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Stable Isotope Switching (SIS): A New Stable Isotope Probing (SIP) Approach to Determine Carbon Flow in the Soil Food Web and Dynamics in Organic Matter Pools

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Running head: ^{13}C Stable Isotope Switching (SIS)

Abstract

Rationale: Recent advances in stable isotope probing (SIP) have allowed direct linkage of microbial population structure and function. This paper details a new development of SIP, Stable Isotope Switching (SIS), which allows the simultaneous assessment of C uptake, turnover and decay, and the elucidation of soil food webs within complex soils or sedimentary matrices.

Methods: SIS utilises a stable isotope labelling approach whereby the ^{13}C-labelled substrate is switched part way through the incubation to a natural abundance substrate. A $^{13}CH_4$ SIS study of landfill cover soils from Odcombe (Somerset, UK) was conducted. C assimilation and dissimilation processes were monitored through bulk elemental analysis-isotope ratio mass spectrometry and compound specific gas chromatography-combustion-isotope ratio mass spectrometry targeting a wide range of biomolecular components including: lipids, proteins and carbohydrates.

Results: Carbon assimilation by primary consumers (methanotrophs) and sequential transport into secondary (Gram negative and positive bacteria) and tertiary consumers (Eukaryotes) was observed. Up to 45% of bacterial membrane lipid C was determined to be directly derived from CH_4 and at the conclusion of the experiment ca. 50% of bulk soil C derived directly from CH_4 was retained within the soil.

Conclusions: This is the first estimate of soil organic carbon derived from CH_4 and is comparable to levels observed in lakes that have high levels of benthic methanogenesis. SIS opens the way for a new generation of SIP studies aimed at elucidating total C dynamics (incorporation, turnover and decay) at the molecular level in a wide range of complex environmental and biological matrices.

Introduction

A range of new techniques, have recently emerged to study environmental microorganisms *in situ* without the need to establish laboratory cultures.[1] Among these so-called '*culture independent* methods' is stable isotope probing (SIP), an approach which involves the incubation of environmental soils or sediments with stable isotope labelled substrates. In

many cases a simple 'pulse chasing' approach is utilised, whereby a short application or single dose of a highly enriched ^{13}C-labelled substrate is applied to complex environmental samples and traced to identify the fate of the substrate. Subsequently, the metabolic activities of microorganisms are assessed through determination of label incorporation into biochemical components of cells of active members of the microbial population including DNA,[4] RNA[5] and phospholipid fatty acids (PLFAs).[1] Of major importance in this area has been the application of highly sensitive detection techniques including gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS)[1,2] and more recently liquid chromatography (LC)-IRMS approaches.[6]

Initial SIP studies focussed on identification of microorganisms that utilised specific substrates, such as soil dwelling methanotrophic bacteria. Interest in soil methanotrophs stems from the fact that such bacteria occur in every soil order and are an important sink for atmospheric CH_4 in well-aerated soils (high affinity methanotrophs)[7] and a highly efficiency filter that consumes >90% of upward diffusing CH_4 (low affinity methanotrophs) in soils where a sub-surface CH_4 source exists (e.g. landfill cover soils) or *in situ* CH_4 production occurs (e.g. natural wetlands) [8,9,10] Notable successes for SIP in this area include the identification of unculturable high affinity methanotrophic bacteria via PLFA ^{13}C-labelling in well-drained non-agricultural soils.[2] Subsequently, SIP has been extended to quantify methanotroph biomass populations through time series $^{13}CH_4$ PLFA labelling.[11] Interestingly, despite their importance as a carbon sink, there has been little study of soil methanotrophs in ecological contexts as a potential source of soil organic matter. The quantity of carbon cycled via high and low affinity methanotrophy in soils is globally significant,[12,13] yet the fate of that carbon remains largely unknown and unquantified. Understanding the sources and stability of organic carbon in soils is a prerequisite for

development of realistic global carbon cycle models that contain fully coupled atmosphere-biosphere-geosphere interactions.

Significantly, it has become apparent that long-term time series $^{13}CH_4$ labelling has the potential to yield a wide range of additional information, including: (i) kinetics of ^{13}C uptake, (ii) mechanisms of C incorporation, and (iii) C flow pathways and turnover in soil. For example, in a time series $^{13}CH_4$-incubation study of methanotrophic bacteria in volcanic soils from Tenerife, Spain high concentrations of ^{13}C-label were incorporated into methanotrophic PLFAs.[14] Due to the high levels of ^{13}C- incorporation of ^{13}C, at later stages of the $^{13}CH_4$ incubation, ^{13}C-label was detected in non-methanotrophic fungal biomarkers (e.g. $C_{18:2}$) providing a clear indication of how this approach could be used to investigate pathways of C flow through soil microbial communities.

In this study we have combined two previous SIP methods i.e. short pulse-chase experiments and long-term continuous labeling approaches to study biosynthesis and C uptake, to enable a comprehensive study of methanotroph C uptake and CH_4 derived C transport and sequestration through the soil food web. A new long-term ^{13}C-labelling approach has been applied whereby $^{13}CH_4$ was switched to CH_4 containing natural abundance levels of ^{13}C and ^{12}C when full labelling was achieved (as indicated by a maximum in the ^{13}C label incorporation curve; Fig. 1). The incubation was then continued to monitor C turnover of the incorporated ^{13}C-label (hence the term ‘stable isotope switching’ (SIS)). This approach allows short, medium and long-term processes involved in the uptake and turnover of C to be investigated in detail. This continuous labelling method utilises flow-through incubation system and differs from pulse labelling experiments,[6] which typically are conducted over much shorter timescales. The ability to supply a stable flow of isotopically labelled gas at a concentration similar to natural environmental conditions limits disruption to the soil

ecosystem and problems associated with selective fertilisation or competitive inhibition of the soil microorganisms.

