CHAPTER ONE

INTRODUCTION

1.1 Significance of petroleum hydrocarbons

Modern industrial society is built and ruled by petroleum hydrocarbons. Petroleum is essential to the current global networked economy, without it, our economic order would cease to function, bringing disaster to many populations. Yet the blessings of hydrocarbons are mixed, there is a growing awareness that imperfect petroleum technologies are changing ecosystems in ways that decrease the ability of these systems to support human populations. For the purposes of this thesis, *environmental pollution* is taken to mean the introduction of chemical compounds that significantly disrupt the equilibrium of an existing well-defined ecosystem. The unintended release of hydrocarbons into the environment can negatively impact human and animal health, and change the characteristics of soils impacting the plant populations they can support (Yu, 2006). Soil hydrocarbons of diesel origin can become embedded in the matrix of soil particles (Belluck et al., 2006). To appreciate the magnitude of unintended hydrocarbon release let us look at some global statistics. In 2003, the world consumption of petroleum was over 63.5 million barrels per day (Jain et al., 2011). The Energy Information Administration (EIA) projects in United States reported that, the world utilization of oil was 98 million barrels per day in 2006. The EIA estimate is that in 2030, the use of oil will reach to 118 million barrels/day (EIA, 2006). One liter of petroleum is enough to render one million gallons of freshwater problematic (Abioye et al., 2010). Sonawdekar (2012) reported that the amount of natural crude oil spill was estimated to be 600,000 metric tons per year with a range of uncertainty of 200,000 metric tons per year. There have been many reports on oil spills worldwide.

For instance, there was a crude oil spill of 0.04 mega tonnes into Prince William Sound, Alaska in 1989. In 2002, the Prestige oil spill occurred 209 km offshore and affected 1,900 km of shoreline in northern and northwestern Spain and western France, dumping 63,000 tonnes of fuel oil (Fernández-Álvarez et al., 2006). As the use of petroleum hydrocarbon products has increased, environmental pollution related to petroleum sources is becoming one of the main causes of soil and water pollution (Abioye et al., 2010). Some developing countries like Iran, which is the first oil-rich country in Middle East region, started oil operations with current production capacities of over 4 million barrels/day of crude oil and 80,000 m^3/day of diesel fuel. There is up to 1.5×10^6 m^3 of soil contaminated around the Tehran refinery due to discharge of crude oil into the environment (Kebria et al., 2009). The rapid transition from an agricultural economy to an industrial economy in some developing countries like Malaysia makes it probable that hydrocarbon environmental pollution is a significant issue. However, there is no statistically significant data on potential soil and water contamination in Malaysia. Much work remains to identify potential contamination sites and to suggest remediation measures (Heng Keng et al., 2009).

Polycyclic aromatic hydrocarbon (PAHs) as a most important source of energy for daily life and the high industrial usage are commonly found as an organic pollutant in the environment (Collins, 2007). Among different kinds of petroleum sources, diesel is one of the important sources of environmental contaminant. Diesel oil hydrocarbons are derived from crude oil refining (Mälkönen, 1995), and diesel is a complex saturated aromatic and aliphatic hydrocarbon (Eriksson et al., 2001; Zanaroli et al., 2010). The presence of a huge number of commercial trucks, private automobiles, ships and boats, locomotives, industrial engines (Roy, 1997), tractors and heavy vehicles has resulted in an increase in the use of diesel fuel. For instance, in UK, there are estimated to be 120 thousand contaminated petrol station sites with an associated remediation cost of two

billion dollar (Collins, 2007) , or in the US petroleum industry spent about one billion dollars in 2001 on remediation (Collins, 2007). It has been estimated that in 2040 contaminated site treatment may cost approximately two trillion dollars (Yu, 2006).

1.2 Risk of spills for the environment and human health

Effects of crude oil pollution in environment will change from one source to another because of crude oil and its derivatives or mixture of organic compounds. It can be different in combination of different sources (Onwurah et al., 2007). Therefore, the potential of remediation techniques will depend on the area where the spill has accrued. Oil spills in the water environment may affect microorganisms physically or by direct toxicity (Onwurah et al., 2007). PAHs are toxic to aquatic organisms because when it is exposed to solar UV radiation, it can produce O_2 via photosensitization, whereby toxic materials are released (Onwurah et al., 2007). There are so many individual constituents of petroleum hydrocarbons, that it is difficult to determine the effect of each constituent within the context of a hydrocarbon mixture. However, aromatic compounds tend to be more toxic than aliphatic compounds.

Crude oil, as a result of PAHs content, interrupts the survival, reproduction, development and growth of organisms. This may increase risk of mortality from infectious diseases (Onwurah et al., 2007)**.** Soil pollution with petroleum spills has resulted in great negative effects on food cycle. For instance, a high concentration of oil pollution that happened on the soil between 1978 and 1979 in Nigeria, affected farm-lands used to grow some of the crops such as cassava, maize, rice and plantain (Onwurah et al., 2007).

Crude oil can affect soil chemistry, germination and growth of plants and fertility but the effects depend on the type of oil spilled (Gavrilescu, 2010; Onwurah et al., 2007). Petroleum and its derivatives in contaminated land can affect some soil

parameters such as the cation exchange capacity and organic matter properties. It is an unimaginable possibility to say that there is a connection between both environmental and human health (Onwurah et al., 2007). Toxic materials in oil may effects on human health via inhibition of nerve synapse function, protein synthesis, damage to plasma membrane and infraction in membrane transport system (Afuwale and Modi, 2012; Onwurah et al., 2007). Light oils contain a high ratio of saturated hydrocarbons; hence, these can be more hazardous than heavy oils (Kauppi et al., 2011). A list was compiled in 1999 on prioritized chemicals based on the frequency of their occurrence at National Priorities List (NPL) sites and their risk towards environment and human health. On this list, PAHs were collectively ranked ninth, and benzo (a) pyrene was ranked eighth (Olson et al., 2003). A number of PAHs have been determined to be probable human carcinogens. Benzene is also of concern because it has been determined to be a known human carcinogen (EPA, 2006). Chronic effects of naphthalene are changes in the nervous system, liver, kidneys, blood and heart, due to their relative insolubility and potential for different chronic effects, like carcinogenicity (Roy, 1997). The Association for Environmental Health and Sciences TPH Working Group also examined toxicity studies for various petroleum hydrocarbon constituents and also developed reference doses and reference concentrations for various hydrocarbon ranges. Some of the hydrocarbon compounds which have been identified as probable human carcinogens such as Benzene, Indeno [1,2,3-c,d]pyrene, Dibenz [a,h] anthracene, Benzo [a] pyrene, Benzo [b] fluoranthene and Benzo [k] fluoranthene (Julia, 2008).

Carcinogenic impacts have been associated with some compounds found in diesel fuels (Roy, 1997). Diesel causes eye and skin irritation in humans, but otherwise its effects on humans are considered to be poorly investigated (Muzyka et al., 2002). Diesel is considered to be harmful and possibly carcinogenic to humans (Työterveyslaitos, 2011), and it contains PAHs that create a risk for human health

because of their carcinogenic and mutagenic properties (Bamforth and Singleton, 2005; Grant et al., 2007).

1.3 Environmental biotechnology to diesel fuel clean up

Environmental biotechnology refers to biotechnology used to remediate contaminated environments. It is a scientific technique that uses living organisms to improve or modify contaminated environment (Onwurah et al., 2007). Therefore, some researchers have briefly described research and strategies for cleaning up oil spills (remediation). Remediation itself is an activity with negative impact on environment and using native materials. This may lead to contradictions between different national environmental quality objectives. According to the United State Environmental Protection Agency 16, polycyclic aromatic hydrocarbons have been reported as carcinogenic and mutagenic compounds (Mancera-López et al., 2008).

A diversity of bioremediation techniques has been developed to increase the biodegradation rate of contaminated sites (Jingchun et al., 2009). Bioaugmentation (application of specifically selected bacteria to contaminated soil) has been well studied before and demonstrated to be useful method in bioremediation of contaminated soil (Jingchun et al., 2009). Some studies have reported various bacteria and fungi species with the capacity of mineralization of organic compounds to degrade PAHs (Mancera-López et al., 2008). More than 200 species of bacteria, yeasts, and fungi have been identified, which are capable of degrading hydrocarbons. In order of importance, these are as follows: (1) heterotrophic bacteria, (2) fungi, (3) aerobic bacteria, (4) actinomycete, (5) phototrophic microbes, and (6) oligotrophic bacteria. Bacteria namely, *Burkholderia* spp., *Bacillus* spp., *Pesodomunas* spp., *Yokenella* spp., *Moraxella* spp., *Acinetobacter* spp., *Stenotrophomonas* spp., *Streptococcus* spp. and *Mycobacterium* (Afuwale and Modi, 2012; Anene and Chika, 2011; Das and

Chandran, 2011), isolated from petroleum contaminated soil have shown the most active factors in petroleum degradation. They serve as initial hydrocarbons degrade (Das and Chandran, 2011). Other organisms such as fungi (*Phanerochaete chrysosporium, Pleurotus ostreatus and Trametes versicolor)* are also capable of degrading the hydrocarbon compounds from the engine oil (Mollea et al., 2005). You-Qing et al., (2008) reported that *Cladosporium* strongly biodegraded diesel oil with a degradation ratio of 34% after 5 days treatment. The tendency of hydrocarbons to microbial degradation can be in order: linear alkanes, branched alkanes, small aromatics and cyclic alkanes, respectively (Das and Chandran, 2011). Some mixtures may not be degraded at all, such as the high molecular weight of polycyclic aromatic hydrocarbons (Atlas and Bragg, 2009; Moneke and Nwangwu, 2011).

Remediation of contaminated sites can be achieved through physical and chemical techniques such as disposal in landfill, incineration, use of chemical oxidants and biological processes (Ayotamuno et al., 2009). Compared to physico-chemical methods, biological processes are thought to be of low environmental risk and low cost but in some cases, longer time is required (Jingchun et al., 2009). Many studies have proved the positive effects of biostimulation in the restoration of total petroleum hydrocarbon contaminated sites (Abioye et al., 2012a; Adesodun and Mbagwu, 2008; Ayotamuno et al., 2009; Hamdi et al., 2007; Kogbara, 2008; Molina-Barahona et al., 2004; Sasek et al., 2003). Biostimulation is a form of bioremediation, which uses an electron acceptor to motivate capable bacteria to degrade environmental pollutants. Biostimulation causes a rapid discharge of major hydrocarbons from environment. It is a cost effective treatment over large areas and it is easy to maintain (Bento et al., 2005; Margesin and Schinner, 2001; Salinas-Martínez et al., 2008). Successful biostimulation has caused degradation of polychlorinated ethylene, petroleum, and reduced uranium and other heavy metals from environment (Miller, 2010). In biostimulation, the addition of

nutrients (nitrogen, carbon, phosphorus and others), oxygen, or other amendments stimulates the activity of naturally-occurring microbes to enhance bioremediation and contaminant desorption from subsurface materials (Salinas-Martínez et al., 2008; Van Deuren et al., 1997). There will be more discussion in the literature review (chapter 2) to evaluate different biological methods.

Numerous factors affecting hydrocarbon biodegradation have been reported (Boopathy, 2000; Das and Chandran, 2011; Khan et al., 2004; Koenigsberg et al., 2005). One of the primary factors that affect the activity of bacteria is accessibility of organic materials to serve as energy source (Boopathy, 2000). Some of the major factors affecting the remediation processes are solubility of contaminants, lack of nutrients in environment, microbial interaction, type of contaminants, physico-chemical bioavailability of pollutants, oxygen diffusion and solubility of organic compounds (Boopathy, 2000). The success of each degradation process depends on substrate (physicochemical characteristics, molecular structure, and concentration), enzyme activities, biomass concentration and population diversity of microbial and a range of environmental factors such as availability of electron acceptors, pH, moisture content, temperature and carbon as energy source (Boopathy, 2000). Numerous factors may limit the rate of degradation. One of important factor is lack of nutrients such as N and P which can affect this process (Abioye et al., 2009). Therefore, the addition of N as organic or inorganic N illustrated an effective method to improve the rate of remediation process (Aspray et al., 2008; Jørgensen et al., 2000; Margesin et al., 2000; Riffaldi et al., 2006; Walworth et al., 2007). Alternatively, amendment with fertilizer as a supplement to remediate, when applied at high concentrations may lead to ground water pollution, prevent microbial activity and increase the salt concentration of ground water (Aspray et al., 2008; Bento et al., 2005; Walworth et al., 2007).

1.4 Problem statement

In today's industrial world, petroleum hydrocarbon is a main compound of our modern life and it is an important source of energy. Fuels derived from crude oil supply more than half of the world's energy (EIA, 2012). There is a rapid rise in petroleum consumption and as a result, annually huge amounts of hydrocarbons are discharging into the environment, either accidentally or deliberately. However, many small spills happen during crude oil recovery, transport, and refining (Bolliger, 2000). The leakage of petroleum hydrocarbons from vehicles onto the road and washing of oil into the coastal environment is becomes a significant source of oil pollution. Diesel, kerosene and Gasoline are used as fuel for cars, ships, trucks and tractors. Although most of the world's nations produce at least minor amounts of oil, the primary areas of oil production are in the Persian Gulf, North and West Africa, the North Sea, and the Gulf of Mexico (Bolliger, 2000). As long as oil is stored, used and transport, there is a potential threat of oil spillage in environment. Oil spillage has been a common problem in shell companies, nations and our environment. During the rapid industrial world, there are many industrial sites in Malaysia which are ability to be contaminated sites and it is going to be significant issues in Malaysia. The United States Environmental Protection Agency reported, there are more than one million underground storage tank sites in the world (EPA, 1995). There are 119,000 confirmed instances of release of petroleum bulk fuels to the groundwater or soil at these underground storage tank sites (EPA, 1995). These releases are significant since the potential hazard of a leaking underground storage tank is that the petroleum or hazardous waste can contaminate the groundwater supplies that serve as drinking water sources for half of all the Americans.

According to the National Research Council (NRC), about 1.3 million tonnes of petroleum are released into the sea annually. United States International Trade Commission (USITC) reported that the global remediation services in 1996 increased

from 25.7 billion US dollar to 29.9 billion US dollar in 2002 (Koplan, 2004). Table 1.1 shows the annual number of oil spills in the world. Remediation of polluted sites by petroleum hydrocarbons started about 20 years ago, but still there are many contaminated sites which need to be decontaminated.

Table 1.1 Annual number of oil spills

Year	**< 700 tonnes oil spills**	**> 700 tonnes oil spills**
2000	21	4
2001	17	3
2002	13	3
2003	17	4
2004	17	5
2005	22	3
2006	13	5
2007	13	4
2008	8	1
2009	7	1
2000s Total	**149**	**33**
Average for decade	**14.9**	**3.3**
2010	4	4
2011	4	1
2010s Total	**8**	**5**
Average	**4**	**2.5**

(ITOPF, 2011)

Until recently, this type of pollution received very little attention in Malaysia. The awareness of this issue is increasing polluted sites in Malaysia and therefore leading to remediation a large number of sites in the near future.

Among those petroleum products, diesel-oil, or fuel oil contaminated soils are more difficult to treat compared to more volatile petroleum products (Chien et al., 2010). We will be particularly concerned with diesel oil, which is a complex mixture of aliphatic and polycyclic aromatic hydrocarbons (PAHs) (Yu, 2006). For most part, diesel comprises of aliphatic hydrocarbons, but it also contains polycyclic aromatic hydrocarbons such as naphthalene, fluorene and phenanthrene. Aromatics consist of a number of rings with a range of one to five (Roy, 1997). Aromatics with more than two rings are mentioned as polyaromatic hydrocarbons (Roy, 1997). Oil pollution will affect soil fertility, food cycle, ground water, marine life, ecosystem and human health. Therefore, after having a full-fledged case study of oil spills, we are able to come up with a suitable solution to remediation of pollution. Conventional remediation technologies are time consuming expensive and environmentally divesting.

The traditional treatment, physical and chemical methods may not remove and degrade the oil thoroughly. Hence, it is unavoidable to use an environmentally friendly technology and low cost method to remediate polluted soils, specifically in developing countries. Biological methods can be most effective in the removal of oil contamination from soil, where physical or chemical methods are not effective. Phytoremediation and bioremediation are suggested as effective methods. In this way, it will be possible to stimulated aerobe/anaerobic biodegradation as a remediation technique for diesel removal in soil. Since, nutrient availability especially nitrogen is the most limiting factor in biodegradation process, it is necessary to apply suitable source of nutrient to both microbes and plants to carry out the decomposition of the oily waste. In this study, organic wastes [used tea leaf (TL), soybean cake (SC) and potato skin (PS)], which are cheap, available and easy to find, were used as supplements, replace to inorganic fertilizers that are expensive and currently used for agriculture purpose, to enhanced biodegradation of diesel fuel contaminated soil.

1.5 Aim and Objectives

The seriousness of diesel fuel pollution in our environment and the possibility of bioremediation and phytoremediation to remediate various types of oil pollution are the driving force behind this study. The aim of this research is to explore the feasibility of using organic wastes (biowastes) to remedy soil, which has been contaminated by diesel fuel. A series of microcosm studies were conducted in a greenhouse and field conditions to compare the potential of different organic waste amendments and the importance of oil-degrading microorganisms in the bioremediation process. In addition, the potential of two different local plants are evaluated on restoration of diesel fuel contaminated soil in phytoremediation process. To achieve this research aim, the following objectives are identified:

1. To evaluate the potential of different organic wastes (tea leaf, soy cake and potato skin) in enhancing biodegradation of diesel fuel in contaminated soil at four different oil concentrations under, laboratory and natural conditions.
2. To isolate and screen potential microorganisms for diesel fuel degradation from contaminated and uncontaminated soil and monitoring the biodegradation process using stable isotope carbon (^{13}C).
3. To comes out soil toxicity test after biostimulation of oil-polluted soil using seed germination test and determine the rate of biodegradation of diesel oil in contaminated soil and to calculate the half-life, using kinetic model.
4. To compare the performance of *Dracaena reflexa* and *Podocarpus polystachyus* plant species in biodegradation of diesel fuel contaminated soil.
5. To determine the uptake rate of heavy metals (Zn and Pb) in diesel fuel contaminated soil by *Dracaena reflexa* and *Podocarpus polystachyus* plants.

1.6 Research plan

The study includes two parts. The first part focus of on bioremediation of diesel fuel from artificially contaminated soil by amendment with three different organic wastes using greenhouse microcosms and natural condition. The second part explored the potential of biowastes to restoration of diesel fuel contaminated soil in phytoremediation process in greenhouse microcosms and natural condition.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of Petroleum Hydrocarbons

A basic knowledge of petroleum chemistry and its properties is necessary for selecting and applying a successful remediation method for petroleum contaminated soil. In this chapter, the properties of TPH and diesel fuel and their behavior are described. Finally, different methods of remediation will be discussed.

2.1.1 Structure and chemistry of PAHs

Petroleum is a natural product, containing a complex mixture of various hydrocarbons, made by the decomposition of plant remains from the carboniferous period under high pressure and temperature (Van Hamme, 2003). The components of crude oil are named petroleum hydrocarbons. Petroleum and its derivatives are organic material with mixtures of liquid, solid, and gaseous hydrocarbons (Brandt, 2006). Petroleum is processed in refineries into a number of products, such as industrial fuel oils, gasoline, kerosene and diesel fuel. All types of petroleum hydrocarbons contain small amounts of different metals such as S, N, Fe, O_2, Va, Ni and Cu (Abdel-Aal, 2003). However, the major ingredients of PHC are two elements; hydrogen and carbon with a range of (11-15%) and (82 - 87%), respectively (Regine, 2003). It also contains oxygen (0 to 0.5%), sulfur (0 to 8%), and nitrogen (0 to 1%) as important minor components. Each type of crude oil has its individual chemical composition depending on its location and origin (Regine, 2003). Petroleum crude oils are the mineral source for many refinery products such as petroleum gas, gasoline, kerosene, fuel oils, lubricating oils, coke, and asphalt (Brandt, 2006). More than 80% of the hydrocarbon components of all types of petroleum products can be arranged as paraffin, asphalt or mixed base (Okoh, 2006). Contents of S, O and N are often higher in comparison with paraffin based crudes, which contain no asphaltic materials (Okoh, 2006). Petroleum

hydrocarbon structural configurations can be divided into two big groups; namely, aliphatics (fatty) and aromatics (fragrant) (Figure 2.1). The aliphatics are divided into four groups; namely, acetylenes, paraffins, olefins (with straight or branched chains) and naphthenes (saturated hydrocarbons with one or more rings cycloalkanes) (Figure 2.1).

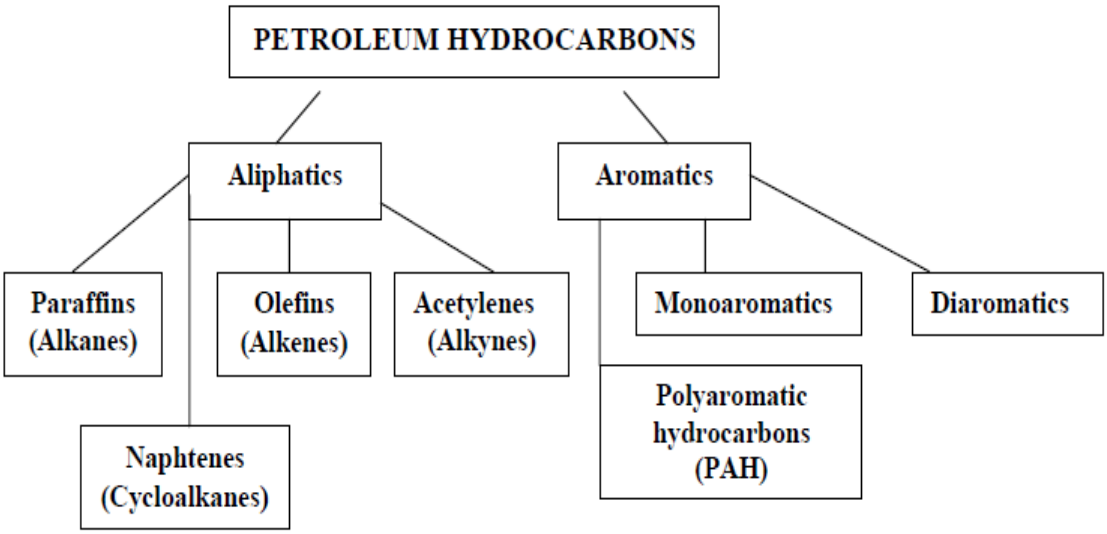


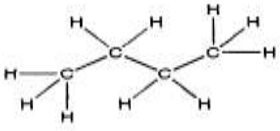
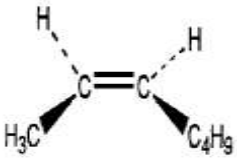
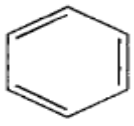
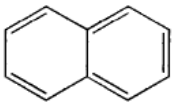
Figure 2.1 Petroleum hydrocarbon structural relationships (Regine, 2003), (modified).

The number of carbon rings in aromatics is one to six which demonstrates high chemical permanence due to double bonds (Brandt, 2006). The aromatic group is divided into monoaromatics (one ring such as benzene, toluene, ethylbenzene and xylene, collectively known as BTEX), diaromatics (benzene rings) and polyaromatic hydrocarbons (compacted aromatic ring structures with more than 2 benzene rings) (Brandt, 2006).

In addition, oil is also characterized by other components. Resins and asphaltenes can consist of a large fraction of heavy fuel oils and crude oils, making those oils very dense and sticky. Refined oils may also have some additives, such as gelling inhibitors,

which are added to diesel fuels (Helton, 2000). Some additives could be of special concern, because they are toxic themselves and significantly change the behavior of the oil products. Chemical structures of various categories of hydrocarbons are shown in Table 2.1.

Table 2.1 Chemical Structures of Various Categories of Hydrocarbons

Category	Description	Example Chemical Structure
Aliphatics		
Alkenes	Carbon chain with single bond between carbon atoms	n-Butane 
Alkynes	Carbon chains with at least one carbon-carbon triple bond (not commonly found in petroleum hydrocarbons)	cis-2-Heptene 
Cycloalkanes	Single-bonded carbon ring structure	Cyclohexane 
Aromatics		
Monoaromatics	Primary structure is the benzene ring made up of six carbon atoms with alternating single and double bonds	Benzene 
PAHs	A compound having two or more benzene rings fused together	Naphthalene 

Source: (Amanda, 2006)

The biodegradability of these particular components is a reflection of their physical state, chemical structure and toxicity (Koleva and Tasheva, 2012). For instance, the most biodegradable petroleum hydrocarbons are *n*-alkanes as a structural group, while $C_5 - C_{10}$ homologues have been shown to be inhibitory to the majority of hydrocarbon degraders (Okoh, 2006).

Another property of petroleum hydrocarbons is having a large number of isomers (same formula with different arrangement of elements). In general, with an increasing number of carbons, the number of isomers will also be increased rapidly. For example, an alkane with six carbon atoms has five isomers. An increase in the number of carbons to 10 can leads to an increase of the number of possible isomers to 75 (Jim et al., 2005).

2.1.2 Diesel fuel toxicity and its composition

The name 'Diesel' was derived from the name of the inventor of the diesel engine and the fuel that runs diesel engines as diesel. Petroleum diesel is a complex combination of thousands of individual compounds, with the carbon numbers between 8 and 22 (2000 to 4000 hydrocarbons) which are generated by the distillation of crude oil, ranging approximately from $C_{10}H_{20}$ to $C_{22}H_{28}$ (EGM, 2011). Diesel oil is of low molecular weight syntheses that are more toxic than long chained hydrocarbons (Kauppi, 2011). The main composition of diesel oil is 75% saturated hydrocarbons from the paraffin family, and 25% aromatic hydrocarbons such as naphthalene and alkyl benzene (EGM, 2011). The more obvious smell of diesel is due to the presence of aromatic hydrocarbons. The boiling point of diesel is in the range of approximately 180 - 360 °C (360 - 680 °F), with a density of about 0.832 kg/l. Table 2.2. Illustrates the carbon chain lengths that affect in the boiling point.

Table 2.2 Relations between carbon numbers and boiling point ranges

Fuel or oil	Carbon number range	Boiling point range (°C)
Petroleum	C4 – C12	40 – 200
Jet fuel	C5 – C14	150 – 275
Kerosene	C6 – C16	150 – 300
Diesel	C8 – C12	1200 – 325
Motor oil	C18 – C34	325 – 600

Source: (Collins, 2007)

The color of diesel fuels varies from colorless to brown with medium volatility and solubility (at 20 °C is about 5 mg L^{-1}) (Table 2.3) (Bacha et al., 2007, Kauppi, 2011).

Table 2.3 Selected diesel fuel hydrocarbons and some of their chemical properties

Compound	Chemical formula	Group	Density 20°C, *g/cm³*	log K_{ow}	Water solubility *mg/L*	B °C	F °C
Naphthalene	C10H8	Aromatic	1.175	3.37	31	218	80
n-Butylcyclohexane	C10H20	Naphthene	0.7992	5.46	-	181	-75
n-Decane	C10H22	n-Paraffin	0.7301	6.25	0.052	174	-30
Anthracene	C14H10	Aromatic	1.251	8.00	8	341	215
n-Pentadecane	C15H32	n-Paraffin	0.7684	8.63	-	271	10
Eicosane	C20H42	n-Paraffin	0.7843	11.27	3E -07	344	36

B = boiling point, F = Freezing point (Bacha et al., 2007; Kauppi, 2011)

Therefore, diesel fuel has been considered as a priority pollutant that exerts bio-hazardous effects on both human and other living organisms in the environment (Kebria

et al., 2009). Diesel hydrocarbon contamination as a result of leakage during transportation, storage, manufacturing, from tanker or storage tank accidents and pipelines may cause considerable damage not only in water intake but also in terrestrial environments. Therefore, diesel hydrocarbon contamination which is a dangerous threat worldwide needs restoration and decontamination. The result of contamination could be increased, especially due to the mobility of oil hydrocarbons which when absorbed into groundwater could pose even a greater threat (UNEPA, 2011).

2.2 Weathering Processes of petroleum sources

When oil is transmitted into the environment, wide varieties of biological processes both chemical and physical begin to transform the discharged oil. In general, these changes are referred to as weathering (Jim, et al., 2005). Realizing the weathering process is important in understanding of oil samples. The weathering process can affect the toxicity and composition of the hydrocarbons (Battelle, 2007). The main weathering processes are biodegradation, dissolution, volatilization, evaporation, and dispersion (EPA, 1999a, 1999b). Although rates of weathering depend on the nature of the environment, there is also great dependence on the chemical and physical properties of the hydrocarbons (Amanda, 2006). Those compounds with lower boiling points will be more volatile, such as gasoline and diesel. The aliphatics are more volatile than aromatic compounds, and volatility decreases with the increase in molecular weight (Amanda, 2006). The consequence of hydrocarbon weathering processes is that the more degradable, soluble and volatile will disappear most rapidly (Amanda, 2006). In contrast, larger PAHs and higher molecular weight will persist in the environment for a long time (Wick et al., 2011).

For example, aliphatics tend to be more volatile. While aromatics tend to be more water soluble than aliphatics. However, when a fuel mixture is released into the

environment, the main water contaminants are likely to be aromatics while aliphatics will be the main air contaminants. In general, volatility and solubility of all compounds will decrease with an increase in molecular weight. Therefore, the more volatile and water soluble substance compounds which have the lowest molecular weight are lost most rapidly from polluted soil (Jim et al., 2005). The rates of weathering or volatilization of individual organic compounds are related by this fact that the fuels are mixtures (ECHC, 2012). For instance, the solubility of pure benzene in water is about 1800 mg L^{-1}. The volatility and solubility of single compounds in petroleum hydrocarbon mixtures are convenient to the volatility or solubility of the compound in its pure condition and its concentration in the mixture. Volatility and solubility of a compound will decrease when the compound is a mixture (EPA, 2000). As indicated above, alkanes tend to be more volatile than aromatics. The trend in volatility by compound class as follow; alkenes = alkanes > aromatics = cycloalkanes (Jim et al., 2005). Considering dissolution and volatilization trends together, one can predict the composition of fuel mixtures after release into the environment. Where volatilization is the dominant process, the loss of lower molecular weight alkanes will be the most significant change in the product. In situations where dissolution overcomes the weathering process, the aromatics will be depleted with benzene and removed most rapidly.

A third process that is usually operative when petroleum mixtures are released into the environment is biodegradation. It has been widely indicated that almost all types of soils and sediments have populations of bacteria and other organisms that are capable of degrading petroleum hydrocarbons. Degradation occurs in both the absence and presence of oxygen. Two important key factors that indicate degradation rates are molecular structure and oxygen supply. Generally, degradation is more rapid under aerobic conditions. Trends in degradation rates according to structure are: 1) *n*-alkanes,

especially in the C_{10} - C_{25} range degrade rapidly, 2) isoalkanes degrade slowly, 3) alkenes degrade more slowly than alkanes, 4) Benzene, toluene, ethylbenzene and xylenes (BTEXs) are metabolized when present in concentrations which are not toxic to the microorganisms, 5) Polycyclic aromatic hydrocarbons (PAHs) degrade more slowly than monoaromatics, and (6) degradation of higher molecular weight cycloalkanes may be very slow. At the initial stages of degradation, the *n*-alkanes are degrading selectively. Over time (weeks or months), they are completely biodegraded. The compounds most easily recognizable in the remaining diesel fuel mixture at this point are the isoprenoids, which include pristane (C_{19}) and phytane (C_{20}). These compounds are alkanes with highly branched structures. These branched structures greatly reduce the rate at which biodegradation occurs.

2.3 Biodegradation of pollution contaminated sites

Due to exploration activities of petroleum hydrocarbon a wide range of pollution takes place in the environment that could result in serious problems for the abiotic and biotic components of the ecosystem (Okoh, 2006). The capability and interaction of animals, plants, and organisms are identified as limiting degradation factors. Most hydrocarbons are insoluble in water, and generally immovable, so their bioavailability is limited in the degradation process (Jim et al., 2005). Biodegradation is defined as the breakdown of organic compounds by Actinomycetes, fungi and bacteria. Microorganisms are provided with metabolic systems to use petroleum as a source of energy and Carbon (Van Hamme et al., 2003). The growth of microorganisms on hydrocarbons depends on the emulsification of the unsolved carbon source in the culture medium (Chrzanowski et al., 2006). The ability of microbes to break down the organic hydrocarbon structure depends on the capacity of microorganisms to degrade organic compounds (Mancera-López et al., 2008). These processes lead to

microorganisms growing properly and metabolizing petroleum. Since *n*-alkanes are completely biodegraded, at the beginning stage of degradation, they are degraded over a period of time (Van Hamme et al., 2003). Pelletier et al., (2004) reported over 90% of n-alkanes degrades in the first six months and most light aromatics (2–3 rings) disappeared during the first year of observation. Pristane (C_{19}) and phytane (C_{20}) are the most easily recognizable compounds in the remaining diesel fuel mixture at this point, which are isoprenoids. The attendant negative outcomes of the physicochemical approach are currently receiving greater attention for the exploitation of the biological alternatives (Okoh, 2006).

2.3.1 Biodegradation of organic compounds by bacteria

Degradation refers to the influence of microorganisms that leads to the breakdown of organic compounds, which are recognized as efficient, economical, and environmentally sound treatment (Jain et al., 2011). In recent years the oil industry has shown considerable interest in the use of microorganisms, especially for controlling and dispersing oil spills using surfactants, bioremediation and oil recovery. The term *"hydrocarbonoclastic"* has been use to describe hydrocarbon-utilizing microorganisms. This specifically relates to microbes, that are capable of degrading hydrocarbons, and all of which share some of the following characteristics (Jim et al., 2005):

i. They are able to extensively degrade partially or fully petroleum based compounds.

ii. They have a capable and efficient hydrocarbon uptake system.

iii. They have receptor sites for binding hydrocarbons.

iv. They are capable of producing surfactants.

v. They are well adapted to the environment, genetically stable with rapid reproduction rates.

vi. They have been selected for their environment.

vii. They must not be pathogenic or produce toxic metabolic products.

Viii. They must have group-specific oxygenases to introduce molecular oxygen into the hydrocarbon and, with relatively few reactions, generate intermediates that subsequently enter common energy-yielding catabolic pathways.

The oil industries, such as transport and oil extraction, seem a threat to the environment because they can cause a huge penetration of petroleum hydrocarbons into the environment (Abioye et al., 2012a; Jim et al., 2005). Due to this reason, there is an interest in finding the best way to degrade petroleum hydrocarbons from the ecosystems. Generally, microorganisms that have the capability to transform organic chemicals or remove them are used for bioremediation of ecosystems that have been polluted by petroleum oil or its fractional compositions (Abioye et al., 2012a; Yanyan et al., 2009). Many studies have used microorganisms and microbial strain to remediate polluted sites (Cunningham et al., 2000; Obuekwe et al., 2009). Some isolated bacteria are effective in degrading diesel oil *(Rhodococcus* sp. and *Acinetobactersp.)* (Gallego et al., 2001), heavy oil *(Pseudomonas* sp.) (Setti et al., 1999) and crude oil *(Candida* sp. and *Rhodococcus* sp.) (Palittapongarnpim et al., 1998). The list of different genera of microorganisms with capabilities to degrade hydrocarbons is shown in Table 2.4.

Table 2.4 Microorganisms reported to utilize petroleum fractions for growth

Yeast	Fungi	Bacteria
Candida	*Absidia*	*Acinetobacter*
Debaryom yces	*Aspergillus*	*Actinomyces*
Endom yces	*Cladosporium*	*Bacillus*
Mycotorula	*Gliocladium*	*Micrococcus*
Rhodotorula	*Penicillium*	*Mycobacterium*
Saccharomyces	*Rhizopus*	*Nocardia*
Torulopsis	*Scolecobasidium*	*Pseudomonas*
Trichosporon	*Trichoderma*	*Streptomyces*
Hansenula	*Syncephalastra*	*Alkaligenes*
Pichia	*Mucor*	*Actinomyces*
	Colletotrichum	*Endomyces*
	Botrytis	

Many hydrocarbons are naturally occurring complex mixtures of organic compounds which are processed by biosynthesis, so it is not surprising that microorganisms have the ability to utilize these compounds. The effects of natural selection mean that for every compound there is at least one microorganism able to at least partially degrade it, if the environmental conditions are favorable. The microorganisms degrade oil and produce intermediate products such as alcohols, phenols, esters, aldehydes, ketones and fatty acids. These in turn are converted into CO_2, water and microbial biomass. This process results in complete mineralization of the pollutant and is clearly the ultimate goal of any bioremediation process. Normal populations of hydrocarbon utilizing microorganisms account for 0.1% of the population but may reach 100% under selective pressure after a spill or prolonged chronic discharges, returning to original levels after the pollutant is removed (Abioye et al., 2012a).

2.4 Mechanism of petroleum hydrocarbon degradation

There are a number of mechanisms known for degradation of organic compounds. During degradation, bacteria are selected for their ability to degrade and this involves several mechanisms to degrade these molecules (Das and Chandran, 2011). The bacteria have to be an electron acceptor for degradation of organic molecules. The mechanism is based on two processes: (i) growth and (ii) cometabolism (Fritsche and Hofrichter, 2005). In the growth part, bacteria uses organic carbon as a source of energy and carbon (mineralization of organic pollutants) (Angelidaki and Sanders, 2004). Co-metabolism is the metabolism of an organic compound in the presence of a growth substrate that is used as the primary carbon and energy source (Fritsche and Hofrichter, 2005).

The degradation mechanism of the hydrocarbon enzyme system can be used in the biodegradation process. Other mechanisms are (1) attachment of microbial cells to the substrates and (2) production of biosurfactants (Das and Chandran, 2011). Some enzymes, for example dioxygenases, monooxygenases and hydroxylases, play an important role in the microbial degradation of oil. Enormous number of bacteria and fungi has the ability to degrade organic pollutants. However, although many bacteria have the ability to degrade the organic pollutants, a single bacterial species does not have the enzymatic capability to metabolize all of the organic compounds in a contaminated soil (Fritsche and Hofrichter, 2005). A mixed microbial community has more powerful biodegradative potential compared to a single species, because the genetic information of more than one organism is necessary to degrade the complex mixtures of organic compounds present in polluted areas (Fritsche and Hofrichter, 2005). In summary, the essential specifications of aerobic microorganisms degrading organic pollutants are as follow (Figure 2.2),

● Metabolic processes for optimizing the contact between the organic pollutants and the microbial cells must be efficient. The chemicals must be accessible to the organisms carrying out biodegrading activities (Fritsche and Hofrichter, 2005).

●Next, the degradation path transforms the organic pollutants systematically into intermediates of the central mediator metabolism (Fritsche and Hofrichter, 2005).

●The initial intracellular attack on organic pollutants is an oxidative process; the activation and incorporation of oxygen is the enzymatic key reaction catalyzed by oxygenases and peroxidases (Fritsche and Hofrichter, 2005).

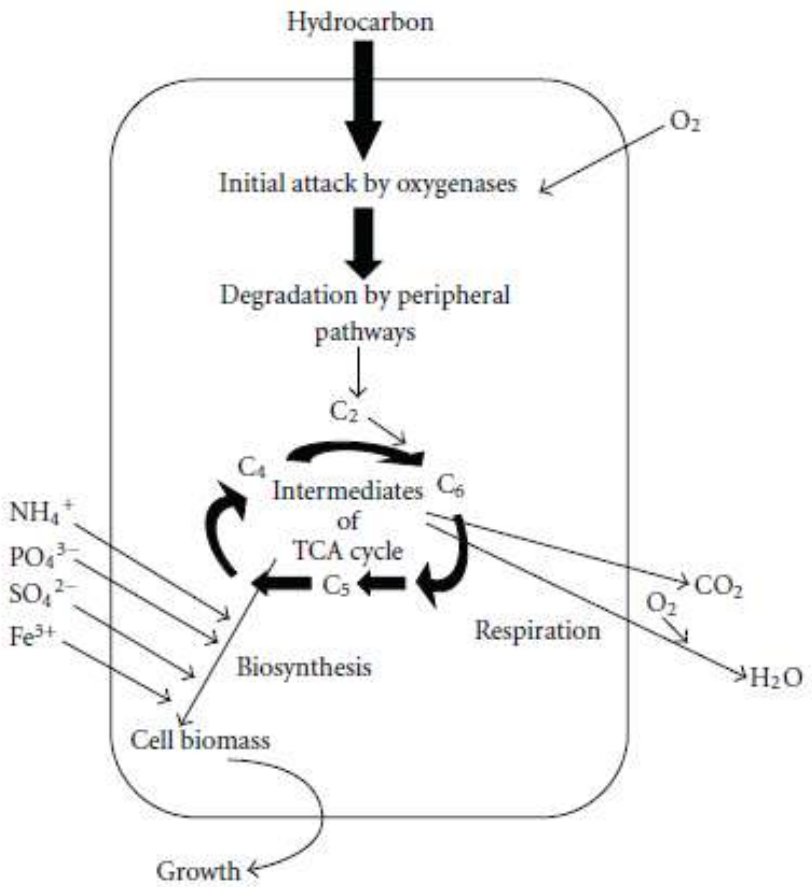


Figure 2.2 Main principle of aerobic degradation of hydrocarbons by microorganisms (Das and Chandran, 2011).

2.4.1 Degradation of hydrocarbon fractions

Microorganisms easily utilize gaseous hydrocarbons. Dry gases are dominated by methane relative to the higher homologs with a dryness coefficient [C1/Σ (C1 – C5)] of > 95%, while wet gases are rich in $C2^+$ components with a dryness coefficient of < 90% (Pallasser, 2000). Whiticar (1994) indicated that microorganisms can metabolize methane, and methanotrophs have been identified in heavily degraded oil reservoirs. Biodegraded gases are usually compositionally dry with very few exceptions.

The C_{6-15}-n-alkane groups are among the most rapidly biodegraded components of oil, although they are also susceptible to removal by extensive water washing. Benzene or toluene is less affected by biodegradation than n-heptane, 3-methylhexane, cyclohe xaneand methylcyclohexane (Masterson et al., 2001). Cyclic and branched-chain alkanes are more stable to biodegradation than linear alkanes (Rojo, 2009). There is a tendency of reduced capability to biodegradation with greater alkyl replacement for alkylcyclohexanes, isoalkanes, alkycyclopentanes and alkylbenzenes (George et al., 2002). The location of methylation has a strong effect on susceptibility to biodegradation. Of the branched alkanes, 2-methylalkanes are more susceptible to degradation than 4- methylalkanes, which in turn are more susceptible than 3-methylalkanes (George et al., 2002). Adjacent methyl group will reduce the susceptibility of an isomer to biodegradation for example, 1,1-dimethylcyclopentane and 1,1-dimethylcyclohexane are the most resistant to the alkylcyclohexanes and alkylcyclopentanes; 1,2,3-trimethylbenzene and 1,2,3,4-tetramethylbenzene are more resistant to biodegradation than other C3- and C4-alkylbenzenes (George et al., 2002). Molecular parameters such as 3-methylpentane/nhexane, 1,1-dimethylcyclopentane/n-heptane, and 3-methylpentane/2-methylpentane thus increase with increasing degree of biodegradation.

2.4.2 Biodegradation of Aromatic compounds

Polycyclic aromatic hydrocarbons (PAHs) which consist of two or more benzene rings are typically more resistant than aliphatic hydrocarbons. Aromatic compounds have a different base on the distillation sites and petroleum refinery. The most important aromatic petroleum hydrocarbons are toluene, benzene, xylene, ethyl benzene, and xylene. Benzene and its properties are important because all aromatic hydrocarbons are derivatives of benzene. Benzene has three double bonds with a six membered ring and it is flat ($C_6 H_6$). It is unsaturated structurally; similar to the cyclic alkenes. However, it is stable and does not participate in reactions that are specific of alkenes. The main differences between aromatic and aliphatic hydrocarbons are providing a useful method for classifying these compounds. For example, the anaerobic degradation process of aromatic hydrocarbons is slow and uncommon compared to aerobic biodegradation (Chakraborty and Coates, 2004; Foght, 2002). In addition, degradation of aliphatic hydrocarbons in aerobic conditions needs oxygen as a terminal electron acceptor. Essentially, aromatic hydrocarbons can support the growth of bacteria when they are present as the sole source of carbon and energy (Boonchan et al., 2000; Field and Alvarez, 2007). Therefore, aromatic hydrocarbons are not as easy to biodegrade compared to branched alkanes which are slightly more readily degradable than the alicyclic hydrocarbons (Cao et al., 2009). A large number of different bacteria and fungi have the ability to metabolize PAH. Degradation of PAH by fungal occurs in two different ways. White rot fungi produce unspecific extracellular ligninolytic enzymes, laccases and peroxidases that initiate a free radical attack by a single electron transfer, leading to the formation of quinines (Pizzul, 2006).

In general, the first step in the aerobic bacterial biodegradation is the hydroxylation of an aromatic ring via a dioxygenase, with the formation of a cis-dihydrodiol (Pizzul, 2006). Polycyclic aromatic hydrocarbons may degrade in the rhizosphere of some plants

and direct plant uptake of pyrene and phenanthrene has been observed (Pizzul, 2006). However, the reason for the degradation of polycyclic aromatic hydrocarbons in the presence of plants is the enhancement of the activity of polycyclic aromatic hydrocarbons degrading microorganism near the roots, where they find an environment rich in nutrients and root exudates (Pizzul, 2006).

2.4.3 Biodegradation of Benzene

Pure benzene (C_6 H_6) is a clear colorless liquid with a boiling point of 80 ^{0}C and melting point of 5.5 ^{0}C (Khan et al., 2004). Benzene is released to water and soils by both natural and industrial sources such as via gasoline leaks from underground storage tanks, hazardous industrial waste sites discharges, and land disposal of benzene containing wastes. In 2004 about 11 metrics of benzene were released to soils from more than 900 processing facilities and domestic manufacturing concerns (PHSA, 2007). Benzene is moderately soluble in water, with a solubility of 1,780 mg L^{-1} at 25 °C. Benzene can transfer to surface water through runoff, to the atmosphere through volatilization and to groundwater because of leaching. Benzene also can accumulate in fruits of plants and leaves. Degradation of benzene by microbe is important for the control of migration of dissolved benzene in the subsurface (Figure 2.3) (Mancini et al., 2003).

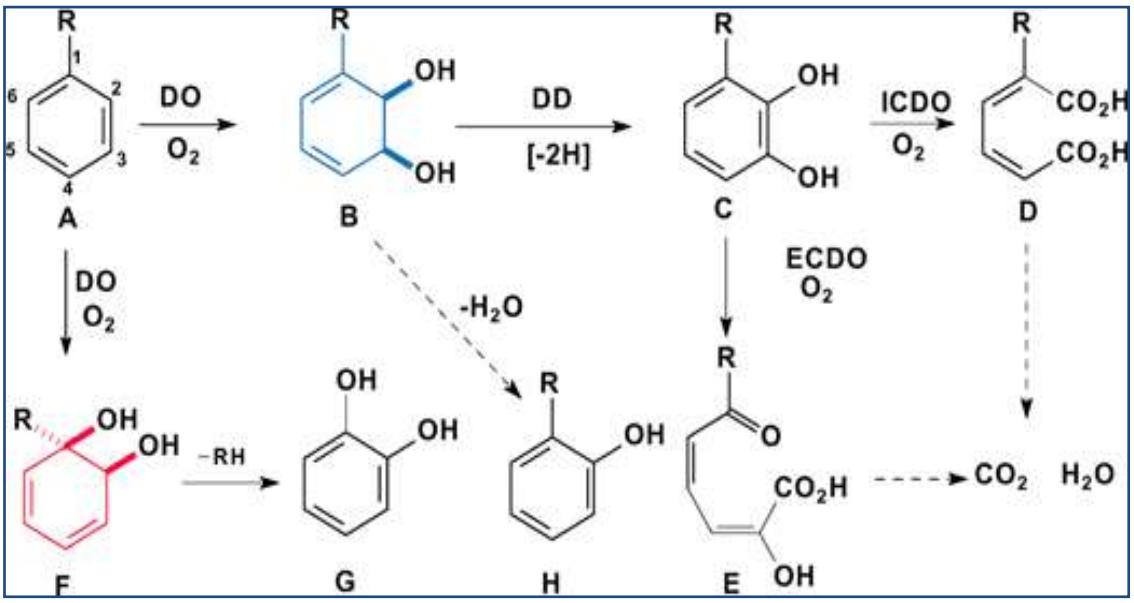


Figure 2.3 Initial steps of bacterial biodegradation pathways for benzene substrates (Boyd and Bugg, 2006).

The successful biodegradation of benzene depends on the abiotic factors and enzymatic capacities of microorganisms. These factors will be effective at suitable growth temperature condition of greater than 15 ^{0}C and the available supplies of fixed forms of phosphorus, molecular oxygen and nitrogen. The benzene ring is the most widely broadcast unit of chemical structure in nature; hence, microorganisms have the ability of degrading aromatic compounds. The benzene ring can be degraded in soil under both anaerobic and aerobic conditions. There are two divergent pathway mechanisms for the biodegradation of benzene. In both methods, the result of the mechanism is the production of catechol which is further catabolized by ortho- and meta- cleavage. Anaerobic degradation of benzene has been reported in soil, water and column studies. Recently, isolates capable of anaerobic benzene degradation have been reviewed (Chakraborty and Coates, 2004; Coates et al., 2002; Kasai et al., 2006) and demonstrated that two Azoarcus strains (DN11 and AN9) and two Dechloromonas strains (RCB and JJ) have the capability to degrade benzene in anaerobic conditions (Weelink et al., 2010). Coates and Achenbach (2004) found that benzene could be

degraded completely within five days. Due to the stability of benzene, anaerobic degradation of benzene is more difficult. The mechanisms of activation and further degradation of benzene are still unknown (Weelink et al., 2010). The initial steps of benzene degradation are carboxylation, methylation, hydroxylation, and subsequent transformation to the central aromatic intermediate benzoyl-CoA (Figure 2.4), which is further degraded to CO_2 (Weelink et al., 2010).

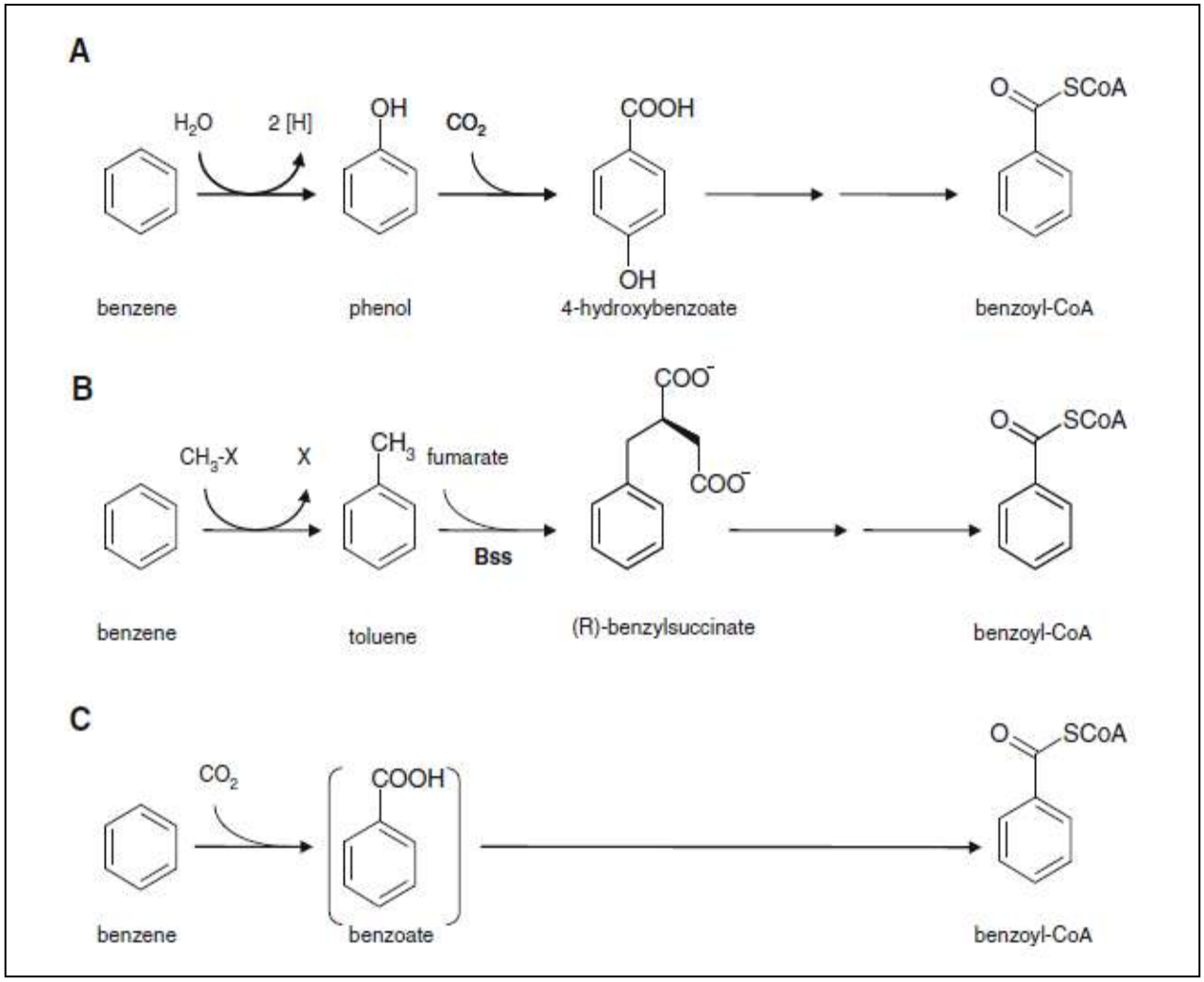


Figure 2.4 Possible mechanisms of benzene degradation under anaerobic conditions a) benzene hydroxylation, b) benzene methylation, c) benzene carboxlation (Weelink et al., 2010).

There are many aerobic metabolic pathways for the degradation of benzene (Jindrová et al., 2002). Aerobic degradation of benzene usually starts by progressive oxidation of the alkyl side chain of the aromatic ring to produce carboxylic acids, or ring oxidation which produces substituted pyrocatechols (Jindrová et al., 2002). Carboxylic acids and pyrocatechols are then transformed to substrates of the citrate cycle through cleavage of the aromatic ring (Jindrová et al., 2002). In aerobic pathways, catechol and protocatechuate are produced as central intermediates, and these compounds are then substrates for ring-cleaving dioxygenases in the central pathways (George et al., 2011). These transformations are mainly based on reactions that are catalysed by oxygenases (Figure 2.5) (George et al., 2011).

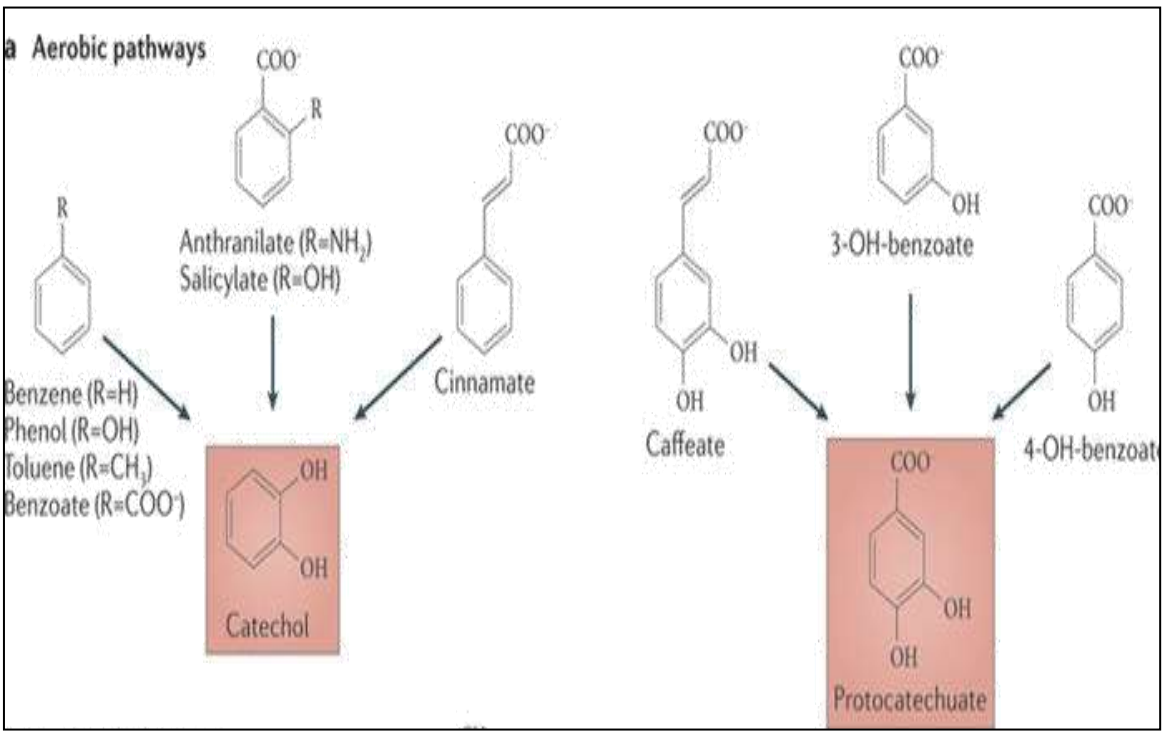


Figure 2.5 Possible mechanisms of benzene degradation under aerobic conditions (George, et al., 2011).

2.4.4 Biodegradation of *n*-alkane

Aliphatic hydrocarbons are more biodegradable than PAHs, due to their being less toxic and higher bioavailability. The n-alkanes (C_6 - C_{15}) are among the most rapidly biodegraded components of oil, although they are also susceptible to removal by extensive water washing. A number of microbial degradation of alkane has been reported (Baek et al., 2006; Colombo et al., 1996; Hidayat and Tachibbana, 2012; Sonia et al., 2002).

Margesin and Schinner (2001) identified *Rhodococcus fascians* as a one of the hydrocarbon degradation bacteria which are able to produced bioemulsifiers when grown with *n*-alkanes as the sole carbon source. Bacteria can uptake and transport soluble alkanes that are dissolved in the liquid phase (Ahmed, 2004). Indeed, bacteria could only utilize solubilized hydrocarbons. There is a different mechanism for biodegradation of aliphatics. Ron and Rosenberg (2002) reported that microbial degradation of aliphatic and aromatic compounds depends on the microorganisms which grow in oil polluted sites and have an important role in the biological treatment of the contamination. One of the limiting factors in this process, especially at low temperature is the bioavailability of many fractions of the oil (Ahmed, 2004). These surface-active materials increase the surface area of hydrophobic substrates and increase their bioavailability, thereby enhancing the growth of bacteria and the rate of bioremediation (Ahmed, 2004). The first sign of biodegradation is 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. Aromatic hydrocarbons are typically more resistant than aliphatic hydrocarbons. In general, branched-chain alkanes and cyclics are more persist out to biodegradation than linear alkanes.

Adjacent methyl group will reduce the susceptibility of an isomer to biodegradation, for example, 1,1-dimethylcyclopentane and 1,1-dimethylcyclohexane are the most resistant of the alkylcyclohexanes and alkylcyclopentanes; 1,2,3-trimethylbenzene and 1,2,3,4- tetramethylbenzene are more resistant to biodegradation than other C3- and C4-alkylbenzenes (George et al., 2002).

One of the alkane degradation pathways, which is most widely accepted, as the first step, is the incorporation of an oxygen atom into the alkane by monooxygenases as described in Figure 2.6 (Ahmed, 2004). The resulting product is an alcohol that is converted by the following alcohol dehydrogenase into an aldehyde and to an alkanoic acid that is finally channeled into the oxidation pathway (Ahmed, 2004).

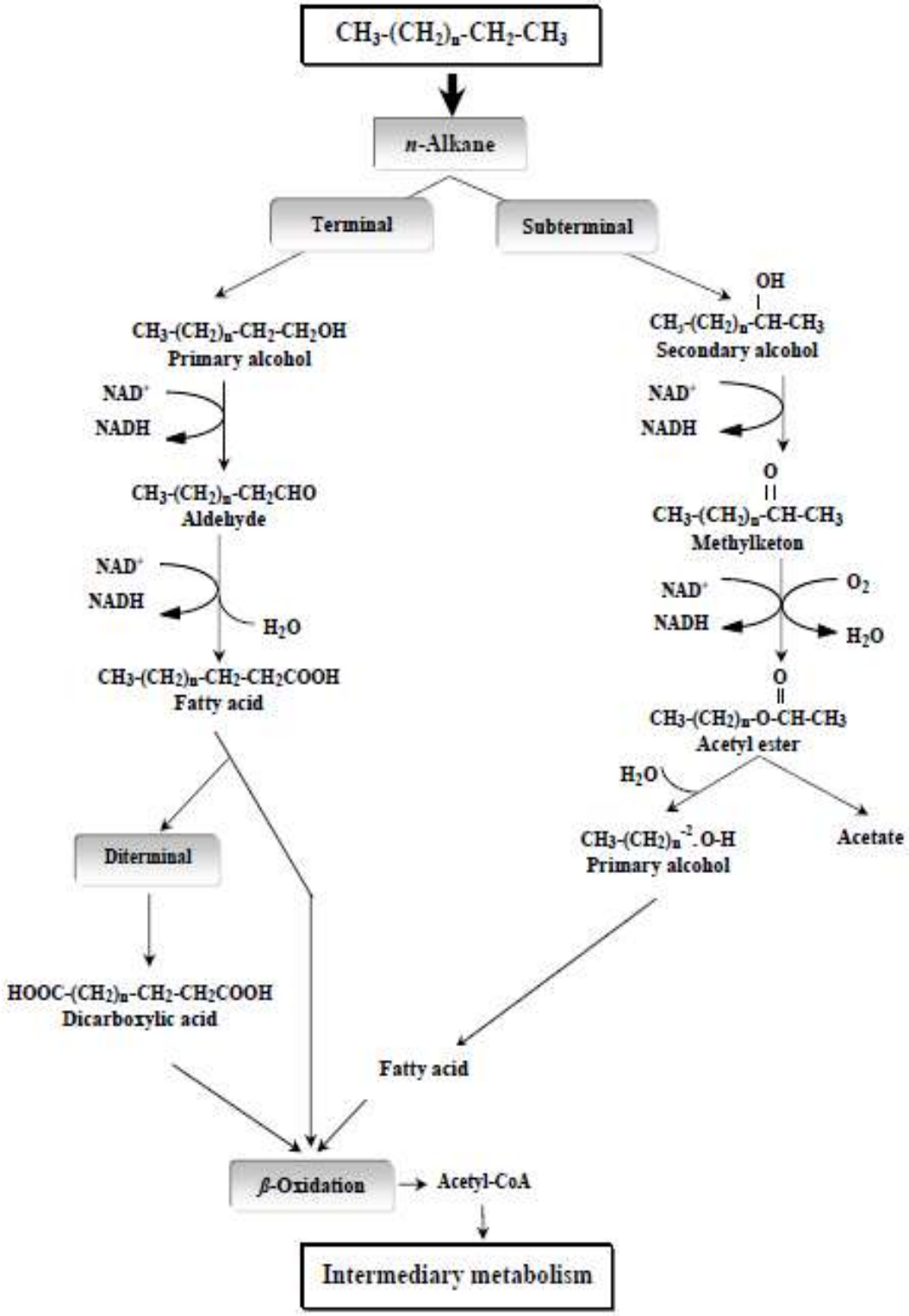


Figure 2.6 Basic metabolism of n-alkanes degradation (Ahmed, 2004).

2.5 Factors affecting biodegradation

Numerous parameters influence the biodegradation process of hydrocarbons. Some environmental conditions such as pH, temperature, oxygen, nutrients, and soil moisture can influence biodegradation results (Gavrilescu, 2010; MSMG and MSMD, 2012). In addition, microbial population and availability of pollutants to the microbial population play a role in the degradation process. Air availability, pH, nutrient levels, and moisture contents are initial controlling factors (Boopathy, 2000; Chaillan et al., 2006). Generally, the greatest degradation happens in a range of pH of 6.5-7.5 and temperature of (20 - 30 ^{0}C) (depends on the microbial species). Microbial growth and activity are readily affected by pH, temperature, and moisture (Harekrushna and Kumar, 2012). The efficiency of the biodegradation process depends on the provision of optimal conditions to improve the degradation process (Figure 2.7)

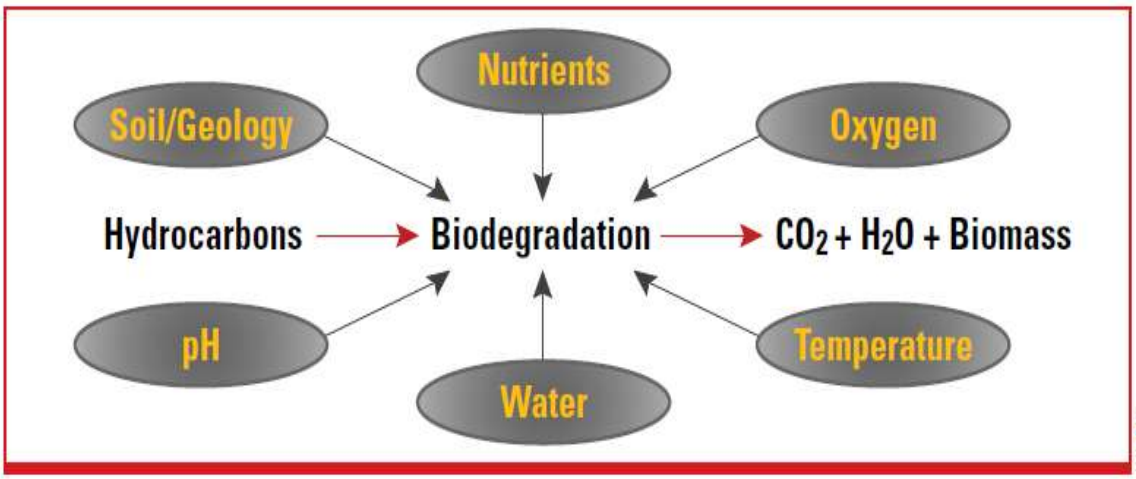


Figure 2.7 Factors affecting biodegradation process.

Some factors influencing the biodegradation process are described in the following sections.

2.5.1 Oxygen

The amount of oxygen determines whether the system is anaerobic or aerobic. In some cases, it is possible to introduce hydrogen peroxide or magnesium peroxide for increasing the amount of oxygen in the soil. A primary step in the catabolism of hydrocarbons by bacteria and fungi involves the oxidation of the substrate by oxygenase for which molecular oxygen is required. Aerobic conditions are necessary for this path of microbial oxidation of hydrocarbons in the contaminated areas. The availability of oxygen in the soil is dependent on the rate of microbial oxygen consumption, the presence of utilizable substrate and the type of soil (Bossert and Bartha, 1984). Aerobic bacteria are recognized as able to rapidly degrade hydrocarbons because of their degradative abilities in the presence of oxygen such as *Pseudomonas, Mycobacterium*, *Sphingomonas* and *Rhodococcus* (Juwarkar et al., 2010). These microbes have often been reported to degrade pesticides and hydrocarbons, both alkanes and polyaromatic compounds (MSMG and MSMD, 2012). The bacteria in the aerobic experiment were able to degrade 20 - 25% of the organic material and 90 - 95% of the alkanes (Van, 2011). In the 50-day anaerobic experiment, 15 - 18% of the organic material and only 20 - 25% of the alkenes were degraded (Van, 2011). Delivering air or oxygen to contaminated soils may be difficult for a number of reasons: the soil porosity may not be favorable 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 because of conditions that do not favor 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.

Although anaerobic microorganisms have the potential to metabolize organic contaminants, and do so in many field situations, oxygen often an integral part in the oxidation of many organic pollutants, including hydrocarbons, because molecular oxygen is required to oxidize the carbon moiety.

2.5.2 Soil properties

Physicochemical properties of soil are another important factor in increasing the speed of the degradation process. Some materials such as organic carbon can be applied in the reclamation of soil structure in order to improve delivery of air, water and nutrients. In environments, evaporation of volatile organic compound (VOCs) was observed from all soil types, especially at the freshly polluted site. Then, the decrease in concentration of oil will be as a result of microbial degradation than volatilization. Binding of soil particles has important role in biodegradation rate, but cannot be measured (Kauppi, 2011).

2.5.3 Nutrient availability

The nutrient status has direct impact on biodegradation and microbial activity (Jain et al., 2011). Nutrient serves as the sole source of electron donor, carbon and energy. Basically, fungus and bacteria need nutrient, vitamin and amino acid for growing. Nitrogen and phosphorus, and in some cases iron, are important elements needed for cellular metabolism which could become a limiting factor and thus affect the biodegradation processes (Das and Chandran, 2011). Atlas and Bragg (2009) reported that availability of nutrients, especially N and P in the degradation process of a spill occurred in freshwater and marine. Therefore, addition of nutrients has been done in reclamation and enhancement of biodegradation (Zhu et al., 2004). On the other hand,

many authors have reported the negative impact of high N-P-K levels of fertilizers on biodegradation of organic pollutants, especially on aromatic compounds (Okoh, 2006). Use of organic fertilizers, composts, poultry manure, banana skins, melon shell, wood chips, rice husk mixtures, soy cake, sewage sludge mushroom and animal droppings in the degradation process was studied by earlier researchers (Abioye et al., 2009; Adesodun and Mbagwu, 2008; Dadrasnia and Agamuthu, 2011; Hickman and Reid, 2008; Ijah and Antai, 2003a; Park et al., 2001). Bento et al. (2005) reported addition of N and P increased the degradation rate from 16% to more than 90 % in contaminated soil.

Abioye et al. (2009) indicated that melon shells as a source of nutrients has the ability to degrade crude oil contaminated soil 30% higher than unamended polluted soil in the same period. It is observed that the addition of spent mushroom compost (SMC) to the contaminated medium reduced the toxicity, added enzymes, microorganisms, and nutrients for the microorganisms involved in degradation of PAHs (Lau et al., 2003). 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. 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 (Lee et al., 2003; Pandey and Fulekar, 2012). Depending on the nature of the impacted environment, some of these nutrients could become limiting, hence, the addition of nutrients is necessary to enhance the biodegradation of oil pollutants (Kim et al., 2005; Okoh, 2006). Frederic et al., (2005), observed that addition of commercial fertilizers containing N and P to hydrocarbon contaminated soil increased the microbial population, he also reported a 77 – 95% loss of total alkanes, and 80% loss of PAHs in hydrocarbons contaminated soil within a period of 180 days. In another the study he used poultry manure (PM) for the enhancement of biodegradation of a substance (Okoh,

2006). However, excessive nutrient concentrations can prevent the biodegradation activity (Okoh, 2006). Many authors have reported the negative effects of high concentration of N-P and K levels on the biodegradation of hydrocarbons (Chaîneau et al., 2005), and more especially on the aromatics.

2.5.4 Moisture

Available water is necessary for the living microorganisms which can affect the microbial activity and growth. So irrigation is needed to achieve the optimal moisture level (Harekrushna and Kumar, 2012). The water holding capacity (WHC) recommended bioremediation process may range from 25% – 28% (Pandey and Fulekar, 2012). However, increasing soil moisture has been found to positively affect the removal of polycyclic aromatic hydrocarbon from soil. Gong et al., (2005) demonstrated the significant effect of moisture on sunflower extraction of PAH from a manufactured gas plant (MGP) soil. Previous works illustrated the greatly effect of moisture on the reduced organic adsorption capacities (Thibaud et al., 1993).

2.5.5 Temperature

Temperature can affect biodegradation due to change in metabolic activity of microbes (Eriksson et al., 2001). This factor has an important role in the diversity of the microbial flora, its physiology, and microbial metabolism. Temperature can affect the rate of biodegradation as well as the chemical and physical properties of oil (Margesin and Schinner, 2001). Generally, microbial activity will reduce at low temperatures (Baraniecki et al., 2002; Delille, 2000; Eckford et al., 2002; Gibb et al., 2001). Also, the bioavailability of some substances, such as polyaromatic hydrocarbons and aliphatics, is dependent on the temperature. Diffusion rates of organic compounds are increased by rising temperature (Northcott and Jones, 2000). Thus, higher molecular reaction rates

due to smaller boundary layers are expected at elevated temperatures (Pelletier et al., 2004). Although hydrocarbon biodegradation can happen over a wide range of temperatures, the rate of biodegradation will reduce with decreasing temperatures (Okoh, 2006). The highest range of degradation in soil environment happens between 30 - 40^{o}C and 20-30 ^{o}C in water environment (Das and Chandran, 2011). Although, many microorganisms in cold climates can survive and grow at temperatures below 5 ^{0}C, but it is essential that contaminated sites be at the optimum temperature for bioremediation to progress successfully. In addition, the solubility and bioavailability of a contaminant will increase as temperature increases, and oxygen solubility will be reduced (Margesin and Schinner, 1999). For example, a selection of *Rhodococcus* species that were isolated from an Antarctic soil was able to successfully degrade a number of *n*-alkane at –2 ^{o}C but was severely inhibited at a higher temperature (Bej et al., 2001). In addition, the PAHs naphthalene and phenanthrene were successfully degraded from crude oil in seawater at temperatures as low as 0 ^{o}C (Bamforth and Singleton, 2005).

Coulon et al., (2005) found that a high percentage of alkane degradation (77 - 95%) occurred at 10 ^{0}C. Low temperatures retard the rate of volatilization of low-molecular-weight hydrocarbons, some of which are toxic to microorganisms (Namkoong et al., 2002). Atlas et al., (1981) reported the highest rate of crude oil degradation with mixed microbial culture in sandy soil that occurred at 3 ^{0}C with 48% compared with 21% at 22 ^{0}C in the same condition. Temperature is often not the major limiting factor for hydrocarbon degradation in the environment especially in tropical climate, except that it relates to other factors such as the physical state of the oil or whether water is available for microbial growth (Atlas, 1981).

2.5.6 Acidity or Alkalinity

pH is important to achieve optimal degradation conditions. Soil pH also affects the activity of enzymes due to the pH sensitivity of amino acid groups, which is essential for binding and catalysis (Dick et al., 2000). If the soil is too acidic it is possible to increase pH by adding lime (Harekrushna and Kumar, 2012). High pH, as can be observed in some soils, would have a negative influence on the ability of microbial populations to degrade hydrocarbon (Lakshmi and Velan, 2011). Most fungi can tolerate slightly acidic conditions but heterotrophic bacteria live in pH 7.0. Brajesh et al. (2003) observed a low degradation rate in the two acidic soils, with half-lives of 224 and 58 days at pH 4.7 and 5.7, respectively. Kastner et al. (1998) reported that the changes of the pH from 5.2 to 7.0 enabled PAHs degradation. Verstraete et al., (1976) reported a high rate of degradation in a range of pH 4.5 to 7.4, but when pH was further raised to 8.5, the rate of biodegradation was decreased significantly. Stapleton et al. (1998) indicate a high rate of the biodegradation of aromatic compounds in acidic environments. In addition, Dibble and Bartha (1979) reported an optimal range of pH (5.0 to 7.8) for the mineralization of oily sludge in soil.

2.5.7 Bioavailability of hydrocarbon

Bioavailability has been defined as the accessibility of a chemical for assimilation and possible toxicity (Puglisi et al., 2007). Bioavailability or bioaccessibility has also been defined as the level to which a substance is free to move on to or into an organism and it is known that bioavailability differs between species and organisms. The bioavailability of organic contaminants is the main factor determining their fate, ecological risk, toxicity, and losses in the environment (Oleszczuk, 2009). It provides information on the actual risk relating to the presence of the contaminants. Two main

factors that determine the amount of a chemical that is bioavailable are the rate of transfer of the compound from the soil to the living cell and the rate of uptake and metabolism (Semple et al., 2003). The bioavailability of a chemical is determined by the rate of mass transfer relative to the intrinsic activity of the soil biota (Semple et al., 2003). Bioavailability controls biodegradation because microbial cells must consume energy to induce catabolic gene systems used in biodegradation (Madsen, 2002). There are a number of approaches to determine bioavailability according to the solid or liquid phase of the contamination media. One of main key that affects microbial degradation is the hydrophobicity of diesel oil, which limits its transfer to the cell surfaces of microorganisms (Lee et al., 2006; Schein et al., 2009). This limitation may be overcome either by growing surfactant producing microorganisms or by an addition of surface-active agents. This indicates there is an increase in the bioavailability of diesel oil to microorganisms. Several constraints can limit the bioavailability of organic compounds in the environment. These are low aqueous solubility, sorption, micropore exclusion, and content of organic carbon (Froehner et al., 2012; Huesemann et al., 2004). Puglisi et al. (2007) reported that phenanthrene degradation is usually lower in soils with high organic matter due to higher adsorption and lower diffusion to the water phase, while in soils with low levels of organic matter, variations in biodegradation may instead, be related to the amount of clay in the soils. The rate of transfer is determined by the equilibrium and actual concentration in the bulk phase and aqueous phase. This is central to the concept of bioavailability as it relates to biodegradation.

2.5.8 Chemical properties of hydrocarbon

When the suitability of a cleanup approach is to be evaluated, the biodegradability and composition of the petroleum hydrocarbon pollutant is the most important consideration (Okoh, 2006). Compositional heterogeneity among different crude oils

and refined products influenced the overall rate of biodegradation of both the oil and its component fractions (Meredith et al., 2000; Okoh, 2006). Biodegradability is essentially impacted by the composition of the oil pollutant (Jain et al., 2011). For example, kerosene can be completely degraded, under suitable conditions (Okoh, 2006), but in the case of heavy asphaltic-naphthenic crude oils, only a maximum of about 11% is biodegradable even under suitable conditions (Okoh, 2006). Okoh et al., (2003) indicated that between 8 and 30% of the crude oil was degraded in polluted soil by mixing bacterial consortium for 15 days. He also noted that heavier crude oils are more generally complicated 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 (Okoh, 2006). Fedorak and Westlake (1981) also reported a high rapid attack of aromatic hydrocarbons during the degradation of crude oil by microbial populations from a fresh site.

2.5.9 Concentration of Petroleum hydrocarbon

Concentration of petroleum hydrocarbon determines to a greater extent the rate of breakdown of the hydrocarbons from the environment (Abioye et al., 2012a). Concentration of hydrocarbon has an important role in its biodegradability and level of toxicity for the degrading by organisms. High concentration of hydrocarbon can be affecting the activity of microorganisms. Concentrations of 1 to 100 μg/ml of water or 1 to 100 μg/g of soil or sediment are not generally considered to be toxic to common heterotrophic bacteria and fungi. Ijah and Antai, (2003b) indicated a high rate of degradation of hydrocarbons in soil contaminated with low concentration (10% and 20%) compared to those contaminated with the high concentration (30 and 40%) of crude oil within a period of one year. Rahman et al., (2002) reported that with the increase of concentration oil from 1 to 10%, biodegradation is decreased from 78% to

52%. Indeed, high concentrations of hydrocarbons can be associated with increased amount of heavy oil in polluted sites which lead to inhibition of biodegradation through limitation in nutrients. Table 2.5 shows the major factors that affect the biodegradation process.

Table 2.5 Factors affecting the biodegradation process.

Parameters	Condition required	Optimum value for oil degradation
Temperature (^{0}C)	14 - 45	20 - 30
Nutrient content	N and P for microbial growth	C: N: P = 100: 1: 1
Oxygen content	Minimum 10%	10 - 40%
Soil pH	5.5 – 8.5	6.5 - 8
Soil moisture	25 – 28%	30 - 90%
Type of soil	–	Silt or low clay

Source (Gavrilescu, 2010).

2.6 Remediation techniques

Contamination poses serious environmental risks, including surface and groundwater contamination, and risks to human health and safety (Balasubramaniam et al., 2007). Thus, the remediation of contaminated soil is an essential practice (Amro, 2004). Traditional technologies such as chemical and physical treatments are not very effective and often expensive. In addition, microbial-based remediation has become more popular in recent decades (Hamby, 1996).

These recent systems are based on the stimulation of aerobic bacteria populations to degrade contaminants, which is done by increasing oxygen flux and adding nutrients to

the contaminated zone. All remediation techniques are used to remove pollutants either in-situ (in place) or ex situ (other sites for treatment). Table 2.6 shows different treatment methods which have been employed for the remediation of soils (Hamby, 1996). According to the Office of Technology Assessment, conventional methods typically recover no more than 15% of the oil after a major oil spill (Zahed et al., 2006). Abioye et al., (2010) estimated that bioremediation was able to remove 92 % of lubricating oil from contaminated soil in three months. Most remediation technologies are site specific and the selection of appropriate technologies is often a difficult, but extremely important step in the successful remediation of a contaminated site (Khan et al., 2004). Therefore, the successful treatment of a contaminated site depends on proper selection, design, and adjustment of the remediation technology's operations based on the properties of the contaminants and soils, and on the performance of the system (Khan et al., 2004).

Table 2.6 Treatment techniques used for soil remediation

Techniques	References
▪ Physical treatments	
- Capping	(Melanie, 2005; Sun et al., 2010)
- Electro kinetics	(Vane and Zang, 1997)
- Soil washing	(Conte et al., 2005; López-Vizcaíno et al., 2012)
- Stabilization	(Fleri and Whetstone, 2007)
▪ Chemical treatments	
- Oxidation	(Ferguson et al., 2004; Lemaire et al.,2011)
- Chemical immobilization	(Saad, 2009)
- Photo degradation	(Jia and Chu, 2009; Villa et al,, 2010)
- Peroxide remediation	(Qi et al., 2004)
▪ Biological treatment	
- Bioremediation	(Dadrasnia and Agamuthu, 2013; Zouboulis and Moussas, 2011)
- Phytoremediation	(Agamuthu et al., 2010; Peng et al., 2009)

Some of the different techniques used in remediating contaminated soil are discussed below.

2.6.1 Current clean up techniques

Physical treatment techniques in remediation of contaminated sites are those methods which do not change the physico-chemical properties of soil, including soil washing, extraction, capping or incineration and chemical treatment techniques such as oxidation, steam extraction, stabilization and chemical extraction (Riser-Roberts, 1998). Table 2.7 describes some limitations and benefits of chemical and physical treatment methods.

