KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

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DEPARTMENT OF ENVIRONMENTAL SCIENCE

EFFECTS OF POULTRY DROPPINGS, COMPOST & NATURAL RUBBER SLUDGE ON BIOREMEDIATION OF PETROLEUM HYDROCARBON CONTAMINATED SOIL

A Thesis submitted to the Department of Environmental Science of the Kwame Nkrumah University of Science and Technology in partial fulfilment of the requirement for the Award of Master of Science Degree in Environmental Science.

By

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DECLARATION

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

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ABSTRACT

This study aimed at using bioremediation, as the primary method of decontamination for a soil contaminated with waste petroleum oil in a mining area. The soil was obtained from Adamus Resources Ltd (ADL), a mining company located in the Western Region of Ghana. The experiment was carried out in the Laboratory using three levels of nitrogen concentrations of 0.2%, 0.4%, and 0.6% from three different organic nutrient supplements namely: Compost(C), Poultry droppings (PD), and natural Rubber processing sludge (RS). A baseline study was carried out on the soil as well as the three organic nutrient supplements and the result of the baseline study shows that the soil contains only 0.03% nitrogen and 0.12% phosphorus with a C: N ratio of 80:3 indicating that the soil lacks the required level of nitrogen for optimum bioremediation to take place. Approximately 1kg composite samples of the oil contaminated soil were placed in plastic containers and were then treated with 0.2%N C, 0.2%N PD, 0.2%N RS, 0.4%N C, 0.4%N PD, 0.4%N RS, 0.6%N C, 0.6%N PD, 0.6%N RS and the one without nutrient supplement serving as the control. During the eight weeks incubation period, the pH, microbial counts, TPH, as well as nutrient levels were monitored periodically. At the end of the eight weeks of incubation, 95.41%, 99.06%, 93.53%, 91.82%, 92.13%, 90.92%, 83.17%, 81.33%, 86.55 % of the 1009.12 mg/kg initial TPH concentration in the contaminated soil, were degraded in the soil samples treated with 0.2%N C, 0.2%N PD, 0.2%N RS, 0.4%N C, 0.4%N PD, 0.4%N RS, 0.6%N C, 0.6%N PD, 0.6%N RS respectively and these were significantly higher at $p < 0.05$ relative to the 50.20% recorded by the control soil. It was evident that the addition of the organic nutrient supplements positively aided the biodegradation of the petroleum hydrocarbons in the soil. Among the different N concentrations, the 0.2 % N concentrations recorded the best results in all the three organic nutrient supplements and the poultry droppings was the best among the other amendment materials.

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CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Advances in science and technology since the industrial revolution has increasingly enabled human to exploit natural resources. However, this has generated unprecedented disturbances in global elemental cycles (Trabalka & Reichle, 1986). The relatively sudden introduction of xenobiotic chemicals or the massive relocation of natural material to different environmental compartments can often overwhelm the self cleaning capacity of recipient ecosystems and thus result in the accumulation of pollutants to problematic or even harmful levels. In addition to minimising the impact of future incidents by controlling contaminant input, pollutant decay should be accelerated to remedy existing problems (Bartha, 1986).

The production, processing, storage, transportation, as well as unintentional spillage of crude oil and petroleum distillates has contributed to the release of hydrocarbons into the environment. Crude oil is a naturally occurring complex mixture of hydrocarbon and non hydrocarbon compounds which possesses a measurable toxicity towards living systems (Nelson-Smith, 1973). The increase in demand for crude oil as a source of energy and as a primary raw material for industries such as the mining industries has resulted in an increase in its production, transportation and refining, which in turn has resulted in gross pollution of the environment (Gutnick and Rosenberg, 1977). The single largest source of petroleum pollution is routine, low-level discharge such as urban runoff, cleaning operations, and oil treatment of roads for dust control by mining companies. These sources together account for 90% of total anthropogenic petroleum pollution (Bartha, 1986). This has resulted in large

number of polluted sites and enormous volumetric quantities of soil, which have been contaminated with hazardous substances. Soil contamination can cause extensive damage to the local ecosystem by accumulating in the tissue of animals and plants and by causing death or mutation to the progeny thereof. Such contamination can also present a serious health threat to humans, and, in extreme cases, can render the contaminated area unsuitable for agriculture and human habitation (Riffaldi *et al.*, 2006).

1.2 Statement of Problem

The use of heavy machines by mining companies requires the use of a lot of petroleum products. Waste oil contamination of soil in mining sites occurs in all mining companies and Adamus Resources ltd, Ghana's newest Gold builder as they call themselves is no exception. The hydrocarbon-contaminated soils are generally treated by secure landfill, incineration, indirect thermal treatment, aeration, vacuum extraction, soil rinsing with organic solvents, and conventional bioremediation (Alexander, 1999). These physico-chemical methods may be prohibitively expensive due to the relatively large volumes of contaminated soil usually involved. Bioremediation is an alternative approach where biological organisms are used for converting the chemical pollutants to less toxic or non-toxic compounds (Ellis, 2000). Biodegradation of chemical pollutants often depend on indigenous and/or augmented micro-organisms (such as bacteria and fungi) to utilise the organic pollutants as food (carbon) source (Walworth *et al.*, 2006). Conditions for bioremediation are optimized by soil water conditioning, aeration, pH, temperature, and nutrient addition. Many works have reported the use of inorganic fertilizers to stimulate indigenous microbial population/activity in soil contaminated with petroleum hydrocarbons (Okolo *et al*,

1993). But these inorganic fertilizers have their own side effects on the soil microbes and groundwater. There is therefore the need to explore organic sources of nutrients for the bioremediation process.

The processing of natural rubber latex into intermediate products such as rubber sheets, crumb rubber and others generates large volumes of wastewater with pollution potential and large deposit of sludge in the waste pit. The Ghana Rubber Estates Ltd (GREL) produces such natural rubber processing sludge in volumes and its disposal is the company's number one problem. The bad odour from the rubber sludge is causing air pollution and even passengers in vehicles passing by always complained of this. Laboratory analysis of the rubber sludge shows that it contains a lot of nutrients such as N, P, S, etc and can therefore be a good source of nutrient for bioremediation. This study seeks to compare the effect of poultry droppings, compost, and natural rubber processing sludge on the degradation of petroleum hydrocarbons in soil.

1.3 Objective of the Study

The main aim of this study is to assess the potential of poultry droppings, compost, and natural rubber processing sludge as sources of nutrient to enhance the rate of biodegradation of petroleum hydrocarbons in oil contaminated soils.

1.4 Specific Objectives

Specifically, this study seeks to:

i. Establish the baseline concentration levels of the contaminated soil as well as the nutrient (N, P, K) levels of the amendment materials.

ii. Determine the right quantities of each of poultry droppings, compost and the rubber sludge required to degrade a given amount of petroleum hydrocarbons in a soil.

iii. Determine TPH content, pH, Bacterial count, Nitrogen and Carbon content during the degradation process.

1.5 Justification of the Study

As a result of the recent agitation by the youth of the mining areas in the western region of Ghana against the operations of the mining companies due to the gross pollution of lands and water bodies in their communities, it is expedient that more research is carry out on the operations of these mining companies. The operation of Adamus Resources limited, a new mining company in the Nzema Areas of the Western Region of Ghana is one of such companies whose waste management practices needs to be looked into. It is a known fact that mining operations involves the use of heavy vehicles which run on petroleum products. During oil change and other maintenance activities, waste oil usually end up in soil and then to water bodies thereby polluting the environment. This happens to be one of the problems facing ARL and the only way they treat their oil contaminated soil is by volatilizations. It is therefore imperative that a better, cheaper and a more environmentally friendly method like bioremediation is experimented and apply to the soil in the field hence this study.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Sources of Soil Contamination

Modern society depends on a variety of synthetic chemicals, and the amount of these chemicals used is huge. Many chemicals, produced on a large scale as parts of the normal activities of industrialized societies, are considered hazardous to humans, plants and animals. The application of highly sensitive analytical techniques to environmental samples has provided society with disturbing information: the air, the water, and the soil are contaminated with a variety of synthetic chemicals. Thus, contamination of soils, sediments, waters, and air with hazardous and toxic chemicals is one of the major problems facing the industrialized world today (Alexander, 1999). Among the most commonly encountered contaminants in soils and waters are hydrocarbons (both aliphatics and aromatics), pesticides, heavy metals and nutrients. These chemicals enter natural environments from various sources. Aromatic hydrocarbons such as BTEX (benzene, toluene, ethylbenzene, and xylene) result from oil refinery waste, leakage from oil pipelines and underground storage tanks or basins, and spills of crude oil in marine environments after accidents at sea. Chlorinated aliphatics such as trichloroethylene (TCE), tetrachloroethylene, perchloroethylene (PCE), and 1,1,1-trichloroethane, are used for degreasing and released to the environments. Industrial chemicals are discharged deliberately or advertently into waters or onto soils following their intended use. By-products of manufacturing operation and pollutants contained in wastes that were inadequately treated are also released to the environments (Alexander, 1999).

Sites that can become contaminated include agricultural areas and vicinity with pesticides, industrial areas with chemical production waste, dumpsites with municipal waste, coal-distillation areas with coal tar, oil refinery areas and groundwater with petroleum hydrocarbons, and wood preservation sites with chlorophenolic compounds (Allard and Neilson, 1997).

Although regulations are strictly enforced in developed countries like the United States and most of the western European countries, these regulations often remain unenforced in many of the developing countries such as Ghana (Allard and Neilson, 1997).

2.2 Fate of Petroleum Hydrocarbons in the Environment

Chemicals released to the environment undergo various dissipation pathways, and the persistence of chemicals in the environment varies widely. Depending on their behaviour in the environment, contaminants are often classified as biodegradable, persistent, or recalcitrant. Factors affecting the local concentration of a contaminant include the amount of compound released, the rate at which the compound is released, its stability in the environment under various conditions, the extent of its dilution, its mobility in a particular environment, and its rate of biological or non-biological degradation (Ellis, 2000; Janssen *et al*., 2001). Oil spills have quite different fates in water and on land. In water, oil, being an immiscible liquid, spreads out over a large area in the form of a thin film depending on the wind, water temperature, and oil viscosity. On land, petroleum hydrocarbons infiltrate vertically downward through the unsaturated soil until they reach the water table where they spread laterally. This report concerns the remediation of petroleum hydrocarbon spills on land where mining operations takes place.

2.3 Chemistry of Petroleum Hydrocarbons

2.3.1 Composition

Hydrocarbons are organic compounds consisting entirely of hydrogen and carbon. Understanding the nature of the contaminant is important in any remediation work. The majority of hydrocarbons found naturally occur in crude oil, where decomposed organic matter provides an abundance of carbon and hydrogen which, when bonded, can catenate to form seemingly limitless chains (McMurry, 2000). Crude oil may contain thousands of diverse chemical compounds including dissolved gases, liquids, and bituminous solids, while refined petroleum products are usually a mixture of defined chemical compounds. Crude petroleum is an extremely complex mixture of fossil material, primarily of plant origin (Speers and Whitehead, 1969). It contains thousands of organic, and a smaller number of inorganic, compounds. Crude oils vary tremendously in chemical composition, in relative concentrations of different chemicals, and in physical properties; no two are alike. Crude oils are refined to produce a wide variety of refined and residual products, mostly fuels that contain a smaller number of chemicals that are usually within a defined boiling point range (McMurry, 2000).

Compounds in petroleum hydrocarbons can be separated into the following categories:

a. Saturated fraction-comprising n-alkanes, branched alkanes, and cyclic alkanes which are all aliphatic compounds. Aliphatic Hydrocarbons have straight or branched chains of carbon atoms with sufficient hydrogen to satisfy the valences requirements of the carbon.

b. Aromatic Hydrocarbons can be identified by the presence of one or more resonance-stabilized six-carbon rings. There are two types of aromatic hydrocarbons: unsubstituted and substituted examples are benzene and toluene.

c. Polycyclic aromatic compounds such as naphthalene, phenanthrene, and benzo(a)pyrene.

d. Polar compounds consisting of nitrogen- (N), sulphur- (S), and oxygen (o) containing substituents, e.g., phenols, pyndine, thiopene, etc.

e. Asphaltic residues consisting of very large complex molecules.

(See Appendix A for examples of chemical structures of each hydrocarbon).

Refined petroleum products contain few asphaltic residues, but may have alkenes or unsaturated aliphatics, among other compounds, formed during the "cracking" process (Bartha, 1986). Hydrocarbons, organic chemicals composed solely of carbon and hydrogen, are by far the most abundant chemicals in crude and refined oils. Variable amounts of organic chemicals containing sulphur, nitrogen, or oxygen also are present in all crude and some refined oils.

There are several factors involved in the degradation of these compounds. These factors can be used as "rules of thumb" for petroleum hydrocarbons.

- Aliphatic hydrocarbons are generally easier to degrade than aromatic compounds
- Straight-chain aliphatic hydrocarbons are easier to degrade than branched-chain hydrocarbons. The introduction of branching into the hydrocarbon molecule hinders biodegradation.

- Saturated hydrocarbons are more easily degraded than unsaturated-hydrocarbons. The presence of carbon-carbon double or triple bonds hinders degradation.
- Long-chain aliphatic hydrocarbons are more easily degraded than short-chain hydrocarbons. Hydrocarbons with chain lengths of less than 9 carbons are difficult to degrade because of their toxicity to microorganisms. Some specialized microorganisms (methanotrophs) can degrade these short-chain hydrocarbons. The optimal chain length for biodegradation appears to be between 10 and 20 carbons." (Baker and Herson, 1994).

2.3.2 Properties of some Petroleum Hydrocarbons

The hydrocarbons in petroleum are aliphatic (saturated), aromatic (unsaturated), or a combination of both (See Appendix A). Aliphatic hydrocarbons, also called alkanes or paraffins, are composed of chains of carbon atoms linked by single covalent bonds. Chemical bonds not occupied by carbon-carbon bonds are occupied by hydrogen atoms. Aliphatic hydrocarbons in petroleum may be normal (a linear chain), branched, or cyclic. They may range in size from methane (CH_4) to at least C78 (a chain of 78 carbons). Some refined oils, particularly light fuels, such as gasoline and kerosene, may contain olefins (aliphatic hydrocarbons containing one or more carbon-carbon double, or occasionally triple bonds) generated during the refining process. Olefins usually represent less than a few percent of the hydrocarbons in fuels (Neff, 1979).

Aromatic hydrocarbons in petroleum are composed of one or more benzene rings, a six-carbon ring containing nine equally shared carbon-carbon covalent bonds. Each carbon atom in benzene is bonded to one hydrogen atom, which can be substituted by a methyl ($-CH_3$), ethyl ($-CH_2-CH_3$), or longer-chain aliphatic group(s) called an alkyl

group(s). Benzene and alkyl benzenes with one or two methyl or ethyl groups (toluene, ethylbenzene, and *m*-, *p*-, and *o*-xylene) are the most abundant aromatic hydrocarbons in most crude and refined oils.

Benzene may be linked to other benzene molecules by single covalent bonds to form compounds such as biphenyl and terphenyl. More frequently, two or more benzenes are fused (sharing two carbons) to form polycyclic aromatic hydrocarbons (also called polynuclear aromatic hydrocarbons [PAHs]) [Neff, 1979]. Naphthalene, composed of two fused benzene rings, has a molecular weight of 128.2 and is the smallest PAH. Coronene, with six condensed, fused rings and a molecular weight of 300.3 is the highest molecular weight PAH considered to have sufficient environmental mobility (aqueous solubility of approximately 0.1 ùg/L: parts per billion [ppb]) to be potentially toxic (Janssen *et al*., 2001).

A large number of products, mostly fuels, are produced from crude oil by refining. Refining involves distillation to isolate oil fractions containing hydrocarbons with different boiling points and stimulation of chemical reactions that convert hydrocarbons in the fractions from one form to another. Refined oil products include gasoline and middle distillate fuels, such as diesel fuel, jet fuel, kerosene, and home heating oil. The petroleum fraction remaining after removal of light and middle distillate fractions is called residual oil, which is used to fuel ships and power plants (bunker fuel) or to produce road paving asphalt. Lubricating oils and petroleum tars also are made from residual oil. Refined and residual petroleum products contain all the chemical classes present in crude petroleum, as well as some compounds produced during refining or added to the finished product to improve its properties

(King, 1992). Each refined or residual product contains primarily crude oil chemicals over a specified boiling point range. Gasoline has an approximate boiling point range from 40 to 205^{o}C; middle distillate fuels have an approximate boiling point range between 175 and 375^{o}C; residual fuel oils are blended from crude oil fractions generally boiling between about 350 and 700^{o}C (King, 1992).

2.4 The Microorganisms

As with any living creature, microorganisms need nutrients to survive. In particular the microorganisms require a carbon (C) source and an energy (E) source. There are also several environmental factors that affect the fate of the organism. These include; temperature, pH, moisture content, amount of substrate present.

2.4.1 Temperature

Temperature affects the microorganism's ability to survive let alone reproduce. If the temperature exceeds the allowable limit then enzyme denaturisation begins and this leads to the inhibition of reproduction and eventually to death. If the temperature drops, reproduction again will come to a halt while the organism focuses its energy on sustaining life. If the temperature drops too low, then the organism will cease to exist. There are ideal temperatures for each microorganism and a small range in which these organisms may survive (Watanabe, 2001).

2.4.2 pH

The pH of the soil and the ground water affect the microorganism in a similar manner as temperature. There is generally a small range of pH in which the organism is capable of sustaining life. If the soil becomes extremely acidic or alkaline then the concentration of microorganism slowly diminishes (Zitrides, 1983).

The optimum pH for biodegradation lies between 6 and 8. However, effective biodegradation can be also found outside this range. Soil pH may affect the solubility, mobility, and ionized forms of contaminants. Microbial activity in the soil is greatly affected by pH, through the availability of nutrients and toxicants and the tolerance of organisms to pH variations (Roberts, 1998). Soil pH can affect the solubility or availability of macro- and micronutrients, the mobility of potentially toxic materials, and the reactivity of minerals (Parr *et al.*, 1983). Hydrocarbon contaminants and soil nutrients can often reduce the pH of the soil. During aerobic degradation of organic molecules, carbonic acid, organic acid intermediates, nitrate and sulphate may accumulate and this can lower the soil pH and inhibit biological activity (Zitrides, 1983).

2.4.3 Moisture Content

A source of water is a necessity for life. For each microorganism there is optimal moisture content for it to grow. Microbes are limited to soluble materials that are transported across their cell membranes into the interior of the cell. The moisture solubilises the substrate and allows the substrate to enter the cell. "For hydrocarbon contaminated soils, moisture level below 50% appear to inhibit degradation"(Cookson, 1995).

2.4.4 Amount of Substrate Present

A source of nutrition is needed. Growth of the microorganism will continue until the substrate is eliminated. Carbon has been said to be the building block of life. Therefore it is no surprise that the presence of carbon is a necessity for microscopic life to flourish. The carbon used to support life may be present in many forms. This is

one of the reasons why microorganisms are an effective means of remediation. The determination of the amount of C used by microorganisms has long been a source of study (Gogoi *et al.*, 2003) .The assimilation of C is an important issue. The percentage of C used by the microbial population reflects the biological efficiency of converting the substrate into biomass. Higher percentages indicate greater efficiency of the organism in the conversion of substrate. The greater the efficiency of conversion the quicker and more complete the remediation of the site by the microorganisms. The determination of the percent used is straightforward in liquid media, but becomes complicated in soils, wastewater, sediments or sewage. The complications exist due to the particulate matter as well as the water insoluble products. An estimate of the assimilated C can be found using the following equation:

$$C_{assimilated} = C_{substrate} \sim C_{mineralized}$$

The assimilated C becomes mineralized further as the cells that are metabolizing the substrate are themselves decomposed or consumed by predators (Alexander, 1994).

2.4.5 Energy Sources

Another key factor in the existence of the microbial population is an E source. Many environmental pollutants represent a novel carbon and energy source for a particular population until they are transformed by the metabolic pathways that are characteristic of heterotrophic microorganisms. In order for the organism to grow on the compound, the compound must be converted to the intermediates that characterize the major metabolic pathways that are characteristic of the heterotrophic microorganisms. Compounds that cannot be modified enzymatically to provide the necessary intermediates will not be able to serve as C and E source. This is due to the fact that the energy yielding and biosynthetic processes are not able to function. This

indicates that the primary phases of the biodegradation process involves the modification of the novel substrate to yield a product that in itself is an intermediate, or a substance that can be further metabolized into an intermediate. This need to convert synthetic molecules to intermediates is common to both aerobes and anaerobes (Alexander, 1994).

If sufficient organic nutrients are not present, then inorganic elements may be used as an energy source. The inorganic elements that may be used are oxygen ($O_{2)}$, nitrogen (N), phosphorous (P) and sulphur (S). For heterotrophic organisms the limiting factor is generally the availability of C. Because C is the limiting element and because it is the element of intense competition, a species that has the unique ability to grow on synthetic molecules has an advantage. As the organisms use these molecules as a C or energy source, the biodegradative process usually will still lead to the mineralization of the other elements in the chemical (Margesin and Schinner, 1997).

Typically microbial cultures produce extracellular surfactants that aid in solubilising hydrocarbons. These surfactants consist of a complex mix of protein, lipids, and carbohydrates. Non-biological surfactants have been used to disperse hydrocarbons (Mishra *et al*., 2001).

2.5 .1 Degradation Pathways

There are several degradation pathways for petroleum hydrocarbons. The purpose of this section is to provide an overview of the common pathways of degradation. Straight chain alkanes are degraded primarily through the oxidation of the terminal methyl group, followed

by cleavage of the molecules between the second and the third carbon in the chain. The initial reaction in the degradation of the straight chain alkanes involves the direct addition of oxygen to the terminal carbon. This forms an alcohol that can subsequently oxidize to a corresponding aldehyde and finally forms a fatty acid. From the fatty acid, a two carbon long intermediate fragment is cleaved. This process is repeated until complete oxidation of the hydrocarbon molecule is achieved. The presence of branching in the molecule will prohibit the cleavage reaction and therefore significantly reduce the molecule's susceptibility to biodegradation (Baker and Herson, 1994).

