Assessing the Role of Soil Chemoautotrophs in Carbon Cycling: An Investigation into Isotopically Labelled Soil Microorganisms

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For the Award of PhD

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Contributions

Chapter II

Fatty acid analysis carried out in collaboration with Dr. Vassilis Kouloumbos and Dr. Brian Moran in the School of Chemical Sciences, Dublin City University, an internal laboratory collaboration. Tomasz Piasecki and Dr. Mirek Macka, School of Chemical Sciences and National Centre for Sensor Research, DCU for the assistance in taking radiometer measurements.

Chapter III

Fatty acid and PLFA extractions and quantification was carried out by Dr. Brian Moran at the School of Chemical Sciences, Dublin City University, an internal laboratory collaboration. DGGE analysis was carried out in cooperation with Dr. Andrew Frazer at the School of Biological Sciences, Queens University Belfast, UK. With the exception of chemical pre-treatment, NMR analysis was carried out by Prof. Andre Simpson and his team at the Department of Chemistry, University of Toronto at Scarborough, Toronto, Canada.

Chapter IV

PLFA extraction and derivitisation was carried out by Dr. Brian Moran at the School of Chemical Sciences, Dublin City University, an internal laboratory collaboration.

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List of Abbreviations

DMOX	4,4-dimethyloxazoline
AS	Abbeyside Soil
AHY	Acid Hydrolysis
A	Adenine
ADP	Adenosine Diphosphate
Al	Aluminium
AOB	Ammonia-Oxidising Bacteria
NH_4Cl	Ammonium Chloride
$[NH_4]_2SO_4$	Ammonium Sulphate
a	Anteiso
As	Arsenic
AES	Atomic Emission Spectroscopy
AMU	Atomic Mass Unit
BHPs	Bacteriohopanepolyols
BHY	Base Hydrolysis
BLAST	Basic Local Alignment Search Tool
br	Branched
Ca	Calcium
$CaCO_3$	Calcium Carbonate
$CaCl_2$	Calcium Chloride
CO_2	Carbon Dioxide
MCO_3^{2-}	Carbonates
H_2CO_3	Carbonic Acid
CRAM	Carboxyl-Rich Acyclic Molecules
CsCl	Cesium Chloride
CSIA	Compound Specific Isotope Analysis
CW	Continuous Wave
CuO	Copper Oxide Oxidation
CP-MAS	Cross Polarisation Magic Angle Spinning
cy	Cyclopropane
C	Cytosine
DGGE	Denaturing Gradient Gel Electrophoresis
dNTP's	Deoxynucleotide Triphosphate
DNA	Deoxyribonucleic acid
DCM	Dichloromethane
H_2	Dihydrogen
DMDS	Dimethyl Disulphide
DMSO	Dimethyl Sulfoxide
N_2	Dinitrogen
O_2	Dioxygen
DS	Double Stranded
EC	Electrical Conductivity
S^0	Elemental Sulphur
ECIC	Environmental Carbon Dioxide Incubation Chamber
EtBr	Ethidium Bromide
FAMEs	Fatty Acid Methyl Esters
Fe^{2+}	Ferrous Iron
FA	Fulvic Acids
γ-HCH	Gamma-hexachlorocyclohexane
GCMS	Gas Chromatography Mass Spectrometry

GMD	Global Monitoring Division
GPP	Gross Primary Production
G	Guanine
G+C	Guanine-Cytosine
HP	Hampstead Park
HSQC	Heteronuclear Single Quantum Coherence
HMW	High Molecular Weight
HPLC	High Performance Liquid Chromatography
HR	High Resolution
HA	Humic Acids
HSs	Humic Substances
HCl	Hydrochloric Acid
HF	Hydrofluoric Acid
H_2S	Hydrogen Sulphide
ICP	Inductively Coupled Plasma
IR	Infra-Red
IPCC	Intergovernmental Panel on Climate Change
IS	Internal Standard
i	Iso
IRMS	Isotope Ratio Mass Spectrometry
KHz	Kilohertz
LCMS	Liquid Chromatography Mass Spectrometry
LMW	Low Molecular Weight
LB	Lysogeny Broth
MAS	Magic Angle Spinning
Mg	Magnesium
PPFD	Maximum Photosynthetic Photon Flux Density
CH_4	Methane
CH_3OH	Methanol
MeOH	Methanol
MeBr	Methyl Bromide
MeCl	Methyl Chloride
MTBE	Methyl Tert-Butyl Ether
MSM	Minimal Salts Medium
MS	Moscow Soil
NOAA	National Oceanic & Atmospheric Administration
NEP	Net Ecosystem Production
NPP	Net Primary Production
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NO_3^-	Nitrate
NMR	Nuclear Magnetic Resonance
OTUs	Operational Taxonomic Units
PPMV	Parts Per Million Per Volume
PLFAs	Phospholipid Fatty Acids
P	Phosphorus
PAR	Photosynthetic Active Radiation
PCBs	Polychlorinated Biphenyls
PAHs	Polycyclic Aromatic Hydrocarbons
PEG	Polyethylene Glycol
PCR	Polymerase Chain Reaction
$S_nO_6^{2-}$	Polythionates
FeS_2	Pyrite

qPCR	Quantitative Polymer Chain Reaction
rf	Radiofrequency
rRNA	Recombinant Ribonucleic Acid
RH	Relative Humidity
ROI	Residue on Ignition
RPM	Revolutions Per Minute
RNA	Ribonucleic Acid
RothC	Rothamsted Carbon Model
SOLID	Signs of Life Detector
Si	Silicon
SS	Single Stranded
SSU	Small Subunit Unit
NaOH	Sodium Hydroxide
NaOMe	Sodium Methoxide
$Na_2S_2O_3$	Sodium Thiosulphate
SOM	Soil Organic Matter
SON	Soil Organic Nitrogen
SPE	Solid Phase Extraction
TSE	Solvent Extraction
SIP	Stable Isotope Probing
SO_4^{2-}	Sulphate
S^{2-}	Sulphide
H_2SO_4	Sulphuric Acid
SELDI	Surface Enhanced Laser Desorption/Ionisation
TS	Teagasc Soil
T-RFLP	Terminal Restriction Fragment Length Polymorphisms
TBA	Tertiary Butyl Alcohol
$[SCN]^-$	Thiocyanate
$S_2O_3^{2-}$	Thiosulphate
T	Thymine
TE	Tris-EDTA
UV	Ultra Violet
USDA	United States Department of Agriculture
USGS	US Geological Survey
WHC	Water Holding Capacity

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Abstract

Recently observed increases in atmospheric CO_2 have created great interest in carbon capture technologies and natural sinks of this major component of the carbon cycle. Humic substances are a large, operationally defined fraction of soil organic matter. It was thought that humic substances consist of cross-linked macromolecular structures forming a distinct class of compounds. However, it was recently concluded by members of my research group that the vast majority of humic material in soils, are a complex mixture of microbial/plant biopolymers and degradation products, and not a distinct chemical category. The postulation that microbial inputs to soil carbon are greatly underestimated was put forward by my research group in 2007. Therefore, I have attempted to demonstrate the inputs made by soil chemoautotrophic bacteria. A method was developed where soil samples were measured for chemoautotrophic activity by subjecting them to a suite of scientific techniques. A growth chamber was used to propagate extant soil chemoautotrophic bacteria from different soils and subjected to an array of chemical and biological analyses. The growth chamber was used to measure CO_2 concentrations and introduce stable isotopic $^{13}CO_2$. Estimations of CO_2 sequestration were made using direct measurements for Irish soils and one Eurasian soil. Isotope labelled DNA was isolated using cesium chloride gradient ultracentrifugation. The dominant chemoautotrophic bacteria uncovered were *Thiobacillus denitrificans* and *Thiobacillus thioparus*. Labelled biomass was isolated and described using GCMS-IRMS and NMR, where an array of PLFAs, protein/peptide, carbohydrates and aliphatics were observed. Finally, an attempt to mimic common agricultural practice was performed to measure soil chemoautotrophic activity. This demonstrated the capability of this approach to benefit carbon flux estimations and hopefully in the future help to elucidate carbon flow into soils for the greater environment.

Chapter I: A Review of Soil Carbon Dynamics, Microbial Sequestration of Atmospheric CO_2 and the Tools used for the Analysis of these Phenomena

1.0 Abstract

Knowledge of soil organic matter transitions is vital to understanding carbon-cycling, soil microbiology and microbially mediated processes. At present, the body of evidence suggests that biogeochemical processes are mediated by microorganisms and yet our understanding of these microbes is still in its infancy. Various tools are at the disposal of scientists to investigate environmental samples but because of the baffling complexity, it is difficult to make accurate estimations of (real-time) *in situ* activity. For this reason, scientists have traditionally prepared and executed experiments in the laboratory (*in vivo*) to understand aspects of a particular system so that collectively, an understanding may eventually be reached. As techniques, technologies and knowledge has progressed, investigators have left the confines of the laboratory to perform their experiments *in situ*. This transition occurred because *in vivo* data was considered limited and in some cases biased. As the concerned project involves environmental samples that will be experimentally assessed within the laboratory, with the overall aim of developing a method that can be applied to any biological CO_2 sequestering system, it was necessary that the topic be fully explored. The following review attempts to document the new and existing knowledge, as well as current techniques used in measuring environmental samples. All the topics presented here are of relevance to the project but because of the wide degree of disciplines and techniques encountered, it was necessary to review each to a sufficient degree but not exhaustively.

1.1 Introduction

Soil, soil organic matter (SOM) and soil microorganisms play a significant role in the terrestrial biosphere of the planet. Of the many chemical and physical actions taking place within soil matrices, the cycling of the elements is a matter of considerable interest to the entire biological community. Soil microorganisms are key players in the fixation and mobilisation of the essential building blocks of terrestrial life, through both heterotrophic and autotrophic metabolic processes, as well as the Earth's biogeochemical cycles (Falkowski *et al*. 2008). The subject of soil chemistry is difficult to define as many disciplines are required to understand even the simplest processes. The research carried out by the internationally respected soil scientist, Prof. David Jenkinson FRS (25th February 1928 – 16th February 2011) was highly influential in setting the patterns of thought for diverse topics such as soil, agriculture and the environment. Prof. Jenkinson was the first to report the isotopic labelling of biomass to study its transformations upon degradation, incidentally using an incubation chamber (Jenkinson, 1971). Jenkinson took the revolutionary step of treating soil microorganisms as a single entity rather than using classic microbiological techniques and coined the term 'soil microbial biomass'. This new approach was akin to studying an entire forest rather than individual trees and has proved to be an excellent aid in interpreting data sets. Prof. Jenkinson was among the first to recognise the significance of the world's carbon stocks in the context of climate change. In response to the new scientific evidence indicating a recent change in the Earth's climate, together with James Rayner they developed the basis of the first mathematical carbon model, the Rothamsted carbon model (RothC; Jenkinson & Rayner, 1977) which had been developed to estimate carbon sequestration in many diverse settings. The RothC model has been used extensively to date (> 632 citations according to ISI Web of Knowledge^{SM}) and has been considerably updated since its inception. The contribution to soil science by Prof. Jenkinson and his colleagues has been beyond measure and it is hoped that the work described in this thesis contributes in some small way to deciphering the complex soil environment.

Certain species of soil bacteria are known to autotrophically fixate mineral forms of gaseous carbon and nitrogen to produce organic cellular matter via various biochemical enzymatic processes. Bacterial species such as cyanobacteria utilise oxygenic photosynthetic biochemical pathways to fixate atmospheric CO_2 and/or N_2 in

soil, freshwater and marine environments (Smith, 1983; Whitton & Potts, 2002; Madigan *et al*. 2009) providing the basis for trophic food webs. Photosynthesis (a biological oxidation processes) is the only known energy transduction process that is not strictly reliant on preformed bond energy (Falkowski & Godfrey, 2008). Other species known to survive autotrophically are the chemolithotrophs. These microorganisms from various genera use inorganic substrates to derive energy for biosynthesis reactions via aerobic or anaerobic respiration (Alfreider *et al*. 2009). These microbes are unique in their ability to derive energy from sources not related to solar activity and can be found in diverse locations both above and below the Earths crust (Pedersen, 2000; Sorokin & Kuenen, 2005; Amend & Teske, 2005; Alfreider *et al*. 2009).

The biological involvement in redox transformations of inorganic substances was first demonstrated by Winogradsky 120 years ago with his landmark work involving sulphur oxidation by the genus, *Beggiatoa* (Winogradsky, 1887). From this pioneering work, a considerable amount of knowledge has been gathered and crucially, the separation of energy and carbon metabolism had been demonstrated. Winogradsky described the significance of inorganic substrates in the central metabolism of an organism that resulted in the fixation of carbon into cellular matter. In regards to the modern world this is an important attribute of these diverse and ubiquitous organisms. As inorganic carbon fixation, resulting in the production of organic compounds, could potentially be of great benefit to the environment and mankind, most notably with the possibility of substantial atmospheric carbon sequestration.

Although a lot of research into autotrophic microorganisms has been carried out over the years, certain modern techniques have yet to be combined to increase potential knowledge in this field. For instance, research into environmentally significant bacterial species is rich and diverse and yet mostly confined to microbiological research laboratories. The combination of instrumental methods and microbiological methods has great potential benefit to both areas of research by combining molecular compound studies with identification techniques, thus linking ecology with function. The sequestration of carbon from the atmosphere and/or from anthropogenic sources is a topic of considerable interest to both the scientific community and the general public. Techniques that can potentially increase our knowledge base, while also providing some insight into carbon dynamics, are of great significance. In this review, it was my aim to

discuss carbon and soil dynamics, environmental microbiology (with a particular context towards soil autotrophs) and some of the techniques employed in measuring these complex, and sometimes baffling natural systems.

1.2 The Global Carbon Cycle and the Pedosphere

"In the sweat of thy face shalt thou eat bread, till thou return unto the ground; for out of it wast thou taken: for dust thou art, and unto dust shalt thou return."

[Genesis 3:19]

1.2.1 Introduction

The constant redistribution of matter through the elemental cycles is of the utmost importance to the environment (Berner, 2004). The global carbon cycle is a combination of naturally occurring processes such as photosynthesis, respiration, decomposition and deposition in which the central element to known life, carbon, is cycled between its major reservoirs e.g. atmosphere, aquatic, terrestrial biomes and living organisms. Carbon, along with various other elements is passed from one reservoir to another via biological, geological or climatic conduits resulting in changes to its chemical state and molecular complexity.

Organic carbon was deposited by carbonaceous comets and other stellar bodies early in the Earth's history (Anders & Owen, 1977; Anders, 1989; Chyba *et al*. 1990). The introduction of this element to the early Earth resulted in a multitude of chemical processes to occur, ultimately resulting in the emergence of life forms possibly as far back as the Haean era (Lunine, 2006). On a lifeless planet elemental cycles would still exist but the influence of life forms such as plants and microorganisms has had a dramatic acceleratory effect on these processes. For example, carbon dioxide (CO_2) in the Earth's atmosphere is removed largely by algae and green plants. It is then transformed into biomass providing the essential first link in the majority of food webs. The opposing process is known as mineralisation, in which the biologically bound compounds are released back into the environment in inorganic forms such as CO_2 or carbonates (MCO_3^{2-}; Manahan, 2000) and especially as carbonic acid (H_2CO_3; Tho & Ha, 1984; Veron *et al*. 2009). Although, small amounts of organic carbonates may also be formed such as dimethyl carbonate and ethylene carbonate (Abbas-Alli & Shaikh, 1996). Methane (CH_4) may also be released as an excreted metabolite gas resulting in further complex biogeochemical interactions. These cycles are more commonly referred

to as biogeochemical cycles due to influences from both the living and the non-living systems (Denman *et al*. 2007). It is usually simpler to describe these interconnected, yet radically different systems as the long-term and the short-term carbon cycles.

The long-term carbon cycle involves the inorganic mineral cycling of atmospheric CO_2. The accumulation of CO_2 in the atmosphere had resulted in dissolved carbonates (CO_3^-) building up in ancient oceans. The weathering of calcium (Ca), magnesium (Mg) and silicon (Si) bearing rocks due to dissolved CO_2 in rain water (resulting in the formation of carbonic acid [HCO_3^-]) lead to the consumption of CO_2 from the atmosphere over long geological periods (Horwath, 2007). The following equation describes this process:

Eqn. 1.0: $$2CO_2 + 3H_2O + CaSiO_3 \rightarrow Ca^{2+} + 2HCO_3^- + H_4SiO_4$$

The products of the above reaction are usually transferred to the ocean in a dissolved state via rivers where the subsequent reaction may occur:

Eqn. 1.1: $$Ca^{2+} + 2HCO_3^- \rightarrow CaCO_3 + CO_2 + H_2O$$

The overall effect of these chemical transformations results in the near permanent geologic storage of carbon via processes such as terrestrial weathering and marine carbonate sedimentation (Berner, 2004). The long-term carbon cycle largely involves the weathering of Ca, Mg and Si bearing rocks and the sedimentation of marine carbonates resulting in eventual subduction of $CaCO_3$ and organic matter. The carbon may eventually be returned to the atmosphere as CO_2 from active volcanic sites and/or tectonic fault lines (Irwin & Barnes, 1980; Giammanco *et al*. 1997; Faria *et al*. 2003). This process of carbon removal and reintroduction over large geological time periods has resulted in the controlled fluctuation of the atmospheric CO_2 content and ultimately the Earth's climate, due to the thermal properties of CO_2.

The evolution of vascular plants has contributed to the long-term carbon cycle due to the deposition of organic material into sediments. The burial of organic matter has led to the accumulation of large volumes of organic carbon deposits which eventually lead to the formation of fossil fuel sources. A good example of this is found in ocean ecosystems, where the "biological pump" takes the relatively small reservoir of

organic carbon (in the form of photosynthesising microorganisms) and moves it to the deep oceans upon their death whereupon sedimentation takes place. The carbonaceous material is oxidised at depth to form carbonates and eventually limestone deposits and other formations occur (Banuri *et al*. 2001; Gebauer *et al*. 2008; Zeebe *et al*. 2008). Also, as the photosynthesisers consume CO_2, the pH of the surrounding water is raised thereby enabling the precipitation of carbonates. The formation of petroleum and natural gas are common examples of this process but much larger amounts of hydrocarbonaceous kerogen (the organic matter content of oil shale), coal and lignite form. Anthropogenic utilisation of these carbon deposits has resulted in the rapid reintroduction of this stored carbon back to the environment in the form of mineralised CO_2 and as carbonaceous by-products (Manahan, 2000).

The short-term carbon cycle is dominated by two principle gases, CO_2 and CH_4. These gases interact with terrestrial and marine organisms providing the basis of various food chains. The functions of photosynthesis, respiration, organic matter and to a lesser extent chemoautotrophy play a fundamental role in the cycling of organic and inorganic carbon. The significance of these two gases has been brought to the forefront of modern environmental, climate and atmospheric research due to the fact that they are greenhouse gases (Le Treut *et al*. 2007). Changes in the short-term carbon cycle lead to fluctuations in the atmospheric composition of these particular gases. These gases absorb outgoing infrared radiation reflected from the Earth's surface, thus trapping heat and potentially increasing the average global surface temperature. Over long periods of time these gases have regulated the temperature of the Earth and acted as a planetary thermostat (Morton, 2007) creating dramatic fluctuations in the climate. It also must be pointed out that fluctuations in the Sun's energy output (Lean & Rind, 1999; Turck-Chièze & Lambert, 2007; Nandy & Martens, 2007; Livingston & Penn, 2009) and variations in the Earth's distance to the Sun have also contributed to climate change events throughout Earth's history (Smith *et al*. 1999; Nisancioglu *et al*. 2009).

Ecosystem carbon cycling involves the conversion of inorganic carbon (CO_2) into organic carbon via the process of gross primary production (GPP). This carbon is captured by plants and free living autotrophic microbes. Some of the carbon is rapidly returned to the atmosphere as respired CO_2, while the remainder is fixed into plant biomass and is known as net primary production (NPP). Net secondary production also takes place where NPP is consumed by heterotrophic fauna and microorganisms.

Therefore, the total stock of carbon in the planetary ecosystem is defined as the GPP minus the respiratory loss of autotrophs and decomposers and is termed the net ecosystem production (NEP; Horwath, 2007). The majority of NPP is consumed by free living microorganisms and fauna. The process of decomposition may take place over days to decades. Depending on the environmental conditions and the type of plant material entering the soil, selective preservation of some recalcitrant plant constituents (lignin) may prevail, leading to the formation of humic substances. Humic substances in the soil represent an important stable carbon pool and can potentially persist for thousands of years (Trumbore & Czimczik, 2008).

Carbon Reservoirs	**Metric tons carbon**	**Actively Cycled**
Atmosphere (CO_2)	6.7×10^{11}	Yes
Ocean		
Biomass	4.0×10^{9}	No
Carbonates	3.8×10^{13}	No
Dissolved and Particulate organics	2.1×10^{12}	Yes
Terrestrial		
Biota	5.0×10^{11}	Yes
Humus	1.2×10^{12}	Yes
Fossil fuel	1.0×10^{13}	Yes
Earth's crust	1.2×10^{17}	No

Carbon Source	**Flux (metric tons carbon / year)**
Fossil fuel combustion	7×10^{9}
Land clearance	3×10^{9}
Forest harvest and decay	6×10^{9}
Forest regrowth	-4×10^{9}
Net uptake via ocean diffusion	-3×10^{9}
Annual flux	9×10^{9}

Table 1.0: Global carbon reservoirs and net carbon flux between major reservoirs. Adapted from: Maier (2009).

Estimations of the global carbon reservoirs demonstrate that prior to 1860 the atmosphere contained approximately 260 ppm CO_2 (Horwath, 2007), but current measurements show that the global concentration to be approximately 390 ppm CO_2 (Tans, 2009). Measurements taken from the Vostok ice core (Petit *et al*. 1999; UNEP/GRID, 2008) have demonstrated the perceived global CO_2 concentration over the past 400,000 years shows a fluctuating pattern over a long geological timescale, with corresponding global average temperature measurements. Various other ice core data have also demonstrated that CO_2 atmospheric concentrations have remained

between 180 ppm (glacial minima) and 300 ppm (glacial maxima) for the last 650, 000 years (Retallack, 2001; Ahrens, 2008). According to Maier (2009), current estimates of stored carbon in the terrestrial environment, accounts for approximately 1.2 x 10^{17} metric tons in comparison to the oceans 4.0 x 10^{13} metric tons (Table 1.0). This estimate indicates the importance of the terrestrial biosphere to carbon storage.

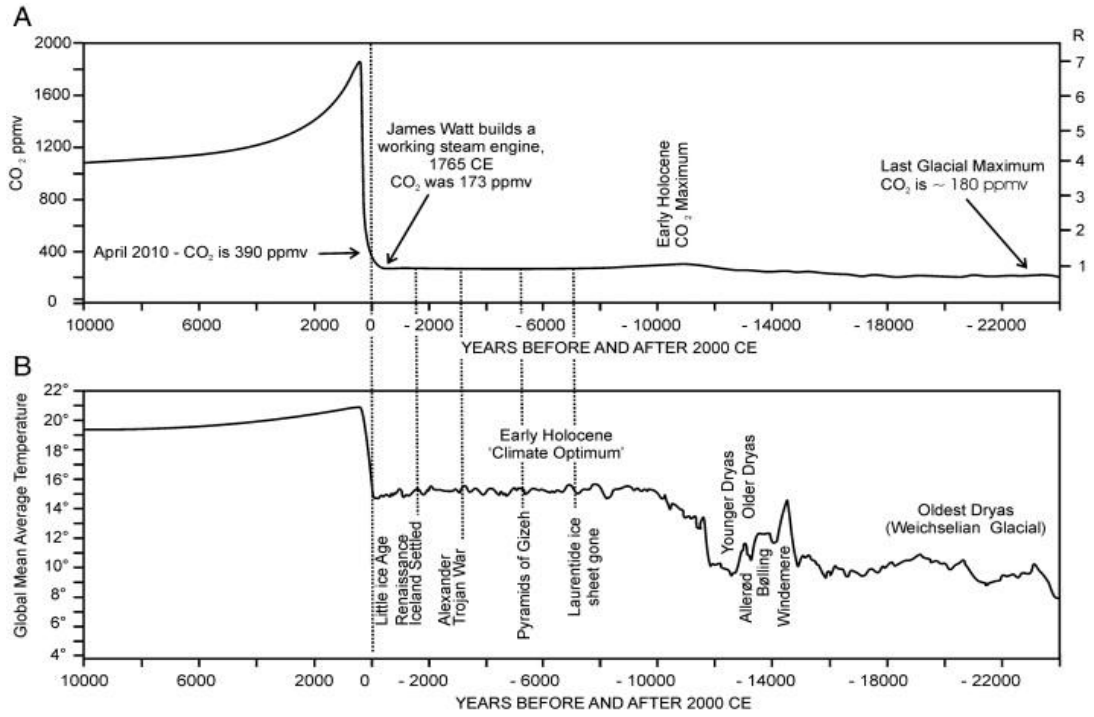


Fig. 1.1: Results of the CLIMBER model for atmospheric CO_2, based upon the burning of 5000 Gtons of fossil fuels in known reserves by 2300. A) Past and future levels of atmospheric CO_2 put into a historical context as far back as 24,000 years ago. B) Past and future global mean annual surface temperatures put into a historical context back to 24,000 years ago. The dotted lines intersecting the two plots indicate important periods and milestones in human history. Image taken from: Hay (2011).

Data similar to that from Fig. 1.1 is currently being used to demonstrate a hypothesis that the planet is undergoing a climatic shift towards higher temperatures with the period 1979-1990 being one of the warmest periods on record (Ahrens, 2008), with CO_2 and CH_4 implicated as a major factor in these estimations of rising surface temperatures (Hussain & Ansari, 2007). It is widely suspected that past anthropogenic activities, such as the industrialisation of Europe and the USA (~1750 AD to present), is largely responsible for this current spike in global atmospheric carbon stocks. CH_4 concentrations have also seen a dramatic rise in atmospheric concentration rising from about 700 ppb in 1775 (Flückiger *et al*. 2002) to 1775 ppb in 2005 (Denman *et al.* 2007) for both hemispheres according to NOAA/GMD data (Manning *et al*. 2011). It appears that the burning of fossil fuels can have an immediate impact on the global carbon cycle

which has led to an accelerated disturbance in the cycling of carbon at the global scale (Horwath, 2007). However, it is now known that weather does vary on long time scales and therefore climate is variable (Keeling, 1997).

There is continual and dynamic exchange of carbon stocks between all of the major biospheres. The carbon cycle consists of a biogenic input/output regime in which living organisms within various ecosystems utilise inorganic carbon within cellular matter. These carbon atoms can then be transferred throughout a complicated food web. The cellular carbon may be transferred from one organic form to another until ultimately degraded into an inorganic form, usually CO_2 and returned to the atmosphere. A simplified version of the carbon cycle may be observed in Fig. 1.2.

The importance of carbon to the biosphere lies in the fact that solar energy is utilised by biological organisms to produce organic carbon. The organic carbon is contained within energy rich molecules that can be biochemically combusted with molecular oxygen (O_2) to regenerate CO_2 and in the process produce energy. This process can occur both biochemically within an organism via aerobic respiration or through chemical combustion (Manahan, 2000).

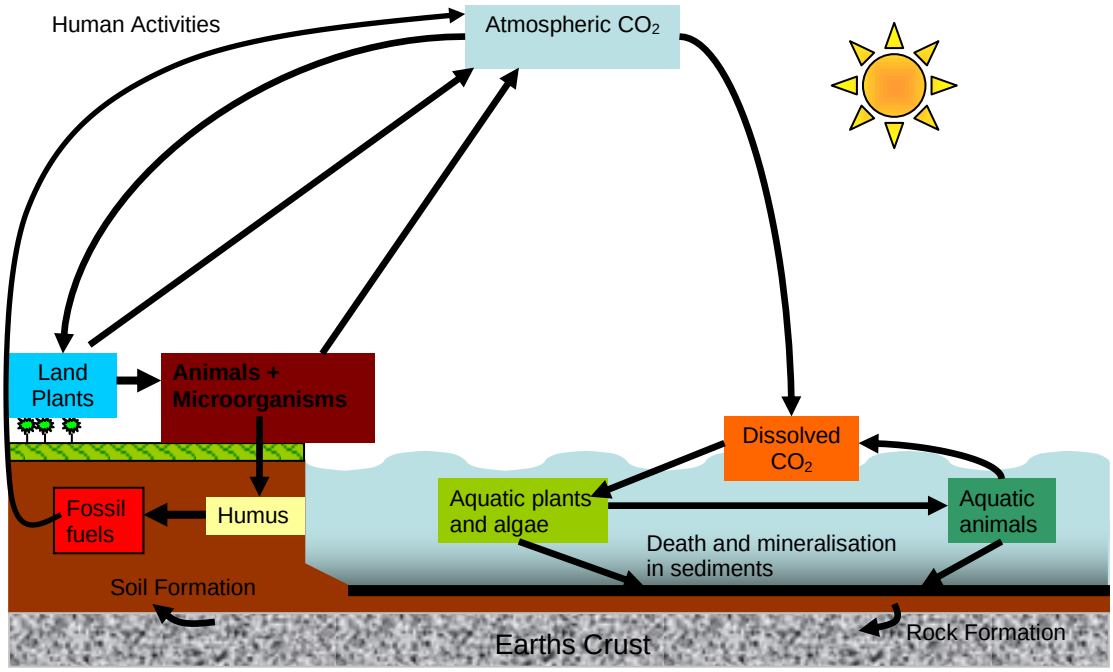


Fig. 1.2: The global carbon cycle. Adapted from: Madigan *et al.* (2009).

The increases of atmospheric CO_2 have been attributed to mostly point sources e.g. fossil fuel combustion and land/forest clearance. Anthropogenic activities in the last 150 years have therefore been attributed to the net transfer of terrestrial carbon to the atmosphere (Falkoski *et al*. 2000). The input of CO_2 to the atmosphere has been partially offset by the continuous net uptake of carbonates as sedimentary rocks within the oceans. According to current knowledge, approximately 20% of the carbon produced in the last 100 years has not been accounted for in the current carbon estimates (Denman *et al.* 2007). It is possible that terrestrial carbon sequestration has been underestimated and that higher rates of photosynthesis and water usage efficiency have been instigated by the higher CO_2 concentrations or higher surface temperatures. The deposition of nitrogenous compounds in the wider environment may also have lead to increased plant primary production (Schlesinger & Andrews, 2000; Trumbore & Czimczik, 2008; Brantley, 2008). However, to counter these assumptions of increases in CO_2 uptake, higher global temperatures would also favour an increase in the rate of decomposition by microorganisms and thus, increasing CO_2 production. It may also be feasible that microbial uptake of CO_2 in the terrestrial ecosystem has been underestimated. Several studies cite that autotrophic microbial uptake is minor when compared to rates of microbial respiration (Miltner *et al*. 2004). The presence of microorganisms has greatly influenced the carbon cycle as they mediate the major chemical reactions. Photosynthetic algae are the dominant species responsible for carbon fixation in aquatic ecosystems. Heterotrophic microorganisms such as chemo-organotrophic bacteria, fungi and protists are chiefly responsible for the degradation of organic and inorganic matter, ultimately resulting in mineralisation.

1.2.2 The Pedosphere

The pedosphere is the thin covering of soil and sediments (naturally occurring, unconsolidated rock particles and organic matter) that overlay most of the habitable terrestrial land masses (Voroney, 2007). Pedology involves the studying and analysing of soils and the various soil processes that influence both its physical and chemical characteristics. Soils are a biologically active and complex mixture of weathered minerals, organic matter, organisms, air and water in which the provisions for life in the terrestrial ecosystem are attained (Brogan *et al*. 2002). Soil is an integral part of the Earth's biosphere and provides a terrestrial link between microorganisms, plants and higher animals (Radojevic & Bashkin, 1999). It is not simply a sum of its mineral and organic matter content but a product of their interactions (Noorallah, 1999). The

pedosphere plays a fundamental role in the cycling of atmospheric gases, nutrients, toxins, metals and the central element to life; carbon. These chemical and physical processes of cycling, transporting and converting compounds to labile or recalcitrant forms are vital for the health of the biological environment including human agricultural processes as well as the global elemental cycles. Due to the long-term time scales involved in the production of soil within the pedosphere, it can be considered a non-renewable natural resource (Hassett & Banwart, 1992) and therefore careful management and protection is required.

The constituents of soil vary widely in regards to components and their abundances thus providing them with various different appearances and structures. Within the soil structure, minerals, organic compounds and microorganisms are among the major solid components of soils and these components are constantly interacting with one another to act as a unified system (Huang *et al*. 1995). The association involving microorganisms interacting with soil minerals and organic matter is depicted in Fig. 1.3.

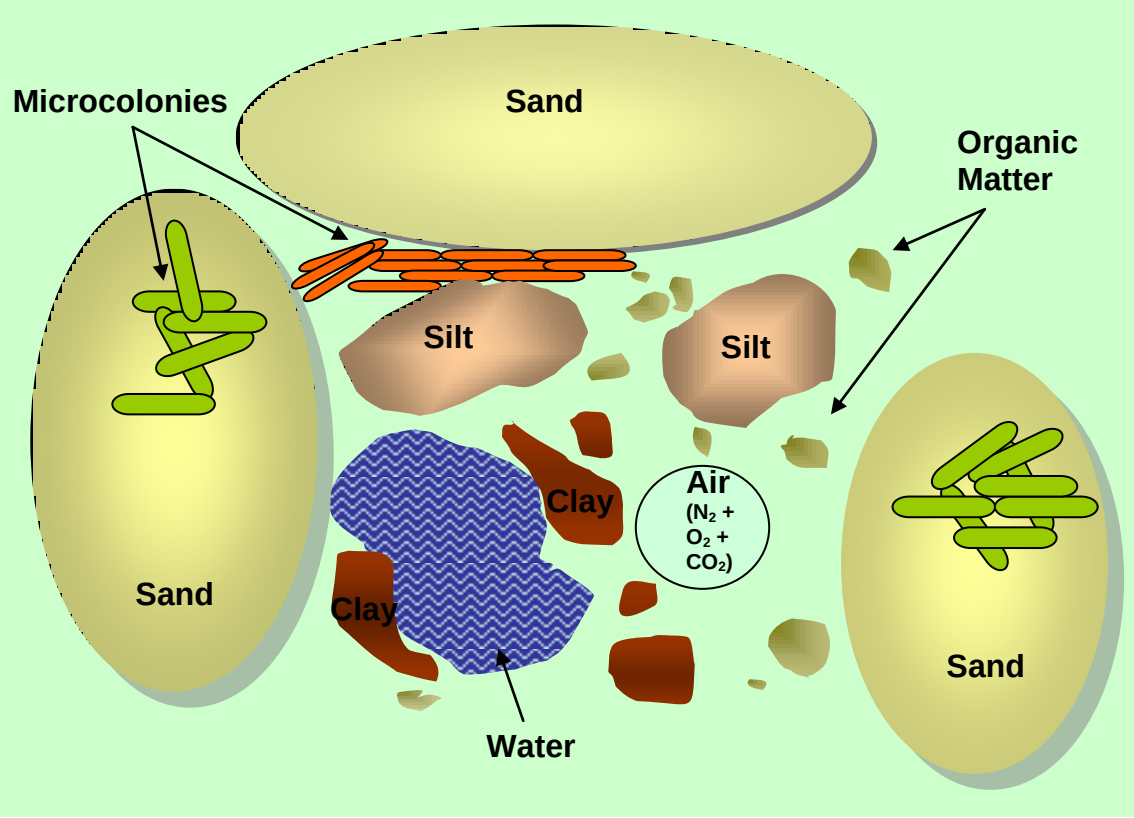


Fig. 1.3: A Soil Microbial Habitat. Taken from: Madigan *et al.* (2009).

According to Lynch (1983), the principal inorganic particle sites of soil are sand, silt and clay. The sizes of these particles in relation to microorganisms are given in Table 1.1. The presence of clay in the soil matrix is an important factor when considering microbiological productivity, due to the electrically charged nature of both clay particles and bacteria equipped with extracellular polysaccharides on their cell wall (Fig. 1.4). Both clay particles and bacterial cells carry a net electronegative charge. The principle clays found in most soils are kaolinite, illite and montmorillonite. These clays carry charges of about 5, 20 and 100 milliequivalents 100 g^{-1} respectively (Fletcher *et al*. 1980). When any surface has become charged it will attract oppositely charged ions from its aqueous surroundings, against the thermal motion of the counter ions (a tendency to evenly distribute ions throughout the solution). Therefore, the region in close proximity to the charged clay particle is richer in counter ions than in the rest of the aqueous phase. As bacterial cells are also electronegatively charged their interaction is governed by a 'bridge' such as a metal ion or interaction is dependent on the cell and the clay becoming polarised (Foster, 1988).

	Diameter or Thickness (µm)
Inorganic constituents	
Sand	50-200
Silt	2-50
Clay	< 2
Microorganisms	
Bacteria	0.5-1.0
Actinomycetes	1.0-1.5
Fungi	0.3-10.0

Table 1.1: Sizes of soil constituents. Adapted from: Lynch (1983).

The surface area of these soil particles are of great importance when considering the density of populations each particle can maintain. Soils have specific surface areas that have a high degree of variability dependent on their texture and mineralogy. Therefore, soil colloids are considered to be surface-active particles. The bacterial population attributable to the total surface area is in fact quite small when compared to the total external surface area of the soil particle and if the entire bacterial population were to be spread out over the surface it would cover only a small area (Table 1.2).

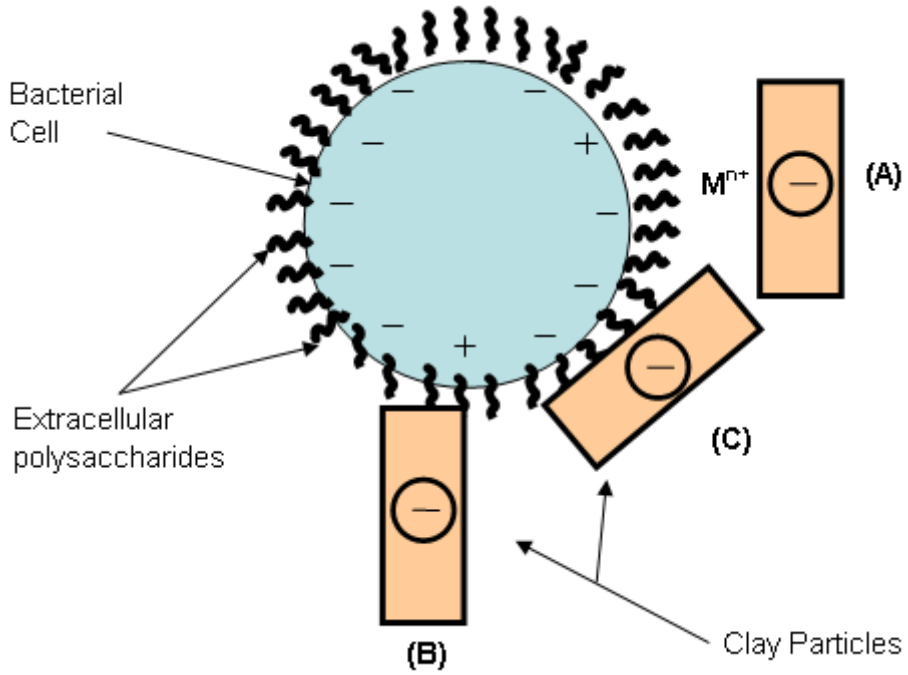


Fig. 1.4: Bacterial cells with a coat of extracellular polysaccharides (EPS) are enveloped by clay particles. Clay particles adhere to the cell surface by bridging through polyvalent cations, represented by M^{n+} (A), although some may be attached directly by electrostatic interactions, either in a face-to-face (B), or edge-to-face (C) association. Adapted from: Theng & Orchard (1995).

Soil carbon consists of a complex mixture of organic and inorganic compounds. Inorganic compounds such as calcium carbonate ($CaCO_3$) are present in the form of minerals and act as buffering agents maintaining soil pH. Organic carbon comes in a wide variety of forms consisting of both living and dead matter. In general, soil organic matter (SOM) is a generalised term that encompasses a large group of compounds, micro- and macroorganisms. In regards to the topic under discussion, SOM shall relate to humic substances (an umbrella term describing a large array of persistent organic molecules), labile organic substrates (carbohydrates, proteins etc) and microorganisms.

Characteristic	Dominant texture		
	Sand*	Silt†	Clay‡
External surface area (m^2 g^{-1})	3	17	60
Total surface area (m^2 g^{-1})	3	55	208
Cation-exchange capacity (cmol kg^{-1})	3.6	13.3	34.5
Porosity (cm^3 g^{-1})	0.21	0.40	0.51
Accessible porosity filled with water at -0.01 MPa (cm^3 g^{-1})	0.07	0.17	0.17
Surfaces developed by bacteria**			
In % external surface area of soil	2.26	0.40	0.11
In % total surface area of soil	2.26	0.12	0.03
Volume of soil bacteria (cm^3 g^{-1} soil)**			
In % soil porosity	0.79	0.41	0.32
In % accessible pore space at -0.01 MPa	2.42	0.97	0.95

* Sandy soil (sand = 79%, silt = 15.3%, clay = 5.7%)
† Orthic luvisol (sand = 33.3%, silt = 50.3%, clay = 16.5%)
‡ Calcisol (Sand = 3%, silt = 28%, clay = 69%)
** A bacterial population of 10^{10} bacteria g^{-1} soil is assumed; with cells being 1 μm long and 0.5 μm in diameter. The total surface area of the bacterial population is then 0.0157 m^2 g^{-1} soil, and the volume of the population is 0.0016 cm^3 g^{-1} soil.

Table 1.2: Selected characteristics of common soil types with different textures in relation to their potential loading capacity for bacteria. Taken from: Huang *et al*. (1995).

Humic substances (HS) are the most widespread and ubiquitous organic material in the terrestrial and aquatic environment and represent the major fraction of SOM (≥ 80%; Schnitzer, 1991). Humic substances have a considerable effect on the colour of soils, usually giving them their dark brown to yellow appearance. Constituents of HS such as humic acids (HA), fulvic acids (FA) and humin are a relatively stable component of SOM and are closely related to the structural and chemical characteristics of the overall organic matter component of soil (Schulten, 1994; Kögel-Knabner, 2000). These classificational names are associated according to the separated fractions. HA is soluble in alkali but insoluble in acid, FA is soluble in both alkali and acid whereas humin is insoluble in either. For this reason these names should be considered operational terms only and used to distinguish the seperatable fractions of HS according to their solubility in aqueous solvents (acid/base; Senesi & Loffredo, 2001). It is indicated that the nature and stability of these substances within the soil, affect carbon and nitrogen cycles and carbon sequestration (Santín *et al*. 2008). HS are produced via a process collectively known as ‘humification’. The decay of plant and animal derived

material by macroorganisms (earthworms, beetles, termites etc.) and microorganisms (fungi, bacteria etc.) result in a series of metabolic pathways that break down the complex organic compounds into simpler molecules. The final product of this breakdown process is 'humus', an important soil colloid (Rose, 2004). Humus plays a vital role in the storage of water and cations due to its negative charge; these are essential characteristics for soil quality and plant growth. There are a number of different pathways proposed for the formation of HS in soil (Fig. 1.5). It is most likely that all of the pathways in Fig. 1.5 play an active role in the formation of HS in soils but depending on various environmental factors, one pathway may be prominent for a particular area. Examples of this can be observed when lignin is the predominant precursor of HS in poorly drained soils or polyphenols synthesised by microorganisms may be predominant in forest soils (Senesi & Loffredo, 2001).

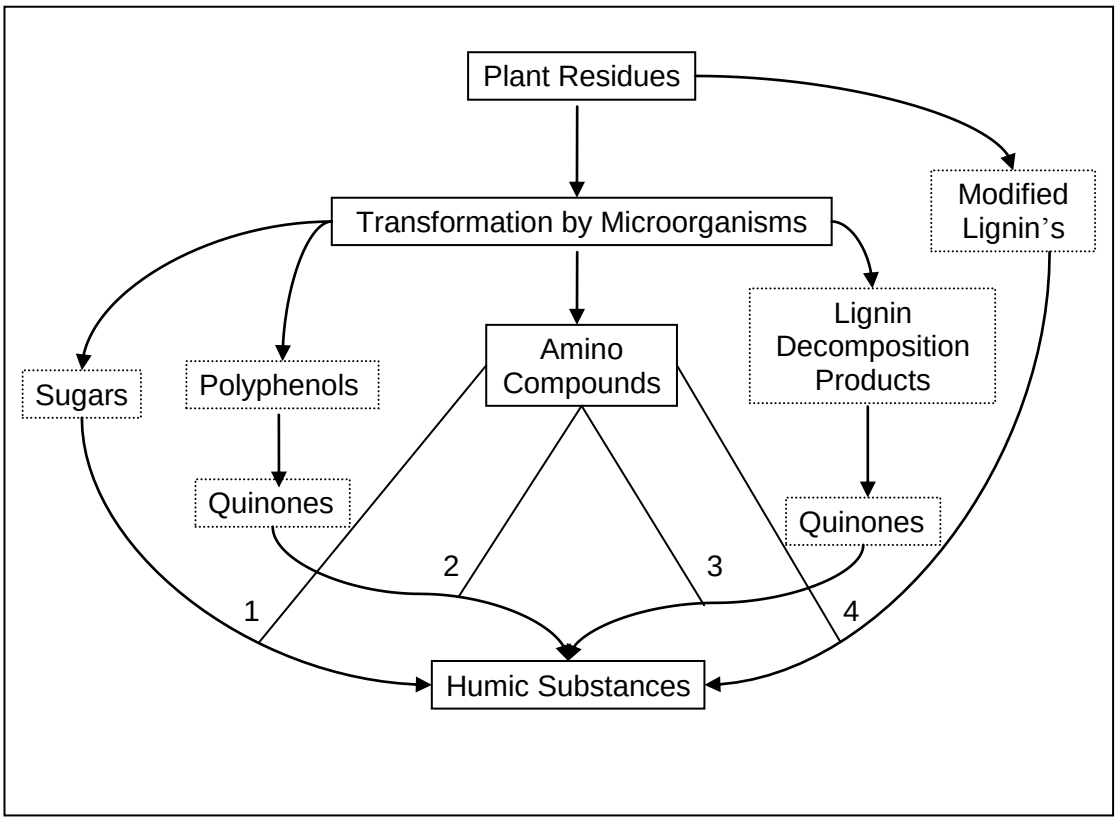


Fig. 1.5: Major pathways proposed for the formation of soil humic substances where pathway 1 represents the reducing of sugars and amino acids formed as by-products of microbial metabolism, presumed to be the only precursors of HS. Pathway 2 represents the so-called 'polyphenol theory' which involves polyphenols and quinones derived from lignin or synthesised by microorganisms (Pathway 3). Pathway 4 represents the so-called 'lignin-protein theory' in which plant lignin acts as the main source of soil HS with the involvement of amino compounds via microbial synthesis. Adapted from: Stevenson (1994); Senesi & Loffredo (2001).

An important study carried out by Schulten (1994) using analytical pyrolysis, namely, Curie-point Py-GCMS, described the chemical building blocks of HS (Fig. 1.6) and provided a molecular chemical basis for the structural modelling of HS in which aromatic rings are connected by long-chain alkyl structures (Schulten & Schnitzer, 1992). Otto & Simpson, (2007) used four different extraction techniques; solvent extraction (TSE), base hydrolysis (BHY), acid hydrolysis (AHY) and copper oxide oxidation (CuO) followed by GC-MS and solid-state ^{13}C cross polarisation magic angle spinning (CP-MAS) NMR, to show the content of SOM. They determined that the predominant classes of SOM were polar and high molecular weight (HMW) compounds. Aliphatic lipids, (62%), phenols and benzyls (17%) were the most abundant species, accompanied by small amounts of low molecular weight (LMW) acids, mono-, disaccharides, terpenoids, steroids, amino acids and amino sugars. The authors point out that the AHY extraction contained organic matter predominantly associated with microorganisms, while the other methods were associated with plant derived materials. According to Jeannotte *et al.* (2008), another useful extraction method for microbially derived fatty acid esters (microbial biomarkers) was a pressurised mixed solvent extraction system. It was observed that using a relatively simple extraction technique and GC analysis the characterisation of the fatty acid content of soils could be achieved, but determining fatty acids from microbial origin proved difficult. It was also highlighted that quantification of the fatty acid content is difficult as spiking methods are susceptible to immediate microbial negative interference due to metabolisation of the spiked material. It was theorised that real soil lipids are relatively stable due to physical and chemical interactions and thus resist microbial breakdown.

It is well known that organic matter and HS have a large positive impact on soil fertility (Kononva, 1966) and biodiversity. The nutritional aspects of SOM and HS are of prime importance and one of the most studied soil fertility functions (Senesi & Loffredo, 2001). The major importance of SOM and HS is the ability to accumulate and dispense nutrients to the surrounding flora and fauna by immobilisation and subsequent mineralisation of complex organic polymers. The release of nitrogen (N), phosphorus (P) and sulphur (S) is carried out mainly by biological processes, whereas the release of macro- (calcium, magnesium and potassium) and micro-nutrient (manganese, copper, iron and zinc) cations are from physio-chemical processes. According to Stevenson (1986), SOM generally contains ≥ 95% of the N and S, and 20-75% of the P in surface

soil. However, according to a recent publication by Simpson *et al.* (2007a) it has been estimated that microbial biomass (alive and dead) accounted for > 80% of the soil N. Through the application of advanced 1-D and 2-D NMR spectroscopy Simpson *et al.* (2007a) also estimated that extractable SOM may in fact consist of mostly microbially derived organic matter. This research concluded that rather than the accepted value of microbial derived soil organic matter being < 5% (Jenkinson & Ladd, 1981; Dalal, 1998) that in fact, the figure should be > 50%.

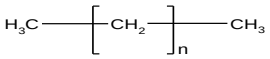
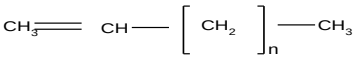
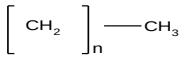
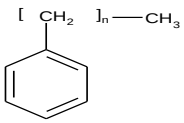
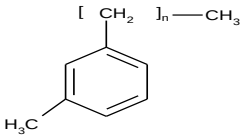
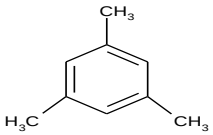
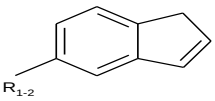
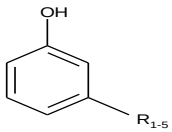
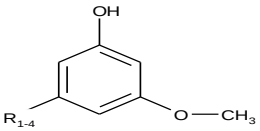
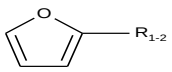
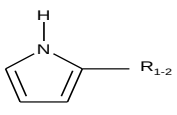
Structure	Name
	Alkanes (n = 2 - 29)
	Olefins (n = 1 - 28)
	Alkylbenzenes (n = 0 - 17)
	Methyl-alkylbenzenes (n = 1 - 11)
	Methyl-substituted benzenes
	Methyl-substituded indenes
	Methyl-substituded phenols
	Methyl-substituted methoxyphenols
	Methyl-substituted furans
	Methyl-substituted pyrroles
	Methyl-substituted pyridine

Fig. 1.6: Principle chemical structures of soil HA identified by Curie-point pyrolysis-gas chromatography / mass spectrometry. Taken from: Schulten & Schnitzer (1992).

1.3 Environmental Microbiology

"The whole history of the world, as at present known, although of a length quite incomprehensible by us, will hereafter be recognised as a mere fragment of time, compared with the ages which have elapsed since the first creature, the progenitor of innumerable extinct and living descendants, was created"

[Charles Darwin (Darwin, 1859)]

1.3.1 Introduction

Environmental microbiology is an umbrella term used to describe various fields of microbiological research, with an emphasis placed upon the study of the physiology and composition of microbiological life forms found in both natural and artificial ecosystems. The origins of environmental microbiology are attributed to the observations made by Antonie van Leeuwenhoek, whose 1677 paper described the creatures which he termed "animalcula" (little animals) discovered by himself in environmental water samples using a homemade microscope (van Leeuwenhoek, 1677). Later, the discovery of autotrophic bacteria by Sergei Winogradsky in the 1800's (Winogradsky, 1887) was a significant event for environmental microbiology as prior to this event, only medical aspects (pathogenic bacteria) had been scientifically investigated. From the pioneering work of these scientists, amongst many others, a large volume of knowledge has been accumulated in regards to environmental ecosystems and the impact that the very small can have on the very large.

In the modern research world, much emphasis has been placed upon the study of prokaryotic microorganisms such as the bacteria and archaea. Prokaryotes form a taxonomic group of their own consisting of single celled organisms, which do not have membrane bounded internal structures. These single celled units of biological life represent a hugely diverse and environmentally significant cluster of organisms. It has been the metabolic activities of these organisms that have altered the biosphere throughout the ages and subsequently produced the O_2 rich atmosphere that we exist in today. Microbes in some as yet unknown form are believed to be the originators of life on this planet (Giulio, 2003; Tirard *et al*. 2010) and it is accepted that they are vital for its continuance as demonstrated by the biogeochemical cycling of the elements (Falkoski *et al*. 2008). It is the chemical complexation and transformation of the elements that are essential for life, which is facilitated for at its basic level, by the actions of microorganisms.

The adaptability and diversity of microbiological life means that locations such as soils, oceans, fluid vents, sewage processing and agricultural sites are areas in which environmental microbiological research has been directed, with important industrial and medical applications being discovered. For instance *Corynebacterium glutamicum* is one of the most important bacterial species to the biotechnology sector with an annual production of more than two million tons of amino acids, mainly L-glutamate and L-lysine (Udaka, 2008). The diversity of microbiological life continues to provide research with ample new discoveries. Microbes can be found in glacial ice sheets (Abyzov *et al*. 2004), within the pores of bedrocks kilometres deep (Pedersen *et al*. 1996) and the fumaroles of volcanic vents (Castaldi & Tedesco, 2005). It would appear that microorganisms can be found active in any site where liquid water can exist including within channels of sea ice where the salt content prevents complete freezing along "brine channels" (Hurst *et al*. 2007). In the context of this project, soil microbiology was the main area of focus, with a special emphasis on the autotrophic prokaryotes such as the sulphur oxidising chemoautotrophic *Thiobacillus* genera (Chemolithotrophic prokaryotes) of the Proteobacteria lineage.

1.3.2 Soil Microbial Diversity

Prokaryotes are best known for their pathogenic properties and ability to thrive in diverse habitats. Only a small proportion of the known species of prokaryotes are in fact pathogens towards higher life forms, such as the plant and animal kingdoms. Most exist in complex food webs or specialist niches where symbiosis, competition, scavenging, parasitism and predation determine the success of individual species. Estimations of the total number of microbial cells on Earth are somewhere in the order of 5×10^{30} cells (Whitman *et al*. 1998). This represents an amount of carbon, within these cells, roughly equal to that found collectively in all the plants on Earth (with plant carbon far exceeding that of animal carbon; Madigan, *et al*. 2009). Interestingly our current level of knowledge on prokaryotes is based upon measurements carried out on > 5000 isolated species (Bull *et al*. 1992; Madsen, 2005). This current estimate represents ~0.1% of the diversity of prokaryotes in the Earth's biosphere (Pace, 1997; Curtis *et al*. 2002), however the possible number of prokaryotes is so large that the task of counting them may be an endless one (Ward, 2002).

The habitats available to life on Earth provide diverse and complex environments where conditions can include extreme variations in temperature, moisture,

salinity, pH, light, pressure and the availability of both organic and inorganic compounds. Each geochemical habitat presents its own varying set of resources available to microorganisms and the expanse of evolutionary time has allowed for the genetic diversity we observe today in all species (Madsen, 2005). It is this genetic diversity that provides prokaryotes the ability to exploit so many habitats and hence, much research is being carried out trying to identify and elucidate these highly industrious and essential microorganisms.

The metabolic diversity of prokaryotes provides for a wide range of enzymatic reaction pathways that can be harnessed in the fields of bioremediation, industrial processes and biotechnology. It is already well established that lactic acid producing bacteria such as the genera *Lactobacillales* have been used by man for centuries in the production of yogurts and cheeses. Recent attention has focused upon the fact that highly polluted areas such as mines, industrial sites and oil spillages can be effectively cleaned up using a low input and cost efficient manner by allowing microbes to perform their natural functions (Watanabe, 2001; Wolicka *et al*. 2009; Santos *et al*. 2011) or by applying genetically modified species to the site of interest (Sayler & Ripp, 2000; Dutta *et al*. 2003). Bioleaching of metals from metal bearing ores (Shi *et al*. 2006; Giaveno *et al*. 2007) and waste streams (Chen *et al*. 2005; Gu & Wong, 2007) are both considered to be a cost effective and an efficient method in the collection of precious or polluting materials from the environment. The biotechnology sectors currently employ microbes to perform a huge number of commercially important processes in the form of chemical products for medicine, industry, agriculture, commodities and recycling. The reader is encouraged to see Gavrilescu & Chisti (2005) for an excellent overview of biotechnology and its applications.

Soil is a huge reservoir of carbon on the planet's surface and subsequently is a perfect home for prokaryotes. Prokaryotes are an essential component of the soil decomposition process, where plant and animal residues are decomposed into small substituents and released into the surrounding food webs as nutrients and organic matter. At present, it is estimated that terrestrial soils contain 2.6×10^{29} cells (Whitman *et al*. 1998). It is generally accepted that SOM consists of approximately 1-5% of live or recently dead microbially derived organic matter (Jenkinson & Ladd, 1981; Dalal, 1998). Evidence has recently come to light that estimated microbial biomass numbers are too low and that in fact extractable microbial biomass contributes > 50% of SOM

(Simpson *et al*. 2007a). The large diversity of bacterial species within soil matrices provides for a complex array of heterotrophic and autotrophic single celled taxonomic groups all competing for space, nutrients, organic matter, light etc; while at the same time competing with a vast array of eukaryotic species such as fungi, protists, algae and macroorganisms. The sheer diversity of species and their role within the soil biosphere places limits on the ability of this review to describe them adequately, therefore, the reader is referred to Buckley & Schmidt (2003), Madsen (2011) and Zhang & Xu (2008) for an extensive introduction into environmental microbiology. The detection and measurement of the activities of autotrophic bacteria within the soil biosphere is the primary concern of the project and therefore will be discussed in more detail.

1.3.3 Bacterial Autotrophy

Autotrophic nutrition involves organisms that can synthesise the organic materials they require from inorganic sources. The primary source of carbon and nitrogen is CO_2 and nitrates (NO_3^-) respectively (Dalal, 2005). Energy requirements can be harvested from sunlight (photosynthesis) or chemical sources (chemosynthesis). As a biological phenomenon, autotrophy is constrained to the green plants (including algae) and autotrophic microorganisms. The work carried out by Winogradsky (1887), where he observed nitrifying and purple sulphur bacteria growing with CO_2 as the sole source of carbon were the first reports of self-sufficiency at the cellular level. Winogradsky had also shown that bacteria are active in the cycling of nitrogen and sulphur, and therefore linked to specific biogeochemical transformations. It was also Winogradsky who proposed the concept of chemolithotrophy; the biochemical oxidation of inorganic substances linked to energy conservation. This was done by careful observations of bacterial nitrification (the oxidation of ammonia to nitrate) and by showing that the organism responsible (the anaerobe *Clostridium pasteurianum*) acquired its carbon from CO_2. Therefore, it was demonstrated that like phototrophic organisms, the nitrifying bacteria were also autotrophic. Winogradsky and his contemporaries such as Beijerinck (who pioneered the enrichment culture technique; Madigan *et al*. 2009) both worked in the field of agricultural microbiology and thus helped in our understanding of microbial processes in the soil, with nitrogen fixation being but one example. The study of soil microorganisms later led to the discovery of the antibiotic Streptomycin and many other commercial products (Smith, 2000).

Bacterial autotrophy has been subdivided over the subsequent years to distinguish between the many diverse groups within the bacterial lineage. For instance, phototrophic prokaryotes can be distinguished according to their metabolic features or choice of habitat, as some species can survive in the presence of O_2 (oxygenic phototrophs) while others can not (anoxygenic phototrophs). Indeed, species such as the cyanobacteria were originally associated more closely to algae because of their ability to produce O_2 during photosynthesis, like that of higher plants and were formally known of as "Blue-Green Algae" within the plant kingdom as the class Cyanophyceae (Hurst *et al*. 2007; White, 2007). The diversity of autotrophic prokaryotes is large and the differences between the physiological groups are distinct, thus enabling these bacteria to exploit many niches. Fig. 1.7 displays the known groups of autotrophs (in coloured boxes) according to the major lineages (phyla) of bacteria according to 16S ribosomal gene sequence.

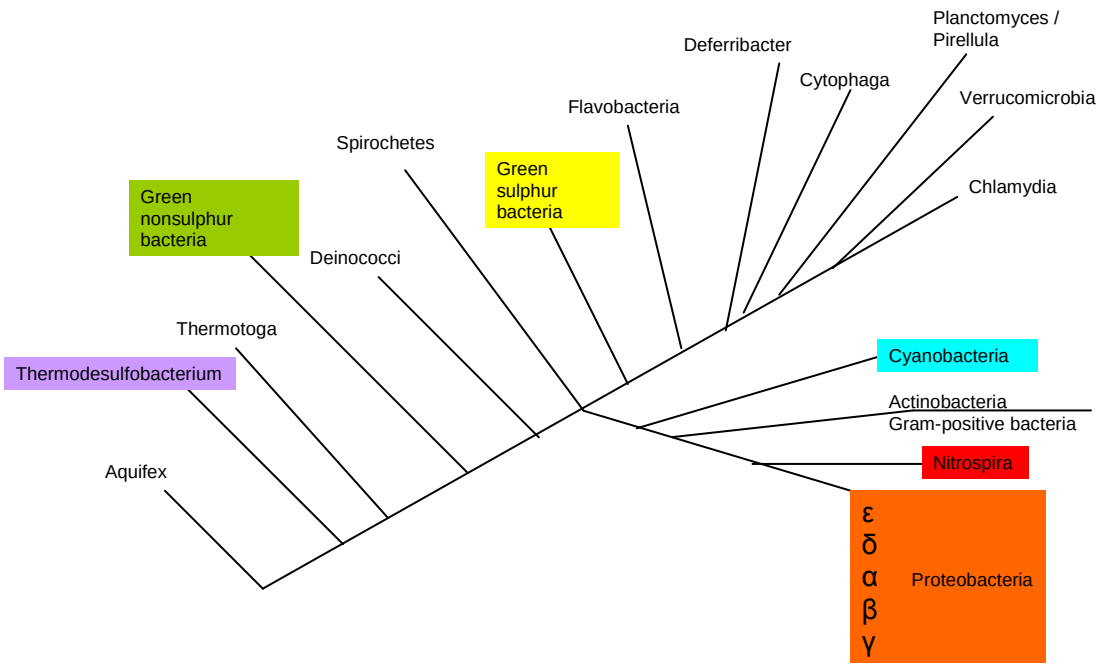


Fig. 1.7: Major lineages of bacteria based on 16S ribosomal RNA gene sequence. Highlighted boxes represent groups containing (but not exclusively) autotrophic species according to lineage affiliation. Adapted from: Madigan *et al.* (2009).

1.3.3.1 Photoautotrophy

Photosynthesis is the integral function to sustaining life on the surface of the planet for both terrestrial and aquatic ecosystems (Bryant & Frigaard, 2006). Although, ecocosms do exist deep under the Earth's crust and in the deep ocean depths, where the ability of

sunlight to reach is completely redundant. The vast majority of life revolves around the ability of the primary producers to convert inorganic carbon molecules from the atmosphere (or as dissolved carbonates in solution) into organic carbon-based compounds, thus sustaining large and complicated food webs (Fig. 1.8).

Photosynthesis involves the conversion of light energy into chemical energy that can then be used for growth. Any organism that can harvest solar radiation to obtain most of their energy requirements are called phototrophic or photosynthetic (Bryant & Frigaard, 2006). Light can be used by these organisms to either drive the phosphorlyation of adenosine diphosphate (ADP) to create adenosine triphosphate (ATP) or drive the transfer of electrons from water ($\Delta E_{m,7}$ = +820 mV) to nicotinamide adenine dinucleotide phosphate ($NADP^+$; $\Delta E_{m,7}$ = -320 mV), otherwise known as the photoreduction of $NADP^+$, or both. Photoreduction of $NADP^+$ involves the oxidation of water molecules to O_2 and the reduction of $NADP^+$ to NADPH (Jones, 1982). These reactions are in fact, in opposite to the electron flow direction during aerobic respiration. For the synthesis of ATP and NADPH to occur, electromagnetic energy must first be absorbed into photosynthetic membranes (via photopigments) and then converted into chemical energy. At the centre of this oxidation reaction are chlorophyll or bacteriochlorophyll molecules. Bacteriochlorophyll is found widely distributed in nature, being identified in green plants, algae and bacteria but not in archaea (White, 2007). Bacteriochlorophyll has also been identified in nonphototrophic bacteria such as Rhizobia species (Kramer *et al*. 1997).

Phototrophic prokaryotes are a wide ranging and diverse group of bacteria that share the ability to use light as an energy source. They can be further classified based upon their physiological differences such as, the ability to produce oxygen, or the source of electrons during biosynthesis. Oxygenic phototrophs such as the *Cyanobacteria*, *Phochloron*, *Prochlorothrix* and the *Prochlorococcus* (White, 2007) are common in nature as well as the anoxygenic phototrophs such as the purple photosynthetic bacteria, green photosynthetic bacteria and *Heliobacter* are also widespread.

1.3.3.2 Cyanobacteria

The cyanobacteria exist in land, fresh water and marine environments, but of particular interest is their presence in the soil matrix. Cyanobacteria are vital in the formation of

soils, as any early colonising species of amassed rock particles would have to be capable of both photosynthesis and nitrogen fixation. The cyanobacteria are usually present in unicellular, colonial and filamentous forms and often show a gliding motility. Importantly, these organisms can also be found as symbionts with certain fungi and lichens, possibly because of their ability to fix nitrogen (N_2) from the atmosphere. The origins of the higher plants and other photosynthetic eukaryotes are attributed to cyanobacteria via the development of a symbiotic relationship between an ancestor of the plant lineage (Thorn & Lynch, 2007). It is theorised that the photosynthetic machinery (e.g. chloroplasts) were then transferred by secondary endosymbiosis of an ancient red algae or green algae species into the erokont and alveolate (algae) or into the excavate and cercozan (protist) lineages, respectively (Thorn & Lynch, 2007).

