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A combined analytical approach to studying carbon dynamics in soils and sediments

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"*We know more about the movement of celestial bodies than the soil underfoot"*

Leonardo Da Vinci

Abstract

Studying the fate of organic carbon in soils and sediments is challenging due mainly to the multiplicity of relationships that exist not only between microbial consortia but between the organic components, nutrients and break down products. Understanding 'what is happening' to carbon in microbial systems would provide valuable information for climate change studies, agricultural planning, pollution studies and more. In this project, a method was developed using gas chromatography-mass spectroscopy (GCMS), isotope ratio mass spectroscopy (IRMS) and nuclear magnetic resonance (NMR) to track ^{13}C in soil biomass. Phospholipid fatty acids (PLFA's) identified using GCMS were used as biomarkers to characterise microbial populations, IRMS data revealed which microbial groups were utilizing ^{13}C labelled substrates. NMR allowed the transformations and fate of the carbon substrates to be determined. As the project progressed and developed the techniques were applied to studying organic pollutants and resident microbes in petroleum polluted sites. The method was also transferred to marine sediments, where it was used to study anthropogenic influence on sea bed organic matter in Dublin Bay. Diversifying the application of these methods has allowed us to investigate the fate organic carbon in a variety of scenarios. In incubated soils amended with CO_2, glucose and acetate, microbial populations varied with carbon input. Studying carbon sequestration in agricultural soil using these techniques proved that sequestration increased significantly with addition of elemental sulphur. In sites polluted with petroleum compounds GCMS was used to qualify and quantify organic pollutants and characterise the microbial populations present. The study of marine sediment in Dublin Bay revealed significant levels of faecal biomarkers throughout the area and also measurable levels of poly-aromatic hydrocarbons (PAH's). The methodology has proven to be adaptable to study carbon compounds in a wide variety of sample types.

Chapter 1

The use of phospholipid fatty acid biomarkers, stable isotopes and NMR to study soil carbon dynamics:

A Literature Review

1.0 Introduction:

Life on Earth depends on the cycling of carbon, the lithosphere is the largest carbon (C) sink with 75,000,000 gigatons (Gt) of C, the oceans contain approximately 38,000Gt C and the terrestrial biosphere contains 2500 Gt C, (Falkowski et al. 2000, Lal 2004). These values are subject to natural and anthropogenic fluxes (Sundquist, Ackerman and Parker 2009). Atmospheric carbon (~590 Gt) in the form of CO_2 exchanges rapidly with the terrestrial biosphere and ocean carbon reservoirs (Lorenz et al. 2007). Much research has been committed to understanding carbon cycling in the marine environment (Jiao et al. 2010), while less effort has been focused on soils (Liang and Balser 2011). Organic carbon represents approximately 60% of global soil carbon while at least 50% of this carbon has traditionally been categorized as the chemically resistant component known as humic substances (HS) (Lal 2004). Therefore, soil organic matter (SOM) contains huge amounts of carbon and plays an important role in regulating anthropogenic changes to the global carbon cycle. SOM is composed of a vast range of organic structures with varying chemical complexity with mean turnover times which range from days to years to millennia (Davidson and Janssens 2006, Amelung 2003, Gleixner et al. 2002b). It plays essential roles in agriculture (Stocking 2003),water quality (Lal et al. 2004), immobilization and transport of nutrients and anthropogenic chemicals (Walker 1995) while also concealing exciting opportunities for the discovery of novel compounds for potential use in industry and medicine (Flaig 1997). This review describes how biomarker analysis combined with stable isotopes and NMR can be used to track the biochemical pathways of particular soil organic matter.

1.1 Rationale

1.1.2 Agriculture

SOM is an important source and sink of carbon and nitrogen, and it plays a vital role in the cycling of these nutrients in terrestrial ecosystems (Belay-Tedla et al. 2009). In the European agricultural community vital grant supports may be refused to farmers who do not comply with certain crop rotation policies or fail to maintain a positive humus balance (Amelung et al. 2008). A high soil organic carbon content is crucial to ensuring soil health and stability (Six, Elliott and Paustian 2000). Carbon sequestration by soil and the natural carbon cycle are not sufficient to offset anthropogenic CO_2 emission into the atmosphere, however deliberate terrestrial sequestration through soil and forest

management has become an accepted method of mitigating global warming (Sundquist, Ackerman and Parker 2009, Six et al. 2002, West and Six. 2007). Maximising SOM however may result in excess nitrate in ground and surface waters from mineralisation of SOM. Advancements in the analysis of transformation and turnover of soil organic carbon (SOC) is therefore relevant to both agriculture and climate change.

1.1.3 Organic Pollution

Petroleum products are amongst the most widely used chemicals in the world. These chemicals are harvested from C reservoirs, known as fossil fuels, which took millennia to form (Sundquist, Ackerman and Parker 2009). The economies of the world are dependent on fossil fuels to power automobiles, heat homes and drive industry. With so much petroleum required contamination of the natural environment is inevitable (Fang, Lovanh and Alvarez 2004a, Sarkar et al. 2005b). Leaking storage tanks, illegal and abandoned petroleum manufacturing sites, poor waste management, spillage during transportation and current industrial practices are just some of the ways contamination can occur. Since the *Exxon Valdez* spill in 1989 much research has been devoted to understanding bioremediation of petroleum compounds in environmental systems (Margesin and Schinner 1997, Jackson and Pardue 1998). Aromatic hydrocarbons and polycyclic aromatic hydrocarbons (PAH's) are major components of petroleum (Seduikiene and Vichackaite 2001, Wang and Fingas 2003). They are persistent organic pollutants (POP's) which threaten water supplies and therefore public health (Fang, Lovanh and Alvarez 2004b, Alvarez 1991). Many mono-aromatic and polyaromatic hydrocarbons are carcinogenic and listed as priority pollutants by the US EPA (Wang and Fingas 2003). Organic matter in soil plays a key function in the partitioning of persistent organic pollutants (Nam et al. 2008). It has been accepted that SOM is the main controlling factor in the sorption of organic chemicals in soil (Lambert 1968, Burgess and Lohmann 2004). Section 1.4.6 of this review looks at how biomarker analysis combined with stable isotopes, can be used to track the degradation pathways of individual organic compounds.

1.1.4 Global Warming and the soil carbon debate

Although it has been widely debated, there is general acceptance that climate change will increase global temperatures by 1.4 to 5.8 °C by the end of the 21st century (Intergovernmental Panel on Climate Change. 2001). Global warming has prompted researchers to investigate the impact this will have on soil organic matter (Biasi et al. 2005, Rusalimova and Barsukov 2006, Smith et al. 2008). Increased temperatures are expected to accelerate the rate of decomposition of soil organic matter, thereby releasing CO_2 to the atmosphere which could result in positive feedback on climate change (Smith et al. 2008, Trumbore, Chadwick and Amundson 1996, Cox et al. 2000). Research at Cranfield University (Bellamy et al. 2005), discovered that between 1978 and 2003 the soils of England and Wales lost an average of 13 million tonnes of carbon a year. The authors suggest this is due to climate change while others would argue it is due to land use. It is unclear from the research where the carbon went, did it leach into water and deeper soils as bicarbonate and organic matter or was it lost to the atmosphere as CO_2? These questions highlight the need for new methods to study the pathways of carbon in soil, this review looks at how biomarkers and stable isotope tracer studies may be employed to this end.

A recent *Nature* article suggests how soils might respond to climate change, an analysis of a five decade record of global soil respiration (R_s) chamber measurements, matched with high resolution historical climate data was presented (Bond-Lamberty and Thomson 2010). This analysis shows that over the past 20 years R_s has increased with global warming. However these results do not necessarily confirm that soils are losing a greater portion of their carbon stores to the atmosphere. Higher temperatures may result in increased plant growth at least in cooler regions (Smith and Fang 2010) this may result in increased R_s as fresh carbon sources are lost to plant growth. Increased R_s may be due to increased carbon cycling rather than just carbon loss from soil C pools to the atmospheric pool (Bond-Lamberty and Thomson 2010). Assessing the balance between soil carbon inputs from increased plant growth, and increased carbon decomposition rates should be a research priority (Smith and Fang 2010). The methodology reviewed in this paper is aimed at better understanding the underlying processes of R_s and the fate of carbon inputs to soil.

The division of soil carbon into two conceptual pools is heavily debated. Labile and recalcitrant carbon pools are differentiated mainly by their varying decomposition

rates (Kleber et al. 2011). It is suggested that the labile carbon pool has a fast turnover time and is composed mainly of carbohydrates and proteins (Rovira and Vallejo 2002, McLauchlan and Hobbie 2004). Recalcitrant carbon is believed to have a long turnover time, chemical recalcitrance has been defined as an inherent chemical property of a molecule rendering it resistant to decomposition, this is usually associated with aromatic structures such as lignin, but also fats, resins and waxes, this soil organic carbon is believed to have been humified (Sollins, Homann and Caldwell 1996). Humic substances (HS) are a large, operationally defined fraction of SOM and represent the largest pool of recalcitrant organic carbon in the terrestrial environment. It has traditionally been thought that HS consist of novel categories of cross-linked macromolecular structures that form a distinct class of chemical compounds (Stevenson 1994). However, there are many questions over the importance of chemical recalcitrance as a stabilization mechanism for soil organic carbon (Kleber et al. 2011). It has been proposed that young SOC does not differ greatly in composition to older SOC (Paul et al. 2006). In recently published work, Kelleher and Simpson (2006), used advanced Nuclear Magnetic Resonance (NMR) approaches to conclude that the vast majority of humic material in soils is a very complex mixture of microbial and plant biopolymers and their degradation products and not a distinct chemical category as is traditionally thought (Kelleher and Simpson 2006). Other studies have shown that there is no evidence to support the theory that humification processes are creating recalcitrant humic substances (Lehmann et al. 2008).

It has been suggested that only heterotrophic utilization of organic labile carbon will increase with global warming, with recalcitrant pools being unaffected, thus the readily available carbon will decline and R_s will decrease (Liski et al. 1999, Rustad 2001, Luo et al. 2001, Melillo et al. 2002). However, there is much debate and this work has been questioned by researchers (Knorr et al. 2005). Since such a large portion of SOM is considered recalcitrant, understanding the temperature sensitivity and potential availability of this carbon pool is vital to climate change modelling (Biasi et al. 2005). Stable isotope studies combined with solid state NMR may provide improved knowledge of soil carbon turnover times thus transforming our interpretation of what is labile and what is recalcitrant.

1.2 Oil hydrocarbons: Anthropogenic soil carbon

1.2.1 Oil and oil spills

Oil a fossil fuel also known as petroleum, is a naturally occurring liquid. The world is powered by petroleum and our over reliance on petroleum means that 2 billion tons of petroleum is refined annually to meet demand (Singh and Lin 2009). There are very few surface seepages of oil, the majority of this resource is located deep below the surface and can only be exploited by drilling. Crude oil is a complex mix of mainly carbon and hydrogen, it contains varying quantities of oxygen, sulphur, nitrogen, metals and other elements (Nadim et al. 2000). With so much petroleum being drilled, transported and stored, accidents, spills and leaks are unavoidable (Sarkar et al. 2005a). Oil contains many toxic and carcinogenic compounds which can persist in the environment for centuries (Fang and Findlay 1996). An oil spill causes extensive damage to the environment and is a danger to human, plant and animal life. Therefore methods to determine the source, extent and fate of petroleum pollutants in the environment are vital (Wang and Fingas 2003).

1.2.2 Target analytes for oil spill identification and characterisation

Petroleum contains thousands of different organic compound, methods used for oil spill analysis can be divided into two categories (Wang and Fingas 2003): non-specific methods and specific. Non specific methods are used for rapid measurement and monitoring of petroleum contamination. These methods (such as EPA method 1614 and 418.1) determine groups or fractions of petroleum hydrocarbons such as total petroleum hydrocarbons (TPH's) (Xie, Barcelona and Fang 1999) and PAH's (Martinez et al. 2004). Specific methods are more focused on individual hydrocarbons and determining the composition of the petroleum spill.

Source-specific target analytes are used to chemically characterise the oil source and the environmental risk of the spill. The major petroleum target analytes are:

- Saturated hydrocarbons C_8 to $C_{40,}$ selected isoprenoids, pristine and phytane.
- Volatile organic carbons (VOC's) such as benzene, toluene, ethylbenzene, ortho, meta and para xylene (BTEX). Also volatile parafins, isoparafins and naphthenes.
- PAH's
- Terpanes and steranes

- Measuring combined TPH's, the unresolved complex mixture (UCM) and carbon isotope ratio ($\delta^{13}C$).

Crude oil is refined into a variety of products with varying chemical and physical properties and therefore different uses:

1. Light distillates: Aviation gas, napha and gasoline/petrol. These distillates usually contain high levels of VOC's such as BTEX, C_3 to C_{12} compounds, naphthalenes and other alkalated benzenes.
2. Mid-range distillates: Kerosene, jet fuel and diesel. C_6 to C_{26} carbons and a variety of PAH's whose composition is indicative of the crude oil feed stock.
3. Heavy Fuels: Numbers 5 and 6 also known as Bunker C, used for marine diesel, home heating and industrial power generation. The chemical composition of this oil fraction can vary greatly as it is often a mix of residual oils and diesel depending on its set purpose. PAH's can be used to determine the source of these spills (Stout et al, 2001).
4. Lubricating oils: Crackcase oil, hydraulic oil, transmission fluid and cutting oil. C18 to C40 carbons – Large UCM and almost no unresolved alkanes.

(Target analyt and distillate information sourced from Wang and Fingas 2003, "Development of oil hydrocarbon fingerprinting and identification techniques)

1.2.3 Aromatic and Poly nuclear aromatic hydrocarbons

1.2.3.1 BTEX compounds

Benzene, toluene and xylene isomers (BTEX) (Figure 1) are monocyclic hydrocarbons which form a major component of gasoline (Singh and Lin 2009).

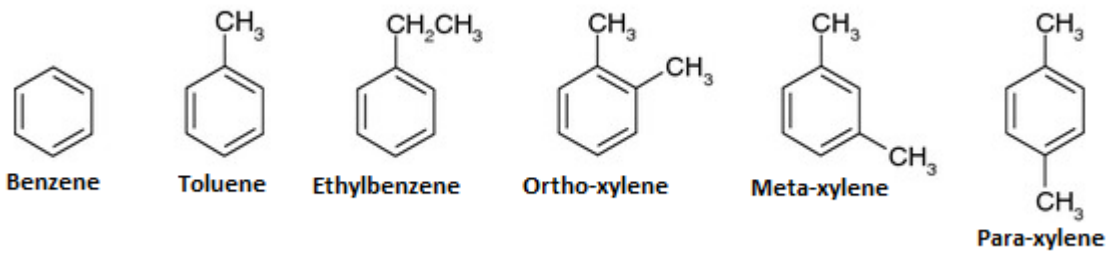

Figure 1: Chemical structures of BTEX

These light weight volatile petroleum products are widely used in industries such as paint, inks, synthetic resins and rubber (Wang and Fingas 2003, Nollet 2001). BTEX compounds are proven to have detrimental effects on human health causing neurological disease and cancer (Chiou, Schmedding and Manes 1982, WHO. 1993, Ezquerro et al. 2004). Compared to other hydrocarbons BTEX compounds have relatively high water solubility and low K_{ow} (See table 1) (EUGRIS. 2012) value thus they are common contaminants of aquifers making them a threat to public health (Alvarez 1991, Tursman and Cork 1992). The major sources of the contamination are leaking underground petrol station tanks and pipes and accidental spills (Tsao, Song and Bartha 1998). BTEX are classified as priority pollutants by the US environmental protection agency (U.S Environmental Protection Agency 1986) and by the European Union. For example a maximum contaminant level (MCL) of 1 μg L^{-1} for benzene in drinking water was established by the European Union in 1998 thus accurate methods for measuring BTEX are required (EU. 1998).

Table 1 Physical and Chemical properties of BTEX compounds

Compound	Mole weight g $mole^{-1}$	Density g ml^{-1}	Boiling point °C	Water solubility mg l^{-1}	Vapor pressure mmHg	Log K_{ow}
Benzene	78	0.88	80.1	1780	76	2.13
Toluene	92	0.87	110.8	535	22	2.69
o-Xylene	106	0.88	144.4	175	5	2.77
m-Xylene	106	0.86	139	135	6	3.20
p-Xylene	106	0.86	138.4	198	6.5	3.15
Ethylbenzene	106	0.87	136.2	152	7	3.15

(EUGRIS – Portal for soil and water management Europe)

1.2.3.2 PAH's

Polycyclic aromatic hydrocarbons (PAH's) are persistent organic pollutants (POP's) widely found in terrestrial and marine environments (Martinez et al. 2004, Raoux et al. 1999, Wilcke 2000, Song et al. 2002, Hung et al. 2009). PAH's have long been known for their carcinogenic potential (Krasnoschekova and Gubergrits 1976) and their adverse effect on marine biota has been well studied (International Agency for Research in Cancer IARC. 1983, Woodhead, Law and Matthiessen 1999, McCready et al. 2000). PAH's are characteristically lipophilic and have low water solubility (See table 2) therefore in aquatic systems they have high affinity for organic matter, they accumulate in sediments and can bio-accumulate in mussels and other aquatic species (Martinez et al. 2004, Knap and Williams 1982, Nendza et al. 1997, Geffard et al.

2003). Due to the potential dangers of this group of chemicals, the US EPA has listed 16 PAH's as priority pollutants (Table 2).

Table 2; Physical and chemical properties of the 16 EPA priority PAH's

Compound	**Molecular weight g/mole**	**Molecular formula**	**Number of rings**	**Solubility in water µg L^{-1}**	**K_{ow}**
Naphthalene	128	$C_{10}H_8$	2	$3.17x10^4$	3.29
Acenaphtylene	152	$C_{12}H_8$	3	$3.4x10^3$	4.07
Acenaphthene	154	$C_{12}H_{10}$	3	$3.93x10^3$	3.98
Fluorene	166	$C_{13}H_{10}$	3	$1.98x10^3$	4.18
Phenanthrene	178	$C_{14}H_{10}$	3	$1.29x10^3$	4.45
Anthracene	178	$C_{14}H_{10}$	3	73	4.45
Flouranthene	202	$C_{16}H_{10}$	4	260	4.90
Pyrene	202	$C_{16}H_{10}$	4	135	4.88
Benzo[*a*]anthracene	228	$C_{18}H_{12}$	4	14	5.61
Crysene	228	$C_{18}H_{12}$	4	2.0	5.16
Benzo[*b*]fluoranthene	252	$C_{20}H_{12}$	5	1.2	6.04
Benzo[*k*]fluoranthene	252	$C_{20}H_{12}$	5	0.76	6.06
Benzo[*a*]pyrene	252	$C_{20}H_{12}$	5	3.8	6.06
Indeno[1,2,3-*cd*]pyrene	276	$C_{22}H_{12}$	6	62	6.58
Dibenzo[*a,h*]anthracene	278	$C_{22}H_{12}$	5	0.5(27°C)	6.84
Benzo[*ghi*]perylene	276	$C_{22}H_{12}$	6	0.26	6.50

(Wang and Fingas 2003, Martinez et al. 2004, Prabhukumar 2010)

Most PAH's in the environment originate from anthropogenic sources, however, some are synthesized by bacteria, plants and fungi from natural products. Some PAH's occur from natural events such as forest fire (Boonyatumanond et al. 2007). The main anthropogenic sources of PAH's are combustion processes such as the burning of coal, automobile engines and biomass mass burning (Simoneit 1984). Urban sources are generally pyrogenic or petrogenic, and both combusted and unburned fossil fuels impart signature PAH's into the environment (Oros and Ross 2004). Unburned fossil fuel/petrogenic PAH's are generally biodegraded faster than combusted fuel derived PAH's (Jones et al. 1986). Neff et al (1979) showed that high temperature combustion emits high MW PAH's with 4 or more rings (Neff 1979). PAH's can enter the environment through atmospheric transfer, and due to their semi-volatilities they are widely distributed (Nollet 2001), they have even been detected in high mountain fish (Dachs et al. 2002). PAH's enter the aquatic environment by gas exchange at air water

interfaces and soot deposition, where they bind to particulate matter in the water column and collect in sediments (Oros and Ross 2004).

As a result of their widespread persistence, PAH's have been prioritised in directives by the European Union since 1976 (EU. 1976). More recent directives (EU. 1980) indicate a maximum residual level of 0.2μg/L in surface water intended to produce drinking water. All over Europe PAH monitoring systems are being set up to monitor levels throughout river basins and there estuaries (Martinez et al. 2004).

1.2.4 Solid-Phase Microextraction for the analysis of Volatile Organic Compounds (VOC's)

Volatile aromatic hydrocarbons are important constituents of petroleum and petroleum derived products (Seduikiene and Vichackaite 2001). VOC's have a high water solubility and thus a common contaminants of ground waters and soils (Alvarez 1991), there is an increasing demand for rapid and reliable analytical techniques for VOC's in environmental matrixes such as soil and water (Seduikiene and Vichackaite 2001). Soil is a complicated matrix which soil naturally contains many organic compounds which can interfere with the analysis of VOC's (Seduikiene and Vichackaite 2001, Ezquerro et al. 2004). Therefore, extraction and purification of VOC's in soil is problematic. Traditional solvent extraction techniques are laborious and use large amounts of solvent (EPA method 8100. 1986) thus they are not efficient or 'green'. Modern super critical fluid extractions and accelerated solvent extraction require expensive equipment. Traditional water preparation methods are also time consuming (Llompart, Li and Fingas 1999). Liquid-Liquid extraction again requires large amounts of solvent and large extractions are prone to contamination. Standard headspace (HS) analysis using a gas tight syringe is only adequate for samples that are significantly contaminated. Purge and trap (PAT) analysis is highly sensitive but requires expensive equipment (Llompart, Li and Fingas 1999).

Solid – phase microextraction (SPME) is a rapid technique developed by Pawliszyn in 1989 (Seduikiene and Vichackaite 2001). It is a solvent free, selective, inexpensive and easily automated extraction technique that simplifies analysis of VOC's and semi-VOC's (Ezquerro et al. 2004, Parnis and Brooks 2001, Munir, Saim and Ghani 2008) in air (Llompart, Li and Fingas 1999, Xiong, Chen and Pawliszyn 2003) water (Llompart, Li and Fingas 1999) and soil (Seduikiene and Vichackaite 2001, Ezquerro et al. 2004).

The SPME apparatus consists of a fused-silica fibre coated with a selective polymeric phase such as polyacrylate, polydimethylsiloxane-divinylbenzene, polydimethylsiloxane, carbowax-divinylbenzene or carboxen-polydimethylsiloxane when exposed to an environmental sample it adsorbs organic compounds. The fibre, which is mounted on a syringe, is then directly transferred to the GC inlet, the compounds desorb from the fibre and are separated by GC capillary column, detection and identification is most commonly achieved with a FID (Seduikiene and Vichackaite 2001, Ezquerro et al. 2004) or a MSD (Llompart, Li and Fingas 1999, Munir, Saim and Ghani 2008). As long as proper conditioning is applied to the fibre prior to each extraction, there should be no carry over from sample to sample and the fibre can be reused again and again. SPME has been utilised for a wide range of organic contaminants in water and soil such as BTEX (Ezquerro et al. 2004), PAH's (Llompart, Li and Fingas 1999, Eriksson et al. 2001) chloro phenols (Wennrich, Popp and Möder 2000) pesticides (Young, Lopez-Avila and Beckert 1996) polychlorinated biphenyls (PCB's) (Llompart, Li and Fingas 1999) and herbicides (Hernandez et al. 2000). SPME has been used to analyse aromatic flavours in wine, it has even been used to analyse chemical warfare agents such as sulphur mustard gas and agent VX (Hernandez et al. 2000, Kimm, Hook and Smith 2002, Hook et al. 2003).

1.2.5 Methods for the analysis of PAH's

As PAHs are persistent priority pollutants that can contaminate water, soils, sediment and accumulate in aquatic filter feeding species such as mussels, clams and oysters, there are many published methods for their analysis (Martinez et al. 2004, Knap and Williams 1982, Nendza et al. 1997, Geffard et al. 2003). The most common instruments used for their analysis are HPLC-UV-Fluorence detection (Miège, Dugay and Hennion 2003) and GC-MS (Marcé and Borrull 2000) analysis. There is a preference for the latter as selective ion monitoring can be utilised (Martinez et al. 2004, Boonyatumanond et al . 2007, Oros and Ross 2004, Macías-Zamora, Mendoza-Vega and Villaescusa-Celaya 2002). They are listed as priority pollutants by the USEPA, thus the EPA have published reference methods for their analysis in water and sediments by HPLC-UV-FL method 550, GC method 610, liquid- solid extraction and GC-MS method 525. Older EPA methods use liquid – liquid extraction for the analysis of PAH's in water, other groups have developed solid phase extraction methods which concentrate the compounds on solid adsorbents (Kiss, Varga-Puchony and Hlavay 1996, Chee, Wong

and Lee 1996). More modern less laborious techniques such as the fore mentioned SPME have also been applied successfully to PAH analysis (Llompart, Li and Fingas 1999, Eriksson et al. 2001).

As for soils and sediments, soxhlet extraction is the bench mark for all other methods (Martinez et al. 2004, Song et al. 2002) innovative techniques include ultrasonic extraction, microwave (Budzinski et al. 1999) and accelerated solvent extraction (ASE) (Boonyatumanond et al. 2007). Many studies look at recovery efficiency of PAH's from matrixes using different extraction techniques (Martinez et al. 2004, Song et al. 2002, Chen, Rao and Lee 1996). Song et al (2002) compared 7 different extraction methods for soils, they concluded that ultrasonic extraction was the most advantageous method as it was less time consuming and used less solvent while providing acceptable recoveries. Martinez et al (2004) also confirmed that for soil ultrasonic extraction was favourable providing 47 to 122% recovery, with better sensitivity for naphthalene compared to soxhlet extraction. %yield greater than 100% is commonly due to human error, e.g when adding small volumes of internal standards to samples for recovery studies. Instrumental error can also occur if instrument reproducibility is poor.

The extraction procedures for PAH's in mussels and other organisms is made more complex by the large amounts of lipids in the organism. Solvent extraction of dried homogenised material is often used (Richardson et al. 2003). Mooibroek et al (2002) compared solvent extraction with microwave-assisted extraction to determine PAH's in worms. They concluded that there was insufficient recovery of less volatile PAH's by microwave extraction (Mooibroek et al. 2002). Martinez et al (2004) compared soxhlet and ultrasonic extraction with ASE for analysis of PAH's in mussels. They showed similar recoveries for each method but soxhlet and ultrasonic extractions did not recover all compounds, the advantage of ASE is that analytes are released from the matrix giving wider range of recovery.

1.2.6 Remediation of soils contaminated with petroleum hydrocarbons (Bioremediation and degradation studies)

As mentioned in section 2.1, soil and aquifer contamination with petroleum products is a major environmental issue. Traditional remediation methods are based on physical and chemical engineering based technologies. Physical treatments for a contaminated site would be disposal to landfill or incineration (Sarkar et al. 2005a), incineration can

lead to air-pollution (Ting, Hu and Tan 1999). Chemical treatment involves the injection of chemicals into the contaminated site, this changes the chemistry of the site to favour breakdown of pollutants or conversion to non-toxic forms (Riser-Roberts 1998). These techniques are often very expensive or use a huge amount of energy, this means they are not ideal when a large area is contaminated (Zhang et al. 2010). Biological treatment usually involves the breakdown of pollutants by microbiological processes (Riser-Roberts 1998). Bioremediation is a cost effective biological treatment for contaminated sites. Bioremediation is defined as the use of living organisms to remove environmental pollutants from soil, water and gasses (Collin 2001). Micro-organisms make up a huge portion of living soil biomass (Curtis, Sloan and Scannell 2002), they can function in the absence of oxygen and other extreme environments (Mishra, Lal and Srinivasan 2001), they are diverse and have a wide range of catalytic mechanisms (Paul et al. 2005). Due to these properties much research has been focused on the search for pollutant degrading micro-organisms, and their application in the field. Recent advances in metagenomics and genome sequencing have helped the search for pollutant degrading genes (Golyshin et al. 2003, Zhao and Poh 2008).

Studies on bioremediation of petroleum contaminated soils have been done since the late 1940's, but it was not until the Exxon Valdez oil spill of 1989 that studies became widespread (Margesin and Schinner 1997, Jackson and Pardue 1998). From the large number of studies conducted almost all have confirmed bioremediation to be a successful treatment of petroleum contaminated sites (Huesemann and Moore 1993, Li, Zhang and Xu 1995, Zhou and Crawford 1995, Liebeg and Cutright 1999).

Bioremediation is generally in-situ or ex situ, In-situ remediation means treating the pollution at the site while ex-situ involves the removal of contaminated material for treatment elsewhere (Aggarwal et al. 1990). There are three types of in-situ bioremediation

(1) Bioattenuation or natural attenuation is the simplest form of bioremediation as it depends on the natural process of degradation, pollutants are broken down or immobilized by the local microbial population (Smets and Pritchard 2003), and also due to some natural chemical reactions and sorption to the soil matrix. Natural attenuation processes are usually contaminant specific, for example they are regarded as a successful treatment of BTEX contaminated sites (Atteia and Guillot 2007). Phytoremediation is a form of natural attenuation by plants – plants can aid in the dissipation of pollutants by removal and promoting microbial growth (Kirka et al.

2005). Zhang et al (2010), showed how the joint action of Pharbitis nil L., an ornamental plant, and its microbial community were a successful remedy for petroleum contaminated soils. In contrast to other bioremediation techniques, monitored natural attenuation (MNA) requires low input and is cost effective. However, bioattuation alone often becomes insufficient as many sites lack the required microbial diversity or the nutrients to sustain high microbial activity (Megharaj et al. 2011).

(2) Biostimulation stimulates the degradation of pollutants by addition of nutrients, proton donors and acceptors, often in the form of fertilizers to boost the potential of the indigenous microbial population (Pankrantz 2001). The indigenous microbial population may not directly degrade the hydrocarbons, but increasing the microbial population is believed to aid in the degradation process (Sarkar et al. 2005a). The degree of microbial activity in a given site depends on the supply of carbon, nitrogen, phosphorus, temperature, oxygen availability, soil pH and the pollutant itself (Carberry and Wik 2001). Traditionally the main focus has been on addition of N and P fertilizers. As carbon is the main component of a fuel spill, it is frequently used as an index of how much N and P to add to the site (Riser-Roberts 1998). More recent studies have looked at carbon addition in the form of glucose, biosolids and compost (Namkoong et al. 2002). These studies did not isolate carbon as a nutrient. The role of carbon supplementation in carbon poor soils was investigated by D.Sarkar et al (2005). They compared two methods of biostimulation, rapid release of inorganic fertilizers and slow release biosolids which added carbon in addition to N and P, with MNA for degradation of hydrocarbons. After eight weeks both biostimulation methods had degraded 96% of hydrocarbons, natural attenuation had 93.8%. The biosolid addition approach saw a two fold increase in microbial mass in the first week. The results suggest that biosolid addition is a more effective stimulant than traditional fertilizer addition because of its ability to supplement carbon. Some biostimulation methods can result in fertilizer induced acidity or nitrate overdosing resulting in a decrease in microbial activity. There is also an issue with supplying stimulants to deeper contaminated soils (McBride 2003).

(3) Bioaugmentation or soil activation is the addition of cultivated microbes to a contaminated site. The microbes can be cultivated from a portion of contaminated soil or from a stock of microbes previously shown to degrade the pollutants in question (Megharaj et al. 2011). Once added to the site the microbes should selectively degrade

the pollutants (Sarkar et al. 2005a). Adding 2% bioremediated soil to a fuel oil contaminated sample has been shown to increased degradation of PAH's (Lamberts et al. 2008). Pre-adaption of cultivated biomass to the target environment is important to improve survival, persistence and degradation potential, this enhances remediation of the site (Megharaj et al. 1997). Pre-exposure of soil to the pollutants invokes the phenomenon of 'soil memory', in which the soil retains specific metabolic capacities which contributes to the subsequent natural attenuation (Reddy and Sethunathan 1983). There are many reports of sites being successfully treated using bioaugmentation (Brunner, Sutherland and Focht 1985). Gilbert and Crowley (1998) found that inoculating PCB contaminated soil with carvone induced bacteria improved biodegradation. Wolika et al (2009) used aerobic microbial communities to successfully degrade BTEX compounds (Gilbert and Crowley 1998, Wolicka et al. 2009).

However, bioaugmentation can fail due to poor microbial efficiency, competitiveness and adaptability relative to the local microbial population. Blasco et al (1995) discovered that bacteria capable of degrading PCB in a lab study failed in in-situ bioaugmentation. Blasco et al (1995,1997) found that the native microbial population formed antibiotic compounds in response to the introduction of specialist PCB degraders. Bioaugmentation is often undesirable especially in protected environments (Megharaj et al. 2011, Blasco et al. 1995, Blasco et al. 1997).

Some bioremediation technologies based on biostimulation and bioaugmentation include bioventing, bioreactor, composting and land farming. Factors which must be taken into account prior to successful bioremediation were proposed by Sebate et al (2004):

- Organic contaminants
- Cost
- Metabolic activity of indigenous microorganisms
- Possible inhibitors
- Influence of nutrients
- Surfactants
- Bioavailability of pollutants
- Appropriate treatments.

(Sebate, Vinas and Solanas 2004)

The ability of the microbial population to degrade the pollutants is dependent on bioavailability of those pollutants (Antizar-Ladislao 2010). Bioavailability is dependent on many factors such as sorption, ionic charge, polarity, pH, partition coefficient values and solubility to name a few. If the pollutants are not bioavailable, surfactants can be added to enhance bioavailability (Laha, Tansel and Ussawarujikulchai 2009).

1.3 Lipid Biomarkers- general intro and culture dependent methods

1.3.1 PLFA's – culture independent methods

Phospholipid fatty acids (PLFA's) are found exclusively in the cell membrane of all living cells and not in storage products (White et al. 1979, Hill et al. 2000). Once a cell dies the cell membrane is rapidly degraded along with the PLFA's, this makes PLFA's a useful indicator of living rather than dead biomass (Hill et al. 2000, Steenwerth et al. 2002). Thanks to this property, PLFA quantities and identities have the potential to communicate rapid changes in soil microbial communities (Zelles. 1999), archae bacteria are an exception as they do not contain fatty acids in their phospholipid membrane (Evershed et al. 2006).

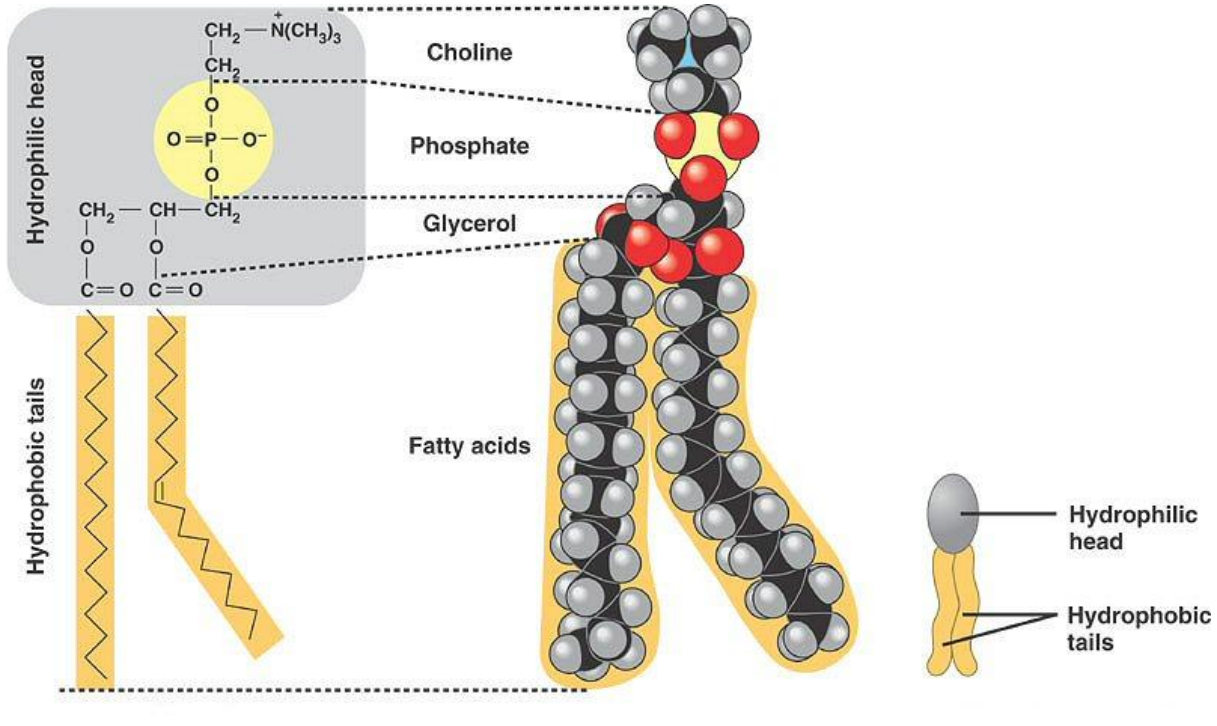

Figure 2 Phospholipid fatty acid structure.(Keller. 2008)

The phospholipids contained in the lipid bilayer of the cell membrane are made up of a phosphorolated polar, hydrophilic head group and a non polar, hydrophobic tail with

two fatty acids (see figure 2). The hydrophilic ends are projected outwards and the hydrophobic tails are projected inwards (see figure 3).

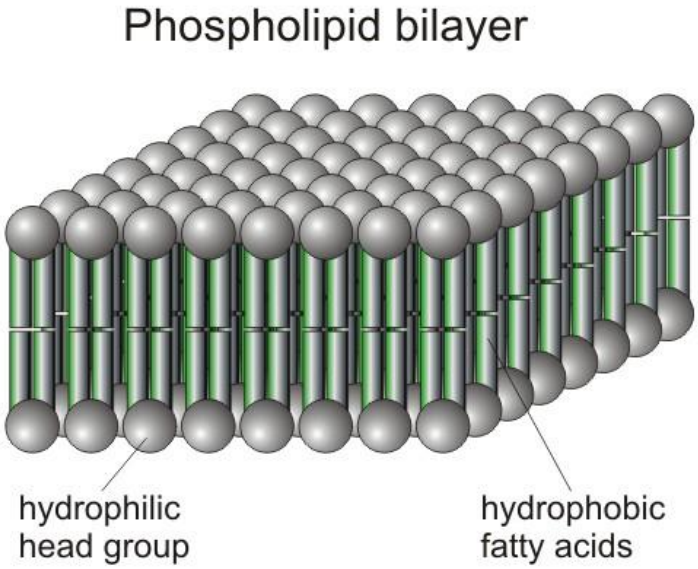

Figure 3: The phospholipid bilayer of cell membranes (McDonald 2012)

This bilayer provides the cell with protection and structure keeping water in the cell while excluding outside water. Proteins dotted along the membrane allow for selective transport of molecules in and out of the cell.

The polar head groups generally have a common structure as seen in figure 2. However, the fatty acid tails vary between organisms in chain length, branching, saturation and unsaturation. It is these variations that allow PLFA analysis to be used as a culture independent method for microbial taxonomy (Hill et al. 2000). Section 1.4.6 discusses further their use when combined with stable isotope studies. PLFA's have been utilized to assess the structure of soil microbial community responses to cropping practices (Zelles et al. 1992, Zelles et al. 1995), pollution (Frostegård, Tunlid and Bååth 1993) and changes in soil quality (Bossio et al. 1998, Petersen et al. 1997).

1.3.2 Analysis and considerations

Soil or sediment samples should be collected using sterilized utensils and glassware. The best way to preserve a sample for PLFA analysis as a snap shot of the microbial community is freezing (Christie 1982). However, freezing causes lysing of the cells and once thawed the PLFA will be rapidly degraded by lipases (Christie 1982). Minimizing the time between removal of samples from the freezer and drying is vital. The PLFA method involves extracting lipids from the samples using organic solvents.

Bligh and Dyer developed a lipid extraction method in 1959, and this protocol still provides the basis for modern extractions (Bligh and Dyer 1959). During the extraction considerations include minimizing light as UV is known to speed up PLFA degradation and do not expose the sample to temperatures over 37°C (Christie 1982). Once dried down the extracts should be stored in an organic solvent such as chloroform until further analysis, storage in alcohols such as methanol will result in loss of double bonds (Christie 1982). Once extracted the PLFA's are separated from the other lipid classes by their polarity. This is achieved using solid phase extraction methods (see table 3, figure 4) such as the one perfected by Pinkart et al (1998). The phospholipid fatty acids are then derivatised to fatty acid methyl esters (FAME) by mild alkaline methanolysis (Guckert et al 1986). Samples are then analysed by GCMS for identification and quantification.

Table 3 Fractionation of lipid classes. (Pinkart, Devereux and Chapman 1998)

Solvent	Portion	Vol (ml)	eluent	Lipid eluted
Chloroform	Neat	5	NL	Neutral lipids
Acetone	Neat	5	PHA	Polyhydroxyalkanoate
Methanol:Chloroform	6:1	2.5	PL	Polar lipids (PLFA)
0.05M sodium acetate in 3a	6:1	2.5	PL	Polar lipids (PLFA)
n-Hexane	Neat	5	ST ES	Steryl esters
n-Hexane:DCM:chloroform	88:10:2	5	Tri	Triglycerides
n-Hexane:ethyl acetate	5:95	5	Strl	Sterols
n-Hexane:ethyl acetate	15:85	5	Di	Diglycerides
Chloroform:methanol	2:1	5	Mono	Monoglycerides

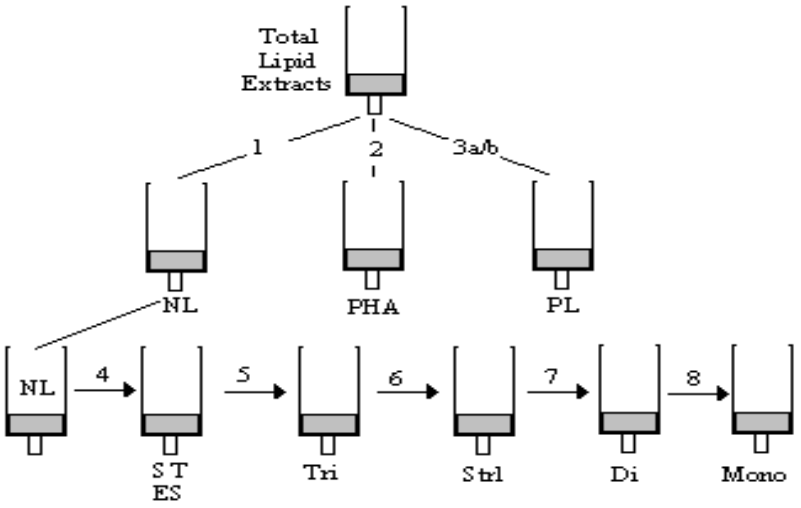

Figure 4 SPE method to separate lipid classes.

A PLFA profile for a given soil samples consists of all extracted PLFA's, present in the chromatogram as FAME with varying chain lengths, levels of double bonds and branching (Leckie 2005). Today's modern MS libraries are such as NIST and Wiley are highly accurate. However, the library results for monounsaturated FA's cannot be reliably reported as double bond position is not always reliable (Nichols, Guckert and White 1986). Double bond position is essential for correct interpretation of FA biomarkers. To provide accurate interpretation a dimethyl disulphide (DMDS) derivatisation is required after mild alkaline methanolysis. This derivatisation adds a CH_3S groups to the carbons either side of the double bond in the fatty acid. The resulting GCMS spectrum will show ions attributed to fragmentation of the compound between the two CH_3S groups (Nichols, Guckert and White 1986).

1.3.3 PLFA interpretation Common signature PLFA's- Table of PLFA and associated consortia, nomenclature explained

Nomenclature is used to simplify and shorten the reporting of FA's (Ruess and Chamberlain 2010). The classification of unsaturated fatty acids is usually based on the position of the double bond(s) from the carboxyl end (Δ end) this gives the systematic name (IUPAC-IUB Commission on Biochemical Nomenclature. 1978). However the most common nomenclature used counts from the methyl end of the FA (ω end). In this short hand or nomenclature the first number is the total carbon number: followed by the number of double bonds. ω is more commonly used to designated the double position from the methyl end. However, it is not unusual to see Δ used (Fang, Lovanh and Alvarez 2004b). There does not seem to be standardised or universal rule for FA nomenclature, there are subtle differences throughout the literature but the most common format is as follows: Double bond geometry is often signified by 'c' for *cis* and 't' for *trans*, 'i' indicates *iso* branching on the second to last carbon while 'a' indicates *anteiso* branching on the third to last carbon. Also '10Me' would indicate a methyl group on the 10th carbon from the carboxyl end. 'cy' indicates a cyclopropane fatty acid (Leckie 2005) (see figure 5).

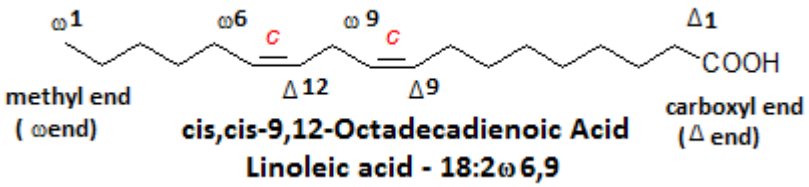

Figure 5. Nomenclature of a dienoic fatty acid.

The presence and abundance of certain signature fatty acids in sample extracts are used as indicators for the presence and abundance of certain microbial species (Hill et al. 2000). However there is seldom an individual biomarker to identify a single microbial strain (Evershed et al. 2006), generally classes of PLFA's are used as biomarkers for microbial populations (Zelles. 1999). Comparing PLFA profiles among samples reflects differences in community composition (White, Stair and Ringelberg 1996). There are many markers used for bacterial species, however there are only a few that are known for fungal species (Leckie 2005). The PLFA 18:2ω6,9 (Figure 5) has been widely used to indicate fungal abundances in microbial communities (Zelles. 1999, Clemmensen et al. 2006). The monounsaturated PLFA 18:1ω9c has also been confirmed as a fungal biomarker (Frostegård and Bååth 1996, Klamer and Bååth 2004). There are many more PLFA indicators for bacteria, see table 4 for common PLFA biomarkers, their predominant origin and references.

1.3.4.1 Misuse and limits of indicator PLFA's

Much of the information of PLFA biomarkers comes from pure cultures (Zelles. 1999). Many publications list the marker PLFA's and from references conclude the organisms present (e.g. table 4). However, as can be seen in table 4 there are often inconsistencies between references in the PLFA markers used for specific microbial groups. Some examples are the use of cy17:0 and cy 19:0 to indicate Gram-negative bacteria, however other sources such as (Schoug et al. 2008) have reported the FA in Gram positive bacteria. Even the well noted fungal markers 18:2ω6,9 and 18:1ω9 have been detected in eucaryotes such as plants (Frostegård, Tunlid and Bååth 2011). Ratios of certain PLFA's such as monounsaturated/cyclopropyl ratios, have been used as stress indicators for microbial communities (Table 4). These stress indicators depend on a bacteria altering their composition is response to stress of starvation. However, there is no way of distinguishing between a shift in membrane composition and a shift in overall microbial population, a change is such ratios will occur with both.