Aromatic hydrocarbons are found mainly in light petroleum products; however, they may be present in all amounts in any petroleum product. Aromatic hydrocarbons are also widely used in industrial solvents. Aromatic hydrocarbons, in general, are very soluble in water and have low boiling points due to their small molecular size. These compounds are also very volatile. There are a large number of different pathways that are used by bacteria to degrade aromatic compounds. Benzene for example is first converted to catechol or protacatechuate. The aromatic nucleus is subsequently opened by one of two pathways: the orthocleavage or the metacleavage pathway. Considering orthocleavage, the aromatic ring of catechol or protocatechuate is opened as a result of the introduction of molecular oxygen into the hydroxyl groups. Acetyl-CoA and succinate are formed as a result of the cleavage. These products can then be further oxidized by the Krebs cycle and the electron transport system. In meta-cleavage also the aromatic ring is opened by the introduction of molecular oxygen, however the cleavage in this case, occurs between a hydroxylated carbon and the adjacent unsubstituted carbon. Acetaldehyde and pyruvate, which can be broken down

by the Krebs cycle and electron transport are the products of the ring cleavage (Baker and Herson, 1994).

The above processes are aerobic in nature. Aerobic degradation is the most common; however, anaerobic degradation can occur. Anaerobic degradation will occur under denitrifying conditions, sulphate-reducing conditions, and methanogenic conditions. The initial step in anaerobic degradation is dissimilar to the aerobic degradation path. The first stage of degradation in an anaerobic system is the hydrogenation of the benzene ring, thus destabilizing the ring. Cleavage through hydration reaction yield aliphatic hydrocarbons that can be further metabolized to the Krebs cycle intermediates as described above. In anaerobic degradation, water acts as the oxygen source for metabolic reactions.

2.5 .2 Biodegradation

Biodegradation can be defined as the biologically catalyzed reduction in complexity of chemicals. Biodegradation of organic contaminants in the natural environment has been extensively studied to evaluate its potential in bioremediation and to understand microbial ecology and physiology (Watanabe, 2001). A number of authors have reviewed the subject of biodegradation of organopollutants over the past decade (Janssen et al., 2001).

In the case of organic compounds, biodegradation frequently leads to the conversion of much of the C, N, P, S, and other elements in the original compound to inorganic products. Such a conversion of organic substrates to inorganic forms, such as CO_2, H_2O and other inorganic compounds is known as mineralization. Consequently,

mineralization is sometimes used as a synonym for *ultimate biodegradation*. In the mineralization of organic compounds by microorganisms, inorganic forms of elements are released to the surrounding environment. Because mineralization results in the total destruction of the parent compound, it is viewed as a beneficial process. Although plant and animal respiration and non-biological processes also play a considerable role in the mineralization process, mineralization of synthetic chemicals appears to result largely or entirely in most environments from microbial activity.

Microorganisms are thought to be ubiquitous, and it has been assumed that there might exist 1000 or more species per gram soil, with accumulative total of 2-3 million bacterial species in the biosphere (Truper, 1992). Natural microbial populations in various habitats contain microorganisms with a diverse array of catabolic activities and an amazing physical versatility (Watanabe *et al*., 1996). A number of microorganisms with ability to degrade a wide variety of compounds have been isolated and characterized (Dickel et al., 1993). Most synthetic products, regardless of their complexity, are degraded by one or more species in some particular environment. Indeed, microorganisms are frequently the sole means of converting synthetic chemicals to inorganic products (Alexander, 1999).

2.5.3 Factors affecting biodegradation rates of chemicals

There has been extensive research to improve biodegradation ability of microorganisms under laboratory conditions, and the rate of microbial biodegradation of a chemical in the environments is known to be affected by a number of physicochemical, biological, and environmental factors. These parameters have been discussed in many recent publications (Alexander, 1999). Among the parameters that appear to be important include the properties of the chemicals to be degraded, the

presence/absence of predators or interspecies competition, the conditions for microbial degradation activity (e.g. presence of nutrients, oxygen, pH, and temperature), and the physicochemical characteristics of the environments (Vogel, 1996).

2.5.3.1 Intrinsic ability of the Microflora at the Site

Since the major pathway by which chemicals are dissipated in the environments is degradation by microorganisms, the presence or absence of microbe(s) or microbial communities with relevant activity affect their rate of degradation. It is well known that many chemicals are not degraded by a single microbial species; but require the cooperation of two or more species. In such cases, transformation that one species alone cannot perform, results in the amount of chemical degraded by the community that is greater than the sum of individual species degradation (Atlas and Bartha, 1992).

2.5.3.2 Properties of Microbial Association

Microorganisms capable of degrading chemicals have central roles in biodegradation, but other organisms also affect the process through increased predation and parasitism of the degrading bacteria and/or a fungal biomass. Although all of these can affect the rate of biodegradation, protozoan grazing is thought to be most effective in controlling the rate of biodegradation. According to Alexander (1999), protozoan grazing is substantial when a prey density is greater than 10^6 to 10^7 bacterial cells per millilitre or per cubic centimetre for nonaqueous environments.

2.5.3.3 Characteristics of Chemicals

Biodegradability of compounds is sometimes related to compound structure and its related physico-chemical characteristics such as solubility and bioavailability. One of the main reasons for the prolonged persistence of hydrophobic organic compounds in the environment is their solubilisation-limited bioavailability, which itself is not intrinsic to the compound but related to the interaction between the compound, the microbes, and soil (Vogel, 1996). It is known that toxicity of chemicals at high concentration affect microbes and their efficiency as degraders, although indigenous microbial population can target constituents over a wide range of concentrations in the environments (Barbeau *et al*., 1997).

2.5.3.4 Availability of Nutrients

The nutrient requirements for microbes are approximately the same as the composition of their cells, and microbial nutrients are categorized into three groups based on the quantity and essential need for them by microorganisms: macro-, micro, and trace nutrients (Sutherson, 1997). The absence of any of these nutrients, in suitable forms and states, in a particular environment will prevent the growth and/or activity of microorganisms. In contrast, enhanced and accelerated biodegradation in fertilized soils have been reported (Margesin and Schinner, 1997). Bioremediation of hydrocarbon-contaminated soils, in particular, is known to be limited by nitrogen (N) and phosphorus (P) since the contaminant, itself, usually functions as a carbon source (Margesin and Schinner, 1997).

Microbial growth may be limited by several elements at the same time and additions of combinations of nutrients can enhance biodegradation (Swindoll *et al*., 1988). Polycyclic aromatic hydrocarbons (PAHs) degradation was found to be optimal when a material containing approximately 75% S, 3% N, and 11% P, was applied to soil (Liebeg and Cutright, 1999).

Although the optimal C: N: P mole ratio for bioremediation applications is thought to be approximately 100:10:1 (Cookson, 1995), there are no specific methods for determining the exact nutrient sources available at a site. In addition, the successful implementation of a nutrient combination at one site for decomposition of one contaminant does not guarantee similar success at a different environment and for a different contaminant.

2.5.3.5 Presence of other Substrates

Natural or contaminated environments characteristically contain more than one organic compound, either natural or synthetic, that can be utilized by microorganisms. The contaminant concentrations may range from quite high to extremely low. Simultaneous metabolism or utilization of several contaminants at the same time is affected by the interaction of the contaminants (Wang *et al*., 1996). Many cases in which one substrate enhances the rate of biodegradation of a second compound have been reported (Millette *et al*., 1995). In contrast, the presence of one contaminant can inhibit the degradation of a second contaminant (Steffensen and Alexander, 1995). The stimulating effect of one contaminant to biodegrade another might result from the greater population size or biomass arising due to additional carbon source. The inhibiting effect might be due to: (1) toxicity of the second contaminant or its

degradation product, (2) competition for limiting nutrients or electron acceptors among microbes, or (3) grazing of degraders by enriched protozoa (Alexander, 1999).

2.5.3.6 Environmental Characteristics

Environmental conditions play a pivotal role in determining biological activity, whether of indigenous microorganisms, cultured indigenous microbes returned to the soil, or exogenous microorganisms introduced to soil. These conditions are classified into two categories: those that reduce the microbial activity such as temperature, humidity, and ionic strength; and those that restrict the mass transfer of the compound to the microorganisms such as clay and organic matter content (Ralebitso *et al*., 2002).

The temperature of the environment is an important factor governing microbial activity and some physical properties of compounds. The optimum temperature for biodegradation of contaminants in temperate climates is generally in the range of 20 ^{o}C to 30 ^{o}C (Atlas, 1992). However, the metabolism of microorganisms can be adapted to work and function optimally at low temperatures (Whyte *et al*., 1996), and there are reports of microbial degradation at low temperatures. Those reports include mineral oil degradation at temperatures below 0^{o}C (Zobell, 1973), biodegradation activities of indigenous soil microorganisms at 10 ^{o}C to 16 ^{o}C from an oil spill in Alaska (Pritchard *et al*., 1992), and the elevated biodegradation of diesel oil by inoculation of cold-adapted indigenous microorganisms in alpine soils under laboratory conditions (Margesin and Schinner, 1997). According to Norris *et al*. (1994), the redox potential must be greater than 50 millivolts for optimal aerobic condition. Christensen et al. (2000) demonstrated that the redox environment forms

the boundaries for attenuation of many compounds. The activities of degrading microorganisms are often restricted to certain redox environments, and degradation processes occur at different rates in different redox environments.

2.5.4 Conditions for Successful Biodegradation by Microorganisms

Because microorganisms are frequently the major cause of contaminant degradation, the absence of a microorganism from a particular environment, or its inability to function, often means that the compound disappears very slowly. However, other factors also are involved and Alexander (1999) has summarized conditions that must be satisfied for successful biodegradation to take place in an environment. The conditions are: (1) organism(s) with proper metabolic activity for the biodegradation of a compound must exist in the environment containing the chemicals, (2) the chemical to be degraded by microorganisms must be exposed to the organism having the requisite enzymes, (3) the functional groups of the compound to be degraded must be exposed if the biodegradation is extracellular, (4) molecules must be transported, either actively or passively, to the internal sites of the cells where the enzyme acts if degradation occurs by intracellular enzymes, and (5) since biodegradation, is a result of microbial activity, environmental conditions must be favourable for microbial growth and activity. If any of the conditions mentioned are not satisfied, it is likely the chemical contaminant will remain undegraded in the soil. It is not certain, at present, how many compounds persist in the environment because of the complete absence of species having the capacity to bring about degradation or the conditions not favourable for microbial biodegradation (Alexander, 1999).

2.5.5 Methods used for Enhanced Biodegradation

2.5.5.1 Enhanced degradation by increasing bioavailability of pollutants

A possible way to enhance bioavailability and, hence, biodegradation of organic contaminants is by increasing the surface area of hydrophobic, water-insoluble substrates. In hydrocarbon degradation, it is essential for bacteria to come in direct contact with the hydrocarbon substrates, which are usually hydrophobic. Therefore, it is not surprising that bacteria growing on hydrocarbons such as petroleum usually produce potent emulsifiers.

A remediation strategy to enhance contact between bacteria and water-insoluble hydrocarbon is the addition of emulsifier to soil. These molecules consist of both a hydrophilic and hydrophobic part, and are often called biosurfactants (Lang and Wullbrandt, 1999).

Bacterial biosurfactants can be classified into two types - low molecular and high molecular weight biosurfactants. There have been numerous reports on biosurfactants produced by microorganisms (Lang and Wullbrandt, 1999), and their role in enhancing bioremediation (Golyshin *et al*., 1999). The net effect of a surfactant on biodegradation depends on the benefits that result from enhanced solubility of target compounds versus the problems caused by a reduction in the adhesion of bacteria to those compounds.

2.5.5.2 Improved biodegradation by augmenting species richness

There are different strategies for increasing the catabolic activity of soil including adding specific microbial strains, introduction of specific mobile genetic elements into microbial strains to enhance biodegradation, or encouraging the activity of highly

diverse microbial communities directly in natural environments. Several tests have shown a positive effect of seeding strains on the degradation of certain compounds in soil. The addition of any species capable of living under the given environmental conditions will give rise to increased biodiversity of the soil for days to months. According to the concept of the carrying capacity of microbial communities, the added populations will generally stabilize at 10^3 cfu/g soil (Vandepitte *et al*., 1995). Hence, inoculation is a valuable approach to broadening the biodegradation potential of soil.

Genetic information can also be transferred from an introduced donor strain to well-established and competitive indigenous bacterial populations of soil. An advantage of this approach is its independence of the long-term survival of the introduced donor strain, which is often the major bottleneck in bioaugmentation processes (Akkermans, 1994). The third strategy is the introduction of an unspecified group of bacteria such as those present in soil, sludge, manure or compost (Barbeau *et al*., 1997). These materials normally contain a high diversity of microorganisms in which the species necessary to destroy the pollutant might be present. Addition of these materials to bioaugment degradation may occur without adaptation to the pollutant because an interaction between different microorganisms may result in improved removal of the contaminant. Moreover, in such samples, the cooperating species might be optimized relative to one another. Using this approach eliminates the difficult task of isolating and characterizing a specific bacterium able to degrade a specific compound.

2. 6 Bioremediation

The term bioremediation has been used to describe the process of purposefully using microorganisms to degrade or remove from the environment hazardous components or wastes (Glazer and Nikaido, 1995). Bioremediation is a resilient and adaptable technology that can be used with a surprising range of treatment approaches to improve removal efficiency and reduce the life cycle cost of a treatment project (Brown *et al*., 1999).

Although the use of bioremediation in the treatment of hazardous waste is a relatively new concept, it is a rapidly growing technology in environmental management, and there have been numerous reports on the application of bioremediation of contaminated sites. Examples of bioremediation include land farming, composting, bioreactors, bioventing, biofilters, bioaugmentation, biostimulation, intrinsic bioremediation, and pump and treat (Boopathy, 2000).

Treatment of contaminated sites rather than disposal is increasingly being emphasized in most industrialized nations since 1970. One factor in the development of bioremediation has been the enhancement of environmental laws and regulations that favour waste treatment rather than waste disposal (Caplan, 1993). Bioremediation has numerous applications including cleanup of soils, waters, lagoons, sludges, and process-waste streams. The shore-line cleanup efforts in Prince William Sound, Alaska, after the Exxon-Valdes oil spill is a good example of large-scale application of bioremediation. At this site, the U.S. Environmental Protection Agency (EPA) and Exxon Company demonstrated effectiveness of bioremediation technology on oil-contaminated beaches (ADEC *et al*., 2005).

A number of bioremediation strategies have been developed to treat contaminated wastes and sites. Selecting the most appropriate strategy to treat a specific site can be guided by considering three basic principles: (a) the amenability of the pollutant to biological transformation to less toxic products (biochemistry), (b) the accessibility of the contaminant to microorganisms (bioavailability), and (c) the opportunity for optimization of biological activity or bioactivity (ADEC *et al*., 2005).

2.6.1 Types of Bioremediation

Based on the place where the contaminated materials are treated, bioremediation technologies can be broadly classified as *ex situ* or *in situ*. *Ex situ* technologies refer to treatments that remove contaminants at a separate treatment facility, while *in situ* bioremediation technologies is the term used for treatments of contaminants in the place itself.

In situ bioremediation, especially by indigenous microbial population, is one of the most attractive features of bioremediation of sites containing readily degradable contaminants. For more recalcitrant compounds, bioaugmentation with adapted or specially designed microbial inoculants is a useful alternative (Vogel, 1996). In situ bioremediation processes currently being utilized in the field are classified into three categories: bioattenuation, biostimulation, and bioaugmentation.

Biostimulation, the artificial creation of an environment that promotes the growth of naturally occurring microorganisms capable of degrading the target contaminants, is the method in which biodegradation by indigenous microorganisms is stimulated and the reaction rates are increased. This option is adopted when there are indigenous microbes with degradation capacity but natural degradation does not occur or the

degradation is too slow. Biostimulation includes supplying the environment with nutrients such as carbon, nitrogen, phosphorus, or other substrates (Dojika *et al*., 1998).

Bioaugmentation is a way to enhance the biodegradative capacities of contaminated sites by inoculation of microorganisms with the desired catalytic capabilities. Bioaugmentation is discussed in detail in section 2.6.1.1. There is another type of bioremediation, which is called bioattenuation (i.e., intrinsic bioremediation by indigenous microorganisms). This is a natural process of degradation, without stimulating indigenous microbial population or inoculation of exogenous microorganisms. Bioattenuation is widely used in the United States as a cleanup method for petroleum-contaminated soil and groundwater at underground storage tank sites (Dojika *et al*., 1998). However, this option is not generally included in the category of in situ bioremediation.

2.6.1.1 Bioaugmentation

Bioaugmentation, in general, is defined as the application of indigenous and exogenous wild-type, or genetically modified organisms to polluted sites or bioreactors in order to accelerate the removal of undesired compounds. There have been numerous reports on feasibility and field application of bioaugmentation as a remediation technology (Vogel, 1996). Microorganisms are thought to be ubiquitous and bioremediation often uses naturally occurring indigenous microorganisms. The addition of supplemental inoculums consisting of either indigenous or non-indigenous microbes can enhance the diversity of the indigenous population and thus increase the degradation rate of target compounds. This is necessary when no microorganism

capable of degradation of target compounds exists in the natural community, or when the activity of the natural community is inhibited.

2.6.1.2 Bioattenuation

While there has been a great deal of work published on the remediation of hydrocarbons in general, there has been very little specific to linear alkylbenzenes. It is worth looking at the more general literature to gain an idea of the general principles (Bregnard *et al.*, 1996).

Hydrocarbons are found in a wide range of environmental settings and anthropogenic sources account for only a small proportion of the overall environmental load. Large volumes of hydrocarbon deposits are found in nature – they are, after all, organic molecules in all senses of the word. It should come as no surprise, therefore, that organisms capable of utilising these materials as both carbon and energy sources should exist (Margesin & Schinner, 1999).

There is a wealth of evidence that hydrocarbons are removed from the environment by biological systems. In studies on soils contaminated with BTEX compounds (benzene, toluene, ethylbenzene and xylene), which are structurally similar to linear alkylbenzenes (LABs). Weidemeier *et al.* (1996) found that volumes of soil known to be contaminated exhibited low oxidation-reduction potential (ORP) and depletion of CO_2, NO_3^- and NH_4^+. All are indicative of aerobic microbial activity. More recently it was shown that the addition of a large amount of carbon source leads to depletion of inorganic nutrients (Margesin & Schinner, 1999).

Weissemann & Kunze (1994) had earlier shown that hydrocarbon contamination of soil led to depletion of nitrogen and that this could be a limiting factor in the rate of removal. A number of studies have demonstrated that rates of removal can be enhanced by the addition of nitrogen (Haigh, 1995); nitrate, oxygen, phosphate and ammonia (Bregnard *et al.,* 1996); water, air and nutrients (Phelps *et al.,* 1994).

The addition of sewage sludge to contaminated soil increases the removal rate of hydrocarbons (Holt & Bernstein, 1992). This may be due to the added nutrients and water in the sludge, the incorporation of air during the mixing process, the bacterial community in the sludge, or a combination of all three. The fact that later studies have shown improvements solely through the addition of nutrients, water and air suggests that the indigenous microbial community possesses the requisite suite of metabolic pathways (Holt & Bernstein, 1992).

While a variety of organisms have been demonstrated to degrade hydrocarbons, remediation of diesel has been shown to be mainly bacterial rather than fungal (Harrison & Betts, 1996). Organisms shown to degrade linear alkylbenzenes (LABs) include *Nocardia amerae* (Bhatia & Singh, 1996) and *Pseudomonas* sp. (Smith and Ratledge, 1989).

Although pure cultures of specific microorganisms have been shown to degrade linear alkylbenzenes (LABs), it is very rare to find a habitat that contains a single species. Bacteria in the environment occur as mixed populations and even under culture conditions, mixed consortia of microorganisms have been shown to be more efficient than any single type (Dave *et al.,* 1994). Even when bacteria that have been selected

for their ability to catabolise a particular material are added to a contaminated soil, there is often no discernible effect on bioremediation rates (Phelps *et al.*, 1994). A healthy soil community will in all probability possess biochemical pathways to utilise most hydrocarbon contaminants and will be better adapted to prevailing conditions than any introduced organism. However, bioaugmentation with *indigenous* microorganisms that have been cultured with elevated levels of the contaminant may enhance *in situ* biodegradation rates (Weber & Corseuil, 1994).

2.6.1.3 Biostimulation

Biostimulation is the manipulation of abiotic factors to optimise conditions for microbial remediation of a contaminant. Activities include the addition of inorganic nutrients such a nitrate and phosphate, and electron acceptors (e.g. oxygen as molecular O_2 in solution or indirectly as H_2O_2).

Perhaps the first engineered bioremediation efforts were those pioneered by Raymond (1974) and described in a review article by Tursman & Cork (1992). Ritter & Scarborough (1995) consider that bioaugmentation is appropriate only for *ex situ* remediation where conditions can be closely controlled (e.g. by land farming or in a bioreactor), while *in situ* remediation is best tackled by biostimulation.

2.6.2 Advantages and disadvantages of bioremediation

Bioremediation, which involves the use of microbes to detoxify and degrade environmental contaminants, has received increasing attention as effective biotechnological approach to clean up a polluted environment. Bioremediation offers several advantages over the conventional chemical or physical technologies,

especially for diluted or widely spread contaminants. These treatment methods have generally been found to be advantageous compared to other treatment methods in the following aspects: (1) The intensity of the process can be adjusted from highly aggressive to passive, (2) the incremental costs for adding a bioremediation component to most types of treatments is lower than other options, (3) the process can be applied to a wide range of purposes, from mass removal to formation of a migration barrier to final polishing, (4) it can be done on site with minimal site disruption, and (5) it can be applied to diluted and widely diffused contaminants (Iwamoto and Nasu, 2001).

Although bioremediation is a generally accepted technology for removal of contaminants from the environments, bioremediation has its limitations and it is still an immature technology. Some chemicals, (i.e., heavy metals, radionuclides, and some chlorinated compounds) are not amenable to biodegradation. It also is not always possible to obtain complete contaminant removal because there might be a threshold concentration below which rates of biodegradation are slow or negligible (Allard and Neilson., 1997).

Bioavailability of pollutants may decrease as biodegradation proceeds, and thus recalcitrant compounds may persist. Secondary effects that develop during bioremediation also must be considered. Biodegradation sometimes generates products with higher toxicities than the parent molecules (Bradley, 2000).

Bioremediation frequently must address multiphasic, heterogeneous environments such as soils, in which the contaminant is present in association with the soil particles, dissolved in soil liquids, and in the soil atmosphere. Because of these complexities,

successful bioremediation is dependent on an interdisciplinary approach involving disciplines such as microbiology, engineering, ecology, and chemistry (Boopathy, 2000).

