decrease in DO levels.

Electrical Conductivity

Biodegradation increases the presence of dissolved ions in solution (Moliterni *et al.*, 2012). As such, an increase in electrical conductivity implied an increase in dissolve ions in solution due to biodegradation of contaminants. Also electrical conductivity was found to be to some extent (day 3 and 5) influenced by increments in temperature and the reason is that, temperature increases the mobility of ions by reducing viscosity. This observation is in conformity with that of Barron and Ashton, (2007) that temperature increases the mobility of ion, thus increases their detection and measurability.

Salinity

Biodegradation increases the presence of dissolved ions in solution (Moliterni *et al.*, 2012). As such the increasing trend in salinity was associated to the increasing concentration of dissolved ions in solution due to biodegradation. Salinity evolution trend showed that, it increased along with increments in electrical conductivity and this is

because they both depend on the quantity of dissolved ions in solution. This finding is in agreement with that observed by McNeil and Cox, (2000) that salinity and conductivity are related and that one can be measured to give an indication of the other. Salinity was also to some extent influenced by temperature (day 3 and 5). Temperature increases the mobility of ions by reducing viscosity and thus increases the measurability of these ions (Barron and Ashton, 2007). This implies that temperature increments on day 3 and 5 possibly caused the increment in salinity values recorded in these days.

Surface Tension

Bacilli sp and *Pseudomonas* sp are known biosurfactant producers and that *Pseudomonas* especially is capable of producing biosurfactants when utilizing hydrocarbons as carbon and energy sources (Rahman *et al*., 2003). Also, bile has the ability to reduce surface tension because of its micelle-forming properties (Vethamuthu *et al*., 1992c). Therefore, the high surface tension reductions recorded in this treatment could be said to be due to synergistic interactions between biosurfactants produced by bacteria and that present in bile.

Microbial Colony Numbers

According to Nilanjana and Preethy (2011), favourable environmental conditions are necessary for successful biodegradation. Thus the increase in microbial colony numbers was interpreted to mean an effective utilization of hydrocarbon contaminants under favourable experimental conditions. Decrease in microbial colony numbers on day 7 however was associated to bacteria encountering unfavourable conditions including toxic by-products of biodegradation process. Biodegradation of certain hydrocarbons produces toxic products which hinders the abilities of certain bacteria (Alexander, 1999).

According to Friedrich (2010), the normal bacteria growth curve follows the order lag, log, stationary and death phases. Therefore the increases and decreases observed in microbial colony numbers were possibly as a result of bacteria going through their normal growth curve.

Percent TPH Degraded

The comparatively very low percentage degradation of 51.11% recorded in this treatment implied that, microbial actions on hydrocarbons were very low in the treatment. This possibly was due to the formation of liquid crystals, gels and macroemulsions (Rouse *et al.*, 2001), these substances hinder biodegradation processes. Also, this low percentage degradation possibly resulted from biochemical interactions between bacteria cells and bile that might have triggered the bactericidal properties of bile by releasing sodium taurodeoxycholate and sodium deoxycholate which are known chemical substances present in bile that inhibit bacterial growth (Sung *et al.*, 1993).

Moreover, it was possible that antagonistic interactions might have resulted from reactions between biosurfactants produced by bacteria and that present in bile when both were concentrated in this treatment, thus affecting the emulsification and solubilizations of hydrocarbon contaminants. Biodegradation of certain hydrocarbons produces toxic products which hinders the abilities of certain bacteria (Alexander, 1999). Thus, it was possible that this treatment produced biodegradation by-products that were largely toxic to bacteria involved in mineralization of hydrocarbons and therefore their capacities were limited.

5.5.8 Nutrients + Bile (1.6 ml) Treatment

pH

pH's initially reduction on day 3 and 5 and subsequently increased on day 7 was associated to the following. Reduction in pH implied the formation of acidic by-products. This is in conformity with Nilanjana and Preethy (2011) that biodegradation of hydrocarbons produces acidic intermediates and by-products. Also accumulation CO_2 possibly contributed to the acidity recorded. This linkage was made because complete mineralization of hydrocarbons according to Adebusoye *et al.* (2006) produces carbon dioxide which reacts with water to form carbonic acids (Adebusoye *et. al.*, 2006).

Increment in pH however was associated to the possibility that, most ions released into solution reacted to form alkaline products such as NaOH and KOH thereby increasing pH. According to Bagherzadeh *et al.* (2008) nutrient addition increases the pH of a given solution (Bagherzadeh *et al.*, 2008). Therefore, the addition of nutrients most possibly also contributed to the increment in pH observed.

Temperature

Microbial respiration is an exothermic redox reaction which releases energy in the form of heat (en.wikipedia.org/wiki/Cellular_respiration). Consequently the increasing trend in temperature recorded from day 1 to 7 in this experiment was related increments in microbial respiration in the treatment process. Also accumulation of CO_2 possibly contributed to temperature increments. Accumulation of CO_2 according to IPCC (2007) increases temperature due to the heat trapping ability of CO_2.

DO

Major degradations of hydrocarbons are carried out by aerobic microorganisms (Leila *et al.,* 2006). Thus the reduction in DO throughout this treatment meant that microorganisms were using more DO for degradation than was supplied. Increments in temperature probably enhanced reductions in DO levels. Temperature increments cause decreases in DO levels (Biology with computers, 2000).

Electrical Conductivity

Biodegradation increases the presence of dissolved ions in solution (Moliterni *et al.*, 2012). As such, an increase in electrical conductivity implied an increase in dissolve ions in solution due to biodegradation of contaminants. Also electrical conductivity was found to be influenced by changes in temperature and the reason is that, temperature increases the mobility of ions by reducing viscosity. This observation is in conformity with that of Barron and Ashton, (2007) that temperature increases the mobility of ions thus increases their detection and measurability.

