ADEBAYO JONATHAN ADEYEMO

BIOREMEDIATION OF BRAZILIAN SOILS CONTAMINATED WITH USED LUBRICATING OIL

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Solos e Nutrição de Plantas, para obtenção do título de *Doctor Scientiae*.

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Maurício Dutra Costa

Ecila Mercês de Albuquerque Villani

Ivo Ribeiro da Silva
(Coorientador)

Sérgio Antonio Fernandes
(Coorientador)

Walter Antônio Pereira Abrahão
(Presidente da Banca)

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"O lord that lends me life, lend me a heart that is replete with thankfulness"

William Shakespeare, Henry VI, Poet II

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BIOGRAPHY

ADEBAYO JONATHAN ADEYEMO, son of John Ademolaju Adeyemo and Eunice Ayodeji Adeyemo was born on 11th of July, 1979, in Aramoko Ekiti, Ekiti State, Nigeria. He obtained a bachelor of Agricultural Technology in Crop, Soil and Pest Management with Second Class Honours (Upper Division) from the Federal University of Technology, Akure, Ondo State, Nigeria on the 6th of December, 2003. He participated in the compulsory National Youth Service Corps (NYSC) after his first Degree between February 2004 and February 2005 as Corp Liaison Officer (CLO) in Okobo, Local Government Area of Akwa Ibom, one of the Oil Producing States of Nigeria, a scheme organized by the Federal Government of Nigeria with a view to the proper encouragement and development of common ties among the youths of Nigeria and the promotion of national unity. His main responsibility was to liaise with the State Governments Coordinator of the scheme in the implementation of developmental projects in the Local Government Area. He started his master's program in the Department of Crop Soil and Pest Management (Soil Management option), in 2006, and was later employed in the same Department during his master´s program as a Teaching Assistant by the University. He obtained his Master´s (M. Agric. Tech) Degree in Agricultural Technology on the 4th of July, 2008. He started his Doctoral program (DSc.) under the fellowship program sponsored by the Third World Academy of Sciences and the National Council for Scientific and Technological Development (TWAS/CNPq) in the Department of Soils, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil, in March 2009. He finished his doctoral program in February 2013.

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ABSTRACT

ADEYEMO, Adebayo Jonathan, D.Sc., Universidade Federal de Viçosa, February 2013. **Bioremediation of brazilian soils contaminated with used lubricating oil.** Adviser: Jaime Wilson Vargas de Mello. Co-advisors: Ivo Ribeiro da Silva and Sergio Antonio Fernandes.

Oil is the most widely distributed source of energy in the world and the largest-scaled environmental pollutant. Oil, oil products, and oil-containing industrial wastes pollution is ranked second place after radioactive pollution on account of their harmful action to ecosystems. Contamination of soil by organic chemicals (mostly hydrocarbons) is prevalent in oil producing and industrialized countries of the world. This may pose a great threat to the environment and human being at large. Different treatment methods have been employed to remediate contaminated soils. Bioremediation, a strategy that uses biological means to degrade, stabilize, and/or remove soil contaminants can be an alternative green technology for remediation of hydrocarbon-contaminated soil. Bioremediation provides an economically beneficial and highly-specific clean-up technology to remove pollutants by targeting individual compounds or their mixtures. The planning of bioremediation strategies for polluted land and water areas should consider the use of the existing indigenous oil oxidizing microorganisms with different affinity to oil fractions and their activation by addition of fertilizers (nitrogen, phosphorus, and potassium). The inoculation of effective microorganisms such as amnite p1300, special bacteria strains consisting a mixture of *Bacillus subtilis, Bacillus megaterium, Pseudomonas putida, Pseudomonas fluorescens, Phanerochaete chrysosporium, Rhodococcus rhodocrous* on a cereal (bran) among others possess significant pollutant degradation potential. It has been shown that these bacteria can use recalcitrant pollutant as energy source. The remediation of polluted soils requires the study of the microorganisms' diversity in the environment and the determination of the ability of different microbes and their consortia to degrade pollutants in the presence of high oil pollution concentration. This is important because it has been observed that the introduction of a single oil-oxidizing strain into the oil-spilled environment does not assure a complete clean-up. This investigation consists of studies with the primary aim of studying the efficacy of commercial bacterial strains for the biodegradation of used lubricating oil in soil. The rates of biodegradation of the used lubricating oil were studied in the laboratory. The model soil

sample of 300 g was contaminated with 1.5, 3.0 and 4.5 % (w/w) of used motor oil at room temperature under laboratory conditions using microcosm of 1 L. The microcosm was used to simulate the comparative effect of spent oil addition and bioremediation using a commercially available hydrocarbon degrading microbial consortium (Amnite P1300) as the bioaugmentation treatment, to enhance biodegradation of hydrocarbons in soils contaminated with different concentrations of used lubricating oil, nutrients amendments ($(NH_4)_2SO_4$ and K_2HPO_4) as biostimulation, unammended soil (natural attenuation) and the control soil treated with sodium azide (NaN_3) on the microbial community in different soil types. Hydrocarbon-utilizing bacterial counts were high in the amended soils with commercial bacterial, nutrients amendments and the natural attenuation (soil alone) compared to control soil treated with sodium azide throughout the period of the study. Oil-contaminated soil amended with Amnite P1300 showed the highest reduction in total petroleum hydrocarbon in all the contamination levels compared to other treatments in all the soil types. The evaluation of soil biological activities as a monitoring tool for the degradation process of the model soils was made via measurements of hydrocarbons degrading bacterial counts (HDB), soil respiration (CO_2), pH and percentage oil degradation. The correlations between these parameters and the levels of the hydrocarbon residues were determined. The residual hydrocarbon content correlated negatively with hydrocarbon utilizers, CO_2 evolution, pH and percentage (%) oil degradation ($P < 0.01$) in the clayey and sandy soils. Complete biodegradation of C_{10} to C_{14} and substantial degradation of C_{15} to C_{28} hydrocarbon fractions and PAHs were recorded in Amnite P1300 amended soils throughout the period of study, thus suggesting the high potential of the product in enhancing biodegradation of hydrocarbons. The results revealed remediation potential of Amnite p1300 to degrade heavy hydrocarbons in used lubricating oil. It is concluded that contamination of soil with oil hydrocarbons has a negative effect on soil ecosystems, and the extent and duration of these effects will depend on the nature of the hydrocarbon contaminant and the soil characteristics. Thus, inhibitory effect of used lubricating oil is more pronounced in sandy soil than clayey soil and show more toxic effect on seed germination and root growth.

RESUMO

ADEYEMO, Adebayo Jonathan, D.Sc., Universidade Federal de Viçosa, fevereiro de 2013. **Biorremediação de solos brasileiros contaminado com óleo lubrificante usado.** Adviser: Jaime Wilson Vargas de Mello. Co-advisors: Ivo Ribeiro da Silva e Sergio Antonio Fernandes.

Petróleo é o recurso energético mais amplamente distribuído no mundo e o mais importante poluente ambiental. Petróleos, derivados de petróleo e os resíduos industriais que contêm petróleo, ocupam o segundo lugar entre os poluentes, perdendo apenas para a poluição radioativa no que se refere a danos causados aos ecossistemas. A contaminação dos solos por compostos orgânicos, principalmente hidrocarbonetos, é comum entre os países que produzem e industrializam petróleo, representando grande ameaça ao homem e ao meio ambiente em geral. Diferentes métodos de tratamento têm sido empregados para eliminar a contaminação dos solos. A biorremediação é uma estratégia que usa meios biológicos para degradar, estabilizar e/ou remover contaminantes do solo, sendo tecnologia ´´verde´´ alternativa na remediação de solos contaminados com hidrocarbonetos. A biorremediação fornece benefícios econômicos e de alta tecnologia para remover concentrações de poluentes, sejam eles individuais ou misturas. O planejamento de estratégias de biorremediação de ambiente terrestres e aquáticos contaminados tem por base a existência de microrganismos naturais que degradam petróleo com diferentes afinidades pelas frações, fertilizantes (nitrogênio, fósforo e potássio). Amnite p1300, o qual consiste de mistura de bacterianas de *Bacillus subtilis, Bacillus megaterium, Pseudomonas putida, Pseudomonas fluorescens, Phanerochaete chrysosporium, Rhodococcus rhodocrous* em cereais (farelo), entre outros possui significativo potencial de degradação de poluentes. Tem sido demonstrado que essas bactérias podem usar poluentes recalcitrantes como fonte de energia. A remediação de poluentes dos solos requer o estudo da diversidade desses microrganismos no ambiente e a determinação da capacidade de diferentes microorganismos e seus diferentes agrupamentos para degradar poluentes na presença de elevadas concentrações de poluição por petróleo. Isto é importante porque tem sido observado que a introdução de uma simples cerpa oxidante de petróleo em ambiente contaminado não assegura limpeza por completo. Este trabalho teve como objetivo principal investigar a eficiência de um produto comercial na biodegradação de óleos lubrificantes usados presentes no solo. A taxa de biodegradação de resíduos de óleo

lubrificante foi estudada em laboratório. Uma amostra de solo modelo de 300 g foi contaminado com 1,5, 3,0 e 4,5 % (w/w) de óleo de motor usado à temperatura ambiente em condições laboratoriais usando microcosmo de 1L. O microcosmo foi usado para simular o efeito comparativo da adição de óleo usado e a bioremediação usando um coquetel microbiano disponível comercialmente que degrada hidrocarbonetos (Amnite p1300) como o tratamento de crescimento microbiano, para elevar a biodegradação de hidrocarbonetos em solos contaminados com diferentes concentrações, adição de nutrientes $(NH_4)_2SO_4$ e K_2HPO_4) como tratamento de bioestimulação, atenuação natural e o solo controle tratado com azida de sódio (NaN_3) sobre a comunidade microbiana em diferentes tipos de solos. A contagem de bactérias que utilizam hidrocarboneto foi elevada nos solos tratados com a mistura de bactérias comercial, com adição de nutrientes e na atenuação natural (apenas solo) comparado com o solo controle tratado com azida de sódio durante o período do estudo. Os solos contaminados com petróleo e adicionadas de Aminte P1300 mostraram elevada redução no total de hidrocarbonetos de petróleo em todos os níveis de contaminação quando comparado com outros tratamentos em todos os tipos de solos. A avaliação da atividade biológica do solo como instrumento para monitorar o processo de degradação dos solos modelos foi feita através da contagem das bactérias degradadoras de hidrocarbonetos (HDB), atividades biológica como (CO_2), pH e porcentagem de degradação do óleo. A correlação entre estes parâmetros e o nível de resíduos de hidrocarbonetos foi determinada. O conteúdo hidrocarboneto resíduos correlacionou se negativamente com hidrocarbonetos utilizados, evolução de CO_2, pH e porcentagem de degradação de óleo ($P < 0,01$). nos solos argilosos e arenosos. A completa biodegradação de C_{10}-C_{14} e a degradação substancial de C_{15}-C_{28} das frações de hidrocarbonetos e PAHs foi registrada com o tratamento de adição de Amnite P1300 nos solos alterados durante o período do estudo. Sugerindo assim elevado potencial do produto em aumentar a biodegradação de hidrocarbonetos. Os resultados revelaram o potencial de remediação de Amnite p1300 na degradação de hidrocarbonetos de cadeia longa, como os óleos lubrificantes usados. Concluímos que a contaminação de solos com hidrocarbonetos tem efeito negativo no solo, e a extensão e duração desses efeitos vai depender da natureza dos hidrocarbonetos contaminantes e das características do solo. Entretanto, o efeito inibitório dos óleos lubrificantes usado é mais pronunciado em solos arenosos do que em solos argilosos e tem efeito tóxico na germinação de sementes e crescimento de raízes.

1. INTRODUCTION

1.1. Background.

Petroleum and its refined products (gasoline, diesel, kerosene, engine oil, jet fuel, etc.) are major resources used for energy requirements in industrial and transportation sectors throughout the world. The lubricating oils represent approximately 2 % of the derivatives of petroleum oil and are used for automotive and industrial purposes. According to their applications, they receive additives (organic and / or inorganic) in their formulation in order to meet the requirements of the different intended applications (ANP, 1999; CEMPRE, 2007). Motor oil is used for lubrication requirements of various kinds of automotive and other engines. During these types of uses, motor oil picks up a number of additional components from engine wear. These include heavy metals, such as lead, chromium, cadmium, and other materials like naphthalene, chlorinated hydrocarbons, sulphur. After the passage of time, oil changes become necessary due to alteration in the viscosity of the oil. Any such oil that becomes unsuitable after use due to contamination, making it unfit for its original purpose, is known as used motor oil (U.M.O) and is required to be suitably disposed of. Thus, any disposal of used oil in soils, surface water and groundwater, sewer systems and waste water disposal or any form of elimination that causes air pollution exceeding the level set by the environmental protection legislation is forbidden (ABNT, 1987; CETESB, 2003; CONAMA, 2005).

The base oil contains C16-C36 hydrocarbons, and more than 75% c-alkanes. The rings number of c-alkanes in the base oil is from 1 to 3 and any ring contains 5 or 6 members. Most of the c-alkanes in the base oil have long alkyl side chains (Koma *et al.*, 2003). The most important characteristic of the lubricating oil for automotive use is its viscosity. Fresh motor oil contains a higher percentage of volatile and water soluble hydrocarbons that would be a concern for acute toxicity to organisms (Boonchan et al., 2000). Used motor oil contains metals and heavy polycyclic aromatic hydrocarbons (PAHs) that could contribute to chronic hazards including mutagenicity and carcinogenicity (Hagwell et al., 1992; Boonchan et al., 2000). As the usage of petroleum hydrocarbon products increased, soil contamination with diesel and engine oils is becoming one of the major environmental problems (Mandri and Lin, 2007). Prolonged exposure to high oil concentration may cause the development of liver or

kidney disease, possible damage to the bone marrow and an increased risk of cancer (Mishra et al., 2001; Propst et al., 1999, Lloyd and Cackette, 2001). In addition, PAHs have a widespread occurrence in various ecosystems that contribute to the persistence of these compounds in the environment (Van Hamme et al., 2003). The illegal dumping of used motor oil is an environmental hazard with global ramifications (Blodgett, 2001). Used oils have the potential to be recycled and re-refined if safely and properly collected, yet in many cases it is poured into an open drain or thrown into the trash where it can contaminate the subsurface soil and ground water. In Africa and some developing countries about 20 million gallons of waste engine oil are generated annually from mechanic workshops and discharged carelessly into the environment (Faboya, 1997; Adegoroye, 1997). Out of which only one liter is enough to contaminate one million gallons of freshwater and create a film that prevents aeration in approximately one thousand square meters of a water body, causing serious damage to fauna and aquatic flora. (USEPA, 1996, Viveiros, 2000; Magalhães et al., 2007). Apart from this, used engine oil renders the environment unsightly and constitutes a potential threat to humans, animals and vegetation (ATSDR, 1997; Edewor et al., 2004; Adelowo et al., 2006). A single automotive oil change is estimated to produce 4–5 L of used oil. The bulk of U.M.O generated in India (about 0.4 million tons (1 ton = 0.907 t) annually) generally goes into undesirable applications and only a very small amount (10 tons annually) is currently re-refined (IIP 1997). A large number of roadside garages drain used oils from automobile engines and there is no record of the next destination of such oils. Major oil-contaminated spots reported are Meethi River at Kurla (Mumbai) and in the Mundka and Rohtak road area near New Delhi (India) (IIP 1997). In the United States, about 500 million gallons of used oil are being disposed indiscriminately every year (Anoliefo and Vwioko 1995: Adesodun and Mbagwu, 2008). Likewise, about 0.1 million tons of U.M.O is reported to be wasted annually in Australia (Kemp 2004).

In Brazil, the production of lubricating oils is approximately one billion liters/year (Conselho em Revista 2005; Magalhães et al., 2007). Of this amount, about 650 million liters/year are consumed by vehicles or other uses, in other words, they are not recoverable. Of the remaining 350 million liters/year used, which are not consumed completely in the

process, 69.2 % is recycled via re- refining, or about 240 million liters /year. Data from 2004 show that in that year approximately 240 million liters were collected i.e., around 24.0 % of the lubricating oil marketed in the country. The volume of used oil collected in 2004, enabled the processing of approximately 170 million liters of re-refined base oil (Magalhães et al. 2007; CEMPRE, 2007). This waste-oil usually contains appreciable amounts of toxic hydrocarbons and heavy metals such as V, Pb, Al, Ni, Fe, Cr and Zn (Whisman et al., 1974).

Table 1. Panorama on consumption and target volumes of oils (million litres / year) of some Latin American countries.

	NO	OU	UOR	RRP	RUP	UOR/NO (%)	RRP/UOR (%)
Argentina	238	119	130	50	80	55	38
Brazil	900	510	390	270	120	43	69
Colombia	150	60	90	56	34	60	62
Equador	56	22	34	14	20	61	41
Mexico	880	570	310	102	208	35	33

Source: Azevedo, 2002

NO - New Oil, OU - oil consumed by use; UOR – used oil residue ; RRP - oil recovered through recycling process ; RUP - oil recovered through unknown process ; % UOR / NO - percentage ratio between the volume of waste oil used to the volume of new oil ; % RRP / UOR -- the ratio between volume of oil recovered by the recycling process on the volume of used oil residue.

Table 1 shows an overview of the volumes in millions liter / year of new oil (NO), oil consumed by use (OU), used oil residue (UOR), oil recovered through recycling process (RRP), oil recovered through unknown process (RUP) , the percentage ratio between the volume of waste oil used on the volume of new oil (% UOR / NO), and the ratio between the amount of oil recovered by the process of Recycling on the volume of

used oil residue (%RRP / UOR) for five Latin American countries , in year 2001 (Azevedo, 2002; Magalhães et al. 2007). It is observed in table 1 above that Brazil has the highest rate of percentage ratio of the volume of oil recovered through recycling process to the volume of used oil residue, RRP/UOR = 69 % . This result shows that among Latin American countries cited in 2001, Brazil had the largest volume of recycled waste oil, but still, the volume of 120 million litres/year presented is considerably high in waste oil recovered through unknown process (RUP). Probably the RUP is associated with improper burning of these residues.

1.2. Chemistry of petroleum hydrocarbon.

Petroleum is defined as any mixture of natural gas, condensate, and crude oil. Crude oil which is a heterogeneous liquid consisting of hydrocarbons comprised almost entirely of the elements hydrogen and carbon in the ratio of about 2 hydrogen atoms to 1 carbon atom (Okoh, 2006). It also contains elements such as N, S, and O, all of which constitute less than 3% (v/v). There are also trace constituents, comprising less than 1% (v/v), including P and heavy metals such as V and Ni. Crude oils could be classified according to their respective distillation residues as paraffins, naphthenes or aromatics and based on the relative proportions of the heavy molecular weight constituents as light, medium or heavy. Also, the composition of crudes may vary with the location and age of an oil field, and may even be depth dependent within an individual well. About 85% of the components of all types of crude oil can be classified as either asphalt base, paraffin base or mixed base. Asphalt base contain little paraffin wax and an asphaltic residue (Atlas, 1981). The S, O and N contents are often relatively higher in comparison with paraffin base crudes, which contain little or no asphaltic materials. Mixed crude oil contains considerable amount of oxides of N and asphalt.

Petroleum oil biodegradation by bacteria can occur under both oxic and anoxic conditions (Zengler et al., 1999), albeit by the action of different consortia of organisms. In the subsurface, oil biodegradation occurs primarily under anoxic conditions, mediated by sulfate reducing bacteria (Holba et al., 1996) or other anaerobes using a variety of other electron acceptors as the oxidant. On a structural basis, the hydrocarbons in crude oil are classified as alkanes (*normal* or *iso*), cycloalkanes, and aromatics. Alkenes, which are the unsaturated analogs of alkanes, are rare in crude oil but occur in many refined petroleum

products as a consequence of the cracking process. Increasing carbon numbers of alkanes (homology), variations in carbon chain branching (*iso*-alkanes), ring condensations, and interclass combinations e.g., phenylalkanes, account for the high numbers of hydrocarbons that occur in crude oil. In addition, smaller amounts of O – (phenols, naphthenic acids), N - (pyridine, pyrrole, indole), and S - (alkylthiol, thiophene) containing compounds, collectively designated as "resins" and partially oxygenated, highly condensed asphaltic fraction occur also in crude but not in refined petroleum (Atlas and Bartha, 1973).

The inherent biodegradability of these individual components is a reflection of their chemical structure, but is also strongly influenced by the physical state and toxicity of the compounds. As an example, while *n*alkanes as a structural group are the most biodegradable petroleum hydrocarbons, the $C_5 – C_{10}$ homologues have been shown to be inhibitory to the majority of hydrocarbon degraders. As solvents, these homologues tend to disrupt lipid membrane structures of microorganisms. Similarly, alkanes in the $C_{20} – C_{40}$ range, often referred to as "waxes", are hydrophobic solids at physiological temperatures. Apparently, it is this physical state that strongly influences their biodegradation (Bartha and Atlas, 1977). Primary attack on intact hydrocarbons always requires the action of oxygenases and, therefore, requires the presence of free O. In the case of alkanes, monooxygenase attack results in the production of alcohol. Most microorganisms attack alkanes terminally whereas some perform sub-terminal oxidation (Figure 1). The alcohol product is oxidised into an aldehyde and finally, to a fatty acid. The latter is degraded further by *beta*-oxidation. Extensive methyl branching interferes with the beta oxidation process and necessitates di terminal attack (Figure 2) or other bypass mechanisms. Therefore, *n*alkanes are degraded more readily than iso alkanes.

Cycloalkanes are transformed by a not fully characterized oxidase system to a corresponding cyclic alcohol, which is dehydrated to ketone. Then, a monooxygenase system lactonises the ring, which is subsequently opened by a lactone hydrolase. These two oxygenase systems usually never occur in the same organisms and hence, the frustrated attempts to isolate pure cultures that grow on cycloalkanes (Bartha 1986b). However, synergistic actions of microbial communities are capable of dealing with degradation of various cycloalkanes quite effectively. As in the case of alkanes, the monocyclic compounds,

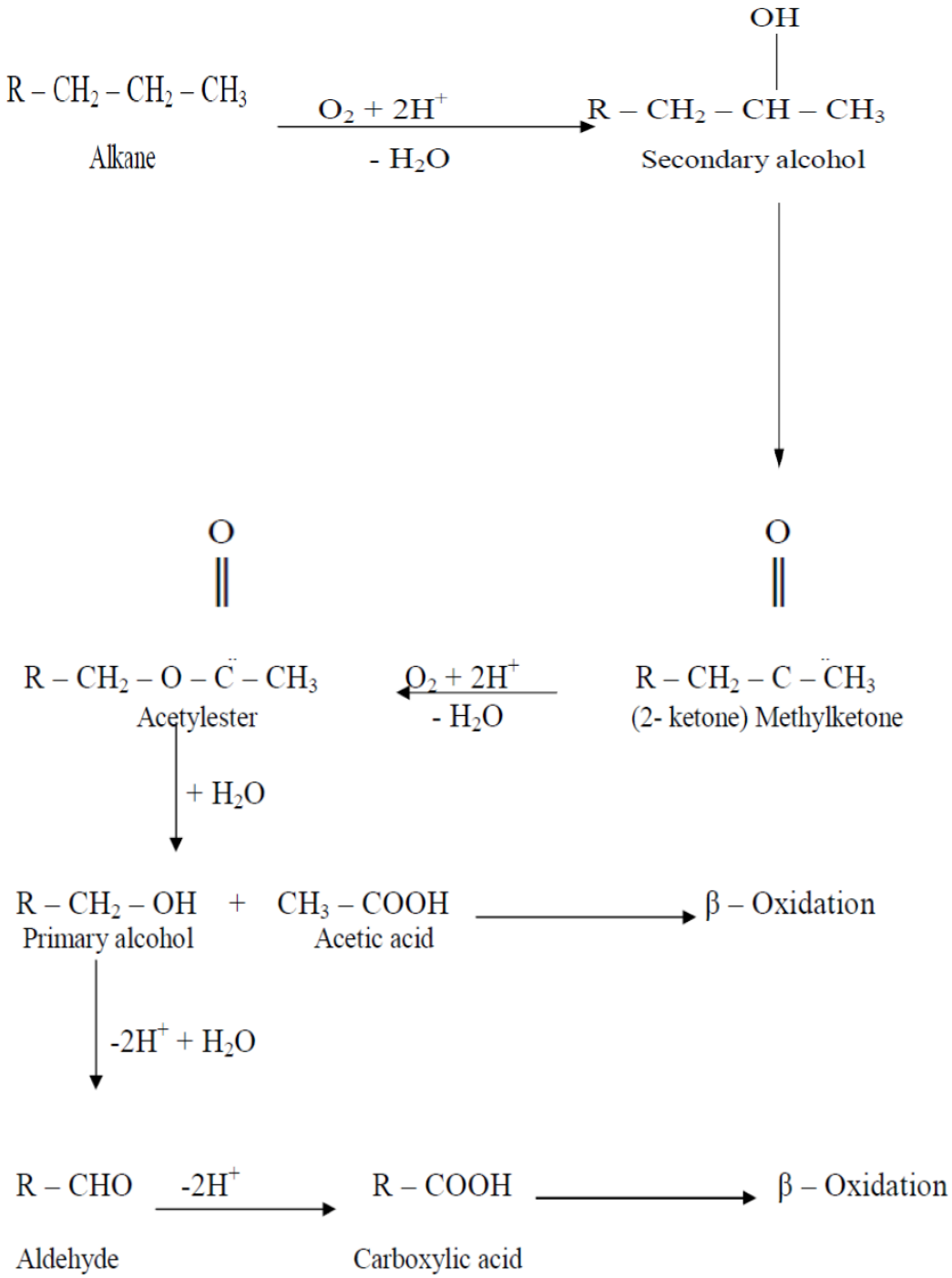

Fig. 1: Pathways, through which sub terminal oxidation of alkanes yield two fatty acid moieties, which are metabolized further by beta-oxidation (Atlas and Bartha, 1998).

cyclopentane, cyclohexane, and cycloheptane have a strong solvent effect on lipid membranes, and are toxic to the majority of hydrocarbon degrading microorganisms. Highly condensed cycloalkane compounds resist biodegradation due to their structure and physical state (Bartha, 1986a).

