USING NITROGEN AND PHOSPHORUS TO STIMULATE MICROBIAL DEGRADATION OF DIESEL OIL IN FOUR GHANAIAN SOILS.

BY

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DECLARATION

I do hereby declare that this thesis has been written by me and that it is the record of my own research work. It has not been presented for another degree elsewhere. Works of other researchers have been duly cited by references to the authors. All assistance received has also been acknowledged.

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DEDICATION

This work is affectionately and humbly dedicated to my wife, Abakisi Priscilla, my children, Gerald and Giselle, my caring Mother and lovely Father, my siblings namely, Patrick, Modesta, Jerremiah and Godfred and to all those who took an interest and encourage me in my academic pursuit.

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ABSTRACT

The amendment of oil-contaminated soils with nutrient elements, especially nitrogen and phosphorus, is a crucial factor for the enhancement of the growth of the indigenous microbes involved in the biodegradation of petroleum hydrocarbons. The present study investigated the(i) effects of nitrogen and phosphorus on diesel oil degradation in four Ghanaian soils, Beach sand (Haplic Arenosol), Toje series (Rhodic Kandiustalfs), Nyankpala series (Plinthic Lixisol), and Oda series (Eutric Gleysol), and (ii) germination and growth of maize and cowpea in diesel oil contaminated soil amended with nitrogen and phosphorus. The soils were contaminated with diesel oil at 10 g oil/kg soil and amended with single super phosphate (P_2O_5) and ammonium nitrate (N) at 0, 30, 60 and 90 kg/ha separately. The treated soils were incubated in the laboratory under room temperature and sampled at 10 days interval for 40 days to monitor the growth of hydrocarbon utilizing bacteria (HUB) and the amount of oil degraded. In the plant culture study, 2 kg of the Toje series was contaminated with diesel oil at 0, 2.5, 5 and 10 g oil/kg soil. The contaminated and uncontaminated soils were amended with N and P, each at a rate of 60kg/ha and maize and cowpea were grown 20 days after contamination. Results showed thatsupplementation of soil with N and P stimulated the growth of HUB and enhanced degradation of diesel oil in the four soils. Increasing the application rate of N and P enhanced both HUB growth and degradation of oil. Although the initial HUB populations of Beach sand and Nyankpala series were lower thanthat of Toje and Oda series, they showed better degradation of the oil. Besides, the HUB populations of the Beach sand and Nyankpala series reached their peaks earlier than Toje and Oda series. The rates of biodegradation of oil in the soils were in the order Beach > Nyankpala > Toje > Oda. These results suggest that (i) the Beach sand and Nyankpala series could contain highly effective HUB useful for the rapid bioremediation of oil-contaminated

soils and (ii) HUB populations whose growth peaks early during bioremediation could be more effective degraders than those that peak later. Results also showed that diesel contamination had adverse impact on nodulation of cowpea and growth of both maize and cowpea. However, addition of nitrogen and phosphorus to the oil contaminatedsoils improved germination, shoot dry matter and nutrient uptake. The effect of nitrogen supplementation in the contaminated soils was pronounced in maize whilst that of phosphorus was pronounced in cowpea. This implies that nutrient elements are important for the growth of crops in oil contaminated soils and nutrient elements are specific to crops grown in oil contaminated soils. Further research should be conducted to isolate and identify effective HUB from Ghanaian soils especially from the Beach sand.

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CHAPTERONE

INTRODUCTION

Petroleum based products are the major source of energy for our industries, transportation construction and agriculture. Over 2 billion tons of petroleum is produced annually worldwide andthe National Research Council estimated that, between 1990 and 1999, over 260,000 tons of oil were released into the natural environment in North America from various sources each year, and over 1,300,000 tons worldwide (National Academy of Sciences, 2002)Environmental contamination with petroleum introduces a myriad of hydrocarbons, causing a variety of problems (Atlas and Philp, 2005).For example, a good percentage of oil spills that occurred on the dry land between 1978 and 1979 in Nigeria, affected farm-lands in which crops such as rice, maize, yams, cassava plantain were cultivated (Onyefulu and Awobajo, 1979). Crude oil affects germination and growth of some plants (Onwurah, 1999). It also affects soil fertility but the scale of impact depends on the quantity and type of oil spilled. Crude oil contamination of land affects certain soil parameters such as the mineral and organic matter content, the cation exchange capacity, redox properties and pH value. As crude oil creates anaerobic condition in the soil, coupled to water logging and acidic metabolites, the result is high accumulation of aluminum and manganese ions, which are toxic to plant growth. Spillages of oil have become a common occurrence. There have being major oil spills the world over, some of which include, Gulf of Mexico oil spill which occurred in the USA in April 2010, Exxon Mobil oil spill in the Niger Delta of Nigeria in May 2010, Mumbai oil spill in India in August 2010 and the Persian Gulf oil spill in Kuwait in January 1991 (Moss, 2010).

Ghana discovered crude oil in 2007 at Cape Three Points in the Western Region and commercial production of crude oil in the country started in late 2010. On December 26, 2009, Ghana experienced its first spillage of about 584 barrels of low-based mud drilling

fluid and the second mud spill of 7 barrels occurred on March 23, 2010 (Ghanaian Daily Graphic, 2010). Ghana is especially vulnerable to oil spills due to the high volume of oil being produced and transported around the coasts. Each major spill incident in the world increases the vulnerability of our fragile environment (Ibe, 2000; Ekpo and Nwankpa, 2005). This is because most spills are often toxic and generally cause deficiency in essential plant nutrients especially available P and total nitrogen.

Apart from phytotoxicity, nitrogen is the major element limiting plant growth in most spills and hydrocarbon contaminated sites (Wyszkowski *et al*., 2004). Crude oil and petroleum are complex mixtures of severalpolycyclic aromatic compounds andother hydrocarbons (Domask, 1984). However, diesel is of significance because it is one of the most extensively used fraction of crude and also it does not volatilised very readily as gasoline (petrol). The major hydrocarbon classes found in diesel fuel (Mackay *et al*., 1985) are the normal alkanes (rapidly degraded), branched alkanes and cycloalkanes (difficult to identify), the isoprenoids (very resistant to biodegradation), the aromatics, (fairly identified and much more soluble than other hydrocarbons), and finally the polar onescontaining mainly sulphur, oxygen and / or nitrogen compounds. Diverse components of crude oil and petroleum such as polycyclic aromatic hydrocarbons ($PAHs_s$) have been found in waterways and in soil as a result of pollution from industrial effluents and petrochemical products (Beckles*et al*., 1998). Contamination of the soil environment by diesel can limit its protective function, upset metabolic activity, unfavourably affect its chemical characteristics, reduce fertility and negatively influence plant production (Gong *et al*., 1996; Wyszkowski *et al*., 2004; Wyszkowski and Wyszkowska, 2005). Contamination of the soil by diesel oil threatens human health and that of the organisms that are dependent on the soil (Aboribo, 2001).

Biodegradation of hydrocarbon-contaminated soils, which exploits the ability of microorganisms to degrade and detoxify organic contaminants, has been established as an efficient, economic, versatile and environmentally sound treatment (Mehrashi *et al*., 2003). Biodegradation is possible because microorganisms have enzyme systems to degrade and utilise diesel oil as a source of carbon and energy (Ijah and Antai, 1988; Ezeji *et al*., 2005; Antai and Mgbomo, 1993). There are two approaches in bioremediation: either the contaminated sites are inoculated with specific hydrocarbon degrading microorganisms (bioaugmentation), or the activity of indigenous organisms is enhanced in situ by addition of appropriate nutrients and inducers (biostimulation) (Morgan and Watkinson, 1989; Sylvestre and Sondossi, 1994). As an alternative to physicochemical clean-up methods that result in negative consequences, bioremediation offers an appealing treatment technology for petroleum hydrocarbon-contaminated soils. Bioremediation processes are effective methods that stimulate the biodegradation in contaminated soils by including additives or improving the availability of materials (Swannell *et al*., 1996).

Biostimulation of indigenous microorganisms by the addition of inorganic nutrients such as nitrogen, phosphorous and potassium that are rapidly depleted because of the high carbon content due to hydrocarbon contamination has been widely used in diesel contaminated soils(Molina-Barahona *et al*., 2004; Perfumo *et al*., 2006). Biostimulation is considered as the most appropriate remediation technique for diesel removal in soil and requires the evaluation of both intrinsic degradation capacities of the autochthonous microflora and the environmental conditions involved in the kinetics of the *in situ* process (Molina-Barahona *et al*., 2004). Nutrient addition stimulates the degradative capabilities of the indigenous microorganismsfound in the soil, as compared to the unamended samples. This allows the microorganisms tobreak down the organic pollutants at a faster rate (Dzantor, 1999; Ausma *et al*., 2002). Creating optimal environmental conditions such as

pH, temperature, oxygen and bioavailability of substrates (contaminants) stimulates the rate of biodegradation. All microbes perform best at optimal nutritional levels of nitrogen, phosphorous, carbon and smaller quantities of other elements such as magnesium, potassium and iron. The microorganisms utilize these nutrients that stimulate the enzymes that enhance biodegradation (Margesin and Schinner, 2001). This process stimulates the numbers and activities of microbial populations, such as bacteria and fungi to effectively degrade the pollutants to harmless products (Dzantor, 1999).Although the potential capability of the indigenous microflora to degrade oil is a function of the physical and chemical properties of the soil and oil, the environmental conditions, and the biota themselves, it is generally accepted that nutrient availability is the most commonlimiting factor (Atlas and Bartha, 1972; Kim *et al*., 2004). In numerous field trials, the feasibility of adding inorganic nutrients on a periodic basis has been demonstrated as a means of sustaining elevated nutrient cones within the sediments for effective bioremediation (Lee and Levy, 1989; Lee and Levy, 1991; Venosa *et al*., 1996). Controlled studies suggest that optimum rates of degradation could be sustained by retaining high but nontoxic, levels of nutrients (Venosa *et al*., 1996; Lee *et al*., 1997). Addition of selected nutrients in the form of organic and/or inorganic fertilizers with electron acceptors such as oxygen and with substrates such as methane, phenol and toluene stimulates pollutant degradation (Thomassin- Lacroix *et al*., 2002; Sarkar *et al*., 2005). Westlake *et al*. (1978) examined the in situ degradation of oil in a soil of the boreal region of the northwest territories of Canada and found that where fertilizer containing nitrogen and phosphorus was applied to the oil, there was a rapid incr3ease in bacterial numbers. This was followed by a rapid disappearance of n-alkanes and isoprenoids and a continuous loss of weight of saturated compounds in the recovered oil. Jobson *et al.* (1974) similarly found that nitrogen and phosphorus addition stimulated hydrocarbon degradation in oil applied to soil. Hunt *et al*.,

(1973) found that fertilizer application to subarctic soils enhanced microbial hydrocarbon degradation. Raymond *et al*. (1976) studied oil biodegradation in soil. Greater oil degradation was found in soils receiving fertilizer application and rototilling than in untreated soils. Advantages of inorganic agricultural fertilizers as bioremediation agents include low cost, availability and ease of application. Furthermore, these organic nutrient formulations may also provide trace elements and other growth factors required by bacteria (Lee and Merlin, 1999).

Most studies on biostimulation and bioremediation involved one soil or soils from the same ecological zone. Bioremediation in Ghanaian soils has received little attention probably because the country has not yet experienced a large scale spillage of crude oil Besides, Ghana has various ecological zones and it would therefore be appropriate to research into the bioremediation in a country that has just discovered oil in commercial quatities.The objectives of this work are to investigate:

1. The effects of nitrogen and phosphorus on diesel oil degradation in four Ghanaian soils.
2. The germination and growth of maize and cowpea in oil contaminated soil amended with nitrogen and phosphorus

Hypothesis:

H_O: Addition of Nitrogen and Phosphorus affects biodegradation of diesel oil in Ghanaian soils

H_A: Addition of Nitrogen and Phosphorus does not affect biodegradation of diesel oil in Ghanaian soils.

CHAPTERTWO

LITERATURE REVIEW

2.1 Origin of Petroleum

Man has been using petroleum since Biblical times, yet the origin of this natural resource remained a mystery for much of man's history. Classical literature is noticeably devoid of insight and even Roger Bacon laments in his 1268 treatise *Opus Tertium* on the lack of discussion on the origins of oils and bitumen by Aristotle and other natural philosophers(Bacon, 1859). Two theories on petroleum emerged during the Renaissance. These theories are the biogenic and abiogenic theories.

Although there is overwhelming evidence for a biogenic origin, some still advocate abiotic theories. The development of the modern abiogenic concept was rooted in the mid-19th century. The prolific French chemist Marcelin-Pierre Berthelot described in 1860 experiments where *n*-alkanes formed during the acid dissolution of steels (Berthelot, 1860). Dmitri Mendeleev reasoned in 1877 that surface waters could percolate deep within the Earth, react with metallic carbides forming acetylene, which could then condense further into larger hydrocarbons (Mendeleev, 1877). Mendeleev's abiotic theory, further refined in 1902 (Mendeleev, 1902) was viewed initially as particularly attractive as it offered an explanation for the growing awareness of the widespread occurrence of petroleum deposits that suggested some sort of deep global process.

Advocates of abiotic origin theories dwindled under the mounting evidence for a biogenic origin of petroleum. By the 1960's, there was little support for an abiotic origin, except among a small group within the Former Soviet Union. First proposed in 1951 by Nikolai Kudryavtsev (Kudryavtsev, 1951) and advanced over the years in numerous Soviet publications (Kenney, 1996) a modernized version of Mendeleev's hypothesis emerged.

This theory relies on a thermodynamic argument, which states that hydrocarbons greater than methane cannot form spontaneously except at the high temperatures and pressures of the lowest most crustal depths.

In the West, astronomers have been the most vocal advocates for abiotic petroleum. Carbonaceous chondrites and other planetary bodies, such as asteroids, comets, and the moons and atmospheres of the Jovian planets, certainly contain hydrocarbons and other organic compounds that were generated by abiotic processes (Cronin, 1988). Sir Fredrick Hoyle reasoned in 1955 that as the Earth was formed from similar materials, there should be vast amounts of abiogenic oil (Hoyle, 1955). In more recent years, Thomas Gold was the strongest promoter for abiotic petroleum (Gold, 1985; Gold, 1999).

Geochemists do not deny the existence of abiogenic hydrocarbons on Earth. Small amounts of abiotic hydrocarbon gases are known to be generated by rock-water interactions involving serpentinization of ultramafic rocks (Sherwood *et al*., 1993 ; McCollom and Seewald, 2001), the thermal decomposition of siderite in the presence of water (McCollom, 2003), and during magma cooling as a result of Fischer–Tropsch type reactions (Potter *et al*., 2001). However, commercial quantities of abiotic petroleum have never been found and the contribution of abiogenic hydrocarbons to the global crustal carbon budget is inconsequential (Sherwood *et al*., 2002).

As fossil evidence emerged during the 18th century that coals were derived from plant remains, many scientists proposed similar origins to explain petroleum. The historic record is somewhat questionable, but Mikhailo Lomonosov is credited by some to have proposed the theory that liquid oil and solid bitumen originate from coal through underground heat and pressure as early as 1757 (Kenney, 1996) and certainly by 1763 (Wellings, 1966). Various biogenic theories emerged during the early 19th century suggesting that petroleum was derived directly from biological remains or through a distillation process (Dott, 1969).

Modern theories that petroleum originated from ancient sedimentary, organic-rich rocks emerged during the 19 century. T.S. Hunt of the Canadian Geological Survey concluded in 1863 that the organic matter in some North American Paleozoic rocks must be derived from marine vegetation or marine animals, and that the transformation of this organic matter to bitumen must be similar to the processes involved in coal formation (Hunt, 1863). Leo Lesquereux, the American father of paleobotany, reached similar conclusions after studying Devonian shales in Pennsylvania (Lesquereux, 1866), as did Newberry in his study of Devonian shales in Ohio (Newberry, 1873). Early 20th century field and chemical studies of the Monterey Formation by the U.S. Geologic Survey provided convincing evidence that the oil was derived from diatoms in the organic-rich shales (Arnold and Anderson, 1907; Clarke, 1916). Similar studies of organic-rich shales conducted in Europe during this time arrived at the same conclusion (Pompeckj, 1901; Schuchert, 1915).

Full ascendancy of the biogenic hypothesis began in the mid-20th century with a convergence of scientific advances in paleontology, geology, and chemistry. In 1936, Alfred Treibs established a link between chlorophyll in living organisms and porphyrins in petroleum (Treibs, 1936). Additional geochemical evidence followed with the discoveries that low to moderate maturity oils still retained hydrocarbon fractions with optical activity (Oakwood *et al*., 1952), that the stable isotopes of carbon of petroleum bear a biological fractionation (Craig, 1953), and that oils contain in addition to porphyrins, a host of hydrocarbons that can be traced back to specific biological precursors (Eglinton and Calvin, 1967). Concurrent with these findings were field studies recognizing that organic-rich strata occur in all petroliferous sedimentary basins, that this sedimentary organic matter (kerogen) is derived from biota, that it has been chemically altered from its initial

state (Forsman and Hunt, 1958) and that oil and gas is produced from this kerogen as the sediments are buried and heated (Tissot, 1969).

2.2 Formation of Petroleum

The accumulation of economic volumes of petroleum (oil and/or gas) in the subsurface requires that several essential geological elements and processes be present at specific time and space (The petroleum system, 1994; Magoon and Beaumont, 1999). Source rocksgenerate and expel petroleum when sufficient thermal energy is imparted to the sedimentary organic matter (kerogen) to break chemical bonds (Magoon and Beaumont, 1999). This heating is induced usually by burial by overburden rock. Once expelled, petroleum migrates either along faults and/or highly permeable strata. Accumulations form only when high porosity strata (reservoirrocks) are charged with migrating petroleum and the petroleum is prevented from further migration. These petroleum trapsare formed only when geologic movements result in subsurface topographies (structural and stratigraphic) that block migration and when the reservoir rocks are covered by low permeability strata called sealrocks (Canfield, 1994). The mere presences of these geological elements are insufficient to form petroleum reserves. Traps must be available at the time of oil expulsion and, once charged, their integrity must be preserved until exploited.

Petroleum sourcerocksare water-deposited sedimentary rocks that contain sufficient amounts of organic matter to generate and expel commercial quantities of oil and/or gas when heated (Tyson, 1995). Such organic-rich strata were deposited throughout Earth's history, in nearly all geologic environments, and in most sedimentary basins. Source rocks, however, typically represent only a minor amount of basinal strata and are formed only when specific conditions exist.

Three general factors control the deposition of organic-rich sediments: productivity, dilution, and preservation (Canfield, 1994; Tyson, 1995). Biological productivity determines the amount of organic matter that is contributed to sediments. Dilution refers to the amount of inorganic minerals that mixes with the organic matter. Once deposited, the organic matter must be preserved in a form that may later generate petroleum. There was once an active debate as to which factor was the most important in forming organic rich sediments (Pedersen and Calvert, 1990). It is now recognized that these three factors are inherently interrelated in a highly complex, and variable manner.

2.3 Composition and Classification of Petroleum.

Petroleum and petroleum products are highly complex and varied mixtures. Hydrocarbons (compounds containing only carbon and hydrogen atoms) compose the majority of the components in petroleum (Weisman, 1998). Crude oil can consist of thousands of individual compounds with hydrocarbons representing from 50 to 98 percent of the total weight of crude oil (Irwin et al, 1998). Crude oil is comprised of both hydrocarbon compounds (accounting for 50–98% of total composition) and non-hydrocarbon compounds (containing sulfur, nitrogen, oxygen, and various trace metals) in a wide array of combinations (Clark and Brown, 1977). When petroleum compounds such as crude oil are released into the environment, the compounds undergo physical, chemical, and biological changes collectively referred to as weathering. The degree to which various types of petroleum hydrocarbons degrade under these changes depends on the physical and chemical properties of the hydrocarbons (Shannon *et al*., 2006).

Petroleum components may be classified into four major groups based on their differential solubility in organic solvents (Leahy and Colwell, 1990).

2.3.1 Saturated Hydrocarbons

They include normal and branched alkanes with structures of C_nH_{2n+2} (aliphatics) and cyclic alkanes with structures of C_nH_{2n} (alicyclics), which range in chain length from one carbon to over 40 carbons. Saturated hydrocarbons usually are the most abundant constituents in crude oils they can make up 15 to 60% of crude. Saturated hydrocarbons are the desired content in crude and what are used to make fuels. The shorter the saturated hydrocarbons are, the lighter the crude is(Xueqing*etal*., 2001).

