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CO₂ uptake by a soil microcosm

Kris M. Hart^a, Seth F. Oppenheimer^b, Brian W. Moran^a, Christopher C.R. Allen^c, Vassilis Kouloumbos^a, Andre J. Simpson^d, Leonid A. Kulakov^c, Leon Barron^e, Brian P. Kelleher^{a,f,*}

^aSchool of Chemical Sciences, Dublin City University, Glasnevin, Dublin 9, Ireland

^bDepartment of Mathematics and Statistics, Mississippi State University, P.O. Drawer MA, Mississippi State, MS 39759, USA

^cThe School of Biological Sciences, Queen's University Belfast, Medical Biology Centre, Lisburn Road, Belfast BT9 5AG, N. Ireland, UK

^dDepartment of Chemistry, Division of Physical and Environmental Science, University of Toronto at Scarborough, 1265 Military Trail, Toronto, Ontario M1C 1A4, Canada

^eAnalytical & Environmental Sciences Division, Kings College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NH, UK

^fThe Irish Separation Science Cluster, Ireland

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ABSTRACT

Sequestration of CO₂ via biological sinks is a matter of great scientific importance due to the potential lowering of atmospheric CO₂. In this study, a custom built incubation chamber was used to cultivate a soil microbial community to instigate chemoautotrophy of a temperate soil. Real-time atmospheric CO₂ concentrations were monitored and estimations of total CO₂ uptake were made. After careful background flux corrections, 4.52 ± 0.05 g CO₂ kg⁻¹ dry soil was sequestered from the chamber atmosphere over 40 h. Using isotopically labelled ¹³CO₂ and GCMS–IRMS, labelled fatty acids were identified after only a short incubation, hence confirming CO₂ sequestration for soil. The results of this *in vivo* study provide the ground work for future studies intending to mimic the *in situ* environment by providing a reliable method for investigating CO₂ uptake by soil microorganisms.

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1. Introduction

Soil carbon is reported to be approximately 3 times the size of the atmospheric pool and 4.5 times that of the biotic pool (Lal, 2004; Schmidt et al., 2011) and thus, it is important to develop and verify management procedures that encourage carbon stabilisation in soil. 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). In contrast to traditional thinking 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 is traditionally thought (Kelleher and 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 to

microbes (as extant organisms or necromass) far exceeds presently accepted values, with large contributions of microbial peptides/proteins found in the HS fraction (Kögel-Knabner, 2002; Kiem and Kögel-Knabner, 2003; Kindler et al., 2006; Simpson et al., 2007; Potthoff et al., 2008). 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. The activity of soil microorganisms still presents itself as a 'black box' due to the low cultivability of microbes, while being the primary agents of biogeochemical change (Madsen, 2005). Methods that enhance our ability to detect and track the flow of environmentally significant compounds through soil, such as the fate of CO₂, should be developed, so that the scientific community can design experiments usually difficult to monitor *in situ*.

Soil microorganisms are key players in the fixation and mobilisation of carbon and nitrogen, through both heterotrophic and autotrophic metabolic processes (Falkowski and 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. Autotrophic microorganisms (bacteria and archaea) capable of growth in the absence of light are generally described as chemoautotrophs (or chemolithotrophs). These prokaryotes use inorganic

* Corresponding author. School of Chemical Sciences, Dublin City University, Glasnevin, Dublin 9, Ireland. Tel.: +353 1 7005134; fax: +353 1 7005503.

E-mail address: brian.kelleher@dcu.ie (B.P. Kelleher).

substrates to derive energy for biosynthesis reactions via aerobic or anaerobic CO₂ 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 and Joffe, 1922; Starkey, 1935; Pedersen, 2000; Amend and Teske, 2005; Sorokin and Kuenen, 2005; Alfreider et al., 2009). Microbial uptake of atmospheric CO₂ via autotrophic processes is a well characterised biological phenomenon, but actual estimations of sequestration rates are rare in the literature (Miltner et al., 2004). These groups of niche microorganisms are suitable for designing initial experiments as the biomass can be controlled, depending on the supply of key nutrients. Also, the autotrophic nature of these particular species allows for the relatively conducive labelling of biomass in order to determine the flow of CO₂ from the atmosphere directly into the SOM fraction.

The purpose of this study is to develop a methodology to detect and quantify the uptake of CO₂ by soil chemoautotrophs under ideal growth conditions using a custom built environmental incubation chamber. Environmental growth chambers have been utilised for this type of study for various related sample types (Fleisher et al., 2008; Ferguson and Williams, 1974; Nakano et al., 2004) but few studies make attempts at quantifying the volume of CO₂ taken up during incubation. The integrity of the data from chamber studies relies heavily on the reliability of measurements (Baker et al., 2004). At present, we were only able to locate a single study in the literature that assessed the accuracy of a sealed chamber when making estimates of CO₂ uptake (Acock and Acock, 1989), with the majority of studies not discussing this experimental aspect despite its relevance to CO₂ uptake determinations (De Morais and Costa, 2007; Ohashi et al., 2005; Pringault et al., 1996). We have therefore developed a mathematical model that takes into account experimental uncertainties such as outgassing and abiotic interactions. The model was used to predict the real-time flux of CO₂ with the aim of estimating CO₂ uptake during the sequestration events of the microbial community.

Here, we incubated soils in the dark, while under elevated ¹²CO₂ and ¹³CO₂ respectively, make estimations of direct ¹²CO₂ uptake and employ compound specific gas chromatography mass spectrometry–isotope ratio mass spectrometry (GCMS–IRMS) to provide evidence of the uptake of CO₂ by soil microorganisms via the production of fatty acids (reported as fatty acid methyl esters [FAMES]). We demonstrate CO₂ uptake by extant soil chemoautotrophic microorganisms that have been provided with a suitable chemical electron donor to observe carbon sequestration. The overall aim of the study was to prepare a working method where soil chemoautotrophy can be induced and a single soil sample may be subjected to a suite of techniques to assist in the elucidation of soil carbon dynamics. It is hoped that the techniques developed herein will allow for investigation into CO₂ sequestering microcosms that attempt to mimic *in situ* conditions.

2. Experimental

2.1. Site details and pre-treatment

Four separate soils were collected for CO₂ uptake measurements and each have been designated an identification name (see Table 1). Only one of the soils (AS) was exposed to ¹³CO₂ in order to demonstrate the sequestration of ¹³C into fatty acids including the blank-control ¹³C incubation. Table 1 provides relevant characteristic information including sampling location and edaphic properties. Surface epipedon (A horizon) samples were collected and transferred aseptically to the laboratory and processed immediately. Roots and large debris were removed manually using aseptic technique. A CHN combustion analyser (Exeter Analytical CE440 elemental analyser) was used to determine the elemental composition and phosphorus (P) analysis by wet digestion according to April and Kokoasse (2009). 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. Permission from the relevant authorities at, Waterford County Council, Dublin City Council and the Botanic Gardens of Moscow State University was acquired.