Prokaryotes convert aromatic hydrocarbons by an initial dioxygenase attack, to *trans*dihydrodiols that are further oxidised to dihydroxy products, e.g., catechol in the case of benzene (Atlas and Bartha, 1998). Eucaryotic microorganisms use monooxygenases, producing benzene 1, 2-oxide from benzene, followed by the addition of water, yielding dihydroxydihydrobenzene (*cis*-dihydrodiol). This is oxidised in turn to catechol, a key intermediate in biodegradation of aromatics, which is then opened by *ortho*- or *meta*cleavage, yielding muconic acid or 2- hydroxymuconic semialdehyde, respectively.

Condensed polycyclic aromatics are degraded, one ring at a time, by a similar mechanism, but biodegradability tend to decline with the increasing number of rings and degree of condensation (Atlas and Bartha, 1998). Aromatics with more than four condensed rings are generally not suitable as substrates for microbial growth, though, they may undergo metabolic transformations. Biodegradation process also declines with the increasing number of alkyl substituent on the aromatic nucleus. Asphaltic tend to increase during biodegradation in relative and sometimes absolute amounts. This would suggest that they not only tend to resist biodegradation but may also be formed from beginning by condensation reactions of biodegradation and photodegradation intermediates. In crude petroleum as well as in refined products, petroleum hydrocarbons occur in complex mixtures and influence each other's biodegradation. The effects may go in negative as well as positive directions. Some *iso*alkanes are apparently spared as long as *n*alkanes are available as substrates, while some condensed aromatics are metabolized only in the presence of more easily utilizable petroleum hydrocarbons, a process referred to as co-metabolism (Wackett, 1996).

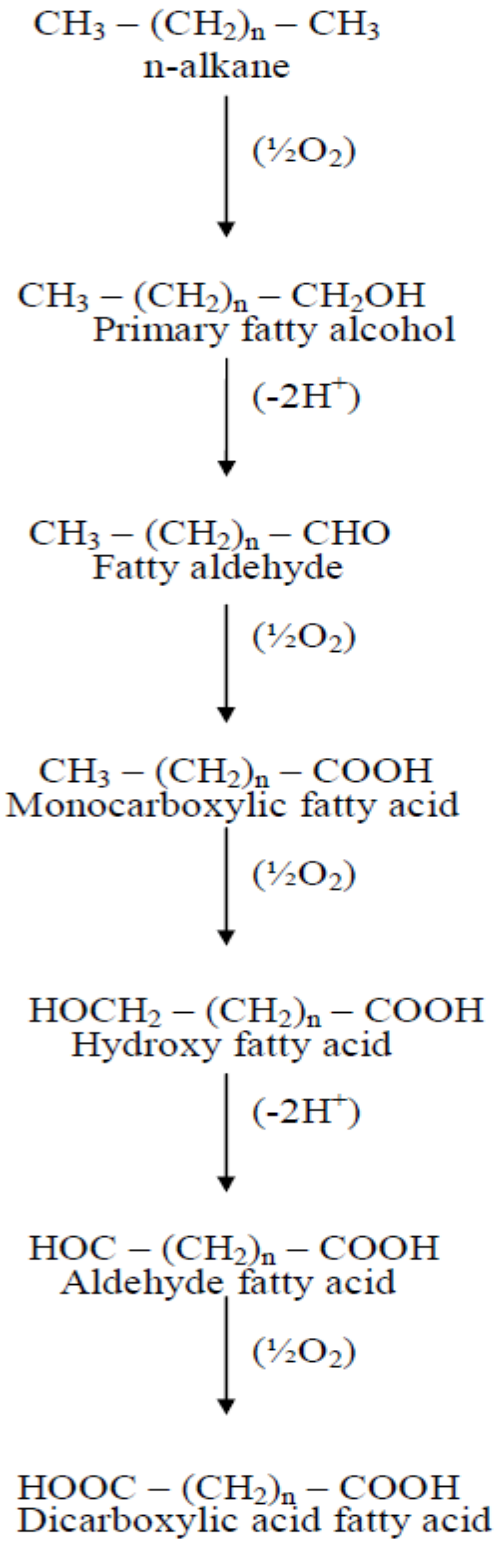

Fig. 2: Pathway of diterminal alkane oxidation (Atlas, 1984)

1.3. Effects of used lubricating oil on human health.

Mineral-based crankcase oils are manufactured using highly refined base oils and contain up to 20% of a variety of additives such as viscosity index improvers, detergents/dispersants, antiwear additives, pour-point depressants, and antioxidants (IARC 1984; Kirk-Othmer 1981). During use, the high temperatures and friction cause changes such as oxidation, nitration, and cracking of polymers in the component chemicals (Vazquez-Duhalt 1989). In addition, a variety of substances such as fuel, water, antifreeze, dust, and various combustion products such as polycyclic aromatic hydrocarbons (PAHs), metals, and metallic oxides accumulate in the oil. The degree of chemical change and accumulation of contaminants in the oil increases with use and varies depending on the type of fuel used and the mechanical properties of the engine. In an engine lubricating system, the required quantity of lubricant is transported where it is needed in the engine. The lubricant protects against wear, reduces friction, cleans the engine of dirt and residue (detergent), protects against corrosion, cools the engine, and seals the pistons (Van Donkelaar 1990). Additives are added to lubricating oils to improve its physical and chemical properties. Consequently, lubricating oils have high additive contents (up to 20%), especially detergents and dispersants which constitute 2-15% of oil weight (Vasquez-Duhalt 1989). However, several of the oil additives are toxic environmental contaminants, e.g., zinc dithiophosphate and zinc diary1 or dialkyl dithiophosphates (ZDTPs); calcium alkyl phenates; magnesium, sodium, and calcium sulphonates; tricresyl phosphates; molybdenum disulfide; heavy metal soaps; and other organometallic compounds that contain heavy metals. Hence, very high levels of zinc and cadmium are found in new motor oil-approximately 1,500 μg/g of zinc and 87 μg/kg of cadmium (Hewstone 1994a; Vasquez-Duhalt 1989). Although ZDTPs have a low acute systemic toxicity, they can cause eye damage and skin irritation (Hewstone 1994a). Prolonged exposure to high concentrations of ZDTPs, calcium alkyl phenates, and magnesium, sodium, and calcium sulphonates had significant effects on the reproductive organs of male rabbits (testicular atrophy and reduction or absence of spermatozoa) which appeared to be species specific. The absorption of tricresyl phosphates caused peripheral nervous system damage, leading to neuromuscular problems (Hewstone 1994a).

In a crankcase-lubricated engine, the oil compartment acts as a sink for heavy molecular incomplete combustion products such as PAHs and their analogs (Scheepers and

Bos 1992). Thus, contaminants such as PAHs, which are formed via combustion, can accumulate in the oil by a factor of up to 1,000. PAHs are known to be highly toxic environmental contaminants with carcinogenic and mutagenic properties. They leave the engine in various ways, such as via particulates, oil leaks, and uncontrolled oil changes, which then accumulates in the environment. One hundred and forty different PAHs have been found in the used oil of crankcase-lubricated engines. These PAHs are also present in much lower quantities in new or fresh oil (Van Donkelaar 1990). Used mineral-based crankcase oil is a complex mixture of metals and PAHs. When motor oils undergo thermal decomposition, gasoline combustion products are formed, significantly increasing the levels of PAHs which contribute to the carcinogenic and mutagenic properties of the oils (Bingham 1988; Ingram et al. 1994). Hence, it is difficult to define the precise composition of used mineral based crankcase oil because of the variety of chemical additives that may be present and the varying degrees of chemical decomposition and contaminant accumulation.

Aromatics hydrocarbons are considered to be the most acutely toxic component of petroleum products, and are also associated with chronic and carcinogenic effects (Anderson et al., 1974). Aromatics are often distinguished by the number of rings they possess, which may range from one to five (Anderson et al., 1974). Lighter, monoaromatics (one ring) compounds include benzene, toluene, ethylbenzene, and xylenes (BTEX). Aromatics with two or more rings are referred to as polyaromatic hydrocarbons (PAHs) (Anderson et al., 1974). Used lubricating oil contains several toxic components including up to 30% aromatic hydrocarbons, with as much as 22 ppm benzo(a)pyrene (a PAH). Upshall et al., (1993) reported that motor oil had a density of 0.828 g/ml and contained 14% aromatics and 65.4% aliphatic (by weight). In their study, the sum of 26 individual PAHs represented 0.17% of the oil, or 1.2% of the aromatic fraction. Chronic effects of naphthalene, a constituent in used motor oil, include changes in the liver and harmful effects on the kidneys, heart, lungs, and nervous system. Due to their relative persistence and potential for various chronic effects (like carcinogenicity), PAHs (and particularly the alkyl PAHs) can contribute to long term (chronic) hazards of jet fuels in contaminated soils, sediments, and groundwater.

1.4. Bioremediation strategies.

The term bioremediation describes the process of contaminant degradation in the environment by biological methods using the metabolic potential of microorganisms to degrade a wide variety of organic compounds (Scragg, 2005). The main advantage of bioremediation is its reduced cost compared to conventional techniques. Besides cost effectiveness, it is a permanent solution, which may lead to complete mineralization of the pollutant. Furthermore, it is a non-invasive technique, leaving the ecosystem intact (Perelo, 2010). Bioremediation can deal with lower concentration of contaminants where the cleanup by physical or chemical methods would not be feasible. The goal of bioremediation is to degrade organic pollutants to concentrations that are undetectable, or if detectable, to concentrations below the limits established as safe or acceptable by regulatory agencies. Bioremediation has been used for the degradation of chemicals in soils, groundwater, wastewater, sludge, industrial wastewater systems, and gases (Okoh and Trejo-Hernandez, 2006). For bioremediation to be effective, microorganisms must enzymatically attack the pollutants and convert them to harmless products. As bioremediation can be effective only where environmental conditions permit microbial growth and activity, its application often involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate (Vidali, 2001). Potential advantages of bioremediation compared to other treatment methods include destruction rather than transfer of the contaminant to another medium; minimal exposure of the on-site workers to the contaminant; longtime protection of public health; and possible reduction in the duration of the remedial process (Okoh and Trejo- Hernandez, 2006). These advantages of the bioremediation systems over the other technologies have been summarized (Leavin and Gealt, 1993) as follows: can be done on site i.e. *in situ* application; keeps site destruction to a minimum; eliminates transportation costs and liabilities; eliminates long-term liability; biological systems are involved, hence often less expensive; and can be coupled with other treatment techniques to form a treatment train. There are three classifications of bioremediation according to Hornung, (1997): *Biotransformation* - the alteration of contaminant molecules into less or nonhazardous molecules *Biodegradation* - the breakdown of organic substances in smaller organic or inorganic molecules *Mineralization* - is the complete biodegradation of organic materials into inorganic constituents such as CO_2 or H_2O. There are three general approaches

to cleaning up contaminated soils:(i) Soil can be excavated from the ground and be either treated or disposed of (Ex-situ treatment), (ii) Soil can be left in the ground and treated in place (in-situ treatment), or (iii) soil can be left in the ground and contained to prevent the contamination from becoming more widespread and reaching plants, animals, or humans (containment and intrinsic remediation), (Jim et al., 2005).

1.4.1. In-situ bioremediation technologies.

In situ bioremediation (ISB) is the use of microorganisms to degrade contaminants in place with the goal of obtaining harmless chemicals as end products (Jim et al., 2005). ISB has the potential to provide advantages such as complete destruction of the contaminant(s), lower risk to site workers, and lower equipment/operating costs (US EPA, 1997). This technology is highly dependent upon external conditions, which is the key to determining whether bioremediation can be performed in situ. The conditions of greatest importance are the physicochemical and chemical conditions that exist in the contaminated soil. These conditions includes dissolved oxygen for aerobic processes, moisture content, pH, nutrient availability especially with regard to nitrogen and phosphorus, temperature, soil composition and concentration of contaminants (Jim et al., 2005). These techniques are generally the most desirable options due to lower cost and fewer disturbances since they provide the treatment in place by avoiding excavation and transport of contaminants. *In situ* treatment is limited by the depth of the soil that can be effectively treated. In many soils effective oxygen diffusion for desirable rates of bioremediation extend to a range of only a few centimeters to about 30 cm into the soil, although depths of 60 cm and greater have been effectively treated in some cases (Vidali, 2001). Accelerated *in situ* bioremediation is where substrate or nutrients are added to an aquifer to stimulate the growth of a target consortium of bacteria. Usually the target bacteria are indigenous; however enriched cultures of bacteria (from other sites) that are highly efficient at degrading a particular contaminant can be introduced into the aquifer (bioaugmentation). Accelerated ISB is used where it is desired to increase the rate of contaminant biotransformation, which may be limited by lack of required nutrients, electron donor or electron acceptor. The type of amendment required depends on the target metabolism for the contaminant of interest. Aerobic ISB may only require the addition of oxygen, while anaerobic ISB often requires the addition of both an electron donor (e.g.,

lactate, benzoate) as well as an electron acceptor (e.g., nitrate, sulfate). Chlorinated solvents, in particular, often require the addition of a carbon substrate to stimulate reductive dechlorination. The goal of accelerated ISB is to increase the biomass throughout the contaminated volume of aquifer, thereby achieving effective biodegradation of dissolved and sorbed contaminant (Wiedemeier et al., 1998). Accelerated in situ bioremediation can be carried out in two ways: biostimulation and bioaugmentation.

1.5. Factors affecting biodegradation of petroleum hydrocarbons.

The fate of a contaminant in the environment is determined by characteristics of the pollutants, the microorganisms, and the environment (Pfaender, Shimp, Palumbo, and Bartholomew, 1985; Madsen, 1991; Tiedje, 1993). The concentration, distribution, function, and structure of the contaminants; the physiology and genetics of the indigenous or introduced microorganisms; and the various soil factors, all result in complex interactions in the subsurface. If one of these critical components is suboptimal for conversion of organic contaminants, biodegradation will be slow or may not take place (Turco and Sadowsky, 1995). Microbial degradation of hydrocarbons in the environment largely depends on the number of biotic and abiotic factors. Some of these factors which will be discussed in detail in this section include: (1) Chemical and physical factor, (2) Biological factor and (3) Soil and Environmental Factor.

1.5.1. Chemical and physical factor.

Organic compounds differ widely in their solubility, from infinitely miscible polar compounds, such as methanol, to extremely low solubility nonpolar compounds, such as polynuclear aromatic hydrocarbons (PAHs) (Horvath, 1982). Many synthetic chemicals have low water solubilities (Stucki and Alexander, 1987). The availability of a compound to an organism will dictate its biodegradability (U.S. EPA, 1985a). Compounds with greater aqueous solubilities are generally more available to degradative enzymes. An example is *cis* -1,2- dichloroethylene, which is preferentially degraded relative to *trans* -1,2-dichloroethylene. This is probably due to "*cis* " being more polar than " *trans* " and, therefore, more water soluble.

The composition and inherent biodegradability of the petroleum hydrocarbon pollutant is the first and most important consideration when the suitability of a cleanup approach is to be evaluated (Okoh, 2006). Compositional heterogeneity among different crude oils and refined products influences the overall rate of biodegradation both of the oil and of its component fractions (Leahly and Colwell, 1990). Biodegradability is inherently influenced by the composition of the oil pollutant. For example, kerosene, which consists almost exclusively of medium chain alkanes, is under suitable conditions, totally biodegradable. Similarly, crude oil is biodegradable quantitatively, but for heavy asphaltic-naphthenic crude oils, only about 11% may be biodegradable within a reasonable time period, even if the conditions are favourable (Bartha, 1986b). Okoh et al., (2002) reported that between 8.8 and 29% of the heavy crude oil *Maya* was biodegraded in soil microcosm by mixed bacterial consortium in 15 days, although major peak components of the oil was reduced by between 6.5 and 70% (Okoh, 2003). Also, about 89% of the same crude oil was biodegraded by axenic culture of *Burkholderia cepacia* RQ1 in shake flask (Okoh et al., 2001) within similar time frame.

Okoh, (2003) noted that heavier crude oils are generally much more difficult to biodegrade than lighter ones, just as heavier crude oils could be suitable for inducing increased selection pressure for the isolation of petroleum hydrocarbon degraders with enhanced efficiency. Also, Okoh et al., (2002) noted that the amount of heavy crude oil metabolized by some bacterial species increased with increasing concentration of starter oil up to 0.6% (w/v), while degradation rates appeared to be more pronounced between the concentrations of 0.4 and 0.6% (w/v) oil.

The physical and chemical nature of the oil pollution is a critical factor in determining rates of biodegradation. Access to the pollutant is a major consideration when trying to degrade hydrocarbons which are, to a greater extent, hydrophobic. The dispersion of crude oil as an oil-in-water emulsion will increase the surface area available for microbial attack and thus increasing the rate of biodegradation. However, water-in-oil, or *"mousse"*, emulsions can form, creating a low surface area to volume ratio, inhibiting biodegradation (Wilkinson et al., 2002). The degree of spreading of oil in water or soil environment determines in part the surface area of oil available for microbial colonization by hydrocarbon-degrading microorganisms; in aquatic systems, the oil normally spreads, forming a thin slick (Atlas, 1981). The degree of spreading is reduced at low temperatures because of the viscosity of the

oil. In soils, petroleum hydrocarbons are absorbed by plant matter and soil particles, limiting its spreading. Similarly *'tar balls'*, which are large aggregates of weathered un-degraded oil, restrict access by microorganisms because of their limited surface area. Auto-oxidation, photooxidation and the removal of low molecular weight hydrocarbons by microbes all aid their formation and tar balls may take thousands of years to degrade (Wilkinson et al., 2002). Hydrocarbon-degrading microorganisms act mainly at the oil-water interface. Such microorganisms can be observed growing over the entire surface of an oil droplet; growth does not appear to occur within oil droplets in the absence of entrained water. Availability of increased surface area will under normal circumstance accelerate biodegradation (Atlas, 1981; Ijah and Antai, 2003b).

Concentration of Petroleum hydrocarbon determines to a greater extent the rate of breakdown of the hydrocarbons from the environment. Concentration of hydrocarbon can affect its biodegradability and toxicity to the degrading organisms. High concentration of hydrocarbon can be inhibitory to microorganisms, and concentration with which inhibition occurs will vary with the compound. Concentrations of 1 to 100µg/ml of water or 1 to 100µg/g of soil or sediment (on dry weight basis) are not generally considered to be toxic to common heterotrophic bacteria and fungi. Ijah and Antai, (2003b) reported high degradation of hydrocarbons in soil contaminated with 10% and 20% crude oil compared to those contaminated with 30 and 40% crude oil which experienced partial degradation of hydrocarbons within a period of 12 months. Another authors reported that percentage of degradation by mixed bacterial consortium decreased from 78% to 52% as the concentration of crude oil was increased from 1 to 10% (Rahman et al., 2002). High concentrations of hydrocarbons can be associated with heavy, undispersed oil slicks in water, causing inhibition of biodegradation by nutrient or oxygen limitation or through toxic effects exerted by volatile hydrocarbons. Fusey and Oudot (1984) reported that contamination of seashore sediments with crude oil above a threshold concentration prevented biodegradation of the oil because of oxygen and/or nutrient limitation.

1.5.2. Biological factor.

Increased persistence of chemicals may result from several types of biological interactions: (1) the biocidal properties of the chemicals to soil microorganisms may preclude their biodegradation, (2) direct inhibition of the adaptive enzymes of effective soil microorganisms, and (3) inhibition of the proliferation processes of effective microorganisms (Kaufman, 1983). Inhibition of microbial degradation may ultimately affect the mobility of a chemical in soil. Bioavailability is extremely important to biodegradation of organic pollutants. It is frequently observed that the rate of removal of compounds from soil is very low even though the compounds are biodegradable, the substrates in these instance may not be in a form that is readily available to the microorganisms (Jim et al., 2005). Biodegradation of hydrophobic pollutants may take place only in the aqueous phase, e.g., naphthalene is utilized by pure cultures of bacteria only in the dissolved state (Wodzinski and Bertolini, 1972). Bouchez et al., (1995) similarly showed that phenanthrene biodegradation occurs only in the aqueous phase. The three main classes of hydrocarbons (aliphatic, alicyclic and aromatic hydrocarbons) vary in their biodegradability according to size and solubility. It is believed that only molecules of hydrocarbons that are dissolved in the aqueous phase are available for intracellular metabolism (Sikkema et al., 1995). The rate at which a particular organic compound dissolves in water is critical to its biodegradability because this governs the rate of transfer to the organism (Jim et al. 2005). The rate of transfer is determined by the equilibrium and actual concentration in the bulk phase and aqueous phase. This central to the concept of bioavailability as it relates to biodegradation.

1.5.3. Soil and environmental factor.

Many microorganisms have specific ecological niches for proliferation and colonization (Daubaras and Chakrabarty, 1992). Metabolism by the indigenous microflora is influenced by soil and environmental factors, such as light, temperature (climate, daily and seasonal temperature fluctuations), pH, presence of cometabolites, reactive radicals, other organic and inorganic compounds, available oxygen and nutrients (nitrogen and phosphorus), as well as the physical state of the oil (Cooney, Silver, and Beck, 1985), moisture content, organic matter, oxidation-reduction potential, attenuation, and soil texture and structure. The environment influences biodegradation by regulating both the bioavailability of the compound

and the activity of the degraders. Often the most important factor limiting rates of biodegradation in the environment is the availability of molecular oxygen (Jim et al., 2005). The initial step in the catabolism of hydrocarbons by bacteria and fungi involves the oxidation of the substrate by oxygenase to which molecular oxygen is required. Aerobic condition is therefore necessary for this route of microbial oxidation of hydrocarbons in the soil environment. The availability of oxygen in the soil is dependent on rates of microbial oxygen consumption, the type of soil, and the presence of utilizable substrate, which can lead to oxygen depletion (Bartha and Bossert, 1984). Delivering air or oxygen to contaminated soils may be difficult for a number of reasons: the soil porosity may not be favourable and therefore mass transfer from the gas phase to the aqueous phase will be limited. Also relatively low solubility of oxygen in water is a primary limiting factor (Jim, et al. 2005). Most contaminated soil may contain large populations of the appropriate microorganisms but can remain contaminated for decades or longer as a result of conditions that do not favour rapid biodegradation of complex pollutants. The complete oxidation of aromatic compounds and hydrocarbons to carbon dioxide is difficult in the absence of molecular oxygen due to the great stability of C – H and C – C bonds.