2.3.2 Aromatic Hydrocarbons

They include monocyclic aromatics (e.g., benzene, toluene, and xylenes) and polycyclic aromatic hydrocarbons (PAHs) (e.g., naphthalene, anthracene, and phenanthrene), which have two or more fused aromatic rings. PAHs are of particular environmental concern because they are potential carcinogens or may be transformed into carcinogens by microbial metabolism. (Xueqing*etal*., 2001). Aromatics hydrocarbons are less abundant than saturatedhydrocarbonsamounting to only a few percent of the total. All aromatic hydrocarbons contain a benzenering.The benzene ring is usually indicated by Fig 2.1.

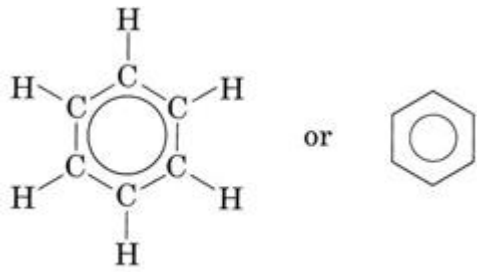
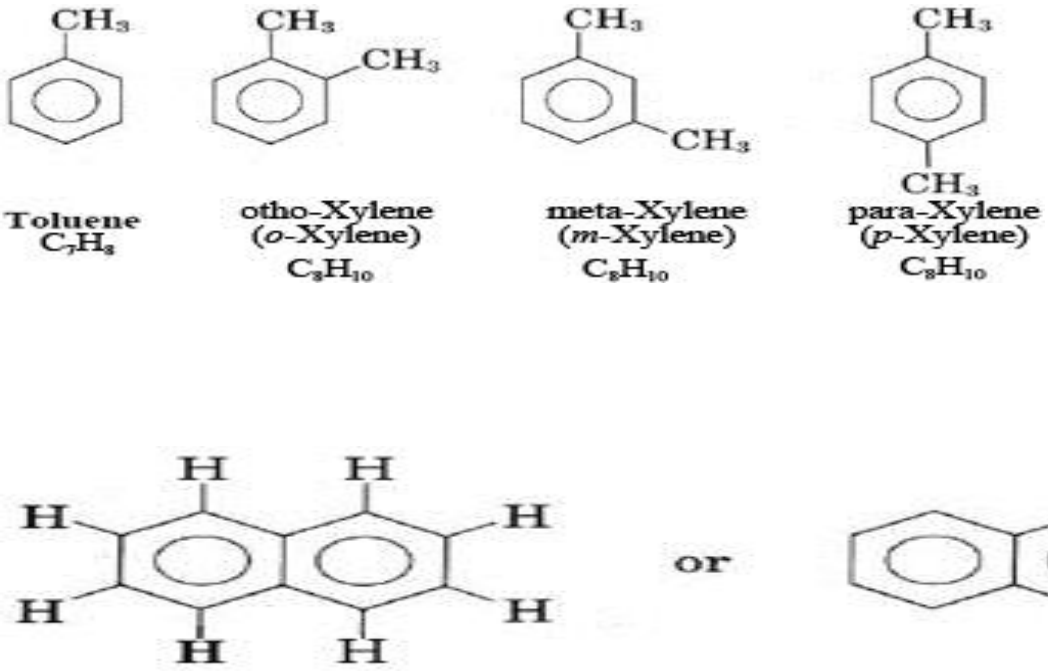


Fig. 2.1 Structure of a benzene ring

Examples of aromatic hydrocarbon in crude oil include:



Naphtthalene

Fig. 2.2Examples of aromatic hydrocarbon in crude oil.

Source: http://chemed.chem.wisc.edu/chempaths/GenChem-Textbook/Aromatic-Hydrocarbons-918.html.

2.3.3 Resins

They include polar compounds containing nitrogen, sulfur, and oxygen (e.g., pyridines and thiophenes). They are often referred to as NSO compounds because they contain atoms of nitrogen, sulphur and oxygen. (Xueqing*et al*., 2001). While the molecular weight of resins is much lower than those of asphaltenes, there is a close relationship between the molecular structures of asphaltenes and resins (Mansoori, 1996; Bunger, 1982; Acevedo *et al*., 1995)

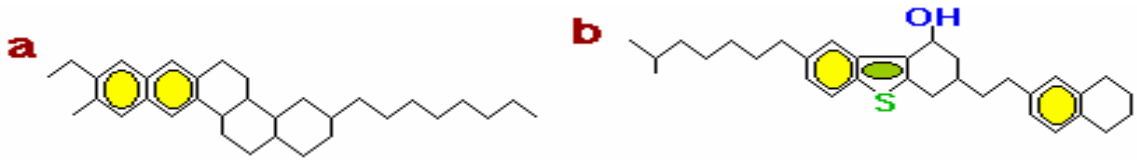


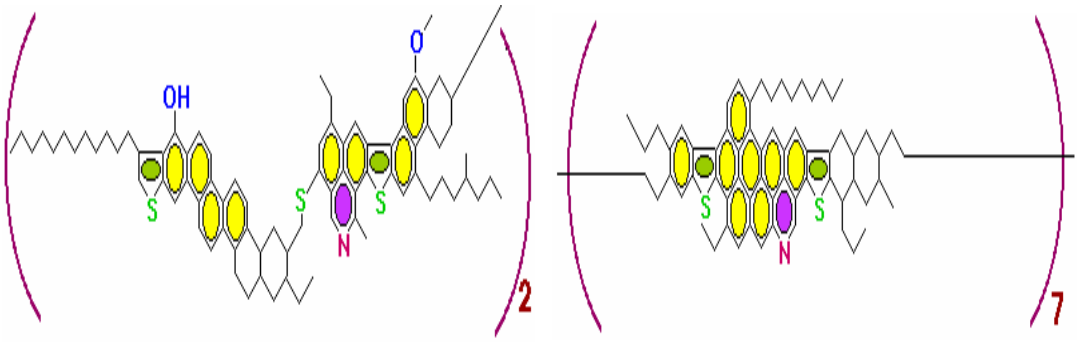
Fig. 2.3 Average molecular structure models of resins: (a) resin fraction of Athabasca tar sand and (b) resin fraction of Athabasca petroleum

Source:Suzuki et al. (1982)

2.3.4 Asphaltenes

Asphaltenes consist of poorly characterized high molecular weight compounds that include both high molecular weight and poorly characterized hydrocarbons and NSOs. Metals such as nickel, vanadium, and iron are also associated with asphaltenes (Xueqing*et al*., 2001).Fig. 2.3 shows four different asphaltene structures separated from different natural petroleum fluids.

(a) (b)



(c) (d)

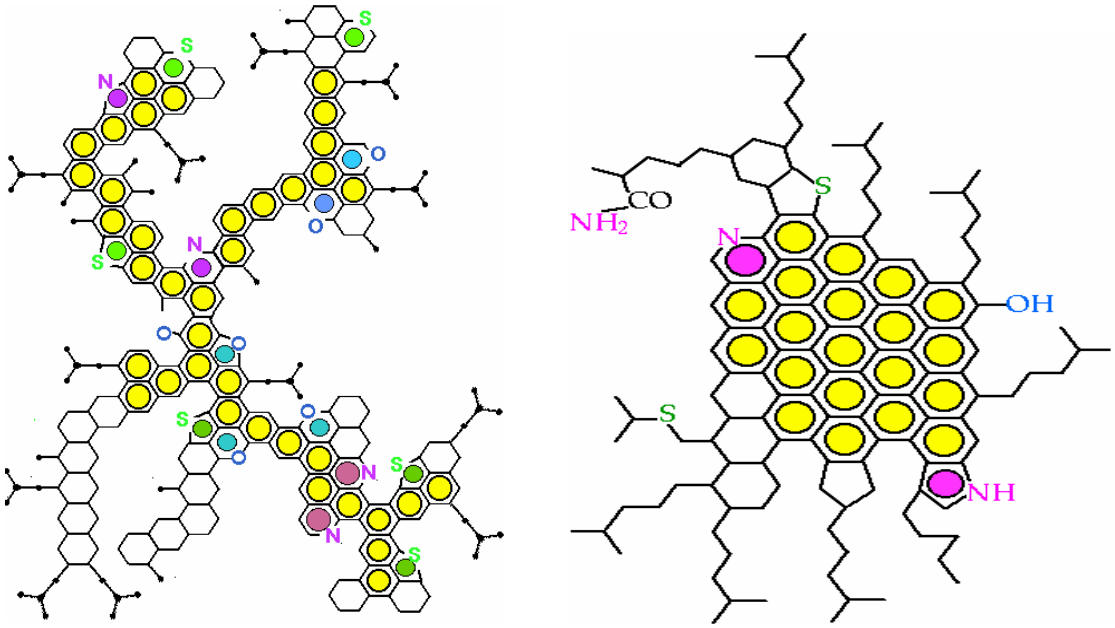


Fig. 2.4 Average molecular structure models of asphaltene: (a) asphaltene fraction of Athabasca tar sand (Canada); (b) asphaltene fraction of Athabasca heavy oil (Canada); (c) asphaltene proposed for Maya crude (Mexico) and (d) asphaltene proposed for 510C Crude Residue (Venezuela)

Note: In these figures ⬡ represents benzene ring and ⬡ represents cyclohexane ring.

Source: (a, b) Suzuki et al. (1982), (c) Altamirano et al. (1986), (d) Carbognani (1992)

2.4 Refined Oil Products

The liquid, or crude oil, portion of petroleum is transformed into commercial form via distillation. Refined petroleum products, such as gasoline, kerosene, jet fuels, fuel oils, and lubricating oils, are derived from crude oil through processes such as catalytic cracking and fractional distillation. These products have physical and chemical characteristics that differ according to the type of crude oil and subsequent refining processes. They contain components of crude oil covering a narrow range of boiling points. In addition, during catalytic cracking operations, oils, can be formed. The concentrations of olefins are as high as 30% in gasoline and about 1% in jet fuel (National Academy Sciences, 1985). A list of chemical compositions of the fractions of crude oils and the refined products is shown in Table 2.1

Table 2.1 Chemical compositions of refined petroleum products

Distillation Fraction	Hydrocarbon types	Range of carbon atoms	Typical refined products
Gasoline and Naptha	Saturates Olefins Aromatics	4-12	Gasoline
Middle distillate	Saturates Olefins Aromatics	10-20	Kerosene Jet fuel Heating oils Diesel oils
Wide-cut gas oil	Saturates Aromatics	18-45	Wax Lubricating oil
Residum	Resins Asphaltenes	>40	Residual oils Asphalt

Source**:** ClarkandBrown **(**1977)

2.4.1 Diesel Oil

Diesel oil hydrocarbons are derived from crude oil refining (Mälkönen, 1995). Diesel is a complex mixture of saturated and aromatic hydrocarbons (Eriksson *et al*., 2001; Zanaroli *et al*., 2010). Diesel oil is a mixture of hydrocarbons such as low molecular weight alkanes and polycyclic aromatic hydrocarbons (PAHs) (Adam and Duncan, 1999). It also contains sulfur, nitrogen and oxygen in low concentrations as well as metals such as lead, nickel, sodium, calcium, copper and uranium (Posthuma, 1970; Westlake, 1982). For the most part, diesel comprises aliphatic hydrocarbons, but it also contains polycyclic aromatic hydrocarbons such as naphthalene, fluorene and phenanthrene (Williams *et al*., 1986; Mälkönen, 1995; Eriksson *et al*., 2001). These aromatic compounds represent 5-30% of diesel oil (TTL, 2011). These low molecular weight compounds that diesel oil contains are usually more toxic than long-chained hydrocarbons, because long-chained ones are less soluble and less bioavailable (Dorn *et al*., 1998). Light oils contain a relatively high proportion of saturated hydrocarbons; hence these can be more toxic than heavy oils (Dorn *et al*., 1998). It is significant to note that diesel is light oil; hence it is very toxic as compared to heavy oils

Diesel fuels vary according to their origin and method of production (ATSDR, 1995). In general, they are similar to heating oil, consisting of aliphatic (mostly paraffins including *n*, isoand cycloparaffins) and aromatic hydrocarbons, including small amounts of organometal constituents such as vanadium and nickel (ATSDR, 1995; Van Hamme *et al*., 2003; Zanaroli *et al*., 2010). Diesel oil contains primary hydrocarbons formed by 9-23 carbon atoms per molecule, and the proportion of their aromatic hydrocarbons reaches 45% of the total composition (Smith, 1990). Some oils contain heavy residues from distillation and thermal cracking along with a variety of additives (organic nitrates, amines, phenols and polymeric substances) (IARC, 1998).

The colour of diesel fuels varies from colourless to brown, and the water solubility in 20°C is about 5 mg L^{-1} and Log K_{ow} 3.3 – 7.06 (ATSDR, 1995). Diesel fuels are therefore partly soluble in water and possibly accumulative in tissues.

The common chemical structure of diesel oil is illustrated in fig. 2.5

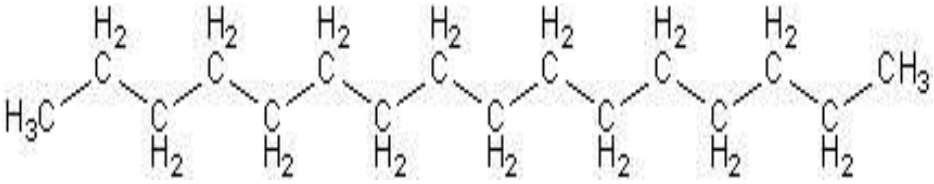


Fig 2.5 Chemical structure of diesel oil.

source: http://www.goshen.edu/chemistry/files/2010/10/diesel-molecule.jpg

2.5 Crude Oil Contamination

With a large demand for crude oil as an energy source, oil contamination occurs quite often as a result of exploration, production, maintenance, transportation, storage and accidental release, leading to significant ecological impact (Allen *et al*., 2007; Paisse *et al*., 2008). Oil contamination is particularly severe and has become a global issue, such as in Kuwait (Al-Sarawi and Massoud, 1998; Al-Hashem *et al*., 2007; Din *et al*., 2008), India (Gogoi *et al*., 2003), Libya (Hamid *et al*., 2008), China (Xiong *et al*., 1997; Liang *et al*., 2009) and the United States (Lundegard and Johnson, 2006).

Petroleum hydrocarbons can cause problems when released into the soil. They can be transported through soil, air or water and can result in fire hazards, human and environmental toxicity, odour and impairment of soil processes such as water retention and nutrient cycling (CCME, 2001). Oil and fuel spills have potential human health and environmental impacts. The properties of petroleum hydrocarbons in contaminated soils depend on the petroleum source, the composition, the degree of processing (crude, blended

or refined) and the extent of weathering due to time spent in the environment (CCME, 2001).

Contamination of the natural environment with petroleum- derived compounds poses an extremely serious problem, and products of the petro-chemical industry, including: aliphatic, ethylenic, naphthene and aromatic hydrocarbons are on top of the list of the most dangerous xenobiotics (Gray *et al*., 1994). Components of the petroleum-derived products, mainly aromatic hydrocarbons (benzene, toluene, xylene), display potential carcinogenic and mutagenic activities (Krah *et al*., 2002). Although they can be biodegraded easily in contrast to man-made compounds, they are also dangerous.

Human health impacts result from environmental exposure as well as from occupational exposure. Health risks depend on the type of petroleum hydrocarbons, and the exposure pathway. Diesel and jet fuel, for example, contain compounds with potentially carcinogenic effects (Hutchenson *et al.*, 1996). Silverman *et al.*, (2012) showed an increase in lung cancer deaths in underground miners. In this study, diesel exhaust exposure was represented by respirable elemental carbon and the study evaluated the exposure-response relationship between diesel exhaust and lung cancer mortality. They showed a strong relationship between diesel exhaust and lung cancer mortality, and heavily exposed workers were three times more likely to die than workers exposed to the lowest amount. Other studies comparing lung cancer and diesel exhaust also found an elevated risk of lung cancer in diesel-truck drivers (Garshick *et al.*, 2008; Steenland *et al.*, 1990).

Alkanes (e.g. – *n*-hexane) may result in mucus membrane irritation or a disruption in central nervous system (CNS) function (Hutchenson *et al.*, 1996). CNS effects and dermal sensitivity are most common from exposure to fuels with lower molecular weights (C5 through C9). Since carbon chain length affects lipid solubility, smaller carbon chains react

more readily with the lipid membrane of nerve cells than longer carbon chains (Hutchenson *et al.,* 1996). Compounds withlarger carbon chains may exhibit mutagenic and carcinogenic properties (Suleiman, 1987). Aromatic compounds (e.g. – benzene) target a variety of systems and organs in humans including the CNS, liver, kidney and hematopoietic system. They are highly lipid soluble, and are readily absorbed through the gastrointestinal tract of mammals (Samata *et al.,* 2002).Many are toxic or mutagenic; benzene is one of the most hazardous due to its carcinogenic properties (Hutchenson *et al.,* 1996).

Diesel vapours or fumes may cause respiratory irritation, resulting in coughing or difficulty in breathing. There may also be irritation to the nose, throat, lungs, respiratory tract and CNS effects including dizziness, headache or loss of coordination. Contact with contaminated soil can cause irritation to the skin if it occurs regularly (MSDS, 2006).

Contamination of soil with diesel oil tends to upset the biological balance of soil (Bundy *et al*., 2002; Caravaca and Rodán, 2003; Delille *et al.*, 2003; Wyszkowska and Kucharski, 2005). It usually alters the succession of microorganisms (Kaplan and Kitts, 2004), which is directly associated with the activity of soil enzymes (Wyszkowska and Kucharski, 2005). Soil microorganisms can deplete important soil nutrients such as nitrogen and thus inhibit root/plant growth (Ellis and Adams, 1961; Volk, 1980; McGill, 1980). In addition, there usually is a high demand for oxygen by soil microorganisms following oil addition to soil and oxygen levels in oil contaminated soil may become depleted, contributing to anaerobic or reducing conditions (Volk, 1980). Several authors have reported that anaerobic or reducing conditions also can result in increased solubilities of manganese and iron in oil contaminated soil (Ellis and Adams, 1961; Schwendinger, 1968; McGill, 1977) to the extent that these potentially phytotoxic elements are absorbed by roots/plants (Gidden, 1976; Volk,

1980). High oil concentrations in soil not only reduce the amount of water and oxygen available for plant growth (Schwendinger, 1968; McGilI, 1980) but also can interfere with soil-plant-water relations through direct physical contact (coating of root tissues) thereby adversely affecting plant growth (Baker, 1971).

Oil spills on terrestrial environments can drastically affect a plant community. Sensitive species may become extinct and tolerant species dominate. Overall plant cover can be reduced and some areas may remain barren this can contribute indirectly to other aspects of habitat deterioration such as sheet erosion (Kinako, 1981). It is generally accepted that lack of vegetation cover, and disrupted soil structure and reduced moisture holding capacity of oil polluted soil all can contribute to erosion. In severe spills, a plant community can be drastically altered or eliminated and the affected area(s) may remain unproductive for several years if no attempt is made to reclaim the site (Freedman and Hutchinson, 1976; Kinako, 1981).

Depending on the spill site, the oil or water soluble constituents may migrate as deep as the water table and form an oil lens or oil pancake above it (Vanloocke *et al.*, 1975). According to Vanloocke *et al.* (1975) less than 1 ppm of oil products is sufficient to give water a foul our and render it unpotable. The application of oil to soil also can create a soil crust, further restricting the infiltration of water and oxygen into the soil profile (McGill and Nyborg, 1975; Volk, 1980).Some workers have observed soil dispersion and disintegration of soil structure after application of oil (EIIis and Adams, 1961; Rowell, 1975) whereas others have observed aggregation of oil-treated soil (Giddens, 1976; Raymond *et al.*, 1976).In addition, it has been reported that oil spills normally increase soil temperature from 0 to 10° C (McGill, 1980) and this is believed to be related to solar warming associated with the

darker colour of the oiled soil (Johnston, 1970; Raymond *et al*., 1976) and to a lack of vegetative cover which would normally shade the soil.

In summary, Oil products, including diesel not only modifies physico-chemical (Tyczkowski, 1993) and biological properties of the soil (Borowiec *et al.,* 1982; Lebkowska *et al*., 1995; Malachowska-jutsza *et al.,* 1997; Olanczuk-Neyman *et al*., 1994), but also contributes to limitations of the productive ability of arable crops. It is known that these compounds are able to affect the quality of surface and ground water and that these products are potentially dangerous for animal and human health.

2.6 Total Petroleum Hydrocarbon (TPH) Measurement

Cumulative concentrations of petroleum hydrocarbons commonly are referred to as total petroleum hydrocarbons (TPH) (Shannon *et al*., 2006). Total petroleum hydrocarbon (TPH) measurements are conducted to determine the total amount of hydrocarbon present in the environment. There are wide varieties of TPH methods. In practice, TPH is defined by the method used to analyse it. Different methods often give different results because they are designed to extract and measure slightly different subsets of petroleum hydrocarbons. No single method gives a precise and accurate measurement of TPH for all types of contamination. The four most commonly used TPH testing methods include gas chromatography (GC), infrared spectrometry (IR), gravimetric analysis, and immunoassay (Weisman, 1998).