Table 2.7 Chemical and physical techniques for oil removal

Methods	Benefits	Limitations
Mechanical separation	- Significant volume reduction - Can be applied in municipal waste management	- It is not effective for some soil types - Must be cleaned up using another method for separated part of contaminated soil
Soil washing	- Fast cleaning method - High efficiency and effective	- High costs of construction - Necessity for transportation of the cleaned up soil
Soil flushing	- Applied *in-situ* - Lack of solid wastes	- Incomplete removal of contaminants
Electroremediation	- Only method for *in-situ* removal - It is applicable to different metals	- Any dissimilarity of the soil body decreases the effectiveness of the method
Extraction and storage	- Short time of excavation - Applied in the case of emergency	- Not applicable in the case of locala and small polluted sites

(Dermont et al., 2008; Pavel and Gavrilescu 2008)

Physical and Chemical treatments are still used as routine methods in many countries like, U.K. (Lessard and Demarco, 2000). On the other hand, in some countries, for example, there is a limitation to the use of these methods especially in the Unites States due to long-term environmental effects (USEPA,1999a,b). Some common physicochemical methods for remediation of soil are discussed below:

i) Soil washing with solvents and water

This *ex-situ* method uses liquids such as water and some solvents in mechanical processes to clean the polluted soils. Solvents are selected based on their ability to solubilized contaminants, and on their health and environmental effects (Khan et al., 2004). There are different soil washing process as, according to the texture of soil and size of soil particles (silt, sand and clay). Since hydrocarbon contaminants have a tendency for sorption and bind to smaller soil particles, such as clay and silt, separating the smaller soil particles from the larger ones reduces the volume of contamination (Khan et al., 2004; Kin, 2008). This technology can be used to clean and recover a large amount of organic pollutants from soil. In Europe, this method is used, extensively, but it has limited use in the United States. The estimated average cost of using this technique is about 170 US dollars per ton, depending on the oil concentration and site conditions. Important notes which are related to soil washing are as follow,

- Mixed waste contamination need a combination of solvents
- In the case of clay soil remediation by washing is very difficult
- Soil washing is more affective for soils which do not have silt or clay particles
- Contaminated soils with a high amount of organic matters and humic acids require pre-treatment
- High costs of making use of the cleaning method and installation.

ii) Soil vapor extraction

The soil vapor extraction (SVE) method known as vacuum extraction or soil venting, is an accepted and cost effective technology for remediating unsaturated contaminated soils (Khan et al., 2004). Other names for SVE include “soil venting”, “soil vacuum extraction”, “vacuum extraction”, “subsurface venting”, “soil gas vapor extraction”, “in situ venting”, “enhanced volatilization”, and “vapor extraction” (Kim et al., 2002). Vacuums are applied through the wells near the source of contamination to evaporate the volatile constituents of the contaminated mass which are subsequently withdrawn through an extraction well (Figure 2.8) (Khan et al., 2004). Then, the extracted vapors are treated before being released into the atmosphere (USEPA, 1995). Some fuels like diesel fuel and heating oil are not rapidly removed by this method. Important notes which are related to the SVE method are as follows:

- SVE can treat large volumes of soil at reasonable costs (Khan et al., 2004).
- Since, this method is in-situ technology and the site problem is minimal.
- SVE is generally not appropriate for sites with a groundwater table located less. than 0.9 m below the land surface (Khan et al., 2004).
- This technique needs only a short time for treatment (USEPA, 1995).
- Of in high concentration it is difficult to achieve the reduction of pollutants.

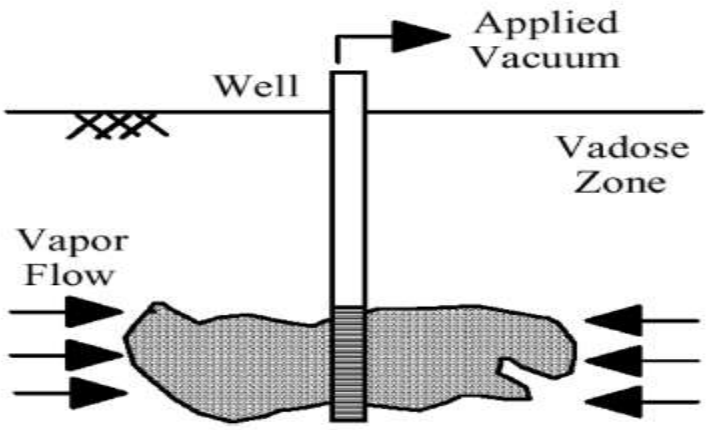


Figure 2.8 Schematic diagram of soil vapor extraction system (Kim et al., 2002).

iii) Solidification/stabilization

Stabilization/solidification is one of the physico-chemical remediation technologies that produced physical changes and relies on the reaction between the soil or waste and a reagent in order to reduce the mobility of the expected contaminants. This method as used in both the physical and chemical processes to reduce potentially adverse impacts on the environment resulting from the disposal of hazardous and mixed wastes (EPA, 1999b). The stabilization technique is used to chemically reduce the soluble in hazardous wastes by converting the contaminants to reduced solubility (Silva et al., 2007). Solidification can be accomplished by a chemical reaction between the waste and solidifying reagents or by mechanical processes (EPA, 1999b).

2.6.2 Biological technology/ Bioremediation

Biological methods are those techniques which depend on the microbial activity to break down and mineralize of contaminates to less to toxic form (Sayara, 2010). 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 (Kumar et al., 2011). Remediation of petroleum-contaminated systems can be obtained by either biological or physicochemical methods. However, the negative consequences of the physicochemical approach are currently directing greater attention to the exploitation of the biological alternatives (Okoh, 2006). 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 pollutants. 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 attack the pollutants and convert them to harmless

products (Sharma, 2012). Potential advantages of bioremediation compared to other treatment methods include destruction rather than transfer of the contaminants to another medium; minimal exposure of the on-site workers to the contaminants; 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). 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. Besides cost effectiveness, it is a permanent solution, which may lead to complete mineralization of the pollutants (Perelo, 2010). Bioremediation can deal with lower concentration of contaminants where the cleanup by physical or chemical methods would not be feasible (Perelo, 2010). In general, biological treatments are considered as cost effective, attractive and environment friendly (Hamdi et al., 2007; Sayara, 2010).

For bioremediation to be successful, the bioremediation methods depend on having the right microbes in the right place with the right environmental factors for degradation to occur (Boopathy, 2000). The right microbes are fungi or bacteria, which have the physiological and metabolic capabilities to degrade the pollutants (Boopathy, 2000). Although bioremediation is being engineered into a novel technology, microorganisms have been used routinely for the treatment and transformation of waste products for at least 100 years (Juwarkar et al., 2010).

There are three classifications of bioremediation according to Leung, (2004):

Biotransformation - the alteration of contaminant molecules into less or nonhazardous Molecules (Leung, 2004).

Biodegradation - the breakdown of organic substances into smaller organic or inorganic

Molecules (Leung, 2004).

Mineralization - the complete biodegradation of organic materials into inorganic constituents such as CO_2 or H_2O (Leung, 2004).

There are three general approaches to cleaning up contaminated soils :(i) Soil can be excavated from the ground and be either treated or disposed off (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).

Bioremediation has many advantages which include:

- This technique can performed in-situ, so there is no transport cost and no soil destroyed.
- This method is a natural process and environmentally friendly.
- It can be coupled with other physical or chemical treatment methods (Boopathy, 2000).
- This method needs less manual supervision.
- Bioremediation needs low capital expenditure compared to other techniques in removing hazardous waste.
- By applying this technique, toxic compounds are removed or destroyed and not just merely separated.

Bioremediation has also some disadvantages such as:

- Bioremediation is limited to those compounds that are biodegradable (Harekrushna and Kumar, 2012), Some of compounds with toxic chemicals such as, radionuclides and heavy metals are not biodegraded by using this method (Boopathy, 2000).
- Soil must have high penetrance, for *in-situ* bioremediation method.

- The bioremediation method is slow and takes time compared to other treatment options such as incineration and excavation.
- This method can be used in all contaminated sites, because some of microbes are sensitive to contaminants.
- Bioremediation cannot be done in some contaminated sites due to the site conditions.

Before starting to use bioremediation techniques, some of the following questions need to be understood:

Does biodegradation occur in the site naturally? Are the contaminated compounds biodegradable? Are environmental and geographic conditions suitable for biodegradation? These questions can be answered by doing site characterization and by treatability studies (Boopathy, 2000).

These days several bioremediation techniques have been applied, which can be carried out either out of or on the sites (Sayara, 2010).

i. *In situ* bioremediation

In situ bioremediation is the use of microorganisms to degrade contaminants in original site with the goal of obtaining harmless chemicals as end products (Jim et al., 2005). In-situ bioremediation refers to the application of biological treatment processes in situ, without the excavation of contaminated soils (Chien et al., 2010). Most often *in situ* bioremediation is applied to the degradation of contaminants in saturated zones. This technology has been developed as a more effective alternative and less costly compared with the methods used to clean up aquifers and soils contaminated with chlorinated solvents, fuel hydrocarbons, toxic metals, explosives and nitrates. *In-situ* bioremediation technology is highly dependent upon external conditions, which are 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 include 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 a lower cost and fewer disturbances since they provide the treatment original site by avoiding excavation and transport of contaminants (Prasad et al., 2012; Vidali, 2001).

In-situ bioremediation treatments are limited by the depth of the soil that can be effectively treated (Laura and Carmen, 2009). In many soils effective oxygen diffusion for desirable rates of bioremediation extend to a depth of only a few centimeters, 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 (PNNL, 2012). Accelerated in situ bioremediation is used where it is desired to increase the rate of contaminant biotransformation, which may be limited by a lack of required electron donor, nutrients or electron acceptor (PNNL, 2012).

Some *in- situ* treatment methods include:

ii. Bioventing

This method can remove and/or remediate contaminated soil under aerobic conditions by providing oxygen to microorganisms in soil, injecting air directly into the residual contamination (Shukla et al., 2010). The bioventing system consists of a blower and a well, which introduce air into soil through the well. This process is similar to soil vapor vacuum extraction, in contrast to soil vapor vacuum extraction, bioventing has a potential remediation method (Cauwenberghe and Rooste, 1998), because it uses low

airflow rates to provide only enough oxygen to keep up microbial activity (Shukla et al., 2010). Bioventing systems promote biodegradation of constituents and minimize volatilization (Sayara, 2010).

iii. Biosparging/air sparging

Air sparging is an in-situ technology in which air is introduced into a saturated and contaminated zone (Hidayat and Tachibbana, 2012). Injected air traverses horizontally and vertically in channels through the soil column, creating an underground stripper that removes contaminants by volatilization (EPA, 2001). Air sparging promotes the growth of aerobic bacteria in a contaminated zone and it may oxidize reduced chemical species (Nadim et al., 2000). Air sparging has been shown to be effective in removing several types of contaminants such as the lighter petroleum compounds (C_3 – C_{10}) and chlorinated solvents (Marley et al., 1992; Reddy et al., 1995). Air sparging can also be explained as a method of site remediation that introduces air (or other gases) into a saturated zone contaminated with volatile organic compounds (VOCs). In addition to volatilization of VOCs, air sparging promotes the growth of aerobic bacteria in saturated zones and may oxidize reduced chemical species (Figure 2.9) (Nadim et al., 2000). Some advantages and disadvantages of air sparging are listed below (Miller, 1996).

- This method can be used to clean contamination below the water table or in the capillary fringe which is in contrast to soil vapor extraction techniques.
- Because of the low operation and maintenance costs of this technology, it may be “particularly effective when large quantities of groundwater must be treated (Miller, 1996).
- This technique cannot be used in confined aquifers

- This method requires detailed pilot testing and monitoring to ensure vapor control and limited migration (EPA, 2011).

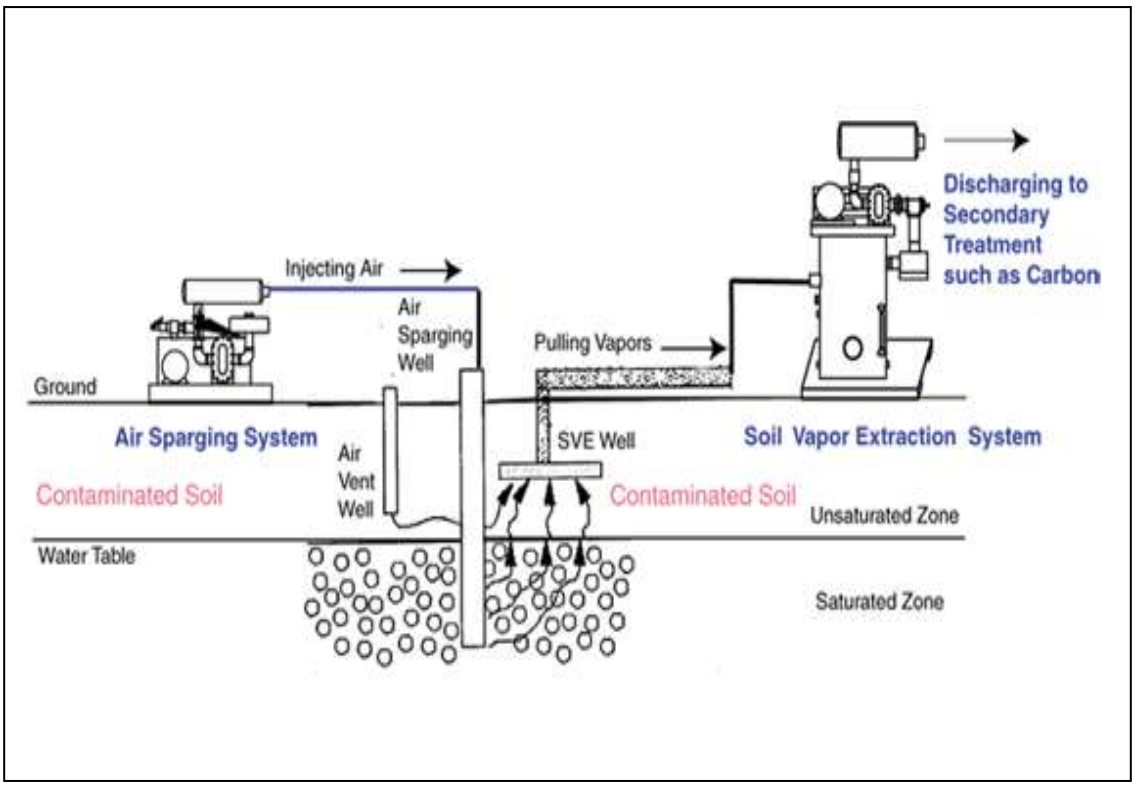


Figure 2.9 Air sparging and soil vapor extraction system.

iv. *Ex situ* bioremediation

This process requires excavation of contaminated soil or pumping of groundwater to facilitate microbial degradation (Kumar et al., 2011). This technique involves the removal of contaminated soil from the ground.

v. Bioreactor

Reactors are one of the important types of ex-situ techniques used to biodegraded water and soil contaminant sites. The bioreactor has become one of the best options for the bioremediation of soils polluted by recalcitrant pollutants under controlled environmental conditions (Robles-González et al., 2008). Bioremediation in reactors

involves the processing of contaminated solid material (soil, sediment, sludge) or water through an engineered containment system (Vidali, 2001). A slurry bioreactor may be defined as a containment vessel and apparatus used to create a three-phase (solid, liquid, and gas) mixing condition to increase the bioremediation rate of soil bound and water-soluble pollutants as a water slurry of the contaminated soil and biomass (usually indigenous microorganisms) is capable of degrading target contaminants (Vidali, 2001). Some limitation of this method is the high operation cost and the soil needs to be excavated. Use of bioreactors in remediation of contaminated soil is reported by many researchers (Leung, 2004; Rehmann et al., 2008; Sharma, 2012).

vi. Land farming

In land farming, contaminated soil is periodically tilled to improve aeration and to promote soil homogeneity for indigenous biodegradative microorganisms for biological degradation (Gan et al., 2009). Since land farming has the potential to reduce monitoring and maintenance costs, as well as cleanup liabilities, it has received much attention as a disposal alternative (Kumar et al., 2011).

vii. Composting and addition of composting materials

A process typically used to degrade solid waste materials, has also recently been studied as a remediation technology for PAH contaminated soils (Gan et al., 2009). This technique involves combining contaminated soil with nonhazardous organic amendants such as agricultural wastes or manure (Kumar et al., 2011). Composting bioremediation strategy is an aerobic process, based on mixing components of composting with the contaminated soil, as the compost matures the pollutants are degraded by the active microflora within the mixture (Semple et al., 2001). Many are based on the application of manure from cows, pigs or chickens (Adesodun and Mbagwu, 2008; Adriana et al.,, 2007; Atagana et al., 2003; Ijah and Ndana, 2003; Sasek et al., 2003). Adriana et al.,

(2007) has reported 63% TPH removal in soil contaminated with petroleum hydrocarbon and amended with raw coffee beans. Sewage sludge is abundant globally, and it has been successfully used as an amendment in bioremediation (Hur and Park, 2003). Virtually, any putrescible material available, such as vegetable (Atagana et al., 2003), spent mushroom compost (SMC) (Eggen, 1999; Lau et al., 2003) and even garden waste (Guerin 2001; Michel et al., 2001) can be used. The use of composting approaches for bioremediation of organic pollutants generally, (Semple et al., 2003) and specifically the use of composting to treat PAHs (Antizar-Ladislao et al., 2004) have been reviewed. Composting is an efficient method that relies on added matrix material and on mixing/aeration, but not on addition of microbial inoculums (Jørgensen et al., 2000). 70% mineral oil biodegradation was recorded by Jørgensen et al., (2000), when bark chips were used as a bulking agent for composting lubricating oil-contaminated oil in a field scale study for a period of five months. Abioye et al., (2009) recorded 75% loss of oil in soil contaminated with crude oil and composted with melon shells for a period of 28 days. Organic wastes like tea leaves, potato skin and soy cake, in earlier studies were found to enhance the biodegradation of diesel oil up to 80% loss of oil within the period of 3 months (Dadrasnia and Agamuthu, 2010). Composting has been indicated to be effective in biodegradation of PAHs at both laboratory and field scales using different types of compost bulking agents such as spent mushroom (Lau et al., 2003) soot waste, green wastes (Antizar-Ladislao et al., 2005) maple leaves and alfalfa (Haderlein et al., 2006). Haderlein et al., (2006) studied the effects of composting to soil by the addition of maple leaves and alfalfa during the mineralization of pyrene and benzopyrene and reported that neither composting nor the addition of compost had any effect on benzopyrene mineralization. In contrast, the pyrene mineralization rate increased dramatically with the amount of time that the soil had been composted (more than 60% mineralization after 20 days). Antizar-Ladislao et al., (2005) used in-vessel

composting technology for the remediation of coal tar contaminated soil and optimized the soil composting temperature at 38 ^{0}C for the most effective degradation. In a related study, solid culture with a small amount of low-quality raw coffee beans was used for total petroleum hydrocarbon removal from a weathered and polluted soil (Adriana et al., 2007).

Amendment of soil contaminated by heavy mineral oil using sawdust, hay and compost was reported by Lee et al., (2008) that after 105 days of experiment the heavy mineral oil was reduced between 18 - 40% from the initial level of contamination of 7490 mg hydrocarbon kg^{-1}, whereas the level of hydrocarbon reduction in non-amended soil was just 9%. The author also observed significantly higher microbial activities in compost amended contaminated soil. Corn and sugar cane residues were reported to stimulate the biodegradation of diesel oil in diesel-contaminated soil by 67% (Molina-Barahona et al., 2004). Ijah et al., (2008) also observed that increase in biodegradation of crude oil in crude oil contaminated soil amended with chicken droppings. They reported 75% of crude oil degradation in soil amended with chicken droppings while only 56.3% degradation was recorded in unamended polluted soil in the 10 weeks of the experiment.

viii. Biopiling

Biopiles are a hybrid of land farming and composting. This system includes biocells, bioheaps, biomounds and compost cells (Khan et al., 2004). This treatment involves the piling of petroleum-contaminated soils into piles or heaps and then simulating aerobic microbial activity by aeration and the addition of minerals, nutrients, and moisture (Khan et al., 2004). This method is effective in a wide range of organic pollutants and the treatment time of this technique is short (6 month to 2 years). The costs of this method depend on the type of contaminant and whether the treatment is pre or post.

Estimated the rang of cost is from 130 - 260 US dollars per cubic yard (FRTR, 1991). Strategies of bioremediation consist of, monitored natural recovery, biostimulation, bioaugmentation and phytoremediation. These strategies can be applied in the combination of biostimulation and phytoremediation. Table 2.8 shows a summary of benefits and limitations of in-situ and ex situ on bioremediation.

Table 2.8 Summary of advantages and disadvantages of in-situ and ex-situ bioremediation processes.

Technology	Examples	Benefits	Limitations
In-situ	Biosparging Bioventing Bioaugmentation	Most cost efficient Treats soil and water Relatively passive Natural attenuation Processes Noninvasive	Extended treatment time Monitoring difficulties Environmental constraints
Ex situ	Land farming Composting Biopiling	Cost efficient Low cost Can be done on site	Mass transfer problem Need to control abiotic loss Bioavailability limitation Space requirements

Source (Vidali, 2001)

2.6.3 Bioremediation strategies

i. Monitored natural recovery

Monitored natural recovery (MNR) is increasingly recognized by the US Environmental Protection Agency (USEPA) as an option for managing or remediating contaminated sediment sites (Magar and Wenning, 2006). NMR is the only bioremediation strategy applied in sediment management currently (Perelo, 2010). This technique includes leaving contaminated sediments in polluted sit and allowing ongoing natural processes such as biological and chemical transformation and aquatic sedimentation to immobilize or degrade the contaminant in-situ, thus reducing its bioavailability (Perelo, 2010). NMR is most effective for low risk sites with a low level of contamination and compared to other techniques this technique is least expensive response action but requires long term monitoring (Perelo, 2010).

ii. Biostimulation approach

This process involves the introduction of nutrients such as organic wastes, fertilizers and organic substances to stimulate the growth of the indigenous species to degrade pollutants. Nutrients need to be added because the input of large quantities of carbon sources tends to result in a rapid depletion of the available pools of major inorganic nutrients such as N and P. Levels of N and P added to stimulate biodegradation at contaminated sites are often estimated from C/N ratios (Lee et al., 2007). Biostimulation aims at enhancing the activities of indigenous microorganisms that are capable of degrading pollutant from soil environment. It is often applied to bioremediation of oil-contaminated soil. In other cases, it is intentional stimulation of resident xenobiotic-degrading bacteria by using electron acceptors, water, nutrients, or electron donors (Widada et al., 2002). Combinations of inorganic nutrients are often more effective than single nutrients (Sutherland, 1992).

Nitrogen is the most commonly used nutrient in a bioremediation project (Liebeg and Cutright, 1999). It is used primarily to support biosynthesis (NH_4^+ and NO_3^-) or as an alternative electron acceptor to oxygen (NO_3^-). Activated sludge has been suggested to be a useful source of N for PAH biodegradation in soils (Juteau et al., 2003). Dried blood acts as a slow release agent of nitrogen (Straube et al., 2003), so does a range of natural materials such as peat, compost and manure (Moore and Chiu, 2001).

iii. Bioaugmentation approach

This process involves the introduction of enzymes into contaminated soil to stimulate degradation of organic pollutants in contaminated zones (Sayara, 2010). It was described by Alexander, (2000) as the inoculation of contaminated soil or water with specific strains or consortia of microorganisms to improve the biodegradation capacity of the system for a specific pollutant organic compound. Bioaugmentation strategies may prove successful especially in the remediation of man-made contaminants, where specialized bacteria with the appropriate catabolic pathways may not be present in the contaminated habitat (Perelo, 2010). Biostimulation aims at enhancing the activities of indigenous microorganisms that are capable of degrading pollutants from the soil environment. It is often applied to the bioremediation of oil-contaminated soil (Das and Chandran, 2011; Kanissery and Sims, 2011). Combinations of inorganic nutrients often are more effective than single nutrients.

Bioaugmentation is a promising and low-cost bioremediation method in which an effective bacterial isolate(s) or microbial consortium capable of degrading xenobiotics is administered to contaminated sites (Ueno et al., 2007). However, the soil environment is very complicated and the degrading ability of exogenously added microorganisms tends to be affected by the physicochemical and biological features of

the soil environment (Ueno et al., 2007). There are three bioaugmentation processes:

First, is to increase the genetic diversity by inoculation of allochthonous microorganisms (Jim et al., 2005), which will lead to an increase in the catabolic potential, whereby the rate of removal of the contaminant(s) by biodegradation will also increase (Dejonghe et al., 2001).

Second, is to take samples from the site and use them as initial inoculates for serial enrichments with the contaminant (s) in question as the sole source of carbon. These inoculums are then returned to the site in large numbers in order to increase the rate of biodegradation. Thirdly, the approach involves the addition of uncharacterized consortia present in materials such as sewage sludge, garden waste and compost (Jim et al., 2005). According to previous researches, bioaugmentation technology has mostly been used for the biodegradation of pure compounds (Mancera-López et al., 2008). The mineralization of high concentrations of organic pollutants has been reported when consecutive inoculations were tested (Schwartz and Scow, 2001). Most bioaugmentation studies have been carried out using filamentous fungi inoculated into model soil systems and using contaminants of low molecular weight PAHs with up to four rings. The interest in these microorganisms is their ability to synthesize relatively unspecific enzymes involved in cellulose and lignin decay that can degrade high molecular weight, complex and more recalcitrant toxic compounds, including aromatic structures (Pe´rez et al., 2002).

Bento et al. (2005) reported a 72.7% light TPH fraction and a 75.2% heavy TPH fraction degradation in diesel contaminated soil bioaugmented with bacterial consortium of *Bacillus cereus, Bacillus sphaericus, Bacillus fusiformis, Bacillus pumilus Acinetobacter junii* and *Pseudomonas* sp. Ying et al. (2010) augmented a PAH-contaminated soil with *Paracoccus* sp. strain HPD-2 and observed a 23% decrease in total PAH concentrations after 28 days, with a decline in average concentration from

9942 to 7638 μg kg^{-1} dry soil. They discovered that the percentage degradation of 3-, 4- and 5(+6) -ring PAHs was 35.1%, 20.7% and 24.3%, respectively (Ying et al., 2010). Bagherzadeh et al., (2008) evaluated the efficiency of pollutant removal by selected microorganisms and reported thus: Five mixed cultures and three single bacteria strains, *Pseudomonas* sp., *Arthrobacter* sp. and *Mycobacterium* sp. were isolated from hydrocarbon-contaminated soils by enrichment on either crude oil or individual hydrocarbons, as the sole carbon source. The strains were selected based on their ability to grow in medium containing crude oil, used engine oil or both. Their ability to degrade hydrocarbon contamination in the environment was investigated using soil samples contaminated with used engine oil. Table 2.9 shows selected literature on bioremediation technique.

Table 2.9 Literature on bioremediation techniques for treating contamination.

Type of Contaminate	Bioremediation techniques	Description	References
Diesel fuel	Biostimulation	Release fertilizer 0, 250, 500, 750 mg N Kg^{-1} The composition of NPK inorganic fertilizer (18: 8: 17)	(Komilis et al., 2010) **(**Silva-Castro et al., 2012)
Petroleum hydrocarbons	Bioventing	Oxygen/air is added to soil vapor phase to stimulate aerobic condition	(FRTR, 2005)
Organic contaminants	Biosparging	oxygen/air is added below groundwater surface	(Doelman and Breedveld, 1999)
Arabian light crude oil	Biostimulation with inoculations	Slow release fertilizer C: N: P (100:10:3)	(Oh et al., 2001)
Crude oil	Natural attenuation and biostimulation	Add fertilizer N-P-K (850-85-240 μg/g respectively)	(Chaîneau et al., 2005)
Some organic and inorganic pollutants	Solidification	Physically bounding or enclosing contaminants within stabilized mass	(FRTR, 2005)

Table 2.9 Cont'd

Type of Contaminate	Bioremediation techniques	Description	References
Petroleum hydrocarbons	Biostimulation	Wood chips 1:1 (v/v) mixed wood chips and sewage sludge 4:1 (v/v)	(Brandt, 2006)
Diesel fuel	Natural attenuation, biostimulation and bioaugmentation	250 mg/kg $(NH4)_2SO_4$ 100 mg/kg K_2HPO_4 40 ml of 2.6 X 10^8 cells ml^{-1} of a bacterial consortium	(Bento et al., 2005)
Lubricating oil	Biostimulation	Banana skin, spent mushroom compost brewery spent grain 10% (w/w)	(Abioye et al., 2012b)
Creosote	Bioagumentation	Organisms *(Photobacterium phosphoreum*)	(Fritsche and Hofrichter, 2005)
Diesel fuel	Biostimulation	Add fertilizer N-P-K C: N: P (100:15:1)	(Mariano et al., 2007)

2.6.4 Cost of bioremediation

Costs data for remediation sites are limited. This section summarizes the available data on bioremediation projects including *in-situ* and *ex-situ* of soil remediation (Table 2.10). In average the cost of enhanced remediation is range from 30 US dollar to 100 US dollar per cubic meter of soil. Many factors can affect the cost such as soil type, quantity and types of contaminant and amendments used (FRTR, 2012).

In-situ bioremediation techniques often costs less compare to other remedial options.

Table 2.10 Data cost for bioremediation projects

Site name	**Contaminants**	**Volume treated (cy)**	**Technology cost ($)**	**Comments**
Hill AFB, US	PHC	1,667	551,000	Direct injection of air (In -situ bioremediation)
Superfund Site, US	PAHs	10,500	2,550,000	Slurry-phase bioreactor system(ex-situ bioremediation)
Bonneville, US	BTEX, PHC	5,000	863,000	Early bioventing application; combined with SVE
Texas Gulf Coast Site	cVOCs	NR	630,000	Recirculation with addition of methanol; anaerobic; intended as a precursor to monitored natural attenuation

NR Not reported
PAHs Polycyclic Aromatic Hydrocarbons
cVOCs Chlorinated Volatile Organic Compound
cy Cubic yards
SVE Soil Vapor Extraction
BTEX Benzene, Toluene, Ethylbenzene

2.6.5 Phytoremediation (Phytotechnology)

Phytoremediation, a green technology, is quite a novel technique which uses plants to remediate contaminated sites such as soil, sediment, surface and groundwater (Kim, et al., 2005). Phytoremediation is relatively easy to implement and is cost-effective at minimal maintenance overheads, and as long as the impacted site can support plant growth, a remediation scheme can be used anywhere (Couto et al., 2012). Phytoremediation appears effective, inexpensive and attractive because in contrast to most other remediation technologies, it is not invasive and, in principle, delivers intact and biologically active soil (Wenzel, 2008).

Phytoremediation has a good image and is often, more cost effective than other techniques (Trapp and Karlson, 2001). Phytotechnology can be used to remediate heavy metals, radioactive materials, and petroleum hydrocarbon. It might be because this method is very slow and takes time (some time more than 10 years), which makes it difficult to evaluate in the early state. Some basic information on the potential application of phytoremediation is as follows,

i. Common and scientific name of plants
ii. Field or laboratory experiment
iii. Morphology and growth form of plant
iv. Evaluated potential of plant survival in high concentrations of hydrocarbon
v. Mechanism of phytoremediation
vi. Types of microorganism which are associated with the plants
vii. Age of plants at first exposure
viii. Availability of requirements for phytoremediation
ix. Contaminated storage sites of plants (i.e. root, steam, leaf or no storage)
x. Cultural information of plants and growth duration

Phytoremediation is not applicable for all phytotoxicity chemicals and where contaminants are in high concentrations or for specific chemicals (Andersen, 2006). Furthermore, phytoremediation is limited to contamination within the depth of the rhizosphere or the depth of influence from evapotranspiration, depending on the most important removal mechanisms in the specific phytoremediation application (Andersen, 2006). Microbe-assisted phytoremediation, including rhizoremediation, appears to be particularly effective for removal and/or degradation of organic contaminants from impacted soils particularly when used in conjunction with appropriate agronomic techniques (Gerhardt et al., 2009). Major drawbacks of phytoremediation include the fact that the detoxification of organic pollutants is often slow and if decomposition is not complete, toxic compounds may accumulate in plant tissue and be released into the environment or enter food chains (Perelo, 2010). Some major advantages and disadvantages of phytoremediation are shown in Table 2.11.

Table 2.11 Advantages and disadvantages of phytoremediation technology

Advantages	Disadvantages
Environmentally friendly	Climate dependant
Many mechanisms for removal	Effectiveness depends on the nature of chemicals
Relatively low cost	Results are variable
Easily maintained and implemented	Limited to sites with lower contaminant concentrations
Faster than natural attenuation	Influenced by soil and climatic conditions of the site
High public acceptance	Effective depth limited by plant roots
Fewer air and water emissions	Effects on food web might be unknown
Several mechanisms for removal	Slower than mechanical treatments
Potential to reduce gas emission	Longer time to remediate
Reduce dust emission	Phytotoxicity limitations

Coupling of phytoremediation of contaminated soil with soil amendments such as organic matter, compost, phosphate, fertilizers, Fe, Mn oxyhydroxides and clay minerals usually reduce the mobility of contaminants in soil.

2.6.6 Costs of phytoremediation

An estimate indicates that the general cost of phytoremediation for one hectare with a depth of 15 cm is about 2,500 to 15,000 US dollars which is based on the cost of 17 to 100 US dollars for each cubic meter. A recent estimate put the cost at approximately 300 US dollars per m^3 per year to phytoremediate a site contaminated with oil and organic compounds using deep-rooted plants and trees (Frick et al., 1999). There are various ways to reduce the cost of phytoremediation, for example, during the *in-situ* phytoremediation process, plants using solar energy as a source of energy helps to reduce the cost of phytoremediation. Maintaining a site for 10 years will help to spread the cost over a longer period.

2.6.7 Methods of phytoremediation application

I. *In-situ* phytoremediation

II. *In-vivo* phytoremediation

III. *In- vitro* phytoremediation

I. *In-situ* phytoremediation

In situ phytoremediation involves placement of live plants in contaminated surface water, soil or sediment, or in soil or sediment that is in contact with contaminated ground water for the purpose of remediation. In this approach, the contaminated material is not removed prior to phytoremediation (Adadzi, 2010; Sun et al., 2011). If the phyto-mechanism consists of only uptake and accumulation as opposed to transformation of a contaminant, the plants may be harvested and removed from the site after remediation for disposal or recovery of the contaminants (Adadzi, 2010; Auxiliadora and Fereres, 2003). A requirement of the in-situ approach is that the contaminant must be physically accessible to the roots.

II. *In-vivo* phytoremediation

For sites where the contaminants are not accessible to the plants, such as the contaminants in deep aquifers, an alternate method of applying phytoremediation is possible (Adadzi, 2010). In this approach, the contaminant is extracted using mechanical means, then it is transferred to a temporary treatment area where it can be exposed to plants selected for optimal phytoremediation (Adadzi, 2010). After treatment, the cleansed water or soil can be returned to its original location and the plants may be harvested for disposal if necessary (Adadzi, 2010). Generally, this approach is more expensive than the in-situ phytoremediation.

III. *In-vitro* phytoremediation

This method is usually via components of live plants, like extracted enzymes. In theory, this approach could be applied in situ under some situations, e.g. applying plant extracts to a contaminated pond or wetland, or through use of an enzyme impregnated porous barrier in a contaminated ground water plume (Adadzi, 2010). Theoretically, this approach is the most expensive method of phytoremediation because of the costs of preparing/extracting the plant enzymes; however, in some plants, such as tarragon, (*Artemisia dracunculas* var *satiya*), exudates are released under stress that could result in reduced production costs (Adadzi, 2010; Susarla et al., 2002).

2.6.8 Mechanisms of phytoremediation

There are various mechanisms by which plants may remediate contaminated sites (Adadzi, 2010). Plants act as solar-driven pumping and filtering systems as they take up contaminants (mainly water soluble) through their roots and transport/translocate them through various plant tissues where they can be sequestered, volatilized or metabolized (Fulekar, 2010). Plants utilize different types of mechanisms for dealing with

environmental pollutants in soil. The mechanisms of phytoremediation include biophysical and biochemical processes like adsorption, transport and translocation, as well as transformation and mineralization by plant enzymes (Figure 2.10) (Pilon-Smits, 2005). Plants have been shown to be able to degrade halogenated compounds like trichloroethylene (TCE) by oxidative degradation pathways, including plant specific dehalogenases (Perelo, 2010). Dehalogenase activity was observed to be maintained after the plants were dead. Enzymes can become bound to the organic matrix of the sediment as where plants die, they decay and are buried in the sediment, thus contributing to the dehalogenase activity observed in organic-rich sediments (Perelo, 2010). A variety of contaminant-degrading enzymes can be found in plants. These include peroxidases, dioxygenases, P450 monooxygenases, laccases, phosphatases, dehalogenases, nitrilases, and nitroreductases (Pilon-Smits, 2005). Phytoremediation is based upon the basic physiological mechanisms taking place in higher plants and associated microorganisms, such as transpiration, photosynthesis, metabolism, and mineral nutrition (Marmiroli et al., 2006).

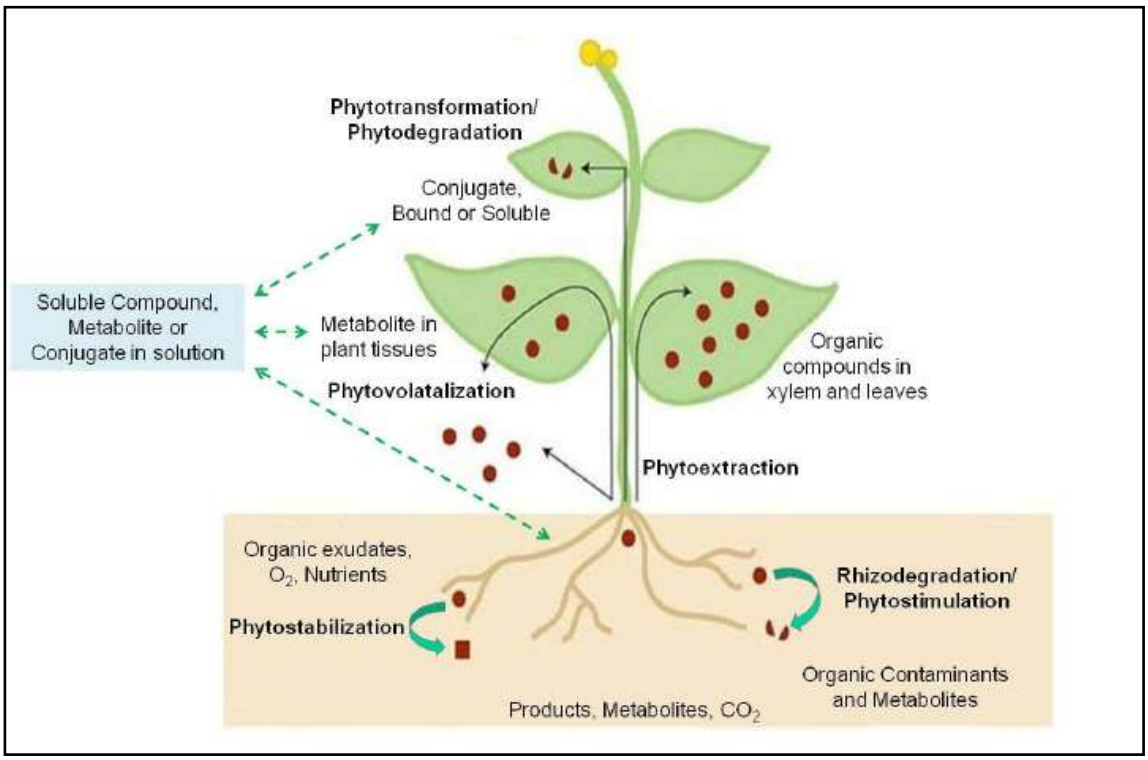


Figure 2.10 Scheme of different mechanisms of contaminant removal by plants: Pollutants in soil and groundwater can be taken up inside plant tissues (phytoextraction) or adsorbed to the roots (rhizofiltration); pollutants inside plant tissues can be transformed by plant enzymes (phytotransformation) or can be volatilized into the atmosphere (phytovolatilization); pollutants in soil can be degraded by microbes in the root zone (rhizosphere bioremediation) or incorporated in soil material (phytostabilization) (Yousaf, 2011).

Plants grow their roots in soils, sediments and water, and roots can take up organic compounds and inorganic substances; roots can stabilize and bind substances on their external surfaces when they interact with microorganisms in the rhizosphere (Marmiroli et al., 2006). Uptaken substances may be transported, stored, converted, or accumulated in the different cells and tissues of the plant (Marmiroli et al., 2006). Finally, aerial parts of the plant may exchange gases with the atmosphere allowing uptake or release of molecules (Marmiroli et al., 2006). Plants often use pathways and enzymes similar to those of mammals, which lead to a breakdown of the oil compounds (Figure 2.11) (Van Aken, 2008).

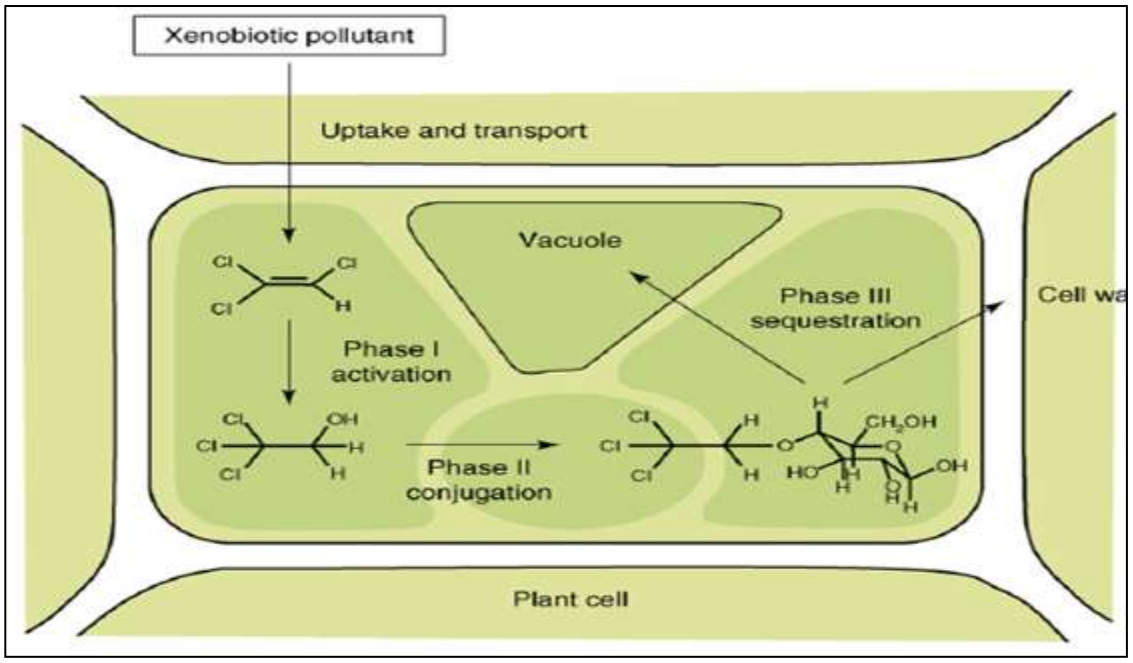


Figure 2.11 hypothetical pathways representing the metabolism of trichoroethylene (TCE) in plant tissues.

i. Phytoaccumulation/ Phytoextraction

Phytoextraction involves the removal and subsequent storage of contaminants by the plant and is often applied to the exclusion and storage of metals that may undergo speciation in plants, but cannot be metabolized (Fulekar, 2010). It can also be explained to mean the ability of plants to take up contaminants into the roots and translocate them into the aboveground shoots or leaves (Figure 2.12). Once a chemical is taken up, the plant may store the chemical and/or its by-products in the plant biomass via lignification (covalent bonding of the chemical or its by-products into the lignin of the plant), sequester it into the cell vacuoles of aboveground tissues (as opposed to in root cells as part of phytosequestration, see Section (Yousaf, 2011). Sorption properties and solubility of organic compounds are major factors that affect the rate of uptake of organic compounds by plants. One characteristic that has been shown to correlate to uptake into a plant is log K_{ow} (octanol-water partition coefficient) (Yousaf, 2011). Specifically, organic chemicals having log K_{ow} values between 1 and 3.5 have been

shown to enter into plants (Yousaf, 2011). Plant roots contain an organic membrane with a lipid bilayer which makes it partially hydrophobic (Figure 2.12). Therefore, hydrophobic chemicals with log K_{ow} more than 3.5 are not sufficiently soluble in the transpiration stream or are bound so strongly to the surface of the roots that they could not be easily translocated into the plant xylem (Yousaf, 2011). On the other hand, chemicals with low log K_{ow} are not sorbet by roots, due to their high polarity.

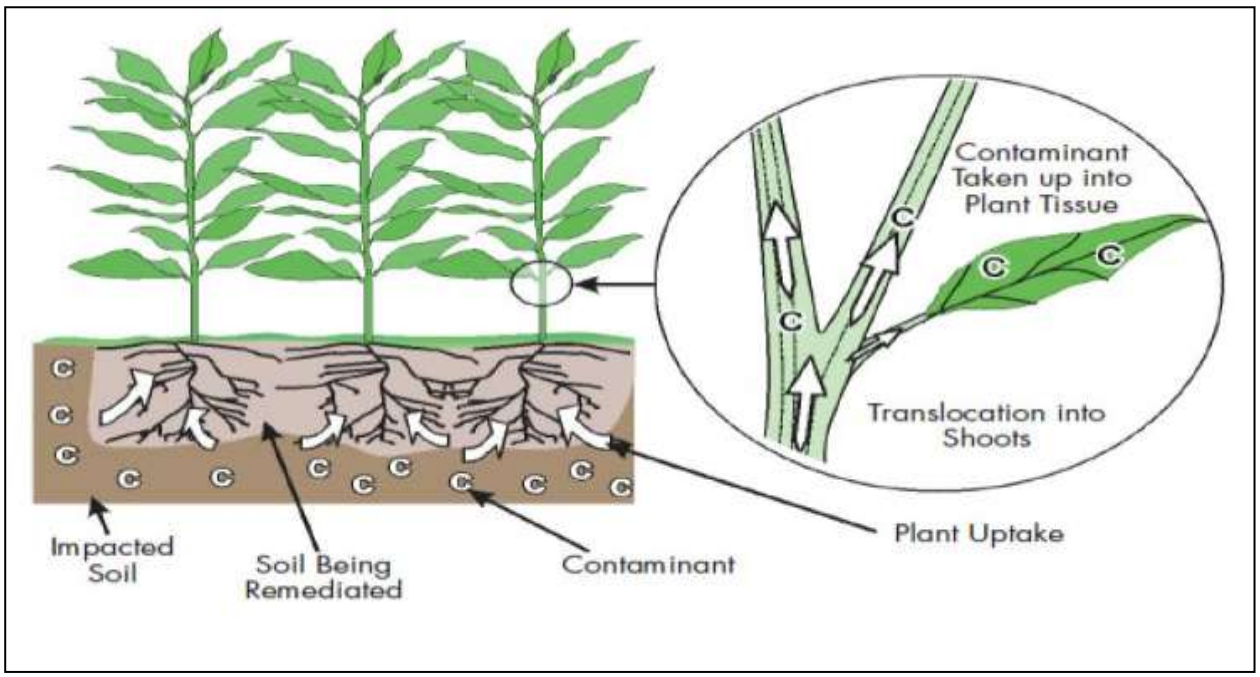


Figure 2.12 Phytoextraction mechanisms.

ii. Phytodegradation/ Phytotransformation

Phytodegradation can be explained as a series of processes that plants utilize to metabolize the contaminants (metabolism within plant). Components of this mechanism are often utilized by plants exposed to herbicides and thus have been researched extensively (Abhilash et al., 2009). Specifically, phytodegradation, also called "phytotransformation," refers to the uptake of contaminants with the subsequent breakdown, mineralization, or metabolization by the plant itself through various internal

enzymatic reactions and metabolic processes (Figure 2.13) (Yousaf, 2011). In the phytodegradation mechanism, plant enzymes are the main key in degradating contaminants such as metals, herbicides, and chlorinated solvents from soil, sediment and groundwater. For accruing phytodegradation, the compounds must be taken up by plants. One study identified 70 organic chemicals which could be taken up and accumulated by trees and plants (Feroz et al., 2012). However, phytodegradation can be limited by root depth. Generally, contaminant degradation due to enzymes produced by a plant can occur in an environment free of microorganisms (for example, an environment in which the microorganisms have been killed by high contaminant levels) (Feroz et al., 2012). Plants are able to grow in sterile soil and also in soil that has concentration levels that are toxic to microorganisms (Feroz et al., 2012). Thus, phytodegradation potentially could occur in soils where biodegradation cannot (Feroz, et al., 2012).

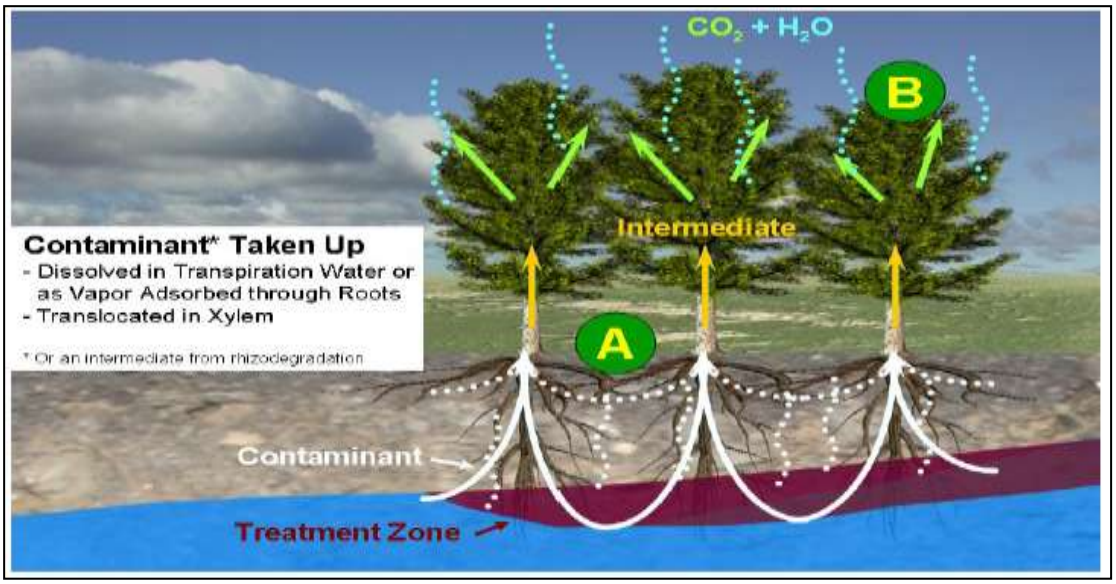


Figure 2.13 Phytodegradation mechanisms. A: enzymatic activity of plant, B: photosynthetic oxidation (Yousaf, 2011).

iii. Phytostabilization

Phytostabilization is the use of plants to immobilize or make insoluble pollutants in contaminated sites by roots or within the root zone (rhizosphere). This mechanism prevents immigration into ground water and reduces the mobility of contaminants. However, hydraulic control to prevent leachate migration can be achieved because of the large quantity of water transpired by plants. At a high level of concentration toxic effects may prevent plants from growing. Therefore, plants should be able to tolerate high levels of contaminants, have high production of root biomass with the ability to immobilize contaminants, and the ability to hold contaminants in the roots (Figure 2.14).

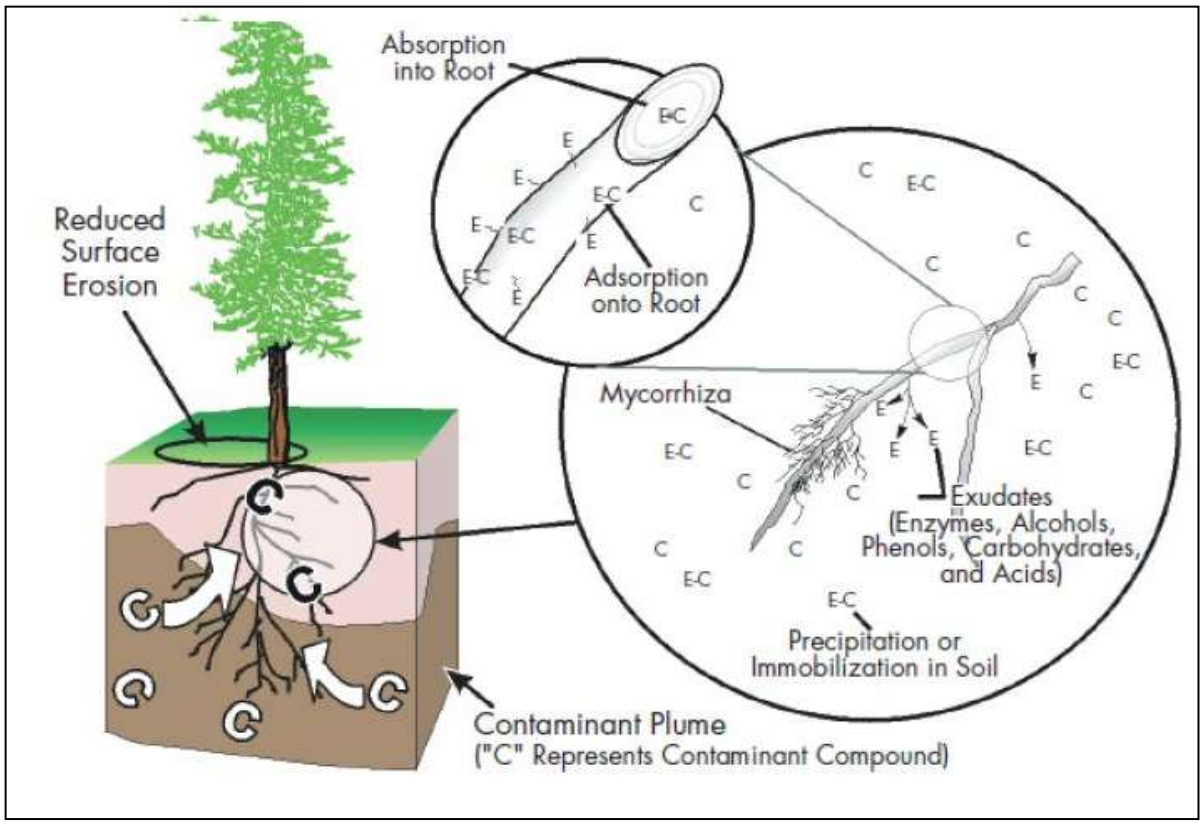


Figure 2.14 Phytostabilization mechanisms.

iv. Rhizodegradation

Rhizodegradation can be described as the transformation of contaminants by resident microbes in the plant rhizosphere (i.e., the microbe-rich zone in intimate contact with the root vascular system) (Abhilash, et al., 2009). The presence of plants on contaminated sites can drastically affect soil redox conditions and organic content (often through the secretion of organic acids from roots), as well as soil moisture (Abhilash, et al., 2009; Fulekar, 2010). Rhizodegradation is also referred to as microbe-assisted phytoremediation or rhizoremediation (Wenzel, 2008). Rhizoremediation is emerging as one of the most effective means by which plants can enhance the remediation of organic contaminants, particularly large recalcitrant compounds.

Complex interactions involving roots, root exudates, rhizosphere soil and microbes do result in degradation of organic contaminants to non-toxic or less-toxic compounds.

As much as 40% of a plant's photosynthate can be deposited in the soil as sugars, organic acids, and larger organic compounds (Kumar et al., 2006). These compounds are commonly used as carbon and energy sources by soil microbes (Chaudhry et al., 2005; Singer et al., 2004). Plant roots can also release degradative enzymes into the rhizosphere. The relationship between the plant root enzymatic and microbial interactions in degrading organic contaminants is shown in Figure 2.15 as described by Rao et al., (2010). Apart from the direct release of degradative enzymes, plants are able to stimulate the activities of microbial degrader organisms/communities (Wenzel, 2008).

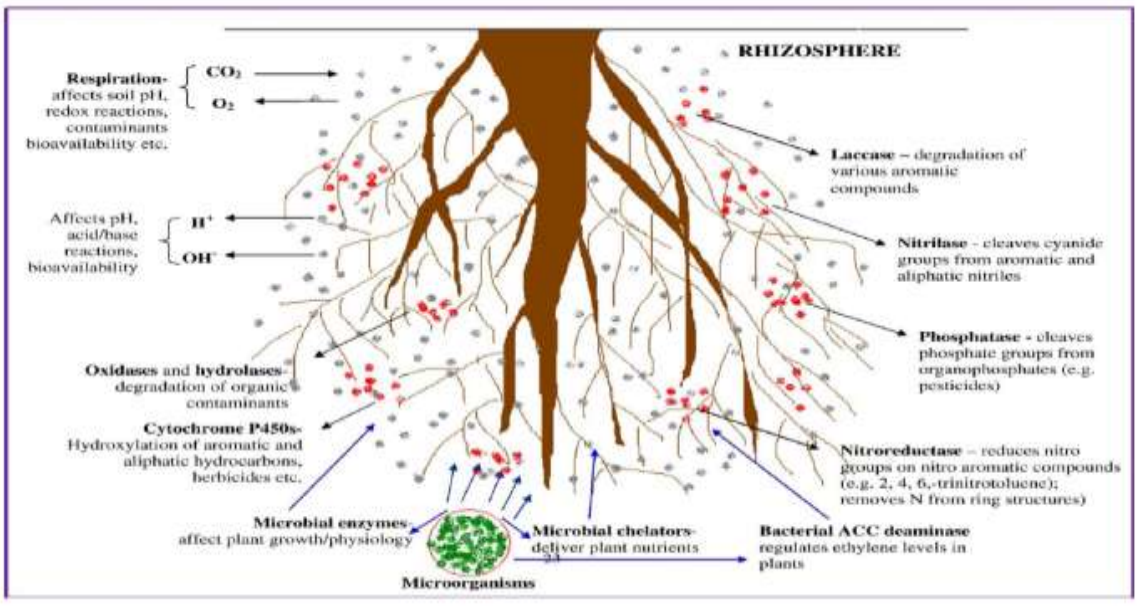


Figure 2.15 Schematic representations of the enzymatic and microbial activities responsible for the enhanced remediation in rhizospheric zone (Rao et al., 2010). Plant–degrader interactions that are thought to be most relevant for the success of rhizodegradation are shown in Figure 2.16 by (Wenzel, 2008). This is important, especially where microorganisms cannot utilize the pollutant as a sole carbon source, for instance, in the aerobic degradation of trichloroethylene (Wenzel, 2008).

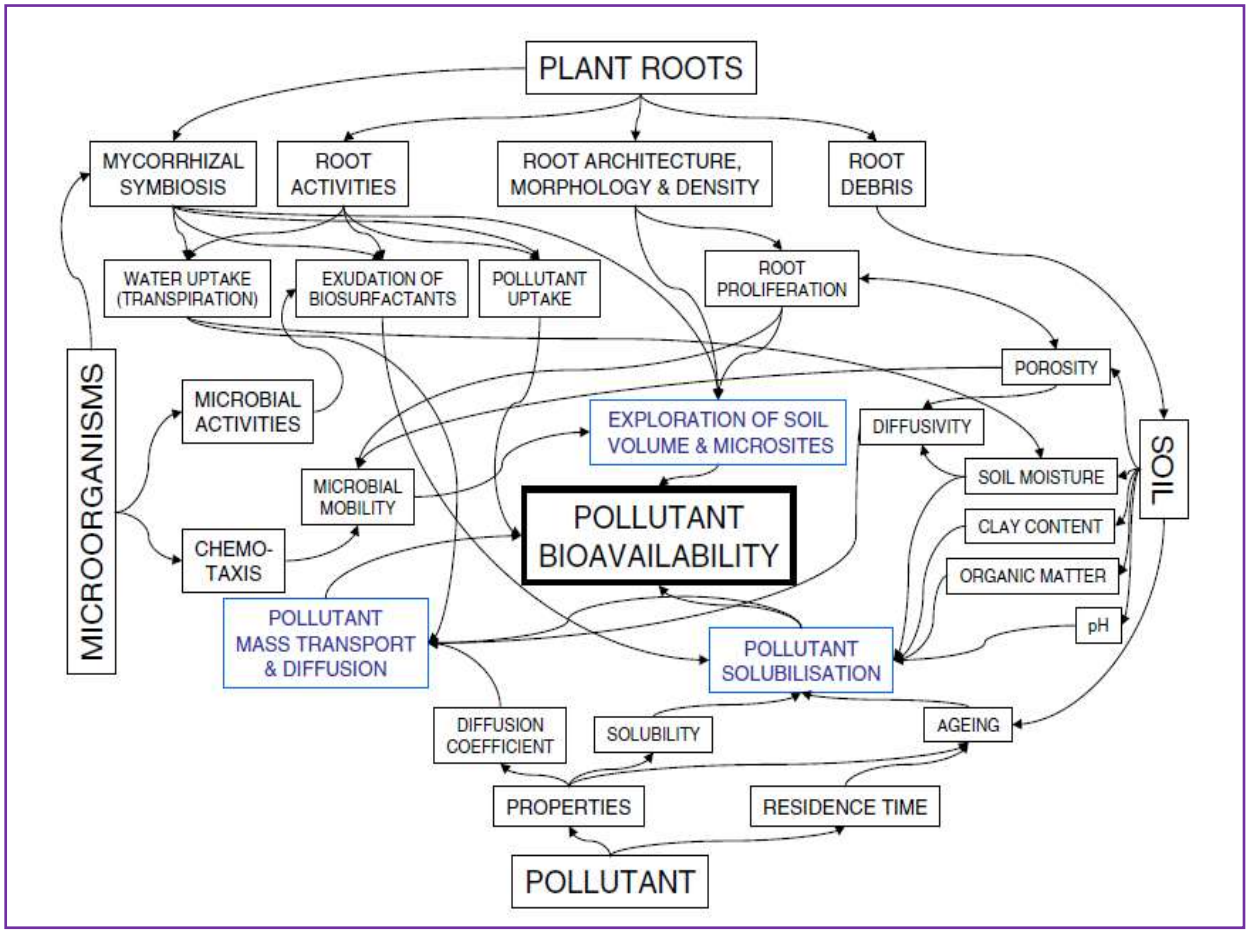


Figure 2.16 Plant-degrader interactions potentially involved in rhizodegradation (solid line arrows indicate positive, dashed line arrows indicate negative influence on the tested targeted process or component) (Wenzel, 2008).

v. Phytovolatilization

Phytovolatilization is one of the main mechanisms which can accrue in the phytoremediation process. Phytovolatilization is the uptake and transpiration of a contaminant by a plant, by the release of the contaminant or a modified form of the

contaminant into the atmosphere from the plant through contaminant uptake, plant metabolism, and plant transpiration (Feroz et al., 2012). In the phytovolatilization process metabolic chemical compounds are released into the atmosphere through plant transpiration (Yousaf, 2011). Table 2.14 indicates a summary of the various phytoremediation processes (EPA, 2000).

Table 2.12 Phytoremediation overview

Mechanisms	Media	Contaminants	Plant used	Results	References
Phytodegradation	Soil, Sediment, Groundwater	Organic compounds, chlorinated solvents phenols, herbicides,	Algae, stonewort, hybrid poplar, bald, cypress, black willow,	Contaminant destruction	White et al., (2006)
Rhizodegradation	Soil	Crude petroleum oil	Vicia faba	47% of total petroleum hydrocarbon was degraded	Diab, (2008)
Phytoextraction	Soil	Aged PAHs from manufacturing.	Rye grass	PAHs removal in 12 months Sweet clover was higher in the presence of plants, 9% to 24% compared to 5% without plant.	Parrish et al., 2004
Rhizodegradation	Soil	Petroleum hydrocarbons	Carex exigua, Panicum virgatum,	70% loss of total petroleum hydrocarbons was recorded after one year growth of these plants in contaminated soil.	Euliss et al., (2008)
Phytovolatilization	Groundwater, soil, Sediment	Chlorinated solvents	Poplars, alfalfa, black locust	Contaminant extraction from media and release and release to air	Singh and Lin (2009)

2.6.9 Influence of Environmental Factors on Phytoremediation

A number of environmental factors affect the phytoremediation process. Water content in soil and wetlands affects plant/microbial growth and the availability of oxygen required for aerobic respiration (Frick et al., 1999). Some other factors such as, type of soil, age and type of plants, nutrients, toxicity of contaminates, water and oxygen availability, chemical properties of soil (pH, CEC), depth of contamination which is important in terms of where contaminants can be treated in the rhizosphere or by plant uptake are important considerations (Kamath et al., 2004). The inorganic mineral nutrients that are most often reported to limit the breakdown of petroleum hydrocarbons in soil are nitrogen and phosphorus (Gaskin, 2010). In some cases, petroleum hydrocarbons are not readily desorbed, and are therefore not available for phytoremediation (Gaskin, 2010).

2.6.10 Interaction between plants and microorganisms

The efficiency of phytoremediation depends mostly on the establishment of robust plant-microbe interactions; however, little is known about how these interactions are influenced by petroleum pollution (Nie et al., 2011). Indeed, interaction between bacteria and plant will affect plant growth either directly or indirectly. Plants, through their 'rhizosphere', could support the hydrocarbon-degrading microbes that assist in phytoremediation in the root zone (Nie et al., 2011). For example, root activities in alfalfa and perennial ryegrass increase the number of rhizobacteria capable of petroleum degradation in the soil (Nie et al., 2011). Then microbes can enhance soil nutrient availability to the plants. Petroleum hydrocarbon is identified as harmful not only for plant growth but also to the microbe's community. In order to better understand the interactions of

petroleum hydrocarbons on microbe-plant there is a need to improve the feasibility and sustainability of phytoremediation.

2.6.11 Plant Selection Criteria

Plant selection is probably one of the most important factors determining the success or failure of the phytotechnology project (Team, 2001). After evaluating the conditions for plant growth at sites, the next stage is to choose the plant which can survive under the site conditions. A basic knowledge about the literature of plants can help to design a phytodegradation project. Some typical information which is needed about plants is the specific and common names, growth habit, tolerance of plants in various conditions such as temperature, diseases and moisture. Native plants and crops can be evaluated as options to choose from the phytodegradation process due to their being suitable for the climatic conditions of the region. Therefore, plants should be native to the area in which they are used and they should be tolerant to weather and soil conditions (Reynoso-Cuevas et al., 2008). As cost is an important factor, plants that require little attention are preferable (Reynoso-Cuevas, et al., 2008). Several types of plants have been identified for their potential for use in the phytoremediation process. A comprehensive list of plants that has proved positive in phytoremediation of organic compounds is listed in Table 2.13. The most common plants are leguminous and grasses that have shown their potential in phytoremediation (Agamuthu, et al., 2010). Grasses are a suitable option to apply in phytoremediation due to the high root surface area (per m^3 of soil) which may penetrate into the soil (depth of up to 3 meter). Leguminous plants also have more advantages compared with non-leguminous, because of their ability of them to fix N compared with other plants for limited supplies of available soil nitrogen at oil-contaminated sites (Frick,

et al., 1999). Some characteristics of plants which make their suitable for remediation of hydrocarbon compounds are as follow:

- Plants high in phytotoxicity.
- Plants able to adapt to different climatic conditions and are able to be destroyed after remediation.
- Plants have with the ability to transfer a high rate of oxygen to steam, root and leaf.
- Plants able to accumulate and absorb toxic substance (Muratova, 2003).

Table 2.13 Plants used for phytoremediation of petroleum hydrocarbon

Plant species	References
carrot (*Daucus carota*)	(Wild and Jones, 1992)
side oats grama (*Bouteloua curtipendula*)	(Aprill and Sims, 1990)
canada wild-rye (*Elymus canadensis*)	(Aprill and Sims, 1990)
soybean (*Glycine max*)	(Dominguez-Rosado and Pichtel, 2005)
alfalfa (*Medicago sativa* L.)	(Muratova et al., 2003)
perennial ryegrass (*Lolium perenne* L.)	(Merini et al., 2009)
Indiangrass (*Sorghastrum nutans*)	(Aprill and Sims, 1990)
winter rye (*Secale cereale* L.)	(Aprill and Sims, 1990)
sorghum (*Sorghum bicolor*)	(Dominguez-Rosado and Pichtel, 2005)
annual ryegrass (*Lolium multiflorum*)	(Sung et al., 2003)
switchgrass (*Panicum virgatum*)	(Aprill and Sims, 1990)
poplar trees (*Populus deltoides x nigra*)	(Jordahl et al., 1997)
Agropyron desertorum	(Sharifi et al., 2007)
Rice (*Oryza sativa* L. Cv.)	(Nwaogu et al., 2012)
Linum usitatissimum	(Sharifi et al., 2007)
Tall fescue *(Festuca arundinacea)*	(Sharifi et al., 2007)
Thuja orientalis	(Harekrushna and Kumar, 2012)
Western wheatgrass (*Agropyron smithii*)	(Aprill and Sims, 1990)
Catalpa ovate	(Harekrushna and Kumar, 2012)
Pinus densiflora	(Harekrushna and Kumar, 2012)
lettuce (*Latuca sativa*)	(Banks et al., 2003)
blue grama (*Bouteloua gracilis*)	(Aprill and Sims, 1990)
Spinach (*Spinacia oleracea L. cv.)*	(Nwaogu et al., 2012)
Sunflower (*Helianthus annu us. L.*)	**(**Nwaogu et al., 2012)
Red clover (*Trifolium pretense L.*)	(Nwaogu et al., 2012)

2.6.12 Plant species used in this study

This study was conducted to select two local plants to remediate diesel fuel in soil. The plants used are described as follow;

i. Dracaena reflexa

Dracaena reflexa, commonly called Reflexed *Dracaena*, Malaysian dracaena, Song of Jamaica, Song of India or Pleomele are spices of from *Dracaena*. This plant (Plate 2.1) can be grown as a small bush. It is a plant with dark green colored leaves with a high drought tolerance which can be grown in part shade. It grows in most tropical and sub tropical regions of Asia and Central America (Gilman, 1999). This plant is easy to grow as it is tolerant to different weather conditions and is also easy to propagate by stem cuttings. *Dracaena* shows a high ability to remove heavy metals like zinc, copper and chromium (Tan et al., 2007). The *Dracaena* is one of the plants used in the NASA Clean air study which has shown to help remove formaldehyde.

Plate 2.1 *Dracaena reflexa.*

Margon, (2011) indicated that Dracaena is helps to filter the air in pollutants. Saiyood et al., (2010) reported that *Dracaena fragrans* and *Dracaena sanderian* as tropical and evergreen plants with fibrous root systems that have the ability to uptake 50% of the bisphenol A (BPA).

ii. Podocarpus polystachyus

Podocarpus polystachyus is a number of the Podocarpus family with the local name of Sea teak. The sea teak is a conifer. This plant is an evergreen shrub or tree that can reach up to a 20 meter hight (Plate 2.2). P. polystachyus is cultivated in Malaysia, Thailand, Singapore and Western New Guinea. Maranho et al. (2006) has indicated that there is a large variability of leaf anatomy related to pollution and petroleum pollution can affect the leaf structure of Podocarpus. In the following study, Maranho et al. (2009) investigated effects of the pollution by petroleum on the tracheids along the stem of Podocarpus lambertii and reported that there is a clear reduction in the length, diameter and cell wall width of the tracheids.

Plate 2.2. *Podocarpus polystachyus.*

According to Burkill (1993), the timber is small but it is still used for building houses, carts and for various other uses. Indeed, the Malay name for the tree is Jati Laut (translating to Sea teak) as well as Setada or Sentada . Burkill notes that medicinal uses possibly ascribed to it include the use of the leaves as an alternative to treat rheumatism and painful joints.

2.7 Biodegradation Kinetics

Many scientists have studied the biodegradation kinetics of organic pollutants. Basically, modeling the bioremediation of contaminated soils can be extremely complicated (Cutright, 1995). Instead, the primary metabolic function, whether bacterial or fungal in nature, is to grow and sustain more of the microorganisms (Maletić et al., 2009). Therefore, the formulation of a kinetic model must start with the active biomass and factors, such as supplemental nutrients and oxygen source that are necessary for subsequent biomass growth (Maletić et al., 2009; Medjor et al., 2012; Pala et al., 2006). Studies of the kinetics of the bioremediation process proceed in two directions: (1) the first is concerned with the factors influencing the amount of transformed compounds with time and (2) the other approach seeks the types of curves describing the transformation and determines which of them fits the degradation of the given compounds by the microbiological culture in the laboratory microcosm and sometimes in the field (Maletić, et al., 2009). Figure 2.17 shows a schematic image of kinetics of biodegradation.

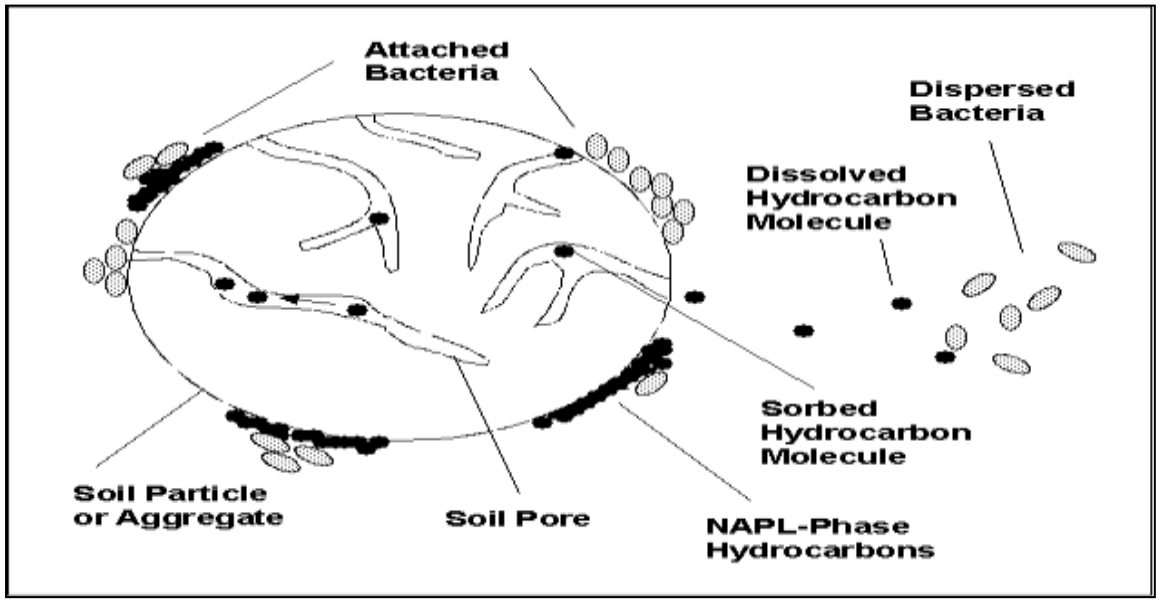


Figure 2.17 Kinetics of Biodegradation

The existing kinetic measurement technique was developed in recent years (Eliosov et al., 2000). A literature survey shows that studies of biodegradation kinetics in the natural environment are often empiric, reflecting only a basic level of knowledge about the microbiological population and its activity in a given environment (Maletić, et al., 2009). One such kinetic model which is used in this study to estimate the biodegradation rate is as follows:

$y = ae^{-kt}$ (Yeung et al., 1997) (Eq 2.1)

where;

y = residual hydrocarbon content in soil (g kg^{-1})

a = initial hydrocarbon content in soil (g kg^{-1}),

k = biodegradation rate constant (d^{-1}) and

t= is time (day)

The half-life was the time after which half of the original amount of substance present had been chemically transformed (Fritsche and Hofrichter, 2005), Half-life was then calculated from the model of Yeung et al., (1997) as

$$\text{Half life} = \ln(2)/k \qquad \text{(Eq 2.2)}$$

This model was based on the assumption that the degradation rate of hydrocarbons positively correlated with the hydrocarbon pool size in soil (Yeung et al., 1997). Another kinetic model often used to determine the rate of biodegradation of contaminants from soil is;

$$\frac{dC}{dt} = k\,Cn \qquad \text{(Eq 2.3)}$$

where C is the concentration of the substrate, t is time, k is the rate constant of the compound degradation and n is a fitting parameter (mostly taken to be unity) (Rončević et al., 2005). Using this model, one can fit the curve of substrate removal by varying n and k until a satisfactory fit is obtained (Rončević et al., 2005). It is evident from the equation that the rate is proportional to the exponent of substrate concentration (Rončević et al., 2005). Researchers involved in kinetic studies do not always report whether the model they used was based on theory or experimental and whether the constants in the equation have a physical meaning or if they just serve as fitting parameters (Rončević, et al., 2005).

Various investigators obtained different values for the rate constant of substrate degradation: for n-alkanes, 0.14 to 0.61 day^{-1} (Holder et al.,1999); for crude oil, 0.0051 to 0.0074 day^{-1} (Seabra et al., 1999); and for PAHs, 0.01 to 0.14 day^{-1} (Hinga, 2003; Holder, et al., 1999; Winningham et al., 1999). Chein et al., (2010) reported the highest first order kinetics TPH decay rate and removal ratio in soil amended with microbial inoculate at

0.015/day and 85%, respectively. Selection of a suitable kinetic model and rate constants are necessary for accurate predictions of the concentrations of hydrocarbons with time in soil after a spill (Rončević, et al., 2005). Kinetic constants are important design parameters to determine the degradation of a substrate in biological treatment systems. The rate of hydrocarbon degradation depends on various factors. Remediation time can be roughly determined from the degradation step of hydrocarbons in the contaminated soil samples (Maletić, et al., 2009). Some experimental studies have shown that biodegradation kinetics can be approximated with first order kinetics (Abioye et al., 2010; Collina et al., 2005; Namkoong, et al., 2002; Rončević, et al., 2005).

First order kinetics such as the well known Michaelis–Menten kinetic model is the most often used equation for representation of the degradation kinetics (Abioye et al., 2012b; Hohener et al., 2003; Pala et al., 2006). Few works have been dedicated to investigate the kinetics of soil bioremediation (Abbassi and Shquirat, 2008). Information on kinetics is extremely important because it characterizes the concentration of the chemical remaining at any time and permits prediction of the levels likely to be present at some future time (Abbassi and Shquirat, 2008). First-order kinetics is commonly used to describe biodegradation in environmental fate models because mathematically the expression can be incorporated easily into the models (Abbassi and Shquirat, 2008). Hwang et al., (2001) reported that the average first order kinetic rate constant of diesel oil was 0.099 per day, during the biodegradation of diesel contaminated soils by using composts. Antizar-Ladislao et al., (2005) investigated the biodegradation of 16 polycyclic aromatic hydrocarbons using laboratory conditions at different temperatures. They found the results of the first order kinetic suitable to describe bioremediation process which ranged between 0.009 day^{-1} at 70 °C and 0.013 day^{-1} at 38°C. Li et al., (2006) used a Luong model to

estimate bioreaction kinetics and found a maximum growth rate of μmax = 0.34 h^{-1} and saturation concentration Ks = 0.041 mM/l.

2.8 Stable isotopes: A tool to monitor biodegradation process

Compound specific isotope analysis (CSIA) is an analytical method that measures the ratios of naturally occurring stable isotopic ratios in environmental samples (EMD, 2011). CSIA is a new approach in environmental investigation settings. Measuring hydrogen, oxygen, nitrogen and carbon isotopes can be useful to get relevant information about environmental remediation such as, the extent of degradation or potential of contaminated sources. Complex compounds are reduced to simple molecules prior to measurement; for example, organic compounds are combusted to CO_2, SO_2, H_2 and N_2 gaseous (Table 2.14). Since the isotopic ratio in the compound is a function of the starting material and the manufacturing process as well as the degradation of that compound after it was made, CSIA has applications in environmental forensics, biodegradation, and abiotic degradation (EMD, 2011).

Table 2.14 Stable isotope fraction ratios

Atom	**Analyzed gas**	**Ratio**
Hydrogen	H_2	$^{2}H/\ ^{1}H$
Chlorine	Cl_2	$^{37}Cl/\ ^{35}Cl$
Carbon	CO_2	$^{13}C/\ ^{12}C$
Oxygen	CO	$^{17}O/\ ^{18}O$
Sulfur	SO_2/H_2S	$^{34}S/\ ^{32}S$
Nitrogen	N_2	$^{15}N/\ ^{14}N$

Isotopic analysis is used in petroleum exploration and geology. All contaminants made of various elements (multiple) and atoms change in isotopic ratios which lead to breaking of bounds between atoms. During the biodegradation of a compound, the chemical process in both biological and abiotic reactions causes change in the isotopic ratios in compounds and CSIA is used to measure these changes. CSIA can be used to gain information, make decisions about monitoring, and remedy selection. It can also answer to some questions such as the ones given

- Has the remediation process occurred?
- Has biological degradation occurred?
- Is there evidence of a slow rate of degradation? (i.e. accumulation)
- Is monitored natural attenuation feasible?

A number of instruments such as the gas chromatograph (GC) and an isotope ratio mass spectrometer (IRMS) are used in laboratory method to measure CSIA. Stable isotope analyses were performed using SERCON GEO 20–20 Continuous Flow Isotope Ratio Mass Spectrometer (CF–IRMS). The continuous flow mass spectrometry offers on–line sample preparation, smaller sample size, faster and simpler analysis and is cost effective compared with the Dual Inlet Isotope Ratio Mass Spectrometer (DI–IRMS). CF–IRMS can be also interfaced with other preparation techniques, including elemental analyzer (EA), the gas chromatography (GC) and recently, the liquid chromatography (LC) (Figure 2.18).

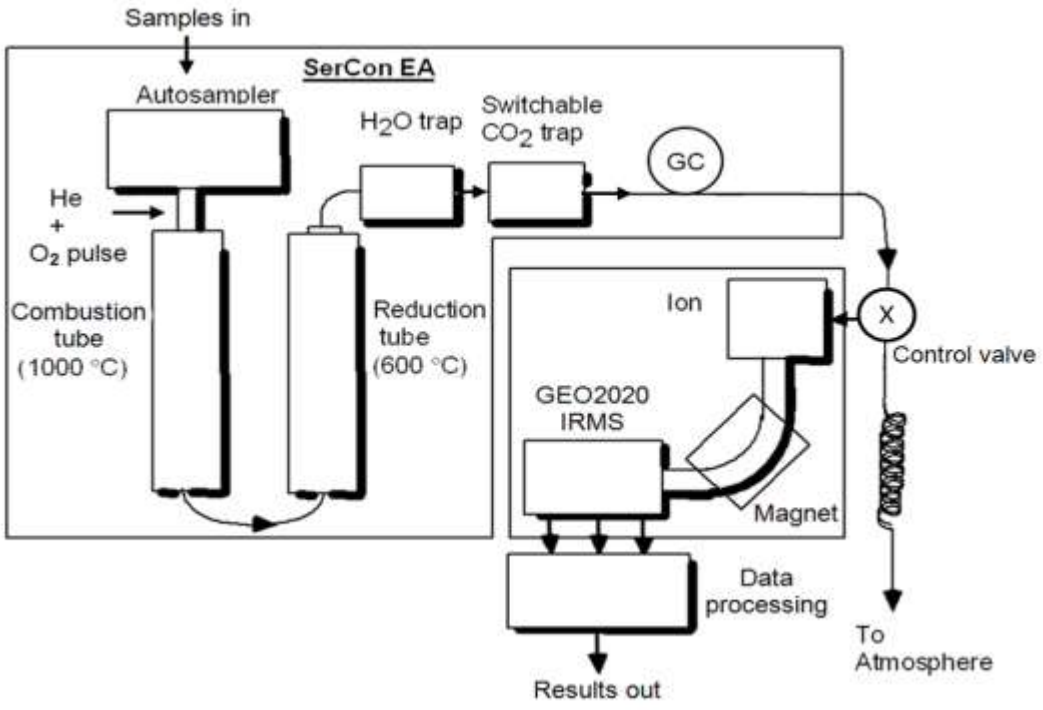


Figure 2.18 Schematic diagram of an elemental analyser (EA) in series with IRMS for the analysis of carbon isotope ratios (SERCON, 2007).

Thus, this method is a very sensitive technique and also since the differences between isotopic ratios are so small, it is more convenient to report them as "per mil" (parts per thousand, or ‰) (EMD, 2011).