Temperature plays very important roles in biodegradation of petroleum hydrocarbons, firstly by its direct effect on the chemistry of the pollutants, and secondly on its effect on the physiology and diversity of the microorganisms (Okoh, 2006). Soil temperature is one of the more important factors controlling microbiological activity and the rate of organic matter decomposition (Sims and Bass, 1984). Temperatures of both air and soil affect the rate of biological degradation processes in the soil, as well as the soil moisture content (JRB Associates, Inc., 1984). Temperature affects the physical nature and composition of the petroleum, the rate of microbial hydrocarbon metabolism, and the composition of the microbial communities (Atlas, 1994). Ambient temperature of an environment affects both the properties of spilled oil and the activity or population of microorganisms (Venosa and Zhu, 2003). Temperature plays a significant role in controlling the nature and extent of microbial hydrocarbon metabolism (Nedwell, 1999; Frederic et al., 2005). Temperature affects the rate of biodegradation, as well as the physical nature and chemical composition of hydrocarbons (Whyte et al., 1998; Rowland et al., 2000). Although microbial activity is generally reduced at low temperatures, many of the components in crude oil and diesel can actually be degraded by

psychrophilic and psychrotrophic microorganisms (Margesin and Schinner, 1999a). The bioavailability of soluble hydrophobic substances, such as aliphatic and polyaromatic hydrocarbons, is temperature dependent. A temperature increase leads to an increase in diffusion rates of organic compounds notably by a decrease of their viscosity (Northcott and Jones, 2000). Thus, higher molecular reaction rates due to smaller boundary layers are expected at elevated temperatures. In counterpart, the increased volatilization and solubility of some hydrocarbons at elevated temperature may enhance their toxicity (Whyte et al., 1998). Such an increase in toxicity may delay the onset of degradation (Leahly and Colwell, 1990). Temperature influences the rate of abiotic weathering process notably evaporation. Temperature can also affect hydrocarbon utilization; bacteria relatively metabolize isoprenoids at 30^{o}C but have difficulty doing so at 40^{o}C. Although many species can withstand freezing and thawing, bacteria cease growth and metabolism altogether at temperature below 12^{o}C due to the formation of intracellular ice (Margesin and Schinner, 1999b). It is essential that contaminated sites be at the optimum temperature for bioremediation to progress successfully, since excessively high or low temperatures sometimes inhibit microbial metabolism. Although hydrocarbon biodegradation can occur over a wide range of temperatures, the rate of biodegradation generally decreases with decreasing temperature. Highest degradation rates generally occur in the range of 30 – 40 ^{o}C in soil environments, 20 – 30^{o}C in some freshwater environments, and 15 – 20 ^{o}C in marine environments (Bartha and Bossert, 1984). In addition, the solubility and bioavailability of a contaminant will increase as temperature increases, and oxygen solubility will be reduced, which will leave less oxygen available for microbial metabolism (Margesin and Schinner, 1999a).

Nutrients are very important ingredients for successful biodegradation of hydrocarbon pollutants, especially N, P and in some cases Fe (Okoh, 2006). Inadequate mineral nutrient, especially N, and P, often limits the growth of hydrocarbon utilizers in water and soils. Addition of N and P to an oil polluted soil has been shown to accelerate the biodegradation of the petroleum in soil (Ijah and Safiyanu, 1997). Addition of a carbon source as a nutrient in contaminated soil is known to enhance the rate of pollutant degradation by stimulating the growth of microorganisms responsible for biodegradation of the pollutant. Depending on the nature of the impacted environment, some of these nutrients could become limiting, hence the

additions of nutrients are necessary to enhance the biodegradation of oil pollutants (Kim et al., 2005). It has been suggested that the addition of carbon in the form of pyruvate stimulates the microbial growth and enhances the rate of PAH degradation. Depending on the nature of the impacted environment, some of these nutrients could become limiting, hence the additions of nutrients are necessary to enhance the biodegradation of oil pollutants (Kim et al., 2005). Pelletier et al., (2004) assessed the effectiveness of fertilizers for crude oil bioremediation in sub-Antarctic intertidal sediments over a one year and observed that chemical, microbial and toxicological parameters demonstrated the effectiveness of various fertilizers in a pristine environment. Frederic et al., (2005), observed that addition of commercial oleophilic fertilizers containing N and P to hydrocarbon contaminated soil increased the hydrocarbon.

Soil pH can be highly variable, ranging from 2.5 in mine spoils to 11.0 in alkaline deserts (Bossert and Bartha, 1984). Most heterotrophic bacteria and fungi favor a pH near neutrality, with fungi being more tolerant to acidic conditions (Atlas, 1988). Extremes in pH, as can be observed in some soils, would 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. Hambrick et al., (1980) found that the rates of microbial mineralization of octadecane and napthalene 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.

Chapter 1

BIOREMEDIATION OF BRAZILIAN SOILS CONTAMINATED WITH USED LUBRICATING OIL

ABSTRACT

Environmental pollution with petroleum and petrochemical products has attracted much attention in recent decades. The use of various kinds of automobiles and machinery vehicles has caused an increase in the use of motor oil and in addition, oil spillages into the environment have become one of the major problems. Spillages of used lubricating oils contaminate natural environment with hydrocarbons. The hydrocarbons spread both vertically and horizontally on both surface and ground-water bodies, soil pore space and adhere/adsorb into surfaces of soil particles. The contamination of groundwater resources by organic chemicals is significant environmental problem that can constitute risks to health of humans and animals. It is believed that remediation methods are favoured and have been used particularly as a secondary treatment option for clean-up of oil spillage. This work has the intent for evaluation and remediation of organic pollutants in particular, spent motor oil contaminated soils via bioremediation. The rates of biodegradation of the used motor oil were studied for a period of 90 days. The model soil of 300 g was contaminated with 1.5 % (w/w) of used motor oil at room temperature under laboratory conditions using microcosm of 1 L. The microcosm was used to simulate the comparative effect of spent oil addition and bioremediation using a commercially available hydrocarbon degrading microbial consortium - Amnite p1300 as the bioaugmentation (T1), nutrients amendments - $(NH_4)_2SO_4$ and K_2HPO_4 (NPK) as biostimulation treatment (T2), unammended soil - natural attenuation as (T3) and the control soil treated with sodium azide (NaN_3) as (T4) on the microbial community in three different soil types (S1, S2 and S3). Hydrocarbon-utilizing bacterial counts were high in the amended soils with treatments T1, T2, and T3 ranging from 3.47×10^6 to 3.27×10^8 CFU/g compared to T4 throughout the 90 days of study. Oil-contaminated soil in T1 showed the highest reduction in total petroleum hydrocarbon with net loss of 36.17 % throughout the 90 days of the experiment compared to other treatments and three soils. The development of the microbial community and its recovery is a useful and sensitive way of monitoring the impact and recovery of used motor oil-contaminated soils. Commercially available microbial-based bioremediation products may be used with some success in tropical soil environments, however soil-specific trials may be required to ensure that the best commercial product is selected. As an alternative, the selective enrichment of indigenous microorganisms may result in similar performance at a reduced cost. The results suggest that different soils have different inherent microbial potential to degrade hydrocarbons under spent oil pollution. This finding should be taken into account in impact and risk assessments of petroleum polluted soils.

Key words: Bioremediation, used lubricating oil, hydrocarbons, microbial consortium, soil types.

Capítulo 1

BIORREMEDIAÇÃO DE SOLOS BRASILEIRO CONTAMINADOS COM ÓLEO LUBRIFICANTE USADO

RESUMO

A poluição ambiental com petróleo e produtos petroquímicos tem atraído muito atenção em décadas recentes. O uso de veículos automotivos e de diversos tipos de maquinaria aumentou o uso de óleo de motor e, assim, com os derramamentos de óleos no ambiente tem-se grande problema. Derrames de óleo lubrificante usado como diesel ou gasolina contaminam o ambiente natural com hidrocarbonetos. Os hidrocarbonetos difundem-se verticalmente e horizontalmente na superfície e nos corpos d'água subterrâneos, nos espaços porosos do solo e se adsorvem às partículas do solo. A contaminação das águas subterrâneas por compostos orgânicos é problema ambiental significativo que pode constituir risco à saúde da humana e animal. Acredita-se que os métodos de remediação são favorecidos e sido usados particularmente como opção de tratamento secundário para a limpeza de derrames de óleo. Este trabalho teve como objetivo avaliar biorremediação solos contaminados com óleos de motor usado. As taxas de biodegradação do óleo de motor usado foram avaliadas por um período de 90 dias. Amostras de trezentos gramas de solo foram contaminadas com 1,5% (w/w) com óleo de motor usado, a temperatura ambiente sob condições laboratoriais usando microcosmos de 1L. O microcosmo foi usado para simular o efeito comparativo da adição de óleo usado e bioremediação utilizando um coquetel de microorganismos disponível comercialmente, para degradar hidrocarbonetos – Amnite p1300 como tratamento de crescimento bacteriano (T1), adição de nutrientes - $(NH_4)_2SO_4$ e K_2HPO_4 (NPK) como tratamento de bioestimulação (T2), solo controle- atenuação natural (T3) e solo controle tratado com azida de sódio (NaN_3) (T4) sob a comunidade microbiana em três diferentes tipos de solo (S1, S2 e S3). O número de bactérias que utilizam hidrocabornetos foi elevado nos tratamentos T1, T2 e T3 variando de 3.47×10^6 a 3.27×10^8 UFC/g comparação ao controle (T4) durante os 90 dias do estudo. Em T1, o solo contaminado com óleo mostrou uma elevada redução no total de hidrocarbonetos do petróleo com degradação liquida de 36,17% ao longo dos 90 dias de experimento, quando comparado aos outros tratamentos e três solos. O desenvolvimento da comunidade microbiana e sua recuperação revelaram ser uma maneira útil e sensível de monitorar o impacto e recuperação dos solos contaminados com óleos de motor usados. Produtos microbianos para bioremediação disponíveis comercialmente podem ser usado com algum sucesso em solos tropicais; entretanto estudos específicos para o tipo de solo são necessários de modo a assegurar a seleção do melhor produto comercial. Como alternativa, o enriquecimento seletivo de microorganismos indígenas pode resultar em desempenho similar a custo reduzido. Os resultados sugerem que diferentes solos apresentam diferentes potenciais microbianos inerentes para degradar hidrocarbonetos sob poluição por óleo usado. Tais descobertas devem ser levadas em consideração na avaliação de impacto e riscos de solos poluídos por petróleo.

Palavras-chave: Biorreme Biorremediação, óleo lubrificante usado, hidrocarbonetos, consórcio microbiano, tipos de solo.

1. Introduction

Despite efforts in some countries to recover and recycle used motor oils, significant amounts of lubricants are input into the environment, particularly in environmentally sensitive applications such as forestry and mining, or through engine losses (Battersby, 2000). Consequently, considerable attention has been given to lubricant biodegradability and persistence in the environment. Therefore, there is a need for effective and environmentally safe cleanup treatments of oil spills. The United State Environmental Protection Agency (40 CFR Part 279) defined "used oil'' as "any oil that has been refined from crude oil or any synthetic oil that has been used and, as a result of such use is contaminated by physical or chemical impurities.'' Used motor oil contains metals and heavy polycyclic aromatic hydrocarbons derived from engine oil - a complex mixture of hydrocarbons and other organic compounds, including some organometallic constituents (Butler and Mason, 1997) that is used to lubricate parts of an automobile engine, in order to smooth engine operation (Hagwell et al., 1992; Boonchan et al., 2000). The rise in consumption of automotive lubricating oil is a worldwide problem and has increased in large used oil volume and its waste. In Brazil, the problems tend to worsen with economic growth, population growth, and rapid industrialization and disregard for environmental health, particularly in relation to used motor oil. The consumption of lubricating oil in Brazil is around 10^6 m^3/year (Conselho em Revista, 2005; Magalhães et al.,2007). Approximately 6.5 x 10^5 m^3/year are consumed in the lubrication process, and from 3.5 x 10^5 m^3/year remainder, only 20% are treated or recycled; therefore, significant volumes of used motor oil are continually discharged into the ecosystems (local environment). Release of hydrocarbons into the environment whether accidentally or due to human activities is a main cause of water and soil pollution (Holliger et al., 1997). These hydrocarbon pollutants usually cause disruptions of natural equilibrium between the living species and their natural environment. The persistent hydrocarbon components are known to have carcinogenic and neurotoxic activities (Reddy and Matthew, 2001; Das and Chandran, 2010). One gallon of used motor oil, improperly disposed of, may contaminate 1 million gallons of fresh water, which is enough to supply 50 people with drinking water for one year. One pint (4 gills or 568.26 cubic centimeters) of used motor oil improperly disposed of can create a one-acre slick on the surface of a body of water and kill floating aquatic organisms (USEPA, 1996).

The uses and unsafe disposal of petroleum hydrocarbon products increase soil contamination, and this has constituted major environmental problems. Therefore, the development of research to remediate soils contaminated with used motor oils, in particular bioremediation, provides an effective and efficient strategy to speed up the clean-up processes (Mandri and Lin, 2007). Various factors including lack of essential nutrients such as nitrogen and phosphorus may limit the rate of petroleum hydrocarbon degradation from contaminated soil. Addition of inorganic nutrients (biostimulation) is therefore needed as an effective approach to enhance the bioremediation process (Semple et al., 2006; Walworth et al., 2007). Also, many microbial strains, each capable of degrading a specific compound, are available commercially for bioremediation (Bragg et al., 1994; Korda et al., 1997; Song et al., 1990). This study aimed at evaluating of the remediation potentials of organic pollutants, in particular, spent motor oil contaminated soils, via bioremediation using commercial microbial consortium. It was also aimed to test a kinetic model to determine the rate of biodegradation of the hydrocarbon in the soil and subsequently determine the half-life of the oil degradation.

2. MATERIAL AND METHODS

2.1. Collection of samples. The soil sample used was collected from three different locations in Minas Gerais State, Brazil, in a hermetic bag and transported to the laboratory for analysis. Used engine oil was collected from a gasoline and car service station close to the Federal University of Viçosa. Amnite P1300 special bacterial strains (Amnite P1300) specially made to degrade used motor oil was obtained from Cleveland Biotech Ltd., UK

2.2. Experimental Design: microcosm set-up description. The model soil of 300 g was contaminated with 1.5 % (w/w) or (15000 mg/kg) of used motor oil at room temperature of (25 ± 1 ^{0}C) under laboratory conditions using microcosm of 1 L. The microcosms were used to simulate the comparative effect of spent oil addition and bioremediation using a commercially available hydrocarbon degrading microbial consortium (Amnite P1300), consisting a mixture of *Bacillus subtilis, Bacillus megaterium, Pseudomonas putida, Pseudomonas fluorescens, Phanerochaete chrysosporium, Rhodococcus rhodocrous* on a cereal (bran) as the bioaugmentation treatment. The microorganisms were conditioned to degrade heavy hydrocarbons. The population numbers in Amnite P1300 was approximately 5

x 10^8 cfu/g of bran. Also, the polluted soils were amended with $(NH_4)_2SO_4$ and K_2HPO_4) as biostimulation. The C:N:P ratio of the nutrient compound was adjusted to 100:7.5:1 (optimum conditions). The same conditions provided in the biostimulation treatment were used in the bioaugmentation treatments plus the addition of Amnite P1300. The unammended soil (natural attenuation), without addition of nutrients and microbial inoculum was included to indicate hydrocarbon degradation capability of microorganisms naturally present in the contaminated soils (i.e. the autochthonous microbes). There was a control soil in which most of the indigenous bacteria were killed by the added biocide sodium azide (NaN_3) (0.3% ww $^{-1}$) to inhibit soil microorganisms and to monitor abiotic hydrocarbon losses on the microbial community in three different soil types. There were six sampling dates; hence 36 microcosms in total were used. Microcosms were arranged in a random order, and rearranged every 2 weeks ± 2 days throughout the duration of the experiment The treatments were set up in triplicate, while the content of each container was tilled every week for aeration, and the moisture content was maintained at 70% (Pramer and Bartha, 1972), water holding capacity by the addition of sterile distilled water every week until the end of the experiment.

2.3. Sampling. Periodic sampling from each microcosm was carried out at 15-day intervals for 90 days. Composite samples were obtained by mixing 10 g of soil collected from different areas of the microcosm for bacteria enumeration and determination of total petroleum hydrocarbon.

2.4. Determination of the physicochemical property of the soil. Table 2 shows the origin and selected physical and chemical characteristics of the noncontaminated soil samples used for the bioremediation studies. Nitrogen content of the soil was determined using the Kjeldahl method, the available phosphorus was determined by colometry after Mehlich 1 extraction and Organic Carbon content was determined using a modifield Walkley-Black (1934) method. The pH was determined using 1:2.5 ratio by weight with distilled water (w/v) after 30-min equilibration using a pH meter and electrode calibrated with pH 4.0 and 7.0 standards. Determinations were made in triplicate.

Table 2: Selected physical and chemical characteristics of the non-contaminated soil samples

Parameters	Soil 1 (S_1)	Soil 2 (S_2)	Soil 3 (S_3)
pH (H_20)	5.20	5.91	4.92
Total Nitrogen (%)	0.43	0.24	0.11
Avail. P (mg/dm^3)	1.00	1.8	0.40
Organic C (dag/kg)	3.50	1.54	0.81
C:N ratio	8.14	6.42	7.56
ECEC ($cmol_c/dm^3$)	3.19	2.29	0.78
Moisture Content (%)	33.80	28.3	11.30
Sand (dag/kg)	11.00	10.00	68.00
Silt (dag/kg)	9.00	22	4.00
Clay (dag/kg)	80.00	68	28.00
Texture	Clayey	Clayey	Clay loamy sand
Soil Type	Red Latosol	Red Lotosol	Red Yellowish Latosol
Parent Material	Sete Lagoas – MG	Cachoeira Dourada - MG	Tres Marias - MG

2.5. Microbial monitoring and enumeration of total aerobic heterotrophic and hydrocarbon-degrading bacteria. The study was conducted at room temperature and monitoring was performed on days 0, 15, 30, 45, 60, 75 and 90. To monitor cell numbers and biodegradation, 1 g of soil was removed from each microcosm at the set times and suspended in 9 mL of saline solution in sterile centrifuge tubes. The mixture was vigorously shaken on a vortex mixer for 3 minutes and then the soil particulates were allowed to settle for 1 min before 0.1 mL of the supernatant was sampled for CFU counts. The number of colony-forming total aerobic heterotrophic bacteria (AHB) was determined by plating three replicate samples from each treatment withdrawn every 15 days. Serially diluted samples (0.1 mL) were plated on nutrient agar medium (Oxoid) supplemented with 10 mg/mL solution of cycloheximide in which 1 mL/L was drawn to suppress the growth of fungi. The oil agar plates were incubated at 30°C for 24 hours, and the colonies were counted. Also, enumeration

of hydrocarbon-degrading bacteria (HDB) was attempted on a mineral medium containing motor oil as the sole carbon source. The mineral medium contained 1.8 g K_2HPO_4, 4.0 g NH_4Cl, 0.2 g $MgSO_4.7H_2O$, 1.2 g KH_2PO_4, 0.01 g $FeSO_4.7H_2O$, 0.1 g NaCl, 20 g agar, one percent (1%) used engine oil in 1,000 mL distilled water, and the medium was adjusted to pH 7.4 (Zajic and Supplission, 1972). The oil agar plates were incubated at 30°C for 7 days before the colonies were counted.

2.6. Extraction of residual oil and analysis of total petroleum hydrocarbons. Total Petroleum Hydrocarbons (TPHs) were extracted according to EPA method 3546 (US EPA, 2007) using the Microwave Automated Reaction System from CEM (Matthews, NC). Briefly, Sodium sulfate (Na_2SO_4) was purified by drying overnight in an oven at 150°C and quickly transferred into a desiccator, Five grams (5 g) of homogenised contaminated soil was weighed out, mixed with 5 g dry anhydrous Na_2SO_4 and ground to less than 1 mm particle sizes, extracted in GreenChem vessels with 25 mL of a 1:1 hexane:acetone mixture according to manufacturer's protocol at 100 °C for 20 minutes. The n-hexane and acetone was filtered through whatman No 1 filter paper to separate the extract from the soil particles, and transferred into 100 mL amber vials through separatory funnel and sequentially rinsed with equal volume of solvent mixture. The solvent were evaporated topartial dryness with a rotary evaporator (Fizatom Rotavapor 801), transferred into 2 mL vials and then dried completely using nitrogen gas. Dried samples were dissolved in 600 µL dichloromethane for gas chromatography analysis. The residual oil was analyzed on Shimadzu GC-17A Chromatograph equipped with a Flame-Ionization Detector (FID) by using fused silica capillary column DB-5 (30 x 0.25 mm), and AOC-17 Shimadzu auto injector complying with Environmental Protection Agency (EPA) standard method 8015 (US EPA 2007). The flow rate of the helium carrier gas was 1.81 mL/min with linear velocity of 38.49 cm/s. The initial temperature was programmed at 40^{o}C and held for 15 min. The temperature was then increased to 280^{o}C at a rate of 10^{o}C /min. The final temperature was held for 31 min. The injector was set in the split mode, the split ratio was set to 1:10; the injection volume was 1 µL and the injector and the detector temperature for GC were maintained at 260 and 280°C, respectively, and the oven temperature was programmed to rise from 40 to 280°C in 10°C/min increments and to hold at 280°C for 31 min. The dry weight of the soil samples was

determined following baking of 10 g of wet soil at > 80 °C for at least 48 hours. Before analyzing the sample extract, a mixture of standards including *n*-alkanes (*n*-decane *n*-C_{10}, *n*-dodecane *n*-C_{12}, *n*-tetradecane *n*-C_{14}, *n*-hexadecane *n*-C_{16}, *n*-octadecane *n*-C_{18}, *n*-eicosane n-C_{20}, *n*-docosane *n*-C_{22}, *n*-tetracosane *n*-C_{24}, *n*-hexacosane *n*-C_{26}, *n*-octacosane *n*-C_{28} and a pure standards containing *n*-triacontane *n*-C_{30}, *n*-dotriacontane *n*-C_{32}, *n*-tetratriacontane *n*-C34, and *n*-hexatriacontane *n*-C_{36}, and a mixture of polycyclic aromatic hydrocarbon consisting of acenaphthene, acenaphthylene, anthracene, benzo (a) anthracene, benzo (a) pyrene, benzo (b) fluoranthene, benzo (g, h, i) perylene, benzo (k) fluoranthene, chrysene, dibenz (a, h) anthracene, fluoranthene,fluorine, indeno (1, 2, 3-cd) pyrene, naphthalene, phenanthrene, pyrene, 1-methylnaphthalene and 2-methylnaphthalene, Supelco) were used for calibration. Five points calibration curves using peak areas were obtained and the response factors were used to determine the concentrations of various hydrocarbons in the sample extract. The total petroleum hydrocarbons were identified and quantified by comparing the peak area of samples with that of the standard of the TPH mixture with reference to the curve derived from standards. Percentage of degradation was calculated by the following expression:

$$\%\, biodegradation = \left[\frac{TPH\ control - TPH\ treatment}{TPH\ control}\right] \times 100 \qquad (1)$$

TPH data were fitted to the first-order kinetics model (Yeung et al., 1997):

$$y = a\, e^{-kt} \qquad (2)$$

where *y* is the residual hydrocarbon content in soil (mg/kg), a is the initial hydrocarbon content in soil (mg/kg), *k* is the biodegradation rate constant (day^{-1}) and *t* is time (days). The biodegradation rate constant *(k),* and half-life *ln(2)/k* of the hydrocarbons in soil during the bioremediation process were calculated from the model using Statistical ® software (Stat Soft. Inc. (2007). The model was used to estimate the rate of biodegradation and half-life of hydrocarbons in soil under each treatment and the model was based on the assumption that the degradation rate of hydrocarbons positively correlated with the hydrocarbon pool size in the soil.

2.7. Statistical Analysis. Statistical analysis of data was carried out using analysis of variance. Means of different treatments were also compared statistically using a General Linear Model (ANOVA) (Tukey test, $p > 0.05$) using statistical 8.0 software (Stat Soft, 2007).

3. RESULTS AND DISCUSSIONS

3.1. Microbial Counts. The aerobic heterotrophic bacterial (AHB) counts in T1 ranged between 1.01×10^8 and 2.4×10^9 CFU/g while T2 and T3 ranged from 1.03×10^8 to 1.7×10^9 and 1.0×10^8 to 3.8×10^8 CFU/g respectively (Fig. 3) in all the three soil types. The treatment T4 had AHB count ranging between 1.27×10^3 and 6.03×10^5 CFU/g. Hydrocarbon Degrading Bacterial (HDB) counts were also higher in oil contaminated soil in T1, T2 and T3 (Fig. 4). The count of HDB in soil amended with T1 was about 2% higher than those amended with T2 and T3. HDB count in soil amended with T1 ranged from 3.6×10^6 to 3.3×10^8 CFU/g, while those amended with T2 and T3 ranged from 3.7×10^6 to 2.6×10^8 and 3.5×10^6 to 5.41×10^7CFU/g, respectively. However, the HDB counts in T4 were 1.07×10^3 to 7.07×10^4 CFU/g lower than T1, T2 and T3. The counts of HDB in soils T1, T2 and T3 were higher compared to that of T4. These results were similar to that obtained by Antai and Mgbomo (1989) whose counts of HDB in hydrocarbon-contaminated soil was 10^8 CFU/g, but higher than that of Ghazali et al. (2004), who obtained 10^7CFU/g; from hydrocarbons degradation in diesel oil polluted soil. The discrepancies in the results may be due to the characteristics and the ecology of the different soil types used for the experiments. The microbial counts of the soils S1 and S2 showed the same pattern. Counts in soils amended with T1 gave highest followed by T2 and T3. Whereas, microbial counts in soil S3 showed different pattern compared with S1 and S2, as T2 gave highest counts compared with T1 and T3. Treatment T4 has the least results in all the soils used for the experiment. This result clearly demonstrates the benefit of bioaugmentation, biostimulation and indigenous microorganisms of used lubricating oil polluted soil. An enhanced comparison between the four treatments investigated in this work is revealed in figure 3 and 4. Treatment T4 is a control system where most of the indigenous bacteria were killed with a biocide (NaN_3).

