The Impact of Nutrient and Biodiesel Amendments on the Biodegradation of Hydrocarbons in Contaminated Soil

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Declaration

I hereby certify that this work is my own, except where otherwise acknowledged, and that this work has not been submitted previously for a degree at this, or any other university.

………………………………………

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Abstract

The effect of nutrient, biodiesel and biochar amendments on the biodegradation of pollutant hydrocarbons was investigated in soil spiked with crude oil and coal tar over periods of up to 180 days. Biodiesel was chosen as an amendment for increasing the bioavailability of hydrocarbon pollutants because of its good solvent properties but low toxicity. Results from laboratory microcosm experiments on soil spiked with weathered and un-weathered tar showed that after 60 days, the concentrations of the potent carcinogen benzo[a]pyrene (BaP) were significantly reduced by 92 and 81%, respectively, in the biodiesel amended samples compared to the 17 and 26% reduction in the controls, and 8 and 34% depletion observed in the nutrient-only amended microcosms, respectively. The 3-ring PAH anthracene was also almost completely biodegraded in all the biodiesel amended experiments. However, phenanthrene degradation was significantly inhibited in these samples as only 0-2% reduction occurred after 180 days as opposed to the losses (>70%) observed in the control and the nutrient amended experiments. Apparent increases in concentrations were observed for some 4-ring PAHs, while the degradation of other 5 and 6-ring compounds was enhanced in the biodiesel amended samples probably due to their increased solubilisation by biodiesel. A stepwise treatment approach conducted on tar spiked soil revealed a higher reduction in BaP (98%) in the biodiesel amended microcosms compared to the control (29%) and phenanthrene depletion was also enhanced by 51% after 60 days of adding biodiesel to soil initially treated with nutrients. A similar trend in PAH degradation was observed for the crude oil spiked soil, but in these experiments the removal of the *n*-alkanes was significantly enhanced by nutrient amendment alone, while degradation of the branched alkanes was increased in the biodiesel treatments. Toxicity assays showed that biodiesel amended microcosms stimulated phosphatase enzyme activity and exhibited a lower toxic response to Microtox *Vibrio fischeri*. In this study, biochar amendment did not significantly reduce residual pollutant concentrations. Overall, the pattern observed in the removal of the PAHs using biodiesel, suggests the cometabolic action of ligninolytic fungi, probably via lignin peroxidases, as also evidenced from the visible growth of moulds after 7-14 days of amendment. The enhanced removal of carcinogenic PAH and the reduced toxicity observed in soil after biodiesel amendment, indicates that this bioremediation technique has potential for full scale field trials.

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Dedication

I dedicate this thesis to The Almighty God, the Creator of the heavens and the earth. You simplified the process for me. I will ever remain faithful and true to You, O God of my salvation.

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List of Abbreviations

Sample Codes

CC	Control sample of crude oil spiked soil
CNT	Nutrient amended sample of crude oil spiked soil
CBN	Biodiesel amended sample of crude oil spiked soil
WC	Control sample of weathered coal tar spiked soil
WNT	Nutrient amended sample of weathered coal tar spiked soil
WBN	Biodiesel amended sample of weathered coal tar spiked soil
TC	Control sample of un-weathered coal tar spiked soil
TNT	Nutrient amended sample of un-weathered coal tar spiked soil
TBN	Biodiesel amended sample of un-weathered coal tar spiked soil
TBN60	Biodiesel amended sample of un-weathered coal tar spiked soil (biodiesel was added after initial incubation of spiked soil with nutrients for 60 days)
TC-B	Biochar treated control of un-weathered coal tar spiked soil
TNT-B	Nutrient and biochar amended sample of un-weathered coal tar spiked soil
TBN-B	Biodiesel and biochar amended sample of un-weathered coal tar spiked soil
TBN60-B	Biodiesel and biochar amended sample of un-weathered coal tar spiked soil (biodiesel was added after initial incubation of spiked soil with nutrients for 60 days)

Acronyms & Other Abbreviations

WHO	World Health Organisation
IPCC	Intergovernmental Panel on Climate Change
PAH	Polyaromatic hydrocarbons
ATSDR	Agency for Toxic Substances and Disease Registry
NTP	National Toxicology Programme
USEPA	United States Environmental Protection Agency
HMW	High molecular weight
LMW	Low molecular weight
IARC	International Agency for Research on Cancer
CMC	Critical micelle concentration
GC-FID	Gas chromatography with flame ionization detection
GC-MS	Gas chromatography-mass spectrometry

GC-MS-MS	Gas chromatography-tandem mass spectrometry
DMSO	dimethylsulfoxide
DCM	Dichloromethane
RME	rapeseed methyl ester
NPK	Nitrogen, Phosphorus, Potassium
ISO	International Organization for Standardization
WHC	Water holding capacity
TOC	Total organic carbon
EOM	Extractable organic matter
TPH	Total petroleum hydrocarbon
FAME	Fatty acid methyl ester
ANOVA	One-Way Analysis of Variance
N	Naphthalene
Acy	Acenaphthylene
Acn	Acenaphthene
2 MN	2-methylnaphthalene
1 MN	1-methylnaphthalene
BP	Biphenyl
Fl	Fluorene
Phe	Phenanthrene
Anth	Anthracene
(3,2,9,1)MP	(3,2,9,1)methylphenanthrene
DBT	Dibenzothiophene
FL	Fluoranthene
PY	Pyrene
B(a)A	Benzo(a)anthracene
Ch	Chrysene
B [b-,k-,j-]F	Benzo [b-,k-,j-]fluoranthene
BeP	Benzo[e]pyrene
BaP	Benzo[a]pyrene
IndPY	Indeno[1,2,3 c-d]pyrene
DBAnth	Dibenzo[a,h]anthracene
BPer	Benzo[g,h,i]perylene.

Chapter 1

INTRODUCTION

1.1 Environmental pollution: a global challenge

Since the first United Nations conference on the environment held in 1972, issues on the environment have remained highlights of global importance (McLin, 1972; Weiss, 1992; WHO, 2000b; Destatte, 2010; Evers *et al.*, 2011; IPCC, 2011; Zhou *et al.*, 2011). The negligent use of the Earth's natural resources, the careless disposal of toxic substances generated from industrial, human and agricultural activities and the poor treatment of wastes, are some of the major anthropogenic activities that have significantly impacted the quality of the environment (Vidali, 2001). These concomitantly contribute to climate change, ozone layer depletion, land, water and atmospheric pollution, threat to global health, and food insecurity (Salter and Ford, 2000; Beniston, 2003; Suk and Davis, 2008; Ericksen *et al.*, 2009; Dimitrov, 2010; Ruane and Sonnino, 2010).

One of the key drivers of environmental pollution is the rising needs of human development and modernisation, which has led to an increasing dependence on energy (MacKenzie, 1998). This high demand for energy that resulted in the transition from the use of renewable forms of energy such as wood, wind etc. to fossil fuels (coal, crude oil and natural gas) has over the years become unsustainable and detrimental to the environment (MacKenzie, 1998; Asif and Muneer, 2007). The release of greenhouse gases from the burning of fossil fuels still remains the dominant cause of global warming and climate change. According to IPCC (2011) report, annual emission of CO_2 from anthropogenic activities increased by about 80% between 1970 and 2004, largely contributing to the observed increase in temperature by 0.2^oC per decade. Air pollution also arising from the inefficient burning of fossil fuels has been reported to affect more than 2 billion of the world's population who cannot afford clean energy, and this can have damaging effects on their health and well-being (Wilkinson *et al.*, 2007). Respiratory related diseases and allergies that result from exposure to emissions from car exhausts, which are more prominent in the urban settlements, are also some of the health hazards associated with fossil fuel consumption (D'Amato *et al.*, 2010).

Fuel spillage is another common occurrence associated with energy consumption. The exposure to fossil fuels within land and marine habitats can cause significant mortality of the organisms living in them, in addition to long term sub-lethal effects on the surviving wildlife that can be attributed to the persistence of toxic pollutants present in these fuels, which delay the recovery of the ecosystem (Peterson *et al.*, 2003). One major group of pollutants found in fossil fuels that are of primary concern because of their associated carcinogenicity and persistence in the environment are the polyaromatic hydrocarbons (PAHs). The presence of high molecular weight PAHs, for example benzo[*a*]pyrene, which is known to be a carcinogenic, teratogenic and toxic compound, can accumulate in the cells of marine and plant organisms and consequently have deleterious effects on humans when it enters the food chain (Juhasz and Naidu, 2000).

1.2 PAH contamination of soil and bioremediation

Polyaromatic hydrocarbons (PAHs) are ubiquitously distributed in both aquatic and terrestrial environments (IARC, 2010). They occur naturally in petroleum and coal and are also produced by anthropogenic activities (WHO, 2000a). The high presence of PAHs in soils is most often due to fossil fuel consumption, storage and disposal, sewage treatment, atmospheric deposition and the use of fertiliser and composts (Cerniglia, 1992; ATSDR, 1995; Macleod and Semple, 2002). Toxicity, carcinogenicity and mutagenicity have been associated with PAHs, especially the high molecular weight (HMW) PAHs (NTP, 2011). This has always been a cause of great concern to regulatory authorities and has led to the development of physical, chemical and biological methods for the clean-up of contaminated sites (Kim *et al.*, 2001; Semple *et al.*, 2003). However, biological treatments (bioremediation) remain the most preferred because they are generally cost effective and environmentally friendly (Kim *et al.*, 2001). The utilization of PAHs as carbon and energy sources by indigenous microorganisms present in the polluted area is one process usually exploited in bioremediation treatments. This is actually the major route by which these compounds are removed from the environment (Haritash and Kaushik, 2009) and is dependent on the level of bioavailability of PAHs to the degrading microorganisms (Lei *et al.*, 2004) .

The removal of high molecular weight (HMW) PAHs from contaminated soils still poses significant challenges to remediation treatments. Efforts made to optimise environmental and microbial factors to favour their degradation are often met with little success when PAHs are not accessible to microbial metabolism (Cerniglia, 1993). Low bioavailability of HMW PAHs can be largely attributed to their hydrophobicity which increases with the number of fused benzene rings. This feature enhances the sorption or sequestration of these compounds to the soil matrix, a process that occurs by slow diffusion of pollutants into the soil organic matter and their subsequent entrapment within the micropores of the soil (Semple *et al.*, 2003); Figure 1.1 shows this physical behaviour of PAH contaminants in the soil.

Sorption of pollutants to soils initially occurs as a fast and reversible process which over a considerably long period of time leaves a residual fraction which becomes resistant to desorption and microbial degradation (Hatzinger and Alexander, 1995). The rate of desorption of PAHs from the soil surface is limited by diffusion (Valderrama *et al.*, 2008) and accelerated by the ability of microorganisms to degrade the PAHs present in the aqueous phase (Semple *et al.*, 2003). In order to facilitate this process, microbial activity could be increased by nutrient addition (biostimulation) (Tejeda-Agredano *et al.*, 2011) or the inoculation of PAH degrading organisms (bioaugmentation) (Mohan *et al.*, 2006). The mass transfer of PAHs from the solid phase to the aqueous phase of the soil where microbial activity occurs can also be enhanced by amendment techniques (Riess *et al.*, 2005). Nonetheless, problems associated with soil amendments relating to toxicity, contaminant mobility and bioavailability are still being encountered (Beesley *et al.*, 2010) thereby necessitating the development of new approaches or the better implementation of existing techniques in bioremediation studies (Gentry *et al.*, 2004).

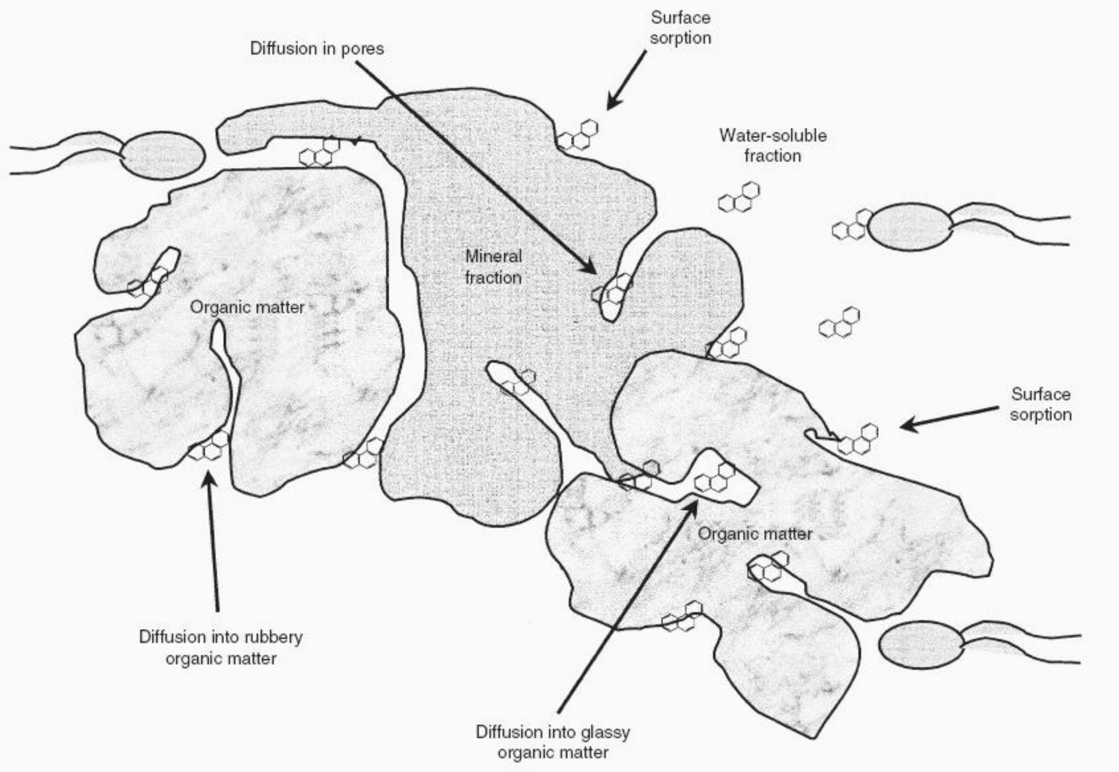


Figure 1.1. Physical behaviour of PAH contaminant within the soil (Source: Semple et al., 2003).

1.3 Increasing PAH bioavailability by solubilisation

The treatment of soils with agents capable of solubilising PAHs present in soil organic matrix is widely used as an effective remediation strategy (Mulligan *et al.*, 2001). Commonly used are the surfactants, which are micelle-forming amphiphilic molecules capable of reducing the soil interfacial tension, increasing the solubility of the hydrophobic organic compounds and transferring the desorbed compounds to the pseudo-aqueous phase for biodegradation (Laha *et al.*, 2009). These compounds are, however, only efficient at concentrations above their critical micelle concentration (CMC) values (Chen *et al.*, 2006). Some of the limitations of surfactant-based techniques include surfactant toxicity and nutrient deficiency due to surfactant degradation (Cuypers *et al.*, 2002). The sorption of surfactants which occurs at concentrations below their CMC values can also cause an increase in the sorption of PAHs which may constitute environmental problems and also reduce their effectiveness (Paria, 2008). PAHs within the micelles may also be unavailable for degradation (Cuypers *et al.*, 2002) and in some cases, the growth and degradation potential of HMW PAH degraders can be inhibited by surfactants. For example, *Stenotrophomonas maltophilia,* a pyrene-degrading organism was found to be inhibited by anionic and ionic surfactants and among the five non-ionic surfactants applied to enhance pyrene degradation, Igepal also showed inhibitory effects (Boonchan *et al.*, 1998).

Alternatively, aqueous-based extraction methods using cyclodextrin has been explored. These biodegradable, cyclic oligosaccharides possess high aqueous affinity due to the presence of functional hydroxyl groups at their torus exterior and have exhibited the potential to decrease soil toxicity in soil bioremediation and enhance PAH degradation by increasing their bioavailability (Gruiz *et al.*, 1996; Fenyvesi *et al.*, 2005). Experiments have also suggested that they could be used as non-exhaustive extraction techniques to assess PAH bioavailability based on their ability to enhance PAH desorption by reducing PAHs in the aqueous phase without increasing the PAH bioavailability or interfering with PAH degradation (Cuypers *et al.*, 2002; Sabaté *et al.*, 2006). However, cyclodextrin stability, their effect on microbial community and mode of contaminant release need to be properly estimated before they are applied as additives in bioremediation studies (Allan *et al.*, 2007).

A more recent approach is the utilization of biodiesel to increase PAH bioavailability. Solubilisation studies conducted with biodiesel, surfactant (Tween 80) and cyclodextrin showed that biodiesel was most efficient in the removal of LMW and HMW PAHs from manufactured gas plant (MGP) and spiked soils compared to the other agents (Wu *et al.*, 2010). Previous research also demonstrated its efficacy in coal tar PAH solubilisation and degradation (Taylor and Jones, 2001), enhancing crude oil degradation by cometabolism (Mudge and Pereira, 1999) as well as clean-up of contaminated shorelines and beaches (Miller and Mudge, 1997) and degradation of residual oil fractions (Fernández-Álvarez *et al.*, 2007). Biodiesel is a non toxic, readily biodegradable fatty acid methyl ester produced from the transesterification of vegetable oil (Pasqualino *et al.*, 2006; Jain and Sharma, 2010). Figure 1.2 shows the transesterification reaction involved in the production process.

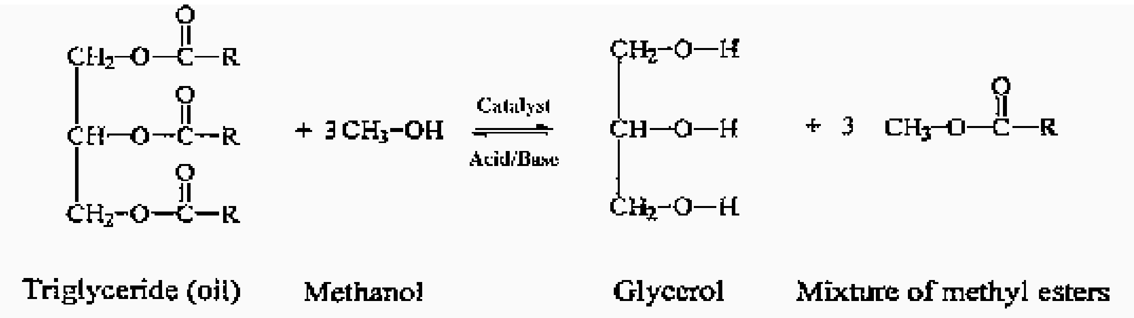


Figure 1.2. Transesterification reaction, R= alkyl group (Jain and Sharma, 2010).

The ability of biodiesel to stimulate degradation of LMW PAHs (two- and three ring PAHs) has been shown by Taylor and Jones (2001). According to their study, there were indications of the enhancement of degradation of four ring PAHs which could not be established due to data variability and insufficient replicates. This study is intended to further investigate the potential of biodiesel in enhancing HMW PAH degradation by increased PAH bioavailability, a possibility that has not been fully explored. Also, the fact that pre-treatment of contaminated soils can enhance the degradation of HMW PAHs by microbial consortia (Li *et al.*, 2008a) coupled with recent knowledge that biodiesel efficiently solubilises HMW PAHs better than surfactants and cyclodextrin (Wu *et al.*, 2010) require that more studies be conducted with biodiesel amendment techniques to investigate the possibilities and processes involved.

1.4 Decreasing PAH bioavailability by sorption

The successful remediation of PAH contaminated soils is also dependent on their microbial status (Juhasz and Naidu, 2000). In bioremediation studies, the possibility that HMW PAH degraders may not be present in the polluted soils or that these degraders may form polar metabolites of higher solubility and mobility that are toxic to the soil environment can occur (Juhasz and Naidu, 2000). In the event of any of these, increased PAH solubility can result in contradictory toxicological effects that can affect the reuse of remediated soils. Soil toxicity can also occur when increased bioavailable concentration is met with reduced microbial uptake (Fent, 2003). In view of these limitations, carbon sorbents could be employed to reduce PAH bioavailability (e.g Zimmerman *et al.*, 2004; Brandli *et al.*, 2008).

The addition of activated carbon as an *in situ* remediation technique can effectively reduce the bioavailability of hydrophobic contaminants (e.g. PAHs and polychlorinated biphenyls – PCBs) in soils and sediments by sorption mechanism, thus reducing their aqueous concentration and bioaccumulation in soil biota (Zimmerman *et al.*, 2004). The strong sorption potential of these carbon particles is enhanced by their high affinity and their characteristic large surface area (Beesley *et al.*, 2010). Studies with marine sediments contaminated with PAHs and PCBs, however, demonstrated that their efficiency in the sequestration of these contaminants is dependent on their dose and particle size (Zimmerman *et al.*, 2005).

An alternative, novel soil amendment technique is the use of biochar (Lehmann *et al.*, 2006). This black carbon is increasingly gaining attention because of its soil conditioning potential, very high organic content, large surface area and ability to also effectively reduce the bioavailability of hydrophobic organic compounds (Beesley *et al.*, 2010). Biochar is recalcitrant to microbial degradation and its sequestration effects can persist for a long period of time thus, increasing its prospects in land reclamation techniques and in the reduction of leachates concentration in polluted sites (Fellet *et al.*, 2011). It has also been useful in the sequestration of greenhouse gases, increasing soil fertility and improving crop production (Lehmann *et al.*, 2006). In this study, biochar is employed as a pollutant-sequestering, vegetable-derived material to reduce PAH bioavailability and the toxicity of residual pollutants in order to facilitate the possible re-use of contaminated land.

1.5 Motivation for this study

1.5.1 Oil pollution in the Niger Delta region of Nigeria

The incessant pollution of land and water by crude oil and petroleum products in the Niger Delta region of Nigeria poses significant threats to its ecosystem (Ekundayo and Obuekwe, 2000; Olajire *et al.*, 2005; Sojinu *et al.*, 2010). This region is known for its increasing oil spills because of its large oil deposits and growing oil and gas related industrial activities. Cases of unattended oil spills are also prevalent in this region which has resulted in the persistence of large amounts of oil in the affected soils thus requiring effective ways of oil removal (Amadi *et al.*, 1996). These sites are poorly and most often never investigated to know the extent of PAH contamination and as a result there is high possibility of accumulation of these pollutants in the food chain by their consumption in drinking water, fish and crops, which could pose a risk to human and other terrestrial and aquatic life (Anyakora *et al.*, 2005a; Ruffin, 2012). Oil pollution from spills and poor waste disposal affect the physico-chemical properties of soils with sometimes also the build-up of high concentrations of heavy metals in the affected sites (Benka-Coker and Ekundayo, 1995; Obiajunwa *et al.*, 2002; Iwegbue *et al.*, 2006). Overall, the sources of livelihood in impacted areas which include farmlands, rivers and forests have been negatively affected (Omokaro, 2009). Figure 1.3 shows a typical example of the extent of damage caused by oil pollution in this region (BBC News, 2010).

Figure 1.3. Oil pollution in the Niger Delta region (BBC News, 2010).

1.5.2 Bioremediation of polluted soils in the Niger Delta

Bioremediation has been suggested by researchers as a better approach for the clean-up of hydrocarbon contaminated soils and the removal of oil from long abandoned spillage sites for this region (Amadi *et al.*, 1996; Mmom and Deekor, 2010). The difficult terrain of its environment also makes mechanical cleaning of spilled oil very challenging, thus bioremediation treatments are considered more applicable alternatives (Benka-Coker and Ekundayo, 1997). In recent years, many efforts have been made in the discovery, assessment and characterisation of polluted sites (Osuji *et al.*, 2004; Anyakora *et al.*, 2005b; Olajire *et al.*, 2005; Osuji and Adesiyan, 2005) and biostimulation and bioaugmentation techniques are the most applied treatments in many studies conducted (Benka-Coker and Ekundayo, 1995; Osuji *et al.*, 2006; Ogbonna *et al.*, 2007; Chikere *et al.*, 2009). The application of PAH bioavailability amendment techniques are, however, yet to be considerably explored to facilitate degradation and removal of spilled oil from polluted soils (Owabor and Aluyor, 2008).

1.6 Research hypotheses

The key hypotheses being tested in this work are, (i) PAH bioavailability amendment techniques will increase the bioavailability and degradation rate of hydrocarbon pollutants and, (ii) biochar can reduce the bioavailability of any residual pollutants after remediation.

1.7 Aim & Objectives

The primary aim of this study is the development of more effective ways to reduce the concentrations of pollutant hydrocarbons in soil and also reduce the toxicities of residual pollutants to levels that enable the re-use of contaminated areas. The objectives of this study are as follows:

- To investigate the biodegradation of crude oil and coal tar hydrocarbon pollutants using biodiesel and nutrient amendment techniques.

- To investigate the utilization of biochar in reducing the bioavailability of residual pollutants after bioremediation.

- To investigate the effect of increased bioavailability of the pollutants on soil toxicity during treatment with the amendments.

Chapter 2

LITERATURE REVIEW

This review focuses on the fate and impact of coal tar and crude oil hydrocarbons in the environment with emphasis on the high molecular weight (HMW) PAHs. The effect of biodiesel and biochar amendment techniques on PAH bioavailability are also discussed.

2.1 Fossil fuels

Fossil fuels provide approximately 80% of the world's energy requirement and this demand is expected to increase as population increases with a further 57% rise in consumption between 2002 and 2025 (Asif and Muneer, 2007). The total energy consumption in 2011 as reported by BP Statistical Review of World Energy (2012) with predictions for 2025 and 2050 (Salameh, 2003; Shell International, 2008) are presented graphically in Figure 2.1. The statistics revealed an increased consumption of natural gas, nuclear, and renewable energy by 35%, 83% and 139%, respectively, from 2025 to 2050. It also predicts a high demand for natural gas by industrialized and emerging economies as compared to crude oil, and a 22% fall in coal consumption by the year 2050.

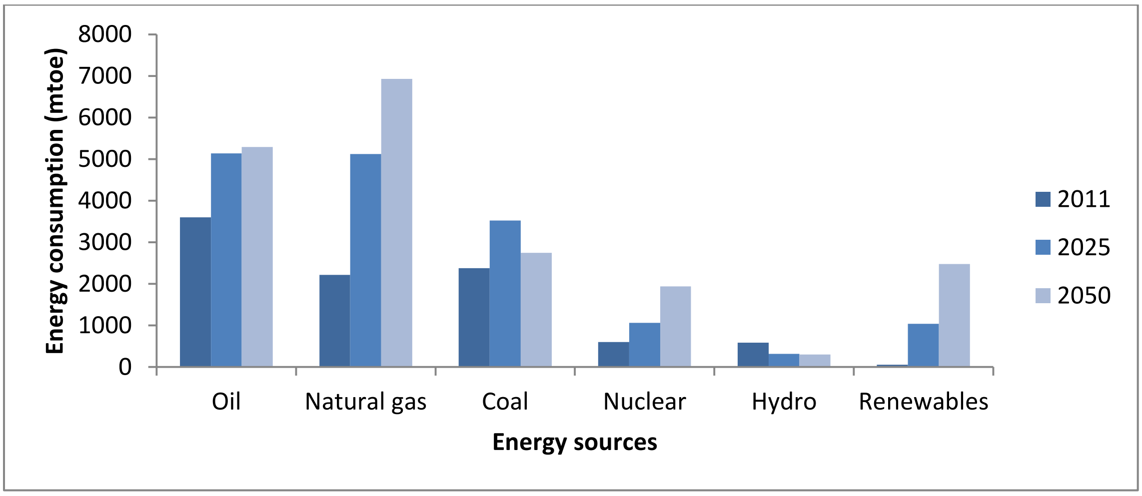


Figure 2.1. Primary energy consumption between 2011-2050 (mtoe) (Source: BP Statistical Review of World Energy, 2012; Shell International, 2008; Salameh, 2003).

2.2 Coal

2.2.1 Formation and structure

Coal is a heterogeneous polymer consisting of carbon, hydrogen, oxygen, sulphur and nitrogen. It is formed by the action of anaerobic microorganisms on plant biomass in converting these materials to peat, which under the higher temperatures and pressures that occur with increasing depth of burial, is then chemically altered to form lignite (low rank coal) coal and finally anthracite - an almost all carbon material with high calorific value (Stach and Murchison, 1982; Killops and Killops, 2009). The chemical composition of coal is dependent on the constituent inorganic matter, the parent material and, the chemical changes it had undergone during the depositional and formation period, which can often be between 200 and 300 million years (Lamb, 1977; Suárez-Ruiz and Crelling, 2008; Stephenson, 2013). Coal diversity is based not only on its chemical composition but also its physical structure in terms of porosity and internal surface area (Wang *et al.*, 2003). To better understand the nature and origin of coal, coal petrologists have attempted a molecular and nomenclature approach in the classification and grouping of coal molecules. Some of these proposed, mostly polyaromatic, molecular representations of lignite, bituminous and anthracite coal are shown in Figure 2.2.

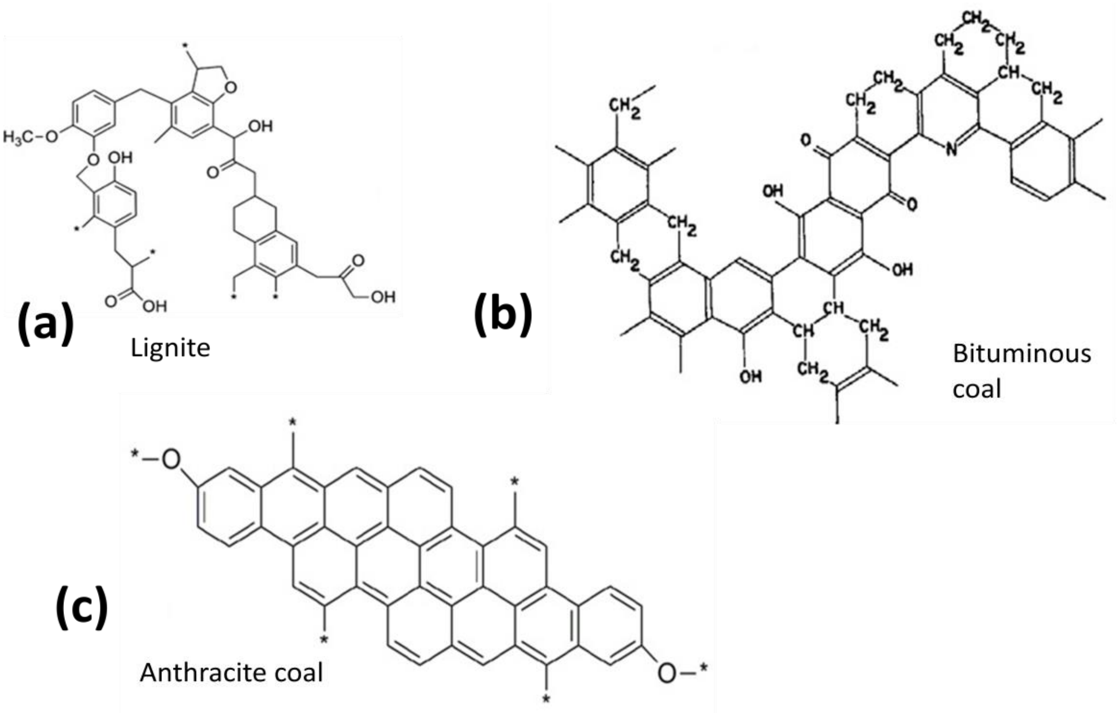


Figure 2.2. Molecular representations of coal (Source: Mathews and Chaffee, 2012).

The petrological components of coal are known as macerals which are broadly grouped into liptinite, inertinite and vitrinite (Stach and Murchison, 1982; Tissot and Welte, 1984; Killops and Killops, 2009). However, according to Scott (2002) the use of these maceral groups as petrographic indices and indicator ratios in order to predict the coals depositional settings still proves difficult and is yet to be widely accepted. Mathews and

Chaffee (2012) also stated that the structural models depicted in Figure 2.2 that illustrate various molecular representations of coal, do not definitively capture the proposed maceral features which make up the coal structure, although it is predicted that with the recent advances in computational technology, analytical techniques coupled with high tech simulations, better and more precise models could emerge.

2.2.2 Uses

Coal is the second most largely consumed fossil fuel after crude oil but its reserves are the more abundant, constituting about 65% of the world's fossil fuel reserves, with oil and gas making up the remaining 35% (Shafiee and Topal, 2009). According to Asif and Muneer (2007), the largest consumer and producer of coal is China with 58% of its energy being supplied by coal. China is followed by USA, the second largest producer of coal then India, Russia and the United Kingdom. Nigeria also has large deposits of lignite and sub-bituminous coal estimated at about 2.7 to 4.0 billion tonnes in reserves; however, the use of coal as source of energy has reduced significantly in the country following the conversion of railway fuel systems to diesel engines and the closure of coal plants during the Nigerian civil war between 1967 and 1970 (Ohimain, 2013). Figure 2.3 below shows the various uses of coal energy and its possible use as a future source of energy in the production of synthetic liquid fuel.

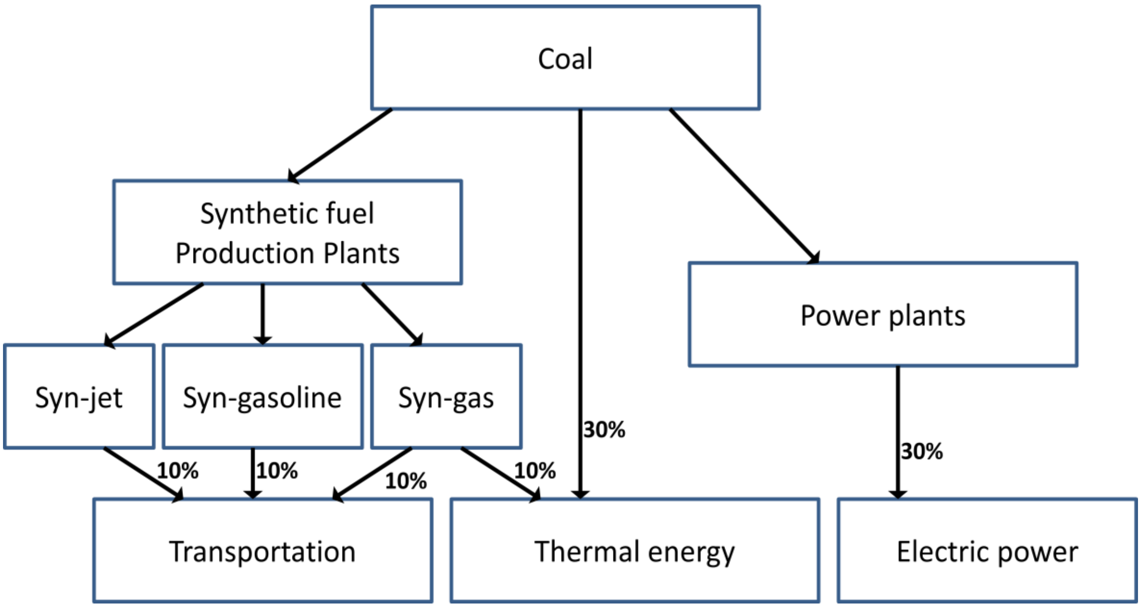


Figure 2.3. Coal/synthetic fossil fuel system (Source: Veziroglu and Sahin, 2008).

The non-fuel use of fossil fuels is also a thriving market that contributes significantly to the manufacturing of other useful products in the industry. In the case of coal, the production of metallurgical coke (for iron production in blast furnaces) by coal carbonization is one of its major non-fuel uses (Song and Schobert, 1996). Coal also serves as a chemical feedstock in the production of carbon materials such as activated carbon, carbon molecular sieves, carbon for phosphoric acid production; it is also used for specialty materials such as fullerene, graphite and diamond production, and for feedstock products derived from coal pyrolysis such as chemicals and gases from coal gasification, coal tars, coal pitch, fertilizers and various other coal by-products (Schobert and Song, 2002).

2.3 Coal tar

Coal tar is among the four types of tar in common use, the others being petroleum tar, wood tar and bituminous tar (Thami and Sarkar, 2002). It remains a notable feedstock for the production of many important aromatic chemicals, accounting for 10-15% of benzene, toluene, ethylbenzene and xylene (BTEX) production and more than 90% of other heavy chemicals (Schobert and Song, 2002). Coal tar, coke and gases are formed by the pyrolysis of coal and this process consists of two distinct phases: the splitting of the weaker C-C bonds between the aromatic units and further reactions of cracking, hydrogenation and assemblage of the primary aromatic units to form the resultant products (Krzesińska *et al.*, 2006). Crude coal tar (the by-product of coal distillation), when subjected to temperatures between 200 – 1300°C, yields various coal tar distillates that are made up of about 10,000 compounds (48% hydrocarbons, 42% carbon and 10% water) (Thami and Sarkar, 2002). Figure 2.4 shows its formation process from coal.

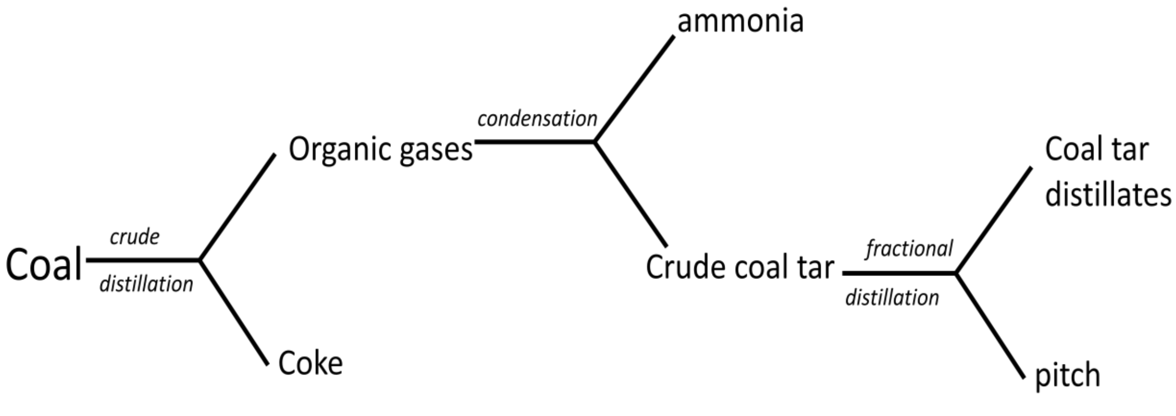


Figure 2.4. Crude coal tar formation from coal (Source: Thami and Sarkar, 2002).

2.3.1 Environmental impact of coal and its products

Coal enrichment in arsenic (mostly in lignite, bituminous and sub-bituminous coals) and mercury (in pulverised coals) can impact negatively on soil, water and air quality

(Yudovich and Ketris, 2005a; Yudovich and Ketris, 2005b). Coal combustion in power plants can result in arsenic poisoning of the surrounding air, while fly ash landfills with arsenic contents can consequently contaminate sediments, soils and natural waters (Yudovich and Ketris, 2005a). Approximately 90% of mercury is volatilised during the burning of coal mine dumps and it is recommended that mercury content be reduced prior to coal combustion and utilization (Yudovich and Ketris, 2005b). Due to the mutagenic and carcinogenic properties of these elements and their compounds, mortality rate increases in settlements with a long history of coal combustion (Yudovich and Ketris, 2005b). Also, an exposure to volatilised fluorine from the domestic use of coal, and selenium by plant uptake when combustion ash was used as soil amendment, was reported in China to cause disease conditions known as fluorosis (bone deformation) and selenosis (hair and nail loss), respectively (Finkelman *et al.*, 2002).

Another major concern of coal utilization is the PAH content. PAHs occur naturally in coals and are most significantly present in coal tar and creosote as compared to other known sources (Mahler *et al.*, 2012). Coal tar and coal pitch are classified as Group 1 carcinogens (IARC, 2010) and studies have shown that products made from these substances for example, paved surfaces coated with coal tar containing 35% coal pitch as sealant, can significantly pose environmental pollution concerns and severely affect the health and wellbeing of humans and other terrestrial and aquatic animals (Mahler *et al.*, 2012). With respect to coal tar toxicity, runoffs from surfaces of roads or pavements coated with coal tar based sealants can increase PAH concentrations in storm water and also cause sublethal effects in aquatic organisms in the presence of ultraviolet radiation (Bommarito *et al.*, 2010; Watts *et al.*, 2010).

2.4 Crude oil

Crude oil (petroleum) is a complex mixture composed of mainly hydrocarbons (carbon and hydrogen) and some amounts of sulphur and organic nitrogen (Prince, 1993; Speight, 2010). Recent studies in “petroleomics” have also revealed over 20,000 distinct heteroatom components of nitrogen, oxygen and sulphur (CHNOS) in petroleum crude oil using ultrahigh-resolution Fourier transform ion cyclotron resonance mass spectrometry (Marshall and Rodgers, 2004). These chemical studies on the characterization of crude oil and its products have increased due to the increasing cost of petroleum, depletion of the preferred light ‘sweet crude’ which has led to a global demand for heavier and high-sulphur sour crude, as well as due to recent improvements in analytical methods (Marshall and Rodgers, 2008).

Crude oil is formed by the catagenesis of kerogen and bitumen, an irreversible process that occurs at increasing temperature, pressure and burial depth in hydrocarbon source rocks and sedimentary basins (Tissot and Welte, 1984; Speight, 2010). Crude oils can be separated into four fractions - saturates, aromatics, resins and asphaltenes (SARA) based on their solubility and polarity properties (Harayama *et al.*, 1999; Fan and Buckley, 2002). Representative hydrocarbons of these groups are shown in Figure 2.5.

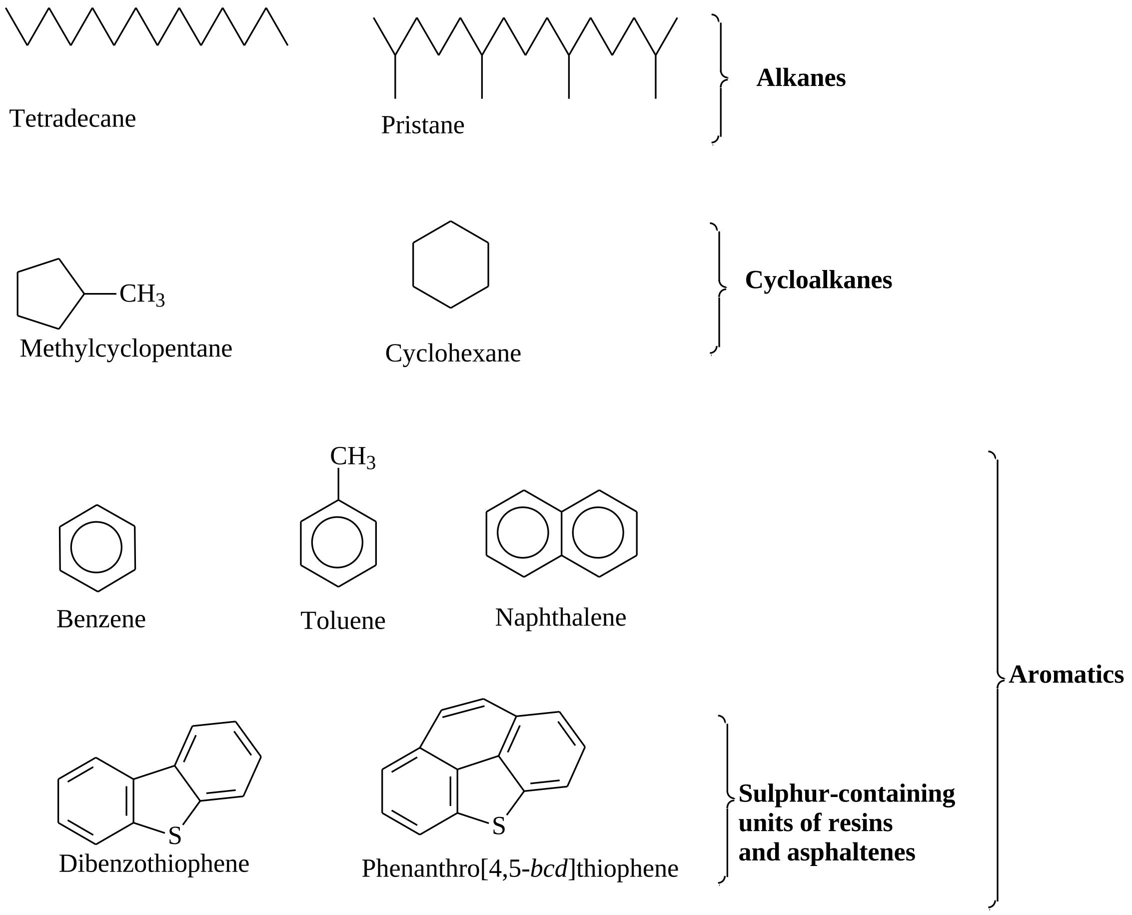


Figure 2.5. Representative hydrocarbons in crude oil (Source: Harayama et al., 2009; Grin'ko et al.,2012).

Saturates are the hydrocarbons with single bonds which can be classified into aliphatic alkanes and cycloalkanes with general formulae C_nH_{2n+2} and C_nH_{2n}, respectively; the aromatics are made up of one or more aromatic rings with benzene as the simplest form; while resins and asphaltenes comprise non-hydrocarbon polar compounds (containing nitrogen, sulphur and/or oxygen) and can easily form complexes with heavy metals (Harayama *et al.*, 1999; Grin'ko *et al.*, 2012). Molecular studies on the components of crude oil are conventionally carried out using simple chromatographic separation techniques followed by high resolution mass spectrometry analysis (Gaspar *et al.*, 2012).

2.4.1 Uses

About 40% of the world's non-solar energy is supplied by crude oil which is consumed by over 200 countries (Hall *et al.*, 2003). In the year 2000, the US Geologic Survey estimated the world's oil reserves to be 1.1 trillion barrels of oil (TBO), consumed at a yearly rate of 0.028 TBO and about half of every barrel of crude oil is used to produce gasoline (Ahlbrandt, 2002; Young, 2006). Other petroleum fractions such as utility fuels (fuel gas and residual oil), jet fuels, heating oils, diesels, gas oils and heavy fuel oils that serve as energy sources or feedstock in petrochemical industries are also distilled from crude oil in petroleum refining industries (Prince, 1993; Harayama *et al.*, 1999; Shah *et al.*, 2011). Figure 2.6 shows the processes involved in the generation of these products in a typical fuel refinery. These processes which include, catalytic reforming, alkylation, isomerisation and fluid catalytic cracking are employed to produce more environmentally friendly products, especially gasoline which is required to have characteristics such as high octane number, low sulphur and arenes content (Ivanov and Argirov, 2005).

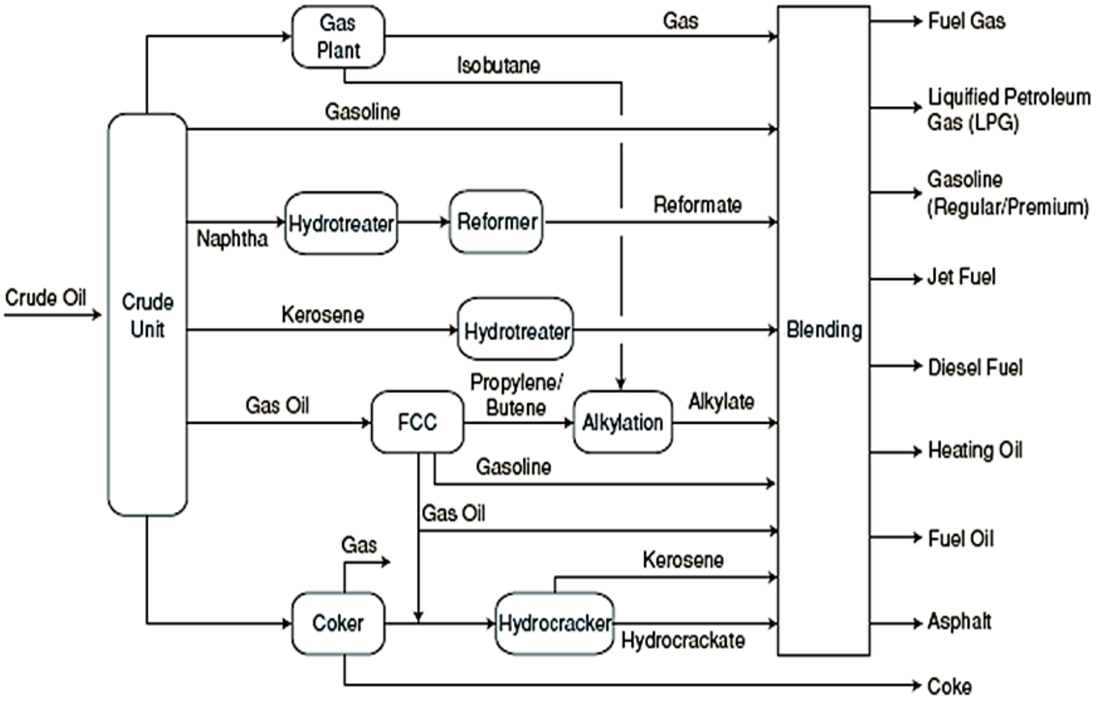


Figure 2.6. Fuel generation process in a typical refinery (Source: Young 2006); FCC= Fluid catalytic cracking unit.

2.5 Environmental impact of crude oil

One of the major concerns of the petroleum industry is the management of the wastes produced in all its key business activities encompassing crude oil extraction, transportation, refining, and marketing (Curran, 1992). These wastes which include oily materials, spent catalysts and chemicals, and other process residuals, if not properly treated and disposed of, become potential threats to the environment thus necessitating the enforcement of stricter environmental regulations (Curran, 1992; Shah *et al.*, 2011). Another obvious problem is the accidental/deliberate discharge of crude oil and its products into land and marine environments from onshore and offshore operations. This consequently results in the contamination of large water bodies (e.g. the Arabian Gulf, the Gulf of Mexico) and the pollution of farmlands, streams and rivers of local communities (e.g. communities in the Niger Delta of Nigeria) (Elhakeem *et al.*, 2007; Ordinioha and Sawyer, 2008). When spillages occur, the exposed population become susceptible to biochemical or physiological changes that occur due to the bioaccumulation of these contaminants in the food chains. This further cascades into genetic and reproduction impairments and other cancer related developments in the affected population (Onwurah *et al.*, 2007).

2.5.1 Soil contamination

The soil is a vital component of the ecosystem whose quality is defined by its continual capacity to sustain life, promote ecological health and maintain biological productivity (Doran, 2002). Soil quality is also an important indicator of environmental quality which can be grossly affected by pollution resulting from various anthropogenic activities (Herrick, 2000; Doran, 2002). The contamination of soil, in addition to water, by petroleum hydrocarbons is a widespread occurrence which affects the environment significantly (Margesin and Schinner, 2001a). According to Morgan and Watkinson (1989), these contaminants enter the soil environment via three major routes: (1) Seepages from underground storage tanks and continuous inputs from domestic wastes and road surfaces, (2) Spills from pipelines, tankers and storage tanks and (3) Slow seepages from natural oil reservoirs. Soil structure and biology can thus be affected as the hydrocarbons accumulate in the soil pores thereby causing a reduction in oxygen and water permeability, altering the binding properties of the clay component, altering soil water holding capacity and consequently affecting major enzymatic pathways of the microbial population (Morgan and Watkinson, 1989).

2.5.2 Fate of crude oil in the environment

Research into the fate of petroleum and its refined products in the environment has gained momentum owing to the growing knowledge that oil contains toxic PAHs that are carcinogenic, teratogenic, mutagenic and capable of endocrine disruption in living organisms (Yamada *et al.*, 2003). According to Morgan and Watkinson (1989), oil spilled on soil, especially from a point source, naturally takes either a lateral flow or vertically downward direction depending on the local environment involved. In a lateral flow scenario, lighter compounds are removed by evaporation and the contaminated area increases (Morgan and Watkinson, 1989). For a vertical downward direction, a multiphase flow of compounds results, which leads to the contamination of the groundwater by the hydrophilic compounds of the oil and the lateral distribution of the hydrophobic components across the water table (Morgan and Watkinson, 1989). These processes are schematically represented in Figure 2.7. Factors such as spill volume, viscosity of the hydrocarbon, temperature, speed of wind, land contour, plant cover and soil composition also affect the rate of movement and distribution of spilled oil (Morgan and Watkinson, 1989).