Our current knowledge of changes in the microbial communities during bioremediation is limited and the microbial community in natural environments is still treated as a black box. The reason for this is that many environmental microorganisms cannot yet be cultured by conventional laboratory techniques. Therefore, attention has to be paid to the application of bioremediation, especially bioaugmentation, because of its unknown effects on the ecosystem. This has led to two essential questions related to bioremediation in the field:

(1) how to clarify the biological contribution to the effectiveness of bioremediation, and

(2) how to access the environmental impact of bioremediation (Iwamoto and Nasu, 2001).

2.6.3 Factors Affecting Biodegradation of Petroleum Hydrocarbons

Chemical and structural factors affecting the biodegradation of the various components of petroleum hydrocarbons were discussed in the preceding pages. However, there are several other factors that can have a significant effect on the biodegradation of these compounds, These factors include photolytic activity, solubility, sorption on solids, presence of surfactants, oxygen, nutrients, temperature, and pH (Payne and Phillips, 1985).

2.6.3.1 Photolytic activity

It is expected that photo-oxidation of the compounds in petroleum hydrocarbons would produce products that are more polar than the parent compounds because of the oxidation. These compounds will be more water soluble and are likely to be more biodegradable (Foght and Westlake, 1984). In oil lenses formed in water after a spill, photo-oxidation may lead to polymerization which may lead to the formation of tarry residues that are difficult to biodegrade (Payne and Phillips, 1985). In terrestrial situations, photooxidation does not play a major mile in the natural degradation processes (Bartha, 1986).

2.6.3.2 Solubility

The solubility of the compounds in petroleum hydrocarbons in water is an important property for evaluating their biodegradation. In general, the higher the aqueous volatility of the compound, the more likely it will biodegrade. A liquid or dissolved aromatic hydrocarbon will be degraded in preference to a solid phase aromatic compound (Foght and Westlake, 1984).

2.6.3.3 Sorption on solids

Hydrophobic compounds having low water volubility tend to concentrate on surfaces. Thus, many compounds present in petroleum hydrocarbons sorb on the particulate matter present (Bartha, 1986). The sorption of these compounds on soils may have varying effects depending on the type of sorbent, the nature of the compound, and its concentration. Subba-Rao and Alexander (1982) found that the degradation of benzylamine sorbed on montmorillonite clay was influenced by the concentration of

benzylamine and clay, while the degradation of benzoate was usually not affected by the clays, montmorillonite, and kaolinite.

Naphthalene biodegradation under denitrifying conditions in soil-water systems was studied by Mikelcic and Luthy (1988). They found that naphthalene sorption-desorption was reversible and rapid compared to the rate of microbial degradation.

2.6.3.4 Presence of surfactants

Surfactants can interact with the compounds present in petroleum hydrocarbons and increase their aqueous solubilities (Ellis *et al*., 1986). Thus, the presence of surfactants, natural or otherwise, may make these compounds available to the microbes for biodegradation. In addition, surfactants can mobilize compounds that are sorbed on the particulate surface, increasing bioavailability of the contaminant.

Some microbes produce biosurfactants to aid in solubilizing compounds that have low solubilities (Lang and Wagner, 1987). These biosurfactants are, generally, glycolipids. They can reduce interracial tension, which produces an emulsion of the compound in water. The finely divided compound in the emulsion results in an increase in the available surface area for contact between cells and the compound, promoting biodegradation (Bury and Miller, 1993). Falatko and Novak (1992) reported that biosurfactants produced by gasoline degrading bacteria increased the volubility of the gasoline compounds.

The surfactants themselves could absorb on soil materials which would reduce their ability to solubilize compounds absorbed on the soils, Oberbremer and Muller-Hurtig (1989) also reported the production of biosurfactants during metabolism of a

hydrocarbon mixture containing tetradecane, pentadecane, hexadecane, pristane, trimethylcyclohexane, phenyldecane, and naphthalene by soil microorganisms.

2.6.3.5 Oxygen

For relatively rapid biodegradation of petroleum hydrocarbons, aerobic conditions are necessary, since anaerobic degradation of these compounds has been demonstrated to be quite slow (Hambrick *et al*., 1980). The initial attack on many of the molecules present in petroleum hydrocarbons is by oxygen through the oxygenase system, as discussed earlier. In subsequent steps, oxygen is the most common electron sink, but in its absence, nitrate or sulphate may act as an electron acceptor to oxidize the partially oxidized intermediates (Bartha, 1986).

2.6.3.6 Nutrients

In water and soil, the growth of petroleum-hydrocarbon-utilizing cells is limited if mineral nutrients, especially N and P, are in short supply (Bartha, 1986). Iron was found to be limiting in clean, offshore seawater, but should not be a limiting factor in most cases (Dibble and Bartha, 1976). In order to prevent nutrient limitations in biological treatment processes, the ratio of C: N: P is kept at 120:10:1 based on the organic carbon content of the feed (Sims *et al*., 1989). In actual practice, during the course of biodegradation, nutrient levels are monitored and kept above a set target level.

2.6.3.7 Temperature

Temperature plays very important roles in biodegradation of petroleum hydrocarbons, firstly by its direct effect on the chemistry of the pollutants, and secondly on its effect on the physiology and diversity of the microbial milieu. Ambient temperature of an

environment affects both the properties of spilled oil and the activity or population of microorganisms (Venosa and Zhu, 2003). At low temperatures, the viscosity of the oil increases, while the volatility of toxic low-molecular weight hydrocarbons is reduced, delaying the onset of biodegradation (Atlas, 1981). Temperature also variously affects the solubility of hydrocarbons (Foght *et al.*, 1996). Although hydrocarbon biodegradation can occur over a wide range of temperatures, the rate of biodegradation generally decreases with decreasing temperature. Highest degradation rates generally occur in the range of 30–40 ^{o}C in soil environments, 20–30 ^{o}C in some freshwater environments, and 15–20 ^{o}C in marine environments (Bossert and Bartha, 1984). However, the bulk of information on hydrocarbon degradation bothers on activities of mesophiles, although significant biodegradations of hydrocarbons have been reported in psychrophilic environments in temperate regions (Pelletier *et al.*, 2004). Nevertheless, documented research on the environmental consequences of terrestrial spills in cold regions is still scarce, even though petroleum contamination is recognized as a significant threat to polar environments. Full-scale in situ remediation of petroleum contaminated soils has not yet been used in Antarctica for example, partly because it has long been assumed that air and soil temperatures are too low for an effective biodegradation (Delille *et al.*, 2004).

Temperature has a profound effect not only on the physical status of the hydrocarbons present, but also on rates of microbial metabolism. In colder conditions, liquid hydrocarbons become waxy solids; soluble hydrocarbons precipitate, and their volubility decreases considerably. This altered physical status affects their bioavailability. Lowering of the temperature slows biodegradation rates significantly.

The QIO (temperature quotient) values for petroleum hydrocarbon biodegradation in soil and in seawater vary from 1.7 to 2.7 (Bartha, 1986).

2.6.3.8 pH

The optimum pH range for the degradation of petroleum hydrocarbons is from 7 to 8.5 in natural waters. In acidic soils liming to pH 7.8 to 8.0 has been reported to be stimulator for the biodegradation of petroleum hydrocarbons (Bartha, 1986).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

3.1.1 Location

The Southern Ashanti Gold Project is centred at 2º14'W and 5º00'N, lying 80 km west northwest of Ghana's largest export port of Takoradi and immediately north of the provincial coastal centre of Axim in the Western Region of the country. The mineral properties concerned comprise seven granted Prospecting Licences (Salman, Salman East, Ankobra, Tumentu, Ebi–Teleku Bokazo, Akanko and Enyinase), and seven Prospecting Licence Applications covering an aggregate area of 450 km^2 (See Figure 3.1).

The Nzema gold project is Adamus's first mine in Ghana, and the first mine in the Nzema East region of Ghana. "Nzema" pronounced "en zimmer" is the name of the people and the local region in which the Project is located. The Ashanti Gold Belt is host to over 100 Moz of gold, with the southern end including the gold mines of AngloGold Ashanti (Iduapriem), Goldfields Ghana (Tarkwa) and Golden Star Resources (Bogoso-Prestea Limited) Nzema includes a series of open pits to exploit deposits along the Ashanti gold belt, in addition to known mineralisation from Anwia and Bokrobo, both of which are located to the west of Salman. Significant deposits, from north to south, include Akanko, Salman North, Teberru, Nugget Hill, Salman Central and Salman South. The selected mining method for the Project is conventional open pit mining including drilling, blasting, loading and hauling operations carried out by a mining contractor (Yeates et al, 2004).

3.1.2 Geology

Prospecting Licences comprising the Southern Ashanti Gold Project straddle a major north northeast trending structure termed the Ankobra Lineament. This feature separates the late Proterozoic volcanic and volcanic-derived lithologies assigned to the Upper Birimian Series to the east, from dominantly sedimentary sequences of the Lower Birimian to the west. The Ankobra Lineament is the southern extension of the Ashanti Shear Zone, which hosts the significant Prestea, Bogoso, Obuasi (Ashanti) and Konongo gold deposits to the north (Figure 3.1).

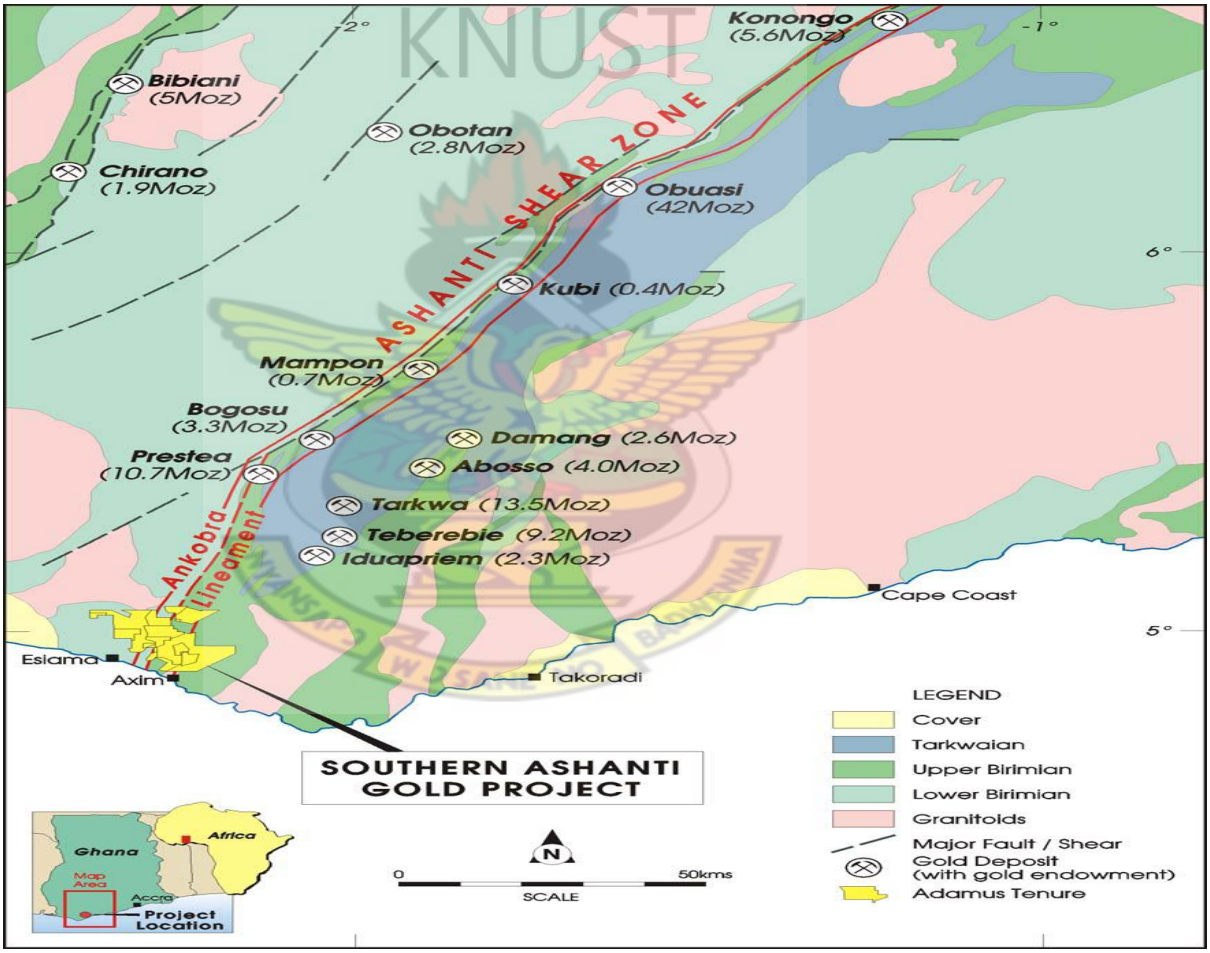


Fig. 3. 1: Project Location and Geological Setting (Yeates *et al*, 2004).

A number of mineralised structures have been identified within the Southern Ashanti Gold Project, the most significant of which is the north-northeast trending Salman lineament, traversing the central part of the concession group, and the Anwia Prospect located within the western (Ebi-Teleku Bokazo) licence (ARL Annual Report, 2010). Mineralisation along the Salman lineament is hosted by a succession of deformed phyllites and greywackes lying immediately east of the Ankobra Lineament. Surface mapping and drilling indicates that the gold mineralisation is mainly associated with a steeply westdipping, highly deformed zone at the contact between a phyllite-dominated sequence in the hanging-wall to the west, and greywacke-dominated metasedimentary sequence in the footwall to the east. The axis of mineralisation appears to lie along this contact, which to the north is intruded by conformable tonalite dykes of the syntectonic Dixcove Suite. Mineralised zones peel off the primary structure, flattening as stockwork zones within the footwall greywacke to the east (Yeates et al, 2004).

3.1.3 Climate

The Southern Ashanti Project is situated in one of the wetter regions in Ghana, with the annual rainfall varying from 1,800 mm to 2,000 mm. Daily temperatures range from 22 ºC to 30 ºC, with humidity averaging 80% (Yeates et al, 2004).

3.1.4 Physiography

The topography varies from broad, flat expanses associated with floodplains of the Ankobra River and tributaries, to heavily dissected hills and ridges rising up to 100 m above sea level (Figure 3.1). The vegetation is dominated by secondary regrowth forest, with isolated remnants of primary tropical forest. Mangroves are locally

developed adjacent to tidal rivers and estuaries. In addition to more traditional subsistence farming activities, palm oil and coconut plantations provide the dominant cash crops in the region (Yeates et al, 2004).

3.2.0 Materials

3.2.1 Soil

The petroleum contaminated soil was collected at random from a heap of petroleum contaminated soil from Adamus Resources Limited (ARL), a mining company at Salma, near Nkroful in the Western Region of Ghana. The bulked soil sample was air-dried and then sieved with a 2 mm sieve to remove stones and other particles. It was then mixed thoroughly to obtain a homogeneous sample. Soil physico-chemical parameters were analyzed before and after treatment with the different nutrient supplements.

3.2.1.1 Physico-Chemical Analysis of the Soil

Particle size analysis was carried out using the hydrometer method (Boyoucos., 1951).

Soil texture was determined by the felt method and was found to be sandy loam. Soil pH was determined using a pH meter according to manufacturer's instruction. The temperature of the soil samples was determined using a mercury thermometer.

Organic carbon was determined in accordance with titration method of Walkey and Black (1934). Total Nitrogen was determined by the Kjedahl digestion and steam distillation method (Black, 1965).

***Determination of Nitrogen By The Kjeldahl* Method**

Digestion

Ten (10) g of air dried sample of contaminated soil was weighed into 500 ml long-necked kjeldahl flask and 10 ml distilled water was added to moisten the sample. One spatulaful of kjeldahl catalyst (mixture of 1 part selenium + 10 parts $CUSO_4$ + 100 parts Na_2SO_4) was then added to the sample, followed by 20 ml conc. H_2SO_4. The Mixture was digested until the solution became clear and colourless. It was allowed to cool in the flask; the fluid was decanted into a 100 ml volumetric flask and make up to the mark with distilled water.

Distillation

An aliquot of 10 ml fluid from the digested sample was transferred by means of a pipette into the Kjedahl distillation flask and 90 ml of distilled water was added to make it up to the 100 ml mark of the distillation flask. 20 ml of 40 % NaOH was added to the content of the distillation flask and distillate was collected over 10 ml of 4 % boric acid. Three drops of the mixed indicator in a 200 ml conical flask was then used to indicate the presence of nitrogen in the sample (a light blue colour shows a positive test).

Titration

Hundred (100) ml of the distillate collected was titrated with 0.1 M HCl till the blue colour changes to grey and then suddenly flashes to pink. A blank test was finally carried out and used in the calculation below.

Calculation

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

% N = 14 × (A-B) × N × 100/(1000 × 1)

Where,

A= volume of standard HCl used in sample titration

B= volume of standard HCl used in blank titration

N= normality of standard HCl

% Crude Protein (CP) = % Total Nitrogen (NT) × 6.25(protein factor)

Determination of Phosphorous

Two (2) g of the soil sample was weighed and placed in a silica crucible and ashed at 550 ^{0}C in a muffle furnace for 4 hours. The ash residue was dissolved in a 4 ml dilute HNO_3, filtered through acid-washed filter paper in a 50 ml volumetric flask and the volume was made up to the mark. The estimation of Phosphorous was carried out in the dry-ashed sample solution with the aid of spectrophotometer. Phosphorus in the form of phosphate ion (PO_4^{-3}) was determined by Ascorbic acid – Molybdate method as follows: 0.25 ml of each serial standard /sample was added to 2.5 ml of colour developing reagent (CDR) (prepared from: 50ml H_2SO_4 + 5 ml PAT + 30 ml OF Am and 15 ml of AA) and incubated at room temperature for 20 minutes. The absorbance was read at 770 nm on the spectrophotometer. A standard or calibration curve plotted from the standard values and an equation of the curve was generated. The concentration of the unknown was then calculated from the equation.

Determination of Potassium

Two (2) g of the soil sample was weighed and placed in a silica crucible and ashed at 550 ^{0}C in a muffle furnace for 4 hours. The ash residue was dissolved in a 4 ml dilute HNO_3, filtered through acid-washed filter paper in a 50 ml volumetric flask and the volume was made up to the mark. The estimation of Potassium was carried out in the dry-ashed sample solution with the aid of spectrophotometer. Six serial standards of 2, 5, 10, 20, 30, 50 mg/l were prepared from a standard stock solution of 100 mg/l K^+. Each serial standard was aspirated starting from the least and the read out was noted. The unknown sample was also aspirated and read out was recorded. A standard or calibration curve plotted from the standard values and an equation of the curve was generated. The concentration of the unknown was then calculated from the equation.

Determination of soil organic matter / organic carbon

Loss in weight on ignition method was used as a direct measure of the OM contained in the soil. 10.0 g of sieved (2 mm) soil was weighed into an ashing vessel (50 ml beaker). The ashing vessel with the soil was placed in a drying oven set at 105 °C and dry for 4 hours. It was removed from the drying oven and placed in a dry atmosphere for it to cool. After cooling, 0.01 g of the soil was weighed (W_1). The ashing vessel with the rest of the soil was placed into a muffle furnace, and the temperature set to 400 °C and allowed in the furnace for 4 hours. The ashing vessel was then removed from the muffle furnace, cooled in a dry atmosphere, and weighed to the nearest 0.01 g (W_2). The percentage of OM is given by:

Percent organic matter (OM) = $(W_1 - W_2)/W_1 \times 100$

Where: W_1 is the weight of soil at 105 °C;

W_2 is the weight of soil at 400 °C.

The percentage of organic (C) in the soil is given by: % OM × 0.58.

3.2.2 Poultry droppings

The Poultry droppings were collected from HASCO poultry farm at Half-Assini in the western region of Ghana. It was air-dried and then pounded into power. The moisture content, pH, organic matter content and nutrient content were all determined just as in the case of the contaminated soil.

3.2.3 Compost

The compost was obtained from Newmont mining company Ltd at Kenyase in the Brong Ahafo region of Ghana. It was air-dried, pounded and then passed through a 2 mm sieve to remove undecomposed materials from it. The moisture content, pH, organic matter content and nutrient content were all determined just as in the case of the contaminated soil as part of the baseline studies.

3.2.4 Natural rubber processing sludge

The Natural rubber processing sludge was obtained from the Ghana Rubber Estate Ltd (GREL) Factory at Apemanim, near Takoradi in the Western Region of Ghana. It was sun-dried, grind and then passed through a 2 mm sieve to obtain fine powder. The moisture content, pH, organic matter content and nutrient content were all determined just as in the case of the contaminated soil as part of the baseline studies.

3.3.0 Methods

3.3.1 Experimental Design

The experimental design consisted of amending 1 kg samples of petroleum oil contaminated soil with three different nutrient supplements all of which were organic materials using three different nitrogen concentrations; 0.2 % N, 0.4 % N and 0.6 % N of each of the poultry droppings, compost, and rubber sludge. Three replicates of each treatment where prepared. The study was laboratory based but was carried out in the open at room temperature.

3.3.2 Bioremediation experiment

Preparation of experimental units

The petroleum contaminated soil was obtained from Adamus Resources Limited (ARL), a mining company at Salma, near Nkroful in the Western Region of Ghana. The bulk soil sample was air-dried to avoid the oil from undergoing volatilization. It was then mixed thoroughly to obtain a homogeneous sample. Approximately 1 kg of the oil-contaminated soil was placed in rectangular plastic trays (20 cm × 14 cm) and 14 cm deep. The first three set of the soil samples were treated with 0.2 % N from each of compost (C), poultry droppings (PD) and natural rubber sludge (RS), another three set of the soil samples were treated with 0.4 % N from each of compost(C), poultry droppings (PD) and natural rubber sludge (RS), while the last three set of soil samples were treated with 0.6 % N from compost (C), poultry droppings (PD) and natural rubber sludge (RS). The control was 1kg oil contaminated soil without any nutrient supplement. Three replicates were prepared for each treatment, making a total of 30 experimental units.

The different soil blends in the various plastic containers were properly mixed to obtain homogeneous units. Some physico-chemical parameters like moisture content, temperature, pH, TPH as well as the nitrogen, phosphorus, and carbon contents were determined as values for day zero (See table 4.1).

3.3.3 Monitoring Process

Moisture management in experimental units

To determine appropriate moisture levels, the moistened soil was compared visually to sealed jars with the same type of soil previously wetted to 40, 50, 60, 70 and 80 % of field capacity. The field capacity of the soil was predetermined by weight difference between completely dry soil and saturated soil which had been allowed to free-drain overnight, according to Zavala et al. (2005).

