BIOREMEDIATION OF OIL-CONTAMINATED SOIL USING EMULSIFIER (LIQUID SOAP), NPK FERTILIZER AND MICROBIAL (*Bacillus sp*) TREATMENT

BY

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DECLARATION

I hereby declare that this thesis entitled "BIOREMEDIATION OF OIL-CONTAMINATED SOIL USING EMULSIFIER (LIQUID SOAP), NPK FERTILIZER AND MICROBIAL (*Bacillus sp*) TREATMENT*"* is a record of my research work under the supervision of Prof.V.O Ajibola and Prof. I.G. Ndukwe in the Department of Chemistry, Ahmadu Bello University, Zaria, Kaduna State Nigeria. All sources of information are duly acknowledged by references. No part of this thesis was previously presented for another degree or diploma at any university or institution.

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Date

CERTIFICATION

This thesis entitled "BIOREMEDIATION OF OIL-CONTAMINATED SOIL USING EMULSIFIER (LIQUID SOAP), NPK FERTILIZER AND MICROBIAL (*Bacillus sp*) TREATMENT*"* by JEREMIAH MAKARAU ILIYA meets the regulations governing the award of degree of M.Sc. in Analytical Chemistry of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This research work is dedicated to God Almighty, my late parents and my family.

ACKNOWLEDGMENT

My greatest thanks go to Almighty God for giving me the wisdom, strength and grace to bring this work to a successful completion.

I express my profound gratitude to my supervisors; Professor V. O. Ajibola and Professor I.G Ndukwe whose constant correction, vast knowledge and useful advice have made this work a success. I sincerely thank the members of staff of Chemistry Department, A.B.U., Zaria especially Professor Uzairu A., Dr S.O Idris among others for their tolerance and meaningful contribution to the success of this work. Mr. Mikailu of NILEST word is not enough to express my gratitude to you for your immense contribution toward the success of this programme. God alone will reward you.

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Abstract

Two different set of soil samples were collected near Kaduna Refining and Petrochemical Company Limited (KRPC), a Subsidiary of Nigerian National Petroleum Corporation (NNPC) Kaduna. The first set of samples labelled (D) was obtained from areas where diesel from the refinery spilled into the environment and the second set of samples labelled (P) was collected from areas where petrol leaked and spilled into the environment. The pH of the soil was found to be 5.9 and 6.2 for D and P samples respectively. The cation exchange capacity (CEC) was higher in sample P than in sample D (32.0 and 30.0 mmol/kg of soil respectively). P has high concentrations of cations (Ca^{2+}, Mg^{2+}, K^{+} and Na^{+} with concentration values 3.6, 1.17, 0.50 and 0.22 mol/kg respectively) because of its high CEC while sample D with a lower CEC has a lower concentration of cation (Ca^{2+}, Mg^{2+}, K^{+} and Na^{+} with concentration values 1.20, 0.27, 0.25 and 0.17 mol/kg respectively). The oil (contaminant) was extracted in dichloromethane and a GC-MS analysis was run to determine the nature (composition) of the oil and the concentration of the contaminant was determined using gravimetric method. Results revealed that the total concentration of the contaminant (oil) before treatment was 398 g/kg and 194 g/kg for sample D and P respectively. The GC-MS results obtained showed that in both samples (D and P) linear and branched alkanes(n-Tetratetracontane, 3,6-Dimethyldecane, n-Pentadecane, 2-Bromodecane, n-Heptadecane etc. and n-Tetradecane, 2-Bromododecane, n-Octadecane, 14-Methyl-8-hexadecenal etc. respectively) were the main contaminants. Bioremediation was initiated and examined by applying separately fertilizer (F), bacteria inoculation (B), emulsifier (E) on the contaminated soil and by combining bacteria inoculation and fertilizer (B and F), fertilizer and emulsifier (F and E), bacteria inoculation and emulsifier (B and E) also a combination of bacteria inoculation, emulsifier and fertilizer (B,E and F) on both sample D and P . Bioremediation was determined by weight lost method throughout the 28days of treatment and the trend of

degradation is thus: (B, E and F) > B > (B and E) > (B and F) > (E and F) > E > F. Results obtained showed that treatment with B,E and F combined together yielded the highest percentage of oil degradation (97%, and 95% for samples P and D respectively), followed by B (96% and 81% for samples D and P respectively). This finding suggests that *Bacillus sp* is a viable microbial strain for bioremediation of oil-contaminated soil when biostimulated by adding fertilizer combined with emulsification (B, E and F). The percentage of oil degraded in sample P and D are almost the same (97 and 95% respectively) and there is significant difference ($P < 0.05$) in the trend of degradation for D and P this may be because, even though the samples contained oil (contaminant) with almost the same composition but differ in the number of carbon chain and other physical factors like density, viscosity etc.

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LIST OF ABBREVIATION

KRPC : Kaduna Refining and Petrochemical Company

NNPC : Nigeria National Petroleum Corporation.

CEC : Cation Exchange Capacity

TPH : Total Petroleum Hydrocarbons

GC : Gas Chromatography

IR : Infrared

ALARP : As Low as Reasonably Practicable.

HOCs : Hydrophobic Organic Contaminant

NAPL : Non Aqueous- Phase Liquid

CMCs : Critical Micelle Concentrations

PAHs : Polycyclic aromatic Hydrocarbons

ISUEIT : Iowa State University Engineering and Information Technology.

PCBs : Polychlorinated Biphenyls.

MS : Mass Spectroscope

BTEX : Benzene, Toluene, Ethylbenzene, Xylenes

PCP : Pentchlorphenol

PCDD : Polychlorinated Dibenzodioxins Chlorinated Hydrocarbons.

PCDF : Polychlorinated Dibenzofurans

FIA : Flame Ionization Analysis.

TNT : 2,4,6- Trinitrotoluene

RDX : IUPAC ID: 1,3,5 – Trinitroperhydro – 1,3,5- triazine

DCM : Dicloromethane

CUCE : Canadian Underwater and Exhibition

NRC : National Research Council

FEPA : Federal Environmental and Pollution Agency.

CHAPTER ONE

INTRODUCTION

1.1 Background Study

One of the major environmental problems in the world today is hydrocarbon contamination resulting from the activities related to the petrochemical industry. In Nigeria, oil pollution problems have been prevalent since the commencement of oil exploration and development of the petroleum industry (Okoh *et al.,* 2001). Accidental release of petroleum products are of particular concern in the environment. Hydrocarbon components have been known to belong to the family of carcinogens and neurotoxic organic pollutants. Currently accepted disposal methods of incineration or burial insecure landfills can become prohibitively expensive when amounts of contaminants are large. The deleterious effect of pollutants on the environment has led to increased awareness and vigilance against contamination of the Niger Delta environment. In relatively recent times in Nigeria, there has been remarkable increase in population, urbanization and industrial activities, (Eze and Okpokwasili, 2010). The release of crude oil into the environment by oil spills is receiving worldwide attention (Millioli *et al.,* 2009). Bioremediation which has been defined as biological response to environmental abuse has continued to receive research attention across the globe (Hammer, 1993). Bioremediation has been described as the use of living microorganisms to degrade environmental pollution. In order words, it is a technology for removing pollutants from the environment thus restoring the original natural environment (Sasikuma and Papmazath, 2003). The long term aim of bioremediation designs is to present cost effective designs which reduces the pollutant to a level referred to as low as reasonable and practicably possible (ALARP). In order to achieve this cost effectiveness, researchers all over the world have begun to pay research attention to the use of organic waste as the source of limiting nutrients for effective bioremediation (Ibiene *et al.,* 2011).

Mechanical and chemical methods generally used to remove hydrocarbons from contaminated sites have limited effectiveness and can be expensive (Das and Chandran, 2011). Bioremediation is the promising technology for the treatment of these contaminated sites since it is cost-effective and will lead to complete mineralization. Bioremediation functions basically on biodegradation, which may refer to complete mineralization of organic contaminants into carbon dioxide, water, inorganic compounds, and cell protein or transformation of complex organic contaminants to other simpler organic compounds by biological agents like microorganisms (Das and Preethy, 2010). Many indigenous microorganisms in water and soil are capable of degrading hydrocarbon contaminants.

Petroleum-based products are the major source of energy for industry and daily life. Leaks and accidental spills occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products. The amount of natural crude oil seepage was estimated to be 600000 metric tons per year with a range of uncertainty of 200,000 metric tons per year (Kvenvolden and Cooper, 2003). Release of hydrocarbons into the environment whether accidentally or due to human activities is one of the main causes of water and soil pollution. Soil contamination with hydrocarbons causes extensive damage of body system since accumulation of pollutants in animals and plant tissue may cause death or mutations (Sheetal, 2012). These oil spills can even cause damage to the sea and shoreline organisms (Rodríguez-Martínez , 2006). The other sources of contamination include service stations, garages, scrap yard, waste treatment plants, saw mills, etc. Many microorganisms have the ability to utilize hydrocarbons as sole sources of carbon as energy for metabolic activities and these micro organisms are ubiquitous and widely distributed in nature (Jyothi *et al.,* 2012). The microbial utilization of hydrocarbons depends on the chemical nature of the compounds within the petroleum mixture and on environmental determinants (Adeline *et al.,* 2009). Hydrocarbons enter into the environment through waste disposal, accidental spills, as

pesticides and via losses during transport, storage, and use. Hydrocarbon (petroleum) degrading bacteria's ability to degrade and/or detoxify organic contaminants has been established as an efficient, economical, versatile and environmentally sound treatment (Atlas, 1981) The extensive use of petroleum products leads to the contamination of almost all compartments of the environment, and biodegradation of the hydrocarbons by natural populations of microorganisms has been reported to be the main process acting in the cleaning of hydrocarbon-polluted environment (Chaillan *et al.,* 2004). Due to extensive increase in environmental pollution, numerous biodegradative bacteria have been isolated in the past, and their physiology, biochemistry, and genetics have been intensively studied**.** Biodegradation, which is the destruction of organic compounds by microorganisms, is carried out largely by diverse bacterial populations, mostly by *Pseudomonas* species (Boboye *et al.,* 2010). The fuel is a complex mixture of normal, branched and cyclic alkanes, and aromatic compounds obtained from the middle-distillate fraction during petroleum separation.

Bioremediation processes rely on the ability of microorganisms present naturally which are highly efficient due to their simplicity and cost-effectiveness when compared to other technologies (Jyoth *et al.,* 2012). Hydrocarbon utilizing microorganisms are ubiquitously distributed in the marine environment following oil spills (Dua *et al.,* 2002).

1.2. Soil contaminated with oil

Soil contaminated with Petroleum is hazardous to human health, pollutes ground water and decreases the agricultural productivity of soils (Wang *et al.,* 2008). The health concern stems primarily from direct contact with the contaminated soil, vapors from the contaminants, and from secondary contamination of water supplies within the soil. The technology commonly used for the soil remediation includes mechanical, burying, evaporation, dispersion and washing (Antai and Mgomo, 1989; Das and Mukherjee, 2011; Johnsen *et al.,* 2005; Colwell

et al., 1973; Leahy and Colwell, 1990). However, these technologies are expensive and can lead to incomplete decomposition of contaminants.

The use of bioremediation process to detoxify or remove pollutants is an evolving method for the removal and degradation of many environmental pollutants including the products of petroleum industry. Bioremediation technology is believed to be non-invasive and relatively cost-effective (Millioli *et al.,* 2009). Biodegradation by natural populations of microorganisms represents one of the primary mechanisms by which petroleum and other hydrocarbon pollutants can be removed from the environment (Rosenberg and Ron, 1996) and is cheaper than other remediation technologies (Vidali, 2001).

The success of oil spill bioremediation depends on the ability to establish and maintain conditions that favor enhanced oil biodegradation rates in the contaminated environment. One important requirement for the degradation of oil is the presence of microorganisms with the appropriate metabolic capabilities. If these microorganisms are present, then optimal rates of growth and hydrocarbon biodegradation can be sustained by ensuring that adequate concentrations of nutrients and oxygen are present.

1.3 Microbial Degradation of Petroleum Hydrocarbon

Biodegradation of petroleum hydrocarbons is a complex process that depends on the nature and on the amount of the hydrocarbons present. Petroleum hydrocarbons can be divided into four classes: the saturates, the aromatics, the asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins), and the resins (pyridines, quinolines, carbazoles, sulfoxides, and amides). Different factors influencing hydrocarbon degradation have been reported by Cooney and Summers, 1976; Chris, 2000). One of the important factors that limit biodegradation of oil pollutants in the environment is their limited availability to microorganisms (Providenti *et al.,* 1995). Hydrocarbons differ in their susceptibility to microbial attack and can be generally

ranked as follows: linear alkanes > branched alkanes > small aromatics > cyclic alkanes (Rosenberg and Ron, 1996). Some compounds, such as the high molecular weight polycyclic aromatic hydrocarbons (PAHs), may not be degraded at all. Microbial degradation is the major and ultimate natural mechanism by which one can clean up the petroleum hydrocarbon pollutants from the environment.

Hydrocarbons biodegraded by bacteria, yeast, and fungi have been reported to have efficiency that ranged from 6% (Jones *et al.,* 1970) to 82% (Pinholt *et al.,* 1979) for soil fungi, 0.13% (Jones *et al.,* 1970) to 50% (Pinholt *et al.,* 1979) for soil bacteria, and 0.003% (Hollaway *et al.,* 1980) to 100% (Mulkins and Stewart, 1974) for marine bacteria. Mixed populations with overall broad enzymatic capacities are required to degrade complex mixtures of hydrocarbons such as crude oil in soil(Bartha and Bossert, 1984), fresh water (Cooney, 1984), and marine environments (Atlas, 1985; Floodgate, 1984).

1.4 Justification for the Research.

In relatively recent times in Nigeria, there has been remarkable increase in population, urbanization and industrial activities (Eze and Okpowasil, 2010). The Kaduna Refinery produces 1.5milion litres of fuel daily and has recently produced 14.989 million litres of petrol, evacuated by 454 trucks , 6 million litres of kerosene have also been produced and evacuated by 184 trucks, another 12 million litres of diesel have been produced and evacuated by 411 trucks, while 13 million litres of low pour fuel oil have been produced within one month. This implies that large volume of oil contaminants (particularly around the oil related companies) are released into the environment. Pollution caused by petroleum and its derivatives is the most prevalent in the refinery environment. The low solubility and adsorption of high molecular weight hydrocarbons limit their availability to microorganisms. Oils are hydrophobic in nature, their availability to bacteria are limited which leads to the

slow degradation because petroleum hydrocarbon compounds bind to soil components, and they are difficult to be removed or degraded. When a major oil spill occurs the supply of carbon is dramatically increased and the availability of nitrogen and phosphorus generally becomes the limiting factor for oil degradation (Atlas, 1984; Leahy and Colwell, 1990), hence there is need to enhance bioremediation. Bioremediation offers several advantages over conventional techniques such as land filling or incineration. Bioremediation can be done on site, is often less expensive and site disruption is minimal, it eliminates waste permanently, eliminates long-term liability, and has greater public acceptance, with regulatory encouragement, and it can be coupled with other physical or chemical treatment methods. The release of crude oil into the environment by oil spill is receiving worldwide attention (Millioli, *et al.,* 2009).

1.5 Aim and objectives

The overall aim of the research is to bioremediate oil contaminated soil using NPK fertilizer, *Bacillus sp* and emulsification by liquid soap.

The specific objectives are to determine the effect of:

1. physicochemical parameters on oil degradation
2. NPK fertilizer as nutrient source in bioremediation
3. microbial seeding on oil degradation.
4. emulsifier (liquid soap) on oil degradation.
5. combination of i-iii above on oil degradation.

CHAPTER TWO

LITERATURE REVIEW

2.1 Oil Clean-up Strategies

As information is gathered, strategies can be developed (and revised) to protect those resources at risk. Though response strategies will vary according to incident-specific conditions, strategies can often be established in spill response planning consistent with response goals (USEPA, 2001). In such a scenario communities exposed to hydrocarbons become adapted, exhibiting selective enrichment and genetic changes that result in increased proportions of hydrocarbon degrading bacteria and bacterial plasmids encoding hydrocarbon catabolic genes (Atlas and Atlas, 1991). Arguably, low temperature will generally increase oil viscosity, inhibit spreading, evaporation, and emulsification, and may extend response windows of opportunity (but some oils may form stable emulsions in low temperatures) (Alfred, 2011). During extreme cold weather, ice may limit the spread of oil and improve the chances of recovery or burning. In high temperature and humidity situations, oil will spread and evaporate faster, increasing fire and explosion potential, accelerating weathering processes, reducing response windows, and impacting equipment deployment times (USEPA, 2001). Reducing overall environmental consequences in an effective and efficient manner usually requires a combination of techniques, including:

i. Natural recovery;
ii. Physical washing;
iii. Physical removal
iv. Physical in situ treatment (including burning); and
v. Chemical or biological treatment (USEPA, 2001)

Oil accidentally released in the environment can have severe negative effects on natural systems. Spills do degrade on their own over time as a result of several natural, physical,

chemical and biological "weathering" processes. The time it takes for an oil spill to breakdown and dissipate naturally depends on several factors, including the volume of the spill and the physical characteristics of the crude, the weather, sea state conditions, and whether the spill remains at sea or is carried onshore, particularly in environments sheltered from wave action, the process can be prolonged. Many crude oil spills can persist within the environment for some time after the spill, breaking up and dissipating slowly and often requiring an active clean-up response. Physical/or mechanical clean-up strategies to contain and recover oil from large spills are typically pursued first, possibly followed by chemical strategies that can speed the dispersion and dilution of the contaminating oil. Overtime, oil hydrocarbons can be partly or completely mineralized. Marine microbial flora with oil-degrading capabilities includes species of bacteria, fungi and yeasts. Such organisms have been found to reside within the water column, sediments, and even in sea ice (Alfred, 2011). Biotech based bioremediation in coastal systems is that it can be incredibly cost effective compared to alternative clean-up strategies. The speed and efficiency with which bioremediated contaminants are broken down depend primarily on three variables: temperature, the concentration of potentially limiting nutrients and the amount of oxygen available in the environment.

2.1.1 Physico-chemical methods

Petroleum products are subjected to various weathering process that alter their chemical and physical nature on entering the marine environment. The chemically and biologically induced changes in the composition of a polluting petroleum hydrocarbon mixture are known collectively as weathering. Microbial degradation plays a major role in the weathering process. Biodegradation of petroleum in natural ecosystems is complex. The evolution of the hydrocarbon mixture depends on the nature of the oil, the microbial community, and a variety

of environmental factors which influence microbial activities (Atlas, 1995). Hydrocarbon biodegradation can occur over a wide range of temperatures, also psychrotrophic, mesophilic, and thermophilic hydrocarbon-utilizing microorganisms have been implicated. Thus ZoBell (1973) and Traxler (1973) reported on hydrocarbon degradation at below 0^0C; Klug and Markovetz (1967) and Mateles *et al*. (1967) reported on hydrocarbon degradation at 70^0C. Expectedly, temperature can have a marked effect on the rates of hydrocarbon degradation, though the effect differs depending on the hydrocarbon composition of a petroleum mixture. Low temperatures retard the rates of volatilization of low-molecular-weight hydrocarbons, some of which are toxic to microorganisms. The presence of such toxic components was found to delay the onset of oil degradation at low temperatures. Temperature often is not the major limiting factor for hydrocarbon degradation in the environment except as it relates to other factors such as the physical state of the oil or whether liquid water is available for microbial growth, but empirical data has shown that at 20^0C, lighter oils had greater abiotic losses and were more susceptible to biodegradation than heavier oils; and in contrast rates of oil mineralization for the heavier oils were significantly lower at 20^0C than for the lighter ones. The light crude oils, however, had toxic volatile components which evaporated only slowly, inhibiting microbial degradation of these oils at 10^0C (Atlas, 1995). It is therefore suggestive that the influence of temperature on hydrocarbon degradation is more complex than simple consideration. Thus the effects are interactive with other factors, such as the quality of the hydrocarbon mixture and the composition of the microbial community.. Though biological approach is less cumbersome and cost effective, physical or mechanical alternatives may become imperative in the event of large magnitude spillage in order to short-circuit time that will be required for intrinsic bioremediation. In large accidental spills, physical or mechanical methods followed by chemical approach are always the first method of choice. The approach involves scooping, the use of skimmers and controlled burning.

Usually, skimmers are attached to ships and once the oil has been picked up, it is separated from the water inside the ship. Materials such as straw, powdered clay saw dust, chopped corncobs stuffed in cloth "sausages", and other organic and inorganic absorbents are used to contain the oil in areas where they can be skimmed. Such methods can offer an appreciable oil clean-up of only 10 to 30%. Subsequently, chemical dispersants such as detergents and solvents are used to get rid of the oil, but these chemicals are toxic to bottom-dwelling (benthic) organisms, shore-dwelling organisms, and open water marine life (Alfred, 2011).

2.1.2 Biological methods

Bioremediation or biorestoration is a managed or spontaneous process in which microbiological processes are used to degrade contaminants to less toxic or non toxic forms, thereby remedying or eliminating environmental contamination. Microorganisms depend on nutrients and carbon to provide the energy needed for their growth and survival. Degradation of natural substances in the marine ecosystems provides the necessary food for the development of microbial populations. Bioremediation technologies harness these natural processes by promoting the enzymatic production and microbial growth necessary to convert the target contaminants to non toxic end products (Atlas, 1995)). The biodegradation of petroleum in the marine environments carried out largely by diverse bacterial populations, the most important (based on frequency of isolation) genera of hydrocarbon utilizes in aquatic environments are *Pseudomonas, Achromobacter, Arthrobacter, Micrococcus, Nocardia, Vibrio, Acinetobacter, Brevibacterium, Corynebacterium, Flavobacterium, Candida, Rhodotorula, and Sporobolomyces* (Atlas and Bertha, 1992).

Bacteria and yeasts appear to be the prevalent hydrocarbon degraders in aquatic ecosystems. In polluted freshwater ecosystems, bacteria, yeasts, and filamentous fungi all appear to be important hydrocarbon degraders (Cooney and Summers, 1976). Comparatively bacteria and

yeasts exhibit decreasing abilities to degrade alkanes with increasing chain length. In contrast a filamentous fungus does not exhibit preferential degradation for particular chain lengths. However, patterns of degradation, i.e., which hydrocarbons could be utilized, were similar for bacteria and fungi, but there is considerable variability among individual organisms. It therefore suggests that the ability to utilize hydrocarbons is widely distributed among diverse microbial populations. Hydrocarbons are naturally occurring organic compounds, and it is not surprising that microorganisms have evolved the ability to utilize these compounds. When natural ecosystems are contaminated with petroleum hydrocarbons, the indigenous microbial communities are likely to contain microbial populations of differing taxonomic relationships which are capable of degrading the contaminating hydrocarbons. Thus**,** the hydrocarbon-biodegrading populations are widely distributed in the world's oceans. Surveys of marine bacteria indicate that hydrocarbon-degrading microorganisms are ubiquitously distributed in the marine environment (Atlas, 1995). Many of these microorganisms possess the enzymatic capability to degrade petroleum hydrocarbons. Some degrade alkanes, others aromatics, and others both paraffinic and aromatic hydrocarbons. Often the normal alkanes in the range C_{10} to C_{26} are viewed as the most readily degraded, but low molecular-weight aromatics, such as benzene, toluene and xylene, which are among the toxic compounds found in petroleum, are also very readily biodegraded by many marine microorganisms. The greater the complexity of the hydrocarbon structure, the slower the rates of degradation (Atlas, 1995).

The major metabolic pathways for hydrocarbon biodegradation are well known. The initial steps in the biodegradation of hydrocarbons by bacteria and fungi involve the oxidation of the substrate by oxygenases, for which molecular oxygen is required. Alkanes are subsequently converted to carboxylic acids that are further biodegraded via oxidation (the central metabolic pathway for the utilization of fatty acids from lipids, which results in formation of acetate which enters the tricarboxylic acid cycle). Aromatic hydrocarbon rings generally are

hydroxylated to form diols; the rings are then cleaved with the formation of catechols which are subsequently degraded to intermediates of the tricarboxylic acid cycle. Interestingly, fungi and bacteria form intermediates with differing stereochemistries. Fungi, like mammalian enzyme systems, form *trans*-diols, whereas bacteria almost always form *cis*-diols (many *trans*-diols are potent carcinogens whereas *cis*diols are not biologically active). Since bacteria are the dominant hydrocarbon degraders in the marine environment, the biodegradation of aromatic hydrocarbon therefore results in detoxification and does not produce potential carcinogens. The complete biodegradation (mineralization) of hydrocarbons therefore produces the non-toxic end products carbon dioxide and water, as well as cell biomass (largely protein) which can be safely assimilated into the food web (Atlas, 1995). While various physico-chemical processes have been developed for treating these pollutants, these approaches are often prohibitively expensive, non-specific or have the potential for introducing secondary contamination. As a result, there has been an increased interest in ecofriendly bio-based treatments commonly known as bioremediation. To date cometabolism is now regarded as one of the most important mechanisms utilized by microorganisms for the transformation of chlorinated aliphatic and aromatic contaminants ((Bouwer and Zehnder, 1993). It is a process whereby microorganisms involved in the metabolism of a growth promoting substrate also transform other organic contaminants (cosubstrates) that are not growth supporting if supplied as sole carbon and energy source. It is an important process in both aerobic and anaerobic environments. Consequently, compounds which otherwise would not be degraded can be enzymatically attacked within the petroleum mixture due to the abilities of the individual microorganisms to grow on other hydrocarbons within the oil (Horvath, 1972). A petroleum hydrocarbon mixture, with its multitude of potential primary substrates, provides an excellent chemical environment in which co-oxidation can occur. Many complex branched and cyclic hydrocarbons undoubtedly are removed as environmental

contaminants after oil spills as a result of cooxidation (Perry, 1979). Hence co-oxidation was hypothesized to be responsible for the degradation of a number of compounds in the oil to account for the similar rates of disappearance of compounds which are normally resistant. Assessing the role of co-oxidation in natural environments is difficult since multiple microbial populations are present. In mixed-population studies, synergism could be an alternative hypothesis to explain the observed results

2.2 Principle of bioremediation

Crude oil is a composite mixture of thousands of different chemical compounds as a result there are different ways to deal with them through microbes and flora. Bioremediation can occur naturally or can be encouraged with addition of microbes and fertilizers. The microbes present in the soil first recognize the oil and its constituents by biosurfactants and bioemulsifiers, and then they attach themselves and use the hydrocarbon present in the petroleum as a source of energy and carbon(NRC, 2012) The low solubility and adsorption of high molecular weight hydrocarbons limit their availability to microorganisms. The addition of biosurfactants enhances the solubility and removal of these contaminants, improving oil biodegradations rates (Oil Spill Intelligence Report, 1997). The constituents of oil differ distinctly in volatility, volubility, and susceptibility to biodegradation. Some compounds are easily degraded, some resist degradation and some are non-biodegradable (Mukred *et al.*, 2008). The biodegradation of different petroleum compounds occurs simultaneously but at different rates because different species of microbes preferentially attack different compounds. This leads to the successive disappearance of individual components of petroleum over time (ISUEIT).