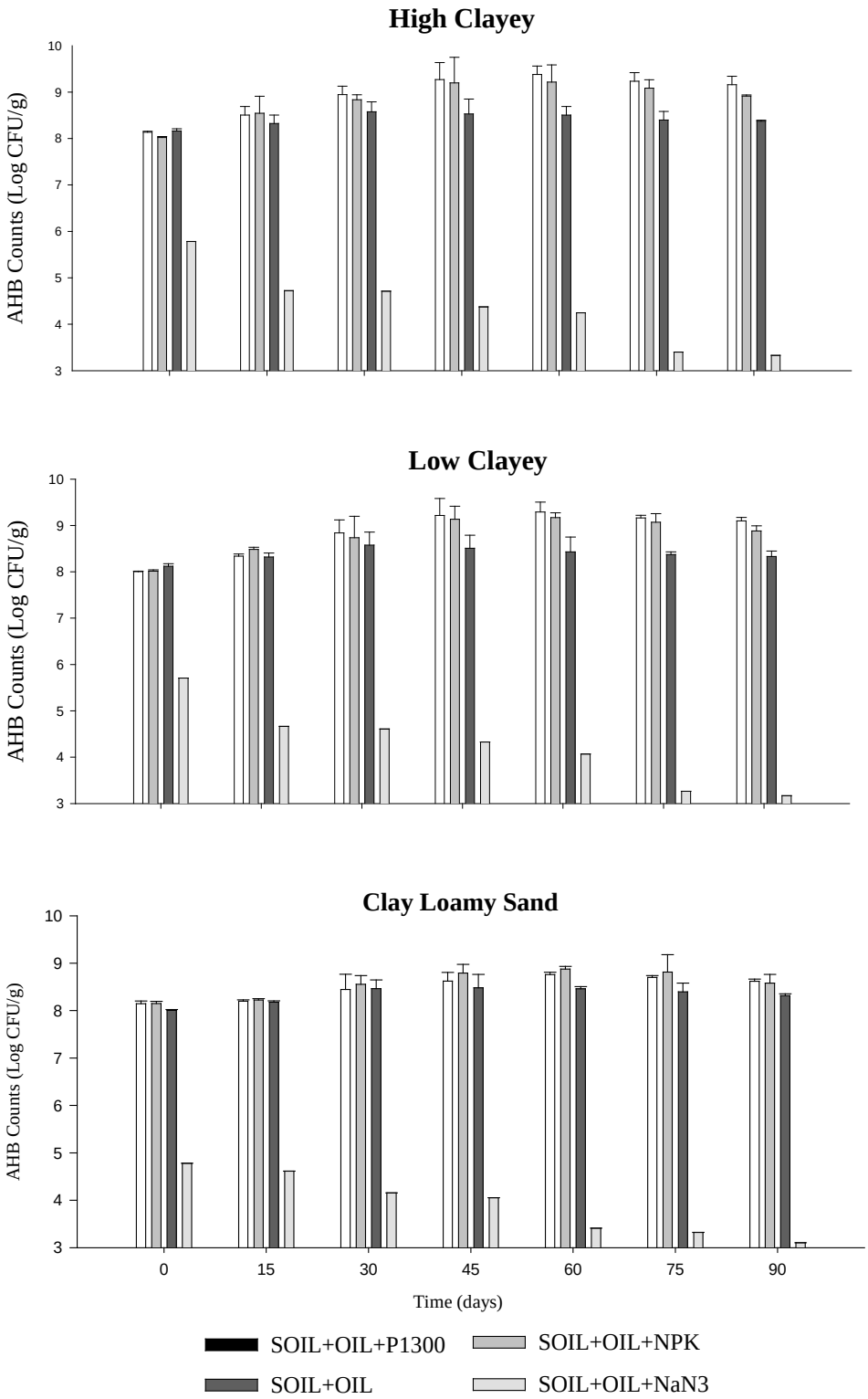

Fig 3: Counts of aerobic heterotrophic bacterial (AHB) population in oil-contaminated soils. Vertical bars indicate standard error of the means SE (n=3)

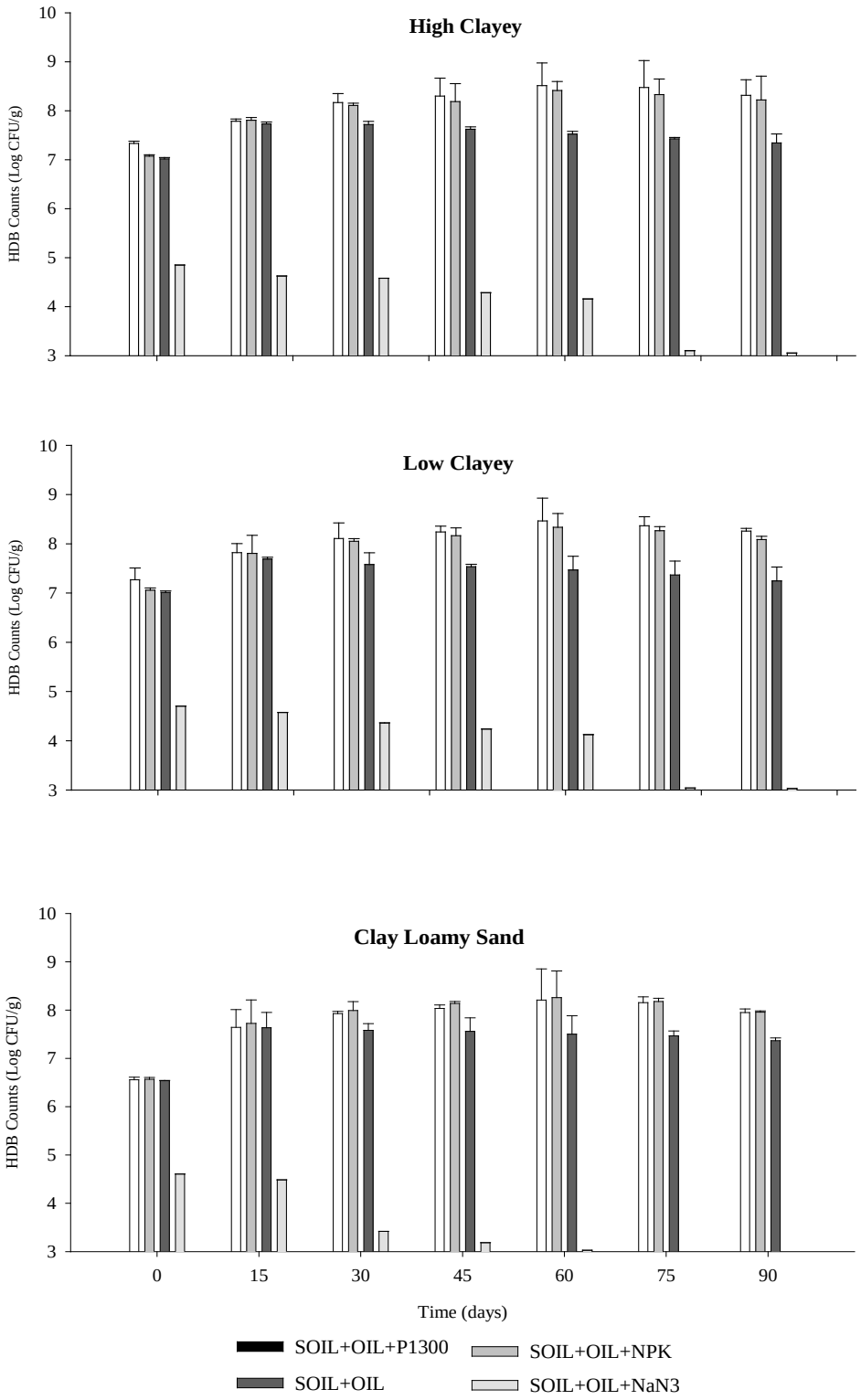

Fig 4: Counts of hydrocarbon degrading bacterial (HDB) population in oil-contaminated soils. Vertical bars indicate standard error of the means SE (n=3)

3.2. Used engine oil hydrocarbon biodegradation. There was a noticeable reduction in the total petroleum hydrocarbon within the first 15 days in all the treatments, but higher reduction was observed at 30 days compared to the control (T4). At the end of the 30 days, there was 49, 69 and 73 % TPH reduction in T3, T1 and T2 respectively, that is, 7,306; 10,278 and 10,881 mg/ kg. TPH reduction was observed in these treatments compared to 27 % (3,991 mg/ kg) TPH reduction in the control soil S1. The same trend was noticed in soils S2 and S3 with T2 (NPK) having the highest TPH reduction (Fig. 5). Because, feeding nutrient solutions containing inorganic substances, such as nitrogen and phosphorus to natural soil bacteria often enhances the ability of the microorganisms to degrade organic molecules into carbon dioxide and water (Stotzky and Norman, 1961a; 1961b). During this period, the added bacteria product acclimatized to their new source of carbon. At the end of 90 days experiment, oil-contaminated soil amended with T1 (Soil + Oil + Amnite P1300) showed the highest reduction in concentration of used engine oil (89%), followed closely by soil amended with T2 (Soil + Oil + NPK) (78%), but no significant differences were observed between the treatments T1 and T2. The results obtained in soil type S3 is lower compared to that of S1 and S2, this may be due to clay contents in these soils which have been shown to offer greater capacity for physicochemical attenuation of contaminants than coarse sands (Pye and Patrick, 1983). However, highest percentage (68%) of TPH reduction was observed in soil amended with T2 in soil S3 at the end of 90 days experiments. The net percentage loss of used oil in the contaminated soils could indicate the effectiveness of each treatment. The highest net percentage loss was observed at 30 days in T2 (45.93%), (40.33%) and (32.58%) followed by that of T1 (41.91%), (36.36%) and (28.83%) and T3 (22.10%), (22.10%) and (10.32%) in soils S1, S2 and S3, respectively (Table 3). However, the net percentage loss of used oil increased from45 days in T1 to the end of the 90 day experiment compared with other treatments.

Table 3: Net percentage loss of total petroleum hydrocarbon in soils during bioremediation

Soil types	Treatments	Time (days)					
		15	30	45	60	75	90
S1	T1	18.53±1.3	41.91±1.4	29.59±0.7	33.50±1.2	34.56±1.0	36.17±0.8
	T2	20.54±1.4	45.93±1.3	29.37±0.8	28.28±0.7	26.88±1.2	25.94±0.8
	T3	7.08±1.3	22.10±1.4	18.10±0.7	17.08±0.4	15.77±1.2	16.13±0.8
S2	T1	15.29±1.0	36.36±0.4	34.21±1.0	31.54±1.7	32.31±1.3	31.66±2.3
	T2	17.21±2.0	40.33±1.2	33.68±0.5	31.19±1.0	31.37±1.2	23.47±2.1
	T3	4.86±1.7	20.77±1.1	15.24±1.9	12.90±1.4	13.48±1.2	10.58±2.1
S3	T1	10.54±2.6	28.83±2.3	21.21±1.8	22.48±2.6	23.59±0.9	23.47±2.1
	T2	10.84±1.1	32.58±1.4	22.88±1.0	23.50±0.8	24.53±0.9	24.99±2.3
	T3	4.86±2.2	10.32±0.4	6.77±0.8	6.24±1.4	5.70±0.8	10.58±2.1

T1=soil+oil+Amnite P1300, T2=soil+oil+$(NH_4)_2SO_4$ and K_2HPO_4), T3=soil+oil alone; S1=Red Latosol, S2 = Red Latosol, S3= Red-Yellowish Latosol; Net % loss = % loss in TPH of oil-contaminated amended soils and oil-contaminated soil alone − % loss in TPH of unamended contaminated control soil with sodium azide.

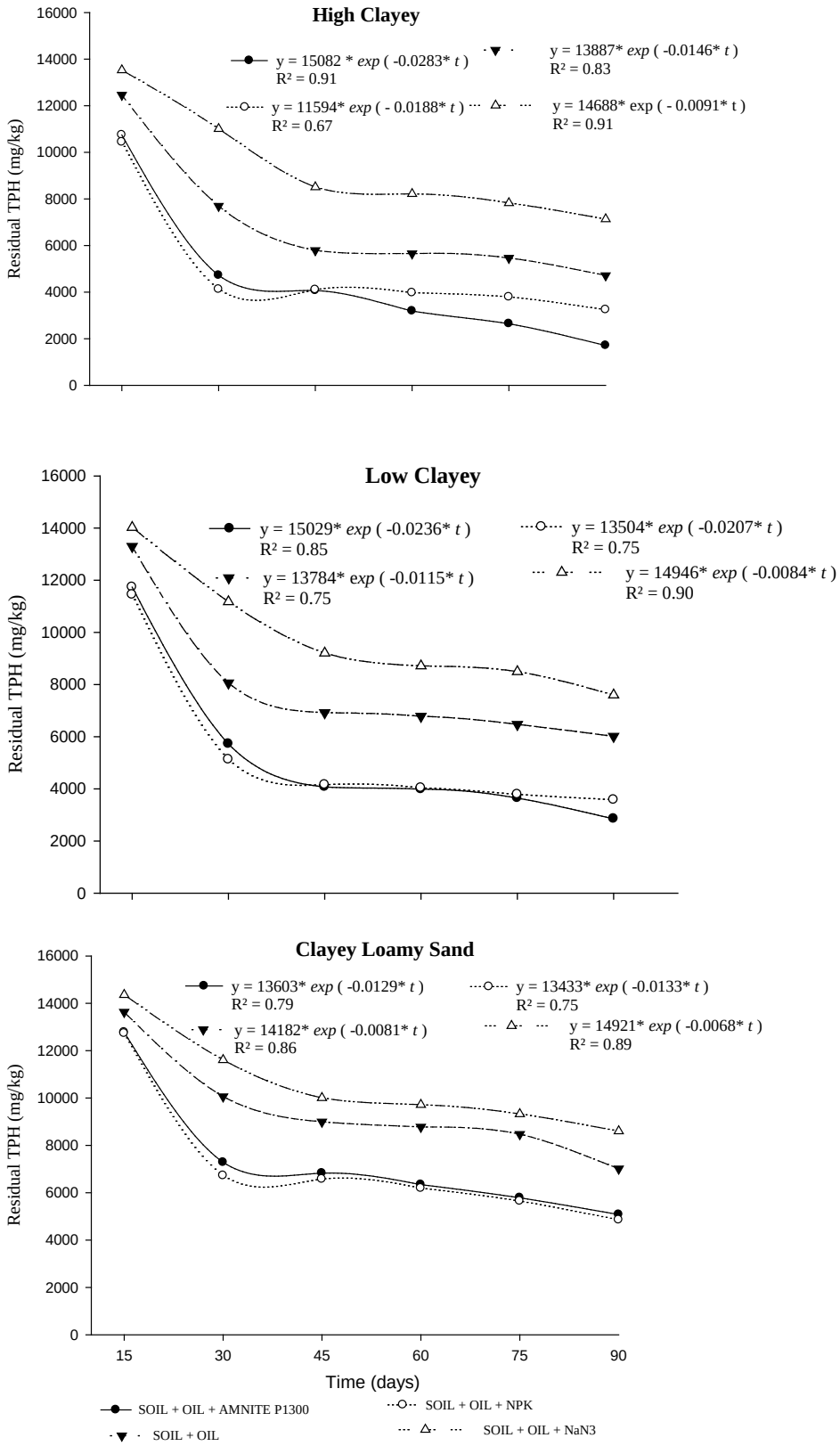

Fig 5. Residual total petroleum hydrocarbons in soils during bioremediation

3.3. Biodegradation rate constant and half-life. The highest biodegradation rates of 0.0283, 0.0236 and 0.0133 day^{-1} and half-lives 24.49, 29.37 and 52.12 days were recorded in T1 in soil types S1 and S2 and T2 in soil S3, respectively. The control T4 showed the least biodegradation rate of 0.0091, 0.0084 and 0.0068 with highest half-lives of 76.17, 82.52 and 101.93 in soils S1, S2 and S3 respectively. The biodegradation rate of used oil in T1 showed the best result for the kinetic parameters in this study, as a result of the added bacterial products, followed by T2 and T3, and this may be due to the bioavailability of the inorganic nutrients to the indigenous bacterial present in the soils (Table 4).

Table 4: Biodegradation rate constant (K) and half-life ($t_{0.5}$) of hydrocarbon in oil-contaminated soils

Treatments	K (day^{-1})			$t_{0.5}$ (days)		
	S1	S2	S3	S1	S2	S3
T1	0.0283 Dc	0.0236Cb	0.0129 Ca	24.49 Aa	29.37 Ab	53.73 Ac
T2	0.0188Cc	0.0207 Cb	0.0133Da	36.87 Aa	33.49 Ab	52.12 Ac
T3	0.0146 Bc	0.0115 Bb	0.0081 Ba	47.48 Ba	60.27 Bb	85.57 Bc
T4	0.0091Ac	0.0084 Ab	0.0068 Aa	76.17 Ca	82.52 Cb	101.93 Cc

T1=soil + oil + Amnite P1300, T2= soil + oil + $(NH_4)_2SO_4$ and K_2HPO_4), T3 = soil + oil alone, T4 = unamended contaminated control soil with sodium azide. S1 = Red Latosol, S2 = Red latosol, S3 = Red-Yellowish Latosol; K = Biodegradation constant (day^{-1}) and H = Half life (days). Values followed by the same capital or small letters are not significant difference between treatments (column) or soil types (row) respectively at the $P < 0.05$ level, while values followed by different capital or small letters indicate significant differences between treatments (column) or soil types (row) respectively at the $P < 0.05$ level.

4. CONCLUSIONS

Hydrocarbon-degrading bacteria counts were higher in the amended soils with treatments T1, T2, and T3 ranging from 3.47×10^6 to 3.27×10^8 CFU/g compared to T4 throughout the 90 days of study. Oil-contaminated soil in T1 showed the highest reduction in total petroleum hydrocarbon with net loss of 36.17 % throughout the 90 days of the experiment compared to other treatments. The development of the microbial community and its recovery is a useful and sensitive way of monitoring the impact and recovery of used lubricating oil-contaminated soils. Commercially available microbial-based bioremediation products showed to be promising in the removal of petroleum hydrocarbons in contaminated clayey soil.

Kinetic model of biodegradation showed the highest biodegradation rate of 0.0283 day^{-1} and least half life of 24.49 days in oil contaminated soil with highest clayey and soil organic matter contents. This reveals the influence of organic matter and its biological roles in the degradation of petroleum hydrocarbons contaminated soils.

Remediation of hydrocarbons contaminated soil is necessary in order to have a safe and healthy environment that will in turn result in healthy lifestyle across the globe. Biological remediation of hydrocarbon and metal contaminated soil offers a better and more environmentally friendly technique that if properly and thoroughly explored can bring our environment into a better place for both plant and animal well-being due to its enormous advantages over other treatment methods. However, despite these enormous advantages of biological treatment method, its potential is yet to be fully utilized in restoration of contaminated soil. This is possibly due to the fact that it takes a long period of time for the complete restoration of the environment; this limitation can however be overcome through nutrient addition and introduction of microbes with biodegradative capability to degrade hydrocarbons.

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Chapter 2

EFFICACY OF MICROBIAL CONSORTIUM IN THE BIODEGRADATION OF USED LUBRICATING OIL CONTAMINATED SOILS OF BRAZIL

ABSTRACT

Experiments were conducted to examine the efficacy of microbial consortium (Amnite p1300) on simulated used lubricating oil contaminated soils of Brazil under laboratory condition for a period of 90 days. The effect of other treatments conditions were also investigated on the microbial survival and used lubricating oil degradation. Artificially contaminated soil types (clayey S_1 and sandy S_2) at a loading rate of 30000 and 45000 mg/kg in a 300 g model with used lubricating oil that was amended with commercially available hydrocarbon degrading microbial consortium: Amnite p1300 as the bioaugmentation (T_1). The effects of other treatments: nutrient amendments - $(NH_4)_2SO_4$ and K_2HPO_4 (NPK) as biostimulation treatment (T_2), unammended soil - natural attenuation as (T_3) and the control soil treated with sodium azide (NaN_3) as (T_4) on the microbial community and the degradation of used engine oil was also investigated. The evaluation of soil biological activities as a monitoring tool for the degradation process of the model soils was made via measurements of hydrocarbons degrading bacterial counts (HDB), soil respiration (CO_2) and pH. The correlations between these parameters and the levels of the hydrocarbon residues were determined. The residual hydrocarbon content correlated negatively with hydrocarbon utilizers, CO_2 evolution and pH in both soils, and the two levels of pollution. At the end of the 90-day experiments, the highest percentage of oil biodegradation (82 and 64 %) was recorded in soils S_1 and S_2, respectively, contaminated with 30000 mg/kg used lubricating oil that was amended with Amnite p1300, while only (68 and 62 %) of oil biodegradation was recorded in soil S_1 and S_2, respectively contaminated with 45000 mg/kg used lubricating oil amended with Amnite p1300. The results revealed remediation potential of Amnite p1300 to degrade heavy hydrocarbons such as used lubricating oil. It is concluded that contamination of soil with oil hydrocarbons has a negative effect on soil ecosystems, and the extent and duration of these effects will depend on the soil characteristics. Thus, inhibitory effect of used lubricating oil is more pronounced in sandy soil than in clayey soil and show more toxic effect on seed germination and root growth. This study has also showed that soil microbiological parameters may be useful tools for assessing the effect of hydrocarbon contamination on soil and environmental health.

Key words: Bioremediation, biodegradation, used lubricating oil, petroleum hydrocarbons, microbial consortium, soil pollution.

Capítulo 2

EFICÁCIA DO CONSÓRCIO MICROBIANO NA BIODEGRADAÇÃO DE ÓLEO LUBRIFICANTES USADOS EM SOLOS CONTAMINADOS DO BRASIL

RESUMO

O experimento foi realizado com o intuito de avaliar a eficiência de consórcio microbiano (Amnite p1300) na degradação de hidrocarbonetos. Uma simulação usando solos contaminados com óleo lubrificante foi realizada em condições laboratoriais por período de 90 dias. O efeito de outros tratamentos também foi investigado através da sobrevivência microbiana e degradação do óleo lubrificante. Os solos (Argiloso S1 e Arenoso S2) foram contaminados artificialmente a uma taxa de 30000 e 45000 mg/kg em 300g de solo contaminado com óleo lubrificante usado, onde foram adicionados microorganismos de uso comercial que degradam hidrocarbonetos: Amnite P1300 como bioaumento (T1). Os efeitos de outros tratamentos: adição de nutrientes - $(NH_4)_2SO_4$ e K_2HPO_4 (NPK) como tratamento de bioestimulação (T2), atenuação natural (T3) e solo tratado com azida de sódio (NaN3) como controle (T4) sobre a comunidade microbiana e a degradação do óleo de motor usado também foram investigados. A avaliação da atividade biológica do solo como instrumento de acompanhamento do processo de degradação dos solos foi realizada pela contagem das bactérias que degradam hidrocarbonetos, respiração do solo (evolução de CO_2) e pH. A correlação entre esses parâmetros e os níveis de resíduos de hidrocarbonetos foi determinada. O conteúdo residual de hidrocarbonetos correlacionou-se negativamente com os hidrocarbonetos utilizados, evolução de CO_2 e pH em ambos os solos, e nos dois níveis de poluição avaliados. Ao final dos 90 dias do experimento, elevadas taxas de biodegradação do óleo (82 e 64%) foram observadas, respectivamente, nos solos S1 e S2 contaminados com 30000 mg/kg de óleo lubrificante usado e tratados com Amnite p1300. Entretanto, apenas 68 e 62% de biodegradação do óleo foi observado respectivamente nos solos S1 e S2 contaminados com 45000 mg/Kg de óleo lubrificante usado e tratados com Amnite P1300. Os resultados revelaram o potencial de remediação de Amnite p1300 na degradação de hidrocarbonetos de cadeia longa como o óleo lubrificante utilizado neste trabalho. Concluímos que a contaminação de solos com hidrocarboneto de óleo usado tem efeito negativo nos ecossistemas, e a extensão e duração efeitos dependem da natureza dos hidrocarbonetos contaminantes e das características do solo. Entretanto, o efeito inibitório do óleo lubrificante usado é mais pronunciado em solos arenoso que argiloso, demostrando efeito tóxico na germinação de sementes e crescimento de raízes. Este estudo também mostrou que os parâmetros microbiológicos do solo podem ser ferramentas úteis na avaliação dos efeito da contaminação de solos por hidrocarbonetos e sobre a saúde ambiental.