The nutrient requirements for cyanobacteria are simple, vitamins are not required, while nitrogen and carbon are sourced from the surrounding space in their inorganic forms. Most cyanobacteria do not grow in the dark although some can grow in the presence of glucose and acetate when light is present but denied CO_2 (photoheterotrophy; Anderson & McIntosh, 1991). There are many metabolic products produced by cyanobacteria that are of practical importance. For instance, during exponential growth phases in water (blooms), neurotoxins are produced leading to the death of surrounding aquatic species and any animals ingesting the water. Species of cyanobacteria are also known to be responsible for the production of some earthy odours and flavours coming from water sources (including drinking water supplies). The most significant compound produced is geosmin (Peter *et al*. 2009; Dzialowski *et al*. 2009).

The small unicellular cyanobacteria that are found in large numbers in the world's oceans are believed to be responsible for a considerable percentage of the annual fixation of CO_2 globally. Therefore, they are not only of benefit to marine food webs but also to the regulation of atmospheric CO_2. The large rates of CO_2 uptake from the atmosphere by cyanobacteria make them interesting to this project. Soil cyanobacterial activity is a worthy scientific pursuit because of their presence in most soils, simple growth requirements and an opportunity to add to the limited knowledge base for this particular group (Zaady *et al*. 2000; Thomas *et al*. 2008).

1.3.3.3 Purple Phototrophic Bacteria

The purple phototrophic bacteria are members of the *Proteobacteria* and are capable of anoxygenic photosynthesis (no release of O_2) or the oxidation of CH_4. Purple bacteria can be sub-divided into phylogenetic, morphological and physiological lines and different genera fall within the *alpha-*, *beta-* or *gammaproteobacteria* (Fig. 1.7).

Purple bacteria contain bacteriochlorophylls and accessory pigments such as caretenoids which give the characteristic purple, red or brown colour. Purple bacteria are further sub-divided as purple sulphur bacteria and purple non-sulphur bacteria. The purple sulphur bacteria utilise hydrogen sulphide (H_2S) as an electron donor (instead of water) for CO_2 reduction during photosynthesis and so do not produce O_2 as a by-product. The sulphide (S^{2-}) is oxidised into elemental sulphur (S^0) which can then be stored within cellular globules as a future energy source, and later oxidised to sulphate (SO_4^{2-}; Prange *et al*. 1999). These bacteria are generally found in anoxic stratified zones within lakes and other aquatic places (where abundant H_2S is present) and geothermally heated springs. The purple non-sulphur bacteria were named so, as it was originally believed that they were unable to grow in the presence of or use S^{2-} as an electron donor for the reduction of CO_2. It has since been discovered that S^{2-} can in fact be used by most species in this group but only at a much lower concentration than the purple sulphur bacteria. The success of this particular group in nature is believed to be attributed to their capacity for photoheterotrophy (where light is the energy source and organic compounds as the carbon source; Ormerod & Sirevag 1983; Madigan *et al*. 2009).

1.3.3.4 Green Photosynthetic Bacteria

The green sulphur phototrophs are strict anaerobes that can use H_2S, S^0, $S_2O_3^{2-}$ or H_2 as the electron donor and CO_2 as the carbon source. Green sulphur bacteria are usually found co-existing with the purple bacteria in S^{2-} rich aquatic environments although they tend to accumulate at different stratified layers or zones. They contain light harvesting pigments housed within special inclusion bodies called chlorosomes, and their reaction centres are different from those of the purple bacteria (White, 2007). Several genera of the green sulphur bacteria are known, and species such as *Chlorobium* are well studied (Eisen *et al*. 2002).

The green non-sulphur bacteria comprise a different taxonomic group to the green sulphur phototrophs. This taxon contains just a few species and most are anoxygenic phototrophs (with the notable exception of the aerobic Gram-negative bacterium, *Thermomicrobium*) and at present all species characterised are thermophillic (Madigan *et al*. 2009). Most of the information available for the green non-sulphur bacteria comes from studies centred upon *Chloroflexus* and they are of particular interest because they form thick microbial mats (mostly in neutral to alkaline hot springs; White, 2007; Madigan *et al*. 2009).

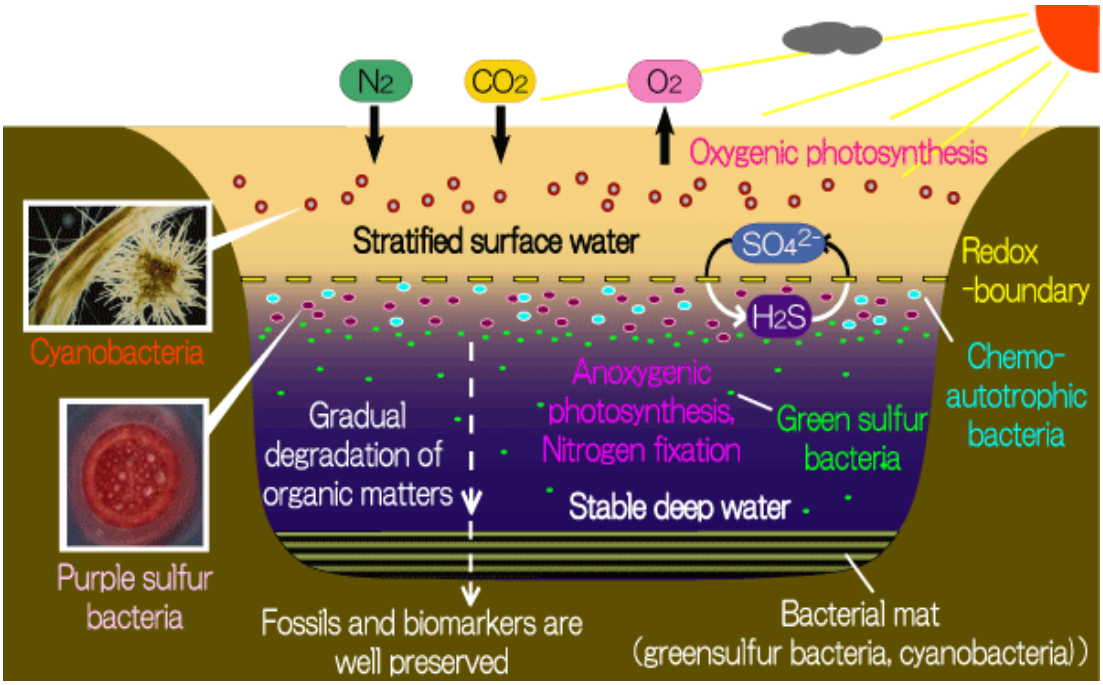


Fig. 1.8: Schematic illustration of a bacterial autotrophic system within a lake. At the redox boundary, bacteria such as the purple-, green sulphur bacteria and chemoautotrophic bacteria form a thick bacterial zone at the redox boundary in lake water. At the bottom of lake, surface sediments are covered by bacterial mats composed of green sulphur bacteria and cyanobacteria. Taken from: JAMSTEC (2003).

1.3.3.5 Chemoautotrophy

Most organisms obtain their energy requirements from the oxidation of organic nutrients (chemoorganotrophs) or by photosynthesis (photoautotrophs). There are also species that can derive metabolic energy via the oxidation of inorganic compounds. These species are known as chemoautotrophs or lithoautotrophs and can be found in both aerobic and anaerobic conditions. These prokaryotes can oxidise inorganic molecules such as H_2, CO, NH_3, NO_2^-, H_2S, S^0, thiosulphate ($S_2O_3^{2-}$) and ferrous iron (Fe^{2+}). These groups of bacteria and archaea are physiologically diverse and exist across

the different taxonomic groups. Many are aerobic with O_2 acting as the terminal electron acceptor, whereas others are facultative anaerobes that can use nitrate or nitrite as the electron acceptor when O_2 is scarce. A small number of chemoautotrophs are obligate anaerobes and can use SO_4^{2-} or CO_2 as the electron acceptor (White, 2007). For all chemoautotrophs the sole source of carbon is CO_2, but they vary in regards to their autotrophic CO_2 fixation pathway (e.g. the Calvin cycle, the acetyl-CoA pathway and the reductive tricarboxylic acid pathway). Some chemoautotrophs are known to be facultatively heterotrophic including all of the H_2 oxidisers (Bowien & Schlegel, 1981), some S^0 oxidisers and some of the Fe^{2+} oxidising bacteria.

The process of obtaining energy from inorganic substrates is less efficient than that carried out by heterotrophs and photosynthesisers. Table 1.3 lists the midpoint potentials of the inorganic substrates at pH 7. Because of the small energy yields between the inorganic electron donor and O_2, Fig. 1.9 demonstrates the cell yields of some of these compounds in comparison to organic substrates.

Compound	E_0'(mV)
CO_2/CO	-540
SO_4^{2-}/HSO_3^-	-516
$H+/H_2$	-414
S^0/HS^-	-270
HSO_3^-/HS^-	-116
NO_3^-/NO_2^-	+420
NO_2^-/NH_3	+440
Fe^{3+}/Fe^{2+}	+772
O_2/H_2O	+818

Table 1.3: Redox potentials of inorganic compounds at pH 7. Adapted from: White (2007).

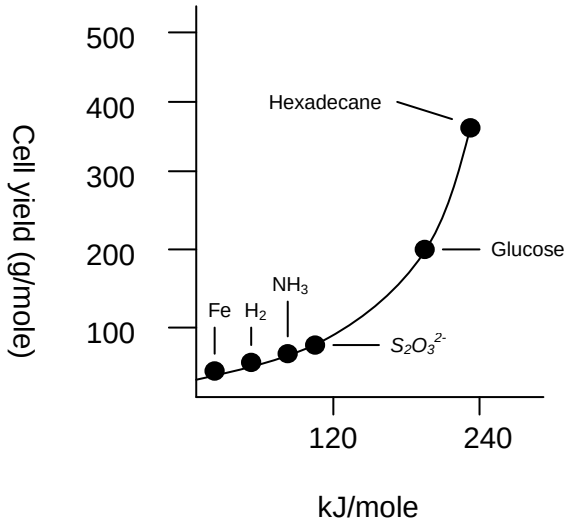


Fig. 1.9: Cell yields versus available energy in organic and inorganic electron donors. Taken from: White (2007).

The oxidation of sulphur containing inorganic molecules in the environment is normally carried out by prokaryotes of the bacteria and archaea domains. Eukaryotic oxidation of sulphur containing inorganic molecules is normally performed by endosymbiosis within worms or mussels at hydrothermal vent systems using prokaryotes (Lane, 2007). Among the many groups of chemoautotrophs presently known, particular attention is to be placed upon the aerobic S^0 oxidisers, namely those found within soil profiles, as these species are pertinent to the work herein. Below is a short account of the current literature in regards to the S^0 oxidising *Proteobacteria* species.

1.3.3.6 Aerobic Sulphur Oxidising Proteobacteria Found Within the Soil Ecosystem

Two broad classes of *Proteobacteria* exist that can oxidise S^0 containing inorganic compounds, those that actively grow at neutral pH (neutrophiles) and those that grow at acidic pH (acidophiles). Some of the acidophiles (pH < 7) also carry the ability to oxidise reduced iron compounds such as Fe^{2+} as an electron donor. The biogeochemical significance of these organisms is centred upon the S^0 cycle with many complex transformations of this nutritional element being facilitated by them. The majority of S^0 on Earth is found within rocks as SO_4^{2-} (gypsum [$CaSO_4.2H_2O$]) and S^- minerals (e.g. pyrite [FeS_2]) but the oceans also represent a significant proportion as SO_4^{2-}. S^0 is readily oxidised by *Thiobacillus* and *Acidithiobacillus* species but due to the insoluble nature of S^0 in aqueous media the bacteria must physically attach themselves to the S^0

crystals. The microbial oxidation of S^0 leads to the formation of sulphuric acid (H_2SO_4) and thus lowers the pH of the surrounding environment. This has been reported as being problematic in waste treatment facilities and leads to the disintegration of concrete sewer pipes (Knight *et al*. 2002). In agricultural settings, S^0 may be added to an alkaline soil to lower the pH as the ubiquitous *Thiobacilli* genera (Chapman, 1990) will readily carry out the acidification process (Janzen & Bettany, 1987; Owen *et al*. 1999; Scherer, 2001). Bacteria of the S^0 cycle are also of great environmental importance when considering their impact on anthropogenic activities, for instance the production and/or consumption of H_2S by SO_4^{2-} reducing bacteria in the agricultural and petroleum industries (Tang *et al*. 2009).

In 1902 the first pure culture of *Thiobacillus* was cultured by Nathansohn and was later named by Beijerinck in 1904 (Smith & Strohl, 1991). As *Thiobacillus* species are the best known and most studied of the S^0 oxidising autotrophs it is useful to use this group to summarise current knowledge. *Thiobacilli* are Gram-negative, small (0.3-0.5 x 0.7-4.0 μm), aerobic rods that can oxidise reduced sulphur compounds leading to the production of H_2SO_4. Energy can be derived from one or more reduced sulphur compounds including S^-, S^0, $S_2O_3^{2-}$, polythionates ($S_nO_6^{2-}$) and thiocyanate ($[SCN]^-$; Kelly & Wood, 2000). All *Thiobacilli* species fix CO_2 using the Calvin-Benson cycle and thus are capable of autotrophic growth (Leduc & Ferroni, 1994). Most soil and water samples contain *Thiobacillus* species that can be isolated in aerated defined media, provided they contain a reduced sulphur compound. An important consideration when culturing these species is the optimum pH as there are three ranges into which *Thiobacilli* fall, 6.5-7.2, 5.5-6.0 and < 4.0 (with some as low as pH 1.5). Most *Thiobacilli* are described as mesophiles with optimum temperatures close to 30^{o}C, with a few species requiring higher temperatures such as *Tb. tepidarius* and *Tb. aquaesulis* falling into the 40-50^{o}C range (Smith & Strohl, 1991). Of the mesophillic aerobic S^0 oxidising bacteria, genera such as *Acidianus, Acidithiobacillus, Aquaspirillium, Aquifex, Bacillus, Beggiatoa, Methylobacterium, Paracoccus, Pseudomonas, Starkeya, Sulfolobus, Thermithiobacillus, Thiobacillus* and *Xanthobacter* have been identified (Friedrich *et al*. 2001).

Thiobacillus species have been identified and isolated from agricultural soils in diverse locations across the world (Chapman, 1990), with importance being placed on the fact that S^0 must be oxidised to SO_4^{2-} to aid plant growth. The work carried out by

Chapman (1990) showed that neutrophillic *Thiobacillus* species were detected in Scottish agricultural soils with small numbers of acidophiles detected. However, sulphur addition to composts and agricultural soils has produced increased counts of acidophilic *Thiobacilli* in other studies (García de la Fuente *et al*. 2007; Madigan *et al*. 2009). Liu *et al*. (2004) demonstrated that by varying the composition of the growth medium, a 2.6 fold increase in H_2SO_4 yield could be achieved and thus greatly enhance bioleaching of heavy metals. Allegretti *et al*. (2006) showed that cultures of *Thiobacillus* can reduce chromium(VI) under acidic and neutral conditions producing an array of sulphur compounds such as polythionates while requiring no external carbon source. The ability of *Thiobacillus* species to reduce and leach metals from soils, sewage sludge and groundwater sources is extensive (Gadd, 2004). A brief account of a study carried out by Kurosawa *et al*. (1993) estimated the CO_2 uptake of *Thiobacillus thiooxidans* JCM 7814 in liquid culture (one of the only such studies locatable in the literature to date). Although the authors acknowledge their difficulty in determining the CO_2 fixation rate, it was estimated that the carbon content of each cell was 48% (comparable to that of algae) with CO_2 as the sole carbon source. The investigators used a fermentor for optimal growth conditions and determined that the specific fixation rate 5.9 g-CO_2 / g-cell.d. This was calculated from the growth rate and carbon content of *Thiobacillus thiooxidans* indicating significant carbon capture potential.

An interesting feature of chemoautotrophs not discussed above, is the possibility of their presence or potential colonisation of other planetary niches such as Mars (Horneck, 2000; Hart *et al*. 2011; Dohm *et al*. 2011). Parro *et al*. (2005) have developed an instrumental technique for the detection of biomarkers specific to chemoautotrophs such as *Leptospirillum ferrooxidans* and *Acidithiobacillus ferrooxidans.* They have developed an immunosensor microarray that is housed within an autonomous and remotely operated instrument called, Signs of Life Detector (SOLID). Using sediment samples taken from acid mine drainage, initial results have proved successful. The use of specific indicating biomolecules is a good means of identification. He *et al*. (2006) have investigated protein expression by *Acidithiobacillus ferrooxidans* when grown under different energy sources, using Surface Enhanced Laser Desorption/Ionisation (SELDI)-ProteinChip technology. This is an affinity based mass spectrometry technique where the proteins of interest are selectively absorbed onto a modified surface of a chip allowing for proteomic analysis. The advantage of this technique is the fact that very little of the sample is required and therefore has great potential for low amounts of

biomass, or dealing with slow growing organisms. *Acidithiobacillus ferrooxidans* were grown on S^0 and Fe^{2+} with 28 differently expressed proteins found.

According to Sakata *et al*. (2008) very little is known about the carbon isotopic variation in the lipids of chemoautotrophic bacteria. Therefore, they have analysed the carbon isotopic variation in the lipids of the ammonia oxidising bacterium *Nitrosomonas europaea*. It was found using GCMS that hopanoids produced by ammonia oxidising bacteria living in fresh water were likely to be depleted in ^{13}C by 26-30 ‰ relative to dissolved CO_2. The results support current theories that suggest that geohopanoids depleted in ^{13}C are derived from ammonia oxidising bacteria.

1.3.4 Conclusion

Environmental microbiology is a wide and diverse field of scientific study. The discoveries being made are continually expanding our knowledge of autotrophic microorganisms and the environments they inhabit. Although it is easy for one to assume that microbiological research should be the reserve of the microbiologist or biotechnologist, a review of the current literature suggests that chemists, namely instrumental chemists, are having a huge impact in this field. The instrumentation available to chemists are facilitating new and exciting discoveries to be made in areas such as biogeochemistry, biocycling, bioremediation and microbiological molecular ecology.

1.4 Stable Isotope Probing

"*Darwin has interested us in the history of nature's technology*"

[Karl Marx (Marx, 1867)]

1.4.1 Introduction

The study of microbial ecology has been greatly advanced by the development of Stable Isotope Probing (SIP). By using stable isotopes such as ^{2}H, ^{13}C and ^{15}N it has become possible to identify nutritional and chemical pathways employed by microorganisms (Whitby *et al*. 2001; Dumont & Murrell, 2005; Cupples *et al*. 2007; Kreuzer-Martin, 2007; Bastias *et al*. 2009). ^{13}C-carbon is currently the most popular choice of stable isotope since carbon is the most common element in DNA and other organic structures. The advantage of SIP to research scientists is that mixed or unknown cultures of

organisms can be identified due to their incorporation of rare isotopes. The first examples of SIP were labelled phospholipid-derived fatty acids (PLFAs; Boschker *et al*. 1998), DNA (Radajewski *et al*. 2000) and RNA (Manefield *et al*. 2002) which established the method as a clear identification tool for identifying target groups of microorganisms that metabolise specific substrates. The incorporation of stable isotopes into cellular material is achieved via incubation of a selected compound that has been artificially enriched with the isotope of choice. After the incubation period, cellular components such as lipids or nucleic acids can be harvested from a sample (e.g. soil, sediment or water) and analysed using various instruments such as Nuclear Magnetic Resonance (NMR) spectrometry (Baldock *et al*. 1990a; Lundberg *et al*. 2001) and Gas Chromatography Mass Spectrometry - Isotope Ratio Mass Spectrometry (GCMS-IRMS; Tillmann *et al*. 2004). Also, the isotopically labelled nucleic acids may be separated from the unlabelled nucleic acids using density gradient ultracentrifugation (Tillmann *et al*. 2004). Once purified, the labelled nucleic acids may be amplified using polymerase chain reaction (PCR) with universal 16S rRNA primers for bacteria and archaea or 18S primers for Eukarya. Once a PCR product has been generated from the extracted nucleic acids ('template DNA'), techniques such as denaturing gradient gel electrophoresis (DGGE) or recombinant clone technology can be used to isolate the DNA of target species (i.e. those that have actively assimilated the applied substrate) and retrieve a DNA sequence fingerprint (Fig. 1.10).

1.4.2 Background and Applications

Although Boschker *et al.* (1998) was the first demonstration of stable isotope labelling of microorganisms, the term SIP originates from Radajewski *et al.* (2000). Using an isotopically enriched substrate it was shown that ^{13}C-labelled microorganisms could be identified from a much larger and diverse group. This was possible because the ^{13}C-DNA could be separated from ^{12}C-DNA using isopycnic density gradient ultracentrifugation in caesium chloride (CsCl) gradients. The buoyant density of DNA always varies depending on its guanine-cytosine (G+C) content but the incorporation of heavy isotopes enhances the buoyant density of labelled DNA considerably (Dumont & Murrell, 2005). Bacterial DNA with a G+C content of 35-70% has a range in density of 1.69-1.73 g cm^{-3}. The calculated buoyant density of ^{13}C-labelled DNA is 1.75-1.79 g cm^{-3} (Radajewski *et al*. 2003), indicating that this labelled DNA will equilibrate at a similar density in the gradient and thus, separate from the unlabelled community DNA under centrifugation. Nitrogen is another element suitable for SIP as ^{15}N-labelled

substrates are readily available, but separation of the labelled downstream material is more challenging. Only 20% heavy isotope enrichment is required for ^{13}C-labelled DNA in order to achieve sufficient separation, unlike ≥ 40% for ^{15}N-labelled DNA (Cadisch *et al*. 2005). This is required because carbon is more abundant in DNA than nitrogen and the shift is a lot less than that of carbon. For the ^{13}C-labelled DNA within the gradient, this shift is 0.036 g ml^{-1}, meanwhile for ^{15}N-labelled DNA the shift is only 0.013-0.016 g ml^{-1} (Cupples *et al*. 2007; Buckley *et al*. 2007). Therefore, the ^{15}N-labelled and unlabelled bands of DNA are close together after ultracentrifugation. Regardless of these difficulties, ^{15}N-labelled-SIP has great potential to elucidate the pathways of nitrogen containing substrates within the environment (O'Malley *et al*. 2007) and the obvious benefits to elucidating aspects of the nitrogen cycle. The addition of ethidium bromide (EtBr) to the density gradient medium (which acts as a staining agent of DNA) allows for clear observation under ultra-violet (UV) radiation. Once illuminated the distinct bands of ^{12}C- and ^{13}C-DNA fluoresce and the buoyant density within the gradient is observable. Radajewski *et al.* (2000) also successfully attempted the separation of ^{2}H-labelled DNA but determined that the separation was half as efficient and hence ^{2}H-labelled substrates are less commonly used.

1.4.3 Detection of Stable Isotope labelled DNA

EtBr is a commonly used staining agent for DNA samples and it can effectively verify a successful separation between stable isotope labelled- and unlabelled-DNA. EtBr staining in SIP experiments is generally limited to DNA that has been extracted from pure culture studies. This is due to the limited volumes that can be retrieved from *in situ* samples, which usually produce a smear of partially labelled DNA (Miller *et al*. 2004; Neufeld *et al*. 2007a) after ultracentrifugation. When an EtBr stained band of labelled DNA is observed under UV fluorescence, it may be extracted from the centrifuge tube using a needle and syringe. The extracts must go through a labour intensive purification process to remove the toxic EtBr. As an alternative, the presence of DNA within the CsCl gradient may be detected using fractionation and subsequent flourometric quantification of total DNA (Tillmann *et al*. 2004) or quantification of 16S rRNA genes in gradient fractions by real-time quantitative PCR (Leigh *et al*. 2007). These two techniques eliminate the hazardous step of preparing and working with concentrated EtBr solutions and offer greater sensitivity. Also, removing the requirement of exposing DNA to UV radiation is an added advantage as that step can result in molecular damage, affecting downstream applications (Neufeld *et al*. 2007b). When it is necessary to

perform gradient fractionation and quantification, an unlabelled control gradient is required to compare the distribution of DNA. The control can be harvested from a laboratory grown mono-culture, such as *Escherichia coli* or generated from unlabelled DNA, taken from the sample at the beginning of the incubation or from a parallel sample incubated with an unlabelled substrate (Uhlík *et al*. 2009). These controls are required to ensure where the unlabelled DNA physically lies in the gradient due to its variability in G+C content. It is an important consideration to note that, a low background of unlabelled DNA, approximately 0.7% is to be expected throughout all of the gradient fractions (Tillmann *et al*. 2004). It is prudent in some experiments to add unlabelled DNA from a mono-culture to act as an internal indicator of separation efficiency so that the relative contamination of the "heavy" DNA can be determined (Singleton *et al*. 2005).

The centrifugation conditions require special attention. Centrifugation conditions of 256,000 g (75,500 RPM) for 16 hours are used when the stable isotope labelled DNA is greatly enriched. However, longer times at lower speeds are recommended for samples that have lower expected isotopic enrichments (such as complex communities; Uhlík *et al*. 2009). A lower speed, leads to a shallower gradient and hence a better separation of the partially labelled DNA due to a higher resolution within a designated range of buoyant densities. This was shown by Hutchens (1991), for ^{13}C-labelled and unlabelled DNA separations (where centrifuge conditions were 140,000 g for 69 hours) and by Cadisch *et al.* (2005) for ^{15}N labelled and unlabelled DNA.

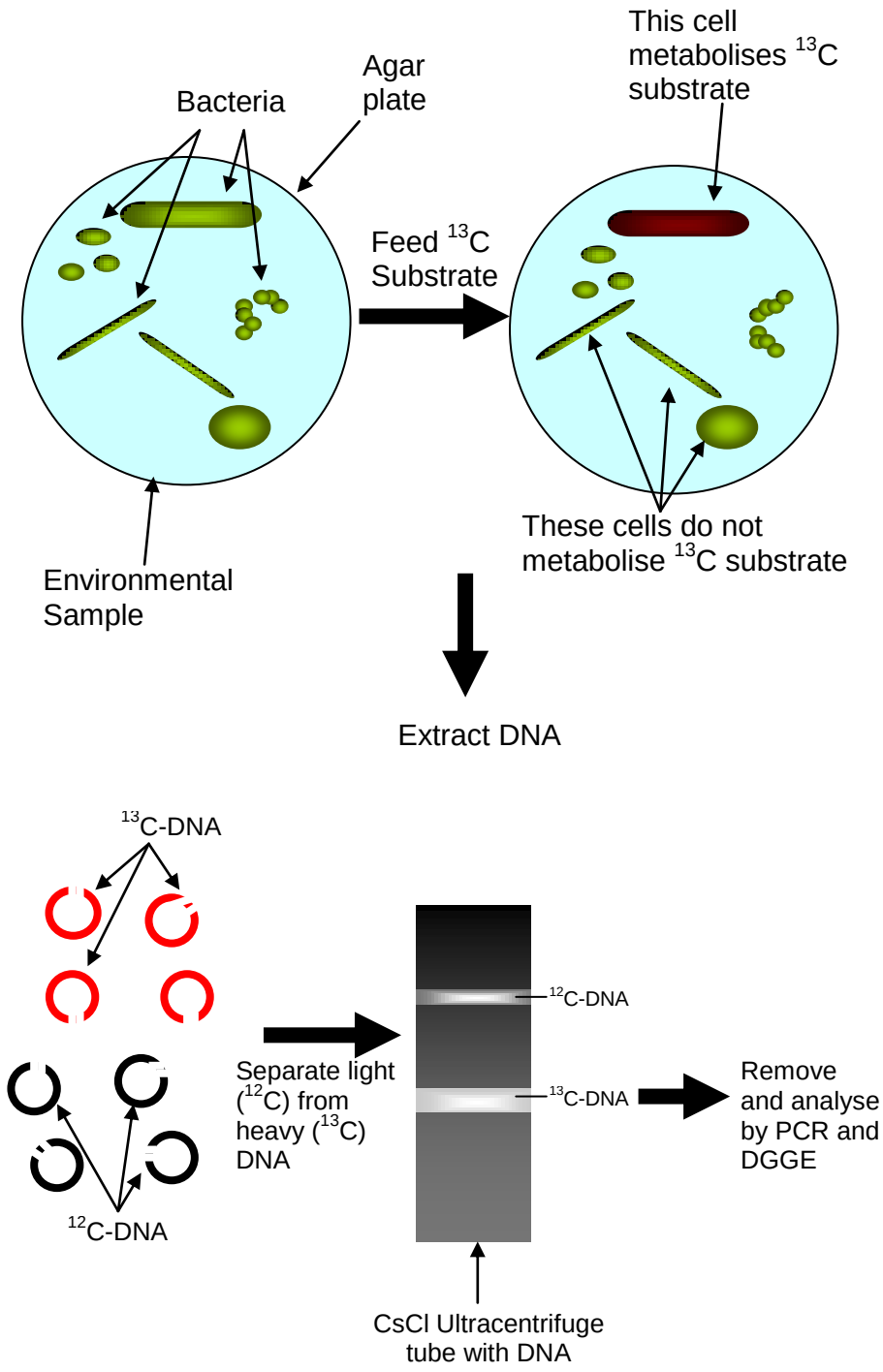


Fig. 1.10: Stable Isotope Probing (SIP). An isotopically labelled ^{13}C-substrate is added to a diverse microbial community in an environmental sample. Organisms that metabolise the substrate incorporate ^{13}C into DNA as they divide. The DNA can be harvested and the heavier ^{13}C DNA can be separated from the lighter ^{12}C DNA. Taken from: Madigan *et al.* (2009).

An important technique for the detection of isotopic enrichment in DNA is liquid chromatography mass spectrometry (LCMS) -IRMS (Chen & Abramson, 1998). Whereas, a simple assay to detect the disappearance of the substrate compound may also be employed. Depending on the type of substrate used, its disappearance may be

quantified using GCMS or HPLC-MS. These techniques have been used by analysts to track the course of the stable isotope from the substrate directly into the DNA (Whitby *et al*. 2001), or by detecting $^{13}CO_2$ production (Padmanabhan *et al*. 2003; DeRito *et al*. 2005; Leigh *et al*. 2007; Manefield *et al*. 2007) or by measuring the decline in labelled substrate (DeRito *et al*. 2005).

1.4.4 Research Potential of DNA-SIP

SIP has been used to successfully label methanotrophs and methylotrophs (McDonald *et al*. 2005) and active root inhabiting communities, through $^{13}CO_2$ labelling of the host plant exudates into microbial RNA (Griffiths *et al*. 2004; Vandenkoornhuyse *et al*. 2007). Ostle *et al.* (2003) found that labelling of microbial DNA and RNA via plant photosynthesis root exudates took place within several hours and an estimated residence time of 15-20 days for ^{13}C-labelled RNA. This indicates the requirement for rapid sampling after applying the labelled compound due to potential carbon turnover and secondary labelled carbon utilisation. The rapidity of root exudates turnover by microbial biomass within the rhizosphere was supported by studies carried out by Gnguyen *et al.* (1999) using $^{14}CO_2$ to label exudates by detection methods other than SIP. The SIP method has been used in field-based studies to track the ^{13}C flow through a phenol degrading microbial community (DeRito *et al*. 2005; Manefield *et al*. 2007). A review by Prosser *et al.* (2006) states that field based nucleic acid SIP operates close to detection limits and that fractionation of the liquid gradient is required before detection of ^{13}C content. A similar study by Tillmann *et al.* (2004), concluded that enhanced detection can be achieved using a fractionation technique of the CsCl gradient to detect labelled DNA and that ~15 μg DNA was the minimum requirement to visualise DNA using EtBr staining and UV illumination. This can be difficult to achieve for environmental samples.

1.4.5 DNA-SIP as a Tool to Investigate Ecological Processes and Bioremediation

The employment of DNA-SIP in bioremediation studies in order to detect the presence of and identify pollutant degrading microorganisms is of significant importance. DNA-SIP is a tool that can be used to link taxonomic identity to function while remaining in the context of the natural community. Initial experiments with single-carbon molecules such as ^{13}C-methanol ($^{13}CH_3OH$) have significantly contributed to the knowledge of carbon flow and cycling, but to date, only in specific environmental niches (Radajewski *et al*. 2000). The detection of these microbes in diverse habitats such as soil, water,

sediments and slurries has revealed that many processes in the environment are carried out by microorganisms not previously implicated with those processes (Morris *et al*. 2002; Jeon *et al*. 2003, 2004; Miller *et al*. 2004; Leigh *et al*. 2007). It is still unknown how much stable isotope is required to be integrated into DNA to successfully separate it from unlabelled DNA, unlike that of mono-culture studies (Dumont & Murrell, 2005; Cadisch *et al*. 2005).

The first example of DNA-SIP (Radajewski *et al*. 2000) used an oak forest soil microcosm. This soil was enriched with $^{13}CH_3OH$ and subsequently the methylotrophs responsible for its degradation were identified. A phylogenetic analysis of the 16S rRNA genes that had been retrieved from the ^{13}C-DNA fraction showed that utilisation of $^{13}CH_3OH$ had been predominantly performed by α-Proteobacteria and also members of the A*cidobacterium* family. This result was not expected as it was thought that a wide group of bacteria assimilate methanol but the results suggested a very small diversity. This may have been due to the acidic conditions of the sample site and possibly the high concentration of methanol used, which may not be suitable conditions for methylotrophs. In a follow up study using $^{13}CH_4$ and $^{13}CH_3OH$, methylotroph populations were shown to consist of distinct populations involved in the consumption of $^{13}CH_4$ (centred around the α- and β- subclasses of the proteobacteria and *Cytophagales*) in comparison to those consuming $^{13}CH_3OH$ (Radajewski *et al*. 2002). Other methylotroph studies involving single-carbon compounds and different sample types have been reported to show diverse groups of pollutant degrading bacteria in the environment (Nercessian *et al*. 2005).

Methane uptake has also been studied to detect the presence of methanotrophs in peat bogs, including the discovery of novel methanotrophic species (Morris *et al*. 2002). Due to the long incubation time (40 days), only DNA with $\geq$ 75% ^{13}C was used for downstream identification techniques to eliminate bacteria that did not assimilate $^{13}CH_4$ as their sole carbon source e.g. cross feeders. Other interesting results from a cave microbial mat (Hutchens *et al*. 2004) suggested that α- and γ-proteobacteria methanotrophs such as *Methylomonas, Methylococcus* and *Methylocystis* were responsible for $^{13}CH_4$ uptake. Interestingly the authors also reported a eukaryotic sequence related to the 18S rRNA gene of the algae *Ochromonas danica*, suggesting that it possibly assimilated $^{13}CH_4$, although it is possible that mineralised carbon

resulting from $^{13}CH_4$ uptake could have been a source of $^{13}CO_2$ and hence, ^{13}C-labelling of a photosynthesising organism.

Autotrophic ammonia-oxidising bacteria were studied in freshwater sediment using incubation with $^{13}CO_2$ (Whitby *et al*. 2001). The authors reported that the ^{12}C-DNA fraction was dominated by *Nitrosospiras* in comparison to the ^{13}C-DNA fraction which contained largely *Nitrosomonad* DNA. This supports the hypothesis that *Nitrosomonads* dominate in laboratory cultures (Stephen *et al*. 1996). DNA-SIP has been used to identify active denitrifying bacteria fed on acetate. The most obvious denitrifiers identified in the ^{13}C-DNA fraction were related to *Comamondadaceae* and *Rhodocyclaceae* families (Ginige *et al*. 2005). A study involving the gas substrates methyl bromide (MeBr) and methyl chloride (MeCl) as primary carbon sources in soil is of notable significance as these gases, which directly contribute to stratospheric ozone depletion, have both natural and anthropogenic sources (Miller *et al*. 2004). DGGE and subsequent 16S rRNA gene amplification of the ^{13}C-DNA microcosm experiment showed that metabolism of these two compounds were carried out by different bacteria. The results showed that the MeBr was mostly consumed by species closely related to *Burkholderia* (not previously known to degrade methyl halides) while the scope of MeCl-degrading bacteria was much more diverse owing to its lower toxicity.

A novel approach taken by Lear *et al.* (2007) was to use DNA-SIP to monitor the activity of microorganisms that transform As(V) to the more mobile and hence hazardous As(III). This approach was novel in the sense that very little research into linking DNA-SIP with metal reducing bacteria is currently available (Wiatrowski & Barkay, 2005). It was reported that species closely related to the dissimilatory As(V)-reducing bacteria *Sulurspirillium* strain NP-4 and *Desulfuotomaculum auripigmentum* were responsible in the contaminated groundwater aquifer for active arsenic reduction. Several studies (not related to the SIP technique) currently show that many species have been identified that perform the reverse reaction, oxidising As(III) to As(V) with both heterotrophic and autotrophic species being responsible (Santini *et al*. 2002; Rhine *et al*. 2006).

Bioremediation studies are extensive throughout many scientific disciplines due to the attention placed on sites contaminated by xenobiotics (especially aromatic compounds) across the terrestrial landscape (Kok *et al*. 2000), deep within the vadose

zone (Konopka & Turco, 1991) and within aquifers (Hutchins, 1991). Various clean up options are being developed, most notably in the biotechnology sector, due to its cost effective nature and possibility of complete elimination of target pollutants (Stenuit *et al*. 2008). Several studies have demonstrated that phytoremediation and rhizoremediation (Demnerová *et al*. 2005; Uhlík *et al*. 2007; Uhlík *et al*. 2009; Gerhardt *et al*. 2009) are environmentally friendly and cost effective treatments for contaminated sites. Many strains of bacteria have been isolated and cultivated in laboratories, which are capable of degrading pollutant molecules (Leigh, 2006). However, the microbes isolated in laboratory cultures may not be representative of *in situ* conditions and may only degrade the pollutant under optimum laboratory conditions (Uhlík *et al*. 2009). Therefore, the use of DNA-SIP to identify the active microorganisms involved in pollution removal is of great importance. For instance, phenol degrading bacteria were observed *in situ* in which relatives of *Pseudomonas, Pantoea, Acinetobacter, Stenotrophomonas and Alcaligenes* were found to be responsible for ^{13}C-phenol degradation (Padmanabhan *et al*. 2003). Another study of ^{13}C-phenol degradation identified *Pseudomonas* as cross feeding organisms and *Kocuria* and *Staphylococcus* as the primary feeders on ^{13}C-phenol (DeRito *et al*. 2005).

The degradation of polycyclic aromatic hydrocarbons (PAHs) is of particular significance due to the widespread nature of this pollutant in soils and sediments. The growth of microbes on several of these environmental pollutants has been performed and isolates have been identified (Schmitt *et al*. 1996; Singleton *et al*. 2005; Rezek *et al*. 2008). Using DNA-SIP, Jeon *et al.* (2003, 2004) and the application of ^{13}C-napthalene to sediment, a previously unknown bacterium was discovered and later designated as *Polaromonas naphthalenivorans* sp. nov. Importantly, the isolate was found to host a distinctive naphthalene dioxygenase gene which was also widespread in the contaminated sediment.

Another group of anthropogenic pollutants that are widespread in the environment are polychlorinated biphenyls (PCBs). These compounds were of significant industrial importance between the 1920s and 1970s until their production was phased out in the US around 1979 (98% of PCB production was in the US; Fiedler, 2001). DNA-SIP was used by several researchers to detect microbes that can metabolise PCBs in conjunction with biphenyl or some plant exudates like flavonoids (Gilbert & Crowley, 1997; Singer *et al*. 2004). Leigh *et al.* (2006) demonstrated the presence of

^{13}C-biphenyl degrading bacteria in root inhabiting pine soils and that the dominant species was *Rhodococcus* sp. A follow up paper combining DNA-SIP with Terminal Restriction Fragment Length Polymorphisms (T-RFLP) analysis was able to identify *Pseudonocardia* sp. as the most abundant biphenyl consumer (Leigh *et al*. 2007). Interestingly various other consumers of biphenyl were discovered including *Nocardiodes*, *Kribella*, *Sphingomonas*, *Variovorax* and *Polaromonas*, where only *Sphingomonas* had previously been implicated in PCB degradation.

1.4.6 Conclusions

DNA-SIP is a powerful technique that helps to elucidate the biochemical pathways of microbiological substrates within both *in vivo* and *in situ* scenarios. The ability to discover which organisms are responsible for particular biogeochemical and biodegradative processes is of significant importance to the scientific community. Also, the results of DNA-SIP studies can also assist in helping to define the laboratory conditions required for as yet uncultivated microorganisms, which can then be isolated and assigned a taxonomic classification. The fact that DNA-SIP can be easily coupled with various other instrumental techniques and metagenomic studies holds immense possibilities for the discovery of new species, functional genes and useful cellular components.

DNA-SIP still has some hurdles to cross such as the fact that some active microorganisms in analysed microcosms may remain undetected due to their slow growth or different rates of substrate utilisation. The issue remains that if longer incubation times are used then microbes detected could be cross feeders or mistaken for cross feeders. Therefore, the technique still requires optimisation, but the current plethora of possible sampling sites, substrates and sample types means that relatively simple discoveries are waiting to be made.

1.5 Polymerase Chain Reaction

"*DNA was the first three-dimensional Xerox machine*"

[Kenneth Boulding (Boulding, 1980)]

1.5.1 What is the Polymerase Chain Reaction (PCR)

PCR is a relatively new scientific technique that has arisen in the last 20 years. It has rapidly been established in medical, microbiological and molecular biology (Bartlett & Stirling, 2003) as well as providing the very basis of the human genome project (Roach *et al*. 1999; The ENCODE Project Consortium, 2007). PCR as a technique was developed from research directly affiliated to the American chemist, Kary Mullis in the spring of 1983 (Mullis & Faloona, 1987; Price, 1989), who claims that PCR as a concept was developed while driving a Honda Civic on Highway 128 from San Francisco to Mendocino (Bartlett & Stirling, 2003). Kary Mullis was awarded the 1993 Nobel Prize for chemistry in receipt of his scientific contribution (Nobelprize.org, 2011).

The PCR concept is based upon several other components that were already in existence. The reproduction of short lengths of single-stranded (SS) DNA (oligonucleotides) and their employment for target specific synthesis to produce copies of DNA using DNA polymerases were already standard practice for most molecular biologists at that time. In its simplest terms, Mullis's idea was to use the apposition of two nucleotides, complementary to opposite strands of the DNA to precisely magnify the region between them (Bartlett & Stirling, 2003). The ability to do this in a repetitive manner in order for each round of replication (polymerase activity) to produce a new copy of template DNA, led to the term 'chain reaction' being applied. The exponential growth of very small amounts of recovered template DNA from a sample has proved to be a huge success and has earned Mullis the title of 'inventor of PCR' as taken from his own words:

> *"In a sense, I put together elements that were already there. You can't make new elements, usually. The new element, if any it was the combination, the way they were used. The fact that I would do it over and over again, and the fact that I would do it in just the way I did, that made it an invention….the legal wording is "presents an unanticipated solution to a long-standing problem" that's an invention and that was clearly PCR."* (Rabinow, 1996)

It must be noted that extensive work carried out by Mullis's colleagues at Cetus Corp. deserve equal credit, as well as the convenient isolation of a thermally stable polymerase from a thermophillic bacterium (*Thermophilus aquaticus*) discovered in a hot spring, had also made the development of PCR possible (Innis *et al*. 1988; Saiki *et al*. 1988). The thermal stability of this enzyme was crucial in the development of PCR as this bacterium proliferates at temperatures of around 95^{o}C and hence, the enzyme does not denature at the denaturation temperatures of a normal PCR cycle. The incorporation of the thermally stable DNA polymerase into the technique solved many problems that had plagued PCR amplification previously. The isolation of *Thermophilus aquaticus*, its subsequent cloning and then purification of the polymerase enzyme, has led to the development of the commercially available Taq polymerase, allowing for PCR amplification cycles to take place without having to open the reaction tube. This crucial fact meant that PCR could be taken from the laborious laboratory practice of moving reaction tubes from one heated water bath to another, to the development of closed DNA thermal cyclers by Cetus and Perkin-Elmer (Bartlett & Stirling, 2003).

PCR has become an essential technique for the majority of biotechnological applications with methods developed in diverse applications such as criminal forensic analysis (Hanson *et al*. 2009; Imbschweiler *et al*. 2009), food science (Scifò *et al*., 2009; Asensio *et al*. 2009), diagnostic medicine (Watzinger *et al*. 2006; Sontakke *et al*. 2009), and environmental/ecological studies (Wintzingerode *et al*. 1997; Bridge & Newsham, 2009; Wakelin *et al*. 2009; Lear & Lewis, 2009).

1.5.2 How Does PCR Work?

PCR is performed by the separation of a double stranded DNA template into two single strands (denaturation), followed by the recombination, under low temperature (annealing), of oligonucleotide primers to the template DNA. Then, the elongation of the primer-template hybrid of DNA is performed by the polymerase enzyme (Thies, 2007). Each of these steps takes place by regulating the reaction temperature for pre-defined periods of time. In general, the temperature is raised between 92-96^{o}C to denature the template DNA and then lowered to 42-65^{o}C so that the primers can anneal to the template. The DNA polymerase enzyme has a temperature activity optimum of 75-80^{o}C (Lawyer *et al*. 1993), so in practice, 72^{o}C is held for a specified time after the annealing of primers. This cycle is replicated anywhere between 20-30 times with each

cycle doubling the number of DNA products (amplicons). After 20 cycles about 1 million copies of a single strand of template DNA has been replicated. The particular reaction times and temperatures chosen for each step are dependent on the original template DNA source and the protocol being used (Fig. 1.11). The annealing step is of critical importance, as lower temperatures are less specific and could allow mismatching of bases to occur when the primer binds to the template (Thies, 2007). The primers used for microbiological ecological studies target for specific DNA sequences, such as those coding for the small subunit (SSU) rRNA genes. Gene sequences of known function or arbitrary primers can also be used to produce a PCR picture.

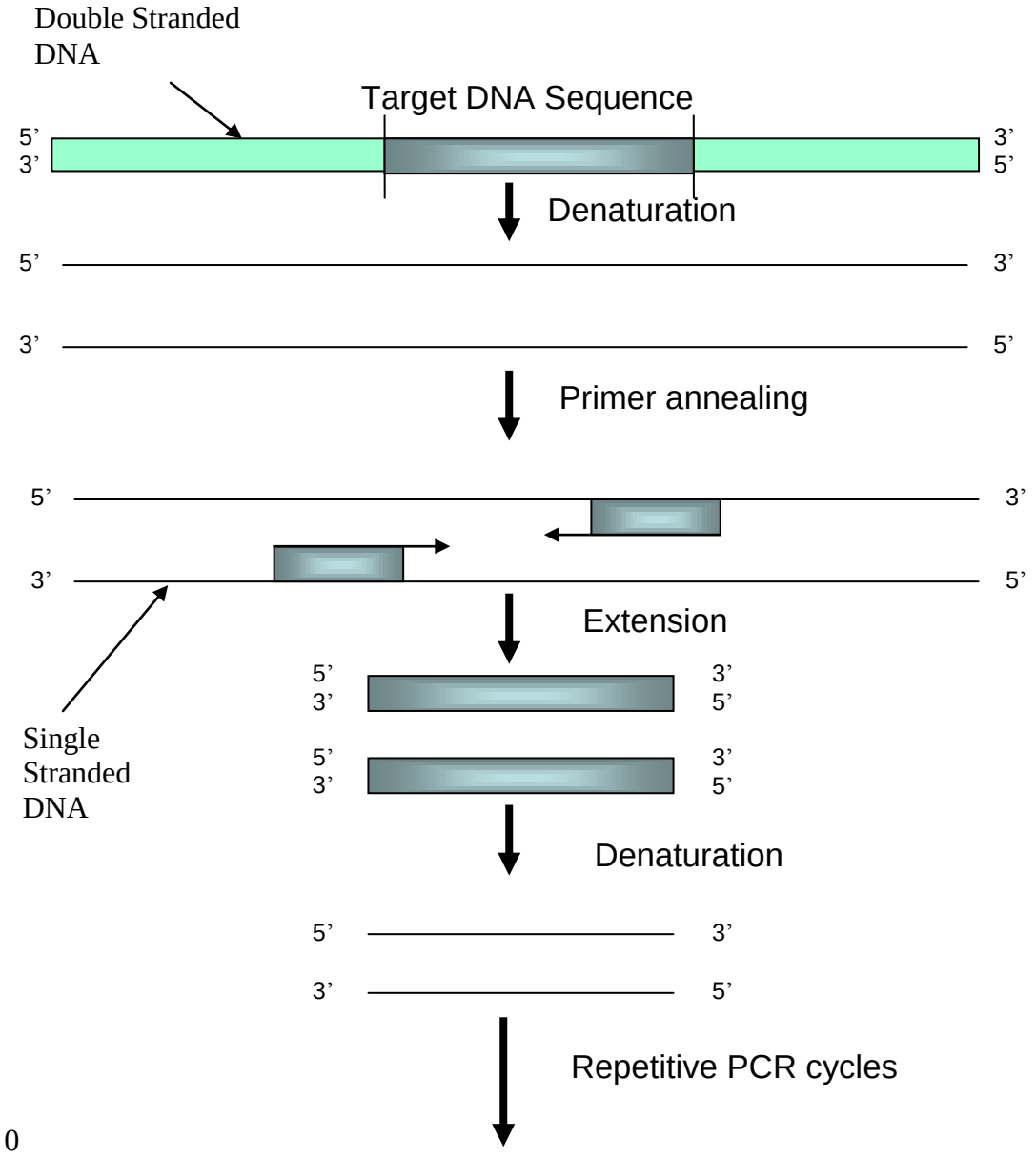


Fig. 1.11: Mechanism of the polymerase chain reaction: DNA template and primer interaction to form copies depending on controlled temperature variations. Adapted from: Thies (2007).

To replicate a strand of DNA using PCR, first one needs the basic building blocks of the DNA molecule e.g. Adenine (A), Thymine (T), Guanine (G) and Cytosine (C). Then the DNA polymerase enzyme arranges these bases in their correct order using a small segment of DNA known as the primer. The primer acts as the unit in which the bases are attached to and act as the template for constructing a new strand. Briefly summarised, the primer is designed for any given PCR and it can determine the efficiency and selectivity of the reaction. Considerations into primer design are amplicon size (amplicons range from 100 to 1000 base pairs in length), G+C content and location (usually only a requirement when a particular form [allele] of a gene is sought; Hyndman & Mitsuhashi, 2003). The important aspect of primers is that they can target different levels of taxonomic resolution. Because ribosomal RNA genes are highly conserved they can discriminate between sequences at the genus level or above (taxonomic rank e.g. phylum, species). The most common targets for working with microbial communities are the rRNA genes due to their relevance in establishing the phylogenetic and taxonomic relationships (Woese *et al*. 1990). These SSU rRNA genes are known as 16S in bacteria and archaea or 18S in eukarya or the large subunit (LSU) rRNA genes are 23S in bacteria and archaea and 28S in eukarya (Thies, 2007). The 16S rRNA primer is the most common and versatile primer used in microbial ecology studies, but more specific primers that target a certain gene are available. Gene specific probes can assist researchers in finding ecologically significant proteins such as those in enzyme production that encode for nitrogen fixation proteins (*nif* H), or encodes the iron protein in nitrogenase reductase (Coelho *et al*. 2009).

1.5.3 Why is PCR Significant to Environmental Microbiology?

The advent of PCR for ecological studies presents a unique opportunity for researchers to probe deeper and in more detail into areas not accessible before. The ability to detect and identify previously uncultured wild bacteria in the laboratory is of immense significance (Zengler *et al*. 2002; Joseph *et al*. 2003). Generic bacterial and archaeal 16S rRNA primers can be applied to almost any sample containing very small amounts of template DNA. Provided no inhibitors or contamination are present, identification of novel species is possible. This opens a whole new window of opportunity for established and new researchers to provide significant impacts into the understanding of the microbiological world (Leininger *et al*. 2006) and identify key natural processes taking place that were attributed elsewhere or even themselves unknown (Madsen, 2005).

Another advantage of PCR based technologies is that relatively small amounts of starting material are required, but it must be acknowledged that this may be a source of bias when considering microbial community studies (Wintzingerode *et al*. 1997). Wintzingerode *et al.* (1997) point out that other sources of bias apply to ecological studies in PCR, such as the preferential amplification of some DNA templates. This is due to the preferred binding of the DNA polymerase at the expense of other DNA templates. Also many species of bacteria contain multiple copies of the operons found in rRNA genes (e.g. *Bacillus* and *Clostridium* species contain 15 copies) (Klappenbach *et al*. 2001); therefore, the sequences observed from these species may be over-represented amongst the cloned DNA fingerprint.

Quantitative PCR (qPCR) or real-time PCR is a recent advancement that allows for the targeting of specific genes in the extracted template DNA to be quantified (Yergeau *et al*. 2007; Plassart *et al*. 2008; Wakelin *et al*. 2009). This method involves fluorogenic probes or dyes used to quantify the number of copies of a desired DNA sequence in the selected sample (Yergeau *et al*. 2007). This innovative approach to PCR has led to the successful quantification of target genes in diverse *in situ* habitats and *in vivo* incubations, thereby exemplifying the ability of soil bacteria to carry out specific functions in the environment (Hermansson & Lindgren, 2001; Kolb *et al*. 2003; Henry *et al*. 2004).

1.6 Denaturing Gradient Gel Electrophoresis (DGGE) and PCR Fingerprinting

"One of the beautiful things about science is that it allows us to bumble along, getting it wrong time after time, and feel perfectly fine as long as we learn something each time."

[Martin A. Schwartz (Schwartz, 2008)]

1.6.1 Introduction to DGGE and PCR Fingerprinting

Once amplified DNA from a sample is achieved using PCR it can be visualised by running it through an electrophoretic gel. The DNA is usually stained with EtBr (or another fluorescent dye with high affinity to DNA) and then the separated products are observed under UV radiation. All nucleic acids are negatively charged (Araya *et al*. 2007) and thus move through the gel towards the positive pole in an electric field. The agarose gel provides resistance to the migration of nucleic acids due to the pore sizes

within the gel matrix and thus larger DNA fragments will travel through the gel slower than smaller fragments (Thies, 2007). A standard molecular weight ladder is usually added to one of the sample lanes so that the size of the PCR products can be estimated during gel analysis. This analysis of PCR amplified nucleic acids is centred upon the presence and distribution of DNA bands of varying sizes within the gel matrix.

Gel electrophoresis relies upon the properties of agarose, as the most popular medium for the electrophoretic separation of nucleic acids, which has a large working range but provides poor resolution (Thies, 2007). Polyacrylamide gels are the preferred matrix for the separation of proteins and DNA fragments up to 2000 bases in length. The polyacrylamide gels separate macromolecules based on the configuration of the molecule as well as size, charge and G+C contents (Weber & Osborn, 1969; Bárány *et al*. 1995). Thus, because of the excellent resolving power of polyacrylamide gels, it is used in PCR fingerprint studies. The techniques that exploit the polyacrylamide gel for the screening of amplified DNA are SS conformation polymorphism (Duthoit *et al*. 2003) and denaturing (or temperature) gradient gel electrophoresis (DGGE or TGGE; Muyzer & Smalla, 1998). Due to the electrophoretic mobility of nucleic acids, these methods can be used to process highly sequence dependent material and can be used in genetic diversity studies to identify target species previously uncultured or isolated.

1.6.2 PCR Fingerprinting

The genetic makeup of microbial communities can be investigated using several methods resulting in a PCR fingerprint (Esseili *et al*. 2008). For the relevance of this review only DGGE will be briefly discussed but for an account of T-RFLP analysis, please see Clement *et al.* (1998) and Schütte *et al*. (2008) and references therein. The advantage of DGGE is that it is relatively quick to perform and allows for high sample throughput. It can be used to target sequences that are considered to be phylogenetically or functionally important (Thies, 2007). DGGE can be used to distinguish PCR products that are of similar length by using extra methods to separate the amplicons into a higher number of bands that can then be used to distinguish community members of a sample pool.

DGGE employs a parallel gradient of denaturing conditions along a polyacrylamide gel. DS DNA (dsDNA) amplicons (PCR amplified) are loaded into wells lined up along the top of the gel. Once an electric current has been activated, the

DNA migrates and the denaturing conditions of the polyacrylamide gel increases. In a polyacrylamide gel the denaturant is usually urea and formamide (Muyzer & Smalla, 1998). With dsDNA being a compact structure it migrates faster than partially denatured DNA. The sequence of a particular DNA fragment will determine the point within the gradient gel that denaturation begins to excessively delay mobility. The sequence of the dsDNA strands affects the duplex stability by both, percentage G+C content and adjoining nucleotide interactions (e.g. CGA is more stable than GAG; Breslauer *et al*. 1986). The gel produces a ladder of bands in each sample lane, dependent on the variability of DNA extracted from the original sample. (Fig. 1.12):

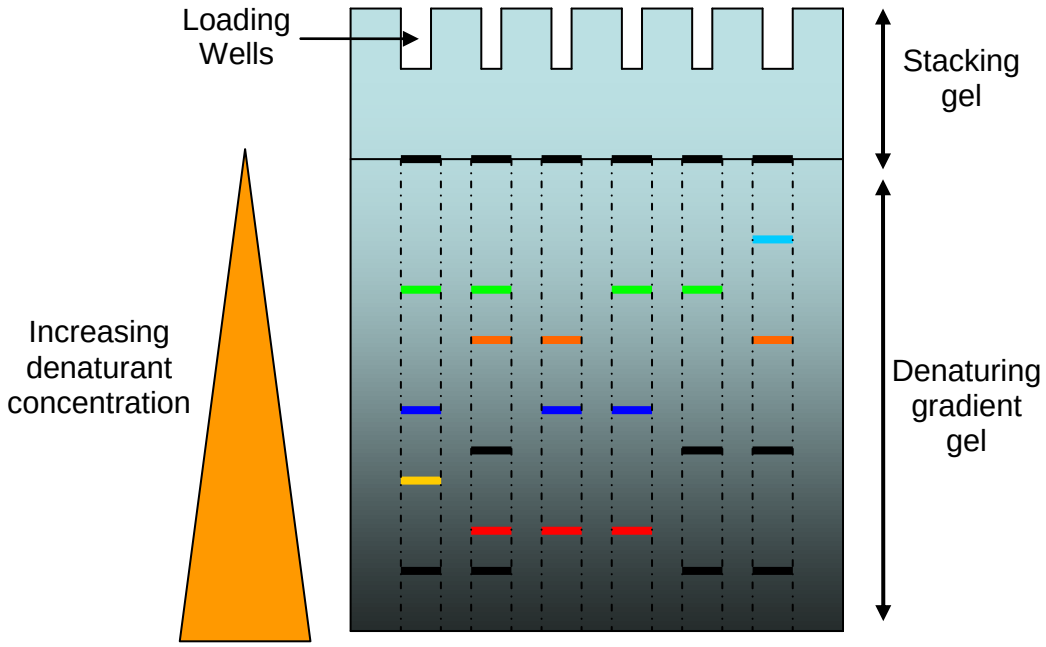


Fig. 1.12: Diagram of a DGGE plate with separated PCR amplified DNA products. PCR amplicons are separated according to their total A+T vs. G+C base pairs and the location of these base pairs to each other. Adapted from: Thies *et al.* (2007).

The ability of DGGE to detect PCR amplicons is high. The sensitivity of the technique is subject to variations in the experimental method and comparison between gels is difficult. DGGE is regularly used as a preliminary screening method to aid recognition of sample diversity. For instance, the resolving power of gel-based analyses is reduced to the number of bands able to "fit" onto the gel and therefore multiple bands may be counted as an individual band. A major advantage in using DGGE to other resolving techniques is that PCR amplicons may be cut out from the gel once separation has occurred, reamplified, cloned and sequenced so that taxonomic and/or phylogenetic data may be retrieved. DGGE is now regularly used in soil microbial ecological studies

to compare the organisation of complicated microbial communities (Viti *et al*. 2006; Esseili *et al*. 2008; Baptista *et al*. 2008; Zhao *et al*. 2008).

1.7 Gas Chromatography – Isotope Ratio Mass Spectrometry

"The range of human knowledge today is so great that we're all specialists and the distance between specialisations has become so great that anyone who seeks to wander freely between them almost has to forego closeness with the people around him."

[Robert M. Pirsig (Pirsig, 1974)]

1.7.1 Introduction to Isotope Ratio Mass Spectrometry

A relatively new analytical technique available to researchers is compound specific analysis using gas chromatography (GC) separation, online combustion of the compounds and analysis by isotope ratio mass spectrometry (IRMS). Gas chromatography mass spectrometry - isotope ratio mass spectrometry (GCMS-IRMS) has become an industrial and research standard instrumental technique because of its ability to measure isotope distribution at natural abundance levels with impressive accuracy and precision. Although GCMS-IRMS has been commercially available since 1990 (Meier-Augenstein, 1999), the rather large cost of the instrumentation had previously limited its availability to most research laboratories (Neufeld *et al*. 2007c; Kuypers & Jørgensen, 2007). In essence IRMS is a very sensitive detector with the ability to provide very precise measurements of isotope ratios with a standard error in a range between four and six figures. Once the detector has been attached to a GC instrument, the analyst is capable of running a highly precise compound specific isotope analysis (CSIA) with special attention to natural isotopic abundance. When using a technique capable of high precision CSIA at levels of natural abundance, information can be attained that indicates the biogenetic relation and possible origin of specific organic compounds (Meier-Augenstein, 1999). The specificity of GCMS-IRMS has been elegantly explained by Jasper *et al.* (2002) using an example where the carbon isotope ratio values of various substances within a combustible liquid solution were analysed. For example, commercial petroleum contains over 100 hydrocarbons and the researchers suggest that by combining the specificities of a number of these compounds, the overall specificity of the technique may be estimated. So if for example, each compound had 67 different isotopic variations and only 5 were measured for their carbon isotopic variations, the combined specificity would be $\sim(1/67)^5$ or 1 in 1.4 billion. This means that for a first order estimate there would be a 1 in 1.4 billion chance

that a randomly analysed petroleum sample would have the same isotopic signature as the one measured. At present GCMS-IRMS is capable of measuring the isotopic abundance (compared to standard reference data) of ^{2}H, ^{13}C, ^{15}N and ^{18}O.

GCMS-IRMS has many applications including food studies (Kelly *et al*. 2005), sporting drug abuse cases (Tsivou *et al*. 2009) and archaeological studies (Charrie´-Duhaut *et al*. 2009). Particular interest is the application of the technique to studies of biochemical processes and especially the assimilation and turnover of nutrients and other biologically important compounds (Heinzle *et al*. 2008).

1.7.2 The Basic Principle of IRMS

Unlike conventional mass spectrometers that provide structural information by scanning the mass range and identifying characteristic fragment ions, the IRMS can acquire highly precise measurements of isotopic abundance. The isotope ratio is achieved by first converting the analyte into a gas that is isotopically representative of the original sample prior to entering the ion source of the IRMS. The measurements take place on a continuous flow isotope ratio reading of $^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$ and these are performed on gases of H_2, CO_2, N_2 and CO respectively. The isotope ratio is determined by comparing the isotopic abundance against a standard or reference gas. This is performed to compensate against mass discriminating effects that will differ between time and different instruments (Meier-Augenstein, 1999).

The natural abundance isotopic ratio is generally reported as delta values (δ). The units are reported per mil ("mil"= 1000) and written ‰. Delta values are calculated using equation 1.2:

$$\delta = \frac{1000\,(R_{sample} - R_{standard})}{R_{standard}}$$

Eqn. 1.2: Where R_{sample} is the ratio of the heavy to the light isotope measured for the sample, and $R_{standard}$ is the equivalent ratio for the standard gas (Barrie & Prosser, 1996).

When comparing a samples delta value to that of the standard (itself calibrated against an international standard) measurements can be made. For instance, if a negative delta value is recorded then the sample is depleted in the heavy isotope relative to the standard. A positive value indicates that the sample is isotopically enriched in the heavy

isotope. Fig. 1.13 is a schematic diagram of a GC-combustion IRMS and is representative of the instrument used in this thesis.

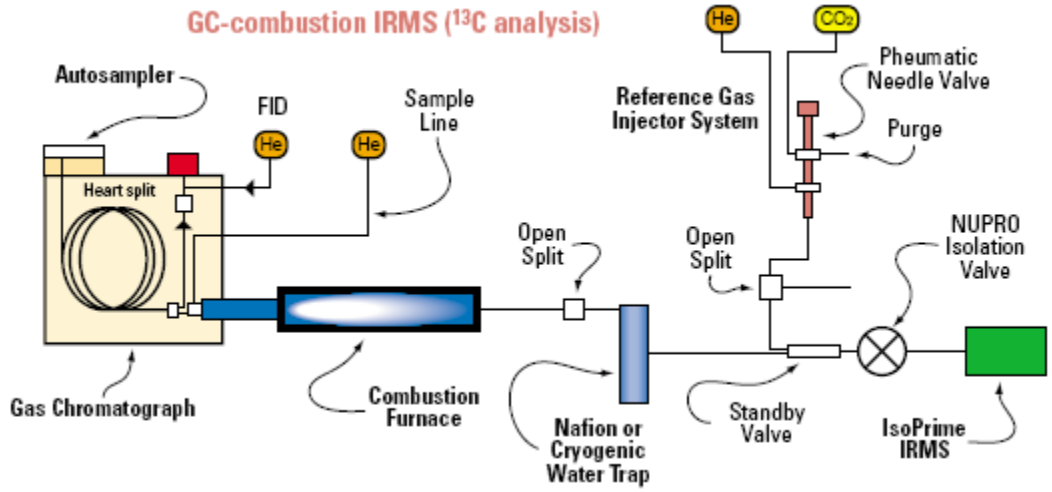


Fig. 1.13: Schematic diagram of an Isoprime GC-IRMS spectrometer. Taken from: Jasper *et al.* (2002).

1.7.3 Environmental Research Using GCMS-IRMS

GCMS-IRMS has wide applications in scientific research but due to its relatively high costs and existing standard procedures, many applications remain at the experimental stage (Benson *et al.* 2006). The diversity of samples and the applications of GCMS-IRMS provide a large scope for scientists in various fields to probe deeper into their particular realms of interest. In regards to this review, tracer studies involving environmental biology will be briefly focused upon. For a more in-depth review into environmental ^{13}C-labelling of microbial lipids the reader is encouraged to read Evershed *et al.* (2006).