Table 4 - Common PLFA's and their predominant origin

Groups of Organisms	PLFA biomarkers	Reference
Procaryotes and Eucaryotes	Straight chain	(Hill et al. 2000, Evershed et al. 2006)
Eucaryotes, cyanobacteria, mosses and higher plants	Long straight chains (>C_{20}) eg 22:0, 24:0	(Zelles. 1999, Ruess et al. 2007)
Bacteria (non-specific)	Saturated straight chain (<C_{20}) 14:0, 16:0, 18:0	(Lechevalier and Lechevalier 1988)
Gram positive bacteria	Iso and antiso branched FA's from C_{14} to $C_{18.}$ Mono unsaturated 18:1ω9	(Zelles. 1999, Zelles et al. 1992, Zelles et al. 1995, O'Leary and Wilkinson 1988)
Gram negative bacteria	cy17:0, cy19:0, 16:1ω5c, 16:1ω7t, 16:1ω7c, 16:1ω9c, 18:1ω5c, 18:1ω7c, 18:1ω9c	(Zelles. 1999, Wilkinson 1988)
Fungi	18:2ω6,9	(Zelles. 1999, Clemmensen et al. 2006)
Higher Fungi	18:3ω3,9,12	(van der Westhuizen et al. 1994)
Arbuscular Mycorrhizal (AM) Fungi	16:1ω5, 18:1ω7, 20:1ω9(*Gigaspora*),	(Olsson et al. 2005, Sakamoto, Iijima and Higuchi 2004)
Actinomycetes	Methyl branching on the 10^{th} carbon, 10Me16:0, 10Me17:0, 10Me18:0	(O'Leary and Wilkinson 1988, Lechevalier 1977)
Sulfate reducers	10Me16:0, 17:1ω6, i17:1ω7	(Hill et al. 2000, Kroppenstedt 19992)
Methane-oxidising bacteria	18:1ω8c, 18:1ω8t, 16:1ω8c, 16:1ω8t, 16:1ω5c, 18:1ω6c	(Hill et al. 2000, Ringelberg et al. 1989)
Protozoa	20:4ω6, 20:3ω6	(Hill et al. 2000, Cavigelli, Robertson and Klug. 1995)
Pseudomonad strains	16:1ω7c, 16:1ω7t, 18:1ω9, cy17:0 and cy19:0	(Fang, Lovanh and Alvarez 2004a)
Microalgae	16:3ω3,6,9	(Hill et al. 2000)
Algae	20:5ω3,6,9,12,15	(Lechevalier and Lechevalier 1988)
Cyanobacteria	18:1ω8c, 18:2ω6,9	(Hill et al. 2000, Zelles. 1999)
Barophilic/psychrophilic bacteria	20:5ω3,6,9,12,15, 20:6ω3,6,9,12,15,18	(Hill et al. 2000)
Zygomycetes	18:3ω6,9,12	Van der Westhuizen et al (1994)
Flavobacterium balustinum	i17:1ω7, Br 2OH-15:0	(Hill et al. 2000)
Stress indicator ratio of monounsaturated FA's to cyclopropyl FA's	16:1ω7c/cy17:0 or 16:1ω8c/cy19:0	(Guckert, Hood and White 1986, Ratledge and Wilkinson 1988)

1.3.4 PLFA- Limitations

Despite the usefulness of PLFA analysis there are some important limitations. Frostegard et al (2011) have published a useful and commonly cited paper on the "use and misuse of PLFA measurements in soils." While they acknowledge the advantages of PLFA's for studying soil microbial communities, they present three notable limitations which should be considered by all analysts utilising the PLFA method:

1.3.4.2 PLFA turnover rates

As mentioned earlier in section 3.1, PLFA's are used to measure rapid responses of soil microbes to changes such as pollution, soil quality, cropping practices etc. This method should be used with caution as there are very few reports analysing turnover in soil and it is difficult to do (Frostegård, Tunlid and Bååth 2011). Growing pure cultures and adding labelled substrates has limited success in mimicking turnover of indigenous populations. PLFA quantities are useful for measuring increases in biomass but measuring decreasing in PLFA's in not as straight forward. Decreasing PLFA's assumes cell death and lipid degradation but these can happen independently of each other in exceptional cases. For example a study of thermophiles in heated peat showed a faster decrease in PLFA's when cooled to 25°C than when cooled to 5°C, when the opposite would be expected (Ranneklev and Bååth 2003). This does not mean for certain that the bacteria survived better at 5°C, it may just be that the dead cells were degraded more slowly at the lower temperature.

1.3.4.3 Use of PLFA's for diversity indices

The use of PLFA data for diversity indices is flawed and should not be attempted (Frostegård, Tunlid and Bååth 2011). This is commonly done by using specific PLFA to represent specific species and then taking the chromatographic peak area as frequency for that species. However there are question marks over the diversity of PLFA, for example fungi lack diversity of PLFA's and only a few markers are known. Therefore, a soil sample with thousands of different fungal species may only contain a few PLFA, this does not accurately represent the fungal diversity in the soil. Other flaws in this method arise from the indices used such as the Shannon diversity index.

1.4 Stable carbon Isotopes - What are they?

The unique identity of a chemical element is established by its atomic number, which is the number of protons in its nucleus. The atomic mass of an element is the sum of the number of protons and neutrons in the atomic nucleus. Some nuclei may have different atomic mass numbers but the same atomic number, these are called isotopes. Since they are the same element with the same atomic number they are chemically similar but differ slightly in physical properties. Carbon has an atomic number of 6 (6 protons), it can have up to 15 isotopes due to different neutron numbers, for example ^{13}C has 6 protons and 7 neutrons. Only 3 of the 15 isotopes are naturally occurring ^{12}C, ^{13}C and ^{14}C. Of these ^{12}C and ^{13}C are stable whereas ^{14}C is radioactive (Phatak 2012, Phatak 2012). The natural abundances of these isotopes is generally agreed to be around 98.89% for ^{12}C, 1.11% for ^{13}C (Boutton 1996) and $<10^{-10}$% for ^{14}C (Goh 1991).

1.4.1 Stable isotope measurement units

There are a few different ways of expressing amounts of more infrequent ^{13}C compared to ^{12}C (Boutton 1991). When ^{13}C levels are at or near natural abundance $\delta^{13}C$ notation is used, when samples are highly enriched ^{13}C is expressed as a percentage. $\delta^{13}C$ (‰) value is calculated from a sample as follows:

$$\delta^{13}C\ (‰) = [Rsample = Rstandard/Rstandard] \times 1000$$

$\delta^{13}C$ is the parts per thousand or per mil (‰) difference between the ^{13}C content of the sample and of the standard. R is the mass $^{45}/_{44}$ ratio of sample or standard.
The ^{13}C atom% expresses the % of ^{13}C per total Carbon atoms:

$$^{13}C\ (atom\ \%) = 100 \times (^{13}C/(^{13}C+^{12}C))$$

^{13}C and ^{12}C are the number of atoms in the sample, the amount of ^{14}C is negligible for this calculation (Amelung et al. 2008).

The atom% excess calculation is useful for calculating the enrichment level of a sample after amending with ^{13}C labelled substrate compared to the ^{13}C background levels prior to ^{13}C addition:

Atom%excess = atom %^{13}C (enriched) – atom %^{13}C (unenriched)

$$= \frac{13C\ x\ 100}{12C+13C}\ enriched - \frac{13C\ x\ 100}{12C+13C} unenriched$$

(Fisher, Haines and Volk 1979).

1.4.2 Analytical techniques

There are different analytical techniques for bulk and compound specific stable isotope analysis (CSIA). For CSIA Isotope ratio mass spectrometry (IRMS) is commonly combined with various separation and injection techniques. For bulk samples, common techniques are elemental analyser – IRMS (EA-IRMS), laser ablation – IRMS (LA-IRMS), liquid digestion – IRMS, Stable Isotope probing (SIP) and secondary ion mass spectrometry (SIMS). For CSIA analytical instrumentation includes GC-C-IRMS, Pyrolysis(Py)-GC-C-IRMS, LC-C-IRMS and thermogravimetry - differential scanning calorimetry – quadropole mass spectrometer – IRMS (TG-DSC-QMS-IRMS which provides simultaneous ^{13}C data, gas analysis, mass balance, energy change for volatile minerals and soil carbon materials). CSIA allows isotopic analysis of specific compounds at low levels in complex samples. CSIA has been applied to the areas environmental analysis, ecology, forensic science and geochemistry (Lopez-Capel, Bol and Manning 2005, Glaser 2005, Herrmann et al. 2007, Philp. 2007, Bernard et al. 2007). The individual compounds are separated by chromatography or a thermal separation step combined to a combustion unit to combust the individual compounds, there ^{13}C content is then measured by IRMS. Some compounds are not volatile enough for analysis by GC and thus require derivatisation. If the derivatising agent contains carbon, a final correction step is required to account for this added C to the individual δ^{13}C values. New derivatisation methods have been developed by Gross and Glaser (2004) which reduce error by reducing the amount of C added (Gross and Glaser 2004).

1.4.3 Fractionation of isotopes

Due to kinetic and thermodynamic processes heavier isotopes are distinguishable from lighter isotopes in most biological systems, this is sometimes referred to as fractionation. For example CO_2 emitted from soil respiration will contain less ^{13}C than the soil that respired it, as the ^{13}C is ‘favoured’ over the ^{12}C (Bol et al. 2003). Thanks to

the biological, chemical and physical fractionation of isotopes, natural $^{13}C/^{12}C$ ratio can provide information on (Amelung et al. 2008):

1. Sources and processes that formed SOM (Amelung et al. 2008, Amelung, Bol and Friedrich 1999, Simpson et al. 1999, Kuzyakov and Bol 2006).
2. Rate of SOM transfer (Huang et al. 1996, Kramer and Gleixner 2006, Krull et al. 2007).
3. Environmental and land management conditions present at the time the SOM was formed (Boutton 1996, Huang et al. 1996, Krull et al. 2007, Krull et al. 2005, Lobe et al. 2005).

It is possible to manipulate natural $\delta^{13}C$ by artificial and natural labelling techniques, which is extremely useful in tracking the fate and turnover of SOM (Petersen et al. 1997, Bol et al. 2004a).

1.4.4 Labelling techniques

Natural labelling: Natural ^{13}C abundance tracer studies are based on the unique physiological differences in the fixation of CO_2 by C3 and C4 plants. C3 plants fix CO_2 to a 3C compound called 3-phosphoglyceric acid, the reaction is catalysed by rubisco and the majority of plants are C3. C4 plants initially fix CO_2 to 4C compounds like malic and aspartic acid and the reaction is catalysed by phosphoenolpyruvate(PEP)-carboxylase, only 1-5% of plants are C4 however, they include important crops like maise and sugarcane (Amelung et al. 2008). Plants with a C3 pathway have $\delta^{13}C$ values from -22 to -32%, plants with C4 pathways have $\delta^{13}C$ values from -9 to -17% (Boutton et al. 1998). This natural difference in isotopic value means carbon derived from C3 plants and C4 plants can be distinguished and traced in SOM using its $\delta^{13}C$ value (Krull et al. 2005, Lobe et al. 2005, Bol et al. 2000, Bol et al. 2004c, Bol et al. 2004b, Gleixner et al. 2002a). C3 and C4 plants can be used to supply naturally labelled substrates as whole tissues or biochemical components derived from the plants, this provides an economic source of ^{13}C labelled substrates (Evershed et al. 2006).

Artificial labelling: Artificial labelling can be divided into three methods based on the amount of ^{13}C enrichment achieved; natural abundance (Thornton et al. 2004, Klumpp, Soussana and Falcimagne 2007) near natural abundance (50-500%)(Evershed et al. 2006) or enriched >500% (Bull et al. 2000, Zak and Kling 2006). Free air CO_2 enrichment (FACE) is an example of an artificial natural abundance labelling technique. In these field scale based experiments CO_2 levels are increased by 100 to 240 ppm

above the normal CO_2 level, all other conditions remain the same. The $\delta^{13}C$ value of the artificially supplied CO_2 (-25 to -70%) is usually different compared to the natural CO_2 (-8%). Thus plants and soil in FACE conditions become labelled compared to those in ambient conditions (Amelung et al. 2008). FACE experiments provide ^{13}C labelled plant tissue which may be used as substrate for enrichment studies (Evershed et al. 2006, Waldrop and Firestone 2004b). In these experiments the fractional input (F*) of C from the ^{13}C source into the soil can be estimated as follows:

$$F^* = (\delta_{final} - \delta_{initial}) / (\delta_{source} - \delta_{initial})$$

(Amelung et al. 2008)

δ_{source} is the $\delta^{13}C$ level of the carbon substrate applied to the system, δ_{final} and $\delta_{initial}$ are the final and initial $\delta^{13}C$ value of the soil carbon pool used in the experiment. FACE experiments are usually limited by the high cost of CO_2 cylinders.

Near natural or high enrichment approaches use heavily labelled plant material (Bromand et al. 2001, Leake et al. 2006) or commercially available organically synthesised ^{13}C labelled substrates (Evershed et al. 2006, Bull et al. 2000, Zak and Kling 2006). Due to the high cost of labelled plant material and commercial substrates most experiments are laboratory based (Evershed et al. 2006). However, as the substrate is highly enriched with ^{13}C, the carbon is easily traced by the sensitive GC/C/IRMS instrument. This allows the fate of particular compounds to be traced in microbial communities and SOM (Evershed et al. 2006). As costs are high artificial labelling experiments are often only run for short periods of time, only soil components such as microbial communities which have high turnover rate are labelled (Amelung et al. 2008).

1.4.5 Incubation experiments

To track the transformation and turnover of specific compounds in SOM by microbial processes, ^{13}C enrichment experiments can be performed in incubation studies using optimal environmental conditions. The conditions provided are ideal form microbial degradation of the costly substrate, thus the chances of a successful experiment in increased. However, because of this they do not usually replicate field conditions, and often both laboratory and field studies are required to thoroughly understand SOM turnover processes. There are a vast number of biomarkers with useful application in marine and terrestrial systems, but very few have been isotopically

investigated (Platzner 1998, Zhang 2002) in incubation studies in agricultural soil, with a few exceptions of PLFA's, alkanes and carbohydrates (Amelung, et al. 2008).

1.4.6 Combining ^{13}C labelling with lipid analysis to access the microbial communities involved in SOM transformation and turnover.

Signature fatty acids can be used for bacterial taxonomy in which specific FA's are employed as taxonomic discriminators (biomarkers) for species identification (Hill et al. 2000). Phospholipid fatty acids (PLFA's) are particularly useful as biomarkers as they are contained solely in the cell membrane. This is important as the cell membrane rapidly degrades after cell death; therefore PLFA's are good indicators of living microbial biomass rather than non-living microbial biomass. When a ^{13}C-labelled substrate is introduced to soil, sediment or culture, microbes utilizing it will incorporate ^{13}C into their PLFA's. ^{13}C analysis of these PLFA's by GC/C/IRMS gives quantitative and chemotaxonomic information on the microbes degrading the substrate. As many microbial populations are uncultivable new culture independent methods are being developed to study these populations. Gene probe methods such as SIP provide phylogenic details of specific groups of microorganisms. CSIA using ^{13}C labeled substrates in incubation studies to enrich PLFA's is complementary to gene probe studies (Hanson,J,R, et al. 1999). These studies allow the microbes responsible for degradation of the isotope-labeled compound to be identified and help characterize the fate of the ^{13}C carbon source in SOM (Evershed et al. 2006).

The combination of CSIA and PLFA biomarker analysis has been used particularly well in the area of identifying methane oxidizing bacteria (MB). Briefly, MB are a known sink for atmospheric and soil born methane. Cultivable MB are categorized into three types, ***I*** more C16 fatty acids, ***II*** more C18 fatty acid and ***X*** share some of the characteristics of ***I*** (Bull et al. 2000). Bull et al (2000) incubated Sitka spruce soil from different soil levels under $^{13}CH_4$ (100ppm and 1.8ppm atmospheric) for 3 weeks at 20°C. They found that type ***II*** MB and some novel methanotrophic bacteria produced br17:0. Knief et al (2003) discovered that incubating diverse upland soil under ^{13}C labeled CH_4 conditions resulted in the label being fixed to branched chain 17:0 PLFA's. Maxfield et al (2006) used a flow chamber for a long term experiment using atmospheric methane conditions (of 2ppm) over 11 weeks. They showed incorporation into PLFA's of between 10 to 50%. However, compared to other studies such as Crossman et al (2005), ^{13}C uptake was slow (Knief, Lipski and Dunfield 2003,

Crossman, Ineson and Evershed 2005, Maxfield, Hornibrook and Evershed 2006, Cébron et al. 2007).

Isotopic analysis combined with biomarker analysis is also used for analyzing natural samples from methane rich environments. Studies have revealed an extreme depletion of ^{13}C associated with sulphate reducing bacteria and Archaea (Elvert, Suess and Whiticar. 1999). As methane is one of the most ^{13}C depleted organic compounds, microbes utilizing methane will have a distinct depletion in ^{13}C also (Whiticar, Faber and Schoell 1986). The ^{13}C levels in sulphur reducing bacteria, archaea and methanotrophs in these methane rich environments are depleted enough to suggest these organisms are involved in methane oxidation (Zhang 2002).

Isotopic techniques are often used to study the organic compounds released by plant roots and their fate in microbial populations. It is estimated that plants release 10 to 40% of the carbon they absorb into the soil through their roots (Macdonald et al. 2004, Singh et al. 2004). Because of this the interphase (rhizosphere) has been termed an "oasis in a desert" from the microbes view (Bertin, Yang and Weston. 2003). Paterson et al (2007) were successfully able to study the microbial utilization of root released carbon compounds using ^{13}C labeled substrates (Paterson et al. 2007).

Similar to studies of MB in methane rich environments, isotopic techniques are used to assess the effect of changing environmental condition on microbial performance. For example, Waldrop and Firestone (2004a) experimented with temperature and nitrogen availability in a study of the decomposition of old carbon (C3) and new carbon (C4) pools. The soil used was from a pineapple plantation and were incubated for 103 days. At temperatures of 20-25°C old carbon was degraded more efficiently than at cooler temperatures. They found that altering N levels did not affect microbial activity. In other short term incubations Waldrop and Firestone (2004b) found that fungi are the predominant sink for newly added carbon, as the fungal biomarker 18:2ω6,9 was labeled with ^{13}C (Waldrop and Firestone 2004b, Waldrop and Firestone 2004a).

Isotope labeling may also help in understanding SOM dynamics in extreme environments where on-site studies may be difficult to organize. For example isotopic techniques applied to tundra soil showed that even at 2.8°C uptake of ^{13}C substrates was rapid (less than 5 days). Substrates used were cellobiose, N-acetylglucosamine and vanillin; again it was found that fungal populations were amongst the first to utilize the

substrate, based on the biomarker 18:1ω9c being labelled. Thus it seems that the cycling of SOM in tundra systems is dominated by fungi (Zak and Kling 2006).

There are many synthesized ^{13}C labeled compounds available from chemical suppliers. Isotopically labeled pollutants such as the BTEX compounds and PAH's can be purchased and used to demonstrate microbial degradation of pollutants. The results often show that only a very specific group of soil micro-organisms degrade the substrate. Hanson et al (1999) found that in a mixed soil microbial population amended with 35ug of toluene only 16 of the 59 (27%) PLFA's in the soil were labeled with ^{13}C, while when using ^{13}C-glucose as a control 91% of PLFA's in the sample had incorporated the ^{13}C from glucose. Although toluene and benzene are structurally similar except for a methyl group studies have shown that different microbial groups are involved in their degradation (Geyer et al. 2005). Other studies found that the ^{13}C enriched PLFA's from a ^{13}C toluene degradation study were similar to the PLFA's of methane-oxidizing bacteria (Pelz et al. 2001). Fang et al (2004) assessed the toluene degradation capabilities of five reference strains of bacteria: Pseudomonas putida mt-2, P.putida F1, burkholderia cepacia G4, B.pickettii PKO1 and P.mendocina KR1. All 5 strains incorporated ^{13}C into their PLFA's. Mellendorf et al (2010) used isotope studies combined with PLFA analysis to determine if phenanthrene degradation in soil was affected by addition of rape seed oil. The addition of rape seed oil increased the total PLFA content of the soil and changed the community profile by encouraging the growth of fungi and gram positive bacteria, this resulted in increased phenanthrene degradation (Mellendorf et al. 2010). Further research that employs isotopic techniques to track the fate of pollutants in soil is required to better understand the microbial communities involved in degradation of specific compounds. The results can then be used to improve bioaugmentation of polluted sites, industrial wastewater treatments and other processes controlled by microbial consortia.

1.5 Nuclear Magnetic Resonance (NMR) Spectroscopy

1.5.1 Environmental applications of NMR Spectroscopy.

"The processes occurring in the top few centimetres of the Earth's surface are the basis of all life on dry land, but the opacity of soil has severely limited our understanding of how it functions." [Andrew Sugden (Sugden, Stone and Ash 2004)]

In a recent article in *Science* soil was described as the most complex biomaterial on Earth (Young and Crawford 2004). As soil organic matter is one of the largest organic carbon pools on the planet, improved understanding of soils response to climate change is needed to determine if this carbon store will be a source or sink for greenhouse gases (Lal 2004). Pollution and contamination of soils is also a major issue of the modern age, the cost of cleaning up contaminated sites in the European Union and America alone was estimated at 1.4 trillion euro in the mid 90's (Okx, Hordijk and Stein. 1996). There are many problems with current remediation techniques, many of the early strategies utilized for PAH's for example, have been shown to be ineffective (Wilson and Jones 1993). Understanding the molecular processes that give rise to persistant organic pollutants in soil is paramount to their remediation (Simpson, McNally and Simpson 2011). Likewise it is critical for sustainable agriculture that we understand the key chemical, physical and biological properties that provide stable soils. NMR spectroscopy is highly effective at analysing complex structures and interactions such as those in soil and it can be applied to a variety of matrices (Simpson, McNally and Simpson 2011). NMR has been proven to be a suitable method for tracking carbon substrates in soil microcosms in a non-invasive approach (Lundberg, Ekblad and Nilsson 2001).

In NMR analysis absorption of electromagnetic radiation in a sample is used to identify molecular species and determine organic structures. The electromagnectic radiation is absorbed by certain nuclei, in the presence of a magnetic field. Only certain atomic isotopes are suitable for NMR analysis and are chosen depending on the compounds of interest. The most commonly encountered within the literature are ^{1}H, ^{13}C, ^{31}P, ^{27}Al, ^{29}Si, ^{17}O and ^{15}N (Hart 2011). Most of the NMR literature focuses on the ^{1}H isotope in liquid-state organic chemistry studies (Lloyd et al. 2007). NMR is a vital tool for synthetic chemists to confirm compound structure. Standard NMR analysis of solids results in broad, poorly resolved spectra but development of a variation of NMR called charged polarised magic angle spinning NMR (CP-MAS-NMR) provides high

resolution spectra of nuclei of atoms such as ^{13}C in solid samples (Simpson, McNally and Simpson 2011).

1.5.2 Principles of NMR Spectroscopy

Its is important to note that information gained in NMR spectroscopy is from studying the nuclei of atoms and not the electrons, as is the case in other spectroscopic techniques such as UV and IR spec. Electrons, protons and neutrons can be imagined as spinning on their axis. In many atoms, such as ^{12}C, these spins cancel each other out and the nucleus of the atom has no overall spin. In other atoms such as ^{1}H and ^{13}C the nuclei do possess an overall spin. These spinning nuclei are also charged, therefore they create a magnetic field. The net spin of a nucleus can be determined as follows:

1. No. Of Neutrons = No. Of Protons ... result in **no spin**
2. No. Of Neutrons + No. Of Protons = odd number ... result **half-integer spin** (i.e.1/2)
3. Odd no. Of Neutrons and Odd no. of Protons ... result **integer spin** (i.e.1)

From quantum mechanics we know that a nucleus with an overall spin '*I*' will have $2I + 1$ possible orientations. Without the influence of an external magnetic field, the nuclei orientations are of equal energy.

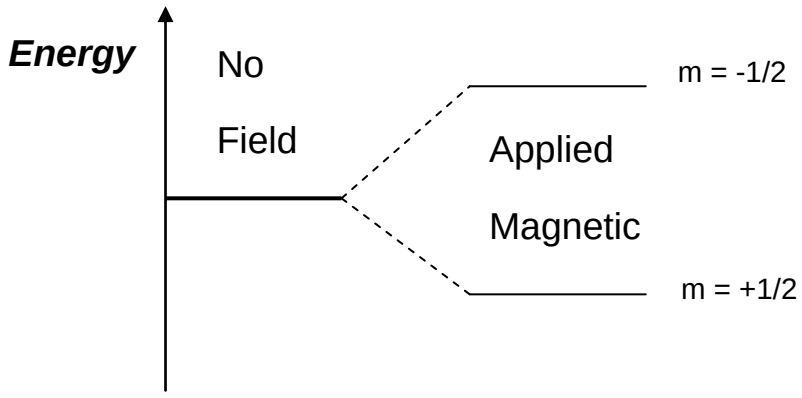

Figure 6 : Energy Levels for a Nucleus with Spin Quantum Number 1/2

Applying an external magnetic field splits the energy levels (Figure 6). The nuclei will align with or against the magnetic field, those aligned with the field have a lower energy level than those aligned against the field. Boltzmann distribution describes why more nuclei remain in the lower energy level. Radiofrequency (rf) energy is applied to excite

nuclei from the lower energy level to the higher one, this is known as a 'flip'. The frequency required is identical to the energy difference between the energy levels. These NMR transitions produce a signal which is measured by a receiver in the instrument (Edwards 2012).

There are two methods of producing this signal: (1) Varying magnetic flux density while transmitter frequency remains constant or (2) varying the transmitter frequency and keeping the magnetic flux density constant. In both the signal received is constantly monitored to develop a spectrum. This is known as the continuous wave (CW) method and was the basis for all early NMR studies but has now been replaced by the pulsed method. With the pulse method all of the nuclei in the sample are excited at once with an rf pulse. To explain it simply; a single pulse is generated by switching on the rf for a very short time. This single pulse contains a band of frequencies, a hard pulse is applied to ensure that all are irradiated equally (Friebolin 2004).

1.5.3 Use of NMR in organic geochemistry.

NMR is possibly the most powerful tool available to analyse complex matrices such as soil and can be used to study soil matrices at different scales of complexity (Simpson, McNally and Simpson 2011). Basic solution-state NMR can be used to determine structural components of soil and provide detailed structural overviews of binding components in complex samples (Kelleher and Simpson 2006, Cook 2004, Kelleher, Simpson and Simpson 2006). Diffusion NMR is more invasive and has been used to study aggregate formation from organic matter in free soil space, such as soil pores (Simpson et al. 2002, Smejkalová and Piccolo 2008). This technique is useful in studying the potentially large colloidal species that are formed (up to a micrometre scale) in the environment (Simpson, McNally and Simpson 2011). NMR can be used to assess the interaction of SOM with clays and other soils. The potential of solid-state and HR-NMR at this scale depends on the ability to decipher how the matrix components are arranged, layered and associated (Wilson and Pugmire 1981, Simpson et al. 2001, Simpson 2006). NMR has also been coupled with magnetic resonance imaging (MRI) to provide a wealth of information on the physical structure of soil columns and movement and transformation of contaminants (Reeves and Chudek 2001, Nestle, Baumann and Niessner 2002).

Kelleher et al (2006) used HR-MAS-NMR to study fate and transformation of plant litter in soil as it plays a key role in carbon and nitrogen cycling in the pedosphere.

HR-MAS-NMR is a powerful spectroscopic tool, especially for studying soil organic matter. The sample is spun in the presence of a solvent at the magic angle. The solvent helps decrease the 1H-1H dipolar interations, the magic angle reduces band broadening. It is used to study samples in their unaltered state so that the structure and reactivity of the different phases can be discerned (Simpson, et al. 2011). Kelleher et al. (2006) utilized ^{13}C and ^{15}N labelled plant material combined with HR-MAS-NMR to show that carbohydrates were rapidly degraded. Hydrolysable and condensed tannin were also readily decomposed while waxes and cuticles remained more stable over the 12 month test period. The most curious finding was that nitrogen from pine material was either selectively preserved or re-structured into novel structures. A follow on study by Spence et al. (2011) applied the same methods to ^{13}C and ^{15}N labelled soil microbial biomass. Similarly, carbohydrates were rapidly degraded, but diffusion edited HR-MAS-NMR showed macromolecular carbohydrates were more resistant to degradation (Spence et al. 2011). Solution state NMR was applied to soil humin by Simpson et al. (2007) to study its structural components. Humin is thought to be the most recalcitrant fraction of soil, but it is also the least understood fraction. Interestingly, the study showed that peptidoglycan was a dominant component of humin indicating a much larger contribution from microorganisms to humin than normally found in humic and fulvic fractions. In the same article Simpson et al. (2007) challenged the accepted estimates on SOM content by claiming that microbes contribution > 50% of the extractable SOM, > ~45% of the soil humin fractions and > 80% of the soil nitrogen (Simpson et al. 2007). ^{13}C labelled glucose transformation was studied in soil by Baldock et al. (1990) using solid-state NMR and it was found that the glucose was converted to carbohydrates, polymethylene and carboxyl carbon (Baldock et al. 1990). Lundberg et al. (2001) carried out a more comprehensive in-situ glucose study of forestry soils using NMR. 50% of the substrate was consumed within 3 days, this coincided with the appearance of olefinic triacyclglycerols. Measurements of soil respiration over the three days indicated that 40% of decomposed glucose was respired as CO_2, 40% was converted into solid state components and the rest as triglycerols. Over the remainder of the 28 day study triacyclglycerol concentration peaked at 13 days and then declined by 60% by the last day. Based on the evidence it was hypothesised that a large number of storage lipids were formed due to glucose degradation by eukaryotic organisms, most likely fungi. The study provides insight into the degradation of a common sugar introduced to soil by plant root interactions. This study is particularly relevant to the current project, were the lipids enriched from ^{13}C glucose degradation are identified.

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Chapter 2

Development of a combined analytical approach to tracking the fate of carbon in soil

Contributors

Dr. Kris Hart (Dublin City University) performed the CO_2 incubation and the glucose assay analysis

Dr. Andre Simpson (University of Toronto Scarborough) performed the NMR analysis

2.0 Abstract

The fate of carbon in soils is poorly understood due mainly to the multiplicity of relationships that exist between microbial consortia, other nutrients and break down products. An understandingof the fate of carbon in soil under varying environments is crucial if we are to develop land management practices which increase carbon sequestration and storage in soil. In this study, a method was developed using gas chromatography-mass spectroscopy (GCMS), compound specific stable isotope ratio mass spectroscopy (IRMS) and nuclear magnetic resonance (NMR) to track ^{13}C labelled carbon from diverse sources in soil. Soil was exposed to carbon in the form of glucose, acetate and carbon dioxide. Microbial populations utilising the carbon substrates were characterized by identifying the phospholipid fatty acid (PLFA) biomarkers labelled with ^{13}C and NMR allowed the transformations of the ^{13}C to be traced. Microbial populations were found to differ with carbon substrate. Fungi were the dominant users of glucose and acetate, while gram negative aerobic bacteria and pseudomonad strains dominated CO_2 uptake. NMR confirmed that the ^{13}C was incorporated in varying quantities into soil organic matter (SOM) components such as lipids, carbohydrates and proteins. This work demonstrates an initial approach to tracking the fate of carbon from varying sources in soil.

2.1 Introduction

The sequestration of carbon in soil as organic matter represents an important option in partially curtailing increasing atmospheric carbon and mitigating carbon loss from soil(Bellamy et al. 2005, King 2011, Gleixner 2013). However, our knowledge of the long-term storage of carbon in soils is limited and the traditional partitioning of soil organic matter (SOM) into stable (recalcitrant) and unstable (labile) pools does not explain the chemical nature and degradation characteristics of soil(Kleber 2010, Schmidt et al. 2011). There is a growing recognition that most recalcitrance in SOM is not as a result of the chemical properties or inaccessibility of soil biomolecules. Instead, there is a realisation that carbon in soil is continuously recycled into new structures by microbial synthesis and that soil carbon dynamics are intimately linked to soil microorganisms(Gleixner 2013, Kiem and Kögel-Knabner 2003, Kindler et al. 2006, Simpson et al. 2007, Liang and Balser 2008, Potthoff et al. 2008, Simpson et al. 2007). Therefore, any attempt to sequester carbon in soil for long periods will have to be coupled to an understanding of the uptake of carbon by soil microorganisms and the fate of this carbon in soil. Studying SOM is of obvious interest in agricultural developmentas a high soil organic carbon content is crucial to ensuring soil health and stability(Six, Elliott and Paustian 2000).In the European agricultural community, grant supports may be refused to farmers who do not comply with certain crop rotation policies or fail to maintain a positive humus balance (Amelung et al. 2008).

Carbon sequestration by soil microbes and the natural carbon cycle are not sufficient to offset anthropogenic CO_2 emission into the atmosphere, however deliberate terrestrial sequestration through soil and forest management has become an accepted method of mitigating global warming(Six et al. 2002, West and Six. 2007, Sundquist, Ackerman and Parker 2009). Maximising SOM however may result in excess nitrate in ground and surface waters from mineralisation of SOM. Advancements in the analysis of transformation and turnover of soil organic carbon (SOC) is therefore relevant to both agriculture and climate change.The monitoring of carbon from different sources in soil is challenging due to the complexity and diversity of soil. Overcoming this challenge would allow us to track how carbon changes over time and thus develop and verify agricultural management options that encourage carbon sequestration in soil.

The use of stable carbon isotopes to trace organic matter within terrestrial ecosystems offers opportunities to study the dynamics of microbial SOM(Schmidt et al. 2011, Dumont, Neufeld and Murrell 2006, Heinzle et al. 2008, Jin and Evans 2010, Hart et al. 2013a). Furthermore, techniques that assess the input of microbe derived carbon to SOM can benefit from a clearer understanding of the products derived from a specific carbon source. In this study, three ^{13}C labelled carbon sources (Glucose-$^{13}C_{6,}$ Sodium acetate-*1*-^{13}C and $^{13}CO_2$) were incorporated into soil samples and analysed by GC-MS coupled to a Compound Specific Stable Isotope Ratio Mass Spectrometer (GCMS-IRMS) and Nuclear Magnetic Resonance (NMR) to assess the fate of the ^{13}C-labelled substrate in soil. NMR and GCMS-IRMS can independently make use of the isotopic enrichment of soil biomass and effectively allows us to link the molecular incorporation of carbon into soil microbes while providing chemotaxonomic information through phospholipid fatty acid analysis (PLFA).

Signature fatty acids (FA) can be used for bacterial taxonomy in which specific FA's are employed as taxonomic discriminators or biomarkers for species identification. PLFA's are particularly useful as biomarkers as they are contained solely in the cell membrane as storage products(Knief, Lipski and Dunfield 2003, Crossman, Ineson and Evershed 2005)(Zelles et al. 1992, Zelles et al. 1995, Hill et al. 2000). This is important as the cell membrane rapidly degrades after cell death; therefore PLFA's are good indicators of living rather than non-living microbial biomass. When a ^{13}C-labelled substrate is introduced to soil, sediment or culture, microbes utilising it will incorporate ^{13}C into their PLFA's. ^{13}C analysis of these PLFA's by IRMS gives quantitative information on the microbes degrading the substrate. The combination of compound specific stable isotope analysis and PLFA biomarker analysis has already been successfully applied to identify methane oxidizing bacteria (Knief, Lipski and Dunfield 2003, Crossman, Ineson and Evershed 2005, Maxfield, Hornibrook and Evershed 2006, Cébron et al. 2007).

NMR spectroscopy can be employed to acquire an overall view of the transformations to carbon once it is taken up by the soil microbes. A significant amount of structural detail about SOM can be provided by NMR spectroscopy and we can use it to describe the molecular characteristics of microbial biomass in soil(Simpson, McNally and Simpson 2011, Spence et al. 2011). Increasing the relative abundance of ^{13}C through isotopic enrichment greatly enhances NMR sensitivity(Kelleher, Simpson and

Simpson 2006) and also intensifies through-bond and through-space couplings that occur between atoms that form the basis of structural identification (Knicker 2002, Ippel et al. 2004).

In recent work within the group NMR was used to observe the formation of lipids, carbohydrates and proteins produced directly from CO_2 utilised by microbial biomass(Hart et al. 2013b). Here we show how NMR can differentiate between ^{13}C labelled carbon from different sources and demonstrate how carbon inputs to SOM can be traced by combining PLFA biomarkers, stable isotope analysis and NMR.

Carbon in the form of glucose, acetate and CO_2 are investigated. Glucose is a common sugar exuded to soil by plant roots, it stimulates microbial decomposition around the roots in a symbiotic relationship between plant and microbe (Lundberg, Ekblad and Nilsson 2001). Acetateis an important product of carbohydrate degradation in anaerobic systems and has been shown to stimulate syntrophic interactions in soil autotrophs and heterotrophs(Handley et al. 2012). Tracing CO_2 fixed non-photosynthetically (by chemoautotrophic microbes) in soils will provide vital detail on the transfer and fate of carbon fixed through this pathway. Although autotrophy is a well-known process, studies that employ stable isotopes to follow the uptake of carbon in soil through this process are rare(Hart et al. 2013b, Miltner et al. 2004, Šantrůčková et al. 2005). In this study we aim to develop methodsthat allow us to follow the fate of C incorporated into SOM by autotrophic (CO_2) and heterotrophic (glucose and acetate) microbes.

2.2 Materials and Methods

2.2.1 Materials

All solvents were ROMIL-SpS super purity solvents (Lennox). D-Glucose-$^{13}C_6$ 99 atom % ^{13}C (Isotech) and Sodium acetate-*1*-^{13}C, 99 atom % ^{13}C (Isotech) were the labelled carbon substrates used for incubations. Non-isotopically labelled D-(+)-Glucose, Sigma Ultra 99.5% GC, and Sodium acetate, minimum 99.0% were sourced from Sigma. Sodium methoxide solution, ACS reagent, 0.5M in methanol (Sigma Aldrich) was used for derivatisation of PLFA to FAME's. Methyl myristate ≥99% (GC) (Sigma) and 5α-Cholestane (Sigma) were used as the specific compound and internal

standard respectively for FAME quantification. Monounsaturated double bond positions were identified using Dimethyl disulphide, 99.0+% (Aldrich).

2.2.2 Soil Incubations

For details on site location, soil pre-treatment, the environmental carbon dioxide incubation chamber (ECIC) and the CO_2 incubation method please see Hart et al (2013a).

2.2.3 Pre-treatment of soil for NMR analysis

All soil samples were freeze dried and subjected to 10% HF treatment to remove silicates and other magnetic minerals. Briefly, ~30 g soil was placed into 250 ml polypropylene centrifuge tubes with 100 ml 10% HF and shaken at 100 reciprocals per minute for 24 hours.Samples were spun down at 6400 rpm for 20 minutes and the supernatant was discarded. The process was repeated x 20. The samples were neutralised by adding sterile, double-distilled water until the supernatant measured pH 6. Neutralised soils were freeze-dried and stored at -80^{o}C until analysis.

2.2.4 Media and incubation conditions for Sodium Acetate and Glucose cultures

A modified M9 minimal salts medium (MSM) (Madigan et al. 2009)was prepared by dissolving 0.5g of potassium phosphate monobasic (H_2KO_4P), 0.5g potassium phosphate dibasic anhydrous (HK_2PO_4), 0.5g of ammonium chloride (NH_4Cl), 0.1g of magnesium sulphate ($MgSO_4$), 0.05g of calcium chloride ($CaCl_2$) and 0.117g of sodium chloride (NaCl) in 1L of Ultra pure water (all chemicals were sourced from Sigma). The media was autoclaved at 121°C for 15 minutes.

The carbon sources used for incubations were ^{12}C and ^{13}C glucose and sodium acetate. Starter cultures were prepared using 100mg of soil, 49ml of M9 MSM and 1ml of 0.25M ^{12}C carbon source (glucose or sodium acetate). Identical control incubations were set up without the addition of 100mg of soil. The starter cultures were incubated at room temperature on a reciprocating shaker at 125 strokes min^{-1} for 48hrs. After 48hrs a 1ml aliquot was removed from each flask and used to inoculate new flasks containing 48ml M9 media and 1ml of 0.25M ^{12}C glucose or sodium acetate, this step was to prevent any possible carryover of soil particles from the original incubations. After another 48hrs the flasks were sub cultured for the final time. Duplicate incubations were set up using 2ml of subculture, 96ml of M9 media and 2ml of 0.25M carbon source

($^{12}C/^{13}C$ Glucose and $^{12}C/^{13}C$ Sodium acetate). Identical controls were prepared for ^{12}C glucose and ^{12}C sodium acetate using sub culture from the control starter incubations. In total there were six incubations for experimental analysis i.e. a ^{12}C and ^{13}C glucose incubation, a ^{12}C and ^{13}C sodium acetate incubation and two control incubations one for ^{12}C glucose and one for ^{12}C sodium acetate. For NMR analysis, the incubations were up scaled to 500ml total volume (10ml culture, 10ml 0.25M substrate and 480ml M9). This provided sufficient biomass for NMR analysis. The cultures were incubated at room temperature on a reciprocal shaker at 125 strokes min^{-1} for 56hrs.

The cultures were sampled at regular intervals over the 56hr period. At each sampling interval 2ml of the microbial broth was filtered using a 0.2um micro syringe, the aliquot was stored at -80°C before thawing for UV analysis. 5ml aliquots were taken and frozen at -80°C in pre-weighed vials prior to freeze-drying for phospholipid fatty acid analysis. The large scale incubation for NMR was processed after 48hrs, the 500ml broth was spun down in a centrifuge and the supernatant was decanted off. The remaining biomass was washed with excess distilled water and centrifuged repeatedly removing any salt ions from the M9 media. The biomass was then freeze-dried ready for NMR analysis.

2.2.5Lipid Extraction

PLFA biomarkers were extracted from the freeze-dried glucose biomass, sodium acetate biomass and CO_2 soil samples using a modified Bligh-Dyer (Bligh and Dyer 1959) extraction utilizing ultra-sonication to enhance recovery. Briefly, 5ml of methanol (MeOH) was added to the freeze dried sample (average <10mg), the sample was sonicated for 15 minutes and centrifuged for 15min at 6000rpm. The solvent was decanted off and filtered under vacuum. The extraction is repeated with 1:1 Methanol:Dichloromethane (DCM) and finally with DCM. The combined extracts were evaporated to near dryness using a rotary evaporator and transferred to a 2ml GC vial and dried under N_2.

2.2.6 Solid Phase extraction and Derivatisation

The PLFA's were separated from the total lipid extracts using solid phase extraction (SPE), (Pinkart, Devereux and Chapman 1998). The aminopropylsilica SPE columns (Alltech, Deerfield, IL) were conditioned under vacuum, with 4ml of (a) Acetone (b) Hexane (c) 4% Acetic Acid (d) Methanol (e) Chloroform. The lipid extracts were dried

down and reconstituted in 400ul of 1:1 MeOH:$CHCl_3$ and transferred to the column. The neutral lipid fraction and the polyhydroxyalkonate fraction were eluted with 5ml of chloroform and 5ml of acetone respectively. The third fraction containing the PLFA's (polar lipid fraction) was eluted with 2.5ml of 6:1 MeOH:$CHCl_3$ and 2.5ml of 0.05M sodium acetate in 6:1 MeOH:$CHCl_3$. The polar lipid fraction was dried under nitrogen and the PLFA's were derivatised to fatty acid methyl esters (FAME) using mild alkaline methanolysis with sodium methoxide (NaOMe). Briefly 50ul of NaOMe and 50ul of $CHCl_3$ was added to the dried extracts in a 2ml GC vial. The sample was vortexed and placed in the oven at 50°C for 10 minutes. The sample was removed from the oven and allowed to cool, 450ul of DI water was added and 70ul of 0.5M HCl. The solution was vortexed and the fatty acid methyl esters were extracted by 2x1ml washings of 4:1 Hexane:$CHCl_3$. Unwanted water was removed using sodium sulphate. The fatty acid methyl ester extract were dried under N_2 and taken up in 100ul hexane containing 100ppm cholestane as an internal standard.

2.2.7 GCMS-IRMS analysis of PLFA's

Samples were analysed using a gas chromatograph (Agilent Model 6890N) mass spectrometer (Agilent Model 5975C Quadropole MS Engine) system equipped with an automatic sampler. This GC was also coupled, via a combustion furnace (GC5) to a continuous flow isotope ratio mass spectrometer (IsoPrime). One ul of each sample was injected and split approximately 50/50 between the MS and IRMS. The column was a fused silica capillary column (30m×0.25mmi.d.) with a film thickness of 0.25 μm (HP-5MS, Agilent).Ultra high purity helium (BIP-X47Sgrade, Air Products) was used as the carrier gas with a flow rate of 1ml min^{-1}. The GCMS was set up as follows; the injector port was set at 250°C with a splitless injection, the initial oven temperature was 60°C for 1.5min and increased at 6°C min^{-1} to 300°C and held for 20min this gave a total run time of 61.5min. The GCMS interface and the Ion source were set at 280°C, filament delay was 10 min. Sample flow to the IRMS was controlled by the heart split valve of the FID, which was set to close after 10 minutes. Closing the valve forced sample through the GC-IRMS transfer line which entered a ceramic combustion furnace (GC5, 650×0.3mmi.d.) packed with a copper oxide/platinum catalyst heated to 850°C. Water was removed from the combustion products by passing the effluent through a nafion membrane; all carbon was reduced to $^{12}CO_2$ and $^{13}CO_2$ prior to entering the IRMS. Reference gas CO_2 of known $\delta^{13}C$ value was introduced from the reference gas injector at the beginning of the run, IRMS system validation was carried out using a stable

isotope reference standard (Mixture B2, Indiana University). Analyt peaks were identified from their mass spectrums using the NIST library. Compounds in the IRMS chromatogram were identified by using their retention time (-40 seconds for IRMS transfer line) to locate the corresponding peak in the GCMS chromatogram.Quantification of fatty acids was performed using a multi-point internal standard method. The standard curve was created using methyl myristate fatty acid methyl ester as the specific compound and cholestane as the internal standard.

2.2.8 Determination of Fatty Acid double bond position

Monounsaturated double bond positions were identified using dimethyl disulphide (DMDS) derivatisation according to Nichols et al. (1986). Briefly, the FAME samples were dried down after their analysis by GCMS. The samples in 50ul of hexane were treated with 100ul of DMDS and 1-2 drops of iodine solution (6% w/v in diethyl ether). The reaction was performed in a standard 2ml GC vial. The sample was placed in the oven at 50°C for 48hrs. After removal from the oven 500ul of hexane was added to the sample, iodine was removed by shaking with 500ul of 5%w/v sodium thiosulphate. The organic layer was removed to a clean GC vial and the aqueous layer was extracted with 4:1 Hexane:Chloroform. The combined organic layers were dried under N2 and reconstituted in 100ul hexane prior to GCMS analysis. The double bond position was identified from the fragmentation between the two CH_3S groups of the DMDS adduct (Nichols, Guckert and White 1986).

2.2.9 UV analysis

Sodium Acetate quantification of the 2ml sub-samples from the ^{12}C and ^{13}C sodium acetate incubations was carried out using a Cary UV 50 scan UV visible spectrometer (Varian). A UV scan of a 0.1M solution of sodium acetate showed the λmax abs to be at 206nm. The instrument was blanked with M9 media to ensure there was no interference from the minimal medium. A standard curve was created using sodium acetate standards of 0.5mM to 6mM, there absorbance was measured in triplicate at 206nm, this resulted in a standard curve with an R^2 value of 0.9963. The samples were analysed in triplicate and their concentration was calculated from the equation of the line.

Glucose quantification was carried out on a Cary UV 50 scan UV visible spectrometer (Varian). AGlucose (HK) Assay Kit (Product Code GAHK-20) was

purchased from Sigma. UV analysis and subsequent calculations were carried out in accordance with the product/manufacturers guidelines.

2.2.10 Solid State ^{13}C NMR Analysis

Samples were packed into 4 mm zirconium oxide rotors with Kel-F rotor caps. ^{13}C cross polarization with magic angle spinning (CP-MAS) NMR spectra were acquired using a Bruker Avance III 500 MHz spectrometer (Bruker Biospin, Canada) equipped with a Bruker 4 mm H-X MAS probe. Spectra were acquired at 298K with a spinning rate of 13 KHz, a ramp-CP contact time of 1 ms, 1 s recycle delay, 8192 scans, 1024 time domain points and ^{1}H decoupling using Spinal64. Spectra were processed using the Bruker Topspin software (version 2.1) with a filling factor of 2 and exponential multiplication resulting in a line broadening of 30 Hz in the final transformed spectrum. Spectral subtractions to produce the difference spectra were performed in the interactive mode of Topspin 2.1.