To demonstrate the potential of this technique we conducted a detailed SIS investigation on soils from the Odcombe landfill site (Somerset, UK) which had a previous study[15] has shown to contain a significant population of methanotrophic bacteria. In this methodological paper we document the details of the SIS approach and summarise the range of data produced by SIS to demonstrate its many potential applications. Full datasets, detailed statistical analyses and consideration of all compound classes investigated will be reported in a subsequent communication.

Experimental

Site - Multiple soil cores (5 cm diameter, 10 cm depth) were collected from two sites at the Odcombe landfill (SW England, 50°56'45"N -2°42'19"W) in March 2007. The landfill consists of a terraced area that was formed by the stacking of waste, which was later capped with sand and clay. The soil cap was sampled from an area of high CH_4 emissions adjacent to a vent previously identified by Crossman *et al.*[15] In this earlier study, conventional PLFA-SIP identified significant populations of low affinity methane oxidising bacteria similar to known Type II methanotrophs, making the cap soil ideally suited for this trial SIS study because of the potential for incorporation of significant amounts of CH_4-derived C into the soil food web. Additionally, landfill cap material is a unique aerobic mineral soil where methanotrophs are a major component of soil microbial biomass, facilitating both rapid and comprehensive labelling of the methanotrophic population and enabling sensitive and selective tracking of the fate of the ^{13}C-signal.

SIS CH_4 incubation

Soils were incubated in a flow through incubation chamber that has been previously described in Maxfield *et al.*[11] All soils were sieved (<2 mm) and incubated in petri dishes (ca. 20 g) over a time course of 154 days. Synthetic air premixed with CH_4 (1.33%) was flowed continuously through the chamber to maintain a steady CH_4 mixing ratio similar to that measured at the landfill site. For the initial 50 days of the incubation 1% of the CH_4 was $^{13}CH_4$ (mixed from > 99% ^{13}C; CK Gas Products Ltd, Hook, UK; equivalent to 133 ppmv $^{13}CH_4$). Following 50 days of incubation the input of $^{13}CH_4$-bearing gas was discontinued and switched to a pre-mixed supply of CH_4 (13300 ppmv) containing natural abundance levels of ^{13}C and ^{12}C. The gas flow rate through the chamber was maintained at 44 mL min^{-1} throughout the entire incubation period, which flushed the entire headspace (63 L) every 24 h. Soil samples were removed in triplicate and from random positions in the incubator at regular intervals during the incubation period at times of 0, 3, 6, 9, 12, 18, 21, 27, 38, 50, 53, 56, 65, 85, 117, 154 days. The initial moisture content of the soil was maintained through the regular addition of double-distilled water (DDW) to each sample (determined gravimetrically). All samples removed from the incubator were stored at -20°C until analysed. Soil samples were freeze-dried and ground prior to extraction and analysis for PLFAs, total amino acids (AAs), total monosaccharides glycolipids (GLFAs), free fatty acids (FFAs), hopanoids, sterols, *n*-alkanes and *n*-alkanols.

Lipid extraction and fractionation

All soils were extracted using a modified Bligh Dyer extraction methodology as described previously.[11] Lipid fractionation was obtained using a modified silicic acid fractionation protocol[16] to yield three fractions: simple lipids, glycolipids and phospholipids. Simple

lipids were further fractioned using silicic acid columns into four fractions; hydrocarbons (eluted with hexane, 4 mL), ketones and wax esters (dichloromethane (DCM), 6 mL), alcohols (DCM/methanol (MeOH), 1:1 *v/v*, 4 mL), and polar poly-functionalised compounds (MeOH, 4 mL). Alcohols and hydrocarbons were further separated by urea adduction to separate cyclic from acyclic components.[17]

Lipid derivitization

The PLFA, GLFA and FFA fractions were methylated with BF_3/MeOH (14% *w/v*) by heating at 70°C for 1 h. Fatty acid methyl esters (FAMES) were dissolved in *n*-hexane for analysis by GC, GC/mass spectrometry (MS) and GC/C/IRMS. All neutral polar lipids were derivatized with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) prior to analysis by GC, GC/MS and GC/C/IRMS.

Extraction and derivatization of carbohydrates

The method of Blakeney *et al.*[18] modified by Docherty *et al.*[19] was employed to prepare alditol acetate derivatives of total monosaccharides. Soils were hydrolysed with H_2SO_4 and the released monosaccharides reduced with $NaBH_4$. Excess $NaBH_4$ was destroyed by the addition of glacial acetic acid to reduce the liberated monosaccharides to their corresponding alditols. The alditols were acetylated by reaction with *N*-methylimidazole and acetic anhydride. A standard mix of rhamnose, fucose, galactose, mannose, xylose, arabinose glucose, inositol and pentaerythritol was prepared according to Docherty *et al.*[19] The DDW (400 μl) was added to the dry monosaccharide standards which were derivatized as above. A

20 μg μl^{-1} internal standard (I.S.) was prepared by dissolving 0.04 g pentaerythritol in 2 ml 1 M NH_3 solution.

Extraction and derivatization of amino acids

An internal standard of nor-leucine (0.2 mg ml^{-1}) was prepared for quantification of AAs. Each AA produced a different GC-FID response which required correction by the application of response factor. The FID response factors were determined from a standard solution of AAs of known concentration according to the method of Corr *et al.*[20] Extracted soil samples were hydrolysed with HCl and purified by ion exchange chromatography[21] using Dowex 50WX8-400 Ion Exchange Resin (Acros Organics, Geel, Belgium). The purified amino acids were derivatized to N-acetyl methyl esters using acetone, triethylamine and acetic anhydride (5:2:1, *v/v*)[20].