The value of ratios is calculated by following this equation (Reinnicke et al., 2012).

$$\delta\ ^{13}C = \frac{(^{13}C/^{12}C_{Sample} - ^{13}C/^{12}C_{Standard})}{^{13}C/^{12}C_{Standard}} \qquad \text{(Eq 2.4)}$$

The natural abundance of stable isotopes of essential elements involved in the biodegradation processes (carbon and oxygen) may be used to monitor (1) the occurrence of in-situ biodegradation, (2) the pathways of degradation, and (3) the rates and extent of biodegradation (Aggarwal et al., 1997). Indeed, one of the primary challenges to determine the efficacy of the bioremediation process as an option to remediate contaminated sites is

monitoring its progress in the subsurface (Conrad and Depaolo, 2004). The primary product during the degradation of organic compounds is CO_2. Increased CO_2 concentrations can indicate that the degradation is accruing. However, hydrocarbon compounds are generally relatively depleted in ^{13}C (low $\delta^{13}C$ values) relative to most other sources of C (Conrad and Depaolo, 2004). Therefore, microbial metabolism of compounds from hydrocarbon tends to produce soil gas CO_2 with low $\delta\ ^{13}C$ values where significant degradation of hydrocarbons is occurs (Conrad and Depaolo, 2004). Monitoring of in situ biotransformation using stable isotopes may be achieved by the analysis of isotopic compositions of the products of degradation or the residual fractions of the contaminant or electron acceptors (Aggarwal et al., 1997). Stable isotope carbon ratio ($^{13}C/^{12}C$) measurements have been successfully demonstrated as a useful technique for monitoring biodegradation pathway of PAHs in several studies (Hunkeler et al., 2001; Kuder et al., 2004; Wang et al., 2005). For example, ^{13}C measurements of chlorinated ethenes and gasoline additives including BETX and MTBE were widely used to identify the gasoline pollution in groundwater (Peng et al., 2004). Recently, CSIA was applied for identifying individual compounds in the bioremediation process. It was also used to distinguish anaerobic and aerobic degradation of chlorinated ethane in situ biodegradation (in field applications) (Chu et al., 2004). Additionally, studies have been done on the application of isotope fractionation in anaerobic processes occurring in situ degradation (Sherwood Lollar et al., 1999; Song et al., 2002; Vieth et al., 2003). Enrichment of ^{13}C during the biodegradation of n-alkane has been demonstrated by (Stahl, 1980) for short chain ($C_1 - C_3$) and long chain ($C_3 - C_6$) by (Lebedew et al., 1969).

2. 9 Heavy metals and PAHs as mixed contaminations

Heavy metals (HMs) are often co-contaminants with organic pollutants such as xylene, benzene, toluene, PAH and ethylbenzene in soil and sediments. USEPA (1999a) reported that more than 40% of the national priority list sites are co contaminated with both PAHs and heavy metals. Heavy metals and polycyclic aromatic hydrocarbon are environmental concerns and must be removed to acceptable levels (Reddy et al., 2011). Although combustion of carbonaceous material is a major source of PAHs, other anthropogenic-related processes, such as gasworks, motor vehicle emissions and smelting, have added toxic heavy metals such as cadmium (Cd), zinc (Zn), Arsenic (As) and lead (Pb) along with PAHs (Thavamani et al., 2012). Food chain contamination is one of the important pathways for the entry of these toxic pollutants into the human body. Consequently, PAHs have often been found to co-exist with heavy metals due to similar pollution sources. Metals- PAHs association was found in industrial places and also in agricultural soils where a strong correlation between PAHs and metals was observed. Chemical and physical methods as well as a combination of them have been used to remediate co-contamination soils. However, information regarding the mechanisms, translocation, combined uptake and accumulation of HMs and PAHs present in soil, sediment and wastewater contamination is still under study (Figure 2.19)

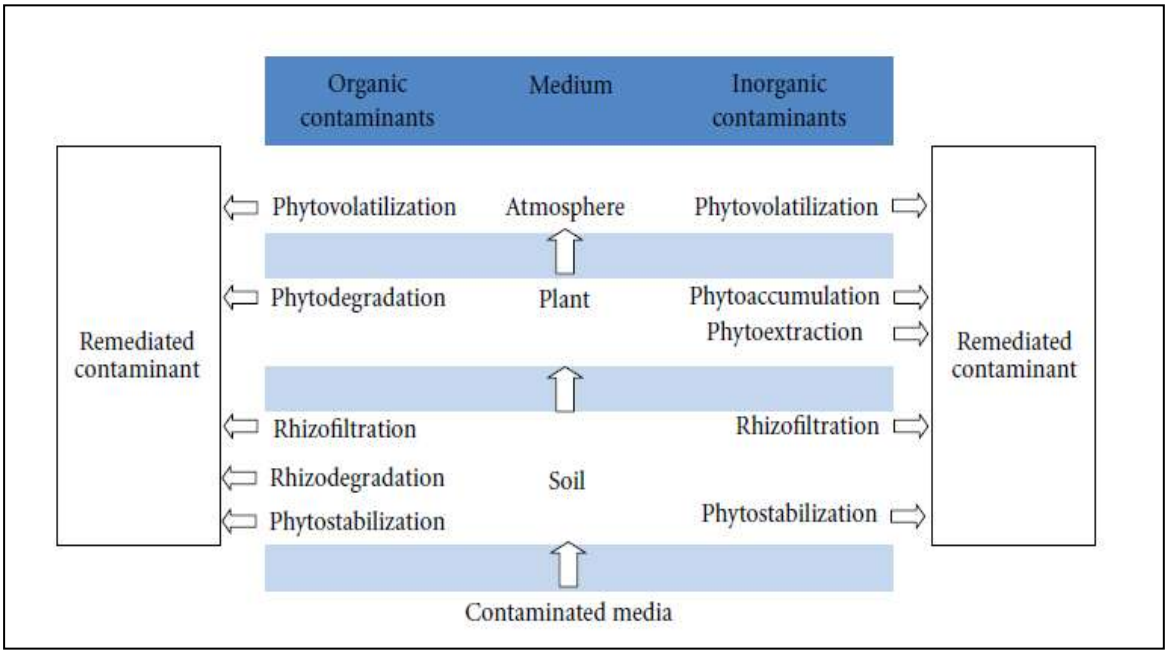


Figure 2.19 Uptake mechanisms on phytoremediation of HMs (Tangahu et al., 2011).

Theoretically, the ability of plants to accumulate and decrease in the soil metals concentration as an operation of metal uptake plays an important role in accessing regulatory acceptance (Wuana and Okieimen 2011). The metal removal can be descripting by the determination of metal concentration in plant tissues.

This approach may be follow up by a number of factors working together during the decontamination of metals. Amount of metal extracted and bioaccumulation factor (BCF) can be used to evaluate the plant's phytoextraction efficiency and calculated according to equation (Ashraf et al., 2012);

$$BCF = \frac{\text{Metal concentration in plant shoot}}{\text{Metal concentration in soil}} \qquad \text{(Eq 2.5)}$$

Cluis (2004) reported that the BCF for hyperaccumulators is more than 1, and in some cases can be increase up to 100. Metal responsive transcription factor is playing an

important role in tolerance of heavy metal stress by plants for transport, HM uptake and detoxification. The transfer capability of heavy metals from soil to the shoots part of vegetables was generally described using the translocation factor (TF) (Li et al., 2010),

$$TF = \frac{\text{Metal concentration in edible part of plant}}{\text{Metal concentration in root of plant}} \quad \text{(Eq 2.6)}$$

This study evaluates the effect of adding organic wastes as an amendment to different concentration of diesel fuel-contaminated soil on nutrient availability, rate of biodegradation and some physicochemical properties of soil. Many researchers have reported using fertilizers and composts to remediate contaminated soil. In addition, no work has been done on diesel fuel-contaminated soil using organic wastes in tropical regions.

CHAPTER THREE

METHODS AND MATERIALS

Biological treatments (Bioremediation and Phytoremediation) were chosen in order to evaluate impacts of organic wastes to increase the microorganism's activity on oil degradation and achieve a suitable option to enhance the diesel-contaminated soil. This study was done in two different environmental situations (laboratory and natural condition) to compare the results of biodegradation process.

3.1 Collection of soil, diesel fuel, organic wastes and plant materials

The soil used in this study, (silty loam), was obtained from the garden section of Asia-Europe Institute, University of Malaya, Kuala Lumpur ($3^{o}07'$ 28.82'' N , 101^{o} 39' 33.86'' F). It was transported to the laboratory and air dried in laboratory conditions at room temperature for a period of one week and then sieved through a 2-mm mesh sieve. The diesel fuel used in this experiment was purchased from a petrol station in Petaling Jaya (shell diesel), Malaysia and its profile was analyzed using gas chromatography mass spectrometry. Organic wastes used in this study were collected from different locations; used tea leaf (TL) and potato skin (PS) were collected from the institute of graduate student's canteen building (IGS), University of Malaya, while the soybean cake (SC) was made in the laboratory. Organic wastes were selected based on their availability and supply in Malaysia. The *Dracaena reflexa* and *Podocarpus polystachyus* plants (eight - month old) were purchased from a greenhouse in Sungai buloh, Selangor, Malaysia. These two plants were selected based on the following factors: high drought tolerance, economic and non-edible plants that are native in Malaysia, tropical and subtropical regions.

3.1.1 Organic wastes used in this study

Three organic wastes (used tea leaf, soy cake and potato skin) were used in this study for amendment of soil contaminated with diesel fuel. These wastes contain appreciable quantities of nutrients that soil microorganisms can use for multiplication in the contaminated soil. The use of such wastes, besides providing alternative substrates, help to solve environmental problems which are caused by their disposal.

i) Spent Tea leaf

Spent tea leaf *(Camellia sinensis L.)* is the residue which is left after preparing tea (Plate 3.1). Nutritional and chemical composition of TL is shown in Table 3.1.

Table 3.1 Dry matter and chemical composition of used tea leaf.

Nutrients (%)	Spent tea
Dry matter	-
Crude protein	25
Ether extracts	3.53
Crude fiber	8.6
Nitrogen free extracts	57
Total Ash	5.87
Calcium	1.5
Phosphorus	0.53

(Konwar and Das, 2011)

Plate 3.1 Tea leaf used in this study.

ii) Soybean cake (SC)

After milk is extracted from soybeans (*Glycine max*), a soy cake is left behind which is called nourishing waste. Soy has been grown for three millennia in Asia and, more recently, has been successfully cultivated around the world. Soybean cake is a product of home industry. It contains high crude fiber and nutrients. For instance, 500,000t soybean cake is equivalent to 30000t of N, 2000t of P_2O_5 and 12000t of K_2O (Krauss, 2000). Organic residue like soybean cake (SB) and powdered rice husk (PR) with higher pH could be combined with industrial wastes to enhance flammable biogas production. Physico-chemical properties and a picture of soybean cake are shown in Table 3.2 and Plate 3.2, respectively. Wankhade and Thakre (2012) indicated that the low yield of flammable biogas from carbonated soft drink sludge could be enhanced significantly when blended with either soybean cake waste or pigging waste.

Table 3.2 Physico-chemical composition of soybean cake

Parameters	soybean cake
Crude nitrogen (%)	2.66
Moisture (%)	62.5
Crude Protein (%)	16.65
Carbohydrate (%)	5.95
Energy (kcalg $^{-1}$)	4.05
Fat content (%)	13.05
Ash (%)	0.45

(Uzodinma et al., 2008)

Plate 3.2 Soy cake used for study.

iii) Potato skin (peel)

The problem of industrial potato waste (IPW) management is a great concern in some developed countries. Therefore, an environmentally friendly solution is under investigation. The major wastes of potato industries is potato peel waste (PPW) and potato starch waste (PSW). The potato peel is a waste of the potato processing plants. While consumption of potatoes has decreased, processed products such as French fries, puree and chips have been growing in popularity (Arapoglou et al., 2009). The waste produced is 90 kg per tonne of influent potatoes which is made up of 50 kg of potato skin, 30 kg of starch and 10 kg of inert substances (Arapoglou et al., 2009). PPW contains cellulose, fragmental sugars, starch and hemicelluloses. As Table 3.3 shows, PPW has a low fermentable reducing sugar (0.6 %) and high starch content (52 % d.w.) Plate 3.3 shows the picture of PPW used for the studies.

Table 3.3 Chemical composition of potato peel wastes

Parameters	% dry weight
Nitrogen % D. M.	1.3
Moisture %	85.06
Protein % D. M.	8
Total carbohydrate % D. M.	58.7
Total soluble sugar % D. M.	1
Reducing sugar % D. M.	0.6
Fat % D. M.	2.6
Ash % D. M.	6.34
Starch % D. M.	52.14

(Arapoglou et al., 2009).

Okeke and Frankenberger (2005) reported that PPW in combination with starch is effective for amylolytic bacteria during the bioreduction of CLO_4^- (perchlorate). The rate of reduction was over 90% when using PPW (2% w/v) over a period of 4 days.

Plate 3.3 Potato skin used for study.

3.2 Physicochemical analysis of soil and organic wastes

Physicochemical properties of soil and organic wastes employed were determined using standard methods. The measurement of soil was done through the hydrometric method. The nitrogen content (of soil used for bioremediation and organic wastes) was done using the Kjeldahl method and the organic carbon was determined using the Furnace method. Phosphorous, was determinated by adopting the American Society for Testing and Material method (ASTM D 5198, Standard Practice for Nitric Acid Digestion of Solid Waste). HANNAHI 8424 model of pH meter was used to determine the pH on the scale 1:2.5 (w/v) soil/distilled water after 30 min equilibrium. All the treatments were set up in triplicates.

3.2.1 Diesel fuel characteristics

Characterization of aromatic and aliphatic fractions of the diesel fuel used in this study was determinated according to USEPA 5035 method. Briefly, gas chromatography coupled with mass spectrometry (QP2010- SHIMADZU), with helium carrier gas (1.79 ml min^{-1}) was used at oven temperature at 50 °C for 1 min, and then increased to 250 °C.

3.3 Biostimulation methodology under laboratory condition

3.3.1 Experimental set- up

The concentration range of oil used as a treatment is within 5-40 % as used by earlier researchers (Ijah and Antai, 2003b; Margesin and Schinner, 1999). 1500 g of fresh soil was filled into clean dry plastic containers, labeled A to E (with volume 3000 cm^3), and contaminated with 5 % (*w/w*) diesel fuel (50,000 mg kg^{-1}) (Appendix B). The samples were mixed daily to provide sufficient aeration. They were also moistened by the addition of water every other day to adjust the water holding capacity at 60% throughout the experimental period. The soil bags were incubated at room temperature (30 ± 2°C). The control (vessel D) with only soil and diesel fuel and an additional control treatment (E) were autoclaved twice (within the same day at 121°C and 15 psi for 1 h) and then 0.5% (w/w) sodium azide was added (as a prisoner) to determine the non-biological loss of diesel fuel from the soil.

3.3.2 Sampling and analysis

The contaminated soil samples were studied at every two-week intervals up to 126 days for chemical and microbiological analyses. Composite samples were taken by mixing five grams of soil collected from five different areas of the microcosm and mixing them well. The following parameters were determined.

i. Measurement of residual total petroleum hydrocarbon in soil

The extent of diesel fuel biodegradation in the soil was determined by accelerating (Richter, 2000) solvent extraction in two replicates of samples formed by suspending 10 g of soil in 20 ml of acetone: n-hexane (1:1 v/v) as a solvent in a 250 ml capacity flask (EPA method 9071). After shaking for 1 hour on an orbital shaker (Model N-Biotek), the solvent-oil mixture was filtered using Whatman number 4 filter paper, and collected in a beaker of known weight and the solvent was completely evaporated under vacuum (70 ^{o}C water bath on rotary evaporation) using rotary evaporator (Model Eyela, N-1100) to approximately 2 ml. The new weight of the beaker consisting of residual oil was recorded. The percentage of degradation of diesel fuel was calculated using the following formula (Ijah and Ukpe, 1992);

% biodegradation = [(TPH control- TPH treatment)/ TPH control] ×100 (Eq 3.1)

where TPH is total petroleum hydrocarbon.

ii. PAHs extraction by hydroxypropyl[b]cyclodextrin (Bioavailability)

The method used hydroxypropyl[b]cyclodextrin (HPCD) to extract the oil from contaminated soils (Oleszczuk, 2009). 5 g of soil samples were filled into Teflon centrifuge tubes and 100 ml of a 50-mM aqueous solution of HPCD was added to it. After shaking the tubes for 20 hours (using orbital shaker, Model N-Biotek-101), the samples were centrifuged for 30 min. The supernatant was discarded, and the residue was shaken with 50 ml deionized water and centrifuged again; the supernatant then was discarded. The PAHs content in the residue was determined after extraction with acetone/hexane in accordance with the method described above (3.5.2.i). The difference between the total PAH content

(as determined in the dichloromethane) and the residue (after extraction with HPCD) was determined as a HPCD or bioaccessible fraction of PAH (Oleszczuk, 2009).

iii. Measurement of dehydrogenase activity

The soil microbial activity was estimated by Dehydrogenase assay. Dehydrogenase activity was determined by monitoring the rate of reduction of 2,3,5-triphenyltetrazolium chloride (INT) as a substrate. One gram moist soil samples were filled in test tubes, mixed with 1.5 mL of 1 M Tris buffer (pH 7.0), and 2 mL of INT solution. Then the test tubes were sealed with screw caps and incubated for 2 h at 40 ^{O}C. After incubation, the developed iodonitrotetrazolium formazan (INTF) was extracted with a mixture of N, N-dimethylformamide and ethanol in volume ratio of 1:1 and measured at 464 nm using a DR /4000 spectrophotometer. The calibration curve was made with iodonitrotetrazolium chloride (INF) in four concentrations (0, 100, 200 and 500 μg INF).

iv. Soil respiration

The total carbon dioxide (CO_2) respired from each of the treatments was determined by sampling the headspace of sealed Wheaton bottles containing sample of soil, oil and the organic supplement. Air samples (1ml) were collected from each bottle at 7, 14, 21, 28, 35 and 42 days, and analyzed using gas chromatography (GC-8A, Shimadzu brand) (Plate 3.4) with a thermal conductivity detector (Miles and Doucette, 2001). The temperatures used for the GC function were detector 110 ^{0}C, injector 110 ^{0}C and column at 130 ^{0}C.

Plate 3.4 Gas Chromatography.

v. Isolation and identification of bacterial diesel degraders

Three replicate samples from each oil polluted soil were withdrawn every two weeks for the enumeration of both heterotrophic and hydrocarbon utilizing bacteria. In order to isolate and enumerate both heterotrophic and hydrocarbon utilizing bacteria, the bacteria enrichment process used a mineral salt medium (MSM) (Vincent et al., 2011). 0.1 ml of serially diluted (1×10^{-1} to 1×10^{-7}) culture solution from one gram hydrocarbon polluted soil samples were plated on nutrient agar medium (Oxide) for isolation of aerobic and heterotrophic bacteria. 50 µg/ml fungazol was used to suppress the growth of fungi. Plates were incubated at 30^{o}C for 24 h after which the colonies were counted. Diesel fuel utilizing bacteria (DUB) in the soil samples were enumerated using oil agar (OA) (Zajic and Supplisson, 1972) ; (1.8 g K_2HPO_4, 1.2 g KH_2PO_4, 4.0 g NH_4Cl, 0.2 g $MgSo_4$.7H2O, 0.1 g NaCl, 0.01 g $FeSO_4.7H_2O$, 20 g agar, 2 ml diesel fuel, 1000 ml distilled water). The oil

agar plates were incubated for five days at 32 ^{o}C before counting the colonies. Bacterial colonies were randomly picked and pure cultures were obtained by repeated sub-culturing on nutrient agar in order to isolate fungal used potato dextrose agar (Difco). The bacterial isolates were characterized based on culture parameters, microscopic techniques (gram straining reaction) and biochemical tests using the Biolog® Microstation system method (Biolog Inc., CA, USA) for identification (Plate 3.5) (Ruan et al., 2005). Organisms isolated for identification were previously re-subcultured two times to ensure that pure strains were obtained. This was eventually followed by the final identification of the organisms using IF-A (inoculation fluid) under Biolog protocol. Ecoplate contains 31 carbon sources with 96 wells to supply carbon and protein for metabolism. The transmittances were adjusted to 95-98% and incubated for 18- 24 hours at 30 ^{o}C. Then Machine reading (Microplate reader) was used to identify the genus and species of samples.

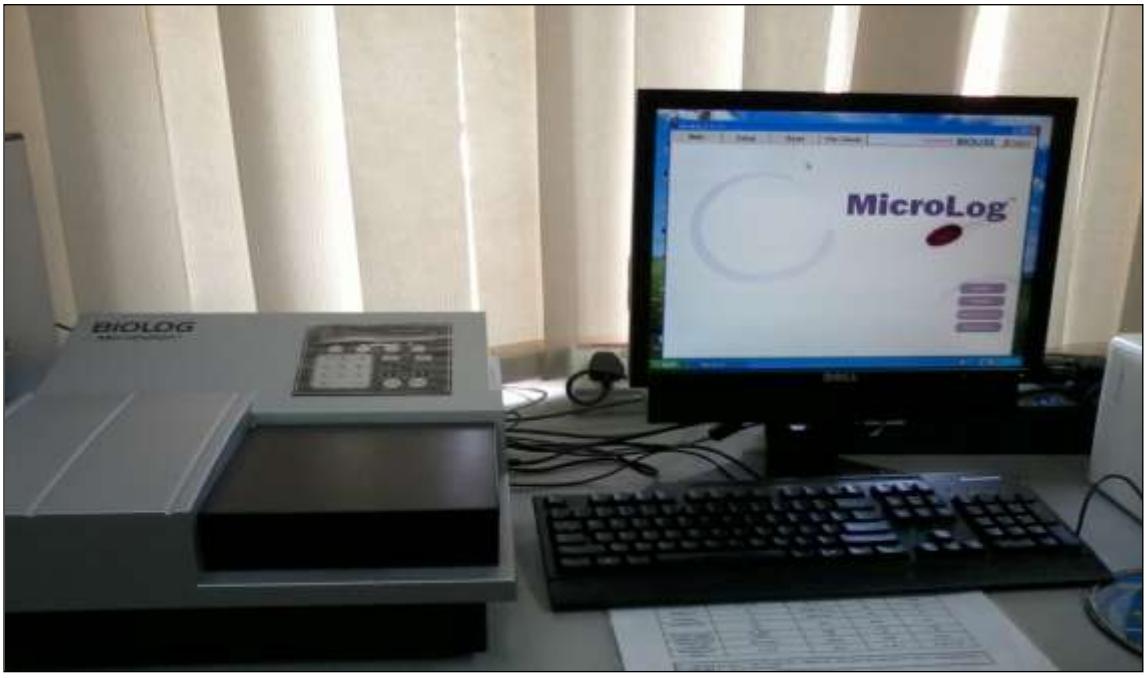


Plate 3.5 Biolog microstation machine.

vi. Measurement of pH value

The pH value of soil was measured in 1:2.5 (w/v) ratios. Ten grams of air-dried soil in 25 ml distilled water was mixed and shaken for 5 minutes, and then allowed to settle for 30 minuets to one hour. After shaking the suspended soil again, pH was measured using standard pH meter (Mettler Toledo AG- Switzerland).

vii. Seed germination toxicity test

The germination test was conducted over a 7-day test period. Seeds of lettuce were obtained commercially. For each soil sample, 100 g of thoroughly mixed remediated soil was placed in Petri dish bottoms. Ten viable seeds of lettuce (*Lactuca sativa L.*) were placed evenly throughout each Petri dish and covered with 15 g of dry sand. Four replicates of the samples were prepared. The moisture content of the soil was maintained at 80% water holding capacity. The Petri dishes were placed in a room with 16 hours light and 8 hours darkness for 7 days. At the end of 7 days, the number of seedlings that emerged from the surface of the sand was counted and recorded (Banks and Schultz, 2005). Germination index of lettuce seed on the remediated soil was calculated (appendix C) using the formula of (Millioli et al., 2009).

viii. Gas Chromatography analysis of residual degraded diesel fuel

Analysis of the residual hydrocarbon in the soil was determined using Gas Chromatography (2010 A) coupled to a trace MS detector (QP2010 Plus). Each extract was transferred to a 2 ml vial and loaded into GC/MS. Helium carrier gas flow was at 1.27 ml min^{-1}. The column oven was initially held at 100 °C for 2 min, increased to 200 °C at a

rate of 10 °C min $^{-1}$, then to 250 °C at 20 °C min $^{-1}$ (held for 5 min) (Padayachee and Lin, 2011). The major hydrocarbon fractions were identified on the basis of their retention time and by comparing them to those of analytical standards.

ix. Biodegradation efficiency calculation

The carbon dioxide analyses were used to estimate the total amount of hydrocarbons mineralized during biodegradation experiments (Mariano et al., 2007). According to the Norm L6.350, 50% of the biodegraded carbon is converted to CO_2 and the other 50% is added to the soil as humus and biomass, and the total biodegraded carbon (Morais and Tornisielo, 2009), and biodegradation efficiency (BE), based on the decrease in the total concentration of hydrocarbons, were calculated using the following equations:

Total biodegraded carbon = 2 × CO_2 produced

$$BE\ (\%) = \frac{\text{Total biodegraded carbon}}{\text{Initial soil organic carbon content}} \times 100 \qquad \text{(Eq 3.3)}$$

The initial soil organic carbon content for each treatment was determined through the carbon mass balance (Morais and Tornisielo, 2009).

x. kinetics of diesel removal and Half- Life

First- order kinetics model is used to express the rate of biodegradation in all of the treatments by the following equation (Chu and Chan, 2003):

$$C_t = C_i \exp(-k\,t) \qquad \text{(Eq 3.4)}$$

Where C_t (mg/g), is the diesel fuel concentration in soil at instant t, C_i (mg/g) is the initial concentration of soil, k is the rate constants of the first order expressed in (day $^{-1}$), and t is

the time (day). The model estimated the biodegradation rate and half- life of hydrocarbons in soil relative to treatments applied.

$$\text{Half life} = \ln 2/k \qquad \text{(Eq 3.5)}$$

To indicate the proportion of the variation explained by the model, the coefficient of multiple determinations (R^2) was calculated;

$$R^2 = 1 - \text{RSS} / \text{CTSS} \qquad \text{(Eq 3.6)}$$

where RSS was the residual sum of squares, and CTSS was the corrected total sum of squares (Bailey and McGill, 2001).

xi. Measurement of stable isotope carbon ($\delta\ ^{13}C$)

Stable isotope analyses were performed using SERCON GEO 20–20 Continuous Flow Isotope Ratio Mass Spectrometer (CF–IRMS). The continuous flow mass spectrometry offers on–line sample preparation, smaller sample size, faster and simpler analysis and is cost effective compared to Dual Inlet Isotope Ratio Mass Spectrometer (DI–IRMS). CF–IRMS was interfaced with an elemental analyzer (EA) and gas chromatography (GC). Sample materials containing carbon were loaded into tin capsules and dropped into a furnace at 1000 ^{o}C in an atmosphere of oxygen. The tin ignited and burned exothermically, and the temperature rose to about 1800 ^{o}C, oxidising the sample. Complete combustion was ensured by passing the combustion products through a bed of chromium oxide at 1000 ^{o}C, using a helium carrier gas. A 15 cm layer of copper oxide followed by a layer of silver wool completed the combustion and removed any sulphur. The gas stream passed into a gas chromatograph where components of interest were separated and then bled into a mass spectrometer where the isotope species were ionised and then separated in a magnetic field (Plate 3.6). These isotopic species were detected separately and from their ratios, the level of ^{13}C calculated. δ values of carbon were calculated as follows:

$$\delta\ (^{13}C)\ (‰) = [\ R_{sample}/R_{standard} - 1] \times 1000 \qquad \text{(Eq 3.7)}$$

where R_{sample} and $R_{standard}$ represent $^{13}C/^{12}C$ ratios of the sample and the international standard.

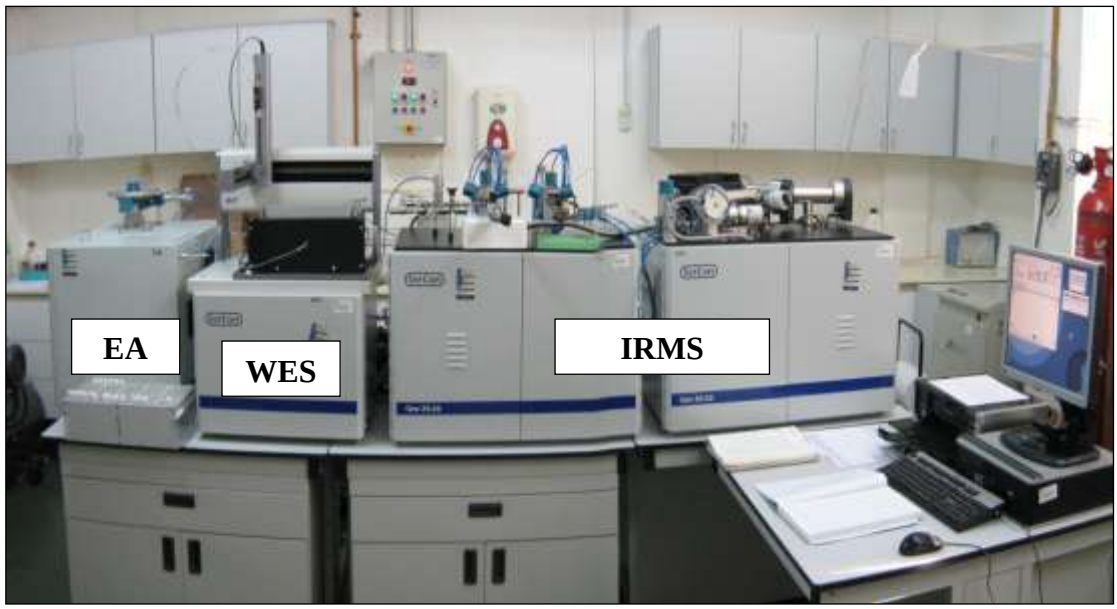


Plate 3.6 Isotope Ratio Mass Spectrometer (IRMS).

3.4 Biostimulation methodology under natural condition

Top soil (0-20 cm) was obtained from the garden section of Asia-Europe Institute, University of Malaya, Kuala Lumpur was air-dried and passed through a 2 mm sieve to remove root materials and stones. Then soil samples (1.5 kg) were artificially polluted with 5%, 10%, 15% and 20% (w/w) diesel fuel and thoroughly mixed. Each of the oil contaminated soil samples were amended with (10% w/w) different organic wastes. Two different control treatments were set up; one control was oil contaminated soil without organic wastes amendment while the second control did not contain organic wastes but the soil was autoclaved in order to determine the oil loss due to the abiotic factor. All samples

were packed into Polythene plastic bags and set up at the experimentation site, exposed to sunlight and rainfall for a period of one year.

3.4.1 Sampling

Replicate samples were withdrawn from each treatment every two months throughout the one-year period of the experiment for the analysis of TPH loss, enumeration of total bacteria and DUB as described in the methods (3.5.2).

3.5 Phytoremediation methodology used in this study

3.5.1 Physicochemical analysis of soil, diesel fuel and organic wastes

Physicochemical properties of soil and organic wastes employed were determined using standard methods. N content was done with the Kjeldahl method and organic carbon was determined using the Furnace method. P, K, Al, Zn, Pb and Cd were determinated using United Environmental Protection Agency method (USEPA 3050 B, 6010B). HANNAHI 8424 model of pH meter was used to determine the pH on the scale 1:2.5 (w/v) soil/distilled water after 30 min equilibrium. All the treatments were set up in triplicates.

3.5.2 Experiment set-up under laboratory and natural conditions

In order to select the range of oil concentration to be used in the phytoremediation experiments, different oil concentrations were evaluated for both plants (*Dracaena reflexa, Podocarpus polystachyus*) to find out at which concentration of diesel fuel, the plants could survive and grow (Appendix D). Control treatment consisting of bags of the plant without diesel fuel or organic wastes was also set up. Additional control treatment comprising of autoclaved soil containing 0.5% (w/w) NaN_3 was also set up to determine non-biological loss of diesel fuel from the soil. All the treatments were set up in triplicate at room

temperature (30 ± 2 ^{o}C). The plants were moderately watered every three days with tap water to prevent leaching from the plastic bags. The same experimental set up was done for field experiment, exposed to sunlight and rainfall for a period of nine months. The appearance of the plants in response to the oil in soil was monitored to determine if there is phytotoxicity of the oil to the plants. The design of the experiment (randomized complete block design) is shown in appendix E. The study at 5 % concentration was monitored for a period of five months in laboratory condition; the reason being that plants could not survive under laboratory conditions because both plants require sunlight to survive. The methodology used for soil preparation and sample analysis was the same for both plants (refer to section 3.7.2).

3.5.3 Sampling and analysis

Soil samples were taken within the rhizosphere zone (1 cm) of plants from each plastic bag every thirty days for analysis of different parameters such as plant biomass, pH, TPH, DUB and AHB counts (see section 3.5.2).

i. Plant biomass

At the completion of the experiment (270 days) plants were uprooted and washed with deionized H_2O and the plants were dried at 75 ^{o}C for 48 hours and weighed (Parrish et al., 2004; Saadati et al., 2012). The plant tissue was extracted in a ratio of 1:1 hexane/acetone in a Soxhlet extractor for ten hours to determine if the roots had absorbed the hydrocarbon from the soil. The extracts were analyzed for hydrocarbons using the gas chromatography with a mass-selective detector (GC/MSD) QP2010A in scan mode. Helium was used as the carrier gas. The GC was equipped with cross-linked 5% phenyl methyl siloxane capillary

column. The temperature was set at 40 °C and raised by 10 °C/min until 300 °C, which was maintained for 8 min.

3.6 Phytoremediation of heavy metals in diesel fuel contaminated soil

3.6.1 Selection of heavy metals concentration

It was considered that the quantity of heavy metals which is added artificially would affect plant growth because plants should be able to survive and tolerate oil and metals. So, the impact of five heavy metals (Cd, Zn, Cu, Mn and Pb) with different concentrations on microbial population was evaluated to select two heavy metals. According to the critical level of heavy metals pollution in Malaysia's soil (Ibarahim, 2009) it was decided to use Cd (10, 20 and 30 mg kg^{-1}), Zn (40, 80 and 120 mg kg^{-1}), Cu (25, 50 and 75 mg kg^{-1}), Mn (75, 150 and 225 mg kg^{-1}) and Pb (30, 60 and 90 mg kg^{-1}). In the experiment, MSM was used as a media. A bio-agent (isolated from diesel contaminated soil) was added to the nutrient broth and kept in an incubator for 24 hours at 30 ^{o}C. One milliliter of bio-agent and microelements were placed into test tube with 10 ml MSM and 0.5 ml oil. The contents of test tubes were analyzed after 2, 4 and 6 days, by taking 1 ml of the solution for analysis. Several dilutions were prepared and incubated at 30 ^{o}C in nutrient agar, and colony forming units (CFUs) were counted after 24 hours (Zukauskaite et al., 2008). Based on preliminary trials, it was decided to use two microelements which have a major impact on the growth of microorganisms, namely Zn (80 mg kg^{-1}) and Pb (60 mg kg^{-1}).

3.6.2 Preparation of co-contaminated soil

Garden soil was taken from a farm in Subang Jaya, Selangor, Malaysia. After being transferred to the laboratory, the soil was air-dried and its chemical and physical characteristics were defined through standard methods (Appendix F). 2 kg of soil was

placed into plastic bags and the desired concentration of Zn (80 mg kg^{-1}) and Pb (60 mg kg^{-1}) in was provided by dissolving zinc-chloride ($ZnCl_2$) and lead-chloride ($PbCl_2$) in 200 ml of distilled water, sprayed layer by layer, and was completely mixed. Then, with the purpose of creating a balance between the different fractions of the elements in the soil, the samples were put through an incubation period of one month. During this period, the moisture of the bags was kept at about (70% ±10) (Saadati et al., 2012). Then soil samples were co-contaminated with 2.5 % diesel oil and thoroughly mixed. 5% of different organic wastes (TL, SC and PS) were also mixed separately with the oil contaminated soil. After mixing the soil, it was allowed to stabilize for four days before transplanting the plants into the contaminated soil. The experiment was carried out in three replicates at room temperature (30 ± 2 ^{0}C). The plants were moderately watered every three days with tap water to prevent leaching from the plastic bags. The design of the experiment is shown in appendix G.

3.6.3 Sampling and analysis of samples

Soil samples were taken from within the rhizosphere zone (1 cm) of plants for each plastic bag on a monthly basis for a period of nine months. Analysis of different parameters such as pH, TPH, DUB and AHB counts was carried out as described in the methods section 3.5.2.

3.6.4 Analysis of heavy metals in soil and plants

The root tissue was extracted with dichloromethane in a Soxhlet extractor for 10 hours to determine if the roots absorbed the hydrocarbons from the soil. The extracts were

analyzed for hydrocarbons using gas chromatography with a mass-selective detector (HP-6890). The GC was equipped with cross-linked 5% phenyl methyl siloxane capillary column; HP-5MS. Helium was used as a carrier gas. The temperature program was started at 40 ^{0}C and raised by 10 ^{0}C/min until 300 ^{0}C, which was maintained for 8 min. The rate of heavy metals in plant tissues was determinated by hot plate wet digestion method (Marin et al., 2011).

Heavy metals (Zn and Pb) in soil were determined by the EPA method 3050B (acid extraction method). Briefly, soil was dried at 40 ^{0}C and ground with a laboratory blender (Waring model). 1 g samples were placed in a 250 ml flask for digestion. Then the samples were heated at 95 ^{o}C with 10 ml of 50% HNO_3 without boiling. This was followed by the addition of 65% HNO_3 until no brown fumes were given up by the samples. Then, gradually, 10 ml of 30% H_2O_2 and 37% HCL were added at 95 ^{o}C for 15 minutes. The digestation obtained was filtered (0.45 μm filter paper) and diluted to 100 ml with deionized water and analyzed with Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP- OES).

3.6.5 Calculations of Translocation factor and Bioconcentartion factor

The transfer capability of heavy metals from soil to the edible part of vegetables was generally described using the translocation factor (TF) (Li et al., 2010),

$$TF = \frac{\text{Metal concentration in edible part of plant}}{\text{Metal concentration in root of plant}} \quad \text{(Eq 3.8)}$$

For plants, the Bioconcentartion factor (BCF) has been used as a measure of the metal accumulation efficiency, whereby value greater than 1 is an indication of plants potential to phytoextraction (Ashraf et al., 2012). BCF was calculated using the following formula,

$$BCF = \frac{\text{Average metal conc. in the whole plant tissue (mg kg}^{-1})}{\text{Metal in the soil (mg kg}^{-1})} \quad \text{(Eq 3.9)}$$

3.6.6 Rate of metal uptake by plants

The rate of uptake of heavy metals (Zn and Pb) by Dracaena and Podocarpus was calculated using the first order kinetic model as follows:

$k = -1/t \ (\ln M/Mo)$ (Eq 3.10)

where;

k = first order rate constant for metal uptake per month

t = time in month

M = mass of residual metal in the soil (mg/kg)

Mo = initial mass of metal in the soil (mg/kg)

3.7 Biodegradation studies with microorganisms isolated

A total of 12 hydrocarbon utilizing bacteria (3 from uncontaminated soil and 9 from contaminated soil) were isolated and identified. Out of all the bacteria identified, 6 bacteria were selected based on the efficient utilization of oil in the preliminary trials and their rapid growth on oil agar for the biodegradation study. The rates and extent of diesel fuel degradation by these six selected microbial isolates were determined using gravimetric analysis (Ijah et al., 2008). The biodegradation studies were carried out by introducing one single colony of isolated bacteria into 10 ml nutrient broth (Merck) and incubated overnight at 30 ^{o}C. After inoculating 2 ml of 24 hour broth culture of each microbial isolates into 100 ml of sterile MSM, then added 0.5 g of diesel fuel in an Erlenmeyer flask. The experiment was set up in triplicates with control flasks which contained 100 ml of sterile mineral salts medium plus 0.5 g of diesel fuel but without added microorganisms. The flasks were incubated in an incubator shaker (Thermo-line, Japan) maintained at 33 ^{0}C at 150 rpm for

35 days. At 7 day intervals, triplicate flasks per organisms plus control flasks were removed from the incubator shaker and the diesel fuel degradation was determined using gravimetric analysis. The solvent was removed by rotary evaporator and the weight of the residual oil was measured and recorded, and the percentage biodegradation of the used lubricating oil was calculated using the formula of Ijah and Ukpe, (1992);

% biodegradation = [(TPH control- TPH treatment)/ TPH control] ×100 (Eq 3.11)

3.8 Statistical analysis

The effects of each factor, including the microbial count and activity, and different species of plants on the concentration of residual diesel fuel in biostimulation and phytoremediation experiments were done by analysis of variance (ANOVA), SPSS version 8. If ANOVA results were significant at $\alpha = 0.05$ (95% confidence level), Duncan test comparison was used to determine the difference among treatments.

CHAPTER FOUR

RESULTS AND DISCUSSION

This study was conducted to investigate the potential of biowaste amendments in the remediation of diesel-contaminated soil and thus the results and discussion of the findings of the study are presented in Chapter four. The chapter is divided into four sections. Section I presents the results and discussion obtained from bioremediation studies under laboratory and natural conditions; Section II presents results and discussion for findings of phytoremediation studies under laboratory and natural conditions; Section III the overall results for both bioremediation and phytoremediation clean-up techniques are compared and discussed; finally in Section IV a general discussion is postulated highlighting the significant results of both clean-up techniques.

4.1 Characterization of soil and amendments

The physicochemical properties of the soils and organic wastes used in the investigation of bioremediation are presented in Table 4.1. The native soil had a natural pH (~7) with low concentration of N (0.8%) compared with SC (1.3%), PS (1.1%) and TL (1.02%). Among the different organic wastes, SC had the highest concentration of N and P. The soil used for bioremediation had C: N ratio of 16.4. This is a low ratio for effective biodegradation of oil in the soil (Gavrilescu, 2010); hence, it needed the addition of biowastes as a source of nutrients. Gavrilescu, (2010) reported that the optimom value of nutrient content for oil degradation and condition requirement for microbial growth is ratio C:N: P = 100:1:1. In addition, Röling et al., (2004) recorded the highest rate of hydrocarbon degradation in those contaminated soil amended with 2.5 g/kg of N, which gave C: N ratio of 300. The

nutrient content, particularly N and P, are the most important parameters to enhance biodegradation of diesel in hydrocarbon-contaminated soil.

Table 4.1 Characteristics of soil and organic wastes used in the project

		Organic Wastes		
Parameters	Soil	TL	SC	PS
Total nitrogen (%)	0.8 ± 0.1	1.02 ± 0.08	1.3 ± 0.1	1.10 ± 0.04
Phosphorus (%)	0.6 ± 0.5	0.7 ± 0.6	0.9 ± 0.9	0.7 ± 0.1
Moisture content (%)	10.2 ± 0.8	34.3 ± 0.5	75.9 ±1.6	62.1 ± 2.0
Organic C (%)	13.1 ± 1.3	55.6 ± 1.2	72.2 ± 0.9	66.3 ± 1.1
pH	7.03 ± 1.5	6.5 ± 1.2	6.8 ± 1.2	6.9 ± 0.5
Silt (%)	70.0 ± 2.5	-	-	-
Sand (%)	20.0 ± 1.8	-	-	-
Clay (%)	10.0± 1.6	-	-	-
Texture	Silty loam	-	-	-

TL: Tea Leaf, SC: Soy Cake, PS: Potato Skin

The moisture content in SC (75.9%) was higher than the native soil (10.2%) and other organic wastes PS (62.1%) and TL (34.3%) (Table 4.1). Moisture could provide a conductive situation for some selected microorganisms that will contribute positively to biodegradation of diesel fuel in contaminated soil.

4.2 Evaluation of bioremediation under lab condition

Total petroleum hydrocarbon residual was determinated for four different oil concentrations (5, 10, 15 and 20 % w/w) with two different quantities of organic waste amendments (5 and 10 % w/w). The experiments were monitored for a period of 126 days, because at the end of 126 days, the percentage of degradation stopped and reached to 95% of the initial amount of all the hydrocarbons.

4.2.1 Biodegradation of diesel fuel (5 % pollution)

The percentage of diesel degradation in soil contaminated at 5% (w/w) with 5% and 10% organic waste amendments are shown in Figures 4.1 and 4.2, respectively. The results show that the rate of oil degradation was higher in the treatments amended with 10% organic wastes compared with 5% organic wastes. Amended soil with 10% and 5% SC recorded 95% and 81% diesel loss, whereas 10% and 5% PS and TL treatments recorded 73%, 68% and 85%, 80% oil loss, respectively.

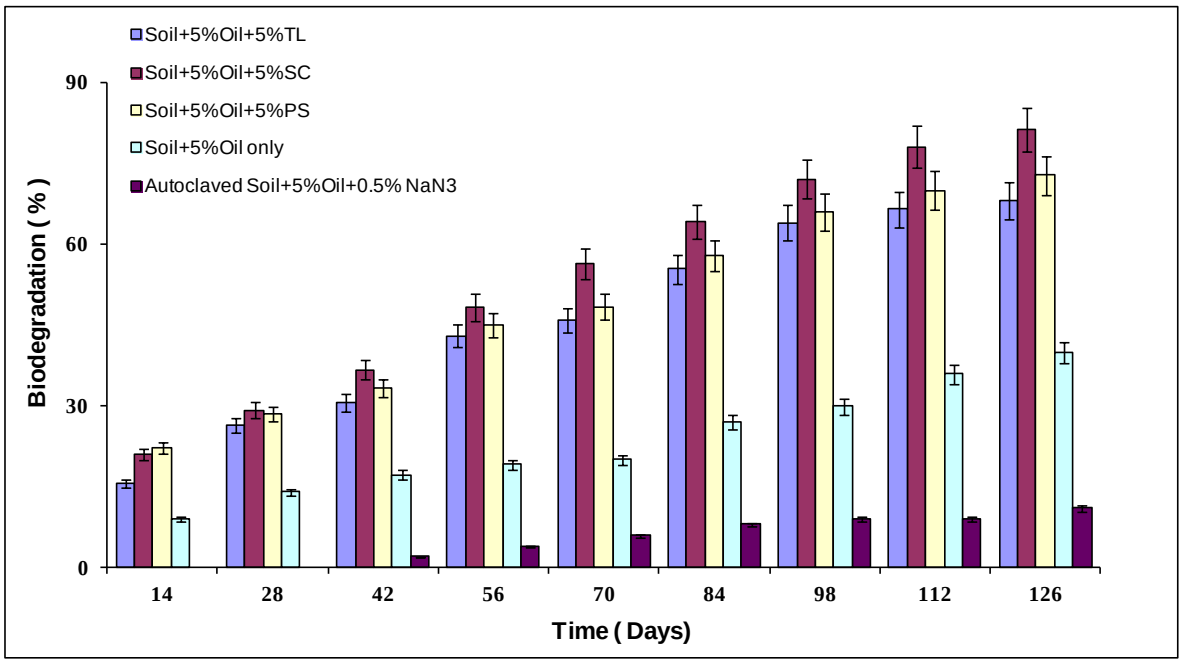


Figure 4.1 Biodegradation of diesel fuel in soil contaminated with 5% oil and amended with 5% organic wastes. (Bars indicate standard error, n = 3).

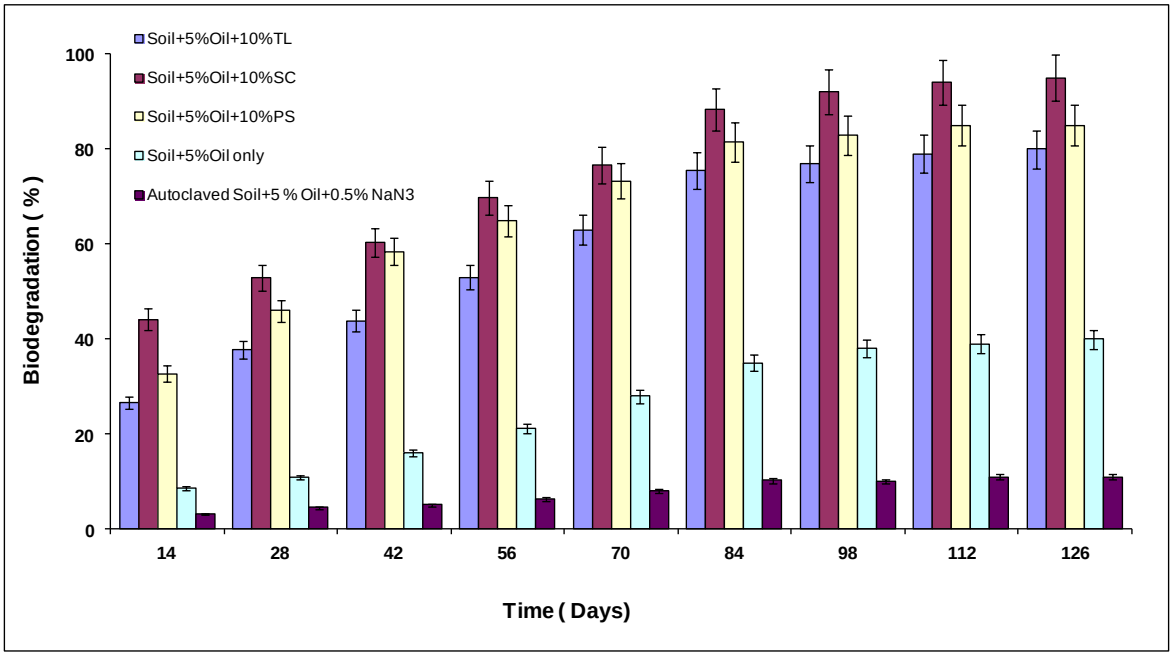


Figure 4.2 Biodegradation of diesel fuel in soil contaminated with 5% oil and amended with 10% organic wastes. (Bars indicate standard error, n = 3).

The rate of biodegradation in unamended soil was 40% which was significantly lower (45%) than soil amended with 10% SC. Similar results were obtained by Van Gestel et al., (2003) who reported 85% diesel oil reduction in contaminated soil amended with different composts (vegetable, fruit and garden waste) at ratio of 1:10 (oil/compost) over a period of 12 weeks. Results indicated high rate of degradation in all the treatments with 5% diesel fuel. The reason could due to the low concentration of oil in the soil, which did not pose serious challenge to affect metabolic activities of microorganisms. Amended soil with 10% and 5% SC had a higher rate of biodegradation at 95% and 81%, respectively; this might be due to the high rate of nutrients (N and P) in SC compared with other organic wastes (Table 4.1). N and P are known as two impotent nutrients to enhance hydrocarbon-utilizing bacteria to breakdown oil compounds and degrade them to carbon dioxide and water (Abioye et al., 2012a; Chaîneau et al., 2005; Padayachee and Lin, 2011).

Only 10% oil degradation was recorded in all autoclaved soil contaminated with 5% oil amended with 0.5% NaN_3 which might be due to some non-biological factors such as photodegradation or evaporation. Van Gestel et al., (2003) observed that the reduction in diesel oil concentration was not only due to degradation, but also possibly due to adsorption to organic substances or volatilization. This is in contrast with findings of Palmroth et al., (2002) who recorded 70% diesel oil loss in autoclaved soil samples during the one-month study. The reason for this difference might be due to degree of sterilization and the poisoned control soil in this study was autoclaved twice, whereas, Palmroth et al., (2002) had not autoclaved the soil and only had added sodium azide (0.5%), so the poisoned control was not sterilized completely.

Table 4.2 shows the net percentage oil loss with 5% diesel fuel and amended with 5% organic wastes. It indicates the effectiveness of each individual amendment compared with the control treatment. The highest net percentage (43.3%) of TPH was recorded in SC

amended soil over a period of 126 days, followed by 34.8% and 30% in those treatments amended with 5% PS and 5% TL, respectively.

The lowest oil loss was recorded from the soil amended with TL compared to those amended with PS and SC. Soil amended with 5% SC recorded a higher net percentage TPH loss (12%), except for the period of 14 days. Table 4.3 shows the net percentage of oil loss with 5% diesel fuel and amended with 10% organic wastes. Throughout this study soil amended with 10% SC recorded the highest net percentage (55%) oil loss. Soil treated with TL recorded the lowest net percentage (40%) of oil loss during the 126 days. Statistical analysis shows a significant difference in net percentage oil loss at $P < 0.05$ confident level in treatments amended with 10% organic wastes, but no significant differences were observed for those treated with 5% organic wastes. Chiu at al., (2009) achieved 54% net percentage oil loss in soil amended with mushroom compost over a period of 22 days, which is similar compared to this result.

Table 4.2 Net (%) loss of TPH in soil amended with 5% diesel fuel with 5% organic waste amendment compared with control

Treatment	Time (days)								
	14	**28**	**42**	**56**	**70**	**84**	**98**	**112**	**126**
Soil+Oil+TL	6.5±1.2	12.5±3.1	13±2.2	24±1.3	26± 3	28±2.4	34±1.9	30.5±3.1	30±1.7
Soil+Oil+SC	12±2.1	15±2.7	19±2.5	29±3.6	36±2.8	37±1.5	42±2.2	42±3.1	43.3±2.7
Soil+Oil+PS	13.2±3	14.5±3.4	16±2.4	25.8±4.1	28.4±3.5	31±2.1	36 ±2.2	34±1.5	34.8±2.4

Net % loss = % loss in TPH (with organic wastes) - % loss in TPH (soil only)

Table 4.3 Net (%) loss of TPH in soil amended with 5% diesel fuel with 10% organic waste amendment compared with control

Treatment	Time (days)								
	14	**28**	**42**	**56**	**70**	**84**	**98**	**112**	**126**
Soil+Oil+TL	17.9±4.8	23±7	25.8±3.7	32±3.5	32±2.1	34.4±2.6	39±2.5	40±3.3	40±2.6
Soil+Oil+SC	35.4±5.6	45±3.1	47.2±3.7	49.6±4.7	48.8±5.3	55.2±4.1	54±2.8	55±3	55±3.7
Soil+Oil+PS	24±3.7	35±5.6	36.4±4.2	36.8±2.3	40±3.4	48.3±6.8	45 ±3.5	46±1.5	45±1.7

Net % loss = % loss in TPH (with organic wastes) - % loss in TPH (soil only)

Statistical analysis (ANOVA) showed that the treatments were significantly different at $P < 0.01$ confidence level for 5% and 10% organic wastes amendment (Table 4.4). Comparison of means revealed that there was no significant difference among treatments amended with organic wastes, while significant difference ($P < 0.01$) was recorded between unamended soil (control) with treatments amended with organic wastes, which proves the positive effect of organic wastes during the biodegradation of diesel oil in the soil (Figure 4.3).

Table 4.4 Analysis of variance for biodegradation of 5% diesel fuel amended with 10% organic wastes

S.V	**SS**	**df**	**MS**
Biodegradation	30047.209	4	7511.802 **
Error	10263.597	40	257.59
Total	40310.806	44	

S.V= Source of variance, MS = Mean square, SS = Sum of square, ** = Significant at 1% level

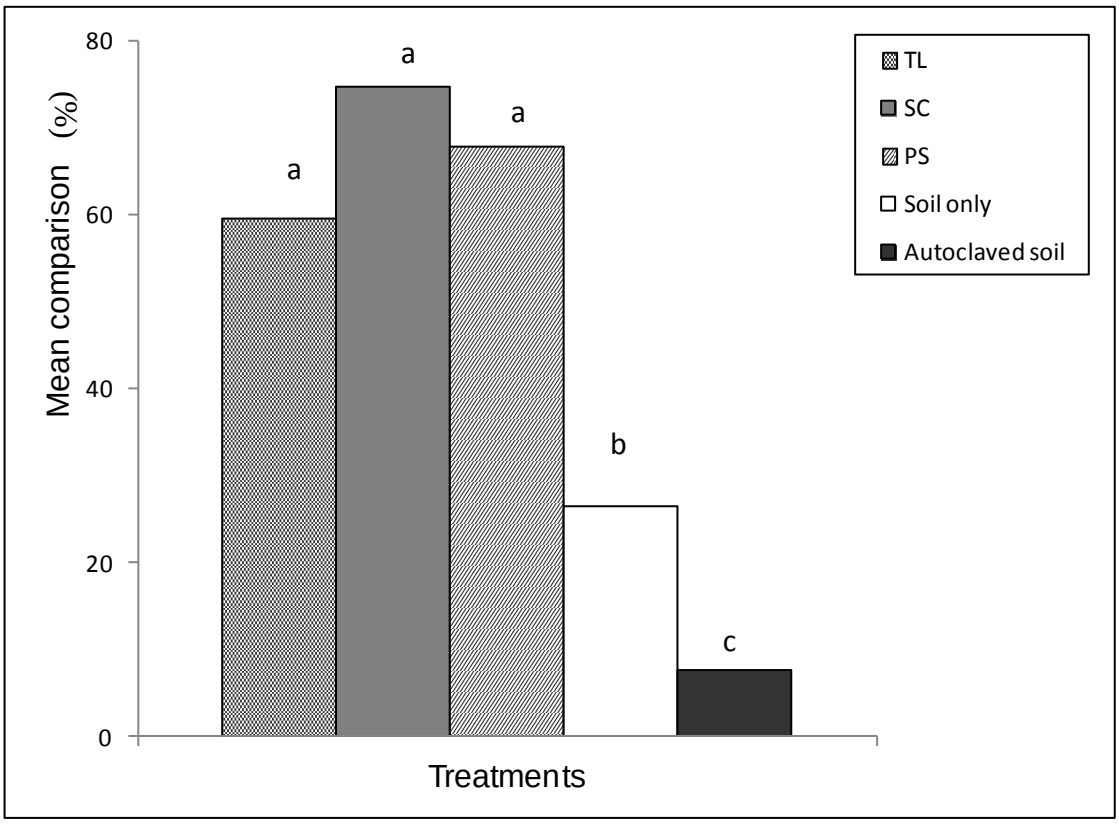


Figure 4.3 Comparison of means of biodegradation in soil polluted with 5% diesel oil amended with 10% organic wastes. The same letter represents no significant difference.

4.2.2 Biodegradation of diesel fuel (10% pollution)

The percentage of diesel fuel degradation in soil polluted with 10% oil concentration with 5% or 10 % organic waste amendments are shown in Figures 4.4 and 4.5, respectively. The results show rapid reduction of over 50% of diesel oil amended with 10% SC during the 56-day period compared with that with 5% amendment with 28% degradation for the same period. However, soil treated with 5% PS and TL recorded the same percentage of biodegradation (25%) at the end of 56 days, where there was a 58% loss of oil in soil amended with 10% SC, while soil amended with 10% TL and PS recorded 39% and 42 % biodegradation, respectively.

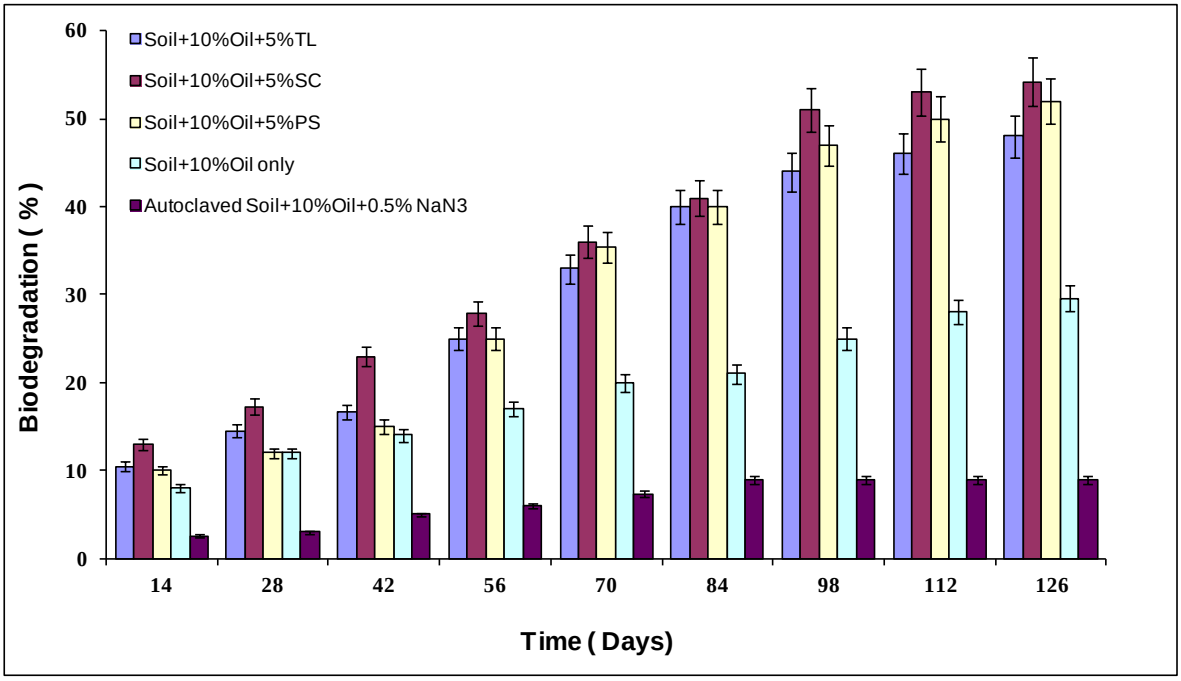


Figure 4.4 Biodegradation of diesel fuel in soil contaminated with 10% oil and amended with 5% organic wastes. (Bars indicate standard error, n = 3).

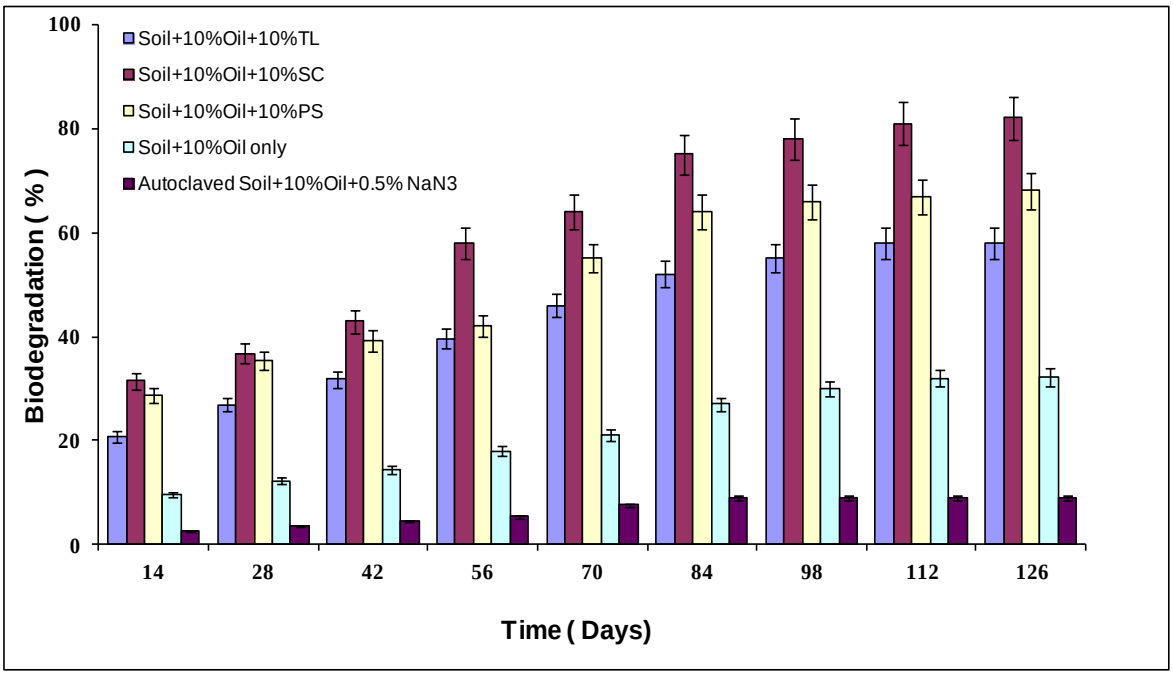


Figure 4.5 Biodegradation of diesel fuel in soil contaminated with 10% oil and amended with 10% organic wastes. (Bars indicate standard error, n = 3).

The reason for the rapid reduction of oil in the first 56 days with 10% organic wastes might be due to the high amount of amendment compared with 5% organic wastes which lead to an increase in the bioavailability of oil for hydrocarbon utilizing bacteria to rapidly degrade compounds and support their metabolic activities. Abioye et al., (2012a) indicated similar results with rapid degradation of lubricating oil within the first 14 days in soil amended with spent mushroom compost. This result is also supported by Singh and Lin (2009) who reported 60% degradation in soil polluted with diesel oil and amended with fertilizers in a period of 30 days of experimentation. According to Bossert and Bartha (1984), a high percentage of biodegradation occurs within the first 90 days of the remediation process. At the end of 126 days, soil treatments amended with 10% SC showed the higher rate of degradation (82%) in soil polluted with 10% diesel oil compared with TL and PS with 58% and 68%, respectively (Figure 4.5). In addition, treatments amended with 5% organic waste and polluted soil with 10% oil, SC showed the highest biodegradation rate (55%) followed by PS and TL treatments which were 52% and 48%, respectively. In unamended control and sterilized control soils, the percentage of degradation recorded were 32% and 9%, respectively. Wellman et al., (2001) recorded similar results with 32% reduction in a control treatment of hydrocarbon concentration over a period of 41 days. Organic wastes and composts with high carbon source have increased ability for oxygen diffusion and mineral nutrients availability which helps bacterial adsorption to the surface of soil. In addition, SC like other amendments probably promoted and enhanced the physico-chemical characteristics of soil to increase the microbial population and adaption in oil polluted soil (Jørgensen et al., 2000).

Statistical analysis shows significant ($P < 0.05$) differences between unamended (control) polluted soil and amended soil with organic wastes in all the treatments, which

proves the positive effect of the organic wastes during the biodegradation of diesel oil in the soil. In contrast, some researchers have indicated that there are no beneficial effects of amendment on degradation of hydrocarbon compounds. Schaefer and Juliane (2007) evaluated the effect of different additives such as brewery and horticultural wastes on TPH degradation at 5000 mg/Kg concentration of crude oil and indicated that the application of these wastes as treatment amended did not enhance the degradation of oil. They assumed that micro-organisms preferred the additives as nutrient sources over the less easily degradable, nitrogen deficient, long-chain crude oil (Schaefer and Juliane, 2007).

The result of net percentage oil loss in soil amended with 5% organic waste and 10% diesel fuel is shown in Table 4.5. Results indicated the higher net percentage oil loss in soil amended with SC (25%) compared to TL (18.4%) and PS (24%).

Statistical analysis does not show significant differences at $\alpha = 5\%$ in the net percentage TPH loss in soil amended with 5% , but there was a significant difference among those treated with 10% organic wastes at $P < 0.05$ confidence level.

Table 4.5 Net (%) loss of TPH in soil amended with 10% diesel fuel with 5% organic waste amendment compared with control

Treatment	Time (days)								
	14	**28**	**42**	**56**	**70**	**84**	**98**	**112**	**126**
Soil+Oil+TL	2.5±0.5	2.5±1	2.6±1.1	8±2.1	13±1.2	16±3.1	15±2	18±1.9	18.4±3.3
Soil+Oil+SC	5±2.2	5.5±0.7	9±0.6	11±1.1	16±1.3	22±1	26±0.8	26±0.5	25±1.2
Soil+Oil+PS	2±0.7	1±0.2	1±0.2	8±0.9	15.4±1.8	19±2.1	22 ±2.5	22±2.1	24±2.3

Net % loss = % loss in TPH (with organic wastes) - % loss in TPH (soil only)

Table 4.6 shows the net percentage of oil loss in soil amended with 10% diesel fuel with 10% organic waste during the bioremediation study. The results are similar with 5% organic wastes (Table 4.5).

Table 4.6 Net (%) loss of TPH in soil amended with 10% diesel fuel with 10% organic waste amendment compared with control

Treatment	Time (days)								
	14	**28**	**42**	**56**	**70**	**84**	**98**	**112**	**126**
Soil+Oil+TL	11±1.8	14. 6±2.8	17.4±2.2	21±3.1	25±1.3	26±2.9	25±3.5	26±2.5	26±3.6
Soil+Oil+SC	22±2.6	24.5±2	28.6±3.1	39.4±3.5	42.8±3.6	48.7±5.2	48±3.5	49±4.1	50±5.5
Soil+Oil+PS	19.1±2	23±2.6	24.8±2.9	23.7±1.8	34±2.4	37±3.3	36 ±2.7	35±3.2	36±3.2

Net % loss = % loss in TPH (with organic wastes) - % loss in TPH (soil only)

Statistical analysis (ANOVA) shows that the treatments inhibited a significant difference at $P < 0.01$ confident level for 5 and 10% organic waste amendments (Table 4.7). Comparison of means revealed that there was a significant difference between treatments amended with different organic wastes (Figure 4.6).

Table 4.7 Analysis of variance for biodegradation of 10% diesel fuel amended with 10% organic wastes

S.V	**SS**	**df**	**MS**
Biodegradation	17809.952	4	4452.488 **
Error	7323.6	40	183.09
Total	25133.552	44	

S.V= Source of variance, MS = Mean square, SS = Sum of square, ** = Significant at 1% level

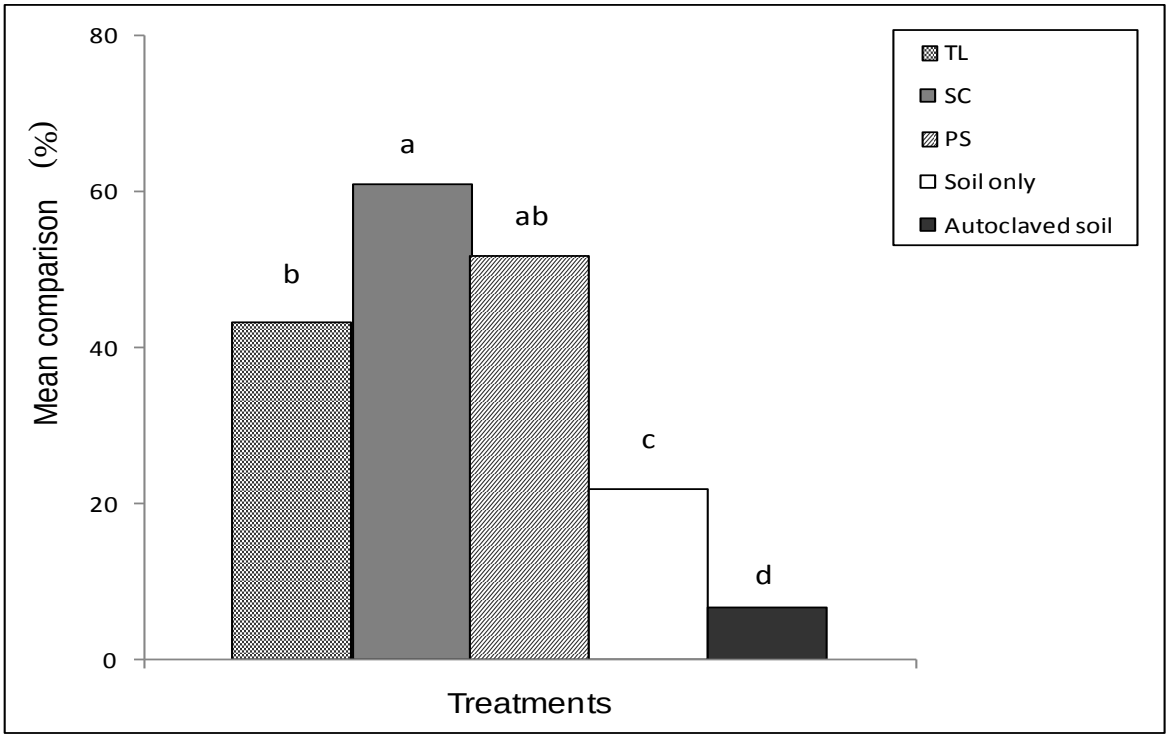


Figure 4.6 Comparison of means of biodegradation in soil polluted with 10% diesel oil amended with 10% organic wastes. The same letter represents no significant difference.

4.2.3 Biodegradation of diesel fuel (15% pollution)

At the end of 126 days, total petroleum hydrocarbon degradation in soil amended with 5% and 10% SC recorded 42% and 55%, respectively (Figures 4.7 and 4.8). Whereas, the soil amended with 10% and 5% TL and PS recorded 33%, 25% and 42%, 40% biodegradation, respectively. This result is similar to Bento et al., (2005) who have reported 72% degradation of light fraction of diesel oil amended with nutrients after six weeks. In contrast, Ijah and Antai (2003b) have indicated a high rate of biodegradation of crude oil in soil polluted with a high amount of crude oil (10% and 20%) within a period of 6 months. However, it was observed that soil amended with 10% organic wastes recorded a higher rate of biodegradation than treatments amended with 5% organic wastes. This might be due to the high amount of nutrient contents, particularly N and P, in soybean cake (Table 4.1)

than the other two wastes, which lead to stimulate indigenous microorganisms to break down the oil.

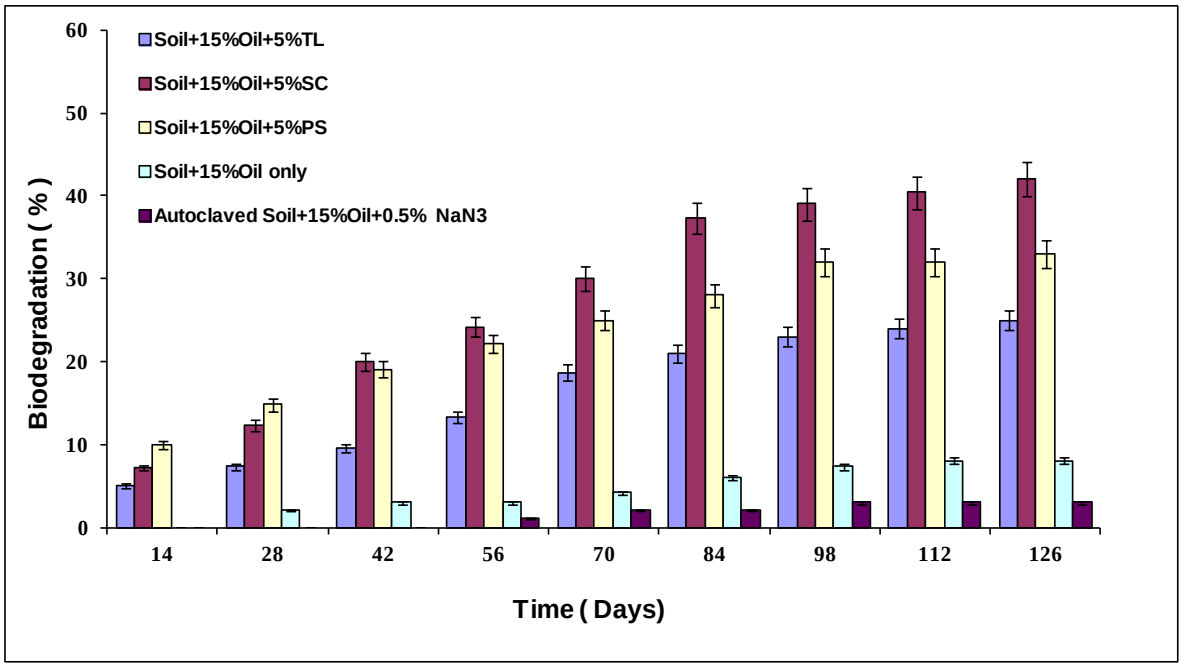


Figure 4.7 Biodegradation of diesel fuel in soil contaminated with 15% oil and amended with 5% organic wastes. (Bars indicate standard error, n = 3).

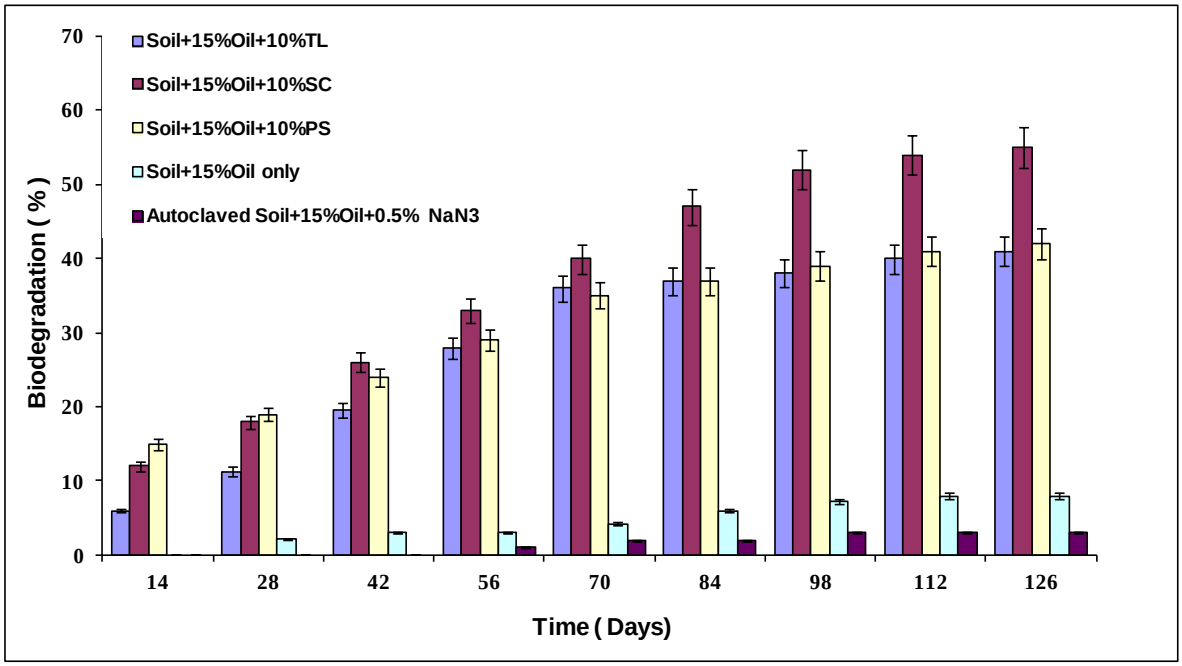


Figure 4.8 Biodegradation of diesel fuel in soil contaminated with 15% oil and amended with 10% organic wastes. (Bars indicate standard error, n = 3).

This result is in conflict with those recorded by Chaîneau et al., (2005) who have reported that a low amount of nutrient addition in soil polluted with crude oil recorded a higher percentage of degradation and assimilation compared with the treatments amended with high nutrients. The differences in these results might be due to the differences in the type of oil used or differences in the soil amendments used. In the Chaîneau et al., (2005) study a mixture of inorganic salts were used but this research used different organic wastes which did not show any toxic effect to the soil and the microorganisms. Abioye et al., (2010) who reported the addition of wastes, such as, brewery-spent grain to lubricating oil-contaminated soil, enhanced the degradation of oil, is in line with the findings of this research. Tables 4.8 and 4.9 show the net percentage TPH loss from soil amended with 10% and 5% organic wastes and polluted with 15% diesel oil. At the end of 28 days soil amended with PS showed a higher net percentage (17%) oil loss compared with other treatments; overall, soil treated with 10% and 5% SC recorded higher net percentage of TPH loss at 47% and 34%, respectively. Statistical analysis does not show significant differences in the net percentage oil loss among those treated at 10% organic waste. However, there was a significant difference among those treated with 5% organic wastes at $P < 0.05$. The results are agreement with the study of Adesodun and Mbagwu (2008) which indicated that there was a significant difference in the net percentage waste lubricating oil loss in soil amended with poultry manure (PM) over in a period of 18 months. The overall observation illustrates that SC is providing a suitable nutrient supplement in stimulating microbial degradation of diesel fuel.

Table 4.8 Net (%) loss of TPH in soil amended with 15% diesel fuel with 10% organic waste amendment compared with control

Treatment	Time (days)								
	14	**28**	**42**	**56**	**70**	**84**	**98**	**112**	**126**
Soil+Oil+TL	6±0.8	9.3±1.2	16.5±1.8	25±3.1	31±2.1	31±2.3	32±1.5	32±3.5	32±1.6
Soil+Oil+SC	12±0.6	16±1.5	23±2.1	30±3.5	35±2.6	41±3.2	46±2.5	46±3.1	47±2.5
Soil+Oil+PS	15±1.3	17±2.1	21±2.2	26±1.8	30±2.1	31±2.6	33 ±2.1	33±2.2	34±3.1

Net % loss = % loss in TPH (with organic wastes) - % loss in TPH (soil only)

Table 4.9 Net (%) loss of TPH in soil amended with 15% diesel fuel with 5% organic waste amendment compared with control

Treatment	Time (days)								
	14	**28**	**42**	**56**	**70**	**84**	**98**	**112**	**126**
Soil+Oil+TL	5±0.6	5.2±2.1	6.5±2.8	10.3±3.1	14.5±2	15±2.2	15.7±2.3	16±1.5	17±2.6
Soil+Oil+SC	7.2±1.6	10.2±1.9	17±1	21.2±2.5	25.8±2.5	31.3±2	31.7±2.5	32.4±3.4	34±3.5
Soil+Oil+PS	10±2.3	12.7±1.1	16±1.9	19.2±3.1	20.8±1.7	22±2.6	24.7±1.1	24±2.7	25±3.1

Net % loss = % loss in TPH (with organic wastes) - % loss in TPH (soil only)

Statistical analysis (ANOVA) shows that the treatments were significantly different in 10% organic waste amendments at $P < 0.01$ confident level (Table 4.10). The result also was significant with 5% organic waste amendments. Comparison of means revealed that there were no significant differences among treatments amended with organic wastes but

there was a significant difference between unamended (control) polluted soil and the amended with organic wastes in all the treatments (Figure 4.9)

Table 4.10 Analysis of variance for biodegradation of 15% diesel fuel amended with 10% organic wastes

S.V	SS	df	MS
Biodegradation	9693.284	4	2423.321 **
Error	4327.533	40	108.188
Total	14020.818	44	

S.V= Source of variance, MS = Mean square, SS = Sum of square, ** = Significant at 1% level

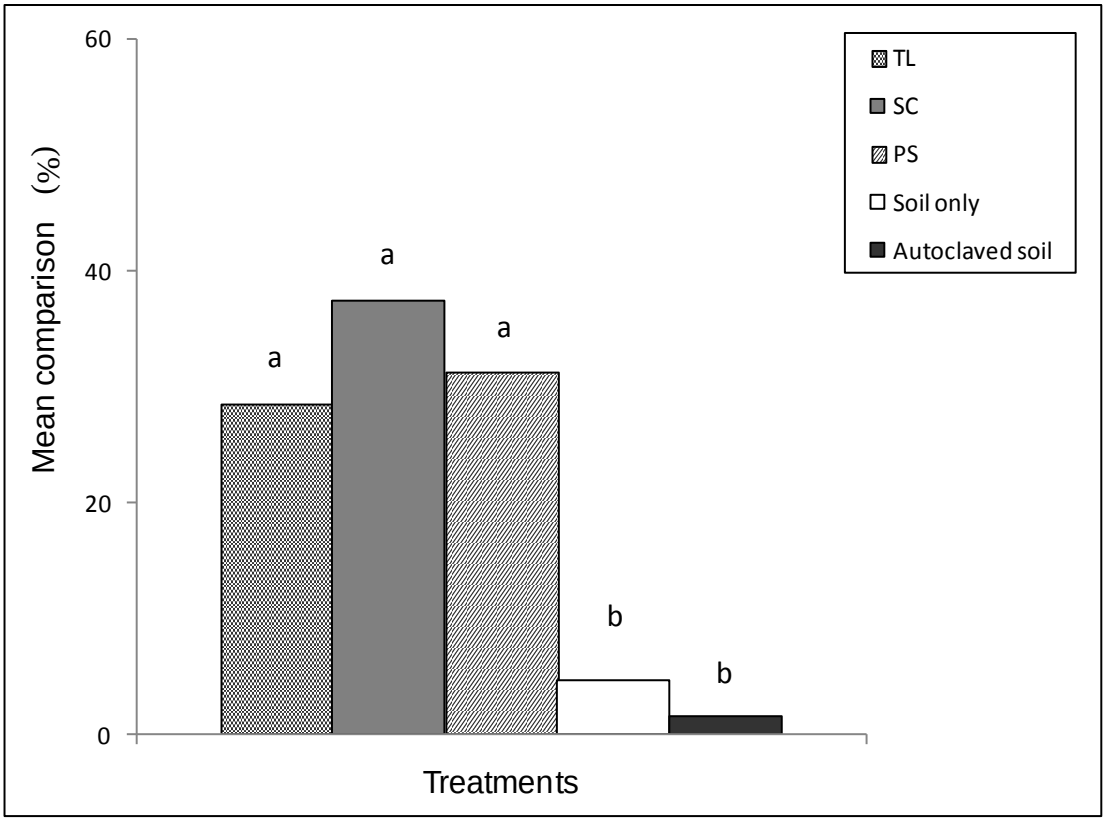


Figure 4.9 Comparison of means of biodegradation in soil polluted with 15% diesel oil amended with 10% organic wastes. The same letter represents no significant difference.