2.2. Environmental carbon dioxide incubation chamber

The environmental carbon dioxide incubation chamber (ECIC) conducts temperature-controlled incubations of environmental samples in the presence of varying concentrations of CO₂. The ECIC consists of two units (Fig. 1). The outer unit controls temperature and houses the onboard CPU. The smaller inner unit has a 40.06 l capacity (inclusive of internal equipment and reaction vessel) and the outer door is sealed using a screw clamp, silicone foam strip and a thin layer of high vacuum grease to create an airtight seal. The ECIC is primarily used to measure and maintain the internal atmospheric concentration of CO₂ over short to long-term incubations while under constant temperature and atmospheric pressure. The inner chamber employs an infra-red (IR) CO₂ detector (GMM220, Vaisala Ltd.) with a detection limit range between 0 and 2000 ppmv (accuracy, including repeatability, non-linearity and calibration uncertainty ±1.5% at 25 °C). The IR detector has been calibrated to detect atmospheric CO₂ and employs a wavenumber (cm⁻¹) detection range between 2270 and 2390 cm⁻¹. The absorbance of ¹³CO₂ in the IR spectrum lies between 2250 and 2290 cm⁻¹ (Gosz et al., 1988) and hence, the ECIC only reports a small percentage of the true concentration of ¹³CO₂ (~20%), and therefore plots of this data have not been used to make quantitative measurements.

A calibration procedure for determining the pumping rate of CO₂ s⁻¹ was performed manually each time a new incubation experiment was performed. Briefly, the liquid CO₂ inlet tube leading into the inner chamber (from a pressurised gas cylinder) was detached and inserted into a 100 ml graduated cylinder. The

Table 1
Soil identifications, sampling locations and some edaphic properties.

Name	Location and sampling date	Soil designation	Coordinates	Moisture (%)	pH	C (%)	H (%)	N (%)	P (%)
AS	Abbeyside, Dungarven, Co. Waterford, Ireland (04/2009)	Acid brown earths	52° 5' 17.36" N, 7° 36' 38.19" W	22.9	7.3	4.25	0.58	0.15	0.21
HS	Alberts College Park (Hampstead Park), Glasnevin, Dublin, Ireland (04/2009)	Grey brown podzolic	53° 22' 54.63" N, 6° 15' 43.72" W	24.5	7.6	8.62	0.97	0.32	0.31
MS	Botanic Gardens of Moscow State University, Moscow, Russian Federation (03/2009)	Histosols cryic	55° 42' 37" N, 37° 31' 87" E	0.45 ^a	6.8	13.08	1.53	0.83	0.22
TS	Oak Park Research Centre, Carlow Town, Co. Carlow, Ireland (09/2008)	Grey brown podzolic	52° 51' 47.24" N, 6° 54' 11.34" W	20.2	6.5	3.61	0.33	0.17	0.56

^a Winter sampling most likely reason for low volume of moisture due to freezing conditions at location, March 2009.

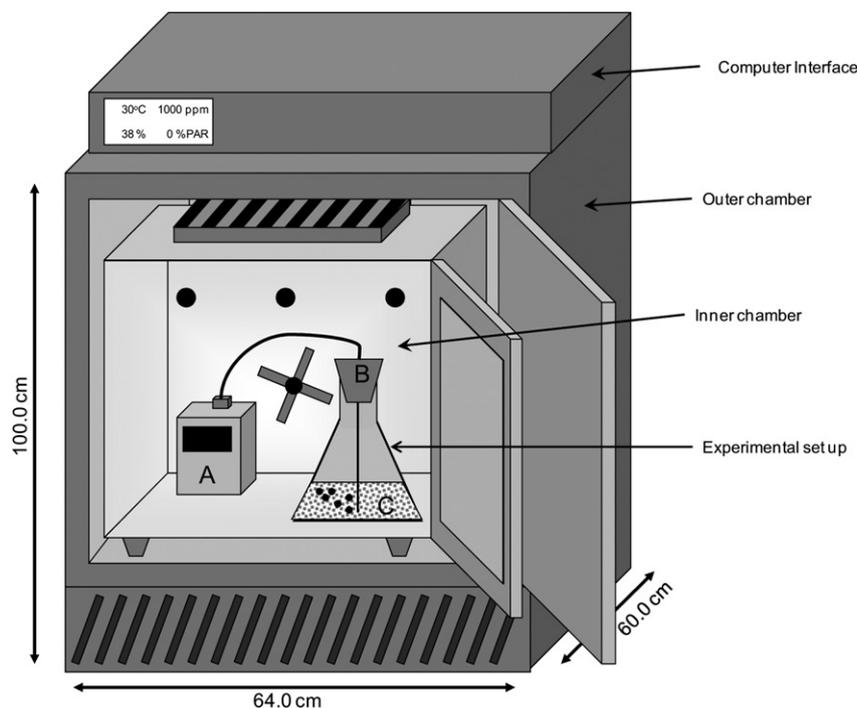


Fig. 1. Schematic diagram of the ECIC. Outer chamber used to control the temperature of the inner chamber via convection of the surrounding air temperature. The outer unit was also used to store the onboard microprocessor and interface unit. Inner chamber (41.5 × 37.0 cm) is the air-tight component and contains the experimental set up. Alphabetically labelled components are thus, A: battery powered air pump. B: Enclosed sample vessel with single entry and exit points for incoming and outgoing atmospheric gases. C: Soil sample and minimal salts medium continuously mixed by bubbling air. The inner unit is used to introduce the sample to the substrate gas and maintain atmospheric concentrations of CO₂. The internal air is circulated using a built-in fan and environmental conditions are monitored using temperature (°C), relative humidity (%RH), PAR (%) and CO₂ sensors.

100 ml graduated cylinder was filled with water and then inverted into a water filled 1000 ml beaker. Using the computer interface, the CO₂ concentration was increased to >500 ppmv. The peristaltic pump injects CO₂ into the graduated cylinder and a volume of water is displaced. The volume is manually recorded and the pump running time (s) noted ($n = 5$). The following calculation was used to determine the input volume per second; Volume H₂O Displaced (ml)/Pump Run Time (s) = cm³ s⁻¹ CO₂.

Introduction of CO₂ into the inner chamber is carried out using a peristaltic pump fed from a pressurised liquid CO₂ cylinder and a pre-programmed concentration setting in parts per million per volume (ppmv) CO₂. Internal measurements of CO₂ are taken every 30 s and relayed to the onboard CPU which maintains the concentration and records the data. The inner chamber houses the detector used to take measurements of internal CO₂ concentrations and provide a controlled environment where CO₂ may be administered to reaction/microcosm vessels.