Palavras-chaves: Biorremediação, biodegradação, óleo lubrificante usados, hidrocarbonetos de petróleo, consócio microbiano, poluição de solo.

1. INTRODUCTION

The rise in consumption of automotive lubricating oil is a worldwide problem and has increased, ending in large used oil volume and its waste. In most of the developing countries, the problems tend to worsen with economic and population growth, rapid industrialization, and disregard for environmental health, particularly in relation to used motor oil. The main component of the base oil is cyclic alkanes (c-alkanes). Long chain hydrocarbon and c-alkanes are known to be recalcitrant to microbial degradation. The base oil contains C_{16}-C_{36} hydrocarbon, and more than 75% c-alkanes. The ring numbers of c-alkanes in the base oil are from 1 to 3 and any ring contains 5 or 6 members. Most of the c-alkanes in the base oil have long alkyl side chains (Koma *et al*., 2003). After a period of usage of lubricating oil to lubricate parts of an automobile engine, in order to smooth engine operation, metals and heavy polycyclic aromatic hydrocarbons (PAHs) get accumulated (Hagwell et al., 1992; Boonchan et al., 2000), and that would contribute to chronic hazard including mutagenicity and carcinogenicity (Butler and Mason, 1997). Prolonged exposure and high oil concentration may cause the development of liver or kidney disease, possible damage to the bone marrow and increased risk of cancer (Mishra *et al*., 2001). In addition, PAHs have a widespread occurrence in various ecosystems that contribute to the persistence of these compounds in the environment (Van Hamme *et al*., 2003). Damage derived from petroleum hydrocarbon contamination will depend on the type and concentration of the contaminant. It has generally been accepted that the susceptibility of hydrocarbons to microbial attack increases in the following order: cyclic alkanes < low-molecular weight aromatics < branched alkanes < n-alkanes (Obbard et al., 2004). The toxicity of an organic pollutant will also depend on the soil characteristics, the higher the soil cation exchange capacity (high organic matter or clay content), the higher the adsorption of the organic pollutant and the lower its toxic effect on the ecosystem (McBride, 1994).

Mechanical methods to reduce hydrocarbon pollution are expensive and time consuming. The cheap, effective and safe method for reducing hydrocarbon pollution could possibly be done through microbial degradation. Biodegradation of complex hydrocarbon usually requires the cooperation of more than a single bacterial species. This is particularly true in pollutants that are made up of many different compounds such as petroleum

compounds and complete mineralization to CO_2 and H_2O is desired. Individual microorganisms can metabolize only a limited range of hydrocarbon substrates, so assemblages of mixed populations with overall broad enzymatic capacities are required to bring the rate and extent of petroleum biodegradation further. Microbes are the main degraders of petroleum hydrocarbons contaminated ecosystems (Leahy and Cowell, 1990). Bioremediation has become an alternative way of remediation of oil polluted sites, where the addition of specific microorganisms (bacteria, cyanobacteria, algae, fungi, protozoa) or enhancement of microorganisms already present can improve biodegradation efficiency in both in-situ or ex-situ procedures (Cookson, 1995). Activities of microorganisms are essential to nutrient cycling in soils, and any effect which pollution has on soil microorganisms will also affect vegetation development, ecosystem functioning and productivity (Bauer et al., 1991; Ladd et al., 1996). Soil microorganisms are very sensitive to any ecosystem perturbation, since their diversity and activity are rapidly altered by such perturbation (Schloter et al., 2003). The measurement of microbiological parameters, such as soil respiration, microbial biomass, provides information on the presence and activity of viable microorganisms as well as on the intensity, kind and duration of the effects of hydrocarbon pollution on soil metabolic activity; such measurements may serve as a good index of the impact of pollution on soil health (Brohon et al., 2001; Eibes et al., 2006). However, results on the effects of hydrocarbon pollution on microbial biomass and activity are not always coincident, probably due to the differences in chemical properties of the hydrocarbon used (Xu and Johnson, 1995). It is also true that contaminants may well serve as organic carbon sources, and an enrichment of oil-degrading microbial populations has been observed in most contaminated ecosystems (Margesin et al., 2000). A better understanding of the effect of hydrocarbon contaminants on plant and soil microorganisms may be of help in assessing the recovery potential of a soil.

Concentration of petroleum hydrocarbon determines to a greater extent the rate of breakdown of the hydrocarbons from soil environment. High concentration of hydrocarbon inhibits microorganisms, and the concentration at which inhibition occurs varied with the compound. Report of (Ijah and Antai, 2003) showed high degradation of hydrocarbons in soil contaminated with 10% and 20% crude oil compared to those contaminated with 30 %

and 40 % crude oil which experienced partial degradation of hydrocarbons within a period of 12 months. Rahman et al., (2002) reported that percentage of degradation by mixed bacterial consortium decreased from 70 % to 52 %, as the concentration of crude oil increased from 2.5 % to 10 %. The work of Rambeloarisoa et al. (1984) also demonstrated that crude oil degradation was inversely proportional to the concentration of oil. High concentrations of hydrocarbons can be associated with heavy, undispersed oil slicks in water, causing inhibition of oil biodegradation due to oxygen limitation or through toxic effects exerted by volatile hydrocarbons on microorganisms. Contamination by oil is generally expected to reduce the biodiversity of the soil microbiota (Atlas et al. 1991). A probable explanation for this is that the addition of large amounts of hydrocarbons selects for a limited number of fast-growing hydrocarbon degraders, which are enriched in the (typically oligotrophic) soil environment under these conditions. However, there is a lack of information on how this may be modulated by differing complex environmental matrices, and in particular, how different soil types and contamination level may affect the development of hydrocarbon-degrading populations. Previous microcosm studies examining the effect of oil contamination on soil microbial communities have focused on a single soil type (e.g. Engelen et al., 1998; Macnaughton et al., 1999). Adeyemo et al., (2012) compared one level (1.5 % w/w) of hydrocarbon contamination in two clayey soils and a sandy soil. Wünsche et al., (1995) also investigated hydrocarbon contamination in two different soil types. However, as one was a historically contaminated refinery soil and one was a contaminated pristine arable soil, it was not possible to separate the effects of soil type and differing oil treatment. It has been shown that even a simple measure of microbial response to oil contamination, such as a toxicity bioassay, gives different responses in different soil types (Marwood et al. 1998), it is therefore very important to understand how different soil types and contamination of hydrocarbons affect the biodegradation of used lubricating oil. The objectives of this work were to investigate the potential use of commercial hydrocarbon degrading bacteria consortium for degrading heavy hydrocarbons in used lubricating oil, and to utilize the commercial bacterial for the possible enhancement of used lubricating oil biodegradation in two different soil types (one clayey and one sandy) differing in their clay

and organic matter content under laboratory conditions at different oil pollution (3.0 % w/w and 4.5 % w/w) concentrations.

2. MATERIAL AND METHODS

2.1. Soil sample. The soil sample used was collected from two locations in Minas Gerais, Brazil with no known history of petroleum product contamination. A single large core was collected for each soil type from the A horizon, not including the surface litter layer, kept in a hermetic bag and transported to the laboratory for analysis. The soil was sieved using a 5 mm diameter stainless sieve. The characteristics which were determined using standard techniques are as listed in Table 1. Used lubricating oil was collected from a gasoline and car service station in close proximity to the Federal University of Viçosa, Viçosa, Minas Gerais, Brazil. Amnite p1300 special microbial consortium specially made to degrade used motor oil were obtained from Cleveland Biotech Ltd., UK.

Table 1: Selected physical and chemical characteristics of the noncontaminated soil samples

Parameters	Soil 1 (S_1)	Soil 2 (S_2)
pH (H_20)	5.20	4.92
Total Nitrogen (%)	0.43	0.11
Avail. P (mg/dm^3)	1.00	0.40
Organic C (dag/kg)	3.50	0.81
C:N ratio	8.14	7.56
ECEC ($cmol_c/dm^3$)	3.19	0.78
Moisture Content (%)	33.80	11.30
Sand (dag/kg)	11.00	68.00
Silt (dag/kg)	9.00	4.00
Clay (dag/kg)	80.00	28.00
Texture	Clayey	Clay loamy sand
Soil Type	Red Latosol	Red Yellowish Latosol
Parent Material	Sete Lagoas - MG	Tres Marias - MG

2.2. Soil analysis. Table 1 shows the origin and selected physical and chemical characteristics of the noncontaminated soil samples used for the bioremediation studies. Nitrogen content of the soil was determined by using Kjeldahl method, the available phosphorus was analysed by colometry after Mehlich 1 extraction and Organic Carbon content was determined using a modifield Walkley-Black (1934) method. The pH was determined using 1:2.5 ratio by weight with distilled water (w/v) after 30-min equilibration using a pH meter and electrode calibrated with pH 4.0 and 7.0 standards. Determinations were made in triplicate.

2.3. Soil microcosms. The artificially contaminated model 300 g of soils were manually mixed with used lubricating oil at room temperature of (25 ± 1 ^{o}C) under laboratory conditions. The soil was spread evenly and thinly in a large glass dish; the oil was added at a level of 30000 mg/kg and 45000 mg/kg dry weight of soil (3 % and 4.5 % w/w) respectively, poured evenly over the surface, and then mixed with a stainless steel spatula for 5 min before transferring to the microcosm (one litre (1L) glass flasks sealed with teflon-lined rubber stoppers). Since it is common for authentically-contaminated soils to have similar or higher oil concentrations (e.g. Saterbak et al. 1999), the concentrations of the added oil was similar to earlier microcosm studies (e.g. Wünsche et al. 1995). The microcosms were used to simulate the comparative effect of spent oil addition and bioremediation using a commercially available hydrocarbon degrading microbial consortium (Amnite p1300), special microbial consortium consisting of a mixture of *Bacillus subtilis, Bacillus megaterium, Pseudomonas putida, Pseudomonas fluorescens, Phanerochaete chrysosporium, Rhodococcus rhodocrous* on a cereal (bran) as the bioaugmentation treatment. The microorganisms were conditioned to degrade heavy hydrocarbons. The concentration of microbial population in Amnite p1300 was approximately 5 x 10^{8} cfu/g of bran. In addition to bioaugmentation using microbial consortium, the polluted soils were amended with ($(NH_4)_2SO_4$ and K_2HPO_4) as biostimulation treatments. The C:N:P ratio of the nutrient compound was adjusted to 100:7.5:1 (optimum conditions), similar conditions provided in the biostimulation treatment were adopted in the bioaugmentation treatments plus the addition of Amnite p1300. The unammended soil without addition of nutrients and bacterial inoculums (natural attenuation), was included to indicate hydrocarbon degradation capability of microorganisms naturally present in the

contaminated soils (i.e. the autochthonous microbes). There was a control soil in which most of the indigenous bacteria were killed by the addition of biocide sodium azide (NaN_3) (0.3% w/w) to inhibit soil microorganisms and to monitor abiotic hydrocarbon losses on the microbial community in two different soil types. There were six sampling dates; hence 48 microcosms in total were used. Microcosms were arranged in a random order, and rearranged every 2 weeks ± 2 days throughout the duration of the experiment. The treatments were set up in triplicate, while the content of each container was tilled every week for aeration, and the moisture content was maintained at 70 % water holding capacity by the addition of sterile distilled water every week throughout the period of the experiment (90 days) (Pramer and Bartha, 1972).

2.4. Gravimetric measurement of used lubricating oil loss in soil. The extents of used lubricating oil biodegradation in soil were also determined gravimetrically after microwave extraction according to the EPA method 3546 (US EPA, 2007) using the Microwave Automated Reaction System from CEM (Matthews, NC). Briefly, Sodium sulfate (Na_2SO_4) was purified by drying overnight in an oven at 150°C and quickly transferred into a desiccators. Five grams (5 g) of homogenised contaminated soil was weighed out, mixed with 5 g dry anhydrous Na_2SO_4 and ground to less than 1 mm particle sizes, extracted in GreenChem vessels with 25 mL of a 1:1 hexane:acetone mixture according to manufacturer's protocol at 100 °C for 20 minutes. The n-hexane and acetone was filtered through whatman No 1 filter paper to separate the extract from the soil particles, and transferred into 100 mL amber vials through separatory funnel and sequentially rinsed with equal volume of solvent mixture. The solvent – oil mixture was later transferred into 100 mL Florentine flask of known weight, the solvent was evaporated into a partial dryness using rotary evaporator (Fizatom Rotavapor 801), and later completely using nitrogen gas. The new weight of the flask (now containing residual oil) was recorded. Percentage biodegradation of used lubricating oil was calculated using the formula of Ijah and Ukpe (1992):

$$\%\ biodegradation\ = \left[\frac{weig\,ht\ of\ \ oil\ \ (control\ \ ) - weig\,ht\ of\ \ oil\ \ (degraded\ \ )}{weig\,ht\ of\ \ oil\ \ (control\ \ )}\right] \times 100 \qquad (1)$$

2.5. Determination of petroleum hydrocarbons in soils. Total Petroleum Hydrocarbons (TPHs) were extracted according to EPA method 3546 (US EPA, 2007) using the Microwave Automated Reaction System from CEM (Matthews, NC). Briefly, sodium sulfate (Na_2SO_4) was purified by drying overnight in an oven at 150°C and quickly transferred into a desiccators. Five grams (5 g) of homogenized contaminated soil was weighed out, mixed with 5 g dry anhydrous Na_2SO_4 and ground to less than 1 mm particle sizes and extracted in a 25 mL GreenChem vessels containing 1:1 hexane : acetone mixture according to manufacturer's protocol, and were kept at 100 °C for 20 minutes. The n-hexane and acetone mixture was filtered through whatman No 1 filter paper to separate the extract from the soil particles, and transferred into 100 mL amber vials through separatory funnel which was sequentially rinsed with equal volume of solvent mixture. The solvent were evaporated to partial dryness with a rotary evaporator (Fizatom Rotavapor 801), transferred into 2 mL vials and then dried completely using nitrogen gas. Dried samples were dissolved in 600 µL dichloromethane for gas chromatography analysis. The residual oil was analyzed on Shimadzu GC-17A chromatograph equipped with a Flame-Ionization Detector (FID) by using fused silica capillary column DB-5 (30 x 0.25 mm), and AOC-17 Shimadzu auto injector complying with Environmental Protection Agency (EPA) standard method 8015 (US EPA 2007). The flow rate of the helium carrier gas was 1.81 mL/min with linear velocity of 38.49 cm/s. The initial temperature was programmed at 40^{o}C and held for 15 min. The temperature was then increased to 280^{o}C at a rate of 10^{o}C /min. The final temperature was held for 31 min. The injector was set in the split mode. The split ratio was set to 1:10; the injection volume was 1 µL and the injector and the detector temperature for GC were maintained at 260 and 280°C respectively and the oven temperature was programmed to rise from 40 to 280°C in 10°C/min increments and to hold at 280°C for 31 min. The dry weight of the soil samples was determined following baking of 10 g of wet soil at > 80 °C for at least 48 hours. Before analyzing the sample extract, a mixture of standards including *n*-alkanes (*n*-decane *n*-C_{10}, *n*-dodecane *n*-C_{12}, *n*-tetradecane *n*-C_{14}, *n*-hexadecane *n*-C_{16}, *n*-octadecane *n*-C_{18}, *n*-eicosane n-C_{20}, *n*-docosane *n*-C_{22}, *n*-tetracosane *n*-C_{24}, *n*-hexacosane *n*-C_{26}, *n*-octacosane *n*-C_{28} and a pure standards containing *n*-triacontane *n*-C_{30}, *n*-dotriacontane *n*-C_{32}, *n*-tetratriacontane *n*-C34, and *n*-hexatriacontane *n*-C_{36}, and a mixture of polycyclic aromatic hydrocarbon consisting of acenaphthene, acenaphthylene, anthracene, benzo (a) anthracene, benzo (a)

pyrene, benzo (b) fluoranthene, benzo (g, h, i) perylene, benzo (k) fluoranthene, chrysene, dibenz (a, h) anthracene, fluoranthene,fluorine, indeno (1, 2, 3-cd) pyrene, naphthalene, phenanthrene, pyrene, 1-methylnaphthalene and 2-methylnaphthalene, Supelco) were used for calibration. Five points calibration curves using peak areas were obtained and the response factors were used to determine the concentrations of various hydrocarbons in the sample extract. The total petroleum hydrocarbons were identified and quantified by comparing the peak area of samples with that of the standard of the TPH mixture with reference to the curve derived from standards. Percentage of degradation was calculated by the following expression:

$$\%\ biodegradation\ = \left[\frac{TPH\ control\ -TPH\ treatment}{TPH\ control}\right] \times 100 \qquad (2)$$

TPH data were fitted to the first-order kinetics model (Yeung et al., 1997):

$$H = H_0\, e^{(-kt)} \qquad (3)$$

Where H is the residual hydrocarbon content in soil (mg/kg), H_0 is the initial hydrocarbon content in soil (mg/kg), k is the biodegradation rate constant (day^{-1}) and t is time (days). This model was based on the assumption that the degradation rate of hydrocarbons positively correlated with the hydrocarbon pool size in soil. The model was used to estimate the rate of biodegradation and half-life of hydrocarbons in soil under each treatment. The biodegradation rate constant (k), and half-life ln(2)/k of the hydrocarbons in soil during the bioremediation process were calculated from the model using Statistical ® software (Stat Soft. Inc. (2007).

2.6. Enumeration of hydrocarbon degrading bacteria. The study was conducted at room temperature and the enumeration was carried out at 0, 15, 30, 45, 60, 75 and 90 days. To monitor cell numbers and biodegradation, 1 g of soil was removed from each microcosm at the set times and suspended in 9 mL of saline solution in sterile centrifuge tubes. The mixture was vigorously shaken on a vortex mixer for 3 minutes and then the soil particulates were allowed to settle for 1 min before 0.1 mL of the supernatant fluids were sampled for CFU counts. The number of colony-forming hydrocarbon-degrading bacteria (HDB) was done by plating three replicate samples from each treatment withdrawn every 15 days on a mineral medium containing used motor oil as the sole carbon source. The mineral medium contained 1.8 g K_2HPO_4, 4.0 g NH_4Cl, 0.2 g $MgSO_4.7H_2O$, 1.2 g KH_2PO_4, 0.01 g $FeSO_4.7H_2O$, 0.1 g

NaCl, 20 g agar, one percent (1%) used engine oil in 1,000 mL distilled water, and the medium was adjusted to pH 7.4 (Zajic and Supplission, 1972). The oil agar plates were incubated at 30°C for 7 days before the colonies were counted.

2.7. Soil respiration. Amended-moist soil corresponding to a 300 g dry soil with water holding capacity (WHC) of approximately 70 % was incubated in triplicate at (25 ± 1 ^{0}C) under laboratory conditions, in one Litre (1L) glass flasks sealed with teflon-lined rubber stoppers. Microbial activity was monitored by analyzing CO_2 accumulation in the headspace by gas chromatography. Headspace samples of 1 cm^3 were taken from microcosms with a Hamilton gastight® syringe and injected into a Gas Chromatograph Model GC- 14B (Shimadzu Crop Kyoto Japan), with a thermal conductivity detector (TCD) at temperature of 150 ° C, injector at 100 º C and Porapak -Q column at 50 º C, using nitrogen as a carrier gas. Respiration rate was expressed as evolved CO_2 in mL CO_2 m^{-2} h^{-1}, and the accumulated CO_2 concentration in mg CO_2/ kg of dried soil. The amount of CO_2 evolved during the mineralization of used lubricating oil was monitored using the accumulated concentration of CO_2 recorded from the CG-TCD. Microcosms were aerated for 15 min after CO_2 measurement to maintain oxygen levels in the system. Microcosms were set up in triplicate in tightly closed glass flasks. To determine metabolic activity in each microcosm, respiration through CO_2 emission monitoring were periodically performed. Each 1-L glass flask contained 300 g moist soils to which used lubricating oil was added. The respiration mean of the blanks was subtracted from the treated microcosms, and the difference in CO_2 production between the blanks and the treated microcosms was used as the amount of CO_2 produced. Analysis of released CO_2 was done in triplicate, and data were subjected to statistical analysis using general linear model of analysis of variance (ANOVA). Significant treatment means were compared using Tukey test at $P > 0.05$ (Statistical Software 8.0: Stat. Soft, 2007), to determine the significance of differences between microcosms.

2.8. Germination toxicity test for the remediated soil. Toxicity of the remediated soils was assessed by germination tests using wheat seeds. Wheat (*Triticum aestivum*) was used in this study owing to its sensitivity to hydrocarbon in soil (Banks and Schultz, 2005). The germination test was conducted over a 7-day test period. Seeds of wheat were obtained

commercially. For each soil sample, 150 g of thoroughly mixed remediated soil was placed in 110 × 110 x 35 mm germination box. Twenty viable seeds of wheat were added and evenly spread into each germination box. Three replicates of the samples were prepared. The moisture of the soil (each soil) was maintained at 80 % water holding capacity. Germination boxes were incubated for 7 days in a plant test chamber with photoperiod of 16 h light and 8 h dark at 20 ^{o}C. At the end of the test, the number of germinated seeds was counted, recorded and compared with the control. Germination index of wheat seed on the remediated soil was calculated using the formula of Millioli et al., (2009):

$$Germination\ index\ (\%) = (\%\ SG)\ \times\ (\%\ GR)/100 \quad (4)$$

$$\%\ SG = \left({}^{\%\ EG}/_{\%\ CG}\right) \times\ 100 \quad (5)$$

$$\%GR = \left({}^{GERm}/_{GERCm}\right)\ \times 100 \quad (6)$$

where % SG = seed germination,

% EG = germination on contaminated soil,

% CG = germination on control soil,

% GR = growth of the root,

GERm = elongation of root on contaminated soil,

GERCm = elongation of root on control soil.

2.9. Statistical data analysis. Data collected were subjected to statistical analysis using general linear model of analysis of variance (ANOVA). Significant treatment means were compared using Tukey test at P > 0.05 (Statistical Software 8.0: Stat. Soft, 2007).

3. RESULTS AND DISCUSSIONS

3.1. Biodegradation of used lubricating oil. Following a better biodegradation extent in our earlier studies using only one contamination level in three types of soils (Adeyemo et al., 2012), the biodegradation of used lubricating oil was studied using Aminte p1300, a commercial bacterial consortium, was investigated. Trends in the biodegradation percentage in the soil contaminated with 3.0% and 4.5% used lubricating oil are shown in Fig.1

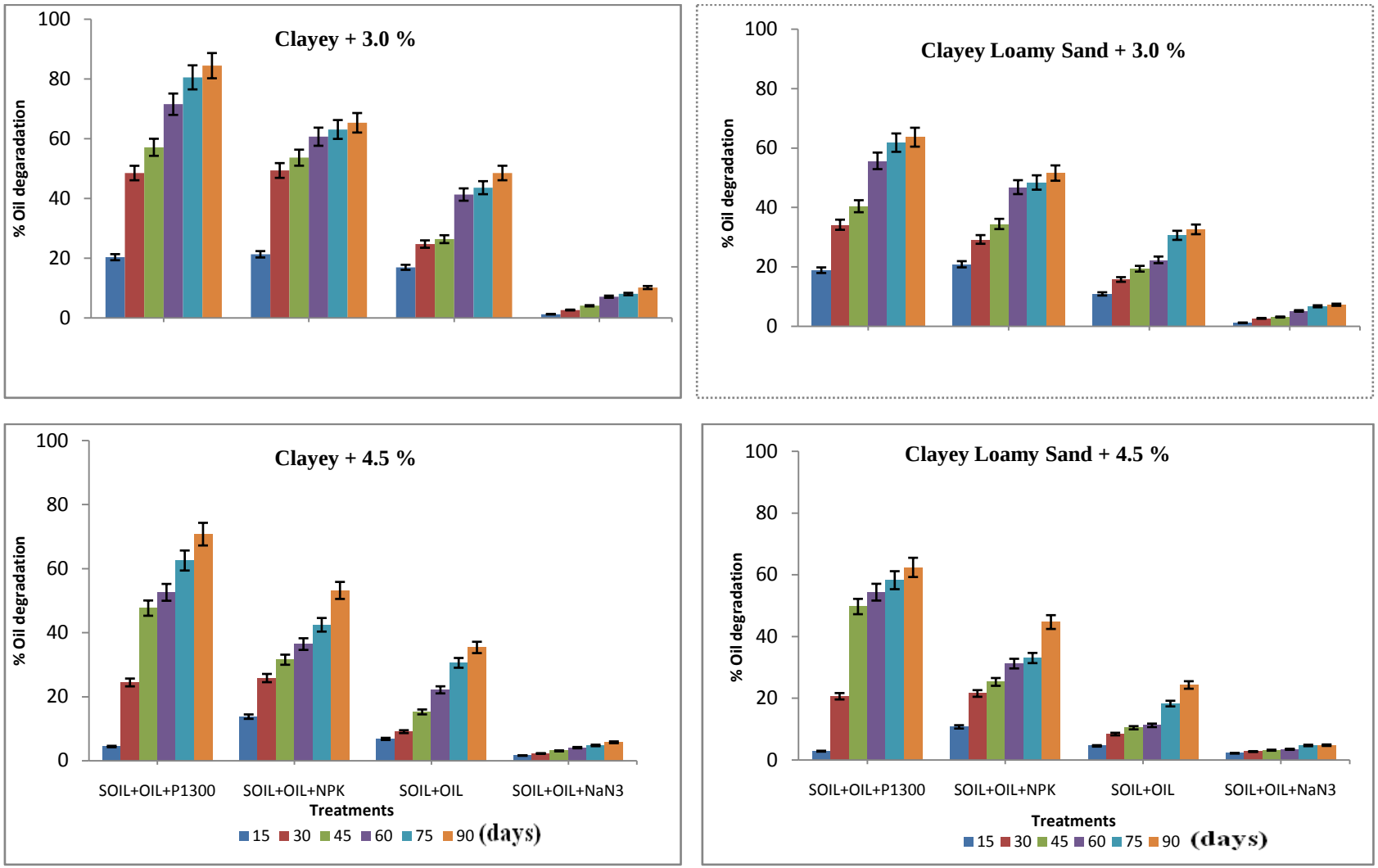


Figure 1: Biodegradation of petroleum hydrocarbon in soil contaminated with 3.5 and 4.5% used lubricating oil in soil 1 (S1) and Soil 2 (S2). Vertical bars indicate standard error of the mean SE (n = 3).