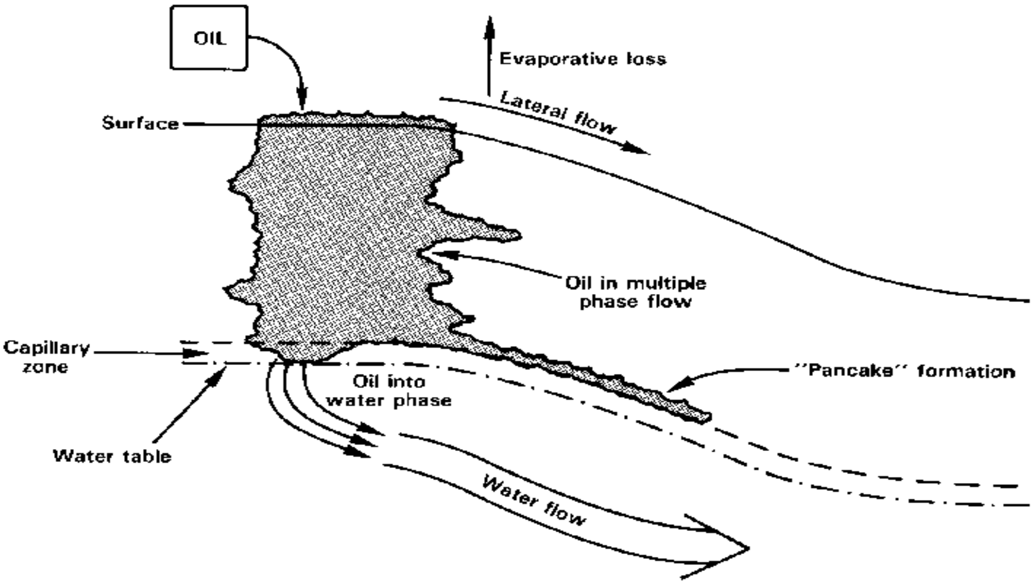
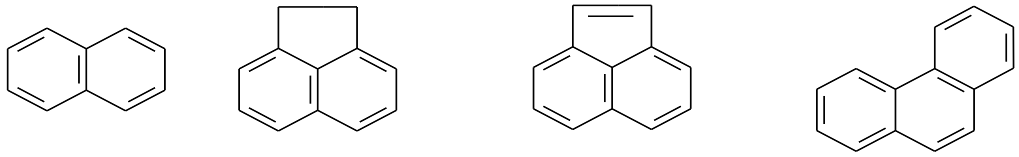


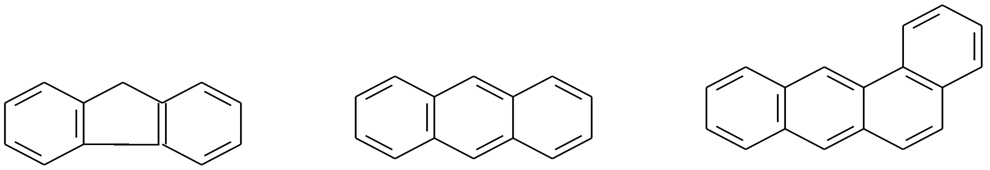
Figure 2.7. Schematic representation of oil movement on the soil surface from point source (Source: Morgan and Watkinson, 1989).

2.6 Polycyclic aromatic hydrocarbons (PAHs)

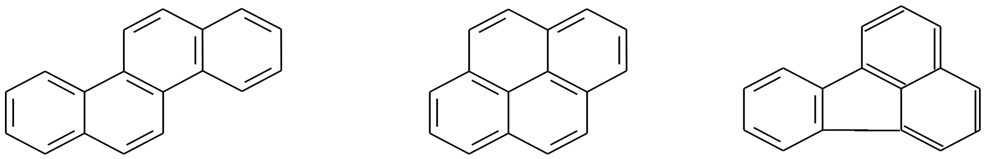
PAHs are a group of compounds with two or more fused benzene rings. They occur naturally in fossil fuels, are formed during the incomplete combustion of organic matter, tobacco smoking, exhaust emissions, volcanic activities, and also found as contaminants in sewage sludge (ATSDR, 1995; WHO, 2000a; IARC, 2010). Among the over 100 known PAHs, a list of 16 priority pollutants have been identified by the United States Environmental Protection Agency (USEPA) as sources of environmental concern due to their persistent nature and carcinogenic potential hence, most cleanup efforts are geared towards reducing these pollutants to safe environmental limits. (OFR, 1982). Figure 2.8 below shows the structure of these 16 PAHs on the USEPA priority pollutants list.



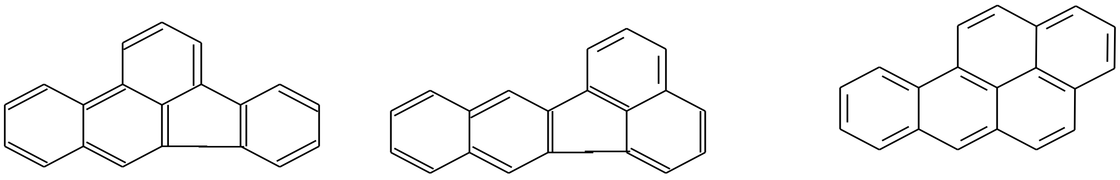
Naphthalene Acenaphthene Acenaphthylene Phenanthrene



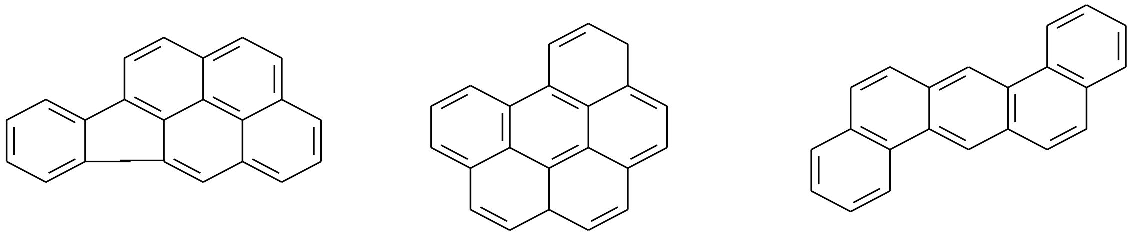
Fluorene Anthracene Benzo[a]anthracene



Chrysene Pyrene Fluoranthene



Benzo[b]fluoranthene Benzo[k]fluoranthene Benzo[a]pyrene



Indeno[1,2.3-cd]pyrene Benzo[g,h,i]perylene Dibenzo[a,h]anthracene

Figure 2.8. Structure of the 16 USEPA priority pollutants (Source: Yang et al., 2004).

The persistence of PAHs is dependent on environmental factors as well as the chemical structure, concentration and level of their bioavailability; these factors also contribute to the level of their toxicity (Bamforth and Singleton, 2005). PAHs are naturally removed from the environment by microbial degradation, a biological process which is being extensively employed in the bioremediation treatments of PAH contaminated sites (Peng *et al.*, 2008).

2.7 Bioremediation of PAH-contaminated soils

Bioremediation is defined as the removal or reduction in concentration of hazardous substances from the environment by using biological systems and processes to accelerate natural biodegradation (OTA, 1991). It is also a process whereby toxic organic pollutants are rendered less harmful by the activity of the existing microbial population within a contaminated environment, under controlled conditions (Bamforth and Singleton, 2005). Micro-organisms are largely responsible for bioremediation due to their relative abundance, vast diversity and potential of utilizing organic substances as sources of nutrients and energy (Dua *et al.*, 2002). Bioremediation techniques for the treatment of PAH-contaminated soils are broadly classified into *in situ* and *ex situ* methods of remediation. The *in situ* treatments include the use of biofilters, biostimulation, bioaugmentation, intrinsic bioremediation and the pump and treat method; *ex situ* techniques include landfarming, composting, biopiling and the use of bioreactors (Boopathy, 2000; Vidali, 2001).

The utilization of hydrocarbons by Gram positive and negative bacteria as carbon and energy sources presents a range of degradation pathways which are initiated by various modes of chemical reactions. PAHs favourably undergo aerobic microbial degradation, a process that is first initiated by dioxygenase enzyme to yield cis-dihydrodiols which are then converted to tricarboxylic acid cycle intermediates (Samanta *et al.*, 2002; Seo *et al.*, 2009). Naphthalene, the most studied PAH, is predominantly biodegraded by the *Pseudomonas* genus including other genera such as *Mycobacterium*, *Corynebacterium*, *Aeromonas*, *Rhodococcus*, and *Bacillus* (Mrozik *et al.*, 2003). In *Pseudomonas* sp. NCIB 9816 and *Pseudomonas putida*, naphthalene is first oxidised by naphthalene dioxygenases to *cis*-1,2-dihydroxy-1,2-dihydronaphthalene which is then converted to 1,2-dihydroxynaphthalene by the action of naphthalene (+)-*cis* –dihydrodiol dehydrogenase. By enzymatic cleavage, 1,2-dihydroxynaphthalene forms *cis*-2-hydroxybenzalpyruvate which is further converted to salicylate and pyruvate by a series of dioxygenases.

Salicylate is then oxidised to catechol by salicalate hydroxylase which finally undergoes *ortho* or *meta* fission, depending on the metabolism of the bacteria (Mrozik *et al.*, 2003). Fungal metabolism of PAHs, by non-ligninolytic and ligninolytic fungi found naturally in soils and woody materials, respectively, has also been reported and the reaction pathways studied (Bamforth and Singleton, 2005). The main pathways of degradation of PAHs by bacteria and fungi are summarized in Figure 2.9.

Anaerobic degradation of PAHs also occurs but the reaction is reported to be slow and the pathways involved are not understood in full details yet, in addition to the fact that studies are relatively complex in terms of culturing the anaerobes involved (Mrozik *et al.*, 2003; Bamforth and Singleton, 2005). These reactions are proposed to be initiated by denitrifying and sulphate reducing microorganisms under anaerobic conditions using nitrate, ferrous iron and sulphate as oxidising agents (Bamforth and Singleton, 2005).

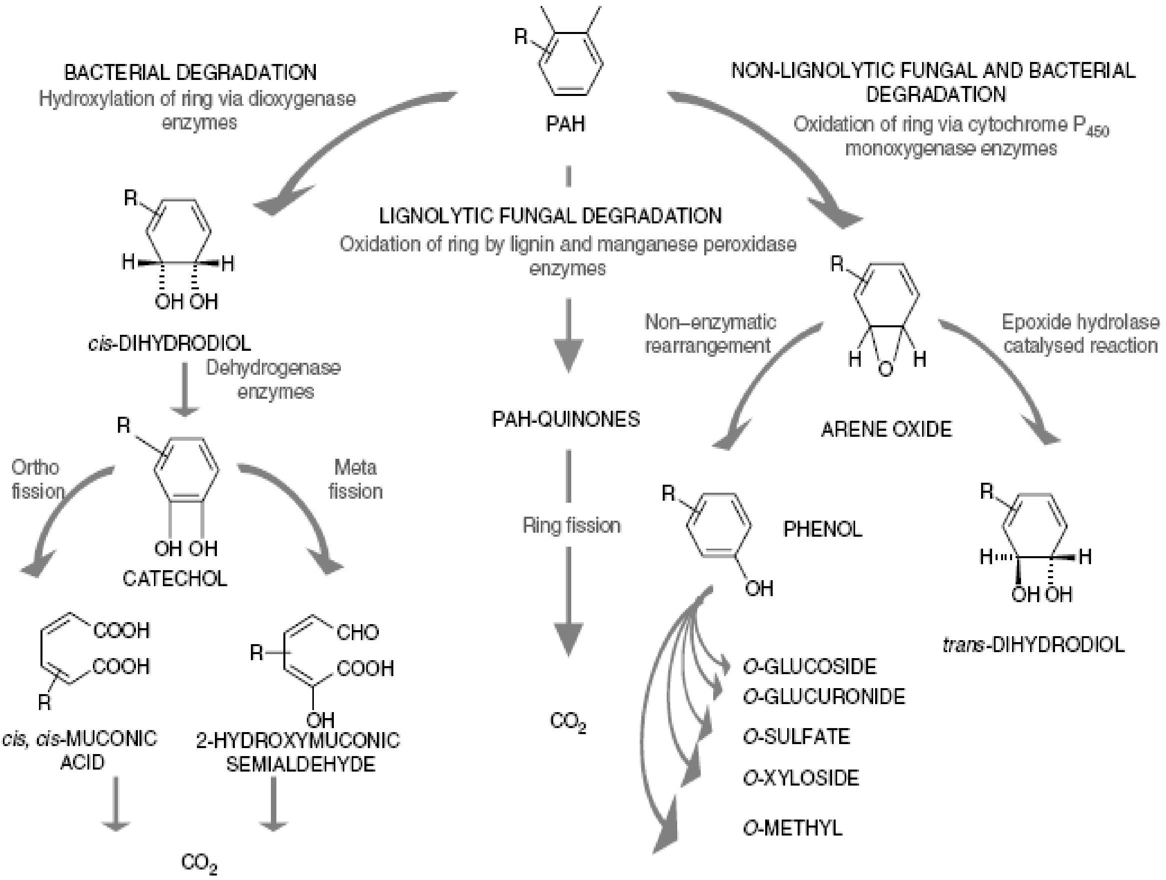


Figure 2.9. The three main pathways for PAH degradation by fungi and bacteria (Source: Bamforth and Singleton, 2005).

2.7.1 Degradation of high molecular weight (HMW) PAHs

High molecular weight PAHs are aromatic compounds with four or more fused benzene rings (Juhasz and Naidu, 2000). These compounds are recalcitrant to microbial degradation, highly persistent in the environment and only a limited number of microorganisms are able to degrade them unlike the low molecular weight PAHs (Cerniglia, 1992; Juhasz and Naidu, 2000). The hydrophobicity of PAHs, which increases with molecular mass, affects the bioavailability and microbial uptake of these organic pollutants and studies have shown that only two-four ring PAHs can be mineralised by bacteria as sources of carbon and energy (Boonchan *et al.*, 2000). PAHs composed of five to seven fused benzene rings can only be partially oxidised by bacteria or cometabolised by fungi and are therefore regarded as major environmental concern (Boonchan *et al.*, 2000; Johnsen *et al.*, 2005) Benzo[a]pyrene is considered a target analyte in environmental studies (Poster *et al.*, 2006) thus its levels in the environment are employed as the basis for the formulation of regulations and clean up endpoints (Juhasz and Naidu, 2000).

Benzo[a]pyrene (BaP) is the most studied of the PAHs and it is among the 16 USEPA priority pollutants. Figure 2.10 shows the proposed microbial degradation of BaP by bacteria. BaP cannot serve solely as a growth substrate for bacteria therefore, significant mineralisation of this pollutant requires the presence of co-substrates as alternate carbon sources (Juhasz and Naidu, 2000). These co-substrates which include low molecular weight PAHs induce competitive and synergistic (cometabolic) effects that are capable of enhancing the degradation of a wide range of PAHs by defined strains (Juhasz *et al.*, 1997a; Peng *et al.*, 2008). Some of the bacteria capable of degrading BaP include *Pseudomonas saccharophilia, Burkholderia cepacia, Sphingomonas paucimobilis, Stenotrophomonas maltophila, Agrobacterium, Bacillus, Rhodococcus, Mycobacterium* and *Flavobacterium* species (Boonchan *et al.*, 2000; Juhasz and Naidu, 2000).

Fungi metabolise HMW PAHs by converting them into detoxified products via a cometabolic transformation process. The process and products of detoxification have been schematically represented in Figure 2.9. *Phanerochaete chrysosporium, Trametes versicolor* and *Pleurotus ostreatus* are some of the most studied species of white rot fungi as well as *Cunninghamella elegans* (a non-ligninolytic fungi) because of their ability to degrade PAHs (Boonchan *et al.*, 2000; Johnsen *et al.*, 2005). *Bjerkandera sp* is another excellent degrader of BaP (83.1% BaP degradation after 28 days incubation)

(Field *et al.*, 1992). It has also been inferred that HMW PAHs present in soil are initially attacked by fungal exoenzymes in order to increase their aqueous solubility and bioavailability before the cometabolites are mineralised by bacterial intracellular enzymes (Johnsen *et al.*, 2005; Peng *et al.*, 2008).

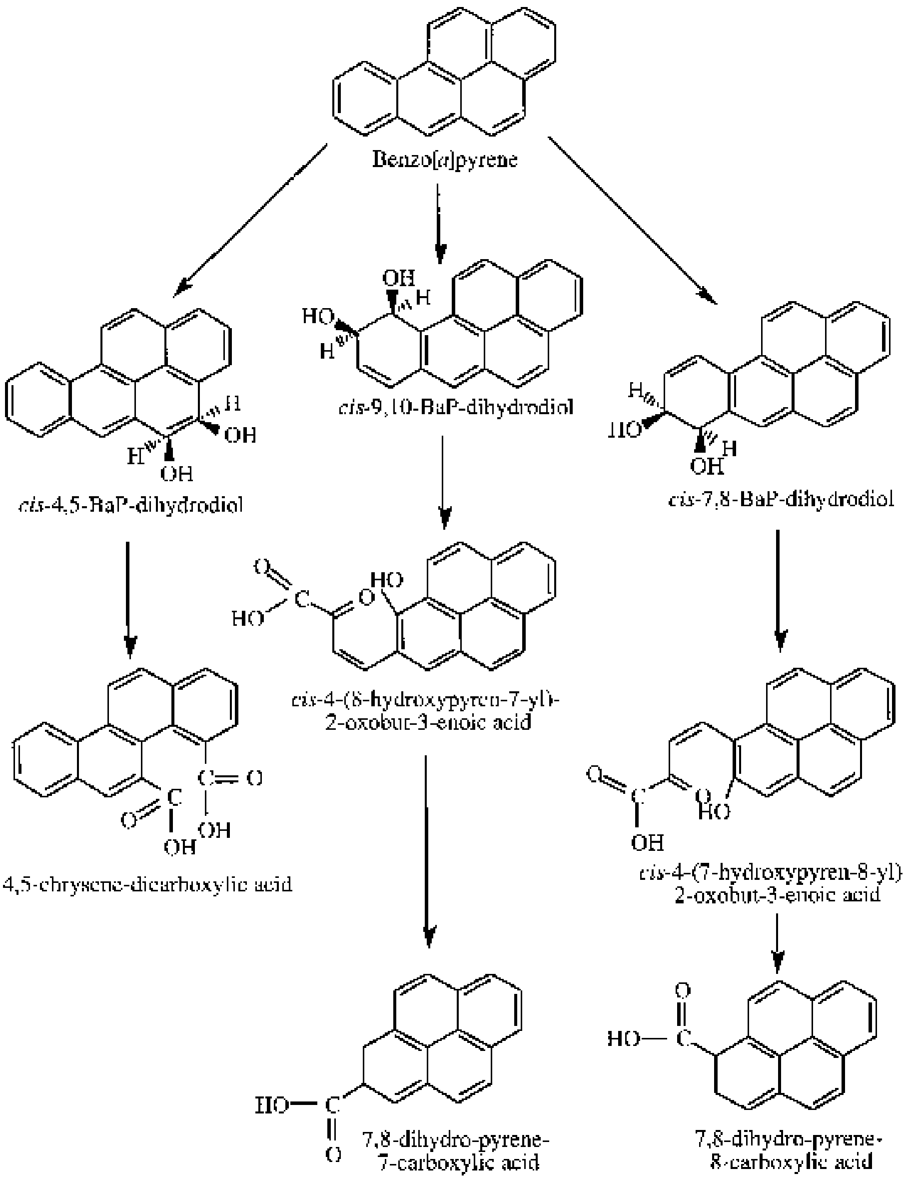


Figure 2.10. Proposed pathway for the bacterial degradation of benzo[a]pyrene (Source: Juhasz and Naidu, 2000).

2.8 Strategies for improving bioremediation of PAH-contaminated soils

Although bioremediation techniques are referred to as environmentally friendly and efficient, the success of the techniques depends on many factors. According to Dua *et al.* (2002), the three guiding principles that govern an effective bioremediation strategy are: the biodegradability of the pollutants involved, the level of bioavailability of these contaminants to microorganisms and the bioactivity optimization potential of the strategy. The degradability of organic pollutants can be influenced by environmental conditions such as soil moisture, soil pH, redox potential, oxygen content, nutrient content and temperature (Wilson and Jones, 1993) as well as the age of the contaminant in the environment (Bamforth and Singleton, 2005). The optimum environmental conditions are presented in Table 2.1 below.

Table 2.1. Optimum conditions for degradation of organic pollutants (Source: Wilson and Jones, 1993)

Parameter	**Conditions required for microbial activity**	**Optimum values for PAH degradation**
Soil moisture	25-85% of water holding capacity	30-90%
Soil pH	5.5-8.5	7.5-7.8
Redox potential	Aerobes and facultative microbes >50mV Anaerobes <50mV	
Oxygen content	Aerobic, minimum air filled pore space of 10% Anaerobic <1% by volume	10-40% O_2
Nutrient content	N and P for microbial growth, C:N:P 120:10:1 (optimum value approx.)	P(5), C:N 60:1 C:P 800:1 Salt concentration <4%
Temperature (^oC)	15-45	20, 24-30

Mohan *et al.* (2006) also described soil, microbial and contaminant associated factors required for pollutant bioavailability, mass transfer and microbial metabolism. These factors are presented schematically in Figure 2.11.

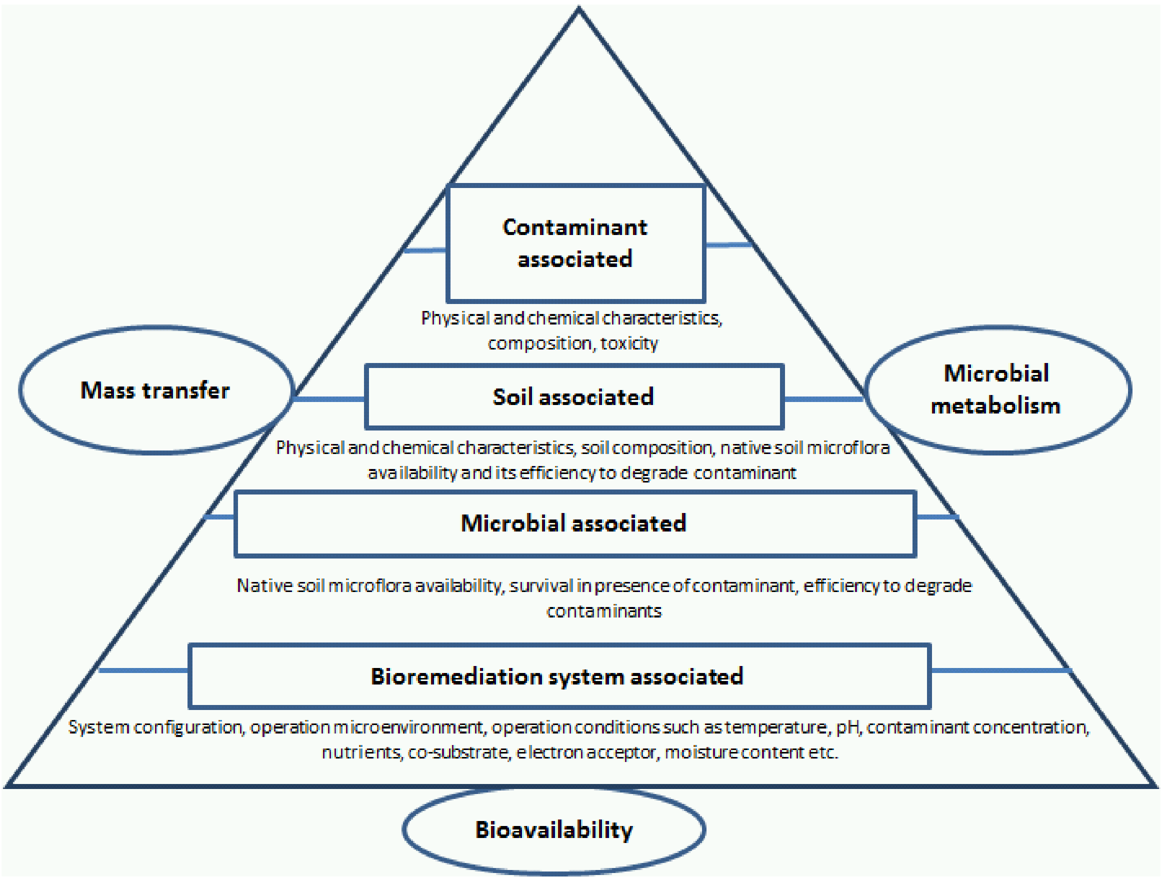


Figure 2.11. Factors associated with the bioremediation efficiency of PAH-contaminated soil (Source: Mohan et al., 2006).

Studies have shown that bioavailability of sorbed organic compounds within the liquid, gaseous and solid interfaces of contaminated soils can be enhanced by the action of surfactants (synthetic and microbial) as agents of emulsification and solubilisation (Volkering *et al*., 1995; Urum *et al*., 2004; Sun *et al*., 2012). The glycolipids which include rhamnolipids, trehalolipids, sophorolipids, surfactin and emulatin are the most studied microbial surfactants and are more preferred to their synthetic counterparts because of their lower toxicity and high biodegradability, although their cost of production is comparatively higher (Desai and Banat, 1997; Banat *et al*., 2010). These surface active compounds also find relevance in industrial applications relating to enhanced oil recovery, de-emulsification, soil remediation and flushing, among others; however with regard to bioremediation, they can elicit stimulatory or inhibitory effects depending on the surfactant chemistry, contaminant, microbial morphology, and other environmental factors such as soil type and characteristics (Singh *et al*., 2007).

The application of multi-disciplinary approaches in the remediation of contaminated sites is another rapidly growing strategy. Genetic engineering of microorganisms via recombinant techniques, genome sequencing and the development of biosensors and probes to identify, isolate and characterize microorganisms with high biodegrading ability are aspects of biotechnology currently employed in the degradation of recalcitrant pollutants (Pieper and Reineke, 2000; Dua *et al.*, 2002; Heidelberg *et al.*, 2002). Also developing is the systems biology approach that employs bioinformatics resources in searching sequence databases, identifying gene functions and constructing metabolic pathways for effective modelling and understanding of complex biological systems (Bansal, 2005; Pazos *et al.*, 2005; Chakraborty *et al.*, 2012). Innovative strategies in monitoring degradation (bioattenuation), supplying nutrients and electron acceptors (biostimulation), inoculation of bacteria with desirable biodegrading potentials (bioaugmentation) and biofilm-mediated approach are also promising *in situ* techniques that are extensively employed in bioremediation (Bento *et al.*, 2005; Mohan *et al.*, 2006; Singh *et al.*, 2006).

2.9 Effects of slow release inorganic fertiliser application on hydrocarbon degradation

The application of organic/inorganic fertilisers as biostimulation agents is primarily aimed at increasing the number and activity of the indigenous microorganisms present within contaminated soils (Mohan *et al.*, 2006). According to Lee *et al.* (2005), the effectiveness of these nutrient formulations is dependent on the application protocol (the form, type and application frequency of the fertiliser), environmental factors, and the characteristics of the oil at a crude oil contaminated site. In their study, they recommended that granular slow release fertilisers be applied when the overlying water temperature is above 15^oC and that at low temperatures, soluble inorganic fertilisers are best suited. This is based on their view that microbial activity can be stimulated but oil degradation may be prolonged due to a delay in nutrient release caused by temperature. Studies by Sarkar *et al.* (2005) also showed that a decline in the increased microbial population can occur after weeks of application of rapid release fertilisers as compared to slow release biosolids due to fertiliser induced acidity and/or ammonia overload.

The application of co-substrates with nutrient supplementation has also shown to positively influence the degradation rate of PAHs. Studies by Taylor and Jones (2001) showed the accelerated degradation of coal tar PAHs when biodiesel and motor diesel treatments were applied together with nutrients. The degradation of recalcitrant 4-6 ring PAHs was also facilitated in oil polluted sediments when chitosan was added with

Osmocote (a slow release fertiliser) in studies carried out by Xu *et al.* (2005). Sunflower oil, oil refinery sludge, municipal sewage are other co-substrates that have been identified to facilitate degradation when added with nutrients in PAH bioremediation experiments (Mohan *et al.*, 2006). Monitored natural attenuation (MNA) is also a relevant inexpensive strategy in the bioremediation of PAHs. Comparative studies of bioremediation treatments have shown that MNA can be more effective than biostimulation techniques, thus proper characterisation of contaminated sites is required before treatments are introduced (Bento *et al.*, 2005).

2.10 Biodiesel amendment technique in PAH bioremediation

Biodiesel is an eco-friendly, renewable energy source which is mainly produced by a base, acid or enzyme catalysed transesterification of oil (triglyceride) and alcohol (methanol or ethanol) (Li *et al.*, 2008b; Jain and Sharma, 2010). Although its use as an alternative fuel for diesel engines, owing to its environmental and economical benefits, has led to debates that bother on food security, biodiesel has been acknowledged as a potential accelerator of PAH degradation in bioremediation studies (Taylor and Jones, 2001; Hill *et al.*, 2006; Fernández-Álvarez *et al.*, 2007). The high biodegradability of biodiesel, which is reported to be about 90-98% mineralisation within 21-28 days under aerobic and anaerobic conditions in both aquatic and soil environments, is one characteristic that favours its use as a non-toxic solvent in PAH degradation (Pasqualino *et al.*, 2006). Also, the effective solubilisation and dispersion of PAHs in biodiesel can increase mass transfer of these pollutants from the contaminated soil or sediment into solution, therefore enhancing their bioavailability and degradation (Taylor and Jones, 2001). This property also promotes its use as a cleanup agent in the removal or recovery of hydrocarbons from contaminated/oil spill sites (Miller and Mudge, 1997; Pereira and Mudge, 2004; Fernández-Álvarez *et al.*, 2007; Gong *et al.*, 2010).

Studies with biodiesel/diesel blends have shown that biodiesel facilitates the degradation of PAHs by cometabolic transformation; a synergistic process whereby microorganisms utilizes a readily biodegradable second substrate as a source of carbon (energy) to degrade the first substrate (Zhang *et al.*, 1998; Pasqualino *et al.*, 2006). In these experiments, the biodegradation rates of diesel for the biodiesel/diesel mixtures were higher compared to those with diesel alone as the sole source of carbon. The results also showed that the blends with higher concentration of biodiesel recorded a higher biodegradation rate for diesel than those with lesser concentrations (Mariano *et al.*, 2008).

As stated by Taylor and Jones (2001), one major drawback that can be associated with the use of biodiesel as a bioremediation agent is the possibility of an induced toxicity that may result after treatment. This can result from the solubilisation of PAHs that invariably increases their bioavailability to the microorganism at concentrations that may inhibit microbial growth and activity. Also highlighted is the production of methanol from the hydrolysis of fatty acid methyl esters which can also inhibit the growth of microorganisms. It was thus suggested, that the volume of biodiesel and degradation conditions be considered before treatment is applied (Taylor and Jones, 2001). Studies by Gong *et al.* (2010) also shows that the efficiency of biodiesel in removing PAHs can be affected by the type of feedstock or raw material used for its production and its level of purity. Their results showed that laboratory synthesized biodiesel produced from soybean oil was more efficient in removing PAHs from the two soil categories (manufactured gas plant and artificially spiked soils) than the purchased waste cooking oil biodiesel which presumably contained impurities and additives.

Biodiesel is commonly produced from rapeseed oil in Europe and this makes up about 80% of the total biofuel production (Demirbas, 2007). In this study, rapeseed methyl ester (RME) was used as the biodiesel in the amendment techniques employed.

2.11 Effects of residual pollutants on possible land reuse

Studies have shown that due to differences in degradation rates of organic pollutants during bioremediation, a residual concentration of hydrocarbons remain undegraded even at optimal biodegradation conditions (Nocentini *et al.*, 2000; Margesin and Schinner, 2001b; Chaillan *et al.*, 2006). This can result in residual toxicity after biodegradation has occurred especially in sites contaminated with mixed pollutants (Nunes-Halldorson *et al.*, 2004). According to Chaineau *et al.* (2003), residual toxicity affects the soil ecosystem in different forms. In their study, although plant growth was reduced in treated soils that were previously spiked with crude oil hydrocarbons, there was no toxic effect on worm survival and seed germination after the 480 days degradation period. The process of reducing the residual concentration of hydrocarbons where toxicity is pronounced, may ultimately determine the duration of the bioremediation process and can as well become the rate limiting step in meeting regulatory requirements and standards (Nocentini *et al.*, 2000).

Bioavailability amendment techniques can also increase the concentration of residual pollutants. According to Fent (2003), the bioavailable fraction of pollutants determines the ecotoxicological effects that occur at contaminated sites especially where there is insufficient microbial uptake of the pollutants at high concentrations. The molecular and observable effects of the induced toxicity at these polluted sites are shown in Figure 2.12.

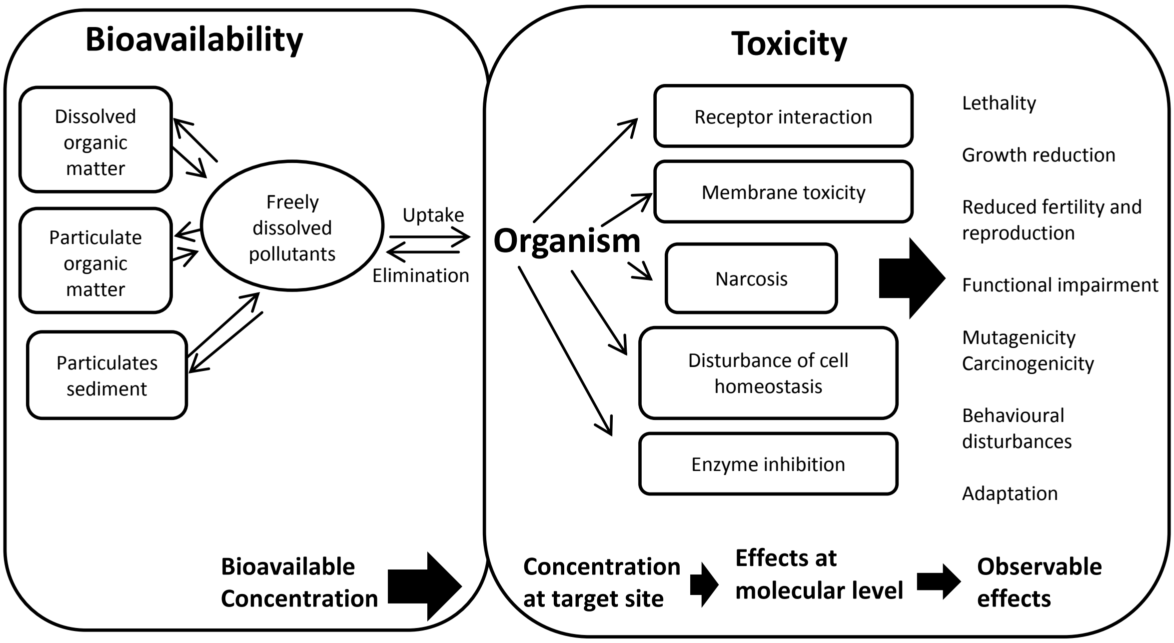


Figure 2.12. Ecotoxicological effects at contaminated sites due to reduced microbial uptake of bioavailable fraction of pollutants (Source: Fent, 2003).

Studies conducted with Inipol, an oleophilic fertilizer, to increase the bioavailability of PAHs in contaminated sub-Antarctic soils revealed an accelerated hydrocarbon degradation at increased temperature which, however, led to an increase in residual toxicity (Coulon *et al.*, 2005). According to Willumsen and Arvin (1999), surfactant-enhanced degradation can also produce metabolites that are toxic compared to their parent compounds thus, resulting in an inhibition in the rate of mineralisation in relation to an achieved increase in PAH solubilisation. This was speculated from the accumulation of coloured products of fluoranthene metabolism in the culture broth containing PAH degrading bacteria *Sphingomonas paucimobilis* and an associated overall decline in the rate of fluoranthene mineralisation.

Sorption agents have been explored in the remediation of contaminated sites as *in situ* treatments to reduce the bioavailability of pollutants. Hale *et al.* (2010) have demonstrated that the use of activated charcoal in the remediation of River Tyne sediments could enhance their quality and potential reuse. Their results showed a greater than 90% reduction in available PAHs in the activated charcoal amended sediments, measured using polyethylene passive samplers, as compared to the unamended treatments. Owing to its microporous structure, high hydrophobicity and large surface area (800-1200 m^2/g), activated charcoal has also been used extensively as an adsorbent in reducing the bioavailability of other inorganic and organic contaminants including metals, polychlorinated biphenyls (PCBs), pesticides and herbicides in water, soil and sediments (Zimmerman *et al.*, 2004; Mohan and Pittman Jr, 2006; Werner *et al.*, 2006; Vasilyeva *et al.*, 2010). However, studies by Jonker *et al.* (2009) have reported ecotoxicological effects of powdered activated charcoal on aquatic invertebrates (*Lumbriculus variegatus, Daphnia magna, and Corophium volutator*) in sediment remediation, although no obvious effect was detected in the indigenous microbial community.

The addition of biochar and green waste compost as amendment techniques in reducing the level of bioavailability of pollutants is also gaining interest for the remediation of polluted soils and sediments (Beesley *et al.*, 2010). Compost amendment techniques have been associated with multiple benefits including heavy metal adsorption, vegetation growth improvement and soil erosion protection; although consequential mobilization of trace metals including arsenic after its application to soil has been reported (Beesley *et al.*, 2010) In this study, however, a biochar amendment technique was employed with the aim of reducing the amount of residual pollutants by sequestration in un-amended, nutrient and biodiesel amended soil microcosms and, its effects investigated.

2.12 Biochar amendment technique in PAH bioremediation

Since the discovery of the *Terra preta* soils within the Amazon Basin, biochar application to soils has attracted much interest as studies are being conducted on various soil types to discover more of its potential benefits (Atkinson *et al.*, 2010; van Zwieten *et al.*, 2010; Bruun *et al.*, 2011; Chen and Yuan, 2011). This is due to the association of the characteristic high fertility and the distinctive microbial biodiversity of these *Terra preta* soils with their varied composition of biochar, when compared to their neighbouring and other tropical soils that were devoid of biochar (Atkinson *et al.*, 2010).

Biochar is a highly porous, carbonaceous material which is aromatic in structure and formed by the pyrolysis of organic matter (Atkinson *et al.*, 2010; Lehmann *et al.*, 2011). Some associated effects of soil amendment with biochar include soil fertility through alkalisation of soil pH; nutrient availability through anion and cation adsorption; change and increase in soil biota composition due to its porous structure and large surface area; and prolonged carbon storage and sequestration associated with its stable molecular structure (Atkinson *et al.*, 2010; Lehmann *et al.*, 2011). The conversion of waste biomass to biochar can also help to mitigate climate change by increasing carbon sequestration and consequently reducing the emission of greenhouse gases to the atmosphere (Macías and Arbestain, 2010). Biochar has also been reported to influence the ecotoxicological impact of organic contaminants by reducing their bioavailability to microbial degradation via carbon sequestration and their subsequent plant uptake, as reported for pesticides in soils where sorption by biochar is more than 2000 times greater than soil sorption capacity (Kookana, 2010).

The efficiency of biochar as a remediation tool in reducing the bioavailability of PAHs and other organic contaminants is dependent on its feedstock (source material) and charring conditions in terms of the oxygen and temperature levels during pyrolysis (Atkinson *et al.*, 2010; Gomez-Eyles *et al.*, 2011). The effect of biochar on soil health was also studied by Gomez-Eyles *et al.* (2011). In their experiment using earthworms together with biochar in the treatment of soils contaminated with PAHs and metals, the results showed that although biochar reduced the accumulation of organic contaminants in the earthworm tissues, there was significant weight loss observed in these organisms. The stability of biochar carbon can be affected by climatic factors despite its recalcitrant nature; therefore its application to temperate soils for agronomic purposes should be properly studied (Atkinson *et al.*, 2010). Biochar can also promote the restoration, re-vegetation and reuse of contaminated soils when incorporated in combination strategies with other amendments such as manure or compost, in order to balance its pollutant sequestration and nutrient retention capabilities with nutrient availability requirements (Beesley *et al.*, 2011).

2.13 Chemical monitoring of PAH bioremediation

The efficacy of bioremediation treatments requires the monitoring of the rate of hydrocarbon degradation which can be demonstrated under controlled conditions by laboratory and *in situ* field experiments (Atlas and Cerniglia, 1995). Thus, a number of conventional chemical and biological methods have been developed to assess both abiotic and biotic parameters in determining the effect and extent of the biodegradation process (Heitzer and Sayler, 1993). In this study, gravimetric and chromatographic techniques were employed as chemical techniques alongside toxicity assays to assess the rate of hydrocarbon degradation and these methods are reviewed.

2.13.1 Gravimetric measurements

The quantitative assessment of petroleum in the environment can be determined by simple, inexpensive gravimetric and compositional analyses (Whittle *et al.,* 1982). They include the determination of the total solvent extractable organic matter (EOM) and the total petroleum hydrocarbons (TPH) present in contaminated samples. These analyses provide information of the total contaminant concentration, and the total amount of saturated and aromatic hydrocarbon fractions in the EOM, respectively; the difference being the polar fraction (Mills *et al.*, 1999). Heavy hydrocarbons, such as coal tar, are also analysed gravimetrically to determine the total tar content which is the measurement needed to estimate the GC-undetectable fraction of a given tar extract (Simell *et al.*, 2000; Li and Suzuki, 2009).

In this study, all analytical tests were conducted on the EOM obtained by Soxhlet extraction, a method commonly used in the analysis of soil PAHs (Hechler *et al.*, 1995). Although this extraction process is time consuming and requires a large volume of solvent, its efficiency is reported to increase with molecular mass i.e. an efficiency range of between 84-100% for PAHs with more than 4 rings and is comparable with other known extraction methods (Lau *et al.*, 2010).

2.13.2 Chromatographic methods

The complexity of environmental samples which can be attributed to the various phases in which they exist has made their analysis difficult, thereby requiring the application of chromatographic techniques for the isolation of their compounds and the separation and detection of multiphase components (Poster *et al.,* 2006). Paper, liquid, column, thin layer, gas and high performance liquid chromatography are some examples of conventional separation methods (Guliyev *et al.*, 2004). Over the years, other modern chromatographic methods mostly based on the conventional approaches but with high sensitivity, speed and resolution have been developed to analyse diverse categories of compounds (Poppe, 1997; Valenzuela *et al.*, 2001; Poster *et al.*, 2006; Huerta-Fontela *et al.*, 2007; Schurig, 2011). These techniques also allow for coupling with detector analysers to enable real time monitoring of analytes (Small *et al.*, 1975).

Gas chromatography with flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS) are common analytical tools used in laboratories to analyse PAHs in environmental samples (Blankenhorn *et al.*, 1992). The latter can be more accurate than the former due to the selectivity of its detector in minimising co-elution interferences and its ability to analyse trace amounts of compounds in complex mixtures (Elhottová *et al.*, 2000; Poster *et al.*, 2006). Specific target analytes such as monocyclic aromatic hydrocarbons, alkylated PAHs and biomarkers are also accurately analysed by GC-MS (Saber *et al.*, 2005). However, fractionation of the sample extract by liquid chromatography for the removal of polar fractions is mostly required to achieve better resolving conditions in both chromatographic methods (Poster *et al.*, 2006).

Limitations of the GC-MS technique are reported for the identification of low level PAHs in wastes mixtures from multiple sources and the analysis of samples that have undergone severe weathering processes (Saber *et al.*, 2005). Thus an improvement on this technique has resulted in the development of other methods which include gas chromatography-tandem mass spectrometry (GC-MS-MS) and gas chromatography-isotope ratio mass spectrometry for complex samples analysis and forensic isotopic measurements, respectively (Natangelo *et al.*, 1999; Saber *et al.*, 2005).

2.14 Toxicity assessment of bioremediated soils

The combination of ecotoxicological analyses with chemical monitoring in assessing hydrocarbon endpoints during remediation treatment is necessary to determine the efficiency of a bioremediation process, assess pollutant bioavailability as well as evaluate the ecological health and soil quality of the bioremediated soils (Płaza *et al.*, 2005; Dawson *et al.*, 2007; Lors *et al.*, 2009). A wide range of bioassays involving microorganisms (biomass-C, enzymatic activity, soil respiration, nitrification), bacteria (luminescence-based biosensors), earthworms (*Eisenia fetida* and *Lumbricus terrestris* experiments) and plants (seed germination, root elongation and plant growth studies) as biological indicators have been employed in the hazard assessment of hydrocarbon contaminated soils (Dawson *et al.*, 2007).

Other bioassays also used to assess bioremediation treatments of contaminated soils include Microtox®, Spirotox, umu-test with S9 activation and Ostracodtoxkit F^{TM} (Płaza *et al.*, 2005). Although a combination of these assays in ecotoxicological analysis can generate a robust dataset, they tend to give differing clean-up endpoints which may need to be transformed into quantitative soil quality indices in order to ascertain ecological health (Dawson *et al.*, 2007). Moreover, in some cases they are unable to identify specific toxic compounds (Płaza *et al.*, 2005). In this study, a soil enzyme activity test (phosphatase assay) and the Microtox® bioassay were used to ascertain the level of toxicity after contaminated soil bioremediation treatment.

2.14.1 Microbial enzyme activity tests

Soil enzymes are synthesized and secreted extracellularly by bacteria and fungi and they play a major role in the total biological activity, nutrient availability, quality and fertility of the soil (Venkatesan and Senthurpandian, 2006). Studies have shown that soil contamination with organic pollutants affects enzymatic activities and the responses of these enzymes differ one from another (Sannino and Gianfreda, 2001). A number of enzyme bioassays utilize dehydrogenase (measures overall microbial activity), urease (hydrolyses urea to carbon dioxide and ammonia), β-glucosidase (catalyses β-glucopyranoside hydrolysis for energy), invertase (involved in soil carbon cycle), phosphatase (maintains soil phosphorus cycle), and arylsulphatase (catalyses the mineralisation of ester sulphate) in the toxicity testing of hydrocarbon remediated soils (Tejada, 2009).

Phosphatases are enzymes that mobilise organic phosphorus by hydrolysing esters and anhydrides of phosphoric acids into soluble inorganic forms needed for bacteria metabolism (López *et al.*, 2006). Inhibitory effects on phosphatase enzyme activity have been observed for insecticides, pesticides and herbicides in soils contaminated with these organic pollutants (Omar and Abdel-Sater, 2001; Sannino and Gianfreda, 2001; Yao *et al.*, 2006). Studies carried out on different soil types (sandy and clayey) also showed that crude petroleum and its refined products (gasoline and diesel) affect the hydrolytic function of phosphatases and other enzymes involved in the nutrient (N, P, C) cycles (Labud *et al.*, 2007). Thus, phosphatase activity can function as a biological indicator to determine ecological stress in polluted soils.

Soil phosphatase activity can be measured by estimating the inorganic phosphate released from soil incubated with organic phosphates (*β*-glycerophosphate, phytin) or the amount of an organic moiety released from soil incubated with β-naphthylphosphate, phenylphosphate or *p*-nitrophenylphosphate (Eivazi and Tabatabai, 1977). The latter approach is reported to be more reliable and sensitive, and the method developed by Tabatabai and Bremner (1969) based on this approach, which estimates the amount of *p*-nitrophenol released when soil is incubated with sodium *p*-nitrophenylphosphate is considered most accurate, rapid and sensitive (Eivazi and Tabatabai, 1977). This method was employed in this study to assess the toxicity level of the bioremediated soils and the efficiency of the amendment techniques applied.

2.14.2 Bioluminescence-based assays

Bacterial bioassays are known to be more efficient, sensitive and cost effective compared to animal, plants and invertebrate based assays, thus they have wide application in ecotoxicological screening and assessments (Parvez *et al.*, 2006). The *Vibrio fischeri* bioluminescence assay has been identified as one of the most sensitive among other bacterial assays in detecting toxicity for a wide range of organic and inorganic substances (Parvez *et al.*, 2006). Its mechanism of action is based on the bacterial bioluminescence pathway, a branch of the electron transport chain which directly correlates electron flow with the metabolic status of the bacteria (Cassells *et al.*, 2000). The light output of the bacteria is therefore reduced in proportion to the concentration of the toxicant on exposure and its intensity can be influenced by factors such as pH, salinity, temperature and the type of the toxicant involved (Cassells *et al.*, 2000).

The bacterial bioluminescence biochemical pathway is catalysed by the enzyme luciferase (Farré and Barceló, 2003) and it is represented by the reaction shown in Equation 2.1 below:

$$\mathrm{FMNH_2 + O_2 + R - CO - H \longrightarrow FMN + R - COOH + H_2O + LIGHT}$$

Equation 2.1. Bioluminescence pathway (Source: Farre and Barcelo, 2003).

In this reaction, reduced flavin mononucleotide ($FMNH_2$) is oxidised by molecular oxygen into flavin nucleotide (FMN) and water (H_2O) in the presence of aldehyde with the emission of a blue-green light of 490nm wavelength. Toxicity is expressed as the effective concentration of a toxicant that reduces the amount of light emitted by 50% (EC_{50}) (Farré and Barceló, 2003). Bioluminescence inhibition is also calculated as:

$$\%I = [1 - (\text{sample light}/\text{control light})] \times 100$$

Equation 2.2. Bioluminescence inhibition (Source: Farre and Barcelo, 2003).

Vibrio fischeri bioluminescence bioassays are commercially available as Microtox, LUMIStox, and ToxAlert which are products of Azur Environmental, Beckman and Merck, respectively (Farré and Barceló, 2003). Although the Microtox bioassay was initially developed for the toxicity testing of aquatic samples, it has also gained application in monitoring acute toxicity in soils, sediments, leachates, industrial effluents, suspended particles and other sample mixtures (Calleja *et al.*, 1986; Kaiser and Palabrica, 1991; Carter *et al.*, 1998; Cassells *et al.*, 2000; Mueller *et al.*, 2003; Matejczyk *et al.*, 2011).

Despite the advantages associated with Microtox bioassay, lower sensitivity to inorganics and highly lipophilic pollutants (Munkittrick *et al.*, 1991), cadmium (Kong *et al.*, 1995) and other heavy metals (Boluda *et al.*, 2011) has been reported. This suggests the need to employ a battery of test methods rather than a single assay in estimating toxicity (Johnson *et al.*, 2004). The poor solubility of some organic substances in the saline solution of the test bacteria is another limitation (Farré and Barceló, 2003). An adequate knowledge of the application of Microtox test method to any given sample is therefore required.

Chapter 3

3.1 MATERIALS AND METHODS

3.1.1 Soil

The soil used for this study was taken from an organic crop field in agricultural land (Nafferton Farm) in Stocksfield, Northumberland. The location, supported by chemical analysis conducted on a sample of it, indicated that there was no previous history of major organic chemical contamination. Prior to use, the soil was passed through a 2mm sieve to remove materials such as leaves, roots and stones.

3.1.2 Crude oil and coal tar

The North Sea crude oil was a medium gravity undegraded oil, originally supplied by BP plc. A slightly weathered coal tar collected from Bowden Close, Durham and a viscous un-weathered sample obtained from Monckton Coke & Chemical Co. Ltd were also used as hydrocarbon pollutants for soil spiking experiments.

3.1.3 Biodiesel, biochar & fertiliser

Biodiesel (rape seed methyl ester, RME- B100) was provided by Dl Oil Plc. Biochar (wood char) prepared at pyrolysis temperatures between 300-500^{o}C was also supplied by DM Jones. For nutrient supplementation, a slow release fertiliser (Westland Feed-All slow release plant food, NPK 14-5-28) was obtained from Homebase Ltd, Newcastle upon Tyne.

3.1.4 Solvents

Dichloromethane, methanol, *n*-hexane, toluene and petroleum ether of reagent grade were redistilled and used as organic solvents in this study. Re-distillation was done in a 30-plate Oldershaw column prior to use. Analytical grade toluene required for phosphatase enzyme assay and dimethylsulfoxide (DMSO) needed for Microtox assay were purchased from Fisher Scientific, UK.

3.1.5 Analytical grade Reagents

Potassium hydroxide, sodium sulphate anhydrous (granular), calcium chloride dihydrate, 4-nitrophenol were supplied by Fisher Scientific, UK. Tris (hydroxymethyl)aminomethane, *D-L*-Malic acid, boric acid and citric acid were purchased from VWR, BDH, UK. Silica 60A (chromatography grade and particle size

70-200 micron) was purchased from Merck, UK. Aluminium oxide was supplied by Merck. Phosphatase substrate was supplied by Sigma-Aldrich, UK. Microtox test kit was purchased from Azur Environmental, UK.

3.1.6 Standards

1,1’-Binaphthyl (Kodak, UK) and squalane (Fluka Chemika) were used as surrogate standards in sample extraction. The internal standards used to quantify the saturated and aromatic hydrocarbons were heptadecylcyclohexane (ICN Ltd, UK) and *p*-terphenyl (Fisher Scientific, UK), respectively, and the Supelco, deuterated internal standard mix, comprising acenaphthene-d10, chrysene-d12, 1,4 dichlorobenzene-d4, naphthalene-d8, perylene-d12, phenanthrene-d10 in dichloromethane, was supplied by Sigma-Aldrich, UK.

3.1.7 Apparatus

All glassware were properly washed and rinsed with deionised water at hot wash cycle of 80^{o}C and then dried in an oven. Glassware was afterwards rinsed with dichloromethane prior to use. Other equipment used included the Soxhlet apparatus, rotary evaporators, heaters, and fume cupboards etc. Instrumental equipment used is described in the appropriate sections below.