4.2.4 Biodegradation of diesel fuel (20% pollution)

At the end of 42 days, the percentage of biodegradation was 11%, 8.5% and 7% in soil amended with 5% SC, PS and TL, respectively (Figure 4.10). Whereas, only 25% biodegradation was recorded in soil amended with 10% SC, and 17% and 19% oil loss with TL and PS amendments was recorded, respectively (Figure 4.11). At the end of 126 days, the rate of biodegradation was too low in all treatments amended with 5% organic wastes. However, maximum percentage of oil loss recorded was 25% and 21% in soil amended with 10% and 5% SC, respectively. The soil amended with oil without organic wastes shows 5% degradation in a period of 126 days. These results are similar to those of Lee et al., (2008) who is reported that the addition of mineral nutrients and organic amendments is a viable choice in the remediation of contaminated soils. They reported a significant reduction in waste lubricating oil achieved by adding manure compost due to it is providing an alternative source of nutrients, especially vitamins, nitrogen and phosphorus.

It is clear that the percentage of degradation was too slow in all the treatments during the first 42 days. The reason for the low rate of biodegradation within this period might be due to the impact of a high concentration of oil on microorganism growth, which leads to negative effects on microbial population in polluted soil. This initial trend of low degradation due to high concentration of oil has also been reported by Abioye et al., (2012a) and Rahman et al., (2002).

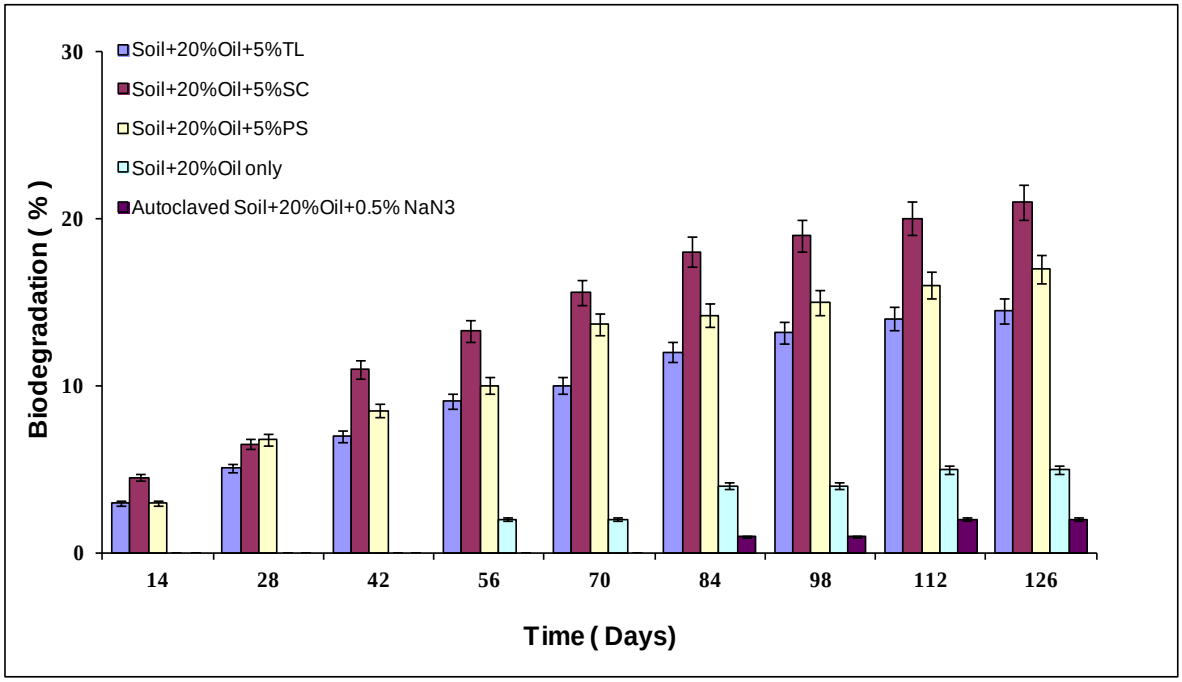


Figure 4.10 Biodegradation of diesel fuel in soil contaminated with 20% oil and amended with 5% organic wastes. (Bars indicate standard error, n = 3).

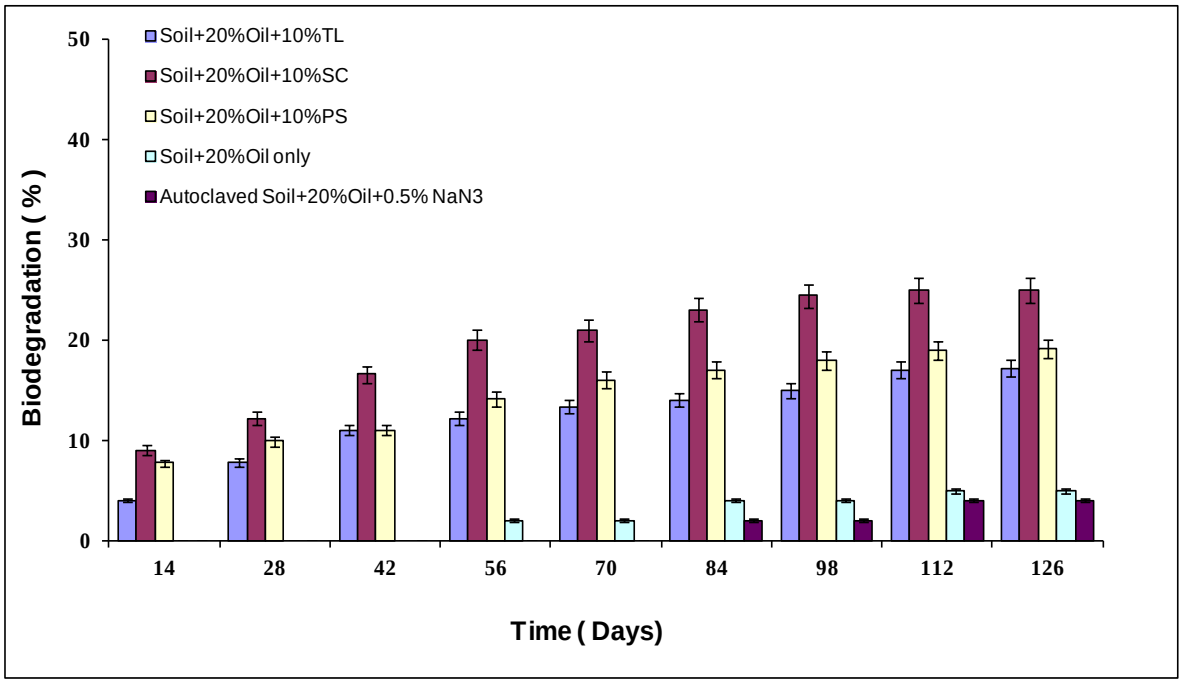


Figure 4.11 Biodegradation of diesel fuel in soil contaminated with 20% oil and amended with 10% organic wastes. (Bars indicate standard error, n = 3).

Tables 4.11 and 4.12 show the net percentage loss of TPH in soil amended with 20% diesel fuel and 10% and 5% organic waste amendments. Statistical analysis shows that there was significant difference ($P < 0.05$) in percentage of biodegradation between soil treated with 10% SC and those amended with 10% PS and TL, but there was no significant difference between treatments with 10% PS and TL. On the other hand, there was a significant difference between soil amended with 5% SC with those amended with 5% TL but the result does not show significant difference between soil amended with 5% PS and TL.

Table 4.11 Net (%) loss of TPH in soil amended with 20% diesel fuel and 10% organic waste amendment compared with control

Treatment	Time (days)								
	14	**28**	**42**	**56**	**70**	**84**	**98**	**112**	**126**
Soil+Oil+TL	4±0.9	7.8±2.6	9.5±1.8	9.2±1	11.3±1.2	11±1.5	11±2.1	17±0.5	12.2±1.6
Soil+Oil+SC	9±1.1	12.2±2.2	16.6±1	18±1.5	19±1.8	19±2.2	20.4±1.8	20±1.4	20±1.1
Soil+Oil+PS	7.7±1.6	10±1.8	11±0.9	12.2±2.1	14±0.7	13±1.6	14±0.8	14±1.7	14.1±0.5

Net % loss = % loss in TPH (with organic wastes) - % loss in TPH (soil only)

Table 4.12 Net (%) loss of TPH in soil amended with 20% diesel fuel with 5% organic waste amendment compared with control

Treatment	Time (days)								
	14	**28**	**42**	**56**	**70**	**84**	**98**	**112**	**126**
Soil+Oil+TL	3±1.2	5.1±1.4	7±0.9	7.1±0.3	8±1.2	8±1.3	9.2±1.1	9±0.4	9.5±0.6
Soil+Oil+SC	4.5±1.3	6.5±0.9	11±1.7	11.3±2	13.6±2.6	14±1.8	15±1.4	15±0.9	16±2.1
Soil+Oil+PS	3±0.7	6.8±1.1	8.5±0.4	8±1.9	9.7±1.1	10.2±0.8	11±1.2	11±1.5	13±0.8

Net % loss = % loss in TPH (with organic wastes) - % loss in TPH (soil only)

Statistical analysis (ANOVA) shows that the treatments were statistically significant for all assessed traits at $P < 0.01$ confidence level in 10% organic waste amendments (Table 4.13). The same result was recorded with 5% organic waste amendments. Comparison of means revealed that there was a significant difference between SC amendment with the other two organic wastes (PS and TL), but it was not significant between TL and PS treatments. However, polluted soil amended with organic wastes had a significant difference with unamended (control) (Figure 4.12).

Table 4.13 Analysis of variance for biodegradation of 20% diesel fuel amended with 10% organic wastes

S.V	**SS**	**df**	**MS**
Biodegradation	2260.609	4	565.152 **
Error	621.209	40	15.53
Total	2881.818	44	

S.V= Source of variance, MS = Mean square, SS = Sum of square, ** = Significant at 1% level

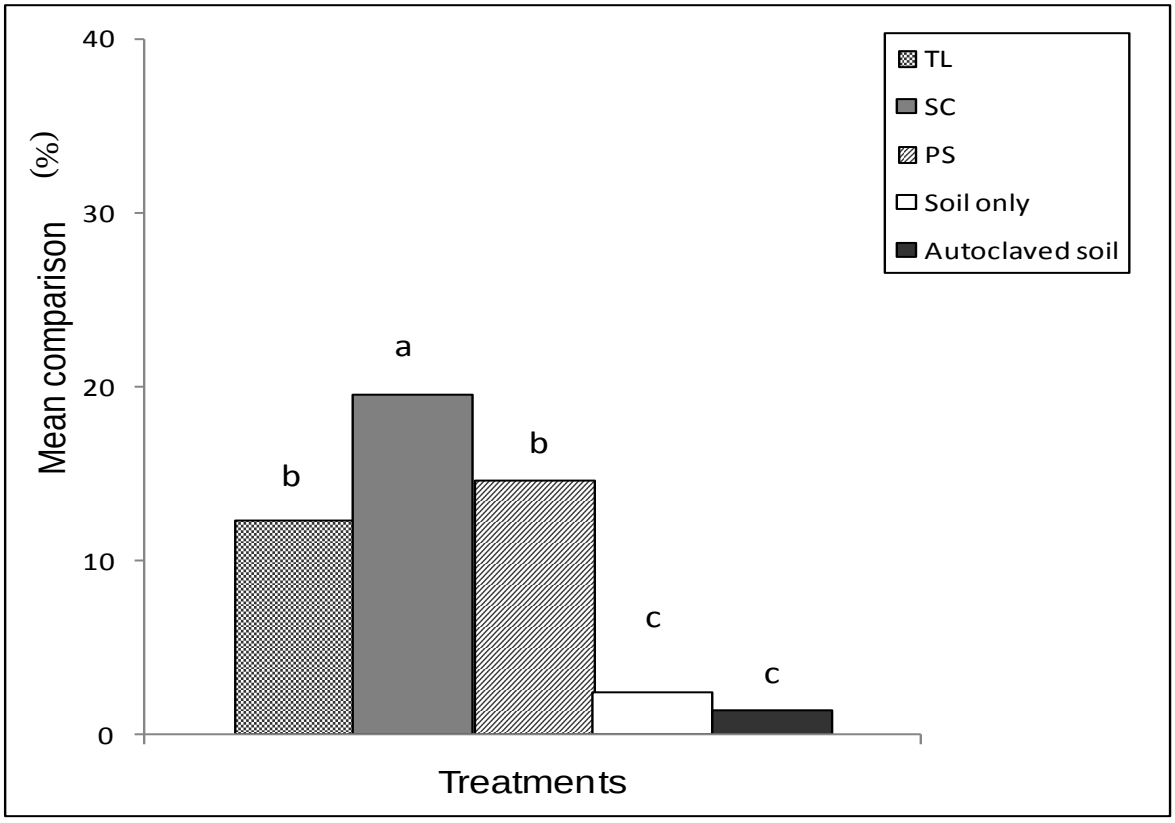


Figure 4.12 Comparison of means of biodegradation in soil polluted with 20% diesel oil amended with 10% organic wastes. The same letter represents no significant difference.

4.2.5 Kinetics model and Half- Life of biodegradation

The first-order kinetic model was used to calculate the rate of biodegradation and it has been reported by many authors (Jørgensen et al., 2000; Namkoong et al., 2002). Tables 4.14 and 4.15 show the biodegradations constant rate in the treatment with 5% and 10% of different organic wastes, respectively. Half-life indicates the length of time it takes to degrade half of the hydrocarbon. The coefficient of determination (R^2) indicates that the model fits well with all the treatments. Data for the sampling periods was combined before this model could be used. The kinetics parameter shows the highest rate of degradation for soil polluted with 5% diesel fuel accrued in soil amended with 10% SC treatment (k = 0.22/day and half-life of 3.05 days) (Table 4.15).

The addition of 5% and 10% SC recorded the highest rate of biodegradation and half-life compared to other organic wastes (PS and TL). It illustrates that SC is the most effective treatment in stimulating biodegradation of soil polluted with diesel fuel throughout the study period. However, this result is different from Abioye et al., (2012a), who found that the highest rate of biodegradation was in 15% lubricating oil-contaminated soil amended with banana skin, while soil amended with brewery spent had the highest percentage of biodegradation during 84 days study. However, by increasing the level of pollution to 15% and 20% diesel oil, SC and PS recorded the same results in the biodegradation rate and half-life at the two different levels of amendments. In soil polluted with 20% diesel oil the highest rate of biodegradation and half-life was in soil treated with SC, which recorded k = 0.021 day $^{-1}$, half-life of 33 days and 0.033 day $^{-1}$, half-life of 21 days in soil amended with 5% and 10% SC, respectively. Soil amended with 5% SC and PS, and polluted with 15% diesel oil indicated the same rate of biodegradation, k = 0.044/day and 0.041/day with half-life of 15.75 days and 16.9 days, respectively.

Table 4.14 Kinetic model and half- life of diesel fuel degradation amended with 5% organic wastes

Treatment	Biodegradation constant (k) day^{-1}	Half- life (days) ($t_{1/2}$)	Coefficients of determination (R^2)
Soil + TL+ 5% Oil	0.069	10.04	0.70
Soil + SC+ 5% Oil	0.114	6.05	0.97
Soil + PS+ 5% Oil	0.098	7.07	0.89
Soil + 5% Oil	0.038	18.24	0.88
Autoclaved soil + 5% Oil	0.005	133.30	0.93
Soil + TL+ 10% Oil	0.050	13.86	0.82
Soil + SC+ 10% Oil	0.061	11.36	0.96
Soil + PS+ 10% Oil	0.051	13.59	0.94
Soil + 10% Oil	0.031	22.35	0.92
Autoclaved soil + 10% Oil	0.006	115.52	0.65
Soil + TL+ 15% Oil	0.024	28.88	0.94
Soil + SC+ 15% Oil	0.044	15.75	0.98
Soil + PS+ 15% Oil	0.041	16.90	0.94
Soil + 15% Oil	0.006	115.52	0.92
Autoclaved soil + 15% Oil	0.001	462.0	0.90
Soil + TL+ 20% Oil	0.014	49.5	0.88
Soil + SC+ 20% Oil	0.021	33.0	0.92
Soil + PS+ 20% Oil	0.020	38.32	0.91
Soil + 20% Oil	0.002	330.0	0.80
Autoclaved soil + 20% Oil	0.001	693.14	0.72

Table 4.15 Kinetic model and half- life of diesel fuel degradation amended with 10% organic wastes

Treatment	Biodegradation constant (k) day^{-1}	Half- life (days) ($t_{1/2}$)	Coefficients of determination (R^2)
Soil + TL+ 5% Oil	0.146	4.74	0.92
Soil + SC+ 5% Oil	0.227	3.05	0.94
Soil + PS+ 5% Oil	0.176	3.93	0.90
Soil + 5% Oil	0.037	18.73	0.87
Autoclaved soil + 5% Oil	0.013	53.31	0.83
Soil + TL+ 10% Oil	0.076	9.12	0.92
Soil + SC+ 10% Oil	0.153	4.53	0.97
Soil + PS+ 10% Oil	0.115	6.02	0.90
Soil + 10% Oil	0.037	18.74	0.94
Autoclaved soil + 10% Oil	0.01	64.78	0.89
Soil + TL+ 15% Oil	0.044	15.75	0.98
Soil + SC+ 15% Oil	0.061	11.36	0.92
Soil + PS+ 15% Oil	0.059	12.37	0.88
Soil + 15% Oil	0.005	128.36	0.69
Autoclaved soil + 15% Oil	0.001	495.10	0.62
Soil + TL+ 20% Oil	0.019	35.36	0.90
Soil + SC+ 20% Oil	0.033	21.0	0.88
Soil + PS+ 20% Oil	0.030	25.5	0.85
Soil + 20% Oil	0.0017	407.73	0.91
Autoclaved soil + 20% Oil	0.0011	630.13	0.98

In soil polluted with 10% diesel oil and amended with 10% SC recorded the biodegradation rate of 0.153/day and half-life of 4.53 days, while the rate of biodegradation and half-life were 0.061/day and 11.36 days respectively in soil amended with 5% SC.

Statistical analysis indicates that there is a significant relationship between the concentration of diesel oil in contaminated soil and the rate of biodegradation. As results show, a higher rate of degradation was recorded in soil polluted with 5% diesel oil compared with 20% diesel oil, which could be attributed to the reduction of the population of microorganism and enzyme activity at different levels of oil pollution. The general trend with soil polluted at a high-level of diesel oil shows that these organic wastes are not efficient enough to stimulate the biodegradation process. These results are similar to the result of research done by Adesodum and Mbagwu (2008) who reported the ineffectiveness of organic wastes with a high level of spent oil pollution which could be attributed to reduction in the activity of the soil microbes at this level of pollution. Many researchers have reported negative effects of petroleum pollution on microflora that lead to decrease in the biodegradation rate and as such bioremediation is a useful method to remediate petroleum hydrocarbon at moderate concentration (Bossert and Bartha, 1984; Schaefer and Juliane, 2007).

The results of soil amended with 5% of different organic wastes also show that SC had the lowest half-life and the highest biodegradation rate in soil contaminated with 20%, 15%, 10% and 5% diesel oil. The half-life were 33 days, 15.75 days, 11.36 days and 6.05 days at 20%, 15%, 10% and 5% diesel oil, respectively (Table 4.13). The reason for the higher rate of biodegradation in soil amended with SC might be the buffering effects of SC and presenting higher quantities of N and P compared with TL and PS, which attributed to its C: N ratio. The result is similar to that of Medjor et al., (2012) who reported that at the

end of 1200 hours of bioremediation of groundwater polluted with diesel oil, first order reaction showed the constant rate of 0.002 hour^{-1}and half-life ($t_{1/2}$) of 346.5 hours. Namkoong et al., (2002) also have indicated that soil treated with sewage sludge with a higher amount of N and P recorded a high biodegradation rate at the end of one month of study. They reported low half-life and high biodegradation rate constant in diesel-contaminated soil with sewage sludge amendment compared with unamended control soil.

4.2.6 Microbial population of soil polluted with 5% diesel fuel

The total colony forming units of aerobic heterotrophic bacteria (AHB) in soil amended with 5% SC and polluted with 5% diesel fuel ranged between 13 × 10 ^{7} colony forming units (CFU)/g and 80× 10^{7} CFU/g while soil treated with 5% PS and TL recorded 11× 10 ^{7} CFU/g to 70×10 ^{7} CFU/g and 17×10^{7} CFU/g to 60 ×10^{7} CFU/g, respectively (Figure 4.13). In control (unamended) treatment AHB had the lowest range from 5 ×10^{7} CFU/g to 51 ×10^{7} CFU/g. The CFU of AHB in soil amended with SC was significantly higher than the control soil.

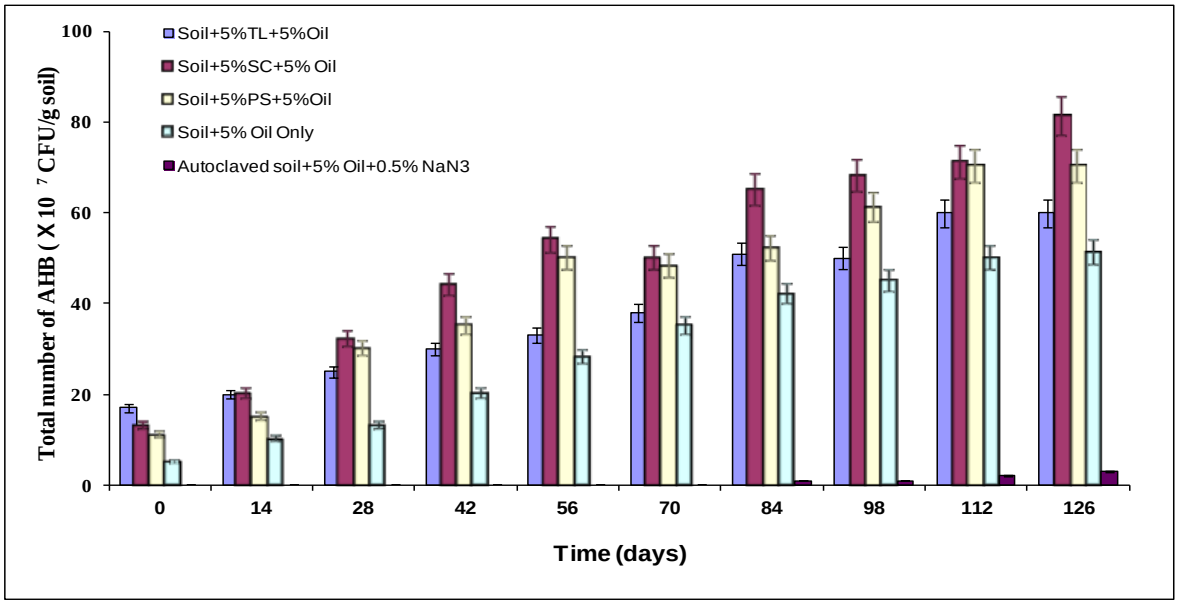


Figure 4.13 Total CFU of aerobic heterotrophic bacterial (AHB) in soil contaminated with 5% diesel fuel amended with 5% organic wastes. Bars indicate standard error (n = 3).

No significant difference was recorded in AHB counts among soils amended with different organic wastes. Figure 4.14, also shows the population of aerobic heterotrophic bacteria in soil contaminated with 5% diesel fuel and amended with 10% organic wastes. Soil amended with 10% SC recorded a higher number of AHB ranging between 31 $\times 10^7$ CFU/g and 263 $\times 10^7$ CFU/g compared with other treatments at the end of 126 days. Results indicate that soil amended with 5% organic wastes shows lower counts than those amended with 10% organic wastes. This finding is similar to Bento et al., (2005) who recorded a higher count of AHB in soil polluted with diesel fuel and amended with crop residues.

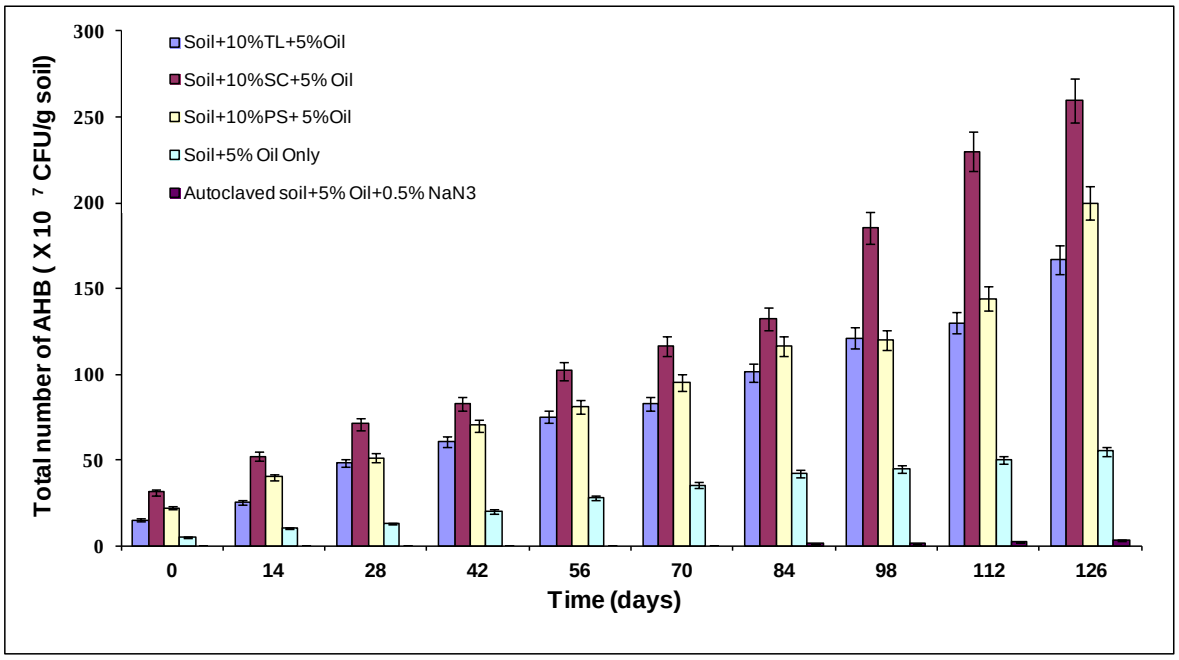


Figure 4.14 Total CFU of aerobic heterotrophic bacterial (AHB) in soil contaminated with 5% diesel fuel amended with 10% organic wastes. Bars indicate standard error (n = 3).

The count of diesel utilizing bacteria (DUB) in soil amended with 5% and 10% organic wastes are shown in Figures 4.15 and 4.16, respectively. The DUB of soil amended with 5% SC was higher than those amended with 5% PS and TL. AHB count ranged between 18

$\times 10^5$ CFU/g and 99 $\times 10^5$ CFU/g while those amended with 5% PS and TL had 83 $\times 10^7$ CFU/g and 75×10^7 CFU/g at the end of 126 days.

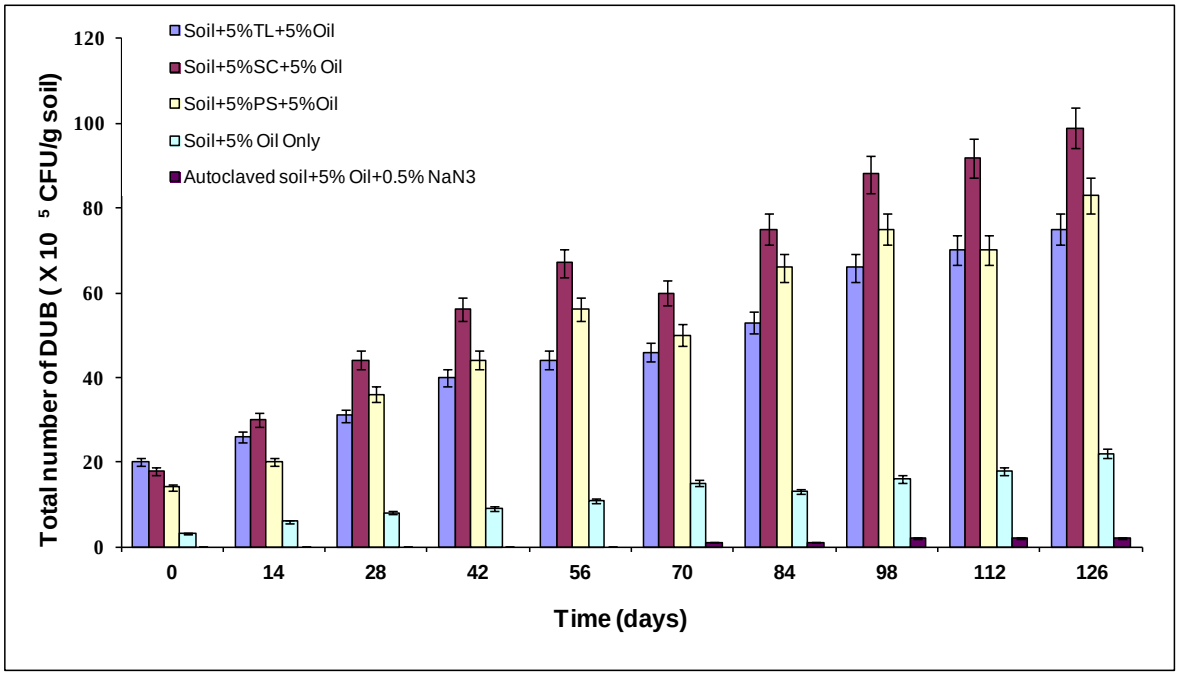


Figure 4.15 Total CFU of diesel utilizing bacterial (DUB) in soil contaminated with 5% diesel fuel amended with 5% organic wastes. Bars indicate standard error (n = 3).

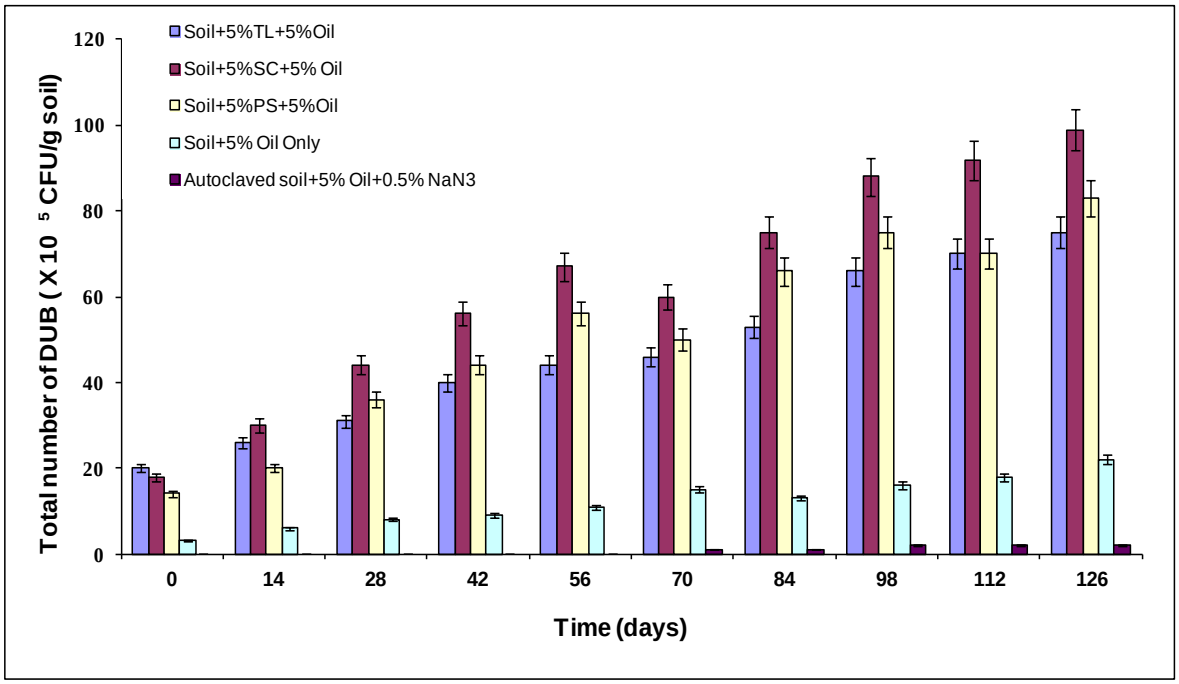


Figure 4.16 Total CFU of diesel utilizing bacterial (DUB) in soil contaminated with 5% diesel fuel amended with 10% organic wastes. Bars indicate standard error (n = 3).

It was observed that there was an increase in AHB population in soil amended with 5% SC from 13 $\times 10^7$ CFU/g to 54 $\times 10^7$ CFU/g during the first eight weeks which began to decrease in week nine. However, the bacteria population continued to increase until the end of the study. There was no bacterial growth detected in all sterilized samples during the 126 days of the study period. In unamended control soil the range was from 3.0×10^7 CFU/g to 22 $\times 10^7$ CFU/g. The result agreed with Padayachee and Lin (2011) who reported an increase in bacterial population from 2.0×10^6 to 3.2×10^6 CFU/ml in soil polluted with diesel oil during the 1st week of all supplemented microcosms amended with fertilizers. Count of DUB in soil amended with 10% SC showed 11% higher than those amended with 10% PS and TL (Figure 4.16). Statistical analysis shows that there was no significant difference between DUB counts on soil amended with 5% and 10% organic wastes at $P < 0.05$ confidence level, but there was significant differences between control soil and soil amended with organic wastes.

4.2.7 Microbial population of soil polluted with 10% diesel fuel

At the end of 126 days, AHB enumeration of 5% SC amended soil showed 17.6% and 71.4% higher than those amended with PS and TL, respectively (Figure 4.17). The count of AHB was between 14 $\times 10^7$ CFU/g and 120 $\times 10^7$ CFU/g in amended soil with 5% SC, while soil amended with 5% PS and TL the ranged of AHB was from 14 $\times 10^7$ CFU/g to 102 $\times 10^7$ CFU/g and 11 $\times 10^7$ CFU/g to 7×10^8 CFU/g, respectively. Therefore, AHB in soil amended with 5% PS was higher on 28th, 56th and 84th days than those amended with 5% SC and TL, but this difference was not significant at $P < 0.05$ confident level. Statistical analysis also shows that there was a significant difference between soil amended with 5% SC with TL,

but there was no significant difference between amended soil with SC and PS at $P < 0.05$ confident level.

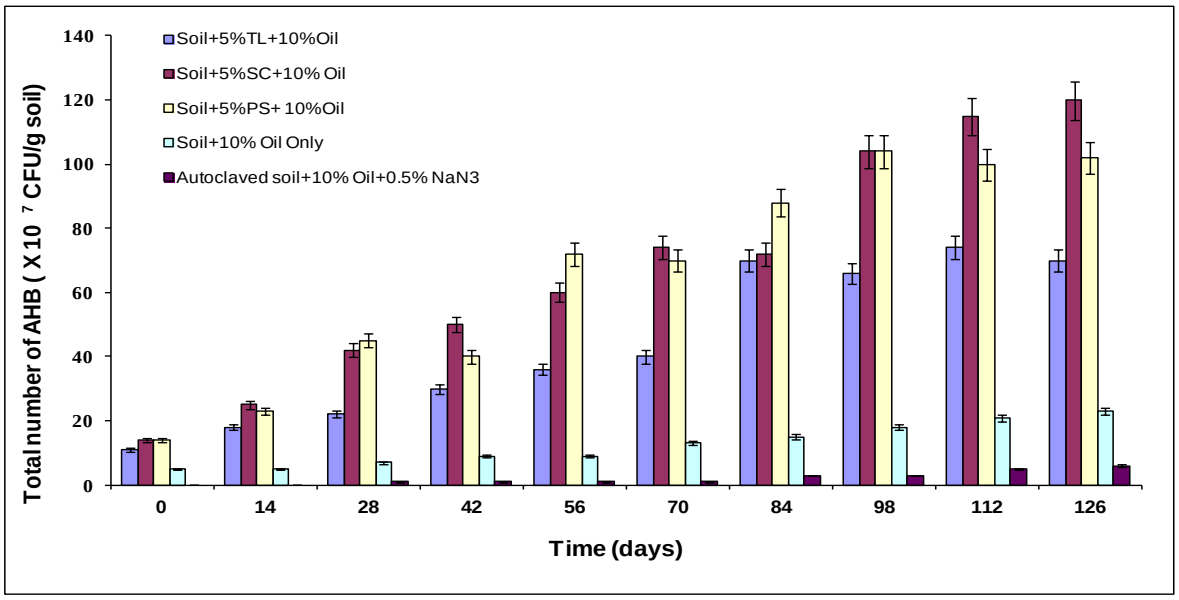


Figure 4.17 Total CFU of aerobic heterotrophic bacterial (AHB) in soil contaminated with 10% diesel fuel amended with 5% organic wastes. Bars indicate standard error (n = 3).

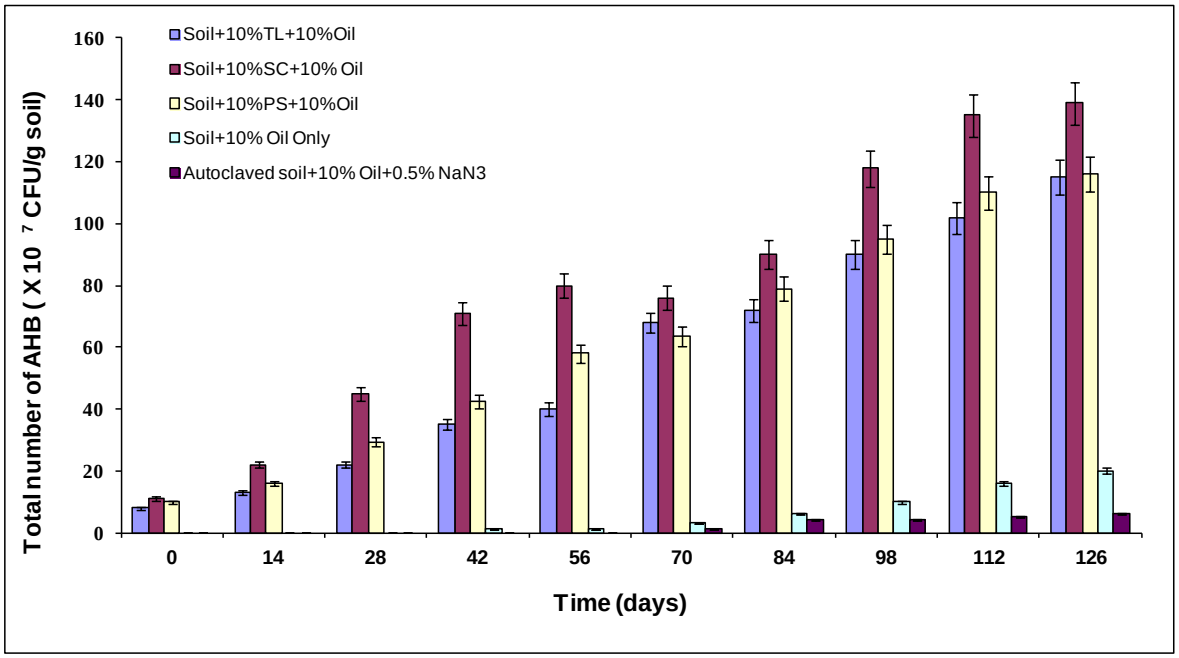


Figure 4.18 Total CFU of aerobic heterotrophic bacterial (AHB) in soil contaminated with 10% diesel fuel amended with 10% organic wastes. Bars indicate standard error (n = 3).

It is observed that soil treated with 10% organic wastes had higher microbial population (Figure 4.18), during the period of study compared with control soil and among the organic waste amended, the AHB count was higher in the order SC > PS > TL throughout the 126 day period of study. The count of AHB in treatments amended with 10% organic wastes was 6 times higher than in the naturally attenatuated soil (unamended) treatments. However, the number of heterotrophic microorganisms in unamended control soil was 2 $\times 10^8$ CFU/g at the end of study, so it shows the lowest number of CFU in control treatments compare to treatments amended with organic wastes. This finding is similar to the finding of Li et al., (2006) who reported that the CFU of AHB in all treatments was significantly ($P < 0.05$) higher than that of the control treatments, and correlated positively with soil residual TPH content during the first 30 days of the incubation.

The CFU of DUB in soil amended with 5% SC ranged from 34 $\times 10^7$ CFU/g to 155 $\times 10^7$ CFU/g (Figure 4.19). However, the DUB count in soil amended with 10% SC ranged from 14 $\times 10^5$ CFU/g to 176 $\times 10^5$ CFU/g (Figure 4.19). It is obvious that DUB count was higher in 10% organic wastes compared with that amended with 5% organic wastes. Similar result has been reported by Abioye et al., (2012a) and Okoh (2006) which indicates that the addition of nutrients to contaminated soil will enhance the rate of biodegradation and stimulate the microbial population. The DUB count was lower in unamended soil (2 $\times 10^5$ CFU/g to 23 $\times 10^5$ CFU/g) compared with those amended with organic wastes. Statistical analysis does not show any significant difference among soil amended with different organic wastes, but there was statistical difference between unamended controls soils with those treated with organic wastes.

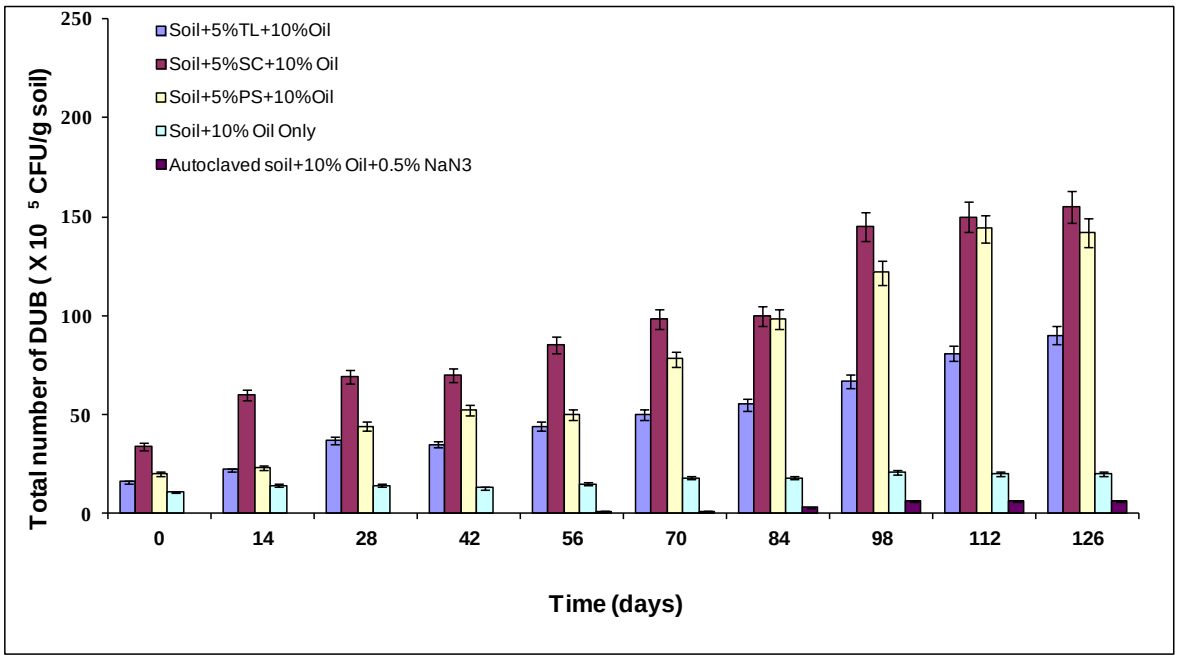


Figure 4.19 Total CFU of diesel utilizing bacterial (DUB) in soil contaminated with 10% diesel fuel amended with 5% organic wastes. Bars indicate standard error (n = 3).

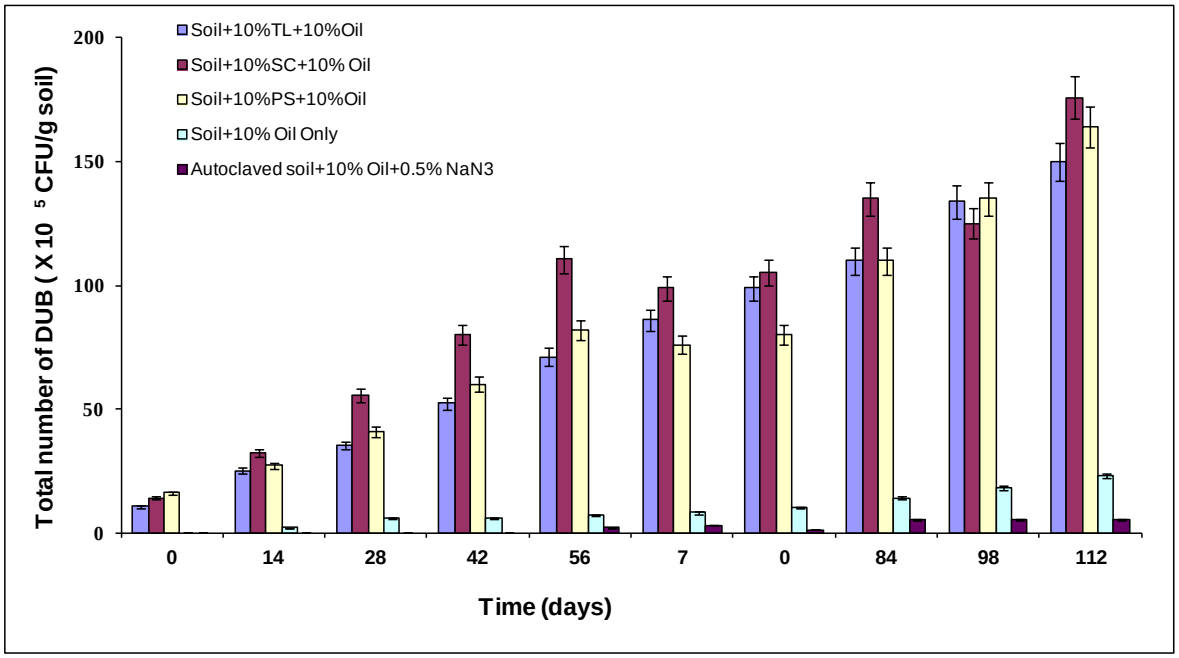


Figure 4.20 Total CFU of diesel utilizing bacterial (DUB) in soil contaminated with 10% diesel fuel amended with 10% organic wastes. Bars indicate standard error (n = 3).

Higher counts of DUB and AHB were recorded in all soil amended with organic wastes compared with unamended polluted soil. This might be due to the ability of amended organic wastes to naturalize the effects of toxic oil on microbial population by providing better physicochemical properties of soil (Abioye et al., 2012b). Amendments might improve the soil aeration condition which is a favored factor for the growth of oil utilizing bacteria species that are only aerobic in nature. In soil treated with sodium azide there were counts of DUB and AHB. This result is similar to the finding of Palmroth et al., (2002) who recorded bacteria counts in control poisoned soil. Higher counts of DUB and AHB demonstrated by soil treated with 10% organic wastes compared with those of 5% amendments might be due to the quantity of organic wastes added which probably provided more nutrients to the soil bacteria than those amended with 5% organic wastes.

4.2.8 Microbial population of soil polluted with 15% diesel fuel

At the end of 126 days, counts of aerobic heterotrophic bacteria in soil amended with 15% diesel oil contaminated and amended with 5% SC ranged from 9 $\times 10^7$ CFU/g to 65$\times 10^7$ CFU/g while soil amended with PS and TL recorded a range of 8 $\times 10^7$ CFU/g to 58 $\times 10^7$ CFU/g and 8 $\times 10^7$ CFU/g to 48 $\times 10^7$ CFU/g, respectively (Figure 4.21). Figure 4.22 also shows the count of AHB in soil polluted with 15% diesel oil and amended with 10% organic wastes. SC amendment had the highest microbial population (102$\times 10^7$ CFU/g) at the end of 126 days. Unamended control soil recorded 15$\times 10^7$ CFU/g microbial population during the period of study. There was no significant difference between aerobic heterotrophic bacteria count of soil amended with 5% and 10% organic wastes; however, there was a significant difference between the counts of AHB in soil treated with supplements and unamended control soils at $P < 0.05$ confidence level.

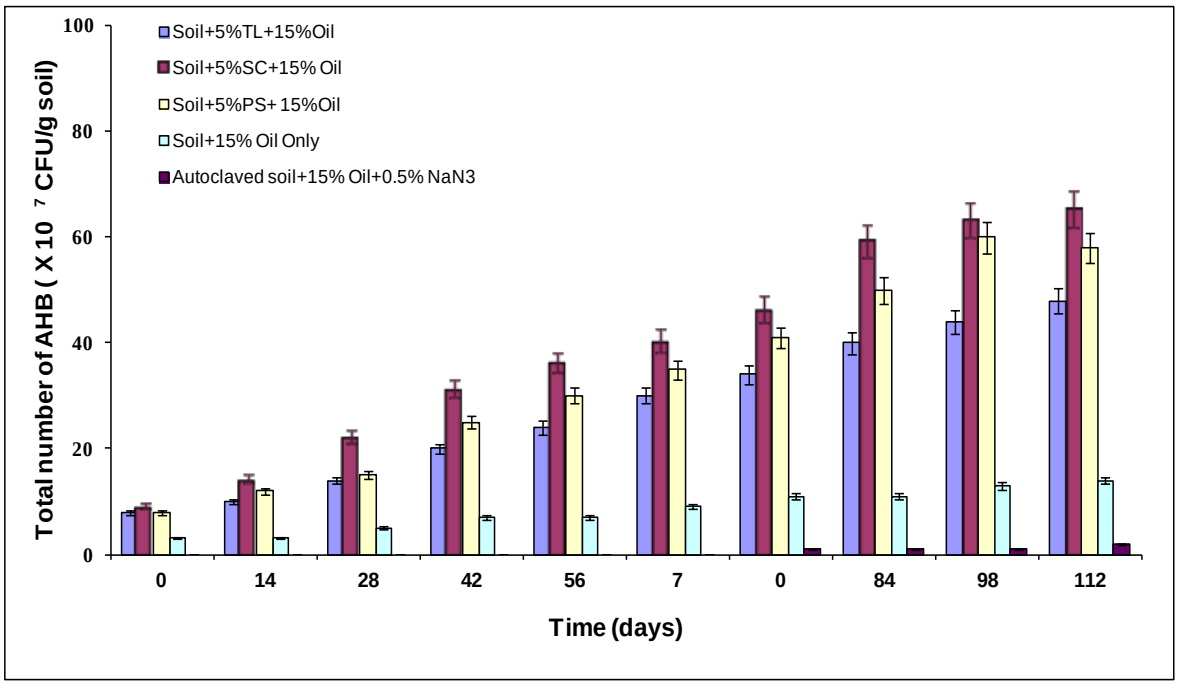


Figure 4.21 Total CFU of aerobic heterotrophic bacterial (AHB) in soil contaminated with 15% diesel fuel amended with 5% organic wastes. Bars indicate standard error (n = 3).

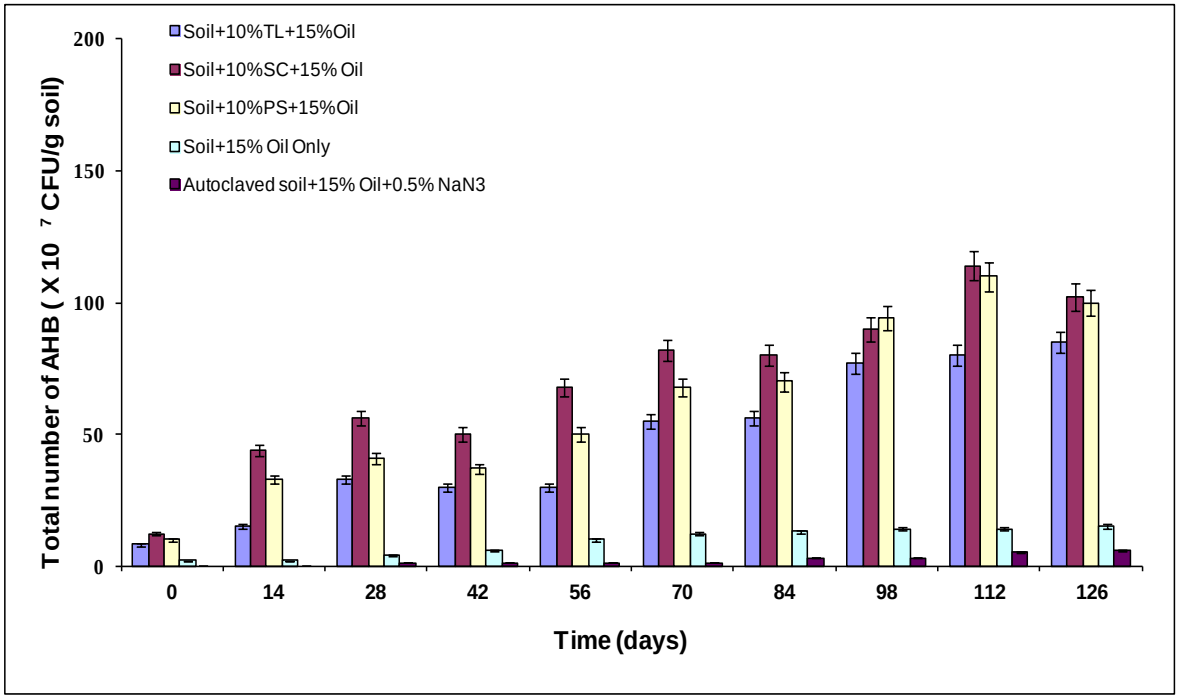


Figure 4.22 Total CFU of aerobic heterotrophic bacterial (AHB) in soil contaminated with 15% diesel fuel amended with 10% organic wastes. Bars indicate standard error (n = 3).

The AHB count in soil treatment with 10% SC was 5.8 times higher than unamended control soil (Figure 4.22). The AHB count in soil amendment with 10% organic waste was twice of the AHB count in soil amended with 5% organic wastes. As mentioned earlier (4.2.6) with the increased amount of organic waste amendments a suitable condition will be provided for microbial growth and increase the microbial population.

The counts of diesel utilizing bacteria in soil treatment with 5 % and 10% organic wastes are shown in Figures 4.23 and 4.24, respectively. The results indicate that the supplementation of organic wastes to 15% diesel fuel contaminated soil enhanced the growth of microorganisms compared with naturally attenuated microcosm. The DUB was highest in soil amended with 10% SC (145 $\times 10^5$ CFU/g) compared with those amended with 10% PS and TL (122 $\times 10^5$ CFU/g and 110 $\times 10^5$ CFU/g, respectively). At the end of the study the number of DUB in soil treated with 10% SC was six-fold more than in unamended soil. There was a significant difference between the population in soils amended with organic wastes and control treatment ($P < 0.05$). However, in the soil treated with sodium azide there was 6 $\times 10^5$ CFU/g microbial population at the end of 126 days. This result is in contrast with et al., (2012a) who reported that there was no microbial growth in poisoned control soil. The reason for this difference might be the differences in microbial ecology of the soil used for these two experiments.

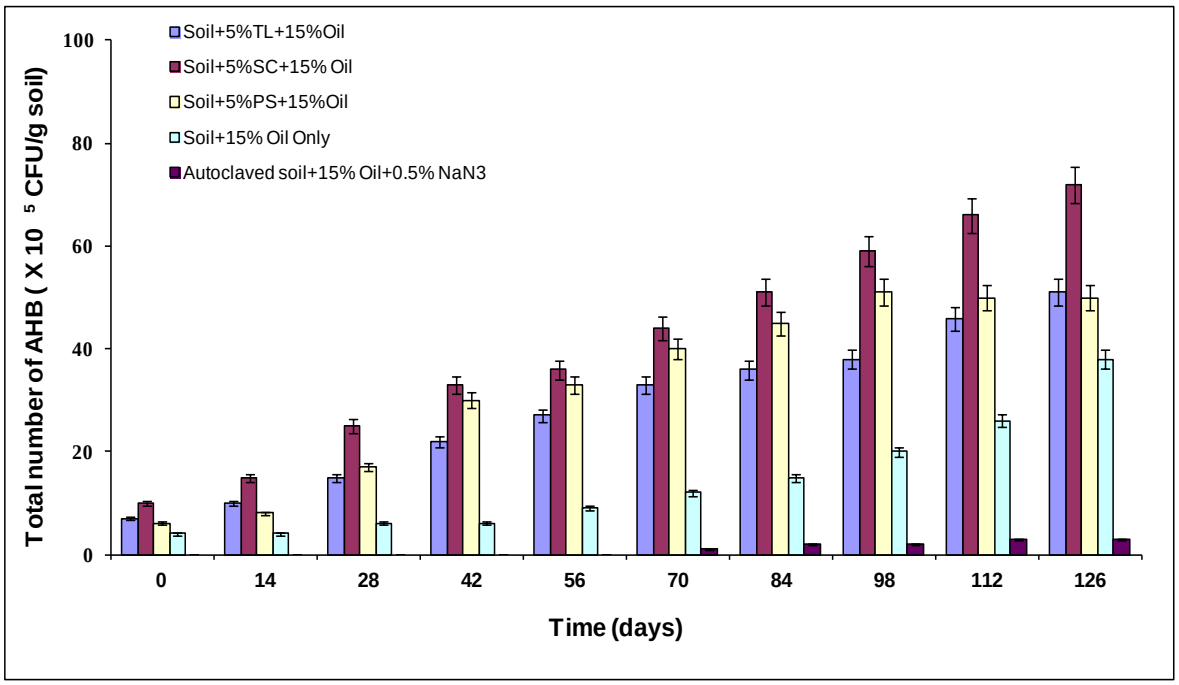


Figure 4.23 Total CFU of diesel utilizing bacterial (DUB) in soil contaminated with 15% diesel fuel amended with 5% organic wastes. Bars indicate standard error (n = 3).

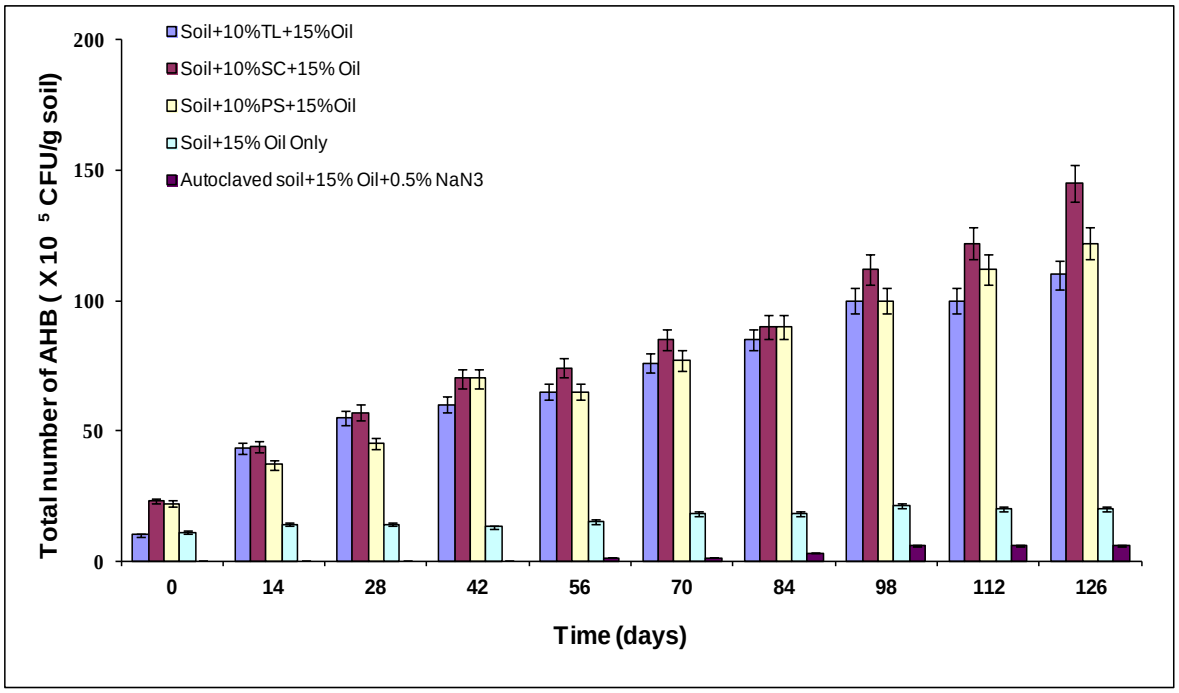


Figure 4.24 Total CFU of diesel utilizing bacterial (DUB) in soil contaminated with 15% diesel fuel amended with 10% organic wastes. Bars indicate standard error (n = 3).

4.2.9 Microbial population of soil polluted with 20% diesel fuel

The counts of AHB in soil contaminated with 20% diesel fuel and amended with 5% SC ranged between 8×10^7 CFU/g and 43×10^7 CFU/g while that of soil amended with 5% PS and TL ranged from 6.0×10^7 CFU/g to 20.0×10^7 CFU/g and 2×10^7 CFU/g to 22×10^7 CFU/g respectively, between 0 and 126 days (Figure 4.25). There was a decrease in the number of colonies on the 72^{nd} day in soil amended with 5% SC, but it continued to grow until the end of 126 days. The count of AHB in soil amended with 5% PS and TL was almost similar during the study. Figure 4.26 also shows the count of AHB in soil contaminated with 20% diesel fuel and amended with 10% organic wastes. The soil amended with 10% SC had the highest range which was from 11×10^7 CFU/g to 65×10^7 CFU/g while the soil treated with PS and TL recorded 11×10^7 CFU/g to 56×10^7 CFU/g and 8×10^7 CFU/g to 38×10^7 CFU/g respectively, in the 126 day period of this study (Figure 4.26). It was noticed that with increased oil concentration in contaminated soil the number of colony forming units decreased. In addition, with the addition of more supplements to the contaminated soil, the number of microbial population increased and that in turn led to an increase in the rate of biodegradation.

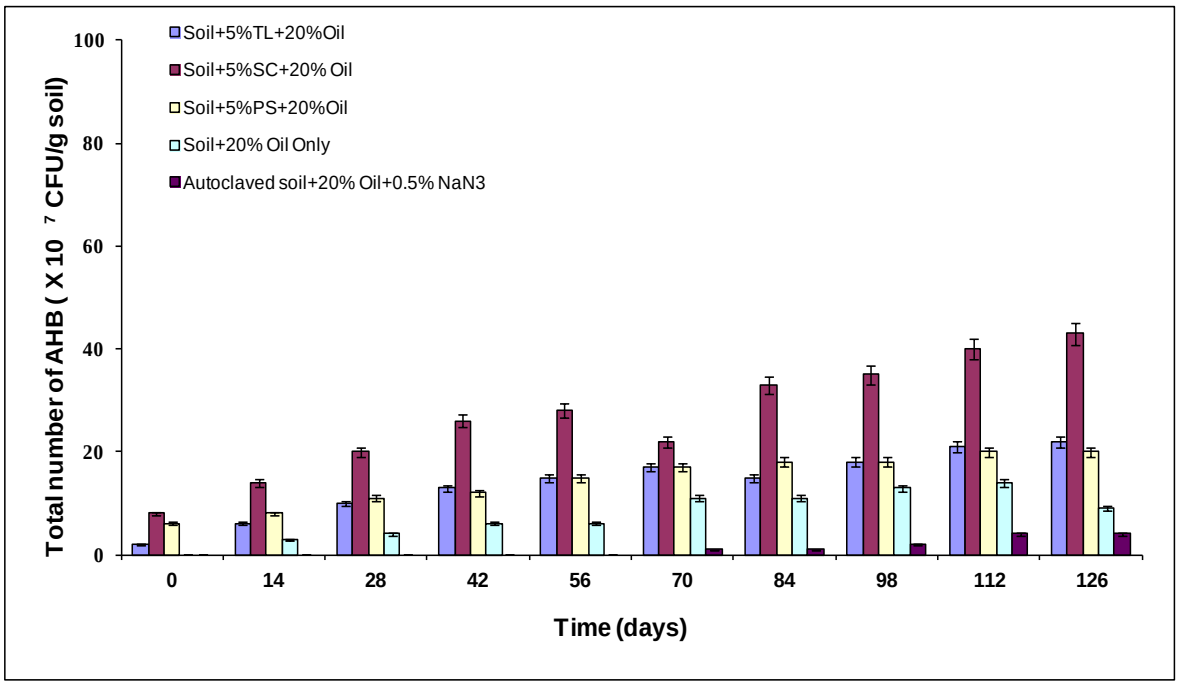


Figure 4.25 Total CFU of aerobic heterotrophic bacterial (AHB) in soil contaminated with 20% diesel fuel amended with 5% organic wastes. Bars indicate standard error (n = 3).

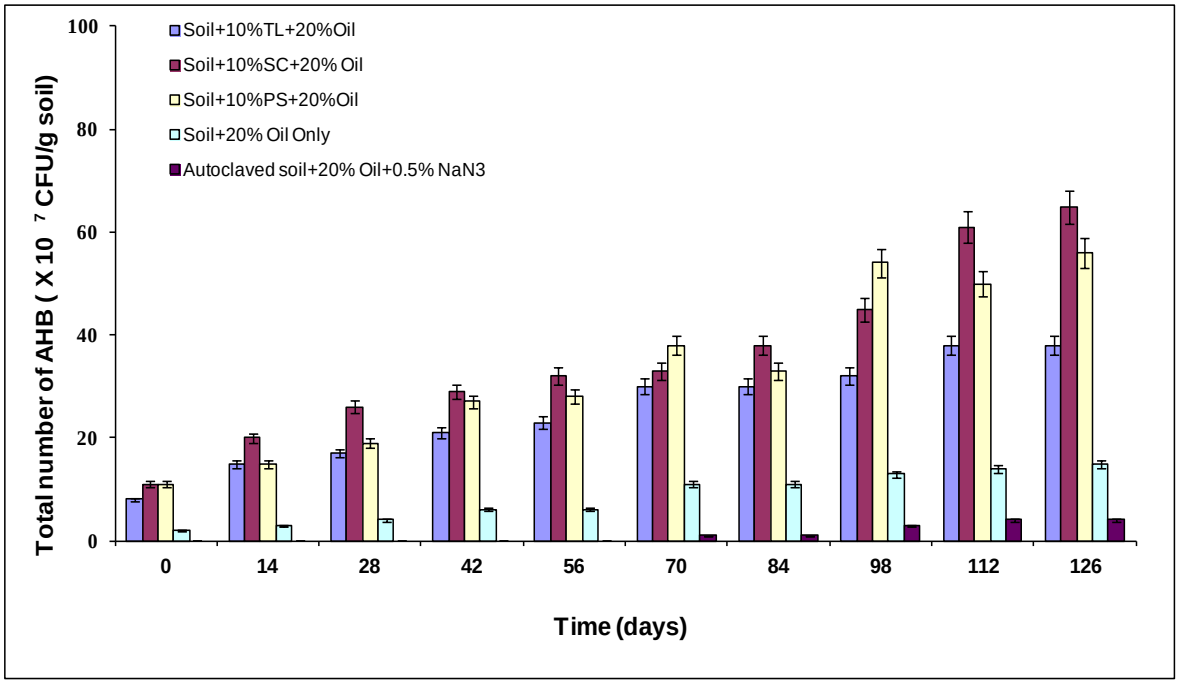


Figure 4.26 Total CFU of aerobic heterotrophic bacterial (AHB) in soil contaminated with 20% diesel fuel amended with 10% organic wastes. Bars indicate standard error (n = 3).

The count of diesel utilizing bacteria in soil contaminated with 20% and amended with 5% SC had a higher range which was from 8 × 10^5 CFU/g to 51 × 10^5 CFU/g compared with soil amended with 5% PS (8 ×10^5 CFU/g to 38 ×10^5 CFU/g) and 5% TL (4 ×10^5 CFU/g to 30.0×10^5 CFU/g) during the 126 days of study (Figure 4.27). Also, the DUB count was the highest in soil amended with 10% SC which ranged between 11 × 10^5 CFU/g and 77 ×10^5 CFU/g while soil amended with 10% PS and TL recorded 13 × 10^5 CFU/g to 60.0 ×10^5 CFU/g and 6 ×10^5 CFU/g to 40.0 × 10^5 CFU/g , respectively (Figure 4.28). The DUB in unamended control soil recorded 16 × 10^5 CFU/g at the end of study. Statistical analysis indicates significant difference between unamended control soil and soil amended with organic wastes at $P < 0.05$ confidence level.

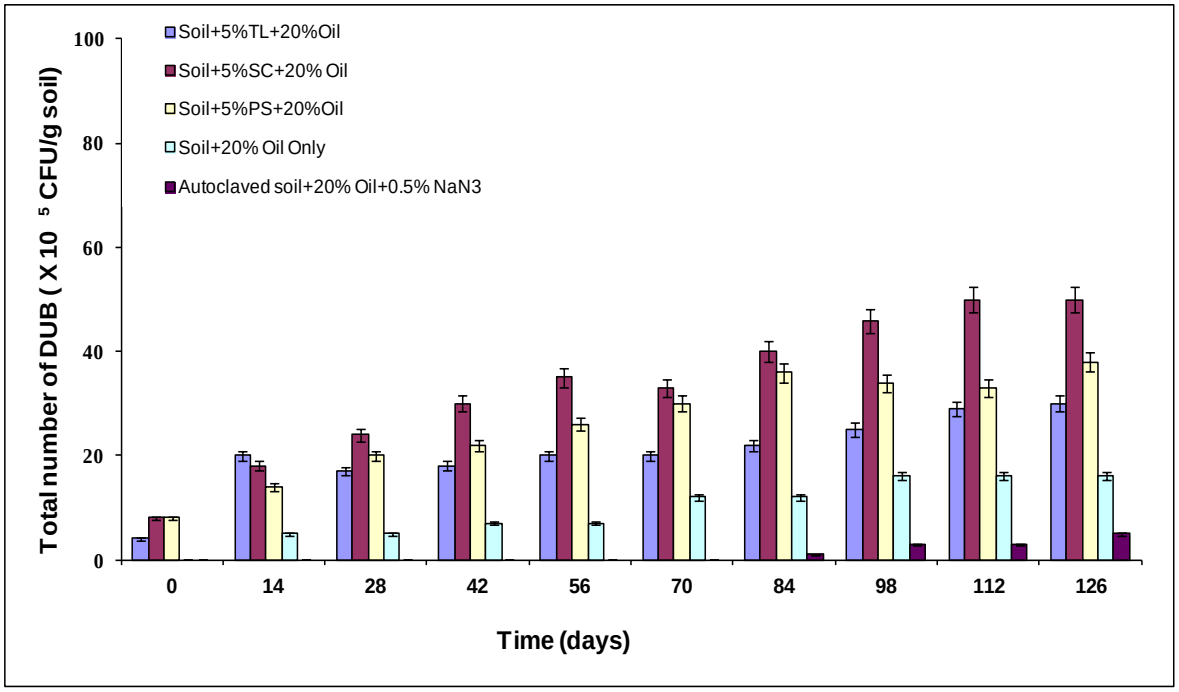


Figure 4.27 Total CFU of diesel utilizing bacterial (DUB) in soil contaminated with 20% diesel fuel amended with 5% organic wastes. Bars indicates standard error (n = 3).

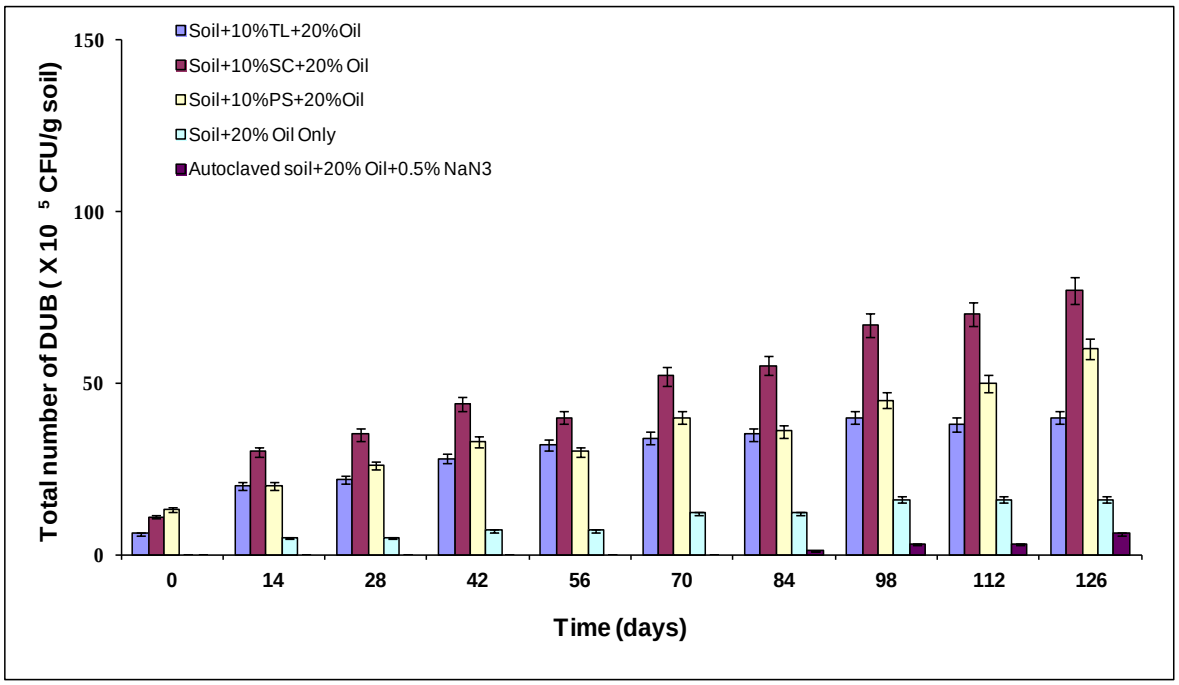


Figure 4.28 Total CFU of diesel utilizing bacterial (DUB) in soil contaminated with 20% diesel fuel amended with 10% organic waste. Bars indicate standard error (n = 3).

The count of DUB in all treatments amended with organic wastes was higher compared with those of unamended control soil and autoclaved poisoned control soil at all the different levels of diesel oil pollution (5%, 10%, 15% and 20%) during the 126 days of this study. These results are similar to those of many researchers who have reported the number of hydrocarbon utilizing bacteria as $\times 10^6$ CFU/g or $\times 10^8$ CFU/g (Antai and Mgbomo, 1993; Ijah and Antai, 2003b). The difference in microbial population in other studies compared with this study might be due to the different type of oil and the microbial ecology of the soil which was used in those studies. However, soil contaminated with limited concentration of diesel fuel (5%) recorded a higher population of DUB and AHB compared with the soil with the higher oil concentration (10%, 15% and 20%). This might be due to the toxicity effects of the higher oil concentration on microbial growth which has negative impacts on microbial enumeration. Rahman et al., (2002) demonstrated that by decreasing

oil concentration from 10% to 1%, the count of microbial population increased. It is noticeable that, those soils polluted with diesel fuel and amendment with organic waste recorded a higher microbial population, particularly in SC treatments. This may be a result of appreciable quantities of nutrients (N and P), especially N which has an important role in biodegradative activities. This reason is supported by Abioye et al., (2009), Adesodun and Mbagwu, (2008) and Ijah and Antai, (2003a).

4.2.10 Bioavailable fraction in total content of analyzed PAHs in diesel fuel contaminated soil

Sites contaminated with petroleum hydrocarbons can be evaluated using *bioassays* (assessments of bioavailability or bioaccessibility by uptake or/ and toxicity to microorganisms). Figure 4.29 indicates the bioavailable fraction in the total content of soil polluted with 5 % diesel fuel. At the 14 days, the bioavailability fractions ranged from 65% to 87% among the various treatments. It was noticeable that in all the treatments, there was a significant decrease in hydroxypropyl[b]cyclodextrin (HPCD) extracted over time. The 10% SC amended treatment and polluted with 5% diesel oil recorded a significant reduction which was from 81% (T 14) to 4.5% (T 126), while this decrease was not significant in those unamended and sterilized soil. The bioavailability of sterilized control soil decreased from 87% to 58% by the 126^{th} day.

This is in total agreement with a previous study that showed the lowest bioavailability of PAHs was in the sewage sludge with the highest organic carbon content (Amir et al., 2005). Amir et al., (2005) indicated that the tendency of change of bioavailability or content of various hydrocarbon compounds during composting is found to be strongly related to the number of their aromatic rings, their structure and molecular weight. In addition, results

illustrated by contaminated soil with 10% diesel fuel there was a significant difference among the different amendments, and bioavailability decreased from 86.6% to 12.2%, 77.1% to 20.3% and 83% to 22.2% in soil treated with 10% SC, TL and PS, respectively (Figure 4.30), while in unamended sterilized soil the differences was not significant. It has been observed that by increasing the soil-pollutant contact time, extractability and bioavailability decreased. Various researchers (Reid et al., 2000; Semple et al., 2001) have reported this phenomenon.

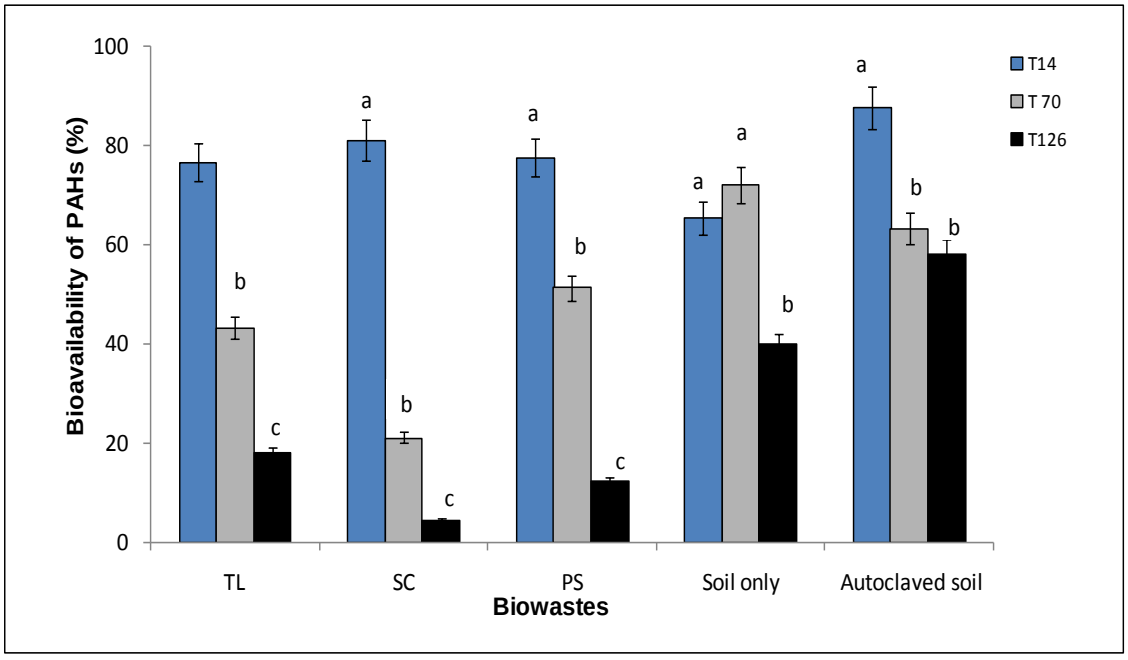


Figure 4.29 Bioavailable fractions in total content of analyzed PAHs in contaminated soil with 5 % diesel fuel and amended with 10% organic wastes. The same letter represents no significant difference ($p > 0.05$).

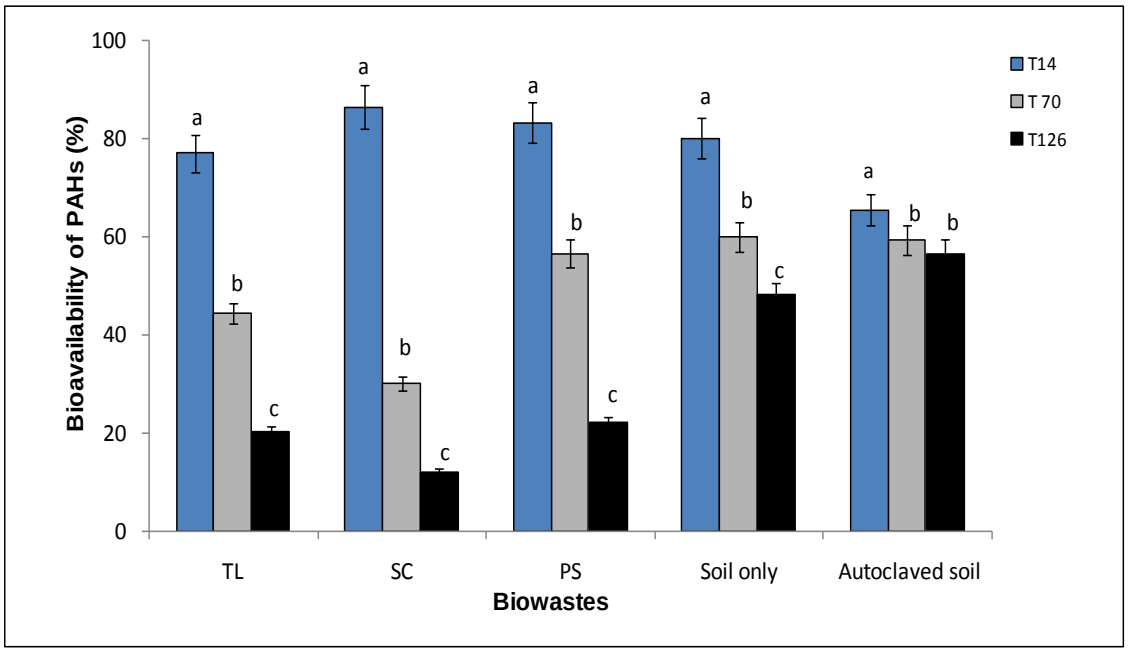


Figure 4.30 Bioavailable fractions in total content of analyzed PAHs in contaminated soil with 10 % diesel fuel and amended with 10% organic wastes. The same letter represents no significant difference ($p > 0.05$).

Guozhong et al., (2011) found that excessive amendment of meat compost that increased the organic carbon content reduced the amount of bioavailable PAH because of sequestration. Some studies showed that soil properties, i.e. organic matter content, matrix nanoporosity and hydrophobicity influenced a decrease/increase of the bioavailable sequestrated fraction (Chung and Alexander, 2002). The result is line with Puglisi et al., (2007) who recorded the percentage of bioavailability of phenanthrene in sterilized control soil which decreased from 87.3 % in 20 days to 47.5% in 240 days, while in non-sterilized soil amended with compost it decreased from 51.1% to 0, at the end of 240 days. This shows that, the bioavailability was significantly reduced in soils amended with compost. In addition, it has been well demonstrated that decomposition of organic hydrocarbons in contaminated soil reduces the bioavailability of organic chemicals and results in a non-degraded residue in the soil (Semple et al., 2003).

A similar trend was observed in the soil polluted with 15% and 20% diesel fuel (Figures 4.31 and 4.32). Statistical analysis carried out did not show any significant difference among treatments in 14 days (T14) at 15% diesel fuel. The percentage of bioavailability had a sharp decrease in soil amended with 10% SC from 80.35 % to 21% in 126 days at 15% diesel fuel. Whereas, in autoclaved control soil it was reduced from 70.2 % to 65% and 89% to 70% at 15 and 20 % diesel fuel, respectively. The results are similar to Semple et al., (2001) who indicated that the mixture of compost-soil reduced pollutant concentration or/and matrix that had less pollutants in a bioavailable form. The impact of organic wastes on the contribution of the potentially bioavailable fraction of the PAH clearly depended on the stage of the experiment and organic waste type (Oleszczuk, 2007). A significant lowering of the potentially bioavailable fraction in soil amended with organic wastes was noted during the biodegradation process of diesel fuel contaminated soil.