The results showed the highest biodegradation percentage (84% and 64%) in soil 1 (S_1) and soil 2 (S_2), respectively, contaminated with 3.0% of the used lubricating oil amended with Amnite p1300 (T_1) compared to (65% and 52%) in T_2; (49% and 33%) in T_3 and (10% and 7%) in T_4 at the end of 90-day experiment. A higher degradation percentage observed in S_1 compared to S_2 in this study might be due to the higher clay contents of S_1. Soils of high clay contents have been shown to offer greater capacity for physicochemical attenuation of contaminants than coarse sands (Pye and Patrick, 1983). The low percentage of contamination with (3.0%) used lubricating oil might be the reason for the relatively high and progressive biodegradation in the soil, because it does not pose a serious challenge to the metabolic activities of soil microorganisms. It may also be due to the mixed bacterial consortium that is present in T_1 that combine individual's effect of the bacterial strains for better degradation (Rahman et al., 2002; Ghazali et al., 2004). With the increase in the concentration of the contaminated soils (4.5%), lower percentage of degradation (71% and 62 %) was observed in S_1 and S_2, respectively, in T_1 compared to the previous level of contamination (3.0%). Similar trends were recorded in soil contaminated with 4.5% in T_2, T_3 and T_4 (53% and 45%), (35% and 24%) and (6% and 5%) respectively. This may be attributed to the toxicity of the oil on the microbial flora of the soil and thus the high concentration of oil which might likely had negative effects on the biodegradative activities of the microbial population in the contaminated soil. (Adesodun and Mbagwu, 2008). Higher degradation was observed in our previous work (Adeyemo et al., 2012) with reduced concentration of the used motor oil in soil following application of microbial consortium (Amnite p1300) with the same treatment. The result is in agreement with the findings of Rahman et al., (2002) who reported decrease in the rate of biodegradation of crude oil, as the concentration of oil increased.

The results of this present study show that highest biodegradation rates were recorded in soil contaminated with 3% oil compared with 4.5%. This high biodegradation rate could be attributed to increase in the activity of soil microbes in the oil polluted soils (Adesodun and Mbagwu, 2008). Amnite p1300 (T1) amended soil recorded highest percentage biodegradation (84% and 64%) in 3% oil-contamination and (71% and 62 %) in 4.5% oil contamination in S1 and S2, respectively, throughout the 90-day period, compared to the poisoned control soil treated with sodium azide which recorded 10% and 7% in 3% oil-

contamination and (6% and 5%) in 4.5% oil contamination in S1 and S2, respectively. This might be due to the combined effect of hydrocarbon-utilizing microbial consortium present in the T1. The vendor (Cleveland Biotech, UK) of the microbial consortium claimed that the consortium (Amnite p1300) had been conditioned to carry out effective and efficient biodegradative activities of heavy petroleum hydrocarbons. Some removal of hydrocarbons was also seen in the soil which was not amended with commercial bacterial consortium. This removal could be attributed to the combined actions of indigenous microbial population stimulated by the addition of nutrients to the polluted soil as well as abiotic weathering. Abiotic weathering processes in polluted soils include evaporation, photochemical oxidation, and adsorption onto particulate material.

3.2. Biodegradation rate and coefficient of determination. The first-order degradation kinetics was reported in earlier studies based on petroleum hydrocarbon degradations (van Gestel et al., 2003; Namkoong et al., 2002; Jørgensen et al., 2000). First-order kinetic model of Yeung et al. (1997) was adopted in this study to evaluate the biodegradation of used lubricating oil in all treatments (T1, T2, T3 and T4) as presented in Table 2. Biodegradation rate constant (k) of the hydrocarbons during the bioremediation process and coefficient of determination r^2 were calculated from the model. Data from three replicates in each treatment from the six sampling periods (two weeks interval) were combined for evaluation using this model. The coefficients of determination (r^2) revealed that the model fits well to all the treatments. The highest biodegradation rates of 0.0201 and 0.0160/day were recorded in soil contaminated with 3.0% amended with Amnite P1300 in S1 and S2, respectively. In soil contaminated with 4.5%, the highest biodegradation rate of 0.0122 and 0.0117/day were also recorded in the treatment amended with Aminte p1300 in S1 and S2 respectively. Poisoned control amended with sodium azide recorded the lowest biodegradation rate of (0.0011 and 0.0010/day) in 3.0% and (0.0007 and 0.0007/day) in 4.5% oil contamination in S1 and S2 respectively. The results show significant relationships between the rate of biodegradation and concentration of oil in the contaminated soil. It is noticeable that biodegradation rate in 4.5% oil contamination were lower compared with 3.0% in both soils. This observation might be attributed to the toxicity of the oil on the microbial biota of the soil. High concentrations of the oil have negatively affected the biodegradative

activities of the microbial population in the contaminated soil. (Adesodun and Mbagwu, 2008).

Table 2: First-order biodegradation parameters of hydrocarbon in used lubricating oil-contaminated soils.

Treatments	K (day^{-1})		R^2	
	S1	S2	S1	S2
3.0%				
T1	0.0201Db	0.0160Da	0.98	0.96
T2	0.0154Cb	0.0126Ca	0.78	0.94
T3	0.0080Bb	0.0062Ba	0.90	0.98
T4	0.0011Ab	0.0010Aa	0.97	0.95
4.5%				
T1	0.0122Da	0.0117Da	0.92	0.89
T2	0.0071Ca	0.0075Ca	0.74	0.93
T3	0.0021Ba	0.0021Ba	0.70	0.82
T4	0.0007Aa	0.0007Aa	0.96	0.74

T1=soil + oil + Amnite P1300, T2= soil + oil + $(NH_4)_2SO_4$ and K_2HPO_4), T3 = soil + oil alone, T4 = unamended contaminated control soil with sodium azide. S1 = Red Latosol, S2 = Red-yellowish latosol; K = Biodegradation constant (day^{-1}) and R^2 = The coefficients of determination. Values followed by the same capital or small letters are not significant difference between treatments (column) or soil types (row) respectively at the $P<0.05$ level, while values followed by different capital or small letters indicate significant differences between treatments (column) or soil types (row), respectively, at the $P<0.05$ level.

There were significant differences in the biodegradation rate in S1 and S2 contaminated with 3.0%. Differences between the two soils S1 and S2, however, were not significant ($P < 0.05$) with respect to the biogradation rate. As the oil contamination increased to 4.5%, the less efficiency of the commercial microbial consortium with heavy application of used lubricating oil could be attributed to reduction in the activity of the soil microbes at this level of pollution in both soils. Bossert and Bartha (1984) stated that sensitivity of soil micro biota to petroleum hydrocarbons is a factor of quantity and quality of oil spilled and previous exposure of the native soil microbes to oil. Although, La-Rue (1977) reported that most microbes can metabolized a wide range of c-compounds, Schwindinger (1968) stated that

beyond 3.0% concentration, oil becomes increasingly deleterious to soil biota and crop growth. Schaefer and Juliane (2007) also concluded that bioremediation is a useful method of soil remediation if pollutant concentrations are moderate.

3.3. Enumeration of hydrocarbon degrading bacteria. The hydrocarbon degrading bacterial (HDB) counts in T1 contaminated with 3 % ranged from (4.5×10^6 to 2.8×10^8 CFU/g) in S1 and (4×10^6 to 2.5×10^8 CFU/g) in S2. The hydrocarbon degrading bacterial counts was slightly higher in S1 than S2. A similar trend was recorded in soil contaminated with 4.5 % in T1, with lower microbial population ranging from (1.28×10^6 to 6.4×10^7 CFU/g) in S1 and (1.08×10^6 to 5.7×10^7 CFU/g) in S2. The reason might be due to its clay contents through surface adsorption and microbial metabolism in this soil which has been shown to offer greater capacity for physicochemical attenuation of contaminants than coarse sands. (Pye and Patrick, 1983). The high clay soil may also contain active organisms whose metabolism results in contaminant attenuation than coarse sands. The low percentage of contamination with (3.0 %) used lubricating oil might be the reason for the relatively high and progressive biodegradation in the soil. The low percentage oil contamination appeared not to pose serious challenge to the metabolic activities of soil microorganisms. The population of hydrocarbons degrading microbial counts were highest in T1 followed by T2 and T3. Control T4 has the least counts in both soils used for the experiment. This result clearly demonstrates the benefit of bioaugmentation of oil polluted soil with Aminte p1300 products. An enhanced comparison between the four treatments investigated in this work is revealed in Fig. 2. In the control treatment (T4), most of the indigenous bacteria were killed with a biocide (NaN_3). The number of hydrocarbon-degrading microorganisms increased with time both at the contamination levels and the two soils. Already after 15 days, the counts of degrading bacterial consortium on used lubricating oil hydrocarbon showed that soil microorganisms adapted rapidly to the hydrocarbon contamination and were able to utilize the spent oil as carbon source (Fig. 2). The counts of hydrocarbon degrading bacteria (HDB) in both level of contamination (3.0% and 4.5%) in T2 were lower than T1, but, appreciably higher compared to T3 and T4. The reason for higher counts of bacteria in T2 soil might be as a result of presence of appreciable quantities of available nutrients added, which are necessary for bacterial biodegradative activities (Abdulsalam et al., 2011).

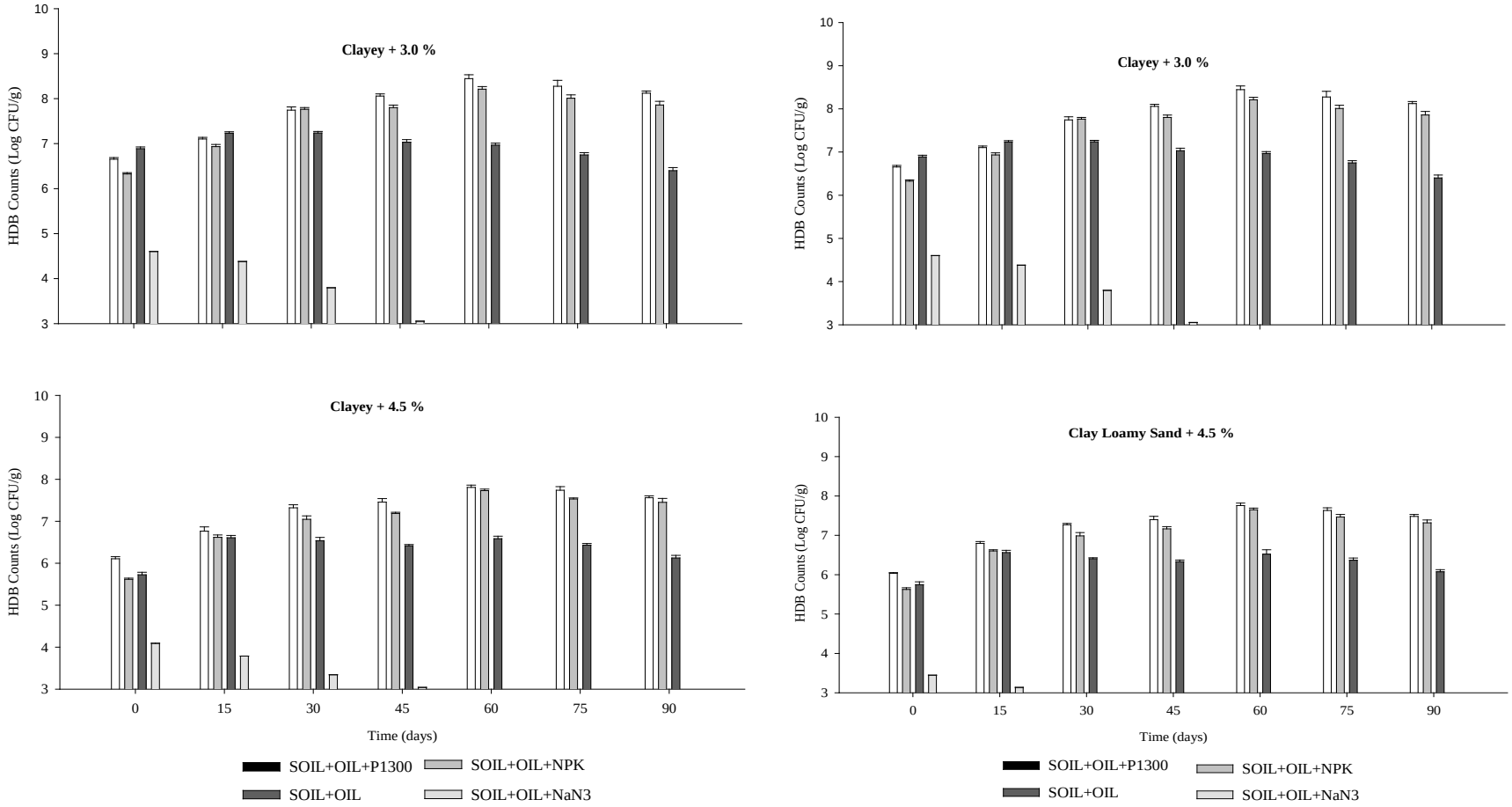


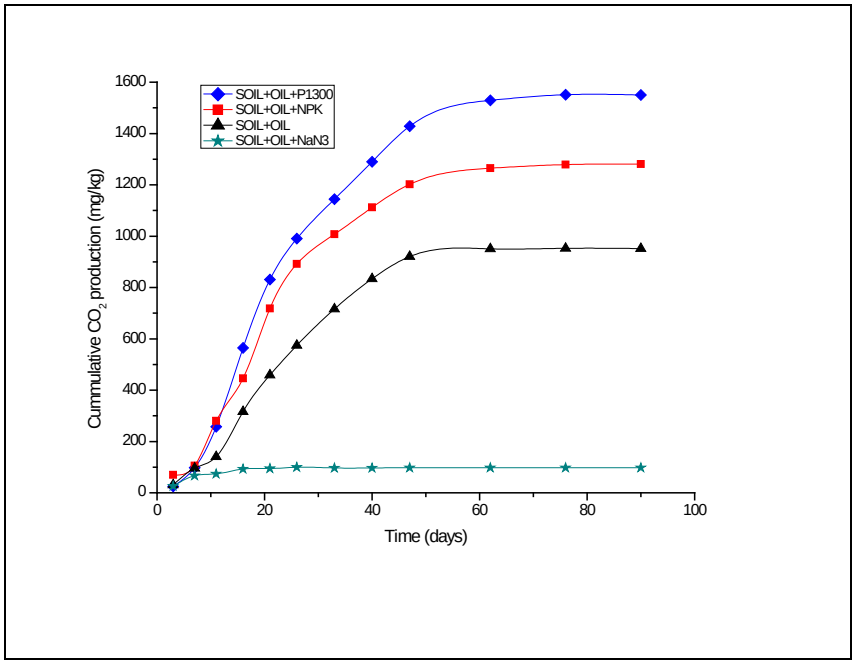
Fig. 2: Hydrocarbon-degrading bacteria (HDB) counts in soils contaminated with 3.0 and 4.5 % used lubricating oil. Vertical bars indicate standard error of the means SE (n = 3)

3.4. Soil respiration (CO_2-evolution) analysis. The values of CO_2 evolved during a 90-day incubation experiment from soil samples at different times in 3.0 % and 4.5 % level of contamination are shown in Fig. 3 and 4 respectively. CO_2 emissions were higher in the clayey soil than in the sandy soil both for the contaminated and the control treatments. This might be due to its higher nutrient contents and microbial metabolism in this clayey soil which has been shown to offer greater capacity for physicochemical attenuation of contaminants than coarse sands. (Pye and Patrick, 1983).

The contaminants at the two loading rates (3 and 4.5 %) assayed, increased soil respiration in both soils, especially in the clayey soil. In the clayey soil, used lubricating oil contaminated soils showed that respiration in 3.0 % loading rates producing the greatest CO_2 emissions. The high amount of CO_2 liberated in clayey soil amended with amnite products and contaminated with 3 % and 4.5 % used lubricating oil is an indication of high utilization of organic carbon nutrients from the petroleum hydrocarbons present in this sample. The exception to this trends was that respiration rate of the samples with higher contamination level of 4.5 % used lubricating oil was lower compared with the soils contaminated with 3 %. This may be due to reduced aeration caused by the very high hydrocarbon concentrations in the soils.

The stimulatory effect of used lubricating oil on soil respiration persisted in both soils throughout the incubation period, and was still noticeable 90 days after contamination (Fig. 3 and 4). The cumulative evolution of CO_2 in the clayey soil (S1) at both level of contamination (3.0% and 4.5 %) in the amended treatments with amnite products (T1) were higher than treatments amended with nutrients (T2) in (3.0% and 4.5 %) level of contamination. The lower values recorded in both soils contaminated with 4.5 % might be due to the toxicity of the spent motor oil to the microorganisms present in the contaminated soils or reduced aeration caused by the very high hydrocarbon concentrations in the soils. Soil respiration (in terms of carbon dioxide - CO_2-evolution) in T1 and T2 were significantly higher than in T3 and T4. The CO_2- evolution in T4 reached maximum values after 30 days, decreasing thereafter and remaining almost constant till the end of the 90- day experiment. Carbon dioxide (CO_2) evolution in T4 indicates that the sodium azide

S1



S2

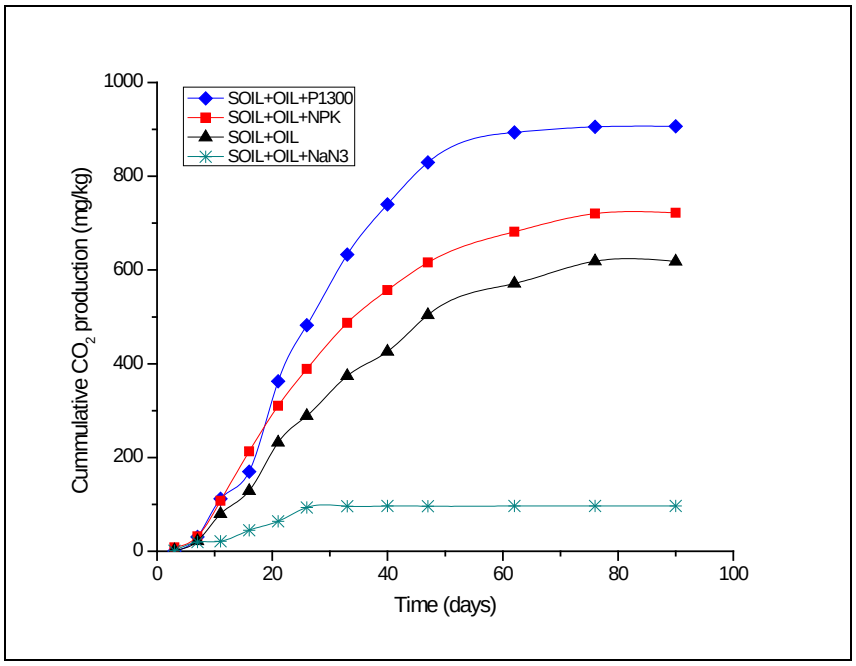
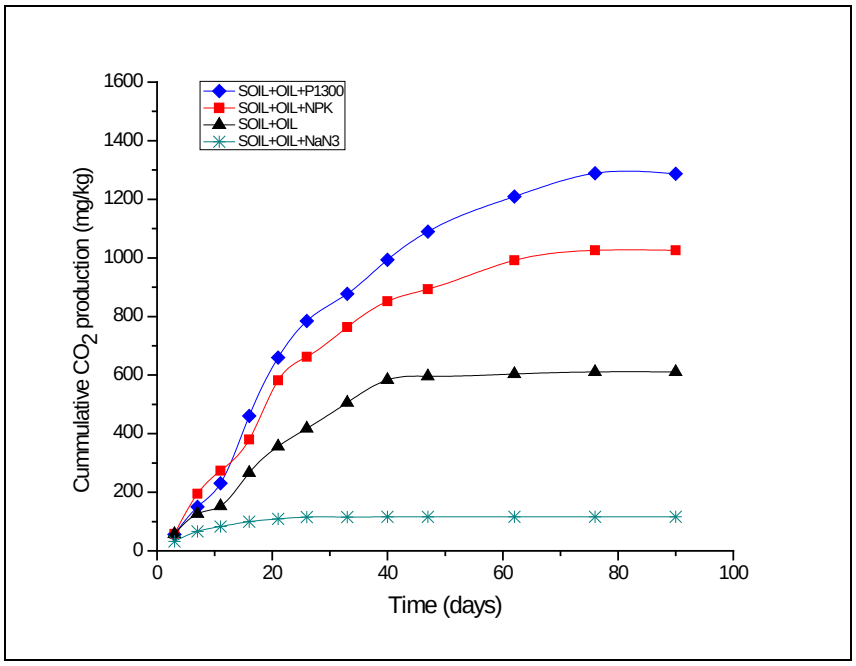


Fig. 3: Cumulative CO_2 production during biodegradation of clayey (S1) and sandy (S2) soils contaminated with 3 % used lubricating oil.

S1



S2

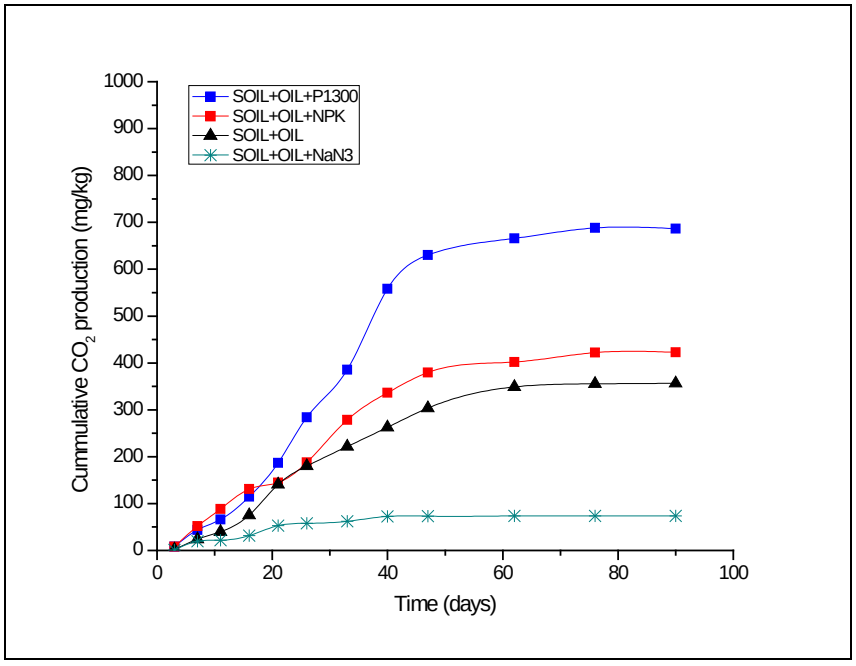


Fig. 4: Cumulative CO_2 production during biodegradation of clayey (S1) and sandy (S2) soils contaminated with 4.5 % used lubricating oil.

biocide used for this experiment was not 100% effective to inhibit the carbon oxidising bacterial metabolism during the experimental period.