2.2.11 High Resolution Magic Angle Spinning (HR-MAS) NMR Analysis

Prior to NMR analysis, samples as well as materials that came into direct contact with the samples (zirconium oxide rotors, Kel-F caps, Kel-F sealing rings, steel spatula, pipette tips) were dried for one week over P_2O_5 under vacuum at room temperature to reduce traces of molecular water that would interfere with NMR spectra. 40mg of dry sample was then weighed directly in a 4 mm zirconium oxide rotor and 60 µL of DMSO-d_6 was added as a swelling solvent. After homogenization using a stainless steel mixing rod, the rotor was doubly sealed using a Kel-F sealing ring and a Kel-F rotor cap. HR-MAS NMR spectra were acquired using a Bruker Avance III 500 MHz spectrometer (Bruker Biospin) equipped with a Bruker 4 mm triple resonance (^{1}H, ^{13}C, ^{15}N) HR-MAS probe with an actively shielded Z gradient and a spinning speed of 6.66 KHz. All HR-MAS experiments were acquired at 298 K. Proton (^{1}H) experiments were acquired with 256 scans, 4096 time domain points and a recycle delay of 2s. Solvent suppression was achieved by presaturation utilizing relaxation gradients and echoes (Simpson et al,2005). ^{1}H HR-MAS spectra were processed with a zero-filling factor of 2 and exponential multiplication, resulting in a line broadening of 2 Hz in the transformed spectrum. ^{1}H–^{13}C Heteronuclear Single Quantum Coherence (HSQC) spectra were collected in phase sensitive mode using Echo/Antiecho-TPPI gradient selection but without sensitivity enhancement. Scans (2048) were collected for each of the 96 increments in the F1 dimension. A relaxation delay of 1 s was employed with 1024 time

domain points collected in F2 and a ^{1}J ^{1}H–^{13}C of 145 Hz. The F2 dimension was multiplied by an exponential function corresponding to a 15 Hz line broadening while the F1 dimension was processed using sine-squared functions with phase shifts of $\pi/2$. Both dimensions were zero-filled by a factor of 2. Quantification from HSQC was done in the multi-integration mode of AMIX 3.8.7 regions where defined as follows: Protein (phenylalanine resonance) ^{1}H 7-7.3 ppm, ^{13}C 125-130 ppm; Lignin (methoxy signal) ^{1}H 3.6-3.8 ppm, ^{13}C 54-58 ppm; Carbohydrates (CH_2 signal) ^{1}H 3.4-3.6 ppm, ^{13}C 58-63 ppm; Lipids (CH_2 β to COOH), ^{1}H 2.1-2.25 ppm, ^{13}C 29-35 ppm. The microbial biomass grown on acetate and glucose, numerous additional lipid signals were observed and the lipid integration region was increase to ^{1}H 2.4-1.9 ppm, ^{13}C 29-35 ppm to account for all lipid signals**.**

2.3 Results and Discussion

Microbial soil cultures were incubated in duplicate with three different carbon substrates; (1) Glucose-$^{13}C_{6,}$ (2) sodium acetate-*1*-^{13}C and (3) $^{13}CO_2$. The end point for the glucose and acetate incubations was determined by visual inspection of the biomass formed in the incubation vessels. After 54 hrs it was determined that biomass had decreased significantly and the incubation was ended. Sub-samples were taken 8 times during the 54 hour incubations. The CO_2 incubation was subsampled at the start (T0) and after 48 hours of incubation (T48). Incubation time was based on previous CO_2 soil incubations by Hart et al. (Detailed tables of lipid biomarkers and quantities over time can be seen in the appendix Tables 1a,2a,3a). The incubation conditions for the glucose and acetate incubations were set up to ensure uptake of the added substrates by microbial consortia. The conditions were ideal for testing and developing the analytical approach but are not representative of environmental conditions. The conditions for the CO_2 incubation however are similar to natural soil conditions in the environment.

Substrate concentrations were measured over the course of the acetate and glucose incubations and total PLFA concentration was used as an indicator of total microbial population in all incubations. Samples were subjected to PLFA, IRMS and high resolution NMR analysis. The PLFA profiles indicate diversity in microbial community composition as a result of varying carbon sources (Figure 1). IRMS allows us to identify isotopically enriched PLFAs that represent the microbes responsible for the degradation of the labelled substrate. Positive $\delta^{13}C$ values for PLFA peaks indicate

that the ^{13}C labelled carbon substrates were utilised by a component of the microbial population. Lastly, NMR data can be used to identify how the label is incorporated into the different chemical categories in the SOM. All techniques can independently make use of the isotopic enrichment (^{13}C) of soil microbial biomass and by combining them we can identify both the key drivers and products of carbon biotransformation.

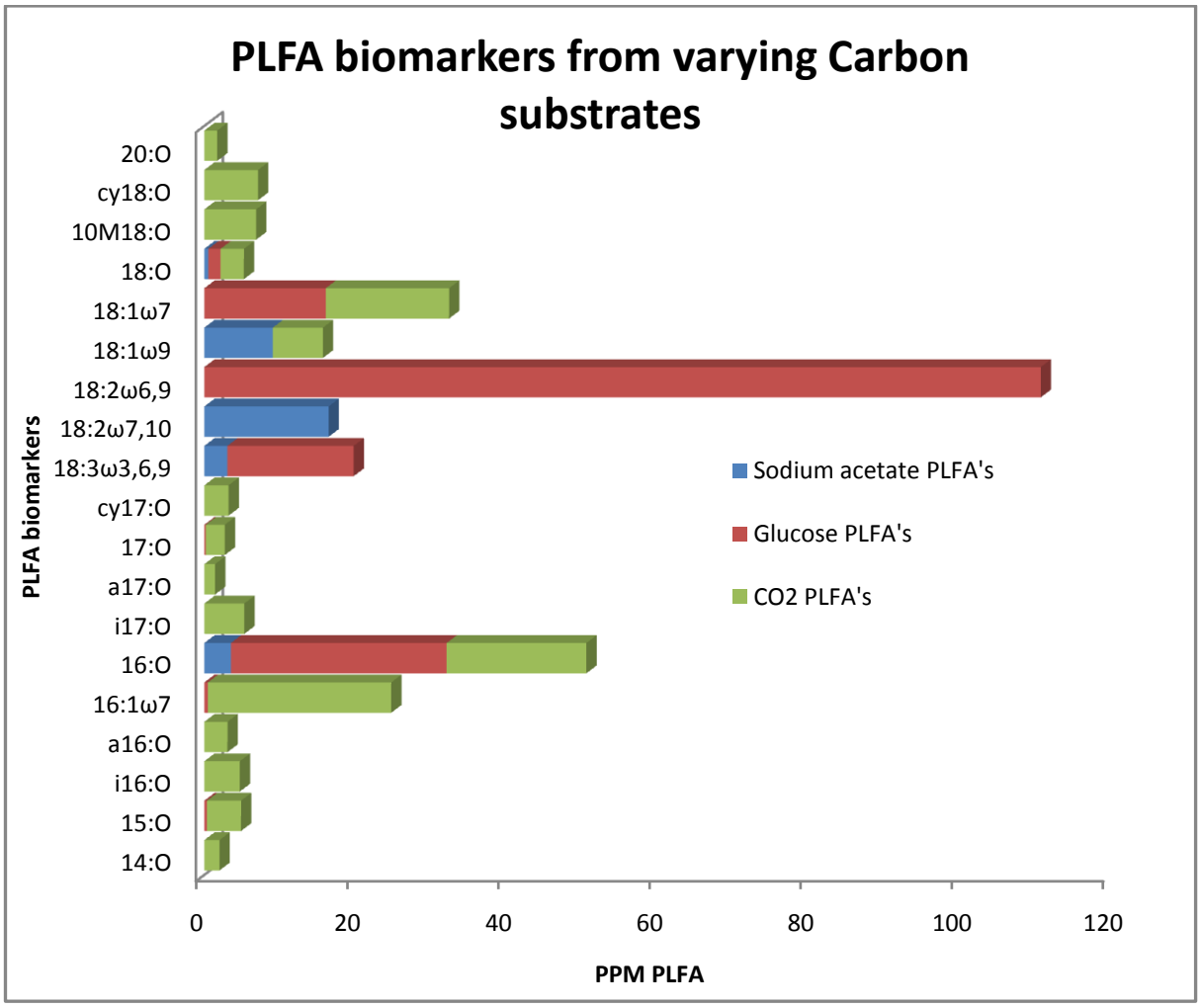

Figure 1: PLFA profiles and relative abundances from varying carbon substrate incubations

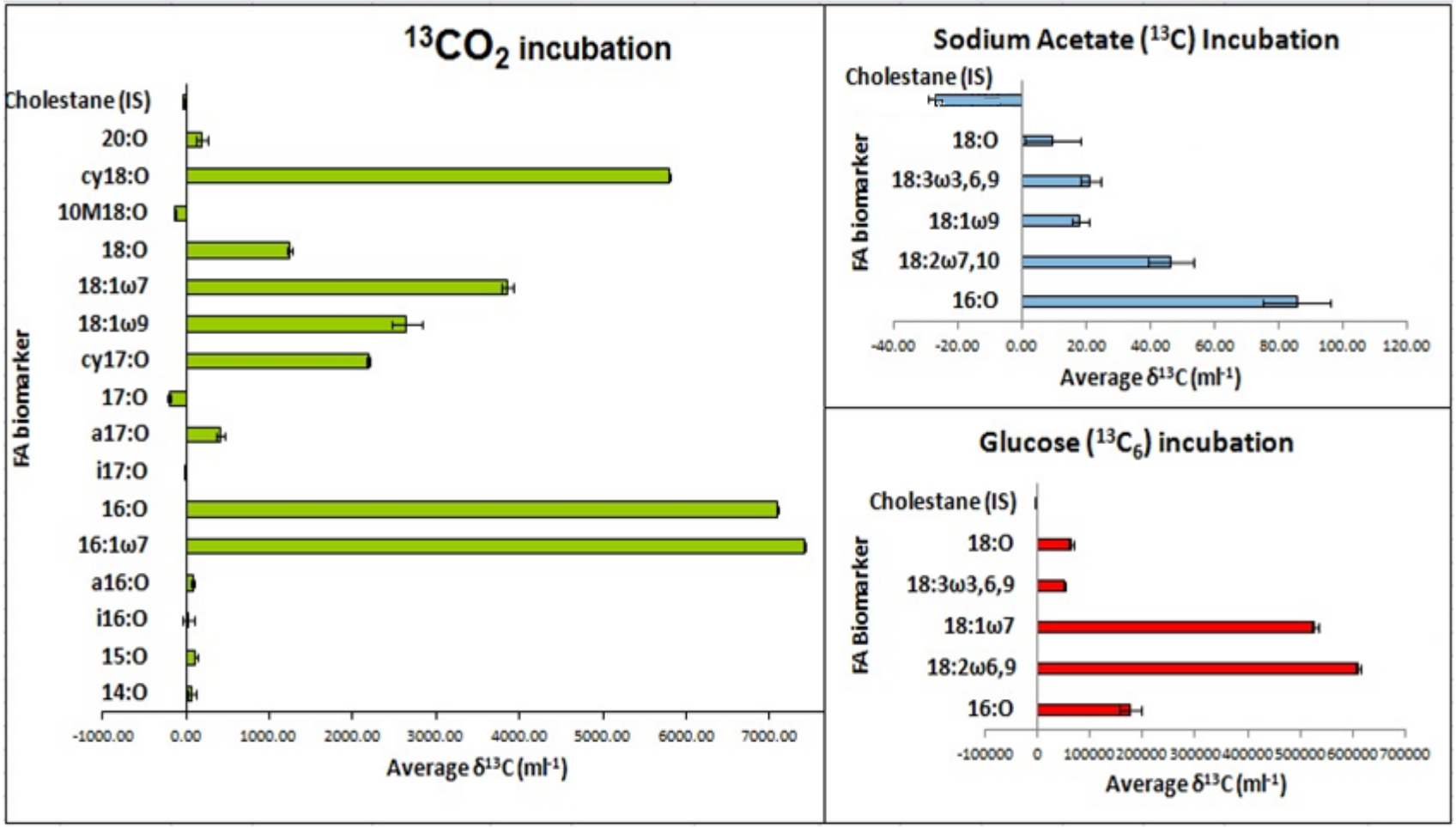

Figure 2: ^{13}C enrichments – Isotope Ratio data displaying Average **δ^{13}C** for PLFA biomarkers found in each incubation. (Lower LOQ = peak height of 0.15 nA)

2.3.1 Glucose incubation

PLFA's extracted and identified from the glucose incubation and their relative abundances can be seen in figure 1. Quantification of glucose over the 54 hours incubation period shows glucose concentration decrease rapidly from 5.55mM at T18 to 0.18mM at T42 (see figure 3 and appendix table 1a). There is a lag phase from T0 to T18 as the microbial population adapts in response to the dramatic increase in available carbon. A decrease in glucose levels between T18 to T42 is inversely correlated to an increase in total PLFA's from 10.117ppm at T24 to 509.811ppm by T42. The glucose quantification and PLFA results from the glucose control confirmed no microbial growth thus glucose levels remained constant (Figure 3). The large increase in PLFA concentration from T24 to T42 is largely due to the appearance of 18:2ω6,9 and 18:3ω3,6,9 which are known biomarkers for fungi (Christie 1982)(Figure 1). Over the 18 hour growth period fungi grow rapidly, during this time there is a sharp decrease in glucose concentration. **Fungi have previously been shown to be the most active microbes in degrading soil glucose** (Lundberg, Ekblad and Nilsson 2001). Several other ^{13}C labeled PLFA's (Figure 2) appear at the growth phase, such as 15:0, 16:1ω7 and 17:0, 16:0 which are ubiquitous in bacteria (Hill et al. 2000). The PLFA profile suggests a mixed microbial population dominated by fungi, which appear to be the main glucose degraders.

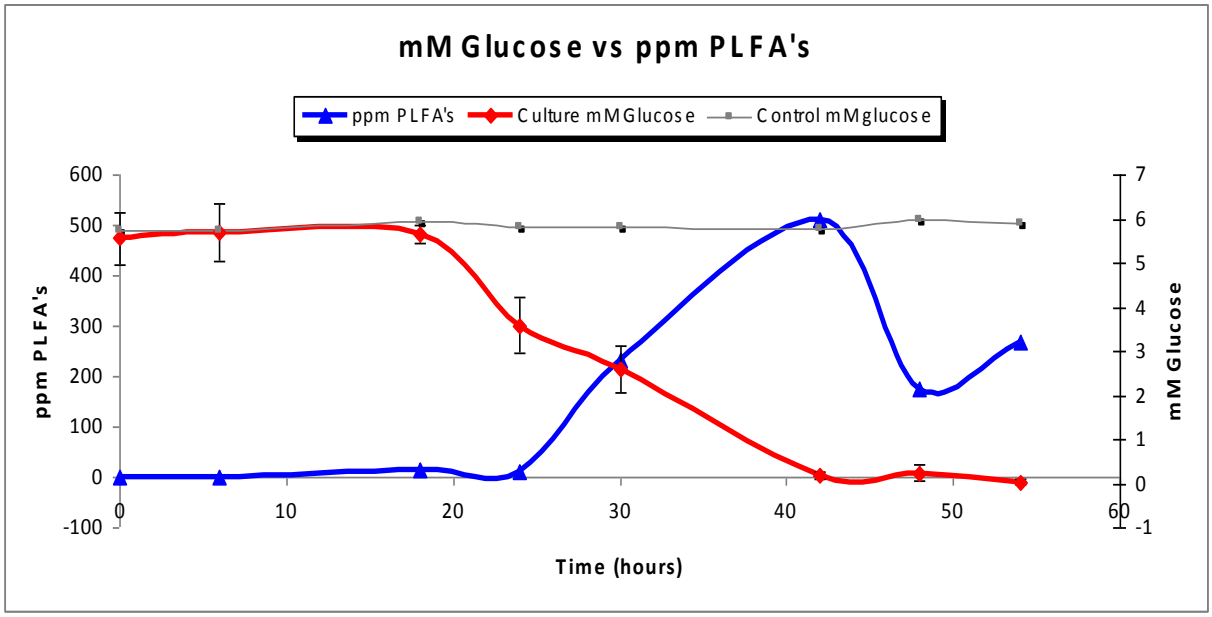

Figure 3: Total PLFA concentration vs **Glucose** concentration over 54 hour incubation at room temperature (~21°C)

At T42 the microbial population is at its peak while the glucose concentration is almost completely depleted. The depletion of glucose results in a decrease in microbial population at T48, most notably the fungiappear to decrease. Although glucose is reduced to only 0.04mM by T54, the PLFA abundance indicates secondary growth. The PLFA profile remains the same from T30 (the growth phase) to T54 with the exception of 18:1ω7, an indicator for the presence of aerobic gram positive bacteria(Hill et al. 2000, Zak and Kling 2006) appearing at T48. At T54 the increase in microbial population is due to fungi but the marker 18:1ω7 also increases. All the PLFAs from the ^{13}C glucose incubation are labeled with ^{13}C and this is confirmed by IRMS analysis (Figure 2). The large $\delta^{13}C$ values especially for the fungal markers verify that the microbial population present is degrading the ^{13}C labelled substrate.

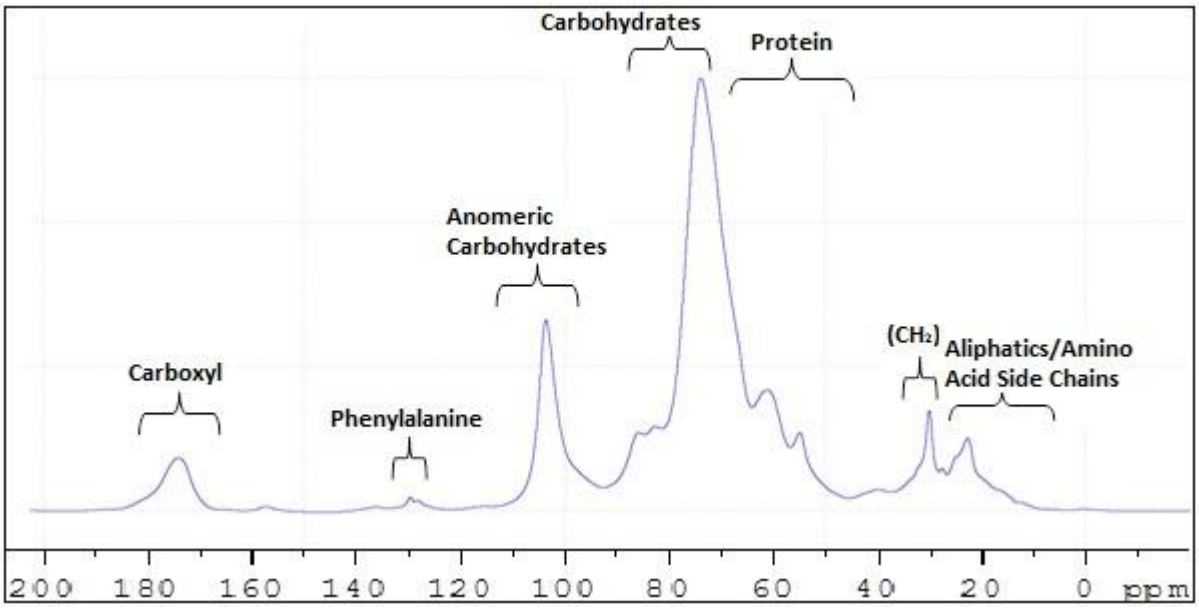

Figure 4: CP-MAS-^{13}C NMR difference spectrum for **Glucose**

2.3.2 Glucose biomass NMR analysis and NMR interpretation

The solid-state CP-MAS-^{13}C NMR difference spectrum (Figure 4) is obtained by subtracting the total organic carbon spectra before and after ^{13}C labelling for each substrate. This quantitative comparison highlights the organic matter that has exclusively been synthesised by microbes that consume each substrate. Only those structural components enriched in ^{13}C appear in the difference spectrum. The total signal increase in the normalized CP-MAS carbon spectrum can be used to estimate the % of total carbon labelled during the growth period. In each case the regions where the

glucose and acetate resonate was not included in the integration to avoid any remaining substrate skewing the results.

After glucose labelling the total signal in the CP-MAS-^{13}C NMR difference spectrum increased approximately 16.5 times which corresponds to ~18% of the carbons becoming labeled. With sodium acetate the carbon signal increased ~15.5 times corresponding to 17% of the carbons becoming labelled.

The NMR data can be used to identify how the label is incorporated into the different chemical categories in SOM. Solid-state NMR provides an overview of the major types of bulk chemical functional groups of SOM but due to the relatively broad lineshapethe extraction of structural information is very challenging and in some cases impossible. However, High Resolution Magic Angle Spinning (HR-MAS NMR) can be employed to overcome challenges associated with pure solid-state NMR analyses. A solvent is added to the analyte and after swelling, the components become NMR observable. HR-MAS NMR allows the analysis of materials that swell, become partially soluble or form true solutions, to be analyzed at resolution close to that observed in solution-state NMR(Kelleher and Simpson 2006, Simpson, Simpson and Soong 2012, Keifer et al. 1996, Millis et al. 1997).Two-dimensional (2-D) HR-MAS NMR (Figure 5) experiments spread signals into a second dimension, thereby decreasing the overlap and providing information as to the connectivity's between nuclei in mixtures(Simpson 2001, Hertkorn et al. 2002).^{1}H–^{13}C Heteronuclear Single Quantum Coherence HR-MAS NMR (HSQC) is an experiment that detects H-C bonds within a structure(Ernst, Bodenhausen and Wokaun 1987). A cross-peak in an HSQC spectrum represents the chemical shift of both carbon and proton atoms in a C-H unit. When considered together, the cross-peaks form a specific pattern or ''molecular fingerprint'' of a specific structure or class of structures. Multidimensional NMR of SOM including detailed assignments of the microbial fraction has been considered in detail in previous publications(Simpson et al. 2007, Ernst, Bodenhausen and Wokaun 1987, Simpson 2002, Simpson, Kingery and Hatcher 2003).

The difference HSQC ^{1}H-^{13}C HR-MAS-NMRspectrum for Glucose biomass(Figure 5) indicates that, the signals from protein increase by only 5%, and the lipids by 367%. When the isotopic enrichment of the label is accounted for this corresponds to approx ~0.05% of the protein and 4% of the lipids labelled over the 48hr

period in the HR-MAS observable fraction. Note the carbohydrates could not be quantified accurately as they overlapped with the glucose carbon source.

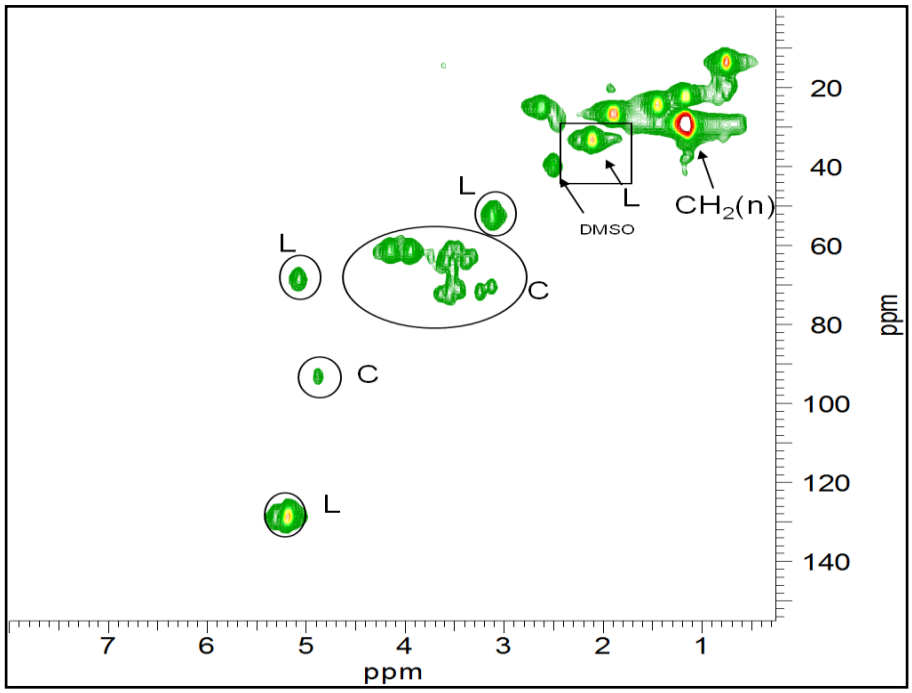

Figure 5: HSQC HR-MAS NMR 2D spectra for microbial biomass grown with ^{13}C labeled Glucose, showing the main organic compounds into which the ^{13}C label was incorporated.

P= Protein C=Carbohydrate L=Lipid

2.3.3Sodium Acetate Incubation

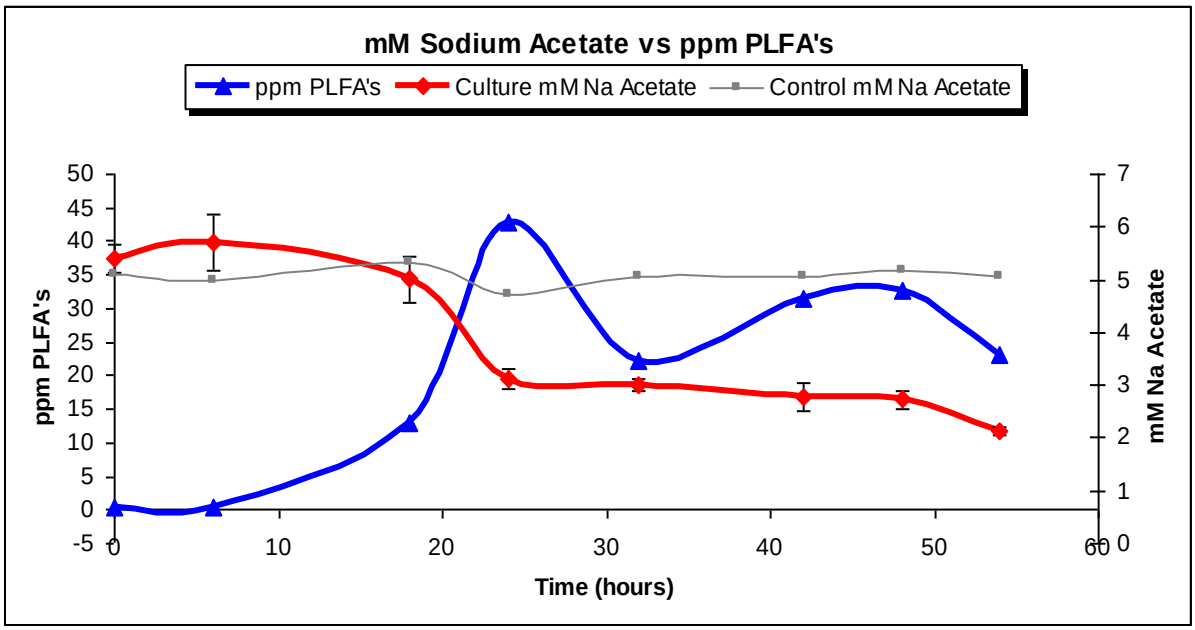

Figure 6: Total PLFA concentration vs Sodium Acetate concentration over 54 hour incubation at room temperature (~21°C)

Quantitative analysis of sodium acetate levels over time (Figure 6) shows steady levels of sodium acetate until T24 where the concentration drops to 3.13mM. This decrease in substrate corresponds to an increase in microbial population confirmed by the increase of total PLFA's from 13 ppm to 42 ppm. PLFA's extracted and identified from the sodium acetate incubation can be seen in Figure 1. The control incubation did not contain any PLFAs and the sodium acetate levels remained constant (Figure 6). 18:2ω7,10, 18:1ω9 and 18:3ω3,6,9 have the highest $\delta^{13}C$ values (Figure 2), these biomarkers are all known fungal markers (Hill et al. 2000, Christie 1982, Zak and Kling 2006). **Fungi were also the dominant microbes identified in acetate rich grasslands by Laughlin et al**. (Laughlin et al. 2009). The ubiquitous bacterial PLFA 16:0 is also prominent at T24 and coincides with the microbial population peak. Sodium acetate levels decrease gradually from T24 to T54 but acetate is not completely depleted over the 54 hours (2.13mM T54 final concentration). After its peak at T24 the microbial population decreases until secondary growth appears to occur at T42 and T48, where PLFA abundance increases. The fungal biomarkers18:2ω7,10 and 18:1ω9 increase in this secondary growth phase but the fungal marker 18:3ω3,6,9 decreases indicating the presence of different fungal communities. The bacterial markers 16:0 and 18:0 increase and decrease simultaneously throughout the incubation, suggesting that they represent the same bacterial population. All the PLFA's from the ^{13}C sodium acetate incubation are labeled with ^{13}C, confirmed by the elevated $\delta^{13}C$ values suggesting that most of the microbial population present utilises the sodium acetate substrate.

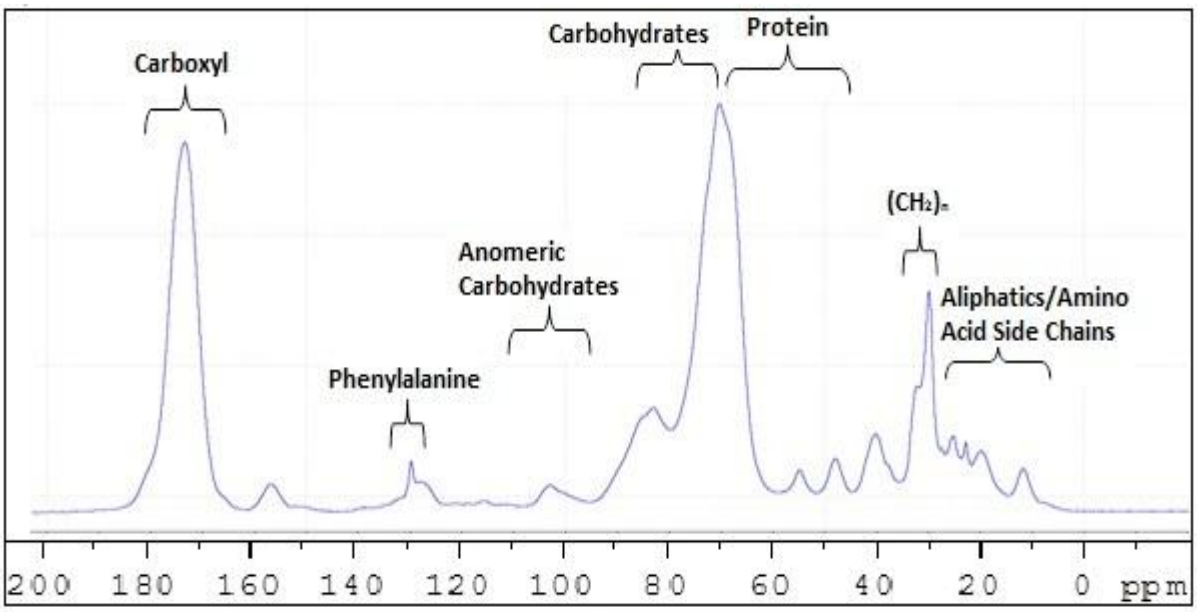

Figure 7: CP-MAS-^{13}C NMR difference spectrum for Sodium Acetate.

3.2.4 Sodium Acetate biomass NMR analysis

The CP-MASNMR (Figure 7) and the HSQC ^{1}H-^{13}C HR-MAS-NMR (Figure 8) spectra for the sodium acetateexperiment showed that the signal for protein increased by 1180% and the carbohydrate region by 800%. Interestingly the lipid signal (used for all quantification in the soil and biomass) decreased by 38% with labelling. Additional lipid signals, however, did arise in the ^{13}C acetate cultured biomass that are not apparent in the glucose cultured biomass, or ^{12}C acetate biomass. By including these signals in the integration the total lipid content of the organic matter rose by 375%. When the natural abundance of ^{13}C and isotopic enrichment of the labelling substrate are considered this corresponds to ~13% of the protein, -0.42% of the main lipids (but 4.13% when additional lipids, specific to acetate labelling are included) and 8.8% of the carbohydrates labelled over the 48hr period in the fraction of material detectable by HR-MAS.

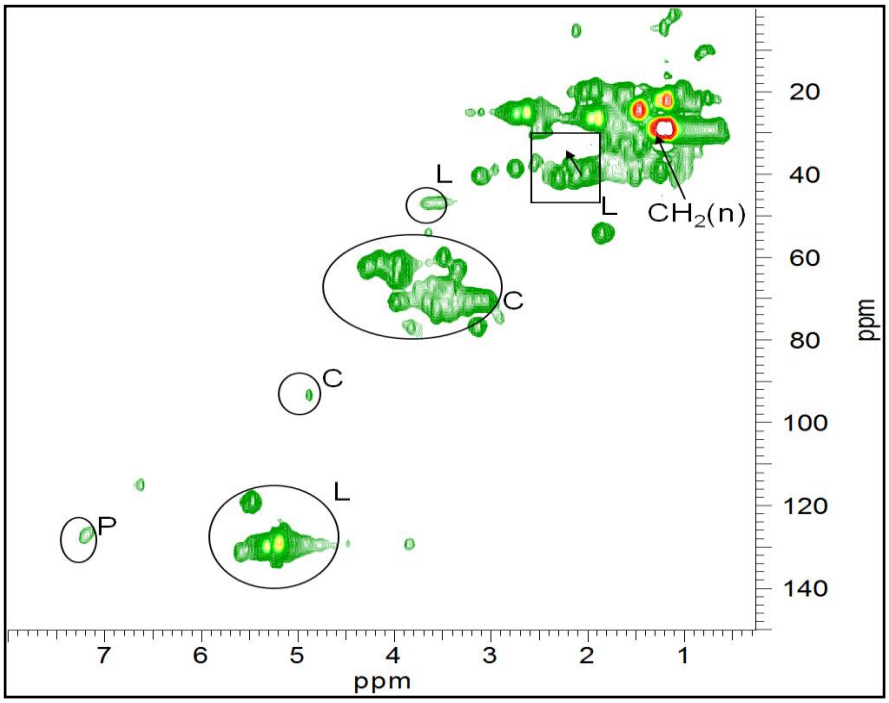

Figure 8: HSQC HR-MAS NMR spectra for microbial biomass grown with ^{13}C labeled Sodium Acetate, showing the main organic components into which the ^{13}C label from ^{13}C-acetate was incorporated was incorporated

P= Protein C=Carbohydrate L=Lipid

2.3.5 CO_2 incubation

The PLFA profile from the CO_2 incubation can be seen in Figure 1. It is clear that the PLFA profile for the the CO_2 incubation is more diverse than that of the microbial microcosms in the sodium acetate and glucose incubation. **However, it is important to point out that the initial soil preparation used for the CO_2 experiment is different to that of the glucose and acetate experiments. In the case of CO_2, a soil slurry was used as described previously (Hart et al. 2013a) while soil cultures were employed for the glucose and acetate studies**. The different approaches facilitate the growth of both heterotrophic and autotrophic soil biomes and allow us to draw initial comparisons between microbial communities that preferentially utilise the varying sources of carbon. Most importantly for this study, it allows us to show that the methodology as outlined here is suitable to follow the fate of carbon from such diverse sources. These differences in incubation may affect the diversity of the microbial populations incorporating the added ^{13}C label. Repeated sub-culturing of the glucose and acetate incubations were done to ensure a population which was utilizing the given substrate. However, this technique is not representative of natural conditions and may lead to less diverse PLFA profile. The conditions of the CO_2 incubations are closer to natural soil conditions and a more diverse PLFA profile is expected.

The anteiso and iso methyl branched FA's (a14:0, i15:0) are found in gram positive bacteria (Zelles et al. 1995, Lechevalier 1977, Zelles. 1999). Neither of these FA's increase over the 48h incubation and IRMS analysis (Figure 2)confirms they have close to natural$\delta^{13}C$ values. This suggests that gram positive bacteria are not thriving in the elevated CO_2 environment. The non-specific bacterial markers 14:0,15:0, and 17:0 (Hill et al. 2000) are not heavily ^{13}C enriched. In contrast, the ubiquitous bacterial FA 16:0has a very high$\delta^{13}C$ value, suggesting that it originates from autotrophic bacteria utilizing inorganic carbon from CO_2 in the chamber. The monounsaturated FA's 16:1ω7 and 18:1ω7 also have high delta values and they increase in abundance over the 48hr incubation. Both FA's are indicators of gram negative aerobic bacteria(Hill et al. 2000, Wilkinson 1988) and 16:1ω7 has been reported in pseudomonad strains of bacteria(Fang, Lovanh and Alvarez 2004). The 18:1ω9 FA is a known fungal biomarker (Zak et al. 1996) and its enrichment with ^{13}C implies that fungi present in the soil slurry are consuming inorganic carbon from CO_2. However, ^{13}C enriched fungal biomarkers

may also indicate that some fungi are simply scavengers or predators that take up the ^{13}C through consumption of autotrophs (Leuders et al. 2006).

Cyclopropyl FA's have been proposed as indicators of starvation stress because these fatty acids increase in species during the stationary phase of growth that follows substrate depletion (Bossio et al. 1998). Two cyclopropyl FA's are found in the profile of the CO_2incubation (cy17:0 and cy18:0), both are ^{13}C enriched which indicates that CO_2 is utilized as a substrate. The quantity of cy17:0 does not change considerably, however as CO_2 decreases in the chamber the concentration of cy18:0 increases significantly, which is in agreement with the theory of substrate starvation proposed by Bossio et al (1998).

The methyl branched FA 10Me18:0 increases in the $^{12}CO_2$ incubation. However, it is not enriched, and has been reported in sulphate reducing bacteria and actinomycetes(Lechevalier 1977, Dowling, N, J, E., Widdel and White 1986, White, Hood and White 1986). This bacterial group does not seem to be utilising CO_2, but it may be surviving on organic carbon present in the slurry and the added $Na_2S_2O_3$. The FA 20:0 which is also not enriched is found in higher plants and mosses and may be present due to plant material that was not fully removed in the pre-treatment process. The total mass of PLFA's increased over the 48hr incubation, due mainly to the increased activity of gram negative bacteria. The elevated delta ^{13}C values of these FA's indicate that the elevated CO_2 levels in the chamber are supporting autotrophic conditions.

2.3.6 CO_2 biomass NMR analysis

A quantitative comparison of the solid state CP-MAS ^{13}C spectra before and after labelling with $^{13}CO_2$ indicate that the total carbon signal in the Hampstead soil increased by 10.8% (Figure 9). When the natural abundance of ^{13}C and isotopic enrichment of the labelling gas are considered, this relates to ~0.11% (or ~ 1 in every 900 carbons) of the total soil carbon being labelled in the 48 h period. It is clear that the 2D HR-MAS spectra (Figure 10) the organic matter is dominated by lipids, carbohydrates and proteins/peptides arising from microbial biomass. Comparison of relative integrals before and after labelling indicates that the intensity of the various sub-components

increased in the order: lipids (100% signal increase), carbohydrates (33% signal increase) and proteins (30% signal increase) and lignin (no change).

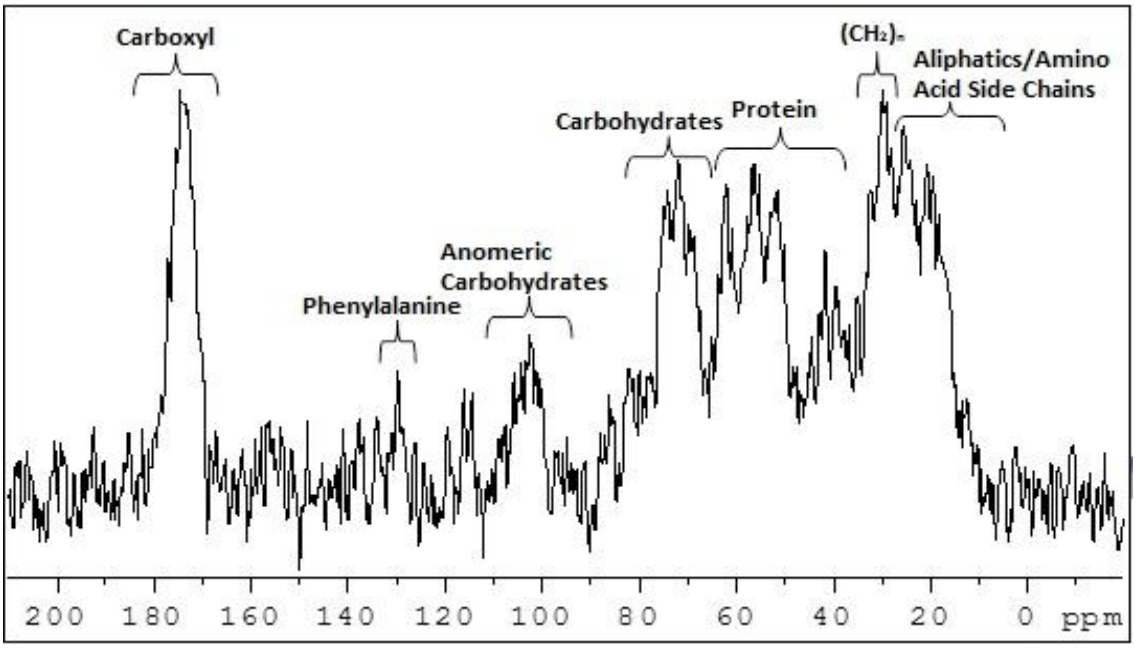

Figure 9: CP-MAS-^{13}C NMR difference spectrum for CO_2

The results indicate that soil microbes are able to directly utilise the atmospheric CO_2 source with a considerable lipid component being stored/used for metabolism, protein/peptide being synthesized for growth, and carbohydrate likely being made for both purposes. As expected the lignin component (as represented by a methoxy carbon crosspeak at ~3.7 – 55 ppm), often the most intense signal in soil organic matter did not change considering this is exclusively synthesised as a structural biopolymer in plants (Kelleher and Simpson 2006).

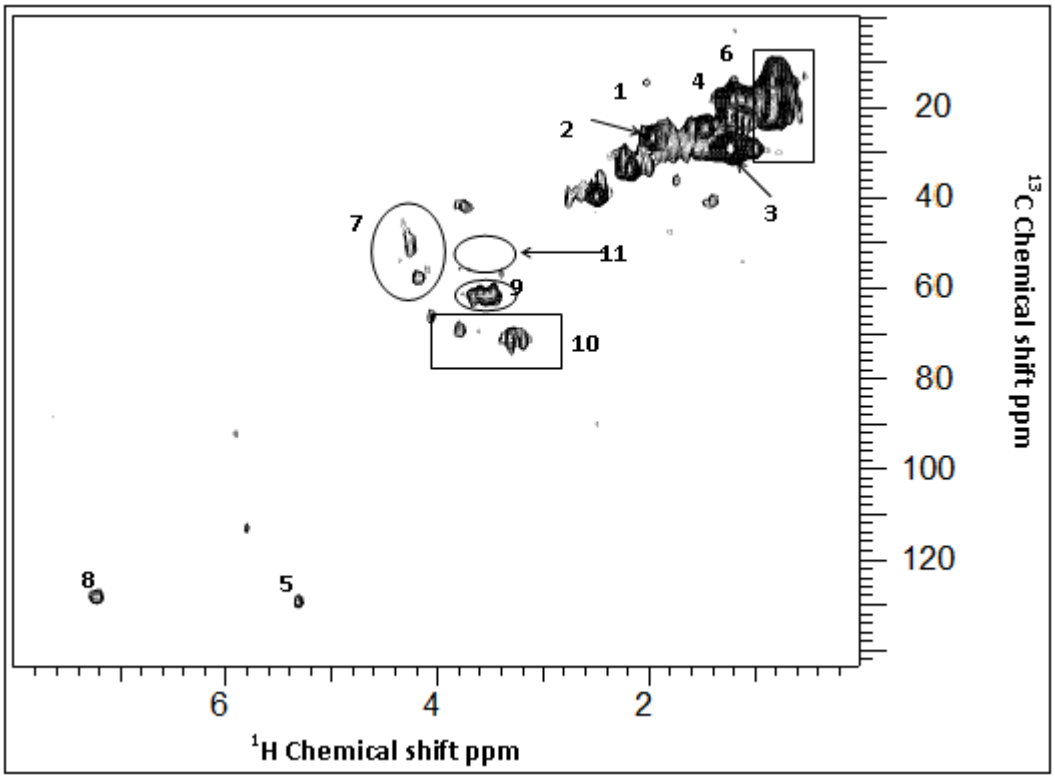

Figure 10: 2D HSQC HR-MAS NMR spectra for microbial biomass grown with ^{13}C labeled CO_2. Assignments are: 1. Acetyl group, very weak, 2. R-CH_2-COOH in lipids, 3. R-$(CH_2)_n$-R main chain in lipids, 4. R-CH_2-CH_2-COOH in lipids, 5.Unsaturation in lipids, 6. Overlap of CH_3 from lipids and CH_3 in amino acids in protein side chains, 7. α H-C units in peptides/proteins, 8. Phenylalanine, 9.CH_2 in carbohydrates, 10.CH in carbohydrates and 11.Lignin methoxyl carbon.

2.3.7 Variation of PLFAs depending on Carbon source

The PLFA profiles in figure 1 confirm that different sources of carbon resulted in varied microbial populations and carbon uptake for each incubation. Glucose was almost completely depleted within 42 hours, while over a third of the acetate still remained after 56 hours. Glucose is a common carbon source for soil heterotrophs and the most common carbohydrate in ubiquitous biopolymers such as cellulose and as such, its rapid metabolism is not surprising(Lundberg, Ekblad and Nilsson 2001). However, the microbial population degrading acetate is fully developed by T24 in contrast the glucose degrading population does not reach its mass peak until T42.

The PLFA profiles of the glucose and acetate incubations differ greatly. 18:3ω3,6,9a fungal marker is the only PLFA common to both incubations. The PLFA profiles show that both substrate degrading populations are dominated by fungi, but the fungal population is dissimilar. There is a greater variety of biomarkers present in the

glucose incubation, reflecting the larger mass of biomass and a more diverse community. Both populations contain 16:0 and 18:0, but this does not directly imply the presence of identical bacteria as these biomarkers are common in a multitude of bacteria.

While the glucose and acetate provided organic carbon for soil heterotrophs, CO_2 in the $^{12}CO_2$ and $^{13}CO_2$ incubations provide inorganic carbon and an environment more conducive to autotrophic microbial communities. Again it is clear in figure 1 that the PLFA profile for the CO_2 incubation differs from the other two. The glucose and acetate incubations have fewer FA's and are less diverse than the CO_2 profile. It is possible that the lack of PLFA diversity in the heterotrophic incubations is due to the repeated sub culturing of the glucose and acetate cultures. This removes the influence of soil minerals replacing them with the M9 media and results in a microbial population of niche organisms that almost exclusively survive on the substrate provided. This dependence is evident in Figures 3 and 6, they show that when the substrate is heavily depleted the microbial population also declines. The CO_2 incubations were performed using a soil slurry containing $Na_2S_2O_3$ as an electron donor. This medium and carbon source results in a more diverse microbial population. The PLFA profile and delta ^{13}C results for the CO_2 incubation confirm not all microbes in the population are dependent on the substrate provided (CO_2) it is probable that the soil slurry contained other carbon sources that support bacterial activity. Several PLFA's are heavily enriched with ^{13}C, the conditions clearly suit some autotrophs which are exploiting the CO_2 in the chamber, and the bacteria thriving appear to be Gram negative aerobic bacteria. Isotopic analysis of the glucose and acetate PLFA's shows that all FA's are enriched with ^{13}C, suggesting that all microbes present are utilizing the organic substrate. The PLFA's from the CO_2 incubation are not as heavily enriched as in the glucose and acetate incubations. The microbial consortia present in the soil slurry are not dependent solely on the carbon substrate.