3.2 Determination of soil physico-chemical properties

3.2.1 pH

Soil pH measurements were conducted in compliance with the protocol BS ISO (2005) as follows: An aliquot (5 mL) of air-dried soil sample was weighed into a 60 mL bottle, to which de-ionised water (25 mL) was then added and the bottle was shaken in an orbital shaker for 1 hour at a speed of 275 ± 10 revs per minute. The bottle was removed from the shaker and the soil-water suspension was allowed to equilibrate for between 1 and 3 hours. The pH-H_2O of the suspension was then measured using a pH meter and electrode which had been calibrated using standard buffer solutions.

3.2.2 Water content

Soil water content was determined by the use of a gravimetric method and expressed as a percentage of the oven-dry mass of the soil sample (ISO, 1993). A sample container was weighed in a balance and recorded as the tare weight (*W1*). Soil sample of about 30 g was then placed in the container, weighed and recorded (wet weight; *W2*). The sample was afterwards placed in the 105°C oven to dry for 24 hours or overnight. The soil sample was then left to cool in the desiccator and the weight was taken and recorded (dry weight; $W3$). All weights were recorded to the nearest 0.1 mg. Triplicate measurements were performed for this analysis and the soil water content was computed with the formula:

$$Water\ content, \% = \frac{100(W2 - W3)}{W3 - W1}$$

Equation 3.1

3.2.3 Water holding capacity (WHC)

Soil water holding capacity was determined by Keen-Razkowski method (Piper, 1966) using a plastic cup (95 mm top diameter, 55 mm bottom diameter and 108 mm height) which was perforated at the bottom for effective water drainage. Filter paper was fixed at the bottom and the cup was weighed. Air dried soil was then poured into the plastic cup to about 1 cm depth. Water was added to the soil until it appeared saturated and began to drain from the bottom of the cup. This was continued for 1 hour to ensure saturation at 100% capacity. The sample was then left overnight for complete drainage of excess water from the soil. The saturated soil sample was afterwards weighed to the nearest 0.1 mg and left to dry overnight in the 105°C oven. The sample was left to cool in the desiccator and the weight of oven-dried soil was taken. Triplicate measurements were performed for this analysis and the percentage water holding capacity was calculated as follows:

$$WHC, \% = \frac{weight\ of\ saturated\ soil - weight\ of\ dry\ soil}{weight\ of\ dry\ soil}\ x\ 100$$

Equation 3.2

3.2.4 Total organic carbon (TOC)

Soil total organic carbon (TOC) was measured using the LECO CS244 Carbon/Sulphur Analyser in compliance with the (ISO (1995)) protocol. Air dried soil was passed through a 0.5 mm sieve. An aliquot (0.1 g) of the soil sample was weighed into a porous crucible and recorded to the nearest 0.1 mg. An empty crucible was also weighed and recorded as 0.1 mg for the method blank The crucibles were then placed in a crucible tray and transferred into the well of the fume cupboard. 1.0 mL of hydrochloric acid 4.0 mol/L was cautiously added to each crucible to remove the carbonate carbon and the acid was allowed to drain from the crucible for a minimum of 4 hours. After rinsing with deionised water (5 mL) five times, the crucibles were afterwards placed in a 65^{o}C oven for 16 to 24 hours and then allowed to cool prior to organic carbon content measurement in the calibrated Carbon/Sulphur Analyser. Calibration was done using a minimum of 5 standards of known carbon content. At the combustion temperature of 1500^{o}C, the carbon present in the sample was oxidised to CO_2, in the presence of oxygen, and was measured by the infra red detector in the analyser. Measurements for the sample and method blank were recorded and the percentage organic carbon content was calculated for the soil sample. Triplicate measurements were performed. The organic carbon content was calculated as follows:

$$Organic\ carbon, \% = C(sample) - C(blank)$$

Equation 3.3

3.2.5 Total nitrogen

An aliquot (100mg) of dried soil was weighed accurately into ceramic crucibles and analysed for nitrogen content using an Elementar VarioMAX CNS analyser. The analysis involves dry combustion at 1145 °C in an oxygen atmosphere. Sulfadiazine (%N = 22.37; %C = 47.99; %S = 12.81) was used as the calibration standard and was analysed at the start and end of the sample sequence and after every 5-10 samples. Raw data was corrected for analytical drift during the analysis using the Elementar software. Triplicate measurements were performed for this analysis.

3.3 Laboratory biodegradation experiment (Phase I)

The purpose of the Phase 1 experiment was to determine the effect of biodiesel amendment on non-biologically degraded samples of crude oil and coal tar.

3.3.1 Preparation of crude oil & weathered tar treatments

Three sets of crude oil and weathered coal tar spiked soil samples were prepared namely; the control (without amendment), nutrient amended (slow release fertiliser added) and biodiesel amended (slow release fertiliser plus biodiesel added). These microcosms were prepared in triplicates and left to undergo biodegradation for the same periods at laboratory conditions and then analysed. The crude oil spiked soil samples were set-up as follows: Aliquots of 500 mg of crude oil were artificially spiked on 20 g of air dried soil samples weighed on aluminium foil. The spiked soil samples were then added to 250 mL brown glass jars containing 30 g of wet soil. 10 g of biodiesel (rapeseed methyl ester) was added to one set of glass jars. 3 g of slow release fertiliser was afterwards added to each jar except the set of control. The water content was finally adjusted to 50% of the soil's water holding capacity and each treatment was mixed thoroughly. All brown jars were covered with aluminium foil perforated with holes to allow for aeration and then stored in cupboards at room temperature. On a weekly basis, water loss was compensated for with the addition of de-ionised water to all treatments and the samples were stirred every 2 weeks.

Weathered coal tar treatments were similar to the above set up with the exception of the initial preparation of the tar sample and the amount of tar spiked on the air-dried soil. The non-viscous coal tar was first dissolved in dichloromethane and aliquots of 250 mg were spiked on 20 g of air-dried soil which were then left in the fume cupboard overnight to allow for solvent evaporation. The rest of the procedure is as stated above. Figure 3.1 shows the schematic representation of the set-up which is also illustrated in Table 3.1. A total of 54 samples were prepared (27 for each hydrocarbon pollutant).

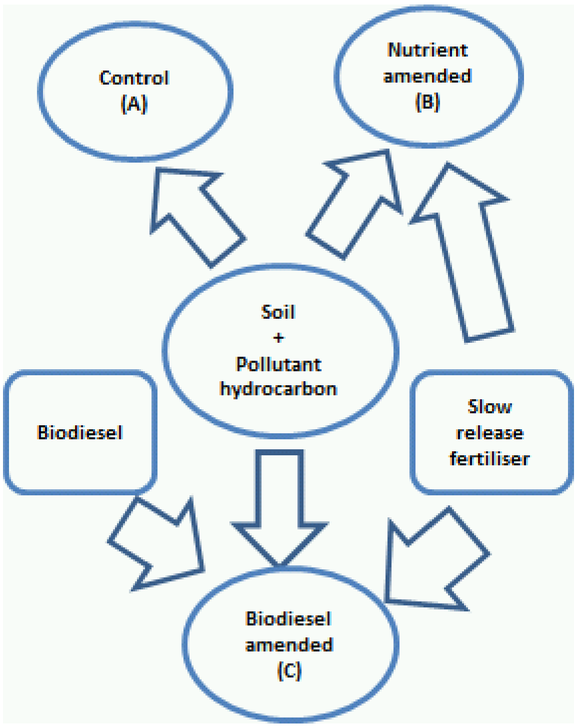


Figure 3.1. Schematic diagram showing the components of the three sets of treatments for the crude oil and tar experiment.

Treatments were collected for analysis at degradation periods of 0, 60 and 180 days as shown in Figure 3.2. On collection, the samples were stored at -20°C before analysis. At the start of analysis, the samples were first freeze dried at -50°C for 48 hours to remove all moisture content and then extracted using the Soxhlet method.

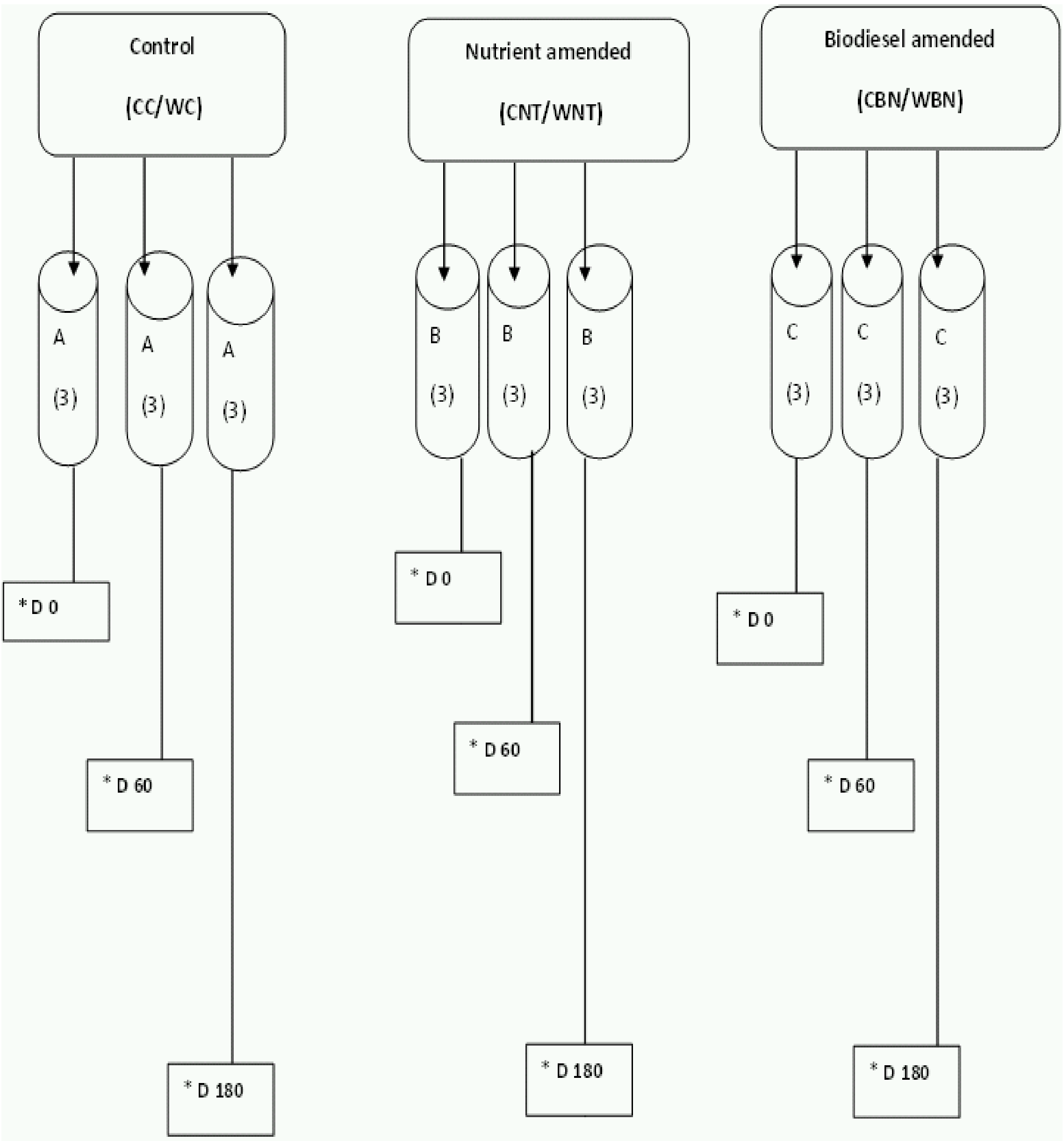


Figure 3.2. Schematic design of the laboratory Phase I biodegradation experiment showing the different incubation periods of the crude oil/weathered tar degradation. (3) = triplicates; D0, D60 and D180 represent incubation periods of 0, 60 and 180 days, respectively; A, B and C represent control, nutrient and biodiesel amended microcosms, respectively.

Table 3.1. Composition of each treatment of the laboratory Phase I biodegradation experiment with their sample codes

Treatment sets	**Sample codes**	**Composition**
		Crude oil
Control	CC	500 mg crude oil + 50 g soil
Nutrient amended	CNT	500 mg crude oil + 50 g soil + 3 g slow release fertilizer
Biodiesel amended	CBN	500mg crude oil + 50 g soil + 3 g slow release fertilizer + 10 g biodiesel
		Weathered coal tar
Control	WC	250 mg tar + 50 g soil
Nutrient amended	WNT	250 mg tar + 50 g soil + 3 g slow release fertilizer
Biodiesel amended	WBN	250 mg tar + 50 g soil + 3 g slow release fertilizer + 10 g biodiesel
Total of 54 samples		

3.4 Sample solvent extraction and extract fractionation

3.4.1 Preparation of materials

The adsorbents (silica gel, alumina), cellulose extraction thimbles (41 mm internal diameter by 123 mm long, Whatman) and cotton wool were cleaned by Soxhlet extraction using 450 mL dichloromethane: methanol (93:7, v/v) for a minimum of 16 hours. After extraction, they were placed in a fume cupboard to dry and the adsorbents were poured into clean glass containers and activated in a 120°C oven for 2 to 3 hours prior to use. The dried thimbles were wrapped in aluminium foil and kept in clean thimble boxes, while the cotton wool was placed in clean jars.

HCl-activated copper turnings were also prepared by adding 30 mL of 2M HCl into a 100 mL conical flask containing copper turnings. The flask was swirled for 2 min and the acid poured into a waste glass container. The copper turnings were then washed for a minimum of six times with distilled water, then methanol to remove the water and finally, dichloromethane. The activated copper turnings were poured into a glass container ready for use. The copper tunings were used to remove elemental sulphur which is readily extracted with hydrocarbons and can affect the analysis of saturated hydrocarbons and the integrity of chromatographic columns (Laflamme, 2011).

3.4.2 Soxhlet extraction of pollutant hydrocarbon spiked soil

The freeze-dried samples were put into pre-extracted thimbles and spiked with known amounts of surrogate standards (1,1'-binaphthyl and squalane), as shown in Table 3.2. The thimbles were then covered with pre-extracted cotton wool and placed in the Soxhlet apparatus. Activated copper tunings and anti-bumping granules were added to the round bottom flask containing the extraction solvent mixture (450 mL) of dichloromethane: methanol (93:7, v/v), and the Soxhlet extraction was done for a minimum of 16 hours.

After Soxhlet extraction, the extracts of the control and nutrient-amended samples were concentrated to 30 mL by rotary evaporation. An aliquot (1 mL) of each extract was transferred to pre-weighed glass vial and evaporated to dryness under stream of nitrogen to constant weight to quantify the extractable organic matter (EOM). The sample extracts were further concentrated to remove all the solvents in order to prevent the interference of methanol during the column elution process. The extracts were then made up to 20 mL in dichloromethane (DCM) and a 2 mL aliquot from each sample was transferred to a glass vial for fractionation.

The biodiesel-amended sample extracts were also concentrated to 30 mL by rotary evaporation. The EOM was then quantified as described earlier and a 1 mL aliquot was transferred to a glass vial for saponification.

3.4.3 Saponification of biodiesel amended soil extracts

Since fatty acid methyl esters can interfere with the identification and quantification of saturated and aromatic hydrocarbons, the process of saponification was carried out to reduce these interferences and to make these hydrocarbon analyses possible (Chandrasekaram, 2009). Extract (1 mL) was transferred to 100 mL round bottom flask containing toluene (6 mL), methanol (6 mL) and deionized water (40 mL). 6.7 g of KOH was added to give a solution of 3 M. However, for the un-degraded Day 0 samples, the solution was made to 9 M. Anti bumping granules were added and the content of the flask was refluxed for 1 hour.

After cooling, the content was decanted into a separating funnel. The flask was rinsed with 1 x 5 mL methanol, 1 x 5 mL mixture of methanol: toluene (50:50, v/v) and 2 x 5 mL toluene and the rinsing were added to the separating funnel. 15 mL of hexane was then added and the separating funnel was shaken and left to stand to allow the separation of the upper organic layer from the lower aqueous layer. The organic layer was thereafter transferred using a pipette to a 100 mL round bottom flask. The process was repeated after two further washings with hexane to complete removal of the organic layer and the content of the flask was dried over granular anhydrous Na_2SO_4. The content was then concentrated to ~2 mL and transferred to a 10 mL glass vial prior to separation on a chromatographic column.

3.4.4 Sample fractionation by column chromatography

The concentrated extracts were all passed through silica gel/alumina columns to separate the saturated and aromatic hydrocarbon fractions. Separations were made using liquid chromatographic columns of 8 mm internal diameter by 24.5 cm long. For all crude oil separations, these fractions were collected in separate 100 mL round bottom flasks, while in the case of the tar extracts, collection was made in one 250 mL round bottom flask to produce the total petroleum hydrocarbon fraction (TPH). The fractionation process is as follows:

A chromatographic column was plugged with a small piece of cotton wool and filled with petroleum ether. Activated silica gel (7 g) was then weighed out in a conical flask and petroleum ether was added with stirring to form slurry. The column was evenly packed with the slurry with gentle tapping and 0.5 g of activated alumina was then added to the column to form a layer just below the solvent (petroleum ether). Each sample extract (previously mixed with alumina and allowed to dry) was added to the column and then eluted first with 70 mL petroleum ether and then 70 mL dichloromethane: petroleum ether solvent mixture (50:50, v/v) to obtain the saturate and aromatic hydrocarbon fractions, respectively. A procedural blank column was also run to check for contamination. The fractions were then concentrated by rotary evaporation to ~2 mL. The saturates (from the crude oil extract) was made up to 5 mL by DCM and 1 mL aliquot was transferred into a GC glass vial. The concentrated aromatic fraction was also transferred to a GC glass vial.

The TPH of the tar extracts were made up to 10 mL with DCM and 1 mL aliquot was transferred into a GC glass vial. The concentrated eluates (saturates, aromatics and TPH) of the saponified crude oil and tar extracts were not diluted by DCM but were all transferred into GC glass vials. Internal standards of known amounts (same as the amount of surrogate standards present, w/v of eluted extract) were then added to each fraction. Heptadecylcyclohexane was added to the saturates, while *p*-terphenyl and deuterated internal standard mix were added to the aromatic fractions. These standards were also added to the vials containing the TPHs. The amounts added are shown in Table 3.2. 50 mg of each surrogate and internal standard was also added to a separate GC vial for relative response factor calculation. Standards were made up to 1 mL by DCM. All standards were also added to the procedural blank. The fractions were analysed afterwards by GC and GC-MS.

Table 3.2. Amounts of surrogate and internal standards added to crude oil and weathered coal tar samples

Standards			
Surrogate standard	*Crude oil*		*Weathered coal tar*
Squalane	1250 µg		87.5 µg
1,1’-binaphthyl	250 µg		5000 µg
Internal standard	Saturates	*Aromatics*	*TPH*
Heptadecylcyclohexane	25 µg	-	8.75 µg
p-terphenyl	-	25 µg	50 µg
Internal standard	-	25 µg	25 µg
Saponified			
Heptadecylcyclohexane	41.7 µg	-	2.9 µg
p-terphenyl	-	8.3 µg	167 µg
Internal standard	-	8.3 µg	167 µg

3.5 Biodiesel composition analysis

The composition of the rapeseed biodiesel used in this study was also determined by GC and GC-MS for the purpose of identifying and quantifying the individual fatty acid methyl esters (FAME) present in the sample. An aliquot (50 mg) of biodiesel was weighed into a clean glass vial and 500 µg of squalane was added. A spatula full of alumina was then added to the sample and mixed. The sample then passed through a silica gel/alumina column by eluting with 70 mL petroleum ether and 70 mL dichloromethane: petroleum ether solvent mixture (50:50, v/v) into a 250 mL round bottom flask. This eluted fraction was then concentrated to 10 mL by rotary evaporation. An aliquot (1 mL) was transferred to a glass GC vial and 50 µg of internal standard (n-heptadecylcyclohexane) was added for quantitative analysis by GC and GC-MS. A procedural blank was also run. Triplicate measurements were conducted for this analysis.

3.6 Gas Chromatography (GC-FID) analysis

GC analyses were carried out on all eluted fractions to check for chromatographic separation, resolution and possible contamination. Identification and quantification was mainly carried out on all aliphatic hydrocarbon fractions of samples. The fatty acid methyl esters present in the biodiesel sample (Section 3.5) were also estimated by GC-FID.

Analysis was performed on a Hewlett-Packard 5890 II instrument coupled to a flame ionisation detector and a split/splitless injector. 1 µL of sample dissolved in DCM was injected by an HP7673 autosampler and separation was performed on a fused silica capillary column (30 m x 0.25 mm i.d) coated with 0.25 µm thick 5% phenylmethyl polysiloxane stationary phase, using hydrogen as the carrier gas (flow 1 mL/min, pressure of 50 kPa, split at 30 mls/min). The GC oven temperature was programmed at $50^{o}C$ for 2 min and ramped at 4 ^{o}C/min to a final temperature of 300 ^{o}C held for 20 min. The acquired data was stored on a LabSystems Atlas laboratory data system for further processing, integration and printing.

3.7 Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis was performed to identify and quantify each major peak from the GC analyses of the aromatic and TPH fractions. The biodiesel FAMEs were also identified by GC-MS. Analysis was performed on a Hewlett-Packard 6890 instrument fitted with a split/splitless injector ($280^{o}C$) and linked to a Hewlett-Packard 5973 MSD which was set with an electron voltage of 70ev, source temperature $230^{o}C$, quadrupole temperature $150^{o}C$, multiplier voltage 1800V and interface temperature $310^{o}C$.

An aliquot (1 µL) of sample dissolved in DCM was injected by an HP7683 autosampler and separation was performed on a fused silica capillary column (30 m x 0.25 mm i.d) coated with 0.25 µm thick 5% phenylmethyl polysiloxane stationary phase. The GC conditions were the same as described above (Section 3.6) except that helium was the carrier gas used. Data acquisition was controlled by a HP Compaq computer using Chemstation software and fraction analyses were usually made in selected ion mode for greater sensitivity. Peaks were identified and labelled after the comparison of their mass spectra with those of the NIST05 library and from their elution order as presented in geochemistry literature.

3.8 Quantification of GC and GC-MS peaks (analytes)

The GC or GC-MS peak area of an analyte is directly proportional to the concentration of that compound and is measured in comparison to the peak area of the internal standard. The analytes were quantified using Equations 3.4- 3.7.

$$Relative\ response\ factor\ = \left(\frac{SS\ peak\ area}{IS\ peak\ area}\right) \times \left(\frac{IS\ weight}{SS\ weight}\right)$$

Equation 3.4

$$\% \, Recovery = \left(\frac{SS\ peak\ area}{IS\ peak\ area}\right) \times \left(\frac{IS\ weight}{RRF}\right) \times \left(\frac{1}{SS\ weight}\right) \times \left(\frac{1}{fraction}\right) \times (100)$$

Equation 3.5

$$X\ (wt) = \left(\frac{X\ peak\ area}{IS\ peak\ area}\right) \times \left(\frac{IS\ weight}{fraction}\right)$$

Equation 3.6

$$X\ (corrected\ wt) = \frac{X\ peak\ area}{IS\ peak\ area} \times \left(\frac{IS\ weight}{fraction}\right) \times \frac{100}{\%\ recovery}$$

Equation 3.7

(*SS = surrogate standard, IS= internal standard and X (wt) = amount of analyte*)

3.9 Laboratory biodegradation experiment with un-weathered tar and biochar (Phase II)

The Phase II biodegradation experiment was conducted on soil samples spiked with fresh un-weathered coal tar. The three main objectives of this laboratory experiment were as follows:

- To determine the effect of biochar amendment on the remediation of coal tar.
- To investigate the extent of coal tar biodegradation using a stepwise amendment approach.
- To assess the effect of amendments on coal tar toxicity.

Result repeatability (i.e. the observed removal of HMW PAHs by biodiesel) was also investigated with respect to the experimental design and laboratory procedures. The analytical protocol followed was the same with the Phase I experiment as earlier described in Sections 3.3 to 3.7.

3.9.1 Preparation of un-weathered tar and biochar amended treatments: (repeatability experiments)

Three sets of un-weathered coal tar spiked soil samples were prepared, namely; the control (without amendment), nutrient amended (slow release fertiliser added) and biodiesel amended (slow release fertiliser plus biodiesel added). These microcosms were prepared in triplicates. The un-weathered coal tar was first dissolved in dichloromethane and aliquots of 250 mg were spiked on 20 g of air dried soil which were then left in the fume cupboard overnight to allow for solvent evaporation. The spiked soil samples were then added to 250 mL brown glass jars containing 30 g of wet soil. Biodiesel (rapeseed methyl ester, 10 g) was added to one set of glass jars. Slow release fertiliser (3 g) was afterwards added to each jar except the set of control. The water content was finally adjusted to 50% of the soil's water holding capacity and each treatment was mixed thoroughly. All brown jars were covered with aluminium foil perforated with holes to allow for aeration and then stored in cupboards at room temperature. On a weekly basis, water loss was compensated for with the addition of de-ionised water to all treatments and the samples were stirred every 2 weeks. The different microcosm treatments were collected at degradation periods of 0, 60 and 90 days and were stored at -20^{o}C prior to analysis.

Biochar amendment was done after samples had undergone a 60-day biodegradation period. Biochar (2.5 g) was added to a set of microcosms of each treatment (i.e. control, nutrient amended and biochar amended samples) and then incubated for 30 days prior to analysis, for comparison with the non-biochar amended treatments. Figure 3.3 shows the schematic design of the set-up which is also illustrated in Table 3.3. A total of 36 samples were set up.

3.9.2 The step-wise amendment technique

This approach involved the addition of biodiesel after 60 days of nutrient supplementation. In other words, the soil micro-organisms were first allowed to utilise the slow release fertiliser to degrade PAHs for duration of 60 days before 10 g of biodiesel was added to the treatments. The biodiesel amended samples were then incubated for additional 60 and 90 days, i.e. sampling was done after a total of 120 and 150 days of incubation, respectively. Also run simultaneously were the control and nutrient amended sets prepared as described in Section 3.9.1 (excluding biodiesel addition) and samples were also analysed after 120 day and 150 day incubation periods.

Biochar amendment was also carried out after the 120-day incubation period. Biochar (2.5 g) was added to a set of microcosms of each treatment (i.e. the control, nutrient amended and biodiesel amended samples) and left for 30 days prior to analysis, for comparison with the non-biochar amended treatments. A total of 30 samples were prepared for this experiment.

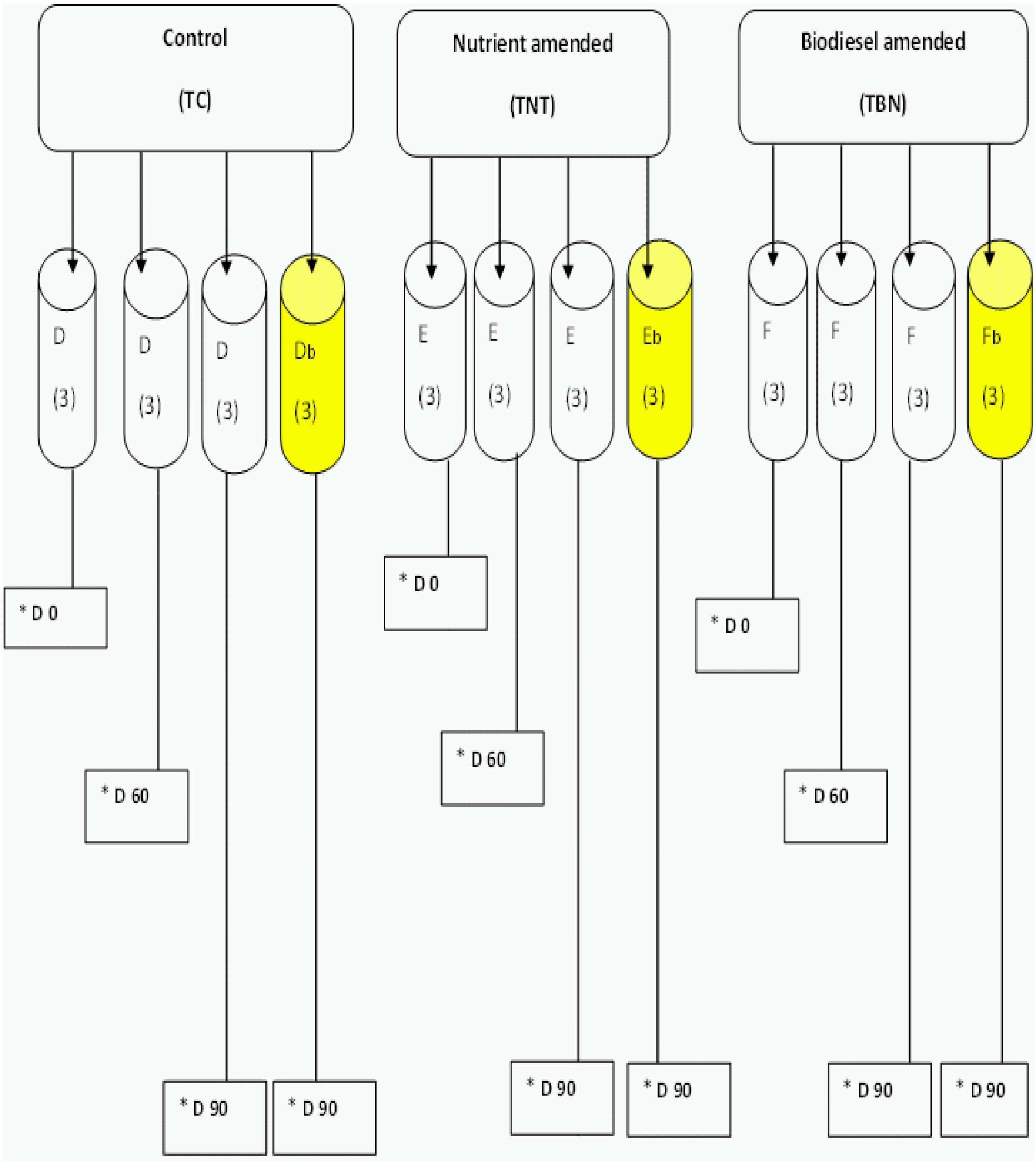


Figure 3.3. Schematic design of the laboratory Phase II (repeatability) experiment showing the different incubation periods of the un-weathered tar degradation. (3) = triplicates; D0, D60 and D90 represent incubation periods of 0, 60 and 90 days, respectively; D, E and F represent control, nutrient and biodiesel amended microcosms, respectively; Db, Eb and Fb are the biochar amended samples for the control, nutrient and biodiesel microcosms, respectively.

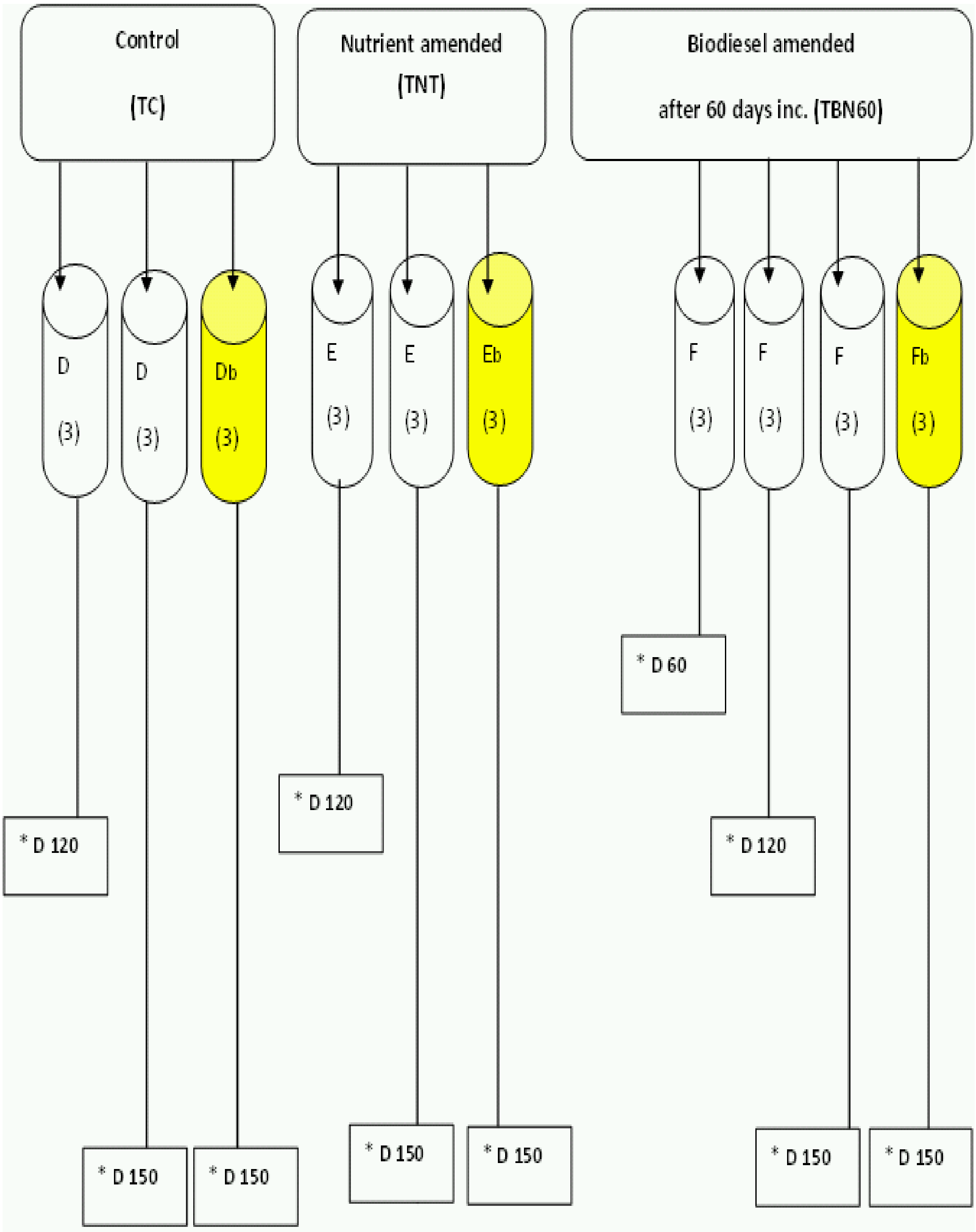


Figure 3.4. Schematic design of the laboratory Phase II (step-wise amendment) experiment showing the different incubation periods of the un-weathered tar degradation. (3) = triplicates; D60, D120 and D150 represent incubation periods of 60 and 120 and 150 days, respectively; D, E and F represent control, nutrient and biodiesel amended microcosms, respectively; Db, Eb and Fb are the biochar amended samples for the control, nutrient and biodiesel microcosms, respectively.

Table 3.3. Composition of each treatment of the Phase II laboratory biodegradation experiment with their sample codes.

Treatment sets	**Sample codes**	**Composition**
		Non biochar category
Control	TC	250 mg un-weathered tar + 50 g soil
Nutrient amended	TNT	250 mg un-weathered tar + 50 g soil + 3 g slow release fertilizer
Biodiesel amended	TBN	250 mg un-weathered tar + 50 g soil + 3g slow release fertilizer + 10 g biodiesel
Biodiesel amended (after 60 days incubation)	TBN60	250 mg un-weathered tar + 50 g soil + 3g slow release fertilizer + 10 g biodiesel
		Biochar category
Control	TC-B	250 mg un-weathered tar + 50 g soil + 2.5 g biochar
Nutrient amended	TNT-B	250 mg un-weathered tar + 50 g soil + 3 g slow release fertilizer + 2.5 g biochar
Biodiesel amended	TBN-B	250 mg un-weathered tar + 50 g soil + 3 g slow release fertilizer + 10 g biodiesel + 2.5 g biochar
Biodiesel amended (after 60 days incubation)	TBN60-B	250 mg un-weathered tar + 50 g soil + 3 g slow release fertilizer + 10 g biodiesel + 2.5 g biochar
Total of 66 samples		

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3.10 Sample analysis

All treatments were analysed as described in Section 3.4. Samples were first freeze-dried and surrogate standards (squalane and 1,1'-binaphthyl) were added in amounts shown in Table 3.4 before Soxhlet extraction. After concentration by rotary evaporation to 30 mL, aliquots of 1 mL were dried to constant weight by a stream of nitrogen to quantify the EOM. Extract solutions were then concentrated to 20 mL and aliquots of 2 mL were passed through chromatographic columns to separate the TPH fractions using 70 ml petroleum ether and 70 mL dichloromethane: petroleum ether solvent mixture (50:50, v/v). The TPH fractions were then concentrated to 10 mL and aliquots of 1 mL were transferred to GC vials. Internal standards (heptadecylcyclohexane, p-terphenyl and a deuterated internal standard mix) were then added (Table 3.4) and the fractions were afterwards analysed by GC and GC-MS.

As earlier described, the biodiesel samples were first saponified (Section 3.4.3) before fractionated by column chromatography. Internal standards were then added to the TPH fractions which were afterwards analysed by GC and GC-MS.

Table 3.4. Amounts of surrogate and internal standards added to un-weathered coal tar samples

Standards	
Surrogate standard	*Coal tar sample*
Squalane	250 µg
1,1'-binaphthyl	7500 µg
Internal standard	*TPH*
n-heptadecylcyclohexane	2.5 - 5 µg
p-terphenyl	75 µg
Internal standard	75 µg
	Saponified
n-heptadecylcyclohexane	9 µg
p-terphenyl	250 µg
Internal standard	250 µg

3.11 Toxicity assessment of un-weathered coal tar treatments

3.11.1 Preparation of treatments

All treatments in the Phase II biodegradation experiments were tested for toxicity. The entire sample set was replicated as described in Sections 3.9.1 and 3.9.2 except that the samples were stored at 4^oC prior to analysis. Additionally, a triplicate set of non-spiked, non-amended 50 g of Nafferton soil was also set up and amended with biochar after 120 days, then analysed after 30 days. This was prepared to check for any form of interference of biochar on the toxicity assays. Soil phosphatase activity was measured in all treatments and thereafter, the Microtox assay was conducted only on selected treatments due to budget constraints.

3.12 Determination of soil phosphatase activity

Soil phosphatase activity was measured by the method developed by Tabatabai and Bremner (1969) using *p*-nitrophenyl phosphate as substrate. This colorimetric method is based on the yellow colour formation of alkaline solutions of *p*-nitrophenol formed as a product of phosphatase activity which is then measured at 400 nm.

3.12.1 Preparation of reagents:

(1) Modified universal buffer (MUB)

12.1 g tris (hydroxymethyl) aminomethane, 11.6 g maleic acid, 14.0 g citric acid, 6.3 g boric acid and 19.5 g NaOH were dissolved in 1 L of deionised water. The stock solution was stored at 4^oC, diluted five times by volume and adjusted to pH 6.5 with 1.0 M HCl or 1.0 M NaOH before use.

(2) *p*-Nitrophenyl phosphate (PNP) solution, 0.115 M

1.927 g of disodium *p*-nitrophenyl phosphate tetrahydrate was dissolved in MUB, and the solution was diluted to 50 mL with MUB and stored at 4^oC.

(3) Calcium chloride, 0.5 M

73.5 g of $CaC1_2.2H_2O$ was dissolved in deionised water and the solution was diluted to 1 L.

(4) Sodium hydroxide, 0.5 M

20 g of NaOH was dissolved in deionised water and the solution was diluted to 1 L.

(5) Standard *p*-nitrophenol solution

1.0 g of *p*-nitrophenol was dissolved in deionised water and the solution was diluted to 1 L and stored at 4^oC.

(6) Toluene (Fisher certified analytical grade).

3.12.2 Procedure

Soil (1 g) was added to a 50 mL Erlenmeyer flask containing 4 mL of MUB, 0.25 mL of toluene and 1 mL of PNP solution. The flask was swirled to mix content, stoppered and placed in an incubator at 37^{o}C. After 1 hour, 1 mL of 0.5 M calcium chloride and 4 mL of 0.5 M sodium hydroxide were added to the flask, and the mixture was centrifuged at 15,000 g/min for 30 min to allow for good separation of the supernatant from the soil suspension. The yellow colour intensity of the supernatant was then measured with a spectrophotometer at absorbance of 400 nm. The *p*-nitrophenol formed was calculated using a calibration graph plotted with readings generated from standards containing 0, 100, 200, 300, 400 and 500 µg of *p*-nitrophenol.

The calibration graph was prepared by diluting 2 mL of the standard *p*-nitrophenol solution to 20 mL in a volumetric flask. Aliquots of 0, 1, 2, 3, 4, and 5 mL of the diluted standard solution were pipetted into centrifuge tubes and volumes were adjusted to 5 mL with deionised water. 1 mL of 0.5M calcium chloride and 4 mL of 0.5 M sodium hydroxide were added to each tube, and the mixture was centrifuged at 4000 rotations/min for 10 min. The yellow colour intensity of the supernatant was then measured with at absorbance of 400 nm and readings were then plotted against the concentration of *p*-nitrophenol.

Controls were also performed for each soil sample to measure any colour formation not derived from *p*-nitrophenol released by phosphatase enzyme activity. The same procedure for the sample analysis as described above was followed except that 1 mL of PNP solution was added to the sample mixture after the additions of 0.5 M calcium chloride and 0.5 M sodium hydroxide (after incubation at 37^{o}C). The mixture was then centrifuged and colour intensity of the supernatant was read at 400 nm.

3.13 Microtox acute toxicity assay

The Microtox toxicity test was carried out using the SDI Model 500 Analyser (Figure 3.5) as specified in the Microtox basic test protocol (Azur Environmental, 1998b; Blaise and Ferard, 2005). Prior to analysis, the tar sample extracts (EOM) were solvent-exchanged into 1 mL of DMSO which is less toxic than the dichloromethane: methanol extractant. Aliquots of 1 µL were then diluted with 10 mL of Microtox diluent before analysis. The concentrations of the solutions ranged between 2 mg/L to 35 mg/L. The

Microtox kit consists of the freeze-dried reagent (stored at -20^{o}C to -25^{o}C), reconstitution solution and diluent (non-toxic 2% NaCl solution), osmotic adjusting solution-OAS (non-toxic 22% NaCl solution) and cuvettes (12 x 50 mm).

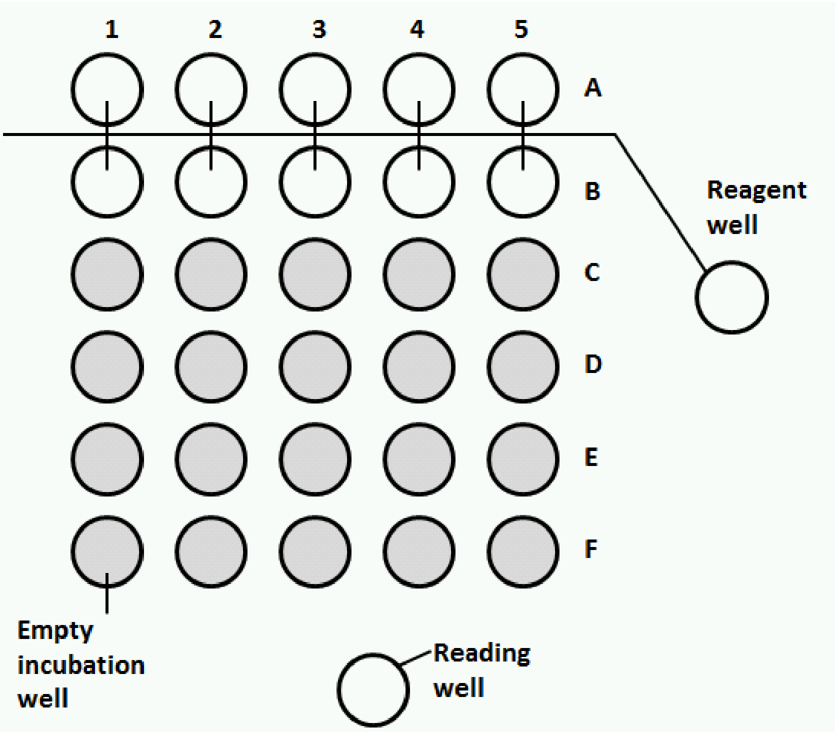


Figure 3.5. Schematic configuration of SDI Model 500 Analyser.

3.13.1 Procedure

Cuvettes were placed in incubator wells A1 to A5, B1 to B5 and reagent well. The incubator wells were temperature controlled at 15^{o} C and the reagent well at 5^{o}C. Reconstitution solution (1,000 µL) was added to reagent well; 500 µL diluent to B1 to B5 and 1000 µL diluent to A1 to A4. An aliquot of the sample (2,500 µL) and 250 µL of osmotic adjusting solution (OAS) were then added to A5, well-mixed and 750 µL of the mixture was afterwards discarded. Serial dilutions of 1:2 of the sample were then made by transferring 1000 µL from A5 to A4, A4 to A3 and A3 to A2. Mixing was made with each transfer and 1000 µL was then discarded from A2 to make it 1000 µL. Samples were allowed to equilibrate for 5 min. The precooled reconstitution solution in the reagent well cuvette was then poured to the freshly opened vial of reagent taken from the freezer. The mixture was swirled in the vial 3 to 4 times and quickly poured back to the cuvette and returned to the reagent well. The reconstituted bacteria solution was then mixed thoroughly by using a pippetor in aspirating and dispensing 500 µL of solution at least 10 times. The solution was used within 3 hours of reconstitution.

The reagent (10 µL) was transferred to B1 through B5. Mixing was done by shaking the cuvettes and the mixture was allowed to equilibrate for 15 min. Zero time light levels (I_0) of B1 through B5 were then read in the reading well as prompted by the computer monitor. The incubation timer was afterwards activated by pressing the spacebar key of the computer and then immediate transfers of 500 µL sample mixture were made from A1 to B1, A2 to B2, A3 to B3, A4 to B4 and A5 to B5. The space bar key of the computer was then pressed again to allow for correction of the incubation time based on the pipetting time. At the end of the incubation time, the light levels of samples were read by placing cuvettes in B1 through B5 in the reading well as prompted by the computer. Readings were taken for 5 min and 15 min incubation time.

The EC_{50} values were determined at the 95% confidence interval by plotting the dose against response on a logarithmic scale which was automatically generated by the Microtox Analyser. Positive control using phenol standard solution (50 mg phenol/500 mL diluent) was initially tested to fall within acceptable limits (EC_{50} between 13 and 26 mg/L) before sample analyses were conducted.

3.14 Statistical methods

Data generated from this study were tested statistically using MINITAB 16.0 software. Comparison of means was conducted using the One-Way Analysis of Variance (ANOVA) test at 95% confidence level followed by the Dunnet’s multiple comparison test set at 5% family error rate. The student *t*-test was also used to compare two different means at 5% level of significance.

Chapter 4

EFFECTS OF NUTRIENT AND BIODIESEL AMENDMENTS ON THE BIODEGRADATION OF CRUDE OIL AND WEATHERED COAL TAR

4.1 Introduction

This Chapter investigates the effect of nutrient (slow release fertiliser-SRF) and biodiesel amendment on the biodegradation of crude oil and weathered coal tar (Phase I biodegradation experiment). These amendment techniques were employed to enhance the natural biodegradation process of the organic pollutants (OTA, 1991); the addition of nutrients (biostimulation) was aimed at increasing the soil microbial biomass and activity, while biodiesel was added as an alternative source of carbon and co-substrate to induce cometabolic effects as well as increase PAH bioavailability by solubilisation (Wilson and Jones, 1993; ATSDR, 1995; Taylor and Jones, 2001; Wu *et al.*, 2010).

The Phase I biodegradation experiment involved the incubation of the hydrocarbon-spiked soil for 60 and 180 days prior to analysis (Figure 3.2 and Table 3.1). In this Chapter, the extent of degradation of the saturated hydrocarbons in the amended and non-amended microcosms of the crude oil spiked experiments is presented followed by the extent of biodegradation of the PAHs, particularly the 16 EPA priority pollutants in both crude oil and weathered tar treatments. Also, results of the preliminary tests conducted on the Nafferton soil and the biodiesel sample used for the experiments are reported.

4.2 Results and Discussion

4.2.1 Soil analysis

The soil from the agricultural site showed a pH of 7.2, which is within the range for optimum microbial activity and PAH biodegradation as suggested by Wilson and Jones (1993) and therefore required no adjustments. Its total organic carbon and nitrogen values were 3.5% and 0.31%, giving a C:N ratio of 11:1, which is also within the range of average C:N ratios of most soils with values between 8 and 17 that are required for optimum microbial activity (Wilson and Jones, 1993). The soil moisture content and water holding capacity were recorded as 21% and 49% of its dry weight, respectively.

4.2.2 Microbial growth in laboratory microcosms

A visible growth of microorganisms (white moulds) was observed in the biodiesel amended samples of the crude oil (CBN) and weathered coal tar (WBN) experiments after 7 days of incubation. After 14 days of incubation, an increased microbial biomass was seen spread over the surface and within pockets beneath the soil surface of the microcosms as shown in Figure 4.1.

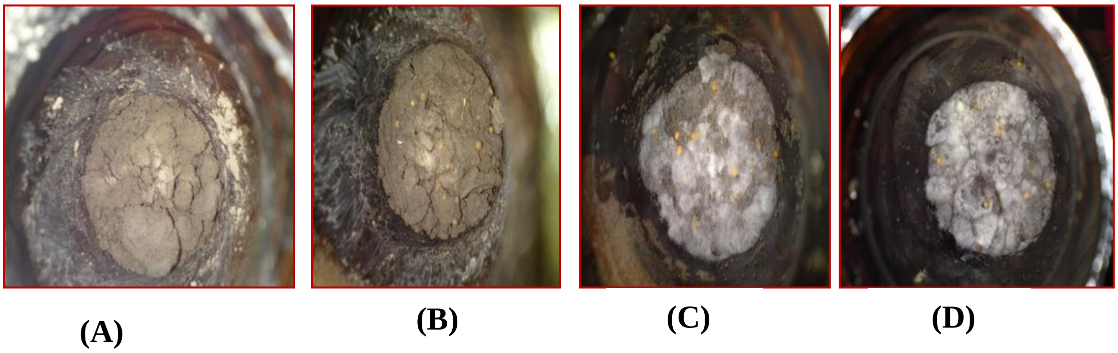

(A) (B) (C) (D)

Figure 4.1. Microbial growth in biodiesel amended experiments after 14 days incubation. A=untreated control (CC); B=nutrient amended (CNT); C= crude oil spiked biodiesel amended sample (CBN); D= coal tar (weathered) spiked biodiesel amended sample (WBN).

The presence of these microorganisms remained noticeable throughout the period of the experiment (180 days). Although no microbiological identification was carried out during the course of this study, it could be inferred that these microbial moulds were a consortium of yeast and fungi that utilise long chain fatty acid methyl esters as sources of carbon. An isolation and identification of these kind of microbial species was carried out by Bücker *et al.* (2011) for sediments contaminated with soybean biodiesel. In their report, *Paecilomyces* sp. (fungi) exhibited the highest microbial biomass on B20 and B100 biodiesel after 60 days incubation period, while the *Rhodotorula* sp. (yeast) showed the highest rate of degradation of the FAMEs after 7 days of incubation. Microbial growth studies done by Sørensen *et al.* (2011) on biodiesel blends (B100 inclusive) also showed that the addition of biodiesel to fuel microcosms increased the microbial biodiversity of the system.

4.2.3 Extractable organic matter (EOM)

The solvent extractable organic matter concentrations (mg g^{-1} dry weight of soil) for all treatments measured at the start of the experiment (day 0) and after 60 and 180 d incubation periods are shown in Table 4.1 and, graphically in Appendix 1. The results showed 75 and 81% decrease in the biodiesel amended samples of crude oil (CBN) and weathered coal tar (WBN) experiments, respectively, after the 60 d incubation period.

This high percentage decrease in the EOM concentrations can largely be attributed to the high degradability of the rapeseed methyl ester (RME) biodiesel component of those microcosms, which could be readily utilized as a source of carbon by the soil microbes. These observed levels of biodiesel degradation also fall within ranges of previous biodegradability studies by Vauhkonen *et al.* (2011) and Demirbaş (2009) which showed between 80-90% decrease and more than 60% degradation after 30 and 28 d degradation period. At the end of the experiment (180 d incubation period), results showed 84 and 88% decrease in EOM concentrations for the crude oil (CBN) and weathered coal tar (WBN) experiments, respectively.

Table 4.1. EOM values for crude oil and weathered coal tar spiked soil

Hydrocarbon pollutant	**Sample Codes**	**Extractable organic matter (EOM, mg/g)**		
		Day 0	**Day 60**	**Day 180**
Crude oil	CC	6.4 ± 0.6	4.7 ± 0.6	3.7 ± 0.3
	CNT	11.0 ± 1.9	6.1 ± 1.1	4.6 ± 0.6
	CBN	155.3 ± 8.5	38.3 ± 6.8	24.8 ± 4.4
Weathered coal tar	WC	4.5 ± 1.2	4.0 ± 0.2	3.7 ± 0.3
	WNT	11.8 ± 2.9	5.0 ± 1.3	4.6 ± 0.6
	WBN	168.4 ± 12.5	31.3 ± 5.9	20.8 ± 13.0

Values are mean (n=3) ± SD. CC=Untreated control, CNT=Nutrient only and CBN=Biodiesel amended samples of crude oil spiked soil; WC= untreated control, WNT=nutrient-amended and WBN= biodiesel amended samples of coal tar spiked soil.