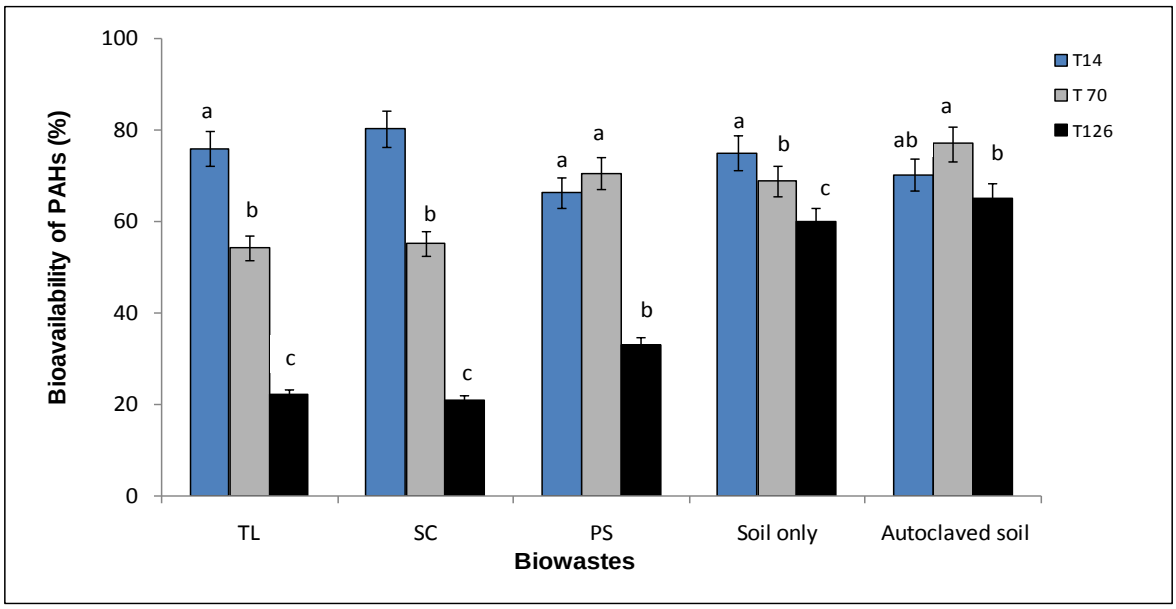


Figure 4.31 Bioavailable fractions in total content of analyzed PAHs in contaminated soil with 15% diesel fuel and amended with 10% organic wastes. The same letter represents no significant difference ($p > 0.05$).

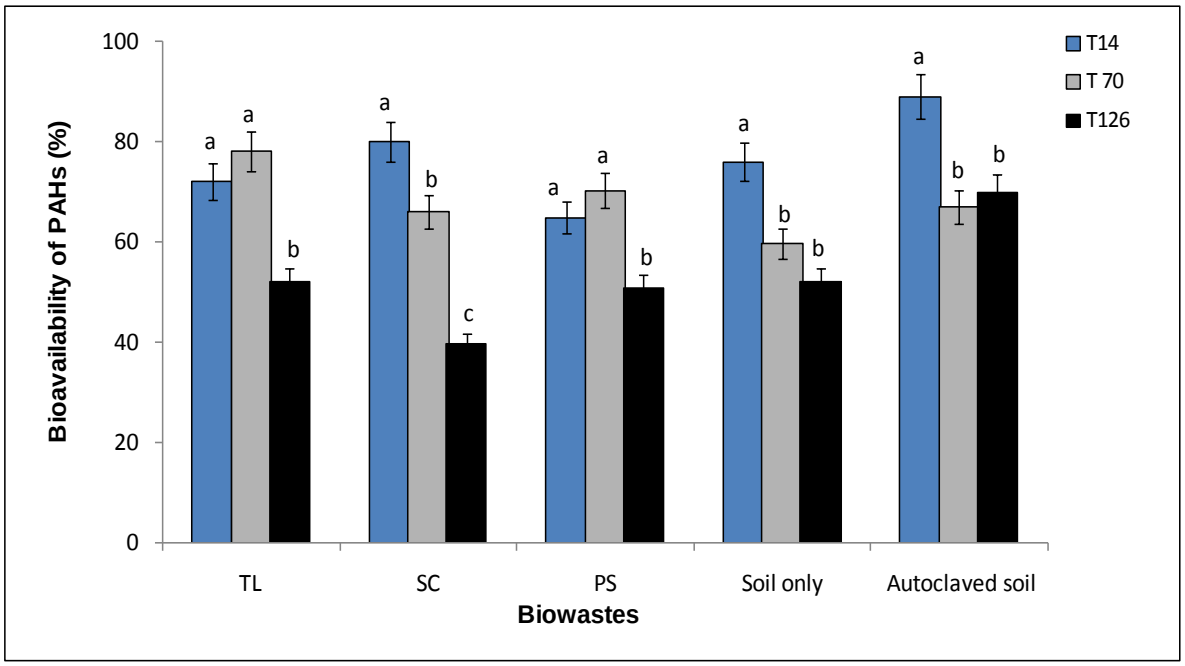


Figure 4.32 Bioavailable fractions in total content of analyzed PAHs in contaminated soil with 20% diesel fuel and amended with 10% organic wastes. The same letter represents no significant difference ($p > 0.05$).

4.2.11 pH of soil contaminated with 5, 10, 15 and 20% diesel fuel

The pH of soil polluted with different concentrations of diesel fuel (5%, 10%, 15% and 20%) and amended with organic wastes (5% and 10%) demonstrated that at the initial stage pH increased slightly, followed by a decrease from 56 day to 84 day period. It increased again to 7.0 throughout the period of the study. Figures 4.33 to 4.40 show the pH assessed during the 126-day period of bioremediation study. Addition of amendment, especially SC and PS, lead to rise in the pH from 6.8 to as high as 8.0 in soil polluted with 15% diesel oil. Soil amended with TL had lower pH which rose from 6.4 to 7.1 in soil treatment with 5% and 10% oil concentration. It might be because the TL naturally has low pH and during the biodegradation of oil in soil amended with TL it always favored slightly acidic or natural pH. This result is in conformity to Ijah et al., (2008) who reported amending hydrocarbon contaminated soil with chicken drop, lead to increased pH of the soil from 6.7 to 7.7.

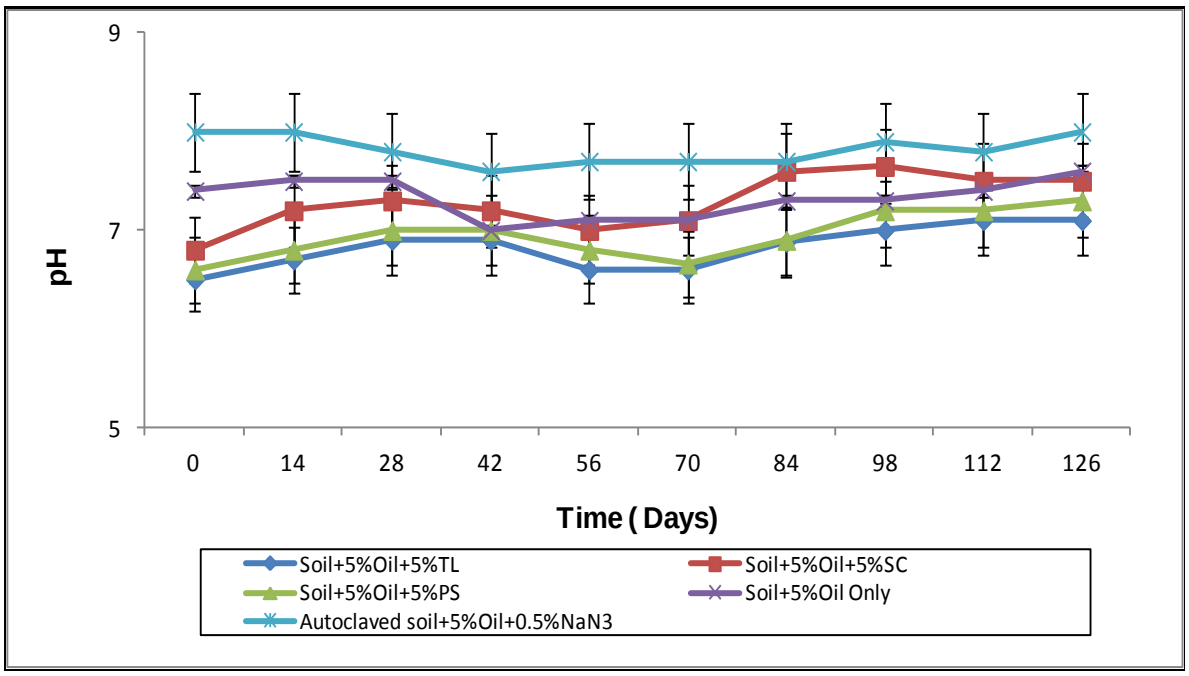


Figure 4.33 pH of soil polluted with 5% diesel fuel and amended with 5% organic wastes. Bars indicate standard error (n = 3).

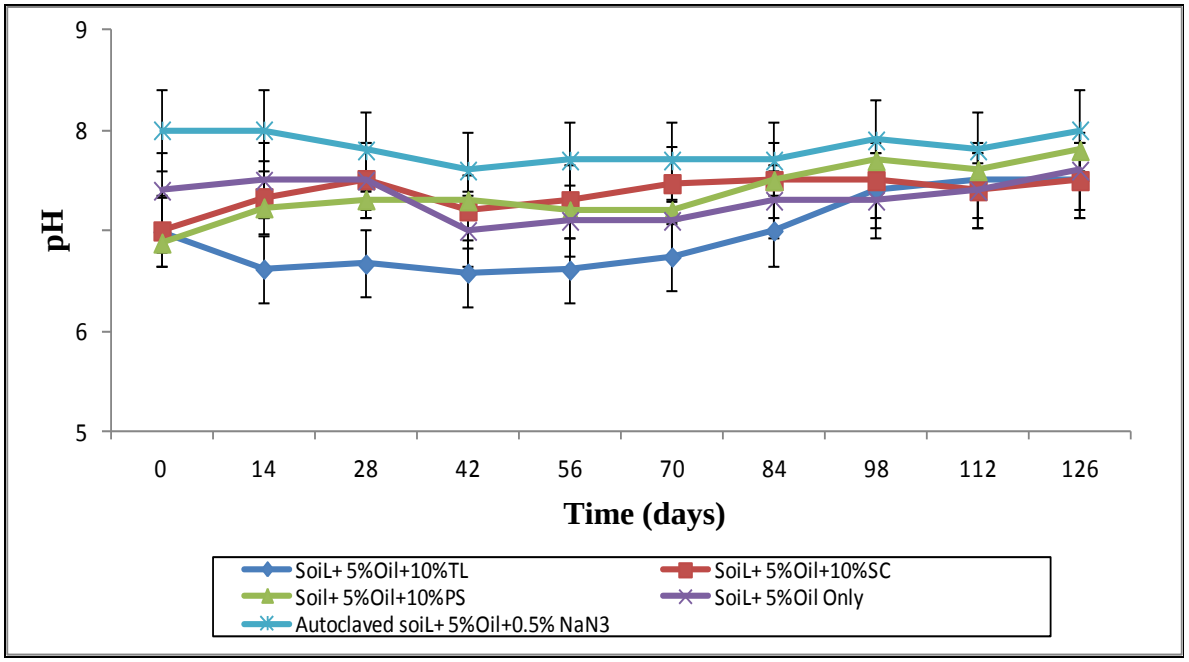


Figure 4.34 pH of soil polluted with 5% diesel fuel and amended with 10% organic wastes. Bars indicate standard error (n = 3).

Initially, the soil pH increased in all the treatments after the soil was polluted with oil and amended with organic wastes. It later decreased from 42 days until 70 days in all the amendments. The reason for the drops in pH between 42 and 70 days might be due to microbial activities during the biodegradation of oil which lead to the accumulation of secondary metabolites that are slightly acidic in nature. The pH of all treatments increased within the last six weeks of study.

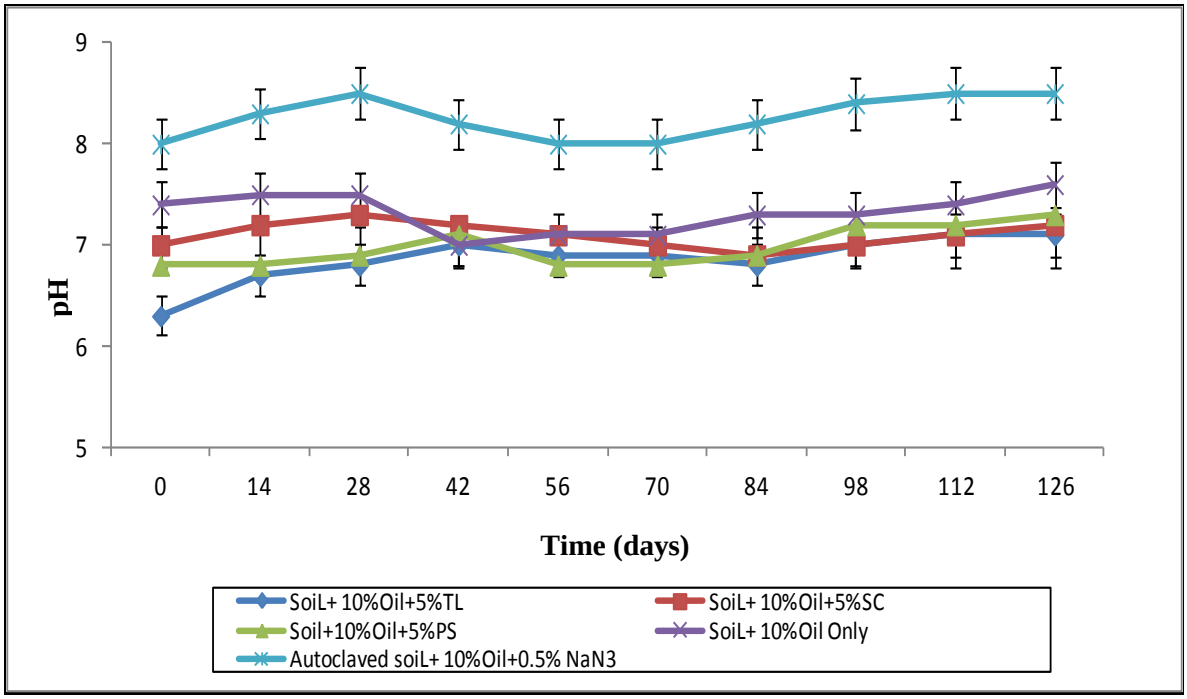


Figure 4.35 pH of soil polluted with 10% diesel fuel and amended with 5% organic wastes. Bars indicates standard error (n = 3).

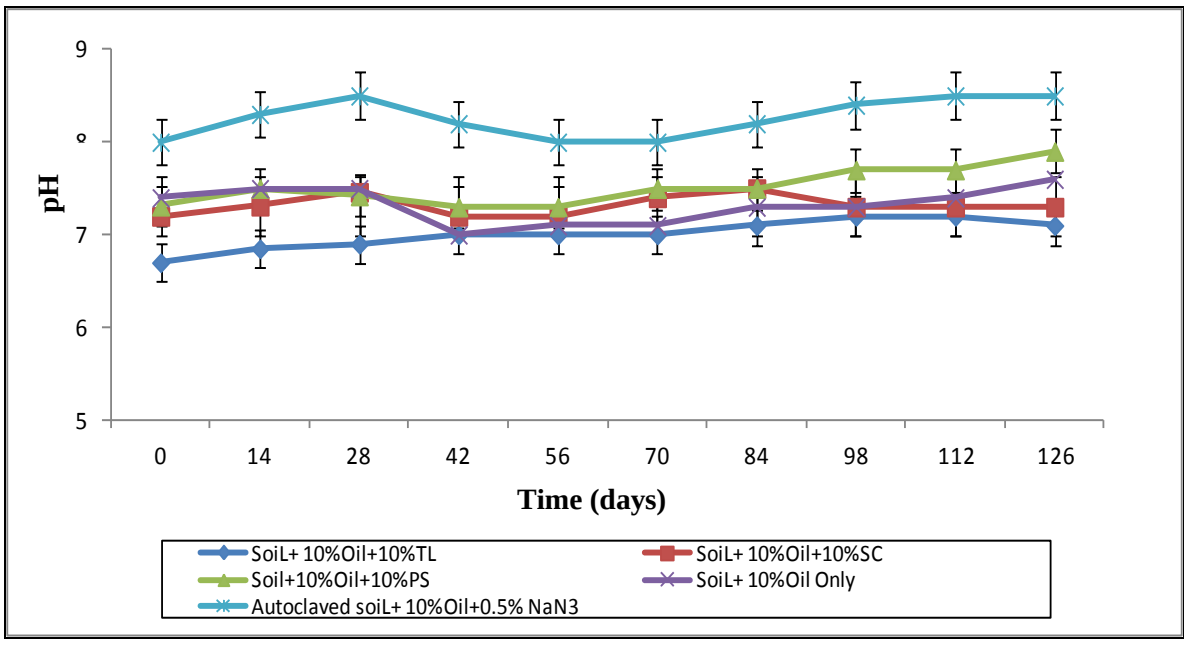


Figure 4.36 pH of soil polluted with 10% diesel fuel and amended with 10% organic wastes. Bars indicate standard error (n = 3).

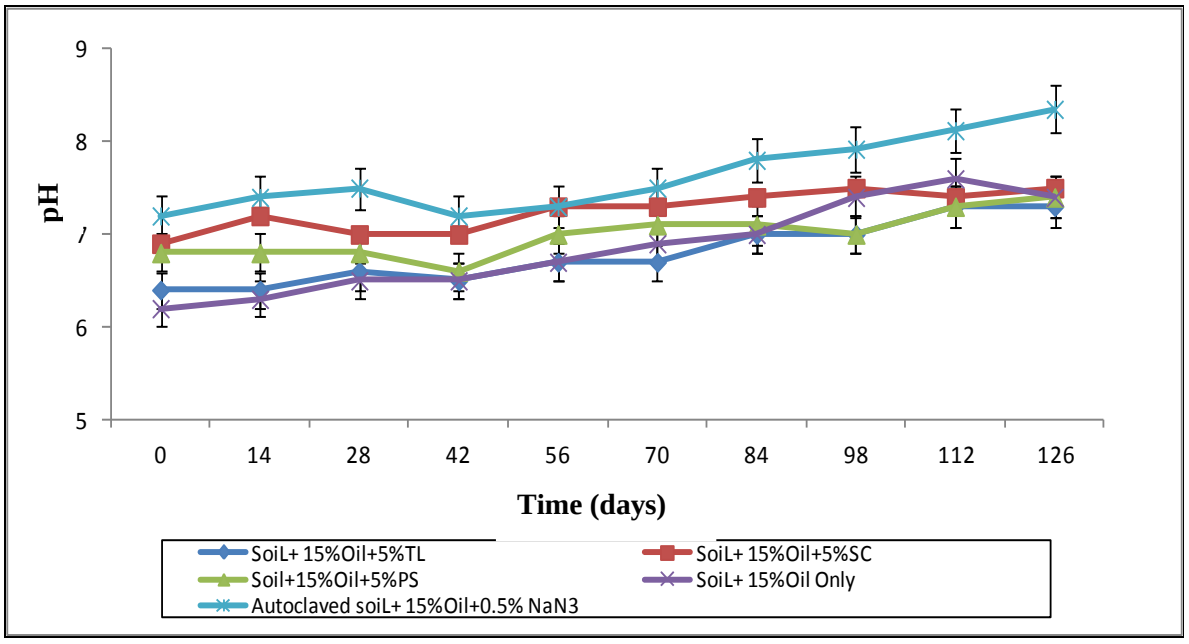


Figure 4.37 pH of soil polluted with 15% diesel fuel and amended with 5% organic wastes. Bars indicate standard error (n = 3).

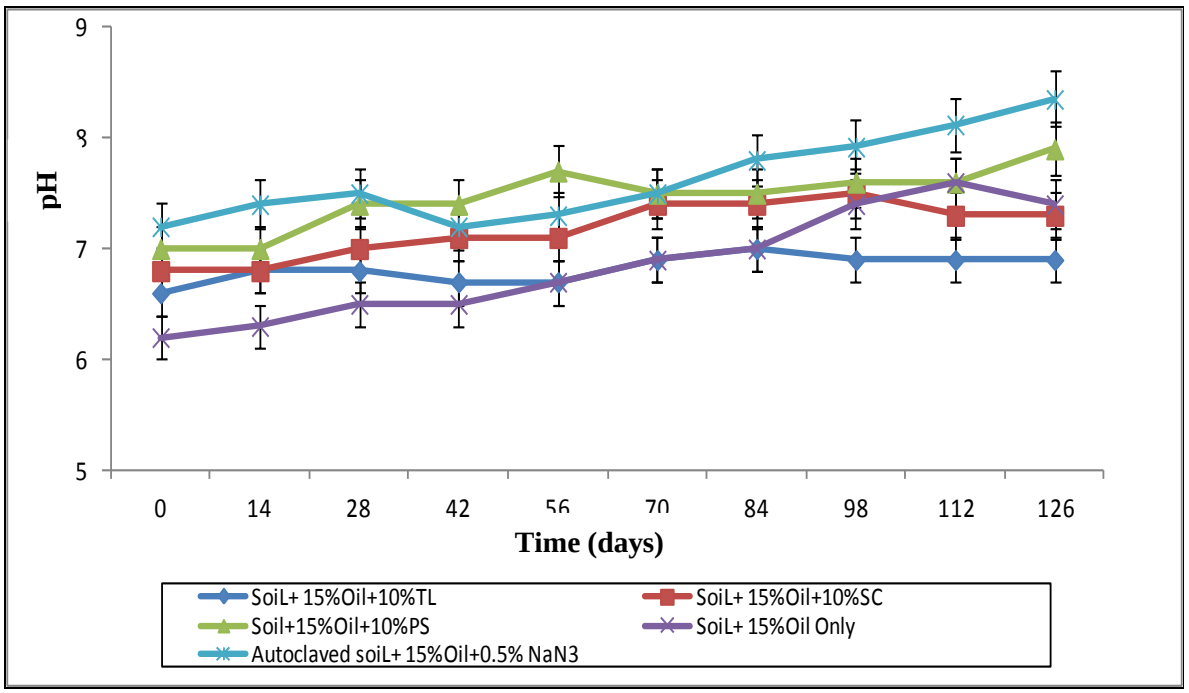


Figure 4.38 pH of soil polluted with 15% diesel fuel and amended with 10% organic wastes. Bars indicate standard error (n = 3).

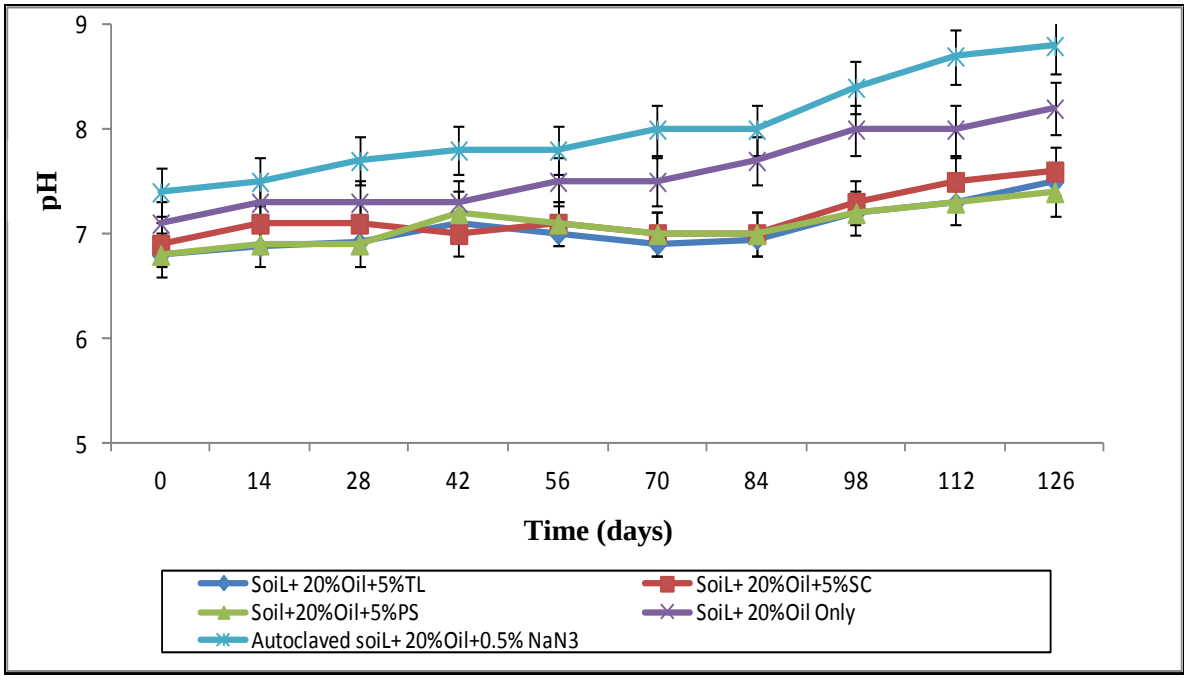


Figure 4.39 pH of soil polluted with 20% diesel fuel and amended with 5% organic wastes. Bars indicate standard error (n = 3).

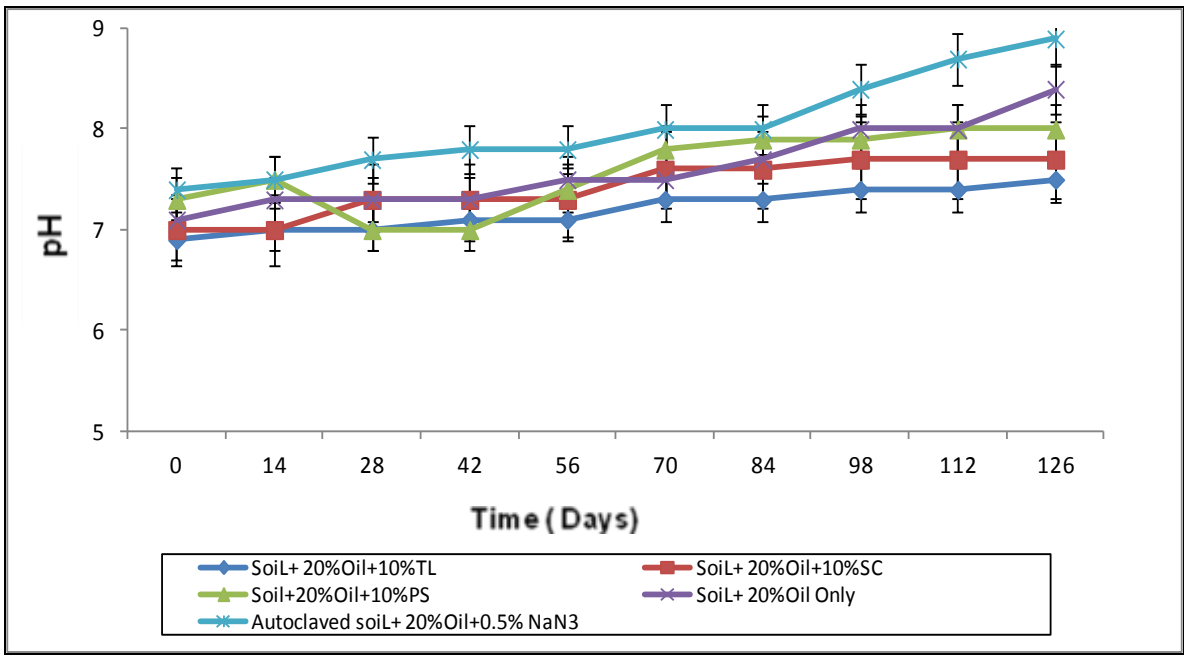


Figure 4.40 pH of soil polluted with 20% diesel fuel and amended with 10% organic wastes. Bars indicate standard error (n = 3).

4.2.12 Seed germination toxicity test

Seed growth and germination test suggested by Millioli et al. (2009) was employed using Lettuce seed (*Lactuca sativa*) is an optimal plant choice and is sensitive to toxic compounds, especially petroleum products (Oleszczuk, 2008). Table 4.16 shows the result of germination toxicity with lettuce after the bioremediation was completed. It is noticeable that soil amended with 10% SC and contaminated with 5% diesel oil had 100% germination over the period of 126 days. While 90%, 60% and 40% germination was recorded in soil amended with 10% SC and polluted with 10%, 15% and 20% diesel oil, respectively. Only 20% and 10% germination was recorded in poisoned control soil contaminated with 5 % and 10% diesel oil, respectively. The results are similar to findings of Saadoun and Al-Ghazawi (2010) who reported a 30% decline in *Cochorus olitorius* seed

germination in soil contamination with diesel fuel at 100 mg/Kg or higher. Statistical analysis revealed a positive correlation between germination toxicity test and percentage of oil loss in contaminated soil. However, it shows that by increasing the quantity of organic waste amendments, a shorter period is needed to remediate petroleum hydrocarbon from soil contamination. Banks and Schultz (2005) illustrated increasing the rate of seed germination by reducing quantity of oil in the soil.

Table 4.16 Seed germination toxicity test (%)

Percentage of oil pollution	Treatments					
	A	B	C	D	E	F
5% organic waste amendments						
5	60±0	90±10	70±6	40±0	20± 0	100
10	40±10	70±6	50±6	40±0	10±0	100
15	30±0	50±0	30±0	20±6	0	100
20	10±6	30±6	20±0	10±0	0	100
10% organic waste amendments						
5	80±0	100	90±6	40±0	20±0	100
10	60±0	90±10	80±0	40±6	10±0	100
15	40±10	60±0	40±0	20±0	0	100
20	20±0	40±0	30±0	10±0	0	100

A= Soil+Oil+TL, B= Soil+Oil+SC, C= Soil+ Oil+PS, D= Soil+Oil,
E= Autoclaved soil+ Oil+NaN_3, F= Unamended soil

Seed germination toxicity index (GI) was used to evaluate the phytotoxicity and rate of detoxification of soil at the end of the bioremediation process. Figure 4.41 shows the GI in soil polluted with 20%, 15%, 10% and 5% diesel fuel and amended with 5% and 10% organic wastes. Soil treated with 10% organic wastes showed that the index was higher than that amended with 5% organic wastes. Results indicated that with the increased rate of oil pollution, the rate of the GI decreased. The negative impact of hydrocarbon on the rate of the GI may be due to toxic effects of hydrocarbons on plants which were inherent to plant growth. This result is similar to Aparna et al., (2010) who reported a decrease in phytotoxicity of sediment as indicated by the increase in the value of the GI. The highest GI was recorded in soil amended with 10% SC (93.34%, 63.33%, 23.23% and 10% at 5, 10, 15 and 20% diesel oil, respectively). This has proven the effectiveness of SC in enhancing biodegradation of diesel contaminated soil. These results match with Mandal et al., (2012) who indicated that the GI increased considerably after remediation of petroleum hydrocarbon contaminated soil. In addition, Oleszczuk (2008) reported that the germination index of *Lepidium sativum* increased after the addition of compost to wastewater sludge during 11 weeks. However, the lowest value of GI was recorded for unamended control treatment soil and soil contaminated with 20% diesel fuel. There was a significant difference in the germination index between control soil and amended soil with organic wastes at $P < 0.05$. Hydrocarbon compounds may affect the root activities in absorption of nutrients or reducing water and gas exchange (Abioye et al., 2012b). It may also enter the seeds and change the metabolic reaction and damage cell membranes and reduce the respiration, transport rate (Abioye et al., 2012b; Adam and Duncan, 2002). This physical barrier was shown to delay seed emergence which could be an additional factor in the overall inhibitory effect of diesel fuel contamination on growth of plants and germination (Shafiq and Iqbal, 2012).

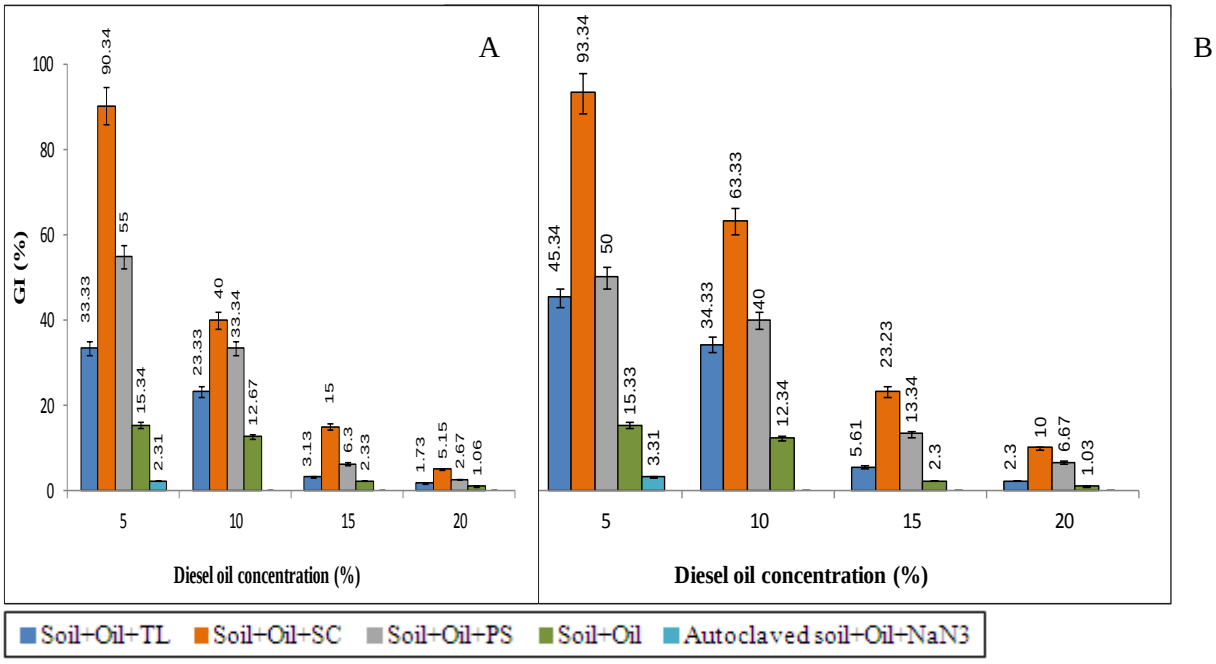


Figure 4.41 Seed germination toxicity index (%) A) amended soil with 5% organic wastes B) amended soil with 10% organic wastes

4.2.13 Monitoring bioremediation using CO_2 produced

The metabolic activity of microorganisms (respiration) in soil amended with 5% and 10% organic wastes and treated with 5%, 10%, 15% and 20% (w/w) diesel fuel is shown in Tables 4.17 to 4.20. In all the treatments, the accumulation of carbon dioxide (CO_2) increased gradually, to the last date of sampling. The cumulative total of CO_2 produced by treatments showed significantly higher than for uncontaminated control soil at $P < 0.05$ confidence level. In 20% oil pollution, the highest CO_2 evolution was by soil amendment with 10% SC was (55.13 mg) and the lowest amount of CO_2 released in unamended control was (37.7 mg) at the end of 42 days, while soil amended with 10% PS and TL librated 52.4 mg and 46.2 mg, respectively (Table 4.17). Obire and Nwaubeta (2001) also reported a progressive increase in the amount of carbon dioxide produced for the first four weeks in the hydrocarbon contaminated soils.

Table 4.17 CO_2 produced (mg/100g) in soil polluted with 20% diesel fuel.

Period (days)	Treatments				
	A	B	C	D	E
10% organic waste amendments					
7	27.12±2.2	35.77±2.4	27.87±2.7	13.65±1.5	0
14	34.56±3.6	42.32±3.7	38.15±2.4	19.16±2.1	0
21	28.22±2.5	46.28±3.5	43.56±4.1	23.17±2.2	1.10±0.4
28	35.05±4.3	50.35±2.1	46.22±3.5	28.54±1.8	2.20±0.3
35	39.10±3.3	52.17±4.2	50.34±3.5	31.24±3.1	3.40±0.5
42	46.22±3.1	55.13±5.4	52.40±3.4	37.70±2.1	4.70±0.8
5% organic waste amendments					
7	11.2±1.8	29.1±2.2	20±0.5	13.65±1.5	0
14	17.8±1.1	30±2.4	22.5±1.7	19.16±2.1	0
21	21.7±2.2	32.2±3.1	27.4±1.6	23.17±2.2	1.1±0.4
28	29.2±1. 5	38.1±2.5	30.4±3.3	28.54±1.8	2.2±0.3
35	32.6±3.8	44.0±5.2	33.3±4.4	31.24±3.1	3.4±0.5
42	36.6±3.3	48.6±2.8	38.9±4.1	37.7±2.1	4.7±0.8

A= Soil+20% Oil+ TL, B= Soil+20% Oil+ SC, C= Soil+20% Oil+ PS,
D= Soil+20% Oil Only, E= Autoclaved soil+20% Oil+ 0.5% NaN_3

In 15% oil pollution the rate of CO_2 production in unamended control soil was 26.7 mg at the end of 42 days which was at a lower rate compared with those amended with organic wastes. CO_2 produced were 44.2 mg, 50.2 mg and 43.8 mg in soil amended with 10% TL, SC and PS, respectively (Table 4.18). After 42 days, lower CO_2 evolution was recorded in soil amended with 5% TL, SC and PS with 41.1 mg, 49.6 mg and 35 mg, respectively. The

differences in all the amounts of CO_2 released were not significant at $P < 0.05$ confidence level. This result is similar to the findings of Oh et al., (2000) who indicated that soil amended with nutrients showed rapid increase in the amount of CO_2 production during the 177 days of incubation compared with a no-nutrient added sample. In soil amended with 5% organic waste, the rate of CO_2 increased gradually, but there was a decrease on the 28th day. The reseaon might be due to a rapid depletion of available nutrients resulting from consumption of a large organic carbon source (Molina-Barahona et al., 2004).

Table 4.18 CO_2 produced (mg/100g) in soil polluted with 15% diesel fuel

Period (days)	Treatments				
	A	B	C	D	E
10% organic waste amendments					
7	13.1±1.3	36.12±5.4	26.7±4.2	7.6±1.1	0
14	24.7±2.2	40.2±2.3	28.5±5.6	13.6±0.7	0
21	26.3±4.4	42.1±6.2	33.6±4.6	18.7±3.1	0
28	32.5±2.5	44.8±5.8	36.05±6.5	25±2.6	2.8±0.3
35	34.8±3.2	47.2±5.4	39.3±6.8	30.2±4.5	3.4±0.8
42	44.2±3.5	50.2±5.6	43.8±6.3	35.5±6.2	3.9±0.2
5% organic waste amendments					
7	11.7±2.1	23.7±2.2	15.6±1.6	7.6±1.1	0
14	25.2±4.6	35.6±4.1	17.6±2.2	13.6±0.7	0
21	36.7±5.5	42.7±4.3	25.2±1.3	18.7±3.1	0
28	28.2±3.2	40.6±3.6	36.7±4.4	25±2.67	2.8±0.3
35	35.7±3.3	47.9±3.1	33.1±5.7	30.2±4.5	3.4±0.8
42	41.1±4.6	49.6±5.2	35±4.5	35.5±6.2	3.9±0.2

A= Soil+15% Oil+ TL, B= Soil+15% Oil+ SC, C= Soil+15% Oil+ PS,
D= Soil+15% Oil Only, E= Autoclaved soil+15% Oil+ 0.5% NaN_3

The amount of CO_2 increased rapidly to the end day of sampling (Table 4.19). About 32.5 mg CO_2 was produced in uncontaminated soil while in soil treated with 10% SC, PS and TL, it was 63.25 mg, 52.7 mg and 55.2 mg, respectively (Table 4.19), while 45.15 mg CO_2 was recorded in soil amended with 5% SC at the end of 42 days. Statistical analysis showed no significant differences in CO_2 released among all the treatments, at $P < 0.05$ confidence level.

Table 4.19 CO_2 produced (mg/100g) in soil polluted with 10% diesel fuel

Period (days)	Treatments				
	A	B	C	D	E
10% organic waste amendments					
7	18.91±1.2	12.50±0.7	15±1.2	5.70±0.4	0
14	22.72±1.9	22.80±1.7	22.50±1.4	15.30±0.5	0
21	36.72±4.1	39.10±2.4	35.60±3.3	21.40±2.3	0
28	42.15 ±1.4	48.80±3.8	46.90± 3.4	28.20±0.9	1.80±0.6
35	50.80±3.2	57.25±2.1	50.30±2.4	30.10±1.2	2 ±0.9
42	55.23±3.5	63.25±5.4	52.70±3.45	32.50±2.6	3.10±1.4
5% organic waste amendments					
7	11.3±1.3	10.2±3.3	9.8±1.1	5.7±0.4	0
14	20.2±4.1	16.8±1.9	5.6±2.3	15.3±0.5	0
21	25.5±1.4	30.1±3.4	25.6±2.4	21.4±2.3	0
28	30.1±2.3	42.3±3.5	37.2±3.6	28.2±0.9	1.8±0.6
35	38.9±4.3	38.5±3.2	41.1±2.9	30.1±1.2	2.2±0.9
42	40.7±3.8	45.1±3.6	43±2.6	32.5±2.6	3.1±1.4

A= Soil+10% Oil+ TL, B= Soil+10% Oil+ SC, C= Soil+10% Oil+ PS,
D= Soil+10% Oil Only, E= Autoclaved soil+10% Oil+ 0.5% NaN_3

The cumulative amount of CO_2 released shows a more distinct effect of organic amendments on TPH degradation. The respiration rate in soil polluted with 5% diesel fuel increased significantly (Table 4.20). The dramatic increase in CO_2 evolution at the early stage was probably due to the rapid degradation of TPH during the same period. In those treatments (TL, SC and PS), where hydrocarbons were added to soil, except for the sterilized contaminated soil (SCS), it was possible to observe significantly higher values of CO_2 release. Since there was total lack of microbial activity in the sterilized soil, the SCS treatment showed the lowest value of respiration. Although total amount of CO_2 evolved for TL was the lowest among the organic amendments experiments, it was 55% and 82% higher than that of soil-only experiment in 5% and 10% TL, respectively.

Table 4.20 CO_2 produced (mg/100g) in soil polluted with 5% diesel fuel

Period (days)	Treatments				
	A	B	C	D	E
10% organic waste amendments					
7	26.2±2.2	16.3±1.4	14.7±2.5	12.3±0.8	0
14	34.1±3.7	32.5±5.3	25.5±2.6	16.6±1.7	0
21	40.4±5.1	45.1±2.5	37.2±3.5	25.7±4.7	2.4±0.4
28	47.5±3.4	51.4±3.3	46.1±4.8	28.4±3.6	3.5±0.2
35	54.8±2.7	57.4±4.2	55.7±6.7	30.1±2.7	4.4±1.5
42	60.3±5.6	65.2±4.5	61.1±5.1	33.13±4.5	5±1.2
5% organic waste amendments					
7	15.2±2.4	22.1±5.1	13.3±1.2	12.3±0.8	0
14	26.8±3.3	28.1±2.2	18.5±2.8	16.67±1.7	0
21	31.2±5.2	38.4±3.7	33.4±5.6	25.7±4.7	2.4±0.4
28	46.2±4.5	49.3±6.5	44.4±6.4	28.4±3.6	3.5±0.2
35	42.6±4.7	45.6±6.5	40.3±3.8	30.1±2.7	4.4±1.5
42	51.3±6.5	58.2±3.5	55.8± 5.4	33.1±4.5	5±1.2

A= Soil+5% Oil+ TL, B= Soil+5% Oil+ SC, C= Soil+5% Oil+ PS,
D= Soil+5% Oil Only, E= Autoclaved soil+5% Oil+ 0.5% NaN_3

The correlation between soil polluted with 20%, 15%, 10% and 5% diesel fuel and CO_2 evolution is shown in Figures 4.42 to 4.45. The results illustrated a positive linear correlation (R^2= 0.8 and above) between soil amended with 5% and 10% organic wastes compared with unamended control soil which showed little correlation. It also indicated

that all treatments amended with 10% organic wastes showed a higher correlation than that amended with 5% organic wastes. High correlation was recorded in soil contaminated with 5% diesel fuel (R^2 = 0.93 – 0.99). Among the treatments amended with organic wastes, the lowest correlation was observed in soil polluted with 15% oil and amended with 5% TL. These results agreed with those of Mancera-Lo´pez et al., (2008) who reported that in bioaugmented systems with *Rhizopus sp.* and *A. sydowii,* a positive correlation of respirometric activity (CO_2 production) with hydrocarbon removal was recorded. However, the positive linear correlation between TPH removal and respirometric activity recorded in all treatments amended with organic wastes. The high correlation is because of the increased mineralized of microorganisms in all the treatments which was due to the increasing in microbial activity that is able to breakdown of the hydrocarbon compounds and thereby releasing CO_2 during this process. Similar findings were observed by Ijah and Anti (2003a), which showed that during bioremediation of crude oil in contaminated soil amended with chicken drop pigs, there was a positive correlation (R^2 = 0.9) between CO_2 evolution and rate of biodegradation in all treatments compared with control soil.

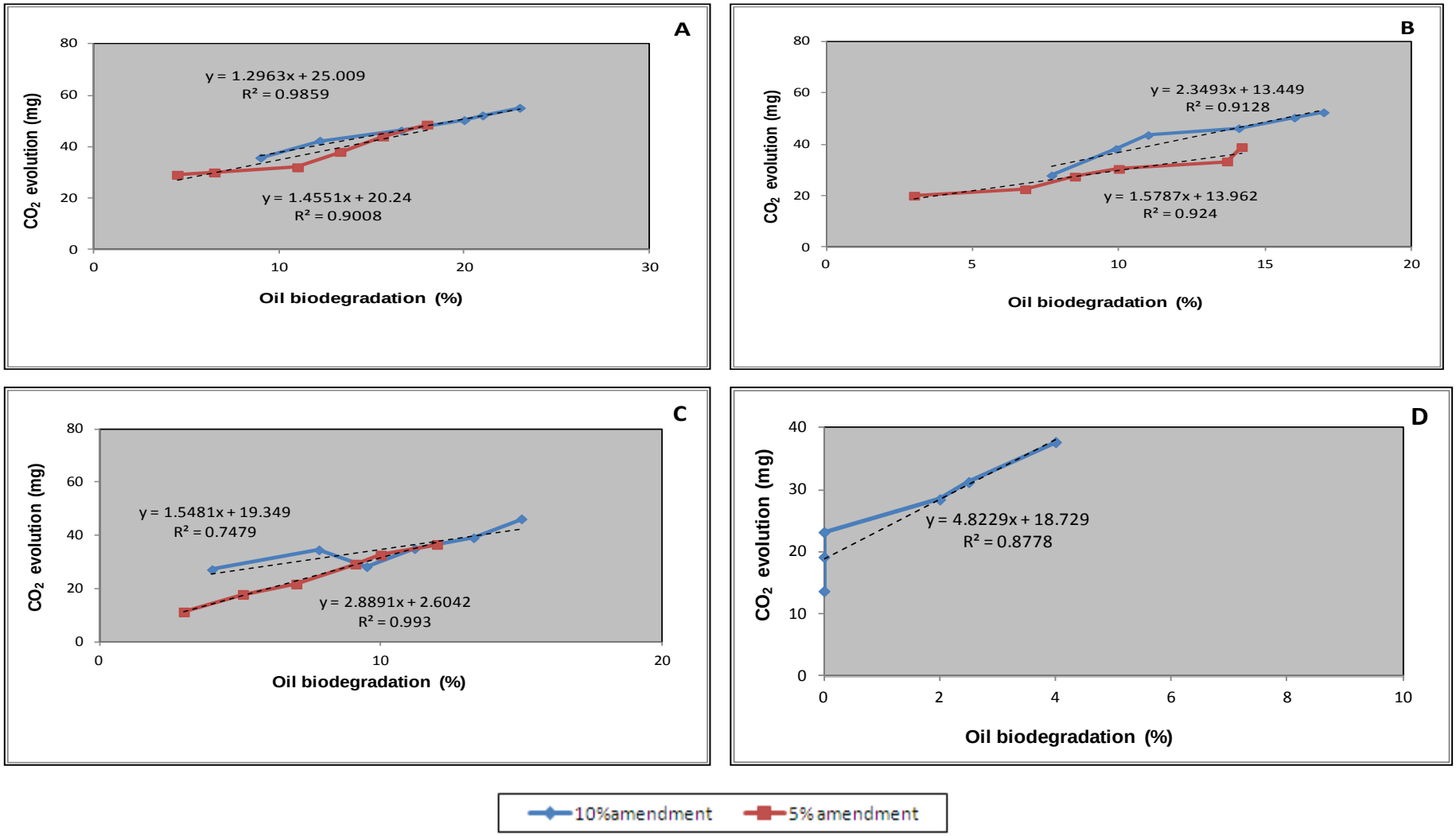


Figure 4.42 Correlation between oil degradation in 20% pollution and CO_2 evolution, A) Soil amended with SC, B) Soil amended with PS, C) Soil amended with TL, D) Unamended control soil

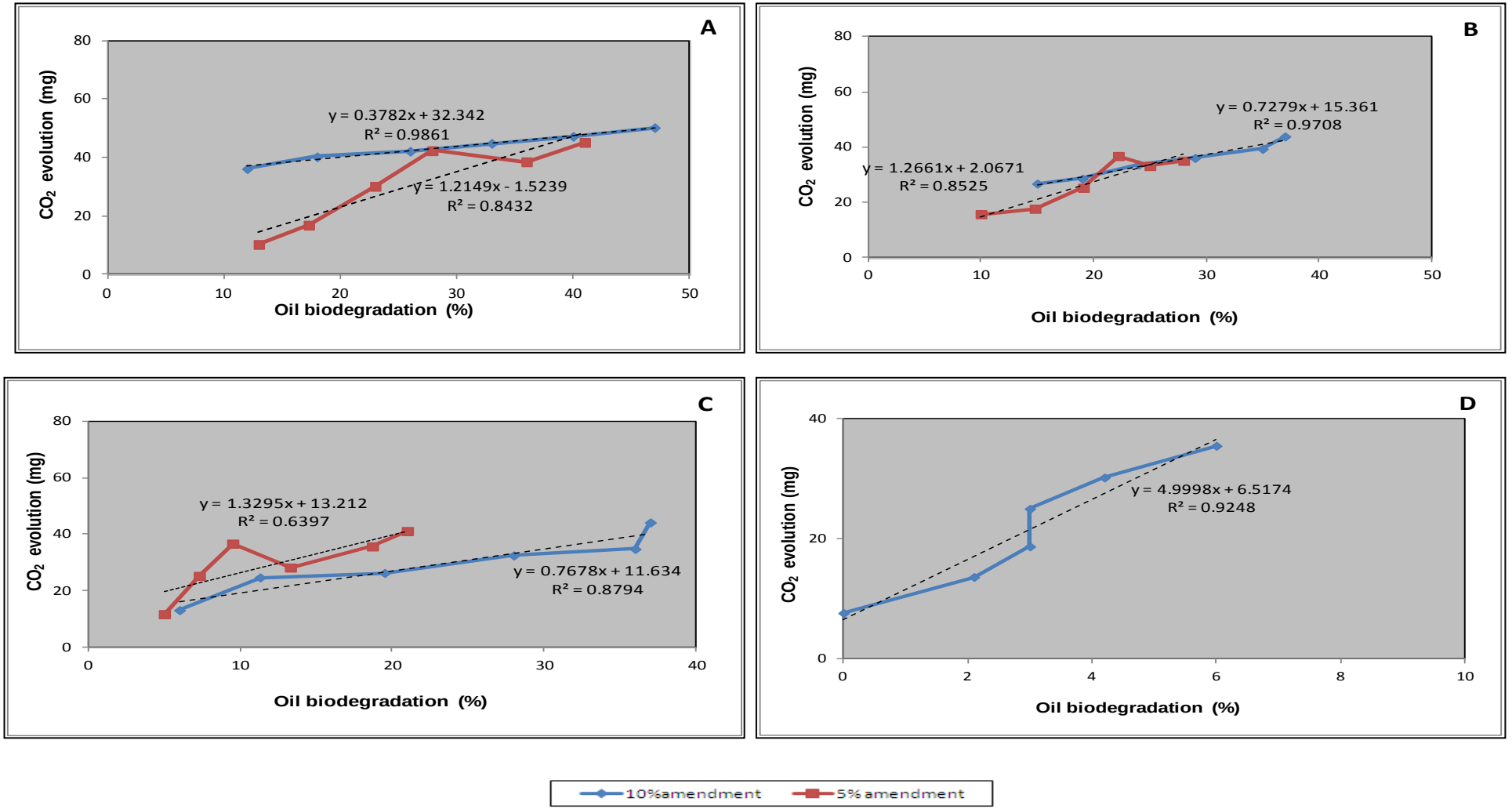


Figure 4.43 Correlation between oil degradation in 15% pollution and CO_2 evolution, A) Soil amended with SC, B) Soil amended with PS, C) Soil amended with TL, D) Unamended control soil

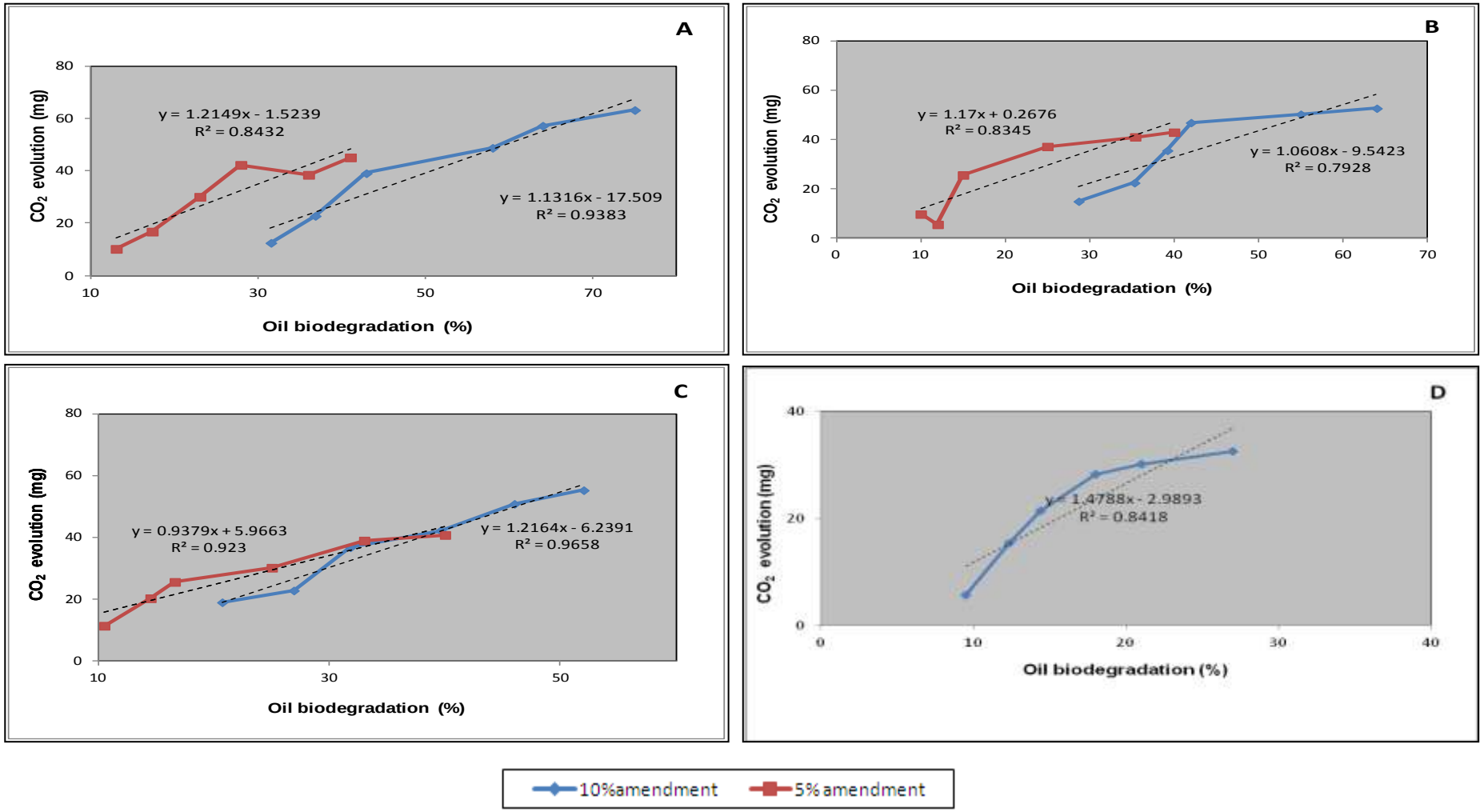


Figure 4.44 Correlation between oil degradation in 10% pollution and CO_2 evolution, A) Soil amended with SC, B) Soil amended with PS, C) Soil amended with TL, D) Unamended control soil

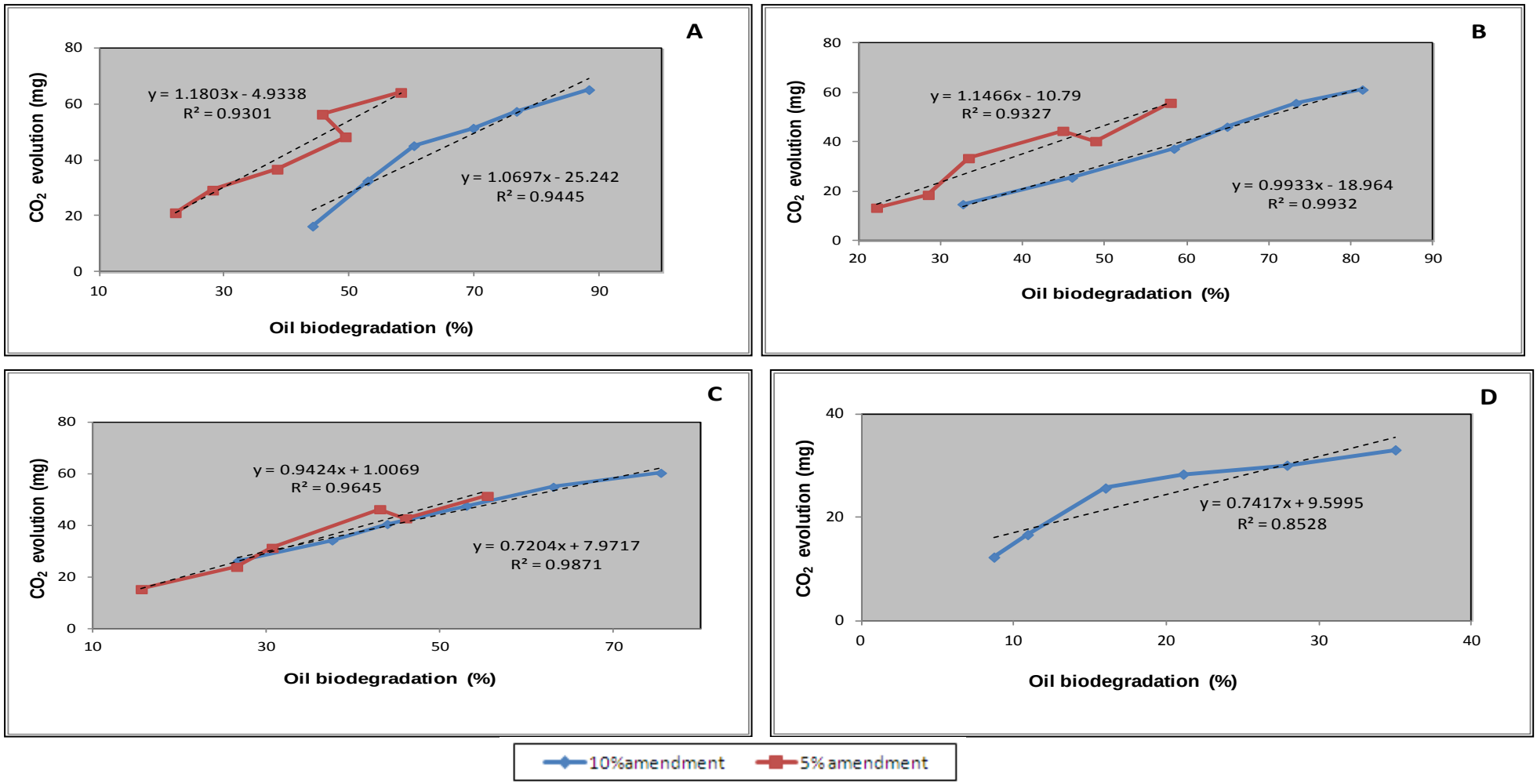


Figure 4.45 Correlation between oil degradation in 5% pollution and CO_2 evolution, A) Soil amended with SC, B) Soil amended with PS, C) Soil amended with TL, D) Unamended control soil

4.2.14 Dehydrogenase activity (DHA)

Biological oxidation activity was investigated based on DH enzyme to evaluate the efficiency of the microbial community to utilize organic compounds. This is as an index of the total oxidative activity in a sample (Aparna et al., 2010). Figure 4.46 shows the DHA in soil contaminated with 5% diesel fuel and treated with 5% and 10% organic wastes. DHA increased with time and then significantly decreased at 70 days in soil amended with 10% organic wastes. The highest microbial activity was recorded by soil amended with 10% SC (200 μINTF/g dw) at 126 days, which is 2.7 fold higher than unamended control soil in the same time. However, in soil amended with 5% TL, SC and PS, 173.2, 190 and 150 μINTF/g dw dehydrogenase activity was recorded at the end of 126 days, respectively. It is observed that by increasing the percentage of organic wastes amendment the rate of DHA is increased compared with the low percentage of amendment. At the end of 126 days DHA diminished in all treatment units since all available organics were degraded. These results agree with the findings of Aparna et al., (2010) who reported that in the biostimulation process with nutrient addition there was increased dehydrogenase activity from 5.8 μg INTF/g dw to 95.6 μg INTF/g dw in the period of 36 days.

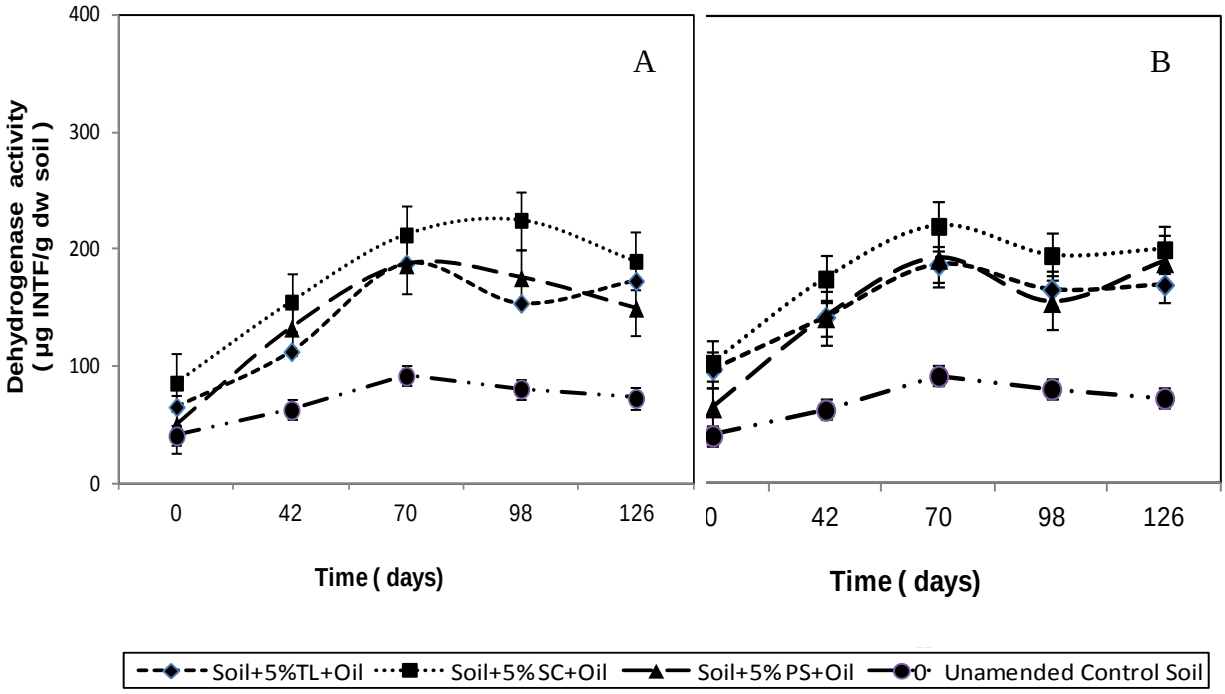


Figure 4.46 Dehydrogenase activity (DHA) in soil polluted with 5% diesel fuel, (A) soil amended with 5% organic wastes, (B) soil amended with 10% organic wastes.

Figure 4.47 illustrates the rate of DHA in soil polluted with 10% diesel fuel and amended with 5% and 10% organic wastes. At the start of the experiment, the DHA was low and not significantly different between treatments. However, over the 42 days the values increased significantly. DHA in soil amended with 10% organic wastes was higher than those amended with 5% organic wastes. At the end of 126 days, DHA recorded in soil treated with 10% SC, TL and PS was 290, 243 and 240 µg INTF/g dw, respectively. This shows that, among different treatment in soil amended with 5% organic waste, SC had the highest rate of DHA which was 2.8 fold higher than the control soil. Figure 4.47 show changes the dehydrogenase activity 15% diesel contaminated soils amended with different organic wastes, respectively.

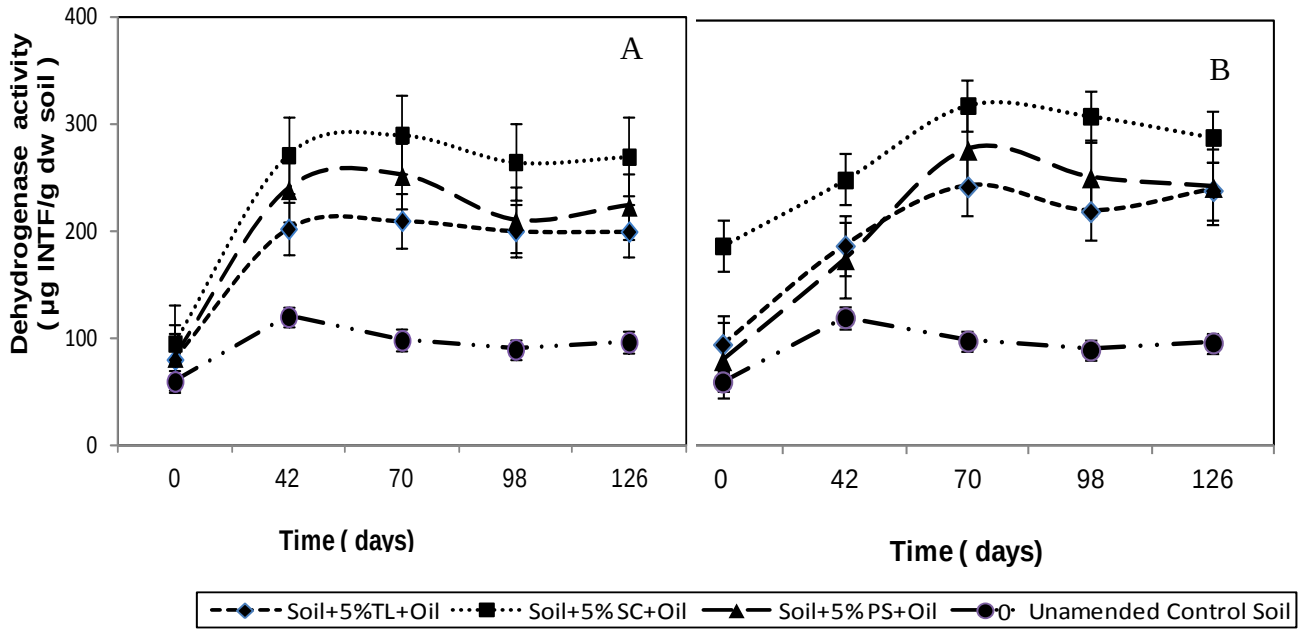


Figure 4.47 Dehydrogenase activity (DHA) in soil polluted with 10% diesel fuel, (A) soil amended with 5% organic wastes, (B) soil amended with 10% organic wastes.

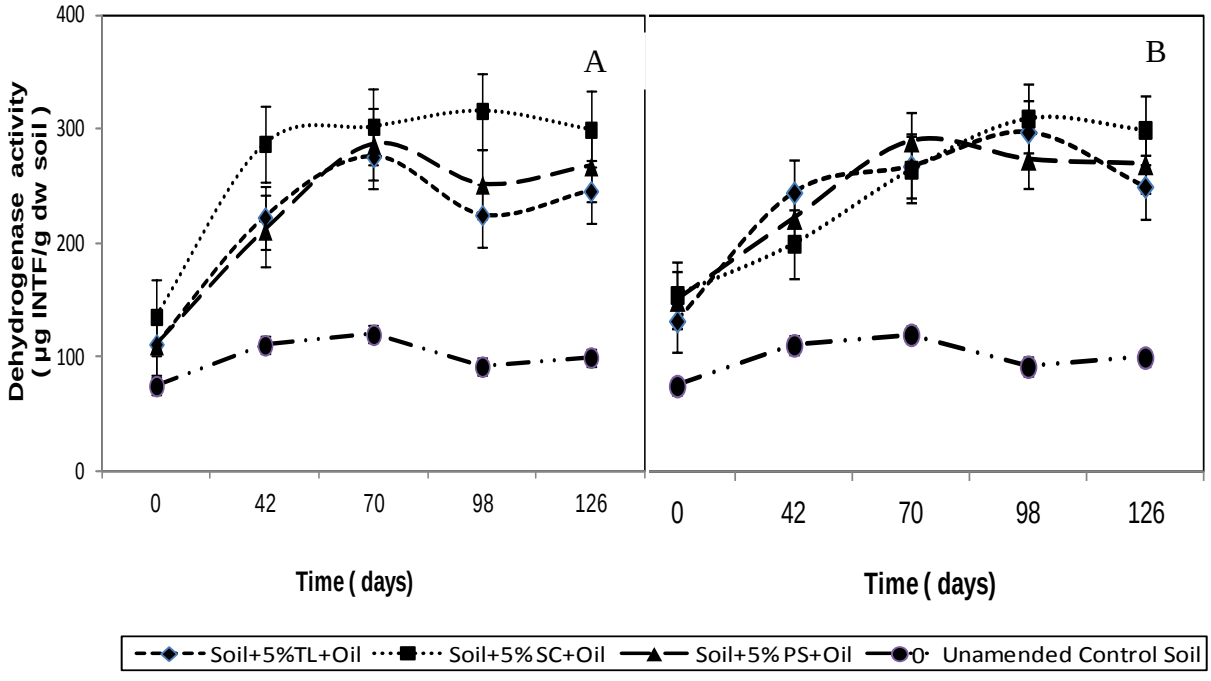


Figure 4.48 Dehydrogenase activity (DHA) in soil polluted with 15% diesel fuel, (A) soil amended with 5% organic wastes, (B) soil amended with 10% organic wastes.

Bento et al., (2003) reported the highest DHA in bioaugmentation of soil contaminated with diesel fuel that was 3.3 fold higher than natural attenuation soil. They also mentioned that the variety of DHA depended on the soil, bioremediation treatment and incubation time. By increasing the incubation period DHA will be increased in contaminated soil. Figure 4.49 show changes the dehydrogenase activity at 20% diesel contaminated soils amended with different organic wastes, respectively. In the range of 14 to 126 days, soil amended with 10% SC presented an increase than other amendments, but with a sharp drop on 98th day. However, soil treated with 5% SC, PS and TL recorded 300, 268 and 246 µg INTF/g dw respectively, compared with unamended controlled soil with 100 µg INTF/g dw. The result contrasts with those of Lee at al., (2011) which demonstrated a significant decrease in DHA during the bioaugmentation and biostimulation process on the 23rd day of study and increased gradually until the end of 40 days due to low water content. This final decrease very likely indicates the lack of optimum growth conditions for microorganisms, when the nutrient source, added only once at the initial phase of the trial, had been exhausted.

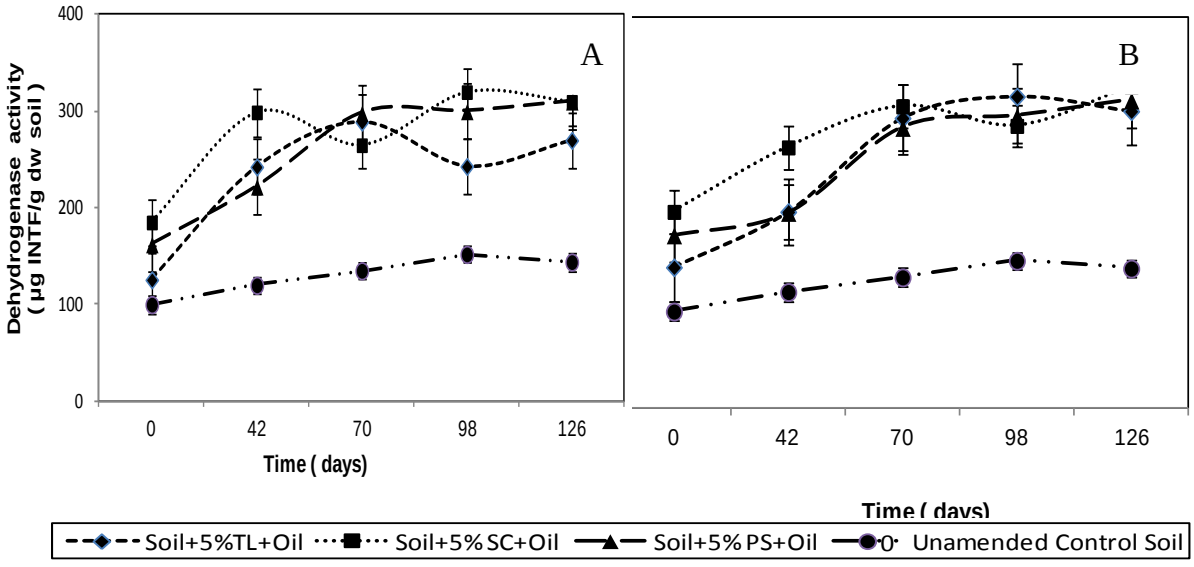


Figure 4.49 Dehydrogenase activity (DHA) in soil polluted with 20% diesel fuel, (A) soil amended with 5% organic wastes, (B) soil amended with 10% organic wastes.

4.2.15 Correlation between CO_2 evolution, Dehydrogenase activity and TPH degradation

Table 4.21 shows the correlation coefficients among TPH degraded, cumulative CO_2 evolved and dehydrogenase activity. High correlations were found between the amount of TPH degraded, the amount of CO_2 evolved, and dehydrogenase activity. Degradation of TPH was significantly related to microbial respiration as measured by CO_2 evolution. Significant positive correlation ($R^2 = 0.93$, $P < 0.01$) was also found between TPH degraded and CO_2 accumulation in soil amended with 10% organic wastes and polluted with 20% diesel oil. The lowest correlation ($R^2 = 0.52$, $P < 0.05$) was observed between dehydrogenase activity and microbial respiration in soil contaminated with 10% diesel oil and amended with 5% organic wastes. This result indicates that the amount of CO_2 evolved and dehydrogenase activity matched well with TPH degradation. The results are similar to the result of Namkoong et al., (2002) who found high correlations ($r = 0.80$ and above) among TPH degradation rate, dehydrogenase activity and the amount of CO_2 evolved in bioremediation of diesel oil contaminated soil using sewage sludge or compost amendments. Also, there was a strong correlation between soil enzymes activity, CO_2-evolution, TPH degradation and microbial counts (Bahrampor and Sarvimoghanlo, 2012; Balba et al., 1998).

Table 4.21 Matrix of correlation coefficients between CO_2 evolution, Dehydrogenase activity and TPH degradation

Treatments	TPH degraded (%)	Cumulative CO_2 (mg/100 gr)	DHA (μg NITF/g dw soil)
5% diesel + 5% organic waste			
TPH degraded	1.00	0.913**	0.825**
Cumulative CO_2	0.913**	1.00	0.832**
DHA	0.825**	0.832**	1.00
5% diesel + 10% organic waste			
TPH degraded	1.00	0.854**	0.903**
Cumulative CO_2	0.854**	1.00	0.839**
DHA	0.903**	0.839**	1.00
10% diesel + 5% organic waste			
TPH degraded	1.00	0.909**	0.564**
Cumulative CO_2	0.909**	1.00	0.522*
DHA	0.564**	0.522*	1.00
10% diesel + 10% organic waste			
TPH degraded	1.00	0.848**	0.861**
Cumulative CO_2	0.848**	1.00	0.757**
DHA	0.861**	0.757**	1.00
15% diesel + 5% organic waste			
TPH degraded	1.00	0.766**	0.824**
Cumulative CO_2	0.766**	1.00	0.820**
DHA	0.824**	0.820**	1.00
15% diesel + 10% organic waste			
TPH degraded	1.00	0.823**	0.903**
Cumulative CO_2	0.823**	1.00	0.735**
DHA	0.903**	0.735**	1.00
20% diesel + 5% organic waste			
TPH degraded	1.00	0.767**	0.902**
Cumulative CO_2	0.767**	1.00	0.671**
DHA	0.902**	0.671**	1.00
20% diesel + 10% organic waste			
TPH degraded	1.00	0.930**	0.907**
Cumulative CO_2	0.930**	1.00	0.839**
DHA	0.907**	0.839**	1.00

** Correlation is significant at the 0.01 level. * Correlation is significant at 0.05 level.

4.2.16 ^{13}C stable isotope analysis

In this study continuous-flow isotope ratio mass spectrometry (CF/IRMS) was used to investigate the $\delta^{13}C$ of dissolved organic carbon as evidence for biodegradation of hydrocarbons. It has been proven to be an excellent environmental detector (St-Jean, 2003). The result of ^{13}C stable isotope analysis in contaminated soil amended with SC was selected to monitor the pathways of degradation. The isotopic composition of CO_2 is shown in Table 4.22. All ^{13}C analyses were conducted in two replicates. Therefore, each data point in the two runs represents the average of the duplicate analysis. This monitoring could be obtained by the analysis of ^{13}C stable isotope compositions of the residual fractions of contaminants. Results indicate that there was an enrichment of ^{13}C and more positive result in all treatments amended with SC during the biodegradation process. The measured $\delta^{13}C$ signature of CO_2 evolved from amended contaminated soil varied between -24 and -28 ‰. This difference in the value of $\delta^{13}C$ indicates that microbial process was involved in the degradation of the diesel fuel fraction leading to the enrichment of $\delta^{13}C$ in diesel fuel residual. The unamended control soil treatment varied between -27 and -29 ‰. Most of the PAHs had stable carbon isotopic ratios in the range of –22 to –30 ‰ (Sun et al., 2003). However, Landmeyer et al., (1996) reported that the value of $\delta^{13}C$, at sites where methanogenesis occurred, ranged from -30 ‰ up to +11.9‰. The small increase in the $\delta^{13}C$ of CO_2 was an expected result that mineralization of microbial biomass or an intermediate product during the degradation of diesel, will lead to a sharp increase in CO_2 concentration. This result is supported by Lollar et al., (2007) who indicated that the ^{13}C enrichment results in microbial degradation of contaminated compounds. They also mentioned that the differences in the measured $\delta^{13}C$ values are attributable to the linearity of the analytical

system. Aggarwal et al., (1997) reported that the changes in the $\delta^{13}C$ of CO_2 generated during the degradation might also result from selective degradation of fractions of diesel oil that may have different isotopic compositions.

Table 4.22 Isotopic Composition ($\delta^{13}C$) of carbon dioxide in biodegradation experiments (‰)

Treatments	Sampling (days)		
	1	70	126
Soil+5% Oil+ 5% SC	-28.15	-27.7	-26.43
Soil+5% Oil+ 10% SC	-28.56	-27.69	-26.71
Soil+5% Oil	-28.11	-27.60	-27.87
Autoclaved Soil+ 5%Oil	-28.23	-27.92	-28.01
Soil+10% Oil+ 5% SC	-26.38	-26.26	-25.84
Soil+10% Oil+ 10% SC	-28.76	-27.02	-26.07
Soil+10% Oil	-28.81	-28.31	-28.07
Autoclaved Soil+ 10%Oil	-28.84	-28.82	-28.75
Soil+15% Oil+ 5% SC	-25.19	-25.71	-25.45
Soil+15% Oil+ 10% SC	-25.05	-25.66	-25.39
Soil+15% Oil	-26.55	-26.63	-26.21
Autoclaved Soil+ 15%Oil	-29.05	-29.26	-28.31
Soil+20% Oil+ 5% SC	-24.97	-24.43	-24.88
Soil+20% Oil+ 10% SC	-28.92	-28.72	-26.77
Soil+20% Oil	-28.42	-28.47	-28.15
Autoclaved Soil+ 20%Oil	-28.53	-28.50	-28.47

- Precision of analysis is ± 0.2 ‰
- Unit is parts per thousands (‰)

4.2.17 Biodegradation Efficiency (BE)

Based on the amount of carbon dioxide produced, the efficiency of biodegradation was calculated. BE of contaminated soil with 5, 10, 15 and 20% diesel fuel concentration and amended with 5 and 10% organic wastes are shown in Figures 4.50 to 4.53. The biodegradation efficiency was evaluated according the CO_2 evolution data. The results clearly indicate that those treatments amended with organic wastes had the best efficiency on the biodegradation of soil contaminated with diesel fuel. These effects were statistically significant for a 95% confidence level ($P < 0.05$) compared with those non-amended contaminated soil. Among the organic wastes, soil treated with 10 % SC showed the highest BE (up to 99%, 96%, 76% and 84%) in soil polluted with 5, 10, 15 and 20% diesel fuel respectively. Positive values in the BE demonstrated that the organic wastes have a favorable effect on the pollutant removal efficiency. Therefore, BE effect was higher in soil amended with 10% compared with 5% organic wastes. The result is in line with Mariano et al., (2007) who reported that the addition of nutrients (fertilizer) to remediate soil contaminated with diesel fuel indicated up to 19% BE during the 50 days of study and this was considered significant compared with controlled treatment. In contrast, Morais and Tornisielo (2009) obtained no significant differences ($P > 0.05$) in the efficiency of biodegradation during the biodegradation process using the inoculums, which indicates no increase in the biodegradation rate. In addition, they reported the contribution of fertilizers plus inoculums, did not affect the increase in efficiency of biodegradation. The reseaon for the different results might be the environmental condition in the process of biodegradation.