Comparison of the three FA profiles shows that only 16:0 and 18:0 are common to all three incubations and this is not surprising as they are ubiquitous in bacteria (Lechevalier et al)(Lechevalier 1977, Lechevalier and Lechevalier 1988). More interestingly the fungal marker 18:1ω9 is found in both the acetate and CO_2 incubation and it is isotopically labeled in both. This may be due to (1) this fungal population may be able to adapt to utilize inorganic and organic carbon depending on its environment or (2) possible cross-feeding at the trophic level could be occurring. The monounsaturated

FA 18:1ω7 is indicative of gram negative aerobic bacteria it appears once glucose is depleted and is also found in the CO_2 incubation where it has a considerably high $\delta^{13}C$ value.

The reduced PLFA diversity in the glucose and acetate incubations, compared to the PLFA profile of the CO_2 soil slurry is almost certainly due to the **sub-culturing** of the soil microcosms. Repeated sub-culturing will eventually lead to a mono-culture like community, composed solely of microbes utilizing the given substrate. Having successfully developed the methods for tracking the ^{13}C label, future glucose and acetate incubation using a soil slurry and removing any sub-culturing would show greater microbial diversity.

2.3.8 NMR confirms ^{13}C incorporation into SOM varies with C source

The ^{12}C difference spectra for both acetate and glucose biomass was tiny in comparison to the ^{13}C spectra (figure 4 and 7), essentially all compounds were labeled and hence the difference is very intense. In the acetate incubation the original lipids decrease, replaced by different lipids as the population grows and becomes dominated by fungi.The glucose incubation does not result in an increase in protein but the acetate does. Protein presence in the acetate NMR spectrum is a good indication that the metabolic pathways of acetate and glucose in soil are very different. Acetate is an important product of central carbohydrate pathways produced in the breakdown of more complex carbohydrates(Handley et al. 2012), it is mainly utilized in the form of acetyl co enzyme A. Acetyl co enzyme A's main function is in the Krebs Cycle, where it is oxidised for energy production in aerobic organisms (Nelson and Cox 2000). The Krebs Cycle produces over 15 adenosine triphosphate (ATP) molecules per pyruvic acid molecule(Ophardt 2012). ATP transports chemical energy within cells. It is used by enzymes and structural proteins in many cellular processes such as the biosynthesis of proteins(Alberts 2002). Before glucose can be utilized in the Krebs cycle to create ATP, it first must be broken down by the ten step metabolic pathway of glycolysis, to 2 pyruvic acid (Pyruvate). Pyruvate is then decarboxylated to acetyl co enzyme A at the start of the Krebs Cycle(Ophardt 2012). As the acetate anion does not have to be converted to pyruvate and is already structurally similar to acetyl co enzyme A, it is quickly oxidised in the Krebs Cycle by aerobes to produce sufficient ATP for protein synthesis. On the other hand glucose first has to be broken down to pyruvate by

glycolysis, before the aerobes can utilise it to produce sufficient ATP for biosynthesis via the Krebs Cycle.

2.4 Conclusion

PLFA biomarker analysis, stable isotope mass spectrometry and NMR were successfully combined to study the uptake and transformations of three different carbon substrates by soil microbial populations.The PLFA profiles (Figure 1/Tables 1a,2a,3a in appendix) show that microbial population varied with carbon substrate. For glucose and sodium acetate all biomarkers were ^{13}C enriched indicating that either the population was dependent solely on the substrate supplied or cross-feeding occurs rapidly and widely among microbial communities. In the CO_2 PLFA profile less than half the PLFAs are enriched highlighting a specific community capable of capturing CO_2 under these conditions. The dominant microbes found to be degrading glucose were gram negative bacteria and fungi. Acetate was degraded almost exclusively by fungi but the biomarkers indicate that it was a different fungal population to that found to be metabolising the glucose. The main groups that used CO_2 as their carbon source were gram negative aerobic bacteria and pseudomonad strains. Fungal PLFA's were also ^{13}C enriched but this may be due to cross feeding. The NMR difference spectra allow us to highlight the components within each biomass sample that increase with labelling due to the uptake of carbon from the different sources. Glucose was shown to be mainly incorporated into lipids and carbohydrates, but did not result in an increase in protein. In contrast, the lipid signal decreased slightly in the sodium acetate incubation but there was a large increase in protein. In the CO_2 incubation, lipids, carbohydrates and proteins were labelled with ^{13}C indicating that, at least in part, they were formed with carbon taken directly from the atmosphere. **To the authors knowledge this is the first time that the metabolic fate of acetate, glucose and CO_2 in soil organic matter has been revealed.** This methodology represents an initial approach that allows us to follow and track the fate of carbon in soil under varying environmental conditions. It can be used to verify whether land management practices result in the loss or sequestration of carbon. The next steps are to apply this approach to real time soil samples over longer time periods.

2.5 Appendix A

Table 1 GLUCOSE incubation PLFA identification and quantity (IRMS LOD <0.15nA)

Glucose Incubation PLFA's identified										*IRMS δ values (‰)*
		Mass of PLFA's (ppm) at Time (T) of sampling in Hours(0,6,18 etc)								
Fatty Acid	**Predominant Origin**	0 hours (T0)	6 hours (T6)	18 hours (T18)	24 hours (T24)	30 hours (T30)	42 hours (T42)	48 hours (T48)	54 hours (T54)	Values at T48
15:0	Common bacterial marker (a)	-	-	-	-	0.196	0.77	0.352	0.659	<LOQ
16:1ω7	Gram negative bacteria(b,j) pseudomomad strains (c) aerobes (a)	-	-	-	-	0.716	1.572	0.475	0.721	<LOQ
16:0	Ubiquitous in Bacteria (c)	0.558	0.173	2.518	1.464	44.592	91.434	28.52	39.897	179647.3
17:0	Bacterial marker (a)	-	-	-	-	0.451	0.766	0.194	0.277	<LOQ
18:2ω6,9	Fungi(d)	-	-	7.804	5.207	122.222	347.822	110.593	166.862	616575
18:1ω7	Aerobes (a) Common bacteria (a,h)Autotrophic bacteria(e) gram negative bacteria (j)	-	-	-	-	-	-	16.057	33.213	533753.9
18:3ω3,6,9	Fungi (d)	-	-	4.495	3.168	57.756	57.182	16.703	21.63	51851.97
18:0	Bacterial marker (f)	-	-	0.251	0.279	4.776	7.693	1.623	2.045	64942.55
18:2ω6,9 ethyl ester	-	-	-	-	-	0.755	2.572	0.653	0.912	<LOQ
	Total PLFA's extracted (ppm)	0.558	0.173	15.068	10.117	231.464	509.811	175.169	266.215	
	mM Glucose	5.55	5.68	5.66	3.59	2.6	0.18	0.23	0.04	

Table 2 SODIUM ACETATE incubation PLFA identification and quantity

Sodium Acetate C12 Incubation PLFA's identified										*IRMS δ values (‰)*
		Mass of PLFA's (ppm) at Time (T) of sampling in Hours(0,6,18 etc)								
Fatty Acid	**Predominant Origin**	0 hours (T0)	6 hours (T6)	18 hours (T18)	24 hours (T24)	30 hours (T30)	42 hours (T42)	48 hours (T48)	54 hours (T54)	Values at T48
16:0	Ubiquitous in Bacteria (c)	0.298	0.194	1.511	5.16	2.032	3.221	3.522	2.495	85.47
18:2ω7,10	Fungi (d)	-	-	6.029	21.688	11.588	15.629	16.441	12.937	46.37
18:1ω9	Fungal(a,j)	-	-	2.611	10.666	5.062	9.566	9.057	5.116	17.96
18:3ω3,6,9	Fungi (a)	-	0.15	2.638	4.779	3.144	2.688	3.05	2.256	21.14
18:0	Bacterial marker (f)	0.114	0.098	0.262	0.674	0.322	0.413	0.532	0.385	9.44
	Total PLFA's extracted (ppm)	0.412	0.443	13.051	42.967	22.148	31.518	32.602	23.188	
	mM Sodium Acetate	5.41	5.71	5.01	3.13	3.02	2.77	2.73	2.13	

Table 3 CO_2 incubation PLFA identification, quantity and δ^{13} values

CO_2 incubation PLFA's identified						*IRMS δ values (‰)*
FA's	***Predominant origin***	***Total T0 PLFA ug/g***	***Stdev***	***Total T48 PLFA ug/g***	***Stdev***	***$^{13}CO_2$ T48***
14:0	Bacterial (non-specific)(f)	0.117	0.011	0.188	0.000	68.18
15:0 *	Bacterial(non specific)(a) anaerobic (g)	0.263	0.017	0.421	0.004	111.42
a14:0	Gram positive bacteria (h)(I)	0.324	0.020	0.438	0.003	27.35
i15:0	Gram positive bacteria(J)(h)(I)	0.157	0.008	0.287	0.011	82.51
16:1ω7 *	Gram negative bacteria(b,j) pseudomomad strains (c) aerobes (a)	1.478	0.058	2.262	0.029	7412.70
16:0 ^ *	Ubiquitous in Bacteria (c,a,f)	1.217	0.018	1.724	0.027	7101.51
a16:0	Gram positive bacteria (h)	0.411	0.021	0.494	0.010	-9.91
i16:0	Gram positive bacteria (j,h,i)	0.081	0.010	0.133	0.003	412.32
17:0 *	Bacterial marker (a) prokaryotes (k)	0.173	0.011	0.235	0.003	-199.77
cy17:0	Pseudomonad strains (c) gram negative (b,h,j) anaerobes (a,g)	0.226	0.011	0.300	0.007	2187.63
18:1ω9^	Fungal (a,j)	0.450	0.025	0.618	0.010	2648.04
18:1ω7*	Aerobes (a) Common bacteria (a,h)Autotrophic bacteria(e) gram negative bacteria (j)	1.197	0.043	1.521	0.015	3855.20
18:0 ^*	Bacteria non specific (F)	0.197	0.015	0.288	0.006	1245.18
10M18:0	Actinomycetes (l,n)Actinobacteria (a)sulphate reducing bacteria (m)	0.259	0.028	0.639	0.007	-123.12
cy18:0	Gram negative (f)	0.464	0.003	0.662	0.012	5798.33
20:0	Microeukaryotes, higher plants and mosses (h)	0.102	0.009	0.159	0.015	190.79
	Total	7.118	0.219	10.368	0.084	

*=appears in glucose incubation also

^=appears in Sodium Acetate incubation also

(a)G.T Hill et al. 2000 (b) Wilkinson et al 1988 (c) J. Fang et al 2004 (d)Christe et al 1988 (e) Hart et al 2011 (f)Lechevalier and Lechevalier et al 1988 (g)Guckert at al 1985 (h)Zelles et al 1995,1999 (i)O'Leary and Wilkinson et al 1988 (j) Zak et al 1996 (k) Dobbs and Guckert 1988 (l) Lechevalier et al 1977 (m) Dowling et al 1986 (n)White et al 1997

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Chapter 3

How does the addition of sulphur to soil influence chemosynthesis and carbon flux?

Contributors

Dr. Paul Flanagan (Queens University Belfast) – performed the 12 week CO2 incubation and also provided the RuBisCO gene analysis and pH data.

Dr. Shane O'Reilly (DCU) – performed the XRF and UV analysis

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3.0 Abstract

Fertilizers are used in agricultural practices worldwide to increase crop yields. The addition of nutrients to soil via fertilizers may impact soil microbial communities, thus influencing the sequestration of atmospheric CO_2 through soil autotrophy. Understanding the impact that nutrients such as sulphur are having on carbon cycling in soil may help in developing land management practices which increase carbon fixation or curtail carbon losses from soil. In this study elemental sulphur was added to agricultural soil and the soil was incubated at average atmospheric temperatures in darkness for twelve weeks under an atmosphere of $^{13}CO_2$. The impact of these conditions on chemoautotrophy was studied by analysis of phospholipid fatty acids (PLFAs), nuclear magnetic resonance (NMR), elemental analysis and molecular biology. **The use of carbon stable isotopes in these experiments augments all aspects of the analysis.**An increase in the $\delta^{13}C$ values of PLFAs confirmed microbial sequestration of $^{13}CO_2$ in both the sulphur treated soil and the control soil with no added sulphur. However, a T-test confirmed that significantly more ^{13}C was incorporated into the PLFAs of the sulphur treated soil. Cross polarisation magic angle spinning (CP-MAS) NMR showed that sulphur addition resulted in a 20 fold increase in ^{13}C fixed to the soil. HeteronuclearSingle Quantum Coherence (HSQC) NMR spectra indicated that the ^{13}C had also been incorporated into lipids, proteins and carbohydrates. A decrease in sulphur levels coupled with an increase in sulphate levels suggested that chemoautotrophs were oxidising sulphur to sulphate and using it as an electron donor to extract energy. The increase in sulphate resulted in a drop in soil pH which lead to the mobilisation of other soil nutrients such as zinc, iron, copper and manganese. Molecular biology using quantitative polymerase chain reaction (qPCR), showed that sulphur addition also stimulated RubisCO gene production, which is a critical pathway for the fixation of CO_2 from the atmosphere.

3.1 Introduction

Soil has been described as the most complex biomaterial on Earth(Young and Crawford 2004). Soil organic matter (SOM) is a major source and sink of carbon (C) and is the biggest carbon pool in the earth's terrestrial ecosystem (1500 – 1600 Pg C) which is approximately 3 times the size of the global atomosphericC pool and 4.5 times the size of the biotic pool(Amundson 2001). Soil plays a key role in C cycling in the terrestrial ecosystemand atmospheric CO_2 has the potential to be stabilised through soil carbon storage(King 2011).SOM plays essential roles in agriculture(Stocking 2003), water quality(Lal et al. 2004), immobilization and transport of nutrients and anthropogenic chemicals(Linn et al. 1993), while also concealing opportunities for the discovery of novel compounds for potential use in industry and medicine(Flaig 1997).Despite this critical role and potential, many uncertainties exist regarding carbon dynamics within the SOM pool (e.g. what are the stable and unstable components), and the role of SOM in carbon sequestration(Kleber 2010). The majority of C fixed to SOMfrom CO_2 is through plant-soil interactions but studies have shown thatsoils void of vegetation still possessed the potential for CO_2 sequestration through bacterial autotrophy (Miltner et al. 2004).Studies from within this group have shown that the contribution of microbial biomass to SOM is seriously underestimated(Kelleher and Simpson 2006, Simpson et al. 2007). Traditionally it was thought that humicsubstances were the single largest component of SOM in agricultural soil, but these studies suggest that bacterial and fungal cellular material are a larger contributor to soil biomass. This is a critical observation as unlike humicsubstances, microbial biomass can play a direct role in releasing or trapping greenhouse gases such as CO_2 and methane. If we have underestimated the contribution of microbes to SOM could we be underestimating their contribution to CO_2 sequestration?

The flux of carbon from soils to the atmosphere through microbial respiration has increased with climate-warming and thaw of permafrost, but whether this reflects a net loss of carbon to the atmosphere that could enhance climate-change is still debated(Hutchinson et al. 2007).Carbon is also being lost from temperate soils at a mean rate of 0.6% p/a(Bellamy et al. 2005). Therefore, the further sequestration of atmospheric carbon in soils would be of mutual benefit to the environment and the agricultural sector.

Chemoautotrophy describes metabolic processes that are used to convert CO_2 into organic materials (biomass) by microbes, using energy obtained from the oxidation of reduced organic or inorganic elements in the soil(Chen et al. 2009). Many of these elements are critical to soil fertility and in agricultural practices these nutrients are controlled through addition of fertilizers. Therefore, nutrient levels are important factors when considering C fixation to soil by chemoautotrophs. Investigating the impact of nutrients applied through agricultural fertilizers on soil C fixation may reveal potential land management strategies which could increase the soil C pool or mitigate loss of C from soil. Here we focus on Sulphur (S) as it is frequentlyapplied to soils in the form of sulphate, whichis rapidly available to plants and crops (Janzen and Bettany 1986).Furthermore it has been reported that soil S levels are decreasing due to the controls introduced regarding global emissions of SO_2 into the atmosphere(Boswell and Friesen 1993, McGrath and Zhao 1995). In the previous chapter and subsequently by Hart et al. (2013), it was shown in a 48 hr CO_2 incubation (Chapter 2) that addition of sodium thiosulphate (Na_2SO_4) as an electron donor had a positive effect on CO_2 sequestration.The fate of elemental S in soil is less well known. S is utilised by certain groups of soil chemoautotrophs,one of these groups, *Thiobacillusspp,* are known to couple sulphur oxidation with carbon fixation (Vishniac and Santer 1957, Suzuki 1965). Could S levels be a limiting factor in the ability of this group of organisms to fix C in soil?

Combining ^{13}C isotope studies withcompound specific stable isotope analysis(CSIA) of phospholipid fatty acids (PLFAs) provides taxonomic and quantitative information on the microorganisms utilising a given ^{13}C labelled substrate (Amelung et al. 2008). The specificity of PLFAs as biomarkers for active microbes is improved by stable isotope studies as microbes utilizing the ^{13}C labelled substrate incorporate ^{13}C into their PLFAs (Evershed et al. 2006). Stable isotope analysis combined with PLFA investigationshave been used previously to track the fate of $^{13}CO_2$ in rhizosphere microbial communities(Treonis et al. 2004, Butler et al. 2003). Nuclear Magnetic Resonance (NMR) can also be used to track the fate of the ^{13}C label as it is incorporated into soil organic matter (Kelleher and Simpson 2006, Kelleher, Simpson and Simpson 2006). The *cbbL* gene, which produces the enzyme RubisCO (Ribulose-1,5-bisphosphate carboxylase/oxygenase)is found in most autotrophic organisms and catalyses the first major step in the CO_2 fixation reaction(Tabita et al. 2007).

In this section the methods developed in chapter 2 are applied to studying the sequestration of CO_2 in agricultural soil through soil autotrophy, over twelve weeks of incubation under average climatic conditions in the Environmental Carbon Dioxide Incubation Chamber (ECIC). The impact of elemental S on microbial CO_2 sequestration to agricultural soil is determined using a combined analytical approach of CSIA, NMR, elemental analysis and molecular biology. The hypothesis for this study is that sulphur addition will stimulate chemoautotrophic fixation of C to agricultural soil.

3.2 Methods

3.2.1 Soil Sampling and Incubation

Soil was collected from the top 10 cm of an arable field run by Teagasc (53.433265 N, -6.322975 W)for research purposes on the outskirts of Dublin, Ireland (15th February 2012). The field was used for crop growing and had previously been fertilized using S in March 2011. Soil pH was 7.23±0.121 other soil properties were total carbon content (%TC) = 4.57, total hydrogen (%TN) = 0.65, total nitrogen (%TN) = 0.31 and total phosphorous (%TP) = 0.14. Soil was transported to the laboratory in DCU within 1 hour of collection.1200 g of soil was divided evenly into two sterile Pyrex dishes. Elemental S (Sigma-Aldrich) was treated under UV light overnight to reduce the potential of microbial contamination. Sulphur was added to one of the soil sub samples (hereafter known as Soil A) at a concentration equivalent to 20 Kg/ha, this was in accordance with guidelines for fertilization of grass and cereal crop soils, suggested by the Sulphur Institute(The Sulphur Institute 2014). No amendments were made to the second soil sub sample (hereafter known as Soil B).

3.2.2 Incubation

The soils were incubated in the ECIC for a period of 12 weeks and exposed to $^{13}CO_2$ at a concentration of ~400 ppm for the duration of the incubation. The experiments were conducted in the dark so as to negate interference of CO_2 flux by photoautotrophs and to stop plant growth. The temperature was set at 11°C and was based on the average temperature of Ireland according to MetÉireann. Chamber CO_2 concentrations were measured throughout the course of the incubation by the internal infra-red CO_2 detector. The ECIC was vented every two days and the $^{13}CO_2$ levels were replenished to original concentrations to avoid dilution of the labelled CO_2 with unlabelled CO_2 which may

result from soil respiration within the chamber. Sub samples were taken at regular intervals over the 12 week incubation for chemical and molecular analysis. Control samples for NMR were created in exactly the same way, except that $^{12}CO_2$ was used. The $^{12}CO_2$ incubated NMR spectra generated during the experimentwere subtracted from $^{13}CO_2$ spectra so that only those structural components enriched in ^{13}C appear. For further details on the ECIC see Hart et al (2013a).

3.2.3 pH analysis

Soil pH was measured throughout the incubation using the 1:5 method (soil:DiH_2O) (Rayment and Higginson 1992). Briefly, 1g of soil was weighed into a clean beaker and 5 mL of DiH_2O was added. A Teflon stirring bar and magnetic stirrer was used to homogenise the mixture. The pH of the sample was measured using a Cyberscan PC300 series pH meter (Eutech Instruments, Singapore) which was calibrated using pH 4, pH 7 and pH 10 (± 0.01) buffer solutions (Fischer Scientific, Dublin, Ireland).

3.2.4 PLFA extraction

PLFAs were analysed prior to incubation (T0) at four weeks (T4), eight weeks (T8) and twelve weeks (T12) which was the end point of the incubation. PLFAs were extracted from freeze dried soil using a modified Bligh-Dyer method(Bligh and Dyer 1959). Briefly, methanol, chloroform and phosphate buffer (pH 7.2) were added to the dry soil (6-8 g) in a ratio of 2:1:0.8 (v/v). The samples were placed on a horizontal shaker for 18 hrs before being centrifuged. The supernatant was decanted and the organic and aqueous phases were split by addition of chloroform and phosphate buffer to achieve a final solvent ratio of 1:1:0.9 (MeOH:$CHCl_3$:phosphate buffer). The organic phase was collected and concentrated by rotary evaporation to ~1mL.

The PLFAs were isolated by a solid phase extraction (SPE) method modified from Pinkart et al (2008). An aliquot of the total organic extracts was added to an aminopropyl cartridge (Alltech 500 mg ultra-clean). Neutral Lipids were eluted with 6 mL of chloroform and glycolipids were eluted with 6 mL of acetone. The PLFAs were eluted with 3 mL of 6:1 MeOH:$CHCl_3$and 3 mL of 0.05 M sodium acetate in 6:1 MeOH:$CHCl_3$, both 3 mL aliquots were combined and concentrated under N_2(Pinkart, Devereux and Chapman 1998). The PLFAs were derivatised to fatty acid methyl esters (FAMEs) using sodium methoxide (100 µl,50°C for 10 min). 5α-cholestane was added to the extracts as an internal standard (IS) at a final concentration of 100 ng/µL. The

double bond position in monounsaturated FAMEs was determined by the formation of dimethyl disulphide adducts(Nichols, Guckert and White 1986). For more details on soild phase extraction and derivatisation techniques see sections 2.2.6.

3.2.5 GC-MS analysis

The gas chromatography mass spectroscopy (GC-MS) and isotope ratio mass spectroscopy (IRMS)data for this chapter was generated after a new Agilent GC (Agilent Model 6890N) was installed in the laboratory. The new GC was interfaced with the Quadropole MS and the older GC remained coupled with the continuous flow IRMS via a combustion furnace (GC-C-IRMS). This simplified the capillary system of the GC-C-IRMS, which had been the cause of a great deal of instrument downtime. Although this meant that simultaneous Quadropole MS and IRMS analysis of a sample was no longer possible, instrument sensitivity and performance was greatly improved. There was significantly less downtime leading to higher productivity in the laboratory overall.

PLFAs were identified and quantified using GC-MS. 1 μL of the derivatised extracts was injected with a 2:1 split onto an Agilent 6890N gas chromatograph (GC) interfaced with an Agilent 5975C Quadropole mass spectrometer (MS) equipped with an autosampler. The GC column used was a HP5-MS fused silica capillary column (30 m x 0.25 mm i.d) with a film thickness of 0.25 μm. The carrier gas was ultra high purity helium (BIP-X47S grade, Air Products) at a flow rate of 1 mL/min. The GC programme was as follows: The injector port was set at 250°C, the initial oven temperature was held at 60°C for 1.5 min and increased at 6°C/min to 300°C and held for 20 min. The GCMS interface and ion source were held at 280 °C. All samples were analyzed in triplicate. PLFA FAMEs were quantified from their total ion chromatograms using multiple point internal standard calibration curves of the response factors oftetradecanoic acid methyl ester to the 5α-cholestane internal standard.The acquired data was processed using Chemstation Software. PLFAs reported were confirmed through a combination of spectral libraries (NIST, Wiley), spectra interpretation, target ion extracted chromatograms, retention times and consulting related literature.

3.2.6 Determination of $\delta^{13}C$ values of PLFAs

Measurements of $\delta^{13}C$ (‰) values of PLFA methyl esters were performed using the continuous flow isotope ratio mass spectrometer (Isoprime) coupled via a

combustion furnace to an Agilent 6890N GC with a HP5-MS column. $\delta^{13}C$ is the parts per thousand difference between the ^{13}C content of the sample and the standard. The international reference standard used is Pee Dee Belemite from South Carolina, U.S (PDB Ratio 45/44 = 0.00112372). A sample with a value of -32‰ for example, has a $^{13}C/^{12}C$ ratio that is 32 parts per thousand (or 3.2%) lower than the PDB standard(Amelung et al. 2008).

Samples were injected using the same GC conditions as described above. The GC column effluent was combusted to CO_2 in the combustion furnace which contained a ceramic tube (GC5, 650x0.3 mm i.d) packed with a copper oxide/platinum catalyst heated to 850°C. The instrument was calibrated using pulses of CO_2 reference gas. $\delta^{13}C$ values are reported against a stable carbon isotope reference standard from Indiana University (n-alkane mixture B2). $\delta^{13}C$ values were also corrected for addition of a methyl group during sodium methoxidederivatisation. Samples were analysed in duplicate and average $\delta^{13}C$ values are reported. Through regular stability and linearity studies using the CO_2 reference gas it was established that $\delta^{13}C$ values could only be reliably reported for analytes with a peak height greater than 0.15 nA. This kept the standard deviation very low at ≤ 1.00‰. PLFA methyl ester peaks were identified using the GC-MS and located in the IRMS chromatogram by their retention times and peak profiles.

3.2.7 Pre-treatment of Soils for NMR analysis

Prior to NMR analysis all soil samples had to be treated rigorously with hydrofluoric acid (HF) to remove inorganic compounds such as iron oxide, calcium carbonate and magnetic minerals like clay in the soil. **Iron oxides** have an unpaired electron that shortens the relaxations of neighbouring protons. This can lead to broadening of ^{13}C resonance lines and can lead to spectra which are almost impossible to interpret. Although all interfering minerals may not be removed completely, as few as 4 treatments with 10% HF have been shown to provide well defined spectra (Goncalves et al, 2003).

Here, 30 g (wet weight) soil was freeze dried. The dried soil was placed in 250 mL polypropylene centrifuge bottles, 80 mL of 1:1HCL(1M):HF(10%) was added to the soil. The mixture was shaken gently on a reciprocating shaker at 90 s.p.m (strokes per min) for 48 hrs. *Note:* For the first washing, bottles were left upright with lids slightly loose as effervescent release of CO_2 was occurring. For all other washings

bottles were shaken horizontally with lids tightened. Samples were centrifuged at 6000 rpm and the supernatant was discarded and stored for professional disposal. 80 mL of 10% HF was added to the bottle and placed on the shaker at 110 s.p.m for 24 hrs. The samples were centrifuged and the supernatant was discarded. The washing with 80 mL 10% HF was repeated nine times. To neutralise the samples after HF treatment they were washed with 100 mL DiH_2O, shaken for 6 hrs, centrifuged and decanted, this was repeated until a sample pH of 6 was achieved (12 washings). At this stage a sub sample of 800 mg (wet weight) was taken and freeze dried, this sample was for solid state analysis by cross polarisation magic angle spinning NMR (CP-MAS).

The remaining soil organic component was exhaustively extracted with NaOH (Kelleher and Simpson 2006) for high resolution magic angle spinning NMR analysis (HR-MAS). Briefly, 90 mL of 0.1 M NaOH was added to the soil, and placed on the shaker at 110 s.p.m for 6 hrs. The samples were centrifuged and the NaOH extract was decanted and stored. This was repeated 5 times. , Amberlite cation exchange resin (SIGMA-ALDRICH) was usedto remove the Na^+ ion. Briefly, the combined NaOH extracts for each sample were vacuum filtered. The ion exchange column containing the resin was pre-treated with 150 mL 10% HCL and 450 mL 0.1 M NaOH, this was repeated ten times, never allowing the resin to dry-out once wetted. The sample was then added to the column and collected drop wise, the removal of Na was confirmed by a pH change from basic to acidic. After each sample the resin was regenerated using the pre-treatment procedure. The collected sample was freeze dried and stored at -20ºC for HR-MAS NMR analysis.

3.2.8 NMR analysis

For details on NMR analysis see sections *2.2.10* and *2.2.11* from previous chapter.

3.2.9 Sulphur and Sulphate analysis

S and other soil micronutrients such as zinc (Zn), copper (Cu), iron (Fe) and manganese (Mn) were measured by X-Ray Fluorescence(XRF) analysis. XRF was performed using a non-destructive portable XRF analyser. The performance of this instrument for soil has previously been assessed (Radu and Diamond 2009, Radu et al. 2013). The **standard error** for the portable XRF is ± 10%. Soil was oven-dried at 50°C for 48 h, ground by mortar and pestle and passed through a 0.85 mm mesh sieve, this step ensured a **homogenous sample**. Prior to sample analysis, an internal instrument

calibration was performed. All samples were analyzed in sample holders using the bulk mode for soil sample according to manufacturer specifications. Each sample was analyzed for 60 s per sample and each sample was analyzed in triplicate.

Sulfate was measured using a $BaCl_2$turbidimetric method for soil(Rump 1999). 1 g of soil was extracted with 1% NaCl solution on a horizontal shaker for 2 hrs. Approximately 60 mg activated charcoal was added and the mixture was filtered. 1 mL of the extract was diluted in 9 mL DiH_2O. Excess $BaCl_2$ powder was added, followed by 2-3 drops of glycerol, and the mixture was stirred for exactly 1 min. The absorbance at 420 nm was measured on a Shimadzu UV1800 UV/Vis spectrophotometer after 2 min and 3 min. The maximum absorbance was taken. Sulfate concentration was calculated by regression (R^2 = 0.997) using a range of sulfate standards from 0 to 75 ppm. The method recovery was assessed using a positive control of Na_2SO_4 dissolved in 1% NaCl (180 ppm sulfate ion), and a negative control was used as a method blank.

3.2.10 Statistical Analysis

T-tests were used to confirm the statistical significance of changes in the mean $\delta^{13}C$ values of soil PLFAs over time, using PAST statistical software(Hammer, Harper and Ryan 2001).

3.2.11 Measuring the cbbL gene for RuBisCo

Quantitative PCR (qPCR) was used to quantify the RuBisCOgene *(cbbL)*, which is associated with atmospheric CO_2 uptake through the RubisCO pathway (Tabita 1999; Badger *et al* 2008). This analysis was carried out by colleagues in the School of Biological Sciences, Queens University Belfast. Briefly, DNA was extracted from 0.3g of soil using a PowerSoil DNA extraction kit (Mobio) according to manufacturer guidelines. DNA was stored at -20°C prior to analysis. All standard PCR reactions were carried out in 25 µL volumes using a DNA Engine DYAD Peltier Thermal Cycler (BioRad). In the case of the 16S rRNA gene amplification, PCR primer pair P1/P2 were used targeting the V3 region of the bacterial 16S gene(Muyzer, de Waal and Uitterlinden 1992). RubisCO genes associated with chemoautotrophic CO_2 fixation were targeted using primer pair *cbbL*f/r (Selesi et al. 2007). The abundance of 16S rRNA and *cbbL* genes was quantified by qPCR using primer pair P1/P2 or *cbbL*f/r, respectively. The thermal cycling program consisted of an initial hotstart at 95°C for 10 min, followed by 35 cycles of 95°C for 20 s, 58°C for 20 s and 72°C for 20 s. Plate

reads were taken following each extension step at 72°C. Gel electrophoresis (Biorad) of products was performed to ensure specificity of product and in the construction of standards. Once product identity was confirmed melting curves were performed at the end of each qPCR run to confirm reaction specificity. All qPCR reactions were performed in an Opticon 3 real-time PCR machine (Biorad) using the Maxima SYBR Green I MasterMix (Fermentas). Each 25µL reaction contained 1 µL of template DNA and a final primer concentration of 0.35µM each. Standard curves, for gene quantification, were generated using purified PCR products from *Pseudomonas putida*(Kenneth, Dhanasekaran and Doherty 2010) or environmental amplicons for 16S rRNA and *cbbL* respectively. Briefly, PCR products from *P. Putida* (DSMZ Culture collection number 8368) or environmental samples were excised from an agarose gel, pooled together and purified using an agarose gel PCR purification kit (Fermentas). DNA concentrations were then measured spectrophotometrically at 260nm using a microcell cuvette (Hellma). Copy numbers were calculated according to the size of the amplicon(Whelan, Russell and Whelan 2003). The standard curves were linear over 5 orders of magnitude with an r^2 value >0.95. Amplification efficiency was calculated as ranging between 91-97%. Results are expressed as copy number per microgram of DNA extracted from each soil sample.

3.3 Results and Discussion

3.3.1 PLFAs

PLFAs were used as biomarkers for the living microbial population of the soil. Incorporation of the ^{13}C label into specific PLFAs was used to classify the microbial species involved in CO_2 sequestration. Up to 61 individual PLFAs were identified in the Teagasc soil at T0 by GCMS analysis indicating a diverse microbial community in the sampled soil. This is in stark contrast to the local park soil (from Hampstead park, studied in chapter two), in which only 20 individual PLFAs were identified. The PLFAs identified and quantified from T0 to T12 can be seen in Table 1a in the appendix of this chapter. Of the 61 individual PLFAs identified, 20 gave a quantifiable response on the IRMS and for the purpose of this study, the focus is only on PLFAs with IRMS peaks higher than 0.15nA (Table 1). T-tests were used to determine if changes in PLFA concentration and $\delta^{13}C$ values were statistically significant. The abundances of the PLFAs reported in Table 1 (as measured by GC-MS in µg/g of soil) do not change

significantly from T0 to T12 in either Soil A or Soil B (P>0.05). As PLFAs are capable of reflecting rapid changes in microbial communities (Evershed et al. 2006), this suggests that the microbial community of the soil does not change greatly from T0 to T12. One point of note from PLFAs not reported in Table 1, is the dramatic decrease in the abundance of polyunsaturated fatty acids 20:4ω6, 20:5ω3 and 20:3ω6, which are often used as biomarkers for species of protozoa (Cavigelli, Robertson and Klug. 1995, Mauclaire et al. 2003, Drigo et al. 2010). These PLFAs decrease by a factor of 10 between T0 and T4, this indicates the incubation conditions do not favour these organisms and that PLFAs can reflect rapid changes in microbial population (this can be seen in Table 1ain the appendix). As the incubation was completed in darkness it is also likely that chemoautotrophs will dominate over photoautotrophs.

An initial indication that addition of S to Soil A had influenced the incorporation of the ^{13}C label into the PLFAs of the soil microbial community can be inferred from differences in the IRMS spectra of Soil A and Soil B at week 12 of the incubation (Figure 1).

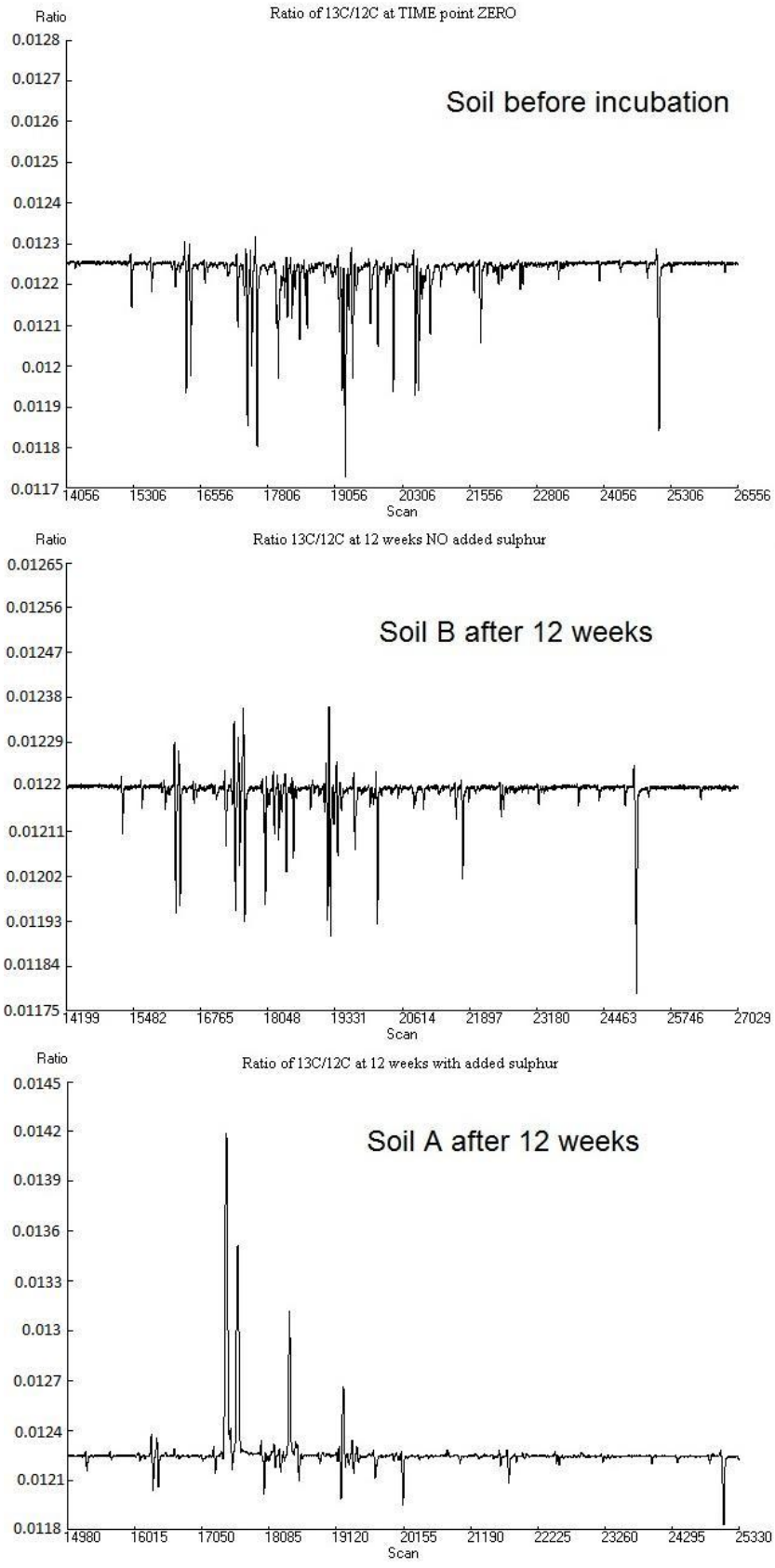

Figure 1: IRMS spectra of PLFA profiles from the $^{13}CO_2$ incubations showing the ratio of mass ion 45 ($^{13}CO_2$) / mass ion 44 ($^{12}CO_2$). Positive peaks indicate ^{13}C enrichment.

Each peak in the IRMS spectra represents an individual PLFA, positive peaks represent compounds enriched in ^{13}C, while negative peaks represent compounds depleted in ^{13}C (natural conditions). The Soil at T0 shows what natural $^{13}C/^{12}C$ ratios look like in an IRMS spectrum of soil, the average natural abundance of ^{13}C isotopes being ~ 1.11% and ^{12}C being ~ 98.89% (Boutton 1996). When compared the T0 IRMS spectrum the $^{13}C/^{12}C$ ratios have changed in both soils over the 12 week incubation. Both the A and B soil have enriched levels of ^{13}C, but the microbial population in Soil A appears to have sequestered more $^{13}CO_2$. The $^{13}C/^{12}C$ ratio values were converted to delta notation ($\delta^{13}C$ ‰), the $\delta^{13}C$ values are reported in table 1. The average $\delta^{13}C$ values of total soil PLFAs are plotted in Figure 2. The higher $\delta^{13}C$ values obtained for PLFAs at weeks 4,8 and 12 confirms incorporation of $^{13}CO_2$ into both Soil A and B. However, Soil A has significantly higher ($P<0.001$) $\delta^{13}C$ values at T4, T8 and T12 compared to the soil B, indicating much higher levels of CO_2 sequestration by soil microbes in Soil A. The average $\delta^{13}C$ values of Soil B increased significantly from -31.74‰ to -21.58‰ between T0 and T4 ($P<0.05$) but then appear to stabilise and do not increase or decrease significantly ($P>0.05$) between T4, T8 or T12. The average $\delta^{13}C$ values of Soil A also increased ($P<0.001$) between T0 and T4.However, in contrast to Soil B, Soil A's $\delta^{13}C$ values do not stagnate and $^{13}CO_2$ incorporation continues to increase ($P<0.05$) from 1.35‰ (T4) to 11.42‰ (T8) and a final value of 29.83‰ (T12). In comparison the B soil has a much lower $\delta^{13}C$ value of -22.43‰ at the end point (T12). The $\delta^{13}C$ values of the PLFAs in the A soil were significantly higher than the B soil by the end of the 12 week incubation($P<0.001$). The addition of sulphur has resulted in increased CO_2 sequestration in Soil A.

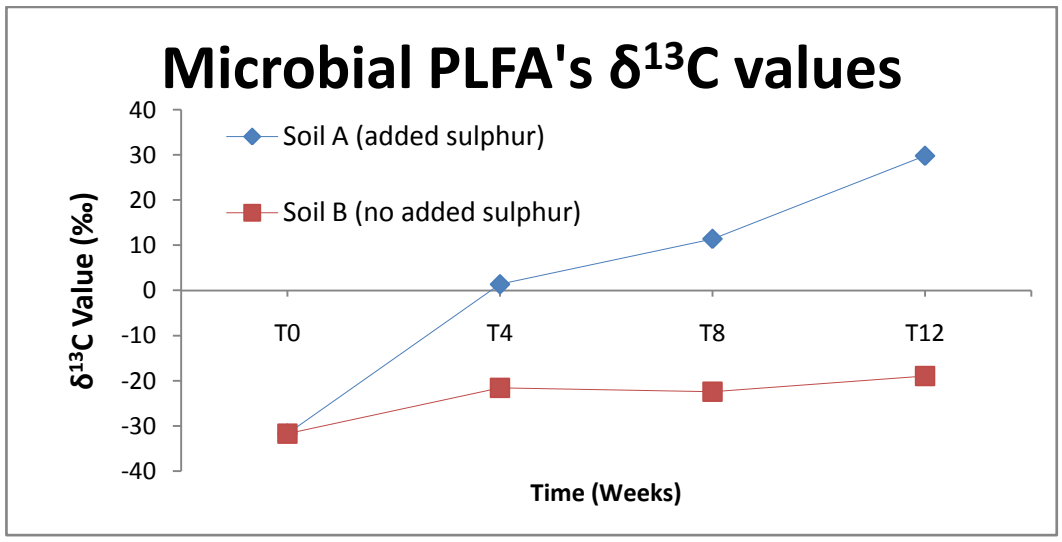

Figure 2: Average $\delta^{13}C$ values (‰) of soil microbial PLFAs (average StDev ≤1.00‰)

Table 1: Major PLFA concentrations (µg/g) and δ13C values in Soil A and Soil B before incubation (T0), weeks 4, 8 and 12.

PLFAs	**Concentration (µg/g soil)**							**δ^{13}C values (‰)**						
		Soil A			Soil B				Soil A			Soil B		
	T0	T4	T8	T12	T4	T8	T12	T0	T4	T8	T12	T4	T8	T12
*i*14:O	0.236	0.279	0.267	0.201	0.251	0.236	0.190	-34.79	-16.60	-30.11	-24.91	-33.01	-33.35	-33.19
*i*15:O	0.784	1.005	0.941	0.735	0.928	0.815	0.729	-32.76	-7.25	-9.51	-0.74	-18.12	-22.82	-18.17
*a*15:O	0.619	0.776	0.721	0.585	0.730	0.622	0.580	-31.80	-11.32	-18.62	-3.40	-24.61	-25.47	-23.27
*i*16:O	0.318	0.351	0.337	0.274	0.322	0.296	0.255	-32.43	-13.56	-19.04	-14.37	-25.89	-26.77	-23.68
16:1ω9	0.201	0.225	0.229	0.189	0.241	0.198	0.195	-22.99	-2.54	-3.92	-11.33	-6.24	-4.06	0.55
16:1ω7	0.968	1.301	1.345	1.233	0.987	0.773	0.846	-34.54	98.26	181.27	285.99	-18.22	-20.46	-8.89
16:1ω5	0.541	0.617	0.560	0.499	0.578	0.491	0.485	-31.81	-0.24	4.81	21.71	-12.23	-10.91	-4.90
16:O	1.276	1.388	1.402	1.223	1.126	1.039	1.017	-32.29	45.59	93.08	184.49	-6.93	-13.10	-5.80
*i*17:1ω7	0.266	0.377	0.361	0.268	0.396	0.310	0.226	-15.06	-5.12	-0.41	16.06	-11.46	-11.02	-18.14
*i*17:O	0.244	0.259	0.238	0.203	0.245	0.208	0.198	-32.51	-7.63	-9.09	-2.41	-19.12	-23.26	-18.20
*a*17:O	0.250	0.262	0.236	0.216	0.249	0.216	0.198	-30.13	-12.54	-22.63	-26.51	-24.52	-25.54	-22.98
cy17:O	0.372	0.489	0.511	0.480	0.438	0.355	0.365	-34.37	22.78	140.45	188.50	-30.89	-29.75	-24.15
br17:O	0.252	0.347	0.338	0.256	0.299	0.263	0.244	-39.66	-14.45	-21.48	-13.68	-33.40	-33.01	-31.19
18:2ω6,9	0.348	0.130	0.155	0.107	0.106	0.085	0.075	-25.42	-11.28	2.67	<LOD	-19.71	-19.04	<LOD
18:1ω9	0.954	0.956	0.901	0.795	0.852	0.738	0.760	-29.81	-10.63	-16.23	-14.33	-23.60	-22.51	-22.72
18:1ω7	1.613	1.598	1.503	1.507	1.341	1.273	1.328	-36.29	11.87	30.54	44.74	-11.79	-13.39	-5.96
18:1ω5	0.194	0.148	0.135	0.125	0.133	0.130	0.116	-33.92	-12.57	-20.96	-17.12	-28.67	-28.26	-25.39
18:O	0.644	0.402	0.363	0.337	0.359	0.387	0.332	-30.79	-5.36	-6.43	3.55	-18.27	-21.85	-16.45
br18:O	0.287	0.370	0.356	0.287	0.319	0.314	0.254	-31.30	-14.81	-23.29	-20.21	-28.68	-27.54	-25.91
cy19:O	0.644	0.679	0.623	0.557	0.630	0.600	0.575	-35.78	-18.16	-31.55	-29.25	-34.45	-33.09	-31.55
Average	0.551	0.598	0.576	0.504	0.526	0.467	0.448	-31.74	1.35	11.42	29.83	-21.58	-22.43	-18.95

Reliable $\delta^{13}C$ values (peak heights >0.15nA) were measured for the 20 PLFAsreported in Table 1. The majority of the ^{13}C label was incorporated into gram negative bacterial PLFAs, (16:1ω7, cy17:0 and 18:1ω7)(Zelles et al. 1995). They had an average $\delta^{13}C$ value of 173.07‰ (+/- 0.33‰) in Soil A after 12 weeks, compared to -8.69‰ in the B soil indicating that sulphur addition has resulted in significantly increased ^{13}C incorporation to these fatty acids. Two of these gram-negative biomarkers; 16:1ω7 and 18:ω7 have been reported as key fatty acids in *Pseudomonas sp*. PS+ (Mauclaire et al. 2003). Molecular markers,18:2ω6 and 18:1ω9are commonly used as indicators of fungal presence (Frostegård and Bååth 1996). The fact that they have incorporated ^{13}C into their fatty acids might indicate that they also are capturing atmospheric CO_2 but we cannot at this stage rule out cross feeding as fungi complete their role as decomposers. The FA 16:1ω5 is a signature biomarker for arbuscularmycorrhizal fungi (AMF)(Olsson et al. 2005), it is highly enriched with a $\delta^{13}C$ value of 21.71‰ (Soil A) and -4.90‰ (Soil B) at T12. AMF have been shown to be a major functional group in the sequestration of plant derived C to rhizosphere microorganisms (Drigo et al. 2010). Although plant derived carbon is not a factor in this study, AMF appear to be incorporating ^{13}C into their PLFAs through a different pathway.