The nutrient-only samples of the crude oil (CNT) and weathered coal tar (WNT) experiments showed 44 and 58% decrease in the EOM concentrations, respectively, while the untreated control experiments (CC and WC) displayed 26% and 11% depletions, respectively, after 60 days. After the 180 d incubation period, 59 and 61% decrease in EOM concentrations were seen in CNT and WNT, respectively, while the CC and WC samples displayed 42 and 19% depletions, respectively.

From the results of the EOM concentrations, the total contaminant level of each treatment set (the control, the nutrient and biodiesel amended samples) after the 60 and 180 d incubation periods may not be predictable because of possible interferences of other biological compounds e.g. the FAMEs and other lipids, plant waxes and chlorophyll, asphaltene compounds, and the NSOs (polar nitrogen-, sulphur-, and oxygen containing compounds, which are extractable along with the crude oil and coal tar hydrocarbons (Mills *et al.*, 1999).

4.2.4 Biodiesel composition and the biodegradation of rapeseed methyl esters (RME)

The types and proportions of the fatty acid methyl esters (FAMEs) in biodiesel is affected by the source of the vegetable oil or animal fat that it is produced from (Stauffer and Byron, 2007). Figure 4.2 shows the chromatogram of the rapeseed biodiesel used in this study, while Table 4.2 reports the percentage chemical composition of the fatty acid methyl esters which was compared with a typical rapeseed oil-based biodiesel reported by Stauffer and Byron (2007). In both cases, the totals do not add up to 100% due to the non-inclusion of compounds of very small proportions. The most abundant compound of the rapeseed biodiesel analysed is shown to be methyl octadecenoate, i.e. the methyl ester of oleic acid ($C_{18:1}$; 84.3%) followed by methyl esters of palmitic, linoleic, stearic and gadoleic acids ($C_{16:0}$, $C_{18:2}$, $C_{18:0}$ and $C_{20:1}$, respectively) in decreasing order of abundance (Table 4.2). Some other FAMEs of <1% composition are also shown in the chromatogram below. The saturated FAMEs constituted only 9.4% of the calculated total of 99.5%.

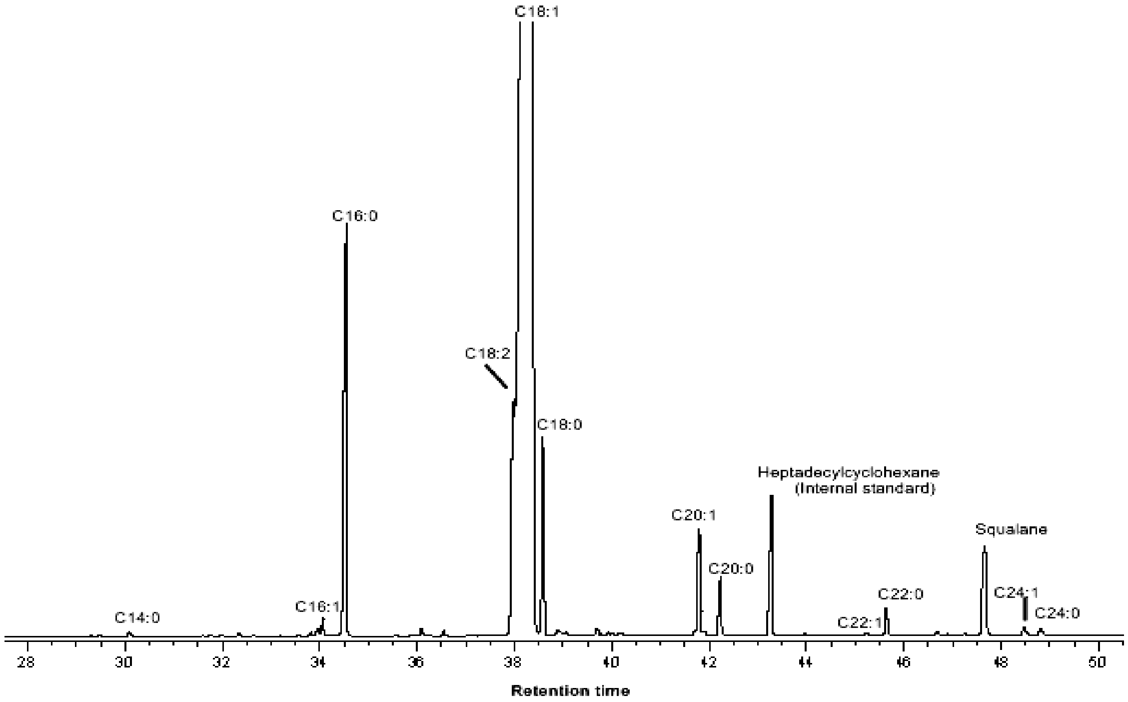


Figure 4.2. Partial gas chromatogram of the rapeseed biodiesel used for this study; the abbreviated names for the fatty acids are represented by the ‘C’ followed by the total number of carbon atoms (including the carboxylic group) present in the chain, and a colon with the number of carbon to carbon double bonds present in the chain.

Table 4.2. Chemical composition of the rapeseed biodiesel used for this study in comparison with typical oil-based rapeseed biodiesel reported by Stauffer & Byron, 2007

Fatty acid methyl esters of rapeseed biodiesel (RME)	**Concentration (%) by mass**	
	RME analysed	**RME (Stauffer & Byron, 2007)**
C14:0	0.1	0.1
C16:0	5.7	4.8
C16:1	0.2	0.2
C18:0	2.3	0.4
C18:1	84.3	61.6
C18:2	4.1	20.6
C18:3	N/A	9.2
C20:0	0.8	0.6
C20:1	1.4	1.4
C22:0	0.4	0.4
C22:1	0.0	0.3
C24:0	0.1	0.1
C24:1	0.1	N/A

Abbreviated names for the fatty acids are represented by 'C' followed by the total number of carbon atoms (including the carboxylic group) present in the chain, and a colon with the number of carbon to carbon double bonds present in the chain.

The removal of these component methyl esters in the biodiesel amended coal tar microcosms at 60 and 90 days incubation periods is shown in Figure 4.3. As seen in the chromatograms, there was complete removal of $C_{18:1}$ after 60 days but traces of $C_{18:2}$ (~3%) and $C_{18:0}$ (~5%) were still seen. Also, a complete disappearance of $C_{16:0}$ and $C_{20:1}$ was also observed. After 90 days, the unsaturated FAME $C_{18:2}$ had completely disappeared from the soil extracts but traces of its saturated isomer $C_{18:0}$ were still present in the coal tar soil extracts.

These results show that the unsaturated FAMEs which constitute more than 90% of the total composition of biodiesel are degraded at a faster rate compared to the saturated methyl esters. This is because unsaturated compounds are less stable and therefore can easily be attacked by microorganisms (Killops and Killops, 2009). Also, the level of stability of compounds increases with saturation (Peters *et al.*, 2005) and this was observed in the preferential degradation of the less saturated C_{18} FAMEs in the order: $C_{18:1}$>$C_{18:2}$>C_{18}, in a pattern that is consistent with previous work by Miller and Mudge (1997). However, the more stable palmitic acid methyl ester ($C_{16:0}$) was observed to have been completely removed at day 60 before the linoleic acid ($C_{18:2}$) and stearic acid

($C_{18:0}$) methyl esters. This may be attributed to the preferential microbial degradation of this methyl ester subsequent to the depletion of the oleic acid methyl ester, which is similar to an observation made by Bücker *et al.* (2011) for the degradation of biodiesel by yeast *Rhodotorula sp.* It is also noteworthy that some of these FAMEs may be extracts from the fatty acid profile of the native soil communities present in the microcosms, including other soil lipids from leaf waxes etc. (Cavigelli *et al.*, 1995; Mills *et al.*, 1999; Schutter and Dick, 2000).

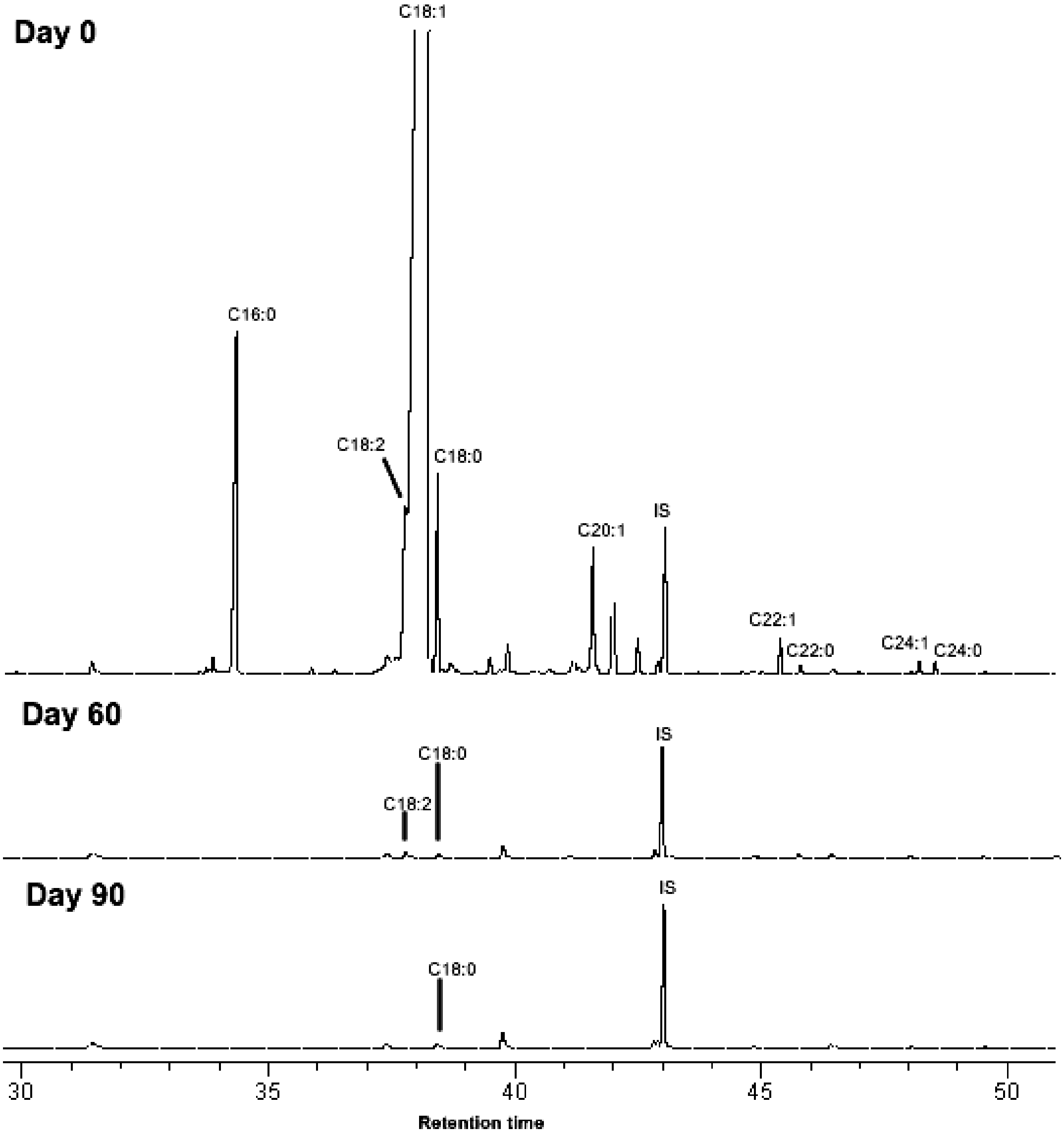


Figure 4.3. Partial gas chromatograms showing the disappearance of the FAMEs in the biodiesel amended extracts of 0, 60 and 90 day incubation periods. The abbreviated names for the fatty acids are represented by 'C' followed by the total number of carbon atoms (including the carboxylic group) present in the chain, and a colon with the number of carbon to carbon double bonds present in the chain.

These results are consistent with the high degradability attributed to biodiesel from previous research (Zhang *et al.*, 1998; Pasqualino *et al.*, 2006; Mariano *et al.*, 2008). However, traces of its methyl esters even after 90 days of addition to the soil may still be able to support microbial growth and activity, thus requiring toxicity analyses to check for any form of induced soil toxicity that could result from the presence of these residual components.

4.2.5 Degradation of crude oil saturated hydrocarbons (*n*-alkanes)

The extent of degradation of the *n*-alkanes (C_{14} to C_{30}) was assessed for all crude oil treatments. After 60 days, the results (Table 4.3) showed that the sum of the *n*-alkanes when compared to their initial concentrations (day 0) showed reductions of 72, 42 and 29% in the nutrient only (CNT), biodiesel amended (CBN) and untreated control (CC) samples, respectively. Continued loss of compounds increased to 92%, 90%, and 84% in the CBN, CNT and CC microcosms, respectively, after 180 days incubation and microbial degradation was evaluated in all the experiments using the conventional biodegradation ratios pristane/*n*C_{17} and phytane/*n*C_{18} (e.g. Jones *et al.*, 1983; Wenger *et al.*, 2002; Wang *et al.*, 2013).

Examination of the chromatograms in Figure 4.4 showed that after 60 days, the branched alkanes pristane and phytane were more abundant relative to *n*-C_{17} and *n*-C_{18} in the CNT samples, as depicted in their increased pristane/*n*C_{17} and phytane/*n*C_{18} ratios of 2.03 and 1.89, respectively; indicating an occurrence of mild to moderate biodegradation (e.g. Peters and Moldowan, 1993; Wenger *et al.*, 2002; Head *et al.*, 2003). In the CBN microcosms, there was only a slight biodegradation of the *n*-alkanes (pristane/*n*C_{17} - 1.09; phytane/*n*C_{18} – 1.06), while the CC samples did not show any appreciable level of microbial biodegradation as the *n*-C_{17} and *n*-C_{18} were still more abundant compared to the branched alkanes (pristane/*n*C_{17} – 0.73; phytane/*n*C_{18} - 0.67) therefore suggesting that any losses in the untreated control experiments could be largely due to very slight biodegradation or evaporation (Peters *et al.*, 2005).

The GC analysis of the alkane fractions for the amended samples (CNT-60 and CBN-60, Figure 4.4) revealed that the biodegradation of compounds followed an already established pattern in which the lower molecular weight *n*-alkanes were preferentially removed before the higher molecular weight compounds (Miller and Mudge, 1997), followed by the isoprenoids (Wenger *et al.*, 2002; Chaîneau *et al.*, 2005; Garcia-Blanco *et al.*, 2007). As shown in the graphs in Appendix 2, the addition of nutrients caused significant depletions in the straight chain *n*-alkanes C_{14} (88%; $p=0.01$) and C_{15}-C_{25} (64-78%; $p \leq 0.03$) relative to the control. *N*-alkanes C_{25}-C_{27} were reduced by more than 50% which was, however, not significant compared to the control ($p>0.05$). Also, significantly higher concentrations were observed for C_{28}-C_{30} ($p \leq 0.03$) relative to the control although depletions were also more than 50%.

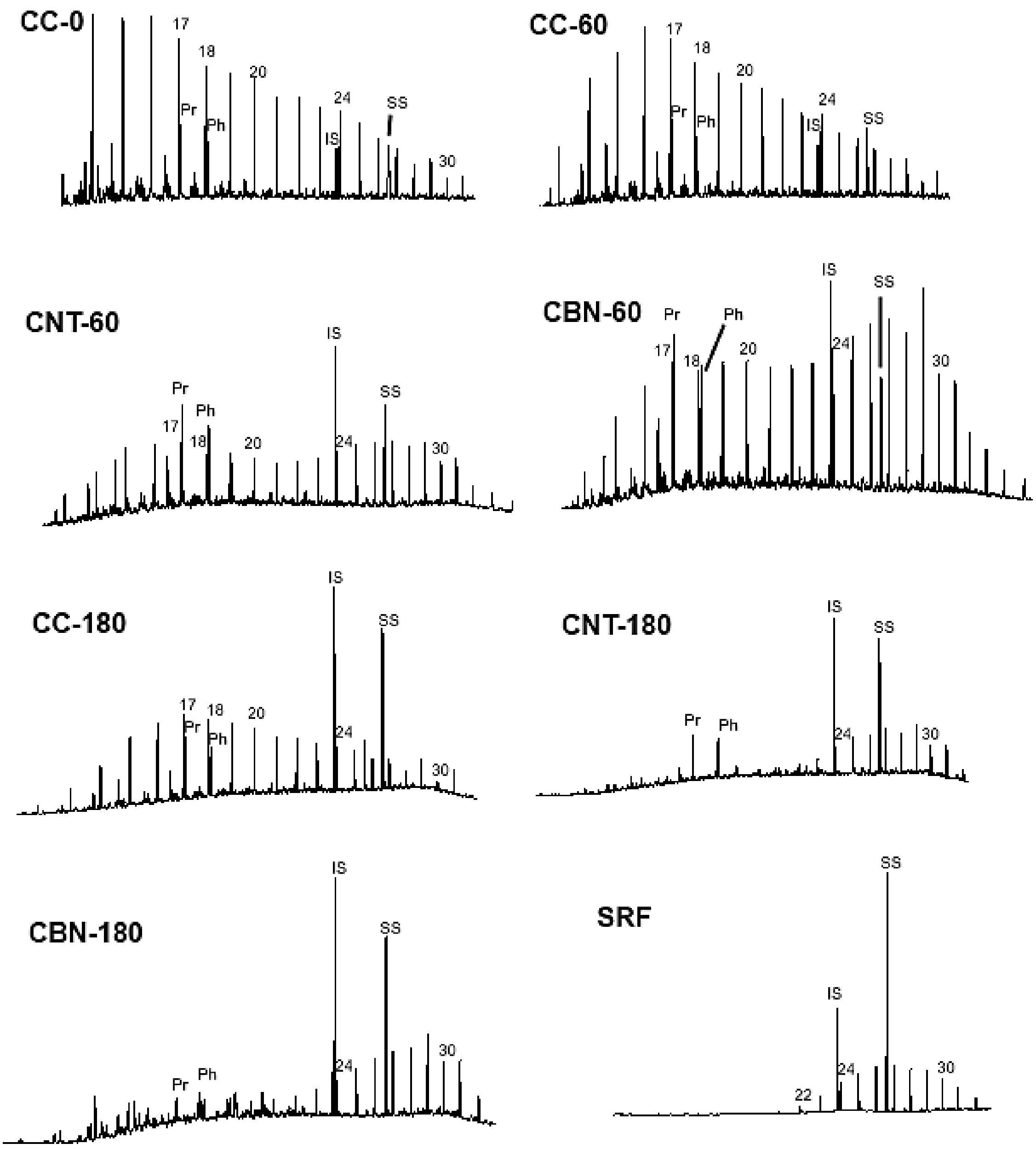


Figure 4.4. Gas chromatograms of the saturated hydrocarbon fractions of the crude oil at the start of the experiment (day 0), at days 60 and 180 for all treatments and also for the extract of the slow release fertiliser (SRF). CC=Untreated control, CNT=Nutrient only, CBN=Biodiesel amended, IS= Internal standard (heptadecylcyclohexane) and SS=Surrogate standard (squalane).

Table 4.3. Concentrations and standard deviations of n-alkanes in crude oil spiked soil after incubation for 0, 60 and 180 days

	Concentrations of *n*-alkanes (µg/g)																	
	CC						CNT						CBN					
	Day 0		**Day 60**		**Day 180**		**Day 0**		**Day 60**		**Day 180**		**Day 0**		**Day 60**		**Day 180**	
	Mean	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**
$n\mathrm{C}_{14}$	62.12	4.7	30.2	4.5	3.88	0.2	64.21	5.8	7.71	2.2	1.04	0.2	56.84	2.4	4.40	3.2	1.57	0.4
$n\mathrm{C}_{15}$	61.56	5.2	43.7	4.1	6.45	0.0	58.05	6.7	12.98	5.0	1.39	0.9	65.69	3.1	17.21	12.6	1.74	0.7
$n\mathrm{C}_{16}$	57.68	4.1	45.1	4.5	8.17	0.1	53.11	8.6	12.14	4.5	1.74	0.7	61.69	3.7	24.73	21.6	2.90	1.9
$n\mathrm{C}_{17}$	54.72	3.9	41.1	4.5	8.85	0.2	49.88	8.1	10.85	4.2	1.88	0.9	53.27	3.7	27.09	21.5	2.24	0.8
Pristane	29.08	1.9	30.0	3.2	8.89	1.0	29.25	0.6	20.81	3.3	8.95	3.1	29.62	1.7	23.09	6.7	7.35	4.6
$n\mathrm{C}_{18}$	46.14	4.0	35.5	3.4	7.62	0.3	40.56	7.2	10.11	3.7	1.12	0.8	45.35	3.7	25.43	19.7	1.50	0.5
Phytane	21.77	1.2	23.9	1.8	7.02	0.8	21.65	0.5	18.06	2.3	7.81	2.8	22.55	2.0	19.48	5.2	6.36	4.2
$n\mathrm{C}_{19}$	41.45	3.2	29.8	3.0	7.58	0.2	36.69	9.2	8.81	3.2	1.95	0.9	38.74	3.1	23.97	16.9	2.13	0.8
$n\mathrm{C}_{20}$	38.41	3.7	28.2	2.4	7.02	0.9	33.25	9.6	8.48	3.1	2.33	1.4	35.68	2.8	23.66	15.3	1.51	0.5
$n\mathrm{C}_{21}$	31.97	2.2	23.6	2.0	6.28	0.3	28.33	8.4	7.49	2.7	1.83	1.0	31.24	2.8	20.85	12.8	1.59	0.5
$n\mathrm{C}_{22}$	31.15	2.9	21.8	2.2	5.54	0.2	26.79	7.5	7.65	2.4	1.98	1.0	29.11	3.1	20.28	12.0	1.88	0.6
$n\mathrm{C}_{23}$	27.17	1.8	19.5	1.7	4.92	0.1	28.68	10.6	8.33	2.5	2.74	0.9	27.87	2.8	20.26	11.1	2.27	0.4
$n\mathrm{C}_{24}$	25.85	1.3	18.2	1.5	4.67	0.2	27.87	7.9	9.98	2.3	4.51	1.0	29.88	3.9	22.28	11.5	3.15	0.6
$n\mathrm{C}_{25}$	22.78	2.6	15.6	1.8	4.49	0.3	29.26	8.4	11.14	1.6	6.34	1.8	29.78	4.8	24.08	11.2	4.20	0.8
$n\mathrm{C}_{26}$	19.10	1.8	13.3	1.1	4.34	1.0	28.29	9.2	11.78	2.0	7.23	1.3	30.19	4.0	24.39	11.0	4.09	0.8
$n\mathrm{C}_{27}$	14.70	1.0	11.3	0.9	3.23	0.2	26.76	8.3	12.27	1.6	6.62	1.0	28.00	4.8	24.10	9.7	5.23	0.9
nC_{28}	10.78	0.8	8.8	0.6	2.34	0.3	24.62	8.1	11.52	1.3	6.60	0.9	25.18	4.4	22.33	8.0	5.77	1.1
$n\mathrm{C}_{29}$	10.95	0.4	8.1	0.8	3.46	0.1	23.39	7.6	11.20	1.4	6.56	0.8	29.43	5.6	26.15	8.8	6.71	1.5
nC_{30}	6.92	0.16	4.1	0.2	1.37	0.2	17.77	6.11	7.76	1.1	4.56	0.7	16.13	3.2	14.82	4.6	5.02	1.4
∑ *n*-alkanes	563.5		397.9		90.2		597.5		170.2		60.4		634.1		366.0		53.5	

CC= untreated control, CNT= nutrient amended and CBN= biodiesel amended microcosms.

Consistent with these results, previous studies (e.g. Swannell *et al.*, 1996; Coulon *et al.*, 2005; Margesin *et al.*, 2012; Fukuhara *et al.*, 2013) have also reported enhanced degradation of aliphatic hydrocarbons when nutrients were added to petroleum contaminated soils and likewise observed the relative recalcitrance of longer chain *n*-alkanes (>20) to degradation that may have resulted from reduced bioavailability of these compounds due to their hydrophobic waxy nature (Sugiura *et al.*, 1997; Tyagi *et al.*, 2011).

The additional amendment with biodiesel in the CBN microcosms did not further enhance degradation in the *n*-alkanes compared to the nutrient-amended samples (CNT) after the 60 days incubation (see Table 4.3, Appendix 2). However, losses seen for C_{14} (92%) and C_{15} (74%) were significant ($p \leq 0.03$) relative to the control. C_{16} was also reduced by 60% but it was not significant relative to the control ($p > 0.05$). Compounds C_{17} - C_{27} were depleted by just between 14-49%. *N*-alkanes C_{28}-C_{30} were also seen to be significantly higher in concentrations in the biodiesel amended samples (CBN) compared to the control ($p \leq 0.04$), hence confirming their recalcitrance to degradation.

These results showed that the rate of degradation of the *n*-alkanes was slowed down in the presence of biodiesel, indicating that the FAMEs were preferentially utilised as sources of carbon during the 60 days incubation period. A similar observation was made in a previous study by DeMello *et al.* (2007) which showed that FAMEs inhibited the initial biodegradation of fossil diesel at the first week of their experiment. Also, it was observed that the fatty acid component of oleophilic fertilisers (e.g. Inipol) were preferentially degraded to crude oil hydrocarbons, but that, however, could be mitigated by further amendment with sugar molasses (Nikolopoulou and Kalogerakis, 2008)

The untreated control (CC) showed 51% decrease in C_{14}, while the rest of the *n*-alkanes, C_{15}-C_{30}, were reduced by between 19-41% after 60 days incubation (Table 4.3, Appendix 2). There was also no loss observed in pristane and phytane compared to the percentage reductions revealed in the amended samples; CNT (pristane-29%, phytane-17%) and CBN (pristane-22%, phytane-14%). The reduced loss in phytane compared to pristane can be attributed to its longer chain length which makes it more recalcitrant to microbial attack. However, the susceptibility of these branched *n*-alkanes to biodegradation reveals their limitations as biomarkers in studies relating to biodegradation and oil spill identification (Obbard *et al.*, 2004; Wang *et al.*, 2013). Based on these results, the rate of biodegradation of the *n*-alkanes in the various microcosm treatments, after 60 days, was observed to occur in the order of CNT>CBN> CC.

After 180 days incubation, the gas chromatograms (Figure 4.4) showed an almost complete removal of C_{14}-C_{23} in the CNT (90-98%) and CBN (92-97%) treatments compared to the CC (82-94%). Losses could still be largely attributed to evaporation in the untreated control (CC-180) as Figure 4.4 showed a relative abundance of the C_{17} and C_{18} *n*-alkanes compared to the branched alkanes. Pristane and phytane were more depleted in the CBN samples (pristane- 75%, phytane-72%) compared to the CNT and CC microcosms which showed similar losses for pristane (69%) and reductions of 64 and 68%, respectively, in phytane (Table 4.3). The higher molecular weight *n*-alkanes C_{24}-C_{26} were also seen to be more reduced in CBN (77-89%) compared to CNT (72-84%) but depletions were not significant ($p=0.05$) compared to the CC samples (68-82%).

The C_{27}-C_{30} *n*-alkanes still showed significantly ($p\leq0.02$) higher concentrations in the CBN and CNT microcosms compared to the control (CC). A possible explanation for this could be that some of these *n*-alkanes were derived from the waxy sealant which comprised the coatings of the slow release fertiliser (SRF) used (e.g. Lee *et al.*, 2005). These waxy *n*-alkanes are composed of C_{22}-C_{32} compounds as seen in the chromatogram for extracts of the SRF granules in Figure 4.4. These compounds were, however, degraded to an extent in the CNT samples after 60 days as concentration levels became almost the same with the control except for C_{28}-C_{30} as earlier mentioned. In the biodiesel samples (CBN), the removal of these compounds was not pronounced until after 180 days incubation period.

The C_{30} *n*-alkane was the least reduced in the CBN microcosms (by 69%) relative to the CC (80%) and CNT (74%) experiments (Table 4.3). Biodiesel treatments (CBN) showed enhanced degradation of the branched *n*-alkanes compared to this high molecular weight n-alkane (C_{30}), indicating that more complex compounds could be degraded in preference to simple molecules if they are made accessible to microbial attack by increased solubilisation. This, however, was not the case in the control and nutrient only treatments which showed the lowest percentage losses for the branched *n*-alkanes compared to C_{30}.

At the end of the experiment (180 day incubation), the residual *n*-alkane concentrations for CBN, CNT and CC were 54, 60 and 90 µg/g soil (Table 4.3), which shows that after the 60 day incubation period which featured the largest disappearance of the FAMEs in the CBN microcosms, the rate of degradation of the *n*-alkanes peaked. The CNT experiments also showed increased loss of aliphatic compounds compared to the control (CC) therefore suggesting that these amendments facilitated the microbial degradation of saturated hydrocarbons.

4.2.6 Degradation of crude oil and weathered coal tar PAHs

The concentrations of the crude oil and weathered coal tar PAHs, and in particular the 16 EPA priority pollutants, were determined at the start of the experiment (day 0) and after 60 d and 180 d incubation periods. After 60 days, nutrient and biodiesel amendment had no significant ($p>0.05$) effect on the degradation of naphthalene (N) in both crude oil and tar experiments relative to the control (Table 4.4 and Table 4.6). The crude oil microcosms showed high reductions of 96% for the nutrient amended (CNT) and untreated control (CC) compared to 89% depletion in the biodiesel treated (CBN) microcosms. The weathered coal tar experiments also showed similar high rates of disappearance in all treatments- 99, 97 and 94% for WNT, WC and WBN microcosms, respectively, as shown in Figure 4.5 and Table 4.6. The maximum mineralisation rate of naphthalene is affected by a number of factors but predominantly depends on temperature (20-30^{o}C) and the presence of oxygen (aerobic conditions) as reported by Bauer and Capone (1985). The addition of nutrients and biodiesel in this work, however, did not cause any inhibitory effects.

Also, in both hydrocarbon pollutant (crude oil and tar) experiments, the methylnaphthalenes and biphenyl were removed to similar extents as the naphthalenes (90-99%; $p>0.05$) in the untreated control and nutrient amended treatments, although comparatively low reductions were observed for the biodiesel amended treatments (methylnaphthalenes- 79-90% and biphenyl 54-63%). Previous studies have shown that naphthalene degrading bacteria can also function in the degradative pathway of biphenyl (Kuhm *et al.*, 1991; Haddock *et al.*, 1993; Uhlik *et al.*, 2012), thus explaining the similarity in degradation rates for both compound groups.

Table 4.4. Concentrations and standard deviations of the 2-3 ring PAHs in the crude oil spiked soil at days 0, 60 and 180

	Concentrations of PAHs (µg/g)																	
	CC						CNT						CBN					
	Day 0		**Day 60**		**Day 180**		**Day 0**		**Day 60**		**Day 180**		**Day 0**		**Day 60**		**Day 180**	
	Mean	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**
Naphthalene	0.22	0.09	0.01	0.01	0.00	0.00	0.27	0.07	0.01	0.00	0.01	0.00	0.08	0.02	0.01	0.01	0.01	0.01
2-methylnaphtalene	0.80	0.44	0.01	0.01	0.05	0.05	0.88	0.33	0.03	0.01	0.06	0.05	0.61	0.04	0.06	0.03	0.43	0.24
1-methylnaphthalene	0.57	0.29	0.01	0.01	0.04	0.04	0.58	0.22	0.02	0.00	0.06	0.04	0.46	0.03	0.06	0.04	0.36	0.22
Biphenyl	1.79	1.26	0.03	0.02	0.04	0.01	1.90	0.92	0.09	0.04	0.06	0.01	0.82	0.19	0.31	0.17	0.42	0.18
Acenaphthylene	BDL	-	-	-	-	-	BDL	-	-	-	-	-	BDL	-	-	-	-	-
Acenaphthene	BDL	-	-	-	-	-	BDL	-	-	-	-	-	BDL	-	-	-	-	-
Fluorene	2.08	0.59	0.14	0.04	0.33	0.26	2.27	0.19	0.12	0.00	0.23	0.29	1.56	0.14	0.40	0.09	0.49	0.08
Phenanthrene	3.14	1.25	0.29	0.02	0.53	0.35	3.28	0.60	0.21	0.01	0.35	0.37	3.29	0.31	2.64	0.55	3.22	0.31
Anthracene	0.08	0.04	0.02	0.01	0.00	0.00	0.08	0.05	0.03	0.01	0.02	0.00	0.07	0.01	0.00	0.00	0.00	0.00
3-methylphenanthrene	1.98	1.11	0.66	0.19	0.05	0.00	1.61	0.11	0.21	0.04	0.05	0.01	1.72	0.17	1.53	0.14	1.44	0.07
2-methylphenanthrene	2.22	1.27	0.39	0.10	0.05	0.01	1.75	0.12	0.21	0.02	0.05	0.01	1.80	0.16	1.45	0.23	1.49	0.09
9-methylphenanthrene	3.47	2.01	1.57	0.35	0.07	0.02	2.64	0.18	0.38	0.05	0.07	0.01	2.66	0.30	2.22	0.21	2.21	0.09
1-methylphenanthrene	2.13	1.08	0.89	0.24	0.05	0.01	1.94	0.31	0.27	0.03	0.05	0.01	2.10	0.15	1.68	0.23	1.72	0.06
Dibenzothiophene	0.37	0.30	0.02	0.01	0.02	0.01	0.30	0.06	0.02	0.01	0.03	0.01	0.30	0.02	0.28	0.07	0.25	0.03

CC= untreated control, CNT=nutrient-amended and CBN= biodiesel amended microcosms. BDL= below detection limit.

Table 4.5. Concentrations and standard deviations of the 4-6 ring PAHs in the crude oil spiked soil at days 0, 60 and 180

	Concentrations of PAHs (µg/g)																	
	CC						CNT						CBN					
	Day 0		**Day 60**		**Day 180**		**Day 0**		**Day 60**		**Day 180**		**Day 0**		**Day 60**		**Day 180**	
	Mean	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**
Fluoranthene	0.35	0.29	0.15	0.01	0.07	0.02	0.31	0.18	0.16	0.04	0.11	0.03	0.31	0.08	0.25	0.02	0.25	0.01
Pyrene	0.30	0.10	0.23	0.04	0.06	0.01	0.36	0.15	0.22	0.02	0.13	0.11	0.38	0.07	0.33	0.03	0.32	0.01
Benzo[a]anthracene	0.11	0.01	0.10	0.02	0.03	0.00	0.15	0.06	0.13	0.03	0.11	0.01	0.22	0.07	0.12	0.01	0.09	0.01
Chrysene	0.82	0.30	0.56	0.04	0.17	0.02	0.69	0.08	0.60	0.03	0.50	0.01	1.00	0.14	0.88	0.03	0.78	0.01
Benzo[b]fluoranthene	0.20	0.02	0.15	0.02	0.07	0.02	0.23	0.08	0.18	0.01	0.13	0.00	0.23	0.06	0.18	0.01	0.15	0.00
Benzo[k]fluoranthene	0.01	0.00	0.01	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Benzo[j]fluoranthene	0.03	0.01	0.01	0.00	0.01	0.00	0.03	0.01	0.02	0.00	0.02	0.01	0.01	0.00	0.01	0.00	0.01	0.00
Benzo[e]pyrene	0.24	0.01	0.19	0.03	0.18	0.02	0.26	0.02	0.23	0.01	0.20	0.02	0.28	0.06	0.23	0.01	0.21	0.01
Benzo[a]pyrene	0.08	0.00	0.05	0.01	0.03	0.01	0.11	0.05	0.06	0.00	0.04	0.00	0.04	0.02	0.02	0.00	0.02	0.00
Indeno[1,2,3-cd]pyrene	0.02	0.01	0.02	0.01	0.01	0.00	0.02	0.00	0.02	0.00	0.02	0.00	0.01	0.00	0.01	0.00	0.01	0.00
Dibenzo[a,h]anthracene	0.04	0.01	0.04	0.01	0.03	0.01	0.06	0.02	0.05	0.00	0.04	0.00	0.03	0.01	0.02	0.00	0.02	0.01
Benzo[g,h,i]perylene	0.09	0.02	0.08	0.02	0.06	0.01	0.11	0.02	0.10	0.01	0.08	0.01	0.08	0.02	0.05	0.00	0.05	0.01
Perylene	BDL	-	-	-	-	-	BDL	-	-	-	-	-	BDL	-	-	-	-	-

CC= untreated control, CNT=nutrient-amended and CBN= biodiesel amended microcosms. BDL= below detection limit.

Table 4.6. Concentrations and standard deviations of the 2-3 ring PAHs in the weathered coal tar spiked soil at days 0, 60 and 180

	Concentrations of PAHs (µg/g)																	
	WC						WNT						WBN					
	Day 0		**Day 60**		**Day 180**		**Day 0**		**Day 60**		**Day 180**		**Day 0**		**Day 60**		**Day 180**	
	Mean	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**
Naphthalene	25.48	4.6	0.68	0.3	0.40	0.0	27.97	3.6	0.17	0.0	1.02	0.7	6.58	2.6	0.42	0.3	0.69	0.1
2-methylnaphtalene	16.03	0.4	0.73	0.5	0.22	0.1	15.54	1.7	0.17	0.0	0.51	0.3	8.33	2.3	1.02	0.7	1.16	0.7
1-methylnaphthalene	8.99	0.2	0.88	0.7	0.20	0.2	8.69	0.9	0.16	0.0	0.47	0.2	4.94	1.3	1.04	0.3	0.88	0.3
Biphenyl	10.55	0.6	0.28	0.2	0.13	0.1	9.77	1.1	0.39	0.1	0.41	0.2	7.41	1.1	3.40	0.4	1.88	2.0
Acenaphthylene	29.93	4.4	9.19	0.4	8.07	0.3	30.41	1.9	9.92	1.5	9.74	1.8	40.19	3.6	7.47	1.1	9.32	0.9
Acenaphthene	2.97	0.4	0.00	0.0	0.00	0.0	2.89	0.3	0.66	0.1	0.00	0.0	2.84	0.1	0.80	0.2	0.85	0.3
Fluorene	59.77	5.6	1.80	0.5	0.51	0.2	56.90	5.6	8.22	2.9	3.39	1.7	40.29	18.5	12.22	4.8	10.59	12.0
Phenanthrene	157.66	23.4	13.79	5.6	2.18	0.4	152.82	3.1	23.20	3.2	10.72	4.4	155.39	1.7	152.63	8.7	168.01	10.7
Anthracene	7.50	1.1	2.08	0.2	0.99	0.0	9.25	1.8	3.27	1.7	2.61	0.3	13.30	14.6	0.00	0.0	0.00	0.0
3-methylphenanthrene	9.75	0.7	1.66	0.5	0.32	0.1	9.11	1.1	3.39	1.1	1.39	0.4	9.05	2.4	9.65	0.6	10.37	1.0
2-methylphenanthrene	10.36	0.9	2.19	0.8	0.47	0.1	10.28	0.1	7.05	1.4	2.21	0.6	14.84	2.8	10.57	0.4	10.90	1.0
9-methylphenanthrene	4.93	0.4	1.61	0.3	0.39	0.1	4.88	0.3	2.85	0.7	1.24	0.3	4.75	1.3	4.41	0.2	4.81	0.4
1-methylphenanthrene	4.81	0.4	1.34	0.3	0.30	0.1	4.67	0.4	2.91	0.7	1.43	0.4	5.88	0.2	4.81	0.0	5.07	0.5
Dibenzothiophene	6.72	1.0	0.47	0.3	0.00	0.0	5.74	0.4	1.23	0.3	0.44	0.2	7.37	0.5	5.26	0.4	5.57	1.1

WC= untreated control, WNT=nutrient-amended and WBN= biodiesel amended microcosms.

Table 4.7. Concentrations and standard deviations of the 4-6 ring PAHs in the weathered coal tar spiked soil at days 0, 60 and 180

	Concentrations of PAHs (µg/g)																	
	WC						WNT						WBN					
	Day 0		**Day 60**		**Day 180**		**Day 0**		**Day 60**		**Day 180**		**Day 0**		**Day 60**		**Day 180**	
	Mean	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**
Fluoranthene	121.07	17.3	63.43	5.6	13.87	1.5	111.72	2.3	103.70	5.3	47.18	12.9	99.41	25.2	106.48	10.9	107.77	2.5
Pyrene	78.48	9.9	65.03	0.3	19.60	2.4	74.22	2.6	71.84	4.1	47.70	11.4	53.29	39.0	65.37	3.9	67.99	1.2
Benzo[a]anthracene	39.61	3.4	31.06	1.0	15.01	2.0	39.63	1.4	34.75	4.8	34.50	1.3	31.93	4.1	17.46	1.2	23.60	2.5
Chrysene	39.53	3.1	34.68	1.7	20.65	2.5	39.73	3.3	36.91	4.5	33.39	1.4	32.62	4.7	31.11	2.1	30.95	0.5
Benzo[b]fluoranthene	50.90	13.2	40.75	7.2	26.70	1.8	37.74	8.1	34.38	8.4	32.48	5.9	44.64	2.9	30.34	2.5	48.17	0.5
Benzo[k]fluoranthene	11.09	9.7	21.04	4.7	19.48	1.9	22.58	4.4	22.22	5.3	20.20	4.2	13.81	1.9	12.82	4.4	0.00	0.0
Benzo[j]fluoranthene	8.25	0.6	7.96	0.4	6.78	0.1	8.35	0.6	7.01	1.3	7.39	0.2	5.52	0.4	3.70	0.1	3.66	0.2
Benzo[e]pyrene	22.36	1.5	22.98	0.9	21.90	0.5	22.23	1.6	20.52	2.4	19.29	0.4	21.29	1.1	17.68	0.8	19.10	2.0
Benzo[a]pyrene	28.22	2.6	23.44	3.4	18.48	0.5	27.04	1.7	19.12	6.2	24.99	0.7	14.35	0.2	1.14	0.6	3.22	1.3
Indeno[1,2,3-cd]pyrene	5.20	2.0	3.83	0.2	3.35	0.1	2.68	2.1	3.30	0.5	3.14	0.2	4.00	0.2	2.74	0.2	3.02	0.3
Dibenzo[a,h]anthracene	14.38	9.4	18.81	0.8	18.74	1.3	18.20	1.5	16.33	2.2	17.03	1.9	19.52	1.2	13.62	0.7	15.16	1.3
Benzo[g,h,i]perylene	18.38	1.4	18.99	1.2	17.77	0.5	18.08	1.4	16.71	2.5	16.17	0.7	17.49	0.9	13.97	0.7	15.88	1.4
Perylene	11.37	4.9	5.93	5.2	7.32	0.2	8.497	1.0	6.912	1.77	7.693	0.5	5.196	0.6	1.5788	0.4	2.8537	0.5

WC= untreated control, WNT=nutrient-amended and WBN= biodiesel amended microcosms.

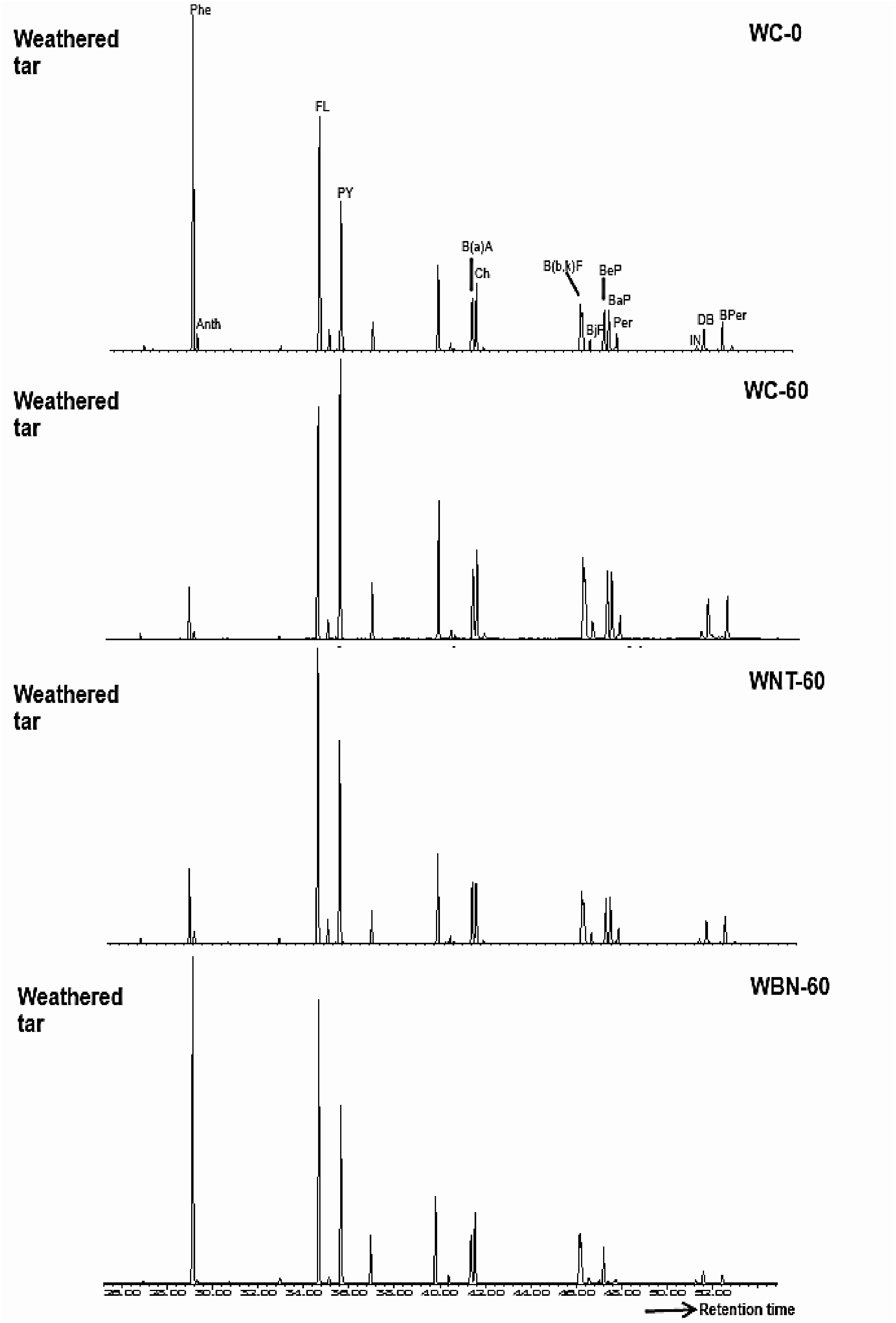


Figure 4.5. Summed ion mass chromatograms (m/z 178+202+228+252+276) of weathered coal tar profile at the start of the experiment (WC-0=untreated control at day 0), and for treatments at days 60 (WNT= Nutrient amended, WBN= Biodiesel amended microcosms);Phe, Phenanthrene; Anth, Anthracene; FL, Fluoranthene; PY, Pyrene; B(a)A, Benzo[a]anthracence; Ch, Chrysene; B(b,k)F, Benzo[b-,k]fluoranthene; BjF, Benzo[j]fluoranthene; BeP, Benzo[e]pyrene; BaP, Benzo[a]pyrene; Per, Perylene; IN, Indeno[1,2,3 c-d] pyrene; DB-Dibenzo[a,h]anthracene; BPer- Benzo[g,h,i]perylene.

A striking feature observed in the degradation of the three-ring PAH, phenanthrene (Phe), on amendment with biodiesel in both the crude oil and coal tar experiments, was that in the former, Phe was only reduced by 20% in the CBN samples compared to 91 and 94% decrease in the CC and CNT microcosms, respectively (Table 4.4). The methylphenanthrenes (MP) were also minimally degraded by between 11-20% compared to 55-83% decreases in the CC and 86-88% reductions in the CNT samples. In the same pattern, the coal tar experiments showed only 2% reduction for Phe in the WBN microcosms relative to 91 and 85% reduction in the WC and WNT treatments, respectively (Figure 4.5 and Table 4.6). The methylphenanthrenes (MP) were also less degraded by between <1%-29% compared to 67-83% decrease in the WC and 31-63% reduction in the WNT samples. However, anthracene (Anth) was completely degraded (100%) in the biodiesel amended samples for both crude oil and tar experiments (i.e. in CBN and WBN samples), while the rate of degradation was observed to be similar in the control (72%) and nutrient amended (64%) samples in both hydrocarbon pollutant experiments.

Following the complete degradation of anthracene (Anth) on amendment with biodiesel, benzo[a]pyrene was significantly depleted by 92% in the coal tar experiments as shown in Table 4.7 and Figure 4.6; and 52% in the crude oil microcosms (Table 4.5). Lower depletions were, however, seen for B[a]P in the WC (17%) and WNT samples (29%) of the coal tar experiments, while 41% reductions was observed for both treatments in the crude oil samples (CC and CNT). As also shown in Figure 4.6 and Table 4.7, perylene (Per) was degraded by 70% in the WBN experiments compared to the 19 and 48% reductions in the WNT and WC samples, respectively. In the crude oil samples, this compound was below detection limits.

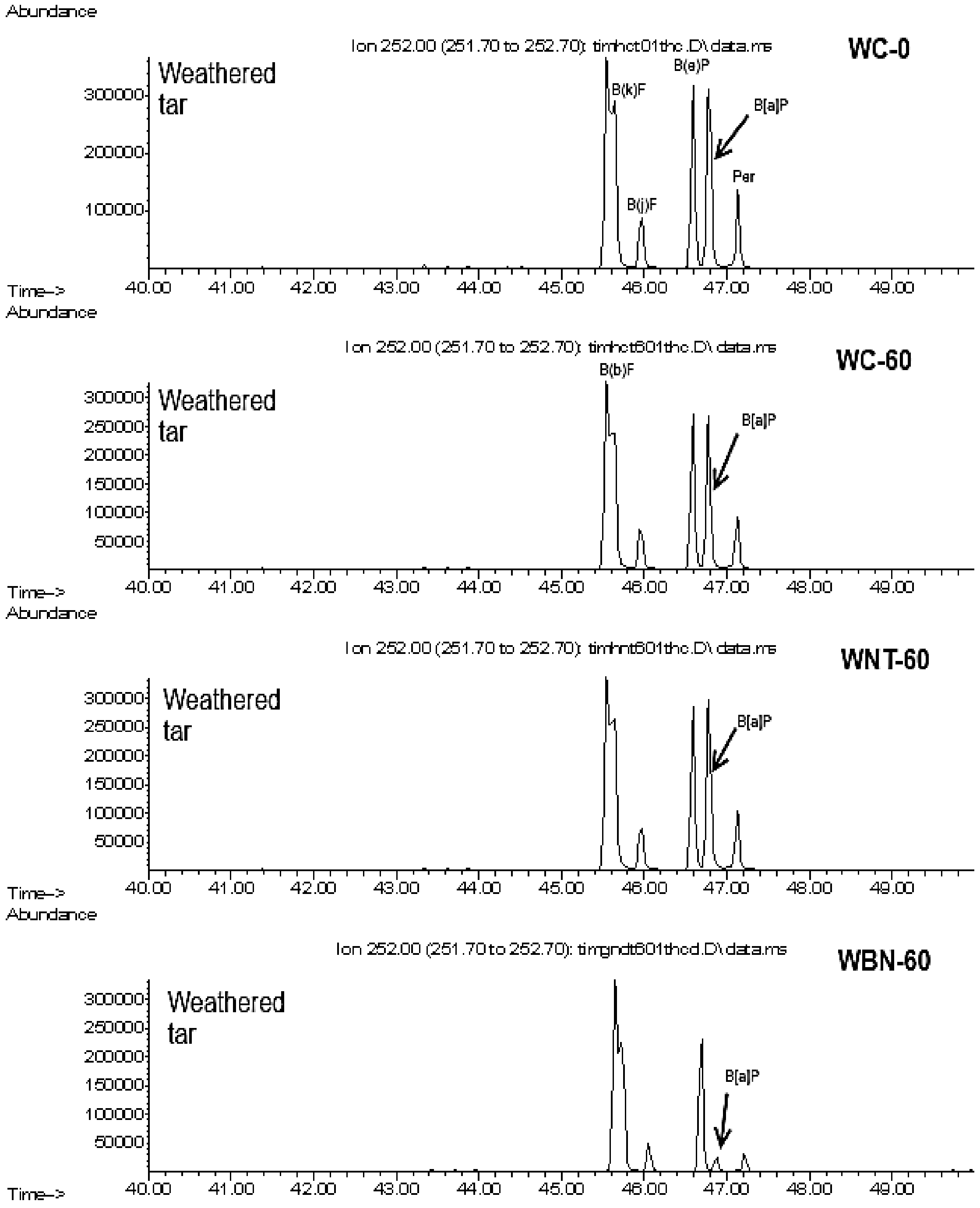


Figure 4.6. Total hydrocarbon fraction m/z 252 mass chromatograms showing the depletion of benzo[a]pyrene in biodiesel amended samples after 60 days (WBN-60) in weathered coal tar microcosms relative to the untreated control (WC-60) and nutrient-amended samples (WNT-60). The relative abundance of this compound at the start of the experiment was also shown in untreated control at day 0 (WC-0). BF, Benzo[b-,k-,j]fluoranthene; B(e)P, Benzo[e]pyrene; B(a)P, Benzo[a]pyrene; Per, Perylene

These results suggest that amendment with biodiesel increased the microbial degradation of the potent carcinogen benzo[a]pyrene (BaP) and anthracene (Anth) relative to other PAHs with the same numbers of rings. A previous study carried out by Collins *et al.* (1996) also reported an enhanced oxidation of BaP and Anth by lacasses (an extracellular ligninolytic enzyme secreted from white rot fungi *Trametes versicolor*) in the presence of the co-oxidant 2,2´-azinobis (3,ethylbenzthiazoline-6-sulfonate)

(ABTS) and a low molecular weight ultrafiltrate from the laccase-containing extracellular culture fluid. This was attributed to the low ionisation potentials of ≤7.45 eV exhibited by B[a]P and Anth and, the similarity in the arrangement of their fused benzene rings. A similar study by Dodor *et al.* (2004) with immobilised laccases also corroborated these findings and further revealed the ability of Tween 80 (an inorganic surfactant) to increase their bioavailability as well as stabilize free laccase enzymes for maximum activity. Collins *et al.* (1996) further reported that phenanthrene and fluorene were not oxidised by laccase enzymes alongside the oxidation of B[a]P and Anth, due to their high ionization potentials of >8.0 eV which, however, is in contrast with findings by Pickard *et al.* (1999) which showed degradation for Phe.