2.3. Minimal salts medium

A modified minimal salts medium (MSM; 0.5 g l⁻¹ K₂HPO₄, 0.5 g l⁻¹ KH₂PO₄, 0.5 g l⁻¹ NH₄Cl, 0.5 g l⁻¹ KCl, 0.10 g l⁻¹ MgSO₄·7H₂O, 0.12 g l⁻¹ NaCl, 0.05 g l⁻¹ CaCl₂·2H₂O; Madigan et al., 2009; Shiers et al., 2005) was prepared and 1 ml of a trace metal solution (0.1 g l⁻¹ ZnSO₄·7H₂O, 0.3 g l⁻¹ MnCl₂·4H₂O, 0.3 g l⁻¹ H₃BO₃, 0.1 g l⁻¹ CuCl₂·2H₂O, 0.2 g l⁻¹ NiCl₂·6H₂O, 0.3 g l⁻¹ NaMoO₄·2H₂O, 1.0 g l⁻¹ FeSO₄·7H₂O) was added. The MSM was made up to 1000 ml and autoclaved at 121 °C for 15 min.

2.4. CO₂ profile soil incubations and ¹³CO₂ isotopic labelling

Soil incubations are identified according to the initials from the soil description and in numerical order according to presentation in this work (e.g., Abbeyside Soil, Experiment 1 (AS1) etc.).

2.4.1. Experiment 1

A known mass of AS1 soil (32.06 g) was transferred into the sterile amber jar (autoclaved at 115 °C for 15 min) and 300 ml of MSM was added aseptically. The sample was placed inside the ECIC (30 ± 0.2 °C, temperature equilibrated for 2 h previously). A sterilised tube was inserted into the jar lid through a prepared hole, while also attached to an autoclaved 0.2 μm gas filter. This was fitted to the outlet port of a battery powered air pump (Agile, A790). The battery air pump was activated (to bubble the internal chamber atmosphere through the sample vessel), and the chamber doors were sealed. No additional CO₂ was added to the chamber and the incubation period remained uninterrupted for 8 days (in the dark). After 8 days the sample was removed from the ECIC. The soil slurry was amended with 6.0 ml (0.2 μm sterile filtered) S₂O₃²⁻ stock solution and 294 ml MSM. The slurry was returned to the ECIC and the previous experimental conditions were repeated.

2.4.2. Experiment 2

Quantification of CO₂ sequestration was performed on incubations AS2, HS2, MS2 and TS2 slurries (30 ± 0.3 g) in sequential order using the following procedure: Slurries were amended with 6.0 ml (0.2 μm sterile filtered) 1000 mM S₂O₃²⁻ stock solution and prepared as above. All samples were incubated individually under dark conditions for 40 h. CO₂ concentration was maintained at 1000 ± 50 ppmv and no sub-sampling took place (i.e. uninterrupted incubations).

2.4.3. Experiment 3

Isotopic labelling of microbial organic matter using ¹³CO₂ took place under similar experimental circumstances as above with these brief exceptions. The ECIC was calibrated using ¹²CO₂ (Industrial grade, AirProducts) to determine the pumping rate (cm³ s⁻¹) required to achieve 1000 ppmv. The calculated pump

time was applied to the $^{13}\text{CO}_2$ (99% atom ^{13}C , Sigma Aldrich) incubation. It was determined that $5.166 \pm 0.2 \text{ cm}^3 \text{ s}^{-1} \text{ CO}_2$ was injected into the chamber each time the pump was activated. The internal atmosphere was vented and replaced with CO_2 -free air (Zero-Air Plus, Airproducts, UK) and then 12 s of pumping time was required to reach 1000 ppmv CO_2 from T_0 . Sequential soil slurries (AS3; $30 \pm 0.5 \text{ g}$, $n = 3$) were prepared as above before sealing the ECIC. CO_2 concentration of $1000 \pm 50 \text{ ppmv}$ was maintained constantly throughout the incubations. AS3 $^{13}\text{CO}_2$ labelled control (designated; AS3_{LC} $^{13}\text{CO}_2$) incubations were carried out verbatim under a 1000 ppmv $^{13}\text{CO}_2$ atmosphere with the exception that slurries were not amended with $\text{Na}_2\text{S}_2\text{O}_3$ (to test if isotopic enrichment was attributable to $\text{S}_2\text{O}_3^{2-}$ mediated reactions or other unknown abiotic soil processes). $^{12}\text{CO}_2$ incubations were performed prior to all isotopic-labelling experiments to provide unenriched spectra for identification purposes when compared to isotopically-labelled FAMES. All isotopic labelling incubations took place over 40 h. 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.

2.5. Analysis of $\text{S}_2\text{O}_3^{2-}$ concentration using ion chromatography

Chromatography separations were performed in duplicate on AS2, HS2 and TS2 slurries as follows. A 10 ml aliquot of supernatant was taken (T_0 and T_{40}) and filtered using a $0.45 \mu\text{m}$ syringe filter to remove particulate matter. Each sample was diluted 1/1000 and stored at $-20 \text{ }^\circ\text{C}$ until analysis. Blank incubations (e.g., no soil inoculum) consisted of sterile 294 ml MSM medium and 6.0 ml ($0.2 \mu\text{m}$ sterile filtered) 1000 mM $\text{S}_2\text{O}_3^{2-}$ stock solution, incubated for 40 h in the ECIC under the same environmental regime as the experimental soils. For chromatographic separations, a Dionex model DX500 ion chromatograph (Dionex Corporation, Sunnyvale, CA, USA) was employed comprising of a GP50 gradient pump at a constant flow rate of 0.30 ml min^{-1} , a CD20 conductivity detector and an EG40 eluent generator with EluGen Potassium Hydroxide cartridge (KOH II EGC) installed. The Rheodyne 7125 injection valve (Rheodyne, Cotati, CA USA) was fitted with a $25 \mu\text{l}$ sample loop for all work. Separations were carried out using a Dionex IonPac AG16 ($2.0 \text{ mm} \times 50 \text{ mm}$) guard and an AS17 ($2.0 \text{ mm} \times 250 \text{ mm}$) analytical column, with a capacity of approximately $42.5 \mu\text{eq}/\text{column}$. All experiments were carried out using suppressed conductivity detection with a Dionex 2 mm bore Atlas suppressor operated at 19 mA in autorecycle mode. For removal of anionic impurities from generated eluents, a Dionex continuously regenerating anion trap column (CR-ATC) was used. For data acquisition, PeakNet 6.60 SP1 data acquisition software (Dionex, Sunnyvale, CA, USA) was used. A five point calibration curve ($0\text{--}20 \text{ mM S}_2\text{O}_3^{2-}$) was prepared (ReagentPlus, Sigma-Aldrich). Calibration and sample determinations were calculated using peak height ($y = 1624x + 0.0002$; $R^2 = 0.9983$).

