Investigating the effects of biochar and activated carbon amendment on the microbial community response in a volatile petroleum hydrocarbon - contaminated gravelly sand

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Abstract

Laboratory batch and long-term column experiments were conducted to investigate the effects of wood-chip biochar and coal-derived activated carbon amendment on the microbiology of a volatile petroleum hydrocarbon (VPH) - contaminated gravelly sand. First, a stable isotope-labelled mono aromatic compound – toluene was used as a model VPH to gain insights into the mineralization of VPHs by soil microorganisms in the presence and absence of biochar or activated carbon. The biodegradation of a mixture of 12 VPHs was subsequently monitored in batch microcosms over a duration of 6-19 days by measuring headspace CO_2 concentration. Further analysis was carried out by characterizing changes in the soil microbial community composition using next generation sequencing techniques – 454 pyrosequencing and Ion torrent sequencing. Increases in the levels of headspace CO_2 in contaminated soil batches as compared to live and abiotic controls to which no VPHs were added indicated a stimulation of microbial activity in the batches through VPH addition. By fitting a maximum specific growth rate of 0.6 h^{-1} (in line with published rates), it was possible to match model predictions of $^{45}CO_2$ and $^{44}CO_2$ concentrations with the experimentally determined data. Half-saturation constants of 4.06 x 10^3 mgL^{-1}, 7.76 x 10^2 mgL^{-1} and 1.83 x 10^2 mgL^{-1} were predicted for soil, soil & BC and soil & AC respectively, much higher than values reported in the literature. Differences in the half-saturation constant suggests that sorbent amendment affects the microbial ecology, by making microorganisms which can utilize substrates at lower concentrations more competitive. Yield coefficients (g biomass-C relative to g (biomass-C + CO_2-C)) compared more closely in the nutrient (N & P) amended soils ranging from 4.83±0.46 in soil and biochar to 7.86±0.72 in unamended soil, than in the batches without nutrients, 4.1±3.1 in soil & BC, 17.7±5.2 in soil and 13.7±4.6 in soil & AC. Sorbent amendment thus reduced yield coefficients, thereby slowing the growth of VPH degrading biomass. Microbial community structure analysis revealed an increase in the relative abundance ranking of members of the genera *Pseudomonas, Pseudoxanthomonas*, and *Arenimonas* by up to 32 folds and in the families *Nocardioidaceae* and *Pseudomonadaceae* by at least 32 folds in sorbent amended and unamended soil batches and columns compared to their initial soil conditions. Consequently, amending soils with 2% BC or AC changed the biokinetics of VPH degradation by rendering VPHs less bioavailable, but did not appear to have any detrimental effects on the VPH degrading bacteria both in the short- and long-term, and may serve as a sustainable, cost-effective approach for enhancing the natural attenuation of VPHs in soil, thus addressing the challenge of petroleum hydrocarbon contamination.

Dedication

To the glory of God the Father for giving me this extremely rare opportunity and to my beloved mum (of blessed memory) for believing in me and for investing the very best in me. I love you mum.

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Glossary of abbreviations

AC	Activated carbon
AMO	Ammonia mono-oxygenases
ANOSIM	Analysis of Similarities
ANOVA	Analysis of Variance
AOM	Amorphous Organic Matter
AOA	Ammonia Oxidizing Archaea
Aro-H	Aromatic hydrocarbons
BC	Biochar
BLAST	Basic Local Alignments Search Tool
BTEX	Benzene, toluene, ethylbenzene, m-xylene
CO_2	Carbon dioxide
Cyc-alk	Cyclic alkane
DMSO	Dimethyl sulfuroxide
DNA	Deoxyribonucleic Acid
FID	Flame Ionisation Detector
GAC	Granular Activated Carbon
GC	Gas Chromatography
HSD	Honestly Significant Difference
ICP-OES	Inductively Coupled Plasma-Optical Emission Spectrometry
MDS	Multi-Dimensional Scaling Plots
MMC	Maximum Membrane Concentration
MS	Mass Spectrometry
NAPL	Non Aqueous Phase Liquid
NOM	Natural Organic Matter
PAC	Powdered Activated Carbon
PCA	Principal Components Analysis
RDP	Ribosomal DataBase
SOM	Soil Organic Matter
VOCs	Volatile Organic Compounds
VPH	Volatile Petroleum Hydrocarbons
OTUs	Operational Taxonomic Units
PAHs	Polynuclear Aromatic Hydrocarbons
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PHs	Petroleum Hydrocarbons
PyC	Pyrogenic Carbon-rich
QIIME	Quantitative Insights Into Microbial Ecology
rRNA	Ribosomal Ribonucleic Acid
Str-alk	Straight chain alkanes
USEPA	United States Environmental Protection Agency
VOCs	Volatile Organic Compounds
WN	With nutrients
WON	Without nutrients

Chapter 1: Introduction, aim and thesis scope

1.1 Introduction

Volatile petroleum hydrocarbons are among the hazardous components of crude oil and because of their readily volatile nature, they have the capacity to migrate across different environmental compartments once they are released from a source. Contaminants released from under the ground due either to leakages from storage tanks or groundwater pollution have been reported to intrude into indoor air resulting in elevated concentrations of these compounds in buildings (U.S.E.P.A., 2002; U.S.E.P.A., 2012). Depending on the depth of contaminant source in the subsurface, the concentration of the pollutants and the degree of natural attenuation of the pollutants in the unsaturated zone, the amount of vapour emanating into the atmosphere may vary from low to high concentrations (U.S.E.P.A., 2012).

Claims from a study by McHugh *et al.* (2004) shows that vapour intrusion from the subsurface is often associated with chlorinated VOCs, at least from the majority of sites so far studied. This has been attributed to the poor biodegradability of these compounds by indigenous microbial communities at impacted sites (McHugh *et al.*, 2004; McHugh *et al.*, 2010). In contrast, VPHs are more readily biodegradable by soil microbial communities present at contaminated sites where oxygen is sufficient to support the growth of these organisms, thus reducing the potential for indoor vapour intrusion by this group of VOCs (McHugh *et al.*, 2010). These claims can be challenged based on reports from studies by Hohener *et al.* (2006) and Pasteris *et al.* (2002) in which other factors such as depth of VPH source underneath the ground and soil porosity were shown to affect the level of oxygen that permeates through the unsaturated zone hence affecting the amount of oxygen that becomes available to the petroleum hydrocarbon degraders at certain depths. Measurements of O_2, CO_2 and VPH profiles in the unsaturated zone of soils containing residual NAPL revealed interesting observations where regions closest to the NAPL source had low O_2 concentrations and, high amounts of CO_2 and VPHs while regions that were further away from the NAPL source had low concentrations of CO_2 and VPHs but high O_2 concentrations further highlighting the role of biodegradation in the attenuation of VPHs in the unsaturated zone (Hohener *et al.*, 2006; Luo *et al.*, 2013). Higher concentrations of non-aqueous phase liquid at the point of release may result in a situation where oxygen utilisation by VPH degraders occurs at a rate that limits the rate of aerobic degradation thereby elevating the potential for indoor VPH intrusion. This may also increase the risk of groundwater contamination

as the vapours may also migrate downwards to the water table (Baehr *et al.*, 1999; Pasteris *et al.*, 2002). In addition to the amount of pollutants present in the soil environment, above the groundwater table, oxygen levels are often sufficiently available from the atmosphere via the soil air-filled pore spaces as a result of which inorganic nutrients e.g. nitrogen and phosphorous become the limiting factor to the biodegradation of VPHs in soil (Hohener *et al.*, 2006; Elazhari-Ali *et al.*, 2013).

Biodegradation of organic pollutants have been investigated using a variety of approaches. One common method that has been employed to study the degradation kinetics of organic compounds involves the use of laboratory batch microcosms. Meynet *et al.* (2012) reported the study of short-term effects of powdered and granulated (GAC) activated carbon on the soil respiration of a PAH impacted soil using batch microcosm experiments. In another study carried out by Bushnaf *et al.* (2011), the first order biodegradation rates of a mixture of 12 volatile petroleum hydrocarbons were determined in a batch experiment in which sandy soils were contaminated with the pollutant mixture. Elazhari-Ali *et al.* (2013) conducted a batch as well as mini-lysimeter study to investigate the effects of biofuels – ethanol and biodiesel on the biodegradation of a VPH mix consisting of straight chain/branched alkanes, cycloalkanes and aromatic hydrocarbons.

In addition to microcosm studies, experiments have been designed in columns to study the effects of vapour migration, sorption and biodegradation on the attenuation of volatile organic compounds. In a study conducted by Jin *et al.* (1994), the effects of toluene vapour migration through soil columns of different lengths containing sterilized, pre-exposed and unexposed soils on the biodegradation of the substrates were investigated. In another study by Hohener *et al.* (2003), volatile petroleum hydrocarbon degradation kinetics were determined using laboratory batch and column experiments. Other column studies include a short-term and long-term experiment to investigate the effect of vapour migration along column lengths, sorbent amendment and microbial degradation on the attenuation of a mixture of 12 volatile petroleum hydrocarbons (Bushnaf *et al.*, 2011). The use of these approaches (laboratory microcosms and columns) as a means of gaining insights into the biodegradation processes taking place in the vadose zone have their advantages as well as their limitations. While laboratory experiments offer the flexibility of controlled conditions, it does not account for the complex and variable environmental conditions under which the attenuation of organic pollutants occurs in most cases.

1.2 Research motivation

In dealing with the challenges of contamination of soil and water bodies, Environmental Engineers have adopted a number of remediation approaches. Hitherto, the attention has been on removing pollutants from the environment, in other words reducing *concentration* of pollutants in the environment. However, there is currently a shift in the paradigm of Environmental regulators from a mere reduction of total pollutant concentration in the environment to a reduction in transfer of pollutants to sensitive ecological receptors (Beesley *et al.*, 2011). This arises from a realization of the possibility for onward transfer of toxic organic and inorganic pollutants that persist in the environment to receptors like plants and humans. As a result of this, there is an increase in the adoption of risk-based approaches in the remediation of organic pollutants from the environment in order to mitigate the *effects* of the pollutants in the environment (Fernandez *et al.*, 2005; Beesley *et al.*, 2011).

In situ stabilization of contaminated soils and sediments which involves the use of sorbents to amend polluted environments is one approach that can be used to accomplish both a reduction in concentration of pollutants in the environment as well as mitigate their effects to receptors within the environment (Ghosh *et al.*, 2011). These bind to pollutants thereby reducing bioavailability of pollutants and exposure of such pollutants to humans and the environment. Examples of sorbents being considered for their sorption capacity are activated carbon (AC) and biochar (Bushnaf *et al.*, 2011; Chen and Yuan, 2011; Meynet *et al.*, 2012).

Quite a number of researches are currently being conducted into the sorption properties of biochars and AC with respect to the remediation of soils and sediments contaminated with organic compounds. However, not much has been done with regards to the effects of sorbent amendment on the microbiology of the soils to which they are being applied, particularly with respect to biodegradation of pollutants (Janssen and Beckingham, 2013). This research therefore intends to investigate the potential trade – offs that may or may not exist between the applications of charred materials to soils contaminated with petroleum hydrocarbons as a remediation approach viz-a-viz the biodegradation of the bioavailable fraction of pollutants in the soil. An attempt is also made at understanding the relationship between microbial diversity in the VPH exposed soil treatments and their functions with respect to pollutant degradation and nutrient cycling (Gray and Head, 2001). Biochar is environmentally friendly in terms of its ability to sequester atmospheric C in the aromatic form for several years in the soil (Glaser *et al.*,

2001; Lehmann and Joseph, 2009) whereas AC is coal derived and adds to the carbon footprint of the soil environment (Sparrevik *et al.*, 2011), but has a higher sorption capacity compared to biochar due to the presence of micro-pores and a greater surface area. Both sorbent materials would be used in the current study.

1.3 Research questions

From the review of literature, there are indications that biochar and activated carbon amendment of hydrocarbon polluted soils influences the sorption of some hydrocarbon fractions more than others. The aromatic hydrocarbon, toluene, for instance has been shown to sorb more to biochar in soils compared to straight chain alkanes e.g. octane and cyclic alkanes (cyclohexane) (Bushnaf *et al.*, 2011). This difference in the binding affinity of compounds to biochar also affects the choices as well as biodegradation rate of the available compounds by soil microorganisms. We therefore hypothesize that by amending soils impacted with petroleum hydrocarbons with biochar and activated carbon, a better sorption of one or two fractions of hydrocarbons selected for this project will be observed and this will likely result in a different biodegradation pattern of the soil microbes. Based on this hypothesis, the following research questions will be investigated:

1. Are there strong negative correlations between sorption of organic pollutants in the soil and biodegradation of organic pollutants?

2. Does biochar or AC amendment of soil alter the growth kinetics of microorganisms in a VPH contaminated soil?

3. What are the effects of biochar or AC amendment and nutrient amendment effects on the bacterial community response in aerobic soil?

1.4 Aim and objectives

The ultimate aim of this research is to assess and to also gain an improved understanding of the effects of biochar and activated carbon amendment of petroleum polluted soils on microbiological processes, with specific interest in pollutant biodegradation, taking place within the soil. In order to achieve this aim, and to provide answers to the aforementioned research questions, the following objectives will be considered:

1. To determine any correlations between sorption of organic compounds by sorbents and biodegradation of organic pollutants in the soil.

2. To determine the effects of biochar and activated carbon amendment on the growth kinetics of microorganisms using $^{13}C_7$-toluene as a model VPH in gravelly sand.

3. To determine the effects of biochar and AC amendment as well as nutrient amendment effects on the bacterial community response following exposure of soil to a VPH NAPL source.

1.5 Thesis scope and structure

To start with, the microbiology of petroleum hydrocarbon degradation is reviewed in chapter 2. Pathways for the metabolism of different classes of petroleum hydrocarbons are discussed and then the chemodynamics of organic pollutants once in the environment are also reviewed. The effects of different processes including sorption, biodegradation, and diffusion on the fate of organic pollutants in the environment are further discussed before considering the conventional remediation approaches. The chapter ends by reviewing a novel *in situ* sorbent amendment as a cost-effective and environmentally friendly remediation strategy and their effects on soil biota.

Chapter 3 presents a chemical analysis of the fate of volatile petroleum hydrocarbons in gravelly sand using $^{13}C_7$ toluene as a model VPH as a way of gaining improved understanding of the effects of sorbent amendment on the growth kinetics of microorganisms in VPH contaminated soil.

As a sequel to Chapter 3, a study of the microbial community structure dynamics upon addition of VPHs to sorbent amended soil using batch experiments were conducted. Chapter 4 considers the effects of different classes of VPHs on the microbial community response in soil without sorbent amendment in the short-term while chapter 5 factors in the effects of inorganic nutrient (N & P) limitation and amending soil with and without biochar and activated carbon on the microbial community response over a duration of 6 days in a batch set-up.

Chapter 6 is a study that investigates the long-term effects of exposure to VPH vapours from a NAPL source containing a mixture of VPHs. The chapter is a follow up study to a previous study (PhD research) by Bushnaf (2014) in which glass columns were packed with soil, or soil & 2% biochar or soil & 2% AC and exposed to pollutant (VPH

mixture) vapours over a duration of 430 days and considers the microbial community response to VPH exposure using next-generation sequencing approaches. Chapter 7 presents the conclusions, any remaining research questions that are yet to be answered and suggestions for future work.
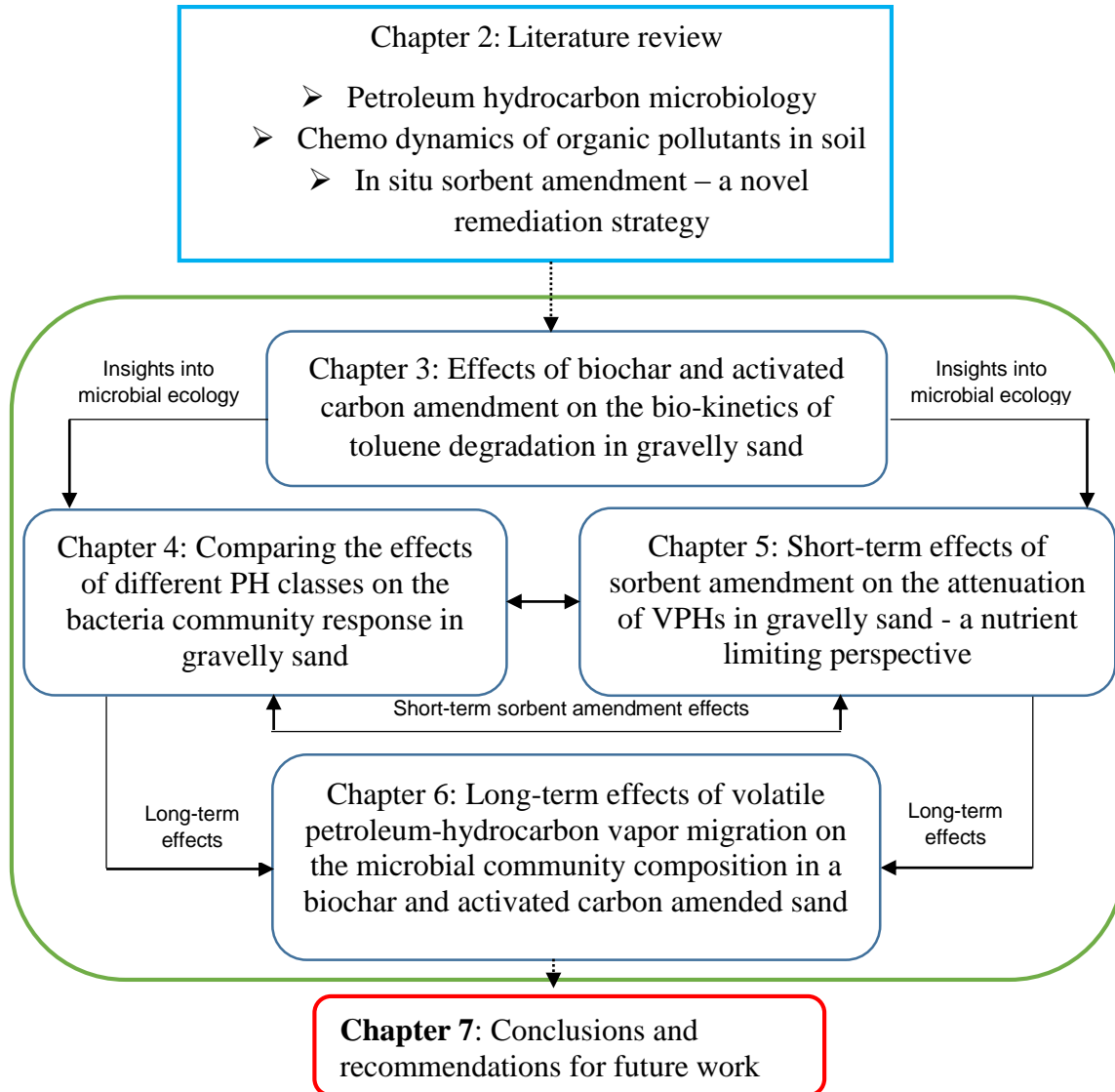
Figure 1.1. Schematic of thesis scope and structure

Chapter 2: Literature review

2.1 Petroleum hydrocarbon degrading microbial communities

Quite a number of bacterial and fungal species have been identified with the capacity to utilize petroleum hydrocarbons as their sole source of carbon and energy even though the percentage of the overall heterotrophic microbial community represented by these organisms (bacteria and fungi) varies according to reports. The most important genera (not an exhaustive list) of petroleum hydrocarbon degrading bacteria isolates based on frequency of isolation from both marine and soil environments include *Achromobacter* (Gojgic-Cvijovic *et al.*, 2012), *Acinetobacter* (Throne-Host *et al.*, 2007), *Nocardia* (Colores *et al.*, 2000; Kalme *et al.*, 2008; Zeinali *et al.*, 2008)*, Pseudomonas* (Asinder and Williams, 1990; Perfumo *et al.*, 2006), *Bacillus* (Gojgic-Cvijovic *et al.*, 2012; Mukherjee and Bordoloi, 2012b) *Arthrobacter* (Jones *et al.*, 1983), *Flavobacterium*, *Micrococcus*, *Alcaligenes* and the *Coryneforms* (Adebusoye *et al.*, 2007). Important fungal genera that have been implicated with petroleum hydrocarbon degradation include *Rhodotorula*, *Aureobasidium*, *Candida* and *Sporobolomyces* from marine environments while *Mortierella*, *Trichoderma*, *Graphium*, *Talaromyces*, *Amorphoteca*, *Neosartorya*, *Candida*, *Yarrowia* and Pichia have been isolated from petroleum hydrocarbon contaminated soils (Chaillan *et al.*, 2004). Others are *Cephalosporin*, *Penicillium* and *Aspergillus* (Chaillan *et al.*, 2004; Singh, 2006).

Other important terrestrial and aquatic members of the microbial community are protozoa and algae. Hitherto, these have not been implicated with the degradation of petroleum hydrocarbons at contaminated sites. Studies by Cerniglia *et al.* (1980) however indicate the capacity of some algae to metabolize the poly-nuclear aromatic hydrocarbon – naphthalene. There are no indications that protozoa have the capacity to utilize any class of petroleum hydrocarbons. In the final analysis, protozoa and algae do not appear to play any significant role in the in situ degradation of petroleum hydrocarbons at contaminated site at least from available reports. The table below summarizes some petroleum hydrocarbon degraders along with their petroleum hydrocarbon substrates:

Substrate	Microorganism
Saturates:	
n-Pentane	*Mycobacterium smegmatis, Norcadia sp.*
n-Hexane	*Mycobacterium smegmatis, Norcadia sp.,* Hyalodendron, Varicosporium, Paecilomyces and Cladosporium.
n-Octane	*Pseudomonas , P. putida, Corynebacterium sp. 7EIC, Nocardia spp.*
n-Decane	*Corynebacterium*
n-Dodecane	*Arthrobacter, Acinetobacter, Pseudomonas putida and yeasts.*
Methylcyclopentane	*P. anaerooleophila*
Methylcyclohexane	*Nocardia petroleophila*
Isooctane	*Pseudomonas sp.,* Acinetobacter
Aromatics:	
Toluene	*Bacillus sp., P. putida, Cunninghamella elegans, Pseudomonas aeruginosa, P. mildenbergii,* methanogens, anerobes, *Methylosinus trichosporium OB3b, Pseudomonas spp., Achromobacter sp., P. anaerooleophila, Azoarccu tolulitycus Tol-4 (anaerobic), Nocardia coralline, P. gladioli BSU 45124, P.putida, Psychrotrophic spp.,* iron reducing bacteria, *Phanerochaete chrysosporium, Microcystis aeruginosa, Seanastrum capricornatum* and *Thauera selenatis* (anaerobe)
m-Xylene	*Pseudomonas putida, Pseudomonas aeruginosa, Phanerochaete chrysosporium,* methanogens and anaerobes.
1, 2, 4-Trimethylbenzene	Yeasts.

Table 2.1. Some Petroleum hydrocarbon degraders and their substrates. Source: Riser-Roberts (1998).

In addition to PHs serving as carbon and energy sources to microorganisms at contaminated sites, other nutrients like nitrogen and phosphorous have been shown to play significant roles in the stimulation of PH degraders. Several studies have been conducted to investigate the strategies for the delivery of these nutrients as well as the optimum amounts required to stimulate microbial activity (Lee, 1995; Boufadel *et al.*, 1999; Obuekwe *et al.*, 2001).

2.1.1 *Metabolism of petroleum hydrocarbons*

As mentioned earlier, PHs have been categorised into three (3) classes on the basis of their structures, viz: alkanes (normal and branched), cycloalkanes and aromatic hydrocarbons. Other classes of PHs such as alkenes and alkynes have been reported to occur in trace amounts in crude oil (Okoh, 2006; Chandra *et al.*, 2012). Studies reveal that n-alkanes are more readily biodegraded than branched (iso) alkanes with the n-alkanes of chain length between C_{10} and C_{25} being more susceptible to microbial attack. Alkanes of shorter chain length ($< C_{10}$) are volatile and easily evaporate during the weathering process due to their low molecular weights and have been reported to be toxic to microbes. Straight chain alkanes of chain length between C_{30} and C_{40} have been shown to support the growth of *Acinetobacter calcoaceticus* and *Nocardioforms* respectively (Radwan *et al.*, 1999).

Terminal oxidation of methyl groups (terminal) produces a primary alcohol which is subsequently oxidised to carboxylic acids and has been reported to occur in *Pseudomonas sp.* and *Acinetobacter sp.* (May and Katapodis, 1990; Lal and Khanna, 1996). Other organisms like *Rhodococcus sp.* have been shown to carry out sub-terminal oxidation of n-alkanes in addition to terminal oxidation in reactions catalysed by monooxygenases. Sub-terminal oxidation of n-alkanes produces a secondary alcohol, then a ketone and finally a fatty acid (Whyte *et al.*, 1998). This pathway (sub-terminal pathway) is not considered as the primary metabolic pathway for most PH degraders (Atlas, 1981).
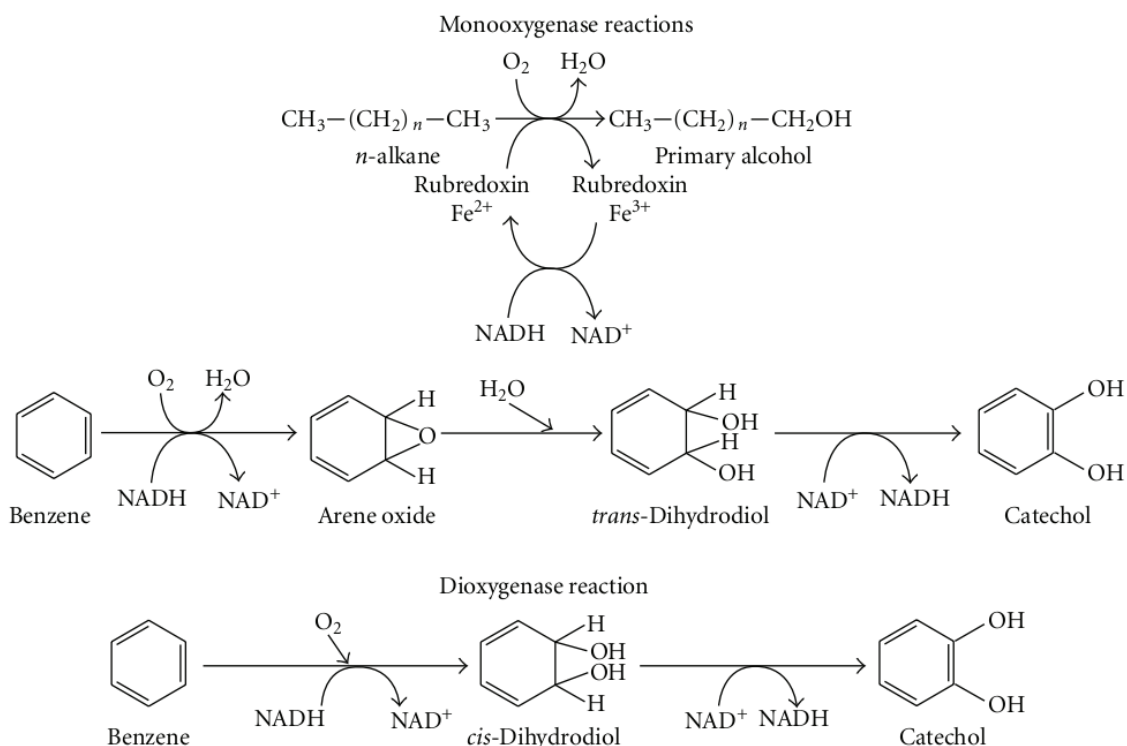
Figure 2.1. Metabolic pathways highlighting the enzymatic processes involved in the metabolism of petroleum hydrocarbons (Das and Chandran, 2010).

Microbial degradation of aliphatic hydrocarbons proceeds with the oxidation of the substrates in a series of reactions catalysed by oxygenases. Oxidation which could either be terminal, sub-terminal or diterminal depending on the organism involved results in the formation of mono and/or dicarboxylic acids which are subsequently metabolised via the β-oxidation pathway of fatty acids. Other minor pathways for the metabolism of PHs are α-oxidation and ω-oxidation pathways (Riser-Roberts, 1998).

In prokaryotes, aromatic hydrocarbons are converted to *cis*-dihydrodiols by the action of dioxygenases, then to dihydroxy compounds such as catechol in the case of benzene. Eukaryotes and fungi oxidise aromatic hydrocarbons using an atom of oxygen (O) in a reaction

catalysed by monooxygenases to produce *trans*-dihydrodiols and finally catechol. Ultimately, catechol undergoes further oxidation to produce intermediates in the citric acid cycle (Riser-Roberts, 1998).

Substrate	Enzyme	Microorganism	Reference
$C_1 - C_8$ alkanes, alkenes, and cycloalkanes	Soluble methane Monooxygenases	*Methylococcus* *Methylosinus* *Methylocystis* *Methylomonas*	(McDonald *et al*., 2006)
$C_5 - C_{16}$ alkanes, fatty acids, alkyl benzenes, cycloalkanes	AlkB related alkane Hydroxylases	*Pseudomonas* *Burkholderia* *Rhodococcus* *Mycobacterium*	(Jan *et al*., 2003)
$C_{10} - C_{16}$ alkanes, fatty acids	Eukaryotic P450	*Candida maltosa* *Candida tropicalis* *Yarrowia lipolytica*	(Iida *et al*., 2006)
$C_5 - C_{16}$ alkanes, cycloalkanes	Baterial P450 oxygenase system	*Acinetobacter* *Caulobacter* *Mycobacterium*	(Van Beilen *et al*., 2006)
$C_{10} - C_{30}$ alkanes	Dioxygenases	*Acinetobacter sp.*	(Maeng *et al*., 1996)

Table 2.2. Some enzymes involved in petroleum hydrocarbon degradation and their substrates.

2.2 Environmental risk assessment: Effects of pollutants versus concentration of pollutants in   the environment

2.2.1 *Chemo dynamics of organic pollutants in the environment*

A major factor that determines the concentration and consequently the effects of pollutants in the environment is the rate at which their chemical and biological transformation occurs. As most pollutants occur in low concentrations in the environment, the process of transformation of these compounds proceeds via first order kinetics. Hence,

$$R \propto [A] \quad\quad\quad (2.1)$$

$$R = K * [A] \quad\quad\quad (2.2)$$

$$\frac{d[A]}{dt} = K * [A] \quad\quad\quad (2.3)$$

where R is the rate of reaction/uptake of pollutant, $[A]$ is the concentration of pollutants and K the proportional change per unit time.

Pollutant uptake by microbial cells occurs via three (3) main mechanisms, namely active transport, passive transport and filtration. The mechanism of uptake depends upon the solubility of the compounds as well as on the size of the molecules. Microbial cytoplasmic membranes serve as barriers to regulate the flux of compounds within the cells. The cell membrane is composed of a phospholipid bilayer and forms a matrix in which transport proteins and enzymes are localised (Sikkema *et al.*, 1995). Phospholipids are made up of a molecule of glycerol attached to two fatty acid moieties and a charged molecule such as inositol, choline, ethanolamine or serine (Nelson and Cox, 2005). The proteins embedded in the lipid bilayer often occur in a folded form such that they invaginate into the membrane forming pores through which compounds can diffuse. Depending on their sizes, neutral compounds, particularly if they are lipophilic in nature, traverse the cytoplasmic membrane without any obstruction by passive diffusion according to Fick's law of diffusion (Shane, 1994). As the molecules increase in size, or become charged, the predominant means of transport across the cytoplasmic membranes switches to the active transport which requires the expenditure of chemical energy (Shane, 1994). Several mechanisms by which hydrocarbons are transported across microbial membranes have been proposed. For example, cyclic hydrocarbons can easily diffuse across the lipid bilayer of the membrane due to their non-polar nature (Sikkema *et al.*, 1995). The uptake of the non-polar PAH naphthalene in a *Pseudomonas* species did not require ATP or any electrical potential suggesting that passive

diffusion was a likely means of transport across the membrane (Bateman *et al.*, 1986). Witholt *et al.* (1990) proposed that the uptake of alkanes was facilitated by the release of the outer membrane lipopolysaccharide which encapsulates droplets of hydrocarbons thereby enhancing mass transfer efficiency. Other studies observed the inclusion of hydrocarbons in bacteria and yeast and in a strain of *Pseudomonas* (Scott and Finnerty, 1976).

Persistence of organic compounds in the environment has been related to two main properties of such compounds; viz: the inaccessibility of compounds to soil microorganisms for biodegradation and their toxicity to soil microbiota. The presence of these persistent compounds in high concentrations in the environment thus makes bioremediation of contaminated sites a serious challenge.

Toxicity of most organic compounds is thought to be correlated to their hydrophobicity as indicated by the logarithm of partition coefficient of those compounds between octanol and water (logP). There are indications that most water-soluble compounds are relatively less toxic to microorganisms. At the same time, the much more lipophilic compounds including some hydrocarbons have been reported not to be toxic to whole cells. In between these two categories are compounds with intermediate hydrophobicity such as alcohols, phenols and aromatic compounds which have been applied as antimicrobial agents in food preservatives, and disinfectants e.t.c. due to their highly cytotoxic nature (Heipieper *et al.*, 1991a). Studies have shown that the toxicity of hydrophobic organic compounds including petroleum hydrocarbons is due to a general, nonspecific effects of these compounds on the fluidity of microbial cytoplasmic membrane as they accumulate in the lipid bilayer and not due to any specific chemical reactions within the bilayer (Cabral, 1991; Saito *et al.*, 1994; Farranate *et al.*, 1995).

In addition to hydrophobicity as a factor that determines toxicity of PHs, highly toxic intermediates of the metabolism of some complex aromatic PHs as well as alkanes could also accumulate within the cytoplasm of microbial cells (Camara *et al.*, 2004). An example of this effect was observed in the biodegradation of *n*-octane during which a toxic intermediate 1-octanol was reported to accumulate within the cells (Chen *et al.*, 1995). The preferential partitioning of hydrocarbons into the lipid bilayer of microorganisms is reportedly the primary cause of toxicity as this process results in the accumulation of the compounds within the lipid bilayer and a subsequent increase in non-specific permeability of the membrane. The molecular structure of hydrophobic compounds also affects their solubility within the membrane. For example, amphiphatic molecules with a similar structure to that of membrane phospholipids will solubilise relatively easily compared to other compounds. Because of this

property, chlorinated derivatives of aromatic compounds e.g. phenolics and other compounds such as alkanols are known to be highly toxic. The composition of membrane phospholipid fatty acid has also been shown to influence the toxicity of hydrocarbon compounds in a study involving artificial membranes (Antunes-Madeira and Madeira, 1989; Weber and de Bont, 1996).

Another mechanism that has been proposed as an explanation for the cytotoxic effects of PHs is a reduction of the energy status of microbial cells (Heipieper and Martinez, 2010). From the foregoing discussion, increased permeabilization of microbial membranes leads to a flux of protons and other ions across the membranes which in turn dissipates the proton motive force (PMF) and the electrical potential of the membranes (Sikkema *et al.*, 1994). In a study by Uribe *et al.* (1990), the functions of the enzyme ATPase as well as other proteins embedded in the membrane and involved in energy transduction was found to be impaired by the effects of organic compounds.

Organic Compound	$\log P_{o/w}$ (mM)	$\log P_{m/b}$ (mM)	$P_{m/b}$ (mM)	Solubility (mg/L)	MMC (mM)	Toxicity
Toluene	2.48	1.77	58.29	6.3	367	+
n-Octane	4.55	3.77	5,936.08	0.0058	34	-
p-Xylene	3.17	2.43	272.21	1.2	327	+
Ethylbenzene	3.03	2.30	199.11	1.6	319	+
Hexane	3.29	2.55	355.88	0.150	53	-
Decane	5.61	4.80	63,343.20	0.00035	22	-
Cyclohexane	3.50	2.76	568.85	0.500	284	+
1-octanol	2.92	2.19	154.90	3.8	588	+
1-Decanol	3.97	3.21	1,621.80	0.23	379	+
1-Hexanol	1.87	1.17	14.80	56.9	841	+
1-Dodecanol	5.02	4.23	16,982.40	0.015	254	+
Naphthalene	3.30	2.56	363.92	0.240	87	-
Phenanthrene	4.46	3.69	4,855.12	0.006	29	-

Table 2.3. Partition coefficient of some petroleum hydrocarbons in octanol/water (logPo/w), membrane buffer (logPm/b), solubility in water at 25 °C and the maximum membrane concentration of organic compounds. + = toxic; - = not toxic. Source: (http://chem.sis.nlm.nih.gov/chemidplus/, 2005).

The effect of pollutants, once in the environment, is determined by their dynamics in the four (4) main compartments of the ecosphere, viz: air, water, soil/sediments and biota. Inter-compartmental transfer rates of pollutants is largely dependent upon the transfer rates occurring at the interface between two compartments. At equilibrium, the rates of transfer of these compounds between the two compartments are equal. Several processes interact in the environment to determine the fate of VOCs in the soil subsurface. The dominant processes include the following:

1. Sorption
2. Volatilisation and
3. Biodegradation

According to Schwarzenbach *et al.* (1993), sorption is defined as "The process by which chemicals become associated with solid phases (either adsorption onto a two-dimensional surface, or absorption into three-dimensional matrix". It is a key process that has been reported to determine the fate and ecotoxicity of organic pollutants in soils and sediments as sorption lengthens the persistence of organic pollutants in the environment. This process reduces availability of the pollutants to microorganisms and consequently their biodegradability but concurrently also reduces the concentration of the compounds in soil pore-water, the ecotoxicity and uptake of the pollutants by soil biota. At equilibrium, the ratio of a substance's total concentration in the sorbed phase (sorbent) to their total concentration in solution is denoted by the solid-water distribution coefficient (K_d) (Schwarzenbach *et al.*, 1993):

$$K_d = \frac{C_s}{C_w} \frac{(mol \cdot Kg^{-1})}{(mol \cdot L^{-1})} \qquad (2.4)$$

where C_s is the compound concentration in the sorbed phase in moles/Kg, C_w is the concentration in solution in moles/L and K_d the solid-water distribution coefficient in L/Kg.

It has been proposed that sorption of organic compounds to soil is a function of the organic matter content of soils. The soil-water distribution coefficient was described by Pasteris *et al.* (2002) according to the following equation:

$$K_d = f_{oc} * K_{oc} \qquad (2.5)$$

where K_d is the solid-water distribution coefficient of the soil or sediment system, f_{oc} is the mass fraction of the more amorphous soil organic carbon and K_{oc} is the sorption coefficient of the organic carbon content of soil/sediment.

More recently, the solid-water distribution coefficient (K_d) of soils and sediments has been normalised to the total organic carbon content of the carbonaceous geosorbents present within the systems. Hence, for unamended soils and sediments containing pyrogenic carbon-rich materials (PyC)(Cornelissen *et al.*, 2005; Hale *et al.*, 2015), equation (2.4) becomes:

$$K_d = f_{oc} * K_{oc} + f_{PyC} * K_{PyC} \qquad (2.6)$$

where f_{PyC} and K_{PyC} are the mass fraction and sorption coefficient of the native carbonaceous geosorbents such as soot, black carbon, and charcoal in soil or sediments. Carbonaceous geosorbents have been shown to possess strong sorption capacities and their presence in soils and sediments can enhance the overall sorption capacities by several orders of magnitude in comparison to pristine soils and sediments (Cornelissen *et al.*, 2005).

Upon addition of biochar or activated carbon to soil, the overall sorption to soil becomes further modified as follows:

$$K_d = f_{oc} * K_{oc} + f_{PyC} * K_{PyC} + f_{AC/BC} * K_{AC/BC} \qquad (2.7)$$

where $f_{AC/BC}$ is the mass fraction and $K_{AC/BC}$ the sorption coefficient of activated carbon or biochar.

The 'dual-mode' sorption theory has been proposed. According to this theory, soil organic matter consists of two organic domains; an amorphous organic matter (AOM) domain also described as the 'soft' domain and the older soil organic matter domain which is also referred to as the 'hard' or 'glassy' domain (Xing and Pignatello, 1997). Both SOM domains have been reported to sorb organic compounds differently and with the passage of time, the AOM domain have been shown to undergo transformation into the 'hard' or 'glassy' state through changes in composition of elements, polarity, condensation and aromaticity (Ran *et al.*, 2007). Worthy of note are the differences between natural organic matter (NOM) and charred organic matter e.g. charcoal, biochar, char e.t.c. with respect to their sorption properties (Smernik, 2009). The sorption capacity of pyrogenic organic matter is reported to be several times higher than that of natural organic matter (Baring *et al.*, 2002; Yang and Sheng, 2003). Also, significant differences exist between pyrogenic and non-pyrogenic organic matter in terms of the mechanism of sorption of the sorbates, reversibility of sorption and the dependence of sorption on the concentration of sorbates. While natural organic matter sorption affinity depends to a less extent on the concentration of the sorbate (as shown by the linear sorption isotherms) and has been described as *absorption* or *partitioning*, the sorption affinity of pyrogenic organic matter depends largely on the concentration of sorbates and

decreases with a rise in the concentration of sorbates. This phenomenon is described as *adsorption* (Smernik, 2009).

As a result of the readily volatile nature of most VOCs, the composition of a mixture of volatile compounds will vary from time to time (Wang *et al.*, 2003). Gas-phase diffusion has been reported to be the predominant process for VOCs migration in the vadose zone (Pasteris *et al.*, 2002; Hohener *et al.*, 2006). Diffusive fluxes for each hydrocarbon compound in a mixture of 13 petroleum hydrocarbons were determined in a lysimeter study conducted by Pasteris *et al.* (2002) using Fick's first law of diffusion as given below:

$$F = -D\frac{\partial C_a}{\partial z} \qquad (2.8)$$

Where *F* represents the vapour diffusive fluxes at the lysimeter surface (g C m^{-2} d^{-1}), $\partial C_a/\partial z$ is the vapour phase concentration gradient at the lysimeter surface (g C m^{-4}), z = 0 and *D,* the effective diffusion coefficient in soil, is the product of the air-filled soil porosity, the tortuosity factor and molecular diffusion coefficient in air (m^2/d). A derivative of equation 2.8 can be obtained according to the methods described by Schwarzenbach *et al.* (1993) assuming a spatially constant diffusion coefficient *D* is taken into account. Equation 2.7 then becomes:

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2} \qquad (2.9)$$

where x and t are distance in meters and time in days. This expression is also known as Fick's second law of diffusion and can be used to describe the transient diffusion of volatile compounds in soil (Schwarzenbach *et al.*, 1993).

A modified version of mathematical model was proposed by Jin *et al.* (1994) and was used to describe the vapour-phase diffusion based on the following assumptions:

1. Diffusion is the predominant transport process of the vapour phase in the subsurface
2. The sorbed and the dissolved phases undergo reversible, linear equilibrium sorption
3. The dissolved and gaseous phases comply with Henry's law
4. The biodegradation reactions obey first order kinetics and
5. Soil diffusion coefficients for the gaseous and dissolved phases are modified from their values in air and water by tortuosity factors according to the methods described by Millington and Quirk (1961):

$$\tau_a = \theta_a{}^{2.33}/n_{tot}{}^2 \tag{2.10}$$

$$\tau_w = \theta_w{}^{2.33}/n_{tot}{}^2 \tag{2.11}$$

where θ_a is the volumetric content of soil air (m^3 gas m^{-3}), θ_w is the volumetric content of soil water (m^3 gas m^{-3}), n_{tot} is the total porosity in the soil. Considering the above equation (2.10 & 2.11), the vapour-phase transport model from (2.9) then becomes:

$$Ra\frac{\partial C_a}{\partial t} = D\frac{\partial^2 C_a}{\partial z^2} - r(C_a) \tag{2.12}$$

where Ra is the capacity factor (m^3 gas m^{-3}) and D the diffusion coefficient are given by:

$$Ra = (\rho_b K_d + \theta_w + \theta_a H)/H \tag{2.13}$$

$$D = (\theta_a \tau_a D_a H + \theta_w \tau_w D_w)/H \tag{2.14}$$

where ρ_b is the soil bulk density (kg m^{-3}), K_d is the distribution between the solid and the dissolved phases (m^3 kg^{-1}), H is the Henry's law dimensionless coefficient (kg m^{-3} air/ kg m^{-3} water), D is the effective diffusion coefficient of any fuel compound in air (m^2/d), D_a is the molecular diffusion coefficient in air (m^2/d), D_w is the molecular diffusion coefficient in water (m^2/d), and $r(C_a)$ is the degradation term (g m^{-3} d^{-1}).

The concept of molecular diffusivities has been proposed based on the Brownian motion of molecules through a medium of interest. The relationship between molecule sizes and diffusivities in different media has also been proposed. According to Schwarzenbach *et al.* (1993), molecules with larger molecular masses and molar volumes both indicative of larger sizes tend to have lower diffusivities as a result of reduced mean free path which also reduces their ability to move through a crowd of other molecules. Other factors such as the viscosity of the medium of interest and the temperature of the molecules have also been suggested to affect molecular diffusivities. Several methods have been used for the quantification of molecular diffusivities of chemicals in gas and aqueous phases. One of such approaches that has been used to estimate the diffusivities of organic molecules in air is the method of Fuller *et al.* (1966):

$$D_a = 10^{-3}\frac{T^{1.75}[(1/m_{air})+(1/m)]^{1/2}}{P[V_{air}{}^{1/3}+V^{1/3}]^2} \quad \text{(cm}^2\text{ s}^{-1}) \tag{2.15}$$

where

T is the absolute temperature (K)

m_{air} is the average molecular mass of air (28.97 g mol^{-1}),

m is the organic chemical molecular mass (g mol^{-1}),

P is the gas phase pressure (atm),

V_{air} is the average molar volume of the gases in air ($\sim$ 20.1cm^3 mol^{-1})

V is the molar volume of the chemical of interest (cm^3 mol^{-1})

2.3 Growth dynamics of heterotrophic microorganisms – The Monod and Contois models

Heterotrophic bacterial growth in soils is a function of the type as well as the diversity of the organisms present in the soil. It also depends on the soil organic matter content. In the absence of any growth inhibition due to contaminant toxicity, the growth of microorganisms can be described by the mathematical model proposed by Monod (1949):

$$\mu = \mu_{max} \frac{S}{K_s + S} \tag{2.16}$$

where μ, and μ_{max} are the specific growth rate (per hour) and maximum specific growth rate (per hour), K_s is the half-velocity constant which is equivalent to the concentration of the nutrients at half the maximum growth rate (mg g^{-1}) and S is the initial substrate concentration (mg g^{-1}soil).

Reports have suggested that the maximum specific growth rate (μ_{max}) of heterotrophic microorganisms depends on the type of organism and the carbon or energy source being utilised while the half-velocity constant is a measure of the affinity of the organism for the limiting nutrient and is an indicator of how fast the maximum specific growth rate can be attained (Chen *et al.*, 2003). For efficient microbial performance, the half-velocity constant must be a minimum value which implies that that the microbes in the soil should be adapted to the soil organic matter or carbon source being introduced.

The Contois equation (Chen *et al.*, 2003) can be used to describe the relationship between biomass formation and organic matter or a carbon source depletion in a heterotrophic bacterial growth as follows:

$$\frac{\partial X}{\partial t} = \frac{\mu_{max} S X}{K_s + S} - k_d X \tag{2.17}$$

$$\frac{\partial S}{\partial t} = -\frac{1}{Y} \left[\frac{\mu_{max} S X}{K_s + S} \right] \tag{2.18}$$

where X is the mass of biomass (mg g^{-1}soil, and K_d is the specific microbial decay rate (mg biomass h^{-1}, Y is the stoichiometric yield coefficient (mg biomass formed mg^{-1} carbon utilised). Since the amount of biomass formed can be expressed in terms of nutrient concentration and vice-versa, it is possible to simulate biomass formation and nutrient depletion as a function of time as follows:

$$S = S_0 - (X - X_0)/Y \qquad (2.19)$$

$$X = X_0 - Y(S_0 - S) \qquad (2.20)$$

where S_0 and X_0 are initial substrate concentration (mg g^{-1}soil) and initial mass of biomass (mg g^{-1}soil) respectively. Substituting for S and X in equations 2.17 and 2.18, we have:

$$\frac{\partial X}{\partial t} = \frac{\mu_{max}X\left[S_0 - \frac{X-X_0}{Y}\right]}{K_s + \left[S_0 - \frac{X-X_0}{Y}\right]} - k_d X \qquad (2.21)$$

$$\frac{\partial S}{\partial t} = -\frac{1}{Y}\left\{\frac{\mu_{max}S[X_0 - Y(S_0 - S)]}{K_s + S}\right\} \qquad (2.22)$$

2.3.1 *The Nitrogen cycle*

Nitrogen is the most abundant element in the atmosphere constituting about 78% of the atmosphere and as such the most likely to limit ongoing natural processes in the ecosystem. Nitrogen in the soil is derived from a range of sources including plants and animal wastes, and from incorporation into the soil by nitrogen fixing bacteria which may either be free living or living in a mutual association with the root nodules of leguminous plants. Atmospheric nitrogen (N_2) is quite stable because of the triple bonds between the nitrogen atoms and needs to be converted to a form that is more readily utilisable by plants and other organisms. Using the energy generated from the oxidation of carbohydrates, and electrons supplied by the nitrogenase enzyme complex, a molecule of nitrogen (N_2) is reduced to two moles of ammonia (NH_3) as follows:

Nitrogenase enzyme complex

$$N_2 + 8H^+ + 8\ e^- + (16\text{-}24)\ ATP \longrightarrow 2NH_3 + H_2 + (16\text{-}24)\ ADP + (16\text{-}24)\ P_i \qquad (2.23)$$
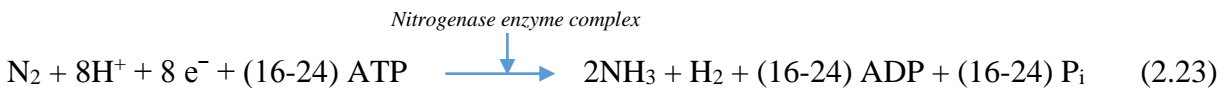
The process of nitrification coverts ammonia into nitrates via a two-step reaction both mediated by nitrifying bacteria. In the first step, ammonia, a chemolithotrophic substrate, is converted to nitrites by the bacterial species *Nitrosomonas* which oxidises ammonia to nitrites followed by a second step in which a different group, *Nitrospira* oxidises nitrites to nitrates as shown below:

$$NH_3 + O_2 + 2\ H^+ + 2\ e^- \xrightarrow{\text{ammonia monooxygenase}} NH_2OH + H_2O \qquad (2.24)$$

$$NH_2OH + H_2O \xrightarrow{\text{hydroxylamine oxidoreductase}} NO_2^- + 5\ H^+ + 4\ e^- \qquad (2.25)$$

$$H_2O + NO_2^- \xrightarrow{\text{nitrite oxidoreductase}} NO_3^- + 2\ H^+ + 2e^- \qquad (2.26)$$
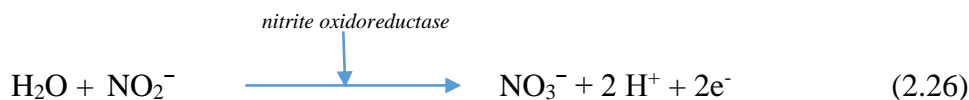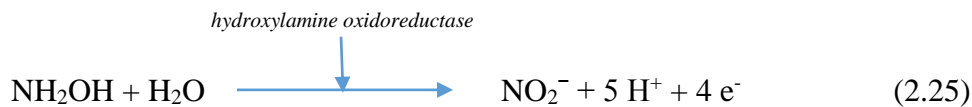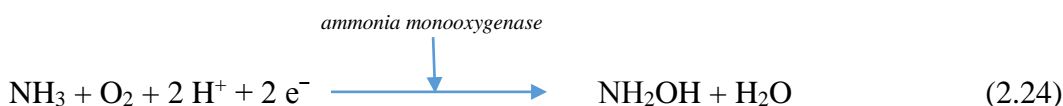
The ammonia monooxygenase enzymes have been implicated in the oxidation of ammonia to hydroxyl amines (2) and these enzymes are reported to be integral membrane proteins (Chen *et al.*, 2003). In the ammonia oxidation step, two electrons are required for the oxidation process and these are generated by the oxidation of hydroxylamines in a reaction catalysed by hydroxylamine oxidoreductase. In total, four electrons are produced in this step but only two reach the terminal electron acceptor (O_2) via the cytochrome oxidases to produce water and energy.

The environmental significance of the nitrogen cycle is the balance created between atmospheric nitrogen and soil nitrogen each year by this process. Intensely fertilised soils from agricultural activities lead to excess accumulation of ammonia and by implication of nitrites and nitrates which are highly water-soluble compounds. These compounds may leach into groundwater and react with organic compounds to form toxic products such as nitrosoamines (Madigan *et al.*, 2009). There are also reports of a blood disorder, methemoglobinemia, caused by the presence of nitrates in drinking water (Slonczewski and Foster, 2014). Amending such excessively fertilised soils with charred materials like biochar or activated carbon may reduce the amount of inorganic nitrogen in these forms that reach groundwater and by extension alleviate any ecotoxicological effects associated with these compounds in solution.

2.4 Modelling the fate of organic contaminants in the environment

More recently, the concept of modelling the fate of organic pollutants in soils and sediments is gaining wider acceptance. Pasteris *et al.* (2002) reported the modeling of vapour phase transport of a mixture of 13 petroleum hydrocarbon compounds to groundwater and to the atmosphere and also monitored the rate of arrival of the persistent components to the groundwater. Bushnaf *et al.* (2011) reported the predictions of a pollutant fate model based on first-order rate biodegradation kinetics of volatile petroleum hydrocarbons in soil pore water of biochar amended soil which did not match experimental values. As a sequel to this study, a more 'robust' mathematical model was developed by Meynet *et al.* (2014) to predict the fate

of VPHs in soils amended with and without biochar based on microbial growth dynamics in addition to physicochemical properties of soil and biochar used in the previous study by Bushnaf *et al.* (2011).

It becomes apparent that mathematical models are an invaluable tool for use not only in predicting the fate of contaminants in the environment given a set of environmental parameters, but can also be used to study several factors and processes that may be interacting to produce specific effects that can be observed. More specifically, predicting pollutant biodegradation viz-a-viz soil microbiology remains a challenge. The advent of new molecular methods empowers us to gain an improved understanding of soil microbial ecology and hence to better investigate the biodegradation pollutants in the soil (Guermouche *et al.*, 2013). Predictive models may be used to augment results from chemical and ecotoxicological tests as it makes room for consideration of the effect of factors that would otherwise not be considered in a laboratory or field experiment.

2.5   Current remediation strategies

Traditionally, the clean-up of organic contaminants in the environment involves a consortium of different approaches depending on the prevailing circumstances. Physical, chemical and biological remediation strategies may be used in concert one with another in order to alleviate pollutant concentration to an 'ecologically' acceptable level (RAAG, 2000).

Physical remediation approaches range from soil washing to incineration, thermal desorption, to soil flushing and encapsulation (Robertson *et al.*, 2003). Soil washing involves cleaning contaminated soils using a combination of solvents with water and some mechanical action to reduce the concentration of pollutants present in the soil (Khan *et al.*, 2004). The choice of solvents used in this approach is based on their potential environmental and health effects and their ability to dissolve certain contaminants. Petroleum hydrocarbons bind more readily to smaller soil particles such as clay and silt. The process of washing separates smaller particles (e.g. silt and clay) from the more coarse particles (e.g. sand), hence, during soil washing, the smaller volume of soil composed mainly of clay and silt and containing most of sorbed hydrocarbons is collected and can be further treated by more suitable methods such bioremediation while the larger faction composed mainly of sand and gravel is considered to be non-hazardous as it contains less amount of pollutants and can be used to refill the excavated site (USEPA, 1996; RAAG, 2000). Another two-step procedure for treating non-volatile and semi-volatile organic compounds e.g PAHs, diesel fuel and fuel oils has been reported by (McBean and Anderson, 1996). Contaminated soils are excavated and placed on polymer linings after which the soil is washed and drained from the bottom (of the polymer

lining) in order to extract the hydrocarbons in aqueous solution. The solid phase can be returned to the original site while the leachate is further treated with conventional wastewater treatment techniques. The merit of this method is that as the two stages are exclusively independent of each other, each stage can be optimised to obtain an improved efficiency of the overall process. For example, a minimum surfactant concentration of 1% is required during the washing process, while concentrations above 2% may affect hydraulic conductivity and could also inhibit microbial activity should the two stages be conducted concurrently. The wash solution can then be treated with a suitable consortium of microorganisms. The thermal desorption process involves the use of heat to treat petroleum-contaminated soils (Molleron, 1994; USEPA, 1995) in an inert atmosphere (oxygen deficient) in order to elevate the vapour pressure of the organic contaminants and cause them to vaporize thereby separating the contaminants from the soil matrix (Wilbourn *et al.*, 1994).

Chemical remediation method include but is not limited to solidification/stabilisation, encapsulation and steam extraction. In situ stabilisation technique aims at reducing the amount of organic and inorganic contaminants available for uptake by ecological receptors by immobilising hazardous substances through physical and chemical methods. Contaminants are converted into less soluble and less toxic forms and encapsulated in a monolithic solid of high structural integrity through this technique (Anderson and Mitchell, 2003).The encapsulation technique involves physical isolation and containment of a contaminated site with the aid of cut-off walls, low permeability caps, grout curtains e.t.c. designed to limit or prevent the transfer of contaminants from the source to non-contaminated environment (Anderson and Mitchell, 2003).

Bioremediation is the use of biological agents such as microorganisms (bacteria and fungi), green plants or their metabolic capacity to remediate contaminated sites and to restore them to their original non-contaminated state (Glazer and Nikaido, 1995). The process of bioremediation has several components to it including the following:

1. Bioattenuation: This is also known as passive remediation or natural attenuation and involves the use of ongoing natural processes to transform the contaminants to a less harmful form and to reduce the transfer of the contaminants to non-contaminated sites (Khan *et al.*, 2004). Natural processes involved in bioattenuation include sorption of contaminants to geologic media, biodegradation by microorganisms and in some cases, reactions of pollutants with naturally-occurring chemicals in the soils. It is a contaminant-specific process and can be used to treat specific compounds e.g. BTEX (Atteia and Guillot, 2006) but not many others. Its advantages include its cost-

effectiveness, non-invasive remediation method and simple technique to perform. On the other hand, it is a slow clean-up approach that may lead to migration of pollutants with time and whose performance can be difficult to predict.

2. Biostimulation: In order to attain optimum microbial activity in contaminated soils and sediments, certain conditions must be met such as optimum temperature, moisture, pH, salinity, oxygen and nutrient availability. Biostimulation is an enhanced form of bioattenuation that involves the stimulation of microbial degradation by introducing the essential nutrients carbon, nitrogen and phosphorous in the correct proportion in order to increase the turnover of chemical pollutants. The proportions of inorganic nutrients required for optimum microbial activity has been reported. Wolicka *et al.* (2009) reported a C: N: P ratio of 100:9:2, 100:10:1 or 250:10:3 during an in situ remediation of BTEX. Also, a 1-5% N by weight of petroleum with a ratio of N: P between 5 and 10:1 was reported by Swannell *et al.* (1996). The process of biostimulation requires the native microbial communities to have the capacity to metabolise the contaminant of interest and for the contaminants to be readily available to the microbes for biodegradation. As nutrients are delivered to soils in solution, the process may increase the chances of pollutant mobility thereby necessitating the treatment of the underlying groundwater. Also, microorganisms may colonise the injection points of the nutrients, resulting in the clogging of nutrients and the injection wells.

3. Bioaugmentation: In some cases, a very slow microbial response to contamination in sites without any prior contamination history might be due to the fact that the indigenous microbial communities lack the metabolic capacity to transform the pollutants, necessitating the introduction of previously adapted microbial inoculum in order to augment the native microorganisms present in the contaminated site. Soils in which microorganisms have been adapted by prior exposure to organic contaminants such as petroleum hydrocarbons can be used to remediate soils newly contaminated with petroleum hydrocarbons. This technique was attempted by Otte *et al.* (1994) in which biomass from a fraction of contaminated soil was cultivated and used as an inoculum to remediate soils contaminated with polychlorinated biphenyls (PCBs) and poly-aromatic hydrocarbons (PAHs).

2.6 In situ sorbent amendment – a novel technique for the remediation of volatile petroleum hydrocarbon contaminated soils and sediments

A more recent approach for remediation of soils and sediments contaminated with organic compounds builds on the concept of the solidification/stabilisation technique. Sorbent

amendment is an in situ soil remediation technique that involves the use of carbon-rich porous materials with a high sorption capacity to amend soils and sediments contaminated with organic and inorganic compounds. Examples of adsorbents that are been investigated for their sorption capacity on organic pollutants are activated carbon (Vasilyeva *et al.*, 2002; Rhodes *et al.*, 2008; Vasilyeva *et al.*, 2010; Ghosh *et al.*, 2011) and biochars (Bushnaf *et al.*, 2011; Gomez-Eyles *et al.*, 2011).

Activated carbon is a synthetic form of carbon produced by subjecting carbonaceous materials such as coal, lignite and peat (Adib *et al.*, 2000; Murillo *et al.*, 2004) to a limited supply of air and subsequently activating by oxidation to eliminate any impurities and to increase total surface area to about $1000m^2$ /g. The resulting material has a complex pore structure and surface functional groups making it an excellent sorbent material for hydrophobic organic compounds.

Biochar is the product of pyrolysis of biomass, a carbon-rich solid material obtained when plant-derived biomass is heated in a limited supply of oxygen at relatively high temperatures ($<700°C$). It is the term used to describe charred materials of biological origin intended for use in improving the quality of soils for agricultural purposes. This distinguishes it from other charred products of non-biological origin referred to as agrichar which can also be used for the same purpose (Lehmann and Joseph, 2009).

Organic pollutants may persist in the environment and this is another serious challenge with respect to remediation. The sorption properties of biochar and activated carbon make them good sorbent materials for immobilising organic pollutants in the soil as indicated by a number of studies (Smernik, 2009; Beesley *et al.*, 2011). The mechanism by which the sorption of organic compounds to biochar occurs has been documented. For example, poly-aromatic hydrocarbons are one group of organics that are strongly adsorbed by biochar through a specific $\pi - \pi$ bonding between the aromatic rings of both the PAH and biochar. In addition, the planar structure of PAHs makes it easy for the compound to fit into the pores of biochar (Baring *et al.*, 2002; Pignatello and Sander, 2005). Other classes of organic compounds that have been shown to be affected by biochar amendment are pesticides (Yu *et al.*, 2010; Lou *et al.*, 2011) and organic solvents.

2.7 Sorbent amendment effect on soil biota

A high level of prokaryote diversity exists in the soil as a result of the high level of heterogeneity and diversity of soil habitats. More specifically, bacteria has been reported to be the dominant life form. In like manner, certain species within the prokaryotic community

(bacteria and archaea) predominate and are present in large numbers in the soil environment because of the presence of nutritional and physico-chemical conditions necessary for their growth and establishment. Important functions of soil bacteria include nutrient cycling (McLeod and Parkinson, 1997; Katterer and Andren; Dominy and Haynes, 2002; Chen *et al.*, 2003), and decomposition of soil organic matter (Barrett and Burke, 2000; Spaccini *et al.*, 2002).

Even though biochar effects on soil biota has not been thoroughly investigated, there's a number of evidences to demonstrate that biochar addition to soil changes soil properties such as pH, water-holding capacity, aeration and nutrient retention capacity. The differences in physical properties between soil and biochar is expected to be responsible for the alteration of soil properties and by extension, soil biota. Biochar with tensile strength less than those of soils can be added to such soils to reduce their tensile strength. Chan *et al.* (2007) demonstrated a reduction in tensile strength of soil from an initial biochar-free value of 64.4KPa – 31KPa after biochar was added to the soil at an amendment rate of 50t biochar ha^{-1}. Plant root elongation and seed germination may be facilitated with reduced soil tensile strength as this condition reduces mechanical interference by soil particles.

Another important soil property that could be affected by biochar addition is soil bulk density. Biochar has been found to contain micro- and macro-pores both of which have been implicated in the reduction of soil bulk density (Downie *et al.*, 2009).

Biochar surface area has also been reported to influence soil surface areas with attendant effects on soil processes like nutrient cycling, water and soil aeration and microbial activity. Sandy soils for example are poor in terms of their ability to retain water and nutrients partly due to the relatively small surface area of their particles. On the other hand, clay soils have very fine particles and a relatively large surface area and therefore able to retain more water than sand. By addition of biochar to such soils, it is possible to create a balance in the net surface areas of different soil types hence improving the fertility of such soils. Soil pH may also increase or decrease depending on the pH and liming value of biochar (Lehmann *et al.*, 2011). The pH values of biochar have been correlated to the pH values of the original feedstock, pyrolysis temperature and the degree of oxidation of the biochar during pyrolysis (Cheng *et al.*, 2006; Chan and Xu, 2009).

The physical properties of biochar such as its porosity and surface areas are thought to create a suitable environment for microorganisms to grow and reproduce. Notable microbes known to inhabit biochar pores and surfaces are bacteria, actinomycetes and arbuscular mycorrhizal

(AM) fungi (Thies and Rillig, 2009). The porous nature of biochar may enhance their capacity to retain moisture which may consequently enhance their habitability to microorganisms. In addition to moisture, other gases such as O_2 and CO_2 may also dissolve in biochar pore water and depending on the available quantity of these gases, microorganisms would proceed to respire aerobically or anerobically (Antal and Gronli, 2003; Thies and Rillig, 2009). Other factors that have been reported to affect microbial activity, abundance and diversity in the soil are temperature and pH. Studies by Fierer and Jackson (2006) reveals that soil pH played a significant role in determining bacterial abundance and diversity with diversity climaxing in neutral soils and at a minimum in acidic soils. Fungal strains are more likely to dominate under extreme pH conditions because of their ability to tolerate wide pH ranges. As a result of this, it is expected that soil pH changes that is brought about by biochar addition to soils would alter the overall ratio of bacteria to fungi as well as the abundance and composition of these microbes in the soil.

Several methods have been used to investigate microbial abundance in soils amended with biochar. These techniques include total genomic DNA extraction (Grossman *et al.*, 2010), substrate induced respiration (SIR) (Steiner *et al.*, 2004), phospholipid fatty-acid (PLFA) extraction (Birk *et al.*, 2009), culturing and plate counting (O'Neill *et al.*, 2009) and fumigation extraction (Jin, 2010). Studies using some commonly occurring mycorrhizal fungi (arbuscular [AM] and ecto-mycchorizal [EM]) showed that there was an increase in the abundance of these organisms around plant root tips upon addition of biochar to soils. The exact mechanism by which this increase occurs is not fully understood but a number of mechanisms have been proposed. These include the sorption of signalling compounds, detoxification of allelochemicals (Warnock *et al.*, 2007), protection of microorganisms (bacteria and fungi) against dessication and from other biota (Saito and Marumoto, 2002; Thies and Rillig, 2009), nutrient and carbon availability (Steiner *et al.*, 2009; Blackwell *et al.*, 2010) and bacterial adhesion to biochar. Bacteria appear to adhere more readily to biochar surfaces than fungi thereby rendering less leachable in soil and consequently increasing their abundance in biochar amended soils (Pietikainen *et al.*, 2000). There are indications of the variable effects of biochar on microbial biomass with respect to different phylotypes and functional groups. Alteration of the soil environmental conditions such as available carbon sources, pH and other abiotic factors by biochar has been reported to cause a change in soil microbial community structures. Studies on soils amended with biochar have shown significant changes in composition and structure of fungal, bacterial and archaeal populations (O'Neill *et al.*, 2009; Grossman *et al.*, 2010).