Interestingly, it has also been shown that ligninolytic metabolism induces inhibitory effects on the mineralisation of non-substrates of lignin peroxidases (LiP) such as Phe, while it enhances the rapid degradation of LiP substrates e.g. B[a]P and pyrene (Hammel *et al.*, 1992). Hammel *et al.* (1991) also demonstrated that the complete mineralisation of anthracene to carbon-dioxide by the fungus *Phanerochaete chrysosorium* can be mediated by ligninolytic enzymes including the LiPs. However, because PAHs are not utilised by fungi as sole sources of carbon, the presence of co-substrates are required for their metabolism (cometabolism) (Cerniglia, 1997). This also occurs for bacteria-mediated biodegradation of PAHs as it has been reported that improved degradation of B[a]P occurred on addition of Phe to bacterial isolates of *Burkholderia cepacia* (Juhasz *et al.*, 1997b), *Pseudomonas saccharophila* P15 (Chen and Aitken, 1999) and *Mycobacterium* sp. strain RJGII-135 (McLellan *et al.*, 2002); leading Juhasz and Naidu (2000) to propose that there possibly could be a link between B[a]P degradation and Phe. Also, relevant to the possible effect of biodiesel on this degradation pattern, Pizzul *et al.* (2007) indicated in their study that an increase from 0.1% of rapeseed oil to 1% inhibited the degradation of Phe and pyrene but enhanced the abiotic transformation of B[a]P.

In view of the above, it therefore could be explained that in the microcosm experiments in this current work, a consortium of bacteria and fungi mediated biodegradation processes were stimulated by the amendment of the hydrocarbon contaminated soil with rapeseed biodiesel, which resulted in the significant degradation of PAH B[a]P and Anth, preferentially to other 5 and 3 ringed PAH, respectively. The inhibitory effect on Phe could be attributed to the presence or activity of ligninolytic enzymes; a lag induced by high nitrogen conditions due to reduced utilisation of the added NPK fertiliser when the FAMEs were preferentially catabolised, or the quantity of biodiesel added to the soil microcosms. Although no genomic analysis was done, these possible effects were investigated to some extent and discussed in the later Chapters.

The degradation of the benzofluoranthenes in the weathered coal tar experiments was also enhanced by the addition of biodiesel. The sum of their concentrations after 60 days relative to their initial amounts showed 27% reduction compared to 0.7% and 7% decrease in the WC and WNT samples, respectively (Table 4.7). In the crude oil experiments, however, depletion was not influenced by amendment with nutrients or biodiesel as almost similar rates of disappearance were observed in all treatments (28%-CC; 25% -CNT; 22% CBN). The relative abundance of these compounds and their increased solubility when biodiesel was added to the tar experiments could probably have increased their biodegradation efficiency compared to the crude oil experiments where amounts were comparatively small (<0.3µg/g of dry soil) and may have included amounts contained in the un-spiked Nafferton soil (~0.03µg/g of dry soil, Appendix 5). It is also possible that the losses in the CC and CNT samples could be due to strong adsorption of these hydrophobic compounds within the soil matrix or organic matter (Morelli *et al.*, 2013). The reduction of benzo[e]pyrene (BeP) was also enhanced in WBN microcosms for coal tar experiments (17%) compared to the 8% decrease in WNT sample. No degradation of this compound was, however, observed in the WC microcosms. On the contrary, the crude oil experiments showed depletions for BeP in all the treatments but the reductions recorded for the amended samples were not significantly different (p=0.22) from the control (CC- 18%; CNT- 9%; CBN-16%).

Biodiesel amendment also enhanced the degradation of the other five and six-ring PAHs, indeno[*1,2,3-cd*]pyrene (IndPY), dibenzo[*a,h*]anthracene (DBAnth) and benzo[*g,h,i*]perylene (BPer), in both hydrocarbon pollutant experiments (Table 4.5, Table 4.7). In the crude oil experiments, these compounds were decreased by 29, 42 and 35%, respectively, in the CBN samples. No depletion of IndPY was noticed in the CC and CNT samples, while DBAnth and BPer were reduced by 11 and 9%, respectively, in CC and, 26 and 15%, respectively, in the CNT microcosms. As earlier mentioned, there is the possibility that some of these compounds measured were from the soil, owing to the relatively small amounts present in the crude oil (<0.15 µg/g of dry soil). The coal tar experiments showed significant decrease (p≤0.026) in IndPY, DBAnth and BPer by 31, 30 and 20%, respectively, in the WBN samples, compared to WC and WNT samples. A reduction of 26% was observed for IndPY in the WC samples, but no depletion was seen for DBAnth and BPer. The WNT microcosms did not show any depletion for IndPY as seen in the crude oil experiments, but 10 and 8% decreases, respectively, were noticed for DBAnth and BPer. Increased bioavailability of these highly recalcitrant PAHs on addition of biodiesel could be implicated for their comparatively higher depletions.

Some other 3-ring PAHs - acenaphthylene (Acy) and acenaphthene (Acn) were below detection limits in the crude oil treatments. Fluorene (Fl) was less depleted in the CBN (75%) microcosms compared to the CC (93%) and CNT (95%) samples. Dibenzothiophene (DBT) degradation was also significantly inhibited (7%; p=0.003) compared to 95 and 94% decreases observed in the CC and CNT samples (Table 4.4). A similar effect ($p \leq 0.021$, Table 4.6) was also noticed for Fl and DBT in the coal tar experiments. The former showed 70% reduction in the WBN samples compared to 98 and 86% decreases in WC and WNT microcosms (p=0.021), respectively; while the latter (DBT) revealed only 29% depletion in the WBN samples compared to 93 and 79% decrease in WC and WNT treatments (p=0.001). These similar effects seen in the degradation of Phe and DBT in all the treatments (inhibitory in the biodiesel amended samples and stimulatory in the control and nutrient amended microcosms) suggest that the same degradative enzymes or metabolic pathway could be involved in the depletion of these compounds. In line with this observation, a study carried out by Denome *et al.* (1993) with pure isolates of *Pseudomonas* strain C18 revealed a singular pathway (DOX) for the metabolism of naphthalene, DBT and Phe encoded in the obtained bacterial DNA.

Biodiesel and nutrient amendments did not also significantly enhance the degradation of the four- ring PAHs, fluoranthene (FL), pyrene (PY), benzo[*a*]anthracene- B(a)A and chrysene (Ch) in the crude oil experiments (Table 4.5). No significant difference ($p \leq 0.28$) was observed in their rate of disappearance in all treatments although comparatively higher concentrations were seen in the CBN microcosms compared to the control (CC), with the exception of B(a)A. It has been suggested that a lag phase can be induced for FL and Ch by competitive inhibition when they are present with other four ring PAHs and this delay in degradation could be elongated in the presence of Phe (Lotfabad and Gray (2002). It is also possible that the presence of biodiesel enhanced the solvent extractability of these compounds thus explaining the increased concentrations of these PAHs in the CBN samples. In the weathered coal tar experiments, no depletion was observed for FL and PY in the WBN microcosms. The effect of addition of nutrient was also negligible compared to the untreated control (WC). B(a)A was, however, most reduced in the CBN treatments by as much as 45% (p=0.001) compared to the WNT (12%) and the WC (22%) samples. The depletion of Ch was observed to a small extent in all treatments, although comparatively low in the amended microcosms (WNT- 7%; WBN-5%) compared to the WC samples (12%). The report by Taylor and Jones (2001) also showed less reductions for four ring PAHs in all treatments including the biodiesel amended samples, compared with the three ringed compounds, consistent with these findings.

After 180 days of incubation, the crude oil experiments revealed no further losses for the 2-ring and 3-ring PAHs in the CBN microcosms, showing that Phe depletion was significantly inhibited by the presence of biodiesel throughout the incubation period (Table 4.4, Appendix 3). Further decreases in concentrations, although to a lesser extent (10-20%), were noticed for DBT and the 4 and 5 ring PAHs but no depletion was observed for B[a]P. No further losses were also seen in the 6-ring PAHs. The CC and CNT samples showed a further 2% reduction for naphthalene, totalling an overall depletion of 98% after 180 days. No further losses were noticed for the other 2 and 3 ring PAHs except anthracene (Anth) which showed an overall 98 and 78% reductions in the CC and CNT samples, respectively, and the methylphenanthrenes (98%). The 4-6 ring PAHs also showed some losses which, however, reduced at increased molecular weight; (7-63% for CC and <1-27% in CNT samples).

In the weathered coal tar experiments (Table 4.6, Table 4.7 and Appendix 4), the WBN microcosms did not show any further reductions in the concentrations of the 2-6 ring PAHs except for biphenyl and fluorene which reduced by 21 and 4%, respectively, after 180 days incubation. In the WC samples, losses were observed for all the PAHs except perylene. The 2-ring PAHs were almost completely removed (i.e. by more than 98%). Acenaphthene and DBT were completely degraded and Phe was reduced by 99%. The 4-ring compounds were further reduced by 35-48% and the 5-ring PAHs exhibited further decreases of between 5-28%. The 6-ring PAHs only showed further reductions of between 1-7%. The reductions in the WNT samples after 180 day incubation were not significant (p>0.05) compared to the WC microcosms. Apart from the losses noticed in the 3-4 ring PAHs (Phe-93%, Acn- 100%, DBT-92%, FL- 58%), no appreciable decreases were seen in the in 5-6 ring PAHs, in fact B[a]P was not further reduced during this period of degradation.

4.3 Conclusions

Laboratory biodegradation experiments on crude oil and weathered tar using nutrient and biodiesel amendments showed that biodiesel enhanced the growth of microorganisms, capable of degrading PAHs, including the potent carcinogen, benzo[a]pyrene. Based on the visual observation of the fungal hyphae and moulds in the microcosms, and the fact that some fungi are known to possess suitable enzymes, it is likely that fungi played a part in the degradation of PAHs. The degradation process is likely to be a cometabolic process as FAMEs were preferentially degraded during the process which may, in turn, have induced the competitive inhibition for phenanthrene (Phe) in favour of anthracene (Anth) degradation. The complete degradation of Anth in both coal tar and crude oil treatments in the presence of biodiesel also suggests the activity of lignin peroxidases (LiPs) because Anth is known to be the simplest PAH that is a substrate of the LiPs. The selective removal of the high molecular weight PAHs especially the 6-ring PAHs may have occurred due to increased solubilisation of these PAHs by biodiesel. This can also be implicated in the apparent increases in concentrations observed for some of the 4-ring PAHs due to biodiesel amendment.

The degradation of *n*-alkanes was significantly enhanced by the addition of nutrients, a process which was achieved to a lesser extent in the presence of biodiesel due to the preferential degradation of the FAMEs. Nevertheless, the branched alkanes were rendered more susceptible to degradation in the presence of biodiesel compared to soil left to remediate themselves or amended with nutrients alone. The effect of nutrient amendment on PAH degradation was, however, negligible, showing that proper aeration and mixing can also enhance PAH removal by naturally occurring microflora existing in polluted soil via natural attenuation. Results obtained from the 180 day incubation experiments suggest that the active window of enhanced degradation for the biodiesel amended treatments seemed to have occurred within the first 60 days of incubation. This is likely due to the high degradability of the FAMEs and their almost complete utilisation as carbon sources within this period. The following Chapter will be investigating the potential of a stepwise amendment technique using biodiesel in degrading both the lower and higher molecular weight PAHs and, the effectiveness of biochar amendment in reducing residual pollutant concentrations in coal tar remediated soil microcosms.

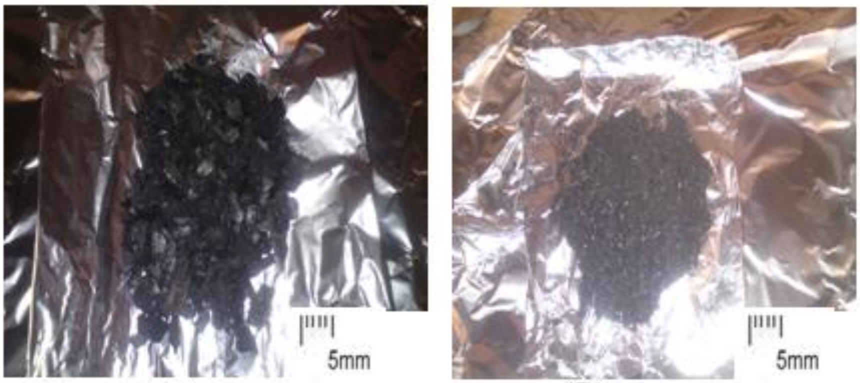
Chapter 5

DEGRADATION OF COAL TAR PAH IN SOIL USING SUCCESSIVE BIODIESEL AND BIOCHAR TREATMENTS

5.1 Introduction

This Chapter investigates the extent of degradation of the 16 EPA priority PAH pollutants in fresh, un-weathered coal tar by biodiesel amendment and the extent of removal of residual PAHs after an amendment with biochar (wood char). As seen in the previous Chapter, the degradation of weathered coal tar and crude oil showed enhanced depletion for the potent carcinogen, benzo[a]pyrene (BaP), complete degradation for anthracene (Anth) but inhibited depletion of phenanthrene (Phe) even up to a 180- day incubation period. The application of the same amendment techniques on fresh, un-weathered coal tar was therefore carried out in order to ascertain the repeatability of these bioremediation methods, especially for the biodiesel amendment technique, as well as its effectiveness on un-weathered coal tar. This formed the first of the 4 major experiments in the second phase (Phase II) of work which entailed using the same methodology described in Chapter 4. However, samples were incubated for only 90 days and analysis was done at 60-d and 90-d intervals (Figure 3.3). This was then followed by the 2nd set of major experiments whereby a 2-step amendment technique was employed for the biodiesel amended experiments as illustrated in Figure 3.4.

The 3rd major set of experiments was set up to evaluate the effectiveness of biochar amendment in reducing residual pollutant concentrations after 30 days of its application to coal tar remediated soil. In these experiments, biochar was added after 60 days of incubation to a separate set of each treatment (i.e. untreated control, nutrient and the biodiesel amended microcosms; Figure 3.3), while another set entailed the addition of biochar after 120 days, in the same manner (Figure 3.4). The concentrations of the 16 USEPA PAHs were then evaluated and compared to the un-amended samples at the end of the experiment i.e. after 90 and 150 days, respectively. The effect of the particle size of biochar on the reduction of PAH concentrations in these two separate experiments was also investigated. Figure 5.1 presents the two types of particle sizes used for the amendment technique. The first particle size (A) which is composed of small fragments >1mm was employed in the repeatability experiments, while the second (B) with particle size ≤ 1mm (obtained by grinding and passing biochar through a 1mm mesh size sieve) was added to the stepwise amendment experiments.



(A) Biochar (particle size > 1mm)

(B) Biochar (particle size ≤ 1mm)

Figure 5.1. The two types of biochar particle sizes used in the biochar amendment technique.

5.2 Results and Discussion

5.2.1 Microbial growth in biodiesel amended laboratory microcosms

A visual inspection of the microbial growth was again conducted for all experimental samples. While no visible growth was observed for the un-amended control (TC) and the nutrient-only (TNT) microcosms, growth appeared in all the biodiesel amended sets (TBN) but only after 2 weeks, unlike the weathered tar where growth was seen after 1 week. This variation may be due to a much longer adaptability period required by the native microflora in the presence of the hydrocarbon pollutant which in this case, is of higher toxicity and higher relative abundance in PAHs compared to the un-weathered coal tar. However, when visible growth occurred, it continued to be visible until the end of the experiment (90 days).

Figure 5.2 presents different durations from 1 week to 90 days of the observed growth pattern and increasing abundance. The growth formation was also similar to the earlier observed moulds in the Phase I experiments as discussed in Chapter 4. It was also evident that when soil was turned after 2 weeks in order to allow for adequate aeration, growth was enhanced with the appearance of dense white-green coloured microbes at the entire surface of the aerated soil after just a week’s interval, as shown in images of the biodiesel amended samples taken after 2 weeks, i.e. from week 3 to 90 days.

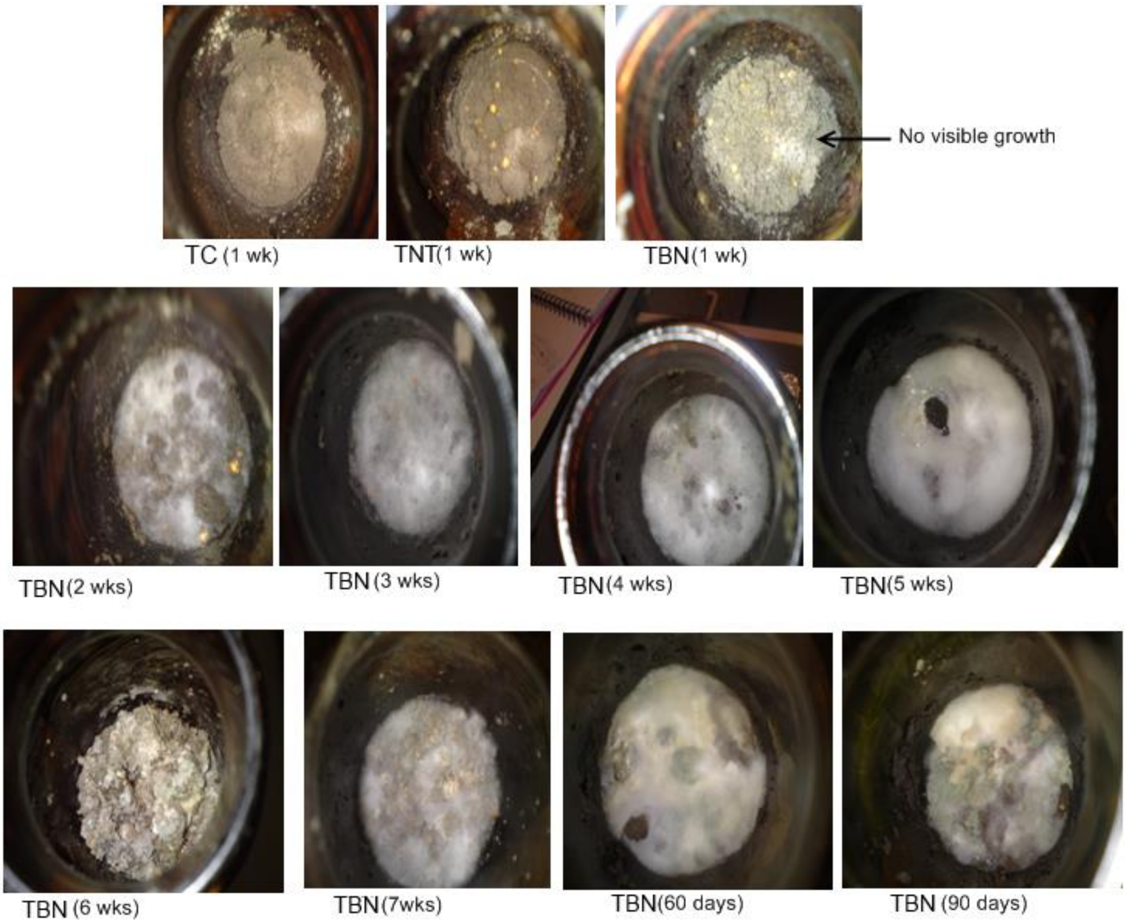


Figure 5.2. Visible microbial growth in biodiesel amended experiments from 2 weeks to 90 days incubation period; also showing no visible growth for all samples at 1 wk period. TC=un-amended control; TNT=nutrient amended; TBN=coal tar (un-weathered) spiked biodiesel amended samples.

5.2.2 Extractable organic matter (EOM) concentrations

After 60 days incubation, the EOM levels in the biodiesel amended (TBN) microcosms were reduced by 80% compared to 39 and 25% depletions displayed by the un-amended control (TC) and nutrient amended (TNT) microcosms, respectively, (Table 5.1, Appendix 6). This large percentage reduction, which is mainly attributed to the high degradability of the FAMEs of the biodiesel component, is very similar to the rate of degradation (81%, Table 4.1) seen previously for the weathered coal tar after 60 days of incubation with biodiesel.

After 90 days, the EOM levels in the TBN microcosms were reduced by 84% (a further 4%). The TC samples did not show further reductions (-3%), while TNT treatments were decreased by 54% (a further 19%). Table 5.1 presents the concentration levels of these various treatments for each incubation period considered which is also depicted graphically in Appendix 6.

Table 5.1. EOM values for un-weathered coal tar spiked soil

Hydrocarbon pollutant	Sample Codes	Extractable organic matter (EOM, mg/g)		
		Day 0	**Day 60**	**Day 90**
Un-weathered coal tar	TC	5.9 ± 1.0	3.6 ± 1.0	3.8 ± 0.1
	TNT	10.4 ± 1.6	6.8 ± 2.7	4.7 ± 0.9
	TBN	162.3 ± 15.0	32.9 ± 7.1	25.5 ± 5.9

Values are mean (n=3) ± SD. TC=un-amended control; TNT=nutrient amended; TBN=biodiesel amended samples.

5.2.3 Degradation of benzo[a]pyrene (BaP) and other PAHs by biodiesel amendment in un-weathered coal tar

After 60 days incubation, the 5-ring PAH, BaP was significantly (p=0.002) depleted by 81% in the TBN microcosms compared to the 26 and 34% depletion in the TC and TNT samples, respectively (Figure 5.3, Table 5.2). Anthracene (Anth) was also almost completely degraded in the presence of biodiesel by 97% relative to 44 and 31% decrease in the un-amended and nutrient microcosms, respectively (p=0.025).

Also consistent with previous data discussed in Chapter 4, phenanthrene (Phe) was only reduced by 3% in the TBN microcosms, while 80% depletion was seen in the TC samples (p=0.001, Figure 5.4). On the contrary, no depletion of Phe was observed in the TNT treatments unlike the weathered tar samples where 85% decrease was recorded after 60days, which was although not significant compared to the un-amended control that showed 91% decrease in concentration (p=0.066, Chapter 4). Since the removal of LMW PAHs was successfully achieved in the un-amended soil samples, it shows that the depletion of Phe was not enhanced by the addition of nutrients. Therefore this inhibitory effect on Phe degradation observed in these set of microcosms may have occurred due to a possible nutrient-induced microbial shift for the degradation of naphthalene which appeared to be the most dominant of the PAHs in the un-weathered coal tar pollutant. Its relative abundance (223 µg/g) is shown to be 8-fold more than the concentration in the weathered tar (28 µg/g) which was reduced by 97% after 60 days. Occurrences of nutrient-induced changes to microbial populations in hydrocarbon polluted sites during bioremediation have been reported by other researchers (MacNaughton *et al.*, 1999; Viñas *et al.*, 2005; Kim *et al.*, 2008).

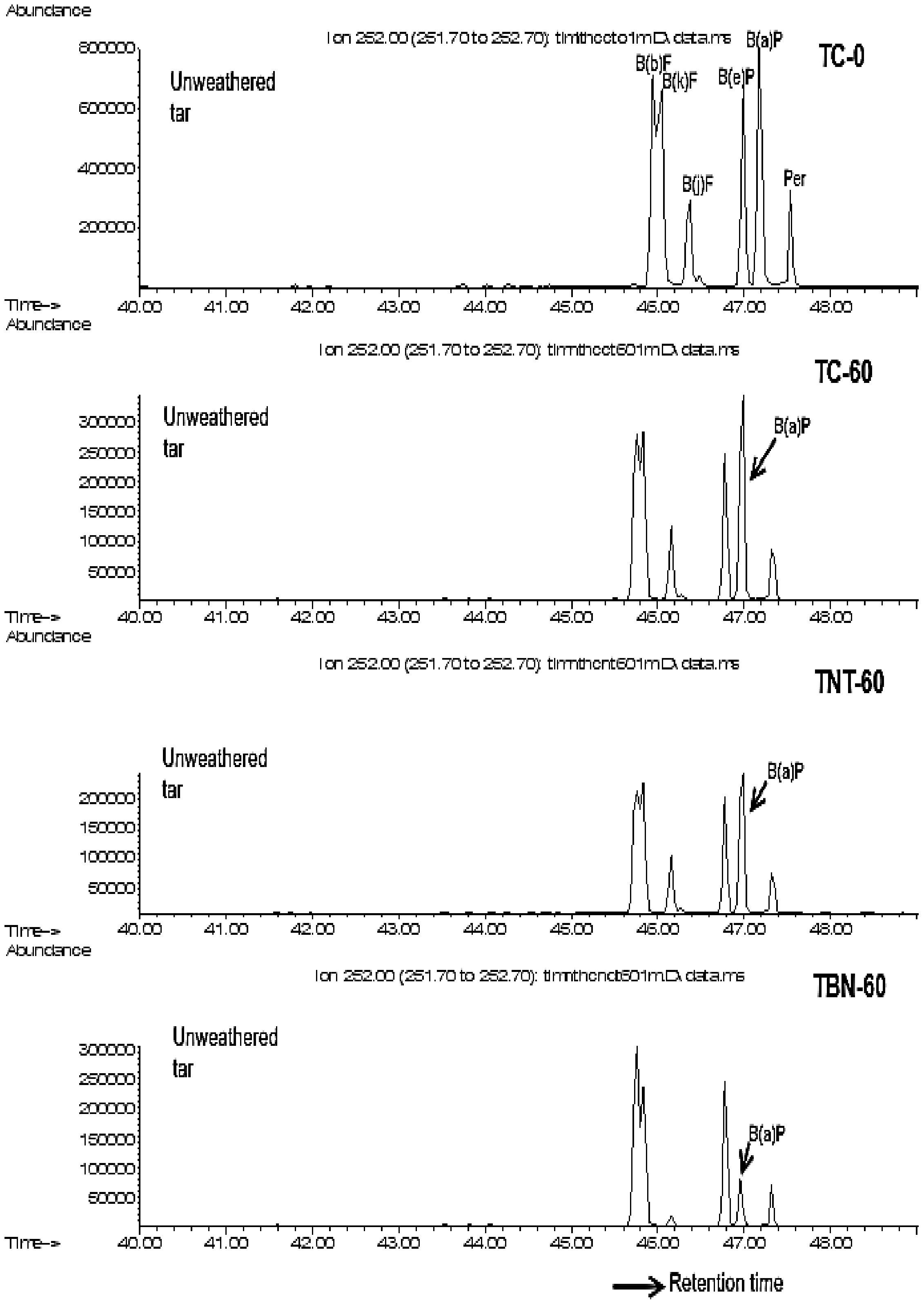


Figure 5.3. GC-MS chromatograms showing depletion of benzo[a] pyrene by biodiesel amendment in un-weathered tar (TBN) after 60 days incubation, relative to control (TC) and nutrient amended (TNT) experiments.

Table 5.2. Concentrations and standard deviations of the 16 EPA priority PAHs in the un-weathered coal tar spiked soil at days 0, 60 and 90

	Concentrations of PAHs (µg/g)																	
	TC						TNT						TBN					
	Day 0		**Day 60**		**Day 90**		**Day 0**		**Day 60**		**Day 90**		**Day 0**		**Day 60**		**Day 90**	
	Mean	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**
Naphthalene	299.50	51.7	1.99	1.1	1.43	0.4	222.69	6.3	6.57	2.2	2.00	0.4	21.87	13.4	44.53	10.5	9.68	14.6
Acenaphthylene	191.47	41.8	22.05	31.4	4.61	2.4	124.36	7.1	72.28	17.5	64.63	10.5	97.83	23.8	76.26	2.2	32.19	19.8
Acenaphthene	14.06	14.1	0.85	1.4	0.14	0.1	5.22	1.0	2.59	0.4	2.10	0.2	4.78	1.1	3.24	0.1	1.63	0.9
Fluorene	88.00	14.3	9.81	13.1	2.97	1.9	71.46	5.1	45.33	7.0	39.83	10.6	62.08	26.4	57.72	0.8	36.00	21.8
Phenanthrene	220.63	45.8	43.51	42.2	27.79	17.1	170.02	14.2	193.94	17.5	141.99	108.0	190.72	8.4	184.47	13.1	160.64	95.7
Anthracene	54.01	7.7	30.26	24.3	3.93	2.4	45.53	5.5	31.19	11.8	7.86	2.9	41.76	7.1	1.17	0.3	1.46	1.6
Fluoranthene	162.40	28.2	131.61	71.4	87.78	44.7	129.36	12.3	173.05	8.7	184.81	19.2	140.00	3.6	149.67	4.9	117.58	55.9
Pyrene	122.31	25.3	124.20	30.0	103.31	44.7	99.80	0.4	125.77	2.5	129.20	10.5	101.15	3.1	100.68	2.3	78.19	44.1
Benzo[a]anthracene	74.16	12.6	54.80	18.4	39.74	11.9	62.47	7.1	41.93	9.0	48.17	5.3	59.66	8.6	34.19	1.9	21.45	11.9
Chrysene	59.53	8.8	46.71	12.9	40.25	9.4	49.23	3.9	35.04	9.8	45.00	3.1	52.62	6.8	42.76	1.1	25.36	14.2
Benzo[b]fluoranthene	60.78	9.2	53.17	10.6	53.00	9.5	52.92	12.5	37.08	16.9	37.49	3.4	54.66	15.0	49.68	6.0	24.04	13.7
Benzo[k]fluoranthene	60.18	11.5	30.40	11.5	28.25	5.5	51.33	3.5	30.18	1.3	40.84	2.8	48.55	5.1	20.18	1.8	18.18	9.8
Benzo[a]pyrene	63.60	8.4	46.77	12.6	35.00	8.7	54.75	6.5	36.04	11.2	34.68	2.7	42.89	7.9	8.06	2.2	4.96	2.5
Indeno[1,2,3-cd]pyrene	10.03	3.7	5.59	1.5	5.78	1.0	8.77	3.4	4.98	1.6	5.23	1.0	7.91	2.3	3.81	0.3	2.20	1.1
Dibenzo[a,h]anthracene	42.11	8.7	27.35	6.3	26.50	4.1	36.16	5.9	23.75	5.4	25.31	5.0	34.23	6.3	20.77	2.0	12.16	5.8
Benzo[g,h,i]perylene	46.72	8.4	28.27	8.4	27.34	4.5	40.15	7.1	24.69	5.6	26.36	5.0	38.41	7.0	21.02	2.1	13.09	6.5

TC= untreated control, TNT=nutrient-amended and TBN= biodiesel amended microcosm.

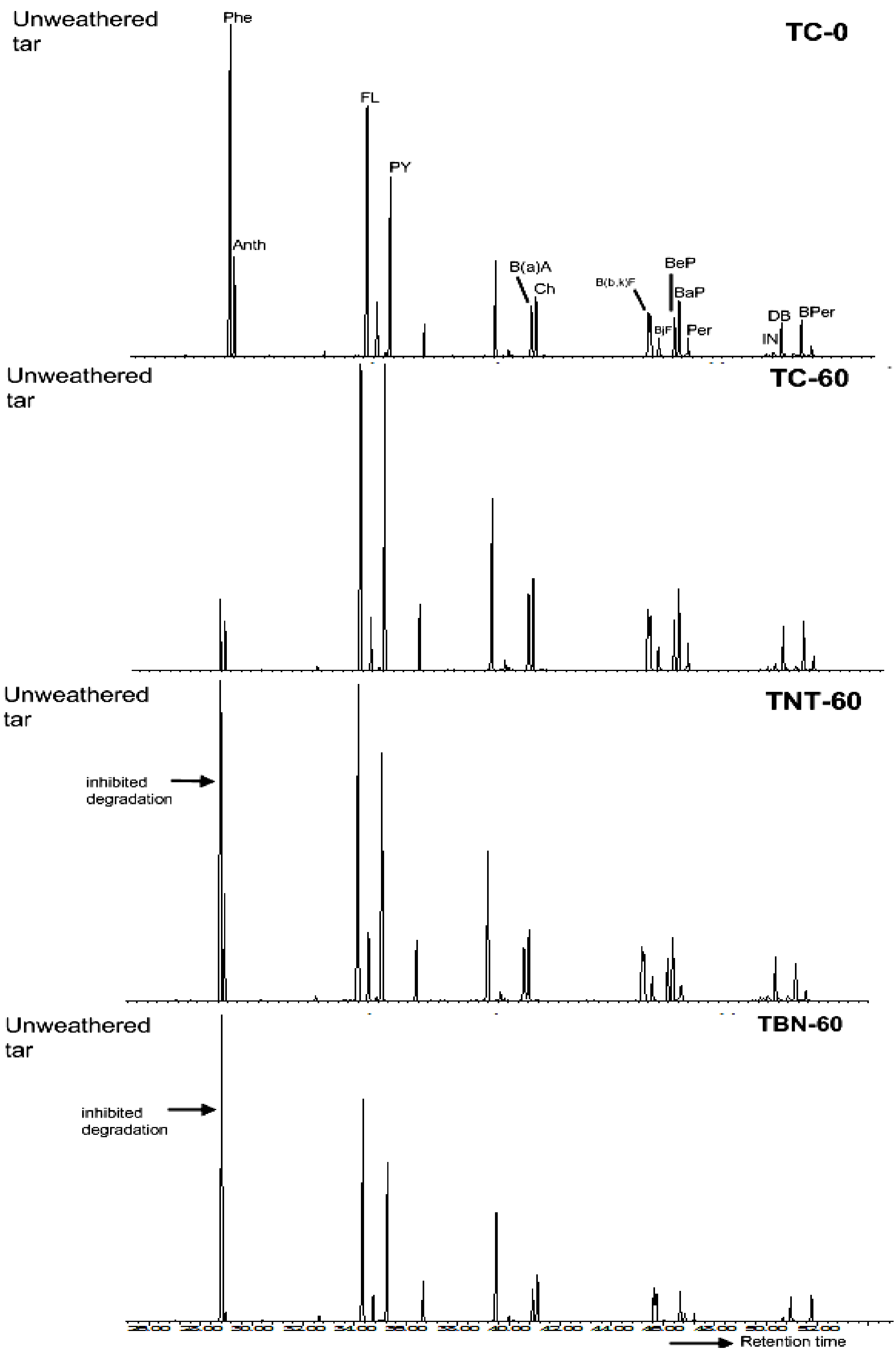


Figure 5.4. Summed ion mass chromatograms (m/z 178+202+228+252+276) of un-weathered coal tar profile at the start of the experiment (TC-0=untreated control at day 0), and for treatments at days 60 (TNT= Nutrient amended, TBN= Biodiesel amended microcosms); Phe, Phenanthrene; Anth, Anthracene; FL, Fluoranthene; PY, Pyrene; B(a)A, Benzo[a]anthracence; Ch, Chrysene; B(b,k)F, Benzo[b-,k]fluoranthene; BjF, Benzo[j]fluoranthene; BeP, Benzo[e]pyrene; BaP, Benzo[a]pyrene; Per, Perylene; IN, Indeno[1,2,3 c-d]pyrene; DB-Dibenzo[a,h]anthracene; BPer- Benzo[g,h,i]perylene.

The possibility of naphthalene toxicity could also have occurred in the nutrient amended treatments. A study carried out by Bouchez *et al.* (1995) reported the inhibitory effect of naphthalene (500 ppm) on the growth of the phenanthrene degrading *Pseudomonas* sp. strain, S Phe Na 1, an occurrence which was, however, relieved when naphthalene was consumed by the *Pseudomonas stutzeri* strain, S Nap Ka 1, that was later added to the medium. Stringfellow and Aitken (1995) also corroborated these findings in their work when they showed that naphthalene and other 2-ring PAHs, 2-methylnaphthalene and 1-methylnaphthalene, strongly interfered with Phe degradation. The inhibition of Phe degradation by naphthalene, even at very low concentrations (5 ppm), has also been reported by Shuttleworth and Cerniglia (1996).

In the TC samples, naphthalene was almost completely removed as the initial concentration was reduced by 99%. Since inhibition of Phe degradation was not observed in the TC samples, it could be inferred that the volatilisation and/or degradation of naphthalene may have occurred more rapidly compared to the nutrient amended microcosms, thereby preventing any form of inhibitory action on the degradation of other LMW PAHs. It is also possible that losses recorded for Phe in these un-amended samples may have also occurred by volatilisation (Kim *et al.*, 2001). In the TBN treatments, the mean concentration of naphthalene after 60 days incubation was, however, significantly ($p<0.01$) higher than the control and Phe inhibition was also observed. It suggests that losses of naphthalene by volatilisation or degradation may have been reduced by the presence of biodiesel, which in turn, inhibited the degradation of other LMW PAHs especially Phe, during the 60 day incubation period.

The results (Table 5.2, Appendix 7) also show that the amendment techniques did not positively affect the rate of degradation of other 3-ring PAHs. Acenaphthylene (Acy) and acenaphthene (Acn) showed significantly ($p \leq 0.02$) higher depletions in the TC samples (88 and 94%, respectively) compared to TNT (42 and 50%, respectively) and TBN microcosms (22 and 32%, respectively). Fluorene (Fl) was also significantly ($p=0.001$) reduced by 89% in the un-amended samples compared to 36 and 7% depletions observed in the nutrient and biodiesel amended microcosms, respectively. Although volatilisation is probably also contributory to the losses observed in the un-amended samples (Rogers *et al.*, 2002), the degrading activities of the indigenous microbes cannot be ruled out. It has been demonstrated previously that autochthonous microorganisms have high degradation potential for three ring PAHs (Fl and Phe) during natural attenuation, as reported for contaminated mangrove sediments (Yu *et al.*, 2005). Bacterial strains (*Sphingomonas* sp.) that are capable of growing solely on Ace and Acn as carbon sources, independent of fertiliser amendments, have also been isolated from former cokeworks sites (Hormisch *et al.*, 2000).

Also consistent with previous results discussed in Chapter 4, no degradation was observed for the 4-ring PAHs, fluoranthene (FL) and pyrene (PY) in the TBN and TNT microcosms. The TC samples showed depletion for FL (19%), while PY was not degraded as well (Table 5.2, Appendix 7). The decrease in benzo[*a*]anthracene – B[a]A and chrysene (Ch) in the treatments was also not significantly ($p>0.05$) different from the un-amended control although depletion of B(a)A was more in the TBN microcosms (43%) compared to TNT (32%) and TC (26%). Degradation of the other five ring PAHs, (benzofluoranthenes) was also not significant ($p>0.05$) in all the experiments, results showed an enhanced depletion of benzo[k]fluoranthene in the presence of biodiesel (58%) compared to the TNT (41%) and TC (49%) samples.

Biodiesel amendment also promoted the degradation of the other five and six-ring PAHs, indeno[*1,2,3-cd*]pyrene (IndPY; 52%), dibenzo[*a,h*]anthracene (DBAnth; 39%) and benzo[*g,h,i*]perylene (BPer; 45%) compared to TNT (43, 34 and 39%, respectively) and TC (44, 35 and 39%, respectively). According to these results, the rate of disappearance of these recalcitrant PAHs was unaffected by the addition of nutrients only. However, their bioavailability to degradation was increased by the addition of biodiesel as was reported in Chapter 4.

After 90 days of incubation, the percentage reductions for the 2-3 ring PAHs were still low in the amended experiments (TBN and TNT) compared to the un-amended control, with exception of naphthalene which had been almost completely removed after 60 days of incubation in the nutrient amended and control samples (Table 5.2, Appendix 7). Phe was reduced to 16% in the nutrient and biodiesel amended experiments, while 87% depletion occurred in the control. The 4-ring PAHs, FL and PY were still not degraded in the TNT samples. However, reductions occurred in the TBN samples by 16 and 23%, respectively, although not significant, compared to the TC experiments where compounds were degraded by 46 and 16%, respectively, ($p>0.05$).

The results (Table 5.2, Appendix 7) also showed that the degradation of the 5-6 ring PAHs were significantly ($p\leq0.03$) enhanced in the biodiesel amended samples compared to the control after 90 days. Benzo [b] and [k] fluoranthene were depleted by

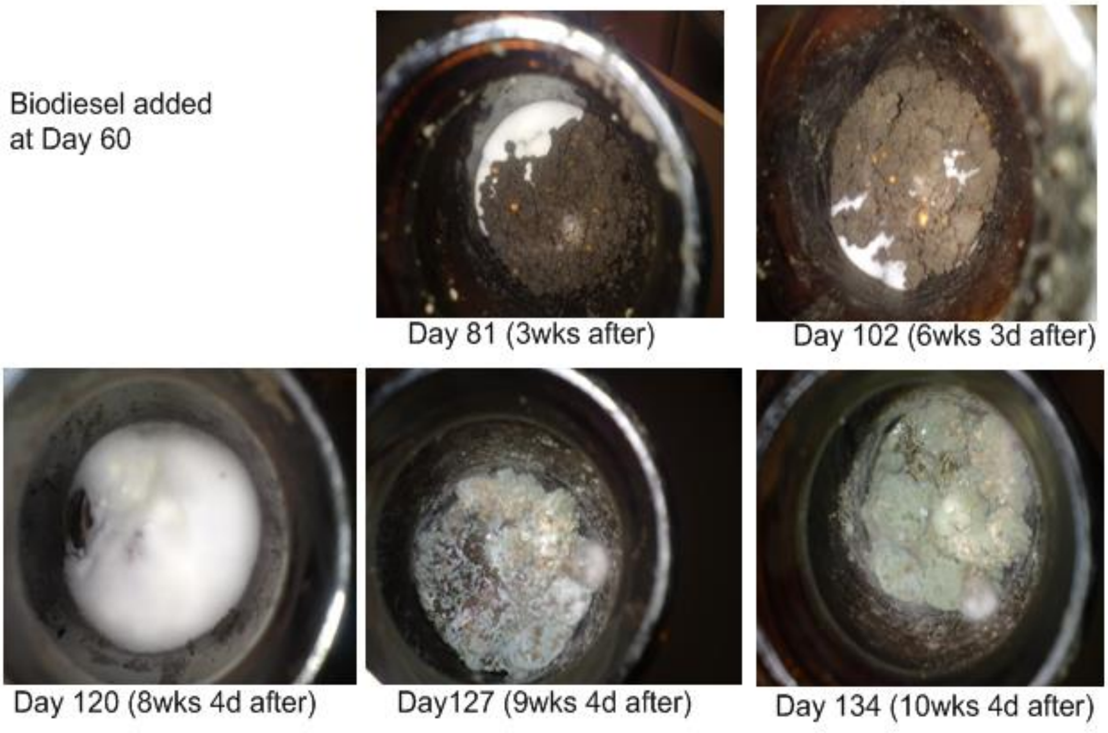
56 and 63%, respectively, compared to 13 and 53%, respectively, in the control. BaP was further reduced to 88% in the TBN microcosms, while 45% depletion was seen in the TC samples. IndPY, DBAnth and BPer were reduced by 72, 64 and 66%, respectively, compared to 35-44% depletions revealed in the TC microcosms. In the TNT samples no further reductions occurred for these recalcitrant PAHs apart from BaP which was further reduced by 2%.

5.2.4 Stepwise degradation of PAHs using biodiesel

As earlier mentioned, this experiment entailed the addition of biodiesel 60 days after initial amendment of the coal tar spiked soil with nutrients. Sampling was done after 60 days (the 120th day after initial spiking) and after a further 30 days (150th day). A visual inspection of the occurrence of visible microbial growth was conducted and the extent of reduction of the extractable organic matter (EOM) within these incubation periods was also investigated. Analysis of degradation of the priority PAHs was also carried out alongside the un-amended control and nutrient amended experiments.

5.2.4.1 Growth of microorganisms

On close inspection, microbial growth became visible in the biodiesel amended samples (TBN60) after 3 weeks of biodiesel application i.e. Day 81 (Figure 5.5 below).



Biodiesel added at Day 60

Day 81 (3wks after)

Day 102 (6wks 3d after)

Day 120 (8wks 4d after)

Day127 (9wks 4d after)

Day 134 (10wks 4d after)

Figure 5.5. Microbial growth on addition of biodiesel after 60 days of nutrient amendment of un-weathered coal tar spiked soil microcosms.

Growth (white-coloured biomass) appeared to concentrate at one side of the soil surface before gradually spreading across the entire soil after about 8 weeks. Growth continued with the appearance of dense green coloured microbes after 60 days of biodiesel addition (Day 120), as was observed for previous biodiesel experiments shown in Figure 5.2.

5.2.4.2 Analysis of EOM levels at the end of incubation periods

Sixty days after biodiesel addition, a decrease of 25% in EOM concentration was recorded for the amended microcosms (TBN60) i.e. day 120 samples (Table 5.3, Appendix 8). This was observed to be considerably lower compared to previous experiments where depletion was 80% (Table 5.1), when biodiesel was added at the start of the experiment and left for the same 60 days before treatment analysis. One possible reason for this could be that there was a prolonged adaptation of the indigenous microorganisms to the newly introduced carbon source (FAMEs) which is evident in the initial slow growth of microbes (Figure 5.5) Also, the slow degradability of biodiesel may have resulted from low nutrient availability. An earlier report by Gong *et al.* (2006) showed, through soil respiration curves, that nutrient supply and aeration were major determinants of the degradability of polyunsaturated fatty acids in sunflower oil, which was employed in the solubilisation and removal of PAHs.

Table 5.3. EOM values for un-weathered coal tar spiked soil

Hydrocarbon pollutant	**Sample Codes**	**Extractable organic matter (EOM, mg/g)**		
		Day 60	**Day 120**	**Day 150**
Un-weathered coal tar	TC	3.6 ± 1.0	3.9 ± 0.4	3.37 ± 0.3
	TNT	6.8 ± 2.7	5.14 ± 1.2	5.02 ± 0.1
	TBN60	189.7 ± 12.3	142.2 ± 98.9	125.7 ± 76.9

Values are mean (n=3) ± SD.

At the end of 150 days incubation period, a further 9% reduction was observed in the TBN60 microcosms which was, however, slightly more than the 4% decrease recorded after an additional 30 days (90 days incubation period) in earlier experiments (Table 5.1). For the TC samples, no further reduction was observed in their EOM concentrations during the 120 days incubation period. However, a 7% reduction was noticed after 150 days. The TNT treatments showed a further reduction of 24%, a rate almost similar to the TBN60 experiments mentioned above. A further 2% decrease was, however, recorded after 150 days (Table 5.3, Appendix 8).

5.2.4.3 Increased degradation of benzo[*a*]pyrene (BaP) in biodiesel amended microcosms by stepwise amendment

As shown in Figure 5.6 and Table 5.4, BaP was significantly ($p=0.007$) reduced by 98% in the TBN60 samples after 120 days following the stepwise amendment technique compared to the control (TC) and nutrient amended microcosms. The chromatograms of each of the triplicate samples (TBN60-120[1], TBN60-120[2], TBN60-120[3]; Figure 5.6) showed a consistently high degradation for this potent carcinogen when treatments were amended with biodiesel after 60 days of nutrient supplementation. The TC samples did not show any further reductions for BaP after the 45% decrease seen in the 90 day samples. However, a 50% decrease was revealed in the TNT microcosms after the 120 days of incubation.

After 150 days, i.e. at the end of the experiment, degradation of BaP further increased by 1% (i.e. a total of 99% decrease) for the TBN60 microcosms with 0.65ppm mean concentration, while no further degradation was observed in the TNT and TC samples and their final mean concentrations showed 42.86 µg/g and 39.53 µg/g, respectively, (Table 5.4).

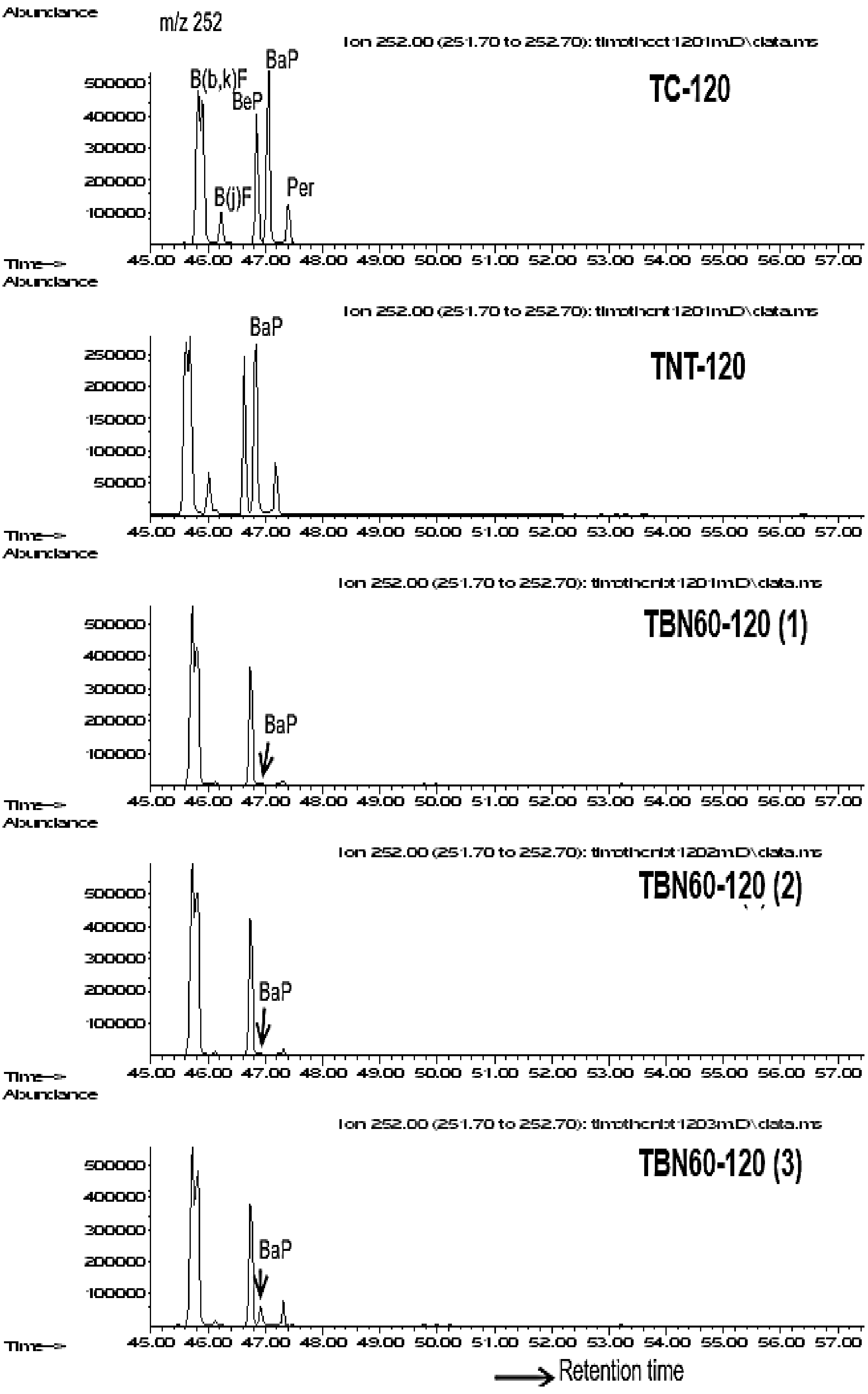


Figure 5.6. GC-MS chromatograms showing increased degradation of benzo(a)pyrene by stepwise amendment using biodiesel (TBN60) compared to the control (TC) and nutrient amended (TNT) microcosms at 120 day incubation period.