After determining the initial moisture content of the various soil blends, the moisture content of each soil blend was raised to 60 % of its field capacity by adding the required amount of distil water. The amount of distil water that was added to each soil blend were calculated using the following relation: If we change X g of soil sample with initial water content a to a final water content b ($b > a$), the following equation can be used to calculate the amount of water (g) to be added: $W_a = \frac{(b-a)X}{(1+a)}$.

The moisture content was maintained at approximately 60 % of field capacity throughout the eight weeks period of the experiment by raising it weekly. Deionized water was then added to the pans to achieve soil moisture content of approximately 60 % of the water holding capacity (Nocentini et al., 2000).

Oxygen management in experimental units

The experiment was an aerobic process and as such the containers were left open to ensure adequate supply of oxygen at all times. Also, the soil samples were regularly stirred and turned three times each day with the help of satirized stainless steel rods.

Temperature measurement in experimental units

These trays were incubated in the open but under a shade at approximately 28 to 30 °C. The mean daily temperatures of each soil blend were taken in the morning (6 am), afternoon (12 noon) and evening (6 pm). There were variations among the different amendment materials and the different treatments but these were not significantly different.

pH measurement in experimental units

The pH of each experimental unit was measured every week. To five grams (5.0 g) of each soil sample (in a sample cell) was added 50 ml of distilled water. The lump of the soil was stirred to form homogenous slurry, then the probe of the pH meter (Jenway 3015 model) was immersed into the sample and allowed to stabilize at 25 ^{o}C and pH of sample was recorded.

The pH Sensor Meter has a single point calibration. The electrode was placed in fresh pH 7 buffer and the Sensor Meter switched on. The "Set pH 7" key was pressed and the reading was allowed to stabilise. After calibration, the Sensor Meter was checked using pH 4 and pH 9 buffer solutions to ensure that the error in the reading was no more than 0.1 pH unit. The calibration was: $\text{pH} = \frac{\text{Raw data value} \times 14}{54560}$ (Crellin, 1999).

3.3.4 Total Petroleum Hydrocarbon (TPH) analysis

Extraction of oil from soil for TPH analysis

Ten (10) g of each soil sample were removed in duplicate from the soil microcosms and mixed with an equal mass of anhydrous sodium sulphate. The mixture was placed in a Whatman cellulose extraction thimble. The oil residue remaining in this sample was extracted with 200 ml of dichloromethane (DCM) for 2 h at a rate of 4 cycles h^{-1} using the Soxhlet apparatus (Helaleh et al., 2001). The DCM fraction was collected in a pre-weighed 250 ml round bottomed flask and the DCM evaporated using a rotary evaporator at 40 °C. The remaining oil residue was quantified by weight to determine the amount of TPH degraded over time. The percentage of TPH degradation was determined using the amount of TPH in the 10 g of contaminated soil sample after the same extraction process at day 0 as 100 %.

Gas chromatography-mass spectroscopic analysis

Samples of soil (100 g each) removed at the initial and final stages of the experiment were analyzed by GC-FID to determine the quantity and composition of the total hydrocarbons. GC-FID analyses of all samples were carried out in the Ghana standard board laboratory, Accra. A Hewlett-Packard 5890 series GC system coupled to a mass spectrophotometer VG TRIO 2000 was used for the analysis. The GC-FID was equipped with a SPB-1701 capillary column (30 m × 0.25 mm i.d × 0.25 μm film thickness) for separation, and helium carrier gas flow was 0.9 μl min^{-1} (set at 100 °C). The injection port temperature was maintained at 250 °C. The headspace was set at 60. kPa. The column oven was initially held at 100 °C for 2 min, increase to 200°C at a rate of 10°C min^{-1}, then to 250 °C at 20 °C min^{-1} (held for 5 min). Data was acquired in the full scan detection mode from 45 to 350 a.m.u at the rate of one scan

per second. The concentration of each carbon length was determined by comparing to a known concentration of the standard.

3.3.5 Microbial analysis

Total viable count of heterotrophic bacterial were isolated and enumerated by pour plate method and growth on plate count agar (PCA). Serial dilutions of 10^{-1} to 10^{10} were prepared by diluting 1 g of the soil blend from each pan into 10 ml of sterilized distilled water. One millilitre aliquots from each of the dilutions were inoculated on Petri dishes with already prepared PCA. The plates were then incubated at 35 ^{o}C for 24 hours. After incubation, all white spots or spread were counted and recorded as total viable counts using the colony counter.

3.3.6 Statistical Analysis

Analysis of variance (ANOVA) was used to determine if the relationships between treatment conditions were statistically significant ($p > 0.05$) at various time points during the experiments. Tukey 99.0% simultaneous confidence intervals were used for this analysis and results were generated using the Student Edition of Statistix 9.0 software.

CHAPTER FOUR

4.0 RESULTS

4.1 Baseline study results

Table 4.1: Baseline parameters of soil and amendment materials

Samples	Nitrogen	Phosphorus	Potassium	Carbon	pH	Moisture	C:N
Oil Contaminated Soil	0.03±0.01	0.12±0.01	0.05±0.02	4.84±0.04	7.21±0.03	1.43±0.02	80:3
Compost	1.47±0.05	0.33±0.05	0.13±0.03	5.91±0.03	7.26±0.16	6.95±0.14	4:1
Poultry Dropping	1.82±0.03	0.29±0.01	0.23±0.03	4.42±0.16	7.98±0.18	5.65±0.03	7:3
Rubber Sludge	3.29±0.03	0.35±0.02	0.10±0.05	5.07±0.02	7.37±0.11	5.00±0.35	3:2
Lsd	0.04	0.04	0.04	0.04	0.04	0.04	
Cv%	1.21	7.26	15.56	0.40	0.29	0.42	

Table 4.1 shows that the oil contaminated soil contained 0.03 % N while the organic nutrient supplements recorded high nitrogen levels. The rubber sludge recorded the highest N content of 3.29 %. Similar trend can be observed for phosphorus. The highest value for Potassium is 0.23 % recorded by the poultry droppings. In term of pH, all materials recorded values a little above 7. The compost recorded the highest value for organic carbon as well as the moisture content.

Based on the above results, the amount of each nutrient source was calculated and added to the 1kg samples of the oil contaminated soil. This brought the nitrogen level of the samples to 0.2%. 0.4% and 0.6%. The C: N ratio of the soil blend was also changed from 80:3 to 100:20 after the addition of the organic nutrient supplements. The C: N ratio is a very critical factor in bioremediation.

4.2 Experimental results

4.2.1 Changes in TPH concentrations during incubation

The soil blends were incubated for eight (8) weeks at a temperature of about 30 ^{O}C. The percentage TPH degradation of the various treatments at the various sampling days is presented in table 4. 2 below:

Table 4.2: Percentage Decrease in TPH during incubation (%)

Treatments	Week 2	Week 4	Week 8
Control	24.67 ± 0.05	46.79 ± 0.02	50.20 ± 0.55
0.2% N C	83.84 ± 0.45	85.94 ± 0.06	95.41 ± 0.30
0.2% N PD	82.93 ± 0.56	98.55 ± 0.20	99.06 ± 0.39
0.2% N RS	73.10 ± 0.46	84.73 ± 0.14	93.53 ± 0.62
0.4% N C	71.51 ± 0.25	84.75 ± 0.52	91.82 ± 0.64
0.4% N PD	51.59 ± 0.73	76.72 ± 0.52	92.13 ± 0.27
0.4% N RS	47.81 ± 0.64	84.45 ± 0.29	90.92 ± 0.60
0.6% N C	45.32 ± 0.34	72.55 ± 0.50	83.17 ± 0.45
0.6% N PD	54.24 ± 0.41	75.48 ± 0.26	81.33 ± 0.51
0.6% N RS	64.47 ± 0.57	83.05 ± 0.43	86.55 ± 0.50

C is Compost, **PD** is Poultry Droppings and **RS** is Rubber Sludge.

4.2.1.1 Comparison of 0.2% N Concentrations at Week 2

As shown in table 2 above, with an initial TPH of 1009.12 mg/kg, there was a sharp reduction of the petroleum hydrocarbons from week zero to week two in all the treatments especially in the soil amended with 0.2 % N using Poultry droppings, compost and Rubber Sludge. The reduction in TPH at week 2 was massive, for example, it decreases from 1009.12 mg/kg to as low as 163.11 mg/kg in the soil treated with compost representing 83.84 %, from 1009.12 mg/kg to 172.25 mg/kg in the soil treated with Poultry droppings representing 82.93% and from 1009.12 mg/kg to 271.49 mg/kg representing 73.10 % in the Rubber Sludge amended soil where as

the control (soil without nutrient supplement) recorded the lowest reduction from 1009.12 mg/kg to 760.20 mg/kg representing only 24.67 %. This trend can be seen in table 4.2.

4.2.1.2 Comparison of 0.4%N Concentration at Week 2

There was general reduction of total petroleum hydrocarbons in the soils treated with 0.4 % N from all the three organic nutrient supplements similar to those of the soils amended with 0.2 % N but of lower quantum. The reduction was from 1009.12 mg/kg to 287.50 mg/kg, 488.56 mg/kg and 526.66 mg/kg representing 71.51 %, 51.59% and 47.81% in the soil amended with 0.4 % N from compost, 0.4 % N from poultry droppings and 0.4 % N from rubber sludge respectively (Table 4.2).

4.2 .1.3 Comparison of 0.6%N Concentration at Week 2

The Percentage TPH degradation of the soil blend treated with 0.6 % N from the compost, poultry droppings and the rubber sludge did not followed the same pattern as in the case of those with 0.2 % N and 0.4 % N. The degradation was rather higher in the soil with 0.6 % N from rubber sludge (64.47 %), followed by the one with 0.6 % N from poultry droppings (54.24 %) and finally the soil with 0.6 % N from compost (45.32 %) as shown in table 4.2.

4.2.1.4 Comparison of 0.2%N Concentration at Week 4

The TPH degradation from week 2 to week 4 was not as quick as was the case from week zero to week 2. For the 0.2 % N concentrations at week 4, the total petroleum hydrocarbons degradation was 98.55% in the poultry droppings, 85.94 % in the compost, and 84.73% in the rubber sludge. These were all significantly different from

the control (soil without nutrient supplement) which recorded just 46.79 % degradation (table 4.2).

4.2.1.5 Comparison of 0.4 % N Concentration at Week 4

The soils treated with 0.4 % N concentration at week 4 recorded the following percentage TPH degradation: 84.75 %, 76.72 % and 84.45 % in soil with compost, poultry droppings and rubber sludge respectively. Though these were significantly different from the 46.79% degradation recorded in the control, the 84.75 % in compost and the 84.45 % in the rubber sludge were not significantly different from each other.

4.2.1.6 Comparison of 0.6 % N Concentration at Week 4

The Percentage TPH degradation of the soil blend treated with 0.6 % N from the compost, poultry droppings and the rubber sludge at week 4 just like in week 2, did not followed the same pattern as in the case of those with 0.2 % N and 0.4 % N. The degradation was also higher in the soil with 0.6 % N from rubber sludge (83.05 %), followed by the one with 0.6 % N from poultry droppings (75.48 %) and finally the soil with 0.6 % N from compost (72.55 %) as showed in table 4.2.

4.2.1.7 Comparison of 0.2 % N Concentration at Week 8

The pattern and level of TPH degradation at week 8 (i.e. the end of the experiment) was similar to the trend recorded at week 4 but of a little lower quantum. At the end of the 60 days incubation, the soil treated with 0.2 % N from poultry droppings recorded the highest percentage TPH degradation (99.06 %) followed by the soil amended with 0.2 % N from compost (95.41 %), and then the soil treated with 0.2 %

N from rubber sludge (93.53 %) while the control recorded a much lower percentage of 50.2 % (table 4.2).

4.2.1.8 Comparison of 0.4 % N Concentration at Week 8

The level of TPH degradation after 60 days of incubation within the soil amended with 0.4 % N were lower than those recorded by soil with 0.2 % N. The percentage TPH degradation however followed the same pattern as those of the soil with 0.2 % N as follows: 92.13 % in the soil amended with 0.4 % N from poultry droppings, 91.82 % in the soil amended with 0.4 % N from compost, and then 90.92 % in the soil amended with 0.4 % N from the rubber sludge (table 4.2).

4.2.1.9 Comparison of 0.6 % N Concentration at Week 8

The Percentage TPH degradation of the soil blend treated with 0.6 % N from the compost, poultry droppings and the rubber sludge at week 8 was just like in week 2 and week 4. The degradation was higher in the soil with 0.6 % N from rubber sludge (86.55 %), followed by the one with 0.6 % N from compost (83.17 %) and finally the soil with 0.6 % N from poultry droppings (81.33 %) as showed in table 4.2.

4.2.2 Microbial count during the 8 weeks of incubation (cfu/g soil)

As part of the monitoring process, the different soil blends were sampled immediately after mixing the components, the soils were analysed to establish the baseline microbial population at week zero. The microbial population of each treatment were monitored at each sampling dates just as was the case for the TPH at week 2, 4 and 8. The results of the microbial populations are presented in table 4.3.

Table 4.3: Microbial count during the incubation (CFU / g soil)

Treatment	Week 0	Week 2	Week 4	Week 8
Control	1.14×10^4	3.60×10^5	3.20×10^5	2.38×10^3
0.2% N C	4.28×10^7	6.50×10^8	6.60×10^9	6.82×10^8
0.2% N PD	4.12×10^7	6.20×10^8	8.68×10^9	9.43×10^8
0.2% N RS	3.29×10^7	5.40×10^8	4.40×10^9	3.90×10^8
0.4% N C	5.38×10^7	5.10×10^7	4.48×10^9	2.20×10^8
0.4% N PD	5.47×10^7	5.18×10^7	3.70×10^8	2.25×10^8
0.4% N RS	4.14×10^7	4.76×10^7	4.27×10^9	1.54×10^8
0.6% NC	5.68×10^7	4.40×10^7	2.72×10^8	7.50×10^7
0.6% N PD	5.76×10^7	6.84×10^7	3.45×10^8	6.15×10^7
0.6% N RS	4.92×10^7	9.23×10^7	4.10×10^9	1.33×10^8
Tukey $HSD_{(0.05)}$	**2.19×10^7**	**4.95×10^6**	**5.27×10^9**	**3.62×10^8**

NB. C is Compost, **PD** is Poultry Droppings and **RS** is Rubber Sludge.

There was a general increase in microbial count in all treatments from week zero to week 4, and then a slight decrease in all treatments at week 8 (Table 4.3). The total microbial counts of the control were found to be lower than all the other treatments at each sampling points. Also, unlike the other treatments, the control started decreasing at week 4 while the others continued to increase until the end of week 8. The control recorded a microbial population of 1.14×10^4 CFU/g soil at week zero, increased to 3.60×10^5 CFU/g soil at week 2 and ended up with 2.38×10^3 CFU/g soil at week 8 (Table 4.3).

The levels of microbial counts were much higher in all the nutrients amended soil compared to the control, for example, the 0.2 % N from the three nutrient sources at week 2, shows that the soil treated with compost recorded the highest microbial count of 6.50×10^8 CFU/ g soil, followed by the soil treated with poultry droppings (6.20 x

10^8 CFU/ g soil) and then the one treated with rubber sludge (5.40 x 10^8 CFU/ g soil). However, at week 4, it was the soil treated with poultry droppings which recorded the highest count of 8.68 x 10^9 CFU/ g soil (the highest in all), followed by the soil treated with compost (6.60 x 10^9 CFU/ g soil) and then the one treated with rubber sludge (4.40 x 10^9 CFU/ g soil). At week 8, the soil treated with poultry droppings once again recorded the highest count of 9.43 x 10^8 CFU/ g soil, followed by the soil treated with compost (6.82 x 10^8 CFU/ g soil), while the soil treated with rubber sludge recorded (3.90 x 10^8 CFU/ g soil).

Table 4.3 above also indicates that, although the soils treated with 0.4 % N recorded a higher microbial count initially compare to their counterparts with 0.2 % N, they all recorded a lower counts at week 2 compare to those of 0.2 % N. The 0.4 % N from poultry droppings recorded the highest count of 5.18 x 10^7 CFU/g soil, followed by the soil treated with compost (5.10 x 10^8 CFU/ g soil), while the soil treated with rubber sludge recorded (4.76 x 10^8 CFU/ g soil). This trend could not be maintained at week 4. At week 4, the poultry droppings rather recorded the lowest counts of 3.70 x 10^8 CFU/ g soil while the compost and the rubber sludge had 4.48 x 10^9 CFU/ g soil and 4.27 x 10^9 CFU/ g soil respectively. The poultry droppings treated soil however ended with microbial population of 2.25 x 10^8 CFU/ g soil followed by the soil treated with compost (2.20 x 10^8 CFU/ g soil), and the soil treated with rubber sludge had 1.54 x 10^8 CFU/ g soil.

Soil samples treated with 0.6 % N recorded higher initial microbial counts than those of the 0.2 % N and 0.4 % N, but recorded lower counts at weeks 2, 4 and 8, compared to their corresponding sources of 0.2 % N and 0.4 % N concentration levels, at except 0.6 % N from rubber sludge and 0.6 % N from compost at week 2 which recorded

much higher values of 9.23 x 10^7 CFU/ g soil and 6.84 x 10^7 CFU/ g soil respectfully. A careful observation shows that 0.6 % N from rubber sludge performed better than the 0.6 % N from poultry droppings and Compost at all sampling days except at week zero (table 4.3).

4.2.3 Percentage Decrease in total nitrogen during 8 weeks of incubation

Table 4.4: Decrease in Total Nitrogen during the incubation (mg/g soil)

Treatment	Week 0	Week 2	Week 4	Week 8
Control	0.03± 0.005	0.05± 0.005	0.04± 0.005	0.02± 0.005
0.2% N C	0.20± 0.005	0.17± 0.005	0.15± 0.005	0.13± 0.005
0.2% N PD	0.20± 0.005	0.18± 0.005	0.14± 0.011	0.12± 0.005
0.2% N RS	0.20± 0.011	0.19± 0.005	0.17± 0.005	0.14± 0.011
0.4% N C	0.40± 0.005	0.34± 0.010	0.31± 0.005	0.26± 0.005
0.4% N PD	0.40± 0.010	0.32± 0.005	0.26± 0.005	0.22± 0.011
0.4% N RS	0.40± 0.005	0.36± 0.005	0.28± 0.005	0.27± 0.010
0.6% N C	0.60± 0.005	0.47± 0.010	0.43± 0.005	0.32± 0.005
0.6% N PD	0.60± 0.005	0.44± 0.011	0.40± 0.020	0.22± 0.005
0.6% N RS	0.60± 0.010	0.42± 0.005	0.32± 0.100	0.29± 0.015

NB. **C** is Compost, **PD** is Poultry Droppings and **RS** is Rubber Sludge.

The Total Kjedahl Nitrogen levels decreased in all treatments as expected, except the control which recorded an increase from 0.03 % in week zero to 0.05 % in week 2. It however decreases to 0.04 % in week 4 and finally ended with 0.02 % in week 8. The trend shows that, among the 0.2 % N concentrations, the poultry droppings had the greatest reduction in week 4 and week 8, while the compost recorded the greatest reduction in week 2. Also in all cases, the 0.2 % N from rubber sludge recorded the lowest reduction.

Reduction in total nitrogen among the 0.4 % N levels followed almost the same pattern as those of the 0.2 % N. The poultry droppings recorded the highest reduction at all times followed by the compost, except in week 4 where the rubber sludge had a greater reduction than the compost. A close look at table 4.4 above indicates that, 0.6 % N from the rubber sludge recorded the highest reduction in total nitrogen in week 2 and week 4 compared to the 0.6 % N from compost and poultry droppings. The 0.6 % N from poultry droppings however recorded the greatest reduction in week 8 (Table 4.4).

4.2.4 Decrease in organic carbon during 8 weeks of incubation

Table 4.5: Decrease in Organic Carbon during the incubation (mg/g soil)

Treatment	Week 0	Week 2	Week 4	Week 8
Control	0.88±0.04	0.84±0.02	0.78±0.06	0.72±0.04
0.2% N C	2.53±0.08	1.45±0.17	1.38±0.15	1.33±0.10
0.2% N PD	2.21±0.10	1.22±0.03	1.22±0.03	1.07±0.05
0.2% N RS	2.34±0.10	1.32±0.04	1.30±0.02	1.19±0.16
0.4% N C	3.78±0.07	2.48±0.10	1.78±0.10	1.71±0.04
0.4% N PD	3.23±0.10	1.91±0.02	1.41±0.05	0.96±0.07
0.4% N RS	3.64±0.10	2.47±0.10	1.55±0.04	1.26±0.10
0.6% N C	3.87±0.17	2.67±0.05	1.95±0.16	1.26±0.21
0.6% N PD	3.41±0.02	2.34±0.04	1.69±0.17	1.12±0.20
0.6% N RS	3.51±0.04	2.41±0.41	1.24±0.20	1.05±0.15

NB. C is Compost, **PD** is Poultry Droppings and **RS** is Rubber Sludge.

During the incubation, soil organic carbon values decreased rather homogeneously with time (Table 4.5). Looking at the initial values (week zero) of each treatment, the control recorded the lowest organic carbon level while the highest organic carbon level was recorded by the 0.6 % N from compost. Also at any given time, the control

had the lowest organic carbon content compared to all other treatments. Furthermore, comparing 0.2 % N concentrations, the results show that the compost at any given time recorded the highest organic carbon, followed by the rubber sludge and then the poultry droppings (Table 4.5). This pattern can also be seen among the 0.4 % N and 0.6 % N levels except in week 4 and week 8 of the 0.6 % N in which the 0.6 % N from rubber sludge recorded higher values than the compost and the poultry droppings. At the end of the experiment, all the treatments recorded significantly lower organic carbon values than the initial values, thus showing an effective decrease during the 60 days incubation period.

4.2.5 Decrease in organic matter during the incubation

As part of the activities for monitoring the rate of bioremediation of the petroleum hydrocarbons, the percentage organic matter content of the soil blends including the control were determined at week 0, 2, 4 and 8, just as the other parameters. The results are presented in table 4.6 below.