Bacterial community composition in biochar-rich soils of the Terra preta were found to vary significantly in comparison with communities from unmodified soils both having similar minerology (Kim *et al.*, 2007a; O'Neill *et al.*, 2009). About 25% diversity was reported amongst bacterial communities in biochar-rich soils against biochar-free soils at the genus, species and family levels of taxonomy (Kim *et al.*, 2007a; O'Neill *et al.*, 2009). Other groups of microorganisms like the archaea and fungi were observed to have a lower diversity in the Terra Preta (biochar amended) in comparison with unamended soils suggesting that different groups of microbes respond quiet differently to biochar amendment. The foregoing effects of biochar on microbial communities could be due to the long-term enrichment of the Terra Preta several hundreds of years with biochar.

Common soil processes affected by biochar addition to soil include denitrification, methane (CH_4) oxidation, carbon mineralisation and nutrient transformation. Carbon mineralisation increased in non-pyrolysed organic matter as a result of higher microbial abundance (Carney and Matson, 2005). Biochars have been reported to contain recalcitrant and labile carbon fractions and an increase in soil respiration (indicated by the evolution of CO_2) that is observed upon the addition of fresh biochars is thought to be due to their highly leachable carbon contents. Biochar also influences nutrient transformation by microorganisms within the soil. Studies on forest soils showed an increase in the activity of nitrogen metabolising enzymes which also resulted in an increased plant uptake of nitrogen from the soil (Lehmann *et al.*, 2003; Deenik *et al.*, 2010). Biochar containing a high mineralisable fraction was reported to increase the immobilisation of nitrogen and hence reduce the amount of nitrogen available for uptake by plants (Deenik *et al.*, 2010). Because of the varying effects of biochars on C and N availability in the soil, emission from microbial processes such as CH_4 and N_2O are often ambiguous. Ethylene, a phytohormone forms the non-aromatic portions of fresh biochars and is produced by microbes in the presence of biochars (Spokas *et al.*, 2010). This is thought to explain the observed reduction of CO_2 and N_2O emissions from biochar amended soils. The exact mechanism by which biochar affects N_2O and CH_4 emissions from the soil is however not clearly understood.

Activated carbon amendment effect on soil biota has been reported. (Meynet *et al.*, 2012) conducted an experiment to investigate the effect of 2% AC amendment of PAH impacted urban soils on the soil microbiota. A bacterial community structure analysis of the powdered or granular (activated carbon amended soils revealed the presence of bacterial taxa that have been reported to degrade PAHs such as *Rhodococcus jostii* RHA-1 and *Rhodococcus erythropolis* in all the soils being investigated. Ultimately, amending PAH impacted soils with

either PAC or GAC did not appear have any detrimental effect on the soil microbiology as the amended soils retained the capacity to degrade PAHs although the degradation effect was most notable in unamended soils. Other studies were also conducted to investigate the effect of AC amendment on soil macrobiota. McLeod *et al.* (2007) reported a survival rate of 100% for the polychaete *Neanthes arenaceodentata* but observed a reduction in their growth rate by 50% following AC amendment.

Chapter 3: Effects of biochar and activated carbon amendment on the bio-kinetics of toluene degradation in gravelly sand

3.1   Introduction

Toluene is a monoaromatic volatile petroleum hydrocarbon compound commonly released into the environment due to its universal presence in fuel and petroleum products, and for its role as a solvent in the manufacture of common products such as plastics, pesticides and synthetic fibre (Jindrova *et al.*, 2002). Due to its toxic nature, and potential to contaminate soil and groundwater, several studies have been conducted to investigate effective approaches for the remediation of this compound from environmental compartments including the use of inorganic nutrients to stimulate microbial degradation (Rosenberg *et al.*, 1996) and in situ sorbent amendment of contaminated soils (Bushnaf *et al.*, 2011) among others. Transformation of VOCs by soil microorganisms also known as biodegradation is increasingly becoming a preferred option because of its cost-effective and environmentally friendly nature in addition to effectively metabolising these compounds to minimum concentrations in the environment.

Microbial transformations of VPHs is a function of the structure of mixed bacterial populations present at contaminated sites as well as their growth kinetics on specific substrates. Most degradation kinetics for pure cultures and bacterial consortia growing on either single substrates e.g. toluene or complex mixtures e.g. benzene, toluene, ethylbenzene and *o*-xylene (BTEX) have been extensively studied (Schirmer *et al.*, 1999; Abuhamed *et al.*, 2004; Littlejohns and Daugulis, 2008). Also, most studies involving the attenuation of organic pollutants have taken into consideration the effects of key processes such as diffusion and sorption of pollutants on biodegradation of the compounds mostly focusing on individual processes e.g diffusion or sorption rather than investigating the effects of two or more processes on the attenuation of contaminants. Quite a few studies have investigated the integrated effects of these processes on the attenuation of contaminants in the environment. Karapanagioti *et al.* (2004) described a model that couples the effects of non-linear sorption with intraparticle diffusion, sorption and biodegradation on the attenuation of organic contaminants in the environment. Hohener *et al.* (2003) also conducted a study to determine the biodegradation kinetics of a mixture of VPHs and the Monod growth kinetics of microbial communities growing on the substrates in batch systems.   In order to advance the design and operation of engineered systems, it is imperative to develop growth models that can quantify kinetic parameters of microorganisms while growing on organic compounds and concurrently considering the complex interactions between chemical processes such as diffusion of

pollutants, volatilization and sorption to soil matrix and biological transformation processes as they affect microbial growth kinetics. Especially the effect of sorption on VPH biodegradation by dynamic microbial communities is still poorly understood. Biochar and activated carbon are currently begin investigated for their sorption capacity on volatile organic compounds and as an innovative approach to mitigating transfer of volatile compounds to ecological receptors and to non-contaminated environments (Bushnaf *et al.*, 2011; Ghosh *et al.*, 2011; Kupryianchyk *et al.*, 2013). Not much has been done, however, on the effects of sorbent amendment on the indigenous microbial community response in contaminated soils.

3.2 Aim

The aim of this study was to investigate for toluene as an exemplary volatile petroleum hydrocarbon compound the effects of sorption by biochar and activated carbon on the pollutant mineralization to CO_2. The insights gained from this study will be used in subsequent chapters to further understand the dynamics of VPH degrading bacterial communities in soils contaminated with a mixture of volatile organic compounds.

3.2.1 *Objectives*

In line with the above study aim, the following objectives were set to be accomplished in the current study:

1. To more reliably distinguish the end product ($^{13}CO_2$) of toluene mineralization from that ($^{12}CO_2$) of other substrates used by the soil microorganisms by introducing into different soil systems a fully stable-isotope labelled compound (toluene - $^{13}C_7$) to serve as a model pollutant.

2. To determine the effects of sorption on the biodegradation of $^{13}C_7$-toluene in biochar and activated carbon amended and unamended sand.

3. To predict the sorption effects of biochar and activated carbon on the $^{13}C_7$–toluene mineralization with a model that considers growth kinetics of VPH degraders in soil.

3.2.2 *Hypotheses*

Based on the study objectives mentioned earlier, the following hypotheses are proposed:

1. As microorganisms tend to adapt to changes in environmental conditions, an increasing preference for utilisation of the introduced substrate – the stable-isotope labelled toluene as carbon source versus other substrates should occur over time.

2. Sorption of organic compounds reduces their bioavailability to soil microorganisms, hence biochar and activated carbon amendment of contaminated soils is expected to slow the rate of toluene mineralization by soil microorganisms.

3. The effect of sorbents can be predicted by assuming that only soil pore water-dissolved toluene is biodegradable, whereas microbial growth kinetics parameters for the substrate toluene would not be altered by addition of biochar or activated carbon.

3.3 Materials and methods

3.3.1 *Soil, biochar and activated carbon*

Previously uncontaminated gravelly sand used for the construction of the Law library of the Newcastle University was obtained for this study. Wet soil was passed through a 2 mm sieve and the resulting soil particle size was mostly in the range of 600 – 2000μm. Total organic carbon (TOC) content of the soil was 1.6±0.1%, total nitrate content 3.9±0.6 µg/g dry weight., nitrite < 1.0 µg/g dry weight and ammonia nitrogen 6.7±0.3 µg/g dry weight. A soil pH value of 7.43 was measured (Bushnaf, 2014).

Biochar produced by fast pyrolysis of woodchips at a temperature of about 800 °C in a fixed bed reactor was obtained from Environmental Power International EPI (Wiltshire, UK) and used for this study. Biochar was ground to a particle size below 163 µm with a total organic carbon (TOC) content of 85±2% and an alkaline pH of 7.83±0.16.

A bitumen-derived activated carbon obtained from Chemviron Carbon Ltd (Lancashire, UK) was ground to particle size below 163 µm, with total organic carbon content of 73±1% and a pH of 7.74±1.00.

3.3.2 *Chemical pollutant*

The chemical preparation consisted of a pure stock (99 atom % isotopic purity) of stable isotope-labelled toluene (heavy isotope – $^{13}C_7$) obtained from Sigma Aldrich (Dorset, UK). The chemical had the following properties: molecular weight of 99.08 by atom percent calculation, a boiling point of 110 °C and melting point of -93 °C and a density of 0.930 g/mL at 25 °C (Sigma Aldrich, UK). The chemical was stored as received in a sealed glass ampule at room temperature.

3.3.3 *Batch experiments*

Batch experiments were conducted at room temperature (20±2 °C) in 65 mL amber coloured vials in order to prevent photolytic degradation of substrates and/or their metabolites during the experiment. Vials were capped with Teflon Mininert caps as illustrated in Figure 3.1. Treatments consisted of soil (15 g d.w.; water content: 0.1 g g^{-1} soil d.w.), soil amended with biochar or activated carbon (15 g, 2% amendment on soil d.w.) to which 5 µL of $^{13}C_7$ toluene was injected through the Mininert valve. The amount of sorbent amendments used was chosen based upon standard application rates previously used in remediation studies (Bushnaf *et al.*, 2011; Meynet *et al.*, 2012). Each treatment consisted of a total of six (6) replicates. Concurrently, two sets of controls were prepared in order to monitor any background respiration that may occur in the course of the experiments. Sterile controls were prepared by autoclaving

three vials (triplicates) containing equal amounts of soil (15 g d.w.) each at 115 °C for 10 minutes while live controls contained only soils also prepared in triplicates. The experiment lasted for 19 days.
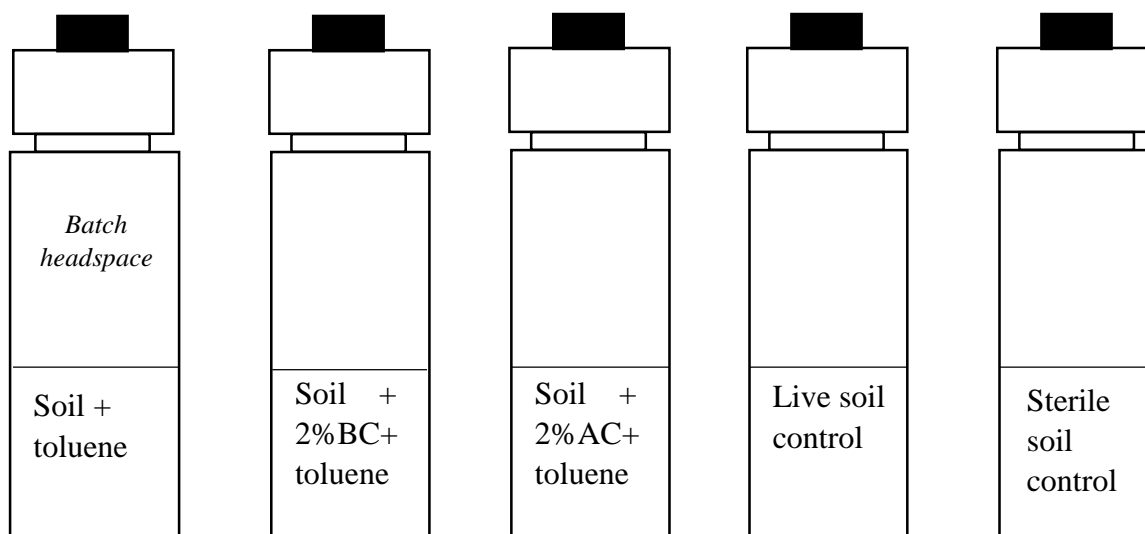
Figure 3.1. A schematic of the batch systems containing soil, soil & biochar, soil & AC, live and sterile soil controls.

3.3.4   *CO₂ analysis by GC-MS analysis, microbial respiration*

In order to monitor microbial respiration in the batches over the duration of the experiments, the concentration of CO_2 in the headspace of each vial was measured using Gas Chromatography. Briefly, headspace gas analysis was conducted on a Fisons 8060 GC using spilt injection (150 °C) linked to a Fisons MD800MS (electron voltage 70eV, filament current 3.6 A, emission current 150 µA, source current 600 µA, source temperature 200 °C, multiplier voltage 500 V, interface temperature 150 °C). The acquisition of data was controlled by a Compaq Deskpro computer using the Xcalibur software in full scan mode (1.0-151.0 amu/sec). The sample was injected in split mode. Separation was performed on a HP-PLOT-Q capillary column (30 mm x 0.32 mm i.d) packed with 20 µm Q phase. The GC was held isothermally at 35 °C with helium as the carrier gas (flow rate 1 mL/min, pressure of 60 kPa, open split at 100 mls/min). The chromatograms of the headspace gas (CO_2) were integrated and quantified and the gas concentrations deduced. Theoretical values of oxygen (O_2) concentrations in the batch headspaces were also calculated before and at the end of the experiments to determine whether batches were still aerobic by the end of the biodegradation experiments. Calculations were done based on the assumptions of the ideal gas law using the following conditions: temperature = 293.15K, atmospheric pressure = 1 atm, volume = 1L and a gas constant R = 0.0821 L atm K^{-1} mol^{-1}.

3.3.5 *CO₂ leakage experiments*

A separate set of batch experiments were conducted in order to quantify an apparent loss of CO_2 via diffusion through the gap between walls of the vials and the Mininert caps (Figure 3.2), and also the distribution of CO_2 between the headspace air and soil. The batches consisted of an empty 65 mL vial, and vials with soil (15 g d.w.), respectively, soil amended with 2% biochar or activated carbon (on soil d.w; 15 g) as described in the preceding section (3.3.3). Batches were prepared in triplicates and autoclaved at 115 °C for 10 minutes in order to prevent CO_2 production from soil microorganisms. A set of empty vials were also prepared in triplicates and autoclaved to serve as controls. All batches were tightly capped and injected with 2 mL of pure CO_2 (CP Grade, BOC Gases, Surrey, UK) followed by monitoring of headspace CO_2 concentrations according to the methods described in section 3.3.4. The leakage experiments lasted for 14 days (2 weeks). A leakage factor was determined based on the assumptions of gas leakage by diffusion according to Fick's first law of diffusion as follows:

$$F = -D\frac{dCa}{dx} \qquad (3.1)$$

where F is the CO_2 mass flux per unit cross-sectional area (A) per unit time, and D is the molecular diffusion coefficient of the moving compound (CO_2) in the gas phase and has the dimension of length squared per time and dx is distance travelled by the gas.

Figure 3.2. An illustration of empty batch vial showing the gap length (dx) and the cross-sectional area (A) of the assumed gap between the Mininert cap and the wall of the vial.

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3.3.6   *Determination of carbon isotope signatures*

The isotopic signature of carbon in each batch was determined by comparing stable isotope ratios (^{13}C:^{12}C) of CO_2 in the respective batch headspaces with that of the reference material VPDB according to the methods described by Diochon and Kellman (2008). Isotopic ratios were reported using the δ notation relative to the VPDB standard (Diochon and Kellman, 2008):

$$\boldsymbol{\delta}\,^{13}C = \left(\frac{R_{sample}}{R_{reference}} - 1\right) 1000 \qquad\qquad (3.2)$$

where R_{sample} and $R_{reference}$ is the ^{13}C:^{12}C of the measured sample and the reference material Vienna Pee Dee Belemnite (VPDB), respectively. The concentrations of mineralisable toluene and soil organic matter were calculated based on the peak areas of CO_2 mass ions (45 and 44 respectively) for each batch.

3.3.7   *GC-FID analysis, headspace VPH measurements*

The headspace concentration of volatile petroleum hydrocarbons in the batches was measured using a HP-7890A Series Gas Chromatograph (Agilent Technologies, Palo Alto, USA). Briefly, 30 μL of samples were injected manually using a Hamilton gas-tight syringe into the machine in split mode, the injector set at 200 °C, flame ionisation detector at 300 °C. Prior to this, a blank sample containing air was ran to ensure that there had not been any previous contamination of the GC columns. This was followed by a calibration of the instrument using different volumes of the pure chemical, ^{13}C$_7$ –labelled toluene. Separation of headspace gas was performed on a fused silica capillary column (30 m x 0.25 mm i.d) coated with 0.25μm dimethyl poly-siloxane (HP-5 phase). The column temperature was maintained at 30 °C for 5 minutes and raised to 120 °C at a rate of 10 °C min^{-1} and then held at this temperature for 6 minutes. The hydrogen carrier gas was set at a flow rate of 1 mL min^{-1}, pressure of 50 kPa and split at 10 mls min^{-1}.

3.3.8   *Sample clean up and quantification of strongly sorbed residuals*

At the end of the biodegradation experiments, on day 19, all vials were uncapped in a fume cupboard and allowed to stand for 48 hours in order to evacuate any remaining volatile petroleum hydrocarbon (toluene - ^{13}C$_7$) present in the batches. Duplicate batches from each treatment were then extracted by adding 30mL of the extraction solvent (Dichloromethane: Pentane mix; 60:40 by volume) to the soil systems. Prior to the addition of the extraction solvents, soils were properly stirred using a clean stirrer each time in order to break up aggregates that stick together. 5μL of toluene (^{12}C$_6$) (Sigma Aldrich, UK) was used to spike each mixture and to serve as an internal standard and the vials containing the soil-solvent mix

were shaken overnight on an orbital shaker. Glass chromatographic columns (45cm x 10mm i.d) were set up and the combined extracts from each batch was passed through separate columns each of which was plugged with glasswool to prevent silica gel from flowing through the columns. Columns were pre-packed with 3.0 g of silica gel topped by a spatula-full of sodium sulphate (Na_2SO_4) (Sigma-Aldrich, Dorset, UK). Silica gel removes humic substances that is contained in the original soil samples which may accumulate in the GC-FID columns while sodium sulphate, a hygroscopic material, removes any water molecules contained in the DCM: Pentane mix. Eluents were collected in 40 mL glass vials from which 1mL of clean extracts were transferred into clean 1mL vials for GC-MS analysis. The compounds in the clean samples were analysed on a HP-5890 series II in split less mode, injector temperature set at 280 °C. The separation of compounds was performed on an Agilent fused silica capillary column (30m x 0.25mm i.d) coated with 0.25 μm dimethyl poly-siloxane (HP-5 phase) (Agilent Technologies, Palo Alto, USA). The GC temperature was programmed from 50-310 °C at 5 °C and held at the final temperature for 20 minutes with hydrogen as the carrier gas (flow rate of 30 mls min^{-1} and initial pressure of 50 kPa).

3.3.9 *Determination of microbial degradation of poorly available substrate after pollutant source removal*

In order to assess the biodegradation of the poorly available substrate at the end of the remediation cycle in different treatments, a second batch experiment was conducted as a follow-up to the initial biodegradation experiment. After evacuating the volatile compounds from the batches over a 48-hours period, duplicate samples from each treatment were pooled into amber-coloured crimp-top vials (37.65 mL) followed by the addition of sterile deionised water (1 mL) to re-moisten the soil environment for enhanced microbial activity. For the sterile and live controls, only one batch each was transferred into the crimp-top vials. The vials were sealed and the aluminium stoppers clamped using a tool. Carbon dioxide production was monitored in batch headspaces on a weekly basis according to the methods in described in section 3.3.4. The experiment lasted for 14 weeks.

3.3.10 *Determination of cation (Ca^{2+} and Mg^{2+}) concentrations in soil systems, ICP-OES analysis*

Soluble cations were extracted from the pore water of different soil systems: soil, soil & biochar and soil & AC by adding 20 mL of deionized water to 20 g of soil or soil amended with 2% biochar or activated carbon (2% on soil d.w.) in 50 mL ultra-high centrifuge tubes with screw caps (VWR Int'l, USA) and shaking the mixtures on an orbital shaker (Stuart, SSL1) for three hours. The resulting suspensions were centrifuged in a 3-16P model centrifuge (Sigma, Germany) at 3000 rpm for 5 minutes. A clear supernatant from each

centrifuge tube was further filtered using a 25 mm syringe filter with a sterile, non-pyrogenic 0.2 μm Supor® membrane in order to remove any suspended particles that might interfere with the analysis. Filtrates were analysed for residual individual cation concentrations using a Varian Vista MPX axial Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) with CCD, operated according to the British Standards methods for the determination of selected elements in the assessment of water quality (Standards, 2007).

3.3.11 *Batch modelling*

A model simulating the kinetic batch sorption and biodegradation test with first-order rate kinetic pollutant sorption by soil particles, radial pollutant intraparticle diffusion in biochar, Monod kinetic pollutant biodegradation and logistic growth of pollutant degrading biomass was used to interpret the batch study data. The model was implemented in Matlab by David Werner, and the equations, numerical solutions and the Matlab codes are provided in the appendix (Appendix A) as supporting information.

3.4   Results and Discussion

3.4.1   *Effects of biochar and activated carbon amendment on biodegradation and sorption of $^{13}C_7$-Toluene*

Soil respiration was monitored in the headspace of batches as an indication of the level of biodegradation taking place within the systems, a technique which has been shown to be effective and reliable in studying the effects of environmental variables on the mineralisation of petroleum hydrocarbons (Pritchard *et al.*, 1992). The heavy isotope containing compound ($^{13}C_7$ - toluene) served as the primary carbon source in this study in order to track the source of carbon dioxide in the batches. Similarly, radiolabelled ^{14}C-hydrocarbons have been used previously in mineralisation studies to monitor the pollutant-derived CO_2 since $^{44}CO_2$ could also be generated from soil organic matter (Mueller *et al.*, 1992; Pritchard *et al.*, 1992). More recently, carbon-specific isotope analysis (CSIA) have been used to gain insights into the various processes involved in the fractionation of carbon isotopes during the biodegradation of volatile petroleum hydrocarbons in the unsaturated zone. Bouchard *et al.* (2008b) conducted an investigation into the fractionation of natural carbon isotopes occurring during the aerobic biodegradation of n-alkanes and aromatic hydrocarbons in contaminated soils. In a separate but related study by Bouchard *et al.* (2008a), the effects of diffusive transport and volatilization of VPHs across a porous medium on the fractionation of carbon isotopes were investigated. Both studies resulted in an improved understanding of potential biodegradation pathways of compounds containing both light and heavy carbon isotopes and the enzymatic processes involved in the transformation of volatile organic compounds. As only a small fraction of the total naturally occurring light isotope ^{12}C is represented by the heavy isotope ^{13}C (ca 1.1%), it is anticipated that most of $^{44}CO_2$ in the current batch study originates from soil organic matter mineralisation, and only a small fraction of the soil organic carbon is mineralised into $^{45}CO_2$. Consequently, most of the $^{45}CO_2$ produced in the batches is expected to originate from the mineralisation of $^{13}C_7$-toluene.

Results from the current study showed that there was an increase in the amount of $^{45}CO_2$ production in the soil systems to which the $^{13}C_7$ toluene was added compared to live and abiotic controls (Figure 3.3). In the soil only batches, there was an initial lag period of about 8 days. This was followed by an increase in the levels of $^{45}CO_2$ to reach a peak concentration of 0.59 g/L on day 16 before entering into a stationary phase. In the soils amended with 2% biochar and activated carbon, there was a shorter lag phase period of about 5-6 days followed by an increase in the levels of headspace $^{45}CO_2$ for the biochar and AC amended soils respectively. The level of $^{45}CO_2$ production was initially higher in the biochar amended soils than in the AC amended soils until the 13th day when $^{45}CO_2$ production in AC amended soils

overtook those in the BC amended soils to reach a peak concentration of 0.021g/L on day 19. The most intensive biodegradation of VPH that followed the longest lag phase in the unamended soils could both be a result of the high concentration of the substrates in the batch headspace indicative of good substrate bioavailability during the first 14 days (Figure 3.4) which is higher than in the biochar and AC amended soils. Good substrate availability means high potential for substrate utilization and growth of substrate degrading microorganisms, but also high potential for toxic effects (Aono *et al.*, 1994; Sikkema *et al.*, 1995; Heipieper and Martinez, 2010), which may explain the long lag phase in the unamended soil. In the biochar and AC amended soil, a short lag phase is likely the result of low substrate concentration reducing toxicity and enabling toluene mineralisation from around day 3 (Figure 3.4), an indication of sorption of the pollutants in the sorbent amended soils affecting soil microbiology.

The maximum cumulative amount of $^{45}CO_2$ produced in the unamended soil was higher than those produced by the biochar amended soil and AC amended soils by a factor of 4.5 and 3.0 respectively. Amending soils with carbonaceous sorbents have been shown to enhance the sorption of organic pollutants from contaminated soils. Studies by Rhodes *et al.* (2008) demonstrated the strong sorption capacity of activated black carbon to phenanthrene thereby reducing its extractability from contaminated soil and to some extent, bioavailability of the pollutant to soil microorganisms. AC was also reported to adsorb VPHs more strongly than biochars in a long-term column study in which sorbent amended and unamended soils were exposed to a NAPL source containing a VPH mixture for over 12 months (Bushnaf, 2014). These findings correspond with the results from the current study (Figure 3.4) in which activated carbon demonstrated a higher sorption capacity over the duration of the experiment compared to biochar amended and unamended soil. This may also explain the lower initial concentration of $^{45}CO_2$ in the AC amended batches.

Theoretically determined oxygen (O_2) levels at the start and the end of the experiments reveal that 24.33%, 7.70% and 9.97% of the initial amount of headspace oxygen for soil, soil & BC and soil & AC respectively had been used up for the mineralization of the substrate. This suggests that the batch systems were in principle, largely aerobic by the end of the experiments. Any levelling off of headspace CO_2 in the batches may therefore not have been as a result of limited supply of oxygen in the batch systems.

In the live and sterile controls, the level of $^{45}CO_2$ production was very low and below the limit of detection of the GC-MS indicating the absence of the stable isotope-labelled carbon source in the control batches.
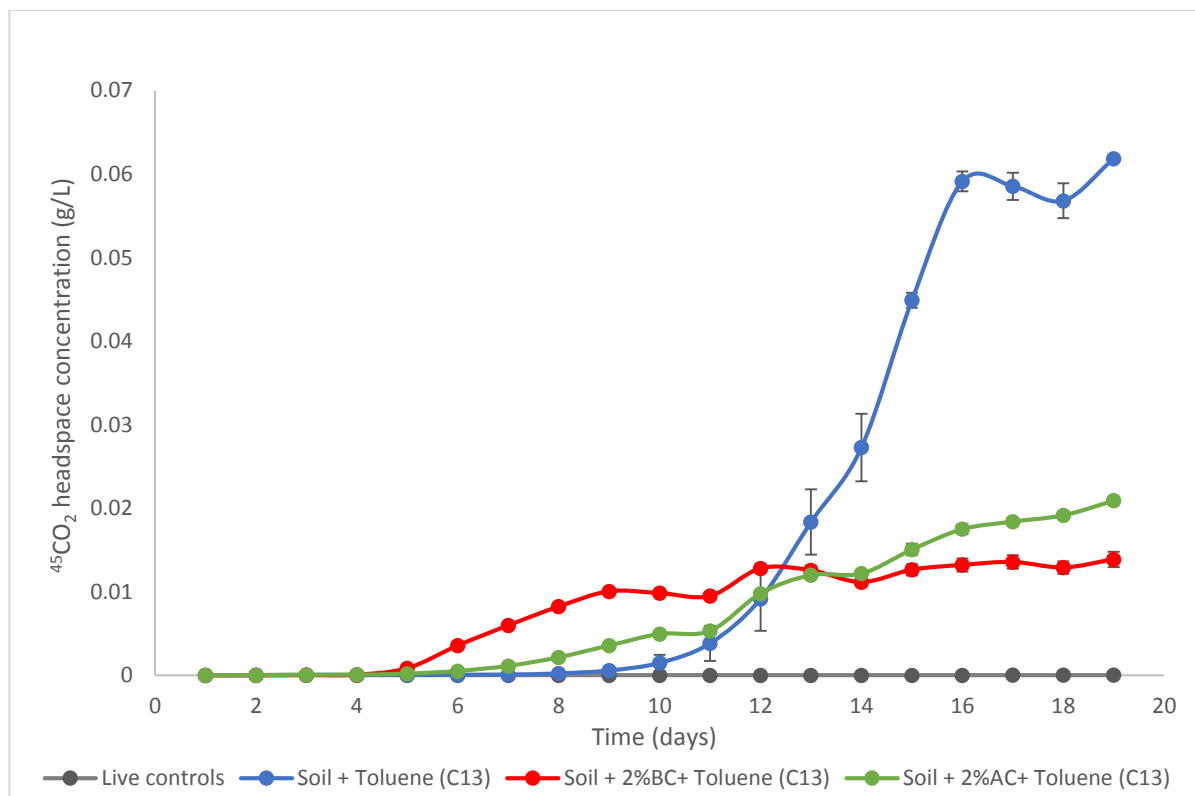
Figure 3.3. Headspace $^{45}CO_2$ concentrations in different soil systems for live controls, unamended soil, soil & biochar and soil & AC. Error bars represent ±1 standard deviation from the mean of six replicates.

Sorption profiles for toluene in the different soil systems as indicated by headspace concentration are shown in Figure 3.4 below. The initial steady-state in toluene headspace concentration of the unamended soil batch from day 3 to around day 11 is an indication of sorption to soil organic matter and can be interpreted to mean that sorption equilibrium between soil, water and air in the batch is attained within this period. A comparison of the effects of sorbent amendment on toluene sorption reveals that amending soils with 2% biochar (represented by red bars) reduced toluene headspace concentration by one order of magnitude compared to unamended soil batch on day 3 whereas for the soil & activated carbon batch, headspace toluene concentration was reduced by up to 2 orders of magnitude right from the onset (day 3) and remained near the limit of detection of the instrument throughout the experiment. Amending soil with 2% biochar was reported to enhance the solid-water distribution coefficient of the soil for toluene by an order of magnitude in a batch study (Bushnaf *et al.*, 2011). These observations also correspond with a long-term column study in which soil-water distribution coefficient of toluene in soil was increased by a factor of 10 in 2% biochar amended soil and by a factor of 100 in 2% activated carbon amended soils (Bushnaf, 2014).

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Activated carbon is typically a stronger sorbent than biochar considering a wide range of feedstock materials from which both sorbents are made (Hale *et al.*, 2015). The presence of pyrogenic carbon-rich materials such as soot, black carbon e.t.c may also influence the sorption of organic pollutants in contaminated soils and sediments (Cornelissen *et al.*, 2005), although sorption efficiency is dependent on the amount of pyrogenic material present in the soil and on the concentration of the pollutants as sorption may be more linear at low pollutant concentrations.

The 'disappearance' of headspace gases in batch studies have been linked to other abiotic processes such as sorption to rubber seals and to leakage from the vials (Hohener, 2010). In order to check the effects of abiotic processes on the 'disappearance' of the CO_2 produced in batch headspaces, leakage experiments were performed and the results are discussed in a following section (Section 3.4.4).
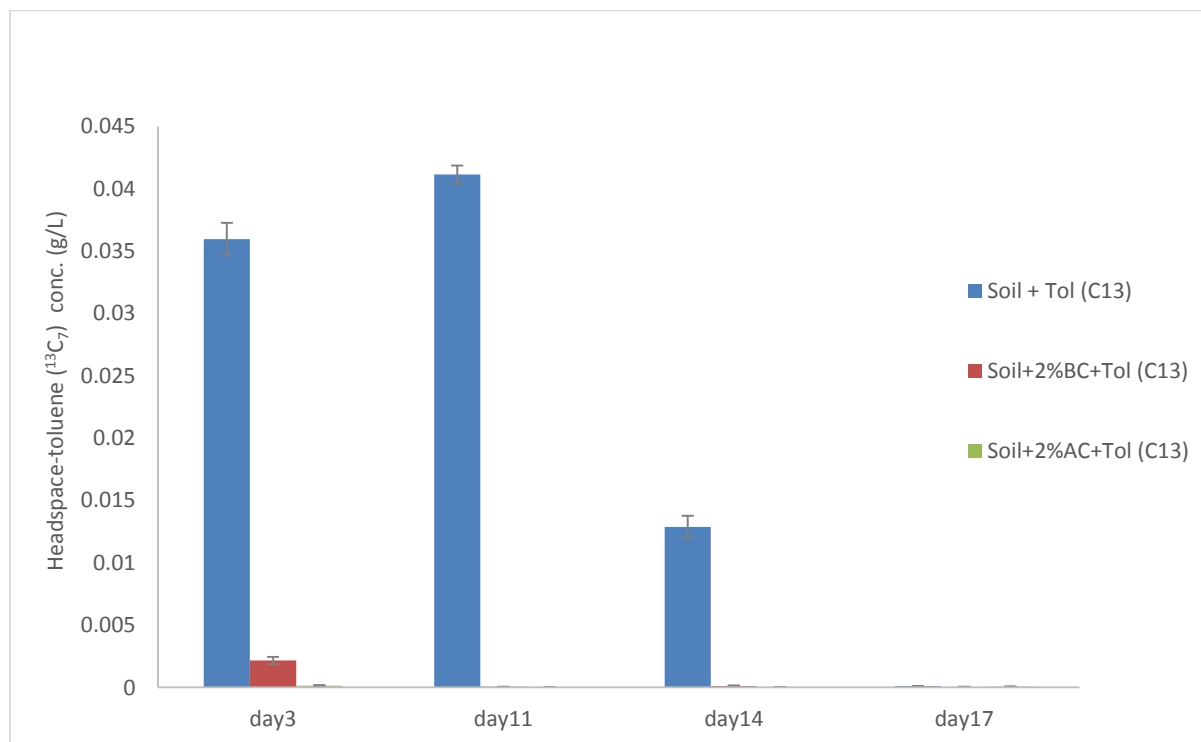
Figure 3.4. Headspace $^{13}C_7$-toluene vapour concentrations (g/L) in the batch systems for soil, soil & biochar and soil & AC measured at different time points during the experiments.

3.4.2   *Effects of substrate availability on the mineralisation of soil organic matter in a batch system*

The lighter isotope – $^{44}CO_2$ in the headspace of batches was concurrently monitored over a 19 – day period as an indication of the level of mineralisation of soil organic matter taking place in the soil systems. In the soil treatments (i.e. amended and unamended soils), an initial lag phase of one day followed by a brief rise in the level of CO_2 production on the 3rd day of the

experiments is noticed. On day 4, a fall in the level of CO_2 production is observed. These observed rise and fall in the levels of headspace CO_2 at this stage (between day 3 and 4) may simply be due to systematic measurement uncertainty, i.e. in the GC-MS calibration as it occurs in all treatments. From about day 4, $^{44}CO_2$ levels start to rise in soils amended with 2% biochar and AC. In the unamended soils, $^{44}CO_2$ production remained approximately constant until the 6th day before rising steadily to reach a maximum concentration on the 19th day. In the live and abiotic controls, the cumulative amount of $^{44}CO_2$ produced never rose above a minimum level indicating that either microbial activity was not stimulated or the batches were sterile (abiotic tests)(Figure 3.5). This shows that $^{13}C_7$-toluene mineralisation stimulated the mineralisation of other ^{12}C-substrates, since $^{45}CO_2$ concentrations rose broadly in line with $^{44}CO_2$ concentrations. Comparing the maximum cumulative amounts of $^{44}CO_2$ produced in the unamended soils on day 19 with that produced by biochar and AC amended soils reveals that $^{44}CO_2$ levels were twice as high in the unamended soils at the end of the experiment. With respect to the controls, $^{44}CO_2$ levels in unamended soils were higher by a factor of 13 and 70 in the live and abiotic controls respectively (Figure 3.5).
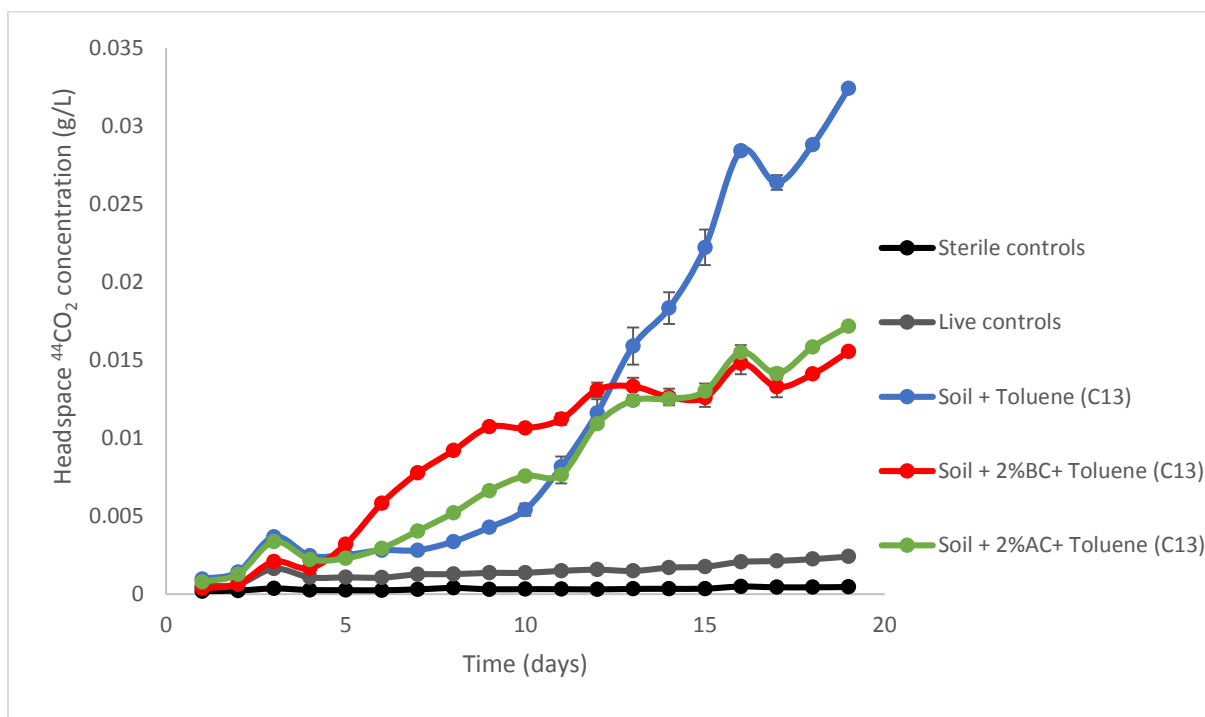
Figure 3.5. Headspace $^{44}CO_2$ concentrations (g/L) in different soil systems for sterile controls, live controls, soil only, soil & biochar and soil & AC. Error bars represent ±1 standard deviation from the mean of measurements (6 replicates).

The lower organic carbon contents of soil compared to the biochar amended and AC amended soils as determined previously (Section 3.3.1) may have been expected to generate a lower amount of $^{44}CO_2$ in the batch headspaces. On the contrary, the maximum cumulative amount of $^{44}CO_2$ produced by soil was twice as much as that produced in the biochar and activated carbon amended soils (Figure 3.5) suggesting that the carbon added in the form of biochar and activated carbon is largely inert i.e. not biodegradable. Soil organic matter is the product of microbial activity on the readily decomposable plant matter (DPM) as well as the recalcitrant plant matter (RPM) composed mainly of cellulose and lignin respectively. It is also made up of soil microbial biomass as well as the exudates of organic carbon being released from plant roots (Standing and Killham, 2007). By percent composition, it is made up of approximately 50-55% C, 5% H, 4.5% N, 33% O, 1% P and 1% S (Horwath, 2007). Biochar consists mainly of a large fraction of stable, aromatised carbon which is not readily biodegradable. Hence, microorganisms are not able to utilise the major part of biochar-C as energy source or the N present within the carbon structure (Lehmann *et al.*, 2011). A fraction of biocharC has, however, been described as labile or volatile and has been shown to readily leach and to be easily mineralised by soil microorganisms. In such cases, there has been indications of microbial activity stimulation as well as increase in abundance (Steiner *et al.*, 2008; Lehmann and Joseph, 2009). The process of mineralisation of soil organic matter releases nutrients such as N in the organic forms which can be subsequently utilised by microorganisms to sustain the process of decomposition. The growth of microbial populations is a process that is thought to be carbon-limited, therefore the introduction of a carbon source to soil systems should stimulate the growth and activity of microorganisms until the limiting factor to their growth becomes the amount of available nitrogen (Garten and Wullschleger, 1999; Garten Jr and Wullschleger, 1999; Garten Jr *et al.*, 2000). Such a situation would result in an intense competition for inorganic nutrients by heterotrophic bacteria where carbon availability is high (Franko *et al.*, 1995). Biochar used in the current study was produced at high temperature (pyrolysis) and therefore would not have contained much labile carbon suggesting that there was not a significant stimulation of the soil microorganisms based on the labile carbon content in the live soil & biochar batch.

The continuous rise in the production of $^{44}CO_2$ on day 19 (Figure 3.5), when the $^{45}CO_2$ production in the unamended soil had largely ceased, can be interpreted to mean that mineralisation of SOM is not yet limited by other factors such as nitrogen availability but that instead, $^{13}C_7$ toluene has been exhausted as an available carbon source(Figure 3.5).

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3.4.3 *Relationship between VPH biodegradation and SOM mineralisation in batch systems*

The $^{45}CO_2$: $^{44}CO_2$ ratios were determined in each soil system over the duration of the experiment as a way of comparing the level of metabolism of carbon sources present in the batches. *An increase in the isotope ratio is indicative of* a higher level of mineralisation of the substrate ($^{13}C_7$ - toluene) compared to the mineralisation of soil organic matter as shown in Figure 3.6. In the biochar and AC amended soils, a rise in the isotope ratio is observed from around day 4 and increases steadily to reach comparable peak values of 1.30 ± 0.03 and 1.02 ± 0.05 respectively. In the unamended soil, a longer lag phase that corresponds to earlier observations made in biodegradation of the substrate (Figure 3.3) is observed. A maximum isotope ratio of 2.22 ± 0.05 was attained on day 17 in the unamended soils. There was a statistically significant strong positive correlation between substrate biodegradation and mineralisation of soil organic matter ($r > 0.9$, $p < 0.01$) in the inoculated batches as indicated by CO_2 production (Figure 3.6) which suggests that toluene degrading microorganisms also utilize other carbon substrates to meet their overall growth requirements. The live soil controls showed a weaker, yet significant correlation ($r = 0.68$, $p < 0.01$) while in the sterile controls in which no $^{45}CO_2$ was detected throughout the experiment, no correlation between substrate biodegradation and SOM mineralisation was observed.

Treatment	Correlation coefficient (r)	P-value	Isotopic signature $\delta^{13}C$ (‰)
Live soil	0.682	1.31×10^{-13}	-159 ± 89
Soil	0.982	1.31×10^{-13}	$199,222\pm14,624$
Soil & BC	0.992	3.64×10^{-16}	$86,030\pm1204$
Soil & AC	0.986	1.46×10^{-13}	$114,614\pm3248$

*Table 3.1. Pearson correlation coefficient (**r**) between measured $^{45}CO_2$ and $^{44}CO_2$ concentrations in the respective soil systems along with their corresponding p-values (95% confidence level) and isotopic signatures.*

Results from a One-Way Analysis of Variance (ANOVA) reveals that the amendment factor had a significant effect ($p < 0.05$) on the carbon isotope signatures of the different soil systems. Considering that the substrate ($^{13}C_7$ toluene) was labelled 100% with the heavy carbon isotope (^{13}C), such high values up to four orders of magnitude higher than the carbon isotope signatures for naturally occurring toluene is expected (Bouchard *et al.*, 2008a; Bouchard *et al.*, 2008b). The process of sorption reduces bioavailability of organic pollutants from soils and sediments thereby hindering biodegradation by indigenous microbial

communities. The bioavailability of soil organic matter is probably less affected by the presence or absence of biochar or AC, since SOM compounds are less mobile than toluene and hence less readily bound by the biochar or AC. This can explain the lower isotopic signature of CO_2-carbon in the biochar and AC amended soil headspace compared to unamended soils (Table 3.1).
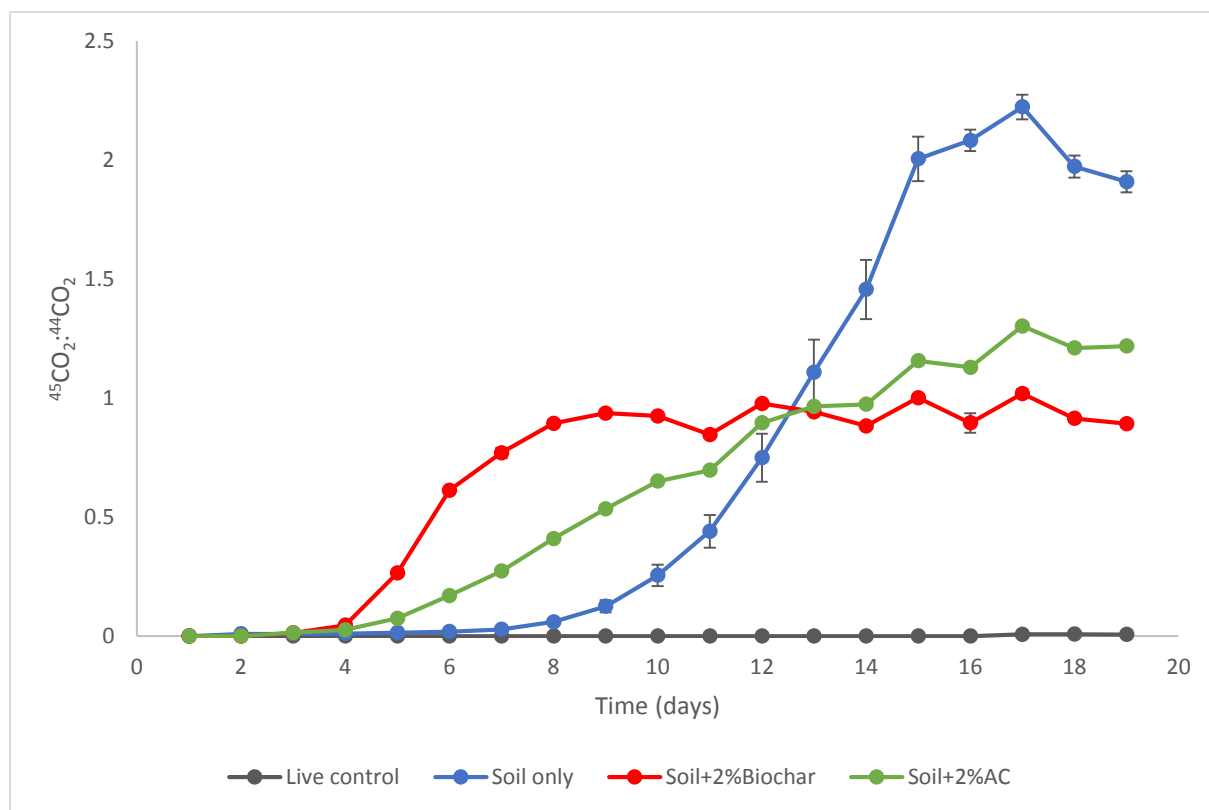
Figure 3.6. Measured $^{45}CO_2$:$^{44}CO_2$ ratios at different time points for the batches sterile controls, live controls, soil only, soil & biochar and soil & AC. Error bars represent ±1 standard deviation from measurements.

A comparison of the percentage toluene-^{13}C (g) converted into $^{45}CO_2$ –C (g) in the different soil systems with respect to time reveals that less than 50% of the initial mass of toluene-^{13}C (g) was metabolised into $^{45}CO_2$ – C in all treatments at the end of the experiment (Figure 3.7). Total CO_2 -C concentration in the batches as determined from headspace CO_2 concentrations and total soil-pore water- dissolved CO_2 revealed that by the 19th day, 28.7±2.4% of toluene-^{13}C (g) had been mineralised to CO_2-C in the unamended soil. In contrast to this, 8.4±1.4% toluene-^{13}C (g) in the biochar amended soil and 11.8±0.7% toluene-^{13}C (g) in AC amended soils had been converted to CO_2-C. Results from quantification of residuals (see Appendix A) after a 48 hours volatilisation in the fume hood shows that at the end of the experiments, on day 19, 96.20%±0.07, 80.48%±1.81 and 64.04%±0.18 of the initially introduced substrate (toluene - $^{13}C_7$) had in principle been bioavailable for either metabolism to CO_2 or biomass

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formation in soil, soil & biochar and soil & AC batches respectively. The relationship between the level of $^{45}CO_2$ production and the amount of substrate available for biodegradation is directly proportional, although the fate of most of the substrate in the batches is not thoroughly accounted for. Given that a generally low biomass yield was determined for all soil systems, it is reasonable to assume that most of the $^{45}CO_2$ produced by the substrate metabolism was lost to other ongoing abiotic processes in the soil systems. It is believed that a significant amount of CO_2 was lost through leakage from the Mininert caps and this became the motivation for subsequent CO_2 leakage experiments in the batches as discussed in the section below (section 3.4.4).
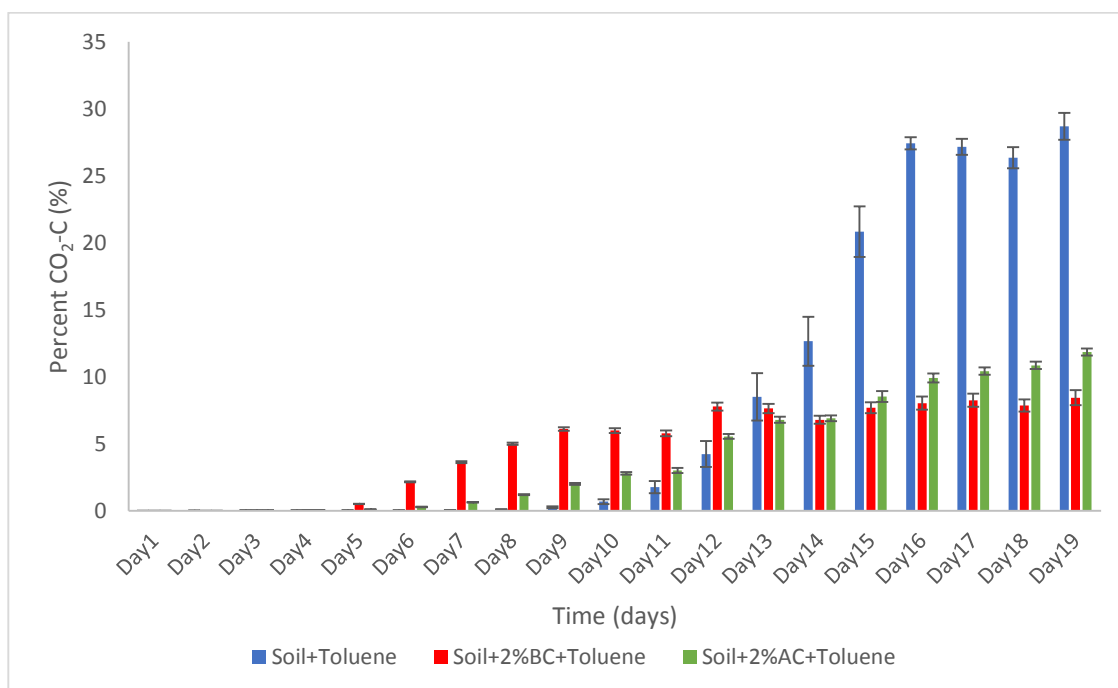
Figure 3.7. Percent $^{45}CO_2$-C (g) relative to $^{13}C_7$-toluene-C (g) produced at different time points for unamended soil, soil & biochar and soil & AC. Percentages represent total $^{45}CO_2$-C in batch headspace and soil pore water assuming that no CO_2 was lost in the batches by leakage. Calculations were based on the assumption that all of CO_2 produced is retained in the batches. Error bars represent ±1 standard deviation from mean of four replicates.

3.4.4 *Quantitative estimation of CO₂ loss from batch microcosms*

Results from CO_2 leakage and dissolution experiments are displayed in the graphs below (Figure 3.8; dotted lines). A comparison of the final concentrations of CO_2, after 14 days, with the initial CO_2 concentrations in the respective soil systems and controls (empty batch) reveals a variation in the reduction of CO_2. The empty batch vials had the highest percentage of CO_2 reduction of 75.0±1.8% followed by the soil & biochar batch in which 72.2±12.5% was reduced by the process of diffusion at the end of the experiment on day 14. In the soil & AC

batch and unamended soils, there was a percentage reduction in CO_2 concentration of $59.1\pm8.6\%$ and $53.8\pm10.5\%$ respectively from the batch headspaces by day 14.

A simulation of the leakage process in which the effects of pH on dissolution of CO_2 in the pore water of the soil systems was also considered is illustrated in Figure 3.8a (solid lines). Based upon the assumptions of CO_2 reduction in the vials via a small gap between the Mininert cap and the glass vial governed by Fick's first law of diffusion, a leakage factor of 5.0×10^{-6} m (gap area/gap length) for leaks via the gaps between the wall of the vials and the valves in the empty batches (grey lines) was determined in the models by fitting predicted values with experimental values using the least sum of squares method. In the unamended soil (represented by blue line graphs), the model predictions for headspace concentrations considering this leakage factor and CO_2 dissolution in soil pore water and at the initial soil pH were in line with the experimental measurements indicating that both dissolution and diffusion controlled the CO_2 concentration in the headspace of the batches. In soils amended with 2% biochar and AC represented by red and green line graphs respectively (Figure 3.8a), predictions were consistently above the measured values throughout the duration of the experiments suggesting that CO_2 loss in these soil systems was affected by factors other than diffusion and dissolution and leakage. Model predictions of Ca^{2+} concentrations at equilibrium with $CaCO_3$, and CO_3^{2-} in comparison with the measured aqueous Ca^{2+} concentrations are displayed in Table 3.2. Predicted and measured values were comparable for soil and soil & AC but not for soil & BC amended batch. A higher measured Ca^{2+} concentration in the soil & BC batch relative to the predicted equilibrium concentration indicates that the pore-water in this batch was oversaturated with regards to CO_2 at equilibrium with calcium carbonate. Further simulations of the leakage experiments assuming that all of the dissolved cations Ca^{2+} and Mg^{2+} precipitated out of the soil pore water solution as carbonates showed that the predictions agreed more closely with the measured values in the soils amended with biochar and AC (Figure 3.8b). This could mean that a high concentration of carbonate ion (CO_3^{2-}) species present in pore water of the soil systems at higher pH values of soil & BC and soil & AC (7.74 and 7.83 respectively) relative to soil pH (7.43) caused a precipitation of carbonate ion in the forms of insoluble $CaCO_{3(s)}$ and $MgCO_{3(s)}$ salts thereby further reducing the concentration of CO_2 in the batch headspaces. In contrast to the soil & biochar and soil & AC batches, the model predictions fell below experimental measurements in the unamended soil (Figure 3.8b) which may be due to the fact that a relatively low pH value (7.43) in the unamended soils did not have any significant effect on carbonate ion speciation in solution compared to the other soil treatments. Results from an ICP-OES analysis of the cation

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concentration in the pore water of the respective soil systems is included in the appendix (Appendix A).

Treatments	Ca^{2+} conc. (aqueous) (g cm^{-3})	Equilibrium Ca^{2+} conc. (atmo) (g cm^{-3})	Sum of squared residuals
Soil	6.49 x 10^{-4}	8.46 x 10^{-4}	1.98 x 10^{-4}
Soil & BC	1.10 x 10^{-3}	1.34 x 10^{-4}	1.15 x 10^{-5}
Soil & AC	4.02 x 10^{-4}	2.03 x 10^{-4}	2.35 x 10^{-6}

Table 3.2. Measured calcium ion concentration and modelled calcium ion concentration at equilibrium with atmospheric CO$_2$ concentrations in different soil systems.
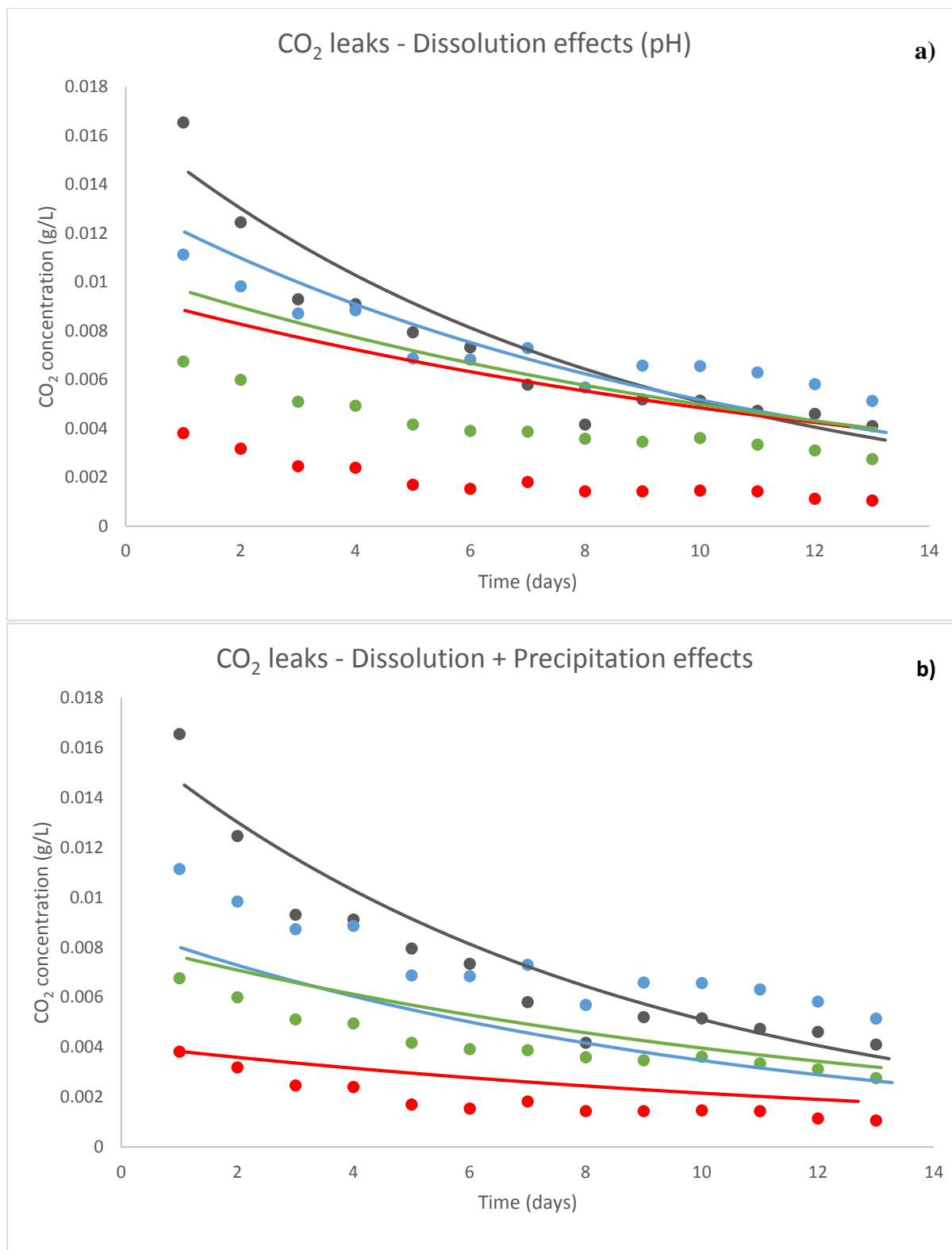
*Figure 3.8. Effects of **a**) CO_2 dissolution in soil pore water on loss of headspace CO_2 and **b**) dissolution and precipitation of CO_2 in forms of insoluble salts on the reduction of CO_2 concentrations for an empty batch vial (measured ●, modelled ▬), unamended soil (measured ●, modelled ▬), soil & biochar (measured ●, modelled ▬) and soil & AC (measured ●, modelled ▬) batches respectively.*

3.4.5   *Predicting biochar and activated carbon amendment effects on the biodegradation of stable-isotope labelled toluene ($^{13}C_7$) in gravelly sand*

The biokinetic model fits to the experimental data of headspace toluene concentration, and CO_2 evolution for soil, soil & biochar and soil & AC are shown in Figure 3.9, Figure 3.10 and Figure 3.11 respectively. At the outset, a biomass maximum specific growth rate of 0.6 h^{-1} in line with published rates (Table 3.3) was assumed for all soil systems. Based on this assumption, the half-saturation constant was varied for the different systems to obtain best fits between experimental and modelled data. Furthermore, the maximum soil biomass carrying capacity was adjusted to explain a levelling off of CO_2 concentrations in soil & biochar and soil & AC systems towards the end of the experiments. Leakage of CO_2 and toluene from the batches was predicted using the leakage factor determined from the empty batch experiment described in the previous section. Growth on ^{13}C toluene and ^{12}C soil organic matter was simulated based on a fixed ratio of ^{13}C to ^{12}C assimilation.

In the soil batches, substrate utilization profiles along with the corresponding predicted biomass growth (Figure 3.9 a & b) showed an initial rapid decline in the headspace concentration of toluene which is followed by a 'fairly' consistent concentration over a period of about ten (10) days. This can be interpreted to mean that sorption of pollutant by soil organic matter at the onset of the experiment attains equilibrium between the soil solids, the soil pore-water and the headspace air quite rapidly. The period between day 1 and 10 (Figure 3.9a) is interpreted in the model as the lag phase in the biomass growth (Figure 3.9b) after which a second dip is observed in the headspace concentration of the substrate due to biodegradation. The period from day 10 onwards (Figure 3.9a) represents a phase of intense biodegradation of toluene which in the model corresponds with a rapid biomass growth (Figure 3.9b) and a concurrent increase in CO_2 levels in the batch headspaces (Figure 3.9c & Figure 3.9d). The long lag phase could be due to the toxic nature of toluene at high concentrations. Toxicity of toluene has been associated with accumulation of the compound into bacterial membranes due to its hydrophobicity and ability to preferentially partition into bacterial cell membranes thereby increasing membrane fluidity and non-specific permeabilization (Aono *et al.*, 1994; Heipieper and Martinez, 2010). This may also explain the relatively longer lag phase during which soil microorganisms acclimate to the new substrate in the unamended soil batch (Figure 3.9b). With respect to CO_2 production in the soil batches, the model fits were in line with experimentally determined data indicating a fairly accurate description of substrate utilization and biomass formation by the Monod kinetics model. A half-saturation constant value of 4.06 x 10^3 mgL^{-1} was predicted by the model for the unamended soil batch (Table 3.3). The predicted value was higher than the

values obtained from most studies on the growth kinetics of toluene degrading microorganisms (Table 3.3). One could fit a lower half-saturation constant to the data by reducing the value of the maximum growth rate from the literature values used in this study (Table 3.3).

In the soil & biochar batch, the substrate degradation profile as predicted by the model (Figure 3.10) reveals a different pattern in which a sharp decline in the headspace concentration of toluene due to biochar-enhanced sorption is followed by a stable concentration almost below the limit of detection over the duration of the experiment. Substrate utilization was slightly over-predicted from around day 10, although this could be due to the difficulty of quantifying head-space concentrations near the detection limit. Biomass growth, however, showed a pattern that corresponds with the Monod growth model but limited by logistic growth (Figure 3.10b). Based upon the model predictions, maximum biomass growth in the biochar amended soils was approximately 3 times lower than the biomass growth in the unamended soil. Model predictions for the production of CO_2 from the mineralization of SOM and the mineralization of toluene in the biochar amended batches (Figure 3.10c & Figure 3.10d) were comparable. The half-saturation constant in the soil & biochar batches had a value of 7.76×10^2 mgL^{-1} and was lower than the prediction in the unamended soil batches by an order of magnitude (Table 3.3). This could be due to sorption of toluene in the biochar amended soil which reduces the concentration of bioavailable substrate to the microorganisms. Consequently, microorganisms which can more effectively utilize low substrate concentrations may have an ecological advantage in the soil & biochar batches. The predicted half-saturation constant was however still much higher in the biochar amended soil than the values obtained from the literature by up to two orders of magnitude (Table 3.3).

In the soil and activated carbon batch, the predicted toluene degradation profiles followed a similar pattern to those in the biochar amended soils (Figure 3.11a). A lower value was predicted for the half-saturation constant of toluene in the activated carbon amended soil compared to the unamended soil and the biochar amended soil batches (Table 3.3). Activated carbon amendment of VPH contaminated soils was shown to enhance the sorption of VPHs more than biochar or unamended soils in a recent batch and column study by Bushnaf (2014). A relatively shorter lag-phase in the growth cycle of the biomass in the AC amended soil can be interpreted to mean that less amount of toluene is bioavailable to soil microorganisms in soil pore water, hence less pollutant toxicity and the length of time required for acclimation to the substrate.

The concurrent increase in the concentrations of both ^{12}C-CO_2 and ^{13}C-CO_2 is a common trend in the batch headspaces of all treatments under investigation (Figure 3.9c&d, Figure 3.10 c&d, and Figure 3.11 c&d). The level of ^{12}C-CO_2 produced in response to the addition of a ^{13}C substrate is remarkable. The cometabolic activity of soil microorganisms on stable-

Model type	Compounds	Parameters	Microorganism	Reference
Monod	Toluene	$K_s = 0.12 \pm 0.02 mgL^{-1}$ $\mu_{max} = 0.86 \pm 0.01 h^{-1}$ $Y_{x/s} = 1.28 \pm 0.01\ g\ g^{-1}$	*Pseudomonas putida* F1	(Reardon *et al.*)
Monod	Toluene	$K_s = 12.22 mgL^{-1}$ $\mu_{max} = 0.68\ h^{-1}$ $Y_{x/s} = 0.71\ g\ g^{-1}$	Bacterial consortium	(Oh *et al.*)
Cometabolism, SKIP	BTEX	$\mu_{max,T} = 0.60\ h^{-1}$ $K_{s,T} = 34.12 mgL^{-1}$ $Y_{x/s,T} = 1.25\ g\ g^{-1}$	Consortium	(Littlejohns and Daugulis)
Andrews	Toluene	$K_s = 0.42 mgL^{-1}$ $\mu_{max} = 3.98\ h^{-1}$ $Y_{x/s} = 0.9\ g\ g^{-1}$	*Pseudomonas putida* 54G	(Mirpuri *et al.*)
Monod	Toluene	$\mu_{max} = 0.6\ h^{-1}$ $K_{s\ (soil)} = 4.06\ x\ 10^3\ mgL^{-1}$ $Y_{x/s\ (soil)} = 0.33\ g\ g^{-1}$ $K_{s\ (soil\ \&\ BC)} = 7.76\ x\ 10^2 mgL^{-1}$ $Y_{x/s\ (soil\ \&\ BC)} = 0.073\ g\ g^{-1}$ $K_{s\ (soil\ \&\ AC)} = 1.83\ x\ 10^2\ mgL^{-1}$ $Y_{x/s\ (soil\ \&\ AC)} = 0.26\ g\ g^{-1}$	Soil VPH degraders	*Current study*

Table 3.3. A summary of the biokinetic parameters used to simulate the biodegradation of toluene and biomass growth of associated microorganisms from the current study compared to values obtained from some published studies. Biomass yields are expressed as dry weight (g) biomass g^{-1} substrate.

isotope labelled toluene and SOM is a likely explanation for this observation. Similar observations were made in studies by Chang *et al.* (1992) and Littlejohns and Daugulis (2008) in which the compound toluene was utilised by microbes as a primary substrate for energy while simultaneously metabolising other secondary, non-growth substrates in the presence of toluene.