3.5. Toxicity and seed germination index. In both soils, the seed germination test was conducted using wheat sown on the oil polluted soils at both 3.0% and 4.5%. Wheat is an important agricultural crops, and its sensitivity to toxic chemicals (mostly petroleum contaminants), has led to its wide use for toxicity tests (Banks and Schultz, 2005). The trends in seed germination on the bacteria-remediated soils that was contaminated with 3.0% and 4.5 % spent oil are shown in Table 3. At the end of the 90-day experiment, the results revealed that 100 %, 80 % and 60 % germination were recorded in 3.0 % soil contamination and 90 %, 60 % and 30 % were recorded in soil contaminated with 4.5 % oil in T1, T2 and T3, respectively, in Soil 1(S1). While 90 %, 70 % and 60 % germination were recorded in 3.0 % soil contamination, 70 %, 60 % and 20 % germination were recorded in soil contaminated with 4.5% oil in T1, T2 and T3, respectively, in Soil 2 (S2). Highest percent germination (100%) was recorded in uncontaminated control in both S1 and S2 at both levels of contamination, while only 30 % and 20 % in S1 and 20% and 10%) in S2 were recorded in poisoned control soils (T4) which were contaminated with 3.0% and 4.5% used lubricating oil, respectively. It is evidenced that the type of soil also influenced the phytotoxic effect, which was more persistent in the sandy soil. The toxicity of an organic pollutant will also depend on the soil characteristics. The higher the soil cation exchange capacity (high organic matter and/or clay content), the higher the adsorption of the organic pollutant and the lower its toxic effect on the ecosystem (McBride, 1994). In the clayey soil (S1), the inhibitory effect was not really pronounced, probably due to the degradation of some of the phytotoxic compounds contained in the hydrocarbons (the resulting metabolites being less phytotoxic). Sequestration and bound-residue formation could also have occurred during organic contaminant degradation in soil, because bound or nonextractable residues (NER) are formed. Part of these residues may be biogenic, because degrading microorganisms assimilate carbon derived from the pollutant and mineralized CO_2 to form cellular components, for example, [fatty acids (FA) and amino acids (AA), which are subsequently stabilized within soil organic matter (SOM) (Scheunert et al., 1985; Karolina et al., 2011). In the sandy soil, the phytotoxicity was higher, perhaps due to a lower degree of hydrocarbon degradation as a

Table 3: Toxicity test based on seed germination (%)

Percentage	Treatments				
Oil pollution	T1	T2	T3	T4	T5
S1 (3%)	100	80	60	30	100
S1 (4.5%)	90	60	30	20	100
S2 (3%)	90	70	60	20	100
S2 (4.5%)	70	60	20	10	100

T1=soil + oil + Amnite P1300, T2= soil + oil + $(NH_4)_2SO_4$ and K_2HPO_4), T3 = soil + oil alone, T4 = unamended contaminated control soil with sodium azide., T5 = uncontaminated soil , S1 = Red Latosol, S2 = Red-yellowish latosol

Table 4: Seed germination toxicity index (%)

Percentage	Treatments			
Oil pollution	T1	T2	T3	T4
S1 (3%)	93.33	49.34	21.20	4.01
S1 (4.5%)	76.71	27.00	7.95	1.93
S2 (3%)	74.97	36.05	16.92	2.50
S2 (4.5%)	52.71	24.60	4.68	0.85

T1=soil + oil + Amnite P1300, T2= soil + oil + $(NH_4)_2SO_4$ and K_2HPO_4), T3 = soil + oil alone, T4 = unamended contaminated control soil with sodium azide., S1 = Red Latosol, S2 = Red-yellowish latosol

consequence of the low microbial biomass content and microbial activity of this soil. The Germination Index (GI) gives an idea of the effect of soil contamination on the seed germination and root growth. Germination index of wheat seed on the remediated soil was calculated using the formula of Millioli et al. (2009). The soils contaminated with used lubricating oil had a stimulatory effect on the seeds germination at higher concentration of 4.5% than 3.0%. Table 4 shows the results of seed germination index in soil contaminated with 3.0% and 4.5% used lubricating oil in all the treatments. Soil treated with Amnite p1300 recorded the highest germination index, 93.33%, and 76.71% in S1 and 74.97 and 52.71% in S2, this result further proved the effectiveness of the commercial bacterial consortium in enhancing biodegradation of hydrocarbon in soil contaminated with used lubricating oil. The

negative effect of hydrocarbons on GI may be attributed to their inherent toxicity and/or to the perturbations they cause in soil and plants due to their hydrophobic properties (Ogboghodo et al., 2004). Hydrocarbons may coat roots, preventing or reducing gas and water exchange and nutrient absorption. They may also enter the seeds and alter the metabolic reactions and/or kill the embryo by direct, acute toxicity. After penetrating the plant tissues, hydrocarbons damage cell membranes and reduce the metabolic transport and respiration rate (Amadi et al., 1992; Xu and Johnson, 1995). Xu and Johnson (1995) indicated that the contaminant could have opposite effects on the plant and microorganism growth. These authors also indicated that the higher the concentration of unsaturated compounds, aromatic, and acids, the more toxic the hydrocarbons are to soil microorganisms.

3.6. Correlations. The results of the regression and correlation analyses demonstrated that several of the investigated soil parameters correlated significantly with the residual soil hydrocarbon content in both soils S1 and S2 following contamination with 3.0% and 4.5% used lubricating oil during the 90-day period of study (Table 5). Hydrocarbon content correlated strongly and negatively with soil pH, the number of hydrocarbon bacteria utilizers ($P < 0.001$), soil respiration (CO_2-evolution) and percentage oil degradation ($P < 0.01$). There were also several significant correlations between other measured soil parameters (Table 5). The Soil pH correlated significantly and positively with hydrocarbon utilizers and soil respiration at both level of contamination in both soils.

Table 5. Correlation matrix (coefficients and significance levels) for the investigation parameters during 90 days biodegradation experiment

Soil Cont. Level	Variables	Hydroc Conc.	pH	HDB	CO_2
	pH	-0.7356***			
S1 (3.0%)	HDB	-0.7889***	0.7266***		
	CO_2	-0.9187***	0.6893***	0.7332***	
	% Oil Degradation	-0.9999**	0.7385***	0.7878***	0.9206**
	pH	-0.5306***			
S1 (4.5%)	HDB	-0.8801**	0.4667***		
	CO_2	-0.8669**	0.4379***	0.7523***	
	% Oil Degradation	-0.9632**	0.5302***	0.8099***	0.9485**
	pH	-0.7384**			
S2 (3.0%)	HDB	-0.7864***	0.7794***		
	CO_2	-0.8433**	0.6599***	0.6045***	
	% Oil Degradation	-0.9940**	0.3207**	0.8100***	0.8099***
	pH	-0.5164***			
S2 (4.5%)	HDB	-0.8975**	0.4164***		
	CO_2	-0.8288**	0.8136***	0.6816***	
	% Oil Degradation	-0.9951**	0.5514***	0.8801**	0.8628**

. Sample number =24 (values were determined with six data points and four soil treatments; each data points represents the mean of three replicates; **P <0.01, ***P<0.001.

3.6.1. Correlation between CO_2 evolution and oil biodegradation. The relationships between oil biodegradation and concentration of CO_2 evolved in soils contaminated with 3 %, and 4.5 % used lubricating oil in different treatments T1, T2, T3 and T4 are shown in Figures 7 to 10. The results of oil biodegradation in soil contaminated with used lubricating oil with 3 and 4.5 % in amended soil with hydrocarbon degrading bacterial consortium products, soil amended with nutrients and unamended soil show strong positive linear correlation with CO_2 evolution, while that of control, where most of the available microorganisms have been killed with sodium azide, shows weak correlation in soil 1 (S1) at both level of pollution 3.0 and 4.5 %. Polluted soil without any amendments (T3) shows better correlation ($r = 0.97$ and 0.99) in both soils S1 and S2, respectively, than those amended with microbial consortium commercial products (Amnite p1300) and nutrient amended soil (T2) at 3.0% oil pollution. This might be due to unavailability of the organic compounds to the bacteria community as a result of the biodegradation that occurred in the amended soils. Commercial microbial consortium amended soil shows strong correlation of (0.96, 0.98) at 3 % and (0.98, 0.88) at 4.5 % in soils 1 and 2 respectively. This might be due to the ability of the microbial consortium products to degrade heavy hydrocarbons effectively. Nutrients amended soils (T2) also had a strong correlation of (0.90, 0.98) at 3 % and (0.98, 0.95) at 4.5 % in soils 1 and 2 respectively. Nutrients availability to the indigenous bacteria might contribute to their population increase and thereby increase degradation of the organic compound. The positive linear correlation between CO_2 evolution and oil biodegradation recorded in most of the treatments can be attributed to the increase in microbial activities in all the treatments which implies that most of the oil breakdown in the contaminated soil can be attributed to microbial degradation due to appreciable release of CO_2 during the process of oil breakdown. These results agrees with the findings of several authors (Ijah and Antai, 2003b; Roling et al., 2004 and Morais and Tornisielo 2009), who reported positive linear correlation of oil biodegradation with high CO_2 evolution in oil contaminated.

3.7. Biodegradation of hydrocarbon fractions. Biodegradation of hydrocarbon fractions present in the used lubricating oil was determined at fifteen days interval for 90 days using GC/FID. The hydrocarbon fractions were divided into three fractions which are: $C_{10} – C_{14}$, $C_{15} – C_{28}$ and $C_{29} – C_{36}$ (Alberdi et al., 2001).

3.7.1 Biodegradation of $C_{10} – C_{14}$ fractions in used lubricating oil. Oil-contaminated soil with 3 % amended with commercial bacterial consortium (Amnite P1300) and Nutrients $(NH_4)_2SO_4$ and K_2HPO_4 recorded complete biodegradation of $C_{10} – C_{14}$ hydrocarbon fractions below the detection limit within ninety days in the clayey soil (S1) compared to unamended and the control soil. In the sandy soil (S2) however, only the treatment with Amnite P1300 recorded complete degradation below the detection limit. There was no complete degradation of the fractions ($C_{10} – C_{14}$) in nutrients amended, unamended and sterile contaminated soils throughout the ninety days periods. Soil contaminated with 4.5% used lubricating oil did not record any oil biodegradation below detection limit in all the treatments within the ninety days of the experiment, but there were appreciable degradation in the soils contaminated with the used lubricating oil amended with Amnite P1300, followed by nutrient amendment and unamended soils, the control soil had the least biodegradation throughout the 90 days period (fig. 5). The sterile polluted soils at 90 days has residual $C_{10} – C_{14}$ fractions of 437.43 and 747.00 mg/kg in clayey soil at 3.0 and 4.5 % level of pollution, and 464 and 817 mg/kg in sandy soil at 3 and 4.5 % level of pollution. The rapid biodegradation of $C_{10} – C_{14}$ fractions has been reported to be among the most rapidly biodegraded components of oil, although they are also susceptible to removal by extensive water washing. Empirically, the first sign of biodegradation are usually n-alkane in the C_{10} to C_{13} range, which probably reflects an optimal carbon number with increasing enthalpy of reaction and decreasing water solubility as the alkane carbon number increases (Palmer, 1993; Masterson, et al., 2001). The results, like those of C_{10} – C14 contaminated at 3.0 % revealed the effectiveness of Amnite P1300 to effect complete degradation of $C_{10} – C_{14}$ fractions in this level of contamination in both soils.

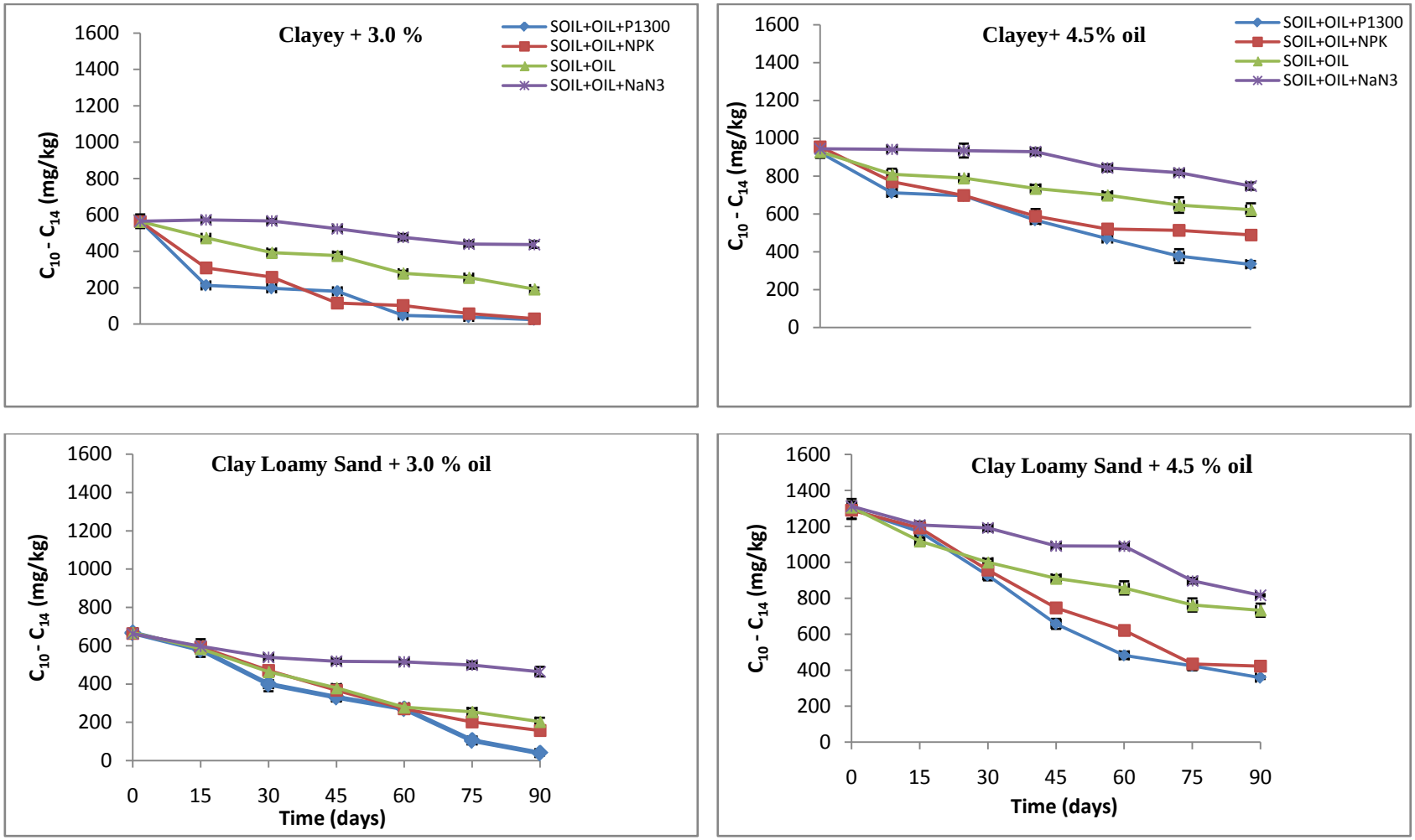


Fig.5: Concentration (mg/kg) of aliphatic hydrocarbon fractions ($C_{10} – C_{14}$) in different soil types (S1) and (S2) contaminated with 3.0 % and 4.5 % used lubricating oil.

This still pointed out its ability to degrade the short chain hydrocarbons fractions better than other treatments applied during the 90 days of the experimental period. The results is similar to that of Chang et al., (2010) who reported a substantial degradation of C_{10}-C_{16} hydrocarbon fraction in aged petroleum hydrocarbon contaminated soil.

3.7.2 Biodegradation of $C_{15} – C_{28}$ fractions in used lubricating oil. The results show that the hydrocarbon fractions $C_{15} – C_{28}$ were not degraded below the detection limit in all the treatments within the experimental periods, however the degree of biodegradation varies greatly based on the percentage of oil pollution and the amendments. The reason for incomplete biodegradation of these hydrocarbon fractions below detection limit might be due to their complex structure, which always posed some significant difficulty to hydrocarbon utilizing bacteria in their complete biodegradation (Peters and Moldowan, 1993). In soil contaminated with 3.0 % oil in both soils, Amnite P1300 amended soil recorded highest biodegradation of $C_{15} – C_{28}$ hydrocarbon fractions from the initial concentration of (16245 and 16348 mg/kg) to (3154 and 3658 mg/kg) in clayey and sandy soil respectively after 90 days of the experimental study. Studies with soil contaminated with 4.5 % oil pollution also revealed that Amnite1300 amended soil has the best treatment where the oil fractions were reduced from the initial concentration of (22954 and 23254 mg/kg) to (9448 and 10748 mg/kg) in clayey and sandy soil respectively after 90 days of the experimental study (fig. 6). The unamended contaminated and control soils recorded very low biodegradation of the $C_{15} – C_{28}$ fractions throughout the 90 days period in both soils at different level of contamination with used lubricating oil. The increase in the biodegradation of $C_{15} – C_{28}$ fractions in soil amended with Amnite P1300 might be due to the ability of the bacterial products conditioned to degrade heavy hydrocarbons.

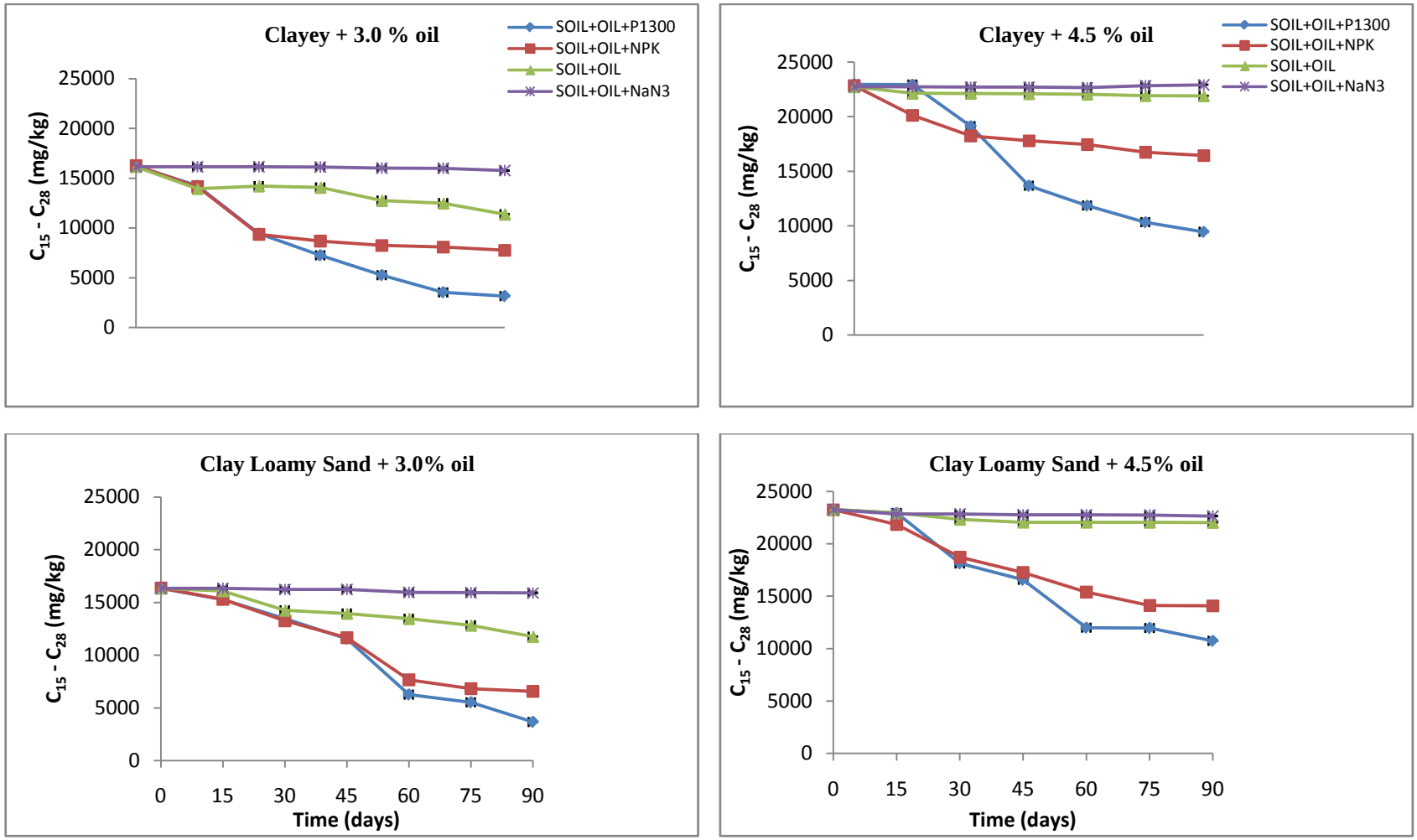


Fig.6: Concentration (mg/kg) of aliphatic hydrocarbon fractions (C15 – C28) in different soil types (S1) and (S2) contaminated with 3.0 % and 4.5 % used lubricating oil.

3.7.3 Biodegradation of C_{29} – C_{36} fractions in used lubricating oil. The results of the study revealed that these fractions of petroleum hydrocarbons were not properly degraded in all the treatments. The incomplete degradation of these hydrocarbon fractions has been reported by different authors that they are not easily degraded by microorganisms in the soil because they are hydrophobic solids at physiological temperature (Alberdi et al., 2001; George et al., 2002). In soil contaminated with 3 % used lubricating oil, soil amended with Amnite P1300 recorded reduction in C_{29} – C_{36} in the concentration from 9441 and 9684 mg/kg to 4566 and 3981 mg/kg in clayey and sandy soil, respectively, after 90 days of the experimental study, whereas in the control contaminated soil, the biodegradation of the hydrocarbon fractions was minimal (reduction from 9321 and 9743 mg/kg to 8816 and 9291 mg/kg in clayey and sandy soil, respectively, after 90 days of the experimental study (fig.7). The same pattern were also recorded in the 4.5 % contaminated level with the highest reduction recorded in the Aminte P1300 amended soil, though lower compare with the degradation recorded in the 3.0 % contaminated level in the hydrocarbon fractions C_{29} – C_{36}. The reason for low biodegradation of these hydrocarbon fractions might also be attributed to the fact that during biodegradation of hydrocarbons in soil or sediments, low molecular weight fractions are known to be degraded first by microorganisms before degrading the higher molecular weight petroleum fractions (Coulon et al., 2004; Sanscartier et al., 2009). Therefore, in this study possibly the low molecular weight fractions were first degraded by indigenous microorganisms before the higher molecular weight, thus, accounting for the low biodegradation of the higher molecular fractions in the range of C_{29} to C_{36}.

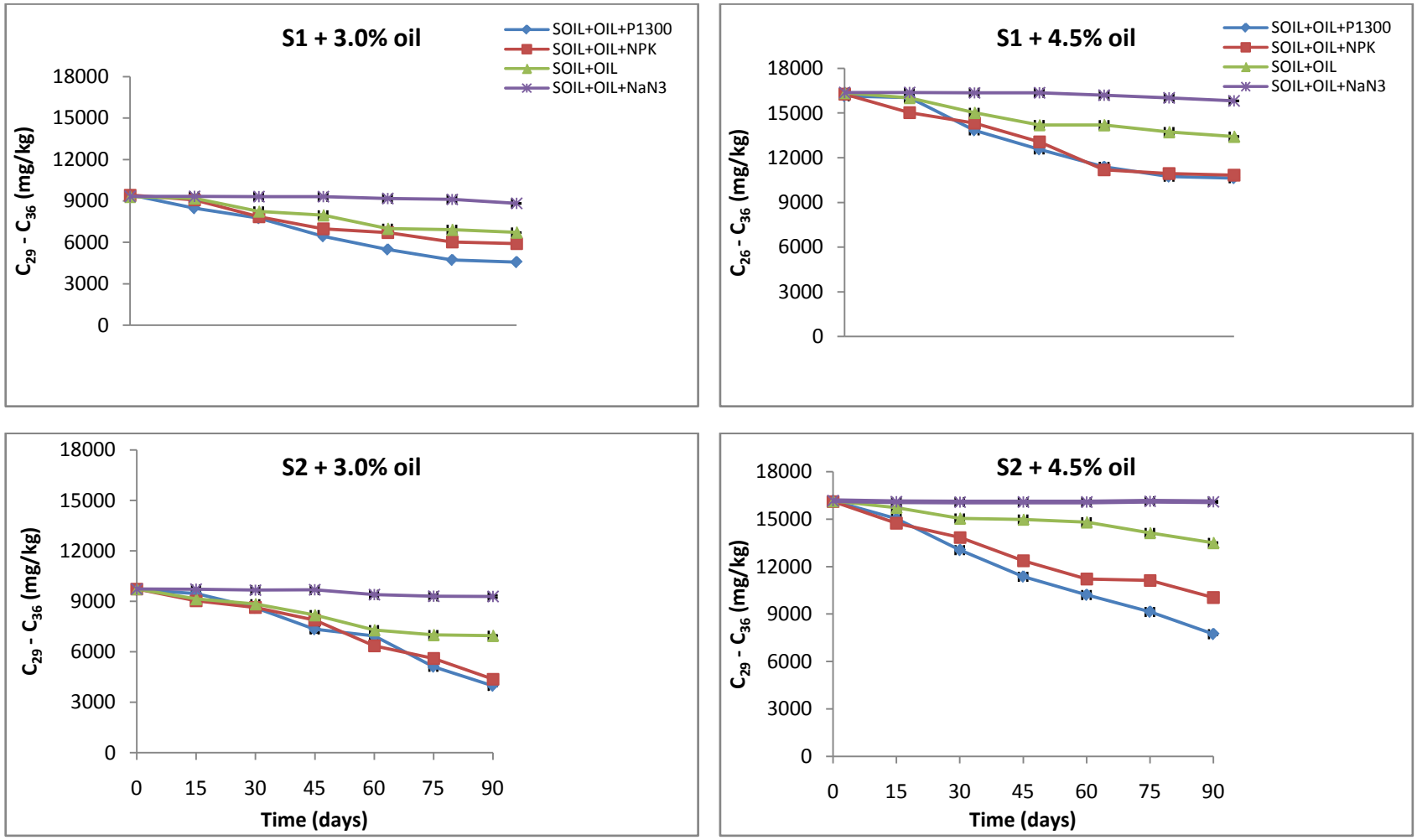


Fig. 7: Concentration (mg/kg) of aliphatic hydrocarbon fractions (C29 – C36) in different soil types (S1) and (S2) contaminated with 3.0 % and 4.5 % used lubricating oil.