Instrumental analyses

GC analysis were performed using a Hewlett-Packard Series 5890 Series II gas chromatograph (Agilent Technologies UK Ltd., Edinburgh, UK) equipped with a flame ionisation detectors (FID) using H_2 carrier gas (10 psi). Non-polar fractions were analysed using a Chrompack CPSil-5CB (50 m x 0.32 mm i.d. x 0.12 μm film thickness). The temperature conditions were 50ºC to 200ºC at 10º C min^{-1}, to 300ºC at 3ºC min^{-1} (held for 20 min). Polar compounds were analysed using a Varian VF23ms (Varian BV, Middelburg, The Netherlands) 50% cyanopropyl equivalent fused-silica column (60 m x 0.32 mm i.d. x 0.25 μm film thickness). The temperature conditions for fatty acid derivatives were 50ºC (2 min) to 100ºC at 15ºC min^{-1}, to 240ºC at 4ºC min^{-1} (held for 20 min). The temperature programme

for monosaccharides was 50ºC (1 min) to 200ºC at 20ºC min^{-1}, to 230ºC at 4ºC min^{-1}, (held for 22 min). The temperature programme for amino acids was 40ºC (1 min) to 120ºC at 15ºC min^{-1}, to 190ºC at 3ºC min^{-1}, to 250ºC at 5ºC min^{-1} (held for 20 min).

GC-MS analyses were performed using a Thermo Finnigan Trace GC-MS (Thermo Fisher Scientific, Hemel Hempstead, UK). All the GC conditions were the same with the exception of helium being used as carrier gas. The interface was held at the maximum oven temperature, the ion source was held at 200°C and the quadropole mass analyser operated in EI mode scanning over the range *m/z* 50-650 at 1.7 scans s^{-1}. The emission current was maintained at 300 μA and electron energy was 70 eV. The data were acquired and analysed using the Excalibur software Version 1.2 (Thermo Fisher Scientific, Hemel Hempstead, UK).

GC-C-IRMS analysis were carried out using a Varian 3500 GC (Varian BV, Middelburg, The Netherlands) coupled to a Finnigan MAT DELTA-S isotope ratio mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK). Analytes in He were combusted to CO_2 using a modified Type I Finnigan MAT combustion interface with a CuO/Pt combustion reactor set at a temperature of 850°C prior to entry into the mass spectrometer source via an open split. The ionisation source electron energy was 100 eV with an electron current of 1 mA. Detection was via 3 Faraday cup collectors set at *m/z* 44, 45 and 46. The chromatographic conditions were the same as those described previously for GC analyses. All samples were analysed in duplicate to verify reliability of $\delta^{13}C$ values. Samples were calibrated against reference CO_2 of known isotopic composition which was introduced directly into the source three times at the beginning and end of every run. Compound specific IRMS performance was determined using a suite of externally calibrated reference fatty acid methyl esters. Analytical precision was <0.5 ‰ (± 1 standard deviation) based upon replicate analysis of reference standards (n=5).

209 **EA-IRMS** analysis of freeze-dried and ground soil samples was conducted using a
210 Eurovector C-N (Eurovector, Milan, Italy) elemental analyser interfaced to an Isoprime
211 (Isoprime Ltd., Manchester, UK) stable isotope ratio mass spectrometer (IRMS). Samples
212 were weighed (1–2 mg), placed into tin capsules and combusted. $\delta^{13}C$ values of the resultant
213 CO_2 from combustion were determined at the NERC Stable Isotope Facility at CEH
214 Lancaster with an analytical precision of <0.15 ‰ (± 1 standard deviation, n=16).

215

216 **Statistical analyses**

217 Two approaches were used to analyse the ^{13}C-label incorporation curves. Linear and non-
218 linear regressions were performed using GraphPad Prism version 5.02 for Windows
219 (GraphPad Software, San Diego, CA, USA), R 2.8.1 (R foundation), and Microsoft Excel
220 (Microsoft, Redmond, WA, USA). Least squares linear regression analysis was applied to %-
221 incorporation data from the first 9 days of the incubation to obtain initial zero-order rate
222 constants, whereas separate non-linear regressions were applied to incorporation data from
223 the first 50 d of the incubation before the switchover to unlabelled CH_4, and the C turnover
224 data from 50 d to 150 d. The equations of the fitted lines were of the form:

225 Incorporation: $$F = F^0 + (P - Y^0) * (1 - \exp(-K * t)) \quad (1)$$

226 Decay: $$F = (F^0 - P) * \exp(-K * t) + P \quad (2)$$

227 Where F is the fraction of excess ^{13}C incorporated into organic material, t is the incubation
228 time in days, R^0 is the initial R value at initial t, *P* is the plateau at maximum ^{13}C
229 incorporation (incorporation) or maximum ^{13}C loss (decay) and K is the first order rate
230 constant expressed in inverse days.

231

Results and Discussion

The SIS approach differs from pulse-chase methods in two key respects: (i) substrate delivery is maintained throughout the experiment at a constant concentration in order to establish equilibrium conditions between substrate and the primary substrate consumer population thus allowing evaluation of substrate cycling purely based on changes in isotopic labelling patterns in different endogenous chemical species, and (ii) the only change in substrate delivery is in its stable isotopic composition, i.e. ^{13}C-enriched to natural abundance, which is switched when isotopic equilibrium with the primary consumers has been established. As a result it is possible to study both assimilation and decay of the ^{13}C-label in a wide range of C pools and molecular species (including biomarker compounds) within a complex ecosystem, in order to provide insights into assimilation pathways, kinetics of turnover and quantitative estimates of pool sizes.