Biomass yields coefficients (g biomass-C g^{-1} substrate-C) ranged from 0.073 g biomass g^{-1} substrate for soil & biochar to 0.33 g g^{-1} for soil and 0.26 g g^{-1} for soil & AC and were much lower than values reported by Elazhari-Ali *et al.* (2013) in a study to investigate the effects of nutrient amendment on the biodegradation of a mixture of 12 VPHs. Yields were determined assuming a biomass carbon content of 100 fg C/cell (Whitman *et al.*, 1998).

Treatments	[b]K_{dec} (h^{-1})	[b]Cb_{max} (Moles biomass-C m^{-3})	[a]Sorption coefficient (m^3 kg^{-1})	[a]Lag phase (hours)	[a]C_{12}/C_{13} assimilation ratio
Soil	6.3 x 10^{-4}	400	7.93 x 10^{-4}	240	0.45
Soil & BC	9.0 x 10^{-5}	11	1.64	96	0.70
Soil & AC	2.7 x 10^{-5}	25	9.32	142	0.63

Table 3.4. Fitted and experimentally determined parameters used to run the logistic growth model of toluene degrading biomass in different soil systems.

a) experimentally determined values   b) fitted values

Other parameters used to predict microbial growth kinetics on toluene in the batches are displayed in Table 3.4. Microbial biomass decay rates (K_{dec}) ranged from 6.3 x 10^{-4} in soil to 9.0 x 10^{-5} and 2.7 x 10^{-5} (h^{-1}) for soil & biochar and soil & AC respectively. These values are comparable with decay rates of 9.0 x 10^{-4} (h^{-1}) and 2.1 x 10^{-3} (h^{-1}) used by (Meynet *et al.*, 2014) to simulate the biodegradation of a mixture of VPHs in column sand amended with or without biochar respectively. In a study by Bauer *et al.* (2008), a higher value for the decay rate, d, of 1.3 x 10^{-2} (h^{-1}) was used to simulate the biodegradation of petroleum hydrocarbons in aquifer material containing *Pseudomonas putida* strains. Maximum biomass carrying capacity (Cb_{max}) was quite variable and somewhat higher for the unamended soil batches than the amended soil batches. This could be due to sorption of nutrients such as NH_4^+ by the biochar and AC. Sorption capacity as indicated by the sorption coefficient was higher for the sorbents (biochar and activated carbon) by about four orders of magnitude compared to soil.
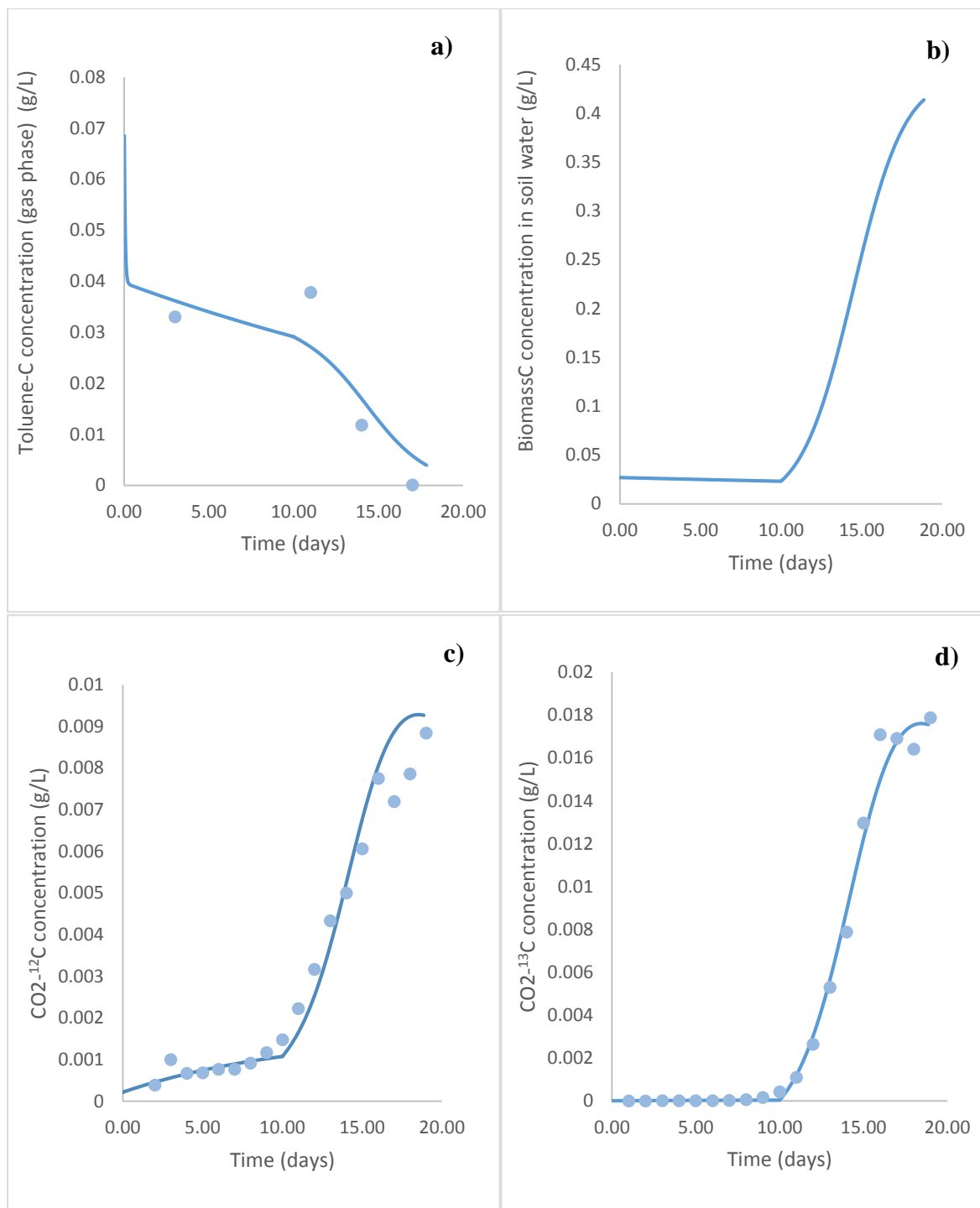
*Figure 3.9. A graph indicating **a)** substrate utilisation **b)** biomass growth in soil pore water **c)** headspace $^{44}CO_2$ concentration and **d)** headspace $^{45}CO_2$ concentration with respect to time in a soil batch. Modelled (line graph) and measured (dotted graph).*
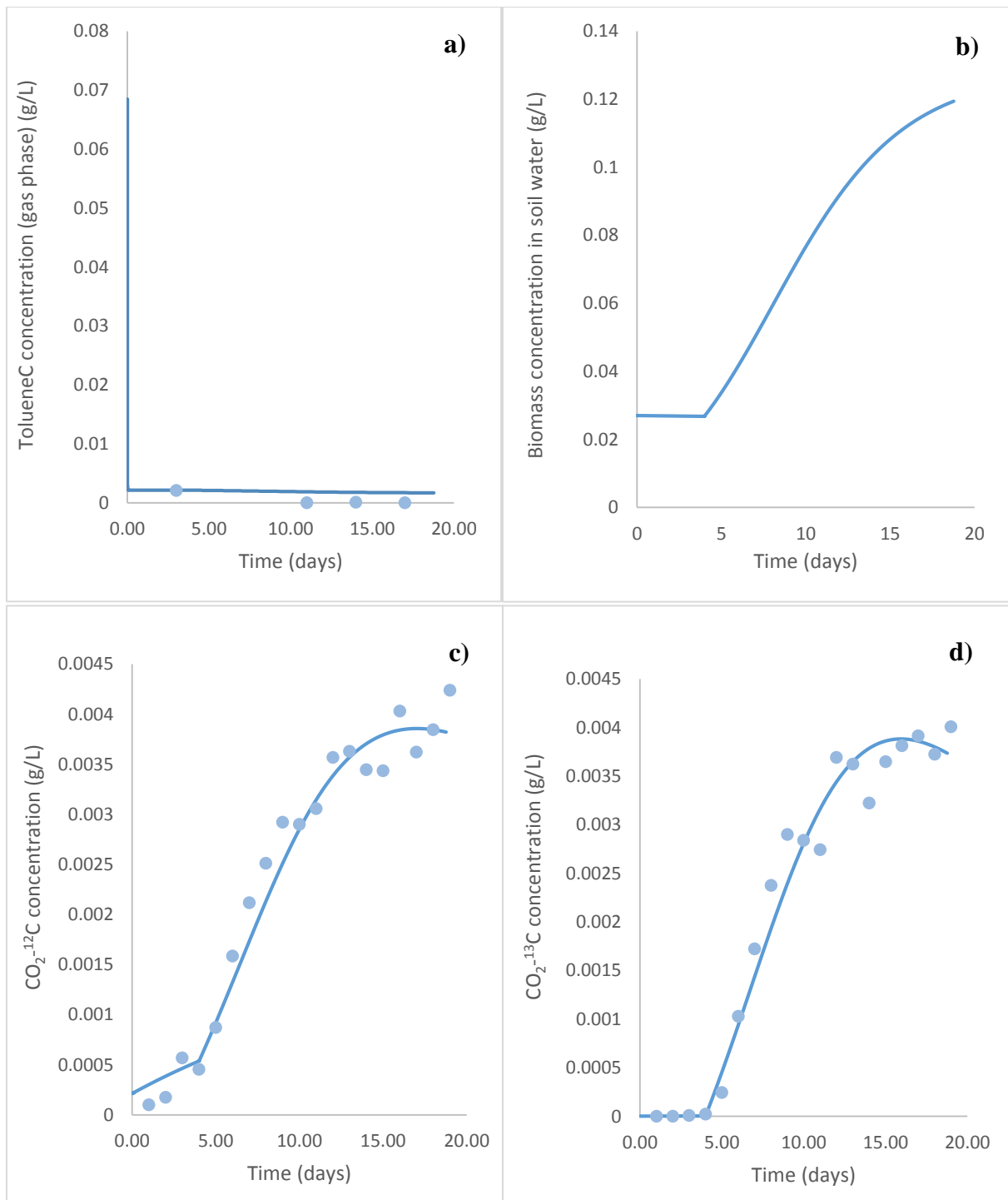
*Figure 3.10. A graph indicating **a)** substrate utilisation **b)** biomass growth in soil pore water **c)** headspace $^{44}CO_2$ concentration and **d)** headspace $^{45}CO_2$ concentration with respect to time in soil + 2%biochar batch. Modelled (line graph) and measured (dotted graph).*
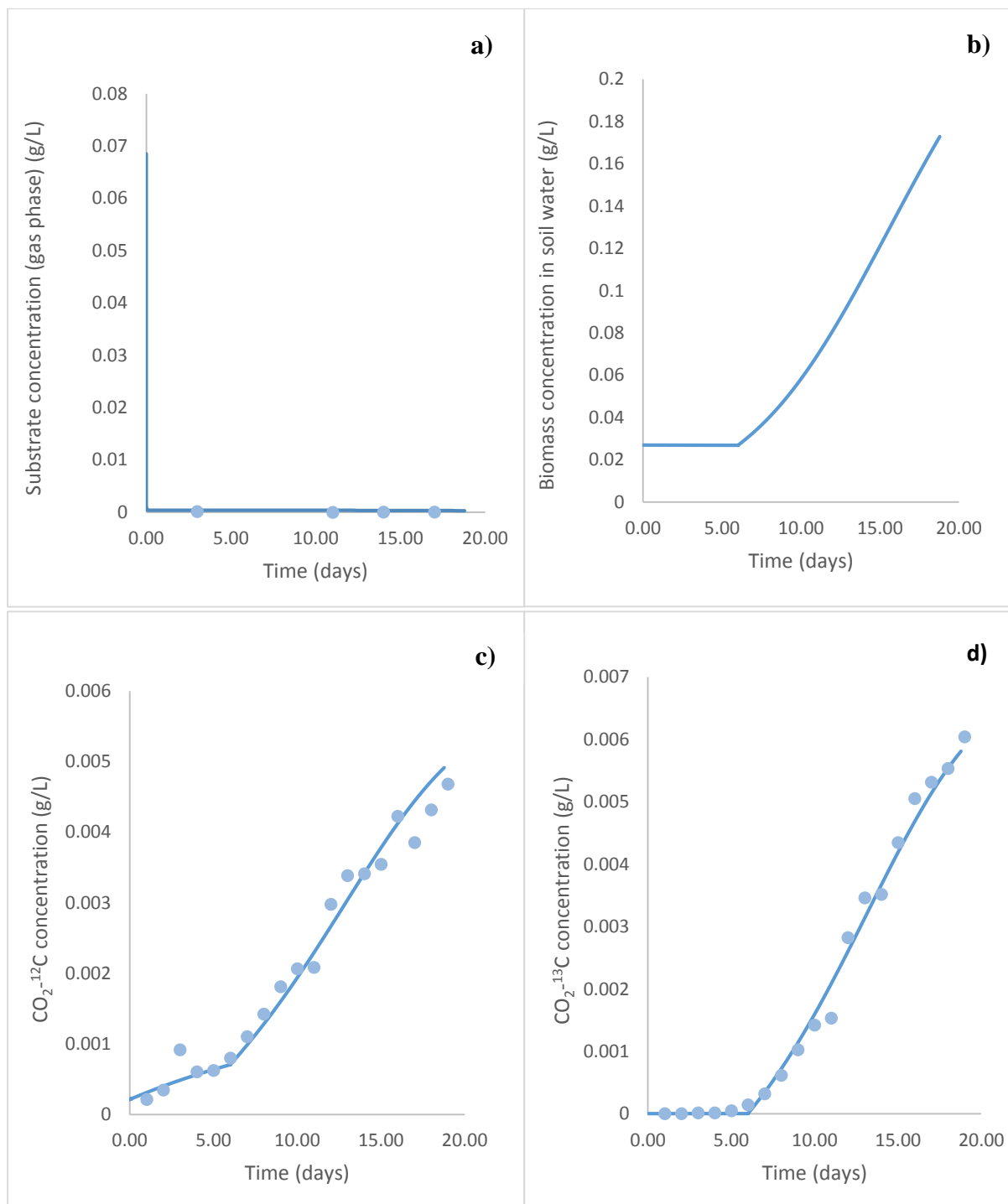
*Figure 3.11. A graph indicating **a)** substrate utilisation **b)** biomass growth in soil pore water **c)** headspace $^{44}CO_2$ concentration and **d)** headspace $^{45}CO_2$ concentration with respect to time in soil + 2% activated carbon batch. Modelled (line graph) and measured (dotted graph).*

3.4.6 *Predicting the biodegradation of poorly available substrate ($^{13}C_7$ - toluene) by microorganisms in different soil systems after evacuation of pollutant source*

Model predictions for the mineralisation of SOM and strongly sorbed toluene post evacuation of the pollutant source based on CO_2 evolution in the different soil systems are shown in Figure 3.12. For the unamended soil (figure 3.12a &b), the experimentally determined ^{12}C-CO_2 production rose slowly in a similar manner to ^{13}C-CO_2 production with a short lag phase. Although soil microorganisms were expected to have acclimated to soil conditions from the previous batch experiments i.e. prior to evacuation of toluene, soil bacteria appeared to require a brief period of lag in growth. This was likely due to the processes of pollutant removal, transfer of soil into smaller vials, addition of deionised water droplet to moisten soil and mixing of soils in order to break up aggregates and to further aerate the soil. ^{12}C and ^{13}C CO_2 production in unamended soil reached maximum concentrations of 0.014 g/L and 0.010 g/L respectively by the 14th week. From the residual toluene analysis conducted prior to the batch (phase II) experiments (Section 3.3.8), it is evident that only a fraction (3.66±0.07%) of the originally introduced substrate was still bound to soil particles after evacuating the batches of toluene vapour. The model slightly over-predicted the concentrations of both ^{12}C and ^{13}C - CO_2 in the unamended soil batch. This may be based on the assumption that the sorbed toluene to soil is relatively rapidly desorbed back into pore-water than is suggested by the experimentally determined values.

In the soil and biochar batch, model predictions for the mineralisation of SOM and toluene are more in line with the measured values (Figure 3.12 c & d). The maximum experimentally determined CO_2 – C concentrations were 0.008 g/L and 0.004 g/L for ^{12}C and ^{13}C respectively. These values are one order of magnitude lower than the values for the unamended soils which may be interpreted to mean that the strongly sorbed pollutants to biochar is more slowly released into soil pore water for biodegradation by microorganisms compared to the soil batches. In the AC amended soil batch, model predictions of ^{12}C and ^{13}C CO_2 were initially higher than the measured values but were eventually aligned for the most part. Maximum ^{12}C and ^{13}C CO_2 concentrations in the soil and AC batch as determined experimentally were 0.014 g/L and 0.016 g/L respectively. The concurrent increase in the concentration of $^{44}CO_2$ in the headspace of the batches could imply ongoing respiration by live microbial biomass from the metabolism of SOM to which they have been previously adapted or that biomass decomposition also generates $^{44}CO_2$ in the respective batches.
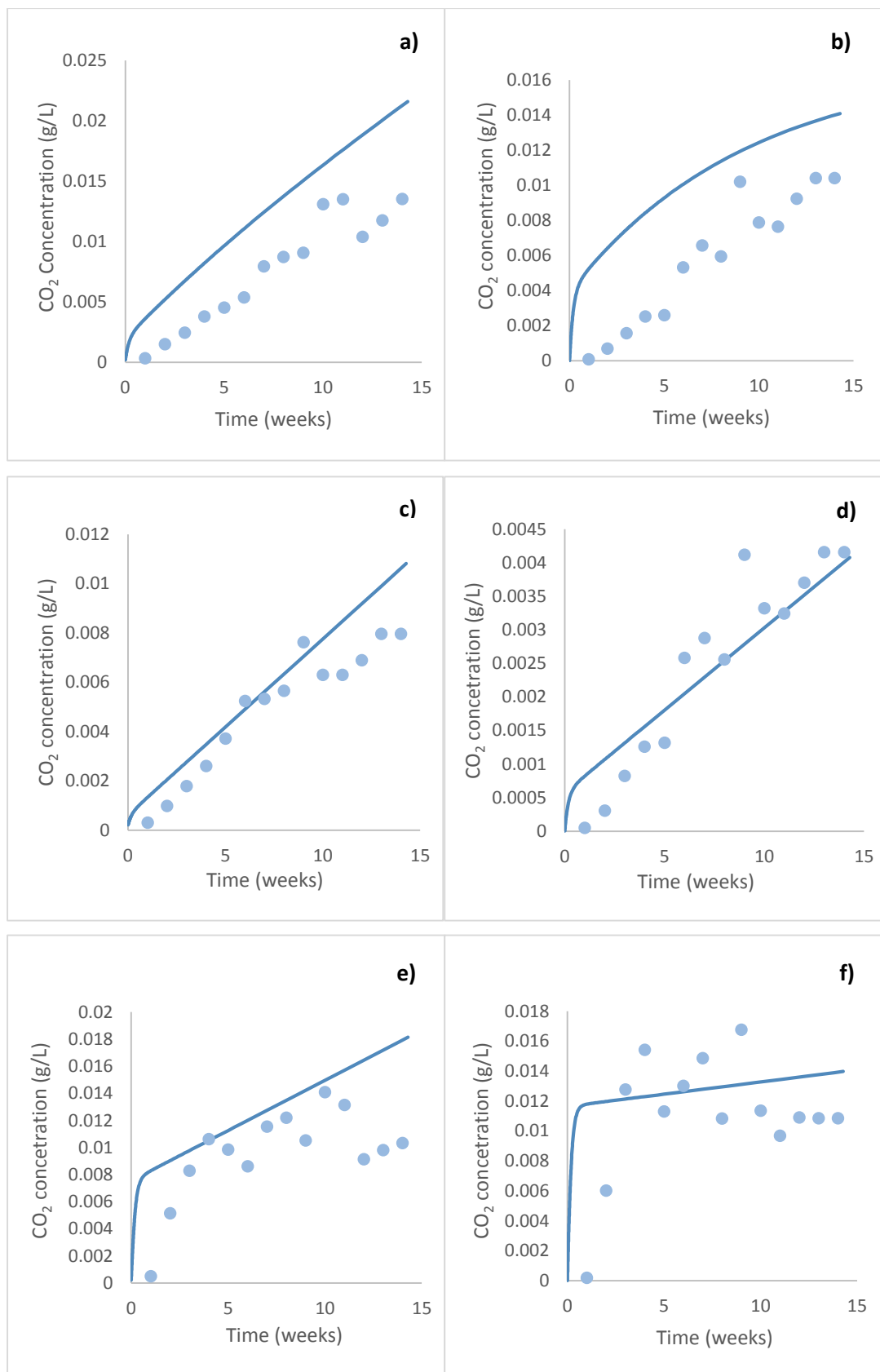
Figure 3.12. A graph illustrating headspace $^{44}CO_2$ and $^{45}CO_2$ concentration with respect to time for soil (a & b), soil & biochar (c & d) and soil & activated carbon (e & f) batches. Modelled (line graph) and measured (dotted graphs).

3.5 Conclusions

The effects of amending soil with and without biochar and activated carbon on the biodegradation of stable-isotope labelled toluene and soil organic matter by indigenous soil microorganisms was investigated. From the foregoing discussion, addition of stable-isotope labelled toluene ($^{13}C_7$) to soil treatments increased the concentrations of headspace $^{45}CO_2$ compared to sterile and live soil controls indicating that microbial activity was enhanced by addition of a carbon source in the form of VPH. Also, there was an increase in the preference for $^{13}C_7$ toluene as carbon source compared to SOM mineralisation in the different soil systems. Carbon-isotope ratios ($^{45}CO_2 : {}^{44}CO_2$) in headspace air for soil & biochar and soil & AC were 1.30 ± 0.03 and 1.02 ± 0.05 (Figure 3.6) respectively as compared to 2.22 ± 0.05 in soil without VPH addition further confirming the increase in the utilisation of $^{13}C_7$ toluene compared to other carbon substrates especially in the unamended soil batch.

Amending soil with either 2% biochar or activated carbon enhanced sorption of the substrate $^{13}C_7$ toluene right from the start of the experiment as indicated by the headspace toluene concentration in the batches (Figure 3.4). Sorption of the pollutants reduced their availability to soil microorganisms and consequently slowed mineralisation of toluene as indicated by the lower CO_2 concentrations in the sorbent amended soil batches (Figure 3.3). Amending soil with activated carbon was previously shown to slow the rate of phenanthrene mineralisation in a study by Rhodes *et al.* (2008).

Model predictions assuming that only soil pore water-dissolved toluene is biodegradable, indicate that biochar and activated carbon amendment also altered the growth kinetics of toluene degrading microorganisms contrary to our proposed hypothesis. By fitting a maximum specific growth rate of 0.6 h^{-1} (in line with published rates), it was possible to match model predictions of $^{45}CO_2$ and $^{44}CO_2$ concentrations with the experimentally determined data (Figure 3.9, Figure 3.10, Figure 3.11). Half-saturation constants of 4.06×10^3 mgL^{-1}, 7.76×10^2 mgL^{-1} and 1.83×10^2 mgL^{-1} were predicted for soil, soil & biochar and soil & AC respectively, much higher than values reported in the literature. The difference between the treatments suggests that amending the soil with biochar and activated carbon thus increased the affinity of microorganisms for the substrate. Differences in the half-saturation constant also suggests that sorbent amendment affects the microbial ecology, by making microorganisms which can utilize substrates at lower concentrations more competitive. Overall, amending soil with 2% biochar or activated carbon hindered the mineralisation of the substrate $^{13}C_7$ toluene due to sorption of the compound and caused a shift in preference for the substrate as indicated by the relatively high SOM mineralisation in the sorbent amended soils.

Microbial growth kinetics parameters (μmax and K_s) are determined by several environmental factors such as soil type, microbial community structure, substrates e.t.c. It is necessary to study the effects of these environmental variables on the biokinetics of petroleum hydrocarbon contaminated soil. A further study was therefore motivated to investigate the effects of VPHs on the microbial community structure and dynamics of the different soil systems in the short and long-term in order to gain a better understanding of their effects on microbial growth kinetics.

Chapter 4: A comparison of the varying effects of different petroleum hydrocarbon classes on the bacteria community response in gravelly sand

4.1 Introduction

Petroleum hydrocarbons may serve as excellent sources of carbon and energy for the growth of microbial biomass in contaminated sites (Galli, 1998). The metabolic pathways for the degradation of PHs have been extensively studied (Van Hamme *et al.*, 2003; Das and Chandran, 2010). Petroleum hydrocarbons are reported to have varying levels of susceptibility to microbial attack and the order of susceptibility of PHs to microbial attack is as follows: cyclic alkanes < monoaromatics < branched alkanes < linear alkanes (Ulrici, 2000). At the same time, some constituents of petroleum may become persistent and toxic once released into the environment. Toxicity of PHs could be a function of their physico-chemical properties such as molecular structures e.g. complex poly-aromatic hydrocarbons (PAHs) tend to persist in the environment compared to mono-aromatic compounds due to their recalcitrant nature, or the solubility of these compounds in aqueous solutions as solubility enhances the bioavailability of organic compounds to indigenous microorganisms and hence their biodegradability. Sikkema *et al.* (1995) conducted a study to investigate the relationship between the hydrophobicity of cyclic hydrocarbons and their toxicity and reported a correlation between compound hydrophobicity and toxicity. In another study by Kabelitz *et al.* (2003), varying chain lengths of aliphatic hydrocarbon metabolites ranging from short chain to intermediate chain length compounds were investigated for their toxic effects on microorganisms. The antimicrobial effects of these compounds were found to be directly proportional to hydrophobic chain length of the molecules underscoring the significance of compound lipophilicity in the determination of toxicity. Above a threshold concentration in the environment, VPHs may exert some deleterious effects on soil microorganisms. The mechanisms by which these effects occur have been previously reviewed (Heipieper *et al.*, 1991b; Kabelitz *et al.*, 2003; Heipieper and Martinez, 2010). Different organisms are reported to show different levels of sensitivies to the same class of compounds. As a result, it is critical to develop an understanding of the response of soil bacteria community to contamination with different classes of PHs under varying environmental conditions as this would enhance the design and development of effective remediation systems.

The use of culture-independent techniques as a means to gaining improved understanding of the microbial community responses to environmental factors is gaining wider acceptance and applicability in the 'ecological' community. The advancements in molecular biology techniques – next-generation sequencing- has enabled an in-depth exploration of large

amounts of sequencing data from environmental samples (Shokalla *et al.*, 2012). Such sequencing data have been used in a variety of environmental applications including studying the effects of soil management types on microbial communities (Nacke *et al.*, 2011) and investigating the health status of an ecosystem by analysing its biodiversity (Hajibabaei *et al.*, 2011).

454-pyrosequencing platform has been widely used within the last decade to conduct metagenomics studies of environmental samples particularly because of the long read lengths of sequences and the relatively short run time of the technique (Shokalla *et al.*, 2012) while the Ion torrent sequencing platform, which is based upon the real-time detection of hydrogen ion concentration, has been developed to utilise one of three different ion chips 314, 316 or 318 each of which is capable of generating up to 10Mb, 100Mb or 1Gb of sequencing data respectively (Shokalla *et al.*, 2012). Such advances may serve as an invaluable tool for gaining insights into the effects of petroleum hydrocarbon contamination on soil microbiology.

4.2   Aim

The aim of this study was to investigate the effects of a non-aqueous phase liquid (NAPL) of different VPH classes (in separate mixtures): straight chain alkanes, aromatic hydrocarbons and cyclic/branched alkanes on the response of the indigenous micro-organisms in a gravelly sand using molecular biology techniques. An aerobic batch experiment was set up in order to monitor the level of biodegradation taking place within different soil systems and control.

4.2.1   *Objectives*

In order to accomplish the aforementioned aim, the following objectives were set:

1. To determine the level of biodegradation of different classes of VPHs compared to an uncontaminated soil by indigenous microorganisms under aerobic batch condition.

2. To determine the effects of different VPH classes on the richness and diversity of microbial communities present within the soil under investigation.

3. To identify pollutant degrading microorganisms for different VPH classes based on their increased abundances in specific treatments.

4. To determine any variation in soil microbial community structures that may arise from the use of different sequencing platforms – 454 pyrosequencing and Ion torrent sequencing to conduct metagenomic studies.

4.2.2 *Hypotheses*

The following hypotheses are proposed:

1. VPHs are known to serve as a carbon and energy source for the growth of certain microorganisms that are able to grow on them. It is therefore expected that the addition of a carbon source in the form of different PH classes will result in varying bacterial responses with respect to the relative biodegradability of straight versus branched/cyclic alkanes versus aromatic hydrocarbons.

2. Also, addition of new substrate (VPH) to the soil should enhance the richness and diversity of microbial communities in the contaminated soils compared to soil in which no VPH was added.

3. It is expected that microbial communities growing on different VPH classes will differ significantly in their species compositions.

4. It is not expected that different sequencing platforms – 454 pyrosequencing and ion torrent sequencing will significantly affect the quality of data generated i.e. type of microbial communities identified from the soil samples. It is, however, expected that a higher amount of data generated by the Ion torrent sequencing platform would result in higher richness and diversity indices of microbial communities compared to the 454-pyrosequencing-derived dataset.

4.3   Materials and methods

4.3.1   *Soil and chemical pollutant mixtures*

The soil used in this chapter is the same as the soil used in chapter 3 (Section 3.3.1). The chemical preparations are as follows: high purity chemicals (Sigma Aldrich, UK) were mixed into three separate classes of petroleum hydrocarbons. The aromatic hydrocarbon mixture consisted of 1 mL each of toluene, m-xylene, and 1, 2, 4-trimethylbenzene (I, 2, 4-TMB). Straight chain hydrocarbon mixture consisted of 1mL each of n-octane, pentane, hexane, decane and dodecane while the cycloalkanes/branched alkane was made up of a mixture of iso-octane, cyclohexane, methylcyclohexane and methylcyclopentane (1 mL each). Each PH mixture was made in transparent glass vials and stored in the dark at room temperature (20 ± 2 °C).

4.3.2   *Batch experiments*

Batch microcosm experiments were performed in amber vials (65 mL, Jencons, a VWR Division, Leicestershire, UK) closed with Teflon Mininert caps (Supelco, Bellefonte, USA) containing 15 g of gravelly sand (water content: 0.1 g g^{-1} soil d.w.) and inoculated with 30 µL of either aromatic hydrocarbon mix or straight chain hydrocarbon mix or a mixture of cycloalkanes/branched alkane. Each treatment was prepared in triplicates and an additional set of live soil controls (without petroleum hydrocarbons) was also prepared in triplicates. The experiments lasted for 14 days (2 weeks).

4.3.3   *Microbial respiration*

Soil respiration was monitored in the batches over a 14 days period by measuring the concentration of headspace CO_2 in each vial containing either soil, or soil inoculated with aromatic hydrocarbon mixture or soil inoculated with aliphatic hydrocarbon mix or soil and alicyclics/branched alkanes. For each soil type, contaminated or non-contaminated, triplicate batches were monitored at room temperature (20 °C) and the analysis of headspace CO_2 was conducted using a Fisons 8060 GC linked to a Fisons MD800 MS with a HP-PLOT-Q capillary column.

4.3.4   *Sample collection and storage*

At the end of the batch experiments, the vials were uncovered and samples were collected in triplicates and stored at -20 °C in filtered-sterile phosphate buffer saline (PBS, Oxoid) 1:1 vol/vol for DNA extraction and PCR amplification. Triplicate samples were also collected from the unamended soil batch and stored for microbial analysis.

4.3.5 *DNA extraction and PCR amplification (454 pyrosequencing library preparation)*

Genomic DNA was extracted from 50 mg of soil (wet weight) using the FastDNA Spin kit according to the manufacturer's instructions (MP Biomedicals, UK). The V4 and most of the V5 regions of 16S rRNA gene was PCR amplified by multiplex PCR reactions (averagely 15 reactions per sample), using primer set 515f (5'-GTGNCAGCMGCCGCGGTAA-3') and 926r (5'-CCGYCAATTYMTTTRAGTTT-3') (Wilhelm *et al.*, 2013), with read length of 400 – 500 base pairs (bp). A unique 8 bp barcode, added to the 5' –end of both the forward and reverse primers through a GA linker, was used to label each sample. The primers were attached to the GS FLX Titanium adapter A (5' - CGTATCGCCTCCCTCGCGCCATCAG – 3') and adapter B (5' – CTATGCGCCTTGCCAGCCCGCTCAG – 3'). Each PCR reaction was performed in a total volume of 25 µL containing 0.5 µL of DNA template, 0.4 µmol L^{-1} of each universal primer, 0.2mmol L^{-1} dNTPs (PCR grade Nucleotide Mix, Roche), FastStart High Fidelity Enzyme Blend (2.5U/reaction), and a final concentration of 1.8 mM $MgCl_2$ in the FastStart High Fidelity Reaction Buffer (Roche Diagnostics GmbH, Mannheim, Germany). The following PCR thermal cycling programme was used: an initial denaturation step of 95 ℃ for 4 minutes followed by 25 cycles of denaturation at 95 ℃ for 1minute, annealing of primers at 55 ℃ for 45 seconds, and elongation at 72 ℃ for 1 minute. The final elongation step was at 72 ℃ for 7 minutes. Multiplex PCR amplicons were pooled together and cleaned using the QIAquick PCR purification kit (QIAGEN, Crawley, UK) according to the manufacturer's instruction. Prior to 454 sequencing of PCR amplicons, the amount of DNA present in clean PCR products were quantified using a Qubit® 2.0 Fluorometer following the manufacture's protocol.

4.3.6 *454 pyrosequencing and Ion torrent sequencing*

Clean PCR amplicons were pooled together (in triplicates) in equimolar concentrations and sequenced on a Roche 454 GS Junior (Macropathology Ltd., Coventry, UK). Sequencing was carried out in a bi-directional manner using unique 8 base-pairs barcoded 515f and 926r primers for both forward and reverse runs. The output data from the sequencing runs in the standard flowgram format (SFF) was filtered for quality and subsequently denoised on a 16 core-computer cluster.

For the ion torrent sequencing, in addition to the sample preparation for PCR, the samples were labelled using a unique 12 base pairs Golay barcode, added to the 5' – end of the forward primers through a GAT spacer, and attached to the Ion adapter A (5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3') while the reverse primers were attached to the Ion adapter trP1 (5'- CCTCTCTATGGGCAGTCGGTGAT-3'). PCR amplicons were

cleaned and size selected using double-sided solid-phase reversible immobilisation (SPRI) beads (Agencourt AMPure XP system, Beckman Coulter). Clean PCR amplicons from samples as described in section 4.3.5 were also pooled in triplicates in equimolar concentrations and sequenced on a Personal Genome Machine (PGM) using a 316 ion chip by the School of Civil Engineering & Geosciences, Environmental Engineering Dept. Newcastle University.

4.3.7 *Microbial community structure analysis*

Briefly, the reads were filtered for quality (filtering criteria: perfect match to sequence barcode/primer, 200bp minimum sequencing length). QIIME Denoiser (Reeder and Knight, 2010) was used to detect and correct sequencing errors and the data were reintegrated into the QIIME pipeline by inflation. The so obtained sequences were clustered into Operational Taxonomic Unit (OTU) at 97% sequence similarity level by the uclust algorithm, a representative sequence from each OTU was selected and taxonomically identified using Greengenes database (McDonald *et al.*, 2012; Werner *et al.*, 2012). Representative sequences and correspondent taxonomic assignment were used to build a table of OTU abundances at different levels of taxonomy. The QIIME (v.1.8.0) pipeline (Caporaso *et al.*, 2010) was used to determine the microbial community diversity within each sample and across the 12 samples. The resulting OTU table at the class level (L3) of taxonomy was imported into PRIMER v6 and log transformed for subsequent beta (β) diversity analysis. The Bray Curtis dissimilarity metric was calculated for L3 OTU table and an average pairwise distance and standard deviation was determined for each pair of sample (Clarke Robert *et al.*, 2006). The resulting Bray Curtis distance matrix was mapped unto a 2 dimensional non-metric multidimensional scaling plot (nMDS) using Primer6.

For alpha diversity analysis in the pyrosequencing-derived dataset, an in silico rarefaction was performed using an OTU table generated in QIIME at a minimum rarefaction depth of 100 in a series of depth and a step-wise increase of 200 sequences, a total number of 10 replicates (multiple rarefactions) at each depth and a maximum rarefaction depth of 8000 in the series of depth. For the Ion torrent data analysis, rarefaction was performed at a minimum depth of 4000 sequences in a series of depths, a step-wise increase of 2000 sequences and a maximum rarefaction depth of 21, 000 in the series of depth. For the diversity within each sample (alpha diversity), the non-parametric species richness estimator Chao1 and the Faith's phylogenetic diversity (PD) index were determined according to the methods described by Chao (1984) and Faith (1992) respectively in QIIME. The Shannon's diversity index (*H′*) was also determined for each sample as a measure of alpha diversity based on derivations made by Shannon and

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Weaver (1949). This index of diversity (Shannon and Weaver) is based on the assumption that biological systems contain information that can be analysed in a similar manner to some coded information and that individual species, if sampled randomly from a large community of species are well represented in the samples (Pielou, 1975; Magurran, 2004). Shannon's index of diversity is given by the equation below:

$$H' = -\sum p_i \ln p_i \qquad\qquad (4.1)$$

where pi is the proportion of individuals present within the *i*th species and is estimated based on a maximum likelihood estimator *ni/N* according to Pielou (1969).

4.3.8 *Statistical analysis*

A 2-Way Analysis of Variance (ANOVA) was performed using Minitab-17 Statistical software (Minitab Ltd., Coventry, UK) on the alpha diversity indices in order to compare the interactive effects of the factors under consideration (VPH classes) on species richness and microbial diversity. Analysis of Similarities (ANOSIM) was conducted on the Pearson' product-moment correlation dissimilarity matrix using PRIMER v6. To compare microbial community structure across all samples based on the relative abundance of OTUs, the dominant genera (relative abundance > 1.0% of the total number of sequences) were square root transformed and a Principal Components Analysis (PCA) performed on the transformed data using XLSTAT for Windows (XLSTAT, 2014). A comparison of the relative abundances of identified bacterial groups between soil treatments (VPH classes) was conducted using Microsoft Excel v2010 (Microsoft, Redmond, USA) for significant effects (p < 0.05).

4.4   Results and Discussion

4.4.1   *Biodegradation of volatile petroleum hydrocarbon mixtures in batch systems*

Biodegradation profiles for the different petroleum hydrocarbon classes are shown in Figure 4.1 below. In the control soil batch, an initial lag phase of approximately 6 days is observed. This was followed by a slow rise in the headspace concentration of CO_2 from day 7 which remained fairly consistent throughout the experiments. Maximum cumulative CO_2 concentration for the control batch was 0.00319 g/L. A similar pattern was observed for the aromatic hydrocarbon contaminated soil batch in which headspace CO_2 rose slowly to reach a comparable maximum concentration of 0.00309 g/L by day 15. In the cyclic and branched alkane contaminated soil batch, headspace CO_2 concentration was slightly higher than in the soil and the aromatic hydrocarbon contaminated batches but never increased by more than one order of magnitude above the background level. In the straight-chain alkane contaminated soil batch, a relatively faster increase in the biodegradation of the compounds was observed as indicated by the rise in headspace CO_2 production in the batches (Figure 4.1) suggesting that soil microbial activity was stimulated the most by the addition of straight chain alkane class of petroleum hydrocarbons in contrast to the other PH classes. CO_2 production in the straight alkane soil batch rose by up to two orders of magnitude to reach a maximum cumulative concentration of 0.112g/L before entering into a stationary phase from around day 13 (Figure 4.1).

The biodegradation of *n*-alkanes depends on their solubility in soil-pore water which is proportional to the carbon chain length of the molecule (Sikkema *et al.*, 1995). Alkanes of intermediate chain lengths ($C_5 - C_{16}$) are less water soluble than the short-chain length compounds ($C_1 - C_4$) rendering them less bioavailable for degradation and less toxic at high concentrations. Therefore, a relatively high concentration of *n*-alkanes of intermediate chain length such as those used in the current batch study may not have been inhibitory to the growth of the microorganisms. More so, they are more readily biodegradable compared to other classes of PHs (Ulrici, 2000). Mono-aromatic hydrocarbons on the other hand are comparatively more soluble in water than *n*-alkanes of intermediate chain length (http://chem.sis.nlm.nih.gov/chemidplus/, 2005). At equivalent concentrations, a higher amount of dissolved aromatic hydrocarbons is expected to result in a more toxic effect than *n*-alkanes. Cyclo-alkanes were reported to be less preferable substrates for microbial growth in a related laboratory column study by Bushnaf (2014).
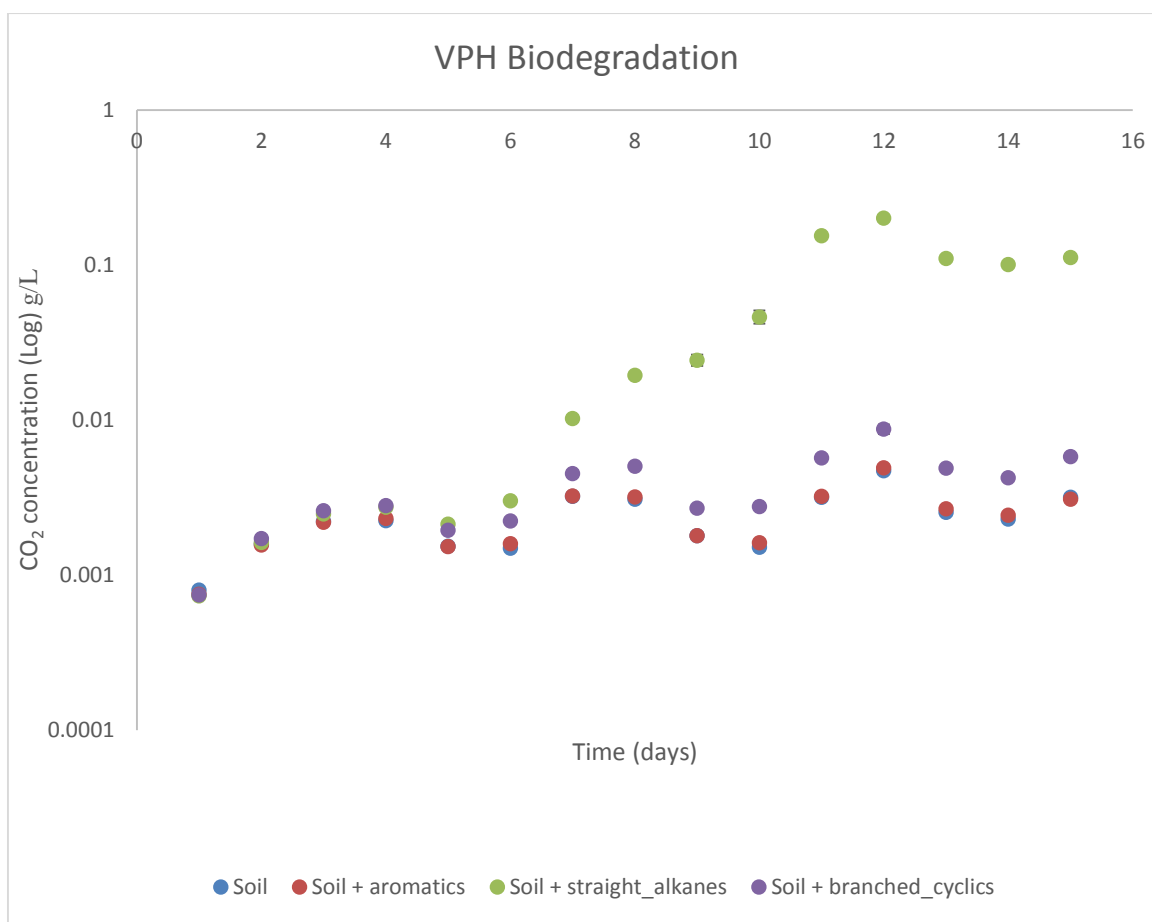
*Figure 4.1. Headspace CO_2 concentration (g/L) in different soil batches for **a)** Soil only **b)** Soil + aromatic H **c)** Soil + straight-chain alkanes and **d)** Soil + cyclic/branched alkanes. Error bars represent ± 1 standard deviation from the mean of 3 replicates.*

4.4.2 *Microbial diversity and species richness*

Microbial diversity and species richness across the different samples as determined by the non-parametric richness estimator (Chao, 1984) and the Faith's Phylogenetic diversity (Faith, 1992) are illustrated in Figure 4.2. For the pyrosequencing dataset (Figure 4.2a), at an even rarefaction depth of 500 sequences, microbial diversity as determined by the Faith's phylogenetic diversity (PD) was the lowest in the Str-alkanes contaminated soil (31.43±0.58). The control soil had a PD value of 43.33±1.34 followed by the soil & aromatic H batch (39.99±1.43) and the soil & cyclic/branched alkane batch (35.77±0.62).

The species richness estimator (Chao1) was higher in the control soil samples compared to the contaminated soils. Chao1 values for soil, soil & aromatic Hs, soil & Str-alkanes, and soil & cyc-alk batches were 797.51±80.14, 733.23±42.01, 580.91±25.33 and 684.49±25.68 respectively. There was a statistically significant difference ($p < 0.05$) between different petroleum hydrocarbon class species richness as indicated by a one-way analysis of variance (ANOVA). A posteriori hypothesis (post-HOC) analysis of the results from the ANOVA using the Tukey's pairwise comparison showed that species richness of the control soils differed significantly from that of straight chain alkane contaminated soils ($p < 0.05$, One way ANOVA) and species richness of straight chain alkane contaminated soils also differed significantly from the species richness of aromatic hydrocarbon contaminated soils ($p < 0.05$, One way ANOVA but was not statistically significantly different from those of the cyclic/branched alkane contaminated soils.

The observed number of species at a 97% sequence similarity level were statistically significantly different between the different classes of PH (ANOVA, $p < 0.05$). More specifically, the control soil samples had the highest observed number of species of 320.50 ± 5.51 followed by the aromatic hydrocarbon contaminated soils with an average observed number of species of 289.10 ± 4.73. The straight chain alkane and cyclic or branched hydrocarbon contaminated soils had an average observed spp. of 229.70 ± 1.97 and 258.57 ± 2.46 respectively. This suggests that contamination of soil with an equivalent dose of different petroleum hydrocarbon classes impacted soil microbiology negatively by significantly reducing bacterial richness with the Str-alkane contaminated soil being the most affected (Chao1 and Observe spp.)(Figure 4.2a). Toxicity of petroleum hydrocarbons was reported to increase with increasing hydrophobicity of the compounds in water. While *n*-alkanes are reported to be relatively more biodegradable by microorganisms compared to other petroleum hydrocarbon classes, a high concentration of the compounds may result in increased uptake of the pollutants and consequently an increased cytotoxic effect.

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With respect to microbial diversity in the pyrosequencing-derived dataset, the Shannon Wiener diversity index ranged from 7.08 ± 0.05 in the straight chain alkane contaminated soils to 8.04 ± 0.03 in the control soils. These values are in the higher range for most soil types as indicated by previous studies (Magurran, 2004; Nacke *et al.*, 2011).

Species richness and microbial diversity as determined in the ion torrent-derived dataset at an even rarefaction depth of 22, 000 sequences across all samples are illustrated in Figure 4.2b. In contrast to the pyrosequencing-derived dataset, there was no statistically significant difference between the species richness estimator (Chao1) of the different soil treatments ($p > 0.05$, One way ANOVA). There was however, a statistically significant difference between the observed number of species in the different classes of petroleum hydrocarbons and control soils with the control soils having the highest observed number of spp. of 4875 ± 188 and the soils contaminated with straight chain alkanes having the least observed number of spp. of 3711 ± 261. In comparison with the pyrosequencing-derived dataset, the average species richness estimator Chao1 in the ion torrent-derived dataset increased by a factor of approximately 10 suggesting that sequencing depth significantly affected the species richness of the soil samples. A one way-ANOVA also revealed a statistically significant difference ($p < 0.05$) between the average number of observed species obtained from the pyrosequencing data (274.5 ± 35.5) and the average value obtained from the ion torrent data (4352 ± 515) which was higher by a factor of approximately 15.
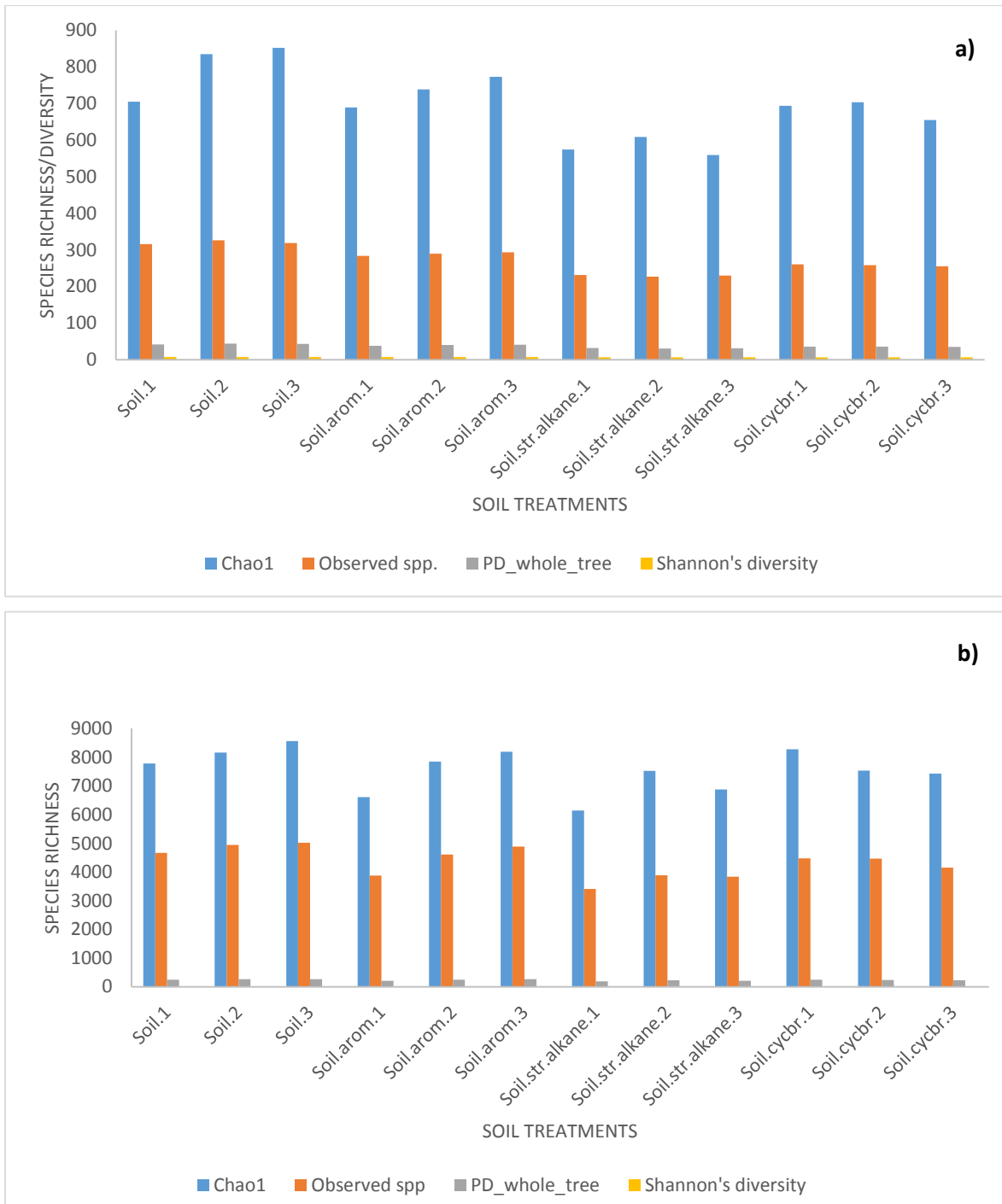
*Figure 4.2. A comparison of alpha diversity indices Chao1, Observed no. of species, Faith's PD and Shannon's diversity index for **a**) 454 sequencing libraries at an even rarefaction depth of 500 sequences and **b**) Ion torrent sequencing library at an even rarefaction depth of 22,000 sequences.*

Rarefaction plots of the observed number of species in the pyrosequencing data and the ion torrent data are shown in the Figure 4.3 below. The results show that by sampling a higher number of sequences, a higher species richness estimate is obtained in both datasets. In the pyrosequencing data, the number of OTUs increased with an increase in the sequencing depth but never attained saturation indicating that the sequences were not exhaustively sampled. Similarly, in the ion torrent data, the rarefaction curve never reached saturation although they were less steep compared to the rarefaction curve from the pyrosequencing data. Several studies on samples from the environment have highlighted the effects of sequencing efforts on the species richness and microbial diversity of such samples (Roesch *et al.*, 2007; Nacke *et al.*, 2011).
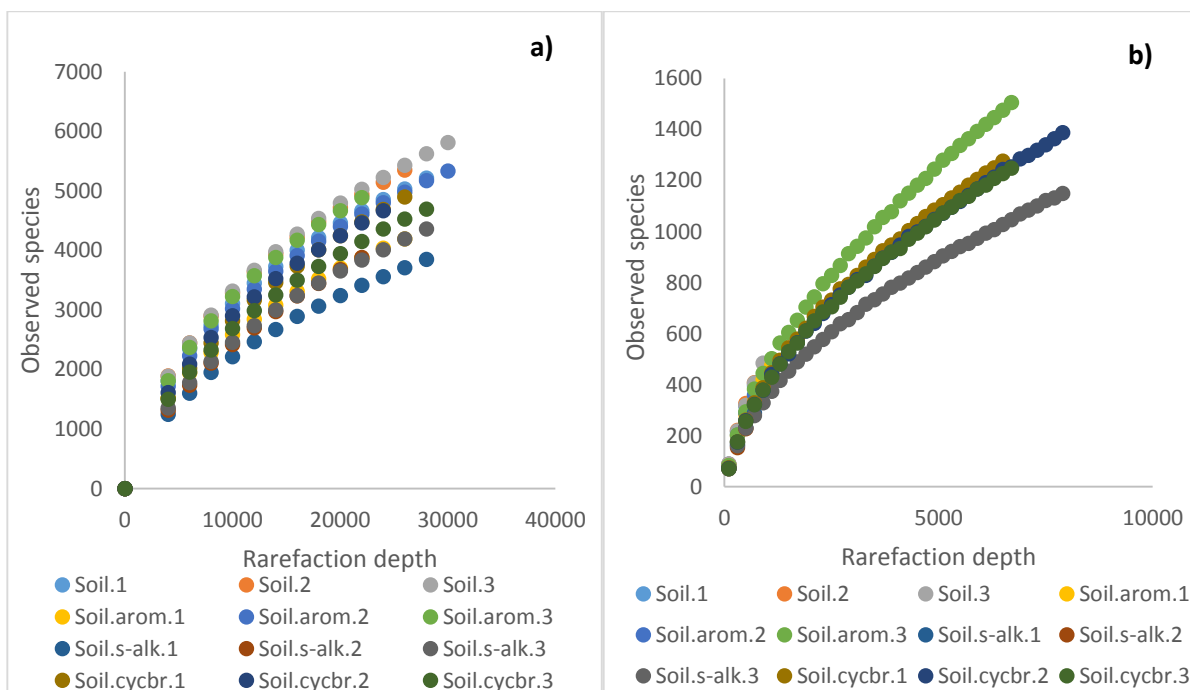
*Figure 4.3. Rarefaction plots showing the effects of sequencing efforts on the observed number of Species for **a**) Ion torrent sequencing and **b**) 454 sequencing platforms.*

4.4.3 *Taxa distribution across samples*

At the phylum level of taxonomy, a total of 39 phyla were identified and classified within the bacterial domain and 2 unclassified phyla within this domain. In the archaea domain there were 3 classified and 1 unclassified domain. The dominant bacteria phyla representing ≥3.0 % of the overall (total) relative abundances in the pyrosequencing-derived data are shown in Figure 4.4a. They are the *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Gemmatimonadetes*, *Nitrospirae*, *Planctomycetes*, *Verrucomicrobia* and the *Proteobacteria* classes *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, *Gammaproteobacteria* representing 4.64, 13.97, 1.38, 7.16, 10.35, 3.93, 1.59, 8.45, 1.43, 13.36, 6.18, 5.89, and 15.27% respectively across the different samples under investigation In the archaea domain, the *Crenarchaeota* was the dominant phyla accounting for 2.64% of the total relative abundance across all samples. The rare bacteria phyla accounting for ≤ 1% of the total relative abundance were the *Armatimonadetes*, *Chlorobi*, GN04, and TM6 representing 0.25, 0.15, 0.17, 0.25% respectively while the rare archaea phyla were the *Euryarchaeota* and the *Parvarchaeota* representing 0.13 and 0.33% respectively of total relative abundance across all samples.

Further analysis of the phyla level taxa revealed a variation in the relative abundances of the different soil treatments. For instance, the *Acidobacteria* had a higher relative abundance in the uncontaminated soils than in all the treated soil samples ($p < 0.05$, 2 sample t-tests). *Actinobacteria* were significantly more abundant in the straight alkane contaminated soils than they were in the uncontaminated soils but were comparable in the other treated soils. *Firmicutes* were significantly higher in relative abundance in the aromatic hydrocarbon contaminated soils than in the uncontaminated and straight alkane and cyclic alkane contaminated soil samples while *Gemmatimonadetes* showed the opposite pattern in which uncontaminated soils had a significantly higher relative abundance compared to the straight chain alkane and cyclic hydrocarbon contaminated soils but not the aromatic hydrocarbon contaminated soil samples. The *Alphaproteobacteria* class did not show any significant variation with respect to their relative abundance across the control and treated soil samples. In contrast, the *Gammaproteobacteria* were significantly more dominant in the Str-alk and Cyc-alk contaminated soils than in the control soils and the Aro-H contaminated soil samples ($p < 0.05$, 2 sample t-tests).

There are no reports to the best our knowledge to indicate that members of the phylum *Acidobacteria* have the potential to degrade any known VPH. On the other hand, members of the phyla *Actinobacteria*, *Firmicutes*, *Gemmatimonadetes*, and *Alphaproteobacteria* have

been isolated and shown to potentially degrade different classes of PHs including alkanes (Engelhardt *et al.*, 2001), *o*-xylene (Morasch *et al.*, 2004), benzene (Li *et al.*, 2006) and gasoline (Robertson *et al.*, 2001). Members of the phylum *Alphaproteobacteria* and *Gammaproteobacteria* were reported from previous studies to dominate bacteria communities in petroleum hydrocarbon contaminated beach sands and marine sediments (Head *et al.*, 2006; Yakimov *et al.*, 2007; Kostka *et al.*, 2011).

Similarly, the ion torrent-derived phyla level taxa summary for the dominant phyla are displayed in the Figure 4.4b below. The taxa distributions reveal an identical pattern to those obtained from the pyrosequencing-derived data as the dominant phyla were retained and did not show any variation. From a qualitative view point, the bacteria community composition did not change between both next-generation sequencing platforms, at least at the phylum level (Figure 4.4) highlighting the reproducibility of results from both next-generation sequencing platforms. From a quantitative view point, however, there were some variations between soil treatments when compared to the taxa distribution from the pyrosequencing-derived data. The *Alphaproteobacteria* were significantly more dominant in the Aro-H contaminated soils than the uncontaminated soil but were more dominant in the uncontaminated soil than in the Str-Alk contaminated soil samples (for both sequencing platforms). The *Gammaproteobacteria* revealed a similar pattern in the ion-torrent generated data where the phylum significantly dominated in the Str-alk and the Cyc-alk contaminated soils compared to the Aro-H contaminated and the control soil samples.
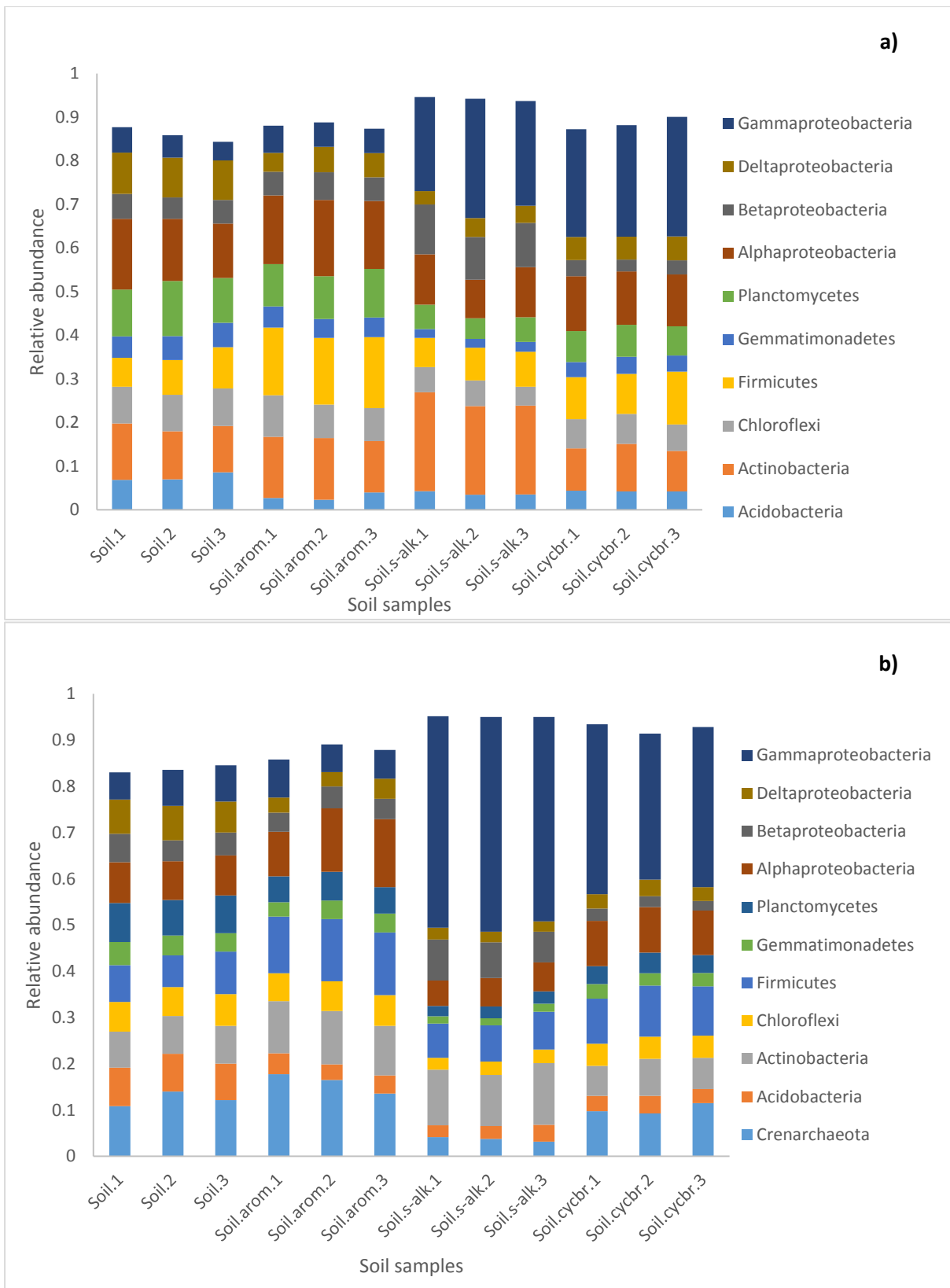
Figure 4.4. Relative abundances of operational taxonomic units (OTUs) accounting for ≥ 3.0% in a) 454 sequencing libraries and b) Ion torrent of all classified sequences obtained from control samples and different soil samples amended with or without biochar or activated carbon.

OTU classification	%Detected			
	Control	Aromatics	Straight alk.	Cyclics/branched
Actinomycetales	**3.5±0.4**	**3.4±0.3**	**15.5±0.2**	**2.7±0.1**
Micrococcaceae	0.6±0.1	0.9±0.1	0.6±0.4	0.6±0.1
Micromonosporaceae	0.7±0.6	0.9±0.1	0.5±0.3	0.6±0.1
Mycobacteriaceae	0.1±0.1	0.2±0.1	0.1±0.1	0.1±0.0
Nocardiaceae	0.1±0.1	0.1±0.1	5.5±0.6	0.1±0.1
Nocardioidaceae	1.0±0.1	0.4±0.1	8.1±0.6	0.3±0.1
Pseudonocardiaceae	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.0
Streptomycetaceae	0.5±0.1	0.6±0.2	0.6±0.2	0.5±0.0
Streptosporangiaceae	0.2±0.1	0.1±0.1	0.1±0.1	0.3±0.0
Bacillales	**5.6±0.4**	**12.1±0.2**	**3.4±0.8**	**7.4±1.3**
Bacillaceae	0.1±0.2	0.3±0.1	0.1±0.0	0.2±0.0
Paenibacillaceae	0.8±0.3	1.6±0.4	0.5±0.3	1.1±0.3
Planococcaceae	1.6±0.4	2.9±0.6	0.6±0.3	1.9±0.4
Thermo-actinomycetaceae	0.2±0.2	0.4±0.2	0.1±0.1	0.3±0.1
Pirellulales	**4.2±0.2**	**5.1±0.8**	**2.3±0.4**	**3.4±0.2**
Pirellulaceae	4.2±0.2	5.1±0.8	2.3±0.4	3.4±0.2
Rhizobiales	**10.6±1.6**	**12.0±1.4**	**5.6±1.4**	**9.3±0.5**
Bradyrhizobiaceae	0.4±0.1	0.3±0.1	0.4±0.2	0.2±0.0
Hyphomicrobiaceae	8.5±0.4	9.7±0.8	3.6±1.0	7.7±0.6
Phyllobacteriaceae	0.3±0.3	0.2±0.1	0.1±0.1	0.1±0.0
Rhizobiaceae	0.2±0.1	0.2±0.2	0.3±0.3	0.1±0.0
Pseudomonadales	**0.5±0.3**	**0.3±0.0**	**16.4±1.8**	**22.2±1.2**
Pseudomonadaceae	0.5±0.3	0.2±0.0	16.3±1.7	22.2±1.2
Xanthomonadales	**3.8±0.6**	**4.2±0.4**	**7.2±1.2**	**2.8±0.3**
Sinobacteraceae	2.4±0.4	2.5±0.4	2.2±0.2	1.8±0.3
Xanthomonadaceae	1.2±0.4	1.6±0.2	5.0±1.1	1.0±0.0

Table 4.1. Summary of bacterial order detected in the highest relative abundance obtained from DNA-derived 454 pyrosequencing libraries of samples from volatile petroleum hydrocarbon contaminated soils.

4.4.4 *OTU functions in petroleum hydrocarbon degradation*

At the order level, most of the sequences associated with the dominant phyla *Actinobacteria*, *Firmicutes*, *Alphaproteobacteria* and *Gammaproteobacteria* across the control and treated soil samples are displayed in Table 4.2. The *Actinomycetales* showed approximately a 5-fold increase in the straight chain alkane contaminated soils compared to the control while the *Bacillales* were twice as high in the aromatic hydrocarbon treated soils than they were in the control samples (Table 4.1). *Pseudomonadales* increased in percentage abundance by a factor of approximately 33 and 44 in the straight chain alkane and cyclic alkane contaminated soils respectively in comparison with the untreated soils while the *Xanthomonadales* increased by a factor of about 2 in the Str-alk contaminated soils compared to the control soils. Most of the sequence affiliated to the order *Actinomycetales* at the family level were the *Micrococcaceae, Micromonosporaceae*, *Mycobacteriaceae, Nocardiaceae, Nocardioidaceae, Pseudonocardiaceae, Streptomycetaceae* and *Streptosporangiaceae*.