One application of IRMS in environmental studies is the investigation of pollutants such as oil spills and other forms of contamination. Schmidt *et al.* (2003) produced a review that provides information on the analysis of organic contaminants in the environment using compound specific isotope analysis (CSIA) as well as quantification of biodegradation products using a range of environmental samples. The fact that microorganisms can utilise and store stable isotopes is of considerable interest when assessing environmental data. For instance, when attempting to determine the origin of biological weapons, CSIA can be employed to determine geo-locations of microorganisms in the same way that they are for human subjects by measuring the stable isotope ratios in water (Phillips *et al.* 2003). Another interesting microbial application was reported by Kreuzer-Martin *et al.* (2004) in which it may be possible to

deduce the growth environment of a particular pathogenic microorganism by determining the relationships between the stable isotopes of carbon, nitrogen and hydrogen in the growth medium. Croft & Pye, (2003) analysed soil samples using IRMS and showed that the carbon and nitrogen isotope ratio data could be used when trying to discern between soil types and soil generated products.

Tracer studies of stable isotopes through food webs have become increasingly popular due to the high precision and sensitivity of GCMS-IRMS. The application of GCMS-IRMS to studies of mammalian metabolism (Meier-Augenstein, 1999; Cunnane *et al*. 1997; Brossard *et al*. 1997; Hamard *et al*. 2009) have helped to elucidate pathways associated with drug metabolism and the formation of cellular components, amongst others. However, GCMS-IRMS has the potential to greatly enhance the tracking of stable isotopes through microbial food webs, which is of considerable importance to the work herein. Heinzle *et al.* (2008) demonstrated in a recent paper that a relatively simple incubation involving the industrially important species *Corynebacterium glutamicum* with an isotopically labelled glucose substrate, can yield significant data from an already well characterised strain of bacteria (Burkovski, 2003; Kirchner & Tauch, 2003) using GCMS-IRMS. The tracer study resulted in a statistically identical relative fractional enrichment of ^{13}C-glucose into proteinogenic amino acids over a range of applied concentrations. The authors also demonstrate the potential of GCMS-IRMS in the quantification of ^{13}C metabolic flux analyses using low labelling degrees of tracer substrates in a large scale bioreactor.

A study focusing on isotopic fractionation of *n*-alkanes under aerobic bio-degradation by Bouchard *et al.* (2008) showed that in alluvial sand, the highest enrichment factor was for propane and the magnitude of the enrichment factor decreased with increasing number of carbon atoms from propane to *n*-decane. It was observed that bio-degradation was limited according to *n*-alkane chain length and that significant carbon isotope fractionation occurs during aerobic bio-degradation. It was inferred that under unsaturated conditions the magnitude of isotope enrichment was connected to molecular size and structure. Another interesting study carried out by McKelvie *et al.* (2007) demonstrated that the presence of ethanol in a MTBE mixture was required under biodegradation conditions. CSIA showed that a substantial volume of tertiary butyl alcohol (TBA) was produced during biodegradation (1200 $\mu g\ l^{-1}$ TBA)

indicating that during the course of the experiment anaerobic breakdown of this by-product did not occur.

A study into the effects of increased anthropogenic nutrient addition to the Mondego estuary by Baeta *et al.* (2009) showed that stable isotopic analysis can be used to track changes in C and N sources in respect to seasonal variations. More specifically, the group concluded that stable isotope values derived from macrophytes and selected grazers are the most useful tracers in the Mondego estuary as little evidence of seasonal variance between primary producers and consumers has been reported. The fact that seasonal variances (temperature, precipitation, nutrients and chlorophyll) did not significantly alter isotopic signatures in the consumers is relevant when considering monitoring purposes, as it frees sampling protocols from seasonal schedules when using IRMS studies. Another marine based study using GCMS-IRMS was carried out by Blumenberg *et al.* (2009) involving Holocene sediment cores taken from the Black Sea. The research group examined the potential for bacteriohopanepolyols (BHPs) to act as environmental markers in marine sediments as these compounds are ubiquitous in organic matter. It was found that tetrafunctionalised BHPs were present within the sediment core and that they were associated with cyanobacteria, pelagic methanotrophic bacteria and purple non-sulphur α-proteobacteria. The results demonstrate that BHPs from microorganisms present in the deeper biogeochemical zones of marine environments are under-represented in comparison to the BHPs present in euphotic zones. BHPs are not considered to be stable biomarkers within sediment cores for living bacteria but still have a use in paleoreconstruction models.

Research into microbial decomposition of ^{13}C compounds is of particular interest and the incorporation of stable isotopes into biomarkers is an extremely useful tool for scientists. Crossman *et al*. (2006) looked at the effect of ammonium sulphate ($[NH_4]_2SO_4$) on populations of CH_4 oxidising bacteria using GCMS-IRMS on PLFAs. CH_4 is an environmentally sensitive gas due to its strong greenhouse properties with soil bacterial oxidation considered an important sink. The researchers reported that previous evidence showed that soil CH_4 oxidation is inhibited in the presence of excessive salt ions. The fact that the target bacterial species are currently not amenable to current culturing methods resulted in the need for *in situ* analysis resulting in data that confirmed the current evidence that salt addition to agricultural lands inhibits CH_4 oxidation. $[NH_4]_2SO_4$ treatment of soils reduced the amount of ^{13}C incorporated into

bacterial PLFAs. Campbell *et al.* (2009) have demonstrated that hydrogen isotopic fractionation occurs during bacterial lipid biosynthesis of H_2 consuming bacteria. The researchers grew pure cultures of *Desulfobacterium autotrophicum* and measured the deuterium to hydrogen isotopic ratios (D/H) of H_2, water and specific fatty acids using GCMS-IRMS. The results suggested that the isotopic content of fatty acids increases with the length of the carbon chain from C_{14} to C_{17} among both the saturated and unsaturated fatty acids. The data shows that a net fractionation of hydrogen isotopes associated with fatty acid biosynthesis in *D. autotrophicum* that is smaller than that in other H_2 consuming bacteria but much greater than in most other photoautotrophs. An application of this data was suggested in the interpretation of sedimentary organic matter when interpreting its sources. The authors concede that a much broader survey of bacterial lipids is required in order to establish the ubiquity and metabolic specificity of H_2 fractionation into biomass.

Tracking carbon as it passes from decomposing plant material and from living roots directly into the soil using GCMS-IRMS was conducted by Elfstrand *et al.* (2008). The experiment was carried out in the field using ^{13}C enriched organic substrates (red-clover green manure and a pulse labelled leek crop) with the aim of determining the incorporation of ^{13}C into fatty acids. The aim of the experiment was to determine if different species of microorganisms and fauna were specific to the decomposing of green manure or the root derived exudates. The GCMS-IRMS data showed that fatty acid markers related to actinomycetes and Gram-positive bacteria were strongly linked to the green manure. The root exudates appear to have been strongly linked to mycorrhizal fungi. In contrast, biomarkers for Gram-negative bacteria were prominent from both substrates and the strongest response was gained from these diverse bacterial groups for PLFAs. This data indicates the specialisation of microbial groups between the two different substrates with the exception of Gram-negative bacteria. However, due to the large variation in isotopic ratios within taxa, the authors could not confirm or deny their hypothesis of carbon specialisation.

Atmospheric enrichment studies involving CO_2 incubation of soils and/or soil derived organic matter deposition via elevated CO_2 conditions are of specific relevance to the work herein. Paterson *et al*. (2008a) investigated atmospheric CO_2 enrichment and nutrient addition to plants, to investigate the microbial carbon sequestration of ^{13}C-depleted compounds. Two CO_2 substrates were used for the study in which one had a

depleted ^{13}C signature (-36.3 ‰) and the other was atmospheric CO_2 (-8.5 ‰). The aim of the experiment was to determine the impact of increased CO_2 concentration and nutrient accessibility by plants on the microbial community. GCMS-IRMS was used to determine the ^{13}C content of soil microbial PLFAs. It was concluded that elevated CO_2 and nutrient concentrations both increased mineralisation of SOM but contrary to the stated hypothesis, these effects were caused by stimulated plant growth and the associated increased area of rhizosphere soil rather than altered balances of microbial assimilation of plant and SOM carbon sources. Another study carried out by Paterson *et al.* (2008b) also centred upon plant inputs to soils using IRMS analysis. Enriched ^{13}C-labelled substrates were added to soils to determine the activity of the microbial population. The mineralisation of the soluble fractions was observed to take place rapidly (< 2 h) and was detected in the bacterial PLFA fraction, whereas in contrast the insoluble additions were mineralised much slower and were recovered in fungal biomarkers. Overall, the additions did not alter the microbial community structure but their fate was dependent on the addition type. The overall conclusion derived from the experiment was that relatively little carbon was transferred between microbial populations and that the majority of carbon loss from the system is from plant respiration.

The fixation of CO_2 into soils is of particular relevance to the work herein. Miltner *et al*. (2004) attempted to elucidate the pathway of carbon sequestered into the soil profile. This was done by analysing the SOM content and its subsequent changes during incubation. A soil was incubated in the dark under $^{13}CO_2$ conditions and found that a significant transfer of ^{13}C-label into the soil organic matter occurred. The enrichment of fatty acids (^{13}C up to 1200 ‰) and amino acids (^{13}C up to 200 ‰) showed that a wide range of autotrophic and heterotrophic microbes mediated the process. Autotrophic inputs to soil carbon were considered to be small if not negligible using their techniques. The authors theorise that a large and as yet undiscovered pool of compounds produced from CO_2 fixation may be present in soil that may explain the route of the "lost" labelled carbon from the experiment.

1.7.4 Conclusion on the Application of GCMS-IRMS for Environmental Research

One of the driving forces behind the development of GCMS-IRMS was the potential to measure the ^{13}C abundance of sedimentary hydrocarbons at a molecular level (Meier-Augenstein, 1999). The majority of work done on IRMS has focused on ^{13}C compounds

of both natural abundance and enrichment/depletion experiments. In recent years attention has focused on $^{2}H/^{1}H$ and $^{15}N/^{14}N$ with considerable progress in fractionation studies for both of these stable isotopes. Commercially available standards exist for $^{18}O/^{16}O$ but routine analysis is still in its infancy. At present, chlorine and bromine isotope analysis is emerging because they are important environmental pollutants (Annable *et al*. 2007; Bernstein *et al*. 2011; Carrizo et al. 2011).

GCMS-IRMS is a technique that has been used in a wide array of applications, but the potential for environmental research is vast and exciting. The sheer diversity of samples that can be processed by a single research team using a single technique provides ample opportunity for new discoveries to be made. The ability to identify labelled compounds from an array of background materials and interferents makes GCMS-IRMS an attractive prospect for environmental researchers as the chance to probe deeper into natural processes has the potential to contribute to the growing body of knowledge accumulated to date. Finally, it has been noticed during the research into this review that more insight into the specific organisms involved in metabolising labelled substrates could be provided by combining GCMS-IRMS labelling studies with molecular DNA analysis (Zhang, 2002).

1.8 Nuclear Magnetic Resonance (NMR) Spectroscopy

"During revolutions scientists see new and different things when looking with familiar instruments in places they have looked before. It is rather as if the professional community had been suddenly transported to another planet where familiar objects are seen in a different light and are joined by unfamiliar ones as well."

[Thomas S. Kuhn (Kuhn, 1962)]

1.8.1 Introduction to Environmental Applications of NMR Spectroscopy

NMR is a suitable method for probing the assimilation of carbon substrates in soil microcosms in a non-invasive manner (Lundberg *et al*. 2001). NMR is a measurement technique where the absorption of electromagnetic radiation within a sample takes place for the purpose of qualitatively assessing the molecular species present and determining organic structures. The general principle involves the absorption of electromagnetic radiation by the magnetic nuclei of certain atoms, while in the presence of an external magnetic field. A limited number of atomic isotopes are available for NMR studies which can be advantageous depending on the study method (e.g. isotope distributions). Many isotopes can be utilised using NMR but routinely ^{1}H, ^{13}C, ^{31}P, ^{27}Al, ^{29}Si, ^{17}O and

^{15}N are commonly encountered within the literature. The majority of NMR work carried out to date has been with the ^{1}H isotope in routine liquid-state organic chemistry studies (Lloyd *et al*. 2007) but an increased awareness of solid-state analysis has become more prevalent in recent times. The analysis of solids using standard NMR techniques provides broad and poorly resolved spectra and therefore a variant of the technique, where the solid is held at a specific orientation (the 'magic angle') to provide high resolution spectra of nuclei of atoms such as ^{13}C. This form of analysis is known as charged polarised magic angle spinning NMR (CP-MAS-NMR) and is useful for measurements of atoms/molecules that have high degrees of spin. The most important components of organic structures when concerned with NMR spectroscopy are ^{1}H and ^{13}C nuclei and although ^{12}C and ^{16}O are also present in organic compounds, they do not possess the required spin and thus do not provide an NMR spectrum.

The project under study involved the analysis of solid-state environmental samples using NMR as a means of tracking the fate of inorganic $^{13}CO_2$ into the soil matrix. The soil samples were prepared as part of a multi-discipline experiment with NMR forming one aspect of the analysis. NMR measurements took place at a research facility at the University of Toronto at Scarbourgh, Toronto, Canada, under the supervision of Dr. Andre Simpson. This collaboration allowed for detailed analysis of the generated data and was a major emphasis for the project as a whole e.g. combination of techniques to study the interactions between a single abiological/biological system.

1.8.2 Basic Principles behind NMR Spectroscopy

In nature, certain nuclei behave as though they are spinning and as nuclei are charged, this creates a magnetic field. These spinning nuclei essentially behave like tiny magnets. When the nuclei are placed between the poles of a strong magnet, they align themselves with or against the external magnetic field. Those nuclei that align with the magnetic field will have a slightly lower energy than those aligned against the field. Energy is applied, in the radiofrequency (rf) range, where it is possible to excite nuclei in a lower energy spin state to the higher energy spin state (otherwise known as a 'flip'; Hart *et al*. 2003). The energy gap between these different spin states is dependent on the strength of the applied magnetic field (e.g. the stronger the field, the larger the energy gap). NMR transitions in the sample produce a signal measured by the receiver channel of the instrument. This only occurs when the resonance condition is satisfied. Two methods of producing this signal are normally applied; varied magnetic flux density with a constant

transmitter frequency or varying the transmitter frequency and keeping the magnetic flux density constant. In both cases the signal received from the sample is monitored constantly to develop the spectrum. This is known as the continuous wave (CW) method as it uses an uninterrupted rf source. The CW method was the basis for all the early NMR experiments but has now been superseded by the pulsed method. In the pulsed method all of the nuclei in the sample are excited simultaneously by an rf pulse. The technical description of this process is complex but essentially a single pulse is generated by switching on the rf for a very short time. The single pulse contains not just the central frequency but a band of frequencies symmetrical around the centre. As the amplitudes of the frequency decrease with increasing distance from the centre frequency, a "hard" pulse is applied to ensure that all the nuclei are irradiated equally i.e. a short pulse of high power (Friebolin, 2004). For a comprehensive discussion of the basics of NMR measurement, please refer to Friebolin (2004).

Solid-state NMR is another powerful approach to elucidating environmental samples. Most solid samples are rigid and the strong dipole interactions arising between proximal nuclei are not averaged to zero unlike liquid samples, which leads to considerable line broadening. This is very relevant to ^{1}H NMR and reduces the chemical shift information. It is for this reason that most environmental studies focusing on SOM involve ^{13}C detection (Forte *et al*. 2006; Salati *et al*. 2008; Simpson *et al*. 2011). ^{13}C is a naturally occurring rare isotope that is on average 1.13% abundant in nature. Homonuclear splitting (^{13}C-^{13}C dipoles) does not dominate and thus provides excellent ^{13}C chemical shift information. ^{1}H-^{13}C dipolar interactions can be suppressed by using high power decoupling. Anisotropy is reduced by spinning the sample at the magic angle (Andrew *et al*. 1959; Franz & Linehan, 1993) otherwise known as magic angle spinning (MAS). The magic angle is a precisely defined angle of 54.7^{o} where any interactions which depend on this second order Legendre polynomial (Bonhomme & Livage, 1998) vanish. Crucially, when sufficient time is left between scans that allow the ^{13}C nuclei to relax (return to equilibrium), then data is generated that can be used to quantify carbon nuclei. A drawback to the MAS-NMR method is the long relaxation times required (seconds to minutes; Mao & Schmidt-Rohr, 2004) which can lead to extremely long measurement periods for environmental samples that usually require thousands of averages for adequate signal to noise ratio (Preston, 2001).

High resolution (HR) MAS-NMR is another powerful tool employed by spectrometrists to investigate environmental samples, but is especially useful for studying organic matter in soils (Colnago *et al*. 2003). HR-MAS-NMR is used to study samples in their unaltered state so that the structures and reactivity of the different phases (solid, semi solid/gel and solution) be discerned (Simpson *et al*. 2011). This recently developed approach can be used to study the gel-like component of a sample and is complementary to solid-state analysis (Maas *et al*. 1996). In the presence of solvent or naturally present water, the sample is spun at the magic angle. The solvent helps to decrease the ^{1}H-^{1}H dipolar interactions, while spinning at the magic angle reduces line broadening. The probes are generally fitted with magic angle gradients permitting solution-state experiments which allows for high resolution data to be collected on the soluble gel phases of a sample that can contain liquid, gel and solid phases. The advantage of studying samples in this state is that the soil/sediment can be analysed in its most biologically active state and that the natural water/solution phase can be measured directly (Simpson *et al*. 2011). The application of HR-MAS-NMR to whole soil was first reported by Simpson *et al*. (2001) where polar structures were observed such as sugars, amino-acids and head groups of long-chain fatty acids. One drawback to HR-MAS-NMR is that ^{1}H is the most commonly used for detection. This isotope gives excellent information on the soluble and gel components, but species in the true solid-state will not be detected by the technique (Simpson *et al*. 2011). The rotor containing the sample may be transferred to a solids probe to study the components that remain as true solids in solution.

1.8.3 Environmental Soil Research Using CP-MAS-NMR

The complex nature of SOM and recalcitrant biopolymers has stifled soil research since its inception, even though this thin band of material sustains all life on dry land (Sugden *et al*. 2004). Environmental matrices such as soil are a complex mixture of minerals, organic matter, micro-, macroorganisms and anthropogenic chemicals. Soil has been described as the most complex biomaterial on Earth (Young, 2004) and NMR is arguably the most powerful tool currently available to evaluate its components (Simpson *et al*. 2011). NMR can be used for the determination of complex structures and their interactions, and can be performed on components with various physical phases (solids, gels, liquids and gases). The multitude of NMR experiments at a molecular level is what makes NMR spectroscopy so valuable to soil scientists. NMR techniques can be used to observe soil matrices at different scales of complexity

(Simpson *et al*. 2011). For example, basic solution-state NMR can be used to elucidate the structural components of soil and provide detailed structural overviews of binding components in complex environmental samples (Simpson *et al*. 2003; Cook, 2004; Kelleher *et al*. 2006; Kelleher & Simpson 2006). Another more invasive form of NMR is known as diffusion NMR that can be used to understand how organic matter forms aggregates in free solution spaces, such as soil pores (Conte & Piccolo, 2002; Simpson *et al*. 2002; Simpson, 2006; Smejkalov & Piccolo, 2008). This technique can be used to study the potentially large colloidal species that are formed (up to the micrometer scale) in the environment, which are central to the reactivity of environmental organic matter (Simpson *et al*. 2011). At a larger scale, NMR can used to assess the association of soil organic matter (SOM) with clays and other soil minerals. The power of solid-state and HR-NMR at this scale lies in the ability to provide information on how the various components are arranged, layered and associated (Wilson *et al*. 1981; Simpson *et al*. 2001; Simpson *et al*. 2002; Simpson *et al*. 2006; Smejkalov & Piccolo, 2008). NMR can also be coupled with imaging technology, such as magnetic resonance imaging (MRI) instruments to uniquely provide a wealth of information about the physical structure of soil columns and the movement and transformations of contaminants (Van As & Van Dusschoten, 1997; Reeves & Chudek, 2001; Nestle *et al*. 2002). The ability of NMR to look into the 'black box' of soil processes using solid and liquid approaches sets it apart from other techniques. A major advantage of solid-state NMR is that very little pre-treatment is required and yet it can provide an arguably unsurpassed level of molecular information, using non-destructive methods. A brief overview from some selected research articles involving solid-state NMR methods shall be reviewed to provide some basis to the power of NMR techniques in soil research.

A study into the fate and transformation of plant litter using HR-MAS-NMR spectroscopy was carried out by Kelleher *et al*. (2006) because of the key role these residues play in the carbon and nitrogen cycles of the pedosphere. The authors claim to present the first application of the prescribed techniques to ^{13}C and ^{15}N labelled plant material. The preliminary study conclusively showed that carbohydrates were rapidly degraded while 2-D HR-MAS-NMR spectra also showed that hydrolysable and condensed tannin structures were readily decomposed. The persistent presence of waxes and cuticles (aliphatic components) showed these components to be more stable over the observation period (12 months). The most significant finding made was the conclusion that nitrogen from pine residue materials was either selectively preserved or

re-structured into what appears to be novel structures. A limitation to this study was the minimal interaction of the labelled biomass with the incubation soil (although due to the expense and limitations of available material for perfectly valid reasons). It is most likely that mineral interactions that took place between SOM in the soil were not prevalent in the experiment and do not completely mimic *in situ* conditions, despite the authors best attempts. A follow on study carried out within the same research group (Spence *et al*. 2011) used the same methods but applied to ^{13}C and ^{15}N labelled soil microbial biomass. A similar rapid loss of carbohydrates was observed however, diffusion edited HR-MAS-NMR revealed that macromolecular carbohydrates were more resistant to degradation. Dissolved protein and peptidoglycogen accumulated in the leachate indicating that the degradation pathways are similar to that of plant material. In a very recent study, McCaul *et al*. (2011) studied the composition of freshwater dissolved organic matter (DOM) extracted from a large lake system on the River Shannon, Rep. of Ireland. As the River Shannon is the largest catchments area within Ireland and Britain (draining a land area of 18,000 km^2) it can be used to identify some of the land based interactions with waterways. 1-D and 2-D NMR was employed to investigate the structural components, where it was reported that carboxyl-rich acyclic molecules (CRAM) were present, which is consistent with previous findings (Lam *et al*. 2007). The presence of phenylalanine was associated with areas influenced by agriculture but significantly not found in areas where Zebra mussels (*Dreissena polymorpha*) dominate. The presence of several microbial biomarkers such as peptidoglycan, lipoproteins and other proteinaceous material indicates that microorganisms contribute to the stable dissolved organic matter pool.

The application of solution-state NMR to elucidate the structural components of soil humin was carried out by Simpson *et al*. (2007b). The importance of this particular chemical class (humin) lies in the fact that it is currently the most recalcitrant and least understood fraction of organic matter. The authors set out to use solution-state NMR in the first attempt at studying the structural components of soil humin. It was found that peptidoglycan, peptides, aliphatic species, carbohydrates and lignin were dominant components. Diffusion edited spectroscopy showed that all species were present as stable aggregates or macromolecules. Interestingly, the large proportion of peptidoglycan indicates a much larger contribution from microorganisms to humin than normally found in humic and fulvic fractions. In the same issue of Environmental Science and Technology, Simpson *et al.* (2007b) follow up the microbial input to humin

hypothesis. The article challenged the currently accepted estimates on SOM content by claiming that microbes contribute > 50% of the extractable SOM, > ~45% of the soil humin fractions and > 80% of the soil nitrogen. The significance of these findings are fundamental to current knowledge, as SOM is intimately linked to nutrient release, nitrogen turnover rates, contaminant fate, soil quality and fertility. The overall conclusion made by the authors is that SOM and soil organic nitrogen (SON) is predominantly of microbial origin and therefore this material (whether living or dead cells) plays an underestimated role in several soil processes. These findings were validated in an independent study carried out by Miltner *et al*. (2009). The conclusion was challenged by Chapman (2008) whose short correspondence argues that Simpson *et al*. (2007a) confuse soil microbial biomass with microbially derived material (necromass). Essentially, Chapman states that nothing new was uncovered in the study and the current estimates are not of microbially derived material but merely the living fraction. Simpson & Simpson (2008) acknowledge in their reply that the incorrect use of the terms "microbial biomass" and "microbial-derived material" was used. Simpson & Simpson (2008) go on to further clarify the aims of the study pointing out that the total microbial contribution is unknown and their research still provides significant insight.

NMR studies concentrating on bacterial inputs to SOM are of particular interest to the project and NMR will be one technique used to elucidate the contribution of soil chemoautotrophs in the logarithmic growth phase. Baldock *et al*. (1990b) provided important initial work in regards to glucose decomposition in soils using solid-state ^{13}C NMR. The soil sample was laced with a ^{13}C-labelled glucose and the fate of the transformed degradants was traced. It was found that most of the substrate was converted into carbohydrates, polymethylene and carboxyl carbon. The authors claim their evidence to be conclusive proof that microbiological elements in the soil are responsible for polymethylene (indicated to be derived from microbial cell membranes) build up. A much more comprehensive ^{13}C NMR study on the consumption of glucose in soils was carried out by Lundberg *et al*. (2001). The *in situ* study was carried out on a forest soil where it was observed that ~50% of the substrate was consumed within three days. The decay of ^{13}C-labelled glucose coincided with the appearance of olefinic triacyclglycerols. Measurements of soil respiration over the three days indicated that 40% of the decomposed glucose was respired as CO_2, 40% was allocated into solid state components and the remainder as triglycerols. For the remainder of the study (28 days),

it was observed that triacyclglycerol concentration peaked after 13 days and then subsequently declined by 60% by the end of the experiment. Based upon this evidence, it was hypothesised that large amounts of storage lipids were formed as a result of glucose degradation by eukaryotic organisms (most likely fungi), and these storage lipids were subsequently consumed once the glucose substrate had become depleted. This elegant study appears to have been slightly overlooked (18 citations) by the research community as it provides an insight into soil respiration of a common sugar introduced into the rhizosphere via plant root interactions. A study by Nieman *et al*. (2007) involved the incubation of cultures of ^{13}C-labelled *Mycobacterium* sp. KMS (grown on [4-^{13}C] pyrene) in the presence of humic acids. The aim was to characterise the chemical nature of the produced residues and evaluate the potential for bonding reactions with humic acid. The application of ^{13}C NMR analysis indicated that the majority of pyrene metabolites were incorporated into cellular material. Some of the remaining metabolites reacted with the added humic material but this did not appear to be the primary fate mechanism. The evidence presented supports previous findings that the humification process is an active contributor to contaminant (PAHs) and toxicity reduction in soil systems.

1.8.4 Conclusion on the Application of NMR Techniques in Environmental Research

As the techniques available to research chemists improve and the sensitivity of NMR increases, so does the potential for opening up the mysterious 'black box' that is soil chemistry. NMR is one of the very few tools currently available that can elucidate the molecular level framework that underlies chemical structures and interactions. As environmental issues have now become quite dominant in the public domain, research funding in this field has steadily increased to unprecedented levels (Simpson *et al*. 2011). However, the majority of NMR research currently involves drug development, materials science, medicine and the industrial chemicals sectors. Only a handful of NMR facilities dedicated to environmental research currently exist. NMR environmental research is still considered to be in its infancy although the scope and capacity for it to bring paradigm changes to our understanding of soil chemistry.

1.9 Project Objectives

To attain an understanding of soil carbon dynamics and the interactions between mineral assemblages, organic matter and the organisms involved in their interactions,

we must first develop methods and strategies that can elucidate specific environmental processes. Mimicking these environmental processes in the laboratory may only have limited benefit when it comes to estimating *in situ* systems due to the requirement for control and repeatability, something that is rare in the natural world. Therefore, it is sometimes necessary to develop first-step protocols where we can produce data from a specific sample type such as soil, to discover what traits can be promoted. The project herein was envisioned to demonstrate that a single soil sample could be incubated to promote microbial autotrophy, and then perform a suite of techniques that had never been previously performed in conjunction with each other.

The first stage of this project was to develop a microcosm where the observation of CO_2 sequestration was not only obvious but quantifiable. Following on from developing a working protocol, the application of a suite of cutting edge techniques to elucidate some of the biological processes was necessary. Finally, it was necessary to provide context for the relevance of this work to the natural world and therefore an experiment was designed to reproduce a common arable practice. Specifically, in chapter II the method development was demonstrated to show how a soil could be manipulated to sequester CO_2 into the organic fraction via stimulation of extant microorganisms of several different soils. This initial work was not intended to demonstrate sequestration capacity but some estimates and comparisons are made to provide context. The objective of chapter III was to demonstrate the capacity for acquiring data on the organisms responsible for carbon capture and the organic inputs to the microcosm from a single sampling event. The emphasis on the single sampling event was important, as it showed that various, highly intensive techniques may be performed on a relatively small amount of material to achieve a greater understanding of environmental processes. As the relevance to environmental systems was limited due to the biases of laboratory bound experiments, the main objective of chapter IV was to demonstrate the applicability of these methods to a currently used agricultural process. In this final experimental section, the application of a common fertiliser to soil was performed to demonstrate that a similar multi-discipline study can help to elucidate biological pathways for carbon sequestration. The incubation method was changed to demonstrate that the techniques can be employed to better mimic the environment and that extreme sample modifications were not always required.

1.10 References

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Chapter II: Carbon Sequestration in a Slurried Soil: Investigating CO_2 Uptake by Soil Microorganisms

2.0 Abstract

Sequestration of CO_2 via biological sinks is a matter of great scientific importance due to their potential to lower atmospheric CO_2 levels and increase soil organic matter. A growing body of evidence suggests that CO_2 uptake via soil dwelling chemoautotrophic bacteria maybe greatly underestimated. In this study, a custom built incubation chamber was used to cultivate a soil microbial community to investigate soil chemoautotrophy. The internal atmospheric CO_2 concentrations were monitored and used to estimate the mass of CO_2 uptake. It was found after careful background corrections that 256.4 µg CO_2 kg^{-1} dry soil was removed from the chamber atmosphere over 16 hours. Comparisons made to photosynthetic controls (i.e. grass and soil algae) whereupon it was observed that the chemoautotrophic fraction sequestered 2.6 and 5.4% of that taken up by grass and soil algae respectively. Using isotopically labelled $^{13}CO_2$ and GCMS-IRMS it was also possible to extract and identify labelled lipids after a short incubation time, hence confirming the CO_2 uptake potential of the soil slurry. Collectively, the results of this study have shown that the role of soil microorganisms in sequestering environmental CO_2 is possibly underestimated. Provided with favourable conditions, chemoautotrophic soil bacteria have the potential to make a significant impact on inorganic carbon sequestration within the environment. The aim of this chapter was to develop the methods necessary to achieve ^{13}C-labelled chemoautotrophic biomass and the calculations necessary to quantify the mass of CO_2 sequestered from the atmosphere. The results of this *in vivo* study have provided the ground work for future studies intending to mimic the *in situ* environment.

2.1 Introduction

The global soil carbon pool (organic and inorganic) is approximately 3 times the size of the atmospheric pool and 4.5 times that of the biotic pool (Lal, 2004). Humic substances (HS) are a large, operationally defined fraction of soil organic matter (SOM). 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, it was recently concluded 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 was traditionally thought (Kelleher & Simpson, 2006). Furthermore, the concept that extractable SOM is comprised mainly of humic materials has also been challenged and it has been shown that the presence of organic material sourced from microbes far exceeds presently accepted values, with large contributions of microbial peptides/proteins found in the HS fraction (Simpson *et al*. 2007). Based on the amount of fresh cellular material in soil extracts, this research group assume it is probable that the contributions of microorganisms in the terrestrial environment are seriously underestimated. If this is the case then efforts to manage soils to increase their carbon storage capacity (as first suggested by the IPCC in 1996 and again in 2001) may be a possible means of slowing the rate of atmospheric CO_2 increase (IPCC, 1996; IPCC, 2001).

If we underestimate the contribution of microorganisms to SOM, do we also underestimate their role in carbon sequestration? In this chapter I attempt to quantify the uptake of CO_2 by soil chemoautotrophs under ideal conditions using a custom built environmental incubation chamber. Biological CO_2 sinks are of global significance and the majority of available data relates to eddy covariance measurements of diverse communities, rather than the direct rate of CO_2 uptake of a particular species (Fierer *et al*. 2009; Fleisher *et al*. 2008; Miltner *et al*. 2004). There are some examples in the literature, such as a report by Tieszen & Johnson (1975) who investigated various vegetation types found widespread across the Arctic Alaskan tundra. They reported maximum photosynthetic rates > 18 x 10^{-4} g CO_2 m^{-2} h^{-1} and confirmed that uptake was related to irradiance exposure and comparable to other species in temperate zones. Dugas *et al*. (1997) measured leaf CO_2 uptake using a leaf chamber on three C_4 grasses and by employing the Bowen ratio/energy balance (a micrometeorological method) determined the rates for three grass types (3.08, 1.21 and 0.76 g CO_2 m^{-2} h^{-1}). The

authors acknowledged that these rates were likely to be underestimated due to the large variations in soil CO_2 flux generated during the canopy chamber experiments. Results were also widely scattered and the authors cite the large variability and a lack of precision. These results show a large variation between sites and species. However, given the large differences and possible calculation errors, these results could suggest that large volumes of substrate CO_2 are being sequestered during a typical growing season when one considers the widespread nature of C_3 and C_4 type grasses (Sage *et al*. 1999).

Soil microorganisms are also key players in the fixation and mobilisation of carbon and nitrogen, through both heterotrophic and autotrophic metabolic processes (Falkowski & Fenchel, 2008). Certain species of soil bacteria are known to autotrophically fixate mineral forms of gaseous carbon and nitrogen to produce organic cellular matter via various biochemical enzymatic processes. Phylum such as cyanobacteria utilise oxygenic photosynthetic biochemical pathways to fixate atmospheric CO_2 and N_2 in soil, freshwater and marine environments (Madigan *et al*. 2009) thus providing the basis for trophic food webs. Another group of bacteria (and archaea) known to survive autotrophically are the chemoautotrophs (chemolithotrophs). These prokaryotes use inorganic substrates to derive energy for biosynthesis reactions via aerobic or anaerobic CO_2 assimilation (Alfreider *et al*. 2009). They are unique in their ability to derive energy from sources not related to solar activity and can be found in diverse locations both above and below the Earth's crust (Waksman & Joffe, 1922; Starkey, 1935; Pedersen, 2000; Amend & Teske, 2005; Sorokin & Kuenen, 2005; Alfreider *et al*. 2009) as well as some groups being considered fairly ubiquitous across soil landscapes, such as the genus *Thiobacillus* (Chapman, 1990; Smith & Strohl, 1991). Microbial uptake of atmospheric CO_2 via autotrophic processes is a well characterised biological phenomenon, but actual estimates of sequestered CO_2 are rare in the literature (Miltner *et al.* 2004). Miltner *et al*. investigated this area and studied the fate of sequestered CO_2 into a soil matrix under dark conditions. The authors incubated soil using an isotopically enriched $^{13}CO_2$ atmosphere over an extended period (61 days) while no additional growth substrate was indicated. It was clearly demonstrated that stable isotope incorporation took place in contrast to fumigated controls and targeted biomolecules displayed evidence of isotopic enrichment. It was shown that 1.3 µmol C g^{-1} soil was incorporated into SOM over the 61 day incubation period. These measurements of SOM accumulation directly from a non-photosynthetic source was

hypothesised to have come from a combination of both autotrophic and heterotrophic growth activities, although the authors clearly point out the limitations of autotrophy within their particular sample. Boyd *et al*. (2009) undertook a study into thermoacidophilic microbial CO_2 uptake and fixation carried out by chemosynthetic thermophiles at a geothermal spring in the Yellowstone National Park. The study was carried out *in situ* and it was calculated that an average of 13.5 ± 0.9 µg C 10^7 $cells^{-1}$ h^{-1} was incorporated over a 2 hour period, but significantly higher rates occurred over the first hour. It was important to note that no additional substrates such as electron donors were incorporated in the experimental set up, but the presence of naturally occurring precipitated S^0 was likely to be the electron donor source. Denaturing Gradient Gel Electrophoresis (DGGE) showed the presence of 14 operational taxonomic units (OTUs), 12 of which were related to the chemoautotroph, *Hydrogenobaculum*. A smaller fraction was affiliated with *Cupriavidus* sp. strain JB1B4 and *Ralstonia taiwanensis* LMG 19424 (both members of the Betaproteobacteria class). Also a sole archaeal 16S rRNA sequence affiliated with *Thermocladium modestius* was detected. All of the chemoautotrophic activity at the site was attributed to the abundance of sulphur flocs, high temperatures (73^oC) and low pH (pH ~3) precluding the dominance of photosynthetic species.

The possibility of atmospheric carbon sequestration occurring in soil is of great interest and hence chemoautotrophy was the target of this study. Environmental growth chambers have been utilised for this type of study for various related sample types (Ferguson & Williams, 1974; Nakanoa *et al*. 2004; Fleisher *et al*. 2008) but few studies make attempts at quantifying the volume of CO_2 taken up during incubation. It should also be noted that a limitation of any environmental chamber study involving gases, is the potential for leaks due to the small molecular size of the substrate gases as well as partial pressure and temperature effects. At present we were only able to locate a single study in the literature that measured the effect of leaks where sealed chambers were utilised in CO_2 uptake studies (Acock & Acock, 1989). The majority of studies do not discuss this experimental aspect despite its relevance to CO_2 uptake determinations (de Morais & Costa, 2007; Ohasi *et al*. 2005; Pringault *et al*. 1996).

In this study, soil was incubated in the dark under the presence of stable isotopic $^{13}CO_2$, estimations of direct CO_2 uptake were made and gas chromatography mass spectrometry-isotope ratio mass spectrometry (GCMS-IRMS) was used to provide

evidence of the uptake of CO_2 by soil microorganisms with a further aim of developing techniques for identifying and elucidating lipid biomarkers. Herein, we demonstrate CO_2 uptake by native soil chemoautotrophic microorganisms that have been provided with a suitable chemical electron donor to observe carbon sequestration. In this environment, carbon fixation far exceeds the CO_2 produced by respiration. By utilising isotopically labelled $^{13}CO_2$ and GCMS-IRMS, the fate of this atmospheric carbon within the autotrophic system was monitored. Incubation of germinated grass under similar environmental conditions was also observed to act as a comparative control for CO_2 uptake estimations. The overall aim of the study was to prepare a working method where soil chemoautotrophy can be induced and subsequently, a single soil sample may be subjected to a suite of techniques to assist in the elucidation of soil carbon dynamics.

2.2 Materials & Methods

2.2.1 Site Details and Pre-treatment

Four soils were used but in the main outlined experiment, the soil designated as 'Abbeyside' was an Acid Brown Earths (Fay & Zhang, 2011), retrieved from an open public area adjacent to St. Augustine's Church, Abbeyside, Dungarvan, Co. Waterford, Ireland (52° 5' 17.36" N 7° 36' 38.19" W). The sampling location was on open grass covered ground within 50 metres of the Atlantic coast (Celtic Sea). All samples were collected and transferred aseptically to the laboratory and processed immediately. Roots and large debris were removed manually using aseptic technique. The soil was partially air dried and then sieved using a sterilised stainless steel mechanical sieve with a ≤ 2 mm aperture size. Sieved soil was stored in an amber jar at 4^{o}C. Accurately weighed aliquots of soil were dried at 104^{o}C for 3 days yielding an average moisture content of 22.9%. A portion of soil was fractionated according to size using a 9 piece aluminium sieve set, range 2000-25 µm (Nickel-Electro, Weston-Super-Mare, United Kingdom) and using the Gradistat soil textural calculator (Blott & Pye, 2001) the soil texture was determined to be a slightly gravelly muddy sand. Using the USDA soil pyramid it was determined that the soil was a loamy sand. A CHN combustion analyser (Exeter Analytical CE440 elemental analyser) was used to determine the soil elemental composition, 4.25% C, 0.58% H, 0.15% N. Phosphorus analysis by wet digestion (April & Kokoasse, 2009) was 0.21% P. Comparison soils were collected aseptically and treated as above. Hampstead Park soil was a Grey Brown Podzolic (Fay & Zhang, 2011), retrieved from an open public area located within Albert College Park

(Hampstead Park), Dublin, Ireland (53° 22' 54.63" N 6° 15' 43.72" W). Soil moisture was 24.5% and soil texture was determined to be slightly very fine gravelly, very coarse silty medium sand. Using the USDA soil pyramid it was determined that the soil was loamy. The CHN soil composition was, 8.62% C, 0.97% H, 0.32% N. Phosphorus analysis by wet digestion was 0.31% P. Teagasc soil was a Grey Brown Podzolic (Conry & Ryan, 1967; Fay & Zhang, 2011), retrieved from a continuous barley crop field at the Oak Park Research Centre, Carlow Town, Co. Carlow, Republic of Ireland (52^{o} 51' 47.24" N, 6^{o} 54' 11.34" W). The soil moisture was 20.2% at time of sampling and the soil texture was determined to be, a very coarse silty medium sand (muddy sand). The CHN soil composition was, 3.61% C, 0.33% H, 0.17% N. Phosphorus analysis by wet digestion was 0.56% P. Moscow soil was taken from the Botanic Gardens of Moscow State University, Moscow, Russian Federation (55^{o} 42' 37" N, 37^{o} 31' 87" E). Soil moisture was 0.45% at the time of sampling (winter sampling most likely reason for low volume of moisture due to desiccation, March 2009) and the soil texture was determined to be, a very coarse silty medium sand. The CHN soil composition was, 13.08% C, 1.53% H, 0.83% N. Phosphorus analysis by wet digestion was 0.22% P. All chemicals and solvents were purchased from Sigma Aldrich. The chemicals were of the highest purity grade available and all solvents used were of PESTANAL® quality.

2.2.2 Environmental Carbon Dioxide Incubation Chamber

The environmental carbon dioxide incubation chamber (ECIC) has the primary function of conducting temperature controlled incubations of environmental samples in the presence of variable concentrations of CO_2 (Fig. 2.0). The chamber houses a smaller inner unit (inclusive of internal equipment, the inner chamber has a 40.06 l capacity) which has been custom designed to be air tight, yet easily accessible (Fig. 2.1). The ECIC was primarily used to measure and maintain the internal atmospheric concentration of CO_2 over short to long term incubations while under constant temperature and atmospheric pressure. The inner chamber employs an infra-red CO_2 detector with a detection limit range between 0-2000 parts per million per volume (ppmv). There was no automated control of the relative humidity (% RH) within the inner chamber but this data was measurable on a real-time basis. Photosynthetic active radiation (PAR) lamps are contained within the inner chamber and lamp strength (%) may be adjusted according to requirements. Maximum lamp strength was determined using a Maya 2000 Pro spectrometer and SpectraSuite software (Ocean Optics Inc.).

Introduction of CO_2 into the inner chamber was carried out using a peristaltic pump fed from a pressurised liquid CO_2 cylinder and a pre-programmed concentration setting in ppmv CO_2. Internal measurements of the CO_2 concentration taken every 30 seconds are relayed to the onboard computer which compares the required to the actual concentration. If the concentration of CO_2 is lower than the programmed value the peristaltic pump activates in short controlled bursts (measured in seconds) until the required concentration was attained. A data logging system is in place where relevant information can be recorded at 30 second intervals and then transferred to a PC for data analysis. The information may be logged according to the operator's requirements and can be readily transferred into user friendly spreadsheets such as Microsoft© Excel. The inner chamber houses the detector used to take measurements of internal CO_2 concentrations and provide a controlled environment where CO_2 may be administered.

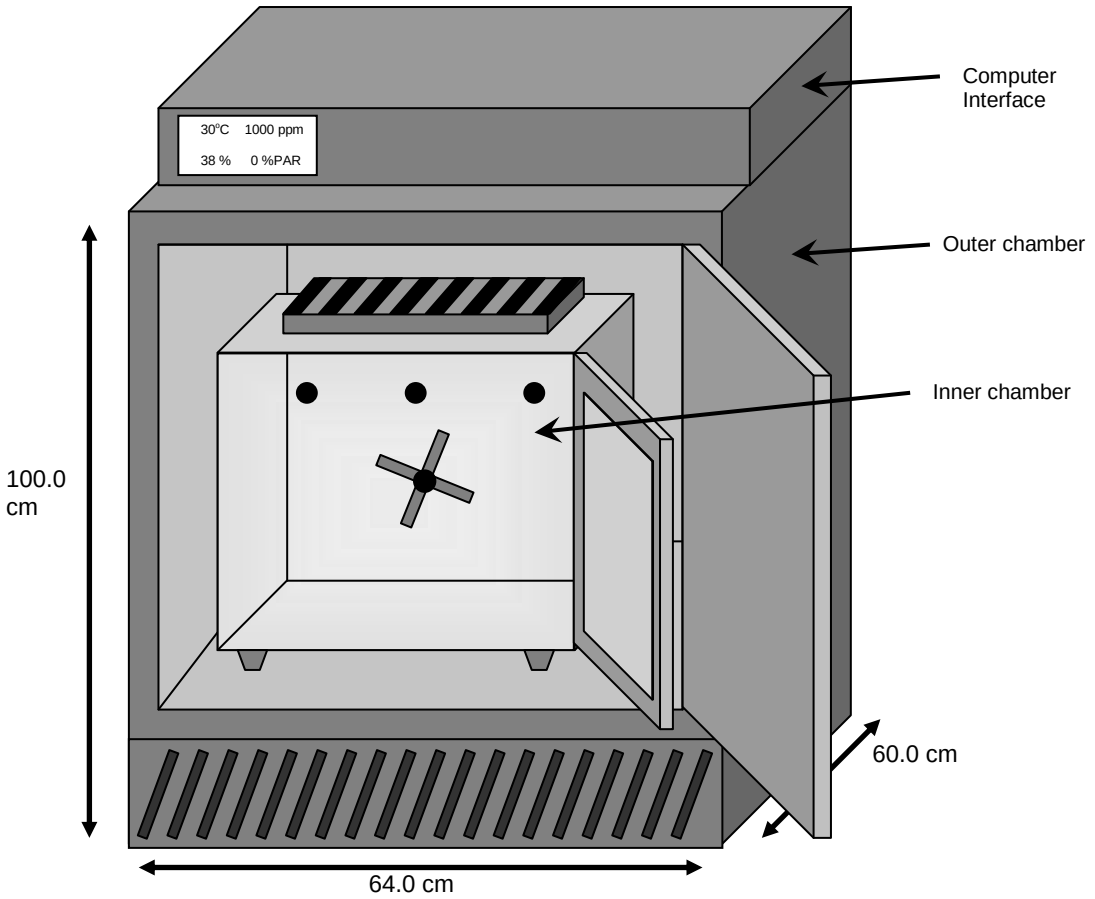

Fig. 2.0: Diagram of the outer chamber component of the ECIC. The outer unit was used to control the temperature of the inner chamber via convection of the surrounding air temperature. The outer unit was also used to store the onboard microprocessor and interface unit.

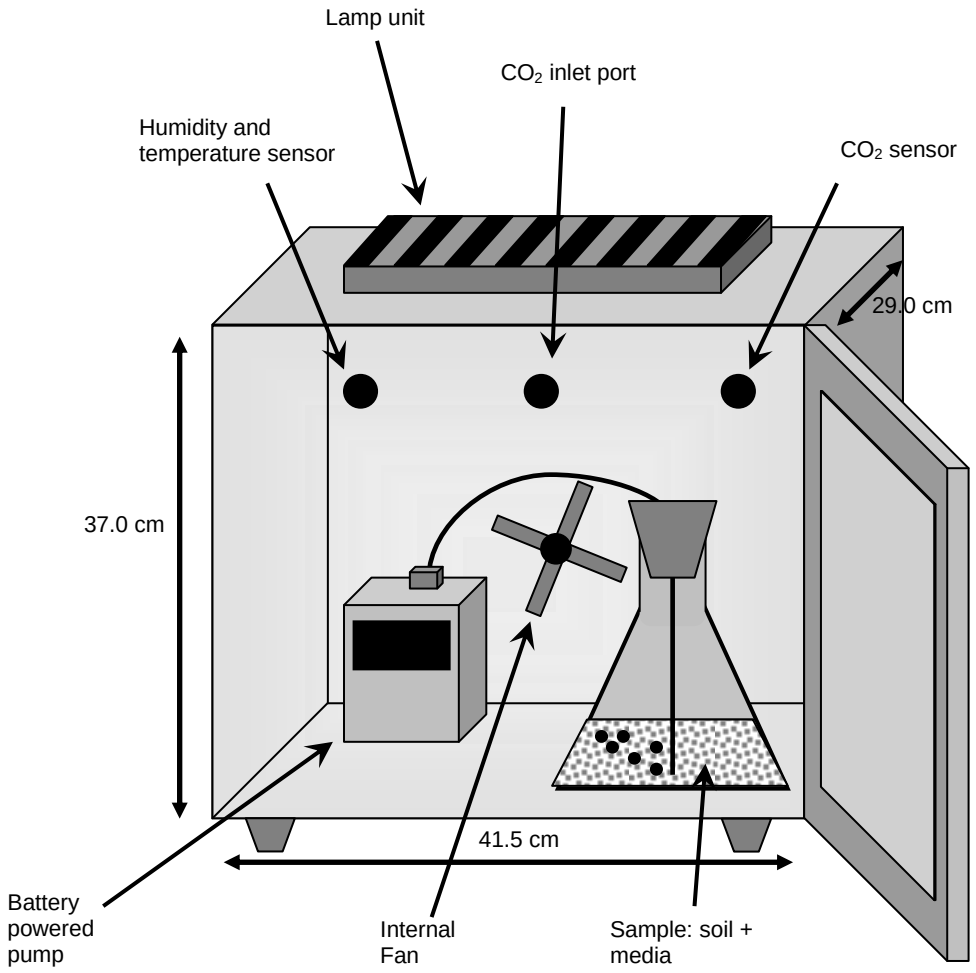

Fig. 2.1: Diagram of the inner chamber component of the ECIC and the experimental setup. The inner unit was used to control and maintain the concentrations of CO_2 (via controlled input of liquid CO_2 using a peristaltic pump) and housing the measurement probes.

2.2.3 Interpreting CO_2 Data Plots of ECIC Incubated Samples

This section is a brief introduction to aid the reader in interpreting the data plots generated by the CO_2 measurements recorded by the ECIC. This section acts as a reference point to avoid confusion when discussing the CO_2 plot data. Fig. 2.2A is an example of an incubation where the chamber door has remained sealed throughout the experiment and internal CO_2 concentrations were continuously monitored. Each data point was recorded at 30 second intervals and therefore, this is high resolution data of the internal chamber atmosphere in real-time. The y-axis displays the concentration of CO_2, measured in ppmv and the x-axis represents the time course of the plot. The identification of an event, such as a spike in concentration (indicated in Fig. 2.2A), can be easily observed, and then the relevant data from the database is extrapolated for quantitative analysis. Fig. 2.2B is an example of an incubation where a series of CO_2

pulses in close succession have been introduced at the beginning of the experiment to achieve a desired starting concentration and then CO_2 flux can be monitored in the presence of the experimental sample through to T_{end} without external influences such as CO_2 pulses or venting (e.g. opening of chamber door).

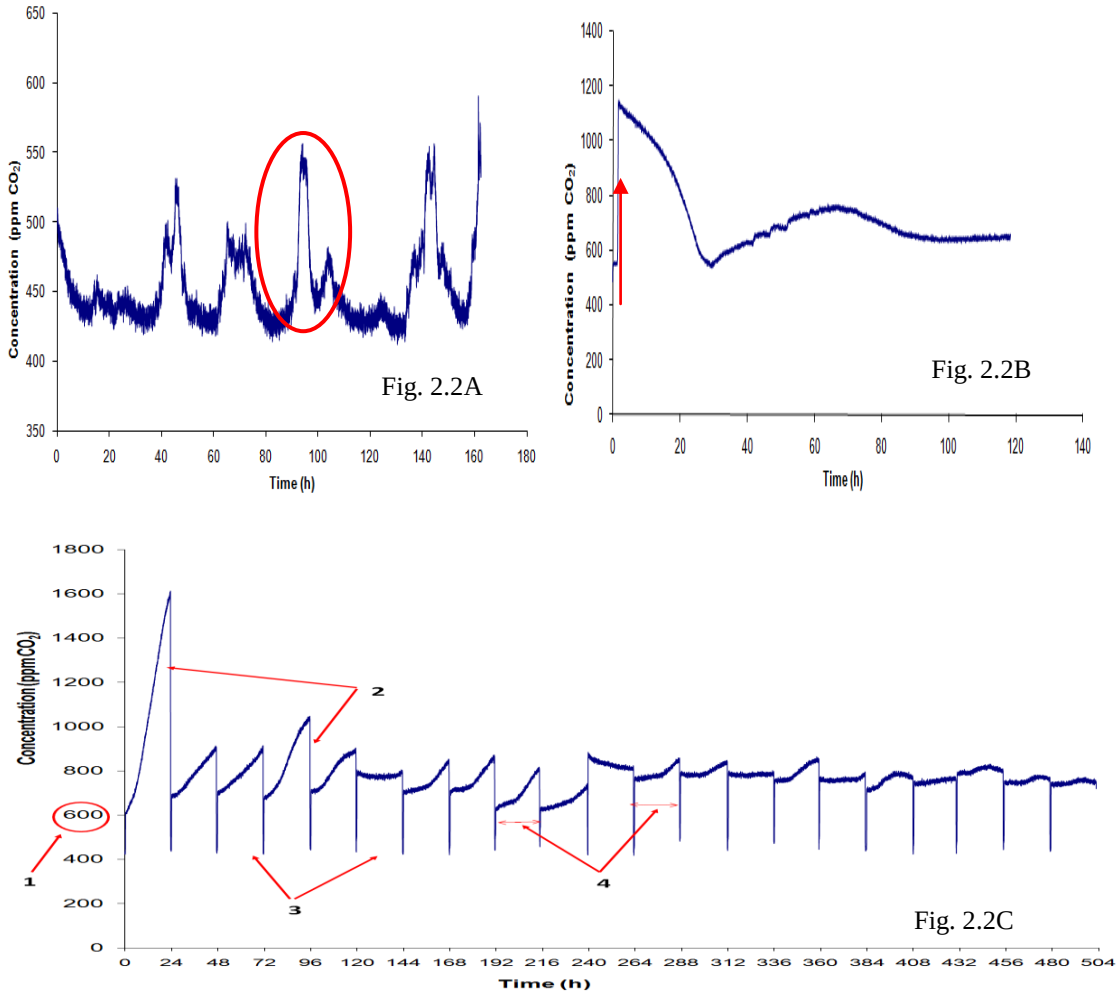

Fig 2.2: A series of example CO_2 data plots describing the typical scenarios encountered when discussing incubations performed using the ECIC and associated data generation. A) Plot showing the basal CO_2 concentration of the ECIC atmosphere in a scenario where no external source or sink of CO_2 influences the incubation. B) A single CO_2 pulse event was observed to demonstrate the rapid (vertical) increase in concentration when the peristaltic pump was activated, followed by a gradual reduction in concentration due to an internal CO_2 sink process. C) A complex plot where large variations in CO_2 are encountered. The pump was active on a daily basis raising CO_2 to a specified concentration, in this instance ≥ 600 ppmv (1), resulting in vertical spikes. Also daily interruptions to the measurements take place when the chamber was opened to the atmosphere resulting in vertical downward spikes where CO_2 equilibrates with the outer atmosphere (2). The downward spikes cease when the internal chamber was either re-sealed or the internal atmosphere reaches equilibrium with the outer atmosphere (3). The periods between the vertical changes in CO_2 concentration (indicated by left to right arrows) are the real-time plots of internal atmospheric CO_2 when the sample interaction can be monitored (4).

Fig 2.2C is a more complicated scenario and more commonly encountered in the work discussed in the project. The numbered arrows indicate significant aspects of the plot that require some explanation. The pump was activated on a daily basis raising CO_2 to a specified concentration, in this instance ≥ 600 ppmv (1), resulting in vertical spikes. Also daily interruptions to the measurements take place when the chamber was opened to the external atmosphere resulting in vertical downward spikes where CO_2 was vented from the ECIC to the outer atmosphere (2). The downward spikes cease when the internal chamber was either re-sealed or the internal atmosphere reaches equilibrium with the outer atmosphere (3). The periods between the vertical changes in CO_2 concentration (indicated by the left to right arrows) are the real-time plots of internal atmospheric CO_2 where the sample interaction can be monitored (4).

2.2.4 Minimal Salts Medium (MSM) Preparation

A modified M9 MSM (0.5 g l^{-1} K_2HPO_4, 0.5 g l^{-1} KH_2PO_4, 0.5 g l^{-1} NH_4Cl, 0.5 g l^{-1} KCl, 0.10 g l^{-1} $MgSO_4.7H_2O$, 0.12 g l^{-1} NaCl, 0.05 g l^{-1} $CaCl.2H_2O$; Madigan *et al*. 2009; Shiers *et al*. 2005) was prepared and 1 ml of a trace metal solution (0.1 g l^{-1} $ZnSO_4.7H_2O$, 0.3 g l^{-1} $MnCl_2.4H_2O$, 0.3 g l^{-1} H_3BO_3, 0.1 g l^{-1} $CuCl_2.2H_2O$, 0.2 g l^{-1} $NiCl_2.6H_2O$, 0.3 g l^{-1} $NaMoO_4.2H_2O$, 1.0 g l^{-1} $FeSO_4.7H_2O$) was added. The MSM was made up to 1000 ml and autoclaved at 121^{o}C for 15 minutes.

2.2.5 $^{12}CO_2$ Profile Soil Incubations

A central hole was inserted into the lid of a 900 ml amber jar, followed by 4 surrounding holes to act as exit vents. The amber jar was then autoclaved at 121^{o}C for 10 minutes. A known weight of soil (32.06 g) was placed into the sterile amber jar and 300 ml of MSM was added aseptically. Dried silica gel (190 g) was placed onto the bottom shelf of the ECIC to reduce excessive humidity. The sample was placed into the ECIC and the temperature set to 30 ± 0.2^{o}C. An autoclaved glass pipette was inserted into the central hole in the jar lid. A clean UV sterilised tube with an autoclaved 0.2 μm gas filter was fitted to the glass pipette and to the outlet port of a battery powered air pump (Agile p/n A790). The battery air pump was activated and the chamber doors were sealed. No additional CO_2 was added to the chamber so that CO_2 flux could be monitored via soil respiration and uptake processes (starting atmospheric concentrations were that of the ambient external environment prior to commencement of incubation). Automated data recording took place every 30 seconds to measure internal environmental conditions (% RH, temp., ppmv CO_2) and the incubation period

remained uninterrupted for 8 days. After the 8 day incubation the sample was removed from the ECIC and the silica gel desiccant replaced. The supernatant was removed and replaced with autoclaved MSM. The 300 ml soil slurry was amended with 6.0 ml (0.2 μm sterile filtered) 1000 mM $Na_2S_2O_3$ stock solution to provide a 20 mM $Na_2S_2O_3$ electron donor source for soil chemoautotrophic species. The soil was returned to the ECIC and the previous experimental conditions were repeated to observe the manifestation of chemoautotrophic conditions and hence CO_2 uptake.

2.2.5 Grass Seedling Incubation

A basal soil incubation was performed to determine the background respiration rates in order to conduct accurate determinations of CO_2 uptake by germinated grass. A 553 g lot of ≤ 2 mm (manually sieved) Abbeyside soil (air dried) was wetted with collected rain water (filter sterilised) and placed into the ECIC for 8 days under 0% PAR, diurnal temperature regime to determine the dark basal respiration. The incubation was repeated with the exception that PAR levels were alternated to resemble diurnal conditions to encourage soil surface algal growth (Table 2.0). Blue Diamond Lea grass seed mixture (29.8% Cornwall, 12.8% Soriento, 13.6% Gilford, 27.7% Montova, 10.6% Navan, 5.5% Huia clover - varieties of *Lolium perenne*, a perennial ryegrass) were conditioned for 4 days at 20^{o}C in 3 ml tap water before planting into the soil sample. The soil mass was placed into a transparent acrylic container (22.1 x 11.5 x 7.3 cm) and evenly spread. The soil was saturated with a 200 ml NPK solution (0.02 M KNO_3, 0.02 M K_2PO_4, 0.05 M NH_3NO_3) and incubated for 3 days in direct sunlight while covered with a clear perforated acrylic lid. Germinated grass was placed into the ECIC for 8 days under a 24 hour diurnal regime under an atmosphere of 800 ppmv CO_2 (Table 2.0) with no additional moisture required. Silica gel (190 g) desiccant was placed into the sealed chamber to absorb excess humidity. After the incubation period all roots, stems and leaves were carefully removed and incinerated (440^{o}C) overnight using a Carbolite muffler furnace to determine residue on ignition (ROI).

Time (min)	Temperature (^{o}C)	PAR (%)[a]
0	20	100
360	18	50
180	12	25
180	10	0
240	10	0
120	12	25
120	16	50
240	20	100

Table 2.0: Automated daily ECIC programme cycle for 7 day diurnal regime. [a]PAR (%): 100% = Maximum Photosynthetic Photon Flux Density (PPFD) 90 µmol m^{-2} s^{-1}

2.2.6 Calculations

High resolution [CO_2] data consisted of measurements taken every 30 seconds. Average CO_2 decay rate values during the experimental events were derived by selecting the CO_2 data points and subtracting the final from the initial recorded value and then dividing by the total time of the CO_2 uptake event (hour). These values were corrected against the pre-determined leak rate of the chamber at that particular pCO_2 (ppmv h^{-1}) depending upon the appropriate sub-period. The resulting value (positive values indicate decreased CO_2 and negative values indicate production of CO_2) could then be converted to mass by multiplication with the total time of the event, the chamber volume (0.041 m^3), and the density of CO_2 (1.977 g l^{-1}). Standard error of ± 14 ppmv CO_2 were calculated by measuring the laboratory atmosphere over 110 hours to determine the stability of the infra-red detector (these measurements were taken over the weekend to avoid rapid changes in CO_2 concentration due to human activity).

2.2.7 Isotopic Labelling Incubation Conditions

Pre-incubated soil (30.05 g) was placed into an autoclaved 900 ml amber jar (prepared as above) and 300 ml MSM was added. The ECIC was programmed to maintain temperature at 30 ± 0.2^{o}C and 190 g of dried silica gel was placed onto the bottom shelf to reduce excessive humidity. The ECIC was calibrated using $^{12}CO_2$ (AirProducts, Industrial grade) and $^{13}CO_2$ (Sigma Aldrich 99% atom ^{13}C). For these calibrations CO_2 was pumped into a filled 100 ml upturned graduated cylinder to measure the volume of water displaced per second of pumping time. The volume (cm^3) of water displaced was divided by the pump running time (seconds) to determine the cm^3 s^{-1} CO_2. The generated figure of 5.166 cm^3 s^{-1} CO_2 was used to determine the volume of CO_2 pumped

into the chamber during incubation. Twelve seconds pumping time was required to reach 1000 ppmv CO_2 from T_0. The 900 ml amber jar containing the soil sample was placed into the ECIC and an autoclaved glass pipette was inserted into the central hole in the jar lid. A clean UV sterilised 30 cm tube with an autoclaved 0.2 μm gas filter was fitted to the glass pipette and to the outlet port of a battery powered air pump. The 300 ml soil slurry was inoculated with 6.0 ml (0.2 μm sterile filtered) 1000 mM $Na_2S_2O_3$ stock solution. The battery air pump was activated and the chamber doors were sealed. The chamber was programmed to achieve a CO_2 concentration of 1000 ± 50 ppmv. All incubations took place in the dark and internal lights were deactivated. For $^{12}CO_2$ and $^{13}CO_2$ experiments, incubations took place over 40 hours. Sub-samples of homogenised soil slurry (40.0 ml) were taken at the start (T_0) and the end of both incubations (T_{40}) for fatty acid analysis using GCMS-IRMS.

2.2.8 Extraction and Analysis of Soil Organic Matter

The SOM was extracted using a modified version of the Bligh & Dyer method (Bligh & Dyer, 1959; Otto & Simpson, 2007) and was carried out in prewashed 40 ml Teflon tubes (Nalgene). After CO_2 incubation in the chamber, a sample of the soil slurry was centrifuged at 6000 rpm (20 minutes). The supernatant (medium) was decanted from the soil and biomass and this remaining solid residue was washed twice with a potassium hydrogen phosphate buffer solution. The soil precipitate was freeze dried (0.90 g $^{12}CO_2$ exp, 1.26 g $^{13}CO_2$ exp, dry weight) before extraction with methanol:dichloromethane (ratio 1:0, 1:1 and 0:1) was performed. The total extracts were filtered, concentrated and reconstituted in 1 ml of MeOH:DCM [50:50] for derivitisation and analysis.

The extracts were analysed by gas chromatography coupled to a quadruple Electron Impact Mass Spectrometer and Isotope Ratio Mass Spectrometry (GCMS-IRMS). The GC column effluent was subsequently split equally between the two detectors. A transmethylation derivitisation was performed to volatise and improve thermal stability of free fatty acids, glycerides and phospholipids. Phospholipids (PLFAs) are important as they are the main component of microbial cell membranes and can be a vital source of lipid biomarkers. In the case of the PLFAs, this procedure cleaves the fatty acid side chain from the glycerol backbone/polar phosphorus head group and methylates to form volatile fatty acid methyl esters (FAMEs). The application of transesterification agents such as sodium methoxide alone is of great value when analysing polyunsaturated fatty acids and those with functional groups other

than double bonds. The method is of little use when looking at mono- or dienoic fatty acids (Christie, 1993). The analysis of derivitised PLFA products provides the information that a compound contains a double bond within its structure but does not indicate as to its location. This was because, during the derivitisation procedure, rearrangement ions move the double bond along the molecule resulting in an average spectrum being displayed to the user upon GCMS analysis. A derivitisation agent that 'locks' the double bond(s) in place, such as dimethyl disulphide (DMDS; Dunkelblum *et al*. 1985) should also be used to complement the analysis (4,4-dimethyloxazoline (DMOX) [Christie, 1993] may also be used as an alternative derivitisation agent). An aliquot of the total extract (200 µl) was evaporated to dryness before derivitisation. The method employed was a transesterification reaction involving sodium methoxide (50 µl; Christie, 1982; Hughes *et al*. 1986). The solution was vortexed and heated for 10 minutes at 50^{o}C. Excess sodium methoxide was quenched with 450 µl deionised water and the resulting NaOH neutralised with 50 µl HCl (0.5 M). The newly formed methyl esters were extracted twice from the aqueous solution with 1 ml of hexane:chloroform [9:1]. The combined extracts were dried over sodium sulphate and evaporated to dryness before reconstitution in 100 µl of a 100 ppm hexane solution of cholestane (internal standard) for GC injection. Determination of monounsaturated fatty acid double-bond position was performed by GCMS analysis of their dimethyl disulphide adducts (Nichols *et al*. 1986).