Table 4.6: Decrease in Organic matter during the incubation (mg/g soil)

Treatment	Week 0	Week 2	Week 4	Week 8
Control	3.41±0.05	3.38±0.05	2.23±0.02	2.10±0.25
0.2% N C	3.67±0.10	2.70±0.10	2.58±0.02	2.50±0.10
0.2% N PD	4.36±0.01	2.33±0.04	2.32±0.03	2.05±0.02
0.2% N RS	3.46±0.02	2.48±0.03	2.44±0.04	2.26±0.02
0.4% N C	5.86±0.04	4.81±0.02	3.27±0.02	3.15±0.02
0.4% N PD	4.48±0.02	3.50±0.03	2.63±0.03	1.85±0.04
0.4% N RS	5.62±0.03	4.41±0.03	2.87±0.02	2.38±0.03
0.6% N C	6.86±0.02	4.87±0.04	3.86±0.02	3.39±0.02
0.6% N PD	5.26±0.01	4.25±0.05	3.13±0.02	2.15±0.02
0.6% N RS	5.39±0.04	4.37±0.03	4.07±0.03	3.38±0.03

NB. C is Compost, **PD** is Poultry Droppings and **RS** is Rubber Sludge.

In all treatments, there was a general decrease in the percentage organic matter from week zero up to week 8 (Table 4.6). The control contains the lowest percentage organic matter at each sampling date as compare to the other treatments. A careful look at table 6 reveals that the 0.6 % N compost recorded the highest percentage organic matter (6.86 %) in week zero while 0.4 % N poultry droppings recorded the lowest percentage organic matter (1.85 %) in week 8. Comparing 0.2 % N levels, the poultry droppings recorded the highest value of 4.36 %, followed by the compost (3.67 %) and then the rubber sludge (3.46 %) at week zero. However, from week 2 to week 8, the compost recorded the highest percentage organic matter followed by the rubber sludge and then the poultry droppings. The trend among 0.4 % N levels differ from that of the 0.2 % N levels, it is the rubber sludge which rather recorded the highest values from week zero to week 4 but had the second highest in week 8. Finally, the pattern among the 0.6 % N concentrations shows that the compost recorded the highest percentage organic matter at each sampling date.

4.2.6 pH values during 8 weeks of incubation

During the 60 days of incubation, the pH values of the soil blends were monitored weekly. This was done by using 10 g soil: 20 ml of distilled water. The results of pH monitoring are shown in table 4.7 below.

Table 4.7: Variation in pH during the 8 weeks of incubation

Treatment	Week 0	Week 2	Week 4	Week 6	Week 8
Control	7.80±0.02	7.62±0.08	7.41±0.05	7.39±0.07	7.26±0.04
0.2% N C	7.26±0.05	6.93±0.03	6.88±0.04	6.85±0.04	6.72±0.04
0.2% N PD	7.98±0.06	7.67±0.11	7.02±0.10	6.92±0.04	6.78±0.04
0.2% N RS	7.52±0.05	7.42±0.05	7.12±0.05	7.02±0.05	6.97±0.02
0.4% N C	6.56±0.07	6.36±0.03	6.07±0.05	5.86±0.02	5.80±0.04
0.4% N PD	8.20±0.13	7.56±0.03	6.74±0.05	6.69±0.03	6.63±0.03
0.4% N RS	7.10±0.02	7.07±0.05	6.47±0.04	6.32±0.04	6.24±0.02
0.6% N C	6.64±0.11	6.51±0.07	6.30±0.04	5.95±0.04	5.86±0.04
0.6% N PD	8.40±0.14	8.20±0.05	7.67±0.03	7.22±0.04	7.18±0.11
0.6% N RS	7.32±0.10	7.10±0.04	6.89±0.03	6.62±0.04	6.45±0.05

NB. C is Compost, **PD** is Poultry Droppings and **RS** is Rubber Sludge.

In this study, the pH of all samples was measured every week, and a general continuous reduction of pH can be seen in the results in table 4.8 above. The pH of the soil samples decreased to as low as 5.86 during 60 days of incubation. The result also shows that the control recorded a pH value of 7.80 in week zero and decreased to 7.26 at the end of week 8, indicating a gradual reduction but remains within a slightly alkaline pH. At the end of the eight weeks of incubation, it was only the control and the 0.6 % N compost which recorded values above 7. A close observation of table 4.7 also reveals that, from week 2 to week 8, all 0.2 % N, 0.4 % N and 0.6 % N compost amended soils recorded pH values less than 7.

4.2.7 Temperature during the first week of Incubation

The mean daily temperature recordings of each sample were determined for the first seven days and the results are presented in table 4.8 below.

Table 4.8: Variation in temperature during the first week of incubation

Treatment	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7
Control	30.7±0.04	32.0	33.0	33.3	33.5	33.7	32.6
0.2% N C	34.2	37.8	38.3	39.2	43.0	37.8	32.8
0.2% N PD	36.3	37.7	38.3	39.3	44.0	37.7	33.7
0.2% N RS	34.0	37.3	38.1	40.7	42.5	38.1	32.6
0.4% N C	36.8	39.3	40.2	44.0	44.3	36.8	33.2
0.4% N PD	38.1	40.3	45.5	45.5	46.6	38.1	33.5
0.4% N RS	37.3	38.5	41.4	42.3	42.5	37.3	33.1
0.6% N C	37.8	39.5	41.2	42.0	42.3	37.8	33.8
0.6% N PD	38.8	44.0	45.2	49.3	50.0	38.8	35.0
0.6% N RS	37.0	39.0	42.2	43.0	43.5	37.0	34.6

NB. C is Compost, **PD** is Poultry Droppings and **RS** is Rubber Sludge.

There was a general increase in temperature from day 1 to day 5 and then a decrease from day 6 to day 7 in all treatments including the control. All treatments recorded the highest temperature values on day 5 and the lowest on day 7. The variations between 0.2 % N levels were not significant and so were those of the 0.4 % N and 0.6 % N levels with the exception of the 0.6 % N poultry droppings which happens to record higher values on all days compare to the compost and the rubber sludge. It can also be observed that almost all treatments recorded temperature values between 32˚C and 35˚C indicating that they have become the same as the atmospheric temperature after day 6.

CHAPTER FIVE

5.0 DISCUSSION

5.1 General TPH reduction

Total petroleum hydrocarbons (TPH) reduction during the eight weeks bioremediation as reported in Table 4.2, shows a large loss of hydrocarbons from 1009.12 mg/kg at the start of the incubation to as low as, 172.25 mg/kg representing 82.93 % TPH degradation in just two weeks. This is certainly due to biological activities and not volatilization, though there could be loss in TPH due to volatilization as a result of the frequent mixing of the soil samples, carried out in order to promote aeration. This however was negligible because the experiment was done at room temperature. Namkoong *et al*. (2000) reported a very small volatilization of TPH (less than 3%) compared with biodegradation. The control soil showed, at the end of the second week, a decrease of about 24.67 % in TPH; such a loss was significantly lower relative to the other treatments which recorded values from 45.32 % to 83.84 % (table 4.2) on the same date. The differences suggest that, the addition of nutrients from the various organic sources has contributed positively to the biodegradation by increasing the microbial community present in the soil. Previous studies suggest that nutrient supplementation stimulates bioremediation by increasing microbial biomass (Walworth *et al*., 2006). In all of these cited reports biostimulation caused a rapid start to bioremediation and this is clearly shown in this study.

5.2 Changes in TPH Content versus Microbial Populations

A general comparison of all the treatments shows that the 0.2 % N levels performed very well in terms of TPH degradation than the 0.4 % N and the 0.6 % N (Table 4.2). This observation can be attributed to the high microbial counts in the 0.2 % N

treatments at all sampling dates except week zero, where the 0.4 % N and 0.6 % N levels had higher counts (Table 4.3). The 0.2 % N amended soils recorded values between 5.40 x 10^8 CFU/g soil to 6.50 x 10^8 CFU/g soil at week 2, where as the rest of the treatments (the 0.4 % N and the 0.6 % N) recorded microbial counts far less, 4.40 x 10^7 CFU/g soil to 9.23 x 10^7 CFU/g soil (Table 4.3). The 0.4 % N and 0.6 % N levels might have supplied higher nitrogen levels to the soil, and that tends to be toxic to the microbes and so led to lesser microbial populations.

Comparing 0.2 % N levels at week 2, the compost and the poultry droppings were not significantly different from each other but at week 4 and 8, they were statistically significant at $p < 0.05$ from the control experiment, with the poultry droppings performing better than the rest. The TPH level was significantly lower in the Poultry Droppings amended soils relative to the other organic amendments (Table 4.2). Similar result was reported by Adesodun and Mbagwu (2007) in the study of effect of some organic wastes on oil polluted soil. This could be attributed to quick net nitrogen mineralization of Poultry Droppings due to its narrow C/N ratio and the variations in the nutrient element composition of the amendments (Table 4.1). The compost performed better than the rubber sludge at week 8 perhaps due to its high fibre content which makes the soil to be friable (increased the soil pores) leading to enough aeration hence greater bioactivity leading to higher TPH degradation. The rapid degradation of hydrocarbons in the compost system was expected since it is rich in nutrients and has high microbial population (Schmitt and Rehn, 2002).

The 0.4 % N concentrations recorded lower TPH degradations relative to the 0.2 % N levels because they contain nitrogen levels higher than the recommended level for effective bioremediation and this leads to the utilization of the nitrogen as energy

source in preference to hydrocarbons. Among the 0.4 % N levels, the compost performed better at week 2 and week 4 but was not significantly different at week 8 compare to the poultry droppings. This trend could be attributed to high temperature recorded by the poultry droppings as high as 46 ^{o}C during the first week of incubation (Table 4.8) which became inhibitory to continued microbial growth, resulting in a decrease in microbial population (Table 4.3). A subsequent decrease in temperature in the seventh day (Table 4.8) resulted in increase in microbial activity at the end of week 2. This phenomenon has been previously reported by Potter *et al*. (1999).

As mentioned above, the soil treated with 0.6 % N recorded lower percentage TPH degradation compare to those of 0.2 % N and 0.4 % N because of the very high nitrogen levels they supplied to the microbes which negatively affected the microbial activity in these soils. Padayachee and Lin, (2011), reports that excess nitrogen can depress microbial activity and petroleum degradation in contaminated soils due to the depression of osmotic soil water potential. A comparison of the percentage TPH degradation among the 0.6 % N levels (Table 4.2) shows that RS > PD > C. The rubber sludge performed better than the others possibly due to the fact that it releases the nitrogen gradually to the microbes hence the higher microbial counts recorded by the 0.6 % N RS at week 2, 4 and 8 than the rest of the other organic nutrient supplement materials (Table 4.2 and 4.3).

5.3 Changes in TPH versus, Total Organic Carbon and Organic Matter

During the incubation, soil organic carbon and organic matter values decreased rather homogeneously with time (Table 4.5 and 4.6). All treatments show higher values than the control (Table 4.6). This is expected because there was no organic material added to the control. The soils treated with compost recorded the highest values at each

sampling date and this can be attributed to the high initial value which is also due to the high fibre content of the compost (Table 4.1). The decrease in organic carbon as well as the organic matter corresponds with the TPH reduction showed in table 4.2 above. At the end of the experiment, organic carbon values were significantly lower than the initial ones, thus showing an effective decrease during the eight weeks incubation period. These results agree with those reported by Ceccanti *et al*. (2003) in a study on the bioremediation of a hydrocarbon contaminated soil.

5.4 Changes in TPH Content versus Total Nitrogen

Table 4.4 above shows that, all treatments recorded a decrease in total Kjedahl nitrogen with the exception of the control which had an increase in week 2 and then decreased in week 4 and week 8. The increase in Nitrogen by the control from 0.03 mg/g soil to 0.05 mg/g soil in week 2 could be due to the decomposition of some plants and animal remains which added some amount of Nitrogen to the soil. The low nitrogen value recorded by the control is obvious because it was devoid of nutrient supplements. All treatments recorded homogeneous reduction in N from week zero to the end of the experiment. The 0.6 % N recorded a massive decrease in N in week 4 (Table 4.4) and this corresponds with the massive reduction in TPH it recorded in that same week (Table 4.2).

5.5 Changes in TPH Content versus variation in pH

Nutrient addition increased the pH of the samples from 7.21 to higher values except those of 0.4 % N compost and 0.6 % N compost in the first day which recorded values below 7 (Table 4.7). This pH increment can be attributed to high metabolic activities possibly resulting in the production of intermediate basic metabolites especially in the

poultry droppings blended soils. Table 4.7 also shows a general decrease in pH in all treatments from week 2 to week 8 from as high as 8.31 in the 0.6 % N poultry droppings to as low as 5.80 in the 0.4 % N compost in week 8 (Table 4.7). The decreases observed in all treatments can be attributed to the degradation of the various organic materials and the hydrocarbons, which might have resulted in the release of acidic intermediate and final products that probably lowered the pH of the mixture (Alexander, 1999). The pH of all the soil blends however remains within the recommended range for optimum bioremediation of 6.5 – 8.5 (Ritter & Scarborough, 1995). This also agrees with the pH value of 7.8 stated by Dibble & Bartha, (1979).

5.6 Changes in TPH Content versus Variation in Temperature

The temperature of the control soil, which ranged between 30.7 °C and 33.7 °C, fluctuated with the daily diurnal air temperatures, which ranged between 30 °C and 33 °C during the experimental period (Table 4.8). Temperatures of all treatments increase from day 1, peaks on day 5 and then decline in day 6 and 7. The highest temperature was recorded by 0.6 % N poultry droppings which rose to 50 °C in day 5 of incubation. Temperatures became relatively stable after the seventh day, fluctuating between 30 °C and 35 °C for the remainder of the experiment (Table 4.8). The large increase in temperatures in the first 5 days could be due to the high initial microbial load, which resulted in high metabolic activities such as decomposition of organic materials (Table 4.3). This phenomenon has been previously reported by Potter *et al*. (1999).

CHAPTER SIX

6.0 CONCLUSION AND RECOMENDATIONS

6.1 Conclusion

The results indicate that the oil content in the oil-contaminated soil has been degraded by more than 81% in 8 weeks in all treatments and as high as 99.06 % by the 0.2 % N from the poultry droppings. The results of the present study shows that, under controlled conditions the rubber sludge (RS), compost(C) and poultry droppings (PD) can effectively accelerate the removal of petroleum hydrocarbons from oil contaminated soils within eight weeks of incubation.

There was no significant effect in the level of TPH degradation by increasing the supplementation frequency.

6.2 Recommendations

i. The use of compost, poultry droppings and natural rubber processing sludge as sources of nutrients (nitrogen, phosphorus, potassium etc) in bioremediation (biostimulation) of petroleum hydrocarbon contamination soil has shown an encouraging results and therefore should to be adopted by the mining companies and fuel filling stations for treating all oil contaminated soils instead of the volatilisation most of them currently practice.

ii. 0.2 % N concentration is recommended for all the three organic nutrient supplements.

iii. The natural rubber processing sludge have high Nitrogen content (3.2 % N per 100g) and can be a very good source of organic fertilizer for farming.

REFERENCES

Akkerman A. D. L., Mirza M. S., Harmsen H. J. M., Blok H. J., Herron P. R., Sessitsch A., Akkermans W. M., (1994). *Molecular ecology of microbes: a review of promises, pitfalls and true progress. FEMS Microbial Rev*; **15**: 185–194.

Alaska Department of Environmental Conservation (ADEC), (2005). *Environmental Monitoring & Assessment Program Implementation Strategy Division of Water.*
(www.dec.state.ak.us/water/wqamp/emap/implementation3_05.doc)
(Last accessed 06/07/10)

Alexander, M., (1999). *Biodegradation and Bioremediation*. Academic Press, San Diego.

Alexander, M. (1994). *Biodegradation and Bioremediation*. Publishers Academic Press, Inc. California, USA, pp. xi.

Allard, A. S., and Neilson, A. H., **(**1997). *Bioremediation of organic waste sites: a critical review of microbiological aspects.* Int. Biodeterior. Biodegrad. 39**:**253–285.

Atlas R. M., (1981). *Microbial degradation of petroleum hydrocarbons: an environmental perspective.* Macmillan Publishing Company, New York, USA, pp. 45:180-209.

Atlas R. M., (1992). *Molecular methods for environmental monitoring and containment of genetically engineered microorganisms.* Biodegradation 3, 137–146.

Atlas R. M., and Bartha R., (1992). *Hydrocarbon biodegradation and oil spill bioremediation.* K. C. Marshall [ed.]. Advances in Microbial Ecology. Plenum Press, New York, pp. 287-238.

Baker, KH, Herson DS (1994). *Bioremediation*. McGraw-Hill, Toronto.

Barbeau, C., L. Deschenes, D. Karamanev, Y. Comeau and R. Samson., (1997). *"Bioremediation of Pentachlorophenol-contaminated soil by bioaugmentation using activated soil*" *Appl. Microbiol. Biotechnol.* 48: 745-752.

Bartha, R., (1986a). *Microbial ecology: fundamentals and applications*. Addisson Wesley Publ., Reading, Mass.

Bartha, R., (1986b). *Biotechnology of Petroleum pollutant biodegradation*. Microb. Ecol. 12:155-172. 1994.

Bhatia, M. & Singh, H.D. (1996). *Biodegradation of commercial linear alkyl benzenes by Nocardia amarae. Journal of Biosciences* **21**, 487-496.

Black, C. A., (1965) *Method of Soil Analysis II*. American Society of Agronomy Madison, WI 573-590.

Boopathy, R., (2000): *Factors Limiting Bioremediation Technologies, Bioresources Technology*, 74,1, 63-67.

Bossert, I.; Bartha, R., (1984). *The Fate of Petroleum in Soil Ecosystems*. In Petroleum Microbiology, RM Atlas (ed.), Macmillan, New York, pp. 453-473.

Boyoucos, L. J. (1951): *Recalibration of hydrometer method of making mechanical analysis of soils*. Agronomy Journal 43(5):434-438.

Bradley, P.M. (2000). *Microbial degradation of chloroethenes in groundwater systems*. Hydrogeology Journal 8, no. 1: 104–111.

Bregnard, T. P. A.; Hohener, P.; Haner, A. & Zeyer, J. (1996): *Degradation of weathered diesel fuel by microorganisms from a contaminated aquifer in aerobic and anaerobic microcosms.* Environmental Toxicology and Chemistry 15, 299-307.

Brown, R., Leahy, M.C., Molnaa, B., (1999): *Bioremediation-a powerful and resilient companion technology*. Pollution Engineering 31(10), 26–29. Canter, L.W., Knox, R.C., 1985. Groundwater Pollution Control, Lewis Publishers, Chelsea, MI.

Bury, S. J., and Miller, C. A. (1993). "*Effect of micellar solubilization on biodegradation rates of hydrocarbons,"* Environ. Sci. Tech. *27(l),* 104-110.

Caplan, J. A., (1993). *The world-wide bioremediation industry: prospects for profit.* Trds. Biotech. 11:320-323.

Ceccanti, B., Garcia, C., Masciandaro, G., Macci, C., Carmignani, A. and Filareto, A., (2003), '*Il ruolo dei lombrichi (Eisenia foetida) nella bioremediation di un suolo contaminato da idrocarburi',* Convegno SISS Qualit`a del suolo, impatto antropico e qualit`a dei prodotti agricoli, Siena 9–12 giugno 2003. 19-20.

Cerniglia, C.E.(1997). *Fungal metabolism of polycyclic aromatic hydrocarbons: past, present and future applications in bioremediation.* Journal of Microbial Biotechnology. 19: 324-327.

Cheston, A.E., (1997). *Bioremediation of joint bays. M.Sc. Thesis.* Cranfield Institute of BioScience and Technology.

Christensen, T.H., P.L. Bjerg, S.A. Banwart, R. Jakobsen, G. Heron, and H.-H. Albrechtsen. (2000). *Characterization of redox conditions in groundwater contaminant plumes*. Journal of Contaminant Hydrology 45, no. 3–4: 165–241.

Cookson, John T. Jnr., (1995), *Bioremediation Engineering: Design and Application.* McGraw Hill, Inc. New York.

Crellin, J. (1999). *Personal Communication*, 19 August 1999 by Crellin. Abington Partners, Bath.

Dave, H., Ramakrishna, C., Bhatt, B.D. & Desai, J.D., (1994). *Biodegradation of slop oil from a petrochemical industry and bioreclamation of slop oil contaminated soil*. World Journal of Microbiology & Biotechnology **10**, 653-656.

Delille, D., Coulon, F., Pelletier, E., (2004). *Effects of temperature warming during a bioremediation study of natural and nutrient-amended hydrocarbon-contaminated sub-Antarctic soils.* Cold Reg. Sci.Tech. 40:61-70.

Dibble, J.T. & Bartha, R., (1979). *Effect of environmental parameters on biodegradation of oil sludge.* Applied Environmental Microbiology. 37, 729-739.

Dibble, J.T.; Bartha, R., (1976). *The effect of iron on the biodegradation of petroleum in seawater.* Appl. Environ. Microb. 31:544-550.

Dickel, O.; Haug, W., Knackmus, H. J., (1993): *Biodegradation of nitrobenzene by a sequential anaerobic–aerobic process.* Biodegradation 4:187–194.

Dojika, M. A., Hugenholtz, P., Haack, S. K., and Pace, N. R., (1998): *Microbial diversity in a hydrocarbon- and chlorinated-solvent contaminated aquifer undergoing intrinsic bioremediation.* Appl. Environ. Microbial. 64, 3869-3877.

Ellis, B. M. L., (2000): *Environmental biotechnology informatics*. Curr Opin Biotechnol 11:232–235.

Ellis, W., Payne, J., and McNabb, J. (1986),*"Treatment of contaminated soils with aqueous surfactants,"* EPA/600/2-85/l 29, Hazardous Waste Research Laboratory, USEPA, Cincinnati, OH. 61-64

Falatko, D. M., and Novak, J. T. (1992). *"Effects of biologically produced surfactants on the mobility and biodegradation of petroleum hydrocarbons,"* Water Environ. Res. 64(2), 163-169.

Foght, J. M., Westlake, D. W. S. (1984). *"Biodegradation of hydrocarbons in freshwater."* Proceedings of the symposium on oil pollution in freshwater. Edmonton, Alberta, Canada, 217-230.

Foght, J. M., Westlake, D, Johnson, W. M., Ridgway, H.F., (1996). *Environmental gasoline-utilising isolates and clinical isolates of Pseudomonas aeruginosa are taxonomically indistinguishable by chemotaxonomic and molecular techniques.* Microbiol. 142: 1333-2340.

Glazer, A. N., Nikaido, H., (1995) "*Microbial insecticides", in Microbial Biotechnology Fundamentals of Applied Microbiology,* (W.H. Freeman and Company, New York), p. 209-229.

Gogoi BK, Dutta NN, Goswami P, Krishna Mohan TR (2003). *A case study of bioremediation of petroleum-hydrocarbon contaminated soil at a crude oil spill site.* Adv in Environ Res., **7**: 767-782.