Salinity

The increasing trend in salinity was also associated to the increasing concentration of dissolved ions in solution due to biodegradation. Biodegradation increases the presence of dissolved ions in solution (Moliterni *et al.*, 2012). Salinity evolution trend showed that, it increased along with increments in electrical conductivity and this is because they both are dependent upon the quantity of dissolved ions in solution. This finding is in agreement with that observed by McNeil and Cox, (2000) that salinity and conductivity are related and that one could be measured to give an indication of the other.

Surface Tension

The rather increasing trend in surface tension recorded in this treatment was explained to be due to high microbial degradation. According to Moliterni *et al.* (2012), high percentage hydrocarbon biodegradation results in surface tension increments. Also, BostrÖm *et al.* (2001) stated that, the increasing presence of dissolved ions increases the surface tension of air-water interface of a given solution as such the presence of large amount of dissolved ions in this treatment possibly contributed to this increasing trend in surface tension.

Microbial Colony Numbers

Favourable environmental conditions are necessary for successful biodegradation (Nilanjana and Preethy, 2011). Therefore the increase in microbial colony numbers from day 1 to 7 implied that, microbes were effectively utilizing hydrocarbon contaminants under favourable experimental conditions for growth.

Percent TPH Degraded

The highest percentage (97.34 %) TPH degradation achieved in this treatment implied that hydrocarbon contaminants were effectively mineralised by microbes and that the mineralization process was enhanced by bile supplied in the treatment. Bile has a solubilising and emulsifying ability (Swapnadip, 2010). This implied that, bile increased the bioavailability of hydrocarbons by increasing their solubilities for easy microbial utilization. The high percentage degradation was also attributed to the large microbial numbers recorded in the treatment. The correlation analysis in figure 4.1 showed that, an increase in microbial number causes a corresponding increase in percentage TPH degraded. Overall, this high percentage TPH degradation implied that, the supply of

nutrients together with bile at a volume of 1.6 ml and under the experimental conditions of this treatment, is recommendable for the realization of a successfully hydrocarbon degradation in a contaminated water of relatively the same volume.

5.5.9 Nutrients + Bile (1.8 ml) Treatment

pH

The change in pH from an initial alkaline medium to an acidic medium was related to an initial concentration of ions that reacted to form alkaline compounds such as NaOH and KOH on day 1 and 3 then followed by the formation of acidic by-products (carboxylic acids) on day 5 and 7. Also the achievement of acidic pH on day 5 and 7 was possibly facilitated by complete aerobic microbial mineralization of some hydrocarbons in the treatment. Complete mineralization of hydrocarbons releases CO_2 which reacts with water to form carbonic acids, thus reducing pH (Adebusoye *et. al.*, 2006).

Temperature

Microbial respiration is an exothermic redox reaction which releases energy in the form of heat (en.wikipedia.org/wiki/Cellular_respiration). Thus the increasing trend in temperature recorded from day 1 to 7 in this experiment was related increments in microbial respiration (activities) in the treatment. The continuous rise in temperature observed was possibly facilitated by the accumulation of CO_2. Accumulation of carbon dioxide increases temperature due to its heat trapping ability (IPCC, 2007).

DO

Major degradations of hydrocarbons are carried out by aerobic microorganisms (Leila *et al.*, 2006). Thus the reduction in DO throughout this treatment implied that

microorganisms were using more DO for degradation than was supplied. Increments in temperature possibly enhanced reductions in DO levels. Temperature increments according to Biology with computers cause decrements in DO levels.

Electrical Conductivity

Biodegradation increases the presence of dissolved ions in solution (Moliterni *et al.*, 2012). As such, an increase in electrical conductivity implied an increase in dissolve ions in solution due to biodegradation of contaminants. Also electrical conductivity was found to be influenced by increments in temperature and the reason is that, temperature increases the mobility of ions by reducing viscosity, thus increases their measurability. This observation is in conformity with that of Barron and Ashton, (2007) that, temperature increases the mobility of ions thus increases their detection and measurability.

Salinity

Biodegradation increases the presence of dissolved ions in solution (Moliterni *et al.*, 2012). Thus the increasing trend in salinity was also associated to the increasing concentration of dissolved ions in solution due to biodegradation. Observations of the trend of salinity showed that, it increased along with increments in electrical conductivity. The reason is that, they both depend on the amount of dissolved ions in solution. This finding is in agreement with that observed by McNeil and Cox, (2000) that salinity and conductivity are related and that one can be measured to give an indication of the other.

Surface Tension

Bacilli sp and *Pseudomonas* sp are known biosurfactant producers and that *Pseudomonas* especially are capable of producing biosurfactants when utilizing hydrocarbons as carbon and energy sources (Rahman *et al*., 2003). Also, bile has the ability to reduce surface tension because of its micelle-forming properties (Vethamuthu *et al*., 1992c). Therefore, the high surface tension reduction was attributable to synergistic interactions between these biosurfactants.

Microbial Numbers

Increase in microbial colony numbers was interpreted to mean an effective utilization of hydrocarbon contaminants under favourable experimental conditions. Favourable environmental conditions are necessary for successful biodegradation (Nilanjana and Preethy). Decrease in microbial colony numbers on day 7 however was associated to microbial colony numbers encountering unfavourable conditions including toxic by-products of biodegradation. Biodegradation of certain hydrocarbons produces toxic products which hinders the abilities of certain bacteria (Alexander, 1999).

The presence of recalcitrant hydrocarbons possibly contributed to the decrements in microbial colony numbers recorded. Some recalcitrant compounds, such as the high molecular weight polycyclic aromatic hydrocarbons (PAHs), may not be degraded at all because of their molecular structures (Atlas and Bragg, 2009). According to Friedrich (2010), the normal bacteria growth curve follows the order lag, log, stationary and death phases. Therefore the increases and decreases observed in microbial colony numbers might have been due to the normal bacterial growth curve.