At the genus level, there was a significant difference with respect to the relative abundances of the OTUs between the controls and the treated soil samples (Table 4.2). The genera *Rhodococcus, Desulfosporosinus*, *Polaromonas*, *Pseudomonas, Mesorhizobium* and *Methylibium* had the highest relative abundances in the Str-Alk treated soils ($p < 0.05$, 2 sample t-Tests) followed by the Aro-H treated soils while *Azomonas* and *Lycinibacillus* were more dominant in the Cyc-alk treated soils than they were in the untreated soils (controls) (Table 4.2). *Pseudonocardia* was more dominant in the aromatic hydrocarbon treated soil compared to the untreated soil ($p < 0.05$, 2 sample t-Tests) (Table 4.2). Members of the genus *Pseudonocardia* were associated with the degradation of the aromatic hydrocarbons toluene and benzene in a compost biofilter study by Juteau *et al.* (1999). *Rhodococcus* is a Gram-positive, aerobic genus belonging to the phylum *Actinobacteria* (Larkin *et al.*, 2010) and has been reported to possess a remarkable range of diverse catabolic genes plus a resilient physiology which explains why it is able to adapt to a wide range of environmental conditions. Other studies by Smits *et al.* (2001) identified the presence of alkane hydroxylase systems within the genome of members of this genus indicating their potential to metabolise this class of hydrocarbons. Members of this genus have also demonstrated the capacity to biodegrade the Aro-H toluene in a bioreactor study (Malhautier *et al.*, 2014). The genus *Desulfosporosinus* belongs to the phylum *Firmicutes* (Prince *et al.*, 2010)**,** members are Gram-positive anerobes and have the capacity to reduce sulfate ions. They have been reported to utilize alternative electron acceptors such as Mn (IV) and Fe (Garten Jr *et al.*) and to

metabolise PHs under anaerobic conditions (Winderl *et al.*, 2010). One study reported the complete metabolism of toluene by the members of this genus (Liu *et al.*, 2004).

Polaromonas are a slow-growing, oligotrophic group of organisms but have been associated with the biodegradation of groundwater contaminants including petroleum hydrocarbons (Mattes *et al.*, 2008). The genus *Pseudomonas* comprises of a metabolically versatile category of microorganisms that can live aerobically or anaerobically on nitrates as an electron acceptor (Palleroni *et al.*, 2010) and are reported to have the potential to metabolise a wide range of organic compounds ranging from alkanes (van Beilen *et al.*, 1994; Mukherjee *et al.*, 2010) to aromatic hydrocarbons – toluene (Assinder and Williams, 1990; Mukherjee *et al.*, 2010), and benzene (Mukherjee *et al.*, 2010) which have been utilized as sole carbon sources under aerobic conditions. Members of this genus are also producers of the biosurfactant, rhamnolipids, which enhances the bioavailability of hydrophobic organic compounds in contaminated environments (Perfumo *et al.*, 2006). The genus *Mesorhizobium* was statistically significantly higher in relative abundance in the Aro-H treated soil than they were in the control soil ($p < 0.05$). Members of genus demonstrated capacity to utilize BTEX and naphthalene in one study (Auffret *et al.*, 2015) and PAHs in another (Jimenez *et al.*, 2011). Other bacterial genera such as *Azomonas* was reported to grow on some metabolites of aromatic hydrocarbons such as benzoate, *p-* Toluate and catechol while simultaneously fixing nitrogen and may thus play some role in the overall degradation of petroleum hydrocarbons (Chen *et al.*, 1993). There was however a significant decrease in the relative abundance of this genus following exposure of soil to all three classes of PHs (Table 4.2) suggesting a likely toxic effects of petroleum hydrocarbons to this bacterial genus. *Lycinibacillus* is a Gram-negative genus belonging to the phylum *Firmicutes*. Members of this genus have been isolated from a mixture of petroleum hydrocarbons – n-hexane, toluene, n-decane, and xylene isomers e.t.c although reported to be less tolerant to PHs compared to other genera (Stancu and Grifoll, 2011).

Genus	Average relative abundances (Petroleum hydrocarbon class effects)				Petroleum hydrocarbon degradation potential
	Soil	Aromatics	Straight Alkanes	Cyclics/branched alkanes	
Rhodococcus	5.04E-04	1.20E-03	4.48E-02	9.30E-04	Alkanes, toluene
Pseudonocardia	5.21E-04	1.78E-03	3.00E-04	9.42E-04	Aliphatic hydrocarbons
Paenibacillus	6.0E-03	1.4E-02	3.8E-03	9.3E-03	*m*-Xylene
Desulfosporosinus	1.06E-03	1.80E-03	1.19E-02	9.95E-04	Toluene
Polaromonas	8.44E-04	1.24E-03	5.11E-02	1.23E-03	Toluene
Pseudomonas	1.13E-03	4.21E-03	3.28E-01	2.77E-01	Alkane degradation, Aromatics (Juteau *et al.*), hexadecane, benzene and toluene
Mesorhizobium	8.48E-04	4.45E-04	1.24E-03	6.54E-04	Benzene, Toluene, Ethylene, m-Xylene. Naphthalene in ONE study and PAHs in another
Methylibium	4.72E-05	1.60E-04	1.34E-03	1.07E-04	Toluene, benzene, ethylbenzene
Azomonas	0.00E+00	2.17E-05	5.50E-04	2.45E-04	Some metabolites of aromatic PH such as benzoate, *p*-Toluate and catechol) while simultaneously fixing N2 and may thus play some role in the overall degradation of PH
Lycinibacillus	5.00E-04	6.76E-04	3.51E-04	7.07E-04	Mixture of PHs – n-hexane, toluene, n-decane, xylene isomers e.t.c although reported to be less tolerant compared to other genera

Table 4.2.   Summary of statistically significant treatment effects (uncontaminated soil batch versus aromatics, straight alkanes and cyclic/branched alkanes soil batches, t-test, two tailed, p <0.05) for OTU identified at the genus level, where members of the genus reportedly degrade petroleum hydrocarbons.

At the species level (OTU L7), a ranking of OTUs in the control soil batch based on their abundances relative to the total OTU abundances and their increases or decreases with the addition of different PH classes are shown in Table 4.3. In this context, OTU ranking is considered to provide an indication of the competitiveness of OTUs relative to other OTUs in the control soil under given conditions (i.e with VPH addition e.t.c.). As microorganisms operate by the principle of the 'survival of the fittest' where they compete with each other for the available resources in their environment (Chen *et al.*, 2003), it is reasonable to infer that organisms with a higher ranking are better acclimated to the conditions of the soil environment prior to any form of treatments.

In the mono-aromatic hydrocarbon contaminated soil, most of the ranked OTUs increased in relative abundance ranking by 2 folds and by 4 folds in one *Pseudomonas* species compared to their initial abundance in the control. A neighbor joining tree is used to show the evolutionary relationship between close and distant relatives of the *Pseudomonas umsongensis* species based on 16S rRNA gene sequence similarity in Figure 4.5. In the soil batch contaminated with straight chain alkanes, ranked OTUs increased by up to 640 folds in an unidentified *Pseudomonas* species. Unidentified species within the genera *Pseudoxanthomonas*, *Rhodococcus*, *Pseudomonas*, *Polaromonas*, and the family *Nocardioidaceae*, and *Pseudomonadaceae* increased in their ranked relative abundances by at least 16 folds compared to the initial conditions in the control batch. In the cyclic and branched alkane treated soil batch, fewer OTUs increased in their ranked relative abundances compared to the straight chain alkane contaminated soil including species within the family *Pseudomonadaceae* and the genus *Pseudomonas* having an increase of at least 16 folds in comparison with the control soil batch (Table 4.3). These results correspond with the chemical data analysis (section 4.4.1) where biodegradation profiles as indicated by headspace CO_2 production were comparable for the control soil and the monoaromatic hydrocarbon contaminated soil batches. In contrast to this, headspace CO_2 production in the straight chain alkane contaminated soil batch was higher than for soil and the monoaromatic treated soil batches by approximately two orders of magnitude by the end of the 15-days experiment. Headspace CO_2 production in the cyclic and branched hydrocarbon treated soil batch was slightly higher than for the control soil batch but did not rise by more than one order of magnitude. The implication of these results is that the PH degrading microbial communities in the soil being investigated responded more positively to the addition of straight chain alkane class of PHs than they did to the addition of other classes of PHs in separate batch systems. Also, the aromatic hydrocarbons showed lower biodegradability

compared to the cyclic and branched chain alkanes. Aromatic hydrocarbons demonstrated a longer lag phase in a previous study (toluene batch study, chapter 3) probably due to their relatively high solubility in water and toxicity to microorganisms while cyclic/branch alkanes have also been shown to have low solubility in water, low biodegradability but high toxicity. The straight chain alkanes were apparently degraded by a more diverse consortium of VPH degrading organisms, than the branched and cyclic alkanes, which are mainly degraded by the *Pseudomonadaceae*. A number of environmental factors might affect the level of metabolism of the different PH classes such as the original soil microbial community composition, soil nutrient composition and the concentration of pollutants added to soils. Typically, most soil and aquatic environments contain a reasonable amount of hydrocarbon degrading microorganisms and have been found to increase significantly in their numbers following contamination with PHs (Head *et al.*, 2006; Kostka *et al.*, 2011).

Taxon (highest level classification)	Relative abundance rank increase for different treatments compared to the soil only control + 2-fold, ++ 4-fold, +++ 8-fold, ++++ 16-fold, +++++ 32-fold, ++++++ 64-fold, +++++++ 128-fold, , ++++++++ 320-fold, +++++++++ 640-fold or greater, equivalent decrease for minus signs			
	Soil Rank	Soil+monoaromatic hydrocarbons	Soil+straight chain alkanes	Soil+branched and cyclic alkanes
g__Rhodococcus	665	+	++++	
g__Rhodococcus	249	+	++++++	+
f__Nocardioidaceae	107	+	++++	
s__Desulfosporosinus meridiei	153	+	++++	
g__Tepidibacter	532		+++++	
g__Phenylobacterium	782		+++++	
g__Polaromonas	185	+	++++++	+
g__Perlucidibaca	749		++++	+
f__Pseudomonadaceae	593	+	+++++++	++++++
f__Pseudomonadaceae	230		+++++	++++
g__Pseudomonas	428	+	+++++++++	++++++++
g__Pseudomonas	232	++	+++++++	++++++++
s__Pseudomonas umsongensis	264	+	++++	++++++
g__Pseudoxanthomonas	600		++++	

Table 4.3. Summary of taxons which showed a minimum 16-fold increase in their relative abundance rank in one of the batch study treatments.
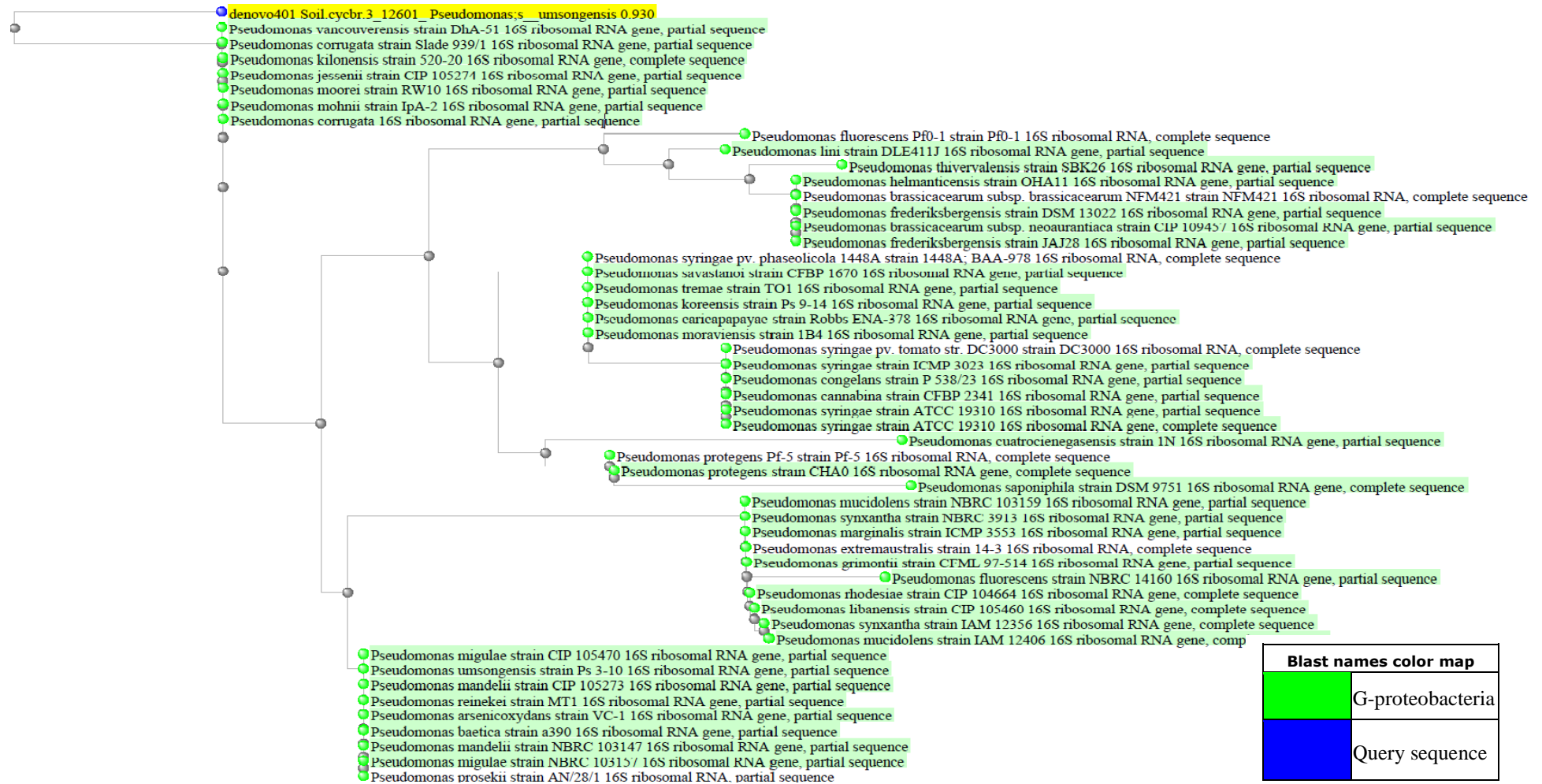
Figure 4.5. Phylogenetic comparison of 16S rRNA gene sequence from an enriched Pseudomonas species (Table 4.3) and closest as well as distant relatives from top 50 BLAST hits (16S rRNA sequences). The bootstrapped neighbor-joining tree was generated in the National Center for Bioinformatics Information (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi?*) based on a maximum sequence dissimilarity of 75%.*

4.4.5   *Inter-relationship between bacterial communities within different soil samples*

A mapping of the Bray-Curtis similarity matrix of the Operational taxonomic units (OTUs) at the class level (Square root transformed) is shown in the Figure 4.6 below. Replicate samples from the same soil treatment type clustered more closely to each other than they did to replicates of samples from other treatments with the exception of a straight-chain alkane treated soil sample which clustered more closely with the cyclic/branched alkane treated soil

samples (Figure 4.6). The pattern of clustering reveals that the petroleum hydrocarbon class was an important factor in shaping the microbial communities within the soil samples. Microbial communities from all samples under investigation clustered at a percentage similarity of 60% while the communities within each replicate clustered at a similarity of 80% indicating a significant level of similarity and reproducibility of results.
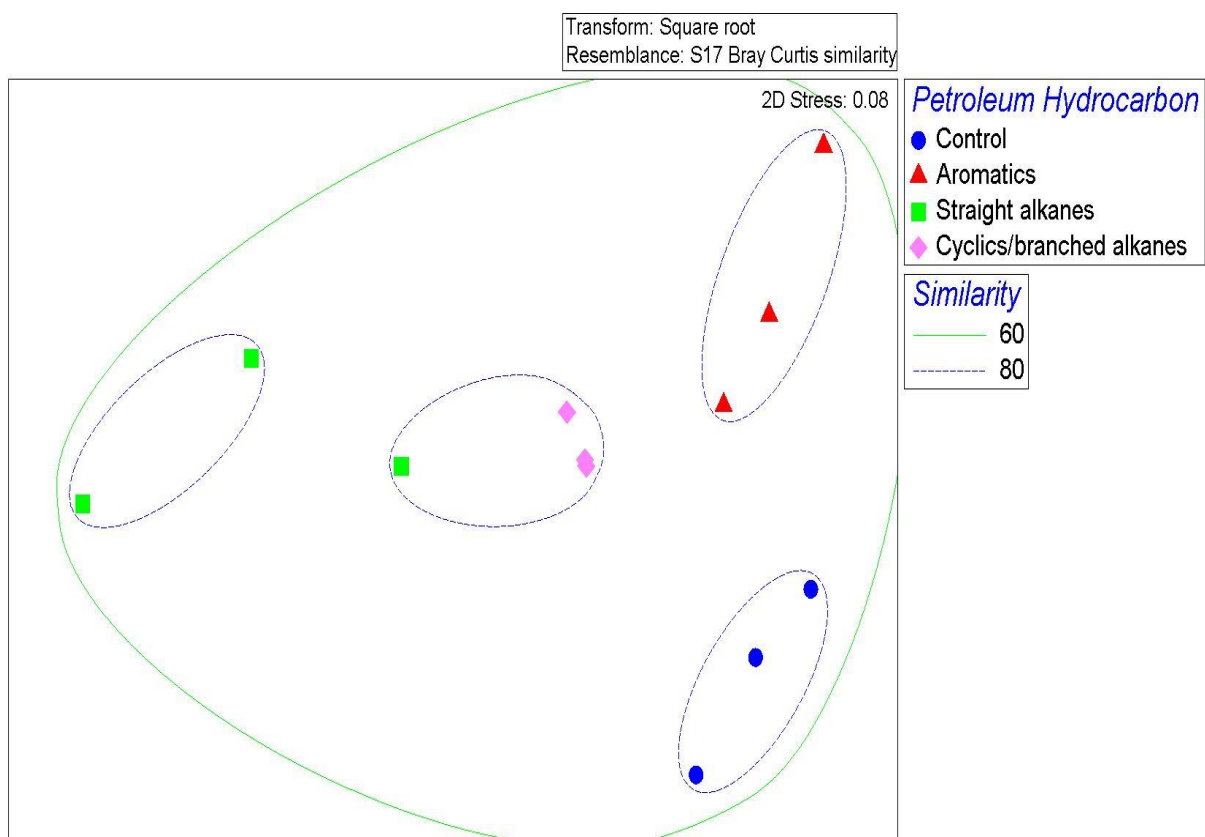
Figure 4.6.   A Nonmetric Multi-Dimensional Scaling (nMDS) of 16S rRNA 454 - pyrosequencing libraries obtained from control soil samples, aromatic hydrocarbon treated soil, straight-chain alkane treated soils and cyclic/branched alkane treated soils   at day 14. Averaged Bray Curtis distance on square root transformed (OTU level 3) data is displayed on the plot.

An analysis of similarities (ANOSIM) was performed between the microbial communities present within each sample based on the Bray-Curtis similarity metric in order to investigate the significance of the factor under consideration. The results showed that the petroleum hydrocarbon class was a statistically significant factor in shaping the microbial communities within different soil treatments (Global $R = 0.861$, $p < 0.01$).
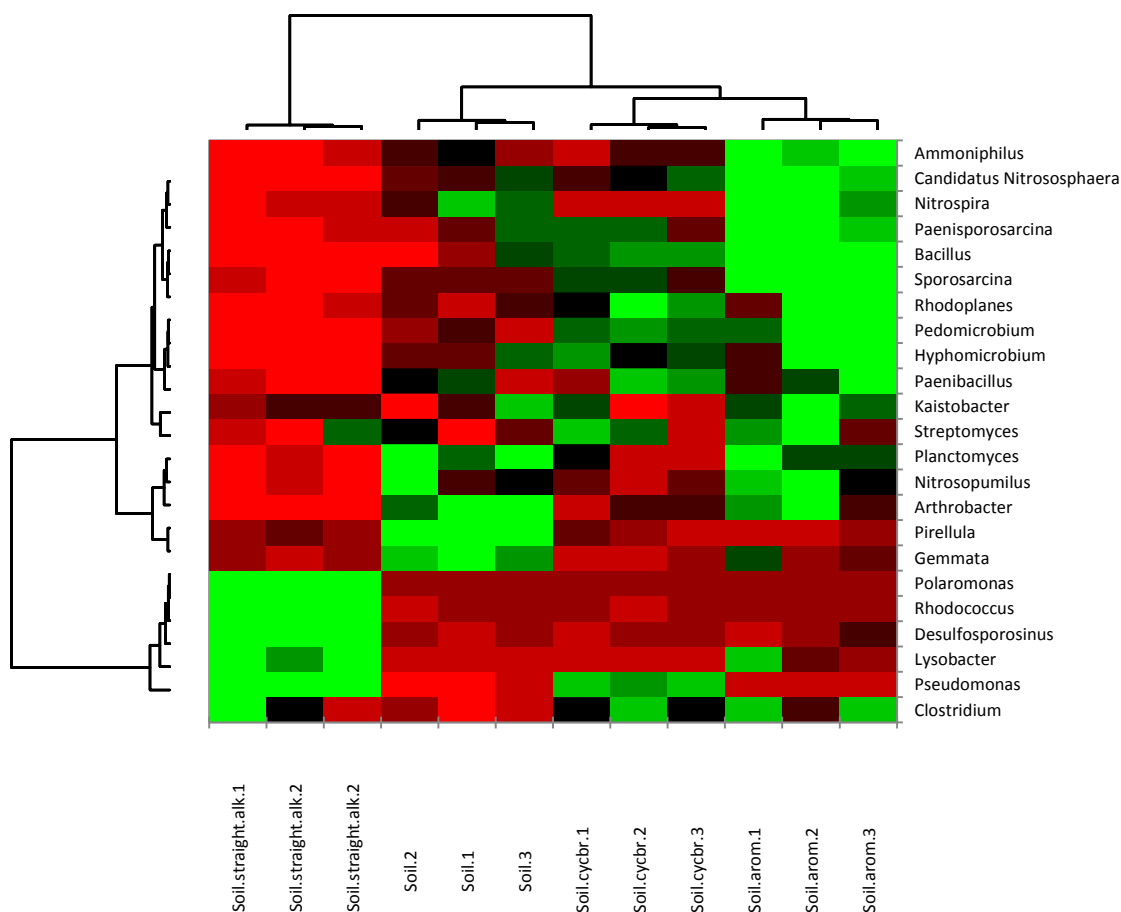
Figure 4.7. A heatmap showing the relationship between relative abundances (> 1.0% of total OTU abundance) and batch samples using dendograms. Colour codes: Green rectangles = high relative abundance, red rectangles = low relative abundance and black rectangles = intermediate abundance. Samples are clustered in columns and OTUs in rows.

Similarly, a clustering of samples based on the most dominant OTUs at the genus level using a heatmap shows that the samples clustered according to petroleum hydrocarbon classes again highlighting the strong effects of PH classes in shaping microbial community structures in contaminated and uncontaminated soils (Figure 4.7). The PH degrading genera *Polaromonas*, *Rhodococcus*, *Desulfosporosinus*, *Lysobacter*, *Pseudomonas* and *Clostridium* were more dominant in the straight chain alkane contaminated soil (indicated by the green

rectangles/squares) but showed the reverse trend in other soil treatments including the control samples. Conversely, the genera *Bacillus*, *Nitrospira*, *Rhodoplanes*, *Nitrosopumilus*, *Planctomyces*, *Hyphomicrobium* and *Candidatus Nitrosospheara* most of which have not been previously associated with petroleum hydrocarbon degradation were more dominant in the aromatic hydrocarbon contaminated soils but less abundant in the straight chain alkane contaminated soil (indicated by red rectangles).

Observations and variables plots of a principal components analysis (PCA) are shown for the dominant bacteria genera (>1% of total relative abundance) for the 454 –pyrosequencing (Figure 4.8a & b) and the Ion torrent (Figure 4.9a &b) derived 16rRNA sequence libraries. A close look at the plots for the pyrosequencing derived data shows a clear pattern of demarcation of the soil samples based on the PH class into different quadrants. The 1st principal component accounted for 67.21% of the variation of the dataset while the 2nd PC accounted for 21.51% variation of the total data. On the 1st PC, the Cyc-alk and Str-alk treated soils were partitioned into the positive axis corresponding to the genera *Pseudomonas*, *Rhodococcus*, *Polaromonas* and *Lysobacter* in the variables plot (Figure 4.8b). The untreated soil and Aro-H treated soils, on the other hand partitioned into the negative axis of the 1st PC corresponding to the genera *Pedomicrobium*, *Rhodoplanes*, and *Candidatus nitrososphaera*. The percentage contribution of individual genus with respect to variation of the data on the 1st PC is as follows: an unidentified genus had the highest contribution of 31.92% followed by the genus *Pseudomonas* with a contribution of 24.20%. The genera *Rhodococcus* and *Polaromonas* contributed 9.33% and 12.93% respectively to the variation of dataset on the 1st PC. More specifically, the increase in the relative abundance of the genus *Pseudomonas* was in the direction of Cyc-alk contaminated soils while *Rhodococcus*, *Polaromonas* and *Lysobacter* increased in their relative abundances in the direction of the straight-chain alkane contaminated soils as indicated by the vector arrow heads (Figure 4.8). On the 2nd PC, the control soil, Aro-H and Straight alkane contaminated soils were partitioned into the negative axis while the Cyc-alk contaminated soil was partitioned into the positive axis of the PC. The genus *Pseudomonas* had the highest contribution of 24.14% to the variation of data while *Polaromonas* had the 2nd highest contribution of 17.70% to data variation on the 2nd PC.

Results of a PCA on the Ion torrent-derived data (Figure 4.9) compared closely with those of the 454 –pyrosequencing data.
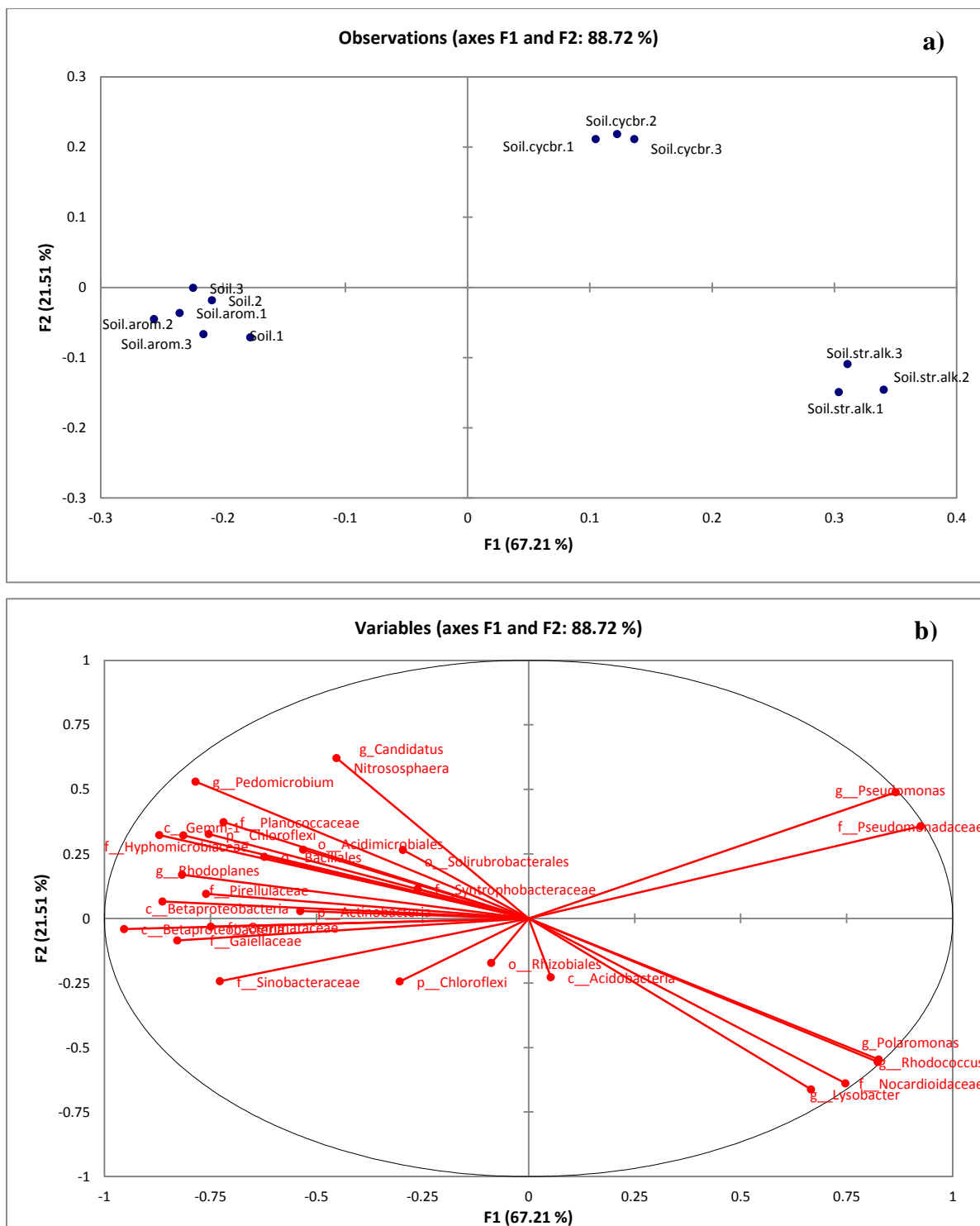
*Figure 4.8. A principal components analysis of 454-pyrosequencing dataset as affected by **a)** petroleum hydrocarbon class **b)** the relative abundances of dominant OTUs (>1.0% of total sequences) at the genus level (square root transformed data). The direction of vectors indicate the direction of change of each variable (OTU relative abundance) so that the observation samples clustered together have similar microbial communities.*
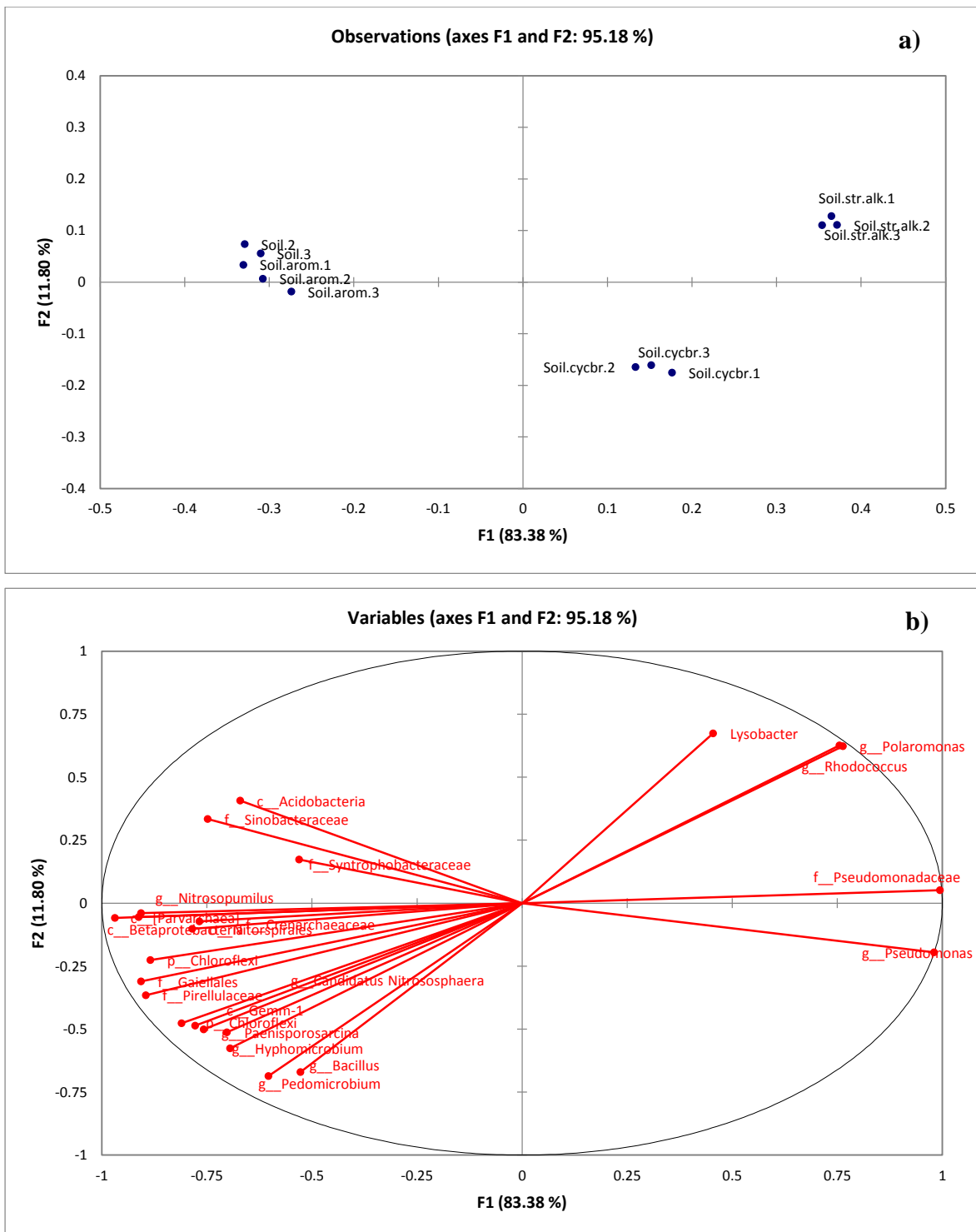
*Figure 4.9. A principal components analysis (PCA) of Ion torrent dataset as affected by **a)** petroleum hydrocarbon class **b)** the relative abundances of dominant OTUs (>1.0% of total sequences) at the genus level (square root transformed data). The direction of vectors indicate the direction of change of each variable (OTU relative abundance) so that the observation samples clustered together have similar microbial communities.*

4.4.6 *General analysis of 454 pyrosequencing and Ion torrent sequencing data output*

Raw pyrosequencing output data from the batch experiments is displayed in Table 4.4. A total of 42,924 sequences passed the quality filtering step out of 71,107 sequences representing approximately 60% of the input sequences. From the total number of quality-filtered sequences, 41,529 sequences were assigned to the domain bacteria out of which 41,305 sequences were classified representing 99.46%. A total of 1391 sequences were assigned to the archaeal domain and below this domain, 1382 sequences were classified representing 99.35%. 4 sequences were not assigned to any domain. Average read length for the pyrosequencing dataset was 378 bp and the number of sequences per sample ranged from 772 to 8684 sequences.

With respect to the Ion Torrent sequencing output, 3, 075 520 raw sequences were originally generated from which a total of 328,867 sequences passed the quality filtering step representing approximately 10% of the input sequences (Appendix B). A total of 286, 085 sequences were assigned to the bacterial domain representing 86.99% of the quality-filtered sequences while 42,691 sequences were assigned to the archaea domain representing 12.98% of the total written sequences. Below the bacterial domain, 285,028 sequences were regarded as classified representing 99.63% while below the archaea domain, 42,540 sequences were classified to represent 99.65% of this domain. Average read length for the Ion torrent sequencing dataset was 318 bp and the number of sequences per sample ranged from 22,119 to 31,792 sequences having an average of 27,405.58± 3020.96 sequences.

The GS junior system is designed to generate to a 100,000 sequence read capacity with an average read length of 400-450bp while the Ion torrent -316 chip platform is reported to generate sequences with average read lengths of 100-200bp (Shokalla *et al.*, 2012). A lower percentage of filtered sequences from the Ion torrent platform at lower quality thresholds (minimum base quality score of 20) compared to the 454 platform (minimum base quality score of 30) suggests that the sequences generated from the Ion torrent sequencing platform was generally of lower quality than those from the 454 GS junior system. However, the higher number of filtered sequences generated by the Ion torrent platform means that microbial diversity can be explored at greater sequencing depths and hence, a more reliable estimate of the sample species richness and diversity can be obtained using the Ion torrent generated sequencing data.

Sample	Petroleum hydrocarbons	No of sequences > 200 bp	No of sequences assigned to domain bacteria	No of sequences classified below bacteria domain level	No of sequences assigned to domain archaea	No of sequences classified below archaea domain level	Sequences not assigned to any domain
Soil.1	N/A	772	750	749	22	22	0
Soil.2	N/A	856	825	818	30	30	1
Soil.3	N/A	1056	976	972	80	80	0
Soil_arom.1	Aromatics	1111	1069	1061	42	41	0
Soil_arom.2	Aromatics	857	839	829	18	18	0
Soil_arom.3	Aromatics	6867	6775	6732	92	90	0
Soil_aliph.1	Straight alk.	746	737	736	9	8	0
Soil_aliph.2	Straight alk.	694	690	689	4	4	0
Soil_aliph.3	Straight alk.	7976	7839	7810	137	137	0
Soil_cyclbr.1	Cyclics/branched alkanes	6523	6198	6160	324	323	1
Soil_cyclbr.2	Cyclics/branched alkanes	8684	8221	8172	462	459	1
Soil_cyclbr.3	Cyclics/branched alkanes	6782	6610	6577	171	170	1

Table 4.4. A summary of the number of sequences > 200 base pairs following quality filtering and assigned to the bacterial and archaea domain per sample in the 454-pyrosequencing generated dataset.

4.5   Conclusions

An investigation of the effects of different volatile petroleum hydrocarbon classes on the soil bacterial community response was conducted in the current chapter. Different classes of PHs had varying effects on the biodegradation of the pollutants by soil microorganisms (Figure 4.1). Headspace CO_2 concentrations were comparable for the soil and the aromatic hydrocarbon contaminated soil batches while in the cyclic/branched alkane contaminated soil, headspace CO_2 concentrations was slightly higher than for uncontaminated soil batch but never rose by more than one order of magnitude (Figure 4.1; purple dotted graph). In the straight chain alkane contaminated soil, CO_2 concentrations rose by up to two orders of magnitude before entering into a lag phase from day 12. These results indicate that the straight-chain alkane class of PHs had the highest stimulatory effect on soil microbial activity with respect to biodegradation of VPHs while the aromatic hydrocarbon class had the lowest stimulatory effect very likely due to toxicity of this PH class at high concentrations. Hence, VPH stimulation of microbial activity varied according to the different PH classes.

Addition of the different classes of PHs also had significant effects on the bacterial species richness and diversity of the control soil batch. A statistically significant decrease in the species richness of the straight-chain alkane contaminated soil compared to the control soil suggests that addition of this class of PHs may exert some deleterious effects on the soil microbial communities. Although microbial activity in the straight-alkane contaminated soil was higher than in the control soil, species richness and diversity for the soil & straight-alkane batch showed the opposite trend compared to the soil batch in the pyrosequencing generated data. Richness and diversity estimates were also lower but not statistically significant ($p > 0.05$, One way ANOVA) for the aromatic hydrocarbon and the cyclic/branched alkane contaminated soil batches. Contrary to our proposed hypothesis, therefore, addition of the different classes of VPHs resulted in a decrease in the microbial species richness and diversity in comparison with the control soil.

Microbial community composition were significantly altered at the species level following addition of different petroleum hydrocarbon classes to the soil in line with our proposed hypothesis. Most of the VPH degrading communities including the genera *Polaromonas*, *Pseudomonas* and *Rhodococcus* showed an increase in their relative abundance ranking by a minimum of 16 folds in the *n*-alkanes contaminated soil batches but by a maximum of 4 folds in the aromatic hydrocarbon contaminated soil. These results corresponds with observations from the chemical data analysis in which CO_2 production was significantly higher in the n-alkane contaminated soil batch than the aromatic hydrocarbon batch. There was apparently a

more diverse consortium of VPH degrading bacteria involved in the degradation of straight chain alkanes than the cyclic/branched alkanes in which the *Pseudomonadaceae* was more dominant.

Although the 454 sequencing platform (Roche 454 GS Junior) generated a considerably lower number of sequences compared to the Ion torrent sequencing platform, the dominant OTUs contributing to sample similarities as determined by different PH classes that were observed from both datasets were identical (Figure 4.8 and Figure 4.9) indicating that the sequencing platform did not significantly alter the major petroleum hydrocarbon degrading bacterial communities in the samples under investigation.

Overall, equivalent concentrations of different PH classes exerted varying effects on the biodegradation of the pollutants and on the microbial community response in the soil under investigation. Sequencing platforms did not appear to significantly alter the microbial community structure of the soil being investigated under the same conditions indicating that the results were repeatable. Further study needs to be conducted on the effects of PHs of different chain lengths such as short, intermediate and long chain alkanes and of different concentrations of the petroleum hydrocarbons on the microbial community response in the soil currently being investigated.

Chapter 5: Short-term effects of sorbent amendment on the attenuation of volatile petroleum hydrocarbons in gravelly sand - a nutrient limiting perspective

5.1 Introduction

In situ amendment of soils and sediments with strong sorbent materials such as biochar and activated carbon is currently being investigated as a cost-effective, environmentally friendly approach for the remediation of PAHs (Cornelissen *et al.*, 2006; Rhodes *et al.*, 2008) and volatile organic compounds (Bushnaf *et al.*, 2011). Inorganic nutrients are essential requirements for microbial growth and survival as they make up biomolecules within the cell. More specifically, the elements nitrogen, phosphorous and sulphur have been reported to represent about 14%, 3% and 1% respectively of microbial dry weight and are constituents of important cell components such as proteins, nucleic acids, and sugars among other biomolecules (Ron and Rosenberg, 2010). Although petroleum hydrocarbons are excellent sources of carbon for biomass formation and for energy, they are quite poor in nitrogen and phosphorous. Hence the need to supplement these nutrients in order to reach the optimum concentrations required for effective attenuation of hydrocarbons from contaminated soils especially nitrogen which is required in high concentrations and can therefore become limiting in the soil subsurface (Hohener *et al.*, 2006; Ron and Rosenberg, 2010).

The standard proportions of nitrogen and phosphorous relative to carbon required for optimum bacterial activity may vary according to different reports: C: N: P ~ 100:10:1 (Litchfield, 1993; Ron and Rosenberg, 2010; Elazhari-Ali *et al.*, 2013); 93:10:1 (Tischer *et al.*, 2014) and 175:13:1 (Fanin *et al.*, 2013). Prolonged presence of petroleum hydrocarbons in the environment may lead to anaerobic conditions where oxygen concentrations become depleted and the process of denitrification reduces the total amounts of nitrogen in the environment making it necessary to introduce the elements for optimum activity (Ron and Rosenberg, 2010). Important nitrogen sources that have been used for bio stimulation include nitrates, ammonia, urea and N_2O (USEPA, 1989). On the other hand, phosphorous is often present in soil subsurface in low amounts and where they occur in high concentrations, have been reported to occur in forms that make them bio unavailable for uptake by microorganisms (USEPA, 1989). The role of microorganisms in the soil phosphorous cycle has been recently reviewed by Richardson and Simpson (2011). They have been reported to mediate the release of P from the pools or forms in which they exist in the soil thereby enhancing their availability for their uptake and utilisation.

Batch studies in which soils were amended with or without 2% biochar and activated carbon were conducted. Sorbent amended and unamended soils were further amended with and without the nutrient solution made of nitrogen and phosphorous and soil respiration was monitored over a period of six (6) days. Addition of biochar and AC to soil was shown to change the partitioning of volatile petroleum hydrocarbons between air, water and soil matrix and this affects the biokinetics of VPH degradation (see Chapter 3 – sorption of $^{13}C_7$ toluene in sorbent amended soil). It is critical to also develop an understanding of the initial effects of sorbent amendment and VPH exposure on the microbial community response, which typically results in the growth of VPH degrading microorganisms as shown in Chapter 4.

5.2   Aim

The main aim of this study was to investigate, using a batch system, the short-term effects of amending soil with or without biochar and activated carbon and with or without inorganic nutrients on the bacterial community response in a volatile petroleum-hydrocarbon contaminated sand.

5.2.1   *Objectives*

The following objectives were set to be accomplished:

1. To study the effects of nutrient amendment on the biodegradation of volatile petroleum hydrocarbons in soil amended with or without 2% biochar or activated carbon.

2. To study the short-term effects of a VPH non-aqueous phase liquid (NAPL) source exposure on the bacterial community response by comparing microbial communities at time t = 0 prior to pollutant exposure with communities on day 6 after exposure to pollutants.

3. To study the short-term effects of inorganic nutrient limitation on microbial community response by amending soil with or without the inorganic nitrogen and phosphorous in addition to the VPHs which serves as a carbon source.

4. To study the effects of the sorbent amendments, biochar and activated carbon on the biodegradation of the VPHs in aerobic sand in the short-term.

5. To study the effects of biochar and activated carbon on the microbial community structure in VPH exposed soils by comparing the communities on day 6, at the end of the experiment with those of unamended soil on day 0.

5.2.2 *Hypotheses*

In line with the above objectives, the following hypotheses are proposed:

1. Nutrient amendment has been shown to stimulate microbial activity, hence, by amending soil with the inorganic nutrients (nitrogen and phosphorous), an increase in the biodegradation of VPHs should occur regardless of sorbent amendment of soil.

2. It is expected that soil microbial community composition would change significantly following exposure to high concentrations of VPHs at the end of the 6 days experiments compared to the initial soil condition on day zero.

3. Nutrient limitation is expected to reduce the rate of metabolism of carbon source present within the batches but not necessarily have a significant effect on the microbial community response in the batches under investigation.

4. Sorption reduces the bioavailability of organic compounds to indigenous microbial communities. Therefore amending soil with 2% biochar or activated carbon should slow the biodegradation of the pollutants.

5. Consequent upon the fourth hypothesis, sorbent amendment should also cause a shift in soil microbial community structure compared to unamended soil.

5.3   Materials and methods

5.3.1   *Soil, biochar and activated carbon*

Soil, biochar and activated carbon are the same as those used in Chapter 3.0 (Section 3.3.1).

5.3.2   *Chemical mixture*

A mixture of 12 major constituents of gasoline was prepared from stock (high purity chemicals) obtained from Sigma-Aldrich (Dorset, UK) with their percentage weight composition chosen based on typical fuel composition. These consisted of three aromatic hydrocarbons namely toluene, 1,2,4-trimethylbenzene and *m*-xylene; five straight chain alkanes namely pentane, hexane, n-octane, decane and dodecane; and four branched chain or cycloalkanes namely iso-octane, methylcyclohexane, methylcyclopentane and cyclohexane. In addition, Sulfur hexafluoride (SF_6) (Sigma-Aldrich, Dorset, UK) was used as a conservative tracer gas.

5.3.3   *Batch experiments*

Batch microcosm experiments were conducted to investigate the effects of nutrient availability on the biodegradation of VPHs. Amber vials (65 mL, Jencons, a VWR Division, Leicestershire, UK) closed with Teflon Mininert valves (Supelco, Bellefonte, USA) contained 15 g (d.w.) of gravelly sand (water content 10% wet weight) without sorbent amendment (soil), and with 2% dry soil weight biochar amendment (live soil & BC) or 2% dry soil weight activated carbon amendment (live soil & AC). 0.03 mL of VPH mixture, equivalent to 0.018 g VPH-carbon was added to the various soil types with and without the addition of 0.0018 g of nitrogen in the form of NH_4Cl and/or 0.00018 g of phosphorus in the form of KH_2PO_4 to study the effect of inorganic nutrient availability on VPH biodegradation (i.e. nitrogen and phosphorus were added together or separately).

5.3.4   *Microbial respiration*

Microbial respiration was monitored in the batches over the duration of the experiment (6 days) by measuring the concentration of headspace CO_2 in each vial containing either soil, or soil amended with 2% biochar or soil amended with 2% activated carbon. For each soil type amended with or without nutrients, triplicate batches were monitored at room temperature (20 °C) and the analysis of headspace CO_2 was conducted using a Fisons 8060 GC linked to a Fisons MD800 MS with a HP-PLOT-Q capillary column.

5.3.5   *Sample collection and storage*

Samples were collected and stored according to the methods described in Chapter 4 (section 4.3.4) for DNA extraction and processing.

5.3.6 *Total cell counts and biomass yield determination*

5 μl of sample slurry stored in ethanol/PBS mixture was added to 995 μl of filtered-sterile phosphate buffer saline (PBS, Oxoid) in 2 mL Eppendorf tubes to make a dilution of 200 times. In order to stain the cells in the samples, 50 μl of SYBR Gold nucleic acid stain in 100 times concentrated dimethyl sulfoxide (DMSO) (Invitrogen Ltd., Paisley, UK) and further diluted 100 times in filtered-sterile phosphate buffer saline (PBS, Oxoid) was added to the sample mixture. The samples were incubated in the dark by wrapping with an aluminium foil at room temperature for 30 minutes. A 13 mm polycarbonate membrane filter (pore size 0.2 μm) (Millipore, Hertfordshire, UK) was aseptically transferred to a sterile Millipore filter holder and the stained samples were filtered using suction from a vacuum pump. The filters were transferred to a microscope glass slide to which a drop of the antifadent Citifluor (Citifluor Ltd., London, UK) was applied for adhesion to the slides. A further drop of Citifluor was added to the top of the filter to enhance adhesion after which a coverslip was placed over the filter. The slides were labelled and placed in a dark room to prevent fluorescence from fading. Viewing of slides was done in immersion oil under a 100 times magnification objective lens of an Olympus BX40 Epi-fluorescence microscope. A dilution that yielded cell counts in the range of 30 – 300 was chosen at the sample preparation step and 20 randomly selected fields of view were counted per slide. Carbon-normalised yield coefficients (g biomass C g^{-1} substrate C) were determined based on the assumption that the average carbon content of bacterial cells is a 100fg of carbon per cell (Whitman et al., 1998).

5.3.7 *DNA extraction and PCR amplification*

DNA extraction and purification was performed as described in Chapter 4 (section 4.3.5).

5.3.8 *454-pyrosequencing*

PCR amplicons from each sample (in duplicates) were pooled together in equimolar amounts and sequenced on a Roche 454 GS FLX+ System by The Centre for Genomic Research (University of Liverpool, UK). A total number of 362,885 sequences passed the initial quality filtering step out of a number of 472,103 sequences that were generated from the pyrosequencing. A UCLUST algorithm (Edgar, 2010) was used to cluster the resulting reads into operational taxonomic units (OTUs) based on a similarity threshold of 97% and the naïve Bayesian RDP classifier of the Ribosomal Database Project was used to assign taxonomy to the cluster at an 80% threshold confidence.

5.3.9 *Microbial community structure analysis*

Sequence analysis was performed in QIIME (v 1.8.0) using the default parameters similar to those used in the previous chapter (Chapter 4.0, Section 4.3.7). An in-silico rarefaction of the

sequences per sample was performed across all samples using the OTU table constructed at an early stage as an input to the QIIME pipeline. A minimum rarefaction depth of 10 in the series of depth, a stepwise increase of 400 sequences, a total number of 10 replicates (multiple rarefactions) at each depth and a maximum rarefaction depth of 19,610 in the series of depth were used to perform the in-silico analysis. For the diversity within each sample (alpha diversity), the non-parametric species richness estimator Chao1 and the Faith's phylogenetic diversity (PD) index were determined according to the methods described by Chao (1984) and Faith (1992) respectively in QIIME. The Shannon's diversity index (H') was also determined for each sample as a measure of alpha diversity based on derivations made by Shannon and Weaver (1949).

5.3.10 *Statistical analysis*

Data analysis in this chapter was performed using the statistical software and methods described in Chapter 4, Section 4.3.8.

5.4   Results and Discussion

5.4.1   *Microbial respiration and total cell counts*

Results of monitoring soil respiration over a six-day period are shown in Figure 5.2. In the soil batch, an initial lag phase of about 3 days is observed (Figure 5.2). This was followed by a rapid increase in the level of headspace CO_2 in the soils from day 4 to reach peak concentrations of 0.127 ± 0.004 g/L and 0.060 ± 0.026 g/L for soil with nutrients and soil without nutrients respectively. In the soil and biochar batch, headspace CO_2 production started to rise on the second day and also increased rapidly to reach a maximum cumulative concentration of 0.132 ± 0.009 g/L for the nutrient amended batch before entering into a stationary phase from day 5 (Figure 5.2). In the soil and biochar batch without nutrient, there was also an increase in the amount of CO_2 production to reach maximum concentrations of 0.082 ± 0.006 but never entered into a stationary phase. In the AC amended soil batch, increase in CO_2 production was slower and rose to reach maximum concentrations of 0.074 ± 0.011 g/L and 0.029 ± 0.001 g/L for the nutrient amended and unamended soils respectively.

Statistical analysis shows that maximum cumulative CO_2 concentrations in the soil & biochar batch differed significantly from that of the soil & AC batch (1-Way ANOVA; Fisher pairwise comparison, $p < 0.05$) but there was no significant difference between the soil and soil & biochar or the soil and soil & AC batches. A comparison of the nutrient factor within each soil systems reveals that microbial activity was significantly higher in the nutrient amended batches than the nutrient unamended batches (2 sample t-tests, $p < 0.05$ in all cases) suggesting that addition of nutrients to the soils stimulated the biodegradation of volatile petroleum hydrocarbons. Nutrient and sorbent amendment factors were both significant (2-Way ANOVA, $p < 0.05$ in both cases) in influencing the level of microbial activity as indicated by the maximum cumulative CO_2 concentrations in the respective batches. There was also a significant interaction between nutrient and sorbent amendment in the determination of microbial activity as indicated by CO_2 production in batch headspaces.

Biostimulation involves the use of stimulants such as inorganic nutrients (e.g. N/P), electron acceptors and other amendments to enhance the metabolic activity of indigenous soil microorganisms (Jukawar *et al.*, 2010). As mentioned previously (Section 4.1), the inorganic nutrients N and P are an essential requirement for the normal growth and functioning of microbes and can either be artificially introduced into the soil or released from the mineralization of soil organic matter which is also serves as a reservoir for inorganic nutrients (Horwath, 2007; Standing and Killham, 2007). On the other hand, petroleum hydrocarbons are excellent sources of carbon for microbial growth but deficient in the

essential nutrients required for growth. The increased level of biodegradation observed in the nutrient amended treatments indicated by increased CO_2 production (Figure 5.2) can be interpreted as an enhanced level of microbial metabolic activity in comparison to soil systems in which a carbon source was added but not a nitrogen or phosphorous source. Supplementing soil systems with inorganic nutrient sources N and P to meet the ratio 100:10:1 (C: N: P) has been shown to enhance the level of biodegradation of organic pollutant in previous studies (Pritchard *et al.*, 1992; Liebeg and Cutright, 1999; Elazhari-Ali *et al.*, 2013).

Batches (Treatments)	Total cell counts (Cells/g of soil)	Yield (Biomass C (g)/Biomass C + CO_2 C) (g)
Control (time zero)		
Soil	$(2.7 \pm 0.14) \times 10^7$	-
Soil +2%BC	$(7.7 \pm 1.50) \times 10^7$	-
Soil +2%AC	$(3.9 \pm 0.74) \times 10^7$	-
With Nutrients		
Soil	$(2.0 \pm 0.15) \times 10^8$	0.079 ± 0.007
Soil +2%BC	$(2.5 \pm 0.35) \times 10^8$	0.062 ± 0.009
Soil +2%AC	$(1.1 \pm 0.09) \times 10^8$	0.048 ± 0.005
Without Nutrients		
Soil	$(1.5 \pm 0.21) \times 10^8$	0.177 ± 0.052
Soil +2%BC	$(1.3 \pm 0.22) \times 10^8$	0.041 ± 0.031
Soil +2%AC	$(1.5 \pm 0.40) \times 10^8$	0.137 ± 0.046

Table 5.1. Average total cell counts and yield measurements.

Carbon-normalized yield coefficients were calculated from the CO_2 production and increase in total cell counts over the first six days of the experiments. Calculated yield coefficients (g biomass-C relative to g (biomass-C + CO_2-C)) compared closely in the nutrient (N & P) amended soils ranging from 0.048 ± 0.005 in soil & AC to 0.062 ± 0.009 and 0.079 ± 0.007 in soil & BC and unamended soil respectively. Calculated yield coefficients were more variable in the batches without nutrients, 0.041 ± 0.031 in soil & BC, 0.177 ± 0.052 in soil and 0.137 ± 0.046 in soil & AC. Amending soil with 2% biochar or AC apparently reduces the yield coefficients for both nutrient amended and unamended batches.

Total bacterial cell numbers for the different soil treatments with and without nutrient amendment are displayed in Table 5.1. Cell numbers ranged from $(1.3 \pm 0.22) \times 10^8$ to $(1.5 \pm 0.40) \times 10^8$ cells/g (d.w.) of soil in treatments without nutrients and from $(1.1 \pm 0.09) \times 10^8$ to $(2.5 \pm 0.35) \times 10^8$ cells/g (d.w.) of soil in nutrients-amended treatments. These values are within typical ranges for most soil types (Whitman *et al.*, 1998; Elazhari-Ali *et al.*, 2013). The

average microbial biomass carbon/g soil (d.w.) was higher for the nutrient amended soils than the nutrient-unamended soils batches with the exception of the soil & activated carbon batches. It therefore appears that the growth of microbial biomass was no longer limited by nutrients but likely by substrates (VPH) availability by the end of the experiment in the soil & AC batch in which the bioavailable substrates would have been greatly reduced due to sorption. A comparison of the total cell numbers/g of soil from the nutrient amended and unamended soil treatments on day 6, at the end of the experiment, with values from live control soils (unamended) at time zero (0), indicates an increase in microbial biomass by a factor of 3.9 and 3.0 in the nutrient amended and nutrient unamended soils respectively, suggesting that nutrient addition also enhanced the growth of VPH degrading microbial biomass regardless of the sorbent amendment.
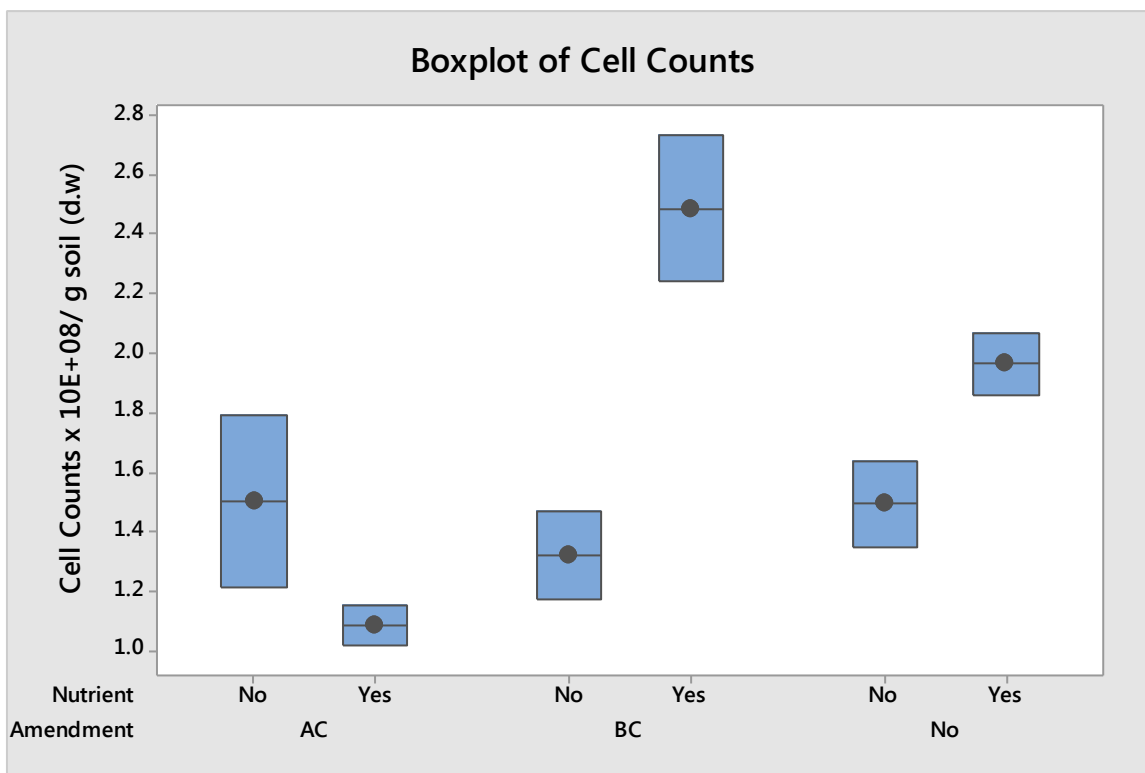
Figure 5.1. Box plots of total cell counts showing the effects of interactions between nutrients and sorbent amendments of soil on cell numbers.
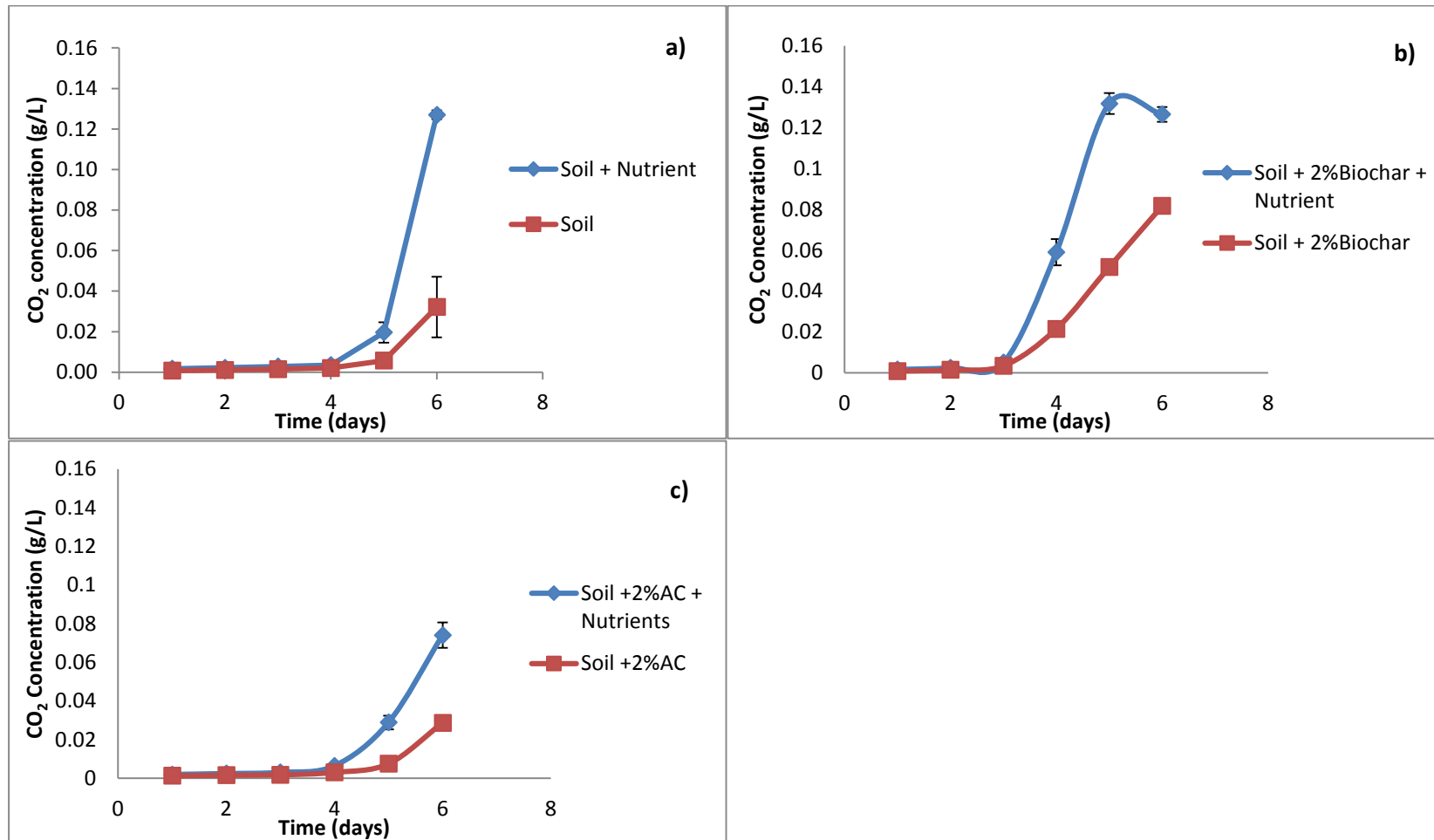
*Figure 5.2. Effects of nutrient amendment on biodegradation of VPHs in **a)** Soil **b)** Soil & Biochar and **c)** Soil & AC batches. With nutrients (blue line graphs), without nutrients (red line graphs). Error bars represent ±1 standard deviation from the mean of duplicate measurements.*

- Soil   **a)**
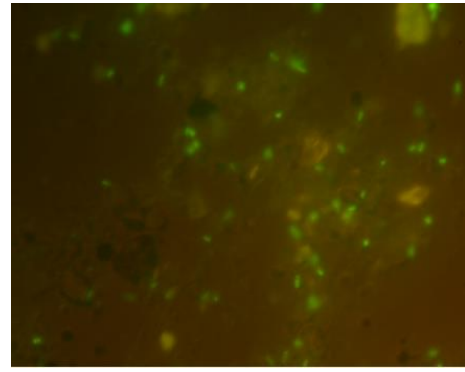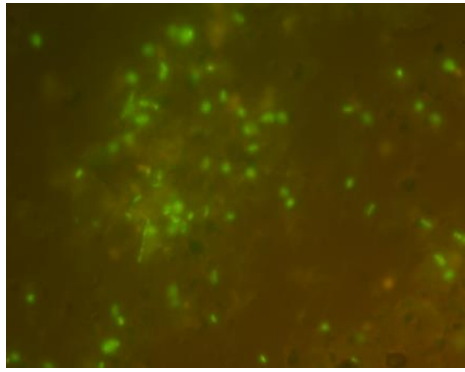
- Soil + 2% biochar

- Soil + 2% AC

- Control (PBS)

- Soil   **b)**

- Soil + 2% biochar

- Soil + 2% AC

10μm
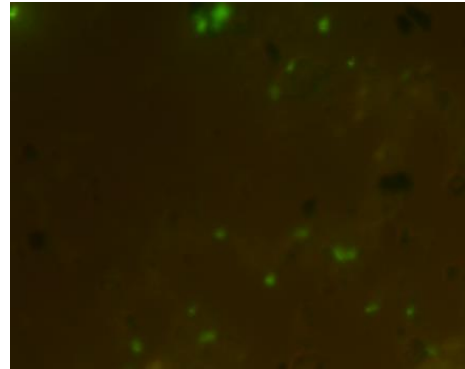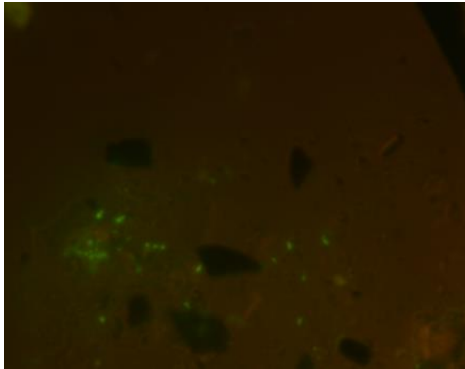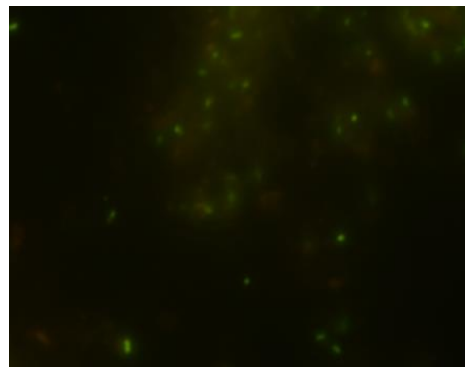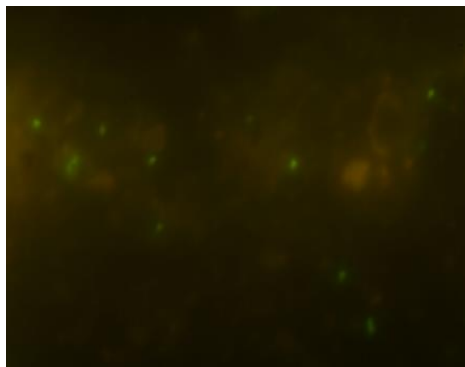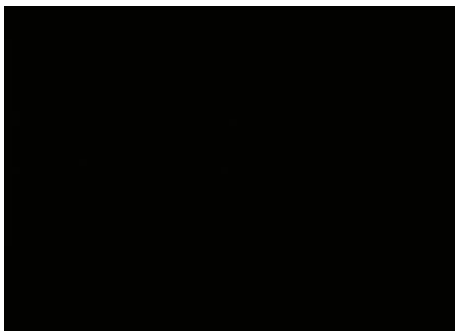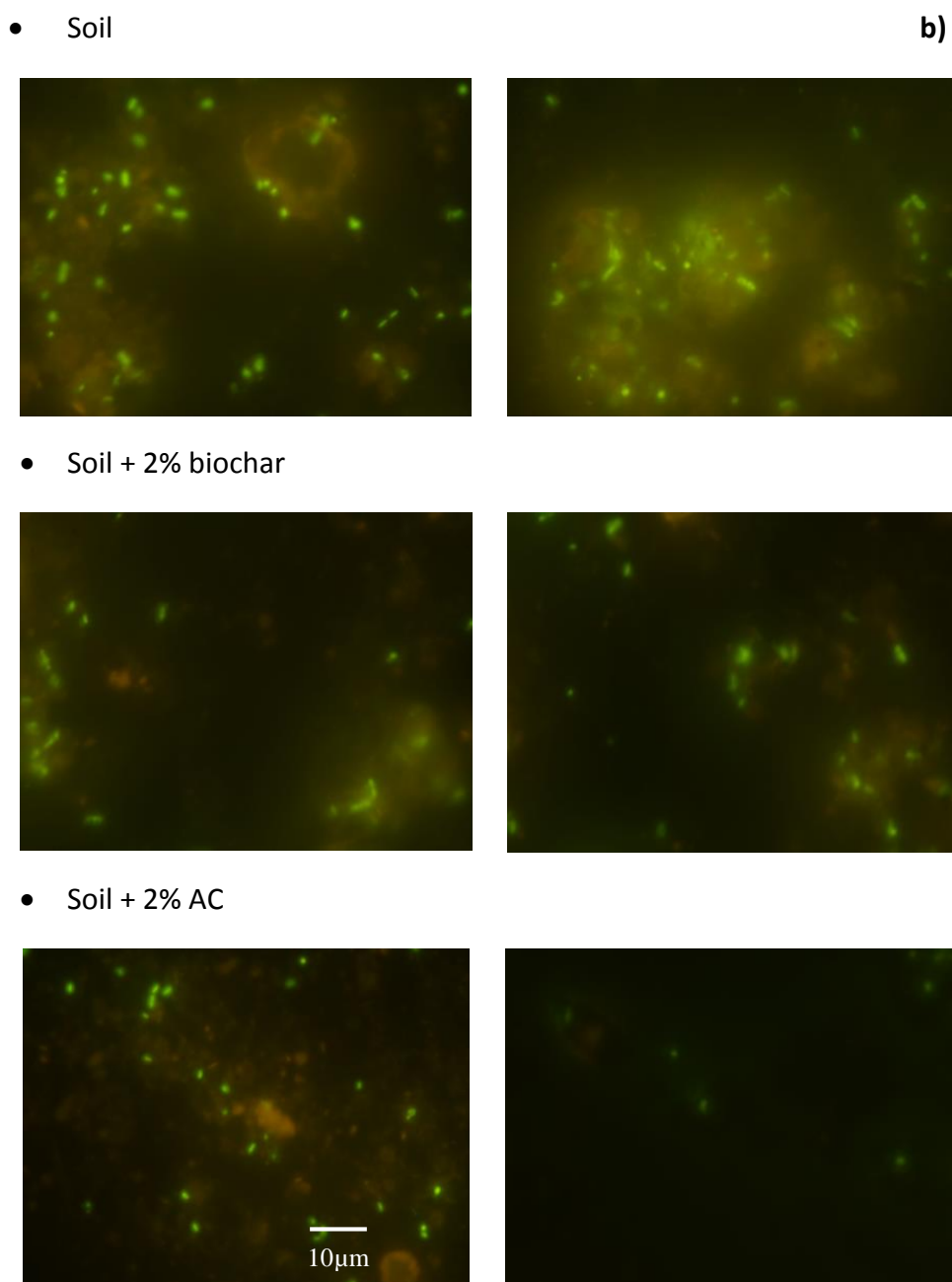
*Figure 5.3. Cell counts images of samples from VPH contaminated soil batches treated **a**) without nutrients and **b**) with nutrients. Images were viewed under an epiflouresence microscope at a magnification of 100X.*

5.4.2 *Analysis of pyrosequencing-generated dataset*

A preliminary analysis of the raw dataset generated from the pyrosequencing process reveals that out of a total of 362,885 sequences that passed the quality filtering step with a minimum read length of 200bp (average read length of 563bp), about 70% of the denoised sequences represented a single taxa (OTU level 7) identified as belonging to the family – the *Pseudomonodacae* across all samples including pure biochar and activated carbon. A further removal of chimeric sequences was performed using the chimera slayer script in QIIME in order to eliminate any chances of the *Pseudomonodacae* family being chimeric. A total of 112 chimeric sequences were detected representing 0.02% of the entire dataset. Due to the likeliness that this single very abundant OTU represents a contaminant or method artifact, the row representing this OTU was manually deleted resulting in a dataset containing 147,882 sequences and a further re-computing of the OTU relative abundances in QIIME was performed using the summarise_taxa.py script. Out of 147,882 sequences, 144,169 sequences were assigned to the bacteria domain representing 97.5% of the denoised/filtered dataset. Of this fraction, 144,144 sequences (99.6%) were classified below the bacteria domain. A total of 3180 sequences were assigned to the archaea domain representing 2.1% of the filtered dataset. 3164 sequences (99.5%) were classified below the archaea domain. A total number of 11 sequences were not assigned to any of the domains. Averagely, each sample contained 12,188.5 sequences (max: 21,044/min: 3333).