Golyshin, P. M., Fredrickson, H. L., Giuliano, L., Rothme, l. R., Timmis, K. N., Yakimov, M. M. (1999). *Effect of novel biosurfactants on biodegradation of polychlorinated biphenyls by pure and mixed bacterial cultures.* New Microbiol. 22, 257-267.

Gutnick, D. L, and E. Rosenberg. (1977). *Oil tankers and pollution: a microbiological approach.* Annu. Rev. Microbiol. 31:379-396.

Haigh, S.D. (1995). *Fate and effects of synthetic lubricants in soil: biodegradation and effects on crops in field studies.* The Science of the Total Environment 168, 71-83.

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

Harrison, A.B. & Betts, W.B. (1996). *Assay of bacterial and fungal activity in diesel contaminated soil using a 14C-glucose utilization method. Letters in Applied Microbiology* **23**, 43-46.

Helaleh Murad, I. H., Tanaka K, Fujii S. I., Korenaga T., (2001): *GC/MS determination of phenolic compounds in soil samples using Soxhlet extraction and derivatization techniques*. Anal. Sci., **17**: 1225-1227.

Herbath, Y. (2000): *The application of in-situ techniques for the bioremediation of soil and groundwater contaminated with cable oil.* Ph.D. thesis. Cranfield Institute of Bioscience and Technology.

Holt, M.S. & Bernstein, S.L. (1992). *Linear alkylbenzenes in sewage sludges and sludge amended soils. Water Research*, **26**, 613-624.

Iwamoto, T., and Nasu, M., (2001). *Current bioremediation practice and perspective*. J. Biosci. Bioeng., **92**, 1-8.

Janssen, D.B., Oppentocht, J. E., Poelarends, G., (2001): *Microbial dehalogenation.* Curr Opin Biotechnol **12**:254–258.

King, R. Barry, Gilbert M. Long, and John K. Sheldon (1992): *Practical Environmental Bioremediation.* Lewis Publisher, New York.

Koussia, E. (1999). *In vitro study of biodegradation of cable oil and the effect of nutrient supplement on biodegradation rate.* M.Sc. Thesis. Cranfield Institute of Bioscience and Technology.

Lang, S., and Wagner, F. (1987). *"Structure and properties of biosurfactants."Biosurfactants and biotechnology.* Marcel Dekker, Inc., New York.

Lang S, Wullbrandt D. (1999). *Rhamnolipids-biosynthesis, microbial production and application potential.* Appl Microbiol Biotechnol **51**:22–23.

Liebeg, E.W., Cutright, T.J., (1999). *The investigation of enhanced bioremediation through the addition of macro and micronutrients in a PAH contaminated soil*. International Biodeterioration and Biodegradation **44**, 55-64.

Margesin R, Schinner F., (1999). *Biological decontamination of oil spills in cold environments.* J. Chem. Technol. Biotechnol., **74**: 381-389.

Margesin, R., Schinner, F., (1997). *Laboratory bioremediation experiments with soil from a diesel-oil contaminated site e significant role of cold-adapted microorganisms and fertilizers.* Journal of Chemical and Biotechnology **70**, 92-98.

McMurry, J., (2000), *"Organic Chemistry"* 5th ed. Cole: Thomson Learning, pp 75-81.

Mikelcic, J. R., and Luthy, R. G. (1988). *"Microbial degradation of acenaphthene and naphthalene under dentrifying conditions in soil-water systems,"*. Appl. Environ. Mirobiol. 54(5), 1188-1198.

Millette, D., J.F. Barker, Y. Comeau, B. J. Butler, E. O. Frind, B. Clement and R. Samson. (1995). *Substrate interaction during aerobic biodegradation of creosote-related compounds: a factorial batch experiment*. Environ. Sci. Technol., **29**: 1944- 1952.

Mishra S., Jyot J. & al. (2001b): *Evaluation of inoculum addition to stimulate in situ bioremediation of oily sludge contaminated soil. Appl. Environ. Microbiol.*, **67**, 1675.

Mishra S., Jyot J. & al. (2001) *In situ bioremediation potential of an oily sludge degrading bacterial consortium. Current Microbiology*, 43, 328.

Namkoong, W., Na, K.J., (2000) *"Effects of recycled wastewater and surfactant on the treatmentefficiency of PAHs-contaminated soil in slurry bioreactor,"* http://www.kowrec.org/journal/9-1/journal9_1_09.htm.

Neff, J.M., (1979). *Polycyclic Aromatic Hydrocarbons in the Aquatic Environments*. Elsevier Applied Science Publishers, London, pp. 469-538.

Nelson-Smith, A. (1973). *Oil Pollution and Marine Ecology*, New York, Plenum Press.

Nocentini, M., Pinelli, D., Fava, F., (2000). *Bioremediation of a soil contaminated by hydrocarbon mixtures: the residual concentration problem.* Chemosphere 41, 1115-1123.

Norris, R.D. (1994). *Handbook of bioremediation*. : CRC Press. Boca Raton, Florida.

Oberbremer, A., and Muller-Hurtig, R. (1989). *"Aerobic stepwise hydrocarbon degradation and formation of biosurfactants by an original soil population in a stirred reactor," Appl. Microbial. Biotechnol.* **31**, *582-586.*

Okolo, J.C., Amadi, E.N, Odu, C.T.I .(2005). Effects of soil treatments containing poultry manure on crude oil degradation in a sandy loam soil. Appl. Ecol. Environ. Res. 3(1) : 47-53.

Parr, J.F., Sikora, L.J., Burge, W.D. (1983). *Factors affecting the degradation and inactivation of waste constituents in soils*. In: Land Treatment of Hazardous Wastes. Parr, J.F., Marsh, P.B., Kla, J.M (Eds). Noyes data corp., Park Ridge, N.J. pp.20-49: 321-337.

Payne, J.R. and C.R. Phillips. (1985). *Petroleum Spills in the Marine Environment: The Chemistry and Formation of Water-in-Oil Emulsions and Tar Balls.* Lewis Publishers, Incorporated, Chelsea, Michigan.

Payne, J. R., and Phillips, C. R. (1985). *"Photochemistry of petroleum in water,"* Environ. Sci. Tech. 19(7), 569-579.

Pelletier E., Delille D, Delille B (2004). *Crude oil bioremediation in sub- Antarctic intertidal sediments: chemistry and toxicity of oiled residues.* Mar. Environ. Res**. 57**: 311-327.

Phelps, T.J., Siegrist, R.L., Korte, N.E., Pickering, D.A., Stronggunderson, J.M., Palumbo, A.V., Walker, J.F., Morrissey, C.M. & Mackowski, R. (1994). *Bioremediation of petroleum hydrocarbons in soil column lysimeters from Kwajalein Island.* Applied Biochemistry and Biotechnology 45/46, pp. 835-845.

Potter CL, Glaser JA, Hermann R, Dosani MA (1999). *Remediation of contaminated East River sediment by composting technology*. In: Leeson, A., Alleman, B. C. (eds) Bioremediation Technologies for Polyclic Aromatic Hydrocarbon Compounds. The Fifth International pp: 1-5.

Pritchard, P. H. *et al.,* (1992), *Oil Spill Bioremediation: Experiences, Lessons, and Resultsfrom the Exxon Valdez Oil Spill in Alaska, Micr*oorganisms *to Combat Pollution*, ed. Rosenberg, E., Kluwer Academic Publishers.

Ralebitso TK, Senior E, Verseveld H. W (2002).; *Microbial aspects of atrazine degradation in natural environments. Biodegradation.*13:11–19. doi: 10.1023/A:1016329628618.

Raymond, R.L. (1974). *Reclamation of hydrocarbon contaminated groundwaters*. *US Patent #3,846,290*. 5th November 1974.

Riffaldi, R., Levi-Minzi, R., Cardelli, R., Palumbo, S., and Saviozzi, A., 2006. *Soil biological activities in monitoring the bioremediation of diesel oil-contaminated soil. Water, Air, and Soil Pollution,* 170, 3-15.

Ritter, W.F. & Scarborough, R.W. (1995). *A review of bioremediation of contaminated soils and groundwater.* Journal of Environmental Science & Health A30**(2)**. pp. 333-357.

Roberts, E.V. 1998. *Remediation of petroleum contaminated soils-biological, physical andchemical processes.* Lewis Publishers. Boca Raton, London, New York Washington, D.C.

Restoration Project 97427 Final Report. Alaska Department of Fish and Game, Division of Wildlife Conservation. Anchorage, AK.

Schmitt, M., Rhen, G., (2002). *Fertilizing Cropland with Poultry Manure*. In Regents of The University of Minnesota. FO-5881 C.

Sims, J. L., Sims, R. C., and Mathews, J. E. (1989). *"Bioremediation of contaminated surface soils,"* EPA/600/9-89/073, Robert S, Kerr Environmental Research Laboratory, USEPA Office of Research and Development, Ada, OK.

Smith, M.R. & Ratledge, C. (1989). Catabolism of alkylbenzenes by *Pseudomonas* sp. NCIB 10643. *Applied Microbiology & Biotechnology*. **32**: 68-75.

Speers, G.C. and Whitehead, E.V. (1969). *Crude petroleum. In: Oroanic Geochemistrv: Methods and Results.* Eglinton, G. and M.T.J. Murphy (Eds.). pp.638 - 675. New York Springer-Verlag,.

Steffensen, W.S. and Alexander, M. (1995). *Role of competition for inorganic nutrients in thebiodegradation of mixtures of substrates. Appl. Environ. Microbiol*., 61(**8**): 2859-2862

Subba-Rao and Alexander, M. (1982). *Effects of sorption on mineralization of low concentrations of aromatic hydrocarbons in lake water*. *Applied Environmental Microbiology* **44**: 659–668.

Sutherson, S., (1997). *Remediation engineering design concepts*, Lewis Publishers, Boca Raton, p. 362.

Swindoll, C. M., A. M. Aelion, and F.K. Pfaender. (1988). *Influence of inorganic and organic nutrients of aerobic biodegradation and on the adaptation response of subsurface microbial communities.* Appl. Environ. Microbiol. V. **54**, pp. 212-227.

Tebbutt, R. (1998). *Natural attenuation of cable oils. M.Sc. Thesis.* Cranfield Institute of Bioscience and Technology.

Trabalka, J.R. & Reichle, D.E. [Eds] (1986). *The changing carbon cycle: A global analysis*, Springer - Verlag.

Truper, H.G.(1992) Prokaryotes: *An overview with respect to biodiversity and environmental importance.* Biodiversity and Conservation. **1**:227-236.

Tursman, J.F. & Cork, D.J. (1992). *Subsurface contaminant bioremediation engineering. Critical Reviews in Environmental Control*. **22**, 1-26.

Vandepitte, V., P. Quataert, H. De Rore, W. Verstraete, (1995) *Evaluation of the Gompertz function to model survival of bacteria introduced into soils*. Soil Biol. Biochem. **27**:365-372.

Venosa, A.D., Zhu, X. (2003). *Biodegradation of Crude Oil Contaminating Marine Shorelines and Freshwater Wetlands.* Spill Sci. Tech. Bull. 8(**2**):163-178.

Vogel, T. M., (1996). *Bioaugmentation as a soil bioremediation approach*. Curr. Opin. Biotechn. **7**:311-316.

Walkey, A. and Black, I. A., (1934). *An examination of Different methods for determining soil organic matter and a proposed modification of the chromic acid titration method.* Soil Science **27**: 29-38.

Walworth, J.; Pond, A.; Snape, I.; Rayner, J.; Ferguson, S.; Harvey, P., (2006). *Nitrogen requirements for maximising petroleum bioremediation in a Sub-Antartic soil.* Cold Regions Sci. Technol., pp. 1-8.

Wang, Y.; Lau, P. C. K. & Button, D. K. (1996). *A marine oligobacterium harboring genes known to be part of aromatic hydrocarbon degradation pathways of soil pseudomonads*. *Appl Environ Microbiol* **62**, 2169–2173.

Watanabe, K. (2001). *Microorganisms relevant to bioremediation*. Curr Opin Biotechnol **12**, 237–241.

Watanabe, K., Hino, S., Takahashi, N., (1996). *Effects of exogenous phenol degrading bacteria on performance and ecosystem of activated sludge.* J. Ferment. Bioeng. **82**: 291–298.

Weber, J. R., W.J. & Corseuil, H. X. (1994). *Inoculation of contaminated subsurface soils with enriched indigenous microbes to enhance bioremediation rates. Water Resources* **28**: 1407-1414.

Weidemeier, T.H., Swanson, M.A., Wilson, J.T., Kampbell, D.H., Miller, R.N. & Hansen, J.E. (1996). *Approximation of biodegradation rate constants for monoaromatic hydrocarbons (BTEX) in ground water.* Ground Water Monitoring and Remediation Summer 1996, 186-194.

Weissemann, S. & Kunze, C. (1994). *Microbial activity in heating oil contaminated soil under field and controlled conditions.* Angew. Bot. **68**, 137-142.

Whyte, A. C., Gloer, J. B., Scott, J. A., and Malloch D. ,(1996), Cercophorins ADC: *Novel Antifungal and cytotoxic metabolites from the coprophilous fungus Cercophora areolata.* J. Nat. Prod. **59**, 765-769.

Yeates, R. S., and Hyde, R. I.,(2004). Qualified persons report on Adamus Resources Limited; R. S. Global. Accra. Pp. 34 and 77.

Zavala, C. J. F., Gavi, R. R. H., Adams, S. H. R., Ferrera-Cerrato, D. J., Palma-López, D. J., Vaquera, H.Y., Domínguez, E. J. M., (2005). *Derrames de petróleo en suelos y adaptación de pastos tropicales en el Activo Cinco Presidentes,* Tabasco. Terra Latinoamericana, **23**: 293-302.

Zitrides, T. (1983). *Biodecontamination of spill sites. Pollut. Eng*. **15**: 25-27.

Zobell, C.E. (1973). *Microbial-facilitated degradation of oil: Present Status, Problems and Perspectives. In the Microbial Degradation of Oil Pollutants*, D.G. Ahearn and S.P. Meyers Pub No. LSU-SG-73-01. Centre for Wetland Resources, Louisiana State University, Baton Rouge. p.3.

APPENDICES

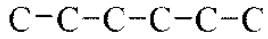
APPENDIX 1 A

Structure of Some Petroleum Hydrocarbons

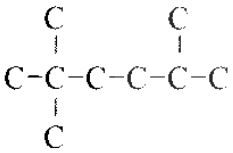
Saturated hydrocarbons

Alkanes

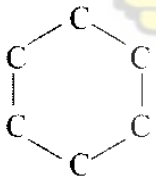
Normal, continuous chain (C_nH_{2n+n})



Iso, branced chain (C_nH_{2n+2})



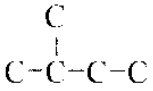
Cyclic, circle of carbons: naphthenes



Unsaturated hydrocarbons

Alkanes

Olefins (C_nH_{2n})



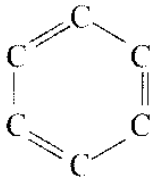
Alkynes

Acetylene (C_2H2)

Arenes

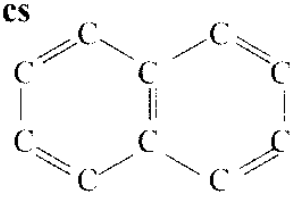
Benzene (C_6H_6)

Toluene (C_7H_8)



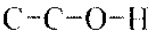
Polynuclear Aromatics

Naphthalene ($C_{10}H_8$)

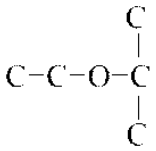


Oxygenates

Ethanol (C_2H_5OH)

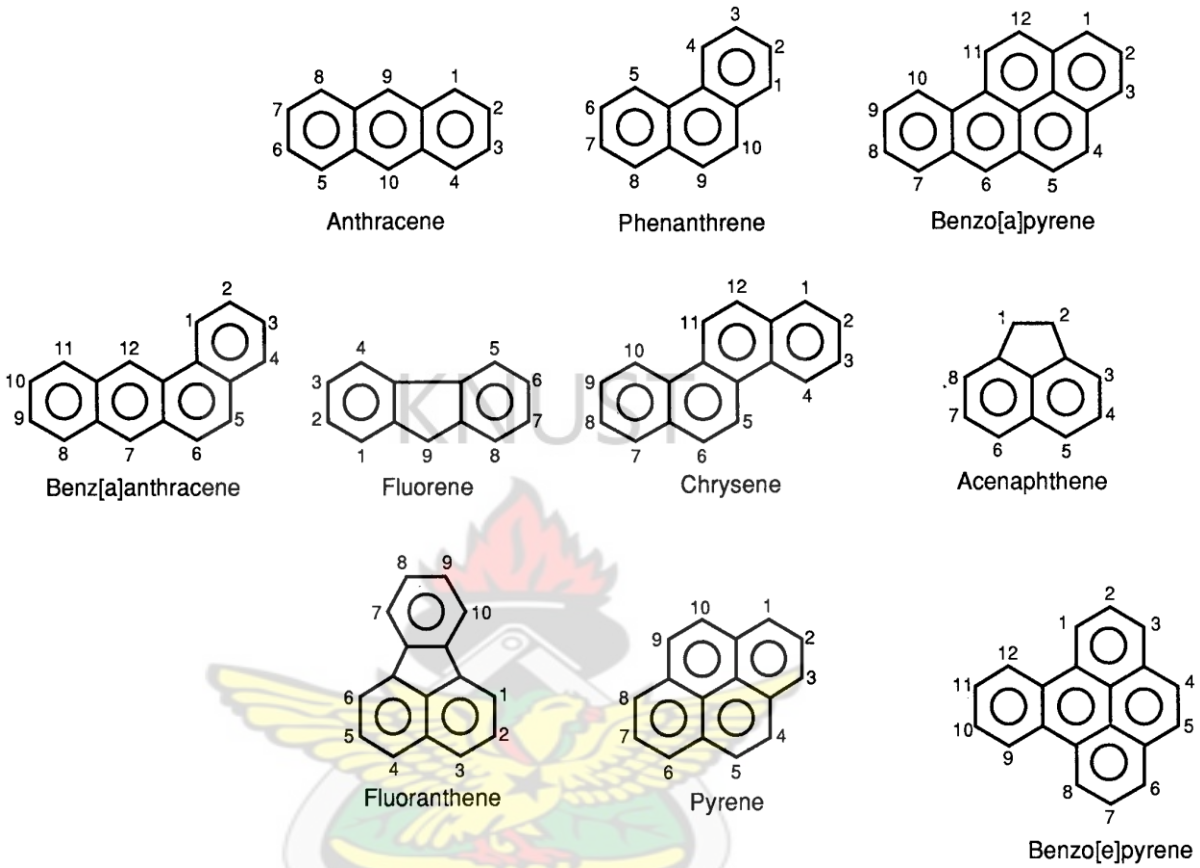


MTBE ($C_4H_9OCH_3$)



APPENDIX 1 B

Structures of Polycyclic Aromatic Hydrocarbons



Structures of polycyclic aromatic hydrocarbons adapted from Cerniglia 1997.

APPENDIX 2 A

Some Plates Showing Microbial Growth

2.1 Microbial growth on control soil

2. 2 Microbial growth on Compost

APPENDIX 2 B

2.3. Microbial growth on rubber sludge

2.4. Microbial growth on Poultry droppings

APPENDIX 3 A

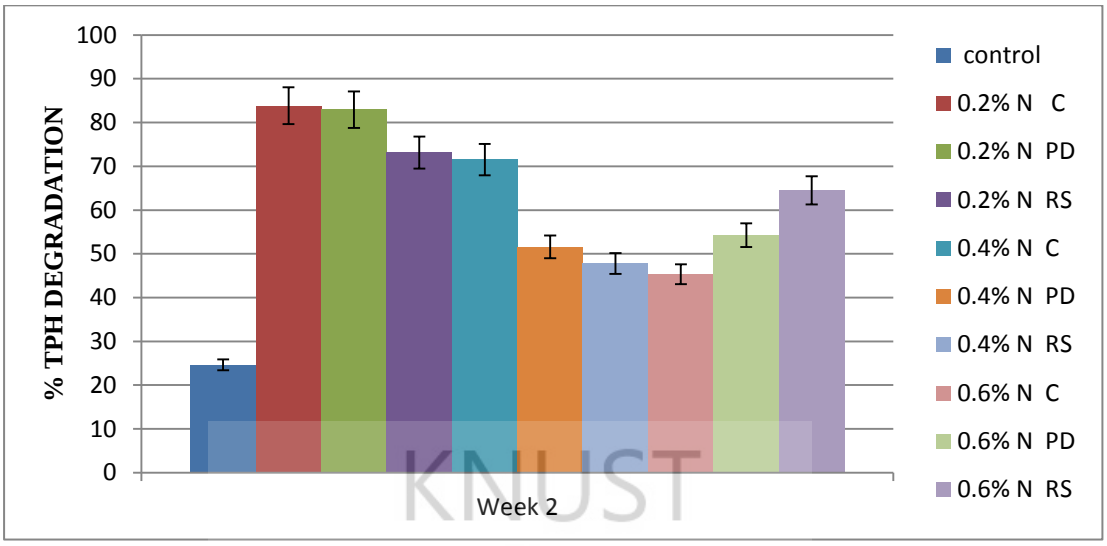
A TPH Concentrations (mg/kg)

Treatment	Week 0	Week 2	Week 4	Week 8	Means
Control	1009.12	760.20 A	536.96 A	502.54 A	**702.21**
0.2% N C	1009.12	163.11 H	141.92 CD	46.28 H	**340.11**
0.2% N PD	1009.12	172.25 H	14.66 D	9.48 I	**301.38**
0.2% N RS	1009.12	271.49 G	154.13 CD	65.31 G	**375.01**
0.4% N C	1009.12	287.50 G	153.89 CD	82.52 F	**383.26**
0.4% N PD	1009.12	488.56 D	234.93 C	79.43 F	**453.01**
0.4% N RS	1009.12	526.66 C	156.94 CD	91.66 E	**446.09**
0.6% N C	1009.12	551.75 B	276.99 BC	169.86 C	**501.93**
0.6% N PD	1009.12	461.77 E	247.47 C	188.39 B	**476.69**
0.6% N RS	1009.12	358.54 F	171.03 C	135.69 D	**418.60**
Tukey $HSD_{(0.05)}$	-	**35.76**	**281.16**	**15.86**	-

C is Compost, **PD** is Poultry Droppings and **RS** is Rubber Sludge.