Iso and anteiso branched C15,C16 and C17 fatty acids are used as biomarkers for gram positive bacteria (Zelles. 1999).Incorporation of ^{13}C from $^{13}CO_2$ into these fatty acids was much lower than gram negative and fungal fatty acids, Saturated PLFAs 16:0 and 18:0 also show high levels of enrichment, especially in Soil A with $\delta^{13}C$ values of 184.49‰ and 3.55‰ respectively. Although these biomarkers are non-specific, 16:0 is an important intermediate in the cellular biosynthesis of fatty acids (Treonis et al. 2004). Iso17:1ω7 has a final $\delta^{13}C$ value of 16.06‰ and has been reported as a biomarker for sulphate reducing bacteria. These biomarker results are similar to the PLFA biomarkers identified in the 48 hrs $^{13}CO_2$ incubation presented in Chapter 2, in which gram negative microbes and fungi were also dominant in ^{13}C incorporation. The FA's 16:1ω7 and 18:1ω7 were also heavily ^{13}C enriched in experiments.

3.3.2 NMR

The CP-MAS-^{13}C NMR difference spectra seen in Figure 3 were obtained by subtracting the NMR spectrum of the $^{12}CO_2$ control incubation from the spectra of Soil A and Soil B. Only components enriched in ^{13}C appear in the CP-MAS-^{13}C difference

spectra, in other words only carbon incorporated through sequestration of the $^{13}CO_2$ is seen.

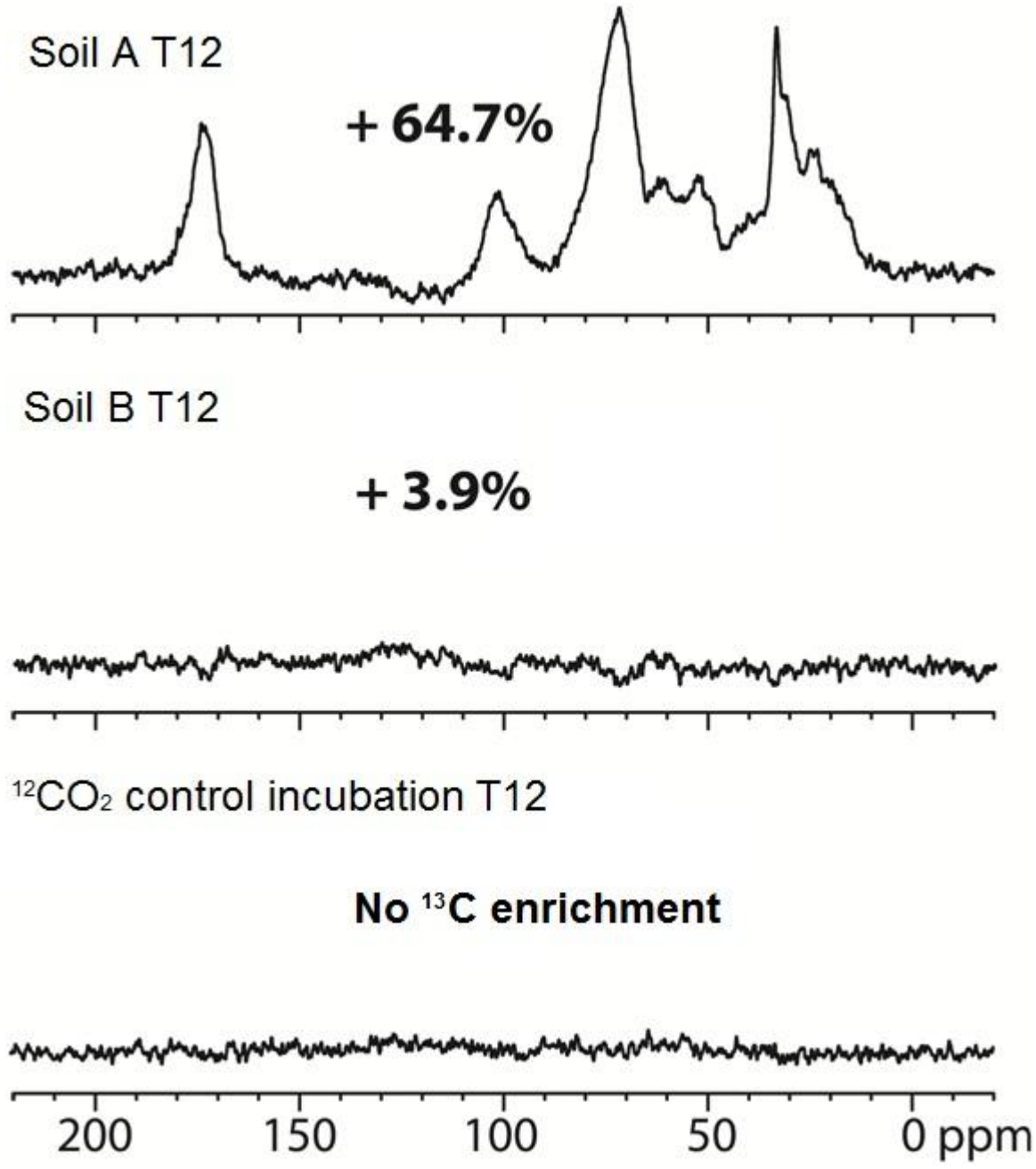

Figure 3: CP-MAS-^{13}C NMR difference spectra showing the total carbon increase due to ^{13}C labeling. The spectrum from the $^{12}CO_2$ control incubation shows no ^{13}C enrichment, Soil B (no added sulphur) shows an increase in ^{13}C of 3.9%, Soil A (with added sulphur) shows an increase in ^{13}C of 64.7%.

A quantitative comparison of the CP-MAS ^{13}C spectrum before and after labeling indicated that the total carbon signal in Soil B increased by ~3.9%. When the natural abundance of ^{13}C and isotopic enrichment of the labeling gas are considered, 3.9%

relates to ~0.04% (or ~ 1 in every 2500 carbons becoming labelled) of the total soil carbon being labeled in the 12 week period. After the addition of sulphur the total carbon signal in Soil A increased by ~64.7%. This corresponds to ~0.7 % (or ~ 1 in every 150 carbons being labelled) of the total soil carbon being labeled in the 12 week period. In simple terms the addition of sulphur to Soil A gave rise to a 20 fold increase in the sequestration of carbon from the atmosphere.

NMR can be used to identify how the label is incorporated into the different chemical categories in the soil organic matter. Due to the relatively broad line-shape of the solids NMR spectra, identification is best done using HR-MAS NMR which can observe H-C units in liquid, gel, and swollen-solid phases, but not true "glassy" solid domains(Kelleher and Simpson 2006, Kelleher, Simpson and Simpson 2006). ^{1}H proton spectrum achieved using the HR-MAS NMR analysis is overlayed with the CP-MAS ^{13}C NMR results seen in Figure 3to create a 2DHeteronuclear Single Quantum Coherence (HSQC) difference spectrum for Soil A (Figure 4). Multidimensional NMR of soil organic matter including detailed assignments of the microbial fractions has been considered in detail in previous publications(Kelleher and Simpson 2006, Simpson et al. 2007, Simpson et al. 2002, Simpson 2006, Simpson et al. 2006, Simpson et al. 2007, Simpson, McNally and Simpson 2011). The only signals present in the difference spectrum are from chemical categories that increased due to the addition of sulphur. It is clear that the spectra are dominated by lipids, carbohydrates and protein/peptides arising from microbial biomass. Comparison of relative integrals before and after labeling indicates that the intensity of the various sub components increased in the order lipids (80 % signal increase), carbohydrate (144% signal increase), protein (121% increase) and lignin (no change, see red circle on Figure 4). When the isotopic enrichment of the label is accounted for this corresponds to ~0.88% of the lipids, 1.6 % of the carbohydrates, 1.33% of the protein the semi solid/gel/liquid phase are labeled over the 12 week period in the HR-MAS observable swellable fraction.

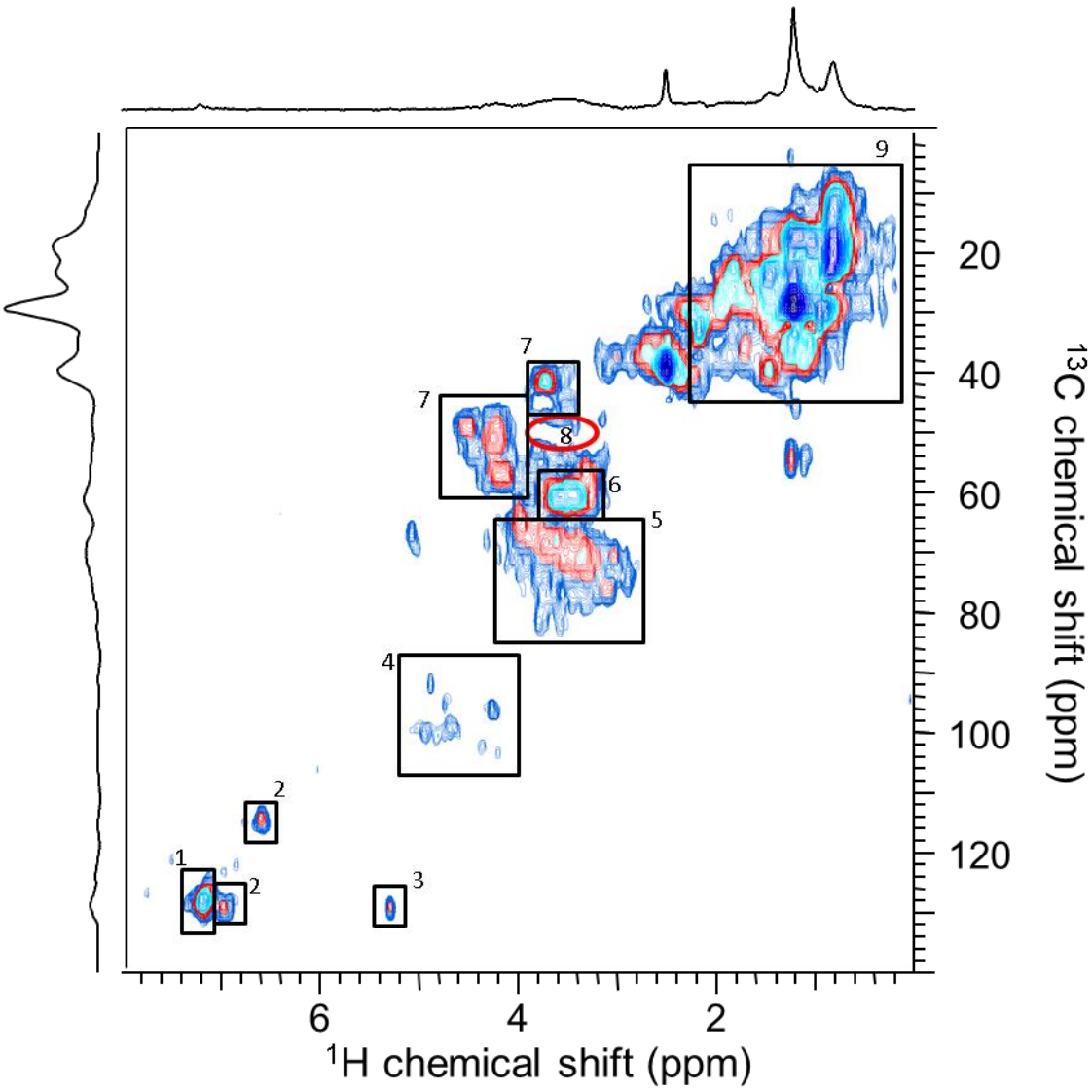

Figure 4 ^{1}H-^{13}CHSQC difference spectrum showing only the components resulting from addition of sulphur in the DMSO swellable fraction. Assignments 1) phenylalanine (from peptide/protein); 2) tyrosine (from peptide/protein); 3) unsaturations (HC=CH) in lipids, 4) anomeric protons (carbohydrates); 5) other CH in carbohydrates; 6) CH_2 in carbohydrates; 7) α-protons in peptides and proteins; 8) methoxyl in lignin; 9) aliphatic linkages including signals from various lipids, and side chain protons in peptides.

The assignments are identified from spectral libraries which have been built up overtime by analyzing varying standards and recording their NMR signals. These results are complementary to the NMR results presented in Chapter 1 and subsequently by Hart et al (2013) in which incorporation of ^{13}C into soil biomass over a 24hr period was traced using the same methods. Over 24hr the majority of the carbon was taken up and stored in the form of lipids as an energy store. From this present study which takes place over a longer period 12 weeks, it is clear the sequestered carbon is transferred to

other microbial components (carbohydrates and proteins) and indeed the microbes are able to grow on carbon sequestered from the atmosphere.

3.3.3 Soil pH, sulphate, sulphur and nutrient levels

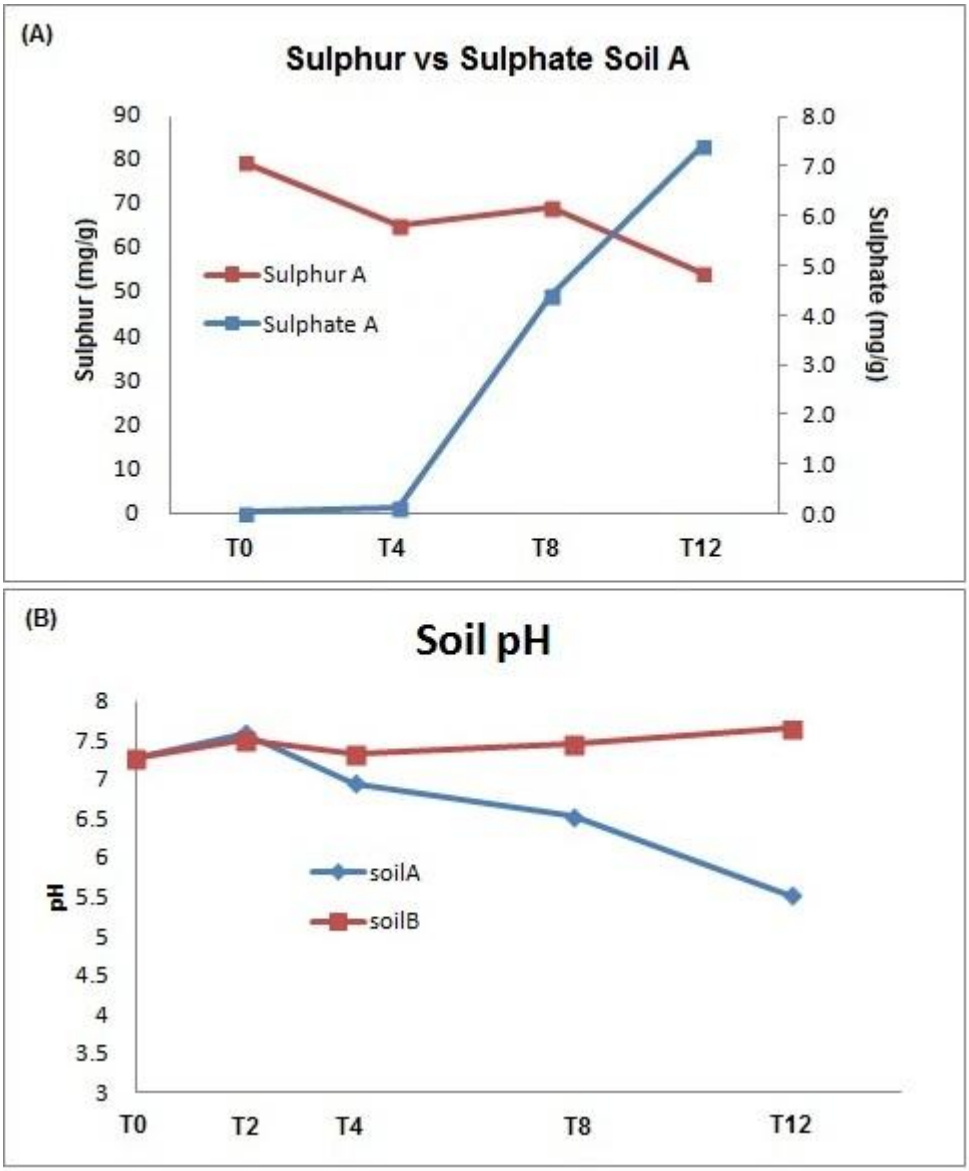

Figure 5: (A) Changes in sulphur (S) (mg/g) and sulphate (SO_4^{2-})(mg/g) in Soil A during the 12 week incubation. (B) Changes in soil pH in Soil A and Soil B during the 12 week incubation.

The pH profile of Soil A dropped by 1.8 units from a pH of 7.3 to a pH of 5.5 from T0 to T12. In contrast, there was no notable change in the soil pH of Soil B (Figure 5). The drop in the pH of Soil Ais potentially as a result of the oxidation of the added sulphur to sulphate (SO_4^{2-})(Yang et al. 2010). The proposed oxidation of sulphur to sulphate is supported by the results shown in Figure 5, where XRF analysis indicates

that over the duration of the experiment the amount of elemental sulphur decreases in soil A, particularly, between weeks 8 to 12 (Note: Sulphur levels in Soil B, to which no sulphur was added were <LOD (<0.1mg/g) of XRF analysis). Further to this, there is a sharp increase in sulphate concentration which is inversely correlated to the drop in sulphur. Sulphate concentration increases from <0.1mg/g at T0 to 7.4 mg/g (Figure 5) soil after 12 weeks of incubation in the ECIC. This data supports the conclusion that elemental sulphur added to Soil Ais being oxidised through to sulphate.This may be the result of a range of microorganisms including well documented bacterial sulphur oxidizers such as Thiobacillusspp(Suzuki 1965, Suzuki, Chan and Takeuchi 1992), as well as fungi (Grayston, Nevell and Wainwright 1986, Grayston 1988, Germida and Janzen 1993) and archaeal groups(Kletzin 1993, Kletzin et al. 2004).

Zn, Cu, Fe and Mn are micronutrients essential for plant growth and development (He *et al* 2005). The drop in pH in Soil A over the 12 week experiment appears to increase theavailability of these micronutrients (Figure 6).This is likely due to a phenomenon known as “micronutrient unlocking” as described by Modaihsh et al (1989) where soil micronutrients become more soluble at lower pH’s, thus their bioavailability increases (Modaihsh, Al-Mustafa and Metwally 1989). **Fe** and **Mn** do not appear to be as strongly coupled to pH as as Zn and Cu are. Fe and Mn are readily oxidised in soil to form oxides, this may affect results of XRF analysis. The abundance and availability of micronutrients in an agricultural soil is critical. However, by changing the pH of soil the application of sulphur may result in more compl exchanges – both beneficial and harmful.

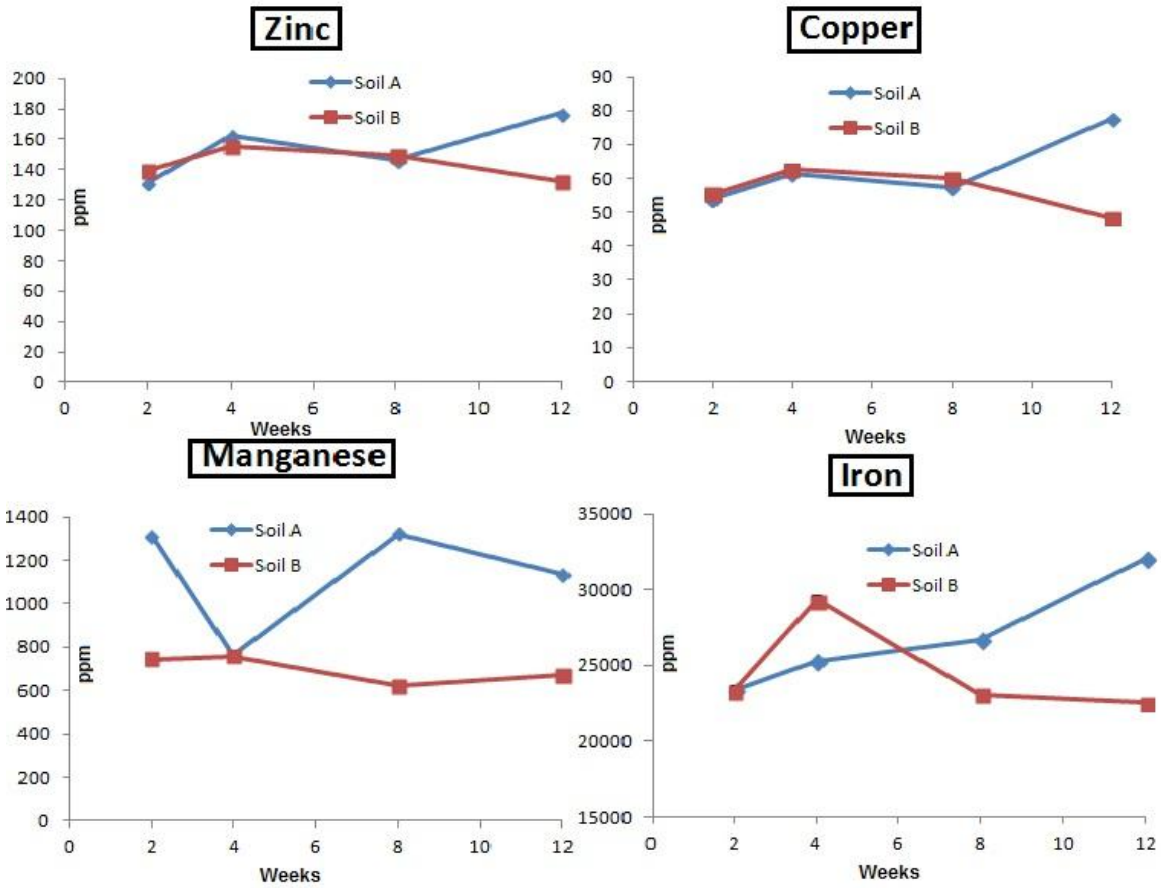

Figure 6: Soil A and Soil B micronutrient concentrations (ppm) over the 12 weeks incubation in the ECIC, measured by XRF.

3.3.4 RubisCO

qPCR was used to quantify *cbbL*which is the gene used to produce the enzyme RubisCO (Figure 7). The presence of the *cbbL* gene in soil indicates that the microbial community present has the potential to sequester CO_2through the RubisCO pathway (Tabita et al. 2007, Badger and Bek 2008). Prior to incubation, *cbbL* was present in the Teagasc soil at a count of 6.2 X $10^3 ng^{-1}$ DNA. Variations in the abundance of the Rubisco gene copies (*cbbL*) were measured in Soil A and Soil B during the 12 week incubation (Figure 7). After two weeks of incubation there was a minor increase in*cbbL*in Soil A (6.4 X 10^3 ng^{-1} DNA), in contrast there was a more notable increase in *cbbL* copies in Soil B(7.9 X 10^3 ng^{-1} DNA). This suggests that the addition of elemental sulphur to Soil A has initially inhibited the microbial populations sequestration of inorganic C. While, the microbial population capable of utilising inorganic C from CO_2, via the RubisCOpathway, have increased in Soil B in response to incubation conditions. After week 2 however, *cbbL* copies stagnate in Soil B but increase rapidly in Soil A reaching 6.4 X 10^3 ng^{-1} DNA by week 8. This data suggests the microbial population

that are sequestering CO_2 through the RubisCO pathway have increased considerably in Soil A. This increase coincides with a significant increase in ^{13}C incorporated into the PLFAs as seen in Figure 2. These result provide strong evidence that the RubisCO pathway is central to the autotrophic fixation of inorganic C from CO_2in Soil A.

At week 12 there is a dramatic drop in *cbbL* gene copies in both Soil A and Soil B. The level falls lower than that measured at T0 suggesting that the microbial population using the RubisCO pathway has collapsed. However, this does not suggest that CO_2 sequestration has stopped as there are many other pathways for CO_2 fixation. ^{13}C incorporation into PLFAs (Figure 2) continues to increase significantly in Soil A up until the end of the incubation (T12), the sequestration of CO_2 continues, possibly through other pathways.

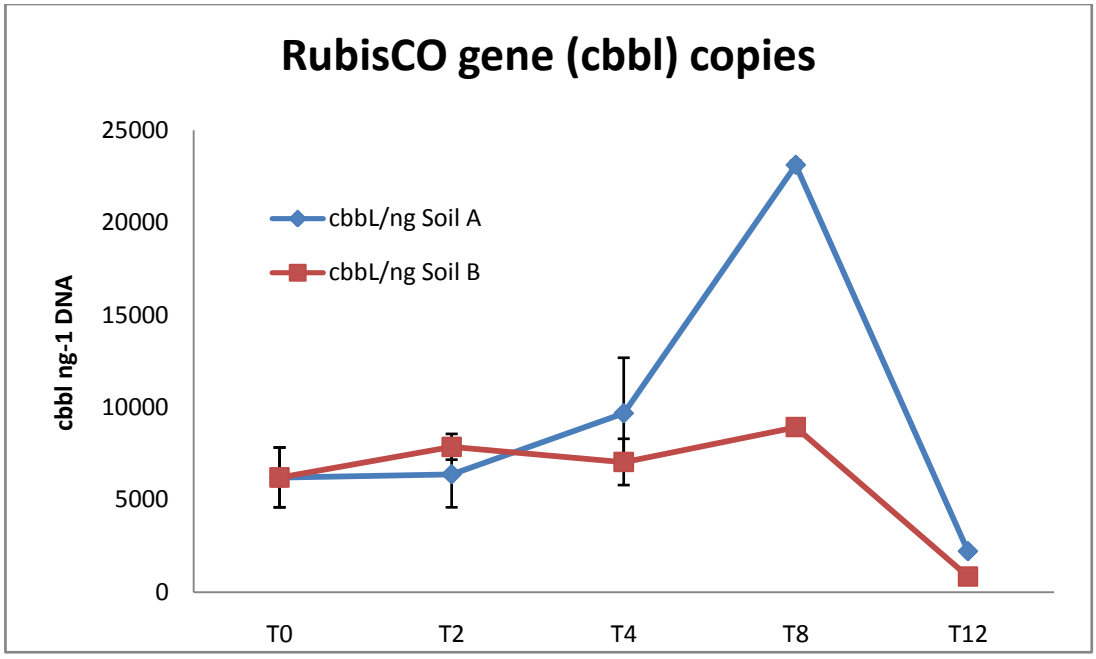

Figure 7: RubisCO gene (*cbbL*) abundance per ng^{-1} DNA extracted was quantified by qPCR at regular intervals over the 12 week incubation period in both Soil A and B. Each data point represents mean abundance of *cbbL* at a given time point. Error bars represent standard deviation. Gene quantification was carried out using triplicate DNA sample.

3.4 Conclusion

To investigate how the addition of elemental sulphur (S^o) effects an agricultural soil, we incubated both an unaltered and a S^o amended arable soil in custom-built, air-tight environmental chambers for 12 weeks The incubations were carried out in both $^{12}CO_2$ and $^{13}CO_2$ atmospheres under close to natural CO_2 concentrations (400ppm). The $^{13}CO_2$ experiments facilitated the combination of NMR and phospholipid fatty acid (PLFA) analysis with compound specific stable isotope mass spectrometry. These techniques can independently make use of the stable isotopic enrichment of soil chemoautotrophs to provide independent evidence of the effect of sulphur addition. RubisCO gene copy (*cbbL*) numbers were quantified and chemical conditions such as pH and metal concentrations were monitored to assess CO_2 uptake pathways and effects of sulphur addition on soil chemistry.

PLFA analysis of the soil chosen for this study revealed a rich diverse microbial community with up to 61 individual PLFAs identified. The significant increase in the $\delta^{13}C$ values of certain PLFAs confirmed that $^{13}CO_2$ was being sequestered by the soil microbial population. ^{13}C IRMS confirmed that $^{13}CO_2$ was sequestered in soils with and without sulphur. However, significantly more ^{13}C was incorporated by the microbial community of the S-amended soil. The addition of sulphur stimulated chemoautotrophy and increased C fixation. The PLFAs enriched with ^{13}C indicate gram negative bacteria and fungi including AMF were the dominant ^{13}C incorporators. This is complementary to the biomarkers identified in the 48 hr CO_2 incubation presented in Chapter 2. The ^{13}C enrichment of PLFAs associated with fungi is interesting as, to the authors knowledge they have not been associated with autotrophy. However, we cannot discount the fact that it may be due to the crossfeeding of fungi on living and dead biomass that includes chemotrophs that have consumed ^{13}C.

Using CP-MAS NMR it was found that sulphur addition to the soil increased ^{13}C fixation from $^{13}CO_2$ 20 fold, with 1 in every 150 carbons becoming labelled, compared to 1 in every 2500 in soil without sulphur addition. The HSQC NMR spectrum was used to identify where the ^{13}C had been incorporated. In contrast to the 48 hr CO_2 incubation presented in Chapter 2, where ^{13}C was mainly incorporated into lipids, results from this 12 week experiment revealed that the ^{13}C signal of carbohydrates and proteins increased by 144% and 121%, respectively. The use of NMR in this way is a powerful ability

which effectively allows us, for the first time, to "see" the biomass produced by microbes as a direct result of CO_2 uptake.

Results of elemental sulphur and sulphate analysis over the course of the 12 week incubation showed that sulphur decreased and sulphate increased, confirming that sulphur was oxidised by microbial populations in the soil to sulphate. This is important as it also coincides with increases in the RuBisCo gene concentration and $^{13}CO_2$ uptake as seen in the PLFA analysis. The oxidation of sulphur to sulphate provides the electron donor for chemosynthetic extraction of energy from chemical bonds and when this happens we see the simultaneous uptake of CO_2 from the atmosphere.

Current methods for capturing CO_2 are either relatively costly or use so much energy that the mitigation benefits become marginal. The process of indirect CO_2 capture by planting forests is unlikely to provide an adequate solution on its own(Hutchinson, et. al. 2007). The sequestration of CO_2 in soil represents a potential solution to rising atmospheric carbon concentrations and decreasing carbon in agricultural soil. Ultimately, if we can fully identify microbial species that efficiently assimilate atmospheric carbon and predict the fate of this carbon under different environmental regimes, measures can be taken to optimise their function in agricultural land – improving agricultural activity while potentially reducing atmospheric CO_2 at the same time.

3.5 Future Work

The next step is to apply this approach to diverse unaltered and amended soils over longer time periods – to ultimately produce a very detailed analysis of the chemoautotrophic 'potential' in agricultural soils. Thus, the broad research aim of future work is to perform a comparative study to fully evaluate the impact of chemoautotrophy for soil management with both different soils found in the Irish agricultural sector AND different inorganic and organic electron donors. We will investigate different soil types (e.g. shallow brown, and podzolic) and assess the influence on CO_2 uptake ofvarying environmental regimes such as the addition of fertilisers or other nutrients, temperature change, moisture content, presence of naturalelectron donors and CO_2 concentration on both quantitative parameters (i.e. carbon flux), and qualitative parameters (i.e. identification of microbial populations involved and associated changes).

3.6 Appendix B Table 1a PLFAs identified and quantified by GCMS (μg/g) in Soil A and Soil B

PLFA		A soil (added sulphur)			B soil (no added sulphur)		
methyl esters	T0	T4	T8	T12	T4	T8	T12
12:O	0.012	0.012	0.013	0.007	0.012	0.012	0.007
i13:O	0.034	0.016	0.017	0.009	0.015	0.015	0.010
a13:O	0.018	0.004	0.005	0.003	0.004	0.005	0.003
13:O	0.007	0.007	0.008	0.005	0.006	0.006	0.004
i14:O	0.236	0.279	0.267	0.201	0.251	0.236	0.190
14:O	0.143	0.146	0.139	0.117	0.122	0.120	0.100
br14:1ω*	0.023	0.032	0.035	0.021	0.038	0.026	0.022
i15:1ω11	0.108	0.121	0.109	0.090	0.097	0.088	0.088
Me14:1	0.039	0.047	0.042	0.033	0.043	0.038	0.033
i15:O	0.784	1.005	0.941	0.735	0.928	0.815	0.729
a15:O	0.619	0.776	0.721	0.585	0.730	0.622	0.580
15:O	0.081	0.087	0.089	0.071	0.077	0.072	0.063
br15:1ω*	0.021	0.025	0.019	0.021	0.026	0.024	0.020
i16:1ω11	0.069	0.071	0.072	0.061	0.075	0.061	0.057
br15:1	0.020	0.025	0.027	0.021	0.031	0.023	0.017
i16:O	0.318	0.351	0.337	0.274	0.322	0.296	0.255
16:1ω9	0.201	0.225	0.229	0.189	0.241	0.198	0.195
16:1ω7	0.968	1.301	1.345	1.233	0.987	0.773	0.846
16:1ω5	0.541	0.617	0.560	0.499	0.578	0.491	0.485
16:O	1.276	1.388	1.402	1.223	1.126	1.039	1.017
br16:O	0.050	0.068	0.065	0.047	0.069	0.056	0.050
i17:1ω7	0.266	0.377	0.361	0.268	0.396	0.310	0.226
10Me16:O	0.538	0.746	0.738	0.529	0.720	0.604	0.515
7Me16:1ω12	0.138	0.018	0.016	0.011	0.010	0.019	0.011
i17:O	0.244	0.259	0.238	0.203	0.245	0.208	0.198
a17:O	0.250	0.262	0.236	0.216	0.249	0.216	0.198
17:1ω8	0.070	0.097	0.084	0.077	0.085	0.072	0.066
cy17:O	0.372	0.489	0.511	0.480	0.438	0.355	0.365
17:O	0.148	0.060	0.064	0.054	0.066	0.051	0.040
br17:O	0.252	0.347	0.338	0.256	0.299	0.263	0.244
7Me17:1ω11	0.120	0.048	0.055	0.041	0.050	0.057	0.038
i18:O	0.030	0.021	0.020	0.016	0.031	0.031	0.014
18:2ω6,9	0.348	0.130	0.155	0.107	0.106	0.085	0.075
18:1ω9	0.954	0.956	0.901	0.795	0.852	0.738	0.760
18:1ω7	1.613	1.598	1.503	1.507	1.341	1.273	1.328
18:1ω5	0.194	0.148	0.135	0.125	0.133	0.130	0.116
18:O	0.644	0.402	0.363	0.337	0.359	0.387	0.332
br18:O	0.287	0.370	0.356	0.287	0.319	0.314	0.254
br18:O	0.085	0.072	0.073	0.065	0.065	0.067	0.045
7Me18:1ω10	0.468	0.041	0.036	0.033	0.043	0.026	0.027
i19:O	0.028	0.017	0.021	0.011	0.027	0.026	0.008
19:1ω*	0.114	0.099	0.084	0.069	0.080	0.080	0.067
cy19:O	0.644	0.679	0.623	0.557	0.630	0.600	0.575
19:O	0.032	0.027	0.029	0.021	0.035	0.030	0.019
20:4ω6	0.873	0.077	0.062	0.064	0.076	0.059	0.043
20:5ω3	0.798	0.063	0.044	0.045	0.045	0.027	0.028
20:3ω7	0.131	0.014	0.012	0.007	0.012	0.005	0.005
20:1ω13	0.458	0.143	0.089	0.094	0.115	0.082	0.089
4,8,12Me18:O	0.074	0.086	0.080	0.063	0.081	0.070	0.068
20:O	0.104	0.124	0.115	0.103	0.104	0.092	0.097
br20:O	0.060	0.060	0.042	0.047	0.058	0.040	0.047
21:O	0.030	0.031	0.031	0.026	0.031	0.024	0.026
22:5ω6	0.080	0.023	0.031	0.029	0.025	0.021	0.006
22:6ω3	0.082	0.020	0.014	0.005	0.022	0.010	0.022
22:4ω6	0.077	0.010	0.003	0.003	0.008	0.001	0.008
22:1ω13	0.032	0.017	0.017	0.011	0.014	0.009	0.011
22:O	0.100	0.116	0.104	0.079	0.087	0.078	0.073
23:O	0.024	0.028	0.023	0.017	0.022	0.015	0.015
24:O	0.084	0.101	0.092	0.065	0.079	0.067	0.064
25:O	0.009	0.011	0.010	0.008	0.011	0.008	0.007
26:O	0.032	0.039	0.041	0.026	0.033	0.030	0.022

3.7 References

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Chapter 4

The occurrence of PAHs and Faecal Sterols in Dublin Bay and their influence on sedimentary microbial communities.

Contributors

Dr. Shane O'Reilly (DCU) – generated the Ocean Data view maps and advised on statistical analysis

Barry Reid (DCU) – assisted with solvent extractions and SPE

Xavier Monteys (Geological Survey of Ireland, Beggars Bush, Haddington Rd, Dublin 4) – provided particle size analysis data.

Bulk physical and chemical analysis was performed by the microanalytical laboratory, University College Dublin.

4.0 Abstract

The interaction of marine pollutants can alter the chemistry and microbiology of coastal environments. To assess organic pollution and its effect on microbial communities, sediment samples were collected from 30 stations in Dublin Bay board the R.V Celtic Voyager (CV10_28). Faecal sterols, polyaromatic hydrocarbons (PAHs) and microbial lipids were extracted from sediments using a classic lipid extraction method and analysed by GC-MS. The procedure allowed for the simultaneous evaluation of faecal sterols, PAH's and their influence on microbial communities. Faecal sterols are used as indicators for sewage contamination in the Bay. Microbial lipids, specifically phospholipid fatty acids (PLFAs), were used as taxonomic indicators for the division and structure of microbial populations in Dublin Bay. The impact of particle size, %TOC, %H and %N were also assessed. Faecal sterols were found to be highest around Howth Head and Dun Laoghaire and this is consistent with historical sewage discharge from the Dublin region. PAH distribution is more strongly associated with areas of deposition with high silt and clay content, suggesting diffuse sources to the Bay such as rainwater run-off and atmospheric deposition. The PAH's ranged from 12 to 3072 ng/g, with 11 stations exceeding the suggested effect range low (ERL) for PAH's in marine sediment's. PAH isomer pair ratios and sterol ratios were also used to determine sources and extent of pollution. PLFA profiles confirmed that sedimentary microbial communities varied from station to station. PLFA's were not impacted by sediment type or depth but were strongly correlated to PAH and faecal sterol levels. Certain biomarkers such as 10Me16:0, i17:0 and a17:0 were more closely associated with PAH polluted sediments, while 16:1ω9, 16:1ω7c, Cy17:0, 18:1ω6, i16:0 and 15:0 all have strong positive correlations with faecal sterols.

4.1 Introduction

Ocean acidification, fossil fuel combustion, waste generation and industrial activity have adversely affected coastal environments for decades. Our understanding of the interactive effects of these pressures on microbes is still in its infancy but it is vital that we know if they combine to alter the microbial detoxification of anthropogenic pollutants (Francisco J. R. C. and Coelho, Ana L. Santos, Joana Coimbra, Adelaide Almeida, ^Angela Cunha, Daniel F. R. Cleary, Ricardo Calado & Newton C. M. Gomes 2013). To begin with, it is important to understand how individual and combined pollutants can affect the structures of microbial communities on the sea-floor.

Hydrocarbon pollution can be from both natural seepage (~47%) and anthropogenic sources (~53%) (Kvenvolden and Cooper 2003). Polycyclic aromatic hydrocarbons (PAH) are a group of compounds most commonly associated with anthropogenic hydrocarbon pollution and are persistent organic pollutants (POPs) widely found in terrestrial and marine environments (Raoux et al. 1999, Wilcke 2000, Song et al. 2002, Martinez et al. 2004, Hung et al. 2009). They have long been known for their carcinogenic potential (Krasnoschekova and Gubergrits 1976) and their adverse effect on marine biota has been well studied (International Agency for Research in Cancer IARC. 1983, Woodhead, Law and Matthiessen 1999, McCready et al. 2000). Benz[a]anthracene, benzo[b]fluoranthene, benzo[a]pyrene, chrysene, dibenz[a,h]anthracene and indeno[1,2,3]pyrene have all been confirmed to have a mutagenic and genotoxic effect on biota (Arcos and Argus , WHO. 1989) and as a result the EPA has listed PAHs as priority pollutants (EU. 1976, EU. 1980). The majority of PAHs are from anthropogenic sources such as combustion of fossil fuels, vehicle emissions, petrochemical spills and burning of biomass (Simoneit 1984). Some PAHs can occur through natural events such as forest fires (Boonyatumanond et al. 2007) and perylene has been suggested as a biologically-created PAH in fresh and marine waters (Aizenshtat 1973, Wakeham 1977, Wakeham,S.G. Shaffner, C. Giger, W. 1980). PAHs enter the marine system by gas exchange at the air water interface, or via dry soot deposition or rainfall/wet deposition. Once in the water they bind to particulate matter and accumulate in sediment (Oros and Ross 2004).

Faecal pollution from treated and untreated sewage can increase problems associated with higher nutrient concentrations (algal blooms) and pathogenic organisms (GESAMP. 2001) (Sutherland et al. 2011). These challenges are likely to worsen as

more people move to coastal areas. In addition, faecal contamination can alter nitrogen and carbon cycling which in turn can influence microbial communities' potential for the detoxification of pollutants such as PAHs (Atlas and Bartha 1972). Certain sterols provide a fingerprint for faecal pollution in marine sediments (Reeves and Patton 2005) and have been used over the last 20 years to indicate and determine the source of sewage contamination in the marine environment (Reeves and Patton 2005, Grimalt et al. 1990, Leeming et al. 1996, Mudge and Lintern 1999, Glassmeyer et al. 2005, Biache and Philp 2013). Traditionally bacterial indicator organisms have been used (e.g. total faecal coliforms) as a measurement of faecal pollution (Nichols et al. 1996) but as they are found in almost all warm blooded animals they are not source specific. In addition, their survival in the environment is short and therefore they are not useful in determining a history of sewage pollution (Biache and Philp 2013). Sterol biomarkers can distinguish between faecal sources and they maintain sufficient structural integrity in the environment to be recognized (Leeming et al. 1996). Sterols are quickly degraded in the aerobic water column but because they are lipophillic they bind strongly to particulate matter and degrade much more slowly once incorporated to sediment (Hatcher and McGillivary 1979). Coprostanol is formed from the biohydrogenation of cholesterol by intestinal microbes. It is the most abundant faecal sterol in human faeces and is used as an indicator of anthropogenic sewage contamination (Leeming et al. 1996). Unlike other sterols such as cholesterol, cholestanol, campesterol, β-sitosterol and stigmastanol which are commonly present in marine sediment from a variety of planktonic and terrestrial sources, coprostanol has not been found to occur in non-sewage contaminated sediments (Nichols et al. 1996, Walker, Wun and Litsky 1982, Elhmalli, Roberts and Evershed 1997, Elhmalli, Roberts and Evershed 2000). Coprostanol will persist under anoxic conditions and can be used for tracing faecal pollution as far back as the early medieval period (Evershed and Bethell 1996). Reliance on coprostanol alone is not sufficient to reveal the overall story of sewage release, therefore coprostanol, cholestanol, epicoprostanol and cholesterol are all measured and subsequent sterol ratios are employed to determine possible faecal sources (Mudge and Lintern 1999, Reeves and Patton 2005).

Although analysis of PAHs and faecal sterols can determine the degree and nature of pollution in environmental samples, these data does not provide any evidence of impact on the living microorganisms in the sediment. The extraction technique required to extract PAHs and faecal sterols from environmental samples simultaneously

extracts PLFAs that can be used as biomarkers for the identification and quantification of broad microbial groups and shifts in microbial community structure (White, Stair and Ringelberg 1996). They have been widely employed for measuring microbial community structures in the environment (White et al. 1979, Zelles et al. 1992, Zelles et al. 1995, Hill et al. 2000) and have also been used as a tool to study microbial response to pollution (Frostegård, Tunlid and Bååth 1993). For example, PLFAs have been employed to determine microbial response to fertilizer treatments in soil (Zhang et al. 2006). Furthermore, specific PLFAs have been recognised as potential biomarkers for hydrocarbon degradation (Aries et al. 2001, Polymenakou et al. 2005, Zhang et al. 2012). However, to the author's knowledge, reports of the impact of sewage discharge on marine microbial populations are rare.

In this study we investigate the presence, concentration and source of PAHs and faecal sterols in 30 sediment sample stations in Dublin Bay. To determine whether these pollutants have a physiological effect on marine microbes, PLFAs were extracted, identified and quantified to investigate the microbial population present in the same sediment samples. Particle size analysis (PSA) and total organic carbon content (%TOC) are also determined to investigate the link between physical parameters, pollutant accumulation and microbial community composition.

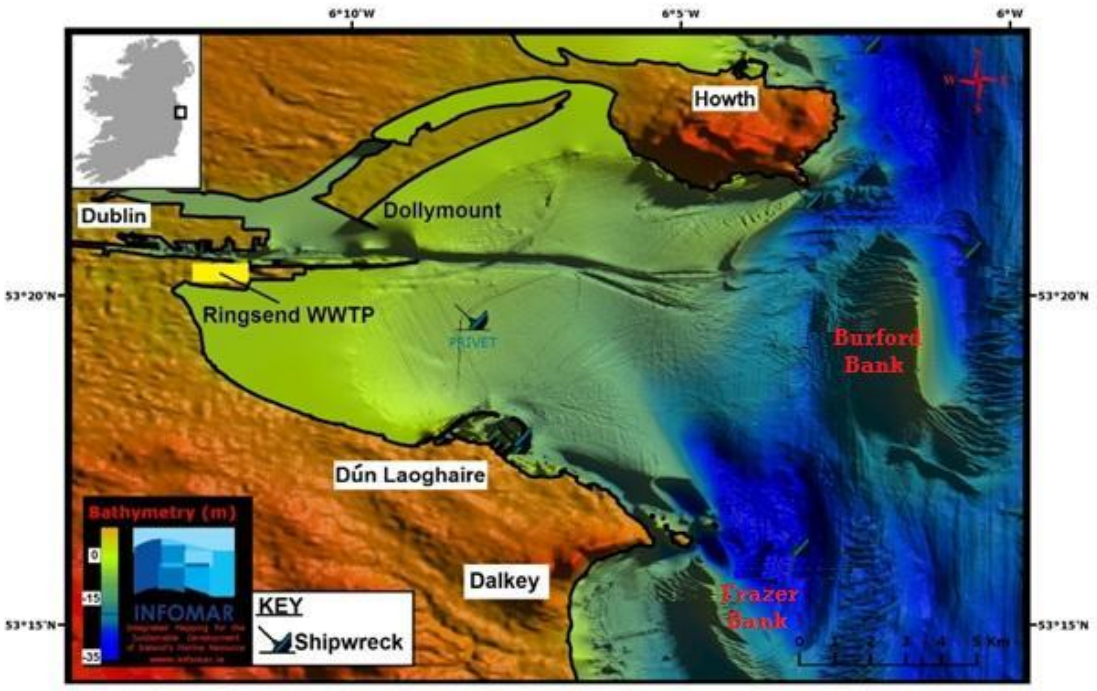

Figure 1 Map of Dublin Bay with sea floor bathymetry (INFOMAR. 2014)

4.2 Materials and Methods

4.2.1 Study Area

Dublin Bay (coordinates; 53°20' N, 6°5 W) is a shallow bay (<35 m) defined by Howth Head to the North and Dalkey Head to the South (Figure 1). The entrance to the Bay is approximately 10.2 km wide and it has a total area of approximately 70 km^2. To the east it opens out into the Irish Sea, while the area immediate to the coastline is dominated by Dublin City with a population of 525,383 (Central Statistics Office 2011). The area surrounding Dublin Bay is the most urbanized region in Ireland. As a result of a high population and industrialisation of the surrounding area, the estuary and the Bay are subject to chemical pollution from a variety of sources. There are two busy marine ports, Dublin and Dun Laoghaire and gas powered energy production sites at Ringsend and North Wall. The estuary receives storm-water run-off from Dublin City and the Ringsend wastewater treatment plant (WWTP) releases treated wastewater into the estuary at Ringsend. The estuary of the R. Liffey acts as a natural division of the city, and to some extent of Dublin Bay also. Poolbeg is a narrow spit of land which juts out into the Bay on either side of the river Liffey. It is a busy port/industrial area with a wastewater treatment plant, gas-fired power station and is the site of proposed municipal solid waste incinerator (Lawlor and McMonagle 2012). The inner part of the Bay is affected by the rise and fall of the tide, with large mud and sand flats exposed at low tide. There are four recognized beaches within the Bay, Dollymount, Merrion, Sandymount and Seapoint. All met the mandatory and guideline values of the EU bathing water directive in 2010 (European Union. 1975). North Bull island is a prominent feature and was formed by sedimentary accumulation due to the construction of the north bull wall in 1821. In 1994 it was recognised as an area of special protection according to the EU Birds Directive (European Union. 1979).