Microorganisms produce enzymes in the presence of carbon sources which are responsible for attacking the hydrocarbon molecules. Many different enzymes and metabolic pathways

are involved in degrading hydrocarbon contained in petroleum. But lack of an appropriate enzyme will either prevent attack or will act as a barrier to complete hydrocarbon degradation (Bijay *et al.,* 2012). Bacteria for hydrocarbon decomposition are commercially available as freeze dried bacteria, which can be used for bioremediation after propagation to a minimum of 2×10^8 CFU/ml. Bacteria that can degrade petroleum products are- *Pseudomonas, Aeromonas, Moraxella, Beijerinckia, Flavobacteria, chrobacteria, Nocardia, Corynebacteria, Atinetobacter, Mycobactena, ,Modococci , Streptomyces, Bacilli, Arthrobacter, Aeromonas, Cyanobacteria etc.*

2.2.1 Nutrients

Although the microorganisms are present in contaminated soil, they cannot necessarily be there in the numbers required for bioremediation of the site. Their growth and activity must be stimulated. Carbon is the most basic form of nutrient required for living organism. In addition to this, the bacteria also need macronutrient like nitrogen and phosphorous to ensure effective degradation of the oil. The optimum nutrient balance required for hydrocarbon remediation is Carbon: Nitrogen: Phosphorus (100:10:4). In general, at least 1 ppm of ammonium nitrogen and 0.4 ppm of orthophosphate needs to be present. Pathways can be influenced by further adjusting volumes of bio-nutrients (U S Congress, 1991). In addition to hydrogen, oxygen, and nitrogen, carbon constitutes about 95% of the weight of cells. The nutritional requirement of carbon to nitrogen ratio is 10:1, and carbon to phosphorous is 30:1.

Table 2.1 Composition of a microbial cell.

Element	Percentage	Element	Percentage
Carbon	50	Sodium	1
Nitrogen	14	Calcium	0.5
Oxygen	20	Magnesium	0.5
Hydrogen	8	Chloride	0.5
Phosphorous	3	Iron	0.2
Sulfur	1	All others	0.3
Potassium	1		

Stainer *et al*. (1986) (as cited in Vidali (2001)).

2.2.2 Optimizing bioremediation performance through nutrients addition

Serious accidental oil spills in the marine environment have provided the impetus for large-scale bioremediation experiments involving addition of nutrients to marine and foreshore environments (Prince, 1992; Atlas and Bartha, 1992; Pritchard *et al.,* 1992). Studies on nutrient addition have provided convincing evidence that it is an efficacious approach to bioremediation of oil pollutants in marine environments. It has been demonstrated to overcome a critical rate-limiting factor in aerobic oil-contaminated marine environments. Rates of stimulation have therefore been typically about 3-5 times the natural rates of oil biodegraded. Greater stimulation might be achieved by higher levels of nutrient addition, but this could risk ecological side effects such as toxicity to marine life and eutrophication with associated algal blooms (Atlas, 1995; Ubalua and Ezeronye, 2007). The use oleophilic micro-emulsion (of Inipol EAP22)with urea as a nitrogen source, laureth phosphate as a phosphate source, and oleic acid as a carbon source and a mixture of slow-release calcium phosphate, ammonium phosphate, and ammonium nitrate within a polymerized vegetable oil coating (customblen) was approved for shoreline treatment and was used as a major part of clean-up effort. Consequently remarkable results were observed on the shorelines treated with Inipol EAP22 and customblen compared to non-bioremediated shorelines (Pritchard *et al.,* 1992). Hence bioremediation treatments are aimed at stimulating pollutant-degrading microorganisms to speed the recovery of contaminated ecosystem to a pre-pollution state in terms of biodiversity and ecosystem function (Röling *et al.,* 2002). Although there is evidence that bioremediation can be used to treat oil-contaminated marine ecosystems effectively, an important limitation of the technology is the difficulty in formulating treatment strategies that will produce a specified outcome in terms of degradation rate and residual contaminant concentration (Head, 1998). Clearly, there is little consensus on how to best optimize nutrient amendment but typically values of around 1-5% N by weight of oil have been used with a ratio of N:P between 5 and 10:1 (Head, 1998). The three types of

fertilizers investigated were: water-soluble nitrogen and phosphorus; slow-release isobutylene diureal; and oleophilic Inipol EAP 22, which contains lauryl phosphate and urea. Oleophilic fertilizer application was followed by dramatic cleansing of the shorelines within a few weeks. Extensive efficacy testing indicated that fertilizer application stimulated the rate of microbial hydrocarbon degradation. Testing for toxicity and ecological effects showed minimal toxicity which was restricted to the immediate region of fertilizer application, and does not result in algal blooms (Atlas and Atlas, 1991). The majority of bioremediation strategies aimed at ameliorating marine oil spills assume that the principal mechanism of hydrocarbon removal is aerobic respiration. Whereas this may be valid for oil spills on coarse pebble or cobble shorelines, oxygen availability is likely to assume greater importance in beaches with fine-grained sediments, such as mudflats or salt marshes.

2.2.3 Electron Acceptor / Oxygen

Although oxygen is not the rate limiting factor, it's one of the most essential elements of microbial degradation of hydrocarbons (Bijay *et al*., 2012). Oxygen is necessary for the initial breakdown of hydrocarbons and succeeding reaction may require it. In the presence of oxygen complete degradation of oil takes place. If large quantities of oil are present, the oxygen in the soil will be depleted very fast causing anaerobic condition.

Anaerobic bacteria will use other electron acceptors like nitrate, iron or sulphate, but the energy yield for the bacteria is less than when oxygen is used as the electron acceptor *(*Braddock *et al.,* 1997*).*The lower energy yield by anaerobic bacteria results in lower degradation and hence a longer period of time is required for remediation (Bijay *et al.,* 2012).

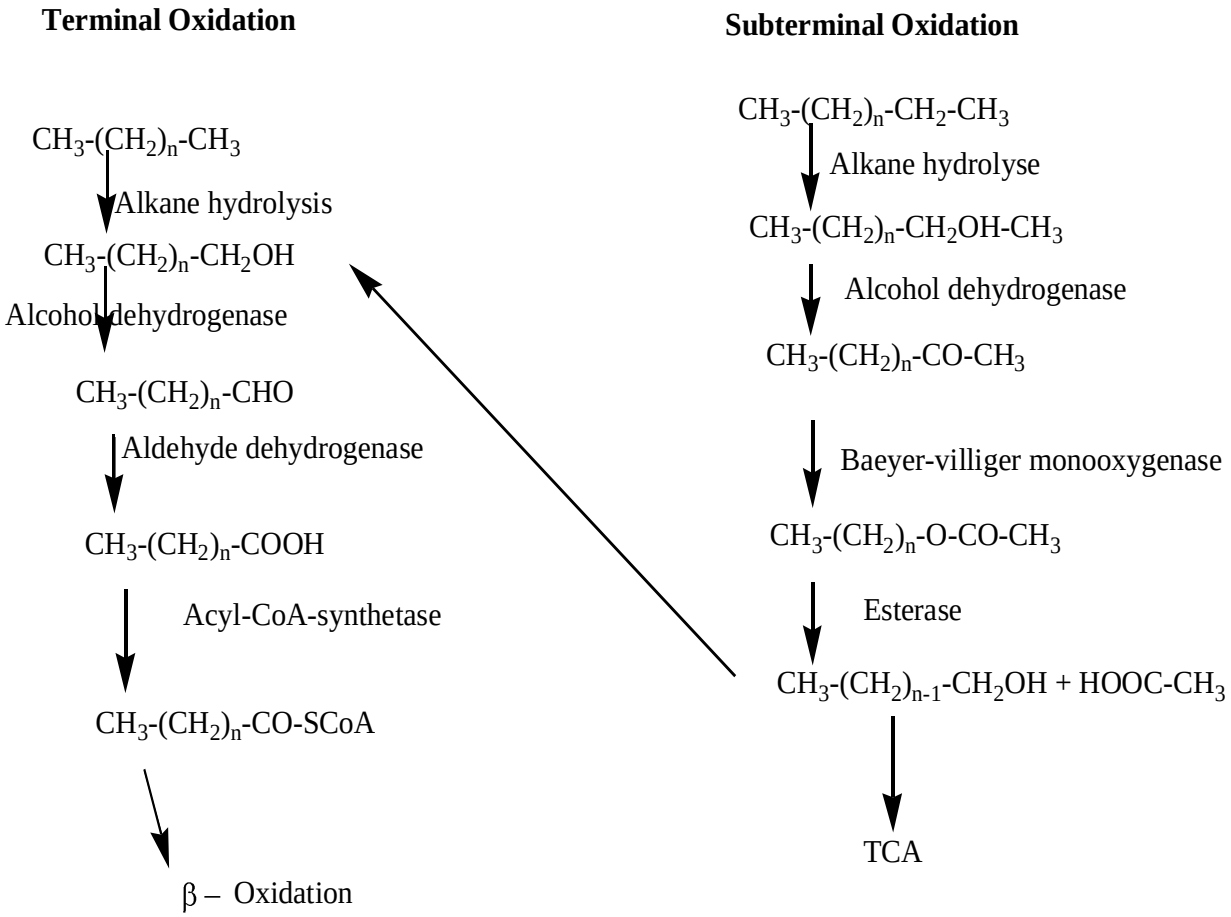


Figure 2.1 Most frequent pathways for the degradation of n-alkanes by terminal and subterminal oxidations

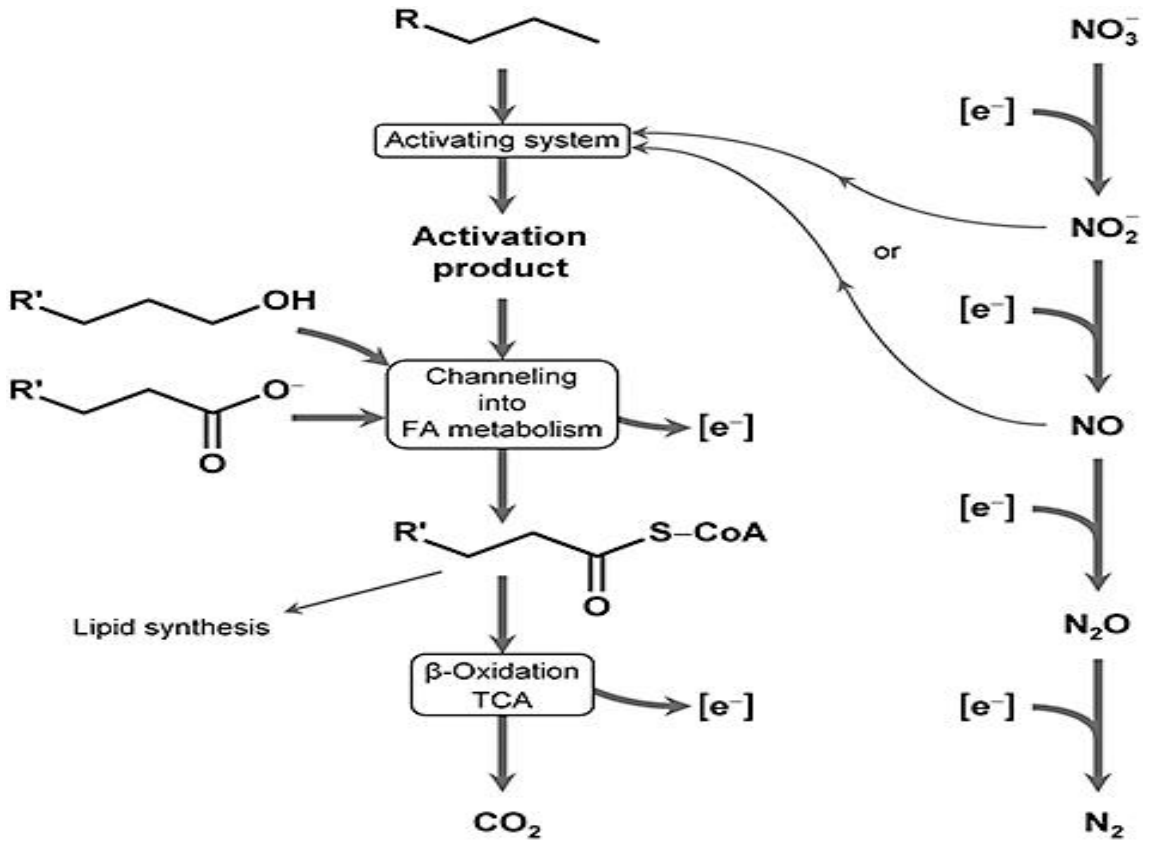


Figure 2.2 Anaerobic pathways for the degradation of alkanes using electron acceptor (nitrate).

2.2.4 Detergent

Oils are hydrophobic in nature, their availability to bacteria is limited which leads to slow degradation. Adding detergent to oil contaminated soil help in desorbing the hydrocarbon and hence boost remediation. Generally microbiologically produced detergents called rhamnolipids are used. They are characterized by having rhamnose moieties and a fatty acid tail (Braddock *et al.,* 1997).

2.2.5 Biosurfactants (Emulsifier)

A number of hydrocarbon-degrading microorganisms produce emulsifying agents and some of these bioemulsifiers have been considered for use in oil cleaning operations. Improvements in biodegradation rates have been achieved by the addition of these surfactants to the contaminated environment. Banat *et al.* (2000), described biosurfactant to be amphiphilic molecules consisting of a hydrophilic part and a hydrophobic part. As a consequence of this property, these molecules tend to concentrate at interfaces thereby decreasing interfacial tension. It has been established that above a certain concentration (critical micelle concentration), stable aggregates of 10 to 200 molecules are formed (called micelles) which can increase the solubility of hydrophobic organic contaminants (HOCs) (Edwards *et al.,* 1991). Biosurfactants of diverse chemical nature and molecular size are produced by the hydrocarbon-degrading microorganisms. These surface-active materials increase the surface area of hydrophobic water-insoluble substrates and increase their bioavailability thereby enhancing the growth of bacteria and the rate of bioremediation. The effect of surfactants on the availability of organic compounds can be explained by three main mechanisms: dispersion of non aqueous-phase liquid (NAPL) organics, leading to an increase in contact area caused by a reduction in the interfacial tension between the aqueous phase and the non aqueous phase; increased apparent solubility of the pollutant caused by the presence of micelles that contain high concentrations of

HOCs (Edwards *et al.*, 1991), and facilitated transport of the pollutant from the solid phase, which can be caused by lowering of the surface tension of the soil particle pore, water interaction of the surfactant with solid interfaces, and interaction of the pollutant with single surfactant molecules (Makkar and Rockne, 2003). Biosurfactants can enhance growth on bound substrates by desorbing them from surfaces or by increasing their apparent water solubility (Marcoux, 2000). Surfactants that lower interfacial tension dramatically are particularly effective in mobilizing bound hydrophobic molecules and making them available for biodegradation. One of the major reasons for the prolonged persistence of high molecular weight hydrophobic compounds is their low water solubility which increases their sorption to surfaces and limits their availability to biodegrading microorganisms. When organic molecules are bound irreversibly to surfaces, biodegradation is inhibited (Van, 1990). Low molecular weight biosurfactants that have low critical micelle concentrations (CMCs) increase the apparent solubility of hydrocarbons by incorporating them into the hydrophobic cavities of micelles (Makkar and Rockne, 2003). Perhaps a more promising solution for bioavailability enhancement may be the use of biosurfactants, whose primary function is to facilitate microbial life in environments dominated by hydrophilic-hydrophobic interfaces (Barkay *et al.*, 1999). Biosurfactants have the ability to solubilize HOCs but have several additional advantages over synthetic surfactants that make them superior candidates in bioremediation schemes (Makkar and Cameotra, 2002; Desai and Banet, 1997). First, biosurfactants are readily biodegradable and therefore pose no additional pollution threat and secondly, most studies indicate that they are completely non toxic to microorganisms and therefore unlikely to inhibit biodegradation of PAHs. Biosurfactant production is potentially less expensive than synthetic surfactants and can be easily achieved *in situ* at the contaminated sites from inexpensive raw materials. They are more effective than synthetic surfactants in increasing the bioavailability of hydrophobic compounds. In addition, they are selective, environmentally friendly and generally less stable

than most synthetic surfactants (Ron and Rosenberg, 2002). For example, high molecular weight bio-emulsifier was recently shown to significantly increase the rate of biodegradation of poly aromatic hydrocarbons (PAHs) by increasing their apparent solubilities 5-20-fold (Rosenberg *et al.,* 1999 and Barkay *et al.,* 1999). Several biosurfactants have found applications in environmental remediation. They include some small well characterized biosurfactants such as *Pseudomonas aeruginosa* rhamnolipids (Providenti *et al.,* 1995; Maier and Soberon-Chavez, 2000), *Candida apicola* sophorose lipids (Hommel *et al.,* 1994), *Rhodococcus erythropolis* trehalose dimyeolipids (Kanga *et al.,* 1997), *Bacillus* sp. Lichenysins (Jenny *et al.,* 1991) and *Bacillus subtlis* surfactin (Makkar and Cameotra, 1997, Awashti *et al.,* 1999). These are potent surfactants, as they reduce surface tension from 72 to less than or equal to 30dynes/cm) and have low CMCs. Bacteria make low molecular weight molecules that efficiently lower surface and interfacial tensions and high molecular weight polymers that bind tightly to surfaces (Desai and Banat, 1997; Rosenberg and Ron, 1997; Rosenberg *et al.,* 1999).

Low molecular weight biosurfactants are generally glycolipids in which carbohydrates are attached to a longchain aliphatic acid or lipopeptides. One of the best studied glycolipids is rhamnolipid, produced by several species of *Pseudomonas.* The high molecular weight bacterial Surfactants are produced by a large number of bacterial species from different genera and are composed of polysaccharides proteins, lipopolysaccharides, lipoproteins or complex mixtures of these biopolymers. They are less effective in reducing interfacial tension but are efficient at coating the oil droplets and preventing their coalescence. They also exhibit considerable substrate specificity. There are at least two ways in which biosurfactants are involved in bioremediation. First is by increasing the surface area of hydrophilic water-insoluble substrates and secondly by increasing the bioavailability of hydrophobic compounds. For bacteria growing on hydrocarbons, the growth rate can be limited by the interfacial surface area between water and oil (Ron and Rosenberg, 2001). When the surface area becomes limiting biomass increases

arithmetically rather than exponentially. If emulsion occurs at, or very close to the cell surface and no mixing occurs at the microscopic level, then each cluster of cells creates its own microenvironment and non overall cell-density dependence could be expected (Ron and Rosenberg, 2002). In effect, the "emulsifier" frees the cell to find fresh substrate. At the same time the depleted oil droplets has been "marked" as used, because it now has a hydrophilic outer surface to which the bacterium cannot attach. The detachment of bacteria from the depleted oil droplets enables them to move to other drops where they metabolize the specific group of utilizable hydrocarbons. Therefore, detachment of bacteria from oil drops results in a more efficient bioremediation. Oil-degrading bacteria can utilize only a limited group of hydrocarbons; hence bacteria attached and growing on an oil droplet become nutrient starved once this group of hydrocarbons is depleted. If the biosurfactant is cell-bound it can the microbial cell surface to become more hydrophobic, depending on its orientation (Ron and Rosenberg, 2002).

2.3 Environmental Requirements

Optimum environmental conditions for the degradation of contaminants are shown in Table 2.2. Microbial growth and activity are readily affected by pH, temperature, and moisture. Although microorganisms have been also isolated in extreme conditions, most of them grow optimally over a narrow range, so that it is important to achieve optimal conditions. If the soil has too much acid it is possible to raise the pH by adding lime. Temperature affects biochemical reactions rates, and the rates of many of them double for each 10 °C rise in temperature. Above a certain temperature, however, the cells die (Pritchard *et al*., 1992). Plastic covering can be used to enhance solar warming in cold season.

Table 2.2 Environmental conditions affecting degradation.

Parameters	Condition required for microbial activity	Optimum value for an oil Degradation
Soil moisture	25–28% of water holding Capacity	30–90%
Soil pH	5.5–8.8	6.5–8.0
Oxygen content	Aerobic, minimum air-filled pore space of 10%	10–40%
Nutrient content	N and p for microbial growth	C:N:P = 100:10:1
Temperature (°C)	15–45	20–30
Contaminants	Not too toxic	Hydrocarbon 5–10% of dry weight of soil
Heavy metals	Total content 2000 ppm	700 ppm
Type of soil	Low clay or silt content	

Source: Vidali (2001)

Available water is essential for all the living organisms, and irrigation is needed to achieve the optimal moisture level. The amount of available oxygen will determine whether the system is aerobic or anaerobic. Hydrocarbons are readily degraded under aerobic conditions, whereas chlorurate compounds are degraded only in anaerobic ones. To increase the oxygen amount in the soil it is possible to till or sparge air. In some cases, hydrogen peroxide or magnesium peroxide can be introduced in the environment. Soil structure controls the effective delivery of air, water, and nutrients. To improve soil structure, materials such as gypsum or organic matter can be applied. Low soil permeability can impede movement of water, nutrients, and oxygen; hence, soils with low permeability may not be appropriate for *in situ* clean-up techniques (Zeyaullah *et al.,* 2009).

2.4 Factors influencing bioremediation of hydrocarbons

The main factors that influence the microbial degradation of hydrocarbons are temperature, pH, moisture level of soil, salinity of environment and chemical composition and concentration of the contaminant hydrocarbons (Desai and Vyas, 2006; Jain *et al.,* 2011; Saleh *et al.,* 2003).

2.4.1 Temperature: It is an important factor that controls the metabolic activity of the degrading microorganisms as well as physical and chemical nature of hydrocarbons. At the mesophilic and thermophilic range of temperatures, it is being found that the enzyme activity of microorganisms increases which helps in increasing the rate of hydrocarbon degradation. Therefore majorly the 30-40^{0}C temperature in the mesophilic range or sometimes 60^{0}C temperatures in thermophilic range is used. Though it is also being observed that some hydrocarbons like, diesel can be degraded at lower temperatures i.e. between 0-10^{0}C. But at low temperatures the viscosity of oil increases which suppresses the spreading of oil on surface which makes the degradation difficult. Moreover in the mesophilic range more variety of

organisms can be available for degradation so mesophilic or thermophilic temperatures are the better choice for bioremediation (Obuekwe *et al.,* 2001).

2.4.2 pH: The choice of pH depends on the microorganisms to be used for the degradation. Fungal strains are found to carry biodegradation at lower pH. Even when indigenous microbial consortium inclusive of fungi, yeast and several bacteria is used they are found to survive at pH 2. Though there are certain bacteria that are alkaliphiles found in alkaline lakes at pH 7.5-10 (Sheetal, 2012).

2.4.3 Salinity: Many isolates are capable of growing at salinity comparable to sea water. The significant hydrocarbon degradation was observed with 0.1-2M NaCl where maximum was at 0.4M i.e. almost equivalent to natural sea water. Though at higher salinity level, the degradation rate was found to decrease (Sheetal, 2012).

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2.4.4 Oxygen: It is one of the basic requirements for the biodegradation. But again the concentration of oxygen used will highly depend on the choice of microorganism used. For aerobic bacteria, stoichiometrically 3.1mg/ml of oxygen is required for the degradation of 1mg/ml hydrocarbons without taking into consideration the total mass of bacteria so it may vary with increasing or decreasing mass of bacteria (Curtis and Lammey, 1998). Even anaerobic biodegradation has proved its importance. Many different types of bacteria are tested and found to be useful in degrading hydrocarbons like benzene, alkanes, toluenes etc.