2.2.9 Analysis by GCMS-IRMS

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), with a split ratio of approximately 50/50. 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 (Air Products, BIP-X47S grade) was used as the carrier gas. The injection port and the GC/MS interface were kept at 250 and 280°C, respectively. The ion source temperature was 280°C. GC oven temperature was 100 to 300°C at a rate of 6°C/minute after 1.5 minute at 100°C. The column head pressure was 69.4 kPa. An aliquot of each sample (1 µl) was loaded into the injection port of the GC using the splitless mode of injection, followed by an elution split after column to both mass spectrometry detectors. The GC effluent was diverted via a heart split valve to a ceramic combustion furnace (GC5, 650

mm X 0.3 mm i.d.) packed with a copper oxide/platinum catalyst heated to 850^{o}C. Water was removed from the combustion products by passing the effluent through a nafion membrane prior to the CO_2 entering the IRMS (Isoprime Ltd, UK). 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). A standard deviation for the instrument was calculated to be ± δ 1.04 over a 10 run sequence of the 15 alkane mixture.

2.2.10 PLFA Nomenclature

Fatty acids are designated by the number of carbon atoms, followed by a colon and the number of double bonds. The positions of double bonds are indicated by the suffix 'ω' and the number of the first double bonded carbon atom from the methyl, or ω, end of the molecule. With branched fatty acids (br), the number before the colon indicates the total number of carbon atoms (not the number in the main chain). A prefix is used to indicate the position of the branch. Iso-branching, indicated by the prefix '*i*', is where the methyl group is at the penultimate carbon atom from the carboxyl group. Anteiso-branched fatty acids, with the prefix '*a*', have the methyl group at the third carbon atom from the carboxyl end. 10ME indicates a methyl group on the tenth carbon atom from the carboxyl end of the molecule. Cyclopropane fatty acids have the prefix '*cy*' (Steer & Harris, 2000). Table 2.0 provides the systematic names and the shorthand designation for a range of saturated fatty acids as a basis for understanding the vast array of fatty acid compounds. This is required as most systematic names despite appendages, are named in relation to the carbon chain backbone.

Systematic Name	Shorthand Designation	Systematic Name	Shorthand Designation
ethanoic	2:0	octadecanoic	18:0
propanoic	3:0	nonadecanoic	19:0
butanoic	4:0	eicosanoic	20:0
pentanoic	5:0	heneicosanoic	21:0
hexanoic	6:0	docosanoic	22:0
heptanoic	7:0	tricosanoic	23:0
octanoic	8:0	tetracosanoic	24:0
nonanoic	9:0	pentacosanoic	25:0
decanoic	10:0.	hexacosanoic	26:0
undecanoic	11:0	heptacosanoic	27:0
dodecanoic	12:0	octacosanoic	28:0
tridecanoic	13:0	nonacosanoic	29:0
tetradecanoic	14:0	triacontanoic	30:0
pentadecanoic	15:0	hentriacontanoic	31:0
hexadecanoic	16:0	dotriacontanoic	32:0
heptadecanoic	17:0		

Table 2.1: List of some common saturated fatty acids and their shorthand designations.

2.3 Results

2.3.1 Chamber Characterisation – Leak Detection

Analysis of the chamber performance under blank conditions (i.e. empty and sealed) determined that a slight leak was present after a series of six replicate incubations were performed. The leak rate was proportional to the CO_2 concentration in the chamber, on which basis, partial pressure correction rates were determined. These were determined in 100 ppmv fractions between 500-1200 ppmv CO_2 (Table 2.2). These correction factors have been applied when determining the uptake volume of CO_2 during perceived chemoautotrophic events. Attempts at finding the site of the leakage was unsuccessful using coloured gases and was most likely attributable to diffusion through the door seal.

Decay Period (ppmv CO_2)	Decay Rate (ppmv hr^{-1} CO_2)	Standard Error (ppmv hr^{-1} CO_2)
1200-1100	16.529	4.361
1100-1000	15.009	4.86
1000-900	13.054	0.764
900-800	9.703	1.625
800-700	6.804	1.423
700-600	5.354	1.055
600-500	2.651	0.493

Table 2.2: Average decay rates extrapolated from CO_2 decay incubations under blank conditions.

2.3.2 Carbon Dioxide Profiles of Soil Before and After Electron Donor Addition

Incubations of soil were performed on pre-incubated samples (exposed to single inoculation of 20 mM $Na_2S_2O_3$ for 8 days) to observe the uninterrupted CO_2 flux within the sealed chamber. This was done to provide a visual plot of data without interruption of the automated pump or operator interference (e.g. opening of doors) as would be observed under the $^{13}CO_2$ labelling experiments. The incubation of fresh soil at 30^{o}C in the MSM under atmospheric concentrations of CO_2 (average indoor atmospheric concentration was determined to be ~450 ppmv CO_2) was performed to map the dynamic flux of CO_2, both under basal (normal background CO_2 flux) and autotrophic induced conditions. These experiments were performed to demonstrate the manifestation of autotrophic conditions in soils. They were not replicated in the ^{13}C-labelling experiment and therefore the $^{12}CO_2$ uptake measurements are also demonstrative to assess the applicability of the technique. The ECIC took regular measurements of certain environmental conditions within the inner unit demonstrating that under favourable conditions (excess nutrients, moisture and constant temperature) the CO_2 concentration was, after an initial increasing phase, static against the perceived background leak average of 7.2 ± 1.1 ppmv h^{-1} CO_2 (Fig. 2.3).

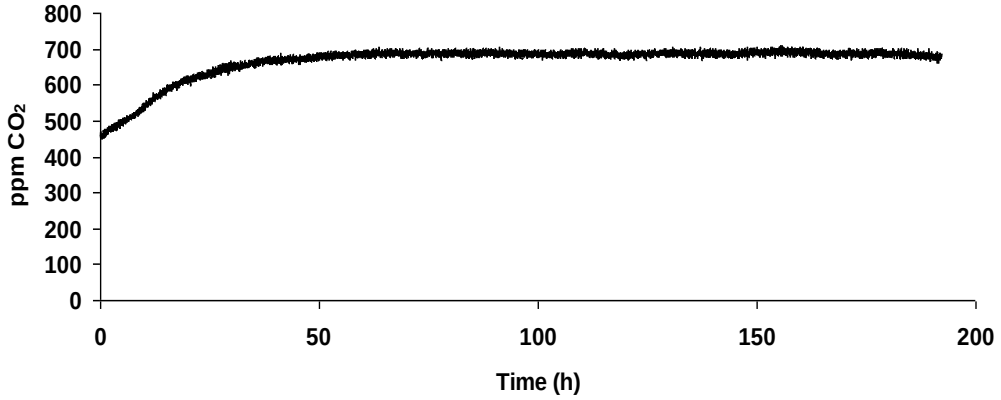

Fig. 2.3: Basal incubation taking place under $^{12}CO_2$ atmosphere over a 192 hour period at 30^{o}C

From the raw data it was extrapolated (after leak correction) that the Abbeyside soil had a negative decay rate of -6.4 ppmv h^{-1} CO_2, indicating that CO_2 production out-competed leaked/sequestered CO_2 (due to the governing effects of partial pressure) over the course of 192 hour (8 day) incubation. Thus, under the conditions provided in the experiment, the metabolic activity of the resident soil microbiota led to net mineralisation of carbonaceous material naturally abundant in the soil matrix (Elberling & Brandt, 2003; Dilustro *et al*. 2005; Jassal *et al*. 2005). This was observable in Fig. 2.3 where the CO_2 gradually increases from 450 ppmv to a maximum concentration of 710 ppmv CO_2, an increase of 260 ppmv over 8 days.

A chemical electron donor was then added to the slurry in an attempt to stimulate the growth and reproduction of chemoautotrophic microorganisms. The electron donor acts as the sole energy source for the abundantly low chemoautotrophs naturally occurring in the soil profile (Smith & Strohl, 1991). The soil incubation employing the chemical electron donor displayed a different pattern of CO_2 fluctuation over a similar experimental period (see Fig. 2.4).

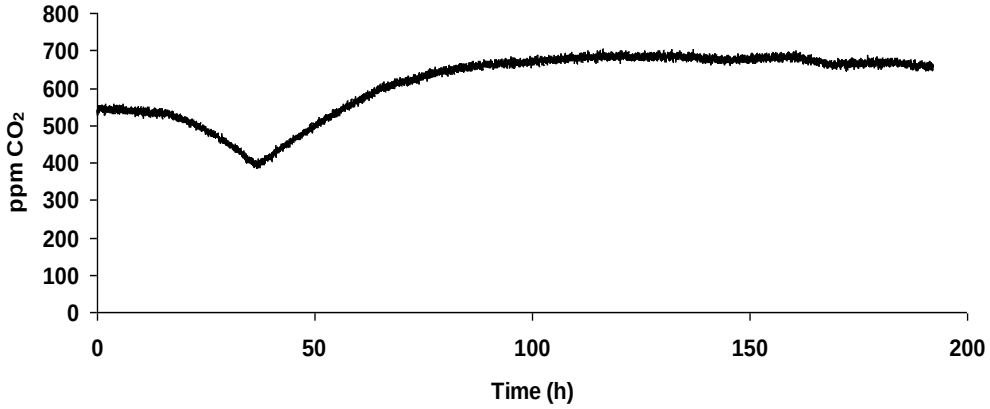

Fig. 2.4: Real-time data plot of a 192 hour soil and electron donor incubation $^{12}CO_2$ profile at 30°C

The $^{12}CO_2$ data plot shows a short lag phase of approximately 16 hours where CO_2 levels remain relatively static. After this initial lag phase, a sharp decline in atmospheric CO_2 for a period of 20 hours was observed, eventually reaching a minimum value of 385 ± 13 ppmv. After the minimum value was observed, the atmospheric concentration of CO_2 rapidly increased to levels resembling that of the basal incubation. Extrapolation (taking into account the CO_2 leaked to the laboratory atmosphere due to partial pressure) of the single decay event showed that CO_2 was sequestered to the soil during this period with an average rate of + 3.6 ppmv h^{-1}, equivalent to 256.4 µg CO_2 kg^{-1} dry soil (1009.3 µg CO_2 m^{-2}). The decay event can be associated only with the new variable entered into the incubation, the chemical electron donor ($S_2O_3^{2-}$), the presence of which provided an environment conducive for bacterial autotrophy to take place. Interpretation of the CO_2 profile observed in (Fig. 2.4) must take into account the effects of both partial pressure dependent leak and the production of mineralised CO_2 from the soil matrix. After about 40 hours of incubation, the efflux of CO_2 from the soil seems to occur at rates exceeding the sum of CO_2 sequestration and the leak rate, until a steady state, where production and losses of CO_2 are equalised. The rapid change in the course of CO_2 after 40 hours of incubation could rather be attributed

either to exhaustion (complete oxidation) of the provided electron donor or a change in phyiso/chemical conditions affecting the active chemoautotrophic microbiota (Fig. 2.4). It must be stressed that the observed uptake of CO_2 into the soil matrix was not intended to demonstrate permanent sequestration of carbon, but the pattern of CO_2 flow through a complex biological matrix where sufficient environmental conditions were temporarily supplied.

Isotopic labelling incubations for the Abbeyside soil were carried out in triplicate (^{12}C and $^{13}CO_2$ respectively) with the $^{12}CO_2$ incubations observed in Fig. 2.5 showing the CO_2 plot maintained at $\geq$ 1000 ppmv. The lack of $^{13}CO_2$ data plots was due to the employment of an IR detector calibrated to detect atmospheric CO_2 and employs a wavenumber (cm^{-1}) detection range between 2270-2390 cm^{-1}. The absorbance of $^{13}CO_2$ in the IR spectrum lies between 2250-2290 cm^{-1} (Gosz *et al*. 1988) and hence the ECIC only reports a small percentage of the true concentration (~20%) and considered to be unreliable. The $^{12}CO_2$ plots are performed prior to the labelling experiments to act as a control for both CO_2 uptake estimations and GCMS-IRMS investigations. To ensure the consistency of the environments to which the microbiota was exposed, the CO_2 atmosphere was kept at approximately 1000 ppmv (0.1% v/v). This allowed for the eventuality that when concentrations dipped below a control threshold of 950 ppmv CO_2, an automated burst of 99% CO_2 was introduced into the chamber via a peristaltic pump (hence the zig zag shape of the CO_2 plots). Extrapolation of the CO_2 plot data for each of the decay periods of the individual incubations was estimated to be 149.7, 4359.0 and 8346.7 µg CO_2 kg^{-1} dry soil (589.2, 17161.6 and 32861.1 µg CO_2 m^{-2}) uptake for incubations A, B and C respectively. The Abbeyside soil was also exposed to an atmosphere of 1000 ppmv $^{13}CO_2$ to act as complimentary evidence of CO_2 sequestration via isotopic enrichment.

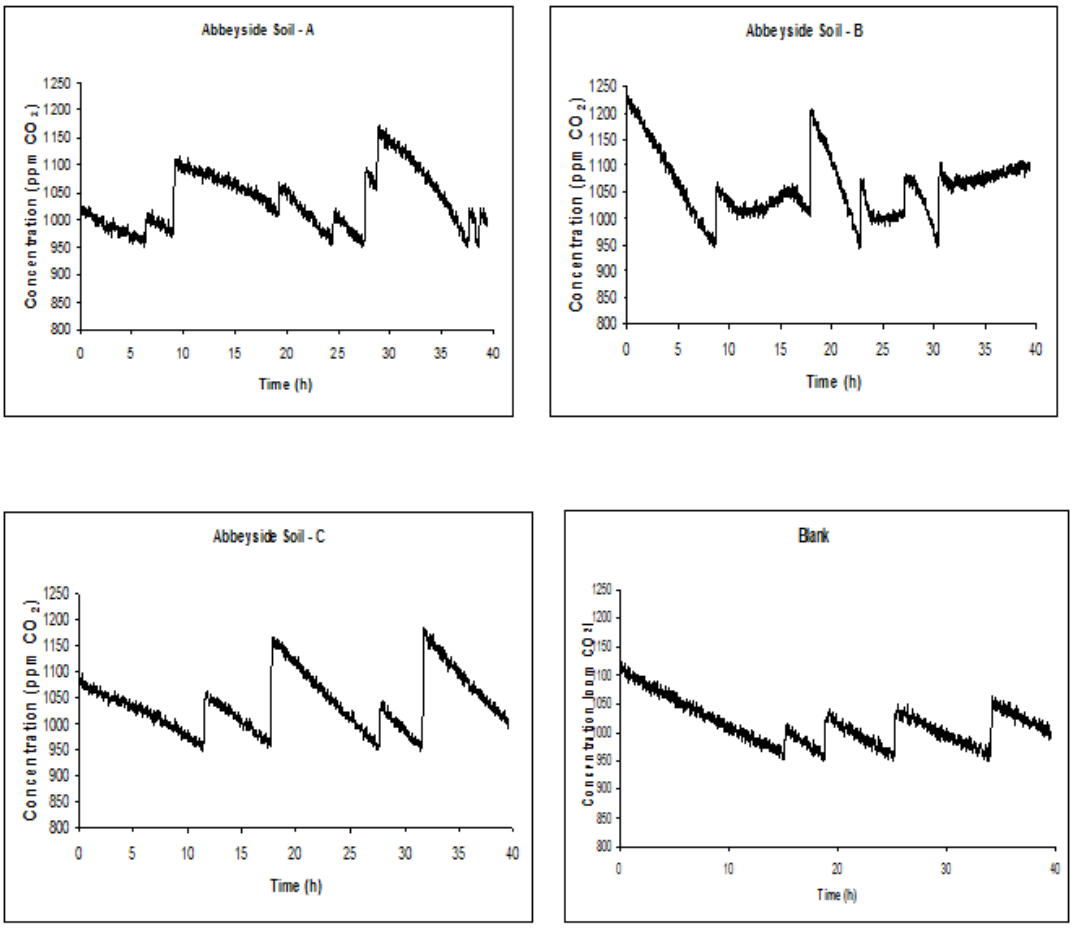

Fig. 2.5: CO_2 plots of Abbeyside soil incubations performed in triplicate prior to isotopic labelling with 99% $^{13}CO_2$

The sequestration of atmospheric CO_2 was further tested on three other soils to ensure the phenomenon of soil chemoautotrophy was not isolated to the Abbeyside site (Fig. 2.6). The blank soil incubation clearly shows the background leak of CO_2 from the chamber according to the partial pressure and the rates of CO_2 decay are measurable. The decay patterns observed for the Hampstead Park and Moscow soils show much more rapid decay plots between CO_2 injection events and are a good visual indicator that CO_2 sequestration was taking place during incubation. Using the partial pressure correction values, the approximate mass of CO_2 sequestered between injection events can be easily deduced. It was possible then to calculate the total fixation over 40 hours, with Hampstead Park and Moscow soils taking up 1261.7 and 684.8 µg CO_2 kg^{-1} dry soil, (4967.2 and 2695.9 µg CO_2 m^2) respectively. The Teagasc soil showed no significant levels of sequestration. This was an interesting observation requiring further investigation as this soil came from a continuous barley crop field and the sample appears to be a net source of CO_2 and devoid of sulphur based chemoautotrophy under the provided conditions. The site was located at the Teagasc agricultural research

facility in County Carlow, Ireland and has undergone continuous barley harvest for approximately 30 years and therefore represents a possible site of low biological diversity due to a lack of crop rotation. This was an interesting negative result as large tracts of land across the world are subject to monocropping, resulting in low species diversity (Nsabimana *et al*. 2004; Dirk van Elsas *et al*. 2002). The data sets in Figs. 2.5 and 2.6 shows that the experiment was repeatable on the Abbeyside soil and on another two unrelated sites using the same methodologies and hence, uptake was not site specific.

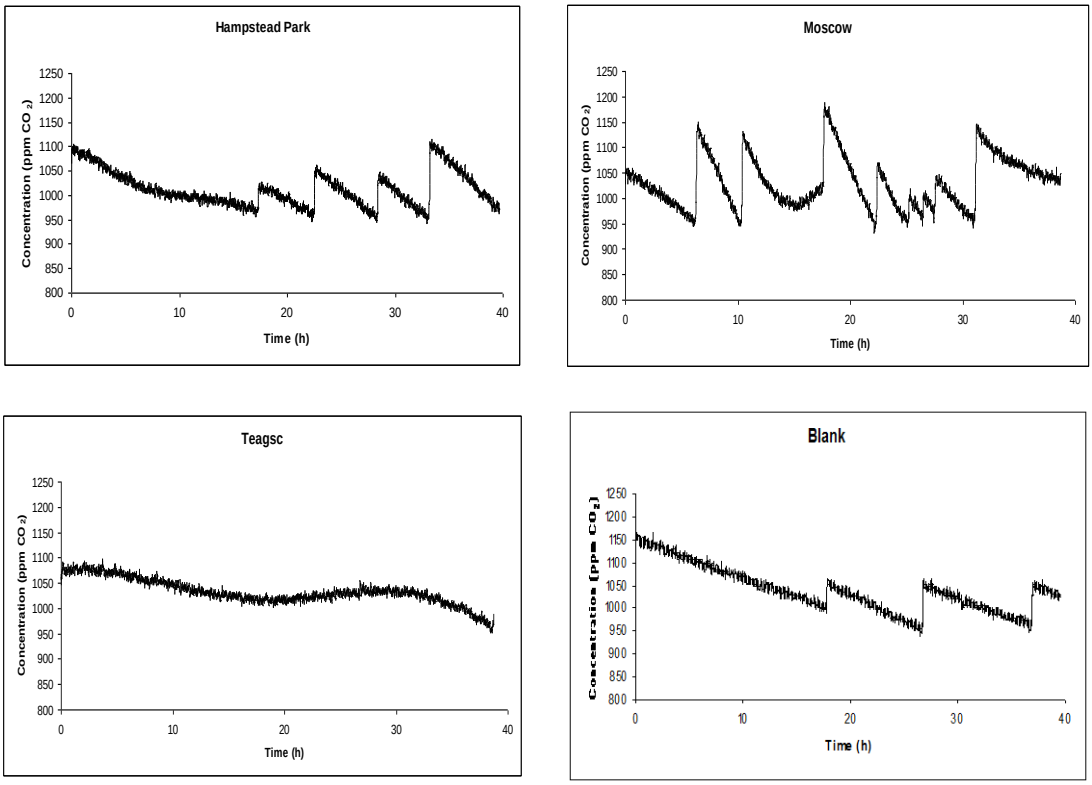

Fig. 2.6: CO_2 data plots for incubated soil slurries exposed to the 20 mM $Na_2S_2O_3$ from three sample sites and an unamended soil (Blank).

2.3.3 Grass Seed Incubation

As a comparison, an agricultural grass (Diamond Lea mixture) was incubated within the ECIC to determine the CO_2 uptake potential. This experiment was carried out as it was important to determine an experimental control under similar conditions prior to forming conclusions as to the CO_2 uptake capacity of the mixed microbial soil cultures. The grass seed incubation study was performed to compare the capacity of the soil's chemoautotrophic microbiota and that of a typical grass growing on the same soil in the presence of excess energy sources (in this case, photons) allowing for optimum uptake

activity. An agricultural grass mixture was used as this is one of the most widespread vegetation types in temperate regions such as Britain and Ireland (Jeffery *et al*. 1995). Little data exists on the contribution of algae on the soil surface to CO_2 sequestration rates (Betting, 1981). When preparing soils it was previously noticed that in the presence of PAR, algal growth over the soil surface was significant. It was estimated that the algal population tends to colonise approximately 5% of the available surface area for our grass seed incubation containers (depending on light exclusion from grass blades). Prior to grass seed incubation, algae were grown across the total soil surface (Fig. 2.7) so that the CO_2 sequestration of soil algae may be estimated and hence, a corrective value may be determined when making assumptions of grass CO_2 uptake (based upon the 5% coverage of algal biomass under normal grass seed incubation conditions). This corrective measure yielded interesting data of its own in regards to rates of CO_2 sequestration by a comparatively smaller biomass community (comprising of a large number of individual algal units). It was observed, that after 8 days incubation under diurnal conditions, the total algal biomass, which colonised approximately 91% of the total soil surface, sequestered 4705.5 µg CO_2 kg^{-1} dry (18525.4 µg CO_2 m^2) soil. It was determined that the 5% contribution to CO_2 uptake for the total surface area colonised by algae during the subsequent grass incubation was 235.3 µg CO_2 kg^{-1} dry soil (926.3 µg CO_2 m^2), which in itself was a significant contributor to CO_2 fixation, but beyond the scope of this particular study for more in-depth analysis.

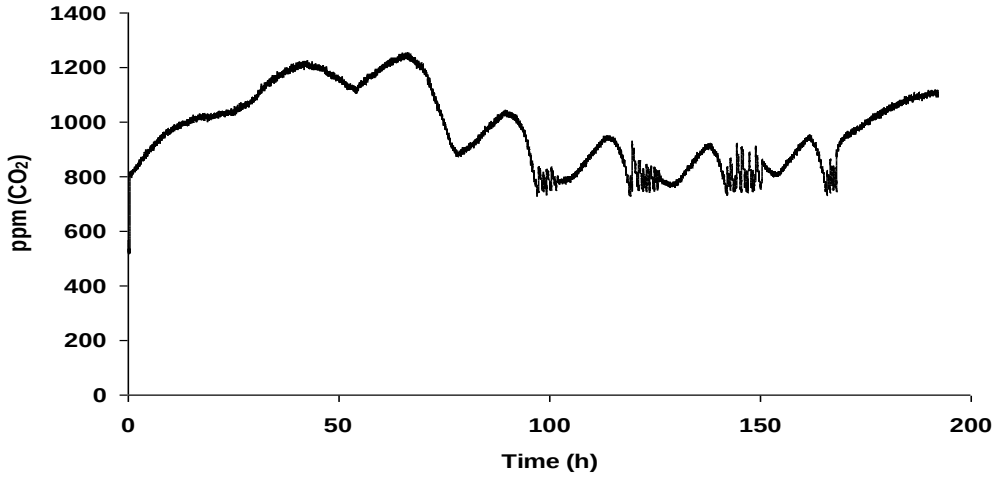

Fig. 2.7: CO_2 (real-time) plot for soil algae under a diurnal regime for 8 days. Please note that the extreme fluctuations at the peak troughs between 95-175 hours are caused by the automated re-introduction of substrate CO_2 to maintain ~800 ppmv CO_2.

The CO_2 flux data for the grass seed incubation (Fig. 2.8) clearly represents the diurnal regime of photosynthesis (25-100% PAR) and night-time respiration (0% PAR) over the course of the study. From the intake and output plots it was possible to determine the overall fixation of atmospheric carbon to organic matter. After 8 days it was determined that 9801.2 µg CO_2 kg^{-1} dry soil (38587.6 µg CO_2 m^2) was sequestered into plant material once algal photosynthetic uptake, nocturnal respiration and soil CO_2 respiration (constant) were taken into account. Based upon the 5% soil coverage of photosynthesising algae during the grass seed experiment, an approximate corrective value of 235.3 µg CO_2 kg^{-1} (926.3 µg CO_2 m^2) has been attributed to these microorganisms and was incorporated into the grass CO_2 uptake calculation. The ROI test revealed that 60.08% of the dry grass solids contained volatile organic compounds.

The grass control was performed to act as a comparative measurement to refer to when discussing microbial uptake of inorganic carbon within soil matrices. This was necessary, as research into the available literature failed to provide adequate data. Our data suggests that provided with adequate conditions, several forms of CO_2 sequestration may be taking place on the terrestrial surface especially in areas heavily fertilised with inorganic fertilisers containing electron donor sources.

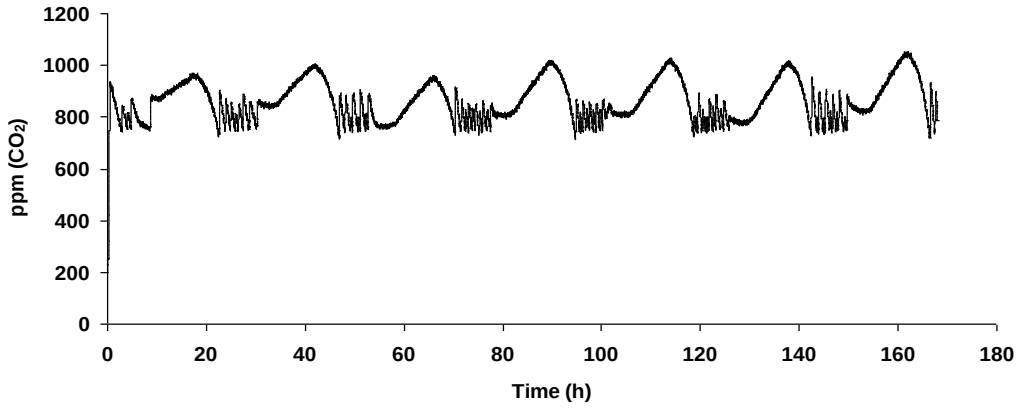

Fig. 2.8: CO_2 (real-time) plot for grass incubated at 800 ppmv CO_2, for 8 days under a diurnal regime.

2.3.4 Isotopic Labelling Incubations

The incubations described so far show the net result of CO_2 uptake and efflux mechanisms. It has previously been demonstrated that CO_2 fixation and subsequent production of organic matter occurs within soil matrices with or without the addition of carbonaceous growth substrates (Miltner *et al*. 2004; Miltner *et al*. 2005; Santruckova *et al*. 2005) and that heterotrophic CO_2 sequestration was possibly a significant factor in these incubation experiments. Also, the presence of organisms such as *Ralstonia*

eutropha which exist as facultative heterotrophs (Pohlmann *et al*. 2007) must be considered as potential sources of carbon fixation through means other than $S_2O_3^{2-}$ oxidation e.g. H_2 chemoautotrophy. In the studies carried out by Miltner *et al*. (2004; 2005) and Santruckova *et al*. (2005), considerable time was required for isotopic incorporation and before quantifiable results could be reported. To investigate the occurrence and effects of heterotrophic CO_2 sequestration during the basal incubation described above, an experiment designed to measure CO_2 sequestration without the presence of an electron donor was performed with 99% isotopically labelled $^{13}CO_2$. The soil was subjected to the same extraction and derivitisation procedure (*vide supra*) as employed for the GCMS-IRMS analysis. Fig. 2.9 shows the ratio spectrum $^{13}CO_2/^{12}CO_2$ (i.e. isotopic ratio) of the NaOMe derivitised extract. The lack of enrichment in the IRMS spectrum indicates that $^{13}CO_2$ was not significantly sequestered in the absence of an electron donor over the 8 day incubation.

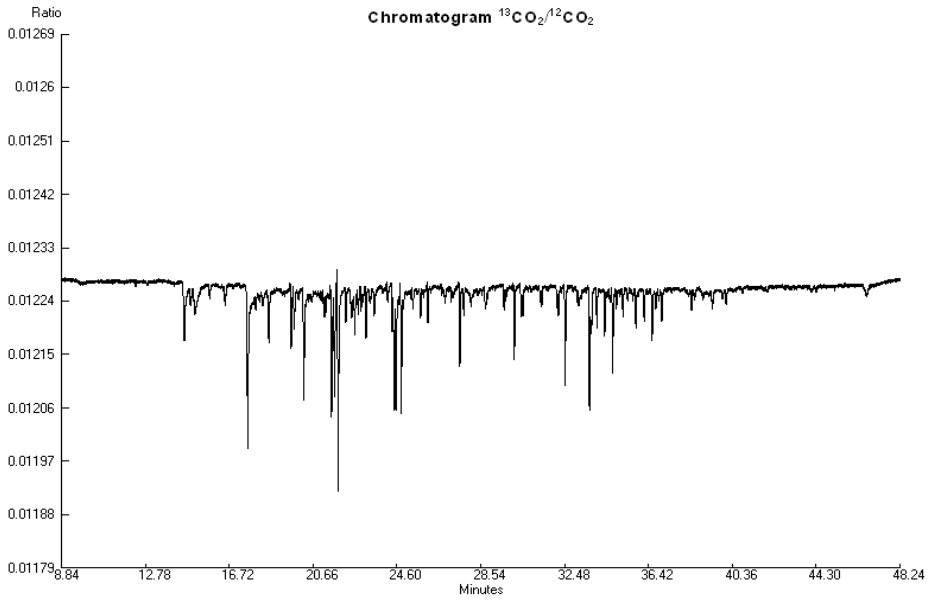

Fig. 2.9: Ratio chromatogram $^{13}CO_2/^{12}CO_2$ of the NaOMe derivitised extract without electron donor ($Na_2S_2O_3$) added during incubation, demonstrating no uptake of labelled CO_2.

2.3.5 Identification of ^{13}C Enriched Lipids from Soil Organic Matter

In all cases of experiments involving potential enrichment of SOM, a control experiment using $^{12}CO_2$ was performed. This served two functions; firstly it demonstrated that the soil organic matter detected in the enriched samples had come about through CO_2 uptake, but secondly and more importantly it provided unenriched spectra for identification and comparison purposes. Figs. 2.10 and 2.11 are IRMS spectrums of the derivitised organics eluting from the GCMS of the $^{12}CO_2$ and $^{13}CO_2$

Abbeyside soil incubations respectively. The figures display the $^{13}C/^{12}C$ ratio of the detectable peaks (^{13}C top and ^{12}C bottom spectrums) as they elute from the GCMS to demonstrate enrichment of certain peaks. This is a first step for the operator to confirm or deny sample enrichment post-incubation. In Fig. 2.10, the $^{13}C/^{12}C$ ratio of the $^{12}CO_2$ incubated soil is the same and therefore the spectrums mirror one another. This was indicative of ^{13}C natural abundance of the soil extracted lipid fraction i.e. even distribution of the rare isotope. In Fig. 2.11, $^{13}C/^{12}C$ ratio of the $^{13}CO_2$ incubated soil was quite different indicating that this soil has become enriched in the stable isotope. The different intensities observed for particular peaks in the top spectrum (^{13}C) in comparison to the same peak in the bottom spectrum (i.e. they are both generated by the same eluted compound) indicates that these particular products were significantly more enriched in ^{13}C than can be explained as natural ^{13}C-abundance.

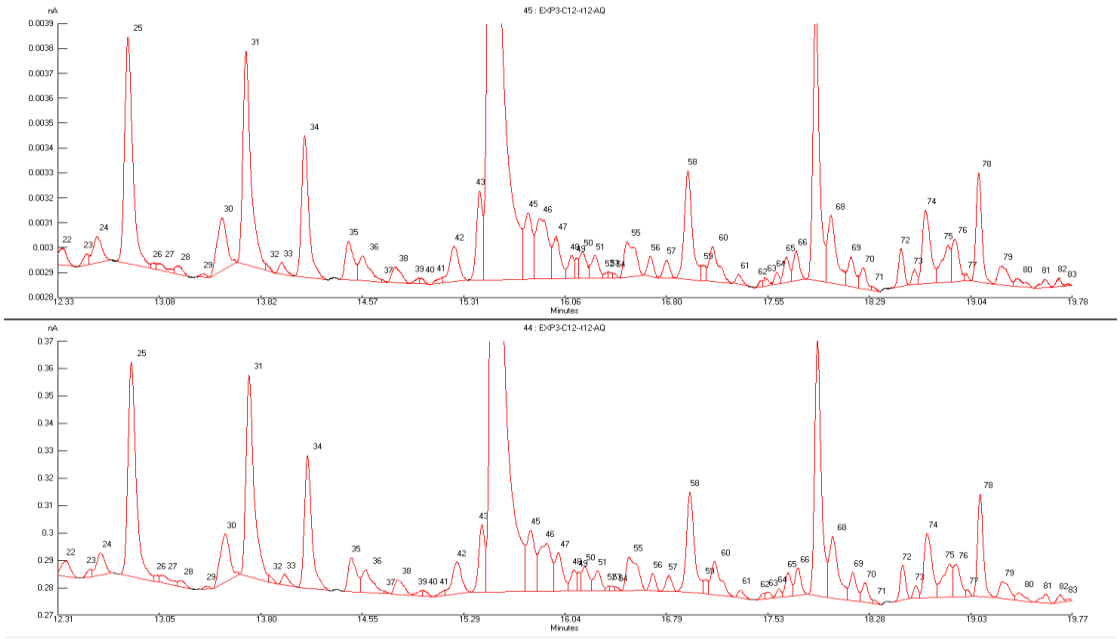

Fig. 2.10: IRMS spectrum of the ^{13}C (top) / ^{12}C (bottom) ratio of detected lipids eluted from the GCMS column from the Abbeyside soil (incubated using 20 mM $Na_2S_2O_3$ and a 1000 ppmv $\underline{^{12}CO_2}$ atmosphere). The $^{13}C/^{12}C$ ratio spectrums resemble each other to such a degree that initial interpretation would suggest that all derivitised lipids for this sample contain the same degree of enrichment (i.e. natural ^{13}C abundance) and therefore no artificial enrichment has taken place.

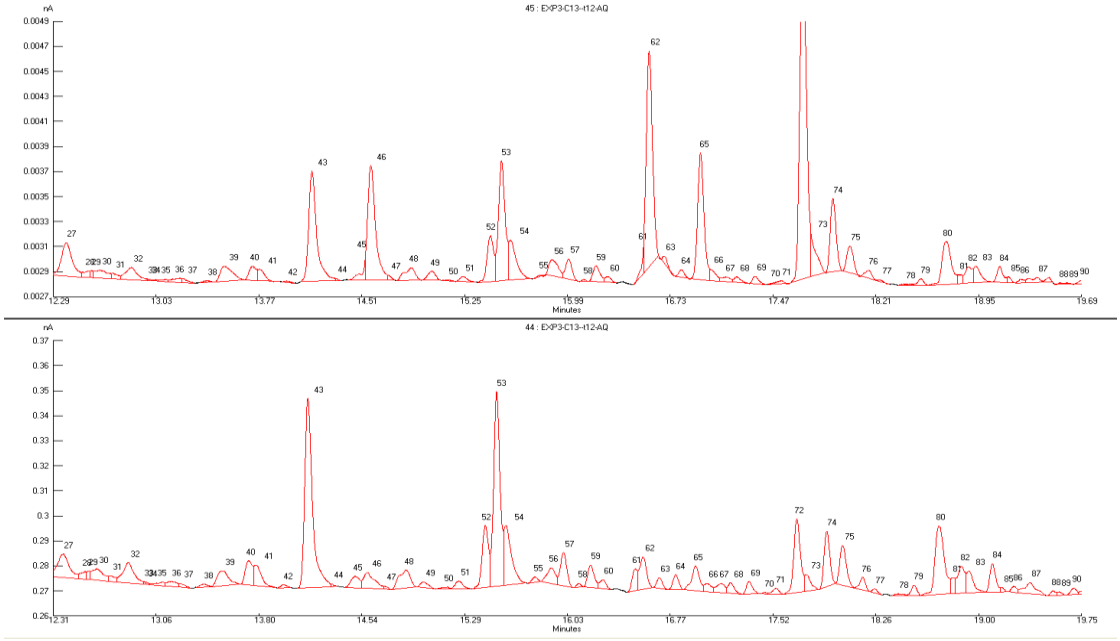

Fig. 2.11: IRMS spectrum of the ^{13}C (top) / ^{12}C (bottom) ratio of lipids eluted from the GCMS column for the Abbeyside soil (incubated using 20mM $Na_2S_2O_3$ and a 1000 ppmv $^{13}CO_2$ atmosphere). Different peak intensities show that the peaks displayed in the top spectrum are ^{13}C-enriched whereas the corresponding peaks on the bottom spectrum are ^{12}C-enriched. Therefore, the initial conclusion was that enrichment levels were not evenly distributed, indicating these peaks (top spectrum) are not of natural ^{13}C-abundance.

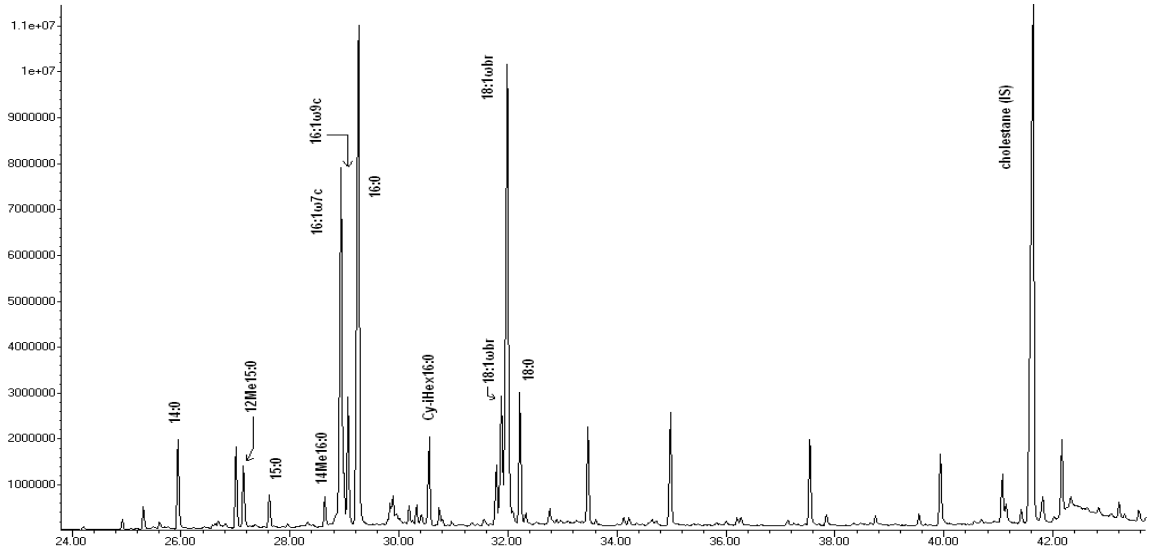

Fig. 2.12: GC chromatogram of NaOMe derivitised extract (Abbeyside soil) identifying ^{13}C-enriched FAMEs. X-axis scale in minutes.

Analysed lipids were selected based on an increased δ ^{13}C value, compared to the corresponding value from the $^{12}CO_2$ incubation. Enriched organics originating from sequestered $^{13}CO_2$ were selected on the basis of a δ value of greater than 50 ‰. Identification of specific lipids was carried out by utilising NIST and Wiley spectral

databases, with a spectral accuracy limit of greater than or equal to 95%. Table 2.3 displays the results of the IRMS analysis for the sodium methoxide derivitised extract while Fig. 2.12 identifies the labelled lipids in the GC chromatogram. Generally, the identified lipids appear to be either saturated or monounsaturated fatty acids. A total of eleven FAMEs were identified as being enriched with the corresponding δ values plotted in Fig. 2.13. In IRMS analysis of a C_{16} fatty acid after derivitisation to the corresponding FAME, 94% of the carbon isotopes measured was from the fatty acid itself and the remaining 6% as a result of the methoxy carbon (Docherty *et al.* 2001). This results in a very minor alteration in the delta (δ) value, especially when dealing with largely enriched lipids as reported here.

	$^{12}CO_2$ incubation		**$^{13}CO_2$ incubation**	
Lipid	**δ ^{13}C**	**st dev (±)**	**δ ^{13}C**	**st dev (±)**
14:0	-30.93	1.63	1766.83	21.97
12Me-15:0	-33.19	0.82	200.97	16.32
15:0	-34.40	0.47	103.27	35.05
*a*16:0	-16.02	2.68	404.69	48.64
16:1ω9	-28.30	2.59	5227.92	19.00
16:1ω11	-32.52	2.30	342.13	41.23
16:0	-26.42	2.11	3877.33	328.12
*Cy*16:0	-31.41	2.08	472.02	18.10
18:1ω9	-25.15	2.37	388.73	20.96
18:1ω11	-33.69	0.96	6732.30	397.01
18:0	-31.79	1.47	457.65	4.91
Cholestane (IS)	-22.40	2.26	-21.82	1.68

Table 2.3: NaOMe derivitised fatty acids extracted from Abbeyside soil after 48 hours exposure to 20 mM $Na_2S_2O_3$ and under atmospheres of differing carbon isotopic composition (1000 ppmv 99% $^{12}CO_2$ and 1000 ppmv 99% $^{13}CO_2$ respectively). δ ^{13}C value of the identified fatty acids as identified using the NIST and Wiley spectral databases.

A large variation in the degree of enrichment can be seen, with the most abundant lipids generally showing the highest levels (Fig. 2.13). The reported average δ ^{13}C value and individual standard deviation for each identified lipid was calculated by running the samples in triplicate. Further evidence of the stability and accuracy of the system can be seen from the δ ^{13}C values of the internal standard, cholestane, which when comparing the delta values from the $^{12}CO_2$ and $^{13}CO_2$ incubation was within our IRMS standard deviation limits.

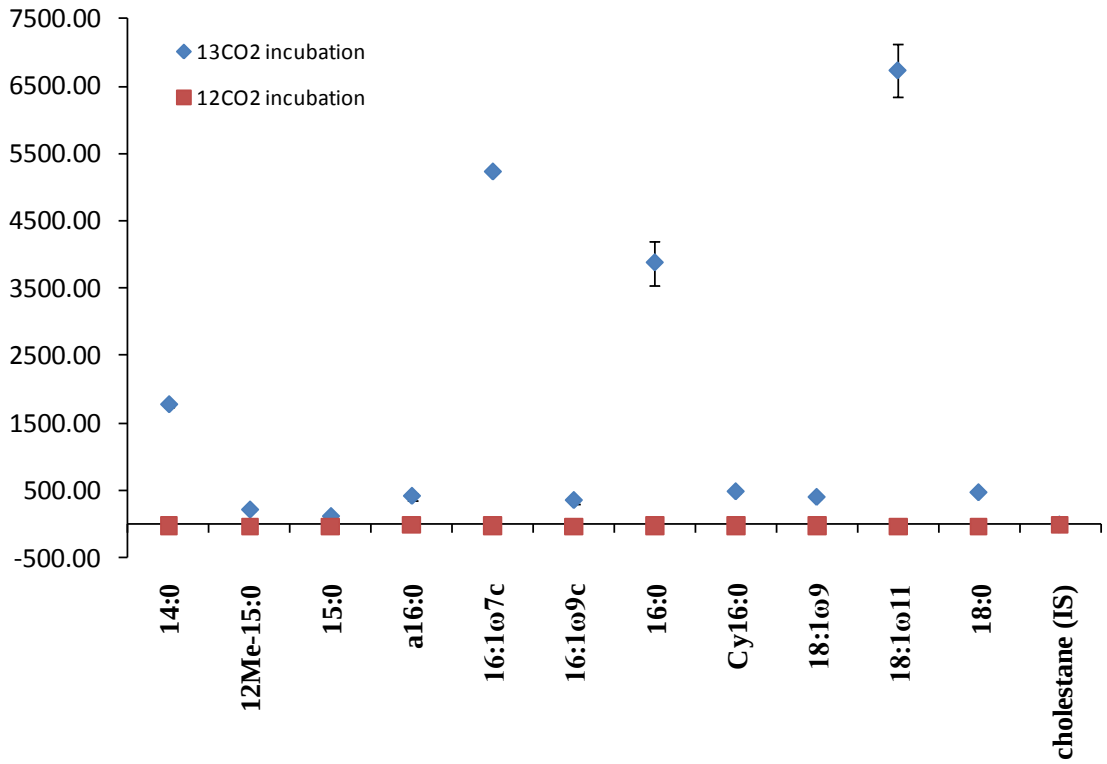

Fig. 2.13: Comparison plot of δ ^{13}C values from identified enriched lipids from $^{12}CO_2$ and $^{13}CO_2$ Abbeyside soil incubations.

2.4 Discussion

The biofixation of CO_2 from a mechanism other than photoautotrophy was of considerable interest. Although the soil sample was provided with conditions conducive for chemoautotrophy and hence no direct comparisons can be made to current *in situ* activity, it is known, that due to land fertilisation practices, biofixation occurs through this chemical pathway, e.g. the additions of sulphur-based fertilisers to agricultural lands (García de la Fuente *et al*. 2007; Yang *et al*. 2010). It was conceivable that CO_2 sequestration may be occurring within the upper zones of the soil matrix, on a slow but continuous basis in conjunction with surface level photosynthesis and hence, the significance of this initial study. When considering phototrophic inputs to CO_2 sequestration by crop plants, (Zhong & Yagi, 2004; Fleisher *et al*. 2008) algae (Smith & Bidwell, 1989; Blair *et al*. 1996), grasslands (Tieszen & Johnson, 1975; Dugas *et al*. 1997; Skinner, 2007), trees (Toivonen & Vidaver, 1988; Holtum & Winter, 2003) and marine higher plants (Ferguson & Williams, 1974; Silva *et al*. 2008) it may seem insignificant to focus on this small group of niche microorganisms, but if they have a continuous input to SOM through direct or indirect means, then further investigations

are required. For instance, Dugas *et al*. (1997) reported that 0.7 g CO_2 m^{-2} h^{-1} was sequestered by the genus *Sorghum*. Comparison of this uptake rate to the one determined in this study (1.2 x 10^{-6} g CO_2 m^{-2} h^{-1}), shows that significant contributions to SOM could be made. This was especially true when considering how many kilograms of wet soil per m^2 of a particular plot could be exposed to favourable growth conditions. The data for the mixed cultures studied would however have to be re-evaluated to take into consideration the changes in the natural climate (inc. temperature, nutrient availability and predation). The CO_2 sequestration potential of hydrogen (H_2) oxidising bacteria in soils has been studied by Dong & Layzell, (2001) and Stein *et al*. (2005) who both determined that soils treated with H_2 were capable of becoming CO_2 sinks. The rates of sequestered CO_2 reported were comparable to the findings in this study, where CO_2 uptake overcame soil respiration.

Provided with favourable conditions it could be inferred that the chemoautotrophic species responsible for the observed CO_2 uptake could have a significant impact on inorganic carbon sequestration, especially when considering the sample location (undisturbed and open grass dominated landscapes as well as arable locations with continuous fertiliser input). That said it was important to note that this experiment took place under optimal conditions for chemoautotrophic growth with an excess of chemical electron donor (supplied in quantities that far exceeds what was normally observed for the discussed environments with the exception of sites using ammonium thiosulphate fertilisers [Graziano & Parente, 1996]). Incubations involving no chemical electron donor showed virtually no CO_2 sequestration during our target time frames (Fig. 2.9) and suggest that although chemoautotrophic microorganisms are present in these soils, the requisite energy sources are not present in significant quantities for the detection of CO_2 uptake under the experimental time frames employed here. However, when one considers agricultural applications of inorganic nutrients such as S^0 and the common practice of spreading manure to land (Oke, 1967; Kotkova *et al*. 2008), the unmeasured effect on CO_2 sequestration has yet to be investigated and the techniques presented here may help to answer this question.

Two questions have been raised during this study; how can we reasonably manage the soil to increase the CO_2 sequestration of the chemoautotrophic fraction of microorganisms and how sustainable was the increase in chemoautotrophic CO_2 assimilation in the soil horizons? Although at present, the answers to both these

questions are outside the scope of this study, it can be hypothesised that chemoautotrophy is already taking place in agricultural settings on a near to continuous basis, but from our understanding, little research has taken place to elucidate the sequestration of CO_2. Acidification, fertilisation and pesticide application involve the addition of sulphur based chemicals directly to the land which are subsequently degraded via microbial mineralisation (biodegradation). To a lesser degree, inorganic forms of sulphur are also continuously being leached into the soil from basic igneous rocks due to weathering and the precipitation of acidic rain water. The acidification of land is a well established agricultural practice required for the growth of various crops requiring acidic soil conditions (Kemmitt *et al*. 2005; Owen *et al*. 1999). Some soils contain natural buffers acquired over geological time and from the surrounding geochemistry, so that natural conditions generally return rapidly, requiring constant application of acidulants. This suggests that the sustainability of acid producing genera such as *Thiobacillus* in higher than naturally occurring levels may be viable in stratified soil horizons. Adequate studies demonstrating the long term effects and potential for human management interventions (additions of buffering agents to maintain pH levels etc.) are also required.

The depth of sulphur penetration and subsequent chemoautotrophy is an interesting aspect to potential studies as it may be expected that little impact to the productive upper horizons would occur with the exception of increased aluminium mobility (Kemmitt *et al*. 2005). Kemmitt *et al*. (2005) point out that human-induced acidification would result in reduced crop yields for most common species but the use of acid tolerant cultivars (Tang *et al*. 2003) may help to solve this issue. Research into agricultural practices is well established and highly valuable to the world economy. The carbon sequestration capacity of chemoautotrophs was also well known, and I propose to investigate the links and potential for carbon sequestration with future investigations into these two topics using the basic experimental methods explored in this study (please see chapter IV).

The detection of a leak from the ECIC, at first appears to be a regrettable set-back for the project as all experiments have become subject to partial pressure effects. Although it was acknowledged as a limiting factor to the conclusions one can draw from the quantitative measurements, it must be stressed that few other researchers, to my knowledge, have investigated the robustness of closed incubation chambers used in

isotopic and quantitative CO_2 measurements (Migné *et al*. 2002; Webster & Payne, 2002; Lou *et al*. 2003; Zhong & Yagi, 2004; Zhong *et al*. 2004; Reth *et al*. 2005; Alavoine *et al*. 2008) with the exception of Ito *et al*. (1980) who acknowledge the presence of a leak but did not provide any measurements or interpretation of its effect on their incubation efficiency. The measurement of the leakage rates at specific partial pressures and the subsequent generation of high resolution correction factors for all quantitative measurements were vital for the reliability of the conclusions drawn in the project.

Chromatograms such as in Fig. 2.12 can provide much information on the fate of sequestered CO_2 and formation mechanisms of organic matter in soil systems. Eleven fatty acid methyl esters were identified from the preliminary GCMS-IRMS analysis. Monounsaturated fatty acids and cyclopropyl fatty acids are common biomarkers for Gram-negative bacteria (Treonis *et al*. 2004) and several straight chain saturated fatty acids (14:0, 16:0 and 18:0) were also found to be highly enriched. Fungal markers that generally contain hydroxyl groups on the fatty acid side chains were not identified (Zelles, 1999). This suggests that crossfeeding of bacterial by-products or labelled biomass by the fungal community did not occur unlike other $^{13}CO_2$ labelling experiments (Jin & Evans, 2010; Butler *et al*. 2003). Further investigation into the lipid profiles of these enriched samples may lead to a means of distinguishing between organisms involved in carbon sequestration and quantification of these lipids may indicate the prevalence of one over the other. Additionally, the fate of newly synthesised organic compounds can be monitored in the soil matrix. In this way we can assess and differentiate between labile and recalcitrant compounds within the soil matrix.

2.6 Conclusions

The overall aim of this study was to prepare a working method in which multiple techniques may be eventually utilised to study carbon uptake in a single soil sample, subjected to various conditional changes. The detectable leak in the chamber may have proved to be an inconvenience, but its reproducible and linear nature led to the calculation of partial pressure dependent correction rates. These correction factors provided reasonably accurate CO_2 flux data. The addition of the chemical electron donor to the soil sample has been shown to be the sole energy source and hence,

essential for the biofixation of CO_2 within the system. The CO_2 fluxes of the incubated samples have been tracked and quantified using high resolution data sets, leading to estimations of inorganic carbon uptake. Specifically, the 8 day soil autotroph enrichment study yielded an uptake value of 256.4 µg CO_2 kg^{-1} dry soil. The short-term 48 hour incubations under elevated CO_2 conditions were determined to sequester 149.7, 4359.0 and 8346.7 µg CO_2 kg^{-1} dry soil. The significance of such sequestration may seem minimal when compared to macroorganisms such as grass, but the global contribution may be more tenable. The isotopic enrichment of Gram-negative prokaryotic biomarkers provided further evidence of incorporation of inorganic atmospheric CO_2 into the soil matrix. Identification of individual microbial species involved in CO_2 uptake, profiling of chemoautotrophic species in different soil samples, and analysis of the fate of organic carbon are only some of further applications that can be envisaged for the current methodology.

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Chapter III: Investigation into the Sequestration of CO_2 by Soil Chemoautotrophic Bacteria Using Advanced Spectroscopic Techniques and Molecular Ecology

3.0 Abstract

Carbon dioxide mitigation technologies and sources of natural sinks is a topic of considerable interest to legislators, planners, farmers and the scientific community (amongst many others) at present. Investigation into methods that reduce CO_2 emissions and/or store the resultant carbon in a form not interactive with the atmosphere has great potential to assist the global population in mitigating the effects of climate change. The aim of this chapter was to apply several cutting edge techniques to determine the activity of chemoautotrophic bacteria after labelling biomass with $^{13}CO_2$. Using an environmental growth chamber to maintain steady conditions, augmented soil was incubated in the presence of isotopically labelled CO_2 to determine the identity of bacterial species responsible for inorganic carbon capture in the dark using stable isotope probing. Post-incubation, the soil was measured to determine the fraction of labelled biomass and biosynthesised compounds using advanced NMR and isotope ratio mass spectrometry. It was found that species closely related to the *Thiobacillus* genus were responsible for soil chemoautotrophy and that a range of lipids (inc. PLFAs), carbohydrates, peptides and aliphatics were being biologically synthesised as a direct result of carbon capture. High resolution infra-red measurements of the growth chamber atmosphere determined that 2018.1 ± 1530.7 µg CO_2 kg^{-1} dry soil were captured over a 48 hour period. Using multiple disciplines to extract information from a complex environmental sample, such as soil, has been shown to have great benefit to elucidating carbon capture dynamics. It is proposed that the methods displayed in this chapter be applied to samples/conditions resembling those of *in situ* scenarios.

3.1 Introduction

The recently observed increases in atmospheric CO_2 and the projected further expansion of global CO_2 production (despite mitigating legislation) has developed a broad scientific interest in global CO_2 sequestration via biological means (McAlpine *et al*. 2010). For instance, reforestation (Reyer *et al*. 2009; Benítez *et al*. 2007), biomass based energy production (Harun & Danquah, 2011; Xie *et al*. 2011) and bio-char addition to soils (Batjes, 1998; Lehmann *et al*. 2006) are among a few of the current practices being investigated alongside large scale emission reduction policies (Leach, 1991). The global soil carbon pool is approximately 3 times the size of the atmospheric pool and 4.5 times that of the biotic pool (Lal, 2004). That said, recent evidence suggests that microbial biomass far exceeds the currently accepted contribution of 5% to soil organic matter (SOM; Jenkinson & Ladd, 1981; Dalal, 1998; Simpson *et al*. 2007a). Simpson *et al* (2007a) have reported that approximately 50% of SOM consists of living or dead microbial cells and subsequently, large contributions of microbial peptides/proteins are found in the humic substances (HS) fraction. Furthermore, the HS that make up a large proportion of long-term SOM in soils, is a complex mixture of microbial and plant biopolymers and their degradation products, and therefore not a distinct chemical category as was traditionally thought (Kelleher & Simpson, 2006). Based on the amount of fresh cellular material in soil extracts, it is probable that the contributions of microorganisms in the terrestrial environment are (seriously) underestimated. If this was the case then efforts to manage soils to increase their carbon storage capacity (as first suggested by the IPCC in 1996 and again in 2001) may be a possible means of slowing the rate of atmospheric CO_2 expansion (IPCC, 1996; IPCC, 2001) by trapping carbon into HS in the soil. This could be a prudent course of action as HS represent an important stable carbon pool and can potentially persist for thousands of years (Trumbore & Czimczik, 2008).

There is wide diversity amongst the soil microbial consortium with each species in a state of constant flux, depending on the current surrounding environment and their place in the food web. Chemoautotrophic bacteria are ubiquitous in most soil types (Chapman, 1990; Smith & Strohl, 1991) and they are unique in their ability to derive energy from inorganic substrates via both aerobic and anaerobic respiration pathways (White, 2007; Alfreider *et al*. 2009). The significance of these particular organisms is that they can continue to sequester CO_2 while in the absence of light and organic matter.

Large scale CO_2 consumption could be taking place in varied and diverse locations (deep sub-surface, aquifers, cave systems, lake beds etc) provided a constant stream of appropriate chemical electron donors are present. Methods to detect the activity of these microorganisms are well established but relatively little data exists in the literature documenting their contribution to CO_2 sequestration and/or SOM formation (Selesi *et al*. 2005; Miltner *et al*. 2005a; Kuparinen & Galvão, 2008; Hart *et al*. 2011a).

The incorporation of stable isotopes into cellular material was achieved via incubation of a selected compound that has been artificially enriched with the isotope of choice. After the incubation period, cellular components such as lipids or nucleic acids can be harvested from a sample (biomass, soil, sediment or water) and analysed using various techniques such as Nuclear Magnetic Resonance (NMR) spectrometry (Lundberg *et al*. 2001; Baldock *et al*. 1990) and/or Gas Chromatography Mass Spectrometry - Isotope Ratio Mass Spectrometry (GCMS-IRMS; Evershed *et al*. 2006). The isotopically labelled nucleic acids may also be extracted from the soil and physically separated from the unlabelled nucleic acids using caesium chloride (CsCl) density gradient ultracentrifugation (Tillmann *et al*. 2004). The ultracentrifugation procedure produces a zone of purified, densely labelled DNA in a separation tube that, once extracted, can help to elucidate the microbiological drivers of a particular process.

The study of molecular ecology has been greatly advanced by the development of stable isotope probing (SIP). Using stable isotopes such as ^{2}H, ^{13}C and ^{15}N it has become possible to identify nutritional and chemical pathways employed by microorganisms (Whitby *et al*. 2001; Dumont & Murrell, 2005; Cupples *et al*. 2007; Kreuzer-Martin, 2007; Bastias *et al*. 2009). The advantage of SIP to research scientists was that mixed or unknown cultures of organisms can be identified due to their incorporation of rare and distinguishable isotopes. The first examples of SIP were labelled phospholipid-derived fatty acids (PLFA; Boschker *et al*. 1998), DNA (Radajewski *et al*. 2000) and RNA (Manefield *et al*. 2002) which established the method as a qualitative tool for identifying target groups of microorganisms that metabolise specific substrates. These techniques can also be used to analyse the incorporation of autotrophically sequestered CO_2 into the soil horizon. The passage of atmospheric carbon through plants to the soil rhizosphere has been demonstrated, resulting in the successful labelling of soil bacteria and fungi (Ostle *et al*. 2003; Griffiths *et al*. 2004; Jin & Evans, 2010; Drigo *et al*. 2010). The rapid incorporation of

photosynthesised carbon from the plant root system into rhizodeposits, resulted in labelling of fungi, followed by bacteria and then later incorporation into microbial grazers such as the protozoans (Jin & Evans 2010; Drigo *et al*. 2010). Techniques used in modern molecular ecology such as PCR probes and molecular cloning are integral to understanding complex environmental processes. Microorganisms are the primary agents of geochemical change but most biogeochemical agents have not yet been cultured because their habitats are poorly understood and difficult to simulate (Madsen, 2005). The employment of PCR-based technologies to extract DNA from both *in vivo* and *in vitro* studies to identify the active species responsible was of prime significance.

Studies into the direct autotrophic labelling of soil microorganisms are uncommon in the literature (Miltner *et al*. 2004), although this was a well known process (Smith & Strohl, 1991). Thus, it proved necessary to undertake a study of the direct ^{13}C-labelling of soil dwelling chemoautotrophic bacteria with the emphasis on instrumental analysis of SOM using GCMS-IRMS and NMR, microbial ecology and direct CO_2 uptake measurements. The essential questions being asked in the chapter are; 'who' was performing CO_2 sequestration? 'what' products are being formed as a direct result of CO_2 uptake? and 'how much' CO_2 was being removed using the soil/slurry approach? I hope to demonstrate a methodological approach with multiple analyses on a single sample to yield high quality and informative data using an *in vivo* approach with the future aim of assisting *in vitro* studies.

3.2 Materials & Methods

3.3.1 Site Details and Pre-treatment

The soil used in the outlined experiment was a Grey Brown Podzolic (Fay & Zhang, 2011), retrieved from an open public area located within Albert College Park (A.K.A. Hampstead Park), Glasnevin, Dublin, Ireland (53° 22' 54.63" N 6° 15' 43.72" W). The sampling location was on open land with a large amount of pine needles present. Samples of the surface epipedon (A horizon) were transferred aseptically to the laboratory and processed immediately. Roots and large debris were removed manually using aseptic techniques. The soil was air dried and then sieved using a sterilised stainless steel mechanical sieve with a ≤ 2 mm aperture size. Sieved soil of the < 2 mm fraction was stored in an amber jar at 4^oC. Accurately weighed aliquots of soil were dried at 104^oC for three days yielding an average moisture content of 24.5%. The soil

water holding capacity (SWC) was determined according to Paetz & Wilke (2005). A portion of soil was fractionated according to size using a 9-piece aluminium sieve set, range 2000-25 µm (Nickel-Electro, Weston-Super-Mare, United Kingdom) and using the Gradistat soil textural calculator (Blott & Pye, 2001) the soil texture was determined to be, a slightly very fine gravelly, very coarse silty medium sand. Using the USDA soil pyramid it was determined that the soil was a sandy loam. A CHN combustion analyser (Exeter Analytical CE440 elemental analyser) was used to determine the soil elemental composition, 8.62% C, 0.97% H, 0.32% N. Phosphorus analysis by wet digestion (April & Kokoasse, 2009) was 0.31% P. Soils were determined for SO_4^{2-} and NO_3^- using the methods laid out by Rump (1999). SO_4^{2-} and NO_3^- were 8.1 ± 0.56 g kg^{-1} and 12.0 ± 0.3 g kg^{-1} respectively. All chemicals and solvents were purchased from Sigma Aldrich. The chemicals were of the highest purity grade available and all solvents were of PESTANAL® quality.

3.3.2 Soil Incubations

Samples were pre-incubated for 14 days to increase microbial biomass under dark autotrophic conditions. A central hole was inserted into the lid of a 900 ml amber jar, followed by four surrounding holes to act as exit vents. The amber jar was then autoclaved at 121°C for 10 minutes. A known weight of dry soil (32.06 g) was placed into the sterile amber jar and 300 ml of autoclaved (121°C / 15 minutes) minimal salts medium (MSM; 0.5 g l^{-1} K_2HPO_4, 0.5 g l^{-1} KH_2PO_4, 0.5 g l^{-1} NH_4Cl, 0.5 g l^{-1} $MgSO_4.7H_2O$, 0.12 g l^{-1} NaCl, 0.05 g l^{-1} $CaCl.2H_2O$ and 1 ml of a trace metal solution [0.1 g l^{-1} $ZnSO_4.7H_2O$, 0.3 g l^{-1} $MnCl_2.4H_2O$, 0.3 g l^{-1} H_3BO_3, 0.1 g l^{-1} $CuCl_2.2H_2O$, 0.2 g l^{-1} $NiCl_2.6H_2O$, 0.3 g l^{-1} $NaMoO_4.2H_2O$, 1.0 g l^{-1} $FeSO_4.7H_2O$]; Shiers *et al*. 2005; Madigan *et al*. 2009) was added aseptically. A stock solution of 1000 mM $Na_2S_2O_3$ was prepared and 6.0 ml (0.2 µm filtered) added to each jar (20 mM $Na_2S_2O_3$). Air was filtered using a 0.2 µm Millex-FG filter unit (Millipore, Molsheim, France) and pumped through the soil solutions for 7 days where upon 250 ml supernatant was removed and replaced with fresh MSM and 6.0 ml (0.2 µm filtered) 1000 mM $Na_2S_2O_3$ for the remaining pre-incubation period.

After the 14 day pre-incubation, MSM and electron donor were aseptically replaced and the incubation jar placed into the environmental carbon dioxide incubation chamber (ECIC). For details on incubation unit please see chapter II, page 89-91; Hart *et al*. (2011a). Dried silica gel (190 g) was placed onto the bottom shelf of the ECIC to

absorb excessive humidity. The ECIC was pre-heated and maintained at 30 ± 0.2^{o}C. A autoclaved glass pipette was inserted into the central hole in the jar lid with a clean UV sterilised tube coupled to an autoclaved 0.2 µm Millex-FG filter unit fitted to the glass pipette and to the outlet port of a battery powered air pump (Agile, p/n A790). The battery air pump was activated and the chamber door was sealed. Automated data entry took place every 30 seconds to record the internal environmental data (% RH, temp., ppmv CO_2) and the incubation period remained uninterrupted for 48 hours. This procedure was repeated for both $^{12}CO_2$ (Air Products 99% CO_2 Industrial grade) and $^{13}CO_2$ (Sigma Aldrich 99% atom ^{13}C) incubations. $^{13}CO_2$-blank incubations were performed in duplicate where soil was treated verbatim as above with the exception that no $Na_2S_2O_3$ was added to the soils at T_0. Estimations of CO_2 sequestration were made using the calculation method and correction factors laid out in chapter II (page. 95, 99; Hart *et al*. 2011a).