5.4.3 *Microbial diversity and species richness*

The non-parametric species richness indicator Chao1 is based upon the number of uncommon species present in a given sample (Chao, 1984) and takes into account the number of singletons as well as doubletons in a sample. In the control samples, on day zero, species richness as estimated by the average Chao1 index value was 1463.93±10.41 OTUs in live soil, 891.72±64.12 OTUs in activated carbon and 1106.40±195.47 in pure biochar.

For the nutrient amended treatments, species richness estimates (Chao1) for soil, soil & biochar and soil & AC were 982.83±27.41, 1102.92±18.63, 1279.39±30.06 respectively and higher but not significantly different from the estimates for nutrient unamended batches (p > 0.05, One-way ANOVA). Richness estimates for nutrient unamended batches ranged from 896.85±194.26 for soil to 1085.37±191.86 for soil & AC. Species richness estimates (Chao1) for the sorbent amended or unamended batches showed that there was no significant difference between the average richness estimates of the soil & AC batch (1182.38±158.49) OTUs and the unamended soil and soil & biochar batches (939.84±123.67 and 1085.19±51.66 respectively).

The number of observed species in the soil controls at time zero had the highest index value of 938.7±5.9 followed by biochar having a value of 695.9±101.4. Activated carbon control had the lowest observed number of species of 630.7±70.6. With respect to nutrient amendment, in the unamended soil batch, the nutrient amended batch had a number of observed species of 656.8±4.8 followed by the unamended soil without nutrients (556.6±130.6). The AC amended soil with nutrients had the highest number of observed species (775.9±12.6) followed by the biochar amended soil with nutrients (762.4±8.0) while the biochar amended soil without nutrient had an observed species of 725.2±31.0. Amending soil with 2% AC and 2% biochar had a significant effect on the observed species ($p < 0.05$, Fisher's pairwise test; One-Way ANOVA) and had average values of 721.88±68.04 and 743.75±28.35 OTUs respectively compared to unamended soil on day 6 (606.73±95.06). There was no significant difference in the observed number of species between soil & biochar and soil & AC batches.

The implication of these results may mean that exposing soil at time zero (control) to a high concentration of non-aqueous phase liquid (NAPL) of VPHs over the duration of the experiment, caused a reduction in the bacterial species richness as indicated by the non-parametric richness estimators (Chao1 and observed species) due to pollutant toxicity. Alternatively, the growth of degrading microorganisms leads to their predominance in the community, meaning that rare species, although still present may not be detected in the analysis.

Statistical analysis of the bacterial richness indices shows that with respect to the indices Chao1 and observed number of species, there was no statistically significant difference between the controls and the soils amended with or without biochar or AC and with or without nutrients (1-Way ANOVA, Tukey Kramer's pair-wise comparison, ($p > 0.05$) (Table 5.2). A comparison of soil treatments at the end of the experiment, showed that amendment was a significant factor in the determination of bacterial species richness. There was thus a significant difference between the unamended soil (with and without nutrients) and soil & activated carbon soil (with and without nutrients) for Chao1 and (1-Way ANOVA, Tukey's pair-wise comparison, $p < 0.05$) observed species (Fisher's pair-wise comparison, $p < 0.05$). Strong sorption of VPHs to the AC amended soil would reduce the amount of bioavailable pollutants to the indigenous microbes in the AC amended soil and consequently their toxicity at high concentrations. On the other hand, in the unamended soil batches (with and without nutrients) by the end of the experiments, there was increased bioavailability of the VPHs leading to increased toxicity at higher concentrations and hence a lower species richness.

Microbial diversity as indicated by the Faith's phylogenetic diversity showed that the control soil (time zero) had the highest diversity with an index of 86.33±0.03. In the biochar and AC controls, the PD index values were 78.95±9.99 and 73.95±8.17 respectively. Amending soil treatments with or without nutrients did not have any significant effect ($p > 0.05$, 2-Way ANOVA) on the sample diversity as indicated by the Faith's PD. The nutrient amended treatments for soil, soil & biochar and soil & AC had PD values of 71.46±0.16, 78.94±0.22 and 75.50±0.21 respectively and were higher than the values for the nutrient unamended treatments (Table 5.2). With respect to sorbent amendment, soil & biochar batch had the highest PD index with a value of 78.53±2.22 followed by the soil & AC batch (73.37±2.47) and the unamended soil batch (67.30±8.40). There was a significant effect of sorbent amendment on microbial diversity ($p < 0.05$, 1-Way ANOVA) between soil and soil & biochar batch. There was no significant difference between soil & biochar and soil & AC batches ($p > 0.05$, 1-Way ANOVA).

A similar trend to the Faith's PD was observed in the Shannon's diversity index with respect to the controls and the sorbent amended soils (with and without nutrient amendment) (Table 5.2).

A 2-Way analysis of variance (ANOVA) of the soil treatments (soil amended with or without the sorbents or nutrients) shows that the amendment factor was a significant factor but not the nutrient factor in determining the species richness (Chao1 and observed species) and microbial diversity (Faith's PD and Shannon's diversity) in the different soil systems. Although there were increases in the richness and diversity indices of all nutrient amended soils compared to nutrient unamended soils (Table 5.2) indicating that the addition of nutrients had a positive effect on the bacterial richness and diversity, these increases were not statistically significant ($p < 0.05$). There was also no significant interaction between the nutrient factor and the amendment factor as it relates to the richness and diversity indices in the different treatments.

Sample I.D	Treatment	Chao1	Observed no of species	Faith's PD	Shannon's
Soil (time 0)	Live control	1463.93±10.41	938.7±5.9	86.33±0.03	8.83±0.04
Biochar	Control	1106.40±195.47	695.9±101.4	78.95±9.99	7.46±0.83
Activated carbon	Control	891.72±64.12	630.7±70.6	73.95±8.17	7.08±1.22
Soil_WON	Without nutrients	896.85±194.26	556.6±130.6	63.14±11.93	5.74±1.29
Soil_WN	With nutrients	982.83±27.41	656.8±4.8	71.46±0.16	7.15±0.02
Soil_2%BC_WON	Without nutrients	1067.46±80.01	725.2±31.0	78.11±3.76	8.24±0.10
Soil_2%BC_WN	With nutrients	1102.92±18.63	762.4±8.0	78.94±0.22	8.47±0.02
Soil_2%AC_WON	Without nutrients	1085.37±191.86	667.9±45.3	71.24±0.17	6.32±0.30
Soil_2%AC_WN	With nutrients	1279.39±30.06	775.9±12.6	75.50±0.21	7.11±0.04

Table 5.2. Average species richness as assessed by the non-parametric estimator Chao1, and Observed number of species and bacterial diversity indices Faith's phylogenetic diversity and Shannon's index (H') determined for the controls (soil, biochar and activated carbon) at time zero and soil treatments with or without biochar or AC and with or without nutrient amendment at the end of the experiment on day 6. Diversity indices were determined at a fixed rarefaction depth of 3210 sequences. Error ranges are calculated as standard deviation from the mean of duplicate measurements.

5.4.4   *Effects of sorbent and nutrient amendment on phylum level taxa distribution across soil samples*

A variation in the relative abundances of the OTUs between the different soil systems is shown in (Figure 5.4). The dominant taxa (OTUs ≥3.0% of all classified sequences) across all soil samples include Actinobacteria, Acidobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Proteobacteria, Planctomycetes and Gemmatimonadetes representing 8.7, 4.6, 4.0, 5.1, 5.9, 54.4, 5.8, and 3.5% of the total OTU abundance in the dataset.

In the initial soil on day 0, the most dominant bacterial phyla were the *Gammaproteobacteria* (11.7±0.9%), *Alphaproteobacteria* (11.35±0.3%) followed by *Actinobacteria* (11.2±1.7%, and *Firmicutes* (8.2±1.3%). In the unamended soils (without biochar or AC), at the end of the experiment on day 6, the most abundant taxa were the *Gammaproteobacteria* (39.4±16.9%), *Betaproteobacteria* (15.7±11.4%), *Alphaproteobacteria* (6.1±2.0%), *Actinobacteria* (5.6±2.0%), and *Planctomycetes* (4.5±1.4%) while in the biochar amended soils, the dominant phyla and *Proteobacterial* classes were *Gammaproteobacteria* (16.1±1.7%), *Betaproteobacteria* (12.1±1.6%), followed by *Alphaproteobacteria* (12.0±0.8%), *Actinobacteria* (10.6±1.2%) and *Planctomycetes* (7.0±0.6%). In the activated carbon amended soils, the dominant taxa were the *Gammaproteobacteria* (38.6±5.8%)*, Actinobacteria* (12.7±4.1%)*, Alphaproteobacteria* (7.0±1.0%) and *Betaproteobacteria* (5.8±0.6%). Most of the dominant bacterial phyla identified in the sorbent amended and unamended soil have been previously detected in soil samples (Kostka *et al.*, 2011; Nacke *et al.*, 2011) and did not appear to vary significantly from the phylum level taxa distribution in the initial soil at time zero. With respect to nutrient amendment, in the unamended soil on day 6, the most dominant phyla in the batches without nutrient were the *Gammaproteobacteria* (40.3±29.2%) followed by the *Betaproteobacteria* (22.3±14.6%), *Alphaproteobacteria* (4.8±2.0%) and *Actinobacteria* (4.2±1.5%) while in the soil batches with nutrients, the most dominant phyla (>3.0% of total OTU abundance) were *Gammaproteobacteria* (38.6±1.0%), *Betaproteobacteria* (9.1±0.8%), *Alphaproteobacteria* (7.4±0.6%) and *Actinobacteria* (6.9±1.5%). In the soil & biochar batch without nutrients, the most dominant *Proteobacterial* class was the *Gammaproteobacteria* (16.7±2.4%) followed by the *Betaproteobacteria* (13.4±1.0%), while in the batches with nutrients the predominant OTUs were *Gammaproteobacteria* (15.6±1.3%) and *Alphaproteobacteria* (11.4±0.3%). In soils amended with 2% activated carbon without nutrients, the most dominant OTUs at the phylum level was the *Gammaproteobacteria* (35.5±0.4%) while in the AC amended soil with nutrients, the most abundant OTUs were *Gammaproteobacteria* (41.8±7.8%) and *Actinobacteria* (12.4±0.4%).
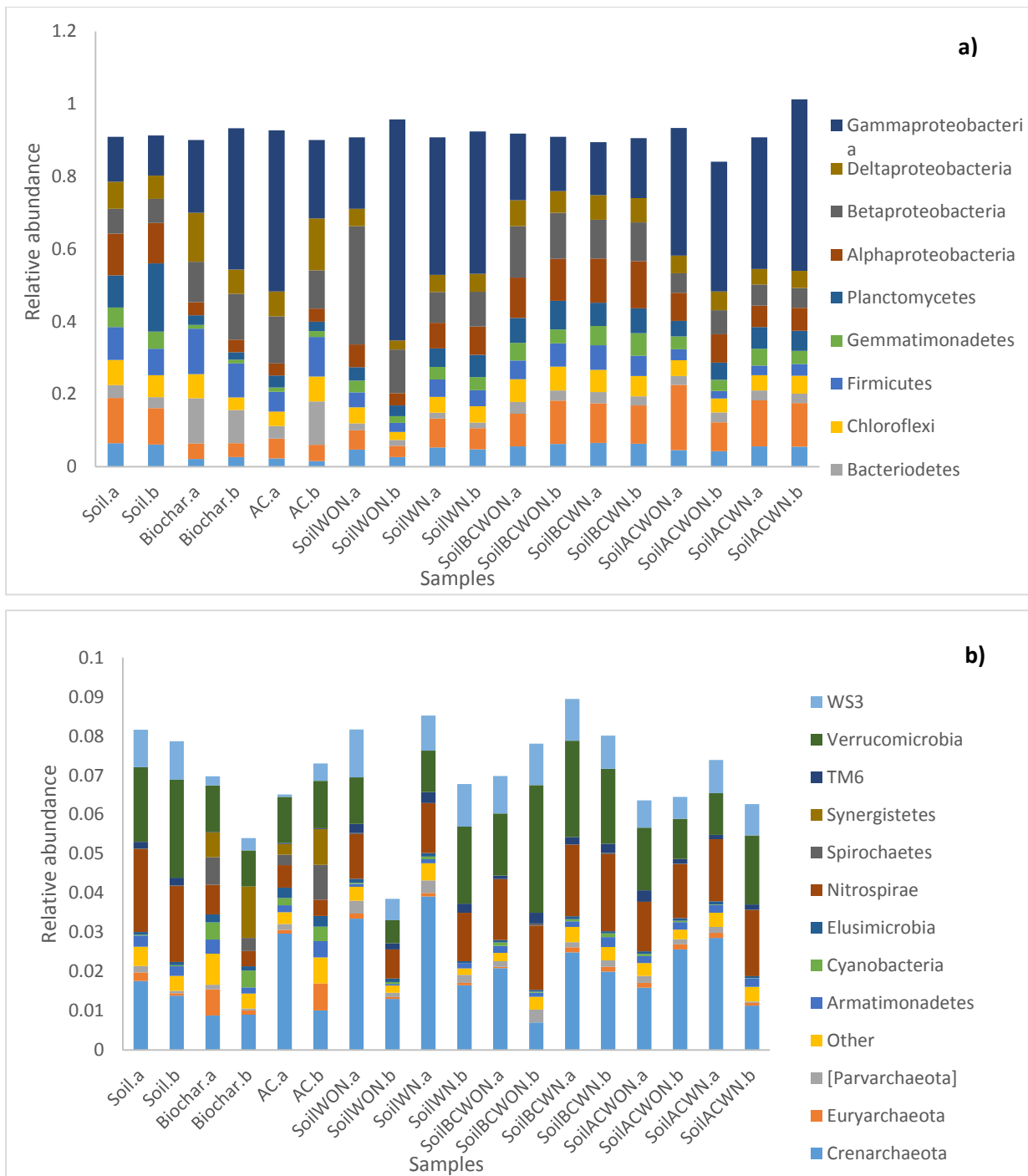
Figure 5.4. Relative abundances of operational taxonomic units (OTUs) accounting for a) ≥ 3.0% and b) ≤ 3.0% of all classified sequences obtained from control samples and different soil samples amended with or without biochar or activated carbon.

At the order level, the most dominant OTU belonging to the *Gammaproteobacteria* in the control soil on day zero was the *Xanthomonadales*. In the biochar and activated carbon controls, the most abundant OTU was the *Pseudomonadales*. Most of the sequences belonging to the most abundant phylum in the unamended soils and to the soil& AC batches (*Gammaproteobacteria*) were related to the *Pseudomonadales* and to *Xanthomonadales*

(*Gamaproteobacteria*) in the biochar amended soils. Members of the order *Xanthomonadales* were reported to grow on toluene and the alkanes as typical growth substrates (Palleroni *et al.*, 2004) while members of the order *Pseudomonadales* have the potential to utilize alkanes as carbon source (Bogan *et al.*, 2003). *Actinobacteria* has also been reported to grow on alkanes (Mikolasch *et al.*, 2003; Dashti *et al.*, 2009).

At the genus level, the most dominant OTU in the soil at time zero was an unidentified member of the order *Bacillales* ($3.64\pm0.46\%$). In the biochar and AC controls, the most dominant OTUs at the genus level was *Pseudomonas* representing $17.30\pm10.08\%$ and $22.52\pm16.87\%$ of the total OTU abundances respectively. In the unamended soil on day 6, at the end of the experiment, the most abundant OTU was *Pseudomonas* representing $19.59\pm14.95\%$. In the soil & biochar batch and the soil & AC batch on day 6, the most abundant OTU at the genus level was *Lysobacter* and an unidentified member of the *Nocardioidaceae* family representing $4.24\pm0.62\%$ and $9.34\pm4.73\%$ respectively.

The different factors under investigation (time, nutrient and amendments) had a significant effect on the relative abundances of the operational taxonomic units. In the unamended soil without nutrients on day 6, the genera *Lamia* and *Mesorhizobium* were more abundant than in the soil on day zero. In the unamended soil with nutrients, the genera *Alicyclobacillus*, and *Lysobacter* were significantly more abundant than in soil on day 0 while and *Achromobacter* showed the opposite pattern ($p < 0.05$). In the soil & biochar batch, the genera *Arenimonas* and *Achromobacter* were relatively more abundant in the batches without nutrient than in the control soil on day zero while *Roseomonas* and *Steroidobacter* showed the reverse pattern. In the soil & biochar with nutrients, *Nocardia*, *Rhodoplanes* and *Phenylobacterium* were significantly more dominant than in the soil at time zero while *Nitrosopumilus* were more abundant in the soil at time zero than in the soil & biochar with nutrients on day 6. In the soil & AC batch without nutrients, the genus *Flavobacterium*, *Streptomyces* and *Phenylobacterium* had a significantly higher relative abundance than on day zero while *Nitrospira* had a higher relative abundance on day zero than on day 6. In the AC amended soil batch with nutrients the genera *Nocardia*, *Roseomonas* and *Phenylobacterium* were significantly higher in relative abundance on day 6 than in soil on day zero.

With respect to sorbent amendments, the genera *Flavobacterium*, *Clostridium*, *Nitrospira*, *Hyphomicrobium*, *Polaromonas*, and *Arenimonas* showed significantly higher relative abundances in the biochar amended soils than the unamended soils (2 sample t-tests; $p < 0.05$) while the genus *Mycoplana* showed the opposite pattern (Table 5.3). *Polaromonas* and *Nitrospira* were significantly higher in activated carbon amended soils than they were in the

unamended soils (2 sample t-tests; $p < 0.05$) whereas *Clostridium* was more dominant in unamended soils than AC amended soils.

5.4.5   *OTU functions viz-a-viz petroleum hydrocarbon degradation*

Paenibacillus is a phylogenetically distinct genera from the rod shaped Bacillus. Members of this group are reported to have a Gram-positive structure but stain negatively in a Gram reaction. There are indications from documented reports that members of this genus have the potential to degrade m-xylene (Ash et al., 1993; Khomenkov et al., 2005; Xie et al., 2010). Mycoplana is a Gram negative, non-spore forming aerobic member of the Brucellaceae family with a strictly respiratory type of metabolism (Garrity et al., 2005). Members of this genera have the ability to grow on L-arabinose, xylose, D-glucose, D-mannose and D-fructose as their sole carbon sources and have been reported to degrade the aromatic PH toluene (Garrity et al., 2005; Velam et al., 2012). Most of the PH degrading bacteria were more dominant in the biochar amended soils than the activated carbon amended soils (Table 5.3). Activated carbon is typically a stronger sorbent material than biochar, it is therefore expected that VPHs are less bioavailable in the AC amended soil, hence less growth of VPH degrading bacteria. Amending soils with biochars have been reported to cause an increase in microbial abundance. Little is known about the mechanisms of action of biochars on microbial composition and abundance although a few proposals have been reviewed in the literature. Lehmann *et al.* (2011) reported that the pH and liming value of biochar may play a significant role in determining soil pH which in turn affects bacterial community composition and structure. In the current study, the pH range between the soil and biochar used was small (ca. 1.82) and hence pH may not be a significant factor affecting microbial abundance in the biochar amended soils. *Clostridium* is a Gram-positive, obligately anerobic, endospore forming bacterial genus and do not carry out dissimilatory sulfate reduction (Collins *et al.*, 1994). There is not much information linking this genus with the potential to degrade petroleum hydrocarbons although some reports suggest that members of this genus were involved in the degradation of toluene in a bioreactor study (Gao *et al.*, 2011). The genus *Hyphomicrobium* is a Gram-negative, facultative methylotrophic and non-spore forming genus belonging to the *Alphaproteobacterial* class (Urakami *et al.*, 1995; Kanamori *et al.*, 2002). Very few reports (just one) indicate the possibility of their involvement in petroleum hydrocarbon degradation (Li *et al.*, 2000). In a study by Young *et al.* (2007), members of the genus *Arenimonas*, a Gram-negative, aerobic genus belonging to the *Gammaproteobacterial* class were isolated from a diesel-oil contaminated soil. No reference was made to their capacity to degrade specific petroleum hydrocarbon compounds.

With respect to nutrient amendment in the soil batch without biochar or activated carbon, *Streptomyces, Geobacter* and *Bacillus* were significantly higher in relative abundance ($p < 0.05$, 2 sample t-test) in nutrient unamended soils than in nutrient amended soils while *Agromyces* was more dominant in soils amended with 2%BC and inorganic nutrients than in biochar amended soils without nutrients. In the activated carbon amended soils, *Hyphomicrobium*, *Cupriavidus*, and *Arenimonas* were more dominant in the nutrient amended soils than the nutrient unamended soils (2 sample t-tests; $p < 0.05$). Members of the *Bacillus* genus are endospore-forming aerobes or facultatively aerobic species that have been reported to possess a wide range of physiologic properties which allows them to thrive in diverse environments (Peter and Turnbull, 1996). Members of this genus have also been shown to degrade the BTEX compounds (Mukherjee and Bordoloi, 2012a). *Cupriavidus* is a Gram-negative, chemolitothrophic group of bacteria in which the ability to nodulate and to fix atmospheric nitrogen (N_2) has been reported (Vandamme and Coenye, 2004; da Silva *et al.*, 2012) but

S.n	Bacteria (Genus)	Average relative abundances (Amendment effects)			Petroleum hydrocarbon degrading potential
		Soil	2% Biochar	2% AC	
1	*Brevibacillus*	2.69E-04	1.14E-03	2.25E-04	Polyethylene
2	*Flavobacterium*	4.53E-04	1.24E-03	5.29E-04	Toluene, Xylene, benzene and hexane
3	*Mycoplana*	5.02E-04	2.20E-04	1.48E-04	Toluene
4	*Paenibacillus*	3.01E-03	6.48E-03	2.60E-03	*m*-xylene
5	*Achromobacter*	1.17E-01	2.83E-02	6.02E-04	Alkanes
6	*Clostridium*	6.34E-05	1.18E-03	4.28E-04	Toluene
7	*Hyphomicrobium*	1.89E-03	4.06E-03	2.11E-03	Petroleum hydrocarbons
8	*Nitrospira*	5.48E-03	8.48E-03	7.03E-03	-
9	*Sphyngopyxis*	1.43E-03	3.37E-04	7.70E-04	Benzene, Toluene, Ethyl-benzene and xylene.
10	*Polaromonas*	6.65E-04	7.14E-03	5.44E-03	Heptane, octane and toluene
11	*Hydrogenophaga*	2.82E-04	1.17E-02	1.91E-03	Benzene, toluene, xylene
12	*Arenimonas*	6.35E-04	2.07E-03	4.96E-04	Diesel oil
13	*Pseudomonas*	1.67E-01	2.91E-02	8.45E-03	Alkanes, aromatics, hexane

Table 5.3. Summary of statistically significant treatment effects (with and without sorbent amendment, t-test, two tailed, p <0.05) for OTU identified at the genus level, where members of the genus reportedly degrade petroleum hydrocarbons.

not the potential to degrade petroleum hydrocarbons while members of the *Bdellovibrio* genus were isolated from a BTEX-fed reactor (Li and Goel, 2012).

At the species level (OTU L7, ranking of OTUs in the control soil at day zero based on their abundances relative to the total OTU abundance in the overall dataset and their corresponding increases or decreases with addition of VPHs and/or nutrients is shown in Table 5.4. Changes in the ranking of the OTUs following VPH addition to soil reveals that bacterial species within the genera *Pseudomonas* and *Achromobacter* and the family *Nocardioidaceae* were higher in ranks amongst the group of known petroleum hydrocarbon degraders.

An increase in the absolute abundances of VPH degrading biomass across all soil treatments (with and without nutrients and sorbent amendment) following exposure of initial soil at time zero to VPH mixture is an indication of growth of soil microorganisms on a carbon source. Most notable VPH degraders were the *Pseudomonas* and the *Polaromonas* genera that showed increases of over a 100 percent in absolute abundances relative to the control to which no VPH was added in all soil treatments (See appendix B; Table 9.9). In addition, the genus *Nitrospira* also demonstrated over 100% increase in absolute abundance in all soil treatments (with or without nutrient or sorbent amendment). The genus *Achromobacter* showed over a 100% increase in absolute abundance compared to their abundance in the control at time zero in all soil treatments except soil & AC batch with nutrients in which they decreased by an order of magnitude (Table 9.9). On the contrary, the genus *Cupriavidus* showed no growth in absolute abundance (absolute abundance = 0.00) following exposure of soil and soil & biochar treatments to VPHs. There was however an increase in absolute abundance by up to two orders of magnitude of this bacterial genus in the soil & AC batch.

Upon amending soil with or without 2% biochar and activated carbon followed by addition of volatile petroleum hydrocarbon mixtures and/or inorganic nutrients to the soil, varying responses of the OTUs relative abundance ranking was observed. Bacterial species belonging to the family *Nocardioidaceae* was shown to increase in relative abundance ranking in the AC amended soils with VPHs with and without nutrients by at least 64 folds but did not show any increase in the soil only or soil & biochar batches. A few bacterial genera belonging to this family were reported to participate in the degradation of petroleum hydrocarbons including the *Nocardiodes* in the degradation of alkanes and crude oil components (Hamamura and Arp, 2000; Hamamura *et al.*, 2006) and the aerobic hydrocarbon degrading genus *Aeromicrobium* (Chaillan *et al.*, 2004). Species within the genus *Achromobacter* also increased in relative abundance ranking by up to 64 folds in the soil and soil & biochar batches relative to the control but never showed any increase in relative abundance ranking in the AC amended soil

batch. One report suggest that an *Achromobacter sp*ecies was isolated from a crude-oil contaminated seawater and was also shown to effectively degrade total n-alkanes and to readily utilize polyaromatic hydrocarbons when incubated at optimum growth conditions (Deng *et al.*, 2014). In another study by Gojgic-Cvijovic *et al.* (2012), members of this genus were part of a consortium of bacteria isolated from a petroleum sludge. Two *Pseudomonas* species were found to increase in their relative abundance ranking relative to the control by at least 16 folds in the soil and VPHs with or without nutrients and by up to 4 folds in the biochar amended soil batches. The genus *Pseudomonas* comprises of a metabolically versatile category of microorganisms that can live aerobically or anerobically on nitrates as an electron acceptor (Palleroni *et al.*, 2010) and are reported to have the potential to metabolise a wide range of organic compounds including alkanes and aromatic hydrocarbon compounds. A *Sphingopyxis* species increased by at least 2 folds in all the soil and soil & biochar batches but not in the soil & AC batch while a *Hydrogenophaga* species demonstrated up to 16 folds increase in the soil & biochar and the soil & AC batches but did not increase in relative abundance in the unamended soil batch, with or without VPH and nutrient addition. Members of the genus *Hydrogenophaga* are reported to have the potential for the degradation of benzene, toluene and to partially degrade *m*- and *p*- xylene (Fahy *et al.*, 2006). Most of the OTUs at the species level of taxonomy that demonstrated a strong response to the addition of VPHs or nutrients were unidentified and may thus be necessary to conduct further analysis by comparing OTU sequences with those of reference databases using other bioinformatics software such as the basic local alignments search tool (BLAST) and the ribosomal database project (RDP).

Taxon (highest level classification)	Relative abundance rank increase for different treatments compared to the soil only control + 2-fold, ++ 4-fold, +++ 8-fold, ++++ 16-fold, +++++ 32-fold, ++++++ 64-fold or greater, equivalent decrease for minus signs						
	Soil d0 Rank	Soil+VPHs	Soil+VPHs +nutrients	Soil+BC+VPHs	Soil+BC+VPHs+ nutrients	Soil+AC+VPHs	Soil+AC+VPHs + nutrients
f__Nocardioidaceae	65			-		++++++	++++++
g__Paenisporosarcina	615	+++++	+++++	++++	++++	+++	
g__Sphingopyxis	615	+	+++	+	+		
o__Burkholderiales	615	++	++	+++	+++	+++	+
g__Achromobacter	93	++++++	++++++	+++++++	++		--
g__Acidovorax	615	++	+++++	++++	++++	++++	+
g__Hydrogenophaga	222			++++	+++	++	+
g__Cupriavidus	562	+	+	+		+	+++
f__Pseudomonadaceae	615	+	++++	+++	+	+	
g__Pseudomonas	24	+++++	+++++	+	++	+	-
s__Pseudomonas__umsongensis	139	+++++	++++	++	++	+	-
g__Arenimonas	615	++	++	+++	+++	+	

Table 5.4. Summary of taxons which showed a minimum 8-fold increase in their relative abundance rank in one of the batch study treatments.

5.4.6 *Relationship between microbial communities in different samples*

A nearest-neighbour joining algorithm was used to cluster the samples into a dendogram (Figure 5.5) based on similarities between bacterial communities in the sorbent and nutrient amended and unamended soil samples. The results revealed that samples from the same treatments (i.e. same nutrients and sorbent amendment) clustered closely to each other than they did to samples from other treatments. This implies that bacterial communities in the samples shifted in response to nutrient and sorbent amendment (ANOSIM test for difference between sorbent amendments, Global $R = 0.364$, $p < 0.05$; for difference between nutrient amendment, Global $R = 0.219$, $p < 0.05$; for difference between time on day zero and day 6, Global $R = 0.778$, $p < 0.05$). Summary statistics of the pair-wise significance between the different samples is included in the (Appendix B). The overall similarity between bacterial communities as determined by the Pearson correlation coefficients was greater than 80% (Figure 5.5) and similarity between duplicate DNA samples from the same treatments (nutrients and sorbent amendment) was even greater (>90% overall similarity) indicating that the methods used are fairly reproducible.
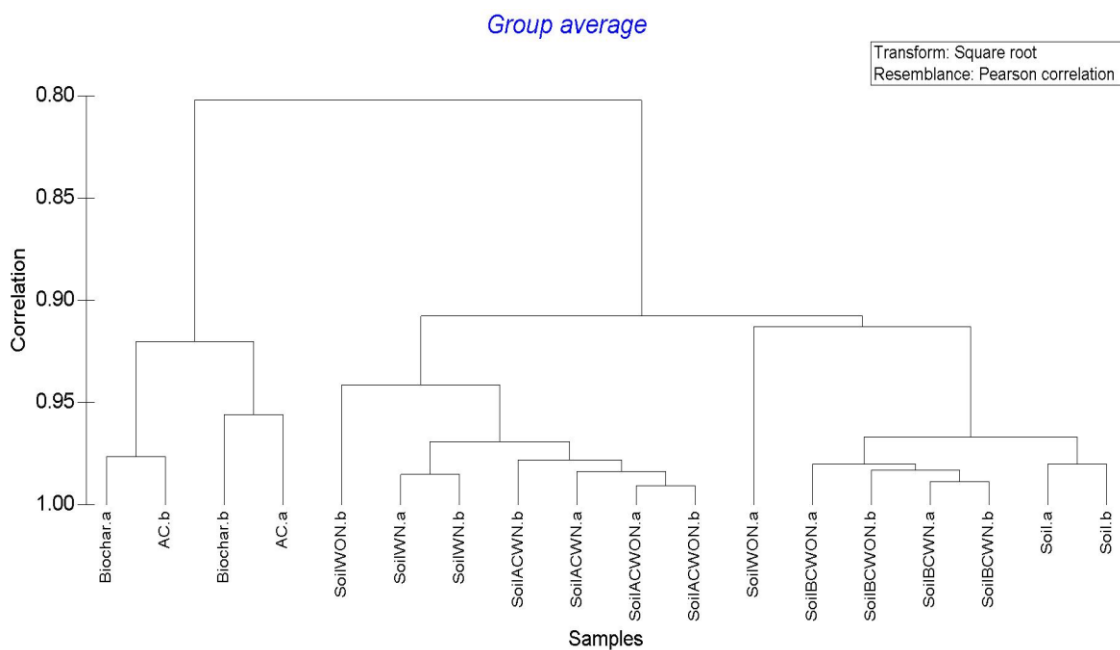
Figure 5.5. A dendogram showing cluster analysis of the similarities (Pearson product-moment correlation coefficient) between the bacterial community compositions of the different samples. OTUs were clustered at class level of taxonomy and square root transformed prior to clustering.

The high percentage similarity between the different treatments and the initial unamended soils (>90%) suggests that amending the soils with inorganic nutrients and either 2% biochar or 2% activated carbon did not have a deleterious impact on the bacterial community

composition of the soil. As the dominant microbial groups at any given point in time and in any particular environment are reported to provide an indication of the key soil processes occurring within that environment, it follows from the results of this study that amending soils with biochar and activated carbon may not have had any potentially strong side effects on soil microbial communities. These observations correspond with findings from other studies in which AC had only minor effects on the soil microbial community response in contaminated soils and sediments (Cho *et al.*, 2009; Cornelissen *et al.*, 2011; Meynet *et al.*, 2012)**.**

Results from a principal components analysis of the most abundant OTUs (>1.0% of total number of sequences) reveals a definite pattern of the samples partitioning into the positive and negative axis of the 1st and 2nd principal components (F1 and F2) with the first principal component accounting for 47.16% and the 2nd principal component accounting for 29.22% of the variability in the entire dataset (Figure 5.6). A close look at the observations (samples) plots viz-a-viz the variables (OTUs relative abundances) plots shows that the genera *Pseudomonas*, *Achromobacter*, and an unidentified OTU contributed positively to the 1st principal component which corresponds to the soils amended with or without nutrients (SoilWN & SoilWON) and the AC and BC controls while *Candidatus Nitrosophaera*, *Lysobacter*, *kaistobacter*, and unidentified genera contributed negatively to the 1st PC corresponding to soils amended with or without BC and AC and with or without nutrients (Figure 5.6). On the other hand, with respect to the second principal component (F2), *Pseudomonas* contributed positively while *Achromobacter*, *Candidatus Nitrosophaera*, *Lysobacter*, and *Kaistobacter* contributed negatively to F2. The highest percentage variation on PC1 was accounted for by the genus *Pseudomonas* (46.45%) followed by *Achromobacter* (18.25%). On PC2, an unidentified OTU accounted for the highest percentage variation on 74.03% followed by the genus *Pseudomonas* accounting for 6.87% of the variation on this PC.
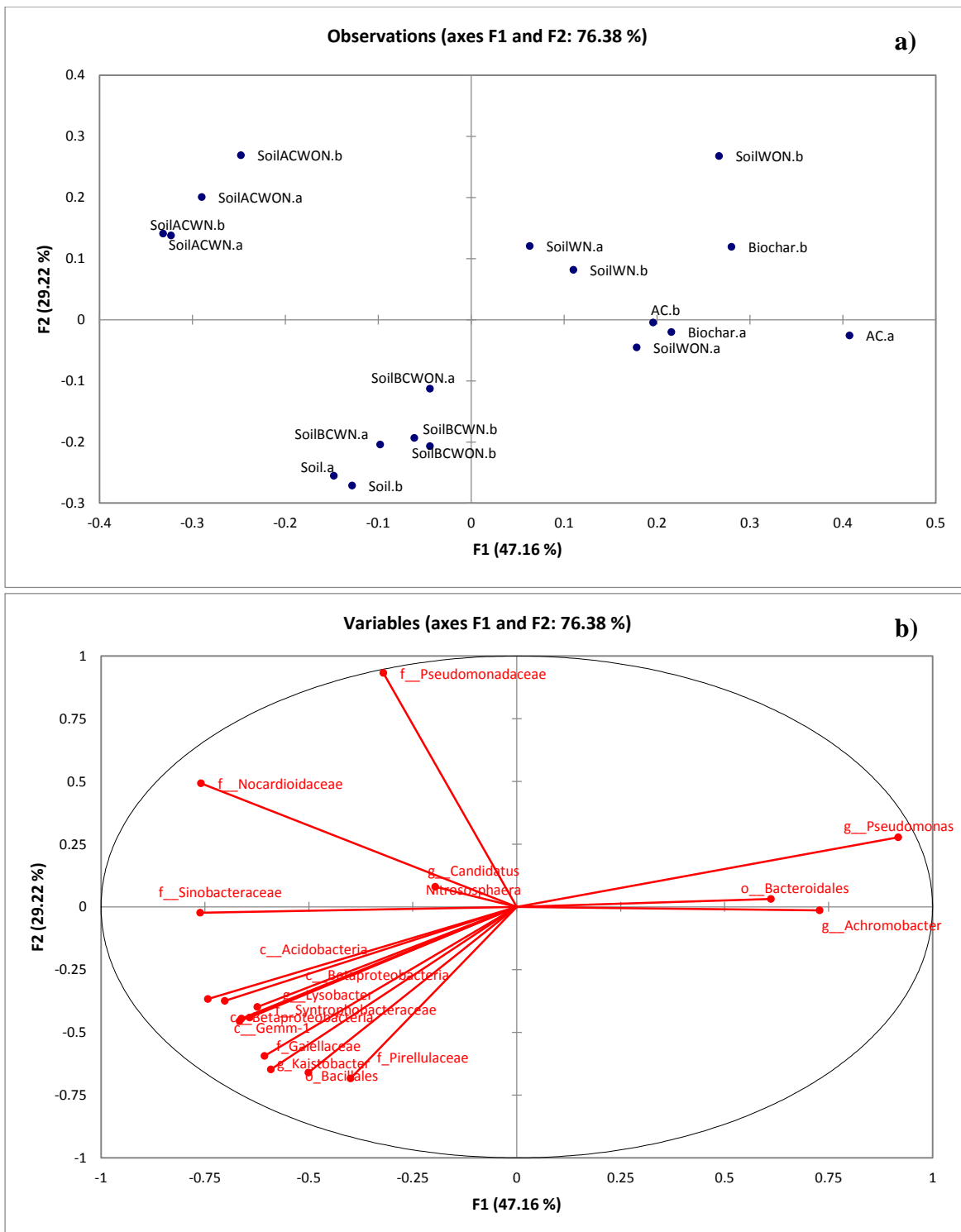
*Figure 5.6. Principal Components Analysis of microbial communities as affected by **a)** sorbent amendment and nutrient amendment based on **b)** the relative abundances of dominant OTUs (>1.0% of total sequences) at the highest level taxononomy (square root transformed data). The direction of vectors indicate the direction of change of each variable (OTU relative abundance) so that the observation samples clustered together have similar microbial communities.*

5.5   Conclusions

The current chapter investigated the short-term effects of amending soil with or without biochar and activated carbon and with or without inorganic nutrients (N and P) on the biodegradation of a mixture of VPHs in sand.

Addition of nutrients to soil batches resulted in an increased evolution of CO_2 compared to batches in which no nutrients were added regardless of sorbent amendment (Figure 5.2). This indicates that soil microbial activity was stimulated by the addition of the inorganic nutrients N and P thereby validating our first hypothesis.

Following exposure to a VPH non-aqueous phase liquid (NAPL) source over a short-term duration of 6 days, results indicated that there was a significant increase in the relative abundances of soil bacterial communities compared to the initial soil conditions on day zero. The genera *Nocardia*, *Rhodoplanes*, and *Sphyngopixis* were significantly higher in relative abundance on day 6, at the end of the experiment compared to the initial soil (day 0) while *Lysobacter*, *Arenimonas* and *Flavobacterium* were more abundant in the nutrient unamended batches on day 6 than in the control soil on day zero. Relative abundance ranking of OTUs in the control soil and increase in the ranking of OTUs also indicated an increase in the ranking of bacterial species within the VPH degrading genera *Pseudomonas* and *Sphyngopixis* and within the families *Nocardioidaceae* and *Pseudomonadaceae* by at least two folds, indicating that over time, exposure to VPH (NAPL) source caused an increase in the relative abundance ranking of respective OTUs. Therefore, over the duration of the experiment, and in line with our proposed hypothesis, microbial community composition was significantly altered following exposure of different soil treatments to VPHs.

Although nutrient limitation affected the rate of metabolism of the carbon source (VPHs) present in the batches as proposed (Section 5.2.2) and as indicated by the concentrations of CO_2 in batch headspaces (Figure 5.2), nutrient amendment also had significant effect on the bacterial community structures (ANOSIM test for difference between nutrient amendment, Global $R = 0.218$, $p < 0.01$). There was also an increase in the relative abundance ranking of the OTUs in the soil on day 0 following amendment with or without nutrient (Table 5.4) by up to 64 folds. Thus, contrary to our hypothesis, the nutrient limited batches also showed an increase in relative abundance of the VPH degrading bacterial communities suggesting that the microbial community response was affected by factors other than inorganic nutrient limitations.

Maximum cumulative headspace CO_2 concentrations were comparable for the soil and the soil & biochar batches (Figure 5.2). Headspace CO_2 concentrations in the soil & AC batch was, however, lower than for soil by up to one order of magnitude indicating the likely effects of low substrate availability on biodegradation of VPHs in the AC amended soil. This confirms the proposed hypothesis that sorbent amendment slows the mineralization of VPHs due to pollutant sorption, especially in the AC amended soil batches.

Amending soils with 2% biochar or AC caused a significant shift in the bacterial community structure as the communities also shifted strongly with sorbent amendment (ANOSIM test for difference between sorbent amendments, Global $R = 0.364$, $p < 0.01$). PH degrading bacterial genera such as *Paenibacillus* and *Flavobacterium* were significantly more abundant in the soil & biochar batches while *Pseudomonas* was more dominant in the unamended soil batch than in the soil & biochar and the soil & AC batches further highlighting the effects of sorbent amendment on the microbial community response on the short term and also validating the fifth proposed hypothesis.

Ultimately, exposing soil to high concentrations of VPHs NAPL source enhanced the biodegradation of petroleum hydrocarbons but also caused a decrease in soil microbial diversity and species richness in the sorbent unamended soil compared to biochar and AC amended soils likely due to toxicity of pollutants at high concentrations. The metabolically versatile bacterial genus *Pseudomonas* appeared to thrive in the unamended soil with VPHs at high concentrations as shown by higher relative abundances than in the sorbent amended batches (Table 5.3) in the short term. Amending soil with biochar and AC clearly had an effect (positive and negative) on the relative abundance ranking of the PH degraders. Further research needs to be conducted on the effects of sorbent materials sourced from different feedstocks on the biodegradation of VPHs in other soil types in order to ascertain the mechanisms by which sorbent amendment affects soil microbial community dynamics in the short term.

Chapter 6: Effects of volatile petroleum hydrocarbon vapour migration on the microbial community composition in a biochar and activated carbon amended sand – a long term study

6.1 Introduction

The fate of volatile petroleum hydrocarbons in the unsaturated zone is currently been investigated. Monitoring the i*n situ* biodegradation of VPHs in the unsaturated zone still remains a challenge as contaminated sites may have to be disturbed thereby disrupting ongoing microbiological processes such as biofilm formation on soil surfaces, alterations in nutrient concentrations and redox conditions e.t.c. Several approaches have been employed to surmount these challenges and to gain an improved understanding of the chemo-dynamics of pollutants in the environment including the use of laboratory batch microcosm and column experiments, and field lysimeter studies.

Batch microcosms have been used previously to study the biodegradation of petroleum hydrocarbons in the unsaturated zone (Hohener *et al.*, 2003; Ostendorf *et al.*, 2007) although a number of limitations have been associated with the use of this approach. One main advantage of laboratory batch experiments is that it offers the flexibility of studying systems in replicates and also to study single and or mixed compound systems (Hohener, 2010). It also allows for the determination of VPH biodegradation rates in moist soils. A major limitation of the batch system is that it is mostly suitable for soils with a high level of petroleum hydrocarbon degradation activities and for compounds whose sorption and partitioning dynamics are properly understood (Hohener, 2010). On the other hand, the use of laboratory columns to study the biodegradation of VPHs is also gaining widespread application particularly for the simulation of vapour migration and attenuation of VPHs in the unsaturated zone (Hohener *et al.*, 2003; Hohener *et al.*, 2006; Bouchard *et al.*, 2008a; Bushnaf *et al.*, 2011). Laboratory column experiments are considered to provide a more reliable simulation of the natural environment with respect to vapour migration in the unsaturated zone of contaminated soils (Kelly *et al.*, 1996).

More recently, *in situ* sorbent amendment of soils and sediments contaminated with organic pollutants is being considered as an innovative approach for mitigating pollutant effects on ecological receptors (Ghosh *et al.*, 2011; Meynet *et al.*, 2012). Laboratory batch microcosms and column studies were also conducted by Bushnaf *et al.* (2011) to investigate the effects of 2% biochar amendment of volatile petroleum hydrocarbon contaminated soil on the attenuation of the pollutants and to determine the sorption and degradation kinetics of volatile compounds. Quite a number of research has been carried out with respect to the

chemodynamics of VPHs in contaminated soils including the determination of biodegradation rates and microbial growth kinetics (Pasteris *et al.*, 2002; Hohener *et al.*, 2003). There are few reports, however, on the effects of organic pollutants on the indigenous microbial community structures present at contaminated sites (Kostka *et al.*, 2011; Guermouche *et al.*, 2013). Fewer reports exist on the effects of sorbent amendment on microbial community structure changes of petroleum hydrocarbon contaminated sites. Sorption reduces the bioavailability of organic pollutants to microorganisms and consequently their rate of biodegradation. It is critical to develop an understanding of the dynamics of microbial communities at contaminated sites as this will further improve the understanding of the behaviour of organic compounds especially as it relates to the use of sorbents for the remediation of VPH contaminated soils.

In a recently concluded laboratory column study by Bushnaf (2014), gravelly sand was amended with and without 2% biochar and activated carbon in separate glass columns in order to investigate the effects of vapour migration along column lengths and sorption to the amendments on biodegradation of a mixture of VPHs. Soil respiration rates at different locations along the column lengths were also monitored during the experiments which lasted for 430 days.

6.2 Aim

The main aim of this study was therefore to investigate the long-term effects of vapour exposure and sorbent amendment on the microbiological communities within the different soil systems. As a follow up to the study by Bushnaf (2014) on the fate and transport of VPHs in long-term column experiments, microbiological analysis was conducted in order to gain an improved understanding of the underpinning phenomena such as changes in microbial community composition in response to VPH exposure and sorbent amendment.

6.2.1 *Objectives*

The following objectives were set in order to accomplish the study aim:

1. To study the long-term effects of VPH exposure on microbial community shifts by comparing microbial communities at time t = 0 prior to pollutant exposure with communities at day 430 after long-term exposure to pollutants.

2. To study the long-term effects of location along column length on microbial communities by comparing locations of the column nearer the NAPL source with locations in the middle and at the end of the column.

3. To study the effects of amendments biochar and activated carbon on the microbial community structure in VPH exposed soils by comparing the communities with those of unamended soil at day 430.

6.2.2 *Hypotheses*

In line with the aim and objectives of this study, the following hypotheses are proposed:

1. Soil microbial communities are expected to change significantly at the end of experiment duration of 430 days following exposure to VPHs even after the source has been exhausted.

2. It is expected that a higher concentration of VPH vapour at the sections of the column nearer the NAPL source (described as column source) would stimulate a higher level of microbial activity and also result in a significantly higher relative abundance of VPH degrading microbial communities at this location across all columns compared to the atmosphere-soil boundary sections of the column.

3. It is also proposed that amending soil with biochar or activated carbon would cause a significant shift in the soil bacterial community structure of the amended soils compared to the unamended soil at the end of the 430 days column study.

6.3 Materials and methods

6.3.1 *Soil, biochar and activated carbon*

Gravelly construction sand used for the construction of the Law library of the Newcastle University was obtained for this study. Biochar obtained from Environmental Power International EPI (Wiltshire, UK) and a bitumen-derived activated carbon obtained from Chemviron Carbon Ltd (Lancashire,UK) as described in Chapter 3, Section 3.3.1 were used to conduct this study.

6.3.2 *Chemical mixture*

The pollutant mixture used in this chapter is the same as the one used in Chapter 5 (Section 5.3.2).

6.3.3 *Column experiments*

The columns described in this section were set up by a previous PhD student (Bushnaf, K 2014) as part of his research. Soil amendments with or without biochar or activated carbon were investigated for their effects on the biodegradation of a VPH NAPL source over the duration of the experiment.

Three glass columns were homogenously packed with gravelly sand, gravelly sand amended with 2% biochar (on soil d.w.), and gravelly sand amended with 2% activated carbon and positioned horizontally at room temperature (Figure 6.1). Each column measured 120 cm in length and had an internal diameter of 7.8 cm. During the first five days after packing, the columns were left undisturbed to monitor background soil respiration after which each column was connected through a curved glass tube of internal diameter 1.1 cm at one end of the column to a 40 mL Teflon vial containing a 20 mL mixture of 12 volatile petroleum hydrocarbons (source).

At the other end of the columns, moist air flowing at a rate of 5±1 mL per minute was used to evacuate the VPH vapours into a fume cupboard in order to attain a near-zero concentration scenario at this end of the columns. Columns were equipped with 7 sampling ports running along the length of the columns and separated from each other by a 15cm distance from where soil pore gases (VPHs and CO_2) were taken and measured as an indication of VPH vapour migration and soil respiration. Sampling ports were sealed with gas chromatography septa (Thermogreen LB-2, Supelco, Bellefonte, USA). The experiment lasted for 430 days.
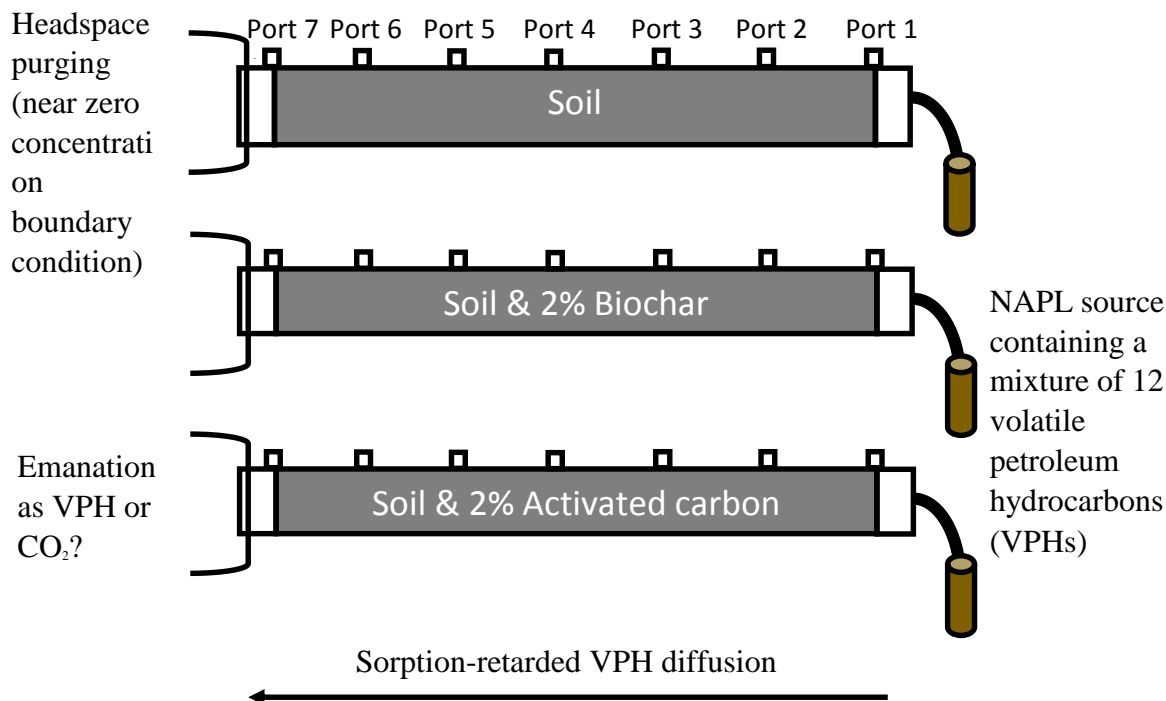
Figure 6.1. A schematic illustration of the column study set-up. VPH and CO2 concentrations were monitored at the different sampling ports for 430 days.

6.3.4   *Sample collection and storage*

At the end of the column experiments, on day 430, the columns were taken apart and samples were collected from three different sections (of equal length) of each column designated as the column source (nearest the NAPL source), column mid (middle part) and the near zero concentration boundary (atmosphere) section corresponding to the part of the column farthest from the NAPL source but nearest the moist air. Samples were collected in duplicates and stored at -20 °C in absolute ethanol: filtered-sterile phosphate buffer saline (PBS, Oxoid) mixed in the ratio 1:1 vol/vol for total cell counts and in filtered-sterile phosphate buffer saline (PBS, Oxoid) 1:1 vol/vol for DNA extraction and PCR amplification. Duplicate samples were also collected from initially unamended soil at time 0 prior to the start of the column experiments and stored as described previously for total cell counts and microbial analysis.

6.3.5   *Total cell counts and biomass growth*

Total cell counts and biomass growth determinations were performed using the same approach as described in Chapter 5, Section 5.3.6.

6.3.6   *DNA extraction and PCR amplification*

DNA extraction, PCR amplification and purification of amplicons were performed according to the methods described in Chapter 5, Section 5.3.7.

6.3.7   *454-pyrosequencing*

PCR amplicons from each sample (in duplicates) were pooled together in equimolar amounts and sequenced on a Roche 454 GS FLX+ System by NewGene Ltd (International Centre for Life, Newcastle upon Tyne, UK). Quality filtering and clustering of sequences into operational taxonomic units (OTUs) were performed as described in Chapter 4, Section 4.3.7.

6.3.8   *Microbial community structure analysis*

The raw sequences were processed using QIIME (v.1.7.0) bioinformatics pipeline using the default parameters described in a previous chapter (Chapter 4, Section 4.3.7). For determination of alpha diversity indices, an in-silico rarefaction of the sequences per sample was performed across all samples using the OTU table constructed at an early stage as an input to the QIIME pipeline. A minimum rarefaction depth of 10 in the series of depth, a stepwise increase of 1842 sequences, a total number of 10 replicates (multiple rarefactions) at each depth and a maximum rarefaction depth of 18,430 in the series of depth were used to perform the in-silico analysis. For the diversity within each sample (alpha diversity), the non-parametric species richness estimator Chao1 and the Faith's phylogenetic diversity (PD) index were determined according to the methods described by Chao (1984) and Faith (1992) respectively in QIIME. The Shannon's diversity index (H') was also determined for each sample as a measure of alpha diversity based on derivations made by Shannon and Weaver (1949).

6.3.9   *Statistical analysis*

Data analysis in this chapter was performed using the statistical software and methods described in Chapter 4, Section 4.3.8.

6.4 Results and Discussion

6.4.1 *Summary of chemical monitoring results from previous column studies (Bushnaf, K., 2014)*

The data used to discuss this section was provided from experiments conducted by Bushnaf (2014). It is included in this chapter to provide background chemical analysis information in order to have a better understanding of the microbiology discussion in the subsequent sections of this chapter.

Results from monitoring cumulative VPH and CO_2 emanation at the near zero concentration boundaries of each column over a period of 430 days are shown in Figure 6.2. A breakthrough of the VPHs at this side of the columns was used as an indication of incomplete attenuation of the pollutants while a breakthrough of CO_2 from this end of the column was considered as an indication of VPH attenuation arising from biodegradation by soil microorganisms. CO_2 fluxes were monitored for the first five days during the experiment and was found to be well below the levels observed after the NAPL sources were connected to the columns, suggesting that the observed CO_2 increase must have been mainly the result of biodegradation of the VPHs.

In the unamended soil and soil & BC columns, there was an initial increase in the emanation of VPH-C at the end of the columns from day 0 to about day 100 after which no further increase in the emanation of VPH-C was observed from around day 100 (Figure 6.2) whereas in the soil & AC column, the VPH-C emanation never rose above a minimum concentration throughout the duration of the experiment. In the soil & activated carbon column (soil & AC), cumulative VPH emanation at the near zero concentration boundary were much lower compared to the soil only and the soil & biochar columns. This can be explained by the higher sorption of VPHs in the soil & AC columns, which corresponds with a higher soil-water partition coefficient in the AC amended soils as determined during this study. With respect to CO_2 emanation fluxes, the soil & AC column initially had the lowest amounts of cumulative CO_2 during the first 200 days of the experiment after which there was a more steady increase in the flux of CO_2 to eventually overtake production levels in the soil and soil & biochar columns by day 430 (Figure 6.2).

By day 430, most constituents of the NAPL sources had been largely exhausted and was remaining mainly the least volatile compounds namely 1,2,4-TMB (0.2-5%), decane (11-31%) and dodecane (56-89%). The soil and activated carbon column had the lowest amount of VPHs remaining in the NAPL source which may imply that the readily volatile VPH constituents sorbed strongly to the activated carbon amended soil due to its higher sorption capacity compared to the soil and soil and biochar columns, thereby enhancing the volatilization of VPHs from the NAPL source. Extractible VPHs from within the columns

were the highest for the soil and activated carbon column and this was dominated by the compound isooctane, making up approximately 84% of the VPH extract from this column. Isooctane was reported as one of the most recalcitrant VPH compounds in a previously conducted soil column study (Hohener *et al.*, 2003).
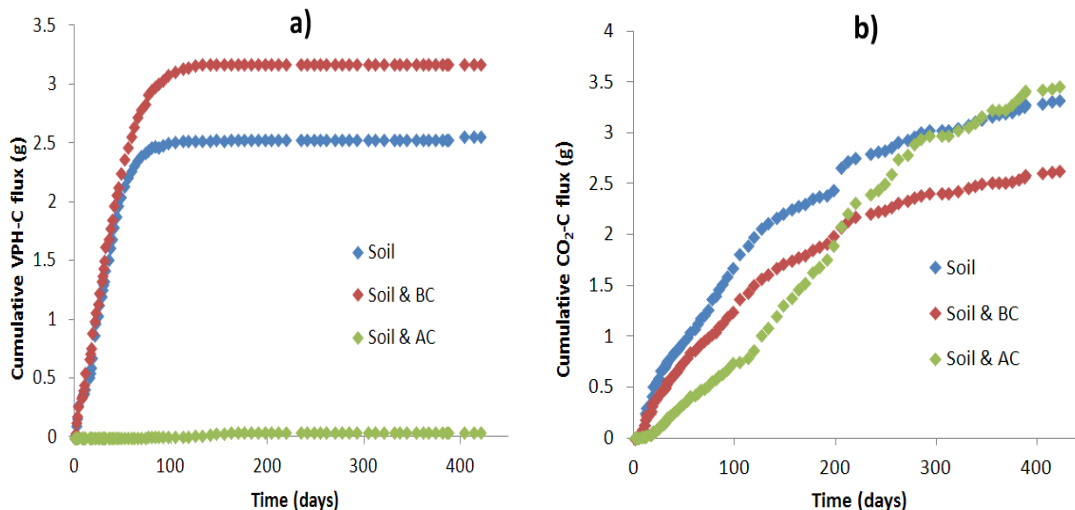
Figure 6.2. Cumulative diffusive VPH and CO_2 carbon flux between ports 6 and 7 (near the zero-concentration boundary side of the soil columns). The cumulative VPH-C flux is a measure for the amount of VPH pollution which has not been attenuated between the source and the measurement point, whereas the cumulative CO_2-C flux is an indicator for the extent of VPH pollution biodegradation within the columns.

A comparison of the amounts of readily available inorganic nutrients at the end of the experiment on day 430 reveals that there was a decrease in the amounts of extractible nutrients compared to the initial soil conditions in all soil systems (Table 6.1). The soil and activated carbon columns were the most depleted in nutrients in comparison with the other soil treatments. Also, ammonium-N, which is more readily assimilated than nitrate-N, was the most depleted inorganic nutrient (>90%) compared to the initial condition in all three columns. It is reasonable to conclude that the observed nutrient depletion in the columns at the end of the experiments can be accounted for by the corresponding growth in microbial biomass when compared with the soil systems at the start of the experiment (Figure 6.3).

Column	Ammonium μgNH_4^+-N g^{-1}	Nitrate μgNO_3^--N g^{-1}	Phosphate $\mu gH_2PO_4^-$-P g^{-1}	NAPLsource (g)	Retained as VPH in soil (g)
	Before column experiments				
Soil	2.9±0.2	8.2±2.0	0.8±0.1	-	-
Soil & 2%BC	5.8±0.5	5.5±0.6	0.2±0.0	-	-
Soil & 2%AC	6.1±1.2	6.5±1.0	0.3±0.0	-	-
	After column experiments				
Soil	0.2±0.0	5.8±1.5	0.4±0.3	1.15±0.09	0.00±0.00
Soil & 2%BC	0.05±0.01	1.8±0.6	0.1±0.0	1.36±0.10	0.01±0.01
Soil & 2%AC	0.05±0.01	0.86±1.30	0.15±0.03	0.65±0.03	0.40±0.05

Table 6.1. Water extractable inorganic soil nutrients and VPH carbon mass balance (in NAPL source and retained as VPH in soil) per gram of soil before and after column experiments on day 430. Initially, 12.5 g VPH-C was present in each source.