Methanotrophic bacteria as primary consumers

Soil PLFA profiles provide general information about soil microbial community structure, diversity and size. PLFA nomenclature indicates the length of the C chain (first number), the number of double bonds (number after the colon), the position of the double bond (ω, counted from the defunctionalised end of the molecule) and geometry (for full details see Zelles).[22] The extremely high abundance of 18:1ω7c observed in the Odcombe vent PLFA profile (Fig. 2) indicates the likely dominance of α-proteobacterial (type II) methanotrophs linked to the high CH_4 flux at the site. This predominance was confirmed by GC-C-IRMS analysis of the PLFA fraction following the $^{13}CH_4$-enriched incubation which also showed with the largest proportion of ^{13}C was incorporated into PLFA 18:1ω7c. The prevalence of methanotrophic bacteria resulted in highly ^{13}C-labelled soils following the $^{13}CH_4/^{12}CH_4$

incubation enabling methanotroph C derived from $^{13}CH_4$ to be traced through the complex soil food web.

Stable Isotope Switching

The extracts from the ^{13}C-labelled soils were analysed by GC-C-IRMS to determine δ^{13}C values to quantify and monitor the fate of metabolised CH_4 across a wide range of compound classes representative of a range of soil biota. Whilst PLFAs are the most commonly studied biomarkers in methanotrophic bacteria SIP studies we and others have shown previously the potential for linking hopanoids with methanotrophic bacteria through $^{13}CH_4$-labelling studies.[23,24,25] However, the purpose of SIS is to move beyond functional taxonomic profiling to explore more fully C cycling and soil microbiological function. Thus, we have conducted a comprehensive survey of methanotroph-derived biochemicals tracing ^{13}C-label into amino acids, carbohydrates, glycolipids, free fatty acids, alcohols, alkanes, hopanoids, sterols and resorcinols (Fig. 3). Different compound classes represent soil C pools of differing stability and recalcitrance. Further, the magnitude of methanotroph C turnover by the soil microbial community suggests that those biochemicals which display little or no ^{13}C-labelling are not closely linked with soil C turnover by soil microorganisms, and thus represent either stable soil C pools receiving little fresh C input, or are soil C pools with a conserved source of C. In addition the total incorporation of ^{13}C into the bulk soil also was assessed. Comparison with this bulk δ^{13}C value indicates the recalcitrance of individual C pools relative to the total pool of soil organic C (Fig. 3).

Figure 4 shows an overview of ^{13}C-label assimilation and dissimilation profiles for a range of selected soil compounds from several of the main compound classes: 16:1ω7c PLFA, C_{31} homohopenol, glucose, valine and the bulk soil. Whilst there are clear differences

in the extent of ^{13}C-labelling of these compound classes the $\delta^{13}C$ curves exhibit the general shape predicted from the experimental design (Fig. 1) with C turnover following a first order rate dissociation curve (Fig. 4, right hand side). However, it is important to note both the differences in the relative uptake of ^{13}C into the different soil compound classes and the wide range of turnover rates which reflects the refractiveness of different biochemicals analysed in this study.

Soil Food Web

As with previous conventional time series labelling studies ^{13}C-label uptake monitored by GC-C-IRMS and expressed by $\delta^{13}C$ values represents the magnitude of ^{13}C-label uptake into a specific compound as a proportion of the total concentration of that compound. Thus, $\delta^{13}C$ values do not indicate the absolute amount of ^{13}C-label present in a specific pool but rather the proportion of that C pool that is derived from the ^{13}C-labelled C source. Hence SIS provides a potential new tool for use in soil food web studies which can be employed to determine the rate of C flow through a soil microbial network and the rate of *in situ* biosynthesis of specific compounds within that network. Figure 4 indicates that a significant proportion of PLFAs within the Odcombe soil were synthesised by methanotrophs directly from CH_4. All PLFAs displayed uptake of the ^{13}C-label indicating both the high magnitude of initial $^{13}CH_4$ incorporation and the extensive redistribution of assimilated C within the soil system. The rates and magnitudes of ^{13}C uptake vary widely indicating that the PLFA producing organisms differed in terms of their usage of CH_4 derived C in biosynthesis, and the biochemical proximity of different PLFAs to the ^{13}C source ($^{13}CH_4$). To more easily visualize PLFA ^{13}C incorporation profiles those data are shown separately (in Fig. 5) highlighting the differences in ^{13}C uptake rate between different groups of PLFA sources.

Despite their lack of taxonomic specificity PLFAs have been widely used to broadly characterize microbial populations[22] and as such PLFAs are an extremely effective tool for identifying active bacterial groups utilizing ^{13}C-labelled tracer compounds. For example, PLFAs commonly linked to methanotrophic bacteria (18:1ω7c, 16:1ω7, 16:1ω5) most rapidly incorporate the ^{13}C-label (Fig 5, panel a). The primary methanotroph PLFA in this soil, 18:1ω7c also reaches a plateau before the $^{13}CH_4$ supply was discontinued following 50 days incubation, indicating maximum ^{13}C-labeling of the methanotroph population and establishment of isotopic equilibrium within the primary consumer population. Primary methanotrophic PLFAs can be separated from PLFAs that incorporated a lower proportion of ^{13}C at a slower rate, the latter likely being indicative of secondary (Fig 5, panel b) and tertiary consumers (Fig 5, panel c). Secondary and tertiary consumers incorporate ^{13}C-label at much reduced rates compared to primary consumers. For example following 18 days incubation under $^{13}CH_4$ primary consumer $\delta^{13}C$ values increased by +100 to +300 ‰ (Fig. 5a), secondary consumer $\delta^{13}C$ values increased by +30 to +60 ‰ (Fig. 5b), and tertiary consumer $\delta^{13}C$ values increased by +10 to +20 ‰ (Fig. 5c). Because microorganisms linked with non-primary methanotroph PLFAs are not obtaining ^{13}C label direct from $^{13}CH_4$, the likely alternative ^{13}C-label redistribution mechanisms are metabolite release and uptake, necromass grazing and direct predation.