3.3.3 pH and Electrical Conductivity:

Separate soil microcosms were prepared in triplicate (and duplicate blanks) to observe any changes in pH and electrical conductivity (this could not be done during the ^{13}C-labelling experiment as it would involve opening the chamber door and disrupting the atmospheric concentrations on a daily basis). The soil microcosms were prepared identically to the main experimental incubation (as stated above) but were removed from the chamber every 24 hours for measurements of pH and electrical conductivity (EC). Blank soil incubations received no exposure to $Na_2S_2O_3$ over the 14 day incubation. Measurements were taken directly from the soil slurry by adding a sterile (autoclaved at 121^{o}C for 15 minutes) Teflon stirring bar and placing the sample jar onto a magnetic stirrer. Once the sample was homogenised, the pH and EC were taken using a Cyberscan PC300 series (Eutech Instruments, Singapore). The probe was pre-sterilised by immersion into a freshly prepared 1.25% sodium hypochlorite solution for 1 hour, and thoroughly rinsed in autoclaved double-distilled water. Calibration was performed immediately prior to measurements using three pH buffer standards (pH 4.00 ± 0.01; pH 7.00 ± 0.01; pH 10.00 ± 0.01, Fisher Scientific, Dublin, Ireland) and two EC standards (1410 ± 0.02 µS/cm; 12,880 ± 0.02 µS/cm, Fisher Scientific, Dublin, Ireland).

3.3.4 Extraction and Analysis of Soil Organic Matter

The SOM was extracted using a modified version of the Bligh & Dyer method (Bligh & Dyer, 1959; Otto & Simpson, 2007) and was carried out in prewashed 40 ml Teflon

tubes (Nalgene). After CO_2 incubation in the chamber, ~40 ml of the soil slurry was centrifuged at 6000 rpm (20 minutes). The supernatant (medium) was decanted from the soil and this remaining solid residue was washed twice with 0.01 M potassium hydrogen phosphate buffer solution. The soil precipitate was freeze dried (0.90 g $^{12}CO_2$ exp, 1.26 g $^{13}CO_2$ exp, dry weight) before extraction with methanol:dichloromethane (ratio 1:0, 1:1 and 0:1). The total extracts were filtered, concentrated and reconstituted in 1.0 ml of MeOH:DCM [50:50] for derivitisation and analysis.

The extracts were analysed by gas chromatography coupled to a quadruple Electron Impact Mass Spectrometer and Isotope Ratio Mass Spectrometry (GCMS-IRMS). The GC column effluent was subsequently split equally between the two detectors. A transmethylation derivitisation was performed to volatise such lipids as free fatty acids and glycerides, but most importantly phospholipids. Phospholipids (PLFAs) are important as they are the main component of microbial cell membranes and can be a vital source of lipid biomarkers. In the case of the PLFAs, this procedure cleaves the fatty acid side chain from the glycerol backbone/polar phosphorus head group and methylates to form volatile fatty acid methyl esters (FAMEs). An aliquot of the total extract (200 µl) was evaporated to dryness before derivitisation. The method employed was a transesterification reaction involving sodium methoxide (50 µl CH_3ONa) (Christie, 1982; Hughes *et al*. 1986). The solution was vortexed and heated for 10 minutes at 50^oC. Excess sodium methoxide was quenched with 450 µl deionised water and the resulting NaOH neutralised with 50 µl HCl (0.5 M). The newly formed methyl esters were extracted twice from the aqueous solution with 1.0 ml of hexane:chloroform [9:1]. The combined extracts were dried over sodium sulphate and evaporated to dryness before reconstitution in 100 µl of a 100 ppm hexane solution of cholestane (internal standard) for GC injection. Determination of monounsaturated fatty acid double-bond position was performed by GCMS analysis of their DMDS adducts (Nichols *et al*. 1986).

3.3.5 Solid Phase Extraction (SPE) of Phospholipid Fatty Acids (PLFAs)

A modified version of the procedure as reported by Pinkart *et al*. (1998) was used for the SPE of microbial lipids from the total lipid extract. The aminopropylsilica columns (Alltech, UltraClean, Aminopropyl 4 ml/500 mg) were placed on the vacuum manifold (Alltech 12-port Vacuum Manifold, Alltech Associates, Deerfield, Illinois, USA). Column conditioning took place at 20 kPa. Aliquots of solvents were passed through the

cartridge to remove air and/or any possible contaminants, with care taken to not let the column packing dry out. The sequence of solvents used was 6.0 ml aliquots of acetone, chloroform, methanol, 5% acetic acid in ether and hexane.

The sample was reconstituted in 100 µl methanol:dichloromethane (50:50). The vacuum pressure was adjusted to 10 kPa before the sample was loaded. The lipid extract was fractionated into three components, neutral lipids, polyhydroxyalkanoates and polar lipids. The neutral lipids were isolated by eluting 6.0 ml chloroform, followed by 6.0 ml acetone for the PAHs. The third fraction containing the polar lipids (PLFAs) was collected using 6.0 ml methanol. These vials were evaporated to dryness under a nitrogen stream before derivitisation. Please refer to section 2.2.10 and Fig. 2.1 for a description of the nomenclature for PLFAs.

3.3.6 Analysis by GCMS-IRMS

Samples were analysed using a gas chromatograph (Agilent Model 6890N) mass spectrometer (GCMS; 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 (IRMS; IsoPrime), with a split ratio of approximately 50/50. 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 (Air Products, BIP-X47S grade) was used as the carrier gas. The injection port and the GCMS interface were kept at 250 and 280°C, respectively. The ion source temperature was 280°C. GC oven temperature was 100 to 300°C at a rate of 6°C/minute after 1.5 minutes at 100°C. The column head pressure was 69.4 kPa. An aliquot of each sample (1 µl) was injected into the injection port of the GC using the splitless mode of injection, followed by an elution split after column to both mass spectrometry detectors. The GC effluent was diverted via a heart split valve to a ceramic combustion furnace (GC5, 650 mm X 0.3 mm i.d.) packed with a copper oxide/platinum catalyst heated to 850^{o}C. Water was removed from the combustion products by passing the effluent through a nafion membrane prior to the CO_2 entering the IRMS (Isoprime Ltd, UK). Reference gas CO_2 of known $\delta\ ^{13}C$ value was introduced from the reference gas injector at the beginning of the sequence. IRMS system validation was carried out using a stable isotope reference standard (Mixture B2, Indiana University). A standard deviation for the instrument was calculated to be ± δ 1.04 over a 10 run sequence of the 15 alkane mixture.

3.3.7 Solid State ^{13}C NMR Analysis:

For solid state ^{13}C analysis, samples were packed into 4 mm zirconium oxide rotors with Kel-F rotor caps. ^{13}C cross polarisation 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 second 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.

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

All soil samples were freeze dried and subjected to 10% hydrofluoric acid (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 6000 RPM for 20 minutes and the supernatant was discarded (waste HF was stored for professional disposal/neutralisation). The process was repeated a total of x20. The samples were neutralised by adding sterile, double-distilled water until the supernatant measured pH ~6 (Gonçalves *et al*. 2003). Neutralised soils were freeze-dried and stored at -80^{o}C until 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 and pipette tips) were dried for one week over phosphorus pentoxide (P_2O_5) under vacuum at room temperature to reduce traces of molecular water that would interfere with NMR spectra. 40 mg 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 homogenisation 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 2 seconds. Solvent suppression was achieved by presaturation utilising

relaxation gradients and echoes (Simpson & Brown, 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 second 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 were 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 1.1-1.33 ppm, ^{13}C 26-32 ppm.

3.3.9. Microbial Analysis - Extraction of DNA from Soil and Ultracentrifugation of Genomic DNA:

DNA from soil slurries was extracted and purified using FASTDNA Spin Kit for Soil (MP Biomedicals, Illkirch, France) according to the manufacturer's instructions. DNA quantification was determined using an ND-100 NanoDrop Spectrometer (NanoDrop Technologies, Wilmington, Delaware, USA). A CsCl gradient ultracentrifugation was performed on approximately 5 µg of DNA. The CsCl gradient was prepared according to Neufeld *et al*. (2007) following the ethidium bromide (EtBr) protocol and gradient fractionation technique. To ensure the formation of the density gradient, two aliquots of control DNA were added to two control tubes. The control DNA was procured through the extraction of *Escherichia coli* LMG 194, grown from batch cultures of 5mM $^{12}C_6H_{12}O_6$ and $^{13}C_6H_{12}O_6$ in LB broth. Ultracentrifuge conditions were 76,000 RPM, 16 hours at 20^{o}C and a total of x12 300 µl CsCl/DNA fractions were taken per sample. EtBr was removed by mixing each fraction with an equal volume of 1-butanol saturated with Tris-EDTA (TE) buffer, centrifuged at 13,000 RPM for 1 minute and the supernatant was discarded. This was repeated for each fraction until the EtBr was no longer visible (approximately three washings). DNA was precipitated by adding 1.0 µl glycogen (to act as a DNA carrier) and 2 equal volumes of 30% polyethylene glycol

(PEG; 46.8 g NaCl and 150.0 g PEG-6000 made up to 500 ml and autoclaved at 121^{o}C for 15 minutes).

3.3.10 Amplification of 16s rRNA and DGGE:

DNA solutions were amplified using Polymerase Chain Reaction (PCR) and 16s rRNA primer pairs (Muyzer *et al*. 1993) with a GC-clamp. PCR conditions using a Peltier thermal cycler – DNA Engine DYAD (Bio-Rad Laboratories, Massachusetts, USA) were as follows: 5 minutes of initial denaturation at 95^{o}C, followed by 33 cycles of 30 seconds denaturation at 95^{o}C, 30 seconds of annealing at 55^{o}C and 1 minute of elongation at 72^{o}C. Aliquots of the PCR products were analysed by 1% (wt/vol) gel electrophoresis (GE; Bio-Sciences, Dublin, Ireland) and visualised by UV excitation after staining with EtBr (10 mg ml^{-1}). Density gradient gel electrophoresis (DGGE) was carried out to assess the diversity and dominance of bacterial DNA within selected fractions and total DNA according to the protocol laid out by Muyzer *et al*. (1993). DGGE gels were visualised under UV and the images processed using Phoretix 1D v10.4:15927/28232 digital software.

3.3.11 Amplification of cbbLr Genes from CsCl Gradient Fraction 5:

Genomic DNA was extracted directly from 0.50 g soil samples and purified using FastDNA spin kit for soil (MP Biomedicals, Illkirch, France) according to the manufacturer's instructions. Amplification of the RubisCO genes via PCR was performed using the primers designed and discussed by Selesi *et al.* (2005). Amplification of the red-like *cbbL* (*cbbL*r) gene was carried out to see if any low abundance autotrophic species may be present other than chemoautotrophs (chemoautotrophs generally use the green-like *cbbL* gene). Amplification conditions were as follows, 100 ng of DNA solution was reacted in 25 µl volumes containing 2.5 µl DreamTaq PCR master mix 2x (Fermentas, GmbH), 200 µM dNTP's (Fermentas, GmbH) and 50 pmol of the reverse and foreword primers (Sigma Aldrich, Haverhill, UK) and 1 U DreamTaq DNA polymerase (Fermentas, GmbH) and nuclease free water (Sigma Aldrich, Dublin, Ireland). PCR cycle conditions were as follows: 4 minutes initial denaturation at 95^{o}C, followed by 32 cycles of 1 minute of denaturation at 95^{o}C, 1 minute annealing at 57^{o}C. The PCR products were subjected to a final extension step for 10 minutes at 72^{o}C. PCR cycle conditions were carried out using a Peltier Thermal Cycler – DNA Engine DYAD (Bio-Rad Laboratories, Massachusetts, USA). Aliquots of the PCR products were analysed in 1.2% (wt/vol) agarose gel (Bio-Sciences, Dublin,

Ireland) by horizontal gel electrophoresis. DNA was visualised by UV excitation after staining with EtBr (10 mg ml^{-1}).

3.3.12 Cloning and Screening of Environmental Clones:

PCR products from the selected samples of the expected size (1,300 kb) were excised and purified from agarose gel using Fermentas GeneJet, Gel extraction kit (Fermentas, York, UK) and a second PCR reaction was performed using the same primers. Lysogeny broth (LB) agar was prepared (tryptone 10 g l^{-1}, yeast extract 5 g l^{-1}, NaCl 10 g l^{-1}, $MgSO_4$ (anhydrous) 2.4 g l^{-1}, KCl 186.04 g l^{-1}, bacteriological agar 1.5% [Hanahan, 1983]), and autoclaved at 121^{o}C for 15 minutes. A 50 mg ml^{-1} ampicillian sodium salt solution (Sigma Aldrich, Dublin, Ireland) in 30% ethanol was prepared and 0.22 µm filtered, prior to applying 50 µl to each LB plate. Purified PCR products were ligated into the vector pJET 1.2/blunt cloning vector (Fermentas, York, UK) using T4 DNA Ligase (and the recommended procedure) and transformed into Bioline α-select chemically competent cells using the procedures laid out in CloneJET PCR Cloning Kit (Fermentas, York, UK). LB plates were incubated overnight at 37^{o}C and selected colonies inoculated into 10 mg ml^{-1} LB broth (6 ml) overnight at 37^{o}C. Plasmid DNA was extracted from 3 ml of all liquid cultures using GE Healthcare plasmidPrep spin mini kit (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions. Restriction enzyme digest of 2.0 µl plasmid DNA, 2.0 µl x10 buffer H (Pharma Biotech, Amersham, UK), 15.5 µl nuclease free water, 0.5 µl Bgl II (Pharma Biotech, Amersham, UK). Samples were incubated at 37^{o}C for 2.5 hours and then heat shocked at 70^{o}C for 15 minutes in a water bath. Restriction fragments were UV visualised using EtBr on a 1% agarose gel.

Plasmid DNA from selected clones was diluted (1/3) and sent for sequence analysis at the University of Dundee (Dundee Sequencing Services, Dundee, United Kingdom) using pJET 2.1 forward (5'-CGACTCACTATAGGGAGAGCGC-3') and reverse (5'-AAGAACATCGATTTTCCATGGCAG-3') primer sets. Sequences were compared to existing deposited sequences at the National Centre for Biotechnology Information Database by BLAST search. All data were checked using Pintail (Ashelford *et al*. 2005) for chimeric sequences. Sequence alignment was carried out using the CAP3 Sequence Assembly Program (Huang & Madan, 1999). Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 5 (Tamura *et al*. 2007, 2011).

3.3.13 Nucleotide Sequence Accession Numbers:

The sequences determined in this study are available at GenBank under accession no. (GenBank Release Date: June 28, 2011): JF968469, JF968470, JF968471, JF968472, JF968473, JF968474, JF968475, JF968476, JF968477, JF968478, JF968479, JF968480, JF968481, JF968482, JF968483, JF968484, JF968485, JF968486, JF968487, JF968488, JF968489 and JF968490.

3.4 Results

3.4.1 Pre-Incubation and Atmospheric CO_2 Flux

To determine the volume (and hence the mass) of CO_2 being sequestered into the soil, high resolution infra-red absorption measurements of the atmospheric CO_2 concentration within the ECIC were made on a continuous basis (every 30 s). Prior to exposure of the ^{13}C isotope to the soil, it was necessary to ensure that the selected soil sample was capable of sequestering CO_2 once provided with the electron donor ($Na_2S_2O_3$). The Hampstead Park soil was taken directly from the sample site and incubated within the ECIC according to the provided protocol (3.3.2 Materials and Methods) over a 17 day period to observe the CO_2 flux. The data plot displayed in Fig. 3.0 shows the fluctuations of CO_2 within the ECIC atmosphere over the prescribed period. It was important to note that for this demonstration experiment, no additional CO_2 was introduced to the chamber, before or during the incubation so that the CO_2 plot was subject to basal soil processes and the determined leak only. Over the course of the incubation it was observed that CO_2 levels increased to a maximum value of 850 ppmv (e.g. initial soil respiration), while concurrently, CO_2 was being leaked to the outer atmosphere at variable rates according to partial pressure (chapter II: Table 2.2). At the CO_2 λ_{max}, a sudden decay in concentration was observed which took place over a 94 hour period. This decay event peaked at a CO_2 λ_{min} value of 270 ppmv. A minimum concentration notably below the external atmospheric CO_2 levels in the Earth's atmosphere, where an average of ~390 ppmv has been generally accepted for 2011 (Tans, 2009). Not taking into consideration that the experiment took place indoors where CO_2 levels are expected to be slightly elevated in enclosed professional establishments (Lee & Chang, 1999; Seppänen *et al*. 1999).

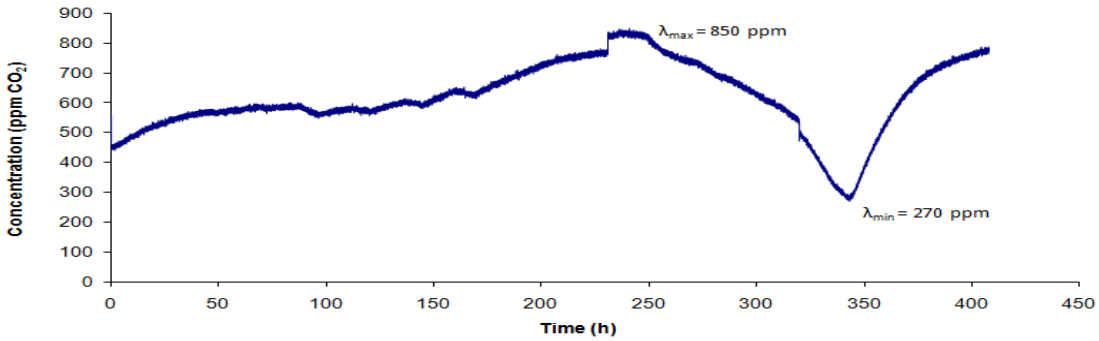


Fig. 3.0: CO_2 plot of Hampstead Park soil incubated at 30°C for 17 days. 30 g soil was exposed to 300 ml MSM and 20 mM $Na_2S_2O_3$ and no additional CO_2 was added to the atmosphere once the ECIC was sealed. Real-time data points were recorded every 30 seconds.

The λ_{min} value of 270 ppmv clearly demonstrates that despite the leak associated with the ECIC, CO_2 was being removed from the chamber atmosphere at a rate faster than it could be replenished by soil respiration or partial pressure from the external atmosphere (e.g. leak in reverse, once the internal CO_2 concentration became negative in comparison to the external atmosphere). Extrapolation of the decay curve observed between point's λ_{max} and λ_{min} (~Day 10 – ~Day 14) was determined to be 0.50 ppmv h^{-1} (leak decay rate corrected). Over the course of this event, it was calculated that 135.8 µg CO_2 kg^{-1} dry soil were sequestered by the soil microcosm during the demonstration experiment.

3.4.2 Soil Incubation, Measurements of Carbon Sequestration and ^{13}C-Labelling of Biomass

The measurement of parameters such as pH and EC in a biological incubation (involving the oxidation of inorganic substances, resulting in salt accumulation and increased acidity) are useful as a means of observing basic chemical changes in the sample. Soils to be placed in the ECIC for CO_2 sequestration studies and isotopic labelling, all underwent pre-incubation/enrichment for 21 days, where after day 7, addition of the electron donor took place. An observable drop in pH for the soils exposed to the electron donor, demonstrated that over the pre-incubation (microbial enrichment) period, a soil that had not been previously exposed to $Na_2S_2O_3$, underwent a significant drop of 1.33 ± 3.4 x 10^{-1} pH units (Fig. 3.1) when compared to the blank soils. The decrease in pH of the experimental soil samples when exposed to a sulphur containing electron donor (such as $Na_2S_2O_3$) was consistent to the known biological activity of sulphur oxidising microorganisms e.g. the production of sulphuric acid (Starkey, 1935; Vogler *et al*. 1942).

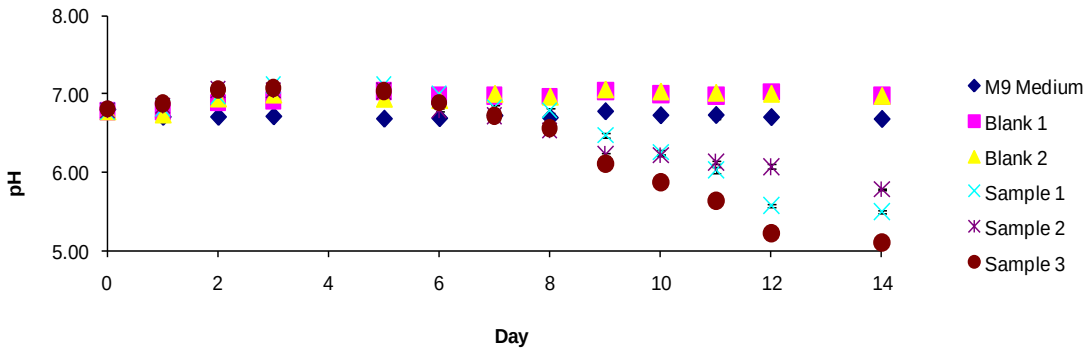


Fig. 3.1: pH plot showing decreasing pH over a 14 day incubation period for Hampstead Park soil exposed to $Na_2S_2O_3$.

An average increase in conductivity of 1.84 ± 4.6 x 10^{-3} mS for the experimental samples demonstrates a significant increase in EC indicating the accumulation of salts over the incubation period. EC measurements correlated with the decreasing pH by increasing over a similar time scale (Fig. 3.2). The gradual increase in EC, in comparison to the steady state observed in the duplicated blanks, indicates the accumulation of salts through the oxidation of $Na_2S_2O_3$ via the ($S_2O_3^{2-} + 2O_2 + H_2O = 2SO_4^{2-} + 2H^+$) reaction pathway.

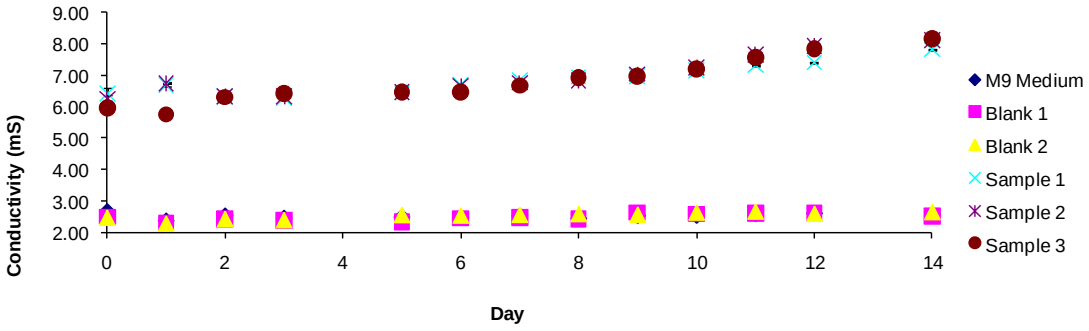


Fig. 3.2: Conductivity plot demonstrating increasing Hampstead Park soil solution EC over a 14 day incubation, after exposure to $Na_2S_2O_3$.

The 21 day incubation period of the Hampstead Park soil used to generate the pH and EC measurements was subsequently used as the source material for the main ^{13}C-labelling experiment. This approach was required because of the necessity to pre-incubate the samples, initially to deplete labile organic matter (following the addition of the MSM and incubation at 30^oC for 7 days) and then to provide conditions suitable for chemoautotrophic growth (e.g. addition of a working concentration of $Na_2S_2O_3$ followed by lag phase growth of extant chemoautotrophs) for the remaining 14 days. The first amendment of electron donor to the soil was subsequently incubated within the

ECIC (simply to maintain constant temperature conditions [30 ± 0.2^{o}C]). This requirement was necessary for meaningful results to be achieved for the ^{13}C-labelling study as Fig. 3.0 clearly demonstrates the lag phase requirements for soils newly amended with $Na_2S_2O_3$ of approximately 10.4 days (please be aware that the data used to generate Fig 3.0 was an independent experiment designed to observe CO_2 fluctuations to a soil newly amended with $Na_2S_2O_3$ and hence, aid in predictions of soil responses). Pre-incubation was a vital aspect to the experiment for two reasons; firstly, to reduce the impact of cross-feeding of labile ^{13}C compounds derived from exudates and/or dead chemoautotrophic primary producers and also to ensure the labelling experiment commences during a period of exponential growth of the target microorganisms. The second reason was vital to the success of DNA-SIP experiments as labelling of DNA only occurs upon cell division (Neufeld *et al*. 2007) and although a wide range of potential chemoautotrophs exist in soils, *Thiobacillus*-like species require approximately 8-10 days of generation time (McGoran *et al*. 1969; Alcántara *et al*. 2004) to reach the exponential growth stage.

After the 21 day pre-incubation, the supernatants were discarded and fresh MSM media and electron donor introduced to each test soil (designated as HP01, HP02 and HP03). The $^{12}CO_2$ incubation (preceding the $^{13}CO_2$ labelling incubation) was carried in triplicate to determine the CO_2 flux and estimate sequestration rates (because $^{13}CO_2$ absorbs IR radiation at a different frequency to $^{12}CO_2$, these measurements were considered unreliable [Gosz *et al*. 1988] and therefore, all CO_2 sequestration rates are based upon the $^{12}CO_2$ plots [Figs. 3.3 and 3.4]). The $^{12}CO_2$ plots taken over 48 hour incubations (Fig. 3.3), show decreasing concentrations of the substrate gas within the ECIC (also the subsequent automated re-introduction of CO_2 when concentrations fall below 950 ppmv appears as a spike in the plots). These plots are difficult to visually interpret because there was little uniformity in the CO_2 activity between samples but that was irrelevant to estimating CO_2 capture. It was worthy of note, that more automated CO_2 injections (required to maintain 1000 ppmv), were required for the experimental samples than the control blanks, indicating that CO_2 consumption was at a greater rate for the $Na_2S_2O_3$ exposed soils. Determinations of CO_2 sequestration were made by extrapolating each decay curve prior to substrate injection (indicated by the sudden spike in CO_2 concentration) and determining the overall decay rate by compiling the data set of active fixation periods i.e. the collective decay rate over 48 hours.

The results showed that significant CO_2 incorporation was taking place after an initial lag phase (Fig. 3.4). Blank incubations (HP04 and HP05), where the Hampstead Park soil was treated the same as the labelled sample, but not exposed to the $Na_2S_2O_3$ electron donor, showed negative uptake rates throughout the incubation period. This indicates that soil respiration was the dominant activity for blank soils. Although, some small positive uptake values were determined for the blank soils during the incubation intervals (Fig. 3.4), the determined standard deviations from the background leak experiment of ± 14 ppmv (chapter II, Table 1.2), demonstrated natural fluctuations in the leak rate and therefore these low uptake rates can be treated with a large degree of caution. Extrapolation of the data from the electron donor exposed soils indicated that positive uptake rates were detected after a lag phase of approximately 16 ± 0.5 hours. The soils continued to sequester atmospheric CO_2 (concurrent to continuous soil respiration as indicated by HP04 and HP05 incubations) for the remainder of the incubation period with an average 60.8 ± 46.3 µg CO_2 30.0 g^{-1} dry soil (2018.1 ± 1530.7 µg CO_2 kg^{-1} dry soil) removed from the atmosphere by T_{48}. It has to be taken into consideration that this figure may be underestimated due to the ongoing sequestration of pore space CO_2 generated though possible mineralisation activities (Miltner *et al*. 2004; Miltner *et al*. 2005a) that are beyond the scope of the experimental design to measure.

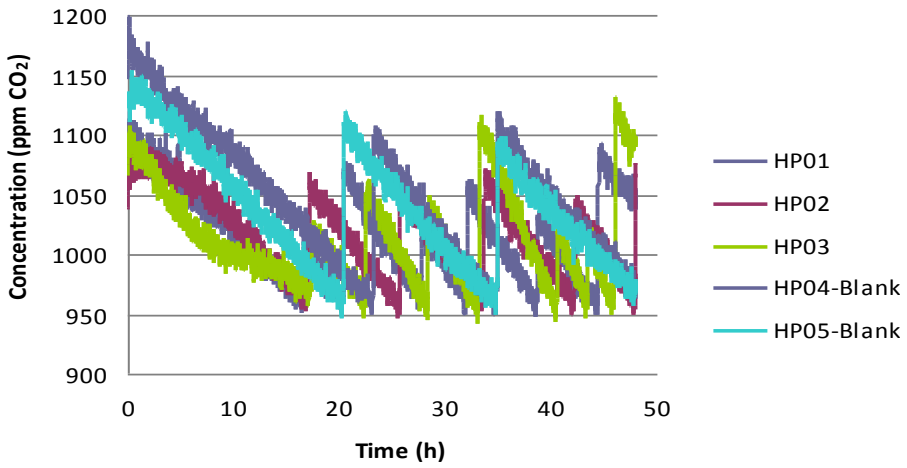
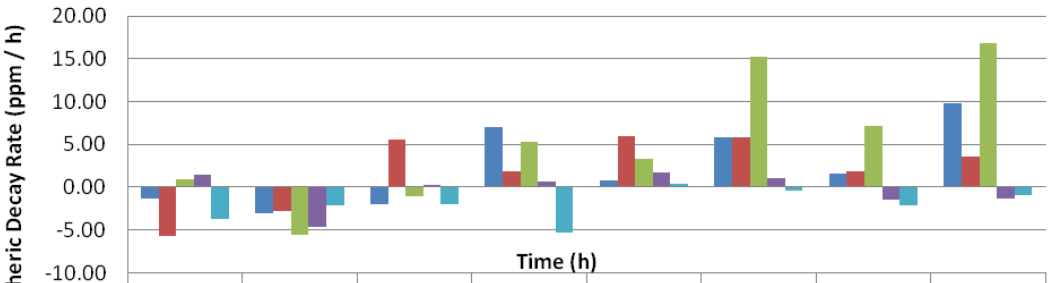


Fig. 3.3: Atmospheric CO_2 real-time data plots during each 48 hour incubation of x3 experimental replicates (HP01, HP02 and HP03) and x2 blanks (HP04 and HP05). Hampstead Park (HP) soil samples incubated within the ECIC under chemoautotrophic conditions for 48 hours. Data points taken every 30 seconds. The plots also show the automated injection of CO_2 back into the system once levels dropped below a 950 ppmv threshold.

It was most likely that CO_2 sequestration continued after T_{48}. To avoid potential cross-feeding amongst the microbiological population (and hence, trophic transfer of

^{13}C), the incubation was limited to 48 hours. This time frame was considered the minimum requirement for sufficient biomass labelling and subsequently, the successful accomplishment of the CsCl ultracentrifugation experiment. Fig. 3.4 clearly shows that the $Na_2S_2O_3$ exposed soils removed CO_2 from the atmosphere at a faster rate than the control blanks over the experimental time frame. It was obvious from the data plots that large variability existed between the experimental samples as HP01, HP02 and HP03 sequestered in total 61.4, 14.2 and 106.9 µg CO_2 30.0 g dry soil, respectively, over 48 hours. All the soils were from the same sampling event, horizon, and were prepared/performed in the same manner. Variability in the results may be due to the unpredictability of complex biological samples or mineral variations from the same horizon (Wirth, 2001), especially when such large inoculua are used. A draw back to the calculation method employed here was the requirement for the inclusion of a soil respiration variable. To estimate ongoing CO_2 evolution during the chemoautotrophic oxidation event in real-time, would be of great benefit to this research.



	0 - 6	6 - 12	12 - 18	18 - 24	24 - 30	30 - 36	36 - 42	42 - 48
HP01	-1.29	-2.99	-2.04	7.00	0.80	5.81	1.63	9.83
HP02	-5.68	-2.83	5.50	1.80	6.00	5.83	1.80	3.64
HP03	0.90	-5.52	-1.04	5.33	3.33	15.18	7.16	16.84
HP04	1.40	-4.59	0.32	0.68	1.66	1.09	-1.49	-1.34
HP05	-3.77	-2.16	-1.99	-5.25	0.35	-0.37	-2.16	-0.87

Fig. 3.4: Atmospheric CO_2 decay rate data for the Hampstead Park soil incubations under chemoautotrophic induced conditions. Negative values indicate soil respiration as the dominant condition for that time interval. HP01, HP02 and HP03 represent soils exposed to 20 mM $Na_2S_2O_3$, where as HP04 and HP05 are soil blanks containing no additional inorganic electron.

3.4.3 GCMS-IRMS Analysis of Extractable Fatty Acids and PLFAs

Organic matter was extracted from aliquots of incubated soil and analysed for lipids and specifically, for phospholipid fatty acids (PLFAs). The total soil extract (termed as 'total lipids' from herein) and isolated PLFAs were both analysed separately with quantification and $\delta\ ^{13}C$ enrichment measured at the commencement (T_0) and cessation (T_{48}) of the incubations. Also, two soils were exposed to $^{13}CO_2$ with no additions of $Na_2S_2O_3$.

The IRMS carbon isotope ratio chromatograms for both the incubations with no additions of chemical electron donor (Fig. 3.5A, B) showed that organic matter was not becoming enriched with ^{13}C during the prescribed incubation period. Two soils were exposed to similar incubation conditions with the only difference being the isotopic composition of the carbon substrate, CO_2. The calibration incubation contained 1000 ppmv $^{12}CO_2$ (98.87% purity assuming an arbitrary value of 1.13% ^{13}C natural abundance [Schwarzenbach *et al*. 2003]) and 20 mM $Na_2S_2O_3$, this has been termed the '$^{12}CO_2$ incubation' and was used to correct for natural abundance and also aided in lipid identification (due to the fragmentation pattern irregularities for ^{13}C enriched compounds). The second incubation contained an atmosphere of 1000 ppmv $^{13}CO_2$ to determine if the soil organic fraction increased in $\delta\ ^{13}C$. Spectra generated by GCMS allowed compound identification while simultaneous $^{13}C/^{12}C$ ratio analysis on each compound was also performed on the IRMS. Fig. 3.6 and 3.7 show the GCMS spectrums for Hampstead Park soil after 48 hour incubation under chemoautotrophic induced conditions. Peaks of interest are noted numerically. The numbers may be referenced with Tables 3.3 and 3.4 for identification.

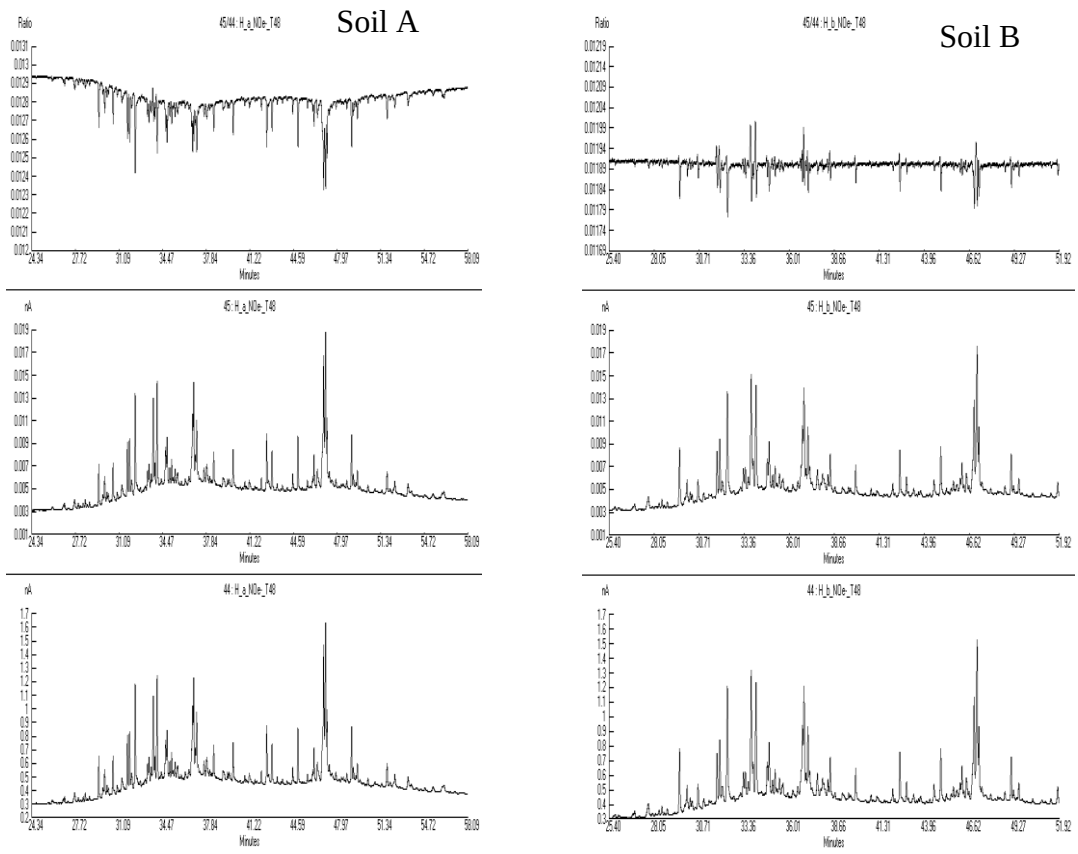


Fig. 3.5: IRMS $^{13}C/^{12}C$ ratio chromatograms for $^{13}CO_2$-blank incubations for Hampstead Park soils A and B. Top chromatogram displays the $^{13}C/^{12}C$ ratio; where downward peaks are predominantly ^{12}C molecules, upward peaks are predominantly ^{13}C molecules and peaks that are both above and below the baseline are naturally abundant ^{13}C molecules. Middle chromatogram displays the abundance of ^{13}C molecules. Bottom chromatogram displays the abundance of ^{12}C molecules. Soil was incubated at 30^{o}C for 48 hours under a 1000 ppmv $^{13}CO_2$ atmosphere but no additions of $Na_2S_2O_3$ were made. Top chromatograms show that no significant δ ^{13}C enriched peaks were present without corresponding (downward) ^{12}C peaks indicating no compounds abundant in ^{13}C isotope.

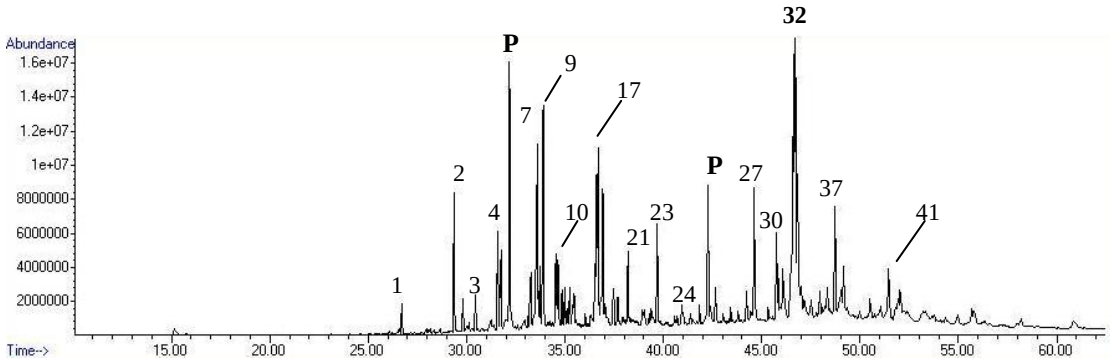


Fig 3.6: Total lipid chromatogram of Hampstead Park soil at T_{48} (Time = minutes). Peak identity was based on elution sequence, please see Table 3.3.

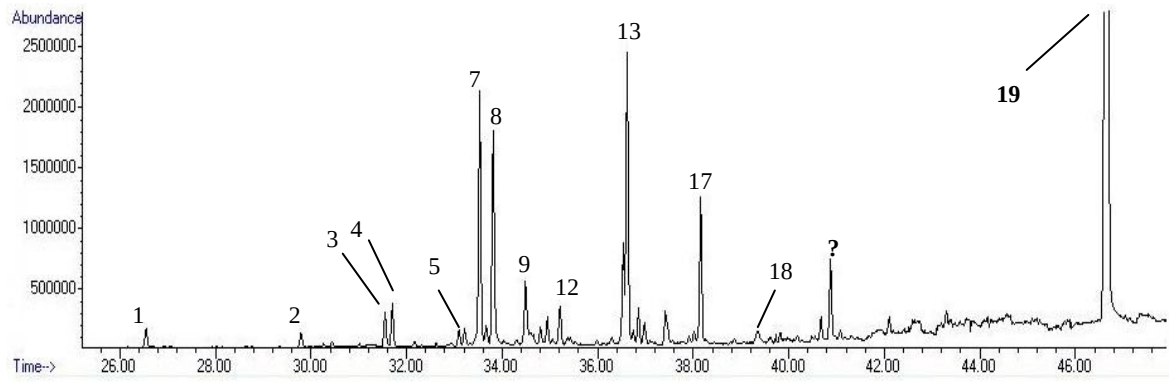


Fig 3.7: PLFAs in the polar lipid fraction (post-SPE) from the Hampstead Park soil lipid extract (Time = minutes). Peak identity was based on elution sequence, please see Table 3.4.

The total lipids extraction and derivitisation revealed that the abundant species observed were normal, branched, mono-, and diunsaturated fatty acids. Saturated fatty acids were the dominant lipid class which ranged from C_{12} to C_{32} and displayed strong even/odd predominance. Odd chained fatty acids were present but of lower abundance to even chained fatty acids. Long-chain fatty acids were present in the total lipid fractions ($\geq C_{20} \leq C_{32}$). Some 'iso' (*i*C_{15} and *i*C_{16}) and 'antiso' (*a*C_{14} and *a*C_{16}) branched fatty acids were observed. Five monounsaturated fatty acids were present, specifically; 16:1ω9, *i*16:1ω9, 16:1ω11, 18:1ω9 and 16:1ω11. A single diunsaturated fatty acid, Methyloctadecadienoate (18:2ω9,12) was identified. Two isotopically labelled Cyclopropanes were identified (*cy*16:0 and *cy*18:0). The neutral lipid, Hexamethyl tetracosahexaene (squalene) was present in the total lipids fraction and was found to be highly enriched in $\delta\ ^{13}C$ (1986.89‰).

The PLFAs SPE fraction revealed that the abundant species to be normal, branched and monounsaturated fatty acids. Saturated fatty acids ranged from C_{14} to C_{20} and displayed strong even/odd predominance. Iso (*i*15:0) and anteiso (*a*14:0 and *a*16:0) branched fatty acids were observed including 10Me-18:0. Monounsaturated fatty acids, 16:1ω9, 18:1ω9 and 18:1ω11 were present and found to be highly enriched in $\delta\ ^{13}C$. Cyclopropanes, *cy*16:0 and *cy*18:0 were present and also enriched in stable isotope. For shorthand names of identified species please see Tables 3.1 and 3.2 for total lipids and PLFAs respectively.

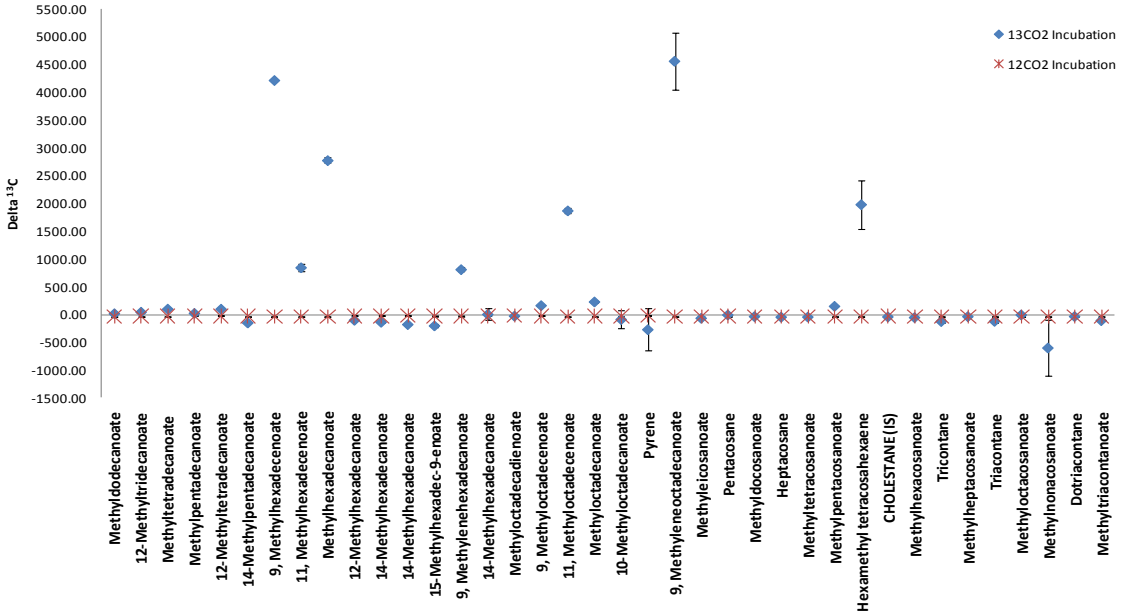


Fig. 3.8: Plot of δ ^{13}C values for total extracted lipids from Hampstead Park soil after 48 hour incubation under either $^{12}CO_2$ and $^{13}CO_2$ atmospheres.

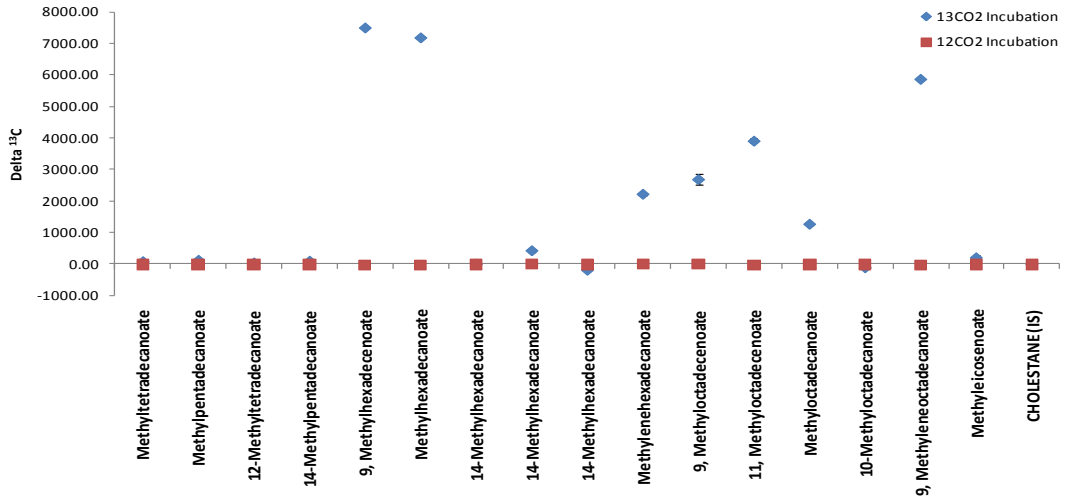


Fig 3.9: Plot of δ ^{13}C values for PLFAs separated from the total lipids using SPE. Hampstead Park soil after 48 hour incubation under either $^{12}CO_2$ and $^{13}CO_2$ atmospheres.

TOTAL LIPID EXTRACT			$^{13}CO_2$		$^{12}CO_2$	
Systematic Name	**Shorthand**	**AMU**	Average delta ^{13}C	st. dev.	Average delta ^{13}C	st. dev.
Methyldodecanoate	12:0	214	21.88	0.96	-32.13	1.30
12-Methyltridecanoate	*i* 13:0	242	49.14	1.67	-31.42	1.06
Methyltetradecanoate	14:0	242	104.46	1.61	-30.60	0.38
Methylpentadecanoate	15:0	256	28.25	0.55	-26.84	0.26
12-Methyltetradecanoate	*a* 14:0	256	103.17	2.65	-27.46	0.21
14-Methylpentadecanoate	*i* 15:0	270	-145.33	1.14	-29.04	0.15
9, Methylhexadecenoate	16:1ω9	268	4223.79	22.52	-34.54	0.07
11, Methylhexadecenoate	16:1ω11	268	850.05	61.89	-34.08	0.14
Methylhexadecanoate	16:0	270	2780.27	48.94	-32.97	0.22
12-Methylhexadecanoate	12Me-16:0	284	-96.71	3.35	-26.43	1.02
14-Methylhexadecanoate	*a* 16:0	284	-132.55	12.23	-26.85	1.47
14-Methylhexadecanoate	*a* 16:0	284	-173.28	9.75	-24.71	0.24
15-Methylhexadec-9-enoate	*i* 16:1ω9	282	-197.71	21.07	-29.36	1.35
9, Methylenehexadecanoate	*cy* 16:0	282	815.91	44.22	-28.32	1.32
14-Methylhexadecanoate	*a* 16:0	284	8.79	98.21	-27.77	0.28
Methyloctadecadienoate	18:2ω9,12	294	-21.50	11.93	-18.11	0.40
9, Methyloctadecenoate	18:1ω9	296	167.52	10.01	-26.36	0.11
11, Methyloctadecenoate	18:1ω11	296	1874.48	49.59	-36.26	0.04
Methyloctadecanoate	18:0	298	233.86	15.24	-30.60	0.57
10-Methyloctadecanoate	10Me-18:0	312	-82.63	162.14	-29.62	0.45
Pyrene	$C_{16}H_{10}$	202	-265.48	376.61	-22.69	0.86
9, Methyleneoctadecanoate	*cy* 18:0	310	4569.90	515.92	-32.27	0.18
Methyleicosanoate	20:0	326	-60.53	3.92	-33.87	0.54
Pentacosane	25:0	352	-1.38	10.81	-30.77	0.76
Methyldocosanoate	22:0	354	-29.59	0.28	-33.46	0.64
Heptacosane	27:0	380	-40.04	6.48	-33.69	0.82
Methyltetracosanoate	24:0	382	-38.36	5.90	-35.61	0.89
Methylpentacosanoate	25:0	396	154.57	3.90	-35.91	0.24
Hexamethyl tetracosahexaene	30:6	410	1986.89	437.77	-33.73	0.25
CHOLESTANE (IS)	**$C_{27}H_{48}$**	**372**	**-35.68**	**3.43**	**-28.19**	**0.31**
Methylhexacosanoate	26:0	410	-47.84	8.55	-38.40	0.28
Tricontane	23:0	422	-118.56	42.24	-38.56	4.28
Methylheptacosanoate	27:0	424	-30.62	0.47	-36.04	0.33
Triacontane	30:0	464	-28.53	35.75	-34.93	2.30
Methyloctacosanoate	28:0	438	0.22	1.82	-38.48	2.84
Methylnonacosanoate	29:0	452	-595.87	505.56	-37.16	2.02
Dotriacontane	32:0	450	-0.55	0.08	-0.55	0.42
Methyltriacontanoate	30:0	466	-102.51	16.05	-37.57	0.70

Table 3.1: Total lipid extract showing δ ^{13}C values for Hampstead Park soil after 48 hour incubation. Cholestane was the internal standard (IS). Compounds displayed according to eluted retention time.

The δ ^{13}C values of total lipids and PLFAs are presented in Tables 3.1 and 3.2 to demonstrate the increase of isotopic enrichment of the $^{13}CO_2$ incubation in comparison to the $^{12}CO_2$ incubation for Hampstead Park soil for some compounds. Increasing isotopic enrichment of the total lipid and PLFA fractions was attributed to the autotrophic substrate source, atmospheric CO_2, being sequestered into the soil horizon and converted into biomass over the course of the two time points.

SPE - PLFAs			$^{13}CO_2$		$^{12}CO_2$	
Systematic Name	**Shorthand**	**AMU**	Average delta ^{13}C	st. dev.	Average delta ^{13}C	st. dev.
Methyltetradecanoate	14:0	242	70.27	53.15	-26.57	3.69
Methylpentadecanoate	15:0	256	114.03	20.79	-27.99	2.31
12-Methyltetradecanoate	*a* 14:0	256	28.95	64.02	-25.04	1.04
14-Methylpentadecanoate	*i* 15:0	270	84.77	23.41	-29.43	5.11
9, Methylhexadecenoate	16:1ω9	268	7503.66	14.27	-38.80	0.38
Methylhexadecanoate	16:0	270	7188.70	11.46	-35.30	0.84
14-Methylhexadecanoate	*a* 16:0	284	-8.76	11.31	-25.02	1.20
14-Methylhexadecanoate	*a* 16:0	284	418.57	48.95	-18.20	12.67
14-Methylhexadecanoate	*a* 16:0	284	-200.92	19.57	-24.22	2.84
Methylenehexadecanoate	*cy* 16:0	282	2215.37	25.46	-19.33	4.26
9, Methyloctadecenoate	18:1ω9	296	2681.35	183.16	-19.51	1.50
11, Methyloctadecenoate	18:1ω11	296	3903.11	67.03	-35.35	0.31
Methyloctadecanoate	18:0	298	1261.51	32.58	-26.14	3.39
10-Methyloctadecanoate	10Me-18:0	312	-123.34	9.00	-30.94	1.99
9, Methyleneoctadecanoate	*cy* 18:0	310	5869.75	16.41	-32.92	3.06
Methyleicosenoate	20:0	324	194.36	67.15	-27.33	8.41
CHOLESTANE (IS)	**$C_{27}H_{48}$**	**372**	**-23.01**	**2.74**	**-23.91**	**0.63**

Table 3.2: PLFA SPE fraction showing δ ^{13}C values isolated from the total lipid extracts of Hampstead Park soils incubated for 48 hours. Compounds displayed according to eluted retention time.

The δ ^{13}C values observed for the two incubations with and without enriched $^{13}CO_2$ reveal several lipids and PLFAs increased in δ ^{13}C abundance from T_0 to T_{48}. Not all compounds increased in isotopic abundance indicating that they were not directly biosynthesised from the CO_2 substrate source. For instance, 14-methylpentadecanoate extracted from the total lipids was considerably depleted although the quantity of this compound (Table 3.3) indicates that it was physically abundant in the sample at both time points. This was not true for the same fatty acid in the PLFA fraction which increased in δ ^{13}C abundance between sampling points. Common fatty acids such as 14-methylhexadecanoate and 10-methyloctadecanoate from the PLFA fraction (Table 3.4) were also depleted in δ ^{13}C at the cessation of the experiment. This was an interesting occurrence as this data could suggest the presence of another taxonomic group(s), but no discernable cross-feeding or participation in autotrophy can be directly confirmed using these techniques, only inferred. The total lipids and PLFA results, demonstrate that δ ^{13}C values for the $^{13}CO_2$ incubations increased significantly over the 48 hour incubation period (but not for all lipids and PLFAs), whereas the corresponding $^{12}CO_2$ incubations remained consistently depleted.

#	Hampstead Park Total Lipids - T_0	ug/g dry soil		Hampstead Park Total Lipids - T_{48}	ug/g dry soil		Difference
		Average	st. dev.		Average	st. dev.	%
1	Methyldodecanoate	33.14	0.87	Methyldodecanoate	29.30	0.28	-11.56
2	12-Methyltridecanoate	35.70	1.03	12-Methyltridecanoate	29.52	0.44	-17.31
3	Methyltetradecanoate	37.70	1.57	Methyltetradecanoate	31.33	0.33	-16.89
4	Methylpentadecanoate	72.02	2.48	Methylpentadecanoate	59.58	1.77	-17.27
5	12-Methyltetradecanoate	61.24	2.25	12-Methyltetradecanoate	49.26	1.73	-19.55
6	14-Methylpentadecanoate	46.87	2.04	14-Methylpentadecanoate	39.76	1.32	-15.16
7	9, Methylhexadecenoate	120.83	8.77	9, Methylhexadecenoate	107.00	2.38	-11.45
8	11, Methylhexadecenoate	45.96	3.01	11, Methylhexadecenoate	37.81	1.44	-17.73
9	Methylhexadecanoate	146.98	3.67	Methylhexadecanoate	128.55	2.07	-12.54
10	12-Methylhexadecanoate	64.55	2.98	12-Methylhexadecanoate	53.56	2.56	-17.03
11	14-Methylhexadecanoate	35.24	1.16	14-Methylhexadecanoate	31.27	0.95	-11.28
12	14-Methylhexadecanoate	36.16	0.63	14-Methylhexadecanoate	30.88	1.09	-14.58
13	15-Methylhexadec-9-enoate	26.28	0.96	15-Methylhexadec-9-enoate	23.14	1.14	-11.93
14	9, Methylenehexadecanoate	38.40	1.82	9, Methylenehexadecanoate	33.31	1.52	-13.25
15	14-Methylhexadecanoate	32.44	1.31	14-Methylhexadecanoate	29.29	1.45	-9.72
16	Methyloctadecadienoate	50.33	2.27	Methyloctadecadienoate	46.28	0.22	-8.05
17	9, Methyloctadecenoate	103.39	4.65	9, Methyloctadecenoate	87.02	2.32	-15.83
18	11, Methyloctadecenoate	100.08	4.50	11, Methyloctadecenoate	84.07	1.74	-16.00
19	Methyloctadecanoate	93.82	4.73	Methyloctadecanoate	69.94	3.30	-25.45
20	10-Methyloctadecanoate	38.15	1.51	10-Methyloctadecanoate	31.93	2.44	-16.30
21	Pyrene	33.79	1.99	Pyrene	31.96	0.66	-5.42
22	9, Methyleneoctadecanoate	45.98	2.03	9, Methyleneoctadecanoate	43.60	1.73	-5.19
23	Methyleicosanoate	67.17	2.37	Methyleicosanoate	54.60	1.08	-18.72
24	Pentacosane	26.21	0.40	Pentacosane	23.43	0.85	-10.59
25	Methyldocosanoate	90.28	2.96	Methyldocosanoate	72.10	1.56	-20.14
26	Heptacosane	30.58	1.84	Heptacosane	27.66	0.39	-9.55
27	Methyltetracosanoate	83.40	1.29	Methyltetracosanoate	68.82	1.04	-17.48
28	Methylpentacosanoate	64.38	1.59	Methylpentacosanoate	58.05	1.41	-9.84
29	Hexamethyl tetracosahexaene	41.19	2.26	Hexamethyl tetracosahexaene	41.58	2.74	0.94
30	Methylhexacosanoate	102.58	4.30	Methylhexacosanoate	80.57	2.47	-21.46
31	Tricontane	29.16	2.12	Tricontane	24.91	1.85	-14.56
32	Methylheptacosanoate	36.37	2.35	Methylheptacosanoate	33.01	2.73	-9.25
33	Triacontane	48.85	2.44	Triacontane	43.09	2.11	-11.79
34	Methyloctacosanoate	44.23	3.92	Methyloctacosanoate	35.41	1.61	-19.96
35	Methylnonacosanoate	33.79	2.49	Methylnonacosanoate	30.51	1.67	-9.71
36	Dotriacontane	80.93	3.91	Dotriacontane	73.85	4.07	-8.75
37	Methyltriacontanoate	35.13	3.22	Methyltriacontanoate	30.61	2.63	-12.89

Table 3.3: Total lipids quantification data showing the mass of total lipids and % difference between sampling points.

Quantification of the total lipids and PLFAs was carried out at T_0 and T_{48} and the average data from both $^{12}CO_2$ and $^{13}CO_2$ incubations (triplicates) are presented for convenience (Tables 3.3 and 3.4). The concentration of a particular molecule from the total lipids would be expected to be higher than the same molecule from the PLFAs fraction as not all fatty acids belong to PLFAs in environmental samples. This was reflected in the soils indicating that a range of fatty acid sources were present. When coupled with $\delta\ ^{13}C$ values, this shows that PLFAs were not the only fatty acid containing compounds being produced as a direct result of CO_2 sequestration. Despite this, the quantification of total lipids and PLFAs has shown that the concentration of nearly each fatty acid derivative decreases despite increasing $\delta\ ^{13}C$ values over the 48 hours incubation (Figs. 3.10 and 3.11). Exceptions, were compounds from the PLFA fraction, where the following increased in mass during the incubation; 9, methylhexadecenoate (+ 27.27%), methylhexadecanoate (+ 17.68%), 11,

methyloctadecenoate (+ 18.74%) and 9, 10-methyleneoctadecanoic acid (+ 11.35%) and could be indicated as biomarkers for $S_2O_3^{2-}$ soil oxidisers.

#	Hampstead Park PLFA's - T_0	ug/g dry soil Average	st. dev.	Hampstead Park PLFA's - T_{48}	ug/g dry soil Average	st. dev.	Difference %
1	Methyltetradecanoate	18.81	0.08	Methyltetradecanoate	17.33	0.04	-7.87
2	Methylpentadecanoate	20.70	0.17	Methylpentadecanoate	19.19	0.08	-7.30
3	12-Methyltetradecanoate	21.41	0.16	12-Methyltetradecanoate	19.56	0.06	-8.65
4	14-Methylpentadecanoate	20.23	0.13	14-Methylpentadecanoate	17.85	0.04	-11.76
5	9, Methylhexadecenoate	28.42	0.48	9, Methylhexadecenoate	36.17	0.25	27.27
6	Methylhexadecanoate	18.75	0.07	Methylhexadecanoate	17.24	0.08	-8.03
7	14-Methylhexadecanoate	21.23	0.15	14-Methylhexadecanoate	20.73	0.19	-2.36
8	14-Methylhexadecanoate	18.62	0.08	14-Methylhexadecanoate	17.17	0.03	-7.81
9	14-Methylhexadecanoate	20.06	0.11	14-Methylhexadecanoate	17.97	0.05	-10.42
10	Methylenehexadecanoate	19.79	0.10	Methylenehexadecanoate	18.77	0.11	-5.15
11	9, Methyloctadecenoate	22.09	0.23	9, Methyloctadecenoate	22.25	0.05	0.71
12	11, Methyloctadecenoate	28.04	0.35	11, Methyloctadecenoate	33.30	0.34	18.74
13	Methyloctadecanoate	19.96	0.16	Methyloctadecanoate	18.52	0.05	-7.22
14	10-Methyloctadecanoate	21.09	0.23	10-Methyloctadecanoate	20.47	0.07	-2.97
15	9, Methyleneoctadecanoate	21.92	0.09	9, Methyleneoctadecanoate	24.40	0.12	11.35
16	Methyleicosenoate	19.16	0.10	Methyleicosenoate	17.39	0.09	-9.23

3.4: PLFA quantification data showing the mass and % difference between sampling points.

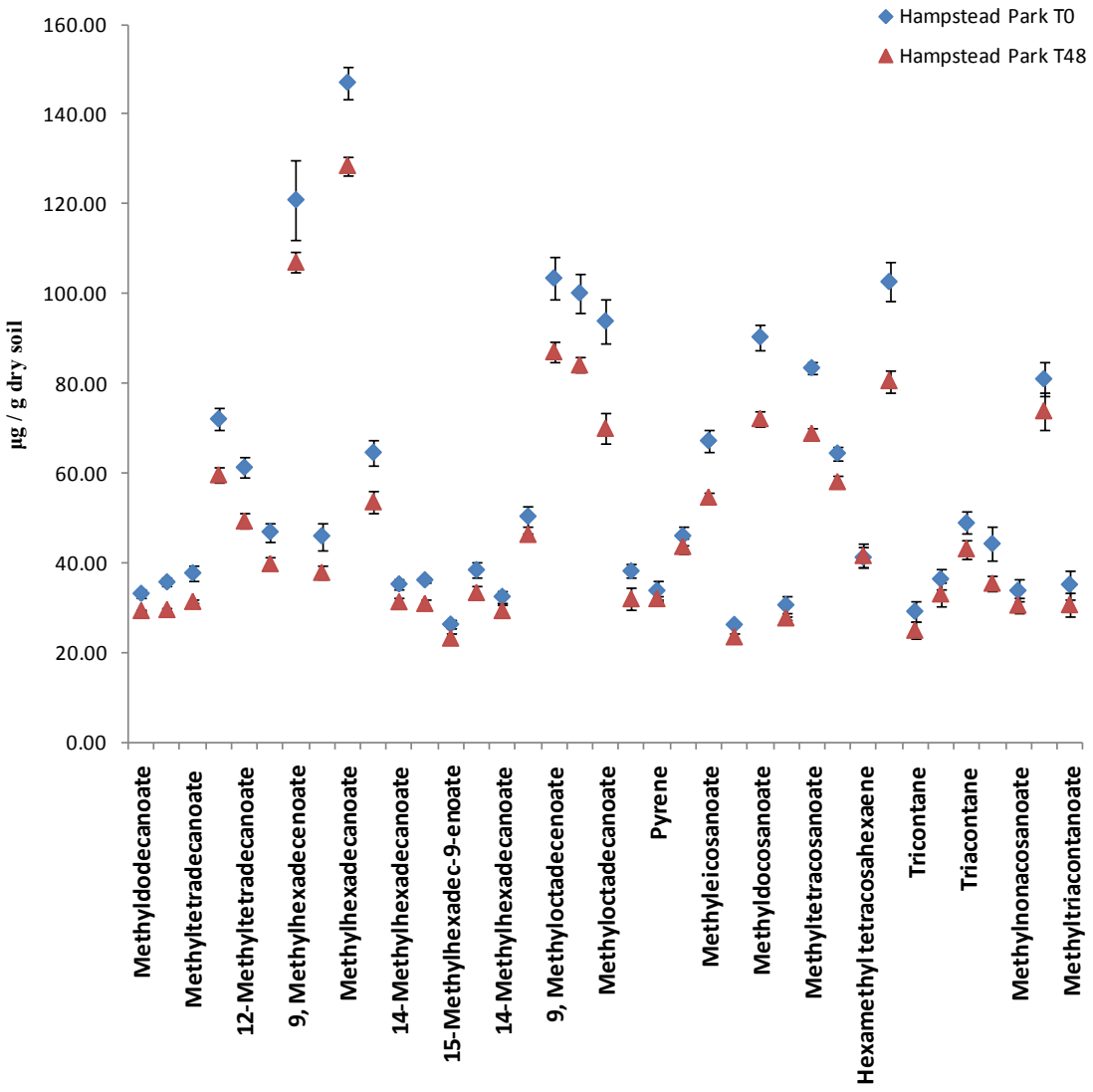


Fig. 3.10: Plot showing the average mass of total lipids extracted from Hampstead Park between time points T_0 and T_{48}, and quantitatively determined using GCMS.