2.6.1 Gas Chromatography (GC) TPH Methods

For GC-based methods, TPH is defined as anything extractable by a solvent or purge gas and detectable by gas chromatography/flame ionization detection (GC/FID) within a specified carbon range. The primary advantage of GC-based methods is that they provide information about the type of petroleum in the sample in addition to measuring the

amount. Identification of product type(s) is not always straightforward, however, it requires an experienced analyst of petroleum products. Detection limits are method- and matrix-dependent and can be as low as 0.5 mg/L in water or 10 mg/kg in soil (Weisman, 1998).

Gas chromatography is a technique that separates mixtures. "A mixture of chemicals is separated into its individual components as the sample travels through a column in the gas chromatograph. Separation is achieved by a combination of factors including boiling point, polarity, and affinity differences among the different components in the sample. The time a compound spends on a specific column is called the retention time and it is reproducible. The retention time is characteristic of a compound under given experimental parameters and specified column. As the separated components elute from the column, they are detected (Swallow *et al*., 1988). The detector signal is proportional to the amount of compound present. Chromatographic columns are commonly used to determine TPH compounds approximately in the order of their boiling points. Compounds are detected with a flame ionization detector, which responds to virtually all compounds that can burn. The sum of all responses within a specified range is equated to a hydrocarbon concentration by reference to standards of known concentration(Weisman, 1998).

2.6.2 Infrared Spectroscopy (IR) TPH Methods

For IR-based methods, TPH is defined as anything extractable by a solvent and can be detected by IR at a specified wavelength. The primary advantage of IR-based TPH methods is that they are simple, quick and inexpensive. Detection limits for a commonly used IR-based TPH method, EPA Method 418.1, are approximately 1 mg/L in water and 10 mg/kg in soil. This TPH method often suffers from poor accuracy and precision, especially for heterogeneous soil samples. IR-based methods give no information on the

type of fuel present, no information about the presence or absence of toxic molecules, and no specific information about potential risk associated with the contamination.

Infrared spectroscopy measures the vibration (stretching and bending) that occurs when a molecule absorbs energy (heat) in the infrared region of the electromagnetic spectrum. Different functional groups and bond types have different IR absorption frequencies and intensities. IR-based TPH methods measure the absorbance of the C-H bond. Most IR-based methods in the United States typically measure the absorbance at a single frequency (usually 2930 cm-1) which corresponds to the stretching of aliphatic CH_2 groups. Some methods, especially in Europe, use multiple frequencies including 2960 cm^{-1} for CH_3 groups and 2900 to 3000 cm^{-1} for aromatic C-H bonds. Samples are extracted with a suitable solvent (i.e., a solvent with no C-H bonds).The absorbance of the extract is measured at the specified frequency and compared to the absorbance of a standard or standards of known petroleum hydrocarbon concentration. The IR absorbance is a measurement of the sum of all the compounds contributing to the TPH result. IR-based TPH methods cannot provide information on the type of hydrocarbon contamination. The extraction solvent for measuring TPH in soil must not contribute any C-H stretching to the measurement. The most frequently specified solvent has been Freon-113. This solvent is no longer being manufactured, in accordance with the Montreal Protocol on Substances that Deplete the Ozone Layer. Some laboratories are depleting stockpiled Freon or redistilling used Freon. Others have switched to alternate solvents. Carbon tetrachloride has been used for IR-based methods (mostly in the European community). It is in limited use in the U.S. because it is a known carcinogen and also affects the ozone layer. Tetrachloroethene (also known as perchloroethylene or PERC) is currently being used by some U.S. laboratories. Solvents such as methanol, methylene chloride, or hexane are not suitable for an IR-based method because they contain C-H bonds (Weisman, 1998). For all

IR-based TPH methods, the C-H absorbance is quantified by comparing it to the absorbance of standards of known concentration. An assumption is made that the standard has an aliphatic-to-aromatic ratio and IR response similar to that of the sample. Consequently, it is important to use a calibration standard as similar to the type of contamination as possible. EPA Method 418.1 specifies a calibration mixture of 15:15:10 *n*-hexadecane: isooctane: chlorobenzene

2.6.3 Gravimetric TPH Methods

Gravimetric methods measure anything extractable by a solvent, not removed during solvent evaporation, and capable of being weighed. Some gravimetric methods include a cleanup step to remove biogenic material. Those that do are considered TPH methods. Those that do not are considered oil and grease (O&G) methods. The advantage of gravimetric methods is that they are simple, quick, and inexpensive. Detection limits are approximately 5-10 mg/L in water and 50 mg/kg in soils. These methods are not especially suitable for measurement of light hydrocarbons that volatilize at temperatures below 70-85°C. They are recommended for TPH measurement only for very oily sludges, for samples containing heavy molecular weight hydrocarbons, or for aqueous samples when hexane is preferred as the solvent. Gravimetric methods give no information on the type of fuel present, no information about the presence or absence of toxic compounds, and no specific information about potential risk associated with the contamination (Weisman, 1998).

TPH compounds are extracted into a suitable solvent. Biogenic polar materials typically may be partially or completely removed with silica gel. The solvent is evaporated and the residue is weighed. This quantity is called TPH or O&G and is reported as a percent of the

total soil sample dry weight. These methods are better suited for heavy oils because they include an evaporation step (Weisman, 1998).

2.6.4 Immunoassay TPH Methods

Immunoassay methods correlate TPH with the response of antibodies to specific petroleum components. A number of different testing kits based on immunoassay technology are available for rapid determination of TPH. The kits are self-contained portable systems designed to conduct analytical work in the field. They include components for sample preparation, instrumentation to read assay results, and immunoassay reagents. Currently, most of these methods measure only aromatics. Immunoassay is used as a screening technique because its precision and accuracy are lower than standard laboratory methods such as GC/FID or IR (Weisman, 1998). Immunoassay measurements may be reported as a range or a single value. Typical detection limits for TPH range from 10-500 mg/kg in soil and 200 to 500 µg/L in water (Weisman, 1998).

Antibodies are made of proteins that recognize and bind with foreign substances (antigens) that invade host animals. Synthetic antibodies have been developed to complex with petroleum constituents. The antibodies in the test kit are immobilized on the walls of a special cell or filter membrane. Water samples are added directly to the cell while soils must be extracted before analysis. A known amount of labelled analyte is added after the sample. The label is typically an enzyme with an affinity for the antibody. The sample analytes compete with the enzyme-labelled analytes for sites on the antibodies. After equilibrium is established, the cell is washed to remove any unreacted sample or labelled enzyme. Colour development reagents that react with the labelled enzyme are added. A solution that stops colour development is added at a specified time, and the optical density

(colour intensity) is measured. Because the colouring agent reacts with the labelled enzyme, samples with high optical density contain low concentrations of analytes. Concentration is inversely proportional to optical density (Weisman, 1998).

2.7 Metabolic Pathway for hydrocarbon biodegradation

Several studies have been performed to determine the metabolic pathway for degradation of hydrocarbon compounds, and there have been a number of reviews on this subject (Donoghue *et al.*, 1976; Foster, 1962; Gibson, 1968; Gibson, 1971; Hopper, 1978).

Hydrocarbons within the saturated fraction include n-alkanes, branched alkanes, and cycloalkanes (naphthenes). The n-alkanes are generally considered the most readily degraded components in a petroleum mixture (Davies and Hughes, 1968; Treccani, 1964). Biodegradation of n-alkanes with molecular weights up to n-C_{44} have been demonstrated (Haines and Alexander, 1974). The biodegradation of n-alkanes (Fig. 2.5) normally proceeds by a monoterminal attack; usually a primary alcohol is formed followed by an aldehyde and a monocarboxylic acid (Foster, 1962; Miller and Johnson, 1966; Ratledge, 1978; Zobell, 1950). Further degradation of the carboxylic acid proceeds with the subsequent formation of two-carbon-unit shorter fatty acids and acetyl coenzyme A, with the eventual liberation of $C0_2$. Fatty acids, some of which are toxic, have been found to accumulate during hydrocarbon biodegradation (Atlas and Bartha, 1973; King and Perry, 1975). Omega (diterminal) oxidation also has been reported (Jurtehuk and Cardini, 1971). Subterminal oxidation sometimes occurs, with formation of a secondary alcohol and subsequent ketone, but this does not appear to be the primary metabolic pathway utilized by most n-alkane-utilizing microorganisms (Markovetz, 1971). The initial steps appear to involve terminal attack to form a carboxylic acid, subterminal dehydrogenation at the

number 10 position to form an unsaturated acid, and splitting of the carbon chain to form a hydroxy acid and an alcohol.

Highly branched isoprenoid alkanes, such as pristane, have been found to undergo omega oxidation, with formation of dicarboxylic acids as the major degradative pathway (McKenna and Kallio, 1971; Pirnik, 1977, Pirnik *et al.*, 1974). Methyl branching generally increases the resistance of hydrocarbons to microbial attack (Fall *et al*., 1979; Pirnik, 1977; Schaeffer *et al.*, 1979). Schaeffer *et al*. (1979), for example, found that terminal branching inhibits biodegradation of hydrocarbons. Methyl branching at the beta position (anteiso-terminus) blocks f-oxidation, requiring an additional strategy, such as alpha oxidation (Beam and Perry, 1973; Lough, 1973), omega oxidation (Pirnik, 1977), or beta alkyl group removal (Cantwell *et al*, 1978; Seubert and Fass, 1964).

Cycloalkanes are particularly resistant to microbial attack (Donoghue *et al.*, 1976; Perry 1979; Trudgill, 1978). Complex alicyclic compounds, such as hopanes (tripentacyclic compounds), are among the most persistent components of petroleum spillages in the environment (Atlas *et al.*, 1981). There have been several reports of the direct oxidative and co-oxidative degradation of both substituted and unsubstituted cycloalkanes. The microbial metabolism of cyclic hydrocarbons and related compounds has been reviewed (Austin *et al*., 1977). Up to six membered condensed ring structures have been reported to be subject to microbial degradation (Cobet and Guard, 1973; Walker *et al*., 1975). Several unsubstituted cycloalkanes, including condensed cycloalkanes, have been reported to be substrates for co-oxidation with formation of a ketone or alcohol (Beam and Perry, 1973; Beam and Perry, 1974; Perry, 1979). Once oxygenated, degradation can proceed with ring cleavage. Degradation of substituted cycloalkanes appears to occur more readily than the degradation of the unsubstituted forms, particularly if there is an n-alkane substituent of adequate chain length (Perry, 1979; Soli, 1973). In such cases, microbial attack normally

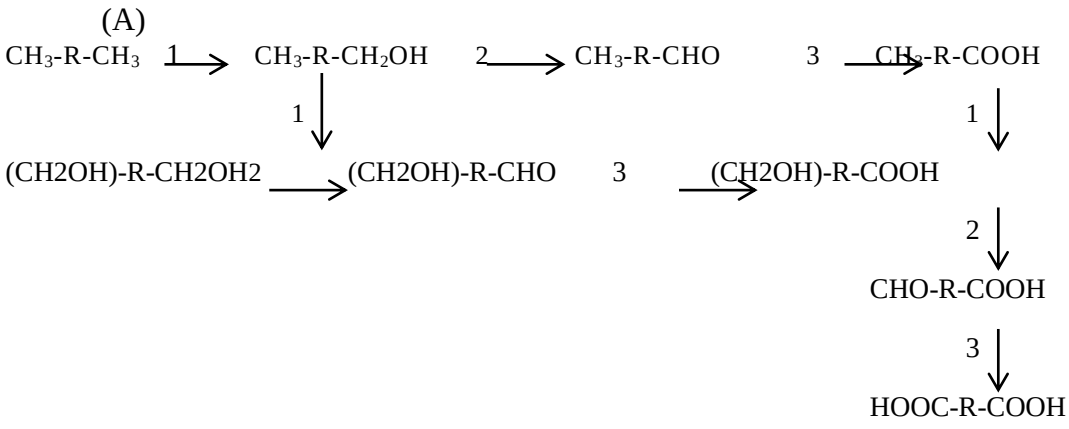
occurs first on the substituted portion, leading to an intermediate product of cyclohexane carboxylic acid or a related compound. A novel pathway for the degradation of cylohexane carboxylic acid involves formation of an aromatic intermediate (Perry, 1979) followed by cleavage of the aromatic ring structure.

The degradation of aromatic hydrocarbons has been reviewed (Cripps and Watkinson, 1978; Gibson, 1977; Hopper, 1978). The bacterial degradation of aromatic compounds normally involves the formation of a diol followed by cleavage and formation of a diacid such as cis, cismuconic acid. In contrast, oxidation of aromatic hydrocarbons in eukaryotic organisms has been found to form a trans-diol (Cerniglia *et al*., 1978; Ferris *et al*., 1976). Cerniglia and Gibson (1979) and Cerniglia *et al*. (1980) also investigated the metabolism of naphthalene by cyanobacteria. They found that naphthalene was oxidized in the light but not in the dark. Scenedesmus strains also were shown to utilize n-heptadecane in the light (mixotrophic growth), but were unable to utilize this alkane in the dark (Masters and Zajic, 1971). The major product formed by Agmenellum and Oscillatoria strains was 1-naphthol (Cerniglia and Gibson, 1979). These organisms also formed cis-1,2-dihydroxy-1,2- dihydronaphthalene and 4-hydroxy-1-tetralene (Cerniglia *et al*., 1980). These results suggest that cyanobacteria have a variety of mechanisms for initiating the oxidation of naphthalene. The Oscillatoria strains also has been found to oxidize biphenyl, indicating that a wider range of aromatic hydrocarbons are subject to oxidation by cyanobacteria

Light aromatic hydrocarbons are subject to evaporation and to microbial degradation in a dissolved state (Kappeller and Wuhrmann, 1978). Extensive methyl substitution can inhibit initial oxidation (Atlas *et al*., 1981; Cripps and Watkinson, 1978). Initial enzymatic attack may be on the alkyl substituent or alternatively directly on the ring (Gibson, 1971). Condensed ring aromatic structures are subject to microbial degradation by a similar

metabolic pathway as monocycic structures (Cripps and Watkinson, 1978; Dean-Raymond and Bartha, 1975; Gibson, 1975; Zobell, 1971); condensed ring aromatic hydrocarbons, however, are relatively resistant to enzymatic attack; for example, Lee and Ryan (1976) found that biodegradation rates were over 1,000 times higher for naphthalene than for benzopyrenes. Structures with four or more condensed rings have been shown to be attacked, in some cases, by co-oxidation or as a result of commensalism (Barnsley, 1975; Gibson, 1975; Walker and Colwell, 1974; Walker *et al.*, 1976).

The metabolic pathways for the degradation of asphaltic components of petroleum are probably least well understood. These are complex structures which are difficult to analyze with current chemical methodology. The degradation of various sulfur-containing components of petroleum has been examined (Hou and Laskin, 1976; Kodama *et al.*, 1970; Walker *et al.*, 1976), but no uniform degradative pathway, comparable to the pathways established for aliphatic and aromatic hydrocarbons, has yet emerged for the asphaltic petroleum components. Advances in determining degradative pathways for asphaltic petroleum components are dependent on improved chemical analytical methodology. The elucidation of the biochemical fate of asphaltic petroleum compounds is a major challenge for future research on petroleum biodegradation.



(B)

R1-(CH2)(CH2)-R2 ⟶ R1-(CH2)(CHOH)-R2 ⟶ R1-(CH2)(CO)-R2

↓

R1-(CH2)O(CO)-R2 R1-COOH + R2-COOH →

(C)R-CH3 → R-CH2OOH → R-(CO)OOH → R-CHO → R-COOH

[D]

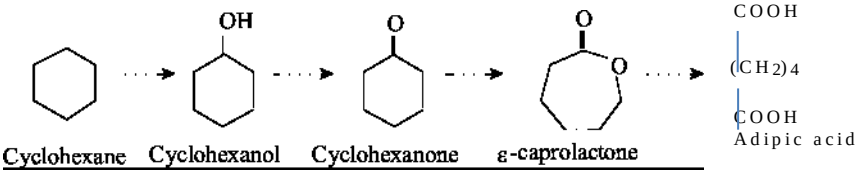


Figure 2.6 Alkane Degradative Pathways

[A] Terminal oxidation of n-alkanes.α-and ω-hydroxylation is catalyzed by the same set of enzymes. With bacteria, steps 1, 2 and 3 are catalyzed by alkane monooxygenase, fatty alcohol dehydrogenase and fatty aldehyde dehydrogenase, respectively. [B] Subterminal oxidation of n-alkanes; [C] n-Alkane degradation via alkyl hydroperoxides; [D] Degradation of cyclohexane. Source :(A-D) Shigeaki *et al.*1999

2.8 Distribution of petroleum degrading microorganism

Hydrocarbon-degrading bacteria and fungi are widely distributed in marine, freshwater, and soil habitats. The literature on actual numbers of hydrocarbon utilizers is confusing because of methodological differences used to enumerate petroleum-degrading microorganisms (Atlas, 1981).

Buckley *et al.* (1976) characterized the distribution of microorganisms in an estuary relative to ambient hydrocarbon concentrations. Although counts were performed on non-hydrocarbon based media, at all but two stations most of the species isolated were able to grow on hydrocarbons, indicating that the ability to utilize hydrocarbons is widespread, even in environments not subjected to high levels of hydrocarbon pollution. Crow *et al.*

(1976) examined the distribution of hydrocarbon utilizers in surface ocean layers and in the underlying water column. They found that populations of hydrocarbonoclastic microorganisms occurred in concentrations 10 to 100 times greater in the surface layer than at a 10- cm depth.

Mulkins-Phillips and Stewart (1974) examined the distribution of hydrocarbon-utilizing bacteria in northwestern Atlantic waters and coastal sediments. The fraction of the total heterotrophic bacteria represented by the hydrocarbon utilizers ranged up to 100%, depending on the area's previous history of oil spillage; most values were less than 10%. They found that the location, numbers, and variety of the microbial hydrocarbon utilizers illustrated their ubiquity and that the broad enzymatic capacity for hydrocarbon degradation indicated the microbial potential for removal or conversion of oil in the environments examined. The presence of hydrocarbon- utilizing microorganisms was demonstrated in sediments and adjacent waters taken from Bermuda, Canadian Northwest Atlantic, and eastern Canadian Arctic marine shorelines (Mulkins-Phillips and Stewart, 1974).

It is clear from a number of studies that the distribution of hydrocarbon-utilizing microorganisms reflects the historical exposure of the environment to hydrocarbons. A large number of laboratory studies have demonstrated sizable increases in populations of hydrocarbon-utilizing microorganisms when environmental samples are exposed to petroleum hydrocarbons (Atlas and Bartha, 1972; Calomiris *et al*., 1976).

Several studies have shown a rise in populations of hydrocarbon-utilizing microorganisms after oil spills. Kator and Herwig (1977) found that within a few days after spillage of South Louisiana crude oil in a coastal estuary in Virginia, levels of petroleum-degrading bacteria rose by several orders of magnitude. The elevated levels of hydrocarbon utilizers

were maintained for over 1 year. Raymond *et al*. (1976) found significant increases in hydrocarbon-utilizing microorganisms in soils receiving hydrocarbons; increased populations were maintained throughout the year. Pinholt *et al.* (1979) examined the microbial changes during oil decomposition in soil. They found an increase from 60 to 82% in oil-utilizing fungi and an increase from 3 to 50% in oil-degrading bacteria after a fuel oil spill. Oppenheimer *et al.* (1977) found a tendency toward higher ratios of hydrocarbon-utilizing bacteria to total viable heterotrophs in the active oil field of the North Sea, probably due to the occurrence of hydrocarbons in the sediments of this region. Gunkel *et al*. (1980) confirmed the occurrence of high numbers of hydrocarbon-utilizing microorganisms in the vicinity of the North Sea oil fields and found a high correlation between concentrations of hydrocarbons and oil-utilizing bacteria in the North Sea.

In experimental field studies in the Arctic, Atlas and co-workers have found large increases in hydrocarbon-utilizing microorganisms in marine (Atlas, 1978; Atlas and Busdosh, 1976; Horowitz and Atlas, 1978), freshwater (Atlas *et al.*, 1976, Horowitz and Atlas 1977), and soil (Sexstone and Atlas, 1977; Sexstone *et al*., 1978) ecosystems; concentrations of hydrocarbon-utilizing microorganisms have been found to rise rapidly and dramatically in response to acute inputs of petroleum hydrocarbons. Bergstein and Vestal (1978), however, found lack of elevated microbial populations in an oil-treated tundra pond unless phosphate also was added.