Furthermore, it was observed following prolonged incubation under $^{13}CH_4$ that a significant proportion of PLFA C was derived from CH_4. Accounting for the fact that the ^{13}C-enriched CH_4 used in this study was only 1% enriched in $^{13}CH_4$, and extrapolating the results to take this dilution into account, following 38 days of the incubation 45% of the C present in the Odcombe soil PLFAs was derived from CH_4. This proportion includes all PLFAs extracted from the soil, which suggests that >45% of total bacterial PLFA C consists of C derived from CH_4. Even allowing for the high abundance of methanotrophs in the Odcombe

soil this is an unexpectedly high proportion of bacterial C after a relatively short incubation periods, suggesting that microbial communities in soil and sedimentary environments where a significant source of CH_4 is present must utilise CH_4 as a major source of C and energy for growth. This high proportion of C is consistent with the appreciable amount of CH_4-derived C that is incorporated into certain lake food webs where CH_4 production is prevalent in anoxic sediments.[26] Whilst there is a wide range of estimates between different lakes, chironomid larvae have been observed as a primary conduit for the trophic transfer of biogenic CH_4 gaining >60% of their C from CH_4[27] and zooplankton in small boreal lakes ~50% through grazing on methanotrophs.[28] In these lake studies trophic C transfer was determined via physical separation of the organisms of interest prior to bulk isotopic measurement by EA-IRMS. The work reported herein is the first assessment of the proportion of CH_4 derived C that is transmitted through microbial food webs, which has been made possible through the application of SIS in combination with GC-C-IRMS analysis of a wide range of soil biochemical components.

C sequestration

In addition to C uptake kinetics, the SIS method also enables the study of rates of C turnover, redistribution and sequestration. Figure 4 (right hand side) shows C turnover of PLFAs following the change from incubation under $^{13}CH_4$ to natural abundance CH_4 and the fitted first order exponential dissociation curves used to determine C turnover rate constants. Box plots of C turnover for each group of compounds analyzed by GC-C-IRMS indicate no observable relationship between C assimilation and turnover (Fig. 3). There is a high degree of variability in both C turnover rates and loss both within and between various compound classes. Although CH_4 is a significant source of C in the Odcombe soils, a large amount of

353 the C utilized in cellular biosynthesis is rapidly lost from the system and not sequestered in
354 long-term C pools. The C pools where C retention is poor include PLFAs, GLFAs, FFAs,
355 carbohydrates, resorcinols and *n*-alkanols, which all lose >60% of their CH_4-derived C after
356 100 days of incubation under natural abundance CH_4. These losses suggest that despite
357 significant initial incorporation of CH_4-derived C, little of the C will be retained long-term
358 within the Odcombe landfill cover soils. The most recalcitrant forms of C include protein-
359 derived amino acids, steroids and hopanoids (Fig. 3). Notably, a wide range of turnover rates
360 were observed for the hopanoids, and the diversity is likely due to functionalised hopanoids
361 being converted to their de-functionalised more stable analogues, indicating long residence
362 times for the pentacylcic hopanoid core structures.

363 First order dissociation curves were fitted to ^{13}C decay curves (Fig. 4) and associated
364 rate constants were used to calculate CH_4-derived C residence times in the Odcombe soils.
365 Total bulk soil ^{13}C had a half-life of 68 days and 47% of the total C derived from CH_4 was
366 retained within the soil after 100 days. Extrapolation of these values indicates that 1 year after
367 SIS just 19% of the CH_4-derived C will remain in the Odcombe soil, with 81% being released
368 primarily as CO_2 via aerobic soil respiration.

369

370 **Conclusions**

371 This study demonstrates that SIS has considerable potential as a new method for determining
372 the kinetics of soil C uptake, turnover, release and sequestration at the molecular level in
373 complex soil and sedimentary matrices. Our findings indicate that in the Odcombe landfill
374 cover soil 45% of bacterial membrane lipid C was directly derived from CH_4 and at the end
375 of the experiment 47% of bulk soil C derived from CH_4 was retained within the soil. In this
376 communication we have demonstrated the potential power of this technique to resolve

discreet C cycling processes within a complex environmental sample. Whilst we have initially focussed on the soil food web in a landfill cover soil, SIS has considerable potential for identifying and elucidating hitherto elusive aspects of nutrient cycling in many different environments using a range of gaseous or liquid substrates, and will lead to new molecular level estimates of ecosystem nutrient dynamics for use in process-based models of element cycling. Previous applications of SIP have utilised a range of ^{13}C-labelled substrates including acetate, glucose, methanol, CO_2 (both directly and indirectly via individual plants; for full overview see Maxfield and Evershed and references therein).[29] The main drawback of the approach is the requirement to incubate soils removed from their natural habitat for long periods, which has the potential to cause changes in the composition of the wider soil microbial community. This issue could be overcome by conducting SIS incubations *in situ* (i.e., in field setting, using an appropriately designed isotope delivery system).[30] The SIS technique could be readily applied to other light rare isotopes including ^{2}H and ^{15}N and we recently developed an aqueous-based re-circulating isotope delivery system that can be employed to conduct SIS experiments using a wide range of different substrates.

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Figure Captions

Figure 1: Schematic representation of stable isotope switching (SIS) experiment based upon previously observed (light grey) and theoretical (dark grey) curves indicating how ^{13}C-label from $^{13}CH_4$ is incorporated by methanotrophs and turned-over within soil ecosystems. The timescale to achieve full labelling of the target population will vary depending upon the isotopically labelled substrate composition, concentration, delivery method and the nature of the environmental sample to be studied.