Table 5.4. Concentrations and standard deviations of the 16 EPA priority PAHs in the un-weathered coal tar spiked soil at days 0, 120 and 150

	Concentrations of PAHs (µg/g)																	
	TC						TNT						TBN		TBN60			
	Day 0		**Day 120**		**Day 150**		**Day 0**		**Day 120**		**Day 150**		**Day 0**		**Day 120**		**Day 150**	
	Mean	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**
Naphthalene	299.50	51.7	1.65	0.3	0.97	0.1	222.69	6.3	1.42	0.2	1.59	0.3	21.87	13.4	1.13	0.4	0.87	0.7
Acenaphthylene	191.47	41.8	8.20	1.6	3.28	0.2	124.36	7.1	31.95	6.0	39.75	14.5	97.83	23.8	5.65	4.4	6.42	4.6
Acenaphthene	14.06	14.1	0.25	0.0	0.04	0.0	5.22	1.0	1.38	0.3	1.50	0.2	4.78	1.1	0.53	0.3	0.53	0.3
Fluorene	88.00	14.3	4.20	0.6	0.68	0.1	71.46	5.1	13.40	8.2	20.72	10.3	62.08	26.4	11.62	6.7	11.23	6.9
Phenanthrene	220.63	45.8	29.75	5.0	5.23	0.6	170.02	14.2	47.71	22.3	60.17	30.7	190.72	8.4	93.68	78.0	118.93	82.2
Anthracene	54.01	7.7	1.99	0.7	4.80	0.6	45.53	5.5	4.66	2.3	19.05	7.7	41.76	7.1	0.00	0.0	0.00	0.0
Fluoranthene	162.40	28.2	94.06	28.1	45.65	2.0	129.36	12.3	199.62	9.0	227.43	6.6	140.00	3.6	124.20	67.2	135.12	61.9
Pyrene	122.31	25.3	90.10	17.0	60.11	5.3	99.80	0.4	139.56	1.7	168.85	5.3	101.15	3.1	70.09	36.5	94.43	48.6
Benzo[a]anthracene	74.16	12.6	41.73	9.0	31.04	1.0	62.47	7.1	33.70	2.6	53.32	1.9	59.66	8.6	13.55	8.0	13.48	7.6
Chrysene	59.53	8.8	45.20	9.3	32.18	0.9	49.23	3.9	32.87	2.1	47.76	1.7	52.62	6.8	32.62	15.2	24.11	12.5
Benzo[b]fluoranthene	60.78	9.2	57.79	18.8	53.81	2.0	52.92	12.5	38.22	2.7	57.46	1.3	54.66	15.0	29.51	14.5	25.47	15.3
Benzo[k]fluoranthene	60.18	11.5	36.85	3.1	26.54	2.2	51.33	3.5	20.74	2.4	30.47	1.1	48.55	5.1	21.48	10.4	14.89	5.7
Benzo[a]pyrene	63.60	8.4	45.45	10.0	39.53	1.4	54.75	6.5	27.31	3.1	42.86	2.3	42.89	7.9	1.07	1.7	0.65	0.5
Indeno[1,2,3-cd]pyrene	10.03	3.7	6.61	1.3	6.55	0.3	8.77	3.4	4.29	0.1	5.93	0.9	7.91	2.3	2.69	1.4	2.09	1.1
Dibenzo[a,h]anthracene	42.11	8.7	30.00	5.0	29.14	2.2	36.16	5.9	19.54	0.4	30.26	1.5	34.23	6.3	13.62	7.1	10.83	5.1
Benzo[g,h,i]perylene	46.72	8.4	31.07	5.0	31.53	2.6	40.15	7.1	21.21	0.7	33.25	0.8	38.41	7.0	14.23	7.5	12.48	6.2

TC= untreated control, TNT=nutrient-mended and TBN60= biodiesel amended (after 60 days of nutrient treatment) microcosms.

5.2.4.4 Improved degradation of phenanthrene in biodiesel amended microcosms by stepwise amendment

Contrary to previous experiments where inhibitory effects on phenanthrene (Phe) degradation was observed for biodiesel amended samples in all previous experiments, a 51% reduction in this 3-ring PAH was revealed in the TBN60 microcosms following the stepwise amendment technique. It therefore suggests that the degradation of Phe gradually began to increase concurrently with the nutrient-limited growth of fungal microbes on the FAMEs of biodiesel introduced after 60 days of incubation. There is also the possibility of the adaptability to Phe by the soil microorganisms after the first 60 days of pre-exposure to this compound which may have reduced the inhibition on Phe degradation, in agreement with the findings of Romero *et al.* (1998).

The earlier suggestion that Phe inhibition in the TNT samples resulted from a nutrient induced microbial shift for naphthalene degradation and/or naphthalene toxicity also seemed to be reinforced following the 72% degradation of Phe observed in these microcosms after 120 days of incubation (Table 5.4). It was observed in these experiments that as naphthalene concentration reduced to 2 µg/g (after 90 days incubation, Table 5.2), a degradation of 16% in Phe was noticed and, when concentrations were reduced to 1.42 µg/g, an increased degradation of 72% was realised (after 120 days incubation). Although these reductions in the amended samples were not significant ($p=0.3$) compared to the control (TC) experiments, no further reduction in Phe was observed in the TC microcosms at this incubation period, as reduction still remained at 87%, which was similar to the percentage decrease recorded after the 90 days incubation period in the control.

At the end of the experiment, Phe was almost completely removed in the TC samples as 98% degradation was recorded. No further reductions were, however, observed in the amended microcosms which goes to confirm the ability of indigenous microorganisms to degrade low molecular weight PAHs independent of any form of amendments.

5.2.4.5 Effects of the step wise amendment technique on other LMW and HMW PAHs in the biodiesel amended microcosms

Although the extent of degradation was not significant ($p>0.05$) compared to the control, high reductions of between 81-95% were observed in the TBN60 microcosms for the 2-3 ring PAHs naphthalene, acenaphthylene, acenaphthene and fluorene, following the stepwise amendment technique after 120 days of incubation (Table 5.4). Depletions of these compounds in the TNT and TC samples were between 74-99% and 95-99%, respectively. Anthracene also showed a complete removal of 100% degradation in the TBN60 microcosms compared to 90 and 96% losses in the TNT and TC samples, respectively.

The 4-ring PAH, benzo[*a*]anthracene – B(a)A, was also significantly (p=0.007) degraded by 77% in the TBN60 microcosms compared to the TNT (46%) and TC (44%) samples (Table 5.4) . Fluoranthene (FL), pyrene (PY) and chrysene (Ch) were, however, not significantly (p>0.05) depleted in the TBN60 microcosms compared to the control. Interestingly, FL and PY still remained un-degraded in the TNT samples even after 120 days of incubation. Reports by Lotfabad and Gray (2002) have shown that Phe can act as an inhibitor for FL and Ch degradation which can in turn inhibit the degradation of PY. This lag in degradation, that seems to be more pronounced in the TNT samples, may indicate the negative effect of competitive inhibition of substrates on their rates of degradation, especially for the more hydrophobic 4- ring PAHs.

The rate of degradation of the 5-ring PAHs, the benzofluoranthenes, were not significantly (p>0.05) different in the TBN60 microcosms compared to the control (Table 5.4). However, Figure 5.6 revealed an almost complete removal of benzo(j) fluoranthene in the TBN60 microcosms relative to the TNT and TC experiments. For the other 5 and 6-ring PAHs, the results showed significantly (p≤0.02) enhanced degradation of indeno[*1,2,3-cd*]pyrene (IndPY), dibenzo[*a,h*]anthracene (DBAnth) and benzo[*g,h,i*]perylene (BPer) for TBN60 microcosms (60-66%) compared to the control (29-34%). Depletions of these compounds were between 46-51% in the TNT treatments which were also lower compared to the biodiesel amended microcosms.

After 150 days of incubation, increased depletions were still seen for the 5-6 ring PAHs in the TBN60 microcosms compared to the other experiments (Table 5.4). The benzo (b)-, and (k)-, fluoranthene were degraded by 53 and 69%, respectively, relative to TNT (-9 and 41%, respectively, and TC samples (11 and 56%, respectively; p≤0.009). Degradation in the HMW PAHs, indeno[*1,2,3-cd*]pyrene (IndPY), dibenzo[*a,h*] anthracene (DBAnth) and benzo[*g,h,i*]perylene (BPer) were reduced by 68-74% in the TBN60 treatments compared to TNT (16-32%) and TC (31-35%) microcosms (p=0.001). The plot of the PAH concentrations for each treatment after the 120 and 150 days incubation periods is presented in Appendix 9.

5.2.5 Biochar amendment analysis

5.2.5.1 Biochar PAH content

The concentration of the 16 USEPA PAHs (1.93 µg/g) in the wood biochar is presented in Table 5.5. In comparison to other studies, PAH compositions and other chemical and physical properties vary for biochars produced even from the same feedstock, depending on the final pyrolysis temperature (Atkinson *et al.*, 2010; Gomez-Eyles *et al.*, 2011; Quilliam *et al.*, 2013). For the biochar used in this study, the three-ring PAH phenanthrene, showed to be the most abundant relative to the other PAHs, with a concentration of 0.44 µg/g.

Table 5.5. PAH concentrations in the biochar used for this study

PAH	Concentration (µg/g)
Naphthalene	0.27 ± 0.06
Acenaphthylene	0.32 ± 0.02
Acenaphthene	0.06 ± 0.00
Fluorene	0.17 ± 0.01
Phenanthrene	0.44 ± 0.04
Anthracene	0.05 ± 0.01
Fluoranthene	0.23 ± 0.02
Pyrene	0.24 ± 0.01
Benzo(a)anthracene	0.03 ± 0.00
Chrysene	0.03 ± 0.00
Benzo[b]fluoranthene	0.03 ± 0.00
Benzo[k}fluoranthene	0.00 ± 0.00
Benzo[a]pyrene	0.02 ± 0.00
Indeno[1,2,3,-cd]pyrene	0.00 ± 0.00
Dibenzo[a,h]anthracene	0.01 ± 0.00
Benzo[g,h,i]perylene	0.02 ± 0.00
Total 16 USEPA PAHS	**1.93 ± 0.16**

Values are mean (n=3) ± SD.

5.2.6 Microbial growth in biodiesel and biochar amended microcosms

In the 30 days of biochar amendment, microbial growth in the biodiesel amended samples visually appeared unaffected. As shown in Figure 5.7, after 7 days of biochar addition visible signs of microbial (mostly fungal) growth appeared in the biodiesel treatments. The microbial growth observed in these treatments occurred due to the utilization of biodiesel as a co-substrate and not primarily due to biochar addition. This was deduced from the biochar control experiments consisting of soil plus biochar only which showed no visible microbial growth of such throughout the 30 day period of incubation. The observation that biochar did not inhibit the growth of these existing microorganisms in the biodiesel amended microcosms increases its potential for use as a remediation tool alongside biodiesel amendment. Studies that show increases in microbial biomass and efficiency on addition of biochar have also been well reported (Atkinson *et al.*, 2010; Lehmann *et al.*, 2011). Recent studies by Sopeña and Bending (2013) also reveal the potential of biochar amendment in protecting the fungal community structure after treating soil with fungicides (azoxystrobin).

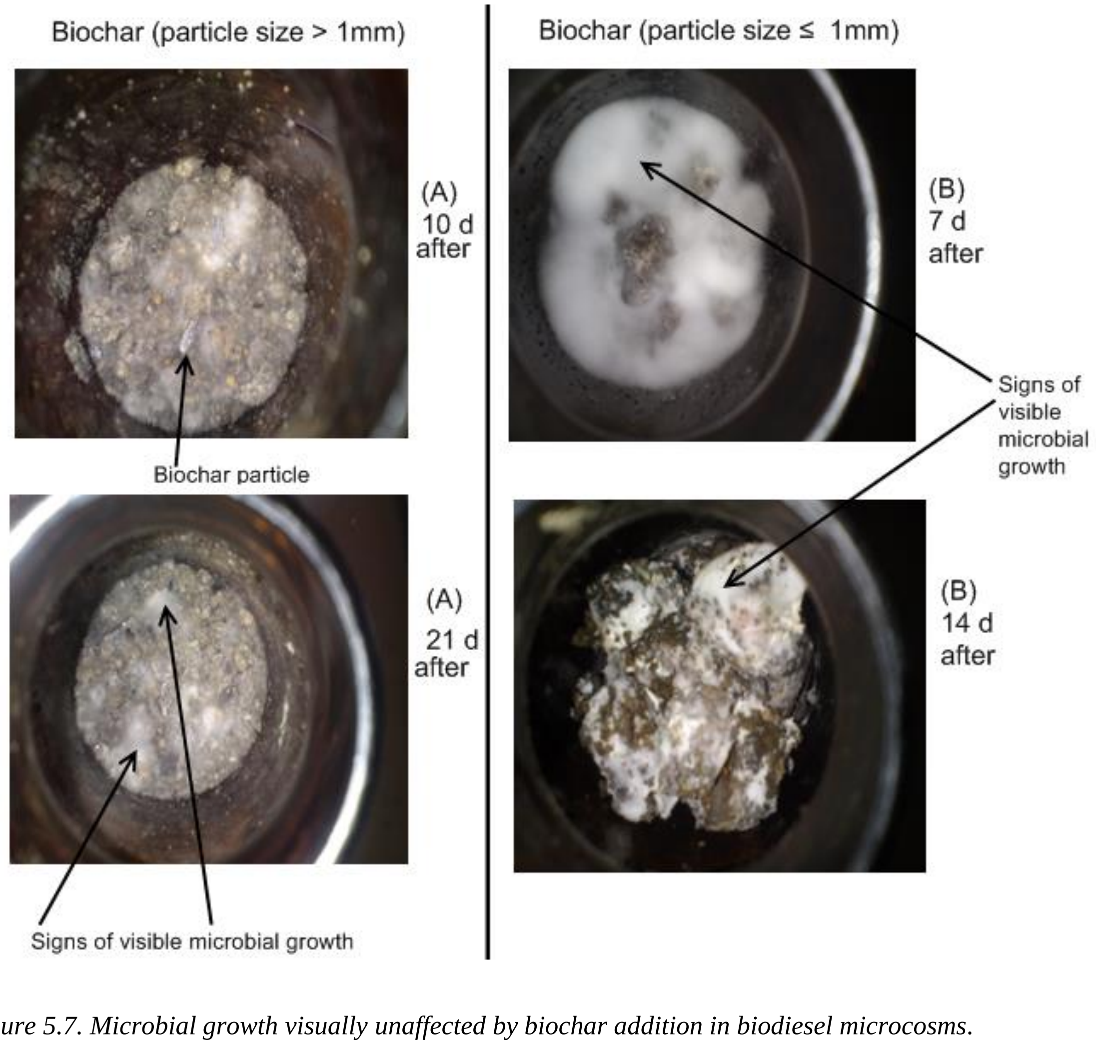


Figure 5.7. Microbial growth visually unaffected by biochar addition in biodiesel microcosms.

5.2.7 Effect of biochar amendment on PAH concentrations

From the GC-MS results (Table 5.6, Table 5.7), no statistically significant differences in PAH concentrations (16 USEPA PAHs; $p>0.05$) were observed between the biochar and non- biochar amended experiments for all three distinct categories (the control, nutrient only and biodiesel amended samples). However, there were clear differences between the results obtained from the use of the two different biochar particle sizes. Higher PAH concentrations, (although not significantly higher, $p>0.05$) were obtained for all the experiments amended with biochar particle size >1mm, than were recorded for all microcosms amended with particle size ≤ 1mm relative to their non-biochar amended categories as shown graphically in Appendix 10.

After 30 days of biochar amendment with particle size >1mm, the biodiesel-amended microcosms (TBN-B) had 48% increased levels in the total 16 USEPA PAHs compared to their non biochar amended samples (TBN), while 19% increase was observed for the nutrient amended experiments (TNT-B) relative to their non-biochar experiments (TNT) (Table 5.6, Appendix 10). In all of these experiments, the PAH groups (2-6 ringed) showed increased concentrations in the biochar amended samples. The relatively higher concentrations noticed in the nutrient treated samples is obviously due to the high amounts of the 3-6 ring PAHs in these samples from their slow biodegradation and also a probable occurrence of relatively increased solvent extractability of these compounds due to the presence of waxy materials in these samples. Increased PAH levels (although less than 1%) were also observed for the untreated control amended with biochar (TC-B) relative to the non-biochar amended category (TC). However, results also showed that the 3 and 6-ring PAHs were reduced by 28 and 1% ($p>0.05$), respectively, in the TC-B experiments.

This relative increase in PAH concentrations observed for the biochar amended experiments compared to the non-amended categories for particle size >1mm suggests two possibilities: (1) the inhibitory effect of the larger biochar particle size distribution, and consequent reduced surface area on its sorption affinity for PAHs and (2) a likely alteration of microbial degradation activity due to a strong adhesion to the soil's degrading microorganisms by its large hydrophobic surfaces. In their study, Zheng *et al.* (2010) showed that biochar of larger particle sizes (greater 0.075 mm) required a longer time for pollutants to reach its microporous region compared to lesser particle sizes. With regards to possible inhibitory activity by biochar, Lehmann and Rondon (2006) also reviewed that the type and amount of biochar added to a soil can affect the microbial composition, population, diversity and activity and that biochar pore geometry and size could also determine the level of growth of certain soil microbial population. There is also the likelihood of PAHs being introduced to the soil on addition of biochar, although in small amounts, as shown in the data presented in Table 5.5.

However, due to the effect of enhanced solubilisation by biodiesel, there is the possibility of increased amounts of PAHs from the biochar which may be the reason for the 48% rise in concentrations in the biochar amended samples compared to the non-biochar category.

The results (Table 5.7) in this study also showed that the amendment with biochar of particle size ≤ 1mm resulted in a 7% reduction (although not significant, $p>0.05$) in the priority PAH groups (2-6 ringed compounds) for the nutrient and biodiesel microcosms (TNT-B and TBN60-B, respectively) compared to their non-biochar experiments (TNT and TBN60, respectively), while a 4% decrease ($p>0.5$) was also observed for the biochar amended controls (TC-B) compared to their non-biochar category (TC). The 2-6 ring PAHs in TBN60-B microcosms were reduced by 4-16% ($p>0.5$), while decrease was seen only for 3-5 ring compounds in TNT-B experiments. The biochar amended control (TC-B) also only showed a 6-7% ($p>0.5$) reduction in the 4-6 ring PAHs.

Despite the lack of statistically significant differences between the biochar and non-biochar amended treatments, the observation of reductions in the PAH groups of the 16 USEPA pollutants in soil amended with finer particles of this carbonaceous material, shows the potential of biochar in soil amendment techniques. It is necessary to point out that the use of solvent extraction in determining the total PAHs in soil before and after biochar treatments, as also employed in similar studies (e.g. Beesley *et al.*, 2010; Gomez-Eyles *et al.*, 2011) may have some impact on the results obtained as there is the likelihood of desorption of the PAHs sorbed to the surfaces of the biochar during extraction, which would also be measured in the analytical protocol. Another factor that could be considered in later work is the contact time or incubation period as the 30-d period chosen for this study did not seem to yield any statistically significant differences in reductions where they occurred. Increased biochar-soil contact time has been reported by other researchers (Beesley *et al.*, 2010; Cao *et al.*, 2011; Oleszczuk *et al.*, 2012) to result in much reduced pollutant concentrations.

Table 5.6. Concentrations and standard deviations of the 16 EPA priority PAHs and summed PAHs in the un-weathered coal tar spiked soil at 90 days incubation

	Concentrations of PAHs (µg/g)											
	Day 90				**Day 90**				**Day 90**			
	TC		**TC-B**		**TNT**		**TNT-B**		**TBN**		**TBN-B**	
	Mean	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**
Naphthalene	1.43	0.4	2.02	0.7	2.00	0.4	2.93	1.0	9.68	14.6	32.83	2.4
Acenaphthylene	4.61	2.4	4.16	2.6	64.63	10.5	77.86	15.6	32.19	19.8	72.56	20.4
Acenaphthene	0.14	0.1	0.20	0.2	2.10	0.2	2.61	0.5	1.63	0.9	2.96	0.6
Fluorene	2.97	1.9	2.40	2.2	39.83	10.6	47.91	10.2	36.00	21.8	52.71	17.6
Phenanthrene	27.79	17.1	18.84	12.4	141.99	108.0	186.42	91.9	160.64	95.7	203.01	41.9
Anthracene	3.93	2.4	2.72	2.0	7.86	2.9	7.84	2.1	1.46	1.6	1.75	1.2
Fluoranthene	87.78	44.7	84.54	47.4	184.81	19.2	207.08	42.1	117.58	55.9	149.96	12.7
Pyrene	103.31	44.7	112.14	35.9	129.20	10.5	152.19	25.9	78.19	44.1	93.99	7.3
Benzo[a]anthracene	39.74	11.9	39.77	12.0	48.17	5.3	56.53	9.1	21.45	11.9	36.38	4.3
Chrysene	40.25	9.4	40.47	8.8	45.00	3.1	53.96	7.3	25.36	14.2	47.48	7.6
Benzo[b]fluoranthene	53.00	9.5	52.88	17.2	37.49	3.4	50.14	7.2	24.04	13.7	48.00	12.6
Benzo[k]fluoranthene	28.25	5.5	32.50	3.2	40.84	2.8	43.54	14.8	18.18	9.8	26.11	6.3
Benzo[a]pyrene	35.00	8.7	37.93	12.2	34.68	2.7	40.00	8.5	4.96	2.5	11.38	5.1
Indeno[1,2,3-cd]pyrene	5.78	1.0	5.58	1.2	5.23	1.0	5.76	0.8	2.20	1.1	4.03	0.5
Dibenzo[a,h]anthracene	26.50	4.1	26.63	4.6	25.31	5.0	29.23	4.1	12.16	5.8	22.21	2.5
Benzo[g,h,i]perylene	27.34	4.5	26.92	4.6	26.36	5.0	30.77	4.9	13.09	6.5	22.10	1.7
sum of 2-ring PAHs	1.43	0.40	2.02	0.71	2.00	0.43	2.93	0.98	9.68	14.61	32.83	2.41
sum of 3-ring PAHs	39.44	23.8	28.32	19.2	256.41	129.3	322.64	114.3	231.93	131.9	332.99	79.7
sum of 4-ring PAHs	271.08	107.0	276.92	103.9	407.18	37.4	469.76	83.2	242.58	121.3	327.81	29.2
sum of 5-ring PAHs	116.25	22.4	123.30	25.9	113.02	7.9	133.68	20.4	47.19	25.0	85.49	15.3
sum of 6-ring PAHs	59.62	9.5	59.13	10.4	56.90	11.0	65.76	9.3	27.45	13.5	48.34	4.6
∑PAH	487.83	161.2	489.69	147.6	835.51	180.8	994.77	216.7	558.83	295.6	827.47	129.8

TC= untreated control, TNT=nutrient-amended and TBN= biodiesel amended microcosms; TC-B, TNT-B and TBN-B are biochar amended samples of the control, nutrient-amended and biodiesel-amended microcosms, respectively.

Table 5.7. Concentrations and standard deviations of the 16 EPA priority PAHs and summed PAHs in the un-weathered coal tar spiked soil at 150 days incubation

	Concentrations of PAHs (µg/g)											
	Day 150				**Day 150**				**Day 150**			
	TC		**TC-B**		**TNT**		**TNT-B**		**TBN60**		**TBN60-B**	
	Mean	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**	**Mean**	**S.d**
Naphthalene	0.97	0.1	1.89	0.3	1.59	0.3	2.48	0.2	0.87	0.7	0.73	0.7
Acenaphthylene	3.28	0.2	3.80	0.7	39.75	14.5	40.21	12.0	6.42	4.6	3.11	2.0
Acenaphthene	0.04	0.0	0.13	0.0	1.50	0.2	1.38	0.3	0.53	0.3	0.34	0.2
Fluorene	0.68	0.1	1.36	0.7	20.72	10.3	16.71	9.6	11.23	6.9	9.06	7.2
Phenanthrene	5.23	0.6	9.56	4.4	60.17	30.7	50.94	23.4	118.93	82.2	106.44	90.8
Anthracene	4.80	0.6	4.34	0.4	19.05	7.7	14.31	5.1	0.00	0.0	0.00	0.0
Fluoranthene	45.65	2.0	45.42	5.1	227.43	6.6	206.16	15.4	135.12	61.9	135.20	64.8
Pyrene	60.11	5.3	56.01	6.0	168.85	5.3	149.99	8.1	94.43	48.6	84.95	42.1
Benzo[a]anthracene	31.04	1.0	28.31	1.3	53.32	1.9	53.15	4.4	13.48	7.6	13.58	7.1
Chrysene	32.18	0.9	29.48	1.4	47.76	1.7	48.53	4.6	24.11	12.5	22.28	11.4
Benzo[b]fluoranthene	53.81	2.0	44.81	8.1	57.46	1.3	56.68	2.9	25.47	15.3	24.01	12.0
Benzo[k]fluoranthene	26.54	2.2	30.43	9.0	30.47	1.1	30.30	2.4	14.89	5.7	13.00	6.7
Benzo[a]pyrene	39.53	1.4	35.96	4.1	42.86	2.3	41.01	3.6	0.65	0.5	0.92	0.5
Indeno[1,2,3-cd]pyrene	6.55	0.3	6.21	0.2	5.93	0.9	6.84	0.5	2.09	1.1	1.86	0.9
Dibenzo[a,h]anthracene	29.14	2.2	27.06	0.8	30.26	1.5	30.77	2.3	10.83	5.1	10.09	4.9
Benzo[g,h,i]perylene	31.53	2.6	29.79	0.8	33.25	0.8	33.15	1.9	12.48	6.2	11.27	5.6
sum of 2-ring PAHs	0.97	0.1	1.89	0.3	1.59	0.3	2.48	0.2	0.87	0.7	0.73	0.7
sum of 3-ring PAHs	14.03	1.1	19.19	6.0	141.19	51.9	123.55	49.4	137.11	93.5	118.94	98.5
sum of 4-ring PAHs	168.99	5.2	159.22	6.5	497.37	10.1	457.83	27.1	267.15	130.4	256.02	124.8
sum of 5-ring PAHs	119.88	5.1	111.21	6.7	130.80	3.7	127.99	8.9	41.01	21.4	37.93	18.9
sum of 6-ring PAHs	67.22	5.1	63.06	1.8	69.44	3.0	70.75	4.6	25.41	12.4	23.22	11.4
∑PAH	371.09	14.8	354.56	5.3	840.39	51.6	782.61	86.8	471.56	238.6	436.84	252.1

TC= untreated control, TNT=nutrient-amended and TBN60= biodiesel amended microcosms after 60 days of nutrient treatment; TC-B, TNT-B and TBN60-B are biochar amended samples of the control, nutrient-amended and biodiesel-amended microcosms, respectively.

5.3 Conclusions

The application of nutrient and biodiesel amendment techniques on the biodegradation of un-weathered tar using nutrient and biodiesel confirmed that biodiesel amendment increased the bioavailability and cometabolic degradation of high molecular weight PAHs especially BaP and other 5 and 6 ring PAHs. Results from the laboratory biodegradation experiments also showed significant depletion of the potent carcinogen, benzo[a]pyrene (BaP), the complete degradation of the 3-ring PAH anthracene (Anth) and the competitive inhibition of phenanthrene (Phe) as was recorded in the crude oil and weathered coal tar experiments in Chapter 4. The application of the stepwise amendment technique where treatments were amended with biodiesel as a successive step after nutrient supplementation, however, relieved the inhibitory effect on Phe as more that 50% reduction was realised for phenanthrene in the biodiesel treated microcosms after 60 days of biodiesel amendment. The inhibition of phenanthrene degradation was possibly due to the relative abundance of naphthalene which had the potential of inducing a toxicity effect which may have been accelerated by the presence of nutrients in causing a microbial shift for naphthalene degraders preferentially to phenanthrene.

Again, the removal of the low molecular weight PAHs (2-3 rings) was better achieved by natural attenuation as degradation was not significantly enhanced by the nutrient and biodiesel amendment techniques. Also, the degradation of the high molecular weight (4-6 ringed) coal tar PAHs was not significantly enhanced by the addition of nutrient alone in these coal tar experiments, resulting in much higher concentrations relative to the other treatments. Biochar amendment did not significantly reduce residual pollutant concentrations in this study. However, the non-interference of biochar in the visible growth of fungal hyphae and moulds in the biodiesel treated microcosms and the observed slight reductions in the PAH groups of the 16 USEPA pollutants in the soil amended with finer particles of biochar, compared to the non-treated microcosms, show the potential of biochar amendment in bioremediation studies. The contact/incubation time, however, may also be crucial in determining the extent of reduction of residual PAHs following biochar amendment. Chapter 6 focuses on the toxicity assessment of the bioremediated soil using the phosphatase method and Microtox bioassay especially for the biodiesel amended microcosms that have shown significant reductions in the potent carcinogen- benzo[a]pyrene.

Chapter 6

TOXICITY EVALUATION OF THE BIODIESEL AMENDMENT TECHNIQUE FOLLOWING THE REMEDIATION OF COAL TAR PAHs

6.1 Introduction

This Chapter investigates the toxicity of the coal tar contaminated soil following the application of PAH bioavailability amendments during bioremediation. Here, the results of the 4th major set of experiments (last of the Phase II experiments) involving the toxicity analyses of the coal tar remediated soil from the repeatability and stepwise amendment experiments are presented and discussed. Soil phosphatase activity was employed as the biological indicator of soil quality and ecological health and was evaluated by the amount of *p*-nitrophenol released when the soil samples were incubated with sodium *p*-nitrophenylphosphate (Tabatabai and Bremner, 1969). The more sensitive Microtox^{Tm} bioluminescence assay was also applied to selected sample sets for data robustness and broader results interpretation (Płaza *et al.*, 2005).

6.2 Results and Discussion

6.2.1 Toxicity assessment of Phase II repeatability experiments by phosphatase enzyme assay

The soil treatments (control, nutrient-amended and biodiesel-amended) were incubated for a maximum of 90 days and amended with biochar after 60 days of incubation (Figure 3.3). Phosphatase activity was then measured at the start of the experiment (day 0) and after 60 and 90 days. According to the results shown in Figure 6.1, the biodiesel amended (TBN) soils showed the highest phosphatase activity compared to the control (TC) and nutrient amended (TNT) microcosms, after 60 and 90 days of coal tar remediation.

Results of the 60-d toxicity evaluation showed a significant ($p=0.00$, student *t*-test) 29-fold increase in enzyme activity in the TBN experiments compared to the insignificant reductions in amounts in the TC and TNT microcosms ($p>0.05$ student *t*-test). This enhanced phosphatase activity following the increased degradation of toxic HMW PAHs (especially benzo[a]pyrene) in these biodiesel amended experiments indicates a high phosphorus mineralisation in these treatments. Although enzyme activity reduced slightly after 90 days of remediation, TBN experiments still exhibited the highest amount of phosphatase activity (22.13 µmol *p*-nitrophenol/g soil/h) which was 10 and 8 times more than the TC (2.19 µmol *p*-nitrophenol/g soil/h) and TNT (2.74 µmol *p*-nitrophenol/g soil/h) microcosms, respectively. These results showed that the untreated control (TC) had the least phosphatase activity at the end of the experiment (p=0.027, ANOVA) compared to the amended treatments. Overall, the soil microcosms can be ranked as TBN>TNT>TC in terms of observed amounts in phosphatase enzyme activity.

A negative correlation (r = -0.96) was also established between phosphatase activity and the extractable organic matter (EOM) concentrations for the TBN treatments as activity increased at decreasing levels of EOM (Table 6.1). However, positive correlations were seen for the TC (r = 0.676) and TNT (r = 0.989) treatments which showed consistent reductions in phosphatase activity even at reduced levels of EOM (Table 6.1). Although several factors influence enzymatic activities, response levels could also be associated with the amount of constituent toxicants, especially the more recalcitrant 5-6 ring PAHs as is the case with the TBN microcosms which had lower concentrations of these compounds compared to the TC and TNT treatments. Baran *et al.* (2004) in their work, also came to a similar interpretation when reporting the differing effects of the types and amounts of PAH on the activities of dehydrogenase, urease, protease and phosphatase enzymes from different pollution sources.

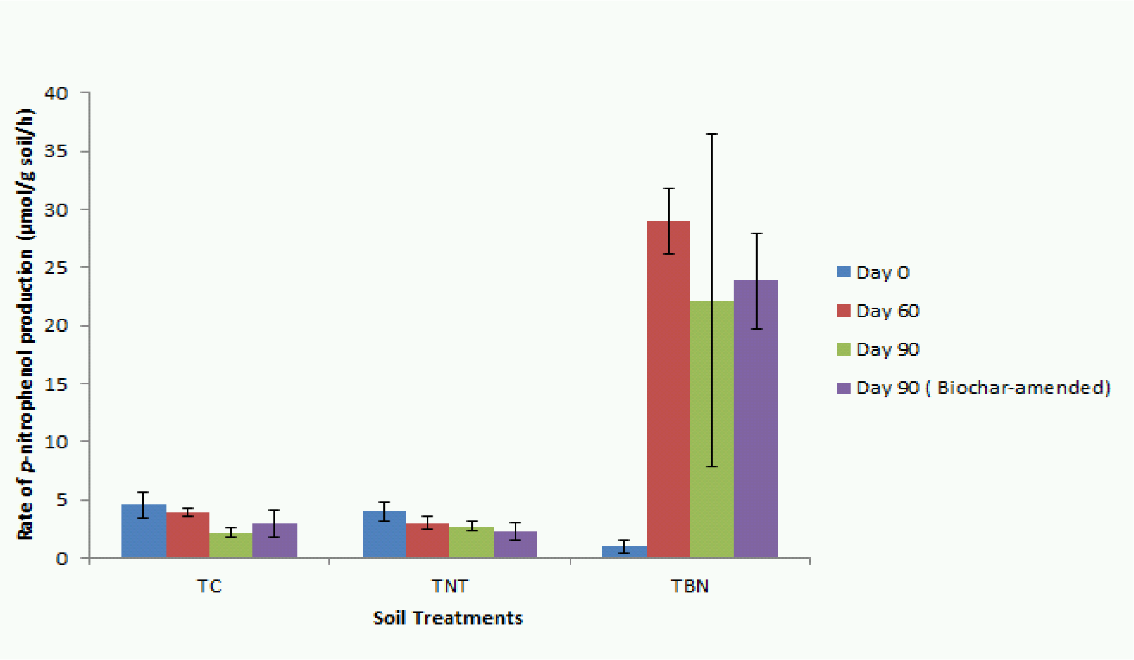


Figure 6.1. Soil phosphatase activity for control (TC), nutrient amended (TNT) and biodiesel amended (TBN) treatments at days 0, 60 and 90 for un-weathered coal tar spiked soil. Effect of biochar amendment on activity is also shown. Error bars indicate +/- one standard deviation from the mean (n=3).

Table 6.1. Correlation data of the phosphatase enzyme activity and the extractable organic matter (EOM) for the control, nutrient and biodiesel amended microcosms of un-weathered coal tar spiked soil

Treatment	Incubation period (days)	Phosphatase enzyme activity (µmol/g soil/h)	Extractable organic matter (EOM, mg/g)	Pearson correlation coefficient (r)
Control	0	4.58 ± 1.14	5.95 ± 1.04	0.676
(TC)	60	3.89 ± 0.34	3.63 ± 1.02	
	90	2.19 ± 0.42	3.80 ± 0.09	
Nutrient- amended	0	4.04 ± 0.80	10.4 ± 1.63	0.989
(TNT)	60	3.03 ± 0.60	6.77 ± 2.66	
	90	2.74 ± 0.45	4.74 ± 0.90	
Biodiesel-amended	0	0.99 ± 0.60	162.34 ± 15.04	-0.959
(TBN)	60	29.00 ± 2.80	32.92 ± 7.06	
	90	22.13 ± 14.27	25.54 ± 5.86	
Control	60	3.89 ± 0.34	3.63 ± 1.02	0.885
(TC)	120	4.76 ± 0.58	3.90 ± 0.42	
	150	3.86 ± 0.30	3.37 ± 0.32	
Nutrient- amended	60	3.03 ± 0.60	6.77 ± 2.66	0.899
(TNT)	120	2.34 ± 0.47	5.14 ± 1.23	
	150	1.66 ± 0.18	5.02 ± 0.12	
Biodiesel-amended	60	2.36 ± 0.25	189.66± 12.29	0.985
(TBN60)	120	0.93 ± 0.66	142.22 ± 98.93	
	150	0.80 ± 0.55	125.72 ± 76.92	

Soil amendment with an additional carbon source and nutrients have also been reported to reduce the impact of PAH toxicity on soil enzymatic activity (Baran *et al.*, 2004). The addition of compost, manure or straw to PAH polluted soils have revealed increases in microbial and enzymatic functions which have been attributed to increased total organic carbon (TOC) on amendment; increased resistance to denaturation as enzymes form stable complexes (enzyme-humus) with the organic matter (in the case of compost) and; the generation of enzyme stimulatory components from the mineralisation process e.g. straw (Ros *et al.*, 2003; Baran *et al.*, 2004; Haritash and Kaushik, 2009; Wyszkowska and Wyszkowski, 2010; Shahsavari *et al.*, 2013). The utilization of biodiesel as an additional carbon source in this study therefore explains the large differences in phosphatase enzyme activity between these samples and the other treatments (TC and TNT) which could also be ascribed to increased TOC content as well as the production of stimulatory enzymatic effects from the degradation of its component fatty acid methyl esters (FAMEs).

Evaluation of the biochar and non-biochar treatments at day 90 revealed no significant differences ($p>0.05$, student *t*-test) in phosphatase enzyme activities between the two categories at the end of the amendment period, although increased amounts in phosphatase levels were noticed in the TC-B and TBN-B samples relative to their un-amended samples, unlike TNT-B when compared to TNT (Table 6.1 and Appendix 12) According to Bailey *et al.* (2011), varying effects of biochar amendment on soil enzyme functions (e.g. stimulatory and inhibitory) do occur and these variations are likely associated with differing biochar-substrate reactions between treatments.

6.2.2 Toxicity assessment of Phase II stepwise amendment experiments by phosphatase enzyme assay

As mentioned in previous Chapters, a stepwise amendment technique was applied to the biodiesel amended microcosms which involved the addition of biodiesel 60 days after nutrient supplementation. The treatments, which also included control as well as nutrient amended microcosms, were then incubated for a further 60 days and biochar was thereafter added and left for a further 30 days for comparison with the non-biochar amended categories (Figure 3.4). Phosphatase activity was measured after the 120 and 150 days incubation periods. From the results presented in Figure 6.2, the biodiesel amended treatments (TBN60) showed the least phosphatase activity compared to the control (TC) and nutrient amended (TNT) samples for both incubation periods.

The day 120 results (Figure 6.2, Table 6.1) showed that enzyme activity was reduced significantly by 61% ($p=0.025$, student *t*-test) in the TBN60 microcosms and 23% decrease ($p>0.05$, student *t*-test) was also observed in the TNT samples compared with their day 60 samples. Although a 22% rise in activity was exhibited by the control (TC) samples at this period of incubation (120 d), it was, however, not statistically different ($p>0.05$, student *t*-test) from the amounts recorded previously for 60 d microcosms. At the end of the experiment (150 d), phosphatase enzyme activity further decreased by 66% in the TBN60 treatments, with results showing significantly reduced amounts (0.8 µmol *p*-nitrophenol/g soil/h, $p=0.011$) compared to results from 60 d (2.36 µmol *p*-nitrophenol/g soil/h, Figure 6.2 and Table 6.1). A further 29% reduction ($p=0.02$, student *t*-test) was observed in the TNT treatments compared to activity recorded at 60 d, while enzyme activity levels dropped by 19% ($p>0.05$, student *t*-test, nearly equal to the initial amounts recorded in day 60, for the TC samples. Overall, the soil microcosms can be ranked as TC>TNT>TBN in terms of observed amounts in phosphatase enzyme activity.

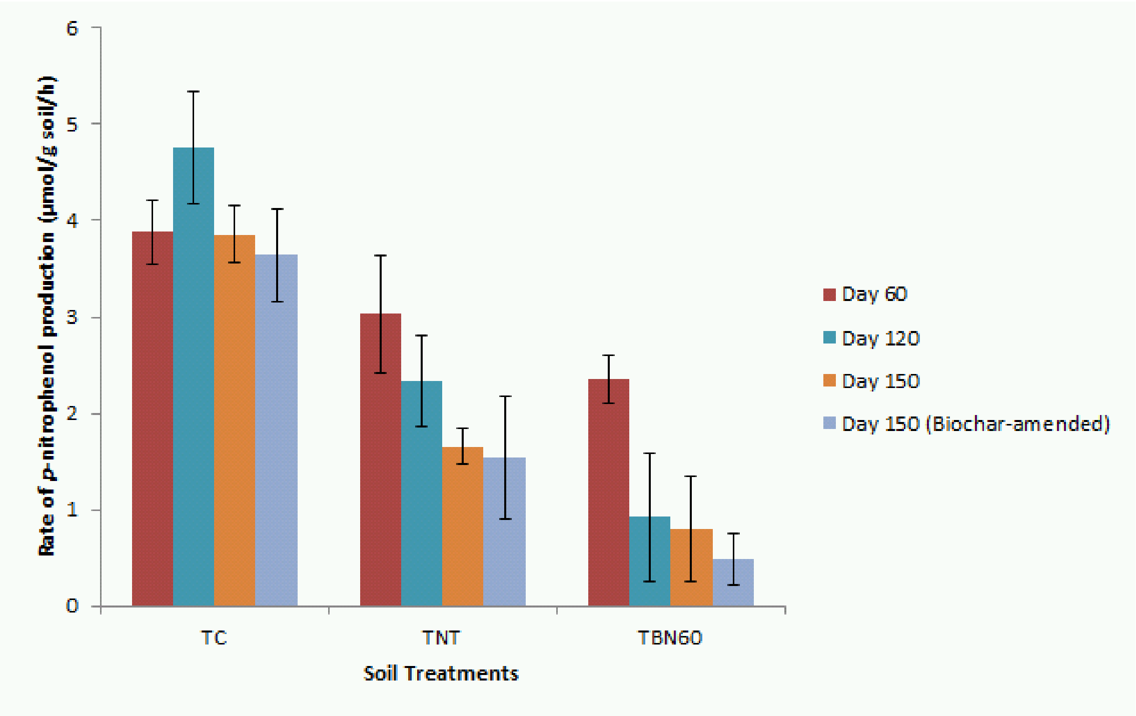


Figure 6.2. Soil phosphatase activity for control (TC), nutrient amended (TNT) and biodiesel amended (TBN60) treatments at days 60, 120 and 150 for un-weathered coal tar spiked soil. Effect of biochar amendment on activity is also shown. Error bars indicate +/- one standard deviation from the mean (n=3).

The decline in the phosphatase enzyme activity observed in the TBN60 treatments after biodiesel addition at 60 d may be due to an overall reduced biological activity in these microcosms, inferred from the high residual EOM levels at the end of the experiment (Table 6.1). This significant reduction following the slow utilization of biodiesel as an additional carbon source, may have resulted from the nutrient-limited growth of microorganisms within these treatments which had probably induced a lower demand for phosphorus and a consequent decline in the release of the phosphatase enzymes. Similar changes in phosphatase activities caused by low nutrient availability that occur during the mineralisation of high C content materials have also been reported e.g. by Albrecht *et al.* (2010b). In their work, the second stage composting of sewage sludge and green waste showed a remarkable decrease in microbial and phosphatase enzyme activity with a consistent reduction in the lipid component (CH_2-groups) of the sludge. Cometabolic degradation of lignin substances by enzymes such as lignin peroxidases, laccases and manganese-dependent peroxidases during the production of humic substances was also observed (Albrecht *et al.*, 2010a). The first stage of composting was, however, characterised by an increase in phosphatase enzymatic activity which was attributed to the high nutrient content of the sewage sludge (Albrecht *et al.*, 2010b).

The negative effect of increased incubation time on phosphatase enzyme activity for organic and nutrient amended treatments inclusive, has also been demonstrated (e.g. Criquet and Braud, 2008). Their study revealed that although available phosphorus (P) increased with time in both amended and un-amended treatments, reduced activities were, however, recorded for all the phosphatase enzymes (acid and alkaline phosphomonoesterases and phosphodiesterases) when incubation period was increased from 25 to 87 days. This same trend was also observed in this study (Figure 6.2) as all treatments exhibited a steady decrease in phosphatase activity when incubation time increased from 60 to 150 days, except for the increase noticed in the control at day 120, which, however, reduced after 150 days of incubation (Figure 6.2, Table 6.1) .

It is also possible that soil toxicity was induced by the residual components of the fatty acid methyl esters (as discussed in Chapter 4) after the 90 days of incubation. The reversibility of the transesterification process produces methanol, a water soluble compound that has been reported to elicit cytotoxic effects by altering acid phosphatase activity in multi-cellular organisms (Da Cruz *et al.*, 2012) and also able to reduce microbial growth at high concentrations in the soil (Luong, 1987). Studies by Sannino and Gianfreda (2001) also corroborate the stimulatory and inhibitory effects of methanol on soil phosphatase enzyme activities. It is also not unlikely that the 60-day pre-exposure of soil enzymes to toxic coal tar PAHs after nutrient supplementation may have consequentially reduced the sensitivity of the phosphatase enzyme activity to stimulation. This can be inferred from the lack of stimulatory effect on addition of biodiesel, even though degradation of LMW and HMW PAHs still occurred in these treatments consequent to the increase in PAH bioavailability.

The phosphatase enzyme activity data in Table 6.1 showed a strong positive relationship with EOM levels for the various incubation periods in all treatments as calculated coefficient values (r) revealed 0.885, 0.899 and 0.985 for the TC, TNT and TBN60 microcosms, respectively. This highlights the observation that at reduced EOM concentrations, enzyme activities were at reduced levels throughout the experiment. Also, no increase in activity was observed when treatments were amended with biochar unlike the slight increases noticed in the TC-B and TBN-B at the 90-day incubation period. Compared to their un-amended categories, activities were reduced, though insignificantly ($p>0.05$) by 5, 7 and 39% in TC-B, TNT-B and TBN60-B treatments, respectively, at the 150-d incubation period (Table 6.1, Appendix 12).

An assessment of the uncontaminated Nafferton soil showed insignificant changes ($p>0.05$) in phosphatase enzyme activity over the 150-day incubation period. The results revealed a 5% increase in activity for the biochar amended soil at the end of the 150-day experiment compared to a 5% decrease revealed in the un-amended soil (Figure 6.3).

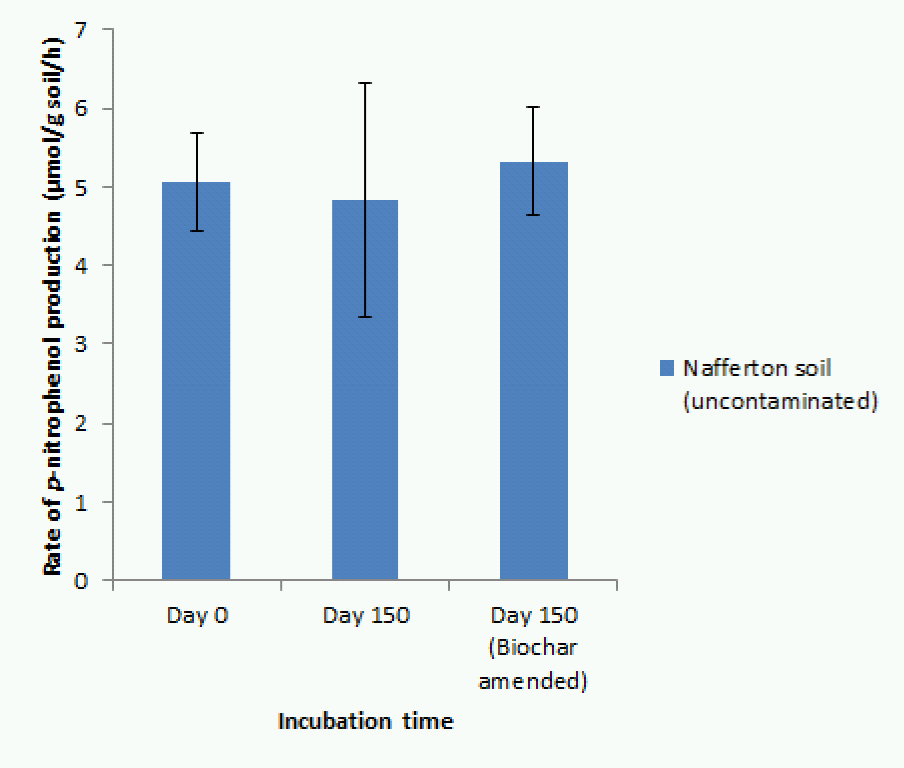


Figure 6.3. Soil phosphatase activity for uncontaminated Nafferton soil at days 0 and 150. Effect of biochar amendment on activity is also shown. Error bars indicate +/- one standard deviation from the mean (n=3).

A comparison of these values with the activities recorded for the control and amended treatments in Figure 6.2 showed that over an increased incubation time, soil amendments (nutrient and stepwise biodiesel amendment techniques) resulted in higher reductions in phosphatase enzyme activity compared to the control. This may be ascribed to resultant changes in microbial community structure, alteration in soil biological/chemical properties over time or an attendant increase in PAH bioavailability (MacNaughton *et al.*, 1999; Taylor and Jones, 2001; Viñas *et al.*, 2005). In the case of the untreated controls, the ageing process of contaminants, acclimatisation of soil microbes to PAHs from long term exposure, and reduced PAH bioavailability could be some contributory factors to their relatively higher enzyme activities at these increased incubation periods (Klimkowicz-Pawlas and Maliszewska-Kordybach, 2003).

6.2.3 Toxicity evaluation by Microtox assay

Seven samples (all in triplicate), were selected for toxicity evaluation using the Microtox bioassay. These included the day 0 (control and biodiesel amended) and day 60 (control, nutrient and biodiesel amended) samples from the repeatability experiments (a total of 5 triplicates) and; the biodiesel amended microcosms for days 60 and 120 (a total of 2 triplicates) from the stepwise amendment experiments. Tests were also conducted for phenol which was used as the positive control. The mean EC_{50} of each sample at incubation time of 5 and 15 minutes is shown in Table 6.2 below.

Table 6.2. The mean EC_{50} of selected control, nutrient- amended and biodiesel amended microcosms from both repeatability and stepwise amendment experiments

		Basic test Microtox EC_{50} (mg/L)	
Sample	Mean concentration (stock, mg/L)	5 min	15 min
[a]TC (day 0)	2.99	0.86 ± 0.11	1.0 ± 0.21
[a]TBN (day 0)	27.1	9.87 ± 1.62	9.55 ± 1.63
[a]TC (day 60)	1.99	1.34 ± 0.41	1.23 ± 0.28
[a]TNT (day 60)	3.5	0.95 ± 0.29	1.15 ± 0.36
[a]TBN (day 60)	5.5	2.12 ± 0.45	1.88 ± 0.36
[b]TBN60 (day 60)	31.6	15.0 ± 3.04	16.21 ± 4.46
[b]TBN60 (day 120)	23.7	6.93 ± 4.47	6.38 ± 4.84
Phenol	100	17.16 ± 1.18	20.21 ± 2.35

EC_{50} values are expressed as mean ± standard deviation; TC= untreated control, TNT=nutrient amended, TBN=biodiesel amended, TBN60=Biodiesel amendment after 60 days of nutrient supplementation. Incubation time is shown in parentheses. a= repeatability experiment, b= stepwise amendment experiment.

At 5 min incubation time, the results showed that biodiesel amendment of PAH contaminated soil can lower soil toxicity as the day 0 microcosms (the repeatability experiments) showed significant reductions by 11 fold (p=0.011, student *t*-test) relative to the untreated control. After 60 days of incubation, the Microtox EC_{50} reduced significantly by 4.7 fold (p=0.001, student *t*-test) for the TBN microcosms as a more toxic response was produced. However, these microcosms still showed the least toxicity (p=0.025, ANOVA) as their mean EC_{50} was the highest (2.12 mg/L,) compared to the untreated control (TC, 1.34 mg/L) and the nutrient amended treatments (TNT, 0.95 mg/L). Although the mean EC_{50} increased for the untreated control after the 60 days incubation, implying a less toxic response compared to the freshly spiked day 0 control, these values were, however, not statistically significant ($p=0.119$, student *t*-test). Data from the 15 min incubation periods did not change significantly ($p>0.05$, student *t*-test) from the corresponding results obtained for the 5 min exposure to *Vibrio fischeri* in all treatments.