2.6. Calculations

High resolution $[\text{CO}_2]$ data consisted of measurements was taken every 30 s. Average CO_2 decay rate values during the experimental events were derived by selecting the CO_2 data and subtracting the final from the initial recorded value and then dividing by the total time of the CO_2 uptake event (h_c). We treat the concentration of CO_2 in the chamber in ppm as a function of time, where time will be measured in minutes, $C(t)$. We consider only two mechanisms that can cause a change in the CO_2 concentration in the chamber, 1) outgassing, 2) some soil/organism action that is either taking up or releasing CO_2 . In this case, the model looks like:

$$\frac{dC}{dt} = h_c(\bar{C} - C(t)) + f(t) \quad (1)$$

Where (\bar{C}) is the external CO_2 concentration (C) is the internal CO_2 concentration, and (f) is an unknown source/sink of CO_2 . External CO_2 concentration (the laboratory atmosphere) varies over time according to unpredictable environmental conditions. The laboratory atmospheric flux of CO_2 (in the vicinity of the ECIC) was measured in three sequential experiments using a pSense portable CO_2 detector (AQ Controls Ltd. Stomnevangen, Sweden), each lasting for 7 days to determine the mean concentration (data not shown).

We treat the external CO_2 concentration as constant using the average external CO_2 flux (data not shown), fixing it at a mean value $\bar{C} = 401.6 \pm 14.1 \text{ ppm}$. Final conversion factor for ppm to g is given as $((xvd)/10 \times 10^5)$. Where, chamber volume (v) is 40.059 l, density (d) of CO_2 is 1.97 g l^{-1} , and x is CO_2 (ppm). Soil respiration was incorporated into the correction factor when determining sequestration rates at a particular partial pressure. Respiration rates are variable for each soil and dependent on current conditions and therefore control soil incubations (i.e. not amended with $\text{Na}_2\text{S}_2\text{O}_3$) were carried out with each different soil to determine respiration rate. For a more detailed description of the mathematical model used to predict the CO_2 sequestration capacity of the microcosm, please see Supplementary data.

2.7. Extraction and analysis of soil organic matter

Total lipids were extracted using a modified version of the Bligh and Dyer (1959) method further developed by Otto and Simpson (2007). Extractions were carried out in pre-washed 40 ml Teflon tubes (Nalgene). This extraction method was selected as it is suitable for low molecular weight compounds extracted from incubation mediums and requires relatively little sample mass (Otto and Simpson, 2007). After incubation, the supernatant and soil were well mixed and a 40.0 ml aliquot was poured into a Teflon tube. After centrifugation at $12,000 \times g$ (20 min) the supernatant was discarded and the procedure repeated with deionised water. The remaining solid residue was washed twice with a 0.02 M dipotassium hydrogen phosphate (K_2HPO_4) buffer solution. The soil precipitate was freeze-dried prior to 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 derivatisation and analysis.

A transmethylation derivatisation was performed to volatilise free fatty acids and phospholipids. Free fatty acids and PLFAs were selected for this experiment to determine the presence of newly formed biomass from a wide as possible array of microbial sources. Although it is well known that PLFAs better represent the living fraction of biomass at the time of sampling than free fatty acids (for instance), the increase in $\delta^{13}\text{C}$ between sampling points should suffice to indicate the living biomass fraction. An aliquot of the total extract (200 μl) was evaporated to dryness before derivatisation. PLFAs were converted to fatty acid methyl esters (FAMES) using 50 μl sodium methoxide (NaOMe) in methanol, resulting in transesterification (Christie, 1982; Hughes et al., 1986). Determination of monounsaturated fatty acid double-bond position was performed by GCMS analysis of their dimethyl disulphide adducts (Nichols et al., 1986).

2.8. Analysis by GCMS–IRMS

FAMES were analysed using a gas chromatograph (Agilent Model 6890N) mass spectrometer (Agilent Model 5975C Quadrupole 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 (BIP-X47S grade, Air Products) 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. The oven temperature of the gas chromatograph was programmed from 100 to 300 °C at a rate of 6 °C/min after 1.5 min at 100 °C. The column head pressure was 69.4 kPa. An aliquot of each sample (1.0 μl) was injected into the injection port of the gas chromatograph using the splitless mode of injection, followed by an elution split after GC column to both mass spectrometer detectors. The GC effluent was diverted via a heart split valve to a ceramic combustion furnace (GC5, 650 mm × 0.3 mm i.d.) packed with a copper oxide/platinum catalyst heated to 850 °C. Water was removed from the combustion products by passing the effluent through a nafion membrane prior to the CO₂ entering the IRMS (Isoprime Ltd, UK). Reference gas CO₂ of known δ¹³C 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). The standard deviation for the instrument was calculated to be ±δ¹³C 1.04‰ over a 10 run sequence of the 15 alkane mixture. Identification of specific lipids was carried out by utilising NIST and Wiley spectral databases, with a spectral accuracy limit of ≥95%. Evidence of the stability and accuracy of the system can be seen from the δ¹³C values of the internal standard, cholestane, which when comparing the delta (δ) values from the ¹²CO₂ and ¹³CO₂ incubation was within our IRMS standard deviation limits (Table 2). Quantification of fatty acids was performed using a ten-point external standard method

Table 2

Enrichment results of NaOMe derivatisations showing delta values (δ) and standard deviations (±δ¹³C) after correction for methylation. Lipid nomenclature: a = anteiso; i = iso; br = branched; cy = cyclopropane; IS = internal standard; ω = location of double bond; nd = non-detected.