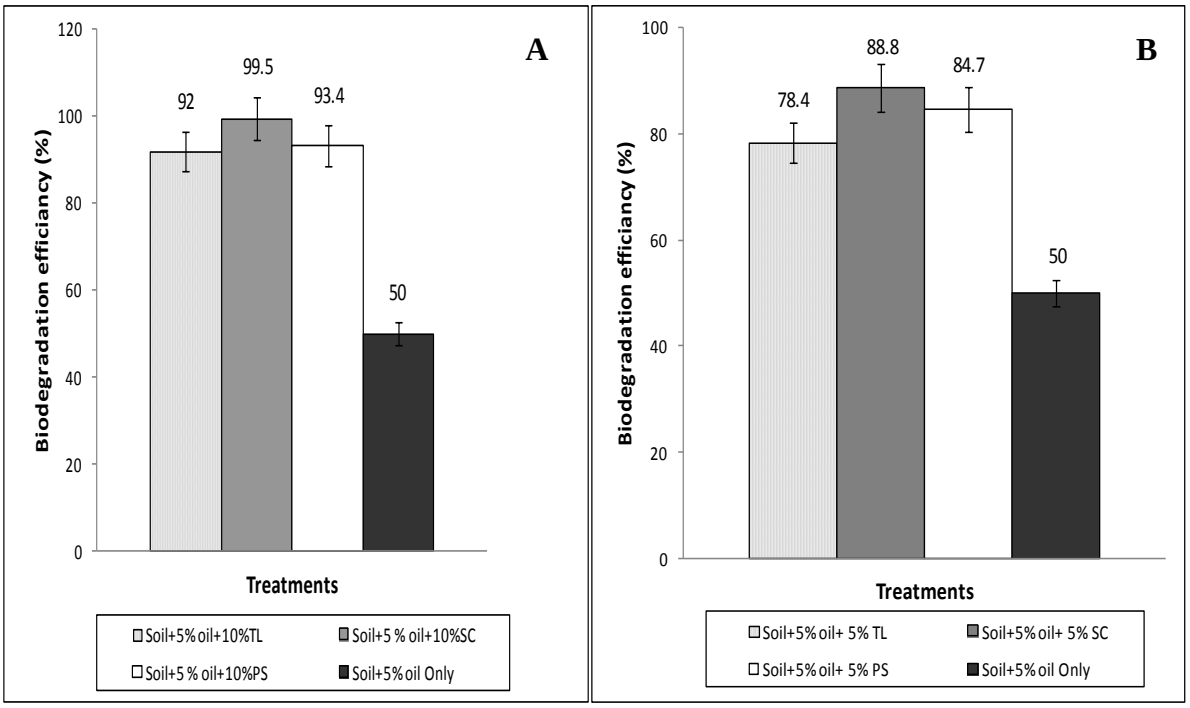


Figure 4.50 Biodegradation efficiency obtained through the respirometric data in soil polluted with 5% diesel oil and amended with A) 10% organic wastes B) 5% organic wastes. Bars indicate standard error (n = 3).

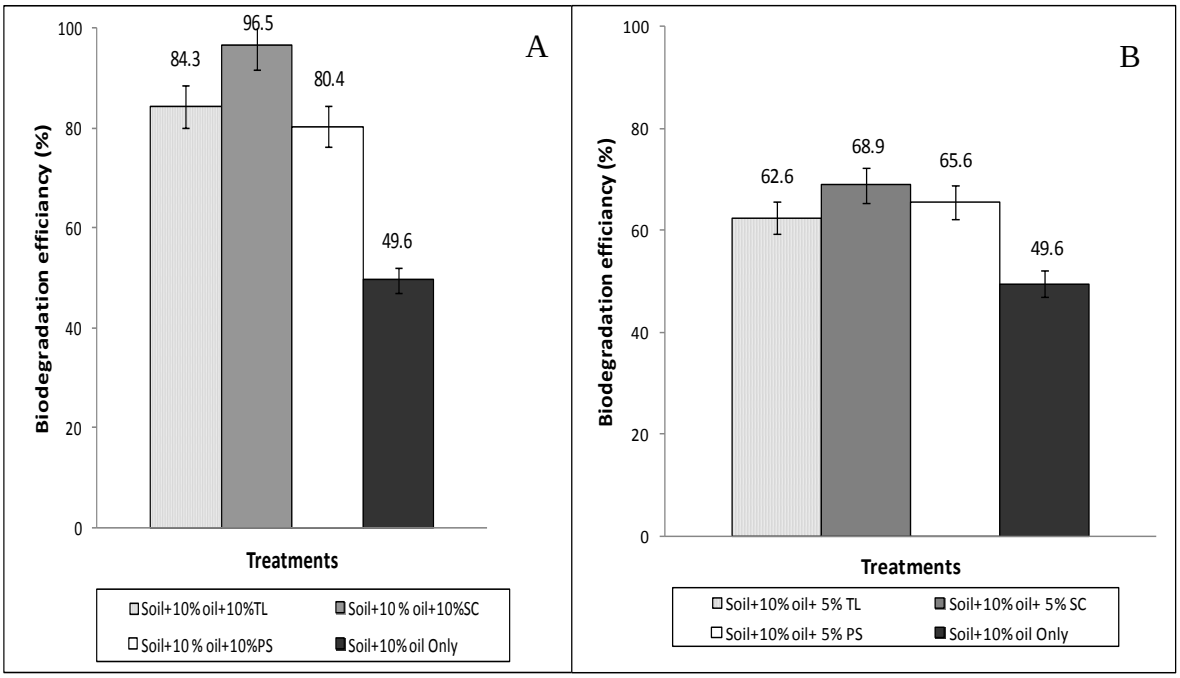


Figure 4.51 Biodegradation efficiency obtained through the respirometric data in soil polluted with 10% diesel oil and amended with A) 10% organic wastes B) 5% organic wastes. Bars indicate standard error (n = 3).

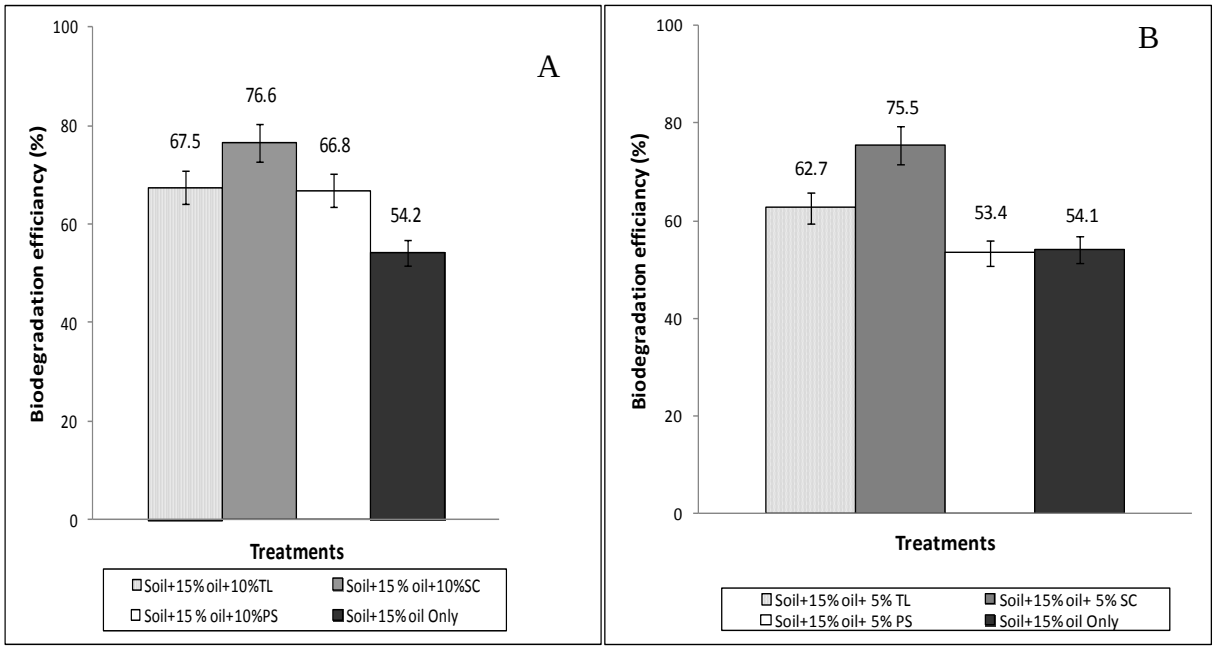


Figure 4.52 Biodegradation efficiency obtained through the respirometric data in soil polluted with 15% diesel oil and amended with A) 10% organic wastes B) 5% organic wastes. Bars indicate standard error (n = 3).

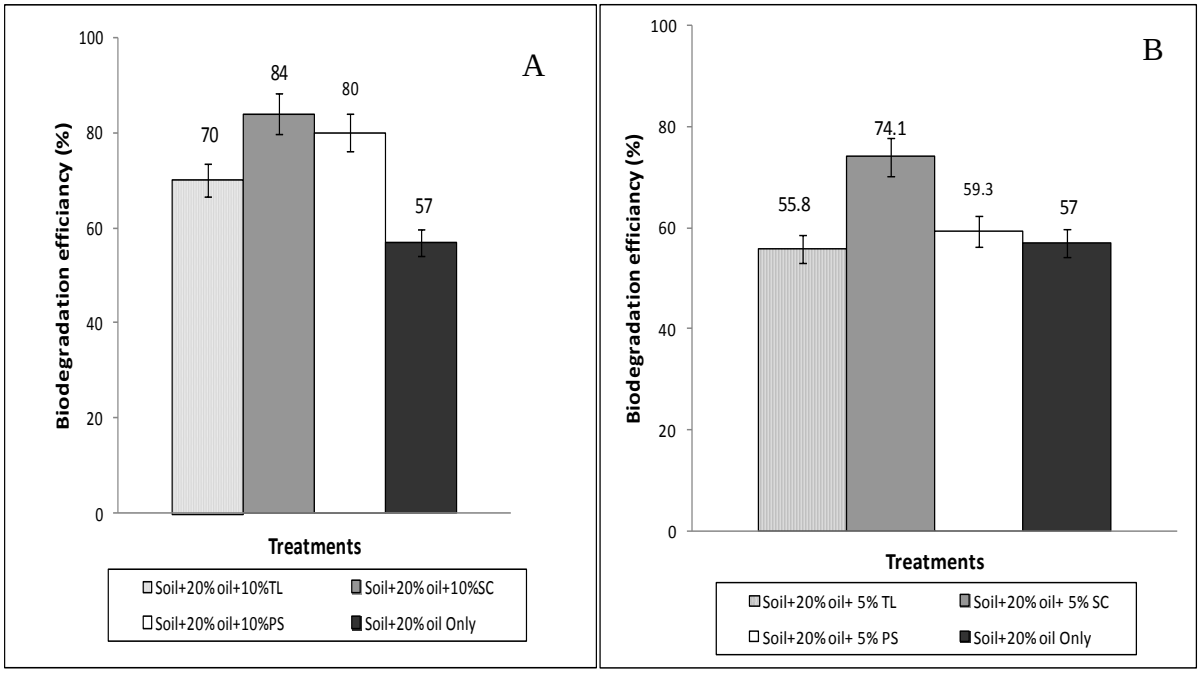


Figure 4.53 Biodegradation efficiency obtained through the respirometric data in soil polluted with 20% diesel oil and amended with A) 10% organic wastes B) 5% organic wastes. Bars indicate standard error (n = 3).

4.3 Results of bioremediation studies under field condition

The bioremediation study of diesel contaminated soil exposed to sunlight and rainfall (under natural condition) was conducted at the pilot site for a period of one year (February 2010 to February 2011). Average rainfall and temperature were 636.9 mm and 33^{o}C, respectively. Only 10% organic waste amendments was used to evaluate biostimulation of diesel contaminated soil under natural condition, due to significant effects of 10% organic waste amendments on biodegradation of diesel fuel under laboratory term.

4.3.1 Biostimulation of diesel fuel contaminated soil

Figures 4.54 to 4.57 show the percentage of biodegradation 5%, 10%, 15% and 20% diesel fuel contaminated soil amendment with 10% organic wastes under natural condition for a period of one year. Soil samples were collected every two months for the determination of the biodegradation rate during the period of study.

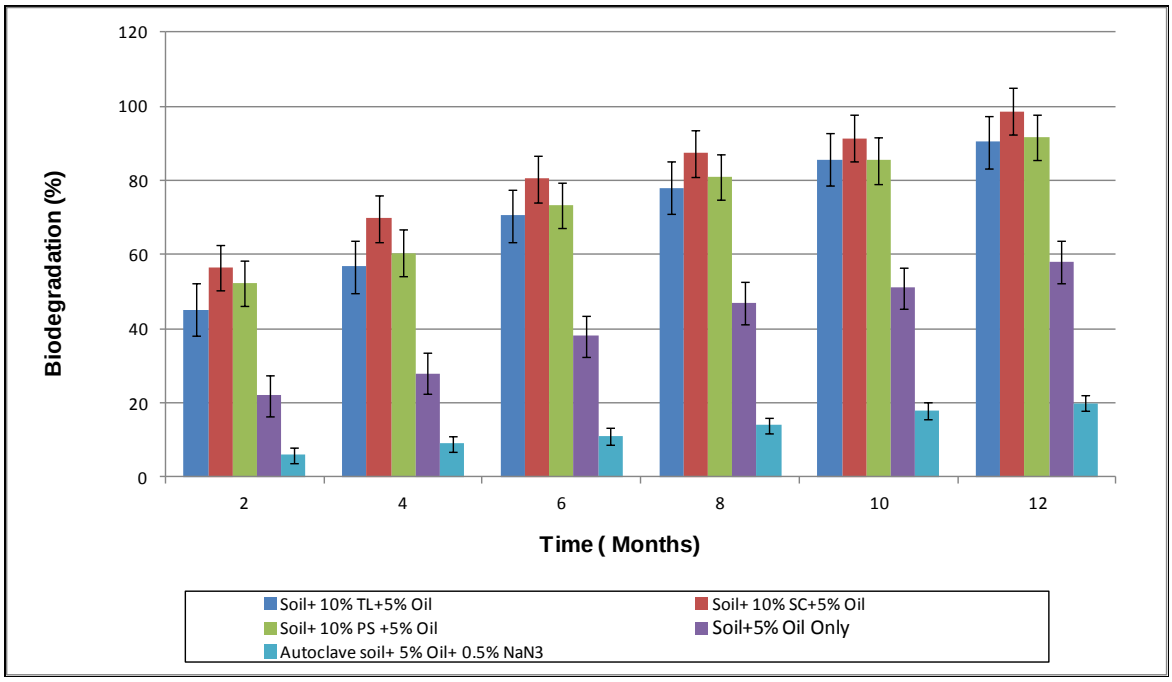


Figure 4.54 Biodegradation of diesel fuel in soil contaminated with 5% oil and amended with 10% organic wastes. (Bars indicate standard error, n = 3).

At the end of one year, the percentage of diesel fuel degradation in soil polluted with 5% oil and amended with 10% TL, SC and PS recorded 90%, 98% and 92%, respectively (Figure 4.54). While the percentage of biodegradation in unamended control soil was 58% which was higher than the rate of biodegradation under laboratory condition. SC treated soil recorded the highest percentage of biodegradation under field condition (98%). The reseaon for the high percentage of biodegradation might be the favorable environmental condition and a longer period of biodegradation under natural condition.

As mentioned earlier (2.5.3) moisture and temperature are known to be important factors in the bioremediation process due to the direct effect on the chemistry of hydrocarbon compounds, and also these two factors can modify the diversity and physiology of microorganisms (Okoh, 2006). In addition, temperature played the main role in the metabolism of microbial hydrocarbon which is attributed for the breakdown of hydrocarbon compounds. In soil polluted with 10% diesel fuel, all treatments amended with organic wastes show a high percentage of biodegradation (Figure 4.55). 78%, 91% and 85% oil loss was recorded in soil contaminated with 10% diesel fuel, at the end of 12 months of study. SC amendment recorded a higher rate of degradation which was almost double the rate of biodegradation compared with unamended control soil (45.6%). The results also indicate a similar result of biodegradation rate in soil contaminated with 15% diesel oil (Figure 4.56). At the end of one year, SC treated soil recorded 72% biodegradation in natural conditions compared with 55% under laboratory condition. Boopathy (2004) reported a significant degradation of diesel fuel under all conditions compared with unsupplemented treatment. At the end of the study, the soil polluted with 20% diesel oil indicated 40%, 51% and 44% oil loss in soil treated with TL, SC and PS, respectively (Figure 4.57), while unamended controlled soil showed 21% biodegradation at the end of 12 months.

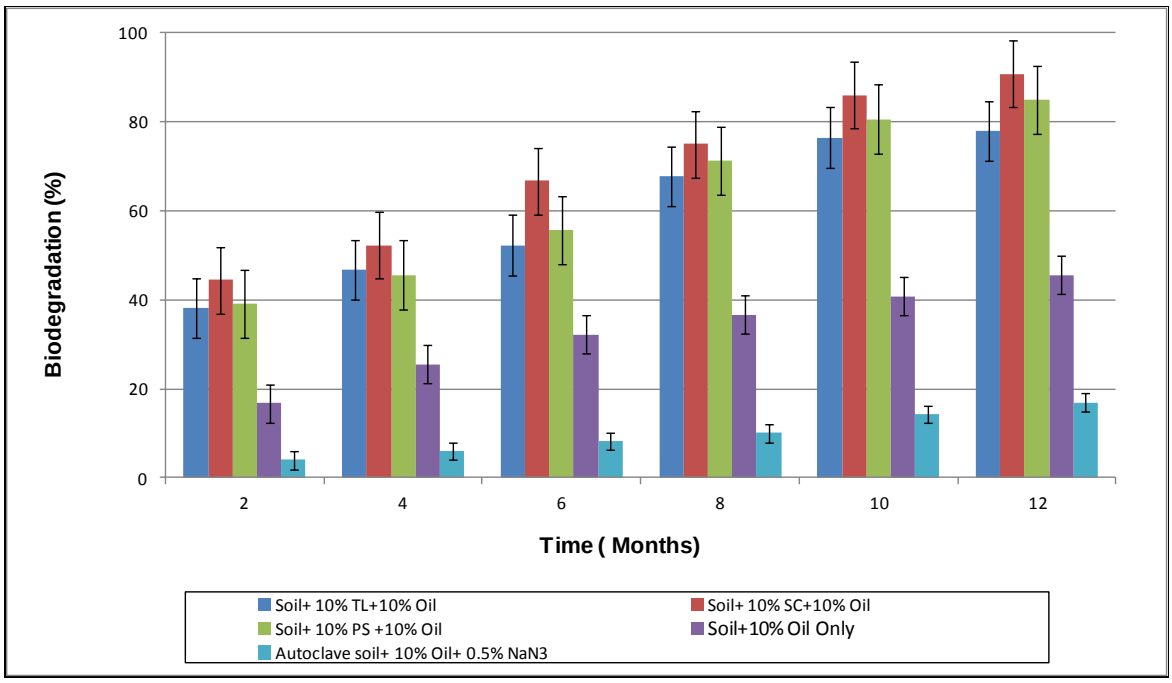


Figure 4.55 Biodegradation of diesel fuel in soil contaminated with 10% oil and amended with 10% organic wastes. (Bars indicate standard error, n = 3).

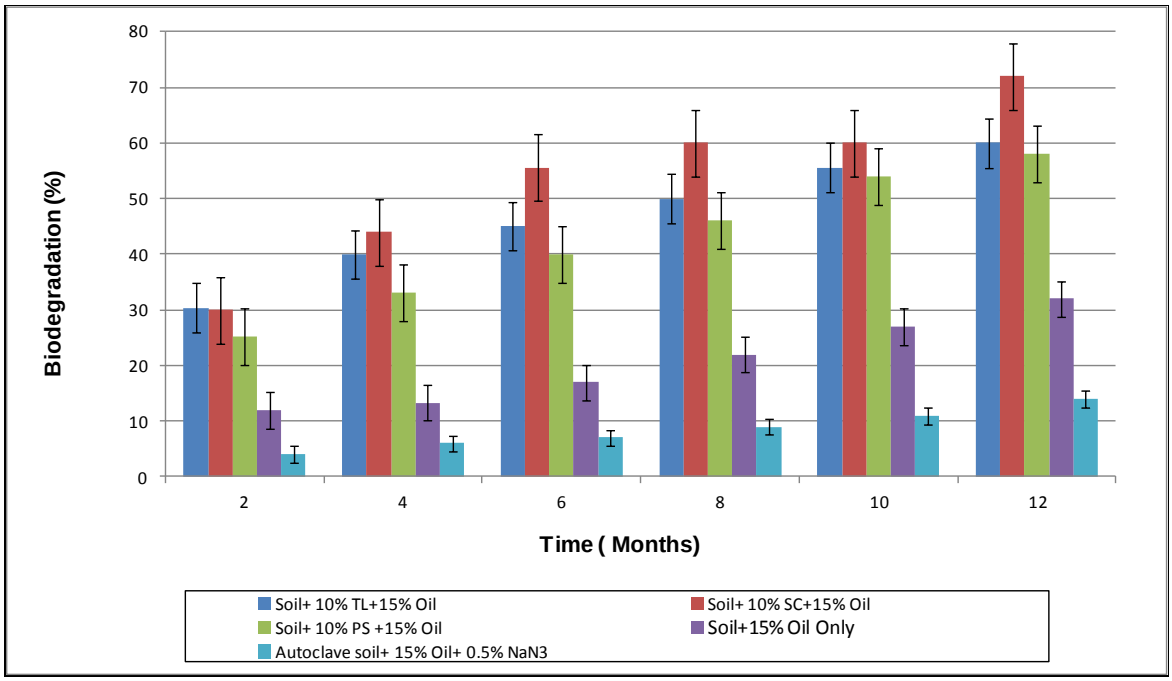


Figure 4.56 Biodegradation of diesel fuel in soil contaminated with 15% oil and amended with 10% organic wastes. (Bars indicate standard error, n = 3).

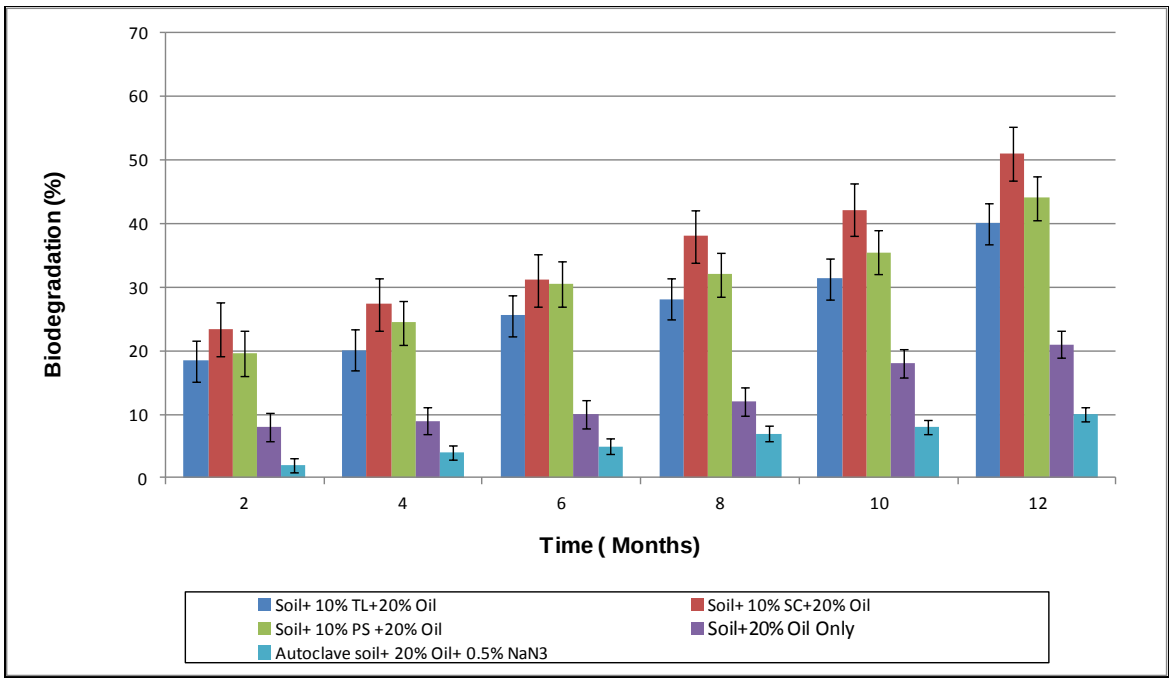


Figure 4.57 Biodegradation of diesel fuel in soil contaminated with 20% oil and amended with 10% organic wastes. (Bars indicates standard error, n = 3).

The result shows an increase in the rate of biodegradation in simulated natural condition. These results demonstrated that there were suitable environmental conditions (exposed to rainfall and sunlight) compared with conditions in the laboratory which resulted in an increase in oil loss of all treatments. For example, the temperature range between 27 to 39 ^{o}C, which is possibly suitable to stimulate the microbial activity in contaminated soil. Many researchers have reported on the significant role of temperature (Boopathy, 2000 and Chaillan et al., 2006). Another reseaon for recording a higher rate of biodegradation in all treatments under natural conditions compared with treatments in laboratory conditions might be due to photodegradation or evaporation of some part of diesel compounds which was in poisoned treatment (autoclaved control soil) with 10%, 14%, 17% and 22% in soil contaminated with 20%, 15%, 10% and 5% diesel fuel, respectively.

4.3.2 Extraction and analysis of residual diesel by GC/MS

The residual oil was analyzed and identified based on their mass spectra and retention times, as indicated by the chromatogram of the remaining diesel after biodegradation tests. Diesel is a complex hydrocarbon compound like aromatics and *n*-alkanes. Microorganisms are known to attack and degrade a specific component as compared with other components of oil (Luo et al., 2012). It has been indicated that the same microorganisms were able to degrade the same compounds to different extents (Obayori et al., 2009). Representatives of GC/MS chromatograms showing the total petroleum hydrocarbon patterns at the 30^{th} day and at the end of one year (day 365) of treatment amended with SC and unamended controlled soil is illustrated in Figure 4.58 – 4.65. Chromatographic analysis gives clearance to estimate the effects of treatments on degradation of diesel fuel in the light (C_{12} – C_{23}) and heavy (C_{23} – C_{40}) fractions (Bento, et al., 2005). As GC/MS chromatogram results in all treatments demonstrated low molecular weight of total petroleum hydrocarbon (C_8- C_{12}) fraction it was quickly degraded and removed due to low concentration and high volatility and degradability, while the high molecular weight of total petroleum hydrocarbon (C > 12) fraction was more hardy for biodegradation (Taccari et al., 2010). The GC/MS results agreed with previous reports showing that biostimulation can lead to a more effective method of degradation of oil from contaminated soil (Bento et al., 2005; Marchal et al., 2003; Olson et al., 1999). Moreover, there are growing evidences that biostimulation of petroleum hydrocarbon contaminated lands using amendments like composts, organic and inorganic wastes are the best approach for bioremediation. Figure 4.58 shows the GC/MS chromatographic profiles of residual diesel fuel in soil polluted with 5% oil and amended with 5% SC on 30^{th} day and at the end of the experiment. Comparison of the chromatograms before and after the biodegradation process demonstrated that the most hydrocarbon fractions had been removed at the end of one-year

compared with unamneded controlled soil. At the end of one year, the hydrocarbon fraction in the range of C_{19} to C_{30} in soil treated with 5% oil and amended with 5% SC shows a higher degradation compared with the start of the experiment. Moreover, in unamended control treatment most fractions remained at the end of one year which shows the positive role of organic wastes to enhance the diesel fuel contaminated soil. These results agree with Xu and Lu (2010) who reported the removal of C_{12} to C_{29} compounds from crude oil polluted soil, at the end of the incubation period. The results of soil contaminated with 5% diesel oil and amended with 10% SC indicated that complete biodegradation of fractions the in the total time of twelve months (Figure 4.59). C_{15} to C_{26} were highly degraded in soil treated with 10% SC at the end of the one-year compared with hydrocarbon components in amended soil with 5% SC which was partially degraded, while there was no complete degradation of the fractions in unamended control soil at the end of the 12^{th} month. However, the significantly higher decay rates in biodegradation of C_{15} - C_{26} fractions in soil amended with organic wastes might be due to their nutrient composition, especially nitrogen and phosphorus, which are very important ingredients for a successful biodegradation of diesel fuel. Depending on the environmental conditions, these nutrients could become limiting factors, therefore the increase of these nutrients are essential to enhance the biodegradation of petroleum hydrocarbon pollutants (Kim et al., 2005; Okoh and Trejo-Hernandez, 2006). This result is similar to a study of Ijah and Anti (2003b) who reported the high hydrocarbon fractions (C_{14} to C_{32}) in contaminated soil with crude oil during the period of 6 to 9 months. The rapid degradation of hydrocarbon fractions in the range of C_8 to C_{14} was recorded in all the treatments, which might be due to the straight structure of compounds that make it easy to degrade. Ghazali et al. (2004) and Erikson et al. (1998) indicated that cyclic or straight aliphatic hydrocarbons below C_{14} require shorter sampling times to give good results of degradation due to their high volatility compared with hydrocarbon with above C_{14} that can be absorbed by soil particles, which make them less volatile (Eriksson et al., 1998; Ghazali et al., 2004).

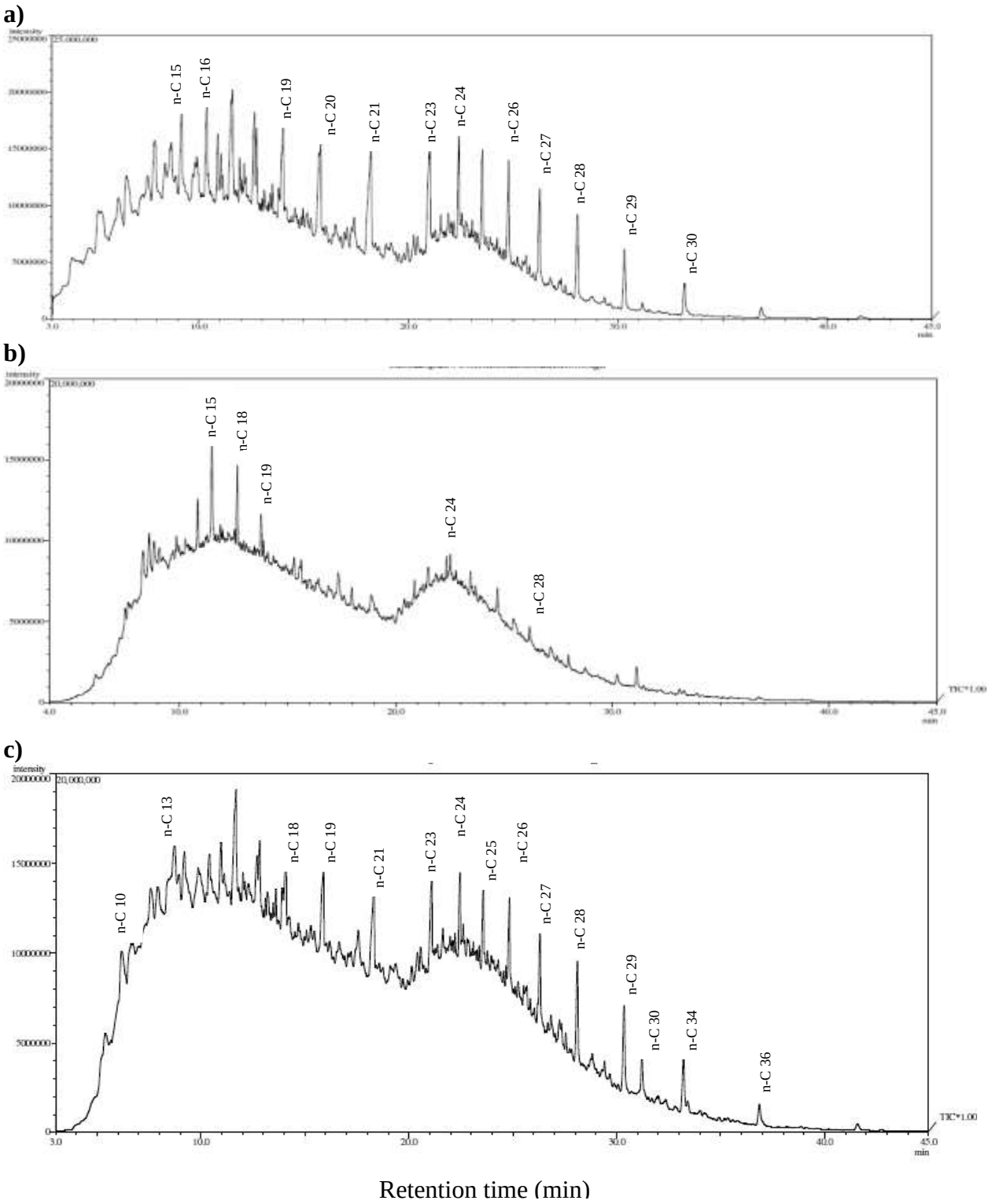
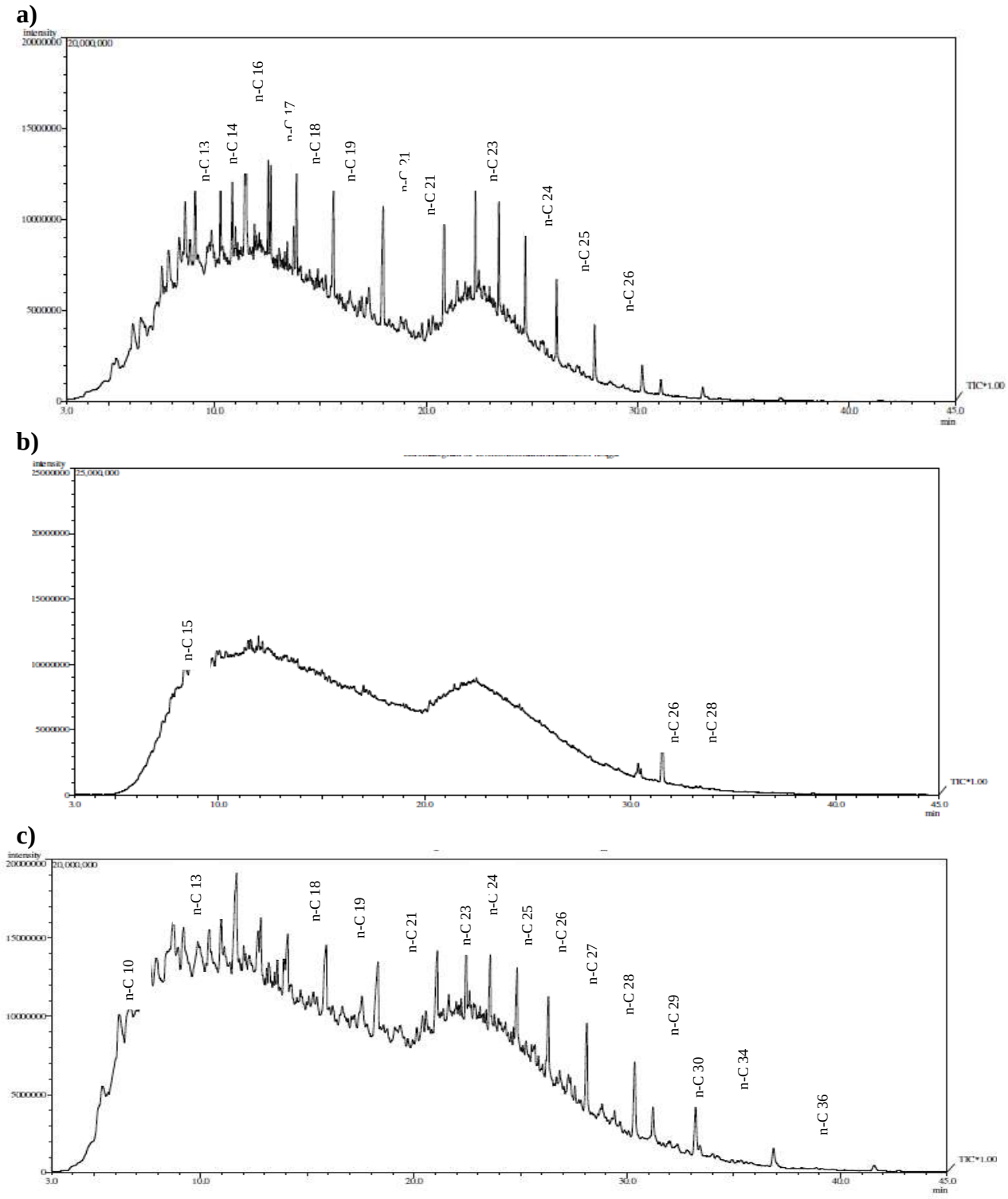


Figure 4.58 Chromatogram of residual diesel fuel in contaminated soil with 5% oil a) amended with 5% SC (T 30) b) amended with 5% SC (T 365) c) unamended control soil (T 365).



Retention time (min)

Figure 4.59 Chromatogram of residual diesel fuel in contaminated soil with 5% oil a) amended with 10% SC (T 30) b) amended with 10% SC (T 365) c) unamended control soil (T 365).

The GC/MS analysis of soil polluted with 10% diesel fuel and amended with 5% and 10% SC are illustrated in Figures 4.60 and 4.61, respectively. The results suggest a significant reduction in diesel content ($C_8 - C_{26}$) in the biostimulation samples compared with the natural attenuation. The effect of biostimulation of diesel oil on the degradation of the light fraction ($C_{12} - C_{23}$) of TPH was higher than the heavy fraction. Bento et al., (2005) observed that the bioaugmentation of soil polluted with diesel oil had the highest degradation in the heavy fractions (75.2%) and light fractions (72.5%) after 12 weeks of study. On the other hand, Seklemova et al., (2001) evaluated the addition of nutrients in forest contaminated soil with diesel oil and found no effects on the degradation of TPH fractions (light or heavy). The reason for differences in results of this study compared with Seklemova study might be attributed to the soils that had varying effects on the degradation of diesel oil. It is obvious that contaminated soil with 10% SC and amended with 5% and 10% SC degraded all the hydrocarbon fractions presented in diesel oil ($C_9 - C_{26}$) compared with unamended control soil. While low degradation of heavy fractions might be due to the structure of the compounds that make them more complex and strong to break down by the enzyme system of microorganisms. However, the results are relevant in that the heavy range of hydrocarbon fractions were properly degraded in contaminated soil with 10% diesel oil and amended with organic wastes within the 365 days of study. Hydrocarbon components of $C_8 - C_{14}$ fractions are known as the most quickly biodegraded components of oil due to the volatility and their simple structure have been reported. However, they are sensitive to removal by water washing (Abioye et al. 2012a; George et al., 2002).

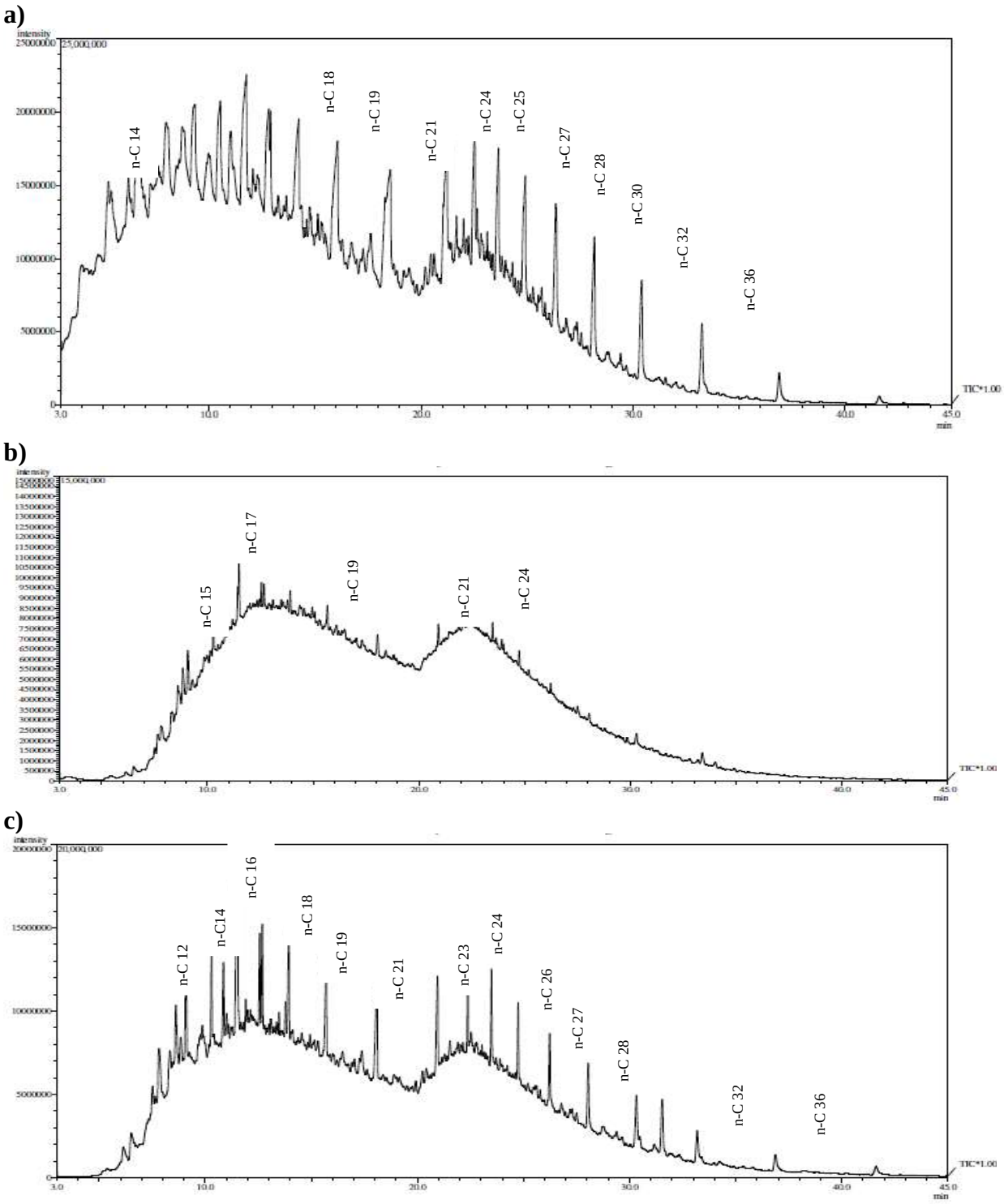


Figure 4.60 Chromatogram of residual diesel fuel in contaminated soil with 10% oil a) amended with 5% SC (T 30) b) amended with 5% SC (T 365) c) unamended control soil (T 365).

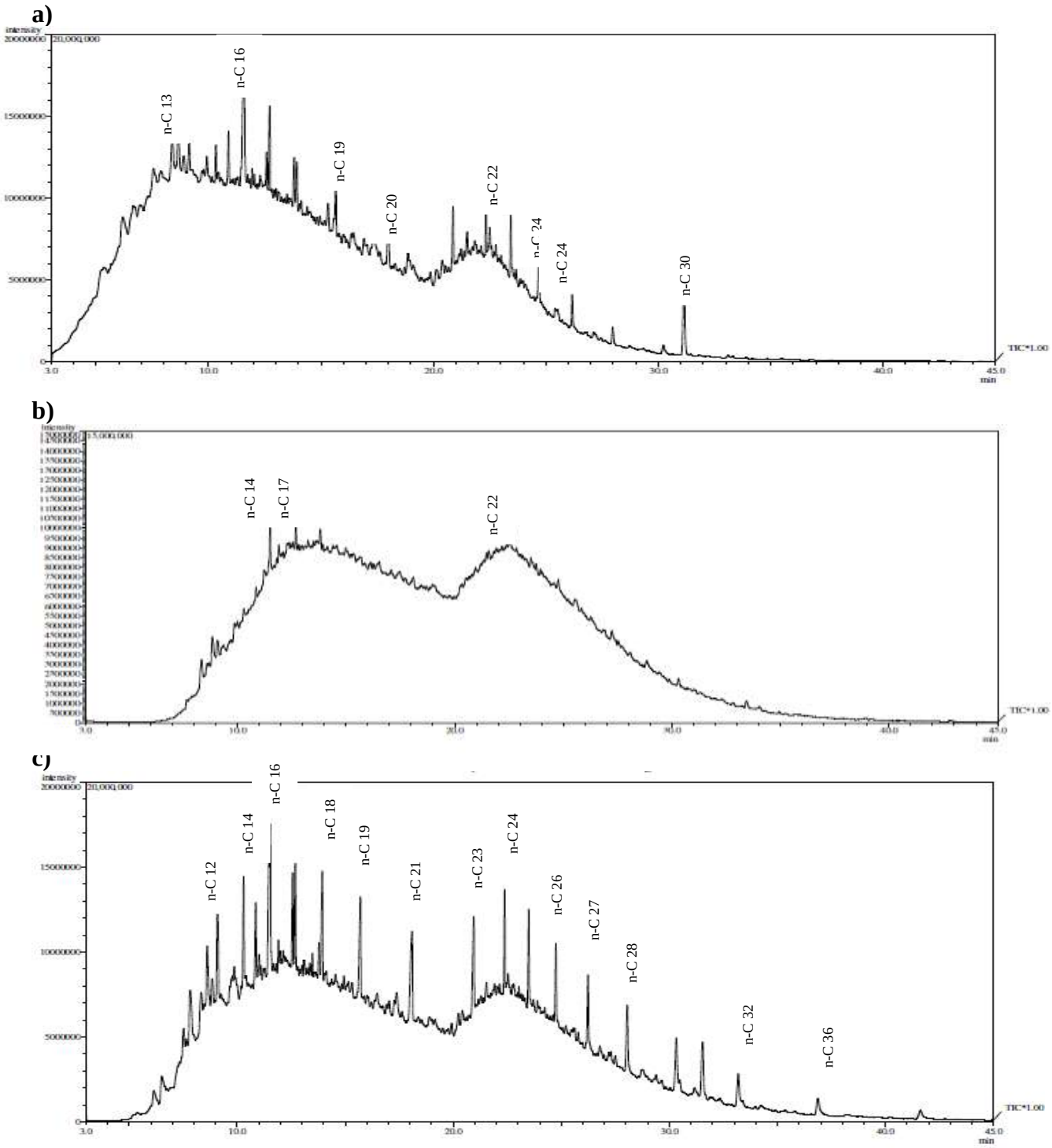


Figure 4.61 Chromatogram of residual diesel fuel in contaminated soil with 10% oil a) amended with 10% SC (T 30) b) amended with 10% SC (T 365) c) unamended control soil (T 365).

The results are similar to the finding of Chang et al., (2010) who discovered remarkable degradation of (C_{10} - C_{16}) hydrocarbon fraction in aged petroleum hydrocarbon contaminated soil. Ijah and Antai, (2003b) also reported that soil contaminated with 10% crude oil C_{14} fraction was completely degraded within the period of one year.

The GC/MS results of biodegradation of hydrocarbon fractions in the soil contaminated with 15% diesel oil are shown in Figures 4.62 and 4.63. The result shows that the contaminated soil amended with 5% SC had slight degradation of the hydrocarbon fractions within the one year of study (Figure 4.62), while soil amended with 10% SC recorded an increase in biodegradation of all fractions. Analysis of the oil extracted from the soil after one-year shows that all hydrocarbon fractions below the C_{13} were completely degraded in soil treated with either 10% and 5% SC compared with controlled soil, while heavy hydrocarbon components were partially degraded in soil after one year. Soil polluted with 20% diesel oil there was no complete degradation of fractions and results do not show any significant difference in removing hydrocarbon components in high concentration of diesel oil (Figures 4.64 and 4.65). The peaks of long-chain petroleum hydrocarbons were relatively higher than those of short chain hydrocarbons. Similar results were achieved by (Huang et al., 2005). The reason for the incomplete hydrocarbon fractions biodegradation in 15% and 20% diesel oil might be attributed to a high poison effect of oil on microorganisms to breakdown the complex structural oil components, which make it significantly difficult for HUB in complete degradation.

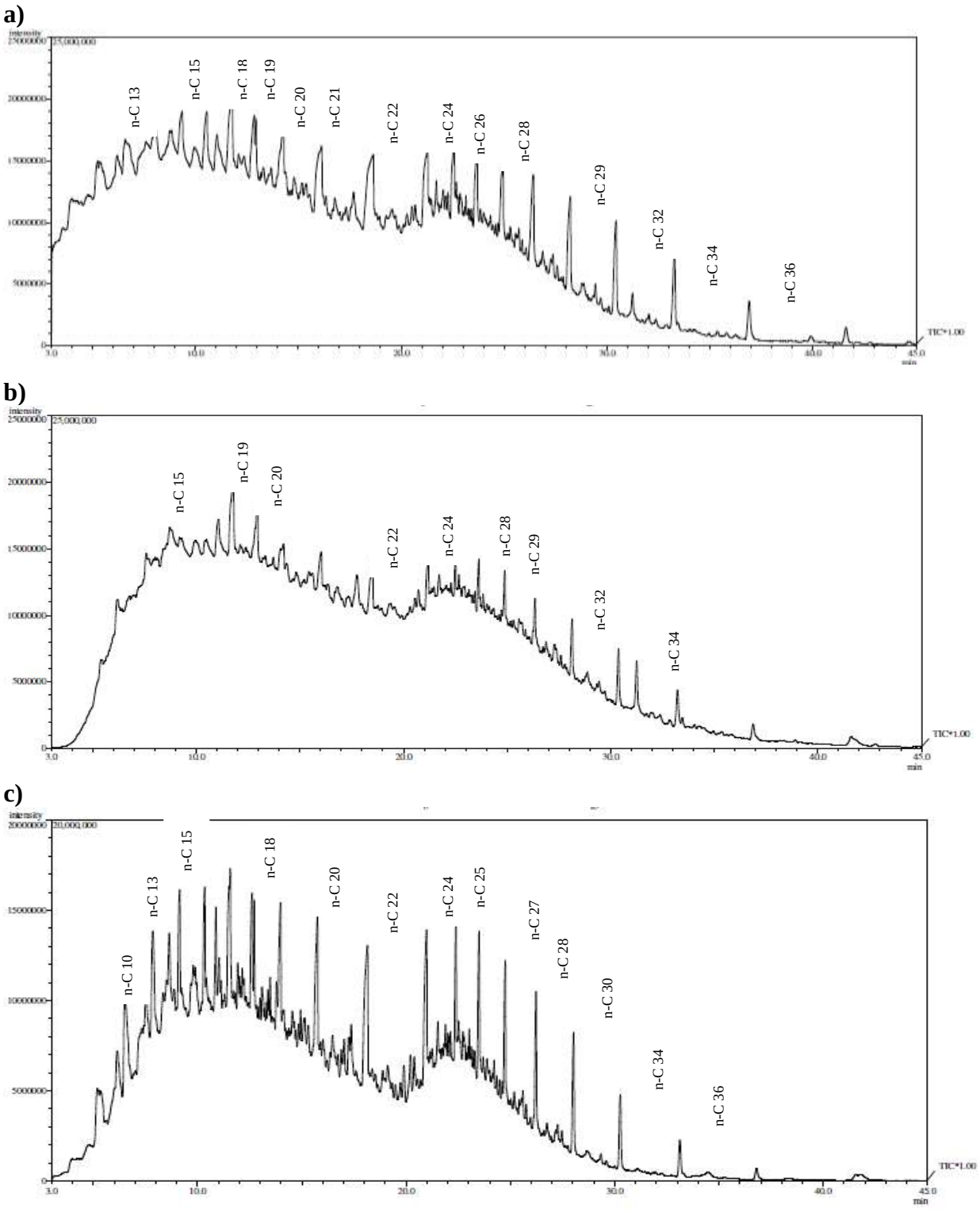


Figure 4.62 Chromatogram of residual diesel fuel in contaminated soil with 15% oil a) amended with 5% SC (T 30) b) amended with 5% SC (T 365) c) unamended control soil (T 365).

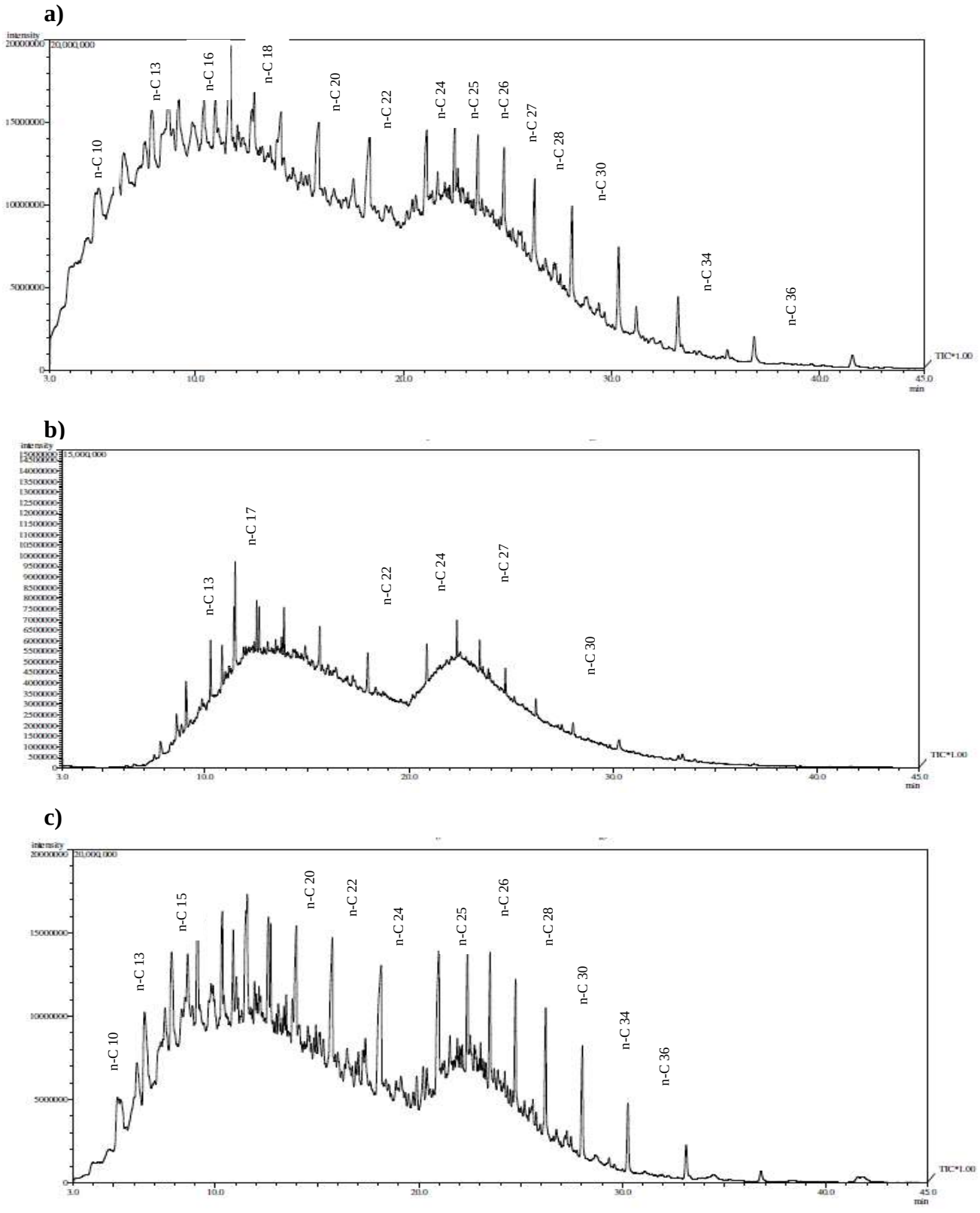


Figure 4.63 Chromatogram of residual diesel fuel in contaminated soil with 15% oil a) amended with 10% SC (T 30) b) amended with 10% SC (T 365) c) unamended control soil (T 365).

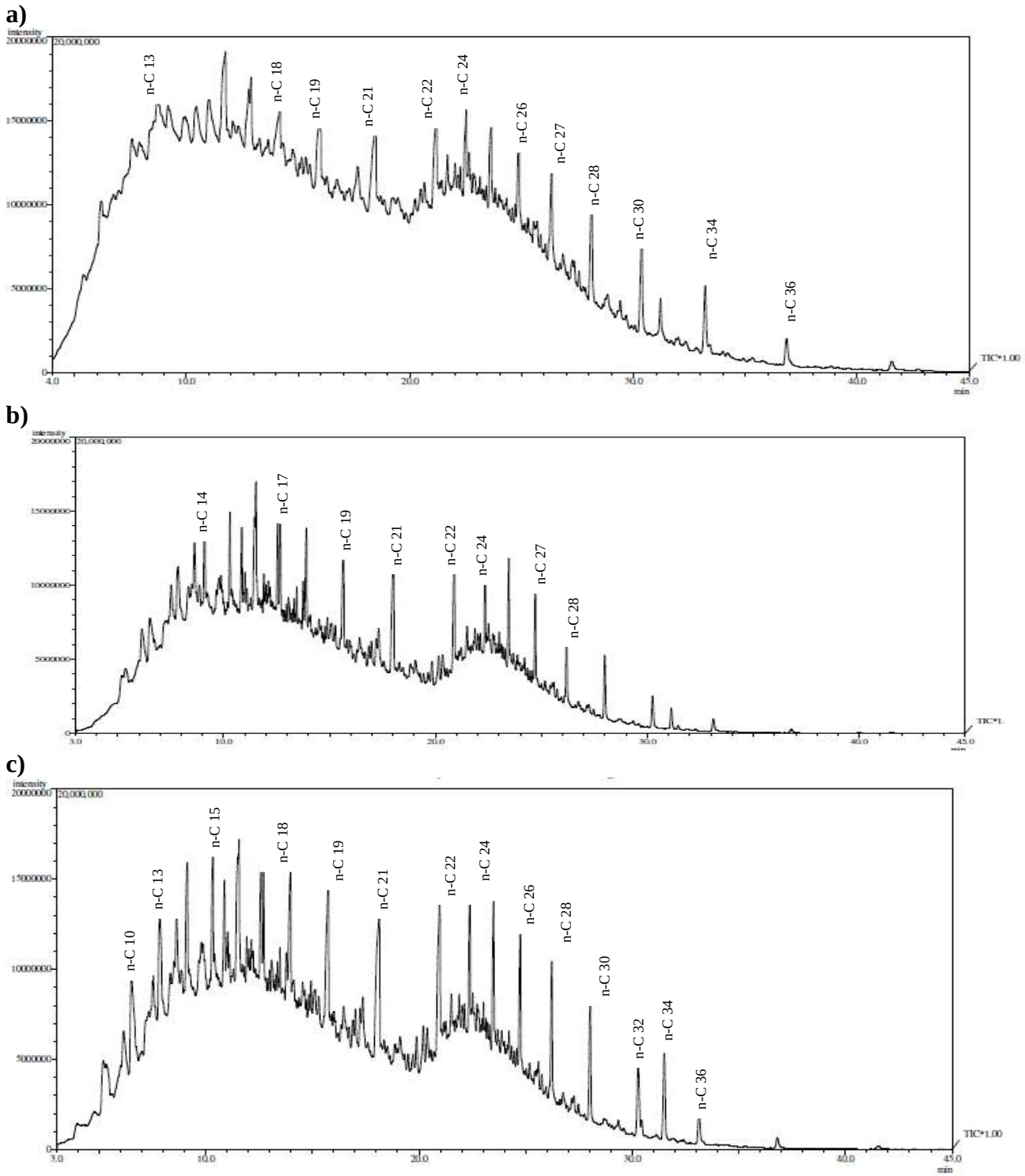


Figure 4.64 Chromatogram of residual diesel fuel in contaminated soil with 20% oil a) amended with 5% SC (T 30) b) amended with 5% SC (T 365) c) unamended control soil (T 365).

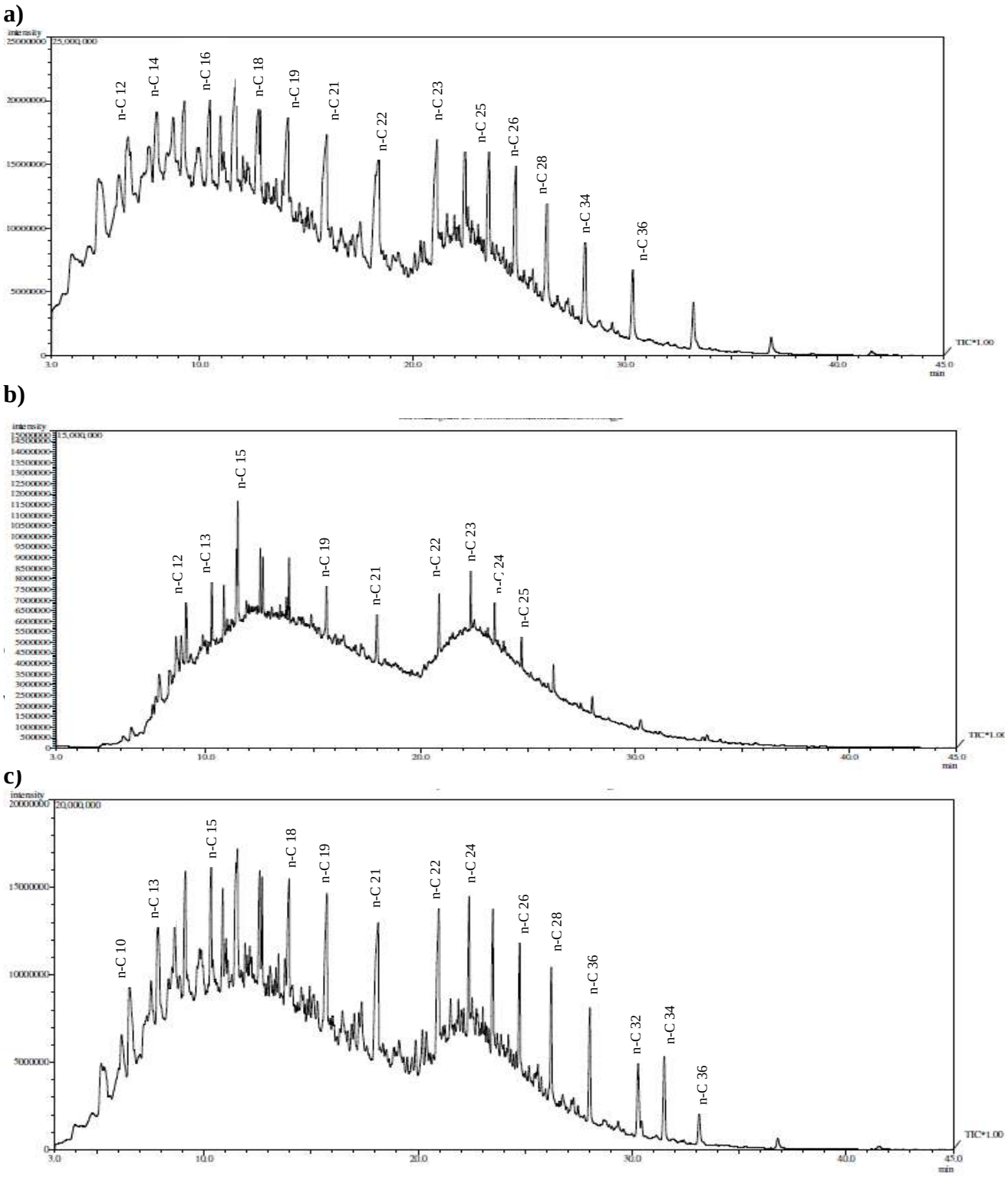


Figure 4.65 Chromatogram of residual diesel fuel in contaminated soil with 20% oil a) amended with 10% SC (T 30) b) amended with 10% SC (T 365) c) unamended control soil (T 365)

4.4 Result of phytodegradation of soil contaminated with diesel oil using *D. reflexa* under laboratory condition

4.4.1 Loss of diesel fuel in soil planted with *D. reflexa*

At the end of 270 days, the percentage of biodegradation in all different amendments and soil polluted with 1 % and 2.5% oil ranged between 13.8% - 98.8% and 11.1% - 90.3%, respectively (Figures 4.66 and 4.67). Oil loss was higher in soil treated with SC (98.8% and 90.3%) followed by soil amended with PS (94% - 85%) and TL (86.1% - 75.2%) in contaminated soil with 1% and 2.5% diesel fuel, respectively. The percentage of biodegradation in contaminated soil containing *Dracaena* without organic wastes recorded 62% and 52.4%, while in control soil without the plant recorded 26.6% and 24.4% oil loss in soil polluted with 1% and 2.5% diesel fuel, respectively during the period of study. About 13.8% and 11.1% oil loss was recorded in autoclaved control soil without plant and polluted with 1% and 2.5% diesel fuel, respectively. The reason for the degradation of oil in autoclaved control soil (without plant) might be some non-biological factors such as volatilization or photodegradation which was recorded in poisoned treatments amended with oil and sodium azide. In addition, the highest rate of biodegradation was recorded in soil amended with SC which is probably due to a higher amount of N and P (Table 4.1), compared with other organic waste amendments. It was also reported by pervious work that soil amended with SC showed a higher rate of degradation (Dadrasnia and Agamuthu, 2010, 2012). This result is similar to the finding of Palmroth et al., (2002) which indicated 60% oil loss in soil polluted with diesel fuel and amended with NPK fertilizer and planted with pine trees at the end of one month. Similarly, Dominguez-Rosado and Pichtel (2005) recorded 67% engine oil loss in contaminated soil planted with mustard and sunflower plants.

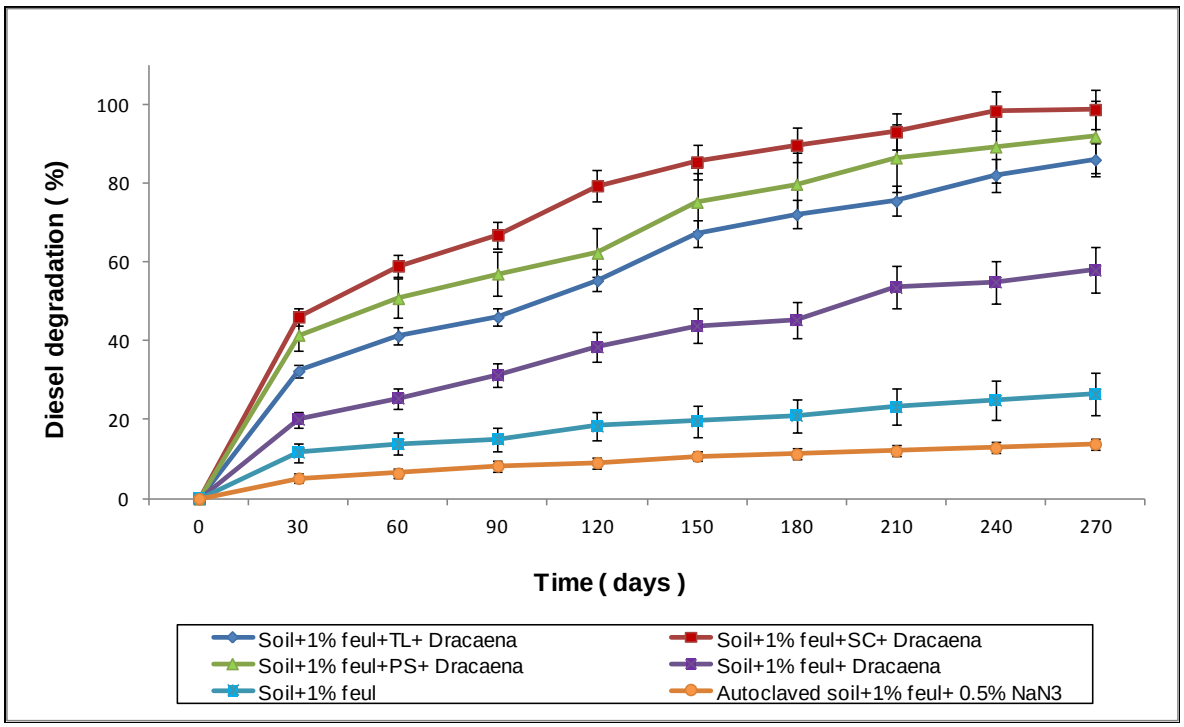


Figure 4.66 Biodegradation of diesel fuel in soil contaminated with 1% oil. Bars indicate standard error (n = 3).

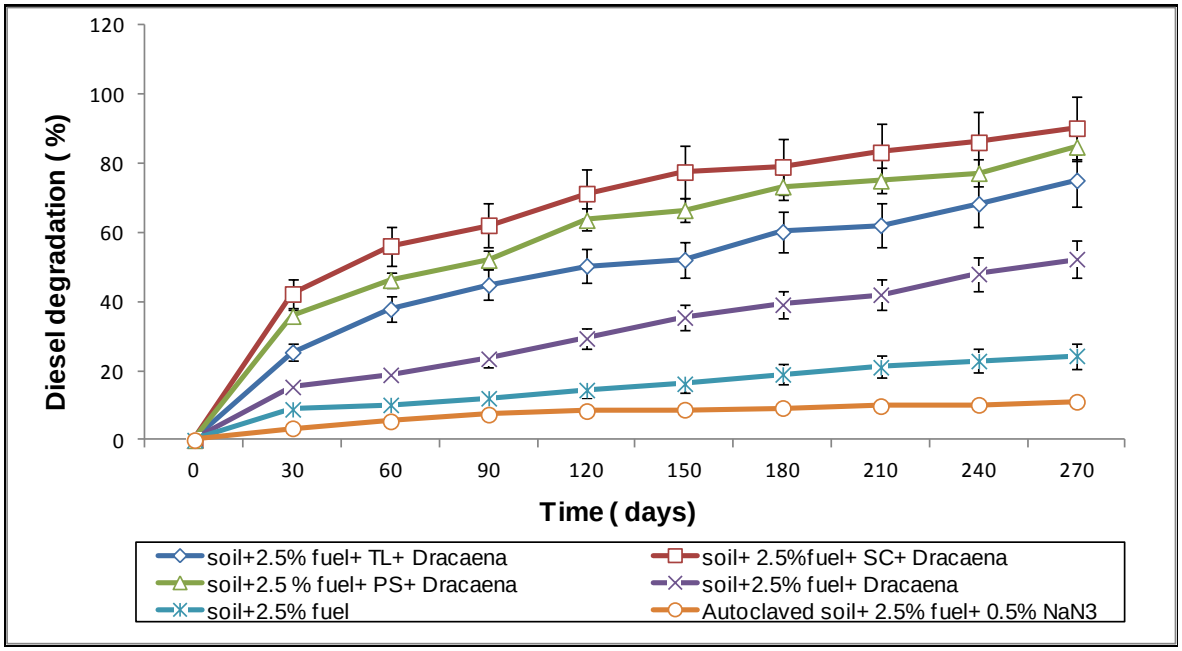


Figure 4.67 Biodegradation of diesel fuel in soil contaminated with 2.5% oil. Bars indicate standard error (n = 3).

A significant difference was observed between soil treated with different organic wastes, unplanted contaminated soil and contaminated soil planted with *Dracaena* at both concentrations ($P < 0.05$). Statistical analysis did not show significant difference in biodegradation of diesel fuel between soils amended with PS and SC amendments but there was significant difference ($P < 0.05$) in the results between soils amended with TL and another two different organic waste amendments. However, the results indicated that in all treatments amended with organic wastes the rate of oil loss was significantly higher than those of unamended and unplanted treatments. This is in agreement with the finding of Vouillamoz and Milke (2001) who indicated that compost addition allowed diesel loss down from 1200 to 200 mg TPH kg^{-1} in contaminated soil planted with ryegrass. Kim et al., (2010) also recorded a significant reduction during the phytodegradation of diesel-contaminated soil at the end of 120 days.

Phytoremediation experiment was monitored for 150 days only for 5% fuel because all the plants at this concentration died within 150 days. At the end of 150 days, oil loss in soil polluted with 5% diesel fuel and amended with SC recorded 19%, followed by soil amended with PS (16.4%) and TL (13.6%), while in contaminated soil without organic waste amendments and planted with *Dracaena* the rate of biodegradation was 8.5%. As Figure 4.68 shows in 120 and 150 days the percentage of oil loss remained stable in all amended treatments.

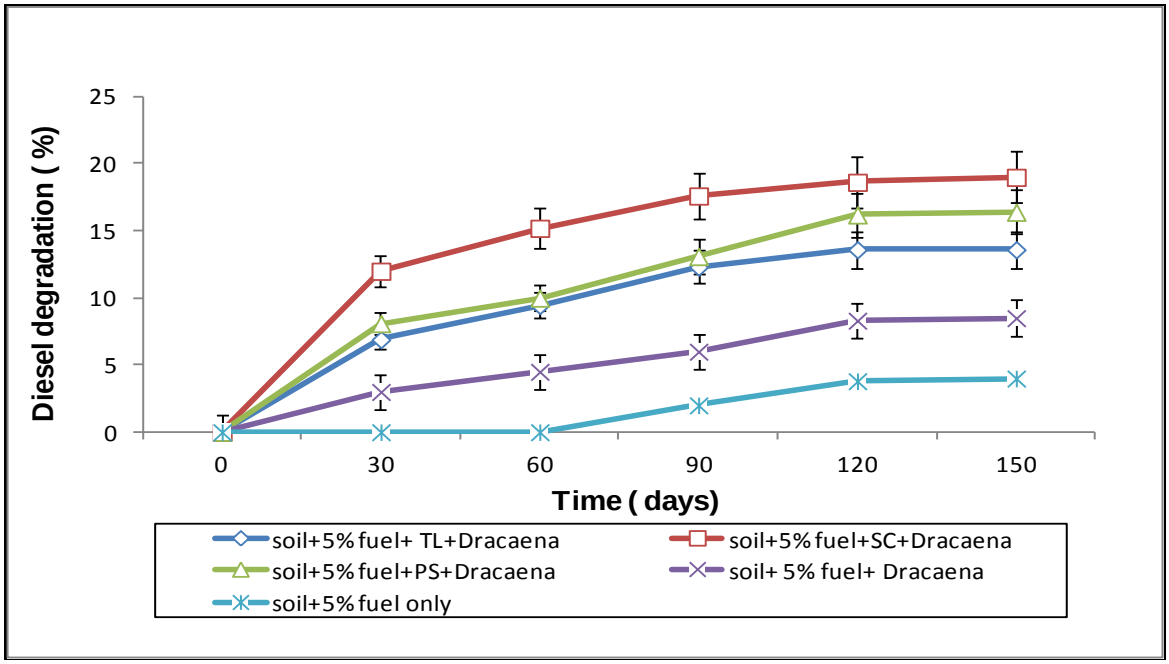


Figure 4.68 Biodegradation of diesel fuel in soil contaminated with 5% oil. Bars indicate standard error (n = 3).

4.4.2 Bacterial count

Aerobic heterotrophic bacteria (AHB) count in *Dracaena* remediated soil amended with SC ranged from 0.78 × 10^9 to 3.38 ×10^9 CFU/g soil, which is 18.8% and 39% higher than that of amended with PS and TL, respectively (Figures 4.69 and 4.70). At the end of 270 days, the count of diesel utilizing bacteria (DUB) soil amended with SC showed a higher microbial population (378 ×10^5 CFU/g soil and 355 ×10^5 CFU/g soil) in soil polluted with 1% and 2.5% diesel fuel, respectively (Figures 4.71 and 4.72). The treatment with only *Dracaena* plant without organic wastes amendment recorded low counts of AHB (172 × 10^5 CFU/g soil and 209 × 10^5 CFU/g soil) in 2.5% and 1% pollution, respectively. Also low counts of DUB and AHB were recorded in soil without plant and organic wastes. However, those treatments amended with SC had a higher number of AHB and DUB in both concentrations of diesel fuel.

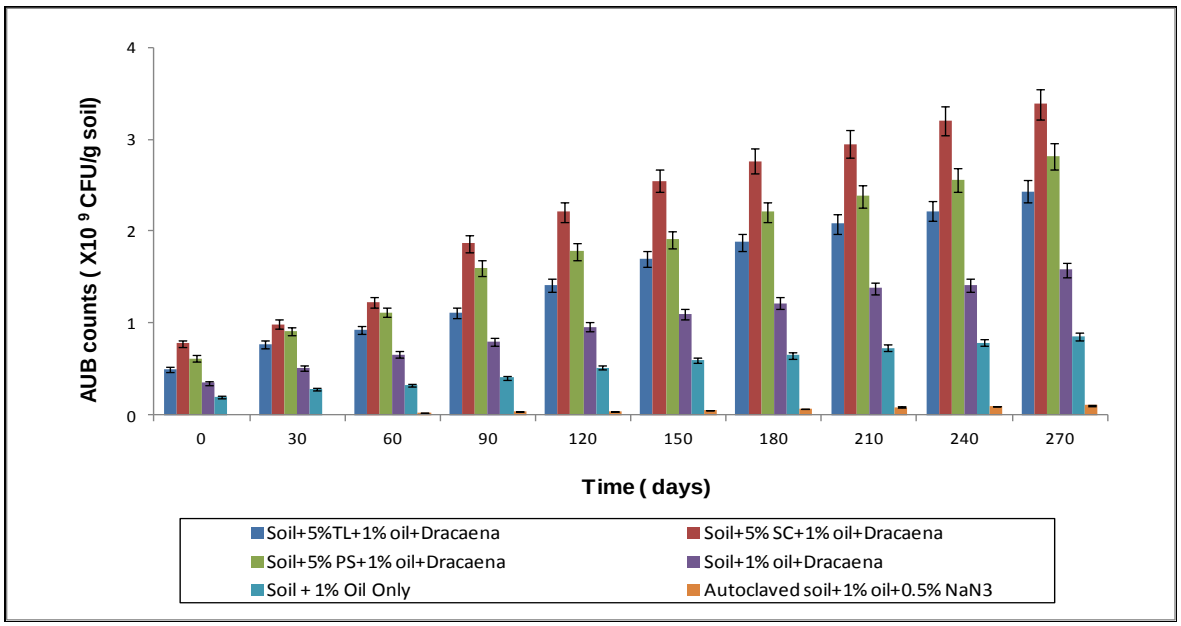


Figure 4.69 Total CFU of aerobic heterotrophic bacterial (AHB) in soil contaminated with 1% diesel fuel. Bars indicate standard error (n = 3).

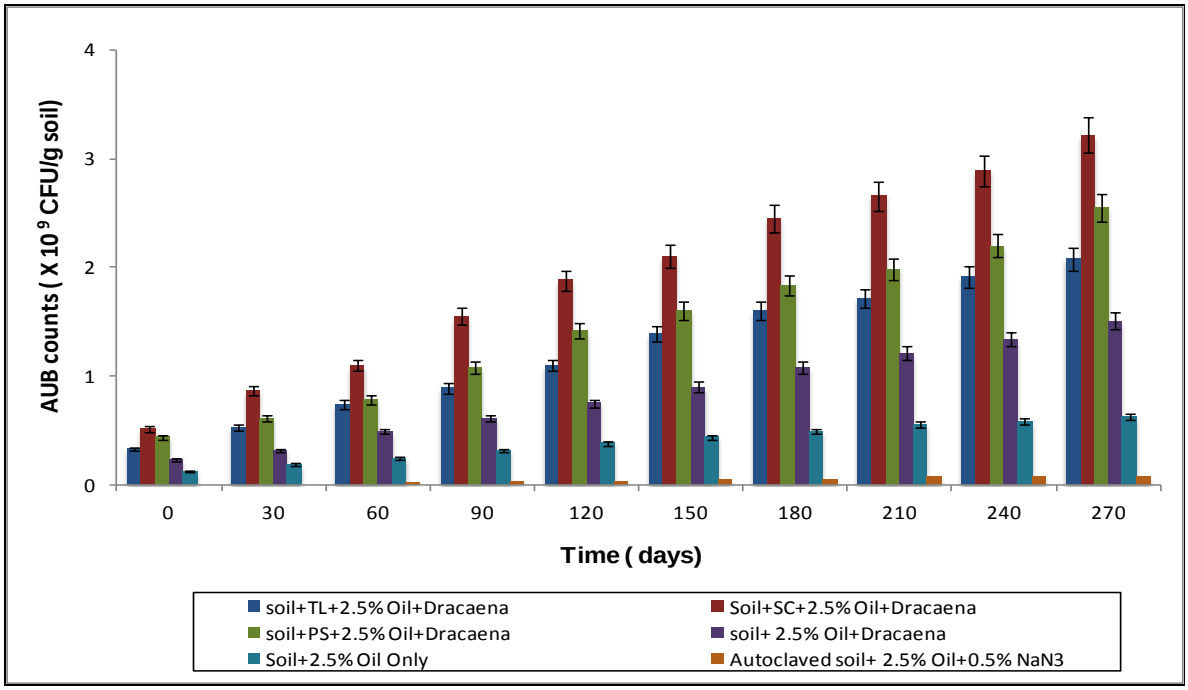


Figure 4.70 Total CFU of aerobic heterotrophic bacterial (AHB) in soil contaminated with 2.5% diesel fuel. Bars indicate standard error (n = 3).

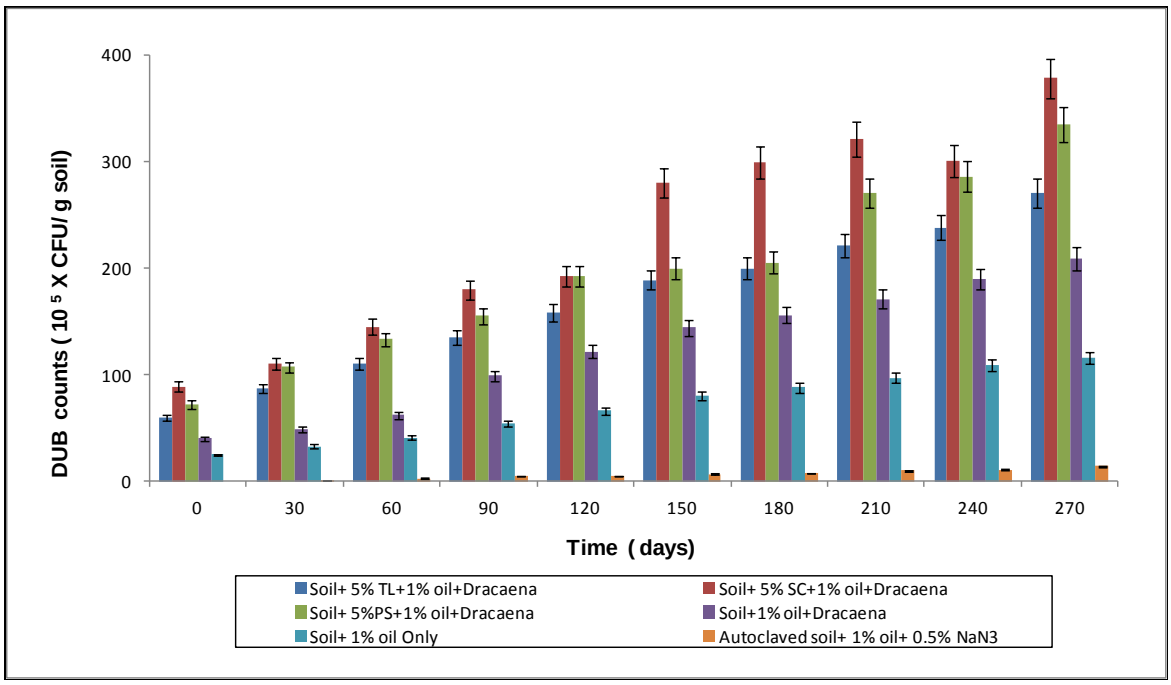


Figure 4.71 Total CFU of diesel utilizing bacterial (DUB) in soil contaminated with 1% diesel fuel. Bars indicate standard error (n = 3).

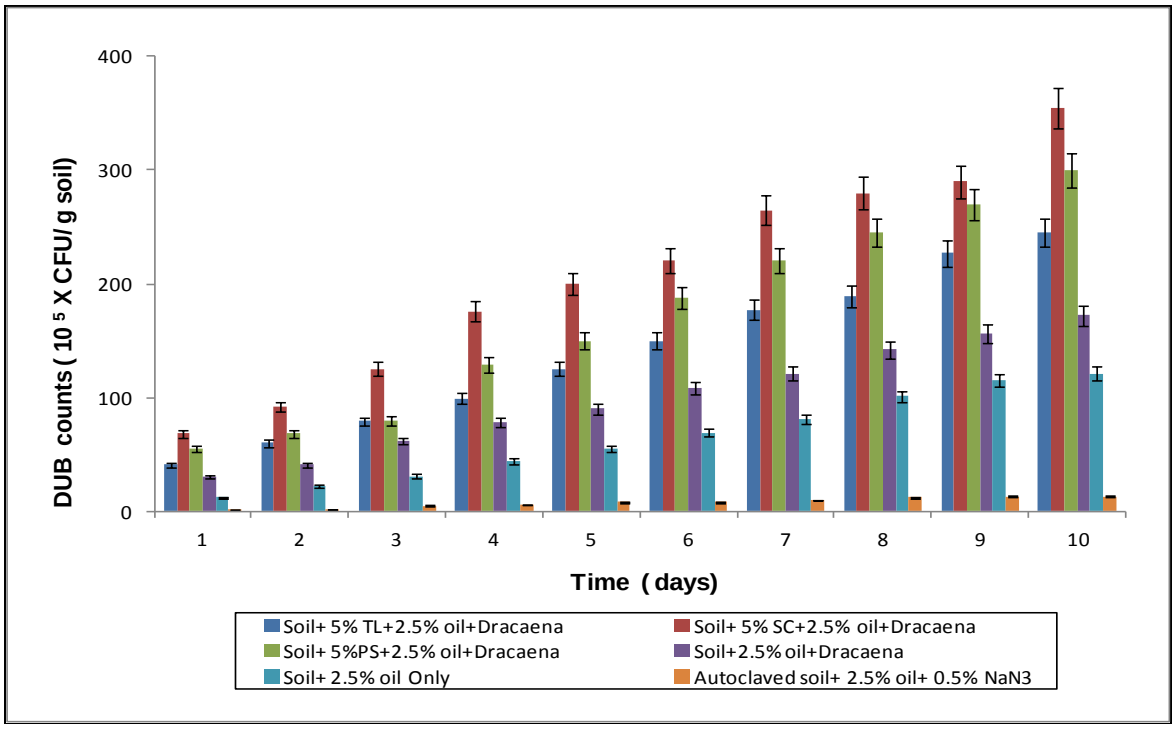


Figure 4.72 Total CFU of diesel utilizing bacterial (DUB) in soil contaminated with 2.5% diesel fuel. Bars indicate standard error (n = 3).

