EMPLOYING THE POWER OF DNA-BASED MICROBIAL COMMUNITY STRUCTURE ANALYSIS FOR THE RATIONAL DESIGN OF HYDROCARBON CONTAMINATED SOIL REMEDIATION

Onotasamiderhi Tarric Igun

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Abstract

The impact of activated carbon (AC) amendments on the biodegradation of crude oil in soil was studied in batch microcosms. AC amendment slowed down biodegradation and removal of hydrocarbon pollutants and was more evident when AC when added from the beginning compared to after 5 months. The microbial sequencing analysis revealed that the overall bacterial community shifted more due to crude oil addition compared to AC amendment at the start and after 5 months. AC amendments reduced and slightly reduced the abundance of hydrocarbon degraders belonging to *Actinobacteria* and classes *Gammaproteobacteria* and Alphaproteobacteria (Such as *Rhodococcus, Marinobacter,* and *Parvibaculum*) in crude oil batches with AC from start and AC after 5 months, respectively. The effect of biofuels on the natural attenuation of toluene was also investigated. $^{13}C/^{12}C$-CO_2 batch production showed that biofuel components were preferentially degraded in blended fuels. Ethanol had a more negative effect on toluene degradation compared to biodiesel, as it was preferentially degraded even with nutrient bio-stimulation. The microbial analysis revealed nutrient effect on the microbial communities with nitrifying bacteria *Nitrospira* seen to make significant gains in OTU ranking and relative abundance due to the nutrient amendment. The microbial community analysis also showed a distinction in microbial communities that degrade biodiesel, ethanol and toluene in the presence or absence of nutrients. For instance the results showed that *Rhodococcus* can degrade toluene in the presence of biodiesel when nutrients are surplus or scarce and can degrade toluene in the presence of ethanol only when nutrients are scarce. Indicating a lower effect of biodiesel on toluene degraders compared to ethanol. *Pseudomonas* was identified as a key ethanol degrader and thrives in presence of both ethanol and toluene when nutrient availability is high but has preference for ethanol as a carbon source. *Nocardia* is main biodiesel degrader when nutrient availability is high or low. This study has shown that the use of DNA microbial community analysis gives a broader insight into microbes involved in the physiological activities and how they are affected by certain treatments and the findings in this study would possibly aid the understanding of the impact of adsorbents on hydrocarbon pollutants and the effect of blended fuels have on the microbiology within soil. It is thereby recommended that during hydrocarbon soil remediation studies/interventions, DNA microbial community analysis should be carried out in conjunction with chemical analysis such as the one carried out in this study as this would inform the proper utilization of the remediation strategies. The work was carried out in batch studies and it is important to repeat it in column, mesocosms or field studies to see if there are any significant changes in the results

Research key words: Activated carbon, Crude oil, Bioremediation, Biofuels, Next generation sequencing

Declaration

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

Onotasamiderhi Tarric Igun

Dedication

To Almighty God for helping and seeing me through this project and to my beloved mother, late Dr. (Mrs.) Igun who invested so much in me. I will always love you mummy

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Glossary of abbreviations

AC	Activated carbon
ANOSIM	Analysis of Similarities
ANOVA	Analysis of variance
API	American Petroleum Institute
ASE	Accelerated solvent extraction
BC	Black carbon
BC	Biochar
BTEX	Benzene, toluene, ethylbenzene and xylenes
CO_2	Carbon dioxide
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
EPA	Environmental Protection Agency
FAD	Flavin adenine dinucleotide
FID	Flame Ionisation Detector
FT-ICR MS	Fourier transform ion cyclotron resonance mass spectrometry
GAC	Granular activated carbon
GC	Gas Chromatography
GS	Genome sequencing
HC	Hydrocarbon compound
HOCs	Hydrophobic organic compounds

NMDS	Non-multi-Dimensional Scaling Plots
MS	Mass Spectrometry
MW	Molecular weight
NADH	Nicotinamide adenine dinucleotide
NGS	Next generation sequencing
OTUs	Operational taxonomic units
PAC	Powdered activated carbon
P.E	Polyethylene
PAH	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyl
PCR	Polymerase Chain Reaction
PGM	Personal Genome machine
QIIME	Quantitative Insights into Microbial Ecology
RDP	Ribosomal DataBase
rRNA	Ribosomal ribonucleic acid
SARA	Saturate, aromatic, resin and asphaltenes
SPMD	Semipermeable membrane devices
TOC	Total organic carbon
USEPA	United States Environmental Protection Agency
VOCs	Volatile Organic Compounds
VPHs	Volatile petroleum hydrocarbon

Chapter 1. Background, aims, objectives, hypothesis and thesis scope

1.1 Background

One of the main causes of environmental pollution is the high dependency of human society on energy brought about by the growing needs of modernization and development (MacKenzie, 1998). Petroleum–based products are currently used in generating a significant proportion of the energy consumed by the industrial and private sectors (Hall *et al.*, 2003; Das and Chandran, 2010), thus causing an increase in the demand for these products. This higher demand has led to increasing levels of petroleum production "activities", supply activities (Hamilton, 2008), which can sometimes lead to environmental pollution (Shabir *et al.*, 2008). Petroleum environmental pollution can occur as a result of the unsuitable industrial disposal of hydrocarbon containing materials (Wilson and Jones, 1993), spills at refineries or oil wells (Holliger *et al.*, 1997) such as BP Deepwater horizon oil well blowout (Crone and Tolstoy, 2010; Bælum *et al.*, 2012) or accidents during transportation such as the Exxon Valdez oil spill (Peterson *et al.*, 2003). In environs which are mainly aquatic, pollutants from Oil spills can be transported from sea surfaces to shorelines by wind or sea waves and can eventually end up in surrounding soil (Osuji and Opiah, 2007).

The pollution of land by hydrocarbon compounds is a common occurrence (Lu *et al.*, 2014) and it is mainly associated with leakages during storage and distribution from vessels such as tanks and drums or pipelines (Nadim *et al.*, 2000; Aislabie *et al.*, 2004). Some other sources of hydrocarbon land contamination include oil seepages from vehicles, generators and incinerators (Aislabie *et al.*, 2004). In 2013, an estimation by US EPA estimated that there were over half a million leaking underground storage tanks in America with most of them containing petroleum products (Lu *et al.*, 2014). The US EPA further states that such leakages from underground storage tanks can affect the indoor air causing raised levels of the contaminants in buildings (EPA, 2012) thus increasing the risk to ecological receptors such as human beings (Bispo *et al.*, 1999; Balasubramaniam *et al.*, 2007). The occurrence of land spills, can lead to a reduction in soil quality (unfit for utilization) by affecting soil properties such as moisture, pH, carbon content and nutrient levels (Aislabie *et al.*, 2004; Shabir *et al.*, 2008). This highlights the importance for land remediation strategies.

1.1.1 Land contamination

There is a linkage between the quality of the environment and the quality of life. Pollution of air, soil and water bodies has been known to affect the environment and also causes a reduction in human life expectancy (Trevelyan, 1952). In recent years there have been issues raised on a global scale regarding contaminated sites. Generally the occurrence of contaminated land can be blamed on past industrial activities and an unawareness of health and environmental impacts linked to the improper production and disposal of hazardous substances (Vidali, 2001). The current awareness of the impacts of land contamination to human health coupled with the need for site redevelopment and utilization has led to rise in government's (internationally) determination to clean up the sites (Vidali, 2001).

In England there are a varying concentrations of hazardous substances such as petroleum hydrocarbons found on land which can range from background levels as a result of human diffuse pollution to higher concentrations due to industrial activities. In some of these cases the land can be identified as contaminated land if there is believed to be a threat to human health or the environment. Contaminated land as defined by Part 2A 1990 Act, is land which the local authorities deem to be in a certain condition due to containing substances that can or causes substantial harm to a human or can or causes substantial pollution of controlled water bodies (Defra, 2012). This legal definition is dependent on the significant levels of risk posed by the contamination at a specific site based on assessments carried out for that site (Hester and Harrison, 2001).

Based on the guidance, there has to be an identifiable contaminate, a receptor and a pathway (route of passage) linking both contaminant and receptor (also termed 'contaminant linkage') for a land to be taken into consideration for possible contamination, and land is considered as contaminated if there is a significant contaminant linkage (i.e. indicates significant risk). (Environmental Agency, 2006). In England and Wales there is believed to be approximately ten thousand contaminated sites and an estimation of 5000 and 20000 sites requiring remediation due to a significant risk to either human health or the environment (Hester and Harrison, 2001).

1.1.2 Remediation approaches for contaminated soils

The remediation of the sites is essential and has led to the advancement in remediation technologies with emphasis on destroying the contaminants instead of the normal disposal methods (Boopathy, 2000). There is an array of techniques utilizing 2 main approaches, which are the in situ remediation, were remediation occurs at site i.e. without moving soil and ex situ, where the contaminated soil is removed from site (excavated) for treatment elsewhere (van Liedekerke *et al.*, 2014). In or ex- situ remediation involves containing the pollution via physical methods and subsequently treating it either chemical and biological treatment methods (Wilson et al., 1986). One such method is natural attenuation also called intrinsic bioremediation. In the US in situ remediation is used preferably over the ex situ remediation as the contaminated site is subjected to very little disruption and it's believed to be cheaper (Palmroth, 2006). However in Europe, ex situ remediation is preferred as the application of in situ remediation on European soils is still poorly understood and it's believed that a faster site remediation is achieved with the ex situ remediation (van Liedekerke *et al.*, 2014).

1.1.3 Natural attenuation

The natural attenuation as a technique is reliant on soil natural process which include biological, chemical or physical to eliminate pollutants (EPA, 1999a). Some of these natural process include sorption, volatilization, biodegradation etc. (EPA, 1999a). It must however be noted that desirable remediation levels may not be achievable in a timely fashion using only natural attenuation (Palmroth, 2006). Natural attenuation has a limited capacity and would require an investigation to ascertain the feasibility of its application at each site and this is because the natural processes involved in natural attenuation may differ between soils or between different locations of the same soil (Yong and Mulligan, 2003). The understanding of the inherent natural attenuation processes within soils to clean pollutants has also aided in the production of designed methods called enhanced natural attenuation, which not only utilize this natural capacity but to also enhance it (Yong and Mulligan, 2003).

1.1.4 Bioremediation

Bioremediation is a process where environmental pollutants such as organic substances are bio-transformed under defined conditions to less hazardous compounds (similar to those produced during the mineralization of organic matter), and the pollutant concentrations are reduced below regulatory defined limits. Example of such innocuous biodegradation products include carbon dioxide, water and nitrate (inorganic compounds) (Mueller *et al.*, 1996; Garbisu and Alkorta,

1999; Paul *et al.*, 2005). The 2 major bioremediation techniques include 1) biostimulation: where there is the addition of nutrients, substrates, oxygen or water to provide a favorable condition for indigenous microorganisms to thrive, and 2) bio-augmentation: the addition of specialized microorganism which possess degrading capabilities in order to enhance the remediation process (Fernández-Luqueño *et al.*, 2011). Microorganisms mineralize pollutants (such as petroleum hydrocarbons), and some specialist microorganisms produce bio-surfactants which help in emulsifying the pollutants thus making them bioavailable for conversion to a non-harmful product (Santhini *et al.*, 2009).

However use of bio-augmentation is limited by exogenous microorganism's inability to compete with the already existing microbial population thereby limiting their growth and population development in real soils. Hence, bio-stimulation is more commonly used as polluted sites often already contain certain indigenous pollutant degrading microorganisms which have developed overtime due to long term exposure to biodegradable pollutants, but will more effectively carry out the biodegradation with the right stimulants and proper treatment management (Vidali, 2001). There are certain factors that can affect the upstream and downstream bio-catalysis process, and these include sorption to solid matrixes, bioavailability, stress, nutrient availability etc. (de Lorenzo, 2008). Pollutant availability impacts on the biodegradation process especially in soils due to its heterogeneous nature (Pignatello, 1989; Mihelcic et al., 1993; Luthy et al., 1994; Alexander, 1995) pollutant aging (Alexander, 1995) or widespread presence of strong sorbents such as chars (Zhang et al., 2005). These pollutant compounds, which may be readily degraded, tend to persist longer in soil or sediments prior to degradation, due to association with the soil matrix, which renders them inaccessible to microorganisms (Pignatello, 1989; Mihelcic et al., 1993; Luthy et al., 1994; Alexander, 1995). Bioavailability in terms of bioremediation is defined as the level to which a contaminant is exposed to biological transformation (Hamelink et al., 1994). Sorption which is known to prevent access of biological organisms to the pollutant and desorption rates are important factors in relation to the pollutant bioavailability (Alexander, 1995; Zhang *et al.*, 1998).

1.1.5 Blended fuels

Increasing levels of greenhouse gas emission resulting from the daily use of fossil fuels is known to affect climate (Teske *et al.*, 2012). This has led to higher utilization of alternative renewable fuels such as biofuels and ethanol (Hill *et al.*, 2006) as single fuels or possibly as blends with fossil fuels (petroleum and diesel) (Elazhari-Ali *et al.*, 2013). Biodiesel and ethanol are known to reduce gas emissions by 41 and 12 % respective in comparison to fossil fuels (Hill

et al., 2006)**.** There is however still the need to understand the impacts the renewable fuel components will have on the natural attenuation or bioremediation of harmful petroleum components such as benzene, toluene, ethylbenzene and xylenes (BTEX), in the event of a blended fuel spill (Da Silva and Alvarez, 2002; Dakhel *et al.*, 2003; Lapinskienė *et al.*, 2006)**.**

1.2 General aims and objectives

The general aim of the project is to use a combination of non-genetic and genetic techniques to offer broader understanding of how the addition of strong carbonaceous sorbents to hydrocarbon polluted soil impacts on the pollutant availability and pollutant biodegradation and also how the presence of renewable fuels can affect the biodegradation of hydrocarbons in bio-stimulated and non-bio-stimulated polluted soils. Many bioremediation approaches of contaminated sites with petroleum hydrocarbons have been designed purely based on chemical measurements (i.e. physico-chemical conditions such a temperature, nutrient concentrations, pH etc required for effective bioremediation) but with better insight and information into what occurs at the microbial community level, the optimization of bioremediation treatments should be facilitated. To be more precise, the specific key hypotheses and objectives of the research are:

Research hypotheses: (i) the addition of adsorbents such as AC can limit the pollutant availability, and hence reduce the rates of pollutant transfer, biodegradation and the abundance of pollutant degrading microorganisms. **(ii)** By varying adsorbent amendment time, the limitation to biodegradation (especially for the easily degradable pollutants) and impacts on hydrocarbon degrading microbes could be reduced **(iii)** Biofuels are more readily biodegradable in comparison to petroleum hydrocarbons and therefore can have a negative impact on the biodegradation of petroleum hydrocarbons by limiting nutrient availability and the abundance of petroleum hydrocarbon degrading microorganisms.

1.3 General research objectives

1. To assess the effects of activated carbon (AC) and AC amendment-time on hydrocarbon compound availability and biodegradation rates.

2. Compare and utilize Next generation sequence (NGS) platforms to analyze and determine the impacts of activated carbon (AC), AC amendment-time and crude oil on diversity and structure indigenous bacterial communities.

3. To elucidate the effects biofuels (presence versus absence of biofuels-ethanol and biodiesel) on the biodegradation of toluene (as a model volatile hydrocarbon) with and without nutrient amendment and also using Next generation sequence (NGS) analysis to determine the impact the substrates have on the microbial community diversity and structure within the soils.

1.4 Structure and scope of the thesis

The thesis is structured into 6 chapters with **Chapter 1** containing a general overview on land pollution and contaminated land remediation approaches. **Chapter 1** also comprises of a summary of research aims, objectives, hypotheses and thesis structure.

Chapter 2. Literature review on impacts and fate of crude oil in the event of a spillage and physico-chemical methods used in assessing contamination and efficacy of natural attenuation. Review of crude oil components, biodegradation and the discussion of effects activated carbon and biofuels on natural attenuation of hydrocarbon compounds. Next generation sequencing (NGS) techniques and their importance in informing and the development of pollution remediation strategies are also discussed. The remaining chapters 3, 4, 5 were written using an IMRAD (introduction, materials and methods, results and discussion) format, which is suitable for the publication of these chapters as scientific papers.

Chapter 3. Comprises microcosm batch experiments and presents results of chemical evaluation of the AC effects and effects of varying sorbent amendment time on the biodegradation of crude oil components. This was carried out using several techniques which includes measuring of carbon dioxide production, polyethylene passive sampling to estimate pollutant availability and water concentrations and quantifying residual soil concentrations. Results reported in chapter 3 are the authors own work.

Chapter 4. Presents results of the evaluation of the effects of crude oil pollution and varying activated carbon amendment time on the microbial community structures within batch samples using 2 different NGS platforms, 454 GS junior pyrosequencing and PGM ion torrent. Results reported in chapter 4 are the authors own work.

Chapter 5. Comprises microcosm batch experiments and presents results of chemical and microbiological evaluation on the effects of ethanol and biodiesel on the biodegradation of stable-isotope labelled toluene with and without bio-stimulation. The chemical evaluation which includes analysis of headspace carbon dioxide and volatile hydrocarbon compounds was performed by M.Sc. student Brenda Mutesi under the supervision of the author. The additional batch experiments for the microbiological assessment and subsequent microbial analysis via Personal genome machine (PGM) ion torrent sequencing was performed by the author.

Chapter 6. Concluding remarks, combined presentation of answers to research hypothesis and future work recommendations.

Chapter 2. Literature review

2.1 Crude oil

2.1.1 Composition of crude oil

Crude oil is derived from aquatic and non-aquatic organic matter, which has formed deposits in rocks (Meinschein, 1959; Speight, 2014). The organic matter is made up of kerogen and bitumen and crude oil is derived from both organic compounds by a process called "catagenesis", which causes the organic matter to crack into smaller molecules due to the exposure to heat, pressure and increasing burial depth (Bjorlykke, 2010; Tissot and Welte, 2013).

Crude oil is an intricate mixture comprising of compounds with varying molecular weights, boiling points and is mainly made of carbon, hydrogen and trace amounts of organic sulphur and nitrogen (Prince, 2008; Speight, 2010). API (American Petroleum Institute) gravity (which is used in measuring the weight of petroleum in comparison to water) and sulfur content (sweet and sour) is normally utilized in distinguishing heavy and light crudes (Young, 2006; Schobert, 2013). Prince, (2008) previously reported that a complete list of molecules found in crude oil are yet to be determined, owing to the fact that most fractions in crude oil are determined based on their physico-chemical properties and there had also been an absence of more advanced analytical procedures to identify molecules. Over the years chemists have mostly utilized various conventional techniques such chromatographic separation and mass spectrometry analysis to characterize and determine crude oil composition (Young, 2006). Recent advances in petroleum research such as the use of FT-ICR MS (Ultra high resolution - Fourier transform ion cyclotron resonance mass spectrometry) has helped in the characterization of crude oil constituents (Marshall and Rodgers, 2004; Gaspar *et al.*, 2012). FT-ICR MS has been used in discovering more than 20,000 different heteroatom compositions of nitrogen, oxygen and sulphur (N,O,S) in crude oil (Marshall and Rodgers, 2004). These recent advances in crude oil-petroleum analysis coupled with the rising cost of crude oil per barrel and a higher demand for heavier and sour crude oil (high sulfur) due to depleting levels of sweet light crude oil, has led to an increased focus on the characterization of crude oil and its derivatives (Marshall and Rodgers, 2008).

Generally crude oil can be separated into four major fractions which are, saturate, aromatic, resin and asphaltenes (SARA). See Figure 2-1(Harayama *et al.*, 1999; Fan and Buckley, 2002).

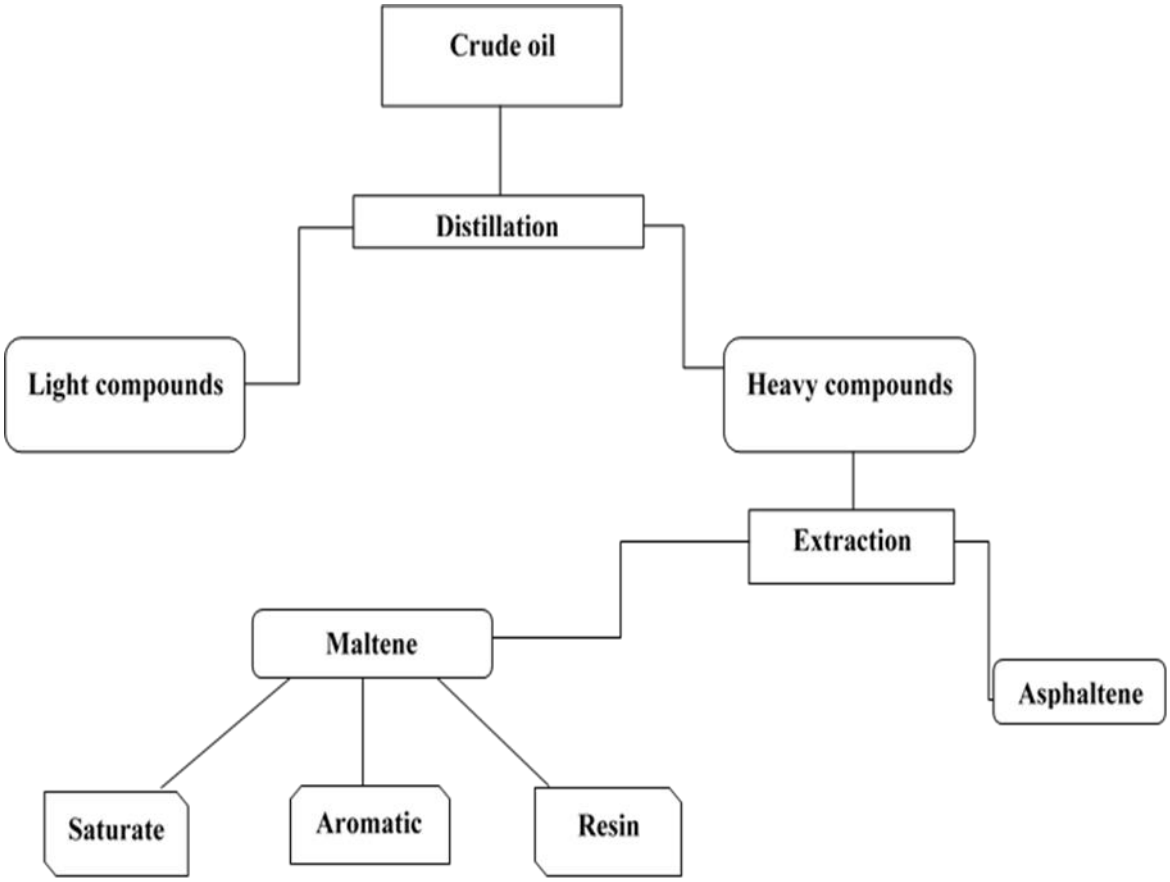


Figure 2-1 Fractionation of crude oil into SARA source: Gaspar et al. (2012)

Aliphatics are a wide range of hydrocarbon compounds and comprises both saturated (alkanes) and unsaturated (alkenes and alkynes) hydrocarbons (Watkinson and Morgan, 1991). The saturated hydrocarbons possess no double bonds and are considered non-polar. Alkane hydrocarbons can be linear or branched (formula: C_nH_{2n+2}) while cyclic or cycloalkanes (e.g. naphthenes) possess a carbon atom ring (formula: C_nH_{2n}) and can have an alkyl substituent(s) (mainly those in crude oil) (Harayama *et al.*, 1999; Fan and Buckley, 2002). See Figure 2-2 for some representative types of alkane compounds found in crude oil.

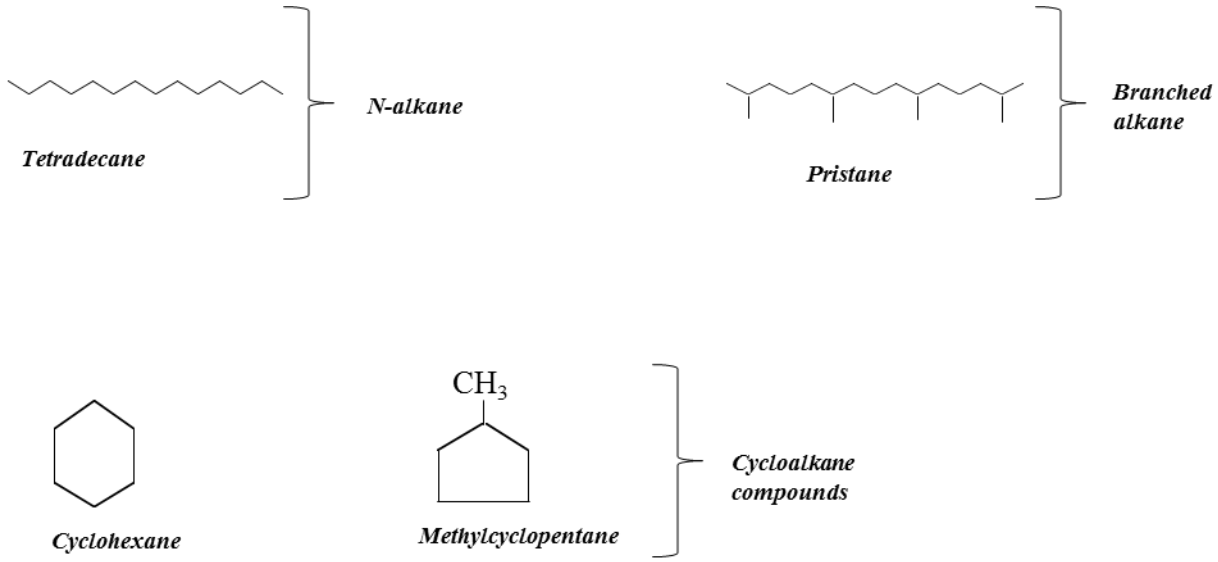


Figure 2-2 Representative alkane molecules, straight, branched and cyclic. Source Harayama et al. (1999).

Aromatics are more polar and possess aromatic ring structures with or without their alkyl group substituent(s). In crude oil there is a higher presence of aromatics with substituted alkyl groups compared to those with alkyl groups (Matar and Hatch, 2001). An important class of aromatics which are found in crude oil are polycyclic aromatic hydrocarbons (PAHs). PAHs in nature are found in rocks and fossil fuels (Lichtfouse *et al.*, 1997; Henner *et al.*, 1999; Lichtfouse *et al.*, 1999). They also occur as a result of partial combustion or pyrolysis of organic matter (Hyötyläinen and Oikari, 2004). Other sources of PAHs include smoke from tobacco (Sakai *et al.*, 2002), volcano eruptions (Srogi, 2007), emissions from automobiles and refinery and power station activities (Omar *et al.*, 2002; Yang *et al.*, 2002; Dyke *et al.*, 2003). PAHs can also be formed from saturated hydrocarbons in the presence of low oxygen and high temperature (over 500^0C) via a process called pyrosynthesis (Ravindra *et al.*, 2008).

PAHs like other hydrocarbons are made up of carbon and hydrogen. PAHs comprise of more than one fused aromatic ring and are classed as either low molecular weight or high molecular weight depending on their molecular structure (Lamichhane *et al.*, 2016). The low molecular weight PAHs comprise of 2-3 aromatic rings while high molecular weight PAHs contains 4 and above, and this impacts on the solubility of PAHs with a decreasing solubility as the molecular mass increases (Heitkamp and Cerniglia, 1989; Balati *et al.*, 2015).

There are over one hundred known PAHs, but those which are commonly found in the environment and are labeled as priority pollutant's by the Environmental Protection Agency

(EPA), possess between 2 e.g. naphthalene to 6 e.g. benzo(ghi)peryene fused benzene ring structures (Zhang *et al.*, 2004; Boehm, 2010).

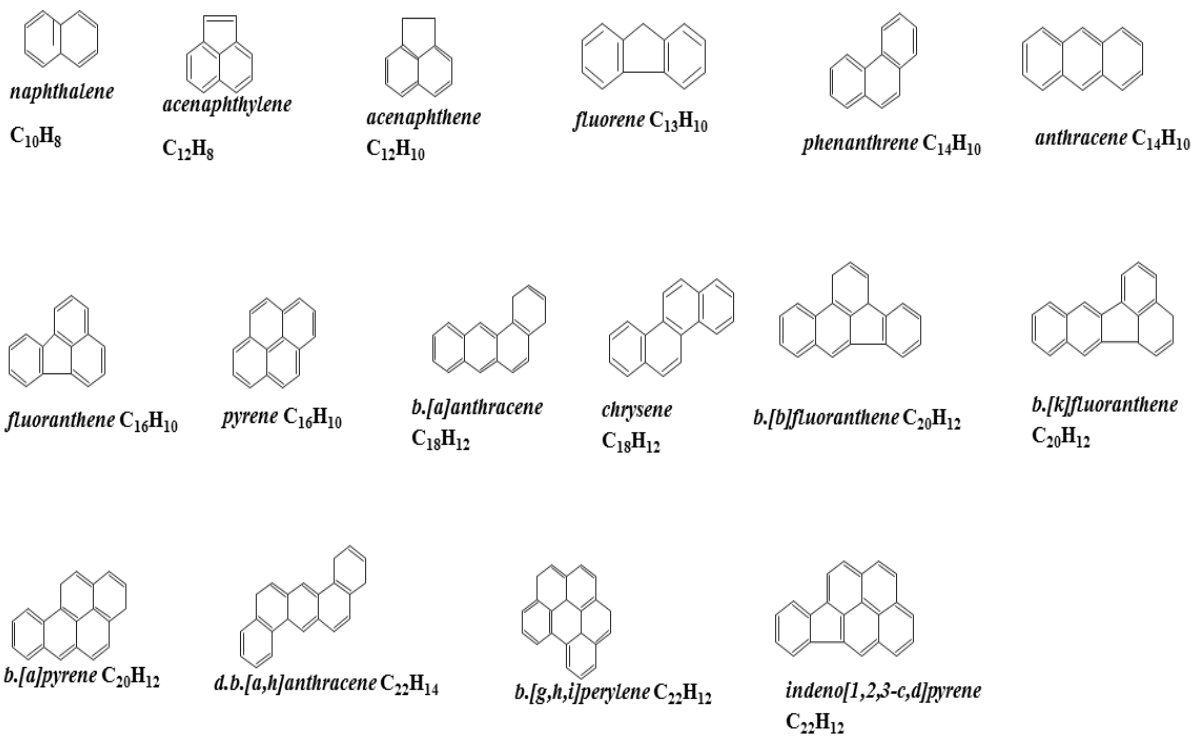
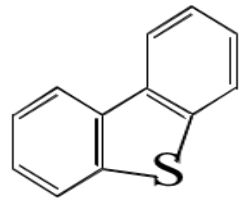


Figure 2-3 USEPA PAHs priority pollutants source Ravindra et al. (2008)

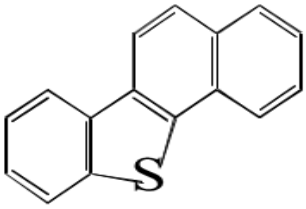
See Figure 2-3 above for the priority 16 USEPA PAHs which are also utilized in this study. Among the 16 USEPA PAHs, six have been identified as carcinogenic, b.[a]anthracene, chrysene, b.[b]fluoranthene, b.[a]pyrene, indeno[1,2,3-c,d]pyrene, d.b.[a,h]anthracene (Ravindra *et al.*, 2008).

Resins and asphaltenes on the other hand, comprise of more polar compounds made up of hydrogen, carbon, small amounts of nitrogen, sulphur and oxygen. Asphaltenes comprise of heavy molecular weight compounds and are insoluble in saturated hydrocarbon solvents like n-heptane but soluble in aromatic hydrocarbon solvents like toluene (Liao *et al.*, 2009), while resins are made up of heterocyclic compounds, acids, sulfoxides, and are soluble in n-heptane (Harayama *et al.*, 1999; Fan and Buckley, 2002). Resins and asphaltenes are useful in the determination of the origin and evolution of organic matter found in rocks (Pelet *et al.*, 1986). They can be produced directly from kerogen, but some reports suggests that they are intermediates obtained during the production of hydrocarbons from kerogen (Pelet *et al.*, 1986).

See Figure 2-4 for sulphur containing constituents of resins and asphaltenes obtained via the complete breakdown of resins and asphaltenes by heat at temperatures 450^0C-650^0C.



Dibenzothiophene



Benzo[b]naphtho[2,1_d]thiophene

Figure 2-4 Constituents of resins and asphaltenes containing Sulphur Grin'ko et al. (2012)

2.1.2 Economic importance and uses of crude oil and petroleum

The global use of crude oil and petroleum as source of fuel has seen an increment by 812 fold between the mid-17th century to 20th century (Hall *et al.*, 2003). This is because the energy generated from petroleum, is of great importance to the economic, social and environmental needs of human populations across the globe (Hall *et al.*, 2001; Tharakan *et al.*, 2001). Currently crude oil is the main source of approximately 40% of the global non-solar energy and it is utilized in over 220 countries (Hall *et al.*, 2003). Diverse petroleum products consumed on a daily basis are generated via the refining of crude oil (Harayama *et al.*, 1999), and these products include gasoline, jet fuels, diesel, gas oils and heating oil (Young, 2006; Prince, 2008). The estimated global consumption of petroleum in 1989 according to the US Energy administration was approximately 1 trillion gallons/year (Prince, 2008), and its believed that the rate of consumption will continue to rise thus leading to the complete consumption of more than half of global recovered oil and oil reserves by 2030 unless more oil is produced to equal demand or there is a reduction in worldwide consumption (Kjärstad and Johnsson, 2009).

2.2 Effects of crude oil on the environment

The increase in demand and utilization of petroleum and its products are indirectly responsible for environmental hydrocarbon pollution (Hall *et al.*, 2003). Petroleum hydrocarbons are amongst the most prevalent contaminants found in soil and also water (Margesin and Schinner, 2001). The petroleum industry has been reported to play a major role in hydrocarbon land pollution due to oil spills (during storage and distribution) and other activities such as gas flaring (Oyem and Oyem, 2013). Petroleum land contamination could ultimately affect ground water, plants and most notably, endanger public health (Oyem and Oyem, 2013) as these chemicals are deemed to possess toxic, mutagenic and carcinogenic properties (Lockard *et al.*, 1982; San Sebastian *et al.*, 2001; Liu *et al.*, 2010). When the pollutant gets into the environment, it can be assimilated and may end up in the tissue of animals and plants (possibly leading to death of biota) and could be accumulated in the food chain thus presenting serious hazard to humans (Onwurah *et al.*, 2007; Lu *et al.*, 2010).. PAHs exposure to human beings has been reported to heighten the risk of getting various forms of cancer (includes lung, breast, prostate, and kidney) and they are known to disrupt immunity and endocrine functions (Lerda, 2010; Siddens *et al.*, 2012; Xu *et al.*, 2013). Land hydrocarbon pollution also causes alterations of soil physiochemical properties such as decreasing soil moisture content, soil porosity, pH and causes increases in soil temperature (affecting soil in areas like the Antarctic) and organic carbon content (Aislabie *et al.*, 2004; Shabir *et al.*, 2008). Some of these alterations to soil physical and chemical properties can limit agricultural crop production via a reduction in nutrient availability and diffusion of oxygen to plant root (Debojit *et al.*, 2011). Several reports have shown the effects hydrocarbon soil contamination can have on crop production. The toxicity caused by hydrocarbon pollution has been shown to slow down the germination of planted seeds and growth of plants such as *Paspalum scrobiculatum* as it reduces water and oxygen supply (Powell, 1997; Ogbo *et al.*, 2009). These contaminants also destroy plant cell membranes causing leaking of cell contents and once entry into the plant is gained they block the stomata and spaces within the cells to prevent or reduce intracellular processes such as nutrient transportation (Baker, 1970). The Nigerian Niger Delta region is globally one of the main oil producing areas. The region boasts of over 30 billion barrels of crude oil and possesses a large reservoir of natural gas of over 180 trillion cubic feet (EIA, 2016). The region also contributes up to 30% of the national gross production (a daily production of 2.5 million Barrels of crude oil) (EIA, 2016). However due to the high level of production activities (such as gas flaring) in the region, there have been reports of PAH soil and sediment contamination and this is believed to affected the regional ecosystem which includes a decrease in crop yield (Sojinu

et al., 2010). This has led rising tensions from the region and a disturbance of the National peace (Sojinu *et al.*, 2010). Similar to hydrocarbon land pollution, the pollution of marine environs by crude oil spills can have widespread effects. There have also been several reports of the effects of marine oil spills on environmental biota. Researchers studied the after effects of the Exxon Valdez spill on physiological and health status of river otters by comparing otters in oiled and non-oil environs. The results showed that otters that were exposed to the oil spill had lower body mass, high levels of biomarkers (such as interleukin-6 immunoreactive, a cancer cell mediator and aspartate aminotransferase which is linked to kidney and liver problems) and change in diet compared to otters that resided in non-oiled environs (Bowyer *et al.*, 2003). A different study analyzed the effects of the deep-water horizon spill, which was known to discharge over 636 million liters of crude oil. The report showed that oil affected the heart formation and caused hydrocarbon cardio-toxicity in developing embryos of pelagic fish, Bluefin tuna, yellow-fin tuna and amberjack (Incardona *et al.*, 2014). Petroleum hydrocarbons pollution in water bodies is also known to affect ability for animals to smell, taste and respond to stimuli thus affecting normal processes like mating, feeding, catching prey or escaping predators (Khan *et al.*, 2005).

2.3 Hydrocarbon soil pollution and environmental fate

To aid in the determination on how effective pollutant clean up strategies proceed, it's vital to understand the fate and actions of pollutants in soil and the environment (Stroud *et al.*, 2007). The soil is an essential part of the environment and it plays an important role in the proper functioning and maintenance of the ecosystem (Glanz, 1995). Soil aids in maintaining the reproductive processes of plants and animals and helps in the sustenance of clean air and water (Doran *et al.*, 1996; Doran, 2002). Therefore, the quality of soil serves as a valuable indicator for environmental quality (Parr *et al.*, 1992) and this quality can be affected in the event of pollution. The sources of hydrocarbons pollutants from which contamination of soil can possibly occur include: (1) leakages from petroleum underground storage tanks and constant contributions from roads and household wastes. These are classed as lower level contributors. (2) Major tanker and pipeline bursts which contribute to soil pollution but are generally classed as minor sources and (3) Leakages from natural oil reserves (Morgan and Atlas, 1989). There has, however, been no comprehensive estimation of complete hydrocarbon soil inputs. Hydrocarbon pollutants upon soil entry can have a negative impacts, this includes effects to the soil structure and biology. Hydrocarbon pollutants can clog-up soil pores, thereby reducing the penetration of oxygen and water, altering the binding of minerals to soil and modifying water holding capacity (Morgan and Atlas, 1989).

Entry of hydrocarbon pollutants into soil could either be from a point source contamination (which includes leakages from tanks or pipelines) or a dispersion over land surfaces (Morgan and Atlas, 1989). The movement of the oil pollutants from point source could be in a lateral or vertical fashion (Morgan and Atlas, 1989) and the flow rate is dependent on amount of oil leaked, the climate conditions and the pollutant and soil properties (Bossert and Bartha, 1984; Gustafson *et al.*, 1997). The lateral flow of the pollutants normally expands the area of contamination while the downward or vertical movement reduces the risk of having a larger surface area of contamination but could lead to contamination of subsoil (Morgan and Atlas, 1989) and ground water (Somers, 1974).

Generally, once a hydrocarbon pollutant attaches to the soil surfaces, its actions in the environment are governed by its physical and chemical properties such as structure, solubility, hydrophobic nature and volatility (Jones *et al.*, 1996; Reid *et al.*, 2000). There are several processes which could occur in the event of soil pollution that determine the fate of organic pollutants and these include the swift removal of freely available fractions of pollutants via leaching, volatilization and microbial degradation (Semple *et al.*, 2013). See Figure 2-5 for a schematic representation of most of the processes. Researchers have previously reported fast early loss of readily available and extractable fractions of organic pollutants through leaching, volatilization and biodegradation (Loehr and Webster, 1996; MacLeod *et al.*, 2001).

Once the readily available fraction has been removed from the soil, the pollutant fractions left behind can form strong bonds (Stokes *et al.*, 2005), some of which are irreversible and the pollutants are only extractable by some organic solvents (Jones *et al.*, 1996; Reid *et al.*, 2000). However, as pollutants remain in the soil over time the bond becomes stronger (Alexander, 1999). Research has shown that increasing pollutant and soil contact time, causes stronger bonds between soil and pollutant fractions and this can lead to pollutant ageing (Hatzinger and Alexander, 1997). This ultimately leads to the pollutant immobilization and reduced risk of toxicity (Nam *et al.*, 1998; Gevao *et al.*, 2001; Allan *et al.*, 2006; Doick *et al.*, 2006). The recalcitrant compound stability is vital in remediation studies as it's also used in assessing the hazard and risks posed by the pollutants (Stokes *et al.*, 2005).

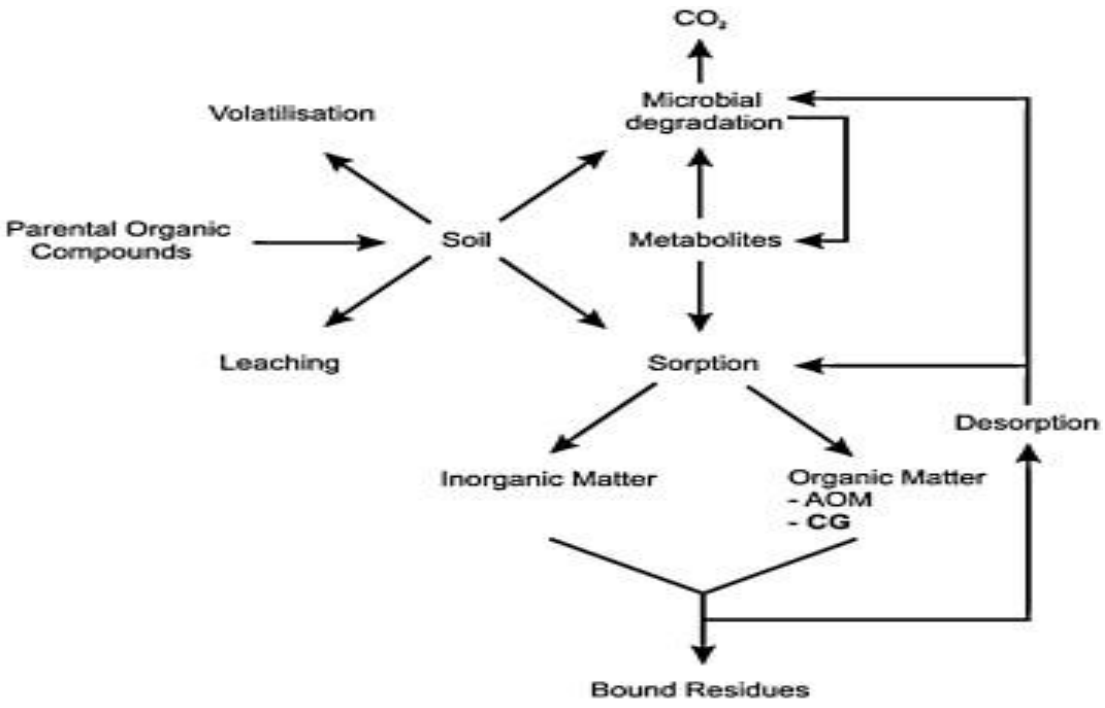


Figure 2-5 Soil and pollutant interactions-fates of pollutant in soil. Source: Semple et al. (2013),

2.4 Sorption and desorption in soil: Basic principles

Soil is made of organic and inorganic materials. The organic (amorphous or geosorbents) component plays a vital role in the fate of hydrocarbon pollutants, as hydrocarbons and other HOCs are known to interact with soil organic components (Schwarzenbach and Westall, 1981; Pignatello and Xing, 1995; Cornelissen *et al.*, 1997a).

Pollutant sequestration (See Figure 2-5) occurs via the movement of contaminants through soil micropores and the binding to the organic matrices of the soil (Hatzinger and Alexander, 1995; Luthy *et al.*, 1997). Sorption is of great importance in remediation studies and has been reported as a limitation in the bioremediation of pollutants such as hydrocarbons due to a reduced transfer of pollutants to microorganisms (Bosma *et al.*, 1996). Sorption basically affects pollutant bioavailability and biotransformation. (Semple *et al.*, 2013). Sorption is also the process known to govern pollutant ageing (Reid *et al.*, 2000).

Sorption entails the phase distribution process whereby pollutants occupy surfaces and interfaces or partition from one phase to another (Huang *et al.*, 2003). Sorption mechanisms comprise of absorption, adsorption and ion exchange (Lamichhane *et al.*, 2016). Sorption is termed adsorption when a pollutant binds on a 2 dimensional solid surface and accumulates between 2 phases (Lamichhane *et al.*, 2016) while absorption is when the molecules permeate

into a 3 dimensional solid matrix. And these molecules interacting with the solid phase could either be in dissolved or vapor state (Schwarzenbach *et al.*, 2005).

The differences in sorption mechanisms in soil can be based on the type of organic matter, as absorption is known to be the mechanism that governs the binding of HOCs to amorphous organic component while the attachment of HOCs to glassy carbon geosorbent (i.e. black carbon) is driven by adsorption (Cornelissen *et al.*, 2005; Koelmans *et al.*, 2006). This double sorption mechanism in dealing with hydrocarbon polluted soil and sediment has been previously examined (Sangiorgi *et al.*, 2014; Liu *et al.*, 2015). Black carbon (BC), such as those produced from partial combustion of materials such as fossil fuels and plants (Koelmans *et al.*, 2006) has been shown to reduce the accumulation pollutants in the environment thus lowering potential risks (Brändli *et al.*, 2008). BC has previously been predicted to produce much higher sorption capacity in soil in comparison to amorphous carbon (Cornelissen *et al.*, 2005). The strength of sorbate-sorbent interaction is also known to be influenced by size, polar nature and planarity of the sorbate (Jonker and Koelmans, 2002), and reports have shown a more extensive sorption to BC when dealing with organic pollutants possesing planar molecular structure due to the nature of black carbon adsorption sites (Cornelissen *et al.*, 2005).

Adsorption interaction forces comprise of (i) physisorption interactions (such as van der Waals forces) which are reversible and physical in nature involving non-specific attachments, and (ii) chemical interactions also called chemisorption (Semple *et al.*, 2013). Both interactions can occur simultaneously or separately (Lamichhane *et al.*, 2016). The van der Waals forces are weak dipolar forces, which are classed as attractive having the ability to adjust to adsorbate surfaces (Senesi, 1992). In the physical adsorption there is no transfer of charges between pollutant and the atoms on the surface of the adsorbent. However, the adsorbate will lose the same amount of energy (energy reduction) expensed on attachment in order to remain on the surface of the adsorbent but will also require the same amount of adsorption energy to desorb from the adsorbent (Prutton, 1994) . For the adsorption to be effective, the van der Waals forces will require the pollutant molecules to be in close proximity to the surface of the solid (Gevao *et al.*, 2000). Chemisorption on the other hand involves the formation of specific stronger and more stable chemical linkages such as covalent, ionic and polar bonds (Prutton, 1994; Hudson, 1998), and this leads to an increased presence of the compound in soil (Berry and Boyd, 1985; Dec and Bollag, 1997). Reports have stated that pollutant ageing in soil can occur either via the movement of the pollutants to stronger adsorption sites or via chemisorption which causes the formation of covalent bonds between the pollutant and soil organic materials (Dec *et al.*, 1997; White *et al.*, 1997). Reports have also stated that the stable linkage formation and recalcitrance

possibily occurs due to a fast sorption and subsequent slow nonstop (sustained) sorption causing the reinforcement of the adsorption linkage (Pignatello and Xing, 1995; Aochi and Farmer, 1997; Xing and Pignatello, 1997).

The processes of sorption and desorption for organic pollutants to adsorbents is known to occur in several phases and reaching equilibrium can take from weeks to years (Alexander, 1995). Kinetic studies have shown that the initial stages are fast (minutes to hours) and subsequently followed by a slow and then a much slower steady prolonged phase of sorption and desorption rates (Alexander, 1995; Xing *et al.*, 1996).

During the rapid sorption, a readily desorbable fraction is created (Comelissen *et al.*, 1999) while the part that desorbs gradually (which also termed the non-labile part) can be subdivided into 2 parts which differ based on their desorption kinetic constants (1^{st} order rate) (Kan *et al.*, 2000). The first part of the non-labile (slow) fraction (i.e. condensed glassy organic matter) has a desorption half-life around 2 to 7 days while the much slower fraction (i.e. sorbents with large surface area like soot) has a desorption half-life of 0.32 – 8.62 years (Kan *et al.*, 2000). In a laboratory and field study examining the sorption and desorption of chlorobenzenes, PCBs and PAH, the rate of desorption from the slow sediment section was around 10-50 times higher than the desorption from the very slow sediment section (Cornelissen *et al.*, 1997b). It has been proposed that the sorption equilibrium state of the 2 non-labile compartments could be defined via the use of Langmuir isotherms as both fraction binding domains differ only in sorption affinity and capacities, while fast binding sites of labile fraction are known to follow to other linear isotherms (van Noort *et al.*, 2003).

2.4.1 Adsorption Isotherm models

The capacity and properties of adsorbents (includes biochar and AC see section 2.8) are important in remediation studies. Adsorption isotherms aid in determining the interactions between HOCs and adsorbents (Lamichhane *et al.*, 2016). The Langmuir isotherm model (equation 2.1) (homogenous adsorption, enthalpy and activation energies remaining constant) is based on the assumption that there is the same solute affinity, adsorption at all adsorption sites and the adsorption activity at one site does not affect the other (Kundu and Gupta, 2006). In Equation 2.1, Qs is the pollutant amount/unit mass of absorbent at an equilibrium state (mg/g), Ae represents the pollutant concentration in the dissolved phase at equilibrium (mg/l), Ma is max adsorption capacity (mg/g) while b is the Langmuir constant (L/mg).

Equation 2.1 $Q_s = \frac{bM_a A_e}{(1 + bA_e)}$

Another model more recently and commonly used to ascertain the adsorption of HOCs in soil is the Freundlich model (Huang et al., 2003) with a majority of studies showing aromatic hydrocarbon adsorption data fitting well with the Freundlich model (Lamichhane et al., 2016). This model is different to the Langmuir model as this describes heterogeneous adsorption with unequal enthalpy and adsorption affinity on adsorbent surfaces (Hameed et al., 2007). The model also assumes that the solutes occupy the stronger adsorption sites first and the total sum of adsorbed solutes equals the sum of adsorption at all adsorption sites (Zeldowitsch, 1934). In Equation 2.2, Qs is the pollutant amount/unit mass of absorbent at an equilibrium state (mg/g), Ae represents the pollutant concentration at equilibrium (mg/l), n is measure heterogeneous adsorption intensity, while Kf is the Freundlich model coefficient or capacity factor.

Equation 2.2 $Q_s = K_f A^n_e$

The linear partitioning method assumes an absence of limited sorption sites on adsorbents (such as amorphous organic substances) for the binding of HOCs compounds even with rising pollutant concentrations (Chiou *et al.*, 1979). And the HOCs will transfer from an aqueous phase to a homogenous, gel-type amorphous soil organic substance (Huang *et al.*, 2003).

Predicting the transport and fate of HCs in soil can be determined using soil:water K_d or soil organic carbon:water (K_{oc}) partitioning coefficient (Mill, 1993). The partitioning model was designed for a systematic and quantitative description of sorption equilibrium (Chiou *et al.*, 1979). In the linear model Equation 2.3, K_d represents partitioning coefficient or phase equilibrium distribution equilibrium coefficient, while Q_s and A_e represent solid and aqueous phase equilibrium concentrations respectively (Huang *et al.*, 2003).

Equation 2.3 $Q_s = K_d A_e$

It is important to note that the type of isotherm utilized is dependent the type, origin of the adsorbent and properties and concentration of the pollutant (Lamichhane *et al.*, 2016).

2.5 Free aqueous concentrations

The readily desorbable fraction can become available for microbial action (Figure 2.6) or could partition to the aqueous phase and then be transported to ecological receptors (Reeves *et al.*, 2004). Assessing freely dissolved pollutant concentrations is one of the main drivers of ecotoxicology studies, as it is the freely dissolved contaminants that make ready contact with biota in the environment (Ehlers and Luthy, 2003) and their measurements could be used in the determination of remediation end points instead of total concentrations (Cornelissen *et al.*, 2005).

Passive sampling can be utilized in detecting levels of freely dissolved pollutants especially for hydrophobic pollutants. Passive sampling procedure is formed on the basis that a contaminant will flow from a medium to a collecting device due differences in chemical potential of the contaminant in both media. The flow will remain until the system reaches a state of equilibrium or the sampling ends. (Górecki and Namieśnik, 2002).

There are a number of passive devices which have been utilized in measuring free aqueous concentrations of organic pollutants (Hale *et al.*, 2010a). One of such devices which has received significant attention is the double phase semipermeable membrane devices (SPMD), which is made up of a non-polar and dense polyethylene tube containing a triolein lipid. This SPMD is non accessible to large aqueous particles (attached to sediments or humic substances) and dissolved organic matter but selects freely dissolved organic contaminants (Huckins *et al.*, 1993). The procedure imitates that of selective permeable barrier (bio membranes) of living organisms and SPMD has been utilized in determining aqueous concentrations chemicals such as polychlorinated biphenyls, pesticides and dinezodioxins and PAHs (Vrana and Schüürmann, 2002).

The use of a single phase polyethylene device made up of thin polyethylene strips is seen as an effective method for in situ sampling of organic compounds (Adams *et al.*, 2007). This polyethylene device was used in the measurement of trace concentrations of phenanthrene, pyrene and biphenyl compounds from Boston harbor water (Adams *et al.*, 2007). Other similar devices are available such as polyoxymethylene strips which were effectively used in

determining the concentrations of freely dissolved PAHs and PCBs during the dredging and disposal of polluted sediments in Norway (Cornelissen *et al.*, 2008). By using these devices, the concentration of dissolved organic pollutants can be determined either via a calibration of device uptake kinetics or estimating the phase partitioning coefficient for specific compounds (Hale *et al.*, 2010a). Acquiring the knowledge of both partition coefficient and concentration ratios of compounds can aid in determining the equilibrium state between compartments (Booij *et al.*, 1998). For example when the P.E is in aqueous solution and at equilibrium, the concentration of truly dissolved HOCs (Wc) can be estimated using the PE-Water partition coefficient constant K_{pe} (Müller *et al.*, 2001; Hale and Werner, 2010) and the measured concentration of the HOCs on the P.E. (PE_C) see Equation 2.4 (Adams *et al.*, 2007). The rate of assimilation by the devices especially with regards to SPMD can be influenced by factors such as design of sampler and molecular properties and also environmental factors like temperature, biofouling, and impedance etc. (Vrana and Schüürmann, 2002).

Equation 2.4 $Wc = PEc/K_{pe}$

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2.6 Remediation approaches

There has been an introduction of remediating approaches to help in the cleanup and management of environmental hydrocarbon pollution and such approaches include bioremediation and more recently, the use of sorbents. However both processes are supposed to be aimed at improving natural attenuation processes.

Bioremediation as a technique utilizes mostly microorganisms such as bacteria and fungi for the remediation of polluted soil and water (Strong and Burgess, 2008). The method is reliant on stimulating the propagation and growth of targeted native microbes or a consortia of microbes within the polluted sites, which are capable of performing the desirable actions (Agarwal, 1998; Stroud *et al.*, 2007). Bioremediation is versatile and an economically viable technique compared to other physiochemical methods (Atlas, 1991; Salanitro *et al.*, 1997). Over the last couple of years, bioremediation of hydrocarbon polluted soils has been an area subjected to intense research were most studies are looking into the developments and improvement of bioremediation strategies to aid in the cleanup of soils contaminated by crude oil and its products (Van Gestel *et al.*, 2003; Xu *et al.*, 2005; Lu *et al.*, 2010). It is effective for the recovery of polluted sites, and has been utilized around the world inclusive of Europe with

different success levels (Kumar *et al.*, 2011). Bioremediation laboratory and field studies have shown effective cleanup of refinery wastes, (Phung and Ross, 1979), oil sludge and pipeline spills (McMillen *et al.*, 1993; Scott *et al.*, 1993; Huesemann, 1994). However, for the process to be a success it requires being able to managed appropriate levels of pollutants, airing and mixing, adding nutrients, pH and moisture monitoring. There are example of such experiments which show that the rate of crude oil removal by bioremediation is dependent on type and concentration of the pollutant and addition of nutrients such as NH_3 and PO_4^{3-} in an optimal C: N: P ratio (Brown and Donnelly, 1983; Atlas, 1984; Leahy and Colwell, 1990; Watkinson and Morgan, 1991; Pollard *et al.*, 1994). All of these provide favorable conditions for the microbes to thrive and aids in optimizing the process (Salanitro *et al.*, 1997).

The more recent approach is the use of carbon-based adsorbents such as biochar (BC) and activated carbon (AC). The use of sorption media which involves the stabilization process has proven valuable in dealing with HOCs pollutants due to its benefits which include being easy to design and operate, low cost and little or no unwanted by products (Tong *et al.*, 2010). These adsorbents sequester and sorb the pollutants thereby reducing available concentrations (Ghosh *et al.*, 2011) by acting as carbon sink (Lehmann *et al.*, 2006). There have been reports on the efficiency of carbonaceous adsorbents on dealing with PAHs polluted soils (Cornelissen *et al.*, 2005; Rhodes *et al.*, 2008) and other pollutants such as polychlorinated biphenyls (Werner *et al.*, 2005; Werner *et al.*, 2009; Vasilyeva *et al.*, 2010), organochlorine pesticides (Hale *et al.*, 2009), and metals (Beesley *et al.*, 2010). The technique holds a lot of promise in the removal of hydrocarbon pollutants owing to the hydrocarbon compounds low solubility in aqueous media (hydrophobic nature) and high binding affinity to solid media (Alcántara *et al.*, 2009; Hu *et al.*, 2014).

2.7 Biodegradation of hydrocarbon compounds (alkanes and PAHs)

The degradation of hydrocarbon compounds by microbes is an intricate process which is dependent on the type and volume of hydrocarbon present and microbes with suitable degrading abilities (Das and Chandran, 2010). Biodegradation of hydrocarbon compounds is the process whereby specific microorganisms assimilate hydrocarbons and convert them into simpler forms which can be further catabolized by the same organism using specific enzymes or depending on the community they may be passed on to other organisms for further degradation (Megharaj *et al.*, 2011). The biodegradation technique is believed to be one of the main natural ways by

which the environment can be remediated of hydrocarbon pollutants (Jones *et al.*, 1983; Atlas, 1991; Amund and Nwokoye, 1993) and has been proven to be effective in dealing with crude oil spill effects in marine soils (Lindstrom *et al.*, 1991; Bragg *et al.*, 1994). Microbes have been reported to generally degrade hydrocarbon compounds in the following order, straight alkanes > branched alkanes > smaller MW aromatics (Perry, 1984; Aliakbari *et al.*, 2014) and bacteria are seen as main degraders of hydrocarbons in the event of a crude oil spill (Rahman *et al.*, 2003; Röling *et al.*, 2004).

Microorganisms utilize hydrocarbons as carbon and energy sources (Aliakbari *et al.*, 2014). As earlier stated alkanes are the most susceptible to microbial degradation and its believed that alkanes with carbon chain lenghts $C_{10} - C_{26}$ are the hydrocarbons most readily used by micorganisms (Blackman, 1986; Atlas, 1995). The degradation of heavier molecular weight alkanes even up to carbon chain lengths C_{44} has been previously reported (Haines and Alexander, 1974; Setti *et al.*, 1993; Varjani *et al.*, 2015). There are numerous reports of microorganisms involved in the aerobic degradation of alkanes, some of which include **Bacteria**-:*Alcanivorax, Pseudomonas Achromobacter, Rhodococcus, Sphingomonas,* **Yeast**-: *Candida, Debaryomyces, Yarrowia* **Fungi:-** *Fusarium, Aspergillus* (Watkinson and Morgan, 1991). Mycobacteria sp are also able to grow and utilize aliphatic hydrocarbon compounds such as propane and other n-alkanes (Kotani *et al.*, 2006). Kotani *et al* (2006) showed that *Mycobacterium sp* was able to utilize n-alkane $C_3 – C_6$, $C_{10} – C_{19}$ and grew on 1 propanol and 1 Butanol while *Pseudonocardia sp* was able to utilize n-alkane $C_2 – C_6$ and $C_{13} – C_{19}$ and grew on 1 propanol, 2 propanol, 1 butanol and 2 butanol. In a separate report, Hamamura, *et al* (2006) showed that *Rhodococcus sp* (*erythropolis* and *coprophilus)* and *Nacardiodes albus* were able to degrade alkanes but showed a preference for the shorter chain length (readily degradable, 15C and below) n-alkanes compared to the longer chain n alkanes (16C and above) (Hamamura *et al.*, 2006b).

Generally during alkane degradation, bacteria (with exception of certain methanotrophs) initially carry out the terminal oxidation of alkanes to alcohols by the use of specialized hydroxylase enzymes systems (Venosa and Zhu, 2003; Aliakbari *et al.*, 2014). See Table 2-1 for a list of some these enzyme classes and organisms that produce them. These oxidizing enzymes act by introducing oxgyen into the n-alkane compound (Van Beilen *et al.*, 2003). There have been reports of the metabolisation of smaller molecular weight alkanes via terminal and subterminal oxidation pathway. Such as the oxidation of propane to propan-1-ol or propan-2-ol or the oxidation of propene to 1-2-epoxypropane by *Rhodococcus rhodochrous* PNKb1(Ashraf *et al.*, 1994). Some methanotrophs have also exhibited similar traits with the

degradation of short chain alkanes (Sullivan *et al.*, 1998) and longer chain alkane degradation via subterminal oxidation has also been reported (Britton, 1984; Whyte *et al.*, 1998). The oxidation of alkanes is assisted by membrane bound proteins, monooxygenase and rubredoxin (van Beilen *et al.*, 1992). The electrons from NADH are transferred to rubredoxin by rubredoxin reductase enzyme via FAD before the electrons are finally transferred to the hydroxylase enzyme (Van Beilen *et al.*, 2003; Van Hamme *et al.*, 2003).

Table 2-1 List of some enzymes which take part in alkane oxidation. Source: Van Beilen et al. (2003)

Class of enzymes	**Alkane Substrate**	**Organism**
AlkB hydroxylases	C5-C16 alkanes	*Alcanivorax, Acinetobacter, Rhodococcus*
Methane/Butane monooxgenase	C2-C8 alkanes	*Pseudomonas sp*
Membrane Methane/Butane monooxgenase	C4-C10 alkanes	*Nocardiodes sp*
Dioxgenase	Propane, isobutane	*Rhodococcus sp*
P450 oxygenase	C4-C16 alkanes	*Acinetobacter sp*

Some genes which encode for different hydroxylase enzymes and are found in certain microbes include alk –B genes which are known to encode for hydroxylases which are responsible for the oxidation of $C_6 - C_{12}$ chain lenght alkane and also chain lenghts longer than C_{16}, while alk-M genes encode for hydroxylases responsible for the oxidation of medium weight alkanes C_8–C_{16} chain (Kohno *et al.*, 2002). After terminal oxidation is complete, the alcohol is converted to an aldehyde by alcohol and aldehyde dehydrogenase and finally to fatty acids prior to transfer into the β- oxidation pathway for further processing by the microbe (Aliakbari *et al.*, 2014; Van Hamme *et al.*, 2003). Alternatively and rarely some fungi and bacteria can oxidize both ends of the alkane compound using fatty acid monooxygenase (ὠ-oxidation) producing dicarboxylic acids (Watkinson and Morgan, 1991; Van Beilen *et al.*, 2003). The subterminal oxidation produces a secondary alcohol which is converted to ketones (Forney and Markovetz, 1970) and further converted to an ester by the Baeyer Villiger enzyme (Forney and Markovetz, 1968; Britton and Markovetz, 1977). Esterase carries out the hydrolysis of the ester to an alcohol and fatty acid (Shum and Markovetz, 1974). The fatty acid is transferred to the β-

oxidation pathway (Van Beilen *et al.*, 2003). Branched alkanes on the other hand are not easily degraded compared to the linear form of alkanes and they are believed to be more recalcitrant to microbial degradation with increasing branching (Britton, 1984). However there have been reports of the degradation of branched alkane compounds such as pristane contained in crude oil by *Brevibacterium erythrogenes* (pristane degraded as diacarboxylic acid*)* (Pirnik *et al.*, 1974) and species belonging to genera *Corynebacterium* (McKenna and Kallio, 1971) and *Rhodococcus* (Nakajima and Sato, 1983) which could follow the β- oxidation. The degradation of branched alkanes phytane and farnesane were also done by *Rhodococcus* through β-oxidation (Harayama *et al.*, 1999). The degradation of small cyclic alkanes occurs via a co – oxidation mechanism, and the pathway involves the enzymatic activity of the Baeyer-Villiger monooxygenase (Cheng *et al.*, 2002). The monooxygenase adds oxygen and cleaves a cycloketone (subterminal oxidation) which gives rise to a clyclo-alcohol and ketone (Harayama *et al.*, 1999). The microbial degradation of small cyclo alkanes have been previously reviewed by researchers (Hasegawa *et al.*, 1983; Trower *et al.*, 1985; Brzostowicz *et al.*, 2000). The degradation of larger cyclo alkanes have also been reported (Schumacher and Fakoussa, 1999). *Rhodococcus spp* was able to degrade cyclodecane by oxidizing it to alcohol and ketone via the enzymatic action of a flavo protein enzyme (Baeyer-Villiger oxygenase) which is specific for larger ring structures. The ketone was converted to an alkanoic acid which can transferred to the β-oxidation cycle (Schumacher and Fakoussa, 1999).

Numerous pathways involved in the degradation of aromatic compounds have been previously explained (Harayama *et al.*, 1999). Microbes degrade PAHs compounds by using dioxygenases which carry out the meta or ortho cleaving of the aromatic ring after introducing single or double hydroxyl groups into the aromatic ring. Monooxygenases catalyse the introduction of a single hydroxyl group while a double hydroxyl is added via enzymatic action of dioxygenases (Harayama *et al.*, 1992; Harayama and Timmis, 2012). A study carried out by Dean-Ross *et al* (2002) showed that *Rhodococcus* sp was able to degrade fluoranthene in the presence of anthracene via the meta cleavage and ortho pathways, producing 2 different metabolites 9 fluorenone-1-carboxylic-acid and fluorine-1-carboxylic acid, respectively, while the fused ring of fluoranthene molecule was cleaved (meta cleavage) to produce 9 fluorenone-1-carboxylic-acid in *Mycobacterium* (Dean-Ross *et al.*, 2002).

During the biodegradation of PAHs, lower molecular weight PAHs like napthalene and phenanthrene are known to be more susceptible to microbial degradation (Harayama *et al.*, 1999). Numerous bacteria have been reported to oxidize napthalene using dioxygenase enzymes such as *Rhodococcus* while a few such as *Mycobacteria* can also use the P_{450}

monoxygenase enzymes (Bamforth and Singleton, 2005). In a study carried out by Samanta et al. (1999), *Arthrobacter sulphureus* preferentially degraded phenanthrene in the presence of other heavier PAHs anthracene, pyrene (Samanta *et al.*, 1999). In similar study *Arthrobacter* sp degraded 92.4 g of fluorene within 37 hours converting it to 30 g/ml of protein. The study also showed the formation of key intermediates such as 9-fluorenone and 3, -4-dihydrocoumarin from the catabolism of fluorene and also indicated that the mineralization of fluorene can follow 3 different routes. The first two routes aid in supporting growth of the *Arthrobacter sp.* The *Arthrobacter* dioxygenase attacked the fluorene compound at position 1,2 leading to acidification and formation of lactone (which is a similar step in the mineralization of naphthalene by *Arthrobacter sp*), this was followed by lactone aromatic ring breakdown or hydrolysis to form 3, -4-dihydrocoumarin, and finally, the formation of 3-hydroxyphenyl and salicylate compounds prior to the complete utilization of fluorene (Casellas *et al.*, 1997).

2.7.1 Effects of nutrients and other bio-stimulation strategies on hydrocarbon biodegradation

The availability of nutrients in the form of nitrogen and phosphorus has been reported to limit the biodegradation of hydrocarbon pollutants in the environment (Walker and Colwell, 1974; Ward and Brock, 1976). The addition of nitrogen and phosphorus helps in enhancing the growth of hydrocarbon degraders leading to increased biodegradation rates (Rosenberg *et al.*, 1996) thus it is vital to have readily available nutrients during hydrocarbon pollution bioremediation (Atlas, 1981; Atagana *et al.*, 2003). Hydrocarbon pollutants cause increased levels of carbon sources which in turn cause a reduction in other nutrients such as nitrogen and phosphorus as they are not found in hydrocarbons (Wolicka *et al.*, 2009). Therefore field and laboratory studies mostly employ the addition of nitrogen and phosphorus in order to balance the nutrients available and enhance microbial degradation (Morgan and Atlas, 1989). There have been several reports on the improvement of hydrocarbon biodegradation due to nutrient bio-stimulation. In a field study by Raymond *et al* (1976), the addition of fertilizer caused a higher reduction in crude oil and petroleum hydrocarbon products and an increased presence of degrading microorganisms compared to untreated soils. The addition of the fertilizer was also seen to reduce pollutant leaching thus reducing hydrocarbon ground water pollution (Raymond *et al.*, 1976). In another laboratory study which looked at improving the parameters for land farming by evaluating the effects of certain conditions such as the addition of fertilizer and micronutrients and sludge loading on the degradation of hydrocarbon pollutants. The results showed an increased degradation of the aromatic fraction and asphalthenes due to fertilizer addition. And similarly, it also showed a reduction in pollutant leaching as there was no recoveries of hydrocarbons or phosphorus in the leachate but slight increase in total organic

carbon possibly due to the hydrocarbon degradation (Dibble and Bartha, 1979). Atlas and Bartha (1972) showed that the addition of nitrate and phosphate together led to a much higher (67% increase) biodegradation and mineralization of petroleum compared to when the nutrient supplements were added separately (Atlas and Bartha, 1972). A recent report also showed an enhancement of hydrocarbon degradation and biomass production by *Pseudomonas, Bacillus, Acinetobacter* and *Stenotrophomonas* in nutrient media with absence of light (Khan *et al.*, 2016), while another study showed reductions in aliphatic and aromatic fractions in nutrient treatment soils (Jiang *et al.*, 2016b). The addition of nutrients has been shown to readily improve hydrocarbon degradation especially when optimal ratios of C: N: P are determined and applied. The addition of inappropriate amounts of C: N: P can show negative effects such as increased toxicity to microbial communities when excess C: N: P is added (Wang *et al.*, 2011). A laboratory microcosm study carried out to determine the impacts of nutrients on the biodegradation of crude oil, showed that the degradation of saturated hydrocarbon compounds was permanently inhibited due to the absence of nutrients while higher levels of nutrient temporally prevented the microbial assimilation of PAHs which was possibly restored due to a gradual reduction in nutrient levels in the soil as other carbon sources were being utilized (Chaineau *et al.*, 2005). Phosphorus is a requirement for the production of cell constituents, nucleic acid and energy in the form of adenine triphosphate in microorganisms. The reported optimum C:N:P is 100: 10:1 (Morgan and Atlas, 1989; Mills and Frankenberger, 1994) while Wolicka et al. (2009) reported C: N: P ratios of 100:9:2 or 250:10:3 in the biodegradation of BTEX and it's been proposed that for remediation of crude oil contaminated soil, C: N: P should be calculated based on the aliphatic hydrocarbon concentrations, as the level of nutrients impacts more on their biodegradation (Chaineau *et al.*, 2005).

In addition to nutrients, there are several factors under natural conditions which can control the rate of hydrocarbon biodegradation; these include temperature, pH, Oxygen etc. Temperature can affect the physico-chemical nature of oils thereby altering the viscosity and solubility of crude oil (Tyagi *et al.*, 2011). Temperature also has effect on microbial metabolic rates and structure of microbial communities (Cerniglia, 1993). The solubility and viscosity of oil increases at low temperature; however this reduces the solubility of oxygen required for biodegradation process thus limiting the activity of aerobic degraders. Low temperature levels also reduces the volatility of smaller hydrocarbon compounds and this enhances their toxicity towards microorganism thereby also causing a delay in the biodegradation process (Cerniglia, 1993). Higher temperatures have been reported to increase hydrocarbon metabolism and by determining the optimum temperature, typically between 30 to 40°C, hydrocarbon

biodegradation is enhanced due to elevated enzymatic activity (Leahy and Colwell, 1990; Cerniglia, 1993; Sihag *et al.*, 2014). Another vital parameter which influences microbial enzymatic activity is pH. Soils are known to have varying pH levels and this can influence the presence of hydrocarbon degrading microbes. For example neutral pH is known to favor mostly heterotrophic bacteria and fungi (Leahy and Colwell, 1990). Pawar (2012) studied the effects of pH on degradation of PAHs and concluded that pH 7.5 was more appropriate for the biodegradation of PAHs in comparison to pH 5 and 6.5. The study also suggested that due to the effects of pH on biodegradation processes, altering soil pH to favorable levels through processes such as liming could enhance bioremediation of hydrocarbon compounds. Oxygen is also an important factor in the biodegradation of petroleum hydrocarbon pollutants in soil. In soils with relatively high hydrocarbon contaminants, the oxygen required can supersede the current oxygen levels within the soil and this can lead to a reduction in biodegradation rates due to rapidly deleting oxygen levels (Davis *et al.*, 1997). The supply of oxygen or aeration is one way of assisting the biodegradation process, as oxidation hydrolysis of intricate hydrocarbon compounds needs to occur prior to their utilization by microorganisms (Wang *et al.*, 2011).

Another cost effective way of improving the biodegradation of hydrocarbon compounds in soil can be done via a bio-stimulation strategy called composting. Composting involves the piling of agricultural and municipal wastes on contaminated soils (Thapa *et al.*, 2012), thereby utilizing microorganisms to remediate or stabilize contaminates (Chen *et al.*, 2015) . The addition of organic wastes such as manure, helps to support the growth and development of microbes thus reducing time required for biodegradation process (Thapa *et al.*, 2012). Other benefits of compositing include improving the quality and fertility of soil and it also aids in reducing cost required for the treatment of agricultural and municipal solid wastes (Chen *et al.*, 2015). Composting application strategies include direct composting, integration of bulking agent, bio-augmentation or the use of surfactants. Most studies have utilized one or a combination of strategies to attain desired results (Chen *et al.*, 2015).

2.7.2 Bio-augmentation

Although most studies have shown that native microbial communities are capable of efficiently degrading hydrocarbon compounds in comparison to introduced strains (Margesin and Schinner, 2001), bio-augmentation strategy (via the introduction of pre-adapted microbes to aid the indigenous microbial communities at site) can be employed to improve the pollutant acclimation time and speed up bioremediation process. This is seen especially in instances

where the response to contamination is slow due to a lack of contaminant prior exposure or the native organisms lack the metabolic ability to degrade the pollutants (Margesin and Schinner, 2001; Vargas *et al.*, 2017). Otte *et al.* (1994) had previously attempted this strategy by cultivating biomass from a fraction of contaminated soil and utilizing it as an inoculum to bio-remediate polychlorinated biphenyls (PCBs) and PAHs contaminated soils. The laboratory results showed a decrease in time required for the removal PCBs and PAHs from soil. Other Studies have previously highlighted the efficacy of bio-augmentation strategies in dealing with hydrocarbon polluted soils. In a comparative study carried out by Bento *et al.* (2005), the removal of total petroleum hydrocarbons in soil by natural attenuation, bio-stimulation and bio-augmentation was examined. Bio-augmentation strategy was shown to have the highest hydrocarbon removal rate from the soil in comparison to the other strategies. In a field study carried out by Andreolli *et al.* (2015), the use of bio-augmentation strategy showed up to 55% removal of toxic hydrocarbon compound from soil and was seen to be more effective than natural attenuation.

2.8 Sorption media

Biochar is a rich carbonaceous compound synthesized from naturally occurring organic materials obtained from agricultural farms, industry and forestry. Some of these materials include wood (Xie *et al.*, 2014; Yargicoglu *et al.*, 2015), wheat (Chun *et al.*, 2004), saw mill and forest remains (Anderson *et al.*, 2013). Biochar is produced via pyrolysis (typically at temperature below 700^0C) of these materials with a limited influx of oxygen thus the final structure comprises of aromatic carbon compound which is of great importance in relation to adsorption. It's been reported to contain low levels of hydrogen and oxygen but is rich in carbon, phosphorus, calcium, magnesium and nitrogen (Lehmann and Joseph, 2015). Biochar is believed to comprise of both glassy and rubbery compartments and adsorption can be linear and non-linear (Chen and Yuan, 2011).