Toxicity evaluation of the stepwise amendment experiments also showed a significant reduction in soil toxicity on amendment with biodiesel after 60 days of nutrient supplementation. The high mean EC_{50} obtained for these TBN60 soil samples (15 mg/L) was consistent with the low toxic response also observed for the repeatability experiments when soil samples were amended with biodiesel (TBN-day 0) (9.87 mg/L, Table 6.2). After an additional 60 days of incubation (120 d), the mean EC_{50} also reduced but, by only 2 fold (6.93 mg/L) which was not statistically different ($p>0.05$ student *t*-test) from the EC_{50} of the 60 d treatments (15 mg/L) which showed lower toxicity. Again, data from the 15 min incubation periods did not change significantly ($p>0.05$, student *t*-test) from the corresponding results obtained for the 5 min exposure to *Vibrio fischeri* in all the treatments. The reference toxicant (phenol) also showed results that fell within acceptable limits: EC_{50} :5 min - between 13 and 26 mg/L (Azur Environmental, 1998a)

According to Baker and Herson (1994), supplemental carbon additions increase soil microbial biomass and the biodegradation rates of contaminants as well as reduce the impact of high concentrations of toxic organics on soil micro-organisms (i.e. lessen the per microbe-toxin concentration), as corroborated by (Banat *et al.*, 2010). This resultant reduction in toxic concentrations may be the reason for the low toxicity responses, evidenced by the high mean EC_{50} values when compared to the control, on amendment of PAH-contaminated soil with biodiesel. Following biodegradation and biodiesel breakdown, toxic response was seen to increase significantly for the repeatability experiments, while increase was not significantly noticed in the stepwise amended treatments. This increased toxicity may have been induced by the possible occurrence of residual toxic intermediates from the cometabolic degradation or transformation of recalcitrant PAHs (Cruz *et al.*, 2013), high acidity from the hydrolysis of the fatty acid methyl esters (Leung *et al.*, 2006) or increased bioavailability of PAHs from the solvency effect of biodiesel (Gong *et al.*, 2010; Wu *et al.*, 2010).

The insignificant rise in toxicity noticed in the stepwise amended experiments was probably due to the presence of substantial amounts of biodiesel in the microcosms after the 60 day amendment period (i.e. 120 d), as only 25% reduction in EOM levels occurred in these samples after the incubation period (Chapter 5). It is possible that a reduction in the impact of PAH toxicity may have occurred steadily in these treatments following the slow degradation of biodiesel. The enhanced degradation of the LMW and HMW PAHs especially phenanthrene and benzo[a]pyrene in these samples (as discussed in Chapter 5) over the increased incubation time of 120 days could also be contributory factors. However, these results have shown that it is possible to achieve increased PAH bioavailability, high removal of LMW and HMW PAHs and an insignificant increase in soil toxicity from the slow breakdown of biodiesel, as was evident in the application of the stepwise amendment technique.

6.3 Conclusions

The toxicity evaluation of the coal tar remediated soil in this study showed that amendment with biodiesel stimulated phosphatase enzyme activity and decreased the impact of PAH toxicity in the soil samples compared to the un-amended (control) and nutrient amended microcosms. Although there was slight reduction in enzyme activity after 90 days of remediation in all treatments, the biodiesel amended microcosms still exhibited the highest amount of phosphatase activity which was 10 and 8 times more than the control and nutrient amended microcosms, respectively. The reduction in enzyme activity following the increased incubation time in these biodiesel experiments may have occurred due to a reduction in the rate of microbial activity, an increase in PAH bioavailability and a possible occurrence of toxic intermediates during biodegradation. Toxicity evaluation of biochar amended microcosms showed insignificant increase in phosphatase activity after 90 days of incubation. At increased incubation time of 150 days, enzyme activities were reduced but not statistically different from the non-biochar amended categories. It therefore suggests that the application of biochar for the adsorption of residual pollutants did not show any significant effect on soil phosphatase enzyme activity.

Results obtained from the Microtox bioassay also showed that the biodiesel treated experiments exhibited lower toxic responses to *Vibrio fischeri* for the different exposure periods (5 and 15 min incubation), compared to the other treatments. Significant reduction in toxicity was also observed when soil samples were amended with biodiesel after 60 days of nutrient supplementation. In these treatments, toxic responses remained low even after an additional 60 days of incubation as the mean EC_{50} values recorded during these periods (at day 120) were statistically insignificant compared to previous day 60 results that indicated lower soil toxicity. The application of this stepwise amendment technique therefore minimised the effect of PAH toxicity from the slow degradation of biodiesel thereby causing a decrease in the amount of toxic pollutants exposed to soil micro-organisms as PAH bioavailability and biodegradation were enhanced in the amended microcosms.

Chapter 7

CONCLUSIONS AND FUTURE WORK

7.1 Conclusions

The primary aim of this study was to develop more effective ways to reduce the concentrations of pollutant hydrocarbons in soil and also reduce the toxicities of residual pollutants in order to enable the re-use of contaminated areas. To this end, the application of nutrient, biodiesel and biochar amendment techniques was investigated in the bioremediation of soil spiked with crude oil and coal tar over periods of up to 180 days in laboratory based experiments. The choice of these soil amendments was informed by the accelerated degradation of toxic pollutants attributed to the use of co-substrates with nutrient supplementation, good solvent properties and low toxicity ascribed to biodiesel and the ability of biochar to reduce the bioavailability of organic contaminants via sequestration. In this work, the three main objectives outlined in Section 1.7 guided the experimental design and approach employed and these are used as the framework in presenting the overall conclusions drawn from this study. Also in this Chapter, further work has been suggested to give a clearer understanding of some underlying factors that may have interplayed in the bioremediation process following soil amendment and to fill the knowledge gaps on some of the propositions made from the findings of this study.

7.1.1 Biodegradation of crude oil and coal tar hydrocarbons using biodiesel and nutrient amendment

In this study, biodiesel amendment enhanced the biodegradation of recalcitrant, high molecular weight PAHs in crude oil and coal tar experiments. Treatments showed significant depletion in benzo[a]pyrene (BaP), a potent carcinogen, and other 5 and 6 ring high molecular weight PAHs, after 60 days of amendment. Anthracene, a three ring PAH was also almost completely degraded in all biodiesel treatments compared to the un-amended and nutrient-amended experiments. However, an inhibitory effect on the biodegradation of phenanthrene was observed on amendment with biodiesel. This similar trend was observed in all crude oil and coal tar experiments following the preferential degradation of the rapeseed fatty acid methyl esters (FAMEs) which is likely to have induced a competitive inhibition on phenanthrene degradation in favour of anthracene.

In all biodiesel treated experiments, a visible growth of fungal hyphae and moulds was observed on the surface and within pockets beneath the soil surface of the microcosms after 7-14 days of amendment and this remained noticeable throughout the periods of the experiments. It is therefore likely that fungi played a part in the degradation of the PAHs. The action of ligninolytic fungi, and most probably their lignin peroxidases (LiPs), may be responsible for the cometabolic degradation of BaP and other 5 and 6 ring compounds following the complete degradation of anthracene - the simplest PAH substrate of the lipid peroxidases, and the preferential degradation of rapeseed methyl esters as additional carbon source. This ligninolytic enzyme degradation of BaP may have also induced the inhibitory effect on the degradation of phenanthrene (a non-substrate of LiPs) in both crude oil and coal tar experiments.

The application of the stepwise amendment approach conducted on un-weathered coal tar experiments further confirmed the ability of biodiesel to promote the selective removal of high molecular weight PAHs, as results also showed significantly higher reductions in BaP (98%) 60 days after adding biodiesel to nutrient treated samples. The inhibitory effect on phenanthrene degradation was also relieved as more than 50% reduction was then recorded for this compound. Significant depletion was also observed for other 5 and 6 ring PAHs compared to the control and nutrient amended microcosms. Apparent increases in concentrations of some hydrophobic 4-ring PAHs also occurred on amendment with biodiesel. This is attributed to increased solubilisation of these compounds and their enhanced solvent extractability in the presence of biodiesel, further confirming the findings of previous work (Wu *et al.*, 2010),

In this study, nutrient amendment did not have significant effect on the degradation of PAHs compared to the controls. Degradation of low molecular weight PAHs were better achieved in the un-amended control compared to the amended experiments, showing the potential of natural attenuation in the removal of these pollutants from contaminated soil. However, degradation of the *n*-alkanes in the crude oil experiments was significantly enhanced by amendment with nutrients only, compared to the control and biodiesel amended samples, although results also revealed that the branched alkanes were more susceptible to degradation in the presence of biodiesel. Since the 5-6 ring PAHs are known to be recalcitrant hydrophobic compounds which are less bioavailable to microbial attack, this study shows that biodiesel can enhance their bioavailability to degradation by increased solubilisation.

7.1.2 Effect of biochar in reducing the bioavailability of residual pollutants after bioremediation

In this study, the application of wood biochar amendment to coal tar remediated soil did not cause any significant reduction in the residual pollutant concentrations when compared to the non-biochar amended categories. Based on results obtained from the two types of biochar particle size used for amendment, the application of finer particle size (<1 mm) of biochar showed about 7% reduction in the concentrations of the 16 USEPA pollutants in both nutrient and biodiesel amended microcosms and 4% decrease in the control compared to their non-biochar treatments after 30 days incubation/contact time. Amendment with larger biochar particle size (>1 mm) did not show any reductions in the sum total of the 16 USEPA pollutant concentrations after the 30-d period in all the treatments (the control, nutrient and biodiesel amended samples) compared to their non-amended categories.

The insignificant effect of biochar amendment in reducing residual pollutant concentrations in this study may probably be due to the relatively short incubation period or contact time allowed for the sequestration process after amendment (i.e. 30 days) and, possibly the unsuitability of the analytical protocol used to measure PAH concentrations after biochar amendment. For the latter, it is possible that PAHs sequestered by biochar may still be measured from the use of an improved solvent extraction process for obtaining the extractable organic matter (EOM) needed for geochemical analysis. Also, the influence of the type and amount of biochar used, including pore size and geometry, cannot be ruled out as these factors could affect the efficiency of the amendment. However, the non-interference of biochar in the visible growth of the fungal hyphae and moulds in the biodiesel amended microcosms and the slight reductions observed in the 16USEPA pollutants on biochar amendment shows the potential of biochar in bioremediation and soil amendment studies.

7.1.3 Effect of increased bioavailability of pollutants on soil toxicity during amendments

Soil toxicity assessment was conducted for all the un-weathered coal tar spiked treatments using the phosphatase enzyme assay, while a selected few, particularly the control and biodiesel amended experiments, were tested using the Microtox bioassay. Results showed that after 60 days of biodiesel amendment, phosphatase enzyme activity increased significantly, indicating a high phosphorus mineralisation in these treatments compared to the control which exhibited the least amount of enzyme activity. Even after 90 days of incubation, phosphatase activity in the biodiesel amended treatments was still 10 and 8 times more than the control and nutrient samples, respectively. It therefore indicates that the increased bioavailability of PAHs particularly the 4-6 ring compounds by biodiesel amendment enhanced their biodegradation thereby lowering toxicity in these soil microcosms compared to the other treatments. However, a decline in phosphatase activity occurred following the stepwise addition of biodiesel to soil initially treated with nutrients. This may be attributed to a toxicity or nutrient-limited reduction in microbial activity from the slow utilization of biodiesel as an additional carbon source in these treatments, thereby inducing a low demand for phosphorus and consequently causing a decline in the release of phosphatase activity.

The Microtox bioassay results also showed lower toxic responses for the biodiesel amended microcosms compared to the control and nutrient amended treatments. Notwithstanding, lower mean EC_{50} values were obtained after 60 days of biodiesel amendment suggesting the possible occurrence of residual toxic intermediates from the cometabolic degradation or transformation of recalcitrant PAHs, high acidity from the hydrolysis of the fatty acid methyl esters or the consequent effect of increased bioavailability of PAHs from the solvency effect of biodiesel. Application of the stepwise amendment technique, however, showed insignificant decrease in the mean EC_{50} values after 60 days of biodiesel amendment which reveals the possibility of achieving increased PAH bioavailability, high removal of LMW and HMW PAHs and an insignificant increase in soil toxicity from the slow degradation of biodiesel.

7.2 Future work

The visible growth of fungal hyphae and moulds was one distinguishing feature observed in all the biodiesel amended experiments. It is therefore important that further studies are conducted for proper identification and classification of these microorganisms. However, based on literature coupled with the consistent results obtained from the geochemical analyses of both crude oil and coal tar degradation regarding the significant removal of substrates of lipid peroxidases- benzo[a]pyrene and anthracene in this work, the presence of ligninolytic fungi has been suggested. Nonetheless, there is the need for proper identification using microbiological or genomic analysis.

Further solubilisation experiments should also be conducted with well-suited analytical protocol in order to quantify the bioavailable fractions of the hydrophobic PAHs after biodiesel amendment over a considerable period of time. This study largely investigated the solvent effect of biodiesel in enhancing the degradation of recalcitrant high molecular weight PAHs by increased solubilisation and consequent increase of their bioavailability to microbial attack. It is therefore pertinent that further studies measuring bioavailability are conducted in order to broaden our knowledge on the dynamics of the biodiesel amendment technique.

From the toxicity evaluation experiments it was proposed that the lower mean EC_{50} values obtained after 60 days of biodiesel amendment may be due to the possible occurrence of residual toxic intermediates from the cometabolic degradation or transformation of recalcitrant PAHs, high acidity from the hydrolysis of the fatty acid methyl esters or the consequent effect of increased bioavailability of PAHs from the solvent effect of biodiesel. It is therefore pertinent that further experiments are conducted to ascertain the most significant factors in all of these possibilities. Also, the testing for methanol concentrations potentially formed from the breakdown of biodiesel, should be conducted in treatments after biodiesel amendment for the different incubation periods.

In this study, biochar amendment was not effective in enhancing the reduction of residual pollutants during bioremediation. This could be due to the type and amount of biochar used, the incubation/contact allowed in the experiments among other factors. Further studies should be conducted using treated wood biochar or other types of biochar, much smaller particle sizes and also allowing for a relatively longer incubation/contact time before analysis in order to check if more significant results can be obtained in this regard.

Due to time constraints, field trials were not conducted in the course of this study. From the laboratory-based experiments conducted in this study, biodiesel amendment has the ability to remove carcinogenic PAH and cause a reduction in soil toxicity and this can further be investigated in full scale field studies.

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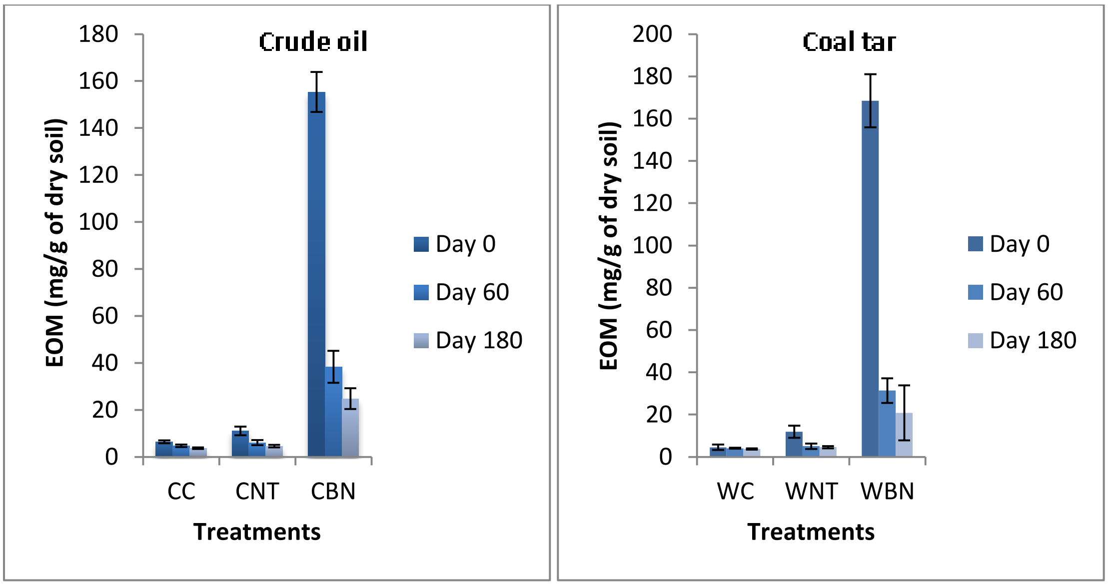
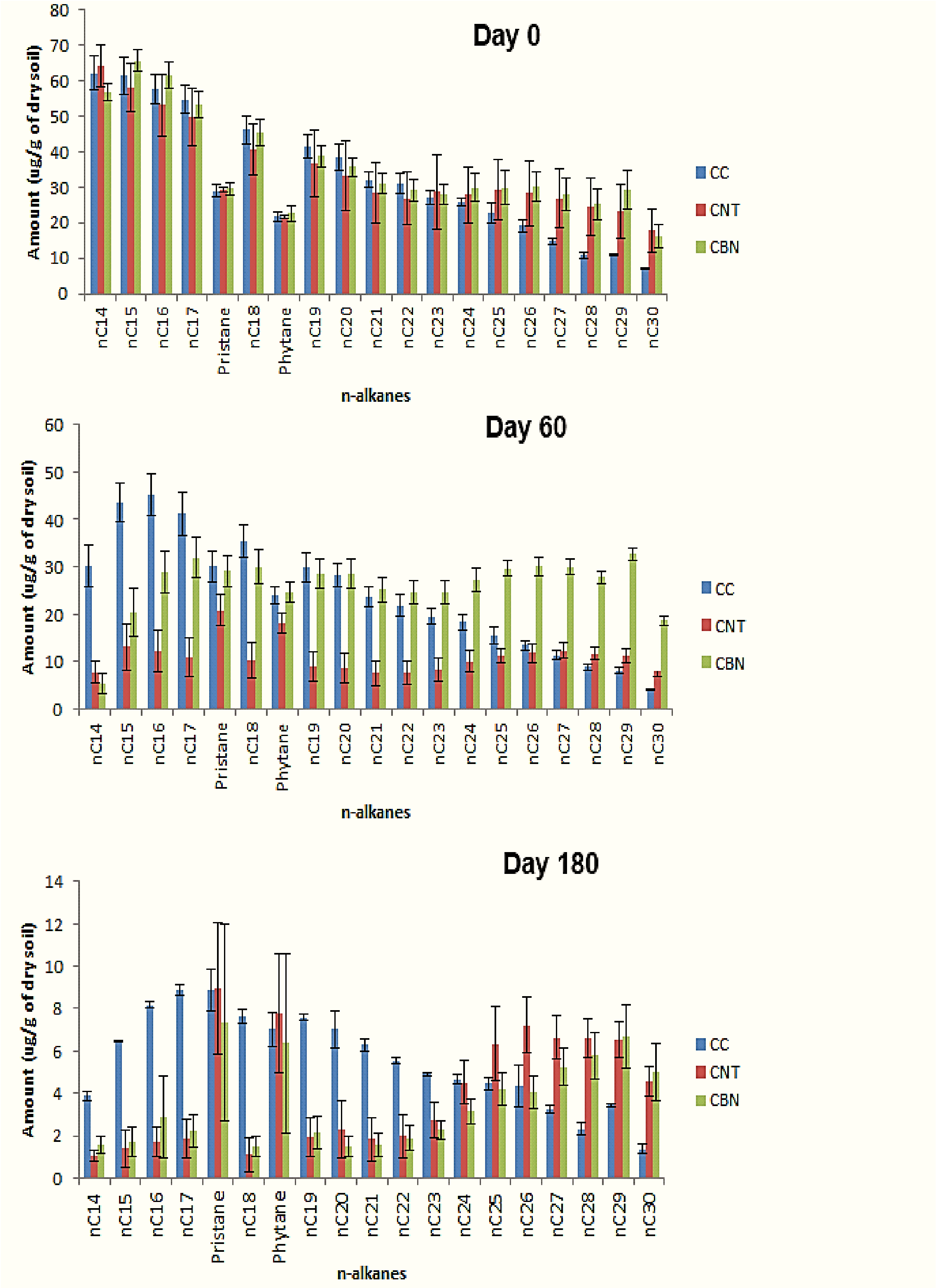
Appendices

Appendix 1. Plot of the EOM concentrations at days 0, 60 and 180 for crude oil and weathered coal tar spiked soil.



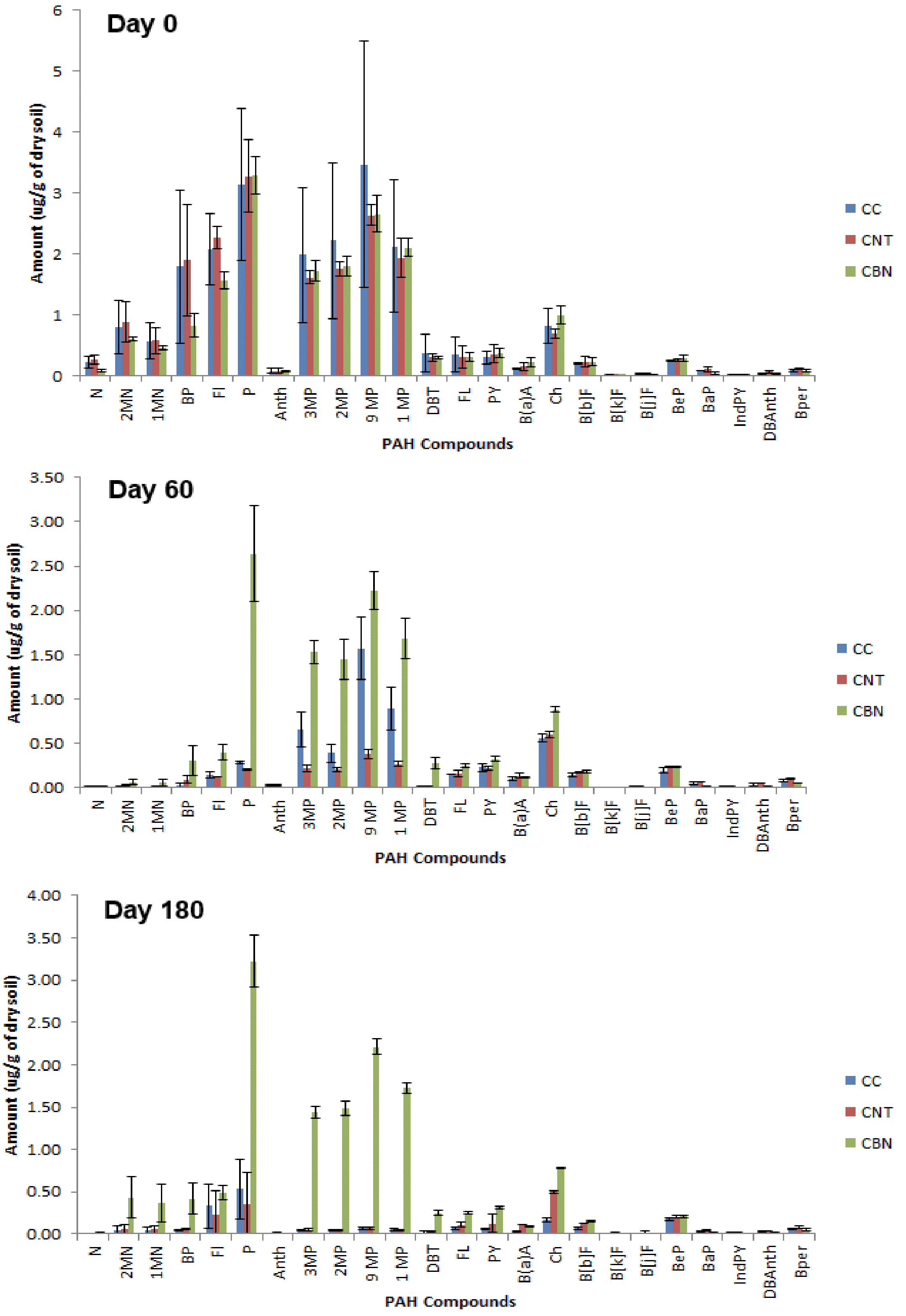
CC=Untreated control, CNT=Nutrient only and CBN=Biodiesel amended samples of crude oil spiked soils; WC= untreated control, WNT=nutrient-amended and WBN= biodiesel amended samples of coal tar spiked soil. Error bars indicate +/- one standard deviation from the mean (n=3).

Appendix 2. Plot of the concentrations of *n*-alkanes in crude oil spiked soil after incubation for 0, 60 and 180 days.



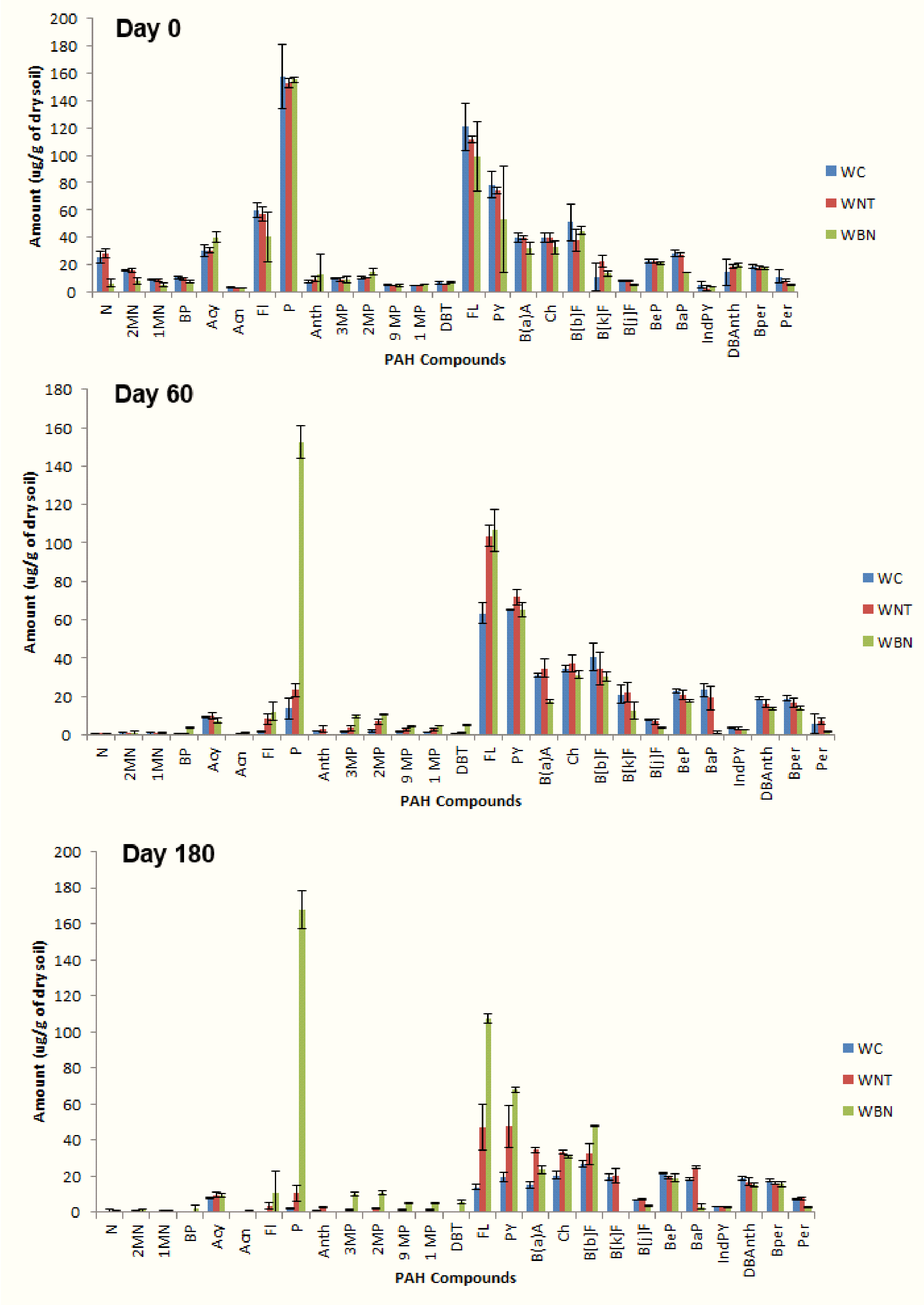
CC=Untreated control, CNT=Nutrient only, CBN=Biodiesel amended. Error bars indicate +/- one standard deviation from the mean (n=3).

Appendix 3. Plot of the PAH concentrations of crude oil spiked soil at days 0, 60 and 180.



CC= untreated control, CNT=nutrient-amended and CBN= biodiesel amended microcosms. Error bars indicate +/- one standard deviation from the mean (n=3). The PAH assignments are: N-Naphthalene; (2-,1-)MN- (2-,1-) methylnaphthalene; BP- Biphenyl; Fl- Fluorene; P- Phenanthrene; Anth-Anthracene; (3,2,9,1)MP- (3,2,9,1) methylphenanthrene; DBT- Dibenzothiophene; FL- Fluoranthene; PY- Pyrene; B(a)A- Benzo(a)anthracene; Ch- Chrysene; B [b-,k-,j-]F- Benzo [b-,k-,j-] fluoranthene; BeP- Benzo(e)pyrene; BaP- Benzo(a)pyrene; IndPY- Indeno(1,2,3 c-d) pyrene; DBAnth- Dibenzo(a,h)anthracene; BPer- Benzo(g,h,i) perylene.

Appendix 4. Plot of the PAH concentrations of coal tar spiked soil at days 0, 60 and 180.



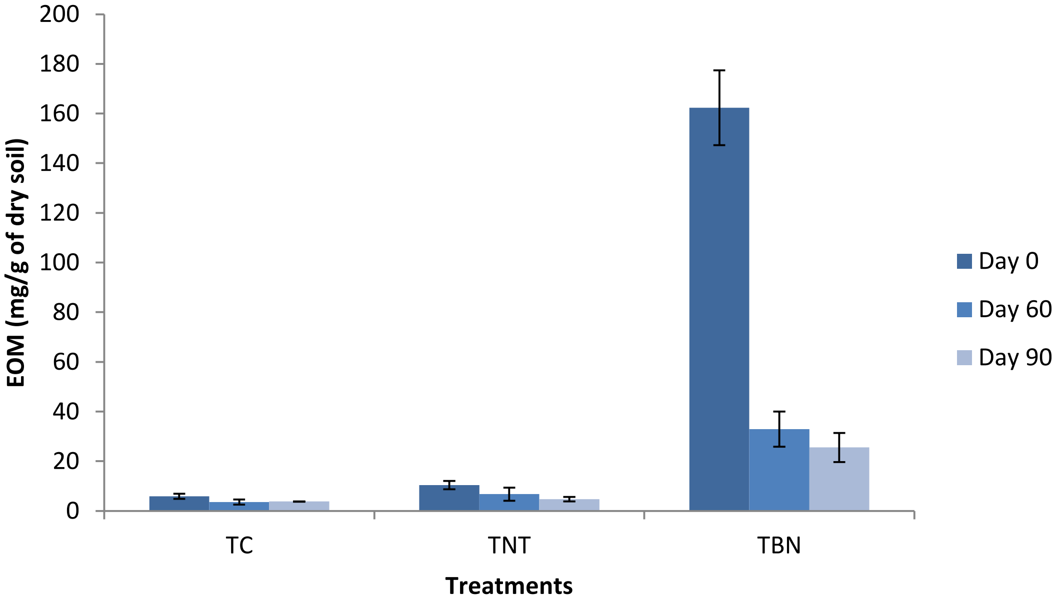
WC= untreated control, WNT=nutrient-amended and WBN= biodiesel amended microcosms. Error bars indicate +/- one standard deviation from the mean (n=3). The PAH assignments are: N-Naphthalene; (2-,1-)MN- (2-,1-) methylnaphthalene; BP- Biphenyl; Fl- Fluorene; P- Phenanthrene; Anth-Anthracene; (3,2,9,1)MP- (3,2,9,1) methylphenanthrene; DBT- Dibenzothiophene; FL- Fluoranthene; PY- Pyrene; B(a)A- Benzo(a)anthracene; Ch- Chrysene; B [b-,k-,j-]F- Benzo [b-,k-,j-] fluoranthene; BeP- Benzo(e)pyrene; BaP- Benzo(a)pyrene; IndPY- Indeno(1,2,3 c-d) pyrene; DBAnth- Dibenzo(a,h)anthracene; BPer- Benzo(g,h,i) perylene.

Appendix 5. Background concentrations of PAHs in the un-spiked Nafferton soil.

Concentrations of PAHs	
Naphthalene	0.00
2-methylnaphtalene	0.00
1-methylnaphthalene	0.00
Biphenyl	0.00
Acenaphthylene	0.00
Acenaphthene	0.00
Fluorene	0.00
Phenanthrene	0.03
Anthracene	0.00
3-methylphenanthrene	0.01
2-methylphenanthrene	0.01
9-methylphenanthrene	0.01
1-methylphenanthrene	0.01
Dibenzothiophene	0.00
Fluoranthene	0.02
Pyrene	0.01
Benzo[a]anthracene	0.03
Chrysene	0.02
Benzo[b]fluoranthene	0.02
Benzo[k]fluoranthene	0.01
Benzo[j]fluoranthene	0.00
Benzo[e]pyrene	0.02
Benzo[a]pyrene	0.01
Indeno[1,2,3-cd]pyrene	0.00
Dibenzo[a,h]anthracene	0.01
Benzo[g,h,i]perylene	0.02
Perylene	0.00

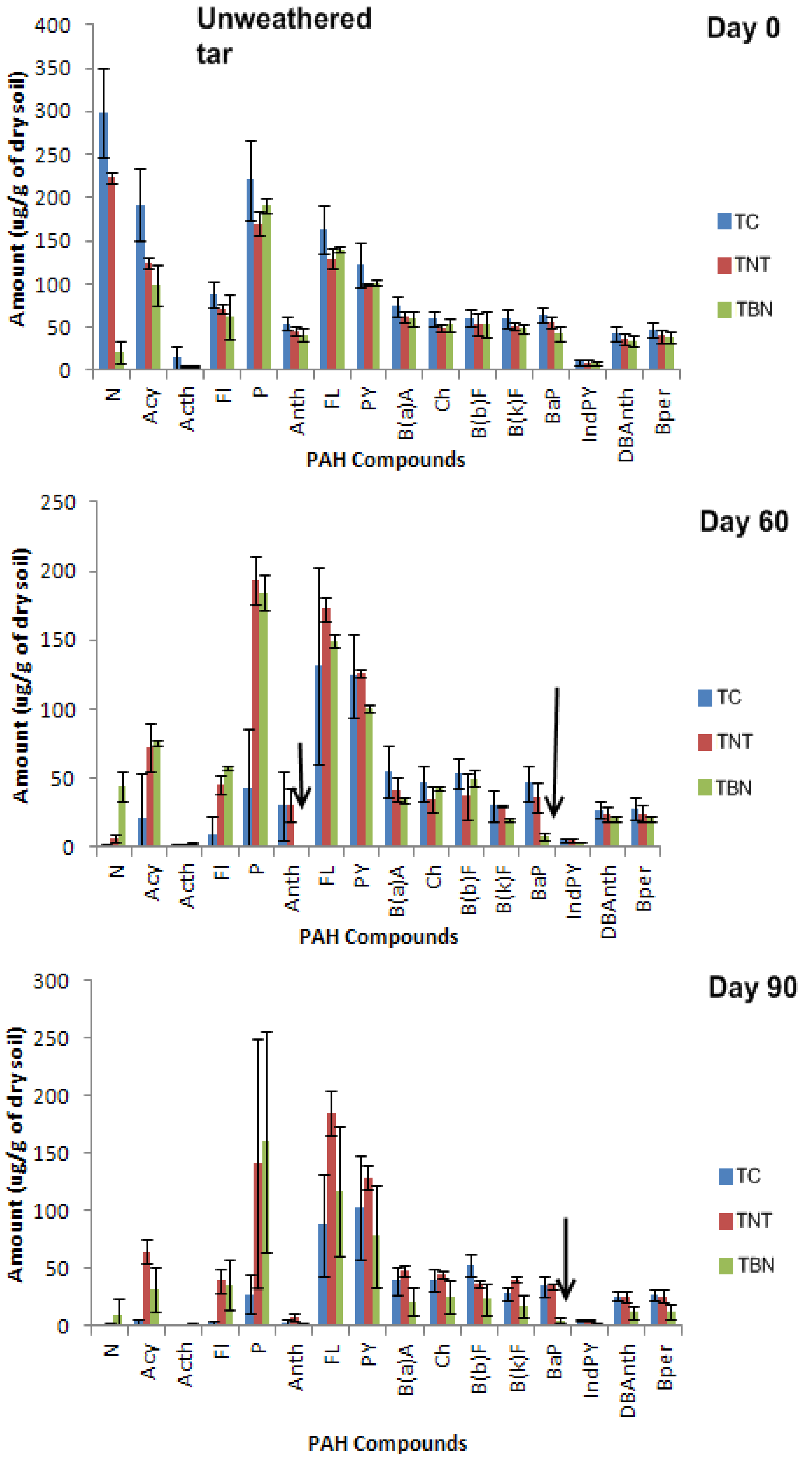
Values represent the concentrations of one replicate of soil sample.

Appendix 6. Plot of the EOM concentrations at days 0, 60 and 90 for un-weathered coal tar spiked soils.



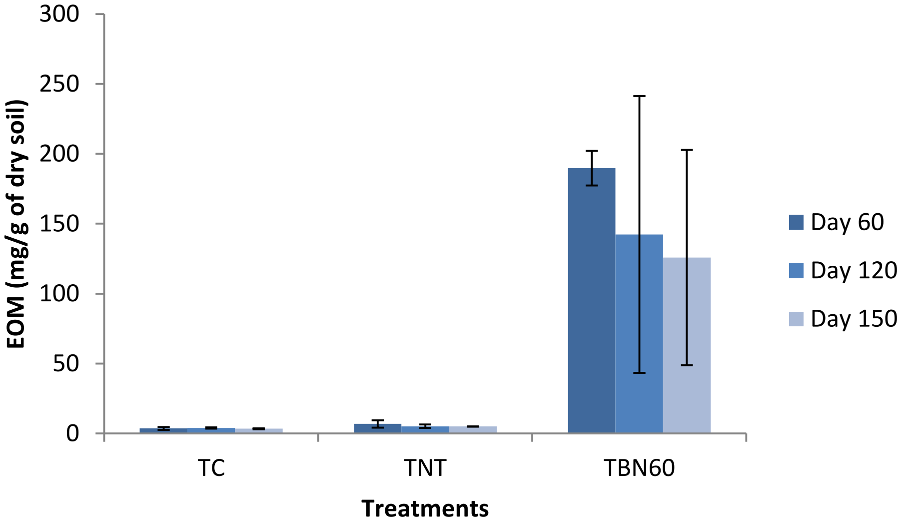
Error bars indicate +/- one standard deviation from the mean (n=3). TC=un-amended control; TNT=nutrient amended; TBN=biodiesel amended samples.

Appendix 7. Plot of the PAH concentrations of un-weathered coal tar spiked soil at days 0, 60 and 90.



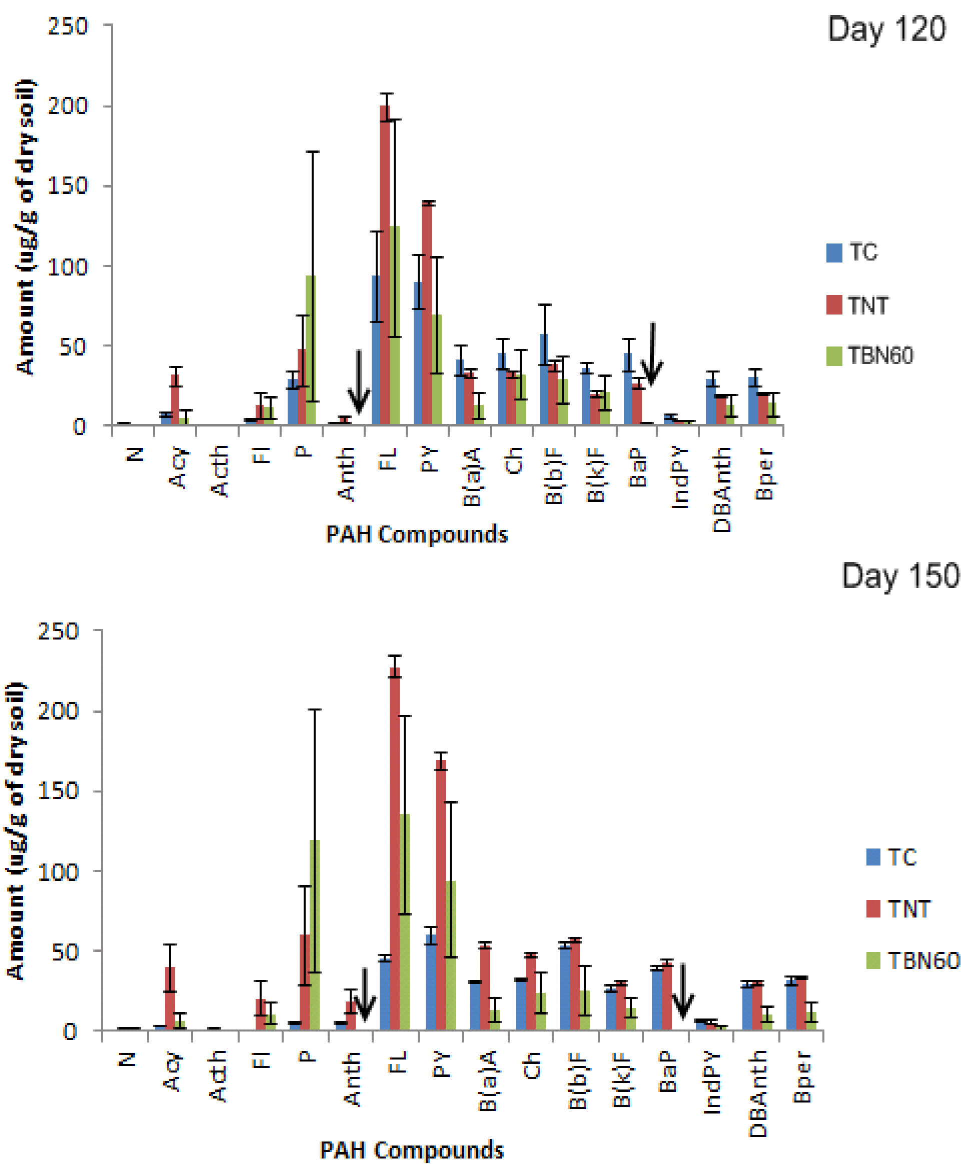
Error bars indicate +/- one standard deviation from the mean (n=3). Significant decrease seen for benzo[a]pyrene (BaP) and anthracene (Anth) in biodiesel amended microcosms (TBN). TC=control; TNT=nutrient amended samples

Appendix 8. Plot of the EOM concentrations for the stepwise amendment experiment.



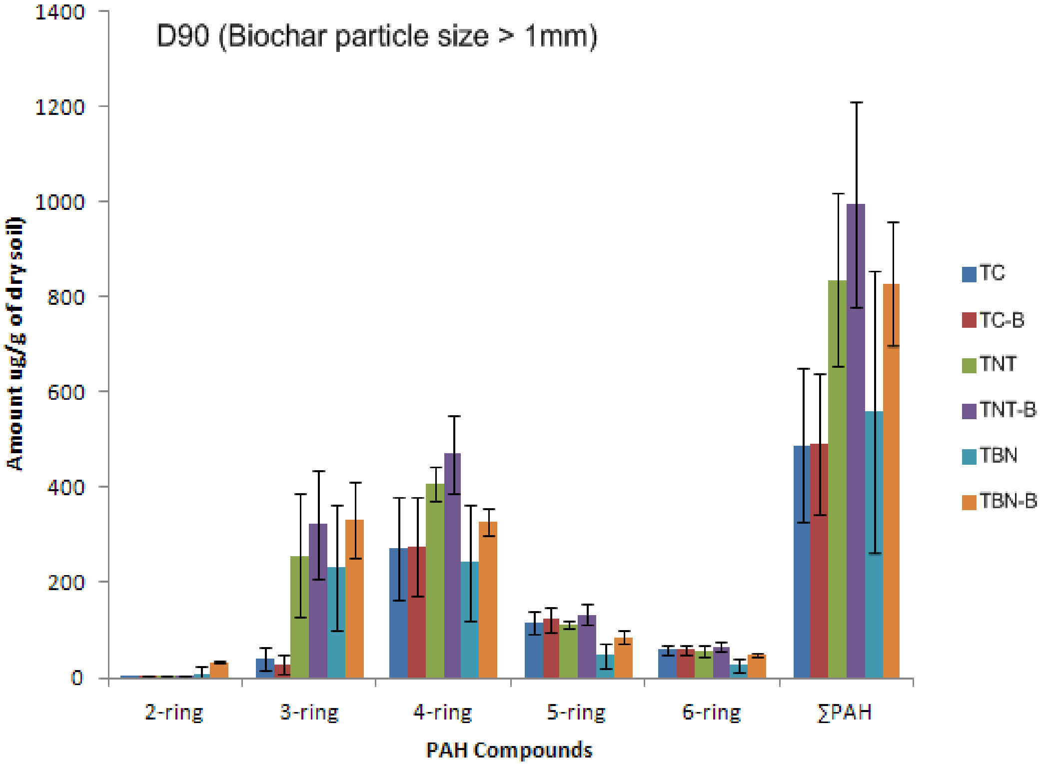
Error bars indicate +/- one standard deviation from the mean (n=3). TC= untreated control; TNT= nutrient-amended; TBN60= biodiesel amended microcosms.

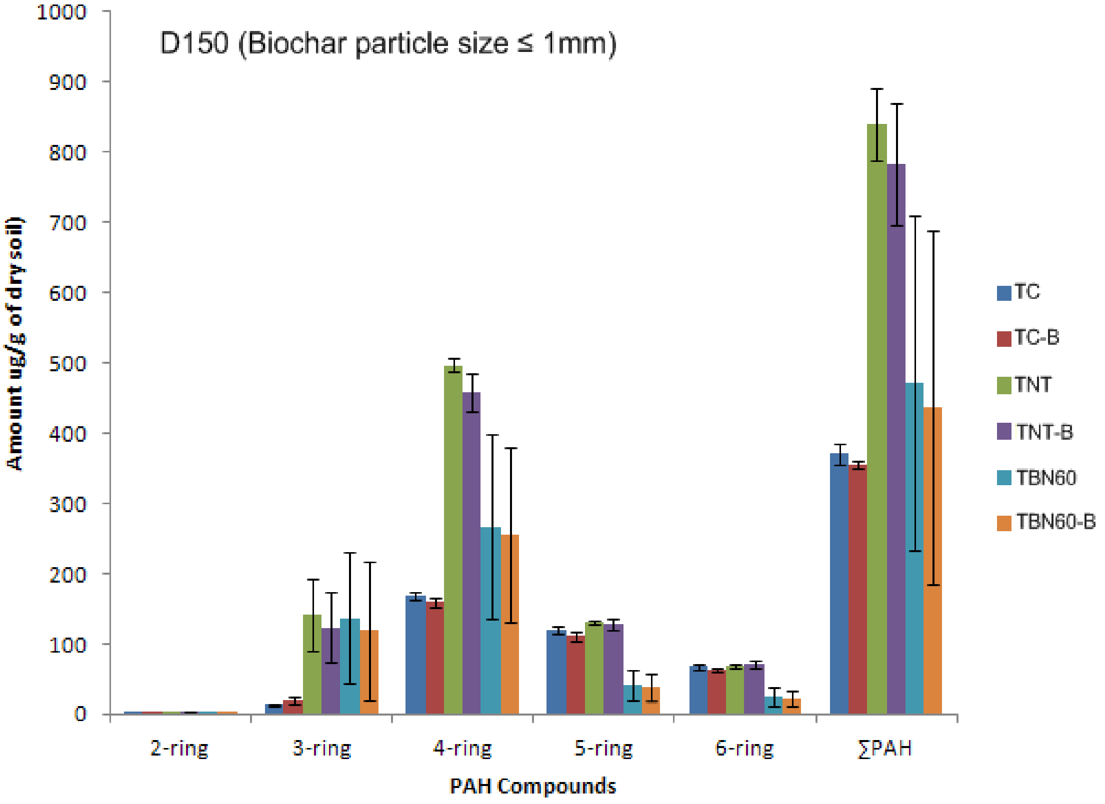
Appendix 9. Plot of the PAH concentrations for 120 and 150 days incubation periods following the stepwise amendment technique conducted for the biodiesel amended microcosms.



Error bars indicate the standard deviation from the mean. Significant reductions seen for benzo[a]pyrene (BaP) and anthracene (Anth) and 6-ring PAHs in biodiesel amended microcosms. TC= untreated control; TNT= nutrient-amended; TBN60= biodiesel amended microcosms of coal tar spiked soil.

Appendix 10. Plot of the concentrations of the 2-6 ringed 16 USEPA PAHs and the sum (ΣPAH) in un-weathered coal tar spiked soil after 90 days (D90) with biochar particle size > 1mm used for amendment; and after 150 days (D150) with biochar particle size ≤ 1mm used for amendment.

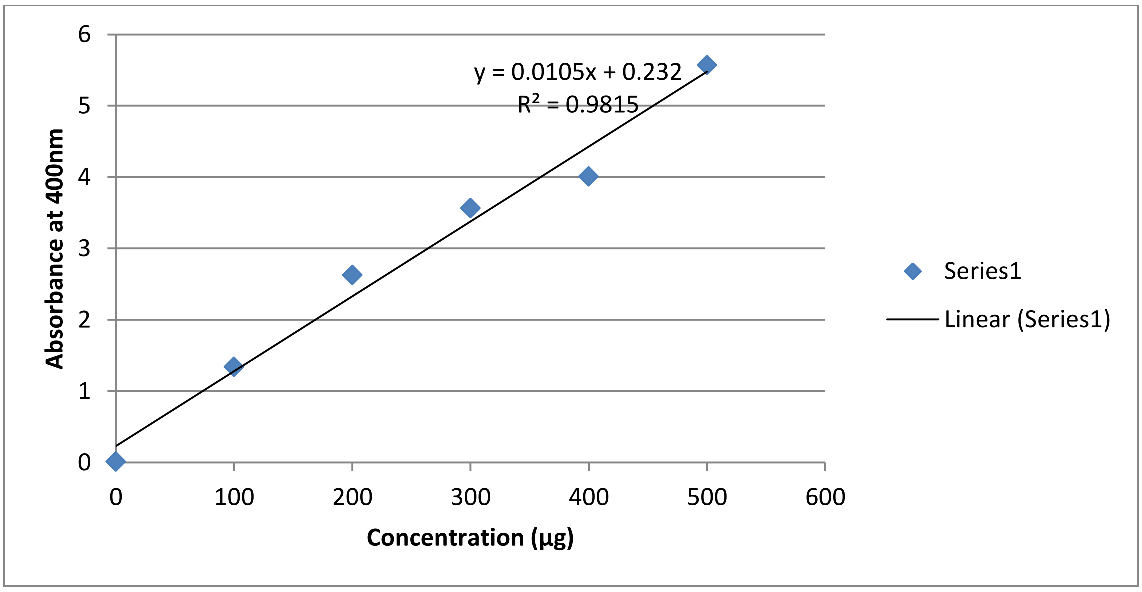




TC= untreated control; TC-B= biochar amended control; TNT= nutrient-amended; TNT-B= nutrient with biochar; TBN, TBN60 = biodiesel amended; and TBN-B, TBN60-B=biodiesel with biochar.

Appendix 11. Calibration data and graph of the *p*-nitrophenol standard for the phosphatase enzyme activity experiments.