The reason for the increase in microbial population of DUB and AHB in contaminated soil with diesel fuel and amended with different organic wastes might be due to the quantities of nutrients in the organic wastes especially N and P that enhanced the multiplication of bacteria in the soil. The results are similar to the findings of Muratova et al., (2003) who reported heterotrophic microorganisms that are able to degrade oil was significantly increased by plant compared with no plant contaminated soil. They observed that the amount of PAH oxidizing microorganisms was seven times higher in the rhizosphere of alfalfa and in the case of reed it was four times lower in the rhizosphere than controlled soil (Muratova et al., 2003). The DUB isolated from the contaminated soil was identified as species of the *Pseudomona sp., Bacillus amyloliquefaciens* and *Micrococcus sp*.. These bacterial species together with root exudates of the *Dracaena* plants possibly helped in the removal of diesel fuel from the soil.

4.4.3 pH of soil in *D. reflexa* remediation under laboratory condition

The pH of soil remediated with the *D. reflexa* at 1%, 2.5% and 5% are shown in Figure 4.73 – 4.75. The pH of the soils ranged from slightly acidic to alkaline (6 to 8.7). The pH of autoclaved control soil was alkaline compared with other treatments. This might be due to the addition of sodium azide, while pH of treatments amended with organic wastes show a slightly acidic condition which might be because plants grow better in soil amended with organic wastes than other treatments. It may be because of high metabolic activities of microorganisms which produce an acidic in condition for rhizosphere. The range of pH was higher in soil polluted with 1% diesel compared with those contaminated soil with 2.5% and 5%, which might be as a result of high microbial activity in treatments amended with

1% diesel oil. It has been proven that microbial activities produce acidic radicals during the degradation process (Singh and Sharma, 2008).

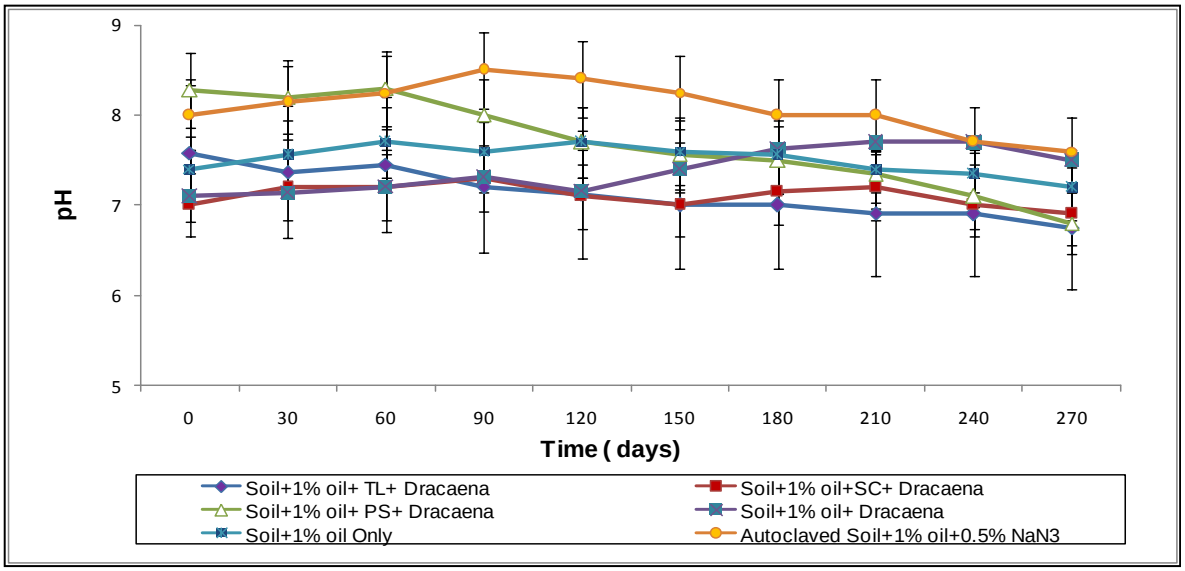


Figure 4.73 pH of soil contaminated with 1% diesel fuel planted with *D. reflexa*. Bars indicate standard error (n = 3).

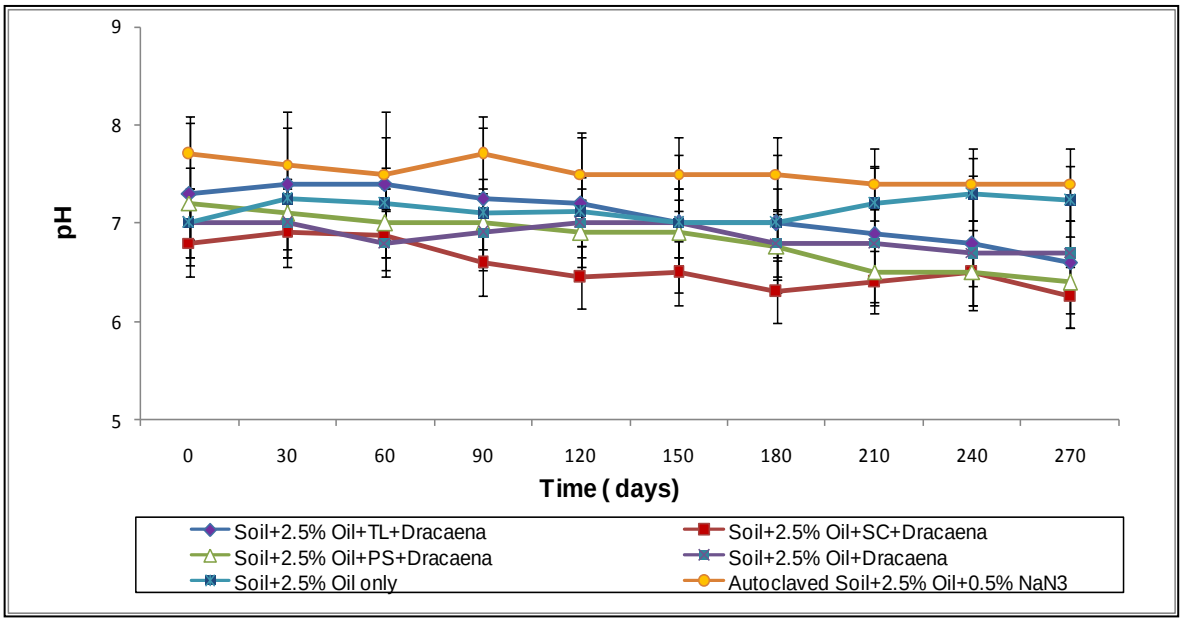


Figure 4.74 pH of soil contaminated with 2.5% diesel fuel planted with *D. reflexa*. Bars indicate standard error (n = 3).

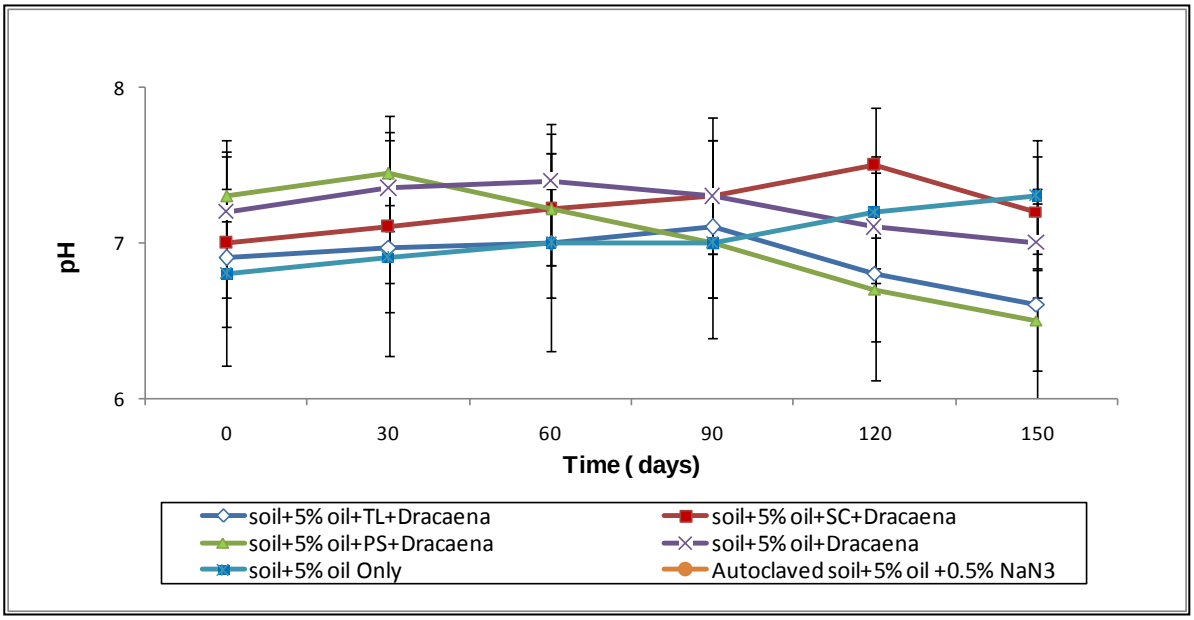


Figure 4.75 pH of soil contaminated with 5% diesel fuel planted with *D. reflexa*. Bars indicate standard error (n = 3).

4.4.4 Plant growth and Biomass production under laboratory condition

The response of the *D. reflexa* plant to 1, 2.5 and 5 % concentrations of diesel was monitored throughout the 270 days of the experiment. No plant death was recorded in the 1% diesel fuel; however, some of the plants in the 2.5% fuel showed signs of phytotoxicity such as yellowing of leaves and stunted growth compared with the controlled. The experiment was monitored for 150 days only for 5% fuel because all the plants at this concentration died within 150 days. The results are in line with the findings of Vouillamoz and Mike (2001), who reported a reduced growth rate in ryegrass planted in diesel-contaminated soil. The pictures of the *D. reflexa* plant are shown in Plate 4.1.

A **B**

C **D**

E

Plate 4.1 *D. reflexa*, A: *Dracaena* amended with TL, B: *Dracaena* amended with SC, C: *Dracaena* amended with PS, D: Control Plant, E: Phytotoxicity effect of oil on *Dracaena*

In order to further assess the endurance of the plant species to petroleum-contaminated soil, the *D. reflexa* was planted in soil containing different concentrations of petroleum contaminants. Different parameters including stem height, fresh weight, dry weight, root length, root weight, and growth rate were measured and recorded. At the initial three weeks of culture, all plants growing in petroleum contaminated soil showed no visible differences in appearance with those in the corresponding controls, although there was a little inhibition of the plants growing in treatment with 5% fuel, compared with those planted in clean soil. After 270- days' exposure, the highest of the *D. reflexa* longitudinal growth was observed in the amendment with SC contaminated soils with 1% and 2.5 % of petroleum hydrocarbons which was 20% and 36% higher than that of the plants growing in clean soil, respectively. The biomass of 2.5% oil contaminated soil (SC amended) was 1.8 and 4.7 times more than treatment amended with PS and TL, respectively. In addition, the result indicated that the biomass of treatment at 1% oil amended with SC was 1.8 and 2.7 times more than the treatment amended with PS and TL, respectively. It was followed by increasing biomass to 1.1 times more in SC amendment at 1% diesel fuel compared with unamended soil. The development of the plants during the 270-day culture period was also evaluated by measuring the dry weight of the plants. It was observed that the biomass of the *D. reflexa* growing in soil amended with SC and 1% diesel did not decrease significantly as compared with that in the corresponding control, although the change in the biomass of the plants depended on the level of petroleum contaminants in soil (Table 4. 23). Noticeably, there was 65% decrease in the biomass of the plants growing in high concentration (5% diesel fuel) of petroleum contaminants; *D. reflexa* species was still alive although some chlorosis was observed in the leaves. Thus, *D. reflexa* is likely to phytoremediate diesel fuel contaminated soil with a concentration $\leq$ 5% (50g/kg) on the basis of the endurance of the species.

Table 4.23 Dry mass of *Dracaena* plant parts at the end of experiment (270 days)

Treatment	Dry weight (g)		
	Leaves	Stem	Roots
A	4.3 ± 0.9	3.0± 0.3	1.2± 0.2
B	8.4± 1.2	9.3± 0.6	5.5±0.1
C	5.8±0.2	4.1±1.6	3.1±0.6
D	1.6±0.7	2.6± 1.1	0.9±0.3
G	1.8±1.2	0.8±0.2	0.5±0.2
H	5.6 ± 1.1	6.7± 0.3	2.3± 0.6
I	3.3 ± 1.4	2.8± 0.7	1.9± 0.1
J	1.1 ± 0.7	1.8± 0.6	0.6± 0.2
M	0.7 ± 0.4	0.4± 0.1	0.4± 0.6
N	2.1 ± 0.8	3.3± 0.2	1.2± 0.2
O	1.7 ± 0.9	1.3± 0.7	0.8± 1.1
P	0.6 ± 0.1	0.2± 0.6	0.4± 0.2
S	7.6 ± 0.2	9.2± 1.5	4.8± 0.3

A, soil + 1% oil + TL; B, soil + 1% oil + SC; C, soil + 1% oil + PS; D, soil + 1% oil only; G, soil + 2.5% oil + TL; H, soil + 2.5% oil + SC; I, soil + 2.5% oil + PS; J, soil + 2.5% oil only; M, soil + 5% oil + TL; N, soil + 5% oil + SC; O, soil + 5% oil + PS; P, soil + 5% oil only; S, control soil i.e. without oil contamination. **(**Values expressed as mean and standard deviation = 3).

4.4.5 Plant uptake of hydrocarbons

Hydrocarbon concentration in shoot and root tissue was analyzed to determine if phytoaccumulation and phytodegradation played a role in diesel fuel removal mechanism. The GC/MS analysis of the plant extract did not show the presence of hydrocarbons in all the treatments. This is in sharp contrast to the results of Palmroth et al., (2002) who observed an uptake of diesel oil by grass root. The difference might be due to the different plants used in these studies; it might also be due to differences in the weather conditions. Palmroth et al., (2002) work was conducted in a cold temperate zone of Finland, while this study was conducted in the tropical zone (Malaysia). Radwan et al., (2000) found that long-chain hydrocarbons accumulated in broad bean (*Vicia faba*), grown in oily soil. The accumulation, especially in the seeds, was thought to pose a risk to human or animal nutrition (Radwan et al., 2000). The result suggests that the mechanism of hydrocarbon removal by the *Dracaena* plants may be via rhizodegradation which has been well documented (Abhilash et al., 2009; Gerhardt et al., 2009). Also, the removal of the oil may be the result of root exudates produced by the *D.reflexa* plant which enhanced the activities of soil microorganisms in mineralizing the oil in the soil. This is supported by the findings of different researchers, who have stated that flavonoids and other compounds released by roots can stimulate growth and activity of hydrocarbon degrading bacteria (Chaudhry et al., 2005; Leigh et al., 2006). In addition, root growth and death are known to promote soil aeration which can enhance oxidative degradation of organic contaminants (Kuiper et al., 2004; Leigh et al., 2002).

4.5 Results of phytodegradation of soil contaminated with diesel using *P.polystachyus* under laboratory conditions

4.5.1 Loss of diesel fuel in soil planted with *P.polystachyus*

The loss of diesel fuel in soil treatment contaminated with 1%, 2.5% and 5% oil are shown in Figures 4.76 to 4.78. The loss of diesel fuel at the end of 270 days in soil contaminated with 2.5% and 1% oil ranged from 12– 84% and 13 – 91%, respectively in all the different treatments. Contaminated soil treated with SC recorded the highest loss of oil (84% and 91%) in 270 days followed by soil treated with PS (72% and 79%) in 2.5% and 1% contaminated soil respectively. The contaminated soil containing only *Podocarpus* plant, without organic wastes treatment recorded 43% and 53% oil loss while control soil without *Podocarpus* plant showed 23% and 26% oil loss in 2.5% and 1% contaminated soil, respectively at the end of 270 days.

About 12% and 13% oil loss in soil contaminated with 2.5% and 1% oil may be due to non biological factors like evaporation; this was recorded in autoclaved soil treated with sodium azide after 270 days. The high loss of oil in soil treated with SC and *Podocarpus* plants may be due to the presence of appreciable nitrogen (1.3%) and phosphorus (0.9%) contents in SC (Table 4.1), and this was also recorded in our previous works, where soil amended with SC recorded 78 % loss of diesel fuel (Dadrasnia and Agamuthu, 2010). A significant difference was observed between soil treated with different organic wastes, unplanted contaminated soil and contaminated soil planted with *Dracaena* at both concentrations ($P < 0.05$). Statistical analysis does not show any significant difference in biodegradation of diesel fuel between soils amended with PS and SC but there was a

significant difference ($P < 0.05$) in the results between soils amended with TL and two other different organic waste amendments.

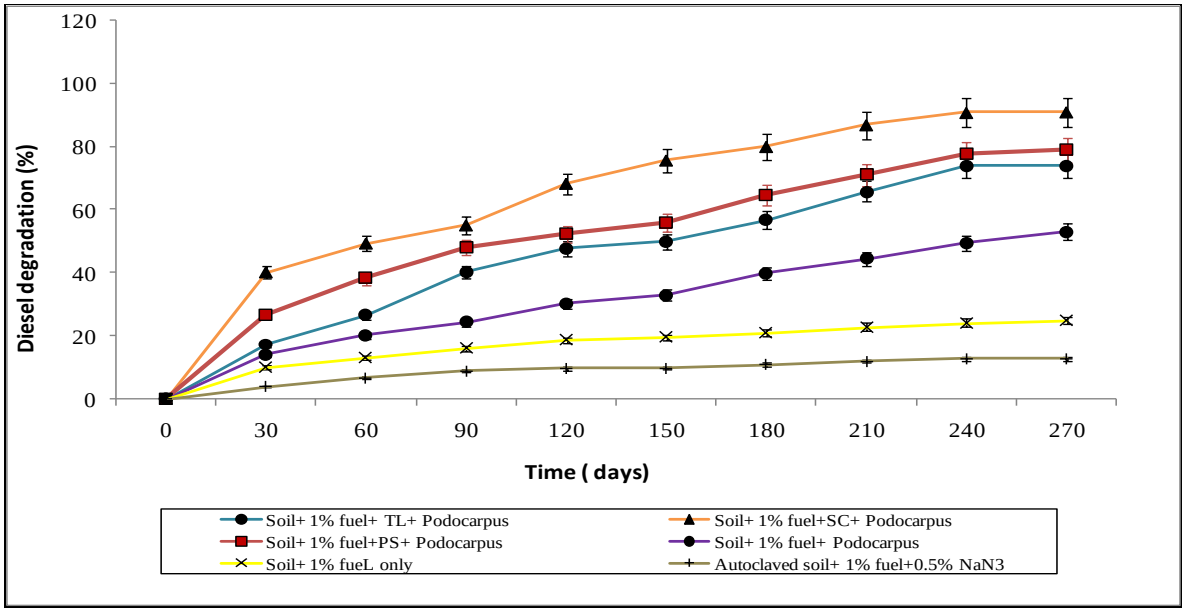


Figure 4.76 Biodegradation of diesel fuel in soil contaminated with 1% oil. Bars indicate standard error (n = 3).

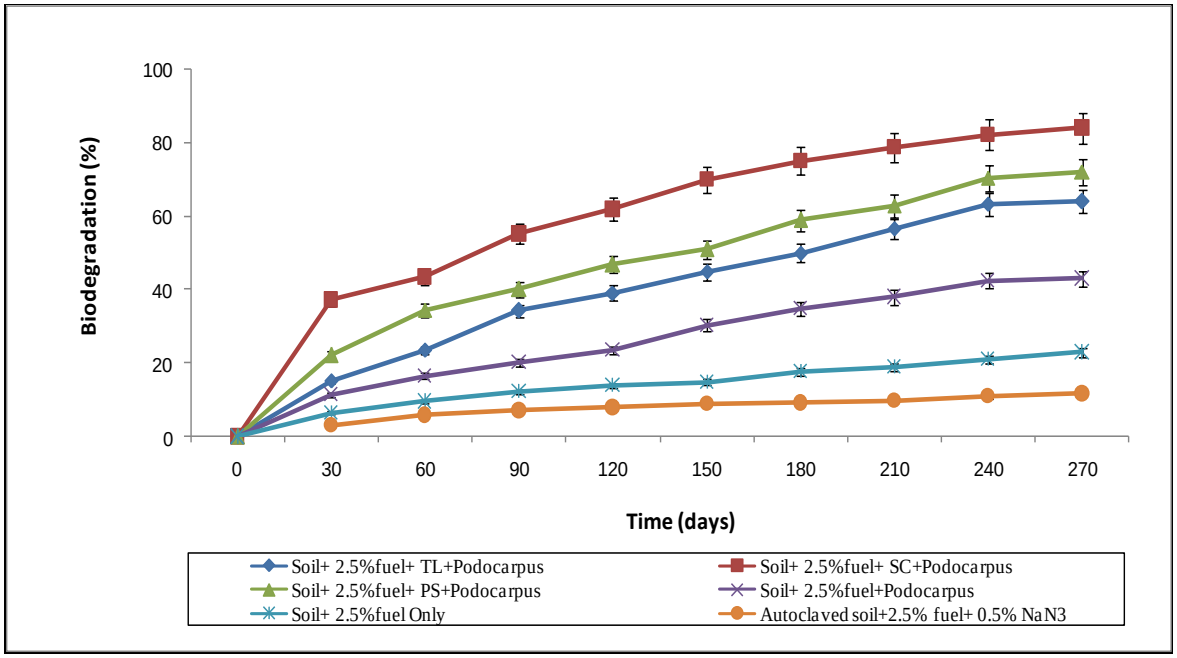


Figure 4.77 Biodegradation of diesel fuel in soil contaminated with 2.5% oil. Bars indicate standard error (n = 3).

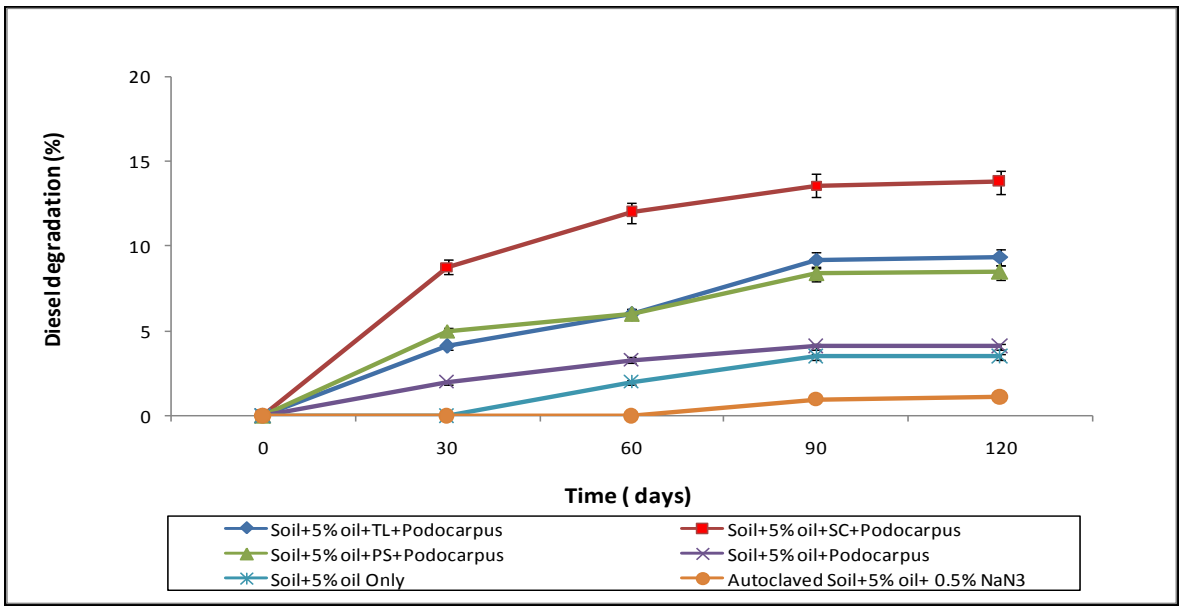


Figure 4.78 Biodegradation of diesel fuel in soil contaminated with 5% oil. Bars indicate standard error (n = 3).

All treatments amended with organic wastes showed rate of oil loss was significantly higher than those in unamended and unplanted treatments. The results are similar to these of Lu et al., (2010) who reported 32% degradation of petroleum in soil planted with *Eleusine indica* and only 5% of PAHs had dissipated in the unvegetated treatments. The finding of Diab (2008) demonstrated the effect of plant roots in biodegradation of diesel oil. He showed that TPH biodegradation was enhanced in the rhizosphere soil of the legume plant (*Vicia faba*) as compared with *Zea mays* and *Triticum aestivuml.* Yateem et al., (2000) investigated the degradation of total petroleum hydrocarbon (TPH) in the rhizospheric and nonrhizospheric soil of three plants, ryegrass (*Lolium perenne),* alfalfa (*Medicaga sativa*) and broad beans (*Vicia faba*), and the result showed that TPH degradation in the soil cultivated with alfalfa and broad beans was 35.8 and 36.6 %, respectively, compared with 24% degradation in ryegrass.

The experiment was monitored for 120 days only for 5% fuel because all the plants at this concentration died within 120 days. Oil loss in soil polluted with 5% diesel fuel and amended with SC recorded 13.8%, followed by soil amended with PS (8.5%) and TL (9.4%), while in contaminated soil without organic waste amendments and planted with *Podocarpus* the rate of biodegradation was 4.1% (Figure 4.77). The percentage of biodegradation in soil amended with TL was slightly higher (0.9%) than those treated with PS.

4.5.2 Bacterial count

The microbial populations of AHB in 1% and 2.5% diesel contaminated soil, planted with *Podocarpus,* are show in Figure 4.79 and 4.80, respectively. The microbial count of AHB recorded 11×10^7 CFU/g soil to 281×10^7 CFU/g soil and 9×10^7 CFU/g soil to 252×10^7 CFU/g soil, in soil contaminated with 1% and 2.5% diesel oil, respectively. Sun et al., (2011) also illustrated an increase in the soil's microbial community during the biodegradation PAH- contaminated soil by alfalfa (*Medicago sativa L.*). The rapid increase in total aerobic hydrocarbon bacteria after contamination of soil could be attributed to the availability of carbon source from the diesel oil (Lawson et al., 2012).

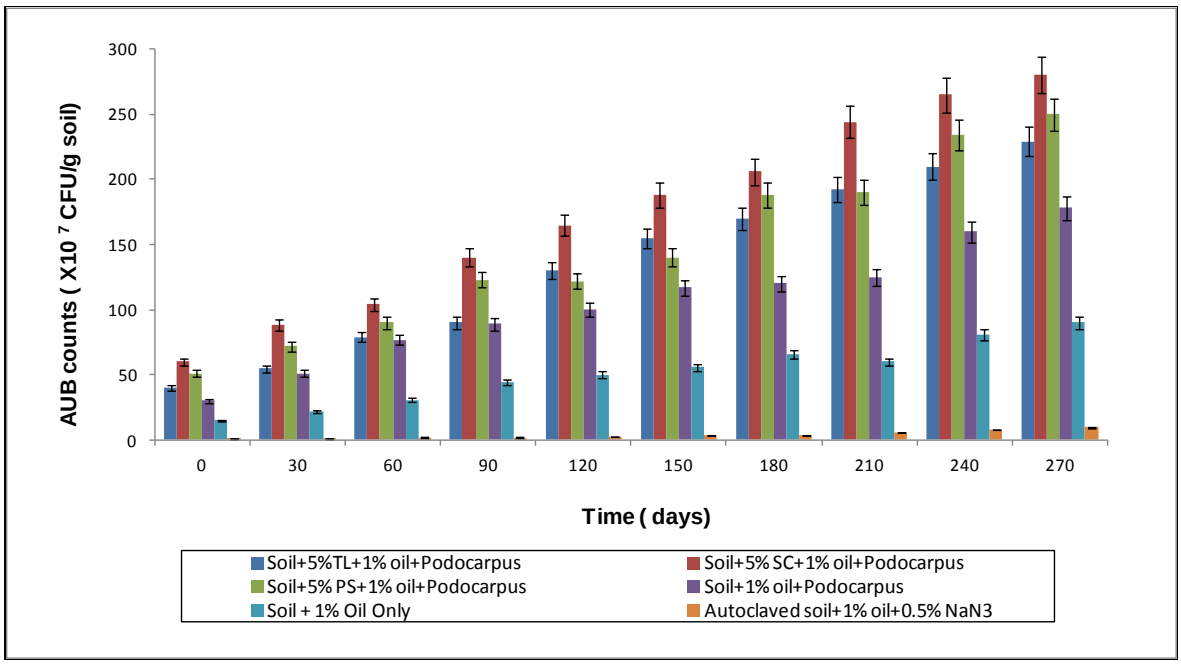


Figure 4.79 Total CFU of aerobic heterotrophic bacterial (AHB) in soil contaminated with 1% diesel fuel. Bars indicate standard error (n = 3).

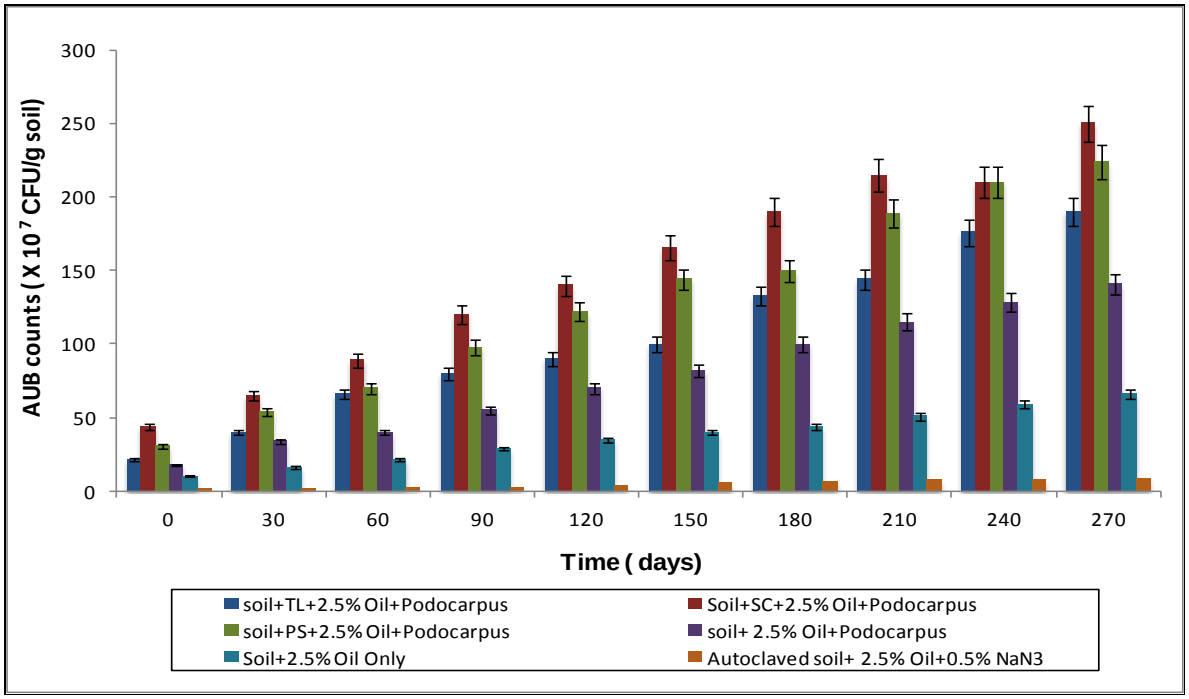


Figure 4.80 Total CFU of aerobic heterotrophic bacterial (AHB) in soil contaminated with 2.5% diesel fuel. Bars indicate standard error (n = 3).

The counts of diesel utilizing bacteria (DUB) in soil contaminated with 1% and 2.5% diesel fuel are shown in Figures 4.81 and 4.82, respectively. Contaminated soil treated with SC and *Podocarpus* remediation shows high counts of DUB (272 $\times 10^5$ CFU/g and 301 $\times$ 10^5 CFU/g) in both soil samples contaminated with 2.5% and 1% oil respectively. This is similar to the findings of Ijah and Antai (2003a), whereas the treatment with only *Podocarpus* plant without organic waste amendments recorded low counts of DUB (150 $\times$ 10^5 CFU/g and 180 $\times$ 10^5 CFU/g) in 2.5% and 1% pollution respectively. The reason for the increase in the counts of DUB in contaminated soil amended with organic wastes might be due to the presence of nutrients in the organic wastes, especially nitrogen and phosphorus that enhanced bacteria population in the soil.

DUB isolated from the contaminated soil was identified as species of *Pseudomonas, Streptococcus sinensis* and *Bacillus amyloliquefaciens*. These bacterial species have been implicated in hydrocarbon degradation by different researchers (Van Hamme et al. 2003; Bento et al., 2005). These bacterial species together with root exudates of plants possibly help in the removal of diesel fuel from the soil.

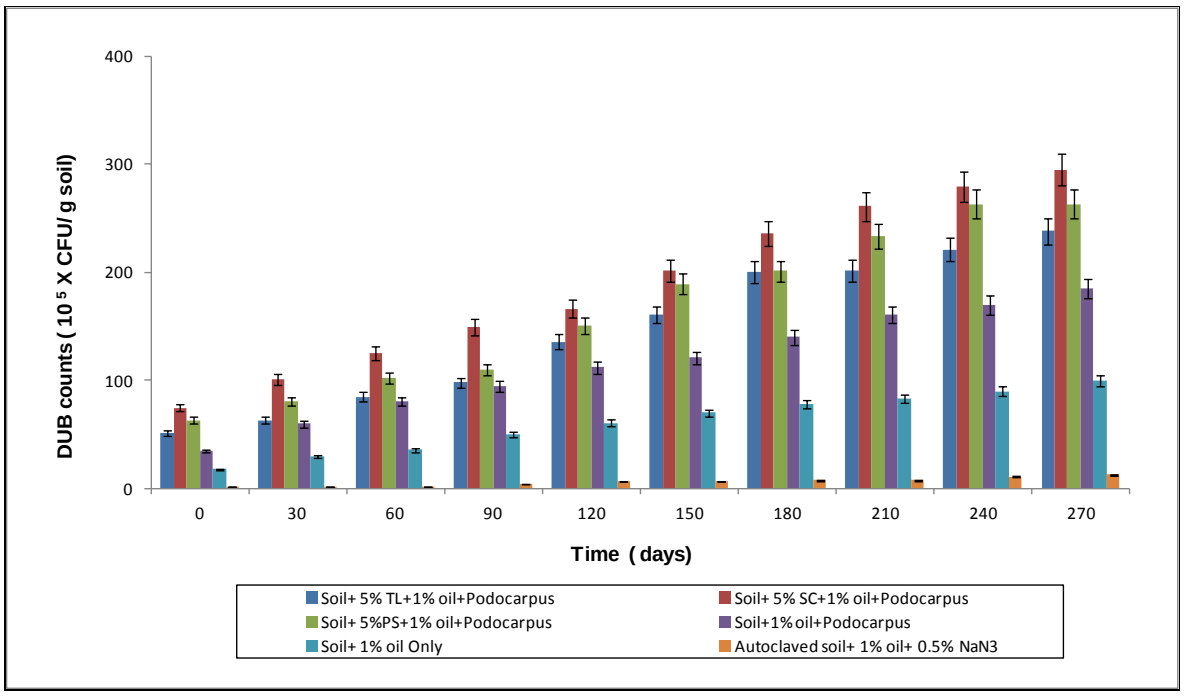


Figure 4.81 Total CFU of diesel utilizing bacterial (DUB) in soil contaminated with 1% diesel fuel. Bars indicate standard error (n = 3).

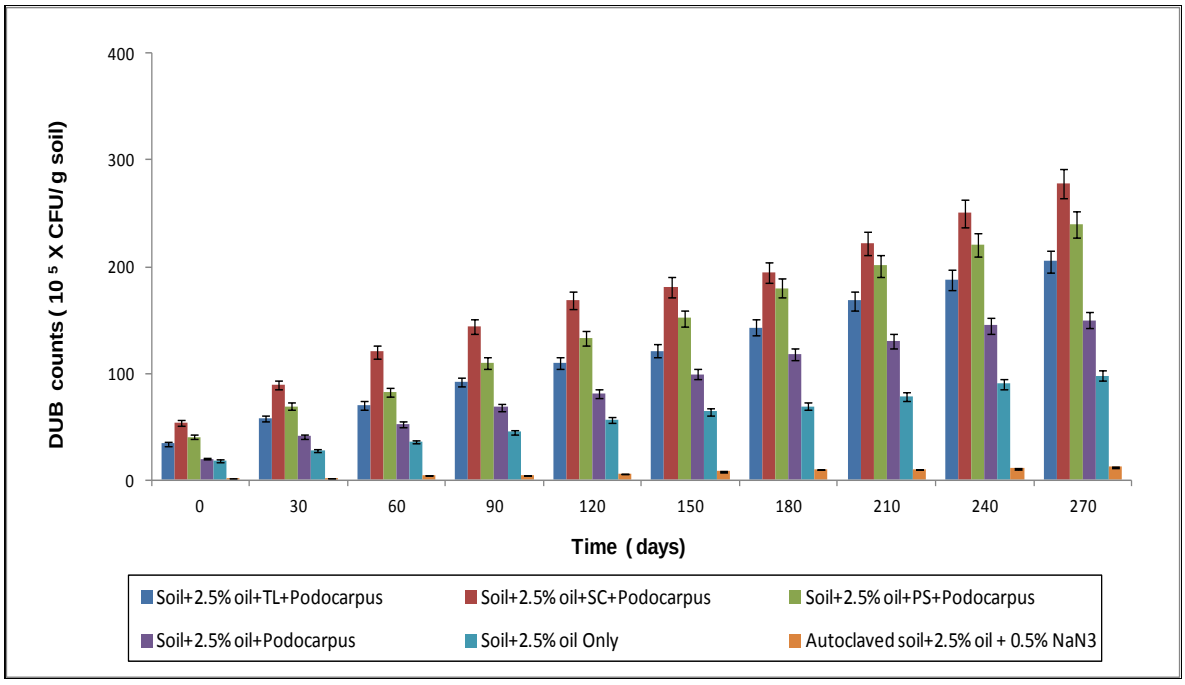


Figure 4.82 Total CFU of diesel utilizing bacterial (DUB) in soil contaminated with 2.5% diesel fuel. Bars indicate standard error (n = 3).

4.5.3 pH of soil in *P.polystachyus* remediation

The pH of the soils varies from slightly acidic to alkaline (6 to 8) (Figures 4.83 and 4.84) during a period of 270 days. The pH of soil amended with SC and TL was slightly acidic compared with other treatments. Decrease in pH might be due to high metabolic activities of microorganisms which produced acidic condition in the rhizosphere (Gadd, 2010). The range of pH was higher in soil polluted with 1% compared with the soil contaminated with 2.5% and 5%, which might be as a result of high microbial activity in treatments amended with 1% diesel oil.

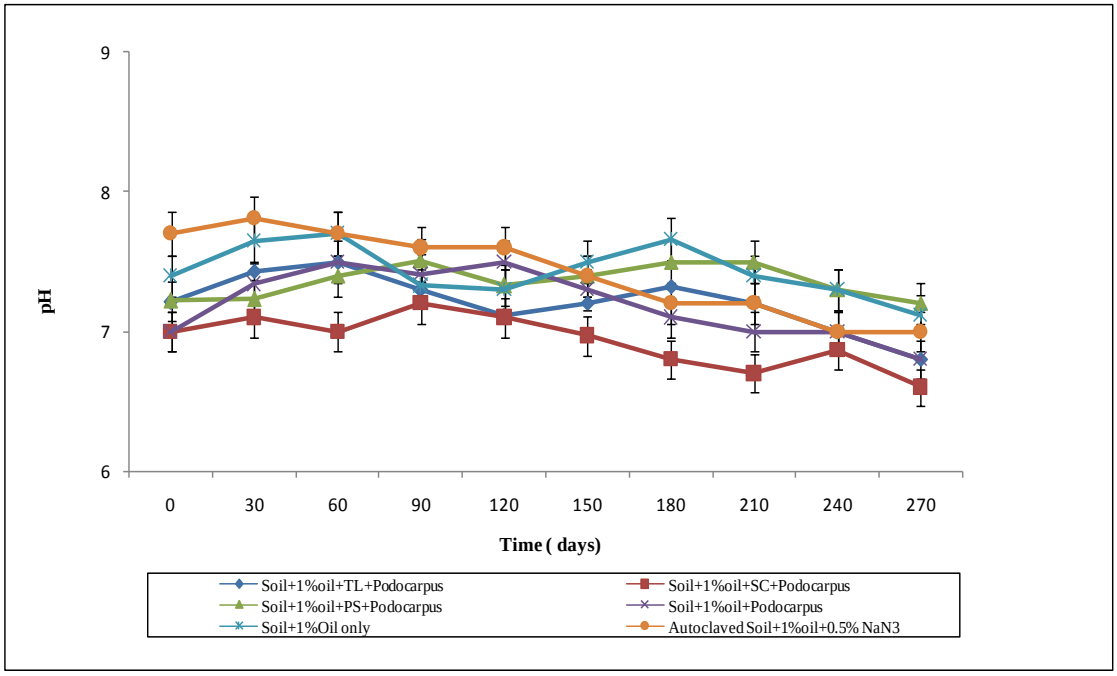


Figure 4.83 pH of soil contaminated with 1% diesel fuel planted with *P.polystachyus*. Bars indicate standard error (n = 3).

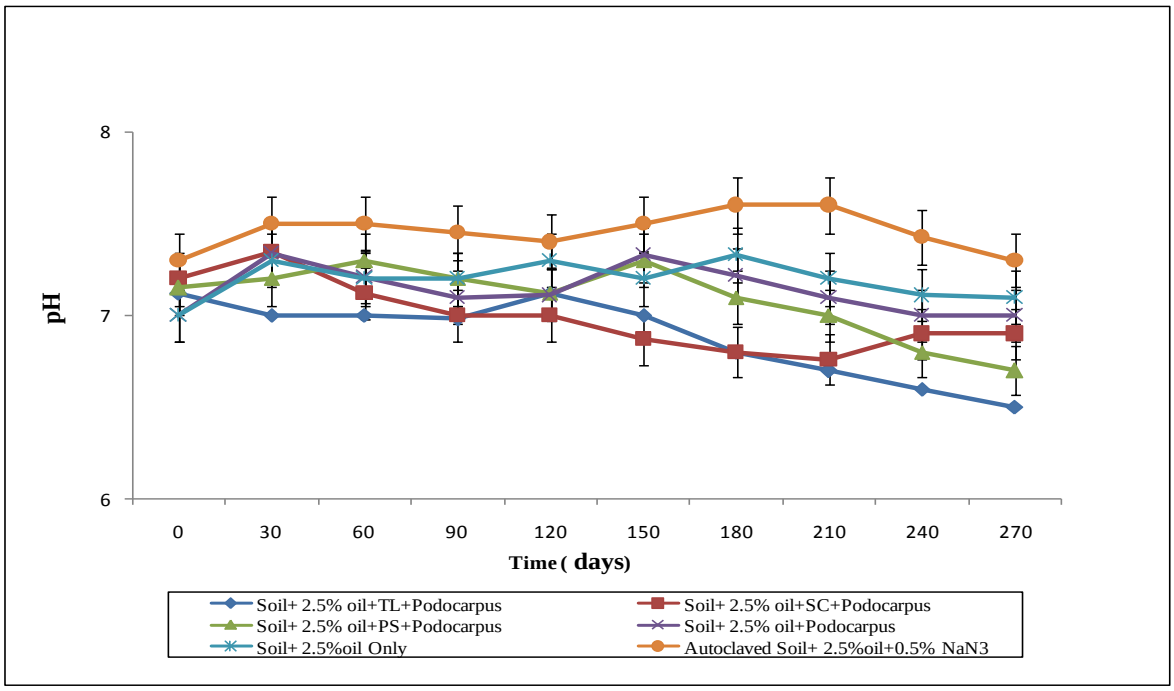


Figure 4.84 pH of soil contaminated with 2.5% diesel fuel planted with *P.polystachyus*. Bars indicates standard error (n = 3).

4.5.4 Plant growth and biomass production under laboratory condition

The appearance of the plants exposed to 1% and 2.5% of diesel were monitored throughout the 270 days of the experiment. No plant death was recorded in the 1% diesel fuel; however, some of the plants in the 2.5% fuel showed signs of phytotoxicity such as yellowing of leaves and stunted growth compared with the control. Plants in soil contaminated with 2.5% diesel oil showed high symptoms of phytotoxicity with death of at least one *Podocarpus* plant recorded in each treatment (data not shown) (Plate 4.2). These results showed that *Podocarpus* could tolerate minimum degree of exposure to hydrocarbons. Dry mass of the *Podocarpus* plants in each treatment was determined at the end of 270 days as shown in Table 4.24.

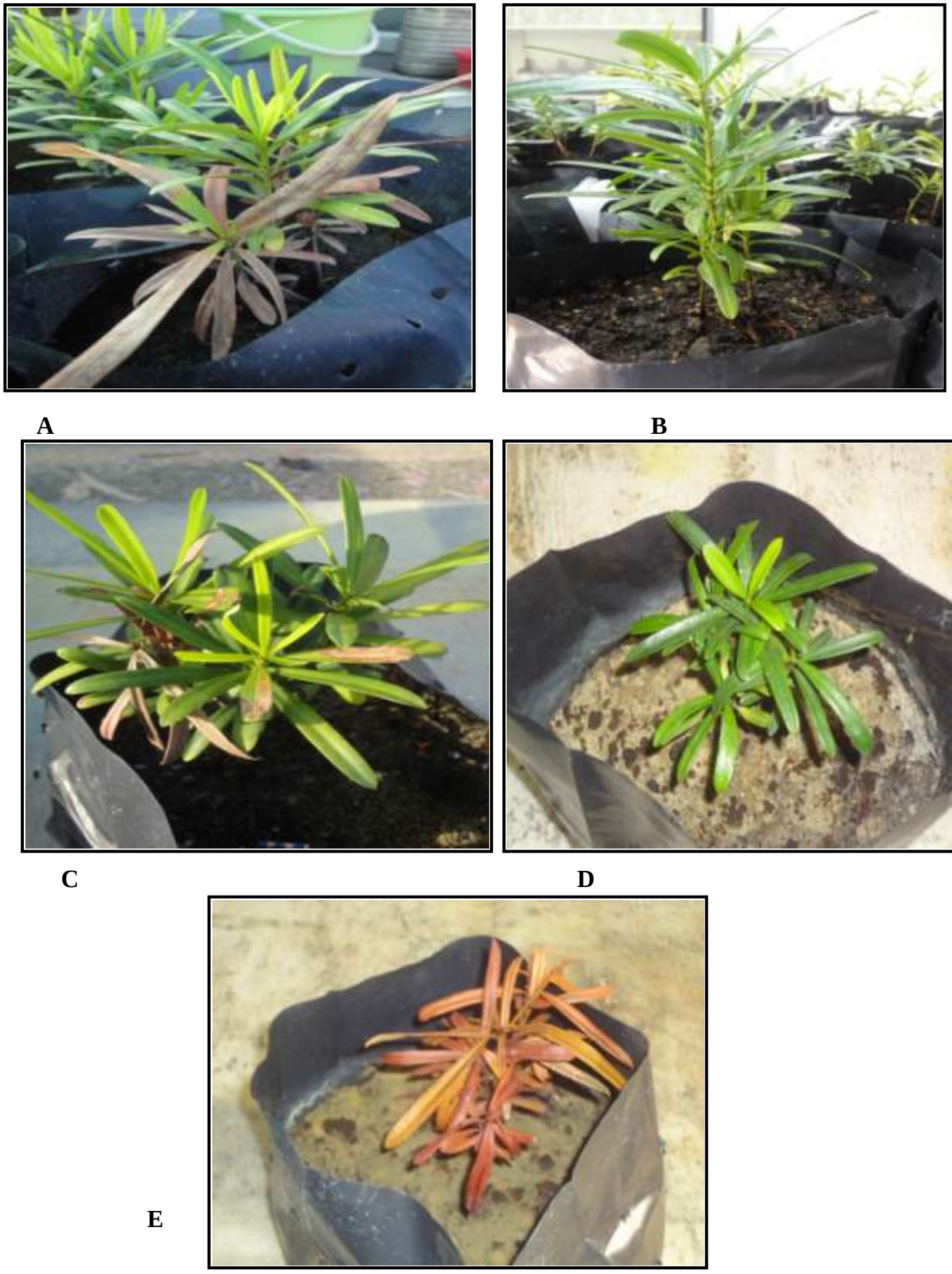


Plate 4.2 *P.polystachyus*, A: *Polystachyus* amended with TL, B: *Polystachyus* amended with SC, C: *Polystachyus* amended with PS, D: Control Plant, E: Phytotoxicity effect of oil on *Polystachyus*

Table 4.24 Dry mass of *Podocarpus* plant parts at the end of experiment (270 days).

	Dry weight (g)		
Treatment	Leaves	Stem	Roots
A	2.5 ± 0.3	1.9 ± 0.4	1.1 ± 0.5
B	4.3 ± 0.8	6.6 ± 0.4	2.9 ± 0.3
C	2.9 ± 0.6	2.7 ± 0.5	2 ± 0.6
D	1.1 ± 0.4	0.9 ± 0.7	0.6 ± 0.5
E	1.0 ± 0.7	0.5 ± 0.2	0.3 ± 0.4
F	3.6 ± 0.8	3.3 ± 0.7	1.8 ± 0.2
G	2.0 ± 1.1	1.7 ± 0.9	0.8 ± 0.3
H	0.7 ± 0.4	0.6 ± 0.1	0.3 ± 0.2
M	4.2 ± 0.4	4 ± 1.1	2.2 ± 0.2

A, soil + 1% oil + TL; B, soil + 1% oil + SC; C, soil + 1% oil + PS; D, soil + 1% oil only; E, soil + 2.5% oil + TL; F, soil + 2.5% oil + SC; G, soil + 2.5% oil + PS; H, soil + 2.5% oil only; M, control soil i.e. without oil contamination. **(**Values expressed as mean and standard deviation = 3).

4.5.5 Plant uptake of hydrocarbons

Podocarpus roots from different treatments were Soxhlet extracted to determine if there was phytoaccumulation of hydrocarbons in the plant roots. The GC/MS analysis of the extract did not show presence of hydrocarbons in all the treatments. This is in sharp contrast to the results of Palmroth et al., (2002), who observed an uptake of diesel oil by grass roots, but agrees with the findings of Chaîneau et al., (2005) who did not observe uptake of hydrocarbons by maize root. The result is also similar to that of Santosh et al., (2009), who observed that the application of organic amendments stabilized As, Cr and Zn in heavy metals contaminated soil and reduced their uptake by plant tissues. The result suggests that the mechanism of hydrocarbon removal by *Podocarpus* plants may be via rhizodegradation or phytovolitilization which has been well documented (Abhilash et al. 2009; Gerhardt et al. 2009). In addition, the removal of the oil may be because of root exudates produced by the plant which enhanced the activities of soil microorganisms in mineralizing the oil in the soil.

4.6 Result of phytodegradation of soil contaminated with diesel using *D.reflexa* under natural condition

4.6.1 Loss of diesel fuel in soil planted with *D.reflexa*

The percentages of oil degradation in contaminated soil in 1% and 2.5% of diesel oil under natural condition are shown in Figures 4.85 and 4.86, respectively. In all the treatments 10 – 80.6% and 13 – 90.8% oil loss were recorded in soil polluted with 2.5 and 1% diesel fuel, respectively. At the end of 270 days, the highest percentage of biodegradation in soil amended with SC recorded (90.8% and 80.6%), followed by TL (82.2% and 70.1%) and soil amended with PS (78.8% and 65.6%) in soil contaminated with 1 and 2.5% diesel fuel. The experiment with soil polluted with 5% diesel oil was conducted for a period of only 180 days under natural condition, because all plants died after 6 months. At the end of 180 days, the results show 24.2%, 23% and 21.6% oil loss in soil amended with SC, TL and PS with 5% diesel fuel, respectively (Figure 4.87).

In soil polluted with 5%, 2.5% and 1% diesel fuel without plant and organic wastes recorded 8.5%, 27.4% and 38.1% oil degradation at the end of experiment. On the other hand, soil contaminated and planted with *Dracaena* without organic wastes shows 11.9%, 46.6% and 66.8% oil loss at 5%, 2.5% and 1% diesel fuel. The results are different from phytoremediation set up under laboratory and natural conditions with the same plants, where soil amended with SC had the highest percentage of oil loss (98.88%, 90.31% and 19) at 1%, 2.5% and 5%. In addition, soil amended with PS showed better results of degradation compared with that amended with TL while at the end of the experiment, under natural condition, the contaminated soil amended with TL had a higher rate of oil loss compared with that amended with the PS. The reason might be due to difference in weather conditions in terms of temperature and rainfall that help *Dracaena* to grow better in those amended with TL than the contaminated soil with PS.

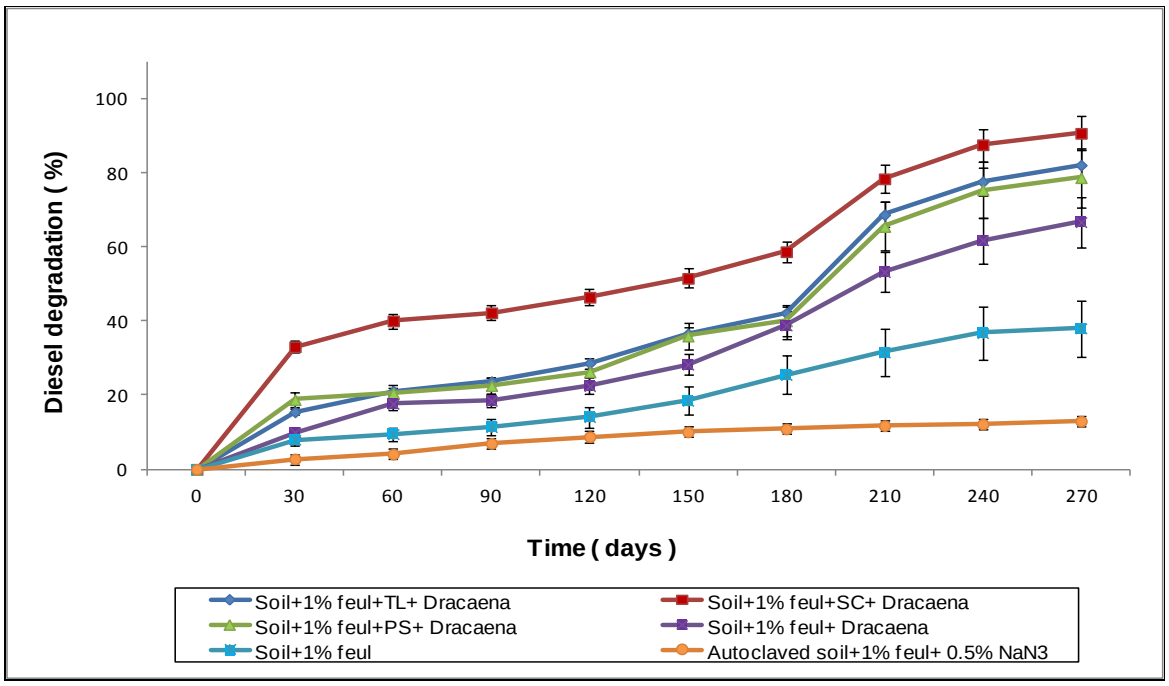


Figure 4.85 Biodegradation of diesel fuel in soil contaminated with 1% oil. Bars indicate standard error (n = 3).

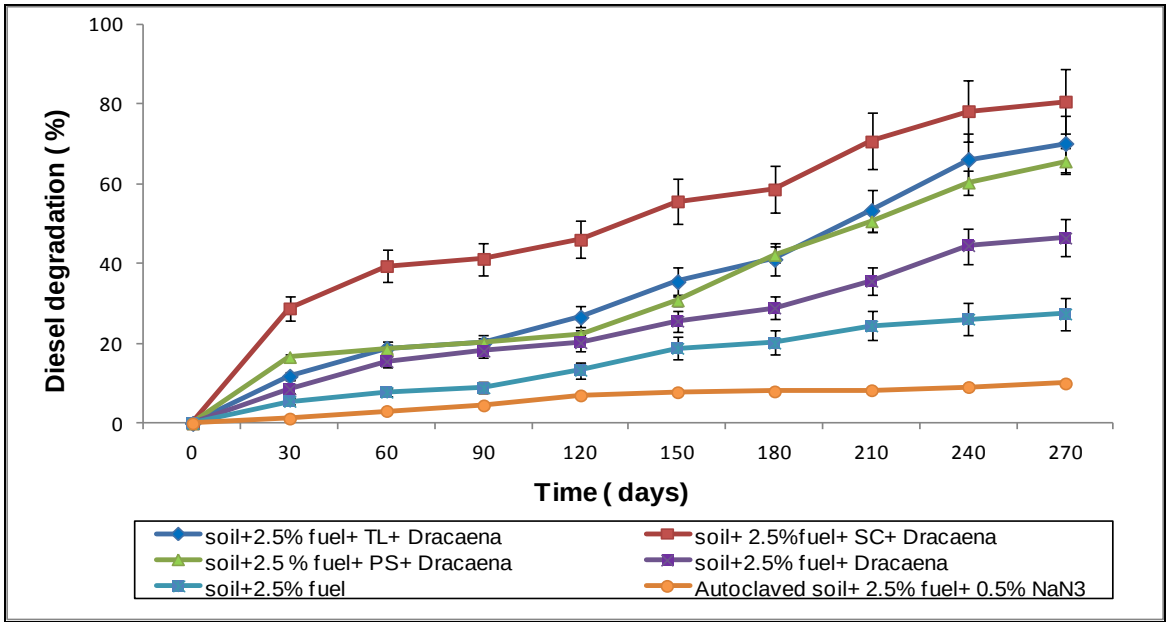


Figure 4.86 Biodegradation of diesel fuel in soil contaminated with 2.5% oil. Bars indicate standard error (n = 3).

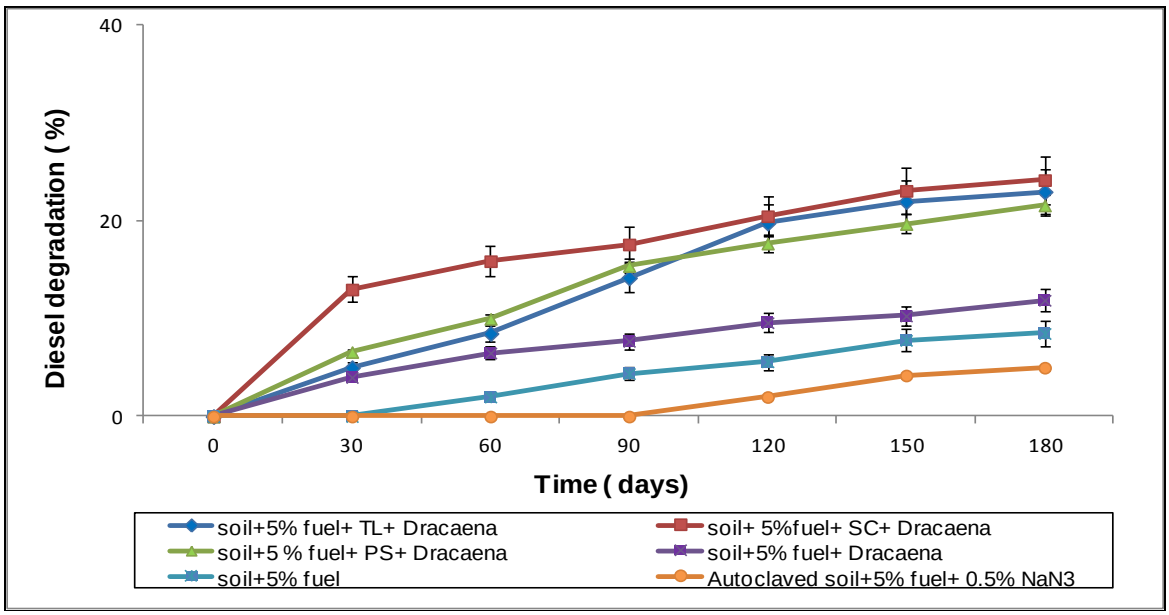


Figure 4.87 Biodegradation of diesel fuel in soil contaminated with 5% oil. Bars indicates standard error (n = 3).

Similar results were reported by many researchers (Abioye et al., 2012b; Dowling and Doty, 2009). It also might be due to the ability of SC and TL to enhance the growth of *Dracaena*. The result is similar to Zand et al., (2010) who investigated the effect of peat amendment with two local plants (maize and tall fescue) on the degradation of petroleum hydrocarbons and reported that tall fescue removed 96.3% of the initial TPHs from contaminated soil. In addition, the results are in agreement with Dominguez-Rosado et al., (2004) who reported 67% of used motor oil remediation in sunflower/mustard, with the addition of NPK fertilizer after 150 days.

At the end of the experiment, 13%, 10% and 5% oil loss were recorded in autoclaved control soil due to non-biological factors, at 1%, 2.5% and 5% diesel fuel, respectively. It was noticed that there was a slow rate of degradation on the 90th and 180th days of diesel fuel, which might be due to no rainfall and high temperature conditions within these months (June - July 2011) and (August - September 2011). The results indicated that addition of organic wastes to contaminated soil planted with *Dracaena* can improve

the rate of biodegradation about 24% higher than those contaminated soil without organic wastes and cultivated with *Dracaena*. The results are similar to the finding of Kim et al., (2010) who illustrated that after 120 days, diesel oil concentration in the planted soil, with fertilizer, was significantly decreased. Statistical analysis does not show any significant difference between soils amended with organic wastes ($P < 0.05$), while there was a significant difference ($P < 0.05$) between contaminated soil with plant and without organic wastes and contaminated soil without plant and organic wastes.

4.6.2 Bacterial count

Microbial population of heterotrophic bacteria (AHB) contaminated soil planted with *Dracaena* under natural condition and exposed to sunlight and rainfall at 1% and 2.5% diesel oil are shown in Figures 4.88- 4.89. AHB count shows 2.02×10^9 CFU/g to 2.98×10^9 CFU/g and 1.91×10^9 CFU/g to 2.78×10^9 CFU/g in soil amended with organic wastes at 1% and 2.5% diesel oil, respectively. Whereas, in contaminated soil with *Dracaena* and without organic wastes the aerobic microbial population was recorded as 1.6×10^9 CFU/g and 1.7×10^9 CFU/g in pollution with 2.5% and 1% diesel oil, respectively. The result of microbial population was similar to the result of phytoremediation set up under laboratory conditions. However, the number of microbial count in soil amended with TL was higher than those contaminated soil amended with PS under natural conditions, which is in contrast to the finding of a laboratory study with the same plant. However, the number of aerobic bacteria in soil polluted with 1% oil and amended with SC was 10.5 and 15.2 times higher than those amended with TL and PS, respectively.

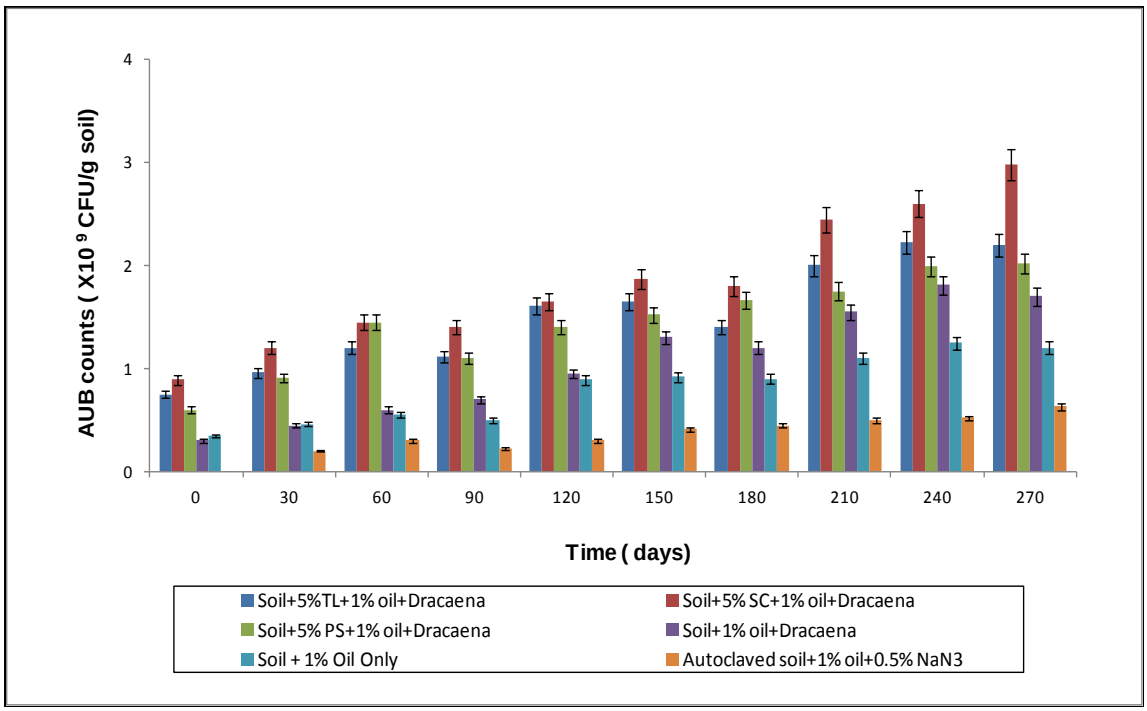


Figure 4.88 Total CFU of aerobic heterotrophic bacterial (AHB) in soil contaminated with 1% diesel fuel. Bars indicate standard error (n = 3).

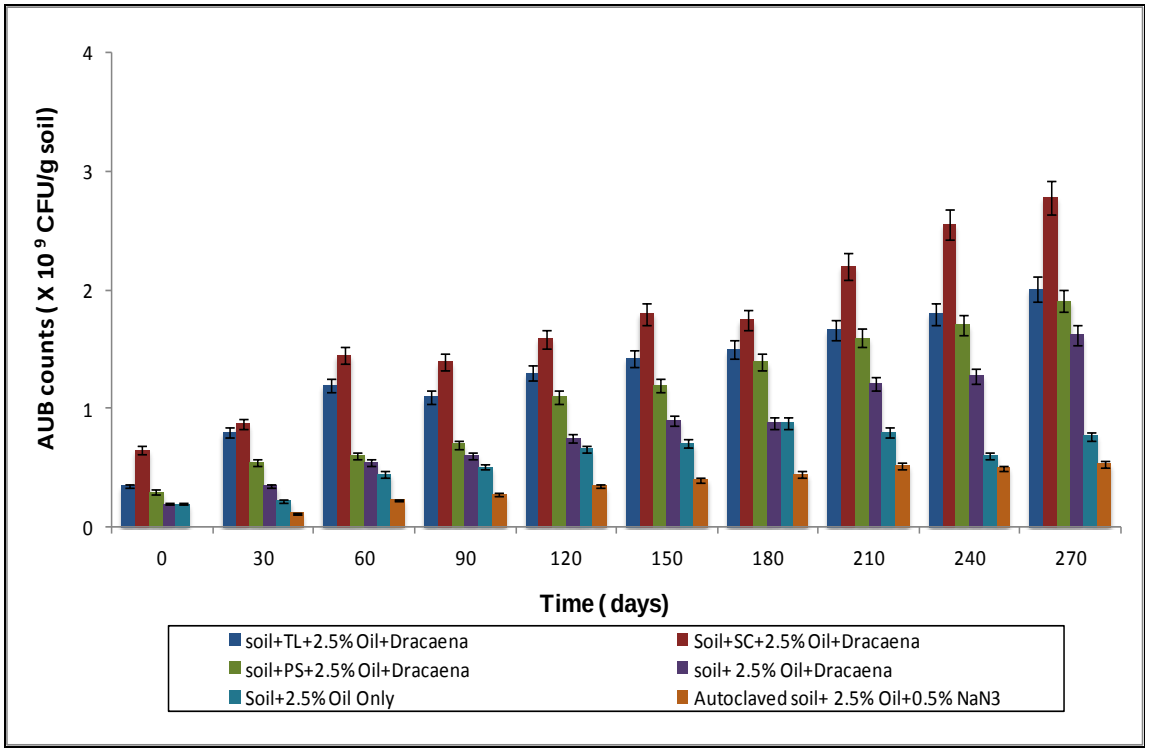


Figure 4.89 Total CFU of aerobic heterotrophic bacterial (AHB) in soil contaminated with 2.5% diesel fuel. Bars indicate standard error (n = 3).

The number of diesel utilizing bacteria (DUB) in soil contaminated with 1% and 2.5 % is shown in Figures 4.90 and 4.91, respectively. Similarly the result for the soil amended with SC under laboratory conditions, showed a higher number of DUB count ranging from 355×10^5 CFU/g to 324×10^5 CFU/g in soil polluted with 1% and 2.5 % diesel oil, respectively. It was observed that the DUB count in polluted soil with *Dracaena* plant and without organic wastes was lower than those of treatments amended with organic wastes, ranging between 198×10^5 CFU/g and 145×10^5 CFU/g at 1% and 2.5% diesel oil, respectively. As mentioned earlier (2.5.3), the reason for the higher number of microbial population in those treated with organic wastes compared with the unamended is due to the availability of nutrients (N and P) in organic wastes which enhances the proliferation of microorganisms in polluted soil. It was noticed that DUB and AHB counts in all treatments had decreased slightly on the 90^{th} and 180^{th} days due to the climatic conditions such as high temperature during that period compared with laboratory conditions. This result was reflected in the percentage of diesel fuel biodegradation in the same period. Since, the same source of soil and plant was used, the microbes isolated from contaminated soil were similar to those isolated from the laboratory experiment. The bacteria were identified as *Pseudomonas citronellolis* and *Bacillus amyloliquefaciens*. These bacteria have been identified by various researchers (Van Hamme et al., 2003; Ijah and Antai, 2003a,b). Several authors have shown the positive effects of bacteria with root exudates to break down the hydrocarbon compounds during the biodegradation of oil (Van Aken et al. 2009; Newman and Reynolds, 2005).

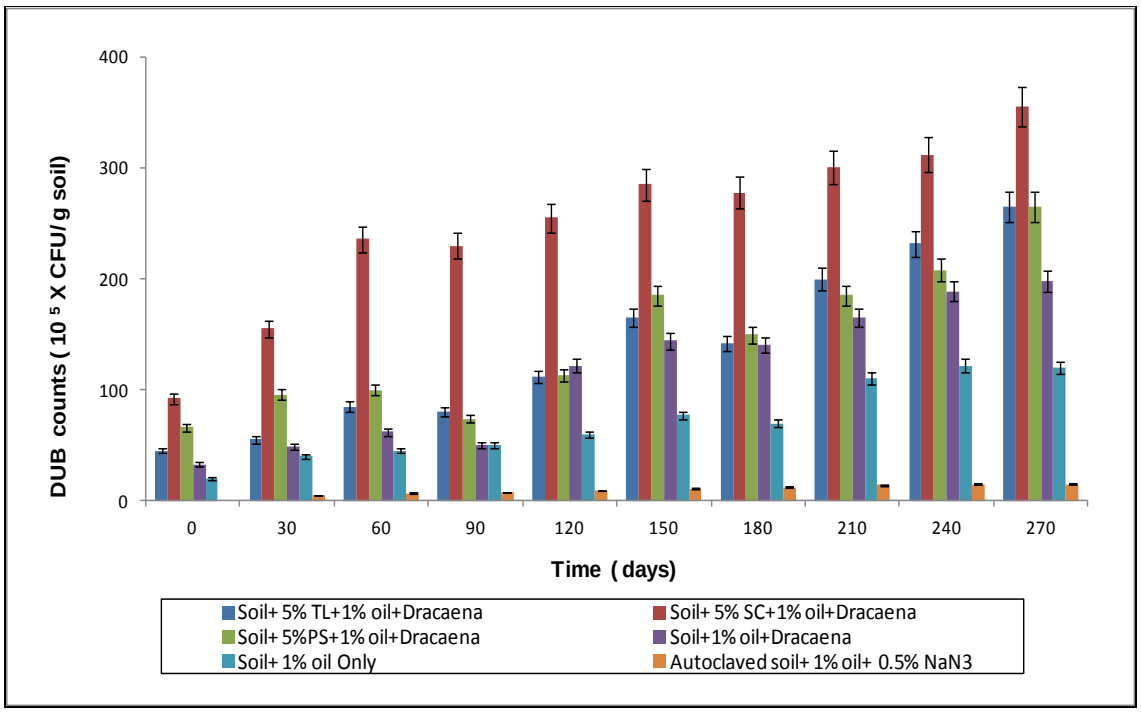


Figure 4.90 Total CFU of diesel utilizing bacterial (DUB) in soil contaminated with 1% diesel fuel. Bars indicate standard error (n = 3).

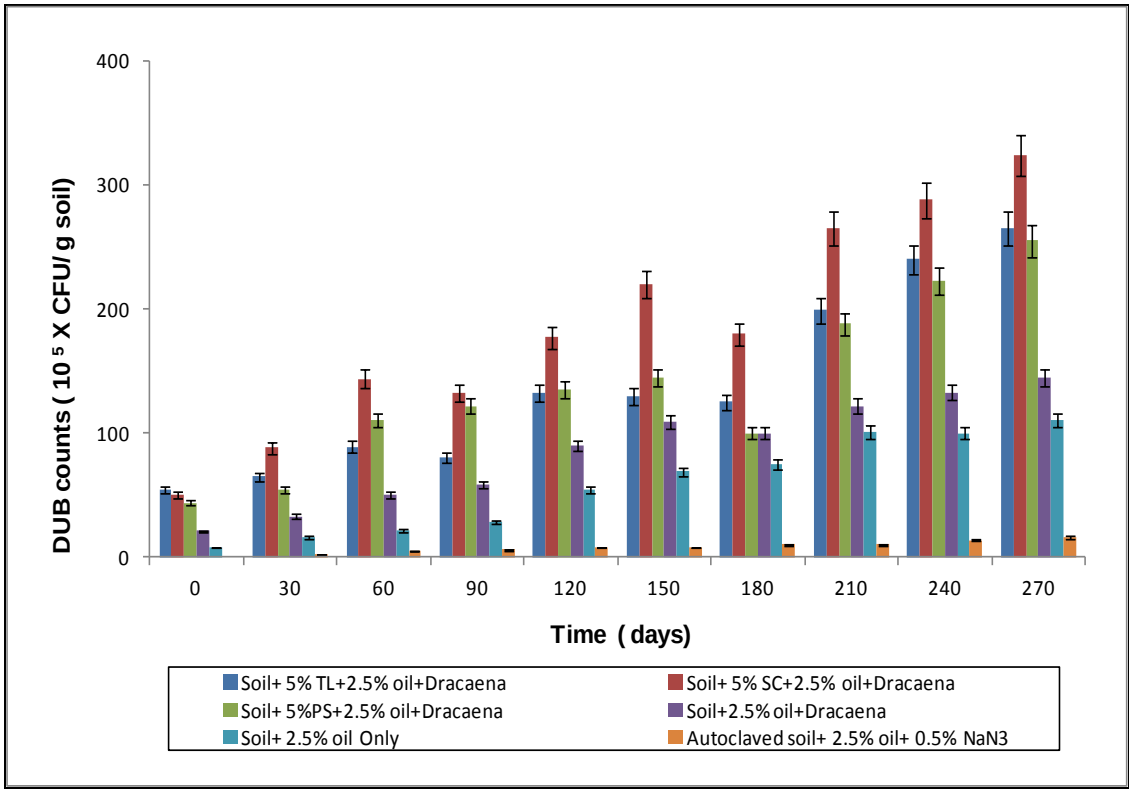


Figure 4.91 Total CFU of diesel utilizing bacterial (DUB) in soil contaminated with 2.5% diesel fuel. Bars indicate standard error (n = 3).

4.6.3 pH of soil in *D.reflexa* remediation under natural condition

pH of remediated soil with *Dracaena* at 1% and 2.5% diesel oil during the period of study are shown in Figures 4.92 and 4.93. The pH of soil treated under natural conditions and exposed to rainfall and sunlight was quite acidic compared with those treatments under laboratory conditions. This finding is similar to Ijah et al., (2008) who reported a varying range of pH during the degradation of petroleum hydrocarbons in the period of study. All treatments amended with organic wastes show more acidic pH compared with those unamended contaminated soil. It may be because of high metabolic activities of microorganisms which produce acidic conditions in the rhizosphere. Gerhardt et al., (2009) reported that plant roots produced more exudates which are slightly acidic in nature.

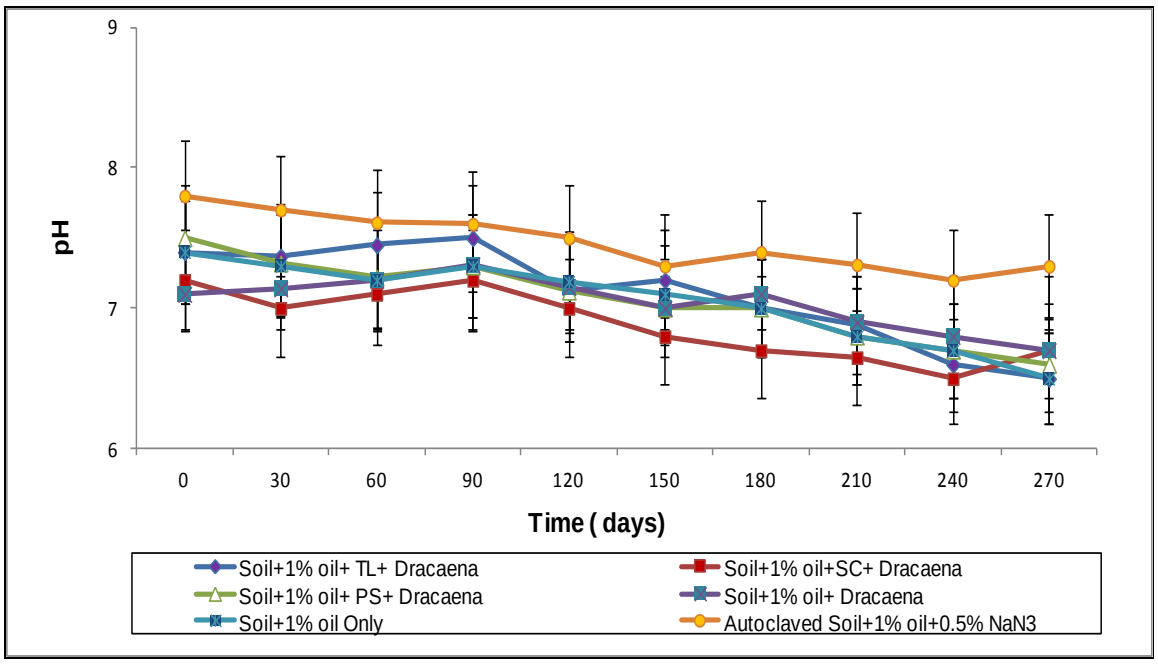


Figure 4.92 pH of soil contaminated with 1% diesel fuel planted with *D. reflexa*. Bars indicate standard error (n = 3).

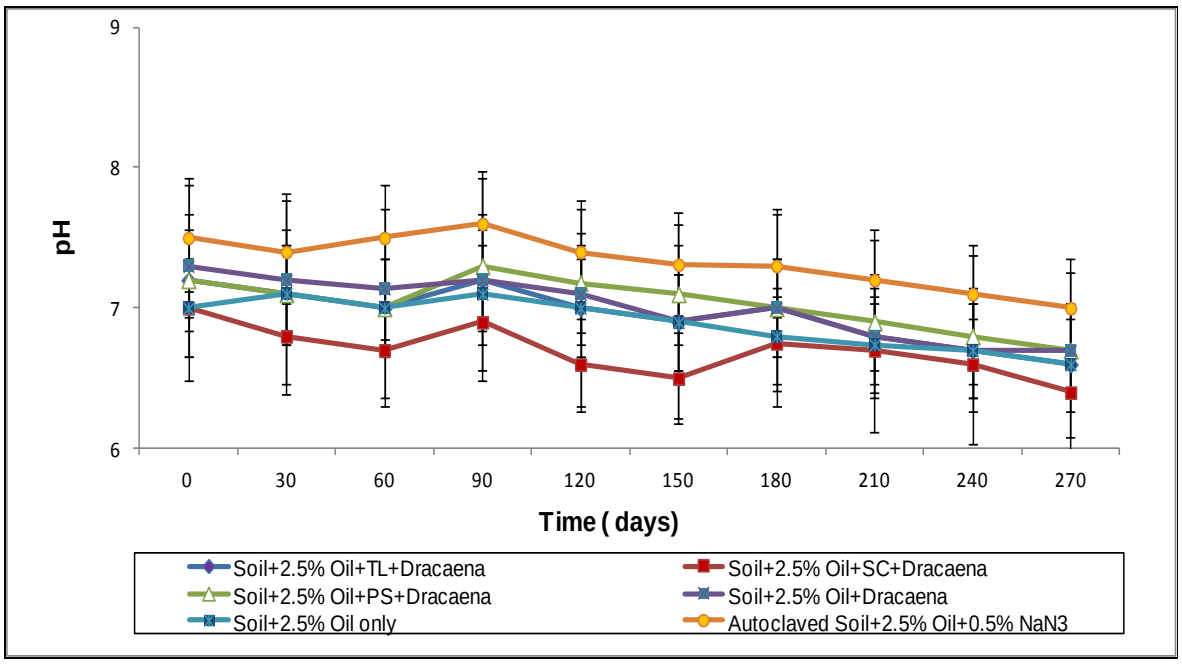


Figure 4.93 pH of soil contaminated with 2.5% diesel fuel planted with *D. reflexa*. Bars indicate standard error (n = 3).

4.6.4 Response of *D. reflexa* to oil pollution under natural condition

The response of the *D. reflexa* exposed to rainfall and sunlight was better than those plants studied under laboratory conditions (29.5 ± 2^0C). The reason might be the fact that all plants exposed to sunlight had higher ability to do the photosynthetic activities compared with those plants in the laboratory. No plant death was recorded at both concentration of diesel oil throughout the 270 days of the study. However, one plant dead in each treatment in the contaminated soil with 5% oil. It demonstrates that plants cannot survive in high concentration of diesel oil. Generally, plants amended with organic wastes grow better than the unamended plants which might be because of the presence of nutrients.