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2.4.5 Nutrients: Nutrients are required to support the biological activity, and hence bioremediation. Microorganisms commonly require carbon, nitrogen and phosphorous for the degradation of hydrocarbons. The amount of various nutrients and ratio of particularly, nutrients

like C, N and P is quite conceivable regarding the success of the bioremediation process. The organic carbon content in hydrocarbon contaminated site is found to be very high attributed to constant input of hydrocarbons. Because of the high carbon content of oil and the low level of other nutrients essential for microbial growth, the rate and extent of degradation are, in general, limited by the low availability of nitrogen and phosphorus. Consequently, growth of hydrocarbon-degrading bacteria and hydrocarbon degradation can be strongly enhanced by fertilization with inorganic N and P. In majority of the treatments the C: N: P ratio is maintained as 120:10:1. Though it may vary as per the need of the microorganisms. Other than these some more nutrients may be added as and when required (Leys *et al.,* 2005).

2.4.6 Chemical composition of petroleum: Petroleum hydrocarbons can be considered of four different types for degradation: (1) saturates, (2) aromatics, (3) asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins), and (4) resins (pyridines, quinolines, carbazoles, sulfoxides, and amides). In general, hydrocarbons have been ranked in the following order of decreasing susceptibility to biodegradation: n-alkanes > branched alkanes > low molecular weight aromatics > cyclic alkanes, with high molecular weight aromatics and polar compounds being extremely recalcitrant. However under some conditions (composition of microbial community, abiotic factors), the order of biodegradation can vary greatly (Das and Chandran, 2011; Obuekwe *et al.,* 2001).

2.4.7 Physical State of hydrocarbons: The bioavailability of hydrocarbons is also dependent on physical state, hydrophobicity, sorption onto soil particles, volatilization and solubility of hydrocarbons greatly affects the extent of biodegradation (Shallu *et al.,* 2014).

2.4.8 Hydrocarbon concentration: The rate of microbial uptake and biodegradation of hydrocarbons is dependent on the solvent solubility of the hydrocarbons. Degradation of hydrocarbons that are highly soluble in water are almost proportional to the concentration but it differs for less aqueous hydrocarbons. Though it needs to be considered that high solubility may become detrimental to the degrading organisms. Usually oil loading greater than 5% leads to decrease in the microbial activity (Del'arco and De Francea, 2001; El-Tarabily, 2002). In addition to this high concentration may disturb C: N: P ratio and create oxygen limitations.

2.5 Advantages and disadvantages of bioremediation

For bioremediation to be successful, the bioremediation methods depend on having the right microbes in the right place with the right environmental factors for degradation to occur. The right microbes are bacteria or fungi, which have the physiological and metabolic capabilities to degrade the pollutants. Bioremediation offers several advantages over conventional techniques such as land filling or incineration. Bioremediation done on site, is often less expensive and site disruption is minimal, it eliminates waste permanently, eliminates long-term liability, and has greater public acceptance, with regulatory encouragement, and it can be coupled with other physical or chemical treatment methods. Bioremediation has also its limitations. Some chemicals are not amenable to biodegradation, for instance, heavy metals, radionuclides and some chlorinated compounds. In some cases, microbial metabolism of contaminants may produce toxic metabolites. Bioremediation is a scientifically intensive procedure which must be tailored to the site-specific conditions, which means one has to do treatability studies on a small scale before the actual clean-up of the sites (Boopathy, 2000).

2.6.0 Strategies and techniques used for monitoring biodegradation

2.6.1 Strategies: Bioremediaiton can be carried out using *in situ* bioremediation, *ex situ* bioremediation or phytoremediation. (Fulekar, 2005; Markandey and Rajvaidya, 2004; Rao, 2005; Rittmann and Mccarty, 2005).

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2.6.2 *In situ* bioremediation of soil: *In situ* techniques do not require excavation of the contaminated soils so may be less expensive, create less dust, and cause less release of contaminants than *ex situ* techniques. Also, it is possible to treat a large volume of soil at once. *In situ* techniques, however, may be slower than ex situ techniques, may be difficult to manage, and are most effective at sites with permeable (sandy or uncompacted) soil (Badiyya, 2013).

2.6.3 Intrinsic bioremediation; Also known as natural attenuation, this type of bioremediation occurs naturally in contaminated soil or water. This natural bioremediation is the work of microorganisms and is seen in petroleum contamination sites, such as old gas stations with leaky underground oil tanks. Application of this technique requires close monitoring of contaminant degradation to ensure that environmental and human health are protected. Intrinsic bioremediation is an option when the naturally occurring rate of contaminant biodegradation is faster than the rate of contaminant migration. These relative rates depend on the type and concentration of contaminant, the microbial community, and the subsurface hydrogeochemical conditions. The ability of native microbes to metabolize the contaminant must be demonstrated either in field tests or in laboratory tests performed on site-specific samples. In addition, analyzing the fate of the contaminants and other reactants and products indicative of biodegradation must continually monitor the effectiveness of intrinsic bioremediation (Sheetal, 2012)

2.6.4 Engineered bioremediation; Engineered bioremediation may be chosen over intrinsic bioremediation because of time and liability. Where an impending property transfer or potential impact of contamination on the local community dictates the need for rapid pollutant removal, engineered bioremediation may be a more appropriate remedy than intrinsic bioremediation. Because engineered bioremediation accelerates biodegradation reaction rates it requires less time than intrinsic bioremediation. The shorter time requirements reduce the liability for costs required to maintain and monitor the site. Since many petroleum hydrocarbons require oxygen for their degradation, the technological emphasis of engineered bioremediation systems in use today has been placed on oxygen supply. Bioremediation systems for soil above the water table usually consist of a set of vacuum pumps to supply air (containing oxygen) and infiltration galleries, trenches, or dry wells to supply moisture (and sometimes specific nutrients). Bioremediation systems for ground water and soil below the water table usually consist of either a set of injection and recovery wells used to circulate oxygen and nutrients dissolved in water or a set of compressors for injecting air. Emerging applications of engineered bioremediation, such as for degradation of chlorinated solvents, will not necessarily be controlled by oxygen. Hence, the supply of other stimulatory materials may require new technological approaches even though the ultimate goal, high biodegradation rates, remains the same (Sheetal, 2012).

2.6.5 Biostimulation: Nutrients and oxygen - in a liquid or gas form - are added to contaminated water or soil to encourage the growth and activity of bacteria already existing in the soil or water. The disappearance of contaminants is monitored to ensure that remediation occurs. (Bento *et al.,* 2005; Sheetal, 2012). The goal of aerobic in situ bioremediation is to supply oxygen and nutrients to the microorganisms in the soil. Aerobic *in situ* techniques can vary in the way they supply oxygen to the organisms that degrade the contaminants. Two such

methods are bioventing and injection of hydrogen peroxide. Oxygen can be provided by pumping air into the soil above the water table (bioventing) or by delivering the oxygen in liquid form as hydrogen peroxide. In situ bioremediation may not work well in clays or in highly layered subsurface environments because oxygen cannot be evenly distributed throughout the treatment area. *In situ* remediation often requires years to reach cleanup goals, depending mainly on how biodegradable specific contaminants are. Less time may be required with easily degraded contaminants.

2.6.6 Injection of hydrogen peroxide: This process delivers oxygen to stimulate the activity of naturally occurring microorganisms by circulating hydrogen peroxide through contaminated soils to speed the bioremediation of organic contaminants. Since it involves putting a chemical (hydrogen peroxide) into the ground (which may eventually seep into the groundwater), this process is used only at sites where the groundwater is already contaminated. A system of pipes or a sprinkler system is typically used to deliver hydrogen peroxide to shallow contaminated soils. Injection wells are used for deeper contaminated soils (Sheetal*, 2012*).

2.6.7 Bioaugmentation: Microorganisms that can clean up a particular contaminant are added to the contaminated soil or water. Bioaugmentation is more commonly and successfully used on contaminants removed from the original site, such as in municipal wastewater treatment facilities. To date, this method has not been very successful when done at the site of the contamination because it is difficult to control site conditions for the optimal growth of the microorganisms added. Scientists are yet to completely understand all the mechanisms involved in bioremediation, and organisms introduced into a foreign environment may have a hard time surviving (Margesin and Schinner, 2001)

2.6.8 *In Situ* bioremediation of groundwater: In situ groundwater bioremediation is a technology that encourages growth and reproduction of indigenous microorganisms to enhance biodegradation of organic constituents in the saturated zone. *In situ* groundwater bioremediation can effectively degrade organic constituents, which are dissolved in groundwater and adsorbed onto the aquifer matrix. In situ bioremediation of groundwater can be combined with other saturated zone remedial technologies (e.g., air sparging) and vadose zone remedial operations (e.g., soil vapor extraction, bioventing) (Mishra *et al.*, 2001).

2.6.9 System design: In-situ bioremediation can be implemented in a number of treatment modes, including: Aerobic (oxygen respiration), anoxic (nitrate respiration), anaerobic (non-oxygen respiration), and co-metabolic. The aerobic mode has been proven most effective in reducing contaminant levels of aliphatic (e.g., hexane) and aromatic petroleum hydrocarbons (e.g., benzene, naphthalene) typically present in gasoline and diesel fuel. In the aerobic treatment mode, groundwater is oxygenated by one of three methods: Direct sparging of air or oxygen through an injection well, saturation of water with air or oxygen prior to re-injection, or addition of hydrogen peroxide directly into an injection well or into reinjected water. Whichever method of oxygenation is used, it is important to ensure that oxygen is being distributed throughout the area of contamination. Anoxic, anaerobic, and co-metabolic modes are sometimes used for remediation of other compounds, such as chlorinated solvents, but are generally slower than aerobic respiration in breaking down petroleum hydrocarbons.

The key parameters that determine the effectiveness of In situ groundwater bioremediation are:

i. Hydraulic conductivity of the aquifer, which controls the distribution of electron acceptors and nutrients in the subsurface,

ii. Biodegradability of the petroleum constituents, which determines both the rate and degree to

which constituents will be degraded by microorganisms.

iii. Location of petroleum contamination in the subsurface. Contaminants must be dissolved in groundwater or adsorbed onto more permeable sediments within the aquifer (Sheetal, 2012).

2.6.10 Application: In situ groundwater bioremediation can be effective for the full range of petroleum hydrocarbons. While there is some notable exceptions like the short-chain, low molecular weight, more water-soluble constituents are degraded more rapidly and to lower residual levels than are long-chain, high molecular weight, less soluble constituents. Recoverable free product should be removed from the subsurface prior to operation of the in-situ groundwater bioremediation system. This will mitigate the major source of contaminants as well as reduce the potential for smearing or spreading high concentrations of contaminants (Sheetal, 2012).

2.6.11 *Ex situ* bioremediation: These techniques involve the excavation or removal of contaminated soil from ground. Ex situ bioremediation can be divided into two main types: Solid phase and Slurry phase ex-situ bioremediation which can be further divided (Pavel and Gavrilescu, 2008; Vidali, 2001; Rao, 2005) .

2.6.11.1 *Ex Situ* Remediation techniques

Ex situ remediation includes techniques such as landfarming, biopiling, and processing by bioreactors along with thermal, chemical, and physical processes (Koning *et al.,* 2000). *Ex situ* remediation is a more thorough remediation technique, but due to the costs associated not only with the remediation processes, but also will the excavation and transportation of the soil, many people are looking towards in situ remediation techniques.

***a. Ex situ* thermal processes** involve the transfer of pollutants from the soil to a gas phase. The pollutants are released by vaporization and the burned at high temperatures. *Ex situ* thermal remediation is completed in 3 steps: soil conditioning, thermal treatment, and exhaust gas purification. (Van Deuren *et al*, 2002). Soil condition is a process in which broken into small grains and sieved in preparation for thermal treatment. Thermal treatment heats the soil in order to transfer volatile pollutants to a gas phase. Heating is done by using a sintering strand, fluid bed, or rotary kiln plants. The soil is usually heated to a low temperature range of 350-550^{o}C. Combustion of the gases occurs over the top of the soil, but the volatile gases are not destroyed. The gases are then burned in an after-burner chamber at approximately 1200^{o}C and dioxins are Destroyed (Koning *et al.,* 2000). *Ex situ* thermal remediation processes are ideal for use when removing petroleum hydrocarbons (TPH), polycyclic aromatic hydrocarbons (PAH), benzene, toluene, ethylbenzene, xylenes (BTEX), phenolic compounds, cyanides, and chlorinated compounds like polychlorinated biphenyls (PCB), pentchlorphenol (PCP), chlorinated hydrocarbons, chlorinated pesticides, polychlorinated dibenzodioxins (PCDD), and polychlorinated dibenzofurans (PCDF) (Koning, *et al.,* 2000).

b. The *ex situ* chemical/physical remediation process known as soil scrubbing uses mechanical energy to separate the pollutants from the soil. The soil is crushed and then separated via sieving. This ensures that the soil sample is homogeneous. The soil is then dispersed in liquid. Water, which is sometimes enhanced with an additive, is used to dissolve the pollutant. The additives are used to overcome the bonding forces between the pollutants and the soil particles. The soil is then separated into two categories: low density and high-density solids. Highly polluted fine particles are then separated out and dewatered. The particles are then rinsed with uncontaminated water. The wastewater and exhaust air are then purified. Soil scrubbing is

most effective when removing BTEX, TPH, PAH, PCB, heavy metals, and dioxins (Koning *et al.,* 2000).

c. Ex situ biological processes include: composting, landfarming, biopiling and the use of bioreactors. Composting consists of excavating the soil and then mixing organics such as wood, hay, manure, and vegetative waste with the contaminated soil (Van Deuren *et al.,* 2002). The organics are chosen based on their ability to provide the proper porosity and carbon and nitrogen balances to aid in the breakdown of contaminants. Maintaining thermophilic temperatures 54 to 65^{o}C is an important part of composting. In most cases, the indigenous microorganisms maintain this temperature while degrading the contaminant. Composting is most effective when removing PAH, TNT, and RDX (Van Deuren *et al.,* 2002). Landfarming is a process in which the soil is excavated and mechanically separated via sieving. The polluted soil is then place in layers no more than 0.4 meters thick. A synthetic, concrete, or clay membrane is then used to cover the contaminated soil layer. Oxygen is added and mixing occurs via plowing, harrowing, or milling. Nutrients and moisture may also be added to aid the remediation process. The pH of the soil is also regulated (keeping it near 7.0) using crushed limestone or agricultural lime (Van Deuren *et al,* 2002). Land farming is most successful in removing PAH and PCP.

2.6.11.2 Advantage of *ex situ* bioremediation processes. Most of the decontaminated soil can be reused. Due to the *ex situ* techniques used to decontaminate the soil, much of the soil cannot be used as filling or agricultural material. The soil can, however, be used for landscaping purposes. If soils are treated with thermal processes or a wet scrubber they may be reused as filling material. A key factor in determining the applicability of soil reuse is the toxicological assessment. Bioassays must be conducted in order to determine the impacts the soil will have on the surrounding area (Koning *et al.,* 2000).

2.6.12 Land farming: It is a simple technique in which contaminated soil is excavated and spread over a prepared bed and periodically turned over until pollutants are degraded. The goal is to stimulate indigenous biodegradative microorganisms and facilitate their aerobic degradation of contaminants. In general, the practice is limited to the treatment of superficial 10–35 cm of soil. Since land farming has the potential to reduce monitoring and maintenance costs, as well as clean-up liabilities, it has received much attention as a disposal alternative. Land farming is a method in which contaminated soil is spread over a prepared bed along with some fertilizers and occasionally rotated. It stimulates the activity of bacteria and enhances the degradation of oil. The criteria for determining proper site location includes: a minimum separation distance of 3feet between the ground surface and the reasonable high groundwater table and the slope of the land does not exceed 8% (Spormann and Widdel, 2000).

2.6.13 Composting: This is a technique that involves combining contaminated soil with nonhazardous organic amendants such as manure or agricultural wastes. The presence of these organic materials supports the development of a rich microbial population and elevated temperature characteristic of composting (Sheetal, 2012). Composting is a process of piling contaminated soil organic substances such as manure or agricultural wastes. The added organic material supports the development of a rich microbial population and elevates temperature of the pile. Stimulation of microbial growth by added nutrients results in effective biodegradation in a relatively short period of time.

2.6.14 Biopiles: This technique is a hybrid of land farming and composting. Essentially, engineered cells are constructed as aerated composted piles. Typically used for treatment of surface contamination with petroleum hydrocarbons they are a refined version of land farming

that tend to control physical losses of the contaminants by leaching and volatilization. Biopiles provide a favorable environment for indigenous aerobic and anaerobic microorganism (Sheetal, 2012).

2.6.15 Bioreactors: This is a type of slurry phase treatment. Slurry reactors or aqueous reactors are used for treatment of contaminated soil and water pumped up from a contaminated plume. Bioremediation in reactors involves the processing of contaminated solid material (soil, sediment, sludge) or water through an engineered containment system. A slurry bioreactor is a containment vessel and apparatus used to create a three-phase (solid, liquid, and gas) mixing condition to increase the bioremediation rate of soil bound and water-soluble pollutants as a water slurry of the contaminated soil and biomass (usually indigenous microorganisms) capable of degrading target contaminants. In general, the rate and extent of bioremediation by bioreactors is better than in situ bioremediation due to better contact between microorganisms and contaminants (Sheetal, 2012).

2.6.16 Phytoremediation: A new and rapidly developing form of bioremediation is phytoremediation, which uses green plants and their associated biota to destroy, remove, contain, or otherwise detoxify environmental contaminants. Four types of destruction and removal reactions can occur in phytoremediation (Fulekar, 2005; Rittmann and Mccarty, 2005).

i. Phytovolatilization is an enhancement of the volatilization process from the soil or through the plant's roots or shoots. Enhanced volatilization can occur via plant transpiration of volatile compounds or transformation of contaminants to more volatile forms.

ii. Phytodegradation involves uptake by the plant and subsequent metabolism by plant enzymes to form benign products.

iii. Phytoextraction involves uptake by the plant and absorption of the contaminant into plant tissue, which subsequently is harvested. Hydrophobic contaminants are most susceptible to photoextraction.

iv. Rhizo (sphere) degradation to benign products is catalyzed by plant enzymes excreted by the roots or by the microorganisms found in the rhizosphere. In addition, phytoremediation can sequester contaminants; a process called phytostabilization, the benefit of phytostabilization is that it makes the contaminants less bioavailable to humans and other receptors. Three forms of phytostabilization are Humification, Lignification, and Aging. The distinctions among the various stabilization, destruction, and removal mechanisms often are not sharp. Phytoremediation is still in its early stages of research and development. Nonetheless, it has promise for the decontamination of petroleum hydrocarbons, a range of chlorinated aliphatics and aromatics (including PCBs), and pesticides. .

2.7 Petroleum hydrocarbon degrading bacteria

Hydrocarbon degrading microorganisms are ubiquitous in nature but are found at relatively higher densities in petroleum contaminated sites. Arctic environments, estuaries, oceans and marine sediments, deep sea, thermal vents, etc. are some of the sites explored for isolating hydrocarbon degrading microorganisms. Hydrocarbon degrading bacteria (aerobic or anaerobic) and fungi (lignolytic) are widely distributed in these habitats. Similarly, hydrocarbon degrading cyanobacteria have been reported but there can be some exceptions to behavior of cyanobacteria (Adebusoye *et al.,* 2006; Gallego, 2001; Oboh *et al.,* 2006; Vidali, 2001).

2.8 Anaerobic Degradation

Most of bioremediation method aims in enhancing oxygen supply to contaminated sites assuming that the principal mechanism of hydrocarbon removal is aerobic respiration. But addition of urea and ammonia-based fertilizers sometimes used for oil-spill bioremediation can potentially exert an oxygen demand due to biological ammonia oxidation (Bijay, 2012). On some sites, mass transfer of oxygen may not be sufficient to replenish oxygen consumed by microbial metabolism, though penetration of oil into deeper sediment layers is also likely to be reduced in fine sediments. Under such conditions anaerobic hydrocarbon degradation may be of relevance. In contrast to the fact that aerobic microbial hydrocarbon metabolism has been extensively investigated, the same is not true about anaerobic hydrocarbon metabolism. The roles of bacteria that participate in these processes under anoxic/anaerobic conditions during biodegradation are not fully understood. Oxygen is not available in all environments where hydrocarbons occur (e.g. in deep sediments, flooded soils, eutrophic lagoons, stagnant fresh and ocean waters and in oil reservoirs) (Riser-Roberts, 1992). Several studies have investigated the question, whether or not the biodegradation of hydrocarbons is possible under anoxic conditions. It was not until the late 1980s that new groups of microorganisms were found to degrade hydrocarbons under strictly anoxic conditions. Studies have confirmed that these microorganisms activate organic compounds by special biochemical mechanisms that differ completely from those employed in aerobic hydrocarbon metabolism (Riser-Roberts, 1992). n-alkanes, branched alkanes, cycloalkanes, and some alkenes have been shown to be degraded under anaerobic conditions. For example, unsubstituted, methyl-substituted, and ethyl-substituted cyclopentenes, cyclopentanes and cyclohexanes were consumed without a substantial lag in the presence of sulfate but rather less effectively under methanogenic conditions. Dimethyl-substituted cyclopentanes and cyclohexanes were biodegraded only in the presence of sulfate

(Widdel and Rabus, 2001). Several laboratory and field studies conducted on biodegradation had clearly demonstrated biodegradation of BTEX (benzene, toluene, ethylbenzene and xylene isomers) compounds. The biodegradation of these individual compounds depends on the terminal electron acceptor. Usually, toluene and *m*, *p*-xylenes degrade under anaerobic conditions. One typical example is benzene degradation. Benzene is cleaved aerobically. On the other hand, it is usually recalcitrant under nitrate-reducing conditions. Benzene cleavage is highly site-specific in the presence of Fe (III) and sulfate terminal electron acceptor. Ethylbenzene degradation was described under aerobic as well as nitrate-reducing conditions. Nevertheless, degradation of this compound is site-specific under Fe (III) and sulfate reducing as well as methanogenic conditions. Utilization of *o*-xylene is enhanced by aerobic conditions, and is site-specific under anaerobic conditions (Schreiber et al., 2004). Hydrocarbons such as alkylbenzenes including m-, o-, and p-xylene, trimethylbenzenes, naphthalene and phenanthrene as well as n-alkanes and branched alkanes can also be metabolized under anaerobic conditions. These reactions may take place under Fe (III)-reducing, denitrifying and sulfate reducing conditions, by anoxygenic photosynthetic bacteria, or in syntrophic consortia of proton- reducing and methanogenic bacteria. Terminal electron acceptors other than oxygen (O_2) shown to be used during this metabolism include sulphate (SO_4^{2-}), nitrate (NO_3^-), sulphur (S) and fumarate in a fermentative oxidation. These microorganisms that use nitrate, ferric iron or sulphate as electron acceptors grow in syntrophic cocultures with other anaerobes (Boopathy, 2004). Most recent studies showed that *n*-hexane was activated under anaerobic conditions at carbon-2 in connection with an addition to fumarate, yielding (1-methylpentyl) succinate. The biochemistry of the degradation of alkylsuccinates has not been elucidated. However, it is expected that these reactions lead to fatty acid metabolism. Toluene has been the most studied hydrocarbon with respect to enzymatic and genetic characterization in the denitrifying bacteria *Azoarcus sp*. In the

proposed pathway, fumarate addition to toluene is mediated by benzylsuccinate synthase to form benzylsuccinate. Series of β-oxidation reactions convert benzylsuccinate to acetyl CoA and benzoyl-CoA which is a central intermediate in the anaerobic degradation of aromatic compounds. Benzoyl-CoA undergoes reductive dearomatization and ring cleavage followed by reactions that again resemble those in the β-oxidation reactions of fatty acids (Widdel and Rabus, 2001). For ethylbenzene, oxidation under denitrifying conditions starts with dehydrogenation by ethylbenzene dehydrogenase to produce 1-phenylethanol. This reaction is followed by oxidation to acetophenone. Acetophenone is carboxylated and after activation yields 3-oxo-3phenylpropionyl-CoA and thiolytic cleavage to acetyl-CoA and benzoyl-CoA. The metabolic pathway for sulfate-reducing bacteria is similar to that of toluene metabolism. In this case (1-phenylethyl) succinate was detected in enrichment cultures of *Azoarcus sp*. indicating ethylbenzene addition to fumarate. There is evidence that anaerobic degradation of *m*-xylene proceeds, in analogy to that of toluene, via *m*-methylbenzylsuccinate to *m*-methylbenzoyl-CoA (Widdel and Rabus, 2001; Riser-Roberts, 1992). PAH are also metabolized under anaerobic conditions. Naphtalene degradation proceeds via carboxylation to form 2-naphtoate (the central intermediate in a pathway analogous to the benzoyl-CoA pathway for monoaromatic compounds) in sulfate-reducing as well as denitrifying bacteria. The identification of other metabolites in a sulphate-reducing enrichment culture indicated the further metabolism of 2-naphthoate (presumably as activated acid) via subsequent reduction of the two rings to yield decalin-2-carboxylate. Alkylnaphthalenes appear to be activated by a mechanism similar to that of toluene (Hamme *et al.*, 2003).