APPENDIX 3 B

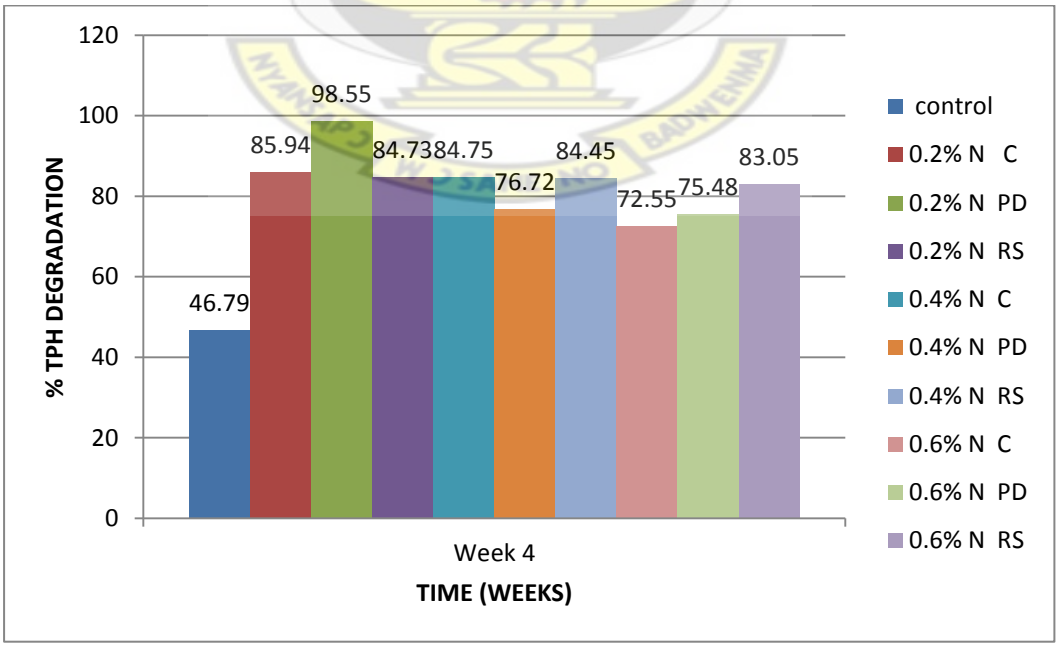
A Graph Showing TPH Degradation (%) At Week 2



C is Compost, **PD** is Poultry Droppings and **RS** is Rubber Sludge.

APPENDIX 3 C

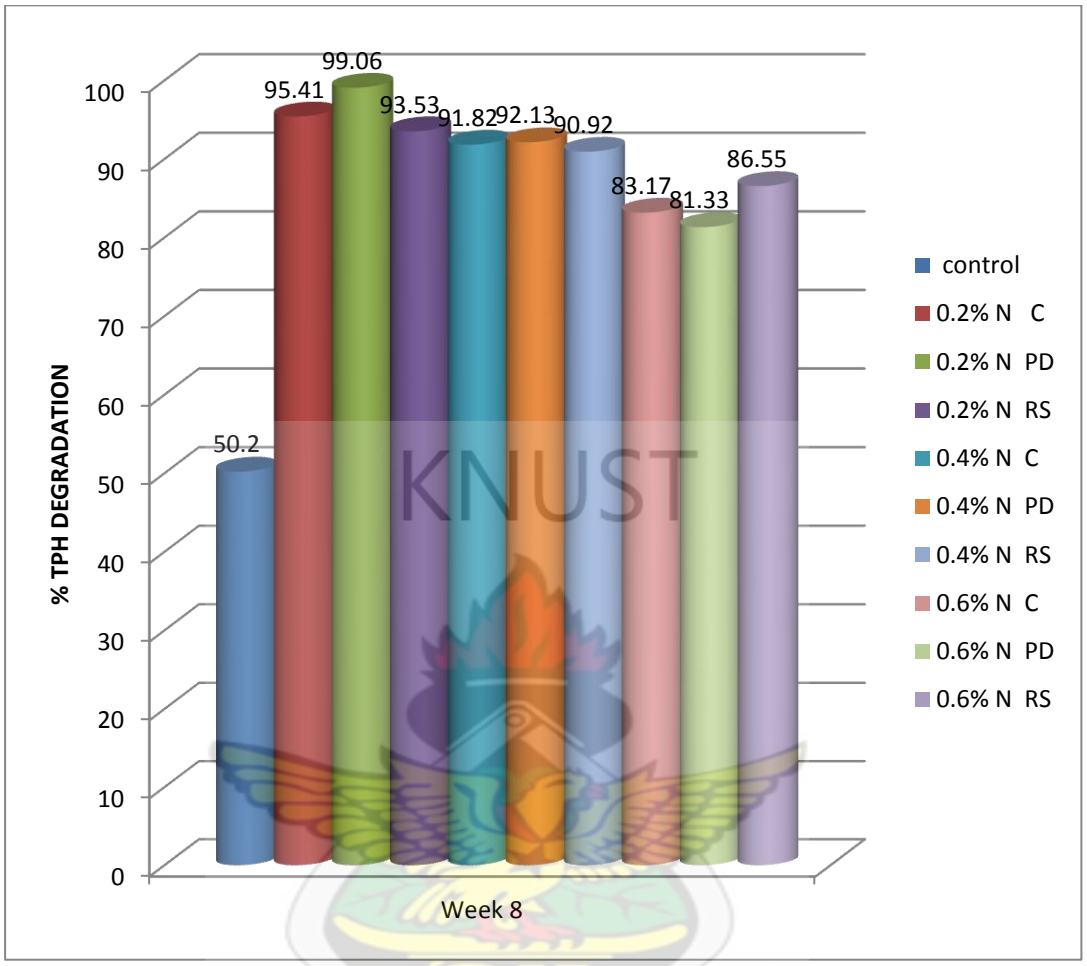
A Graph Showing TPH Degradation (%) At Week 4



C is Compost, **PD** is Poultry Droppings and **RS** is Rubber Sludge.

APPENDIX 3. D

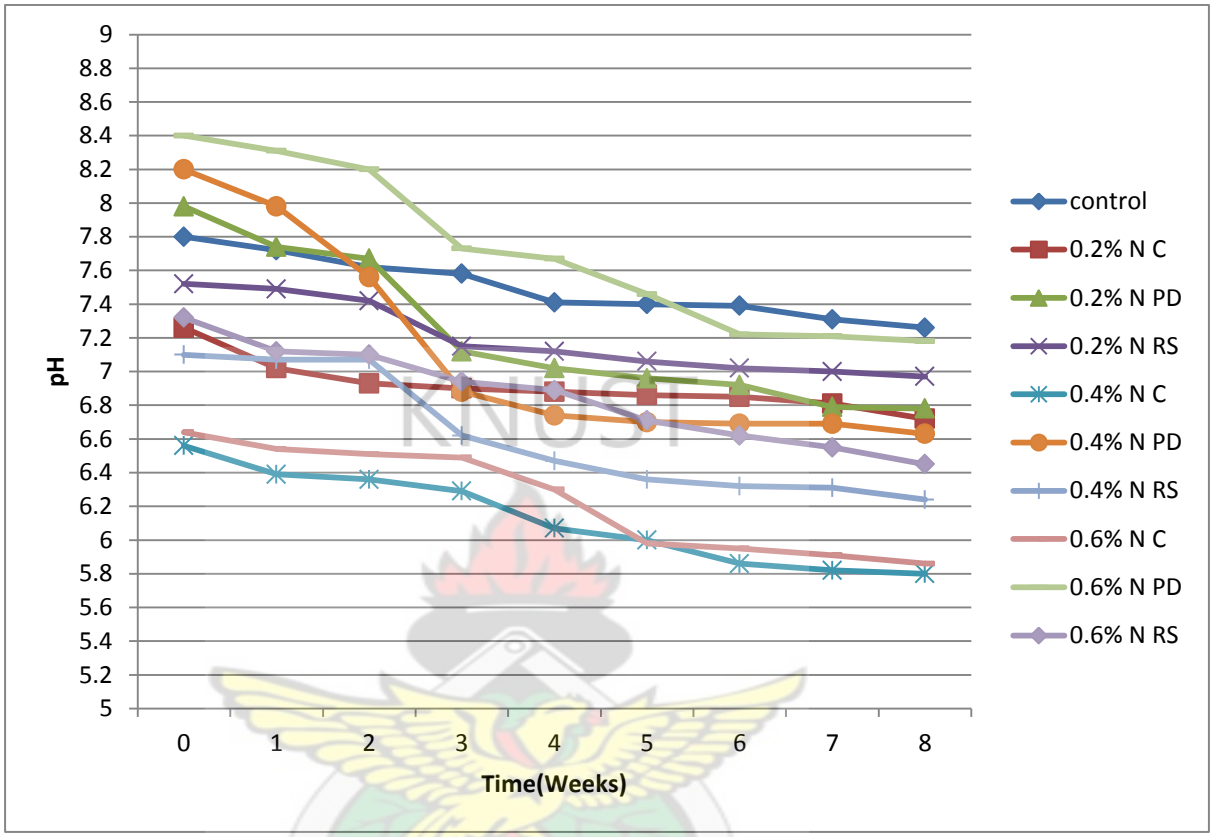
Graph Showing TPH Degradation (%) At Week 8



C is Compost, **PD** is Poultry Droppings and **RS** is Rubber Sludge.

APPENDIX 3. E

Graph Showing Variation In pH during 60 days of incubation.



C is Compost, **PD** is Poultry Droppings and **RS** is Rubber Sludge.

APPENDIX 4 A

ANOVA TABLE FOR TPH DEGRADATION

Student Edition of Statistix 9.0 15/09/2012, 08:36:00 PM

Analysis of Variance Table for WEEK 0

Source	DF	SS	MS	F	P
REP	2	0.00000	0.00000		
CONC	2	0.00000	0.00000	M	M
SOURCE	3	0.00000	0.00000	M	M
CONC*SOURCE	6	0.00000	0.00000	M	M
Error	16	0.00000	0.00000		
Total	29				

Note: SS are marginal (type III) sums of squares

Grand Mean 1009.1

WARNING: The total sum of squares is too small to continue.

The dependent variable may be nearly constant.

Analysis of Variance Table for WEEK 2

Source	DF	SS	MS	F	P
REP	2	268	134		
CONC	2	171444	85722	2131.11	0.0000
SOURCE	3	432305	144102	3582.47	0.0000
CONC*SOURCE	6	195250	32542	809.01	0.0000
Error	16	644	40		
Total	29				

Note: SS are marginal (type III) sums of squares

Grand Mean 463.13 CV 1.37

Analysis of Variance Table for WEEK 4

Source	DF	SS	MS	F	P
REP	2	3993	1996		
CONC	2	42121	21061	8.47	0.0031
SOURCE	3	317075	105692	42.51	0.0000
CONC*SOURCE	6	65752	10959	4.41	0.0081
Error	16	39782	2486		
Total	29				

Note: SS are marginal (type III) sums of squares

Grand Mean 257.47 CV 19.37

Analysis of Variance Table for WEEK 8

Source	DF	SS	MS	F	P
REP	2	42	21		
CONC	2	31881	15940	2015.18	0.0000
SOURCE	3	436104	145368	18377.4	0.0000
CONC*SOURCE	6	16819	2803	354.38	0.0000
Error	16	127	8		
Total	29				

Note: SS are marginal (type III) sums of squares

Grand Mean 196.96 CV 1.43

Analysis of Variance Table for TPH_DEGRA

Source	DF	SS	MS	F	P
REP	2	2.239E-27	1.119E-27		
CONC	2	338.395	169.197	4.9E+30	0.0000
SOURCE	3	4359.99	1453.33	4.2E+31	0.0000
CONC*SOURCE	6	156.129	26.0215	7.5E+29	0.0000
Error	16	5.538E-28	3.461E-29		
Total	29				

Note: SS are marginal (type III) sums of squares

Grand Mean 80.306

WARNING: The model error mean square is too small to continue.

The model may fit the data exactly.

ANOVA MEANS FOR TPH DEGRADATION

Student Edition of Statistix 9.0 15/09/2012, 08:35:17 PM

Tukey HSD All-Pairwise Comparisons Test of WEEK 2 for CONC

CONC	Mean	Homogeneous Groups
3	533.07	A
2	515.58	B
1	340.74	C

Alpha 0.05 Standard Error for Comparison 3.2580

Critical Q Value 3.651 Critical Value for Comparison 8.4101

Error term used: REP*CONC*SOURCE, 16 DF

All 3 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 2 for SOURCE

SOURCE	Mean	Homogeneous Groups
CONTROL	758.64	A
RS	385.56	B
PD	374.19	C
C	334.12	D

Alpha 0.05 Standard Error for Comparison 2.9898 TO 4.2282

Critical Q Value 4.047 Critical Value for Comparison 8.5552 TO 12.099

Error term used: REP*CONC*SOURCE, 16 DF

All 4 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 2 for CONC*SOURCE

CONC	SOURCE	Mean	Homogeneous Groups
3	CONTROL	760.20	A
2	CONTROL	759.60	A
1	CONTROL	756.11	A
3	C	551.75	B
2	RS	526.66	C
2	PD	488.56	D
3	PD	461.77	E
3	RS	358.54	F
2	C	287.50	G
1	RS	271.49	G
1	PD	172.25	H
1	C	163.11	H

Alpha 0.05 Standard Error for Comparison 5.1784 TO 9.4545

Critical Q Value 5.349 Critical Value for Comparison 19.587 TO 35.761

Error term used: REP*CONC*SOURCE, 16 DF

There are 8 groups (A, B, etc.) in which the means

are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 4 for CONC

CONC	Mean	Homogeneous Groups
3	308.11	A
2	261.40	AB
1	202.90	B

Alpha 0.05 Standard Error for Comparison 25.615

Critical Q Value 3.651 Critical Value for Comparison 66.121

Error term used: REP*CONC*SOURCE, 16 DF

There are 2 groups (A and B) in which the means

are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 4 for SOURCE

SOURCE	Mean	Homogeneous Groups
CONTROL	512.56	A
C	190.93	B
PD	165.69	B
RS	160.70	B

Alpha 0.05 Standard Error for Comparison 23.506 TO 33.242

Critical Q Value 4.047 Critical Value for Comparison 67.262 TO 95.123

Error term used: REP*CONC*SOURCE, 16 DF

There are 2 groups (A and B) in which the means

are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 4 for CONC*SOURCE

CONC	SOURCE	Mean	Homogeneous Groups
3	CONTROL	536.96	A
1	CONTROL	500.90	A
2	CONTROL	499.82	AB
3	C	276.99	BC
3	PD	247.47	C
2	PD	234.93	C
3	RS	171.03	C
2	RS	156.94	CD
1	RS	154.13	CD
2	C	153.89	CD
1	C	141.92	CD
1	PD	14.66	D

Alpha 0.05 Standard Error for Comparison 40.714 TO 74.332

Critical Q Value 5.349 Critical Value for Comparison 154.00 TO 281.16

Error term used: REP*CONC*SOURCE, 16 DF

There are 4 groups (A, B, etc.) in which the means

are not significantly different from one another

Tukey HSD All-Pairwise Comparisons Test of WEEK 8 for CONC

CONC	Mean	Homogeneous Groups
3	246.45	A
2	188.52	B
1	155.90	C

Alpha 0.05 Standard Error for Comparison 1.4448

Critical Q Value 3.651 Critical Value for Comparison 3.7295

Error term used: REP*CONC*SOURCE, 16 DF

All 3 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 8 for SOURCE

SOURCE	Mean	Homogeneous Groups
CONTROL	498.30	A
C	99.55	B
RS	97.55	B
PD	92.43	C

Alpha 0.05 Standard Error for Comparison 1.3258 TO 1.8750

Critical Q Value 4.047 Critical Value for Comparison 3.7938 TO 5.3653

Error term used: REP*CONC*SOURCE, 16 DF

There are 3 groups (A, B, etc.) in which the means

are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 8 for CONC*SOURCE

CONC	SOURCE	Mean	Homogeneous Groups
1	CONTROL	502.54	A
2	CONTROL	500.48	A
3	CONTROL	491.87	A
3	PD	188.39	B
3	C	169.86	C
3	RS	135.69	D
2	RS	91.66	E
2	C	82.52	F
2	PD	79.43	F
1	RS	65.31	G
1	C	46.28	H
1	PD	9.48	I

Alpha 0.05 Standard Error for Comparison 2.2964 TO 4.1926

Critical Q Value 5.349 Critical Value for Comparison 8.6860 TO 15.858

Error term used: REP*CONC*SOURCE, 16 DF

There are 9 groups (A, B, etc.) in which the means

are not significantly different from one another.

APPENDIX 4. B

ANOVA TABLE FOR MICROBIAL COUNT

Student Edition of Statistix 9.0 16/09/2012, 09:18:37 AM

Analysis of Variance Table for WEEK 0

Source	DF	SS	MS	F	P
REP	2	1116.67	558.333		
CONC	2	7.379E+08	3.690E+08	3.7E+07	0.0000
SAMPLE	3	7.860E+08	2.620E+08	2.7E+07	0.0000
CONC*SAMPLE	6	1.510E+09	2.517E+08	2.6E+07	0.0000
Error	22	216.667	9.84848		
Total	35	3.034E+09			

Grand Mean 4346.8 CV 0.07

Analysis of Variance Table for WEEK 2

Source	DF	SS	MS	F	P
REP	2	5.556E+12	2.778E+12		
CONC	2	1.781E+19	8.906E+18	3206250	0.0000
SAMPLE	3	2.195E+19	7.318E+18	2634443	0.0000
CONC*SAMPLE	6	4.307E+19	7.178E+18	2583940	0.0000
Error	22	6.111E+13	2.778E+12		
Total	35	8.283E+19			

Grand Mean 1.02E+09 CV 0.16

Analysis of Variance Table for WEEK 4

Source	DF	SS	MS	F	P
REP	2	16.6667	8.33333		
CONC	2	3.990E+18	1.995E+18	2.4E+17	0.0000
SAMPLE	3	4.251E+18	1.417E+18	1.7E+17	0.0000
CONC*SAMPLE	6	1.005E+19	1.675E+18	2.0E+17	0.0000
Error	22	183.333	8.33333		
Total	35	1.829E+19			

Grand Mean 1.05E+09 CV 0.00

WARNING: The total sums of squares is very large compared to the residual sums of squares, indicating a potential round-off error problem.

The model may fit the data exactly.

Analysis of Variance Table for WEEK 8

Source	DF	SS	MS	F	P
REP	2	5555556	2777778		
CONC	2	5.679E+17	2.839E+17	1.0E+11	0.0000
SAMPLE	3	2.771E+17	9.237E+16	3.3E+10	0.0000
CONC*SAMPLE	6	5.770E+17	9.617E+16	3.5E+10	0.0000
Error	22	6.111E+07	2777778		
Total	35	1.422E+18			

Grand Mean 9.38E+07 CV 0.00

WARNING: The total sums of squares is very large compared to the residual sums of squares, indicating a potential round-off error problem.

The model may fit the data exactly.

ANOVA MEANS FOR MICROBIAL COUNT

Student Edition of Statistix 9.0 16/09/2012, 09:18:37 AM

Analysis of Variance Table for WEEK 0

Source	DF	SS	MS	F	P
REP	2	1116.67	558.333		
CONC	2	7.379E+08	3.690E+08	3.7E+07	0.0000
SAMPLE	3	7.860E+08	2.620E+08	2.7E+07	0.0000
CONC*SAMPLE	6	1.510E+09	2.517E+08	2.6E+07	s0.0000
Error	22	216.667	9.84848		
Total	35	3.034E+09			

Grand Mean 4346.8 CV 0.07

Analysis of Variance Table for WEEK 2

Source	DF	SS	MS	F	P
REP	2	5.556E+12	2.778E+12		
CONC	2	1.781E+19	8.906E+18	3206250	0.0000
SAMPLE	3	2.195E+19	7.318E+18	2634443	0.0000
CONC*SAMPLE	6	4.307E+19	7.178E+18	2583940	0.0000
Error	22	6.111E+13	2.778E+12		
Total	35	8.283E+19			

Grand Mean 1.02E+09 CV 0.16

Analysis of Variance Table for WEEK4

Source	DF	SS	MS	F	P
REP	2	16.6667	8.33333		
CONC	2	3.990E+18	1.995E+18	2.4E+17	0.0000
SAMPLE	3	4.251E+18	1.417E+18	1.7E+17	0.0000
CONC*SAMPLE	6	1.005E+19	1.675E+18	2.0E+17	0.0000
Error	22	183.333	8.33333		
Total	35	1.829E+19			

Grand Mean 1.05E+09 CV 0.00

WARNING: The total sums of squares is very large compared to the residual sums of squares, indicating a potential round-off error problem.

The model may fit the data exactly.

Analysis of Variance Table for WEEK 8

Source	DF	SS	MS	F	P
REP	2	5555556	2777778		
CONC	2	5.679E+17	2.839E+17	1.0E+11	0.0000
SAMPLE	3	2.771E+17	9.237E+16	3.3E+10	0.0000
CONC*SAMPLE	6	5.770E+17	9.617E+16	3.5E+10	0.0000
Error	22	6.111E+07	2777778		
Total	35	1.422E+18			

Grand Mean 9.38E+07 CV 0.00

WARNING: The total sums of squares is very large compared to the residual sums of squares, indicating a potential round-off error problem.

The model may fit the data exactly.