Concentration (µg)	Absorbance
0	0.013
100	1.342
200	2.629
300	3.567
400	4.007
500	5.57

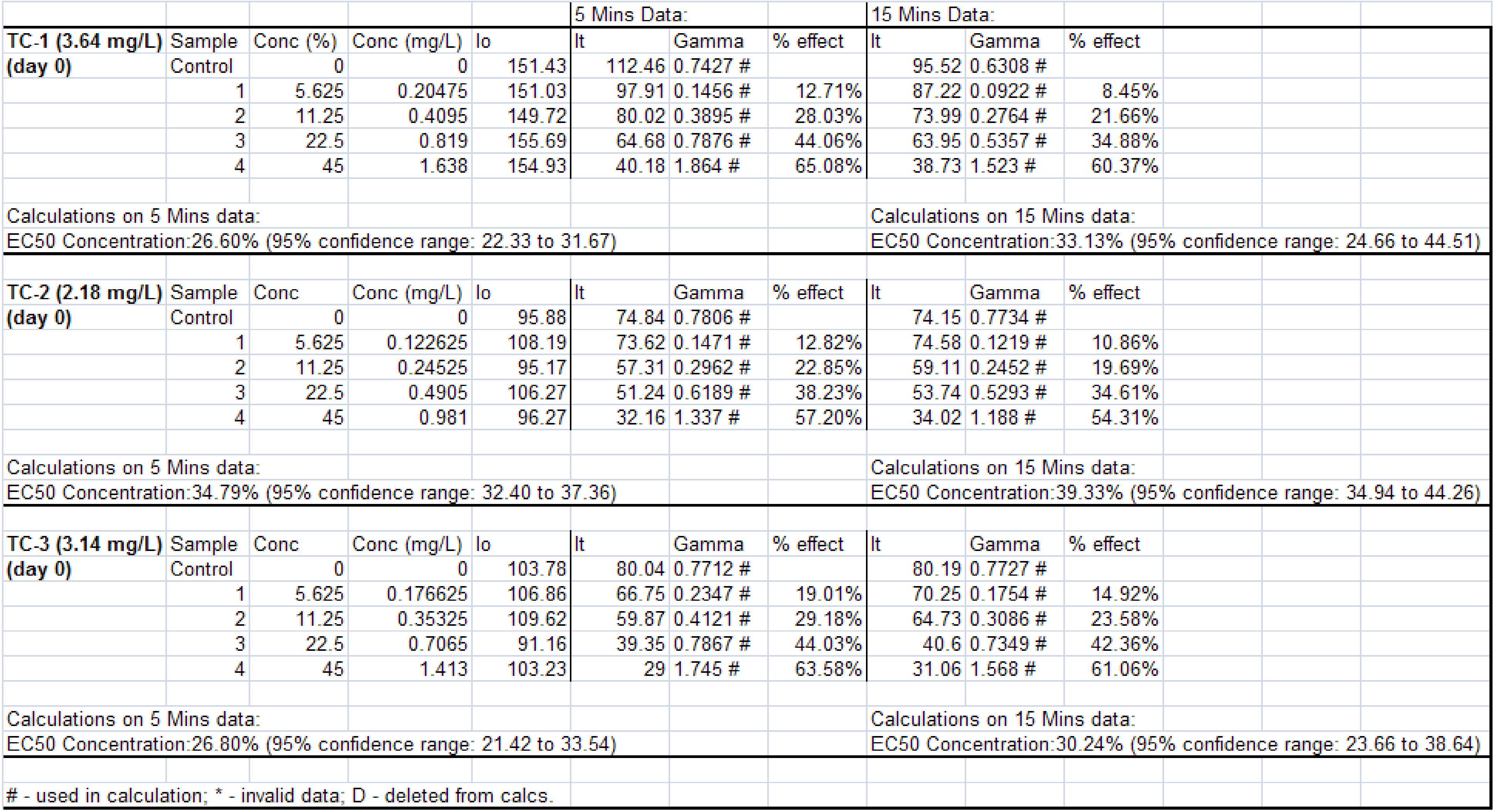


Appendix 12. Phosphatase enzyme activity data for biochar amended microcosms.

Treatment	Incubation period (days)	Phosphatase enzyme activity (µmol/g soil/h)
Control	90	2.96 ± 01.17
(TC-B)	150	3.65 ± 0.48
Nutrient- amended	90	2.27 ± 0.81
(TNT-B)	150	1.55 ± 0.64
Biodiesel-amended		
TBN	90	23.86 ± 4.13
TBN60-B	150	0.49 ± 0.27

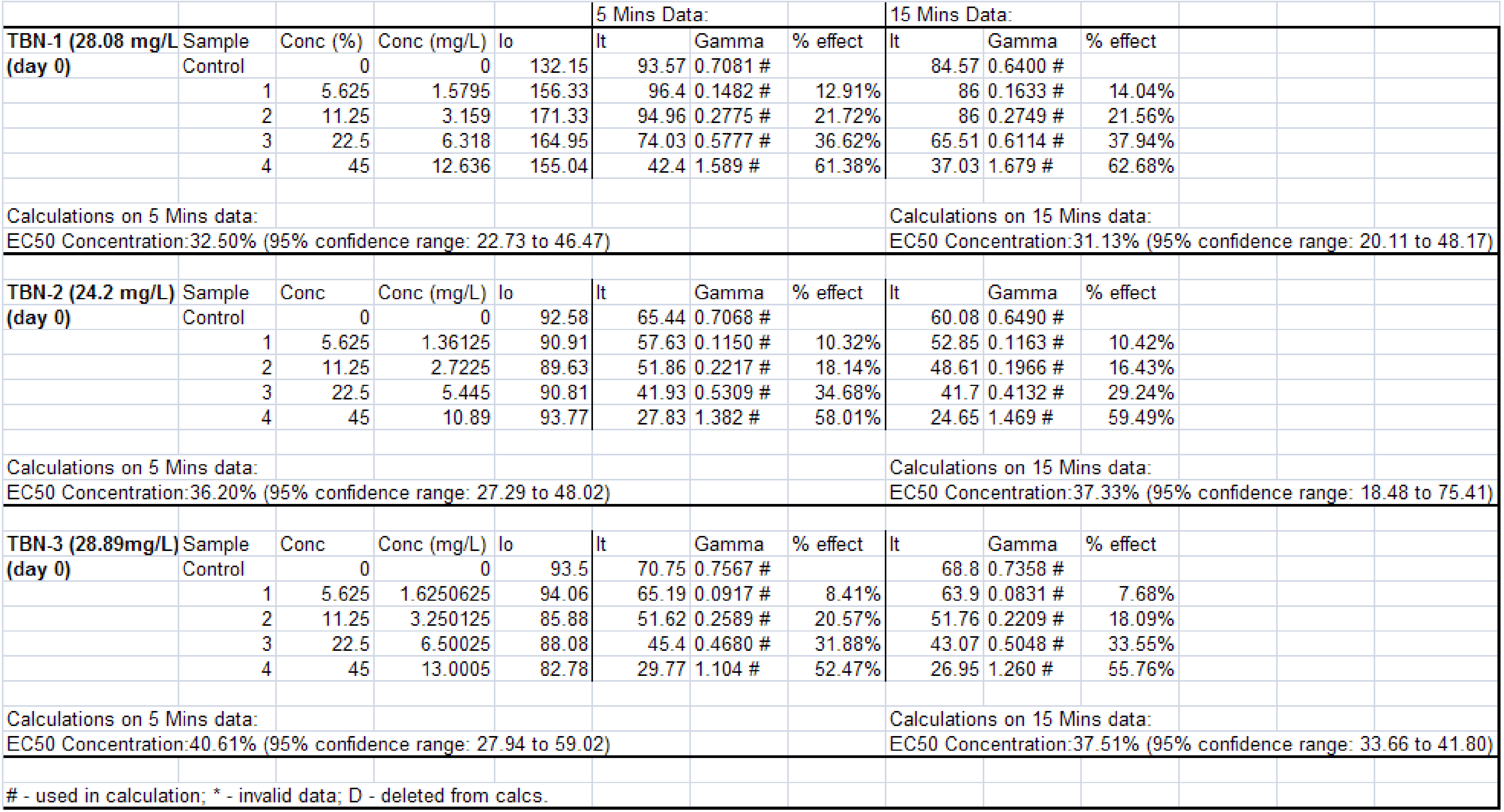
TC-B, TNT-B and TBN-B are biochar amended samples of control, nutrient and biodiesel treated soil, respectively. TNB60-B is biochar sample of biodiesel treated soil after 60 days of nutrient supplementation.

Appendix 13. Microtox data for the control (TC) at day 0 incubation period.



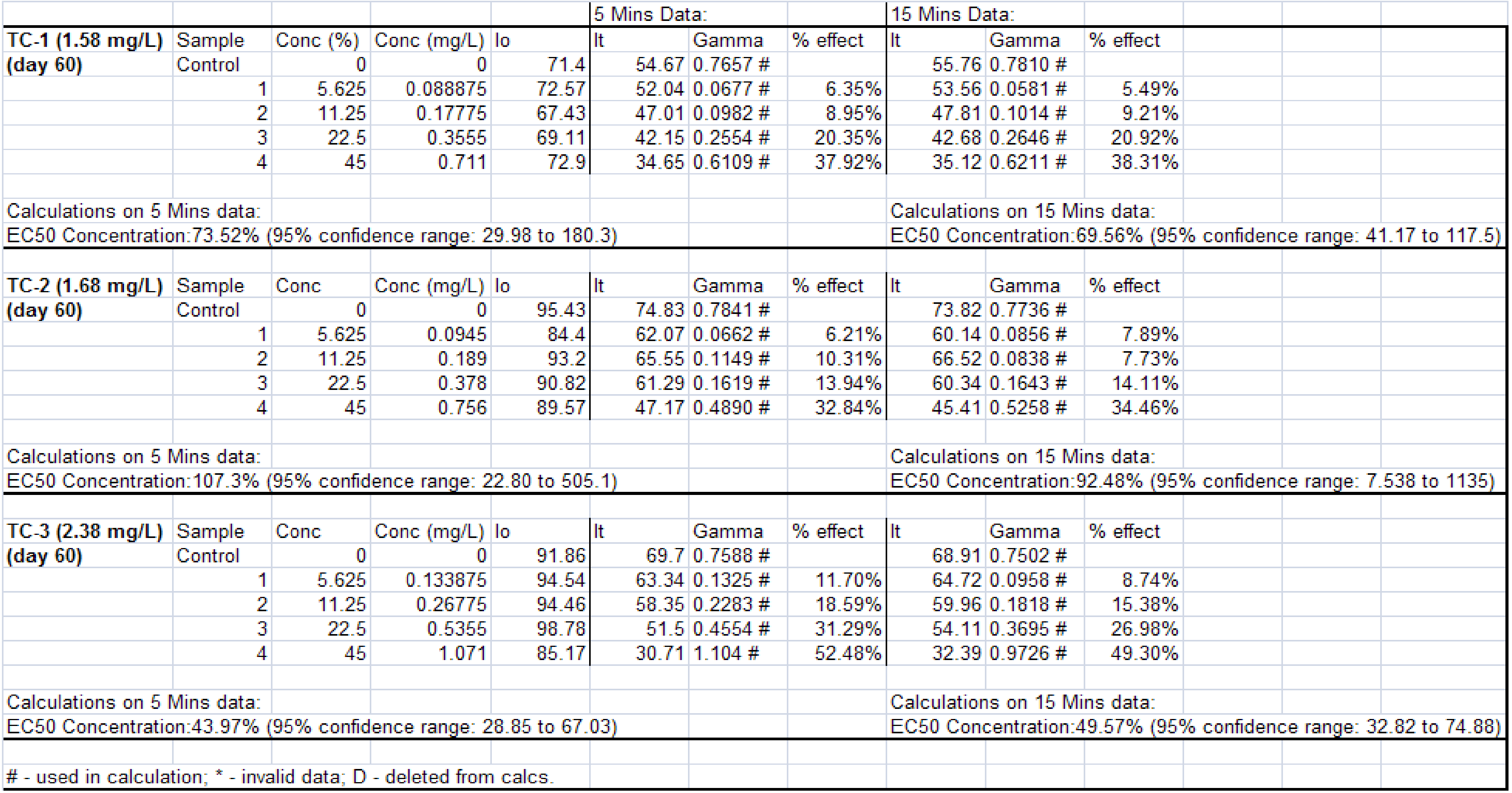
					5 Mins Data:			15 Mins Data:		
TC-1 (3.64 mg/L)	Sample	Conc (%)	Conc (mg/L)	Io	It	Gamma	% effect	It	Gamma	% effect
(day 0)	Control	0	0	151.43	112.46	0.7427 #		95.52	0.6308 #	
	1	5.625	0.20475	151.03	97.91	0.1456 #	12.71%	87.22	0.0922 #	8.45%
	2	11.25	0.4095	149.72	80.02	0.3895 #	28.03%	73.99	0.2764 #	21.66%
	3	22.5	0.819	155.69	64.68	0.7876 #	44.06%	63.95	0.5357 #	34.88%
	4	45	1.638	154.93	40.18	1.864 #	65.08%	38.73	1.523 #	60.37%
Calculations on 5 Mins data:								Calculations on 15 Mins data:		
EC50 Concentration:26.60% (95% confidence range: 22.33 to 31.67)								EC50 Concentration:33.13% (95% confidence range: 24.66 to 44.51)		
TC-2 (2.18 mg/L)	Sample	Conc	Conc (mg/L)	Io	It	Gamma	% effect	It	Gamma	% effect
(day 0)	Control	0	0	95.88	74.84	0.7806 #		74.15	0.7734 #	
	1	5.625	0.122625	108.19	73.62	0.1471 #	12.82%	74.58	0.1219 #	10.86%
	2	11.25	0.24525	95.17	57.31	0.2962 #	22.85%	59.11	0.2452 #	19.69%
	3	22.5	0.4905	106.27	51.24	0.6189 #	38.23%	53.74	0.5293 #	34.61%
	4	45	0.981	96.27	32.16	1.337 #	57.20%	34.02	1.188 #	54.31%
Calculations on 5 Mins data:								Calculations on 15 Mins data:		
EC50 Concentration:34.79% (95% confidence range: 32.40 to 37.36)								EC50 Concentration:39.33% (95% confidence range: 34.94 to 44.26)		
TC-3 (3.14 mg/L)	Sample	Conc	Conc (mg/L)	Io	It	Gamma	% effect	It	Gamma	% effect
(day 0)	Control	0	0	103.78	80.04	0.7712 #		80.19	0.7727 #	
	1	5.625	0.176625	106.86	66.75	0.2347 #	19.01%	70.25	0.1754 #	14.92%
	2	11.25	0.35325	109.62	59.87	0.4121 #	29.18%	64.73	0.3086 #	23.58%
	3	22.5	0.7065	91.16	39.35	0.7867 #	44.03%	40.6	0.7349 #	42.36%
	4	45	1.413	103.23	29	1.745 #	63.58%	31.06	1.568 #	61.06%
Calculations on 5 Mins data:								Calculations on 15 Mins data:		
EC50 Concentration:26.80% (95% confidence range: 21.42 to 33.54)								EC50 Concentration:30.24% (95% confidence range: 23.66 to 38.64)		
# - used in calculation; * - invalid data; D - deleted from calcs.										

Appendix 14. Microtox data for the biodiesel-amended treatments (TBN) at day 0 incubation period.



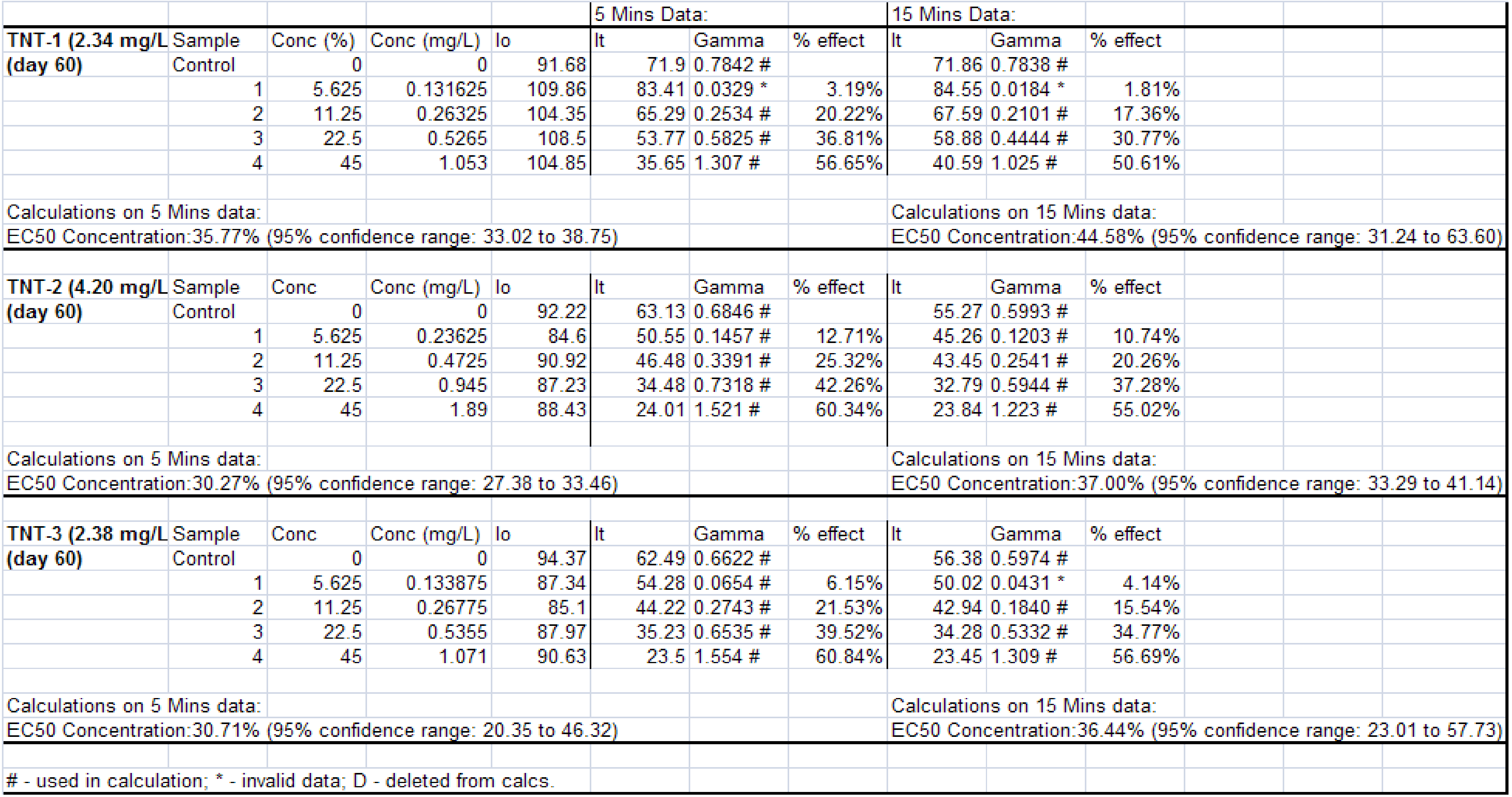
					5 Mins Data:			15 Mins Data:		
TBN-1 (28.08 mg/L	Sample	Conc (%)	Conc (mg/L)	Io	It	Gamma	% effect	It	Gamma	% effect
(day 0)	Control	0	0	132.15	93.57	0.7081 #		84.57	0.6400 #	
	1	5.625	1.5795	156.33	96.4	0.1482 #	12.91%	86	0.1633 #	14.04%
	2	11.25	3.159	171.33	94.96	0.2775 #	21.72%	86	0.2749 #	21.56%
	3	22.5	6.318	164.95	74.03	0.5777 #	36.62%	65.51	0.6114 #	37.94%
	4	45	12.636	155.04	42.4	1.589 #	61.38%	37.03	1.679 #	62.68%
Calculations on 5 Mins data:								Calculations on 15 Mins data:		
EC50 Concentration:32.50% (95% confidence range: 22.73 to 46.47)								EC50 Concentration:31.13% (95% confidence range: 20.11 to 48.17)		
TBN-2 (24.2 mg/L)	Sample	Conc	Conc (mg/L)	Io	It	Gamma	% effect	It	Gamma	% effect
(day 0)	Control	0	0	92.58	65.44	0.7068 #		60.08	0.6490 #	
	1	5.625	1.36125	90.91	57.63	0.1150 #	10.32%	52.85	0.1163 #	10.42%
	2	11.25	2.7225	89.63	51.86	0.2217 #	18.14%	48.61	0.1966 #	16.43%
	3	22.5	5.445	90.81	41.93	0.5309 #	34.68%	41.7	0.4132 #	29.24%
	4	45	10.89	93.77	27.83	1.382 #	58.01%	24.65	1.469 #	59.49%
Calculations on 5 Mins data:								Calculations on 15 Mins data:		
EC50 Concentration:36.20% (95% confidence range: 27.29 to 48.02)								EC50 Concentration:37.33% (95% confidence range: 18.48 to 75.41)		
TBN-3 (28.89mg/L)	Sample	Conc	Conc (mg/L)	Io	It	Gamma	% effect	It	Gamma	% effect
(day 0)	Control	0	0	93.5	70.75	0.7567 #		68.8	0.7358 #	
	1	5.625	1.6250625	94.06	65.19	0.0917 #	8.41%	63.9	0.0831 #	7.68%
	2	11.25	3.250125	85.88	51.62	0.2589 #	20.57%	51.76	0.2209 #	18.09%
	3	22.5	6.50025	88.08	45.4	0.4680 #	31.88%	43.07	0.5048 #	33.55%
	4	45	13.0005	82.78	29.77	1.104 #	52.47%	26.95	1.260 #	55.76%
Calculations on 5 Mins data:								Calculations on 15 Mins data:		
EC50 Concentration:40.61% (95% confidence range: 27.94 to 59.02)								EC50 Concentration:37.51% (95% confidence range: 33.66 to 41.80)		
# - used in calculation; * - invalid data; D - deleted from calcs.										

Appendix 15. Microtox data for the control (TC) at day 60 incubation period.



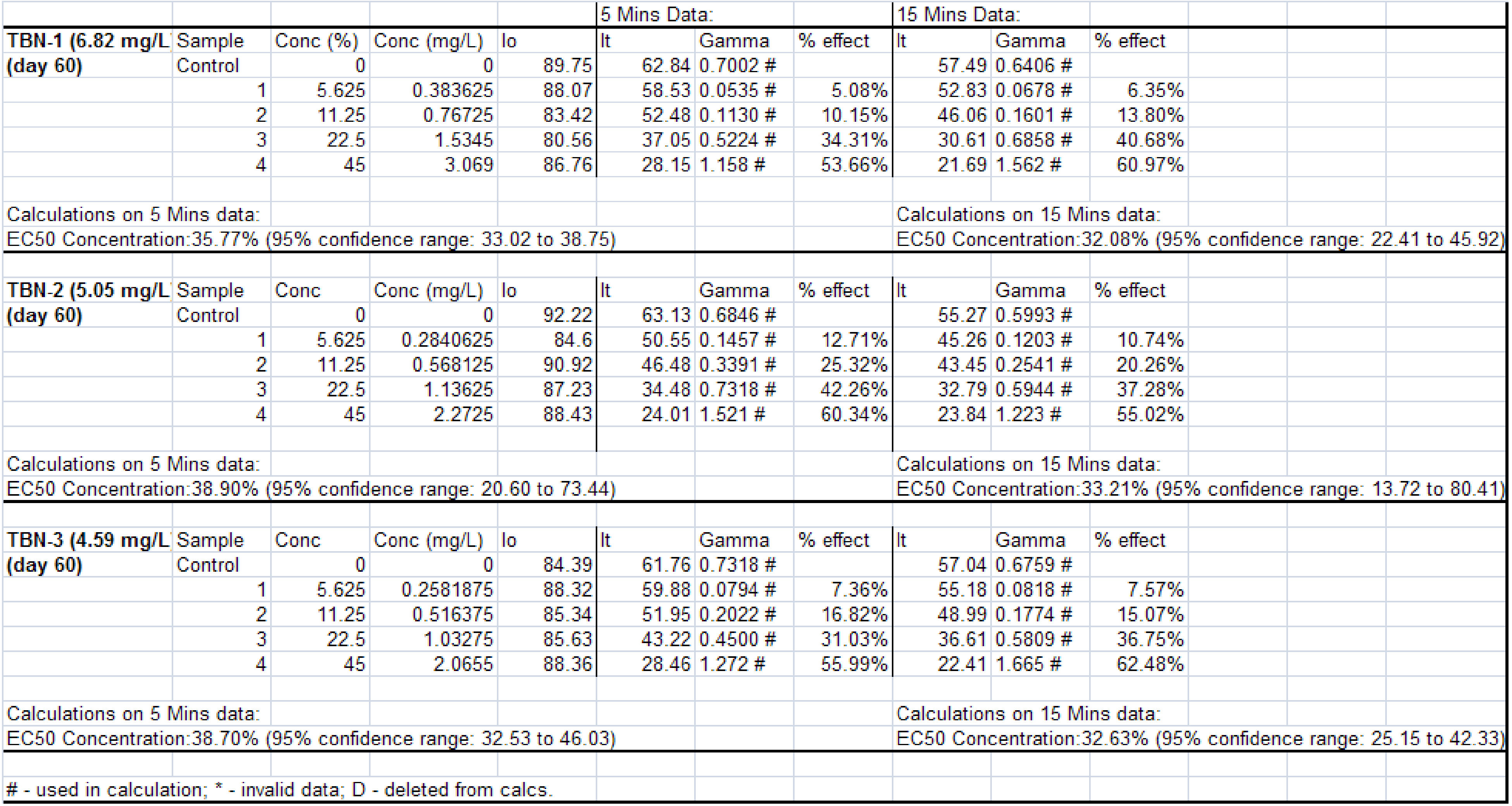
					5 Mins Data:			15 Mins Data:		
TC-1 (1.58 mg/L)	Sample	Conc (%)	Conc (mg/L)	Io	It	Gamma	% effect	It	Gamma	% effect
(day 60)	Control	0	0	71.4	54.67	0.7657 #		55.76	0.7810 #	
	1	5.625	0.088875	72.57	52.04	0.0677 #	6.35%	53.56	0.0581 #	5.49%
	2	11.25	0.17775	67.43	47.01	0.0982 #	8.95%	47.81	0.1014 #	9.21%
	3	22.5	0.3555	69.11	42.15	0.2554 #	20.35%	42.68	0.2646 #	20.92%
	4	45	0.711	72.9	34.65	0.6109 #	37.92%	35.12	0.6211 #	38.31%
Calculations on 5 Mins data:								Calculations on 15 Mins data:		
EC50 Concentration:73.52% (95% confidence range: 29.98 to 180.3)								EC50 Concentration:69.56% (95% confidence range: 41.17 to 117.5)		
TC-2 (1.68 mg/L)	Sample	Conc	Conc (mg/L)	Io	It	Gamma	% effect	It	Gamma	% effect
(day 60)	Control	0	0	95.43	74.83	0.7841 #		73.82	0.7736 #	
	1	5.625	0.0945	84.4	62.07	0.0662 #	6.21%	60.14	0.0856 #	7.89%
	2	11.25	0.189	93.2	65.55	0.1149 #	10.31%	66.52	0.0838 #	7.73%
	3	22.5	0.378	90.82	61.29	0.1619 #	13.94%	60.34	0.1643 #	14.11%
	4	45	0.756	89.57	47.17	0.4890 #	32.84%	45.41	0.5258 #	34.46%
Calculations on 5 Mins data:								Calculations on 15 Mins data:		
EC50 Concentration:107.3% (95% confidence range: 22.80 to 505.1)								EC50 Concentration:92.48% (95% confidence range: 7.538 to 1135)		
TC-3 (2.38 mg/L)	Sample	Conc	Conc (mg/L)	Io	It	Gamma	% effect	It	Gamma	% effect
(day 60)	Control	0	0	91.86	69.7	0.7588 #		68.91	0.7502 #	
	1	5.625	0.133875	94.54	63.34	0.1325 #	11.70%	64.72	0.0958 #	8.74%
	2	11.25	0.26775	94.46	58.35	0.2283 #	18.59%	59.96	0.1818 #	15.38%
	3	22.5	0.5355	98.78	51.5	0.4554 #	31.29%	54.11	0.3695 #	26.98%
	4	45	1.071	85.17	30.71	1.104 #	52.48%	32.39	0.9726 #	49.30%
Calculations on 5 Mins data:								Calculations on 15 Mins data:		
EC50 Concentration:43.97% (95% confidence range: 28.85 to 67.03)								EC50 Concentration:49.57% (95% confidence range: 32.82 to 74.88)		
# - used in calculation; * - invalid data; D - deleted from calcs.										

Appendix 16. Microtox data for the nutrient-amended treatments (TNT) at day 60 incubation period.



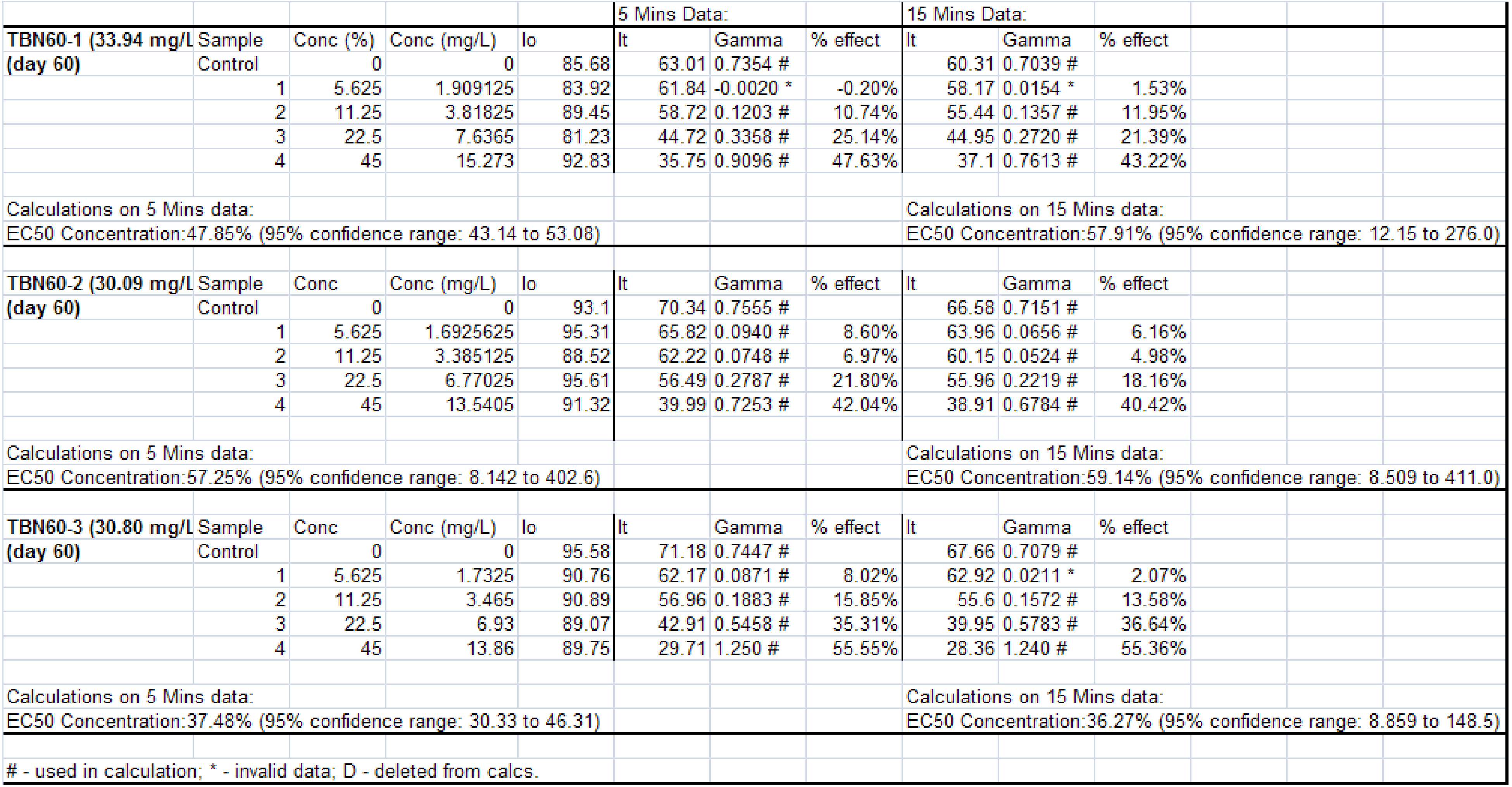
					5 Mins Data:			15 Mins Data:		
TNT-1 (2.34 mg/L	Sample	Conc (%)	Conc (mg/L)	Io	It	Gamma	% effect	It	Gamma	% effect
(day 60)	Control	0	0	91.68	71.9	0.7842 #		71.86	0.7838 #	
	1	5.625	0.131625	109.86	83.41	0.0329 *	3.19%	84.55	0.0184 *	1.81%
	2	11.25	0.26325	104.35	65.29	0.2534 #	20.22%	67.59	0.2101 #	17.36%
	3	22.5	0.5265	108.5	53.77	0.5825 #	36.81%	58.88	0.4444 #	30.77%
	4	45	1.053	104.85	35.65	1.307 #	56.65%	40.59	1.025 #	50.61%
Calculations on 5 Mins data:								Calculations on 15 Mins data:		
EC50 Concentration:35.77% (95% confidence range: 33.02 to 38.75)								EC50 Concentration:44.58% (95% confidence range: 31.24 to 63.60)		
TNT-2 (4.20 mg/L	Sample	Conc	Conc (mg/L)	Io	It	Gamma	% effect	It	Gamma	% effect
(day 60)	Control	0	0	92.22	63.13	0.6846 #		55.27	0.5993 #	
	1	5.625	0.23625	84.6	50.55	0.1457 #	12.71%	45.26	0.1203 #	10.74%
	2	11.25	0.4725	90.92	46.48	0.3391 #	25.32%	43.45	0.2541 #	20.26%
	3	22.5	0.945	87.23	34.48	0.7318 #	42.26%	32.79	0.5944 #	37.28%
	4	45	1.89	88.43	24.01	1.521 #	60.34%	23.84	1.223 #	55.02%
Calculations on 5 Mins data:								Calculations on 15 Mins data:		
EC50 Concentration:30.27% (95% confidence range: 27.38 to 33.46)								EC50 Concentration:37.00% (95% confidence range: 33.29 to 41.14)		
TNT-3 (2.38 mg/L	Sample	Conc	Conc (mg/L)	Io	It	Gamma	% effect	It	Gamma	% effect
(day 60)	Control	0	0	94.37	62.49	0.6622 #		56.38	0.5974 #	
	1	5.625	0.133875	87.34	54.28	0.0654 #	6.15%	50.02	0.0431 *	4.14%
	2	11.25	0.26775	85.1	44.22	0.2743 #	21.53%	42.94	0.1840 #	15.54%
	3	22.5	0.5355	87.97	35.23	0.6535 #	39.52%	34.28	0.5332 #	34.77%
	4	45	1.071	90.63	23.5	1.554 #	60.84%	23.45	1.309 #	56.69%
Calculations on 5 Mins data:								Calculations on 15 Mins data:		
EC50 Concentration:30.71% (95% confidence range: 20.35 to 46.32)								EC50 Concentration:36.44% (95% confidence range: 23.01 to 57.73)		
# - used in calculation; * - invalid data; D - deleted from calcs.										

Appendix 17. Microtox data for the biodiesel-amended treatments (TBN) at day 60 incubation period.



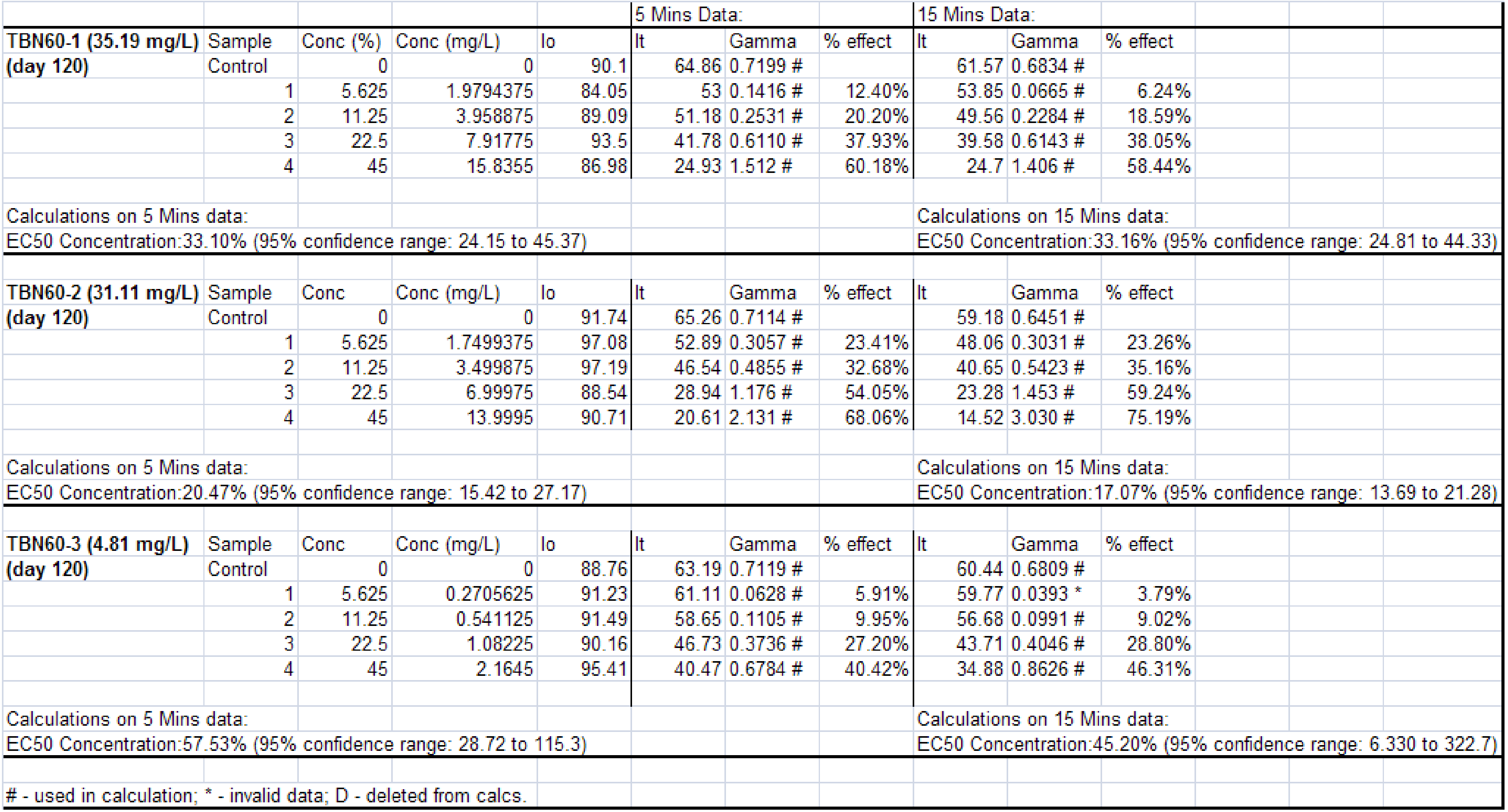
					5 Mins Data:			15 Mins Data:		
TBN-1 (6.82 mg/L	Sample	Conc (%)	Conc (mg/L)	Io	It	Gamma	% effect	It	Gamma	% effect
(day 60)	Control	0	0	89.75	62.84	0.7002 #		57.49	0.6406 #	
	1	5.625	0.383625	88.07	58.53	0.0535 #	5.08%	52.83	0.0678 #	6.35%
	2	11.25	0.76725	83.42	52.48	0.1130 #	10.15%	46.06	0.1601 #	13.80%
	3	22.5	1.5345	80.56	37.05	0.5224 #	34.31%	30.61	0.6858 #	40.68%
	4	45	3.069	86.76	28.15	1.158 #	53.66%	21.69	1.562 #	60.97%
Calculations on 5 Mins data:								Calculations on 15 Mins data:		
EC50 Concentration:35.77% (95% confidence range: 33.02 to 38.75)								EC50 Concentration:32.08% (95% confidence range: 22.41 to 45.92)		
TBN-2 (5.05 mg/L	Sample	Conc	Conc (mg/L)	Io	It	Gamma	% effect	It	Gamma	% effect
(day 60)	Control	0	0	92.22	63.13	0.6846 #		55.27	0.5993 #	
	1	5.625	0.2840625	84.6	50.55	0.1457 #	12.71%	45.26	0.1203 #	10.74%
	2	11.25	0.568125	90.92	46.48	0.3391 #	25.32%	43.45	0.2541 #	20.26%
	3	22.5	1.13625	87.23	34.48	0.7318 #	42.26%	32.79	0.5944 #	37.28%
	4	45	2.2725	88.43	24.01	1.521 #	60.34%	23.84	1.223 #	55.02%
Calculations on 5 Mins data:								Calculations on 15 Mins data:		
EC50 Concentration:38.90% (95% confidence range: 20.60 to 73.44)								EC50 Concentration:33.21% (95% confidence range: 13.72 to 80.41)		
TBN-3 (4.59 mg/L	Sample	Conc	Conc (mg/L)	Io	It	Gamma	% effect	It	Gamma	% effect
(day 60)	Control	0	0	84.39	61.76	0.7318 #		57.04	0.6759 #	
	1	5.625	0.2581875	88.32	59.88	0.0794 #	7.36%	55.18	0.0818 #	7.57%
	2	11.25	0.516375	85.34	51.95	0.2022 #	16.82%	48.99	0.1774 #	15.07%
	3	22.5	1.03275	85.63	43.22	0.4500 #	31.03%	36.61	0.5809 #	36.75%
	4	45	2.0655	88.36	28.46	1.272 #	55.99%	22.41	1.665 #	62.48%
Calculations on 5 Mins data:								Calculations on 15 Mins data:		
EC50 Concentration:38.70% (95% confidence range: 32.53 to 46.03)								EC50 Concentration:32.63% (95% confidence range: 25.15 to 42.33)		
# - used in calculation; * - invalid data; D - deleted from calcs.										

Appendix 18. Microtox data for the biodiesel-amended treatments (TBN60) at day 60 incubation period.



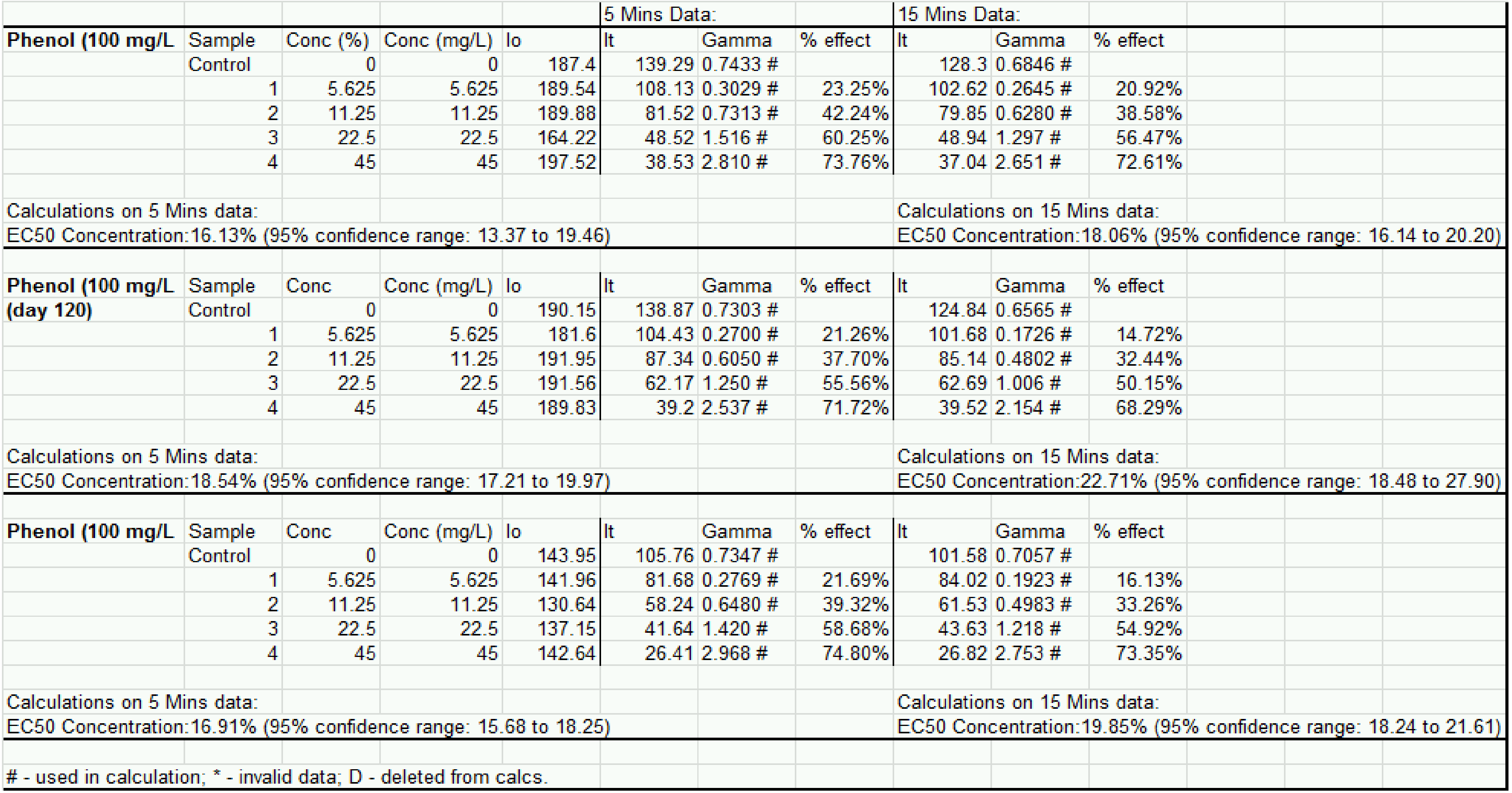
					5 Mins Data:			15 Mins Data:		
TBN60-1 (33.94 mg/L	Sample	Conc (%)	Conc (mg/L)	Io	It	Gamma	% effect	It	Gamma	% effect
(day 60)	Control	0	0	85.68	63.01	0.7354 #		60.31	0.7039 #	
	1	5.625	1.909125	83.92	61.84	-0.0020 *	-0.20%	58.17	0.0154 *	1.53%
	2	11.25	3.81825	89.45	58.72	0.1203 #	10.74%	55.44	0.1357 #	11.95%
	3	22.5	7.6365	81.23	44.72	0.3358 #	25.14%	44.95	0.2720 #	21.39%
	4	45	15.273	92.83	35.75	0.9096 #	47.63%	37.1	0.7613 #	43.22%
Calculations on 5 Mins data:								Calculations on 15 Mins data:		
EC50 Concentration:47.85% (95% confidence range: 43.14 to 53.08)								EC50 Concentration:57.91% (95% confidence range: 12.15 to 276.0)		
TBN60-2 (30.09 mg/L	Sample	Conc	Conc (mg/L)	Io	It	Gamma	% effect	It	Gamma	% effect
(day 60)	Control	0	0	93.1	70.34	0.7555 #		66.58	0.7151 #	
	1	5.625	1.6925625	95.31	65.82	0.0940 #	8.60%	63.96	0.0656 #	6.16%
	2	11.25	3.385125	88.52	62.22	0.0748 #	6.97%	60.15	0.0524 #	4.98%
	3	22.5	6.77025	95.61	56.49	0.2787 #	21.80%	55.96	0.2219 #	18.16%
	4	45	13.5405	91.32	39.99	0.7253 #	42.04%	38.91	0.6784 #	40.42%
Calculations on 5 Mins data:								Calculations on 15 Mins data:		
EC50 Concentration:57.25% (95% confidence range: 8.142 to 402.6)								EC50 Concentration:59.14% (95% confidence range: 8.509 to 411.0)		
TBN60-3 (30.80 mg/L	Sample	Conc	Conc (mg/L)	Io	It	Gamma	% effect	It	Gamma	% effect
(day 60)	Control	0	0	95.58	71.18	0.7447 #		67.66	0.7079 #	
	1	5.625	1.7325	90.76	62.17	0.0871 #	8.02%	62.92	0.0211 *	2.07%
	2	11.25	3.465	90.89	56.96	0.1883 #	15.85%	55.6	0.1572 #	13.58%
	3	22.5	6.93	89.07	42.91	0.5458 #	35.31%	39.95	0.5783 #	36.64%
	4	45	13.86	89.75	29.71	1.250 #	55.55%	28.36	1.240 #	55.36%
Calculations on 5 Mins data:								Calculations on 15 Mins data:		
EC50 Concentration:37.48% (95% confidence range: 30.33 to 46.31)								EC50 Concentration:36.27% (95% confidence range: 8.859 to 148.5)		
# - used in calculation; * - invalid data; D - deleted from calcs.										

Appendix 19. Microtox data for the biodiesel-amended treatments (TBN60) at day 120 incubation period.



					5 Mins Data:			15 Mins Data:		
TBN60-1 (35.19 mg/L)	Sample	Conc (%)	Conc (mg/L)	Io	It	Gamma	% effect	It	Gamma	% effect
(day 120)	Control	0	0	90.1	64.86	0.7199 #		61.57	0.6834 #	
	1	5.625	1.9794375	84.05	53	0.1416 #	12.40%	53.85	0.0665 #	6.24%
	2	11.25	3.958875	89.09	51.18	0.2531 #	20.20%	49.56	0.2284 #	18.59%
	3	22.5	7.91775	93.5	41.78	0.6110 #	37.93%	39.58	0.6143 #	38.05%
	4	45	15.8355	86.98	24.93	1.512 #	60.18%	24.7	1.406 #	58.44%
Calculations on 5 Mins data:								Calculations on 15 Mins data:		
EC50 Concentration:33.10% (95% confidence range: 24.15 to 45.37)								EC50 Concentration:33.16% (95% confidence range: 24.81 to 44.33)		
TBN60-2 (31.11 mg/L)	Sample	Conc	Conc (mg/L)	Io	It	Gamma	% effect	It	Gamma	% effect
(day 120)	Control	0	0	91.74	65.26	0.7114 #		59.18	0.6451 #	
	1	5.625	1.7499375	97.08	52.89	0.3057 #	23.41%	48.06	0.3031 #	23.26%
	2	11.25	3.499875	97.19	46.54	0.4855 #	32.68%	40.65	0.5423 #	35.16%
	3	22.5	6.99975	88.54	28.94	1.176 #	54.05%	23.28	1.453 #	59.24%
	4	45	13.9995	90.71	20.61	2.131 #	68.06%	14.52	3.030 #	75.19%
Calculations on 5 Mins data:								Calculations on 15 Mins data:		
EC50 Concentration:20.47% (95% confidence range: 15.42 to 27.17)								EC50 Concentration:17.07% (95% confidence range: 13.69 to 21.28)		
TBN60-3 (4.81 mg/L)	Sample	Conc	Conc (mg/L)	Io	It	Gamma	% effect	It	Gamma	% effect
(day 120)	Control	0	0	88.76	63.19	0.7119 #		60.44	0.6809 #	
	1	5.625	0.2705625	91.23	61.11	0.0628 #	5.91%	59.77	0.0393 *	3.79%
	2	11.25	0.541125	91.49	58.65	0.1105 #	9.95%	56.68	0.0991 #	9.02%
	3	22.5	1.08225	90.16	46.73	0.3736 #	27.20%	43.71	0.4046 #	28.80%
	4	45	2.1645	95.41	40.47	0.6784 #	40.42%	34.88	0.8626 #	46.31%
Calculations on 5 Mins data:								Calculations on 15 Mins data:		
EC50 Concentration:57.53% (95% confidence range: 28.72 to 115.3)								EC50 Concentration:45.20% (95% confidence range: 6.330 to 322.7)		
# - used in calculation; * - invalid data; D - deleted from calcs.										

Appendix 20. Microtox data for phenol (reference toxicant/positive control).



					5 Mins Data:			15 Mins Data:		
Phenol (100 mg/L	Sample	Conc (%)	Conc (mg/L)	Io	It	Gamma	% effect	It	Gamma	% effect
	Control	0	0	187.4	139.29	0.7433 #		128.3	0.6846 #	
	1	5.625	5.625	189.54	108.13	0.3029 #	23.25%	102.62	0.2645 #	20.92%
	2	11.25	11.25	189.88	81.52	0.7313 #	42.24%	79.85	0.6280 #	38.58%
	3	22.5	22.5	164.22	48.52	1.516 #	60.25%	48.94	1.297 #	56.47%
	4	45	45	197.52	38.53	2.810 #	73.76%	37.04	2.651 #	72.61%
Calculations on 5 Mins data:								Calculations on 15 Mins data:		
EC50 Concentration:16.13% (95% confidence range: 13.37 to 19.46)								EC50 Concentration:18.06% (95% confidence range: 16.14 to 20.20)		
Phenol (100 mg/L	Sample	Conc	Conc (mg/L)	Io	It	Gamma	% effect	It	Gamma	% effect
(day 120)	Control	0	0	190.15	138.87	0.7303 #		124.84	0.6565 #	
	1	5.625	5.625	181.6	104.43	0.2700 #	21.26%	101.68	0.1726 #	14.72%
	2	11.25	11.25	191.95	87.34	0.6050 #	37.70%	85.14	0.4802 #	32.44%
	3	22.5	22.5	191.56	62.17	1.250 #	55.56%	62.69	1.006 #	50.15%
	4	45	45	189.83	39.2	2.537 #	71.72%	39.52	2.154 #	68.29%
Calculations on 5 Mins data:								Calculations on 15 Mins data:		
EC50 Concentration:18.54% (95% confidence range: 17.21 to 19.97)								EC50 Concentration:22.71% (95% confidence range: 18.48 to 27.90)		
Phenol (100 mg/L	Sample	Conc	Conc (mg/L)	Io	It	Gamma	% effect	It	Gamma	% effect
	Control	0	0	143.95	105.76	0.7347 #		101.58	0.7057 #	
	1	5.625	5.625	141.96	81.68	0.2769 #	21.69%	84.02	0.1923 #	16.13%
	2	11.25	11.25	130.64	58.24	0.6480 #	39.32%	61.53	0.4983 #	33.26%
	3	22.5	22.5	137.15	41.64	1.420 #	58.68%	43.63	1.218 #	54.92%
	4	45	45	142.64	26.41	2.968 #	74.80%	26.82	2.753 #	73.35%
Calculations on 5 Mins data:								Calculations on 15 Mins data:		
EC50 Concentration:16.91% (95% confidence range: 15.68 to 18.25)								EC50 Concentration:19.85% (95% confidence range: 18.24 to 21.61)		
# - used in calculation; * - invalid data; D - deleted from calcs.										