Percent TPH Degraded

86.36% though a high percent degradation was lower compared to some treatments (Bile 1.2, 1.4 1.6, 1.8 ml, Nutrient + Bile, 1.2, 1.6 ml and control treatment) in the study. This implied that increasing volume of bile to 1.8 ml did not produce a corresponding increase in percentage TPH degraded. The possible reasons for this relatively lower percentage degradation are that; this treatment possibly produced biodegradation by-products that were largely toxic to bacteria involved in mineralization of hydrocarbons. Biodegradation of some class of hydrocarbons produces toxic products which hinders the abilities of most bacteria (Booyjzsen, 2007). Additionally this outcome was related to possible biochemical interactions between bacteria cells and bile that might have triggered the bactericidal properties of bile by releasing sodium taurodeoxycholate and sodium deoxycholate which are growth (Sung *et al*., 1993).

5.6 Relationship between Percent TPH Degraded and Volume of Bile Supplied

The reasons assigned to the medium correlation between volume of bile added and percent TPH degraded are that, the treatments with relatively higher bile volumes but lower percentage TPH degradation possibly produced biodegradation by-products that were largely toxic to bacteria involved in mineralization of hydrocarbons.

Also there was the possibility that, antagonistic interactions might have occurred between biosurfactants produced by bacteria and that present in bile in these treatments, thus retarding degradation processes. Moreover this can be related to possible biochemical reactions between bacteria cells and bile that possibly triggered the bactericidal properties of bile by releasing sodium taurodeoxycholate and sodium deoxycholate which are

known chemical substances present in bile that inhibit bacterial growth (Sung *et al.*, 1993). Therefore it was concluded that, increasing the concentration/quantity of biosurfactants is not an assurance of a corresponding increase in biodegradation of a hydrocarbon contaminated medium (water).

This observation is similar to conclusions drawn by Rouse *et al.* (2001) that certain surfactants inhibit biodegradation. They demonstrated this phenomenon by using anionic DiPhenyloxide DiSulfonate Surfactants (DPDS) (C_{12} and C_{16} alkyl moieties) with naphthalene in aqueous systems to find the influence of these surfactants on biodegradation of naphthalene. The overall trend for both C_{12}- and C_{16}-DPDS assays indicated decreasing efficiency in degradation of naphthalene with increasing surfactant concentration.

Also the observation that, nutrient medium treatment with no bile addition produced a higher percentage degradation than the treatments, Bile (1.4 ml), Nutrients + Bile (1.2 ml), Nutrients + Bile (1.4 ml) and Nutrients + Bile (1.8 ml) implied that, the mere supply of bile is not an assurance of a higher percentage degradation but rather, a measured amount should be dispensed depending on the prevailing experimental conditions.

5.7 Relationship between TPH Degraded and Microbial Colony Numbers

The strong relationship realized between microbial colony numbers and TPH degraded was attributed to the fact that *Bacillus* sp and *Pseudomonas* sp isolated in the study were effectively utilizing the hydrocarbon contaminants as carbon source for their multiplications (growth). These bacteria have been isolated at oil contaminated sites and are known petroleum hydrocarbon degraders (Chaillan *et al.*, 2004).

Summarily, the deduction made was that, the more the microbial biomass, the more hydrocarbons are biodegraded and the lesser the residual TPH recorded.

CHAPTER SIX

6.0 Conclusions and Recommendations

6.1 Conclusions

Based on the microscopy investigations and biochemical analysis carried out in the study, microorganisms involved in biodegradation were characterized as members of *Pseudomonas* sp and *Bacillus* sp group of bacteria.

The high percentage TPH degradation recorded in nutrient medium treatment (without bile) implied that, successful biodegradation can be achieved with the supply of nutrients only.

It was established that, the supply and increments in quantities of bile did not necessarily result in an increase in percentage TPH degraded.

It was also realized that, the mere combination of bile and nutrients does not increase the mineralization capacities of hydrocarbon degrading microbes.

An increase in microbial numbers however reflected an increase in percentage TPH biodegraded.

6.2 Recommendations

The following are recommended for further research.

- A study should be carried out where different masses of dewatered bile are used to assess the percentage TPH degradation in a similar setup used in the present study.

This would give information as to whether dewatering reduces the bactericidal property of bile or aggravates it. Based on this, the right state of bile can be recommended for bioremediation.

- A study should be conducted to find out the percentage TPH degraded in each sampling day so as to establish the individual percentage contributions of the various sampling days to the total percentage hydrocarbon degraded at the end of each treatment period.

- A comparative study should be conducted between bile and a commercial biosurfactant to evaluate the efficiency of bile against the commercial product in enhancing biodegradation of petroleum hydrocarbon contaminants.

- A research should also be conducted to find out the effect of bile on biodegradation of petroleum hydrocarbons contaminants under anaerobic conditions.

- A 16S DNA analysis should be conducted to find out the exact species of *Pseudomonas* and *Bicilli* that were involved in biodegradation in this study.