APPENDIX 4. C

ANOVA TABLE FOR NITROGEN CONTENT

Student Edition of Statistix 9.0 08/09/2012, 05:09:09 PM

Analysis of Variance Table for WEEK 0

Source	DF	SS	MS	F	P
CONC	2	0.54000	0.27000	211.30	0.0000
SAMPLE	3	0.96730	0.32243	252.34	0.0000
CONC*SAMPLE	6	0.18000	0.03000	23.48	0.0000
Error	24	0.03067	0.00128		
Total	35	1.71797			

Grand Mean 0.3154 CV 11.33

Analysis of Variance Table for WEEK 2

Source	DF	SS	MS	F	P
CONC	2	0.14591	0.07295	44.67	0.0000
SAMPLE	3	0.42795	0.14265	87.34	0.0000
CONC*SAMPLE	6	0.10002	0.01667	10.21	0.0000
Error	24	0.03920	0.00163		
Total	35	0.71307			

Grand Mean 0.2360 CV 17.12

Analysis of Variance Table for WEEK 4

Source	DF	SS	MS	F	P
CONC	2	0.13301	0.06651	40.72	0.0000
SAMPLE	3	0.43146	0.14382	88.05	0.0000
CONC*SAMPLE	6	0.06907	0.01151	7.05	0.0002
Error	24	0.03920	0.00163		
Total	35	0.67274			

Grand Mean 0.2229 CV 18.13

Analysis of Variance Table for WEEK 8

Source	DF	SS	MS	F	P
CONC	2	0.08583	0.04292	26.28	0.0000
SAMPLE	3	0.34729	0.11576	70.87	0.0000
CONC*SAMPLE	6	0.04953	0.00826	5.05	0.0018
Error	24	0.03920	0.00163		
Total	35	0.52185			
Grand Mean	0.1896	CV	21.32		

ANOVA MEANS FOR NITROGEN CONTENT

Student Edition of Statistix 9.0 08/09/2012, 05:09:30 PM

Tukey HSD All-Pairwise Comparisons Test of WEEK0 for CONC

CONC	Mean	Homogeneous Groups
3	0.4652	A
2	0.3156	B
1	0.1652	C

Alpha 0.05 Standard Error for Comparison 0.0146

Critical Q Value 3.533 Critical Value for Comparison 0.0365

Error term used: Error, 24 DF

All 3 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK0 for SAMPLE

SAMPLE	Mean	Homogeneous Groups
C	0.4100	A
PD	0.4100	A
RS	0.4100	A
CONTROL	0.0314	B

Alpha 0.05 Standard Error for Comparison 0.0169

Critical Q Value 3.902 Critical Value for Comparison 0.0465

Error term used: Error, 24 DF

There are 2 groups (A and B) in which the means

are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK0 for CONC*SAMPLE

CONC	SAMPLE	Mean	Homogeneous Groups
3	C	0.6100	A
3	PD	0.6100	A
3	RS	0.6100	A
2	C	0.4100	B
2	PD	0.4100	B
2	RS	0.4100	B
1	C	0.2100	C
1	PD	0.2100	C
1	RS	0.2100	C
2	CONTROL	0.0323	D
1	CONTROL	0.0310	D
3	CONTROL	0.0310	D

Alpha 0.05 Standard Error for Comparison 0.0292

Critical Q Value 5.098 Critical Value for Comparison 0.1052

Error term used: Error, 24 DF

There are 4 groups (A, B, etc.) in which the means

are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 2 for CONC

CONC	Mean	Homogeneous Groups
3	0.3059	A
2	0.2502	B
1	0.1519	C

Alpha 0.05 Standard Error for Comparison 0.0165

Critical Q Value 3.533 Critical Value for Comparison 0.0412

Error term used: Error, 24 DF

All 3 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 2 for SAMPLE

SAMPLE	Mean	Homogeneous Groups
RS	0.3090	A
C	0.3050	A
PD	0.2820	A
CONTROL	0.0480	B

Alpha 0.05 Standard Error for Comparison 0.0191

Critical Q Value 3.902 Critical Value for Comparison 0.0526

Error term used: Error, 24 DF

There are 2 groups (A and B) in which the means are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 2 for CONC*SAMPLE

CONC	SAMPLE	Mean	Homogeneous Groups
3	C	0.4337	A
3	PD	0.4127	AB
2	RS	0.3967	AB
3	RS	0.3287	ABC
2	C	0.3127	BCD
2	PD	0.2447	CDE
1	RS	0.2017	DE
1	PD	0.1887	E
1	C	0.1687	E
1	CONTROL	0.0487	F
3	CONTROL	0.0487	F
2	CONTROL	0.0467	F

Alpha 0.05 Standard Error for Comparison 0.0330

Critical Q Value 5.098 Critical Value for Comparison 0.1190

Error term used: Error, 24 DF

There are 6 groups (A, B, etc.) in which the means are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 4 for CONC

CONC	Mean	Homogeneous Groups
3	0.2954	A
2	0.2267	B
1	0.1467	C

Alpha 0.05 Standard Error for Comparison 0.0165

Critical Q Value 3.533 Critical Value for Comparison 0.0412

Error term used: Error, 24 DF

All 3 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 4 for SAMPLE

SAMPLE	Mean	Homogeneous Groups
C	0.3333	A
RS	0.2657	B
PD	0.2517	B
CONTROL	0.0410	C

Alpha 0.05 Standard Error for Comparison 0.0191

Critical Q Value 3.902 Critical Value for Comparison 0.0526

Error term used: Error, 24 DF

There are 3 groups (A, B, etc.) in which the means

are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 4 for CONC*SAMPLE

CONC	SAMPLE	Mean	Homogeneous Groups
3	C	0.4267	A
3	PD	0.3987	A
2	C	0.3567	AB
3	RS	0.3147	ABC
2	RS	0.2797	BC
2	PD	0.2307	CD
1	C	0.2167	CD
1	RS	0.2027	CD
1	PD	0.1257	DE
1	CONTROL	0.0417	E
3	CONTROL	0.0417	E
2	CONTROL	0.0397	E

Alpha 0.05 Standard Error for Comparison 0.0330

Critical Q Value 5.098 Critical Value for Comparison 0.1190

Error term used: Error, 24 DF

There are 5 groups (A, B, etc.) in which the means

are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 8 for CONC

CONC	Mean	Homogeneous Groups
3	0.2409	A
2	0.2039	A
1	0.1239	B

Alpha 0.05 Standard Error for Comparison 0.0165

Critical Q Value 3.533 Critical Value for Comparison 0.0412

Error term used: Error, 24 DF

There are 2 groups (A and B) in which the means

are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 8 for SAMPLE

SAMPLE	Mean	Homogeneous Groups
C	0.2983	A
RS	0.2400	B
PD	0.1860	C
CONTROL	0.0340	D

Alpha 0.05 Standard Error for Comparison 0.0191

Critical Q Value 3.902 Critical Value for Comparison 0.0526

Error term used: Error, 24 DF

All 4 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 8 for CONC*SAMPLE

CONC	SAMPLE	Mean	Homogeneous Groups
3	C	0.4197	A
3	RS	0.2937	B
2	C	0.2867	B
2	RS	0.2727	B
2	PD	0.2237	BC
3	PD	0.2157	BC
1	C	0.1887	BC
1	RS	0.1537	C
1	PD	0.1187	CD
1	CONTROL	0.0347	D
3	CONTROL	0.0347	D
2	CONTROL	0.0327	D

Alpha 0.05 Standard Error for Comparison 0.0330

Critical Q Value 5.098 Critical Value for Comparison 0.1190

Error term used: Error, 24 DF

There are 4 groups (A, B, etc.) in which the means

are not significantly different from one another.

APPENDIX 4. D

ANOVA TABLE FOR ORGANIC CARBON CONTENT

Student Edition of Statistix 9.0 08/09/2012, 03:15:20 PM

Analysis of Variance Table for WEEK 0

Source	DF	SS	MS	F	P
REP	2	1.4194	0.70972		
CONC	2	6.2358	3.11791	985388	0.0000
SAMPLE	3	23.9743	7.99144	2525625	0.0000
CONC*SAMPLE	6	2.9201	0.48668	153811	0.0000
Error	22	0.0001	3.164E-06		
Total	35	34.5497			
Grand Mean	2.6717 CV 0.07				

Analysis of Variance Table for WEEK 2

Source	DF	SS	MS	F	P
REP	2	1.41928	0.70964		
CONC	2	5.78459	2.89230	1838442	0.0000
SAMPLE	3	5.17766	1.72589	1097033	0.0000
CONC*SAMPLE	6	2.75512	0.45919	291875	0.0000
Error	22	3.461E-05	1.573E-06		
Total	35	15.1367			
Grand Mean	1.8494 CV 0.07				

Analysis of Variance Table for WEEK 4

Source	DF	SS	MS	F	P
REP	2	1.41674	0.70837		
CONC	2	1.62794	0.81397	246620	0.0000
SAMPLE	3	1.32677	0.44226	133997	0.0000
CONC*SAMPLE	6	1.34491	0.22415	67914.2	0.0000
Error	22	0.00007	3.301E-06		
Total	35	5.71643			

Grand Mean 1.4709 CV 0.12

Analysis of Variance Table for WEEK 8

Source	DF	SS	MS	F	P
REP	2	1.42216	0.71108		
CONC	2	0.58103	0.29052	194004	0.0000
SAMPLE	3	1.10150	0.36717	245191	0.0000
CONC*SAMPLE	6	1.55623	0.25937	173206	0.0000
Error	22	0.00003	1.497E-06		
Total	35	4.66095			

Grand Mean 1.2599 CV 0.10

ANOVA MEANS FOR ORGANIC CARBON CONTENT

Student Edition of Statistix 9.0 08/09/2012, 03:15:44 PM

Tukey HSD All-Pairwise Comparisons Test of WEEK 0 for CONC

CONC	Mean	Homogeneous Groups
3	3.0420	A
2	2.8828	B
1	2.0903	C

Alpha 0.05 Standard Error for Comparison 7.262E-04

Critical Q Value 3.554 Critical Value for Comparison 1.825E-03

Error term used: REP*CONC*SAMPLE, 22 DF

All 3 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK0 for SAMPLE

SAMPLE	Mean	Homogeneous Groups
C	3.3259	A
RS	3.1629	B
PD	2.9171	C
CONTROL	1.2809	D

Alpha 0.05 Standard Error for Comparison 8.385E-04

Critical Q Value 3.928 Critical Value for Comparison 2.329E-03

Error term used: REP*CONC*SAMPLE, 22 DF

All 4 means are significantly different from one another

Tukey HSD All-Pairwise Comparisons Test of WEEK 0 for CONC*SAMPLE

CONC	SAMPLE	Mean	Homogeneous Groups
2	C	3.7783	A
3	C	3.6687	B
2	RS	3.6407	C
3	RS	3.5093	D
3	PD	3.3093	E
2	PD	3.2307	F
1	C	2.5307	G
1	RS	2.3387	H
1	PD	2.2113	I
3	CONTROL	1.6807	J
1	CONTROL	1.2807	K
2	CONTROL	0.8813	L

Alpha 0.05 Standard Error for Comparison 1.452E-03

Critical Q Value 5.142 Critical Value for Comparison 5.281E-03

Error term used: REP*CONC*SAMPLE, 22 DF

All 12 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 2 for CONC

CONC	Mean	Homogeneous Groups
3	2.2653	A
2	1.9749	B
1	1.3078	C

Alpha 0.05 Standard Error for Comparison 5.121E-04

Critical Q Value 3.554 Critical Value for Comparison 1.286E-03

Error term used: REP*CONC*SAMPLE, 22 DF

All 3 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 2 for SAMPLE

SAMPLE	Mean	Homogeneous Groups
C	2.1997	A
RS	2.1333	B
PD	1.8244	C
CONTROL	1.2400	D

Alpha 0.05 Standard Error for Comparison 5.913E-04

Critical Q Value 3.928 Critical Value for Comparison 1.642E-03

Error term used: REP*CONC*SAMPLE, 22 DF

All 4 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 2 for CONC*SAMPLE

CONC	SAMPLE	Mean	Homogeneous Groups
2	RS	2.6700	A
3	C	2.6700	A
2	C	2.4803	B
3	RS	2.4100	C
3	PD	2.3413	D
2	PD	1.9093	E
3	CONTROL	1.6400	F
1	C	1.4487	G
1	RS	1.3200	H
1	CONTROL	1.2400	I
1	PD	1.2227	J
2	CONTROL	0.8400	K

Alpha 0.05 Standard Error for Comparison 1.024E-03

Critical Q Value 5.142 Critical Value for Comparison 3.724E-03

Error term used: REP*CONC*SAMPLE, 22 DF

There are 11 groups (A, B, etc.) in which the means

are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 4 for CONC

CONC	Mean	Homogeneous Groups
3	**1.7649**	**A**
2	**1.3784**	**B**
1	**1.2692**	**C**

Alpha 0.05 Standard Error for Comparison 7.417E-04

Critical Q Value 3.554 Critical Value for Comparison 1.864E-03

Error term used: REP*CONC*SAMPLE, 22 DF

All 3 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK4 for SAMPLE

SAMPLE	Mean	Homogeneous Groups
RS	1.6961	A
C	1.5690	B
PD	1.4410	C
CONTROL	1.1773	D

Alpha 0.05 Standard Error for Comparison 8.564E-04

Critical Q Value 3.928 Critical Value for Comparison 2.378E-03

Error term used: REP*CONC*SAMPLE, 22 DF

All 4 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 4 for CONC*SAMPLE

CONC	SAMPLE	Mean	Homogeneous Groups
3	RS	2.2410	A
2	C	1.7793	B
3	PD	1.6910	C
3	CONTROL	1.5787	D
2	RS	1.5490	E
3	C	1.5490	E
2	PD	1.4093	F
1	C	1.3787	G
1	RS	1.2983	H
1	PD	1.2227	I
1	CONTROL	1.1773	J
2	CONTROL	0.7760	K

Alpha 0.05 Standard Error for Comparison 1.483E-03

Critical Q Value 5.142 Critical Value for Comparison 5.394E-03

Error term used: REP*CONC*SAMPLE, 22 DF

There are 11 groups (A, B, etc.) in which the means

are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 8 for CONC

CONC	Mean	Homogeneous Groups
3	1.4393	A
1	1.1773	B
2	1.1629	C

Alpha 0.05 Standard Error for Comparison 4.996E-04

Critical Q Value 3.554 Critical Value for Comparison 1.255E-03

Error term used: REP*CONC*SAMPLE, 22 DF

All 3 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 8 for SAMPLE

SAMPLE	Mean	Homogeneous Groups
C	1.4332	A
RS	1.4329	A
CONTROL	1.1213	B
PD	1.0520	C

Alpha 0.05 Standard Error for Comparison 5.769E-04

Critical Q Value 3.928 Critical Value for Comparison 1.602E-03

Error term used: REP*CONC*SAMPLE, 22 DF

There are 3 groups (A, B, etc.) in which the means

are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK8 for CONC*SAMPLE

CONC	SAMPLE	Mean	Homogeneous Groups
3	RS	1.8493	A
2	C	1.7090	B
3	CONTROL	1.5207	C
1	C	1.3307	D
2	RS	1.2600	E
3	C	1.2600	E
1	RS	1.1893	F
3	PD	1.1273	G
1	CONTROL	1.1213	H
1	PD	1.0680	I
2	PD	0.9607	J
2	CONTROL	0.7220	K

Alpha 0.05 Standard Error for Comparison 9.992E-04

Critical Q Value 5.142 Critical Value for Comparison 3.633E-03

Error term used: REP*CONC*SAMPLE, 22 DF

There are 11 groups (A, B, etc.) in which the means

are not significantly different from one another.

APPENDIX 4. E

ANOVA TABLE FOR ORGANIC MATTER CONTENT

Student Edition of Statistix 9.0 08/09/2012, 03:11:22 PM

Analysis of Variance Table for WEEK0

Source	DF	SS	MS	F	P
REP	2	0.5400	0.27000		
CONC	2	15.0263	7.51314	1892624	0.0000
SAMPLE	3	18.5064	6.16880	1553973	0.0000
CONC*SAMPLE	6	11.6953	1.94921	491023	0.0000
Error	22	0.0001	3.970E-06		
Total	35	45.7681			

Grand Mean 4.5873 CV 0.04

Analysis of Variance Table for WEEK2

Source	DF	SS	MS	F	P
REP	2	0.54000	0.27000		
CONC	2	16.3536	8.17679	4429559	0.0000
SAMPLE	3	3.15487	1.05162	569689	0.0000
CONC*SAMPLE	6	6.78400	1.13067	612509	0.0000
Error	22	4.061E-05	1.846E-06		
Total	35	26.8325			

Grand Mean 3.6599 CV 0.04

Analysis of Variance Table for WEEK 4

Source	DF	SS	MS	F	P
REP	2	0.5400	0.27000		
CONC	2	6.0233	3.01163	671511	0.0000
SAMPLE	3	5.6747	1.89158	421770	0.0000
CONC*SAMPLE	6	1.9615	0.32692	72893.9	0.0000
Error	22	0.0001	4.485E-06		
Total	35	14.1996			
Grand Mean	2.8220	CV 0.08			

Analysis of Variance Table for WEEK8

Source	DF	SS	MS	F	P
REP	2	0.5400	0.27000		
CONC	2	2.3109	1.15545	432067	0.0000
SAMPLE	3	5.8416	1.94721	728134	0.0000
CONC*SAMPLE	6	1.6322	0.27203	101722	0.0000
Error	22	0.0001	2.674E-06		
Total	35	10.3248			
Grand Mean	2.4607	CV 0.07			

ANOVA MEANS FOR ORGANIC MATTER CONTENT

Student Edition of Statistix 9.0 08/09/2012, 03:12:01 PM

Tukey HSD All-Pairwise Comparisons Test of WEEK 0 for CONC

CONC	Mean	Homogeneous Groups
3	5.2798	A
2	4.7573	B
1	3.7249	C

Alpha 0.05 Standard Error for Comparison 8.134E-04

Critical Q Value 3.554 Critical Value for Comparison 2.044E-03

Error term used: REP*CONC*SAMPLE, 22 DF

All 3 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK0 for SAMPLE

SAMPLE	Mean	Homogeneous Groups
C	5.3368	A
RS	4.9022	B
PD	4.6990	C
CONTROL	3.4113	D

Alpha 0.05 Standard Error for Comparison 9.392E-04

Critical Q Value 3.928 Critical Value for Comparison 2.608E-03

Error term used: REP*CONC*SAMPLE, 22 DF

All 4 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 0 for CONC*SAMPLE

CONC	SAMPLE	Mean	Homogeneous Groups
3	C	6.8597	A
2	RS	5.8583	B
2	C	5.4797	C
3	RS	5.3897	D
3	PD	5.2587	E
2	PD	4.4797	F
1	PD	4.3587	G
1	C	3.6710	H
3	CONTROL	3.6113	I
1	RS	3.4587	J
1	CONTROL	3.4113	K
2	CONTROL	3.2113	L

Alpha 0.05 Standard Error for Comparison 1.627E-03

Critical Q Value 5.142 Critical Value for Comparison 5.915E-03

Error term used: REP*CONC*SAMPLE, 22 DF

All 12 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 2 for CONC

CONC	Mean	Homogeneous Groups
3	4.2673	A
2	3.9923	B
1	2.7200	C

Alpha 0.05 Standard Error for Comparison 5.547E-04

Critical Q Value 3.554 Critical Value for Comparison 1.393E-03

Error term used: REP*CONC*SAMPLE, 22 DF

All 3 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 2 for SAMPLE

SAMPLE	Mean	Homogeneous Groups
C	4.0173	A
RS	3.8870	B
CONTROL	3.3793	C
PD	3.3558	D

Alpha 0.05 Standard Error for Comparison 6.405E-04

Critical Q Value 3.928 Critical Value for Comparison 1.779E-03

Error term used: REP*CONC*SAMPLE, 22 DF

All 4 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 2 for CONC*SAMPLE

CONC	SAMPLE	Mean	Homogeneous Groups
3	C	4.8703	A
2	RS	4.8100	B
2	C	4.4813	C
3	RS	4.3700	D
3	PD	4.2493	E
3	CONTROL	3.5793	F
2	PD	3.4987	G
1	CONTROL	3.3793	H
2	CONTROL	3.1793	I
1	C	2.7003	J
1	RS	2.4810	K
1	PD	2.3193	L

Alpha 0.05 Standard Error for Comparison 1.109E-03

Critical Q Value 5.142 Critical Value for Comparison 4.034E-03

Error term used: REP*CONC*SAMPLE, 22 DF

All 12 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 4 for CONC

CONC	Mean	Homogeneous Groups
3	3.3723	A
2	2.7012	B
1	2.3925	C

Alpha 0.05 Standard Error for Comparison 8.646E-04

Critical Q Value 3.554 Critical Value for Comparison 2.172E-03

Error term used: REP*CONC*SAMPLE, 22 DF

All 3 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 4 for SAMPLE

SAMPLE	Mean	Homogeneous Groups
C	3.2363	A
RS	3.1274	B
PD	2.6929	C
CONTROL	2.2313	D

Alpha 0.05 Standard Error for Comparison 9.983E-04

Critical Q Value 3.928 Critical Value for Comparison 2.773E-03

Error term used: REP*CONC*SAMPLE, 22 DF

All 4 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 4 for CONC*SAMPLE

CONC	SAMPLE	Mean	Homogeneous Groups
3	RS	4.0713	A
3	C	3.8583	B
2	C	3.2707	C
3	PD	3.1283	D
2	RS	2.8713	E
2	PD	2.6313	F
1	C	2.5800	G
1	RS	2.4397	H
3	CONTROL	2.4313	I
1	PD	2.3190	J
1	CONTROL	2.2313	K
2	CONTROL	2.0313	L

Alpha 0.05 Standard Error for Comparison 1.729E-03

Critical Q Value 5.142 Critical Value for Comparison 6.288E-03

Error term used: REP*CONC*SAMPLE, 22 DF

All 12 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 8 for CONC

CONC	Mean	Homogeneous Groups
3	2.8151	A
2	2.3297	B
1	2.2375	C

Alpha 0.05 Standard Error for Comparison 6.676E-04

Critical Q Value 3.554 Critical Value for Comparison 1.678E-03

Error term used: REP*CONC*SAMPLE, 22 DF

All 3 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 8 for SAMPLE

SAMPLE	Mean	Homogeneous Groups
C	3.0101	A
RS	2.6767	B
CONTROL	2.1407	C
PD	2.0156	D

Alpha 0.05 Standard Error for Comparison 7.709E-04

Critical Q Value 3.928 Critical Value for Comparison 2.141E-03

Error term used: REP*CONC*SAMPLE, 22 DF

All 4 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of WEEK 8 for CONC*SAMPLE

CONC	SAMPLE	Mean	Homogeneous Groups
3	RS	3.3910	A
3	C	3.3793	B
2	C	3.1497	C
1	C	2.5013	D
2	RS	2.3800	E
3	CONTROL	2.3407	F
1	RS	2.2590	G
3	PD	2.1493	H
1	CONTROL	2.1407	I
1	PD	2.0490	J
2	CONTROL	1.9407	K
2	PD	1.8483	L

Alpha 0.05 Standard Error for Comparison 1.335E-03

Critical Q Value 5.142 Critical Value for Comparison 4.855E-03

Error term used: REP*CONC*SAMPLE, 22 DF

All 12 means are significantly different from one another.

APPENDIX 5. A

PHOTOGRAPH OF THE EXPERIMENTAL SET-UP

APPENDIX 5. B

Kjedahl apparatus for determining Total Nitrogen