Lipid	¹² CO ₂ AS3 T ₄₀		¹³ CO ₂ AS3 T ₄₀		¹³ CO ₂ AS3 control T ₄₀	
	Average δ ¹³ C (‰)	st dev (±‰)	Average δ ¹³ C (‰)	st dev (±‰)	Average δ ¹³ C (‰)	st dev (±‰)
12:0	-33.0	6.6	-33.6	0.5	-31.8	5.4
14:0	-25.5	5.1	1652.6	11.5	-27.8	1.0
i14:0	-27.5	2.2	46.3	0.8	-26.4	1.2
a14:0	-24.9	0.7	188.8	0.8	nd	–
15:0	-21.0	7.8	153.1	11.5	nd	–
16:1ω7	-32.9	8.1	578.6	20.2	nd	–
16:1ω5	-28.4	3.1	3718.9	63.2	nd	–
16:0	-25.6	0.9	413.0	296.6	-29.0	0.6
i16:1ω8	-26.6	5.0	3718.9	296.6	-39.1	0.5
br16	-27.2	3.7	283.4	215.1	nd	–
a16:1ω8	-29.9	5.2	411.0	280.6	nd	–
a16:1ω6	-37.2	10.0	306.7	175.0	nd	–
i16:0	-27.7	7.7	488.6	53.1	nd	–
a16:0	-26.3	10.3	-19.0	5.5	nd	–
cy16:0	-21.0	6.1	3705.4	295.2	-35.0	6.2
17:0	-30.5	5.5	516.1	5.7	-36.01	0.4
i17:0	-36.4	1.4	217.0	32.3	nd	–
a17:0	-36.2	2.9	265.1	18.0	nd	–
18:2ω9,12	-36.6	3.1	324.9	291.9	-36.7	0.5
18:1ω9	-34.1	2.0	363.2	28.1	-37.9	4.5
18:1ω7	-32.9	3.1	602.3	33.7	nd	–
18:0	-32.4	5.6	26.3	10.6	-33.3	4.7
cy18:0	-34.9	2.4	-33.0	1.1	-40.2	4.4
19:0	-29.7	8.2	-35.0	0.9	-35.3	1.4
20:4ω5, 8,11,14	-31.8	3.5	-26.8	3.7	-39.3	4.2
20:5ω5, 8,11,14,17	-19.5	4.7	-26.5	0.8	-39.0	3.4
20:0	-36.8	1.7	-34.0	0.2	nd	–
21:0	-42.4	2.1	-40.5	0.9	nd	–
Cholestane (IS)	-34.6	0.5	-35.8	2.4	-35.7	2.0

($R^2 = 0.9935$). Response factors were calculated using nonadecanoic acid methyl ester (Sigma-Aldrich) and a cholestane internal standard (Sigma-Aldrich). The δ values for fatty acids were calculated from triplicate samples and were corrected after IRMS analysis. This was done because after derivitisation to the corresponding FAME, 94% of the carbon isotope measured comes 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 δ value, especially when dealing with largely enriched lipids as reported here.

2.9. Statistical analysis

Analyses of variance (ANOVA) were used to assess the amount of individual FAMEs (μg g⁻¹ dry soil) between sampling points. ANOVA were also used to determine the incorporation of ¹³C label into individual FAMEs for each CO₂ treatment. Data was tested for normality using the Shapiro–Wilk statistic and transformed when necessary. Only 22 of the total 28 FAMEs were used as the amount derived from replicates ($n = 3$) allowed for identification but too low to quantify. All statistical tests were performed using SigmaPlot version 11 (Systat Software, Inc.).

3. Results

3.1. Carbon dioxide flux before and after the addition of the electron donor – experiment 1

Slurry experiments performed using AS1 (exposure to 20 mM S₂O₃²⁻; 8 days) were carried out to observe the uninterrupted CO₂ flux within the sealed chamber. This was done to provide a visual plot of CO₂ data without interruption of the automated pump or operator interference (e.g., unsealing of access doors). The incubation of fresh soil at 30 °C under atmospheric concentrations of CO₂ (average indoor atmospheric concentration was determined to be 430 ± 14 ppmv CO₂) was performed to map the dynamic flux of CO₂, both under basal (normal background CO₂ flux) and chemically altered conditions (i.e. addition of S₂O₃²⁻). This was carried out to demonstrate the manifestation of chemoautotrophic conditions in soil and hence, uptake measurements are only demonstrative to assess the applicability of the technique. During the incubation from Fig. 2, it was observable that under these conditions the course of CO₂ concentration was static, after an initial increase of approximately 200 ppmv (T₀–T₃₀; Fig. 2) most likely derived from soil respiration.

From the CO₂ plot shown in Fig. 2 it was extrapolated that AS1 had a decay rate of +6.4 ± 0.2 ppmv h⁻¹ CO₂, indicating that CO₂ production over-took the rate of CO₂ 'loss' from the system (e.g., outgassing, physical absorption and hetero-, autotrophic

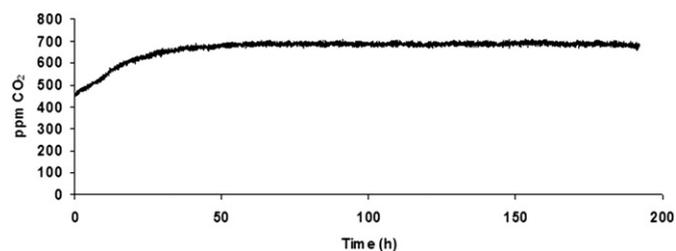


Fig. 2. AS1 basal incubation (no addition of electron donors) taking place under an ambient CO₂ atmosphere. Incubation took place over a 192 h period at 30 °C, soil was immersed in 300 ml MSM. The real-time plot demonstrates an increase in CO₂ production early during the incubation indicative of rapid respiration due to changes in soil environmental conditions (e.g., wetting). CO₂ production equilibrates ≥30 h and remains stable until incubation end.

sequestration) over the course of 192 h (8 day) incubation. Thus, under the favourable 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 and Brandt, 2003; Dilustro et al., 2005; Jassal et al., 2005). This was observable in Fig. 2 where the CO₂ gradually increases from 450 ppmv to a maximum concentration of 710 ppmv CO₂, an increase of 260 ppmv over 8 days.

A chemical electron donor was then added to a soil slurry in an attempt at stimulating the growth and reproduction of extant chemoautotrophic microorganisms. The electron donor acts as the sole energy source for the abundantly low chemoautotrophs naturally occurring in the soil profile (Smith and Strohl, 1991). The soil incubation employing the chemical electron donor displayed a different pattern of CO₂ fluctuation over a similar experimental period (see Fig. 3).