The main riverine input to the Bay comes from the river Liffey (Wilson 2003). Other significant rivers entering the Bay include the Dodder and the Tolka. A rapid dispersion/absorption of nutrients is expected in the Bay due to the dynamic tidal currents, up to 0.5$m.s^{-1}$ (McMahon and Silke 1998).

The main sewage input to Dublin Bay currently comes from the Ringsend wastewater treatment plant (WWTP) at Poolbeg with primary, secondary and partial tertiary treatment (UV screening) of sewage from Dublin City, North and South Dublin. The plant has operated in its current capacity since 2003. Prior to the construction of a

pumping station at Dun Laoghaire and an underwater pipeline to Ringsend in 1991, wastewater from this area was discharged to the Bay after only basic screening. In 2001 a similar underwater pipeline was completed to bring wastewater from North Dublin to the Ringsend WWTP, prior to this wastewater was discharged virtually untreated from the nose of Howth Head. The Liffey is designated as a sensitive body of water under the Urban Wastewater Directive but the WWTP's current discharges do not meet the requirements of the directive. The EPA licence granted to the plant requires it to meet the standards set by the directive by 2015 (Lawlor and McMonagle 2012) .

Reports of OM input to Dublin Bay are scarce, but a previous study found that hydrocarbon and PAH levels were highest in the Tolka estuary and inner harbour but were not notably elevated elsewhere (Choiseul, Wilson and Nixon 1998). In 2009 a large scale survey of urban soils in Dublin (URGE report – Urban geochemistry in Europe) showed that the soils of Dublin inner city were found to contain high levels of all 16 PAHs determined in the study (Andersson et al. 2011). The results of this study may be useful in determining if urban run-off is a source of PAH's to Dublin Bay.

4.2.2 Sample collection and storage.

Samples were collected on-board the *R.V Celtic Voyager* between the 2nd and 7th of June 2010 (cruise number CV10_28). Surface sediments were collected from 30 sample stations at the entrance of Dublin Bay, in a sampling grid lying between Howth Head in the North and Dalkey Head to the South. Grab samples were taken using a Day Grab. Sediment (top 5cm) for organic analysis was sub-sampled into 125 mL furnace glass jars with Teflon-lined lids. Additional sub-samples were placed in plastic bags for particle size analysis (PSA). All scoops and spatulas used for sub-sampling were Teflon coated and solvent rinsed between sample stations. Two boxcores (BC79 and BC81) taken on the same sampling cruise in the Irish Sea to the North of Dublin Bay were sub sampled (top 5cm) and used as controls. All samples were frozen on-board and transported the short distance to the Laboratory, for storage at -20°C.

4.2.3 Particle size and elemental analysis

Particle size analysis (PSA) was performed on 23 Dublin Bay samples taken on the CV10_28 cruise and 27 samples from a previous cruise (CV08_03 in 2008) were also plotted to improve coverage of the sample area. Laser Granulometry was used (Malvern MS2000) for fractions <1000µm and dry sieving for fractions >1000 µm.

Percentage per size class calculated using the MS2000 were converted to total sample percentages and integrated with the >1000 µm data (dry sieving results). Total organic carbon (TOC) and total nitrogen (TN) were measured in 22 samples. Inorganic carbon was removed by addition of 6M HCL. Dried sediment was analyzed using an Exeter Analytical CE440 elemental analyser.

4.2.4 Extraction of PLFA's. PAH's and faecal sterols

Sediment samples were freeze-dried and sieved (mesh size no.20). Three 10 g (dry-weight) sub-samples were taken from each station/grab sample and subjected to replicate extractions. The 10 g of sediment was transferred to a 50 mL screw cap PTFE centrifuge tube. A solution of two deuterated internal standards was added (Phenanthrene-d_{10} and Perylene d_{-12}). Samples were extracted using a modified, monophasic Bligh and Dyer extraction (White et al. 1979, Bligh and Dyer 1959, Fang and Findlay 1996). Briefly, a mixture of dichloromethane, methanol and 50mM phosphate buffer (pH 7.4) was added to the sediment in a ratio of 1:2:0.8 for a total volume of 38 mL. The tubes were capped and placed on a horizontal shaker at 140 strokes/min for 6-8 hours in darkness. The samples were then centrifuged at 6000 rpm for 15 min. The supernatant was decanted into clean separating funnels. The total lipid extracts (TLEs) were split from other components by addition of DCM and phosphate buffer, so that the final ratio of DCM-MeOH-phosphate buffer was 1:1:0.9. The funnel was inverted and vented several times and left to stand in darkness overnight (~16hrs). The lower organic phase was separated off and filtered into a round bottom flask through a glass fibre filter paper containing Na_2SO_4. The extract was rotary evaporated to near dryness (~1 mL), transferred to a 2 mL GC vial and dried fully under N_2. The TLE's were re-suspended in 1ml chloroform and stored at -20°C for further processing.

4.2.5 Fractionation and Derivatisation

The total lipid extracts were separated into neutral lipids (NLs) and polar lipids (PLs), the PAHs and faecal sterols were eluted in the neutral lipid fraction and PLFAs were contained in the polar lipid fraction. This was achieved using a modified solid phase extraction (SPE) method of Pinkart et al, 1998 (Pinkart, Devereux and Chapman 1998). Briefly, the aminopropylsilica SPE columns (Alltech, Deerfield, IL) were cleaned under vacuum, with 5 mL of (a) Acetone (b) Hexane (c) 4% Acetic Acid (d) Methanol and conditioned with 5 mL of chloroform. The dried TLE's were suspended in 500 µl 1:1 MeOH:DCM and loaded onto the SPE column. NLs were eluted with 6ml chloroform,

glycolipids were removed using 6 mL acetone, PLs were eluted using 3 mL 6:1 MeOH:$CHCl_3$ and 3 mL 0.05M sodium acetate in 6:1 MeOH:$CHCl_3$. The NL's and PL's were retained, dried under N_2, and transferred to clean 2 mL GC vials and suspended in 1 mL $CHCl_3$. The NL's were desulphurised by addition of a spatula tip of activated copper powder and gentle shaking overnight.

The faecal sterols in the NL fraction were derivatised using BSTFA (N,O-Bistrifluoroacetamide) to replace the hydroxyl group with a more stable trimethylsilyl group. Briefly, 90 µL of BSTFA and 10 µL of pyridine were added to the NL extracts, the vial was capped, vortexed and placed in the oven at 70°C for 2.5 hrs. The phospholipid fatty acids in the PL fraction were derivatised to fatty acid methyl esters (FAMEs) using mild alkaline methanolysis with sodium methoxide (NaOMe). Briefly, 100 µL of NaOMe and 100 µL of 1:1 MeOH:toluene was added to the dried extracts in a 2 mL GC vial. The sample was vortexed and placed in the oven at 50°C for 30 min. The sample was removed from the oven and allowed to cool, 200 µL of DI water was added and 140 µL of 0.5M HCl. The solution was vortexed and the fatty acid methyl esters were extracted by 2x1 mL washings of 4:1 Hexane:$CHCl_3$. Unwanted water was removed using Na_2SO_4 . Both the NL's and PL's were dried completely under N_2 and resuspended in 100 µL of hexane containing 100ppm 5-α-Cholestane as an internal standard, ready for injection. Double bond position in monounsaturated FAME's was confirmed by the formation of dimethyldisulphide (DMDS) adducts according to Nichols et al, 1986. Briefly, the previously NaOMe derivatised PL's were dried under N_2. 50 µL hexane ,100 µL DMDS and 1-2 drops of iodine solution (6% w/v in diethyl ether) was added to the FAME's. The solution was vortexed and placed in the oven at 50°C for 48 hrs (Nichols, Guckert and White 1986, Nichols, Guckert and White 1986). 500 µL of hexane and 500 µL of sodium thiosulphate was added, the DMDS adducts were extracted with 2x1 mL washings of 4:1 Hexane:$CHCl_3$ The combined organic layers were dried under N_2 and reconstituted in 100 µL hexane prior to GCMS analysis.

4.2.6 GC-MS analysis

Samples were analysed using a gas chromatograph (Agilent Model 6890N) mass spectrometer (Agilent Model 5975C Quadropole MS Engine) (GCMS) system equipped with an automatic sampler. The column was a fused silica capillary column (30 m×0.25 mm i.d.) with a film thickness of 0.25 µm (HP-5MS, Agilent). Ultra high purity helium (BIP-X47Sgrade, Air Products) was used as the carrier gas with a flow rate of 1 mL

min^{-1}. The GCMS was set up as follows; the injector port was set at 250°C with a 2:1 split injection, the initial oven temperature was 60°C for 1.5min and increased at 6°C min^{-1} to 300°C and held for 20min this gave a total run time of 61.5min. The GCMS interface and the Ion source were set at 300°C and 230°C respectively. Filament delay was 10 min. 1 μL of sample was injected using the 2:1 split. The MS was operated in electron impact mode with ionisation energy of 70eV and a mass scan range set from 30 to 650 Da.

The acquired data was processed using Chemstation Software. All PLFAs, PAHs and faecal sterols reported were confirmed through a combination of spectral libraries (NIST, Wiley), spectra interpretation, target ion extracted chromatograms, retention times and consulting related literature. Compounds were quantified from their total ion currents (TIC) using multiple point internal standard (5α-cholestane) calibration curves. The specific compounds used for the internal standard calibrations curves were tetradecanoic acid methyl ester, nonadecane, stigmasterol, phenanthrene - d_{10} and perylene - d_{12}.

4.2.7 Statistical Analysis

Principle component analysis (PCA) was performed on all multi-variable data from Dublin Bay using PAST software (v1.75) (Hammer, Harper and Ryan 2001) in an attempt to simplify the data and determine meaningful associations between components. For PCA all individual components were normailsed to values between 0 and 1. Zero values were replaced by half the minimum values. Principle component scatter diagrams and loading values were used to determine the most significant biomarkers. Any statistically significant correlation values between PSA, PAH's, Sterols, %TOC and the dominant FA biomarkers were determined using Pearson's Correlations in PAST. R^2 values > 0.5 with P values < 0.05 were considered significant. Distribution maps were created using Ocean Data View (Schlitzer 2002).

4.3 Results

4.3.1 PSA and elemental analysis

The distribution of sediment type (%clay, %silt) n = 50 and %TOC n = 20 in Dublin Bay can be seen in Figure 2. This area of the Bay is dominated by sand 78%, followed by silt 12%, with clay and gravel accounting for 5% each. The finer silts and clays are expected to settle in areas of deposition where currents are weaker, while sands and gravel may indicate sites with strong tidal currents and low deposition.

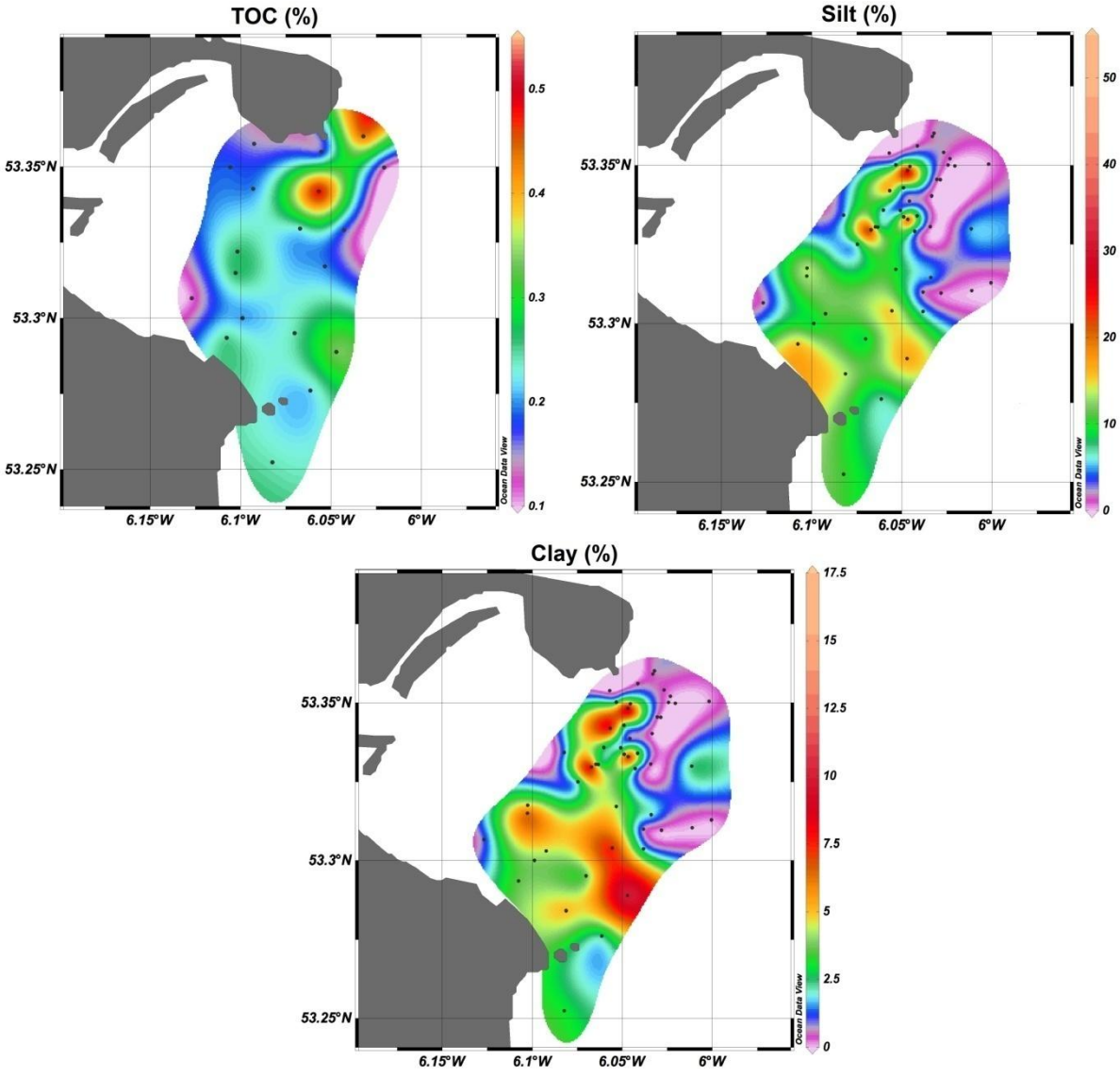

Figure 2: Spatial distribution of Total organic carbon (TOC), Silt and clay in the marine sediments of Dublin Bay. Maps generated using Ocean Data View software.

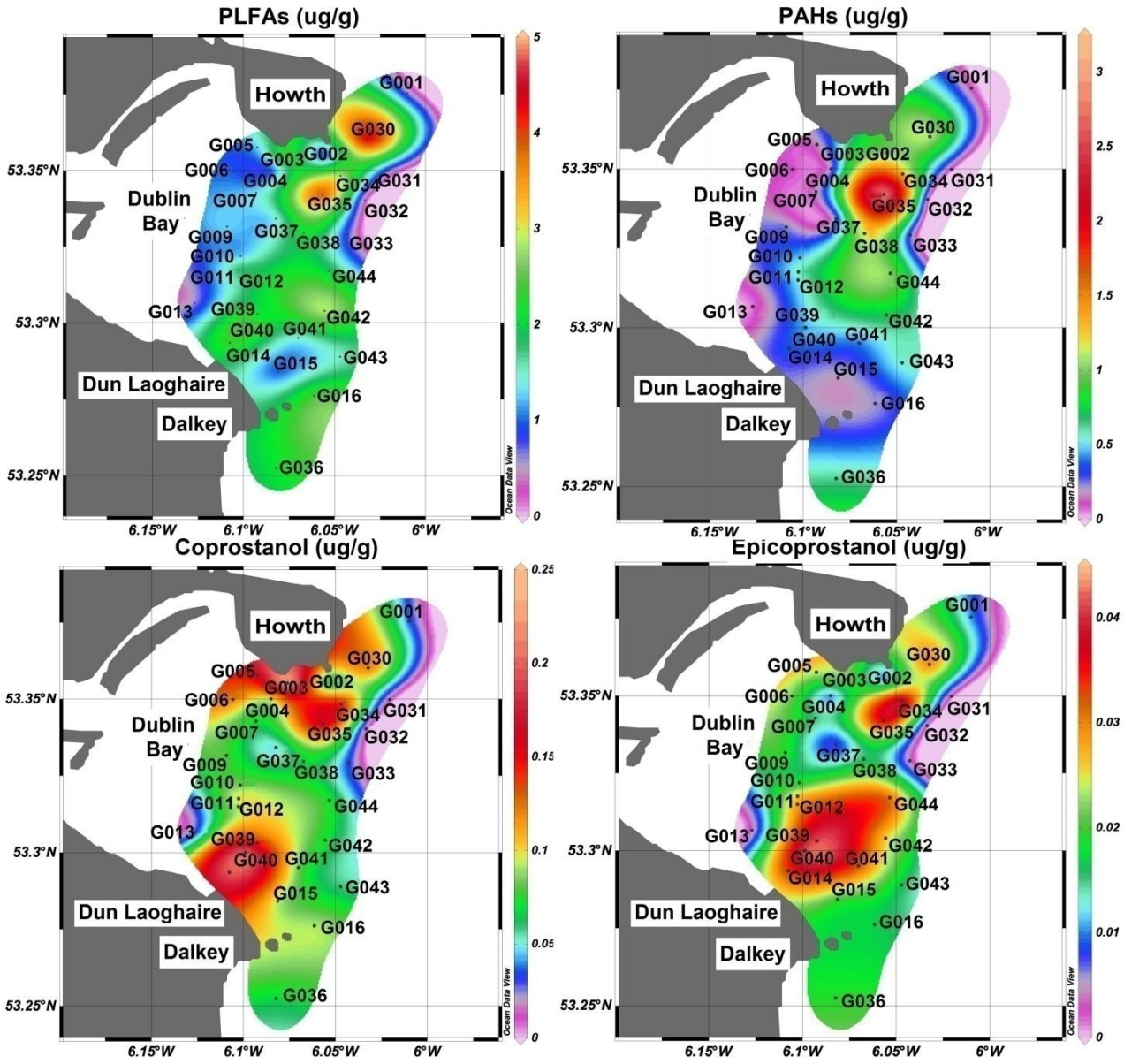

Figure 3*:* Spatial distributions of PLFA's, PAH's and faecal sterols (µg/g) in the sediments of Dublin Bay. Maps generated using Ocean Data View software.

4.3.2 PAHs

The percentage recovery of the deuterated internal standards ranged from 70.5% to 105.9%. 32 sediment samples were analysed and 15 individual PAHs were identified and quantified using GCMS (Table 1). The concentration of individual PAHs ranged from 0.003 µg/g to 0.429 µg/g dry weight. The limit of detection (LOD) was established analytically to be 0.0015 µg/g dry weight. The average concentration for PAHs in the sampling area is 0.540 µg/g. This is below the concentration level often used as a warning for possible PAH induced

Table 1: Average PAH concentrations from triplicate analysis of Dublin Bay Grab samples and Irish Sea Control samples Boxcore(BC)79 and 81 (μg/g dry weight) Blank spaces indicate concentration below the Limit of Detection (0.0012μg/g).

Sample	PAH's (μg/g)															
	Ace	F	Phe	An	Fl	Py	B[a]A	Chry	B[b]F	B[k]F	B [e]P	B[a]P	Per	InP	BP	PAH (totals)
G001			0.012		0.016	0.026	0.007	0.015						0.003		0.080
G002			0.029	0.014	0.139	0.135	0.085	0.105	0.106	0.052	0.024	0.035	0.089	0.057	0.051	0.920
G003			0.055	0.044	0.087	0.095	0.053	0.070	0.046	0.030	0.020	0.077	0.058	0.053	0.059	0.746
G004			0.007	0.007	0.017	0.024	0.011	0.022						0.004		0.093
G005			0.017	0.024	0.018	0.020	0.009	0.030	0.020							0.139
G006			0.016	0.025	0.020	0.022	0.008	0.020	0.020							0.132
G007			0.016		0.017	0.031	0.010	0.036	0.025	0.016						0.151
G009			0.018	0.025	0.030	0.047	0.015	0.043	0.025	0.011				0.028	0.032	0.274
G010			0.027	0.029	0.034	0.047	0.022	0.062	0.028	0.031		0.096	0.044	0.019	0.009	0.449
G011	0.003	0.005	0.027	0.043	0.058	0.075	0.032	0.080	0.066	0.043		0.050	0.044	0.057	0.033	0.616
G012			0.018	0.042	0.065	0.058	0.025	0.062	0.031	0.017		0.049	0.031	0.049	0.023	0.468
G013			0.009		0.008	0.011										0.028
G014			0.029		0.041	0.070	0.013	0.049	0.047	0.029			0.031	0.025	0.027	0.361
G015					0.019	0.027	0.010	0.018	0.028	0.016		0.018		0.024	0.013	0.173
G016			0.020	0.033	0.026	0.050	0.013	0.031	0.017			0.007		0.005	0.004	0.208
G030		0.012	0.065	0.058	0.122	0.161	0.083	0.080	0.092	0.061	0.033	0.099	0.075	0.115	0.092	1.149
G031			0.015		0.012	0.016	0.004	0.014								0.060
G032						0.012										0.012
G033			0.024	0.000	0.045	0.039	0.023	0.035	0.026							0.192
G034	0.006	0.008	0.102	0.081	0.173	0.178	0.059	0.091	0.094	0.077	0.029	0.144	0.083	0.076	0.067	1.266
G035	0.060		0.148	0.178	0.259	0.429	0.150	0.179	0.231	0.154	0.119	0.290	0.336	0.260	0.279	3.072
G036	0.007	0.006	0.038	0.063	0.062	0.077	0.028	0.071	0.042	0.032	0.026	0.070	0.047	0.026	0.023	0.618
G037			0.020		0.025	0.022										0.068
G038	0.003	0.004	0.086	0.063	0.200	0.191	0.073	0.137	0.098	0.071	0.030	0.073	0.087	0.066	0.065	1.247
G039	0.003		0.032	0.046	0.050	0.070	0.019	0.093	0.033	0.026	0.010	0.033	0.036	0.054	0.036	0.541
G040	0.003	0.004	0.025	0.054	0.028	0.057	0.022	0.060	0.029	0.018		0.029	0.028	0.047	0.034	0.437
G041	0.003		0.024		0.031	0.072	0.014	0.054	0.029	0.019		0.049	0.035	0.048		0.378
G042	0.004	0.004	0.030	0.054	0.046	0.048	0.021	0.042	0.041	0.043		0.069	0.049	0.065	0.041	0.557
G043	0.006	0.012	0.055	0.067	0.084	0.102	0.024	0.100	0.040	0.037	0.012	0.048	0.040	0.048	0.031	0.706
G044	0.008	0.010	0.060	0.108	0.152	0.140	0.051	0.113	0.062	0.060	0.024	0.083	0.061	0.058	0.060	1.049
BC79					0.004	0.003										0.007
BC81			0.004	0.004	0.019	0.015	0.041	0.025								0.107

biological effects on marine biota, as the proposed effect range low (ERL) and effect range medium (ERM) are 0.552 μg/g and 3.160ug/g respectively (Long, Field and MacDonald 1998). Although the average PAH levels for the Bay are below the ERL, PAHs are not evenly distributed throughout the sampling area. 11 individual sample stations exceed the ERL (Table 1) with G035 having a total PAH concentration of 3.072 μg/g which is close to the ERM. The boxcore control sample BC79 had the lowest PAH concentration at 0.007 μg/g. The distribution of PAHs in the Dublin Bay sample area is plotted using Ocean Data View and can be seen in figure 3.

Close to 75% of the PAH's identified in the Bay are 4 and 5 ring PAHs. The following PAH isomer pair ratios were applied to the data to elucidate the possible sources of PAHs in Dublin Bay: Fluoranthene/fluoranthene + pyrene(Fl/Fl+Py), benz[a]anthracene/ benz[a]anthracene + chrysene (BaA/228), anthracene/anthracene + phenanthrene (An/178) and indeno[1,2,3-c,d]pyrene/ indeno[1,2,3-c,d]pyrene + benzo[g,h,i]perylene (IP/IP+BghiP). These ratios were utilised successfully by Oros et al (2004) to determine the sources of PAHs in San Francisco Bay. The PAHs isomer pair ratios were compared to ratios from several PAH sources previously compiled by Yunker et al (2002). Sources are determined as follows: IP/IP+BghiP ratio <0.20 - petroleum, 0.20-0.50 petroleum combustion, >0.50 - combustion of coal, grasses and wood. An/178 ratio <0.10 unburned petroleum sources, > 0.10 - combustion source. BaA/228 ratio <0.20 petroleum, 0.20-0.35 - petroleum and combustion, >0.35 - combustion. FL/FL+Py ratio <0.40 - petroleum, 0.40-0.50 - petroleum combustion, >0.50 - combustion of coal grasses and wood (Yunker et al. 2002).

The PAH isomer pair ratios IP/IP+BghiP, An/178 and BaA/228 are plotted against FL/FL+Py in Figure 4, to show how PAH ratios from Dublin Bay sediments compare to potential source ratios (Figure 4 based on the work of Oros et al (2004). The PAH ratios were also applied to the results from the URGE report from the GSI (2011) to establish if PAH sources to Dublin's urban soils and marine sediments are similar. Figure 3 shows that the isomer pair ratios for the individual Dublin Bay sample sites are comparable and fall within a narrow range. The average ratio results for the Bay are: IP/IP+BghiP = 0.56, indicating PAH's primarily originate from combustion of coal or biomass. An/178 = 0.55, indicating a dominance of combustion sources. BaA/228 = 0.31, signifying a mixed influence of unburned petroleum and combustion sources. FL/FL+Py = 0.44, indicative of petroleum combustion.

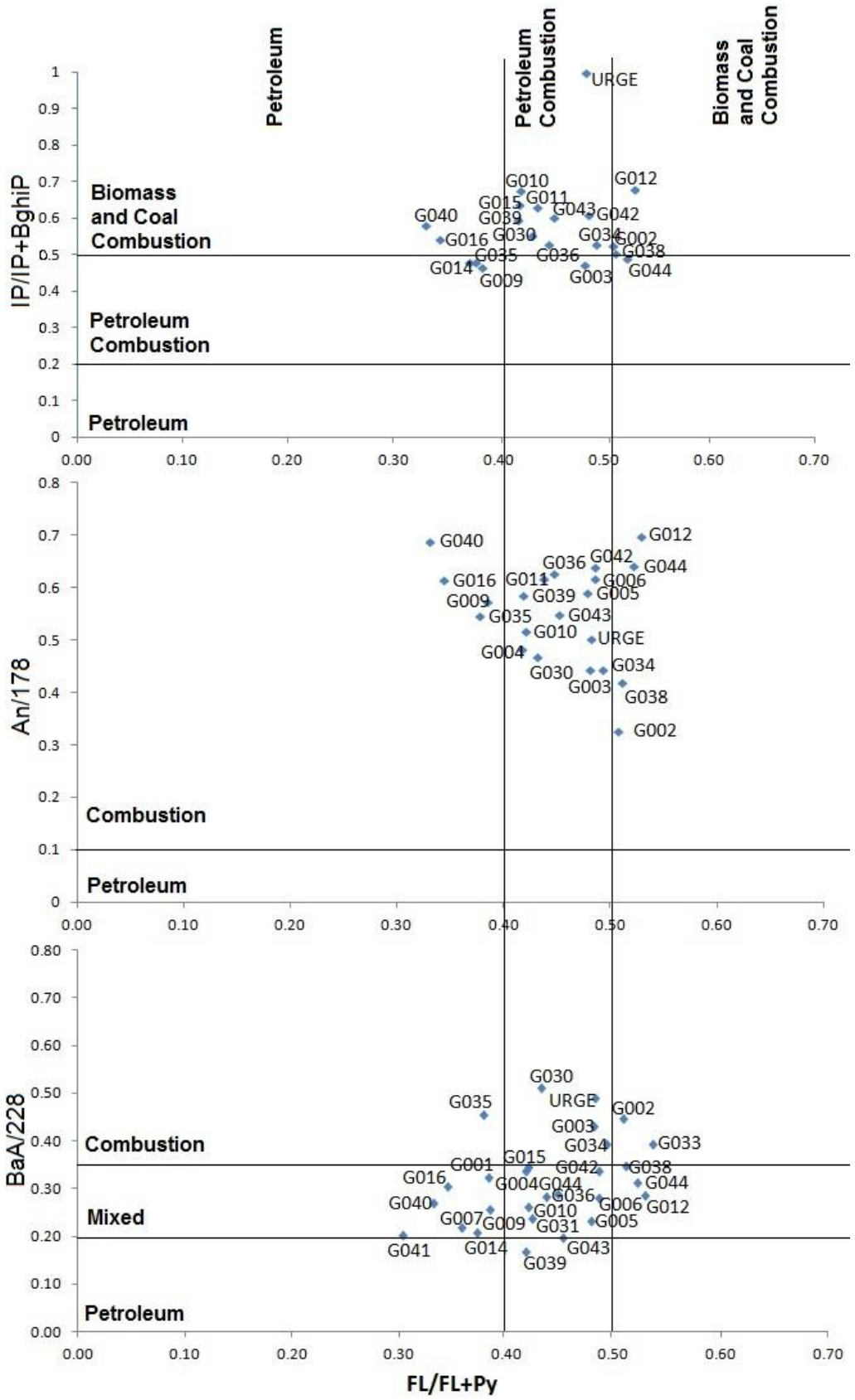

Figure 4 PAH isomer pair ratios plot for source identification (Yunker et al 2002, Oros et al 2004) Data points represent samples sites.

4.3.3 Faecal Sterols

Although up to 20 individual sterols were noted in the GC-MS chromatograms of the Dublin Bay samples, for the purpose of this paper only coprostanol (5β-cholestan-3β-ol), epicoprostanol (5β-cholestan-3α-ol), cholestanol (5α-cholestan-3β-ol) and cholesterol (cholest-5-en-3β-ol) were quantified (Table 2). Coprostanol and epicoprostanol were chosen as they are commonly utilised as indicators of sewage input. Cholesterol and cholestanol can be used in sterol ratios with the faecal sterols, helping to elucidate the source and other characteristics of sewage matter in sediments (Sterols ratios seen in Table 2) (Reeves and Patton 2005, Mudge and Lintern 1999, Writer et al. 1995). Sterol concentrations range from 0.001 μg/g to 4.219 μg/g dry weight (LOD = 0.0009 μg/g). Sterols are unevenly distributed throughout the Bay, the distribution of coprostanol and epicoprostanol in the sampling area can be seen in Figure 3. The concentration of coprostanol is used as an indicator of human faecal input in marine environments (Mudge and Lintern 1999). It is highest around Howth Head in the North of the Bay and Dun Laoghaire in the South (Figure 3). The 5*β*/(5*β*+5α) stanol ratio which was calculated as follows: (coprostanol + epi-coprostanol) / (coprostanol + epi-coprostanol + colestanol), was used to determine the extent of sewage pollution, with values >0.7 considered significantly polluted, and values <0.3 are considered uncontaminated (Mudge, S.M. & Ball, A.S. 2006). Samples with ratios between these values cannot be categorized by this stanol ratio alone. Therefore, the stanol ratio results were cross-plotted with a coprostanol to cholesterol ratio for which values greater than 0.2 can be considered contaminated with faecal matter (Figure 5) (Grimalt et al. 1990). The control samples taken from outside the Bay (BC79 and BC81) are not contaminated according to the plot. Interestingly, G030 appears unpolluted in Figure 5, despite having a coprostanol concentration of 0.128 μg/g. However, the concentrations of cholesterol and cholestanol are notably higher at this site resulting in a lowering of the ratio values. This may indicate a biogenic/marine input of cholesterol also resulting in high levels of its primary degradation product cholestanol at this site. Other useful sterol ratios shown in Table 2 are cholestanol/cholesterol and epi-coprostanol/coprostanol. Bacteria in the environment convert cholesterol to cholestanol, which usually occurs in anaerobic reducing environments. Thus the greater the cholestanol/cholesterol ratio values the more reducing the environment (Mudge, S.M. & Ball, A.S. 2006). Coprostanol is converted to epi-coprostanol during sewage treatment or conversion happens slowly in the environment, the ratio epi-coprostanol/coprostanol

Table 2: Average Sterol concentrations of Dublin Bay samples and Irish Sea control sites (μg/g dry weight) and sterol ratios used. Blank spaces indicate concentration below the Limit of Detection (0.001 μg/g).

Sample	Sterols (μg/g)					Sterol ratios			
	Coprostanol	Epicoprostanol	Cholestanol	Cholesterol	Total	Cholestanol/	Epi/	copro/	5β/(5β+5α)
G001	0.035	0.010	0.066	0.174	0.323	0.38	0.28	0.20	0.40
G002	0.041	0.005	0.033	0.087	0.175	0.38	0.13	0.47	0.58
G003	0.250	0.024	0.161	0.505	1.184	0.32	0.10	0.49	0.63
G004	0.077	0.007	0.100	0.297	0.533	0.34	0.09	0.26	0.46
G005	0.153	0.024	0.101	0.227	0.599	0.45	0.16	0.67	0.64
G006	0.125	0.022	0.078	0.144	0.410	0.54	0.18	0.87	0.65
G007	0.069	0.016	0.099	0.242	0.455	0.41	0.24	0.29	0.46
G009	0.081	0.020	0.153	0.206	0.447	0.74	0.24	0.39	0.40
G010	0.081	0.017	0.160	0.156	0.453	1.02	0.21	0.52	0.38
G011	0.110	0.028	0.167	0.200	0.513	0.83	0.25	0.55	0.45
G012	0.086	0.028	0.157	0.134	0.369	1.17	0.33	0.64	0.42
G013	0.020	0.002	0.030	0.101	0.167	0.29	0.11	0.20	0.43
G014	0.197	0.034	0.240	0.628	1.199	0.38	0.17	0.31	0.49
G015	0.089	0.019	0.102	0.295	0.548	0.35	0.21	0.30	0.51
G016	0.093	0.017	0.125	0.479	0.818	0.26	0.18	0.19	0.47
G030	0.128	0.031	0.456	4.219	5.981	0.11	0.24	0.03	0.26
G031	0.008	0.001	0.013	0.041	0.066	0.30	0.12	0.18	0.40
G032	0.005		0.006	0.053	0.066	0.12	0.11	0.09	0.45
G033	0.032	0.003	0.026	0.066	0.125	0.39	0.11	0.48	0.58
G034	0.160	0.043	0.218	0.841	1.359	0.26	0.26	0.19	0.48
G035	0.168	0.039	0.173	1.156	1.568	0.15	0.23	0.15	0.54
G036	0.066	0.019	0.127	0.172	0.383	0.74	0.29	0.38	0.40
G037	0.042	0.005	0.057	0.264	0.377	0.22	0.13	0.16	0.45
G038	0.066	0.016	0.084	0.219	0.432	0.38	0.24	0.30	0.49
G039	0.132	0.041	0.185	0.245	0.589	0.75	0.31	0.54	0.48
G040	0.215	0.042	0.203	0.385	0.963	0.53	0.19	0.56	0.56
G041	0.080	0.033	0.109	0.195	0.417	0.56	0.41	0.41	0.51
G042	0.059	0.016	0.139	0.167	0.362	0.83	0.27	0.35	0.35
G043	0.071	0.025	0.178	0.598	0.879	0.30	0.36	0.12	0.35
G044	0.073	0.032	0.173	0.245	0.533	0.71	0.44	0.30	0.38
BC79	0.023		0.054	0.165	0.259	0.33		0.14	0.30
BC81	0.030		0.098	0.462	0.662	0.21		0.06	0.23

can indicate the level of sewage treatment or older sewage pollution (Grimalt et al. 1990, Mudge and Lintern 1999, McCalley, Cooke and Nickless 1981). The sites that appear to be contaminated with sewage around Howth Head and Dun Laoghaire have low epi-coprostanol/coprostanol ratio values indicating that the sewage present here is untreated or older pollution.

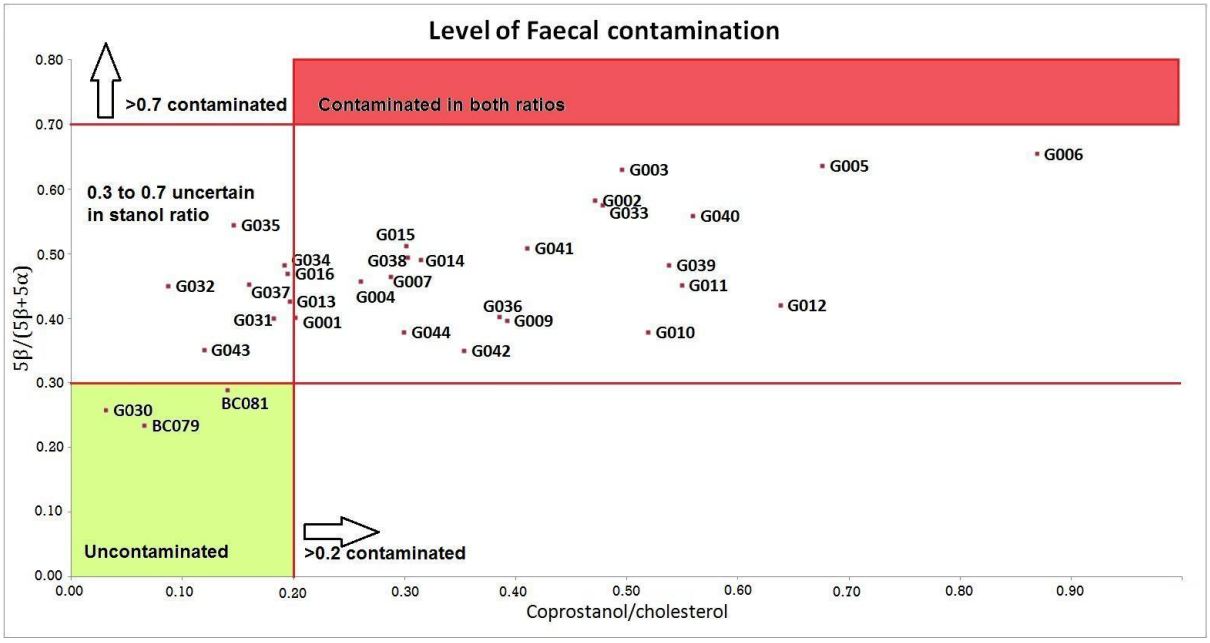

Figure 5 Sterol ratio crossplot. 5β/(5β+5α) stanol ratio values >0.7 considered significantly polluted, <0.3 coprostanol/cholesterol values >0.2 can be considered contaminated with faecal matter (Grimalt et al. 1990, Mudge, S.M. & Ball, A.S. 2006) Data points represent sample sites.

4.3.4 PLFAs

Between the Dublin Bay sediments and two control samples from the Irish Sea, sixty-seven PLFAs were identified. PLFA distribution can be seen in Figure 3. The average PLFA composition of Dublin Bay was composed of: monounsaturated fatty acids (MUFAs) = 37.2%, saturated fatty acids (SFAs) = 29.4%, branched fatty acids (brFAs) = 18.2%, polyunsaturated fatty acids (PUFAs) = 12.3%, branched monounsaturated fatty acids (brMUFAs) = 1.6% and cyclo-propyl fatty acids (cycFA's) = 1.2%. Carbon chain length ranged from C_{12} to C_{24}. Total PLFA abundance as a measure of microbial biomass ranged from 136.9 ng/g dw (G032) to 4664.1 ng/g dw (G030) in Dublin Bay. The highest level of microbial biomass was found in the control sample BC81, with a total PLFA concentration of 7492.5 ng/g dw (Figure 6).

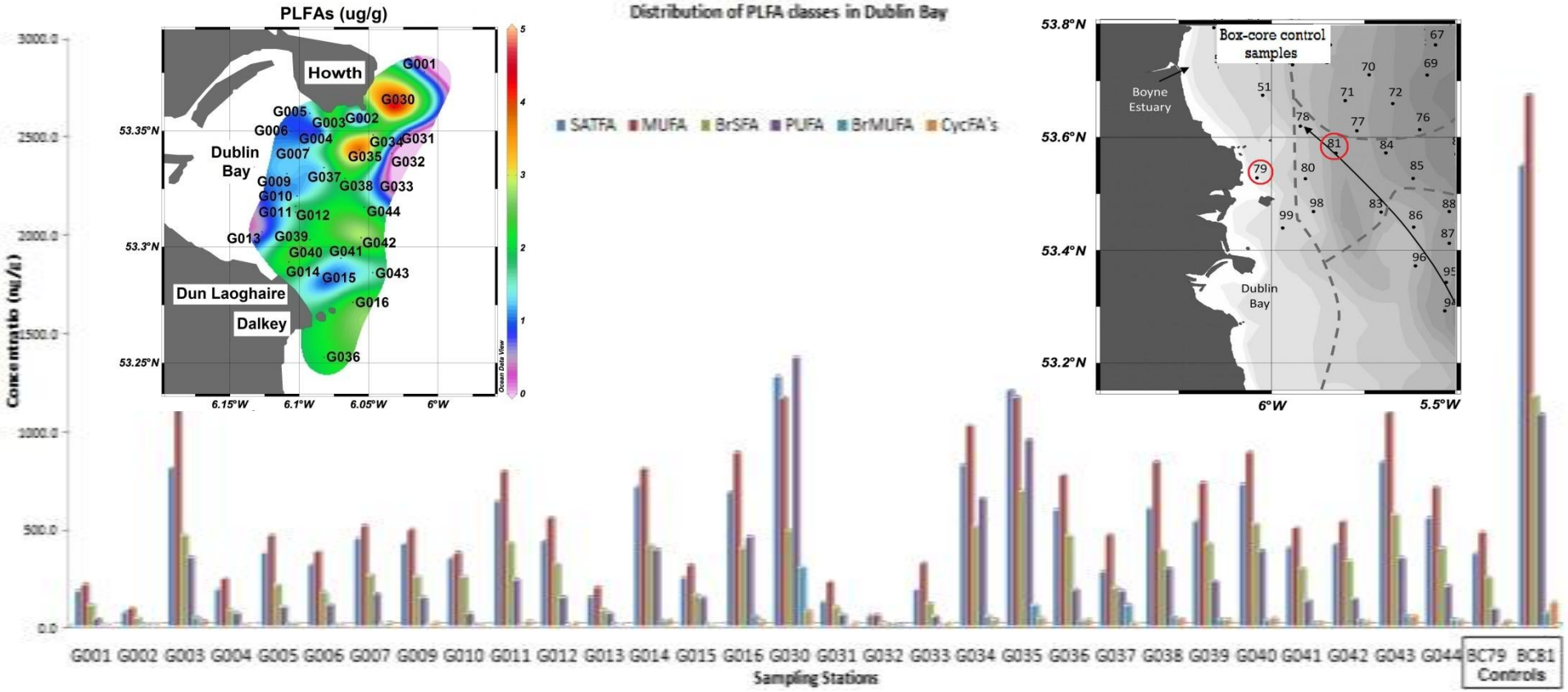

Figure 6: Distribution of major PLFA classes (ng/g dw) identified in the sediment of Dublin Bay and control samples from the Western Irish Sea.

Monounsaturated fatty acids usually originate from bacteria and microalgae (Rütters et al. 2002, Widenfalk et al. 2008). The major MUFAs are 16:1ω7, 18:1ω9 and 18:1ω7. There is a dominance of even numbered over odd numbered SFAs. The major SFAs are 16:0 and 18:0, these PLFAs are ubiquitous in organisms (White et al. 1979). Long-chain saturated PLFAs (C20-C24) only account for 1.6% of total PLFAs. 16:1ω7, 18:1ω9, 18:1ω7, 16:0 and 18:0 account for an average 44.5% of the total PLFAs in the Dublin Bay. These are commonly reported as dominant PLFAs in marine sediments (Aries et al. 2001, Polymenakou et al. 2006). brFAs are used as indicators of bacterial biomass in marine environments (O'Leary and Wilkinson 1988, Chikaraishi and Naraoka 2003) with methyl branching on the 10^{th} carbon used as a specific biomarker for sulphate reducing bacteria (Matsui and Ringelberg 2004). The dominant brFAs were i15:0, a15:0, i16:0, i17:0, a17:0 and 10Me16:0. PUFAs in marine sediment are likely as a result of eukaryotic communities (White et al. 1979, Carrie, Mitchell and Black 1998) such as benthic microeukaryotes of phyto- and zooplankton input from the water column. The PUFAs in Dublin Bay ranged from C_{18} to C_{22}, and a polyunsaturated C_{16} was identified in the controls. The main PUFAs in Dublin Bay were 20:4, 20:5 and 22:5. In G030 PUFAs are notably higher than any other site, making up 29.3% of total PLFA's here. This suggests there is a significant input from marine eukaryotes at this site. brMUFAs such as 7Me16:1w8 and i19:1w9 are only minor components of the PLFA profile of the Bay but are strongly correlated to PUFA ($R^2 = 0.87$), which could mean they are linked to eukaryotic communities in the marine environment (Christie 2014). cycFAs such as cy17:0 and cy18:0 were also detected in the sediments at low abundances.