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APPENDIX A. Results of TPH Analysis for Nutrient Medium Treatment

Print Date: Sun Aug 26 15:38:58 2012 Page 1 of 1

Title : C:\star\data\2012\student project\knust student\2012-08-20 TPH\nd.run
Run File : C:\star\data\2012\student project\knust student\2012-08-20 tph\control-front.mth
Method File : c:\star\data\2012\student project\knust student\2012-08-20 tph\control-front.mth
Sample ID : ND

Injection Date: 8/20/2012 3:33 PM Calculation Date: 8/26/2012 3:36 PM

Operator :
Workstation: OS
Instrument : Varian CP-3800 GC
Channel : Front = FID

Detector Type: 3800 (10 Volts)
Bus Address : 44
Sample Rate : 10.00 Hz
Run Time : 54.945 min

** GC Workstation Version 6.41 ** 02460-3090-C65-01F4 **

Run Mode : Analysis
Peak Measurement: Peak Area
Calculation Type: External Standard

Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Group	Status Codes
1	C11	0.1044	7.364	-0.178	1255	BB	0.4	0	
2	C12	0.0042	9.632	0.037	53	BB	0.7	0	
3	C16	0.0273	18.990	-0.006	287	BB	0.0	0	
4	C25	0.0702	36.003	0.111	413	BB	0.0	0	
5	C28	1.7471	40.366	0.068	6152	BB	0.0	0	
6	C29	0.4021	41.609	-0.055	1070	BB	0.0	0	
7	C30	1.0954	42.918	-0.054	2308	BB	4.2	0	
8	C32	1.8106	45.944	0.021	2122	BB	4.4	0	
9	C33	1.4723	47.843	0.070	1408	BB	4.1	0	
	Group 0	6.7336		0.014	15068				
	Totals:	6.7336		0.014	15068				

Total Unidentified Counts : 0 counts

Detected Peaks: 11 Rejected Peaks: 2 Identified Peaks: 9

Multiplier: 1 Divisor: 1 Unidentified Peak Factor: 0

Baseline Offset: -80 microVolts LSB: 1 microVolts

Noise (used): 75 microVolts - monitored before this run

Tray: 2 Vial: 2 Injection Number: 1 Volume: 1.00 uL

APPENDIX B. Results of TPH Analysis for Bile (1.2 ml) Treatment

rint Date: Sun Aug 26 15:20:03 2012 Page 1 of 1

itle :
lun File : C:\star\data\2012\student project\knust student\2012-08-20 TPH\b 1.run
lethod File : C:\star\Methods\Alkanes.mth
lample ID : B 1

njection Date: 8/20/2012 5:30 PM Calculation Date: 8/26/2012 3:03 PM

perator :
orkstation: OS
nstrument : Varian CP-3800 GC
hannel : Front - FID

Detector Type: 3800 (10 Volts)
Bus Address : 44
Sample Rate : 10.00 Hz
Run Time : 54.945 min

• GC Workstation Version 6.41 •• 02460-3090-C65-01F4 ••

un Mode : Analysis
eak Measurement: Peak Area
alculation Type: External Standard

Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Group	Status Codes
1	C12	0.0107	9.451	-0.144	135	BB	0.2	0	
2	C13	0.0168	12.074	0.164	203	BB	1.9	0	
3	C14	0.1024	14.231	-0.151	1208	BB	0.0	0	
4	C16	0.0540	19.170	0.039	568	VB	3.9	0	
5	C19	0.0288	25.340	-0.174	251	BB	3.4	0	
6	C23	0.0227	32.609	-0.163	167	BB	0.0	0	
7	C24	0.1433	34.331	-0.069	952	BB	0.3	0	
8	C25	0.0132	35.664	-0.298	78	BB	1.1	0	
9	C26	0.0355	37.372	-0.096	183	VB	0.0	0	
10	C27	0.0279	38.903	-0.011	114	VP	8.1	0	
11	C28	0.1878	40.226	-0.089	661	PV	3.3	0	
12	C29	0.1621	41.666	0.002	431	VV	1.7	0	
13	C30	0.3377	42.967	-0.005	711	VV	0.0	0	
14	C31	0.9607	44.378	0.013	1545	VV	3.5	0	
15	C32	0.6804	45.953	-0.021	797	VB	3.0	0	
16	C33	1.0166	47.835	-0.034	972	VV	1.6	0	
17	C34	0.6923	50.136	0.013	515	VB	3.0	0	
18	C35	1.2495	52.859	0.039	702	BB	0.0	0	
	Group 0	5.7424		-0.985	10193				
	Totals:	5.7424		-0.985	10193				

otal Unidentified Counts : 22147 counts

etected Peaks: 69 Rejected Peaks: 7 Identified Peaks: 18

ultiplier: 1 Divisor: 1 Unidentified Peak Factor: 0

aseline Offset: -34 microVolts LSB: 1 microVolts

oise (used): 53 microVolts - monitored before this run

ray: 2 Vial: 4 Injection Number: 1 Volume: 1.00 uL

APPENDIX C. Results of TPH Analysis for Bile (1.4 ml) Treatment

Print Date: Sun Aug 26 15:20:12 2012 Page 1 of 1

Title :
Run File : C:\star\data\2012\student project\knust student\2012-08-20 TPH\b 2.run
Method File : C:\star\Methods\Alkanes.mth
Sample ID : B 2

Injection Date: 8/20/2012 6:28 PM Calculation Date: 8/26/2012 3:03 PM

Operator :
Workstation: OS
Instrument : Varian CP-3800 GC
Channel : Front = FID

Detector Type: 3800 (10 Volts)
Bus Address : 44
Sample Rate : 10.00 Hz
Run Time : 54.945 min

** GC Workstation Version 6.41 ** 02460-3090-C65-01F4 **

Run Mode : Analysis
Peak Measurement: Peak Area
Calculation Type: External Standard

Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Group	Status Codes
1	C14	0.1314	14.213	-0.169	1551	BB	0.0	0	
2	C15	0.0694	16.646	-0.150	782	BB	0.0	0	
3	C16	0.0901	19.162	0.031	948	VB	4.9	0	
4	C19	0.0821	25.341	-0.173	716	BB	3.1	0	
5	C23	0.1204	32.599	-0.173	887	BB	3.6	0	
6	C25	0.2120	35.987	0.025	1248	PB	0.0	0	
7	C26	0.3479	37.377	-0.091	1796	BB	0.0	0	
8	C27	0.1278	38.842	-0.072	522	BB	0.0	0	
9	C28	6.4145	40.307	-0.008	22586	BB	10.3	0	
10	C29	0.0983	41.607	-0.057	261	BB	5.1	0	
11	C30	0.3627	42.928	-0.044	764	BB	6.5	0	
12	C31	0.1084	44.313	-0.052	174	BB	2.1	0	
	Group 0	8.1650		-0.933	32235				
	Totals:	8.1650		-0.933	32235				

Total Unidentified Counts : 2406 counts

Detected Peaks: 19 Rejected Peaks: 3 Identified Peaks: 12

Multiplier: 1 Divisor: 1 Unidentified Peak Factor: 0

Baseline Offset: -14 microVolts LSB: 1 microVolts

Noise (used): 58 microVolts - monitored before this run

Tray: 2 Vial: 5 Injection Number: 1 Volume: 1.00 uL

APPENDIX D. Results of TPH Analysis for Bile (1.6 ml) Treatment

```
rint Date: Sun Aug 26 15:20:19 2012                    Page 1 of 1

itle.        :
un File      : C:\star\data\2012\student project\knust student\2012-08-20 TPH\b 3.run
ethod File   : C:\star\Methods\Alkanes.mth
ample ID     : B 3

njection Date: 8/20/2012 7:27 PM     Calculation Date: 8/26/2012 3:03 PM

perator      :                              Detector Type: 3800 (10 Volts)
orkstation: OS                              Bus Address  : 44
nstrument  : Varian CP-3800 GC              Sample Rate  : 10.00 Hz
hannel     : Front = FID                    Run Time     : 54.945 min

* GC Workstation Version 6.41 ** 02460-3090-C65-01F4 **

un Mode           : Analysis
eak Measurement: Peak Area
alculation Type: External Standard
```

Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Group	Status Codes
1	C12	0.0197	9.304	-0.291	249	BB	0.2	0	
2	C14	0.1124	14.214	-0.168	1327	BB	0.0	0	
3	C16	0.0830	18.991	-0.140	873	BB	3.9	0	
4	C23	0.0273	32.601	-0.171	201	BB	3.3	0	
	Group 0	0.2424		-0.770	2650				
	Totals:	0.2424		-0.770	2650				

```
otal Unidentified Counts :          645 counts

etected Peaks: 8          Rejected Peaks: 3          Identified Peaks: 4

ultiplier: 1          Divisor: 1          Unidentified Peak Factor: 0

aseline Offset: -32 microVolts          LSB:    1 microVolts

oise (used): 52 microVolts - monitored before this run

ray: 2  Vial: 6          Injection Number: 1          Volume: 1.00 uL

********************************************************************************
```

APPENDIX E. Results of TPH Analysis for Bile (1.8 ml) Treatment

Print Date: Sun Aug 26 15:20:28 2012 Page 1 of 1

Title :
Run File : C:\star\data\2012\student project\knust student\2012-08-20 TPH\b 4.run
Method File : C:\star\Methods\Alkanes.mth
Sample ID : B 4

Injection Date: 8/20/2012 8:25 PM Calculation Date: 8/26/2012 3:03 PM

Operator :
Workstation: OS
Instrument : Varian CP-3800 GC
Channel : Front = FID

Detector Type: 3800 (10 Volts)
Bus Address : 44
Sample Rate : 10.00 Hz
Run Time : 54.945 min

** GC Workstation Version 6.41 ** 02460-3090-C65-01F4 **

Run Mode : Analysis
Peak Measurement: Peak Area
Calculation Type: External Standard

Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Group	Status Codes
1	C14	0.0947	14.217	-0.165	1118	BB	0.0	0	
2	C16	0.1275	19.163	0.032	1341	VB	3.9	0	
3	C19	0.1528	25.338	-0.176	1333	BB	3.4	0	
4	C23	0.2102	32.592	-0.180	1548	BB	3.4	0	
5	C25	0.1413	36.008	0.046	832	BB	0.0	0	
6	C28	2.6689	40.356	0.041	9397	PB	0.0	0	
	Group 0	3.3954		-0.402	15569				
	Totals:	3.3954		-0.402	15569				

Total Unidentified Counts : 3179 counts

Detected Peaks: 12 Rejected Peaks: 3 Identified Peaks: 6

Multiplier: 1 Divisor: 1 Unidentified Peak Factor: 0

Baseline Offset: -41 microVolts LSB: 1 microVolts

Noise (used): 57 microVolts - monitored before this run

Tray: 2 Vial: 7 Injection Number: 1 Volume: 1.00 uL

APPENDIX F. Results of TPH Analysis for Nutrient + Bile (1.2 ml) Treatment

Print Date: Sun Aug 26 15:20:37 2012 Page 1 of 1

Title :
Run File : C:\star\data\2012\student project\knust student\2012-08-20 TPH\b & n 1.run
Method File : C:\star\Methods\Alkanes.mth
Sample ID : B & N 1

Injection Date: 8/20/2012 9:23 PM Calculation Date: 8/26/2012 3:04 PM

Operator :
Workstation: OS
Instrument : Varian CP-3800 GC
Channel : Front = FID

Detector Type: 3800 (10 Volts)
Bus Address : 44
Sample Rate : 10.00 Hz
Run Time : 54.945 min

** GC Workstation Version 6.41 ** 02460-3090-C65-01F4 **

Run Mode : Analysis
Peak Measurement: Peak Area
Calculation Type: External Standard

Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Group	Status Codes
1	C12	0.0351	9.549	-0.046	444	BB	0.3	0	
2	C13	0.0264	11.688	-0.222	319	BB	0.3	0	
3	C14	0.2469	14.206	-0.176	2914	BB	7.1	0	
4	C15	0.0611	16.643	-0.153	688	BB	4.2	0	
5	C16	0.1544	19.161	0.030	1625	VB	4.1	0	
6	C17	0.0203	21.485	0.124	198	BB	2.0	0	
7	C18	0.1259	23.428	-0.059	1174	VB	9.0	0	
8	C19	0.2166	25.410	-0.104	1889	BB	11.4	0	
9	C20	0.3367	27.338	-0.111	2761	BB	5.0	0	
10	C21	0.2771	29.180	-0.121	2215	BB	4.2	0	
11	C22	0.3055	30.946	-0.127	2368	BB	3.9	0	
12	C23	0.1395	32.776	0.004	1027	VB	0.0	0	
13	C24	0.0103	34.349	-0.051	69	VB	0.0	0	
14	C25	0.4956	35.940	-0.022	2917	VB	5.2	0	
15	C26	0.7249	37.356	-0.112	3742	BB	6.0	0	
16	C27	0.3779	38.817	-0.097	1545	BV	4.7	0	
17	C28	17.5804	40.270	-0.045	61902	BB	7.5	0	
18	C29	0.3465	41.651	-0.013	922	BB	8.6	0	
19	C30	0.5682	43.024	0.052	1197	BB	0.0	0	
20	C31	0.2956	44.299	-0.067	475	BV	4.8	0	
21	C32	3.2649	45.835	-0.139	3826	BB	5.9	0	
22	C33	1.8008	47.907	0.038	1722	VB	0.0	0	
23	C34	0.2567	50.098	-0.025	191	VB	0.0	0	
	Group 0	27.6673		-1.442	96130				
	Totals:	27.6673		-1.442	96130				

Total Unidentified Counts : 18787 counts

Detected Peaks: 37 Rejected Peaks: 1 Identified Peaks: 23

Multiplier: 1 Divisor: 1 Unidentified Peak Factor: 0

Baseline Offset: -86 microVolts LSB: 1 microVolts

Noise (used): 41 microVolts - monitored before this run

Tray: 2 Vial: 8 Injection Number: 1 Volume: 1.00 uL

APPENDIX G. Results of TPH Analysis for Nutrient + Bile (1.4 ml) Treatment

Print Date: Sun Aug 26 15:20:52 2012 Page 1 of 1

Title :
Run File : C:\star\data\2012\student project\knust student\2012-08-20 TPH\b & n 2.run
Method File : C:\star\Methods\Alkanes.mth
Sample ID : B & N 2

Injection Date: 8/20/2012 10:22 PM Calculation Date: 8/26/2012 3:04 PM

Operator :
Workstation: OS
Instrument : Varian CP-3800 GC
Channel : Front = FID

Detector Type: 3800 (10 Volts)
Bus Address : 44
Sample Rate : 10.00 Hz
Run Time : 54.945 min

** GC Workstation Version 6.41 ** 02460-3090-C65-01F4 **

Run Mode : Analysis
Peak Measurement: Peak Area
Calculation Type: External Standard

Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Group	Status Codes
1	C16	0.0416	19.160	0.029	438	BB	2.7	0	
2	C19	0.0522	25.332	-0.182	455	BB	2.6	0	
3	C23	0.0689	32.590	-0.182	507	BB	3.2	0	
4	C28	0.1446	40.199	-0.116	509	BB	0.0	0	
5	C29	0.0752	41.665	0.001	200	VB	0.0	0	
6	C30	0.2441	43.021	0.049	514	VV	0.0	0	
7	C31	7.2560	44.302	-0.063	11669	PB	4.4	0	
8	C32	20.0049	45.927	-0.047	23445	VP	4.9	0	
9	C33	30.9399	47.830	-0.039	29589	VB	5.9	0	
10	C34	34.7650	50.075	-0.048	25838	BB	7.1	0	
11	C35	37.4218	52.788	-0.032	21035	VB	8.4	0	
	Group 0	131.0142		-0.630	114199				
	Totals:	131.0142		-0.630	114199				

Total Unidentified Counts : 11769 counts

Detected Peaks: 46 Rejected Peaks: 3 Identified Peaks: 11

Multiplier: 1 Divisor: 1 Unidentified Peak Factor: 0

Baseline Offset: -66 microVolts LSB: 1 microVolts

Noise (used): 57 microVolts - monitored before this run

Tray: 2 Vial: 9 Injection Number: 1 Volume: 1.00 uL

APPENDIX H. Results of TPH Analysis for Nutrient + Bile (1.6 ml) Treatment

rint Date: Sun Aug 26 15:20:59 2012 Page 1 of 1

itle :
un File : C:\star\data\2012\student project\knust student\2012-08-20 TPH\b & n 3.run
ethod File : C:\star\Methods\Alkanes.mth
ample ID : B & N 3

njection Date: 8/20/2012 11:20 PM Calculation Date: 8/26/2012 3:04 PM

perator :
orkstation: OS
nstrument : Varian CP-3800 GC
hannel : Front = FID

Detector Type: 3800 (10 Volts)
Bus Address : 44
Sample Rate : 10.00 Hz
Run Time : 54.945 min