The general aim for the production and application of biochar is the management of the environment and under this broad aim are objectives such improving soil quality, managing waste, alleviation of climate change, and producing renewable energy which are all interconnected with societal and monetary benefits (See Figure 2-6) (Lehmann and Joseph, 2015). Pollutants such as hydrocarbon compounds have been reported to affect soil properties and decline soil fertility (Akpan *et al.*, 2013), however addition of biochar is believed to improve soil fertility, texture, porosity, plant-biota interactions and water holding capacity (Atkinson *et al.*, 2010; Lehmann *et al.*, 2011). Such changes occur as result of the dissimilar

physical nature of the soil and biochar (Lehmann *et al.*, 2011) and soil biota such as microorganisms which partake in vital soil processes such as nutrient cycling (Steiner *et al.*, 2008) which will most likely be affected by the changes. Biochar creates an environment for certain microorganisms to thrive. For example the addition of biochar to clay helps in reducing the tensile strength of the soil thus improving fungal nutrient mining (Warnock *et al.*, 2007) and biota transportation through soil (Lehmann *et al.*, 2011). More nutrient is retained in soil with higher water holding capacity because as there is a reduction in water filtration through soil pores, nutrient leaching (like nitrate) also reduces (Glaser *et al.*, 2002). There is the improvement of soil structure by improving soil aggregate stability and reducing the disaggregation impacts of wetting and drying cycles (erosion) on the soil (Piccolo *et al.*, 1997).

There have been many reports on changes to soil microbial community structure and abundance induced by the presence of biochar (Pietikäinen *et al.*, 2000; Kim *et al.*, 2007; Grossman *et al.*, 2010). It is also known that biochar addition can increase soil microbial biomass (Liang *et al.*, 2010) and microbial reproduction rates (Pietikäinen *et al.*, 2000). A good example is the increase of mycorrhizal fungi (arbuscular and ectomycorrhizal) which colonize the plant roots (such as larch and wheat) due to the addition of biochar (Makoto *et al.*, 2010; Solaiman *et al.*, 2010). The addition of biochar could also increase in the abundance of bacteria in the soil, as they are known to adhere unto biochar surfaces making them less leachable (Pietikäinen *et al.*, 2000). However this increase in abundance which gives rise to shift in the community structure is normally brought about by the dominace of a particular microbial group as the increases do not occur evenly across all phylotypes (Lehmann *et al.*, 2011).

Environmental changes such as global warming has been brought about by increasing populations and industrial activities (Barrow, 2012). By adding biochar to soil it's been reported that climate change can be mitigated via the sequestration of carbon and its storage in a stable form for many years and also reduction of greenhouse gas emissions (Lehmann *et al.*, 2008; Macías and Arbestain, 2010). Biochar has shown promise as an effective adsorbent with regards to hydrocarbon remediation due to its surface area and structure (Yargicoglu *et al.*, 2015; Lamichhane *et al.*, 2016), and there have been several reports on its efficiency in dealing with hydrocarbon pollutants. A field trial study showed that amendment of soils with biochar caused a reduction in the concentration of heavy molecular weight PAHs (4-5 ring structures) and lower molecular weight PAHs (4-5 ring structures) by more than 50% and 40 % respectively thus lowering the toxic effect to microbes and plants (Beesley *et al.*, 2010). Biochar has also

been reported to cause a reduction of up to 57% of freely dissolved PAHs in sewage sludge (Oleszczuk *et al.*, 2012) while Wang et al also demonstrated the removal of up to 60% PAHs from solution using wooden biochar (Wang *et al.*, 2006).

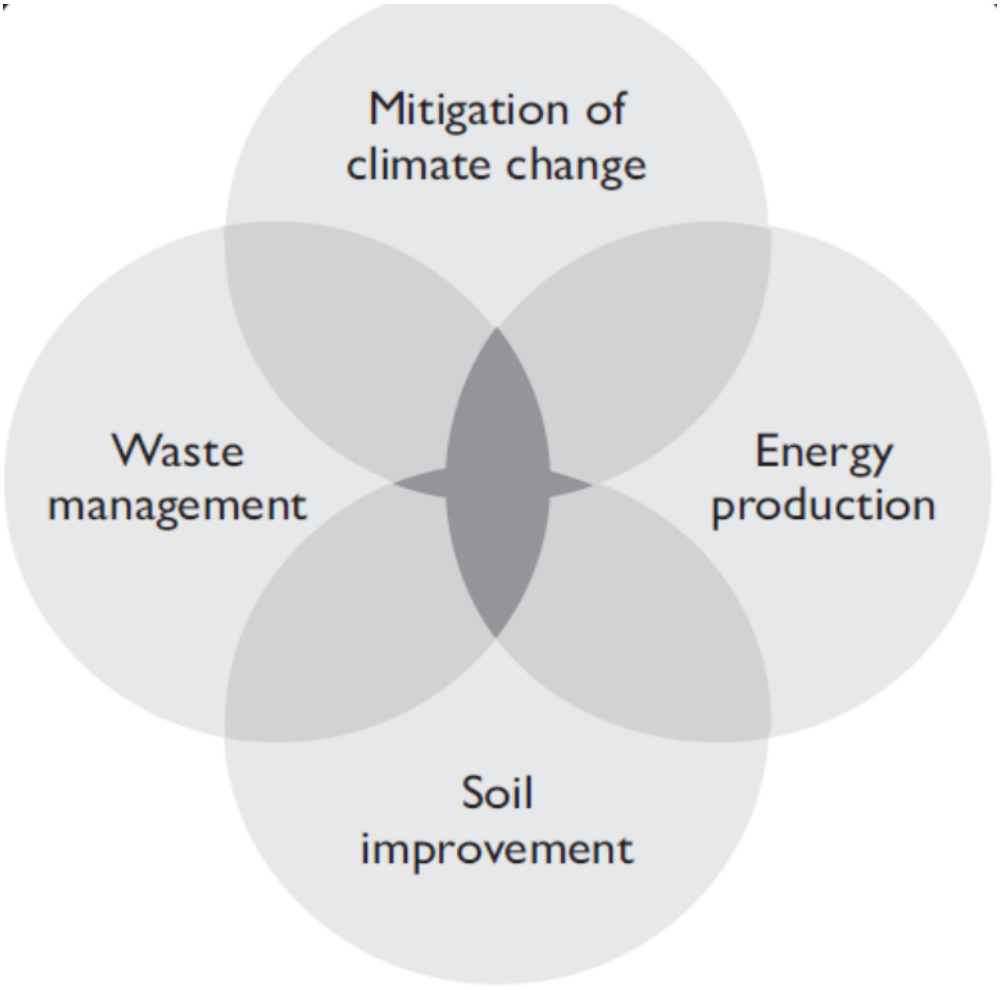

Figure 2-6 Benefits of biochar production and application Source: Lehmann and Joseph (2015)

It's also been reported that biochar feed stock, production method, pollutant concentration and ageing all govern the sorption capacity of biochar (Wang *et al.*, 2006). In a report biochar produced from wheat straw was used in the removal of over 90% of PAHs, i.e. phenanthrene, fluoranthene, and pyrene, from aqueous solution and the results also showed that the percentage recovery increased with higher dose of biochar. Biochar production techniques in the same report impacted on the removal rate as there was higher removal with biochar produced at higher pyrolysis temperature compared to those produced at lower temperatures (Li *et al.*, 2014). The increase in sorption for PAHs with increasing production temperature has also been reported in other reports (Chen *et al.*, 2008; Chen and Yuan, 2011). It is believed that increasing pyrolytic temperature increases sorption capacity via the enhancement of sorbent carbon content and surface microporosity (Bornemann *et al.*, 2007; Chen and Yuan, 2011).

Similar to biochar, activated carbon (AC) is a carbonaceous material obtained via the pyrolysis of different carbonaceous materials which include agricultural precursors such as coconut shells (Alyani and Amin, 2008), sugar cane bagasse and wood (Ioannidou and Zabaniotou, 2007). The production occurs at temperature below 1000 ^{0}C and can involve a 2 step process of carbonization (<800 ^{0}C) with limited oxygen and activation (950-1000^{0}C). The resultant product property is dependent on the material and activating agent utilized (Bansal and Goyal, 2005). The process enhances carbonyl content but removes organic compounds and nutrients from AC (Warnock *et al.*, 2007). There are mainly 2 forms of activated carbon (although others forms such as cloth are synthesized), the granular activated carbon (GAC) which possesses large internal surface area and micropores and the powdered or finely crushed form (PAC) with larger pores and lesser internal surface area (Bansal and Goyal, 2005). This high surface area and microporosity are some of the properties which have made AC one of the most frequently utilized adsorbents in the immobilization of HOCs (Burchell, 1999; Ioannidou and Zabaniotou, 2007) and several studies have shown the potential AC in the reduction of hydrocarbon concentrations. A field AC amendment study using 2% PAC and granulated AC and passive samplers for measurement of free aqueous concentrations showed that PAC was better than GAC in the removal of PAHs with removal efficiencies of 93%, 76 % and 84%, 69% respectively at 17 and 28 months (Hale *et al.*, 2012). Similarly varying dosages of AC (0.5 - 2%) was used in significantly reducing aqueous concentration (up to 95%) of PAHs in sewage sludge in an experiment carried by Oleszczuk et al., (2012). AC synthesized from soya bean was able to remove phenanthrene, naphthalene and acenaphthene at very high rates and the removal was optimal at higher carbon concentrations and carbonization temperatures (Kong *et al.*, 2011). AC has also been reported to be effective in the removal of alkanes as reported in a modelled study using coconut shell AC. The results of the study showed the AC had high removal capacity for alkanes (methane, butane and ethane) (Walton *et al.*, 2005).

In terms of AC effects to soil biota there have been reports that sorbents reduce microbial bio-accessibility (Rhodes *et al.*, 2008), meaning that AC has previously has negative impacts on the microbiology of the soil with respect to the rate of HCs degradation. Field study experimental results showed, however, equal abundance of PAHs degraders *Rhodococcus jostii* RHA-1 and *Rhodococcus erythropolis* in soils with and without PAC and GAC addition, thus the AC amended soils retain the ability to biodegrade PAHs (Meynet *et al.*, 2012).

2.9 The impacts of adding biofuels components on the fate of HCs

The use of biofuels offers benefits which have made them an attractive fuel alternative to petroleum (Bozbas, 2008; Demirbas, 2009a; Escobar *et al.*, 2009). Some of these benefits include: reduction in enviromental (climate change) (Escobar *et al.*, 2009), financial and socioeconomic effects (Demirbas, 2009b). Biofuels are synthesized from natural materials such as corn, wheat and oil seeds and 2 of the most commonly utilised biofuels are ethanol and biodiesel (Demirbas, 2008). Ethanol can be used in its pure form, or more typically it is mixed with gasoline (leading to gashol) to boost octane and reduce air pollution caused by unleaded gasoline, while biodiesel which also can be used in pure form or can be mixed with petroleum diesel, is used in automobiles and electric generators (Demirbas, 2008; Demirbas, 2009b). Inspite of the various environmental benefits of biofuels, there are concerns that in the event of a spill, the co-existence of biofuels and petroleum in soil gives rise to longer petroleum vapor plumes at contaminated sites (Cagliari *et al.*, 2010). Cagliari *et al*., (2010) also reports that the volatilzation or evaporation of benzene is not solely affected by ethanol concentration, but other aromatic compounds play a vital role, while toluene and xylene evaporation is dependant on ethanol concentration. There are also reports of higher ground water pollution risks due to the presence of ethanol, as ethanol can act as a cosolvent, thus increasing BTEX dissolution into water from gasohol (Powers *et al.*, 2001). Its been suggested that the transport of BTEX in moving groundwater can be sorbed on carbon materials in aquifer thus reducting the rate of transportation, but it's not clear if the presence of ethanol reduces sorption and facilitates BTEX transportation (Alvarez, 1996). When compounds like benzene get into ground water in considerable concentrations they can pose a risk to important water bodies (Cagliari *et al*., 2010). The aerobic biodegradation of BTEX may become more limited due to the reported preferential degradation of ethanol in gasoline and exhaustion of electron acceptors (Alvarez, 1996).

The inibitory effects of biodiesel on the removal of hydrocarbons compounds have also been reported (Corseuil *et al.*, 2011). In a batch microcosm experiment carried out by Corseuil *et al*., (2011) the time required for the removal of benzene and toluene increased from 25 to 34 days in the presence of biodiesel and with only 45% of benzene removal. The report also assumed that biodiesel is more favorable to limit migration over a small region and induced a longer removal inhibitory effect on other HCs in comparison to ethanol due to its lower mobility and solubility and it is also less biodegradable. However this claim still requires further investigation. Elazhari *et al*., (2013) also reported the effects of biofuels on the volatile hydrocarbon degradation. The report showed a tempory competition between biofuel and VPH

degraders in the presence of limited nutrients thus leading to a reduction in aromatic compound degradation. The results also showed ethanol to be more easily degradable than biodiesel as ethanol had a larger impact on the degradation of the monoaromatic compounds. The biodegradation of toluene however increased with decreasing levels of ethanol (Elazhari-Ali *et al.*, 2013).

2.10 Microbial sequence analysis and its importance

The characterization of bacterial population within polluted sites and the knowledge of microbial degrading capabilities would offer valuable additional understanding for enhancing the remediation and management of such environs (Brito *et al.*, 2006a). The recent advancement in microbial ecological studies have offered the ability to utilize culture-independent methods for the identification and characterization of microbial diversity at environmental sites (Van Hamme *et al.*, 2003; Brito *et al.*, 2006a). The genomic DNA analysis helps in cutting out the need for cultivation and isolating specimens which can be laborious (Shokralla *et al.*, 2012; Czaplicki and Gunsch, 2016). Genomic molecular techniques are used in the identification and defining of community structures and the technique is based on the analyses of 16S rRNA genes (Brito *et al.*, 2006a). The 16S rRNA gene is the most frequently used species proxy in microbial community analysis (Cardenas and Tiedje, 2008), as its universally found in bacteria. 16S rRNA genes are known to comprise of 9 hypervariable regions (V1-V9) and flanking conserved regions (Chakravorty *et al.*, 2007). The conserved regions are quite similar between bacterial species while the hypervariable region possess varying sequence diversity in bacteria species and can be targeted in the taxonomy identification of bacteria (Chakravorty *et al.*, 2007). Hypervariable regions of the 16S rRNA gene and nucleotide barcodes (aid in sample identification) can be combined with primers in the surveying of environmental samples with numerous sequences at a particular time (Cardenas and Tiedje, 2008). The primers utilized can be specific and used in targeting certain taxa whilst others normally classed as universal primers can target all prokaryotic genes (Baker *et al.*, 2003). Due to the sequence diversity within 16S rRNA hypervariable regions, a single hypervariable cannot be utilized in distinguishing all bacteria (Chakravorty *et al.*, 2007), and its believed more than one hypervariable region should be targeted. These hypervariable regions, which are between 100-350bp, possess information, which have been utilized in microbial classification by databases such as Ribosomal Database Project (RDP), Greengenes and Silva (Cardenas and Tiedje, 2008). Cole et al., (2009) stated that as of March 2010, RDP had over 1 million bacterial and 50 thousand archaeal archived 16SrRNA gene sequences some of which were obtained from cultured and uncultured prokaryotic organisms. The use 16S rRNA genes

has its limitations which include low evolution rate, inability to link organism functions and variation in copy numbers, however it's the only molecular marker located in all prokaryotic organisms that possess enough genetic information which could be utilized in differentiating closely related microorganisms (Schloss *et al.*, 2011).

2.10.1 Advancement of DNA sequencing

The advancement in orthodox sequencing technologies, such as the 1st generation Sanger sequencing which utilizes a chain termination technology (Sanger *et al.*, 1977) gave rise to broader scale biosynthetic projects with wider application range (Shokralla *et al.*, 2012). The use of the Sanger sequencing was labor intensive (manual) and requiring radioactive primers (Liu *et al.*, 2012). This led to the generation of the first automated sequencer, the Applied Biosystems AB370 (Liu *et al.*, 2012) and after a series of modifications which included switching separation technique from slab based to capillary electrophoresis separation technique and increasing the number of samples that can be analyzed (higher parallelism) (Zhou *et al.*, 2010), it was used in sequencing the human genome (Hutchison, 2007). However, major limitation in the use of the system included low throughput and high cost, notably the human genome project which took approximately 10 years and cost 3 billion dollars (Schadt *et al.*, 2010). The dependence on the separation electrophoresis led to the inability to attain higher parallelism, speed and reduction of costs and highlighted the crucial need for new generation of systems in DNA sequencing (Zhou *et al.*, 2010).

2.10.2 Next generation sequencing platforms

There is the need for the ability to read DNA from a series of DNA templates in parallel during the recovery of DNA sequences from environmental samples and next generation sequencing technologies have the ability to perform this task at low costs (Shokralla *et al.*, 2012). The principle behind the high throughput NGS technology entails the sequencing of up to millions of DNA molecules at the same time in a parallel manner (Pareek *et al.*, 2011; Shokralla *et al.*, 2012). The sequenceing can be done in a step by step repetitive fashion or in a real-time continous fashion and each template which has been individually sequenced is totaled with the sum of sequences generated (Pareek *et al.*, 2011). There has been an increase recently in the number of studies utilizing NGS platfoms and although these technologies differ in terms of chemistry, addition of bases, detection tools (Shokralla *et al.*, 2012), template size, read length, through put and genome coverage (Metzker, 2010), they all broadly share a reliance on

combination strategies of library preparation, sequencing, imaging, alignment and assembly procedures (Metzker, 2010).

The next generation sequencing platforms are categorised in 2 main classes, namely the PCR and non PCR based technologies (Shokralla *et al.*, 2012). The former includes Roche 454Genome Sequencer (GS), illumina Genome sequence (HiSeq 2000), AB SOLID and Ion personal Genome Machine (PGM Life Technologies) (Shendure and Ji, 2008). Alternative systems are also offered by Roche and illumina companies which are the Roche 454 GS junior and illumina MiSeq. These machines (inclusive of the PGM machine) are bench top smaller size machines which are cheaper to run (Loman *et al.*, 2012). The non PCR based platfoms include HeliScope and PacBio RS SMRT systems. The differences between some of these systems in terms of running costs, read lengths, sequencing out puts, run times, advantages and limitations have been reviewed by Shokralla *et al*., (2012) and Liu *et al* (2012).

2.10.3 Importance of Next generation sequencing in remediation studies

An understanding of the interactions between microbes and changes in the environment caused by natural or anthropogenic sources is an important aspect of remediation studies (Abram, 2015). Microorganisms are well-known for colonizing polluted or harsh environments (Bundy *et al.*, 2009; Jones *et al.*, 2014b). Microbial communities are an important parameter in the monitoring of polluted sites (Beale *et al.*, 2017) and for bioremediation (natural attenuation, bio-stimulation or bio-augmentation) to be successful it is important to have an understanding of the physico-chemical nature of the contaminated site and also comprehensive description of microbial communities (structure) which participate in important physiological activities (Desai *et al.*, 2010; George *et al.*, 2010). NGS is powerful tool that offers a broad understanding into novel organisms in a high-throughput manner thus allowing the detailed study of complex microbial communities in their natural environments (Solomon *et al.*, 2014). This ability to gain in-depth insight of the microbial communities residing at contaminated sites is vital, as it would help in determining the microbial responses to contamination and will also aid in evaluating the effects of bioremediation interventions on the native microorganisms (such as to enrichment or augmentation target groups of microorganisms) either via the detection of changes to the community structure or the monitoring of indicator microbes (Yang *et al.*, 2012; Techtmann and Hazen, 2016). For example, microorganisms are the "primary responders" in the event of an oil spill, and the community structure can be altered after contamination, due to the varying abundance different constituents within complex petroleum hydrocarbon mixtures (Mendelssohn *et al.*, 2012). The successive degradation of these petroleum components and

presence of toxic intermediate compounds, leads to changes in type of microorganism present or absent at a particular contaminated site (Mendelssohn *et al.,* 2012). This type alteration or changes can be studied or monitored via the use NGS technologies.The use of such metagenomics' technique is known to offer a more impartial analysis of the microbial communities within polluted environs which includes the capture of microbes that are hard to culture thus having the potential to inform the proper utilization of remediation strategies to achieve a speedy clean-up of polluted sites in a minimally invasive manner (Techtmann and Hazen, 2016). In addition, metagenomics' approaches also assist in the comprehension of certain bioremediation processes which can aid in informing efforts to improve the efficiency of bioremediation (Techtmann and Hazen, 2016).

More recently NGS platforms have been utilized in environmental studies related to hydrocarbon pollutants. A study by Kostka *et al.* (2011) examined the bacterial communities within beach sands which were affected by the deep water horizon spill. The study was able to carry out the identification and characterization of the predominant bacteria communities, which play vital roles in hydrocarbon degradation and its believed that these organisms could be utilized as exemplary and indicator microorganism's in future ecological investigation of microbial hydrocarbon degraders in the Gulf of Mexico. King *et al.* (2015) also reports on power of NGS technologies which was very useful in the identification and characterization of hydrocarbon degrading communities at an exceptional resolution level after the deep water horizon spill. The extensive analysis of the bacterial communities obtained from NGS results showed an abundance hydrocarbon degrading taxa with specific reference to *Gammaproteobactria* class within the Gulf of Mexico and also highlighted a succession of changes to the microbial communities depending on oil flow rate, physico-chemical conditions and changes to oil composition.

In another study by Yergeau *et al.,* (2012) researchers utilized Roche 454 sequencing and PGM ion torrent (314 and 316 chip) in elucidation of microbial communities in the Athabasca environment, impacted by oil-sand mining. The results revealed a difference in microbial communities between samples taken from rivers close to oil sands and those taken further away. From samples taken in close proximity, researchers were able to identify a dominance of *Proteobacteria* (with dominant classes *Alphaproteobacteria* and Betaproteobacteria) while *Bacteroidetes*, *Firmicutes,* and *Chloroflexi* were also abundant and given recognition as potential hydrocarbon pollutant bio indicators. The report also showed the reliability of ion torrent in the sequencing of libraries from environmental samples. In a separate report, 454 pyrosequencing was used in identifying the bacterial communities found in crude oil pipeline

permafrost soils. 84834 reads and 14448 operational taxonomic units (OTUs) were identified. A majority of the bacteria belong to *Proteobacteria* made up of classes *Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria* and *Gamaproteobacteria*. The researchers were also able to identify known model hydrocarbon degraders *Rhodococcus, Pseudomonas and Sphingomonas* (Yang et al., 2012*).* Pyrosequencing and DGGE were used in studying bacterial and fungi microbial communities in bio-stimulated and bio-augmented creosote polluted soils. Pyrosequencing results showed the abundance of bacteria species belonging to *Gammaproteobacteria* and fungal genera *Fusarium* and *Scedosporiun.* However the sequencing data also revealed major changes in bacterial community composition due to the addition of a mobilizing agent while the proliferation of *Actinobacteria and Bacteriodetes* being affected by the presence of a nonionic surfactant (Lladó *et al.*, 2015). Reports such as these have shown the significance in carrying out bioremediation assays in combination with use of NGS technologies.

Chapter 3. Physico-chemical evaluation- Impacts of activated carbon amendment time on the fate of crude oil in soil bioremediation experiments.

3.1 Introduction

Crude oil spills resulting in soil pollution can have far-reaching consequences, and the effects range from animals to human health and this has been a major issue over the last couple of years (Khan *et al.*, 2005; Atlas and Hazen, 2011; Ordinioha and Brisibe, 2013). Consequently, there is a need for effective remediation strategies. Bioremediation, as a remediation strategy is readily utilized due to its low cost and no adverse environmental effects (Atlas, 1991; Margesin and Schinner, 2001; Maletić *et al.*, 2013). The efficacy of this technique on hydrocarbon-contaminated soils has already been demonstrated in laboratory experiments (Mukherjee and Bordoloi, 2011) and field trails (Xu and Lu, 2010; Beškoski *et al.*, 2011). However, PAHs and other complex recalcitrant compounds are harder to biodegrade than other crude oil components (Atlas, 1995), because of their chemical stability and limited bioavailability thereby increasing their persistence in the environment (Shin *et al.*, 2006) and possible long-term risks to environmental receptors (Huesemann *et al.*, 2004)

The use of strong adsorbents such as activated carbon (AC) and biochar has recently gained a lot of interest in the clean-up of hydrophobic organic compound (HOC) polluted environments due to their perceived economic and environmental benefits (Bhatnagar and Sillanpää, 2010; Xi and Chen, 2014). AC has been shown to reduce the bioavailable concentrations of HOCs such as dissolved concentrations of PAHs and thus being able to limit mobility as well as environmental bioavailability and toxicity. (Zimmerman *et al.*, 2004; Brändli *et al.*, 2008; Yang *et al.*, 2009; Hale *et al.*, 2012).

When added to soil or sediment, AC strongly binds or sorbs these compounds causing the pollutants to transfer from the polluted matrix to the AC surface thus limiting the transfer into the environment or uptake by organisms and plants (Ehlers and Luthy, 2003; Denyes *et al.*, 2013). Examples of some organisms, which have been used to demonstrate reduced pollutant bioavailability following AC amendment, are earthworms, clams (*Macoma balthic),* and the gastropod Hinia (McLeod *et al.*, 2004; Cornelissen *et al.*, 2006; Jakob *et al.*, 2012). AC has also been shown to improve plant growth (Jakob *et al.*, 2012) such as *Raphanus sativus* in hydrocarbon polluted/unpolluted soils in comparison to other compounds such as charcoal and compost (Marchal *et al.*, 2014) and shown to reduce plant pollutant uptake from soil (Denyes *et al.*, 2013).

Although researchers have highlighted the importance of strong adsorbents in particularly AC in the removal of organic pollutants due its hydrophobic nature, absorptive capability, surface area and structure (pore size) (Zimmerman *et al.*, 2005; Vasilyeva *et al.*, 2006; Awoyemi, 2011; Patmont *et al.*, 2015), there have been varying reports on the effects of strong sorbents on beneficial microbial activities such as HOCs degradation. Some reports have suggested that the use of sorbents such as AC, limits not only the pollutant ecotoxicity, but also microbial bio-accessibility which in turn slows down the pollutant biodegradation and this could possibly enhance the persistence of HOCs pollutants (Rhodes *et al.*, 2008; Rhodes *et al.*, 2010). This reduced bioavailability could lead to a shift in the microbial community structure and function brought about by the possible loss of HOCs degraders from the predominant microbial community and/or a metabolic switch to other forms of carbon substrates over HOCs. Both scenarios could potentially lead to a reduction in pollution attenuation via biodegradation (Meynet *et al.*, 2012). On the other hand, Meynet *et al.* (2012) also showed that AC amendment had little negative effects on bacterial community structure and activities in a PAH polluted urban soil. Others also have stated that at high concentrations, adsorbents aid in overcoming toxic effects of HOCs to microbial activity (Vasilyeva *et al.*, 2010) and also improved the biodegradation of short-chain alkanes and volatile hydrocarbons through better retention in soil (Bushnaf *et al.*, 2011). All of these contrary observations coupled with evidence that addition of adsorbents generally improves soil quality (Glaser *et al.*, 2002; Major, 2010) has led to the need for further investigation into the effects of adsorbent amendment (such as the addition AC) on the biodegradation of HOCs, notably hydrocarbon compounds.

This chapter investigates and reports the chemical evaluation of activated carbon amendment effects and the effects of varying activated carbon amendment addition times on the remediation and biodegradation of petroleum hydrocarbons originating from crude oil. This strategy was employed to realize complementary benefits of adsorption and biodegradation and to help determine the conditions for the optimal use of adsorbents (based on timing of the addition) in crude oil contaminated soil remediation (Qin *et al.*, 2013). It is believed that microbial degradation of certain hydrocarbon compounds (especially easily biodegradable) in soils is rapid at the initial stage (Alexander, 1995) and the addition of AC could possibly inhibit the degradation of these readily biodegradable compounds, however on the other hand AC addition could also help in reducing risks if compounds are toxic at high concentrations, poorly bioavailable, or otherwise not easily biodegradable.

A series of microcosm for biodegradation and sorption experiments were set up, involving the incubation of soils spiked with crude oil and with/without AC amendment. Also, results of

preliminary tests carried out on the crude oil used for the experiments are reported in this study. To determine the effects of sorbent addition time, the adsorbent (2% powdered coconut shell activated carbon) amendment was carried out at 2 different time points, i.e. at the beginning of the treatment and after 5 months. The rationale behind the addition the of AC at month 5 was to reduce effect of sorption on the initial biodegradation process and by giving ample time for the microbes to degrade their most prefered substrates i.e. the readily available and easily biodegrable fractions before adding AC to stabilize poorly degradable residuals. By doing this, the biodegradation should not be affected as much as when AC is added from the beginning. Five months were chosen as a reasonable time period to allow the removal of readily biodegradable crude oil components to occur as some reports have shown the fast removal these components (de Jonge *et al.*, 1997; Lepo and Cripe, 1999; Huesemann *et al.*, 2004; Sabaté *et al.*, 2004; Farahat and El-Gendy, 2008). Furthermore, the addition of AC (2% PAC, dry sediment) was previously shown to effectively reduce concentrations of PAHs in sediments (Hale *et al.*, 2012) and reports have suggested that for AC sorption to be effective, the ideal contact time is between 1 to 6 months (Zimmerman *et al.*, 2004; Millward *et al.*, 2005), so microcosms were incubated for a total period of one year. The initial addition of nutrients is necessary to sustain microbial biodegradation activities (Das and Chandran, 2010) and the addition of nitrogen and phosphorus to achieve a carbon: nitrogen: phosphorus (C: N: P) ratio of 100:10:1 has been previously shown to enhance soil hydrocarbon microbial degradation, (Chandra *et al.*, 2013).

3.2 Objectives

The specific objectives of this chapter are:

1. To determine the effect AC amendment on crude oil mineralization to CO_2
2. To determine the effect AC amendment on petroleum hydrocarbon volatilization from soil
3. Comparing the effect of varying AC amendment times and biodegradation processes are in the reduction of petroleum hydrocarbon residual soil concentrations
4. Comparing how effective varying AC amendment times and biodegradation processes are in the reduction of the petroleum hydrocarbon availability and transfer in soil (as measured by P.E uptake, aqueous concentrations, and soil-water partitioning coefficients K_D)

3.3 Materials and methods

The rate of crude oil biodegradation and volatilization was obtained via carbon dioxide and hydrocarbon volatilization (VPHs i.e. aliphatic and aromatic fractions) monitoring respectively. The distribution/partitioning of the different crude oil components between the aqueous and solid phase was determined with the help of polyethylene passive samplers and ASE extraction and the potential risks assessed accordingly. Most of the tests or investigations carried out in this chapter were done in the laboratory, but could possibly be applicable to field bioremediation studies. Laboratory tests are usually done as preliminary assays to evaluate the feasibility of a remediation treatment strategy. In the laboratory the amount of soil used is little in comparison to field studies, however the results obtained from the laboratory such as degradation rates and pollutants remaining (residual concentrations) may be useful in predicting attainable rates and field studies concentrations. (Aichberger *et al.*, 2005).

3.3.1 Soil physico-chemical properties: TOC, total nitrogen, pH

The surface soil used for the setup of the batch system was obtained from Exhibition Park, Newcastle, during a park redevelopment. The total carbon (organic and inorganic) was measured on a LECO carbon analyzer (LECO, 1996). The total carbon measured for the soil was 3.34 %, organic carbon was 2.33%, and inorganic carbon was 1.01%, nitrogen 0.132%, Sulphur was 0.05% and pH was 7.88.

3.3.2 Soil water content

Soil water content was established using a gravimetric technique (ISO, 1993). See Equation 3.1. An empty vial was initially tarred and weight recorded (M1). 20g of soil used for each vial in the batch system was weighed and recorded as ($M2_{(wet\ weight)}$), The soil was then placed in a ceramic crucible and dried in an oven (at 105 °C) for 24 hours. It was allowed to cool before re-weighing and the value was recorded as $M3_{(wet\ weight)}$. The soil moisture/water is usually denoted a % ratio of water to soil dry weight. The soil water content was determined using formula:

Equation 3.1 **% Water = (100(M2-M3))/ (M3-M1)**

3.3.3 Activated carbon

Coconut shell activated carbon (AC) was produced by Norit and sorbent property characterisation has been carried out according to Han *et al.* (2015). The activated carbon surface area was 975 m^2 g^{-1}, open surface area was 40 m^2 g^{-1}, pore volume was 0.47 cm^3 g^{-1}, micro-pore volume was 0.43 cm^3 g^{-1} , pore size was 37.1 Å.

3.3.4 Crude oil

The North Sea crude oil was provided by Dr. Martin Jones and originally supplied by BP plc. 0.5 ml of the crude oil was added to the soil. The API gravity of the crude oil was 39 degrees and specific gravity (density) was 0.8224. The mass of crude oil added to 20g of soil was 0.4112g giving a concentration of 20,560 mg/kg of soil.

3.3.5 Standards

Carbon dioxide (CO_2): Gas mixture, UN195 gas standard (1000psi pressure and 112DA size) accuracy +/- 2%, compressed gas N.O.S, class 2.2. (Scientific and technical gases L.T.D). EPA 610 polycyclic aromatic hydrocarbons mix in methanol, from SUPELCO. Volatile petroleum hydrocarbon (VPHs, See Table 3.3 for concentrations) The VPH mixture made up of 12 major constituents of gasoline or kerosene. The original VPH standard mix comprised of 12 fuel compounds of 99% purity. They included straight (also isomers), cyclo, branched alkanes and aromatic hydrocarbon. N-pentane C_5H_{12}, n-hexane $C_7H_{14,}$ n-octane $C_8H_{18,}$ n-decane $C_{10}H_{22,}$ n-dodecane $C_{12}H_{26,}$ cyclohexane $C_6H_{12,}$ Isoocatane $C_8H_{18,}$ methylcyclohexane $C_7H_{14,}$ methylcyclopentane C_6H_{12} m-xylene $C_8H_{10,}$ 1,2,4- trimethylbenzene $C_9H_{12,}$ Toluene C_7H_8 and was supplied by Sigma Aldrich, Dorset, UK.

3.3.6 Nutrient preparation

A nutrient solution containing adequate quantities of N (NH_4CL) and P (KH_2PO_4) to give a C: N: P ratio of 100:10:1 was prepared. The nutrient solution was prepared by dissolving 13.356g of NH_4Cl (ammonium chloride) and 1.533g of KH_2PO_4 in 50 mL distilled water (See Appendix A for calculations). The nutrient solution was autoclaved before 0.5 mL each was dispensed

into all amber vials giving a nutrient N concentration of 0.013% (w/w %), 0.194% (w/v %) and P concentration of 0.011% (w/w %), 0.169 % (w/v %) in soil and soil solution respectively.

3.3.7 Batch experiment 1: Microcosm experimental design (Crude oil biodegradation and sorption)

To measure the biodegradation of crude oil and the effect of activated carbon on crude degradation, batch experiments were carried out at room temperature (20±2°C) for 12 months. The experiment was set up in clean 64 mL brown amber vials and was most of the time stoppered with foam plugs, and only stoppered with Teflon Mininert valves (Supelco, Bellefonte, USA, Figure 3-1) when measuring CO_2. All the batches consisted of 20g of soil (wet weight) and 0.5 ml of nutrients. A conscious decision was taken not use sterile controls in the experiment as experience shows that is re-growth even after double autoclaving. The nutrient solution contained N (NH_4Cl) and P (KH_2PO_4) to give C: N: P ratio of 100:10:1. The nutrient solution was carefully dispensed using 1ml pipette (Gilson Pipetman, UK). 1 g of activated carbon was measured and added directly to sorbent amendment vials while 0.5 mL of crude oil was added to each crude oil batch sample with a 1 mL pipette (Hamilton, USA, NEVADA). The microcosm contents were mixed initially and then twice a month as it is believed efficacy of AC treatments can be improved by proper distribution (AC- soil contact) of AC particles (Cho et al., 2009) and soil-water content was maintained by controlling the weight of the vials (Qin et al., 2013). In real applications, occasional mixing can for example be achieved by turning windrows. Treatments were as follows: crude oil with activated carbon amendment at time zero (colour: red), crude oil with activated carbon amendment after 5 months (colour: green) and crude oil no activated carbon amendment (colour: yellow). (See Table 3-1 for full description and Figure 3-2 for coding system). The controls were set up, with and without activated carbon amendment (colour: blue and black respectively) and both without crude oil. All treatment and controls were set up in triplicates and stored in the laboratory. The colour coding system will be used throughout this chapter to differentiate between each treatment and help with easy identification.

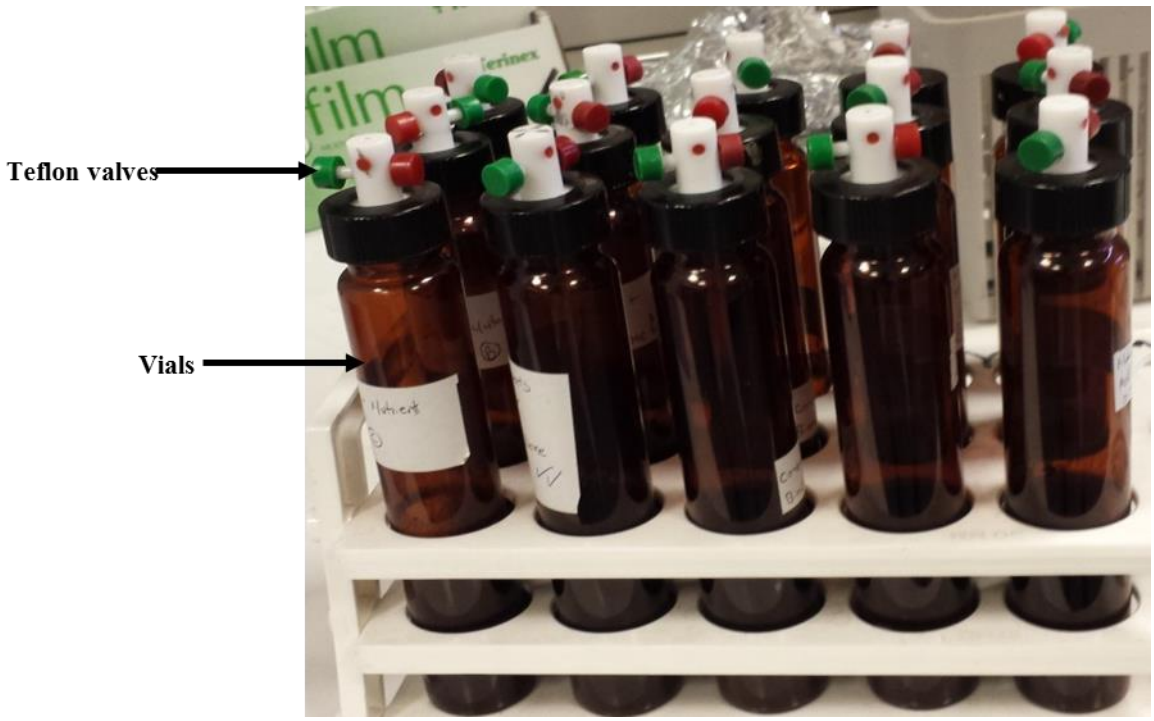

Figure 3-1 showing the batch experiment. 64 mL amber vials stoppered with Teflon mininert valves.

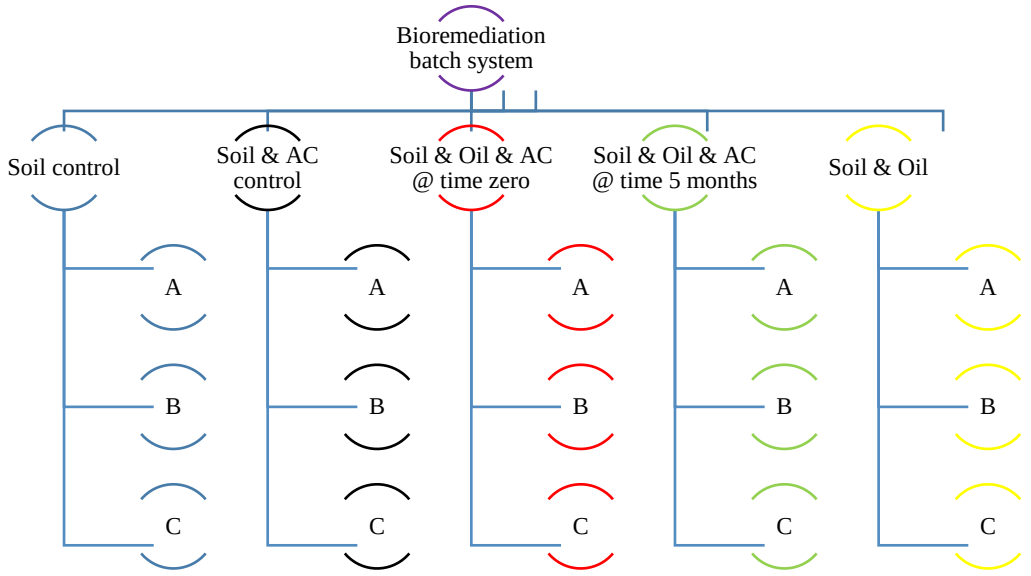

Figure 3-2 color-coded schematic representation of the batch experiment design showing the various triplicates

Table 3-1 Full description of treatment used in batch experiment. Concentration in volumes added: Crude oil 20,560 mg/kg of soil, nutrient added: P concentration of 0.011% (w/w %), 0.169 % (w/v %) and N concentration of 0.013% (w/w %), 0.194% (w/v %), Soil water content was 1.35 ml

SAMPLE ID	SOIL MASS (g)	NUTRIENT (mL)	MASS OF ACTIVATED CARBON (g)	VOLUME OF CRUDE OIL (mL)
Soil control	20	0.5	0	0
Soil & AC control	20	0.5	1	0
Soil & Oil & AC @ time zero	20	0.5	1	0.5
Soil & Oil & AC @ time 5 months	20	0.5	1	0.5
Soil & Oil	20	0.5	0	0.5

3.3.8 Analytical methods

3.3.8.1 Carbon dioxide quantification: Gas chromatography mass spectrometry (GC-MS) measurement

In order to determine the difference in the rate of biodegradation/mineralization between the various treatments, carbon dioxide CO_2 was measured. For the crude oil batch samples, carbon dioxide measurement was preformed over 2 days per week for 52 weeks. Airtight Teflon miniert valves replaced the foam plugs for over the 2 days of measurement to allow the buildup of carbon dioxide in the vial headspace. The measurement was carried out on a 8060 Gas Chromatography attached to a Fisons MD800 Mass spectrometry. The settings are as follows:

70eV, filament current: 3.6A, Emission current: 150μA, current source: 600μA, Temperature: 150^0C, multiplier voltage: 300V, at 3 turns). Gases were separated with a HP-PLOT-Q capillary column (30m x 0.32 mm i.d) packed with 20 μm Q phase (Agilent Technologies, Palo Alto, USA) and the GC is sustained isothermally at 35^0C, with helium carrier gas (30mL/min flow rate, 65 kPa pressure, split at 100 mL/min). Both standard and sample injections were performed with 100 μL Hamilton gas tight syringe. Integrated chromatograms (ion or mass: 44 representing carbon dioxide was entered) were viewed in full scan mode on Xcalibur software. Gas mixture, UN195 gas standard (1000psi pressure and 112DA size) accuracy +/- 2%, compressed gas N.O.S, class 2.2. (Scientific and technical gases L.T.D) was used for calibration. Prior to carbon dioxide measurement, test standard injections were carried out using various standard volumes to check the peak production (elution from column) and determine adequate time intervals between injections. The calibration standard curve was produced with GCMS using 20 μL, 40 μL, 60 μL, 80 μL and 100 μL injection volumes of 1% carbon dioxide UN195 gas standard. Standard volumes were injected (2 minute interval) in split mode while the samples volume injections were 100 μL (3 minute interval). The peak areas and retention times were collated and the ideal gas law formula (Equation 3.2) was used in determining the mass of carbon dioxide in the standard.

Equation 3.2 $PV=nRT$

V= Volume

P= Pressure

R= ideal law gas constant

T= Temperature

n= number of moles

3.3.8.2 Determining carbon dioxide production per batch/week

A linear regression analysis (least squares regression) was carried out using the mass of CO_2 and peak areas (relative abundances) for the standards and this generated a linear curve, which was forced through the origin. The regression equation (y=mx, where m= slope/gradient and

y= abundance or area) and volume of sample injected was used in estimating the concentration of CO_2 in the each sample grams per liter (g/L).

Equation 3.3 Carbon dioxide g/L $= \frac{\text{Sample peak areas}}{\text{slope} \times \text{volume of sample injected}}$

The real volume of the vial (V_{vial}) was determined by adding water to an empty clean vial prior to weighing it. The vial real air volume ($V_{v\text{-}air}$/mL) was calculated by subtracting the volumes of all the constituents of the treatment (water content, nutrient, crude oil, soil and activated carbon) from the real volume of the vial (Equation 3.4).

Equation 3.4 $V_{v\text{-}air} = V_{vial} - V_{water} - V_{Nutrient} - V_{Crude\ oil} - V_{soil} - V_{activated\ carbon}$

Thus mass (µg) of CO_2 produced per gram of soil per week was estimated using the following formulas Equation 3.5, Equation 3.6 and Equation 3.7

Equation 3.5 Mass of CO_2 (µg)/vial = CO_2 concentration × vial real air volume

Equation 3.6 $CO_2\ (\mu g)/g\ \text{of soil} = \frac{\text{Mass of } CO_2\ (\mu g)/\text{vial}}{\text{Mass of soil (Dry weight)}}$

Equation 3.7 $\text{slope (m)} = \frac{\Delta\ \text{Mass of } CO_2\ (\mu g)/g\ \text{of soil}}{\Delta\ \text{Time}}$

3.3.8.3 Volatile petroleum hydrocarbon (VPHs) analysis and quantification, GC-FID measurement (Head space injections) – Crude oil batch system

GC-FID measured was carried out similar to the procedure described in Bushnaf *et al.* (2011). The gas chromatography flamed ionization detection (GC-FID) of VPHs in the batch system at 3 months, 6 months and 9 months was carried out on an Agilent 7870A gas chromatograph (Agilent Technologies, Palo Alto, USA). The flame ionization detector temperature was set at 300 ^{0}C, GC column temperature was set at 30 ^{0}C for 5 min (steadily increased by 10 ^{0}C to 120 ^{0}C every minute) and then kept at 6 minutes for a 20 minute total run (Frequency at 50Hz, hydrogen carrier gas, 2 ml/min flow rate, pressure at 50kPa). The capillary column (30 m x 0.249 mm i.d) covered by a 0.25 µm film (dimethyl poly-siloxane, HP-5 phase, Agilent

Technologies, Palo Alto, USA) was responsible for compound separation. Injections (60µL) were carried out using a Hamilton syringe via the split mode injector port (ratio of 1:10, at temperature 200^0C). Prior to standard and sample injections, a blank burn-off and machine calibration was performed. The standard calibration was carried out via the injection (Hamilton syringe, Nevada USA) of diluted headspace (1ml from the headspace of vial containing the Vphs mixture into a 42ml vial) concentrations (Volume: 20 µL, 40 µL, 60 µL, 80 µL, 100 µL) from 42 mL vials stoppered with a mininert valve containing a mixture of 12 liquid VPHs. Test runs was initially carried out to detect VPHs peaks in the standard and produced similar retention times (

Table 3-2).

Table 3-2 VPHs retention times (mins)

Number	Compound	Retention time (Minutes)
1	n-pentane	1.39
2	n-hexane	1.78
3	methylcyclopentane	1.99
4	cyclohexane	2.31
5	isooctane	2.62
6	methylcyclohexane	3.21
7	toluene	4.31
8	n-octane	5.53
9	m-xylene	7.52
10	1,2,4 TBM	10.31
11	n-decane	10.49
12	n-dodecane	13.70

The GC chromatograms were obtained and viewed with the Thermo-atlas laboratory software. The peak areas for each compound in standard and samples

Table 3-3 Molecular weights, quantities and concentration in mixture [a] calculated using mole number. [b] calculated using mole fraction and Raoult's law. [c] calculated using the ideal gas law. [d] calculated using M. weight and [c]. [e] calculated using 1ml/42 (standard headspace dilution)

Compound	Volume of compound	Concentration	M. Weight	Density	Purity	Vapor pressure at 20^0C	Mole [a] fraction	Vapor [b] pressure at 20^0C	Conc. of [c] compound	Conc. of [d] compound	Conc [e]. in std
	ml	% (v/v)	(g/ mole)	(g/ml)	%	(Pure liquid) Atm		(solution) Atm	(mole/ liter)	(g/ liter)	(ug/ ul)
n-pentane	5	5	72	0.626	99	0.560500	0.061468	0.034453	0.001422	0.102583	0.002442
n-hexane	9	9	86	0.66	95	0.171100	0.093716	0.016035	0.000662	0.057027	0.001358
n-octane	8	8	114	0.703	99	0.014500	0.069760	0.001012	0.000042	0.004768	0.000114
n-decane	14	14	142	0.73	99	0.001180	0.101770	0.000120	0.000005	0.000705	0.000017
n-dodecane	6	6	170	0.748	99	0.000099	0.037332	0.000004	0.000000	0.000026	0.000001
methylcyclopentane	8	8	84	0.692	99.8	0.183400	0.093957	0.017232	0.000711	0.059848	0.001425
methylcyclohexane	12	12	98	0.77	99	0.048470	0.133342	0.006463	0.000267	0.026188	0.000624
cyclohexane	8	8	84	0.779	99.5	0.102000	0.105452	0.010756	0.000444	0.037357	0.000889
isooctane	15	15	114	0.692	99.8	0.053900	0.129794	0.006996	0.000289	0.032979	0.000785
toluene	4	4	92	0.865	99.8	0.028950	0.053637	0.001553	0.000064	0.005904	0.000141
m-xylene	5	5	106	0.862	99	0.008110	0.057525	0.000467	0.000019	0.002044	0.000049
1,2,4 trimethylbenzene	6	6	120	0.889	98	0.001900	0.062247	0.000118	0.000005	0.000587	0.000014

The mass (µg) of the respective compounds in the injected standard was calculated based on the diluted concentration and volume of standard injected (20, 40, 60, 80, 100 µL). The diluted concentration values were obtained based on a series of calculation using the compounds molecular weights, quantities and concentration in the original mixture (Table 3-3). The regression coefficient and equation was deduced by plotting the mass concentration (µg) of the

respective compounds in standards versus response and then utilized in calculating the concentrations of compounds (µg/µL) in sample headspace injected.

3.3.8.4 Volatile petroleum hydrocarbon (VPHs) analysis: polyurethane foam plug experiment

At month 11, the extraction and cleanup of extracts of volatile hydrocarbon compounds (VPHs) from polyurethane foam plugs was carried out similar to previously described methods (EPA, 1999b). The polyurethane foam plugs were placed in separate conical flasks (covered with aluminum foil paper) and a double extraction (2x25ml each) over 48 hours was carried out using hexane (Sigma Aldrich, UK). The extracts were decanted into 64ml brown amber vials and covered with white Teflon-lined screw caps. The cleanup of sample extracts was performed using clean glass columns (2.5 mm) packed with a ball of glass wool, silica gel 3g (±0.1) and sodium sulphate (1cm). The use of column chromatography to clean sample extracts before commencement of the GC analysis aids in eliminating interferences and the silica gel is known to act as a sorbent for contaminants (EPA, 1999b). The columns were initially rinsed with hexane before the adding of 2x20 ml of the hexane extracts to the column. 15ml of hexane was added to the column and the rationale here is that the alkane fraction will migrate down the column with the hexane. 15 ml of hexane: acetone (50:20) was then added to wash the column before eluting the aromatic fraction with hexane: acetone (80:20). Elutants were collected in a 40ml brown amber vial and concentrated to 1ml prior to analysis on the gas chromatograph with flamed ionization detection (3.3.8.3) and gas chromatography mass spectrometry (Section 3.3.8.5) for alkane and aromatic fractions (PAHs) respectively. PAHs quantification was calibrated with varying concentrations of 16 USEPA standards (Supleco, EPA 610 aromatic mixture) while that of the alkanes carried out using varying concentration of diluted 12 VPHs. Internal standards of squalene (25µL) and phenanthrene D10 were used for PAHs quantification and squalene (5µL) for alkane quantification.

3.3.8.5 Gas chromatography mass spectrometry (GCMS) analysis for alkanes and PAHs

GCMS was carried out on a Hewlett-Packard 7890A GC fit with a split/split less injector (temperature: 2800C), connected to a Hewlett-Packard 5975inertXLMSD. To enhance the sensitivity, data acquisition was in full scan and SIM (selected ion mode) (50-550 amu/sec or 30 ions 0.7cps 35ms dwell). The program was set and the compounds were separated by a built in fused silica capillary column (30 m x 0.25 mm i.d) with a 0.25 µm film thickness HP-5 phase

coating (Agilent LTD, Wokingham, Berkshire, UK). The GC temperature was set at 50 ^{0}C-300 ^{0}C/min and held at 300^{0}C for 20 minutes with helium as the carrier gas. 1 µL of the sample was injected by a HP7683 automatic sampler in a split/pulse mode with an initial pressure of 150 kpa held for 1 minute, split less and thereafter the pressure was 50 kpa with a split flow rate of 30 ml/min.

3.3.8.6 Determining hydrocarbon free aqueous concentrations and bioavailability using polyethylene passive samplers after 52 weeks.

Passive samplers are known to amass HOC in polluted waters till equilibrium is reached and have been employed in accurately determining freely dissolved concentrations of sparsely water-soluble pollutants (Jonker and Koelmans, 2002). To investigate the availability of hydrocarbons at the end of the batch experiment, polyethylene (P.E) sheets gotten as plastic bags (VWR international Ltd, Leicestershire, UK) were cut into thin strips (300±0.1mg) and cleaned with hexane: acetone (50:50) for 24 hours in aluminum covered glass beakers in the fume cupboard. The soils in the microcosms were mixed properly and a 5 ml sub-sample was stored away in the freezer (at -20^0 C) for microbial analysis. The remaining soil was weighed; 40 ml of distilled water and one sampler (PE strip) was added to each vial. To stop biodegradation, a biocidal chemical, sodium azide (0.4 ml, 1%, Sigma Aldrich) was added to each vial which were capped with Teflon-lined lids. The vials were rolled up together, place lying horizontally and equilibrated for 4 weeks by continuous agitation on an orbital shaker at 130 rpm (Han *et al.*, 2015). At the end of this period, the samplers were obtained from the batches and rinsed in distilled water, hydrochloric acid and propanol (only strips from crude oil batches) and dried with a paper towel (Han *et al.*, 2015). A 2x extraction was carried out over 48 hours by agitation with 10 ml hexane: acetone (80:20). Squalene (25µL) was used as internal standard. The extracts were concentrated, fractions separated using column chromatography as in section 3.3.8.4 and quantified on the GC-MS. The aqueous pollutant concentrations (W_C) was obtained using the P.E concentrations (PEc) and the PE to water partitioning coefficient (Kpe), Equation 3.8. (Hale *et al.*, 2010a; Hale and Werner, 2010).

Equation 3.8 $K_{pe} = PEc / Wc$

3.3.8.7 Determination of alkane and polycyclic aromatic hydrocarbon (PAH) residual concentrations after 52 weeks**.**

At the end of the experiment and after sample separation the samples were inspected for presence of any rocks, grounded and mixed thoroughly again. 5 g of soil was mixed thoroughly with furnaced sea sand (to increase moisture absorption) in a foil paper prior to transfer into ASE extractor cells. Cellulose filter paper was placed at the top and bottom of the extractor cell, topped up with some more sea sand (furnaced) at both ends (mixed soil sample added in the middle) prior to sealing with preassembled extractor caps. This was to ensure the cells were tightly packed (Wang et al., 1999). Extraction was carried out on a Dionex ASE machine with hexane: acetone (50:50) Extraction program condition was: 5 minute heating up time, 5 minute static period, 1500 psi cell pressure, 100^0C oven temperature, 100% flush volume, 120 seconds purge time, 3 cycles and 40ml of extraction solvent. Extracts were collected in corresponding clear cleaned 64 ml glass vials sealed with Teflon septum (sterile) caps (Wang et al., 1999). Activated copper was added to each extract to remove sulphur. Prior to extract clean up and concentrating, cyclohexane was added twice to 1ml of each sample (15 ml mark) and blown down to 1ml. Clean up and separation of fractions using column chromatography as in section 3.3.8.4. However the aromatic fraction was eluted using hexane: dichloromethane (60:40). The samples were re-concentrated again and transferred to GC vials prior to the addition of internal standard squalene (25 µL). Both aliphatic and aromatic fractions were quantified on the GC-MS (section 3.3.8.5). Varying concentrations of total petroleum hydrocarbons (TPH, C10-C19, Sigma-Aldrich) and 16 USEPA standards (Supleco, EPA 610 aromatic mixture in methanol) were used in calibration of alkane and PAHs quantifications. The residual soil concentrations obtained (Cs) and estimated water concentrations (Wc) (from section 3.3.8.6) was used in estimating the solid-water distribution coefficient (KD) as described by Zimmerman et al. (2004) and according to Equation 3.9.

Equation 3.9 $$K_d = \log \frac{C_s}{Wc}$$

3.3.8.8 Analysis of crude oil used in batch experiment (courtesy of Dr. David Werner)

A known volume of North Sea crude oil supplied by Dr. Martin Jones (Newcastle University) separated into aliphatic and aromatic fractions using column chromatography similar to that described in section 3.3.8.4 and then quantified by GCMS.

3.3.8.9 Quality assurance and control

Quality assurance was performed while carrying out the experiments to ensure the maintenance of high standards. The microcosms were mixed twice every month with a stainless steel spatula for aeration for 12 months. Water was added to the batch when there was evidence of drying out (reduced weight). The soil in the vials was mixed properly (to keep the soil moist) and the foam plug replaced prior to re-weighing the vials. The vials were kept away from sunlight during mixing and amber colored vials was used to prevent photolysis. The vials where immediately shut with airtight Teflon-lined caps after opening to avoid vaporizations, or foam plug traps were used to quantify the amount of volatilization. To reduce sorption to apparatus glass equipment or stainless steel tools were used instead of plastic equipment. In all chemical analyses, internal standards were used to help correct for any loss of analyte while standards were used to calibrate the quantification of compounds. Batch experiment was done in triplicates to improve the significance of the experimental results and appropriate controls were run in parallel with the treatments. During the measurement of carbon dioxide and volatile petroleum hydrocarbons, the 100 µL Hamilton syringe was checked for blockage prior to each injection by injecting laboratory air into a beaker containing water. A gas detector was used to check for gas leaks from the injector port of the GC before all experimental runs, otherwise the septum was replaced. The septum for the GC-MS injection port was also changed after every run to prevent gas (headspace sample) leakage during injections. The Teflon mininert valves were quickly opened and closed, before and after inserting the Hamilton syringe to minimize any gas headspace loses. The university laboratory health and safety regulations where adhered to. This includes wearing protective laboratory coats, gloves, working in fume cabinets (when handling hazardous substances) and proper disposal of waste products. A COSHH (control of substance hazardous to health) form was also filled and signed for all hazardous substances used during the experiment work.

3.3.8.10 Statistical analysis

Student *T-test* was performed on Microsoft excel version 2010 (Microsoft, Redmond, USA) using 95% confidence, two-tailed, independent samples to compare the effects of the various

treatments via the differences in CO_2 production, residual, bioavailable and free aqueous pollutant concentrations.

3.4 Results and discussion

3.4.1 Activated carbon impacts on the rate of crude oil mineralization (carbon dioxide measurements and percentage of crude oil carbon mineralized to CO_2 carbon)

The CO_2 monitoring over 12 month period showed there were clear differences in CO_2 emanating from the various batches right from the onset (See Week 2, Figure 3-3). The microbial activity based on the CO_2 respiration rates was significantly higher (t-test; $p < 0.05$) for both crude oil batches with AC amendment to occur only after 5 months (Soil & oil & AC at time 5 months) and without AC amendment (Soil & oil) (Sample values: 16.8±3.5 and 13.2±2.0 CO_2 mg/batch of soil respectively**)** in comparison to Control Soil (4.1±1.7 CO_2 mg/batch) and Control Soil & AC at time zero 1.9±0.1 CO_2 mg/batch).The above results indicate that degradation of crude oil starts almost immediately in the batches bio-stimulated and spiked with crude oil and no AC amendment. This is consistent with reports which shown no lag phase (Kim *et al.*, 2005) or short lag phase for the depletion fraction of crude oil fractions and mineralization to CO_2 with nutrient bio-stimulation (Hamamura *et al.*, 2006a; Singh *et al.*, 2015).

The results (Figure 3-3) highlighted the effect of the AC amendment on the microbial respiration. At week 5, there was a significant difference (t-test p value: < 0.05) in CO_2 production in crude oil batches 'Soil & oil & AC at time 5 months' and 'Soil & oil' compared to crude oil batches with AC amendment at the beginning 'Soil & oil & AC at time zero. There was no significant difference (t-test; $p = >0.05$) in CO_2 production at week 5 between Soil & oil & AC at time zero and controls or between controls 'Soil' and 'Soil & AC at time zero' as a result of the addition of AC in the absence of crude oil.

The CO_2 production from crude oil batches with amendment at 5 months and without AC amendment were initially comparable, as expected, since treatments were identical up to the sorbent addition in month 5. Upon the addition of AC, the CO_2 emanating from 'Soil & oil' batches slightly surpasses (not significant, t-test; p = >0.05) the CO_2 produced from 'Soil & oil & AC at time 5 months' between week 26 and 30 (Figure 3-3 Week 30). The positive effects of adding sorbents at later stages of crude oil biodegradation have been previously reported. Qin *et al.* (2013) showed that amending crude oil polluted soils with sorbents (such as biochar) midway through the study was better than amendment at the beginning as the amendment midway through promoted the bioremediation process via having a positive infleuence on the biodegradation process. Although the effect of AC amendment is not statistically significantly evident here, it is nonetheless believed that biodegradation in 'Soil & oil & AC at time 5 months' is slightly hindered due to the addition of AC. The results at 52 weeks also confirms the hindrance due to the addition of AC, as there was higher CO_2 production (not significant, t-test; p = >0.05) from 'Soil & oil' compared to 'Soil & oil & AC at time 5 months' (approximately 8% more) treatment with the former being slightly higher (115±15 and 124±2.7 mg/batch respectively). However, in batch 'Soil & oil & AC at time zero' at 52 weeks (Figure 3-3) the CO_2 production was significantly lower compared to Soil & oil' or 'Soil & oil & AC at time 5 months' (t-test; p = <0.05). The results for CO_2 production at 52 weeks for batch 'Soil & oil & AC at time zero' was approximately less by 52% and 48%, respectively, when compared with that of 'Soil & oil' and 'Soil & oil & AC at time 5 months' and this fits well with lower removal of alkanes and low molecular weight PAHs from batch 'Soil & oil & AC at time zero' (Figure 3-5 and Figure 3-6). These findings are in line with other reports that confirm that the addition of AC reduces or hinders the rate of hydrocarbon mineralization to CO_2 by limiting microbial access (slows down biodegradation process) (Rhodes *et al.*, 2008; Rhodes *et al.*, 2010; Oyelami *et al.*, 2014) and this evident especially when added from the start.

Surprisingly, the CO_2 production rate in 'Soil & oil' also slows down around week 31 even after the addition of nutrients, addition of water to prevent drying out and the manually mixing of the soil in the batches. Previous reports have shown that effective mixing of treatment soils can help increase biodegradation activity and drastically reduce bioremediation time (Eriksson *et al.*, 2000). A possible reason for the reduction in biodegradation rate is due the presence of not easily biodegradable fractions such as heavier and weathered aromatic (PAHs) fractions in the soil. Analysis of the soil hydrocarbon (PAHs) concentrations after 12 months (Figure 3-6) supports this claim

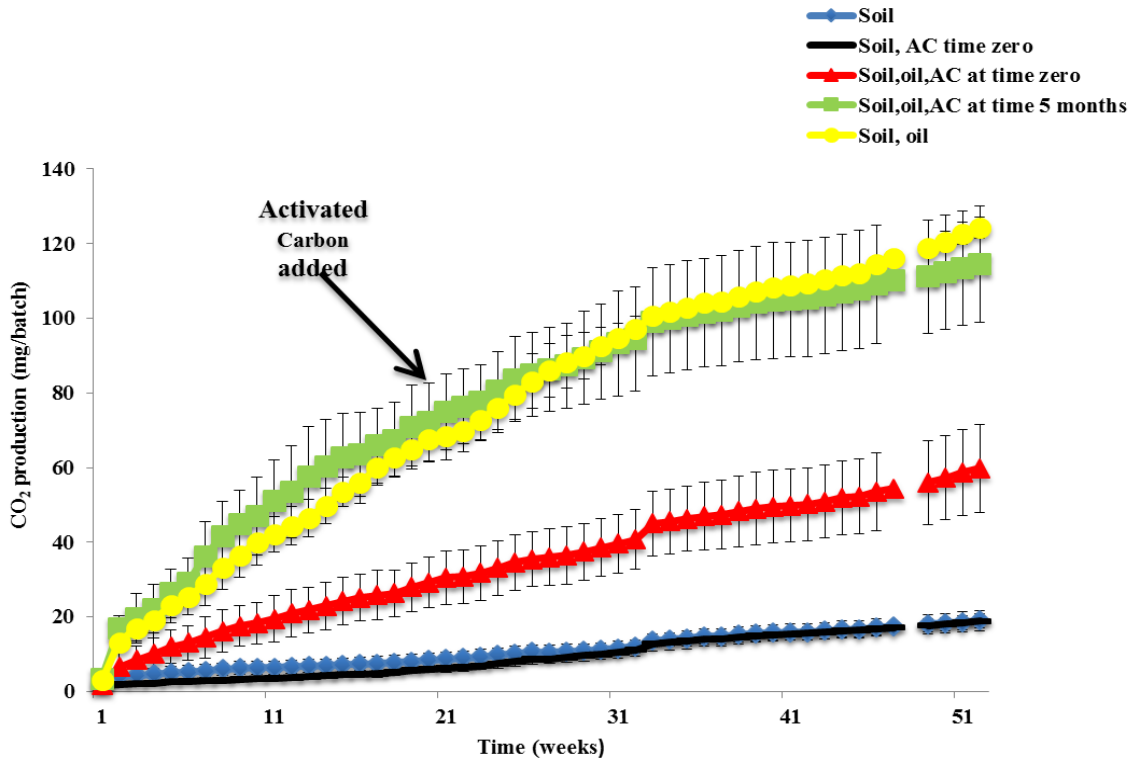

Figure 3-3 CO_2 emanating from sediment batch system. AC indicates activated carbon amendment. Controls: Soil, Soil & AC at time zero. Crude oil spiked vials: Soil&oil&AC at time zero, Soil&oil&AC at time 5 months, Soil&oil. Error bars represent ± 1 standard deviation (SD=3) from the mean of three replicates

3.4.2 Soil residual hydrocarbon concentrations after biodegradation and activated carbon amendment: saturated (n-alkanes) and aromatic (polycyclic) hydrocarbon compounds

The initial concentration of pollutants in the soil was not measured because the soil was obtained from Exhibition Park (Newcastle upon Tyne) which is a play area or park for children and there was no obvious hydrocarbon source nearby. However measurements after 52 week indicated the presence of PAHs from urban pollution in the control soil (see below). However the crude oil (North Sea) used in the study was analyzed to determine the concentrations and amounts of 10 aliphatic (alkanes, between C_{10}-C_{28}) and 16 aromatic (US EPA PAHs) hydrocarbons added to the oil polluted batches. The aromatic fraction contributed to 15.9% of the total measured hydrocarbon concentration (mg/ml) and was mainly comprised of 2-6 ring PAHs compounds (16 PAHs priority pollutants, US EPA) (Appendix A). The concentration of the individual compounds in crude oil added to each batch (0.5ml) was also determined (Figure 3-4). The results for the aromatic fraction also showed a dominating presence (93.66% for total PAHs mass µg) of 2, 3 and 4 ring PAHs, 45.5% naphthalene (38.3±1.0µg/g), 13.9% fluorene (11.8±0.2µg/g), 29.5% phenanthrene (24.8±0.2µg/g) and 4.76% chrysene (4±0.2µg). The

presence of the 10 alkanes was also detected and showed a decrease in concentration and mass the higher the complexity and molecular mass of the compound. In comparison with the 16 PAHs, there was a higher concentration of the 10 alkanes which contributed approximately 84.02% of total measured hydrocarbon mass in the crude oil. This is in line with reports for the SARA distribution of the North Sea light condensate crude oil recording around 82.7% for saturates, 13.4 % for aromatics and 3.9 for Resins.(Aske, 2002). Tissot and Welte (1984) also indicated that on average most crude oil have a higher percentage of saturated compounds compared to aromatics, and the composition varies from oil to oil and is dependent on origin (source) and state of the reservoir. Determining the abundance these component in the crude oil mixture is important as it helps in understanding the biodegradability of the crude oil and behavior of the individual crude oil constituents in the batch system and also the efficacy of the treatment in the removal of the pollutant and reduction of potential risks.

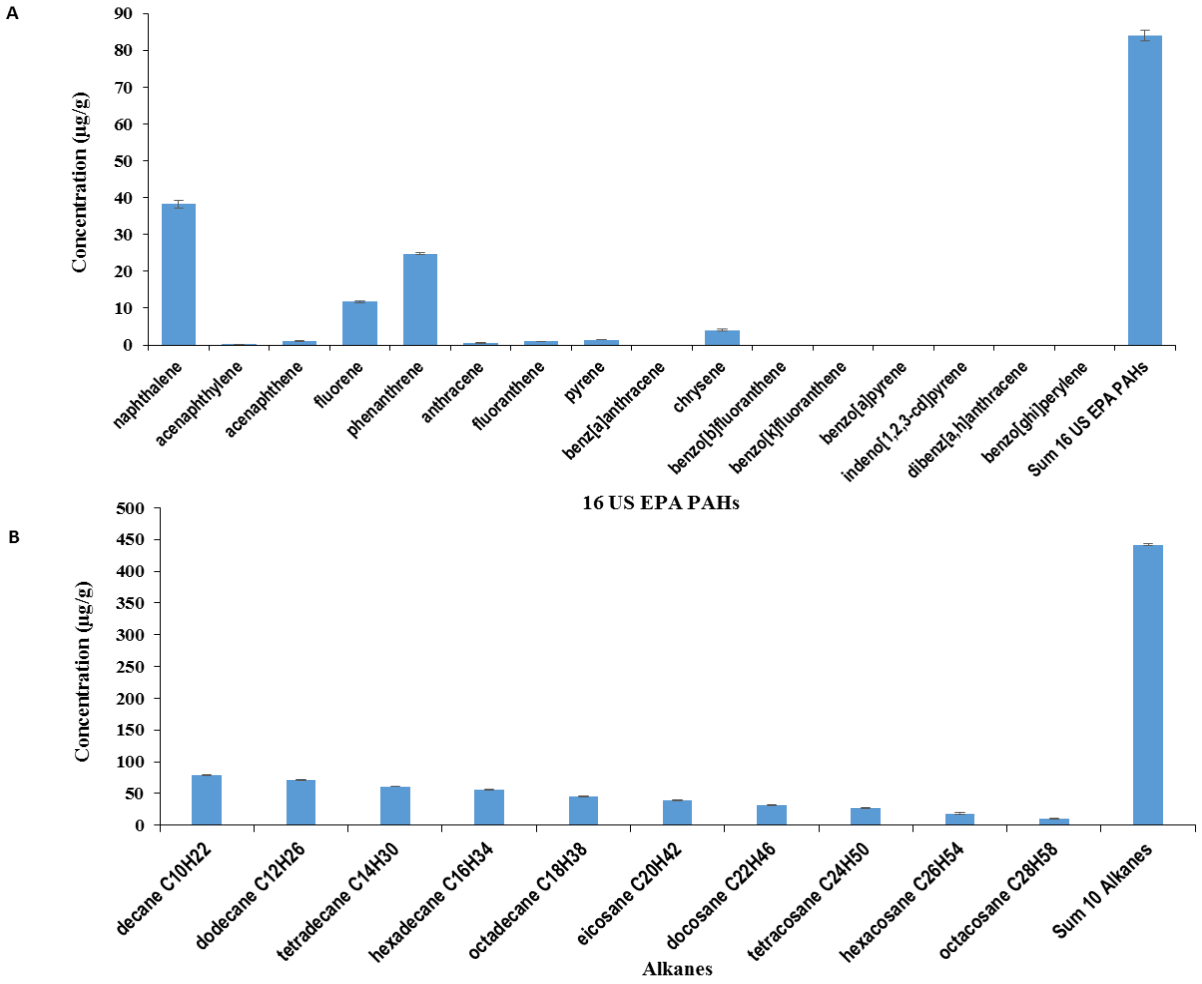

Figure 3-4 Concentration of hydrocarbon compounds in 0.5ml of crude oil (0.4112 grams of crude oil) added to soil batch system. A: 16 US EPA PAHs, B: 10 alkanes. Compounds with no visible bars were below detection limit. Error bars ± 1 standard deviation (SD, n=3)

After 52 weeks, ASE was used to assess the end point concentrations for each treatment. The results (Figure 3-5 A) showed higher concentrations of sum of 10 alkanes decane to octacosane (µg/g) recovered from batches 'Soil & oil & AC at time zero' (313±31.3 µg/g) in comparison to controls and crude oil batches with AC amendment at time 5 months and no AC amendment (values 0.5±0.4, 0.2±0.1, 2.2±1.5 and 3.0±1.0 µg/g respectively). A similar pattern was also seen from the individual alkane (C_{10}-C_{28} µg/g) concentrations values (Figure 3-5 B) with microcosms with AC amendment at time zero having a higher concentration compared to the other treatment batches. In comparison to the total amount of C10-C_{28} alkanes (442±1.8 µg/g) added to each batch (Figure 3-4B) this showed a saturated fraction % removal of approximately 29%, 99% and 99% for Soil & oil & AC at time zero', 'Soil & oil & AC at time 5 months' and Soil & oil respectively.

These findings indicate that when AC is added at the beginning, it is effective in binding hydrocarbons thus making them biologically less available for biodegradation (Rhodes *et al.*, 2008; Rhodes *et al.*, 2010; Oyelami *et al.*, 2014). In contrast, in treatment crude oil batch samples with AC amendment at time 5 months and no AC amendment, the removal of saturated aliphatic hydrocarbons was equally effective, and the loss of alkanes occurred as a result of volatilization and biodegradation of the volatile fraction and microbial biodegradation of the non-volatile fraction. The alkane solid phase results, mirrors closely what is seen in the CO_2 results and are in line with other studies that have reported simliar high alkane removals (degradation) with biostimulation in soil with values dropping within control range (removal efficiency between 70 to over 90%) (de Jonge *et al.*, 1997; Huesemann *et al.*, 2004) and a rapid degradation also of volatile petroleum hydrocarbon compounds in soil (Bushnaf *et al.*, 2011).