6.4.2 *Total cell counts*

Results from total bacterial cell counts are shown in Figure 6.3. There was an increase in total cell numbers in all three columns compared with the initial soil conditions at time zero (represented by purple coloured bars in Figure 6.3) which corresponded with a decrease in the readily available nutrients at the end of the 430 days experiment (Table 6.1). Biomass growth as indicated by cell carbon per column increased by a factor of 6.26, 1.95 and 4.17 in soil, soil & biochar and soil & activated carbon in comparison with the control soil at time zero. There was a statistically significant difference between the total cell numbers at time zero and day 430 for the unamended soil and soil & activated carbon columns ($p < 0.05$, One-way ANOVA; Tukey's test) but not for the soil & biochar column ($p > 0.05$, One-way ANOVA; Tukey's test). Average cell numbers per gram of soil on day 430 was comparable for soil ($1.71 \times 10^8 \pm 5.10 \times 10^7$), soil & biochar ($1.51 \times 10^8 \pm 4.64 \times 10^7$) and soil & activated carbon ($1.61 \times 10^8 \pm 3.16 \times 10^7$) and broadly consistent with the idea of a comparable soil biomass carrying capacity in gravelly sand with and without biochar and activated carbon amendment. Biomass growth have been demonstrated by cells in soil systems to which carbon sources were introduced in the form of volatile petroleum hydrocarbons (Elazhari-Ali *et al.*, 2013). With respect to column locations, there was no observed definite pattern in the variation of cell numbers and this may be due to experimental uncertainties.
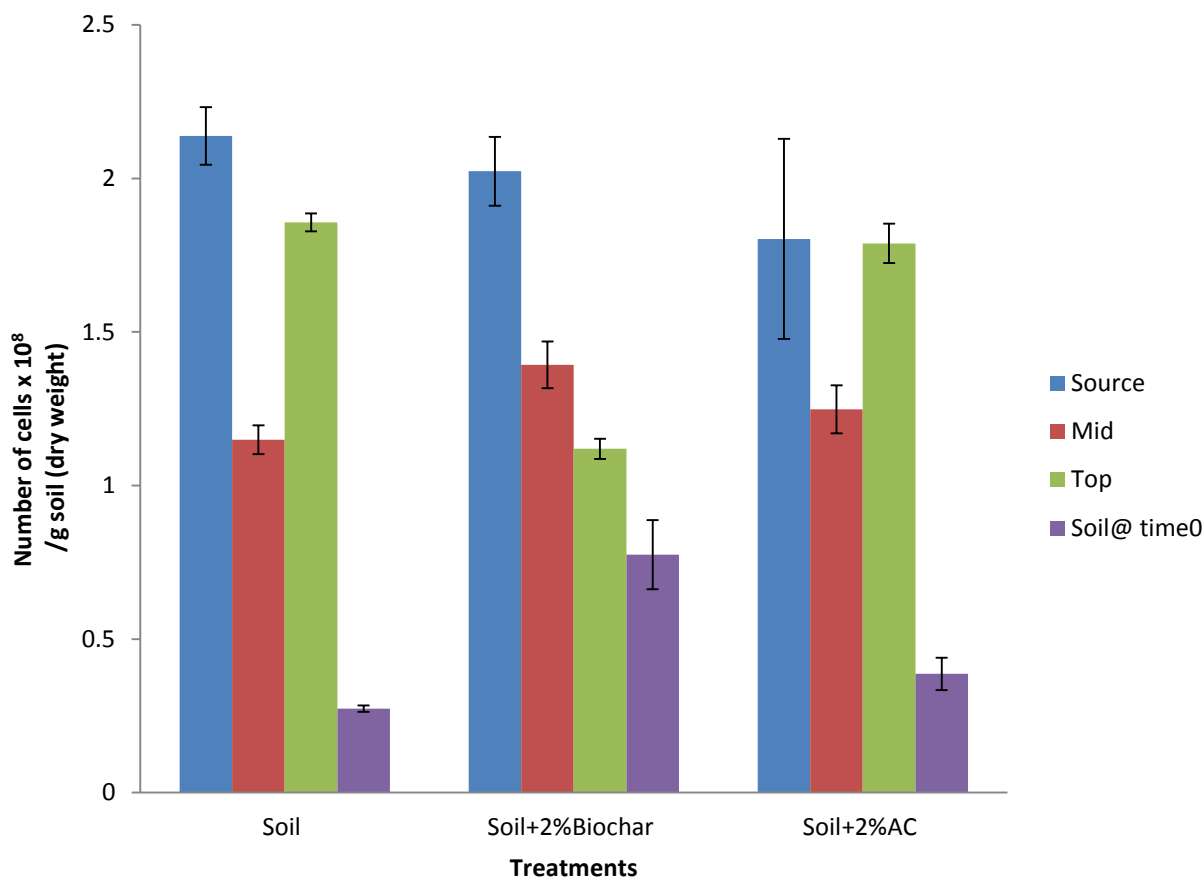
Figure 6.3. Total bacterial cell numbers per gram of soil (d.w.) determined for controls prior to experiments (purple bars) and for each section of the different amendments indicated by blue bars for the source section of the columns, red bars representing the mid- section of columns and green bars representing the atmosphere side of columns. Error bars represent ± 1 standard deviation from the mean of duplicate samples.

6.4.3 *Preliminary analysis of pyrosequencing dataset*

A total of 365,166 sequences passed the initial quality filtering step with a minimum read length of 200bp (average read length of 447 bp per read), representing approximately 60% of the original number of reads generated from the pyrosequencing process. Out of this number, 357,892 sequences were assigned to the bacterial domain representing 98.01% while 7086 sequences were assigned to the archaea domain representing 1.94%. 324,035 sequences were classified below the bacterial domain (91%) while 6921 sequences were classified below the archaea domain (98%). A total of 188 sequences did not fall into any domain. On the average, each sample contained 18,258 sequences (min: 4068/max: 24,176). A total of 12,265 OTUs were generated from the OTU picking step.

6.4.4 *Microbial diversity and species richness*

Microbial diversity and species richness index values are displayed in Table 6.2. Species richness as determined by the non-parametric richness estimator, Chao1, ranged from 4805.48 to 4774.34 in the control soils on day 0.

With respect to column locations, in the unamended soil column, samples from the column source section had the highest species richness value of 5520.24±812.31 OTUs when a comparison of averages is done followed by samples from the column near zero concentration boundary of the unamended soil (4313.17±75.91). In the biochar amended soil column, the atmosphere side of the column had a higher richness index of (4956.67±190.35) compared to the source section of the column (4418.94±19.90) while in the soil & AC column, samples from the near zero concentration boundary section of the column had a lower species richness estimate of 3797.69±2.32 compared to the source section of the soil & AC column (4453.58±125.59).

With respect to sorbent amendment, the unamended soil column had an average richness index (Chao1) value of 4916.70±841.16 while soils amended with 2% biochar had an average index value of 4687.80±329.54. For the soil and activated carbon column, the average species richness value was 4125.64±385. There was no statistically significant difference ($p > 0.05$, Tukey's HSD test) between species richness index (Chao1) of the control soil at time zero and the soils from the columns on day430 suggesting that exposure to VPHs in the long term did not negatively impact on the species richness of the initial soil condition. Neither location nor amendment was a significant factor in the determination of species richness based on the non-parametric estimator Chao1 ($p > 0.05$, Tukey's HSD test).

Average number of observed species for initial soil at time zero was 2689.75±24.11 OTUs. In the unamended soil column on day 430, average number of observed species in source section of the column was 3049.45±30.88 followed by the atmosphere side of the column (2326.35±70.22). In the soil & biochar column, average number of observed species for the source and atmosphere sides of the column were 2476.35±21.85 and 2620.50±26.16 respectively. The number of observed species were however lower for the soil & activated carbon columns source (2385.9±53.88) and atmosphere (2093.25±50.70) sides at the end of the experiment on day 430. Statistical analysis of the results showed that there was no significant difference between the number of observed species at time zero and on day 430 for the treated soil columns. With respect to sorbent amendment, the number of observed species in the unamended soil column on day 430 was 2687.90±453.77 OTUs while in the soil and biochar column on day 430, there were 2548.43±85.52 OTUs on the average. Species richness

as determined by the number of observed species revealed that there was an average of 2239.58±174.28 OTUs in the soil and AC column. Amending soil with 2% biochar or activated carbon did not have a significant effect on the species richness as determined by observed species richness estimator ($p > 0.05$, One-way ANOVA).

Microbial diversity within different soil samples were determined based on the Shannon's index of diversity. Results show that soil samples at time zero had an average index of 9.43±0.05. For the different soil treatments unamended soil column, the source side of the unamended soil column had a Shannon's index of diversity 9.72±0.19 followed by the atmosphere side of the unamended soil column (9.04±0.19). In the soil & biochar column, the source side of column had a higher diversity index of 9.22±0.04 compared to the atmosphere side of the soil & biochar column (9.21±0.11) while the soil & AC column source and atmosphere sides had average diversity index values of 8.97±0.06 and 8.48±0.14 respectively. With respect to sorbent amendment, the average Shannon's diversity index values for unamended soil, soil & biochar and soil & AC on day 430 were 9.38±0.42, 9.21±0.07 and 8.72±0.30 respectively. There was no statistically significant difference ($p > 0.05$, One Way ANOVA) between the diversity index of the treated soils (amendments) at the end of the 430 days experiment with the diversity in soil at time zero. Column location did not also have a significant effect on microbial diversity as in dicated by the Shannon's index of diversity ($p > 0.05$, One Way ANOVA). Sorbent amendment, however, had a significant effect on the Shannon's index of diversity between the unamended soil and the soil & AC treatments on day 430 ($p < 0.05$, Tukey's pair-wise comparison).

Amendment	Sample	Chao1	Observed spp.	Faith's PD	Shannon's index
NA	Soil_time0	4789.91±22.02	2689.75±24.11	221.76±0.31	9.43±0.05
Unamended	Soil_source	5520.24±812.31	3049.45±30.88	260.32±29.55	9.72±0.19
Unamended	Soil_atm	4313.17±75.91	2326.35±70.22	203.14±2.40	9.04±0.19
Biochar	Soil+2%BC_source	4418.94±19.90	2476.35±21.85	213.48±0.36	9.22±0.04
Biochar	Soil+2%BC_atm	4956.67±190.35	2620.50±26.16	229.17±1.02	9.21±0.11
Activated carbon	Soil+2%AC_source	4453.58±125.59	2385.9±53.88	203.35±2.34	8.97±0.06
Activated carbon	Soil+2%AC_atm	3797.69±2.32	2093.25±50.70	182.47±5.35	8.48±0.14

Table 6.2. Average species richness as assessed by the non-parametric estimator Chao1, and Observed number of species and bacterial diversity indices Faith's phylogenetic diversity and Shannon's index (H') determined for the source and near-zero concentration sections of the columns containing soil, soil & biochar and soil & AC. Diversity indices were determined at a fixed rarefaction depth of 16,588 sequences. Error ranges are calculated as standard deviation from the mean of duplicate samples.

Results of the Faith's phylogenetic diversity index for samples from the source and near zero concentration boundary sections of all three columns as determined in QIIME are displayed in Table 6.2. Average Faith's PD index for initial soil at time zero was 221.76±0.31. In the unamended soil columns at the end of the experiment on day 430, average PD index for the column source section was 260.32±29.55 and higher than for the atmosphere side of the column (203.14±2.40). For samples from the soil and biochar columns on day 430, average PD index for the source and atmosphere sides of the column were 213.48±0.36 and 229.17±1.02 respectively. Soil samples from the soil and activated carbon column atmosphere side had the lowest PD index value of 182.47±5.35 compared to the initial soil at time zero.

Average Faith's PD index values for the unamended soil, soil & biochar and the soil & AC columns were 231.73±37.19, 221.33±9.08 and 192.91±12.52 respectively. There was no statistically significant difference between the PD index for soils at time zero and soils from the treated columns on day 430. There was also no significant difference between soil, soil & biochar and soil & activated carbon columns with respect to their PD index values ($p > 0.05$ in all cases, One-way ANOVA).

Phylogenetic diversity is a measure of alpha diversity that takes into account the sum of the entire branch lengths in a phylogenetic tree leading to each taxa within a sample community. As a measure of diversity, it is not sensitive to changes in the number of a particular species in a dataset. In order words, addition of a new individual to the community does not affect the index value. It is however, sensitive to sequencing effort and increasing the sequencing depth of a given sample tends to affect the PD index of that community (Lozupone and Knight, 2007). This is consistent with findings from the current study in which phylogenetic diversity increases correspondingly with increase in sequencing depth of samples. Rarefaction curves of the PD index did not level off (Figure 6.4) indicating that the sequences were not exhaustively sampled and the introduction of a new sequence would have resulted in an increase in the average branch length of the tree of sequences leading from that OTU.

A comparison of the mean values for the species richness estimators (Chao1 and Observed spp) and bacterial diversity (Faith's PD and Shannon's diversity index) of the different column locations showed that location was a significant factor in determining bacterial diversity ($p < 0.05$, 2 Way-ANOVA) but was not a statistically significant factor in the
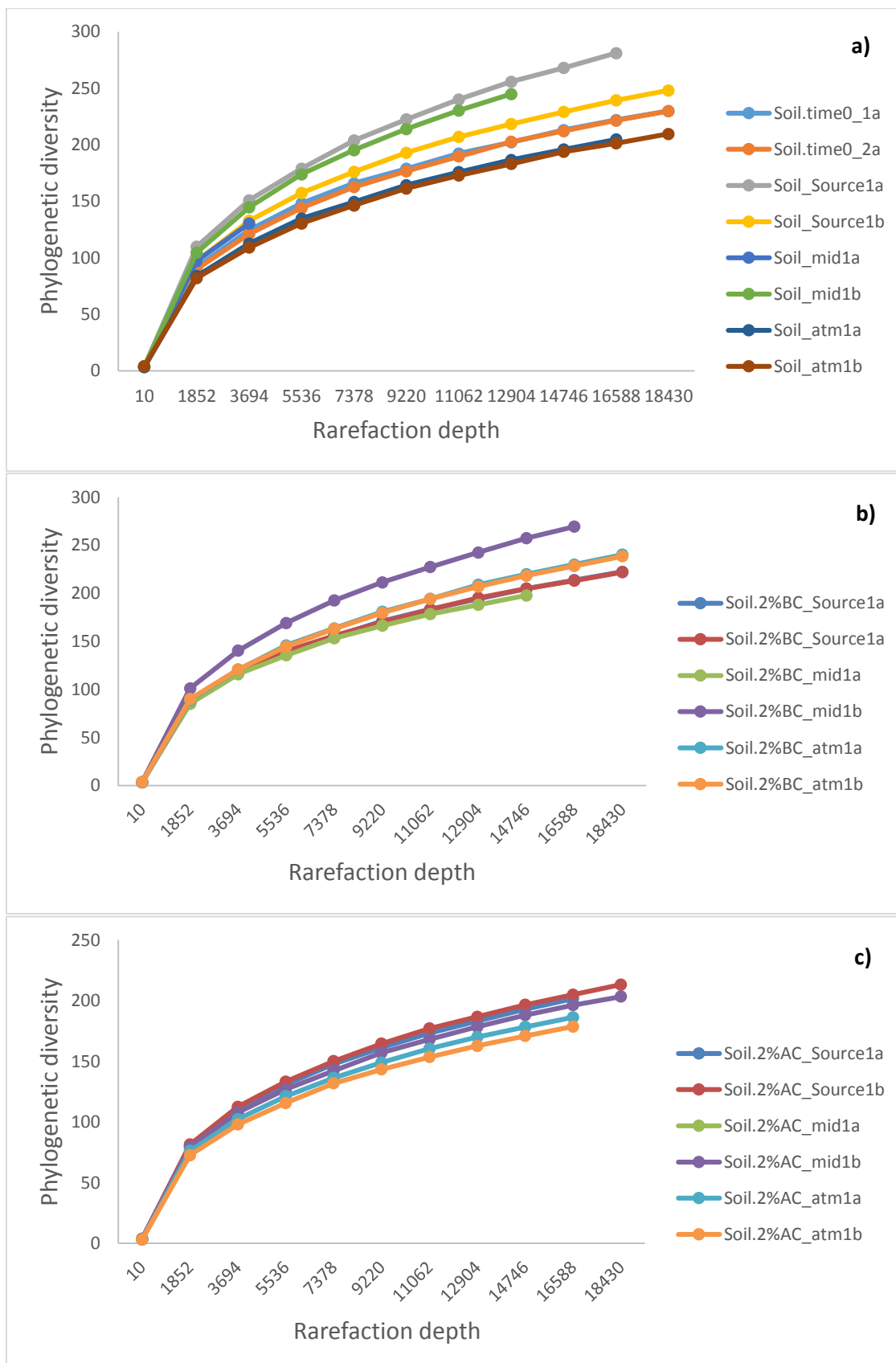
*Figure 6.4. Rarefaction curves indicating the effects of sequencing efforts (depth) on the phylogenetic diversity of **a)** unamended soil and control soils at time t = 0 **b)** soils amended with 2%BC and **c)** soils amended with 2%AC.*
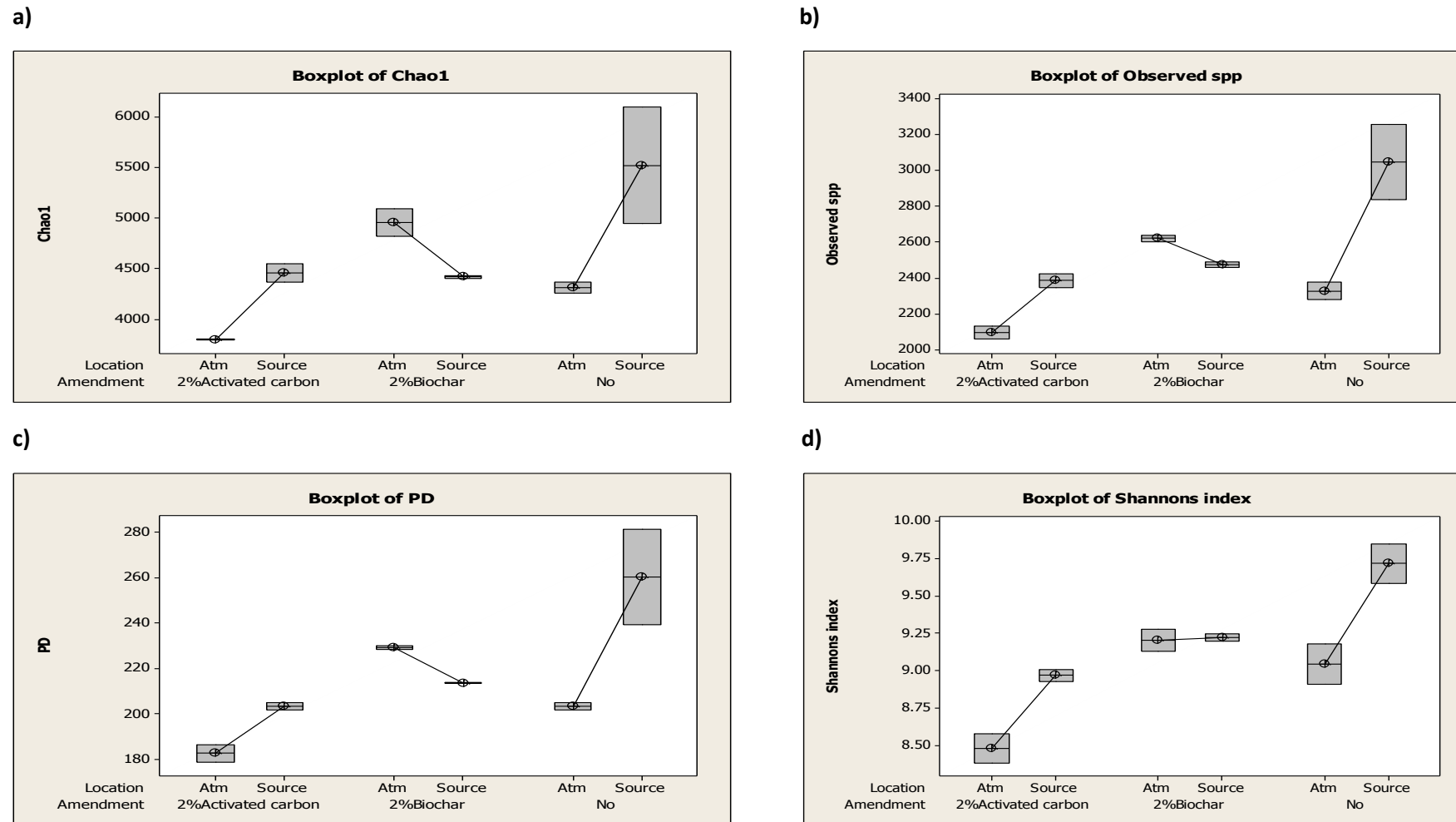
Figure 6.5. Boxplots of bacterial diversity and species richness indicating data spread within replicates, comparing the mean values of replicates from each treatment and showing the effects of interaction of column locations and sorbent amendment on the diversity indices.

estimation of species richness as predicted by the non-parametric estimator Chao1 (2 Way-ANOVA, $p > 0.05$). There was also a significant effect of the interaction of the factors, amendment and location along the columns on the species richness estimators and diversity indices (2 Way-ANOVA, $p < 0.05$).

6.4.5 *Taxa distribution across different samples*

A total of 50 phyla were identified from the classified sequences and 2 unidentified phyla within the bacterial domain. In the archaea domain, there were 2 identified phyla and 1 unidentified phylum. The dominant bacterial phyla ($> 1\%$ of total relative abundance) were the *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Gemmatimonadetes*, *Nitrospirae*, *Planctomycetes*, *Verrucomicrobia* and the *Proteobacterial* classes *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, *Gammaproteobacteria* representing 5.16, 8.93, 5.75, 5.70, 4.39, 4.16, 1.92, 7.51, 1.89, 14.85, 11.59, 9.72, and 12.80% respectively of the abundance across all samples. These phyla have been regularly identified in soil microbial communities from previous studies (Janssen, 2006; Roesch *et al.*, 2007). In the archaea domain, the most abundant phyla was the *Crenarchaeota* representing 1.64% of total relative abundance across all samples. The rare phyla ($< 1.0\%$) were the *Armatimonadetes*, *Chlorobi*, *Cyanobacteria*, *Elusimicrobia*, TM6, and WS3 representing 0.27, 0.37, 0.10, 0.14, 0.21 and 0.89 % of the total abundance respectively across all samples and within the archaea domain, *Euryarchaeota* representing 0.31% of the total abundance.

In the control soil, at time zero, the most dominant *Proteobacterial* OTU at the class level was *Alphaproteobacteria* representing 13.15%. The second most dominant taxa was *Gammaproteobacteria* constituting 11.60% and *Deltaproteobacteria* making up 8.68% of the total abundance. In the unamended soil column, the most abundant OTU was the *Alphaproteobacteria* representing 15.07% of total OTU abundance on day 430. In the soil & biochar column and soil & activated carbon, the most abundant OTU representing 15.61% and 14.42% respectively of the total OTU abundance was the *Alphaproteobacteria* in the respective columns.

With respect to column location in the unamended soils, the most dominant taxa was *Alphaproteobacteria* from the near zero concentration boundary of the columns representing 20.27% of the relative abundances from this column. For soils amended with 2% biochar and 2% activated carbon, the most dominant taxa was *Alphaproteobacteria* from the column source (16.43%) and column near zero concentration boundary (16.57%) sections respectively. The most abundant taxa across all samples investigated during this study was the

Proteobacterial class – *Alphaproteobacteria* constituting 14.85% followed by *Gammaproteobacteria* which accounted for 12.81% of the total relative abundance across all samples.

The most dominant OTUs with respect to amendment for the unamended soil and the soil & biochar columns was the *Alphaproteobacteria* representing 15.07±4.50 and 15.61±0.80% of the total OTU abundance while in the soil & AC column, the most dominant OTU was the *Betaproteobacteria* representing 15.50± 0.87 of the total OTU abundance in the dataset.

There was a variation in the relative abundances of the dominant phyla between different amendments. *Acidobacteria* showed a significantly higher relative abundance in the unamended soils than biochar amended soils while *Actinobacteria* showed the opposite pattern. *Euryarchaeota*, *Actinobacteria*, *Armatimonadetes*, *Bacteriodetes*, and *Gemmatimonadetes* were significantly higher in relative abundance in biochar amended soils compared to the activated carbon amended soils. *Acidobateria*, *Armatimonadetes*, *Bacteriodetes*, *Chloroflexi*, *Cyanobacteria*, *Elusimicrobia*, *Firmicutes,* and *Fusobacteria* had significantly higher relative abundance in the unamended soils compared to the activated carbon amended soil while *Actinobacteria* showed the opposite pattern (p < 0.05 in all cases, 2 sample t-tests).
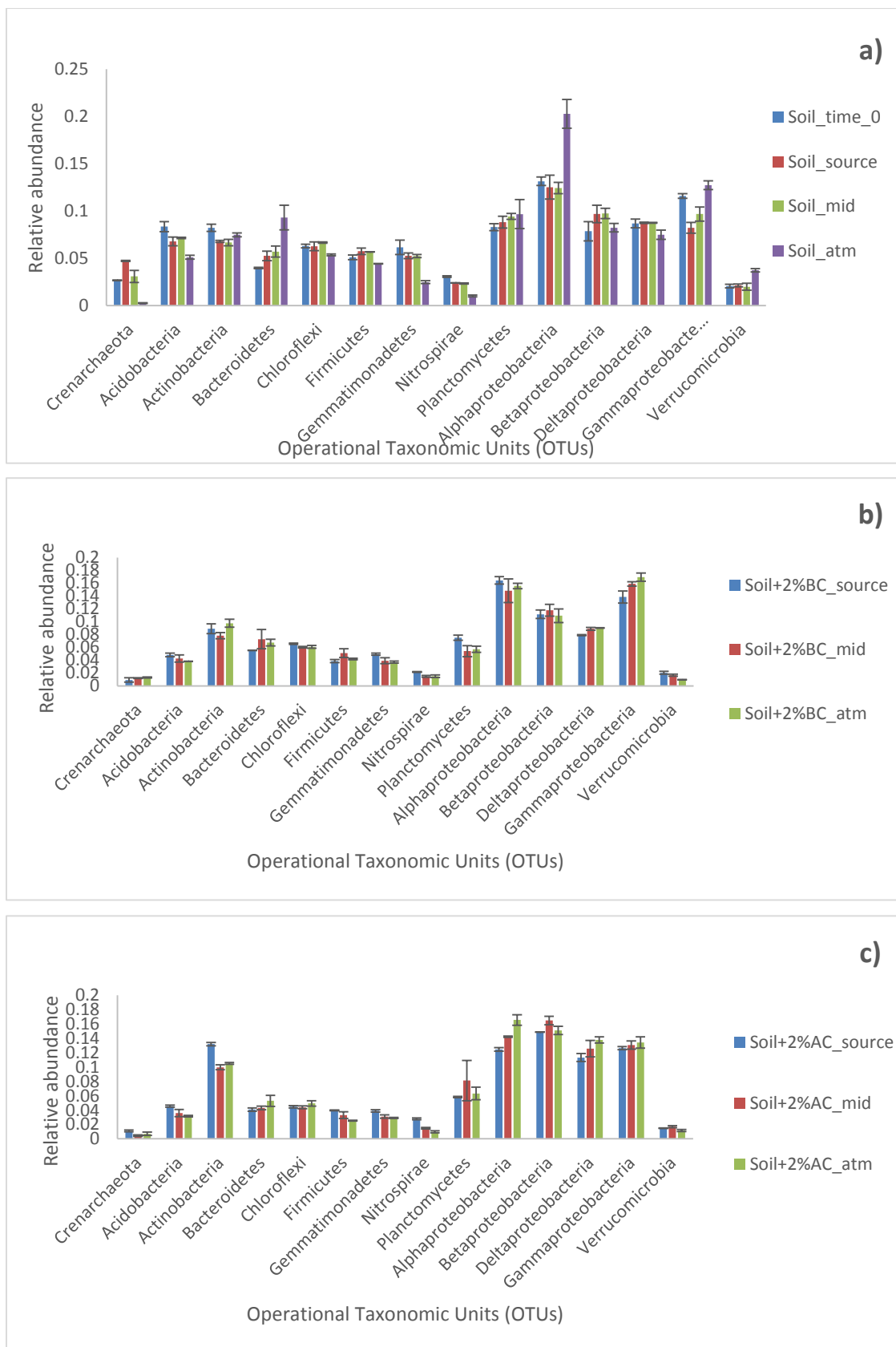
*Figure 6.6. Relative abundances of dominant phyla and proteobacterial classes from **a)** soil **b)** soil & biochar and **c)** soil & AC libraries in which 16S rDNA sequences were classified based on nearest neighbour in Greengenes database (greengenes.secondgenome.com).*

6.4.6 *Relationship between microbial communities in different samples*

A mapping of the average Bray Curtis dissimilarity matrix (log transformed data) unto a 2D ordination space shows a pattern of the microbial community response to the environmental factors under investigation (Figure 6.7). The pattern of grouping of the samples (78% similarity) reveal a pronounced response of microbial communities to the amendments with the biochar amended soil samples clustering more closely to each other than to the activated carbon amended soils and vice versa. The unamended soils clustered more closely with the control samples (initial soil at time 0) with the exception of samples from the near zero concentration boundary of the unamended soils. Clustering of all samples was performed at a 60% similarity.
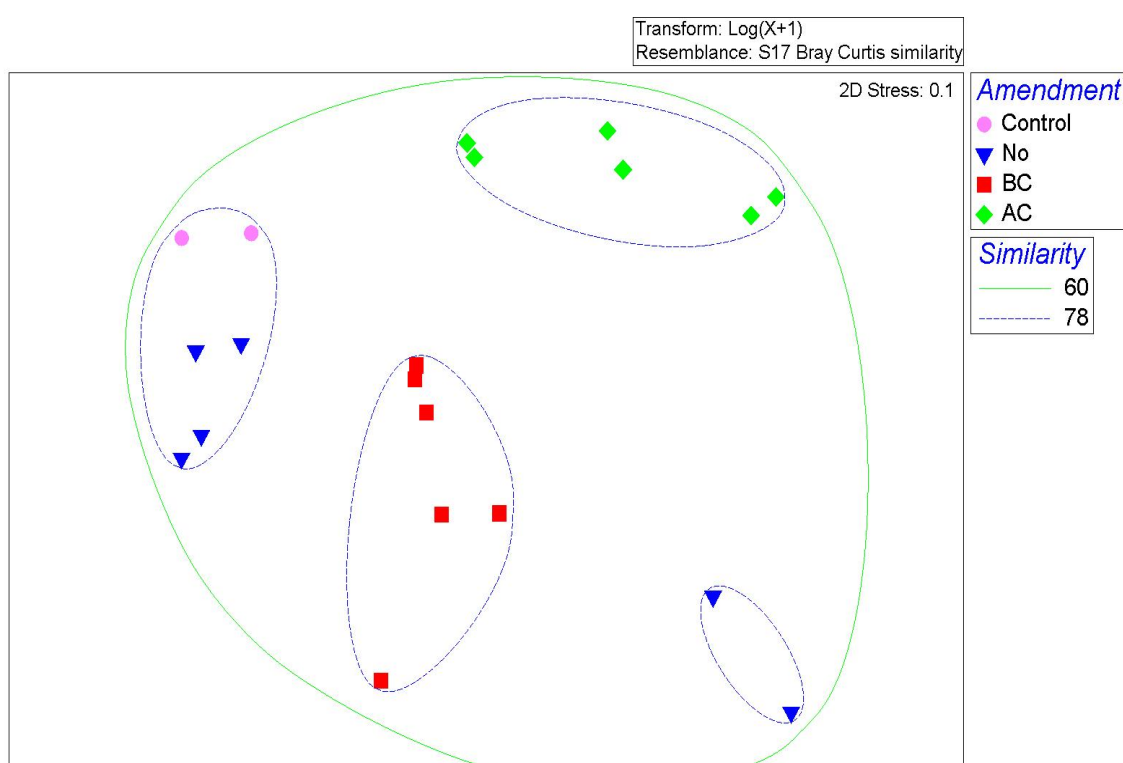
Figure 6.7. Multidimensional Scaling plot of 16S rRNA pyrosequence libraries obtained from control soil samples, unamended soil, soil & BC and soil & AC at day 430. Averaged Bray Curtis distance on log (x+1) transformed data at the OTU level 3 (class level of taxonomy) is displayed on the plot.

An analysis of similarity (ANOSIM) was carried out to investigate the statistically significant factors that contributed to the shaping of the microbial communities. At higher levels of taxonomy (class level), the results showed that neither location along the columns nor time had any significant effect on the microbial communities within the different samples. There was nevertheless a detectable effect of sorbent amendment on the

relative abundance distribution of operational taxonomic units between the treatments (ANOSIM test for difference between amendments, Global R= 0.631, p < 0.05).

A further analysis of sample similarities was performed using a principal components analysis (PCA) to investigate the effects of the relative abundances of the OTUs on similarities between different samples. At the species level, a comparison of the column locations with respect to relative abundances of the most abundant OTUs (> 1.0% of total relative abundance) revealed distinct patterns in which samples were partitioned into both positive and negative axis of the 1st and 2nd principal components (accounting for 74.87% variability of all samples) (Figure 6.8).
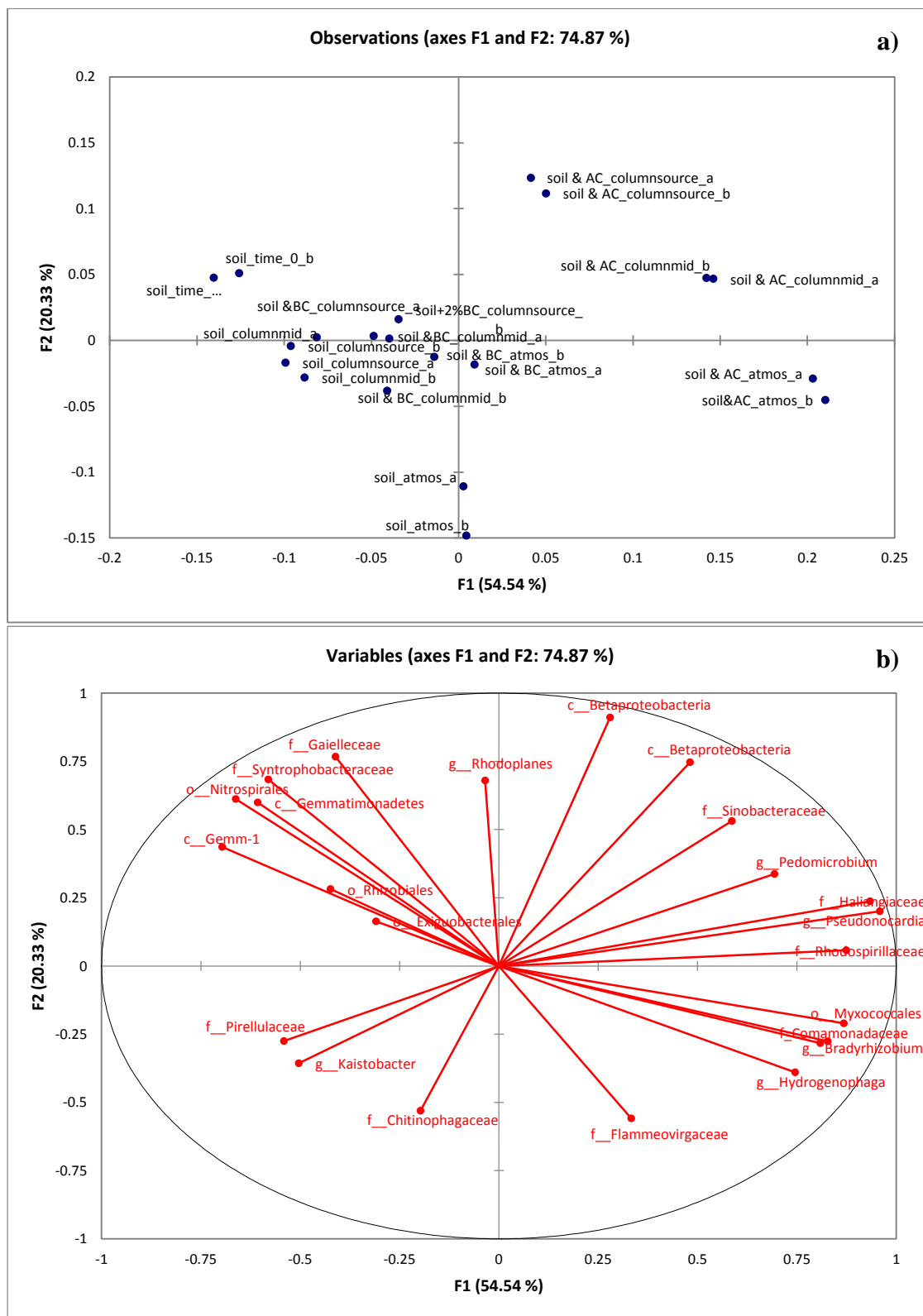
*Figure 6.8. Principal Components Analysis of microbial communities as affected by **a)** sorbent amendment and column locations based on **b)** the relative abundances of dominant OTUs (>1.0% of total sequences) at the species level (square root transformed data). The direction of vectors indicate the direction of change of each variable (OTU relative abundance) so that the observation samples clustered together have similar microbial communities.*

The samples partitioned according to column locations suggesting a significant relationship between microbial communities from the same locations than from other locations. In terms of percentage contribution of each OTU relative abundance to the principal components, the genus *Pseudonocardia* had the highest contribution of 30.19% to the first principal component followed by an unidentified genus representing 16.18% of the first principal component. The genera *Bradyrhizobium*, *Paedomicrobium*, *Rhodoplanes* and *Hydrogenophaga* represented 3.18, 1.41, 0.002 and 12.52% of the first PC and 0.95, 0.89, 2.11 and 9.15% of the second PC respectively. A principal components analysis of the most abundant OTUs (variables) also showed a pattern that corresponds to the pattern on the PCA of the samples (observations). Based upon a 2-sample t-Test, the genera *Pseudonocardia* and *Bradyrhizobium* were more abundant in the activated carbon amended soils than in the biochar amended or unamended soils (p < 0.05). The genus *Bradyrhizobium* is a nitrogen – fixing root nodule bacteria and was among several genera isolated from a biologically active granular activated carbon filter following filtration of disinfected drinking water in studies conducted by Niemi *et al.* (2009).

6.4.7   *Microbial functions, petroleum hydrocarbon degradation and nitrogen cycling*

At the order level of taxonomy, the most abundant OTU in the control soil at time zero was the *Xanthomonadales* with a percentage abundance of 9.59% followed by the *Rhizobiales* with an abundance of 7.27%. The most abundant OTU in the source side and middle of the unamended soil column was the *Rhizobiales* representing 7.06 and 7.39% respectively of the total abundance in the soil column while at the soil-atmosphere boundary of the column, the most dominant OTU was the *Xanthomonadales* representing 9.42% of total abundance. In the unamended soil column, most of sequences belonging to the *Alphaproteobacteria* were affiliated to *Rhizobiales*. Members of this order have been reported to possess the potential to degrade toluene (Zengler *et al.*, 1999), straight-chain alkanes (Schleheck *et al.*, 2004) and gasoline (Prantera *et al.*, 2002).

In the biochar amended soil column, the most dominant OTU at the order level from the source, middle and atmospheric sides of the column was the *Xanthomonadales* representing 9.97%, 9.03%, and 9.34% respectively.

In the activated carbon amended soil column, the most abundant OTU from the source and middle sides of the column was the *Xanthomonadales* representing 10.07% and 10.22% of the total OTU abundance in this column while in the atmosphere side of the column, the dominant OTU was the *Rhizobiales* representing 10.34% of the total abundance. In the biochar and activated carbon amended soil columns, the dominant order within the

Gammaproteobacteria was the *Xanthomonadales* which appear to play a significant role in the biodegradation of petroleum hydrocarbons particularly of linear alkanes and aromatic HC. They have been reported to thrive on hexane (Friedrich and Lipski, 2008), toluene (Juteau *et al.*, 1999; Kim *et al.*, 2007b), alkanes (Yutse *et al.*, 2000; Palleroni *et al.*, 2004) and polyaromatic hydrocarbons (Hamann *et al.*, 1999; Juhasz *et al.*, 2000).

At the genus level, an unidentified OTU belonging to the *Gammaproteobacteria* had the highest total relative abundance of 5.08% across all samples. Correspondingly, members of the dominant *Proteobacterial* subclasses - *Alphaproteobacteria* and *Gammaproteobacteria* were reported to be key players in the biodegradation of petroleum hydrocarbons in a meta-analysis of 16S rDNA gene libraries obtained from the oiled Pensacola beach samples of the Deep-water Horizon spill in the Gulf of Mexico (Kostka *et al.*, 2011). Both classes are predominantly made up of Gram-negative organisms and have been reported to contain hydrocarbon-degrading bacterial genera (Kostka *et al.*, 2011).

A comparison of the relative abundances of the operational taxonomic units at the genus level revealed that the factors under consideration (column location and amendment) had a significant effect on the relative abundances of the OTUs. With respect to location along column lengths, the genera *Sphingobium, Sphingopyxis, Norcadioides, Pseudonocardia*, and *Methylibium* were relatively higher ($p < 0.05$, 2 sample t-test) at the column source (across all three columns) than they were at the near zero concentration boundary side of the columns whereas *Polaromonas* and *Alkanindiges* showed the opposite pattern (Table 6.3). *Sphingomonads* are a group of Gram-negative obligate aerobes that are reported to exist in a wide range of different environments and have been shown to degrade mono- and particularly poly-aromatic hydrocarbon compounds (Kawasaki and Kertesz, 2012). The ability to degrade hexane by a *Sphingobium* species has also been demonstrated (Liang and Lloyd-Jones, 2010). *Norcadioides*, a Gram-positive bacterial genus is reported to

contain two distinct monooxygenase genes required for the oxidation of alkanes and genus members have been shown to be involved in decane degradation (Hamamura *et al.*, 2001). Members of the genus *Pseudonocardia* have a high G+C content according to documented reports and have also been implicated with the degradation of the aromatics - toluene, benzene and 1,4-dioxane (Juteau *et al.*, 1999). Other reports indicate the potential of members to degrade aliphatic hydrocarbons (Balows *et al.*, 1992). *Polaromonas* is an oligotrophic group, moderately psychrophilic and slow growing. Members of genus have been reported to grow on the n-alkanes heptane and octane. There are also evidences to show their ability to metabolise the aromatic HC – toluene from a microcosm experiment (Sun *et al.*, 2010). *Alkanindiges* are aerobic cocci that thrive on straight-chain aliphatic hydrocarbons such as hexadecane and heptadecane although growth is not supported by short-chain compounds ($<C_{15}$) with decane as an exception according to documented reports (Ron and Rosenberg, 2010). Amending the soils with 2% biochar or activated carbon had a significant effect on the relative abundances of the operational taxonomic units at the genus level ($p < 0.05$; t tests). The genera *Pseudonocardia* and *Streptomyces* differed significantly in relative abundance in the following order (Soil < BC < AC) (Table 6.3) while the genera *Nitrosopumilus* and *Clostridium* showed the reverse pattern (Soil > BC > AC) in their relative abundances.

With regards to column locations, the VPH concentrations were expected to be higher at the side of the column nearer the NAPL source for soil, soil & biochar and soil & AC. It was therefore expected that a relatively higher abundance of VPH degraders would be detected at this side of the columns. This coincided with the results from this study in which most VPH degraders were significantly more dominant at the source side of all three columns than at the atmosphere boundaries of the respective columns. From the chemical analysis results (Table 6.1), the soil and AC column had the highest amount of VPH residual which may account for the relatively high amount of cumulative CO_2 in this column. Concurrently, most of the measured VPH residuals might have been strongly sorbed to the AC particles, thus rendering them unavailable to microorganisms for biodegradation. Nevertheless, there was a preponderance of petroleum hydrocarbon degrading bacterial communities in the AC amended soil at the end of the experiment (Table 6.3).

A summary of OTU absolute abundance increase or decrease relative to controls (soil at time zero) are displayed on table 9.17 & 9.18 (Appendix B). The *Nocardioides* grew in absolute abundance by upto an order of magnitude in all column locations (soil, soil & BC and soil &AC) following exposure to VPHs and after 430 days compared to the initial condition

Genus	Average relative abundances (location effects)		Average relative abundances (amendment effects)			Petroleum hydrocarbon degradation potential of genus members
	Source	Atmo	Soil	2% BC	2% AC	
Nitrosopumilus	-	-	9.01E-03	2.24E-03	4.21E-04	Naphthalene, xylene, toluene,
Sphingomonads - Sphingomonas - Sphingobium - Sphingopyxis	- 1.2E-02 1.4E-03	- 4.2E-05 1.3E-04	9.93E-05 8.83E-03 3.97E-04	4.23E-04 1.71E-03 6.70E-04	1.59E-04 1.97E-03 7.51E-04	BTEX, naphthalene, anthracene, phenyl Ability to degrade hexane
Nocardioides	4.4E-03	1.9E-03	-	-	-	Alkanes
Pseudonocardia	1.9E-02	1.1E-02	4.48E-03	5.39E-03	3.31E-02	Toluene, benzene Aliphatic hydrocarbons
Streptomyces	-	-	2.39E-03	3.63E-03	4.87E-03	Alkanes, PAHs, benzene, xylene, cyclohexane,
Flavobacterium	-	-	6.08E-04	1.04E-03	3.91E-04	Toluene, xylene, benzene, hexane, crude oil, gasoline, kerosene
Paenibacillus	-	-	2.75E-03	2.23E-03	1.66E-03	*m*-Xylene
Methylibium	5.3E-03	3.1E-03	-	-	-	Toluene, benzene, ethylbenzene
Clostridium	-	-	5.09E-03	4.95E-03	2.38E-03	Toluene
Polaromonas	2.7E-03	5.0E-03	-	-	-	*n*-alkanes, heptanes, octane, toluene
Alkanindiges	2.4E-05	1.2E-04	-	-	-	Decane, hexadecane and heptadecane
Pseudoxanthomonas	-	-	4.06E-03	2.30E-03	3.19E-03	BTEX (benzene, toluene, ethylbenzene, *o*-, *m*-, and *p*- xylene) compounds.

Table 6.3. Summary of statistically significant treatment effects (location source vs. atmospheric boundary side and/or sorbent amendment, t-test, two tailed, p < 0.05) for OTU identified at the genus level, where members of the genus reportedly degrade petroleum hydrocarbons.

(Table 9.17). *Sphingomonas* showed an increase in absolute abundance compared to soil on day 0 by at least one order of magnitude in all column locations and amendments with the exception of the soil & AC column (atmosphere side) in which no growth of the VPH degrading biomass was observed (Table 9.17). *Pseudoxanthomonas* also demonstrated growth in absolute abundance in all soil columns (locations and amendments) by at least an order of magnitude. On the other hand, the genera *Pseudonocardia* and *Flavobacterium* only increased by 3 orders of magnitude in the soil & BC columns atmosphere and mid locations respectively but did not show any growth in the other column locations (for sorbent amended and unamended soil).

In addition to the nitrifying bacteria *Nitrosomonas* and *Nitrobacter*, there are confirmed reports that the *Nitrosopumilus* genus within the Archaea domain also contain the ammonia monooxygenase (**AMO)** genes (Könneke *et al.*, 2005). The *Nitrosopumilus* are a group of Gram-negative, chemolithotrophic organisms that are mesophilic in nature (Yagi *et al.*, 2010). Results from the current study revealed a significant effect ($p < 0.05$, 2 sample t-test) of sorbent amendment on the relative abundance of this genus (Table 6.3). Table 6.1 shows measurements of the inorganic nitrogen contents of the different soil systems in the forms of NH_4^+ and NO_3^- as measured before and after the column experiments. Results from these experiments showed a reduction in the amounts of inorganic nitrogen (NH_4^+) at the end of the experiment (at day 430) in the following order: Soil < Soil & BC < Soil & AC indicative of sorption effects in the amended soil systems. Correspondingly, the ammonia oxidising archaea (AOA) were significantly higher in relative abundance in the initial unamended soil (time zero) as well as the unamended soil (day 430) compared to soils amended with 2% biochar or activated carbon on day 430 (Figure 6.9a).

A close look at the absolute abundances (Figure 6.9b) of the members of the archeae domain reveals that there was significant growth in the abundance of the species on day 430 especially in the soil only column compared to the soil at time 0. This may imply that while amending soil with biochar or AC hindered the growth of the organisms in comparison with unamended soil due to sorption of inorganic nutrients N & P, exposing the soil systems to the VPH mixture also enhanced their growth especially in the unamended soil column.
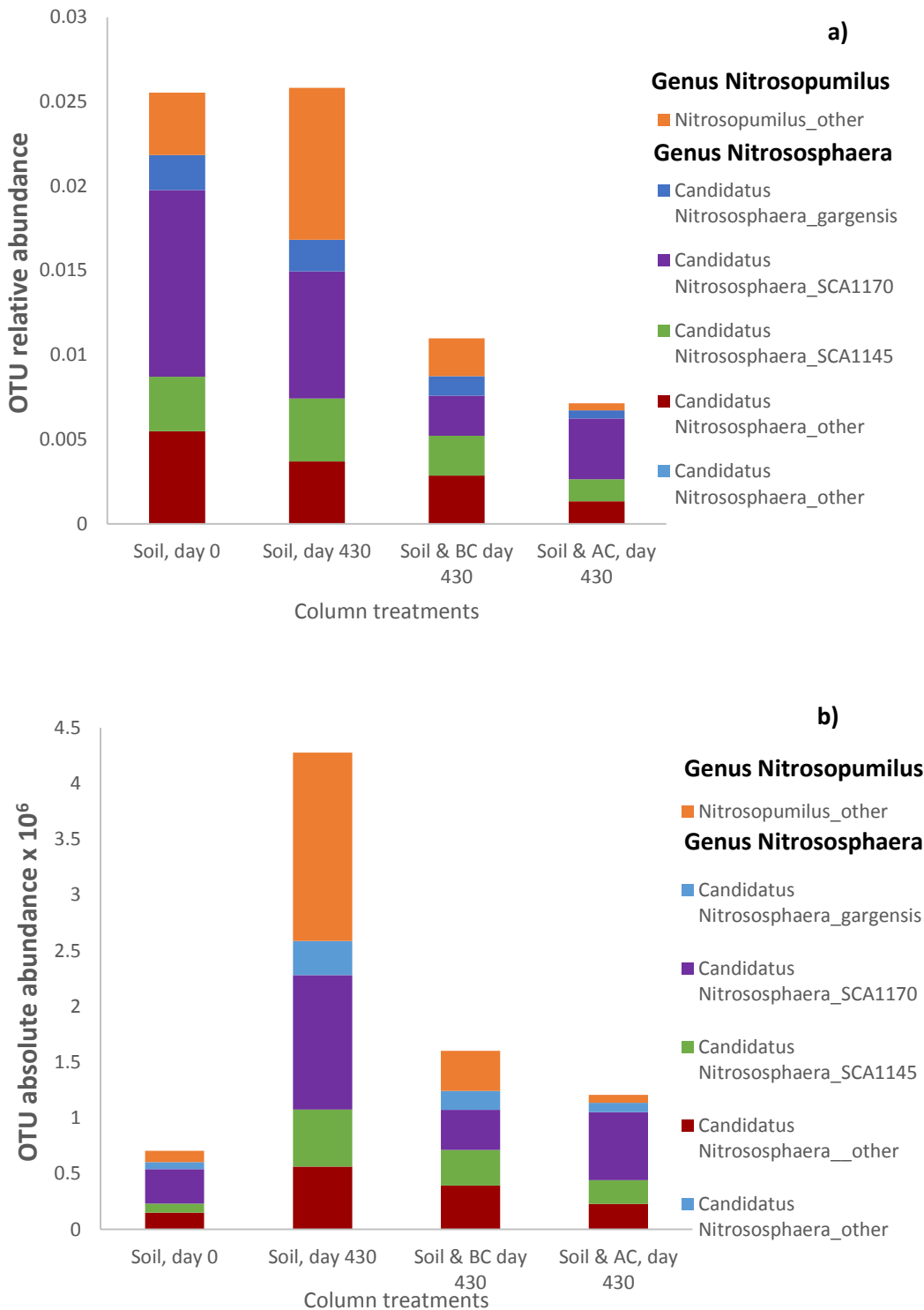
*Figure 6.9. Biochar (BC) and activated carbon (AC) effects on the **a)** relative abundances and **b)** absolute abundances of ammonia-oxidizing archaea (AOA) in pyrosequencing generated 16S sequence libraries of column studies, average of two replicates for soil, day 0, and six replicates for the other samples. AOA were much more abundant in the 16S sequence libraries than ammonia oxidizing bacteria (i.e. total relative abundance for all bacteria from the order of Nitrosomonadales was < 0.0001 in all soils).*

A ranking of operational taxonomic units (OTUs) at the species level of taxonomy in the control soil on day zero (Soil d0) based on their abundances relative to the total OTU abundance in the overall dataset and their increase or decrease with respect to sorbent amendment or column locations is displayed in the table below (Table 6.4). The results reveal that species within the genus *Nitrosopumilus*, increased in their relative abundance ranking by 16 folds at the source side of the unamended soil column in comparison with the initial soil condition but decreased by up to 4 folds in the biochar or AC amended soil columns. This observation may be due to inorganic nutrient availability (ammonia, nitrates and phosphates) in the unamended soil column compared to the sorbent amended soil columns as discussed above. Bacterial species within the *Norcadioidaceae* family increased by up to 8 folds in the AC amended soil and 2 folds increase in the unamended and the biochar amended soils respectively. A *Pseudonocardia* species increased by at least 2, 4 and 32 folds in relative abundance ranking in the unamended soil, biochar amended and AC amended soils respectively. Members of this genus were previously associated with the degradation of aromatic and aliphatic hydrocarbons (Juteau *et al.*, 1999). A relatively high cumulative CO_2 production in the AC amended soil column (Figure 6.2) may also be attributed to the abundance of this OTU compared to the other soil treatments as they have been reported to demonstrate potential to degrade different classes of petroleum hydrocarbons (Table 6.3). Within the order *Bacteroidales*, an opposite pattern to the *Pseudonocardia* species is observed in which at least 8 folds increase in relative abundance ranking of OTU and up to 32 folds increases in the unamended soil and the biochar amended soils was observed and a maximum 2-folds increase in the AC amended soil. *Chloroflexi* species increased by minimum 4-folds in the soil and soil & biochar columns and by 8 folds in the soil & AC column in their relative abundance ranking. Species within the family *Comamonadaceae* and the genus *Hydrogenophaga* also increased in their relative abundance ranking by a minimum of 2 folds in all the column locations being considered during this study indicating a positive response to the exposure of the VPHs regardless of locations (and sorbent amendment) of the columns. Members of the *Comamonadaceae* family were reported to harbour aerobic toluene degraders particularly within the genus *Methylibium* (Nakatsu *et al.*, 2006). Other species within the *Chromatiaceae* increased by maximum of 16 folds in the unamended soil and the soil & biochar columns but increased by only 2 folds in the AC amended soils while within the *Pseudomonadaceae* family, there was also an increase in species relative abundance ranking a by maximum of 8 and 32 folds in the unamended soil and the biochar amended soils respectively and a 2 folds decrease in relative abundance ranking in the AC amended soil.

Taxon (highest level classification)	Relative abundance rank increase for different treatments compared to the soil only control + 2-fold, ++ 4-fold, +++ 8-fold, ++++ 16-fold, +++++ 32-fold, ++++++ 64-fold or greater, equivalent decrease for minus signs									
	Soil d0 Rank	Soil+VPHs d430 source	Soil+VPHs d430 middle	Soil+VPHs d430 atmos	Soil+BC+VPHs d430 source	Soil+BC+VPHs d430 middle	Soil+BC+VPHs d430 atmos	Soil+AC+VPHs d430 source	Soil+AC+VPHs d430 middle	Soil+AC+VPHs d430 atmos
g__Nitrosopumilus	72	++++		---			-	-	--	--
g__Microbacterium	458			+++	+		+			
f__Nocardioidaceae	94			+			+	+++		+
g__Aeromicrobium	495			+	+	+	+		+	+++
g__Pseudonocardia	204	+	+	++	++	++	+++	+++++	+++++	++++++
o__Bacteroidales	629	+++++	+++	+++	+++	++++++	+++++	+	+	
f__Chitinophagaceae	86			++++		+				+
f__Flammeovirgaceae	9			+++	+	++	+	+		+
c__Chloroflexi	458	++	++	++	++	++	++	+++	+++	+++
f__Phyllobacteriaceae	131			+++			+		+	++
g__Oleomonas	629			+++++						+
g__Sphingobium	80	---	--	++++	---	---	+	---	---	+
f__Comamonadaceae	79	++	++	+++	+	++	++	++	+++	+++
g__Hydrogenophaga	116	+	++	++++	++++	++++	+++++	++	+++	+++++
o__Myxococcales	17			+++	-	-			++	++++
f__Haliangiaceae	36				+	+	++	++	++	+++
g__Nannocystis	420	+	+		-	-	-	++++	+++	++
f__Alteromonadaceae	629	+	+		+	++	+++		++	+
g__HB2-32-21	272	++	++++	++	+++	++++	++++	+	++++	+++++
f__Chromatiaceae	629	++++	+++	++	++	++++	++++	+		
o__PYR10d3	181			+++		+	+++++			
f__Pseudomonadaceae	166	+	+++	+	+++	+++++	++++	-	-	
f__Sinobacteraceae	105			++++	+	+		+	++	+
g__Pseudoxanthomonas	235			+++	+	+	++	+	++	++

Table 6.4. Summary of taxons which showed a minimum 8-fold increase in their relative abundance rank in one of the column study treatments.

A species within the genus *Pseudoxanthomonas* increased in their relative abundance ranking by 8 folds in the unamended soil and by a maximum of 4 folds in the biochar and AC amended soils.

Results from the species level correspond with the variation in the OTU relative abundances at the genus level (Table 6.4). Although most of the species that showed an increase in their relative abundances compared to the day zero (Soil d0) soil ranking are unidentified, the results reveal a pattern in which the dominant OTUs at the species level of taxonomy are linked to the dominant OTUs at the genus level.

6.5 Conclusions

A long-term column study was conducted to investigate the effects of sorption and biodegradation on the attenuation of volatile petroleum hydrocarbons in sand. Results from microbiological analysis shows that bacterial communities responded to the factors (i.e. time, location and amendment) under investigation.

A ranking of the relative abundances of the OTUs in the control soil at time zero revealed an increase following exposure to VPH vapour from a NAPL source over the duration of the experiment (430 days). Most VPH degrading OTUs including species from the family *Nocardioidaceae, Comamonadaceae*, *Pseudomonadaceae* and the from genera *Sphingobium*, *Pseudoxanthomonas* and *Pseudonocardia* increased in their relative abundance rankings by a minimum 2 folds upon exposure to VPH vapours after 430 days in the respective soil treatments suggesting that with time, there was an increase not just in the activity of microorganisms but also in their relative abundances.

With respect to column locations, the genera *Sphingomonads*, *Nocardiodes*, *Pseudonocardia* and *Methylibium* were significantly higher in relative abundances at the column locations nearer the NAPL source than at the near zero concentration boundary side of the columns while *Polaromonas* and *Alkanindiges* showed the reverse pattern. Contrary to our proposed hypothesis, VPH degrading OTUs also increased significantly in their relative abundances at the atmosphere side of the columns.

Similarly, amending soil with 2% biochar or AC significantly affected microbial communities in the respective columns. The genera *Paenibacillus*, *Clostridium*, and *Pseudoxanthomonas* were significantly more dominant in the unamended soil column than in the sorbent amended soil columns. The genera *Pseudonocardia* and *Streptomyces* were significantly higher in relative abundance in the soil & AC column than in the unamended soil or soil & biochar columns. At the class level of taxonomy (OTU level 3), a one way analysis of similarities (ANOSIM) revealed that the amendment factor had a significant effect on shaping the microbial communities in the respective columns. At the species level, OTU relative abundance ranking also increased in the sorbent amended columns following exposure to VPH vapours in the genera *Pseudonocardia* and *Hydrogenophaga* both of which have been reported to degrade petroleum hydrocarbons from previous studies. In addition to the effect on the relative abundances of VPH degrading OTUs, sorbent amendment also significantly reduced the amounts of inorganic nutrients ammonium

(NH_4^+), nitrates (NO_3^-), and phosphates (PO_4^{2-}) in the respective soil columns compared to the initial soil concentrations. These reductions coincided with a reduction in the relative abundances of ammonia oxidising archaea (AOA) in the sorbent amended soil columns. Hence, sorbent amendment significantly altered the microbial communities of the different soil systems with respect to VPH degradation and nutrient cycling.

Overall, amending soil with 2% biochar or activated carbon over a long term altered the microbial community composition of soil by causing an increase in the relative abundance ranking of most of the VPH degrading OTUs and did not appear to negatively impact soils with regards to bacterial community dynamics of VPH degradation in the long term.

Chapter 7: Overall conclusions and recommendations for future work

7.1 Effects of biochar and activated carbon amendment on pollutant toxicity to soil VPH degrading bacteria

Amending soil with 2% biochar or activated carbon enhanced the sorption of the pollutants thereby reducing their bioavailability to soil microorganisms for biodegradation and their mobility in soil. At higher VPH ($^{13}C_7$ – toluene) concentrations, therefore, reduced pollutant bioavailability in the sorbent amended soil batches was observed to shorten the lag phase in the growth cycle of soil bacteria in comparison with the unamended soil batch in which a lag phase twice as long was observed. In contrast, addition of different VPH classes to soil batches without sorbent amendment revealed that straight chain alkanes had the highest stimulatory effect on soil bacteria as indicated by CO_2 production while the aromatic hydrocarbons had the lowest stimulatory effect on microbial activity suggesting that barring any sorption effects, at equivalent concentrations of VPH, aromatic hydrocarbons exerted the most toxic effects on soil microorganisms the straight chain or cylic/branched alkane class of VPHs. Sorbent amendment apparently reduces pollutant toxicity, based on the CO_2 levels in batch headspaces, to soil bacteria especially for the mono-aromatic hydrocarbons by reducing the concentration of VPH that ultimately becomes available to soil VPH degraders. Amending soil with biochar or AC therefore enhanced sorption of the pollutants, and reduced their bioavailability to microbial activity thereby reducing their toxicity at high concentrations on the short-term.

7.2 Effects of biochar and activated carbon amendment on the growth of VPH degrading bacteria

Growth of VPH degrading bacteria as indicated by the evolution of CO_2 was slowed with biochar or activated carbon amendment in the short-term batch study. In a batch study in which soil was amended with or without 2% biochar or AC and with or without nutrients, microbial growth was slowed in the sorbent amended batches especially in the soil & AC batches with nutrients. Maximum cumulative headspace CO_2 concentration was the least in the soil & AC batch compared to unamended soil and the soil & biochar batches on the short term. Total bacterial cell numbers were the lowest in the soil & AC batch with nutrients suggesting that microbial growth was no longer limited by the nutrient availability, but likely by the substrate (VPH) availability, which has been greatly reduced due to sorption by the AC. On the long-term, sorbent amendment was observed to initially slow the metabolism of VPHs especially in the soil & AC column. CO_2 concentrations rose steadily in the soil & AC column to reach similar levels as in the unamended soil column by day 430. This can be interpreted to mean that higher sorption of VPH by AC retained a higher concentration of the

pollutant in the soil & AC pores. Desorption of pollutants back into pore water over time would result in a higher level of biodegradation of the pollutants in the soil & AC column.

On the other hand, an increase in absolute abundances (by over a 100%) of the petroleum hydrocarbon degrading bacterial genera *Nocardioides*, *Methylibium*, *Alkanindiges* and *Pseudoxanthomonas* in the long-term column studies and the genera *Pseudomonas*, *Arenimonas* and *Polaromonas* in the batch studies following exposure of the control (soil at time 0) to VPHs suggested that VPH exposure enhanced the growth of petroleum hydrocarbon degraders in the long and short term respectively.

7.3   Effects of biochar and activated carbon amendment on microbial ecology in VPH-contaminated gravelly sand

The effects of sorbent amendment on soil microbial ecology was also observed. Different VPH degrading bacteria were observed to thrive in the batch and column soils amended with or without sorbent and/or nutrient amendment. In the short-term batch study with AC amendment and with or without nutrient amendment, bacterial species within the family *Nocardioidaceae* showed a 64-fold increase in their relative abundance ranking following addition of VPHs over a 6-days duration. Other species within the family *Pseudomonadaceae* and the genera *Pseudomonas* and *Achromobacter* also showed a minimum 2-fold increase in soil batches amended with or without biochar or AC and in the nutrient amended or unamended soil. Yield coefficients (g biomass-C g^{-1} substrate) as determined for the VPH degrading bacterial biomass at the end of the batch study following VPH addition showed that sorbent amendment reduced biomass yields for both nutrient amended and unamended batches suggesting that soil bacteria had a limited amount of VPHs to metabolise in the sorbent amended batches compared to the unamended soil batches. In the column study, species within the family *Nocardioidaceae* increased in their relative abundance ranking by a maximum of 8-folds in the soil & AC column. Members of the *Pseudomonadaceae* family also increased in relative abundance ranking by up to 32 folds in the soil & biochar column while members of the genera *Pseudonocardia, Pseudoxanthomonas* and *Hydrogenophaga* increased in their abundance ranking in the soil, soil & biochar and soil & AC columns following exposure to VPHs over the duration of the experiment (430days). In addition to changes in the microbial community structure, sorbent amendment also affected the amount of available inorganic nutrients in soil at the end of the column studies. A decrease in nutrient concentrations of NH_4^+, NO_3^-, and PO_4^{2-} from their initial conditions on day 0 (Table 6.1) notably in biochar and AC amended soil columns coincided with a decrease in the relative abundances of the ammonia oxidizing archaea (AOA) group organisms. Hence, although sorbent amendment reduces the bioavailability of VPHs to soil bacteria, they did not appear to

have any negative effect on the growth of VPH degrading bacteria both in the short- and long-term. Sorption, however, reduces the yields of VPH degrading biomass and exerts a growth limiting effect on microorganisms involved in nutrient cycling on the long-term.

7.4   Broader implications of the current research

Amending soil with biochar and AC mitigated the biodegradation of VPHs as demonstrated by sorption of toluene ($^{13}C_7$) compared to unamended soil. This observation was in line with observations from a subsequent batch study in which sorbent amendment especially with AC reduced the level of biodegradation of a consortium of VPHs in the short term. On the contrary, biodegradation as indicated by CO_2 production was higher in the soil & AC treatments than in the unamended and soil & biochar batches at the end of the long term column studies. The implications of these observations could mean that sorption of VPHs to sorbent in the short term reduces their availability for biodegradation while in the long term, strongly bound pollutants to biochar and particularly to AC amended soils, slowly desorbs back into soil pore water as previously described, thereby enhancing their availability to microorganisms for biodegradation. In the short term batch studies, amending soil with 2% biochar or AC also affected microbial ecology by exerting varying effects on microbial biomass yield coefficients. Average yield coefficients were lower for AC amended soil than the unamended soil and soil & biochar with or without nutrient amendments. From the microbiological viewpoint, a more diverse consortium of bacteria was involved in the degradation of straight chain and cyclic alkanes than was required for the degradation of aromatic hydrocarbons in batch systems without sorbent amendment. In the short term batch study systems, members of the genus *Pseudomonas* and the species *Pseudomonas umsongensis* were enriched across all treatments that were exposed to VPHs but not in the long-term column studies. This observation is likely to imply that at high pollutant concentration and in the short term, members of the genus *Pseudomonas* have the capacity to thrive on the selection of VPHs used in the current study. Petroleum hydrocarbon degrading members of the family *Nocardioidaceae* and *Pseudomonadaceae* and the genus *Pseudoxanthomonas* were enriched in all treatments (with or without sorbent/nutrient amendment) relative to their abundances in the controls in both short term batch and long term column studies indicating their potential to grow both in the short term and in the long term when most of the VPHs in the NAPL source has been largely exhausted. In the column studies, the increase in absolute abundances of the species within the ammonia oxidizing archeae (AOA) compared to soil at time zero especially in the unamended soil column suggested that exposing this group of microbes to VPHs over the duration of 430 days enhanced their growth although growth was apparently limited by inorganic nutrient

availability as demonstrated in the soil & biochar and soil & AC columns at the end of the experiment.