GC Workstation Version 6.41 ** 02460-3090-C65-01F4 **

un Mode : Analysis
eak Measurement: Peak Area
alculation Type: External Standard

Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Group	Status Codes
1	C13	0.0343	11.754	-0.156	414	BB	0.2	0	
2	C19	0.0346	25.331	-0.183	301	BB	2.8	0	
3	C23	0.0510	32.592	-0.180	376	BB	2.5	0	
4	C28	0.0891	40.193	-0.122	314	BB	2.4	0	
	Group 0	0.2090		-0.641	1405				
	Totals:	0.2090		-0.641	1405				

otal Unidentified Counts : 418 counts

etected Peaks: 7 Rejected Peaks: 2 Identified Peaks: 4

ultiplier: 1 Divisor: 1 Unidentified Peak Factor: 0

aseline Offset: -15 microVolts LSB: 1 microVolts

oise (used): 74 microVolts - monitored before this run

ray: 2 Vial: 10 Injection Number: 1 Volume: 1.00 uL

APPENDIX I. Results of TPH Analysis for Nutrient + Bile (1.8 ml) Treatment

rint Date: Sun Aug 26 15:21:07 2012 Page 1 of 1

itle :
un File : C:\star\data\2012\student project\knust student\2012-08-20 TPH\b & n 4.run
ethod File : C:\star\Methods\Alkanes.mth
ample ID : B & N 4

njection Date: 8/21/2012 12:19 AM Calculation Date: 8/26/2012 3:04 PM

perator :
orkstation: OS
nstrument : Varian CP-3800 GC
hannel : Front = FID

Detector Type: 3800 (10 Volts)
Bus Address : 44
Sample Rate : 10.00 Hz
Run Time : 54.945 min

• GC Workstation Version 6.41 ** 02460-3090-C65-01F4 **

un Mode : Analysis
eak Measurement: Peak Area
alculation Type: External Standard

Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Group	Status Codes
1	C12	0.0796	9.324	-0.271	1008	BB	0.5	0	
2	C14	0.3953	14.212	-0.170	4665	BB	7.5	0	
3	C15	0.0636	16.642	-0.154	717	BB	4.8	0	
4	C16	0.2370	19.160	0.028	2494	VB	3.6	0	
5	C17	0.0680	21.231	-0.130	663	BB	3.9	0	
6	C18	0.1751	23.418	-0.069	1632	VB	8.3	0	
7	C19	0.1461	25.403	-0.111	1274	VB	10.3	0	
8	C20	0.3879	27.334	-0.115	3181	BB	4.9	0	
9	C21	0.2799	29.181	-0.120	2237	BB	4.2	0	
10	C22	0.3281	30.945	-0.128	2543	BB	3.7	0	
11	C23	0.1080	32.758	-0.014	795	VB	0.0	0	
12	C24	0.2490	34.271	-0.129	1654	BB	3.6	0	
13	C25	0.6071	35.943	-0.019	3573	VV	4.8	0	
14	C26	0.7612	37.353	-0.115	3930	BB	7.6	0	
15	C27	0.4710	38.819	-0.096	1925	BB	5.4	0	
16	C28	20.8599	40.266	-0.049	73449	BB	7.0	0	
17	C29	0.5338	41.640	-0.024	1420	BB	7.4	0	
18	C30	0.3858	42.919	-0.053	813	BV	5.1	0	
19	C31	2.2696	44.479	0.114	3650	BB	4.9	0	
20	C32	1.6800	45.842	-0.132	1969	BB	5.0	0	
21	C33	1.1873	47.907	0.038	1135	VB	0.0	0	
	Group 0	31.2733		-1.719	114727				
	Totals:	31.2733		-1.719	114727				

otal Unidentified Counts : 14525 counts

etected Peaks: 34 Rejected Peaks: 3 Identified Peaks: 21

ultiplier: 1 Divisor: 1 Unidentified Peak Factor: 0

aseline Offset: -33 microVolts LSB: 1 microVolts

oise (used): 62 microVolts - monitored before this run

ray: 2 Vial: 11 Injection Number: 1 Volume: 1.00 uL

APPENDIX J. Formula for Determining Microbial Colony Numbers

Microbial numbers in Colony Forming Units (CFU) was obtained using the following formula ;

$$\text{CFU/ml} = \frac{\Sigma(number\ of\ colonies)}{ml} \text{ x number of dilutions}$$

APPENDIX K. Protocols for Determining Phosphorous and Nitrogen Content of a given Sample

Determination of Phosphorus as Phosphate Ion (Po4-3)

Digestion process

Put 2g of sample in a furnace at temperature 550 °C for 4hrs, add 4ml of concentrated HCl and 4ml of water and mixed, filter through acid wash filter paper into 100ml volumetric flask.

Phosphorus in phosphate ion (PO_4^{-3}) can be determined by Ascorbic acid – Molybdate method

Principle

Ammonium Molybdate and Potassium Anumonyl Tatrate (PAT) react with phosphate ions in strong acidic medium to form a complex. By reduction with ascorbic acid, an intense blue colour is formed which is measured with a spectrophometer

Reagents

1.0.1M Ascorbic Acid (A.A)

2.4% solution of Ammonium Molybdate (AM)

3.2.5M H_2SO_4

4.0.28% PAT

Working Colour Developing Reagent (CDR)

The CDR is prepared as ff;

50ml H_2SO4 + 5ml PAT + 30ml OF Am and 15ml of AA

6 serial standards are prepared from pure phosphate compound.

Protocol

1. Pick 0.25ml of each serial standard /sample.

2. Add 2.5ml of CDR.

3. Incubate at room temperature for 20minutes.

4. Read absorbance at 770nm on the spectrophotometer.

Crude Protein / Nitrogen Determination by Kjeldahl Method

Reagents

Mix indicator reagent as follows: Dissolve 0.2g of methylene red in 100ml 95% ethyl alcohol. Also dissolve 0.1g methylene blue in 50ml 95% ethyl alcohol and add the 2 solutions

Chemicals Needed

Boric acid (4%), 40% NaOH, 0.1M HCL, Concentrated H_2SO_4

Preparing Kjeldahl catalyst

Mix 1 part selenium + 10 parts $CuSO_4$ + 100 parts Na_2SO4

Digestion

Weigh 10g air dry sample into 500ml long-necked kjeldahl flask and 10ml distilled water to moisten the sample. Add 1 spatula full of kjeldahl catalyst (mixture of 1 part selenium + 10 parts $CuSO_4$ + 100 parts Na_2SO_4), followed by 20ml concentrated H_2SO_4. Digest until the solution clear and colourless. Allow the flask to cool, decant the fluid into a 100ml volumetric flask and make up to the mark with distilled water.