Figure 2: Partial gas chromatogram of the Odcombe landfill soil PLFA fraction (T_0 d). Where 1, C_{19} alkane; 2, *i*14:0; 3, *a*14:0; 4, *i*15:0; 5, *a*15:0; 6, 15:0; 7, *i*16:0; 8, 16:0; 9, 16:1ω7, 16:1ω5; 10, *i*17:0; 11, *a*17:0; 12, 17:0; 13, 17:1ω8; 14, 18:0; 15, 18:1ω7; 16, 18:2; 17, 20:0; 18, 18:3; 19, 11-CH_3O-17:0; 20, 22:0; 21, br23:0; 22, 23:0; 23, 24:0. See text for explanation of PLFA nomenclature.[26]

Figure 3: Top panel - ^{13}C-label uptake rate (zero order) from $^{13}CH_4$ into a wide range of soil biochemicals; Bottom panel - ^{13}C-label turnover (first order) following SIS to natural abundance. Whiskers represent the lowest data point still within 1.5 of the interquartile range of the lower quartile, and the highest data point still within 1.5 of the interquartile range of the upper quartile.

Figure 4: Mean $\delta^{13}C$ values for bulk C and selected components extracted from Odcombe landfill soil following incubation under 1.3% CH_4. Error bars represent ±1 standard deviation (n = 3). Left hand side = 1% enriched in $^{13}CH_4$. Right hand side = CH_4 with a natural abundance of ^{13}C and ^{12}C. C decay curves were fit to a first order dissociation rate constant (Equation 2)

517 **Figure 5:** ^{13}C-label incorporation by selected PLFAs. (a) primary consumers, (b) secondary
518 consumers, and (c) tertiary consumers. Note the different y-axis scales for plots a, b and c.
519 Error bars represent ±1 standard deviation from the mean (n = 3).

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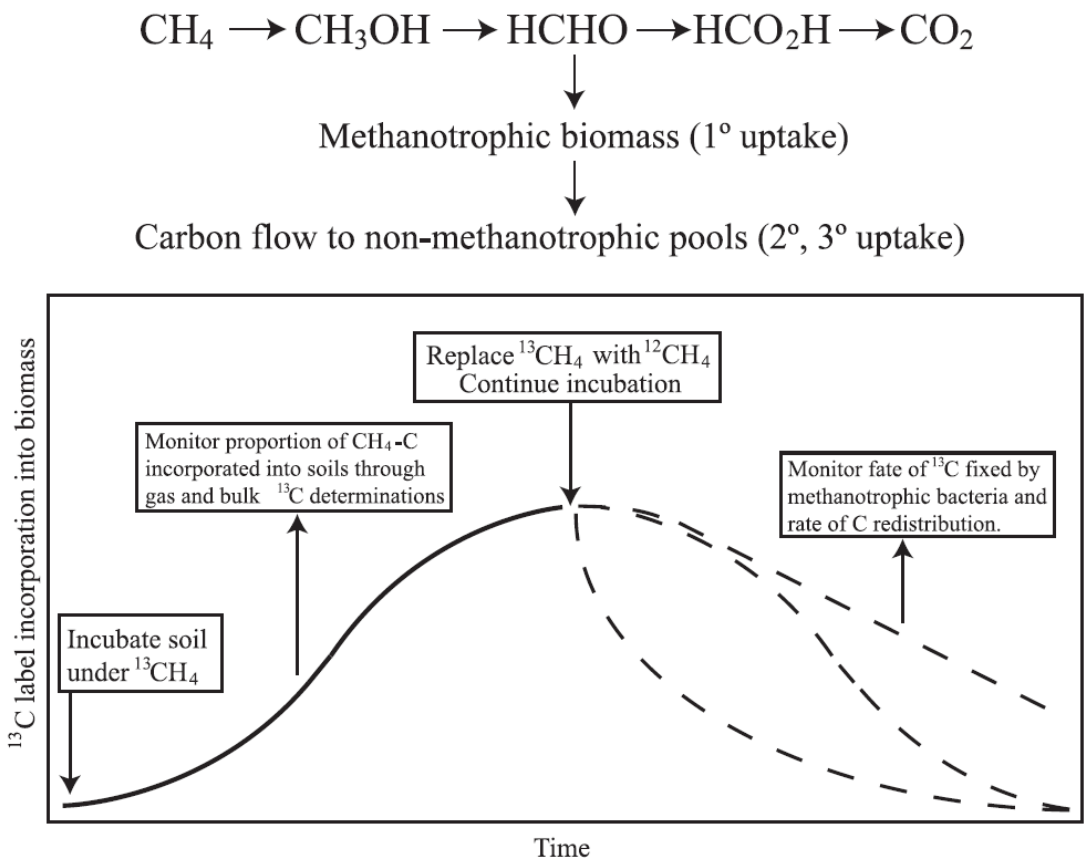

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537 Figure 1: Maxfield *et al.*

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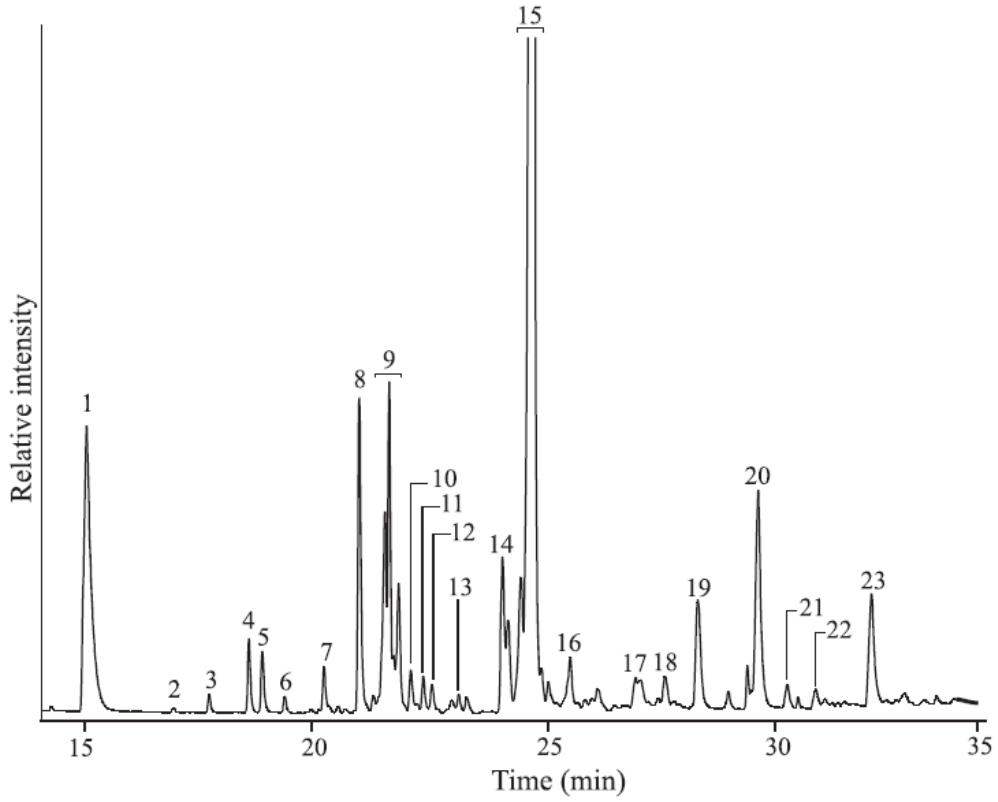

