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¹³C-Labeling of lipids to investigate microbial communities in the environment

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The introduction of ¹³C-labelled substrates to soils, sediments or cultures followed by ¹³C analysis of phospholipid fatty acids (PLFAs) provides quantitative and chemotaxonomic information for the groups of microorganisms utilizing a given substrate. Gas chromatography-combustion-isotope ratio mass spectrometry has provided the high precision necessary to measure small isotopic changes (differences in the relative abundances of ¹³C to ¹²C expressed as $\delta^{13}\text{C}$ values) for nanogram amounts of individual compounds, such as microbial PLFAs. This methodology constitutes a powerful new culture-independent method for investigating microbial communities in the environment. The information obtained is highly complementary to that obtained from gene-probe-based methods, and considerable possibilities exist to extend this methodology to include other biochemical components of microorganisms.

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Introduction

The complexity of the microbial populations in sedimentary environments is universally acknowledged, with major challenges to their study arising from the unculturable nature of the major proportion of such populations [1]. This places considerable emphasis on the use of indirect approaches (e.g. field- or laboratory-based CO₂ flux measurements) and/or culture-independent methods, to investigate the nature and functional ecology of such microbes. Gene probes provide phylogenetic information or identify specific groups of microorganisms (reviewed elsewhere in this issue). Complementary to these approaches are compound-specific stable isotope methods based on microbial lipids. This approach has

become possible as a result of the development of gas chromatography-combustion-isotope ratio mass spectrometry (GC/C/IRMS). During the past 15 years very extensive use has been made of $\delta^{13}\text{C}$ values (relative abundance of ¹³C to ¹²C compared to international standard of defined stable carbon isotope composition) of microbial lipid biomarkers (e.g. bacteriohopanes and isoprenoidal lipids) preserved in geologic sediments, to reconstruct ancient environments [2]. An emerging area of application of this methodology is in the investigation of living microbial populations in the environment.

As in the investigation of ancient environments, the use of compound-specific stable isotope approaches to study living microbial populations rests on matching specific compounds, termed biomarkers, to a particular group(s) of organism(s). An essential difference is that if the presence of a given biomarker is taken to indicate the activity of a given microbial group then, on cell death, that biomarker must be rapidly removed from the system. This principle underpins the use of PLFAs to evaluate microbial communities in the environment [3]. Phospholipids are the major components of the cell membranes of living organisms and, crucially, they only remain intact in viable cells [4]; PLFA concentrations and distributions therefore have the capacity to reflect rapid changes in microbial populations. Table 1 summarizes the broad affiliations of PLFAs with known source organisms. Significantly, the specificity of PLFA analysis for assessing the activity of microbial communities in the environment is greatly increased by the use of ¹³C-labelled substrates in conjunction with GC/C/IRMS. This review highlights recent advances in the use of ¹³C-labelling of lipids to study microbial communities in the environment. Topics covered include the ¹³C-labelling of microbes, laboratory and instrumental methods used to determine $\delta^{13}\text{C}$ values of individual lipids and an overview of recent applications of these approaches.

Labelling approaches

Labelling approaches involve adding a ¹³C-labelled substrate to a microcosm, sediment, soil or culture medium, then following its fate temporally and/or spatially. Substrates labelled at natural abundance (i.e. those derived from plants having C₃ or C₄ photosynthetic pathways) provide opportunities for using either whole tissues or biochemical components derived therefrom, as economical and abundant sources of ¹³C-labelled substrate [5,6]. Likewise, free-air carbon dioxide enrichment

Table 1

Classes of PLFAs associated with particular taxonomic or functional groups of microorganisms.