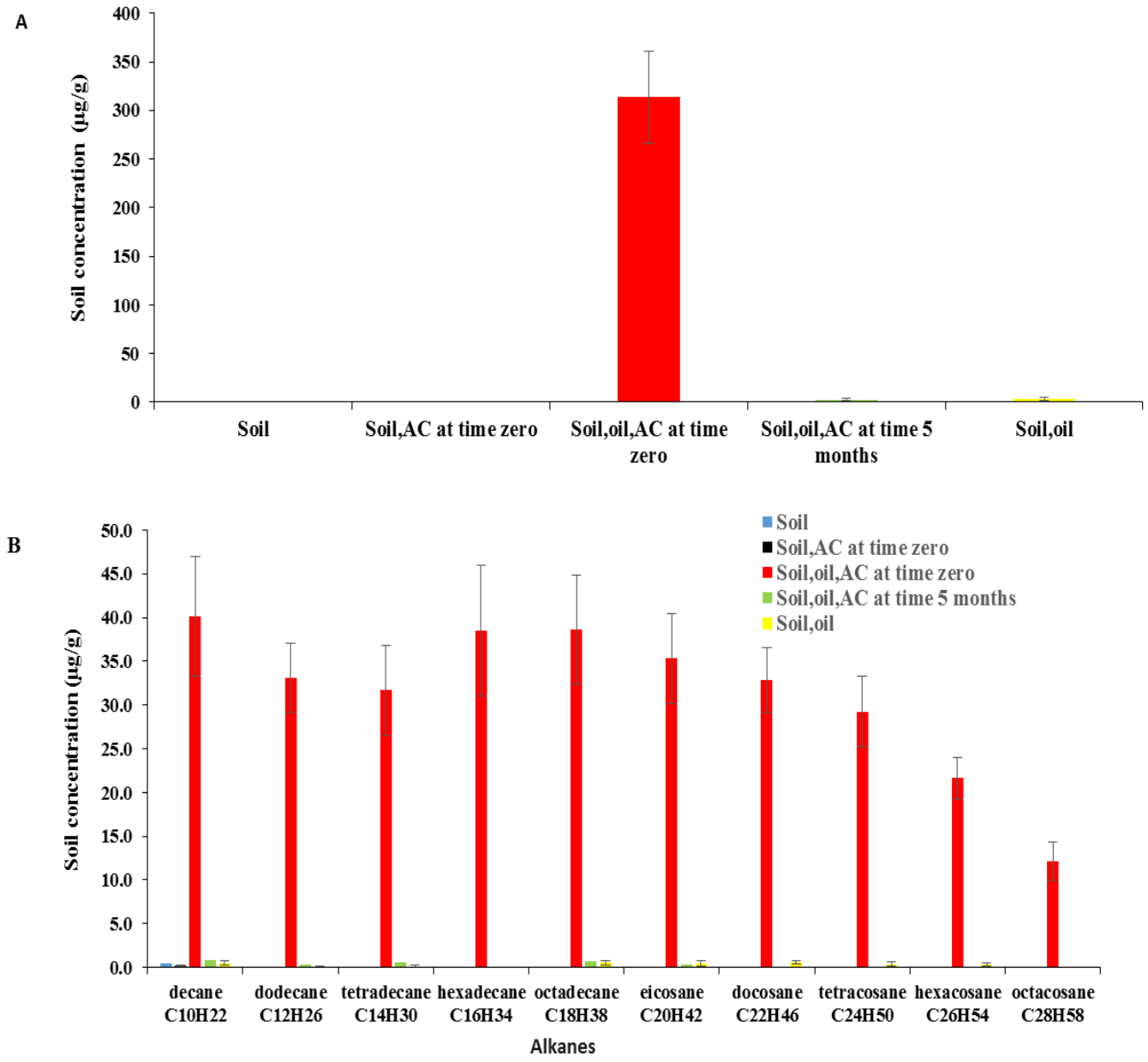

Figure 3-5 Soil concentration of alkanes after 52 weeks of biodegradation and AC amendments. Average alkane concentrations detected in batches. A: sum of the alkane concentrations per treatment, B: concentration of individual alkane compounds per treatment. Error bars: represent ±1 standard deviation (SD=3) from the mean of three replicates

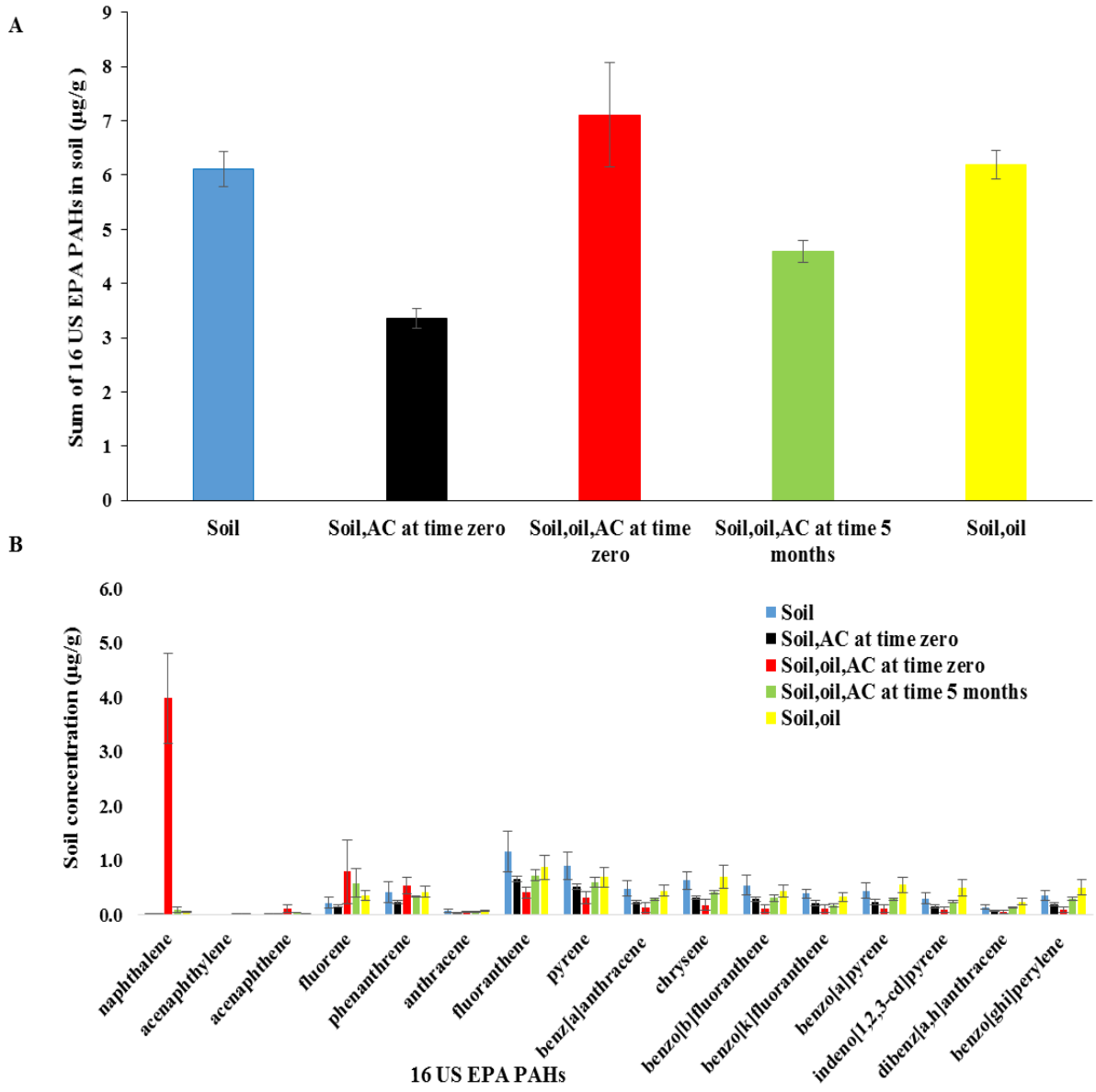

Figure 3-6 Soil concentration of PAHs after 52 weeks of biodegradation or AC amendments. Average PAHs concentrations detected in batches. A: sum of the PAHs concentrations per treatment, B: concentration of individual PAHs compounds per treatment. Error bars: represent ±1 standard deviation (SD=3) from the mean of three replicates

For the more complex or recalcitrant compounds (aromatic fraction) in the soil, the sediment ASE extraction revealed the presence of several heavier molecular weight PAHs in the Control Soil, which were very low, or below detection limit in crude oil analysis (fluorene to benzo[ghi]perylene), (Figure 3-6 and Figure 3-4 A). These findings are not unexpected since the soil was obtained from the Exhibition Park in Newcastle upon Tyne and there have been previous reports of background PAHs contamination in sediments and soils in and around the northeast of England (Law et al., 1997; Woodhead et al., 1999). These PAHs compounds are believed to have been aged over time (de Jonge *et al.*, 2008), thus becoming a soot-like

substance with very low availability for mass transfer (Alexander, 1995; Bosma *et al.*, 1996; Carmichael *et al.*, 1996; Alexander, 1999; Reid *et al.*, 2000; Jonker and Koelmans, 2002). This also indicates these heavier molecular weight PAHs present in crude oil spiked batches (Figure 3-6 B) most likely would have originated from the soil and not the crude oil.

The average sum of PAHs concentration recovered µg/g (Figure 3-6 A) from the batch samples showed there was no significant difference (t-test; $p = >0.05$, although AC seemed to reduce the extraction efficiency in Soil & AC at time zero between controls and crude oil batches. The results were comparable, as some of the values for crude oil batches were within control range. However, there was a difference (t-test; $p < 0.05$) and noticeable effect with varying the time addition of AC, with microcosms with AC addition at time zero having a 38.5% higher PAHs (due to naphthalene concentration) concentration in compared to AC addition after 5 months. For the profile of the individual PAHs compounds (Acenaphthene - Benzo(ghi)perylene) (Figure 3-6 B), there was a noticeable sorption effect on the heavier molecular PAH compounds in 'Soil & oil & AC at time zero', compared to other treatments (as the AC seems to slightly inhibit the extraction of these compounds) and is a possible indication that the addition of AC at the start causes the compounds to become even more strongly bound to the point where they can no longer be extracted and quantified via ASE. Although the methods employed and objectives differed to this study, Oyelami *et al.* (2014) reported that the reduction in both the mineralization and extraction of phenanthrene from treated and controls soils samples was as a result of AC amendment and also increased with AC-soil pollutant contact time. Other reports have also shown reductive effects of PAHs and other HOCs extractability due sorbent amendment (Vasilyeva *et al.*, 2006; Beesley *et al.*, 2010; Vasilyeva *et al.*, 2010; Hale *et al.*, 2012; Brennan *et al.*, 2014). This reduced PAH extractability even with very rigorous methods would of course also imply a significant risk reduction.

The average sum of PAHs fraction recovered from the soil (µg/g) for all treatments, Controls: Soil, Soil & AC at time zero', and crude oil batches 'Soil & oil & AC at time zero', 'Soil & oil & AC at time 5 months' and Soil & oil were 6.1±1.8, 3.3±0.3, 6.9±2.3, 4.6±0.3 and 6.1±1.4 respectively and in comparison to total amount of PAHs (Figure 3-4 A, 84±1.5 µg/g). This possibly indicates that the smaller molecular weight PAHs such as naphthalene, phenanthrene etc were biodegraded especially in batches were AC was not added at the start. Smaller molecular weight PAHs like naphthalene, but also phenanthrene, etc, are more readily biodegradable than larger molecular weight PAHs. (Juhasz and Naidu, 2000; Yu *et al.*, 2005).

Most of the PAHs recovered form 'Soil & oil & AC at time zero' microcosms as earlier stated was attributed to naphthalene (Figure 3-6 B and Figure 3-7) with concentration values of 4.0±0.8 µg/g (80±16 µg) contributing approximately 57 % of total sum of PAHs obtained from these batches. The results in this study show that naphthalene originates from the crude oil and binds readily to AC and AC added at time zero was significantly effective in limiting the volatilization or mineralization of naphthalene (Figure 3-7). This is in comparison to the other crude oil treatments where naphthalene had seemingly been removed via biodegradation. The higher CO_2 production (Figure 3-3) from crude oil batches 'Soil & oil & AC at time 5 months' and Soil & oil compared to 'Soil & oil & AC at time zero', also confirms this. This also further confirms that sorbent amendment can limit biodegradation via enhanced sorption and reduction in bioavailability (Quilliam *et al.,* 2013). This finding is in contrast to reports by Qin *et al.* (2013) , as the sorbent amendment at beginning in this study was more effective for the binding of hydrocarbon molecules (Alkanes and PAHs) in comparison to adding AC mid-way through.

Previous reports have shown lower molecular weight PAHs to correspond more readily to AC treatment (Lebo *et al.,* 2003; Brändli *et al.,* 2008; Choi *et al.,* 2012) due to faster kinetics in desorbing and binding to AC surface compared to higher molecular weight compounds (Zimmerman *et al.,* 2004; Zimmerman *et al.,* 2005; Hale and Werner, 2010; Choi *et al.,* 2012). Lesser sorption would have probably have aided the biodegradation of the lower molecular weight PAHs in AC non-mended batches. It is also believed naphthalene would bind more rapidly to AC because of its higher concentration in the North sea crude oil (Figure 3-4 A) and there is a higher availability of naphthalene PAHs compounds to interact with AC active sites in comparison to other PAHs, due to lesser molecular sieving effects for smaller molecules (Lamichhane *et al.,* 2016). Several Reports have indicated higher initial concentration of HOCs favors or increases the adsorption to sorbents (Ping *et al.,* 2006; Awoyemi, 2011; Gupta, 2015) and that PAHs concentration and structure are important factors in rate of their adsorption to adsorbents (Liu *et al.,* 2014).

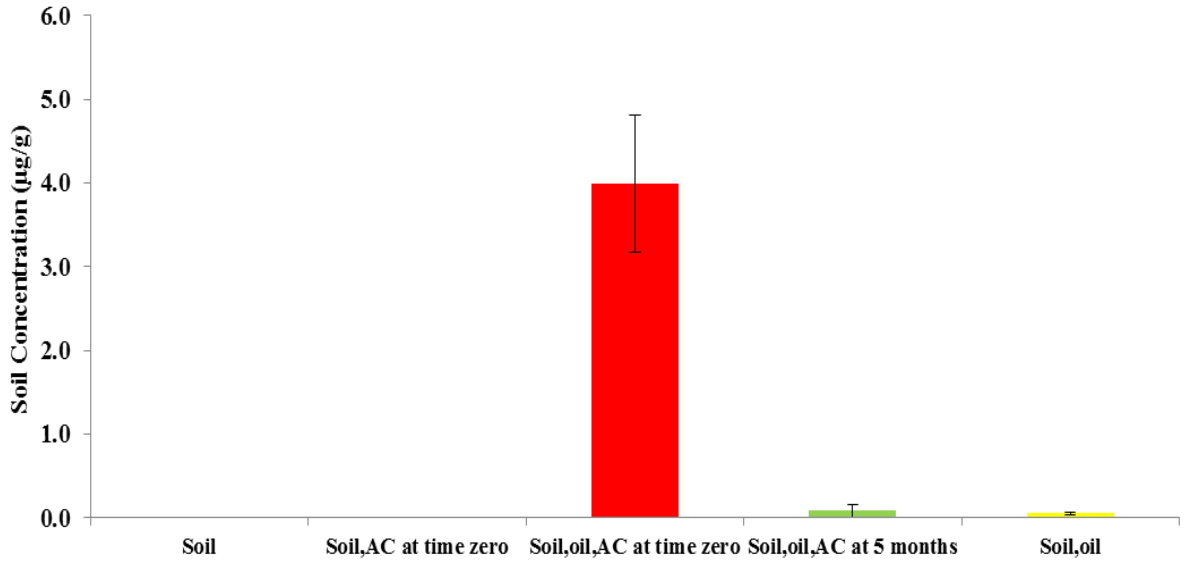

Figure 3-7 Average soil concentrations of naphthalene detected from the crude oil added to the batches. Error bars: represent ±1 standard deviation (SD=3) from the mean of three replicates

3.4.3 Volatile hydrocarbon concentration (head space injections and polyurethane foam plug

The headspace concentrations and volatilization of hydrocarbons (via the foam plug method) was measured in the batch system (vials), however the concentrations were very low, close to detection limit and no trends were visible. Very low headspace and foam plug concentration are a possible indication that the volatilization in the batch system was effectively controlled by the combined action of sorption and also biodegradation. In AC amended batches the AC was able to reduce the volatilization of the compounds due to sorbent amendment (Bushnaf *et al.*, 2011) while the compounds were quickly removed by biodegradation in the un-amended crude oil batches (Höhener *et al.*, 2003). It was previously shown that the removal of these volatile compounds occurs very early during monitoring, possibly within a matter of days (Bushnaf *et al.*, 2011; Meynet *et al.*, 2014).

3.4.4 Hydrocarbon Polyethylene concentrations, free aqueous hydrocarbon concentrations and hydrocarbon phase partitioning (estimated Log Kd) after biodegradation and activated carbon amendment.

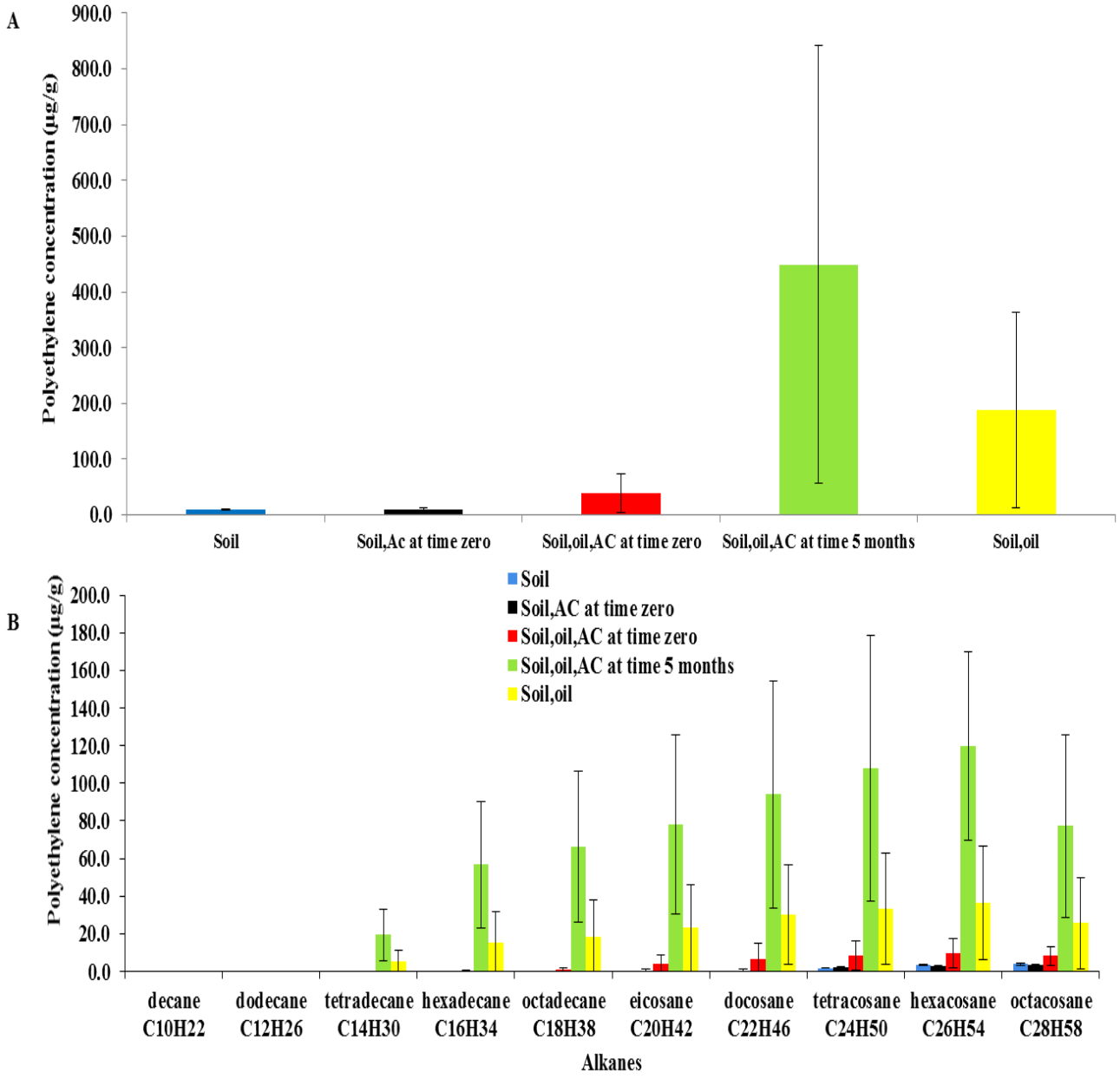

Figure 3-8 Polyethylene samplers uptake of alkanes after 52 weeks of biodegradation or AC amendments. Average alkane polyethylene concentrations detected in batches. A: sum of the alkane concentrations per treatment, B: concentration of individual alkane compounds per treatment. Error bars: represent ±1 standard deviation (SD=3) from the mean of three replicates

Polyethylene samplers (P.E) were utilised in comparing the differences in hydrocarbon availability for the treatments after the batch experiments. P.E samplers are known to mainly accumulate truly dissolved HOCs compounds (Hale *et al.*, 2012) and the compounds obtained are believed to be potentially available for uptake by plants, soil-dwelling organisms (Hawthorne *et al.*, 2007) and microbes, including pollution biodegrading microbes. The results for the availability of the alkanes (Figure 3-8) showed that the alkanes were more readily accumulated by the P.E samplers for the treatment 'Soil & oil & AC at time 5 months' compared the controls and the other crude oil amended batches. This is indicates that the addition of AC at 5 months was not as effective in reducing the availability of the residual crude oil alkane fraction in comparison to batches with AC addition from the beginning and/or bioremediation over a period of twelve instead of only five months. In Soil & oil & AC at time Zero' (Figure 3-8 A and B) the reduction in availability of alkane compounds strongly bound to AC is believed to explain the very low uptake by P.E (See soil concentrations, Figure 3-5), while biodegradation is believed to be responsible for the low P.E alkane uptake in the Soil & oil system. The fouling of AC by crude oil biodegradation metabolites might explain the lesser AC effectiveness as an alkane sorbent, if added after 5 months instead of at the beginning.

From the aromatic fraction availability results (Figure 3-9), the addition of AC at time zero was also able to significantly (t-test; $p = < 0.05$) reduce the P.E accumulation of 16 EPA PAHs (average sum and some of the individual heavier MW PAHs) in treatments 'Soil & oil & AC at time Zero' and Soil & AC at time Zero' in comparison to the other two treatments. Previous studies have shown that amendment with 2% powdered activated carbon (PAC) can dramatically reduce freely dissolved concentrations of PAHs (Brändli *et al.*, 2008; Hale and Werner, 2010; Choi *et al.*, 2012; Hale *et al.*, 2012). Hale *et al.* (2010b) in batch studies, showed sorbent amendment (2% PAC), was more effective in the further reduction of already strongly bound or aged PAHs in River Tyne sediment in comparison to one month bio stimulation and bio augmentation treatments. Several studies have shown the potential AC in the reduction of dissolved hydrocarbon concentrations (Zimmerman *et al.*, 2004; Cornelissen *et al.*, 2011; Oleszczuk *et al.*, 2012) and PAHs bioavailability (Jakob *et al.*, 2012). From the results for the individual compounds, all the treatments showed low availability concentrations of smaller MW (Naphthalene – Phenanthrene). The crude oil batches with no AC amendment 'Soil & oil' had the highest P.E concentration of heavier PAHs concentration, which indicates that were not readily biodegraded and perhaps also solubilized from the soil by the crude oil addition, thus becoming a higher risk. It also indicates a microbial preference for straight chain alkanes and lower MW aromatics (Perry, 1984; Ulrici, 2000) over heavier PAHs (Atlas and Bragg, 2009).

The high standard deviation in Soil & oil batches is due to one of the three replicates having a much higher uptake of PAHs compared to other replicates. The batches amended with AC at 5 months (Soil & oil & AC at time 5 months) showed a higher reduction in PAHs availability (sum and individual, although not significant) in comparison Soil & oil, and this occurred through the combined action of sorption and biodegradation. A possible reason for the lesser effectiveness or attenuation of availability compared to AC amendment at the start is possibly due to the build-up of partially oxidized compounds (metabolites) within the soil, which can compete for or block (foul) the PAHs binding sites of the AC particles, or enhance PAHs solubility in soil porewater via the surfactant effect (Koelmans *et al.*, 2009; Rhodes *et al.*, 2010).

The results so far show that the addition of AC from the start was most effective in reducing freely dissolved hydrocarbon especially with regards to the toxic PAHs compounds (Figure 3-9). Figure 3-10 (A, B and C) shows the more effective reduction of some known toxicological relevant (carcinogenic) PAHs in Soil & oil & AC at time zero batches compared to crude oil spiked batches without AC and AC amendment at 5 months. The fact that results for Soil & oil & AC at time zero showed a higher soil concentration (especially for the alkanes) (Figure 3-5 and Figure 3-6) and a marked reduction in bioavailable hydrocarbon concentrations (Figure 3-8 and Figure 3-9) even with the rigorous extraction methods employed in the study is an indication that the compounds bind very strongly to the AC adsorbent and are unlikely to pose significant risks (Beesley *et al.*, 2010). In addition to the effects of AC, the AC addition to strongly weathered PAH pollution in controls (Soil & AC at time zero) is effective in further reducing the already very low availability of the PAHs in the soil.

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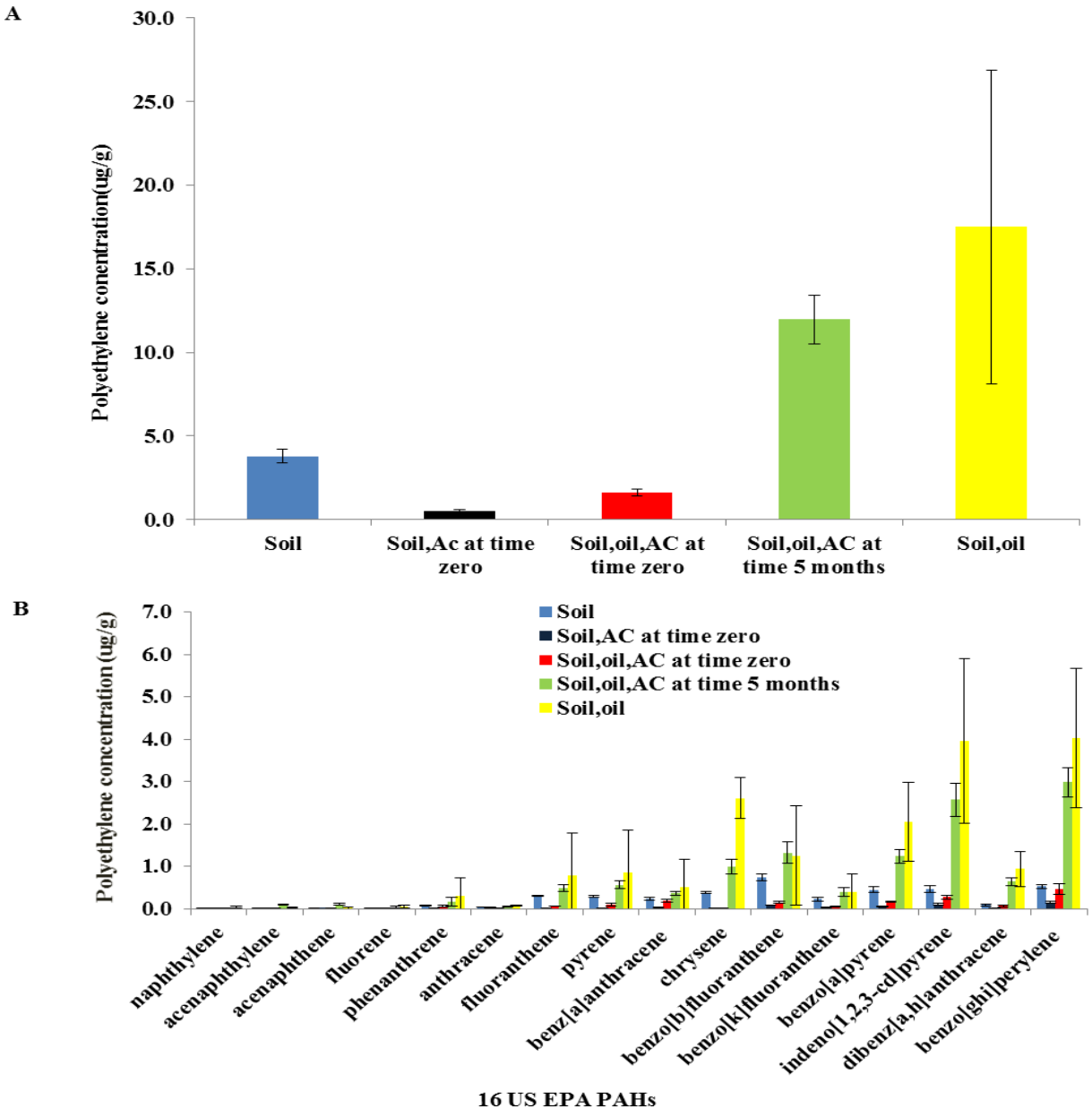

Figure 3-9 Polyethylene samplers uptake of PAHs after 52 weeks of biodegradation or AC amendments. Average PAHs polyethylene concentrations detected in batches. A: sum of PAHs compound concentrations per treatment, B: concentration of individual PAHs compounds per treatment. Error bars: represent ±1 standard deviation (SD=3) from the mean of three replicates

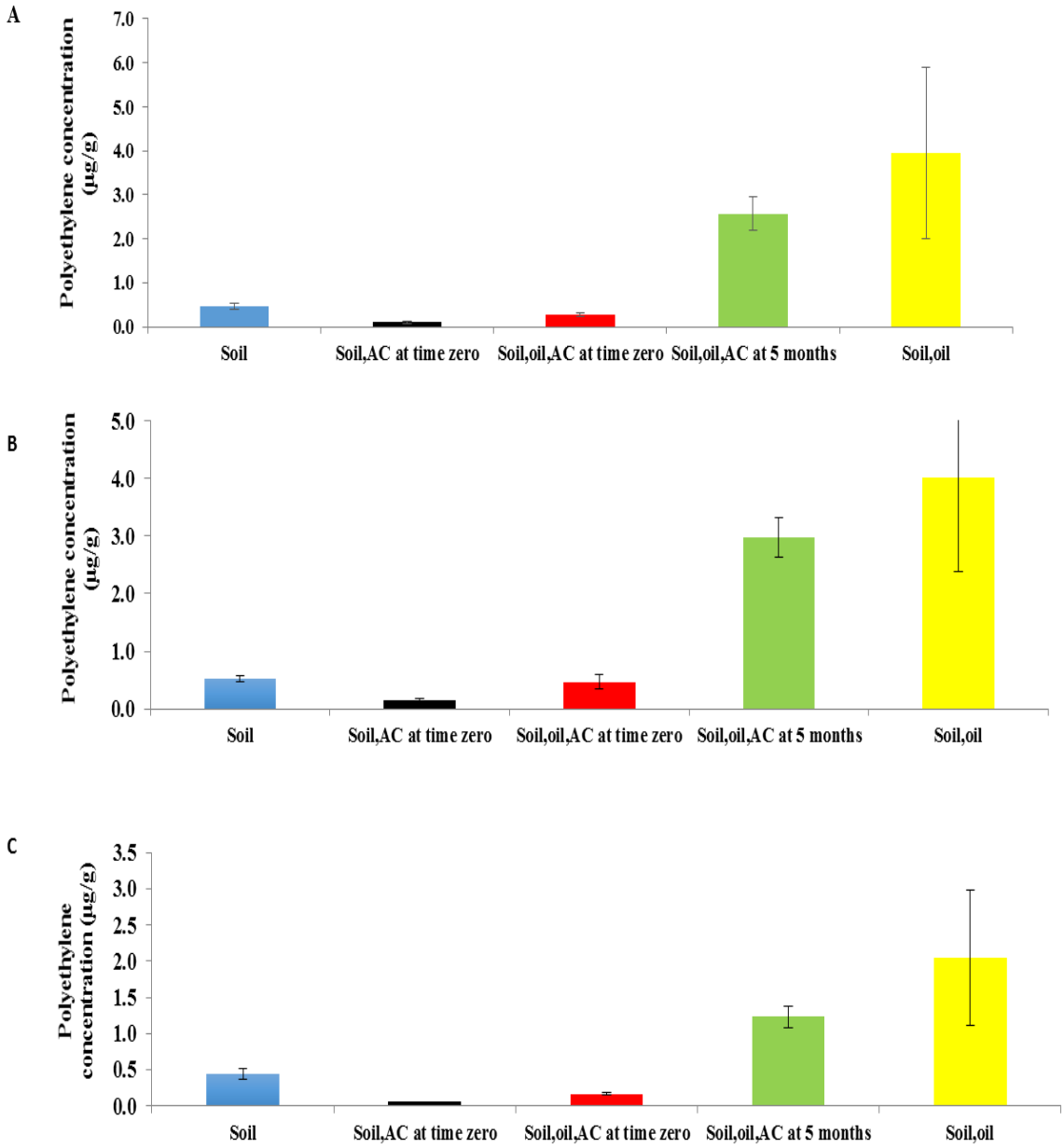

Figure 3-10 Average P.E concentrations of 3 polycyclic aromatic hydrocarbons. A: benzo(a)pyrene,(5 ring PAH), B: indeno(1,2,3-cd)pyrene (6 ring PAH) C: benzo(ghi)perylene (6 ring PAH) from batches. Error bars: represent ±1 standard deviation (SD=3) from the mean of three replicates

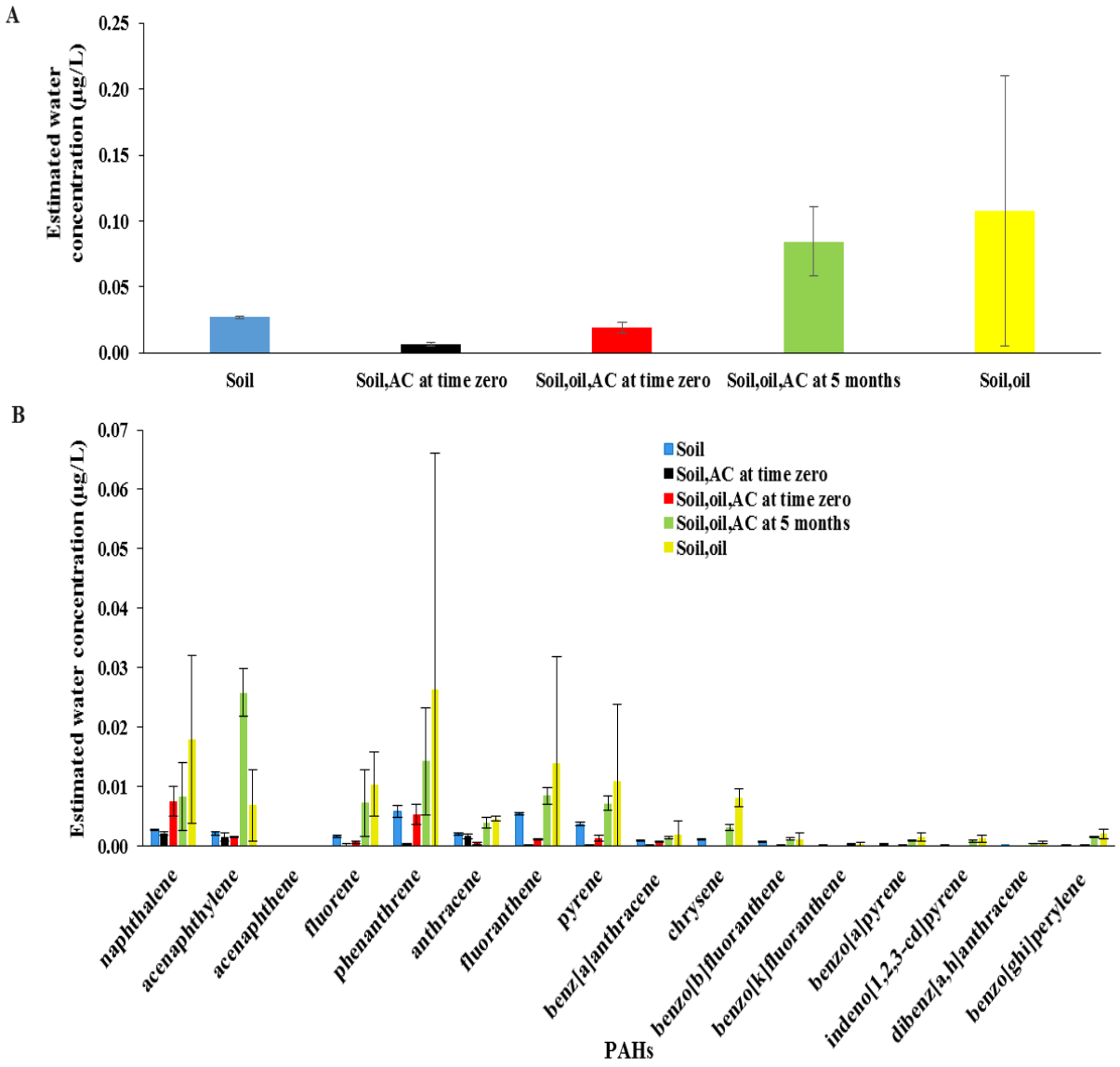

Figure 3-11 Free aqueous concentration of PAHs after 52 weeks of biodegradation or AC amendments. Average PAHs concentrations detected in batches. A: sum of the PAHs concentrations per treatment, B: concentration of individual PAHs compounds per treatment. Error bars: represent ±1 standard deviation (SD=3) from the mean of three replicates

The free aqueous PAHs concentrations (WC) (Figure 3-11) was obtained using the P.E PAHs concentrations (PEC) and P.E-water partitioning coefficient (KPE) (Hale et al., 2010a; Hale and Werner, 2010). The free aqueous concentrations were not estimated for the saturated fraction due to unavailable P.E – water partitioning coefficient values in the literature. The free aqueous concentration is predominantly made up of smaller molecular weight compounds giving a different emphasis to the results. The results show a decrease (although not significant) in free aqueous concentrations for the PAHs (Figure 3-11 average sum and individual) in treatments in 'Soil & oil & AC at time Zero' and Soil & AC at time Zero' compared to the other

crude oil treatments. Indicating that AC was able to reduce the transfer of the compounds into the aqueous phase, where they are available for leaching and posing a potential groundwater pollution risk. For example naphthalene that has the highest soil concentrations for 'Soil & oil & AC at time Zero', but also has the lowest aqueous concentration (although not statistically significantly) in comparison to other crude oil batches. This is also similar for some of the lower MW compounds such as fluorene and phenanthrene.

The sediment–water partitioning coefficients K_d estimation uses soil and aqueous PAHs concentrations (Figure 3-12) and is defined as the proportion of the pollutant concentration existing in both solid and aqueous phases at equilibrium (EPA, 2004). Compounds with larger K_d are known to migrate slowly and this reduces the tendency of the pollutant reaching the receptor in turn lowering potential risks (Krupka *et al.*, 1999; Mayer *et al.*, 2016). AC amendment batches from the start had K_d values ranged between 3.7±0.1 and 7.4±0.1 cm^3/g for Control Soil & AC at time Zero' and between 3.7±0.1 and 6.5±0.1 cm^3/g for Soil & oil & AC at time Zero'. The K_d values for Control Soil ranged between 3.7±0.1 and 6.4±0.2 cm^3/g. Similar K_d values have been previously reported (Hale and Werner, 2010) and is an indication that the AC amendment enhances K_d. The addition of AC also seems to enhance the K_d for naphthalene in soil 'Soil & oil &AC at time Zero' and slightly in 'Soil & oil & AC at time 5 months' and is possibly due to the high concentration of naphthalene in the crude oil. In general, the K_d values for batches amended with AC from the start were larger in comparison to crude oil batches with no AC amended and AC amendment at 5 months (range from 3.2±0.1 and 5.7±0.2 cm^3/g, range, 2.7±0.1 and 5.8±0.2 cm^3/g). Therefore, going by the availability and aqueous concentrations results, it shows that in certain situations adsorbents such as AC acts as a significant sink for hydrophobic pollutants (Mayer *et al.*, 2016) which leads to reduced mobility in soil and lower potential risks of groundwater and air pollution. In addition the results also shows that the addition of AC to the original soil (Control Soil & AC at time zero) significantly increased the binding of native soil PAHs, thus resulting in the highest K_d values. The addition and biodegradation of crude oil adds other compounds which can compete with the native soil PAHs for AC sorption sites, thus the K_d values follow the order – Soil & AC at time zero > Soil & oil & AC at time zero > Soil & oil & AC at time 5 months.

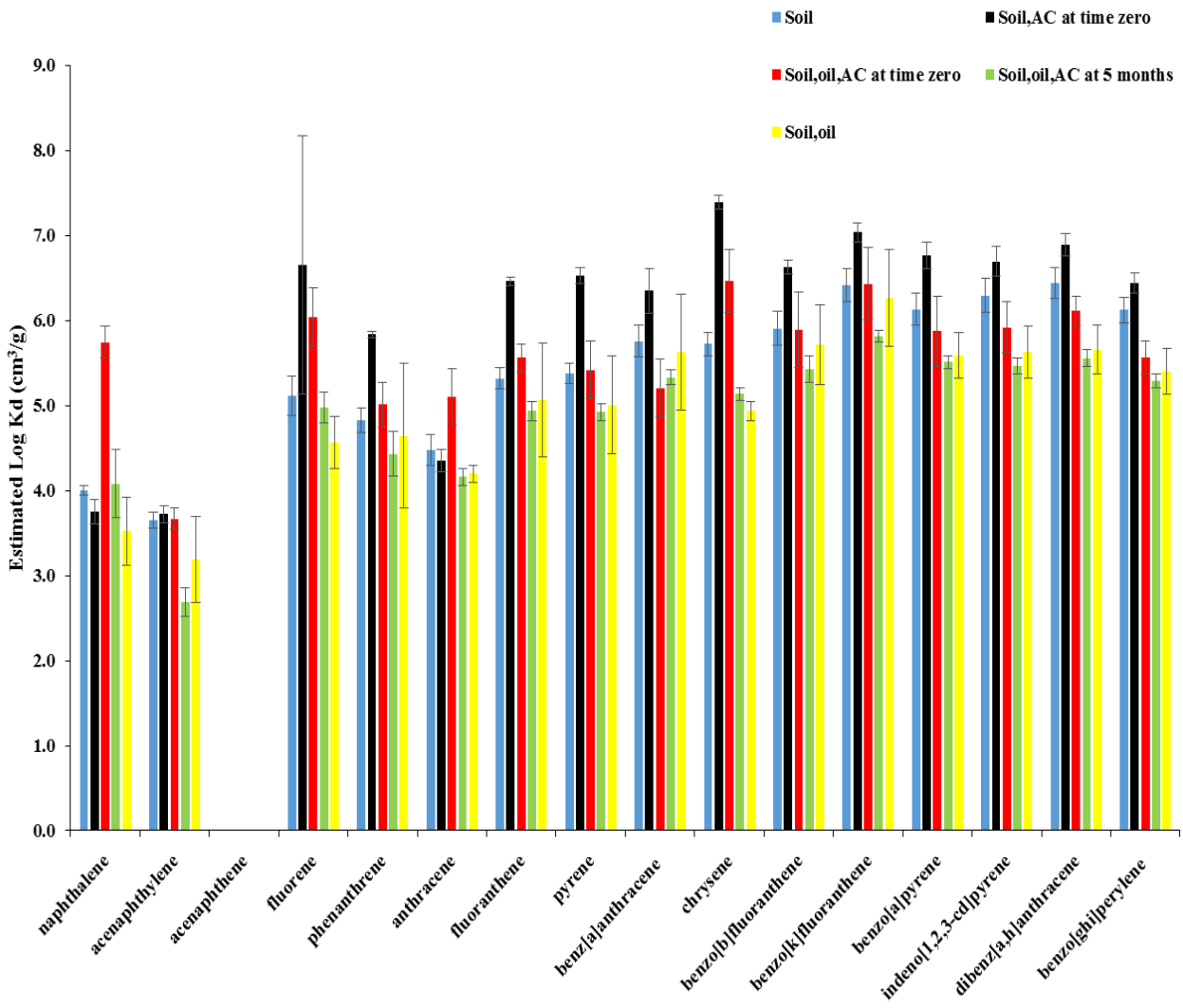

Figure 3-12 Estimated Log Kd values of 16 US EPA polycyclic aromatic hydrocarbons obtained using P.E and soil PAHs concentrations. Error bars: represent 1 standard deviation (SD=3) from the mean of three replicates

3.5 Conclusion

The study on the effect of AC on crude oil biodegradation has shown that AC amendment slows down biodegradation rate and more so if AC is added at the beginning compared to amendment after 5 months. And as illustrated by the ASE results and higher K_D values, this is hindrance to biodegradation occurs due sorption of the pollutants leading a reduced pollutant availability. The CO_2 evolution and ASE results has shown that by adding AC after 5 months there was an avoidance of the hindrance of the mineralization of the easily degradable crude oil fractions such as alkanes and naphthalene. These finding are in line with the general proposed hypothesis 1 and 2 (chapter 1 page 5). However another key finding of this study, shows that AC amendment at 5 months wasn't as effective as AC amendment from the beginning in reducing P.E uptake of PAHs and the subsequent free aqueous concentrations and this was possibly due to fouling by metabolites. The results show that important trade-offs exist between the negative effects of AC on biodegradation of alkanes and small molecular weight PAHs and its benefits in reducing hydrocarbon bioavailability for the less biodegradable compounds such as weathered and heavier molecular weight PAHs.

Therefore, AC should ideally be added after the biodegradation has been completed as much as possibly, including the biodegradation of metabolites, to stabilize the least biodegradable residuals such as those persisting for decades in the urban soil. During the biodegradation process, the contaminant mobility should be controlled with additional measures such as vapour barriers to minimize volatile emissions and liners to minimize leaching risks. Perhaps, crude oil contaminated soil could be "sandwiched" between layers of AC amended soils acting as volatilization and leaching barriers until the biodegradation process has been completed, at which point the AC amended layers could be mixed with the bio-remediated soil to stabilize residuals. The addition of AC to a layer (1.25 cm) of soil in an *in situ* modelled sediment study, showed that AC acted as a barrier by isolating and reducing the PCB mobility in comparison to sand (only) caps (Murphy *et al.*, 2006). To further reinforce the results above, as most of the results generated are based on chemical assessments, there still a need for further insight on how AC amendment affects the predominant bacterial community structure and function with respect to crude oil biodegradation.

Chapter 4. Impacts of activated carbon amendment time on soil microbial crude oil biodegradation in soil: An assessment using two 16s rRNA sequencing methods.

4.1 Introduction

There are certain factors that determine the occurrence of effective bioremediation of HOCs and two of these include the presence of microorganisms with HOCs catabolic abilities (Das and Chandran, 2010) and the presence of bioaccessible and biodegradable pollutants (Reid *et al.*, 2000). Certain bacteria possess the ability to degrade hydrocarbon pollutants either aerobically or anaerobically and will preferentially attack hydrocarbons with smaller molecular weight over heavier hydrocarbons (Perry, 1984; Ulrici, 2000). Reports have shown that consortia of mixed bacteria have a higher degrading efficacy for hydrocarbons compared to single organisms (Jacques *et al.*, 2008; Zhang *et al.*, 2010). This is due to the synergistic effects of mixed consortia (Boopathy, 2000), however this synergistic ability could be affected or at risk by the addition of adsorbents (such as AC), as adsorbent amendment has been reported to reduce mineralisation of HOCs by limiting the pollutant bioaccessibility (Rhodes *et al.*, 2008; Rhodes *et al.*, 2010) , thus potentially disadvantaging hydrocarbon degrading microorganisms, or inhibiting the induction of hydrocarbon metabolism.

16s RNA analysis has become a vital tool in microbial identification (Sogin *et al.*, 2006; Flanagan *et al.*, 2007) and in the elucidation of microbial diversity in intricate environmental samples (Shokralla *et al.*, 2012). DNA sequencing has evolved over the years (Liu *et al.*, 2012) and the use of next generation sequencing platforms have been utilised in the recovery of vital DNA sequence information from ecological samples which can then be compared with sequences of already known organisms to infer organism identity (Shokralla *et al.*, 2012). NGS platforms particularly PCR based methods have been utilised in various pollution related studies, some of which include the determination of the impacts of varying biochar addition time on microbial communities structures in crude oil polluted soils (Qin *et al.*, 2013), the treatability of water body pollution with granulated activated carbon biofilms (Islam *et al.*, 2015) and utilization of NGS platforms (454 and ion torrent) in the identification of predominant microbes in Athabasca river and how oil sands mining impacts on the microbial community structure with the river (Yergeau *et al.*, 2012a).

454 pyrosequencing has been preferentially used for analysis of environmental samples (metagenomics) due to generation of longer sequences leading to a higher probability in

sequence matches. This sequencing method employs the detection of light emission generated by the DNA nucleotide incorporation and enzymatic action of luciferase (Shokralla *et al.*, 2012). However 454 platform producer Roche has withdrawn its support for the 454 platform (Fordyce *et al.*, 2015) due its NGS project's inability to compete with projects of other sequencing platforms such as Illumina and Ion torrent (Hollmer, 2013). Ion torrent does not require light detection or a camera but detects a change in concentration of hydrogen ions (pH change) due the addition of nucleotides in the DNA strand while Illumina which is a third common platform for NGS uses a sequencing by synthesis method which is followed by a bridge amplification (Liu *et al.*, 2012; Shokralla *et al.*, 2012).

Employing such powerful and fast sequencing tools (such as PGM ion torrent and 454) for the characterization of bacterial populations would help in determining the impacts AC and crude oil on predominant microbial community (determined via detection of changes to the community structure, for example richness, diversity and abundance changes) and this would be useful in better understanding physicochemical observations, like those described in the previous chapter. This can help optimize remediation approaches especially with regards to the utilization of activated carbon.

This chapter investigates and reports the evaluation of the effects of crude oil pollution and varying activated carbon amendment times on the microbial community structures within batch samples. The study will attempt to compare NGS platforms, 454 pyrosequencing and PGM ion torrent outputs and to our knowledge is this is the first study looking at how the varied PAC amendment time affects microbial community using 2 different NGS platforms.

4.2 Objectives

The specific objectives are

1. Determination of the effects of crude oil pollution and PAC amendment time on the richness and diversity of microbial communities within soil batch systems.

2. Studying microbial community abundance changes to aid in the identification of HC utilizing microbes which benefit from crude oil pollution and also determining how AC amendment affects them.

3. Comparing 2 different NGS (bench top) platforms, 454 GS junior and PGM Ion torrent, to ascertain that the differences in soil microbial communities observed are not dependent on the choice of the NGS platforms used by the investigators.

4.3 Materials and Methods

4.3.1 Soil sample preparation and storage

As briefly mentioned (see chapter 3 section 3.3.8.6), at the end of the batch experiment and prior to passive sampling and residual concentration assessment (section 3.3.8.6 and 3.3.87 respectively) the vials were uncapped and soil samples collected (in triplicate) from the batches and also from the untreated (original soil) in duplicate. The collected samples were mixed properly and stored away in the freezer (at -20^0 C, in PBS, Oxoid) for microbial analysis.

4.3.2 DNA extraction and polymerase chain reaction (PCR) amplification of 16srRNA gene

DNA extraction: The Fast-DNA Spin kit was utilized in extracting the genomic DNA and was carried out with slight modifications to the manufacturer's instructions (MP Biomedicals, UK). 2 ml of soil in PBS was transferred into an Eppendorf tube and centrifuged for 2 mins at 13000 rpm. 0.5 g of the pellet was added to a provided matrix E lysis tube containing soil lysis buffer solution (sodium phosphate and MT buffer). The sample pellets were lysed with Hybaid Ribolyser, followed by centrifugation for 10 mins at 13000 rpm. 1 ml of supernatant was transferred to sterile 2 ml Eppendorf tube followed by the addition of 1 ml DNA binding buffer. The Eppendorf tube containing sample and binding buffer was mixed by inverting for 2 mins and allowed to stand for a further 3 mins to allow DNA bind to the matrix. 500-600 µl of supernatant was discarded once the binding buffer had settled at the bottom of the tube and the step was repeated with the remaining 1 ml DNA sample. 700 µl of DNA-binding buffer mixture was transferred to a spin filter and centrifuged for 1min at 13000 rpm. The flow through was decanted and the step repeated with remaining DNA-binding buffer mixture. 500 µl of SEWS-M DNA wash solution was added to the spin filter, centrifuged for 1 min at 13000 rpm and further centrifuged for 2 mins at 13000 rpm to dry the matrix of residual DNA wash solution. The spin filter was transferred to a sterile Eppendorf tube prior to the addition of 50 µl DNA elution buffer and centrifugation for 1min at 14000 rpm. The eluted or extracted DNA was transferred to a sterile Eppendorf tube and stored at -20^0 C.

Polymerase chain reaction (PCR) for 454 and PGM ion torrent: Prior to PCR amplification of the V4 and V5 regions of the 16S rRNA gene, the sets of primers used (515f**:** 5'-GTGNCAGCMGCCGCGGTAA-3', 926r: 5'-CCGYCAATTYMTTTRAGTTT-3' for 454 and ion torrent) were diluted 1:10 in molecular biology grade water. Set primers were chosen as they have been previously utilized by other studies in targeting V4 and V5 regions (Quince *et al.*, 2011). Unique barcodes, 8 base pairs (454, 5'- end for forward and reverse via a GA linker) and 12 base pair (ion torrent, Golay barcode, 5'- end for forward via a GAT barcode spacer) were added. The barcoding utilization aids the sequencing of multiple samples and it's normally used for easy identification of sequences belonging to a specific sample (Parameswaran *et al.*, 2007). GS FLX titanium adapter A (5' - CGTATCGCCTCCCTCGCGCCATCAG – 3') and adapter B (5' – CTATGCGCCTTGCCAGCCCGCTCAG – 3') were attached to primers for the 454 run while A (5'- CCATCTCATCCCTGCGTGTCTCCGACTCAG-3') and Ion adapter trP1 (5'-CCTCTCTATGGGCAGTCGGTGAT-3') adapters were attached to forward and reverse primers (respectively) for the ion torrent run. For the multiplex PCR reactions, master mixes were made up containing fast start high fidelity (HF) buffer (containing $MgCl_2$), fast start HF enzyme blend (Roche diagnostic, Germany), PCR grade nucleotide mix (dNTPs, Roche) and nuclease water see Table 4-1 for complete list and volumes for the 25µl reaction. The barcoded diluted primer and DNA were added separately to each PCR reaction tube.

Table 4-1 25 µl PCR reaction mixture used as a guide in the preparation of master mixes for multiplex PCR reactions (average of 20 reactions per sample)

Reagent	Concentration	Volume (µL)
High fidelity buffer	18mM $MgCl_2$	2.5
High fidelity enzyme blend		0.25
Forward and Reverse primers	0.4µmol/L	0.5 (each)
dNTPs	0.2 mmol/L	0.5
DNA template		0.5
Nuclease free water		20.25

For the PCR program, there was an initial denaturation step at 95°C for 4 min, followed by a three step program of 25 repeated cycles. This 3 step program consists of a denaturation at 95°C for 1 min, primer annealing at 55°C for 45 s and extension at 72°C for 1 min. A subsequent

final extension step of 72°C for 7 min was done after the 25 cycles. The amplified products were stored at 4°C if used on the same day or -20^0 C if utilized later.

4.3.3 Purification, quantification and Agarose gel electrophoresis

Agarose gel electrophoresis: Agarose gel electrophoresis was used to check for the PCR product (insert) after the PCR step and purification step. An agarose gel-casting tray was set up by inserting combs in the tray and using autoclave tape to seal both ends of the tray. To make 1X TAE buffer, 2 ml TAE buffer (2M tris acetate, 0.05M EDTA, pH 8.3, Eppendorf scientific, New York, USA) was mixed with 98 ml of distilled water. 2g of agarose powder was measured and added to 1X TAE buffer in a conical flask and subsequently melted by heating for 3 minutes in a microwave. Nancy fluorescent DNA dye was added after allowing agarose gel mixture to cool to 60 0C. The agarose gel mixture was gently poured into the prepared casting tray and allowed to set for 30 minutes. Upon setting the autoclave tape and comb were removed and the casting tray was transferred into an electrophoresis tank containing approximately 800 µL of 1XTAE buffer. A Loading buffer was applied to all samples being checked and the samples and PCR reference maker (fragment sizes- 2000, 1500, 1000, 750, 500, 300, 150 and 50 base pairs) were gently pipetted into the wells of the 1XTAE gel. The electrophoresis was run for 45 minutes at 100V. The stained PCR products were visualised on a UV illuminator linked to a Bio-Rad fluor-S multi imager (Bio-Rad, UK).

The positive multiplex amplified samples (Agarose gel checked) were pooled and cleaned using a QIAquick purification kit for 454, and a Agencourt AM Pure XP purification kit for the ion torrent.

<u>Purification with QIAquick purification kit (QIAGEN, Crawley, UK) for 454 amplicon library:</u>
Buffer PB (5x sample volume) was added to pooled PCR samples in a 2 ml sterile Eppendorf tube and mixed manually by pipetting. The Buffer PB+sample mixture was transferred to a QIAquick column and centrifuged for 1 min at 13000 rpm (multiple columns were used when using the mini-elute QIAquick spin columns and subsequently pooled together in the next step). The flowthrough was discarded and 750 µl of P.E buffer was added to the QIAquick column and further centrifuged for 1 min at 13000 rpm. The QIAquick column was placed in a sterile 1.5 ml Eppendorf and DNA was eluted by adding 30 µl EB buffer (or 11µl when using the

mini-elute QIAquick spin columns) and centrifuged for 1 min at 13000 rpm. The purified DNA was stored at -20^0 C.

Agencourt AM Pure XP purification for ion torrent: This procedure uses an Agencourt AM Pure XP reagent containing magnetic beads (Beckman Coulter, USA) in the purification of samples. Briefly, for a double sided purification of DNA insert (removal of fragments <300 and >600 base pairs) 0.6 x sample volume (at the beginning) and 1.1 x sample volume (midway through the procedure) of AM Pure XP reagent was added directly to an Eppendorf tube containing pooled samples and mixed manually by pipetting. The tubes were allowed to stand and incubate for 5 mins at room temperature. The tubes were placed in a Dyna Mag $^{TM-2}$ magnetic rack for 2-5 mins thus causing a separation of the beads from the solution. The supernatant was discarded carefully without disrupting the beads. With the tubes still on the magnetic rack, 40µl of freshly prepared 70% ethanol (wash) was added and samples were incubated at room temperature for 30 seconds (The wash was repeated). Residual ethanol was removed with smaller volume pipettes and tubes allowed to dry on the magnet at room temperature for 5 mins. The tubes were removed from the magnet, 20 µl of TE buffer was added to each tube to disperse beads before mixing by manually pipetting. The tubes were placed on the magnet for another 1 min. Upon separation; the purified amplicons (supernatant) were carefully transferred to a sterile 200 µl Eppendorf tube and stored at -20^0 C.

Quantification: PCR amplicon quantification was done on a Qubit 1.0 fluorometer (Invitrogen, USA) using Qubit dsDNA HS assay kit (Invitrogen, USA). Briefly, working assay solution was prepared by doing a 1:200 dilution of Qubit standard reagent in Qubit standard buffer. 1 µl of clean PCR product was added to 199 µl of working solution in a sterile tube and mixed by vortexing. The pre calibrated Qubit 1.0 fluorometer was used in determining the DNA concentrations by selecting the dsDNA high sensitivity assay type on Qubit 1.0 fluorometer.

4.3.4 454/Roche pyrosequencing and ion torrent sequencing

The purified samples were diluted due to variation between sample concentrations. This enabled the pooling of samples in equimolar concentration. The pooled amplicon libraries for 454 were sequenced on a Roche 454 GS junior (Macropathology, Ltd, Coventry. UK). The sequencing was performed bi-directionally (from 515f and other from 926r) for forward and reverse runs, thus generating an output data in a standard flow gram format (SFF). The pooled amplicon libraries for the ion torrent run were sequenced at the School of Civil Engineering &

Geosciences, Newcastle University on a PGM (personal genome machine) ion torrent (316 ion chip) sequencer generating a Fastq file.

4.3.5 Quality assurance and control

Quality assurance was performed while carrying out the experiments to ensure the maintenance of high standards. All reagents and extracted DNA were kept on ice until used to prevent denaturation. The Eppendorf tubes (200 µl, 1.5 ml and 2ml) and all plastic ware were always autoclaved before use to eliminate contaminants and were kept in a beaker sealed with thin foil paper. To reduce the risk of contamination during the preparation samples and mixing of reagents for both PCR and purification steps, the procedures were mainly carried out in the microbiology safety cabinet. Eppendorf tubes were mainly stored in the drying cabinet, sealed with foil paper in a beaker and only opened in the microbiology safety cabinet to prevent contamination. Prior to working in the microbiology safety cabinet, the cabinet and everything to be transferred (includes tubes, tube racks, beakers and pipettes) into the cabinet were decontaminated by cleaning with 70% ethanol. All tubes and other plastic ware were clearly labeled with water resistant permanent markers to allow for easy identification. Test runs were always done for PCR and amplification steps prior to the final run to determine how effective the dilutions or slight modifications to manufacturers procedures were. Agarose gel checks for the presence of the desired insert were always carried out after every amplification and purification step. The Nancy DNA fluorescent DNA stain dye was kept at 4^0C and stored away from light to prevent false results during electrophoresis check and when the melted gel is poured into the casting tray all bubbles were eliminated with a sterile pipette tip. Extra care was taken when using UV illuminator linked to a Bio-Rad fluor-S multi imager (Bio-Rad, UK) as to prevent exposure to ultra violet light. The ultra violet light was switched off before accessing the UV illuminator. The university microbiology laboratory health and safety regulations were adhered to and this includes wearing protective laboratory coats, gloves, working in the laboratory and safety cabinets and proper disposal of waste products. To prevent cross contamination, 2 separate gloves were used when working between safety cabinets and laboratory benches. Gloves and laboratory coats were taken off when exiting the laboratory and fresh sterile gloves worn during entry. Prior to the commencement of the microbial work a Bio COSHH risk assessment was carried out and necessary forms completed and signed for all hazardous substances used during the experiment work.

4.3.6 Qiime sequence analysis and microbial community analysis

The reads for 454 and ion torrent sequencing were initially demultiplexed (according to the barcodes utilized) and quality filtered using the 'split_libraries.py' command. 3 mapping files were used, 2 files for the 454 demultiplexing (due to the bidirectional sequencing), one for the forward and one for the reverse read. For the quality filtering criteria (Appendix B), a minimum sequencing length was set at 200 nucleotides for 454 and 100 for ion torrent as the 454 GS junior machine is known to generate sequences of length 400-450 bp, while the ion torrent with a 316 chip typically generates sequences with length between 100-200bp (Shokralla et al., 2012). A minimum quality score of 20 was used, no ambiguous bases (N) exceeding limit of 6 were allowed, exact matches to barcodes in mapping files and sequences containing homopolymers longer than 6 were discarded. To prevent problems when merging reads due to similar barcodes on both read sets, an 'n' parameter was used during the demultiplexing of the reverse reads which is a start index for the split library command. The raw sequences for the 454 run forward and reverse were denoised separately (Reeder and Knight, 2010) using a cluster of 15 CPUs to correct for sequencing errors and to prevent a high amount of erroneous Operational Taxonomic Units (OTU). The denoised sequence files were inflated, reverse 454 reads were reverse complemented (to ensure similar sequence orientation with forward reads) and merged (concatenated) with forward reads to give a single file prior to reintegration into the QIIME pipeline. After quality filtering, clustering of sequences for both 454 (concatenated data and just the forward dataset) and ion torrent data into OTUs was done at 97% similarity using the Uclust algorithm (Edgar, 2010). Representative sequences from each OTU were taxonomy assigned (at 50% and also at 80% cut off the sequences) with the RDP classifier (Wang et al., 2007) and aligned using PyNAST (Caporaso et al., 2009). The 50% cut off was used to help increase the number of sequences used to perform the taxonomy assignment. Chimera sequences were identified and subsequently removed using the QIIME's Chimera Slayer (Haas et al, 2011). The sequences were filtered using a Lanemask file, which excised columns containing gaps and variable regions. The representative aligned filtered sequences were used in generating a Newick phylogenetic tree using FastTree2 (Price et al, 2010) for further analysis. OTU abundance tables for each sample at different taxonomic levels were generated using selected representative sequences and their taxonomic assignments. The QIIME pipeline (Caporaso et al., 2010) was utilized in assessing diversity within each sample (α- diversity). To allow comparison between sequencing methods (ion torrent and 454), rarefaction was done at a minimum (starting) depth of 100 per sample, a sequence step-wise

increment of 100 sequences per depth, and 10 iterations (replicates) per depth. A maximum depth of 1500 was chosen (based on the sample with smallest OTU sequence count) to allow even sampling across the samples. For α- diversity, the Chao1 (non-parametric qualitative richness estimator) and Faith's phylogenetic diversity (PD) index (Chao, 1984., Faith, 1992) were determined. The Faith PD index works by collating phylogenetic tree branch lengths as a degree of diversity and basically a newly identified closely related OTU induces a small increment in diversity while a less related OTU produces a higher increase. The comparison of microbial communities between samples (β- diversity) was carried out using PRIMER v6 software (Primer-E Ltd., Plymouth, UK). The Qiime generated OTU table (class level) was log transformed and used for the diversity analysis which includes Bray Curtis dissimilarity metric calculation, a pairwise distance (average) and standard deviation (Clarke Robert et al., 2006). The output of the Bray Curtis analysis was used in generating a non-metric multidimensional scaling plot (nMDS) and further integrated with the chemical data presented in chapter 3. For statistical analysis, an Analysis of Similarities (ANOSIM) and a Nested ANOSIM was carried using a Pearson' product moment correlation dissimilarity matrix (Appendix B). Student *T-test* was performed on Microsoft excel version 2010 (Microsoft, Redmond, USA) to compare the effects of treatments on the α- diversity matrices for the various sequence runs and was also used for comparing the differences ($p < 0.05$) in relative OTU abundances for bacteria across various samples. Correlation analysis between ion torrent and 454 dataset (at phylum and genus level) also was performed on Microsoft excel version 2010 (Microsoft, Redmond, USA) to compare and determine the similarity and difference between data sets. PCA (principal component analysis) was carried out on XLSTAT software (XLSTAT, 2014) using the most abundant organisms (> 0.5% total sequences, log transformed OTU table). Spearman ranked correlation was performed on IBM SPSS statistics for windows to establish the relationship between data taxonomy assigned at bootstrap cut off of 50% and 80%.

4.4 Results and discussion

4.4.1 Quality filtering and OTU selection for 454 GS Junior pyrosequencing and Ion torrent sequencing

Most of the results (charts and tables) presented in this chapter were generated using the 50% RDP taxonomy assigned data. The 50% cut off data was used as the RDP taxonomy assignments at 50% cut off has been reported to be sufficient enough to accurately classify sequences at the genus level while still providing assignments for a higher percentage of sequences in comparison to cut off at 80% (Claesson *et al.*, 2009). Claesson *et al.* (2009) study

showed that for V4 reads (which is one of the targeted hypervariable regions in this study) at a RDP classifier bootstrap cut off of 50%, the fraction of sequences classified to genus was 97% while at 80% cut off was 87.9%. The fraction of sequences correctly classified to genus was 94.5% at 50% cut off and 95.7% at 80% cut off (a difference of 1.2%). In addition the 50% RDP cut off data was confidently utilized as the results from the spearman correlation analysis performed at genus level (454 GS and PGM ion torrent data), comparing RDP taxonomy assigned data at 50% and at 80% cut off, showed that there little difference between the 2 cut off datasets (Rho values generally above R^2 0.8 with significant correlation at 0.01 level) (See Appendix section D for tables 7.4-7.15 and scatter plots 7.15-7.26 showing comparisons. For example O.Soil.1_50% versus O.Soil.1_80%). This is also an indication, that changing % threshold from 80 to 50 had little effect on the microbial communities.

Of the initial 69888 sequences for 454 GS junior and considering forward reads, a total of 46,241 (66.2%) sequences passed the demultiplexing quality filtering while 52,189 (74.7%) passed the quality filtering considering reverse reads (Table 4-2). The average read length for the forward and reverse reads were 384 and 388 bp respectively while the number of sequences for the samples ranged between 821-14878 and 744-14878 respectively (Table 4-2). For the merged (concatenated) reads, 5023 sequences were culled via denoising leaving a combined total (forward and reverse) of 93,407 from 98,430. From the 93407 combined quality sequences, the bacteria domain was assigned 87,729 (93.9%) sequences and 87,474 (99.7%) of the assigned sequences were classified. The archaea domain was assigned 1620 (1.7%) sequences and 1592 (98.3%) of the assigned sequences were classified while 4058 sequences remained unassigned representing 4.3 %.

For the ion torrent, of the initial 3,111,945 sequences, 814,387 (26.17 %) sequences passed the quality filtering (Appendix B), 512,061 (62.8%) were assigned to the bacterial domain and 511,134 (99.8%) of the assigned sequences were classified. The archaea domain was assigned 36,195 (4.4%) sequences and 35,208 (97.2%) of the assigned sequences were classified. A total of 266,131 sequences were unassigned representing 32.7 %. The average read length for ion torrent was 277 bp and the number of sequences for the samples ranged between 13159 and 60597. At a minimum quality score of 20, the ion torrent a had lower percentage of sequences passing the quality filtering and a higher percentage of unassigned sequences which is an indication that the ion torrent probably generated sequences of lower quality in comparison to the 454 generated sequences.

Table 4-2 Quality filtering criteria and results for 454 bidirectional pyrosequencing data. Also contained barcodes used for forward and reverse runs

Quality filtering	Forward Reads	Reverse reads
Inputted raw sequences	69888	69888
No. sequences < 200 bp (removed)	543	543
No. ambiguous bases (removed)	12	12
Av. Sequence length	384	388
No. Max homopolymer failed sequences (removed)	2788	2788
No. sequences with correct barcodes	2650	2992
No. sequences with not correct barcodes (removed)	20270	14322
No. sequences failed minimum score (removed)	34	34
No. of sequences passed filtering	46241	52189
Samples	No. Sequences/barcodes	
O.soil 1	3365/TATATGCG	1668/TATAGCTC
O.soil 2	1668/TATAGCTC	4270/TATCTCGT
Soil 1	4270/TATCTCGT	7618/TATCAGAC
Soil 2	7618/TATCAGAC	14878/TATCGTCA
Soil 3	14878/TATCGTCA	9340/TATGCGTA
Soil & AC 1	986/TATGCTAG	1809/TACTACGA
Soil & AC 2	1126/TACTATCG	1008/TACTCTAC
Soil & AC 3	1233/TACTCAGT	1077/TACATGCT
Soil & Oil & AC 1	1028/TACACGTA	2243/TACGTATC
Soil & Oil & AC 2	2243/TACGTATG	1266/TAGTCACA
Soil & Oil & AC 3	1154/TACGACAT	1158/TAGTGTGT
Soil & Oil & AC at 5 months 1	1807/TAGTACGA	1050/TCTACTCT
Soil & Oil & AC at 5 months 2	1048/TAGTGTAC	1045/TCTAGAGA
Soil & Oil & AC at 5 months 3	1005/TAGACTCT	1031/TCTCACTA
Soil & Oil 1	1169/TCTACTGA	1114/TCTGTCAC
Soil & Oil 2	821/TCTCTATC	774/TCATAGTC
Soil & Oil 3	822/TCTCGCAG	840/TCATCTGA
Total	46241	52189

4.4.2 Microbial diversity and richness

The rarefaction curves were produced to assess the richness and diversity within samples. The **α**- diversity within a given sample set normally begins with a steep slope (indicating detection or discovery of species per sampling depth), which begins to level or flatten out with time as a lower number of additional species is being detected (Wooley *et al.*, 2010). For the current data, Chao1 and Faith PD index matrices were chosen. Chao1 as a qualitative richness estimator takes into account the number of singletons (rare species) and doubletons observed (Chao, 1984) while the divergence method, Faith PD adds up the entire length of branches in a phylogenetic tree leading to members of the community (Faith, 1992). By using both methods a broader picture (based on richness and phylogenetic relationship) of the diversity within samples could be determined (Lozupone and Knight, 2008). From the **α**- diversity results (Figure 4-1 and Figure 4-2) there is a noticeable difference between samples, and also between the 2 sequencing platforms. For the 454 GS junior data set, there is a significant higher richness (p value <0.05) in original soil (Osoil) and Control Soil (i.e. soil and nutrients) in comparison to other treatments at various sequencing depths, Figure 4-1 A and at a single rarefaction depth of 1400 sequences (Figure 4-3 A). The PD estimator results (Figure 4-2 A and Figure 4-3 B) also showed that the original soil (Osoil) and Control Soil had a slightly higher (p value >0.05) phylogenetic diversity than other treatment samples

Comparing sequencing platforms, the richness estimator values (Overall) per treatment sample was higher for the ion torrent compared to 454 GS junior system (both are bench top instruments)(Figure 4-1) and this is also evident at a single rarefaction depth of 1400 sequences (Figure 4-3 A). At a depth of 1400 sequences, species richness was significantly higher (p value 0.001) for the ion torrent compared to 454 GS junior data with sample treatment values from 1916±24 -3616±42 compared to 871±17-1555±26 respectively. This was expected, as ion torrent are generally known to produce more sequences and much higher throughput (more OTUs) compared to 454 (Loman *et al.*, 2012; Indugu *et al.*, 2016). When the phylogenetic diversity (PD whole tree) results between the 2 platforms at different rarefactions depths and at rarefaction depth of 1400 sequences (Figure 4-2 & Figure 4-3 B) were compared, the 454 data is seen to have higher diversity compared to the ion torrent data. This is probably because of the greater read length of the 454 (Brulc *et al.*, 2009; Indugu *et al.*, 2016). The sample treatment PD estimator values at rarefaction depth of 1400 sequences ranged from 51±0.5-70±0.2 for 454 data and 38±0.1-53±0.2 for ion torrent data.

Interestingly, the sample order for OTU richness and phylogenetic diversity was different for the ion torrent and 454 GS Junior data. Although the Control Soil & AC at time zero exhibits the highest OTU richness and diversity, Figure 4-1B, Figure 4-2 B and Figure 4-3 in the ion torrent data, it falls to the middle for the 454 GS junior derived data, with original soil (Osoil) being the highest (Figure 4-1 A, Figure 4-2 A and Figure 4-3). The marked difference in sample order between the two platforms in terms of α-diversity could possibly be due to the differences in error correction methods. This indicates that the denoising possibly changed the order of OTU richness and phylogenetic diversity of the 454 data.

Ion torrent is known to have a higher error rate in comparison to the 454-platform (Laehnemann *et al.*, 2016), and with the extra denoising step for 454 data and with other quality filtering steps (Kunin *et al.*, 2010) such as removal of chimeras (demultiplexing and chimera detection was also done for ion torrent data) there would probably have been a higher reduction in number of artefacts thus producing the more accurate data (Quince *et al.*, 2009; Reeder and Knight, 2010). Denoising of ion torrent data for environmental samples has been previously attempted by researchers (using a developed pyronoise algorithm) (Puente-Sánchez *et al.*, 2015), but the denoising of the ion torrent data in this study was not carried out, as most of the denoising flowgram algorithms are more specific for 454- derived data. In addition, Indugu *et al.* (2016) concluded that differences between 454 and ion torrent sequences results could be due to differences in the approach and in the study researchers showed comparable results (diversity and community studies) by using the same DNA extraction, purification and bioinformatics methods. In this study and as explained earlier, the Agencourt AM Pure XP reagent and Dyna Mag $^{TM-2}$ magnetic rack were utilised in amplicon library purification preparation for ion torrent while either the QIAquick spin columns or mini-elute QIAquick spin columns was used in amplicon library purification preparation for 454 GS junior .Looking at treatment effects, rarefaction curves (Figure 4-1 and Figure 4-2) the Soil & oil on average seemed to have the lowest richness and diversity for both sequencing derived data sets. This is probably due to an enhanced cytotoxic as well as growth related effects as a result of the higher concentration, uptake and biodegradation of easily biodegradable compounds (alkanes) from the crude oil. Hydrocarbon contamination (containing easily degradable substrates) has been previously shown to impact negatively on soil microbial richness and diversity, as a few specialist hydrocarbon degrading microorganisms become predominant in the soil microbial community (Sutton *et al.*, 2013). The results for both sequencing platforms also showed that AC amendment time did not significantly (t-test, 2 sided, unpaired, $p > 0.05$) affect the richness or

diversity within crude oil polluted batches (Soil & oil & AC at time zero, Soil & oil & AC at time 5 months were compared).