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547 Figure 2: Maxfield *et al.*

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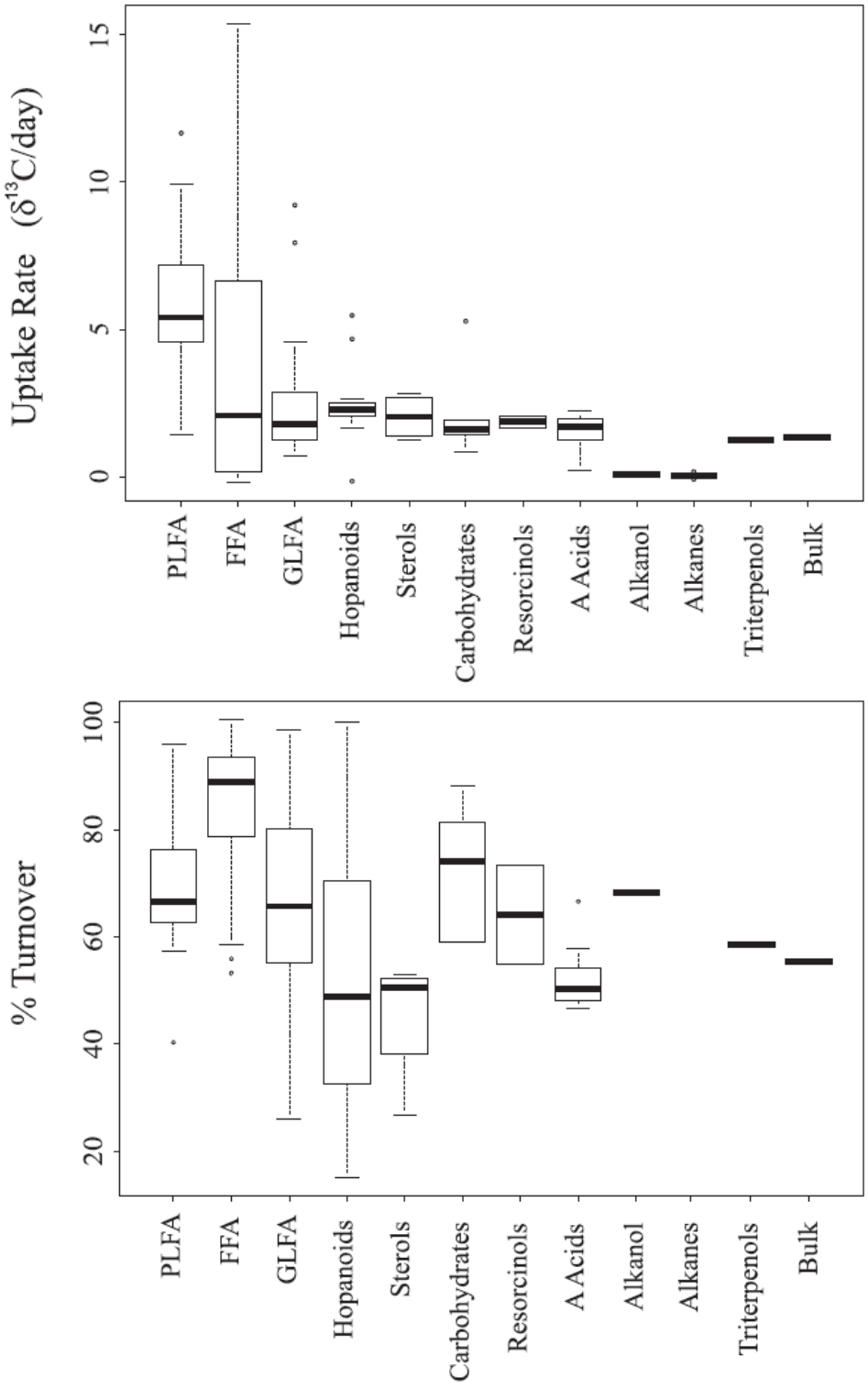
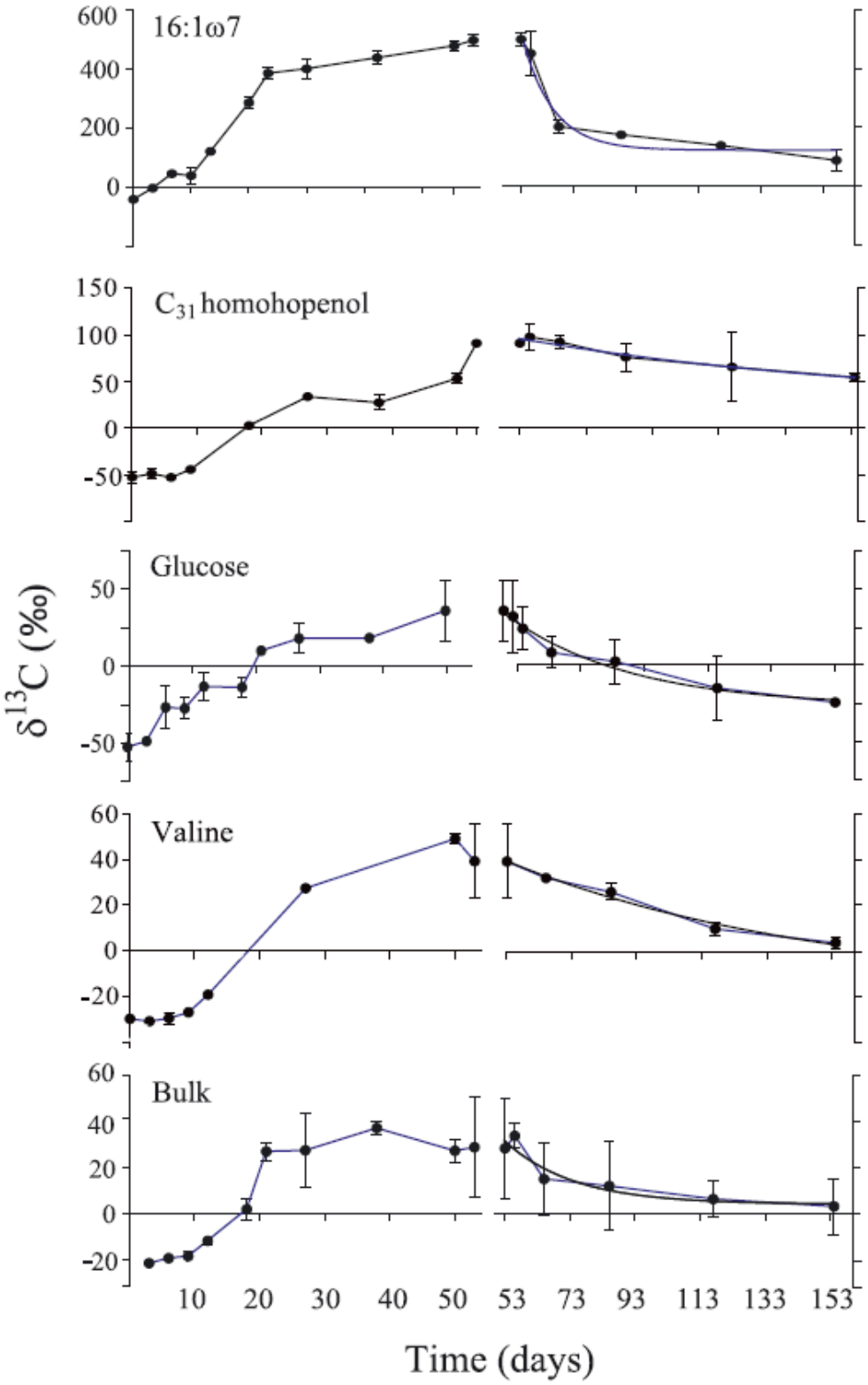