In general, population levels of hydrocarbon utilizers and their proportions within the microbial community appear to be a sensitive index of environmental exposure to hydrocarbons. In unpolluted ecosystems, hydrocarbon utilizers generally constitute less than 0.1% of the microbial community but in oil-polluted ecosystems they can constitute up to 100% of the viable microorganisms. The degree of elevation above unpolluted

compared reference sites appears to quantitatively reflect the degree or extent of exposure of that ecosystem to hydrocarbon contaminants (Atlas, 1981).

Hydrocarbons in the environment are biodegraded primarily by the bacteria and fungi. Although ubiquitous in terrestrial (Atlas *et al*., 1980; Jones and Edington, 1968) and aquatic (Buckley *et al*., 1976; Mulkins-Phillips and Steward, 1974; Ward and Brock, 1976) ecosystems, the fraction of the total heterotrophic community represented by the hydrocarbon-utilizing bacteria and fungi is highly variable, with reported frequencies ranging from 6% (Jones *et al.*, 1970) to 82% (Pinholt *et al.*, 1979) for soil fungi, 0.13% (Jones *et al.*, 1970) to 50% (Pinholt *et al*., 1979) for soil bacteria, and 0.003% (Hollaway *et al*., 1980) to 100% (Mulkins-Phillips and Steward, 1974) for marine bacteria. Individual organisms can metabolize only a limited range of hydrocarbon substrates (Britton, 1984), so that assemblages of mixed populations with overall broad enzymatic capacities are required to degrade complex mixtures of hydrocarbons such as crude oil in soil (Bossert and Bartha, 1984), freshwater (Cooney, 1984), and marine (Atlas, 1981; Floodgate, 1984) environments.

The ability to degrade and/or utilize hydrocarbon substrates is exhibited by a wide variety of bacterial and fungal genera. Floodgate (1984) listed 25 genera of hydrocarbon-degrading bacteria and 27 genera of hydrocarbon-degrading fungi which have been isolated from the marine environment; a similar compilation by Bossert and Bartha (1984) for soil isolates includes 22 genera of bacteria and 31 genera of fungi. Based on the number of published reports, the most important hydrocarbon-degrading bacteria in both marine and soil environments are Achromobacter, Acinetobacter, Alcaligenes, Arthrobacter, Bacillus, Flavobacterium, Nocardia, and Pseudomonas spp. and the coryneforms; the importance of hydrocarbon-degrading strains of the salt requiring Vibrio spp. is limited to the marine environment. Austin *et al*. (1977), in a numerical taxonomy

study of petroleum degrading bacteria from Chesapeake Bay water and sediment, found that Pseudomonas, Micrococcus, and Nocardia spp., members of the family Enterobacteriaceae, actinomycetes, and coryneforms made up 95% of the isolates. Among the fungi, Aureobasidium, Candida, Rhodotorula, and Sporobolomyces spp. are the most common marine isolates and Trichoderma and Mortierella spp. are the most common soil isolates. Hydrocarbon-degrading Aspergillus and Penicillium spp. have been frequently isolated from both environments. Based on the work of Kirk and Gordon (1988), the truly marine, beach-adapted genera Corollospora, Dendryphiella, Lulworthia, and Varicosporina should be added to the list.

2.9 Methods of enumerating and identifying petroleum degrading microorganisms

Despite the intricacies, tools are being developed in an attempt to better appreciate microbial abundance and distribution in natural environments in the hopes of associating community structures with ecosystem functions. Approaches to cataloguing microbial diversity and community function can be broadly divided into culture-dependent and culture-independent methods, both of which may include genetic characterization techniques.

2.9.1 Culture Based Techniques

Microbial counts are often used to monitor the bioremediation process. In general, the more microbes, the more quickly the contaminants will be degraded. Correlating an increase in the number of contaminant-degrading bacteria above normal field conditions is one indicator that bioremediation is taking place. Analysis of the microbial communities that take part in in-situ hydrocarbon biodegradation activities has been a challenge to microbiologists (Macnaughton *et al*., 1999). The reason for this is that most (~90 to 99%)

of the species making up competent degrading communities do not form colonies when current laboratory-based culture techniques are used (Rollins and Colwell, 1986; Rozsak and Colwell, 1987; Wilkinson, 1988). The techniques are briefly described below.

2.9.1.1 Plate count

Plate count is a traditional technique, which quantifies the number of bacteria capable of growing on a prescribed set of nutrients and substrates in a solid medium, by counting the colonies formed (National Research Council, 1993). The general procedure involves (1) making the solid medium or gel from a liquid solution with appropriate nutrients and substrates, using a solidifying agent like agar, (2) Spreading a sample containing the bacteria of interest thinly over the surface of the gel in plates, (3) Incubating the plates, (4) counting the bacterial colonies formed. Each colony is assumed to have arisen from a single bacterial cell.

A number of studies have used hydrocarbon incorporated into either agar-based or silica-based media to enumerate hydrocarbon-degrading microorganisms (Horowitz and Atlas, 1978; Sexstone and Atlas, 1977; Walker and Colwell, 1976). However, other researchers reported that plate counts are unsuitable for enumerating hydrocarbon-utilizing microorganisms because many marine bacteria can grow and produce micro-colonies on small amounts of organic matter existing in the solid media, resulting in the counting of non-hydrocarbon utilizers (Atlas, 1981; Higashihara, *et al*., 1978). Plate count also underestimates the number and diversity of bacteria because of the difficulty in enriching viable colonies from environmental samples. Culturable techniques have been found to be inferior to techniques that do not rely on viable culturing for enumeration (Macnaughton *et al*., 1999).

2.9.1.2 Most-probable-number (MPN) procedures

MPN procedures have been viewed as a more reliable method for enumerating hydrocarbon-utilizing microorganisms because such procedures eliminate the need for a solidifying agent and permit direct assessment of the ability to actually utilize hydrocarbons (Atlas, 1981; Wrenn and Venosa, 1996). MPN procedures use liquid nutrient media in test tubes or microtiter plates and hydrocarbons as the sole carbon source. The enumeration is carried out through a statistical analysis based on the numbers of a series of diluted liquid samples that show evidence of bacterial growth. This evidence of bacterial growth can be established based on turbidity, release of $^{14}CO_2$ from radiolabeled hydrocarbons, disruption of oil sheen, and reduction of dyes (Rice and Hemmingsen, 1997). Either statistical tables (Eaton *et al*., 1995) or a computer program (Klee, 1993) can be used to determine the MPN. Most existing MPN procedures use crude oil or a refined petroleum product as the selected hydrocarbons, which cannot distinguish different groups of hydrocarbon degraders.

2.9.2 Culture-Independent Techniques

The main challenge for accurate analysis of hydrocarbon-degraders using existing culture-based techniques is that most these species are not able to be cultured (Atlas & Bartha, 1987, Macnaughton *et al*., 1999). The emergent culture-independent molecular techniques have made it possible to identify the diversity and composition of uncultivated microbial communities and to enumerate bacteria in more precise ways.

2.9.2.1 Phospholipid fatty acid (PLFA) analysis

Phospholipid fatty acid (PLFA) analysis is based on the characteristic “signature” of fatty acids present in the membranes of all cells (National Research Council, 1993). The

distribution of fatty acids is unique and stable. Therefore, it can be used as an identifying index. Determination of biomass through analysis of the extractable lipids avoids culture bias. This technique also provides a quantitative means to measure viable biomass, community composition, and nutritional status (White *et al*., 1998).

Phospholipids can be extracted from the sample and the phosphate can be measured by colorimetric techniques (Findlay *et al*., 1989). The results can represent the amounts of viable cells and biological activities in the sample. A more powerful PLFA method involves extraction and separation of lipid classes into neutral-, glyco-, and polar-lipid fractions, followed by quantitative analysis using gas chromatography/mass spectrometry (GC/MS) (Macnaughton *et al*., 1999; White *et al*., 1998). This procedure can quantitatively determine the characteristics of microbial communities. However, PLFA analysis cannot identify species composition.

2.9.2.2 Nucleic Acid-Based Molecular Techniques

Nucleic acid-based molecular techniques can identify bacterial species by the unique sequence of molecular codes in their genes. One of the most useful methods for determining the diversity of bacterial communities is denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al*., 1993). The method provides a means of separating the PCR (polymerase chain reaction) products from mixed cultures based on the melting properties of the DNA. Usually the 16s rDNA portion of the bacterial genome is targeted for PCR amplification, since this region is commonly used for bacterial identification. PCR primers can be designed to detect a broad range of bacteria (universal) or can be designed just for a specific group of interest. The DGGE gel is made of acrylamide, and contains a gradient of formamide and urea, which both act to denature, or

pull the strands of the DNA apart. The PCR products are loaded onto the gel, and a voltage is established across the gel for several hours. As DNA, which carries a net negative charge, is carried through the gel, it encounters an increasing gradient of denaturant, which causes the DNA chains to separate and the effective size of the molecules increases, causing movement through the gel to cease. The end result is a DNA banding pattern, where DNA requiring more chemical potential to denature travels further and DNA requiring less chemical potential to denature stays near the top of the gel. Each DNA band approximately corresponds to the presence of one kind of organism in the mixed culture. This banding pattern is sometimes referred to as a "community fingerprint," and allows for a quick approximation of number of bacterial species (diversity) present. Bands can be excised for sequencing analysis, and sequences can be compared to the Ribosomal Database Project (Maidak *et al*., 2000) containing the 16S rDNA sequences of currently known organisms.

The use of DGGE for quantitative purposes is still not well established. However, it can be used in conjunction with other quantitative methods such as PLFA analysis to provide insight into microbial species distribution. The PLFA-DGGE techniques were successfully used in determination of microbial population changes during the Delaware field study on oil bioremediation (Macnaughton *et al*., 1999).

2.10 Microbial Biodegradation and Bioremediation

Microbial biodegradation is the process of breakdown of generally complex, organic pollutants (contaminants) to smaller, simpler products by the activities of microorganisms (Sharma, 2010). These organic substances serve as the microbial food source or substrate. Biodegradation in fact involves a series of biological degradation steps (pathways) that finally result into the oxidation of the parent compound and often into energy generation.

Complete biodegradation or mineralisation involves oxidation of parent compound to form carbon dioxide and water, a process providing both carbon and energy for growth and reproduction of microbial cells (Sharma, 2010).

Bioremediation is 'the use of living organisms (primarily microorganisms) for removal of a pollutant from the biosphere' (Sharma, 2010). Bioremediation has been defined as "the act of adding materials to contaminated environments to cause an acceleration of the natural biodegradation processes" (Office of Technology Assessment, 1991). This technology is based on the premise that a large percentage of oil components are readily biodegradable in nature (Atlas, 1984, 1981; Prince, 1993). It relies on biological processes to minimise an unwanted environmental impact of the pollutants. The microorganisms, in particular have the abilities to degrade, detoxify and even accumulate the harmful organic as well as inorganic compounds (Sharma, 2010)

Although conventional methods, such as physical removal, are the first response option, they rarely achieve complete clean-up of oil spills. According to the Office of Technology Assessment (1990), current mechanical methods typically recover no more than 10-15 percent of the oil after a major spill. Bioremediation has emerged as one of the most promising secondary treatment options for oil removal since its successful application after the 1989 Exxon Valdez spill (Bragg *et al*., 1994; Prince *et. al*., 1994).

During bioremediation, microbes utilize chemical contaminants in the soil as an energy source and, through oxidation-reduction reactions, metabolize the target contaminant into useable energy for microbes. By-products (metabolites) released back into the environment are typically in a less toxic form than the parent contaminants. For example, petroleum hydrocarbons can be degraded by microorganisms in the presence of oxygen through aerobic respiration. The hydrocarbon loses electrons and is oxidized while oxygen

gains electrons and is reduced. The result is formation of carbon dioxide and water (Nester *et al*., 2001). When oxygen is limited in supply or absent, as in saturated or anaerobic soils or lake sediment, anaerobic (without oxygen) respiration prevails. Generally, inorganic compounds such as nitrate, sulfate, ferric iron, manganese, or carbon dioxide serve as terminal electron acceptors to facilitate biodegradation (State of Mississippi, Department of Environmental Quality, 1998).

Bioremediation strategies are based on the application of various methodologies to increase the rate or extent of the biodegradation process. The success of oil spill bioremediation depends on our ability to optimize various physical, chemical, and biological conditions in the contaminated environment. The success of oil spill bioremediation depends on our ability to establish and maintain conditions that favour enhanced oil biodegradation rates in the contaminated environment.

Diesel oil bioremediation in soil can be promoted by stimulation of the indigenous microorganisms, by introducing nutrients and oxygen into the soil (biostimulation) (Seklemova *et al*., 2001) or through inoculation of an enriched microbial consortium into soil (bioaugmentation) (Richard and Vogel, 1999; Barathi and Vasudevan, 2001).

2.10.1 Bioaugmentation

Bioaugmentationinvolves introduction of exogenic microorganisms (sourced from outside the soil environment) capable of detoxifying a particular contaminant, sometimes employing genetically altered microorganisms (Biobasics, 2006). Although hydrocarbon-degrading microorganisms are widespread in nature, bioaugmentation has been considered as a potential strategy for oil-bioremediation since the 1970s. The rationale for adding oil-degrading microorganisms is that indigenous microbial populations may not be capable of degrading the wide range of potential substrates present in complex mixtures such as

petroleum (Leahy and Colwell, 1990). Other conditions under which bioaugmentation may be considered are when the indigenous hydrocarbon-degrading population is low, the speed of decontamination is the primary factor, and when seeding may reduce the lag period to start the bioremediation process (Forsyth *et al*., 1995).

2.10.2 Biostimulation

Biostimulation also utilizes indigenous microbial populations to remediate contaminated soils. Biostimulation involves the addition of rate-limiting nutrients to accelerate the biodegradation process. In most ecosystems that have been heavily contaminated with hydrocarbons, nutrients are likely the limiting factors in oil biodegradation. Biostimulation consists of adding nutrients and other substances to soil to catalyze natural attenuation processes. The main purpose of bench-scale treatability studies is to determine the type, concentration, and frequency of addition of amendments needed for maximum stimulation in the field (Venosa, 1998). Most laboratory experiments have shown that addition of growth limiting nutrients, namely nitrogen and phosphorus, has enhanced the rate of oil biodegradation. However, the optimal nutrient types and concentrations vary widely depending on the oil properties and the environmental conditions. Both laboratory studies and field tests have shown that bioremediation, biostimulation in particular, can enhance oil biodegradation on contaminated shorelines (Prince, 1993; Swannell, *et al*., 1996). Recent field studies have also demonstrated that biostimulation is a more effective approach because the addition of hydrocarbon degrading microorganisms will not enhance oil degradation more than simple nutrient addition (Lee *et al*, 1997; Venosa *et al*., 1996).

2.11. Advantages and Disadvantages of Bioremediation

Bioremediation has several advantages over conventional technologies. First, the application of bioremediation is relatively inexpensive. For example, during the cleanup of the Exxon Valdez spill, the cost of bioremediating 120 km of shoreline was less than one day's costs for physical washing (Atlas and Cerniglia, 1995). Bioremediation is also a more environmentally benign technology since it involves the eventual degradation of oil to mineral products (such as carbon dioxide and water), while physical and chemical methods typically transfer the contaminant from one environmental compartment to another. Since it is based on natural processes and is less intrusive and disruptive to the contaminated site, this "green technology" may also be more acceptable to the general public.

Bioremediation like other technologies also has its limitations. Bioremediation involves highly heterogeneous and complex processes. The success of oil bioremediation depends on having the appropriate microorganisms in place under suitable environmental conditions. Its operational use can be limited by the composition of the oil spilled. Bioremediation is also a relatively slow process, requiring weeks to months to take effect, which may not be feasible when immediate cleanup is demanded. Concerns also arise about potential adverse effects associated with the application of bioremediation agents. These include the toxicity of bioremediation agents themselves and metabolic by-products of oil degradation and possible eutrophic effects associated with nutrient enrichment (Swannell *et al*., 1996). Bioremediation has been proven to be a cost-effective treatment tool, if used properly, in cleaning certain oil-contaminated environments. Few detrimental treatment effects have been observed in actual field operations.

2.12 Factors affecting biodegradation of petroleum hydrocarbons

2.12.1 pH

Soil pH is important because most microbial species can survive only within a certain pH range. Furthermore, soil pH can affect availability of nutrients. Soil pH can be highly variable, ranging from 2.5 in mine spoils to 11.0 in alkaline deserts (Bossert and Bartha, 1984). Biodegradation of petroleum hydrocarbons is optimal at pH 7 (neutral); the acceptable range is pH 6 – 8 (US EPA, 2006; "Landfarming"; State of Mississippi, Department of Environmental Quality, 1998).

Most heterotrophic bacteria and fungi favor a pH near neutrality, with fungi being more tolerant of acidic conditions (Atlas, 1988). Extremes in pH, as can be observed in some soils, would therefore be expected to have a negative influence on the ability of microbial populations to degrade hydrocarbons. Verstraete *et al*. (1976) reported a near doubling of rates of biodegradation of gasoline in an acidic (pH 4.5) soil by adjusting the pH to 7.4. Rates dropped significantly, however, when the pH was further raised to 8.5. Similarly, Dibble and Bartha (1979) observed an optimal pH of 7.8, in the range 5.0 to 7.8, for the mineralization of oily sludge in soil. The pH of sediments in special environments such as salt marshes may be as low as 5.0 in some cases (Patrick and DeLaune, 1977). Hambrick *et al*. (1980) found the rates of microbial mineralization of octadecane and naphthalene to be depressed at this pH compared with pH 6.5. Octadecane mineralization rates increased further when the pH was raised from 6.5 to 8.0, whereas naphthalene mineralization rates did not.

2.12.2 Temperature

The ambient temperature of an environment affects both the properties of spilled oil and the activity or population of microorganisms. 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 influences rate of biodegradation by controlling rate of enzymatic reactions within microorganisms. Generally, "speed of enzymatic reactions in the cell approximately doubles for each 10 ºC rise in temperature" (Nester *et al*., 2001). There is an upper limit to the temperature that microorganisms can withstand. Most bacteria found in soil, including many bacteria that degrade petroleum hydrocarbons, are mesophiles which have an optimum temperature ranging from 25 - 45 ºC (Nester *et al*., 2001). Thermophilic bacteria (those which survive and thrive at relatively high temperatures) which are normally found in hot springs and compost heaps exist indigenously in cool soil environments and can be activated to degrade hydrocarbons with an increase in temperature to 60 ºC. This finding "suggested an intrinsic potential for natural attenuation in cool soils through thermally enhanced bioremediation techniques" (Perfumo *et al*., 2007).

Some hydrocarbons are more soluble at lower temperatures (e.g., short-chain alkanes), and some low-molecular-weight aromatics are more soluble at the higher temperature (Foght and Westlake, 1987). 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 to 40°C in soil environments, 20 to 30°C in some freshwater environments, and 15 to 20°C in marine environments (Bossert and Bartha, 1984; Cooney, 1984; Jordan and Payne, 1980).

The effect of temperature is also complicated by other factors such as the composition of the microbial population. In environments where a psychrophilic population has been established, degradation can occur at significant rates under cold conditions. Hydrocarbon biodegradation has been observed at temperature as low as 0-2°C in seawater and –1.1°C

in a soil. Colwell *et al*. (1978) reported greater degradation of Metula crude oil at 3°C than at 22°C with a mixed culture in beach sand samples. Westlake *et al*. (1974) also found that bacteria capable of degradation at 4°C would metabolize oil at 30°C, but those populations that developed at 30°C had a limited activity at 4°C.

2.12.3 Oxygen

The initial steps in the catabolism of aliphatic (Singer and Finnerty, 1984), cyclic (Perry, 1984), and aromatic (Cerniglia, 1984) hydrocarbons by bacteria and fungi involve the oxidation of the substrate by oxygenases, for which molecular oxygen is required. Aerobic conditions are therefore necessary for this route of microbial oxidation of hydrocarbons in the environment.

The availability of oxygen in soils is dependent on rates of microbial oxygen consumption, the type of soil, whether the soil is waterlogged, and the presence of utilizable substrates which can lead to oxygen depletion (Bossert and Bartha, 1984). The concentration of oxygen has been identified as the rate-limiting variable in the biodegradation of petroleum in soil (von Wedel *et al*., 1988) and of gasoline in groundwater (Jamison *et al*., 1975).