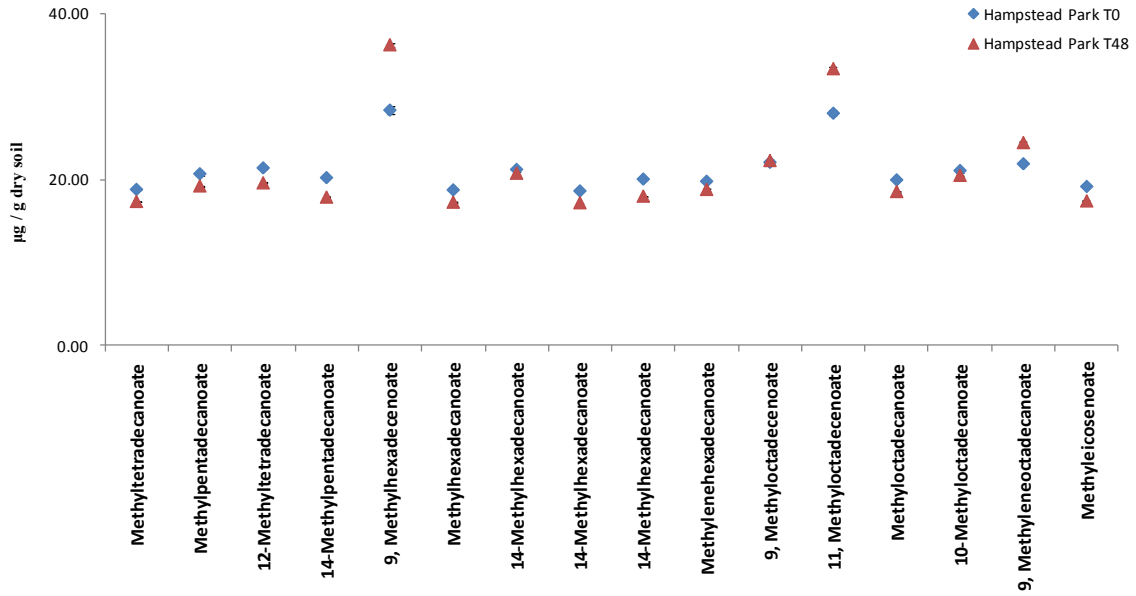


Fig. 3.11: Plot showing the average mass of PLFAs extracted from Hampstead Park between time points T_0 and T_{48}, and quantitatively determined using GCMS.

3.4.4 NMR

A quantitative comparison of the ^{13}C CP-MAS spectra before and after the addition of electron donor, indicated that the total carbon signal in the Hampstead Park soil increased by 10.8%. 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 hour period. The NMR data can be used to identify how the label was incorporated into the different chemical categories in the soil organic matter. Due to the relatively broad lineshape of the solids NMR spectra identification was best done using ^{1}H-^{13}C HR-MAS-NMR which can observe H-C units in liquid, gel, and swollen-solid phases, but not true "glassy" solid domains (Kelleher & Simpson, 2006). The CP-MAS difference spectrum highlights the components in the SOM that increase with labelling. While accurate quantification of each chemical sub-category was not possible due to spectral overlap, general assignments based on knowledge from the HR-MAS-NMR and previous works (see Simpson *et al*. (2010) and references therein), are possible (see Fig. 3.12) all of which are consistent with the microbial signature increasing within the soil organic matter pool with ^{13}C labelling.

Fig. 3.13 shows the heteronuclear singular quantum coherence (HSQC) difference spectrum between the labelled and non-labelled samples. Multidimensional NMR of SOM including detailed assignments of the microbial fraction has been

considered in detail in previous publications (Kelleher *et al*. 2006; Kelleher & Simpson, 2006; Simpson 2002; Simpson *et al*. 2003; Simpson *et al*. 2007a; Simpson *et al*. 2007b; Simpson *et al*. 2010). The only signals present in the difference spectrum are from chemical categories that increased during the ^{13}C labelling incubation. It was clear that the spectra are dominated by lipids, carbohydrates and protein/peptide 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 of lipids (100% signal increase), carbohydrate (33% signal increase), protein (30% increase) and lignin (no change, see red circle on Fig. 3.13). 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 synthesised for growth, and carbohydrate likely being made for both purposes. As expected the lignin component in soil did not change, considering this was exclusively synthesised as a structural biopolymer in plants.

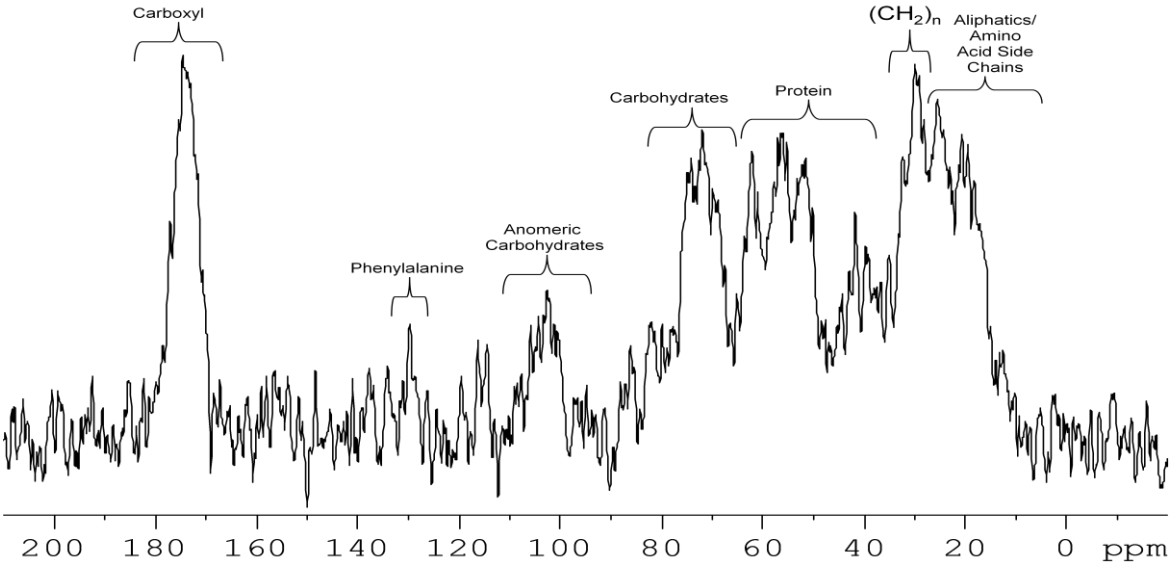


Fig. 3.12: Solid phase ^{13}C CP-MAS-NMR difference spectrum of Hampstead Park soil after 48 hour incubation under $^{13}CO_2$ conditions. The spectrum has been blank corrected by subtraction of the concurrent ^{13}C CP-MAS of the $^{12}CO_2$ incubation. Spectrum shows the presence of biological components that, after $^{12}CO_2$ blank correction, demonstrate the presence of labelled biomaterial.

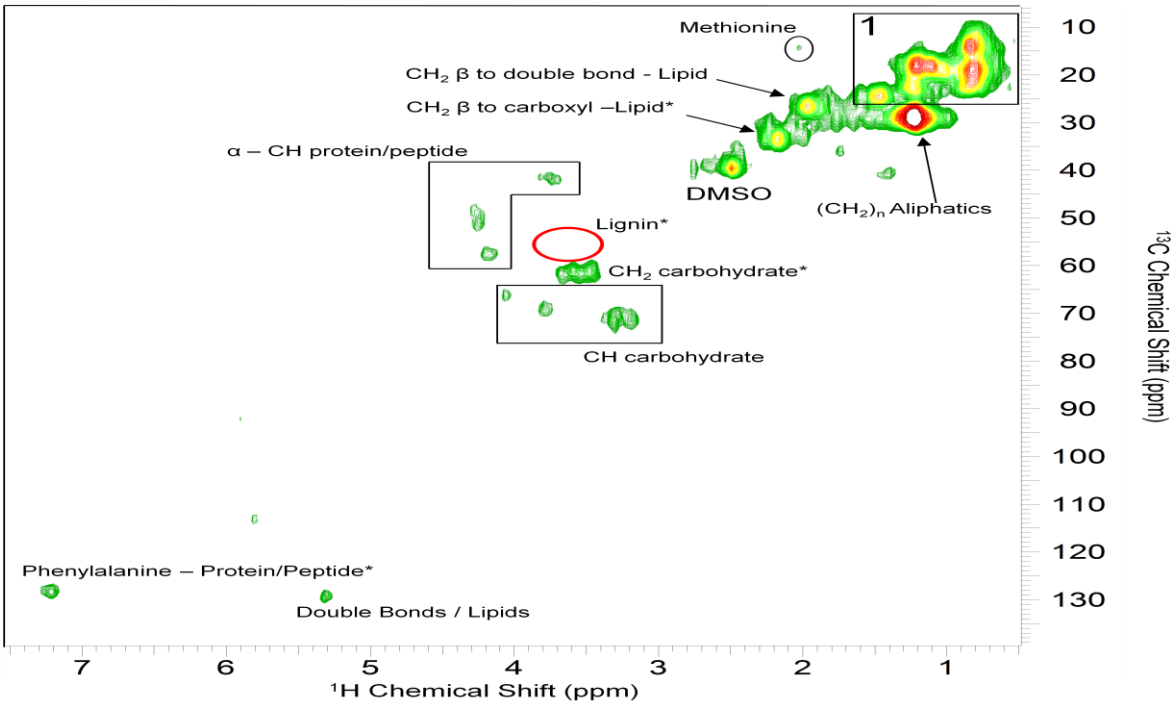


Fig. 3.13: HSQC ^{1}H-^{13}C HR-MAS-NMR spectrum showing Hampstead Park soil after 48 hour incubation under chemoautotrophic conditions and exposed to $^{13}CO_2$. The spectrum has been blank corrected by subtraction of the concurrent HSQC of the $^{12}CO_2$ incubation. The spectra were dominated by lipids, carbohydrates and protein/peptide arising from microbial biomass.

3.4.5 Analysis of the DNA-SIP Fractions post-Ultracentrifugation

The analysis of isotopically labelled nucleic acids was difficult because of mixture with the more abundant ^{12}C isotope. Techniques such as isopycnic CsCl gradient ultracentrifugation (from this point onwards designated as DNA-SIP) are used to separate the heavier ^{13}C-DNA from the ^{12}C-DNA. The control study has demonstrated the applicability of the technique to observe the formation of DNA bands and to ensure the technique was in control and successful (Fig. 3.14).

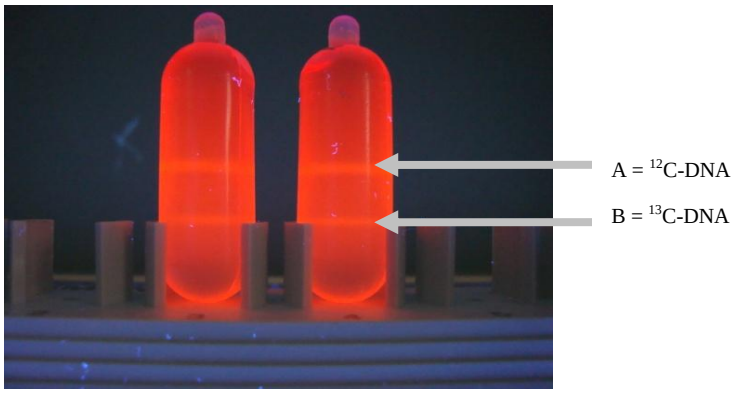


Fig. 3.14: Ultracentrifuge tubes containing CsCl solution and both isotopically labelled and unlabelled *E. coli* LMG 194 DNA (control experiment). The image shows DNA undergoing fluorescence while in the

presence of EtBr and UV radiation. The presence of two distinct DNA bands (marked by arrows A and B) show the degree of separation ^{13}C-labelled DNA will undergo once subjected to long-term ultracentrifugation (16 hours and 76, 000 RPM).

DNA-SIP was used after the soil was exposed to 20 mM $Na_2S_2O_3$ and $^{13}CO_2$, to assess the putative chemoautotrophic bacterial population. DNA-SIP is a labour intensive procedure with down-stream DNA-based applications that take considerable periods of time to accomplish and therefore DNA-SIP was carried out only on HP01, rather than all three viable soils. This is standard practice for RNA- and DNA-SIP experiments (Prof. Andrew Whitely, Centre for Ecology and Hydrology; personal communication). After 48 hour incubation the total DNA was extracted (23.7 and 20.3 µg DNA 500 mg^{-1} wet soil respectively for $^{12}CO_2$ and $^{13}CO_2$ treatments) and 5 µg DNA was subjected to isopycnic ultracentrifugation using a CsCl gradient. Post-ultracentrifugation, the 3.9 ml tube was fractionated into 300 µl fractions (x12 fractions per 3.7 ml tube). Once the DNA from each 300 µl fraction had been precipitated and purified, it was PCR amplified and UV visualised on an agarose gel (Fig. 3.15).

The purified DNA from the two incubations has shown clear differences in their DNA profile. The extracted DNA for the $^{12}CO_2$ incubation (Fig. 3.15: row A) was clustered around fractions 7, 8 and 9. The DNA from the $^{13}CO_2$ incubation (Fig. 3.15: row B) has spread between a wider range, e.g. fractions 5, 6, 7, 8 and 9. Assuming that the DNA from Fig. 3.15 row A was composed of predominantly ^{12}C and acts as the proxy, then only fractions (represented in Fig. 3.15 [B]) 5 and 6 would be considered ‘labelled’ with ^{13}C. Fraction 7 from the $^{13}CO_2$ incubation may however also contain a homogenous mixture of both ^{12}C- and ^{13}C-labelled DNA.

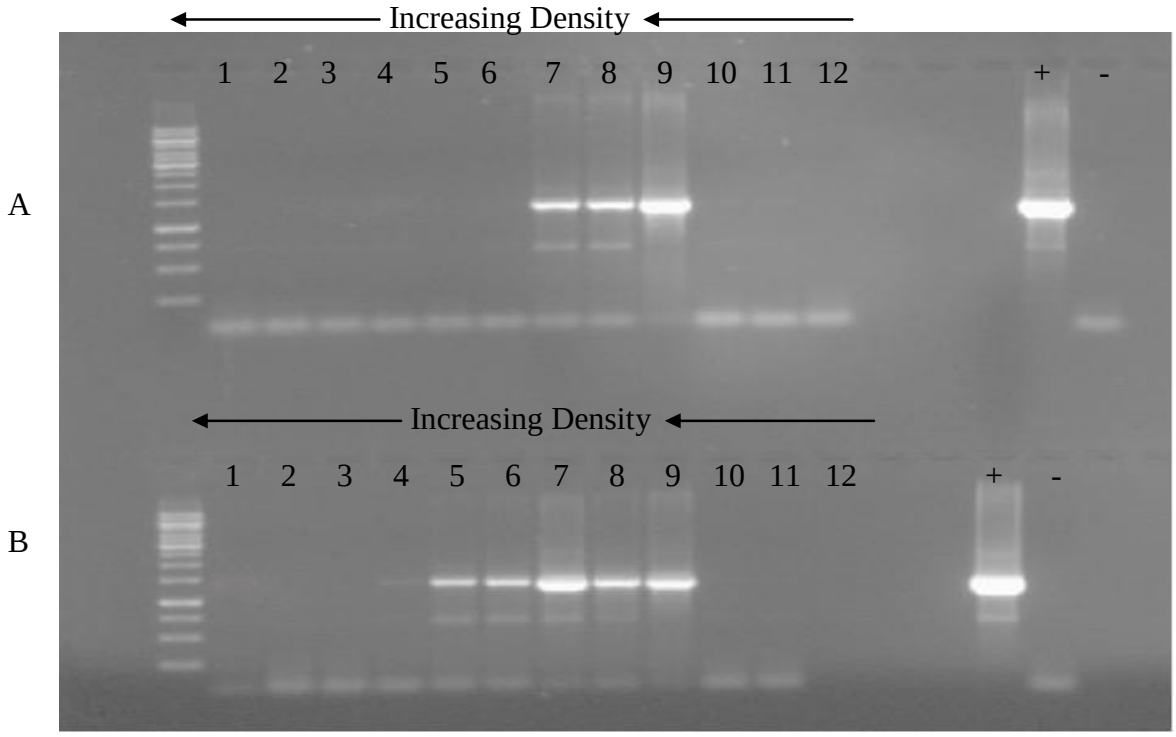


Fig. 3.15. Gel electrophoresis analysis of 16s rRNA PCR amplified extracted Hampstead Park soil DNA after 48 hours incubation under chemoautotrophic conditions. DNA was separated according to its isopycnic correlation to CsCl gradient induced under ultracentrifugation. Positive (+) and negative (-) controls were performed using *Pseudomonas putida* G7 PCR product and ultra-pure autoclaved water. A) Hampstead Park soil exposed to 99% $^{12}CO_2$ for 48 hours, run against a 1 kb ladder. B) Hampstead Park soil exposed to 99% $^{13}CO_2$ for 48 hours, run against a 1 kb ladder. The observable presence of DNA in fractions 5 and 6 for the $^{13}CO_2$ gradient fractions provides first step evidence of higher density DNA penetration and indicates ^{13}C incorporation into genomic DNA.

DGGE analysis of the $^{13}CO_2$ incubated DNA indicated low diversity amongst the fractions (Fig. 3.16). The 16S rRNA amplified DNA taken from fractions 5-9 showed similar homology to one another (with the exception of fraction 4 where no PCR product was present) but interestingly the unamplified total ^{12}C- and ^{13}C-DNA taken from the pre-ultracentrifuged soil extracts contained two bands not prevalent in the other PCR amplified lanes (fractions 5-9). The relevance of these bands remains unknown as due to time and budget constraints, the majority of work was carried out on the post-ultracentrifuged samples rather than the soil DNA extract. However, the total DNA samples (pre-ultracentrifugation) were cloned into recombinant *E. coli* to assess the presence of viable DNA fragments for downstream 16S rRNA amplification of the ultracentrifuged DNA (see Table 3.5). Table 3.5 demonstrates both the presence of viable DNA fragments after the DNA extraction procedure, but also the presence of known soil chemoautotrophic species such as *Thiobacillus denitrificans*. The

significance of their presence in the soil sample at T_{48} was obvious considering the nature of the incubation they were retrieved from, but it was vital the same *Thiobacillus*-like sequences are located in the denser fractions of the CsCl tube post-ultracentrifugation.

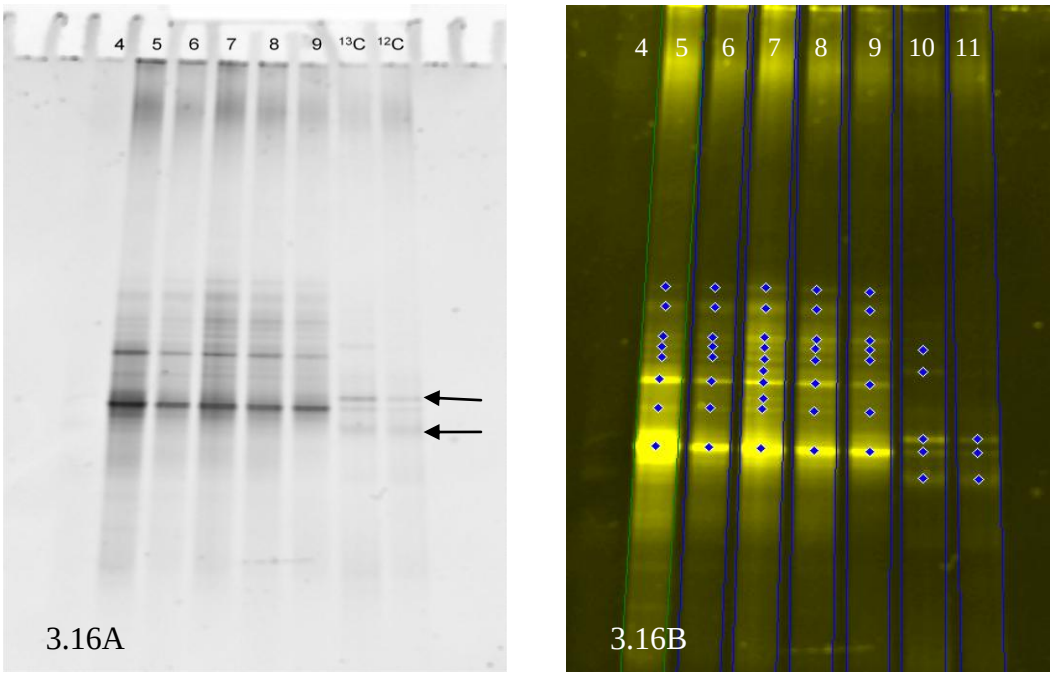


Fig. 3.16: DGGE images of Hampstead Park soil DNA after 48 hours exposure to $^{13}CO_2$ under chemoautotrophic conditions. Lanes 4-9 consist of 16s rRNA amplified DNA taken from corresponding fractions of the CsCl gradient (ultracentrifugation procedure). Lanes ^{13}C and ^{12}C are the unamplified DNA extracted directly from the soil after the incubation term ended (pre-ultracentrifugation). Arrows in Fig. 3.16A indicate DNA bands in the unamplified total DNA not observed in the fractionated PCR Amplicons. Fig. 3.16B has been modified to show the band distinctions more clearly and ♦ indicates location of bands.

Once the image had been digitised using Phoretix 1D software, a dendrogram could be created to indicate the similarity between lanes (Fig. 3.17). The dendrogram shows the relationship between each fraction. Fractions 5, 6 and 7 show close homology and fractions 8 and 9 are also closely related to one another. These results are consistent with an assumption that different classes of organisms would be located at different points along the CsCl tube post-ultracentrifugation. The close homology of the Total-DNA samples (^{12}C and ^{13}C) was also reassuring as the original soil samples were incubated under the same conditions and therefore bacterial biodiversity between them would not be expected.

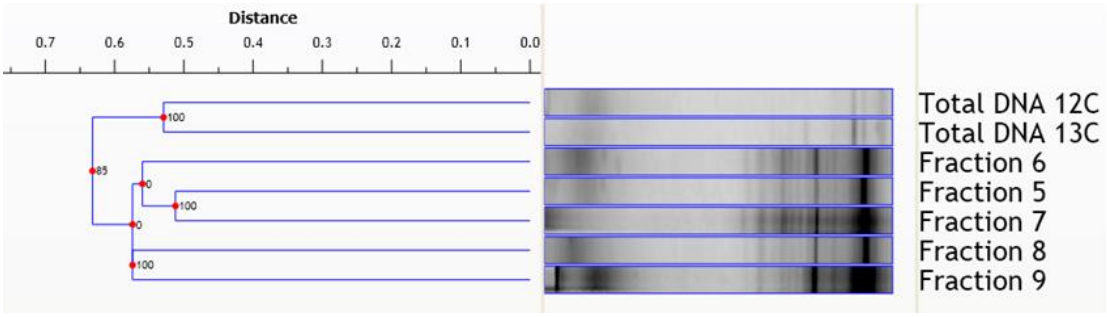


Fig. 3.17: DGGE dendrogram; Single Linkage (Jeffery's X). The digitised version of the DGGE image shows the similarity between the fractions. The accompanying tree shows % relatedness with 100 indicating the lanes are most similar (but not necessarily identical). Distance ruler indicates the euclidean distance.

Based upon the differing buoyant densities of the extracted DNA, it was feasible that deeper penetration into the salt gradient indicates isopycnic correlation. Thus, bacterial species that autotrophically assimilate $^{13}CO_2$ and incorporate the stable isotope into their DNA would be located in the denser regions of the gradient (< fraction 7) to those species that do not assimilate the abiotic carbon source (Radajewski *et al*. 2000; Neufeld *et al*. 2007). This was observed for the Hampstead Park soil incubation after DNA-SIP was successfully carried out and demonstrated with UV visualisation (Fig. 3.15).

The DNA from the total ^{13}C-DNA extract (pre-ultracentrifugation) and fractions 5, 6, 7, 8 and 9 of the $^{13}CO_2$ incubation was cloned into recombinant *E. coli* cells. The data was used to gain insight into the bacterial ecology of the enrichment sample. The PCR amplified plasmid DNA from selected *E. coli* colonies (of the correct fragment size and alignment) was sequenced to provide an overview of each fraction (Table 3.6). No viable DNA was found in fractions 1-4 and 10-12. These fractions represent zones where the DNA presence was extremely low to zero or the DNA was lost during the salt removal and recovery stage of the DNA-SIP protocol.

The identification of chemoautotrophic species by comparison to the BLAST database is a standard method in ecological microbiology. It was necessary to look at all the retrieved sequences and group them according to CsCl fraction to ensure they cluster together. Their homology was compared statistically to produce a phylogenetic tree (Fig. 3.18). It was thus prudent to first discuss the relevant clusters of organisms depending on their location in the CsCl gradient to assess their significance. The

identification of known chemoautotrophic bacteria and their location within the CsCl gradient has been displayed in Table 3.7. Table 3.7 and the accompanying diagram clearly show that the majority of chemoautotrophs were located within fraction 7, but also single sequences were located in fractions 5 and 6. The closest sequence matches for chemoautotrophic bacteria were all from the *Thiobacillus* genera, specifically *Thiobacillus denitrificans*. This particular organism was interesting as not only is it one of the first bacteria to be linked to sulphur-based chemoautotrophy (Beijerinck, 1904), but unlike others of the *Thiobacillus* genera who perform aerobic respiration, *T. denitrificans* can use nitrate as the electron acceptor under anaerobic conditions if necessary (facultative anaerobe; Smith & Strohl, 1991). A single *T. denitrificans* strain was also observed in fraction 9 indicating this particular isolate was not potentially ^{13}C-DNA labelled. A single chemoautotrophic isolate was not a surprising find as the chamber atmosphere was not exclusively $^{13}CO_2$ at T_0 (approximately 200 ppmv $^{12}CO_2$) and soil respiration processes during the incubation period would have contributed to the $^{12}CO_2$ substrate pool (Trumbore, 2000; Flechard *et al*. 2007). The largest percentage of closest matches to known chemoautotrophic sequences was found in fraction 7 (16.7% of the total number of submitted sequences).

Of the 24 complete 16S rRNA sequences (combined forward and reverse 16S rRNA sequences) retrieved from the DNA-SIP cloning experiment, the dominant sequence related to *Rhodanobacter lindaniclasticus* (33.3%). *R. lindaniclasticus* has been described as a Gram-negative, aerobic, chemo-organotroph and attains its maximum growth rate at 30^{o}C (Nalin *et al*. 1999). It was one of the very few known organisms capable of degrading gamma-hexachlorocyclohexane (lindane; Nalin *et al*. 1999) but is also known for the rapid removal of benzo[α]pyrene (Kanaly *et al*. 2002) and the denitrification of acidic soils (van den Heuvel *et al*. 2010). *R. lindaniclasticus* is an important soil microbe in bioremediation and relatively new to science since its classification in 1999 (Nalin *et al*. 1999).

Fig. 3.18 shows the phylogenetic relationships between all the retrieved sequences from the DNA-SIP experiment and clearly shows that the organism-matches made from the BLAST search database cluster together. The numbers placed next to each leaf of the phylogenetic tree represents the bootstrap value i.e. the confidence limits. For example, a value of 100 between sequences *Thiobacillus denitrificans* ATCC25259 (Fraction 6) and *Thiobacillus denitrificans* ATCC25259 (Fraction 7)(3)

means that they were directly related in 100% of bootstrap replications (1000 bootstrap replications per analysis were used). Please see Semple & Steel (2003) for a comprehensive discussion of phylogenetics and Felsenstein (1985) for the importance of bootstrap analysis. The *Thiobacillus*-like sequences have been placed in what is called a monophyletic clade (group containing the most common ancestor of the given set of taxa and all the descendents of that common ancestor [Holmes, 2003]) in 100% of the bootstrap replications. The phylogenetic tree contains no outlier sequences with the exception of species that have no genetic relatives to cluster towards (e.g. *Adhaeribacter terreus* strain DNG6 [Fraction 6]). This initial statistically-based analysis, indicates that organisms with similar BLAST search matches are indeed phylogenetically related to one-another.

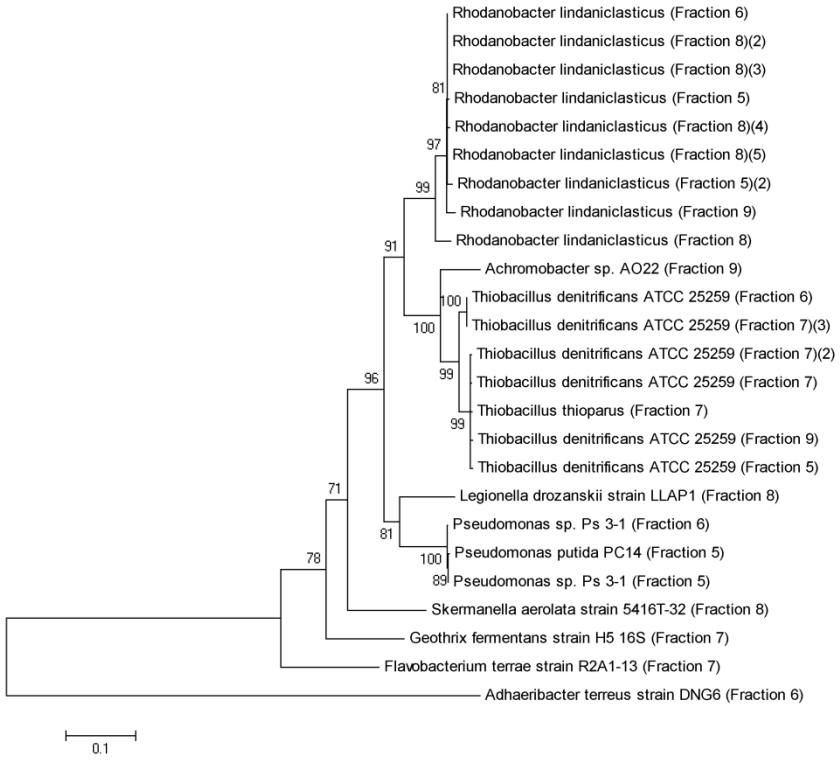


Fig. 3.18: Molecular Phylogenetic analysis by Maximum Likelihood method (1000 Bootstrap replications). This represents the phylogenetic similarity of the total number of sequences determined after fractionation of the CsCl isopycnic ultracentrifugation (Hampstead Park $^{13}CO_2$). The bar represents 0.1 changes per nucleotide or amino acid.

The next logical step to ensure that the DNA-SIP sequences are closely related to the indicated species (as according to BLAST); a molecular phylogenetic analysis tree was prepared, as before in Fig. 3.18, but with imported sequences from pure culture studies (Fig. 3.19) to ensure the DNA-SIP sequences cluster with their assumed species. In Fig. 3.19 bracket bars indicate the different clusters of sequences (DNA-SIP

sequences are now de-noted with an i.d. name correlated with Table 3.6 to indicate the closest resemblance match) and each cluster will be briefly explored according to its assigned number.

Cluster 1 (Fig. 3.19) contains the imported sequences relating to *T. denitrificans* and *T. thioparus* (but not *Acidithiobacillus thiooxidans*) and crucially the single *T. thioparus* (HP14F7) from the DNA-SIP study aligns closely to the *T. thioparus* imported sequences as does the *T. denitrificans* respectively. This was an important observation as it now conclusively shows that these sequences are highly likely to belong to the *Thiobacillus* genus and could possibly be *T. denitrificans* and/or *T. thioparus*. It must be noted that HP5F5 was not present in cluster 1 but this shall be explored further in the analysis of cluster 7. *T. denitrificans* and *T. thioparus* are soil chemoautotrophic species that are known to be active under the experimental conditions provided in the incubation experiment (Kelly & Wood, 2000; Beller *et al*. 2006). Their presence was highly significant. This was especially pertinent when considering the locations within the CsCl gradient where the majority of these sequences were observed in (Fractions 7 and 6).

Cluster 2 (Fig. 3.19) contains 2 imported *Achromobacter* sequences and a single DNA-SIP sequence (HP23F9). Closest match comparison using BLAST search indicated that HP23F9 had a high homology to *Achromobacter* sp. AO22 (99%). *Achromobacter* sp. AO22 is a heavy-metal tolerant species isolated from lead contaminated soil (Ng *et al*. 2009). It has not been reported to significantly sequester CO_2 and was not isolated from denser CsCl fractions.

Cluster 3 (Fig. 3.19) was represented by the imported sequences relating to *Rhodanobacter*. Six of the *R. lindaniclasticus*-like sequences have clustered together with the imported model sequences. We can assume that sequences HP1F5, HP2F5, HP16F8, HP18F8, HP19F8 and HP20F8 are most likely to be from the genera *Rhodanobacter* with a very close homology to *R. lindaniclasticus.* The remaining *R. lindaniclasticus*-like sequences have clustered together (cluster 8) and these shall be discussed in the relevant section.

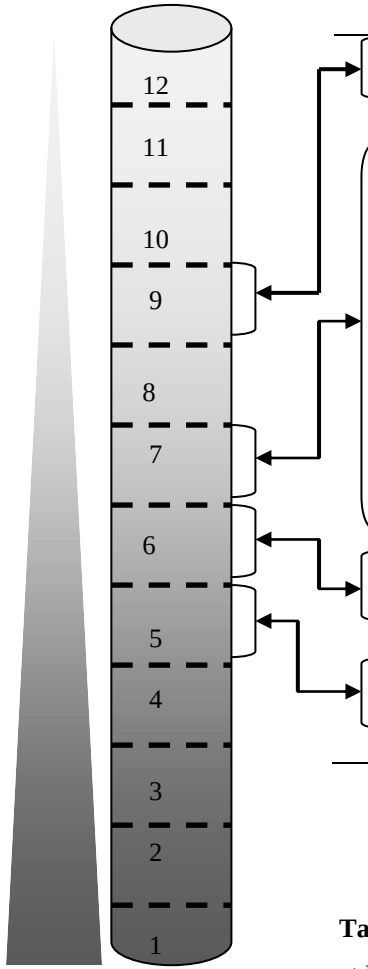
Sample Name	Accession No.	Closest Match / Description	Max Score	Total score	Query Coverage (%)	E Value	Max Identity (%)	Reference
HP T-DNA 1	CP001339.1	*Thioalkalivibrio* sp. HL-EbGR7, complete genome	1142	1142	91	0	84	Muyzer *et al*. (2011)
HP T-DNA 2	FJ605268.1	*Rhodanobacter* sp. DCY45 16S ribosomal RNA gene, partial sequence	394	394	99	2^{E-106}	89	Bui *et al*. (2010)
HP T-DNA 3	AY934488	*Hyphomicrobium* sp. WG6 16S ribosomal RNA gene, partial sequence	1740	1740	86	0	95	Borodina *et al*. (2005)
HP T-DNA 4	CP000116.1	*Thiobacillus denitrificans* ATCC 25259, complete genome	2333	4666	99	2^{E-106}	98	Beller *et al*. (2006)
HP T-DNA 5	NR_036779	*Geothrix fermentans* strain H5 16S ribosomal RNA, partial sequence	1216	1216	95	0	83	Lonergan *et al*. (1996)
HP T-DNA 6	NR_036779	*Geothrix fermentans* strain H5 16S ribosomal RNA, partial sequence	1251	1251	97	0	84	Lonergan *et al*. (1996)
HP T-DNA 7	NR_028745.1	*Thioalkalivibrio denitrificans* strain ALJD 16S ribosomal RNA, partial sequence	1550	1550	98	0	88	Sorokin *et al*. (2001)
HP T-DNA 8	CP000116.1	*Thiobacillus denitrificans* ATCC 25259, complete genome	2318	4637	99	0	98	Beller *et al*. (2006)

Table 3.5: Closest match to complete 16S rRNA sequences retrieved from soil extracted DNA (T-DNA) prior to ultracentrifugation. Closest match descriptions taken from the BLAST database. E value represents the number of hits that are expected due to randomness.

Fraction	Sample Name	Accession No.	Closest Match / Description	Max Score	Total score	Query Coverage (%)	E Value	Max Identity (%)	Reference
5	HP1F5	L76222.1	*Rhodanobacter lindaniclasticus* 16S ribosomal RNA gene, complete sequence	2418	2418	100	0	99	Thomas *et al.* (1996)
5	HP2F5	L76222.1	*Rhodanobacter lindaniclasticus* 16S ribosomal RNA gene, complete sequence	2300	2300	100	0	98	Thomas *et al.* (1996)
5	HP3F5	AY973266.1	*Pseudomonas putida* strain PC14 16S ribosomal RNA gene, complete sequence	2381	2381	100	0	99	Heinaru *et al.* (2000)
5	HP4F5	AF468453.1	*Pseudomonas* sp. Ps 3-1 16S ribosomal RNA gene, partial sequence	2376	2376	100	0	99	Kwon *et al.* (2003)
5	HP5F5	CP000116.1	*Thiobacillus denitrificans* ATCC 25259, complete genome	2278	4557	99	0	97	Beller *et al.* (2006)
6	HP6F6	CP000116.1	*Thiobacillus denitrificans* ATCC 25259, complete genome	2226	4453	100	0	96	Beller *et al.* (2006)
6	HP7F6	L76222.1	*Rhodanobacter lindaniclasticus* 16S ribosomal RNA gene, complete sequence	2426	2426	99	0	99	Thomas *et al.* (1996)
6	HP8F6	AF468453.1	*Pseudomonas* sp. Ps 3-1 16S ribosomal RNA gene, partial sequence	2372	2372	100	0	99	Kwon *et al.* (2003)
6	HP9F6	EU682684.1	*Adhaeribacter terreus* strain DNG6 16S ribosomal RNA gene, partial sequence	1776	1776	90	0	95	Zhang *et al.* (2009)
7	HP10F7	NR_036779.1	*Geothrix fermentans* strain H5 16S ribosomal RNA, partial sequence	1326	1326	96	0	82	Lonergan *et al.* (1996)
7	HP11F7	CP000116.1	*Thiobacillus denitrificans* ATCC 25259, complete genome	2302	4604	99	0	98	Beller *et al.* (2006)
7	HP12F7	CP000116.1	*Thiobacillus denitrificans* ATCC 25259, complete genome	2309	4619	99	0	98	Beller *et al.* (2006)
7	HP13F7	EF117329.1	*Flavobacterium terrae* strain R2A1-13 16S ribosomal RNA gene, partial sequence	2062	2062	97	0	94	Weon *et al.* (2007a)
7	HP14F7	AF005628.1	*Thiobacillus thioparus* 16S ribosomal RNA gene, complete sequence	2250	2250	99	0	96	Vlasceanu *et al.* (1997)
7	HP15F7	CP000116.1	*Thiobacillus denitrificans* ATCC 25259, complete genome	2210	4420	99	0	96	Beller *et al.* (2006)

8	HP16F8	L76222.1	*Rhodanobacter lindaniclasticus* 16S ribosomal RNA gene, complete sequence	2118	2118	99	0	94	Thomas *et al.* (1996)
8	HP17F8	NR_036803.1	*Legionella drozanskii* strain LLAP1 16S ribosomal RNA, partial sequence	2078	2078	99	0	93	Birtles *et al.* (1996)
8	HP18F8	L76222.1	*Rhodanobacter lindaniclasticus* 16S ribosomal RNA gene, complete sequence	2397	2397	99	0	99	Thomas *et al.* (1996)
8	HP19F8	L76222.1	*Rhodanobacter lindaniclasticus* 16S ribosomal RNA gene, complete sequence	2421	2421	99	0	99	Thomas *et al.* (1996)
8	HP20F8	L76222.1	*Rhodanobacter lindaniclasticus* 16S ribosomal RNA gene, complete sequence	2388	2388	99	0	99	Thomas *et al.* (1996)
8	HP21F8	DQ672568.1	*Skermanella aerolata* strain 5416T-32 16S ribosomal RNA gene, partial sequence	2131	2131	99	0	96	Weon *et al.* (2007b)
9	HP22F9	L76222.1	*Rhodanobacter lindaniclasticus* 16S ribosomal RNA gene, complete sequence	2343	2343	99	0	98	Thomas *et al.* (1996)
9	HP23F9	EU696789.1	*Achromobacter* sp. AO22 16S ribosomal RNA gene, partial sequence	2396	2396	99	0	99	Ng *et al.* (2009)
9	HP24F9	CP000116.1	*Thiobacillus denitrificans* ATCC 25259, complete genome	2273	4547	100	0	97	Beller *et al.* (2006)

Table 3.6: Closest match to complete 16S rRNA sequences retrieved from the central fractions of the DNA-SIP experiment. Closest match descriptions taken from the BLAST database. Sample names are denoted as follows; Hampstead Park soil (HP), clone number in order, fraction number (F_n).



Accession Number	Description	Max Score	Query Coverage (%)	E Value	Max Identity (%)
CP000116.1	*Thiobacillus denitrificans* ATCC 25259, complete genome	2273	100	0.0	97
CP000116.1	*Thiobacillus denitrificans* ATCC 25259, complete genome	4420	99	0.0	96
CP000116.1	*Thiobacillus denitrificans* ATCC 25259, complete genome	2309	99	0.0	98
CP000116.1	*Thiobacillus denitrificans* ATCC 25259, complete genome	2302	100	0.0	98
AF005628.1	*Thiobacillus thioparus* 16S ribosomal RNA gene, complete sequence	2250	99	0	96
CP000116.1	*Thiobacillus denitrificans* ATCC 25259, complete genome	2226	100	0.0	96
CP000116.1	*Thiobacillus denitrificans* ATCC 25259, complete genome	2278	99	0.0	97

Table 3.7: Location of bacterial 16S sequences within the CsCl gradient that displayed chemoautotrophic-like resemblance to deposited sequences at NCBI.

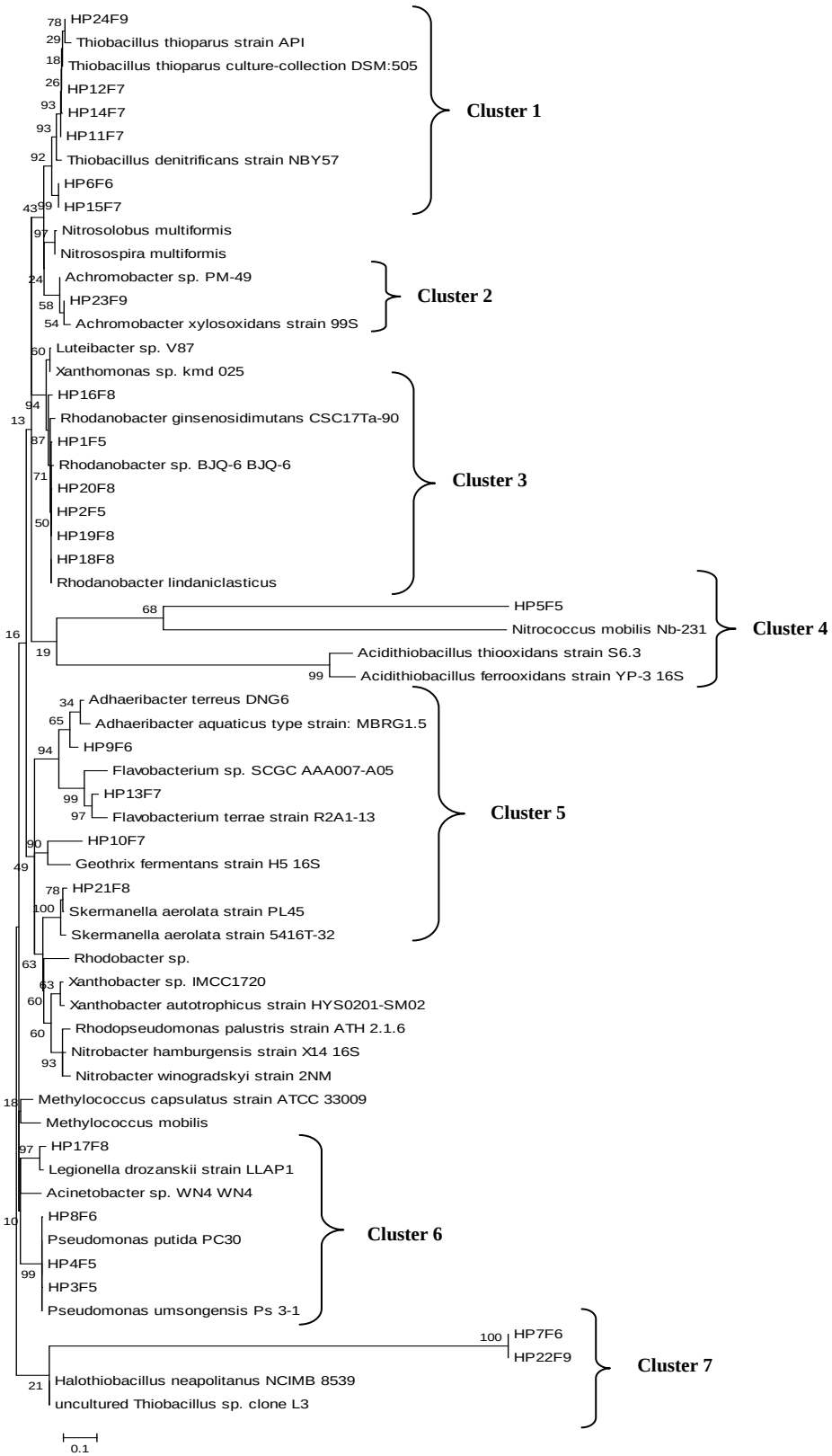


Fig. 3.19: Molecular Phylogenetic analysis by Maximum Likelihood method (1000 Bootstrap). Consensus tree of gene sequences reflecting the relationships of the 16S gene fragments recovered from the CsCl gradients and cloned into recombinant *E. coli*. The sequence isolates are designated with "HP", followed by a number and then the corresponding fraction (F_n) they were located within the CsCl gradient. An encompassing collection of organisms representing some of the major lineages of bacteria were used as outgroups for tree calculations. The bar represents 0.8 changes per nucleotide or amino acid.

Cluster 4 (Fig. 3.19) was represented by three imported sequences who all can chemoautotrophically fix CO_2 as well as a single sequence from the DNA-SIP experiment (HP5F5). The homology between HP5F5 and the imported sequences are relatively low with the closest homology with *Nitrococcus mobilis* Nb-231 (68% bootstrap value). A BLAST search for the closest match indicated that HP5F5 bears high resemblance to *T. denitrificans* (97%) but its failure to group into cluster 1 indicates this to be uncertain. The evidence suggests the sequence bears close homology to other chemoautotrophic strains and further investigation was required (Fig. 3.20).

Cluster 5 (Fig. 3.19) contained several different imported sequences of distantly related bacterial genera. The genera *Adhaeribacter* was represented by two imported sequences from the bacterial strains *A. terreus* and *A. aquaticus*. HP9F6 clustered together with these *Adhaeribacter* strains. BLAST analysis showed that HP9F6 had a closest match to *A. terreus* (95%). The genus *Adhaeribacter* is relatively new to science as it was only created in 2005 (Rickard *et al*. 2005) as a member of the *Flexibacteraceae* family. It is a Gram-negative, non-motile, aerobic chemo-organotroph that is known to produce copious amounts of extracellular fibrillar material (Zhang *et al*. 2009). No evidence of chemoautotrophic growth has been uncovered in the current literature. The *Flavobacterium* genus was represented by two imported sequences. SIP-DNA sequence, HP13F7 shows a close homology to this genus with high bootstrap values and a close sequence match to *F. terrae* in the BLAST database (94%). These common soil bacteria are Gram-negative, aerobic and chemo-organotrophs (some species are known parasites to cold water fish; Weon *et al*. 2007a). A single imported sequence was selected from the GenBank database to represent the bacterial strain *Geothrix fermentans* in the phylogenetic analysis as this organism is not currently well represented in the archives. HP10F7 was the only DNA-SIP sequence to cluster together with *G. fermentans* and indeed the BLAST analysis shows that these two strains are similar (82%). Although the % match was considered to be low, their correlation within the phylogenetic tree suggests they are closely related and likely to be of the same genus. Little research has been performed on *G. fermentans* and it has been placed within its own genus. It is found in terrestrial sub-surface locations and is a Gram-negative chemo-organotroph capable of reducing Fe(III) (Coates *et al*. 1999).

Cluster 6 (Fig. 3.19) was represented by the imported sequences relating to *Pseudomonad's*. It was quickly ascertained that all of the DNA-SIP sequences believed to be *Pseudomonas* have clustered in this region. Although only three *Pseudomonas* species have been observed in the experiment, it was worth noting that the soil bacterium, *P. putida* is a fast growing, non-fastidious species that displays a very diverse metabolism and its presence in the denser CsCl gradients (F5 and F6) was worthy of speculation. As it was likely the *Pseudomonas*-like species from the DNA-SIP experiment were ^{13}C labelled it was possible they may have been assimilating biomass or cell exudates from the primary producers e.g. *T. denitrificans*. Another possibility was that the *Pseudomonas*-like species could have been directly involved in chemoautotrophic growth. The facultatively autotrophic bacterium *P. oxalacticus* when under heterotrophic stress will grow autotrophically (Shively *et al*. 1998) and please note that the soil cultures these samples were taken from were pre-incubated to reduce background levels of labile organic carbon. It was worthwhile to further investigate this by comparison to a phylogenetic tree containing more autotrophic imported sequences. This will be explored further once the other clusters have been assessed (Fig. 3.20). A single imported sequence of the bacterial strain *Legionella drozanski* was also present in cluster 6. This sequence had high correlation to HP17F8. Analysis of the BLAST database showed that HP17F8 indeed had a high affinity (93%) to that strain and therefore we can be confident that this sequence is a close relative to the imported sequence. *L. drozanski* is a Gram-negative, parasite of free-living amoebae (Adeleke *et al*. 2001) and was likely to be not involved in autotrophic growth. Its position in the CsCl gradient (Fraction 8) indicates that it was most likely to be predominantly ^{12}C-labelled further compounds this.

Cluster 7 **(Fig. 3.19)** contains two SIP-DNA strains (HP7F6 and HP22F9) that are highly similar to one another (100% bootstrap value) but highly divergent to the other members of the phylogenetic tree. The closest genera within the confines of the tree are *Halothiobacillus neopolitanus* NCIMB 8539 and uncultured *Thiobacillus* sp. clone L3. HP7F6 and HP22F9 are still quite divergent from these chemoautotrophic species but are more related to them than *R. lindaniclasticus* as the BLAST search database indicates (99 and 98% respectively).

The anomaly that was observed in cluster 4 (where HP5F5 did not cluster with the other supposed *Thiobacillus*-like sequences), required that a new phylogenetic tree

be constructed. This tree contained imported sequences from mainly autotrophic genera (although some non-autotrophs are included such as *Rhodanobacter*). DNA-SIP sequences from clusters 1, 4, 6 (with the exception of sequence **HP17F8)** and 7 were compiled with 59 imported sequences from the BLAST database (a fairly representative range of autotrophic genera), in an attempt at resolving the likely genera of the outlier sequences HP5F5, HP7F6 and HP22F9. Sequences with a high homology to the *Pseudomonas*-like genera (HP3F5, HP4F5 and HP8F6) were also included to see if clustering with any autotrophic genera occurs, namely the closely related facultative heterotroph, *Wautersia oxalatica* (also known as *Ralstonia eutropha* in the literature). The sequences of cluster 1 were added as a control to ensure they remained within the *T. denitrificans* and *T. thioparus* clade.

The molecular phylogenetic tree in Fig. 3.20 was prepared in an attempt to resolve the issue of outliers observed in **Fig. 3.19** and unless otherwise stated, all mention of DNA-SIP sequences in this paragraph relate to interpretations of Fig. 3.20 only. It was first prudent to note that sequences originally observed in cluster 1 (**Fig. 3.19**), have remained within the *T. denitrificans* and *T. thioparus* clade in Fig. 3.20, further compounding the likelihood of the DNA-SIP sequences in this cluster being closely related to the imported sequences. HP7F6 has aligned itself with the imported *Rhodanobacter* sequences indicating it to be more closely related to that genus, than any of the surrounding autotrophic genera (this correlates with the BLAST database match). The second *Rhodanobacter*-like sequence, HP22F9, did not cluster towards any of the imported sequences but did closely align itself with the *Pseudomonas*-like sequences (HP3F5, HP4F5 and HP8F6). This was interesting as it appears these four sequences have more in common with each other than any of the autotrophic genera. The sequence, HP5F5 has aligned itself with *Acidiferrobacter thiooxidans* (47% bootstrap replications). *A. thiooxidans* (GenBank accession number: AF387301) is an extremely new addition to the GenBank database and Hallberg *et al*. (2011) describe the organism as a thermo-tolerant autotrophic species capable of growing on Fe(II), S^0, S^{2-}, $S_2O_3^{2-}$ or $S_4O_6^{2-}$. This important connection with a novel organism was of interest to the experiment as it indicates that HP5F5 may in fact be itself a novel organism closely related to *A. thiooxydans*.

PCR products that had been amplified by application of the *cbbL*r gene primers were used to construct a clone library. Of the 40 prepared clones, only 15 contained the

correct size fragment (800 bp). Sequence analysis of all the retrieved plasmid DNA revealed that a single species was amplified, although its closest match within the GenBank database was of low probability ($\mu \approx 84\%$). Table 3.8 clearly shows that a single dominant organism was amplified using the *cbbL*r gene with close homology to *Nitrosospira multiformis* ATCC 25196.

The clustering of the sequences into two distinct groups (Fig. 3.21) indicates that more than one closely related species employing the red-like *cbbL*r gene may be present although no sufficient match was found in the Genbank database to indicate the alternative genus/species. *N. multiformis* is a known ammonia oxidiser, found in the soil rhizosphere and is known to fixate CO_2 via the form I (red-like) RubisCO enzyme pathway (Norton *et al*. 2008). The dominance of the *N. multiformis*-like sequences retrieved from the *cbbL*r study are unlikely to bear any semblance to the actual abundance of these species in the CsCl fraction from which they were sampled (Fraction 5), as PCR amplification cannot be used to estimate population density. The study does demonstrate the presence of a species closely related to a well known chemoautotrophic ammonia-oxidising bacteria (AOB) and further, the lack of other bacterial species harbouring the *cbbL*r gene (the majority of *Thiobacilli* species use the green-like *cbbL* gene in autotrophic carbon fixation). As no measurements of ammonia within the incubated soil sample were made (although NH_4Cl was a major constituent in the MSM) it was difficult to make any assumptions of the activity of AOB. It is known that most species of *Nitrosospira* are acidophiles (Jordan *et al*. 2005) and as the experiment progressed over the 48 hour incubation very little change in the pH was observed (Fig. 3.1) but conditions were slightly acidic.

Fig. 3.20: Molecular Phylogenetic analysis by Maximum Likelihood method (1000 Bootstrap). Consensus tree of gene sequences reflecting the relationships of the 16S gene fragments of selected sequences from the DNA-SIP study and compared to isolated strains of known autotrophic bacteria. The sequence isolates are designated with "HP", followed by a number and then the corresponding fraction (F_n) they were located within the CsCl gradient. An encompassing collection of organisms representing some of the major lineages of bacteria were used as outgroups for tree calculations. The bar represents 0.2 changes per nucleotide or amino acid.

#	Accession Number	Closest Match / Description	Max Score	Total Score	Query Coverage	E Value	Max Identity	Reference
1	CP000103	*Nitrosospira multiformis* ATCC 25196, complete genome	848	848	98	0	84	Norton *et al.* (2008)
2	CP000103	*Nitrosospira multiformis* ATCC 25196, complete genome	843	843	99	0	86	Norton *et al.* (2008)
3	CP000103	*Nitrosospira multiformis* ATCC 25196, complete genome	868	868	100	0	84	Norton *et al.* (2008)
4	CP000103	*Nitrosospira multiformis* ATCC 25196, complete genome	877	877	99	0	84	Norton *et al.* (2008)
5	CP000103	*Nitrosospira multiformis* ATCC 25196, complete genome	834	834	98	0	84	Norton *et al.* (2008)
6	CP000103	*Nitrosospira multiformis* ATCC 25196, complete genome	848	848	98	0	84	Norton *et al.* (2008)
7	CP000103	*Nitrosospira multiformis* ATCC 25196, complete genome	875	875	99	0	84	Norton *et al.* (2008)
8	CP000103	*Nitrosospira multiformis* ATCC 25196, complete genome	888	888	99	0	84	Norton *et al.* (2008)
9	CP000103	*Nitrosospira multiformis* ATCC 25196, complete genome	866	866	99	0	84	Norton *et al.* (2008)
10	CP000103	*Nitrosospira multiformis* ATCC 25196, complete genome	798	798	99	0	84	Norton *et al.* (2008)
11	CP000103	*Nitrosospira multiformis* ATCC 25196, complete genome	863	863	99	0	84	Norton *et al.* (2008)
12	CP000103	*Nitrosospira multiformis* ATCC 25196, complete genome	821	821	99	0	84	Norton *et al.* (2008)
13	CP000103	*Nitrosospira multiformis* ATCC 25196, complete genome	877	877	99	0	84	Norton *et al.* (2008)
14	CP000103	*Nitrosospira multiformis* ATCC 25196, complete genome	886	886	99	0	84	Norton *et al.* (2008)
15	CP000103	*Nitrosospira multiformis* ATCC 25196, complete genome	888	888	99	0	84	Norton *et al.* (2008)

Table 3.8: Phylogenetic affiliation of dominant clones from PCR amplified *cbbL*r genes. DNA extracted from fraction 5 of the Hampstead Park $^{13}CO_2$ exposed soil incubation at T_{48} after ultracentrifugation and subsequent cloning into recombinant *E. coli*.

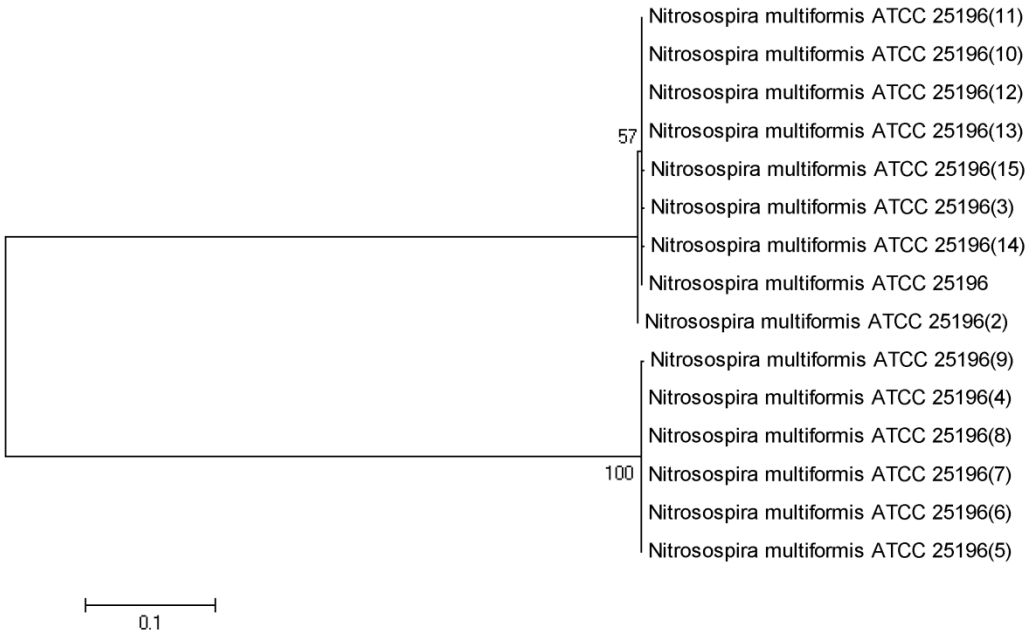


Fig. 3.21: Phylogenetic analysis of *cbbL*r genes amplified from DNA extracted from fraction 5 of the Hampstead Park $^{13}CO_2$ exposed soil incubation at T_{48} after ultracentrifugation. The bar represents 0.1 changes per nucleotide and subsequent cloning into recombinant *E.coli*.

3.5 Discussion

3.5.1 Soil Incubation, Measurements of Carbon Sequestration and ^{13}C-Labelling of Biomass

The ability for a soil picked at random to harbour chemoautotrophic communities was not remarkable due to their ubiquitous spread in the environment (Chapman, 1990) but their low abundance was demonstrated by the need for pre-incubation, as observed in Fig 3.0. The plot in Fig. 3.0 demonstrated the capacity for CO_2 sequestration within a relatively small volume of soil, upon receiving an energy source required for chemoautotrophy. The decay curve observed at ~250 hours was a clear indicator of sudden activity, where ultimately the λ_{min} CO_2 level dropped below the average concentration accepted for the Earth's atmosphere (Tans, 2009). This suggests that a sudden change in activity within the sample took place, leading to consumption of atmospheric CO_2 (within the ECIC) until, for an undisclosed reason, sequestration ceased and CO_2 reintroduction took place (most likely to be microbial respiration). The CO_2 plot observed in Fig. 3.0 showed an interesting pattern, which can be inversly correlated with the growth phase pattern of microbes. For instance, between T_0-T_{250} h, a steadily increasing level of CO_2 was observed despite the presence of the electron donor (chemoautotrophic bacterial lag phase). A sudden drop in CO_2 levels (the substrate

source for chemoautotrophic growth) between T_{250}-T_{344} resembles the logarithmic growth phase of $S_2O_3^{2-}$ oxidising microorganisms. A short stationary phase was observed of 1.5 hours and then a rapid reintroduction of CO_2 to the atmosphere (death phase?) for the remainder of the incubation. This interpration was open to scrutiny due to lack of evidence for this particular incubation, but based upon its CO_2 plot, a working method for rapid CO_2 uptake could be devised to ensure that ^{13}C-labelling experiments begin when the supposed $S_2O_3^{2-}$ oxidisers could be in the logarithmic growth phase. This premise was important for, not only the great expense of purified ^{13}C substrates, but also the need for actively dividing microorganisms for down-stream applications e.g. incorporation of ^{13}C into complex molecules such as nucleic acids (Neufeld *et al*. 2007).

The establishment of soil cultures conducive to $S_2O_3^{2-}$ oxidation was achieved by incubating the soils prior to the main experiments (pre-incubation). The biochemistry of bacterial, aerobic $S_2O_3^{2-}$ oxidation lead us to hypothesise that the production of SO_4^{2-} and hence H_2SO_4 would most likely occur over the course of the pre-incubation and hence, act as an indicator of soil enrichment. The pH and EC results (Figs. 3.1 and 3.2) of freshly incubated soil demonstrated an inverse relationship to each other over the 14 day incubation, which was consistent with existing research (Harrison, 1984; Johnson & Hallberg, 2009). The pH and EC plot over the initial 6 days showed little variation, which was consistent with the initial lag phase observed in Fig. 3.0 and others (Alcántara *et al*. 2004; González-Sánchez *et al*. 2008), where approxiamtely 8-10 days gestation time was required for soil generated chemoautotrophic cultures. The basic soil chemistry changed over the course of the pre-incubation, that is, the pH dropped by ~1.33 units and EC increased by 1.84 mS. The time trend of these observations takes place in a manner consistent with the hypothesis that $S_2O_3^{2-}$ oxidation took place after an initial lag phase of approximately 6 days (no measurements of CO_2 uptake were recorded at this time).

The capacity to measure the removal of CO_2 from the chamber atmosphere was an important aspect to the project. The CO_2 sequestration values calculated for the Hampstead Park soils demonstrate significant activity taking place, although little uniformity was observed. The average uptake value for the (main) three incubated soils (under a $^{12}CO_2$ atmosphere) was 75.8 ± 32.8 µg CO_2 30.14 g^{-1} dry soil 40 h^{-1} (2018.2 ± 1530.7 µg CO_2 kg^{-1} dry soil). The large standard deviation encountered demonstrates

the variablilty of the results due to the unpredictability of biological systems. All three experimental samples did show a positive uptake trend, as observed in Fig. 3.4. This was an important demonstration that inorganic carbon was being removed from the inner chamber atmosphere at a rate faster than can be explained by the determined leak rates (inclusive of partial pressure). It was prudent to consider that the 'missing' CO_2 may have been incorporated into the soil system (Shively & Barton, 1991; Beller *et al*. 2006; González-Sánchez *et al*. 2008; Hart *et al*. 2011a) as it was the only object present in the chamber capable of doing so. Before moving on, it must be pointed out that the CO_2 decay rate averages calculated for each of the soil experiments are themselves based on averages of each CO_2 decline trend observed in Fig. 3.4 and therefore some error can be expected. Each decay trend in the CO_2 plots of Fig 3.3 are corrected against the average leak rates (where CO_2 was flowing out of the system as goverened by partial pressure). The effects of the leak, take into account partial pressure effects but this system was limited. The limitation stems from the need to use managable correction factors, i.e. 100 ppmv fractions. The recorded CO_2 data could be used to make more accurate predictions if compared against an algorithmic model that could determine the leak rate and/or the respiration rate at every measurement point between 1200-500 ppmv (as these are the points the leak rates have been measured against). This subject was part of on-going work in collaboration with Dr. Seth Oppenheimer, Dept. of Mathematics and Statistics, University of Mississippi where we hope to model CO_2 sequestration, leakage rates and soil respiration at any point, simultaneously throughout the incubation.

3.5.2 GCMS-IRMS Analysis of Extractable Fatty Acids and PLFAs

PLFAs have been used as taxonomic markers for the quantification and classification of microorganisms for a long time (Tunlid & White, 1992; Frostegård & Bååth, 1996; Zelles, 1997, 1999). PLFAs can be used as biomarkers for Gram-positive (odd-chained and iso/anteiso fatty acids) and Gram-negative bacteria (mono-unsaturated and cyclic fatty acids), fungi (octadecadienoic acid) and general membrane lipids (C_{16} and C_{18}). For the extracted samples a strong predominance for even-numbered, straight chain fatty acids and cyclopropanes indicating the dominant presence of Gram-negative bacteria (Kandeler, 2007). The presence of iso/anteiso methyl-branched fatty acids indicates the presence of Gram-positive bacteria (Zelles, 1997, 1999) although, only three out of seven of the iso/anteiso methyl-branched fatty acids (total lipids) were enriched with $\delta\ ^{13}C$. Detection of the sulphate-reducing bacteria PLFA biomarker, 10-

methyloctadecanoate (Dowling *et al*. 1986; Kerger *et al*. 1986) and 2-hexylcyclopropane octanoate indicates the active presence of Gram-negative chemoautotrophic species (Kerger *et al*. 1986). The lack of > C_{20} straight iso/anteiso methyl branched PLFAs indicates no higher plant input to the samples (Ruess & Chamberlain, 2010) which demonstrates that light was excluded from the chamber during incubations. Also, no significant detection of 5, methylhexadecenoate (16:1ω5), a biomarker for cyanobacteria (Kandeler, 2007), further compounds this assumption. The detection of a single diunsaturated fatty acid, Methyloctadecadienoate (18:2ω9,12) indicated the presence of eukaryotes (fungal) or cyanobacteria in the total lipids fraction. The fact that it remained depleted in δ ^{13}C, under $^{13}CO_2$ conditions, indicates that it was likely the organism(s) responsible were not active in inorganic carbon capture. Hexamethyl tetracosahexaene (more commonly known as squalene) was extracted in the total lipids fraction and found to be highly enriched in δ ^{13}C. Squalene is a critical intermediate isoprenoid for all domains of life (Peters *et al*. 2007). Although, squalene is utilised as an intermediate by all organisms in its uncyclised form, it is more frequently reported in bacteria (Amdur *et al*. 1978). It is used as a biosynthetic precursor for both hopanoids and steroids and indicates the contribution of bacteria to the enriched organic carbon pool (Hart *et al*. 2011b). The compounds observed indicate a wide degree of microbial diversity present in the soils (expected for a large environmental inoculum like the one used in this experiment). The distribution of ^{13}C label throughout the organic matter was likely to be directly attributed to chemoautotrophy and some limited crossfeeding.