Ultimately, although amending soil with biochar or activated carbon apparently reduces the amount of pollutant that is available for biodegradation by soil microorganisms as shown by CO_2 production in the short term, in the long term, a slow release of adsorbed pollutants into soil pore water may imply that pollutants increasingly become available thereby stimulating biodegradation of pollutants in soil pore water. An increase in absolute abundances and relative abundance ranking of VPH degraders in biochar and AC amended soils from both batch and column studies following exposure to VPHs suggests that sorbent amendment may not significantly limit the growth of microorganisms with respect to PH degradation.

7.5   Recommendations for future work

From the foregoing study, a number of questions are yet to be answered and should form a basis for future research in the area of *in situ* sorbent (biochar and activated carbon) amendment as an innovative approach for the remediation of soils contaminated volatile petroleum hydrocarbons.

Further considerations should be given to the effects of other VPHs, as single compounds on the growth kinetics of microorganisms in the soil under investigation. Biomass yields can be determined for the growth of soil microorganisms on single VPH substrates and a comparison with growth on complex substrate mixtures conducted. Also, dual combinations of the PHs e.g toluene and 1,2,4-TMB, *n*-octane and hexane e.t.c. can be studied to deduce the effects of interactions such as enhancements, inhibition and other types of interactions on the growth kinetics parameters of the organisms in the soil being studied. Finally, a mixture of all 12 VPHs and the effects of their interactions on the kinetics of VPH degrading bacteria would be an interesting means of gaining a better understanding of how the microbial community dynamics affect kinetic parameters of microorganisms growing on the pollutant mixture.

To further understand the effects of nutrient availability and sorbent amendment on the microbial community response, more work should be done to investigate the effects of other inorganic nitrogen and phosphorous sources on the biodegradation of the pollutants. Also, varying concentrations of the nutrients should be used to assess their stimulatory or inhibitory effects on soil microbial activity. In addition, varying concentrations of different classes of VPHs should be used to ascertain a threshold for toxicity of pollutants to microbial communities in the soil. Consideration should also be given to the effects of other chemical properties such as structural formulae of compounds. For instance, hydrophobicity of straight

chain alkanes is a function of the carbon chain lengths which in turn determines the solubility and bioavailability of the compounds in soil pore water.

For long-term studies, sampling intermittently would be challenging and also disrupt ongoing soil processes but may also give more insight into community dynamics at intervals during the long term experiments. Samples of the different soil treatments collected at different time intervals can be used to assess processes such as nutrient and VPH availability and how these affect the dynamics of microbial communities at such time intervals.

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Appendix

Appendix A

Batch biodegradation model:

The batch experiments were simulated by assuming Monod kinetic growth of microbial biomass following the addition of a fully substituted ^{13}C substrate ($^{13}C_7$ toluene).

Parameters are expressed in SI units of mole, second, kg, meters, except for the hydrogen ion concentration $[H^+]$ which is expressed in moles per litre. Substrate, biomass and CO_2 concentrations are expressed on a carbon-normalized basis (mole substrate C per m^3 or kg, mole biomass C per m^3, mole CO_2 C per m^3).

t (s)	Time
r (m)	Radial distance from the BC particle centre
C^{sub}_{air} (moles C m^{-3})	Substrate concentration in soil air and headspace air
C^{sub}_{water} (moles C m^{-3})	Substrate concentration in soil porewater
C^{sub}_{soil} (moles C kg^{-1})	Substrate concentration associated with soil solids
C^{sub}_{BC} (moles C kg^{-1})	Substrate concentration associated with the BC solid matrix
$C^{sub}_{BC,ippw}$ (moles C m^{-3})	Substrate concentration in the BC intraparticle porewater
C^{bio}_{water} (moles C m^{-3})	Biomass concentration in soil porewater
C^{CO2}_{air} (moles C m^{-3})	CO_2 concentration in soil and headspace air
C^{CO2}_{water} (moles C m^{-3})	CO_2 concentration in water
C^{H2CO3}_{water} (moles C m^{-3})	Carbonic acid concentration in water
C^{H2CO3*}_{water} (moles C m^{-3})	Apparent carbonic acid concentration in water
C^{HCO3-}_{water} (moles C m^{-3})	Hydrogen carbonate concentration in water
C^{CO3--}_{water} (moles C m^{-3})	Carbonate concentration in water
$[H^+]$ (moles per litre)	Hydrogen ion concentration in water
$C^{CO2,13C}_{air}$ (moles C m^{-3})	^{13}C-CO_2 concentration in soil and headspace air
$C^{CO2,12C}_{air}$ (moles C m^{-3})	^{12}C-CO_2 concentration in soil and headspace air
$C^{CO2,13C}_{atmos}$ (moles C m^{-3})	^{13}C-CO_2 concentration in the atmosphere
$C^{CO2,12C}_{atmos}$ (moles C m^{-3})	^{12}C-CO_2 concentration in the atmosphere
H^{sub} (-)	Dimensionless Henry constant for the substrate
K^{sub}_{soil} (m^{3}kg^{-1})	Soil solid-water partitioning coefficient for the substrate
K^{sub}_{BC} (m^{3}kg^{-1})	BC solid-water partitioning coefficient for the substrate
H^{CO2} (-)	Dimensionless Henry constant for CO2
K^{CO2}_h (-)	Hydration constant for CO2
H^{CO2}_* (-)	Apparent dimensionless Henry constant for CO2

K_1 (moles per litre)	Apparent carbonic acid dissociation constant
K_2 (moles per litre)	Hydrogen carbonate dissociation constant
V_w (m^3)	Soil porewater volume in the batch
Msoil (kg)	Dry soil mass in the batch
M_{BC} (kg)	Dry BC mass in the batch
R_{BC} (-)	BC particle radius
$N_{p,BC}$ (-)	Number of BC particles in the batch
θ_w (-)	Water-filled BC intraparticle porosity
d_{BC} (kg m^{-3})	Solid density of the BC skeleton
τ (-)	BC pore network tortuosity factor
$D^{sub}_{eff,BC}$ (m^{2}s^{-1})	The effective diffusion coefficient of the substrate in the water-filled BC pore network
D^{sub}_{aq} (m^{2}s^{-1})	The molecular diffusion coefficient of the substrate in water
D^{sub}_{air} (m^{2}s^{-1})	The molecular diffusion coefficient of the substrate in air
$D^{CO2,13C}_{air}$ (m^{2}s^{-1})	The molecular diffusion coefficient of ^{13}C CO_2 in air
$D^{CO2,12C}_{air}$ (m^{2}s^{-1})	The molecular diffusion coefficient of ^{12}C CO_2 in air
k_{sorb} (s^{-1})	First-order kinetic sorption rate
L_f (m)	Leakage factor, gap area divided by the gap length
μ^{sub}_{max} (s^{-1})	Maximum specific biomass growth rate
K^{sub}_S (moles C m^{-3})	The half-velocity constant
Y^{sub} (moles C moles^{-1} C)	Yield coefficient
$C^{bio}_{water,max}$ (moles C m^{-3})	Maximum biomass concentration in soil porewater
dec (s^{-1})	First-order biomass decay rate
f (-)	^{12}C to ^{13}C assimilation ratio
$BGR^{CO2,12C}_{soil}$ (moles C kg^{-1}s^{-1})	Background soil respiration rate
nbc	Number of BC shells (discretization)
$V_{BC,i}$ (m^3)	Volume of BC shell i
$CF_{i=1..nbc}$ (m^3)	Capacity factor for the substrate concentration in BC intraparticle porewater
$CF_{i=nbc+1}$ (m^3)	Capacity factor for the substrate concentration in soil porewater
$CF_{i=nbc+2}$ (kg)	Capacity factor for the substrate concentration in soil solids
$CF_{i=nbc+3}$ (m^3)	Capacity factor for the biomass concentration in soil porewater

$CF_{i=nbc+4}$ (m^3)	Capacity factor for the ^{12}C CO_2 concentration in soil porewater
$CF_{i=nbc+5}$ (m^3)	Capacity factor for the ^{13}C CO_2 concentration in soil porewater

Table 9.1. Independent and dependant variables and parameters and their dimensions

Description of the thermodynamic equilibrium in the batch system:

1. Substrate

The substrate concentration in the batch headspace, C^{sub}_{air}, is related to the substrate concentration in soil pore water, C^{sub}_{water}, by Henry's law

$$H^{sub} = \frac{C^{sub}_{air}}{C^{sub}_{water}}$$

(9.1)

where H^{sub} is the dimensionless Henry's law constant.

When sorption equilibrium has been established, the substrate concentration in soil pore water is related to the substrate concentration of the soil particles, C^{sub}_{soil}, by the partitioning coefficient, K^{sub}_{soil},

$$K^{sub}_{soil} = \frac{C^{sub}_{soil}}{C^{sub}_{water}}$$

(9.2)

The model also considers soil amendment with a porous black carbon (BC) sorbent material such as biochar or activated carbon. Within BC particles, the substrate concentration in the intraparticle pore water, C^{sub}_{ippw}, is related to the substrate concentration of the BC solid matrix, C^{sub}_{BC}, by the partitioning coefficient, K^{sub}_{BC},

$$K^{sub}_{BC} = \frac{C^{sub}_{BC}}{C^{sub}_{ippw}}$$

(9.3)

2. CO_2

The CO_2 concentration in the batch headspace air, C^{CO2}_{air}, is related to the dissolved CO_2 concentration in soil pore water, C^{CO2}_{water}, by Henry's law

$$H^{CO2} = \frac{C^{CO2}_{air}}{C^{CO2}_{water}}$$

(9.4)

The equilibrium between dissolved CO_2 and carbonic acid (H_2CO_3) is described by a hydration equilibrium constant, K^{CO2}_h,

$$K^{CO2}_h = \frac{C^{CO2}_{air}}{C^{CO2}_{water}} \tag{9.5}$$

The sum of dissolved CO_2 and H_2CO_3 concentrations will be referred to as the apparent carbonic acid concentration in water, C^{H2CO3*}_{water},

$$C^{H2CO3*}_{water} = C^{H2CO3}_{water} + C^{CO2}_{water} \tag{9.6}$$

and the modified Henry's law constant relates the CO_2 concentration in the batch headspace air to the apparent carbonic acid concentration in soil pore water

$$H^{CO2}_* = \frac{C^{CO2}_{air}}{C^{H2CO3*}_{water}} \tag{9.7}$$

The apparent carbonic acid dissociation constant, K_1, relates the apparent carbonic acid concentration in soil pore water to the hydrogen carbonate (or bicarbonate) concentration in soil pore water, C^{HCO3-}_{water},

$$K_1 = \frac{C^{HCO3-}_{water} \cdot [H^+]}{C^{H2CO3*}_{water}} \tag{9.8}$$

where $[H^+]$ is the molar hydrogen ion concentration, or 10^{-pH}.

The hydrogen carbonate dissociation constant, K_2, relates the hydrogen carbonate concentration in soil pore water to the carbonate concentration in soil pore water, C^{CO3-}_{water},

$$K_2 = \frac{C^{CO3--}_{water} [H^+]}{C^{HCO3-}_{water}} \tag{9.9}$$

Differential equations:

1. Substrate

The following partial differential equation governs the substrate concentration in BC intraparticle pore water

$$\left(\theta_w + (1 - \theta_w)d_{BC}K^{sub}_{BC}\right) d/dt \, C^{sub}_{BC,ippw} = \frac{D^{sub}_{eff,BC}}{r^2} \cdot \partial/\partial r \, r^2 \, \partial/\partial r \, C^{sub}_{BC,ippw} \tag{9.10}$$

where θ_w is the water-filled intraparticle BC porosity, d_{BC} is the skeletal solid density of the BC, r is the radial distance from the BC particle centre, and t the time.

The effective diffusion coefficient of the substrate in the BC pore network is defined as

$$D_{eff,BC}^{sub} = \theta_w \tau D_{aq}^{sub} \qquad\qquad (9.11)$$

where τ is the BC pore network tortuosity factor, and D^{sub}_{aq} is the molecular diffusion coefficient of the substrate in water.

Boundary conditions:

A no net flux boundary condition is enforced at r=0 in the BC particle core

$$\partial/\partial r\, C_{BC,ippw}^{sub}\Big|_{r=0} = 0$$

$$(9.12)$$

and the substrate concentration in BC intraparticle pore water on the BC particle surface is equal to the substrate concentration in soil porewater (no external aqueous film mass transfer resistance)

$$C_{BC,ippw}^{sub}\Big|_{r=R_{BC}} = C_{water}^{sub}$$

$$(9.13)$$

Assuming instantaneous exchange of the substrate between headspace air and soil pore water, the following differential equation governs the substrate concentration in soil pore water:

$$(V_{air}H^{sub} + V_{water}) \cdot d/dt\, C_{water}^{sub} = +r_1 - r_2 - r_3 - r_4$$

$$(9.14)$$

where V_{air} is the volume of air in the batch and V_w is the volume of water in the batch.

The BC particle to soil porewater mass transfer rate, r_1, is described by

$$r_1 = -N_{p,BC} \cdot 4\pi R_{BC}^2 \cdot D_{eff,BC}^{sub} \cdot \partial/\partial r\, C_{BC,ippw}^{sub}\Big|_{r=R_{BC}}$$

$$(9.15)$$

where $N_{p,BC}$ is the number of BC particles, and R_{BC} is the BC particle radius.

The soil pore water to soil particles mass transfer rate, r_2, is described by

$$r_2 = -V_w k_{sorb}\left(\frac{C_{soil}^{sub}}{K_{soil}^{sub}} - C_{water}^{sub}\right)$$

$$(9.16)$$

where k_{sorb} is a first-order kinetic sorption rate.

The batch air to outside air mass transfer rate, r$_3$, which is assumed to be due to leakage through a small gap between the cap and the glass vial is described by

$$r_3 = -L_f \cdot D_{air}^{sub} \cdot H^{sub} \cdot \left(0 - C_{water}^{sub}\right)$$

(9.17)

where L$_f$ is a leakage factor approximately equal to the cross-sectional area of the gap divided by the gap length, and D^{sub}$_{air}$ is the molecular diffusion coefficient of the substrate in air.

The substrate mass utilization rate due to biodegradation, r$_4$, is described by assuming Monod kinetics limited by logistic growth according to

$$r_4 = V_w \mu_{max}^{sub} \cdot \frac{C_{water}^{sub}}{K_S^{sub} + C_{water}^{sub}} \cdot \frac{1}{Y^{sub}} \cdot \frac{C_{water,max}^{biomass} - C_{water}^{biomass}}{C_{water,max}^{biomass}} \cdot C_{water}^{biomass}$$

(9.18)

where μ^{sub}$_{max}$ is the maximum specific growth rate, K^{sub}$_S$ is the half-velocity constant, Y^{sub} is the yield coefficient, C^{biomass}$_{water}$ is the concentration of the biomass in soil pore water, and C^{biomass}$_{water,max}$ is the maximum concentration of the biomass in soil pore water. Equation 18 assumes that only substrate dissolved in soil pore water is biodegradable, and the substrate degrading biomass is dissolved in or in direct contact with the soil pore water.

The following differential equation governs the substrate concentration in soil particles

$$M_{soil} \cdot {}^d\!/_{dt} C_{soil}^{sub} = +r_2$$

(9.19)

where M$_{soil}$ is the dry mass of soil in the batch. The soil pore water to soil particles mass transfer rate r$_2$ has been describe above.

2. Biomass

The following differential equation governs the growth and decay of ^{13}C biomass on the ^{13}C substrate

$$V_w \, {}^d\!/_{dt} C_{water}^{biomass,13C} = V_w \left(\mu_{max}^{sub} \cdot \frac{C_{water}^{sub}}{K_S^{sub} + C_{water}^{sub}} \cdot \frac{C_{water,max}^{biomass} - C_{water}^{biomass}}{C_{water,max}^{biomass}} - dec \right) \cdot C_{water}^{biomass}$$

(9.20)

where dec is the biomass first-order decay rate.

Based on the assumption that the assimilation of ^{13}C from the ^{13}C substrate enables a proportional assimilation of ^{12}C from other carbon substrates, the growth and decay of ^{12}C biomass is described by

$$V_w \, {}^d\!/_{dt} \, C_{water}^{biomass,12C} = f \cdot V_w \, {}^d\!/_{dt} \, C_{water}^{biomass,13C}$$

(9.21)

where f is the ^{12}C to ^{13}C assimilation ratio.

The total biomass concentration then changes according to

$$V_w \, {}^d\!/_{dt} \, C_{water}^{biomass} = (1+f) \cdot V_w \left(\mu_{max}^{sub} \cdot \frac{C_{water}^{sub}}{K_S^{sub}+C_{water}^{sub}} \cdot \frac{C_{water,max}^{biomass}-C_{water}^{biomass}}{C_{water,max}^{biomass}} - dec \right) \cdot C_{water}^{biomass}$$

eq 22.

3. CO$_2$

Assuming instantaneous exchange of CO_2 between headspace air and soil pore water, a constant soil pH, no carbonate dissolution or precipitation, and a constant background soil respiration by microorganisms other than the substrate degraders releasing additional ^{12}C CO_2 from soil organic carbon metabolization, the following differential equations govern the concentration of ^{13}C CO_2 and ^{12}C CO_2 in batch headspace air

$$\left(V_a + V_w \cdot \frac{1}{H_*^{CO2}} \left(1 + \frac{K_1}{10^{-pH}} + \frac{K_1 K_2}{10^{-2pH}} \right) \right) {}^d\!/_{dt} \, C_{air}^{CO2,13} = +r_5^{13C} - r_6^{13C}$$

(9.23)

$$\left(V_a + V_w \cdot \frac{1}{H_*^{CO2}} \left(1 + \frac{K_1}{10^{-pH}} + \frac{K_1 K_2}{10^{-2pH}} \right) \right) {}^d\!/_{dt} \, C_{air}^{CO2,12C} = +r_5^{12C} - r_6^{12C} + r_7^{12C}$$

(9.24)

For ^{13}C-CO$_2$, the net ^{13}C-CO$_2$ mass production rate, r_5, is described by

$$r_5^{13C} = V_w \left(\mu_{max}^{sub} \cdot \frac{C_{water}^{sub}}{K_S^{sub}+C_{water}^{sub}} \cdot \frac{C_{water,max}^{biomass}-C_{water}^{biomass}}{C_{water,max}^{biomass}} \cdot \frac{1-Y^{sub}}{Y^{sub}} - \frac{1}{1+f} \cdot dec \right) \cdot C_{water}^{biomass}$$

(9.25)

Based on the assumption that the ^{12}C substrates are assimilated with the same yield as the ^{13}C substrate, the net ^{12}C-CO$_2$ mass production rate is described by

$$r_5^{12C} = f \cdot r_5^{13C}$$

(9.26)

The batch air to outside air mass transfer rate, r_6, which is assumed to be due to leakage through a small gap between the cap and the glass vial is described by

$$r_6^{13C} = -L_f \cdot D_{air}^{CO2,13C} \cdot \left(C_{atmos}^{CO2,13C} - C_{air}^{CO2,12C} \right)$$

$$(9.27)$$

$$r_6^{12C} = -L_f \cdot D_{air}^{CO2,12C} \cdot \left(C_{atmos}^{CO2,12C} - C_{air}^{CO2,12C} \right)$$

$$(9.28)$$

for ^{13}C-CO_2 and ^{12}C-CO_2 respectively, where $C^{CO2,13C}_{atmos}$ and $C^{CO2,12C}_{atmos}$ is the $^{13}CO_2$ and $^{12}CO_2$ concentration in the atmosphere respectively.

The additional ^{12}C-CO_2 mass production by the respiration of soil microorganisms other than the substrate degraders is described by

$$r_7^{12C} = M_{soil} \cdot BGR_{soil}^{CO2,12C}$$

$$(9.28)$$

where $BGR^{CO2,12C}_{soil}$ is the background soil respiration rate.

Numerical solution:

The differential equations are solved with Matlab © using the differential equation solver ode15. In order to use this solver, the system of partial and ordinary differential equations is transformed into a system of only time-dependant ordinary differential equations using the method of lines. The BC particles are discretized into nbc concentric shells, where C_1 is the substrate concentration in the BC intraparticle porewater in the BC particle core, and C_{nbc} is the substrate concentration in the BC intraparticle porewater in the outermost BC shell (Figure 1a).

C_{nbc+1} is the substrate concentration in soil porewater (Figure 1b). C_{nbc+2} is the substrate concentration for the soil solids. C_{nbc+3} is the biomass concentration in soil porewater. C_{nbc+4} is the ^{13}C-CO_2 concentration in headspace and soil air. C_{nbc+5} is the ^{12}C-CO_2 concentration in headspace and soil air.

a) Discretion of the BC particles

b) Concentration vector

i = nbc

i = ...
i = 2
i = 1

dr=R_{BC}/nbc

R_{BC}

r

R_{BC} = Biochar radius

nbc = number of discrete shells
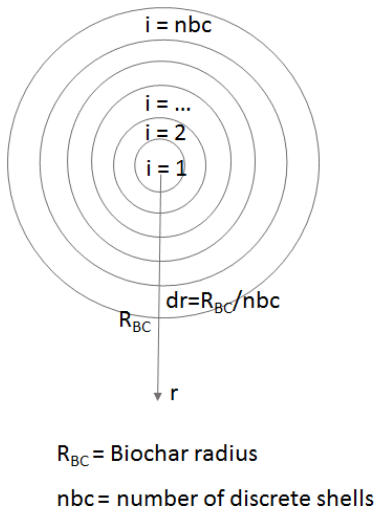
C_1 — Substrate concentration in intraparticle porewater in the innermost BC shell (moles C m^{-3})

$\vdots$

C_{nbc} — Substrate concentration in intraparticle porewater in the outermost BC shell (moles C m^{-3})

C_{nbc+1} — Substrate concentration in soil porewater (moles C m^{-3})

C_{nbc+2} — Substrate concentration in soil solids (moles C kg^{-1})

C_{nbc+3} — Biomass concentration in soil porewater (moles C m^{-3})

C_{nbc+4} — ^{12}C CO_2 concentration in soil air and batch headspace (moles C m^{-3})

C_{nbc+5} — ^{13}C CO_2 concentration in soil air and batch headspace (moles C m^{-3})

Figure 9.1. Discretization of the BC particles and concentration vector

For each BC shell i, the shell volume $V_{BC,i}$ is

$$V_{BC,i} = \frac{4}{3}\pi\left((idr)^3 - ((i-1)dr)^3\right) = \frac{4}{3}\pi dr^3(3i^2 - 3i + 1) = \frac{4}{3}\pi R_{BC}^3 \frac{(3i^2 - 3i + 1)}{nbc^3}$$

(9.29)

For each BC shell i, the outer shell surface area is

$$A_{BC,i} = 4\pi i^2 dr^2 = 4\pi \frac{i^2}{nbc^2} R_{BC}^2$$

(9.30)

The total number of BC particles, $N_{p,BC}$, can be calculated from the mass of BC in the batch, M_{BC},

$$N_{p,BC} = \frac{M_{BC}}{(1-\theta_{BC})d_{BC}} \cdot \frac{1}{\frac{4}{3}\pi R_{BC}^3}$$

(9.31)

The capacity factor CF_i for the substrate concentration in intraparticle porewater of the shells i of all BC particles is

$$CF_i = N_{p,BC}V_{BC,i}(\theta_{BC} + (1 - \theta_{BC})d_{BC}K_{BC})$$

(9.32)

The total substrate mass transfer rate from all BC shells i to shells i+1 due to substrate diffusion in intraparticle porewater is

$$r_{ipd,i} = -\frac{N_{p,BC}A_{BC,i}D_{eff,BC}^{sub}}{dr}(C_{i+1} - C_i) = -\frac{nbc \cdot N_{p,BC}A_{BC,i}D_{eff,BC}^{sub}}{R_{BC}}(C_{i+1} - C_i)$$

(9.33)

For the outermost shell surface areas, it has been assumed that there is no aqueous film mass transfer resistance, and the substrate concentration at the interface is equal to the soil porewater concentration, thus the diffusion distance dr is reduced by half

$$r_{ipd,nbc} = -2 \cdot \frac{nbc \cdot N_{t,BC}A_{BC,i}D_{eff,BC}^{sub}}{R_{BC}}(C_{nbc+1} - C_{nbc})$$

(9.34)

The following ordinary differential equations describes the concentration changes in the intraparticle BC porewater in the particle core which are due to the substrate intraparticle diffusion

$$d/_{dt}\, C_1 = -\frac{r_{ipd,1}}{CF_1}$$

(9.35)

and for i=2 … nbc

$$d/_{dt}\, C_i = -\frac{r_{ipd,i} - r_{ipd,i-1}}{CF_i}$$

(9.36)

The capacity factor CF for the substrate concentration in soil pore water, which is assumed to be in instantaneous equilibrium with the substrate concentration in soil air and headspace air, is

$$CF_{nbc+1} = (V_{air}H^{sub} + V_{water})$$

(9.37)

The substrate mass transfer rate from soil porewater to soil solids is

$$r_{sorb} = -V_w k_{sorb}\left(\frac{C_{nbc+2}}{K_{soil}^{sub}} - C_{nbc+1}\right)$$

(9.38)

The batch air to outside air mass transfer rate, r_{leak}, which is assumed to be due to leakage through a small gap between the cap and the glass vial is described by

$$r_{leak,sub} = L_f \cdot D_{air}^{sub} \cdot H^{sub} \cdot C_{nbc+1}$$

(9.39)

The substrate mass removal rate from the soil pore water due to biodegradation is

$$r_{deg} = V_w \mu_{max}^{sub} \cdot \frac{C_{nbc+1}}{K_S^{sub} + C_{nbc+1}} \cdot \frac{C_{water,max}^{biomass} - C_{nbc+3}}{C_{water,max}^{biomass}} \cdot \frac{1}{Y^{sub}} \cdot C_{nbc+3}$$

(9.40).

The following ordinary differential equation describes the concentration changes in the soil porewater

$$\frac{d}{dt} C_{nbc+1} = \frac{r_{ipd,nbc} - r_{leak,sub} - r_{sorb} - r_{deg}}{CF_{nbc+1}}$$

(9.41)

The capacity factor CF_{nbc+2} for the substrate concentration in soil solids is

$$CF_{nbc+2} = M_{soil}$$

(9.42).

The following ordinary differential equation describes the concentration changes in the soil solids

$$\frac{d}{dt} C_{nbc+2} = \frac{+r_{sorb}}{CF_{nbc+2}}$$

(9.43)

The capacity factor CF_{nbc+3} for the biomass concentration in soil porewater is

$$CF_{nbc+3} = V_w$$

(9.44)

The net biomass mass production rate is

$$r_{prod,biomass} = V_w \left((1+f) \cdot \mu_{max}^{sub} \cdot \frac{C_{nbc+1}}{K_S^{sub} + C_{nbc+1}} \cdot \frac{C_{water,max}^{biomass} - C_{nbc+3}}{C_{water,max}^{biomass}} - dec \right) \cdot C_{nbc+3}$$

(9.45)

The following ordinary differential equation describes the biomass concentration change in the soil porewater

$$\frac{d}{dt} C_{nbc+3} = \frac{+r_{prod,biomass}}{CF_{nbc+3}}$$

(9.46)

Assuming instantaneous equilibrium between soil porewater, soil air and headspace air, and instantaneous CO_2 hydration and proton exchange, stable soil pH, and no formation or dissolution of carbonates, the capacity factor CF_{nbc+4} for the ^{13}C-CO_2 concentration in soil air and headspace air is

$$CF_{nbc+4} = V_a + V_w \cdot \frac{1}{H_*^{CO2}}\left(1 + \frac{K_1}{10^{-pH}} + \frac{K_1 K_2}{10^{-2pH}}\right) \tag{9.47}$$

The batch air to outside air mass transfer rate, $r_{leak,CO2}$ is

$$r_{leak,CO2,C13} = -L_f \cdot D_{air}^{CO2,C13} \cdot \left(C_{atmos}^{CO2,13C} - C_{nbc+4}\right) \tag{9.48}$$

The ^{13}C-CO_2 mass production rate is

$$r_{prod,CO2,C13} = V_w \left(\mu_{max}^{sub} \cdot \frac{C_{nbc+1}}{K_S^{sub}+C_{nbc+1}} \cdot \frac{C_{water,max}^{biomass}-C_{nbc+3}}{C_{water,max}^{biomass}} \cdot \frac{1-Y^{sub}}{Y^{sub}} - \frac{1}{1+f} \cdot dec\right) \cdot C_{nbc+3}$$

(9.49).

The following ordinary differential equation describes the ^{13}C-CO_2 concentration change in the soil air and headspace air

$$d/_{dt} C_{nbc+4} = \frac{+r_{prod,CO2,13C} - r_{leak,CO2,13C}}{CF_{nbc+4}} \tag{9.50}$$

The capacity factor CF_{nbc+5} for the ^{12}C-CO_2 concentration in soil air and headspace air is

$$CF_{nbc+5} = V_a + V_w \cdot \frac{1}{H_*^{CO2}}\left(1 + \frac{K_1}{10^{-pH}} + \frac{K_1 K_2}{10^{-2pH}}\right) \tag{9.51}$$

The batch air to outside air mass transfer rate, $r_{leak,CO2}$, which is assumed to be due to leakage through a small gap between the cap and the glass vial is

$$r_{leak,CO2,C12} = -L_f \cdot D_{air}^{CO2,C12} \cdot \left(C_{atmos}^{CO2,12C} - C_{nbc+5}\right) \tag{9.52}.$$

The ^{12}C-CO_2 mass production rate from the substrate degrading biomass is

$$r_{prod,CO2,C12} = f \cdot V_w \left(\mu_{max}^{sub} \cdot \frac{C_{nbc+1}}{K_S^{sub}+C_{nbc+1}} \cdot \frac{C_{water,max}^{biomass}-C_{nbc+3}}{C_{water,max}^{biomass}} \cdot \frac{1-Y^{sub}}{Y^{sub}} - \frac{1}{1+f} \cdot dec\right) \cdot C_{nbc+3}$$

(9.53).

The ^{12}C-CO_2 mass production rate from the rest of the soil biomass is

$$r_{bgr,CO2,C12} = M_{soil} \cdot BGR_{soil}^{CO2,12C} \tag{9.54}.$$

The following ordinary differential equation describes the ^{12}C-CO_2 concentration change in the soil air and headspace air

$$d/_{dt} C_{nbc+5} = \frac{+r_{prod,CO2,12C} - r_{leak,CO2,12C} + r_{bgr,CO2,C12}}{CF_{nbc+5}} \tag{9.55}.$$

This system of ordinary differential equations 35,36,41,43,46,50,55 describes the temporal change in the concentrations, d/dt $C_{i=1..nbc+5}$ as a function of the concentrations $C_{i=1..nbc+5}$.

Matlab © implementation:

1. Batch model script (for AC amended soil)

```
clear all
close all
clc


% Simulates a kinetic batch sorption and biodegradation test with radial
% intraparticle diffusion for porous black carbon particles (biochar or AC)
% first-order kinetic sorption for soil, and Monod kinetic growth of
% pollutant degrading biomass on a 13C substrate.
% t: Time [s]
% c: Vector of dependant variables
%   1: Substrate conc in porewater, BC particle core [moles substrate C/m3]
%   2: Substrate conc in porewater, next BC shell [moles substrate C/m3]
%    ...
%   nbc: Substrate concentration in porewater, outermost BC shell
%   nbc+1: Substrate concentration in soil porewater [moles substrate C/m3]
%   nbc+2: Substrate concentration in soil solids [moles substrate C/g]
%   nbc+3: Biomass concentration in soil porewater [moles biomass C/m3]
%   nbc+4: 13CO2 concentration in headspace air [moles CO2 13C/m3]
%   nbc+5: 12CO2 concentration in headspace air [moles CO2 12C/m3]


%% Set parameters


% Batch
% Total volume batch, Vbatch [m3]
Vbatch = 65/10^6;
% Leakage factor (gap area/gap length) [m]
Lf = 5.3*10^-6;
% Soil
% Dry soil mass in batch, Msoil [kg]
Msoil = 15/1000;
% Soil water content, WCsoil [m3 water per kg dry soil]
WCsoil  = 0.1*1000/10^6;
% Solid density soil particles, dsoil [kg/m3]
dsoil = 2.5/1000*10^6;
```

% BC (Biochar/AC)

% Mass of biochar added, Mbc [kg]

Mbc = 0.02*Msoil;

% Radius of the particles, Rbc [m]

Rbc = 50*10^-6;

% Solid density of the particles, dbc [kg/m3]

dbc = 1.8/1000*10^6;

% Intraparticle porosity of the particles, pbc [m3/m3]

pbc = 0.57;

% Tortuosity factor of the particles, tortbc [-]

tortbc = pbc;

% Soil pH (with BC amendment if present)

pH = 7.74;

% Pollutant

% Molecular diffusion coefficient of the pollutant in water, Daq [m2/s]

Daq = 0.00027/106^0.71/10^4;

% Molecular diffusion coefficient of the pollutant in air, Dair [m2/s]

Dair = 0.078/10^4;

% Soil sorption coefficient, Ksoil [m3/kg]

Ksoil =7.9*10^-4;

% Soil sorption first-order uptake rate water, ksoil -> solid [1/s]

ksoil = 0.001;

% BC sorption coefficient Kbc [m3/kg]

Kbc = 9.32;

% Dimensionless Henry coefficient H

% [moles substrate C per m3 air/moles substrate C per m3 water]

H = 0.26;

% Biomass and biodegradation

% Monod half-rate constant, KS [moles/m3]

KS = 11.9415;

% Monod maximum growth rate, umax [1/s]

```
umax = 0.6/3600;

% Yield coefficient, Y [moles biomass C/moles substrate C]

Y = 0.135;

%   Decay rate, kdec [1/s]

kdec = 7.50e-09;

%   Lag phase, lag [s]

lag = 6*24*3600;

%   Maximum soil biomass carrying capacity, Cbmax [moles biomass C/m3 soil water]

Cbmax = 25;

% Cell carbon content [moles biomass C/cell]

Ccell = 100*10^-15/12;

% C12/C13 assimilation ratio [-]

C12_C13_ratio = 0.68;


% CO2

% Atmospheric concentration [moles CO2-C/m3]

C12_atm = 400*10^-6/0.0224;

C13_atm = 400*10^-6/0.0224*0.013;

% Acid constants CO2 [moles/L]

K1 = 4.45*10^-7;

K2 = 4.69*10^-11;

% Dimensionless Henry constant air-water CO2

% [moles CO2 C per m3 air/moles CO2 C per m3 water]

H_CO2 = 0.034/0.0404;

% Molecular diffusion coefficient in air [m2/s]

Dair_12CO2 = 0.17/10^4;

Dair_13CO2 = 0.17/10^4;

% Background soil respiration [moles CO2-C/s]

BGResp = 10^-11;


% Discretization

%   Number of particle shells, nbc [-]

nbc = 31;

 %% Initial conditions and duration
```

% Mass of substrate added to the batch [moles substrate C]

MP0 = 0.867*0.005/12;

% Initial substrate concentration in soil pore water [moles/m3]

Cw0 = MP0/(WCsoil*Msoil+(Vbatch-WCsoil*Msoil-Msoil/dsoil)*H);

% Initial cell count [cells/kg of wet soil]

CC0 = 2.7*10^10;

% Initial biomass [moles biomass C/m3 of soil pore water]

Cb0 = CC0*(1+WCsoil/1000)/WCsoil*Ccell;

c0 = zeros(nbc+5,1);

c0(nbc+1,1)=Cw0;

c0(nbc+3,1)=Cb0;

c0(nbc+4,1)=C13_atm;

c0(nbc+5,1)=C12_atm;

% Duration of the experiment [s]

duration = 20*24*60*60;

tspan = [0 duration];

%% Calculated parameters

% Leakage multiplier for the substrate (Gap area/Gap length*Dair*H) [m3/s]

Lm_Substrate = Lf*Dair*H;

% Leakage factor for CO2 (Gap area/Gap length*Dair) [m3/s]

Lm_12CO2 = Lf*Dair_12CO2;

Lm_13CO2 = Lf*Dair_13CO2;

% Effective diffusivity [m2/s]

Deffbc = tortbc*pbc*Daq;

% Shell thickness [m]

drbc = Rbc/nbc;

% Number of particles [-]

Np = Mbc/dbc/(1-pbc)/4*3/pi/Rbc^3;

% Extraparticular soil water volume [m3]

Vwsoil = WCsoil*Msoil;

% Extraparticular soil air volume [m3]

Vasoil = Vbatch-Vwsoil-Msoil/dsoil;

%% Calculate areas, volumes and capacities

% Radii of the biochar shells, including the innermost radius of zero

rvec=[0:nbc]'*drbc;

% Corresponding areas times the number of particles [m2]

Avec=4*pi*rvec.^2*Np;

% Volumes of the shells times the number of particles [m3]

Vshell=4/3*pi*(rvec(2:end).^3-rvec(1:end-1).^3)*Np;

% Volume of water in the shells [m3]

Vwshell=Vshell*pbc;

% Compute the mass of solids for all shells [kg]

Mshell=Vshell*(1-pbc)*dbc;

% Compute the capacity for each element [m3] or [kg]

Capacity=[Vwshell+Mshell*Kbc;Vwsoil+Vasoil*H;Msoil;Vwsoil;...

Vasoil+Vwsoil/H_CO2*(1+K1/10^-pH+K1*K2/10^-(2*pH));...

Vasoil+Vwsoil/H_CO2*(1+K1/10^-pH+K1*K2/10^-(2*pH))];

%% determine sparsity pattern

ivec=nan(nbc+5+2*nbc+8,1);

jvec=nan(nbc+5+2*nbc+8,1);

avec=ones(nbc+5+2*nbc+8,1);

% self relationship

index = 0;

add = nbc+5;

ivec(index+1:index+add)=1:nbc+5;

jvec(index+1:index+add)=1:nbc+5;

index = index+add;

% biochar shell relationships to outer neighbor and soil porewater

add = nbc;

ivec(index+1:index+add)=1:nbc;

jvec(index+1:index+add)=2:nbc+1;

index = index+add;

% biochar shell relationships to inner neighbor and soil porewater

```
add = nbc;
ivec(index+1:index+add)=2:nbc+1;
jvec(index+1:index+add)=1:nbc;
index = index+add;
% soil porewater -> solid relationship
add = 1;
ivec(index+1:index+add)=nbc+1;
jvec(index+1:index+add)=nbc+2;
index = index+add;
% soil solid -> soil porewater relationship
add = 1;
ivec(index+1:index+add)=nbc+2;
jvec(index+1:index+add)=nbc+1;
index = index+add;
% soil porewater -> biomass relationship
add = 1;
ivec(index+1:index+add)=nbc+1;
jvec(index+1:index+add)=nbc+3;
index = index+add;
% biomass -> soil porewater relationship
add = 1;
ivec(index+1:index+add)=nbc+3;
jvec(index+1:index+add)=nbc+1;
index = index+add;
% additional relationship 13CO2 -> pollutant in soil water
add = 1;
ivec(index+1:index+add)=nbc+4;
jvec(index+1:index+add)=nbc+1;
index = index+add;
% additional relationship 13CO2 -> biomass in soil water
add = 1;
ivec(index+1:index+add)=nbc+4;
jvec(index+1:index+add)=nbc+3;
index = index+add;
% additional relationship 12CO2 -> pollutant in soil water
```

```
add = 1;
ivec(index+1:index+add)=nbc+5;
jvec(index+1:index+add)=nbc+1;
index = index+add;
% additional relationship 12CO2 -> biomass in soil water
add = 1;
ivec(index+1:index+add)=nbc+5;
jvec(index+1:index+add)=nbc+3;
index = index+add;
% make a sparse matrix of that
sparsepat=sparse(ivec,jvec,avec);


% set options (absolute and relative tolerance, sparsity pattern,
%          enforce non-negativity for all concentrations)
options=odeset('abstol',1e-13,'reltol',1e-7,'Jpattern',sparsepat,...
          'nonnegative',[1:nbc+5]);


%% Solving the system of differential equations
[time,concentrations] = ode15s(@GeorgeBatchDGLV3,tspan,c0,options,...
   Lm_Substrate,Lm_13CO2,Lm_12CO2,C12_atm,C13_atm,drbc,Deffbc,Avec,...
   Vwsoil,ksoil,Ksoil,lag,umax,KS,Y,kdec,Cbmax,C12_C13_ratio,BGResp,...
   Capacity,nbc);


%% Extracting solutions and plotting
% Toluene prediction water [g C/L]
Cw=concentrations(:,nbc+1)*12/1000;
% Toluene prediction air [g C/cm3]
Ca=concentrations(:,nbc+1)*H*12/1000;
% Toluene prediction BC intraparticle porewater concentration [g C/L]
IntraPartCw=concentrations(:,1:nbc)*12/1000;
% Toluene prediction concentration soil [g C/kg]
Csoil=concentrations(:,nbc+2)*12;
% Biomass prediction [g C/L]
Cb=concentrations(:,nbc+3)*12/1000;
% 13C CO2 prediction concentration in air [g C/L]
```

```
C13co2=concentrations(:,nbc+4)*12/1000;
% 12C CO2 prediction concentration in air [g C/L]
C12co2=concentrations(:,nbc+5)*12/1000;


% Experimental data
% Toluene data [time in s Conc in Toluene-C g/cm3]
Tol_data = [3*24*3600 0.000126333;...
    11*24*3600 0.0000162357;...
    14*24*3600 0.0000364628;...
    17*24*3600 0.0000572168];


% 13C CO2 data [time in s Conc in CO2 13-C g/cm3]
C13co2_data = [2*24*3600 0.0000000000;...
    3*24*3600 0.0000136668;...
    4*24*3600 0.0000165964;...
    5*24*3600 0.0000492275;...
    6*24*3600 0.000144001;...
    7*24*3600 0.000318779;...
    8*24*3600 0.000616431;...
    9*24*3600 0.001024461;...
    10*24*3600 0.001423464;...
    11*24*3600 0.001533097;...
    12*24*3600 0.002824531;...
    13*24*3600 0.003461073;...
    14*24*3600 0.003516627;...
    15*24*3600 0.004348111;...
    16*24*3600 0.005053128;...
    17*24*3600 0.0053148;...
    18*24*3600 0.005533387;...
    19*24*3600 0.006039331];


% 12C CO2 data [time in s Conc in CO2 12-C g/cm3]
C12co2_data = [2*24*3600 0.000345635;...
    3*24*3600 0.000914131;...
    4*24*3600 0.000603741;...
```

```
    5*24*3600 0.000623199;...
    6*24*3600 0.000798527;...
    7*24*3600 0.001100869;...
    8*24*3600 0.001420499;...
    9*24*3600 0.001808232;...
    10*24*3600 0.002063057;...
    11*24*3600 0.002084425;...
    12*24*3600 0.002975414;...
    13*24*3600 0.003382422;...
    14*24*3600 0.003408841;...
    15*24*3600 0.003544204;...
    16*24*3600 0.004225365;...
    17*24*3600 0.003853549;...
    18*24*3600 0.004315954;...
    19*24*3600 0.00468257];


% Calculate the sum of squared residuals for data fitting
PredictionsToluene = interp1(time,Ca,Tol_data(:,1));
ResidualsToluene = PredictionsToluene-Tol_data(:,2);
SquaredResidualsToluene = ResidualsToluene.*ResidualsToluene;


PredictionsC13co2 = interp1(time,C13co2,C13co2_data(:,1));
ResidualsC13co2 = PredictionsC13co2-C13co2_data(:,2);
SquaredResidualsC13co2 = ResidualsC13co2.*ResidualsC13co2;


PredictionsC12co2 = interp1(time,C12co2,C12co2_data(:,1));
ResidualsC12co2 = PredictionsC12co2-C12co2_data(:,2);
SquaredResidualsC12co2 = ResidualsC12co2.*ResidualsC12co2;


SumSquaredResiduals = sum(SquaredResidualsToluene)+...
    sum(SquaredResidualsC12co2)+sum(SquaredResidualsC13co2)


subplot(2,2,1);
plot(time/86400,Ca,Tol_data(:,1)/86400,Tol_data(:,2),'d');
xlabel('t [d]');
```

```
ylabel('c [g/L]');
title('Substrate C concentration in soil air')


subplot(2,2,2);
plot(time/86400,Cb);
xlabel('t [d]');
ylabel('c [g/L]');
title('Biomass C concentration in soil water')


subplot(2,2,3);
plot(time/86400,C13co2,C13co2_data(:,1)/86400,C13co2_data(:,2),'d');
xlabel('t [d]');
ylabel('c [g/L]');
title('Carbon dioxide 13C concentration')


subplot(2,2,4);
plot(time/86400,C12co2,C12co2_data(:,1)/86400,C12co2_data(:,2),'d');
xlabel('t [d]');
ylabel('c [g/L]');
title('Carbon dioxide 12C concentration')
```

2. Function

```
function dcdt = GeorgeBatchDGLV3(t,c, Lm_Substrate,Lm_13CO2,Lm_12CO2,...
   C12_atm,C13_atm,drbc,Deffbc,Avec,Vwsoil,ksoil,Ksoil,lag,umax,KS,Y,...
   kdec,Cbmax,C12_C13_ratio,BGResp,Capacity,nbc)


% Compute the leakage mass transfer rates
r_leak_substrate = Lm_Substrate*c(nbc+1);
r_leak_13C = -Lm_13CO2*(C13_atm-c(nbc+4));
r_leak_12C = -Lm_12CO2*(C12_atm-c(nbc+5));
if Capacity(nbc) > 0
% Extend the BC porewater concentration vector at the inside
cext=[c(1);c(1:nbc+1)];
% Compute the total diffusive mass transfer rate at all BC interfaces
```

```
r_ipd = -diff(cext)/drbc*Deffbc.*Avec;
% For mass transfer from the the outermost shell to the free water, you
% only have to cover half the distance
r_ipd(end)=r_ipd(end)*2;
else
r_ipd = zeros(nbc+1,1);
end
if Capacity(nbc+2) > 0
% Sorption by the soil with first order kinetics
r_sorption = -ksoil*Vwsoil*(c(nbc+2)./Ksoil-c(nbc+1));
else
r_sorption = 0;
end
% Biomass-C production rate, Monod, Logistic
r_prod_13C = 0;
r_prod_12C = 0;
if t > lag
r_prod_13C = umax*c(nbc+1)/(KS+c(nbc+1))*c(nbc+3)*(Cbmax-c(nbc+3))/...
    Cbmax*Vwsoil;
r_prod_12C = C12_C13_ratio*r_prod_13C;
end
% Biomass-C decay rate
r_dec_13C = 1/(1+C12_C13_ratio)*kdec*c(nbc+3)*Vwsoil;
r_dec_12C = C12_C13_ratio/(1+C12_C13_ratio)*kdec*c(nbc+3)*Vwsoil;
% Substrate-C mass removal by degradation rate
r_deg_13C = r_prod_13C/Y;
% CO2-C production
r_prod_13C_CO2 = r_prod_13C/Y*(1-Y)+r_dec_13C;
r_prod_12C_CO2 = r_prod_12C/Y*(1-Y)+r_dec_12C+BGResp;
% Mass balance for all shells and the extraparticular water
if Capacity(nbc) > 0 && Capacity(nbc+2) > 0
dcdt=[-diff(r_ipd);r_ipd(end)-r_deg_13C-r_sorption-r_leak_substrate;...
    r_sorption;r_prod_13C-r_dec_13C+r_prod_12C-r_dec_12C;...
    r_prod_13C_CO2-r_leak_13C;r_prod_12C_CO2-r_leak_12C]./Capacity;
elseif Capacity(nbc) == 0 && Capacity(nbc+2) > 0
```

```
dcdt=[zeros(nbc,1);-r_deg_13C-r_sorption-r_leak_substrate;r_sorption;...
    r_prod_13C-r_dec_13C+r_prod_12C-r_dec_12C;r_prod_13C_CO2-r_leak_13C;...
    r_prod_12C_CO2-r_leak_12C]./[ones(nbc,1);Capacity(nbc+1:nbc+5)];
elseif Capacity(nbc) > 0 && Capacity(nbc+2) == 0
dcdt=[-diff(r_ipd);-r_deg_13C-r_leak_substrate;0;...
    r_prod_13C-r_dec_13C+r_prod_12C-r_dec_12C;r_prod_13C_CO2-r_leak_13C;...
    r_prod_12C_CO2-r_leak_12C]./[Capacity(1:nbc+1);1;...
    Capacity(nbc+3:nbc+5)];
else
dcdt=[zeros(nbc+3,1);-r_leak_13C;-r_leak_12C]./[ones(nbc+3,1);...
    Capacity(nbc+4:nbc+5)];
end
```

```
File        :M:\MS\georgevhcsplit4sep14\dwgvocsoil1sp.D
Operator    : p.d
Acquired    :  4 Sep 2014  12:51     using AcqMethod DWVHCSC2SPL.M
Instrument  :   5975C
Sample Name: vhc
Misc Info   : soil 1 voc 1/1000ul inj ss mh sc split
Vial Number: 3
```
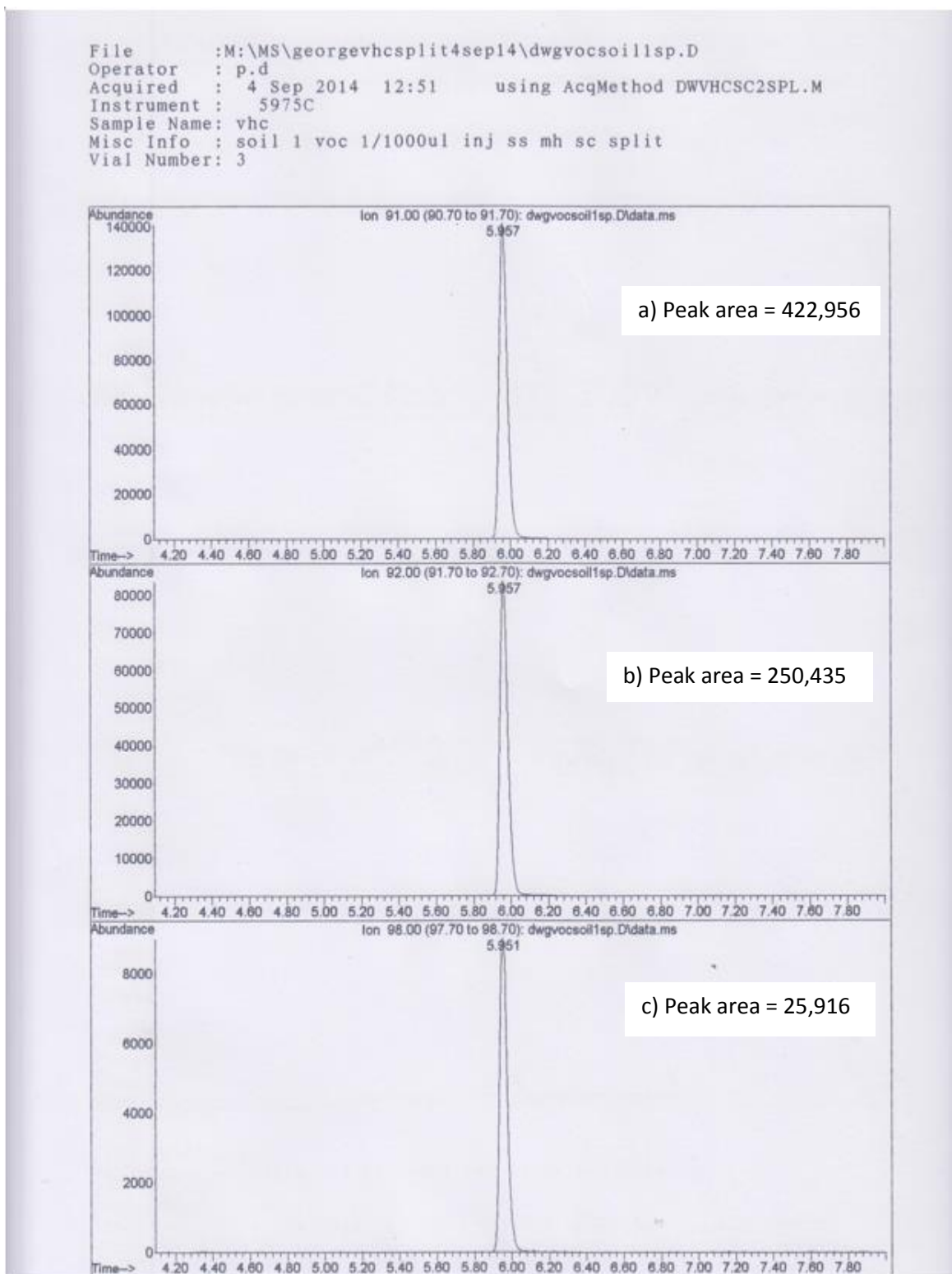

*Figure 9.2. Gas Chromatography Mass Spectrometry peaks of $^{12}C_6$ toluene internal standard extracts for **a**) ion 91 **b**) ion 92 and $^{13}C_7$ toluene **c**) ion 98 from soil batch (1) at the end of biodegradation experiments.*

```
File        :M:\MS\georgevhcsplit4sep14\dwgvocsoil2sp.D
Operator    : p.d
Acquired    : 4 Sep 2014  13:43    using AcqMethod DWVHCSC2SPL.M
Instrument  :  5975C
Sample Name: vhc
Misc Info   : soil 2 voc 1/1000ul inj ss mh sc split
Vial Number: 4
```
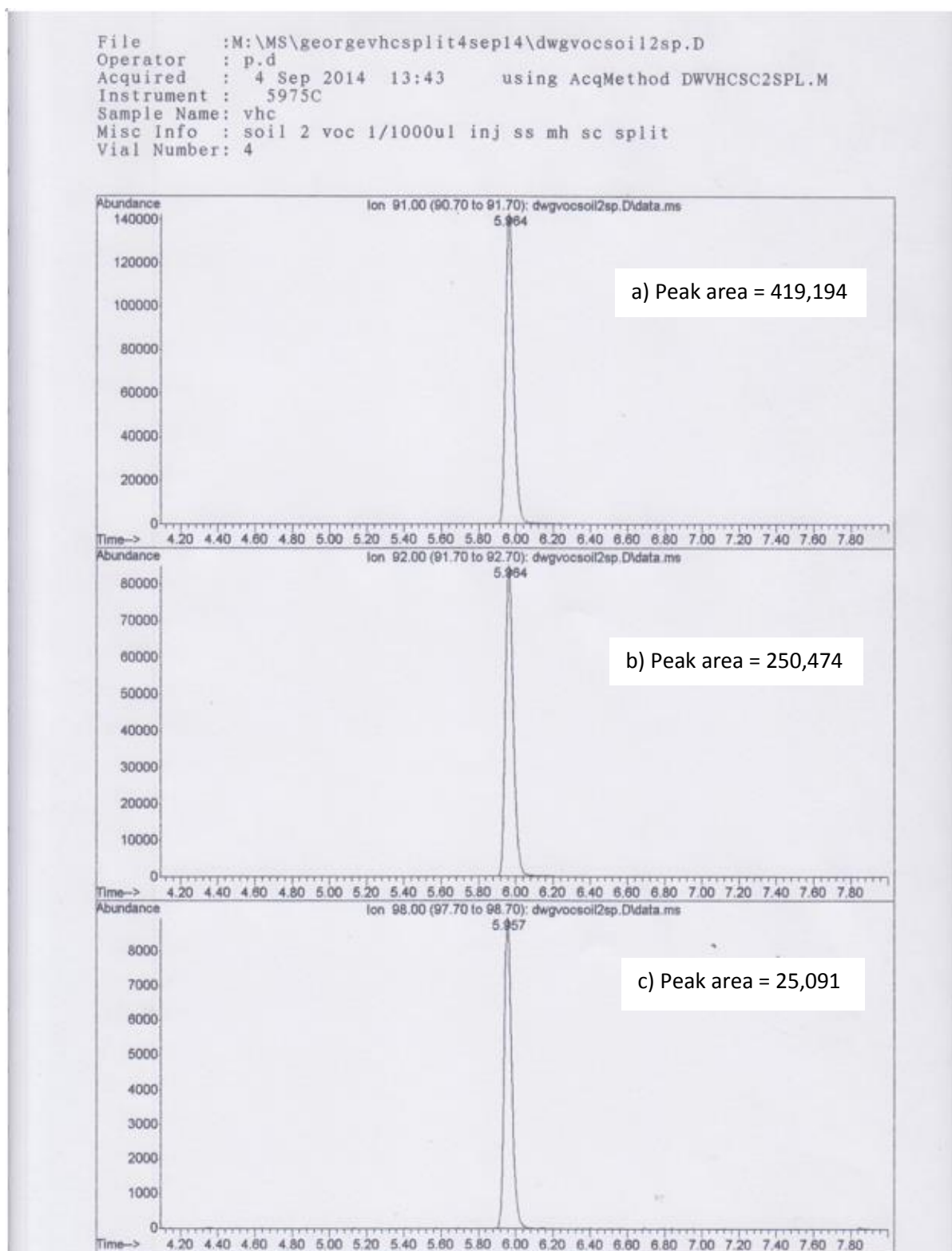

*Figure 9.3. Gas Chromatography Mass Spectrometry peaks of $^{12}C_6$ toluene internal standard extracts for **a)** ion 91 **b)** ion 92 and $^{13}C_7$ toluene **c)** ion 98 from soil batch (1) at the end of biodegradation experiments.*
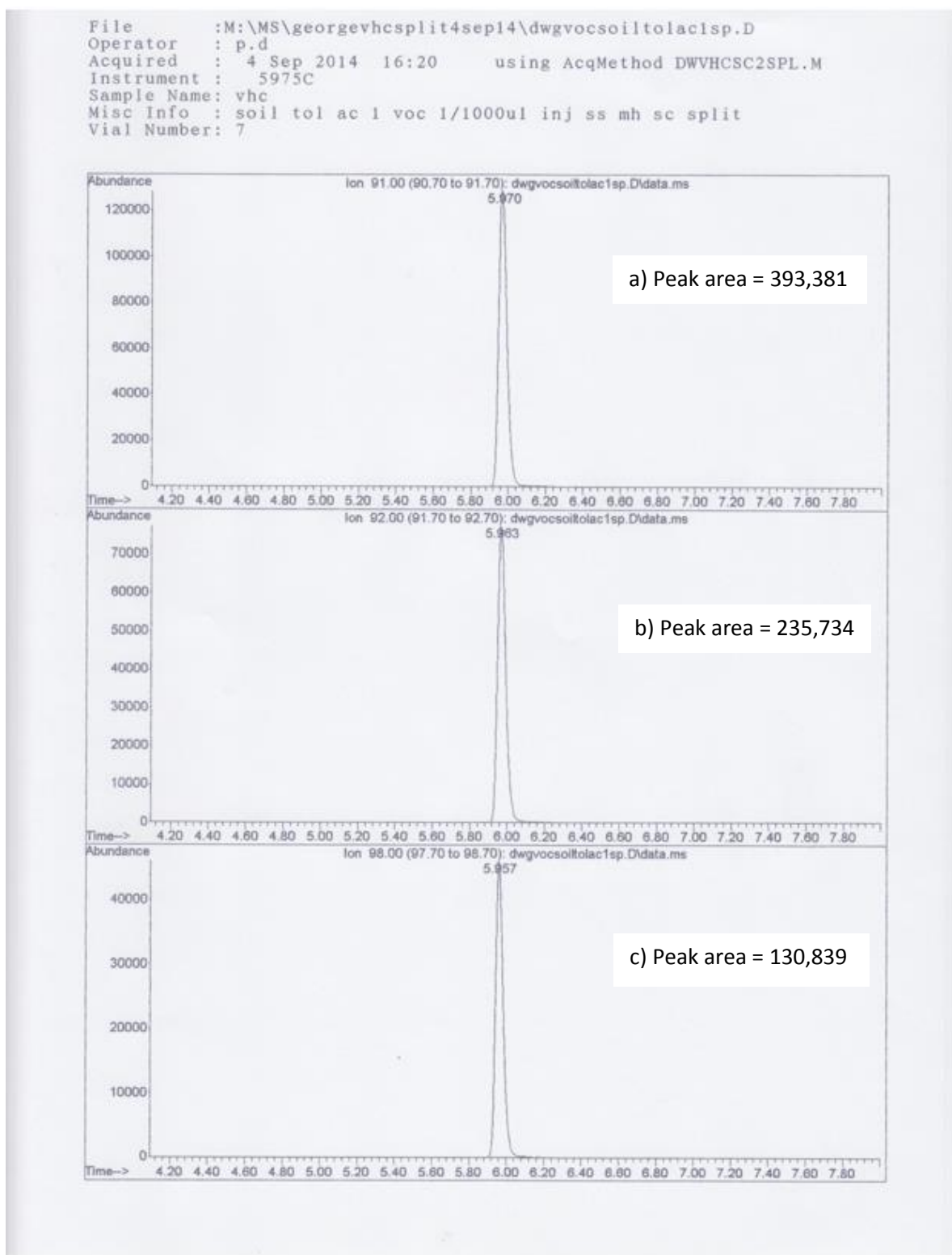
*Figure 9.4. Gas Chromatography Mass Spectrometry peaks of $^{12}C_6$ toluene internal standard extracts for **a**) ion 91 **b**) ion 92 and $^{13}C_7$ toluene **c**) ion 98 from soil & biochar batch (1) at the end of biodegradation experiments.*
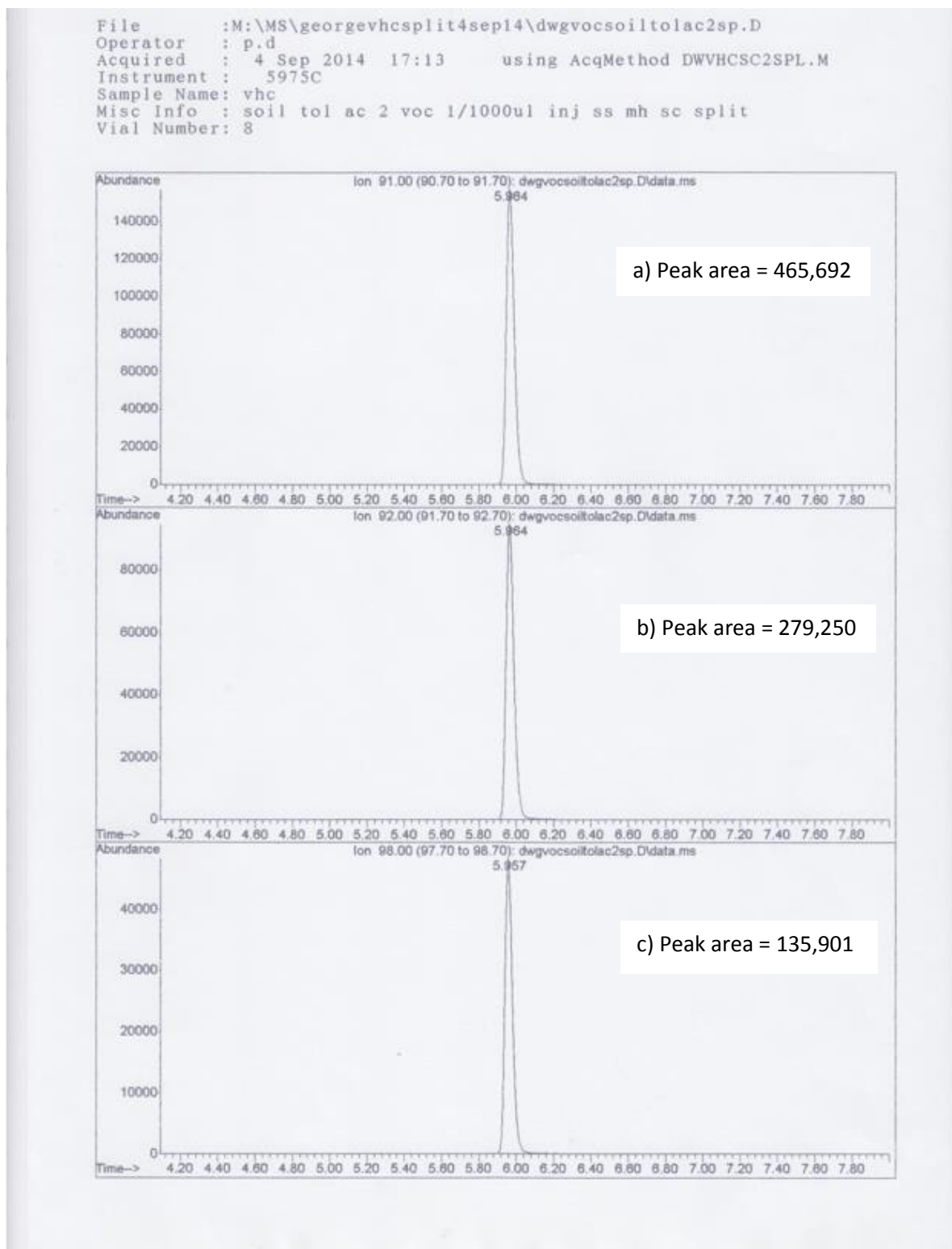
*Figure 9.5. Gas Chromatography Mass Spectrometry peaks of $^{12}C_6$ toluene internal standard extracts for **a)** ion 91 **b)** ion 92 and $^{13}C_7$ toluene **c)** ion 98 from soil & biochar batch (2) at the end of biodegradation experiments.*

```
File        :M:\MS\georgevhcsplit4sep14\dwgvocsoilac2sp.D
Operator    : p.d
Acquired    : 4 Sep 2014  15:28      using AcqMethod DWVHCSC2SPL.M
Instrument  :   5975C
Sample Name: vhc
Misc Info   : soil ac 2 voc 1/1000ul inj ss mh sc split
Vial Number: 6
```
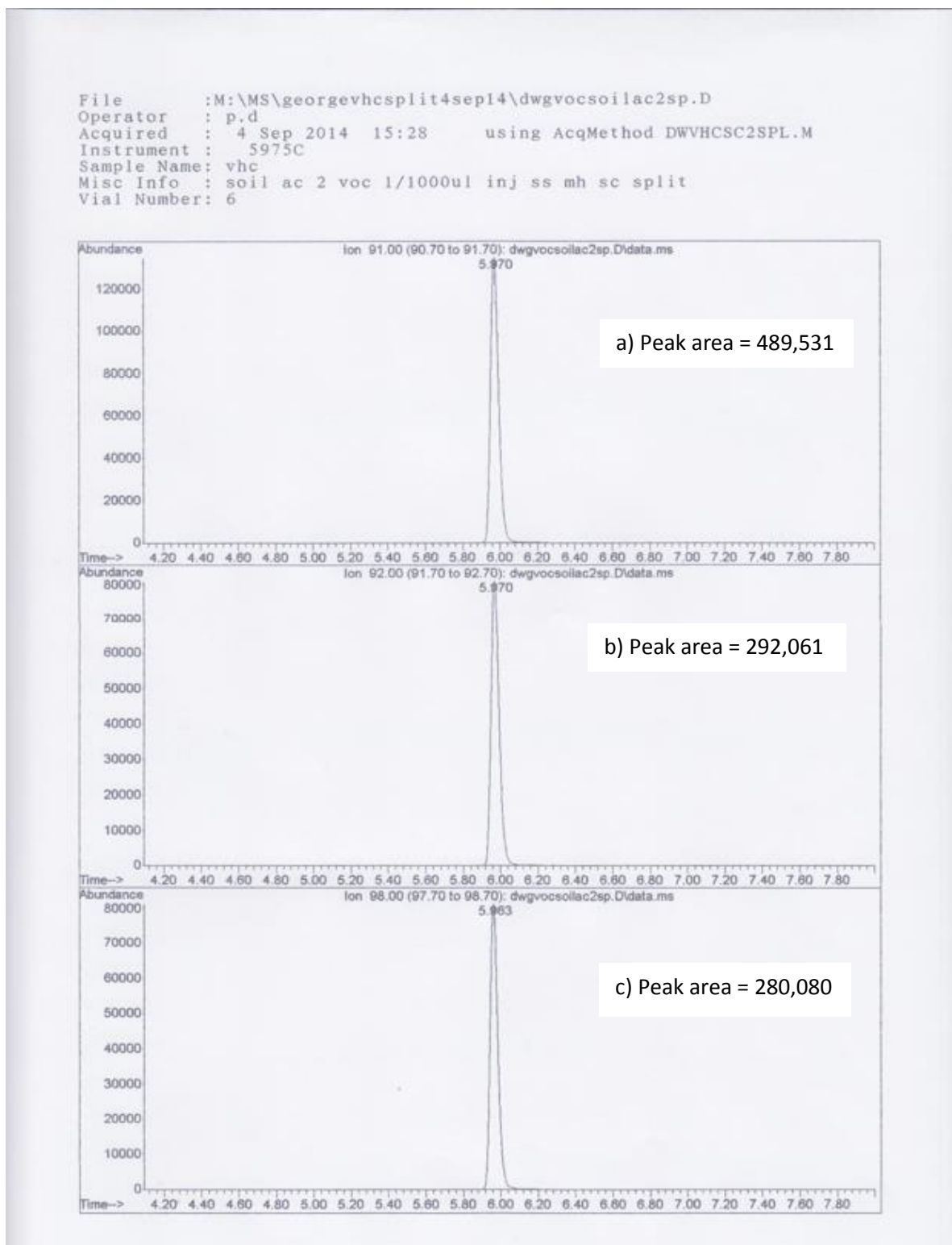

*Figure 9.6. Gas Chromatography Mass Spectrometry peaks of $^{12}C_6$ toluene internal standard extracts for **a**) ion 91 **b**) ion 92 and $^{13}C_7$ toluene **c**) ion 98 from soil & activated carbon batch (1) at the end of biodegradation experiments.*

```
File        :M:\MS\georgevhcsplit4sep14\dwgvocsoilac1sp.D
Operator    : p.d
Acquired    : 4 Sep 2014  14:36    using AcqMethod DWVHCSC2SPL.M
Instrument  : 5975C
Sample Name: vhc
Misc Info   : soil ac 1 voc 1/1000ul inj ss mh sc split
Vial Number: 5
```
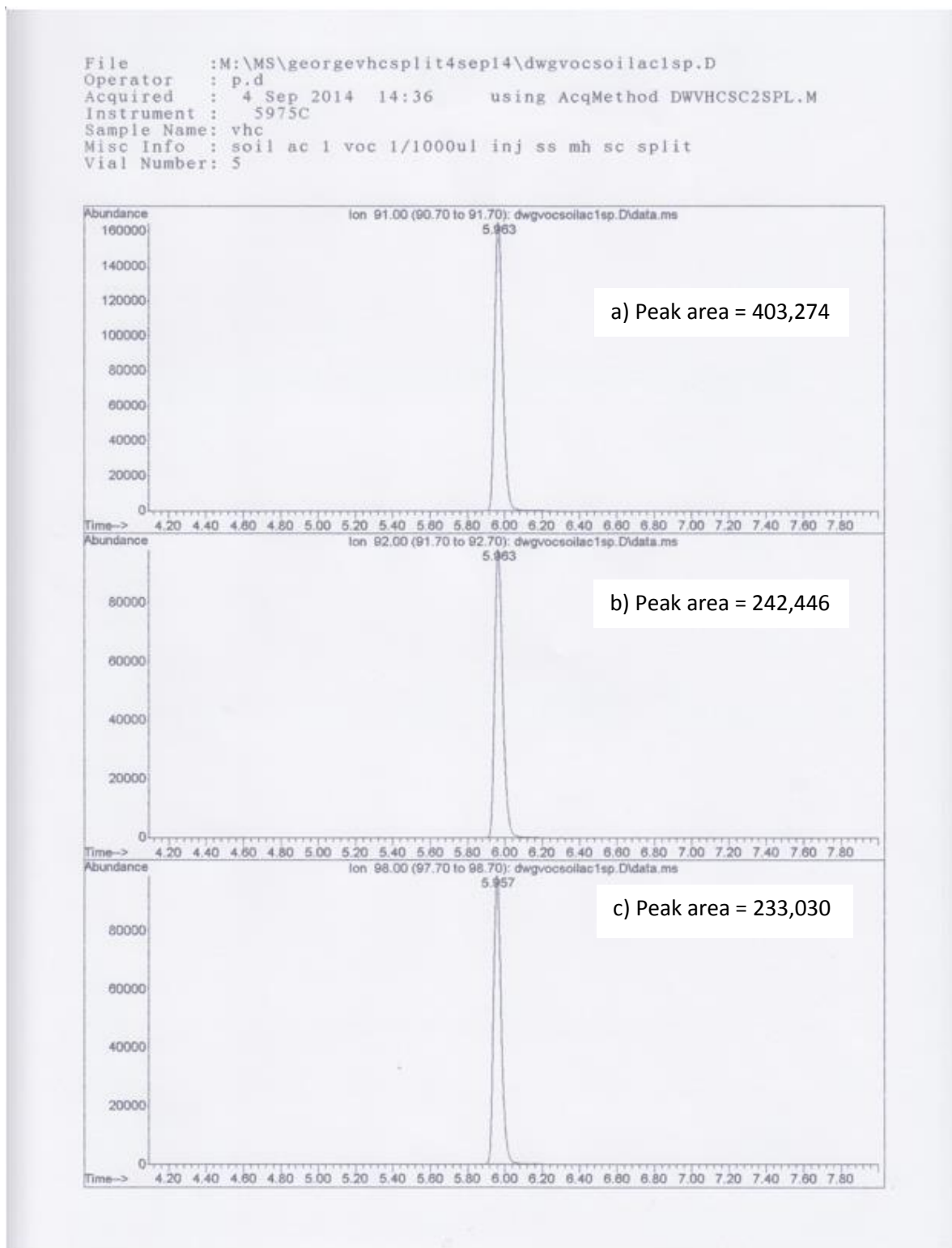

*Figure 9.7. Gas Chromatography Mass Spectrometry peaks of $^{12}C_6$ toluene internal standard extracts for **a**) ion 91 **b**) ion 92 and $^{13}C_7$ toluene **c**) ion 98 from soil & activated carbon batch (2) at the end of biodegradation experiments.*

Sample label	Calcium	Magnesium	Sodium	Potassium	Zinc	Aluminium	Silicon
Blank	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Standard	60.0±36.1	20.0±10.0	10.0±5.0	20.0±10.0	4.0±2.0	2.0±1.0	10.0±5.0
Soil	64.9±0.5	9.6±0.1	15.4±0.4	4.3±0.0	0.0±0.0	0.0±0.0	6.3±0.1
Soil + 2% Biochar	113.5±15.2	13.4±2.2	15.6±1.8	6.2±0.4	0.0±0.0	0.0±0.0	5.4±0.8
Soil + 2% AC	40.2±0.3	6.1±0.0	12.9±0.0	3.4±0.0	0.0±0.0	0.0±0.0	5.9±0.0

Table 9.2. Metal ion concentrations (mg/L) in pore-water extracted from soil, soil & biochar and soil & AC. Measurements were determined using an Inductively Coupled Plasma- Optical Emission Spectrometer (ICP-OES).