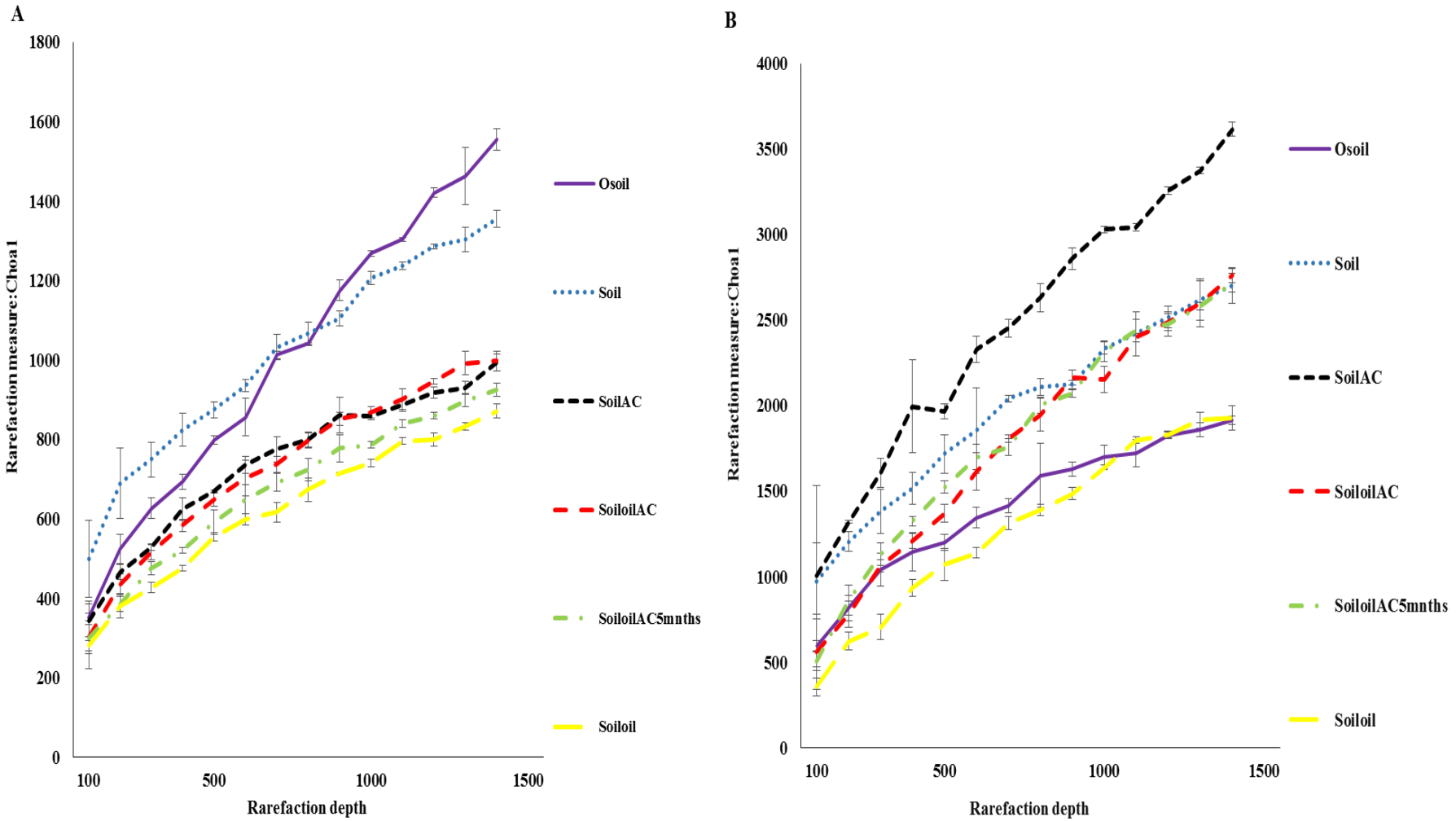

Figure 4-1 Rarefaction curves for soil treatment samples, 454 GS junior system (A) and Ion torrent (B) sequence run. Chao1 alpha diversity metric utilized. Error bars represent ±1 standard deviation (SD=3) from the mean of 3 replicates

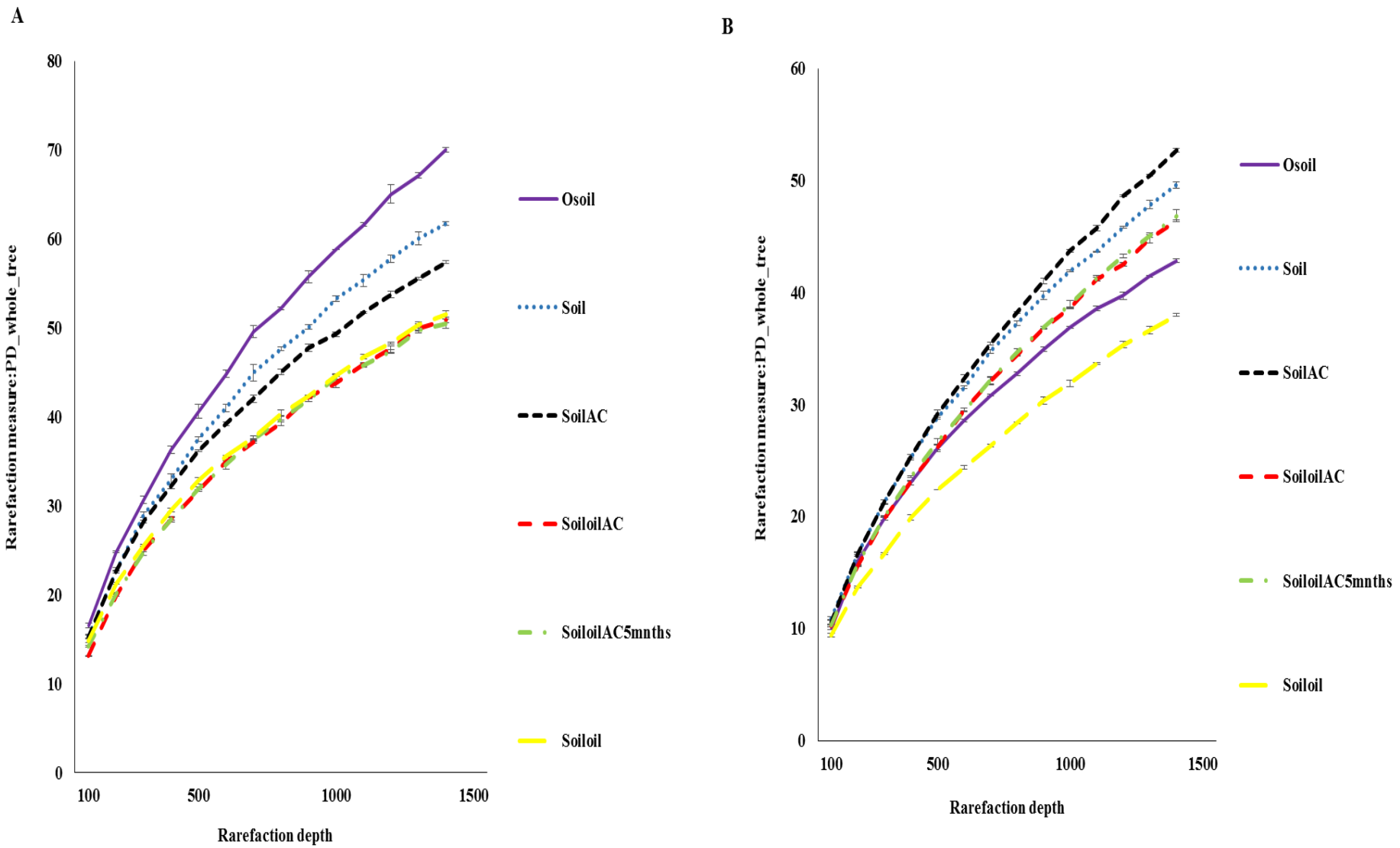

Figure 4-2 Rarefaction curves for soil treatment samples, 454 GS junior system (A) and Ion torrent (B) sequence run. PD_whole_tree alpha diversity metric utilized. Error bars represent ±1 standard deviation (SD=3) from the mean of 3 replicates

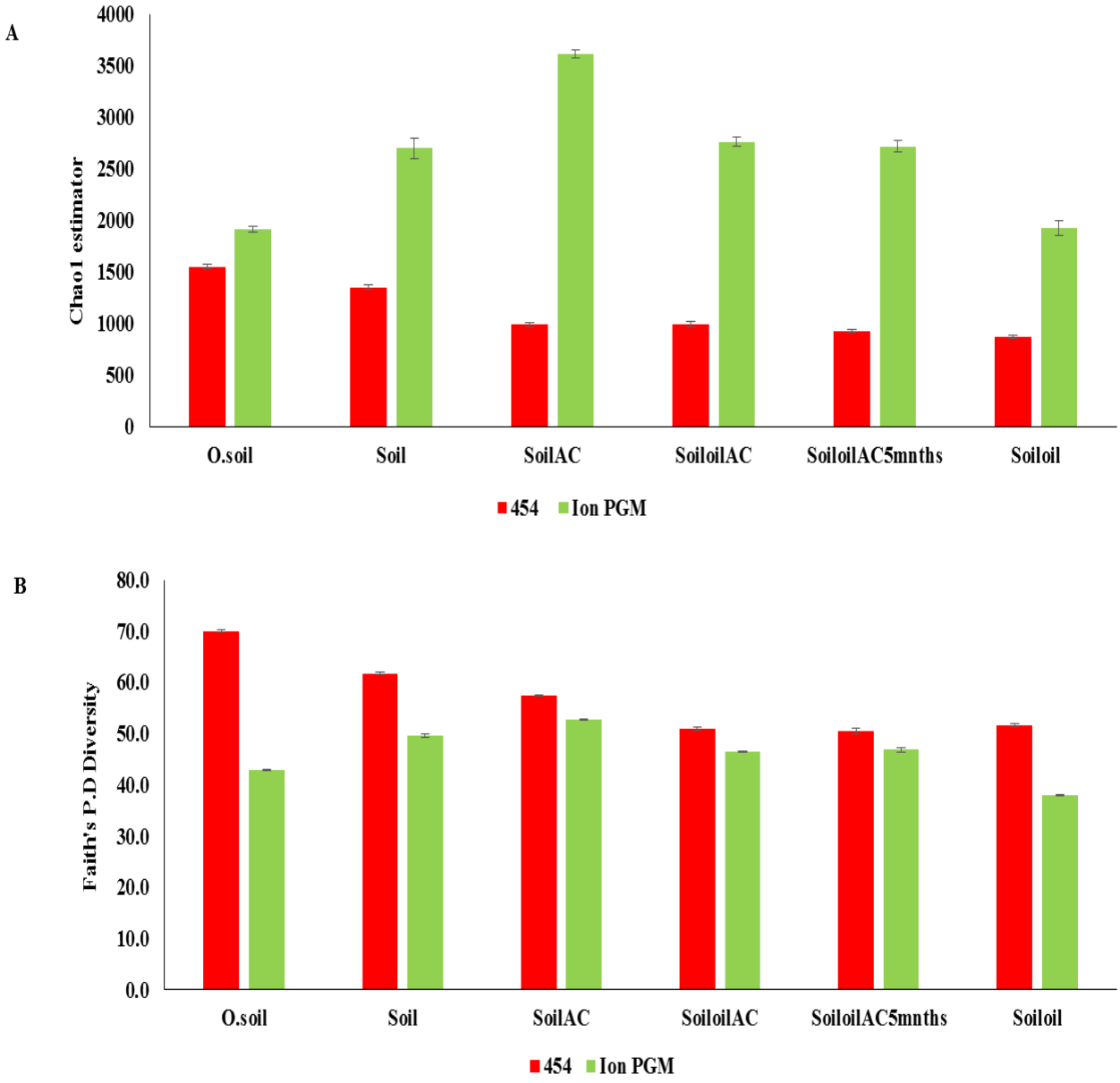

Figure 4-3 Comparing average treatment diversity at single rarefaction depth (1400) between 454 GS junior and Ion torrent sequencing libraries. Utilising Chao1 (A) and P.D_whole tree (B) estimators. Error bars representing SD from mean of three replicates

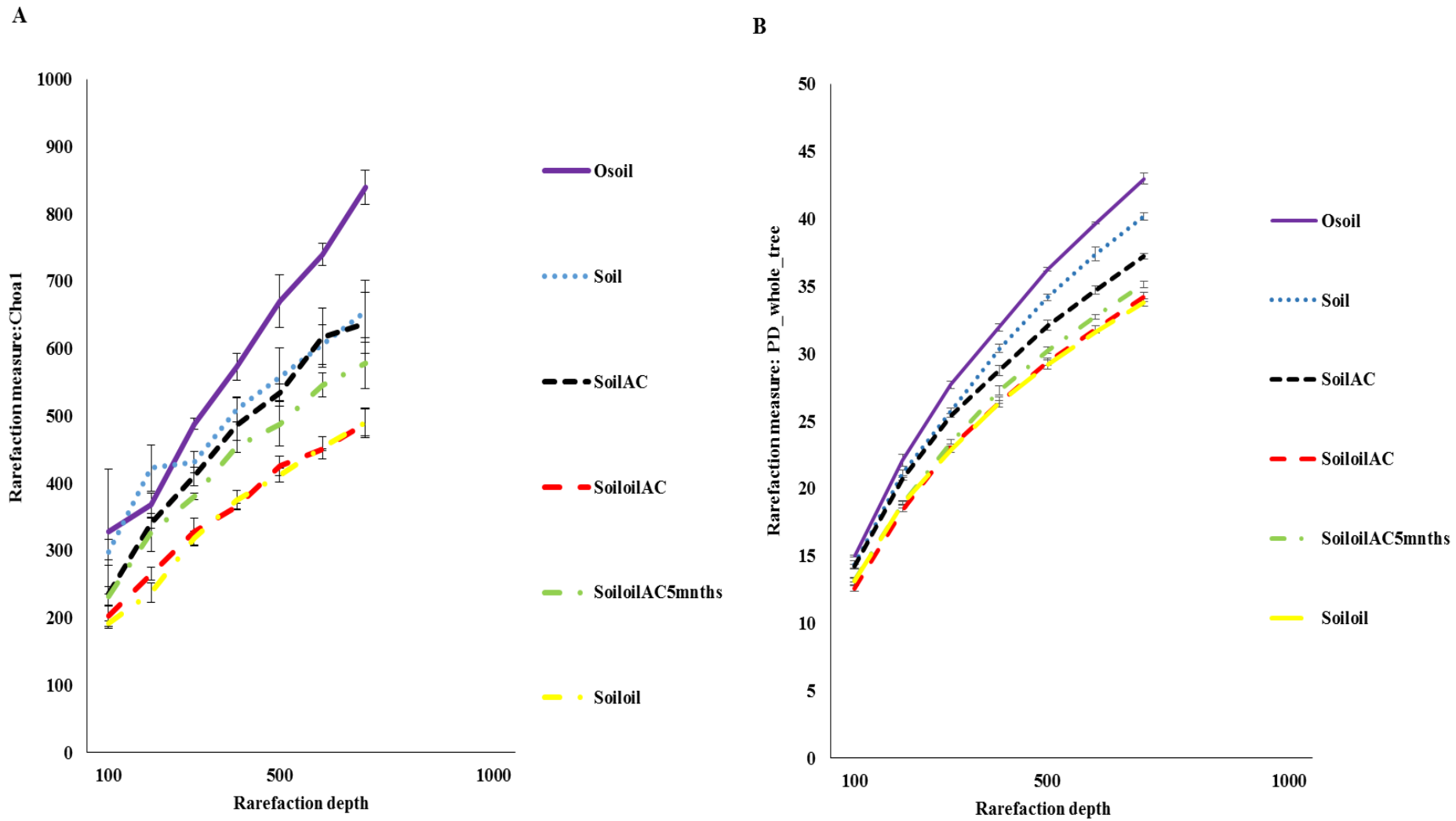

Figure 4-4 Rarefaction curves for soil treatment samples, 454 forward reads only (A) Choa1 estimator (B) PD_whole_tree estimator. Error bars represent ±1 standard deviation (SD=3) from the mean of 3 replicates

The alpha diversity for the forward reads was also observed using the same estimators Choa1 and PD_whole_tree (Figure 4-4) to see if the differences in results from the 2 platforms was as a result of the merging (concatenating) of reads. The results using forward reads showed a similar pattern to the concatenated alpha diversity results (Figure 4-1 A and Figure 4-2 A with original soil (O.soil) samples having highest values followed by Control Soil. Soil & oil & AC at time 5 months moving to 4th position was the only noticeable difference and it's seen to have slightly higher values compared to crude oil batches with AC amendment from the start and no AC.

4.4.3 Taxonomic summary compositions and distribution outputs

For the 454 GS junior run (at phylum level), within the bacteria domain there were 39 classified phyla and 1 unclassified while there were 2 classified and 1 unclassified belonging to the archaea domain. The ion torrent derived taxonomic summary results showed 43 classified and 1 unclassified phyla within the bacteria domain and 2 classified and 1 unclassified phyla within the archaea domain (See Appendix Section E tables 7.16 to 7.21 for full data set). The taxonomic summaries for the 2 sequencing platforms (at phyla level, taxonomy assigned at 50% cutoff) for the most abundant bacteria and archaea (representing ≥3.0 % of relative abundances Figure 4-5) were similar in terms of the identification of organisms per sample but they varied in the sample order of abundances. Across the various samples under study, the identifiable taxa included Actinobacteria, Bacteroidetes, Chloroflexi, Planomycetes, Firmicutes and Proteobacteria broken up in to the classes of: Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Gammaproteobacteria representing 16.7, 11.1, 7.5, 8.1, 3.0, 16.3, 7.0, 3.0, and 9.0% for ion torrent data, respectively, while 454 GS junior had 14.3, 17.2, 6.8, 10.1, 3.7, 18.5, 1.66, 2.50, 13.29% in these classes. Acidobacteria was more dominant in ion torrent data with a percentage of 4.7 (Figure 4-5 B) in comparison to 454 data with a percentage of 2.2 (not shown in Figure 4-5 A). Another highlighted difference between the phylum taxonomic summaries of the 2 runs was the higher presence (6.0 %) of Crenarchaeota belonging to the archaea domain which was much lower (1.7%) for the 454 run across all samples (not shown in Figure 4-5 A). There were differences in relative abundances between treatments. The relative abundance of Gammaproteobacteria in Soil & oil was significantly higher (t-test, 2 sided, unpaired, $p < 0.05$) in comparison to Soil & oil & AC amendment batches and the controls (Control Soil & AC, Control Soil and O.soil i.e. original soil, for the 2 sequencing platforms. Members belonging to the phylum *Gammaproteobacteria* have previously been shown to

degrade hydrocarbon compounds (Hara *et al.*, 2003b; Shahriari Moghadam *et al.*, 2014; Singh *et al.*, 2015).

Crenarchaeota, present in the Ion torrent run, was a lot higher (t-test, 2 sided, unpaired, p value <0.001) in AC amendment batches and crude oil polluted (but not if only AC was added, Control Soil & AC at time zero) as compared to Soil & oil, Control Soil and original soil (O.Soil) (Figure 4-5 B). Indicating that the organisms belonging to *Crenarchaeota* phylum benefited more from the addition of activated carbon in the presence of crude oil. The relative abundance for *Acidobacteria* and *Betaproteobacteria* (more evident for ion torrent) for both runs was higher in the controls (Control Soil, Control Soil & AC at time zero and original soil-O.soil) as compared to the crude oil polluted batches. There was no significant difference between treatment samples (t-test, 2 sided, unpaired p >0.05) for *Alphaproteobacteria* class, *Planomycetes*, *Deltaproteobacteria* and *Actinobacteria* for samples of both sequencing platforms as the relative abundances were comparable. For *Chloroflexi* there no significant difference when Soil & oil batches and AC amended crude oil batches were compared and also no significant difference when AC amended crude oil batches and controls were compared but there was significant difference (t-test, 2 sided, unpaired p <0.05) when Soil & oil batches were compared with controls batches. The crude oil only batches had a lower relative abundance in comparison to the controls for both sequencing derived data.

Noticeable differences in relative abundances patterns between the sequencing derived data were also seen for *Firmicutes* and *Bacteroidetes*, and may have arisen due to differences in error correction methods as previously explained in section 4.4.2. For the 454, *Firmicutes* had a higher relative abundance (t-test, 2 sided, unpaired p <0.05) in Soil & oil compared to AC amended batches while the reverse was the case for the ion torrent with *Firmicutes* having a slightly higher abundance (non-significant) in AC amended batches in comparison to Soil & oil. *Bacteroidetes*, also showed conflicting patterns for both sequencing runs with significantly higher relative abundances (t-test, 2 sided, unpaired p <0.05) in controls and AC amendment batches compared (Figure 4-5 A) to Soil & oil for the 454 derived data while for the ion torrent derived results (Figure 4-5 B) *Bacteroidetes*, showed a higher relative abundances in crude oil batches with or without AC in comparison to control samples.

The taxonomic summaries for the 2 sequencing platforms (at phyla level, taxonomy assigned at 80% cutoff) for the most abundant bacteria and archaea (representing ≥3.0 % of relative abundances Figure 4-6) shows similar relative abundance patterns to the results presented in

Figure 4-5 and thus further highlights that there is little difference between data obtained at 50% and 80% RDP assign taxonomy.

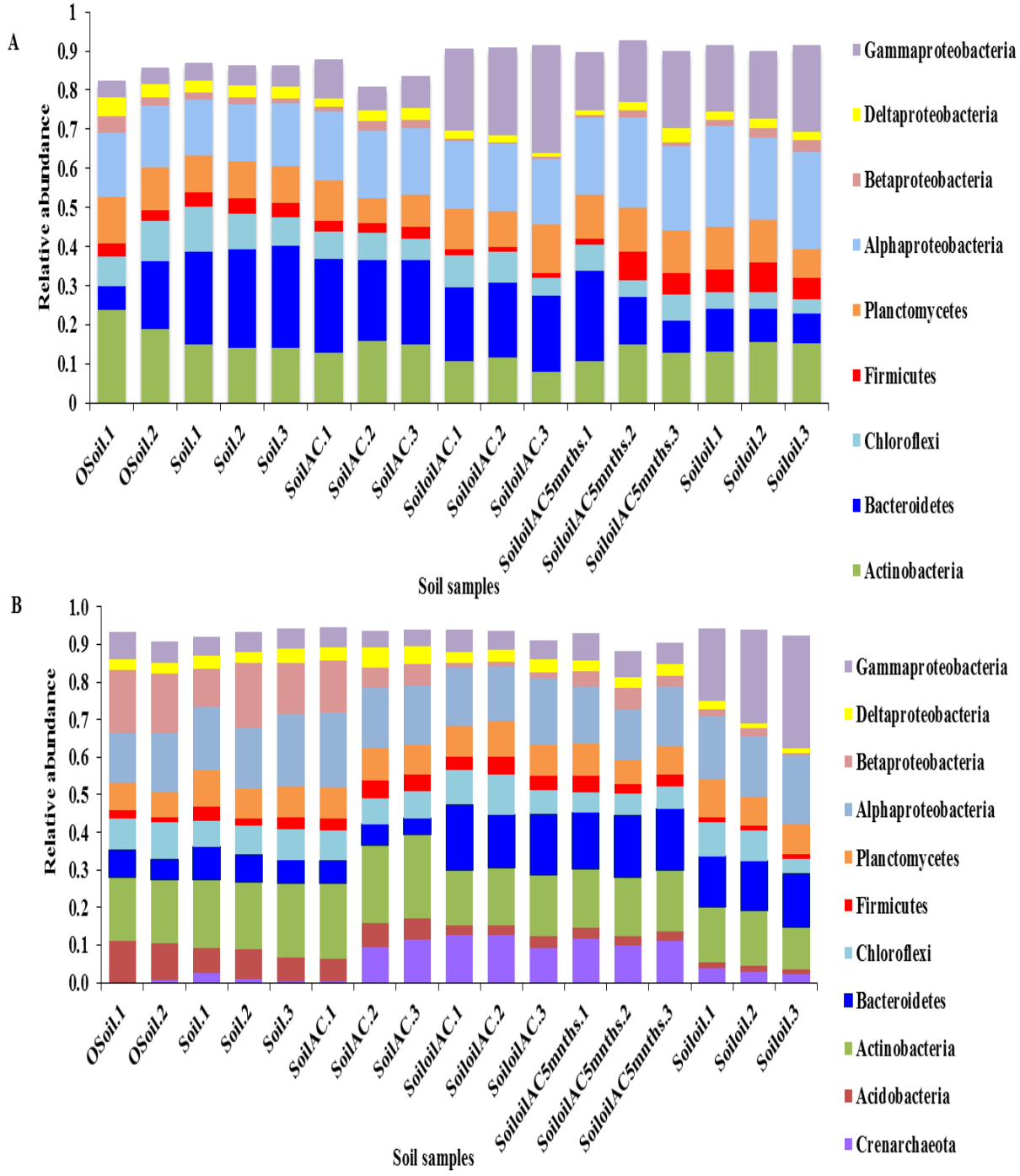

Figure 4-5. Relative abundance of phylogenetic groups in treatment soils, taxonomy assigned at 50% bootstrap cutoff using RDP classifier. Control. AC amended and AC un-amended crude oil soils. OTUs accounting for ≥ 3.0% of all classified sequences across all samples. A). 454 GS junior sequencing libraries B) Ion torrent sequencing libraries

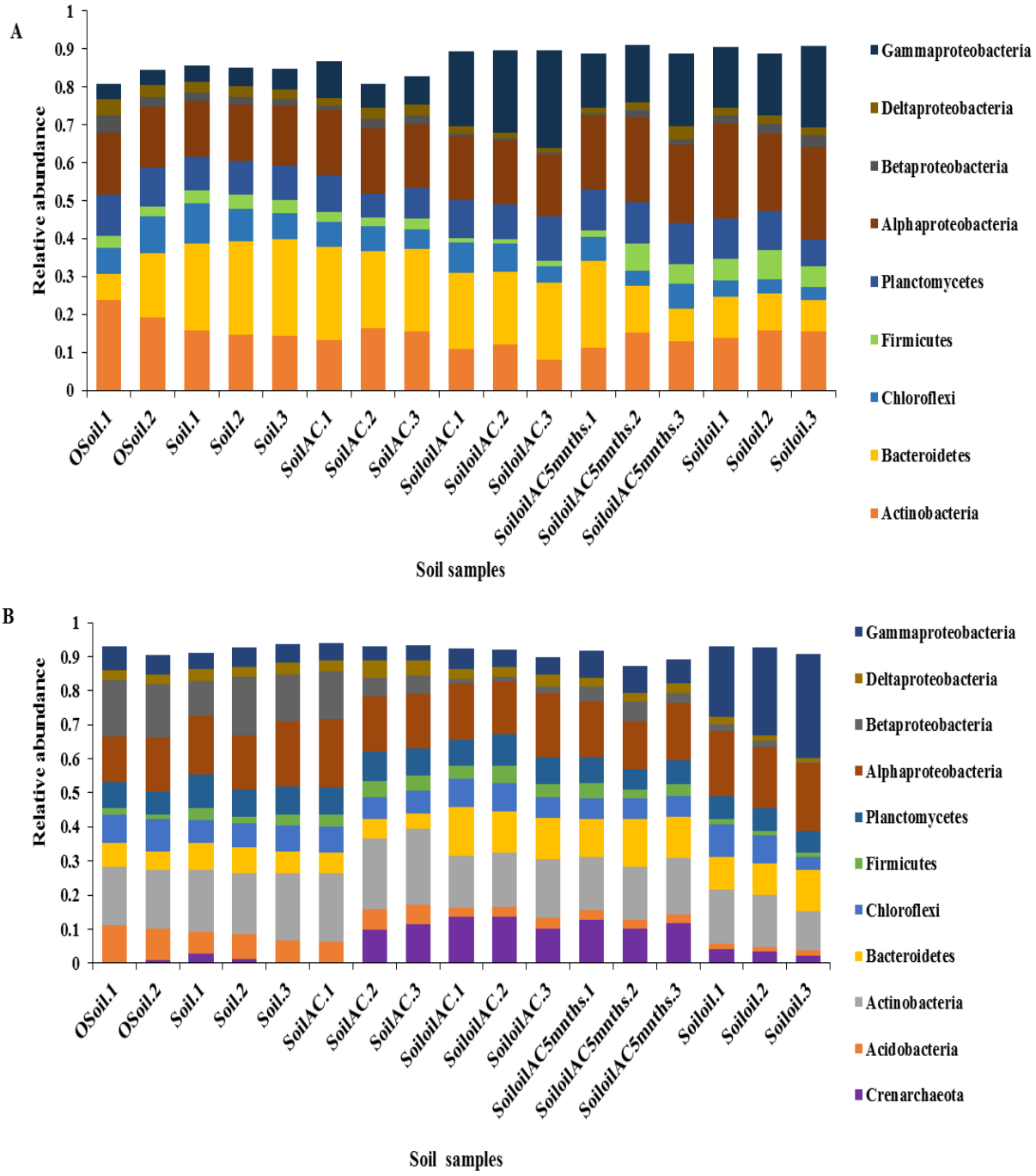

Figure 4-6. Relative abundance of phylogenetic groups in treatment soils, taxonomy assigned at 80% bootstrap cutoff using RDP classifier. Control. AC amended and AC un-amended crude oil soils. OTUs accounting for ≥ 3.0% of all classified sequences across all samples. A). 454 GS junior sequencing libraries B) Ion torrent sequencing libraries

The observation of treatment effects at order level (Figure 4-7) for 454 (Figure 4-7A) showed that most of the OTUs at belonged to the dominant phyla of *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Planomycetes*, *Firmicutes* and especially the classes of *Alphaproteobacteria*, *Gammaproteobacteria* (Figure 4-5). Generally for the treatment effect the 454 GS junior data showed that on average, *Actinomycetales, Gaiellales, Bacillales. Pirellulales, Rhizobiales, Alteromonadales* were generally higher in Soil & oil and Soil & oil & AC at time 5 months batches in comparison to Soil & oil & AC at time zero batches (The addition of AC at the beginning may have reduced their abundance) while *Actinomycetales, Bacillales, Alteromonadales* were higher in Soil & oil and Soil & oil & AC at time 5 months batches compared to controls (Control Soil and Control Soil & AC at time zero) batches. However with regards to significant differences, *Actinomycetales* which comprises of sequences for (at the family level) *Microbacteriaceae, Micrococcaceae, Mycobacteriaceae, Nocardiaceae, Nocardioidaceae, Streptomycetaceae* were significantly more abundant (t-test, 2 sided, unpaired $p< 0.05$) in Soil & oil batches in comparison to controls (Control Soil and Control Soil & AC at time zero) and Soil & oil & AC at time zero batches. Similarly, *Bacillales* was also shown to have a higher abundance (t-test, 2 sided, unpaired $p< 0.05$) in Soil & oil in comparison to Soil & oil & AC at time zero, Soil & oil & AC at time 5 months and controls. Thus indicating the addition of crude oil and nutrients alone was quite beneficial to organisms belonging to these order and AC amendment (especially from the start) impacted on their abundance. There were no significant difference between Soil & oil & AC at time zero and Soil & oil & AC at time 5 months for most of the identified OTUs at order level (apart from *Bacillales* and *Alteromonadales*). *Alteromonadales*, which comprises of some known hydrocarbon degraders, was higher in crude oil batches with or without AC in comparison to controls O.soil and Soil (ie. Soil + nutrients), while *Xanthomonadales* (also known to possess potent HC degraders), was slightly higher (Not significant t-test, 2 sided, unpaired $p > 0.05$) in Soil & oil in comparison to other treatments. *Flavobacteriales* seemed to benefit more from AC amendment and nutrient bio-stimulation with higher abundances seen in Control Soil, Control Soil &AC at time zero, Soil & oil & AC at time zero and Soil & oil & AC at time 5 months. The comparison of sequencing result outputs (454 and ion torrent) at order level OTUs showed some differences in the sample order of abundances (Figure 4-7 A & B). Ion torrent results also show the presence of *Nitrososphaerales* and *Burkholderiales* which belong to class *Thaumarchaeota* (an *Archaea*) and *Betaproteobacteria* respectively. Order *Nitrososphaerales* which comprises of a host of ammonia oxidizing organisms (Zhang et al., 2005) was highest in AC amendment soils. Compared to 454 data (Figure 4-7 A), the relatively high abundance of *Nitrososphaerales* in ion torrent results (Figure 4-7 B) seems to lead a reduction in abundance

or presence of organisms such as *Flavobacteriales* and also *Alteromonadales*, *Xanthomonadales* and *Actinomycetales* (which comprise of known hydrocarbon degraders) within Soil & oil & AC at time zero and Soil & oil & AC at time 5 months. Thus leading to similar community abundance profiles within those samples. AC and crude oil proved detrimental to the *Burkholderiales* with significant (t-test, 2 sided, unpaired $p < 0.05$) lower abundances in AC amended or crude oil batches compared to controls O.soil and Control Soil. Order *Gaiellales* seems to have a low abundance in Soil & oil batches for both sequencing runs (Figure 4-7) and similar to 454 GS junior results relative abundances of *Xanthomonadales, Alteromonadales* and *Actinomycetales* was high in Soil & oil batches (Figure 4-7B)

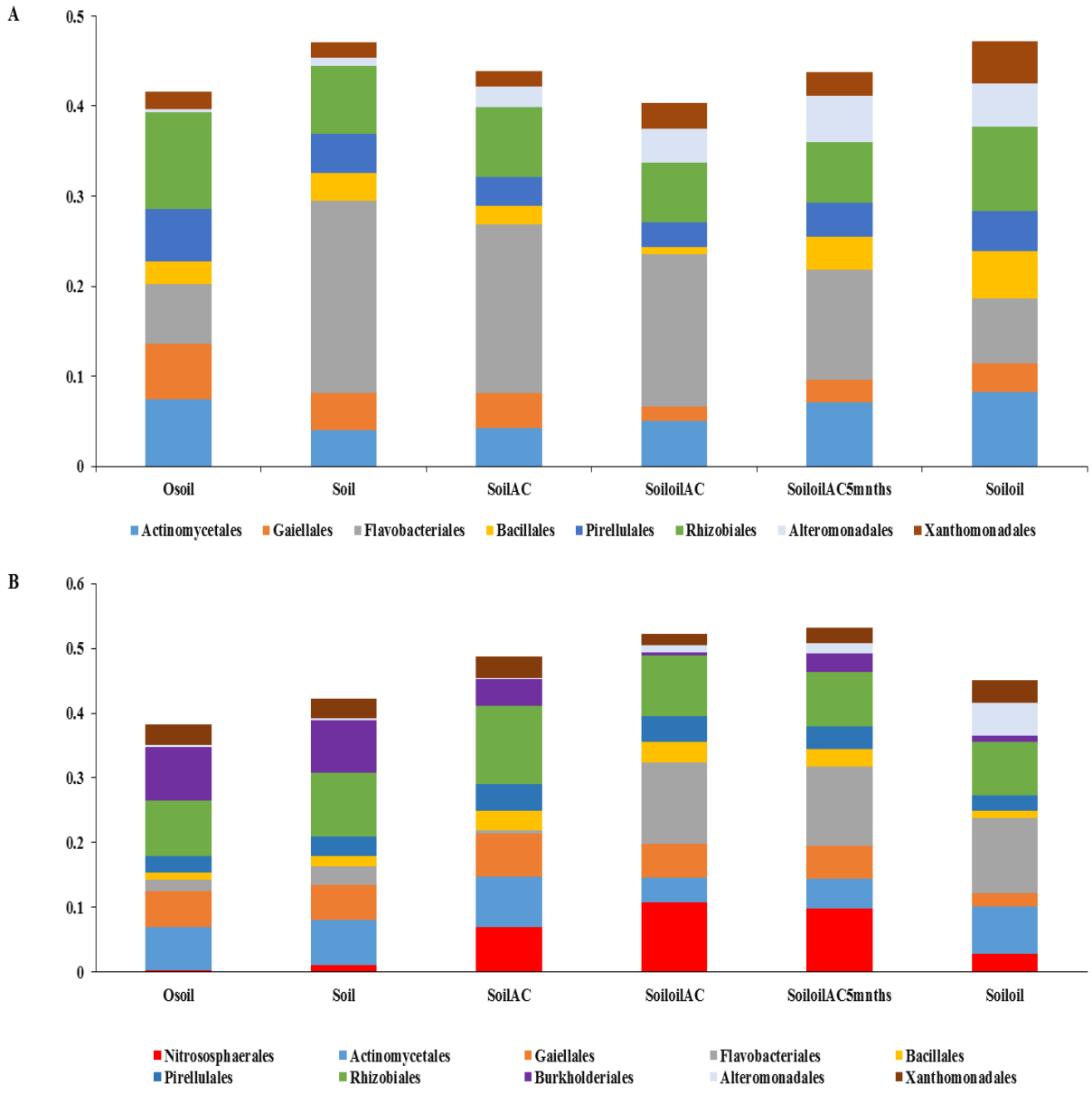

Figure 4-7 Average relative abundance of replicate treatment soils. Order level accounting for >2.0% off all classified sequences (A: 454, B: PGM ion torrent)

4.4.4 Identification of OTU functions in crude oil spiked and sorbent amended soils

The sources cited below which show the detection of hydrocarbon degrading organisms similar to those detected in this study, were taken from a range of studies carried out using samples in diverse environs.

The overall relative abundance at genus level for the 454 GS junior[a] platform showed that there were some differences for OTUs that could potentially degrade crude oil when Soil & oil & AC at time 5 months and Soil & oil were compared to controls (Soil and Soil & AC at time zero) (Table *4-3*) Some these OTUs include *Parvibaculum, Marinobacter, Salinibacterium, Arenibacter, Luteimonas,* Alcanivorax and *Rhodococcus* .The higher presence or relative abundance of these genera in Soil & oil and Soil & oil & AC at time 5 months was probably induced by the presence of crude oil and nutrients and the addition of AC after 5 months didn't significantly affect the competitiveness of bacteria belonging to these genera. The result also correlates with higher carbon dioxide production in batches Soil & oil & AC at time 5 months and Soil & oil (See Chapter 3 Figure 3-3) as result of higher crude oil degradation. *Parvibaculum,* an *Alphaproteobacteria,* have been previously detected in a microcosm study which attempted to reveal microbial diversity in PAH polluted soils (Sipilä *et al.*, 2008). One of the species belonging to *Parvibaculum, P Hydrocarbonoclasticum* (*Parvibaculum Hydrocarbonoclasticum.)* has been depicted as an alkanotroph (ability to degrade and utilise alkanes) and in one study was able thrive on and utilise n-alkanes by encoding and producing an oxidising cytochrome P450 enzyme to catalyse the oxidation of n-alkanes (Austin *et al.*, 2013). In another study, which investigated the effect of the deep-water horizon spill on microbes in sediment and water samples, the 454 pyrosequencing results showed a very high abundance of *Parvibaculum* in contaminated sediment samples and the authors suggested that *Parvibaculum species* possess hydrocarbon degrading abilities using both alkanes and PAHs as carbon and energy sources (Looper *et al.*, 2013).

The genus *Luteimonas* belonging to the class *Gammaproteobacteria,* has been previously linked to the specific degradation or mineralization of and Benzo (a) pyrene (Jones *et al.*, 2014a), while *Luteimonas Mephitis* which was detected at species level in this study with a significantly higher abundance (t-test, 2 sided, unpaired $p < 0.05$) in Soil & oil & AC at time 5 months and Soil & oil in comparison to other batches (Figure 4-8 B) has also been previously detected in land polluted by PAHs (Bacosa and Inoue, 2015). *Salinibacterium is an Actinobacteria* and has been previously shown to utilise PAHs such as phenanthrene and pyrene (Isaac *et al.*, 2013). *Marinobacter* belongs to class *Gammaproteobacteria,* and is a gram negative rod shaped

bacterium (Shahriari Moghadam *et al.*, 2014) and is involved in the degradation of a wide range hydrocarbon compounds which includes crude oil (Shahriari Moghadam *et al.*, 2014) and naphthalene (Hedlund *et al.*, 2001). *Rhodococcus* is a gram positive, rod shaped *Actinobacteria* which has been reported to degrade crude oil compounds efficiently in the presence of nutrients (Sharma and Pant, 2001). Some of the compounds *Rhodococcus* degrades that are found in crude oil include a range of saturated hydrocarbons (Hamamura *et al.*, 2006b; de Carvalho, 2012) and PAHs such as naphthalene (Alquati *et al.*, 2005). The simultaneous degradation of different PAHs compounds by *Rhodococcus species* has been reported to occur via the Meta cleavage and ortho pathways (Dean-Ross *et al.*, 2002). The 454 GS junior [a] results in this study highlighted some negative effects of AC amendment on potential degraders such as *Salinibacterium* and *Muricauda*, with significant higher relative abundances (t-test, 2 sided, unpaired $p < 0.05$) in Soil & oil and Soil & oil & AC at time 5 months compared Soil & oil & AC at time zero (Table 4-3). In a laboratory study conducted by Jiménez *et al.* (2011) to detect hydrocarbon potential degraders of crude oil in marine samples, *Muricauda* species were able to grow on aromatic and aliphatic compounds and were also able to transform PAHs. There was also a significant difference (t-test, 2 sided, unpaired $p < 0.05$) for relative abundances of *Marinobacter* between Soil & oil & AC at time 5 months and Soil & oil & AC at time zero with the former having significantly higher abundance. Further comparing the effects of AC amendment at the genus level, another genera that possesses hydrocarbon-degrading abilities is *Alcanivorax. Alcanivorax* is a *Gammaproteobacteria*, and, according to the translation of its Latin name, is a voracious alkane degrader (both straight and branched chain) (Hara *et al.*, 2003b; Hara *et al.*, 2003a). *Alcanivorax* was detected at slightly higher relative abundances (t-test, 2 sided, unpaired $p > 0.05$) in Soil & oil and Soil & oil & AC at time 5 months compared to Soil & oil & AC at time zero (Table 4-3), thus indicating that the addition of AC from the start was slightly reducing the presence of this hydrocarbon degrader. One study showed high levels of *Alcanivorax species* in the presence of crude oil and nutrients (Singh *et al.*, 2015), however it must be noted that *Alcanivorax species* are only predominant at initial or early stages of crude oil pollution (kasai *et al.*, 2005; Jiménez *et al.*, 2011). One member of the genera, *Alcanivorax, A venustensis* was also detected at species level for both concatenated and forward reads (only) (Figure 4-8 A) and was shown to have a slightly higher relative abundance (t-test, 2 sided, unpaired $p > 0.05$) in Soil & oil in comparison to the other treatments. The insignificant difference between Soil & oil and other treatments (see error bars) was due to relative abundance differences between replicates of the same sample (Figure 4-8 A). *A venustensis* has been previously shown to utilise alkane fractions such as hexadecane and tetradecane (Fernández-Martínez *et al.*, 2003). The higher abundance of alkane degraders such as

Marinobacter, Parvibaculum, Alcanivorax in Soil & oil and Soil & oil & AC at time 5 months batches also correlates with the higher removal of alkanes seen from the results of residual soil concentrations (see chapter 3 Figure 3-5)

For the ion torrent data [b] all the genera mentioned (Table 4-3) were detected, however the relative abundances were lower compared to 454. For ion torrent sequencing only *Marinobacter* and *Arenibacte*r were significantly higher in crude oil treatment batches (with or without AC) compared to the controls. *A venustensis* detected in 454 was not identified at species level in ion torrent data and a much lower relative abundance was detected across all samples for *Luteimonas Mephitis* in ion torrent data compared to 454 (Figure 4-8 B)

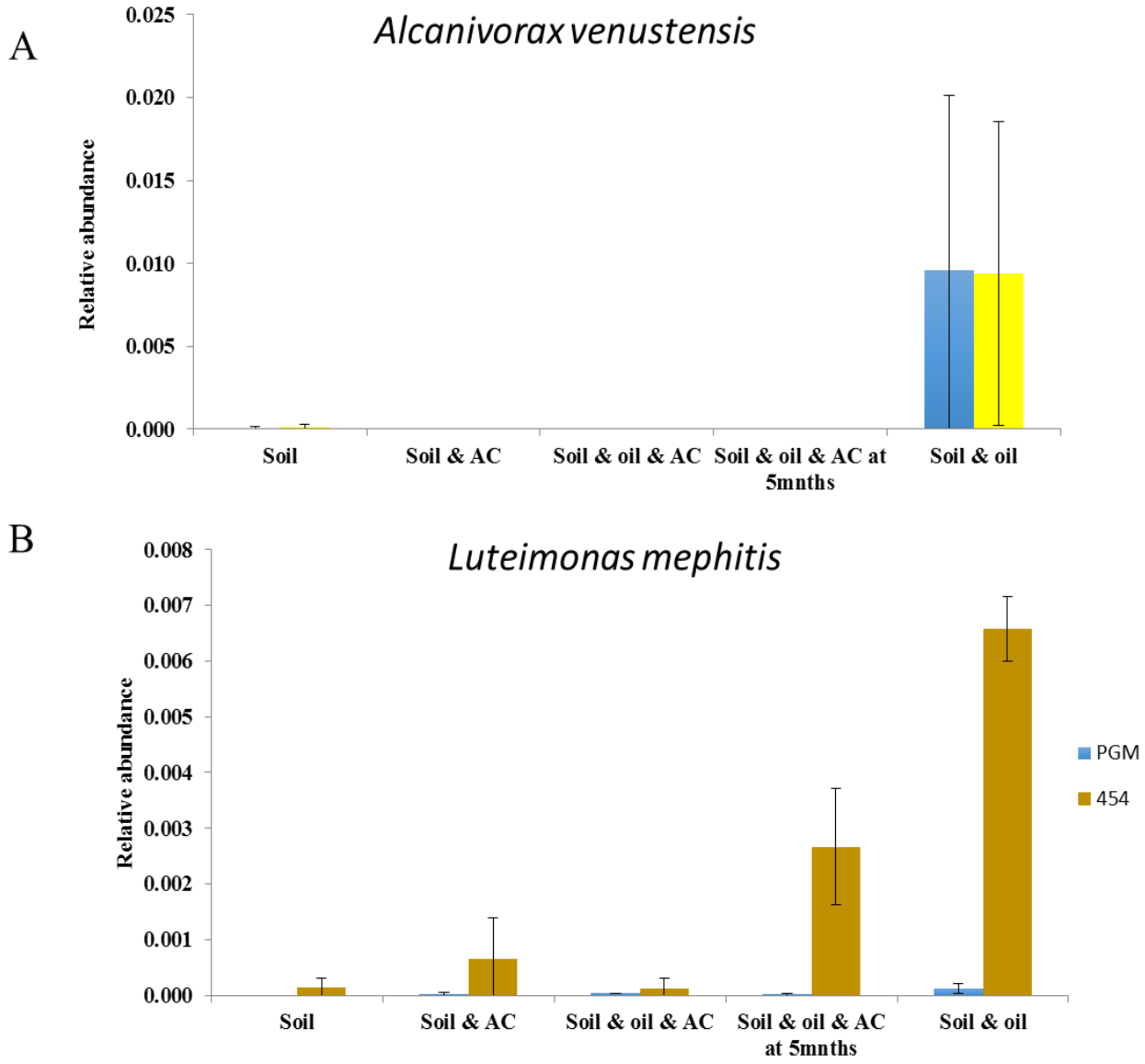

Figure 4-8 Average relative abundance of hydrocarbon degrading Species detected in crude oil batch samples. A) A. venustensis concatenated reads (blue) and forward reads only (yellow) from 454 GS junior B) L. mephitis detected from 454 GS junior and PGM Ion torrent sequencing

It is surprising that the sequencing results for the soil batch samples showed the presence of microorganisms which are predominantly found in marine environments such *Marinobacter and Alcanivorax* (Hara et al., 2003a; Shahriari Moghadam et al., 2014). As stated in chapter 3 section 3.3.1, the soil was obtained from the exhibition park Newcastle city, during the park redevelopment. Newcastle upon Tyne is close to Tyneside and Northumberland coastlines and the presence these marine organism could possibly be due to a wider dispersion of these microorganisms. It believed that microbes disperse freely as they do not undergo geographic isolation or speciation (Papke *et al.*, 2003). There is a common assumption or theory which has been in place from early 20th century regarding the existence and dispersal of microorganisms, 'everything is everywhere but the environment selects'(Baas-Becking, 1934; De Wit and Bouvier, 2006) and is based on the principle that species of microbes will be everywhere but the environment decides if the microbes thrive (O'Malley, 2007). The soil from the exhibition park was enriched with nutrients and crude oil creating an environment for these marine organisms to thrive or grow (See Figure 4-8 A, Soil and Soil & oil batches both containing nutrients but no AC). Recent reports have shown that it is not impossible to detect high abundance of these marine organisms in hydrocarbon polluted soils. In a recent study by Sheng *et al.* (2016), soil samples collected in field close to crude oil wells showed a high abundance of *Alcanivorax* within hydrocarbon polluted soil samples.

The increase or decrease in rank abundance due to varying treatment effects was determined at species level relative to the original soil (O.soil i.e. untreated soil) OTUs ranked abundance (Table 4-4 and Table 4-5). The higher the ranking of OTUs in a particular soil treatment indicates better acclimatisation to the treatment conditions, i.e. a gain in competitiveness as compared to the other species originally present in soil. In Soil & oil and Soil & oil & AC at time 5 months (454 GS junior data Table 4-4), there was an increase in OTU ranking by 4 folds and 16 respectively as compared to the original soil microbial community for *Arthrobacter psychrolactophilus. Arthrobacter psychrolactophilus* hasn't been previously detected in hydrocarbon polluted soils but previous 16srRNA sequence analysis showed that the organism has sequence similarities with *Arthrobacter oxydans* (94.4 %, which is related to other *Arthrobacter* hydrocarbon degraders) and *Arthrobacter* polychromogenes (94.3%) (Loveland-Curtze *et al.*, 1999). The addition of crude oil only or crude oil and AC after 5 months seems a more favorable condition for *Arthrobacter psychrolactophilus*, as there was no increase OTU ranking in Soil & oil & AC at time zero, Control Soil & AC at time zero and Control Soil (Table 4-4).

The addition of crude oil only or crude oil and AC seemed a more favorable condition for *Rhodococcus equi* with an 8 fold increase for both Soil & oil and Soil & oil & AC at time 5 months respectively and a 4 fold increase in Soil & oil & AC at time zero (Table 4-4). In comparison to the initial abundance in the O.soil sample. No increase in *Rhodococcus equi* relative abundance was recorded in Control Soil & AC and Control Soil. *Rhodococcus equi* has previously been shown to grow on and remove (uptake) both alkanes (Bouchez-Naïtali *et al.*, 2001) and PAHs (Fijałkowska *et al.*, 1998). *Rhodococcus* possesses a hydrophobic cell wall enabling it to accumulate hydrocarbons from the environment, which may be particularly beneficial in AC amended soils (Van Hamme and Ward, 2001; De Carvalho *et al.*, 2014). *A venustensis* an alkane degrader (Fernández-Martínez *et al.*, 2003) showed ranked increase of up to 8 fold and 32 fold in Soil & oil & AC at time 5 months and Soil & oil respectively but only a 2 fold increase was detected for Control Soil & AC at time zero and Soil & oil & AC at time zero in comparison to initial abundance in the O.soil sample. *Altererythrobacter epoxidivorans* a gram negative organism normally found in marine sediments (Li *et al.*, 2016), also showed 4 and 8 fold increase in Soil & Oil & AC at 5 months and Soil & Oil respectively and only a 2 fold increase in the other treatment batches. *A. epoxidivorans* has been reported to utilize benzo (a) pyrene as a main source of carbon and energy by encoding for 22 enzymes (15 to oxidize the aromatic ring and 2 responsible for re-aromatization) which carry out the degradation (Li *et al.*, 2016). Other species which have shown increase in ranked abundance but haven't been previously implicated in hydrocarbon degradation include *Muricola jejuensis. Muricola jejuensis is* a marine organism which was first isolated in Korea and known to degrade carbohydrate, lipid and gelatinous compounds (Kahng *et al.*, 2010). The results showed a dramatic *Muricola jejuensis* ranked abundance increase of 32 fold and 128 fold in Soil & oil & AC at time 5 months and Soil & oil respectively. Class *Alphaproteobacteria* that contains some key players involved in hydrocarbon degradation such *Rhodobacteraceae (Kostka et al., 2011)* has an O.soil ranking of 105 but increases by 32 fold in Soil & oil & AC at time zero and up 64 fold in both Soil & oil & AC at time 5 months and Soil & oil. There were also ranked abundance increases for *Oceanicella actignis* (an *Alphaproteobacteria* 4 and 8 fold) and *Marinobacter bryozoorum* (16 fold) in Soil & oil & AC at time 5 months and Soil & oil respectively while an increase by only 2 and 4 fold were detected for Soil & oil & AC at time zero. Unidentified species within *Flavobacterium, Skermanella,* increased in ranked abundance in Soil & oil & AC at time 5 months and Soil & oil compared to other treatments while unidentified organisms belonging to the family of *Hahellaceae,* increased in ranked abundance (16 fold) in Soil & Oil compared to other treatments. *Hahellaceae* is known to contain organisms which are involved in the degradation of aromatic compounds (Pieper, 2009). Genus *Pedomicrobium, which*

belongs to family hypomicrobiaceae, seems to benefit from the addition of AC and crude oil, but increases significantly (32 fold) with the addition of just nutrients and crude oil (Soil & oil). *Pedomicrobium* has previously been detected microcosm hydrocarbon polluted soils and has been suggested to posses hydrocarbonoclastic abilities (de la Cueva *et al.,* 2016). The findings generally correspond with the higher carbon dioxide production in Chapter 3 Figure 3-3 and greater alkane removal (chapter 3 Figure 3-5) in Soil & Oil & AC at 5 months and Soil & Oil batches and basically shows that the hydrocarbon degrading organisms reacted more positively to addition of crude oil with no AC or AC amendment after 5 months when Soil & Oil & AC at 5 months and Soil & Oil are compared to Soil & Oil & AC and controls. However genus *Owenweeksia* and species *Nocardia concava* which have not been previously linked with hydrocarbon degradation increase by 16 fold and 8 fold respectively in Soil & oil & AC at time zero.

The ranked abundance result for Ion torrent (Table 4-5) gives a slightly different picture. *Candidatus Nitrososphaera_SCA1145,* which has a ranked abundance of 525 in, O.soil increases up 32 fold in Soil & oil batches and more than 64 fold in AC amended batches. *Candidatus Nitrososphaera* are known ammonia oxidizing bacteria (Jiang *et al.*, 2010) and would benefit from the addition of nutrients substrates. *Aeromicrobium* which belongs to family *Nocardioidaceae* and has been previously linked with the degradation of hydrocarbon compounds (Chaillan *et al.,* 2004) seems to makes more gains with the addition of crude oil (Soil & oil) with no AC amendment. *Muricola jejuensis* ranked abundance in O.soil is lower for ion torrent compared to 454 GS junior results (Table 4-4). Similar to the 454 GS junior results (Table 4-4), *Muricola jejuensis* ranked abundance also dramatically increases in Soil & oil & AC at time 5 months and Soil & oil but increases in Soil & oil & AC at time zero was also recorded which was not seen in 454 GS junior results. Also *Marinobacter bryozoorum* which belongs to a genera containing hydrocarbon degrading species has a much lower ranked abundance in O.soil (625) for ion torrent results (Table 4-5) compared to 454 GS junior results (107) (Table 4-4). *Marinobacter bryozoorum* only increases by 8 fold in Soil & oil batches and 2 fold in Soil & oil & AC at time zero with no increase recorded in Soil & oil & AC at time 5 months. Class *Alphaproteobacteria* increased by 4 fold in Soil & oil & AC at time zero and Soil & oil & AC at time 5 months but increased by 64 fold in Soil & oil batches. The ion torrent results however showed a dramatic *Nocardia, Rhodococcus, Arenibacter* and *Parvibaculum* (known hydrocarbon degraders) (Alquati *et al.*, 2005; Gutierrez *et al.,* 2014) ranked abundance increase in Soil & oil batches but not in AC amended batches. Other genera such as

Microbulbifer, Aequorivita, Fluviicola and specie Rhodoplane elegans seem to benefit from the addition of crude oil or crude oil plus AC

Table 4-3. Some potential hydrocarbon degraders detected from amplicon sequence libraries. [a] denotes 454 GS junior while [b] PGM ion torrent

Taxon level: Genus	Average OTU relative abundances					
	Soil (control 1)	SoilAC (control 2)	SoiloilAC	SoiloilAC 5 months	Soiloil	Previous reported HC degradation activities
Luteimonas	3.14E-03 [a]	3.66E-03 [a]	8.26E-03 [a]	1.15E-02 [a]	1.97E-02 [a]	Benzo (a) pyrene(Jones *et al.*) Luteimonas Mephitis detected in PAH mixtures(Bacosa and Inoue)
Marinobacter	3.99E-03 [a] 8.71E-06 [b]	7.66E-03 [a] 6.67E-05 [b]	8.12E-03 [a] 3.26E-03 [b]	2.44E-02 [a] 3.07E-03 [b]	1.87E-02 [a] 8.99E-03 [b]	Crude oil (Shahriari Moghadam *et al.*). Naphthalene(Hedlund *et al.*), Flouranthene, chrysene, benzo (a) anthracene (Vila *et al.*). Aliphatic HC hexane, pristine, eicosane (Brito *et al.*). Hexadecane (Fernández-Martínez *et al.*; Gerdes *et al.*), Utilization of tetradecane (Fernández-Martínez *et al.*)
Parvibaculum	9.50E-05 [a] 0.00E-00 [b]	1.82E-03 [a] 1.21E-04 [b]	7.47E-03 [a] 2.25E-04 [b]	1.19E-02 [a] 1.42E-05 [b]	1.95E-02 [a] 7.17E-03 [b]	Alkanes (Austin *et al.*) and PAHs (Sipilä *et al.*; Lai *et al.*)
Rhodococcus	1.31E-03 [a] 7.71E-04 [b]	3.01E-03 [a] 1.07E-03 [b]	1.04E-02 [a] 7.12E-04 [b]	2.42E-02 [a] 1.31E-03 [b]	1.62E-02 [a] 1.75E-02 [b]	Crude oil fractions- aliphatic and aromatic (Sharma and Pant). Efficient removal of naphthalene (Alquati *et al.*). Hexadecane (Hamamura *et al.*) Fluoranthene and pyrene (Dean-Ross *et al.*)
Salinibacterium	7.34E-04 [a]	3.05E-04 [a]	7.52E-04 [a]	5.77E-03 [a]	1.62E-02 [a]	Utilize phenathrene and pyrene under aerobic conditions (Isaac *et al.*)
Muricauda	0.00E+00 [a]	1.17E-03 [a]	0.00E+00 [a]	1.56E-02 [a]	2.43E-02 [a]	Naphthalene, phenanthrene, dibenzothiophenes and carbazoles (Jiménez *et al.*)
Arenibacter	5.39E-04 [a] 0.00E-00 [b]	3.39E-03 [a] 0.00E-00 [b]	2.75E-03 [a] 2.06E-04 [b]	1.33E-02 [a] 4.04E-05 [b]	8.37E-03 [a] 2.88E-03 [b]	Utilization naphthalene over phenanthrene and emulsification of n-tetradecane and crude oil(Gutierrez *et al.*)
Alcanivorax	7.75E-05 [a]	1.60E-04 [a]	1.12E-04 [a]	2.79E-03 [a]	2.12E-02 [a]	Aliphatic hydrocarbons (branched and unbranched alkanes) (Hara *et al.*). Alcanivorax venustensis utilized- Hexadecane and tetradecane (Fernández-Martínez *et al.*).

Table 4-4 Showing taxon increase or decrease in ranked relative abundance when compared to the untreated soil.454 GS junior sequencing data

Taxon (Highest level classification)	Relative abundance rank increase for different treatments compared to the original soil.+ 2-fold, ++ 4-fold, +++ 8-fold, ++++ 16-fold, +++++ 32-fold, ++++++ 64-fold or greater (and vice versa for reduction (minus -)					
	O.Soil	Soil	SoilAC	SoiloilAC	SoiloilAC 5 months	Soiloil
s__Arthrobacter psychrolactophilus	129		-		++	++++
s__Rhodococcus equi	99			++	+++	+++
s__Nocardia concava	219	-		++++	++	+
g__Owenweeksia	400	++	++	+++	+	+
f__Flavobacteriaceae	174	++	++++	++++	+++++	++
g__Flavobacterium	337	+	++	++	++++	+++
s__Muricola jejuensis	476		++		+++++	+++++++
c__Chloroplast	476		+	++	+	++++
c__Alphaproteobacteria	105	+	+++	+++++	+++++++	++++++
f__Rhizobiaceae	337	+	+	+	++	+++
s__Oceanicella actignis	272		+	+	++	+++
g__Inquilinus	476	+	+	+++	+++	+++
g__Skermanella	476				+	+++
s__Altererythrobacter epoxidivorans	138	+	+	+	++	+++
s__Marinobacter bryozoorum	107	+	++	++	++++	++++
s__Alcanivorax venustensis	476		+	+	+++	+++++
f__Hahellaceae	476					++++
g__Acinetobacter	69	-	++	++++++	+++++	++++++
g__Luteimonas	175		-	++	++	+++
g_Pedomicrobium	202	-	+	+++	+++	+++++

Table 4-5 Showing taxon increase or decrease in ranked relative abundance when compared to the untreated soil. Ion torrent sequencing data

Taxon (Highest level classification)	Relative abundance rank increase for different treatments compared to the original soil.+ 2-fold, ++ 4-fold, +++ 8-fold, ++++ 16-fold, +++++ 32-fold, ++++++ 64-fold or greater (and vice versa for reduction (minus -)					
	O.Soil	Soil	SoilAC	SoiloilAC	SoiloilAC 5 months	Soiloil
s__Candidatus Nitrososphaera_SCA1145	525	++++	+++++++	+++++++	+++++++	+++++
s__Candidatus Nitrososphaera_SCA1170	73		++	+++	++	+
g__Iamia	247	++	+	+	+	+++
g__Nocardia	625	-	+	+	+	++++++
g__Rhodococcus	475	+	++	+	++	++++++
g__Aeromicrobium	625			+	++	+++
g__Fluviicola	625	++		+++	+++	++++
g__Aequorivita	625		-	++	+++	++++
g__Arenibacter	625	-	-	+		++++
s__Muricola_jejuensis	504			+++++	++++++	++++++
s__Sediminibacter_furfurosus	417	+++++	-	+++++++++	++++++	+++++++
g__Nitrospira	14		-	----	--	----
f__Phycisphaeraceae	625	-		+++++	++++	+++++
c__Alphaproteobacteria	108	+	-	++	++	++++++
g__Parvibaculum	339	--			-	++++
s__Rhodoplane_elegans	625		+	++	++++	+++
s__Constrictibacter_antarcticus	625	-	-			+++
g__Novosphingobium	20		-	----	----	----
g__Marinobacter	625			++++	+++	+++++
s__Marinobacter_bryozoorum	625	-	-	+		++
g__Microbulbifer	625	-		++++	+++++	++++

4.4.5 Bacteria community structure analysis with respect to treatment effects

The effect of treatments on the overall community structure was further assessed via the Bray Curtis similarity analysis (Figure 4-9 and Figure 4-10). For the Bray Curtis similarity, the closeness of data points reveals the similarity between samples and bacterial communities for those samples. For the effects of crude oil on the microbial communities with the ion torrent platform, the NMDS plot (Figure 4-9 A) shows clustering of the replicates of each sample that contain crude oil (Figure 4-9 A). The communities for all samples had a percentage similarity of 60% while the communities with crude oil samples (with and without AC) clustered together at percentage similarity of 75% (Figure 4-9 A). The microbial communities within control samples, O.soil, Control Soil and Control Soil & AC also had a comparable percentage similarity of 75% (Figure 4-9 A). The communities within Soil & oil replicates clustered at 85% and the same significant levels of clustering was also seen for communities between replicate samples of Soil & oil & AC at time zero and Soil & oil & AC at time 5 months. The clear demarcation between control samples and crude oil spiked samples signify that crude oil was an important factor in determining community structure and has a major influence in shaping the microbial communities within batch samples. The results (Figure 4-10 A) show that AC amendment was not as significant as crude oil in shaping the microbial communities as Control Soil & AC at time zero had a percentage similarity of 75% with original soil (Osoil) and Control Soil. This is because AC is a stable form of carbon not readily utilized by microorganisms. The results (Figure 4-10 A) also show that AC amendment times had very little effect on the microbial communities in crude oil batches as there was no clear demarcation in clustering between replicates of crude oil batches with AC amendment either at time zero or at 5 months. To support the results above, a dendogram for clustering similarity was generated (Figure 4-11) and also shows the individual replicate samples of crude oil batches for Ion torrent (Figure 4-11 A) clustering well together compared to control samples without crude oil.

For the 454 GS junior platform (Figure 4-9 B), the crude oil addition was also more effective in shaping the microbial communities within samples in comparison AC amendment. The communities for all samples had a percentage similarity of 60% but the results between the 2 platforms differed slightly as the ion torrent data showed clearer clustering according to treatments. The communities within crude oil sample replicates Soil & oil clustered at similarity of 80% with 2 replicates of Soil & oil & AC at time 5 months while replicates Soil & oil & AC at time zero also clustered at percentage similarity of 80% with 1 replicates of Soil & Oil & AC at 5 months (Figure 4-9 B). The controls Soil & AC at time zero, O.soil and Soil clustered together at a similarity of 80% with the exception of one replicate from O.soil and one replicate

from Soil &AC at time zero. The dendogram (Figure 4-11 B) for clustering similarity was also showed Soil & oil samples clustering together with 2 replicates of Soil & oil & AC at time 5 months while Soil & oil & AC at time zero samples clustered with 1 replicate of Soil & oil & AC at time 5 months and Control Soil & AC at time zero.

An ANOSIM (analysis of similarities, based on Bray Curtis similarity coefficient) was carried out on Primer V6 software (Primer-E Ltd, Plymouth, UK) for the sequencing data to determine the significance of factors crude oil, AC and nutrients addition. The results for the ion torrent and 454 generated data both confirmed that crude oil as a factor had a significant effect on the microbial community structure with global R= 0.906, $p < 0.05$ and R= 0.646, $p < 0.05$ respectively. The results also confirmed AC wasn't a significant factor in shaping the microbial communities with global R= 0.079, p = 0.19 and R= 0.145, p =0.085 Ion torrent and 454 GS junior respectively. On the other hand the nested ANOSIM (Appendix B) looking at AC amendment as a factor within crude oil amended batches confirmed that AC amendment had a significant effect on the microbial communities within crude oil amended batches for both platforms (454 GS junior global R= 0.542, p = 0.003, Ion torrent global R= 0.590, p = 0.002). The nutrients was shown to have a much more significant effect in shaping the communities with global R= 0.405., p =0.022 and R= 0.505, p =0.015 Ion torrent and 454 GS junior respectively.

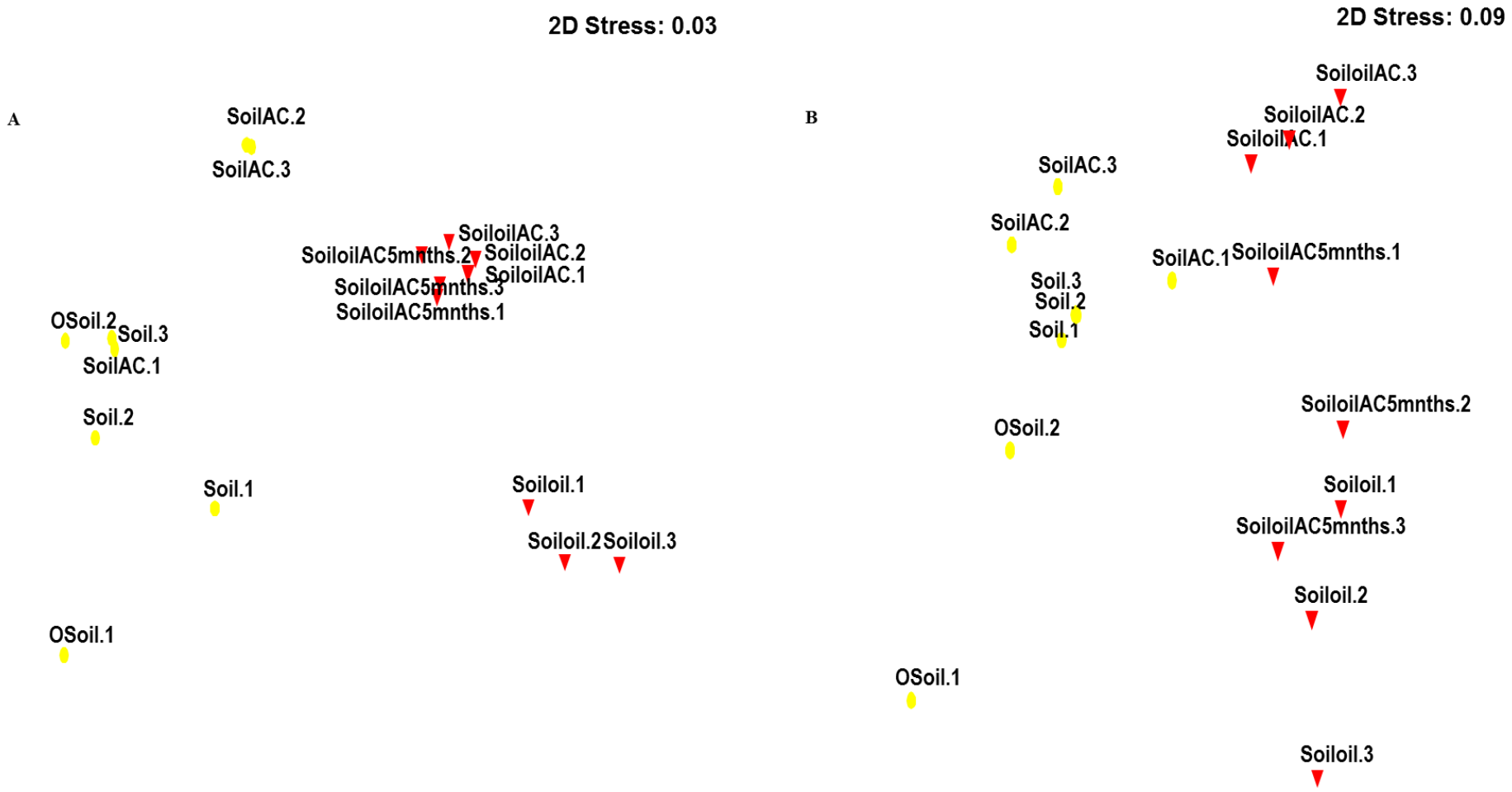

Figure 4-9 A multi-dimensional scaling for crude oil effects (A) Ion torrent platform (B) 454 GS junior platform. Analysis was based on Bray Curtis distance using square root transformed OTU table at level 3, Crude oil, No crude oil

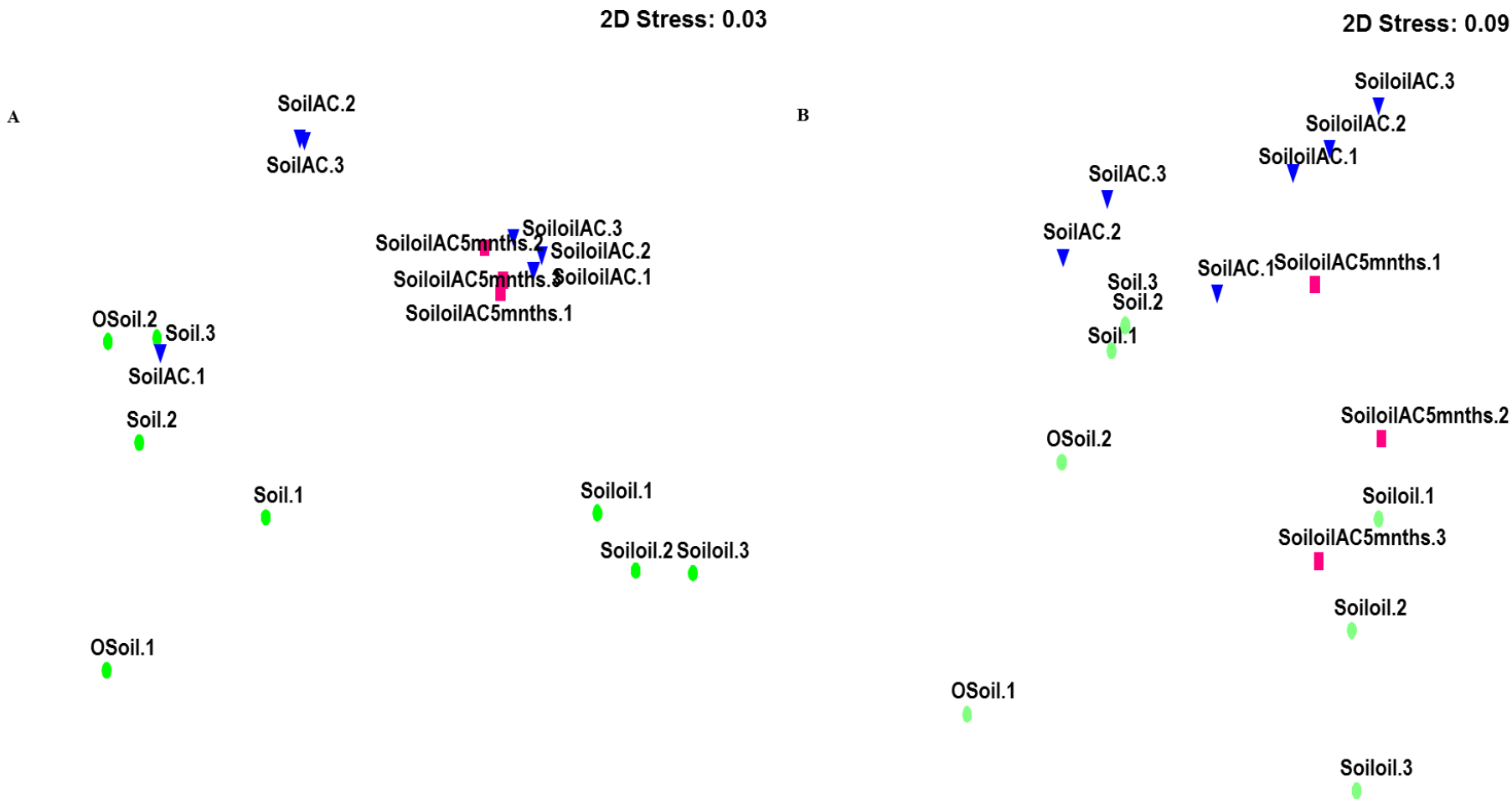

Figure 4-10 A multi-dimensional scaling for adsorbent amendment (AC)effects (A) Ion torrent platform (B) 454 GS junior platform. Analysis was based on Bray Curtis distance using square root transformed OTU table at level 3, No AC, AC @ t=5 months, AC @ t=zero

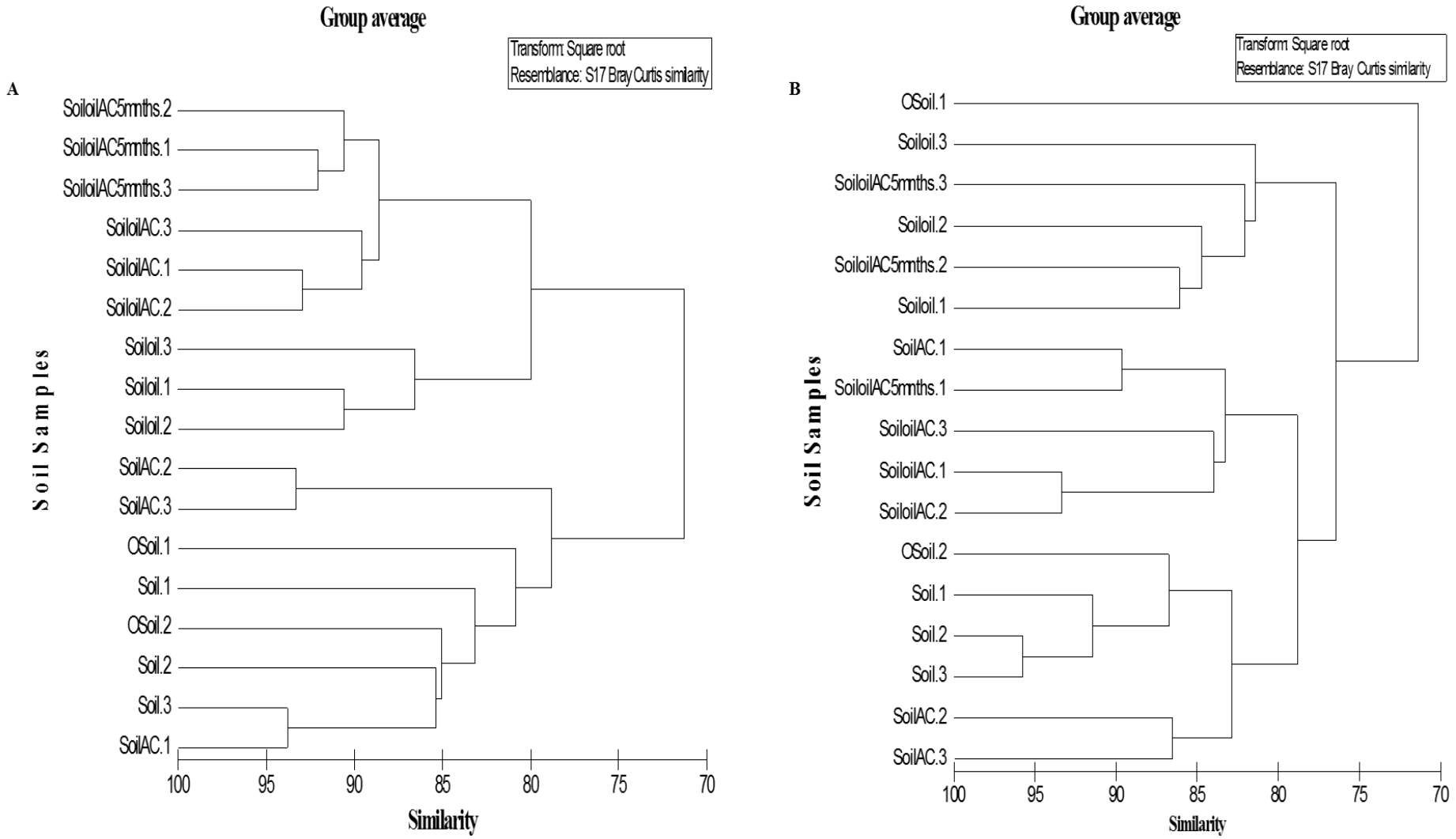

Figure 4-11 Clustering analysis (A) Ion torrent platform (B) 454 GS junior platform. Analysis was based on Bray Curtis distance using square root transformed OTU table at level 3