Anaerobic oil degradation has been shown in some studies to occur only at negligible rates, as reviewed by Atlas (1981), leading to the conclusion that the environmental importance of anaerobic hydrocarbon degradation can be discounted. However, recent studies have shown that anaerobic hydrocarbon metabolism may be an important process in certain conditions (Head and Swannell, 1999). The biodegradation of some aromatic hydrocarbons, such as BTEX compounds, has been clearly demonstrated to occur under a variety of anaerobic conditions (Krumholz *et al*., 1996; Leahy and Colwell, 1990). Studies have also demonstrated that in some marine sediments, PAHs and alkanes can be degraded under sulfate-reducing conditions at similar rates to those under aerobic conditions

(Caldwell *et al*., 1998; Coates *et al*., 1997). The importance of anaerobic biodegradation of oil in the environment still requires further studies.

2.12.4 Nutrients

Assuming that microbes are present, nutrient availability, especially of nitrogen and phosphorus appears to be the most common limiting factor (Pritchard *et al*., 1992; Rosenberg *et al*., 1992). Since microorganisms require nitrogen and phosphorus for incorporation into biomass, the availability of these nutrients within the same area as the hydrocarbons is critical. Researchers examining the fate of large oil spills have thus properly concluded in many cases that concentrations of N and P are limiting with respect to rates of hydrocarbon biodegradation.

Nitrogen and phosphorus may also be limiting in soils, and adjustment of carbon/nitrogen/phosphorus ratios by the addition of nitrogen and phosphorus fertilizers like urea-phosphate, N-P-K fertilizers, and ammonium and phosphate salts has been demonstrated in several studies (Dibble and Bartha, 1979; Jamison *et al*., 1975; Jobson *et al*., 1974; Verstraete *et al*., 1976) to stimulates the acceleration of the biodegradation of crude oil or gasoline in soil and groundwater. Again laboratory and field experiments with organic fertilizers, including fish bones, fish or animal meal, have also shown success (Rhykerd *et al*., 1999; Vasudavan and Rajaram, 2001; Venosa *et al* 1996). Other investigators observed no increase in biodegradation rates (Lehtomaki and Niemela 1975) or an increase only after a delay of several months to a year (Odu, 1978; Raymond *et al*., 1976) when fertilizer amendments were used. These seemingly contradictory results have been attributed by Bossert and Bartha (1984) to the variable and complex composition of soils and to other factors such as nitrogen reserves and the presence of nitrogen-fixing bacteria.

2.12.5 Other factors

Other important factors affecting biodegradation of petroleum hydrocarbons include chemical composition of the oil or hydrocarbons, physical state of the oil or hydrocarbons, concentration of the oil or hydrocarbons, salinity, pressure and water activity.

2.13 Effect of diesel contamination oil on plant growth and germination

The effects of crude oil on the growth and performance of plants have been reported by many researchers. The response of plants to diesel oil contamination is unambiguously negative (Chaineau *et al*., 1997; Salanitro *et al*., 2004). These effects have been observed to occur due to the interference of the plant uptake of nutrients by crude oil and the unfavourable soil structure due to pollution with crude oil as well as soil air, water and chemical properties of the contaminated soil (Plice, 1948; Gudin and Syratt, 1975; McGill and Rowell, 1977; Caravaca and Rodán, 2003; Iwanow *et al.*, 1994).

Diesel oil can cause chronic or acute effects in plants. Many authors have reported a lower rate of germination in soil contaminated by petroleum or its derivatives (Amakiri and Onofeghara, 1984; Adam and Duncan, 1999, 2002; Vavrek and Campbell, 2002; Achuba, 2006). According to Baker (1970) and Adam and Duncan (2002), petroleum hydrocarbons may form a film on the seed, preventing the entry of oxygen and water.

The most common and important symptoms observed in the plants contaminated with oil and its byproducts include the erosion of the epicuticular wax (Baker, 1970), degradation of chlorophyll (Baker, 1970; Malallah *et al*., 1996 and 1998), alterations in the stomatal mechanism (Baker, 1970), reduction in photosynthesis and respiration (Baker, 1970), increase in the production of stress-related phytohormones , accumulation of toxic substances or their byproducts in vegetal tissue (Baker, 1970), decrease in size and less production of biomass (Proffitt *et al*., 1995; Green *et al*., 1996; Kuhn *et al*., 1998. Essien

and John (2010) reported that, Crop growth, indicated by root elongation, diminished to 7.4 ± 0.64 cm in polluted soil compared to13.47 ± 6.40 cm in the control soil (Unpolluted soil).

In some cases after an oil spill, an increase in the production of biomass and in the growth and development of some species can be observed. This likely occurs for three reasons: i) the oil may kill some organisms present in the soil, increasing the organic matter available; ii) some compounds that regulate the plant growth could be present in the oil; and iii) nitrogen fixation may increase with the presence of oil in the soil (Baker, 1971). Some earlier studies suggest that straw fertilization can be a means alleviating the effects produced by diesel oil and petrol on biochemical properties of soil and on the growth and development of plants (Wyszkowska and Kucharski 2000).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Soil sampling and Preparations

Soil samples were randomly taken from four different agroecological zones which had no previous history of petroleum or petroleum product contamination. The soils sampled were Toje series classified asRhodic Kandiustalf(Eze, 2008), Oda series classified as Eutric Gleysol(Owusu-Bennoah *et al;* 2000), Nyankpala series classified as Plinthic Acrisol(Nartey,1994), and Beach sand classified as Haplic Arenosol by FAO (1988). These soils were selected for the study taking into consideration their locations within the agroecological zones of Ghana. The soil from the beach was selected because current oil drilling in Ghana is offshore; hence soils at the beach are at a high risk of contamination when there is spillage. Oda series is a forest soil sampled from Forest and Horticultural Crops Research Centre (FOHCREC), Kade, Toje series is a coastal savannah soil sampled from the University of Ghana Demonstration Farm at Legon. The soil from the beach is also a coastal savannah soil sampled from the beach at Bortianor in Accra, and the Nyankpala series is a Guinea savannah soil sampled from Nyankpala. The soils were collected from the Ap horizon at a depth of 0-20 cm and transported to the laboratory. The soils were air dried, crushed and passed through a 2 mm sieve and stored for laboratory analyses and experiments.

3.2 Laboratory Analyses

3.2.1 Particle Size Analysis

The particle size analysis of the soil was determined using the Bouyoucos Hydrometer method modified by Day (1965). Forty (40) grammes of the air-dried and sieved soil sample were weighed into a beaker followed by the addition of 60 ml of 6% H_2O_2 in order

to destroy the organic matter. Hundred (100) ml of 5% Calgon (sodium hexametaphosphate) solution was added. The suspension was shaken on a mechanical shaker for two hours. The suspension was transferred into a graduated sedimentation cylinder and was brought to the 1000 ml mark with distilled water. At a constant temperature, a plunger was inserted into the cylinder and moved up and down several times to mix the suspension thoroughly. Five (5) minutes after the plunger was removed, a hydrometer was gently lowered into the content and the scale at the top of the meniscus was noted and it represented the reading for silt and clay. A second reading was taken after 5 hours to represent that of clay. The suspension was poured from the sedimentation cylinder into a 47-micron sieve and effluent discarded. Tap water was run through the sediment in the sieve to wash off most of the fine material. The sand particles left in the sieve were transferred into a moisture can, oven dried for 24 hours and dry weight determined. Blank hydrometer readings of sodium hexametaphosphate solution at 5 minutes and 5 hours were taken. The percentage of Sand, Silt and Clay were calculated based on the oven dry weight of the soil sample taken as follows

$$\text{Clay (\%)} + \text{Silt (\%)} = \frac{\text{Hydrometer reading at 5mins}}{\text{sample weight (g)}} \times 100 \quad ----------[1]$$

$$\text{Clay (\%)} = \frac{\text{Hydrometer reading at 5 hours}}{\text{sample weight (g)}} \times 100 \quad ----------[2]$$

Silt (%) = [1] – [2] ……. [3]

Sand (%) = 100 – [1] ……..... [4]

3.2.2 Soil pH Determination

The pH of the soil was determined using a HANNA pH 213 microprocessor pH meter in distilled water at soil to water ratio of 1:1. Twenty (20) grams of soil was weighed in duplicates into 50 ml beakers (Dole, 1941; Bates, 1954). Twenty (20) millilitres of distilled water was added and the soil-liquid suspension was stirred for 30 minutes. The suspension was allowed to stand for an hour for equilibration. The pH metre was standardised using buffer solutions of pH 4.0 and 7.0. The electrode was inserted into the suspension to measure the pH of the samples.

3.2.3 Determination of Soil Organic Carbon

Organic carbon was determined by the wet combustion method of Walkley and Black (1934). A half gram (0.5 g) of air-dried and sieved soil (0.5 mm sieve) sample was weighed into a conical flask. Ten (10) ml of 0.167 M potassium dichromate ($K_2Cr_2O_7$) and 20 ml of concentrated sulphuric acid (H_2SO_4) were added. The flask was swirled to ensure that all the soil particles were in contact with the solution and digested. The content of the flask was allowed to settle for 30 minutes. The unreduced potassium dichromate ($K_2Cr_2O_7$) remaining in solution after the oxidation of the oxidizable organic material in the soil sample was titrated with 0.2 M ferrous ammonium sulphate after adding 10 ml orthophosphoric acid and 2 ml of barium diphenylalanine sulphate (an indicator) until colour change from a dirty brown colour to a bright green end point. Standardization of the potassium dichromate ($K_2Cr_2O_7$) with the ferrous ammonium sulphate was done. The titre value was used to calculate the percent carbon (%C) as:

$$\%C = \frac{[0.3x\,(10 - XN)\,]}{W} \times 1.33 \quad ----------[5]$$

X = Titre value of the ferrous ammonium sulphate

N = Molarity of the ferrous ammonium sulphate (0.2M)

W = Weight of the soil sample

3.2.4 Determination of Total Nitrogen

The Kjedahl (1883) method was used in the determination of total nitrogen. Two (2) grams of soil was weighed into 300 ml Kjedahl flask and a tablet of a digestion accelerator (selenium catalyst) was added. This was followed by addition of 5 ml of concentrated H_2SO_4. The mixture was digested until the digest became clear. The flask was then cooled and its content transferred into a 100 ml volumetric flask. The content was made to the 100 ml mark with distilled water. An aliquot of 5 ml of the digest was taken into a Markham distillation apparatus and 10 ml of 40% NaOH was added and the mixture distilled. The distillate (liberated ammonia) was collected in 5 ml of 2% boric acid (H_3BO_3). Three drops of a mixed indicator containing methylene blue and methyl red were added to the solution and then back titrated with 0.01M HCl from green to reddish end point. The percent N was calculated as follows:

$$\%N = \frac{0.01 \times \text{titre volume} \times 0.014 \times \text{volume of extract} \times 100}{Soil\ Sample\ weight\ (g) x\ volume\ of\ aliquot\ (mL)} \ldots\ldots\ldots\ldots\ldots (6)$$

Where

0.01 = Molarity of HCl

0.014 = Milliequivalent of Nitrogen

3.2.5 Available Phosphorus Determination

Available P of the Beach soil was determined using the method of Watanabe and Olsen (1965). Ten grams of the fine earth fraction was weighed into extraction bottles. A 100 ml sodium bicarbonate (pH 8.5) was added to the samples in the extraction bottles; it was then capped and shaken for 30 min on a mechanical shaker. The extracts were filtered using Whatman's No. 125 filter paper to obtain clear solution. A 10 ml aliquot was taken into a test tube and then in drop-wise, 1 ml of 1.5 M H_2SO_4 was added to decolourise the solution by settling the organic matter in it. It was then left in a refrigerator to cool for few minutes. The extracts in the test tubes were centrifuged, and gently decanted for colour development and phosphorus analysis. The concentration of P in the extracts was then determined using the Murphy and Riley method (1962) described as followed. An aliquot of 1mL of the sample solution was pipetted into a 50 ml volumetric flask and a drop each of P-nitrophenol and ammonium hydroxide were added. Then, 8 ml of a solution containing concentrated sulphuric acid, ammonium molybdate, potassium antimony tartrate, and ascorbic acid were added. The content was topped up to the 50 ml mark with distilled water. The concentration of phosphorus was then determined on a Philips' UV spectrophotometer at a wavelength of 712 nm. Available phosphorus content of the soil was calculated as;

$$P(mg/kg) = \frac{(spectrometer\ reading - blank\ reading) \times volume\ of\ extract}{Volume\ of\ aliquot \times sample\ weight} \quad \ldots\ldots\ldots (7)$$

Available P of the other soils (Toje, Oda and Nyankpala) was determined using Bray 1 method. Five (5) grams of soil was weighed into a centrifuge tube in duplicates. Fifty (50) millilitres of Bray solution (0.03 N NH_4F +0.025 N HCl) was added (Bray and Kurtz,

1945). The tubes were shaken end-over-end on a mechanical shaker for 5 min and were then centrifuged at 2500 rpm for 5 min. The suspensions were each filtered through a No. 42 Whatman filter paper into a 50 ml Erlemeyer flask. Phosphorus in the filtrate was determined using the molybdate-ascorbic acid method as follows:

An aliquot of 1 ml was transferred into a 50 ml Erlemeyer flask and about 30 ml of distilled water was added in duplicate. The pH was adjusted using P-nitrophenol indicator and neutralised with a few drops of 4 M NH_4OH until the solution turned yellow. Five (5) millilitres of a mixture of ascorbic acid, ammonium molybdate, antimony potassium tartarate and concentrated H_2SO_4 (reagent A) was added and made to volume (50 ml) with distilled water. The solution was mixed thoroughly by shaking and allowed to stand for about 50 min for the colour to stabilise. A blank was prepared with distilled water and 5 ml of reagent B(1.056 g of ascorbic acid in 200 ml of reagent A).

The concentration of phosphorus was then determined on a Philips' UV spectrophotometer at a wavelength of 712 nm. Available phosphorus content of the soil was calculated with equation (7).

3.2.6 Exchangeable Cations (Na, K, Ca and Mg)

Ten grammes (10 g) of soil was weighed into an extraction bottle and 100 ml of 1 M ammonium acetate (NH_4OAc) was added and shaken for 30 minutes. The suspension was allowed to settle, after which it was decanted and filtered. The filtered solutions (aliquots) were used for the determination of Ca, Mg, K and Na. The concentrations of potassium (K) and sodium (Na) were determined using the flame photometer (Chapman, 1965).

3.2.6.1 Potassium (K) Determination

The flame photometer was standardized such that 10 mg/kg of K gave 100 full scale deflections. The flame photometer after standardization was used to determine the concentration of potassium in 10 ml aliquot. The result was used in the calculation of the amount of potassium present in the soil as shown in the formula below.

$$\text{Exchangeable K (cmol/kg soil)} = \frac{\text{R} \times \text{V} \times 100}{\text{Weight of soil} \times 39.1} - - - - - - [8]$$

Where R= Flame Photometer reading for K (ppm)

39.1=Molecular weight of Potassium

V= Volume of extract (100 ml)

3.2.6.2 Sodium (Na) Determination

The flame photometer was standardized in a way that 10 mg/kg of Na gave 100 full scale deflections. After the standardization of the photometer, the concentration of sodium in 10mL aliquot was determined. The result was then used in the calculation of the amount of sodium (Na) present in the soil as shown by the formula below.

$$\text{Exchangeable Na (cmol/kg soil)} = \frac{\text{R} \times \text{V} \times 100}{\text{Weight of soil} \times 23} - - - - - [9]$$

Where R= Flame photometer reading for Sodium (ppm)

V= Volume of extract (100 ml)

23=Molecular weight of Sodium

3.2.6.3 Calcium (Ca) Determination

To a 10 ml aliquot of the sample solution, 10 ml of 10% KOH and 1ml triethanolamine (TEA) were added. Three drops of 1M KCN solution and a few crystals of cal-red indicator were then added after which the mixture was titrated with 0.02M EDTA solution from red to blue end point. The titre value was used in the calculation of calcium as shown below.

$$\text{Ca}\ (cmol/kg) = \frac{\text{Titre value} \times \text{N} \times \text{Vol.of extract} \times 100\ (\text{meq}/100\ \text{g})}{\text{Vol. of aliquot} \times \text{Weight of soil}} \quad \ldots\ldots\ldots\ldots(10)$$

Where N = Molarity of EDTA

3.2.6.4 Magnesium (Mg) Determination

To a 10 ml aliquot of the sample solution, 5 ml of ammonium chloride – ammonium hydroxide buffer solution was added followed by 1ml of triethanolamine. Three drops of 1M KCN solution and a few drops of Eriochrome black T solutions were added after which the mixture was titrated with 0.02M EDTA solution from red to blue end point. The end point titre value determines the amount of calcium and magnesium in the solution. The titre value of magnesium was then determined by subtracting the value obtained for calcium above from the new titre value obtained. The titre value of magnesium was then used for the calculation of the concentration of magnesium (Mg) as shown below.

$$\text{Mg}\ (cmol/kg) = \frac{\text{Titre value} \times \text{N} \times \text{Vol.of extract} \times 100\ (\text{meq}/100\ \text{g})}{\text{Vol. of aliquot} \times \text{Weight of soil}} \quad \ldots\ldots\ldots\ldots(11)$$

Where N = Molarity of EDTA

3.2.7 Cation Exchange Capacity (CEC)

Ten (10) g of soil was weighed into an extraction bottle and 100 ml of 1N NH_4Ac solution was added and shaken on a mechanical shaker for 30 minutes. The suspension was filtered through a No. 42 Whatman filter paper and and the samples leached four times with 25 ml of alcohol (ethanol) to wash off excess ammonium. The residue was again leached four times with 25 ml acidified KCl. Ten (10) ml of the decanted leachate (filtrate) was pipetted into a Kjeldahl flask and 10 ml of 40% NaOH, and about 100 ml of distilled water was added. The solution was distilled and the distillate collected over 5 ml of boric acid to which three drops of methyl red and methylene blue mixture had been added. The distillate was back-titrated against 0.01M HCl until the end point. The titre value was used to determine the CEC of the soil.

$$CEC\ (cmol/kg) = \frac{volume\ of\ extract\ x\ molarity\ HCl\ x\ titre\ value\ x\ 10}{volume\ of\ aliquot\ used} \quad \ldots\ldots\ldots\ldots (12)$$

3.3 Experiment 1

The sieved soils were contaminated with diesel oil at 10g oil/kg of soil. The contaminated soils were amended with ammonium nitrate as source of nitrogen and single superphosphate as source of phosphorous(P_2O_5) at 0,30,60 and 90 kg/ha. The treatments were replicated four times and arranged in a complete randomised design. The contaminated soils were left to stand in the laboratory under room temperature. Samples were taken at intervals of 10 days for hydrocarbon utilising bacterial (HUB) population and quantity of diesel oil degraded.

3.3.1 Hydrocarbon utilising Bacterial Population Count

The hydrocarbon utilising bacterial count was estimated using the modified mineral salts agar medium of Mills et al. (1978) and modified vapour phase transfer technique of Okpokwasili and Amanchkwu (1988). One (1) g of soil was sampled, tenfold serial dilution was prepared. One (1) ml of appropriate dilution was plated onto the modified mineral salts medium containing 10 g NaCl, 0.42 g $MgSO_4.7H_2O$, 0.29 g KCl, 0.53 g KH_2PO_4, 0.42 g NH_4NO_4, and 15 g agar in 1L distilled water (adjusted pH = 6.8). The modified vapour transfer technique involves spreading 0.5 ml of diesel (serving as carbon source) onto the mineral salts agar medium after setting and allowed to stand for about one hour (1 hr) for the sterilised diesel oil to diffuse into the agar medium before incubating at room temperature for 5 to 7 days. The number of colonies formed was used to estimate the hydrocarbon utilising bacterial population.

3.3.2 Amount of Diesel Oil Degraded

Residual diesel oil in the contaminated soils was extracted using a modified method of Abu and Ogiji (1996). Soil samples were air dried to constant weights, 5 g was placed into small plastic containers and 10 ml chloroform was added. Residual oil was extracted by gently shaking the containers for 5 min at 150 rpm. Each extract was filtered through cotton wool in a funnel and collected in a clean glass container, closed immediately and analysed for diesel oil content. Quantitative determination of diesel oil the extracts was employed as described by Udeme and Antai (1988). A standard curve of absorbance (520 nm) against varying concentrations of diesel oil in chloroform was drawn after taking readings from a spectrophotomer (Phillips PU 8620 spectrophotometer). The diesel oil concentrations were calculated from the standard curve. The residual diesel oil data were fitted to the first order kinetics model to calculate the biodegradation rate.

$$C = C_o e^{-kt} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots.(13)$$

Where C is the hydrocarbon content in the soil (gkg^{-1}) at time t, C_o is the initial hydrocarbon content in soil (gkg^{-1}), k is the biodegradation rate constant (g oil d^{-1}), e is the specific degradation constant and t is time.