Previous studies investigated a phenanthrene-adapted anaerobic group capable of degrading phenanthrene in river sediment**.** These studies explored the effects of several factors such as sludge source, the presence of individual or mixed PAHs, pH and the addition of electron donors

on PAH degradation rates. The results show that the order of degradation rates for PAHs in municipal sludge under anaerobic conditions is: phenanthrene > pyrene > anthracene > fluorene > acenaphthene. In petrochemical sludge the order is acenaphthene > fluorene > phenanthrene > anthracene > pyrene (Chang *et al.*, 2001, 2003; Meckenstock *et al.*, 2004). The mechanism by which anaerobic benzene degradation occurs is unclear. Anaerobic degradation of benzene has not yet been described in detail because, except for two *Dechloromonas* strains, no pure cultures of bacteria have yet been isolated for study (Riser-Roberts, 1992).

It is obvious that the degradation of petroleum and refined products proceeds much faster in the presence of oxygen than under anoxic conditions. Furthermore, aerobic microorganisms degrade a larger range of hydrocarbon compounds than anaerobic. Aerobic bioremediation processes are very effective in treating hydrocarbon contamination, however they are often expensive (e.g. hydrogen peroxide). For this reason anaerobic biodegradation provides cost-effective and advantageous in situ bioremediation technology that can be used for the decontamination of soil, sediment, and ground water contaminated with petroleum hydrocarbons. It was observed that benzene, toluene, ethylbenzene, and xylene are degradable without oxygen in contaminated groundwater (Johnson *et al.*, 2003; Coates *et al.*, 2002).

2.9 Techniques for studying oil degradation.

2.9.1 Respirometry The rate of carbon dioxide production and oxygen consumption is measured in a soil with a soil respirometer or an electrolytic respirometry apparatus. The technique appears to provide accurate estimates of microbial activity in soils with low organic matter content(Sheetal, 2012).

2.9.2 Gas Chromatography (GC): This technique identifies and quantifies contaminants by separating a complex organic mixture into its components. The sample extract is injected into a heated chamber and then is carried through column by an inert gas (mobile phase). The analytes pass through absorbent material (stationary phase). From the chromatogram and the information contained in the report, numerous components can be accurately identified and quantified. This technique can be used alone or in combination with Mass Spectroscopy (MS) or Flame Ionization Analysis (FIA) of separated analytes (Mittal, 2009; Sharma and Rehman, 2009).

2.9.3 Luminescence techniques

Luminescence arises from stimulation, either thermal or optical, of minerals that have been previously exposed to ionising radiation. During exposure, radiation energy is accumulated and stored in the crystal lattice; this energy is stored in the form of electrons that have been trapped at defects in the lattice. During stimulation, the trapped charge is released and as a result the luminescence signal becomes zero. Radiation-induced luminescence should be distinguished from other luminescence phenomena, e.g. photoluminescence, phosphorescence, etc. which are not dose dependent and thus not relevant to dating or dosimetry. Reporter gene cassettes containing luciferase genes and contaminant-specific promoter sequences are inserted into a bacterial host. Their expression is induced by contaminant of interest. Measurements of light emitted by such systems reflect bacterial metabolic activity in real time. This technique has limited application in multi-species oil-degrading microbial communities and under field conditions (Sheetal, 2012).

2.9.4 Fluorescence analysis

During fluorescence analysis of a compound, excitation and emission spectra are recorded. These spectra can be manipulated and used for the identification of the compound. This technique is

use for fingerprinting and has become a part of a basic and routine analytical procedure in analysis following oil spill in coastal waters. Although valuable as a rapid means of discrimination between some suspect sources this method's discriminating capacity is severely limited (Oil Degradation Monitoring at Metamicrobe.com).

2.9.5 Determination of Total Petroleum Hydrocarbons (TPH) by Infrared Spectrometry (IR) and Gas Chromatography (TPH-IR and TPH-GC)

The sample of oil is solvent-extracted and total mass of dissolved hydrocarbons is subsequently quantified by comparing the infrared absorption of the extraction liquid against that of control hydrocarbon mixture. This method is characterized by low technical complexity and quick quantification of oil contaminants (Mittal and Singh, 2009; Sharma and Rehman, 2009).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Basic Materials Used

Erlenmeyer flask

Washed bottle

Gas jar

Beakers

Kjeldahl flask

Universal bottle

Liquid soap (Emulsifier)

Fertilizer (N P K)

Masking tape

Petri- dish

Spatula

Syringe

Tissue paper

Measuring cylinder

Funnels

Volumetric flask

Burettes

Asbestos sheets

pH meter

Moisture can

Desiccator etc.

3.2 Sample Location

The site chosen for the experiment is the Kaduna refinery depots area of Kaduna State, where a huge volume of oil-effluent is spilled. Two different sets of samples were collected near Kaduna Refining and Petrochemical Company Limited (KRPC), a Subsidiary of Nigerian National Petroleum Corporation (NNPC) Kaduna (Lat:$10^0$25'18.81'' and Long: $7^0$29'14.63''). The samples areas were selected because of their closeness to where petroleum products are dispensed into tanker trucks.

3.3 Sample collection

The sampling strategy employed in this study is the stratified method. The sampling field was divided into sectors or quadrants and a total of fifteen (15) samples were collected according to proportionality rule (i.e. taking more samples in areas with high concentration of contaminant) (Venosa *et al.,* 1996). This method was combined with the composite method which involves the mixing of sampling units to form a single sample (Venosa *et al.*, 1996)**.**

The top 2cm layer of the sample core was collected and used for oil analysis using a sterilize spatula. The first set of samples labelled (D) was obtained from areas where diesel from the refinery spilled into the environment and the second set of samples labelled (P) was collected from areas where petrol leaked and spilled into the environment. To avoid cross contamination or chemical and biological changes in samples, clean and proper PVC bags in tin cans and sampling bottles were used for storage.



Plate 3.1 KRPC, Kaduna Aerial view.

3.4 Sample preparation

Samples were prepared through hand picking of dirt, stone, irons etc., air dried and sieved using 2mm sieves prior to physicochemical parameters analysis.

3.5 Cultured Medium preparation

The medium used for culturing the sample was blood agar base. The medium was prepared by weighing 40g of the blood agar into 1000 ml distilled water in a volumetric flask and heated to dissolve, sterilized at 121^{o}C for 15mins and 20 ml were poured into a Petri dish and was allowed to cool and solidify (as described by manufacturer).

3.6 Sample Culturing

The sample (0.1g) was weighed and introduced into sterile test tube containing 10ml of normal saline (0.8g of normal salt in 10ml of distilled water) and shaken properly.

A loop full of the sample from the normal saline was sub-cultured onto a plate of the prepared medium (blood agar) and the sample was smeared and streaked on the surface of the medium, incubation of the cultured medium was made anaerobically (introducing sample in an anaerobic jar and introducing a gas pack kit to generate carbon (IV) oxide) at 37^{o}C for 48hrs after which the cultured medium was observed for bacteria colony growth and morphology (i.e. size and colour). The bacteria colonies were Gram stained, the gram reaction of the colonies was determined.

3.7 Gram staining

The Gram staining was prepared by picking a colony of the bacteria and smeared on a glass slide, air dried and heat fix. This was brought to the staining rack in a sink and flooded with

crystal violet and allowed to stand for 1 min , washed with water and flooded with Grams iodine and allowed to stand for 1min and washed with water. This was decolorized with ethanol for 10 secs and washed with water and later counter stained with safranin for 30secs and washed with water and blot dried using filter paper. This was viewed on a microscope using oil immersion magnification (x100) for Gram reaction, shape and cell arrangement. Biochemical activities of the colonies were carried out (oxidase and catalase).

3.8 Preparation of Reagents

3.8.1 Preparation of methyl orange indicator

Methyl orange sodium salt (50mg) was dissolved in 100ml distilled water which contained 15.2ml of 0.01 M HCl solution. The solution was heated mildly and allowed to cool before it was filtered.

3.8.2 Preparation of Calcimeter Bernard filling solution

$CaCl_2.6H_2O$ (200g) was dissolved in 100ml distilled water followed by the addition of 3 drops of methyl orange indicator and drops of 4M solution of hydrochloric acid were added until the solution became red-orange.

3.8.3 Preparation of Solution of 4M HCl

The 4 M solution was prepared by adding slowly 82.117 ml of stock HCl solution(12.178 M based on a density of 1.2 g/ml, a formula weight of 36.46 g/mol, and a concentration of 37% w/w) to 100ml deionized water in a 250 volumetric flask and adjusted to 250 ml with deionized water.

3.8.4 Preparation of 0.16M Potassium dichromate ($K_2Cr_2O_7$)

*Potassium dichromate (0.16*M*) was prepared by dissolving* 12.26g of oven-dried, desiccated Potassium dichromate in approximately 150ml of distilled water and diluted to the mark in a 250ml volumetric flask.

3.8.5 Preparation of 1.0 M Ferrous Sulphate ($FeSO_4 \cdot 7H_2O$)

Ferrous Sulphate (556.04 g) was dissolved in approximately 1500 ml of distilled water and 30ml of concentrated Sulphuric acid(18M) was carefully added, cooled and diluted to 2000ml in a volumetric flask.

3.8.6 Preparation of Ortho-Phenanthroline Ferrous Complex Indicator

O-phenanthroline (3.71g) and $FeSO_4 \cdot 7H_2O$ (1.74g) were slowly dissolved in 250 ml of water in a volumetric flask.

3.8.7 Preparation of 0.01M $CaCl_2.2H_2O$

The $CaCl_2.2H_2O$ (1.47g) was dissolves in distilled water and made up to 1litre in a 1000 ml volumetric flask.

3.9 Moisture content determination

The moisture can was heated in an oven to drive off any moisture and cool in a desiccator and the weight of the cool moisture can was taken. 10g of the sample was weighed into the dry can and placed in an oven at 110^oC over night after which the can with the sample was placed in a desiccator and cooled for 1hour and its weight taken immediately.

The % moisture = (Weight of sample and can – Weight of oven dry sample and can x 100) / (Weight of oven dry sample and can)

3.10 pH Determination

Soil sample (10g) was weighed into a 50ml beaker, and distilled water (20ml) added and the suspension was stirred for 30min. The suspension was allowed to stand for 30min undisturbed and the electrode was immersed about half way into the mixture, the pH meter reading taken after 30s. This was repeated using 0.01M $CaCl_2.2H_2O$ solution. The pH meter was standardized before the readings of the soil samples were taken.

3.11 Determination of total nitrogen in soil

Mesh soil (2g) was weighed into 800ml Kjeldahl digestion flask and 10ml of distilled water added and allowed to stand for 30 minutes. Kjeldahl catalyst mixture (5g) was added and 15ml of 18.6M sulphuric acid added and the flask was heated cautiously on digestion stand until frothing ceased, and the heating was increased until the digest was clear. Heating was continued for another one hour. The flask was cooled and 100ml of distilled water was added slowly and shaken. Three chips of granulated zinc were added. Boric acid (2%, 30ml) was measured into 250ml Eelenmeyer flask and the flask placed under condenser. NaOH (75ml of 10M) was added slowly into the digest in the Kjeldahl flask and it was immediately connected to the distilling apparatus. The distillate (125ml) with NH_3 liberated was titrated with standard acid after addition of four drops of mixed indicator.

3.12 Determination of Cation Exchange Capacity (CEC) by Ammonium Saturation

The 2mm sieved soil (10g) were weigh into a 100 ml plastic beaker and 40 ml of 1M ammonium acetate was added, stirred and allowed to stay overnight. The soil was then filtered with light suction using a 55 mm buchner funnel and before the soil dry, it was leached with 1M ammonium acetate to a volume of 250 ml. The leachate from the soil was tested with 3 drops of

ammonium oxalate and heated which gave white precipitates indicating that Ca is present; the soil was further leached until the leachate is calcium free. The soil was leached again four times with 25 ml portion of 1 M ammonium chloride and once with 25 ml of 0.25 M ammonium chloride. The electrolytes present in the soil were washed out with 200 ml of isopropyl alcohol. The leachate was tested for chloride with 0.1 M silver nitrate till its negligible and the soil is allow to drained thoroughly. The soil is further leached gradually with acidified 250 ml 0.1M sodium chloride. 50 ml of 2% boric acid was measured into a 250 ml conical flask and 3drops of mixed indicators were added. The acidified sodium chloride leachate were poured into a 500 ml Kjeldahl flask and connected to the still. 10 ml of 1 M sodium hydroxide was added into the flask and distilled over the boric acid in the conical flask. 150 ml of the distillate was collected and titrated with a standard acid (0.1M HCl). A blank was prepared by taken 50 ml of boric acid and mixed indicator which was titrated with the standard acid (0.1M HCl). The CEC was calculated in cmol per kg of soil thus:

CEC (cmol/kg) = (AT – BT) x C x 100 / Weight of soil.

Where AT is the actual titre value, BT is blank titre value, and C is the concentration of the standard acid (0.1M)

3.13 Chloride Determination

Sample (10g) was weigh into a 100 ml conical flask and 40 ml of distilled water added stirred and left overnight for extraction to occur. 20 ml of the extract was pipette into a 100 ml conical flask and 3 drops of potassium chromate indicator were added and titrated with 0.01 M silver nitrate solution. The concentration of chloride was calculated in mg/kg thus;

Cl^- (mg/kg) = (1000 X T X Ev X 0.01M $AgNO_3$ / Wt X Aliquote Taken).

Where T is the titre value, Ev is the extraction volume (40ml) and Wt is the weight of sample (10g).

3.14 Determination of Carbonate and Bicarbonate

Sample (10g) was weighed into a 100ml conical flask and 40ml of distilled water added stirred and left overnight for extraction to occur. 20ml of the extract was pipette into a 100ml conical flask and 3drops of phenolphthalein indicator were. This was to be titrated with 0.02M H_2SO_4 to obtain a titre value which will be used to calculate the concentration of carbonate if the colour changes to pink when indicator is added but the colour remain unchanged indicating that carbonate is zero. But when methyl orange indicator was added, the colour changes to yellow and was titrated to a red colored end point which indicate that bicarbonate is present. Its concentration was calculated thus;

HCO_3^- (mg/kg) = (1000 x 0.02 x T x Ev) / (Wt x Aliquote)

CO_3^{2-} (mg/kg) = (1000 x 0.02 x T x Ev) / (Wt x Aliquote)

Where T is the titre value, Ev is the extraction volume (40 ml) and Wt the weight of sample (10g).

3.15 Organic Carbon Determination

Soil sample (10g) was weigh into a 250 ml Erlenmeyer flask and 10 ml of 0.5 M K_2CrO_7 was added using pipette and the flask was swirl gently to disperse the soil and 10 ml of 18 M H_2SO4 was added rapidly using measuring cylinder and swirled for 1min and the flask was allow to stay on an asbestos sheet for 30 mins. 100 ml of distilled water was added to the flask and allowed to cool and 5 drops of the indicator (O- phenanthroline ferrous sulphate) was added and it was

titrated with ammonium ferrous sulphate solution till the colour change from blue to green then to red. A blank determination was done in same manner but without soil. The organic carbon was calculated thus;

Organic carbon (g/kg) = (BT – AT) x 0.3 x M x F / (Wt x 10)

Where BT is the blank titre value, AT is actual titre value, M is concentration of ammonium sulphate and F is the correction factor (1.33) (Nelson and Sommers, 1982).

3.16 Determination of Exchangeable Bases (Mg, Ca, K and Na)

The 2mm sieved soil (10g) was weighed into a funnel fitted over 100 ml volumetric flasks. The soil in the funnel was leached with 1M ammonium acetate solution into a volume of 100ml (leaching was done for about 2hours with about 10ml of the acetate at each washing) the funnel was remove and the flask corked and shaken thoroughly. Na and K was determined by flame emission while Ca and Mg was determine by atomic absorption spectrometry using the leachate.

3.17 Soil Particle Size Analysis

The 2mm sieved soil (100g) was weighed into a 250 ml plastic beaker and 100 ml of 50% calgon (37g/ml sodium hexametaphosphate) was added and stirred with a glass rod. 100ml of distilled water was added and stirred and allowed to stay for 30mins with occasional stirring. This was transferred into a mixing cup and stirred again for 5mins and transferred to a 1litre measuring cylinder made to the 1000ml mark with distilled water and mixed vigorously using a long handle plunger and ensuring that the sediment at the bottom is thoroughly disturbed and the hydrometer reading was taking at 40 sec and 2 hour (as recommended by Bouyoucos for the 20µm and 2µm fraction respectively). A blank was prepared by taken 100ml of 50% calgon solution into a 1litre cylinder and made up to the mark with distilled water and the blank

hydrometer reading was taken at 40 sec and 2 hours. The temperature of the suspension and the blank was also taken. The suspension was poured through a 0.2 mm sieve and the sand grains retained in the sieve were washed with distilled water and the washing transferred into a beaker and dried to in an oven at 105^{o}C. The weight of the sand was finally taken and recorded which gave the coarse sand fraction.

C**alculations**

% Clay = (R – B + 0.36T) x 2. %Silt = (R – B + 0.36T) x 2 - % Clay.

%Sand = 100 – (% Clay + %Silt) based on the international system of textural classification.

Where R is the hydrometer reading in soil suspensions (g/l), B is the hydrometer reading in the blank (g/l) 0.36 is a factor for every 20^{o}C rise in temperature and T is room temperature minus 20

3.18 Phosphorus determination

The prepared sample solution (25ml) was transferred into a 50ml volumetric flask, 5ml of the prepared molybdate solution and 2ml of hydrazine sulphate solution were added and the solution was diluted to the graduation mark with distilled water and mixed thoroughly. The flask was immersed in a boiling water bath for 10min which was later removed and cooled rapidly using ice blocks. The flask was shaken again and the meniscus was adjusted using distilled water and using 1cm cell the transmittance or absorbance was measured at 830nm with a reagent blank (Eaton *et al.,* 1995; Tan, 1996).

3.19 The Experimental Sets.

The material used in preparing the experimental sets are; fertilizer(NPK 20, 10, 10), Bacterial culture (1.0×10^{8} CFU/ml) and emulsifier (liquid soap) .

Pure cultures of indigenous micro organisms capable of degrading oil were obtained by

enrichment technique from the effluent polluted soil which had been contaminated over a period of time. The pure isolates (cultured), fertilizer and emulsifier were applied on the polluted soil samples as shown in table 3.1.

3.20 Extraction of oil for GC-MS analysis

The oil was extracted by solvent extraction using dichloromethane (DCM). Soil sample (20g) was extracted with 20ml of DCM and the extract was evaporated to dryness. The dry residue was re-dissolve in 20ml of DCM then evaporated to dryness. The final residue was re-dissolved in 5ml of DCM and analyzed by GC and chromatograms were obtained.

3.21 Biodegradation of Crude Oil

The amount of crude oil degraded in each soil sample was determined by the weight loss method of Bossert and Bartha (1984). This was done by suspending 3g of soil in 10ml of diethyl ether in a universal bottle and shaken vigorously to extract the oil. The solvent-oil mixture was transferred slowly into a beaker of known weight. This was repeated until all the oil was extracted from the soil. The solvent-oil mixture was exposed at room temperature overnight to allow the solvent to evaporate completely. The new weight of the beaker containing the residual oil was taken and the percentage of oil degraded calculated (Ijah and Ukpe, 1992).

$$\%\ \text{of oil degraded(eliminate)} = \frac{\text{Original oil concentration} - \text{Final oil concentration}}{\text{Original oil concentration}} \times 100$$

The original oil concentration is the initial concentration of oil (weight of extracted oil in g/g) obtained before applying anything on the contaminated soil. While the final concentration of oil is the concentration of oil extracted on day 7, 14, 21 and 28 after the treatment has been applied on the contaminated soil (weight in g/g).

3.22 The emulsifier (Liquid soap)

The soap used was made using a sulphurnated linear alkane, sodium hydroxide and sodium trioxocarbonate (as indicated by the manufacturer).

3.23 Statistical Tool

A simple T-test analysis was used to determine the significant difference in the oil degradation trend between P and D after; (1) inoculation with *Bacillus sp,* (2) adding emulsifier and *Bacillus sp* inoculation (3) adding fertilizer, adding emulsifier and *Bacillus sp* inoculation using IBM SPSS statistics 20.

Table 3.1. Experimental Set Design (Ibiene *et al.*, 2011; Ijah and Ukpe, 1992)

Experimental set	Test experiment
A	Polluted Soil sample(200g) + Fertilizer(1.0g)
B	Polluted Soil sample(200g) + Bacterial culture(0.25ml)
C	Polluted Soil sample(200g) + Bacterial culture(0.25ml) + Fertilizer(1g)
D	Polluted Soil sample(200g) + Emulsifier(1ml) + Bacterial culture(0.25ml)
E	Polluted Soil sample(200g) +Emulsifier(1ml) + Bacterial culture(0.25ml)+Fertilizer(1g)
F	Polluted soil sample(200g) + Emulsifier(1ml) + Fertilizer(1g)
G	Polluted soil sample(200g) + Emulsifier(1ml)
H(CONTROL)	Polluted Soil sample(200g) + no amendment

CHAPTER FOUR

RESULTS

4.1 Physicochemical Parameters.

Results for Physicochemical parameters of the soil samples are given in Table 4.1. The average pH of samples D and P was found to be 5.9 and 6.2 respectively and textural content of the soil is sand. The organic content was found to be 28.30 and 37 g/kg for D and P respectively. The cation exchange capacity (CEC) was determined and was found to be 30.0 and 32.0 mmol/kg for D and P respectively. The concentration of total nitrogen was determined and was found to be 3.30 and 10.5 g/kg for D and P respectively and the concentration of available phosphorus in D and P was found to be 1.33 and 11.36 g/kg respectively.

4.2 The Percentage of Oil (Contaminant) Degraded With Time

Figures 4.1 to 4.18 shows the extend of degradation of oil (contaminant) in soil samples (D and P) expressed in percentages with time when inoculated with bacteria, emulsified with liquid soap and stimulated by adding NPK fertilizer separately and by combination of these treatments . These results were obtained by determining the weight loss of oil with time.

Figure 4.1 shows the percentage of oil degraded with time in the control (sample D without treatment). The initial concentration of the sample was determined and after 7days 3% of the initial concentration was degraded in the sample and 7days later 58% of the oil concentration was found to be degraded and after these 14days the degradation of the oil was very slow.