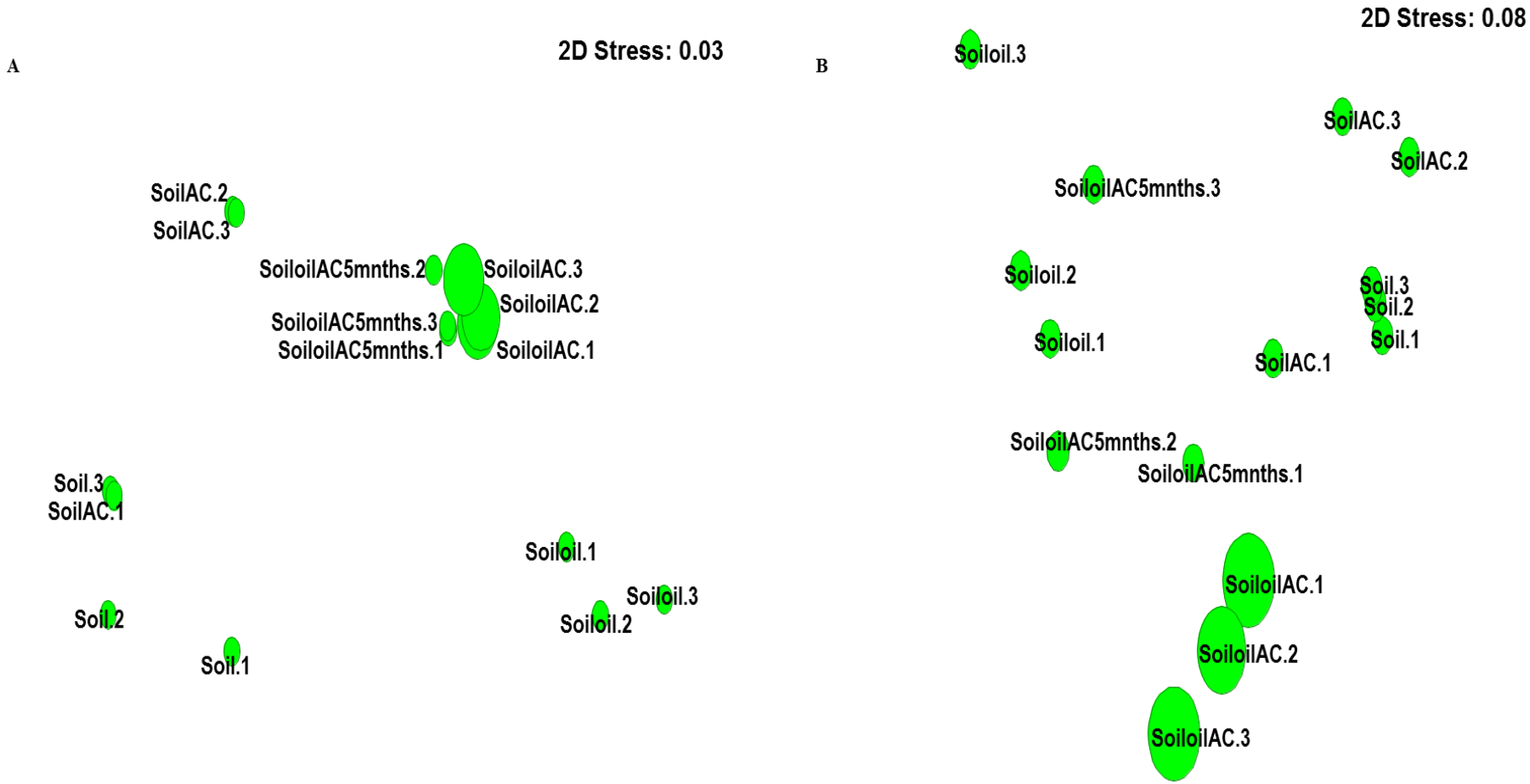

Figure 4-12 Integrated multi-dimensional scaling plot. Green bubbles indicate residual alkane soil concentration (µg/g) in treatment batches after 52 weeks (A) Ion torrent platform (B) 454 GS junior platform

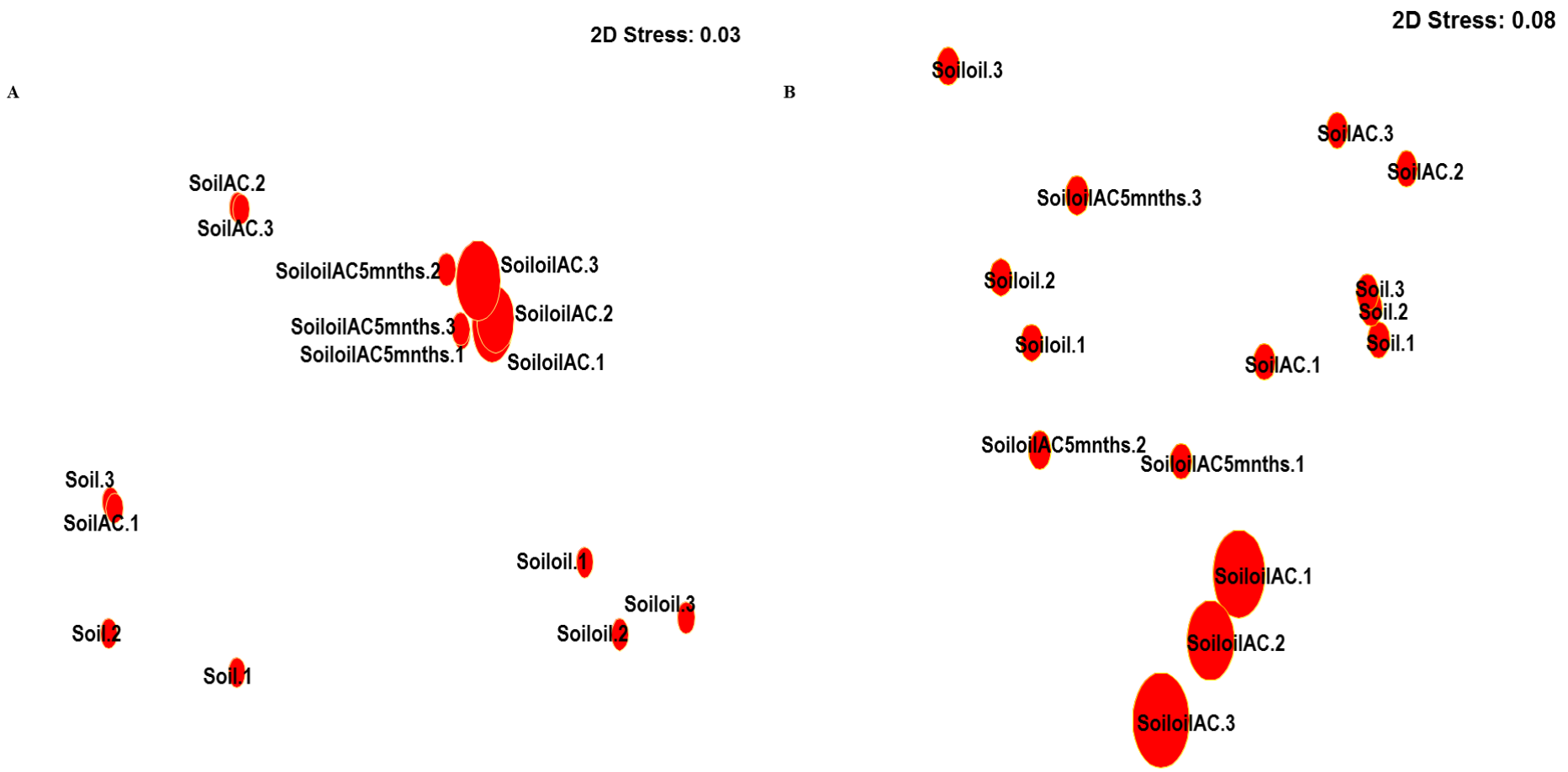

Figure 4-13 Integrated multi-dimensional scaling plot. Red bubbles indicate residual naphthalene soil concentration (µg/g) in treatment batches after 52 weeks (A) Ion torrent platform (B) 454 GS junior platform

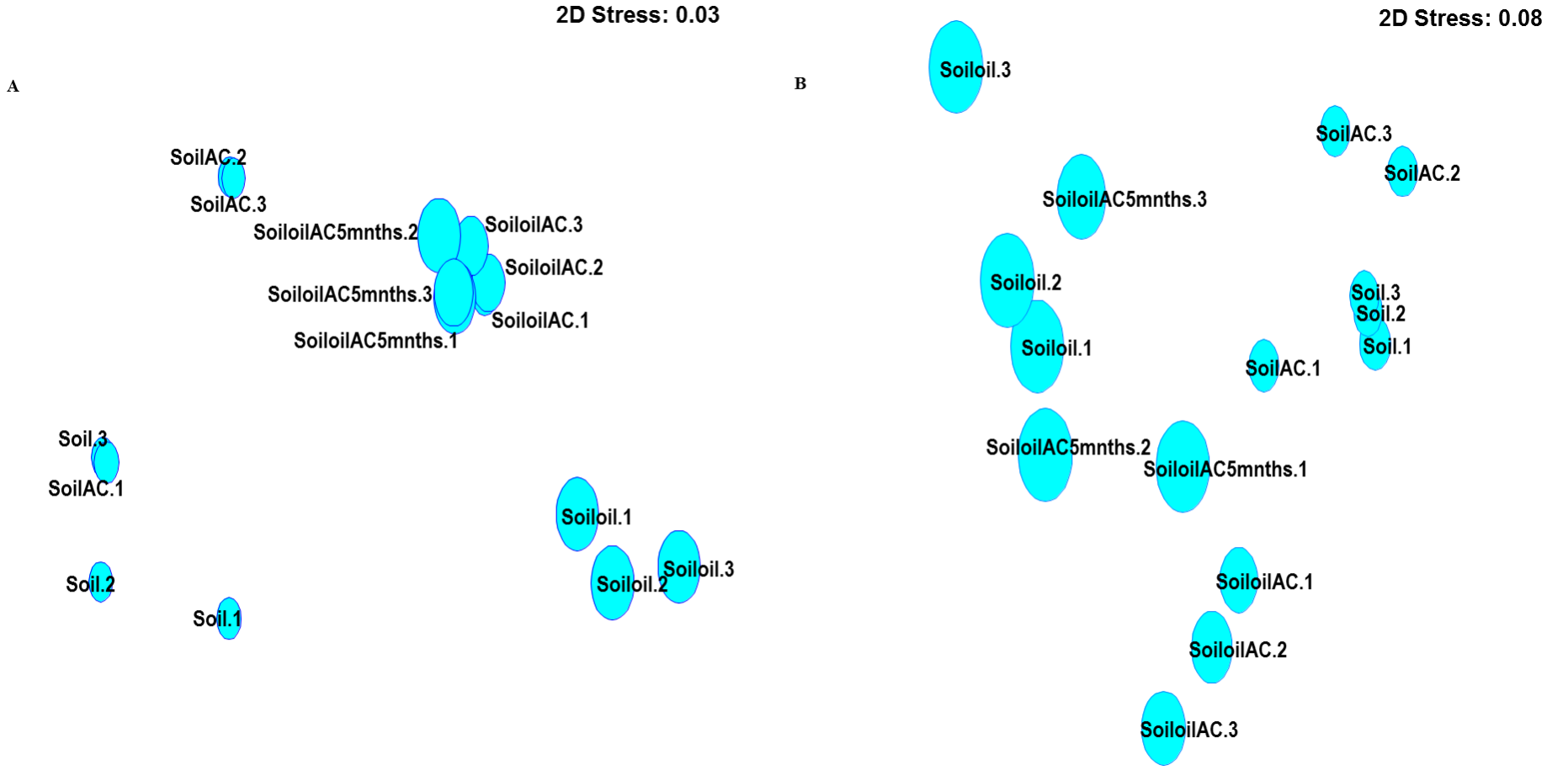

Figure 4-14 Integrated multi-dimensional scaling plot. Blue bubbles indicate CO_2 produced (mg/batch) in treatment batches after 52 weeks (A) Ion torrent platform (B) 454 GS junior platform

The integrated data (Figure 4-12, Figure 4-13 and Figure 4-14) was done by linking the microbial communities (Figure 4-9) to the physiochemical data (chapter 3) on an NMDS plot. The larger bubbles represent higher concentration while the smaller bubbles a lower concentration of carbon dioxide mg/batch (Figure 4-14), residual alkane µg/g (Figure 4-12) and residual naphthalene µg/g (Figure 4-13) for the various batches. The results for the integrated data shows lower alkane and LMW PAHs (naphthalene) concentration (Figure 4-12, Figure 4-13) and higher carbon dioxide production (Figure 4-14) from batches Soil & oil & AC at time 5 months and Soil & oil compared to Soil & Oil & AC for both platforms. This correlates with the higher relative abundance of some hydrocarbon degraders in batches Soil & oil & AC at time 5 months and Soil & oil compared to Soil & Oil & AC for both platforms. Some of such organism detected for ion torrent data include *Rhodococcus* belonging to phylum *Actinobacteria* while 454 GS junior data showed higher relative abundances in Soil & oil & AC at time 5 months and Soil & oil for *Rhodococcus, Salinibacterium* (both belonging to *Actinobacteria*), *Alcanivorax*, *Marinobacter Luteimonas* (belonging *Gammaproteobacteria)* and *Parvibaculum* (belonging to Alpha*proteobacteria*) (Also see Table 4-3). As expected the controls all show lower carbon dioxide production and residual alkane and naphthalene concentrations. This suggests that the hydrocarbon degrading organisms within the microbial community had a more positive reaction to addition of crude oil with no AC or AC amendment after 5 months compared to the other batch conditions and AC amendment effect on hydrocarbon degrading microbial activity was higher for Soil & Oil & AC batches.

The heat map (Figure 4-15) is an illustration of relative abundance relationships between samples at genus level for the 454 GS junior sequences run. The results also show a demarcation as the crude oil samples (with or without AC) clustered together on one side of the heat map and samples with no crude oil on the other side, thus illustrating strong effects of crude oil in shaping the microbial communities. The hydrocarbon-degrading genus *Alcanivorax,* was fairly dominant (high to fairly high to intermediate abundance seen by bright green, green and one black squares) in Soil & oil samples, but less dominant or low in most cases in the other treatments. The AC in crude oil samples amended with AC at the start and at 5 months held back the abundance increase of *Alcanivorax* following crude oil addition which is also illustrated in Figure 4-8 A. Other hydrocarbon degrading genera *Marinobacter*, *Rhodococcus, Luteimonas, Muricauda* were also shown to be more dominant in crude oil batches without AC and AC amendment at 5 months (with the exception of one replicate of Soil & oil &AC at 5 months which showed a low abundance of *Luteimonas*) as compared to in controls and Soil & oil & AC. *Arenibacter* and *Salinibacterium* are also abundant in Soil & oil & AC at time 5

months and Soil & oil in comparison to Soil & oil & AC at time zero. This indicates that the higher elevated presence of these genera occurred as a result of the crude oil pollution and the addition of AC from the start caused a lower increase in their abundance compared to AC addition after 5 months. However genera such as *Sediminibacter* were more dominant in controls and Soil & oil & AC at time zero, *Nocardia* is abundant in Soil & oil & AC at time zero and Soil & oil & AC at time 5 months while *Rhodoplanes* was more dominant in control samples only. *Muricola* was also more dominate in batches with AC amended from the start. *Muricola* and *Rhodoplanes* and were less abundant (see red rectangles) in Soil & oil and Soil & oil & AC at 5 months.

For the heat map for the Ion torrent run (Figure 4-16) *Muricola was abundant* Soil & oil & AC at time 5 months and Soil & oil in comparison to other treatment batches. The known hydrocarbon degraders, *Marinobacter, Nocardia, Rhodococcus and Mycobacterium,* where mainly abundant in Soil & oil batches also highlighting the effect of AC amendment on hydrocarbon degraders. Only *Candidatus Nitrososphaera* and *Sediminibacter* were abundant in Soil & oil & AC at time zero and Soil & oil & AC at time 5 months while *Rhodoplanes, Hyphomicrobium, Nocardioides and Bradyrhizobium* were mainly abundant in Soil & AC at time zero

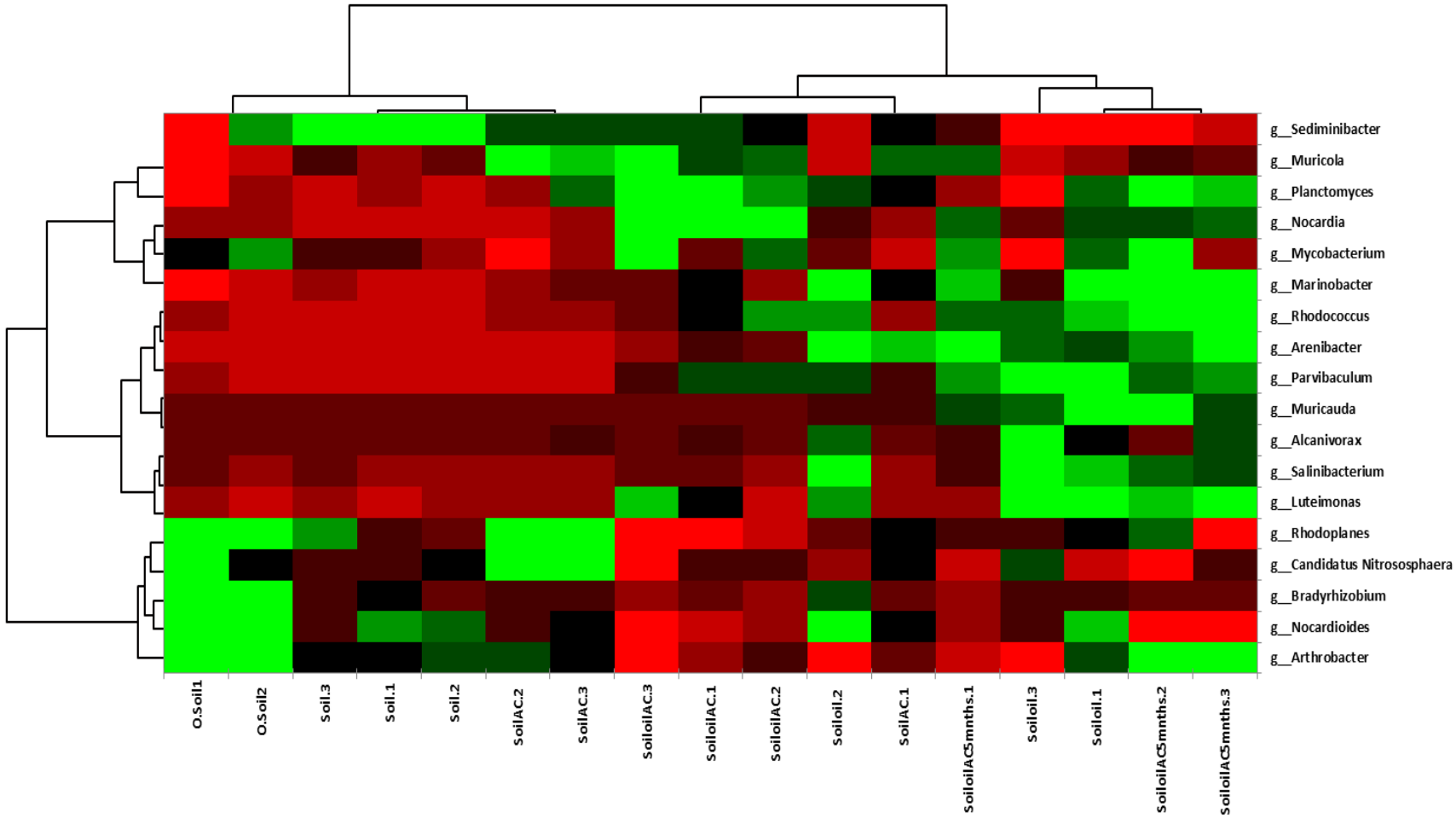

Figure 4-15 Heatmap with dendograms illustrating treatment effects. Sum of OTU abundance ≥0.5% of bacteria identifiable at genus level for 454 GS Junior sequence data. Samples in columns and OTUs in rows. Colour coding: low, intermediate and high abundance- Red triangles, black triangles, green triangles respectively

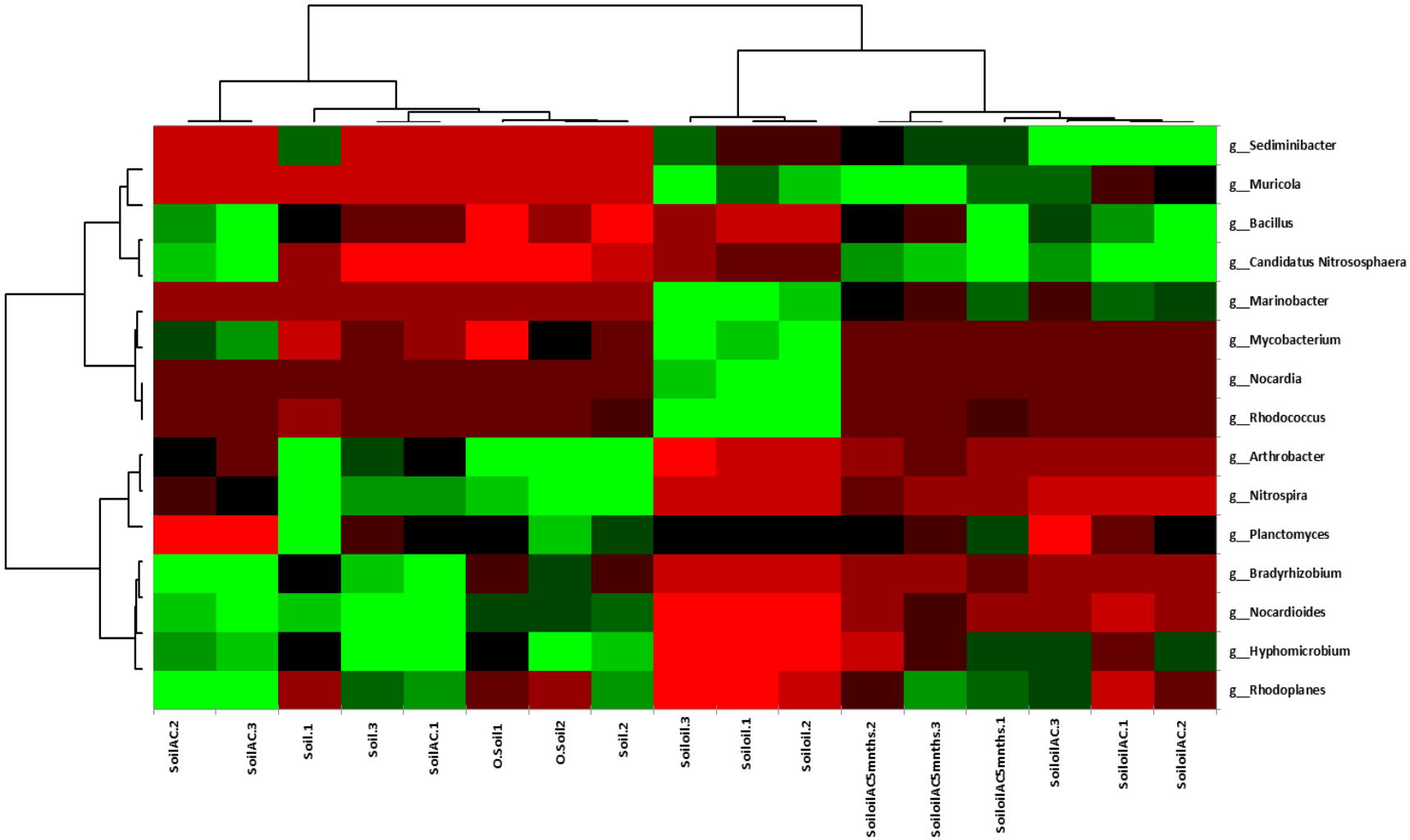

Figure 4-16 Heatmap with dendograms illustrating treatment effects. Sum of OTU abundance ≥0.5% for bacteria identifiable at genus level for Ion torrent sequence data. Samples in columns and OTUs in rows. Colour coding: low, intermediate and high abundance- Red triangles, black triangles, green triangles respectively

PCA (principal component analysis plots of observations and variables) showing pattern of demarcation based on treatment effects was generated using dominant OTUs (> 1% total sequences) at genera for both 454 GS Junior and Ion torrent data (Figure 4-17 and Figure 4-18 respectively). For the 454 GS Junior data the soil samples partitioned into F1 and F2 PC quadrants based on treatment effects. The PCs both accounted for 66.30% data variability, PC1 accounting for 44.76% and PC2 accounting for 21.55%. On PC1, the Soil & oil, Soil & oil & AC at time zero and Soil & oil & AC at time 5mnths demarcated to the negative axis (Figure 4-17 B) with organisms *Luteimonas, Marinobacter, Rhodococcus, Alphaproteobacteria, Alteromonadaceae, Luteimonas* and *Gammaproteobacteria* increase in relative abundance in the direction of Soil & oil and Soil & oil & AC at time 5mnths (Figure 4-17 A) while *Muricola, Phycisphaerales, Planctomyces,* relative abundance increase more in the direction of Soil & oil & AC at time zero. (Figure 4-17 A). The controls O.soil, Soil and Soil & AC demarcated to the positive axis of PC1. Organisms such as *Candidatus nitrososphaera, Rhodoplanes Gaiellaceae, Solirubrobacterales, Chloroflexi* etc which relative abundance are in the direction of O.soil contributed 5.87%, 5.81%, 8.62%, 8.29%, in terms of data variability on PC1 while other key contributors *Marinobacter, Rhodococcus, Alphaproteobacteria, Alteromonadaceae* and *Gammaproteobacteria* increase in their relative abundance in the direction Soil & oil and Soil & oil & AC at time 5mnths and contributed 5.58%, 5.55%, 8.32%, 7.08% and 6.57% respectively. On PC2 (Figure 4-17 B), O.soil, Soil & oil & AC at time 5 months and Soil & oil demarcated to negative axis (with the exception of one replicate of Soil & oil & AC at time 5 months) while Soil & oil & AC at time zero, Soil & AC at time zero and Soil demarcated to the positive axis of PC2. *Flavobacteriaceae, Muricola, Sediminibacter, Bacillales, Pirellulaceae* contributed the highest to data variability for PC2 with 16.05%, 10.2% 11.0%, 10.59% and 13.46% respectively while *Luteimonas* which relative abundance increase in the direction of Soil & oil contributed 8.68%.

For the Ion torrent data (Figure 4-18), both PCs accounted for 69.76% data variability, PC1 accounting for 41.46 % and PC2 accounting for 28.30%. On PC1, the O.soil, Soil and Soil & AC demarcated to the positive axis while Soil & oil & AC at time 5 months, Soil & oil & AC at time zero and Soil & oil demarcated to negative axis (Figure 4-18 B). The key contributors include *Acidobacteria, Muricola, Sediminibacter, Chitinophagaceae, Anaerolineae, Chloroflexi, Nitrospira, Bradyrhizobium, Hyphomicrobium, Sinobacteraceae* with values 6.85%, 5.65%, 4.80%, 8.07%, 7.27%, 8.26%, 7.33%, 4.46%, 6.37%, 7.82% respectively while class *Gammaproteobacteria* and known hydrocarbon degraders *Rhodococcus and Mycobacterium* only

contributed 2.84 %, 2.90 % and 2.19 % respectively with their relative abundance increasing in the direction of Soil & oil (Figure 4-18 A). On PC2, AC amended samples (apart from one replicate of Soil & AC at time zero) demarcated to positive axis while O.soil, Soil and Soil & oil demarcated to negative axis of PC2. The key contributors include *Candidatus nitrososphaera, Acidimicrobiales Solirubrobacterales, Cytophagaceae, Bacillus, Hyphomicrobiaceae,* with values 9.71 %, 5.31 %, 6.99 %, 7.21 %, 8.83 %, 7.35 %, respectively and relative abundances increasing in the direction of Soil & oil & AC at time 5 months and Soil & oil & AC at time zero while other key contributors *Gaiellaceae and* Rhodoplanes with values 8.64 % and 5.67 % increase in the direction of Soil & AC at time zero (Figure 4-18 A). *Gammaproteobacteria* and *Rhodococcus* are also key contributors (similar to 454 GS junior data) with values 7.33% and 6.72% and move in the direction of Soil & oil.

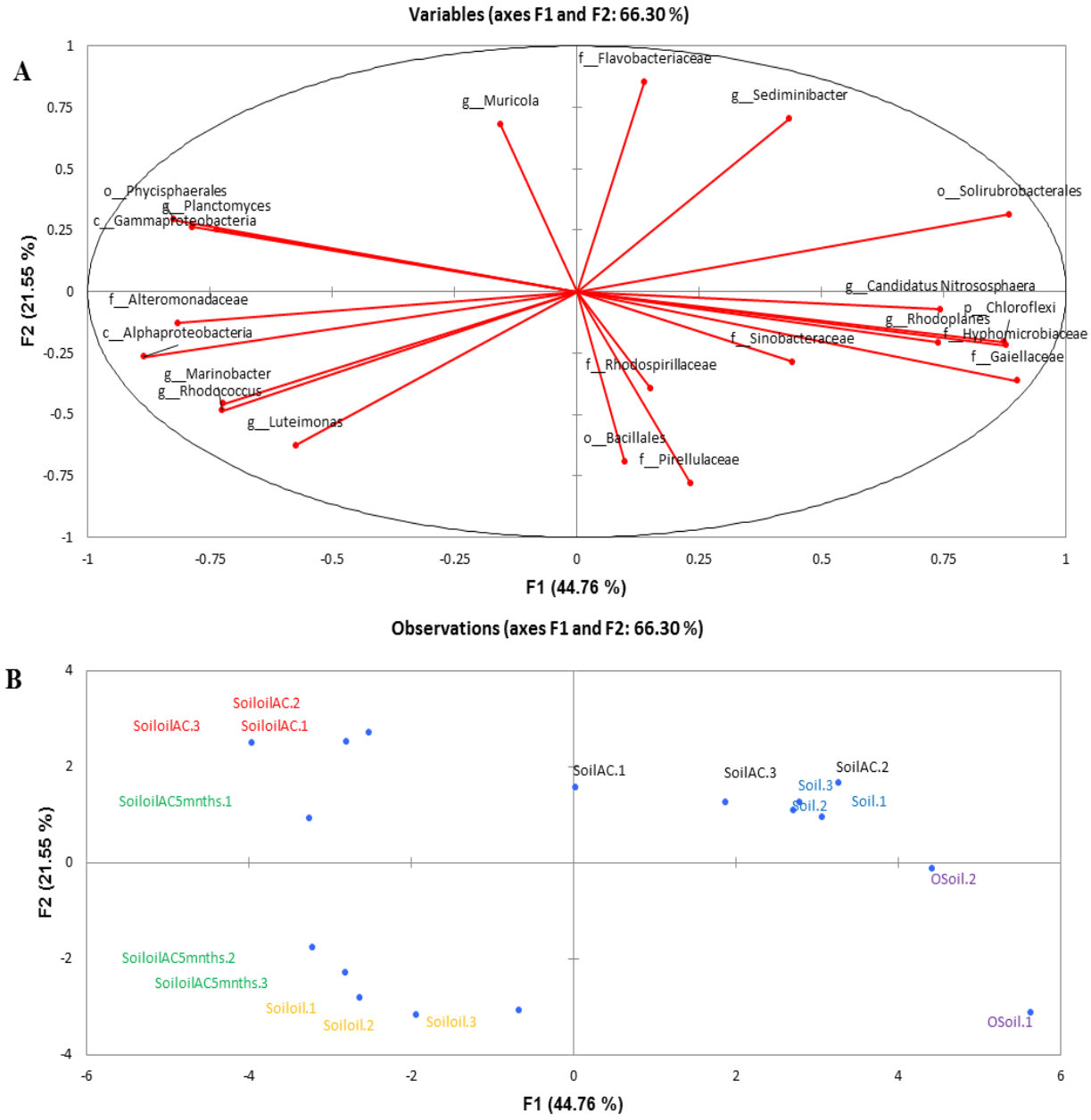

Figure 4-17 PCA analysis of 454 GS Junior data. (A) Dominant OTUs relative abundance (> 0.5% of total sequences at genus level) and (B) Treatments. Vectors indicate change in OTU abundance and corresponds to similar sample clustering

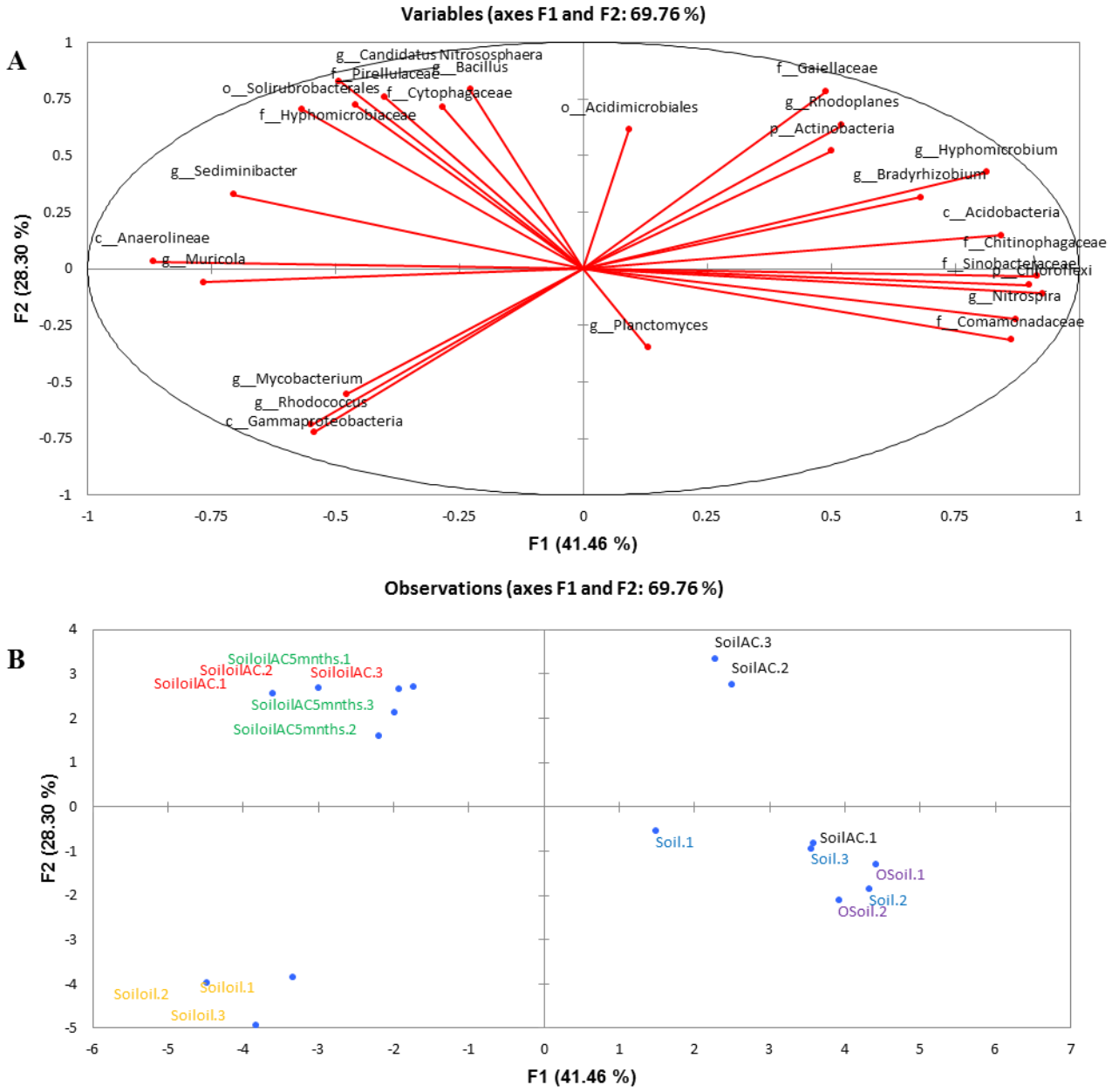

Figure 4-18PCA analysis of Ion torrent data. (A) Dominant OTUs relative abundance (> 0.5% of total sequences at genus level) and (B) Treatments. Vectors indicate change in OTU abundance and corresponds to similar sample clustering

4.5 Conclusion

The data generated for the 454 GS junior and Ion torrent generally showed that there was little differences between phylogenetic compositions at phylum level (R^2=0.8915, see Appendix D Figure 7.13 and 7.14 for correlation analysis done at phylum and genus levels). The very abundant species which happen to be the most relevant in crude oil degradation, for example some belonging to phylum *Actinobacteria* and class *Gammaproteobacteria,* the Ion torrent data is quite congruent with 454 GS junior data. For the less abundant OTUs, there are significant differences between the platforms. For example there is a higher abundance of *Archaea* (*Crenarchaeota* 5% more) and *Betaproteobacteria* (6%) (See Figure 4-5, Figure 4-6 taxonomy assigned data at 50% and 80%) in ion torrent data compared to 454 GS junior, so diversity measures give different results according to which platform being utilized. A sample to sample comparison between platforms also showed some dissimilarities in phylogenetic compositions from order Figure 4-7 and beyond. For example some genera detected (See Table 4-3), had higher average relative OTU abundances in 454 GS junior data compared to ion torrent. The correlation analysis also showed that 454 GS junior and ion torrent data were quite dissimilar at genus level (R^2=0.146, see Appendix D Figure 7.14). This could possibly be because of the higher amount of OTUs belonging *Archaea* and *Betaproteobacteria* taxonomic groups (Complete OTUs belonging to *Archaea* were 8, complete OTUs belonging to *Betaproteobacteria* were 48) and higher incomplete OTUs detected at genus level in Ion torrent which were lower in 454 GS Junior data (Complete OTUs belonging to *Archaea* were 3, complete OTUs belonging to *Betaproteobacteria* were 17) due to better quality filtering. Another advantage of the 454, in addition to the better bioinformatics pipeline, is greater read length, so species can be more reliably identified.

However for proper comparative evaluation of end results from 2 different sequencing platforms, the bioinformatics and possibly the purification approach should be as similar as possible. For the 454 GS junior data, which is possibly the less erroneous data, the addition of AC and crude oil had a significant effect on the α- diversity species richness with the untreated (O.soil) and nutrient amended only (Soil) batches, having much higher values for the α- diversity estimators in comparison to the other soil batches. The decrease in richness for crude oil batches contradicts the hypothesis that crude oil addition will increase richness and possibly diversity. The decrease possibly occurred due to a cytotoxic effect caused by higher hydrocarbon degradation, as well as

the selective growth of a few specialist bacteria, which degrade petroleum hydrocarbons, resulting in an uneven community structure.

In line with objective 2 of the thesis and 3rd chapter objective, (both 454 GS junior and Ion torrent) the addition of crude oil was seen to be a major factor in the shaping of microbial communities and it altered the community composition with significant higher abundances for known hydrocarbon (HC) degrading genera *Parvibaculum*, *Marinobacter*, *Salinibacterium*, *Arenibacter*, *Luteimonas*, *Rhodococcus* and species *Rhodococcus equi A venustensis* *Altererythrobacter epoxidivoran* and *Luteimonas Mephitis* in Soil & oil batches. AC amendment (at the beginning or midway through) did not significantly alter the structure of the general bacterial community within the soils (ANOSIM), however AC amendment in crude oil batches had significant effect on the bacterial community (also see nested ANOSIM) and seems to reduce abundance increases of potential hydrocarbon degraders, and this is in line with one of the general predicted hypothesis in Chapter 1. This corresponds well with the CO_2 production, alkane and naphthalene removal results in chapter 3 (Also see integrated data Figure 4-12, Figure 4-13 and Figure 4-14). The slight reduction overall in the abundances of potential hydrocarbon degraders is more evident in Soil & oil & AC batches compared to Soil & oil & AC at 5 months batches, and AC addition, through the sorption of petroleum hydrocarbons, slows down the biodegradation of hydrocarbon compounds (such as alkane and naphthalene, Figure 4-12, Figure 4-13), thus leading to lower CO_2 production rates (Figure 4-14). As previously stated in chapter 3, natural attenuation is a viable treatment option as seen by the effective removal of crude oil fractions such as saturated (Figure 3.7) and lower molecular weight PAHs (Figure 3.8) by the identified hydrocarbon degraders in bioremediation batches. It's believed that AC which is shown here to reduce the abundance of potential HC degraders, should added after the biodegradation of readily biodegradable compounds has been completed to stabilize the poorly biodegradable residuals.

Chapter 5. Microbiological assessment of the effects of biofuels (ethanol and biodiesel) on the biodegradation of a hydrocarbon compound (toluene)

The physico-chemical results presented in sections 5.1.9 of this chapter, which includes $^{12}CO_2$, and $^{13}CO_2$ measurements were obtained from batch experiments carried out by Brenda Shartsi Mutesi (Masters Student)

5.1 Introduction

Climate change occurs due to inherent changes of climatic systems or as a result of human activities (anthropogenic). The release of greenhouse gases such as CO_2 (emitted via the burning of fossil fuels) causes excessive increases in the planet's temperature which is also known as global warming (Escobar *et al.,* 2009). Global warming has been reportedly linked to approximately one hundred thousand deaths per year due to its effects on the ecosystem as the ever increasing temperatures could lead threats of starvation, flooding (changes in rain fall patterns and intensity and increased ocean levels), reduced water supply and disease outbreak like malaria. (Escobar *et al.*, 2009). Taking into account all these problems associated with the use of fossil fuels, interest is growing in the utilisation of renewable biomass generated fuels such as biofuels as a source of energy (Demirbas, 2009a; Escobar *et al.*, 2009). The EU renewable energy policy also stated the need for renewable energy production to displace fossil fuels energy production and utilisation, as this will aid in improving sustainability and reducing emissions of greenhouse gases (Howes, 2010). Biomass based biofuels are believed to be readily accessible and seen as a more reliable and sustainable fuel with less pollution emissions in comparison to fossil based fuels (Demirbas, 2007).

Over the years, low proportions of biofuels have been mixed with petroleum or diesel (Elazhari-Ali *et al.*, 2013) comprising of toxic aromatic compounds such as benzene and toluene etc. which could be a risk in event of a spill (Corseuil *et al.*, 2011). Microbial fuel biodegradation mitigates the risks posed by hydrocarbon spills, and with reports of biofuels in blended fuels being more susceptible to microbial degradation compared to the hydrocarbon components, there have been worries of an inhibition of the degradation of hydrocarbons by microbial communities when blended with either ethanol (Da Silva and Alvarez, 2002) or biodiesel (Lapinskienė et al., 2006).

It is therefore vital to elucidate the effects of biofuels on the biodegradation of hydrocarbon components (Dakhel et al., 2003) and also determine the impacts they have on the indigenous microbial community structure, as little is known about the microbiology of blended fuel degradation in soil (Österreicher-Cunha et al., 2004). Using both chemical and microbial molecular sequencing methods, this chapter investigates the effects of biofuels (ethanol and biodiesel) on the degradation of toluene in soils with and without inorganic nutrient amendments.

5.2 Objectives

The specific objectives are to

1. Use $^{12}CO_2$ and $^{13}CO_2$ production profiles in the determination of impacts of ethanol and biodiesel on the degradation rates of ^{13}C toluene and thus to derive the effects nutrients have on the degradation of single and blended fuels.
2. Use microbial molecular methods such as ion torrent sequencing and quantitative PCR analysis to determine (by studying differences in microbial diversity, richness, community structure and abundance) the impacts nutrient, single and blended fuels on the indigenous microbial communities.
3. Identify potential HC and other compound utilizing microbes and ascertain how the presence or absence fuels or nutrient amendment affects the relative abundance.

5.3 Materials and methods

5.3.1 Chemicals and materials

Soil properties

The soil used for the set-up of the batch experiment was previously obtained from construction site at Newcastle university campus and was examined by Elazhari-Ali et al. (2013). The dry weight soil was analyzed by Derwent side environmental testing services, Durham UK.

Table 5-1 Characteristics of soil utilized

Property	Soil
Sand	87.26%,
Silt	11.33%
Clay	1.42%.
Ammonical nitrogen	7.3 mg/kg
Phosphorus	550 mg/kg
TOC	1570 mg/kg.

Biodiesel, ethanol and toluene

Biodiesel and ethanol composition were previously reported by Elazhari-Ali et al. (2013). Toluene $^{13}C_7$ (purity 99%, mw: 99.08 g/mol, density 0.930 g/mL at 25^0C) or ordinary toluene was supplied by Sigma Aldrich, Gillingham, UK and possessed purity above 99%.

5.3.2 Nutrient preparation

A mixture of nutrient mineral water containing quantities of nitrogen (NH_4Cl) and P (KH_2PO_4) to give a C: N: P ratio of 100:10:1 was prepared by dissolving 42.7 mg of NH_4CL (ammonium chloride) and 4.9 mg of KH_2PO_4 in 10 mL distilled water (See Appendix A for calculations of mass of C, N and P added). The nutrient solution was autoclaved before 0.3 mL each was dispensed into all amber vials giving a nutrient N concentration of 0.052% (w/w %), 0.58 % (w/v %) and P concentration of 0.045% (w/w %), 0.51% (w/v %) in soil and soil solution respectively. Toluene served as carbon source) for microbial growth batch experiment design.

A batch experiment was set up for biodegradation and microbial analysis by replicating that of Brenda Shartsi Mutesi (Master's student dissertation project 2014). The batch experiment was carried out at room temperature (20±2°C). Batch samples were prepared in triplicates in clean 40 mL brown amber vials stoppered with Teflon mininert valves (Supelco, Bellefonte, USA). Each vial contained 15 g of sieved sandy soil and 50% of the batch samples were amended with 0.3 mL of nutrient solution (C: N: P ratio: 100:10:1). For the full composition of vials see Table 5-2. Biodiesel and ethanol was added separately while toluene was dispensed via the Teflon mininert valves to reduce volatilization. To maintain a balance of carbon content in blended fuel batches, equal volumes of each fuel were added. The nutrient solution was added as previously stated in section 5.1.2. Controls were also set up in triplicates, with and without nutrients and with or without fuels added. The batch experiment was kept in the laboratory at room temperature for 30 days.

Table 5-2 Description of constituents of hydrocarbon and biofuels batch experiment

SAMPLE ID	SOIL MASS (g)	NUTRIENT (mL)	VOLUME OF SINGLE FUEL (µL)
Soil	15	0	0
Soil and nutrient	15	0.3	0
Soil and toluene	15	0	4
Soil and toluene and nutrient	15	0.3	4
Soil and biodiesel	15	0	4
Soil and biodiesel and nutrient	15	0.3	4
Soil and ethanol	15	0	4
Soil and ethanol and nutrient	15	0.3	4
Soil and toluene and biodiesel	15	0	2
Soil and toluene and biodiesel and nutrient	15	0.3	2
Soil and toluene and ethanol	15	0	2
Soil and toluene and ethanol and nutrient	15	0.3	2

5.3.3 $^{12}CO_2$ and $^{13}CO_2$, quantification, GC-MS measurement

Carbon dioxide measurements and the results for the biofuels and hydrocarbon batches was performed and provided by Brenda Shartsi Mutesi (Master's dissertation project, 2014). $^{13}C_7$ labeled toluene (Sigma Aldrich, Gillingham, UK) was utilized to aid in the separation and identification of $^{12}CO_2$ and $^{13}CO_2$. Carbon dioxide in vial headspace was measured by GC-MS similar to the GC-MS measurement reported in chapter 3 section 3.1.4.1. Integrated chromatograms (ion or mass: 44 representing $^{12}CO_2$ and 45 representing $^{13}CO_2$) were viewed in full scan mode on Xcalibur software.

5.3.4 Soil sample preparation and storage

After 30 days, the batch experiment was stopped and the 36 vials were uncapped and samples collected and stored for microbial analysis as reported in chapter 4 section 4.3.1.

5.3.5 DNA extraction and PCR

The DNA extraction and PCR amplification for PGM Ion torrent sequencing was carried out as described in chapter 4 section 4.3.2.

5.3.6 Amplicon library preparation and PGM ion torrent sequencing.

The amplified DNA from 36 samples were purified and quantified as described in chapter 4 section 4.3.3 while Ion torrent sequencing was carried out as described in chapter 4 section 4.3.4.

5.3.7 Quantitative PCR (qPCR) analysis

The quantitative Real Time PCR (qPCR) was used in the estimation of the total bacterial gene copy numbers in the various treatment soils. For the quantitative Real Time PCR (qPCR) analysis, primers 338F and 1046R were used (Thermo Fisher Scientific). The Real Time PCR was done on a BioRad CFX 96 TM Real-Time system containing a C1000 TM thermal cycler and a BioRad CFX manager software. The 10 µL reaction mixture each contained 5 µL of SsoFast EvaGreen Supermix (BioRad Laboratories, UK), 0.5 µL each of forward and reverse primes (diluted 1/10), 1 µL of water (filter sterilized), 3 µL template DNA (diluted 1/10, 1/100 and 1/1000) or standard or water (no template controls-NTC). The NTC was used to ensure the mix is free from contaminants. The stock solution of standards were pre-prepared containing 10^9 copies concentration of plasmid per µL and the stock solution was diluted in series of $10^9 – 10^1$ gene copies per µL to give a calibration curve. The standard curve efficiency has to be between 90 and 110% and have an $r^2 \geq 0.99$ to pass the qPCR run. The dilutions of standard, primers and DNA samples was carried out

manually in the microbiology safety cabinet The total required volumes for all reagents was supplied and final mixing was performed by the Qiagility robot (QIAGEN, UK). See appendix section (Appendix C) for a full description of 96 well plate set up. Each sample amplification was carried out in triplicate, and the run was repeated twice (1st run was a test run) using the following qPCR program: heated to 98°C for 3 mins, DNA denaturation at 98°C for 5 secs and primer annealing at 60°C for 10 secs (39 cycles for denaturation and annealing). This was then followed by melting curve at temperature increments of 0.5°C every 5 secs from 65°C to 95°C.

The gene copy number per gram of soil was calculated using Equation 5.1

Equation 5.1 $G_{cs} = SQ * df * V_{DNA} * Ws$

Where G_{cs} is the gene copy number, SQ represents starting quantity, df is the DNA dilution factor i.e. 1/10, 1/100, 1/1000. V_{DNA} is the volume (µL) of extracted DNA and Ws is weight of soil in grams from which the DNA was extracted. Each replicate of the samples were run 3 times, an average of the 3 runs per replicate was determined.

5.3.8 QIIME, microbial community and statistical analysis

The ion torrent sequencing reads were processed on QIIME and analyzed as described in chapter 4 section 4.1.6. For the α- diversity, default rarefaction minimum (starting) depth of 10 per sample and a sequence step-wise increment of 1940 sequences per depth, and 10 iterations (replicates) per depth was used. A maximum depth of 19410 was chosen (based on the sample with smallest OTU sequence count) to allow even sampling across the samples. The comparison of microbial communities between samples (β- diversit) was also carried out using PRIMER v6 software (Primer-E Ltd., Plymouth, UK) as described in chapter 4 section 4.1.6. The QIIME generated OTU table (genera level 6) was log transformed and used for the diversity as described in chapter 4 section 4.1.6. Heat map was generated on XLSTAT software (XLSTAT, 2016) using the most abundant identifiable genera (> 0.5 % at Level 6, log transformed OTU table). For statistical analysis, one way Analysis of Similarities (ANOSIM) (Appendix B) was carried using a Pearson' product moment correlation dissimilarity matrix (Appendix B) and a 2 way nested ANOSIM was done considering biodiesel and ethanol as factors within toluene amended batches. Student *T-test* was performed on Microsoft excel version 2010 (Microsoft, Redmond, USA) using 95%confidence,

two-tailed, independent samples to compare the effects of treatments on α- diversity matrices, differences in relative OTU abundances for identifiable bacteria across various samples and differences in CO_2 production.

5.4 Results and discussion

5.4.1 Carbon dioxide ($^{12}CO_2$ and $^{13}CO_2$) production

The amount of $^{12}CO_2$ and $^{13}CO_2$ produced (mg) (Figure 5-1) was used to determine the effects of biofuels on the biodegradation of toluene. The rationale here is that there will be a production of $^{13}CO_2$ via the biodegradation of isotope labelled $^{13}C_7$ toluene while the biodegradation of biofuels; ethanol and biodiesel would mainly produce $^{12}CO_2$. The $^{12}CO_2$ production from Soil & ethanol batches with or without nutrients showed that ethanol was readily degraded (Figure 5-1). Soil & ethanol and Soil & ethanol & nutrients had the highest $^{12}CO_2$ amount produced with values of 0.92±0.38 mg and 1.62 ±0.08 mg (0.002±0.0 mg/g/d and 0.004±0.0 mg/g/d, daily mineralization rate see Appendix F, Figure 7.27) respectively. The nutrient addition induced a 1.75 times increase (t.test, 2 sided, unpaired, $p < 0.05$) in the production of the $^{12}CO_2$ in Soil & ethanol batches (Figure 5-1 A and B). In comparison to the controls (Soil and Soil nutrient), the $^{12}CO_2$ produced from the Soil & ethanol batches and Soil & ethanol & nutrient batches was 11.9 times and 19.74 times higher respectively, showing mineralization of the added ethanol. A significant difference in $^{12}CO_2$ production was also observed between Soil & toluene and Soil & toluene & nutrient batches and between Soil & toluene& ethanol and Soil & toluene& ethanol & nutrient batches (Figure 5-1). The $^{12}CO_2$ production from Soil & toluene batches ($^{13}C_7$ toluene) was low and comparable with or within range of $^{12}CO_2$ production from Soil batches (control) (Figure 5-1 A). In contrast, the $^{12}CO_2$ production from Soil & toluene & ethanol was significantly higher (t.test, 2 sided, unpaired, $p < 0.05$) than $^{12}CO_2$ produced from Soil & toluene batches and Soil batches (control). Nutrient amendment was generally also seen to increase $^{12}CO_2$ production as Soil & toluene & ethanol & nutrient had a 1.37 times $^{12}CO_2$ production increment compared to Soil & toluene & ethanol batches while Soil & toluene & nutrient batches had a 4.6 times $^{12}CO_2$ production increment in comparison to Soil & toluene batches. As expected, the results showed that there was a lower production of $^{12}CO_2$ (Figure 5-1 A) for Soil & toluene & ethanol and Soil & toluene & ethanol & nutrient batches compared to (t.test, 2 sided, unpaired, $p < 0.05$) that of Soil & ethanol and Soil & ethanol & nutrient

batches, respectively. And this is possibly because there is less ethanol in the blended fuel batches, 2 uL versus 4 uL for ethanol only batches.

In the absence of nutrient amendment, biodiesel; was seemly seen to be less biodegradable than ethanol when comparing $^{12}CO_2$ production profiles for Soil & ethanol batches and Soil & toluene & ethanol batches with Soil & biodiesel and Soil & toluene & biodiesel batches (Figure 5-1 A). The results (Figure 5-1 A) shows that $^{12}CO_2$ production from Soil & biodiesel batches was 0.13 ± 0.09 mg (daily rate of 0.00029±0.0 mg/g/d) while that of Soil & toluene & biodiesel batches was 0.08 ± 0.03 mg (daily rate of 0.0009±0.0 mg/g/d). However upon nutrient amendment, $^{12}CO_2$ production (Figure 5-1 B) increased to 0.5 ± 0.3 mg and 0.8 ± 0.19 mg for Soil & biodiesel & nutrient and Soil & toluene & biodiesel & nutrient batches respectively (daily rate of 0.001±00 and 0.002±00 mg/g/d). The results so far indicate that nutrient amendment enhances the biodegradation of both ethanol and biodiesel, seen by enhancement of $^{12}CO_2$ production.

Similarly, looking at the $^{13}CO_2$ production profiles from toluene spiked batches with and without nutrient amendment (Figure 5-1A &B respectively), the $^{13}CO_2$ production over 30 days from Soil & toluene (only) batches was 0.09 ± 0.08 mg (Figure 5-1A, no nutrient added) while the production from mixed batches, Soil & toluene & ethanol and Soil & toluene & biodiesel batches (no nutrient added) was much lower and comparable with each other (Figure 5-1 A). However upon nutrient amendment (Figure 5-1 B), the $^{13}CO_2$ production from Soil & toluene & biodiesel & nutrient batches was much higher (t.test, 2 sided, unpaired, $p < 0.05$) than $^{13}CO_2$ from Soil & toluene & biodiesel batches. The sum of $^{13}CO_2$ from Soil & toluene & biodiesel & nutrient reached approximately 1.25 ± 0.29 mg (Figure 5-1 B, and a daily mineralization rate of 0.003±00mg/g/d see Appendix F) and was comparable (t.test, 2 sided, unpaired, $p>0.05$) with $^{13}CO_2$ production from Soil & toluene & nutrient batches which had 1.26 ± 0.18 mg of $^{13}CO_2$ production (Figure 5-1 B). Thus indicating that biodiesel effect on the biodegradation of toluene was reduced with nutrient amendment. The nutrient amendment had a lower effect on the biodegradation of toluene in the presence of ethanol (compared to presence of biodiesel) but $^{13}CO_2$ production was still significantly (t.test, 2 sided, unpaired, $p<0.05$) increased from 0.01±0.007 mg in Soil & toluene & ethanol batches to 0.15 ± 0.09 mg (Figure 5-1A & B) in Soil & toluene & ethanol & nutrient.

Generally from the carbon dioxide results (Figure 5-1), it shows that in the absence of nutrient amendment, only ethanol is readily biodegraded. Nutrient amendment facilitates toluene and biodiesel degradation, but toluene degradation is inhibited in the presence of ethanol (up to 88 %

reduction in $^{13}CO_2$ production compared to toluene only or toluene plus biodiesel nutrient amended batches). Ethanol seems to have a higher effect on toluene degradation compared to biodiesel and the biodegradation of toluene is significantly improved with nutrient amendment. These findings are similar and in line with those reported by Elazhari-Ali *et al.* (2013). To further illustrate this effect, the $^{13}C/^{12}C$-CO_2 ratio (Figure 5-2), was calculated by comparing the concentrations of $^{13}CO_2$ over $^{12}CO_2$. As the blended fuels were mixed in a 1:1 v:v ratio, a $^{13}C/^{12}C$-CO_2 ratio of about 1 would indicate equal preference for the biofuel and toluene component of the binary mixture. Without nutrient addition, the $^{13}C/^{12}C$-CO_2 ratio was significantly below 1, even for the pure toluene fuel addition to soil, indicating that without nutrient amendment, the biofuel, or even ordinary soil organic carbon, is a preferred substrate as compared to toluene. With nutrient addition, $^{13}C/^{12}C$-CO_2 ratio was above 3 for Soil & toluene & nutrient batches, indicating a preference for toluene over soil organic carbon, if nutrients are sufficiently abundant, and the ratio was generally above 1 for Soil & toluene & biodiesel & nutrient batches, indicating preference for toluene over biodiesel if nutrients are sufficiently available. $^{13}C/^{12}C$-CO_2 ratio for Soil & toluene & ethanol batches (with or without nutrients) was, on the other hand, below 1 indicating that ethanol was preferentially degraded and had a more hindering effect on toluene degradation compared to biodiesel. These findings are similar to reports by Elazhari-Ali *et al.* (2013) where ethanol was more degradable than biodiesel and had a larger impact on the degradation of monoaromatic petroleum hydrocarbons.

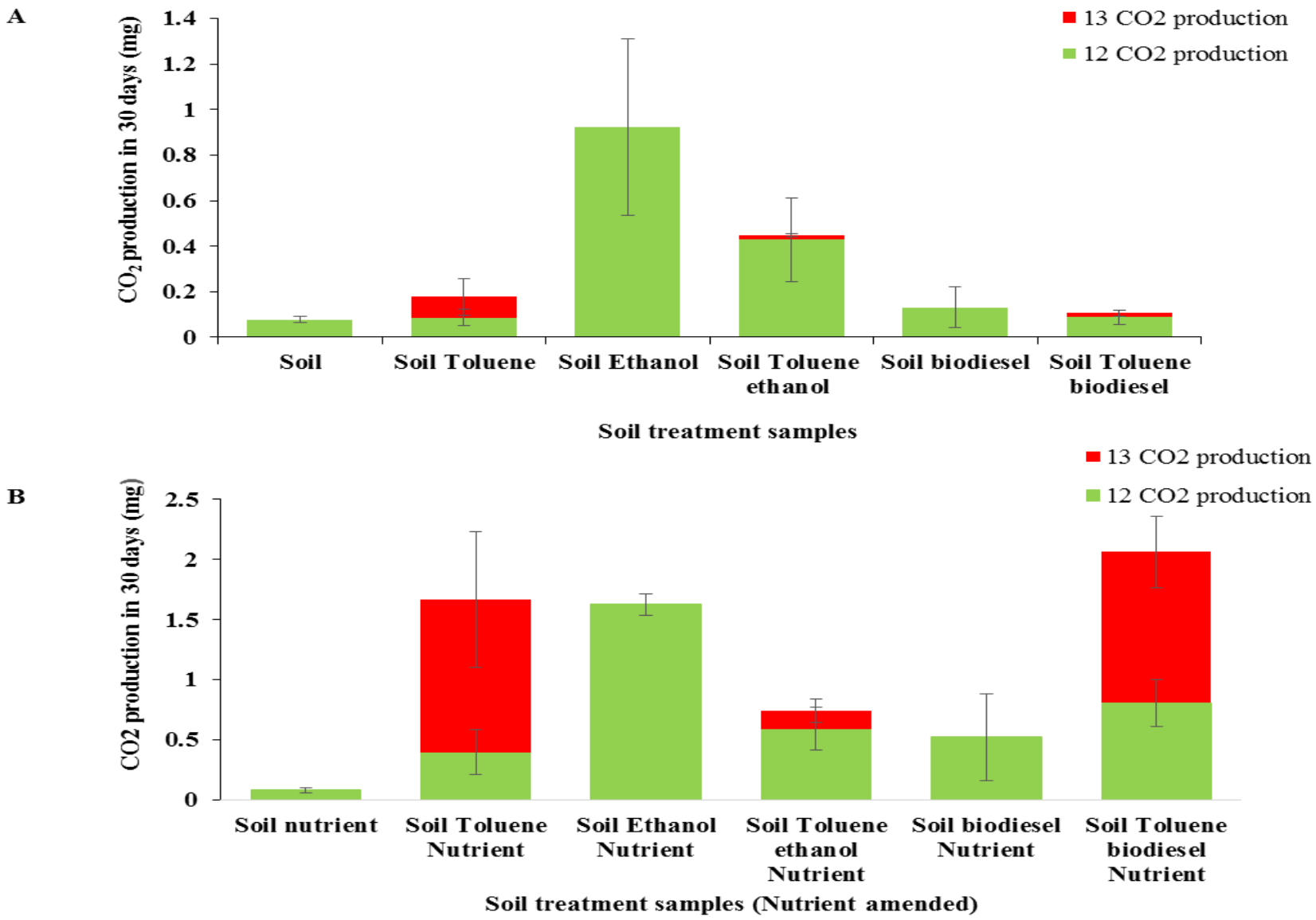

Figure 5-1 $^{12}CO_2$ and $^{13}CO_2$ production (mg). A) No nutrient B) Nutrient amended

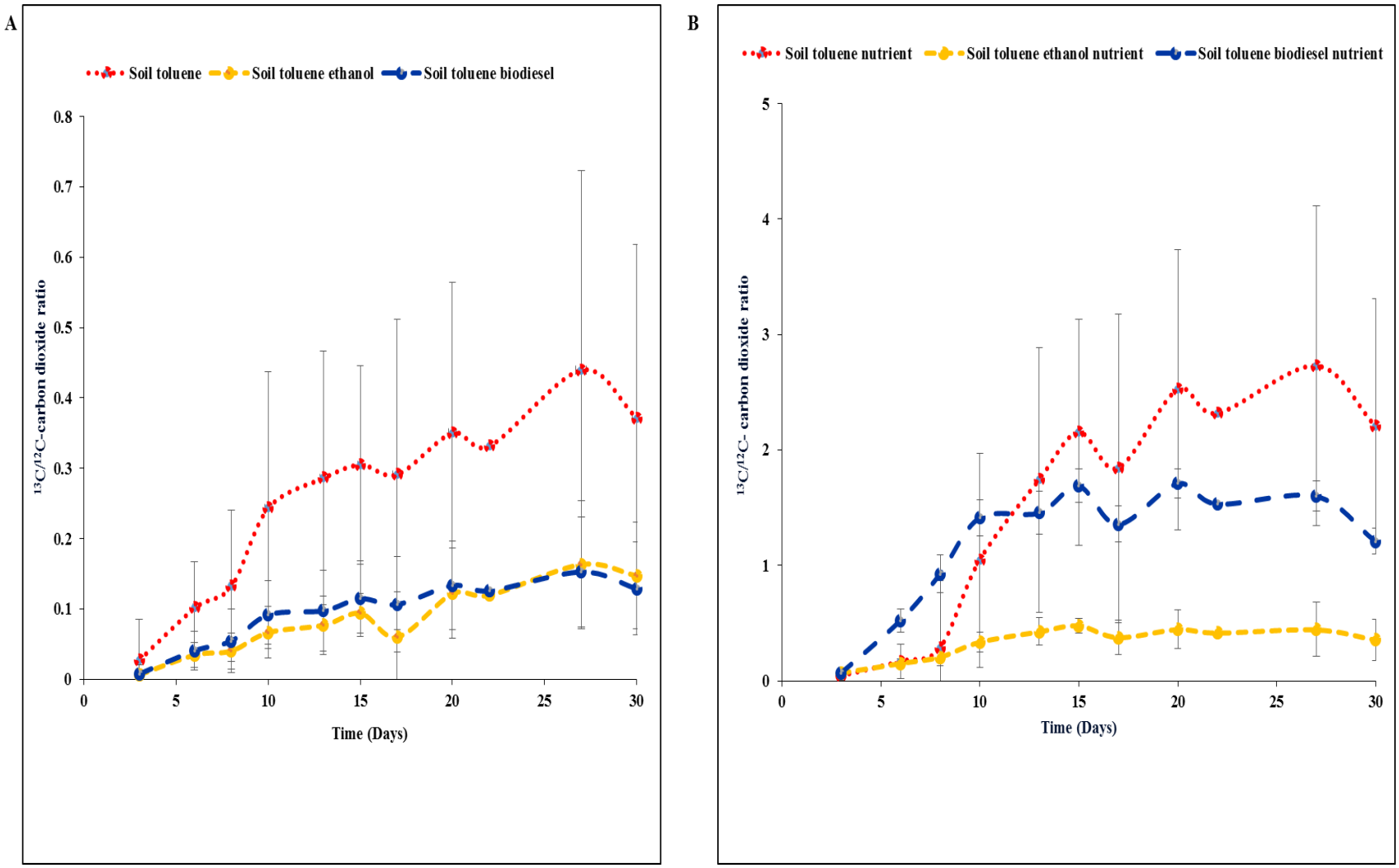

Figure 5-2 Estimation of production ratio of $^{13}C/^{12}C$-Carbon dioxide in toluene only and toluene and biofuels mixed batches. (A) $^{13}C/^{12}C$-Carbon dioxide production ratio (B) $^{13}C/^{12}C$-Carbon dioxide production ratio with nutrient amendment. Error bars representing: 1 standard error

5.4.2 Quality filtering and selection of OTUs

Of the initial 3075520 sequences for Ion torrent reads, a total of 1655496 (53.8%) sequences passed the demultiplexing quality filtering (Appendix B). The average read length for ion torrent was 276 bp and the number of sequences for the samples ranged between 27465 and 60203. From the 1655496 quality filtered sequences, the bacteria domain was assigned 1637486 (98.9%) sequences and 1619498 (98.9%) of the assigned sequences were classified. The archaea domain was assigned 17978 (approximately 1 %) sequences and 17850 (99%) assigned sequences were classified.

5.4.3 Treatment effects on diversity and richness

For the richness and diversity analysis, Chao1 and Faith PD index matrices were utilized. For more details on both estimators please refer to chapter 4 section 4.4.2. The results (rarefaction curves, Figure 5-3) for both estimators showed similar sample patterns with regards to treatment effects and also showed that there were no significant differences for the alpha-biodiversity between samples ($p>0.05$, one way ANOVA). The Controls (Soil and Soil & nutrients batches) and Soil & toluene & biodiesel batches had the highest values for both estimators thus indicating a statistically insignificant trend towards higher richness and diversity (t.test, 2 sided, unpaired, $p>0.05$) within those batches in comparison to other treatments soils. Chao1 and Faith PD index estimate values for the batches with single fuels with no nutrient amendment (Soil & toluene, Soil & biodiesel and Soil & ethanol) were next in line (followed very closely) with higher richness and diversity. Ethanol has previously been reported to increase microbial richness and diversity due to its readily biodegradable nature and utilization by a large number of microbes (Ma *et al.*, 2013). The results generally show that even at sampling depth of 19410, new species were still being discovered, as indicated by the steep slope for the treatment samples, however there is slight demarcation between biodiesel single fuel batches (black) and Toluene and ethanol mixed batches (yellow) as the slopes begins to slightly level or flatten out (Wooley et al., 2010). The blending of ethanol and toluene with or with no nutrient amendment was seen to reduce the richness and diversity. The slightly lower richness and diversity was initially believed to probably occur due to competition for nutrient resources (Da Silva and Alvarez, 2002) but even with the addition of nutrients, Soil & toluene & ethanol & nutrients has the lowest richness and diversity. Interestingly, Soil & ethanol & nutrient, Soil & toluene & nutrient and Soil & toluene & biodiesel & nutrient batches also had slightly lower richness and diversity which indicates nutrient amendment slightly reduces richness and diversity

in those samples. This is in line with findings in the earlier chapters that growth of VPH degraders means that other organisms are relatively less frequently detected, resulting in lesser diversity. Generally the results show biodiesel has a slightly lesser effect on richness and diversity within samples when compared to ethanol.

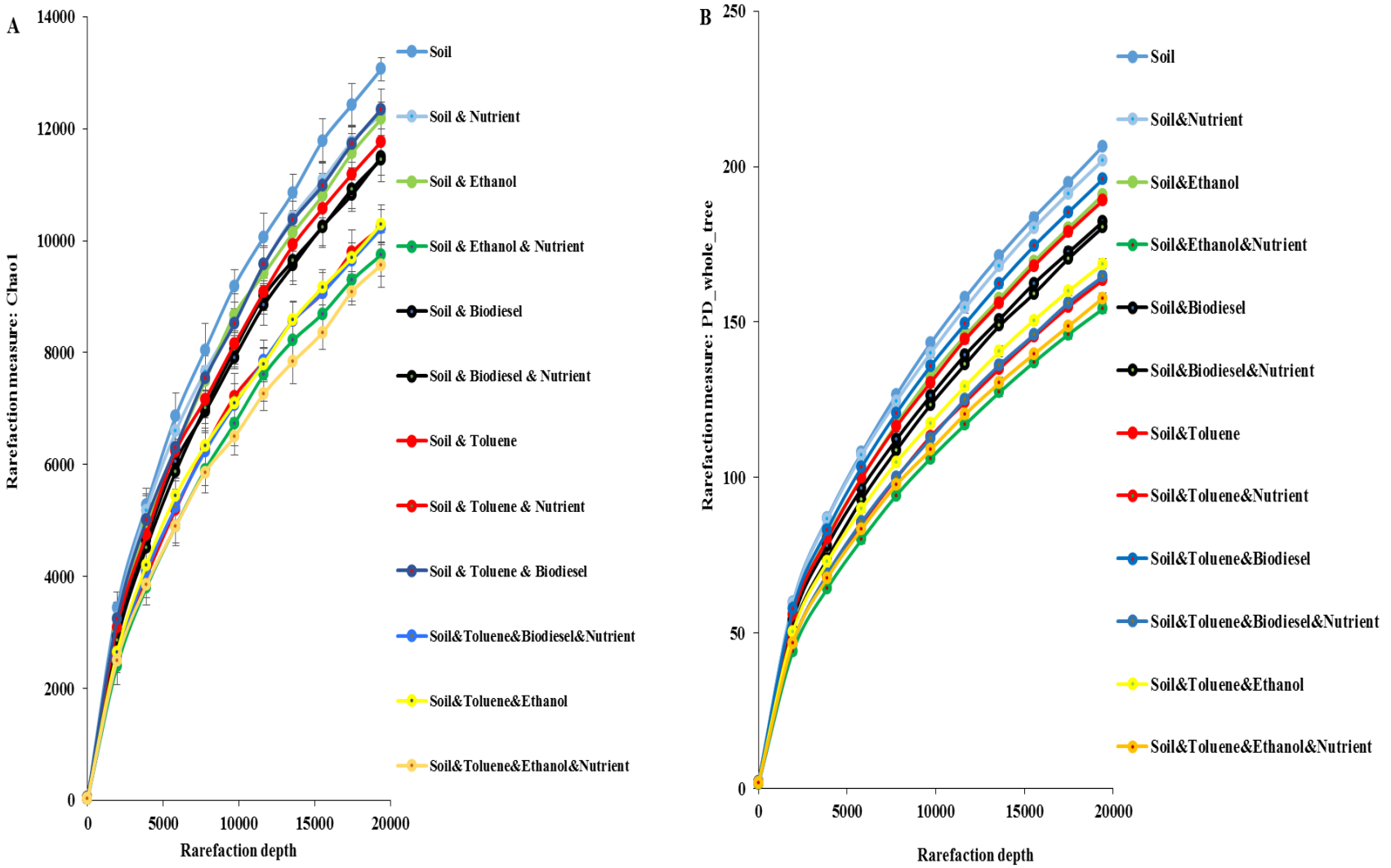

Figure 5-3 Rarefaction curves for treatment samples. (A) Chao1 estimator (B) P.D_whole_tree estimator. Error bars represent ±1 standard deviation (SD=3) from the mean of 3 replicates

5.4.4 Taxonomy composition and OTU detection and functions

For the taxonomic compositions at phylum level, within the bacteria domain there were 42 classified and 1 unclassified phyla while there were 3 classified and 1 unclassified phyla belonging to the archaea domain The taxonomic summaries for the ion torrent sequencing platform (at phyla level) for the most abundant bacteria and archaea (representing ≥2.0 % of overall relative abundances) across the various samples under study is shown in (Figure 5-4). The identifiable taxa include *Actinobacteria, Bacteroidetes, Chloroflexi, Planomycetes, Gemmatimonadetes, Acidobacteria* and *Proteobacteria* broken up in to the classes of: *Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, and Gammaproteobacteria representing* 19.5, 5.9, 7.5, 4.0, 5.4, 4.6, 7.4, 11.2, 2.1 and 22.6%, respectively.

For the treatment effects at phylum and class level, the results showed that ethanol plus nutrient amendment possibly affects the abundance of *Alphaproteobacteria,* as the relative abundance was lower (t.test, 2 sided, unpaired, $p<0.05$) in Soil & toluene & ethanol & nutrient batches and Soil & ethanol & nutrient batches compared to other treatment soils (Figure 5-4). *Betaproteobacteria* seems to benefit more from the addition of both biodiesel and nutrients as there is an increase (t.test, 2 sided, unpaired, $p<0.05$) in its relative abundance in Soil & biodiesel & nutrients batches compared to controls and other treatment soils (Figure 5-4). This indicates that class *Betaproteobacteria* possibly plays a role in the degradation biodiesel when nutrient availability is high and in the absence of toluene. The relative abundances of *Deltaproteobacteria, Planomycetes* and *Acidobacteria* (Figure 5-4 and Figure 5-5) were lower in batches with single or blended fuels with nutrient amendment (t.test, 2 sided, unpaired, $p<0.05$) compared to controls and treatment batches with no nutrient amendment. There was a similar result seen for *Bacteroidetes* (Figure 5-4 and Figure 5-6) in single or blended fuels with nutrient amendment with the exception of Soil & biodiesel & nutrients. Thus indicating that these phyla and class (i.e. *Deltaproteobacteria, Planomycetes Acidobacteria)* of bacteria might not have really benefitted from nutrient amendment. It also shows that *Bacteroidetes* thrives better than the other phyla groups (*Deltaproteobacteria, Planomycetes Acidobacteria*) when biodiesel is added together with nutrients. There was also no significant difference between the relative abundances of *Bacteroidetes* in Soil & toluene & biodiesel batches and Soil & toluene & biodiesel & nutrients batches indicating that *Bacteroidetes*

does well in biodiesel and toluene blends with or without nutrient amendment. Compared to Soil (control), Soil & toluene, Soil & toluene & biodiesel and Soil & toluene & ethanol batches, the relative abundance of *Chloroflexi* and *Gemmatimonadetes*, (Figure 5-4 and Figure 5-6) was lower in Soil & ethanol batches and Soil & biodiesel batches (t.test, 2 sided, unpaired, $p<0.05$) and significantly lower in all single and blended fuel batches with nutrient amendment (t.test, 2 sided, unpaired, $p<0.001$). The results also show that *Chloroflexi* (Figure 5-4 and Figure 5-6) seems to benefit from the addition of toluene as a single fuel and does well when toluene is blended with ethanol or biodiesel with no nutrients added. Thus indicating *Chloroflexi's* possible involvement in the degradation of toluene when nutrients are scarce. *Chloroflexi* has been previously linked with degradation of toluene (Fowler *et al.*, 2014) while both *Chloroflexi* and *Gemmatimonadetes* have both been linked with the biodegradation of PAHs (Muangchinda *et al.*, 2015).