4.3.5 Statistical analysis

PCA was used to simplify the multivariate dataset and reveal key variables (PLFAs, PAHs, sterols etc.) that explain variation in the study region and between stations. To identify potential geochemical relationships between PAHs, Sterols, PLFAs, particle size, TOC and TN in Dublin Bay, the data was plotted in a PCA scatter diagram (Figure 7). The first two components explained 74.5% of the variance (59.3% and 15.2% respectively). The plot revealed three distinct groupings/loadings: Short-chained bacterial ($<C_{20}$) MUFAs and brFAs, faecal sterols, %TOC and %TN are loaded together towards the right of the plot. PAH's, %clay, %silt and %mud are closely grouped to the bottom left. PUFA, brMUFAs biomarkers for marine algae and plankton are loaded to

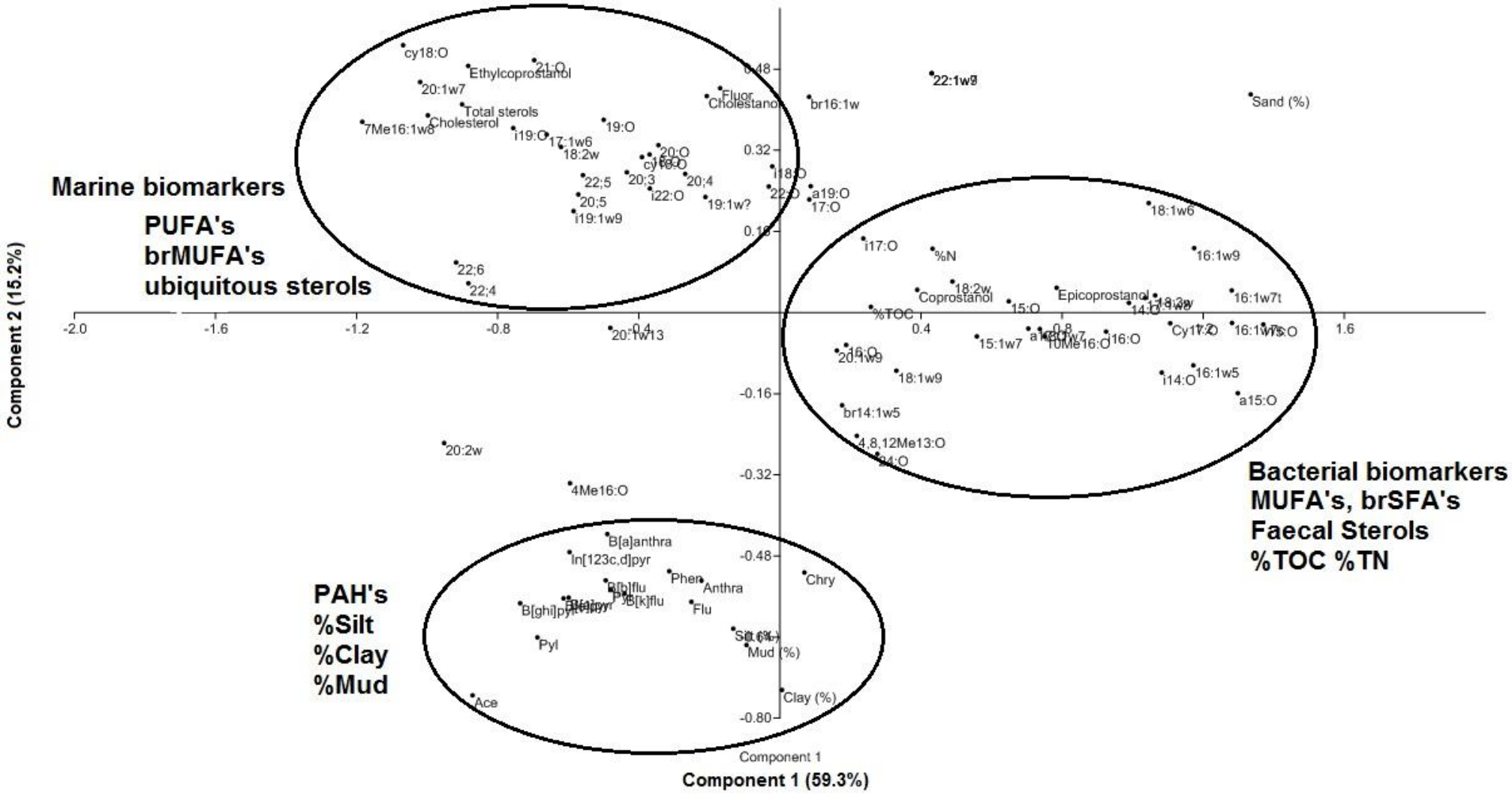

Figure 7: PCA scatter plot of components 1 and 2 from all Dublin Bay data including Individual Sterols, PAH's, PLFA biomarkers, particle size /sediment type, %TOC and %TN.

the top left of the diagram with the ubiquitous sterols. Some components do not appear to have a clear association with any of these groups as 25.5% of variance is unaccounted for by components 1 and 2 reflecting the complexity of organic matter input and distribution in this large sampling area.

PCA was also used to characterize the individual stations according to their PLFA profiles (Figure 8). Component 1 accounted for 66.1% of variance, stations with high PLFA/microbial abundance are loaded to the right and stations with low abundance are loaded to the left. Component 2 explained 14.5% of the variance, stations positively loaded on component 2 are more influenced by PUFAs, indicating the importance of marine algae at these sites. Stations negatively loaded on component 2 are relatively enriched in MUFAs and BrFA, indicating a bacterial influence at these sites.

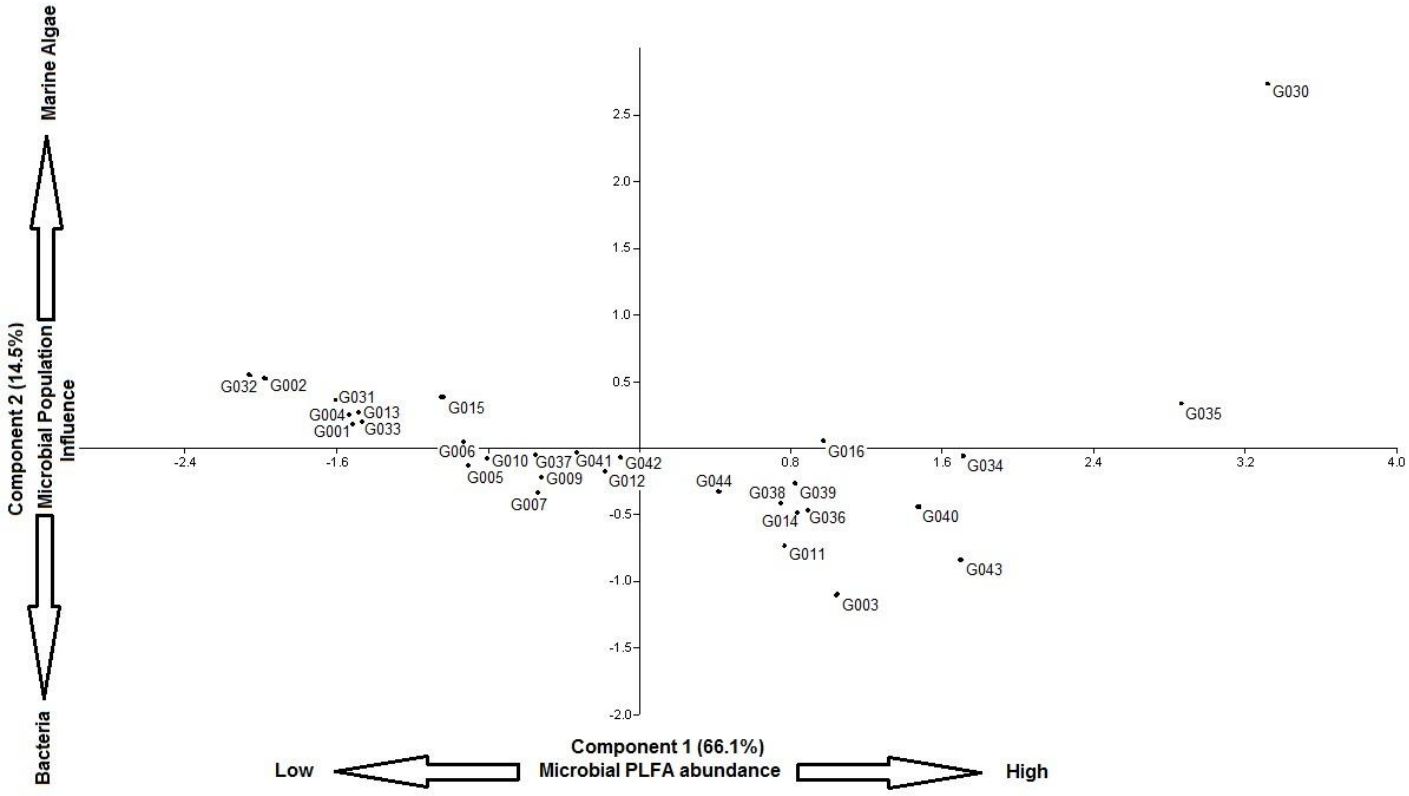

Figure 8: PCA scatter plot of component 1 and 2 for PLFA profiles of sampling sites. Data points represent sample sites.

Pearson correlations were carried out on all data from Dublin Bay (Table 2a appendix). PAHs strongly positively correlated with the clay portion of sediment with a significant, $R^2 = 0.59$, $P < 0.05$. The faecal sterols coprostanol and epi-coprostanol are

not strongly associated with any sediment type. Both PAHs and sterols have a negative correlation with sand. Coprostanol and epi-coprostanol are significantly correlated to each other but there is no significant correlation with PAHs suggesting different sources. Total PLFAs, PAHs and sterols are all strongly correlated with TOC, while TOC and Total PLFAs do not appear dependent on sediment type perhaps being more influenced by organic matter input. The main sewage biomarkers coprostanol and epi-coprostanol are not significantly correlated to the ubiquitious sterol cholesterol, likely due to marine algae and plankton being the dominant origin of cholesterol.

The individual dominant PLFAs were determined by their PCA loading values (appendix A). The major PLFAs were correlated against sewage sterols and PAH data to determine if anthropogenic organic matter input impacted microbial profiles in the Bay. The PUFAs of marine algae and plankton were strongly correlated with cholesterol. Bacterial PLFAs with significantly strong correlations to PAHs are 10Me16:0, i17:0 and a17:0. Bacterial markers more closely correlated with faecal sterols are 16:1ω9, 16:1ω7c, Cy17:0, 18:1ω6, i16:0 and 15:0. (Additional Pearson correlation data can be seen in the Appendix section)

4.4 Discussion

The main focus of this study was to determine the distribution and possible sources of PAH's and faecal sterols in Dublin Bay, and whether these anthropogenic inputs impact sedimentary microbial communities.

The average PAH concentration of the Bay is 0.540 μg/g. This represents a relatively clean Bay, considering the ERL set out by Long et al (1998), is 0.552 μg/g. However, there are 11 stations which exceed this value. There is a strong positive correlation between PAH abundance and clay, and also to silt, which suggests that PAHs source and fate are associated with the prevailing hydrographic and depositional regime of the Bay. The majority of the samples exceeding the ERL are to be found in the deeper part of the Bay (~24 m), west of the Burford Bank (Figure 1). It is also notable that the seabed channel formed by the R. Liffey appears to flow into this area of the Bay. In this deeper area currents are expected to weaken, resulting in a depositional environment. This would allow particulate matter and potentially PAHs transported from the R. Liffey to sink and accumulate here and would also explain why G035 located in this area has the highest PAH concentration (3.072 μg/g) in the Bay. It also

explains the high silt content (20.6%) in this area. PAHs may persist for longer periods here than in shallower sites where there is higher tidal energy.

4-5 ring PAHs make up 75% of the PAHs in the Bay and suggests that combusted fossil fuels are the main PAH source. However, it could also indicate that the pollution is older/historic pollution with lower weight PAHs having degraded faster (Simo, Grimalt and Albaiges 1997). According to the PAH isomer ratios (Figure 4), the major source of PAHs to the Bay is the combustion of fossil fuels. There is a minor influence of unburned petroleum on the PAH profiles of the Bay. Applying the ratios to the results from the URGE report indicates that the main PAH sources in Dublin's urban soils are also combustion sources dominated by coal and biomass combustion with almost no influence from unburned petroleum. The likely combustion sources are (1) Vehicle emissions from high levels of traffic in Dublin City and (2) power generating stations burning fossil fuels at Poolbeg. Historically, coal was used for energy production at Poolbeg and the coal industry around Dublin's docklands produced organic contamination and by-products such as clinker coal were used as fill material for land reclamation (Andersson et al. 2011).

Levels of faecal sterols are low in the sample area when compared to studies of samples taken near sewage outfall points (Reeves and Patton 2005, Grimalt et al. 1990). The presence of coprostanol in the environment is a clear indicator of faecal input (Biache and Philp 2013). The ratio cross-plot (Figure 5) is used as an indicator of the level of sewage pollution. The plot shows no sites were classified as contaminated by both ratios. However, considering that the nearest active sewage discharge point at Ringsend WWTP is 7-10km away, the levels of faecal contamination in this area of the Bay are unusual. The elevated levels and ratio values found in samples around Howth Head and Dun Laoghaire are consistent with former sewage discharge points. Until 1993 sewage was discharged with only rudimental screening at points along the Dun Laoghaire coast and up until 2001 sewage was discharged virtually untreated from the nose of Howth Head (Lawlor and McMonagle 2012). It is likely that the elevated faecal sterol levels in these areas are primarily a result of historical sewage discharge.

We know that sewage formally released from Dun Laoghaire and Howth was largely untreated, therefore the majority of epi-coprostanol in this area can be attributed bacterial activity in the environment (Mudge and Lintern 1999, McCalley, Cooke and Nickless 1981). Epi-coprostanol levels and ratio values are again highest around Howth

and Dun Laoghaire. Samples near Dun Laoghaire have the highest ratio values (Table 2), which corresponds with the older age of the sewage pollution here compared to sewage sterols around Howth Head. The cholestanol/cholesterol ratio is also useful for relative comparisons between samples, higher values indicating reducing environments (Mudge, S.M. & Ball, A.S. 2006). G010, G011 and G012 have the highest cholestanol/cholesterol ratio values. The most unpolluted samples according to coprostanol values and the ratio cross plot are the controls BC79 and BC81 from the Irish Sea. Although G030 has a substantial coprostanol value (Table 2) it is uncontaminated according to the ratio cross-plot (Figure 5). The cholesterol concentration at this site is several orders of magnitude higher than the average for the Bay. This skews the ratio calculations for the ratio cross plot. High cholesterol values can be explained by the PLFA profile of this site, there is a distinct dominance of PUFAs of marine microalgae and zooplankton. Cholesterol is a principle sterol encountered in marine algae (Volkman 1986), macro fauna and zooplankton (Belt et al. 2000). It was also noted on visual inspection at the time of sampling that G030 had a rich benthic community, starfish, sea cucumbers, sea urchins and crabs were all observed in a single grab.

The PLFA profile of the Bay is presented in Figure 6 and describes microbial community composition using the most abundant PLFA groups. Changes in PLFA profiles between sampling stations shows there is variation in microbial community structure. PCA is useful in showing how the PLFA profiles of sampling stations vary (Figure 8). Stations are separated by their microbial/PLFA abundances on component 1 and separated by their community structure on component 2.

PCA was also valuable when establishing if there was any relationship between the large amounts of multi-variable data from Dublin Bay. PCA results (Figure 7) showed three main groupings, these potential associations were further analysed using Pearson's correlations. PAHs are significantly correlated to clay and silt content, suggesting they are accumulating in areas of deposition. Faecal sterols are not correlated to any specific sediment type. There is no significant relationship between PAHs and faecal sterols in the Bay, suggesting they have different sources. As PAHs are settling in depositional zones, a diffuse source such as dry/wet atmospheric deposition or rain water run-off from land are likely. The faecal sterols appear to accumulate near point sources such as the former outfall points of Howth Head and Dun Laoghaire. TOC is strongly correlated to PLFAs, PAHs and total sterols. However, it is not associated with

silt or clay content which is often reported (Reeves and Patton 2005, Hatcher and McGillivary 1979, Bachtiar, Coakly and Risk 1996). TOC in Dublin Bay seems to be impacted by the sources of organic matter. Microbial PLFAs are not affected by depth or sediment type but are significantly positively correlated with PAHs and faecal sterols. Some individual microbial PLFAs show a degree of preference for sites contaminated with PAHs or faecal sterols. Biomarkers such as 10Me16:0, reported in sulphate reducing bacteria and i17:0 and a17:0, which have been previously reported to increase in marine hydrocarbon degrading bacteria (Aries et al. 2001, Matsui and Ringelberg 2004), have significantly stronger correlations with PAH's. 16:1ω9, 16:1ω7c, Cy17:0, 18:1ω6, i16:0 and 15:0 all have strong positive correlations with faecal sterols.

4.5 Conclusion

There are substantial levels of PAH's in the sediment of the Bay, with 10 samples exceeding the recommended ERL. From the dominance of 4-5 ring PAH's and the ratios used it is clear that the primary source of PAHs is the combustion of fossil fuels. Their distribution in the Bay is dictated by depositional processes suggesting their route to the marine environment is through diffuse sources such as dry or wet atmospheric deposition. The high levels of PAHs at station G035 corresponds to where the channel cut in the sea-bed by the R. Liffey terminates, suggesting the R. Liffey is a potential transport route of PAHs to this area of the Bay. The levels of faecal sterols in the Bay are low in comparison to other studies of harbours and Bays (Reeves and Patton 2005, Grimalt et al. 1990). The levels of coprostanol are a definite indicator of human faecal input in these settings. With the active sewage outfall at Ringsend over 7km away, the faecal sterol fingerprint here is as a result of the history of sewage release from Howth and Dun Laoghaire. Although outfall from these points ended in 2001 and 1993 respectively, there is still a significant faecal sterol presence in the sediments. Faecal sterols appear to have accumulated near these former point sources and are less impacted by natural hydrographic and depositional processes. From the PLFA profiles it is clear that microbial communities vary from station to station. Sedimentary microbial communities do appear to be impacted by anthropogenic pollution. Some individual PLFA's appear to be present specifically in response to PAH contamination or sewage contamination.

The data collected gives an indication of the impact that anthropogenic inputs are having on marine sediment in Dublin Bay. The information presented by this study may be useful in making informed **planning decisions** regarding sewage discharge and waste management systems for urban coastlines. For example, the particle size data indicating the sediment type, is useful in determining if a specific area is one of deposition or strong tidal movement. Discharging sewage waste and urban run-off into areas of deposition, with high silt and clay content, would not be advised as organic compounds settle quickly here and persist in sediments. Discharge, if necessary, should be released into zones with strong tidal currents, shown by high sand content. Strong currents mean the organic compounds are dispersed and spend longer in the oxygenated water column, where there degrade more quickly.

The PAH and faecal sterol data can be used to indicate areas which are already subjected to pollution from waste discharge. These areas could be avoided when planning future sewage and run-off points. In Dublin Bay area to avoid would be around Howth Head, Dun Laoghaire and the area to the North of the Bay (G030) where the R.Liffey appears to deposit high levels of PAH's.

4.6 Future Work

In the future we would like to apply the methods used in Dublin Bay to study anthropogenic impact on sedimentary microbial populations in other urbanised coastal zones. Perhaps, taking other persistent organic pollutants into account, such as polychlorinated biphenyls. Some of the PLFAs reported here appeared to be correlated to PAHs and other to faecal pollution. It would be interesting to determine how specific these biomarkers are for microbes in polluted sediment. Are these PLFAs suitable as indicators of microbial populations degrading specific pollutants? A stable isotope incubation study using a ^{13}C labelled pollutant of interest may be the best way to confirm this.

4.7 Appendix C

Table 1a Principle component analysis loading values of PLFA's from sediment

PLFA variable	PC 1	PC 2	PC 3
*i*14:O	0.142	-0.181	0.102
14:O	0.162	-0.157	0.103
br14:1ω5	0.084	-0.111	0.465
4,8,12Me13:O	0.141	-0.079	-0.215
*i*15:O	0.155	-0.193	0.049
*a*15:O	0.153	-0.197	-0.088
15:1ω7	0.077	-0.105	0.296
15:O	0.156	-0.106	-0.062
*i*16:O	0.182	-0.147	-0.068
16:1ω9	0.108	-0.137	0.148
16:1ω7c	0.141	-0.186	0.086
16:1ω7t	0.118	-0.133	0.244
16:1ω5	0.166	-0.193	-0.064
16:O	0.148	-0.010	-0.033
br16:1	0.050	0.014	0.345
10Me16:O	0.134	-0.050	-0.124
7Me16:1ω8	0.072	0.220	0.118
*i*17:O	0.166	0.041	-0.043
*a*17:O	0.166	-0.064	-0.146
Cy17:O	0.180	-0.165	0.023
17:1ω8	0.178	-0.176	0.008
17:1ω6	0.096	0.130	0.061
17:O	0.147	0.052	0.024
18:2ω	0.121	0.140	0.047
18:2ω	0.158	-0.037	-0.003
18:3ω	0.174	-0.135	0.008
*i*18:O	0.142	0.053	0.048
4Me16:O	0.104	0.053	-0.055
18:1ω9	0.146	-0.049	-0.003
18:1ω7	0.170	-0.112	0.052
18:1ω6	0.210	-0.090	-0.054
18:O	0.125	0.108	0.045
*i*19:1ω9	0.128	0.185	-0.044
*i*19:O	0.107	0.177	0.022
*a*19:O	0.177	0.122	-0.152
cy18:O	0.128	0.135	0.030
cy18:O	0.050	0.220	0.133
19:1	0.134	0.128	-0.087
19:O	0.107	0.133	0.021
20;4	0.141	0.089	0.027
20;5	0.133	0.137	0.028
20;3	0.127	0.145	0.064
20:2	0.069	0.202	0.178
20:1ω13	0.116	0.071	-0.282
20:1ω9	0.121	0.006	-0.197
20:1ω7	0.078	0.190	0.113
20:O	0.119	0.103	0.046
21:O	0.095	0.150	0.083
22;5	0.127	0.176	-0.049
22;6	0.121	0.168	0.133
22;4	0.122	0.221	-0.044
*i*22:O	0.145	0.151	-0.029
22:O	0.160	0.081	-0.047
24:O	0.138	-0.022	-0.265

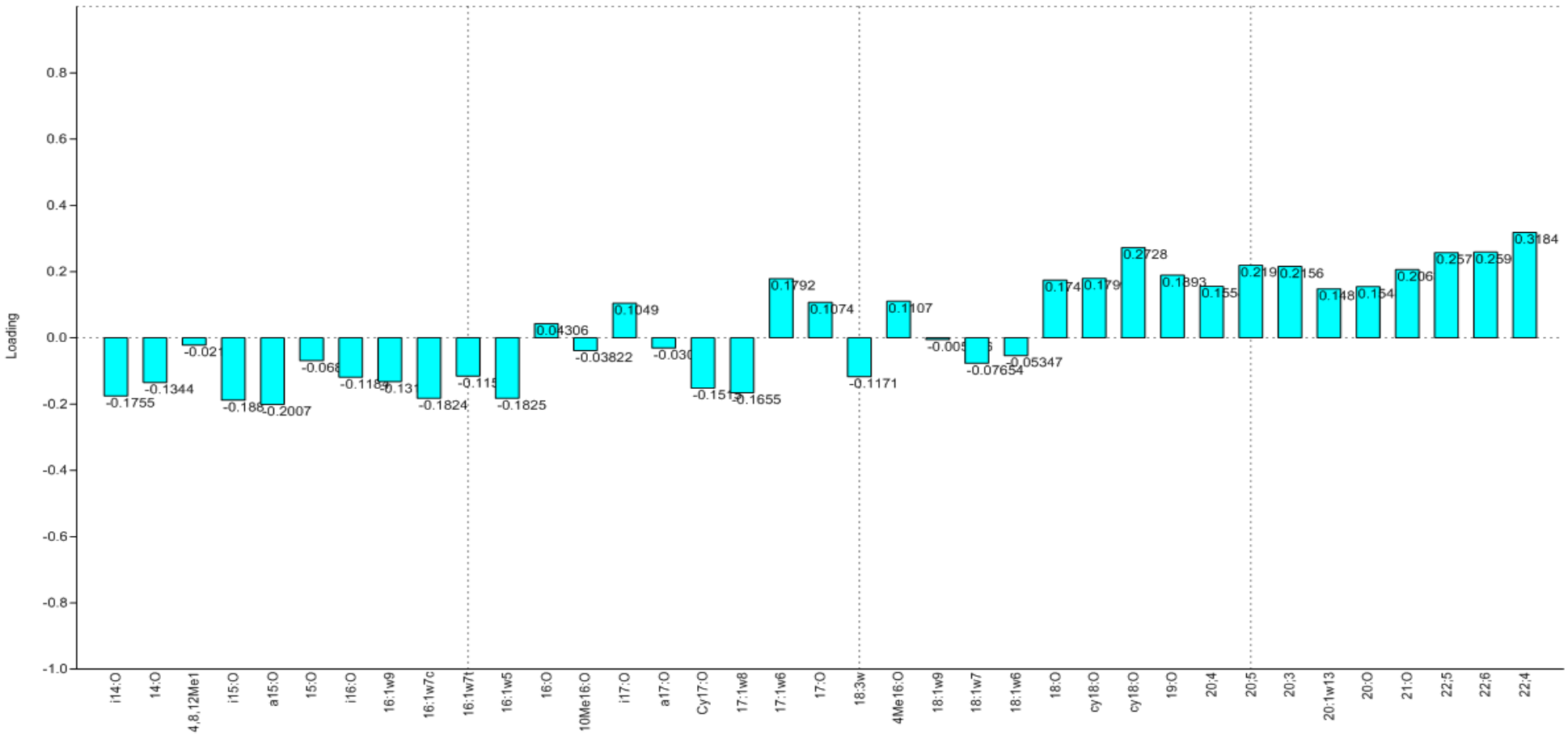

Figure 1a: PCA data – Showing PLFA loading values for component 2 (seen in table 1a). Showing two main groupings - Short chained bacterial PLFA's appear negatively loaded and longer chain marine algae biomarkers appear to be positively loaded

Table 2a: Pearson's correlation results: R^2 values - bottom left. P values - top right.

	Total PLFA's	Total PAH's	Coprostanol	Epicoprostanol	Cholesterol	Cholestanol	Total sterols	Clay (%)	Silt (%)	Mud (%)	Sand (%)	%TOC	%N
Total PLFA	0	0.004	0.006	0.007	0.001	0.000	0.001	0.376	0.340	0.342	0.052	0.000	0.004
Total PAH's	0.710	0	0.167	0.068	0.202	0.244	0.200	0.026	0.059	0.043	0.037	0.001	0.009
Coprostanol	0.697	0.391	0	0.000	0.218	0.013	0.177	0.534	0.423	0.448	0.583	0.067	0.161
Epicoprostanol	0.684	0.501	0.858	0	0.237	0.009	0.188	0.328	0.345	0.331	0.533	0.028	0.057
Cholesterol	0.785	0.363	0.351	0.338	0	0.000	0.000	0.658	0.680	0.668	0.160	0.009	0.010
Cholestanol	0.829	0.333	0.644	0.671	0.865	0	0.000	0.972	0.925	0.938	0.239	0.007	0.008
Total sterols	0.798	0.365	0.383	0.374	0.999	0.888	0	0.687	0.712	0.700	0.161	0.008	0.009
Clay (%)	0.257	0.591	0.182	0.283	-0.130	0.010	-0.118	0	0.000	0.000	0.005	0.135	0.185
Silt (%)	0.276	0.516	0.233	0.273	-0.121	0.028	-0.109	0.928	0	0.000	0.001	0.291	0.382
Mud (%)	0.274	0.547	0.221	0.281	-0.126	0.023	-0.113	0.964	0.994	0	0.001	0.230	0.308
Sand (%)	-0.529	-0.560	-0.161	-0.182	-0.397	-0.337	-0.396	-0.707	-0.786	-0.774	0	0.093	0.108
%TOC	0.834	0.791	0.502	0.584	0.665	0.683	0.675	0.419	0.304	0.343	-0.466	0	0.000
%N	0.722	0.668	0.396	0.520	0.659	0.674	0.668	0.376	0.253	0.294	-0.448	0.953	0

Table 3a: Pearson correlation data – Main bacterial biomarkers correlated with pollutant sources.

	SATFA	MUFA	BrSFA	PUFA	BrMUFA	CycFA's	Coprostanol	Epicoprostanol	Total sewage	Cholestero	Cholestanol	Total sterol	Total PAH	Depth (M)
SATFA	0	1.52E-17	7.44E-14	1.28E-11	2.09E-05	3.85E-12	5.77E-05	2.48E-06	1.35E-05	1.95E-05	1.18E-08	5.79E-06	1.19E-05	0.18695
MUFA	0.9634	0	2.28E-17	2.15E-07	0.00075	8.75E-11	3.19E-05	2.51E-06	7.59E-06	0.001937	1.84E-06	0.0007223	0.000146	0.22355
BrSFA	0.93189	0.96229	0	9.94E-06	0.00924	8.53E-09	8.87E-05	4.82E-08	1.26E-05	0.018948	9.10E-06	0.0096857	2.74E-05	0.24547
PUFA	0.90022	0.78948	0.71267	0	2.50E-10	1.76E-09	0.0046274	0.0011615	0.0024606	2.93E-11	4.09E-08	2.48E-11	5.01E-05	0.074643
BrMUFA	0.69416	0.58177	0.46719	0.87519	0	4.39E-07	0.2045	0.11817	0.17007	2.23E-14	6.57E-06	5.09E-13	0.014374	0.10809
CycFA's	0.90877	0.88472	0.8365	0.85519	0.77717	0	0.007262	0.00013966	0.0027878	1.04E-06	7.58E-09	3.61E-07	0.001061	0.088634
Coprostanol	0.6666	0.68304	0.65399	0.50281	0.23843	0.48003	0	7.42E-07	9.49E-28	0.10278	0.00045965	0.031723	0.04531	0.30542
Epicoprostanol	0.74376	0.74345	0.81293	0.56431	0.29142	0.64003	0.76761	0	8.45E-09	0.066121	5.54E-06	0.027608	0.003474	0.48021
Total sewage	0.70514	0.71904	0.70688	0.53234	0.25718	0.52671	0.99325	0.83662	0	0.083191	0.0001228	0.024838	0.024909	0.45582
Cholesterol	0.69598	0.54287	0.42589	0.89382	0.93766	0.76128	0.3037	0.33987	0.3215	0	6.53E-08	6.76E-28	0.025758	0.14162
Cholestanol	0.83235	0.74987	0.71476	0.81531	0.72238	0.83798	0.5998	0.72627	0.64406	0.80841	0	2.57E-09	0.020546	0.5025
Total sterols	0.72526	0.583	0.46464	0.89514	0.9215	0.78062	0.39292	0.40211	0.40896	0.99342	0.85096	0	0.03149	0.21402
Total PAH's	0.70834	0.63866	0.68717	0.6706	0.44237	0.56794	0.36816	0.51654	0.40877	0.40662	0.42091	0.39341	0	0.24472
Depth (M)	0.24769	0.22898	0.21876	0.3303	0.29931	0.31626	-0.19356	0.134	-0.14148	0.27483	0.12734	0.23364	0.2191	0

Table 4a Pearson correlation Data – PLFA classes vs pollutants and depth

	i14:0	14:0	i15:0	a15:0	15:0	i16:0	16:1w9	16:1w7c	16:1w7t	16:1w5	16:0	10Me16:0	i17:0	a17:0	Cy17:0	17:1w8	18:3w	18:1w9	18:1w7	18:1w6	Total pah's	Coprostanol	Epicoprostanol	Total sewage
i14:0	0	1.48E-20	8.34E-20	1.61E-10	2.36E-05	5.83E-11	7.60E-14	2.01E-17	5.98E-06	1.11E-11	9.91E-08	0.0003814	9.95E-06	1.11E-06	4.60E-12	1.06E-10	2.49E-10	1.60E-09	3.75E-13	6.60E-09	0.0053149	4.13E-05	0.00011652	1.95E-05
14:0	0.97783	0	5.78E-18	1.43E-10	4.22E-06	1.50E-12	3.60E-12	1.47E-16	1.16E-06	2.42E-12	1.87E-09	0.0006274	3.17E-07	9.12E-08	2.39E-13	2.05E-11	1.77E-11	3.54E-11	3.60E-15	4.96E-10	0.0033146	1.31E-05	1.65E-05	4.38E-06
i15:0	0.97489	0.96587	0	6.92E-15	8.75E-07	2.63E-15	3.01E-14	7.35E-22	9.56E-07	8.24E-16	1.41E-08	1.63E-05	7.06E-07	4.01E-09	1.22E-16	9.29E-14	2.84E-13	1.44E-10	8.77E-16	8.94E-12	0.0040724	7.16E-05	8.41E-06	2.01E-05
a15:0	0.87928	0.88037	0.9428	0	5.59E-07	4.75E-16	2.07E-07	5.39E-11	8.53E-07	1.13E-15	2.61E-07	1.45E-06	1.57E-06	1.12E-12	4.16E-14	1.48E-13	6.03E-12	1.14E-08	1.91E-10	8.98E-12	0.0033054	0.00083444	1.70E-07	0.00014883
15:0	0.69108	0.73232	0.7645	0.77282	0	1.41E-09	3.37E-05	5.35E-06	0.0003551	1.47E-08	2.45E-07	6.95E-06	1.04E-06	3.61E-08	1.41E-07	2.98E-08	4.19E-06	1.70E-07	2.61E-07	3.70E-07	0.0048555	0.00025092	1.99E-05	8.16E-05
i16:0	0.88819	0.91495	0.94671	0.95297	0.85762	0	4.58E-09	4.86E-12	1.01E-06	8.16E-19	8.90E-12	7.81E-07	2.87E-10	1.60E-15	2.66E-16	4.56E-15	9.68E-14	5.58E-14	6.77E-16	3.94E-16	0.0004012	4.53E-05	1.06E-07	6.97E-06
16:1w9	0.93178	0.90923	0.93629	0.79016	0.68162	0.84418	0	6.79E-17	2.01E-05	2.62E-09	1.42E-06	0.0005286	3.16E-05	2.40E-05	1.17E-10	2.98E-09	2.15E-09	2.99E-08	1.31E-12	5.00E-08	0.023032	8.69E-05	0.0012137	6.83E-05
16:1w7c	0.96264	0.95683	0.98215	0.88885	0.72703	0.90718	0.95919	0	2.86E-06	4.01E-13	6.05E-08	8.69E-05	4.16E-06	3.73E-07	1.99E-15	4.31E-12	5.79E-12	1.12E-09	3.75E-15	6.73E-10	0.0082642	3.94E-05	4.14E-05	1.53E-05
16:1w7t	0.72453	0.75913	0.7628	0.76498	0.60899	0.76177	0.69523	0.74076	0	4.87E-06	0.0001451	0.0013216	0.0001286	1.60E-05	2.67E-07	1.11E-06	1.80E-06	1.30E-05	2.13E-06	6.11E-06	0.056607	0.014618	0.0025579	0.0081367
16:1w5	0.90125	0.91187	0.95104	0.94991	0.82944	0.97038	0.85072	0.92288	0.72915	0	6.15E-09	2.86E-06	2.12E-07	1.77E-11	1.48E-16	1.09E-15	3.83E-12	1.40E-10	8.77E-13	4.63E-12	0.0017131	7.94E-05	3.58E-07	1.43E-05
16:0	0.80201	0.85454	0.83002	0.78623	0.7873	0.90289	0.75512	0.80956	0.63882	0.84058	0	3.45E-06	5.79E-17	1.73E-11	8.80E-10	4.73E-08	4.14E-09	3.12E-22	1.45E-14	2.86E-11	4.92E-07	4.85E-05	2.97E-06	1.17E-05
10Me16:0	0.60647	0.58833	0.70052	0.75469	0.72108	0.76664	0.5947	0.6546	0.55903	0.74069	0.73672	0	5.40E-07	3.44E-09	4.59E-07	4.37E-07	4.17E-06	1.09E-06	3.05E-06	1.02E-07	0.000327	0.060529	0.00024565	0.024809
i17:0	0.71265	0.78288	0.76852	0.75316	0.76121	0.8739	0.68332	0.73263	0.64262	0.78976	0.95966	0.77345	0	6.27E-13	8.48E-09	1.47E-07	1.57E-08	5.86E-14	6.27E-11	1.21E-12	5.71E-06	0.00044674	1.39E-06	9.85E-05
a17:0	0.75985	0.80331	0.84574	0.91678	0.81708	0.94861	0.69065	0.78007	0.70092	0.89778	0.89795	0.84756	0.92027	0	8.82E-12	3.56E-11	1.00E-10	4.15E-12	8.34E-11	5.95E-16	4.50E-05	0.00088343	5.26E-09	0.00012075
Cy17:0	0.90755	0.92576	0.95741	0.93474	0.79647	0.95492	0.88213	0.94778	0.78587	0.95679	0.86267	0.77637	0.83658	0.90295	0	7.64E-19	2.04E-16	6.17E-12	4.34E-15	1.37E-14	0.0016143	0.00015578	1.17E-06	3.32E-05
17:1w8	0.883	0.89664	0.93077	0.92834	0.81981	0.94452	0.84922	0.908	0.76001	0.95002	0.8132	0.77727	0.7958	0.89226	0.97052	0	1.28E-12	7.30E-10	3.68E-12	5.61E-13	0.0056265	0.0001651	2.62E-06	3.91E-05
18:3w	0.87521	0.89774	0.92481	0.90567	0.7325	0.93056	0.85295	0.90595	0.75033	0.90881	0.84536	0.73258	0.82858	0.88351	0.95578	0.91593	0	9.10E-11	3.94E-13	8.62E-15	0.0010959	0.0011774	6.21E-06	0.00031889
18:1w9	0.85625	0.89231	0.8803	0.83279	0.7934	0.93332	0.81978	0.86013	0.70623	0.88054	0.98321	0.7602	0.93308	0.90825	0.90551	0.86462	0.88437	0	6.89E-19	1.44E-12	1.76E-06	3.89E-05	4.51E-06	1.02E-05
18:1w7	0.92325	0.94547	0.95082	0.8777	0.78625	0.95174	0.9158	0.9453	0.74694	0.91827	0.93961	0.73936	0.88757	0.88513	0.94472	0.90908	0.92297	0.97074	0	5.77E-14	7.54E-05	1.23E-05	3.10E-06	3.11E-06
18:1w6	0.83971	0.86853	0.90285	0.90282	0.78023	0.95361	0.81239	0.86544	0.72404	0.90751	0.89402	0.80164	0.91628	0.9522	0.93985	0.92093	0.94187	0.91521	0.93315	0	0.000533	8.17E-05	1.54E-08	1.06E-05
Total PAH's	0.49597	0.51873	0.509	0.51887	0.50045	0.60468	0.41376	0.47321	0.35178	0.54815	0.77515	0.61187	0.72559	0.67361	0.55068	0.49312	0.56665	0.75082	0.65883	0.5944	0	0.04531	0.003474	0.024909
Coprostanol	0.67598	0.70606	0.66035	0.57744	0.62093	0.67344	0.6546	0.6773	0.44139	0.65731	0.67152	0.34669	0.60082	0.57521	0.63656	0.6347	0.56375	0.67763	0.70744	0.65646	0.36816	0	7.42E-07	9.49E-28
Epicoprostanol	0.64568	0.70015	0.71664	0.79336	0.69541	0.80095	0.56252	0.67595	0.5306	0.78077	0.73995	0.62164	0.75553	0.8425	0.75884	0.74257	0.72366	0.73086	0.73898	0.82886	0.51654	0.76761	0	8.45E-09
Total sewage	0.69602	0.73152	0.6952	0.63801	0.65647	0.721	0.66174	0.70216	0.47404	0.70372	0.70869	0.40903	0.65082	0.64458	0.68201	0.67749	0.61274	0.71198	0.73895	0.71109	0.40877	0.99325	0.83662	0

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Chapter 5

Methods for characterising petroleum polluted sites and the potential for biodegradation.

Contributors

Dr. Paul Flanagan (QUB) – assisted and advised during the setting up of the ^{13}C benzene incubations

Dr. Chris Allen and his group (QUB) – supplied the gene ratio data

5.0 Abstract

Fossil fuel, its refined products and the by-products of its combustion are the most frequently discovered persistent organic pollutants (POPs) in the environment. Effective methods of analysis are required to accurately assess and respond to a petroleum pollution incident. Here sediment samples suspected of being contaminated with varying levels and sources of petroleum related pollutants are characterised. Solvent extractions and solid phase micro extraction (SPME) were used to extract volatile, semi-volatile and non-volatile, aromatic and polyaromatic hydrocarbons (PAHs). These compounds were used as source specific target analytes to characterise the contaminated sites. Coal ash pollution was confirmed at one site based on the levels and profile of PAHs detected. BTEX contamination was confirmed at a second site, which was confirmed using SPME to extract volatile compounds. The potential for in-situ biodegradation was also considered, using phospholipid fatty acids as biomarkers to elucidate the microbial community composition in the polluted samples. Individual PLFAs which have previously been reported for hydrocarbon degraders were identified. Gram positive bacteria were found to dominate polluted samples. Compound specific stable carbon isotope incubations were also investigated as a method for determining if in-situ bioremediation was possible in the BTEX polluted site. Benzene levels decreased over the 200 day incubation but loss of benzene from the abiotic controls and no measurable levels of ^{13}C enrichment meant that biodegradation could not be confirmed. However, the failure of this isotopic study allowed the limitation of the method to be assessed. Gene ratio results supplied by colleagues in Queens University Belfast were compared to PAH concentration levels in a variety of samples, these gene ratio showed potential as diagnostic tools for petroleum polluted sites.

5.1 Introduction

Petroleum pollutants can enter the environment through a variety of sources such as spills, dumping of waste material like ash or through by-products of the combustion of fossil fuels. Petroleum pollution incidents cause significant damage to human health, natural resources, marine and terrestrial life. Petroleum contains thousands of different organic compounds (Wang and Fingas 2003) many of which are persistent in the environment due to their hydrophobic nature (Knap and Williams 1982, Nendza et al. 1997). It is important to characterise the type of petroleum pollution present in the environment, so that an accurate assessment of the fate, long term impact and potential clean-up of the pollutants can be made on a case to case basis. In this study target analytes were chosen, BTEX (benzene, toluene, ethyl benzene and the xylene isomers) and polyaromatic hydrocarbons (PAHs) were used to determine the type and level of petroleum pollution in the samples.

BTEX compounds are proven to have detrimental effects on human health causing neurological disease and cancer (Chiou, Schmedding and Manes 1982, WHO. 1993, Ezquerro et al. 2004). BTEX compounds are made up of a single benzene ring with varying positioning and levels of alkyl groups, they are volatile, making them a major component of the lighter petroleum distillates such as petrol/gasoline (Wang and Fingas 2003). Soil and sediment contamination arises usually from leaking underground petrol station tanks, pipes and accidental spills (Tsao, Song and Bartha 1998). BTEX are listed as priority pollutants (U.S Environmental Protection Agency 1986) and due to their relatively high water solubility can be particularly damaging to aquifers (EUGRIS. 2012). PAHs, as studied in the previous chapter are common constituents of petroleum products and wood preservatives such as creosote (Cerniglia 1992). They are also generated through the incomplete combustion of fossil fuels, high levels of PAHs are found is automobile exhaust fumes, smoke stack emissions from power stations and left over coal ash. PAHs are known for their carcinogenic potential (Krasnoschekova and Gubergrits 1976).

The most common methods used for petroleum pollution analysis are Gas Chromatography (GC) methods (Wang and Fingas 2003). In this study capillary GC-mass spectrometry (GC-MS) is used to separate, identify and quantify the multitude of organic compounds present in the contaminated samples. Due to the volatility of some target analytes, solid phase micro-extraction (SPME) was used to extract and identify

the lighter more volatile petroleum compounds. SPME has a wide range of applications in environmental analysis: BTEX compounds (Ezquerro et al. 2004), PAH's (Llompart, Li and Fingas 1999, Eriksson et al. 2001) chloro phenols (Wennrich, Popp and Möder 2000) pesticides (Young, Lopez-Avila and Beckert 1996) polychlorinated biphenyls (PCB's) (Llompart, Li and Fingas 1999) and herbicides (Hernandez et al. 2000).

Phospholipid fatty acids (PLFAs) are employed for bacterial taxonomy, to elucidate the microbial communities present in the samples. It is generally accepted that only 1% of microbial diversity in soil can be cultured in the laboratory (Torsvik and Ovreas 2002) while the PLFA technique represents a culture independent method of microbial species identification. The microbial community of soil plays an important role in the removal of contaminants such as PAHs (Muckian et al. 2009) and therefore the identification of the microbes already present in a polluted site is the first step in determining if bioremediation is viable (MacNaughton et al. 1999). PLFAs have been used previously to study changes in soil microbial community in petroleum polluted soil (Zhang et al. 2012) and soil under different fertilizer treatments (Zhang, Wang and Yao 2007). In this study PLFA analysis was also combined with compound specific stable isotope analysis (CSIA) using ^{13}C labelled benzene as the substrate in anaerobic incubations of soil from a polluted site. The analysis of PLFAs combined with ^{13}C labelled tracers allows the tracking of microbes biodegrading the benzene (Fang, Lovanh and Alvarez 2004). This method was successfully applied in Chapters 2 and 3 to trace acetate, glucose and CO_2, but in this study some limitations of the technique were exposed.

The above methods were applied to samples from sites suspected of being polluted with petroleum compounds. Samples were characterised according to their pollutant profiles, microbes were classified using PLFAs, and stable isotope incubations were used to assess the degradation and fate of pollutants. Finally, in collaboration with colleagues from Queens University Belfast (QUB), specific gene ratios were compared to the level of PAHs in varying samples. These gene ratios are proposed a diagnostic tool for petroleum polluted sites.

5.2 Methods

5.2.1 Samples

Soil samples from polluted sites were sourced through industrial partners in QUESTOR (a QUB/DCU industrial/academic initiative). Two sites contaminated with hydrocarbons were sourced (Note: Information, such as site locations and usage history is not provided here due to confidentiality agreements with the industrial partners):

Sites:

1. BTEX site.

Soil/sediment samples were taken from a site in the UK reported to be contaminated with BTEX (2 samples). The site had previously been subject to an accidental spill of light weight petroleum product. The soil/sediment from this site was of a sandy nature.

1. Inner Plume (IP) sample: a corer was used to take a sediment sample from 6m depth, **inside the petroleum spill zone**.
2. Outer Plume (OP) sample: sediment sample also taken at 6m depth but **outside of the spill zone.**

2.Coal Ash site.

Soil samples were taken from a site in Portadown N. Ireland, which had been used for dumping of coal ash (3 samples initially taken):

1. S1 grab sample of soil from the top 10 cm of the site with suspected high levels of PAH's due to coal ash dumping
2. S2 grab sample of soil from the top 10 cm of the site also suspected to have high levels of PAH's due to coal ash
3. Control sample taken from an area of the site which was not expected to contain coal ash waste.

Additional soil samples were taken to test the hypothesis that **Gene ratios** can be used as a diagnostic tool for PAH's degradation. The samples studied ranged in levels of pollution from very low to very high. To facilitate this, soil samples were taken from the Viso Valley in the Italian Alps. This remote, high mountainous location represented a potentially PAHs free, pristine soil. However, PAHs can occur through natural events such as forest fires (Boonyatumanond et al. 2007), perylene has also been suggested as a biologically-created PAH found in otherwise pristine fresh and marine waters (Aizenshtat 1973, Wakeham 1977, Wakeham,S.G. Shaffner, C. Giger, W. 1980).

1. Sludge sample (SS) a soil sample from the Portadown site taken at 6 m depth heavily contaminated with PAHs from coal ash.
2. Viso 10A sample taken from the Viso Valley in the Italian Alps taken as a potentially pristine sample with low or no PAH contamination.
3. Viso 10Cox also taken as a pristine sample from the Viso Valley.

5.2.2 Extraction of Phospholipid Fatty Acids and PAHs

Samples were weighed into solvent washed Teflon centrifuge tubes and freeze-dried for 24hrs prior to extraction. Samples were extracted using ultrasonication assisted solvent extraction. Solvent was added in a ratio of 2.5:1, 2.5ml of solvent for every gram of sample (e.g. 6g was extracted with 15ml of solvent). Methanol was added to the sample, it was sonicated for 15 min and then centrifuged for 15 min at 6000 rpm. The supernatant was decanted and filtered through a glass fibre filter paper. The solvent extraction of the remaining sample was repeated with 1:1 Methanol:Dichhloromethane, Dichloromethane and Hexane. The combined extracts were then roto-evaporated to ~1ml and transferred to a clean 2ml GC vial. The extract was evaporated to complete dryness under $N_{2,}$ 1ml of chloroform was added and the extracts were stored at 4°C for further processing.

For PAH analysis the extracts did not require further processing such as SPE or derivatisation. The extracts were dried completely, some samples required dilution as high levels of pollutants were a potential danger for the GC-MS system. Dried extracts were resuspended in 100ul of hexane containing 100ppm cholestane as an internal standard and analysed by GC-MS.

5.2.3 Determining the LOD for PAHs

The limit of detection (LOD) for PAHs was determined by creating a calibration curve of low concentration of deuterated PAHs, naphthalene, acenaphthalene, anthracene and perylene. Their concentrations ranged from 0.12 to 20 ppm. All standards gave a quantifiable response at 0.12 ppm, except for perylene which was only measured in the next 0.6 ppm standard. Baseline noise was also taken into account, it was measured using Chemstation Software, this value was x3 for the LOD, and subbed into the equation of the line for each standard. 0.15 ppm was found to be the best average LOD for the range of PAH compound studied. (See figure 1a in appendix).