3.4 Experiment 2

The effect of remediating diesel oil contaminated soil with nitrogen and phosphorus on the germination and growth of maize and cowpea was investigated by setting up a pot experiment in a screenhouse. These test crops are mainly consumed cereal and legume, respectively in the country. The pot experiment was conducted at the Ecological Laboratory's screenhouse of the University of Ghana, Legon. Two (2) kilograms of the Toje series was weighed into each pot. The soils were contaminated with diesel oil at 0 (C0), 2.5 (C1), 5 (C2) and 10 g soil/kg soil (C3). The contaminated and uncontaminated soils were amendedwith Nitrogen (N60) and phosphorus (P_2O_5) (P60) at a rate of 60kg/ha, and control (T0) where no fertilizer was applied. The pots wereallowed to stand in the screenhouse for 20 days in order to allow degradation to take place. After 20 days, the test crops maize and cowpea were planted in the treated soils at 3 replicates for 4 weeks. One week after planting (7 days), % germination was determined and 4 weeks after planting (4WAP) shoot dry weights, N and P uptake and nodule number (cowpea) were determined.

3.5 Analysis of Plant Materials

One-tenth of a gram (0.1g) of milled plant sample was weighed into a 500ml conical flask and five (5) ml of concentrated sulphuric acid (H_2SO_4) was added to oxidize the organic matter and drops of hydrogen peroxide (H_2O_2) was used to digest the plant sample completely. The digest was allowed to cool and later transferred into a 100 ml volumetric

flask and made to the 100 ml mark with distilled water. The solution was shaken thoroughly to ensure homogeneous mixture. A 5 ml aliquot of the digest was used to determine the total N as described under 3.2.4. Also an aliquot of 1 ml was used to determine the total P using the ammonium molybdate-ascorbic acid method as described under 3.2.6. Total N and P uptake were calculated as follows:

$$Total\ N\ uptake\ (g/plant) = \frac{\%N\ concentration\ in\ plant\ x\ Shoot\ dry\ matter\ weight\ \ (g/plant)}{100} \ldots\ldots\ldots (14)$$

$$Total\ P\ uptake\ (g/plant) = \frac{\%P\ concentration\ in\ plant\ x\ Shoot\ dry\ matter\ weight\ \ (g/plant)}{100} \ldots\ldots\ldots (15)$$

3.6 Statistical analyses

Analysis of variance (ANOVA) was used to test for significant differences among the means at 5% probability level using GenStat statistical software package (9^{th} edition).

CHAPTER FOUR

RESULTS

4.1 Physical and chemical properties of the soils used for the study

Data on some selected important physical and chemical properties of the soil series used for the study are shown in Table 4.1. The particle size analysis showed that the Toje series is a clay loam according to the USDA (2003) system of classification. The texture of the Oda and Nyankpala series was classified as sandy loam. The texture of theBeach sandwas sand.The Oda soil had higher organic carbon content compared to the other soils. The pH of Toje, Oda, and Nyankpalawere moderate to acidic whiles that of Beach sand was neutral. Oda soil had relatively high available phosphorus and total nitrogen whereas that of Toje, Nyankpala and Beach sand had low values. The CEC value for Toje was higher than the other soils.

Table 4.1 Some Physico-chemical properties of the soils used

Soil properties	Oda	Toje	Nyankpala	Beach Sand
Sand (%)	62.65	61.48	50.03	99.13
Silt (%)	32.35	13.52	44.97	0.87
Clay (%)	5.00	25.00	5.00	0.00
Texture	sl	cl	sl	s
pH	5.6	5.3	4.8	7.0
OC (%)	0.72	0.56	0.34	0.11
Total N (%)	0.16	0.12	0.07	0.02
Available P (mg/kg)	8.23	4.68	1.92	0.10
Na (cmol/kg)	0.19	0.30	0.23	3.04
K (cmol/kg)	1.86	0.82	1.02	0.09
Mg (cmol/kg)	1.21	0.54	3.48	9.28
Ca (cmol/kg)	3.48	1.03	2.51	10.36
CEC (cmol/kg)	5.72	11.68	6.14	0.21

sl = sandy loam cl = clay loam s = sand

4.2 Response of HUB population to nitrogen and phosphorus amendment

The effects of nitrogen on the population of hydrocarbon utilising bacteria (HUB) in the four contaminated soils are shown in Fig. 4.1.The bacterial growth pattern was similar in the four soils. HUB populations grew to peak values and then declined. The Beach sand and Nyankpala series reached their peak values 20 days after incubation (DAI) while those in the Oda and Toje series reached their peak values 30 DAI. The initial HUB populations in Nyankpala, Oda, Toje and Beach sand were 5.9 x 10^5, 1.1 x 10^7, 8.4 x 10^6 and 3.3 x 10^2cfu/g soil, respectively. Increasing the application rate of N resulted in increased HUB populations. At 30 and 40 DAI the nitrogen treatments were not significantly ($p > 0.05$) different in the four soils. Response of HUB to phosphorus application followed the same trend as the response to nitrogen application as shown in Fig. 4.2.

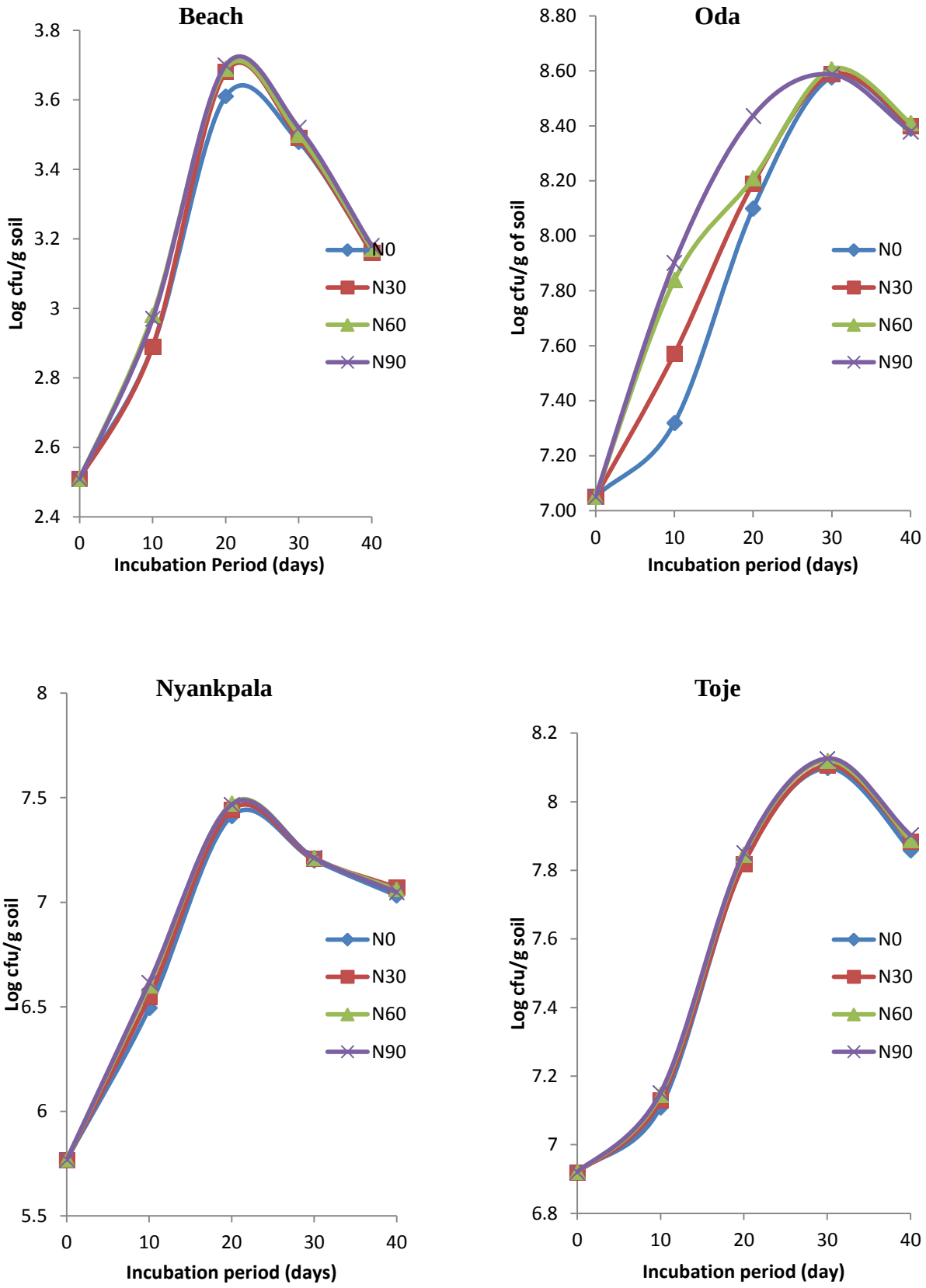


Fig 4.1 Response of HUB populations to different rates of nitrogen

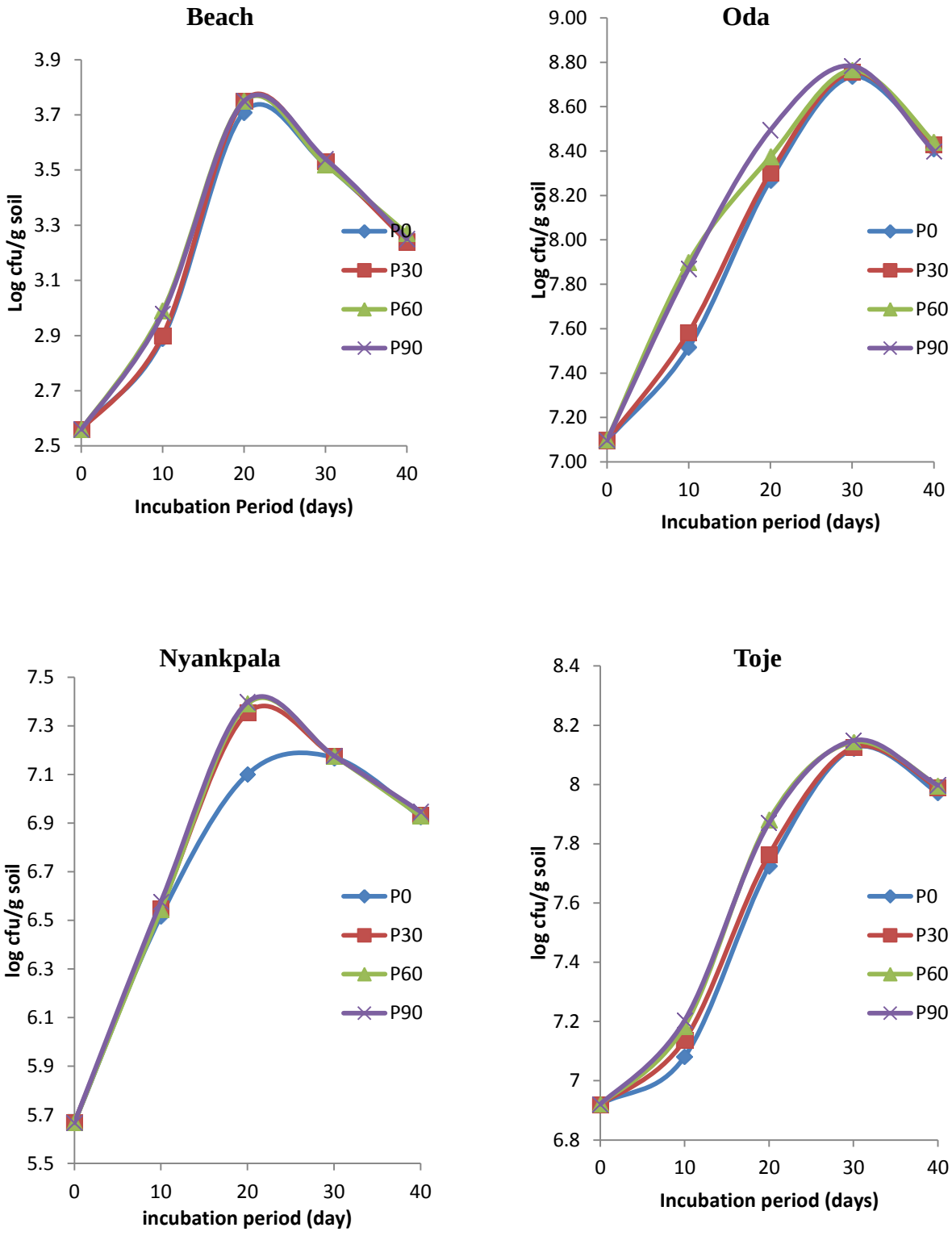


Fig 4.2 Response of HUB populations to different rates of phosphorus

4.3 Effects of nitrogen and phosphorus on Diesel oil Degradation

The effects of N and P on the amount of diesel oil degraded in the various contaminated soils are shown in Fig. 4.3 and 4.4. There was a general increase in the amount of diesel oil degraded from 10 DAI to 40 DAI for all the soils as indicated by the cumulative amount of diesel oil degraded in the soils. The results also showed that as rate of fertilizer application increased, the amount of diesel degraded increased. At 40 DAI the cumulative amount of diesel oil degraded in the four contaminated soils was in the order 90 > 60 > 30 > 0 N or P_2O_5 kg /ha. Significant ($p < 0.05$) differences among the treatments were observed between 10 and 20 DAI, but not at 30 and 40 DAI.

Using first-order kinetics the rates of biodegradation of diesel oil in the various treatments were determined and presented in Table 4.2. Results showed that increasing application rate of nitrogen and phosphorus resulted in increasedrate of biodegradation.

Table 4.2 Biodegradation rate constant (g oil/d) of the various treatments.

Treatment	Toje	Nyankpala	Oda	Beach Sand
N0	0.0191a	0.0224a	0.0169a	0.0270a
N30	0.0200a	0.0236b	0.0172ab	0.0290a
N60	0.0204a	0.0241c	0.0176b	0.0300a
N90	0.0210a	0.0244c	0.0185c	0.0310a
LSD(p = 5%)	0.0023	0.0005	0.0005	0.0046
P0	0.0191a	0.0227a	0.0187a	0.0294a
P30	0.0194a	0.0235b	0.0191a	0.0314b
P60	0.0205b	0.0243c	0.0192a	0.0322c
P90	0.0217c	0.0259d	0.0200b	0.0334d
LSD(p = 5%)	0.0004	0.0006	0.0007	0.0005

***** Means having subscript in common within each column are not significantly different at 5% probability.

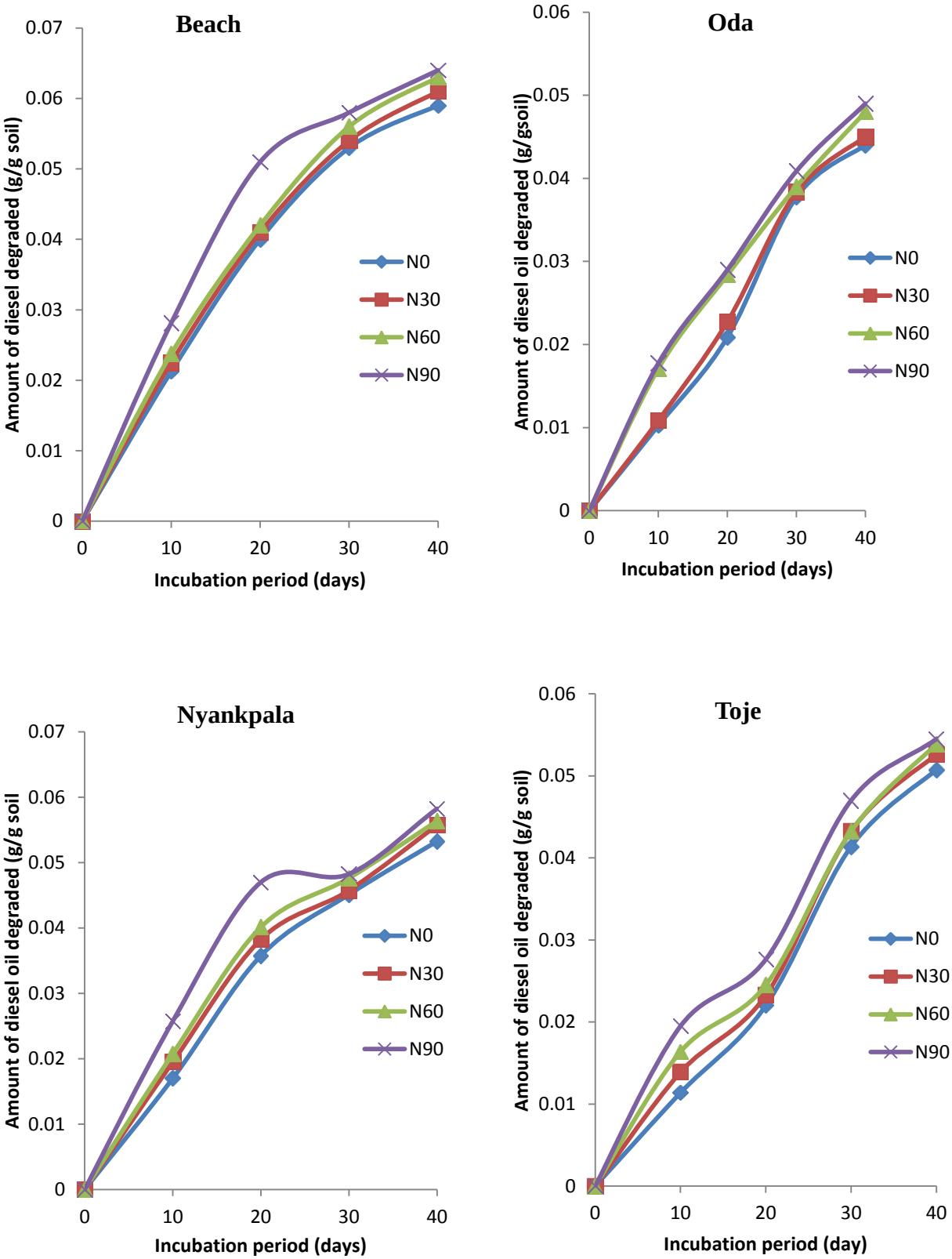


Fig. 4.3 Effect of nitrogen amendment on the degradation of diesel oil

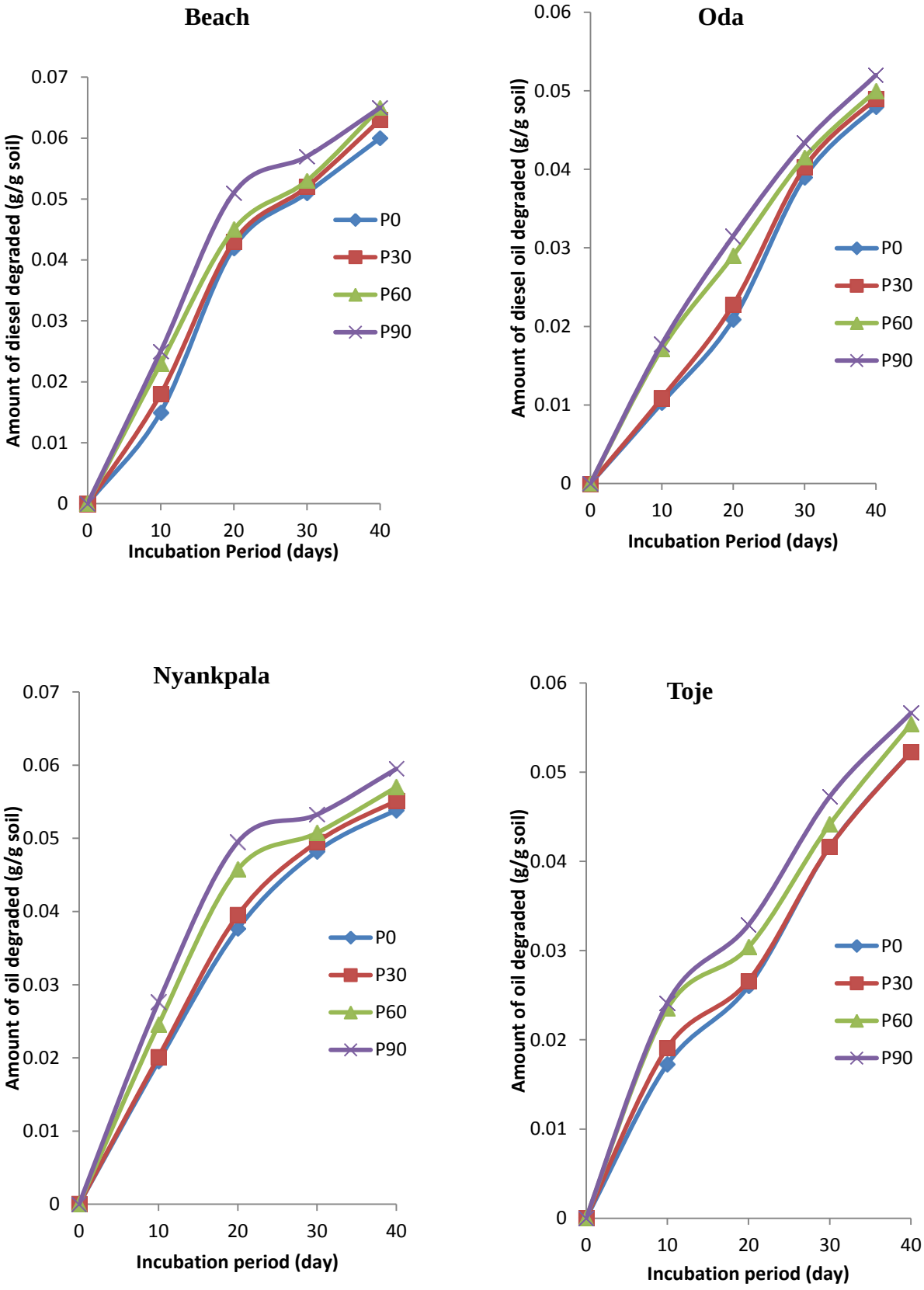


Fig 4.4 Effect of phosphorus amendment on the degradation of diesel oil in the four soils

However, there were no significant ($p > 0.05$) differences in the rates of degradation within the N treatments in the Toje series and Beach sand. Generally the rate of biodegradation in the contaminated soils was in the order Beach >Nyankpala>Toje>Oda.