A higher abundance of *Gammaproteobacteria* was seen (t.test, 2 sided, unpaired, $p<0.05$) in Soil & biodiesel & nutrient batches and Soil & toluene & biodiesel & nutrient batches which had significantly higher abundance (t.test, 2 sided, unpaired, $p<0.001$) in Soil & ethanol & nutrient batches and Soil & toluene & ethanol & nutrient batches when compared to control batches (Soil and Soil & nutrients) and single or blended fuel batches (with no nutrients added) (Figure 5-4). The addition of nutrients to Soil & Toluene batches had little impact (t.test, 2 sided, unpaired, $p>0.05$) on the abundance of Class *Gammaproteobacteria* as amendment only increased the abundance slightly. This shows that *Gammaproteobacteria* seems to benefit from the addition of biofuels and biofuels and hydrocarbon blends when there is high nutrient availability (Figure 5-4). Thus indicating Class *Gammaproteobacteria* possible involvement in the degradation of biofuels in the absence or presence of hydrocarbons when nutrients are readily available. *Gammaproteobacteria* contains organisms such as *Pseudomonas* which have been connected with the biodegradation of toluene (Cápiro *et al.*, 2008; Gupta *et al.*, 2008). *Pseudomonas* is a well know BTX degrader and has been previously detected in ethanol and toluene blends (Cápiro *et al.*, 2008; Ma *et al.*, 2013). Another organism belonging to *Gammaproteobacteria* is *Pseudoxanthomonas* which has been linked with the degradation of hydrocarbons such as phenanthrene (Patel *et al.*, 2012) and BTEX (Choi *et al.*, 2013). The relative abundance of *Actinobacteria* in Soil & toluene was much higher (t.test, 2 sided, unpaired, $p<0.001$) compared to controls batches (Soil and Soil & nutrients), ethanol or biodiesel batches (with nutrient amendment) and toluene and biofuel blended batches (with nutrient amendment) (Figure 5-4). The addition of nutrients to Soil & toluene batches slightly increased the abundance of *Actinobacteria* (t.test, 2 sided, unpaired, $p>0.05$) when comparing Soil

& toluene batches and Soil & toluene & nutrient batches. However, the abundance of *Actinobacteria* in Soil & toluene & nutrients batches was still seen to be higher (t.test, 2 sided, unpaired, $p<0.05$) than the abundance in Soil & toluene & ethanol & nutrients batches but not significantly (t.test, 2 sided, unpaired, $p>0.05$) higher than the abundance in Soil & toluene & biodiesel & nutrients batches. Similarly, the abundance of *Actinobacteria* in Soil & toluene batches was higher compared to Soil & ethanol batches (t.test, 2 sided, unpaired, $p<0.05$) but not significantly higher compared to the abundance in Soil & biodiesel batches (t.test, 2 sided, unpaired, $p>0.05$). The results at phylum level generally show that nutrient amendment did not significantly lead to an increase in *Actinobacteria* abundance and it also showed that ethanol seems to have negative effect on *Actinobacteria* abundance in the presence of toluene (with or without nutrient amendment) while biodiesel has a lesser effect. *Actinobacteria* also seems to benefit from the addition toluene and possibly biodiesel and indicates possible involvement in the degradation of both toluene and biodiesel. *Actinobacteria* contains organisms such as, *Rhodococcus and Pseudonocardia* which have been previously shown to degrade toluene (Juteau *et al.*, 1999). It also contains *Nocardia* which has been previously reported to use toluene and benzene as carbon sources (Jamison *et al.*, 1969).

The observation of treatment effects going down to the order level (Figure 5-7) showed that most of the OTUs at 2% relative abundance across all samples belonged to the dominant phyla of *Actinobacteria, Bacteroidetes*, *Planomycetes,* and *proteobacteria* classes: *Alphaproteobacteria*, *Gammaproteobacteria* (Figure 5-4). Generally for the treatment effect, the results showed that on average, *Pseudomonadales* which belongs to class *Gammaproteobacteria* was higher (t.test, 2 sided, unpaired, $p<0.05$) in Soil & ethanol & nutrient and Soil & toluene & ethanol & nutrient batches compared to Soil & toluene & biodiesel & nutrient batches and significantly higher (t.test, 2 sided, unpaired, $p<0.001$) in comparison to the control (Soil and Soil & nutrient) and the other single fuel batches (with and without nutrient amendment) and blended fuel batches without nutrient amendment. This indicates that *Pseudomonadales* seems to benefit from the addition of ethanol and nutrients and can possibly degrade ethanol in the presence of toluene when nutrient availability is high. *Actinomycetales,* which belongs to *Actinobacteria,* was higher in Soil & toluene and Soil & toluene & nutrients batches in comparison to Soil & ethanol batches, Soil & toluene & ethanol batches (both with our without nutrients) and controls (t.test, 2 sided, unpaired, $p<0.05$). However, there were no significant difference (t.test, 2 sided, unpaired, $p>0.05$) for *Actinomycetales* when Soil & toluene and Soil & toluene & nutrients batches were compared to

Soil & biodiesel or Soil & toluene & biodiesel batches. This result show that *Actinomycetales* probably does better with the addition of biodiesel than ethanol especially when no nutrients are added as *Actinomycetales* abundance in Soil & toluene was higher (t.test, 2 sided, unpaired, $p<0.05$) than in Soil & biodiesel & nutrients and Soil & toluene & biodiesel & nutrients. This also indicates *Actinomycetales* possible involvement in degradation of toluene and biodiesel and ethanol limits its abundance even with nutrient addition. Order *Burkholderiales* and *Xanthomonadales* seem to benefit from the addition of biodiesel, as they were higher (t.test, 2 sided, unpaired, $p<0.05$) in Soil & biodiesel & nutrient batches in comparison to controls and other treatment soils.

The increase or decrease in rank relative abundance due to varying treatment effects was determined at species level relative to the Soil sample (control) OTUs ranked relative abundance (Table 5-3,). The sample representations are as follows: +N (Soil & nutrient), +E (Soil & ethanol), +E+N (Soil & ethanol & nutrient, +B (Soil & biodiesel), +B+N (Soil & biodiesel & nutrient), +T (Soil & toluene), +T+N (Soil & toluene & nutrient), +B+T (Soil & toluene & biodiesel), +B+T+N (Soil & toluene & biodiesel & nutrient), +E+T (Soil & toluene & ethanol), +E+T+N (Soil & toluene & ethanol & nutrient.

An OTU that gained significantly in its relative abundance ranking in one of the treatments is an OTU, which has a competitive advantage under these specific conditions (i.e. with biofuel, toluene, and nutrient addition). Unsurprisingly, then, nitrifying bacteria *Nitrosomonadaceae* and *Nitrospira* are seen to make significant gains in OTU ranking and relative abundance due to the nutrient amendment. *Nitrospira*, which belongs to phylum *Nitrospirae* increased by 4 folds in Soil & nutrient batches, 2 folds in Soil & ethanol & nutrient batches and 2 folds in Soil & biodiesel & nutrient batches. *Nitrosomonadaceae* which belongs to class *Betaproteobacteria*, increased by 8 folds in Soil & nutrient batches, 8 folds in Soil & ethanol & nutrient batches and 2 folds each in Soil & biodiesel & nutrient, Soil & toluene & nutrient, Soil & toluene & biodiesel & nutrient and Soil & toluene & ethanol & nutrient. Both *Nitrosomonadaceae* and *Nitrospira* benefit from the ammonia (NH_4Cl) in the nutrient added to the batches. Organisms which belong to *Nitrosomonadaceae* and *Nitrospira* are of great importance in nitrogen cycling (Arp *et al.*, 2007). Ammonia oxidizing bacteria mostly belong to class *Nitrosomonadaceae* and are known to convert ammonia to nitrite while the nitrite oxidizing bacteria some of which are found in class *Nitrospira* converts nitrite to nitrate (Arp *et al.*, 2007). The family *Nocardioidaceae* which includes genera *Aeromicrobium* makes more gains with the addition of biodiesel and has been previously linked

with the degradation of hydrocarbon compounds (Chaillan *et al.*, 2004). Indicating that *Nocardioidaceae* possibly plays a role in biodiesel degradation when nutrients are scarce. *Nocardiaceae*, which includes the genera *Rhodococcus* and *Nocardia*, makes significant gains due to addition of biodiesel or toluene and biodiesel blends but hardly any gains with addition of ethanol. Thus indicating *Rhodococcus* possible involvement in the degradation of toluene while *Nocardia* is possibly involved in the biodiesel degradation. The biodiesel used in this study comprises of fatty acid methyl esters (FAME) such as palmitic acid (Demirbas, 2009c) which can be hydrolyzed to methanol and fatty acids (Stolz *et al.*, 2002; Sousa *et al.*, 2007; Aktas *et al.*, 2010). *Nocardia species* are hydrocarbon degraders and cells are known to grow on a variety of PAHs (Zeinali *et al.*, 2007), however they are also know to degrade fatty acids (Taylor and Wain, 1962) and seem to benefit from the addition of biodiesel. *Methylophilaceae* and *Methylotenera mobilis* also benefit from the addition of biodiesel and nutrients as they are seen to increase by 4 fold and 16 fold respectively in Soil & biodiesel & nutrient batches. *Methylotenera Methylophilaceae, which belongs to class Betaproteobacteria,* includes specie *Methylotenera mobilis*. *Methylotenera* are known to thrive in diverse environs (Lapidus *et al.*, 2011) and are main consumers of methanol in situ (Kalyuhznaya *et al.*, 2009). It has also been reported that nitrate stimulates the growth of *Methylotenera spps such as Methylotenera mobilis* as the organism was only able to grow on methanol in the presence of nitrate but not in the presence of ammonium (Kalyuhznaya *et al.*, 2009). The fact there are nitrite-oxidizing bacteria such as *Nitrospira* present could be a reason why *Methylotenera mobilis* makes significant gains with biodiesel and nutrient addition.

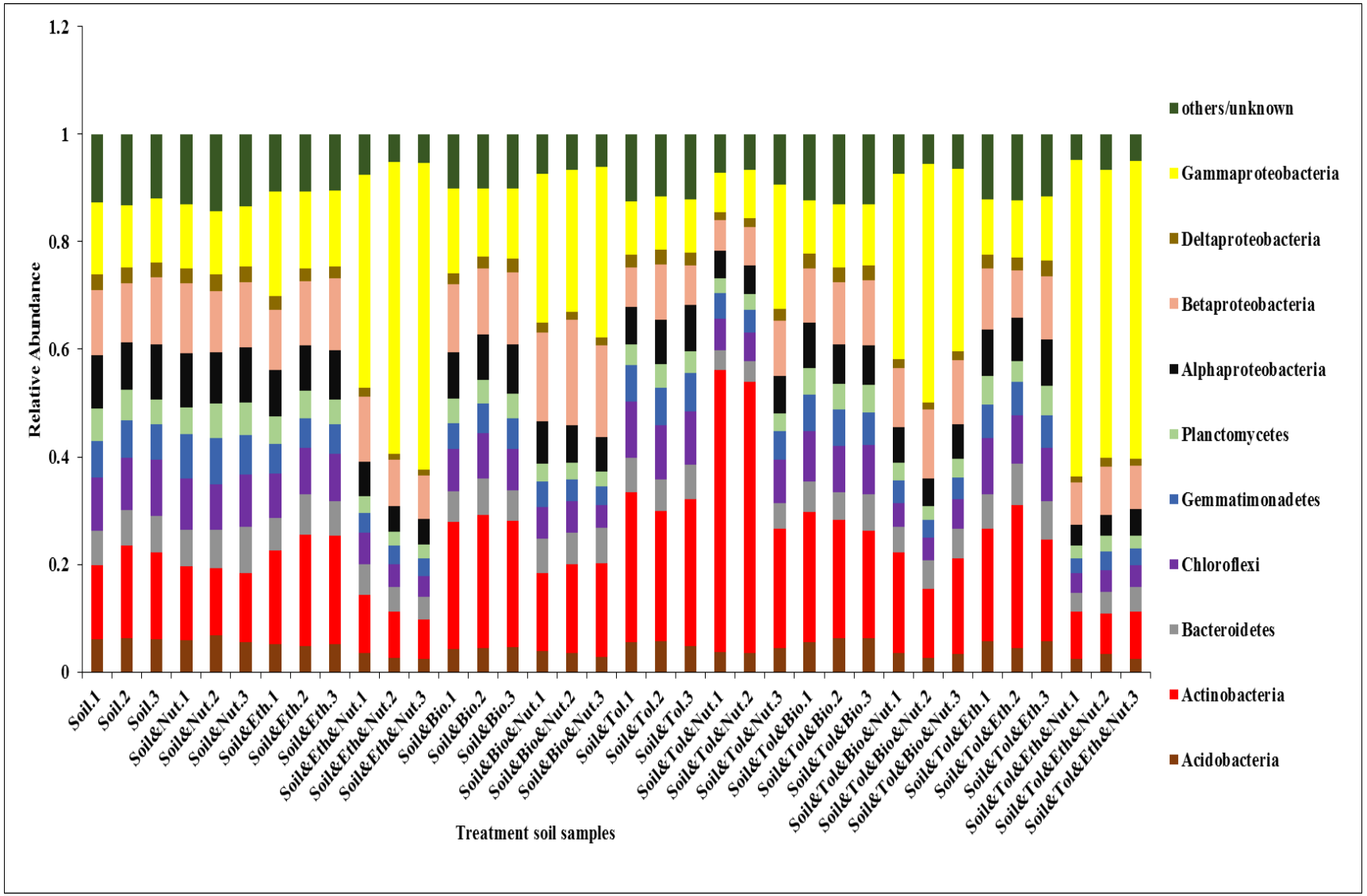

Figure 5-4 Relative abundance of phylogenetic groups in treatment soils. Phyla accounting for ≥ 2.0% of all classified sequences

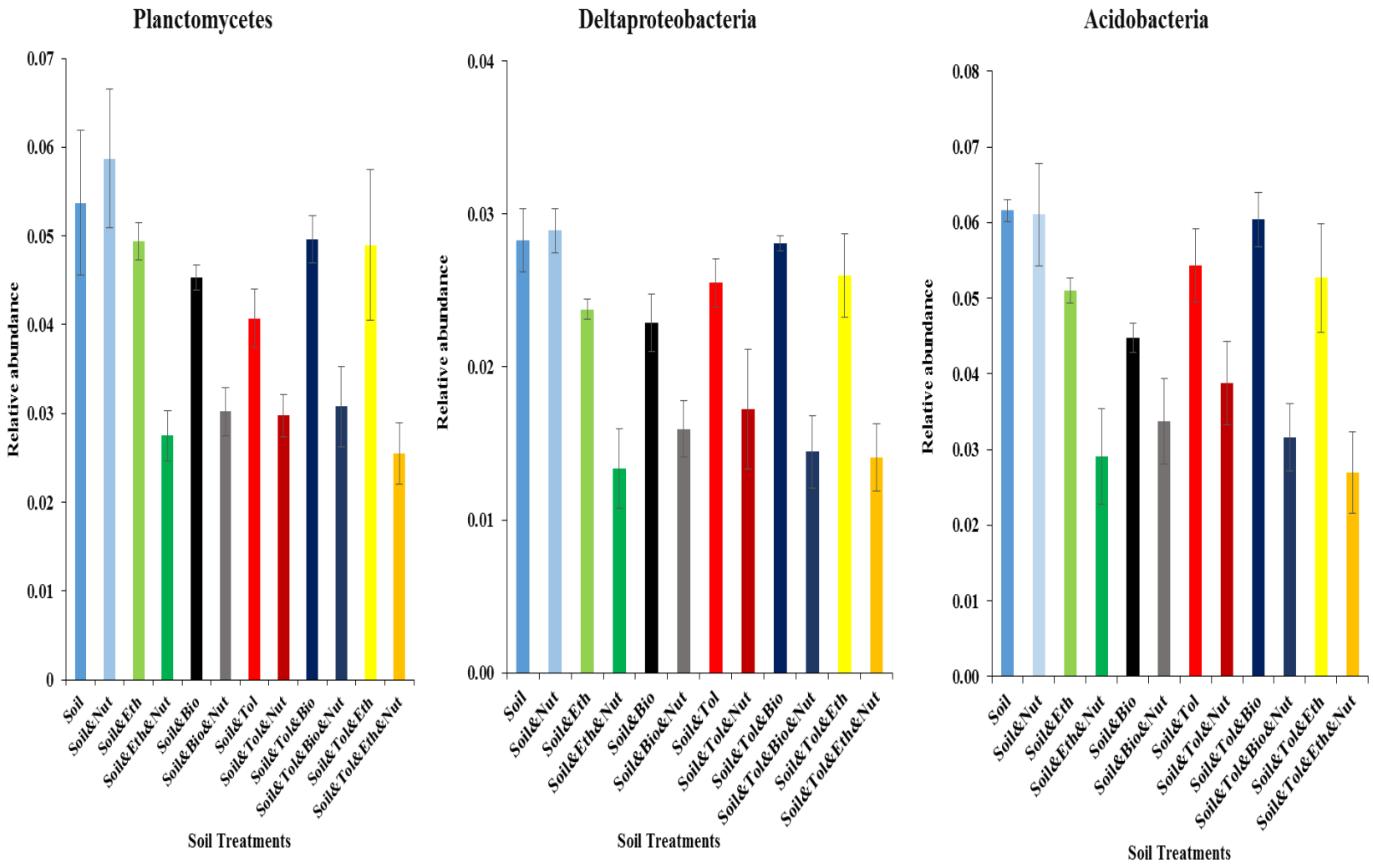

Figure 5-5 Treatment effects. Average relative abundance of organisms detected at phylum level Error bars represent ±1 standard deviation (SD=3) from the mean of 3 replicates

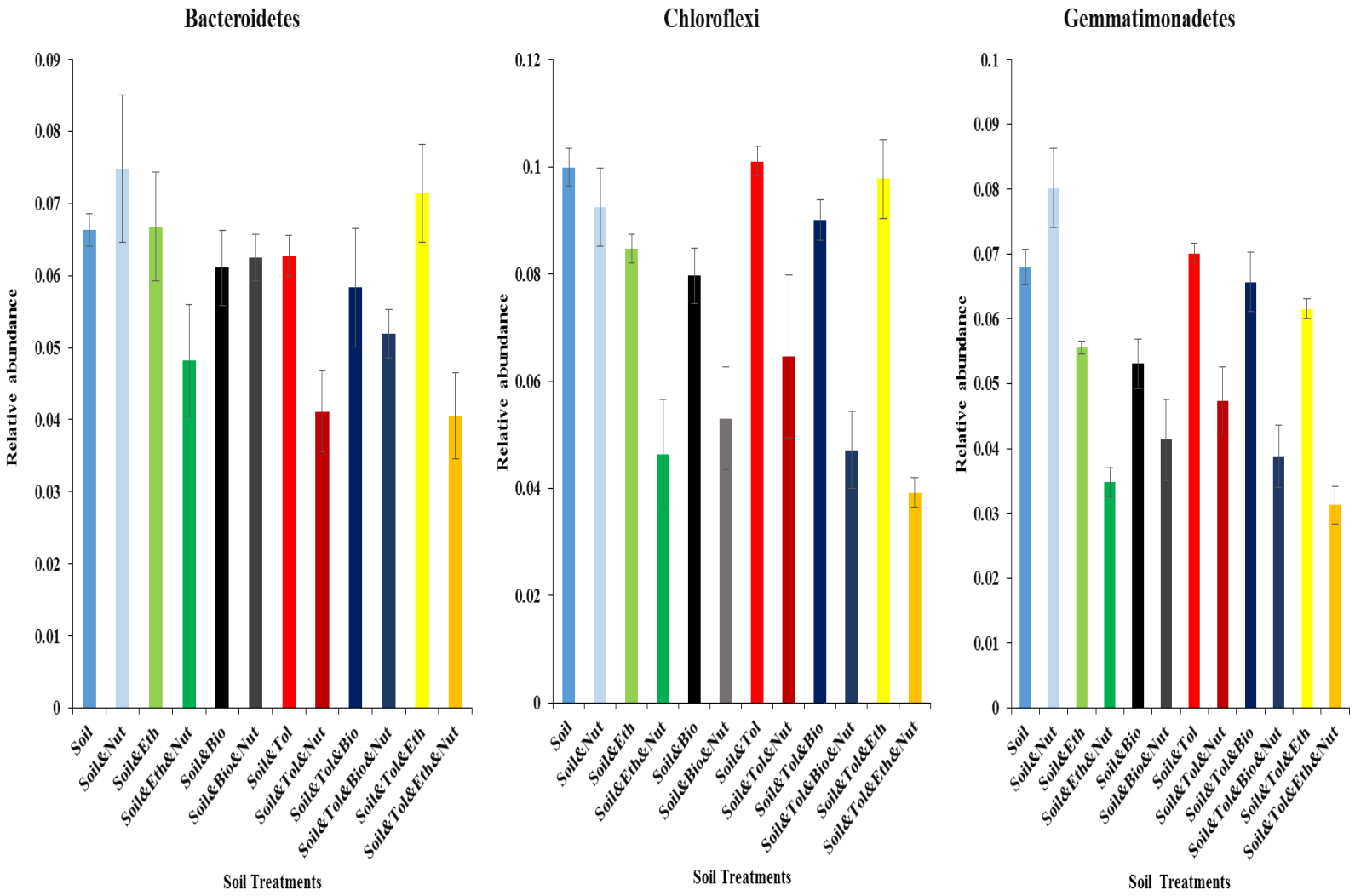

Figure 5-6 Treatment effects. Average relative abundance of organisms detected at phylum level Error bars represent ±1 standard deviation (SD=3) from the mean of 3 replicates

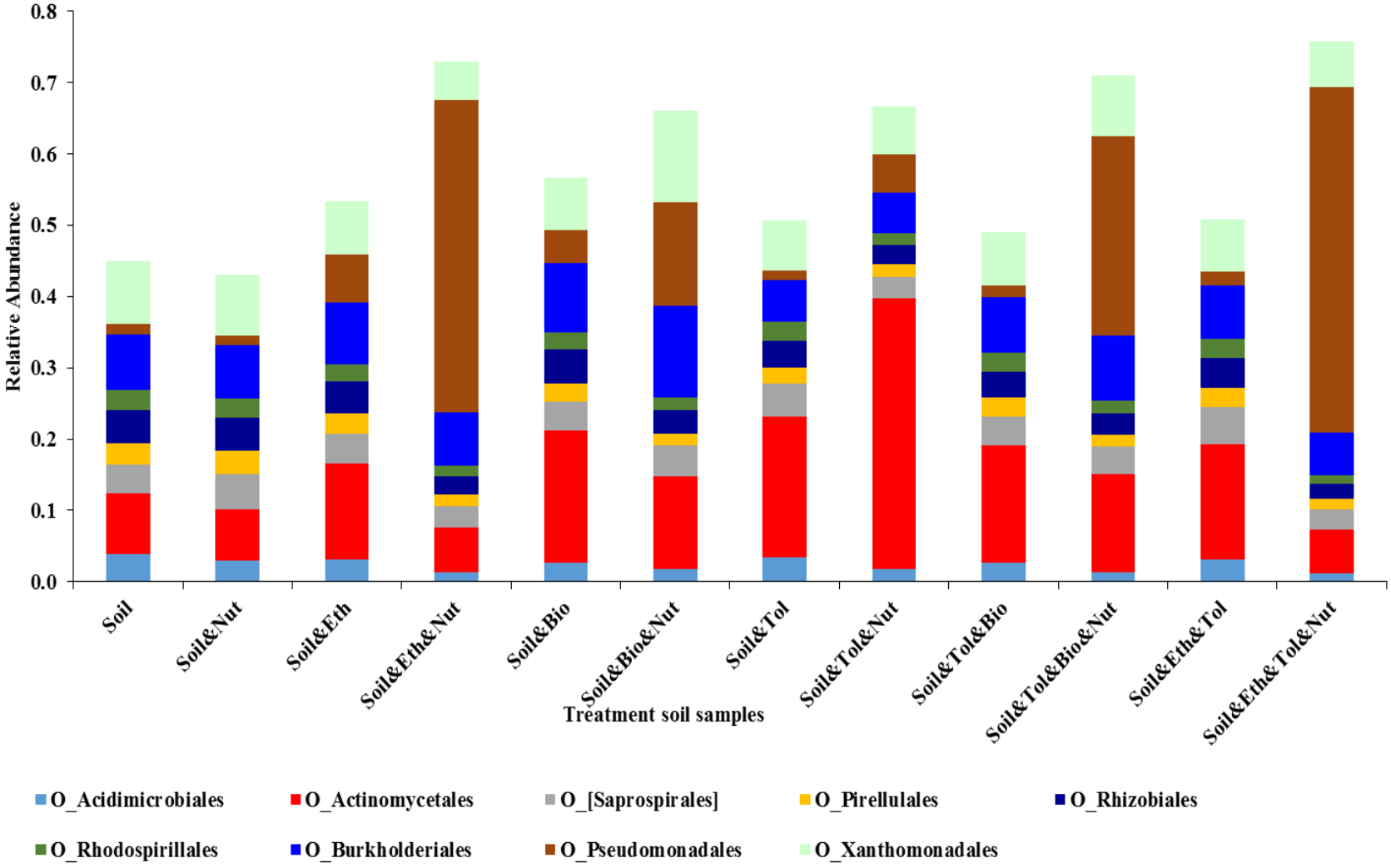

Figure 5-7 Average relative abundance of replicate treatment soils. Order level accounting for > 2.0% of all classified sequences

Other organism that benefitted from biodiesel addition includes *Hydrogenophaga* and the family *Xanthomonadaceae* that consist of the genera *Lysobacter* and *Pseudoxanthomonas*. *Hydrogenophaga* species are known to produce Polyhydroxyalkanoates (PHA) (Tanamool *et al.*, 2011) and methanol and fatty acids are known precursors or substrates used in the production of PHA (Jiang *et al.*, 2016a). This is probably why it increased up to 4 fold with the addition of biodiesel and nutrients. *Pseudoxanthomonas* also increases by 4 fold with the addition of biodiesel and nutrients. *Pseudoxanthomonas* has been previously linked to the degradation of PHAs (Volova *et al.*, 2016) and PAHs and has been detected media containing diesel (Restrepo-Florez, 2013). The results in Table 5-3 shows that genera *Rhodococcus and Pseudonocardia* which belong to phylum *Actinobacteria* are seen to make gains in OTU ranking and relative abundance due to the addition of toluene. *Rhodococcus* increased when toluene was added to a batch as it increased by 32 folds in Soil & toluene batches and up to 64 folds in Soil & toluene & nutrient batches. *Rhodococcus* species are known to utilize a range of hydrocarbons as carbon sources (Song *et al.*, 2011) one such detected in this study is *Rhodococcus globerulus* which increased 8 folds (each) in both Soil & toluene batches and Soil & toluene & nutrient batches. *Rhodococcus globerulus* has been shown to degrade biphenyls (Asturias and Timmis, 1993) and was detected in soil contaminated with benzene, toluene, styrene, xylene, dicyclopentadiene and naphthalene and was able to utilize styrene as source of carbon and energy (Greene *et al.*, 2000). *Rhodococcus* also increased by 8 folds in Soil & toluene & biodiesel (with and without nutrient amendment) and by 16 fold in Soil & toluene & ethanol batches. No increase was seen in Soil & toluene & ethanol & nutrients batches for *Rhodococcus globerulus or Rhodococcus*. Thus indicating that *Rhodococcus* can degrade toluene in the presence of biodiesel when nutrients are surplus or scarce and can degrade toluene in the presence of ethanol when nutrients are scarce. The results also show that *Rhodococcus* can compete with *Pseudomonadaceae* members in Soil & toluene & ethanol when nutrients are scarce, but not if nutrients are abundant. *Pseudonocardia* has a Soil (control) ranking of 22 increased by 4 fold in both Soil & toluene and Soil & toluene & biodiesel batches and it also seems to do better without nutrient addition. *Pseudonocardia* has been shown to degrade both toluene and xylene (Marek *et al.*, 2001) and host of compounds which include phenol, trichloroethane, benzothiphene sulfones etc (Lee *et al.*, 2004). The family of the *Pseudomonadaceae*, which includes Pseudomonas, benefit the most from the ethanol or biodiesel addition and make significant gains in ethanol amended batches and biodiesel batches (both with and without nutrients).

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											
	Soil Rank	+N	+E	+E+N	+B	+B+N	+T	+T+N	+B+T	+B+T+N	+E+T	+E+T+N
o__Actinomycetales	86				+	+	+	++	+	+		
f__Intrasporangiaceae	84		+	+	+	+	+	++	+	++	++	+
s__Agrococcus jenensi	670				+		+	+	+	+	+++	+
g__Agromyces	266		+		+	+	+	+	+	++	++	+
f__Micrococcaceae	27		+	++	+		+	++	++	+	+	++
f__Nocardiaceae	539				+	++	++	+++	+	++	+	
g__Nocardia*	579				+	++				+		
g__Nocardia*	203				+++	+++++	+		+	++++		
g__Rhodococcus*	183						+++	++++++	+	+++	+	
g__Rhodococcus*	24						+++++	+++++	++	++	+++	
s__Rhodococcus globerulus	524						++	++	+	+	+	
f__Nocardioidaceae	40	-	+		++	+	+	+	++	+	+	+
f__Nocardioidaceae	69	-	+		++	+	+	+	++	+		
g__Aeromicrobium*	6	-	+	-	++	-		--	-	--	-	-
g__Pseudonocardia*	22		+		+		++	+	++		+	+
g__Nitrospira	229	++		+		+						
g__Hydrogenophaga	8	-		+	+	++	--			+		+
f__Methylophilaceae	321					++				+		
s__Methylotenera mobilis	213		+		+	++++				++		
f__Nitrosomonadaceae	379	++		+		+		++		+		+
f__Nitrosomonadaceae	392	+++		+++		++				++	+	++
f__Pseudomonadaceae	25		++++	+++++	+++	+++++		++++		++++	+++	++++
f__Pseudomonadaceae	136			++++++		+				++++++	++++++	+++++
g__Pseudomonas*	104		++	+++++	++	++++		+		++++	+	++++
g__Pseudomonas*	470			++		+			+	++	+	++
f__Sinobacteraceae	19						+	+	+	++	+	+
f__Xanthomonadaceae	46		-	+		++			-	+		
g__Lysobacter	39	+		+		+++			-	+		-
g__Pseudoxanthomonas*	539	+	+	+	+	++	+	+	+	++	+	+
g__Thermomonas	66			+		++		+		+		

Table 5-3 Increase and decrease in ranked relative abundance compared to untreated soils

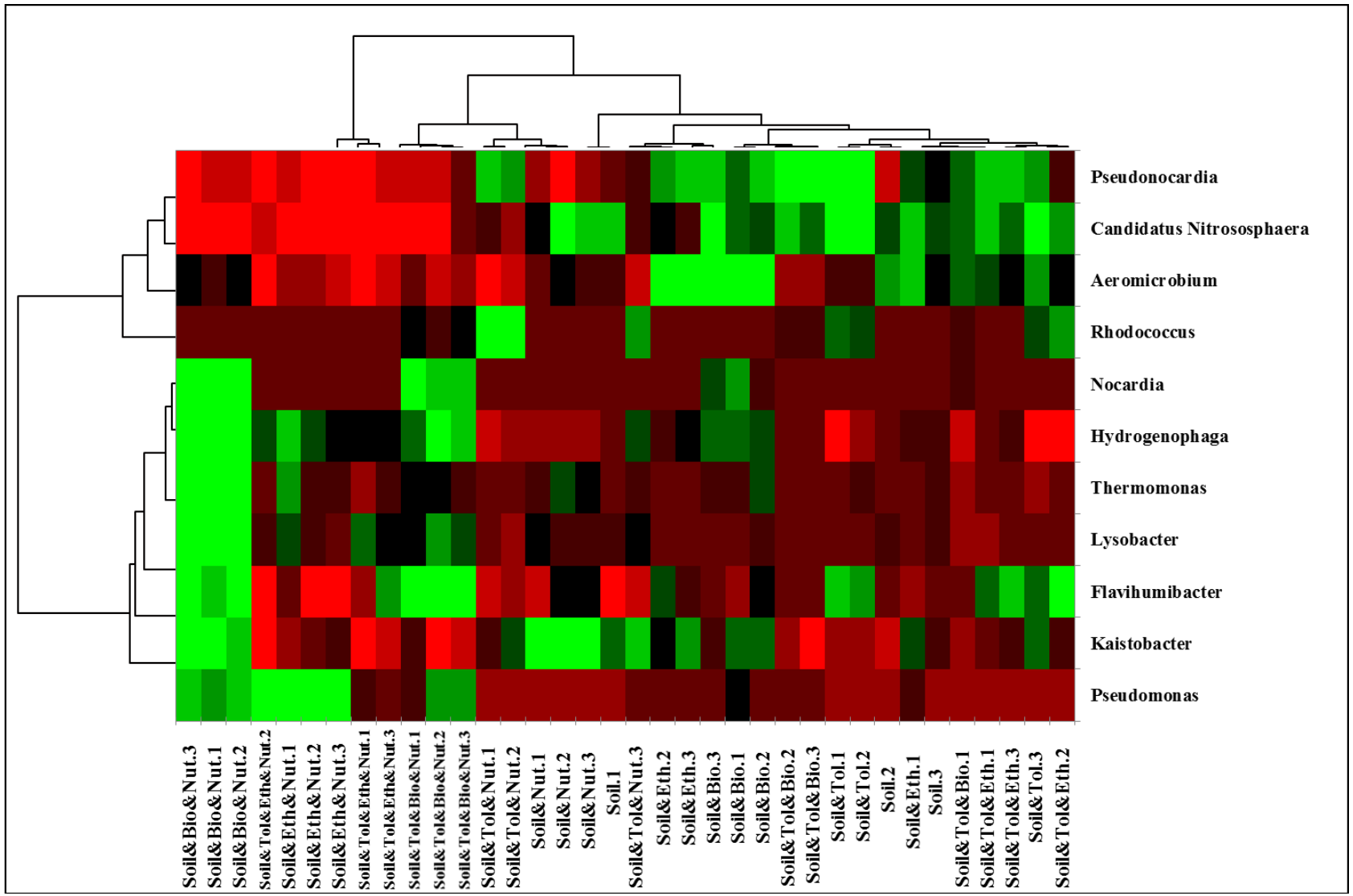

Figure 5-8 Heatmap with dendograms illustrating treatment effects (OTU abundance >0.5 at genus level). Samples in columns and OTUs in rows. Colour coding: low, intermediate and high abundance- Red triangles, black triangles, green triangles respectively

Pseudomonadaceae also possess the ability to compete with members of *Nocardiaceae* in toluene or biodiesel batches when nutrient are added. *Pseudomonas* has been shown to utilise a variety of hydrocarbon compounds such as hexadecane, n alkanes (Barathi and Vasudevan, 2001) and toluene (Zylstra and Gibson, 1989). *Pseudomonas* is also known to oxidise ethanol to acetic acid (Madigan *et al.,* 1997).

5.4.5 Treatment effects on bacteria communities

The heat map (Figure 5-8) is an illustration of relative abundance relationships between samples detected at genus level. The results show a demarcation, as the samples with nutrient amendments clustered together on one side of the heat map and samples with no nutrients on the other side (with the exception of Soil), thus illustrating strong effects of nutrients in shaping the microbial communities. Similar to the results for the ranked abundances, *Pseudomonas* is highly abundant in Soil & ethanol & nutrients batches and fairly abundant in Soil & biodiesel & nutrient batches. Thus indicating possible ethanol and biodiesel degradation when nutrient availability is high. *Pseudomonas* has been previously linked with the biodegradation of methylesters in diesel and biodiesel blends (Owsianiak et al., 2009). *Pseudomonas* abundance is low in biodiesel or ethanol batches (single or blended fuel) with no nutrients added. *Pseudomonas* is fairly abundant in biodiesel blended fuel batches (2 replicates samples of Soil & toluene & biodiesel & nutrients) upon nutrient amendment. This indicates that nutrient amendment improves *Pseudomonas* abundance in toluene & biodiesel blended batches but not in all Soil & toluene & ethanol & nutrients batches as the abundance remains low even with nutrient amendment. Also similar to the results for the ranked abundances, *Pseudonocardia* is seen to be abundant in batches which have toluene and seems to be more abundant in samples with no nutrient amendment, *Rhodococcus* is also more abundant in Soil & toluene & nutrient compared to other treatment samples. *Nocardia, Hydrogenophaga, Thermomonas, Flavihumibacter, Kaistobacter* were all highly abundant in Soil & biodiesel & nutrient batches while *Nocardia, Hydrogenophaga Flavihumibacter* was highly abundant in Soil & toluene & biodiesel & nutrients batches in comparison Soil & toluene & ethanol & nutrients batches. *Flavihumibacter* belongs to family *Chitinophagaceae* and which contains species believed to be of great importance in bioremediation as it can utilise diverse carbon sources which includes succinic acid mono-methyl esters (Lee *et al.*, 2014). Some species of *Flavihumibacter* are known to utilise 4-hydroxyphenlyacetic acid (Zhou *et al.*, 2015) which is an

anti-oxidant compound found in rape seed and olive oil (Papadopoulos and Boskou, 1991). The composition of biodiesel used in this study has been previously reported by Elazhari-Ali et al. (2013) and contains methyl ester from rapeseed oil. *Kaistobacter* also seems abundant in Soil & nutrient (control) samples while *Aeromicrobium* is abundant in single biofuel batches with no nutrients. *Kaistobacter* belongs to *Sphingomonadaceae* and *Sphingomonadaceae* has been detected in diesel contaminated soils in extreme temperature condition with or with nutrient amendment (Yergeau *et al.*, 2012b).

The treatment effects on the general microbial community structure were determined using Bray Curtis similarity analysis (Figure 5-9 and Figure 5-10). For effects of nutrients on the general microbial communities, the NMDS plot (Figure 5-9 A) shows good clustering of the replicates of each sample with nutrient amendment (with exception of Soil & nutrients and Soil & toluene & nutrients batches). The communities for all samples had a percentage similarity of 75% while the communities for nutrient amended batches clustered together at percentage similarity of 80% (Figure 5-9 A). For effects of toluene, ethanol or biodiesel on the general microbial communities, the NMDS plot (Figure 5-9 B, Figure 5-10 A & B), the communities for all samples had a percentage similarity of 75% but there was no clear distinctive demarcation between samples with or without toluene, biodiesel or ethanol. The clearer and more distinctive demarcation between nutrient amended and nutrient un-amended batches (Figure 5-9 A) signify that nutrient was an important factor in determining community structure and has a major influence in shaping the microbial communities within batch samples. To back the results above, a dendogram for clustering similarity was also generated (Figure 5-11) and also shows (with exception of Soil & nutrients and Soil & toluene & nutrients batches) the samples of batches with nutrient amendment (including replicates of Soil & nutrients and Soil & toluene & nutrients batches replicates) clustered to one side and individual replicates of the nutrient amended batches clustered well together compared to batches with no nutrients added. Interestingly toluene seems to have a strong effect in batches without nutrient addition as (with exception of one replicate each for Soil & toluene and Soil & toluene & ethanol) toluene batches (single and blended) cluster to one side. Also it's possible that toluene had a stronger effect than the nutrient amendment in Soil & toluene & nutrients batches. The ANOSIM (analysis of similarities, based on Bray Curtis similarity coefficient) was carried out on Primer V6 software (Primer-E Ltd, Plymouth, UK) determining the significance of factors toluene, biodiesel, ethanol and nutrients addition. The results confirmed that nutrient as a factor had a significant effect on the microbial community structure with global R= 0.546, p= 0.001. The

results also confirmed that although nutrient amendment had the most effect, toluene was also significant in shaping the microbial communities with global R= 0.116, p = 0.02. The results also confirmed ethanol had more effect in shaping the microbial communities compared to biodiesel with global R= 0.138, p = 0.034 for ethanol and R= 0.043, p = 0.189 for biodiesel. The nested ANOSIM looking at ethanol or biodiesel as a factor within toluene amended batches confirmed that ethanol had a significant effect on the microbial communities within toluene and ethanol blended batches (global R= 0.174, p = 0.025) while biodiesel had a non-significant effect on the microbial communities within toluene and biodiesel blended batches (R= 0.08, p = 0.13).

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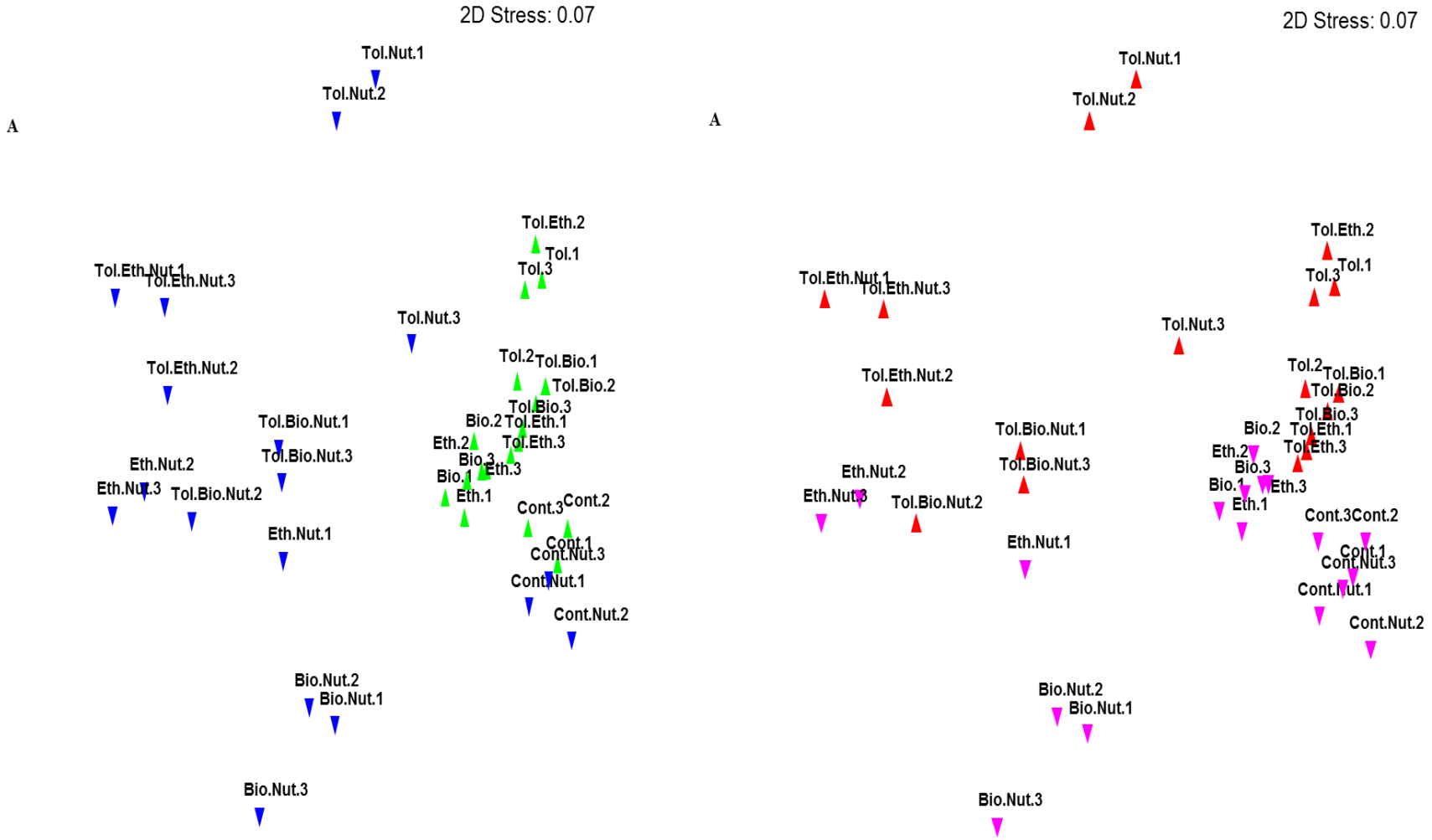

Figure 5-9 A multi-dimensional scaling: Treatment effect on bacterial community in soil batches (Ion torrent libraries). Analysis was based on Bray Curtis distance using square root transformed OTU table at level 6. A) Nutrient ▼, No Nutrient ▲ B) Toluene ▲, No Toluene ▼

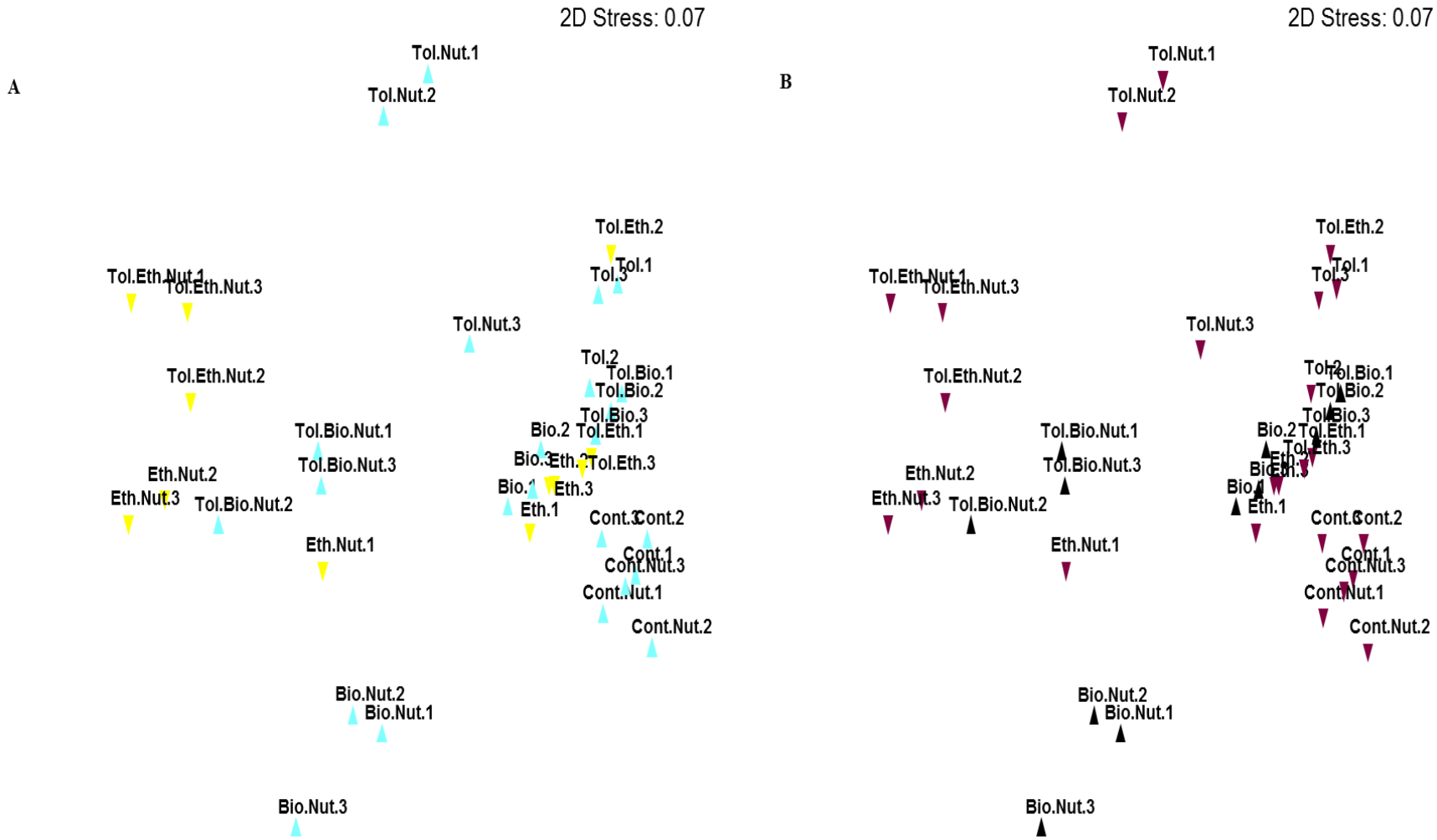

Figure 5-10 A multi-dimensional scaling: Treatment effect on bacterial community in soil batches (Ion torrent libraries). Analysis was based on Bray Curtis distance using square root transformed OTU table at level 6. A) Ethanol▼, No Ethanol▲ B) Biodiesel▲, No Biodiesel▼

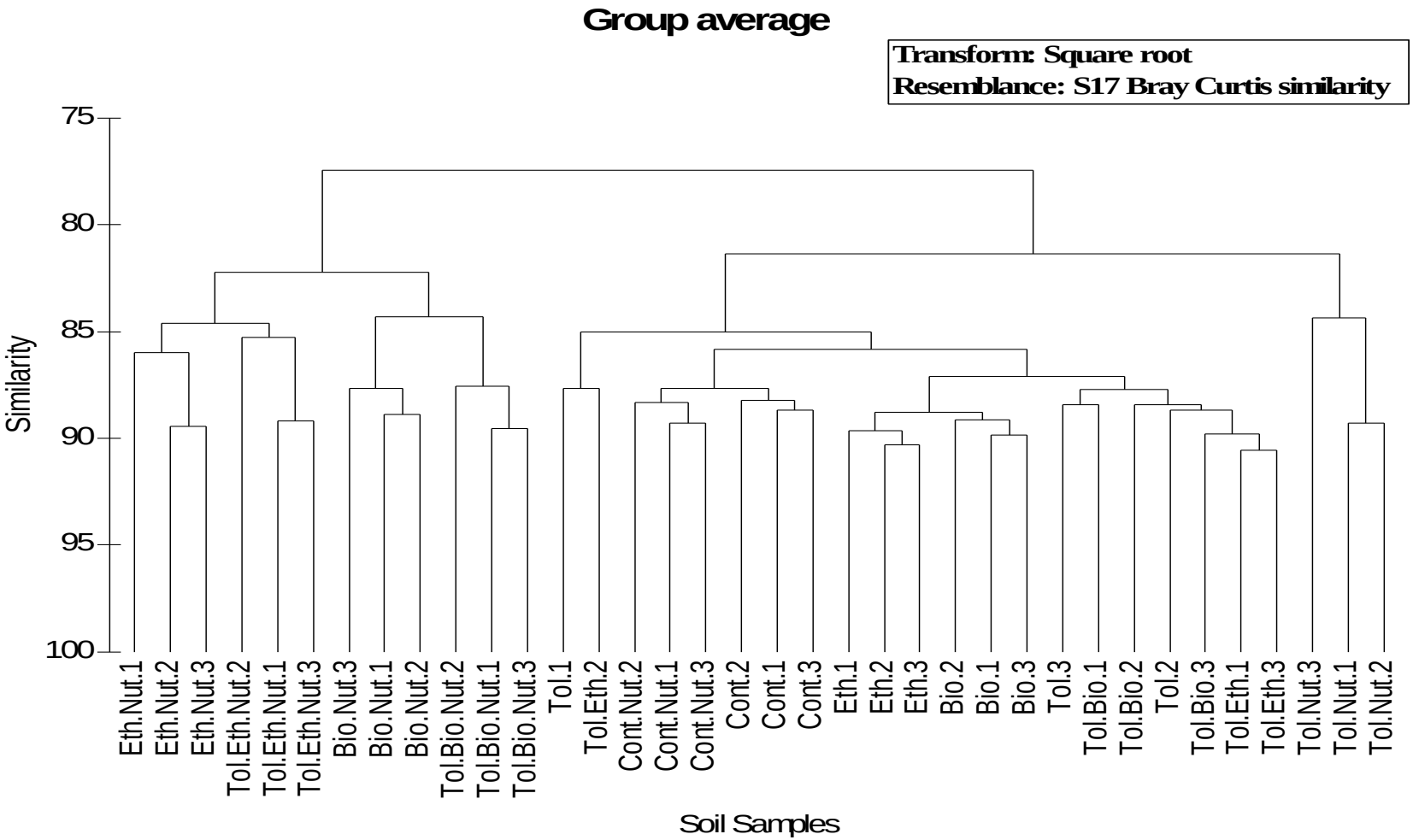

Figure 5-11 Hierarchical cluster analysis for treatment samples. Analysis was based on Bray Curtis distance using a square root transformed OTU table at Level 6

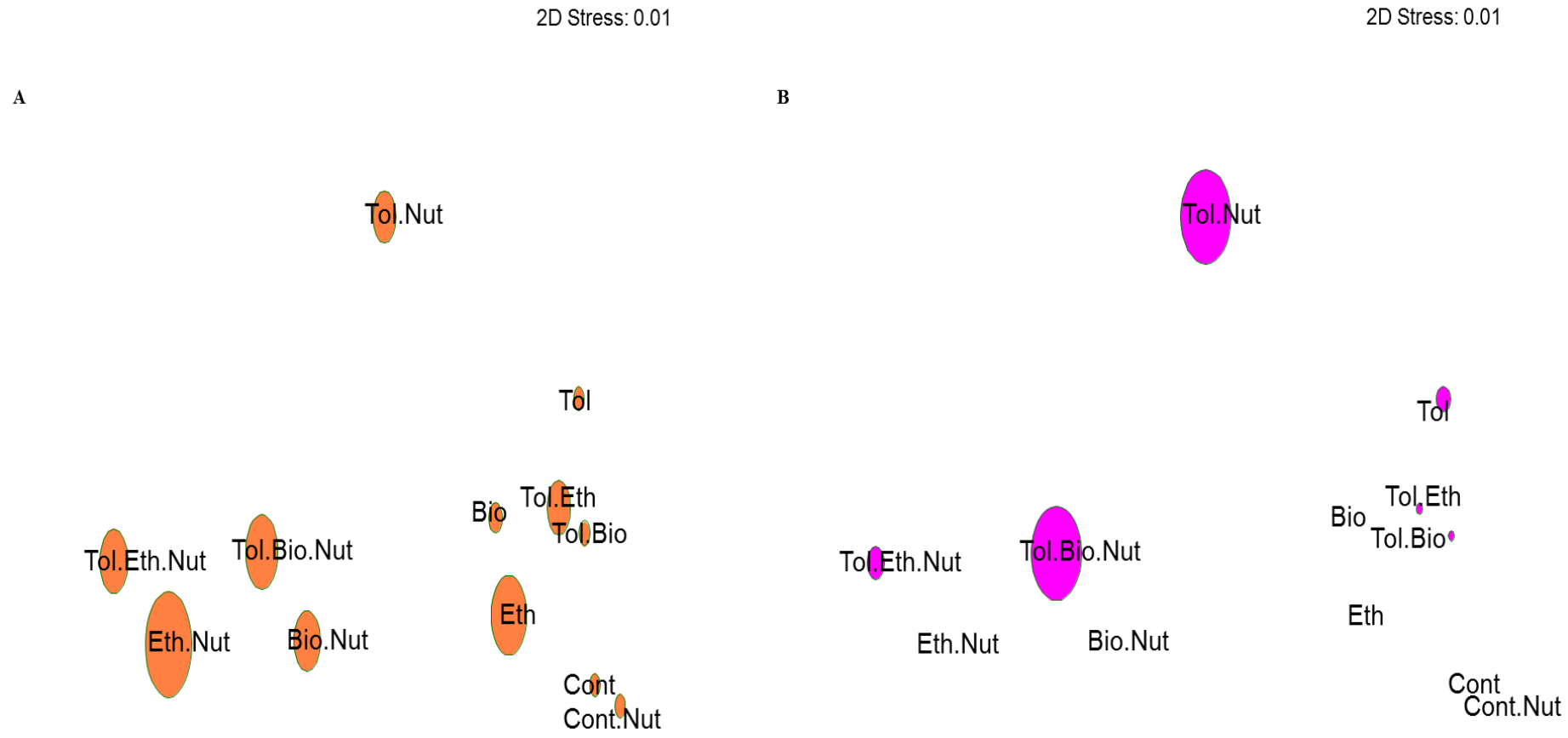

Figure 5-12 Integrated multi-dimensional scaling plot. A). $^{12}CO_2$, B) $^{13}CO_2$ (replicate average) produced (mg/batch) in treatment batches after 30 days Ion torrent platform

The integration data Figure 5-12, links $^{12}CO_2$, and $^{13}CO_2$ production with microbial communities within the batches. The larger bubbles signify higher $^{12}CO_2$, and $^{13}CO_2$ production (mg/batch) from the respective batches. The integrated data (Figure 5-12 A) showing higher $^{12}CO_2$ in single fuel batches, Soil & ethanol, Soil & ethanol & nutrients, Soil & biodiesel & nutrient and in mixed fuel batches, Soil & toluene & ethanol & nutrient batches and Soil & toluene & biodiesel & nutrient. This corresponds to high increase in relative abundances for organisms (See Table 5-3) such as *Pseudomonas* in Soil & ethanol and Soil & ethanol & nutrients batches and Soil & toluene & ethanol & nutrient batches. *Nocardia, lysobacter* and *Pseudomonas* are high in Soil & biodiesel & nutrient batches and *Nocardia, Pseudomonas* and *Rhodococcus* in Soil & toluene & biodiesel & nutrient. The $^{13}CO_2$ production (Figure 5-12 A) which is higher in Soil & toluene & nutrient correlates with higher relative abundance of *Rhodococcus.* Soil & toluene & biodiesel & nutrient which comprise of *Nocardia, Pseudomonas* and *Rhodococcus* also showing high $^{13}CO_2$ production.

5.4.6 Quantitative PCR analysis and estimated absolute abundance

For the quantitative PCR analysis, the efficiencies of the standards was determined and gave a linear standard curve and r^2 value was greater than 0.99 (Appendix C) and magnitude of $10^8 – 10^1$ gene copies per µL for all standards. The real time quantification results were expressed as gene copy numbers per gram of wet soil and the results in Figure 5-13 represent the average of replicates per sample. The results showed that with the addition of nutrients only there was no increase in bacterial cell numbers as there was no difference (t.test, 2 sided, unpaired, $p>0.05$) between Soil and Soil & nutrient batches while addition of single biofuel as carbon or growth substrates with or without nutrients (Soil & ethanol, Soil & biodiesel, Soil & ethanol& nutrients and Soil & biodiesel & nutrients) caused an increase (t.test, 2 sided, unpaired, $p=0.03$) in bacterial cell compared to Soil and Soil & nutrient batches. The addition of toluene or toluene & nutrients (Soil & toluene and Soil & toluene &nutrients) did not lead to a significant increase (t.test, 2 sided, unpaired, $p> 0.05$) in bacterial cell numbers, although measured gene copy numbers slightly increased from $6.0 \times 10^8 (\pm 2.0 \times 10^8)$ for both Soil and Soil & nutrient batches to $1.0 \times 10^9 (\pm 1.0 \times 10^8)$ for Soil & toluene and Soil & toluene &nutrients (Figure 5-13). The addition of blended fuels (Toluene & Biodiesel or toluene & Biodiesel) as extra growth substrates in combination with nutrients lead to highly significant increases (t.test, 2 sided, unpaired, $p<0.05$) in comparison to single fuel batches (with and without nutrient amendment) and blended fuel batches (without nutrient amendment).

Assuming that each bacterial cell contained 1 gene copy an estimated absolute abundance of organisms within the various batches was determined by multiplying total gene copy number per batch by relative abundance of the most dominant genera (Figure 5-14 and Figure 5-15). Comparing the differences between relative abundance and estimated absolute abundances, no major change was noticed between the averages of relative and absolute abundances patterns (Figure 5-15) for *Gammaproteobacteria* organisms (*Pseudomonas* and *Lysobacter)* or for *Nocardia, Rhodococcus,* and *Pseudonocardia.* However looking at the individual replicate samples in the heat map (Figure 5-15) and comparing it with heat map for the relative abundances (Figure 5-9) there are some noticeable differences. For instance compared to the heat map using relative abundances there was a reordering of positions of treatment samples and genera based on the estimated absolute abundance. There were also changes in abundances for genera within some samples. For Soil & toluene & ethanol batches, *Kaistobacter Aeromicrobium* seem to increase from low abundance for relative abundance results to higher abundance for the estimated absolute abundance results while no changes in abundance patterns was seen for Soil & toluene & biodiesel and Soil & ethanol & nutrients samples. The abundance of *Candidatus Nitrososphaera*, *Aeromicrobium* and *Kaistobacter* seem to reduce in abundance in Soil batches and Soil & nutrient batches for the estimated absolute abundance compared to pattern seen for the relative abundance. *Kaistobacter* is also seen to reduce in Soil & ethanol batches while *Kaistobacter* and *Hydrogenophaga* reduce in abundance for the estimated absolute abundance. For Soil & toluene & ethanol & nutrient batches which is seen to have the highest average gene copy number (Figure 5-13) the abundance of *Pseudomonas*, *Kaistobacter, Flavihumibacter, lysobacter* and *Thermomonas* generally increased for the estimated absolute abundance results. From the results it shows that absolute abundance changes emphasizes nutrient effects, whereas relative abundance changes more sensitively reflect the effects of the carbon substrate.

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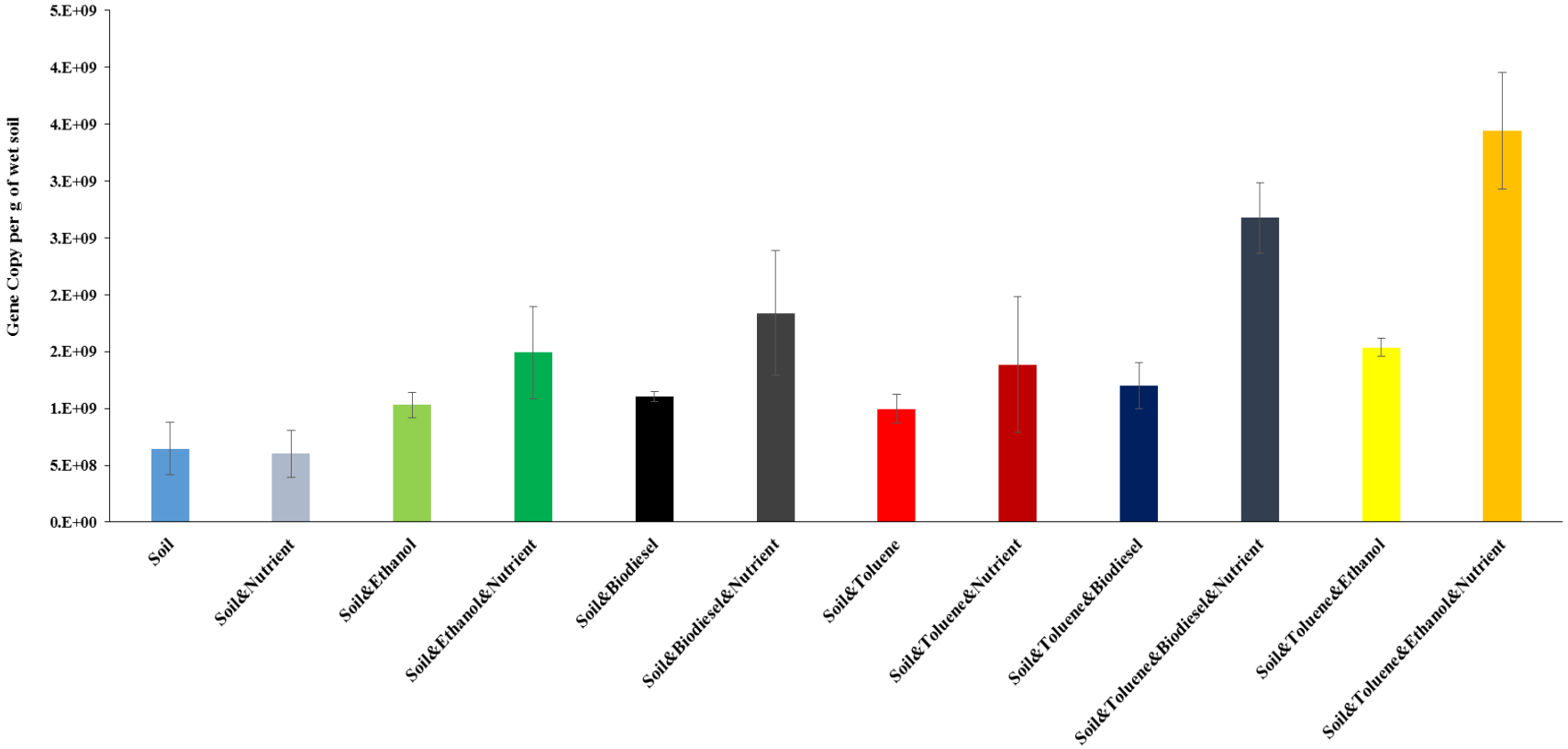

Figure 5-13 . Average gene copy number per gram of wet soil for DNA extracted from triplicate treatment samples. Error bars represent ±1 standard deviation (SD=3) from the mean of 3 replicates

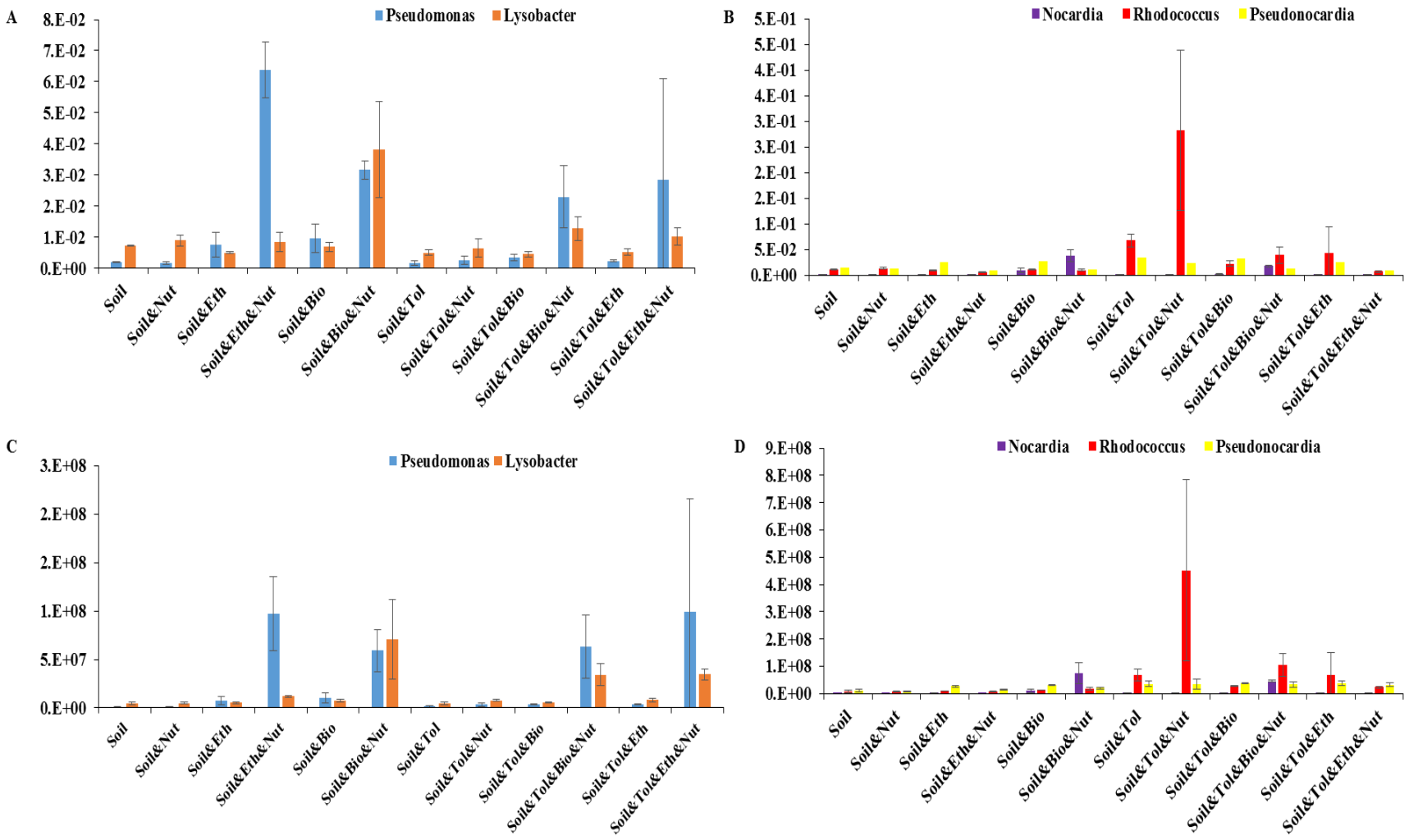

Figure 5-14 showing comparison of average relative abundance (A and B) and average estimated absolute abundance (C and D) of some dominant genera. Error bars represent ±1 standard deviation (SD=3) from the mean of 3 replicates

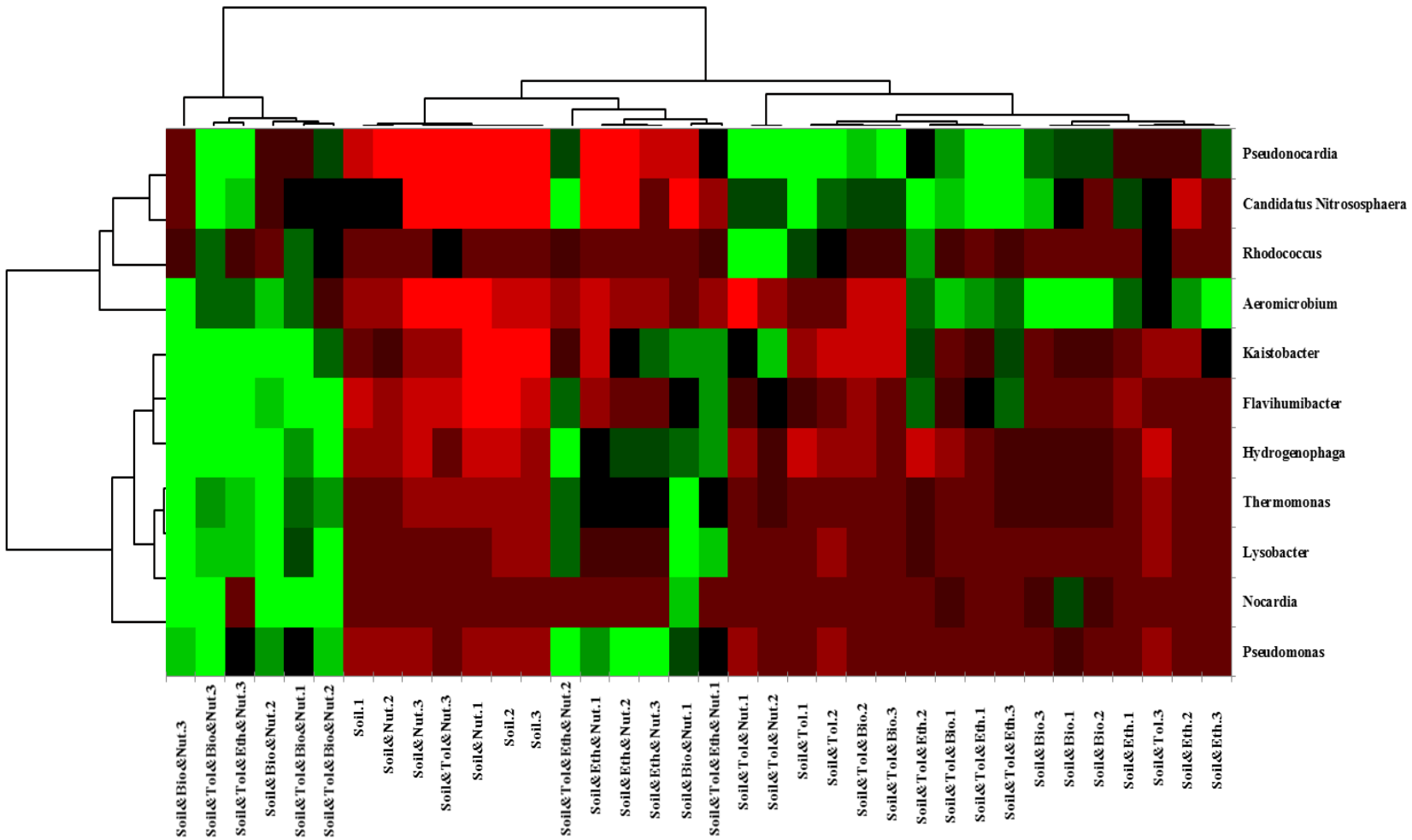

Figure 5-15 Heatmap with dendograms for estimated absolute abundance. Samples in columns and OTUs in rows. Colour coding: low, intermediate and high abundance- Red triangles, black triangles, green triangles respectively.

5.5 Conclusion

In line of the proposed hypothesis, the results for the carbon dioxide production has shown that ethanol hinders toluene biodegradation as it is preferentially metabolised, whereas biodiesel has a much lesser impact on the degradation of toluene. $^{13}CO_2$ production was improved in biofuels and toluene batches (especially for toluene and biodiesel blended batches) by adding nutrients thus indicating that nutrient amendment facilitated toluene degradation, but an inhibitory effect was seen if ethanol was present. Nutrient amendment was also seen to have a significant effect in shaping the microbial communities. The addition of nutrients and extra growth substrates in the form of fuels was also seen to enhance total bacterial cell numbers, however this also led to differences in communities within treatment soil batches.

In terms of differences in microbial communities degrading biodiesel, ethanol and toluene, *Rhodococcus* (Belongs to *Actinobacteria)* is seen as the main beneficiary when either only toluene (with or without nutrients added) or when both toluene and ethanol are present and nutrients are scarce. In toluene and ethanol batches when nutrients are scarce there seems to be a preference for ethanol over toluene degradation as evidenced by the higher production of $^{12}CO_2$ over $^{13}CO_2$ (See Figure 5-2, integrated Figure 5-12).. *Pseudomonas* (which belongs to class *Gammaproteobacteria*) is seen as one of the main ethanol degraders as it be benefits from addition of ethanol in the absence or presence of toluene when nutrients are high. And even with the addition of nutrients to improve toluene degradation in Toluene & ethanol batches there is still remains a higher relative abundance of *Pseudomonas* in Soil & Toluene & ethanol & nutrients batches leading to a higher production of $^{12}CO_2$ over $^{13}CO_2$ (See integrated Figure 5-12). The results above thus confirm that ethanol seems to hinder toluene biodegradation as ethanol is preferentially metabolised in presence of toluene. Other organism which is seen to benefit from the addition of toluene when there is limited nutrient is Pseudonocardia (Belongs to *Actinobacteria*). *Nocardia* (Belongs to *Actinobacteria*) is seen as one of the main biodiesel degrader's when nutrient availability is high or low and is also present in high abundance together with *Rhodococcus, Pseudoxanthomonas* and *Pseudomonas* in biodiesel and toluene batches when nutrient availability is high. This leads to a much higher production of $^{13}CO_2$ over $^{12}CO_2$ (See integrated Figure 5-12), thus indicating a higher degradation of toluene and a reduction of the effect of biodiesel due to added nutrients.

There have been studies such as one carried out by Wilson *et al.* (2016) which have shown that ethanol limits the biodegradation of toluene, the study however did not examine the microbial communities within the samples. To the best of our knowledge, this is the first study to investigate the effects of biofuels (ethanol and biodiesel) on the degradation of toluene with the utilization of a next generation sequencing platform. And the utilization of DNA community analysis in this study has provided awareness of the microbes which play a vital role in degradation of such fuel types. This study highlights the importance of employing DNA microbial community analysis in remediation studies/interventions as the findings in this study would possibly aid understanding of the impact blended fuels have on the microbiology within soil. It is thereby recommended that DNA microbial community analysis should be carried out in conjunction with chemical analysis such as the one carried out in this study to give an insight into the physiological activities and help to determine the participants of such activities.

Chapter 6. Conclusions and recommendations

6.1 The effects of activated carbon and activated carbon amendment time on the removal of hydrocarbon pollutants (crude oil)

In order to help optimize the remediation of crude oil polluted soils an objective of this study was access the effects of sorbents and sorbent amendment time (AC) on the biodegradation of crude oil. The addition of 2% powdered AC at the start of the study to batches (with no crude and spiked with crude oil) was seen to enhance the sorption of pollutants, both native and easy degradable fractions of crude oil thereby limiting the pollutant availability to microbial biodegradation and removal. The CO_2 production and ASE results showed that the biodegradable fractions (alkanes and smaller molecular weight PAHs) were readily removed from bioremediation batches containing nutrients and no AC. However in terms of bioavailability studies (P.E), the bioremediation batch set up had the highest potential risk, despite of the greatest reduction in the total pollutant concentration in soil, due to the microbes inability to degrade heavier PAHs, which were originally present in the urban soil and may have been mobilized by the added crude oil. By carrying out AC amendment after 5 months, the concomitant action of using both bioremediation or natural attenuation to first remove the readily biodegradable fractions then subsequently utilizing AC amendment stabilize residuals was tested. This was done in order to maximize the benefits of either process. By amending the crude oil spiked soil with 2% AC after 5 months the readily biodegradable fractions were also readily removed up until AC was added, and the effect of the AC addition was on crude oil mineralization was hardly noticeable. Surprisingly, the study also showed that AC amendment after 5 months was not as effective as AC amendment from the start in reducing potential risks (i.e. lowering P.E and water PAHs concentrations). This inability to reduce potential risks probably occurred due to fouling of the AC by partially oxidized compounds or metabolites of the crude oil biodegradation.

The study has illustrated that the addition of sorbents such as AC can limit the pollutant availability, and hence slow down the rate of pollutant biodegradation and this is correlates with what previous studies have reported (Rhodes *et al.*, 2008; Rhodes *et al.*, 2010; Oyelami *et al.*, 2014). The results of this study also highlights the importance of choosing the right time for the AC addition. And with regards to AC amendment after 5 months it's believed that AC was added too early and the

strategy might be more effective if more time had been given for the natural attenuation process to be completed.