Appendix B

Sample	Petroleum hydrocarbons	No of sequences > 200 bp	No of sequences assigned to domain bacteria	No of sequences classified below domain level	No of sequences assigned to domain archaea	No of sequences classified below domain level	Sequences not assigned to any domain
Soil.1	N/A	28509	24361	24246	4146	4120	2
Soil.2	N/A	27360	22114	21984	5224	5190	22
Soil.3	N/A	31792	26846	26627	4938	4925	8
Arom.1	Aromatics	26042	20359	20266	5669	5647	14
Arom.2	Aromatics	30756	24957	24823	5792	5781	7
Arom.3	Aromatics	23758	19923	19876	3830	3824	5
Straightalk.1	Straight alkanes	29687	28219	28170	1467	1467	1
Straightalk.2	Straight alkanes	22119	21114	21078	1000	998	5
Straightalk.3	Straight alkanes	29937	28773	28712	1160	1160	4
Cyclbr.1	Cyc/branched alks	26116	23168	23101	2947	2935	1
Cyclbr.2	Cyc/branched alk	24129	21393	21347	2723	2709	13
Cyclbr.3	Cyc/branched alk	28662	24858	24798	3795	3784	9

Table 9.3.A summary of the number of sequences > 200 base pairs following quality filtering and assigned to the bacterial and archaea domain per sample in the Ion torrent (PGM) - generated dataset.

a)

Taxon level OTU	Soil	Soil & aromatics	Soil & Str. alkanes	Soil & cyc/branched
Acidobacteria	7.5±1.0	3.0±0.9	3.8±0.5	4.3±0.1
Actinobacteria	11.51.3	13.3±1.3	21.1±1.3	10.0±0.8
Chloroflexi	8.5±0.1	8.3±1.1	5.3±0.9	6.5±0.4
Firmicutes	8.0±1.4	15.7±0.5	7.4±0.7	10.3±1.6
Gemmatimonadetes	5.3±0.4	4.6±0.3	2.1±0.1	3.7±0.2
Planctomycetes	11.2±1.3	10.2±0.8	5.3±0.5	7.0±0.3
Alphaproteobacteria	14.3±1.8	16.3±1.1	10.6±1.6	12.2±0.4
Betaproteobacteria	5.3±0.4	5.7±0.5	10.4±0.8	3.2±0.5
Deltaproteobacteria	9.2±0.2	5.2±0.8	3.8±0.6	5.3±0.1
Gammaproteobacteria	5.1±0.8	5.8±0.4	24.3±2.9	25.9±1.4

b)

Taxon level OTU	Soil	Soil & aromatics	Soil & Str. alkanes	Soil & cyc/branched
Crenarchaeota	12.3±1.6	15.9±2.2	3.7±0.5	10.2±1.2
Acidobacteria	8.1±0.2	4.0±0.6	3.0±0.6	3.4±0.4
Actinobacteria	8.0±0.2	11.2±0.4	12.1±1.1	7.1±0.8
Chloroflexi	6.5±0.3	6.4±0.3	2.8±0.2	4.8±0.0
Firmicutes	8.0±1.2	13.1±0.7	7.8±0.4	10.5±0.6
Gemmatimonadetes	4.4±0.5	3.7±0.6	1.6±0.1	2.9±0.2
Planctomycetes	8.1±0.4	5.9±0.3	2.5±0.2	4.1±0.3
Alphaproteobacteria	8.6±0.2	12.7±2.7	6.0±0.4	9.8±0.1
Betaproteobacteria	5.2±0.9	4.4±0.3	7.7±1.1	2.3±0.3
Deltaproteobacteria	7.2±0.4	3.6±0.7	2.4±0.2	3.2±0.3
Gammaproteobacteria	7.2±1.1	6.8±1.2	45.5±1.1	34.3±2.6

*Table 9.4. A summary of the percentage abundances of dominant OTUs (>3.0% of total relative abundance) at the phylum level of taxonomy for the different soil treatments for **a)** 454-pyrosequencing data and **b)** Ion torrent datasets respectively.*
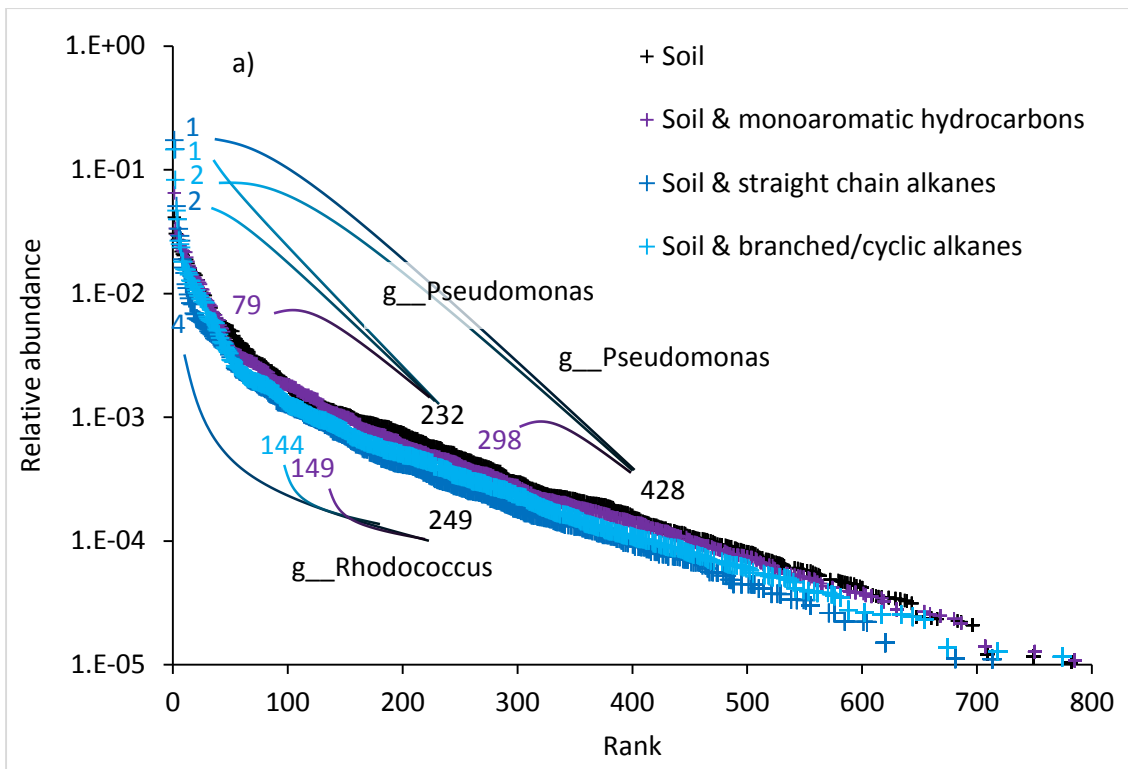
Figure 9.8. A graph showing the increase in OTU relative abundances versus their relative abundance ranking in the control (uncontaminated soil) following addition of different petroleum hydrocarbon classes to soil.
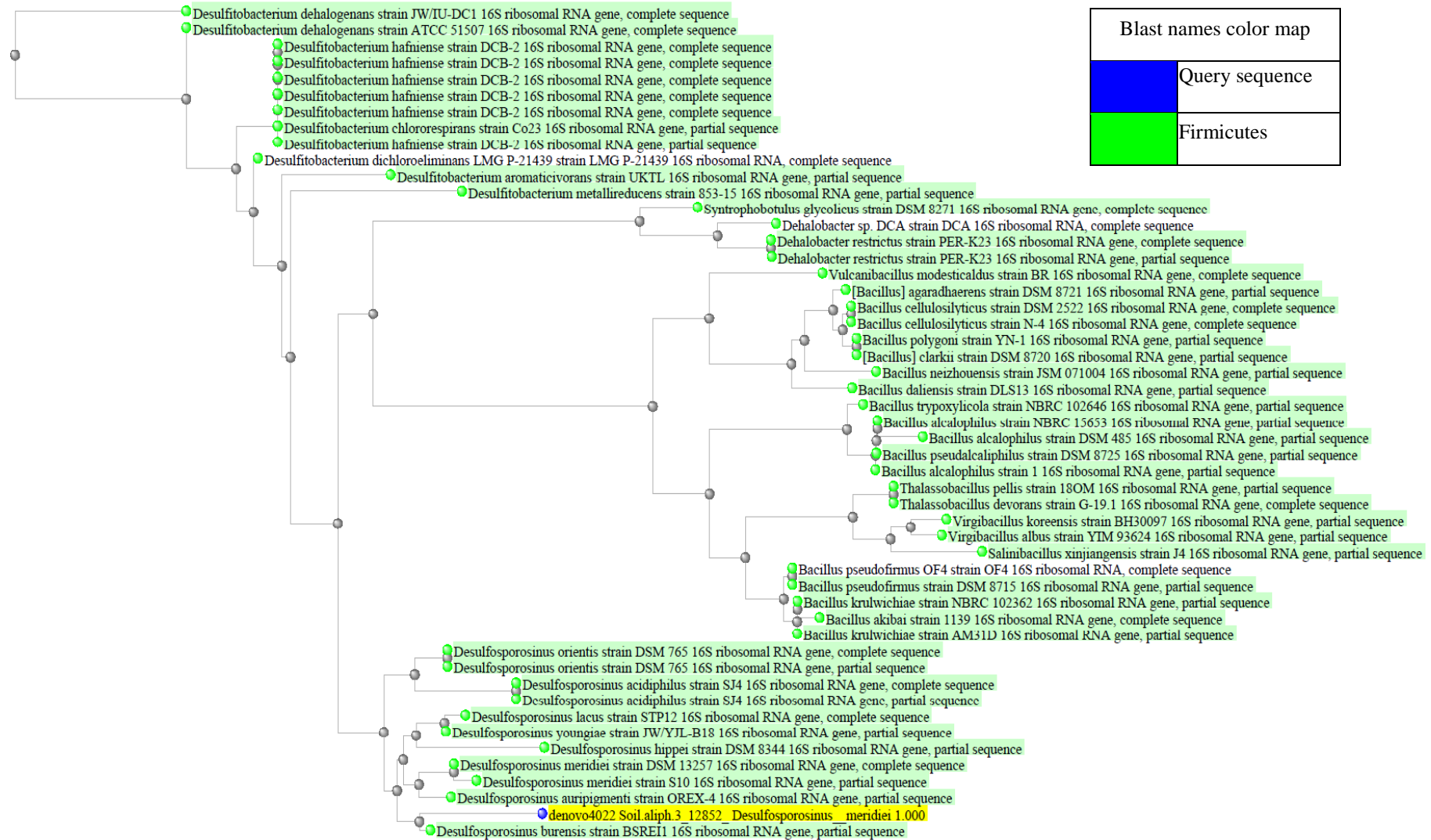
Figure 9.9. Phylogenetic comparison of 16S rRNA gene sequence from an enriched Desulfosporosinus meridiei (Table 4.3) and closest as well as distant relatives from top 50 BLAST hits (16S rRNA sequences).

Target organisms (Species)	Strain	% Similarity	Ribosomal RNA gene sequence	Source/Reference
Pseudomonas corrugata	-	94	Partial sequence	Myung et al. (2010)
Psuedomonas mohnii	IpA-2	94	Partial sequence	Camara et al. (2007)
Psuedomonas moorei	RW10	94	Partial sequence	Camara et al. (2007)
Psuedomonas jessenii	CIP 105274	94	Partial sequence	Verhille et al. (1999)
Pseudomonas kilonensis	520-20	94	Complete sequence	Sikorski et al. (2001)
Pseudomonas mucidolens	IAM 12406	95	Complete sequence	Anzai et al. (1997)
P. saponiphila	DSM 9751	95	Complete sequence	Lang et al. (2010)
Pseudomonas cuatrocienegasensis	1N	93	Partial sequence	Escalante et al. (2009)
Pseudomonas fluorescens	Pf0-1	93	Complete sequence	Silby et al. (2009)
Pseudomonas fluorescens	NBRC 14160	93	Partial sequence	Unpublished
Desulfosporosinus burensis	BSREI1	98	Partial sequence	Mayeux et al. (2013)
Desulfosporosinus meridiei	DSM 13257	98	Complete sequence	Pester et al. (2012)
Desulfosporosinus lacus	STP12	98	Complete sequence	Ramamoorthy et al. (2006)
D. orientis	DSM 765	98	Complete sequence	Pester et al. (2012)
Desulfosporosinus youngiae	JW/YJL-B18	98	Partial sequence	Lee et al. (2009)
Bacillus pseudofirmus	DSM 8715	91	Partial sequence	Nielsen et al. (1994)
Bacillus akibai	1139	90	Complete sequence	Nogi et al. (2005)
Bacillus alcalophilus	DSM 485	90	Partial sequence	Ash et al. (1991)
Salinibacillus xinjiangensis	J4	90	Partial sequence	Yang et al. (2009)
Virgibacillus albus	YIM 93624	90	Partial sequence	Zang et al. (2012)

Table 9.5. A summary of BLAST returned hits (close and distant relatives) of the enriched Pseudomonas umsongensis and Desulfosporosinus meridiei from one of the batch studies.

Taxon level OTU	Soil	Biochar	Activated carbon
Acidobacteria	6.3±0.3	2.4±0.4	1.9±0.5
Actinobacteria	11.2±1.7	4.1±0.3	5.0±0.7
Bacteriodetes	3.3±0.4	10.8±2.4	7.7±6.0
Chloroflexi	6.5±0.6	5.1±2.2	5.4±2.0
Firmicutes	8.2±1.3	11.0±2.2	8.2±4.0
Gemmatimonadetes	5.1±0.4	1.0±0.0	1.4±0.2
Planctomycetes	13.8±7.0	2.4±04	3.0±0.5
Alphaproteobacteria	11.4±0.3	3.5±0.1	3.5±0.2
Betaproteobacteria	6.8±0.2	11.9±1.1	11.8±1.7
Deltaproteobacteria	6.9±0.7	10.1±4.8	10.6±5.3
Gammaproteobacteria	11.7±0.9	29.5±13.4	33.0±16.1

Table 9.6. A summary of the percentage abundances of dominant OTUs (>1.0% of total relative abundance) at the phylum level of taxonomy for the batch study controls: soil, pure biochar (BC) and activated carbon (AC) for 454-pyrosequencing data.

Taxon level OTU	SoilWON	SoilWN	Soil & BCWON	Soil & BCWN	Soil & ACWON	Soil & ACWN
Acidobacteria	3.7±1.5	5.0±0.4	5.9±0.5	6.4±0.2	4.4±0.2	5.6±0.1
Actinobacteria	4.2±1.5	6.9±1.5	10.5±2.1	10.7±0.3	13.0±7.1	12.4±0.4
Bacteriodetes	1.8±0.2	1.6±0.0	3.0±0.3	2.8±0.4	2.6±0.2	2.6±0.1
Chloroflexi	3.3±1.6	4.4±0.1	6.4±0.2	5.9±0.4	4.1±0.3	4.6±0.5
Firmicutes	3.3±1.1	4.6±0.3	5.8±0.9	6.2±0.8	2.5±0.7	3.0±0.4
Gemmatimonadetes	2.6±1.0	3.5±0.1	4.4±0.7	5.7±0.6	3.3±0.3	4.1±0.7
Planctomycetes	3.3±0.5	5.6±0.7	7.4±0.7	6.6±0.3	4.5±0.3	5.7±0.3
Alphaproteobacteria	4.8±2.0	7.4±0.6	11.4±0.3	12.5±0.5	7.8±0.1	6.1±0.3
Betaproteobacteria	22.3±14.6	9.0±0.8	13.4±1.0	10.7±0.0	6.0±0.9	5.6±0.3
Deltaproteobacteria	3.7±1.6	4.8±0.2	6.6±0.8	6.7±0.0	5.0±0.2	4.5±0.3
Gammaproteobacteria	40.3±29.2	38.6±1.0	16.7±2.4	15.6±1.3	35.5±0.4	41.8±7.7

Table 9.7. A summary of the percentage abundances of dominant OTUs (>1.0% of total relative abundance) at the phylum level of taxonomy for the different soil treatments, with and without sorbent and nutrient amendment for a batch study 454-pyrosequencing data.

OTU classification	%Detected		
	Soil	Biochar	Activated carbon.
Actinomycetales	**3.7±0.6**	**2.0±0.1**	**2.1±0.3**
Micrococcaceae	0.7±0.1	0.3±0.1	0.1±0.0
Micromonosporaceae	0.6±0.1	0.2±0.1	0.1±0.1
Mycobacteriaceae	0.2±0.1	0.6±0.2	0.4±0.1
Nocardioidaceae	8.0±0.1	0.2±0.0	0.3±0.2
Streptomycetaceae	0.6±0.2	0.1±0.0	0.2±0.1
Bacillales	**6.0±1.0**	**1.2±0.6**	**0.8±0.2**
Bacillaceae	0.4±0.1	0.5±0.5	0.1±0.1
Paenibacillaceae	1.1±0.2	0.1±0.1	0.2±0.1
Planococcaceae	0.3±0.1	0.1±0.0	0.1±0.0
Thermoactinomycetaceae	0.4±0.1	0.1±0.1	0.0±0.1
Pirellulales	**5.9±2.8**	**1.2±0.1**	**1.1±0.3**
Pirellulaceae	5.9±2.8	1.2±0.1	1.1±0.3
Rhizobiales	**5.3±0.5**	**1.4±0.1**	**1.4±0.1**
Bradyrhizobiaceae	0.5±0.1	0.2±0.1	0.2±0.1
Hyphomicrobiaceae	3.3±0.4	0.6±0.0	0.5±0.2
Phyllobacteriaceae	0.2±0.0	0.2±0.1	0.1±0.1
Pseudomonadales	**2.5±0.1**	**24.0±13.6**	**26.0±14.7**
Pseudomonadaceae	0.5±0.3	23.9±13.3	25.9±14.6
Moraxellaceae	0.0±0.0	0.2±0.1	0.1±0.1
Xanthomonadales	**8.2±0.7**	**2.2±0.7**	**2.7±0.8**
Sinobacteraceae	2.4±0.4	1.2±0.2	2.2±0.2
Xanthomonadaceae	1.2±0.4	0.9±0.6	5.0±1.1
Syntrophobacterales	**3.2±0.3**	**3.4±2.0**	**3.4±1.7**
Syntrophaceae	0.0±0.0	1.8±1.2	1.6±1.3
Syntrophobacteraceae	3.2±0.3	1.0±0.4	1.3±0.1
Syntrophorhabdaceae	0.0±0.0	0.6±0.4	0.5±0.5

*Table 9.8. Summary of bacterial order detected in the highest relative abundance obtained from DNA-derived pyrosequencing libraries of samples from control samples **a**) Soil **b**) Biochar and **c**) Activated carbon.*

OTU classification	%Detected						
	SoilWON	SoilWN	SoilBCWON	SoilBCWN	SoilACWON	SoilACWN	.
Actinomycetales	**1.9±0.8**	**2.1±1.3**	**5.0±1.4**	**4.5±0.2**	**9.4±6.7**	**8.5±0.8**	
Micrococcaceae	0.5±0.3	0.5±0.4	2.7±1.1	2.1±0.3	0.5±0.1	0.6±0.1	
Micromonosporaceae	0.4±0.1	0.4±0.1	0.5±0.2	0.5±0.1	0.2±0.0	0.4±0.1	
Micobacteriaceae	0.1±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.1±0.0	0.1±0.1	
Nocardioidaceae	0.3±0.0	1.0±0.6	0.5±0.0	0.8±0.1	7.9±6.6	6.6±0.9	
Streptomycetaceae	0.4±0.3	0.3±0.1	0.5±0.1	0.7±0.1	0.3±0.2	0.4±0.0	
Bacillales	**2.5±1.0**	**3.5±0.3**	**4.4±0.7**	**4.8±0.5**	**1.9±0.6**	**2.0±0.2**	
Bacillaceae	0.1±0.0	0.0±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.2±0.1	
Paenibacillaceae	0.4±0.1	0.7±0.0	0.8±0.3	1.0±0.1	0.4±0.0	0.4±0.1	
Planococcaceae	0.8±0.3	1.1±0.1	1.0±0.1	1.1±0.0	0.3±0.3	0.1±0.0	
Thermoactinomycetaceae	0.1±0.0	0.2±0.1	0.3±0.0	0.2±0.0	0.1±0.0	0.1±0.0	
Pirellulales	**1.6±0.4**	**2.7±0.5**	**3.6±0.6**	**3.2±0.1**	**2.0±0.3**	**2.2±0.2**	
Pirellulaceae	1.6±0.4	2.6±0.4	3.6±0.6	3.2±0.1	2.0±0.3	2.2±0.2	
Rhizobiales	**2.4±0.7**	**3.6±0.4**	**5.1±0.0**	**5.4±0.1**	**3.0±0.3**	**4.2±0.2**	
Bradyrhizobiaceae	0.3±0.1	0.2±0.1	0.7±0.2	0.5±0.0	0.4±0.1	0.4±0.1	
Hyphomicrobiaceae	1.4±0.4	2.2±0.6	2.4±0.3	3.4±0.1	1.8±0.4	2.4±0.1	
Phyllobacteriaceae	0.1±0.0	0.1±0.1	0.3±0.0	0.1±0.1	0.1±0.1	0.2±0.0	
Pseudomonadales	**35.6±30.8**	**31.75±0.4**	**7.9±2.9**	**5.7±0.7**	**35.2±8.7**	**24.5±1.0**	
Pseudomonadaceae	35.6±30.8	31.7±0.3	7.8±2.9	5.7±0.8	34.5±9.6	24.4±1.1	
Moraxellaceae	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.7±0.9	0.2±0.1	
Xanthomonadales	**4.0±1.2**	**6.0±0.6**	**7.9±0.8**	**8.8±0.5**	**5.7±0.7**	**9.9±0.5**	
Sinobacteraceae	1.9±0.5	2.6±0.9	3.0±0.3	3.9±0.2	3.0±0.5	7.0±0.2	
Xanthomonadaceae	1.9±0.6	3.2±0.4	4.8±0.9	4.6±0.7	2.6±0.1	2.7±0.4	

Syntrophobacterales	**2.1±0.9**	**2.6±0.1**	**3.5±0.2**	**3.4±0.2**	**2.3±0.2**	**2.7±0.0**
Syntrophaceae	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Syntrophobacteraceae	2.1±0.9	2.6±0.1	3.5±0.2	3.4±0.2	2.3±0.2	2.7±0.0
Syntrophorhabdaceae	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

Table 9.9. Summary of bacterial order detected in the highest relative abundance obtained from DNA-derived pyrosequencing libraries of samples from volatile petroleum hydrocarbon contaminated soil batches with and without sorbent amendment and with or without nutrient amendment.

Taxon (Genus level classification)	Absolute abundance increase/decrease for different treatments compared to the soil only control						
	Soil d0 Rank	Soil+VPHs	Soil+VPHs +nutrients	Soil+BC+VPHs	Soil+BC+VPHs+ nutrients	Soil+AC+VPHs	Soil+AC+VPHs+ nutrients
Brevibacillus	3.18E+04	4.15E+04	6.12E+04	1.77E+05	2.49E+05	8.54E+04	1.49E+04
Sphingopyxis	4.64E+03	0.00E+00	5.79E+05	4.21E+04	9.52E+04	1.55E+05	1.19E+05
Flavobacterium	1.93E+04	1.03E+05	6.12E+04	1.52E+05	3.51E+05	1.56E+05	5.16E+04
Achromobacter	6.50E+04	3.10E+07	9.54E+06	5.77E+06	3.72E+06	2.69E+05	5.43E+03
Acidovorax	0.00E+00	8.07E+04	1.38E+06	8.56E+05	1.98E+06	6.39E+05	1.56E+04
Hydrogenophaga	1.59E+04	4.15E+04	6.44E+04	1.72E+06	2.78E+06	5.18E+05	2.05E+05
Cupriavidus	6.60E+02	0.00E+00	0.00E+00	0.00E+00	0.00E+00	4.75E+04	3.53E+05
Mycoplana	5.32E+03	4.15E+04	1.59E+05	0.00E+00	1.12E+05	4.75E+04	1.49E+04
Pseudomonas	3.28E+05	3.54E+07	3.05E+07	3.88E+06	7.66E+06	3.14E+06	4.24E+05
Paenibacillus	1.83E+05	4.03E+05	7.51E+05	7.64E+05	1.87E+06	5.89E+05	3.71E+05
Arenimonas	3.06E+04	8.29E+04	1.62E+05	2.95E+05	5.05E+05	9.12E+04	8.21E+04
Polaromonas	4.58E+04	1.20E+05	1.28E+05	1.13E+06	1.54E+06	8.03E+05	1.03E+06
Hyphomicrobium	9.50E+04	2.93E+05	4.46E+05	4.18E+05	1.29E+06	3.54E+05	3.89E+05
Nitrospira	1.54E+05	8.47E+05	1.27E+06	1.18E+06	2.12E+06	1.52E+06	1.03E+06

Table 9.10. Absolute abundance data of OTUs where there was statistically significant difference between different treatments (sorbent and nutrient amendments) and increase in relative abundance ranking by at least 2 folds.

Factors	R Statistic	Significance level (%)
Time		
Global test	0.778	0.01
Pair-wise test		
Soil_d6, SoilBC_d6	0.625	0.03
Soil_d6, SoilAC_d6	0.406	0.03
SoilBC_d6, SoilAC_d6	1.000	0.03
Nutrient		
Global test	0.218	0.01
Pair-wise test		
NA, No_nutrients	0.298	0.04
NA, Nutrients	0.359	0.02
Amendment		
Global test	0.349	0.00
Pair-wise test		
No amendment, BC	0.625	0.03
No amendment, AC	0.406	0.03
Biochar, AC	1.000	0.03

Table 9.11. A summary of analysis of similarities (ANOSIM) R statistic and significance level for the factors time, nutrient and sorbent amendment in a short-term batch study on the effects of nutrients and sorbent amendment on microbial communities in VPH contaminated sand.
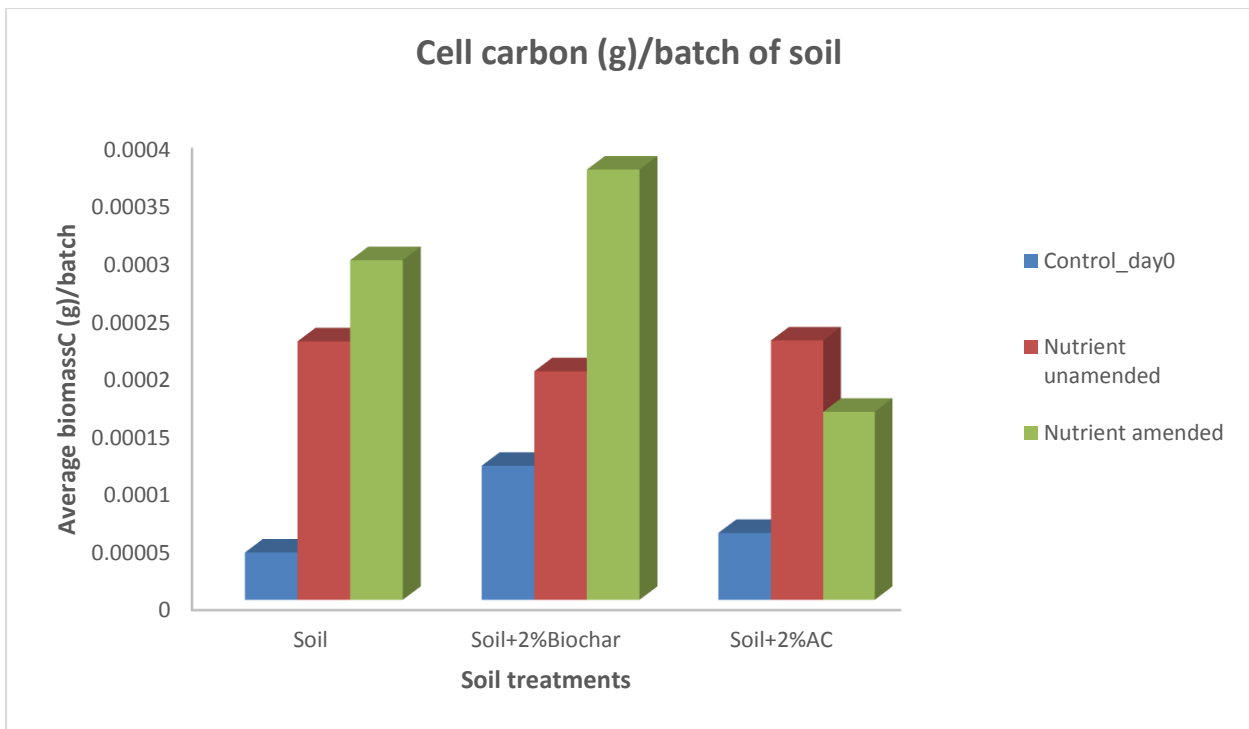
Figure 9.10. A graph showing biomass carbon (g) per batch of soil (g) in the respective batches with or without biochar or AC and with or without nutrient amendment.

Figure 9.11. Relative operational taxonomic unit (OTU) abundance distribution and phyla associations in ranked order for a) controls b) VPH + nutrient effects c) Biochar, VPHs & nutrient effects and d) AC, VPHs & nutrient effects.
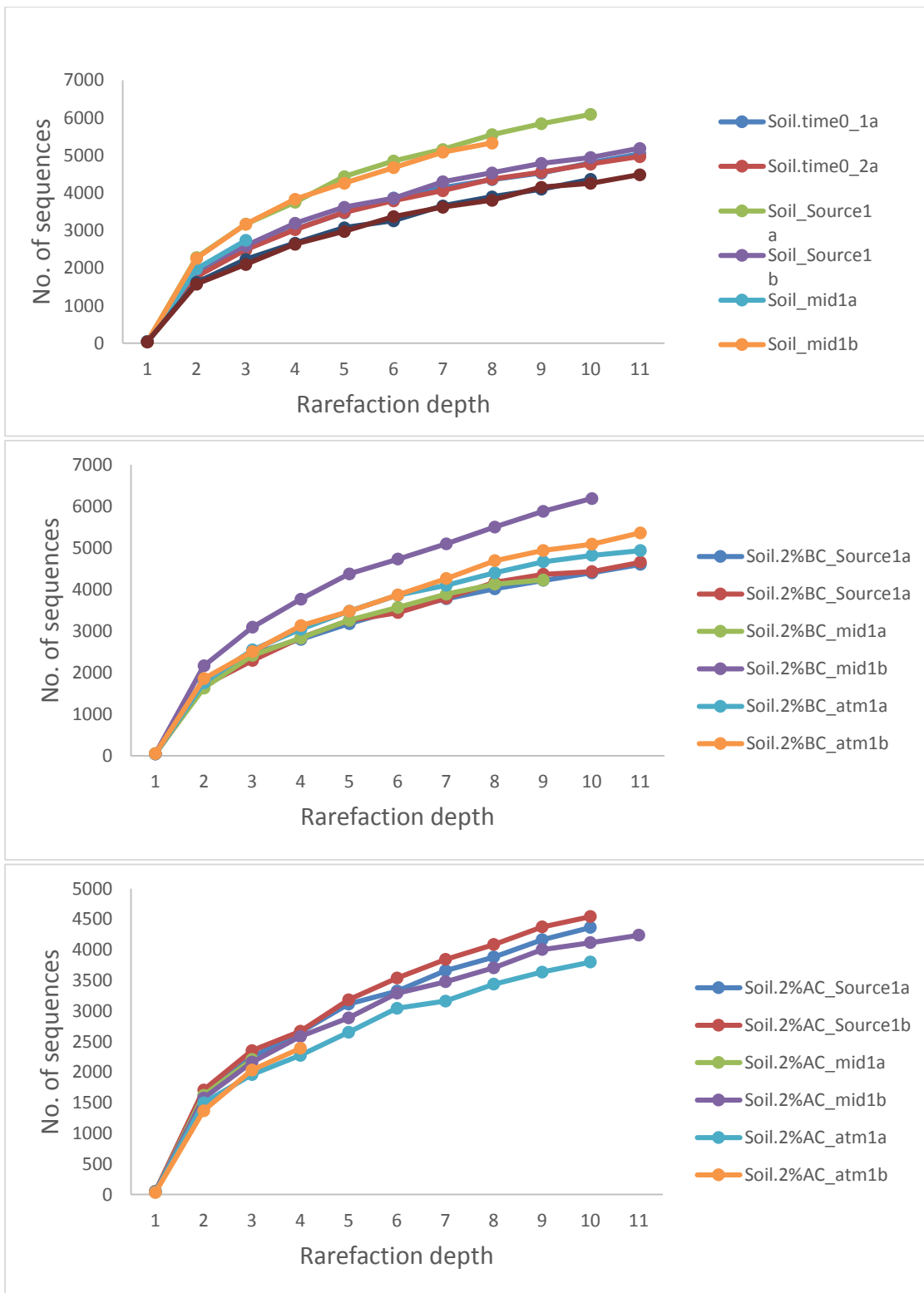
*Figure 9.12. Rarefaction curves indicating the effects of sequencing efforts (depth) on the species richness (Chao1) of **a)** unamended soil and control soils at time t = 0 **b)** soils amended with 2%BC and **c)** soils amended with 2%AC.*

Taxon level OTU	Soil	Soil_source	Soil_mid	Soil_atmos
p__Crenarchaeota	2.7±0.0	4.7±0.1	3.1±0.9	0.3±0.1
p__Acidobacteria	8.4±0.8	6.8±0.7	7.2±0.1	5.1±0.3
p__Actinobacteria	8.2±0.6	6.8±0.2	6.7±0.5	7.5±0.3
P__Bacteriodetes	4.0±0.1	5.3±0.7	5.7±0.8	9.3±1.8
p__Chloroflexi	6.3±0.3	6.3±0.6	6.7±0.1	5.4±0.1
p__Firmicutes	5.1±0.3	5.7±0.5	5.7±0.0	4.4±0.0
p__Gemmatimonadetes	6.2±1.1	5.3±0.4	5.2±0.2	2.5±0.2
p__Nitrospirae	3.1±0.1	2.4±0.0	2.3±0.1	1.0±0.1
p__Planctomycetes	8.3±0.5	8.8±0.9	9.4±0.4	9.7±2.2
c__Alphaproteobacteria	13.1±0.6	12.5±1.8	12.4±0.8	20.3±2.2
c__Betaproteobacteria	7.9±1.4	9.7±1.3	9.7±0.8	8.2±0.6
c__Deltaproteobacteria	8.7±0.6	8.7±0.1	8.7±0.0	7.5±0.7
c__Gammaproteobacteria	11.6±0.3	8.2±0.8	9.7±1.0	12.7±0.6
p__Verrucomicrobia	2.1±0.3	2.1±0.2	2.0±0.5	3.7±0.3

Table 9.12. A summary of the percentage abundances of dominant OTUs (>1.0% of total relative abundance) at the phylum level of taxonomy for the different soil column locations and control at time zero.

Taxon level OTU	Soil & BC_source	Soil & BC_mid	Soil & BC_atmos
Crenarchaeota	0.9±0.5	1.2±0.1	1.3±0.1
Acidobacteria	4.8±0.4	4.3±0.8	3.8±0.0
Actinobacteria	8.9±1.1	7.8±0.7	9.8±0.9
Bacteriodetes	5.5±0.0	7.3±2.1	6.8±0.7
Chloroflexi	6.5±0.1	6.0±0.2	6.1±0.3
Firmicutes	3.9±0.3	5.1±1.0	4.2±0.2
Gemmatimonadetes	4.9±0.2	3.9±0.7	3.7±0.2
Nitrospirae	2.2±0.1	1.5±0.2	1.5±0.3
Planctomycetes	7.5±0.6	5.4±1.2	5.7±0.7
Alphaproteobacteria	16.4±0.8	14.8±2.6	15.6±0.6
Betaproteobacteria	11.2±0.9	11.8±1.3	10.9±1.5

Deltaproteobacteria	7.9±0.1	8.9±0.3	9.0±0.0
Gammaproteobacteria	13.8±1.3	15.9±0.5	16.9±0.9
Verrucomicrobia	2.0±0.3	1.7±0.2	1.0±0.0

Table 9.13. A summary of the percentage abundances of dominant OTUs (>1.0% of total relative abundance) at the phylum level of taxonomy for the different soil & biochar column locations.

Taxon level OTU	Soil & AC_source	Soil & AC_mid	Soil & AC_atmos
Crenarchaeota	1.1±0.2	0.5±0.2	0.7±0.3
Acidobacteria	4.6±0.2	3.6±0.7	3.2±0.2
ctinobacteria	13.2±0.4	10.0±0.5	10.5±0.2
Bacteriodetes	4.1±0.3	4.3±0.3	5.3±1.1
Chloroflexi	4.5±0.2	4.4±0.3	4.9±0.5
Firmicutes	4.0±0.1	3.3±0.7	2.5±0.1
Gemmatimonadetes	3.9±0.2	3.1±0.3	2.9±0.1
Nitrospirae	2.8±0.2	1.5±0.1	1.0±0.2
Planctomycetes	5.8±0.1	8.1±4.0	6.3±1.2
Alphaproteobacteria	12.5±0.4	14.2±0.1	16.6±1.0
Betaproteobacteria	14.9±0.0	16.5±0.9	15.1±0.8
Deltaproteobacteria	11.3±0.8	12.6±1.6	13.8±0.6
Gammaproteobacteria	12.6±0.3	13.1±0.8	13.4±1.1
Verrucomicrobia	1.5±0.1	1.7±0.2	1.2±0.2

Table 9.14. A summary of the percentage abundances of dominant OTUs (>1.0% of total relative abundance) at the phylum level of taxonomy for the different soil & activated carbon column locations.
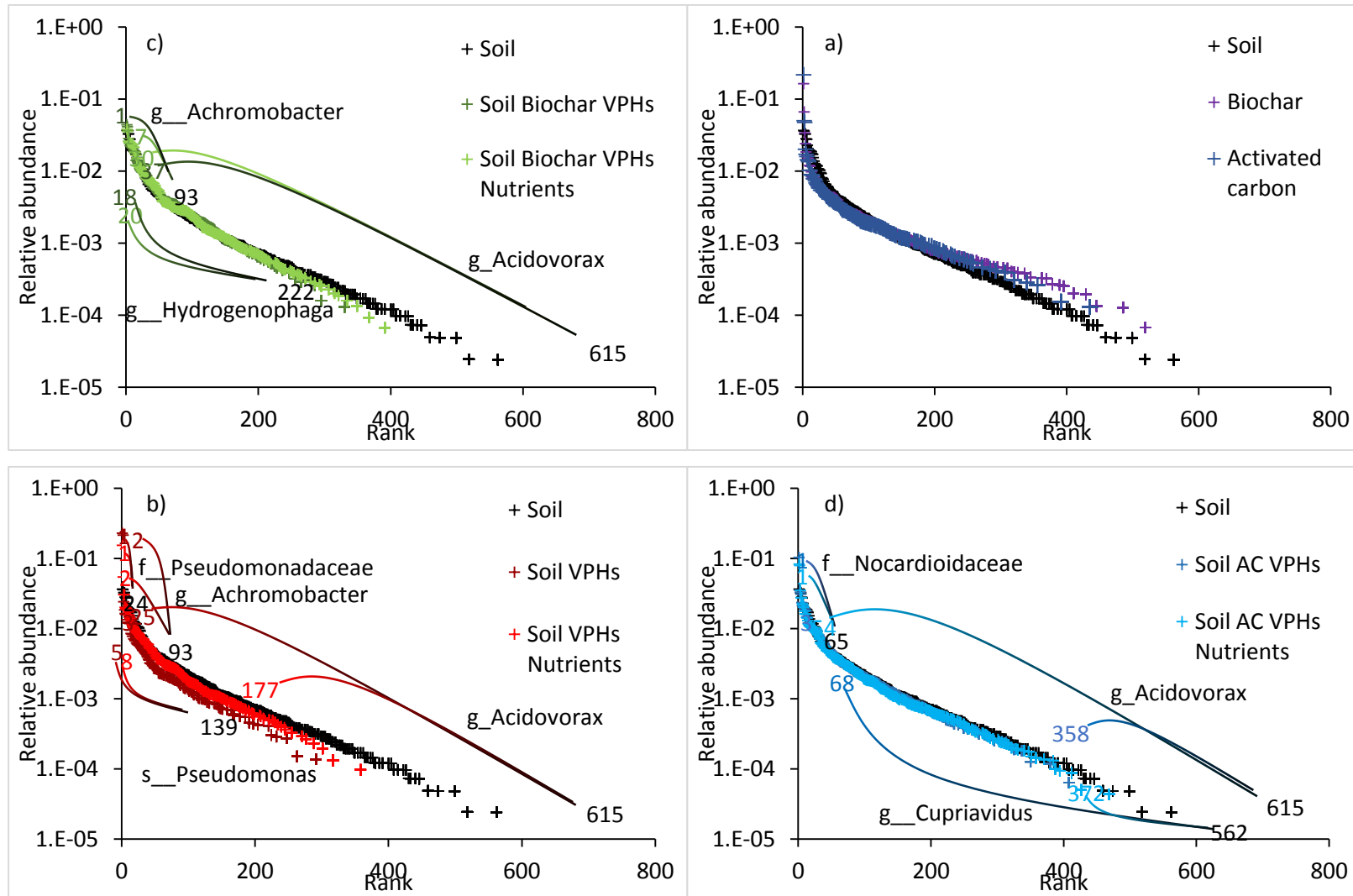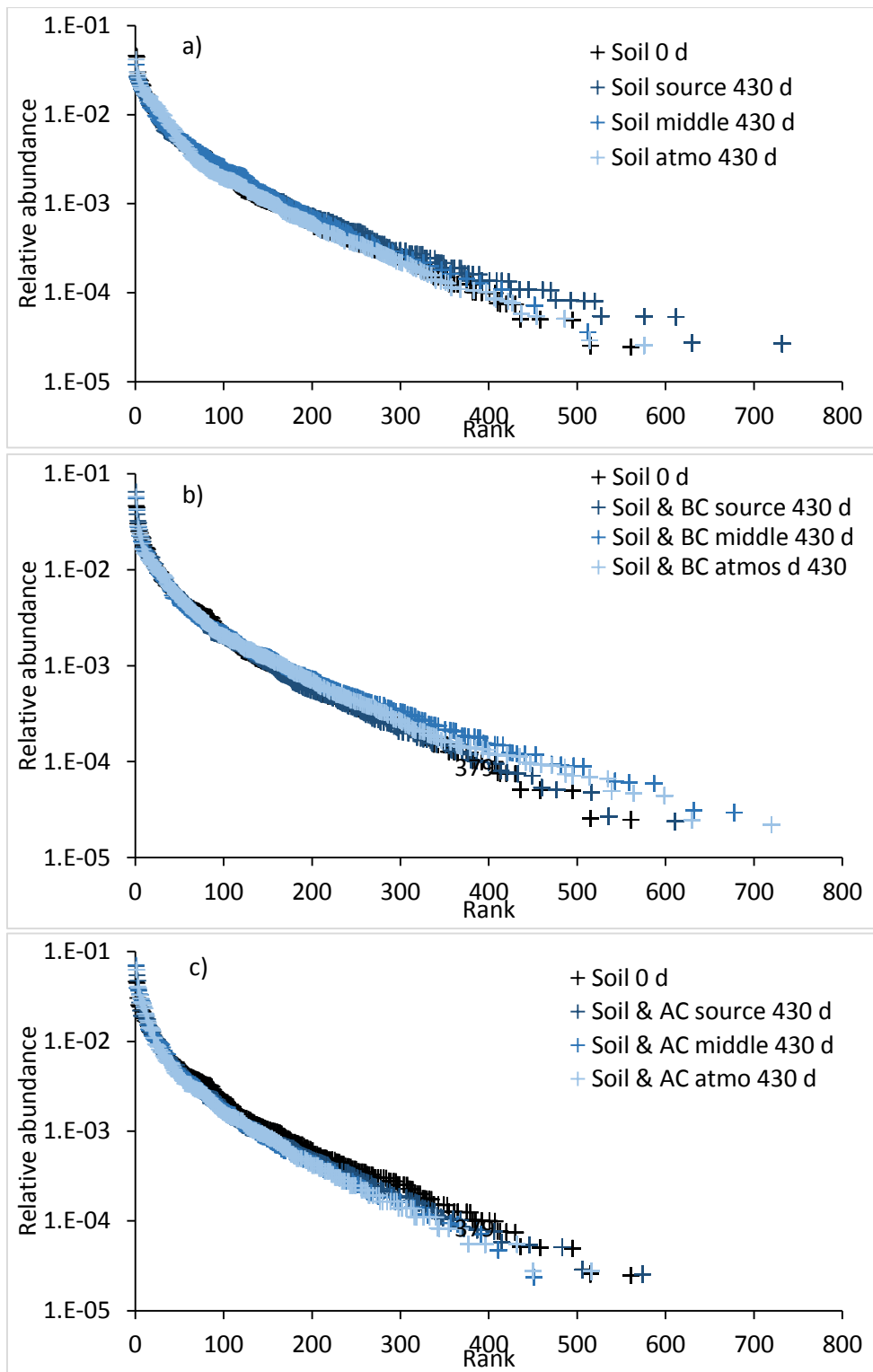
Figure 9.13. Relative operational taxonomic unit (OTU) abundance distribution and phyla associations in ranked order for a) Soil b) Soil & Biochar and c) Soil & activated carbon columns.

OTU classification	%Detected			
	Soil (d0)	Source	Mid	Atmosphere
Actinomycetales	**2.5±0.3**	**2.7±0.1**	**2.7±0.3**	**4.4±0.0**
Micrococcaceae	0.6±0.1	0.8±0.0	0.6±0.1	0.5±0.0
Micromonosporaceae	0.4±0.0	0.3±0.0	0.2±0.1	0.3±0.1
Mycobacteriaceae	0.1±0.0	0.1±0.0	0.1±0.1	0.8±0.1
Nocardioidaceae	0.5±0.1	0.4±0.1	0.6±0.0	1.1±0.0
Streptomycetaceae	0.5±0.0	0.3 ±0.0	0.3±0.0	0.3±0.1
Streptosporangiaceae	0.1±0.0	0.1±0.0	0.1±0.1	0.1±0.0
Sphingobacteriales	**3.8±0.1**	**4.0±0.5**	**5.0±0.1**	**8.5±1.6**
Chitinophagaceae	1.4±0.1	1.8±0.3	2.6±0.3	3.7±0.6
Flammeovirgaceae	2.1±0.0	1.7±0.1	1.9±0.1	4.2±0.9
Pirellulales	**3.4±0.6**	**3.4±0.4**	**3.4±0.4**	**3.9±0.7**
Pirellulaceae	3.4±0.6	3.4±0.4	3.4±0.4	3.9±0.7
Rhizobiales	**7.3±0.6**	**7.1±1.4**	**7.4±0.2**	**8.5±1.1**
Bradyrhizobiaceae	0.5±0.1	0.9±0.2	1.6±0.5	0.9±0.1
Hyphomicrobiaceae	4.4±0.3	4.0±0.6	4.4±0.5	3.4±0.2
Phyllobacteriaceae	0.2±0.1	0.1±0.1	0.3±0.0	1.4±0.2
Rhodobiaceae	0.2±0.1	0.3±0.1	0.3±0.1	0.2±0.0
Burkholderiales	**1.4±0.3**	**3.5±0.7**	**3.5±0.1**	**4.9±0.1**
Alcaligenaceae	0.2±0.0	0.3±0.1	0.2±0.1	0.3±0.0
Comamonadaceae	0.9±0.2	3.0±0.5	2.9±0.1	4.4±0.2
Oxalobacteraceae	0.3±0.1	0.3±0.1	0.3±0.1	0.1±0.0
Myxococcales	**2.7±0.3**	**2.8±0.3**	**3.0±0.2**	**4.4±0.3**
Haliangiaceae	0.7±0.1	0.6±0.2	0.7±0.0	0.9±0.2
Nannocystaceae	0.2±0.1	0.3±0.0	0.3±0.0	0.2±0.0
Polyangiaceae	0.1±0.0	0.1±0.0	0.1±0.1	0.1±0.0
Xanthomonadales	**9.6±0.9**	**4.8±0.1**	**4.9±0.8**	**9.4±0.2**
Sinobacteraceae	5.1±0.6	3.1±0.3	2.9±0.4	5.4±0.4
Xanthomonadaceae	3.4±0.2	0.9±0.2	1.2±0.4	3.8±0.7
Syntrophobacterales	**4.5±0.2**	**3.8±0.0**	**3.7±0.1**	**2.2±0.2**
Syntrophaceae	0.0±0.0	0.1±0.1	0.0±0.1	0.0±0.0
Syntrophobacteraceae	4.5±0.2	3.7±0.2	3.7±0.2	2.1±0.2
Desulfobacteraceae	0.0±0.0	0.1±0.1	0.0±0.1	0.0±0.0

Table 9.15. Bacterial order detected in the highest relative abundance from DNA-derived pyrosequencing libraries of samples from soil (day 0) and VPH contaminated soil column (d430).

OTU classification	%Detected		
	Source	Mid	Atmosphere
Actinomycetales	**3.5±0.3**	**5.2±0.6**	**7.3±0.3**
Micrococcaceae	0.9±0.3	0.8±0.2	0.8±0.1
Micromonosporaceae	0.4±0.0	0.3±0.1	0.4±0.2
Mycobacteriaceae	0.1±0.0	0.1±0.0	0.2±0.1
Nocardioidaceae	0.6±0.0	0.7±0.0	1.3±0.1
Streptomycetaceae	0.4±0.1	0.4±0.1	0.4±0.0
Streptosporangiaceae	0.0±0.0	0.1±0.0	0.1±0.0
Sphingobacteriales	**5.1±0.6**	**5.3±0.7**	**3.9±0.4**
Chitinophagaceae	1.7±0.2	1.9±0.2	2.5±0.3
Flammeovirgaceae	3.1±0.2	2.8±0.9	2.4±0.4
Pirellulales	**3.2±0.1**	**2.5±0.6**	**2.6±0.3**
Pirellulaceae	3.2±0.1	2.5±0.6	2.6±0.3
Rhizobiales	**6.2±1.2**	**6.7±0.6**	**7.8±0.4**
Bradyrhizobiaceae	0.9±0.1	0.8±0.2	0.8±0.0
Hyphomicrobiaceae	4.1±0.0	3.4±0.7	3.7±0.2
Phyllobacteriaceae	0.2±0.0	0.3±0.1	0.5±0.1
Rhodobiaceae	0.3±0.0	0.2±0.0	0.2±0.0
Burkholderiales	**4.9±1.4**	**5.0±1.0**	**3.5±0.1**
Alcaligenaceae	0.3±0.0	0.3±0.1	0.2±0.0
Comamonadaceae	3.4±0.5	4.3±1.4	4.4±0.1
Oxalobacteraceae	0.3±0.0	0.4±0.1	0.3±0.0
Myxococcales	**2.7±0.3**	**3.3±0.1**	**5.2±0.2**
Haliangiaceae	1.3±0.0	1.4±0.2	1.5±0.3
Nannocystaceae	0.1±0.0	0.1±0.1	0.1±0.0
Polyangiaceae	0.1±0.1	0.1±0.0	0.0±0.0
Xanthomonadales	**9.0±0.4**	**9.3±1.1**	**10.1±0.2**
Sinobacteraceae	7.2±0.5	6.4±0.1	6.3±0.7
Xanthomonadaceae	1.6±0.1	1.6±0.1	2.1±0.2
Syntrophobacterales	**4.5±0.1**	**4.5±0.2**	**4.8±0.4**
Syntrophaceae	0.0±0.0	0.1±0.2	0.1±0.0
Syntrophobacteraceae	3.8±0.1	4.2±0.3	4.3±0.1
Desulfobacteraceae	0.0±0.0	0.1±0.2	0.1±0.0

Table 9.16. Bacterial order detected in the highest relative abundance from DNA-derived pyrosequencing libraries of samples from VPH contaminated soil & biochar column (day 430).

OTU classification	%Detected		
	Source	Mid	Atmosphere
Actinomycetales	**7.3±0.3**	**6.0±0.4**	**7.2±0.2**
Micrococcaceae	0.5±0.1	0.3±0.1	0.4±0.0
Micromonosporaceae	0.5±0.1	0.7±0.2	0.5±0.0
Mycobacteriaceae	0.1±0.0	0.1±0.0	0.2±0.1
Nocardioidaceae	2.3±0.1	0.6±0.0	1.1±0.2
Streptomycetaceae	0.6±0.1	0.5±0.1	0.5±0.1
Streptosporangiaceae	0.1±0.0	0.0±0.0	0.1±0.0
Sphingobacteriales	**3.9±0.4**	**4.2±0.4**	**5.1±1.1**
Chitinophagaceae	1.4±0.3	1.5±0.1	1.8±0.4
Flammeovirgaceae	2.2±0.1	2.4±0.2	3.0±0.6
Pirellulales	**2.3±0.2**	**3.1±1.6**	**2.6±0.6**
Pirellulaceae	2.3±0.2	3.1±1.6	2.6±0.6
Rhizobiales	**7.8±0.4**	**9.3±0.6**	**10.3±0.6**
Bradyrhizobiaceae	1.2±0.1	1.6±0.1	1.9±0.2
Hyphomicrobiaceae	4.4±0.4	5.3±0.0	5.7±0.4
Phyllobacteriaceae	0.2±0.0	0.3±0.0	0.9±0.1
Rhodobiaceae	0.2±0.1	0.2±0.0	0.2±0.0
Burkholderiales	**3.5±0.1**	**6.4±0.1**	**8.0±0.8**
Alcaligenaceae	0.2±0.0	0.2±0.0	0.1±0.0
Comamonadaceae	3.0±0.1	5.9±0.1	7.7±0.7
Oxalobacteraceae	0.3±0.0	0.2±0.0	0.1±0.0
Myxococcales	**5.2±0.2**	**7.6±0.7**	**10.3±0.3**
Haliangiaceae	1.9±0.0	3.0±0.4	3.3±0.0
Nannocystaceae	0.8±0.2	0.4±0.0	0.4±0.0
Polyangiaceae	0.1±0.0	0.2±0.0	0.1±0.0
Xanthomonadales	**10.1±0.2**	**10.2±0.4**	**9.4±0.8**
Sinobacteraceae	7.5±0.4	8.0±0.5	6.8±0.2
Xanthomonadaceae	2.1±0.2	1.7±0.2	2.3±0.6
Syntrophobacterales	**4.8±0.4**	**3.4±0.6**	**1.7±0.1**
Syntrophaceae	0.0±0.0	0.0±0.0	0.0±0.0
Syntrophobacteraceae	4.7±0.4	3.3±0.6	1.7±0.1
Desulfobacteraceae	0.0±0.0	0.0±0.0	0.0±0.0

Table 9.17. Bacterial order detected in the highest relative abundance obtained from DNA-derived pyrosequencing libraries of samples from VPH contaminated soil & AC column (day 430).

Taxon (Genus level classification)	Average absolute abundances for different treatments compared to the soil only @ time t = 0 following exposure to VPHs									
	Soil d0	Soil+VPHs d430 source	Soil+VPHs d430 middle	Soil+VPHs d430 atmos	Soil+BC+VPHs d430 source	Soil+BC+VPHs d430 middle	Soil+BC+VPHs d430 atmos	Soil+AC+VPHs d430 source	Soil+AC+VPHs d430 middle	Soil+AC+VPHs d430 atmos
Nitrosopumilus	1.01E+05	4.68E+06	5.85E+05	9.53E+03	5.44E+05	4.17E+05	1.17E+05	1.52E+05	3.53E+04	2.46E+04
Sphingomonas	6.99E+02	2.34E+04	3.35E+04	4.77E+03	1.50E+04	2.55E+04	2.75E+03	3.49E+04	5.88E+03	0.00E+00
Sphingobium	0.00E+00	0.00E+00	4.15E+03	2.62E+06	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.47E+04
Sphingopyxis	1.38E+03	3.47E+04	8.30E+03	2.04E+04	2.04E+04	8.67E+03	1.02E+04	1.43E+04	2.94E+03	9.81E+03
Nocardioides	8.24E+03	4.64E+04	1.66E+04	4.83E+04	7.45E+04	5.95E+04	4.89E+04	9.22E+04	3.49E+04	9.82E+04
Pseudonocardia	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.75E+03	0.00E+00	0.00E+00	0.00E+00
Flavobacterium	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	4.33E+03	0.00E+00	0.00E+00	0.00E+00	0.00E+00
Paenibacillus	2.27E+04	4.04E+04	1.66E+04	5.24E+04	2.59E+04	2.55E+04	1.81E+04	2.01E+04	5.88E+03	3.44E+04
Methylibium	4.15E+03	5.87E+03	0.00E+00	2.16E+05	3.61E+04	7.23E+04	1.47E+05	3.44E+04	3.46E+04	3.19E+05
Clostridium	6.78E+02	1.16E+04	0.00E+00	0.00E+00	4.80E+03	8.44E+03	2.47E+03	1.49E+04	0.00E+00	4.90E+03
Polaromonas	0.00E+00	1.17E+04	0.00E+00	0.00E+00	0.00E+00	8.20E+03	2.47E+03	0.00E+00	0.00E+00	0.00E+00
Alkanindiges	6.78E+02	2.94E+04	8.30E+03	4.77E+03	0.00E+00	8.20E+03	0.00E+00	0.00E+00	1.14E+04	0.00E+00
Pseudoxanthomonas	7.26E+04	5.82E+05	4.10E+05	2.67E+05	2.54E+05	4.55E+05	3.16E+05	2.96E+05	7.28E+04	2.40E+05
Nannocystis	0.00E+00	1.76E+04	0.00E+00	9.53E+03	0.00E+00	1.23E+04	5.51E+03	0.00E+00	0.00E+00	0.00E+00

Table 9.18. Absolute abundance data of OTUs where there was statistically significant difference between different treatments (column locations) and increase in relative abundance ranking by at least 2 folds.

Genus	Average absolute abundances	Average absolute abundances following exposure of soil treatments to VPHs at the end of the experiment (amendment effects)		
	Soil_day0 (Control)	Soil_day430	Soil + 2% BC_day430	Soil + 2% AC_day430
Nitrosopumilus	1.01E+05	1.76E+06	3.59E+05	7.05E+04
Candidatus Nitrososphaera	5.98E+05	2.75E+06	1.24E+06	1.14E+06
Sphingomonads - Sphingomonas - Sphingobium - Sphingopyxis	6.99E+02 0.00E+00 1.38E+03	2.05E+04 8.73E+05 2.11E+04	1.44E+04 0.00E+00 1.31E+04	1.36E+04 4.91E+03 9.02E+03
Nocardioides	8.24E+03	3.71E+04	6.10E+04	7.51E+04
Pseudonocardia	0.00E+00	0.00E+00	9.18E+02	0.00E+00
Streptomyces	0.00E+00	9.79E+03	8.61E+04	4.97E+04
Flavobacterium	0.00E+00	0.00E+00	1.44E+03	0.00E+00
Paenibacillus	2.27E+04	3.65E+04	2.32E+04	2.01E+04
Methylibium	4.15E+03	7.41E+04	8.51E+04	1.29E+05
Clostridium	6.78E+02	3.87E+03	5.24E+03	6.60E+03
Polaromonas	0.00E+00	3.92E+03	3.56E+03	0.00E+00
Alkanindiges	6.78E+02	1.41E+04	2.73E+03	3.81E+03
Pseudoxanthomonas	7.26E+04	4.19E+05	3.42E+05	2.03E+05

Table 9.19. Absolute abundance data of OTUs where there was statistically significant difference between different treatments (amendments) and increase in relative abundance ranking by at least 2 folds.)