3.8. Biodegradation of PAHs in used lubricating oil. The results from this study demonstrate degradation of eight PAHs present in the used lubricating oils during the biodegradation studies, using a microbial consortium present in Amnite P1300 as an amendment, addition of nutrients, unmended and the control soil with sodium azide to degrade soils contaminated with used lubricating oil. These PAHs have been identified by US Environmental protection Agency (EPA) as priority pollutants. Figure (8 to 11) show the results (concentration in mg/kg) of different residual PAHs present in the used lubricating oil in each treatment during the bioremediation studies. Pruell and Quinn (1988) detected sixteen PAHs with 2- to 6-rings in used lubricating oils. In other similar studies, twenty-five PAHs with 2- to 6-rings were recorded by Östman and Colmsjö (1989), and as many as eighty-four were reported by Grimmer *et al*. (1981) and Paschke *et al*. (1992). The following eight PAHs with 2- to 4-rings (Naphtalene (Nap), Acenaphthylene (Can), Acenaphthene (Anth), Fluorene (Flu), Phenanthrene (Phr), Anthracene (Ant), Fluoranthene (Flt) and Pyrene (Pyr) were the main PAHs detected in our studies during the bioremediation experiment in soils contaminated with used lubricating oil. These contrasting results demonstrated the difficulty of detecting individual PAHs in used lubricating oils whose matrices become complex after high temperature combustion.

The quantity and the composition of PAHs in the laboratory incubations were determined at the start of the experiment, and every 30 days thereafter till the end of the incubation. After 90 days of incubation, Naphthalene, Acenaphthylene and Acenaphthene were depleted in all the treatments and the abiotic control samples in both soils and contamination levels below the detection limit of 0.5 mg/kg, indicating abiotic removal, probably due to volatilization. It has been observed that PAHs resistance to oxidation, reduction, and vapourization increases with increasing molecular weight, whereas the aqueous solubility of these compounds decreases (Taylor and Jones, 2000). The results of biodegradation of different PAHs of higher molecular weights within the period of the study revealed the degradation of fluorene below the detection limit of 0.5 mg/kg in Amnite P1300 (T1), Nutrients amended (T2) and unamended (T3), in both soils and contamination levels, whereas, limited degradation were recorded in the control soils (T4) in both contamination levels within the period of the experiment. Complete degradation of phenanthrene and

anthracene was only achieved in T1 and T2, while the two PAHs were not completely degraded in T3 and T4 in both soils and contamination levels.

Complete degradation of fluoranthene and pyrene below the detection limit was also revealed in T1, the effect of the addition of the nutrients was negligible in the degradation of these two PAHs. However, it was clear that two – to four-ring aromatic compounds in the used lubricating oil had been substantially degraded in Amnite P1300 amended soils at both contamination levels, while other treatments did not record complete degradation of fluoranthene and pyrene after 90 days of the experimental study. The reason for complete degradation of PAHs recorded in soil amended with Amnite P1300 might be due to the soil texture improvement from possible increased in oxygen transfer as a result of the increase in the bacterial consortium present in the contaminated soil. Singh and Lin (2008) asserted that individual microorganisms can metabolize only a limited range of hydrocarbon substrates, so assemblages of mixed populations with overall broad enzymatic capacities are required to increase the rate and extent of PAHs biodegradation. Also, loss of PAHs recorded in the sterile polluted soil might be due to different processes such as volatilization, adsorption, photolysis or chemical degradation which are known to contribute to PAHs degradation in contaminated soil (Haritash and Kaushik, 2009).

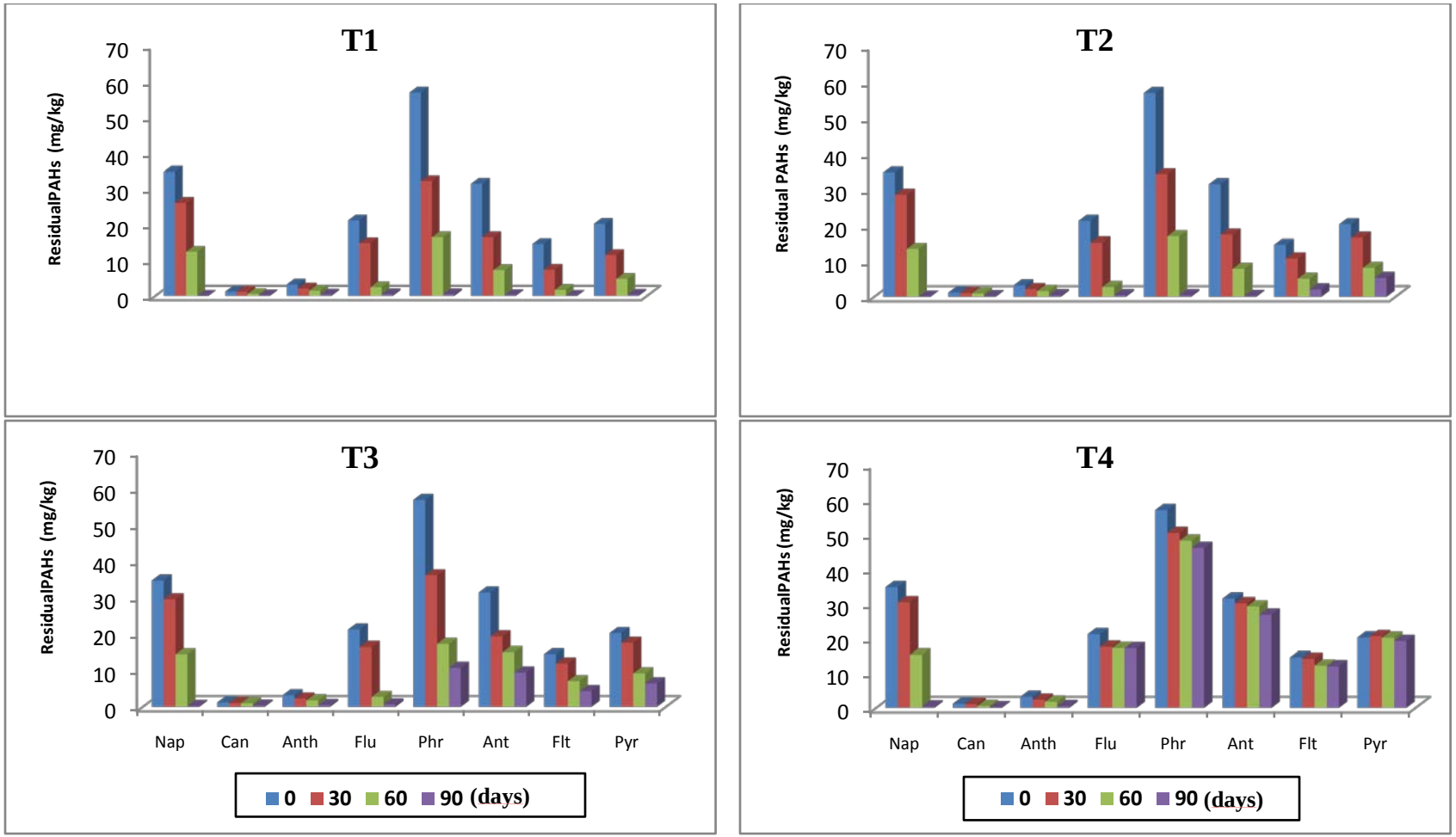


Fig.8: Concentration (mg/kg) of Polycyclic Aromatic Hydrocarbons (PAHs) in Soil 1 (S1) contaminated with 3.0 % used lubricating oil. PAHs present are: Nap - Naphtalene ; Can – Acenaphthylene; Anth – Acenaphthene; Flu – Fluorene; Phr – Phenanthrene; Ant – Anthracene; Flt - Fluoranthene and Pyr – Pyrene.

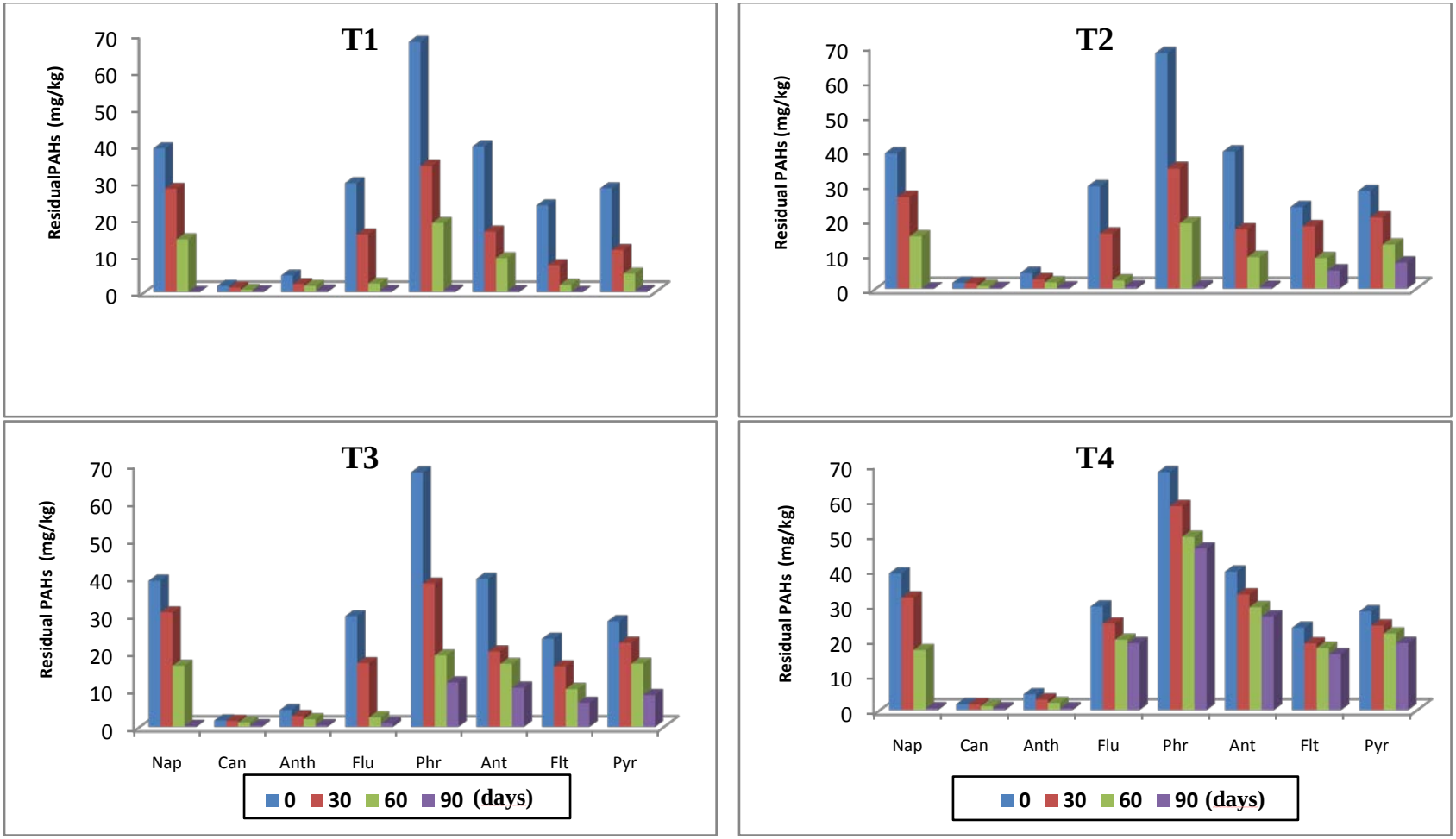


Fig.9: Concentration (mg/kg) of Polycyclic Aromatic Hydrocarbons (PAHs) in Soil 1 (S1) contaminated with 4.5 % used lubricating oil. PAHs present are: Nap - Naphtalene ; Can – Acenaphthylene; Anth – Acenaphthene; Flu – Fluorene; Phr – Phenanthrene; Ant – Anthracene; Flt - Fluoranthene and Pyr – Pyrene.

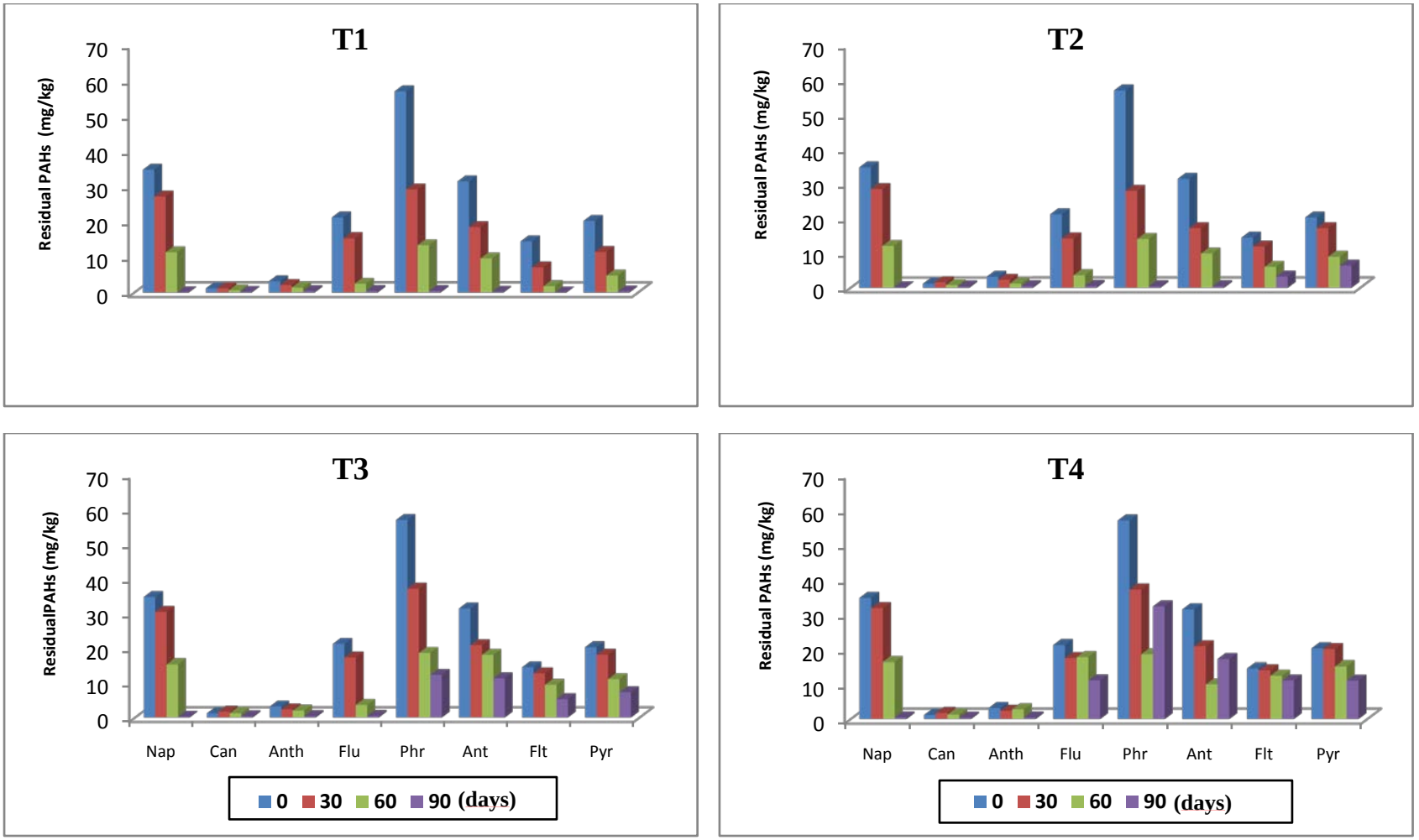


Fig.10: Concentration (mg/kg) of Polycyclic Aromatic Hydrocarbons (PAHs) in Soil2 (S2) contaminated with 3.0 % used lubricating oil. PAHs present are: Nap - Naphtalene ; Can – Acenaphthylene; Anth – Acenaphthene; Flu – Fluorene; Phr – Phenanthrene; Ant – Anthracene; Flt- Fluoranthene and Pyr – Pyrene.

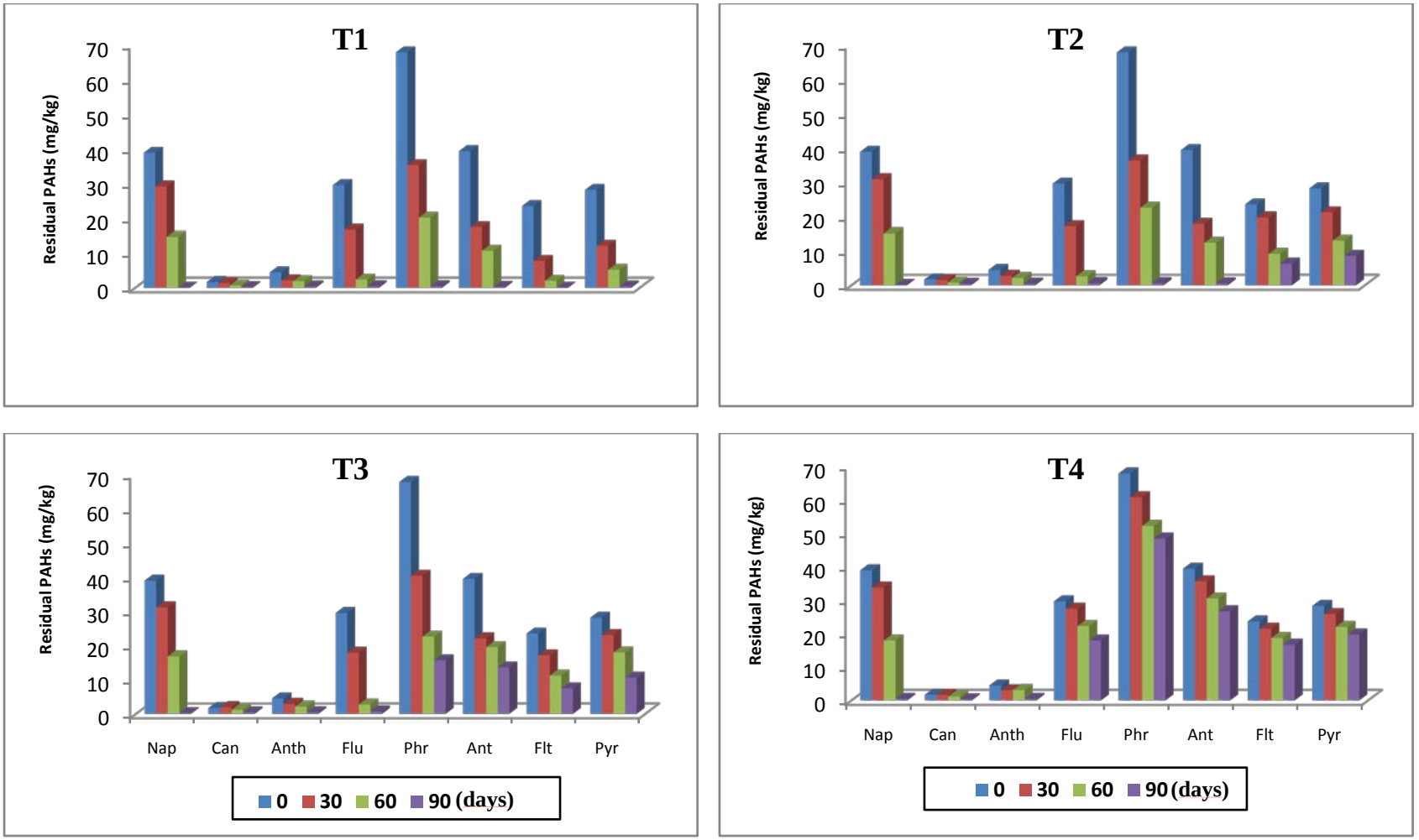


Fig.11: Concentration (mg/kg) of Polycyclic Aromatic Hydrocarbons (PAHs) in Soil 2 (S2) contaminated with 4.5 % used lubricating oil. PAHs present are: Nap - Naphtalene ; Can – Acenaphthylene; Anth – Acenaphthene; Flu – Fluorene; Phr – Phenanthrene; Ant – Anthracene; Flt- Fluoranthene and Pyr – Pyrene.

4. CONCLUSIONS

The present study clearly showed efficiency of mixed microbial consortium in the degradation of used lubricating oil components. Hence we suggest the use of the mixed microbial consortium for bioremediation of used lubricating oil contaminated sites.

The toxic effect of used lubricating oil is more pronounced in sandy soil than clayey soil. The influence of the soil characteristics on the toxic effects of hydrocarbons has also been revealed in this study, soils with a higher organic matter and clay content being less affected by this kind of contamination.

Hydrocarbons degrading bacteria (HDB) counts were higher in clayey soil, at highest population of (2.8×10^8 CFU/g) at day 60, in 3.0% contamination level, though the population reduces as the contamination level increases. This study has also shown that soil microbiological parameters may be useful tools for assessing the effect of hydrocarbon contamination on soil wellbeing.

The contaminants at the two loading rates (3 and 4.5%) assayed, increased soil respiration in both soils, especially in the clayey soil. The used lubricating oil contaminated clayey soils showed that respiration in 3.0 % loading rates producing the greatest CO_2 emissions. The higher amount of CO_2 liberated in clayey soil amended with amnite products and contaminated with 3 % and 4.5 % used lubricating oil is an indication of high utilization of organic carbon nutrients from the petroleum hydrocarbons present in this sample.

The bioaugmentation approach (Amnite P1300) gave the best result in this study as the concentration of the hydrocarbon contaminated soil increases and this can be used to develop a safe and economical full-scale treatment technology for soils contaminated with used lubricating oil. However, laboratory studies can produce overly optimistic results under controlled conditions, making it difficult to predict the performance of bioremediation strategies in the field where there is less control of environmental parameters and increased soil heterogeneity. An upgrade to field scale trials is therefore warranted to corroborate with the conclusions drawn from this study prior to undertaking field scale ventures. Also, the types of soil and hydrocarbon concentrations may determine the rate and extent of hydrocarbon remediation.

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GENERAL CONCLUSIONS

The results of the experiments demonstrate that the microbial consortium product (Amnite P1300) utilised has achieved a substantial decrease in hydrocarbons content, as evidenced by monitoring the Total Petroleum Hydrocarbons (TPH) in used lubricating oil, effecting an elimination of approximately 89 % of the original content.

Kinetic model of biodegradation showed the highest rate of $0.0283day^{-1}$ and least half life of 24.49 days in oil contaminated clayey soil. The biodegradation rate of used lubricating oil in clayey contaminated soil is therefore evident. Though, the biodegradation rate reduces as the concentration of the pollution increases, but this clearly points out that, clayey soil contaminated with used lubricating oil degraded faster than sandy soil when amended with microbial consortium.

The correlations between the parameters investigated and the levels of the hydrocarbon residues were determined. The residual hydrocarbon content correlated negatively with percentage of hydrocarbon degradation, hydrocarbon utilizers, CO_2 evolution and pH in all the soil types, and the levels of pollution.

The influence of the soil characteristics on the toxic effects of hydrocarbons has also been revealed in this study, soils with a higher organic matter and clay content being less affected by this kind of contamination. Thus, inhibitory effect of used lubricating oil is more pronounced in sandy soil than in clayey soil and show more toxic effect on seed germination and root growth. The results of this study therefore, indicate that Amnite P1300 is an efficacious product facilitating the biodegradation and elimination of hydrocarbon compounds from used lubrication oil contamination in different soil types, but there is a recalcitrant fraction of the hydrocarbons that remain in soil after the evaluated time period. The shorter chain aliphatic hydrocarbons are more degraded than the longer chain aliphatic hydrocarbons in both soils.

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