6.2 The effects of biofuels on the biodegradation of petroleum hydrocarbons with and without nutrient amendment.

The study set out to evaluate effects of biofuels (ethanol and biodiesel) on the biodegradation of hydrocarbon components (toluene). The $^{12}CO_2$ evolution in ethanol only batches was the highest thus indicating that ethanol was more readily degraded in comparison to biodiesel. $^{13}CO_2$ was the highest in toluene only batches. The addition nutrient was also seen to enhance CO_2 in all fuel batches, which similar to a lot of previous studies suggests that nutrient amendment promotes the biodegradation of fuel compounds (Pritchard *et al.*, 1992). With equal volume binary mixtures of biofuels and $^{13}C_7$ stable isotope labelled toluene there was generally more $^{12}CO_2$ produced in comparison to $^{13}CO_2$ (as indicated $^{13}CO_2/^{12}CO_2$ ratios) with no nutrients, indicating that the microbes were possibly showing a preference for the degradation biofuels (especially ethanol) or soil organic carbon over toluene. And this is in line with one of the proposed thesis hypothesis (Chapter 1), which suggests that biofuels such as ethanol are more readily biodegradable in comparison to hydrocarbons and can hinder the biodegradation of toluene in the absence of surplus nutrients. This is correlates with a previous report which showed that presence of biofuel led to a reduction in the biodegradation of mononaromatic hydrocarbons due low nutrient availability (Elazhari-Ali *et al.*, 2013). The addition of nutrients was seen to significantly improve the biodegradation of toluene. The $^{13}CO_2/^{12}CO_2$ ratio above 1 suggested a preference for toluene over biodiesel with nutrient amendment. Although the $^{13}CO_2$ production was improved in ethanol and toluene batches, the $^{13}CO_2/^{12}CO_2$ ratio was still below 1 indicating a preference for the degradation of ethanol over toluene, also with nutrient amendment. The results suggest distinct biofuels effects on the biodegradation of hydrocarbons with ethanol having a bigger impact effect than biodiesel and this is similar to findings previously reported by Elazhari-Ali *et al.* (2013).

6.3 The use of next generation sequencing (NGS) in elucidating the effects the treatments (crude oil, biofuels, sorbents and nutrients) have on microbial community structures within the soils

For the study on effects of AC on crude oil biodegradation, the use of next generation sequencing technique helped in elucidating the effects of crude oil and AC. The addition crude oil was seen as a major factor in shaping the microbial communities as illustrated by ANOSIM. The NGS techniques were used detecting hydrocarbon-degrading communities and showed high relative abundances for organisms belonging to phyla *Actinobacteria* and classes *Gammaproteobacteria* and Alphaproteobacteria (Such as *Rhodococcus*, *Marinobacter*, and *Parvibaculum*). The addition of AC was shown by nested ANOSIM and ranked abundance results to reduce the abundance of such organisms with slightly more relative abundance decreases seen for hydrocarbon degraders due to AC amendment from the start compared to AC amendment after 5 months. The microbial communities within various batches was also linked to the chemical data, thus showing higher production of CO_2 evolution and lower residual concentrations of alkanes and naphthalene in crude oil batches with AC amendment after 5 months and no AC where there are higher abundances of potential hydrocarbon degraders. A previous report has also shown a reduction in *Actinobacteria* and *Gammaproteobacteria* in crude oil contaminated soil due to adsorbent (biochar) amendment (Qin *et al.*, 2013). As previously stated, it's believed that AC which is shown in this study to reduce the abundance of potential HC degraders (thereby reducing the removal or biodegradation of hydrocarbon compounds), should be added after the biodegradation of readily biodegradable compounds has been completed to stabilize the poorly biodegradable residuals. Comparing the outcomes between the 454 GS Junior and Ion Torrent platforms, the study showed that the results from both platforms were only comparable at the phyla level (R^2=0.8915, see Appendix D). The platforms showed some dissimilarities in phylogenetic compositions from order and beyond and were quite dissimilar at genus level (R^2=0.146). One of such differences was the higher presence of organisms belonging to *Archaea* and *Betaproteobacteria* detected for the ion torrent data that were lower in 454 GS junior data. This suggests that more work is needed to improve the reliability of NGS data curation and interpretation methods.

For the effects of biofuels on hydrocarbon degradation, the NGS results showed that nutrient amendment was significant in shaping the microbial communities and organisms such as *Nitrosomonadaceae* and *Nitrospira* were detected having increased abundances due to nutrient amendment. The results also showed differences in microbial communities in responses to the type

of fuel added i.e. ethanol, biodiesel, and toluene as single fuels or in blends identifying organisms which have gained in competitiveness. For example ethanol plus nutrient amendment possibly affects the abundance of *Alphaproteobacteria*. *Betaproteobacteria* possibly plays a role in the degradation biodiesel when nutrient availability is high and in the absence of toluene. *Deltaproteobacteria*, *Planomycetes*, *Acidobacteria* and *Bacteroidetes* were lower in batches with single or blended fuels with nutrient. However, *Bacteroidetes* thrives better than the other phyla groups (*Deltaproteobacteria, Planomycetes Acidobacteria*) when biodiesel is added together with nutrients. The results also showed that *Chloroflexi* seems to benefit from the addition of toluene as a single fuel and does well when toluene is blended with ethanol or biodiesel with no nutrients added. *Gammaproteobacteria* seems to benefit from the addition of biofuels and biofuels and hydrocarbon blends when there is high nutrient availability. The results at phylum level generally show that nutrient amendment did not significantly lead to an increase in *Actinobacteria* abundance and it also showed that ethanol seems to have negative effect on *Actinobacteria* abundance in the presence of toluene (with or without nutrient amendment) while biodiesel has a lesser effect. Drilling down to the genus level, *Rhodococcus* (which belongs to *Actinobacteria)* is seen as the main organism involved in the degradation of toluene in the presence ethanol and low nutrient availability while it can also degrade toluene in the presence of biodiesel when nutrients are surplus or scarce. *Pseudomonas* is seen as one of the main ethanol degraders, while *Nocardia* is involved in the degradation of biodiesel. These results are contrast to Elazhari-Ali *et al.* (2013) reports which showed a dominance of *Pseudomonas* in fuel mixtures of monaromatic and biofuels (ethanol and biodiesel). It is important to state denaturating gradient gel electrophoresis (DGGE) was used was used in that study which further highlights the power of NGS technologies. Both sets of results i.e. for effects of AC on crude oil biodegradation and effects of biofuels on hydrocarbon degradation show unique microbial communities within the soils as a result of the type of treatment added and this could have implications in the design of soil remediation.

6.4 Recommendations

The research has shown the use of a multidisciplinary approach, which can possibly be applied in the remediation studies and most notably highlighted the usefulness of NGS techniques in the identification of microbes in environmental samples and elucidating the impacts of certain treatments on these microbes. 16S rRNA gene analysis has been used for analyzing and detection of bacterial communities at various taxonomic levels and can be applied or used alongside other

monitoring methods, during the treatment of environmental samples as this study has shown. However, there is still too much dependency of study outcomes on the NGS platform used as this study has tried to elucidate to compare and highlight the similarities and differences between 2 NGS platforms. Moving forward, the results generated from the sequencing platforms (mainly ion torrent as the support for 454 has been withdrawn) used in this study could also be compared with other platforms such as Illumina to determine any further differences.

For the effects of AC on crude oil biodegradation and effects of biofuels on hydrocarbon degradation, the work in this thesis was done in batch studies and it could be worth trying do repeat them in column, mesocosms or field studies and carrying out similar microbial analysis to see if there are any significant changes in the results obtained from this study. For the effects of AC on crude oil biodegradation, a longer column and batch study should be done and enough time should be given for the biodegradation process to be completed before the addition of AC to stabilize the non-degradable residuals.

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Chapter 7. Appendix

Appendix A

Example of nutrient calculation for crude oil and AC batches batches

Calculation for Mass of C, N and P to be added to crude oil batch system

Mass of soil in each vial=20g

Volume of crude oil added to each vial= 0.5ml

Crude oil density = 0.8224g/mL

Mass of crude oil added: Density x volume = 0.8224 x 0.5 = 0.4112g

Percentage of C in crude oil = 85%

Mass of C added: 0.4112g x 0.85 = 0.3495g

C:N:P=100:10:1, C=0.3495g :N=0.03495 :P=0.003495

Mass of NH_4CL added: N= 14,Molar mass of NH_4CL= 53.5, = 0.03495 x 53÷14 = 0.13356g

Mass of KH_2PO_4 added: P= 31,Molar mass of KH_2PO_4= 136, = 0.003495 x 136÷31= 0.01533g

Example of nutrient calculation for biofuels and hydrocarbon batches

Volume of Toluene to be added: 4ul

Toluene density: 0.920g/ml

Molecular weight: 92.14g/Mol

Mass of Toluene: Volume x Density

= 4/1000 x 0.920 = 0.00368g of Toluene

Fraction of mass which is Carbon=

7x12/92.14= 0.9116561

Mass of Carbon added to samples = 0.00368g x0.9116561

= 0.003355g

C: N: P = 100: 10: 1

Therefore C=0.003355: N= 0.0003355: P= 0.00003355.

N=14, NH4CL= 53.5.

Mass of NH4CL to be added= 0.0003355x53.5/14= 0.0012821g

P=31, KH2PO4= 136

Mass of KH2PO4 to be added= 0.00003355x136/31= 0.000147187g

Add N and P in 0.3 mL of water per batch

Stock solution concentration = 0.0012821g NH4Cl/0.3 mL = 0.004273 g NH4Cl per mL and 0.000147187g KH2PO4/0.3 mL = 0.0004906 g KH2PO4 per mL

18 batches, 0.3 mL each = 6 mL, so prepare 10 mL of stock solution

Added 42.7 mg of NH4Cl and 4.9 mg of KH2PO4 to 10 mL of water and added 0.3 mL of this stock solution to each batch.

w/w and w/v % calculation For example added 0.5 mL/50mL*14 g (N/mole)/53.5 (NH4Cl/mole) = 0.00262 g of N added per batch. Divide by the g of soil per batch to report N concentration in (w/w), divide by the volume of water in each batch to get the N concentration in soil solution (w/v).

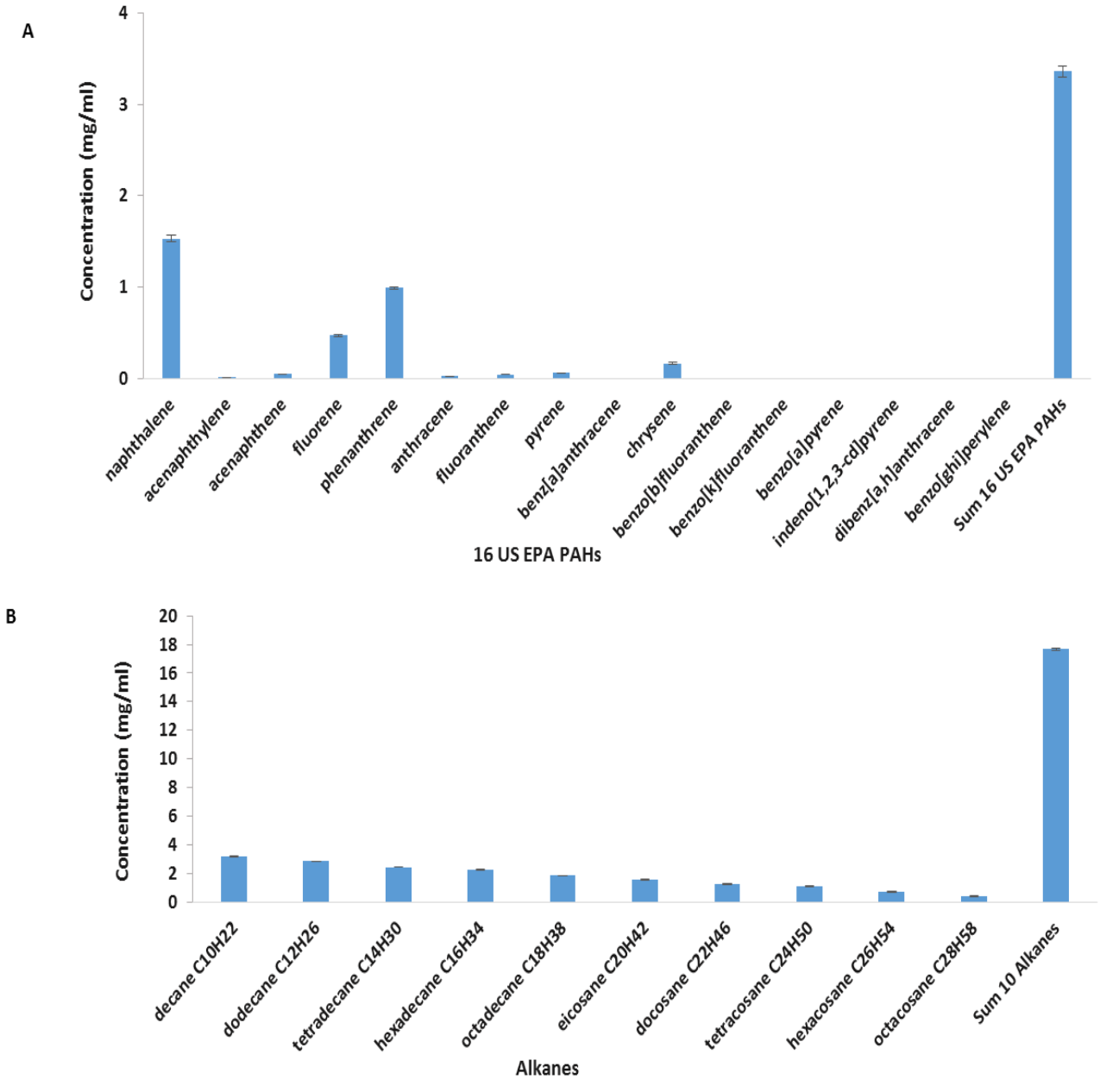

Figure 7.1 Analysis of crude oil (North Sea) used in batch experiment. A: 16 US EPA PAHs, B: 10 alkanes. Compounds with no visible bars were below detection limit. Error bars ± 1 standard deviation (SD, n=3

Appendix B

Split library quality filtering results for bidirectional 454 GS junior (Forward and reverse) reads for crude oil batch studies

Number raw input seqs 69888

Length outside bounds of 200 and 1000: 543
Num ambiguous bases exceeds limit of 6 :12
Missing Qual Score 0
Mean qual score below minimum of 20: 34
Max homopolymer run exceeds limit of 6: 2788
Num mismatches in primer exceeds limit of 100: 0

Number of sequences with identifiable barcode but without identifiable reverse primer: 17731

-z truncate_only option enabled; sequences without a discernible reverse primer as well as sequences with a valid barcode not found in the mapping file may still be written.

Sequence length details for all sequences passing quality filters:
Raw len min/max/avg 200.0/588.0/430.2
Wrote len min/max/avg 171.0/559.0/384.0

Barcodes corrected/not 2650/20270
Uncorrected barcodes will not be written to the output fasta file.
Corrected barcodes will be written with the appropriate barcode category.
Corrected but unassigned sequences will not be written unless --retain_unassigned_reads is enabled.

Total valid barcodes that are not in mapping file 0
Sequences associated with valid barcodes that are not in the mapping file will not be written.

Barcodes in mapping file
Num Samples 17
Sample ct min/max/mean: 821 / 14878 / 2720.06

SampleSequence Count		Barcode
A.B5	14878	TATCGTCA
A.B4	7618	TATCAGAC
A.B3	4270	TATCTCGT
A.B1	3365	TATATGCG
A.B10	2243	TACGTATG
A.B12	1807	TAGTACGA
A.B2	1668	TATAGCTC
A.B8	1233	TACTCAGT

A.B15 1169 TCTACTGA
A.B11 1154 TACGACAT
A.B7 1126 TACTATCG
A.B13 1048 TAGTGTAC
A.B9 1028 TACACGTA
A.B14 1005 TAGACTCT
A.B6 986 TATGCTAG
A.B17 822 TCTCGCAG
A.B16 821 TCTCTATC

Total number seqs written 46241

Number raw input seqs 69888

Length outside bounds of 200 and 1000 :543
Num ambiguous bases exceeds limit of 6 :12
Missing Qual Score 0
Mean qual score below minimum of 20: 34
Max homopolymer run exceeds limit of 6: 2788
Num mismatches in primer exceeds limit of 100: 0

Number of sequences with identifiable barcode but without identifiable reverse primer: 26980

-z truncate_only option enabled; sequences without a discernible reverse primer as well as sequences with a valid barcode not found in the mapping file may still be written.

Sequence length details for all sequences passing quality filters:
Raw len min/max/avg 217.0/586.0/430.3
Wrote len min/max/avg 189.0/558.0/388.3

Barcodes corrected/not 2992/14322
Uncorrected barcodes will not be written to the output fasta file.
Corrected barcodes will be written with the appropriate barcode category.
Corrected but unassigned sequences will not be written unless --retain_unassigned_reads is enabled.

Total valid barcodes that are not in mapping file 0
Sequences associated with valid barcodes that are not in the mapping file will not be written.

Barcodes in mapping file
Num Samples 17
Sample ct min/max/mean: 774 / 14878 / 3069.94
SampleSequence Count Barcode
A.B4 14878 TATCGTCA
A.B5 9340 TATGCGTA
A.B3 7618 TATCAGAC
A.B2 4270 TATCTCGT
A.B9 2243 TACGTATC

A.B6 1809 TACTACGA
A.B1 1668 TATAGCTC
A.B10 1266 TAGTCACA
A.B11 1158 TAGTGTGT
A.B15 1114 TCTGTCAC
A.B8 1077 TACATGCT
A.B12 1050 TCTACTCT
A.B13 1045 TCTAGAGA
A.B14 1031 TCTCACTA
A.B7 1008 TACTCTAC
A.B17 840 TCATCTGA
A.B16 774 TCATAGTC

Total number seqs written 52189

Split library quality filtering results for PGM ion torrent reads for crude oil batch studies

Number raw input seqs 3111945

Length outside bounds of 100 and 1000: 767585
Num ambiguous bases exceeds limit of 6: 0
Missing Qual Score 0
Mean qual score below minimum of 20: 39022
Max homopolymer run exceeds limit of 6 : 118960
Num mismatches in primer exceeds limit of 100: 0

Sequence length details for all sequences passing quality filters:
Raw len min/max/avg 100.0/544.0/308.4
Wrote len min/max/avg 69.0/513.0/277.4

Barcodes corrected/not 1256199/134236
Uncorrected barcodes will not be written to the output fasta file.
Corrected barcodes will be written with the appropriate barcode category.
Corrected but unassigned sequences will not be written unless --retain_unassigned_reads is enabled.

Barcodes in mapping file
Num Samples 17
Sample ct min/max/mean: 13159 / 60597 / 47905.12
SampleSequence Count Barcode
A.B15 60597 ACCTGTCTCTCT
A.B17 58839 ACGAGTGCTATC
A.B13 58359 ACCGCAGAGTCA
A.B9 54932 ACATTCAGCGCA
A.B12 54515 ACCAGCGACTAG

A.B16 54037 ACGACGTCTTAG
A.B14 53800 ACCTCGATCAGA
A.B10 52235 ACCACATACATC
A.B8 51184 ACATGTCACGTG
A.B6 50313 ACATCACTTAGC
A.B5 46912 ACAGTTGCGCGA
A.B7 43680 ACATGATCGTTC
A.B11 43308 ACCAGACGATGC
A.B4 41517 ACAGTGCTTCAT
A.B3 41286 ACAGCTAGCTTG
A.B2 35714 ACAGCAGTGGTC
A.B1 13159 ACAGAGTCGGCT

Total number seqs written 814387

Split library quality filtering results for PGM ion torrent reads for biofuels and hydrocarbon batch studies

Number raw input seqs 3075520

Length outside bounds of 100 and 1000 : 588024
Num ambiguous bases exceeds limit of 6: 0
Missing Qual Score 0
Mean qual score below minimum of 20: 28591
Max homopolymer run exceeds limit of 6 :185941
Num mismatches in primer exceeds limit of 100: 0

Sequence length details for all sequences passing quality filters:
Raw len min/max/avg 100.0/518.0/307.1
Wrote len min/max/avg 69.0/487.0/276.1

Barcodes corrected/not 529986/131083
Uncorrected barcodes will not be written to the output fasta file.
Corrected barcodes will be written with the appropriate barcode category.
Corrected but unassigned sequences will not be written unless --retain_unassigned_reads is enabled.

Barcodes in mapping file
Num Samples 36
Sample ct min/max/mean: 27465 / 60203 / 45986.00
SampleSequence Count Barcode
A.B24 60203 ACCACATACATC
A.B23 57121 ACATTCAGCGCA

A.B29 54428 ACCTGTCTCTCT
A.B18 54028 ACAGTGCTTCAT
A.B21 53444 ACATGATCGTTC
A.B30 49729 ACGACGTCTTAG
A.B22 49714 ACATGTCACGTG
A.B17 49275 ACAGCTAGCTTG
A.B7 49099 AATCGTGACTCG
A.B33 49018 ACGCAACTGCTA
A.B13 48910 ACACTGTTCATG
A.B8 48872 ACACACTATGGC
A.B34 48759 ACGCGATACTGG
A.B6 48603 AATCAGTCTCGT
A.B10 48461 ACACGAGCCACA
A.B32 48027 ACGATGCGACCA
A.B19 47995 ACAGTTGCGCGA
A.B27 47634 ACCGCAGAGTCA
A.B35 47119 ACGCGCAGATAC
A.B31 47100 ACGCTATCTGGA
A.B9 46268 ACACATGTCTAC
A.B36 46033 ACGAGTGCTATC
A.B28 45081 ACCTCGATCAGA
A.B3 44982 AACTGTGCGTAC
A.B12 44686 ACACTAGATCCG
A.B16 44149 ACAGCAGTGGTC
A.B25 43535 ACCAGACGATGC
A.B11 43335 ACACGGTGTCTA
A.B15 42473 ACAGAGTCGGCT
A.B14 42183 ACAGACCACTCA
A.B2 40156 AACTCGTCGATG
A.B20 38600 ACATCACTTAGC
A.B26 37629 ACCAGCGACTAG
A.B4 33330 AAGAGATGTCGA
A.B1 28052 AACGCACGCTAG
A.B5 27465 AAGCTGCAGTCG

Total number seqs written 1655496

ANOSIM for 454 GS junior Crude oil batch samples

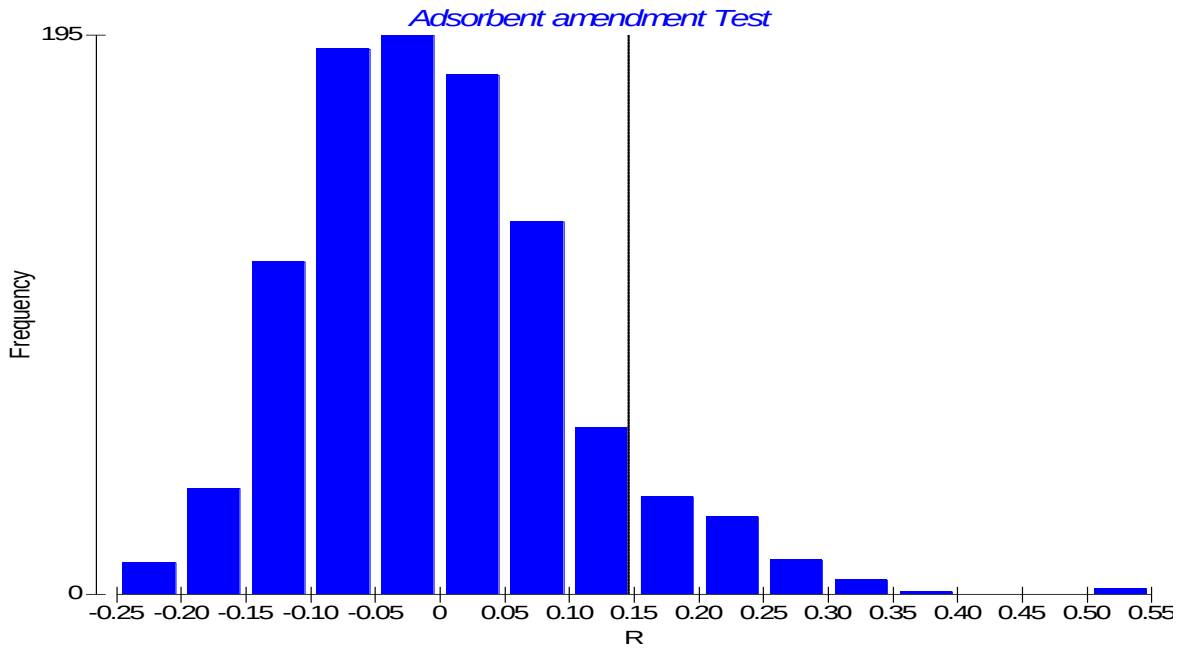

Figure 7.2 Analysis of similarities to determine effect of AC on microbial communities in batch samples

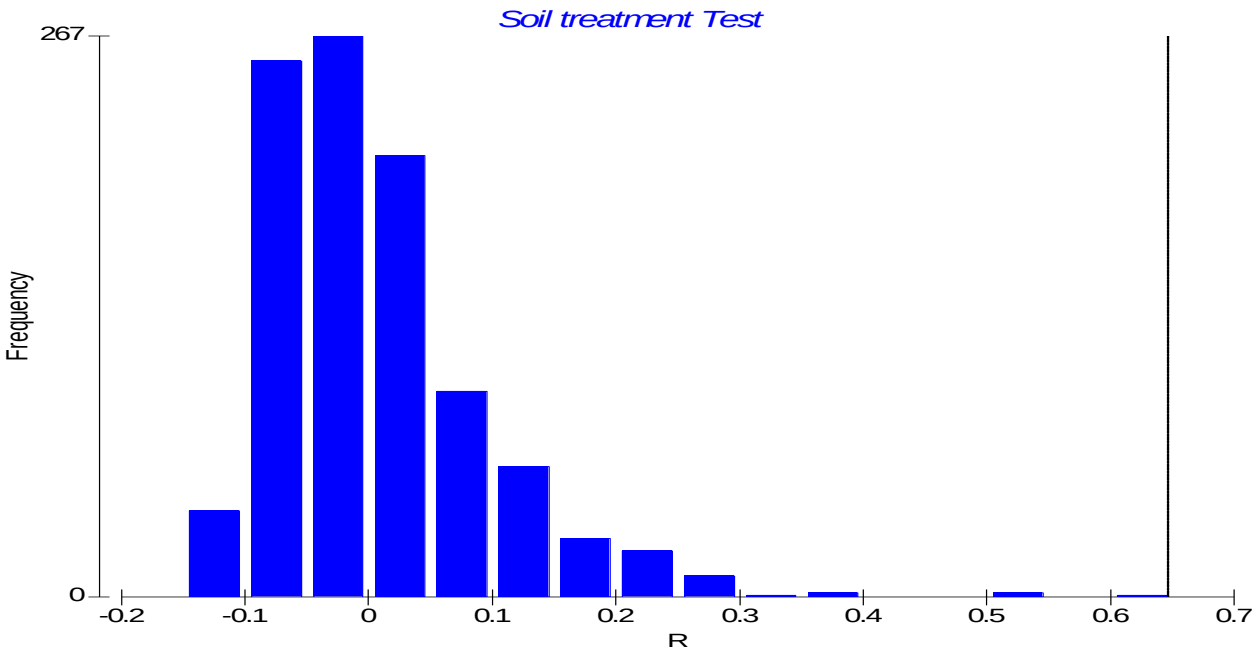

Figure 7.3 Analysis of similarities to determine effect of crude oil on microbial communities in batch samples

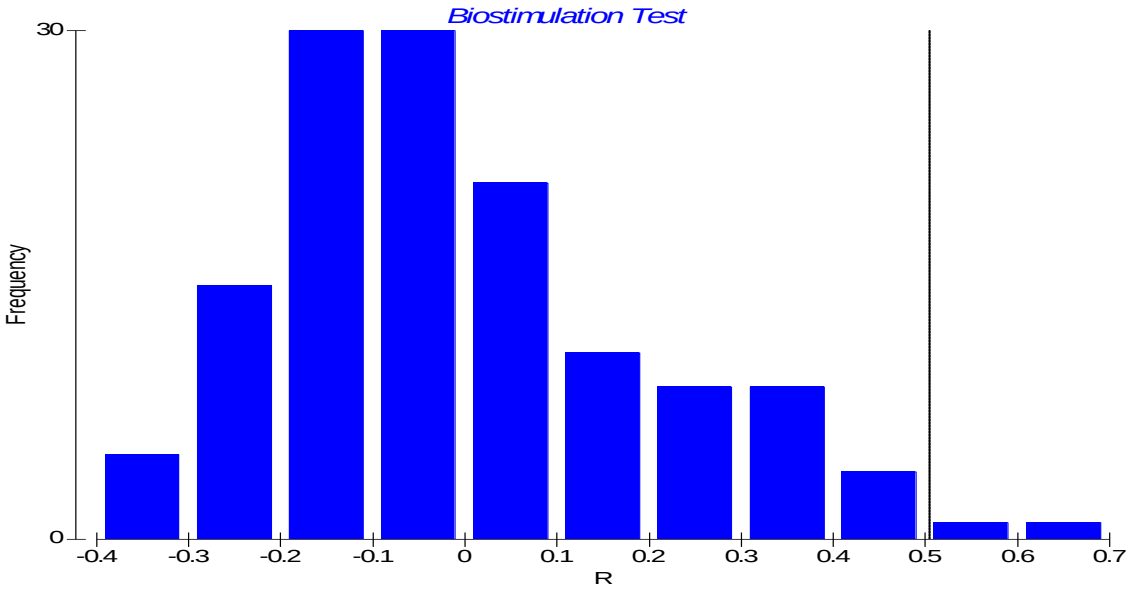

Figure 7.4 Analysis of similarities to determine effect of nutrient on microbial communities in batch samples

ANOSIM

Nested Analysis of Similarities 454 GS junior Crude oil batch samples

Two-Way Nested Analysis

Resemblance worksheet
Name: Resem1
Data type: Similarity
Selection: All

Factor Values
Factors: Adsorbent amendment(Soil treatment)
AC unamended(No Crude oil)
AC amended (t=0)(No Crude oil)
AC amended (t=0)(Crude oil)
AC amended (t=5)(Crude oil)
AC unamended(Crude oil)

Factor Groups

Sample	Adsorbent amendment(Soil treatment)
OSoil.1	AC unamended(No Crude oil)
OSoil.2	AC unamended(No Crude oil)
Soil.1	AC unamended(No Crude oil)
Soil.2	AC unamended(No Crude oil)
Soil.3	AC unamended(No Crude oil)
SoilAC.1	AC amended (t=0)(No Crude oil)
SoilAC.2	AC amended (t=0)(No Crude oil)
SoilAC.3	AC amended (t=0)(No Crude oil)
SoiloilAC.1	AC amended (t=0)(Crude oil)
SoiloilAC.2	AC amended (t=0)(Crude oil)
SoiloilAC.3	AC amended (t=0)(Crude oil)
SoiloilAC5mnths.1	AC amended (t=5)(Crude oil)
SoiloilAC5mnths.2	AC amended (t=5)(Crude oil)
SoiloilAC5mnths.3	AC amended (t=5)(Crude oil)
Soiloil.1	AC unamended(Crude oil)
Soiloil.2	AC unamended(Crude oil)
Soiloil.3	AC unamended(Crude oil)

TESTS FOR DIFFERENCES BETWEEN Adsorbent amendment GROUPS
(across all Soil treatment groups)
Global Test
Sample statistic (Global R): 0.542
Significance level of sample statistic: 0.3%
Number of permutations: 999 (Random sample from 15680)
Number of permuted statistics greater than or equal to Global R: 2

ANOSIM for PGM ion torrent crude oil batch samples

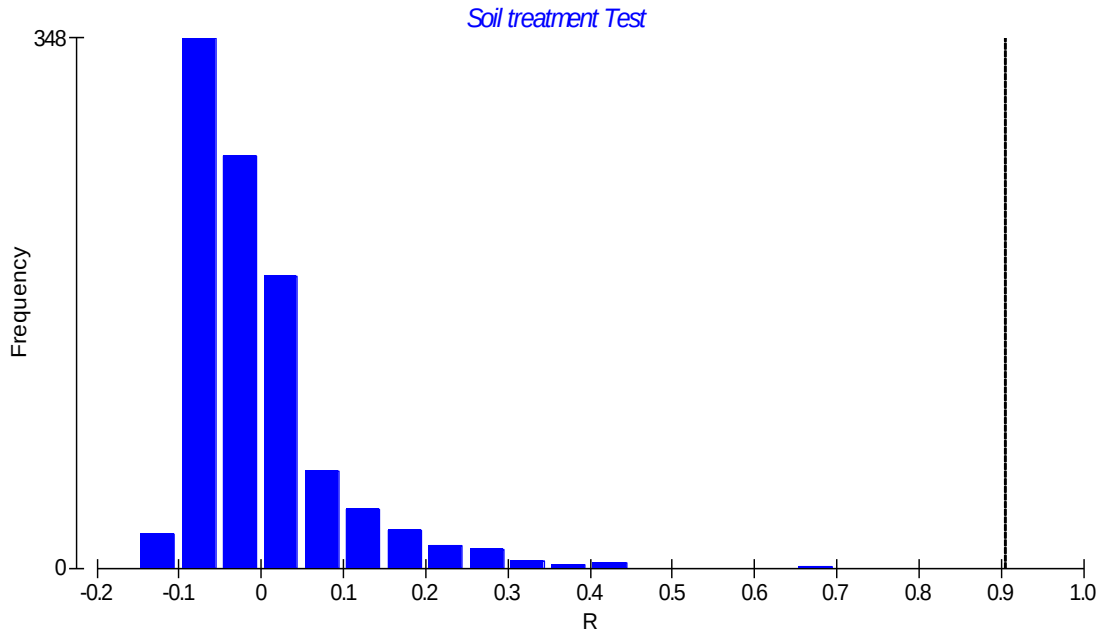

Figure 7.5 Analysis of similarities to determine effect of crude oil on microbial communities in batch samples

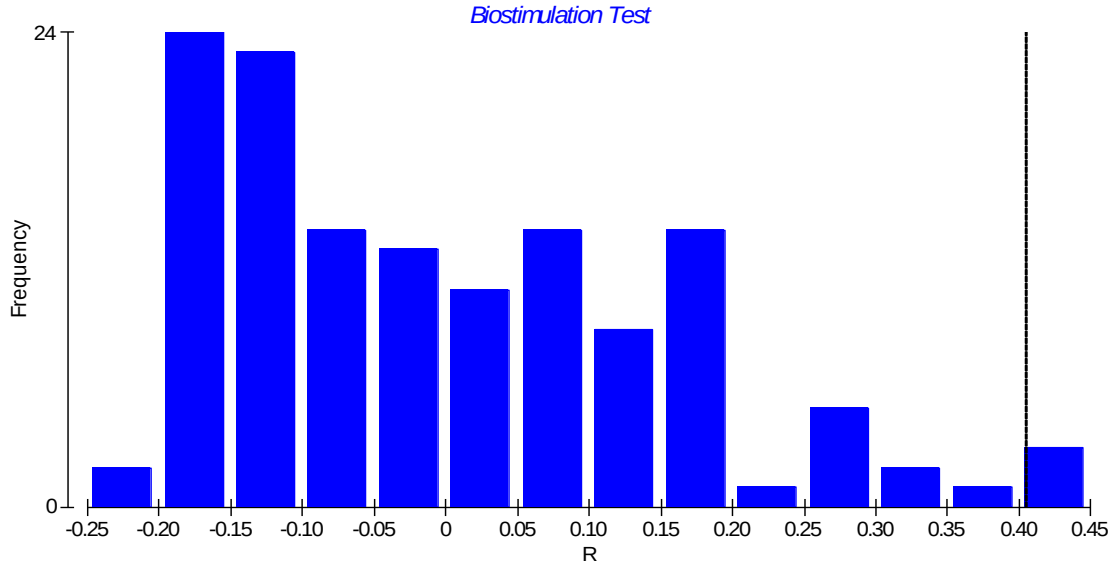

Figure 7.6 Analysis of similarities to determine effect of nutrient on microbial communities in batch samples

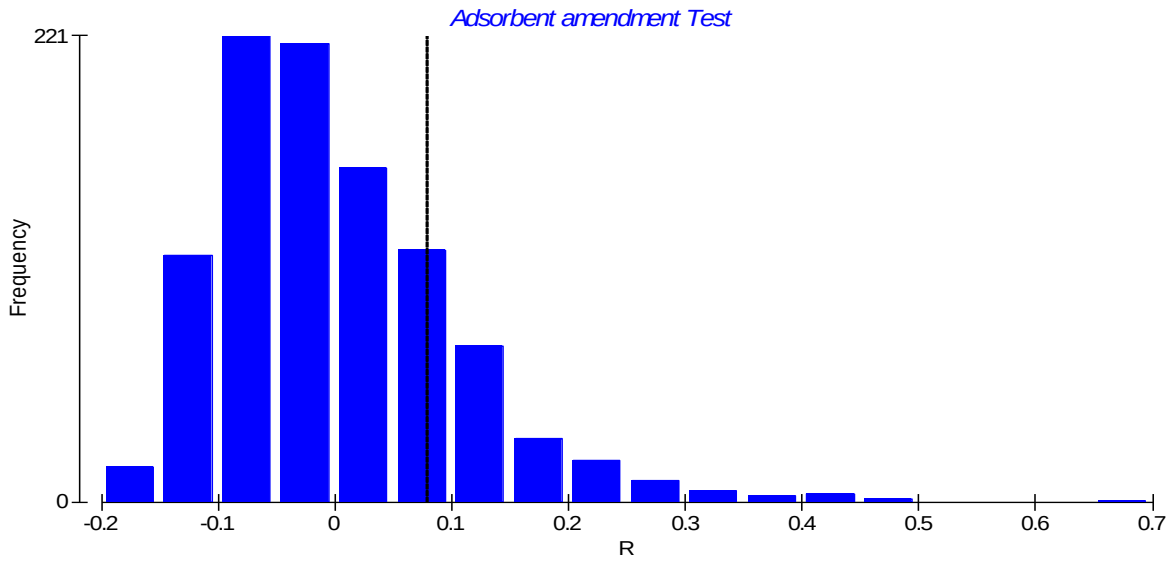

Figure 7.7 Analysis of similarities to determine effect of AC on microbial communities in batch samples

ANOSIM

Analysis of Similarities

Nested Analysis of Similarities Ion torrent Crude oil batch samples

Resemblance worksheet
Name: Resem1
Data type: Similarity
Selection: All

Factor Values
Factors: Adsorbent amendment(Soil treatment)
AC unamended(No Crude oil)
AC amended (t=0)(No Crude oil)
AC amended (t=0)(Crude oil)
AC amended (t=5)(Crude oil)
AC unamended(Crude oil)

Factor Groups

Sample	Adsorbent amendment(Soil treatment)
OSoil.1	AC unamended(No Crude oil)
OSoil.2	AC unamended(No Crude oil)
Soil.1	AC unamended(No Crude oil)
Soil.2	AC unamended(No Crude oil)
Soil.3	AC unamended(No Crude oil)
SoilAC.1	AC amended (t=0)(No Crude oil)
SoilAC.2	AC amended (t=0)(No Crude oil)
SoilAC.3	AC amended (t=0)(No Crude oil)
SoiloilAC.1	AC amended (t=0)(Crude oil)
SoiloilAC.2	AC amended (t=0)(Crude oil)
SoiloilAC.3	AC amended (t=0)(Crude oil)
SoiloilAC5mnths.1	AC amended (t=5)(Crude oil)

```
SoiloilAC5mnths.2  AC amended (t=5)(Crude oil)
SoiloilAC5mnths.3  AC amended (t=5)(Crude oil)
Soiloil.1          AC unamended(Crude oil)
Soiloil.2          AC unamended(Crude oil)
Soiloil.3          AC unamended(Crude oil)

TESTS FOR DIFFERENCES BETWEEN Adsorbent amendment GROUPS
(across all Soil treatment groups)
Global Test
Sample statistic (Global R): 0.59
Significance level of sample statistic: 0.2%
Number of permutations: 999 (Random sample from 15680)
Number of permuted statistics greater than or equal to Global R: 1
```

ANOSIM for PGM ion torrent Biofuel and hydrocarbon batch samples

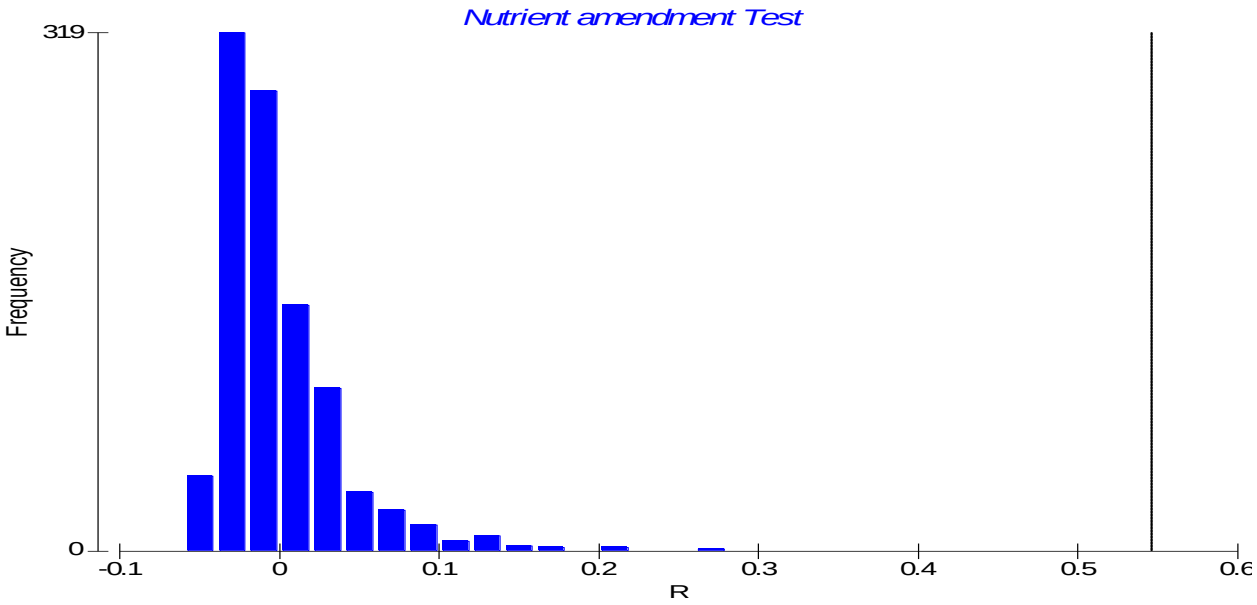

Figure 7.8 Analysis of similarities to determine effect of nutrient on microbial communities in batch samples

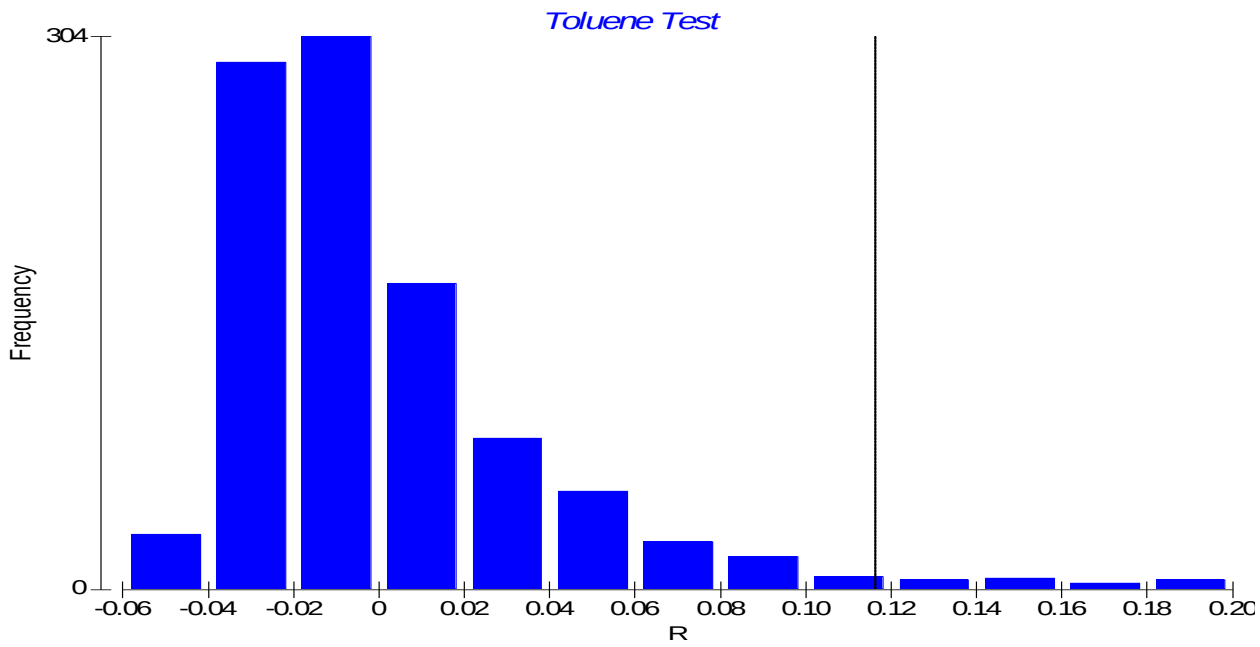

Figure 7.9 Analysis of similarities to determine effect of toluene on microbial communities in batch samples

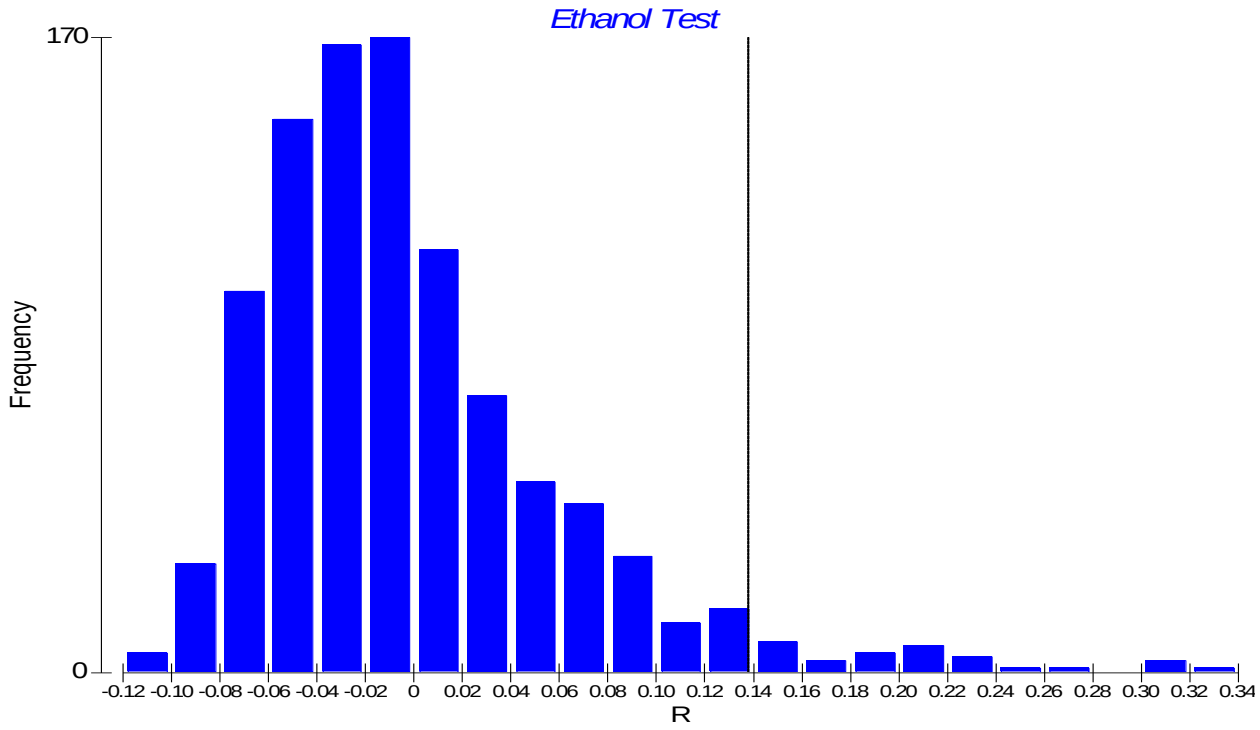

Figure 7.10 Analysis of similarities to determine effect of ethanol on microbial communities in batch samples

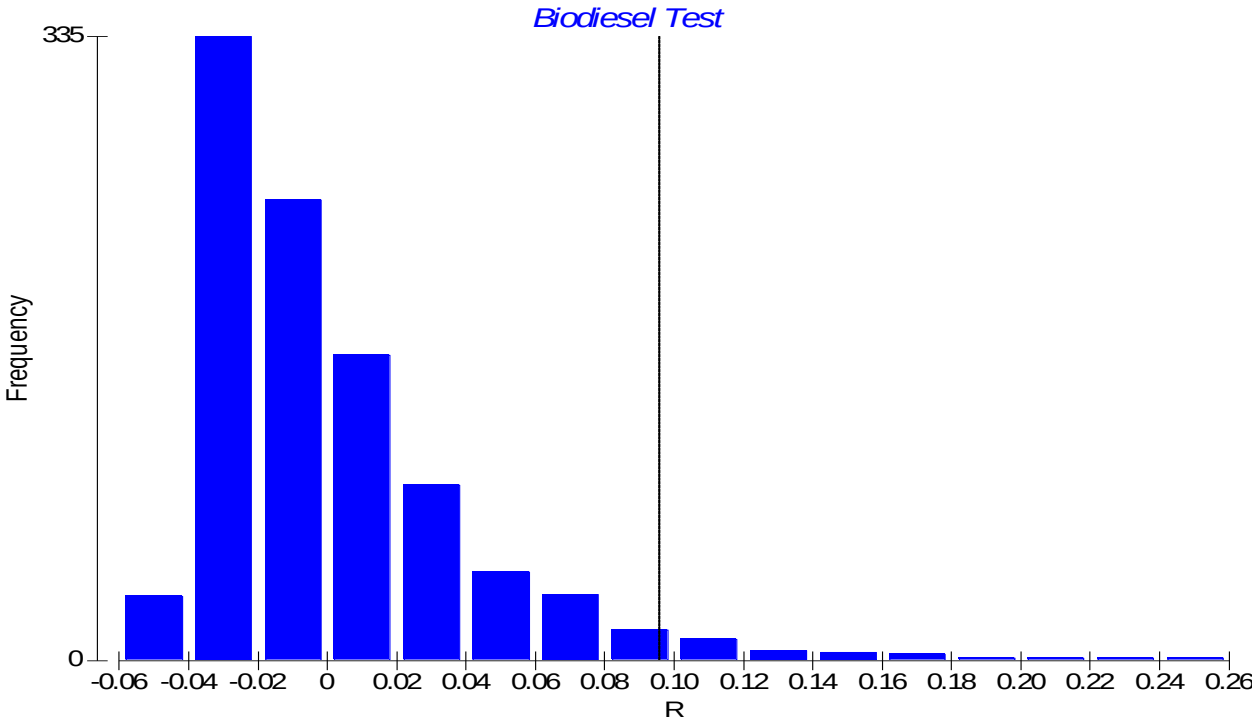

Figure 7.11 Analysis of similarities to determine effect of biodiesel on microbial communities in batch samples

Appendix C

Full description of qPCR 96 well plate set up

Table 7.1 qPCR set for 1st run

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 10^8	Std 10^8	Std 10^8	Std 10^7	Std 10^7	Std 10^7	Std 10^6	Std 10^6	Std 10^6	Std 10^5	Std 10^5	Std 10^5
B	Std 10^4	Std 10^4	Std 10^4	Std 10^3	Std 10^3	Std 10^3	Std 10^2	Std 10^2	Std 10^2	NTC	NTC	NTC
C	Cont. (1A) (1/100)	Cont. (1A) (1/100)	Cont. (1A) (1/100	Cont. (1A) (1/1000)	Cont. (1A) (1/1000)	Cont. (1A) (1/1000)	Cont. (1B) (1/100)	Cont. (1B) (1/100)	Cont. (1B) (1/100)	Cont. (1B) (1/1000)	Cont. (1B) (1/1000)	Cont. (1B) (1/1000)
D	Cont. (1c) (1/100)	Cont. (1c) (1/100)	Cont. (1c) (1/100)	Cont. (1c) (1/1000)	Cont. (1c) (1/1000)	Cont. (1c) (1/1000)	Cont. (2A) (1/100)	Cont. (2A) (1/100)	Cont. (2A) (1/100)	Cont. (2A) (1/1000)	Cont. (2A) (1/1000)	Cont. (2A) (1/1000)
E	Cont. (2B) (1/100)	Cont. (2B) (1/100)	Cont. (2B) (1/100)	Cont. (2B) (1/1000)	Cont. (2B) (1/1000)	Cont. (2B) (1/1000)	Cont. (2c) (1/100)	Cont. (2c) (1/100)	Cont. (2c) (1/100)	Cont. (2c) (1/1000)	Cont. (2c) (1/1000)	Cont. (2c) (1/1000)
F	Soil + eth (A) (1/100)	Soil + eth (A) (1/100)	Soil + eth (A) (1/100)	Soil + eth (A) (1/1000)	Soil + eth (A) (1/1000	Soil + eth (A) (1/1000	Soil + eth (B) (1/100)	Soil + eth (B) (1/100)	Soil + eth (B) (1/100)	Soil + eth (B) (1/1000)	Soil + eth (B) (1/1000)	Soil + eth (B) (1/1000)
G	Soil + eth (C) (1/100)	Soil + eth (C) (1/100)	Soil + eth (C) (1/100)	Soil + eth (C) (1/1000)	Soil + eth (C) (1/1000)	Soil + eth (C) (1/1000)	Soil + eth+ Nut (A) (1/100)	Soil + eth+ Nut (A) (1/100)	Soil + eth+ Nut (A) (1/100)	Soil + eth+ Nut (A) (1/1000)	Soil + eth+ Nut (A) (1/1000)	Soil + eth+ Nut (A) (1/1000)
H	Soil + eth+ Nut (B) (1/100)	Soil + eth+ Nut (B) (1/100)	Soil + eth+ Nut (B) (1/100)	Soil + eth+ Nut (B) (1/1000)	Soil + eth+ Nut (B) (1/1000)	Soil + eth+ Nut (B) (1/1000)	Soil + eth+ Nut (C) (1/100)	Soil + eth+ Nut (C) (1/100)	Soil + eth+ Nut (C) (1/100)	Soil + eth+ Nut (C) (1/1000)	Soil + eth+ Nut (C) (1/1000)	Soil + eth+ Nut (C) (1/1000)

Table 7.2 qPCR set for 2nd run

	1	2	3	4	5	6	7	8	9	10	11	12
A	Soil + Bio (A) (1/100)	Soil + Bio (A) (1/100)	Soil + Bio (A) (1/100)	Soil + Bio (A) (1/1000)	Soil + Bio (A) (1/1000)	Soil + Bio (A) (1/1000)	Soil + Bio (B) (1/100)	Soil + Bio (B) (1/100)	Soil + Bio (B) (1/100)	Soil + Bio (B) (1/1000)	Soil + Bio (B) (1/1000)	Soil + Bio (B) (1/1000)
B	Soil + Bio (C) (1/100)	Soil + Bio (C) (1/100)	Soil + Bio (C) (1/100)	Soil + Bio (C) (1/1000)	Soil + Bio (C) (1/1000)	Soil + Bio (C) (1/1000)	Soil + Bio+Nut (A) (1/100)	Soil + Bio+Nut (A) (1/100)	Soil + Bio+Nut (A) (1/100)	Soil + Bio+Nut (A) (1/1000)	Soil + Bio+Nut (A) (1/1000)	Soil + Bio+Nut (A) (1/1000)
C	Soil + Bio+Nut (B) (1/100)	Soil + Bio+Nut (B) (1/100)	Soil + Bio+Nut (B) (1/100)	Soil + Bio+Nut (B) (1/1000)	Soil + Bio+Nut (B) (1/1000)	Soil + Bio+Nut (B) (1/1000)	Soil + Bio+Nut (C) (1/100)	Soil + Bio+Nut (C) (1/100)	Soil + Bio+Nut (C) (1/100)	Soil + Bio+Nut (C) (1/1000)	Soil + Bio+Nut (C) (1/1000)	Soil + Bio+Nut (C) (1/1000)
D	Soil + Tol (A) (1/100)	Soil + Tol (A) (1/100)	Soil + Tol (A) (1/100)	Soil + Tol (A) (1/1000)	Soil + Tol (A) (1/1000)	Soil + Tol (A) (1/1000)	Soil + Tol (B) (1/100)	Soil + Tol (B) (1/100)	Soil + Tol (B) (1/100)	Soil + Tol (B) (1/1000)	Soil + Tol (B) (1/1000)	Soil + Tol (B) (1/1000)
E	Soil + Tol (C) (1/100)	Soil + Tol (C) (1/100)	Soil + Tol (C) (1/100)	Soil + Tol (C) (1/1000)	Soil + Tol (C) (1/1000)	Soil + Tol (C) (1/1000)	Soil + Tol+ Nut(A) (1/100)	Soil + Tol+ Nut(A) (1/100)	Soil + Tol+ Nut(A) (1/100)	Soil + Tol+ Nut(A) (1/1000)	Soil + Tol+ Nut(A) (1/1000)	Soil + Tol+ Nut(A) (1/1000)
F	Soil + Tol+ Nut(B) (1/100)	Soil + Tol+ Nut(B) (1/100)	Soil + Tol+ Nut(B) (1/100)	Soil + Tol+ Nut(B) (1/1000)	Soil + Tol+ Nut(B) (1/1000	Soil + Tol+ Nut(B) (1/1000)	Soil + Tol+ Nut(C) (1/100)	Soil + Tol+ Nut(C) (1/100)	Soil + Tol+ Nut(C) (1/100)	Soil + Tol+ Nut(C) (1/1000)	Soil + Tol+ Nut(C) (1/1000)	Soil + Tol+ Nut(C) (1/1000)
G	Soil + Tol+ Bio(A) (1/100)	Soil + Tol+ Bio(A) (1/100)	Soil + Tol+ Bio(A) (1/100)	Soil + Tol+ Bio(A) (1/1000)	Soil + Tol+ Bio(A) (1/1000)	Soil + Tol+ Bio(A) (1/1000)	Soil + Tol+ Bio(B) (1/100)	Soil + Tol+ Bio(B) (1/100)	Soil + Tol+ Bio(B) (1/100)	Soil + Tol+ Bio(B) (1/1000)	Soil + Tol+ Bio(B) (1/1000)	Soil + Tol+ Bio(B) (1/1000)
H	Soil + Tol+ Bio(C) (1/100)	Soil + Tol+ Bio(C) (1/100)	Soil + Tol+ Bio(C) (1/100)	Soil + Tol+ Bio(C) (1/1000)	Soil + Tol+ Bio(C) (1/1000)	Soil + Tol+ Bio(C) (1/1000)	Soil + Tol+ Bio+Nut (A) (1/100)	Soil + Tol+ Bio+Nut (A) (1/100)	Soil + Tol+ Bio+Nut (A) (1/100)	Soil + Tol+ Bio+Nut (A) (1/1000)	Soil + Tol+ Bio+Nut (A) (1/1000)	Soil + Tol+ Bio+Nut (A) (1/1000)

Table 7.3 qPCR set for 3rd run

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	Soil + Tol+ Bio+Nut (B) (1/100)	Soil + Tol+ Bio+Nut (B) (1/100)	Soil + Tol+ Bio+Nut (B) (1/100)	Soil + Tol+ Bio+Nut (B) (1/1000)	Soil + Tol+ Bio+Nut (B) (1/1000)	Soil + Tol+ Bio+Nut (B) (1/1000)	Soil + Tol+ Bio+Nut (C) (1/100)	Soil + Tol+ Bio+Nut (C) (1/100)	Soil + Tol+ Bio+Nut (C) (1/100)	Soil + Tol+ Bio+Nut (C) (1/1000)	Soil + Tol+ Bio+Nut (C) (1/1000)	Soil + Tol+ Bio+Nut (C) (1/1000)
B	Soil + Tol+Etha (A) (1/100)	Soil + Tol+Etha (A) (1/100)	Soil + Tol+Etha (A) (1/100)	Soil + Tol+Etha (A) (1/1000)	Soil + Tol+Etha (A) (1/1000)	Soil + Tol+Etha (A) (1/1000)	Soil + Tol+Etha (B) (1/100)	Soil + Tol+Etha (B) (1/100)	Soil + Tol+Etha (B) (1/100)	Soil + Tol+Etha (B) (1/1000)	Soil + Tol+Etha (B) (1/1000)	Soil + Tol+Etha (B) (1/1000)
C	Soil + Tol+Etha (C) (1/100)	Soil + Tol+Etha (C) (1/100)	Soil + Tol+Etha (C) (1/100)	Soil + Tol+Etha (C) (1/1000)	Soil + Tol+Etha (C) (1/1000)	Soil + Tol+Etha (C) (1/1000)	Soil + Tol+Etha +Nut (A) (1/100)	Soil + Tol+Etha +Nut (A) (1/100)	Soil + Tol+Etha +Nut (A) (1/100)	Soil + Tol+Etha +Nut (A) (1/1000)	Soil + Tol+Etha +Nut (A) (1/1000)	Soil + Tol+Etha +Nut (A) (1/1000)
D	Soil + Tol+Etha +Nut (B) (1/100)	Soil + Tol+Etha +Nut (B) (1/100)	Soil + Tol+Etha +Nut (B) (1/100)	Soil + Tol+Etha +Nut (B) (1/1000)	Soil + Tol+Etha +Nut (B) (1/1000)	Soil + Tol+Etha +Nut (B) (1/1000)	Soil + Tol+Etha +Nut (C) (1/100)	Soil + Tol+Etha +Nut (C) (1/100)	Soil + Tol+Etha +Nut (C) (1/100)	Soil + Tol+Etha +Nut (C) (1/1000)	Soil + Tol+Etha +Nut (C) (1/1000)	Soil + Tol+Etha +Nut (C) (1/1000)

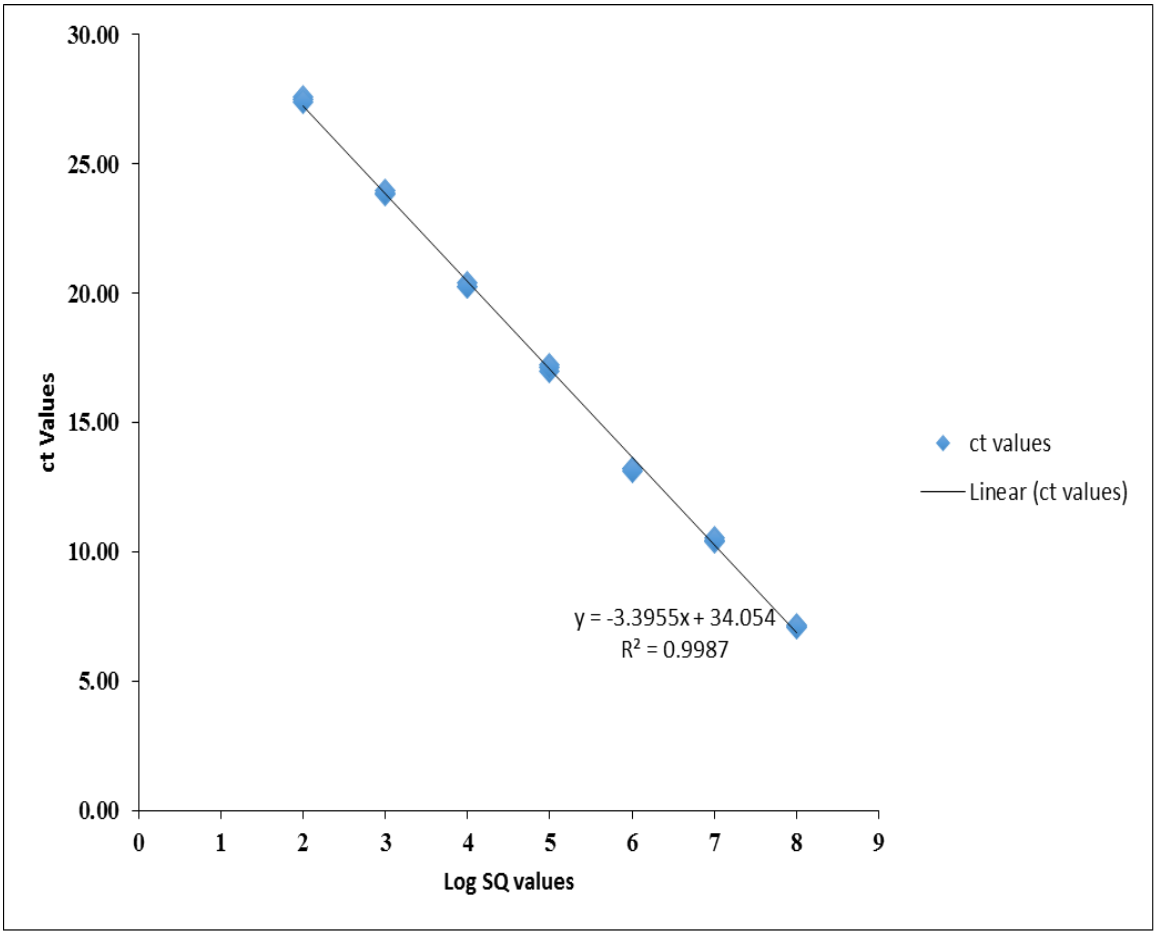

Figure 7.12 Standard calibration curve for first run of qPCR analysis

Appendix D

Correlation analysis performed on Microsoft excel version 2010 (Microsoft, Redmond, USA)

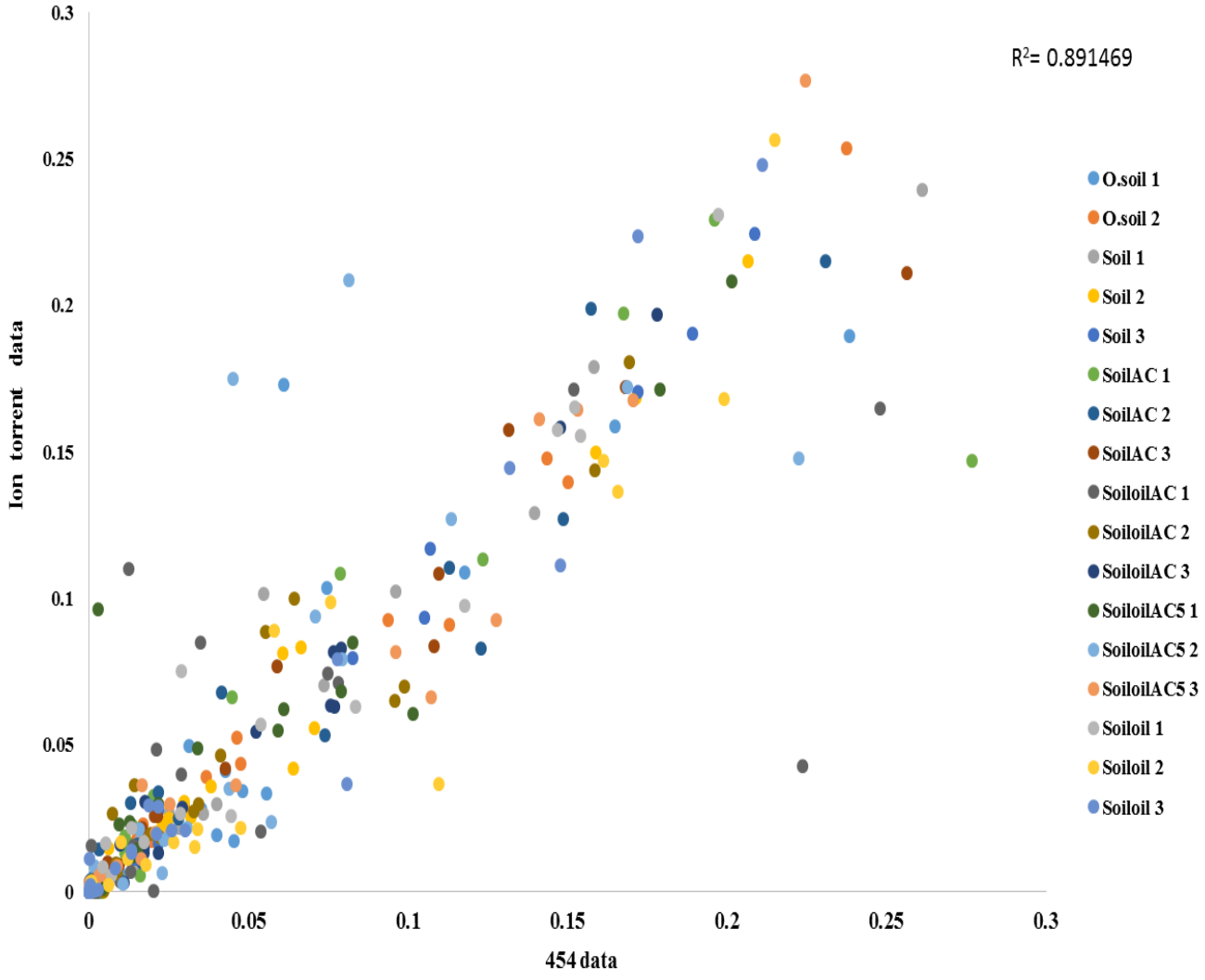

Figure 7.13 Correlation analysis between 454 and ion torrent data (50% bootstrap cut off) at phylum level. Insert correlation R^2 value

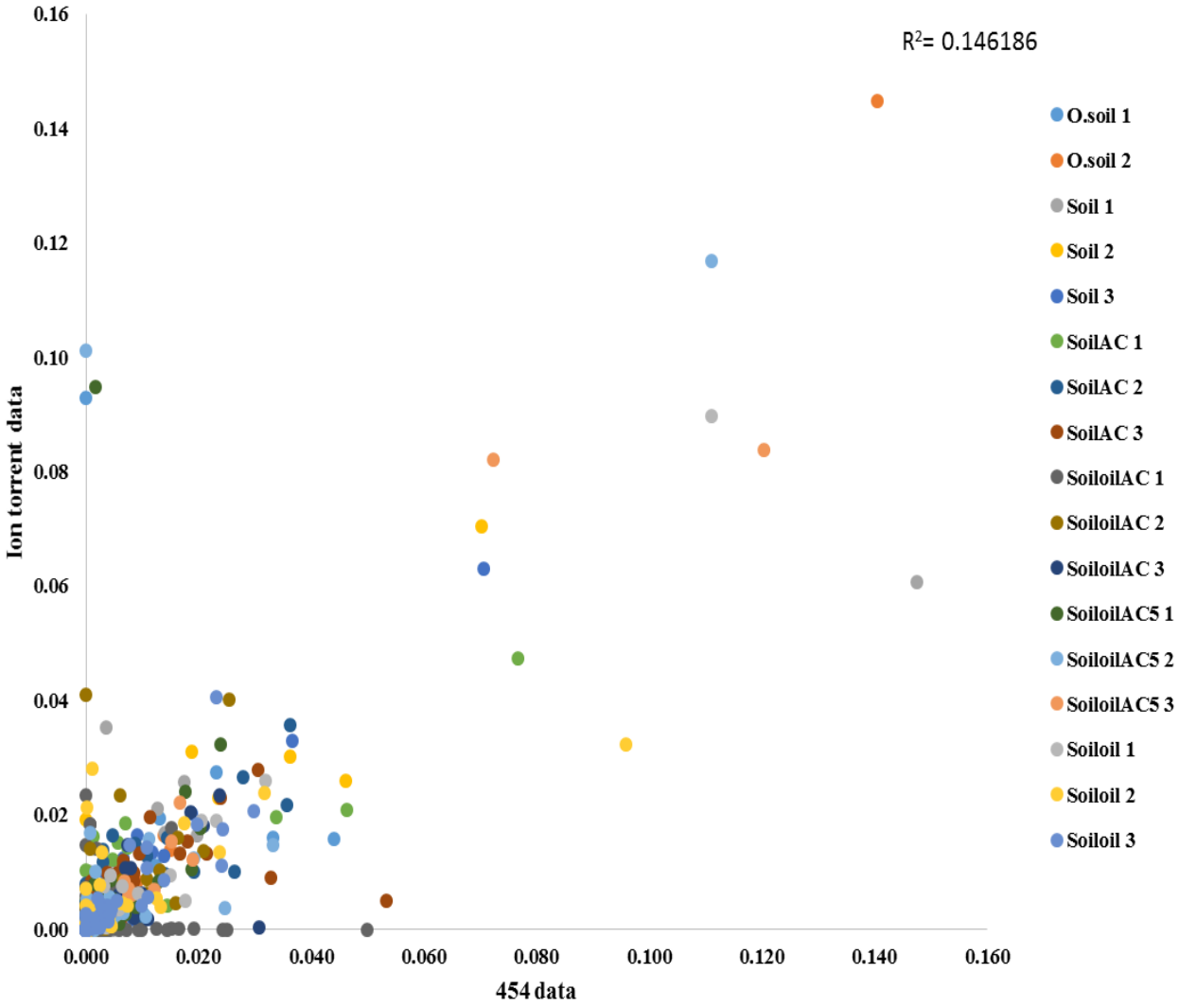

Figure 7.14 Correlation analysis between 454 and ion torrent data (50% bootstrap cut off) at genus level. Insert correlation R^2 value

Spearman correlation (Rho) performed on IBM SPSS Statistics

Table 7.4 Spearman Rho Correlations performed at genus level (Control: Original Soil i.e. Soil only, assigned taxonomy at 50% and 80% bootstrap cutoff) 454 Pyrosequencing data

Correlations

			OSoil.1_50%	OSoil.1_80%	OSoil.2_50%	OSoil.2_80%
Spearman's rho	OSoil.1_50%	Correlation Coefficient	1.000	.919**	.648**	.639**
		Sig. (2-tailed)	.	.000	.000	.000
		N	193	193	193	193
	OSoil.1_80%	Correlation Coefficient	.919**	1.000	.675**	.676**
		Sig. (2-tailed)	.000	.	.000	.000
		N	193	193	193	193
	OSoil.2_50%	Correlation Coefficient	.648**	.675**	1.000	.969**
		Sig. (2-tailed)	.000	.000	.	.000
		N	193	193	193	193
	OSoil.2_80%	Correlation Coefficient	.639**	.676**	.969**	1.000
		Sig. (2-tailed)	.000	.000	.000	.
		N	193	193	193	193

**. Correlation is significant at the 0.01 level (2-tailed).

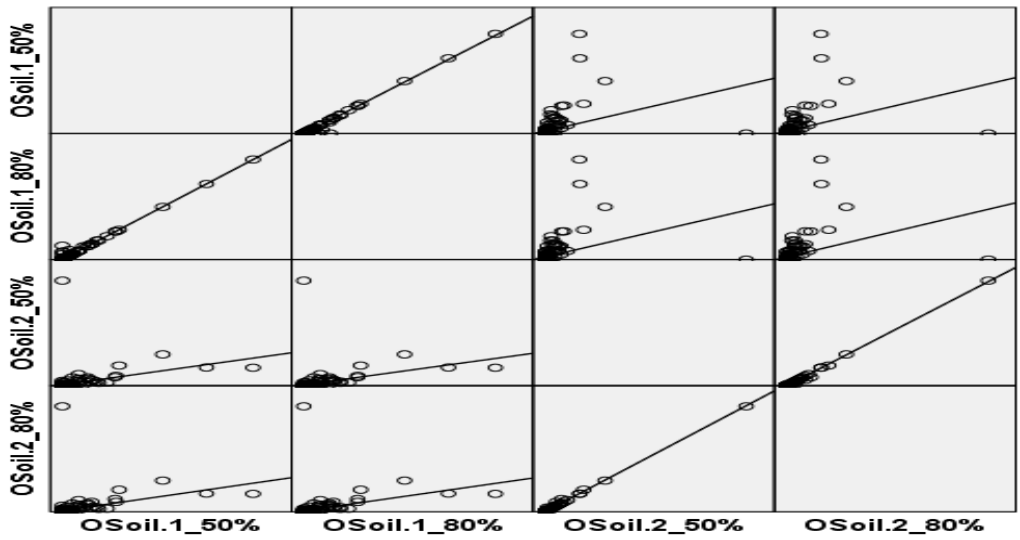

Figure 7.15 Spearman Rho Correlations performed at genus level (Control: Original Soil i.e. Soil only, assigned taxonomy at 50% and 80% bootstrap cutoff) 454 Pyrosequencing data

Table 7.5 Spearman Rho Correlations performed at genus level (Control: Soil + nutrients, assigned taxonomy at 50% and 80% bootstrap cutoff) 454 Pyrosequencing data