Following a short lag phase of approximately 16 h where CO₂ levels remain relatively static, a sharp decline in atmospheric CO₂ for a period of 20 h was observed, eventually reaching a minimum of 385 ± 13 ppmv. After the minimum value was observed, the atmospheric concentration of CO₂ rapidly increased to levels resembling that of the basal incubation. Extrapolation of the single decay event showed that CO₂ was sequestered to the soil during this period with an average +3.6 ppmv h⁻¹, equivalent to 5.82 g CO₂ kg⁻¹ dry soil. The decay event can be associated only with the new variable entered into the incubation, the chemical electron donor (S₂O₃²⁻), the presence of which provided an environment conducive for microbial chemoautotrophy to take place. After about 40 h of incubation, the efflux of CO₂ from soil seems to occur at rates exceeding the sum of CO₂ sequestration, where eventually a steady state was achieved. It must be stressed that the observed uptake of CO₂ into the soil matrix was not intended to demonstrate permanent sequestration of carbon, but the pattern of CO₂ flow through a complex biological matrix where sufficient environmental conditions were temporarily supplied. The results suggest that chemoautotrophic microorganisms can be stimulated through the addition of chemical electron donors to induce net CO₂ uptake (albeit temporarily). The CO₂ flux activity observed here in experiment 1 was used as the rationale for running subsequent incubations for 40 h. This was because an initial lag phase (~16 h) + active growth phase (~20 h) was required to ensure sufficient time had been provided to the chemoautotrophic population to oxidise the supplied electron donor and hence, consume sufficient CO₂ to be quantifiable. To allow for unpredictable variances in microbial responses, 40 h was selected to ensure the electron donor had been predominantly oxidised without excessive time for the mineralisation of chemoautotrophic biomass prior to quantification.

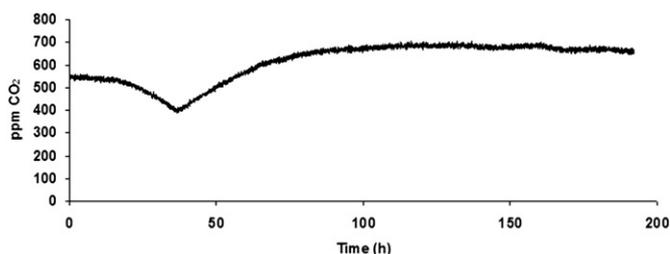


Fig. 3. Real-time ¹²CO₂ data plot of the test soil, AS1 after exposure to the electron donor. Incubation carried out for 192 h at 30 °C, soil was immersed in 300 ml MSM amended with 20 mM Na₂S₂O₃. Plot demonstrates a rapid decay profile between ~15.3 and ~36.6 h, indicative of CO₂ consumption into the soil microcosm. The plot demonstrates that >36.6 h CO₂ concentrations began to increase rapidly until ~86.1 h where upon relative equilibrium was achieved until the incubation was stopped.

3.2. CO₂ sequestration – experiment 2

To ensure the consistency of the substrate (i.e. CO₂) to which the autotrophic microbiota was exposed, the CO₂ atmosphere was artificially maintained at ~1000 ppmv CO₂ (0.1% v/v). For the AS2 sample, CO₂ consumption during the 40 h incubation was determined to be 4.52 ± 0.05 g CO₂ kg⁻¹ dry soil. The sequestration of atmospheric CO₂ was further tested on three other soils to ensure the phenomenon of soil chemoautotrophy was not isolated to the AS site. The decay patterns observed for the HS2 and MS2 samples showed similar decay plots and are a good indicator that CO₂ sequestration was taking place during incubation. It was possible to calculate total fixation over 40 h, with HS2 and MS2 samples taking up an average 4.49 ± 0.06 and 4.40 ± 0.05 g CO₂ kg⁻¹ dry soil, respectively. The TS2 demonstrated no detectable levels of sequestration indicating that respiration was the dominant process taking place despite exposure to S₂O₃²⁻. Similar uptake results were observed for three of the four soils shown here, indicating that initial electron donor concentration was a limiting factor for extant populations.

3.3. Isotopic labelling incubations – experiment 3

Sample AS3 was exposed to ¹³CO₂ to act as complimentary evidence of CO₂ sequestration via isotopic enrichment of membrane lipids. To investigate the potential for heterotrophic CO₂ sequestration leading to false positive assumptions, an incubation termed AS3_{LC} was carried out. The AS3_{LC} was incubated under the presence of ¹³CO₂ but devoid of applied electron donor. AS3_{LC} was subjected to GCMS-IRMS analysis to determine fatty acid content and δ ¹³C labelling after 40 h. A lack of enrichment in the IRMS spectrum for all identified FAMES indicated that ¹³CO₂ was not significantly sequestered in the absence of an electron donor over the 40 h incubation (Table 2). Also, 14 fatty acid molecules were absent from the ¹³CO₂ control soil in comparison to the S₂O₃²⁻-exposed soils (Table 2). Significant ¹³C enrichment was observed for the electron donor amended AS3 slurry when incubated under ¹³CO₂ (P = < 0.001) in comparison to the unlabelled (¹²CO₂) AS3 slurry (Table 2).

3.3.1. FAME profile

A total of 28 normal, branched, mono- and poly-unsaturated fatty acids are reported using GCMS. Saturated fatty acids ranged from 12:0 to 21:0 and no predominance towards even and odd chained molecules was obvious. Long-chain (>C₂₀) fatty acids were present up to C₃₂ but corresponding IRMS peaks were beyond the threshold limits and therefore not reported further. Mono-unsaturated fatty acids ranged from 16:0 to 18:0, with a maximum at 18:1ω9. Double bonds were localised to position ω7 and ω5 for 16:1, ω6 and ω8 for 17:1, ω7 and ω9 for 18:1 fatty acids. Several “iso” and “anteiso” branched fatty acids were detected ranging from 13:0 to 17:0. Cyclopropanes (cy16 and cy18) were observed in the “iso” branched position, although only cy16:0 was enriched in δ ¹³C. All Fatty acids >18:0 were depleted in δ ¹³C for all incubations and most likely relate to background organic matter of higher plant origin. The diunsaturated fatty acid 18:2ω9,12 was enriched in δ ¹³C while the tetra- and penta- unsaturated fatty acids, 20:4ω5,8,11,14 and 20:5ω5,8,11,14,17 remained depleted. The majority of the alkanes, branched and monounsaturated fatty acids displayed strong isotopic enrichment as displayed in Table 2.