The enrichment of fatty acids with ^{13}C during the course of the experiment was intended to further demonstrate the hypothesis that chemoautotrophy was stimulated after addition of $Na_2S_2O_3$. Fatty acid profiles provided by GCMS-IRMS, have clearly demonstrated the enrichment of the stable isotope into biomass over the course of the experiment. The incorporation of the isotopic label into lipids and specifically PLFAs has shown that CO_2 was the primary carbon source for chemoautotrophic microorganisms (as a lack of plant biomarkers indicated no photosynthetic activity). Biomarker evidence was tentative but the identification of a 10-methyl branched (C_{18}) and the cyclopropyl ring containing PLFAs, indicate the active presence of sulphur activated bacteria (Ruess & Chamberlain, 2010).

Employing biomarker methods for soil samples is difficult as the lipid composition of terrestial species is less known to that of marine species (Kattner *et al*. 2003; Stübing *et al*. 2003; Stevens *et al*. 2004a,b). Many of the lipids observed such as straight chain fatty acids (e.g. 16:0, and 18:0) are common across taxa from diverse locations. This similarity was caused by the uniform biosynthesis of fatty acid production within the animal, plant and microbial cell. For instance, acetyl-CoA is used as the primer and the carbon chain is elongated by the condensation of malonyl-CoA to the primer. This process yeilds palmitic acid (16:0) as a major lipid product (Weete, 1980; Ratledge & Wilkinson, 1988) which was a dominant lipid observed in this experiment. The high levels of 16:0 PLFA that were observed along with considerable δ ^{13}C enrichment, back up this statement and indicate that chemoautotrophic bacteria were producing this PLFA during the active growth phase.

The quantification of lipids and specifically PLFAs has shown that the mass of nearly all fatty acids decreased between the two sampling points. This was a strange observation, as one would expect biomass to increase during the growth phase considering that a growth stimulant had been applied to an environmental sample (Lundberg *et al*. 2001; Lu *et al*. 2004; Leuders *et al*. 2006). The increasing δ ^{13}C values showed that active growth was taking place (considering that PLFAs are immediate storage compounds produced by microbes) in the soil once it was exposed to $^{13}CO_2$ and a suitable electron donor. DNA-SIP evidence indicates that chemoautotrophic bacteria were present and the increasing accumulation of ^{13}C in the organic matter pool shows that these organisms were growing, and importantly, this was expected to be at the short-term expense of other microbial species. It was feasible that other species may reduce their growth rate and/or possibly die as the chemoautotrophic fraction compete for nutrients/oxygen and alter the surrounding chemical environment, such as the production of waste by-products (acidulants). The fatty acids of the originally more numerous basal species go into recession due to rapid enzymatic degradation of these specific compounds once the cell expires (Ruess & Chamberlain, 2010). The assumption that the biomass, of actively growing chemoautotrophic species would off-set basal PLFA decreases, can be argued due to the slow doubling time of *Thiobacillus*-like species (the dominant chemoautotroph observed) where it has been estimated no more than two generations could have evolved over the prescribed incubation period (Kingma & Silver, 1980; Pronk *et al*. 1992; Alcántara *et al*. 2004; González-sánchez *et al*. 2008). It was also possible that handling of the sample soil may also effect fatty acid

concentrations. Petersen & Klug (1994) conclusively showed that sieving, storage and incubation temperature all caused significant downward shifts of PLFAs, although these soils had been pre-incubated under similar conditions to that of the experiment to stabilise the soils and enrich the microbial population.

The observation of a polyaromatic hydrocarbon (PAH) in the extractable organic matter was worthy of note. Pyrene was quantified and appeared to be stable in the sample. Measurement of the $\delta\ ^{13}C$ values were depleted for both time points which implies that it had no involvement in direct carbon capture. It has been reported that PAH accumulation in agricultural soils is traditionally due to atmospheric deposition, slow degradation and that soils in industrialised nations contain similar levels (Jones *et al*. 1989), except in the case of hydrocarbon spillages (Schwark, 2010). Sources in the literature intimate that natural sources of PAHs in soils exist such as volcanoes and forest fires (Nam *et al*. 2009; Ma *et al*. 2010) but this was highly unlikely for the sampling site in urban Dublin. Presence of *R. lindaniclasticus* throughout the gradient fractions of SIP-DNA experiment was of interest as these organisms are known as rapid degraders of pyrene and other PAHs (Kanaly *et al*. 2002). Presence of pyrene was an oddity worthy of mention but was highly unlikely to have any connection to soil chemoautotrophy.

3.5.3 NMR

The ^{1}H-^{13}C HSQC HR-MAS-NMR analysis of the organic extracts has provided an excellent overview of the ^{13}C labelled contents. As the spectra observed in Figs. 3.12 and 3.13 were both subtracted against the natural abundance of ^{13}C in the incubated samples (using the $^{12}CO_2$ incubated soils), we are observing the direct incorporation of atmospheric carbon into biological compounds. The location of lipids in the HSQC spectrum (Fig. 3.13) was presumed by the detection of the polar head groups of long-chain fatty acids (Simpson *et al*. 2001). The presence of the polar head group is directly complimentary to the GCMS-IRMS analysis of PLFAs (where the polar head group was cleaved off the molecule so that the fatty acid side-chain may be analysed using GCMS) and confirms that phospholipids were extracted and derivitised using SPE and sodium methoxide, respectively.

Methionine is one of the three sulphur containing proteinogenic amino acids (the others are cysteine and cystine) and it is a common intermediate in the biosynthesis of

phospholipids in the *Thiobacilli* genera (Barridge & Shively, 1968). Phenylalanine is a common amino acid expressed by bacteria such as *Corynebacterium glutamicum* and *E. coli* (de Boer & Dijkhuizen, 1990; Sprenger, 2007) amongst others. Phenylalanine was also identified using ^{1}H-^{13}C HSQC HR-MAS-NMR. Other than conflicting reports of phenylalanine being a growth inhibitor to some autotrophic stains (Kelly, 1967; Johnson & Vishniac 1970), phenylalanine is an amino acid required by bacteria for growth but not all species produce it *de novo*. The abundance of phenylalanine in the sample is represented by its strong presence in the HSQC spectrum, and is likely because of its close association with proteins and peptides.

3.5.4 Analysis of the DNA-SIP Fractions post-Ultracentrifugation

DNA-SIP is a powerful tool used to identify the functional capabilities of unculturable microorganisms as they exist in microbial communities (Buckley *et al*. 2007). The principle that any species directly assimilating the isotopically labelled substrate, will upon cell division, incorporate the stable isotope into nucleic acids (and therefore become enriched) was a vital aspect in determining the species responsible. The denser fraction of DNA (isotopically labelled) that was observed to penetrate deeper into the CsCl gradient (Figs. 3.14 and 3.15) was an ideal example of the expected separation of $^{13}C/^{12}C$ DNA, as fully labelled ^{13}C-DNA increases in buyont density by 0.036 g ml^{-1} (Birnie & Rickwood, 1978). This was not observed for the Hampstead Park soil DNA-SIP experiment as it is well known that once extracted, environmental samples contain small quantities of DNA (low to zero visibility of bands post-ultracentrifugation) and the DNA smears across the gradient due to isotopic mixing (Radajewski *et al*. 2000; Neufeld *et al*. 2007; Buckley *et al*. 2007).

The distribution of nucleic acids across the CsCl gradient was demonstrated by the PCR amplification of recovered DNA from each fractionated portion of the CsCl gradient (Fig. 3.15). The presense of PCR amplicons in the electrophoresis gel from fractions 5 and 6 for the $^{13}CO_2$ incubation, in contrast to no visible amplicons in the $^{12}CO_2$ incubation for the same fraction number (i.e. buyont density), are a clear indicator of isopycnic separation of 'heavy' DNA from the remaining unlabelled community DNA. The PCR amplicon fragments retrieved from five fractions of the $^{13}CO_2$ incubation were then cloned into recombinant *E. coli* to give an indication of the diversity of species present and their location in the CsCl gradient. The distribution of organisms whose DNA sequence was highly similar to *Thiobacillus*-like species were

mostly clustered in Fraction 7. However, single sequences were observed in both fractions 5 and 6, indicating the presence of *Thiobacillus*-like species in the densest fractions. A single *Thiobacillus*-like sequence was also located in fraction 9 which may be interpreted as being unlabelled.

Cluster analysis of these sequences using a phylogenetic tree, showed that all of the *Thiobacillus*-like sequences grouped together (Fig. 3.18) as did any other dominant groups, such a *Rhodanobacter*-like sequences. When the retrieved sequences were compared to imported sequences from the BLAST database, HP5F5 did have close homology to the imported sequences and clustered away from the *Thiobacillus*-like group (**Fig. 3.19**; cluster 1). This analomy was further investigated by statistically comparing all the *Thiobacillus*-like sequences in another phylogenetic tree using imported sequences of mostly autotrophic bacteria (Fig. 3.20). The aim of this exercise was to determine if HP5F5 shared homology with any of these other known autotrophs to give some indication of the genera. HP5F5 branched off with only one relative, *Acidiferrobacter thiooxydans*. This particular organism is relatively new to science having only recently been isolated and issued within a set genera (Hallberg *et al*. 2011). It was originally named *Thiobacillus ferroxidans* m-1 25 years ago but it has recently been determined that it is more closely related to *Ectothiorhodospira spp*. genera and is in fact, only distantly related to the *Acidithiobacilli* genera (Hallberg *et al*. 2011). The clustering of HP5F5 with this particular strain indicates it is more closely related to the family *Ectothiorhodospiraceae* of the Gammaproteobacteria than the *Thiobacillus* genus, as indicated by the initial BLAST search (Table 3.6). The tentative identification of HP5F5 as *T. denitrificans*, has not stood up to scrutiny, while its presence in the fraction of highest density and still being closely related to only known chemoautotrophic species indicates that it may in fact be a novel bacterium, active to some degree in the chemoautotrophic oxidation of $S_2O_3^{2-}$.

For the remaining sequences retrieved from the CsCl gradient, the dominant strain match was *Rhodanobacter lindaniclasticus*. The *R. lindaniclasticus*-like sequences accounted for 33.3% of all the viable strains which was significant. *R. lindaniclasticus* is an aerobic chemo-organotroph, that coincidentally has the same G+C ratio as *T. denitrificans* (63 mol%). These Gram-negative species are commonly isolated from soil habitats and are known to be degraders of Gamma-hexachlorocyclohexane (γ-HCH) or lindane as well as PAHs, various sugars and amino

acids with an optimum growth rate at 30^{o}C (Nalin *et al*. 1999). Within the *Xanthomonadaceae* family, *Rhodanobacter thiooxydans* (G+C of 64.6 mol%) has been documented to oxidise $S_2O_3^{2-}$ under aerobic conditions, although it does not derive its carbon requirements autotrophically (Lee *et al*. 2007). The only isolate currently locatable in GenBank of this species was added to the phylogenetic tree (Fig. 3.20) and although the *Rhodanobacter*-like sequences were closely related to *R. thiooxydans*, they were still more closely alighned with *R. lindaniclasticus*. The interesting biochemistry of *R. thiooxydans* does indicate that, the possibility exists that $S_2O_3^{2-}$ oxidation, as an heterotrophic energy source, may be taking place by members of the *Xanthomonadaceae* genera although at present this remains purely speculative.

Gene specific primers were used on CsCl fraction 5, in an attempt to isolate the presence of bacteria that have the ability to fixate CO_2 through the RubisCO enzymatic pathway. RubisCO is a bifunctional enzyme that controls the reduction of CO_2 and the oxygenolysis of ribulose-1,5-bisphosphate (Selesi *et al*. 2005) and hence, a major component of the Calvin-Benson cycle. This enzyme is responsible for the vast majority of photosynthetic carbon fixation and nearly all primary production is linked to it. There are multiple natural forms of RubisCO, which differ in structure, catalytic property and O_2 sensitivity (Tabita, 1988). The type used in this study is classified as 'form I', which occurs in both photo- and chemoautotrophs. Form I RubisCO is a hexadecamer and it is composed of eight large and eight small subunits. The large subunit of form I RubisCO is encoded by the *cbbL* gene (Kusian & Bowien, 1997) and is large enough (1,400 bp) to be used in meaningful phylogenetic analyses (Selesi *et al*. 2005; Kamimura *et al*. 2010; Tourova *et al*. 2009). Two types of form I RubisCO exist, the red- and green-like types. The red-like type occurs in non-green algae and α- and β-Proteobacteria. The green-like type contains sequences from plants, algae and α- β- and γ-Proteobacteria (Tourova *et al*. 2005) including the *Thiobacillus* genera (Kellermann & Griebler, 2009; Utåker *et al*. 2002). Due to time constraints it was decided to only amplify the sequence fragments with the red-like primer. The aim of this experiment was to observe if any other types of bacteria containing the *cbbL*r gene were present in the sample, regardless of any potential metabolic impacts. The PCR amplified fragments were cloned into recombinant *E. coli* and expressed fragments of correct size (800 bp) were subsequently sequenced (Table 3.8).

All 15 of the retrieved sequences were shown to be similar (closest match) to *Nitrosospira multiformis* ATCC 25196. This indicates the dominant presence of *N. multiformis*-like species in the enriched sample for organisms containing the *cbbL*r gene. This observation does not intimate the abundance of this species or even its influence on this particular microcosm. *N. multiformis* ATCC 25196 is an AOB capable of chemoautotrophic growth and it is commonly isolated from aerobic soils (Norton *et al*. 2008). Bacterial ammonia oxidisers gain energy though oxidation of ammonia to nitrite and carbon by fixation of CO_2 (Schramm *et al*. 1998). AOB (including *N. multiformis*) are readily enriched from most soils and are ubiquitous (Zhang *et al*. 2010; Avrahami & Bohannan, 2007). The formation of two distinct clusters in Fig. 3.21 indicated that two *N. multiformis*-like groups of sequences were expressed. The two groups are highly related to one-another and suggested that at least two species of the *Nitrosospira*-like genera were amplified in the PCR reaction. As this amplification experiment was only aimed at qualitatively assessing the presence of *cbbL*r genes it was not prudent to make any more assumptions at this point, but future analyses should consider the employment of quantitative PCR (qPCR) to assess the abundance of amplified species when using specific primers and hence, the abundance of template DNA may be provided at the sampling events.

The presence of known chemo-organotrophic bacteria in the denser fractions may have been caused by several factors. The G+C contents of organisms in complex communities can differ over a wide range of buyont densities (Buckley *et al*. 2007) and this can help to explain the smear effect, as not all DNA in the heavy fractions may be ^{13}C-labelled (Manefield *et al*. 2002; Leuders *et al*. 2004). Another consideration was the capacity for cross-feeding from non-target functional groups, be it from trophic cascade effects or direct microbial predation (Leuders *et al*. 2006). In regards to cross-feeding, the isotopic signature of nucleic acids from these non-target microbes, as a natural consequence of label dilution, would be less than that of the primary producers until the flow of label saturates the community (Buckley *et al*. 2007). This scenario has been minimised in the discussed SIP incubation, as the incubation was performed over the least amount of time possible (Dumont & Murrell, 2005) only allowing for a few generations of *Thiobacillus*-like species to divide (Alcántara *et al*. 2004) and hence reduction of available ^{13}C-labelled biomass. Known microbial predators such as *Myxobacteria* (Reichenbach, 1999), *Lysobacter* (Reichenbach, 1992a) and *Cytophagles* (Reichenbach, 1992b) were not located in any of the fractions, although this was not

conclusive as more cloned sequences would be necessary to rule them out. Another possibility for nucleic acid enrichment of stable isotope was the fixation of CO_2 by heterotrophic soil bacteria (Miltner *et al*. 2004; Miltner *et al*. 2005a; Šantrůčková *et al*. 2005). The assimilation of CO_2 via heterotrophic respiration is a well known process performed by prokaryotes and eukaryotes alike. The impact that this phenomenon has upon the experimental method under scrutiny was low, as it is known that the process takes long periods to accumulate enough isotope in biomass to become detectable, even through sensitive techniques such as GCMS-IRMS (Miltner *et al*. 2004; Miltner *et al*. 2005b) and therefore not considered to be a major impact on this study.

3.6 Conclusions

The introduction states the essential questions being asked in the project to be: who was performing CO_2 sequestration under the chosen conditions of incubation? what products are being formed as a direct result of CO_2 uptake? and how much was being removed? These questions have all been addressed by the work presented as follows. The question of 'who' has been resolved using CsCl gradient ultracentrifugation and DNA-SIP molecular biology. Native species of the chemoautotrophic genera were labelled with the stable isotope ^{13}C. Labelled DNA showed that *Thiobacillus denitrificans, Thiobacillus thioparus* and an unidentified species with close homology to *Acidiferrobacter thiooxydans*, were actively sequestering CO_2 from the chamber atmosphere directly into the soil sample. Using sensitive techniques such as GCMS-IRMS and ^{13}C CP-MAS-NMR, it was possilbe to address the question of 'what' products were directly related to CO_2 sequestration and biomass production. NMR provided an overview of the enriched biomass to show that large amounts of lipids, proteins/peptides, carbohydrates and aliphatics were produced (amongst smaller amounts of other biomolecules). More invasive analytical investigations using GCMS-IRMS identified the bacterial PLFA fraction directly involved in chemoautotrophic growth. Various bacterial PLFAs were observed (amongst many other extractable lipid-like compounds) to be enriched in ^{13}C isotope and although no specific biomarkers for chemoautotrophic species could be validated, no specific fungal, algal or higher plant biomarkers were detected. 'How much' CO_2 was being removed from the atmosphere of the inner chamber has been determined mathematically. It was estimated that 75.8 ± 32.8 µg CO_2 30.14 g^{-1} dry soil 40 h^{-1} were removed on average for three 48 hour incubations. The large standard deviation demonstrates large data fluctuations when

dealing with diverse biological communities although every care was taken in keeping the conditions as stable and reproducible as possible.

The answer to these questions was the fundamental driving force behind the entire project and applying both molecular biology and instrumental analytical measurements, I feel they have been answered for this type of environmental sample. The development of the methods applied here can now be pioneered on scenarios that closely resemble *in situ* conditions such as existing agricultural practices and other natural CO_2 sequestration inputs to large scale systems such as the soil or marine environments.

3.7 References

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Chapter IV: Carbon Dioxide Fixation Associated with the Biological Oxidation of Elemental Sulphur in Soil

4.0 Abstract

Plant nutrition requires various elements to be biologically available as well as specific physio-chemical environments to promote healthy and efficient growth. Sulphur, in the form of sulphates is one such element and in recent years agriculturally important crops such as wheat have shown signs of sulphur deficiency. Sulphur is imported into soil horizons by the weathering of mineral bearing rocks, deposition by anthropogenic pollution, volcanic disturbances, seaspray and agricultural fertilisers. The conversion reactions from elemental sulphur to sulphates are mediated by a consortium of microbes, some of which are known to be chemoautotrophic. As chemoautotrophs fixate atmospheric CO_2 during growth, and large tracts of land are exposed to regular inputs of elemental sulphur, it has been noticed that no measurements of the fate of CO_2 have been reported previously. Therefore, I have attempted to apply similar methods to those used in the previous chapters to quantify the sequestration of CO_2 and identify PLFAs associated with bacterial chemoautotrophy. Attempts at quantifying CO_2 sequestration from a soil were inconclusive but the identification of *Thiobacillus denitrificans* and *Thiobacillus thioparus* using *cbbL* gene specific PCR primers, confirmed the presence of chemoautotrophic bacteria. This was significant, as very little molecular biology has been used to confirm the microbiological activity of agricultural sulphur oxidation in the pedosphere. Also, to track the fate of assimilated carbon, samples were exposed to an atmosphere containing $^{13}CO_2$. GCMS-IRMS analysis has confirmed the sequestration of atmospheric CO_2 by demonstrating continuous increases in $\delta\ ^{13}C$ values for a range of PLFAs associated with Gram-negative bacteria.

4.1 Introduction

The application of elemental sulphur (S^0) to agricultural lands for the purposes of fertilisation, acidification and/or as a fungicide are widespread and well known (Zhao *et al*. 1999; Owen *et al*. 1999; Scherer, 2001). S^0 is applied to the land surface to provide sulphates (SO_4^{2-}) for plant growth and in doing so, acidifies the immediate environment through the production of sulphuric acid (H_2SO_4; Nevell & Wainwright, 1987; Friedrich *et al.* 2001; Weindenfeld, 2011). This is especially important in areas with alkaline soils or where alkaline composts are applied, as certain minerals are insoluble at a pH > 7 (García de la Fuente *et al*. 2007). A good example of this practice is the addition of S^0 to calcareous soils where relatively insoluble rock phosphate is applied as a phosphorus source (Aria *et al*. 2010). Worldwide use of S^0-based fertilisers has been growing since the successful reductions in emissions of sulphur particles and sulphur dioxide (SO_2) to the atmosphere in the 1970's (Zhao *et al*. 1999; Blake-Kalff *et al*. 2000) and the indirect benefit such emissions had to plant nutrient requirements (Hassett & Banwart, 1992). The increase in S^0 fertiliser demand was attributed to the observation of sulphur deficiency in many crops where S^0 is an essential growth nutrient, whose functions in the plant are closely related to that of nitrogen (Naeem *et al.* 2008; Ceccotti, 1996).

According to the US Geological Survey (USGS), estimations of worldwide S^0 production for 2006 was 65.7 million tons with agricultural chemicals accounting for approximately 60% of reported S^0 demand, (US Geological Survey, 2009). The input of SO_4^{2-} (the biologically accessible form to plants) to soils from natural sources such as volcano's (Wallace, 2003) is difficult to estimate, but Lein (1983) estimated between 27 Tg S^0 yr^{-1} (non-eruptive phase) and 1.4 Tg S^0 yr^{-1} (during eruptive phases), both estimates are based on the degassing of lava. Berresheim & Jaeschke (1983) estimated that between ~8 Tg S^0/yr for non-eruptive phases and ~1 Tg S^0 yr^{-1} for eruptive phases. The recent volcanic eruption at Eyjafjallajökull on the southern fringe of Iceland received a lot of scientific attention as the ash cloud reached densely populated areas of Northern Europe (Colette *et al*. 2011). It was observed that this relatively short event released significant contributions of SO_4^{2-} into the atmosphere over Northern Europe (Flentje *et al*. 2010). Volcanic related sulphur deposits are generally localised and therefore only furnish a small fraction of the world's agricultural sulphur demand (Ceccotti *et al*. 1998) and hence anthropogenic inputs are now required on an on-going basis. S^0 containing fertilisers are particularly used in areas where high rainfall and

coarse textured soils suffer from excessive leaching in agricultural sites (Boswell & Friesen, 1993).

The most concentrated sulphur-containing fertiliser available is S^0 and its ability to satisfy agricultural nutrient demands, depends on the speed it can be oxidised to SO_4^{2-} and thus become available to plants (Wiedenfeld, 2011). It is known that the efficacy of S^0 oxidation is dependent on the particle size (Lefroy *et al.* 1997), surrounding soil temperature and moisture levels (Jaggi *et al.* 2005). Optimum oxidation rates are attained at the maximum water holding capacity (WHC) and between 4 to 40^oC (Janzen & Bettany, 1987a; Weir, 1975).

The process of S^0 oxidation in soils is mediated through the actions of a consortium of microbes, one group of which, the chemoautotrophic *Thiobacilli* are well known and ubiquitous in soil environments (Robertson & Kuenen, 2006; Chapman, 1990). An important and well researched aspect of the chemoautotrophic bacteria is the ability to source carbon directly from the air in the form of CO_2 and produce biomass via the reductive Calvin-Benson cycle (Trudinger, 1956; Smith & Strohl, 1991; Leduc & Ferroni, 1994). S^0 is oxidised to H_2SO_4 ($2S^0 + 3O_2 + 2H_2O \rightarrow 2H_2SO_4$) which in turn reacts with carbonate containing minerals to release CO_2 ($H_2SO_4 + CaCO_3 \rightarrow CaSO_4 + H_2O + CO_2$) resulting in mineral weathering and contributing to the microbial foodweb. García de la Fuente *et al*. (2007) demonstrated that an immediate increase in autotrophic activity was stimulated through the addition of S^0 to soil and this activity was mostly attributed to species of the *Thiobacillus* genus. However, a more recent study by Yang *et al*. (2010), argued that the chemoautotrophic fraction of the microbial consortia is only active during a relatively short period of the S^0 oxidation process. The researchers postulated that the complete oxidation of S^0 took place over a long-term basis with aerobic heterotrophic S^0-oxidising bacteria taking a dominant role after approximately 8 weeks. The authors claim that the oxidation rate decreases over time due to bacteria-substrate interaction. *Thiobacillus* species consume the electron donor at the crystalline surface (decreasing the fractal dimension at the small scale), eventually leading to less surface area for the microorganisms to colonise. It was thus prudent to limit studies of S^0-soil chemoautotrophy to 4 ± 2 weeks as demonstrated by Yang *et al*. (2010).

An interesting observation by Seidel *et al*. (2006) reported that biological S^0 (the microbiological waste by-product from sulphide (S^{2-}) containing waste-waters being

one example) displayed a clear increased bioavailability for *Thiobacilli* rather than the technical grade S^0. If this waste by-product was to be redirected towards agricultural requirements, a clear cost saving could be achieved. A study by Stamford *et al*. (2002) involved the inoculation of S^0 and *Thiobacillus* on soil to measure the effects on salinity. A reduction in electrical conductivity (EC) over a two week period was observed. It was speculated that the dissociated H^+ ion may displace Na^+ from clay particles, allowing salts to be leached from the immediate area; however, the production of SO_4^{2-} in the soil and its specific effect on the EC was not discussed. It has also been observed that greater oxidation of S^0 takes place at depths of up to 15 cm and oxidation rates of between 3.4-26 μg S^0 cm^{-2} d^{-1} were reported in the literature (Neilsen *et al*. 1993).

The fixation of CO_2 is a subject of great interest and the topics only briefly discussed above are well known and widely documented (Janzen & Bettany, 1987b; Friedrich *et al*. 2001; Vidyalakshmi *et al*. 2009). Although the autotrophic microbial assimilation of CO_2 is now well understood, little information is available that describes the fate of assimilated CO_2 in the field under autotrophic conditions following S^0 oxidation (García de la Fuente *et al*. 2007; Islam *et al*. 2009; Yang *et al*. 2010). In fact, the contribution of chemoautotrophy to CO_2 capture in agricultural settings has been overlooked (Martens *et al*. 2005; Post *et al*. 2009). A preliminary search of the literature indicates that polymerase chain reaction (PCR) based techniques, used to investigate environmental samples is wide-spread and diverse, but very little interest has been placed upon the oxidation of applied S^0 to agricultural soils and hence the species responsible was assumed to be *Thiobacillus* by many researchers (García de la Fuente *et al*. 2007; Yang *et al*. 2010).

I hope to apply these advanced techniques to highlight the possible contributions of this common biologically mediated soil reaction to carbon sequestration. The approach outlined below, hopes to further elucidate the production of organic matter in the soil profile by tracking the fate of the rare stable isotope ^{13}C. Isotopically enriched $^{13}CO_2$ was used to track the fixation of inorganic carbon into phospholipid fatty acids (PLFAs). Molecular ecology techniques, that specifically target the microorganisms thought to be largely responsible for soil S^0 oxidation, will be used to assess their diversity. Incubations of soil will take place within an environmentally controlled incubation chamber (Hart *et al*. 2011a) with the express purpose of maintaining

optimum growth temperatures for target organisms and maintaining a constant concentration of CO_2 with the aim of quantifying substrate uptake.

4.2 Materials & Methods

4.2.1 Site Details and Pre-treatment

The soil used in the outlined experiments was a Grey Brown Podzolic (Fay & Zhang, 2011), retrieved from an open public area located within Albert College Park (Hampstead Park), Glasnevin, Dublin, Ireland (53° 22' 54.63" N 6° 15' 43.72" W). The sampling location was on open land with a large amount of pine needles present. Samples were transferred aseptically to the laboratory and processed immediately. Roots and large debris were removed manually using aseptic techniques. The soil was air dried and then size fractionated using an autoclaved stainless steel mechanical sieve with a 2 mm aperture size. Sieved soil was stored in an amber jar at 4^{o}C. Accurately weighed aliquots of soil were dried at 104^{o}C for 3 days yielding an average moisture content of 24.5%. The soil water holding capacity (SWC) was determined according to Paetz & Wilke (2005). A portion of soil was fractionated according to size using a 9 piece aluminium sieve set, range 2000-25 µm (Nickel-Electro, Weston-Super-Mare, United Kingdom) and using the Gradistat soil textural calculator (Blott & Pye, 2001), the soil texture was determined to be "a slightly very fine gravelly, very coarse silty medium sand". Using the USDA soil pyramid it was determined that the soil was a "sandy loam". A CHN combustion analyser (Exeter Analytical CE440 elemental analyser) was used to determine the soil elemental composition, 8.62% C, 0.97% H, 0.32% N. Phosphorus analysis by wet digestion (April & Kokoasse, 2009) was 0.31% P. Soils were determined for SO_4^{2-} and NO_3^- using the methods laid out by Rump (1999). SO_4^{2-} and NO_3^- were 8.1 ± 0.56 g kg^{-1} and 12.0 ± 0.3 g kg^{-1} respectively. All chemicals and solvents where purchased from Sigma Aldrich. The chemicals were of the highest purity grade available and all solvents were of PESTANAL® quality.

4.2.2 Incubation Conditions

Colloidal S^0 (Sigma Aldrich, Steinheim, Germany) was oven dried at 104^{o}C for 48 hours and then passed through a sterile ≤ 400 µm sieve. All glassware, reagents (where appropriate) and equipment were autoclaved at 121^{o}C for 15 minutes and subsequently dried at 110^{o}C in a dry oven. The air dried soil was transferred in 100 g lots to 1000 ml Pyrex recrytallising dishes and 1.2 g of S^0 (≤ 250 µm) was added to each dish, with the

exception of the designated blank incubations, and homogenised by shaking (Janzen, 1990). The experimental soils (S^0 amended) to be exposed to $^{12}CO_2$ and $^{13}CO_2$ were designated 'Soil HP1' and 'Soil HP2' respectively (i.e. Hampstead Park 1 and 2). A 0.01 M $CaCl_2$ solution was used to maintain 80% SWC on a daily basis (gravimetric analysis). The total mass of the dish, soil, S^0 and $CaCl_2$ was determined at T_0 using a top pan balance. The pH and EC of the soils were measured using a Eutech Cyberscan PC 300. Incubations were carried out within a temperature controlled incubation unit (Hart *et al*. 2011a) maintaining 30^{o}C, 600 ppmv CO_2 and no source of internal/external light (i.e. photons). For the $^{13}CO_2$ (Sigma Aldrich; 99% $^{13}CO_2$) experiment, the inner incubation chamber was aerated prior to commencement, including each time the door was opened, using CO_2-Free high purity air (Air Products; PR Zero plus). This was done to prevent isotope dilution with atmospheric $^{12}CO_2$ to an acceptable degree. Daily sub-sampling took place in duplicate using aseptic technique and ~1 g soil samples were taken for pH and EC measurements (Cyberscan PC300 series, Eutech Instruments, Singapore). Several 0.5 g aseptic subsamples were taken for microbiological analysis. The total mass was measured each day to assess SWC and hence replenish when levels fell below 80% SWC. To assess the effect of 0.01M $CaCl_2$ on the daily EC measurements, a separate incubation was carried out at 30^{o}C with daily measurements of pH and EC to determine if any significant change occurred. For this control sample, SWC was maintained at 80% against moisture evaporation (but no S^0 or $^{13}CO_2$ was applied). Further; two control blanks were prepared as follows; Blank 1 was incubated in the same manner as the above experimental samples under 600 ppmv $^{13}CO_2$ but no S^0 was added and Blank 2 acted as an abiotic control where the soil had undergone x5 daily autoclave sterilisations (121^{o}C / 15 minutes) and 1.2 g sterile S^0 added.

4.2.3 S^0 Determination by Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES):

Concentration of S^0 was determined every 2 days (odd day regime; Day 1, 3 etc) by chloroform extraction and digestion of 1 ± 0.1 g soil samples using the protocols laid out by Zhao *et al*. (1994, 1996). Sample digestion was carried out using a temperature controlled dry oven (Binder, Tuttlingen, Germany). Analysis was carried out on duplicated incubation samples using a Varian, Liberty 220 ICP-AES and premier grade argon (Air Products, Dublin) using the 182.034 nm wavelength under vacuum. A 999 ± 2 mg l^{-1} S^0 ICP standard (Fluka, TraceCERT ultra) was used to prepare calibration curves.

4.2.4 Extraction and Analysis of Soil Organic Matter (SOM)

The SOM was extracted using a modified version of the Bligh & Dyer method (Bligh & Dyer, 1959; Otto & Simpson, 2007) and was carried out in prewashed 40 ml Teflon tubes (Nalgene). After CO_2 incubation in the chamber, a sample of the soil slurry was centrifuged at 6000 rpm (20 minutes). The supernatant (medium) was decanted from the soil and this remaining solid residue was washed twice with a potassium hydrogen phosphate buffer solution. The soil precipitate was freeze-dried (0.90 g $^{12}CO_2$ exp, 1.26 g $^{13}CO_2$ exp, dry weight) before extraction with methanol:dichloromethane (ratio 1:0, 1:1 and 0:1) was performed. The total extracts were filtered, concentrated and reconstituted in 1 ml of MeOH:DCM [50:50] for derivitisation and analysis.

The extracts were analysed by gas chromatography coupled to a quadruple Electron Impact Mass Spectrometer and Isotope Ratio Mass Spectrometry (GCMS-IRMS). The GC column effluent was subsequently split equally between the two detectors. A transmethylation derivitisation was performed to volatise such lipids as free fatty acids and glycerides, but most importantly phospholipids. Phospholipids (PLFAs) are important as they are the main component of microbial cell membranes and can be a vital source of lipid biomarkers. In the case of the PLFAs, this procedure cleaves the fatty acid side chain from the glycerol backbone/polar phosphorus head group and methylates to form volatile fatty acid methyl esters (FAMEs). An aliquot of the total extract (200 µl) was evaporated to dryness before derivitisation. The method employed was a transesterification reaction involving sodium methoxide (50 µl; Christie, 1982; Hughes *et al*. 1986). The solution was vortexed and heated for 10 minutes at 50^{o}C. Excess sodium methoxide was quenched with 450 µl deionised water and the resulting NaOH neutralised with 50 µl HCl (0.5 M). The newly formed methyl esters were extracted twice from the aqueous solution with 1.0 ml of hexane:chloroform [9:1]. The combined extracts were dried over sodium sulphate and evaporated to dryness before reconstitution in 100 µl of a 100 ppm hexane solution of Cholestane (internal standard) for GC injection. Determination of monounsaturated fatty acid double-bond position was performed by GCMS analysis of their dimethyl disulphide adducts (Nichols *et al*. 1986).

4.2.5 Solid Phase Extraction (SPE) of Phospholipid Fatty Acids (PLFAs)

A modified version of the procedure as reported by Pinkart *et al*. (1998) was used for the SPE of microbial lipids from the total lipid extract. The aminopropylsilica columns

(Alltech, UltraClean, Aminopropyl 4.0 ml/500 mg) were placed on the vacuum manifold (Alltech 12-port Vacuum Manifold, Alltech Associates, Deerfield, Illinois, USA). Column conditioning took place at 20 kPa. Aliquots of solvents were passed through the cartridge to remove air and/or any possible contaminants, with care taken to not let the column packing dry out. The sequence of solvents used was acetone, chloroform, methanol, 5% acetic acid in ether and hexane. Aliquots of 6.0 ml for each solvent were used.

The sample to be separated was reconstituted in 100 µl methanol:dichloromethane (50:50). The vacuum pressure was adjusted to 10 kPa before the sample was loaded. The lipid extract was fractionated into three components, neutral lipids, polyhydroxyalkanoates and polar lipids. The neutral lipids were isolated by eluting 6.0 ml chloroform, followed by 6.0 ml acetone for the PAHs. The third fraction containing the polar lipids (PLFAs) was collected using 6.0 ml methanol. These vials were evaporated to dryness under a nitrogen stream before the appropriate derivitisation technique employed. Please refer to section 2.2.10 and Fig. 2.1 for a description of the nomenclature for PLFAs.

4.2.6 Analysis by GCMS-IRMS

Samples were analysed using a gas chromatograph (GC; 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), with a split ratio of approximately 50/50. 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 (Air Products, BIP-X47S grade) was used as the carrier gas. The injection port and the GCMS interface were kept at 250 and 280°C, respectively. The ion source temperature was 280°C. The oven temperature of the gas chromatograph was programmed from 100 to 300°C at a rate of 6°C/minute after 1.5 minutes at 100°C. The column head pressure was 69.4 kPa. An aliquot of each sample (1 µl) was injected into the injection port of the GC using the splitless mode of injection, followed by an elution split after column to both mass spectrometry detectors. The GC effluent was diverted via a heart split valve to a ceramic combustion furnace (GC5, 650 mm X 0.3 mm i.d.) packed with a copper oxide/platinum catalyst heated to 850^{o}C. Water was removed from the combustion products by passing the effluent through a nafion membrane prior to the CO_2 entering

the IRMS (Isoprime Ltd, UK). 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). A standard deviation for the instrument was calculated to be ± δ 1.04 over a 10 run sequence of the 15 alkane mixture.

4.2.7 PCR and Cloning:

A limited analysis of the diversity of green-like and red-like RubisCO genes was performed on the incubated soils to assess if the gene diversity changed over the period of the incubation. Two different primer sets were used to target the green-like and red-like phylogenetic groups of *cbbL* genes. Genomic DNA was extracted directly from 0.50 g soil samples and purified using FastDNA spin kit for soil (MP Biomedicals, Illkirch, France) according to the manufacturer's instructions. Amplification of the RubisCO genes via PCR was performed using the primers designed and discussed by Selesi *et al.* (2005). Amplification conditions were as follows, 100 ng of DNA solution was reacted in 25 µl volumes containing 2.5 µl DreamTaq PCR master mix 2x (Fermentas, GmbH), 200 µM dNTP's (Fermentas, GmbH) and 50 pmol of the reverse and foreword primers (Sigma Aldrich, Haverhill, UK) and 1 U DreamTaq DNA polymerase (Fermentas, GmbH) and nuclease free water (Sigma Aldrich, Dublin, Ireland). PCR cycle conditions were as follows: 4 minutes initial denaturation at 95^{o}C, followed by 32 cycles of 1 minute of denaturation at 95^{o}C, 1 minute annealing at 57^{o}C, for the red-like and 67^{o}C for the green-like *cbbL* primers, and 1 minute elongation at 72^{o}C. The PCR products were subjected to a final extension step for 10 minutes at 72^{o}C. PCR cycle conditions were carried out using a Peltier Thermal Cycler (USA, Massachusetts). Aliquots of the PCR products were analysed in 1.2% (wt/vol) agarose gel (Bio-Sciences, Dublin, Ireland) by horizontal gel electrophoresis. DNA was visualised by UV excitation after staining with ethidium bromide (10 mg ml^{-1}). For cloning, PCR products from the selected samples of the expected size (1,100 bp for green-like and 800 bp for red-like *cbbL*) were excised and purified from agarose gel using Fermentas GeneJet, Gel extraction kit (Fermentas, York, United Kingdom) and a second PCR reaction was performed. Lysogeny broth (LB) agar was prepared (tryptone 10 g l^{-1}, yeast extract 5 g l^{-1}, NaCl 10 g l^{-1}, $MgSO_4$ (anhydrous) 2.4 g l^{-1}, KCl 186.04 g l^{-1}, bacteriological agar 1.5% [Hanahan, 1983]), and autoclaved at 121^{o}C for 15 minutes. A 50 mg ml^{-1} ampicillin sodium salt solution (Sigma Aldrich) in 30% ethanol was prepared and 0.22 µm filtered prior to applying 50 µl to each LB plate. Purified PCR

products were ligated into the vector pJET using 1.2/blunt cloning vector (Fermentas, York, United Kingdom), T4 DNA Ligase and the recommended procedure. Ligated products were transformed into Bioline α-select chemically competent *Escherichia coli* cells using the procedures laid out in CloneJET PCR Cloning Kit (Fermentas, York, United Kingdom). LB plates were incubated overnight at 37^{o}C and selected colonies inoculated into 10 mg ml^{-1} LB broth overnight at 37^{o}C. Plasmid DNA was extracted from all liquid cultures using GE Healthcare plasmidPrep spin mini kit (GE Healthcare, Little Chalfont, United Kingdom) according to the manufacturer's instructions. Restriction enzyme digest of 2.0 µl plasmid DNA, 2.0 µl x10 buffer H (Pharma Biotech, Amersham, United Kingdom), 15.5 µl nuclease free water, 0.5 µl BG1 II (Pharma Biotech, Amersham, United Kingdom). Samples were incubated at 37^{o}C for 2.5 hours and then heat shocked at 70^{o}C for 15 minutes in a water bath. Restriction fragments were visualised (UV fluorescence) on a 1% agarose gel to ensure correct fragment size.

Plasmid DNA from selected clones was diluted and sent for sequence analysis at the University of Dundee (Dundee Sequencing Services, Dundee, United Kingdom) using pJET 2.1 forward (5'-CGACTCACTATAGGGAGAGCGC-3') and reverse (5'-AAGAACATCGATTTTCCATGGCAG-3') primer sets. Sequences were compared to existing sequences at the National Centre for Biotechnology Information Database by BLAST search. All sequence data was checked using Pintail (Ashelford *et al*. 2005) for chimeric sequences. Sequence alignment was carried out using the CAP3 Sequence Assembly Program (Huang & Madan, 1999). Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 5 (Tamura *et al*. 2007, 2011).

4.3 Results

4.3.1 Soil pH and Electrical Conductivity

Measurements of the soil pH (Fig. 4.0) showed a gradual increase in soil acidity for the soils exposed to granulated S^0 in comparison to the blank (which remained constant at pH 8.0 ± 0.2). Both soils exposed to S^0 showed similar patterns of pH reduction with little activity for the first 5 days of incubation (initial lag phase) followed by a gradual and constant decline from slightly basic to slightly acidic soil environments. Soil pH decreased by 1.3 and 1.9 pH units for the $^{12}CO_2$ and $^{13}CO_2$ incubations respectively. Considering the short length of the incubation period (21 days) this shows that S^0 had an

acidulant effect. It was likely that the small drop in pH would be persistent in this soil and would potentially increase over time as more S^0 was oxidised (Wiedenfeld, 2011).

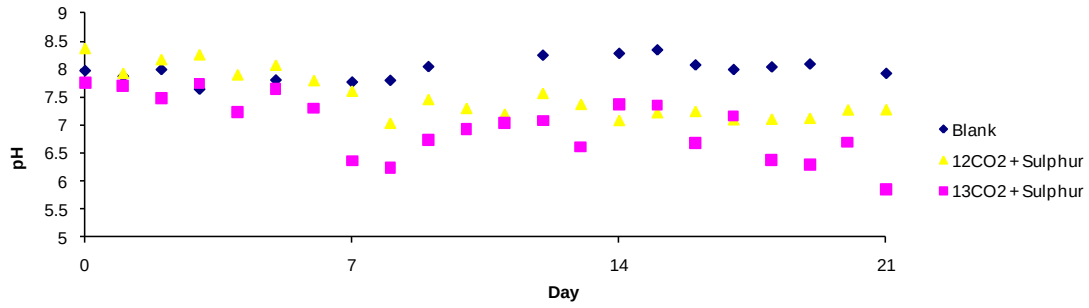


Fig. 4.0: pH measurements of incubated Hampstead Park soil exposed to S^0 over a 21 day period. Incubations carried out at 600 ppmv CO_2, 30°C and SWC maintained at 80%.

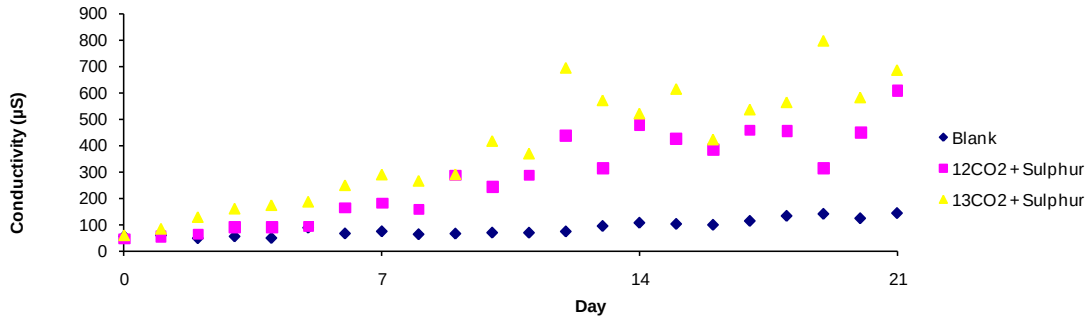


Fig. 4.1: EC measurements of incubated Hampstead Park soil exposed to S^0 over a 21 day period. Incubations carried out at 600 ppmv CO_2, 30°C and SWC maintained at 80%.

The application of S^0 has resulted in a net increase in EC to the experimental soil samples as observed in Fig. 4.1. The regular addition of 0.01 M $CaCl_2$ buffer (to maintain 80% SWC) resulted in an increase of EC by 93.5 ± 1.2 µS over the 21 days incubation. The experimental soils increase by an average of 650.9 ± 126.2 µS over the same period (according to the blank data). The increasing EC display a clear build up of ionic species in the soil medium most likely due to the assumed oxidation of S^0 to SO_4^{2-}.

4.3.2 ICP-AES Determination of S^0

Extraction and digestion of S^0 from soil was carried out efficiently and provided a high recovery rate (86.8 and 97.2% S^0 for soils HP1 and HP2 respectively) of initial substrate for both soil incubations according to day 0 extractions and measurements (e.g. immediately after initial inoculation). The generation of a calibration curve using a S^0 standard demonstrated the instrument stability (Fig. 4.2) and a detection range within

the required parameters (150 - ≤ 1 ppm). Absorption wavelength 182.034 nm (R^2 = 0.9998) was chosen for the analysis, although no major advantage was obvious.

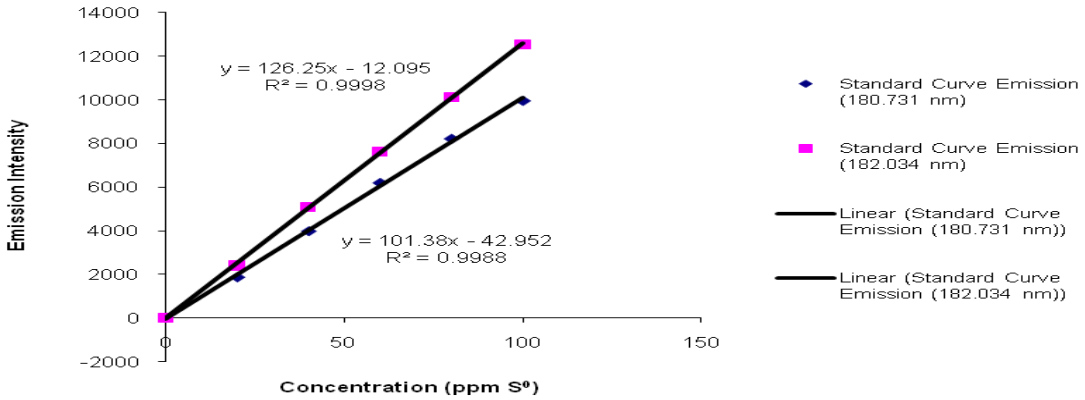


Fig. 4.2: Standard curve for quantitative ICP spectroscopy using emission lines 180.731 and 182.034 nm.

The rate of S^0 oxidation was perceived to be low but a clear declining trend was observed for both S^0 inoculated samples (Table 4.0 and Fig. 4.3). Blank 2 (abiotic soil control) was extracted and measured to ensure that no chemical or physical attribute of the soil was responsible for the declining trends, considering that leaching was not a factor in the experimental set up. The abiotic blank extractions showed no significant fluctuations of S^0 during the sampling regime with the concentration remaining at 148.6 ± 1.6 mg^{-1} S^0 kg throughout the experiment.

The data observed for both incubations showed an average removal of 10.65 ± 1.1 mg^{-1} S^0 kg^{-1} from the soil matrix over the 21 day period and hence only 7.1% of the total added substrate was converted to sulphur salts (mainly SO_4^{2-} and possibly small amounts of organic-S), these results are comparable to those observed for some New Zealand (Watkinson, 1989) and Canadian soils where it was believed the surface area of S^0 particles was considered a large factor in microbial oxidation capacity (Janzen & Bettany, 1987b). The observation of S^0 removal, no matter how slight, was a positive indication of aerobic S^0 oxidation by extant soil microorganisms.

Sample Name	Intensity 1	Intensity 2	Intensity 3	Average	SD	Concentration (ppm S^0)	SD
Blank 2 Day 1	20689.222	20499.795	20558.359	20582.45867	96.98578521	150.9343756	12.86320424
Blank 2 Day 3	20185.349	20588.254	20514.025	20429.20933	214.4257045	149.7205195	13.79342142
Blank 2 Day 5	20320.879	20422.105	20400.398	20381.12733	53.29347722	149.3396719	12.51712655
Blank 2 Day 7	20521.057	19889.875	20366.355	20259.09567	328.977359	148.3730845	14.70076126
Blank 2 Day 9	20588.014	20344.020	20369.254	20433.76267	134.1800807	149.7565855	13.15781252
Blank 2 Day 11	20552.274	20514.099	20399.354	20488.57567	79.59090406	150.1907479	12.725423
Blank 2 Day 13	20458.205	20512.233	20433.596	20468.01133	40.22521066	150.027862	12.41361553
Blank 2 Day 15	20453.014	20489.586	20402.369	20448.323	43.79732164	149.8719149	12.44190948
Blank 2 Day 17	20366.654	20342.258	20477.205	20395.37233	71.91126619	149.4525036	12.66459419
Blank 2 Day 19	20147.259	20452.534	20573.357	20391.05	219.6021131	149.4182673	13.83442268
Blank 2 Day 21	20255.225	20485.298	21001.255	20580.59267	382.0353326	150.9195954	15.12102244
Soil HP1 Day 1	18577.413	18799.102	18977.722	18784.746	200.5402755	136.6950647	13.68343783
Soil HP1 Day 3	18529.529	18431.657	18398.269	18453.15167	68.21885679	134.0685776	12.63534738
Soil HP1 Day 5	18369.876	18525.332	18537.896	18477.70133	93.59054656	134.2630304	12.83631126
Soil HP1 Day 7	18554.454	18575.568	18557.333	18562.45167	11.44992622	134.9343201	12.18569248
Soil HP1 Day 9	18425.298	18754.467	18554.897	18578.22067	165.8193376	135.0592231	13.4084205
Soil HP1 Day 11	18154.521	18201.251	18227.014	18194.262	36.74837892	132.0179663	12.38607627
Soil HP1 Day 13	18147.298	18099.968	18114.369	18120.545	24.2618931	131.4340693	12.28717341
Soil HP1 Day 15	18205.548	18214.014	18187.497	18202.353	13.54414416	132.0820535	12.20228035
Soil HP1 Day 17	18089.589	18001.322	18077.888	18056.26633	47.94150784	130.9249314	12.47473472
Soil HP1 Day 19	18024.259	18035.659	17919.547	17993.155	64.0007285	130.4250396	12.60193646
Soil HP1 Day 21	17807.278	17864.358	17988.589	17886.74167	92.70486007	129.5821617	12.82929592
Soil HP2 Day 1	20458.545	20655.326	20648.225	20587.36533	111.618165	150.9732403	12.97910428
Soil HP2 Day 3	20599.568	20589.389	20499.876	20562.94433	54.85539183	150.7798066	12.52949815
Soil HP2 Day 5	20614.522	20598.257	20608.219	20606.99933	8.200807655	151.1287571	12.15995689
Soil HP2 Day 7	20577.471	20563.990	20559.544	20567.00167	9.33525224	150.8119439	12.16894259
Soil HP2 Day 9	20501.000	20511.524	20499.335	20503.953	6.609318194	150.3125485	12.14735104
Soil HP2 Day 11	20474.254	20465.633	20433.904	20457.93033	21.24921011	149.9480125	12.26331058
Soil HP2 Day 13	20454.284	20444.205	20482.557	20460.34867	19.88225622	149.9671677	12.25248322
Soil HP2 Day 15	20324.258	20311.014	20344.253	20326.50833	16.73337326	148.9070462	12.22754157
Soil HP2 Day 17	20220.255	20298.221	20274.104	20264.19333	39.91666662	148.413462	12.41117162
Soil HP2 Day 19	20201.140	20210.289	20199.589	20203.67267	5.7821536	147.9340904	12.14079924
Soil HP2 Day 21	20041.257	20048.252	20057.999	20049.16933	8.408612632	146.7103017	12.16160287

Table 4.0: ICP emission intensity values (in triplicate) measured at emission intensity 182.034 nm. Mass calculation of S^0 in soil over the 21 day incubation period based upon on average emission intensity. Quantitation determined according to slope y =126.25x-12.095 (Fig. 4.2).

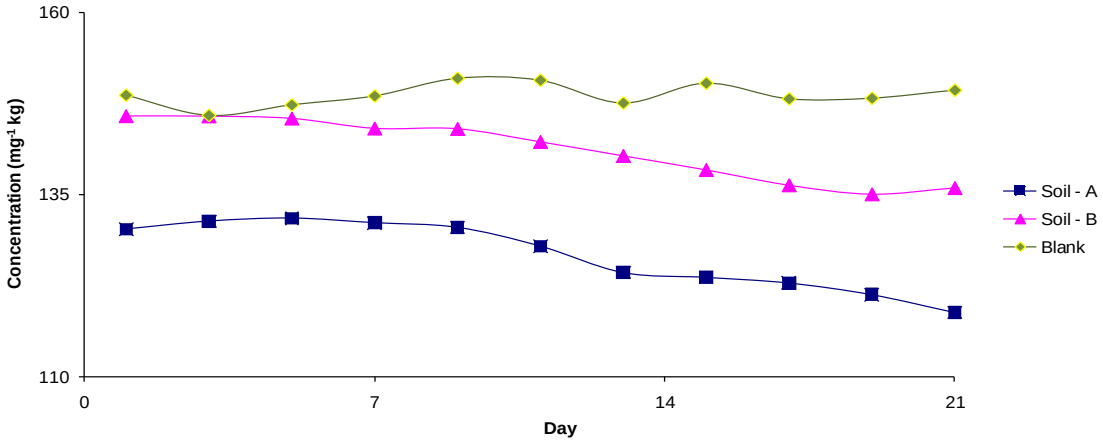


Fig. 4.3: Soil extractable S^0 plots from incubated Hampstead Park soil over 21 days. The S^0 content of both samples decreased by an average of 10.65 ± 1.1 mg^{-1} S^0 kg^{-1} dry soil as determined by ICP-AES.

4.3.3 Atmospheric CO_2 Plots

The soils were all incubated within the ECIC for a period of 21 days with daily sub-sampling (HP1, HP2, Blank 1 and Blank 2). The concentration of CO_2 substrate gas was monitored within the internal atmosphere of the ECIC with the ultimate aim of

determining the mass of CO_2 sequestered into the soil, if any. The data plot for HP2 and blank 2 were not analysed or displayed as the incubation took place under $^{13}CO_2$ conditions and this isotopic molecule falls primarily out of the IR absorption detectors range (Gosz *et al*. 1988). Blank 2 was an abiotic control of sterile soil to show that ^{13}C sequestration (GCMS-IRMS) did not take place in the absence of the microbiological soil fraction. The data plots in Fig. 4.4 are difficult to visually interpret because of the elevated CO_2 levels (≥ 600 ppmv), partial pressure effects (see chapter II, pages 91-93, 98-99 for interpretation of the CO_2 plots and partial pressure effects on the ECIC respectively) and the disruption caused by daily sub-sampling. The initial large increase in CO_2 on the first day of incubation (T_0-T_{24}) was attributed to the rewetting of air-dried soil. The rewetting of dry soil has been well documented to cause rapid soil respiration from both biotic and abiotic sources (Fischer, 2009; Thomson *et al*. 2010; Butterly *et al*. 2010). The HP1 CO_2 data plot in Fig. 4.4 shows that between the pulse events and daily sampling, an increasing trend in concentration occurred with the exception of day 10-11. This observation indicates that soil respiration was the dominant factor influencing atmospheric concentrations of CO_2 and not soil sequestration.

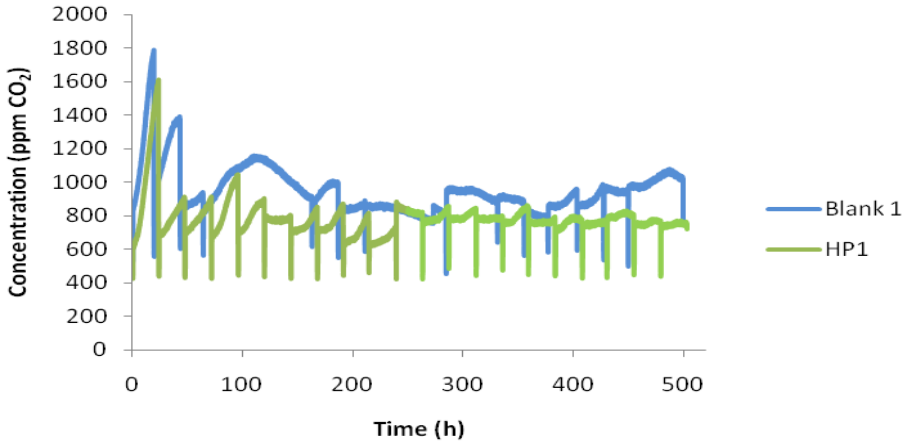


Fig. 4.4: Data plot showing atmospheric CO_2 concentraion (ppmv) of the ECIC during incubation of Hampstead Park soil not exposed to S^0 (Blank 1) and Hampstead Park soil, exposed to S^0 (HP1). Data points taken every 30 seconds for 500 hours (~21 days) to demonstrate CO_2 flux. Rapid increases in CO_2 are pump events where substrate gas was introduced to attain incubation concentrations of ≥ 600 ppmv, with the exception of between T_0-T_{48} for Blank 1 and T_0-T_{24} for HP1 (soil respiration due to re-wetting of dry soil). Rapid decreases in CO_2 (vertical trends) are due to exposure to the external atmosphere for sub-sampling (Blank 1 = 14 events; HP1 = 21 events) resulting in atmospheric equilibriation.

During the course of the 21 day incubation, HP1 required a total input volume of 1077 cm^3 CO_2 to achieve and maintain 600 ppmv (average peristaltic pump input rate = 5.44 cm^3 CO_2 s^{-1} and a total pump running time of 198 seconds). This introduced

volume was not inclusive of the CO_2 already present in the chamber atmosphere prior to sealing of the chamber door. Extrapolation of the daily decay curves (using the calculation method described in chapter II, page 95), and correcting for partial pressure on the ECIC internal concentration of CO_2, it was determined that no significant sequestration was detectable for the S^0 fertilised Hampstead Park soil using this method.

4.3.4 Extraction and Analysis of Phospholipid Fatty Acids (PLFAs)

Sub-samples of soil were taken at five regular intervals to measure the incorporation of ^{13}C into biological material using GCMS-IRMS. The hypothesis, that S^0 provides energy for growth of chemoautotrophic microorganisms in soil, which in turn sequester atmospheric CO_2 was tested. This would be indicated if during the incubation, under the presence of $^{13}CO_2$, build up of isotopic label in the extractable organic matter took place over time. SOM was extracted, and the PLFA fraction was separated using SPE. PLFAs were isolated to represent the living microbial community (as PLFAs rapidly break down upon cell death) and because these biomarkers represent a large proportion of the cell biomass (White *et al*. 1979; Frostegård & Bååth, 1996; Alvarez & Steinbüchel, 2002; Frostegård *et al*. 2011).

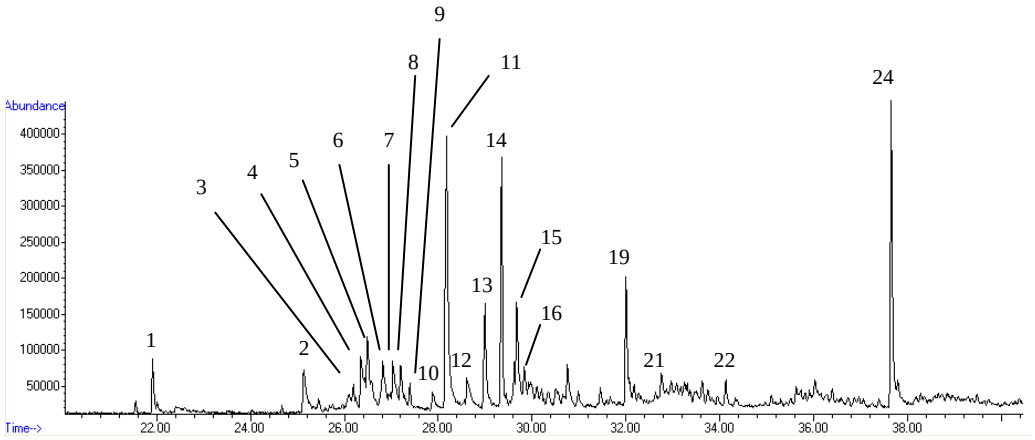


Fig. 4.5: GCMS chromatogram of Blank 1 extractable organics following NaOMe derivitisation (Time = minutes). Hampstead Park Soil was incubated for 21 days with no additional S^0 under a 600 ppmv $^{13}CO_2$ atmosphere. The soil was extracted for total lipids and PLFAs were separated using SPE to determine ^{13}C incorporation into PLFAs by means other than S^0 oxidation mediated chemoautotrophy. Please see Table 4.1 for peak identification by numbered sequence.

The extraction of PLFAs from the soil, designated as Blank 1, resulted in a chromatogram (Fig. 4.5) which clearly shows the presence of extractable organic matter after perfoming SPE to isolate the PLFA fraction. Identification of some of the major peaks (Table 4.1) resulted in only two abundant PLFAs (peak No's 13 and 19) both of

these were saturated fatty acids (15:0 and 14Me-18:0 respectively) and are commonly isolated in soil microbial studies (Kramer & Gleixner, 2006; Hanif *et al*. 2010). The majority of peaks were unknown, possibly due to plastizers leached from laboratory equipment during sample preparation (McDonald *et al*. 2008). The unknown compounds were of low peak area and most likely to be small amounts of contaminant compounds leached during the SPE procedure. It was likely that small amounts of PLFAs of varying composition were also extracted but masked by the impurities or the peak areas were too small for positive identification. The isotope ratio chromatogram shown in Fig. 4.6 clearly shows (top chromatogram of the $^{13}C/^{12}C$ ratio) that none of these peaks had a ^{13}C abundance higher than natural occuring (as these are incorporated into the ^{12}C peaks as well). The $^{13}C/^{12}C$ ratio observed in the GCMS-IRMS chromatogram, Blank 1 demonstrated no significant CO_2 seqestration over the 21 day incubation.

Peak No.	RT (min)	Description	AMU
1	21.913	2,6-Di-*tert* -butylphenol	206
2	25.137	Unknown	208
3	26.191	Unknown	221
4	26.355	Unknown	208
5	26.429	Unknown	208
6	26.490	Unknown	222
7	26.821	Unknown	222
8	27.035	Unknown	222
9	27.201	Unknown	236
10	27.396	Unknown	429
11	28.179	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	236
12	28.617	Unknown	236
13	28.991	14-Methylpentadecanoate	270
14	29.348	Methyl-3-(3,5-diterbutyl-4-hydroxyphenyl) propionate	292
15	29.624	Unknown	248
16	29.673	Phthalate	278
17	29.834	Siloxane	503
18	30.754	Phthalate	248
19	32.000	Methyloctadecanoate	535
20	32.074	Unknown	281
21	32.755	Unknown	429
22	34.126	Siloxane	429
23	37.652	Phthalate	279
24	37.793	Phthalate	429

Table 4.1: Identified peaks from Blank 1 after 21 days incubation. The Hampstead Park soil was incubated under an atmosphere of 600 ppmv $^{13}CO_2$ but no additional S^0 was added.