Figure 3: Maxfield *et al.*



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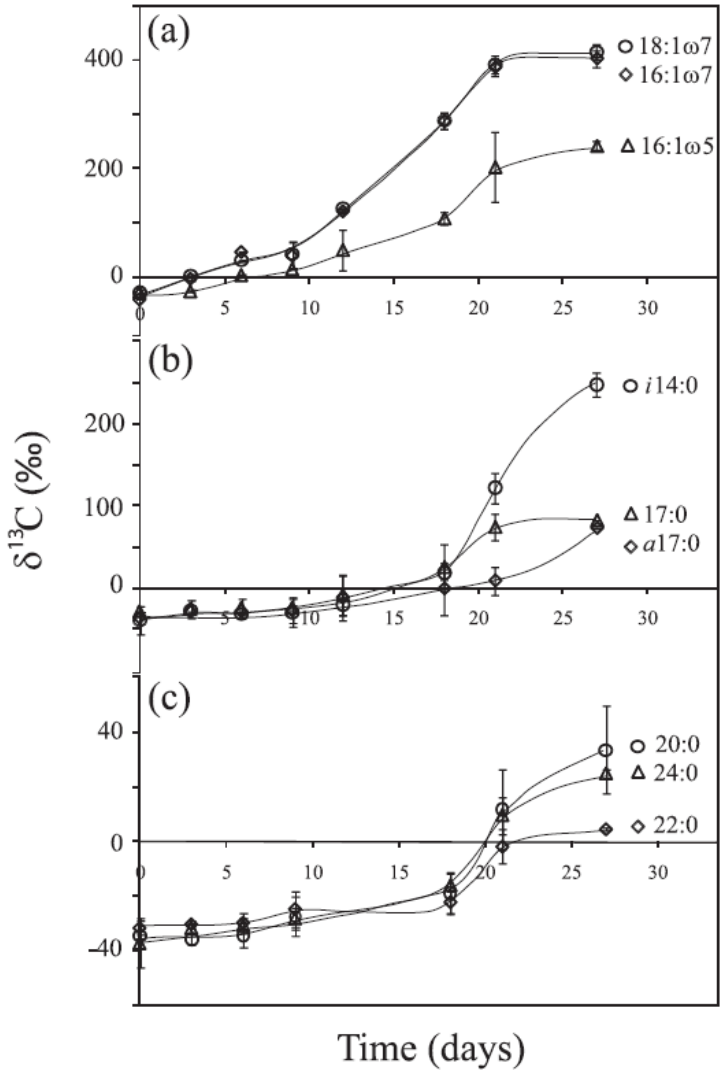
561 Figure 4: Maxfield *et al.*



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563 Figure 5: Maxfield *et al.*