3.3.2. FAME quantification

The quantification of FAMES was carried out to determine any significant changes in lipid biomass before and after exposure to the electron donor (e.g., Fig. 4). Ten fatty acids significantly

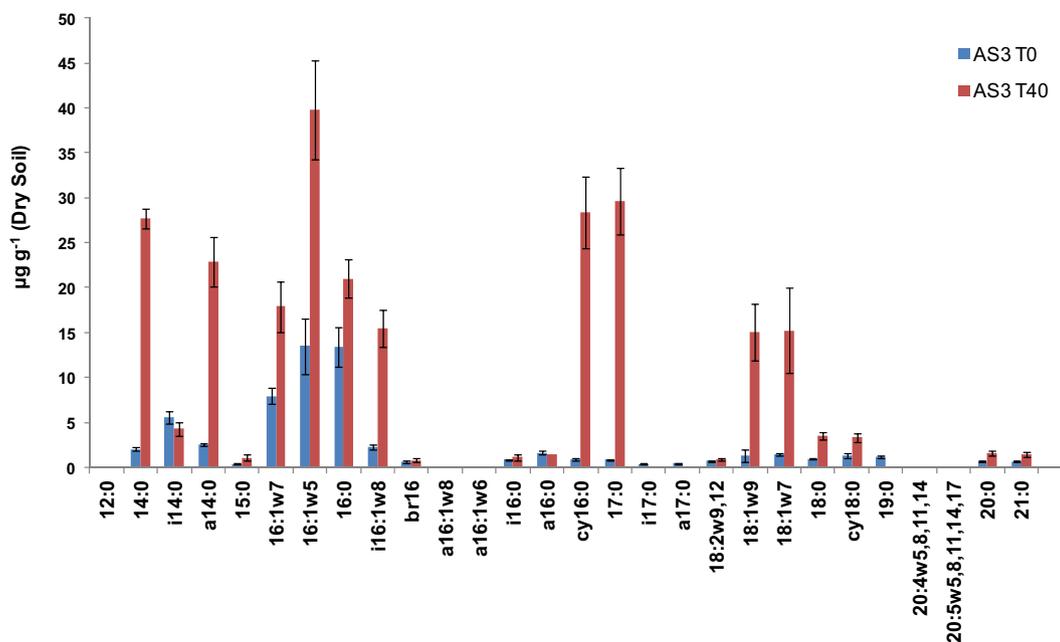


Fig. 4. Quantification of extractable FAMES ($\mu\text{g g}^{-1}$) for AS3 (freeze dried; $n = 3$) before and after 40 h exposure to $\text{S}_2\text{O}_3^{2-}$ and 1000 ppmv CO_2 and 20 mM $\text{Na}_2\text{S}_2\text{O}_3$. Reported fatty acids along the x-axis are given in order of elution from the GC column. Several FAMES were significantly increased in mass over the course of the incubation ($P < 0.05$). Not all identified FAMES could be quantified as some GCMS peaks were too low to accurately report.

increased in mass over time ($P < 0.05$) with correspondingly high $\delta^{13}\text{C}$ values (e.g., 14:0, a14:0, 16:1w7, 16:1w5, 16:0, i16:1w8, cy16:0, 17:0, 18:1w9 and 18:1w7). Two fatty acids showed an increase in mass, but only slight enrichment could be detected for 18:0 ($\delta^{13}\text{C}$ $26.3 \pm 10.6\text{‰}$), whereas, cy18:0 remained depleted ($\delta^{13}\text{C}$ $-33.0 \pm 1.1\text{‰}$). Nine other fatty acids were not significantly altered in mass ($P < 0.05$) and remained at similar levels for both time points (e.g., 12:0, i14:0, 15:0, br16:0, i16:0, a16:0, 18:2w9,12, 19:0, 20:0 and 21:0) although, four of these (underlined) were enriched in the stable isotope. Total mass of all extractable FAMES at T_0 was $6.1 \pm 1.0 \mu\text{g g}^{-1}$, whereas, at T_{40} the total mass was $25.2 \pm 3.5 \mu\text{g g}^{-1}$. This results in a total production of $19.1 \pm 2.5 \mu\text{g g}^{-1}$ (extractable) FAMES (an increase of 75.7% extractable FAMES in 40 h).

3.4. Ion chromatography determination of electron donor

The oxidation of $\text{S}_2\text{O}_3^{2-}$ was tested during the course of the incubation using ion chromatography (IC). The concentration of the $\text{S}_2\text{O}_3^{2-}$ ion in the slurry was determined using duplicated samples at T_0 and T_{40} for each soil. Fig. 5 clearly demonstrates that for the AS2 and HS2 samples, $\text{S}_2\text{O}_3^{2-}$ was removed from solution over the course of 40 h. However, the concentration of the electron donor remained static for the TS2 incubations. Control blanks consisting of $\text{S}_2\text{O}_3^{2-}$ in sterile M9 solution (a and b respectively), also remained static showing that the electron donor was stable under the prescribed conditions. The IC results (Fig. 5) were consistent with the uptake data where consumption of CO_2 was taking place concurrently with $\text{S}_2\text{O}_3^{2-}$ oxidation. Also, CO_2 concentration remained largely constant when $\text{S}_2\text{O}_3^{2-}$ remains unchanged.

4. Discussion

4.1. CO_2 sequestration

The so called “demonstration incubation”; AS1, displayed net sequestration of CO_2 during an event precipitated by the addition of

a chemical electron donor. AS1 was used to visualise the removal of CO_2 from the chamber atmosphere during the relatively short growth phase of the microorganisms. Net soil CO_2 sequestration was measured for all the test soils, except for TS2, despite ongoing respiration from all the microcosms. The experimental samples AS2, HS2 and MS2 demonstrated that $\text{S}_2\text{O}_3^{2-}$ mediated chemoautotrophy and was not only measurable but also repeatable.

It has previously been demonstrated that direct 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, 2005; Santruckova et al., 2005) and that heterotrophic CO_2 sequestration was possibly a significant factor in these incubation experiments. However, in the studies

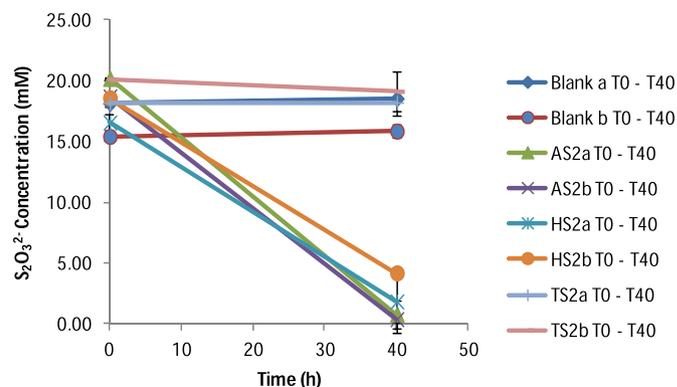


Fig. 5. Concentration of $\text{S}_2\text{O}_3^{2-}$ in soil slurry suspensions (CO_2 quantification experiment). Determined using IC and duplicated samples. Only two sampling time points were available due to the incubation chamber being sealed during the experimental period. For duplicated soils; AS2 and HS2, oxidation of $\text{S}_2\text{O}_3^{2-}$ was evident and in most cases almost complete after 40 h. Soil TS2 demonstrated no discernible oxidation of $\text{S}_2\text{O}_3^{2-}$ and (reflecting the CO_2 uptake data) indicates that under the prescribed conditions, autotrophy was not induced for this sample. Key: Blanks a, b = MSM medium with 20 mM $\text{S}_2\text{O}_3^{2-}$ electron donor, AS2a, b = Abbeyside soil, HS2a, b = Hampstead Park, TS2a, b = Teagasc Soil. Lettering (a, b) refer to replicates from each soil sample.

carried out by Miltner et al. (2004, 2005) and Santruckova et al. (2005), considerable time was required for isotopic incorporation before quantifiable results could be reported. The incubations carried out here were relatively short and net CO₂ sequestration appears to have been dependent upon the addition of a chemical electron donor. Additionally, the ¹³CO₂ control incubation demonstrated that incorporation of δ ¹³C was not detectable after the 40 h period. This indicates that heterotrophic uptake of CO₂ was unlikely to be a source of false-positive results not accounted for in the estimations.