Correlations

			Soil.1_50%	Soil.1_80%	Soil.2_50%	Soil.2_80%	Soil.3_50%	Soil.3_80%
Spearman's rho	Soil.1_50%	Correlation Coefficient	1.000	.947**	.829**	.805**	.715**	.749**
		Sig. (2-tailed)	.	.000	.000	.000	.000	.000
		N	193	193	193	193	193	193
	Soil.1_80%	Correlation Coefficient	.947**	1.000	.800**	.838**	.681**	.800**
		Sig. (2-tailed)	.000	.	.000	.000	.000	.000
		N	193	193	193	193	193	193
	Soil.2_50%	Correlation Coefficient	.829**	.800**	1.000	.913**	.864**	.854**
		Sig. (2-tailed)	.000	.000	.	.000	.000	.000
		N	193	193	193	193	193	193
	Soil.2_80%	Correlation Coefficient	.805**	.838**	.913**	1.000	.801**	.909**
		Sig. (2-tailed)	.000	.000	.000	.	.000	.000
		N	193	193	193	193	193	193
	Soil.3_50%	Correlation Coefficient	.715**	.681**	.864**	.801**	1.000	.867**
		Sig. (2-tailed)	.000	.000	.000	.000	.	.000
		N	193	193	193	193	193	193
	Soil.3_80%	Correlation Coefficient	.749**	.800**	.854**	.909**	.867**	1.000
		Sig. (2-tailed)	.000	.000	.000	.000	.000	.
		N	193	193	193	193	193	193

**. Correlation is significant at the 0.01 level (2-tailed).

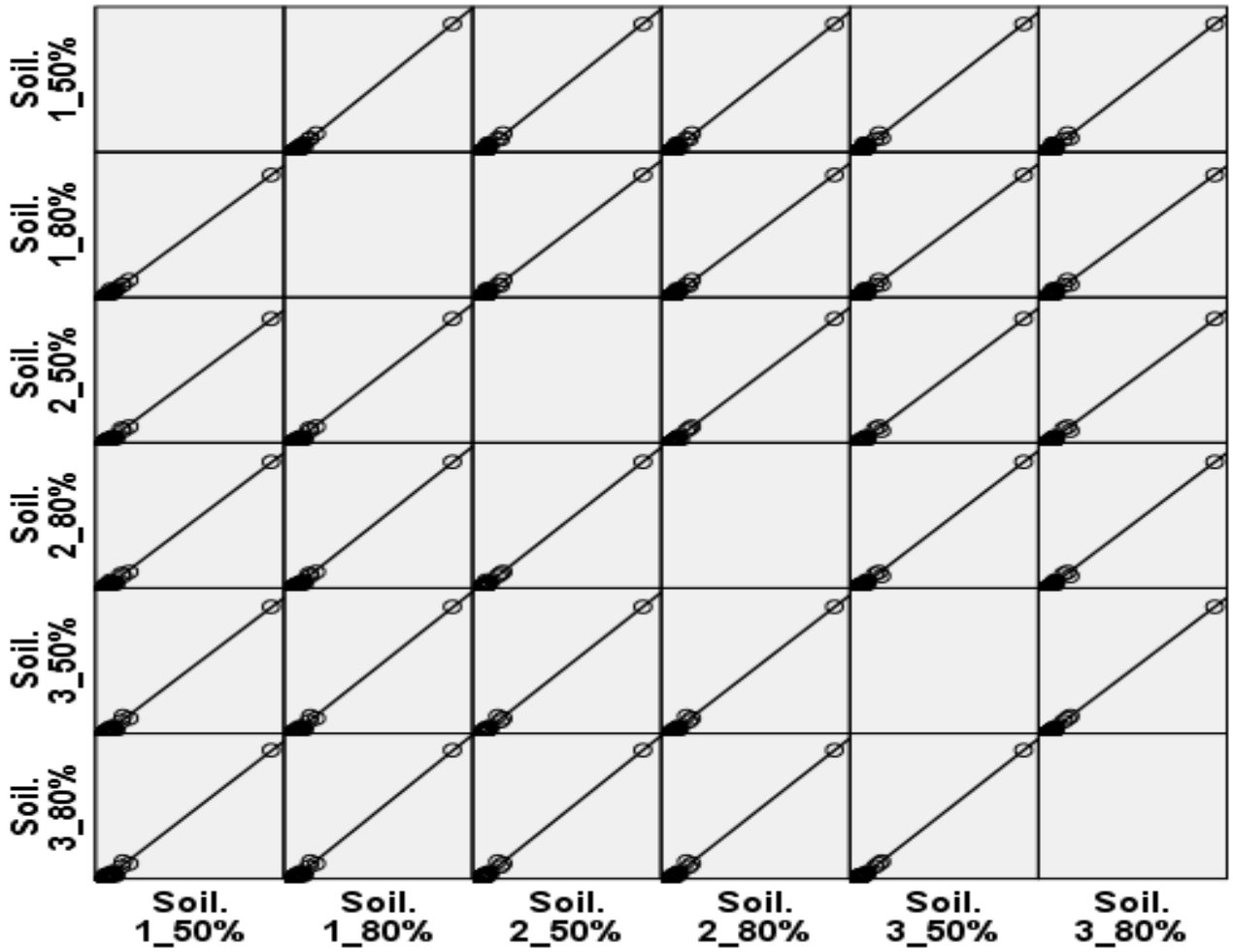

Figure 7.16 Spearman Rho Correlations performed at genus level (Control: Soil + nutrients, assigned taxonomy at 50% and 80% bootstrap cutoff) 454 Pyrosequencing data

Table 7.6 Spearman Rho Correlations performed at genus level (Control: Soil + nutrients + AC, assigned taxonomy at 50% and 80% bootstrap cutoff) 454 Pyrosequencing data

Correlations

			SoilAC.1_50%	SoilAC.1_80%	SoilAC.2_50%	SoilAC.2_80%	SoilAC.3_50%	SoilAC.3_80%
Spearman's rho	SoilAC.1_50%	Correlation Coefficient	1.000	.937**	.691**	.637**	.604**	.562**
		Sig. (2-tailed)	.	.000	.000	.000	.000	.000
		N	193	193	193	193	193	193
	SoilAC.1_80%	Correlation Coefficient	.937**	1.000	.661**	.711**	.564**	.634**
		Sig. (2-tailed)	.000	.	.000	.000	.000	.000
		N	193	193	193	193	193	193
	SoilAC.2_50%	Correlation Coefficient	.691**	.661**	1.000	.906**	.763**	.708**
		Sig. (2-tailed)	.000	.000	.	.000	.000	.000
		N	193	193	193	193	193	193
	SoilAC.2_80%	Correlation Coefficient	.637**	.711**	.906**	1.000	.692**	.768**
		Sig. (2-tailed)	.000	.000	.000	.	.000	.000
		N	193	193	193	193	193	193
	SoilAC.3_50%	Correlation Coefficient	.604**	.564**	.763**	.692**	1.000	.908**
		Sig. (2-tailed)	.000	.000	.000	.000	.	.000
		N	193	193	193	193	193	193
	SoilAC.3_80%	Correlation Coefficient	.562**	.634**	.708**	.768**	.908**	1.000
		Sig. (2-tailed)	.000	.000	.000	.000	.000	.
		N	193	193	193	193	193	193

**. Correlation is significant at the 0.01 level (2-tailed).

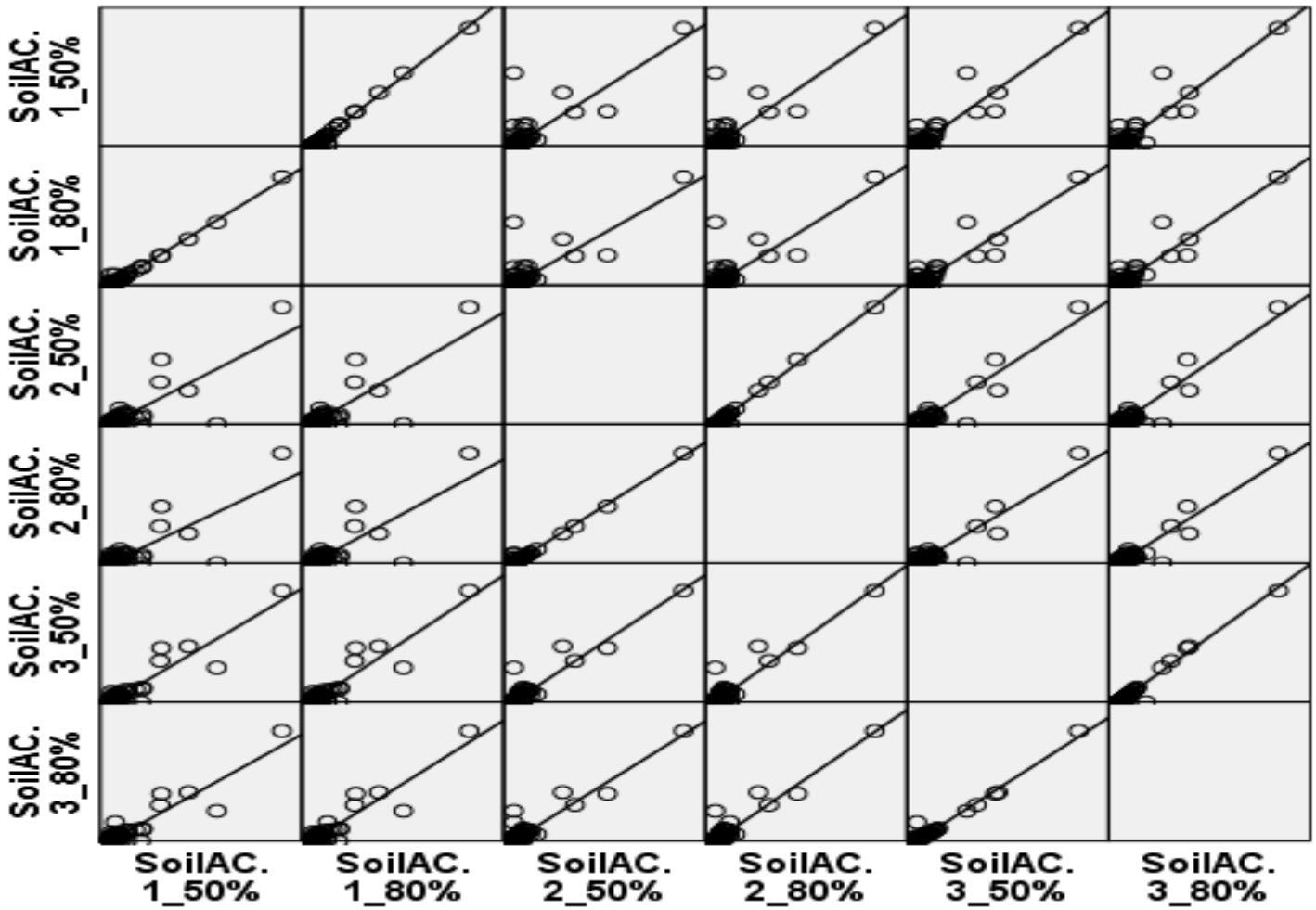

Figure 7.17 Spearman Rho Correlations performed at genus level (Control: Soil + nutrients + AC, assigned taxonomy at 50% and 80% bootstrap cutoff) 454 Pyrosequencing data

Table 7.7 Spearman Rho Correlations performed at genus level (Soil + nutrients + Oil + AC, assigned taxonomy at 50% and 80% bootstrap cutoff) 454 Pyrosequencing data

Correlations

			SoiloilAC.1_50%	SoiloilAC.1_80%	SoiloilAC.2_50%	SoiloilAC.2_80%	SoiloilAC.3_50%	SoiloilAC.3_80%
Spearman's rho	SoiloilAC.1_50%	Correlation Coefficient	1.000	.987**	.867**	.874**	.751**	.711**
		Sig. (2-tailed)	.	.000	.000	.000	.000	.000
		N	193	193	193	193	193	193
	SoiloilAC.1_80%	Correlation Coefficient	.987**	1.000	.856**	.878**	.745**	.720**
		Sig. (2-tailed)	.000	.	.000	.000	.000	.000
		N	193	193	193	193	193	193
	SoiloilAC.2_50%	Correlation Coefficient	.867**	.856**	1.000	.967**	.750**	.728**
		Sig. (2-tailed)	.000	.000	.	.000	.000	.000
		N	193	193	193	193	193	193
	SoiloilAC.2_80%	Correlation Coefficient	.874**	.878**	.967**	1.000	.728**	.724**
		Sig. (2-tailed)	.000	.000	.000	.	.000	.000
		N	193	193	193	193	193	193
	SoiloilAC.3_50%	Correlation Coefficient	.751**	.745**	.750**	.728**	1.000	.960**
		Sig. (2-tailed)	.000	.000	.000	.000	.	.000
		N	193	193	193	193	193	193
	SoiloilAC.3_80%	Correlation Coefficient	.711**	.720**	.728**	.724**	.960**	1.000
		Sig. (2-tailed)	.000	.000	.000	.000	.000	.
		N	193	193	193	193	193	193

**. Correlation is significant at the 0.01 level (2-tailed).

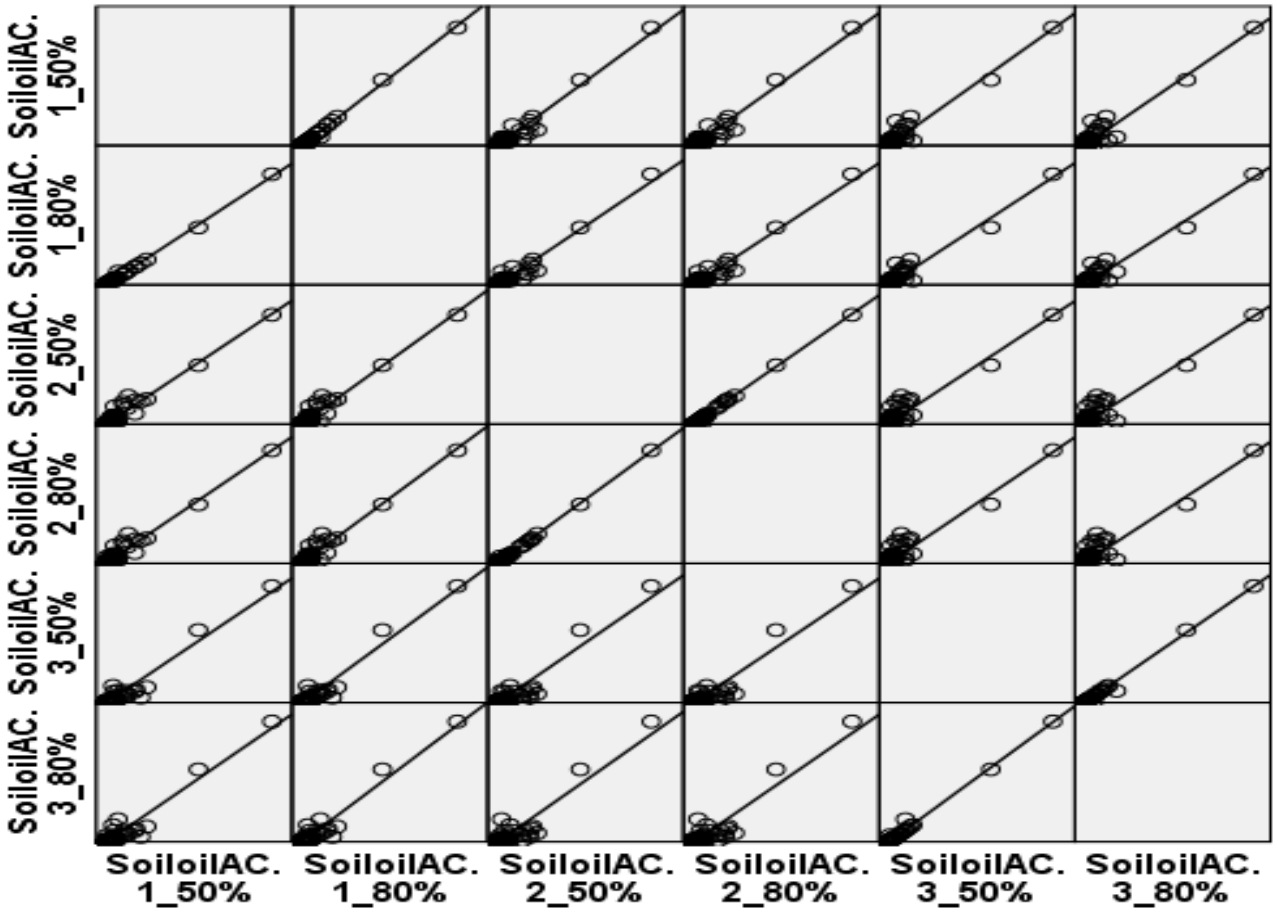

Figure 7.18 Spearman Rho Correlations performed at genus level (Soil + nutrients + Oil + AC, assigned taxonomy at 50% and 80% bootstrap cutoff) 454 Pyrosequencing data

Table 7.8 Spearman Rho Correlations performed at genus level (Soil + nutrients + Oil + AC at 5 months, assigned taxonomy at 50% and 80% bootstrap cutoff) 454 Pyrosequencing data

Correlations

			SoiloilAC5_1_50%	SoiloilAC5_1_80%	SoiloilAC5_2_50%	SoiloilAC5_2_80%	SoiloilAC5_3_50%	SoiloilAC5_3_80%
Spearman's rho	SoiloilAC5_1_50%	Correlation Coefficient	1.000	.940**	.706**	.682**	.693**	.671**
		Sig. (2-tailed)	.	.000	.000	.000	.000	.000
		N	193	193	193	193	193	193
	SoiloilAC5_1_80%	Correlation Coefficient	.940**	1.000	.667**	.693**	.654**	.703**
		Sig. (2-tailed)	.000	.	.000	.000	.000	.000
		N	193	193	193	193	193	193
	SoiloilAC5_2_50%	Correlation Coefficient	.706**	.667**	1.000	.937**	.759**	.702**
		Sig. (2-tailed)	.000	.000	.	.000	.000	.000
		N	193	193	193	193	193	193
	SoiloilAC5_2_80%	Correlation Coefficient	.682**	.693**	.937**	1.000	.719**	.751**
		Sig. (2-tailed)	.000	.000	.000	.	.000	.000
		N	193	193	193	193	193	193
	SoiloilAC5_3_50%	Correlation Coefficient	.693**	.654**	.759**	.719**	1.000	.935**
		Sig. (2-tailed)	.000	.000	.000	.000	.	.000
		N	193	193	193	193	193	193
	SoiloilAC5_3_80%	Correlation Coefficient	.671**	.703**	.702**	.751**	.935**	1.000
		Sig. (2-tailed)	.000	.000	.000	.000	.000	.
		N	193	193	193	193	193	193

**. Correlation is significant at the 0.01 level (2-tailed).

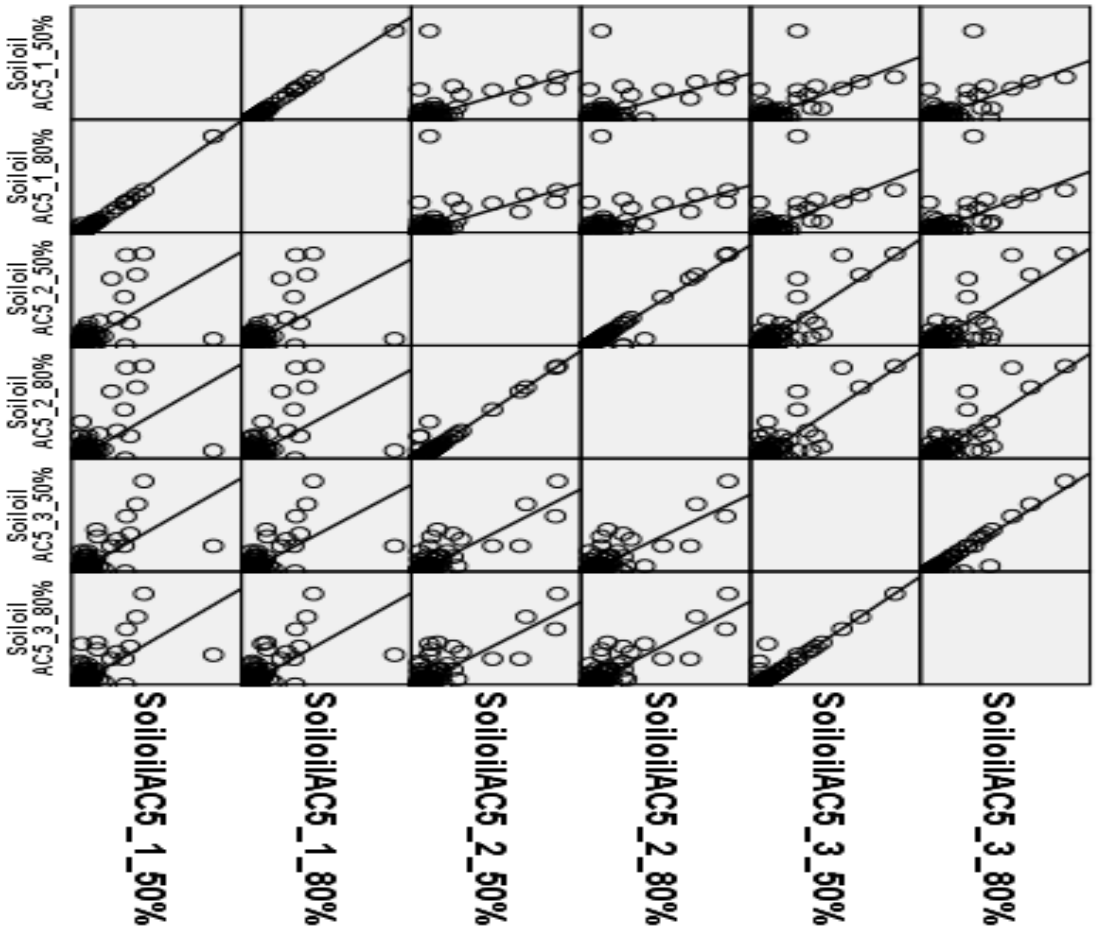

Figure 7.19 Spearman Rho Correlations performed at genus level (Soil + nutrients + Oil + AC at 5 months, assigned taxonomy at 50% and 80% bootstrap cutoff) 454 Pyrosequencing data

Table 7.9 Spearman Rho Correlations performed at genus level (Soil + nutrients + Oil, assigned taxonomy at 50% and 80% bootstrap cutoff) 454 data

Correlations

			Soiloil.1_50%	Soiloil.1_80%	Soiloil.2_50%	Soiloil.2_80%	Soiloil.3_50%	Soiloil.3_80%
Spearman's rho	Soiloil.1_50%	Correlation Coefficient	1.000	.947**	.765**	.748**	.767**	.744**
		Sig. (2-tailed)	.	.000	.000	.000	.000	.000
		N	193	193	193	193	193	193
	Soiloil.1_80%	Correlation Coefficient	.947**	1.000	.717**	.783**	.717**	.776**
		Sig. (2-tailed)	.000	.	.000	.000	.000	.000
		N	193	193	193	193	193	193
	Soiloil.2_50%	Correlation Coefficient	.765**	.717**	1.000	.915**	.774**	.729**
		Sig. (2-tailed)	.000	.000	.	.000	.000	.000
		N	193	193	193	193	193	193
	Soiloil.2_80%	Correlation Coefficient	.748**	.783**	.915**	1.000	.746**	.805**
		Sig. (2-tailed)	.000	.000	.000	.	.000	.000
		N	193	193	193	193	193	193
	Soiloil.3_50%	Correlation Coefficient	.767**	.717**	.774**	.746**	1.000	.937**
		Sig. (2-tailed)	.000	.000	.000	.000	.	.000
		N	193	193	193	193	193	193
	Soiloil.3_80%	Correlation Coefficient	.744**	.776**	.729**	.805**	.937**	1.000
		Sig. (2-tailed)	.000	.000	.000	.000	.000	.
		N	193	193	193	193	193	193

**. Correlation is significant at the 0.01 level (2-tailed).

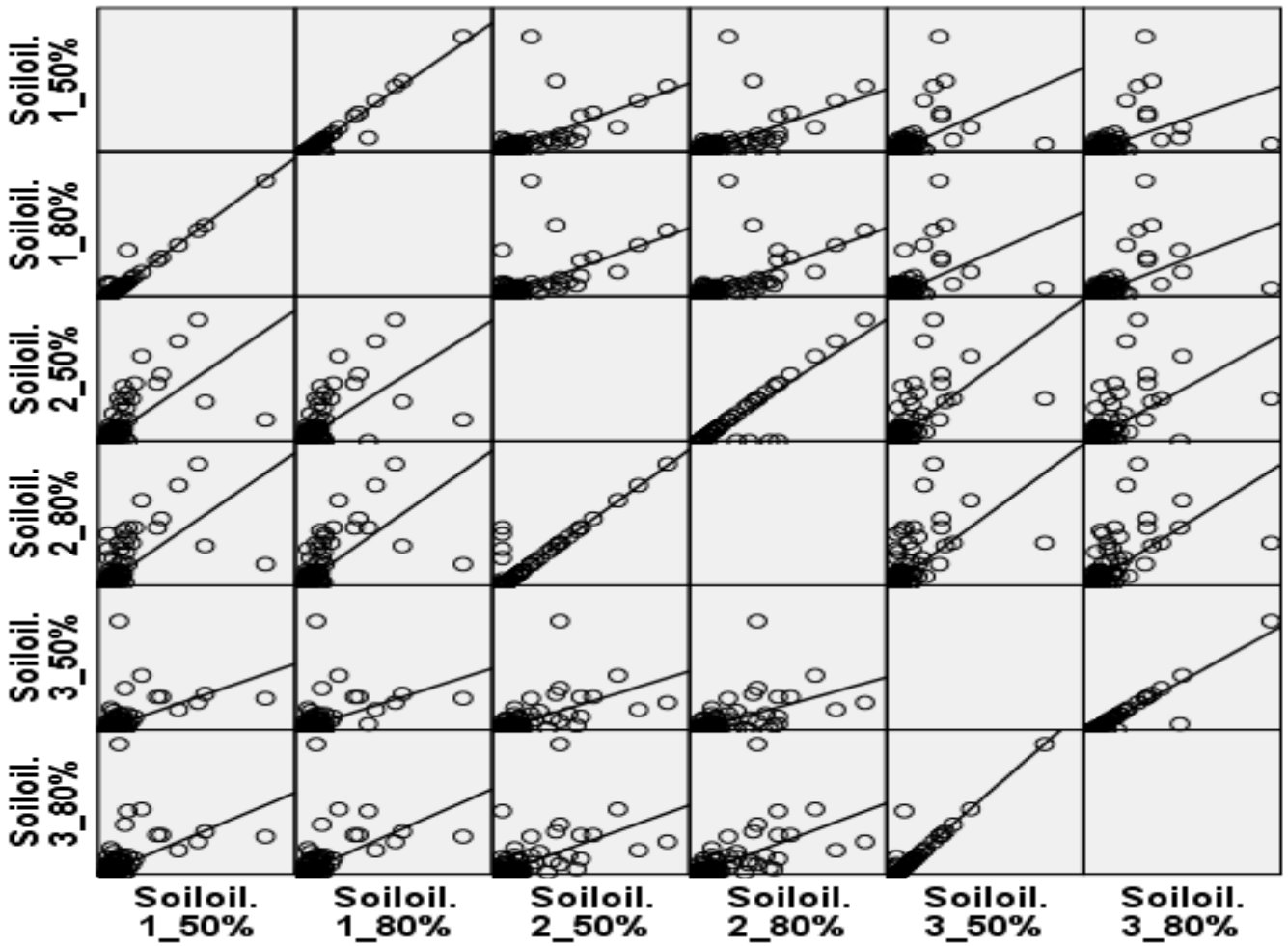

Figure 7.20 Spearman Rho Correlations performed at genus level (Soil + nutrients + Oil, assigned taxonomy at 50% and 80% bootstrap cutoff) 454 Pyrosequencing data

Table 7.10 Spearman Rho Correlations performed at genus level (Control: Original Soil i.e. Soil only, assigned taxonomy at 50% and 80% bootstrap cutoff) PGM Ion torrent data

Correlations

			OSoil.1_50%	OSoil.1_80%	OSoil.2_50%	OSoil.2_80%
Spearman's rho	OSoil.1_50%	Correlation Coefficient	1.000	.926**	.629**	.607**
		Sig. (2-tailed)	.	.000	.000	.000
		N	369	369	369	369
	OSoil.1_80%	Correlation Coefficient	.926**	1.000	.602**	.620**
		Sig. (2-tailed)	.000	.	.000	.000
		N	369	369	369	369
	OSoil.2_50%	Correlation Coefficient	.629**	.602**	1.000	.909**
		Sig. (2-tailed)	.000	.000	.	.000
		N	369	369	369	369
	OSoil.2_80%	Correlation Coefficient	.607**	.620**	.909**	1.000
		Sig. (2-tailed)	.000	.000	.000	.
		N	369	369	369	369

**. Correlation is significant at the 0.01 level (2-tailed).

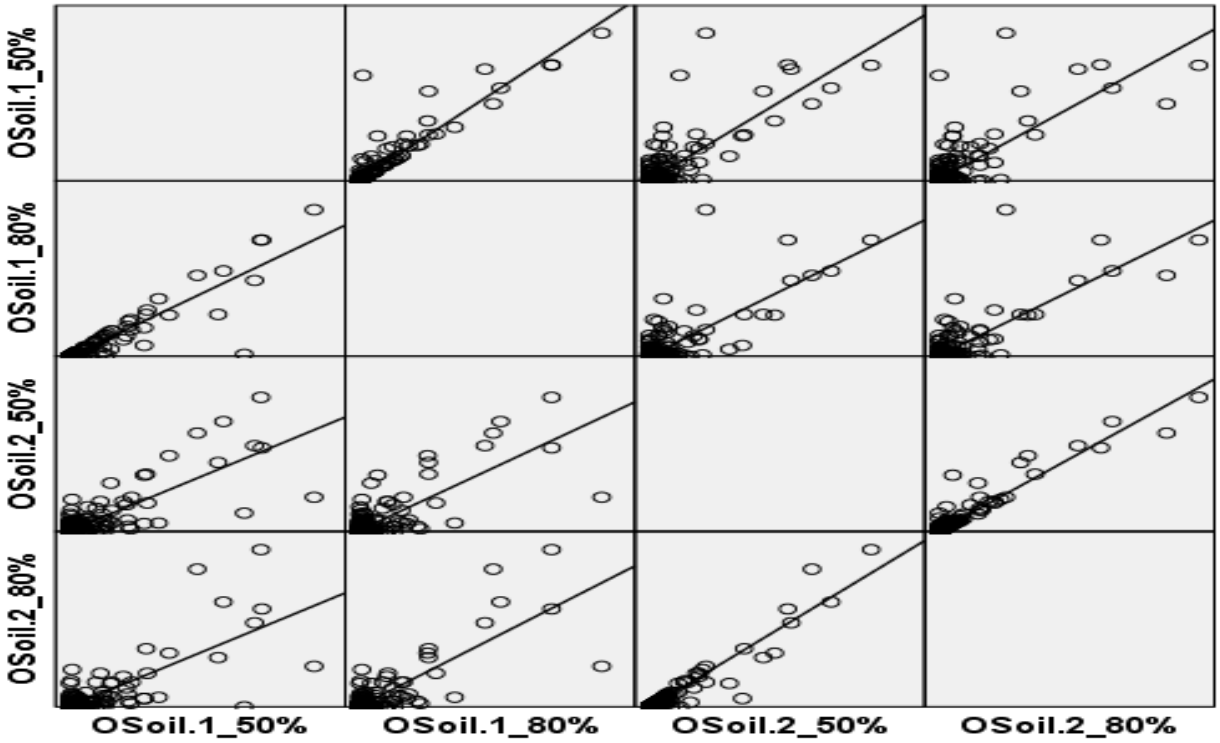

Figure 7.21 Spearman Rho Correlations performed at genus level (Control: Original Soil i.e. Soil only, assigned taxonomy at 50% and 80% bootstrap cutoff) PGM Ion torrent data

Table 7.11 Spearman Rho Correlations performed at genus level (Control: Soil + nutrients, assigned taxonomy at 50% and 80% bootstrap cutoff) PGM Ion torrent data

Correlations

			Soil.1_50%	Soil.1_80%	Soil.2_50%	Soil.2_80%	Soil.3_50%	Soil.3_80%
Spearman's rho	Soil.1_50%	Correlation Coefficient	1.000	.888**	.646**	.612**	.656**	.607**
		Sig. (2-tailed)	.	.000	.000	.000	.000	.000
		N	369	369	369	369	369	369
	Soil.1_80%	Correlation Coefficient	.888**	1.000	.557**	.601**	.561**	.607**
		Sig. (2-tailed)	.000	.	.000	.000	.000	.000
		N	369	369	369	369	369	369
	Soil.2_50%	Correlation Coefficient	.646**	.557**	1.000	.931**	.653**	.617**
		Sig. (2-tailed)	.000	.000	.	.000	.000	.000
		N	369	369	369	369	369	369
	Soil.2_80%	Correlation Coefficient	.612**	.601**	.931**	1.000	.615**	.634**
		Sig. (2-tailed)	.000	.000	.000	.	.000	.000
		N	369	369	369	369	369	369
	Soil.3_50%	Correlation Coefficient	.656**	.561**	.653**	.615**	1.000	.934**
		Sig. (2-tailed)	.000	.000	.000	.000	.	.000
		N	369	369	369	369	369	369
	Soil.3_80%	Correlation Coefficient	.607**	.607**	.617**	.634**	.934**	1.000
		Sig. (2-tailed)	.000	.000	.000	.000	.000	.
		N	369	369	369	369	369	369

**. Correlation is significant at the 0.01 level (2-tailed).

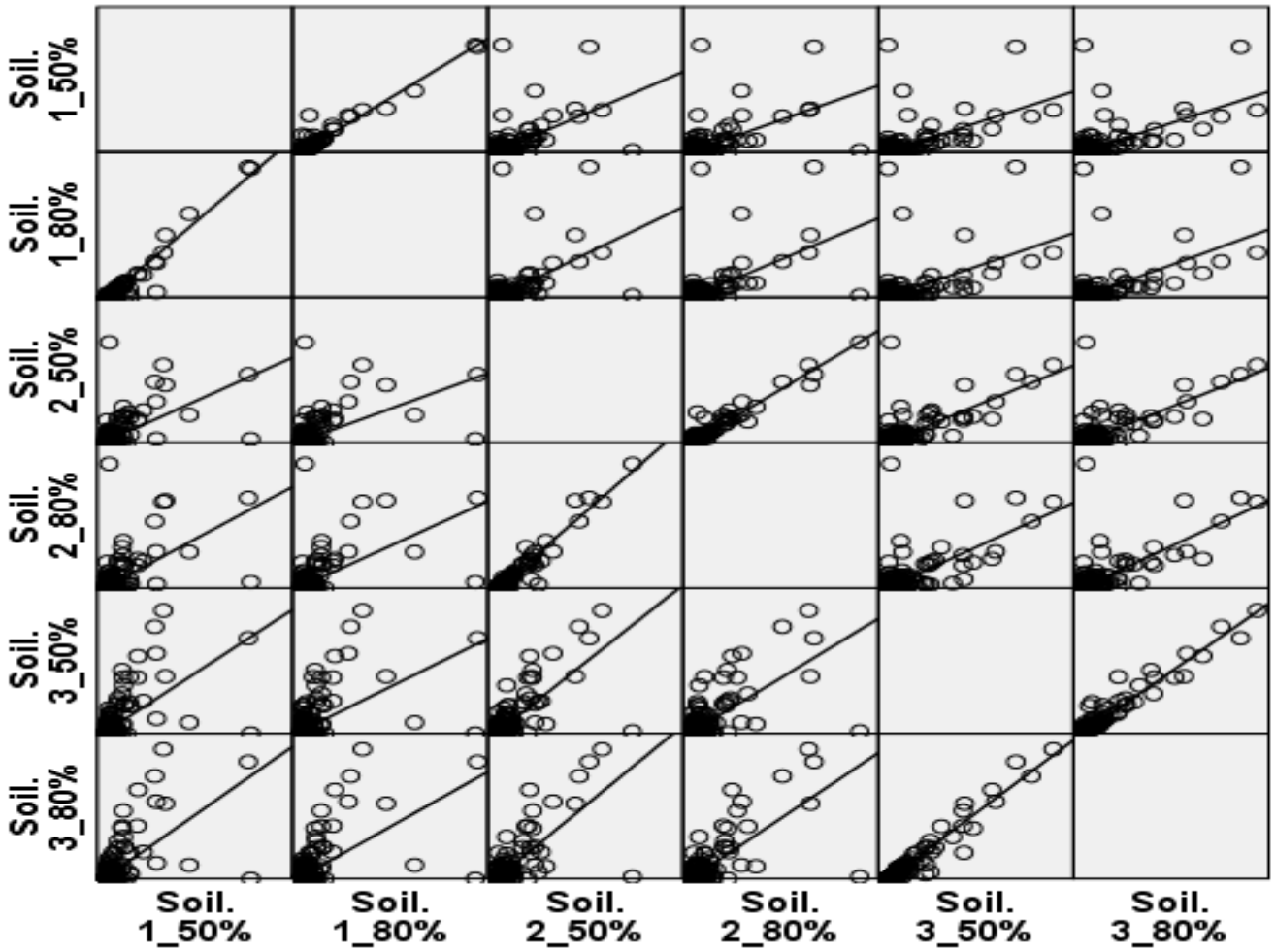

Figure 7.22 Spearman Rho Correlations performed at genus level (Control: Soil + nutrients, assigned taxonomy at 50% and 80% bootstrap cutoff) PGM Ion torrent data

Table 7.12 Spearman Rho Correlations performed at genus level (Control: Soil + nutrients + AC, assigned taxonomy at 50% and 80% bootstrap cutoff) PGM Ion torrent data

Correlations

			SoilAC.1_50%	SoilAC.1_80%	SoilAC.2_50%	SoilAC.2_80%	SoilAC.3_50%	SoilAC.3_80%
Spearman's rho	SoilAC.1_50%	Correlation Coefficient	1.000	.907**	.674**	.599**	.688**	.602**
		Sig. (2-tailed)	.	.000	.000	.000	.000	.000
		N	369	369	369	369	369	369
	SoilAC.1_80%	Correlation Coefficient	.907**	1.000	.615**	.622**	.625**	.639**
		Sig. (2-tailed)	.000	.	.000	.000	.000	.000
		N	369	369	369	369	369	369
	SoilAC.2_50%	Correlation Coefficient	.674**	.615**	1.000	.925**	.891**	.827**
		Sig. (2-tailed)	.000	.000	.	.000	.000	.000
		N	369	369	369	369	369	369
	SoilAC.2_80%	Correlation Coefficient	.599**	.622**	.925**	1.000	.831**	.868**
		Sig. (2-tailed)	.000	.000	.000	.	.000	.000
		N	369	369	369	369	369	369
	SoilAC.3_50%	Correlation Coefficient	.688**	.625**	.891**	.831**	1.000	.915**
		Sig. (2-tailed)	.000	.000	.000	.000	.	.000
		N	369	369	369	369	369	369
	SoilAC.3_80%	Correlation Coefficient	.602**	.639**	.827**	.868**	.915**	1.000
		Sig. (2-tailed)	.000	.000	.000	.000	.000	.
		N	369	369	369	369	369	369

**. Correlation is significant at the 0.01 level (2-tailed).

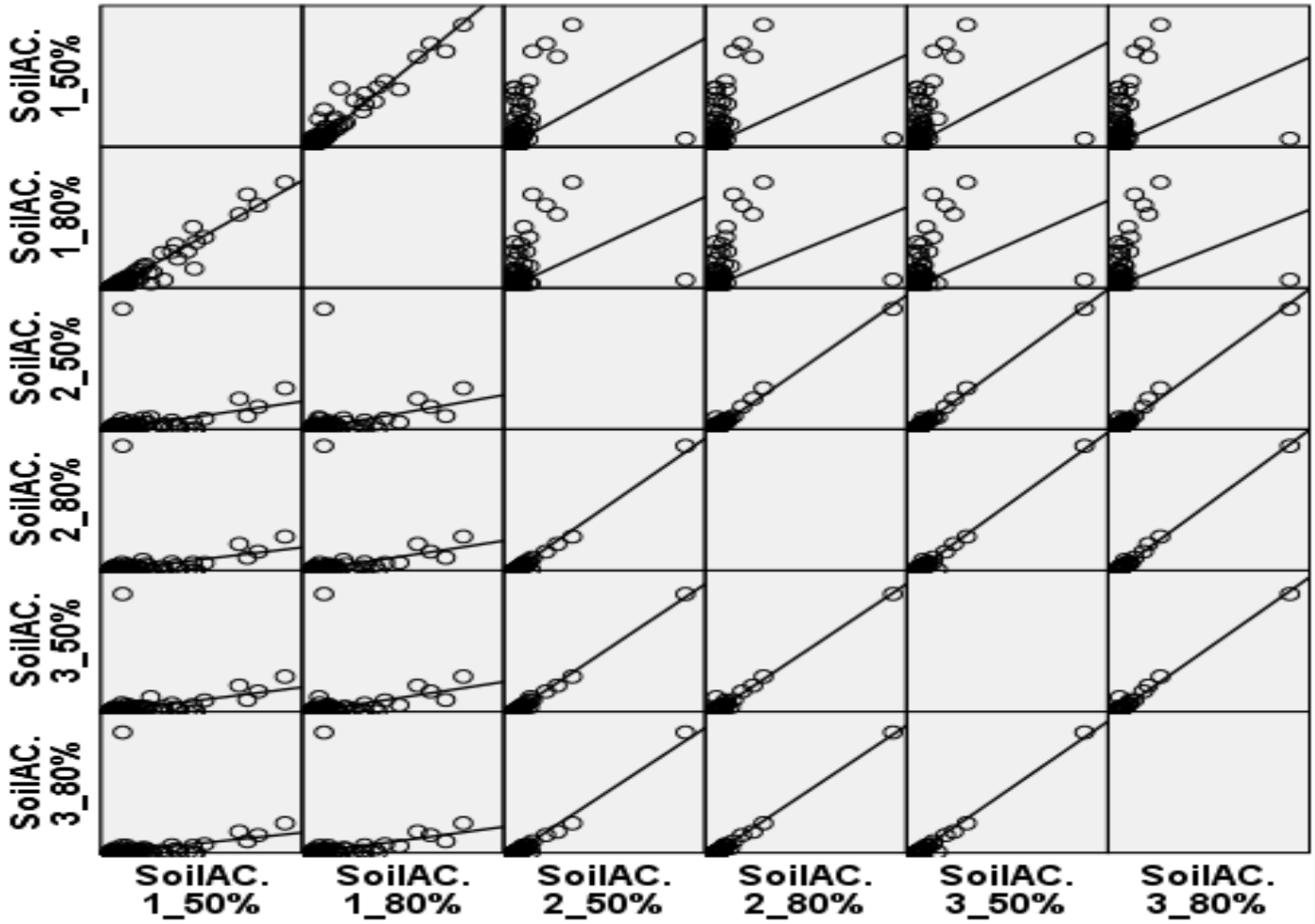

Figure 7.23 Spearman Rho Correlations performed at genus level (Control: Soil + nutrients + AC, assigned taxonomy at 50% and 80% bootstrap cutoff) PGM Ion torrent data

Table 7.13 Spearman Rho Correlations performed at genus level (Soil + nutrients + Oil + AC, assigned taxonomy at 50% and 80% bootstrap cutoff) PGM Ion torrent data

Correlations

			SoiloilAC.1_50%	SoiloilAC.1_80%	SoiloilAC.2_50%	SoiloilAC.2_80%	SoiloilAC.3_50%	SoiloilAC.3_80%
Spearman's rho	SoiloilAC.1_50%	Correlation Coefficient	1.000	.932**	.824**	.762**	.799**	.791**
		Sig. (2-tailed)	.	.000	.000	.000	.000	.000
		N	369	369	369	369	369	369
	SoiloilAC.1_80%	Correlation Coefficient	.932**	1.000	.772**	.794**	.761**	.816**
		Sig. (2-tailed)	.000	.	.000	.000	.000	.000
		N	369	369	369	369	369	369
	SoiloilAC.2_50%	Correlation Coefficient	.824**	.772**	1.000	.914**	.781**	.756**
		Sig. (2-tailed)	.000	.000	.	.000	.000	.000
		N	369	369	369	369	369	369
	SoiloilAC.2_80%	Correlation Coefficient	.762**	.794**	.914**	1.000	.744**	.778**
		Sig. (2-tailed)	.000	.000	.000	.	.000	.000
		N	369	369	369	369	369	369
	SoiloilAC.3_50%	Correlation Coefficient	.799**	.761**	.781**	.744**	1.000	.937**
		Sig. (2-tailed)	.000	.000	.000	.000	.	.000
		N	369	369	369	369	369	369
	SoiloilAC.3_80%	Correlation Coefficient	.791**	.816**	.756**	.778**	.937**	1.000
		Sig. (2-tailed)	.000	.000	.000	.000	.000	.
		N	369	369	369	369	369	369

**. Correlation is significant at the 0.01 level (2-tailed).

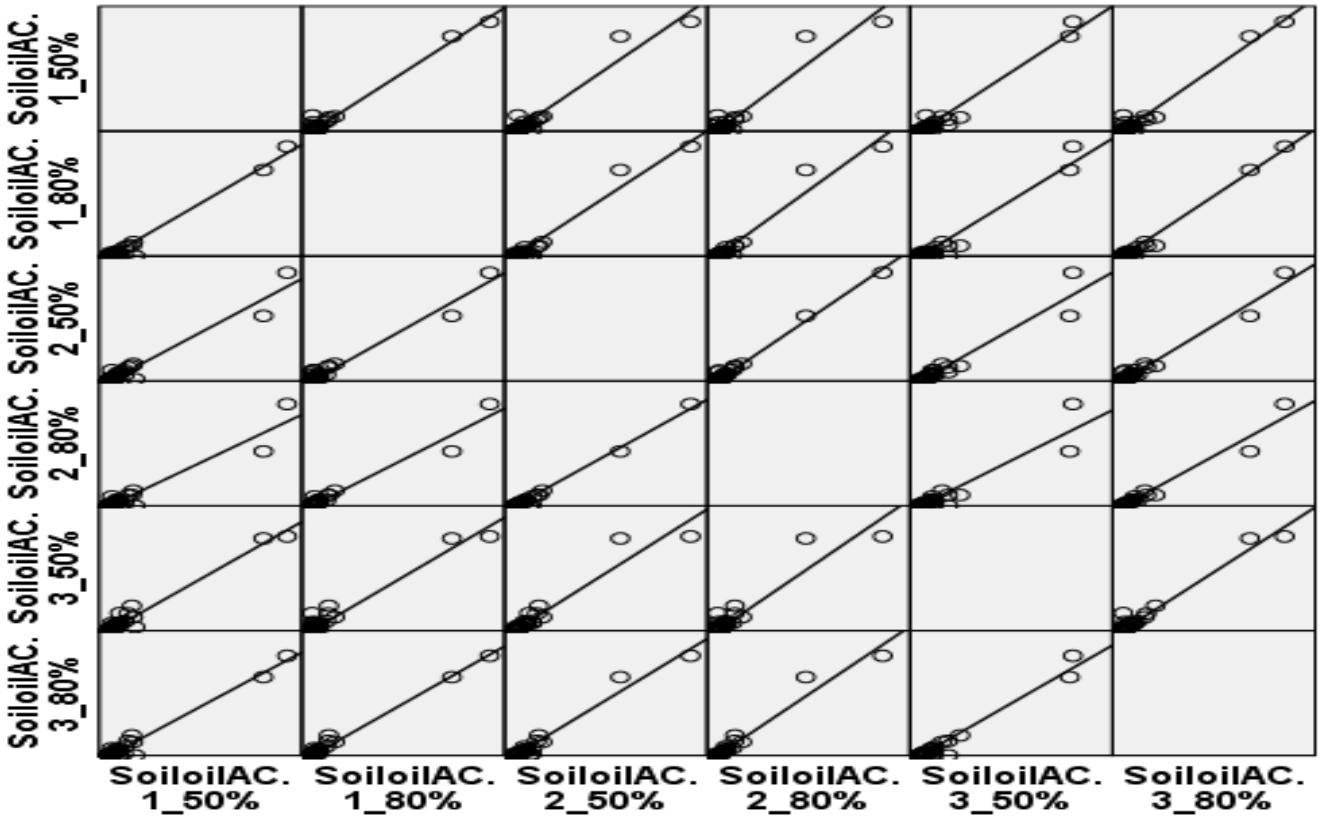

Figure 7.24 Spearman Rho Correlations performed at genus level (Soil + nutrients + Oil + AC, assigned taxonomy at 50% and 80% bootstrap cutoff) PGM Ion torrent data

Table 7.14 Spearman Rho Correlations performed at genus level (Soil + nutrients + Oil + AC at 5 months, assigned taxonomy at 50% and 80% bootstrap cutoff) PGM Ion torrent data

Correlations

			SoiloilAC5_1_50%	SoiloilAC5_1_80%	SoiloilAC5_2_50%	SoiloilAC5_2_80%	SoiloilAC5_3_50%	SoiloilAC5_3_80%
Spearman's rho	SoiloilAC5_1_50%	Correlation Coefficient	1.000	.919**	.818**	.753**	.841**	.781**
		Sig. (2-tailed)	.	.000	.000	.000	.000	.000
		N	369	369	369	369	369	369
	SoiloilAC5_1_80%	Correlation Coefficient	.919**	1.000	.741**	.794**	.802**	.820**
		Sig. (2-tailed)	.000	.	.000	.000	.000	.000
		N	369	369	369	369	369	369
	SoiloilAC5_2_50%	Correlation Coefficient	.818**	.741**	1.000	.842**	.818**	.732**
		Sig. (2-tailed)	.000	.000	.	.000	.000	.000
		N	369	369	369	369	369	369
	SoiloilAC5_2_80%	Correlation Coefficient	.753**	.794**	.842**	1.000	.746**	.784**
		Sig. (2-tailed)	.000	.000	.000	.	.000	.000
		N	369	369	369	369	369	369
	SoiloilAC5_3_50%	Correlation Coefficient	.841**	.802**	.818**	.746**	1.000	.922**
		Sig. (2-tailed)	.000	.000	.000	.000	.	.000
		N	369	369	369	369	369	369
	SoiloilAC5_3_80%	Correlation Coefficient	.781**	.820**	.732**	.784**	.922**	1.000
		Sig. (2-tailed)	.000	.000	.000	.000	.000	.
		N	369	369	369	369	369	369

**. Correlation is significant at the 0.01 level (2-tailed).

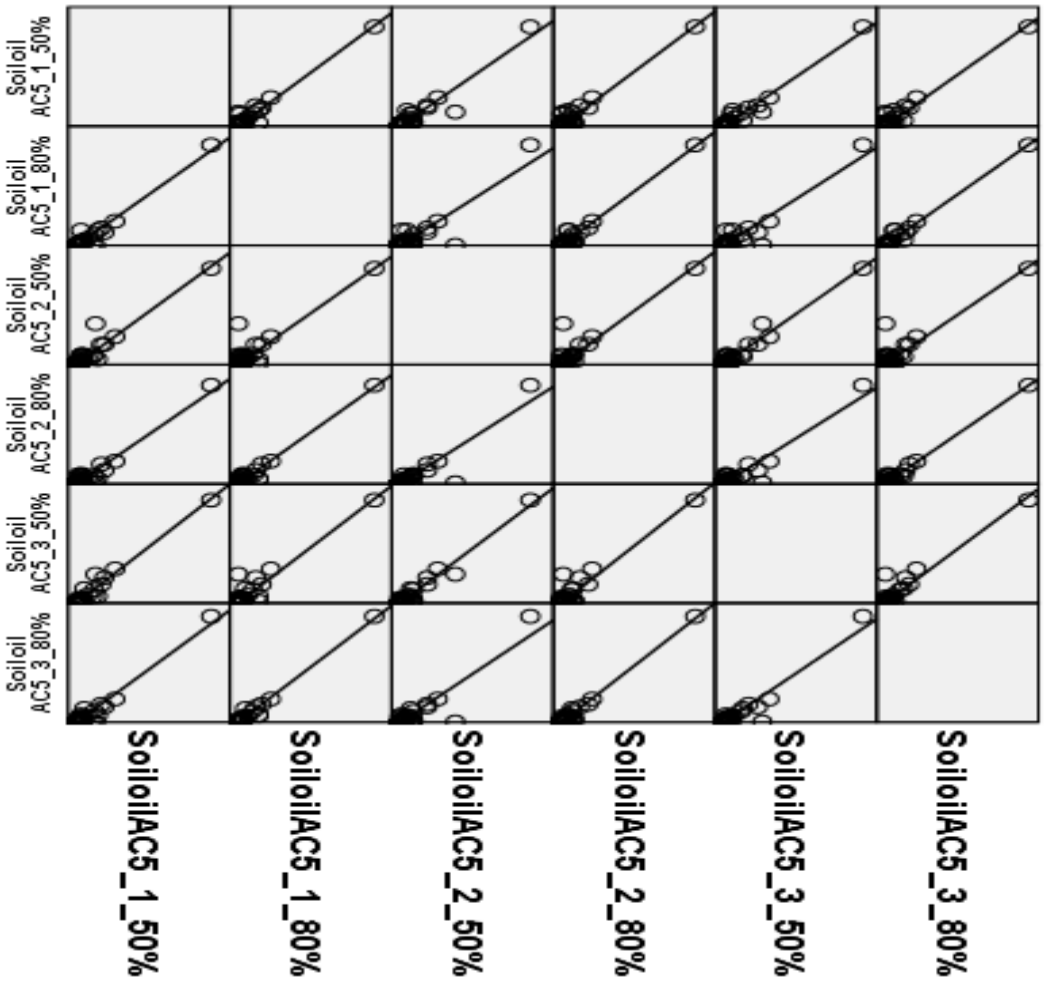

Figure 7.25 Spearman Rho Correlations performed at genus level (Soil + nutrients + Oil + AC at 5 months, assigned taxonomy at 50% and 80% bootstrap cutoff) PGM Ion torrent data

Table 7.15 Spearman Rho Correlations performed at genus level (Soil + nutrients + Oil, assigned taxonomy at 50% and 80% bootstrap cutoff) PGM Ion torrent data

Correlations

			Soiloil.1_50%	Soiloil.1_80%	Soiloil.2_50%	Soiloil.2_80%	Soiloil.3_50%	Soiloil.3_80%
Spearman's rho	Soiloil.1_50%	Correlation Coefficient	1.000	.894**	.817**	.760**	.726**	.696**
		Sig. (2-tailed)	.	.000	.000	.000	.000	.000
		N	369	369	369	369	369	369
	Soiloil.1_80%	Correlation Coefficient	.894**	1.000	.765**	.791**	.639**	.729**
		Sig. (2-tailed)	.000	.	.000	.000	.000	.000
		N	369	369	369	369	369	369
	Soiloil.2_50%	Correlation Coefficient	.817**	.765**	1.000	.913**	.772**	.751**
		Sig. (2-tailed)	.000	.000	.	.000	.000	.000
		N	369	369	369	369	369	369
	Soiloil.2_80%	Correlation Coefficient	.760**	.791**	.913**	1.000	.718**	.775**
		Sig. (2-tailed)	.000	.000	.000	.	.000	.000
		N	369	369	369	369	369	369
	Soiloil.3_50%	Correlation Coefficient	.726**	.639**	.772**	.718**	1.000	.819**
		Sig. (2-tailed)	.000	.000	.000	.000	.	.000
		N	369	369	369	369	369	369
	Soiloil.3_80%	Correlation Coefficient	.696**	.729**	.751**	.775**	.819**	1.000
		Sig. (2-tailed)	.000	.000	.000	.000	.000	.
		N	369	369	369	369	369	369

**. Correlation is significant at the 0.01 level (2-tailed).

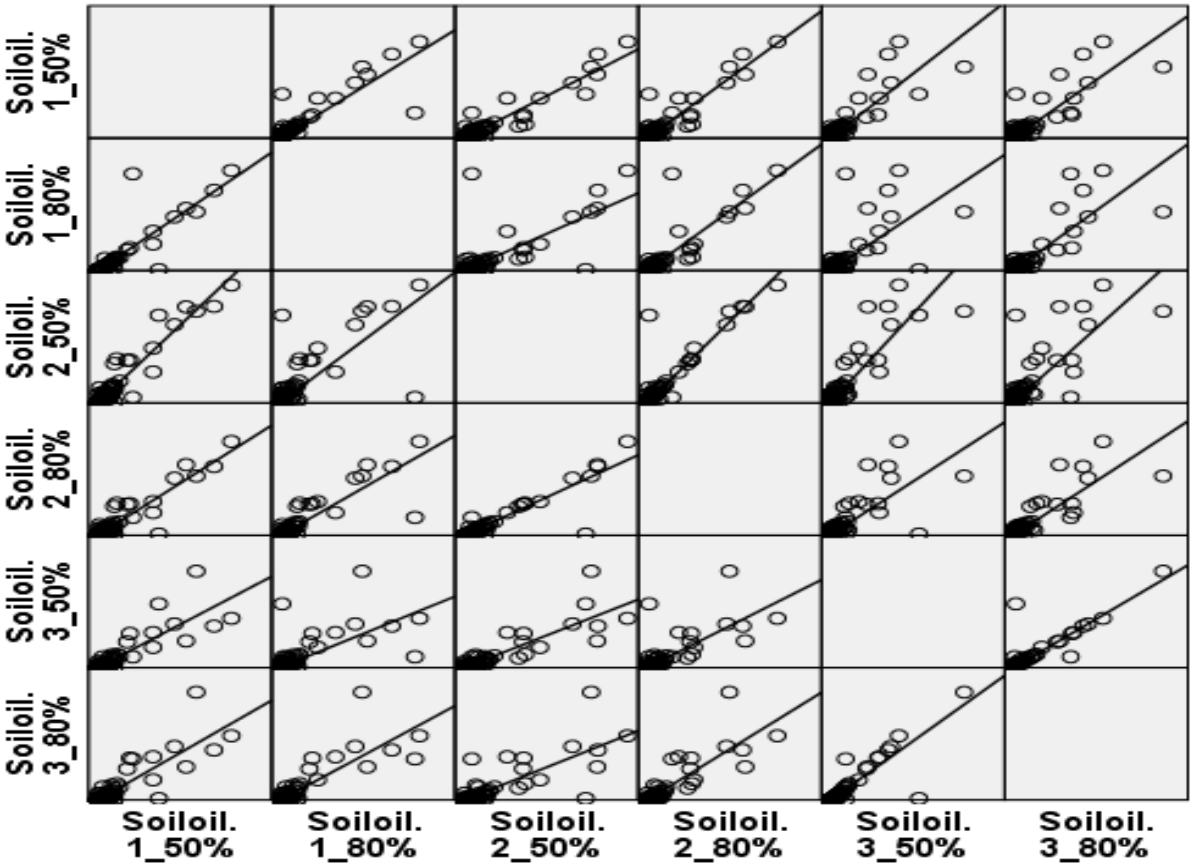

Figure 7.26 Spearman Rho Correlations performed at genus level (Soil + nutrients + Oil, assigned taxonomy at 50% and 80% bootstrap cutoff) PGM Ion torrent data

Appendix E

Table 7.16 Full 454 Pyrosequencing data at Phylum level (Sum relative abundances)

Taxon	Osoil	Soil	SoilAC	SoiloilAC	SoiloilAC5months	Soiloil
Unclassified;Other	0.0E+00	4.6E-05	0.0E+00	0.0E+00	0.0E+00	0.0E+00
k__Archaea;Other	0.0E+00	9.4E-05	0.0E+00	0.0E+00	0.0E+00	0.0E+00
k__Archaea;p__Crenarchaeota	6.3E-02	4.9E-02	9.2E-02	3.2E-02	2.3E-02	3.6E-02
k__Archaea;p__Euryarchaeota	0.0E+00	2.4E-04	2.3E-03	2.3E-03	9.1E-04	0.0E+00
k__Archaea;p__[Parvarchaeota]	2.3E-04	8.0E-04	4.2E-03	0.0E+00	0.0E+00	4.8E-04
k__Bacteria;Other	3.4E-03	6.6E-03	4.2E-03	7.2E-03	1.3E-03	3.1E-03
k__Bacteria;p__	4.3E-04	9.4E-05	0.0E+00	0.0E+00	0.0E+00	0.0E+00
k__Bacteria;p__AD3	0.0E+00	1.9E-04	8.7E-04	3.4E-04	9.0E-04	0.0E+00
k__Bacteria;p__Acidobacteria	8.9E-02	7.3E-02	7.8E-02	3.7E-02	5.0E-02	4.1E-02
k__Bacteria;p__Actinobacteria	4.3E-01	4.3E-01	4.4E-01	3.0E-01	3.8E-01	4.4E-01
k__Bacteria;p__Armatimonadetes	1.5E-03	2.8E-03	4.8E-03	1.6E-03	1.9E-03	1.7E-03
k__Bacteria;p__BRC1	2.8E-03	3.8E-03	5.2E-04	1.6E-03	0.0E+00	1.4E-03
k__Bacteria;p__Bacteroidetes	2.3E-01	7.5E-01	6.6E-01	5.8E-01	4.4E-01	2.7E-01
k__Bacteria;p__Chlamydiae	4.3E-04	1.0E-03	1.5E-03	0.0E+00	0.0E+00	7.0E-04
k__Bacteria;p__Chlorobi	7.0E-03	2.4E-02	3.1E-02	6.5E-02	1.2E-02	1.4E-02
k__Bacteria;p__Chloroflexi	1.8E-01	2.8E-01	2.0E-01	2.1E-01	1.8E-01	1.2E-01
k__Bacteria;p__Cyanobacteria	1.1E-03	2.1E-03	3.5E-03	4.1E-03	1.3E-03	2.8E-02
k__Bacteria;p__Elusimicrobia	1.8E-03	2.9E-04	0.0E+00	0.0E+00	5.3E-04	7.0E-04
k__Bacteria;p__FBP	6.6E-04	9.5E-05	0.0E+00	0.0E+00	0.0E+00	0.0E+00
k__Bacteria;p__FCPU426	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	1.8E-03

Table 7.17 Continuation 454 Pyrosequencing data at Phylum level (Sum of replicate relative abundances)

	Osoil	Soil	SoilAC	SoiloilAC	SoiloilAC5months	Soiloil
k__Bacteria;p__Fibrobacteres	1.1E-03	7.1E-04	9.2E-04	0.0E+00	3.8E-04	4.8E-04
k__Bacteria;p__Firmicutes	6.3E-02	1.1E-01	7.8E-02	3.6E-02	1.4E-01	1.9E-01
k__Bacteria;p__GAL15	1.9E-04	9.4E-05	0.0E+00	0.0E+00	0.0E+00	0.0E+00
k__Bacteria;p__GN02	0.0E+00	0.0E+00	9.2E-04	6.5E-04	0.0E+00	0.0E+00
k__Bacteria;p__GN04	1.9E-04	9.4E-05	0.0E+00	0.0E+00	0.0E+00	0.0E+00
k__Bacteria;p__Gemmatimonadetes	3.2E-02	6.5E-02	1.3E-01	4.7E-02	7.6E-02	4.3E-02
k__Bacteria;p__Kazan-3B-28	0.0E+00	2.4E-04	0.0E+00	6.5E-04	0.0E+00	0.0E+00
k__Bacteria;p__MVP-21	4.3E-04	1.4E-04	0.0E+00	3.1E-04	0.0E+00	0.0E+00
k__Bacteria;p__NKB19	1.4E-03	5.4E-03	2.9E-03	4.6E-03	4.3E-03	0.0E+00
k__Bacteria;p__Nitrospirae	1.6E-02	8.7E-03	1.0E-02	1.9E-03	5.4E-03	2.3E-03
k__Bacteria;p__OD1	4.7E-04	5.2E-04	1.7E-03	3.4E-04	7.6E-04	4.5E-03
k__Bacteria;p__OP11	2.3E-04	6.6E-04	4.8E-04	1.4E-03	3.8E-04	2.1E-03
k__Bacteria;p__OP3	1.9E-04	7.6E-04	0.0E+00	0.0E+00	0.0E+00	4.8E-04
k__Bacteria;p__PAUC34f	0.0E+00	9.5E-05	0.0E+00	0.0E+00	0.0E+00	0.0E+00
k__Bacteria;p__Planctomycetes	2.3E-01	2.8E-01	2.5E-01	3.2E-01	3.4E-01	2.9E-01
k__Bacteria;p__Proteobacteria	5.5E-01	7.4E-01	9.0E-01	1.3E+00	1.3E+00	1.4E+00
k__Bacteria;p__SBR1093	0.0E+00	2.1E-03	1.7E-03	6.4E-03	6.4E-03	4.8E-04

Table 7.18 Continuation 454 Pyrosequencing data at Phylum level (Sum of replicate relative abundances)

	Osoil	Soil	SoilAC	SoiloilAC	SoiloilAC5months	Soiloil
k__Bacteria;p__Spirochaetes	2.3E-04	3.3E-04	3.9E-04	0.0E+00	0.0E+00	7.0E-04
k__Bacteria;p__TM6	3.8E-03	4.4E-03	4.7E-03	2.9E-03	2.8E-03	5.9E-03
k__Bacteria;p__TM7	9.0E-04	6.6E-04	0.0E+00	0.0E+00	0.0E+00	1.9E-03
k__Bacteria;p__Tenericutes	2.3E-04	9.5E-05	4.8E-04	4.7E-04	1.1E-03	6.6E-04
k__Bacteria;p__Verrucomicrobia	8.1E-02	1.3E-01	8.0E-02	4.0E-02	7.6E-02	6.0E-02
k__Bacteria;p__WPS-2	1.9E-04	1.4E-04	9.6E-04	3.7E-03	0.0E+00	0.0E+00
k__Bacteria;p__WS2	1.6E-03	3.6E-03	1.4E-03	3.0E-03	3.8E-04	0.0E+00
k__Bacteria;p__WS3	6.2E-03	9.2E-03	8.3E-03	4.0E-03	1.7E-03	4.8E-03
k__Bacteria;p__WS4	0.0E+00	4.3E-04	4.8E-04	4.7E-04	1.1E-03	0.0E+00
k__Bacteria;p__[Thermi]	3.8E-04	1.9E-03	1.2E-03	1.1E-03	5.1E-03	1.2E-02

Table 7.19 PGM Ion torrent data at Phylum level (Sum of replicate relative abundances)

Taxon	Osoil	Soil	SoilAC	SoiloilAC	SoiloilAC5mnths	Soiloil
k__Archaea;p__Crenarchaeota	7.6E-03	4.1E-02	2.1E-01	3.5E-01	3.2E-01	8.8E-02
k__Archaea;p__Euryarchaeota	3.3E-03	7.9E-04	1.3E-03	1.4E-03	1.6E-03	4.9E-04
k__Archaea;p__[Parvarchaeota]	7.1E-04	9.5E-05	0.0E+00	3.0E-03	1.6E-03	0.0E+00
k__Bacteria;Other	1.7E-03	4.2E-03	3.5E-03	4.4E-03	4.7E-03	5.8E-03
k__Bacteria;p__AD3	0.0E+00	9.5E-05	2.8E-04	1.3E-04	2.2E-04	5.0E-05
k__Bacteria;p__Acidobacteria	2.1E-01	2.1E-01	1.8E-01	7.9E-02	8.2E-02	4.4E-02
k__Bacteria;p__Actinobacteria	3.4E-01	5.6E-01	6.3E-01	4.7E-01	4.7E-01	4.1E-01
k__Bacteria;p__Armatimonadetes	1.7E-03	4.7E-03	3.8E-03	1.8E-03	2.0E-03	7.2E-04
k__Bacteria;p__BRC1	1.9E-04	2.3E-04	1.2E-03	3.0E-03	2.0E-03	1.6E-03
k__Bacteria;p__Bacteroidetes	1.3E-01	2.3E-01	1.6E-01	4.8E-01	4.8E-01	4.1E-01
k__Bacteria;p__Chlamydiae	0.0E+00	0.0E+00	2.4E-04	5.3E-04	7.5E-04	6.5E-04
k__Bacteria;p__Chlorobi	1.2E-02	1.2E-02	4.9E-03	2.6E-02	2.5E-02	5.2E-02
k__Bacteria;p__Chloroflexi	1.8E-01	2.3E-01	2.2E-01	2.7E-01	1.7E-01	2.1E-01
k__Bacteria;p__Cyanobacteria	7.4E-03	1.5E-02	6.4E-03	3.8E-03	1.6E-03	2.8E-03
k__Bacteria;p__Elusimicrobia	2.4E-03	6.9E-04	2.3E-03	1.1E-04	2.0E-04	1.4E-04
k__Bacteria;p__FBP	0.0E+00	6.4E-04	6.2E-04	2.7E-05	0.0E+00	0.0E+00
k__Bacteria;p__FCPU426	0.0E+00	0.0E+00	6.3E-05	0.0E+00	0.0E+00	2.6E-05
k__Bacteria;p__Fibrobacteres	8.3E-05	4.9E-04	6.0E-04	4.9E-04	2.8E-04	5.2E-05
k__Bacteria;p__Firmicutes	3.5E-02	8.5E-02	1.3E-01	1.2E-01	1.0E-01	4.1E-02
k__Bacteria;p__GAL15	3.3E-04	1.1E-04	2.9E-05	5.4E-05	2.0E-04	0.0E+00
k__Bacteria;p__GN02	0.0E+00	0.0E+00	1.2E-04	2.9E-05	8.9E-05	0.0E+00
k__Bacteria;p__GN04	2.5E-04	0.0E+00	6.0E-05	5.4E-05	0.0E+00	5.0E-05

Table 7.20 Continuation of PGM Ion torrent data at Phylum level (Sum of replicate relative abundances)

Taxon	Osoil	Soil	SoilAC	SoiloilAC	SoiloilAC5mnths	Soiloil
k__Bacteria;p__Gemmatimonadetes	4.9E-02	5.8E-02	4.5E-02	7.4E-02	1.5E-01	7.0E-02
k__Bacteria;p__Kazan-3B-28	0.0E+00	0.0E+00	0.0E+00	2.9E-05	0.0E+00	2.6E-05
k__Bacteria;p__MVP-21	0.0E+00	0.0E+00	1.8E-04	9.5E-05	2.3E-04	1.2E-04
k__Bacteria;p__NC10	2.5E-03	1.5E-03	0.0E+00	0.0E+00	0.0E+00	0.0E+00
k__Bacteria;p__NKB19	0.0E+00	2.8E-04	3.6E-04	5.9E-03	2.9E-03	4.2E-03
k__Bacteria;p__Nitrospirae	3.8E-02	5.3E-02	3.6E-02	1.1E-02	1.9E-02	4.3E-03
k__Bacteria;p__OD1	8.3E-05	7.0E-05	2.6E-04	3.9E-04	8.6E-04	2.7E-04
k__Bacteria;p__OP11	4.8E-04	5.8E-04	5.3E-04	4.3E-04	6.4E-04	4.3E-04
k__Bacteria;p__OP3	2.1E-04	2.4E-04	1.2E-04	2.9E-04	1.0E-04	0.0E+00
k__Bacteria;p__OP8	0.0E+00	1.3E-04	0.0E+00	7.2E-05	0.0E+00	0.0E+00
k__Bacteria;p__Planctomycetes	1.4E-01	2.6E-01	2.5E-01	2.6E-01	2.2E-01	2.6E-01
k__Bacteria;p__Proteobacteria	8.1E-01	1.2E+00	1.0E+00	7.8E-01	8.7E-01	1.4E+00
k__Bacteria;p__SBR1093	0.0E+00	3.2E-05	9.8E-05	1.1E-03	6.5E-04	1.7E-03
k__Bacteria;p__SR1	0.0E+00	6.4E-05	0.0E+00	0.0E+00	0.0E+00	0.0E+00
k__Bacteria;p__Spirochaetes	2.4E-03	4.1E-04	2.1E-04	2.7E-04	1.7E-04	2.4E-04
k__Bacteria;p__Synergistetes	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.1E-04	0.0E+00
k__Bacteria;p__TM6	0.0E+00	3.9E-04	2.0E-03	2.8E-03	3.7E-03	3.2E-03
k__Bacteria;p__TM7	4.5E-03	8.3E-04	2.6E-04	1.1E-04	4.8E-04	7.9E-04
k__Bacteria;p__Tenericutes	0.0E+00	1.9E-04	1.6E-04	1.2E-04	1.1E-04	0.0E+00

Table 7.21 Continuation of PGM Ion torrent data at Phylum level (Sum of replicate relative abundances)

Taxon	Osoil	Soil	SoilAC	SoiloilAC	SoiloilAC5mnths	Soiloil
k__Bacteria;p__Verrucomicrobia	1.5E-02	3.5E-02	6.0E-02	5.9E-02	5.2E-02	2.5E-02
k__Bacteria;p__WPS-2	1.7E-04	4.1E-04	8.0E-04	8.4E-05	9.3E-04	1.2E-02
k__Bacteria;p__WS2	0.0E+00	2.9E-04	8.7E-05	4.2E-03	1.8E-03	4.1E-03
k__Bacteria;p__WS3	3.9E-03	5.5E-03	4.0E-03	5.7E-03	2.7E-03	1.3E-03
k__Bacteria;p__WS4	0.0E+00	2.4E-04	2.6E-04	2.6E-04	3.8E-04	1.9E-04
k__Bacteria;p__WS5	0.0E+00	6.4E-05	9.1E-05	0.0E+00	0.0E+00	0.0E+00
k__Bacteria;p__[Thermi]	2.1E-03	9.4E-04	7.0E-04	3.5E-04	3.1E-04	2.4E-04

Appendix F

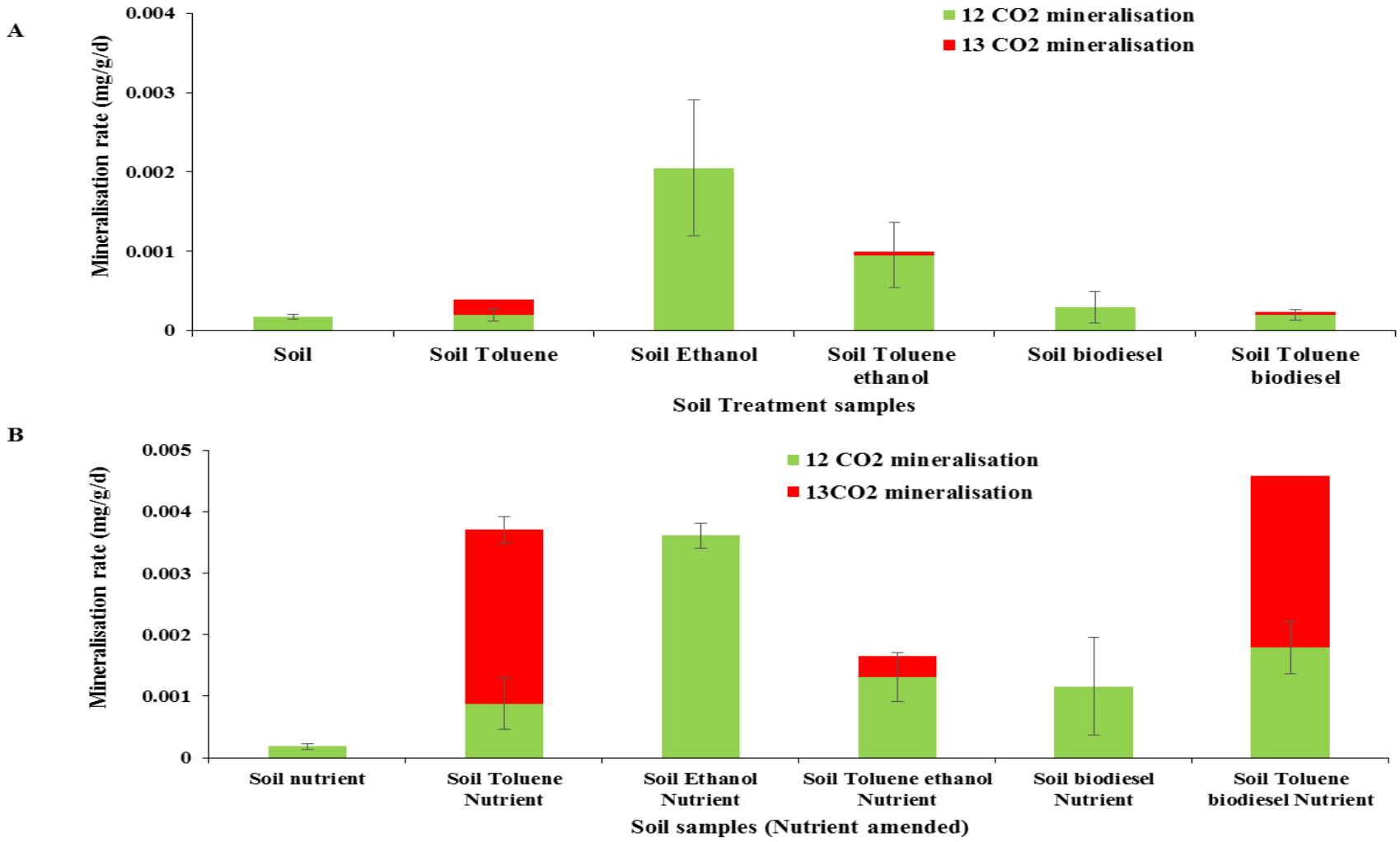

Figure 7.27 Daily Fuel mineralization rate to 12CO2 and 13CO2 (mg/day). A) No nutrient B) Nutrient amended