Table 4.1: Measured Physicochemical Parameters of Soil Samples

Parameters	Contaminated with Diesel(D)	Contaminated with Petrol (P)
Moisture content (%)	0.50	1.25
Sand (g/kg)	940	900
Silt (g/kg)	20	60
Clay (g/kg)	40	40
Textural class	Sand	Sand
pH	5.9	6.2
Organic content (g/kg)	28.30	37.00
Nitrogen (g/kg)	3.30	10.50
Available phosphorus (mg/kg)	1.33	11.36
Calcium (cmol/kg)	1.20	3.60
Magnesium (cmol/kg)	0.27	1.17
Potassium (cmol/kg)	0.25	0.50
Sodium (cmol/kg)	0.17	0.22
CEC (cmol/kg)	3.00	3.20
Carbonate (mg/kg)	0.00	0.00
Bicarbonate (mg/kg)	0.80	1.20
Chloride (mg/kg)	0.60	0.80

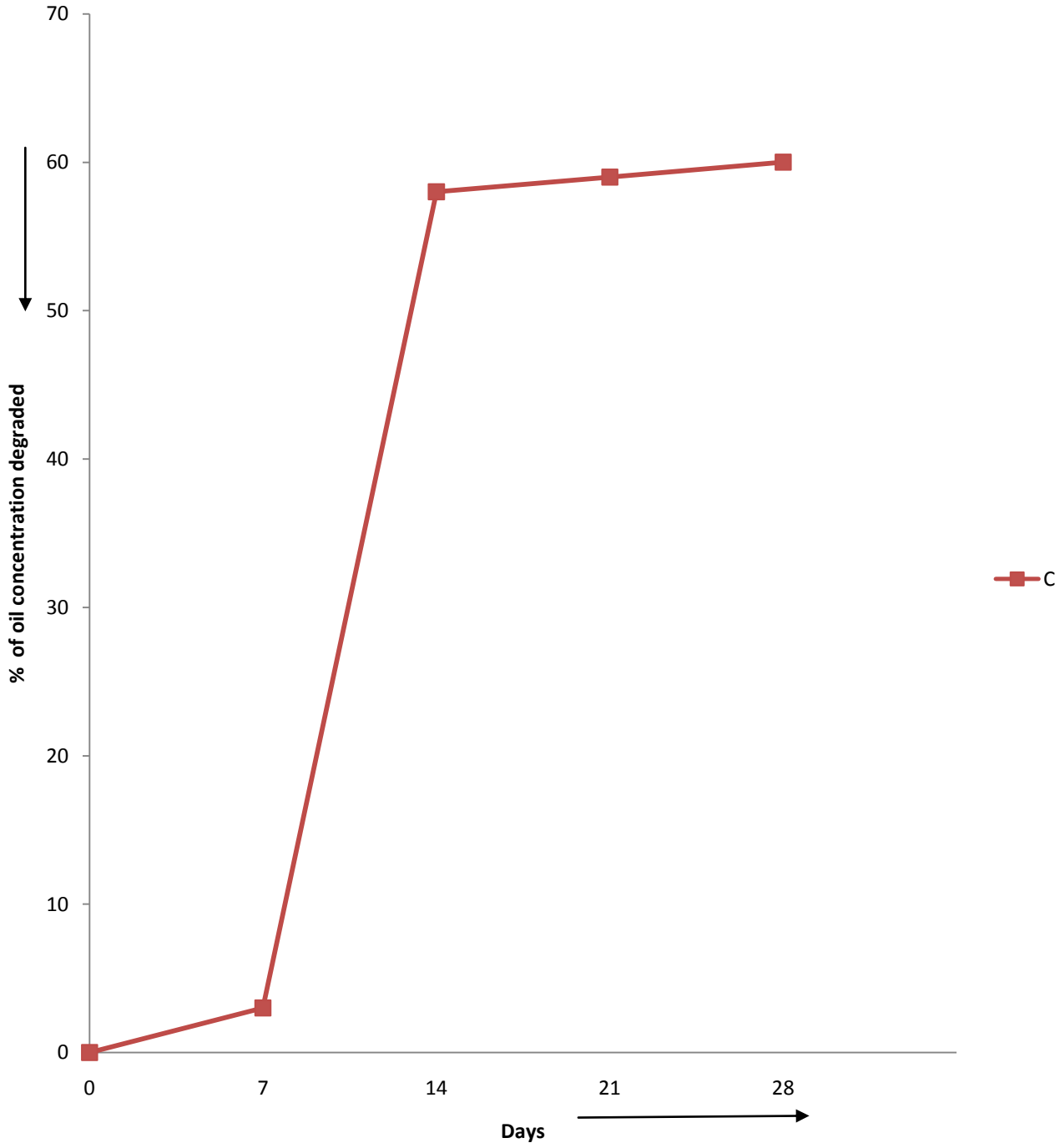


Figure 4.1 The rate of oil degradation with time (CONTROL)

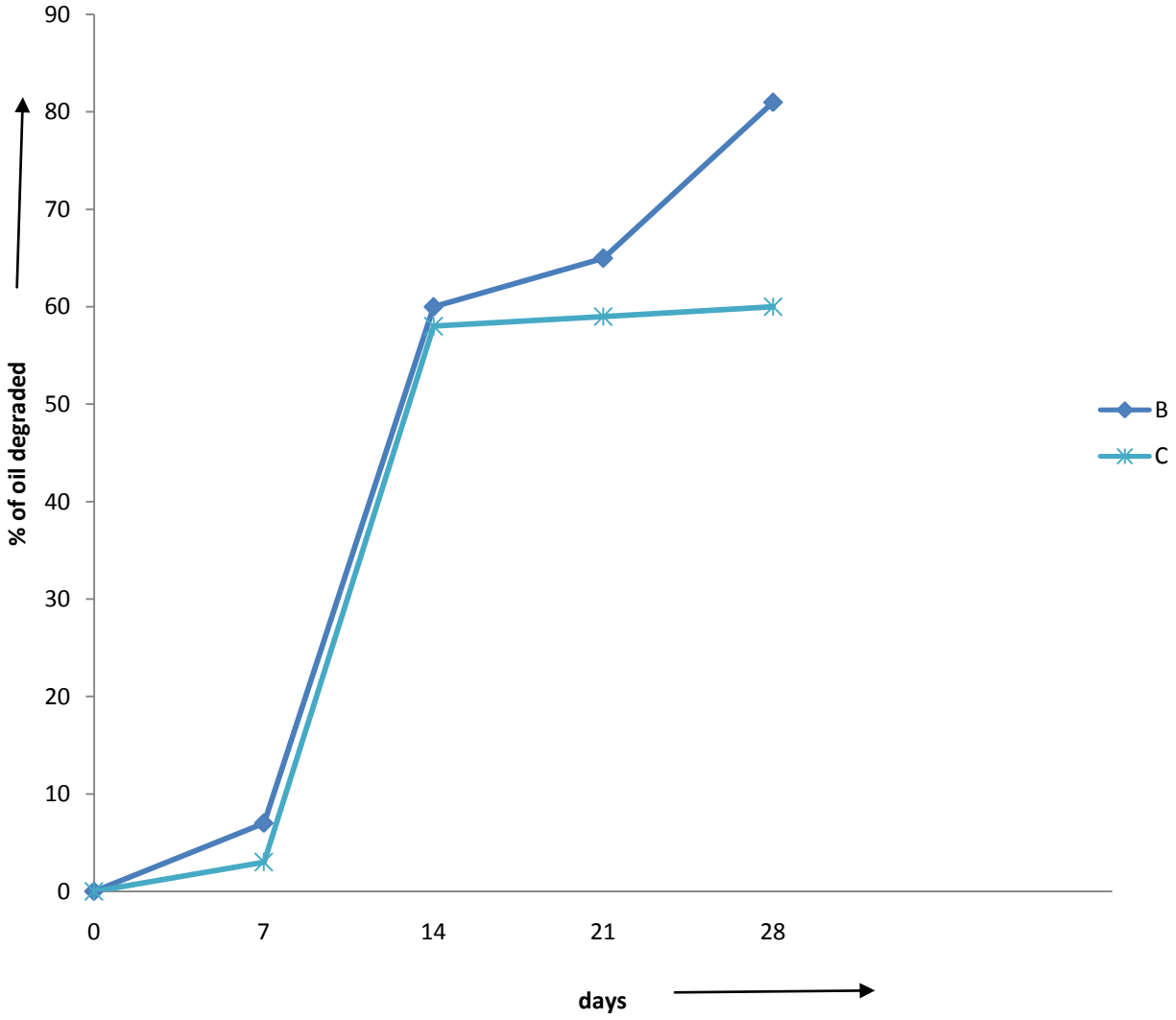
C = control

Figures 4.2 and 4.3 are a display of the percentage of oil degraded with time when sample sets are inoculated with Bacillus sp (B) only for sample P and D respectively.

In Figure4.2 (inoculation of P with bacteria), 7% of the oil was degraded after 7days of inoculation, and 7days later the percentage of oil degraded increased to 60%. This high increase in degradation futher increased to 64% after another 7day and later increased to 81%. While in Figure4.3 (inoculation of D with bacteria) 7% of the oil was degraded after 7days and 7days later the percentage increased to 88%, after another 7days the percentage increased to 94% after which there was no further increased in degradation of oil.

Figure 4.4 shows the percentage of oil degraded with time when sample (D) was treated with NPK fertilizer (F) only. The percentage of oil degraded when fertilizer was added after 7days was 20% which is above the control and 7days later the percentage was less than the control and after then no further significant degradation was observed.

Figures 4.5 and 4.6 show the percentage of oil degraded with time when sample sets (D and P respectively) are emulsified (E) only. In Figure 4.5 the percentage of oil degraded on day 7, is 13% and on day 14 the percentage increased to 61% after which the degradation rate decreased giving no further high percentage even after 14days later. Figure 4.6 the concentration of oil degraded on day 7 is 2% which is lower than the concentration recorded in the control on day 7, but on day 14 it increased to 73% and on day 21 it increased further to 88%.



B- Bacteria, C- Control.

Figure 4.2 The percentage of oil degraded (eliminated) from sample P using Bacteria(B) (bioaugmentation) with time.

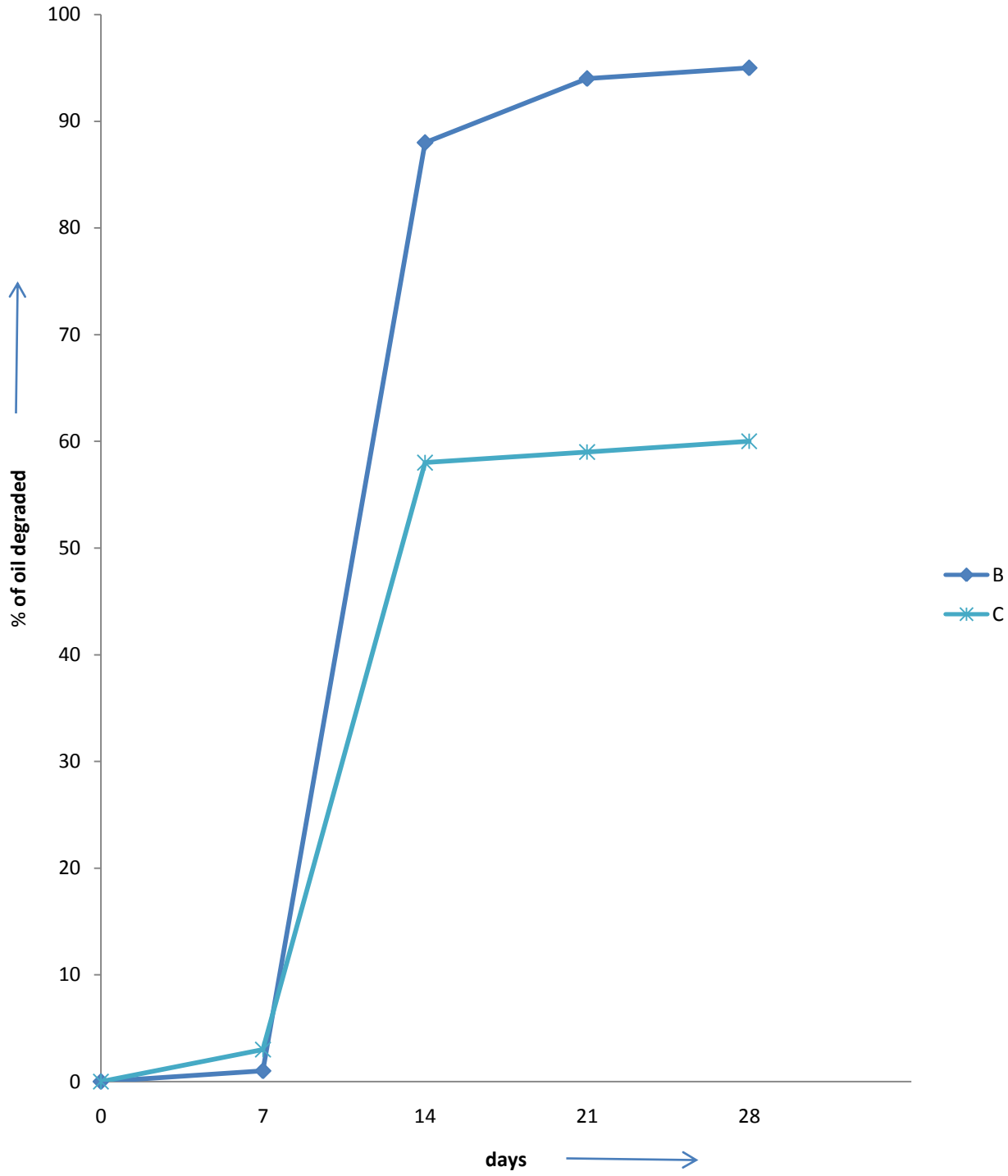
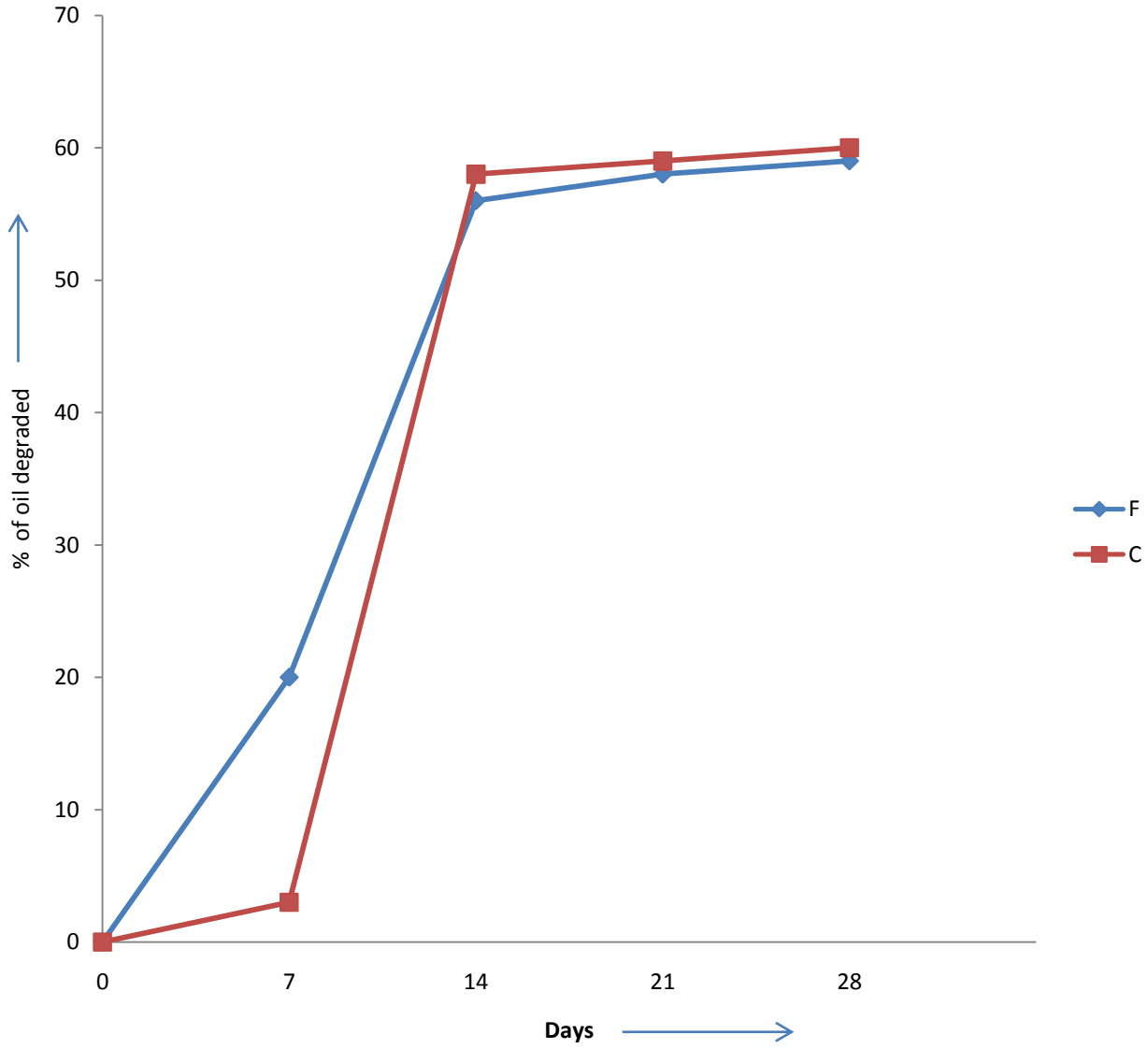


Figure 4.3 The percentage of oil degraded (eliminated) using Bacteria in sample D (bioaugmentation) with time.

C – control, B - bacteria



F- Fertilizer, C- Control.

Figure 4.4 The percentage of oil degraded (eliminated) from sample D using fertilizer (biostimulation) with time.

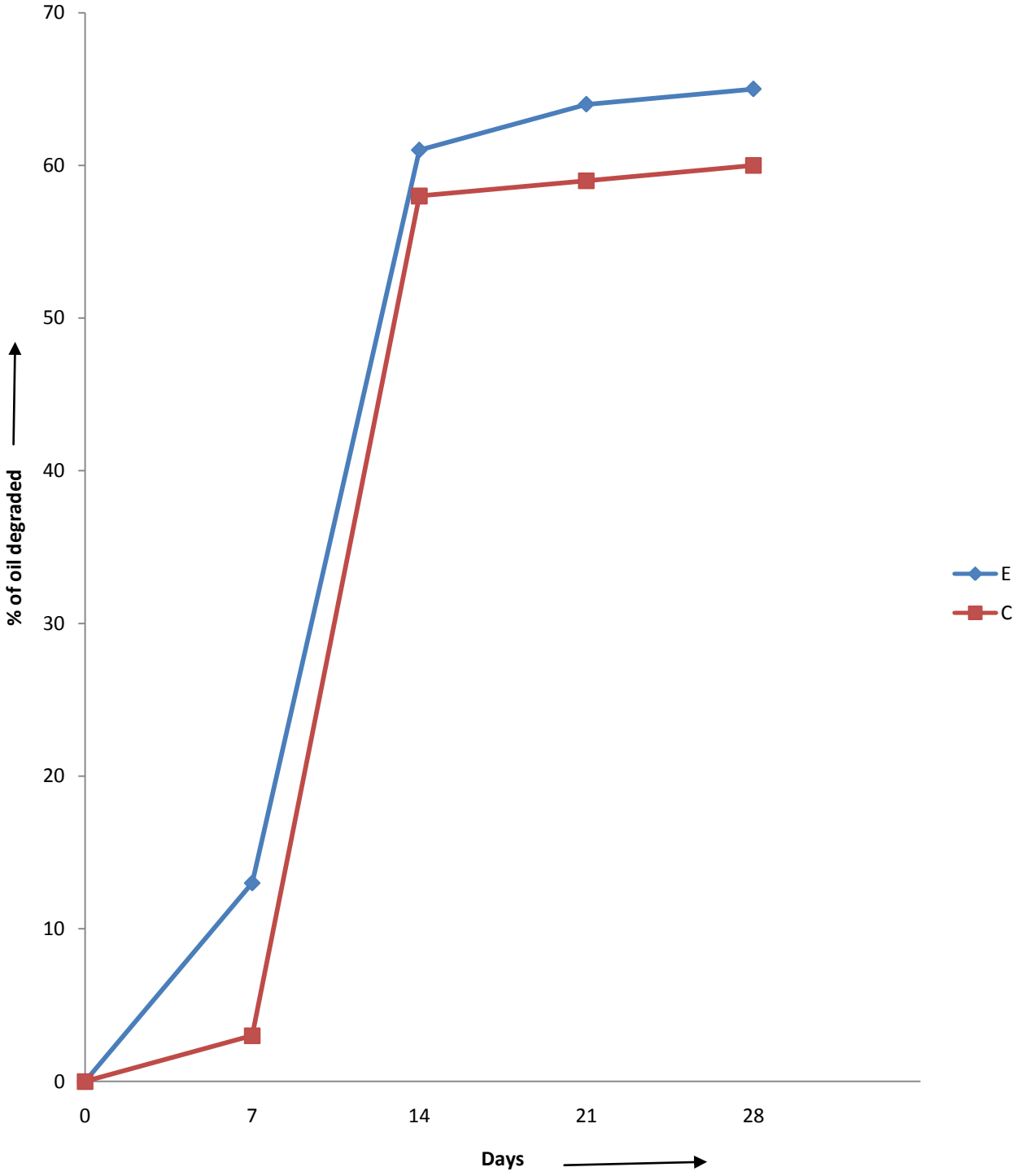


Figure 4.5 The percentage of oil degraded from sample D with time using emulsifier (E). E = emulsifier, C = control

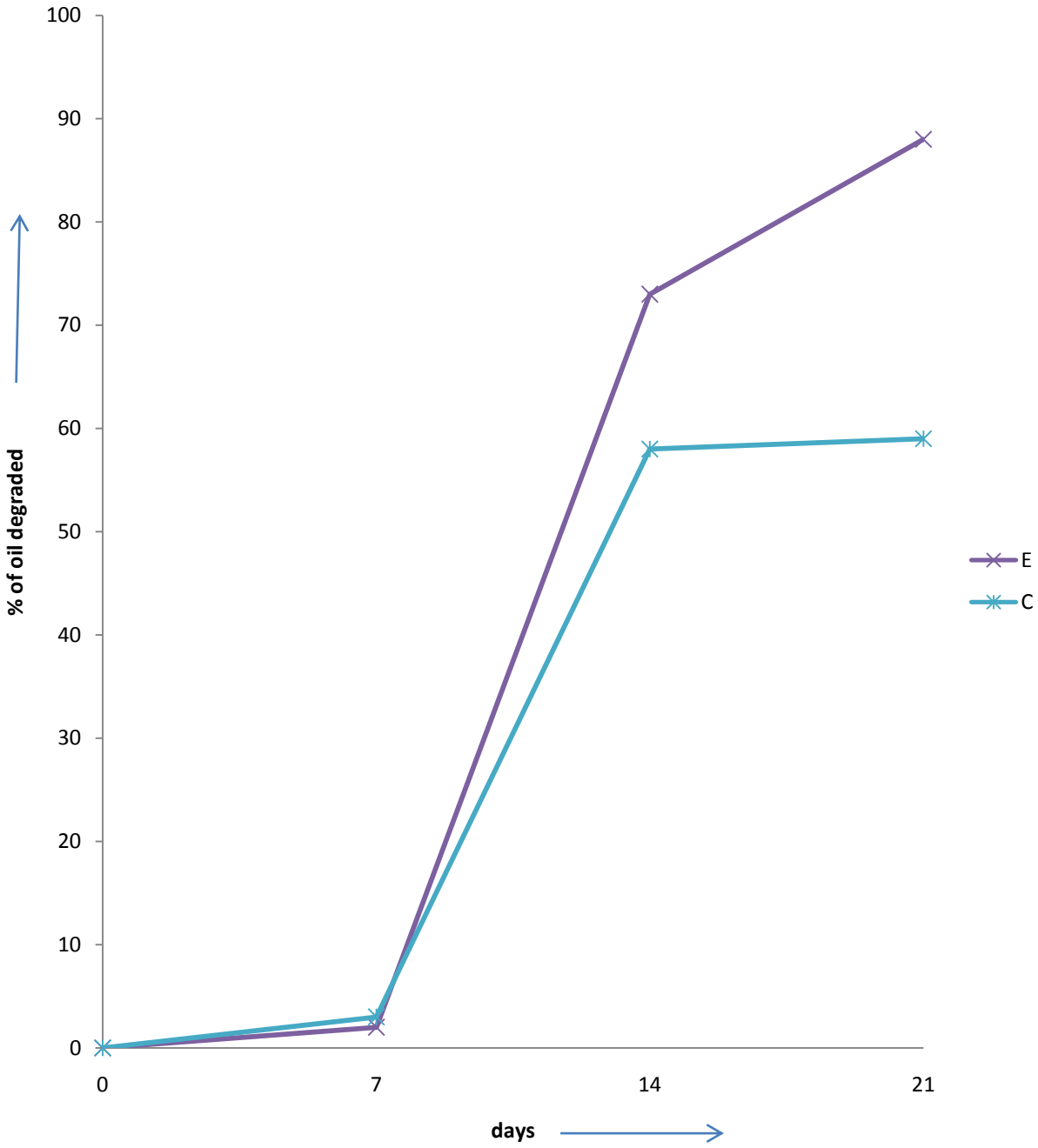


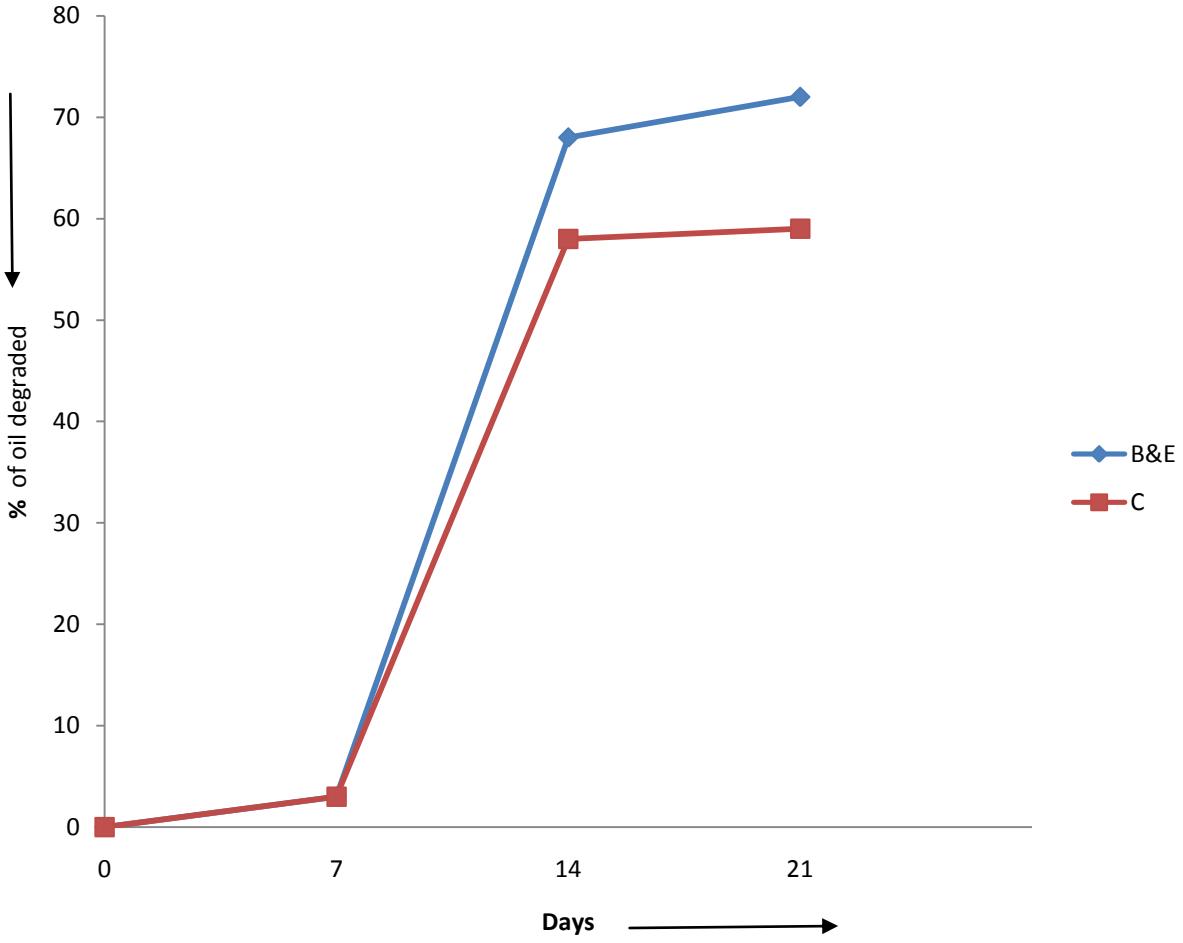
Figure 4.6 The percentage of oil degraded from sample P with time using emulsifier . E = emulsifier, C = control

Figures 4.7 and 4.8 show the percentage of oil degraded from the sample sets (D and P respectively) with time when emulsified and inoculated with *Bacillus sp* (B and E). In Figure4.7, 3% of the oil was degraded on day 7 and on day 14 the percentage increased to 68% and on day 21 it increased slightly to 72%. While in Figure4.8, the concentration of oil degraded on day 7 is 6% and it increased to 88% on day 14. Thereafter there was no significant degradation occurring.

