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CO₂ uptake by a soil microcosm

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A B S T R A C T

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 \(^{13}\)CO₂ 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...
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 chemosynthetic microorganisms 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; Nakanoa 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 (Acocq and Acocq, 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 chemosynthetic 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 chemosynthetic activity 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 ¹³CO₂ into fatty acids including the blank-control ¹²CO₂ 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 Kokossa (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 (GM220, Vaisalia 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 2390 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° 9′ 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 podzol | 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 | 0.45a | 6.8   | 13.08 | 1.53  | 0.83  | 0.22  |
| TS                | Oak Park Research Centre, Carlow Town, Co. Carlow, Ireland (09/2008) | Grey brown podzol | 52° 51′ 47.24″ N, 6° 54′ 11.34″ W | 20.2 | 6.5   | 3.61  | 0.33  | 0.17  | 0.56  |

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

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 pre-programmed concentration setting in parts per million per volume (ppmv) CO2. Internal air is circulated using a built-in fan and environmental conditions are monitored using temperature (°C), relative humidity (%RH), PAR (%) and CO2 sensors.

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 CaCl·2H₂O; Madigan et al., 2009; Shiels 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 H₂BO₃, 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) S₂O₃⁡²⁻ 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 S₂O₃⁡²⁻ 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 $^{13}$CO\(_2\) (99% atom $^{13}$C, Sigma Aldrich) incubation. It was determined that 5.166 ± 0.2 cm\(^2\) s\(^{-1}\) 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 (AS\(_3\); 30 ± 0.5 g, n = 3) were prepared as above before sealing the ECIC. CO\(_2\) concentration of 1000 ± 50 ppmv was maintained constantly throughout the incubations. AS\(_3\) $^{13}$CO\(_2\) labelled control (designated; AS\(_3\)C $^{13}$CO\(_2\)) incubations were carried out verbatim under a 1000 ppmv $^{13}$CO\(_2\) atmosphere with the exception that slurries were not amended with Na\(_2\)S\(_2\)O\(_3\) (to test if isotopic enrichment was attributable to $S_2O_3^{2-}$ mediated reactions or other unknown abiotic soil processes). $^{13}$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–IRM.

2.5. Analysis of $S_2O_3^{2-}$ 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 µm syringe filter to remove particulate matter. Each sample was diluted 1/1000 and stored at −20 °C until analysis. Blank incubations (e.g., no soil inoculum) consisted of sterile 294 ml MSM medium and 6.0 ml (0.2 µm sterile filtered) 1000 mM $S_2O_3^{2-}$ 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 µl sample loop for all work. Separations were carried out using a Dionex IonPac AG16 (2.0 mm × 50 mm) guard and an AS17 (2.0 mm × 250 mm) analytical column, with a capacity of approximately 42.5 µeq./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 (0–20 mM $S_2O_3^{2-}$) 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 [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. Stomnevaegen, 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 $\bar{C} = 401.6 ± 14.1$ ppm. Final conversion factor for ppm to g is given as ([x2d]/10 × 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 Na\(_2\)S\(_2\)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 x 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\(_2\)HPO\(_4\)) buffer solution. The soil precipitate was freeze-dried prior to extraction with methanol: dichloromethane (ratio 1:1, 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 volatise 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 $\delta^{13}$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 (NaO\(_\text{Me}\)) 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–IRM

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 (Isoprine 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 ±0.3 ‰, respectively, 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

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<th>Lipid</th>
<th>12CO₂ AS1 T₀</th>
<th>12CO₂ AS3 T₀</th>
<th>13CO₂ AS3 control T₀</th>
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<tr>
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<td>±δ¹³C (‰)</td>
<td>Average δ¹³C (‰)</td>
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<td>−36.8</td>
<td>1.7</td>
<td>−34.0</td>
</tr>
<tr>
<td>21:0</td>
<td>−42.4</td>
<td>2.1</td>
<td>−40.5</td>
</tr>
<tr>
<td>Cholestane (IS)</td>
<td>−34.6</td>
<td>0.5</td>
<td>−35.8</td>
</tr>
</tbody>
</table>

### 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–Wilks 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 of 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 carbonate 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 ppm by to a maximum concentration of 710 ppm by CO2, an increase of 260 ppm by over 8 days.