4.7 Phytodegradation of soil contaminated with diesel using *P.polystachyus* under natural condition

4.7.1 Loss of diesel fuel in soil planted with *P.polystachyus*

At the end of 270 days, the percentage of oil loss in soil treated with SC and *Podocarpus* recorded 85.5% and 77.6% in contaminated soil with 1% and 2.5% diesel fuel, respectively (Figures 4.954 and 4.95). Polluted soil treated with SC had the highest rate of degradation while, the percentage of degradation in contaminated soil with 1% and 2.5% diesel fuel and amended with PS and TL recorded 67.2%, 58% and 72%, 60%, respectively. However, at the end of 270 days, contaminated soil without organic waste amendments with *Podocarpus* had 44.6% and 33.5% at 1% and 2.5% diesel fuel, respectively. At the end of the experiment, 13% and 9.6% oil loss was recorded in autoclaved control soil including sodium azide with 1% and 2.5% diesel fuel that might be due to some non-biological factors such as photodegradation or evaporation. High rate of degradation recorded in soil amended with organic wastes (TL, SC and PS) compared with unamended treatments was because of available appreciable nutrients such as N and P in the organic wastes that enhanced the microbial growth in the rhizosphere area of plants. In addition, it might be also as a result of the fact that contaminated soil loosens the compactness due to mixing organic wastes with polluted soil before transferring plants, which make it more sufficient for bacteria aeration in the soil. The result is line with Palmroth et al., (2002) who reported 60% oil loss in diesel-contaminated soil with the addition of NPK fertilizer and planted with pine tree during one month. Statistical analysis of oil degradation in all the treatments treated with organic wastes dose not show significant difference at $P < 0.05$ significant level, while there was significant difference ($P < 0.05$) between the treatments amended without organic waste with organic waste. However, the results prove the fact that organic

wastes positively attributed to the biodegradation of the diesel oil from the contaminated soil.

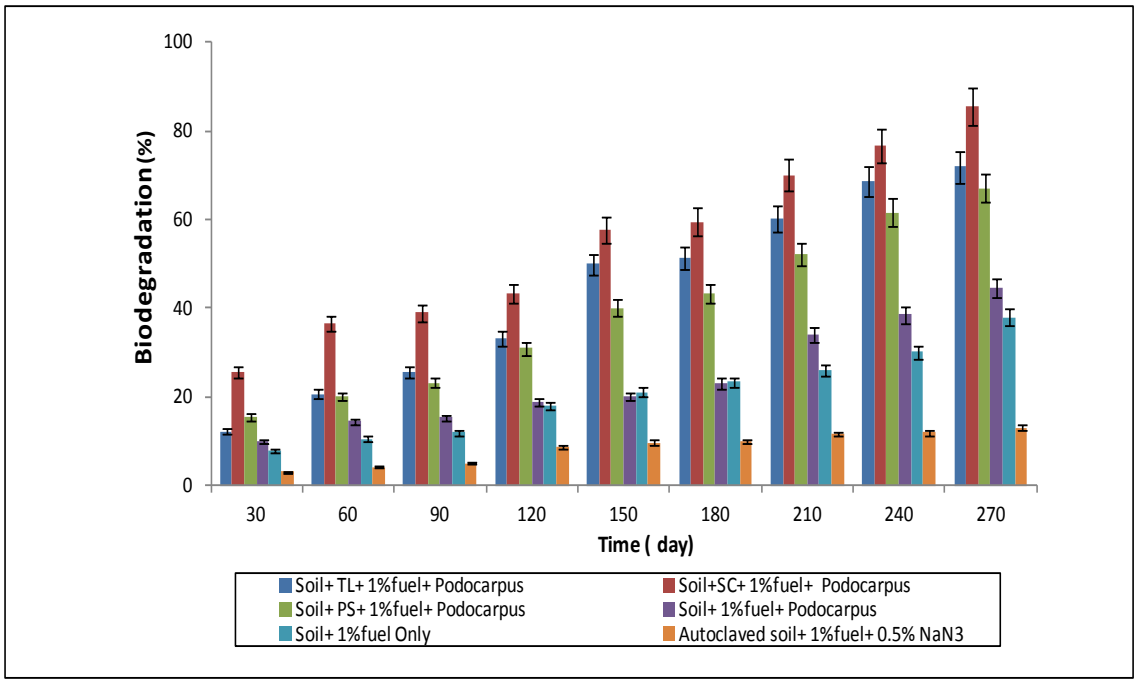


Figure 4.94 Biodegradation of diesel fuel in soil contaminated with 1% oil. Bars indicate standard error (n = 3).

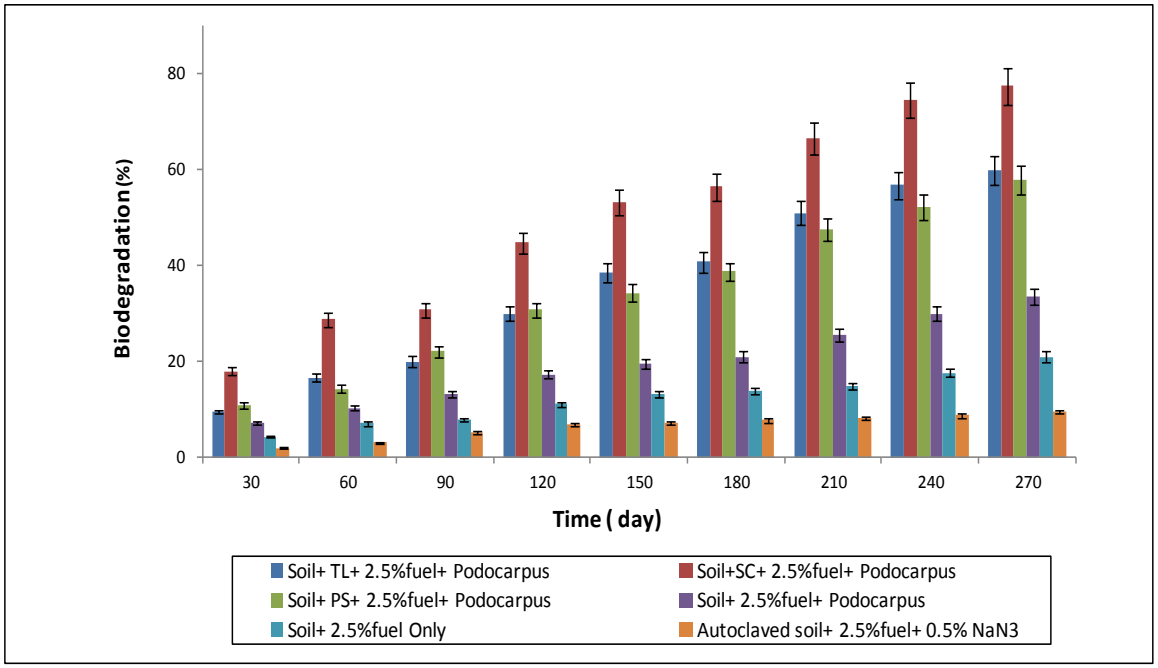


Figure 4.95 Biodegradation of diesel fuel in soil contaminated with 2.5% oil. Bars indicate standard error (n = 3).

The monitoring experiment at 5% diesel oil planted with *P. polystachyus* was conducted for a period of 180 days as all the plants died at this concentration. However, contaminated soil amended with SC, TL and PS recorded 20.8%, 15.6% and 16.1% degradation in 5% diesel oil at the end of six months (Figure 4.96). The result revealed that those treated with *Podocarpus*, without organic wastes, the percentage of degradation at 5% oil is significantly low (10%) compared with those amended with organic wastes. Vouillamoz and Milke (2001) illustrated the positive effects of compost addition on the rate of degradation during the phytoremediation of diesel fuel in contaminated soil. The percentage of degradation at 5% oil with *Podocarpus* under natural conditions was slightly higher compared with phytoremediation set up with 5% oil under laboratory condition. The reason might be that the outdoors experiment lasted 270 days with exposure to sunlight and rainfall compared to laboratory condition, without sunlight but with artificial light.

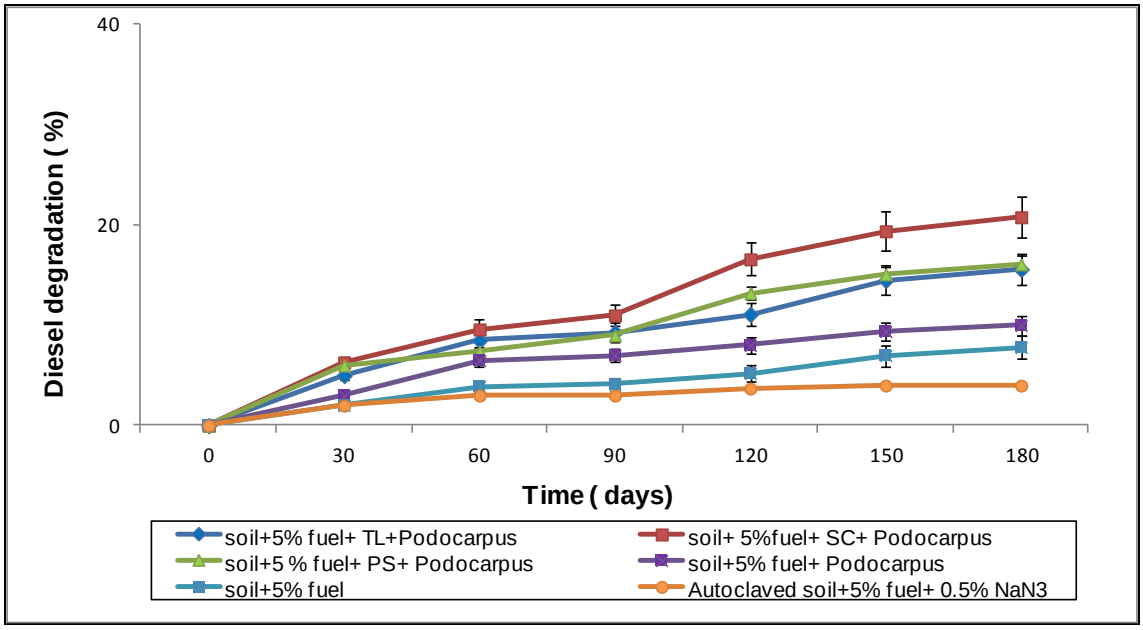


Figure 4.96 Biodegradation of diesel fuel in soil contaminated with 5% oil. Bars indicate standard error (n = 3).

4.7.2 Bacterial count

The microbial population of AHB in *Podocarpus*, contaminated with diesel oil at 1% and 2.5% under natural condition are shown in Figures 4.97 and 4.98, respectively. The count of AHB in all amended soil treated with organic wastes ranged from 212 × 10^7 CFU/g to 255 × 10^7 CFU/g and 177 × 10^7 CFU/g to 232 × 10^7 CFU/g in 1% and 2.5% oil, respectively. Similar to phytoremediation under laboratory conditions, soil amended with SC recorded the highest AHB count (255× 10^7 CFU/g and 232× 10^7 CFU/g), followed by TL amended (231× 10^7 CFU/g and 185× 10^7 CFU/g) and those of amended with PS that recorded 212× 10^7 CFU/g and 177× 10^7 CFU/g in 1% and 2.5% oil, respectively, whereas those treatments with *Podocarpus* alone recorded 161× 10^7 CFU/g and 141× 10^7 CFU/g in contaminated soil with 1% and 2.5% diesel oil, respectively. The DUB counts of contaminated soil amended with SC had the highest population compared with other treatments. The count of DUB in all amended soil treated with organic wastes ranged from 229 × 10^5 CFU/g to 281 × 10^5 CFU/g and 205 × 10^5 CFU/g to 278 × 10^5 CFU/g in 1% and 2.5% oil, respectively. Those treatments amended with SC ranged between 281× 10^7 CFU/g and 278× 10^7 CFU/g with 1% and 2.5% diesel oil, respectively (Figures 4.99 and 4.100). The bacteria isolated and identified was similar to the species of isolated from the *Dracaena* experiment which were *Bacillus sp.* and *Pseudomonas sp.* . These species might be attributed to plant roots to degrade diesel oil in rhizosphere zone of the plant. These bacteria species have been reported by Madhuri and Rangaswamy (2009) who indicated microorganisms have the ability to degrade petroleum hydrocarbon fractions. This result is similar to the finding of phytoremediation with the *D. reflexa*.

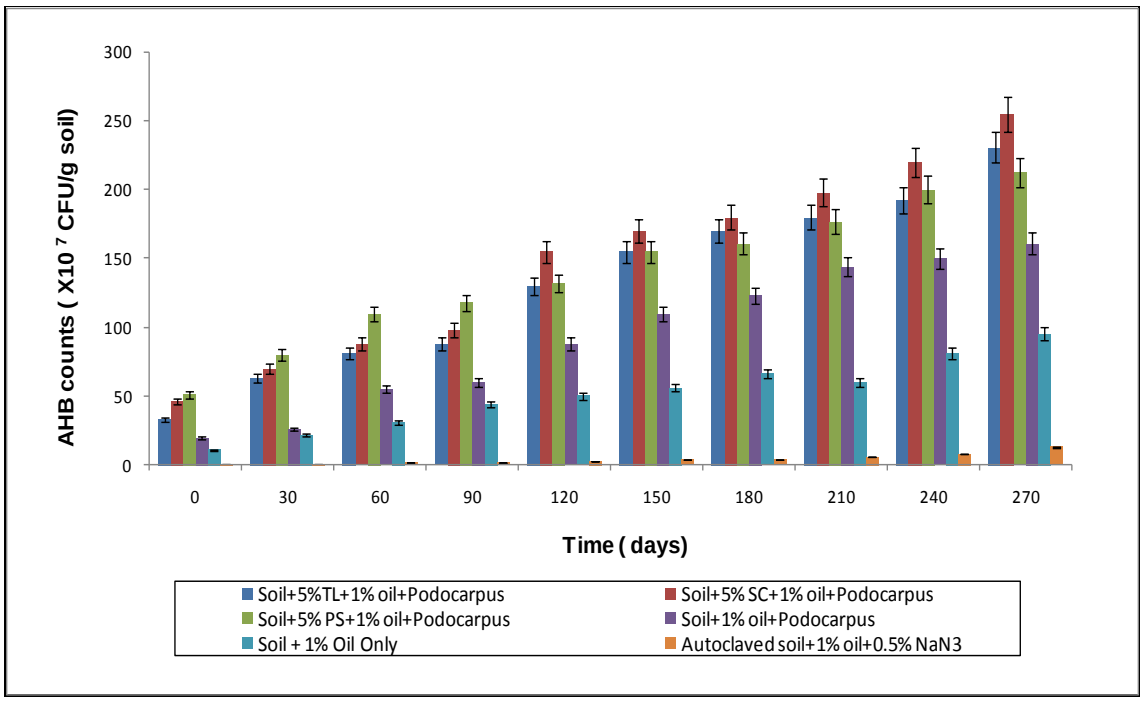


Figure 4.97 Total CFU of aerobic heterotrophic bacterial (AHB) in soil contaminated with 1% diesel fuel. Bars indicate standard error (n = 3).

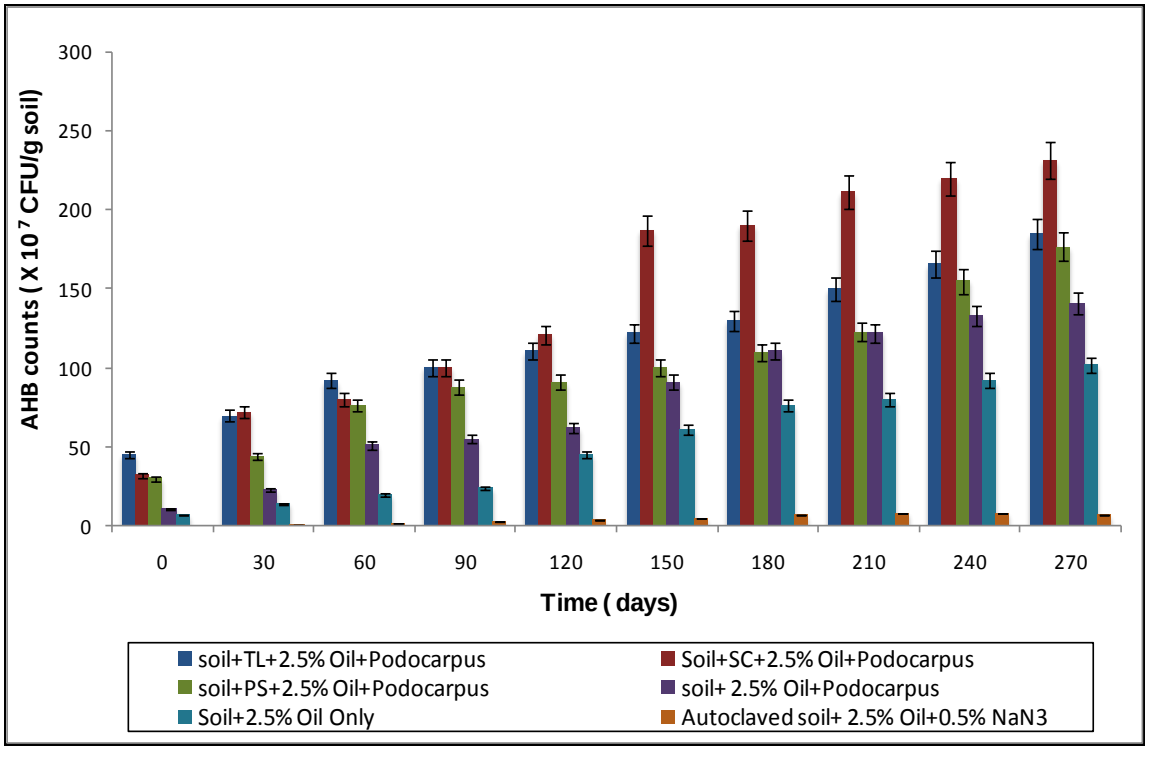


Figure 4.98 Total CFU of aerobic heterotrophic bacterial (AHB) in soil contaminated with 2.5% diesel fuel. Bars indicate standard error (n = 3).

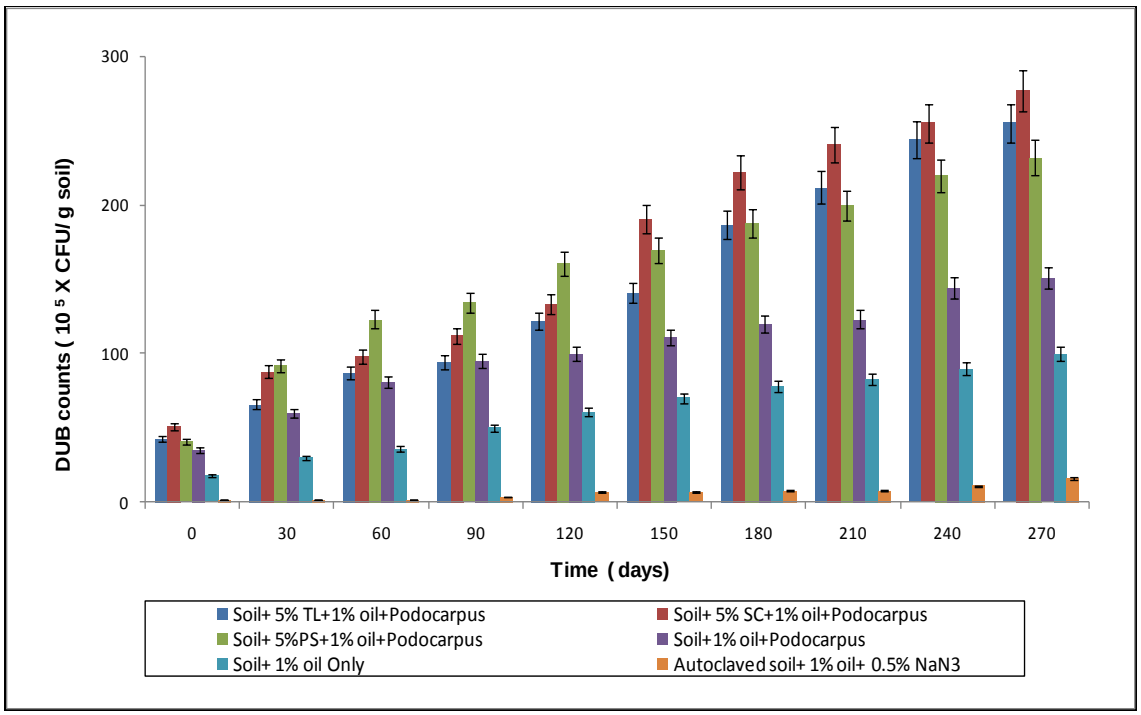


Figure 4.99 Total CFU of diesel utilizing bacterial (DUB) in soil contaminated with 1% diesel fuel. Bars indicate standard error (n = 3).

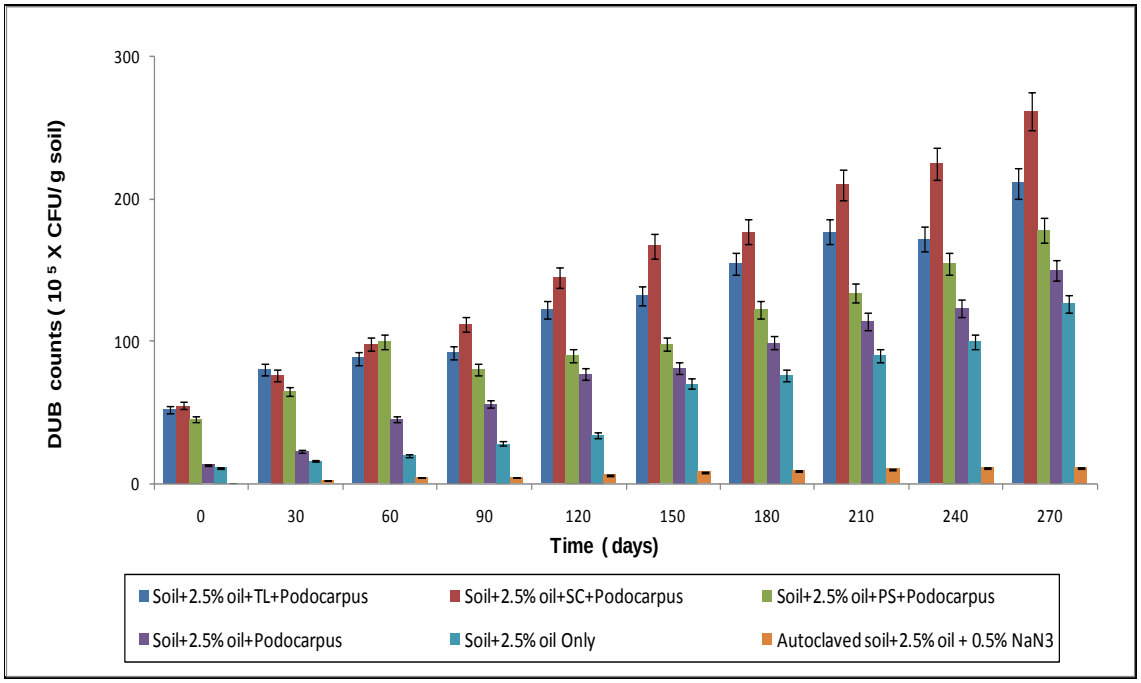


Figure 4.100 Total CFU of diesel utilizing bacterial (DUB) in soil contaminated with 2.5% diesel fuel. Bars indicate standard error (n = 3).

4.7.3 pH of soil in *P.polystachyus* remediation

Figures 4.101 and 4.102 show the pH of remediated soil with *Podocarpus* at 1% and 2.5% oil during the period of the study. The pH of soil treated under natural conditions, and exposed to rainfall and sunlight was quite acidic compared with those treated under laboratory condition. This finding is similar to that of Ijah et al., (2008) who reported a varying range of pH during the degradation of petroleum hydrocarbons during period of study. All treatments amended with organic wastes show more acidic pH compared with those unamended contaminated soil. This may be because of high metabolic activities of microorganisms which produced acidic conditions in the rhizosphere. Gerhardt et al., (2009) reported that plant roots produced more exudates which are slightly acidic in nature.

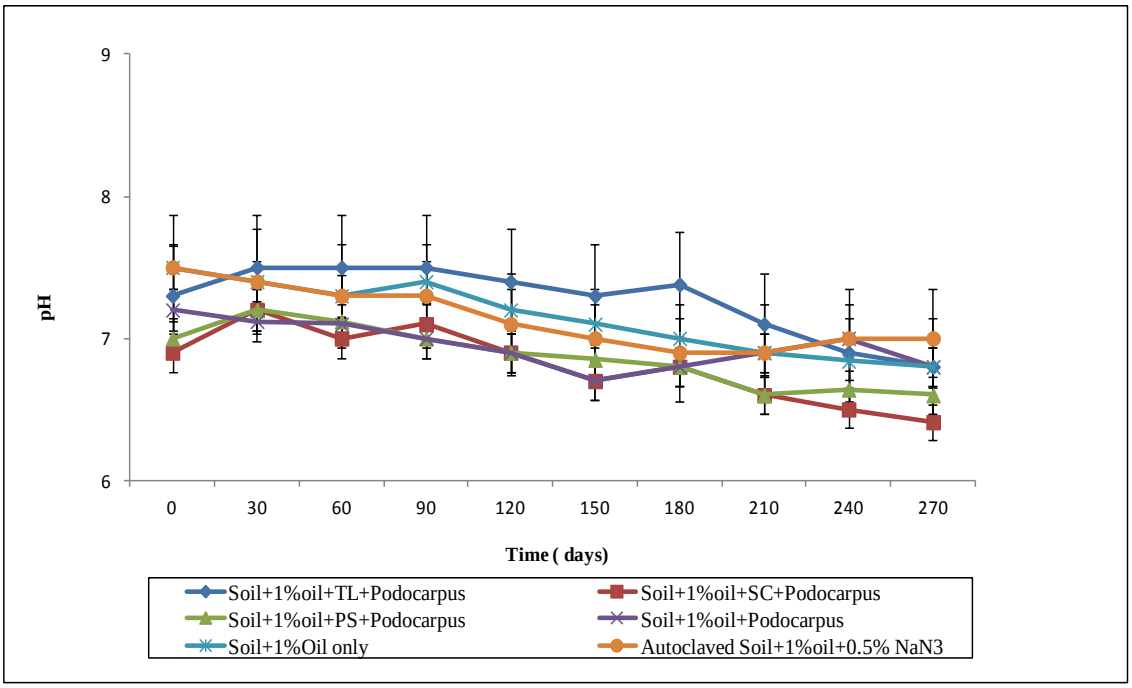


Figure 4.101 pH of soil contaminated with 1% diesel fuel planted with *P.polystachyus*. Bars indicate standard error (n = 3).

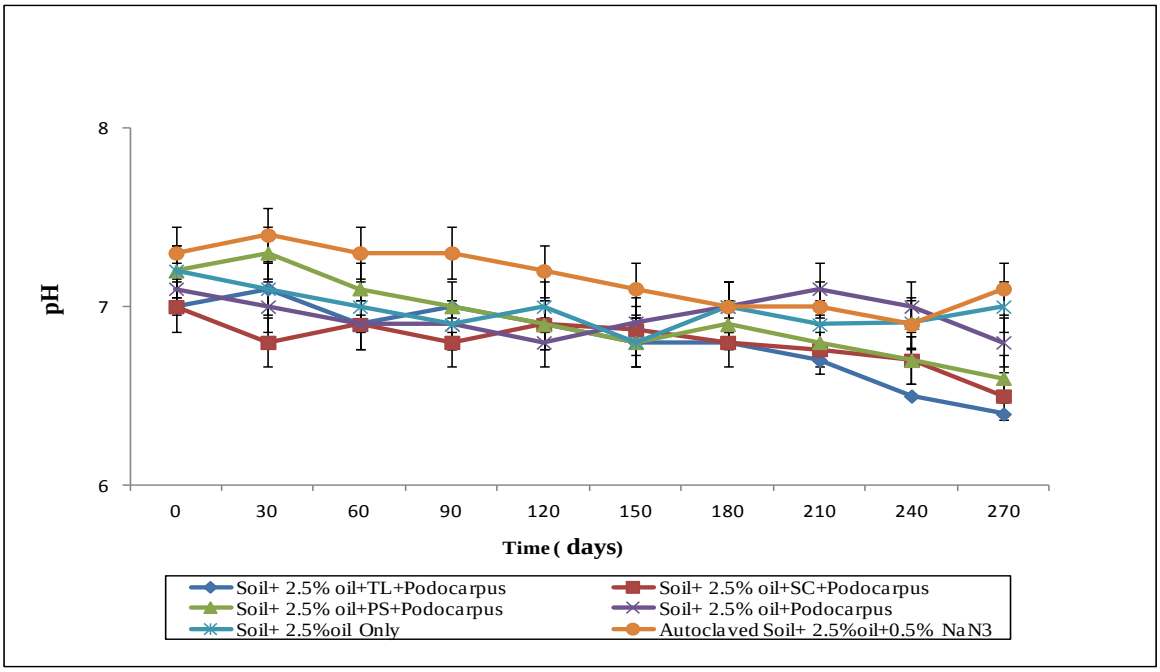


Figure 4.102 pH of soil contaminated with 2.5% diesel fuel planted with *P.polystachyus.* Bars indicate standard error (n = 3).

4.7.4 Bioaccumulation of heavy metals by *Dracaena reflexa*

The transformation and bioaccumulation potential of plants are the key factor controlling the efficiency of remediation technology. The heavy metal concentrations of diesel fuel, unpolluted soil and contaminated soil with 2.5 % diesel oil before remediation are shown in Table 4.25. Diesel fuel contained the lowest concentration of zinc (Zn) and lead (Pb) compared to unplanted polluted soil. However, soil used for phytoremediation had 18.1(mg/kg) and 19.2 (mg/kg) Zn and Pb, respectively. After soil was artificially polluted with 2.5% diesel oil and 80 (mg/kg) Zn or 60 (mg/kg) Pb, the amount of Zn and Pb reached 74.52 (mg/kg) and 55.3 (mg/kg), respectively. After harvesting plants from different treatments, roots, leaves and stems were dried at 40 ^{0}C for 3 days, then ground, and 0.5 g digested with mixture of acids were analyzed with ICP- OES to determine the accumulation of metals in the soil and plant.

Table 4.25 Heavy metal concentration of diesel fuel, soil contaminated with 2.5% oil and unpolluted soil before remediation.

	Heavy metals (mg/kg)*	
Substrate	Zn	Pb
Diesel fuel	0.84	0.03
Unpolluted soil	18.1	19.2
Soil + 2.5% oil	19.32	19.4
Soil+2.5% oil+ 80 ppm Zn	74.52	-
Soil+2.5% oil+ 60 ppm Pb	-	55.3

* mg/kg = ppm

Based on preliminary trials (3.8.1), it was decided to use two microelements which have a major impact on the growth of microorganisms, namely Zn (80 mg kg^{-1}) and Pb (60 mg kg^{-1}). The residue of Zn and Pb concentrations in the soil polluted with 80 ppm Zn and 60 ppm Pb and planted with the *D. reflexa* in different treatments are shown in Tables 4.26 and 4.27. Zn accumulation in soil of those amended with organic wastes ranged from 32.5 mg/Kg to 41.03 mg/Kg, while in polluted control soil with the *Dracaena* and without organic wastes recorded 58.1 mg/Kg. Pb accumulation in treated soil amended with organic wastes recorded a range of between 21.1 mg/Kg and 34.2 mg/Kg, whereas in polluted and unamended treatment there was a higher quantity of Pb. Translocation of Zn and Pb from the roots of the *Dracaena* plant to the leaves and stems was recorded in all the treatments.

Table 4.26 Residual Zn concentration in soil remediated with *Dracaena* and polluted with 80 ppm Zn after 6 months

Treatments	Heavy metal (mg/Kg)
	Zn
Soil+2.5% oil + TL+ Dracaena	32.5
Soil+2.5% oil + SC + Dracaena	34.2
Soil+2.5% oil + PS + Dracaena	41.0
Soil+2.5% oil + Dracaena	58.1
Soil without oil+ Dracaena	14.3

Table 4.27 Residual Pb concentration in soil remediated with *Dracaena* and polluted with 60 ppm Pb after 6 months

Treatments	Heavy metal (mg/Kg)
	Pb
Soil+2.5% oil + TL+ Dracaena	21.1
Soil+2.5% oil + SC + Dracaena	28.2
Soil+2.5% oil + PS + Dracaena	34.2
Soil+2.5% oil + Dracaena	45.4
Soil without oil+ Dracaena	ND

ND: Not detected

At the end of 180 days, appreciable quantities of Zn and Pb were detected to accumulate in *Dracaena* roots, stem and leaves (Tables 4.28 and 4.29). There is accumulation of Zn

and Pb in roots and steams. The result is in conflict to the finding of Palmorth et al., (2007) which demonstrated that in contaminated soil with weathered hydrocarbons and amended with fertilizers (NPK) and biowaste composts; there was no accumulation of heavy metals in plant contextures. The differences discovered in the results might be due to the use of different sources of hydrocarbon and differences in the soil and plants used in both experiments. They used poplar grasses, clover and pine while in this study *Dracaena reflexa* was used.

In addition, the differences can be because of using freshly contaminated soil in this study while Palmroth et al., (2007) had used weathered hydrocarbon contaminated soil. All of these parameters can attribute to the different results between the two studies.

Table 4.28 Zn contents with *D. reflexa* in soil contaminated with 80 ppm Zn

Treatments	Zn (mg/Kg)		
	Roots	Stems	Leaves
Soil+2.5% oil + TL+ Dracaena	15.1	10.9	ND
Soil+2.5% oil + SC + Dracaena	16.53	12.2	6.32
Soil+2.5% oil + PS + Dracaena	12.2	8.25	4.2
Soil+2.5% oil + Dracaena	3.4	1.8	ND
Soil+ Dracaena	1.4	ND	0.08

ND: Not detected

Table 4.29 Pb contents in *D. reflexa* in soil contaminated with 60 ppm Pb

Treatments	Pb (mg/Kg)		
	Roots	Stems	Leaves
Soil+2.5% oil + TL+ Dracaena	16.70	9.8 0	3.50
Soil+2.5% oil + SC + Dracaena	13.83	6.70	ND
Soil+2.5% oil + PS + Dracaena	10.10	7.60	ND
Soil+2.5% oil + Dracaena	1.10	ND	0.005
Soil without oil+ Dracaena	0.8 0	0.065	ND

ND: Not detected

There was bioaccumulation of Zn and Pb in *Dracaena* roots compared with stems and leaves in different treatments. No accumulation of Pb was recorded in leaves of all treatments (Table 4.29). Soil treated and amended with TL recorded the highest accumulation of Pb in tissues, however, soil amended with TL and SC recorded close result in accumulation of Zn in tissues, which was probably due to the large amounts of fiber and proteins contained in the tea waste. The result is supported by Zuorro and Lavecchia (2010) who reported that removal efficient of up to 98% of Pb^{2+} in lead contaminated soil using spent tea leaves. Accumulation of Zn in *Dracaena* roots in the polluted soil amended with organic waste ranged from 12.2 mg/Kg to 16.53 mg/Kg. Accumulation of Zn in treatments amended with SC shows a higher amount (16.53 mg/Kg) compared with the other two organic wastes amendment. In addition, the accumulation of Pb in *Dracaena* roots in the polluted soil amended with organic waste ranged from 10.1 mg/Kg to 16.7 mg/Kg, while in soil amended with TL recorded a higher rate of accumulation (16.7 mg/Kg) and in unpolluted controlled soil without *Dracaena* had 0.8 mg/Kg. However, accumulation of Zn in plant tissue was higher than Pb; this might be because of higher quantity of Zn in polluted soil compared with Pb

polluted soil (Table 4.26). A small quantity of Pb was detected in *Dracaena* leaves; however, 3.5 mg/Kg Pb was recorded in treatment amended with TL. Accumulation of Pb was minimal in leaves (0.005 mg/Kg) compared with the quantity in roots (1.1 mg/Kg), in control soil with *Dracaena* polluted with 2.5% diesel fuel (Table 4.28). In general, from the results obtained and described above, *Dracaena reflexa* obviously has the ability to accumulate heavy metals in tissues. Thus, the accumulation of Pb and Zn is remarkable in *Dracaena* plant amended with organic wastes than with unamended control soil and treated with *Dracaena* without organic wastes. Organic waste amendments might provide a suitable condition to increase the bioavailability of metals in hydrocarbon polluted soil through to enhance the capability of the plant to uptake these metals in different plant tissues. The result agrees with the finding of Tan et al., (2007) who reported that *D. reflexa* has ability to accumulate Zn and Cd in different tissues in polluted soil with Zn and Cd under greenhouse conditions. However, the result is in contrast to Clemente et al., (2006) and Walker et al., (2004) who reported that the mobility and bioavailability of heavy metals could be reduced in the case of fresh contamination by adding organic amendments and vegetation. The accumulation of Zn and Pb in *Dracaena* tissues indicated that the *D. reflexa* could be a sink for bioavailable Zn and Pb. In addition, low concentration of Pb in *Dracaena* might be due to the lack of transport mechanisms for Pb in *Dracaena* tissues. This is similar to the result of Blaylock et al., (1997) who found that the mobility and translocation of Pb from roots to leaves is very slow.

4.7.5 Translocation and bioconcentration factors of Zn and Pb in *D. reflexa*

Table 4.30 shows the translocation factor (TF) in the edible part of the plant and the bioconcentration factor (BCF) of Zn in all the treatments. In polluted soil with 80 ppm Zn, the highest BCF was observed in amended soil with SC, while the lowest was, in

the soil without plant and uncontaminated (0.019). According to Santosh et al., (2009), BCF is the measurement of the metal accumulation efficiency and indicates the capacity of metal accumulation in relation to plant biomass. The bioconcentration factor was higher in those treatments amended with organic wastes compared with the unamended control soil. This might be because of available nutrients for the plant growth that produced high plant biomass, thereby persuading bioaccumulation of the metals in the plant tissues more than those of the unamended control treatments. Statistical analysis shows a significant difference between TF in stems and leaves ($P < 0.05$). The highest TF in leaves and steam was observed in soil amended with SC (Table 4.30).

Table 4.30 Bioconcentration and translocation factors of Zn in *Dracaena* remediated soil

Treatments	Zinc (Zn)		
	BCF	TF (in stem)	TF (in leaves)
Soil+2.5% oil + TL+ Dracaena	0.348	0.720	0.000
Soil+2.5% oil + SC + Dracaena	0.470	0.730	0.380
Soil+2.5% oil + PS + Dracaena	0.329	0.670	0.330
Soil+2.5% oil + Dracaena	0.069	0.529	0.000
Soil without oil + Dracaena	0.019	0.000	0.057

Table 4.31 shows the translocation factor (TF) in the edible part of the plant and Bioconcentration factor (BCF) of Pb in all the treatments. In polluted soil with 60 ppm Pb, the highest BCF was observed in amended soil with TL, while the lowest was, in the soil without a plant and uncontaminated (0.002). Statistical analysis does not show significant different between amendment treatments TF in stems and leaves at $P < 0.05$.

The highest TF in leaves was recorded in soil amended with TL, while the highest TF in stems was observed in soil amended with PS (Table 4.31). The result is in contrast to the result of Adesodun et al., (2010) who reported that TF of Zn in soil remediate with sunflower and contaminated with Zn was more than 1. The differences in the results recorded in the two studies might be because of the use of different plants for phytoremediation.

The results disagree with Santosh et al., (2009) who found that the application of dairy sludge significantly reduced the extractable As, Cr and Zn concentration in soil, while the application of organic amendment stabilized As, Cr and Zn and reduced their uptake in plant tissue. The differences in the results might be due to the use different of plants; Santosh et al., (2009) used *Jatropha* in their study and their treatments were not polluted with hydrocarbon compounds.

Table 4.31 Bioconcentration and translocation factors of Pb in *Dracaena* remediated soil

Treatments	Lead (Pb)		
	BCF	TF (in stem)	TF (in leaves)
Soil+2.5% oil + TL+ Dracaena	0.54	0.58	0.20
Soil+2.5% oil + SC + Dracaena	0.37	0.48	0.00
Soil+2.5% oil + PS + Dracaena	0.32	0.75	0.00
Soil+2.5% oil + Dracaena	0.01	0.00	0.00
Soil without oil + Dracaena	0.00	0.08	0.00

4.7.6 Rate of metal uptake by *Dracaena reflexa*

Table 4.32 reveals the rate of heavy metal uptake from soil by *Dracaena*. Within the 180 day period treatments amended with organic waste recorded the highest rate of metal uptake than the unamended soil. However, treated soil amended with TL recorded a higher rate of uptake in Zn and Pb contamination, followed by waste amended soil with SC and PS. The reason for this higher uptake rate by amended soil compared to control treatments can be attributed to the speed of the growth of *Dracaena* in these treatments that was much faster and it grew taller than plants in controls. The result is similar to the findings of Chen et al., (2011) who reported that using Shougang slag (SG slag) as an amendment in heavy metal contaminated soil increased the rate of adsorption capacity which led to increase in rate constant of Zn, Cd, Cu and Pb compared with those amended with Baoshan slag (BS slag).

Table 4.32 Rate of metals uptake from soil by *Dracaena*

Treatments	Rate of uptake (monthly)	
	Zn	Pb
Soil+2.5% oil + TL+ Dracaena	0.138	0.160
Soil+2.5% oil + SC + Dracaena	0.129	0.112
Soil+2.5% oil + PS + Dracaena	0.099	0.080
Soil+2.5% oil + Dracaena	0.041	0.032
Soil without oil + Dracaena	0.098	0.000

4.7.7 Bioaccumulation of heavy metals by *Podocarpus polystachyus*

Residual Zn and Pb concentrations in the soil polluted with 80 ppm Zn and 60 ppm Pb and planted with *P. polystachyus* in different treatments are shown in Tables 4.33 and 4.34. Zn accumulation in those amended with organic wastes ranged from 28.7 mg/Kg to 37.1 mg/Kg, while in polluted control soil with *Podocarpus* and without organic wastes recorded 57.3 mg/Kg. Pb accumulation in treated soil amended with organic wastes recorded a range between 34.6 mg/Kg and 37.1 mg/Kg, whereas in polluted and unamended treatment it shows a higher quantity of Pb. Translocation of Zn and Pb from the root of *Podocarpus* plant to the leaves and stems was recorded in all the treatments.

Table 4.33 Residual Zn concentration in soil remediated with *Podocarpus* and polluted with 80 ppm Zn after 6 months

Treatments	Heavy metal (mg/Kg)
	Zn
Soil+2.5% oil + TL+ Podocarpus	36.6
Soil+2.5% oil + SC + Podocarpus	28.7
Soil+2.5% oil + PS + Podocarpus	37.1
Soil+2.5% oil + Podocarpus	57.3
Soil without oil + Podocarpus	15.2

Table 4.34 Residual Pb concentration in soil remediated with *Podocarpus* and polluted with 60 ppm Pb after 6 months

Treatments	Heavy metal (mg/Kg)
	Pb
Soil+2.5% oil + TL+ Podocarpus	36.2
Soil+2.5% oil + SC + Podocarpus	34.6
Soil+2.5% oil + PS + Podocarpus	37.1
Soil+2.5% oil + Podocarpus	41.2
Soil without oil + Podocarpus	15.8

ND: Not detected

At the end of 180 days, quantities of Zn and Pb were determined to detect the accumulation in *Podocarpus* roots, stem and leaves (Table 4.35 and 4.36). Heavy metal uptake demonstrated the accumulation of Zn in the root and stem of *Podocarpus* while there was no accumulation of Zn in leaves of *Podocarpus* in all the treatments. The accumulation of Zn in the leaves of *Podocarpus* ranged from 1.1 mg/kg to 2.01 mg/kg in those planted amended soil with organic wastes, whereas in the roots of *Podocarpus* the range was between 10.4 mg/kg to 13.7 mg/kg. Soil amended with SC and planted with *Podocarpus* recorded the highest Zn and Pb in stems and roots compared with other organic wastes. However, the result of Pb contaminated soil also revealed that there was no accumulation of metals in *Podocarpus* leaves (Table 4.37). The result of Zn and Pb accumulation is in line with the study of Hassinen et al., (2009), who recorded metal accumulation in the plant parts of hybrid aspen which planting on a metal contaminated sites, in the first year of study.

Table 4.35 Zn contents in soil of *P. polystachyus* planted and contaminated with 80 ppm Zn

Treatments	Zn (mg/Kg)		
	Roots	Stems	Leaves
Soil+2.5% oil + TL+ Podocarpus	11.8	6.73	1. 2
Soil+2.5% oil + SC + Podocarpus	13.7	10. 8	2.0
Soil+2.5% oil + PS + Podocarpus	10.4	8.7	1.1
Soil+2.5% oil + Podocarpus	4.4	ND	ND
Soil without oil + Podocarpus	6.1	ND	0.6

ND: Not detected

Detection of Pb was in the roots and stems of *Podocarpus* and only 0.8 mg/kg and 0.5 mg/kg were detected in leaves of *Podocarpus* in soil amended with TL and PS in soil amended, respectively (Table 4.36). The trace of Pb in roots of plants was from 7.21 mg/kg to 9.8 mg/kg in soil amended with organic wastes, while 0.1 mg/kg Pb was detected in roots of treatment with *Podocarpus* without organic waste amendments. However, no trace of Pb was detected in leaves, possibly that is why there is no report on phytoremediation of heavy metals with *Podocarpus*. The result is in agree to Mun et al., (2008) who discovered the bioaccumulation of Pb in stems and roots of the *H. cannabinus*. They reported that there is a hyperaccomulation of Pb in those amendments with added fertilizers compared with the ones without amendments.

Table 4.36 Pb contents in soil of *P. polystachyus* planted and contaminated with 60 ppm Pb

Treatments	Pb (mg/Kg)		
	Roots	Stems	Leaves
Soil+2.5% oil + TL+ Podocarpus	9.8	6.3	0.8
Soil+2.5% oil + SC + Podocarpus	7.4	5.2	0.6
Soil+2.5% oil + PS + Podocarpus	7.2	4.3	0.5
Soil+2.5% oil + Podocarpus	0.1	0.0	ND
Soil without oil+ Podocarpus	0.9	ND	ND

ND: Not detected

The result of heavy metal accumulation in *Podocarpus* tissues (Table 4.36) is similar to the finding of Hassinen et al., (2009) who recorded that hybrid aspen has the ability to accumulate Zn and Fe from contaminated sites. Addition of organic amendments could promote plant growth, as well as, improve the absorption of metals and accumulation in the plant tissues. This might also be due to the quantity of nutrients in organic wastes which lead to enhance the ability of fibrous roots to growth. The results are in contrast to Tordoff et al., (2000) and Walker et al., (2004) who illustrated the significantly reduced uptake of metals such as As, Cr and Zn in contaminated soil by *Jatropha curcas* with the application of dairy sludge. The differences in the two studies might be because of different ecology and environmental factors of the soil which was utilized for phytoremediation purposes or the use of different organic amendments and plants (Abioye et al., 2010).

4.7.8 Translocation and Bioconcentration factors of metals in *P. polystachyus*

The bioconcentration factor (BCF) and translocation factor (TF) of Zn and Pb with *Podocarpus* is shown in Tables 4.37 and 4.38. Among the different treatments the highest TF (stem) belongs to soil amended with PS (0.83) and in TF (leave) the highest was recorded in contaminated soil amended with SC (0.14). However, the highest BCF (0.35) was recorded in contaminated soil amended with SC and 2.5% diesel fuel. The finding is contrast with the TF (stem and leave) Zn recorded with *Dracaena* and it disagrees with the result of Santosh et al., (2009) who demonstrated the high TF in those treatments without organic waste amendments. The reason for different rate of uptake by plants is due to the differences in amendments and also may be difference in plants physiological system used in this study. The result of the bioconcentration factor (BCF) and translocation factor (TF) of Pb in *Podocarpus* is shown in Table 4.39.

The highest TF in stem was recorded in soil amended with PS with 0.83. There was significant difference in TF in Pb contaminated soil amended with organic wastes and unamended controlled soil at $P < 0.05$ confidence level. However, there was no significant difference between treatments in BCF. The results indicate that *Podocarpus* has the ability to be used for phytodegradation of hydrocarbons while bioaccumulation of metals was low in *Podocarpus* tissues.

Table 4.37 Bioconcentration and translocation factors of Zinc in *Podocarpus* remediated soil

Treatments	Zinc (Zn)		
	BCF	TF (in stem)	TF (in leaves)
Soil+2.5% oil + TL+ Podocarpus	0.26	0.57	0.10
Soil+2.5% oil + SC + Podocarpus	0.35	0.78	0.14
Soil+2.5% oil + PS + Podocarpus	0.27	0.83	0.10
Soil+2.5% oil + Podocarpus	0.06	0.00	0.00
Soil without oil + Podocarpus	0.09	0.00	0.09

Table 4.38 Bioconcentration and translocation factors of Lead in *Podocarpus* remediated soil

Treatments	Lead (Pb)		
	BCF	TF (in stem)	TF (in leaves)
Soil+2.5% oil + TL+ Podocarpus	0.30	0.64	0.08
Soil+2.5% oil + SC + Podocarpus	0.23	0.70	0.08
Soil+2.5% oil + PS + Podocarpus	0.21	0.60	0.07
Soil+2.5% oil + Podocarpus	0.00	0.50	0.00
Soil without oil + Podocarpus	0.01	0.00	0.00

4.7.9 Rate of metals uptake by *Podocarpus polystachyus*

Table 4.39 reveals the rate of heavy metal uptake in remediation of soil by *Podocarpus*. Within 180 day period of study treatments amended with organic wastes recorded the highest rate of metals uptake than the unamended soil. However, treated soil amended with SC recorded a higher rate of uptake in Zn contamination, followed by amended soil with PS and TL but in Pb contaminated soil the rate of uptake was as follows SC > TL > PS. This shows the potential of organic waste amendments to enhance phytoremediation of the oil and at the same time bioaccumulation of metals. The result is line with the finding of the uptake rate in soil with *Dracaena* plant. The rate of uptake in *Dracaena* contaminated soil was higher than *Podocarpus* contaminated soil. The reason might be the differences in physiology of the plants or the faster growth and higher biomass of *Dracaena* plant compared with *Podocarpus*.

Table 4.39 Rate of constant metals uptake in remediate soil with *Podocarpus*

Treatments	Rate of uptake (monthly)	
	Zn	Pb
Soil+2.5% oil + TL+ Podocarpus	0.118	0.071
Soil+2.5% oil + SC + Podocarpus	0.159	0.078
Soil+2.5% oil + PS + Podocarpus	0.116	0.066
Soil+2.5% oil + Podocarpus	0.043	0.050
Soil without oil + Podocarpus	0.029	0.032

4.8 Biodegradation test using bacteria isolated from diesel-contaminated soil

Five bacteria were selected for biodegradation study based on the efficient utilization of oil in the preliminary trials and their rapid growth in oil agar. The five microbial isolates were identified as species *Stenotrophomonas acidaminiphila, Bacillus lichenifomis, Brevibacillus parabrevis, Ochrobactrum tritici, Pesedomonas citronellolis.* These bacterial species have been identified by many reserchers (Zahra et al., 2006; Zanaroli et al., 2010). Table 4.40 shows the result of the percentage of biodegradation by these isolated bacteria during the 25 day incubating. Results indicate the appreciable percentage of biodegradation in microbial isolates compared to the percentage of oil loss in control flask (without microbial). The results illustrate the rapid reduction of oil in the first week of incubation. In flask incubated with *Bacillus lichenifomis* and *Peseudomonas citronellolis* recorded 24.7% and 24.6% biodegradation, respectively in 7^{th} day compared with 1.2% recorded in control flask. At the end of the incubation period higher percentage of degradation was recorded for *Bacillus lichenifomis* (45.8%) follow by *Peseudomonas citronellolisc* (40.6%), *Brevibacillus parabrevis* (33.2%), *Ochrobactrum tritici* (30.5%) and *Stenotrophomonas acidaminiphila* (28.4%). Out of 5 bacteria isolated *Bacillus licheniformis* recorded higher percentage of degradation compared with other bacteria. The reseaon might be due to the higher ability of Bacillus for biodegradation than other bacteria or might be because of the existance of effective biodegrative enzymes in Bacillus isolates. The result is supported by Kohsari et al., (2010) who reported that *Bacillus licheniformis* culture could efficiently reduce 35% of quinoline in aqueous media. They also indicated that resting cell of *B.licheniformis* were capable of removing 25% total nitrogen from 5% crude oil. The result is similar with Ayoub and Ghaemi (2003) that *B. licheniformis* was highly effective in the degradation of cyclo- and iso- alkanes between C_{20} - $C_{30.}$

Table 4.40 Percentage of diesel fuel biodegradation by microbial isolates

Oil biodegradation (%)					
Microbial isolates (days)	**7**	**14**	**21**	**28**	**35**
Bacillus licheniformis	24.7	28.5	33.6	38.9	45.8
Brevibacillus parabrevis	21.1	24.4	26.7	30.8	33.2
Ochrobactrum tritici	20.3	22.2	25.5	28.1	30.5
Peseudomonas citronellolis	24.6	26.4	30.4	36.6	40.6
Stenotrophomonas acidaminiphila	15.5	17.1	20.3	25.5	28.4
Control	1.2	1.8	2	2.2	3

In addition, Luo et al., (2012) reported that *Psedomunas sp.* degraded 75.9% of diesel oil from the bilge water within 10 days of incubation at optimal condition. The difference oil biodegradation might probably due to the different media pollution, which Luo et al., (2012) evaluated oil degradation in water pollution while we studied soil pollution with diesel oil. At the end of incubation period, *Brevibacillus parabrevis* recorded 33.2% degradation. The results from Bao et al., (2012) reflected that *Brevibacillus parabrevis* degraded 99% crude oil within 14 days and *B. parabrevis* could adapt to high concentration of petroleum environment with better biodegradation potential. The difference in result of degradation might be due to different oil used for the studies, and diesel oil probably inhibits the growth of the organisms and subsequently reduced the rate of oil biodegradation compared to the results of Bao et al., (2012). *Stenotrophomonas acidaminiphila* rcorded 28.4% degradation at the end of 25 days. It might possibly be due to non production of biosurfactants by the isolated strains (Walter et al., 2010).

4.9 Comparison of results of phytoremediation between *Dracaena* and *Podocarpus*

After 270 days, *D. reflexa* recorded higher rate of oil degradation compare with *P. polystachyus.* Total percentage of oil loss recorded by *Dracaena* was 98.8%, 90.3% and 19% in soil polluted with 1, 2.5 and 5% diesel oil, respectively. *P. polystachyus* remediated 91%, 85% and 13.8% with 1, 2.5 and 5% diesel oil, respectively. It can be concluded that *Dracaena* will be good for remediation of low and medium concentration of diesel fuel contaminated soil for a period of nine months compare with *Podocarpus*. *Dracaena* has potential to develop and grow as shrub and survive for a several years. On the other hand, results demonstrated that both plants are not suitable for remediation of high level of diesel fuel contamination in a short term of remediation.

4.10 General discussion

The main objective of this research was to evaluate the potential of biowastes (organic waste amendments) to enhance the biodegradation (biostimulation) using two local plants (*Dracaena* and *Podocarpus*) for phytoremediation of diesel fuel contaminated soil. Four different concentrations (5, 10, 15 and 20% w/w) of diesel fuel were used for biostimulation studies within 126 days and 365 days under laboratory and natural conditions, respectively. SC showed the highest potential in enhancing the biodegradation of oil at all the diesel fuel pollution levels compared with PS and TL amendments. SC amendment enhanced the biodegradation of diesel fuel by 95% to 25% oil loss ranged for 5 to 20% diesel fuel. The reason for the high potential by SC might be due to its high nutrients level (especially N) compared to other amendments utilized in this study. The results obtained demonstrated the potential of organic wastes for oil bioremediation in the order SC > PS > TL.

Two different rate of organic waste amendments (10% and 5%) were tested at four different levels of oil pollution (5, 10, 15 and 20% w/w) in order to evaluate which

amendments will show the best degradation rate on diesel fuel. Treatments amended with 10% biowastes recorded the highest percentage of degradation compared to polluted soil amended with 5% biowastes. In natural condition study, contaminated soil amended with SC aided an enhanced stimulation of hydrocarbon degradation and it showed high potential to degraded different level of the hydrocarbon fractions (C_{12} to C_{20}) at the end of one-year study.

Two local plants namely, *Dracaena* and *Podocarpus* were used and monitored for 270 days under laboratory and natural conditions. The results of GC/MS of the two plants tissues did not show accumulation of oil; it is concluded that the mechanisms of phytoremediation by the plants possibly is via the phytovolitilization or rhizodegradation. It is supported by the fact that more number of bacteria is found in rhizosphere zone of plant roots than in unplanted contaminated soil. Comparison of the results of plants on phytoremediation shows the higher percentage of biodegradation under laboratory condition (98.8%) than those under natural condition (90.8%).

Phytodegradation of soil polluted with 5% diesel oil with *Podocarpus* was conducted 120 days (four months). At the end of the four months studies, at 5% diesel oil contamination, *13.8*% and 18.6% oil loss were recorded with *Podocarpus* and *Dracaena*, respectively.

In overall, higher growth rate of *D. reflexa* resulted probably enhanced abundant fibrous root which in turn might have positively led to higher rate of oil biodegradation in the contaminated soil. Phytoremediation of co-contaminated soil with heavy metals (80 ppm Zn and 60 ppm Pb) and 2.5% diesel oil was monitored for a period of 6 months (180 days). *Dracaena* did not show accumulation of hydrocarbon in its tissues, while it is recorded appreciable bioaccumulation of heavy metals (Zn and Pb) in plant root and stem. These results demonstrated that *Dracaena* has high potential for remediation of hydrocarbon and heavy metals contaminated soil. The result agrees with the finding of

Tan et al., (2007) who reported that *D. reflexa* has ability to accumulate Zn and Cd in different tissues in polluted soil with Zn and Cd under greenhouse conditions.

SC was more effective in phytodegradation studies with *Dracaena* and *Podocarpus*, while TL was more effective in accumulation of heavy metals (Zn and Pb) in phytoremediation studies of co-contamination soil with 2.5% diesel oil. The differences in the activities of these three different organic wastes might be due to differences in the structure and physiological systems of both plants which make it suitable for remediation process. It is concluded that to develope the better application of remediation methods for the removal of diesel fuel from soil, addition of biowastes has many essential advantages and potentially useful, due to their specificity and cost effective option. Based on the present studies, addition of nutrients (especially N and P) in the form of organic waste amendments as a cheap and available option to the contamination system may enhance the removal efficiencies further. The improvement of rhizoremediation of diesel fuel in soil by the native plants with respect to nutrients availability would benefit from further investigation.

CHAPTER FIVE
CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

In the bioremediation study, a significant reduction in diesel fuel (45%) was achieved by adding soycake. A 20 to 55 net percentage oil loss was recorded with supplementation of organic wastes compared with the control soil. Biodegradation, with addition of amendments (TL, SC and PS), is the ideal option for remediation of hydrocarbon contaminated soil. DUB and AHB counts in all the soils amended with different biomass were higher compared with that of unamended control soil. *Bacillus licheniformis*, recorded higher percentage of degradation (45.8%) compared to other five bacteria isolated. The results of monitoring ^{13}C stable isotope illustrates enrichment value of $\delta^{13}C$ in treatments amended with organic wastes compared to control soil which exhibited mineralization of microbial biomass during the degradation of diesel contaminated soil. Germination toxicity test with lettuce seed (*Lactuca sativa*) (after bioremediation process) was higher in soil polluted with 5% and 10% diesel fuel and soil amended with 10% organic wastes. However, treatments amended with 15% and 20% oil recorded the highest toxicity (10%– 40%) of seed germination for remediated soil.

The first order kinetic model shows the highest rate of degradation for soil polluted with 5% diesel fuel accrued in soil amended with 10% SC treatment (k = 0.22/day and half-life of 3.05 days). Phytoremediation study with D. *reflexa* and P. *polystachyus* demonastrated possitive effects on enhancing the reduction of diesel fuel from contaminated soil compared to the unplanted control soil. Furthermore, addition of

organic residual, especially SC, to the diesel-contaminated soil enhanced the growth of plants and propagation of microbial population in the soil. No accumulation of hydrocarbon was found in the plants tissues, indicating that oil loss from the polluted soil might be via phytovolitilization or rhizodegradation mechanisms. Findings of the study concluded that the decrease in total petroleum hydrocarbon was attributable to increased diesel utilizing bacterial population in the rhizosphere.

Both plants showed the ability of bioaccomulation of Zn and Pb present in the soil in their roots and stems. First order kinetic model shows that treated soil amended with TL recorded a higher rate of uptake of Zn and Pb, with 0.14 and 0.16 mg kg^{-1}per month by *Dracaena*. The study the ability of SC and TL with plants for remediation of heavy metals (Zn and Pb) and hydrocarbons in contaminated soil. It is conclusive that these two species are hypertolerant to the presence of heavy metals in soil. This provides an optional method in removing metals and diesel fuel contaminants from soil while assisting the growth of economically viable plants like *Dracaena* which is being used for NASA study as an air cleaner.

5.2 RECOMMENDATIONS

- In this study three different organic wastes were utilized, it is recommended using other available organic wastes which may give different results.
- Further studies should be done using higher levels of Organic wastes.
- Older contaminated soils are more difficult to remediate than freshly contaminated ones. However, it is recommended that bioremediation and phytoremediation is done on older soils.

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APPENDIX A

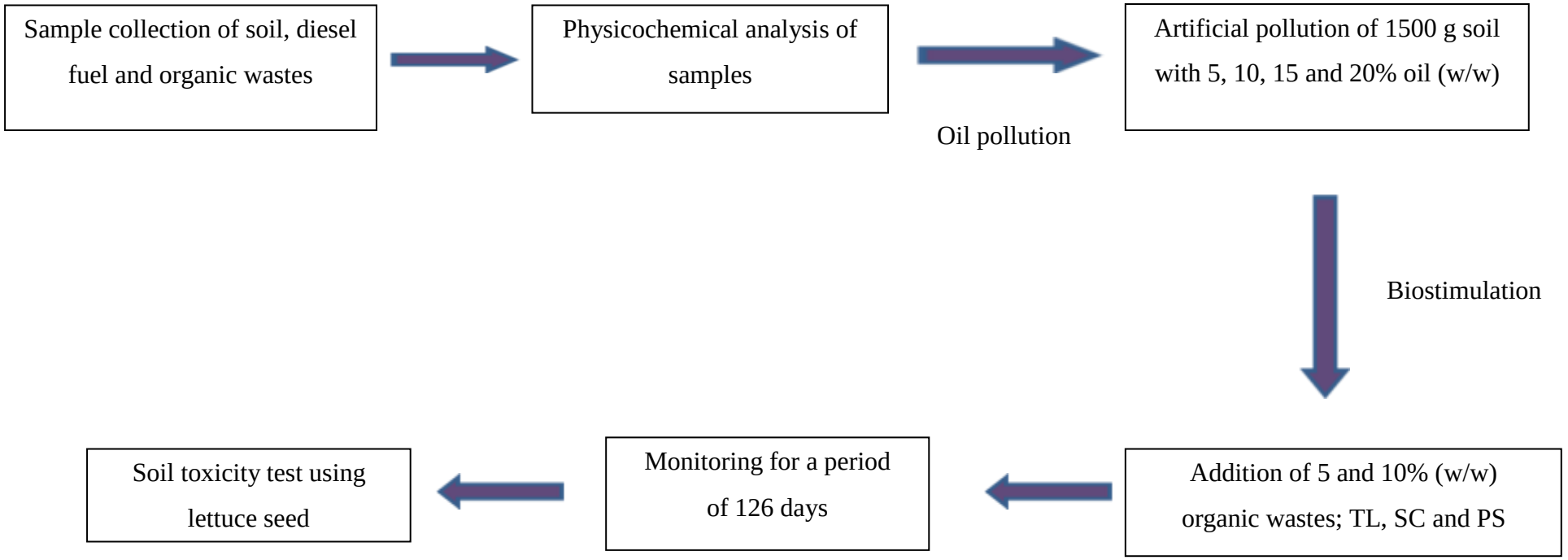
Chemical composition of Diesel fuel*

Compound Detected	Result
Alkanes	1791 ppm
Naphthalene	56 ppm

*** GC-MS Screening**

APPENDIX B

Methodology for biostimulation



APPENDIX C

$$\text{Germination index (\%)} = \frac{(\%\ SG) \times (\%GR)}{100} \qquad \text{(Eq 3.2)}$$

% SG = (% EG/%CG) x 100

% GR = (GERm/GERCm) x 100

where % SG = seed germination,

% GR = growth of the root,

% EG = germination in contaminated soil,

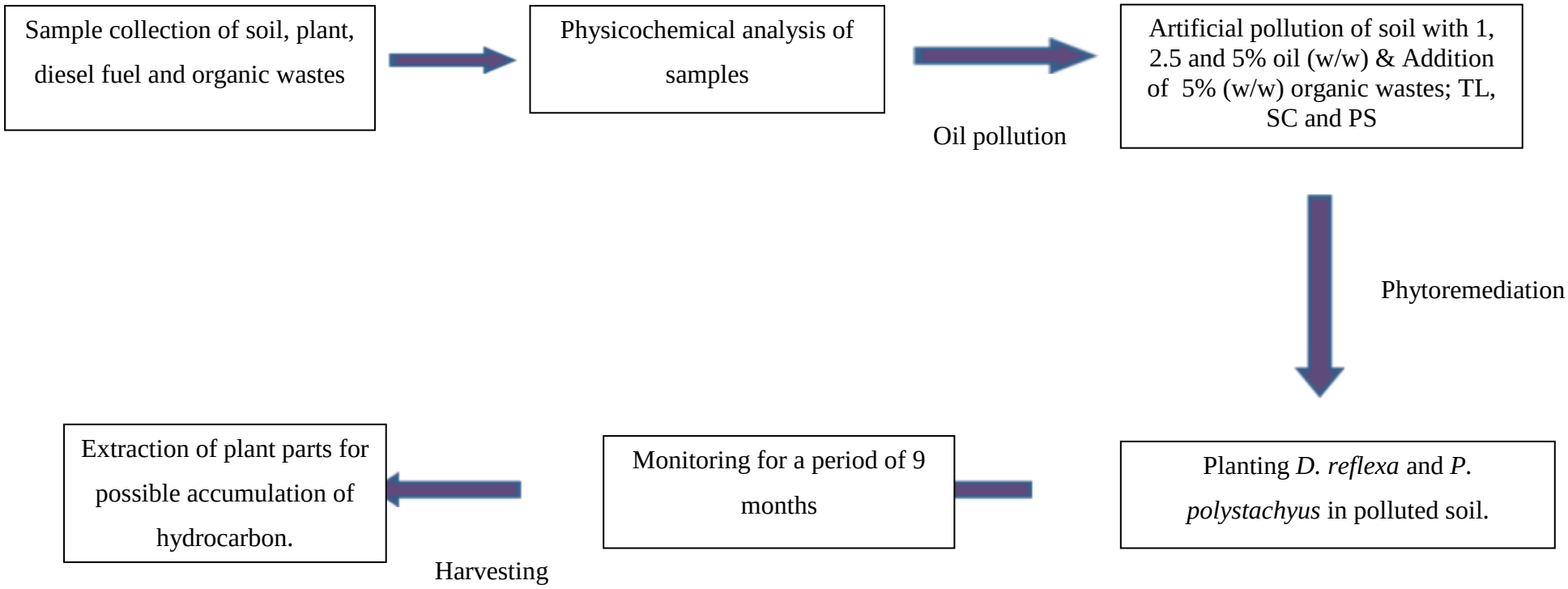
% CG = germination in control soil,

GERm = elongation of root in contaminated soil,

GERCm = elongation of root in control soil.

APPENDIX D

Methodology for phytodegradation



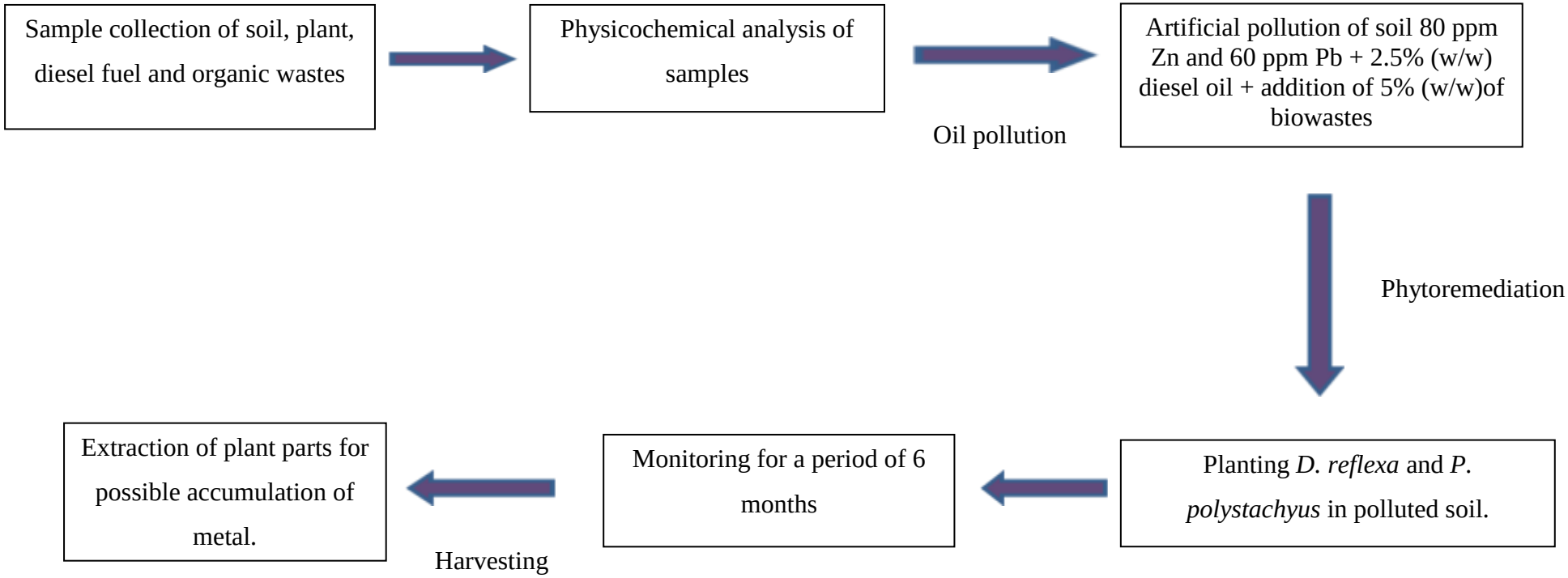
APPENDIX E

Experimental Design for phytoremediation study under laboratory and natural conditions

Treatment	Details of Treatment
A	2 Kg soil + 1 % oil + 5% TL + Dracaena/Podocarpus
B	2 Kg soil + 1 % oil + 5% SC + Dracaena/Podocarpus
C	2 Kg soil + 1% oil + 5% PS + Dracaena/Podocarpus
D	2 Kg soil + 1% oil + Dracaena/Podocarpus
E	2 Kg soil + 1% oil only
F	2 Kg autoclaved soil + 1 % oil + 0.5% NaN_3
G	2 Kg soil + 2.5 % oil + 5% TL + Dracaena/Podocarpus
H	2 Kg soil + 2.5 % oil + 5% SC + Dracaena/Podocarpus
I	2 Kg soil + 2.5 % oil + 5% PS+ Dracaena/Podocarpus
J	2 Kg soil + 2.5 % oil + Dracaena/Podocarpus
K	2 Kg soil + 2.5 % oil only
L	2 Kg autoclaved soil + 2.5 % oil + 0.5% NaN_3
M	2 Kg soil + 5 % oil + 5% TL + Dracaena/Podocarpus
N	2 Kg soil + 5 % oil + 5% SC + Dracaena/Podocarpus
O	2 Kg soil + 5 % oil + 5% PS+ Dracaena/Podocarpus
P	2 Kg soil + 5 % oil + Dracaena/Podocarpus
Q	2 Kg soil +5 % oil only
R	2 Kg autoclaved soil + 5 % oil + 0.5% NaN_3
S	2 Kg soil without oil + Dracaena/Podocarpus

APPENDIX F

Methodology for phytodegradation of co-contaminated soil



APPENDIX G

Experimental Design for phytoremediation of co-contaminated soil

Treatment	Details of Treatment
A	2 Kg soil + 2.5 % oil + 5% TL + 60 ppm Pb + Dracaena or Podocarpus
B	2 Kg soil + 2.5 % oil + 5% SC + 60 ppm Pb + Dracaena or Podocarpus
C	2 Kg soil + 2.5 % oil + 5% PS+ 60 ppm Pb + Dracaena or Podocarpus
D	2 Kg soil + 2.5 % oil + 60 ppm Pb + Dracaena or Podocarpus
E	2 Kg soil + 60 ppm Pb + + Dracaena or Podocarpus
F	2 Kg soil + 60 ppm Pb + 2.5 % oil only
G	2 Kg autoclaved soil + 2.5 % oil + 60 ppm Pb + 0.5% NaN_3
H	2 Kg soil + 2.5 % oil + 5% TL + 80 ppm Zn + Dracaena or Podocarpus
I	2 Kg soil + 2.5 % oil + 5% SC + 80 ppm Zn + Dracaena or Podocarpus
J	2 Kg soil + 2.5 % oil + 5% PS+ 80 ppm Zn + Dracaena or Podocarpus
K	2 Kg soil + 2.5 % oil + 80 ppm Zn + Dracaena or Podocarpus
L	2 Kg soil + 80 ppm Zn + + Dracaena or Podocarpus
M	2 Kg soil + 80 ppm Zn + 2.5 % oil only
N	2 Kg autoclaved soil + 2.5 % oil + 80 ppm Zn + 0.5% NaN_3
O	2 Kg soil + Dracaena or Podocarpus

Note: 1) Only 2.5% oil was used in co-contaminated soil

2) 60 ppm Pb and 80 ppm Zn were used since they had major impact on the microbial growth.

APPENDIX H

PUBLICATIONS

H_1. Published and accepted papers

- Dadrasnia A. and Agamuthu, P. (2013). Dynamics of diesel fuel degradation in contaminated soil using organic wastes. *International J. Envi Sci & Tech.* 10: 769–778.
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Dynamics of diesel fuel degradation in contaminated soil using organic wastes

Abstract

Bioremediation is an effective measure in dealing with such contamination, particularly those from petroleum hydrocarbon sources. The effect of soil amendments on diesel fuel degradation in soil was studied. Diesel fuel was introduced into the soil at the concentration of 5 % (w/w) and mixed with three different organic wastes tea leaf, soy cake, and potato skin, for a period of three months. Within 84 days 35% oil loss was recorded in the unamended polluted soil while 88%, 81% and 75% oil loss were recorded in the soil amended with soy cake, potato skin and tea leaf, respectively. Diesel fuel utilizing bacteria counts were high in all organic wastes amended treatments, ranging from 111×10^6 to 152×10^6 Colony Forming Unit/gram of soil, compared to the unamended control soil which gave 31×10^6 CFU/g. The bacterial count was significantly high compared to unamended soil. The diesel fuel utilizing bacteria isolated from the oil contaminated soil belongs to *Bacillus licheniformis, Ochrobactrum tritici* and *Staphylococcus sp*. Oil–polluted soil amended with soy cake recorded the highest oil biodegradation with a net loss of 53%, compared to the other treatments. Dehydrogenase enzyme activity, which was assessed by 2,3,5-triphenyltetrazolium chloride technique, correlated significantly with the total petroleum hydrocarbons degradation and accumulation of CO_2. First-order kinetic model revealed that soy cake was the best of the three organic wastes used, with biodegradation rate constant of 0.148 day^{-1} and half-life of 4.68 days. The results showed there is potential for soy cake, potato skin and tea leaf to enhance biodegradation of diesel in oil contaminated soil.

Key words: Bioremediation, Diesel fuel, Hydrocarbon, Organic waste

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Potential of biowastes to remediate diesel fuel contaminated soil

Abstract

The unintended release of hydrocarbons into the environment can negatively impact human and animal health, and could further change the characteristics of soils. The aim of the present work was to investigate the rate of biodegradation at 10 and 20% diesel fuel in contaminated soil amended with 10% of three different organic wastes (tea leaf, soy cake, and potato skin) for a period of 126-days. 82 and 25% oil loss was recorded in soil amended with soy cake at 10% and 20 % oil pollution, respectively. Diesel fuel utilizing bacteria counts were high in all organic wastes amended treatments, ranging from 150×10^6 to 176×10^6 CFU/g of soil, compared with the unamended control soil which gave 23×10^6 CFU/g. Dehydrogenase activity in soil was markedly enhanced by the application of organic wastes. Diesel oil composition monitored by GC/MS indicated complete degradation of n-C_9 – C_{12}. First-order kinetic model showed that among the three organic wastes used, soy cake had the highest biodegradation rate constant of 0.153 day^{-1} at 10% oil pollution, while biodegradation rate was 0.033 day^{-1} at 20% oil pollution. The results showed there is potential for soy cake, potato skin and tea leaf to enhance biodegradation of diesel in contaminated soil.

Key words: Bioremediation, Diesel fuel, Hydrocarbon, Organic waste

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Bioavailability and bioremediation of diesel fuel-contaminated soil using organic wastes as supplement

Abstract

Soil and surface water contamination by organic compounds is a common occurrence in most developing countries. This caused harmful effects on the environment and human beings. Bioremediation can be an alternative green technology for remediation of such hydrocarbon-contaminated soil. Bioremediation of soil contaminated with 5% and 10% (w/w) diesel fuel amended with 10% soy cake (SC), potato skin (PS) and tea leaf (TL) was studied for a period of 84 days, under laboratory condition. At the end of 84 days, the highest percentage of oil biodegradation (88%) was recorded in soil contaminated with 5% diesel fuel and amended with SC, while only 75% of oil biodegradation was recorded in soil contaminated with 10% diesel fuel and amended with SC. Bioavailability which was assessed by the hydroxypropyl cyclodextrin (HPCD) extraction method showed that bioavailability reduced in soil amended with organic wastes. Results of first order kinetic model to determine the rate of biodegradation of diesel fuel revealed that soil amended with SC recorded the highest kinetic rate of oil biodegradation 0.148 day^{-1} and 0.103 day^{-1} in 5% and 10% oil pollution. The results of this study demonstrated the potential of SC as a good substrate to enhance remediation of hydrocarbon contaminated soil at low pollution concentration.

Key words: Bioavailability, bioremediation, diesel fuel, organic waste

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Enhanced Degradation of Diesel-Contaminated Soil Using Organic Wastes

Abstract

This study was carried out to enhance the biodégradation of diesel fuel in soil contaminated with 10 %(w/w) diesel fuel amended with 10% tea leaf (TL), soy cake (SC), potato skin (PS) for a period of 3 months under laboratory condition. At the end of 84 days, the highest percentage of oil biodégradation (76%) was recorded in soil amended with SC; 64% and 53% were recorded with soil amended with PS and TL respectively, while only 27% of oil degraded in control treatment. Hydrocarbon utilizing bacteria (HUB) counts were high in all organic wastes amended treatments, ranging from 45×10^{6} CFU/g to 90×10^{6} CFU/g of soil compared to unamended control soil (4×10^{6} CFU/g to 8×10^{6} CFU/g of soil). The count in amended soil was significantly different at ($P > 0.05$) compared to unamended soil. The results obtained showed 90%, 80% and 60% seed germination in remediated soil contaminated with 10% diesel fuel and amended with SC, PS and TL respectively, over the period of 84 days. The results show the high potential of SC for enhanced biodégradation of hydrocarbon in oil contaminated soil.

Key words: Degradation; Diesel; Hydrocarbon; Organic waste

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Diesel fuel degradation from contaminated soil by *Dracaena reflexa* using organic waste supplementation

Abstract

The phytoremediation potential of *Dracaena reflexa* to remediate diesel contaminated soil was determined in a greenhouse study. *D.reflexa* was planted in soil contaminated with different concentrations of diesel fuel (1, 2.5 and 5% w/w). 5% (w/w) of three different organic wastes [tea leaf (TL), soy cake (SC) and potato skin (PS)] were mixed with the soil and monitored for 270 days. The results of the biodegradation of oil and its fractions showed a reduction of 90 and 98% of total petroleum hydrocarbons (TPHs) in soil amended with SC, at 2.5% and 1% fuel, respectively. It was observed that in the non-cultivated polluted soil the TPHs, were reduced by 24 -27%. Soil amended with SC provided the greatest diesel fuel loss when compared to other organic waste supplements. *D. reflexa* roots did not accumulate hydrocarbons from the soil, but the number of hydrocarbon utilizing bacteria was high in the rhizosphere, thus suggesting that the mechanism of the oil degradation was via rhizodegradation. This study has shown that *D.reflexa* amended with organic wastes has a potential for biodegrading hydrocarbon-contaminated soil.

Key words: Phytoremediation, Rhizodegradation, Degradation, *Dracaena reflexa.*

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Organic Wastes to Enhance Phytotreatment of Diesel-Contaminated Soil

Abstract

Toxic inorganic and organic chemicals are major contributors to environmental contamination and poses major health risk to human population. In this work, *Dracaena reflexa* and *Podocarpus polystachyus* were investigated for their potential to remove hydrocarbon from 2.5 and 1% diesel fuel contaminated soil amended individually with 5% organic wastes [Tea Leaf (TL), Soy Cake (SC), and Potato Skin (PS)] for a period of 270 days. Loss of 90 % and 99% oil was recorded in soil contaminated with 2.5 and 1% oil with SC amendment, respectively, compared with 52 % and 62% in unamended soil with *D. reflexa* at the end of 270 days. Similarly, 84 and 91% oil loss was recorded for *P. polystachyus* amended with organic wastes in 2.5 and 1% oil, respectively. Diesel fuel disappeared more rapidly in the soil amendment with SC than in other organic wastes supplementation. It was evident that plants did not accumulate hydrocarbon from the soil, while the number of hydrocarbon utilizing bacteria was high in the rhizosphere, thus suggesting that the mechanism of the oil degradation was rhizodegradation. Kinetic model result indicated a high rate of degradation in soil amendment with SC at 1 % with Dracaena compare to other treatments. Thus, a positive relationship was observed between diesel hydrocarbon degradation with plant biomass production. *D. reflexa* with organic wastes amendment has a greater potential of restoring hydrocarbon-contaminated soil compared to *P. polystachyus* plant.

Keywords: Phytodegradation, biowastes, Total petroleum hydrocarbon, *D. reflexa, P. polystachyus*

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Bioremediation of diesel fuel contaminated soil by *Podocarpus polystachyus* enhanced with organic wastes

Abstract

A greenhouse experiment was performed to evaluate the effectiveness of *Podocarpus polystachyus* in phytoremediation of soil contaminated with 1% and 2.5% w/w diesel fuel. This research was aimed at assessing the potential of 5% (w/w) of three organic waste amendments (biowastes)[tea leaf (TL), soy cake (SC) and potato skin (PS)] to enhance degradation of diesel in contaminated soils for a period of 270 days. Addition of biowastes, especially SC, to contaminated soil planted with P. *polystachyus* rapidly increased the rate of removal of diesel fuel by 90% and 84% in soil contaminated with 1% and 2.5% oil, respectively. Loss of diesel fuel at 53% and 43% were recorded in treatments without organic waste amendment and planted with P. *polystachyus* in 1% and 2.5% contamination, respectively. Diesel fuel degradation was more rapid in the soil amendment with SC than in other organic waste amendments. P. *polystachyus* roots did not accumulate oil from the contaminated soil, but the number of hydrocarbon utilizing bacteria (HUB) was high in the rhizosphere, thus suggesting that the mechanism of the oil degradation was via rhizodegradation or phytovolatilization. P. *polystachyus* with organic waste amendment has potential in restoring hydrocarbon-contaminated soil.

Key words: Phytoremediation, *Podocarpus polystachyus*, Diesel fuel, Organic waste amendments