Figure 4.9 shows the percentage of oil degraded in D with time using *Bacillus sp* and NPK fertilizer (E and F). On day7 the concentration of the oil degraded is 12%, on day 14 the concentration increased to 56% this further increased to 77% on day 28

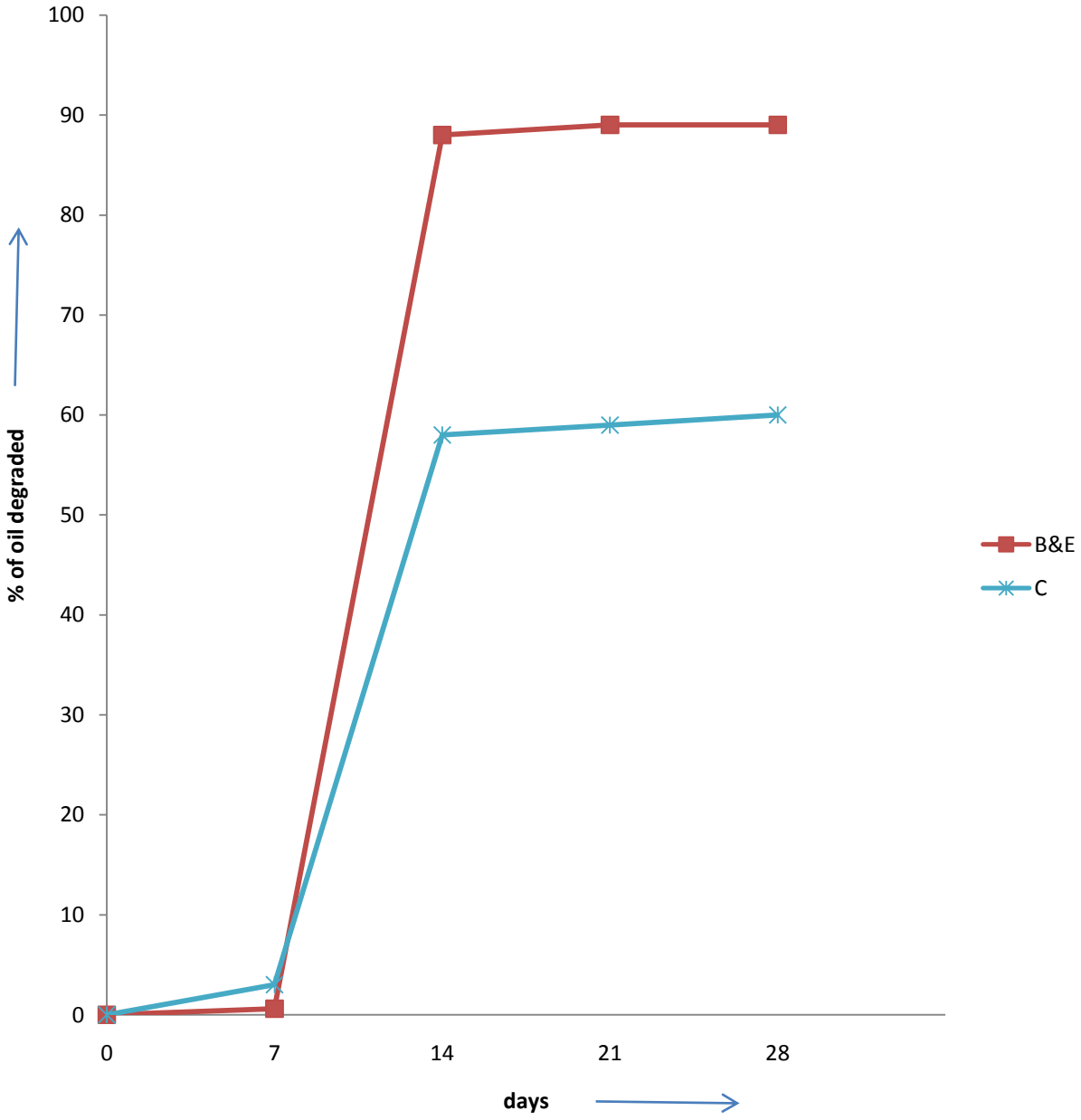
Figure 4.10 shows the percentage of oil degraded with time when sample (D) is inoculated with *Bacillus sp* and NPK fertilizer added (B and F). On day 7 the concentration of oil degraded is 23% which happen to be the highest concentration recorded on day 7 in all the experimental sets. On day 14 the percentage increased to 45% and on day 21 it increased further to 63% after then no further significant degradation was recorded.

Figures 4.11 and 4.12 show the percentage of oil degraded with time when samples (D and P respectively) are treated with Emulsifier, NPK fertilizer and inoculated with *Bacillus sp* (B, E and F). In Fig4.11 (sample D) the concentration of oil degraded after 7days is 5%, and on day 14 the concentration increased to 50% this further increased to 95% after 28days. Fig4.12 (sample P) shows that on day 7 the concentration of oil degraded is 20% and on day 14 this concentration increased to 84%, on day 28 the concentration further increased to 97%.



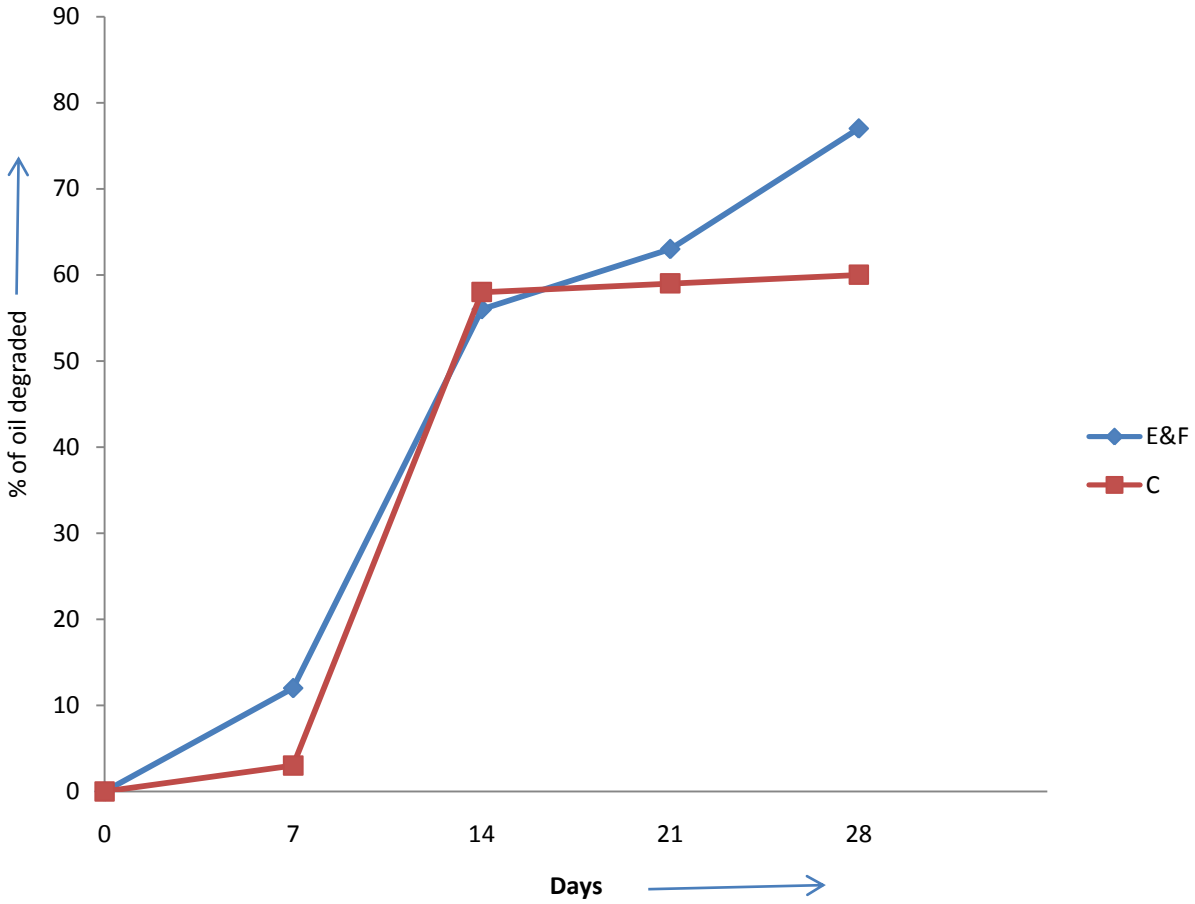
B-Bacteria, E-Emulsifier, C-Control.

Figure.4.7 The percentage of oil degraded (eliminated) from sample D using bacteria and emulsifier in combination.



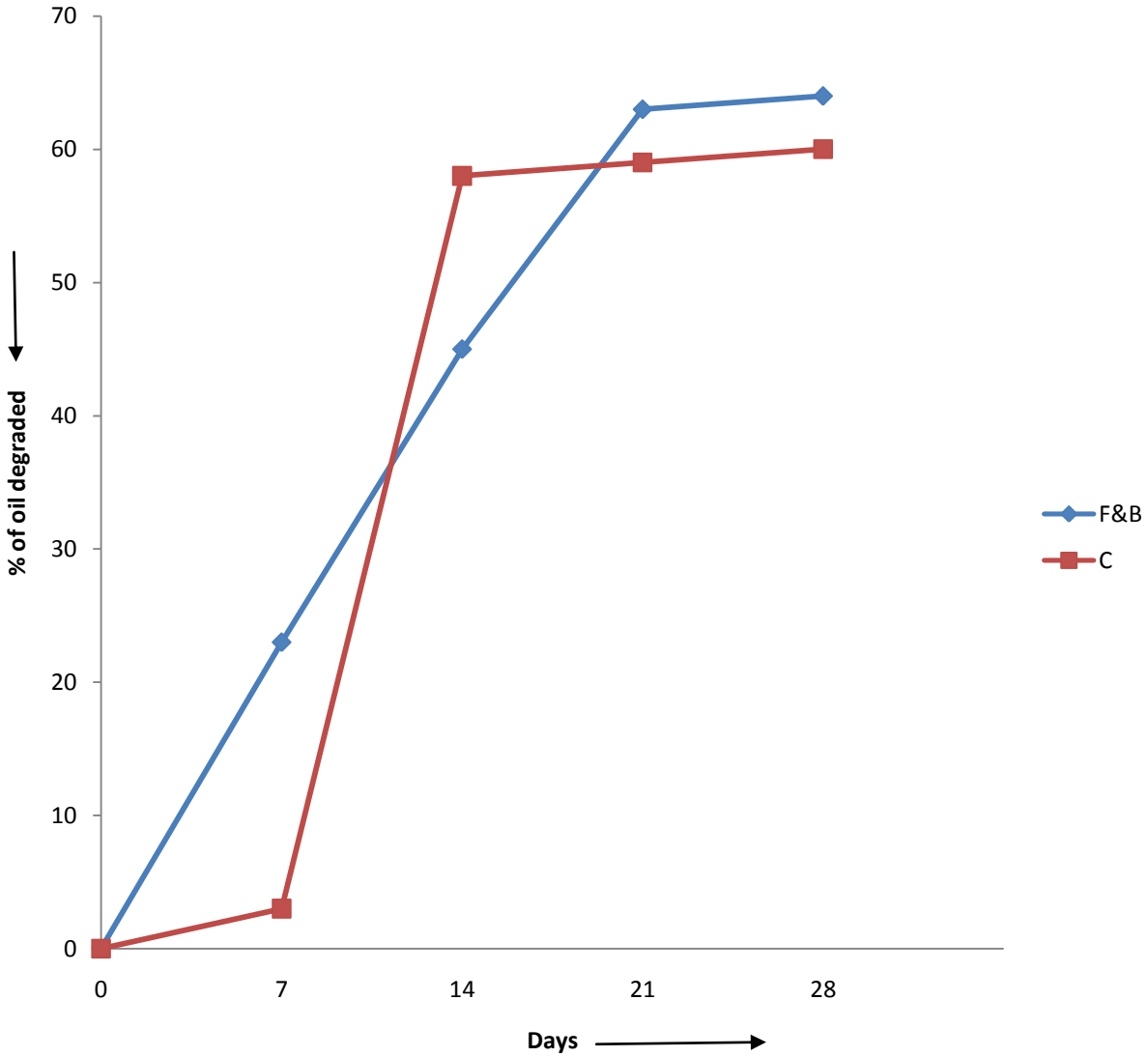
B-Bacteria, E-Emulsifier, C-Control.

Figure 4.8 The percentage of oil degraded (eliminated) from sample P using bacteria and emulsifier in combination.



E = emulsifier, F = fertilizer, C = control

Figure 4.9 Rate of oil degradation in Sample D treated with Fertilizer and Emulsifier



B-Bacteria, F- Fertilizer C- Control

Figure 4.10. The percentage of oil degraded (eliminated) from sample D with time using bacteria and fertilizer in combination.

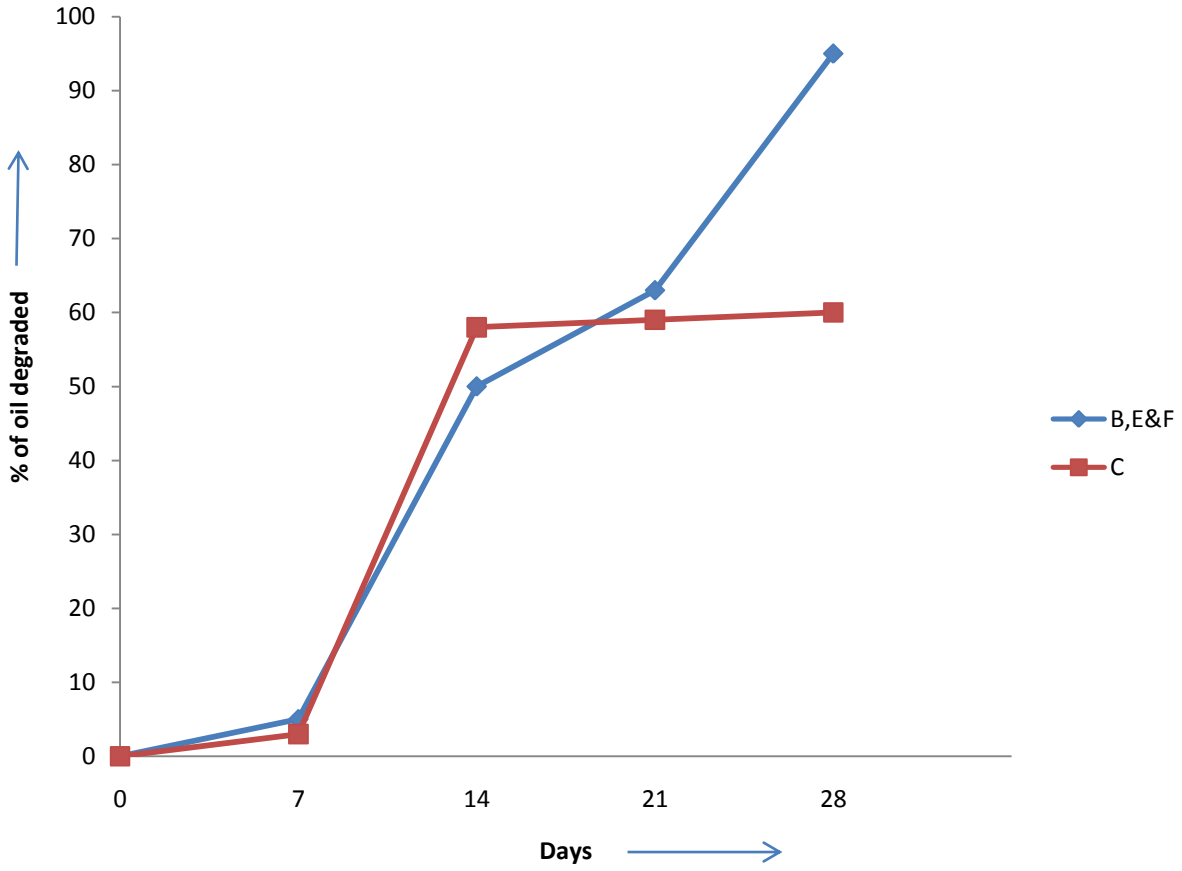


Figure 4.11. Percentage of oil degraded with time for sample D using Emulsifier, Bacillus sp and NPK Fertilizer (B, E and F). C = control.

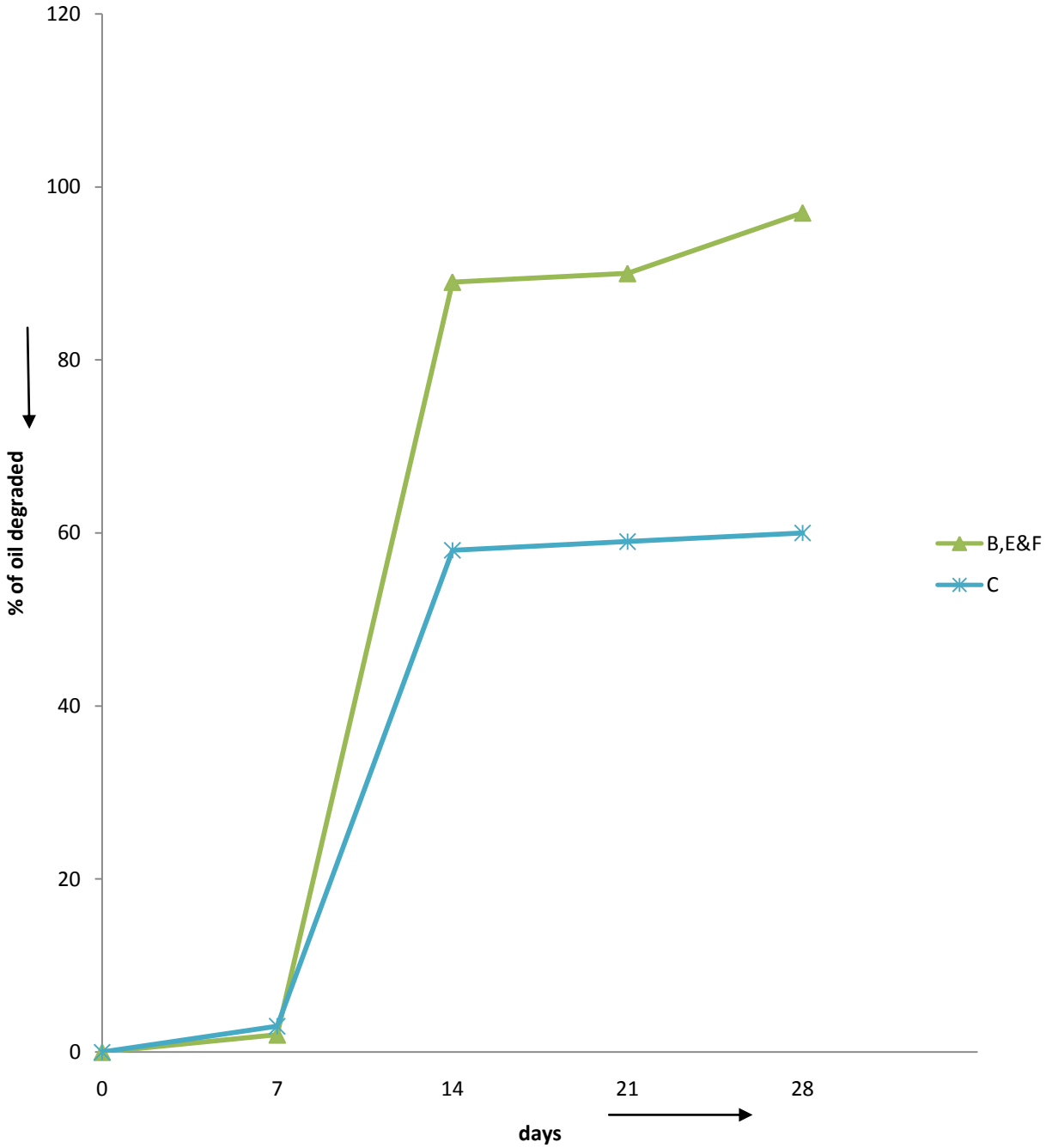
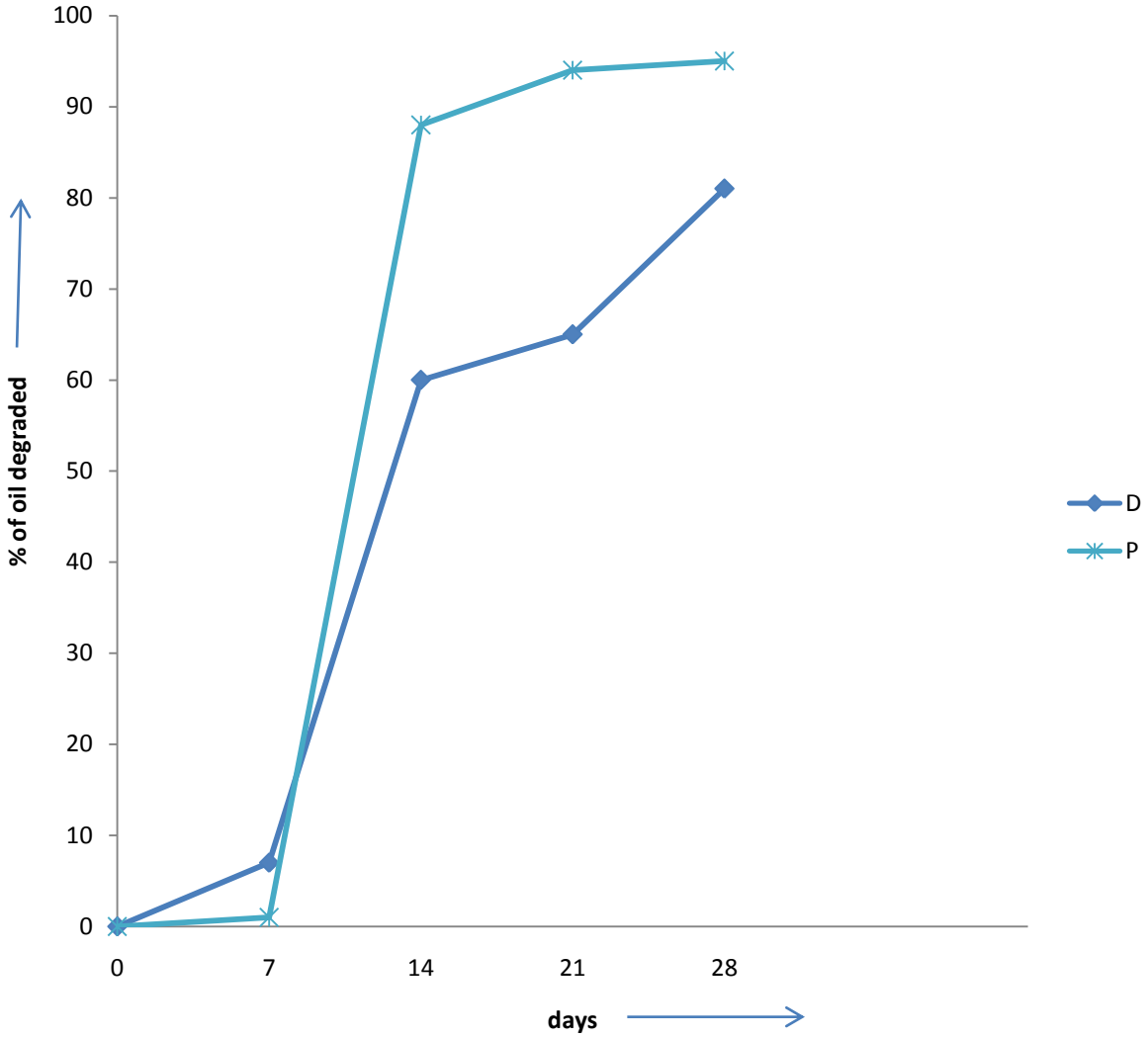


Figure 4.12 The percentage of oil degraded (eliminated) from sample P with time using Bacteria, Emulsifier and Fertilizer (B, E and F). C = control.

Figure 4.13 compares the trend in rate of degradation of oil in sample P and D when inoculated with Bacillus sp. On day 7 both samples had 7% of their contaminant degraded, on day 14 the concentration degraded increases to 88% in sample P while in D it increased to only 60% which later increase to 65 while sample P it further shoots up to 94%. At the last week of the experiment the degradation was slow in sample P (94 -95%) while in sample D it shoots up (65 – 81%) The trend of degradation in sample D and P is different ($P<0.05$).