A chemical electron donor was then added to a soil slurry in an attempt to stimulate 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 (S\(_2\)O\(_3\)\(^{-2}\)), the presence of which provided an environment conducive for microbial chemoautotrophy to take place. After approximately 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 be sufficiently high to enable the electron donor (e.g., Fig. 4). Ten fatty acids significantly

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 S\(_2\)O\(_3\)\(^{-2}\). 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 \(^{13}\)CO2 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\(_{13C}\) was carried out. The AS3\(_{13C}\) was incubated under the presence of \(^{13}\)CO2 but devoid of applied electron donor. AS3\(_{13C}\) was subjected to GCMS-IRMS analysis to determine fatty acid content and \(^{13}\)C labelling after 40 h. A lack of enrichment in the IRMS spectrum for all identified FAMEs indicated that \(^{13}\)CO2 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 \(^{13}\)CO2 control soil in comparison to the S\(_2\)O\(_3\)\(^{-2}\) exposed soils (Table 2). Significant \(^{13}\)C enrichment was observed for the electron donor amended AS3 slurry when incubated under \(^{13}\)CO2 (\(P = < 0.001\)) in comparison to the unlabelled (\(^{12}\)CO2) 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 observed. Long-chain (>C\(_{20}\)) fatty acids were present up to C\(_{32}\) but corresponding IRMS peaks were beyond the range of the IRMS spectrometer. The TS2 demonstrated no detectable levels of sequestration leading to false positive assumptions, an incubation termed AS3\(_{13C}\), was carried out. The AS3\(_{13C}\) was incubated under the presence of \(^{13}\)CO2 but devoid of applied electron donor. AS3\(_{13C}\) was subjected to GCMS-IRMS analysis to determine fatty acid content and \(^{13}\)C labelling after 40 h. A lack of enrichment in the IRMS spectrum for all identified FAMEs indicated that \(^{13}\)CO2, 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 \(^{13}\)CO2 control soil in comparison to the S\(_2\)O\(_3\)\(^{-2}\) exposed soils (Table 2). Significant \(^{13}\)C enrichment was observed for the electron donor amended AS3 slurry when incubated under \(^{13}\)CO2 (\(P = < 0.001\)) in comparison to the unlabelled (\(^{12}\)CO2) AS3 slurry (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
increased in mass over time ($P = < 0.05$) with correspondingly high $\delta^{13}$C values (e.g., 14:0, a14:0, 16:1ω7, 16:1ω5, 16:0, i16:1ω8, cy16:0, 17:0, 18:1ω9 and 18:1ω7). Two fatty acids showed an increase in mass, but only slight enrichment could be detected for 18:0 ($\delta^{13}$C 26.3 ± 10.6‰), whereas, cy18:0 remained depleted ($\delta^{13}$C −33.0 ± 1.1‰). 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, 18:2ω9,12, 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 ± 1.0 µg g$^{-1}$, whereas, at $T_{40}$ the total mass was 25.2 ± 3.5 µg g$^{-1}$. This results in a total production of 19.1 ± 2.5 µg 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 S$_2$O$_3^{2-}$ was tested during the course of the incubation using ion chromatography (IC). The concentration of the S$_2$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, S$_2$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 S$_2$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 S$_2$O$_3^{2-}$ oxidation. Also, CO$_2$ concentration remained largely constant when S$_2$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 S$_2$O$_3^{2-}$-mediated chemosynthesis 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...
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 Båth, 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; Stöbinger 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 chemautotrophic 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. Although, a large proportion of the fatty acids were enriched, we observed that the monounsaturated fatty acids (16:1, 16:1, α16:1 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 chemoaotrophic growth under the prescribed conditions. The fungal and plant biomarker 18:2o9,12, 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 protozoan- (Vestle and White, 1989; Rann 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 chemautotrophy 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 18:1o9 is common to both fungi and Gram-positive bacteria (Báth, 2003; Vestle and White, 1989). Further, enrichment of general fungal biomarkers such as 16:1o5 (Olsson et al., 1995, 2003), 18:1o7 (Olsson, 1999) and 18:2o9,12 (Frostegård and Båth, 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 chemautotrophic 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 microbial 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) chemotaurotrophic 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.

References