4.2. Isotopic labelling incubations

The identification of specific fatty acids displaying strong ¹³C enrichment is of relevance to inorganic carbon sequestering microorganisms. FAMES have been used as taxonomic markers for the quantification and classification of microorganisms for a long time (Tunlid and White, 1992; Frostegård and Bååth, 1996; Zelles, 1997, 1999), although, employing biomarker methods for soil samples is difficult as the lipid composition of terrestrial species is less known to that of marine species (Kattner et al., 2003; Stübing et al., 2003; Stevens et al., 2004a,b). Although PLFAs are a more useful biomarker as they closely represent living material (PLFAs are rapidly turned over in soils upon cell death (Ruess and Chamberlain, 2010)), we have performed short controlled incubations that show the enriched biomaterial was sourced from the recent growth of chemoautotrophic microorganisms and possibly some opportunistic cross-feeding.

Enriched FAMES can be used as biomarkers for Gram-positive (odd-chained and iso/anteiso fatty acids) and Gram-negative bacteria (monounsaturated and cyclic fatty acids), fungi (octadecadienoic acid) and general membrane lipids (16:0 and 18:0). For the extracted sample (AS3), no obvious predominance for even or odd-numbered, straight chain fatty acids, suggests the presence of both Gram-positive and -negative bacteria (Kandeler, 2007). The presence of iso/anteiso methyl-branched fatty acids indicates an active presence of Gram-positive bacteria (Zelles, 1997, 1999) and the distribution of branched fatty acids hints at a wide biodiversity (Jin and Evans, 2010). The dominance of short straight-chains (<20:0) and the highly isotopically labelled cyclopropane, cy16:0, indicated that Gram-negative bacteria were the dominant genera's. Although, a large proportion of the fatty acids were enriched, we observed that the monounsaturated fatty acids (16:1, i16:1, a16:1 and 18:1) and the cyclopropane (cy16:0) were considerably more enriched than others. These fatty acids are characteristic of Gram-negative bacteria (Zelles, 1999) suggesting they were more active in carbon assimilation (Miltner et al., 2004).

Quantification of FAMES from the AS3 incubations ($n = 3$) has shown that the majority of lipids increase in mass over the two time points. Ten fatty acids were observed to have significantly increased in mass over time with considerable δ enrichment, indicating that these particular FAMES may be significant to chemoautotrophic growth under the prescribed conditions. The fungal and plant biomarker 18:2ω9,12, did not increase significantly, although it still became considerably enriched (most visually identifiable roots had been removed from the soil prior to dark incubation). This indicates that rapid fungal crossfeeding may have been taking place. It has been shown previously that fungal species tend to be primary feeders at the expense of autotrophic metabolism within the rhizosphere (Butler et al., 2003; Jin and Evans, 2010) prior to widespread degradation of biomass and exudates by the remaining microbiological community. The lack of enrichment and low mass of polyenoic fatty acids for the sampling events, indicates a limited input of plant- and possibly protazoan- (Vestle and White, 1989; Rønn et al., 2002; Treonis et al., 2004) derived fatty acids.

The enrichment of fatty acids with ¹³C during the course of the experiment was intended to further demonstrate the hypothesis that chemoautotrophy was stimulated after addition of Na₂S₂O₃ and hence, the applicability of the prescribed method for delivering high value data. The presence of short chained FAMES (≤C₂₀) indicates microbial input to SOM (Ruess and Chamberlain, 2010; Hart et al., 2011) rather than fresh inputs by higher plant material. A lack of FAMES with enriched fatty acid chains >20:0 or polyunsaturated fatty acids, indicates that no higher plant (including algae) or fungal activity was directly related to carbon capture (Ruess and Chamberlain, 2010). Several monounsaturated FAMES were observed that are common across taxa, but 18:1ω9 is common to both fungi and Gram-positive bacteria (Bååth, 2003; Vestle and White, 1989). Further, enrichment of general fungal biomarkers such as 16:1ω5 (Olsson et al., 1995, 2003), 18:1ω7 (Olsson, 1999) and 18:2ω9,12 (Frostegård and Bååth, 1996; Zelles, 1999; Zechmeister-Bolternstern et al., 2011) indicates that fungi (e.g., ectomycorrhizal and arbuscular mycorrhizal etc.) were possibly actively consuming biomass and/or exudates of the S₂O₃²⁻ activated consortium (cross-feeding?). The incorporation of the isotopic label into lipids and specifically PLFAs, has shown that CO₂ was an important carbon source for chemoautotrophic microorganisms (as a lack of plant biomarkers indicated no photosynthetic activity). The outlined method has proven to be successful for the stable isotopic labelling of biomass involving trace gases as the substrate. This is particularly relevant for microbiological studies involving environmentally significant processes involving gaseous nutrients and carbon substrates.

5. Conclusions

The overall aim of this study was to prepare a working method in which multiple techniques may eventually be employed to study carbon uptake by a single soil sample that has been subjected to various conditional changes. This aim was fully achieved and the ECIC has been demonstrated as fit for purpose. The addition of a chemical electron donor to soil was essential for the biofixation of CO₂ within this particular system. The CO₂ fluxes of the incubation samples have been tracked and quantified using high resolution data sets, leading to estimations of inorganic carbon uptake. Specifically, the 40 h soil (samples AS2 and AS3 respectively) chemoautotrophic enrichment study yielded an uptake value of 4.52 ± 0.05 g CO₂ kg⁻¹ dry soil, where, 19.1 ± 2.5 μg g⁻¹ of lipid membrane material was attributed to direct CO₂ consumption. The isotopic enrichment of lipids provided further evidence of the incorporation of inorganic atmospheric CO₂ into the soil matrix. The next goal is to apply this methodology to study microcosms that more closely mimic *in situ* conditions to assess carbon uptake under different environments. Identification of microbial species involved in CO₂ uptake, profiling of microbial species in different soil samples, and analysis of the fate of organic carbon are only some of the further applications envisaged for the current methodology.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.soilbio.2012.10.036>.

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