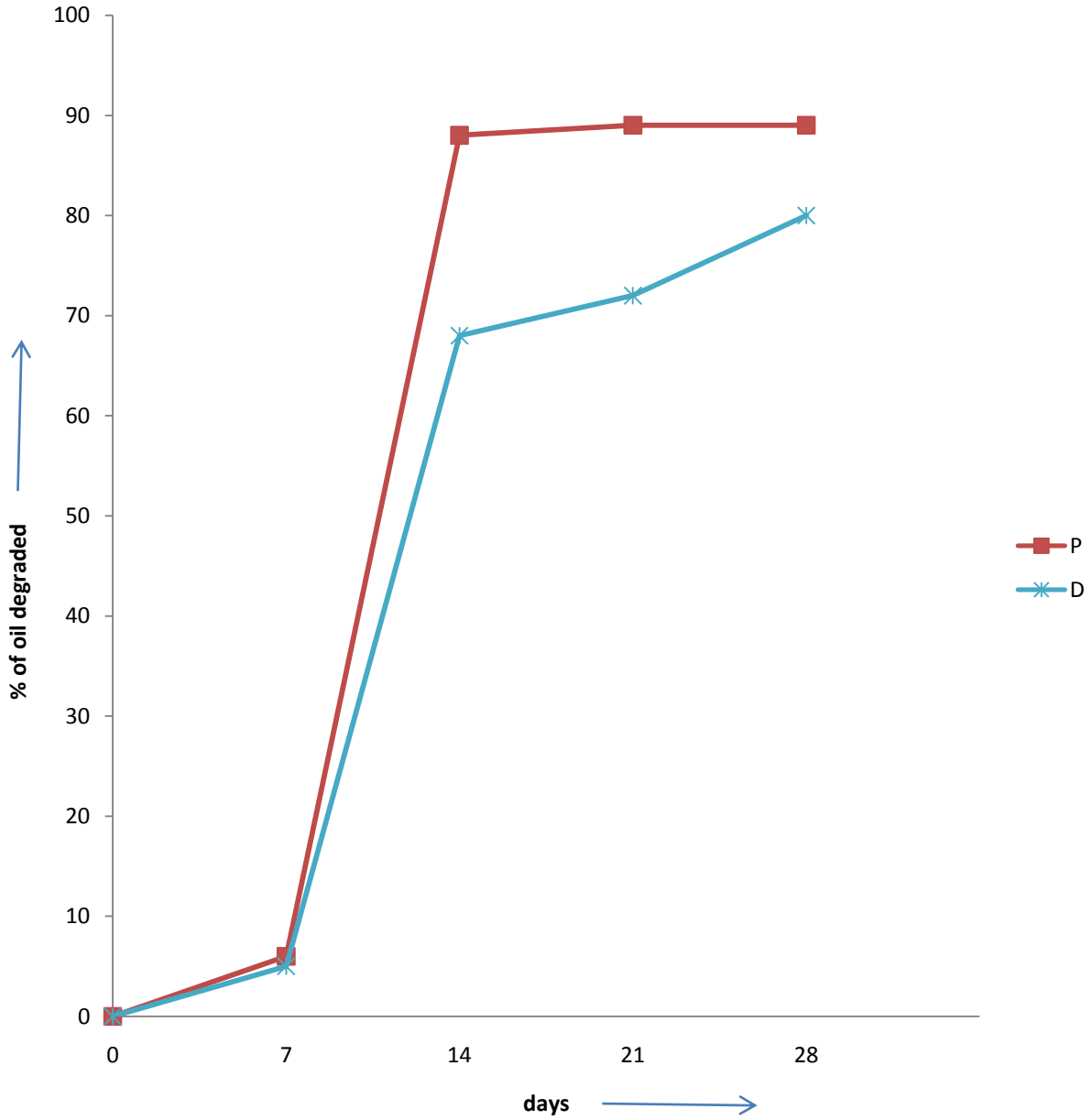
Figure 4.14 compares the trend in rate of degradation of oil in sample P and D when inoculated with Bacillus sp and Emulsified (B and E). There is a statistical difference ($P<0.05$) in the rate of degradation of oil between D and P.

Figure 4.15 compares the trend of degradation of oil when treated with Emulsifier, Fertilizer and inoculation with Bacillus sp (B, E and F) on sample P and D. There is a significant difference ($P<0.05$) in the trend of degradation between D and P even though the have almost the same percentages after 28days (97 and 95% respectively).



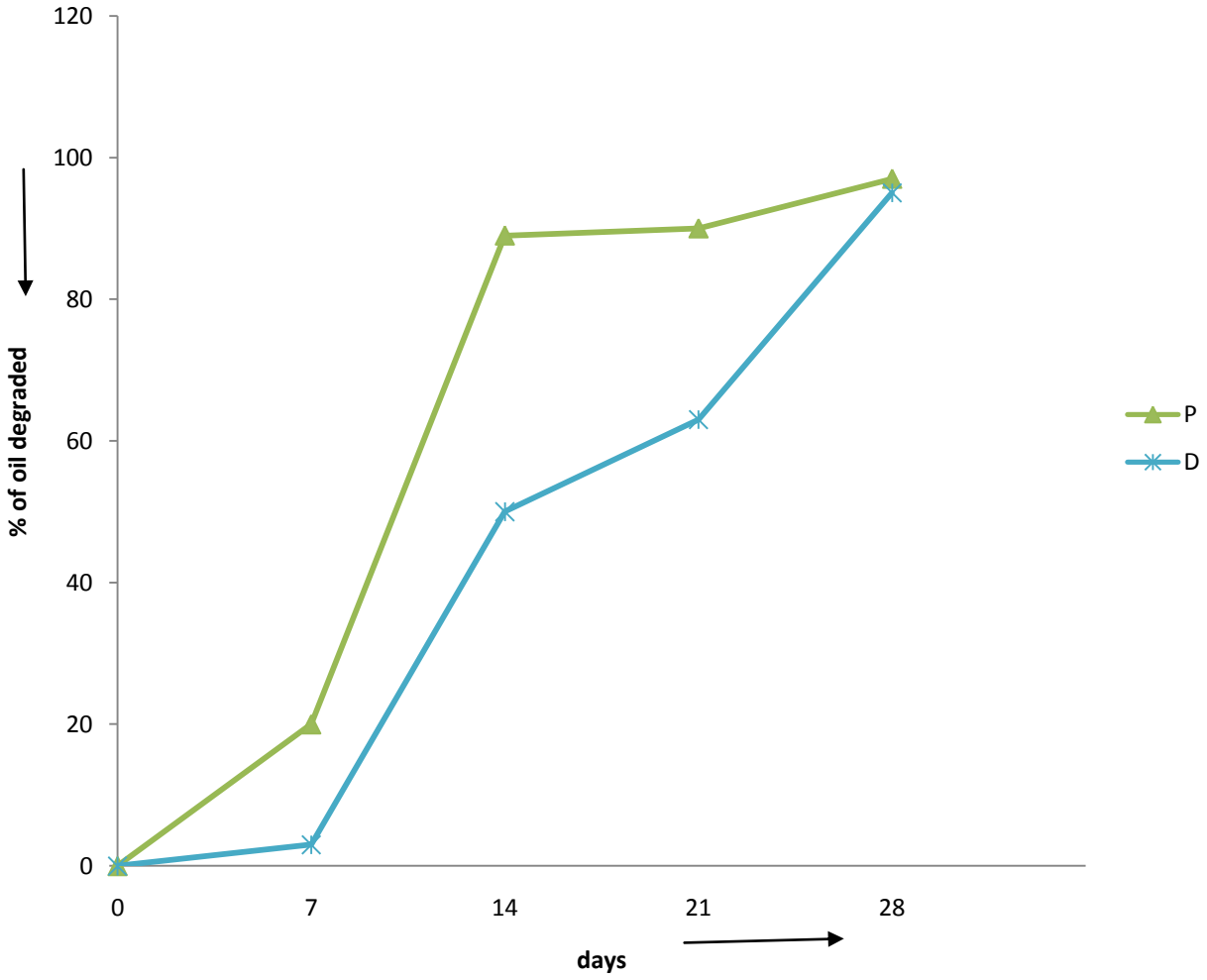
D = sample contaminated with diesel oil, P = sample contaminated with petrol

Figure 4.13 The rate of oil degradation with time from sample D and P when inoculated with *Bacillus Sp.*



D = sample contaminated with diesel oil, P = sample contaminated with petrol

Figure 4.14 The percentage of oil degraded (eliminated) using bacteria combined with emulsifier on sample D and P



D = sample contaminated with diesel oil, P = sample contaminated with petrol

Figure 4.15 The percentage of oil degraded (eliminated) using bacteria in combination with fertilizer and emulsifier.

Figures 4.16 and 4.17 are chromatograms of samples extract for sample D and P respectively. The chromatogram in Figure 4.16 (sample D chromatogram) has strong peaks 2, 3, 4, 5, 8, 9, 10, 11, 12, 13, 15 and 16 which correspond to the following compounds:3-Ethyl-3-methyldecane, 3,7-Dimethylnonane, 2,2-Dimethylpentane, sulphorus acid-2-ethylhexyl pentadecyl ester, 2-Bromo dodecane, 3,6-Dimethyldecane, n-Pentadecane, 2-Bromodecane, n-Heptadecane, n-Tetracosane, n-Heneicosane and n-Tetratetracontane repectively while the chromatogram in Figure 4.17 shows strong peak 6, 8, 10, 11, 20 and 22 which correspond to n-Pentadecane, n-Tetradecane, 2-Bromododecane, n-Octadecane, Glycerol-1,3-dipalmitate and 14-Methyl-8-hexadecenal respectively. This shows that D and P composed mainly of saturates (linear and brached alkanes).

4.3 Gram stain: After observing the stained colonies, the cells were discovered to be Gram positive, the shape was *Baccilus* and were arranged in clustered form. The biochemical test shows that the colonies are catalase positive, oxidase negative and they are motile.

GCMS-QP2010 PLUS
SHIMADZU,JAPAN

NARICT, ZARIA
GCMS ANALYSIS

ILIYA JEREMIAH (SAMPLE-D)

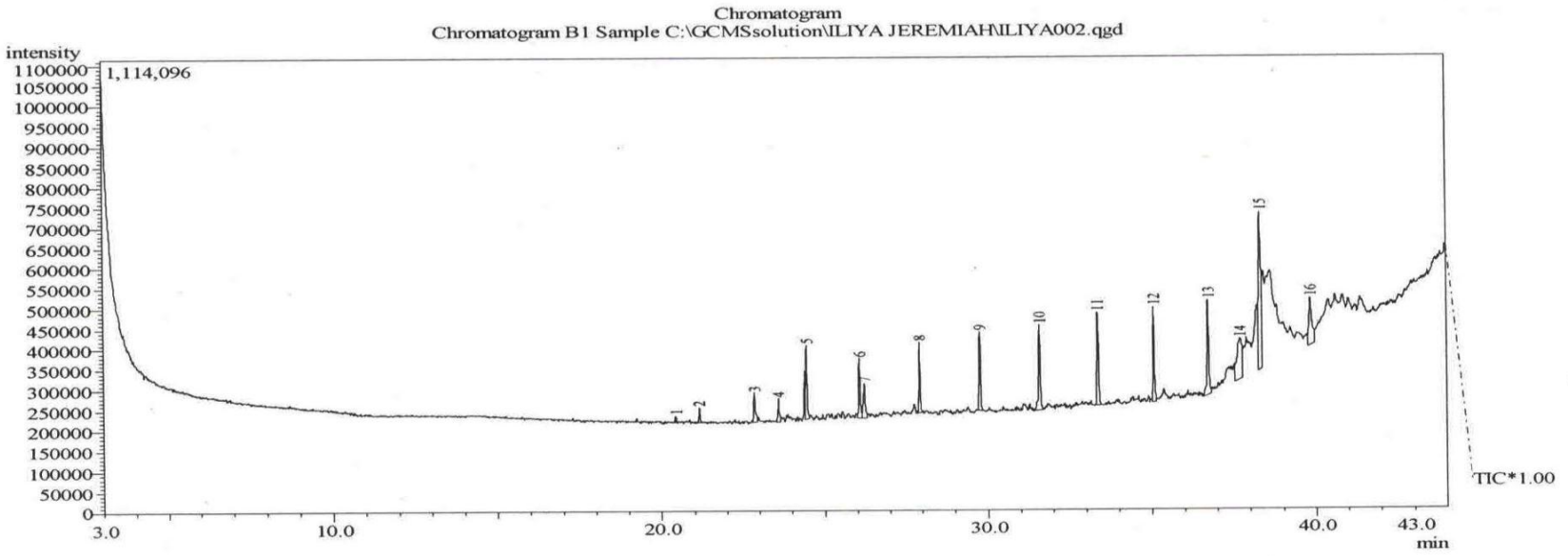


Figure 4.16 Chromatogram of Sample D Extract

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GCMS ANALYSIS

ILIYA JEREMIAH (SAMPLE-P)

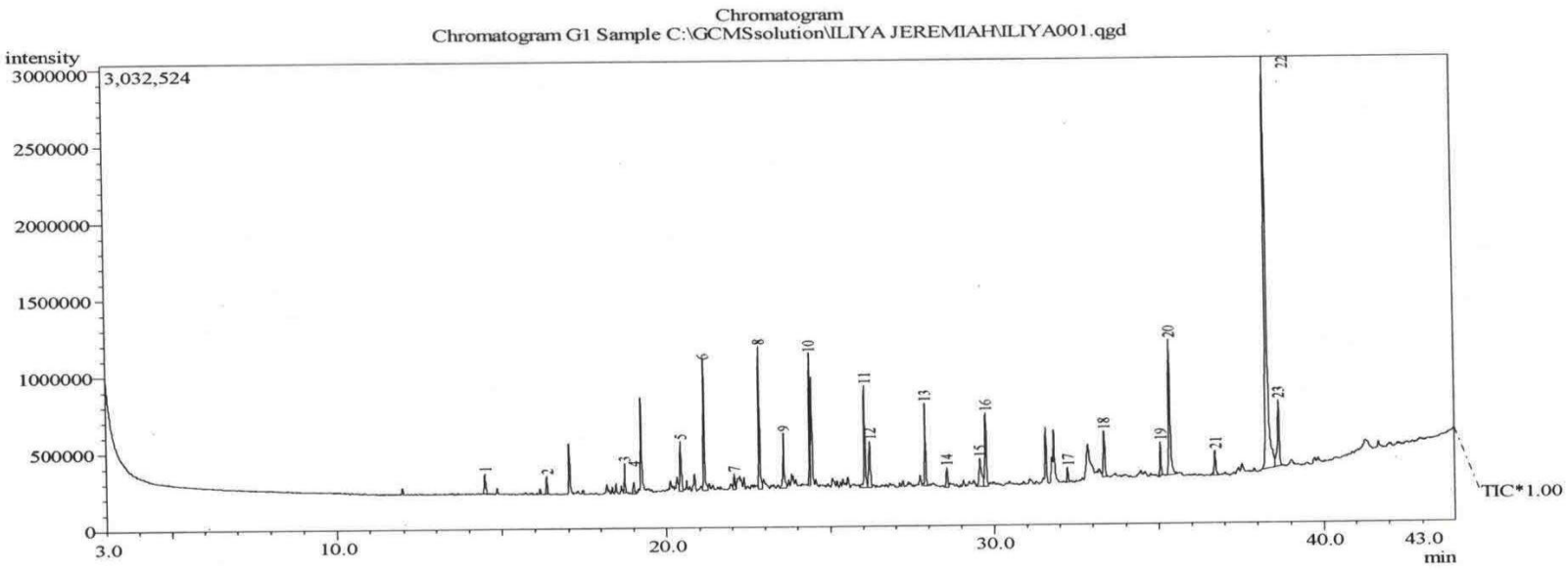


Figure 4.17 Chromatogram of Sample P Extract

CHAPTER FIVE

DISCUSSION

5.1 Physicochemical Properties of Soil

The results of the physicochemical analysis of the contaminated soils in Table 4.1 show that the textural content of the soil is sandy with a pH value ranging from 5.9- 6.2. This is consistent with the report of Onyeiwu (2008) who carried out a similar research on the same sample site. The pH range is optimum for bioremediation (Vidali, 2001) because it falls within the range 5.5-8.8 necessary for microbial growth and activities hence pH adjustment before treatment is not necessary. Studies have also shown that degradation of oil increases with increasing pH and that optimum degradation occurs under slightly alkaline conditions (Dibble and Bartha, 1979; Foght and Westlake, 1987). Vidali (2001) reported an optimum pH required for microbial activity to be 5.5 – 5.8. The pH range (5.2-5.8 and 5.8-6.5) for sample D and P respectively are both slightly lower than the 7.4 value reported by Achi *et al.* (2011). The reduction in pH value found in this study could be due to the increase in contaminant concentration and the nature of the contaminant.

The organic content in the samples were found to be averagely 28.30 and 37.00 g/kg for sample D and P respectively against the 14.67 g/kg value reported by Achi *et al* (2011) in his control site (a non oil contaminated site) within Kaduna metropolis and the CEC value of 30.0 and 32.0 mmol/kg for sample D and P respectively against the reported value of 13.07 mmol/kg (Achi *et al.,* 2011). The high percentage organic carbon content in the study sites is probably due to spillage of oil into the sites, which consist mainly of hydrocarbon (Akoto *et al.,* 2008). The CEC of soils is related to the nature and quality of soil and organic carbon contents. Hence, high percentage organic carbon from these sites could be responsible for the high CEC value. According to Awode *et al*.(2008), CEC of soil is more greatly influenced by organic matter,

hence CEC tends to be higher in the study sites than in the control sites. All study sites had comparatively higher mean cation exchange capacity than the mean CEC of 8.14 mmol/kg reported by Isirimah (1987). The samples have average moisture content of 0.5 and 1.25% for sample D and P respectively and higher CEC usually indicates that more organic matter is present in the soil, high CEC soils generally have greater water holding capacity than low CEC soils this is consistent with the results in Table 4.1 (high CEC in P show also high % moisture content when compared with sample D). Soils with a low CEC are more likely to develop deficiencies in potassium (K^+), magnesium (Mg^{2+}) and other cations The addition of organic matter will increase the CEC of a soil but requires many years to take effect (CUCE, 2007). Sample P has higher CEC thus higher concentration of cations (Ca^{2+}, Mg^{2+}, K^+ and Na^+ with values 3.6, 1.17, 0.50 and 0.22 mol/kg respectively) while sample D with a lower CEC has a lower concentration of cation (Ca^{2+}, Mg^{2+}, K^+ and Na^+ with values 1.20, 0.27, 0.25 and 0.17 mol/kg respectively). This implied that soil mineral concentration is affected by discharge of hydrocarbons to the soil. The concentration of total nitrogen in the samples was found to be 3.30 and 10.50 mg/kg for sample D and P respectively. The concentration of phosphorus was found to be 1.33 and 11.36 mg/kg for sample D and P respectively. These provide adequate nutrient level for the microbial growth. The high concentration of nitrogen and phosphorus in sample P is capable of enhancing the natural degradation of sample contaminant since P and N are nutrient to microbes the biostimulate the existing indigenous organisms.

5.2 Gram stain: After observing the stained colonies, the cells were discovered to be Gram positive, the shape was *Baccilus* and were arranged in clustered form. The biochemical test shows that the colonies are catalase positive, oxidase negative and they are motile. Showing that its bacillus strain

5.3 Degradation of oil (contaminant) by Natural processes.

Figure 4.1 shows the rate of degradation of soil contaminant (oil) with time without treatment. There are lot of natural degradation processes taking place on organic compounds pollutants which include evaporation, degradation by indigenous microorganisms etc. The results show that after 7days 3% of the oil was degraded and after 14days 58% was degraded after which degradation process reduced until no significant further degradation was noticed. This could be due to complete utilization of existing nutrients in the soil required for microbial growth and activities and/or decrease in microbial population. Because of the high carbon content of oil and the low level of other nutrients essential for microbial growth, the rate and extent of degradation are, in general, limited by the low availability of nitrogen and phosphorus (Leys *et al.*, 2005). Obere and Nwaubeta (2002) reported a decline in microbial population as biodegradation progresses especially when the light hydrocarbons are exhausted.

5.4 Effect of inoculation with *Bacillus sp* (B) only (bioaugmentation).

Figure 4.2 (sample P treatment) shows that 7% of the oil has been degraded and this is slightly higher than the control this shows the effect of increasing microbial population, 14days after inoculation the concentration increased to 60%. This further increased to 81% after 28days of inoculation. Similar trend was obtained in sample D (Figure 4.3), 88% of the oil was degraded in just 14days and after 28days it increased to 96% this indicates that the rate and extend of degradation of oil in sample D is higher than in P, this could be as a result of the nature of the oil. In both P and D the period in which the highest concentration of oil degradation is the first 14days. This is consistent with the report of Abdulatif *et al* (2015), that the highest

hydrocarbon concentration reduction occurred within the first 15 days of treatment or inoculation with microbes.

5.5 Effect of Adding Fertilizer (F) Only (Biostimulation)

From Figure4.4 addition of NPK fertilizer to the sample (D) resulted in 20% degradation of the contaminant after 7days. After 14 days 56% was degraded from the samples this is even below the control value and the next 14 days only yielded 3% degradation of the contaminant. This could be as a result of inhibition of microbial activities by the fertilizer. When a major oil spill occurs the supply of carbon is dramatically increased and the availability of nitrogen and phosphorus generally becomes the basic factor for oil degradation (Atlas, 1984; Leahy and Colwell, 1990). However the effect of adding nutrient to soil rich in nutrients may yield an insignificant result as shown in the graph ($P > 0.05$) it will inhibit degradation of hydrocarbons by microbes as reported by Padayachee and Lin (2011) that fertilizer supplementation had an inhibitory effect on the microbial population in contaminated sea sand. Most nitrogen fertilizers are composed of highly water soluble nitrate and/or ammonium salts which quickly dissolve into soil pore water. This increases the salt concentration of the soil solution and lowers the soil osmotic potential (the portion of the soil water potential energy attributable to dissolved solutes), which can inhibit microbial activity. An osmotic potential drop of 0.50MPa can reduce microbial petroleum degradation by roughly 50% (Braddock *et al.*, 1997; Walworth *et al*., 1997). In this research after 28 days of adding only fertilizer to the sample, only 59% of the oil concentration was degraded slightly below the control (60%). This indicates that there was a reduction in degradation process when only fertilizer was added to the sample which could be as a result of its negative effect on the indigenous microbes.

5.6 Effect of Emulsification (E) of Sample on Degradation of its Contaminant (Oil)

One of the important factors that limit biodegradation of oil pollutants in the environment is their limited availability to microorganisms. Adding detergent to the oil contaminated soil helps in desorbing the hydrocarbon and also decreases interfacial surface tension (Fiechter, 1992; Georgiou *et al*., 1992;Rouse *et al*., 1994 ; Lin,1996; Shafi and Khanna, 1995; Volkering *et al*.,1998;Karanth *et al*., 1999). This fact was substantiated in results obtained when 0.25 ml of emulsifier was applied on the sample as seen in Fig.4.5. When emulsifier was added, 13% of the oil was degraded after 7days. This increased to 61% and 65% after 14 and 21 days respectively. This high percentage degradation was obtained though not higher than when inoculation was done but still make a significant ($p < 0.05$) effect on the degradation of the contaminant. Similar result was obtained in sample P (Figure 4.6) where 3%, 74%, 88% concentration was degraded after 7, 14 and 21 days respectively which is even higher than obtained in sample D. This could be due to the slight difference in the composition of the oil (contaminant) and their different responses to the emulsifier.

5.7 Effects of combining Bacteria inoculation and Emulsification (B and E) on soil contaminant

Oils are hydrophobic in nature, their availability to bacteria are limited which leads to the slow degradation because petroleum hydrocarbon compounds bind to soil components, and they are difficult to be removed or degraded (Fiechter, 1992; Georgiou *et al*., 1992;Rouse *et al*., 1994 ; Lin,1996; Shafi and Khanna, 1995; Volkering *et al*.,1998;Karanth *et al*., 1999). Therefore, the essence of emulsification before inoculation is to increase the availability of oil particles to

enable microbes in the soil degrade the contaminant (oil). This is shown in Figures4.7 and 4.8. In Figure4.7 the result shows that after inoculation with bacteria and adding fertilizer on sample D, 5% of the oil was degraded, 14 days after the concentration increased to 68% which shows a progression in degradation throughout the 28days of treatment. A similar trend of progression in degradation was obtained in sample P as shown in Figure 4.8 where 89% of the oil was degraded after 28days of treatment and the trend shows a progression in degradation as long as oil particles are available for microbes.

5.8 Effect of Emulsification and Addition of Fertilizer (E and F)

Figure 4.9 shows the percentage of oil degraded with time when samples are emulsified and NPK fertilizer is added to sample D. After 7 days of treatment 12% of the contaminant concentration was degraded, this increased to 56% after 14 days, which further 63 and 77% after 21 and 28 days respectively. This gave a higher percentage compared to when only fertilizer was applied. This indicates that effect of inhibition of degradation of oil by nutrients (Padayachee and Lin, 2011) can be suppressed by emulsification.