4.4 Germination response to Nitrogen and Phosphorus application

The response of cowpea and maize to N and P application is shown in Fig. 4.5. In all the test crops, increasing diesel oil concentration resulted in a decrease in the proportion of seeds that germinated. In the uncontaminated soil (C0), N and P enhanced germination of both maize and cowpea. Generally, although the application of N and P at 60 kg/ha enhanced germination of both test crops at all levels of diesel contamination, the effect wassignificantly ($p < 0.05$) only in cowpea. The influence of N and P on germination in the contaminated soils was pronounced in maize and cowpea, respectively.

4.5 Effect of Diesel oil Remediation on Shoot Dry Weight

The shoot dry weights (SDW) of the test crops grown in contaminated and uncontaminated soils are shown in Fig. 4.6. Generally, SDW decreased with increasing level of oil contamination. The average percent reductions in SDW in the contaminated soils compared to the uncontaminated-unamended treatment (C0T0), Nitrogen treatment C0N60) and Phosphorus treatment (C0P60) were 82.7%, 82.6% and 79.87%, respectively for cowpea and 53.31%, 50.24% and 52.2%, respectively for the maize.In the uncontaminated soil (C0), P and N application significantly ($p < 0.05$) increased SDW in cowpea and maize, respectively. Although the application of the two plant nutrient elements increased SDW of the test crops in the contaminated soils, the effect was not significant ($p > 0.05$). Results also showed that the influence of N was more pronounced in the maize than in cowpea, while the influence of P was more pronounced in cowpea than in maize.

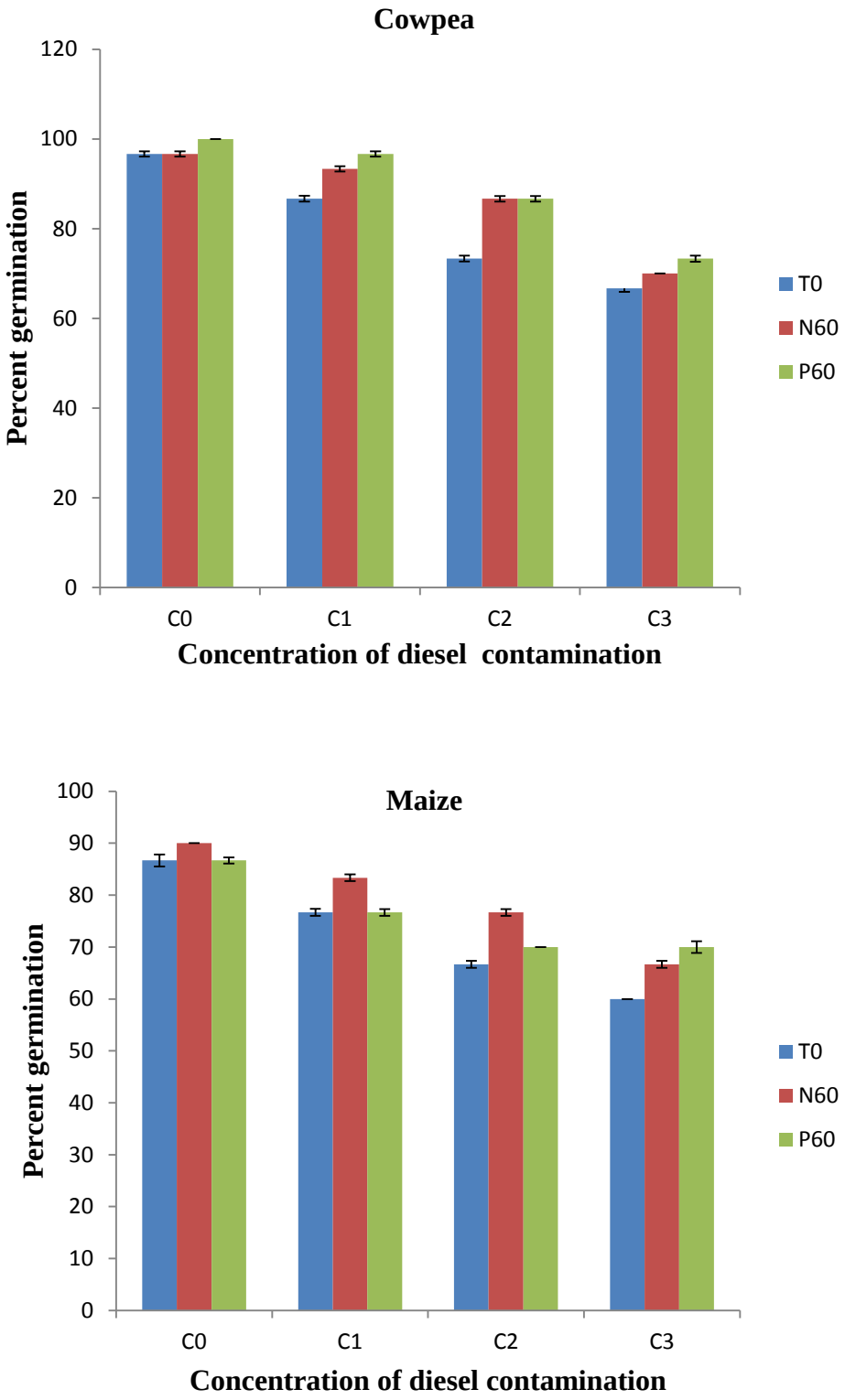


Fig. 4.5 Germination of cowpea and maize in contaminated and uncontaminated soils amended with N and P

Note:

- Bars in the figure represent standard errors.
- The soils were contaminated with diesel oil at 0 (C0), 2.5 (C1), 5 (C2) and 10 g oil/kg soil (C3).

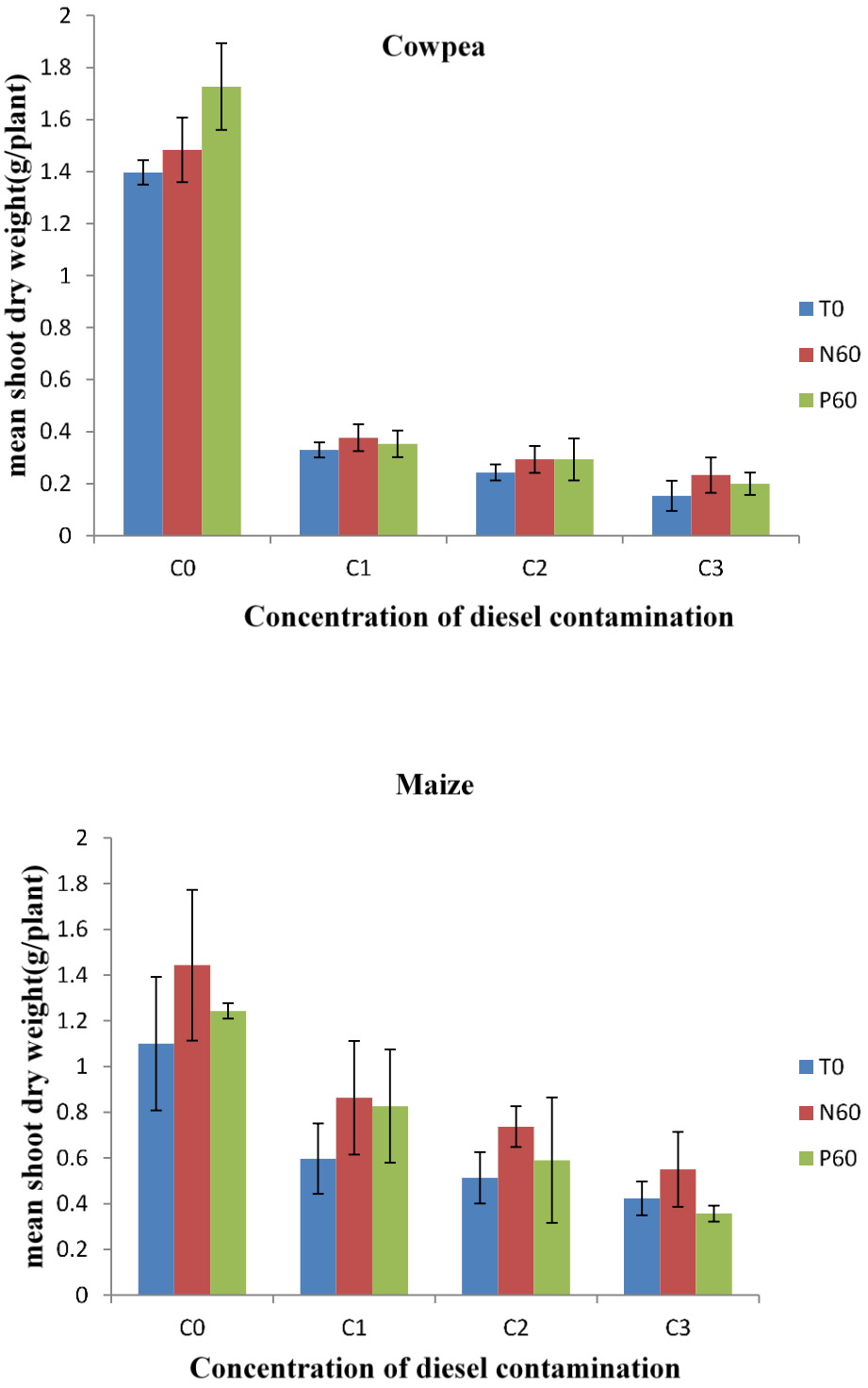


Fig. 4.6 Shoot dry weights of cowpea and maize in contaminated and uncontaminated soils amended with N and P

Note:

- Bars in the figure represent standard errors.
- The soils were contaminated with diesel oil at 0 (C0), 2.5 (C1), 5 (C2) and 10 g oil/kg soil (C3).

4.6 Total N and P Uptake of Test Crops

Total N and P uptake of the test crops varied among the different treatments at 4 weeks after planting (Fig. 4.7 and Fig. 4.8). Results showed that uptake generally decreased with increasing level of diesel in soil. In the uncontaminated soil (C0), N uptake in cowpea increased when N and P were added, although, significant ($p < 0.05$) increase was observed for only soils treated with P. In maize, N uptake significantly ($p < 0.05$) increased when the uncontaminated soil was treated with N and P, with the influence of N application being more pronounced than with P application. Nitrogen uptake by cowpea in the contaminated soils treated with N and Preduced by an average of 82.48% and 88.84% respectively, the contaminated soil untreated with N and P recorded 85.83% average reduction in N uptake. The P uptake by cowpea was reduced by 85.32% and 88.56% in the N and P treated soils respectively,the contaminated soil not treated with N and P recorded 87.61% reduction in P uptake.In cowpea, P uptake was only enhanced by N and P application but there was no significant ($p > 0.05$) difference between the treatments in the contaminated soils.In maize, N uptake for the N treated soil, P treated soil and the unamended soil with N and Pin the oil contaminated soils reduced by an average of 57.94%, 59.89% and 61.35%, respectively.N uptake generally was enhanced by the application of N and P, however, N application caused more N uptake by the maize plant than P application. The P uptake by maize was reduced by 62.43% and 64.57% in the N and P treated soils respectively and the untreated soil recorded 65.80% reduction in P uptake.

4.7 Nodule Formation

Results on nodule formation in cowpea are shown in Table 4.3. Results showed that no nodule was formed inthe oil contaminated soils; neither the addition of N and P couldremedy the effect of the oil. The addition of N in the uncontaminated soil decreased nodule number, but not significantly ($p > 0.05$) different from the control. However, the application of P significantly ($p < 0.05$) increased nodule number.

Table 4.3 Effects of diesel oil contamination on nodule count.

Treatment	C0	C1	C2	C3
T0	17a	0	0	0
N60	15a	0	0	0
P60	21b	0	0	0
LSD	3.263			
cv	18.5%			

***** Means having subscript in common within each column do not have any significant difference at 5% probability.

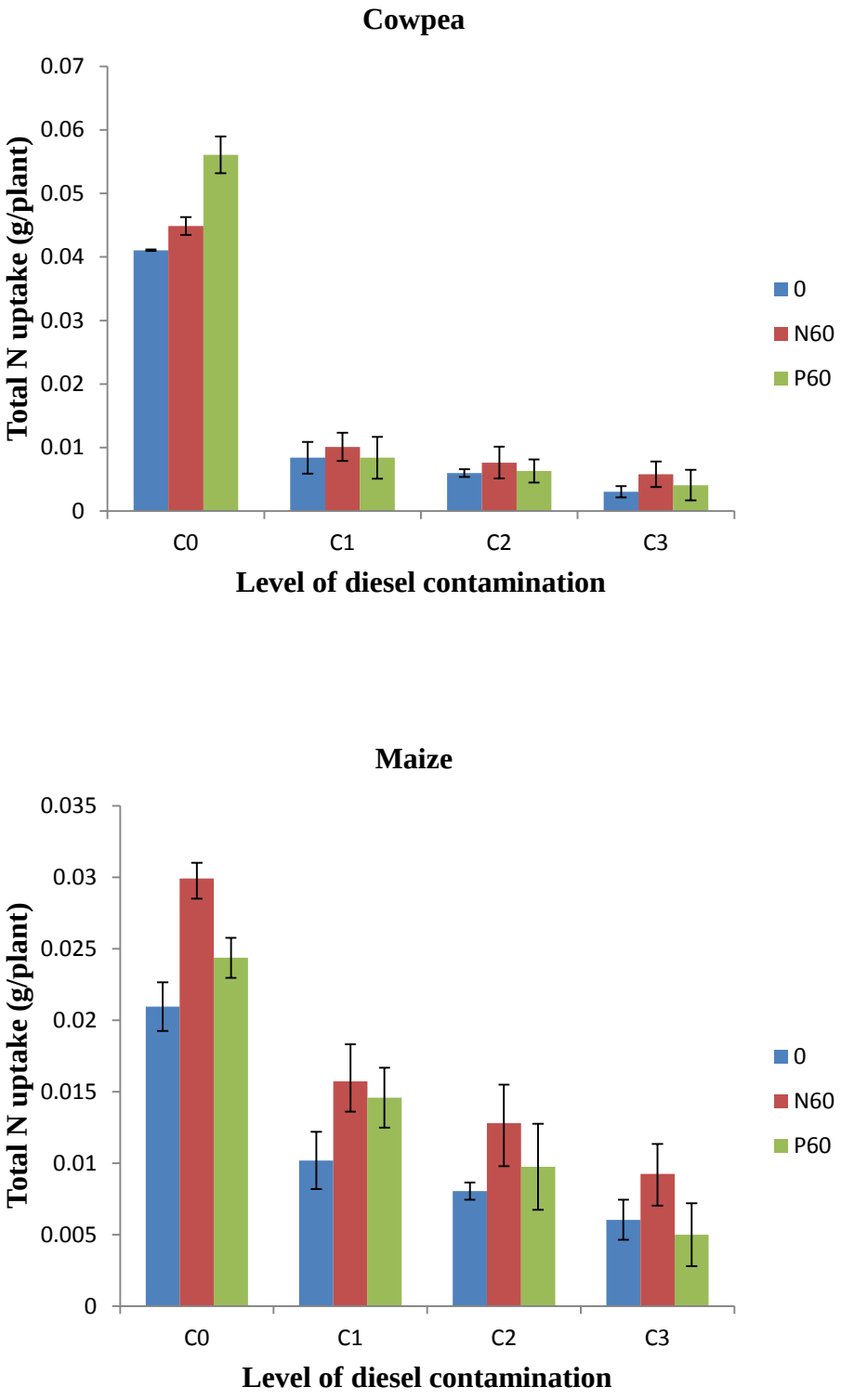
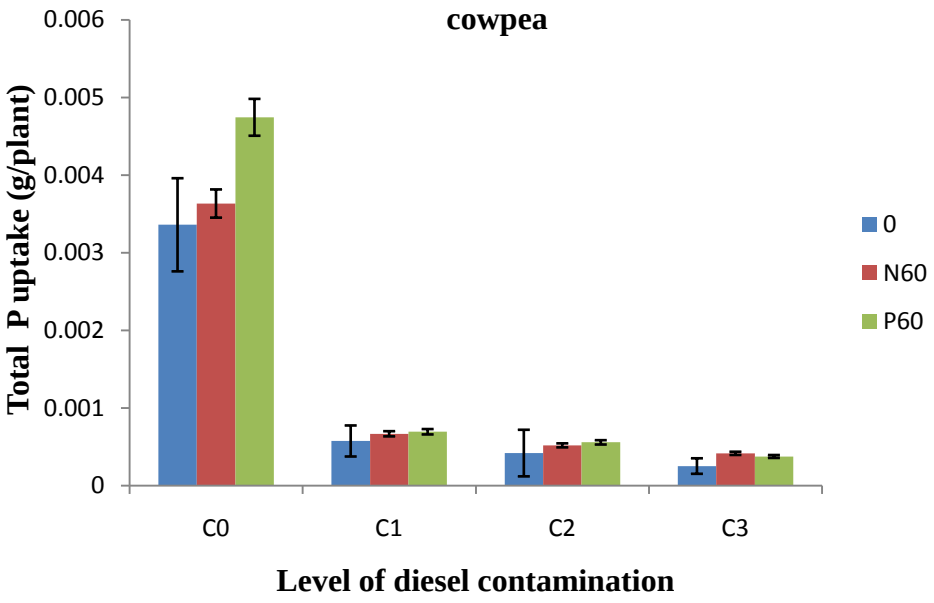


Fig. 4.7 Total N uptake of cowpea and maize in contaminated and uncontaminated soils amended with N and P

Note:

- Bars in the figure represent standard errors.
- The soils were contaminated with diesel oil at 0 (C0), 2.5 (C1), 5 (C2) and 10 g oil/kg soil (C3).



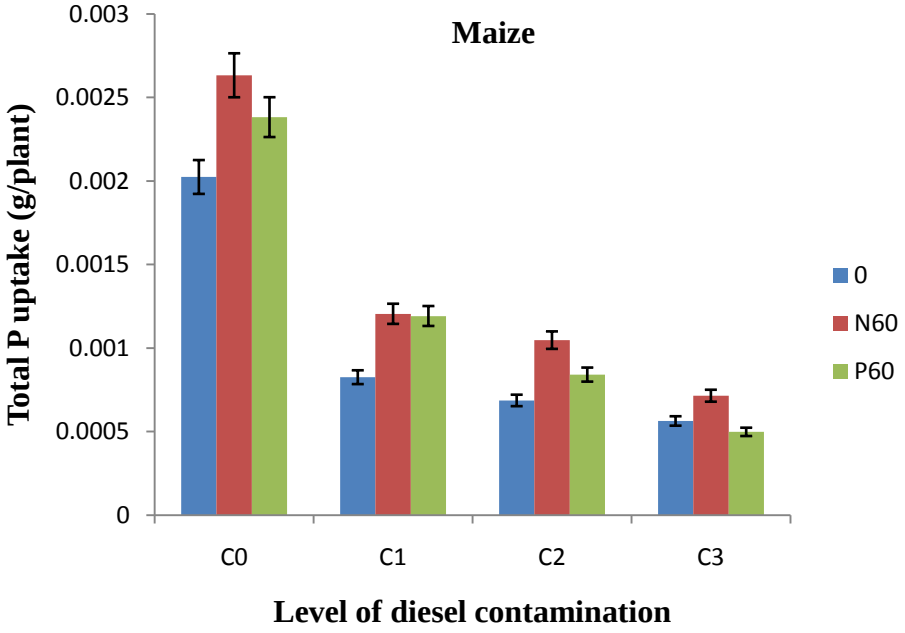


Fig. 4.8 Total P uptake of cowpea and maize in contaminated and uncontaminated soils amended with N and P

Note:

- Bars in the figure represent standard errors.
- The soils were contaminated with diesel oil at 0 (C0), 2.5 (C1), 5 (C2) and 10 g oil/kg soil (C3).