5.2.4 Solid phase extraction (SPE) and derivatisation.

The total extracts were fractionated into neutral and polar lipids using solid phase extraction (Pinkart, Devereux and Chapman 1998). PLFAs were separated from petroleum compounds, this simplified the resulting chromatograms and reduced co-elution. Full details of SPE can be seen in section *2.2.6*. PLFA extracts were derivatised to fatty acid methyl esters (FAMEs) by sodium methoxide derivatisation also described in section *2.2.6*. The derivatised extracts were dried completely and taken up in 100 µl hexane containing 100ppm cholestane as an internal standard.

5.2.5 Solid Phase micro extraction (SPME)

SPME was used qualitatively to characterise the volatile and semi-volatile hydrocarbons in the polluted samples. Briefly, 1g of sample was weighed into a glass vial and then crimp sealed. The sample was placed in the oven for 10 minutes at 60°C and then placed in a water bath at 60°C and the poltdimethylsiloxane (PDMS) fibre (Supleco) was exposed to the sample for an absorption time of 30 minutes. The fibre was then inserted into the inlet of the GCMS at 250°C and the volatiles were desorbed for 1 minute. GCMS acquisition parameters were as follows: Inlet 250°C, Splitless injection, no solvent delay. Column oven: initial temp 45°C hold for 5 minutes, ramp at 10°C/min to 225°C hold for 1 min. Total run time 25 minutes. The SPME fibre was conditioned in the oven at 60°C for 15min prior to the next sample analysis.

5.2.6 Multi-discipline approach to studying benzene degradation in a BTEX polluted site.

Samples from the BTEX site were incubated at 23°C with ^{13}C labelled benzene as the carbon substrate. A control sample from outside the spill and local park soil was also incubated to serve as a control. Some preliminary plating and plate counts showed that microbial populations from the site survived/grew on benzene substrate. The microcosms were performed in triplicate, under aerobic and anaerobic conditions. 1g of sample was amended with 1mM of ^{13}C benzene and 4ml of M9 media (section 2.2.4) was added. 30g microcosms for PLFA and NMR analysis were also set up. Samples were crimp sealed in headspace vials, anaerobic microcosms were performed in a customized glove box flushed with N_2 gas, and an oxygen metre ensured no/low levels of O_2.

Gas chromatography flame ionisation detection (GC-FID Agilent 6890N) and direct headspace analysis was used to measure benzene concentrations and monitor degradation over time. Briefly, a gas tight syringe was used to directly inject 10ul from the sample headspace at room temperature onto the GC with a HP5 column. The GC-FID set up was as follows: Splitless injection, Flow rate = 1 mL/min, Injector = 280°C, Oven Program: Isothermal 50°C for 3 min, Detector temperature = 280°C. Direct headspace (D-HS) analysis was preferred to SPME as only benzene levels were required and D-HS analysis using the GC-FID rather than the GC-MS was sufficient. A standard calibration curve for benzene was created by spiking a control soil (heated to 120°C for 48hrs to remove potential volatile contaminants) with benzene ranging from 0.1 to 1.2 mM.

The microcosms were sampled destructively in triplicate over 200 days, once gas analysis was complete samples were frozen for further analysis including:

- DNA extraction and sequencing (identifying specific microbes at Queens University Belfast)
- Phospholipid fatty acid extractions and GCMS analysis (indicator of microbial population present)
- Isotope ratio mass spectroscopy (See section 2.2.7) (to identify ^{13}C enriched fatty acids)

- Nuclear magnetic resonance spectroscopy to determine the transformation and fate of the benzene in the microcosms.

5.2.7 Gene Ratio analysis

Microbiology was performed in Queens University Belfast. To estimate the biodegradation potential of bacterial consortiums in contaminated and pristine environments samples were collected and a library of primers that specifically target the functional genes involved in aerobic and anaerobic degradation of aromatic compounds were tested. Extracted DNA preparations were analysed by PCRs (polymerase chain reaction) using a broad range of primers. To quantify and distinguish PAH-degrading bacterial populations in contaminated and non-contaminated environments and also to estimate the biodegradation potential of bacterial communities' real time PCR assays were employed. Quantitative or real-time PCR is now widely applied in microbial ecology to quantify the abundance and expression of taxonomic and functional gene markers within the environment (Fierer and Jackson 2006, Anderson and Cairney 2004). Based upon the results of general PCR, sets of primers were chosen for quantification of 16S rRNA gene copy number and also quantification of bacteria capable of aerobic (PAH-RHDá-GN) and anaerobic (bamA) degradation of PAHs in soil and water samples.

5.3 Results

5.3.1 Portadown site:

The average PAH values of triplicate extractions, are given in Table 1. S1 and S2 as expected were contaminated with PAHs. However, the control was also contaminated with PAHs. Although not a PAH, cyclic octatomic sulphur, an allotrope of sulphur was noted to be significant in S1, S2 and the control. It is often found in high levels in coal and its waste products such as coal ash.

Table 1 Portadown site samples S1, S2 and the control. PAH concentrations in ug/g.

PAH	**S1 µg/g**	**S2 µg/g**	**Control µg/g**
Biphenyl	0.54	-	-
Acenaphthene	0.11	-	-
Fluorene	0.48	-	-
C0-Dibenzothiophene	0.45	-	-
C0-Phenanthrene	4.09	0.02	0.07
Anthracene	1.58	-	0.28
Fluoranthene	7.46	0.28	0.51
Pyrene	6.10	0.26	0.43
Benz[a]anthracene	3.79	0.19	0.31
C0-Chrysene	3.83	0.21	0.35
Benzo[b]fluoranthene	3.78	0.21	0.47
Benzo[k]fluoranthene	2.61	0.24	0.46
Benzo[e]pyrene	1.30	0.09	0.12
Benzo[a]pyrene	4.14	0.24	0.34
Perylene	1.28	0.06	0.09
Dibenz[a,h]anthracene	1.00	0.06	0.13
Indeno[1,2,3-cd]pyrene	2.73	0.16	0.33
Benzo[g,h,i]perylene	2.51	0.17	0.33
Total	47.80	2.17	4.23

- blank cells indicate values < LOD (0.15ppm)

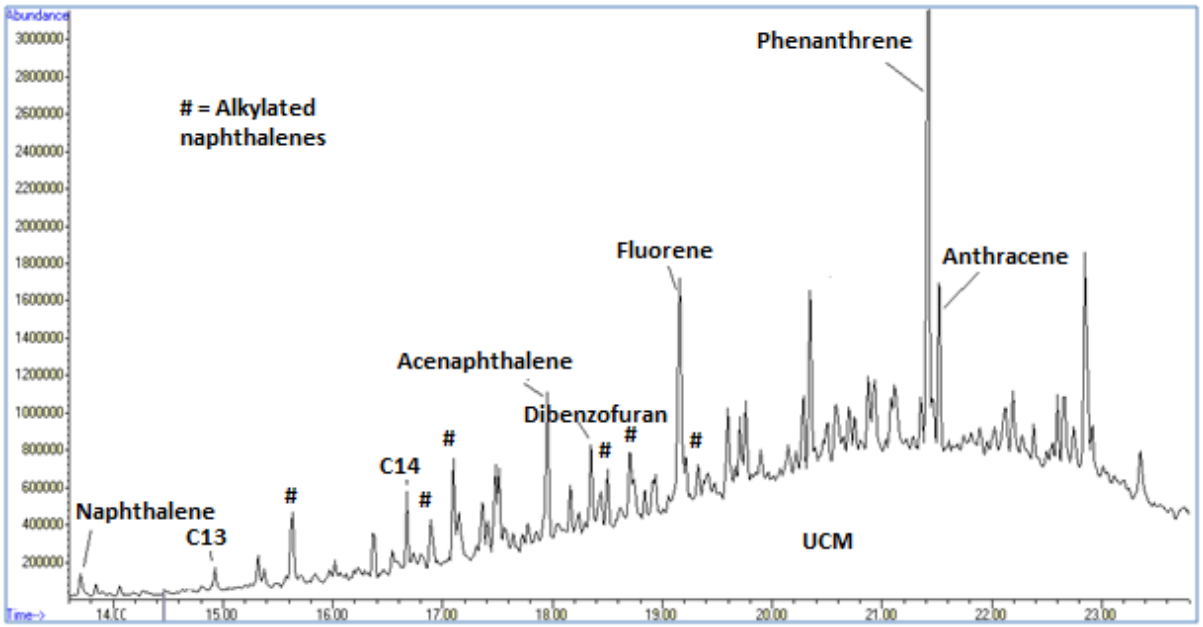

Figure 1 Solid phase micro extraction (SPME) GC-MS chromatogram of **S1** at 60°C Volatile and semi volatile hydrocarbons are extracted at this temperature.

SPME was used qualitatively to identify volatile and semi-volatile PAHs that may not be visible in the solvent extracted chromatogram of sample S1 (Figure 1). It confirmed that no BTEX compounds were detectable in S1 and that the majority of PAHs present are extracted by the solvent extraction, naphthalene being the most notable exception. The raised baseline of the UCM (unresolved complex mixture) is typical of a sample heavily contaminated with hydrocarbons (Wang and Fingas 2003).

PLFAs were used to characterise the microbial population present in the Portadown site (Table 2). The saturated fatty acid (FA) 16:0 is ubiquitous in bacteria often used to compare abundance of bacterial biomass between samples (White, Hood and White 1986, Zelles et al. 1995). The S1 sample contains the most biomass per gram followed by the control sample and S2. Iso and anteiso branched FA's indicate that gram negative bacteria dominate in PAH polluted samples (Hill et al. 2000).

Table 2 Portadown PLFA biomarkers identified and quantified in S1,S2 and the control.

	S1	**S2**	**Control**
FAME Biomarkers	**ng/g**		
14:0	7.48	1.90	4.75
i15:0	57.83	11.50	25.80
a15:0	47.75	11.07	27.20
15:0	4.32	1.28	3.46
i16:0	14.47	6.67	8.78
16:1ω7	49.85	8.36	12.39
16:1ω5	5.33	1.44	1.90
16:0	62.01	17.04	46.33
i17:0	3.59		2.95
a17:0	7.50		9.04
cy17:0	14.52		8.98
17:0	1.55		1.46
Tetra Me 16:0	4.84		
i18:1ω12			
18:2ω6,9			17.27
18:1ω11	13.13	6.85	3.25
18:1ω7	31.89		12.34
18:0	8.35	4.75	11.54
cy19:0	6.28		7.53
Total	**340.70**	**70.85**	**204.98**

The FA profiles of the S1 and control suggest similar populations, while S2 which also contains the lowest levels of PAH's, is considerably different. Notably the cy17:0 and cy19:0 FA's used in stress indicator ratios and to mark anaerobes, are absent (Zelles. 1999, Zelles et al. 1992). The populations are dominated by gram positive bacteria. The common fungal marker 18:2ω6,9 is only present in the control sample (Sakamoto, Iijima and Higuchi 2004). Although S1 has high levels of PAH's its FA's shown it has the largest bacterial population.

5.3.2 BTEX site

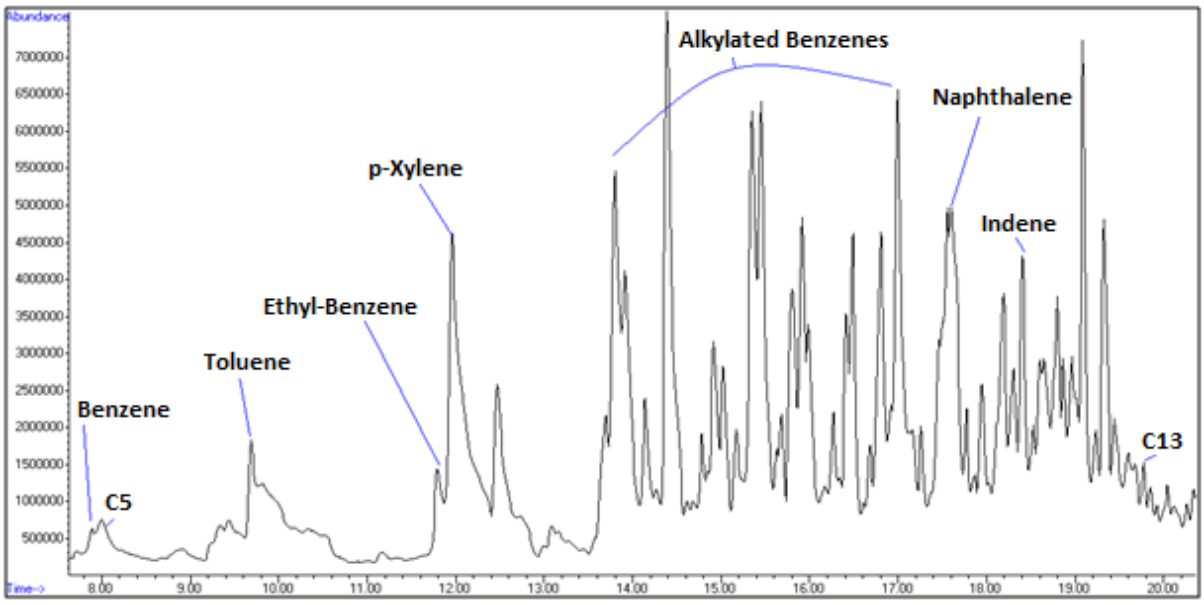

Figure 2 SPME GC-MS chromatogram of volatile compounds at room temp from BTEX IP sample

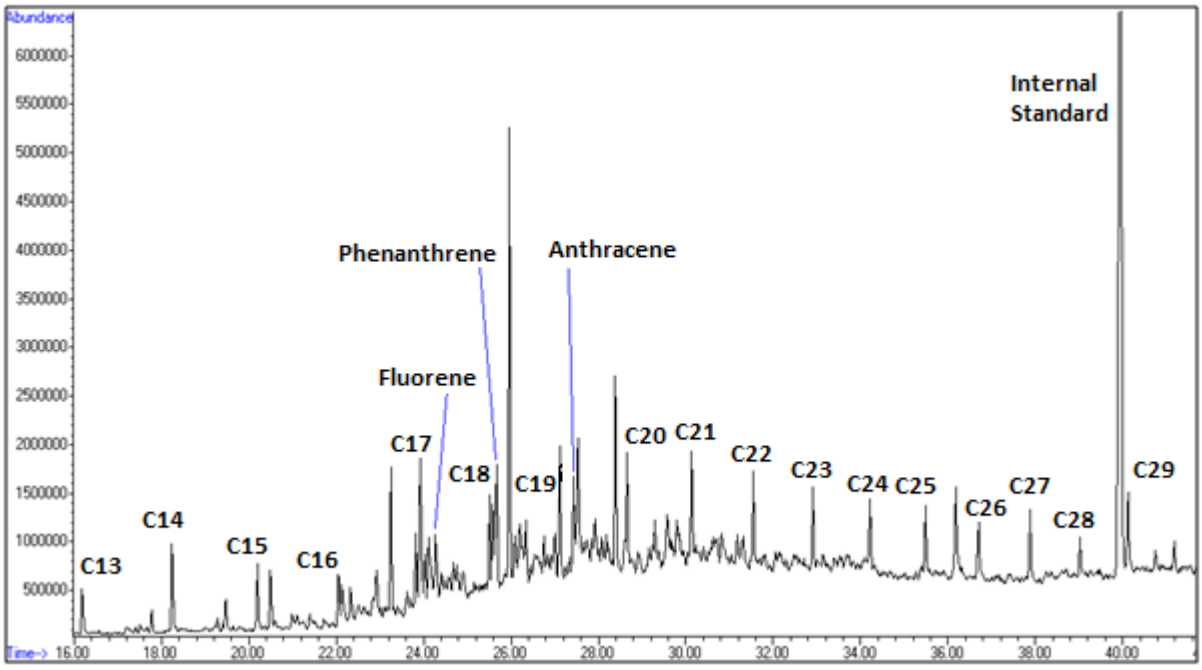

Figure 3 Solvent extracted PAHs and hydrocarbons from the IP sample of the BTEX site.

Benzene, toluene, ethyl benzene and p-xylene were all identified in the inner plume volatiles along with napthalene and indene (Figure 2) when combined with the solvent extracted compounds (Figure 3) a spill of light petroleum distillate is likely. The alkylated benzene compounds are also strong indicators that this is a gasoline spill (Wang and Fingas 2003). The volatiles range from C5 to C13 and the solvent

extractable hydrocarbons range from C13 to C29. By combining the results from both the whole range of organic pollutants can be identified.

Table 3 Solvent extracted PAHs from BTEX site quantified in ug/g. (Std dev ±0.01ug/g)

PAH	**IP** µg/g	**OP** µg/g
Fluorene	0.08	-
Phenanthrene	0.09	-
Fluoranthrene	0.02	-
Pyrene	0.07	-
TOTAL	0.26	-

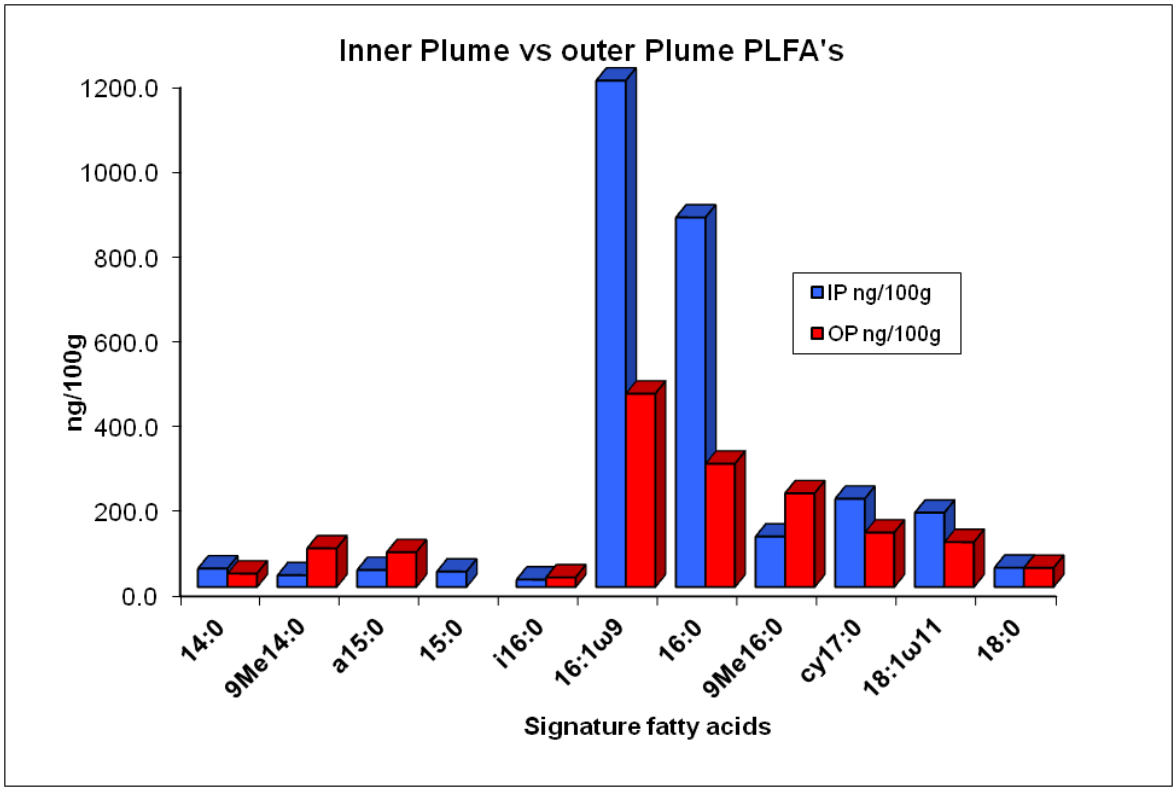

Figure 4: PLFA biomarkers extracted from the BTEX IP and OP.

Table 4 Total PLFA quantity used to compare microbial abundance between samples.

	IP ng/100g	**OP ng/100g**
Total Quantity of FA's	2778.5	1473.9

The greater abundance of PLFAs extracted from within the plume (IP) (Table 4) suggest a greater microbial population exists within the spill site. Elevated levels of organic pollutant such as PAH's and hydrocarbons were not present outside the spill

(OP). Due to the low organic matter in the site's sandy sediment PLFAs were only extractable at ng/100g level (Figure 4). The higher level of organic compounds inside the spill (IP) correlates with higher microbial population. 16:1ω9 and Cy17:0 have been reported in toluene degrading pseudomonad strains (Fang, Lovanh and Alvarez 2004). These biomarkers are notably higher in the IP. The saturated iso and anteiso methyl branched FA's a15:0 and i16:0 are biomarkers for gram positive bacteria (Zelles et al. 1992). Saturated mid chain methyl branched FA's (9Me14:0 and 9Me16:0) are found in mixed microbial populations, 9Me14:0 has been reported in lichenised fungi (Hill et al. 2000).

5.3.3 Multi-discipline approach to studying benzene degradation in a BTEX polluted site.

The anaerobic microcosms set up using the BTEX samples IP and OP were analysed for benzene concentration over 200 days. The samples taken at ~6m depth were expected to be anaerobic; therefore anaerobic degradation of benzene was of interest. Abiotic controls only containing the media and 1mM benzene were expected to maintain stable benzene levels throughout the study. However, the benzene levels in all incubations drop significantly, with roughly ¾ of the benzene lost (figure 5).

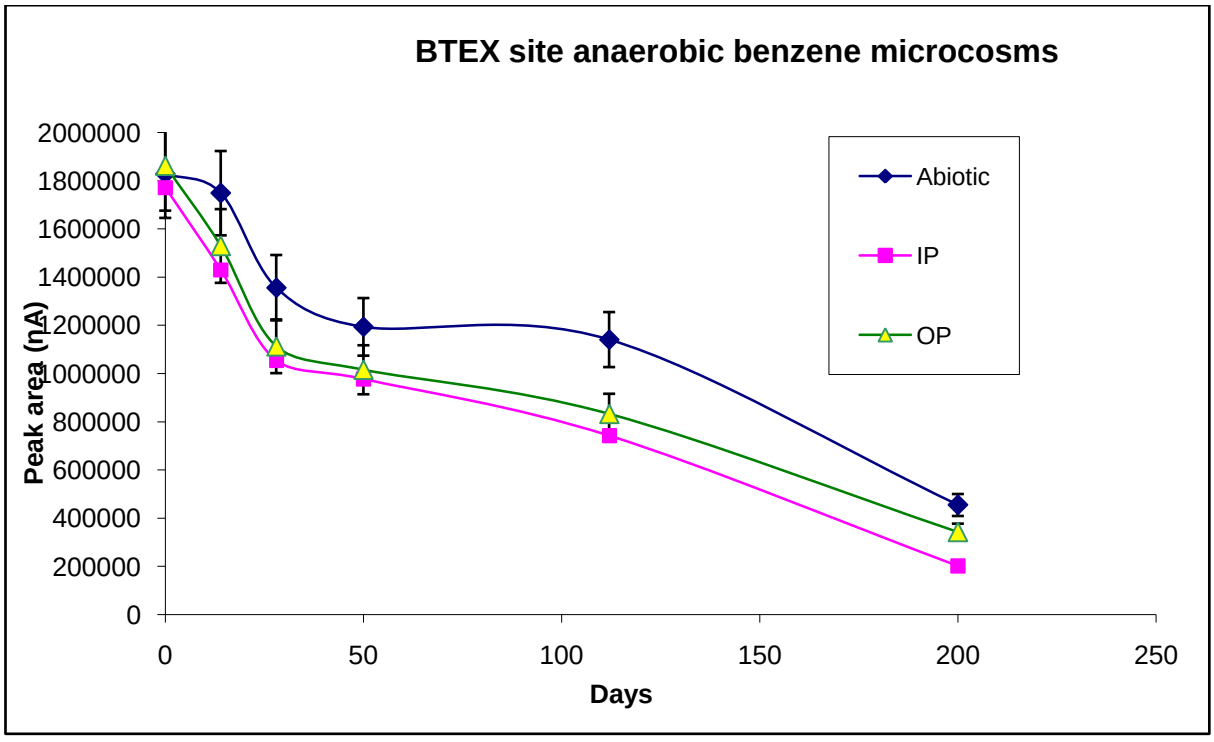

Figure 5 Benzene measured by GC-FID over 200 days of anaerobic incubation of IP, OP and abiotic samples.

There is marginally less benzene in the OP and IP microcosms at 200 days, and when this is normalised against the abiotic (Figure 6) control it indicates that biodegradation may be the cause of lower benzene levels in the OP and IP.

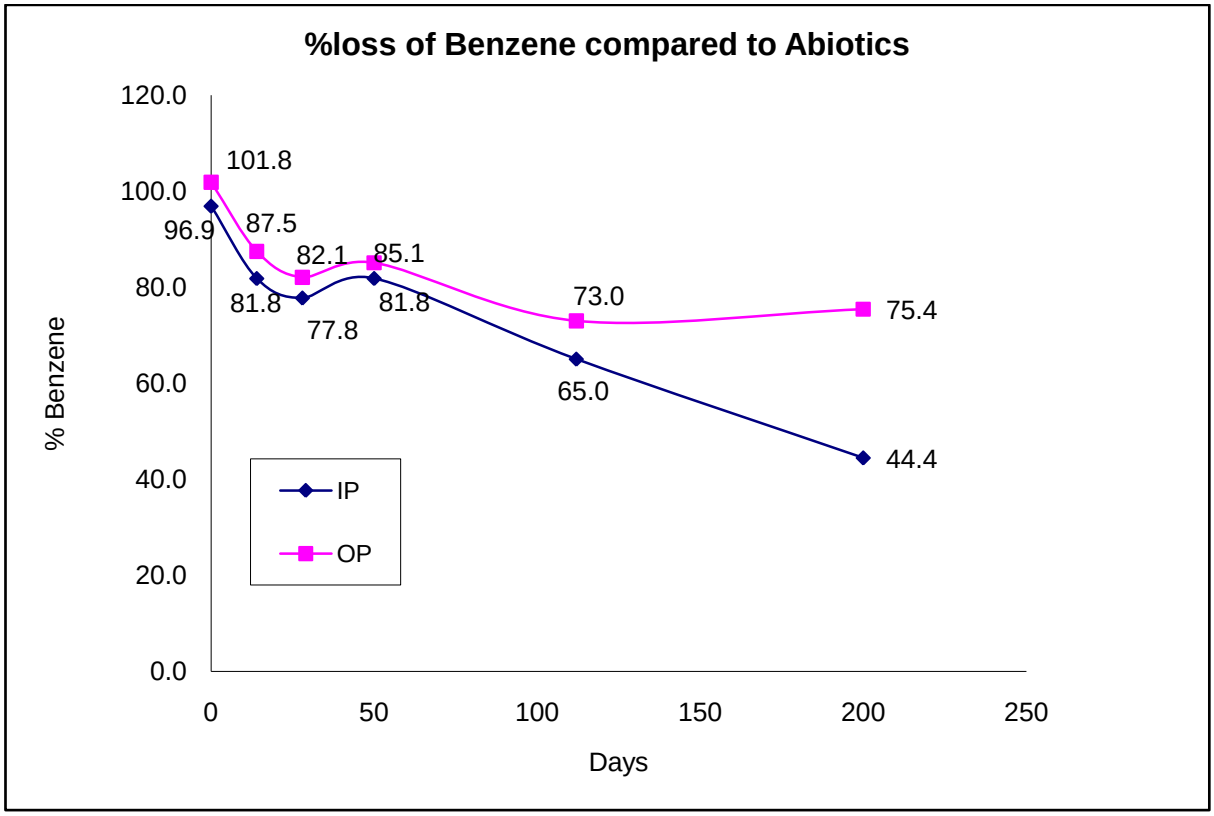

Figure 6 Benzene levels over the 200 days of anaerobic incubation normalized against abiotic controls

Analysis of figure 6 would suggest that biodegradation has occurred. However the significant loss of benzene from the abiotic controls indicated that it is not the only reason for loss. There are **two potential reasons for this significant loss of benzene** in the abiotic microcosms. **1.** Although every effort was made to avoid microbial contamination of the abiotic controls, accidental contamination may have resulted in the growth of a microbial population capable of degrading the added benzene. No microbial growth was visible in the abiotic controls but this does not confirm that bacteria were not present. **2.** Due to the high volatility of benzene it is possible that the large loss of benzene from the microcosms was due to evaporation from the headspace vial. The crimp seal vials used may not have been suitable for anaerobic incubations over such a lengthy time-scale (i.e. 200 days).

It was planned to investigate biodegradation of the ^{13}C benzene through IRMS identification of enriched PLFAs. However, extraction of the 30g microcosms for IP and OP samples did not produce any measurable PLFAs. DNA also proved difficult or

not possible to extract. It must be noted that the PLFA profile produced for IP and OP samples in figure 4 was only achieved by extracting 100g, and due to insufficient sample this quantity was not practical for the stable isotope incubations. Failure to extract labelled PLFAs meant that processing the samples for NMR was not justifiable. In hindsight these samples were not suitable for this type of carbon stable isotope incubation study mainly because the levels of microbial biomass present were too low.

5.3.4 Gene Ratio work in collaboration with Queens University Belfast.

Table 5 Samples analysed for GN/bamA gene ratio and PAHs

Sample	PAHs µg/g	GN/bamA gene ratio
Porta SS	52.71	14.73
Porta S1	47.80	7.73
Porta Control	4.23	91.67
Alps Viso 10 B	0.04	272.33
Alps Viso 10 Cox	0.02	457.00

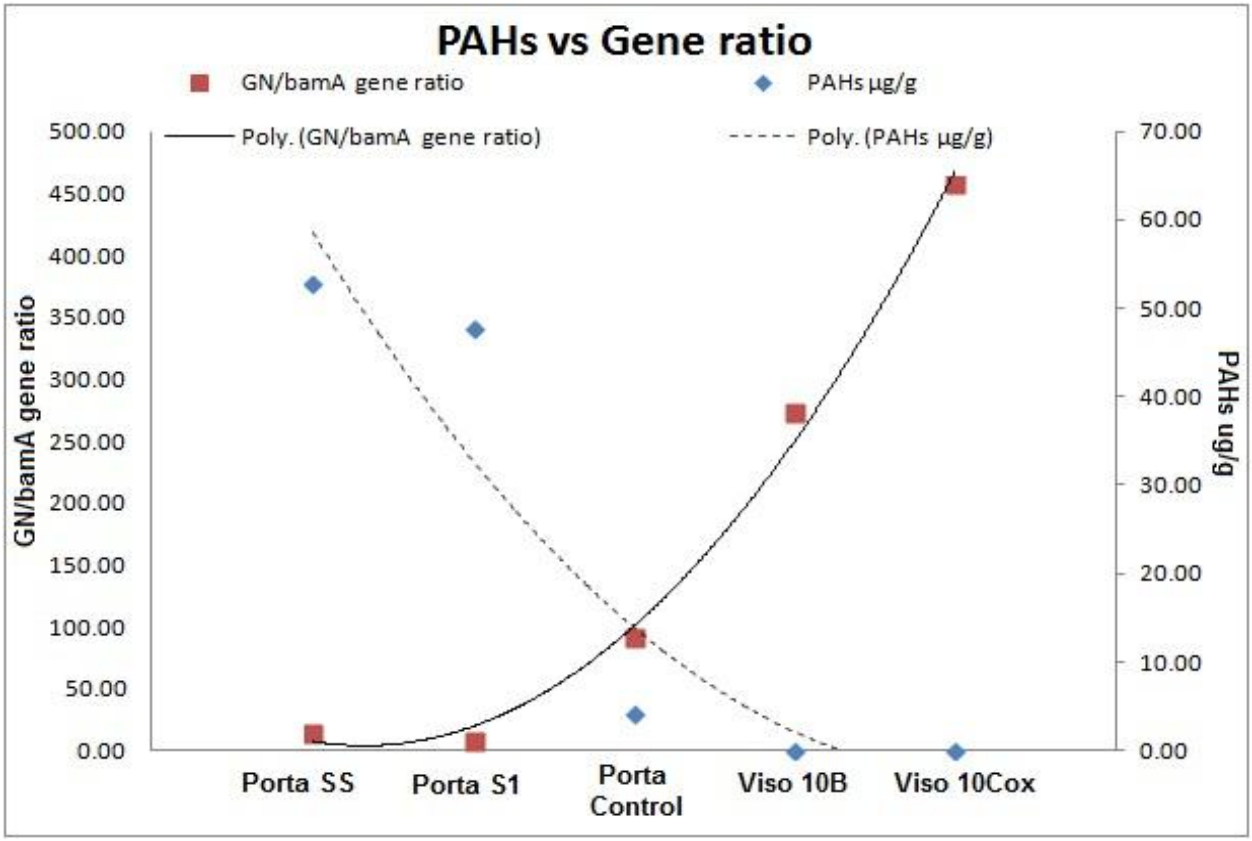

Figure 7 PAH concentration in soil samples vs GN/bamA gene ratio

Here we investigated the potential of the GN gene to bamA gene ratio as a diagnostic tool to quickly assess PAH contamination and the possible presence of PAH degraders

(Table 5). The GN/bamA ratio is inversely correlated to PAH contamination level in Figure 7. Results of real-time PCR showed that aerobic and anaerobic degradation of aromatic compounds is actively occurring in the soil at all analysed Portadown sites. The Alps samples are 'pristine' soils expected to have a ratio of over 100.

5.4 Discussion

5.4.1 Portadown and BTEX sites

All the PAHs extracted from Portadown samples are listed as priority pollutants by the U.S EPA (U.S Environmental Protection Agency 1986). The PAHs extracted from samples S1, S2 and the control from the Portadown site are dominated by heavier 4-5 ring PAHs and is consistent with coal ash contamination (Zhou et al. 2005). SPME to identify volatile PAHs in S1 showed that naphthalene was the lowest molecular weight aromatic compound present, no BTEX compounds were detected. Only solvent extractable PAH's were quantified. The control sample which was expected to be free of PAHs, was found to contain 4.23 μg/g of PAHs, which is significant considering the effect range medium in marine environments is 3.160 μg/g (Long, Field and MacDonald 1998). The use of this sample as a control for the Portadown site is therefore questionable although PAHs have been found in pristine samples from all over the world (Xiao et al. 2010, Westgate and Wania 2010).

The presence of benzene, toluene, ethyl benzene and p-xylene in the IP sample confirmed that the site was contaminated with BTEX compounds. The IP sample also contained naphthalene and indene in the volatiles extracted by SPME. The solvent extraction showed that only low weight PAHs were present in the IP. Their total concentration was 0.26 μg/g (Table 3), which is relatively low when compared to the Portadown site (Table 1). These results suggest a spill of a light distillate petroleum product likely to be gasoline. The OP sample did not contain any BTEX compounds or PAH's which makes it is a suitable control for this site.

The PLFA profiles of the site help categorise the microbial population present. S1 the most heavily polluted sample has a significant gram positive population indicated by the iso and anteiso branched FA's. It has been noted in other studies on the biodegradation of hydrocarbons and PAHs that gram positive bacteria dominate (Zhang et al. 2012, Uyttebroek et al. 2006).

To determine the PLFA profile of the BTEX site samples, a relatively large amount of sediment had to be extracted (100g). Despite this, the PLFA's were only measurable in ng/100g. This low level of microbial population may be due to low organic matter and the sandy nature of the samples. The PLFA profile of the samples indicates that there is a larger microbial population in the IP. This may be due to the higher levels of organic pollutants in the IP sample. Similar to the Portadown site, iso and anteiso branched FA's indicated a gram positive population. The FA 16:1ω9 has been reported in toluene degrading bacteria (Fang, Lovanh and Alvarez 2004), it is significantly higher in the IP.

The variation in PLFAs in the polluted samples from both sites suggests that the microbial population present is potentially impacted by the classification of petroleum pollution. Variations in the PLFA of bacterial cell membranes may represent a response to such pollution.

5.4.2 ^{13}C benzene incubation study

Sediment from the BTEX polluted site was chosen for stable carbon isotope incubation studies using benzene as the sole carbon source. Similar studies with different substrates (toluene) have been successful (Fang et al. 2004) but studies with the more carcinogenic benzene are rare. Initially, plate counts were performed using microbes from the BTEX site cultured on Agar. These plate counts looked promising as microbes grew in benzene amended agar, with higher counts occurring from IP microbes. Measuring benzene in the anaerobic microcosms over the 200 day incubation showed that benzene decreased significantly in the IP, OP and abiotic samples. The loss of benzene in the abiotic controls was deduced to be the loss of some volatile benzene through the lid of the crimp sealed headspace vials as no microbial growth appeared in the controls. When the benzene values of the IP and OP incubations were normalised as a % of the abiotic values, it could be proposed that biodegradation was occurring in both samples. However, no measurable PLFAs were extracted from the 30g incubation. This meant that ^{13}C incorporation could not be confirmed or disproved by IRMS. It was known prior to setting up the microcosms that microbial abundance was low in these samples as 100g had to be extracted to provide ng of PLFAs. It was hoped that incubation of 30 g of sediment in mineral rich M9 media would stimulate sufficient microbial growth to extract PLFAs. However, the low carbon content meant that it would have been very difficult to generate NMR spectra. The low microbial abundance combined with the

significant decrease in benzene in the abiotic controls signified that little could be inferred from the experiment. The success of the carbon stable isotope incubation studies in Chapter 2.0 and Chapter 3.0 was not replicated in this study. However, the failure of the study did provided valuable experience for future studies. The reasons for failure were summarised as:

1. The volatility of Benzene: As benzene is highly volatile it is easily lost due to evaporation or seepage through the seal of the headspace vials (as seen in the abiotic controls). Improved seals on incubation vials are required; alternatively a less volatile PAH such as phenanthrene or fluoranthene could be used to study the biodegradation of petroleum pollutants (Muckian et al. 2009).

2. Low microbial biomass of samples: The samples used had very low levels of PLFA's (ng/100g) resulting in the samples not being suitable for compounds specific IRMS analysis. A site with a richer soil microbial population is required.

3. Age of samples: The samples were stored for 2 years at 4°C prior to setting the incubations. This was out of our control and access to fresh sample proved to be very difficult despite assurances from our collaborating industries. In this storage time the microbial community could have changed or reduced so that the samples are no longer an accurate representation of the site. A polluted site with permission for regular access is desirable.

5.4.3 Gene Ratios

In all analysed samples Gram-positive structural gene markers prevail over Gram-negative, this is reflected by the PLFA results. It is known from the literature that GP PAH degraders dominant in older PAH-polluted sites (Uyttebroek et al., 2006). Analysing the PCR results for SS,S1, Control, Viso 10A and Viso 10Cox, suggest that the ratio of PAH-RHDá-GN (aerobic) gene relative to bamA (anaerobic) gene copy negatively correlate with the PAH-contamination level in the environmental samples. The GN:BamA ratio could be used as a diagnostic test method PAH pollution in soil,

pristine, uncontaminated soils having a ratio above 100 and contaminated soils having a ratio below 100.

5.5 Conclusion

GCMS analysis was successfully used to determine the type and level of petroleum pollution in contaminated and control sites. The BTEX site was confirmed to contain BTEX in the IP sample but not in the OP sample. The Portadown site was found to be polluted with high levels of PAHs. Individual PLFAs were identified in the samples which have previously been associated with biodegradation of petroleum pollutants. Ideally more samples from each site taken over a longer time period would have allowed for a more complete investigation of in-situ biodegradation of petroleum pollutants but access to both sites was limited by the companies involved. The ^{13}C incubations were unsuccessful but identifying the limitations of this method is useful for future studies. It was found that the ratio of aerobic (*PAH-RHDá-GN*) gene to anaerobic (*bamA*) gene copy number had potential to be used as a diagnostic tool to assess PAH-contamination level and possible biodegradation in an environment.

5.6 Future Work

In the future we would like to apply the methods used here to the site of a recent pollution event, where access for sampling over a year or more would be available. The limitations of the stable isotope incubations found here would provide experience for future studies of pollutant biodegradation in soils. If appropriate samples with sufficient microbial biomass and pollutant levels were available, a PAH such as phenanthrene labelled with ^{13}C would be less volatile to work with and would still provide information on microbes degrading aromatic compounds.

5.7 Appendix D

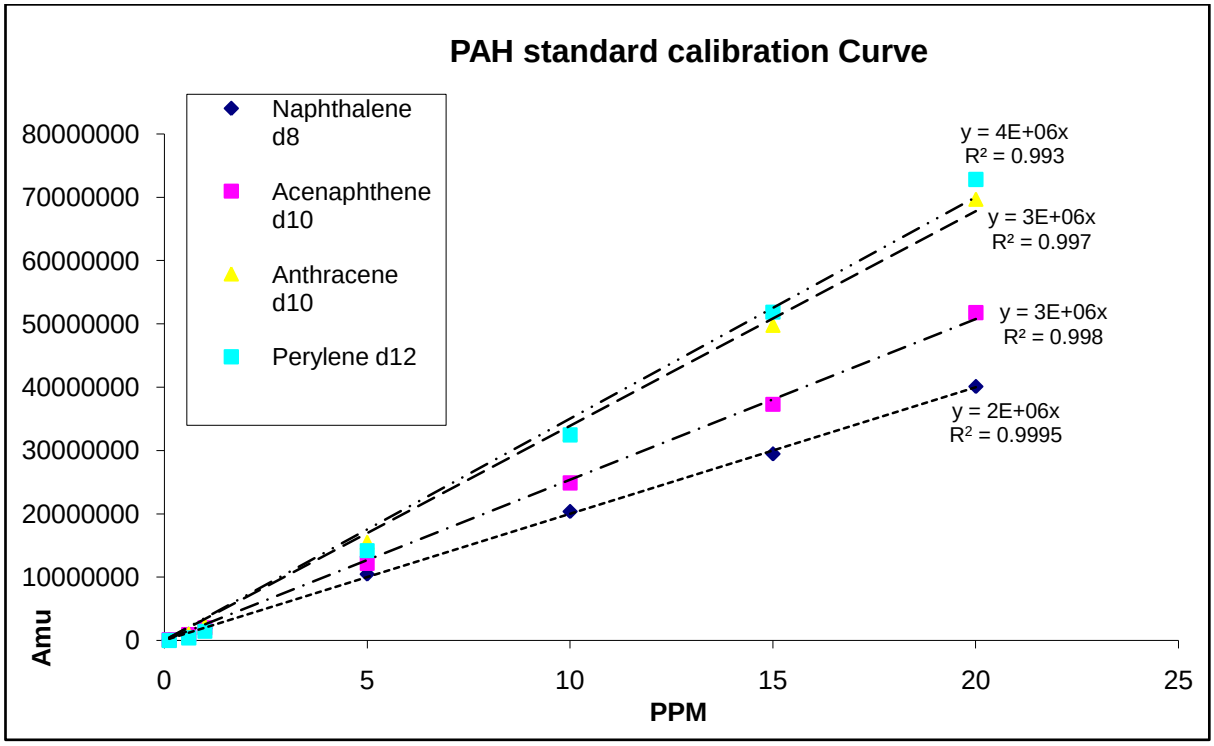

Figure 1a PAH calibration curves to determine LOD.

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Chapter 6

Rationale and Outcomes

Carbon is present in all known life forms and its cycling is crucial to Earths biosphere, it forms a diversity of inorganic and organic compounds, with almost 10 million compounds identified to date. Anthropogenic activities impact the environment around us, this impact is often measured by our "carbon footprint". Scientist have calculated our impact on climate due to increased CO_2 levels and the detrimental effects of carbon rich organic pollutants on terrestrial and marine ecosystems is well documented. However, "what is happening" to CO_2 and pollutants in soil is not well known. The work presented in this thesis represents for the first time, an approach to 1. Identifying and quantifying specific carbon compounds in soil, 2. Identifying the microbial populations degrading these compounds and 3. Actually determining what happens to this carbon in the long term.

The approach utilises GCMS, IRMS, compound specific stable isotopes, microbial taxonomy, biomarkers, XRF, elemental analysis, NMR analysis as well as complimentary microbiology techniques. This multidiscipline, holistic approach can provide and has provided a window into the complex interactions and fates of carbon in soil. The application of the approach is diverse, in this thesis alone it has allowed us to study a wide range of carbon compounds on land and in the sea.

As the thesis progressed the methods were adapted and developed further. Allowing the study varying pollutants and biomarkers which tell us more about how aspects of mankind's "carbon footprint" are impacting soils and sediments.

Chapter 2 gave an insight into the fate of glucose, acetate and carbon sequestered from CO_2 by soil microbial communities. Increasing CO_2 levels due to anthropogenic activities is one of the most critical issues facing mankind. Soil is a known store of carbon but have climate modellers underestimated the ability of microbial biomass in soil to fix excess CO_2? Results in chapter Chapter 3 would suggest so. The impact of another anthropogenic activity, agriculture and fertilizer practices, on soil carbon dynamics is assessed. Addition of sulphur as a fertilizer, to soil stimulated autotrophic activity and NMR confirmed that carbon sequestration increased 20 fold. This is highly significant and confirms that autotrophic activity in soil plays a crucial role in fixation of excess CO_2.

In Chapter 4 the approach was used to determine the impact of petroleum pollutants and sewage discharge on marine sediment of Dublin Bay. Biomarker analysis allowed us to determine the pollution state of the Bay. Microbial populations around

Howth Head and Dun Laoghaire were significantly affected by sewage discharge when compared to less affected areas. PAH levels were notably high with the R.Liffey appearing to be a diffuse source for pollutants entering the marine system. The results showed certain areas of the bay are under stress from waste discharge and urban runoff, action needs to be taken to improve treatment and capture of urban waste. The approach was also successful in identifying bacterial biomarkers which may be specific for the type of pollutant present, this warrants further research into potential pollutant degrading bacteria.

Oil spills are one of the most environmentally devastating incidents caused by human activities. Oil contains thousands of organic compounds many of which are damaging to plant, animals and humans. In chapter 5 we explore ways in which the developed approach can improve our assessment of spill sites. Can tracking carbon and using stable isotope studies reveal biodegradation and potential clean up approaches? The results in chapter 5 are not as desired due mainly lack of access to appropriate samples. However, this approach to spill sites represents a potential commercial application, through the development of biodegradation techniques and tools.

Overall the approach, methods and results presented in this thesis represent a novel and incisive way of assessing the impact of our "carbon footprint" on the world around us.

Poster Presentations

From Chapter 2

QUESTOR conference, Belfast, N. Ireland. May 2011

Conference on Analytical Sciences Ireland, Dublin. February 2011

25th International Meeting Organic Geochemistry (IMOG), Interlaken, Switzerland. September 2011

From Chapter 3

QUESTOR Conference, Dublin, Ireland. May 2013

From Chapter 4

Geoscience Seminar, Dublin, Ireland. November 2013

From Chapter 5

QUESTOR Conference, Cranfield, England. May 2012

Oral Presentations

From Chapter 2

QUESTOR conference, Belfast, N. Ireland. May 2010

From Chapter 3

Irish Mass Spec Society Conference, Dublin, Ireland. May 2014 (Note: Awarded prize for best student presentation)

From Chapter 4

Environ 2014 Conference, Dublin, Ireland. February 2014

Publications

From Chapter 2

Hart, K., Kulakova, A., Allen, C., Simpson, A., Oppenheimer, S., Masoom, H., Courtier, M.D., Soong, R., Kulakov, L., Murphy, B. and Kelleher, B. 2013. **Tracking the fate of microbially sequestered carbon dioxide in soil organic matter.** *Environmental Science and Technology*, 47(10), pp.5128-5137.

From Chapter 4

O'Reilly, S.S., Szpak, M.T., Flanagan, P.V., Monteys, X., Murphy, B.T., Jordan, S.F., Allen, C.C.R., Simpson, A.J., Mulligan, S.M., Sandron, S. and Kelleher, B.P. 2014. **Biomarkers reveal the effects of hydrography on the sources and fate of marine and terrestrial organic matter in the western irish sea.** *Estuarine, Coastal and Shelf Science*, 136(0), pp.157-171.

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From Undergrad

Wharton, M., Geary, M., O'Connor, N. and Murphy, B. 2011. **A rapid high performance liquid chromatographic (HPLC) method for the simultaneous determination of seven UV filters found in sunscreen and cosmetics.** . *International Journal of Cosmetic Science*, 33(2), pp.164-170.