5.9 Effect of inoculation with bacteria and adding fertilizer (B and F)

The specific inhibitory effects of excess nitrogen include an increased lag phase (Huntjens *et al.*, 1986; Ferguson *et al.*, 2003) and preferential inhibition of aromatic degradation (Fayad and Overton, 1995). Genouw *et al.* (1994) found that addition of 4000 mg N kg^{-1} of soil inhibited microbial degradation of an oil sludge. This report is substantiated in the result shown in Figure 4.10 when fertilizer was added and bacteria were inoculated on sample D. Within 7 days of treatment 23% of the contaminant was degraded which happened to be the highest

concentration degraded in the first 7days which could be as a result of bioaugmentation of the microbes in the soil, this increased to 45%, 62%, 64% after 7days interval which shows that after the first two weeks of applying bacteria and adding fertilizer a reasonable concentration was degraded and after which no further degradation occurred. This may be attributed to inhibition of microbial activities in the soil contaminant by the high concentration of NPK fertilizer (1g/200g of soil) which even contained less concentration of nitrogen compared to the concentration (4000 mg N kg^{-1} of soil) reported by Genouw *et al.* (1994) .

5.10 Effect of Combining Emulsifier, Fertilizer and Bacillus sp (B, E and F)

The microbes present in the soil first recognize the oil and its constituents by biosurfactants and bio emulsifiers, and then they attach themselves and use the hydrocarbon present in the petroleum as a source of energy (NRC, 2012) The low solubility and adsorption of high molecular weight hydrocarbons limit their availability to microorganisms. The addition of biosurfactants enhances the solubility and removal of these contaminants, improving oil biodegradations rates (Oil Spill Intelligence Report, 1997). The application of urea resulted not only in a rapid multiplication of bacteria but also accelerated the disappearance of the hydrocarbons and known oil-degrading bacteria are added to supplement the existing microbial population (Garg *et al*., 1994; Prince, 1993). The observation above was substantiated in Figure 4.11 and 4.12. Figure 4.11 (sample D) showed that 3% of the oil concentration was degraded after 7days of treatment with B, E and F and 14days of treatment 59% was degraded, after 21day this increased to 63% which further increased to 95% after 28days. This shows a progression in degradation of the oil. While in Figure 4.12 (sample P), a similar trend was found. 20% of the oil was degraded after 7days, 89% was degraded after 14days which is very

high compared to the concentration obtained in sample D (Figure 4.11), after 21days 90% was obtained and after 28 days from the time of applying B, E and F, 97% of the oil was degraded which is the most effective. In both sample D and P a very high percentage of almost 100% degradation of the contaminant was achieved within just about 30days. It indicates that when microbial population is increased and the emulsification of the oil is done with increase in nitrogen and phosphorus ratio through addition of NPK a large volume of the contaminant will be eradicated within 28 days. Abdulatif *et al.* (2015) reported that after 30 days of treatment, total petroleum hydrocarbon (TPH) concentration had decreased significantly.

5.11 Effect of Oil concentration and composition (nature of contaminant) of the sample.

The average concentration of the oil from the samples collected was determined and found to be 398 and 194g/kg for sample D and P respectively before treatment and these values are high. Onyeiwu (2008) reported 320-868 mg/l in 2007. The maximum concentration of oil allowed for discharge into inland water by the National guidelines and standards for pollution abasement in industries in Nigeria is 10mg/l (FEPA, 1991).

The low concentration of oil (194g/kg) in P when compared with sample D (398g/kg) makes oil degradation in sample P faster (high slope) as shown in Figure 4.13 – 4.15 since degradation of oil is affected by volume, density and viscosity of oil (Atlas, 1984; Leahy and Colwell, 1990). This is also shown in their trend of degradation. Figure 4.16 and 4.17 is Chromatograms of sample D and P extract respectively. The chromatogram in figure 4.16 shows strong peak 2, 3, 4, 5, 8, 9, 10, 11, 12, 13, 15 and 16 which correspond to the following compounds: 3-Ethyl-3-methyldecane, 3,7-Dimethylnonane, 2,2-Dimethylpentane, sulphorus acid 2-ethylhexyl pentadecyl ester, 2-Bromo dodecane, 3,6-Dimethyldecane, n-Pentadecane, 2-Bromodecane, n-

Heptadecane, n-Tetracosane, n-Heneicosane and n-Tetratetracontane repectively while the chromatogram in Figure 4.17 shows strong peak 6, 8, 10, 11, 20 and 22 corresponding to n-Pentadecane, n-Tetradecane, 2-Bromododecane, n-Octadecane, Glycerol-1,3-dipalmitate and 14-Methyl-8-hexadecenal respectively. The hydrocarbons present mainly are linear and branch alkanes (saturates).

Petroleum hydrocarbons contain a complex mixture of compounds; all the components of petroleum do not degrade at the same rate. The rate by which microorganisms degrade hydrocarbons depends upon their chemical structure and concentration. Petroleum hydrocarbons can be categorized into four fractions: saturates, aromatics, resins and asphaltene. Of the various petroleum fractions, n-alkanes of intermediate length (C_{10}-C_{25}) are the preferred substrates for microorganisms and tend to be the most readily degradable, whereas shorter chain compounds are rather more toxic. Longer chain alkanes (C_{25}-C_{40}) are hydrophobic solids and consequently are difficult to degrade due to their poor water solubility and bioavailability, and branched chain alkanes and cycloalkanes are also degraded more slowly than the corresponding normal alkanes. (Balba *et al.*, 1998; Loeher *et al*., 2001; Ivančev-Tumbas *et al*., 2004; Brassington *et al*., 2007; Stroud *et al.*, 2007).

Microbial degradation of crude oil or oil waste depends on a variety of factors such as the physical conditions, nature, concentration and ratios of various structural classes of hydrocarbons present, the bioavailability of the substrate, and the properties of the biological system involved (Winter *et al*,1993; Ko and Lebeault,1999, Sugiura *et al.*,1997; VanHamme and Ward,1999; Yuste *et al.*,2000). A generalized sequence of petroleum components in order of decreasing biodegradability is represented as follows (Huesemann,1995): *n*-alkanes > branched-chain alkanes > branched alkenes>low-molecular-weight *n*-alkyl aromatics

>monoaromatics> cyclic alkanes > polynuclear aromatics > asphaltenes with high molecular weight aromatics and polar compounds being extremely difficult to degrade. Since the contaminants are mainly saturates, degradation of contaminants will be easier and faster this accounts for the high percentage oil degraded in both samples P and D. However the composition of microbial community, abiotic factors etc, can alter the order of biodegradation greatly (Das and Chandran, 2011; Obuekwe , 2001).

The trend shown in figures 4.13, 4.14 and 4.15 is a clear indication that there is significant difference ($P < 0.05$) in rate of degradation of oil in both samples D and P. This may probably be due physical property factor difference between P and D (though both samples have mainly the same chemical composition) since degradation of oil is also affected by volume, density and viscosity of oil (Atlas, 1984; Leahy and Colwell, 1990).

CHAPTER SIX

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

The physical state of petroleum hydrocarbons has marked effect on their biodegradation and their degradation from oil spills seem to be highly dependent on localized environmental conditions. The ability of microorganisms to degrade hydrocarbons is being taken advantage of in major bioremediation efforts. Seeding with microbes (B), adding nutrients (F), emulsification (E), a combination of bacteria and emulsifier (B and E), combination of bacteria and fertilizer (B and F), combination of emulsifier and fertilizer (E and F), a combination of bacteria, emulsifier and fertilizer (B, E and F) have all been used in bioremediation efforts in this research for 28days. The most convincing demonstration of bioremediation efficiency resulted from combination of bacteria, fertilizer and emulsifier (B, E and F) on the contaminated soil having the highest percentage of oil degraded or eliminated (97% and 95% for sample P and D respectively) followed by bacteria inoculation (B) only on the contaminated soil(96%). The analysis also reveals that bioremediation of the oil contaminated soil became effective after a minimum of 14 days for each sample set.

6.2 Conclusion

Based on the finding of this research (within the limit of experimental error and estimations) it was concluded that a better result of bioremediation can be achieved by improving on the conventional method of bioremediation (bioaugmentation and biostimulation) when emulsifier is added. The trend in degradation is given thus: (B, E and F) > B > (B and E) > (E and F) > (B and F) > E > F. This research also showed that a noticeable degradation occurred after 14 days of treatment.

6.3 Recommendations on further studies

i. The importance of anaerobic biodegradation of oil in the environment requires further studies.

ii. More studies are required to understand the effect of salinity on oil biodegradation

iii. Investigate the potential of bioremediation in treating buried oil.

iv. Further research is still required to determine the effectiveness of bioremediation in fine sediments

v. The effect of climate change on oil degradation.

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APENDICES

Apendix A Percentage oil degraded with time in sample D

Days	F	B	F&B	B&E	B,E &F	E&F	E	C
7	20	7	23	3	5	12	13	3
14	56	88	45	68	50	56	61	58
21	58	94	63	72	63	63	64	59
28	59	95	64	73	95	77	65	60

Appendix B Percentage of oil degraded with time in sample P

DAYS	B	E	B & E	B, E & F	C
Day 7	7	2	6	20	3
Day 14	60	73	88	84	58
Day 21	64	88	89	90	59
Day 28	81	89	89	97	60

Appendix C The percentage (%) of oil degraded (eliminated) from the samples (D and P) using bacteria combine with emulsifier

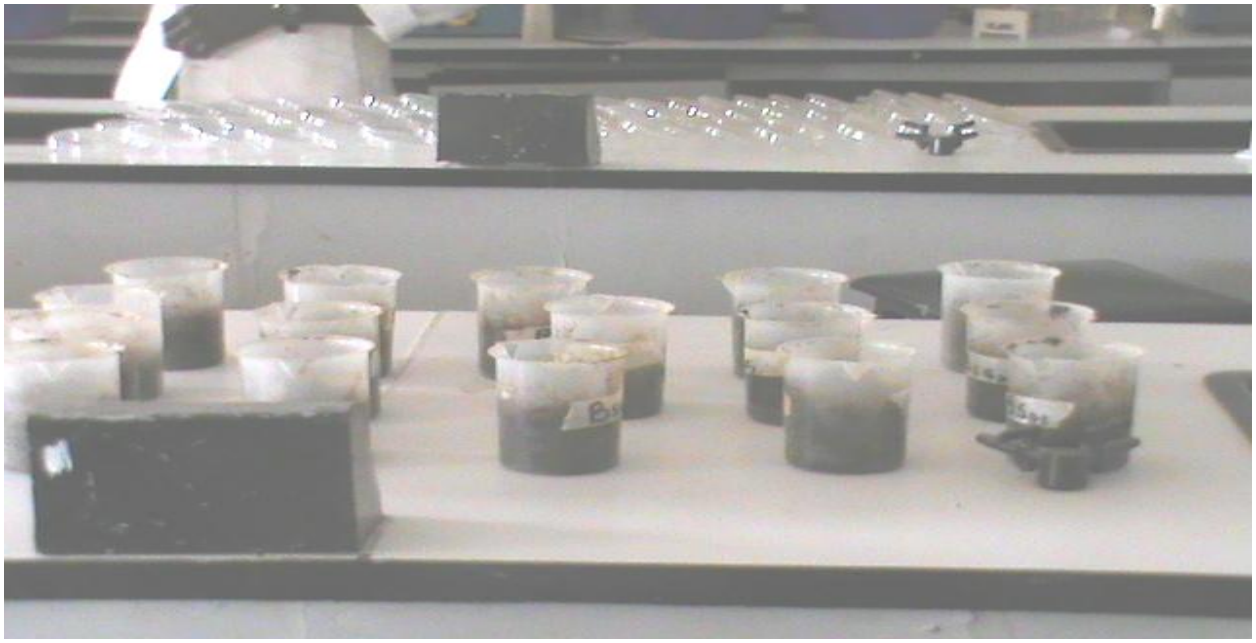
Days	P	D
7	6	5
14	88	68
21	89	72
28	89	80

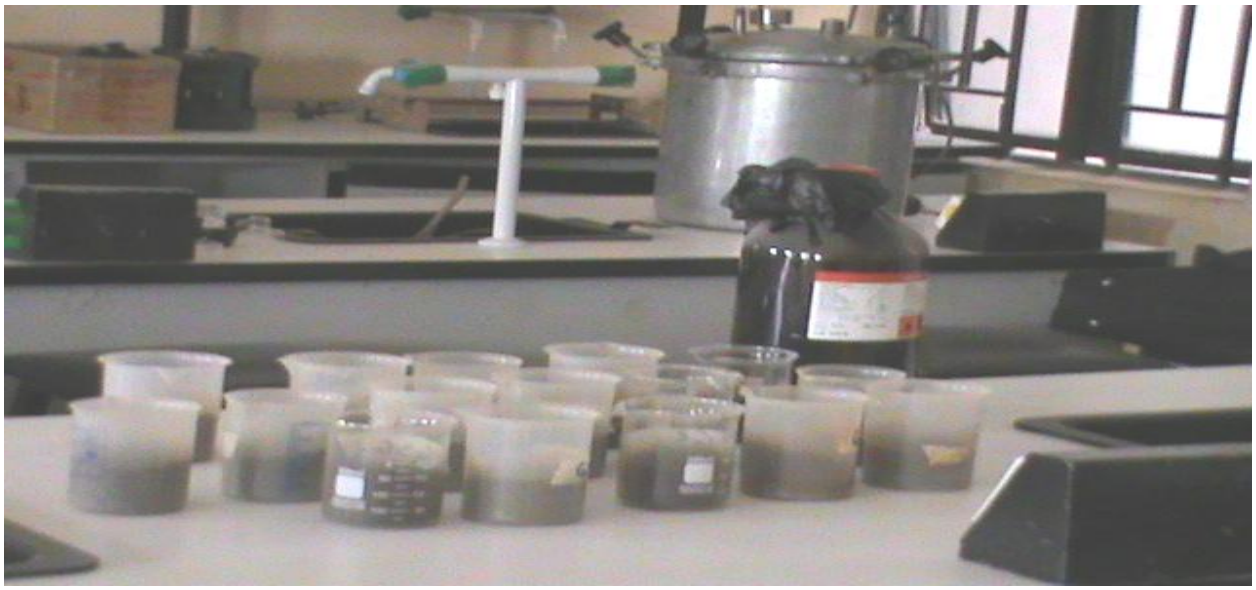
Appendix D The percentage (%) of oil degraded (eliminated) using bacteria only on D and P.

DAYS	P	D
Day 7	7	7
Day 14	60	88
Day 21	65	94
Day 28	81	95

Appendix E The percentage (%) of oil degraded (eliminated) from the samples (P and D) using bacteria combine with fertilizer and emulsifier

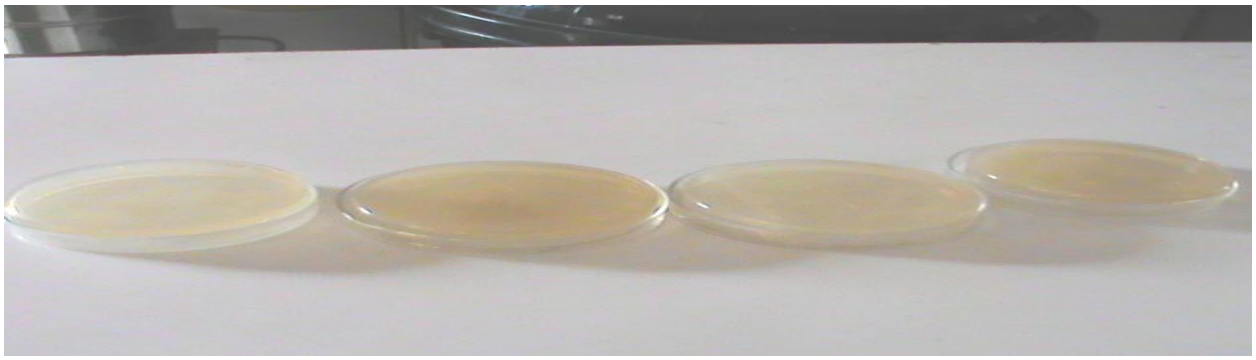
DAYS	P	D
7	2	3
14	89	50
21	90	63
28	97	95





Appendix F Sample sets





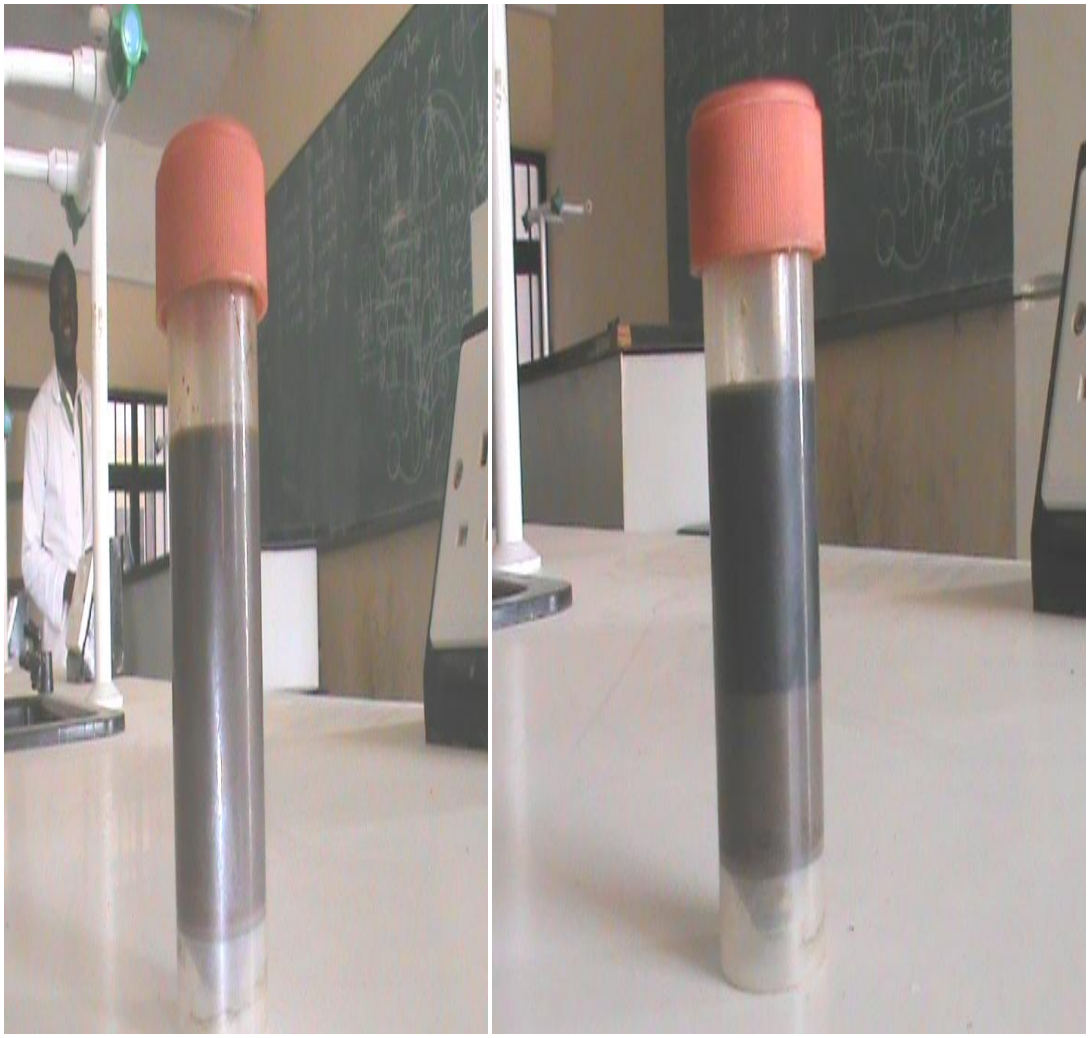


Appendix G **Incubated isolate and nutrient broths**

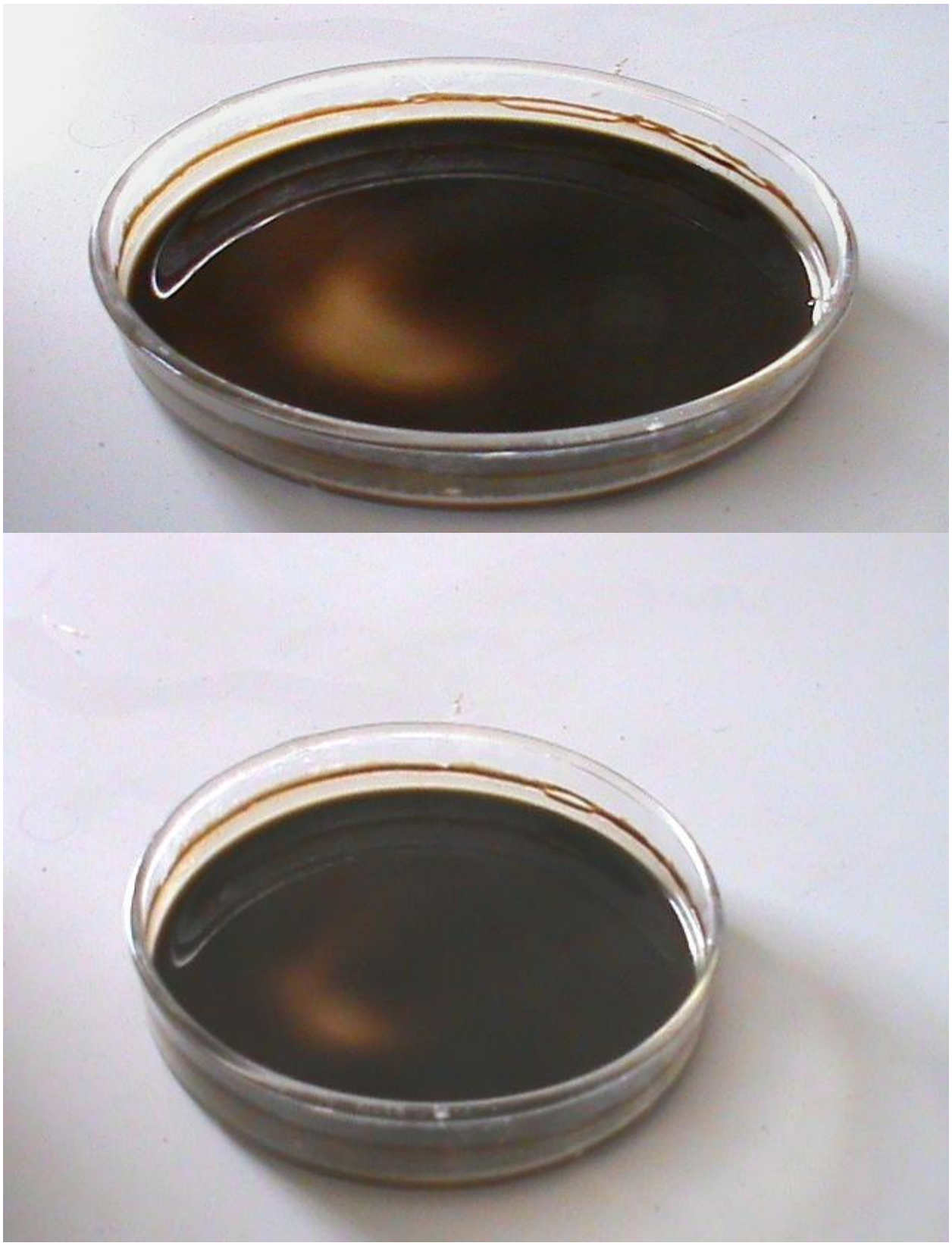




Appendix H Soil samples.



Appendix I Mixture of oil, solvent and soil forming two layers.



Appendix J Extracted oil exposed for solvent to evaporate.





Appendix K Plastic petri-dishes for extracting oil.



Appendix L Set of samples being prepared.

Apendix M. Statistical Analysis: T-test result comparing the trend in the degradation of oil in sample P and D

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	BP	53.250000	4	32.1078910	16.0539455
	BD	71.000000	4	42.7784993	21.3892496

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	BP & BD	4	.973	.027

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	BP - BD	-17.7500000	13.6717470	6.8358735	-39.5048004	4.0048004	-2.597	3	.081

Where BP is result obtained when Bacteria is applied on sample P,

While BD is result obtained when Bacteria is applied on sample D.

P = 0.081 / 2 = 0.041, P < 0.05.

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	BEP	68.000000	4	41.3360214	20.6680107
	BED	56.250000	4	34.5289733	17.2644867

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	BEP & BED	4	.991	.009

Paired Samples Test

	Paired Differences					t	df	Sig. (2-tailed)
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
				Lower	Upper			
BEP - BED	11.7500000	8.5391256	4.2695628	-1.8376544	25.3376544	2.752	3	.071

Where BEFP is result obtained when Bacteria and Emulsifier are applied on sample P,

While BEFD is result obtained when Bacteria and Emulsifier are applied on sample D.

$P = 0.071 / 2 = 0.036$, $P < 0.05$.

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	BEFP	74.000000	4	36.1754982	18.0877491
	BEFD	52.750000	4	38.1783097	19.0891549

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	BEFP & BEFD	4	.913	.087

Paired Samples Test

	Paired Differences					t	df	Sig. (2-tailed)
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
				Lower	Upper			
Pair 1 BEFP - BEFD	21.250000	15.671099	7.8355493	-3.6862150	46.1862150	2.712	3	.073

Where BEFP is result obtained when Bacteria, Emulsifier and Fertilizer is applied on sample P

While BEFD is result obtained when Bacteria, Emulsifier and Fertilizer is applied on sample D

$P = 0.073 / 2 = 0.036$, $P < 0.05$.