Distillation

Transfer an aliquot of 10ml fluid from the digested sample by means of a pipette into kjeldahl distillation flask. Add 90ml of distilled water to make it up to 100ml in the distillation flask. Add or dispense 20ml of 40% NaOH to the content of the distillation flask. Collect distillate over 10ml of 4% boric acid and 3 drops of mixed indicator in a 200ml conical flask. The presence of nitrogen gives a light blue colour.

Titration

Titrate collected distillate (about 100ml) with 0.1N HCL till the blue colour changes to grey and then suddenly flashes to pink.

CALCULATION

Weigh sample used, considering the dilution and the aliquot taken for distillation.

10g x 10ml/100ml = 1g

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

Where,

A = volume of standard HCL used in sample titration

B = volume of standard HCL used in blank titration

N = normality of standard HCL

$$\%\ \text{Crude Protein (CP)} = \frac{\%\ Total\ Nitrogen\ (NT)\ x\ 6.25\ (protein\ factor)}{1}$$

APPENDIX L. Table of ANOVA Alpha Values Determined

PARAMETER	ALPHA VALUE (0.05; 95% confidence intervals)	SIGNIFICANTLY DIFFERENT? (P<0.05)
pH	0.2005	No
Temperature	0.9917	No
DO	0.3654	No
Electrical Conductivity	0.0001	Yes
Salinity	0.0001	Yes
Surface Tension	0.0001	Yes
Microbial Colony Numbers	0.0089	Yes
TPH	0.0001	Yes

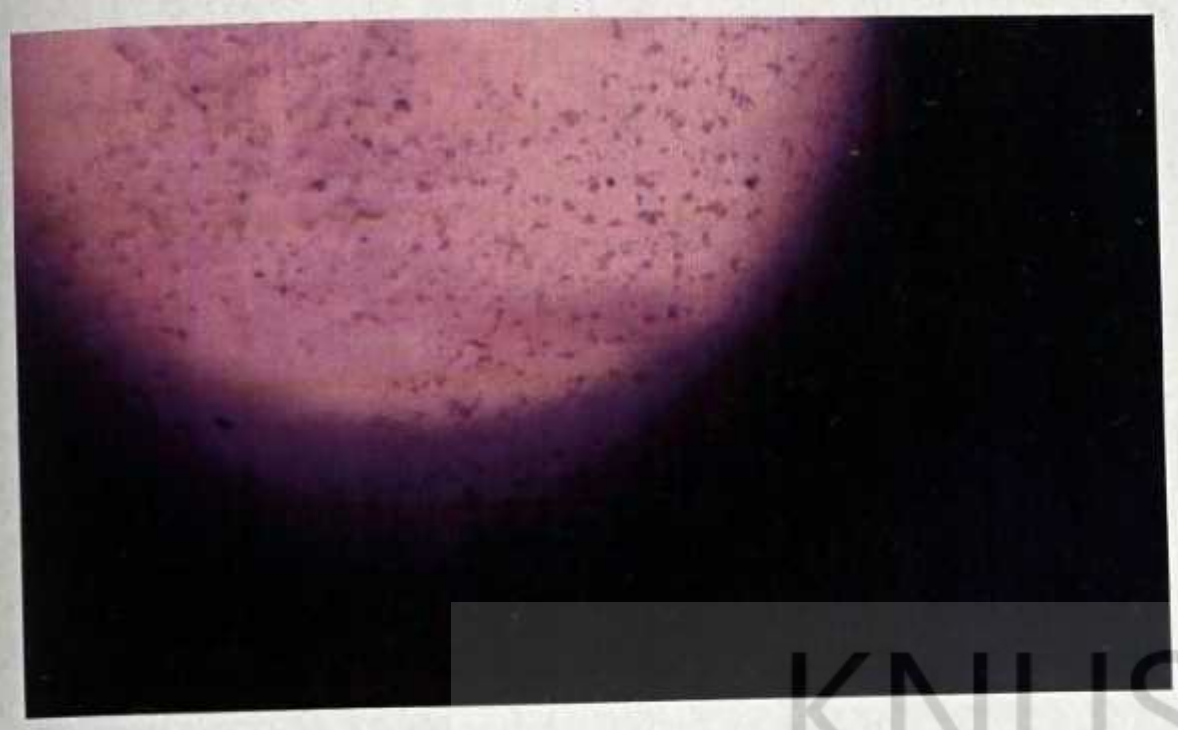
APPENDIX M. Conditions of Simulated Water before Biodegradation

Parameter / Material	pH	Temp (°C)	DO (mg/l)	EC (µS/cm)	Sal (PSU)	ST (mN/m)	MC x10^{10} (CFU/ml)
Contaminated water	7.11 ±0.07	26.40 ±0.09	7.01 ±0.08	912.00 ±0.01	0.20 ±0.01	67.50 ±0.04	3.00 ±1.74

These values changed to the respective day 1 values in each treatment after the 'conditioning' runs in the treatments.

APPENDIX N/PLATE 1. Gram Positive *Bacillus* Sp. under Microscope

APPENDIX O/ PLATE 2. Gram Negative *Pseudomonas* sp under Microscope



APPENDIX P/PLATE 3. Oil before Biodegradation

APPENDIX Q/PLATE4 . Oil after Biodegradation

APPENDIX R/ PLATE 5. Bioreactor Setup