CHAPTER FIVE

DISCUSSION

5.1 HydrocarbonUtilizing Bacteria growth

Bacteria capable of degrading oil, known as HUB, exist in many soils.Their populations vary in different soils, and the addition of petroleum contaminants stimulate the growth of HUB bacteria, since the petroleum acts as an energy source, even sometimes as a selective medium for them.It is therefore not suprising that addition of diesel in this study enhanced the growth of those organisms capable of using diesel for growth. HUB bacteria besides energy source require other nutrients for their growth. These nutrients they acquire from the soil in which they are growing. Several studies (Ellis and Adams, 1961; Volk, 1980; McGill, 1980) have shown that the amounts of N and P present in many oil contaminated soils are limited, and unable to supply soil bacteria with adequate N and P for optimum growth. Amelioration of soil with these nutrients in such cases has resulted in increased populations of bacteria in soil establishing that N and P are essential nutrient elements for achieving high numbers of bacteria in soil.Since degradation of contaminants in the soil is often linked to numbers of the degrading bacteria, increasing the number of bacteria acting on a particular substrate under any given set of conditions, will result in faster degradation. This was exactly what was observed; addition of either N or P increased both the population of HUB, with the concomitant decrease in the levels of petroleum remaining in the soil, a measure of the extent and rate of the degradation of the contaminant diesel by the HUB organism.This finding agrees with studies by Chorom *et al.* (2010), who observed that application of fertilizers to crude oil polluted soil had a significant effect on soil bacteria growth; and that the average bacterial growth in the treatment samples had a significant difference from bacterial growth in the control. Studies by several researchers

(Ijah and Antai (2003); Adesodun and Mbagwu (2008); Nakasaki *et al.*, (1992); Joo *et al.*, (2001); Joo *et al.*, (2007) also indicated that, nitrogen and phosphorus are necessary nutrients for biodegradation activities. Microorganisms require phosphorus as phospholipids in synthesising cell membranes, as components of nucleic acids and for sugar phosphorylation (Andrew and Jackson, 1996). Microorganisms also exploit nitrate sources to meet their protein and nucleic acid requirements (Odokuma and Akponah, 2010). The HUB growth increased in the first few weeks and decreased thereafter with time. Sang-Hwan *et al.* (2007)made a similar observation and concluded that hydrocarbon degrading bacteria population increased rapidly during the first 30 days of 105 days testing period. However, with increasing time, due to soil resistant components with high chain and with less remaining nutrients, thegrowth of bacteria and oil degradation decreased (Schaefer and Juliane, 2007). The non-significant difference in counts as time elapse was probably because of the depletion of these inorganic nutrients as time went on.The differences in the initial HUB count in the four soils could be attributed to the nutrient status of the soils.

Oda and Toje soils recorded high initial HUB population and HUB population growth peaks at 30 DAI because they contained high organic matter and soil nutrients that encouraged and sustained bacterial growth much longer than Nyankpala and Beach sand. The low P and N and organic carbon levels in the Nyankpala and Beach sand could be the reason for the initial low HUB population and early HUB population growth peaks at 20 DAI for these two soils.

5.2 Diesel oil Degradation

Nutrient addition is the most common method for stimulating bioremediation of oil contaminated environments. Nutrients especially are believed to be among the most important factors limiting microbial growth and thus oil biodegradation. Biostimulation of

soil microorganisms by the addition of nitrogen and phosphorus resulted in the degradation of diesel oil. The reason for increased biodegradation of oil in the amended soils as compared to the unamended soil could be attributed to the increase in HUB counts, subsequently leading to an increase in the biodegradation of the contaminant (diesel oil). Nutrients are very important ingredients for successful biodegradation of hydrocarbon pollutants, especially nitrogen, phosphorus and in some cases iron (Cooney, 1984). When a major oil spill occurs, the supply of carbon is dramatically increased and the availability of phosphorus and nitrogen generally becomes the limiting factor (Atlas, 1984). Hence the addition of nutrients is necessary to enhance the biodegradation of oil pollutants (Choi *et al*., 2002; Kim *et al*., 2004). Pelletier *et al*. (2004) assessed the effectiveness of fertilizers for crude oil bioremediation in sub-Antartic intertidal sediments over a one-year period and observed that chemical, microbial and toxicological parameters demonstrated the effectiveness of various fertilizers in a pristine environment. Previous studies(Lee and Levy, 1989; Lee and Levy, 1991; Lee *et al*., 1993)have demonstrated the effectiveness of inorganic fertilizer additions to stimulate the biodegradation rates of oil stranded within sandy beach sediments. However, excessive nutrient concentrations could inhibit the biodegradation activity (Chaillan *et al*., 2006); and several authors have higlighted the negative effects of high NPK levels on the biodegradation of hydrocarbons (Oudot *et al*., 1998; Chaineau *et al*., 2005) and more especially on the aromatics (Carmichael and Pfaender, 1997). Regardless of the type of nutrient applied, the rate of oil degradation was only sensitive to input of nutrients during the first 20 days. The oil content in all the treatments decreased with time; however the treatments containing N and P had the lowest oil content at the end of the 40 days, thus indicating that N and P played a more vital role in the utilisation of diesel oil by the microorganisms. The Beach sand had the highest amount of diesel oil degraded among all the four soils. The highbiodegradation

recorded in the Beach sand could be due to free movement or circulation of air. Aerobic conditions are generally considered necessary for extensive degradation of oil hydrocarbons in the soil environment since major degradative pathways for both saturates and aromatics involve oxygenases (Al- Aubaidy, 2004). Studies by Essam *et al*. (2012) demonstrated that aeration has an influential role in speed and efficiency of the degradation process. These results also agree with the study of (Okpokwasili and Oton, 2006.). Some other researchers have also shown increase in biodegradation of petroleum with the addition of fertilizer and ventilation (Ramsay *et al*., 2000; Chorom *et al.*, 2010).

The best two degrading soils were Beach sand and Nyankpala. Results indicated that their initial HUB were lower than that of Oda and Toje but they were capable of degrading the oil more than the later soils. The difference in the degradation could be due to the presence of effective HUB species in the Beach sand and Nyankpala. This confirms the assertion by Leahy and Colwell (1990) that oil degradation does not depend on only the population of HUB in the environment but appears to be a function of the ecosystem, local environmental conditions and to an extent on the genetic make-up of the microbial population of the HUB. According to Spain and van Veld (1983) and Spain *et al.* (1980), the mechanisms by which HUB can effectively degrade oil are (i) induction and/or depression of specific enzymes, (ii) genetic changes which result in new metabolic capabilities, and (iii) selective enrichment of organisms able to transform the compound or compounds of interest.

5.3 Germination oftest crops

The germination results showed that increasing concentration of diesel oil adversely affected germination. These results support the findings of Horne (1978) who stated that heavy crude oil pollution lead to poor germination due to poor soil aeration. Bossert and

Bartha (1985) also observed a low germination of corn seeds in soil contaminated by crude oil. Similar results were reported by Gallegos-Martinez *et al*. (2000) who found a reduction in germination between 30-90% in the tropical native Mexican species subjected to soil contamination with crude oil.

Campbell and Vavrek (1999) also showed that the number of seedlings germinating from seeds in oil contaminated wetland soil declined relative to the uncontaminated soils. According to Adam and Duncan (2002), diesel oil has volatile components that contain light hydrocarbons capable of entering easily through the plant cell walls. These small hydrocarbon molecules that penetrate the plants can be phytotoxic, explaining the decrease in seed germination (Ogbo, 2009). Fresh crude oil has a coagulatory effect on the soil, binding the soil particles into water impregnable soil that seriously impair water drainage and oxygen diffusion (Atuanya, 1987). According to Bona *et al*. (2011), planting in diesel contaminated soil could allow diesel oil to cover the seeds producing a barrier to the passage of water and possibly gases to the interior of seeds. Salanitro *et al*.(2004) also reported that, the decrease in germination as diesel oil concentration increases could not just be due to the contaminant concentration, but also to the hydrocarbon type, plant species and reduction in oxygen transfer between the seed and the surrounding environment. According to Kirk *et al*, 2002, rye grass showed a 50% inhibition of germination with petroleum oil contamination and thus quite tolerant of the contaminant; results of this study showed minimum percent germination of 60%, indicating that the test crops are tolerant of diesel oil contamination.

The enhanced germination rate recorded for the test crops grown in a soil treated with N and P as compared to the control could be attributed to the fact that the fertilizer has enhanced the rate of degradation of the diesel in the soil and has therefore remediated the soil to enable the seeds to germinate. The P and N promoted microbial degradation of

hydrocarbons because P and N are important for microbial growth as indicated in the previous discussion.

5.4 Shoot Dry Matter

The plants grown in the uncontaminated soil had higher shoot dry matter (SDM) than those from the contaminated soil. This shows that crude oil contamination inhibited plant growth and it is similar to the findings of Baker (1970), Akinola *et al.*(2004), Merckl *et al.*(2004) and Agbogidi *et al*. (2006; 2007). The toxicity of petroleum hydrocarbons at higher concentrations has been linked to displacement of nutrients and nutrient uptake (Amadi *et al*., 1993); reduction in available phosphorus and total nitrogen (Baker-Coker and Ekumdayo, 1995) and interference with soil chemotaxis by crude oil (Rosenburg *et al.*, 1992) culminating in growth retardation (Travern, 1992).

Phosphorus is among the most needed elements for crop production in many tropical soils. Phosphorus, although not required in large quantities, is critical to cowpea yield because of its multiple effects on nutrition (Adetunji, 1995). P deficiency is the most limiting soil fertility factor for cowpea production (Reamaekers, 2001). Muleba and Ezumah, (1985) have reported that phosphorus application influences the content of other nutrients in leaves and seeds. The significant higher SDM in the Phosphorus (P60) treatment is not surprising, this is so because it is a scientifically established fact that phosphorus fertilization increases nodulation (Luse *etal*., 1975;Agboola and Obigbesan 1977) and that legume yield is positively influenced by the root nodulation capacity (Madukwe *et al*., 2008). Ramolemane *et al*. (1996) also reported that nodulation and dry matter accumulation by Bambara groundnut increased with phosphorus application. Because of its multiple effects on plant nutrition (not only on nodulation), phosphorus fertilizer is recommended to increase yield (Reamaekers, 2001). Smyth and Cravo (Mokwunye *et*

*al.*1986) working on Xanthic Hapludox near Manaus, Brazil reported that for cowpea critical levels for soil P was 60 kg·P·ha^{-1}.

Nitrogen is an integral component of many compounds, including chlorophyll and enzymes essential for plant growth processes. The corn plant requires N soon after germination to initiate the growth of stems, leaves and ear structures. Inadequate N availability during the first 2 to 6 weeks after planting can result in reduced yield potentials (Jones, 1985). Uptake is relatively slow during the first month (Ikisan. 2010).Nitrogen is important for carbohydrate use within plants and stimulates root growth and development as well as the uptake of other nutrients (Brady and Weil, 2001). Nitrogen deficiency in maize at any stage of the growth especially at tasseling and silking stage will lead to virtual crop failure (Ikisan. 2010).Results showedthat the soils that were treated with N had higher SDW as compared to the soil that was not treated (T0).According to Walker *et al*. (2001), availability of N in the soil directly affects the relative growth rate of plants. Agbogidi *et al*. (2007a) reported that, petroleum products are known to reduce nitrogen availability in the soil.According to Wyszkowski and Ziolkowska (2008) proper growth of cultivated plants is dependent on the content of nutrients in the soil. The low SDW in all the contaminated treatments could be due to the disruption of absorption of nutrients by petroleum products, decrease in soil pH and increasing acidity content of the polluted soil (Njoku *et al.,* 2008). Nutrients are essential to plant growth and development; hence reduction in their bioavailability will lead to a reduction in plant growth.

5.5 Total N and P Uptake of Test Crops

Uptake of N and P in the test plants generally decreased as the level of diesel oil contamination increased. However, at any giving concentration of diesel contamination, the uptake of N and P were generally high in plant grown in soil treated with N and P as

compared to those grown in the control soil (untreated soil).The low shoot N and P in the plants grown in the contaminated soil could be attributed or associated with the poor absorption of nutrients by the roots of the plants in the contaminated soil.Reduction in shoot N and shoot P content could be the result of formation of an oil layer over the roots, which might have disrupted the cell membrane or caused the limited absorption of these nutrients.This observation confirms the findings of Kayode *et al.,* (2008) who reported that the presence of engine oil in the soil holds the soil too compact for the roots of the plants to penetrate and so affected the extent of root growth and nutrient absorption. Due to the insufficient nutrient absorbed by the plants grown in the contaminated soil, it was observed that the shoot N and Shoot P as well as SDM decreased as the concentration of diesel oil increased.

It is generally accepted that available soil nutrients, notably nitrogen are immobilised by soil microorganisms following oil application (Ellis and Adams 1961; Schwendinger 1968; Giddens 1976; McGill 1977). Extractable phosphorus levels may also become depressed (Udo and Fayemi, 1975; Loynachan 1979). The reduced nitrogen and phosphorus availability in the diesel oil contaminated soils could also be the result of the reduced shoot N and P uptake.Another reason for the low shoot P and N content could be that the diesel oil could have created a soil crust, further restricting the infiltration of water and oxygen and other nutrients from the soil to the plant (McGill and Nyborg 1975; Volk 1980).

5.6 Nodule Formation

There were no nodules recorded for cowpea grown in the contaminated soils even addition of phosphorus and nitrogen could not remedy the situation. This finding agrees with a study by John *et al*. (2011) which revealed that symbiotic nitrogen fixing bacteria

associated with legumes in wetlands are very sensitive to crude oil pollution. Non nodulation possibly could be due to the unfavourable conditions provided by the oil for nodulation. The diesel oil might have also affected the survival or multiplication of rhizobia by altering the soil properties and hence decreasing their chances of invasion into the roots and subsequent nodulation process. Contamination of soil by crude oil could lead to a depression of microbial density and activities even in case of relatively light contamination (Odu, 1972). The non-development of root nodules from plants grown in the diesel contaminated soils could be attributed to the possibility of toxicity by the diesel oil contamination.

Results showed that addition of phosphorus however significantly increasd nodule numbers in the uncontaminated. This could be attributed to the fact that phosphorus stimulated root and plant growth, initiated nodule formation as well as influenced the general efficiency of the rhizobium-legume symbiosis. This result confirms the findings of Luse *etal.* (1975), Agboola and Obigbesan (1977), who reported increases in the number of nodules in cowpea due to phosphorus application. This indicates that P is essential for nodule formation in cowpea. Application of N in the uncontaminated soil reduced nodule number.The reduction in nodule number observed in the nitrogen amended treatment was not suprising as it is well known that high soil Nitrogen, particularly mineral Nitrogen, during initial growth retards nodule formation (Ezedinma 1964; Tewari, 1965).

CHAPTER SIX

CONCLUSION AND RECOMENDATION

Biostimulation of indigenous microorganisms to enhance the degradation of diesel in some Ghanaian soils with N and P fertilizer showed promising results. The fertilizer addition significantly stimulated the degradation of diesel oil in all the four soils used in the present study. The study revealed that Beach sand and Nyankpala series, with initial low HUB populations recordedthe highest degradation.These two soils also had HUB populations growth peaks earlier (20 days fter incubation) than the other two soils (Oda and Toje series) whose HUB population growth peaks later (30 days after incubation). These results suggest that (i) the Beach sand and Nyankpala series could contain effective HUB needed for bioremediation of oil-contaminated soils (ii) soils whose HUB populations growth peaks early during bioremediation could be effective degraders.

The study also showed that diesel contamination has adverse impact on nodulaion in cowpea and growth of cowpea and maize. However addition of nitrogenand phosphorus to the contaminatedsoils improved germination, shoot dry matter and nutrient uptake. The effect of nitrogen in the contaminated soils was pronounced in maize while that of phosphorus was pronounced in cowpea. This implies that nutrient elements are important for the growth of crops in oil contaminated soils and nutrient elements are specific to crops grown in oil contaminated soils. Further research work should be conducted to:

I. Isolate and identify effective HUB from Ghanaian soils especially Beach sand
II. Determine the optimum application rate of nitrogen and phosphorus fertilizers required for optimum growth of maize and cowpea in oil contaminated soils.

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APPENDIX

Germination of Cowpea and Maize

Variate: Germ_COWPEA

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trt	11	46.2222	4.2020	15.13	<.001
Residual	24	6.6667	0.2778		
Total	35	52.8889			

Significant at 5%: LSD.= 0.8882

Variate: Germ_MAIZE

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trt	11	29.4167	2.6742	6.88	<.001
Residual	24	9.3333	0.3889		
Total	35	38.7500			

Significant at 5%: LSD.=1.051

SDW of cowpea and maize

Variate: SDW_COWPEA

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trt	11	11.028231	1.002566	153.39	<.001
Residual	24	0.156867	0.006536		
Total	35	11.185097			

Significant at 5%: LSD.= 0.1362

Variate: SDW_MAIZE

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trt	11	3.80130	0.34557	9.77	<.001
Residual	24	0.84880	0.03537		

Total	35	4.65010			

Significant at 5%: LSD.= 0.317

SHOOT N UPTAKE

Variate: N_g_shoot_COWPEA

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trt	11	1.166E-02	1.060E-03	2203.29	<.001
Residual	24	1.154E-05	4.810E-07		
Total	35	1.167E-02			

Significant at 5%: LSD.= 0.001169

Variate: N_g_shoot_MAIZE

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trt	11	1.945E-03	1.768E-04	315.16	<.001
Residual	24	1.347E-05	5.610E-07		
Total	35	1.958E-03			

Significant at 5%: LSD.= 0.001262

Shoot P uptake

Variate: P_g_shoot_COWPEA

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trt	11	8.246E-05	7.496E-06	298.36	<.001
Residual	24	6.030E-07	2.513E-08		
Total	35	8.306E-05			

Significant at 5%:LSD= 0.0002671

Variate: P_g_shoot_MAIZE

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trt	11	1.744E-05	1.585E-06	63.09	<.001

Residual	24	6.030E-07	2.512E-08
Total	35	1.804E-05	

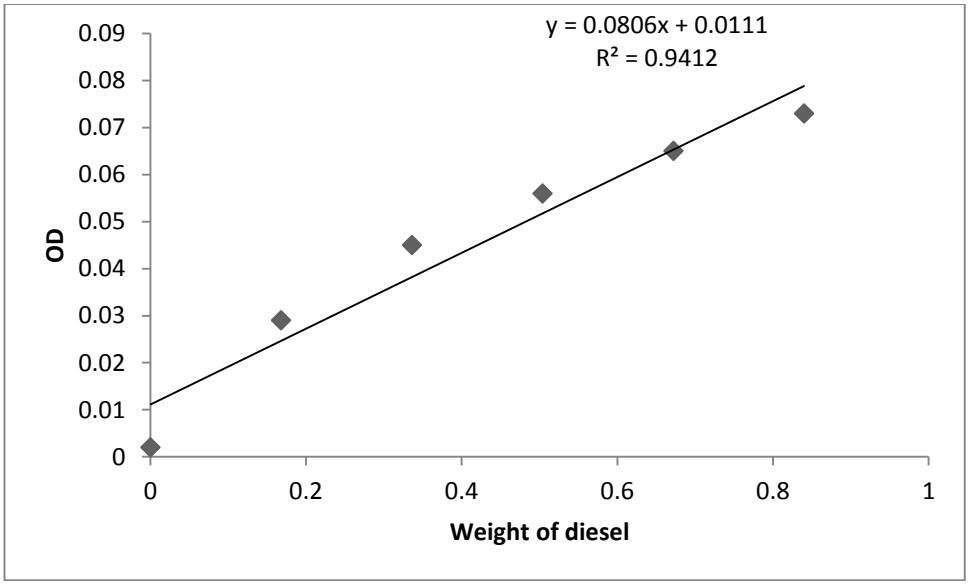
Significant at 5%: LSD = 0.0002671

Nodule Count

Variate: Nodule_no

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trt	2	56.000	28.000	10.50	0.011
Residual	6	16.000	2.667		
Total	8	72.000			

Significant at 5%: l.s.d. = 3.263



Standard curve for determining the amount of diesel oil degraded.