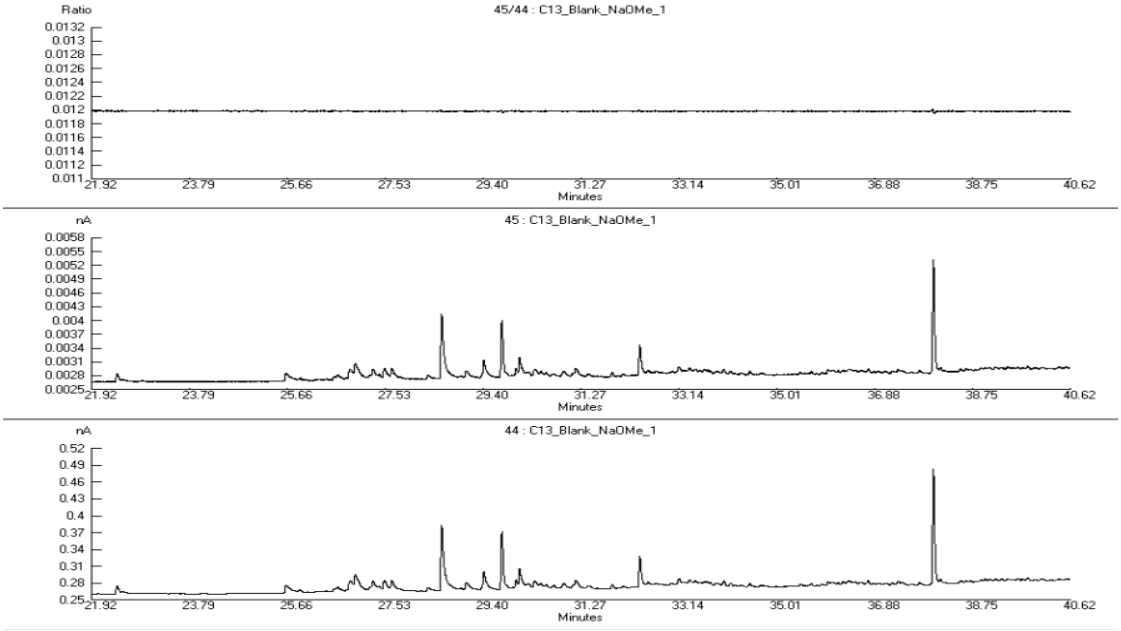


Fig. 4.6: IRMS $^{13}C/^{12}C$ ratio chromatogram showing Blank 1 (Hampstead Park soil) after 21 days incubation under 600 ppmv $^{13}CO_2$ atmosphere but no S^0 electron donor. Top chromatogram shows the ratio difference between ^{13}C and ^{12}C (45/44), middle and bottom chromatograms show the ^{13}C (45) and ^{12}C (44) peaks respectively as they are eluted from the GCMS (retention time in minutes)

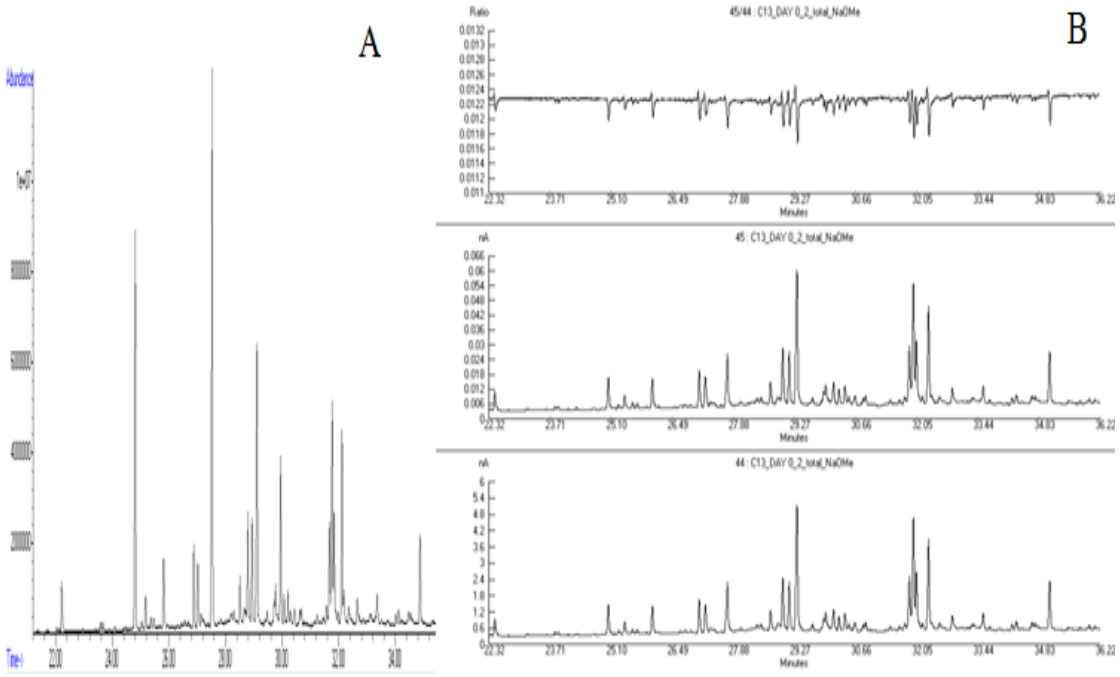


Fig. 4.7: A) GCMS chromatogram showing the total peaks of extractable PLFAs at day 0 from Hampstead Park soil exposed to S^0 and 600 ppmv $^{13}CO_2$ (Y-axis = abundance; X-axis = retention time in minutes). B) IRMS chromatogram showing the $^{13}C/^{12}C$ (45/44) ratio of Hampstead Park soil incubated in the presence of S^0 and 600 ppmv $^{13}CO_2$ at day 0. Top chromatogram shows the ratio difference between ^{13}C and ^{12}C (45/44), middle and bottom chromatograms show the ^{13}C (45) and ^{12}C (44) peaks respectively as they are eluted from the GCMS (X-axis = retention time in minutes).

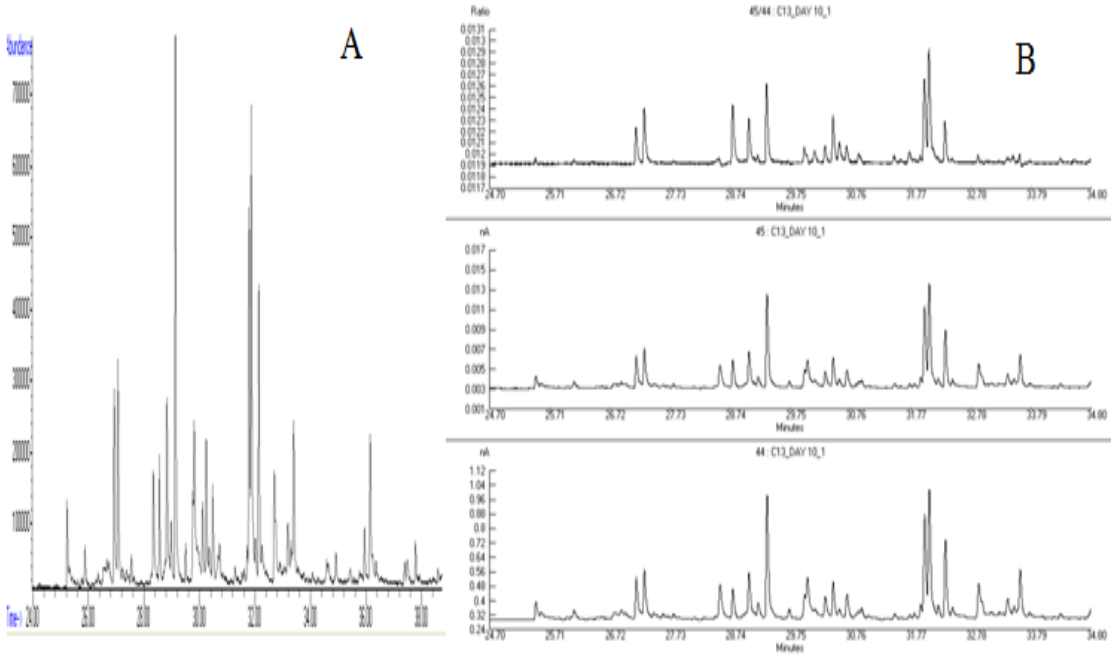


Fig. 4.8: A) GCMS chromatogram showing the total peaks of extractable PLFAs at day 10 from Hampstead Park soil exposed to S^0 and 600 ppmv $^{13}CO_2$ (Y-axis = abundance; X-axis = retention time in minutes). B) IRMS chromatogram showing the $^{13}C/^{12}C$ (45/44) ratio of Hampstead Park soil incubated in the presence of S^0 and 600 ppmv $^{13}CO_2$ at day 10. Top chromatogram shows the ratio difference between ^{13}C and ^{12}C (45/44), middle and bottom chromatograms show the ^{13}C (45) and ^{12}C (44) peaks respectively as they are eluted from the GCMS (X-axis = retention time in minutes).

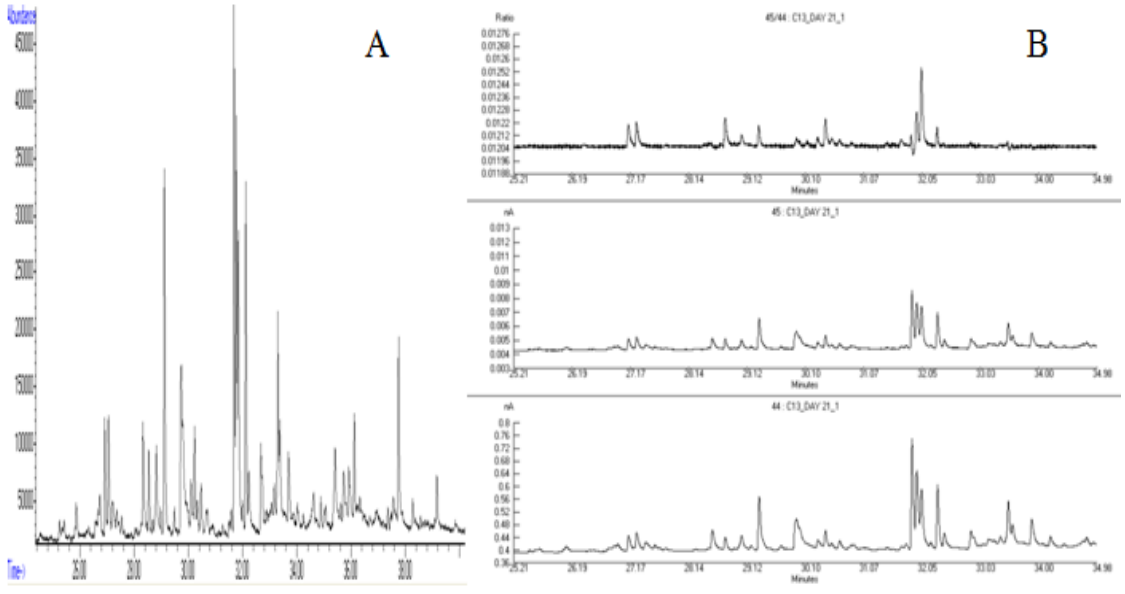


Fig. 4.9: A) GCMS chromatogram showing the total peaks of extractable PLFAs at day 21 from Hampstead Park soil exposed to S^0 and 600 ppmv $^{13}CO_2$ (Y-axis = abundance; X-axis = retention time in minutes). B) IRMS chromatogram showing the $^{13}C/^{12}C$ (45/44) ratio of Hampstead Park soil incubated in the presence of S^0 and 600 ppmv $^{13}CO_2$ at day 21. Top chromatogram shows the ratio difference between ^{13}C and ^{12}C (45/44), middle and bottom chromatograms show the ^{13}C (45) and ^{12}C (44) peaks respectively as they are eluted from the GCMS (X-axis = retention time in minutes).

The gradual increase in $^{13}C/^{12}C$ ratio was obvious (Figs. 4.7B, 4.8B and 4.9B). At day 0, no ^{13}C peaks of significance were greater than natural abundance (peaks with corresponding downward peaks are of no significance and represent molecules consisting of predominantly ^{12}C isotope), whereas, days 10 and 21 show clear enrichment of delta (δ) ^{13}C over natural abundance ratios. The top chromatogram in Figs. 4.8B and 4.9B show peaks that have been significantly enriched and using the retention time, the identity of the peaks can be ascertained by cross-reference with the accompanying GCMS chromatogram (Figs. 4.8A and 4.9A). Sub-samples were taken at several points during the incubation to provide a time study demonstrating δ ^{13}C incorporation into PLFAs. For the five dates selected, a corresponding table is provided (Tables 4.2, 4.3, 4.4, 4.5 and 4.6) and the δ ^{13}C values for PLFAs for each sampling event have been presented in Fig. 4.10.

Day 0			$^{13}CO_2$			$^{12}CO_2$
Systematic Name	**Shorthand**	**AMU**	**RT (Sec)**	**Major Area**	**delta ^{13}C**	**delta ^{13}C**
Methyltetradecanoate	14:0	242	1556.8	2.9913E-10	**-13.35**	**NA**
Methyltetradecanoate	14:0	256	1626.0	1.2723E-10	**-31.98**	**-22.77**
Methylpentadecanoate	15:0	256	1634.2	1.5464E-10	**-34.65**	**-27.36**
Methylpentadecanoate	15:0	270	1723.6	7.1376E-11	**-18.96**	**-31.28**
9, Methylhexadecenoate	16:1ω9	268	1739.1	1.7422E-10	**-20.63**	**-28.60**
11, Methylhexadecenoate	16:1ω11	268	1743.8	1.3847E-09	**-17.57**	**-27.14**
Methylhexadecanoate	16:0	270	1756.9	5.9317E-10	**-27.79**	**-25.12**
Methylhexadecanoate	16:0	284	1816.4	6.0429E-11	**-25.15**	**-25.22**
Methylheptadecanoate	17:0	284	1824.1	1.129E-10	**-22.29**	**-25.07**
9, Methylenehexadecanoic acid	*Cy* 16:0	282	1838.1	9.3371E-11	**-28.51**	**-25.21**
9, Methyloctadecenoate	18:1ω9	296	1915.7	5.4786E-10	**-24.38**	**-24.95**
11, Methyloctadecenoate	18:1ω11	296	1920.4	7.415E-10	**-31.60**	**-28.55**
Methyloctadecanoate	18:0	298	1937.0	2.3863E-10	**-22.91**	**-23.59**
Methyloctadecanoate	18:0	312	1970.5	2.1233E-10	**-19.89**	**-21.62**
Methyloctadecanoate	18:0	312	2000.2	6.8068E-11	**-41.47**	**-28.28**

Table 4.2: PLFAs extracted and qualitatively assessed using GCMS-IRMS for day 0 incubation of Hampstead Park soil exposed to S^0 and $^{13}CO_2$ for 21 days. AMU = Atomic Mass Unit, RT = Retention Time.

Day 6			$^{13}CO_2$			$^{12}CO_2$
Systematic Name	**Shorthand**	**AMU**	**RT (Sec)**	**Major Area**	**delta ^{13}C**	**delta ^{13}C**
Methyltetradecanoate	14:0	242	1559.8	7.158E-11	**-18.19**	**-30.10**
Methyltetradecanoate	14:0	256	1626.2	1.0782E-10	**79.36**	**-24.20**
Methylpentadecanoate	15:0	256	1634.2	2.1091E-10	**40.64**	**-27.27**
Methylpentadecanoate	15:0	270	1723.4	7.3134E-11	**82.40**	**-32.14**
9, Methylhexadecenoate	16:1ω9	268	1739.4	2.8087E-10	**35.54**	**-5.30**
11, Methylhexadecenoate	16:1ω11	268	1748.9	8.5499E-11	**42.94**	**-26.91**
Methylhexadecanoate	16:0	270	1757.3	5.9008E-10	**75.08**	**3.63**
Methylhexadecanoate	16:0	284	1816.2	2.6562E-10	**-4.33**	**-25.44**
Methylheptadecanoate	17:0	284	1824.1	2.1709E-10	**68.28**	**-27.15**
9, Methylenehexadecanoic acid	*Cy* 16:0	282	1838.2	1.6618E-10	**-7.53**	**31.35**
9, Methyloctadecenoate	18:1ω9	296	1915.8	6.4732E-10	**-2.26**	**-24.66**
11, Methyloctadecenoate	18:1ω11	296	1920.6	8.3945E-10	**121.89**	**2.11**
Methyloctadecanoate	18:0	298	1937.1	2.6857E-10	**50.75**	**-20.73**
Methyloctadecanoate	18:0	312	1970.6	2.237E-10	**-9.96**	**-21.34**
Methyloctadecanoate	18:0	312	2000.2	1.0414E-10	**-12.32**	**-25.40**

Table 4.3: PLFAs extracted and qualitatively assessed using GCMS-IRMS for day 6 incubation of Hampstead Park soil exposed to S^0 and $^{13}CO_2$ for 21 days. AMU = Atomic Mass Unit, RT = Retention Time.

Day 10			$^{13}CO_2$			$^{12}CO_2$
Systematic Name	**Shorthand**	**AMU**	**RT (Sec)**	**Major Area**	**delta ^{13}C**	**delta ^{13}C**
Methyltetradecanoate	14:0	242	1528.1	2.206E-10	**4.89**	**-31.14**
Methyltetradecanoate	14:0	256	1629.3	7.0309E-10	**127.42**	**-25.81**
Methylpentadecanoate	15:0	256	1637.6	7.684E-10	**201.09**	**-25.32**
Methylpentadecanoate	15:0	270	1726.6	4.2701E-10	**389.43**	**-26.23**
9, Methylhexadecenoate	16:1ω9	268	1743.0	8.082E-10	**146.59**	**-26.58**
11, Methylhexadecenoate	16:1ω11	268	1752.2	2.3334E-10	**19.37**	**-25.95**
Methylhexadecanoate	16:0	270	1761.2	1.9637E-09	**115.45**	**-25.77**
Methylhexadecanoate	16:0	284	1819.9	3.0109E-10	**95.21**	**-25.40**
Methylheptadecanoate	17:0	284	1827.8	4.6265E-10	**225.95**	**-24.27**
9, Methylenehexadecanoic acid	*Cy* 16:0	282	1841.8	3.8413E-10	**86.43**	**-26.23**
9, Methyloctadecenoate	18:1ω9	296	1920.1	1.3996E-09	**171.94**	**-25.32**
11, Methyloctadecenoate	18:1ω11	296	1924.8	1.8538E-09	**202.34**	**-29.35**
Methyloctadecanoate	18:0	298	1941.1	1.052E-09	**89.79**	**-25.60**
Methyloctadecanoate	18:0	312	1974.7	5.0542E-10	**5.62**	**-21.59**
Methyloctadecanoate	18:0	312	2003.8	2.5917E-10	**41.76**	**-27.49**

Table 4.4: PLFAs extracted and qualitatively assessed using GCMS-IRMS for day 10 incubation of Hampstead Park soil exposed to S^0 and $^{13}CO_2$ for 21 days. AMU = Atomic Mass Unit, RT = Retention Time.

Day 16			$^{13}CO_2$			$^{12}CO_2$
Systematic Name	**Shorthand**	**AMU**	**RT (Sec)**	**Major Area**	**delta ^{13}C**	**delta ^{13}C**
Methyltetradecanoate	14:0	242	1528.3	2.3805E-11	**69.41**	**-25.75**
Methyltetradecanoate	14:0	256	1627.1	1.6385E-10	**227.84**	**-27.06**
Methylpentadecanoate	15:0	256	1635.3	2.1268E-10	**176.19**	**-28.30**
Methylpentadecanoate	15:0	270	1724.0	1.6802E-10	**420.23**	**-27.61**
9, Methylhexadecenoate	16:1ω9	268	1740.4	2.7203E-10	**152.05**	**-29.14**
11, Methylhexadecenoate	16:1ω11	268	1749.9	9.979E-11	**51.78**	**-28.41**
Methylhexadecanoate	16:0	270	1758.1	7.7943E-10	**111.16**	**-27.34**
Methylhexadecanoate	16:0	284	1816.9	9.888E-11	**161.45**	**-29.31**
Methylheptadecanoate	17:0	284	1824.7	2.3467E-10	**239.17**	**-24.87**
9, Methylenehexadecanoic acid	*Cy* 16:0	282	1838.7	1.3941E-10	**97.62**	**-27.38**
9, Methyloctadecenoate	18:1ω9	296	1916.6	6.7257E-10	**151.70**	**-25.70**
11, Methyloctadecenoate	18:1ω11	296	1921.3	1.0929E-09	**254.83**	**-29.95**
Methyloctadecanoate	18:0	298	1937.8	3.6815E-10	**164.55**	**-24.79**
Methyloctadecanoate	18:0	312	1971.2	2.9513E-10	**15.52**	**-22.60**
Methyloctadecanoate	18:0	312	2000.7	1.3208E-10	**50.48**	**-28.35**

Table 4.5: PLFAs extracted and qualitatively assessed using GCMS-IRMS for day 16 incubation of Hampstead Park soil exposed to S^0 and $^{13}CO_2$ for 21 days. AMU = Atomic Mass Unit, RT = Retention Time.

Day 21			$^{13}CO_2$			$^{12}CO_2$
Systematic Name	**Shorthand**	**AMU**	**RT (Sec)**	**Major Area**	**delta ^{13}C**	**delta ^{13}C**
Methyltetradecanoate	14:0	242	1528.4	2.343E-11	**81.59**	**-32.43**
Methyltetradecanoate	14:0	256	1628.0	1.7401E-10	**259.60**	**-28.07**
Methylpentadecanoate	15:0	256	1636.3	2.6006E-10	**206.66**	**-27.22**
Methylpentadecanoate	15:0	270	1725.3	1.0748E-10	**504.30**	**-25.19**
9, Methylhexadecenoate	16:1ω9	268	1741.7	1.9488E-10	**106.71**	**-26.64**
11, Methylhexadecenoate	16:1ω11	268	1751.3	4.0627E-11	**68.04**	**-25.06**
Methylhexadecanoate	16:0	270	1759.4	5.7571E-10	**28.17**	**-25.60**
Methylhexadecanoate	16:0	284	1818.4	1.4795E-10	**86.22**	**-23.12**
Methylheptadecanoate	17:0	284	1826.1	1.9101E-10	**275.86**	**-26.33**
9, Methylenehexadecanoic acid	*Cy* 16:0	282	1840.4	1.2303E-10	**94.24**	**-19.98**
9, Methyloctadecenoate	18:1ω9	296	1918.0	7.3605E-10	**74.31**	**-29.64**
11, Methyloctadecenoate	18:1ω11	296	1922.7	6.4414E-10	**254.80**	**-19.37**
Methyloctadecanoate	18:0	298	1938.9	5.2787E-10	**39.75**	**-27.27**
Methyloctadecanoate	18:0	312	1972.7	1.5913E-10	**23.95**	**-24.98**
Methyloctadecanoate	18:0	312	2002.1	1.493E-10	**12.22**	**-25.94**

Table 4.6: PLFAs extracted and qualitatively assessed using GCMS-IRMS for day 21 incubation of Hampstead Park soil exposed to S^0 and $^{13}CO_2$ for 21 days. AMU = Atomic Mass Unit, RT = Retention Time.

Normal and monounsaturated fatty acid methyl esters were abundant in all samples. Saturated fatty acids ranged from C_{14} to C_{18} and displayed strong even/odd predominance. No long chain fatty acids (> C_{20}) were observed and therefore short chain fatty acids are the dominant product. Monounsaturated fatty acids were observed,

specifically; 16:1ω9, 16:1ω11, 18:1ω9 and 18:1ω11. The depleted δ ^{13}C values measured for the $^{12}CO_2$ incubations throughout the experimental period show that the isotopic abundance of δ ^{13}C in the sample lipids remained consistent with natural abundance. The extractions for the $^{12}CO_2$ exposed soil yielded the same variations of PLFAs (although depleted in ^{13}C) as the $^{13}CO_2$ exposed soils which indicates that similar microbiota were present and therefore the soil conditions were similar during both incubations (as they were run concurrently and not parallel). The δ ^{13}C values for the $^{13}CO_2$ exposed soil increased over time, suggesting continual production of PLFA producing organisms and hence, CO_2 sequestration. Fig. 4.10 demonstrates the increase in δ ^{13}C for the reported PLFAs and shows the higher enrichment of certain molecules over others. For instance, methylpentadecanoate (15:0) showed the highest level of enrichment in comparison to the second most enriched molecule (exclusive of days 0 and 6).

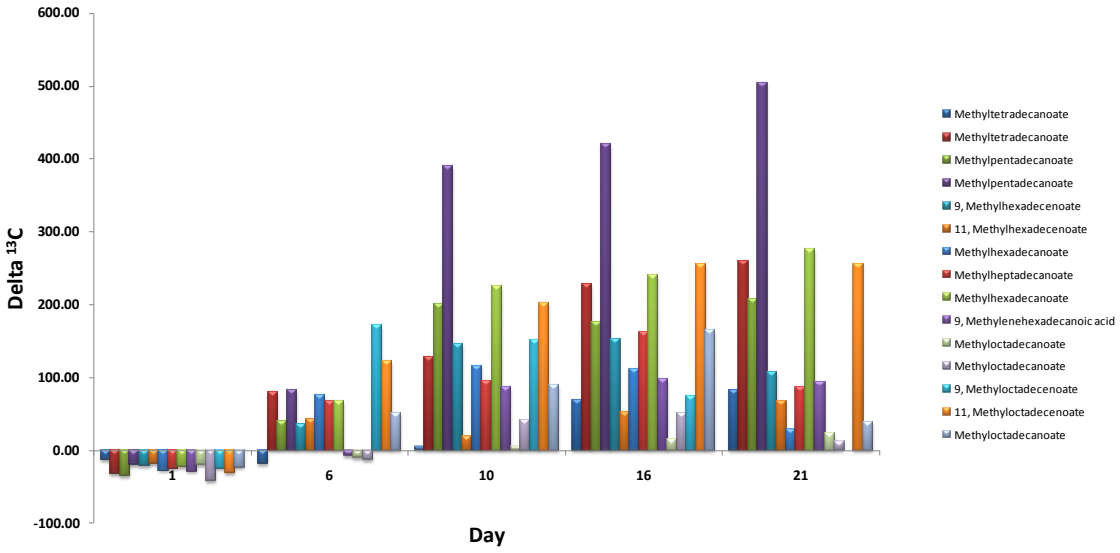


Fig. 4.10: Column chart showing the Delta ^{13}C of PLFAs over the selected sub-sample periods for the incubation of Hampstead Park soil (S^0 inoculated) over 21 days under a 600 ppmv $^{13}CO_2$ atmosphere. Chart shows the ^{13}C enrichment of each PLFA increasing against time.

4.3.5 Diversity of Green-like and Red-like RubisCO Form I Large-Subunit Genes

The results showed a low diversity of autotrophic activity utilising this normally abundant gene (RubisCO is the most abundant protein of Earth [Ellis, 1979]). Fig 4.11 shows the presence of DNA extracted directly from soil. The PCR products shown in Fig 4.12 showed that amplification of soil extracted DNA was successful when using the ribulose-1,5-biphosphate carboxylase/oxygenase (RubisCO) form I large-subunit genes as a functional biomarker. The green-like *cbbL* (*cbbL*g) PCR amplicons on day

21 all showed close homology to *Thiobacillus*-like species (Table 4.7), indicating that chemoautotrophic oxidation of S^0 was taking place. The presence of *Thiobacillus*-like species was encouraging as these species are well known S^0 oxidisers in environmental samples (Trudinger, 1956; Chapman, 1990; Smith & Strohl, 1991; Yang *et al*. 2010). The molecular phylogenetic tree has shown that the sequences are closely related with very low bootstrap errors (Fig. 4.13). No viable amplicons for the *cbbL*g primers could be detected at day 0, despite numerous attempts, which may indicate that these organisms were of extremely low abundance at the start of the experiment.

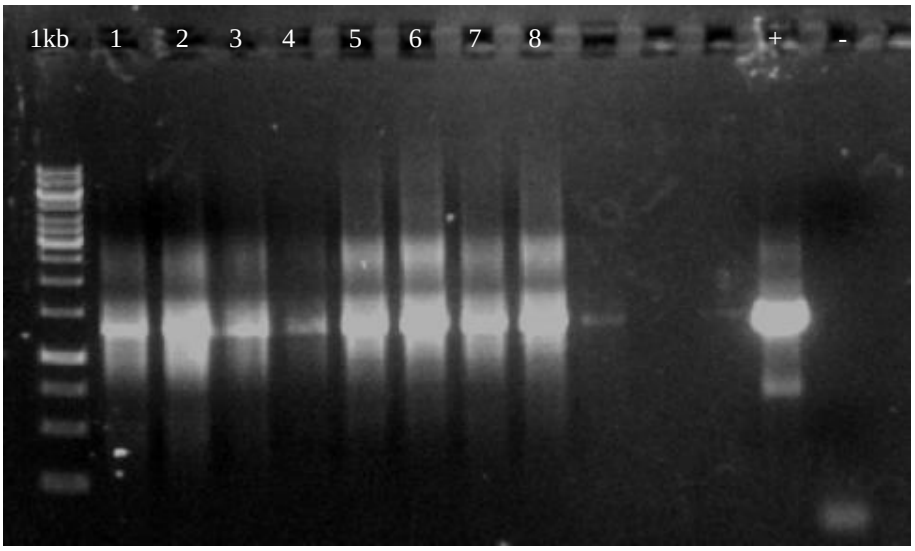
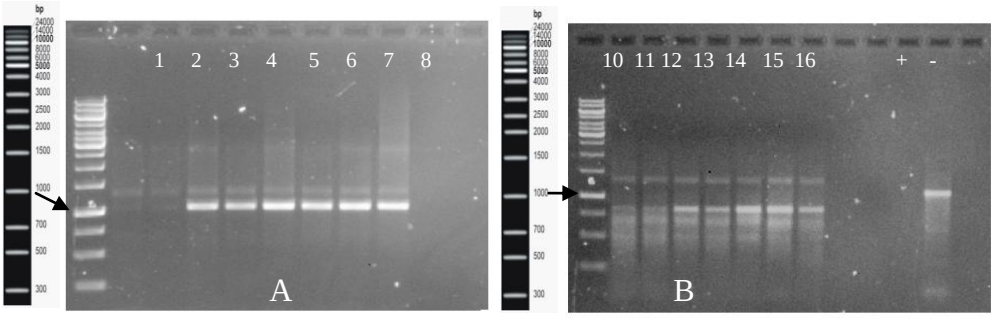


Fig. 4.11: Agarose gel showing total DNA extracted directly from Hampstead Park soil incubated in the presence of S^0 for 21 days. 1-2 = Day 0 HP1 and HP2; 3-4 = Day 6 HP1 and HP2; 5-6 = Day 16 HP1 and HP2; 7-8 = Day 21 HP1 and HP2. Positive control (+) consisted of *Pseudomonas putida* G7 (1/20 dilution) DNA and negative control (-) was nuclease-free water (Sigma Aldrich, Dublin).



Figs. 4.12: Agarose gels (1.1%) of PCR amplified soil DNA taken from S^0 incubated microcosms. A) *cbbL*g fragment of approximately 1200 bp (run against a 1 kb ladder) 1-2 = Day 0 HP1 and HP2; 3-4 = Day 6 HP1 and HP2; 5-6 = Day 16 HP1 and HP2; 7-8 = Day 21 HP1 and HP2. B) *cbbL*r fragment of approximately 800 bp (run against a 1 kb ladder) 9-10 = Day 0 HP1 and HP2; 11-12 = Day 6 HP1 and HP2; 13-14 = Day 16 HP1 and HP2; 15-16 = Day 21 HP1 and HP2. Positive (+) and negative (-) controls

were *Pseudomonas putida* G7 and nuclease-free water respectively. Arrows indicate location of the 1000 bp DNA fragment for reference.

The red-like *cbbL* (*cbbL*r*)* gene probe also provided few viable clones in which the correct fragment size had been inserted. For the viable sequences retrieved, only x4 closest matches to known species were attributed and all were of low homology (Table 4.8). The low % homology observed for both *cbbL* gene types was a strong indication that the amplified DNA sequences were from species under-represented in the GenBank database and possibly of novel species. This finding was not surprising as the diversity of soil microbes is still largely unknown (Rondon *et al*. 1999, and references therein) and the amplification of specific genes does not indicate activity, but merely their presence in the sample. *Rhodopseudomonas palustris* is a purple non-sulphur phototrophic bacterium commonly isolated from soil and water samples (Oda *et al*. 2003). *R. palustris* is an anaerobic facultative phototroph commonly isolated from organic waste digesters (Jung *et al*. 1999; Nagadomi *et al*. 2000; Kim *et al*. 2004) and the phylogenetic tree suggests these two sequences are of high homology to one another (Fig. 4.14). *Nitrosospira multiformis* is an ammonia oxidising chemoautotroph commonly found in soil environments (Norton *et al*. 2008). This organism (formerly known as *Nitrosolobus multiformis*) grows strongly attached to the soil particle surface (Aakra *et al*. 2000) and in close association with nitrite oxidising bacteria such as the *Nitrobacter* genera. The closest match to the third organism was *Pseudomonas aeruginosa*, a common bacterium that thrives in most natural and man-made habitats. No records of *P. aeruginosa* carrying the *cbbL* gene could be located in the literature but due to the low % similarity of the BLAST search to this species and the high similarity of other genes found in *Pseudonomads* such as *cbbQ* genes (Yokoyama *et al*. 1995), the likelihood of the sequence being directly related to *P. aeruginosa* was low.

Sample Name	Accession No.	Closest Match / Description	Max Score	Total score	Query Coverage (%)	E Value	Max Identity (%)	Reference
HPt21Green6.1	EU746410	*Thiobacillus thiophilus* strain D24TN	1370	1370	93	0	92	Kellermann & Griebler, (2009)
HPt21Green6.2	CP000116	*Thiobacillus denitrificans* ATCC 25259, complete genome	1333	1333	100	0	89	Beller *et al*. (2006)
HPt21Green6.3	CP000116	*Thiobacillus denitrificans* ATCC 25259, complete genome(2)	1353	1353	99	0	89	Beller *et al*. (2006)
HPt21Green6.7	CP000116	*Thiobacillus denitrificans* ATCC 25259, complete genome(3)	1261	1261	100	0	88	Beller *et al*. (2006)

Table 4.7: Closest match sequence table for *cbbL*g amplicons for DNA extracted from soil/S^0 incubations after 21 days incubation. **The sequence isolates are designated with "HP", followed by a time (t) value and then the corresponding *cbbL* gene type (green) and the clone number.**

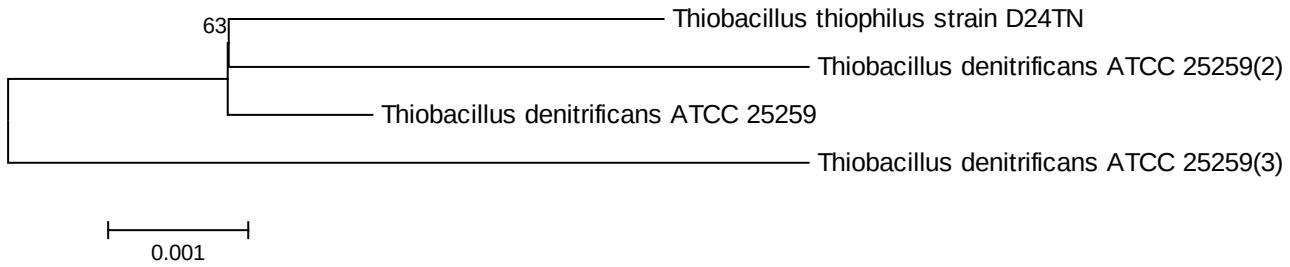


Fig. 4.13: Molecular Phylogenetic analysis by Maximum Likelihood method (1000 Bootstrap). Consensus tree of gene sequences reflecting the relationships of the 16S gene fragments of selected sequences from the S^0 oxidation study using the *cbbL*g forward and reverse primers. The bar represents 0.1 changes per nucleotide or amino acid.

Sample Name	Accession No.	Closest Match / Description	Max Score	Total score	Query Coverage (%)	E Value	Max Identity (%)	Reference
HPt21 Red4.1	BX572597	*Rhodopseudomonas palustris* CGA009 complete genome	809	809	95	0	85	Larimer *et al*. (2004)
HPt21 Red4.5	CP000103	*Nitrosospira multiformis* ATCC 25196, complete genome	800	800	100	0	83	Norton *et al*. (2008)
HPt21 Red4.13	CP000103	*Nitrosospira multiformis* ATCC 25196, complete genome(2)	834	834	100	0	83	Norton *et al*. (2008)
HPt21 Red4.18	CP000744	*Pseudomonas aeruginosa* PA7, complete genome	93.3	93.3	14	2.00^{E-15}	79	Roy *et al*. (2010)
HPt21 Red4.30	BX572597	*Rhodopseudomonas palustris* CGA009 complete genome(2)	753	753	99	0	82	Larimer *et al*. (2004)

Table 4.8: Closest match sequence table for *cbbL*r amplicons for DNA extracted from soil/S^0 incubations after 21 days incubation. **The sequence isolates are designated with "HP", followed by a time (t) value and then the corresponding *cbbL* gene type (red) and the clone number.**

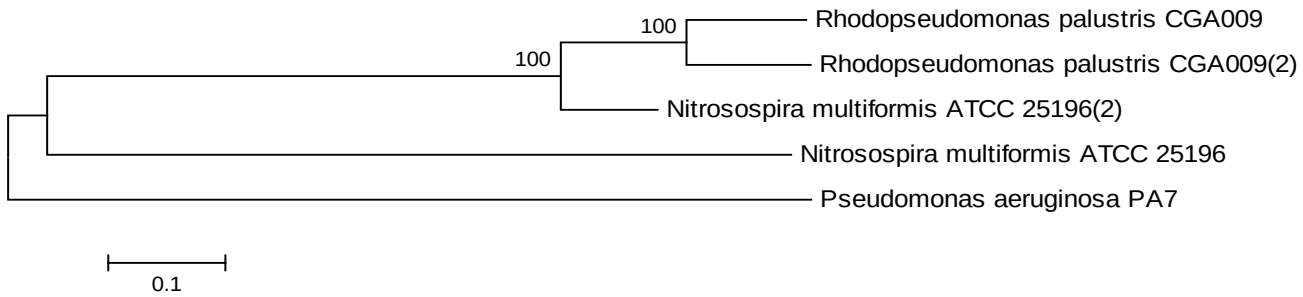


Fig. 4.14: **Molecular Phylogenetic analysis by Maximum Likelihood method (1000 Bootstrap). Consensus tree of gene sequences reflecting the relationships of the 16S gene fragments of selected sequences from the S^0 oxidation study using the *cbbL*r forward and reverse primers.** The bar represents 0.1 changes per nucleotide or amino acid.

4.4 Discussion

4.4.1 Soil pH and Electrical Conductivity

The results strongly correlate with existing evidence that S^0 was rapidly oxidised to SO_4^{2-} resulting in the inverse relationship observed between a declining pH and increasing EC (Janzen & Bettany, 1987c; Chapman, 1989; Watkinson, 1989; García de la Fuente *et al*. 2007; Yang *et al*. 2010). The increasing acidity of the soils over the relatively short incubations was significant, 1.3 and 1.9 pH units, considering that a relatively small amount of the S^0 was oxidised in comparison to the total application. The experimental conditions were designed to maximise CO_2 uptake potential with optimal temperature and the SWC being constantly maintained for the benefit of oxidising microorganisms. The S^0 particle size may have restricted the available surface area for microorganisms to exploit. The average particle size used in the experiment was ≤ 250 µm but Janzen & Bettany (1987b) found that particle size was crucial to oxidative capacity and maximum rates were observed when particle size was ~2 µm. However, the results shown in this work are still in line with the findings of Janzen & Bettany (1987b) for similar particle sizes and therefore a great potential for CO_2 uptake measurements using highly micronised S^0 was possible. The increases in EC demonstrate the increased build up of ionic species in the soil while the blank incubation reflects the contribution of the buffer used to maintain 80% SWC (daily additions). In an agricultural setting, the oxidation of S^0 leads to SO_4^{2-} accumulation and EC can serve as a measure of soluble nutrients (anions and cations) in the rhizosphere (Eigenberg *et al*. 2002) but it was beyond the scope of this study to observe if the EC would subsequently decrease after crop growth and subsequent harvesting (e.g. salt removal via translocation).

The pH and EC study would have greatly benefited from some statistical analysis such as a multivariate regression. This could be done where pH and EC are the bivariate dependent (response) variables and time is the independent (explanatory) variable. A study using continuous data, such as those provided by daily measurements, could also be analysed using a bivariate time series analysis. These approaches would help in assisting the visual interpretations made to confirm the significance of the changes in sample pH and EC. However, these approaches are complex and required considerable resources to complete and therefore were

considered beyond the scope of this particular study. The pH and EC measurements are considered to be complimentary to the overall analysis but without further statistical analysis their use is limited.

4.4.2 ICP-AES Determination of S^0

The incubation period lasted for 21 days but it was most likely that S^0 oxidation continues after this period as shown by Yang *et al*. (2010); García de la Fuente *et al*. (2007); Chapman (1989) and Nevell & Wainwright (1987). The removal of S^0 from the soil was much higher than that observed by Yang *et al.* (2010) over a similar time frame and temperature. Yang *et al*. (2010) used a higher soil inoculant and a lower substrate concentration and S^0 concentrations were determined according to SO_4^{2-} increases in concentration rather than real time quantitative analyses such as ICP-AES. The constant decay trend of extractable S^0 from the soil system indicates that, biological oxidation took place resulting in the production of SO_4^{2-}.

4.4.3 Atmospheric CO_2 Plots

The methods applied in chapters II and III for determining CO_2 sequestration of a soil chemoautotrophic community failed to detect significant rates of atmospheric capture for this particular incubation method. The lack of quantifiable determinations are unfortunate and the reasons are not immediately clear when one considers the physio-chemical data (pH, conductivity and ICP) and the PLFA analysis carried out using GCMS-IRMS which suggest otherwise. Increases in $\delta\ ^{13}C$ abundance, for specific PLFAs over the course of the incubation, suggests that CO_2 was being actively removed from the chamber atmosphere at an undefined rate. The results from chapters II and III suggest that some soils (including the soil used in this study) readily sequestrated CO_2 at a measurable rate using the onboard CO_2 IR detector and yet despite the strong evidence that chemoautotrophy may have been taking place, only soil respiration was obvious (Fig. 4.4). It was possible that active CO_2 sequestration may have been taking place in the sample but the rate of soil respiration was masking the ability to determine the uptake rate. This was clearly a limitation of the current CO_2 sequestration calculation method. Although the use of S^0 in chemoautotrophic studies is well known and documented (Smith & Strohl, 1991; and references therein) I have been unable to locate any research reports detailing CO_2 sequestration rates by

soil bacteria using S^0 as the terminal electron donor, and therefore any comparisons as to the expected uptake are not possible.

Several differences in the incubation method were applied to the soil in this experiment in comparison to those explored previously in this project. As the applicability to *in situ* environments was highly important to the project, an incubation resembling agricultural conditions was designed. The soil remained at ~80% saturation using a buffer solution. This was in contrast to the previous incubation methods (in preceding chapters) where enrichment cultures were grown in aerated liquid soil slurries giving the microbial population a well mixed, oxygenated environment to flourish. The removal of the minimal salts medium (Shiers *et al*. 2005; Madigan *et al*. 2009) would also effect microbial species as the minerals provided in this solution are required for exponential growth. The exposed soils were incubated at 30^{o}C (optimal growth temperature of most chemoautotrophic species [Smith & Strohl, 1991]) and considerable moisture loss was encountered every 24 hours requiring constant rewetting of the soil to ~80% WHC. This led to an accumulation of $CaCl_2$ in the soil as blank 2 indicates in the EC measurements (Fig. 4.1). The use of a sterile 0.01 M $CaCl_2$ buffer was considered to be prudent (Barron *et al.* 2010) as it was important to avoid adding wetting solutions of unknown composition to the sample (such as tap water) but also important to not add solutions that would impact negatively on the microbiological population due to osmotic stress e.g. deionised water (Csonka, 1989). The constant drying out and re-wetting of the soil may have also had a retardant effect on the ability of the extant microbial community to continue a S^0 based exponential growth phase unabated (Lund & Goksøyr, 1980).

The use of S^0 as the electron donor has to be considered as a possible reason why CO_2 sequestration was not measurable within the ECIC. S^0 was selected as the electron donor for this experiment because of its active use in agriculture rather than $Na_2S_2O_3$. The significance of the electron donor lies in the fact that all chemoautotrophic bacteria must carry out reversed electron transport (with the exception of hydrogen and carbon monoxide oxidisers [White, 2007]) to generate NADH for biosynthesis. This was because the electron donor is more electropositive than the NAD^+/NADH couple and hence reversed electron flow is required. The reversed electron flow is governed by the energy potential of the electron donor and

because of the relatively small gap between oxygen and most inorganic electron donors, the energy yields are quite low (see chapter I, Table 1.4). As the redox potential of S^0 oxidation is lower than that of $S_2O_3^{2-}$ oxidation, it is logical to assume that cell yields will also be lower (Kelly, 1982; Leduc & Ferroni, 1994; White, 2007). In addition, the insoluble nature of S^0 in aqueous solutions restricts its bioavailability to microorganisms that must first travel to the particles location and then physically attach themselves to the surface, rather than absorb it, in a solution state (Lefroy *et al.* 1997). As the work carried out by Janzen & Bettany (1987c) Watkinson (1989) and Yang *et al*. (2010) reported, as the S^0 particle size decreases, the available surface area to bacteria increases and thus leads to increased SO_4^{2-} release (Fox *et al*. 1964) i.e. increased biological oxidation of S^0.

4.4.4 Extraction and Analysis of Phospholipid Fatty Acids (PLFAs)

The extraction of PLFAs from soils is a technique rapidly gaining popularity with the scientific community to assess microbial influences on soil chemistry and with the number of peer-reviewed publications growing exponentially (Frostgård *et al*. 2011), the technique will be relied upon heavily in the future. The use of stable isotopes as tracers in microbial ecology studies is extensive (Boschker & Middleburg, 2002) and the use of deliberately added tracers for isotopic analysis provides a unique ability to link microbial identity with function. The results presented here clearly showed that at the beginning of the experiment (day 0), the $\delta\ ^{13}C$ signature of all extracted PLFAs were similar for both $^{12}CO_2$ and $^{13}CO_2$ experiments. As the $^{13}CO_2$ incubation progressed, the level of enrichment in the PLFAs continued to increase until the cessation of the experiment while $\delta\ ^{13}C$ for the $^{12}CO_2$ incubation remained depleted. No quantification of the PLFAs has been presented in this work as the GCMS-IRMS analysis was only intended to act as an indicator of CO_2 sequestration. The PLFA evidence presented here supports the main hypothesis that soil S^0 oxidation was a biologically driven process.

The significance that certain PLFAs became much more enriched in ^{13}C over others was difficult to put into context as all the straight chain alkanes observed are between C_{14} - C_{20} and, the most commonly found in environmental samples (Finean & Mitchell, 1981; Morgan & Winstanley, 1997). The presence of only short chained

PLFAs ($\leq C_{20}$) indicates microbial input to SOM (Ruess & Chamberlain, 2010; Hart *et al*. 2011b) rather than fresh inputs by higher plant material. A lack of PLFAs with enriched fatty acid chains > C_{20} or polyunsaturated species, indicates that no higher plant (including algae) or fungal activity was directly related to carbon capture (Ruess & Chamberlain, 2010). Several monounsaturated PLFAs were observed but these are common across taxa, but 18:1ω9 is common to fungi and Gram-positive bacteria (Bååth, 2003; Vestal & White, 1989). Further, the lack of general fungal biomarkers such as 16:1ω5 (Olsson *et al*. 1995; 2003), 18:1ω7 (Olsson, 1999) and 18:2ω6,9 (Frostegård & Bååth, 1996; Zelles, 1999) indicates that etomycorrhizal and arbuscular mycorrhizal fungi were not actively consuming biomass and/or exudates of the S^0 activated consortium. The lack of iso/anteiso branched fatty acids for the S^0 incubated samples was interesting as this may suggest very little input by Gram-positive bacteria to these soils (Zelles, 1997; 1999). The dominance of short straight-chains (< C_{20}) and cyclopropane PLFA's indicates that Gram-negative bacteria were the dominant genera's. Also, the significant $\delta\ ^{13}C$ measurements made for 18:1ω11 further compounds the evidence for Gram-negative activity as this is a known pre-cursor molecule for the production of cyclopropanes (Grogan & Cronan, 1997; Zechmeister-Bolternstern *et al*. 2011). Biomarker PLFAs reported for *Thiobacillus*-like species such as *i*17:1ω5, 10Me18:1ω6 and 11Me18:1ω6 (Kerger *et al*. 1986; Piotrowska-Seget & Mrozik, 2003) were not observed in this experiment but the chemoautotrophic genera from environmental samples are not very well represented in the literature and therefore significance of these particular biomarkers was tentative at present.

Comparison of the PLFAs from the $Na_2S_2O_3$ incubated samples from chapter III, where extensive iso/anteiso branched fatty acids were detected, indicates a different microbial consortium (Gram-positive bacterial biomarkers) for that experiment. The relevance was significant when one considers the differences in the incubation methods between these experiments, using soils sourced from the same location. The lack of introduced nutrients (M9 MSM), water saturation and electron donor source for the S^0 incubated soil may be responsible for the difference in PLFA profiles. This implies that different incubation methods that target the same consortia of CO_2 sequestering organisms could have implications for future soil manipulation studies. This was pertinent for microbial-stress studies, where soils may undergo

anthropogenic manipulation, which results in the target activity (in this case, CO_2 capture) yet with only the promotion of a small consortium of microorganisms. It was important to point out that the established paradigm, that different environmental conditions produce different PLFA profiles, was not under dispute but, that it was extremely pertinent to this type of study. The samples discussed in this chapter were intended to resemble *in situ* conditions of an agricultural setting. The experiment better represents the consortia of extant chemoautotrophic microbes currently active in the environment during relatively short periods (in comparison to the soil slurry technique employed in chapters II and III).

The stable isotopic labelling of PLFAs was obvious in the experiment as each of the selected time points demonstrates (Fig. 4.10). Specific PLFAs gradually increased in $\delta\ ^{13}C$ over time, with the five most commonly observed PLFA molecules (15:0, 16:0, 17:0 and 18:1ω11) reaching an average of + 300.24 ‰. The five selected PLFAs in this instance were simply chosen because of their dominant $\delta\ ^{13}C$ values ($\geq$ 200 ‰) but, this further compound the previous findings of their ubiquitous spread in environmental samples (Finean & Mitchell, 1981; Morgan & Winstanley, 1997) and indicates the contribution to this organic matter pool by chemoautotrophic species. The PLFAs with lower $\delta\ ^{13}C$ values ($\leq$ 200 ‰) are arguably of more interest. For instance, methyltetradecanoate (14:0) did not provide positive $\delta\ ^{13}C$ values until day 10. An increase in $\delta\ ^{13}C$ until day 21 was observed, but this delayed appearance could infer secondary feeding by organisms expressing this particular PLFA or other chemoautotrophs becoming active after a lag phase greater than 6 days. This was a difficult assumption to back up without a greater spread of biomarkers available for analysis, which may be present in the extractions but masked in the GCMS chromatogram by other dominating peaks.

The fractionation of ^{13}C by bacteria during synthesis reactions (Hayes, 2001) must also be taken into consideration when observing $\delta\ ^{13}C$ values and making any assumptions on activity. Autotrophic bacteria fractionate stable isotopes and this is reflected in their PLFA profiles (Cowie *et al*. 2009). Hayes (2001) reports that lipids are in general, depleted in ^{13}C by 1-6 ‰ compared to total biomass. Using bacterial PLFAs like *i*15:0 and *a*15:0 from a mixed culture, Boschker *et al*. (1999) determined that they were depleted between 4-6 ‰ relative to the substrate, and has subsequently

used this as a sufficient correction factor. Different lipids contain different fractionation ratios and at present, the reasons why, are largely unknown (Boschker & Middleburg, 2002). Bacterial fractionation of stable isotopes has not been accounted for in these samples as no suitable correction factor could be located in the literature (specifically for chemoautotrophic bacteria exposed to atmospheres containing 99% ^{13}C-CO_2). This is most likely because fractionation was not considered to be an operational issue when stimulating isotope levels via an artificially enriched substrate. By adding the isotopic pulse into a trophic food web, the difficulties presented by natural abundance are no longer of significant concern (Ruess & Chamberlain, 2010).

The identification of cyclopropanes (*cy*16:0 and *cy*18:0) was worthy of note as these can sometimes be a misinterpretation from the mass spectrum. This may happen because under electron bombardment in the mass spectrometer the cyclopropyl fatty acids can undergo rearrangement to produce double bonds and become indistinguishable from monoenoic fatty acids such as 9, methylheptadecenoate (Christie & Holman, 1966). This essentially means that a library search of a peak may provide a high percentage match to a cyclopropyl fatty acid when it is in fact a monoenoic fatty acid and vice versa. To distinguish these two molecules, basic data such as retention time and AMU are required. The operator must note the retention time where the molecule was eluted from the GC column in accordance with the reported AMU. If the reported retention time for the assumed cyclopropane was much later than that of the straight chained monoenoic fatty acids previously eluted (despite similar AMU's), then one can be confident in a correct identification. This was because monoenoic and cyclopropyl fatty acids have very different retention times (Christie, 1993) despite their similar AMU values. Therefore, a high degree of confidence can be placed on the identification of the Gram-negative bacterial biomarkers, *cy*16:0 and *cy*18:0, which became significantly $\delta\ ^{13}C$ enriched.

The $\delta\ ^{13}C$ enrichment data demonstrates that growth of microorganisms was actively taking place throughout the experiment and that a relatively short lag-phase of between 0-6 days was required to detect significant growth. Comparison of this data to the *cbbL*g gene PCR amplification, assumes the presence of a well known S^0 oxidising bacteria (*Thiobacillus denitrificans*) in the soil and therefore, a possible point source for $^{13}CO_2$ sequestration under the prescribed conditions. This experiment

was a good indication that chemoautotrophic bacteria were actively assimilating CO_2 for the majority of the experiment, which was in agreement with the results of other researchers (Garciá de la Fuente *et al*. 2007; Yang *et al*. 2010). Based upon the conclusions made by Yang *et al*. (2010), it would be an interesting aspect to follow up the experiment with a much longer incubation to observe if the PLFA profiles and the microbial ecology change significantly. An incubation period of 60 days would be a more appropriate time scale but due to the size limitations of the ECIC, the number of incubation vessels required (minimum of four) and the overall project coming to an end, it was not possible at this time.

4.4.5 Diversity of Green-like and Red-like RubisCO Form I Large-Subunit Genes

The amplification of the *cbbL* gene from the incubated soil has provided evidence for the presence of chemoautotrophic genes after 21 days. Daily sampling took place with the aim of observing species diversity over the course of the experiment but due to time constraints it was not possible to extract, purify and clone the DNA from all of these samples and regrettably, the decision was taken to only perform the experiment on days 0 and 21. The cloning experiment for day 0 provided negative results for both of the *cbbL* green- and red-like PCR probes, although DNA was successfully extracted from all sub-samples and amplified using the RubisCO *cbbL* gene specific primers. It was worthy of comment, that the amplification of both types of *cbbL* genes for day 0 provided faintly fluorescent products indicating the low abundance of the amplicons for time 0 (however, this was by no means a quantitative assessment). The *cbbL*g probe has shown that *Thiobacillus*-like species were present in the soil after it had been infused with the inorganic electron donor. S^0 oxidation by *Thiobacillus* and other chemoautotrophic soil bacteria, is a well known process (Friedrich *et al*. 2001), but the literature lacks evidence confirming the active oxidation of S^0 by this genus using modern molecular methods (most studies employed ‘defined medium growth’ and ‘most probable number’ techniques to identify and enumerate chemoautotrophs), especially in regards to common agricultural practices (Watkinson, 1989; Knights *et al*. 2001; Stamford *et al*. 2002; García de la Fuente *et al*. 2007; Stamford *et al*. 2007; Stamford *et al*. 2008; Yang *et al*. 2010).

At present, bioremediation (Norlund *et al*. 2009; Boonchayaanant *et al*. 2009) and bioleaching (Liang *et al*. 2009; Sonnleitner *et al*. 2011) receive the most in depth analyses using gene probes. Very little of the current evidence that *Thiobacillus was* the chief organism responsible for agricultural S^0 oxidation has been adequately tested using modern molecular ecological techniques. The experiment has provided a limited view of the potential types of soil bacteria present at the culmination of the incubation capable of CO_2 fixation. This information, although not conclusive, demonstrates the potential to perform PCR-based probes of soil microbiology to ascertain sources of CO_2 consumption during widespread and regularly employed agricultural practices.

4.5 Conclusions

Though oxidation of S^0 took place in the soil, not all of the proposed aims were met. For instance, the quantification of CO_2 sequestration from the atmosphere was an important aspect to the experiment. This was not achieved as the CO_2 flux was masked by soil CO_2 respiration. Thus, detection of chemoautotrophic microbial activity (based upon the oxidation of the S^0 electron donor) was too subtle to measure using IR absorption. PLFA profiles have demonstrated that CO_2 was used as an on-going substrate source during the incubation, as $\delta\ ^{13}C$ values increased consistently over time producing a range of PLFAs associated with Gram-negative bacteria. The accompanying ICP, pH and EC data all indicated that S^0 was actively being aerobically oxidised to produce an acidifying compound (most likely H_2SO_4) and this biochemical process was presumed to be the source of biological CO_2 fixation. Few conclusions can be made from the PLFA data in regards to species diversity due to the commonality of the molecules observed, but it was clear from the $\delta\ ^{13}C$ values that chemoautotrophy was an active biological process, when S^0 was applied.

It was apparent that further investigations are required into understanding the complex relationships between soil biotic and abiotic components. For instance, further intergration of geomorphological and geological investigations are required to assertain the presence of electron donor bearing minerals, such as pyrite or monazite in the test soils. Additional, PCR techniques should be used to investigate the contribution to soil S^0 oxidation by heterotrophic bacteria and eukaryotic organisms (fungi) as their contribution has also been documented in the literature (Vidyalakshmi

et al. 2009; Yang *et al*. 2010). Further work using molecular biology techniques should include CsCl gradient ultracentrifugation, as this would greatly enhance our undertanding using cutting edge protocols and instrumentation. To my knowledge, no measurements or estimations of CO_2 sequestration resulting from agricultural S^0 oxidation have been carried out in either *in vivo* or *in vitro* studies. This implies that a significant gap in the knowledge of CO_2 transfer between the atmosphere and the pedosphere exists. This unique opportunity to provide applicable data to soil carbon dynamics and atmospheric flux ratios was the primary driving force behind this experiment and a natural follow up to the work presented in chapters II and III, but due to the long incubation times of samples and the fact they could only be performed sequentially has prevented this from being accomplished (*tempus edax rerum*). Current work not presented in this thesis hopes to elucidate carbon sequestration to a greater degree by accurately modelling the soil flux using an algorithmic approach. At present, the groundwork for predicting soil chemoautotrophic CO_2 sequestration has been successfully demonstrated using the described techniques and the advances molecular ecology can bring to this particular subject are highly significant.

4.6 References

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5.0 General Conclusions & Future Work

5.1 General Conclusions

The importance of developing methods that measure carbon sequestration in the environment is a topic of great significance. Soil is currently thought to be a potential agent for greater carbon capture. Although, greater understanding of pedosphere dynamics must be attained before this may come to fruition. The deposition of organic compounds in the soil is a complex process and the discussed project only investigated the role of one group of primary producers. Understanding of this critical first step in naturally occuring carbon capture, is fundamental if the scientific community are to attain a complete understanding of the carbon cycle and eventually quantify the volumes transferred between biospheres.

The project aims were to develop techniques that could accurately measure carbon sequestration and thus link CO_2 to soil biomass. An enrichment culture was propogated, and subsequently measured by several cutting edge techniques. In chapter II the methodology to incubate a soil, calculate sequestration and qualitativley determine ^{13}C incorporation into cellular matter was demonstrated on several soils. With the establishment of a working method, chapter III demonstrated that a suite of investigative techniques could be used to elucidate the path of carbon taken from the atmosphere and transformed into organic matter as well as identifying the key bacterial species involved. The measurements made in chapter III are limited for climate modelling, but clearly demonstrate the power of the developed method to investigate environmental processes. The incubation performed in chapter IV was designed to mimic a currently used agricultural practice and therefore demonstrate the applicability of the developed methods to the natural world. The application of S^0 to soil landscapes is well established and understood from a chemical and biochemical aspect, but little work has been done using modern molecular ecological techniques to identify the key species responsible and no estimates of CO_2 sequestration have been proposed. Application of the measurement techniques developed in chapter II was a prime opportunity to contribute to the 'missing carbon' question in climate models. Mixed success was achieved, as carbon sequestration could not be determined for this

incubation type, but identification of chemoautotrophs using modern molecular biological techniques was achieved leaving scope for future investigations.

The project has conclusively shown that by using the custom built environmental incubation chamber (ECIC) and applying the techniques outlined in the preceeding chapters, a greater understanding of carbon flux and definitive measurements can be achieved. It is in my opinion that the methods and data discussed in this project can be used on an array of sample types to make credible estimations of biogeochemical activity. The relevance of this achivement should not be overlooked as despite the ongoing achievements in soil biology, soil chemistry and soil physics, these disciplines remain largely independent fields of research. This is doomed to failure as no single discipline will be able to fully understand the complex interactions taking place in complex biomaterials such as soil. Future scientific endevours to dicipher the non-renewable resource that is soil, will have to employ techniques from a wide range of investigative disciplines and I hope this project will contribute in some small way.

5.2 Future Work

In the future it would be prudent to study CO_2 sequestration in environmental samples resembling *in situ* conditions such as the agricultural S^0 fertiliser study. I believe a study into comparing typical soil samples under different stress factors will help to maximise CO_2 capture so that it can be quantified using the ECIC. If successful, proposals could be made for augmenting common agricultural practices to enhance soil carbon capture with not only the obvious benefit towards climate change mitigation and climate model scenarios, but economic benefits from the carbon trading scheme could be reaped.

Currently, a mathematical model is being developed, using the measurements made by the ECIC onboard instruments, to make highly accurate predictions of atmospheric CO_2 flux. This algorithm approach will be used to estimate, to a high degree of certainty, the mass of CO_2 at any point during the incubation and hence provide atmospheric flux plots to describe biological activity in a non-invasive manner. This achievement is hoped to bring greater confidence when providing data

to the greater scientific community. It may also be used to determine respiration rates as well as assimilation rates of CO_2 and therefore could resolve the undetermined CO_2 uptake rate for S^0 oxidation.

The application of molecular biology to soil studies has provided obvious benefits but at present only a limited view of the microbial community was observed. In the future it was hoped to apply quantitative PCR to the extractable DNA samples to determine the amount present at the time of sampling. Also, PCR probes of other extant lineages of soil organisms should be investigated, espcially for archaea and fungi. Archaea are increasingly being implicated in significant biogeological processes and it was possible their impact on CO_2 capture was highly underestimated. Qualitative assessment of soil fungi (despite it being well established that these organisms display no autotrophic traits) during carbon capture studies was also vital to assess the effects of potential cross-feeding, respiration rates and potential for nutrient/electron donor utilisation (as some fungi have been implicated in S^0 oxidation). Time studys over extended periods, following ^{13}C-pulse events is highly recommended to help elucidate the ultimate residence time of assimilated carbon directly sourced from CO_2 sequestration.

Conferences/Symposiums Attended

- Society for General Microbiology (SGM), Autumn Meeting Trinity College, Dublin, Ireland, 8-11th Sep. 2008.
- American Geophysical Union (AGU), Joint Assembly - The Meeting of the Americas, Toronto, Ontario, Canada, 24-27th May 2009.
- International Meeting on Organic Geochemistry (IMOG), 24th International Meeting on Organic Geochemistry, Bremen, Germany, 6-11th Sep. 2009.
- INFOMAR – Seabed 10 Ireland's Marine Mapping Strategy – A Ten Year Celebration: The Irish National Seabed Survey (INSS) and INFOMAR Programmes. Dublin, Ireland. 6-7th Oct. 2009.
- Society of Chemical Industry (SCI), Green Chemistry in Ireland: Including Highlights of Environmental Technology Projects Funded by the EPA, Dublin, Ireland, 15th April 2010.
- The Questor Meeting, Belfast, N. Ireland, 17th May 2010.
- European Commission, Social Media Conference: Communicating Environmental Issues Online, Dublin, Ireland, 4th Sep. 2010.
- 8th Annual EPA Postgraduate Seminar, Dublin, Ireland 11th Nov. 2010.
- Conference on Analytical Sciences Ireland 2011 – 6th CASi, Dublin, 21-22nd Feb. 2011.

Poster Presentations

- Kris M. Hart, Vasilelios Kouloumpos, Brian Moran, Brian P. Kelleher, Christopher CR. Allen & Andre Simpson (2009) *Ecological and Molecular Study of Soil Chemoautotrophic Microorganisms via the Uptake of Atmospheric* $^{13}CO_2$. American Geophysical Union, Joint Assembly - The Meeting of the Americas, Toronto, Ontario, Canada, 24-27th May 2009.

- Kris M. Hart, Vasilelios Kouloumpos, Brian Moran, Brian P. Kelleher, Christopher CR. Allen & Andre Simpson (2009) *Sequestration of Carbon Dioxide by Chemoautotrophic Soil Microorganisms*. IMOG, 24th International Meeting on Organic Geochemistry, Bremen, Germany, 6-11th Sep. 2009.

- Brian W. Moran, Kris Hart, Vasilelios Kouloumpos & Christopher CR. Allen (2010) *Sequestration of Carbon Dioxide by Chemoautotrophic Soil Microorganisms*. Environment Ireland 2010, Dublin, Ireland, 14th Sep. 2010.

- Kris M. Hart, Brian W. Moran, Christopher CR. Allen, Andre J. Simpson & Brian P. Kelleher (2011) *The Analysis of Soil Chemoautotrophy Using Molecular and Instrumental Techniques via the Uptake of $^{13}CO_2$*. Conference on Analytical Sciences Ireland, Dublin, 21-22nd February 2011.

- Brian T. Murphy, Christopher CR. Allen, Leonid Kulakov, Larkin Mike, Brian W. Moran, Kris M. Hart & Brian P. Kelleher (2011) *The Use of Isotopic Techniques to Track the Fate of Organic Pollutants in Environmental Systems*. Conference on Analytical Sciences Ireland, Dublin, 21-22nd February 2011.

Oral Presentations

- Kris M. Hart, Brian P. Kelleher and Christopher CR. Allen. *Stress induced molecular and ecological changes in soil autotrophs: carbon capture and novel compound prospecting* - Society of Chemical Industry, Green Chemistry in Ireland: Including highlights of Environmental Technology Projects Funded by the EPA, Dublin, Ireland, 15th April 2010.

- Kris M. Hart, Brian W. Moran, Andre J. Simpson, Christopher CR. Allen, Brian P. Kelleher. *Carbon capture by soil autotrophs* – 8th Annual EPA Postgraduate Seminar, Dublin, Ireland 11th November 2010 – INVITED LECTURE.

- Kris M. Hart, Brian W. Moran, Andre J. Simpson, Christopher CR. Allen, Seth Oppenheimer, Brian P. Kelleher. *CO_2 uptake by soil chemoautotrophs: identification, quantification and characterisation*. International Symposium on Interactions of Soil Minerals with Organic Components and Microorganisms – 3rd Intercongress Symposium of Commission 2.5, IUSS. 26th June -1st July 2011.

Publications Originating from this Thesis

- Hart KM., Moran BW., Kouloumbos V., Allen CCR., Kulakov LA., Simpson AJ., McNally DJ. & Kelleher BP*. (2011) An approach to the investigation of CO_2 uptake by soil microorganisms. *Biogeosciences Discussions* **8**, 9235-9281.

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Expeditions

- Bantry Bay and Dunmanus Bay - Pockmark ground-truthing survey in Dunmanus Bay, Co. Cork, Cruise Leg: CV0923, Celtic Voyager, 22-28th April 2008.