PLFA	Taxonomic or functional group ^a	Ref.
Saturated straight chain >C ₂₀	Prokaryotes, eukaryotes Eukaryotes, mosses, higher plants	[36]
Branched chain	Gram-positive bacteria	[37]
iso/anteiso and others	Gram-positive bacteria	[38,39]
10Me	Gram-negative bacteria: <i>Cytophaga</i> , <i>Acetobacter</i> , <i>Flavobacterium</i> Actinomycetes, sulfate reducers	[40,41]
Cyclopropyl	Gram-negative bacteria: <i>Rhodospirillum</i> , <i>Cromatium/Legionella</i> Anaerobic Gram-positive bacteria: <i>Clostridium</i> , <i>Bifidobacterium</i>	[42]
Monounsaturated	Gram-negative aerobes, obligate anaerobes	[43]
18:1ω8c	Methanotrophic bacteria	[44]
Polyunsaturated	Eukaryotes and cyanobacteria	[45]

^a The PLFA listed is indicative of the group of organisms (or is isolated from them). Table adapted from [3].

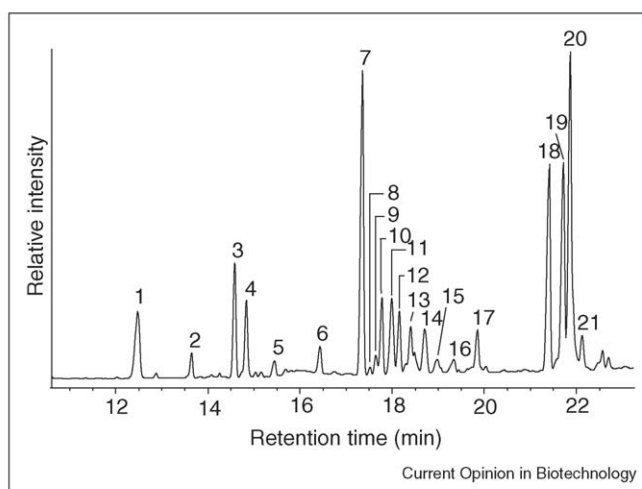
experiments (FACE) provide further useful sources of ¹³C-labelled plant tissues (e.g. δ¹³C = -43‰ [7[•]]). Despite the high sensitivities of the GC/C/IRMS instrument, the small difference (~15 to 20 ‰) between such plant tissues and their respective ecosystems imposes practical limits on detecting the fate of label. An alternative approach is to use highly ¹³C-labelled substrates produced by raising plants or algae on ¹³CO₂ or H¹³CO₃⁻, respectively [8[•]]. Similarly, methanotrophic bacteria can be cultured on ¹³CH₄ [9–12,13[•],14^{••},15[•],16[•],17^{••},18^{••}]. Another approach is to employ ¹³C-labelled substrates produced by organic synthesis. A wide range of ¹³C-labelled compounds are available from commercial suppliers. Universally ¹³C-labelled synthetic compounds can be expensive, thereby restricting their use in field scale studies, however, the high sensitivities of GC/C/IRMS

[19[•]] offer considerable potential. Significantly, ¹³C-labelling at specific positions in substrates raises opportunities for determining the fate of particular moieties or functional groups during assimilation by microbial communities. A novel approach to ¹³C-labelling of below-ground soil microbial communities demonstrated by Ineson and co-workers [20] involves the exposure of photosynthesising plants, either in the field or laboratory, to ¹³CO₂, resulting in ¹³C-labelled root exudates being delivered directly to the rhizosphere.

PLFA analyses and GC/C/IRMS

Analyses of PLFAs from ¹³C-labelling studies proceed as for PLFA ‘fingerprinting’ [3]. Figure 1 shows a typical PLFA profile obtained for a forest soil. The distribution of PLFAs is complex revealing >50 individual PLFAs,

Figure 1



Gas chromatogram of PLFAs extracted from soil from an oak woodland. Peaks are numbered: 1) C₁₉ alkane (IS; internal standard), 2) 14:0, 3) i15:0, 4) a15:0, 5) 15:0, 6) i16:0, 7) 16:0, 8) 16:1ω11c, 9) 16:1ω9c, 10) 16:1ω7c, 11) 10Me16:0, 12) 16:1ω5c, 13) i17:0, 14) a17:0, 15) br17:1 ω 8c, 16) 17:0, 17) 17:1ω 6c, 18) 18:0, 19) 18:1ω9c, 20) 18:1ω7c, 21) 18:1ω5c.

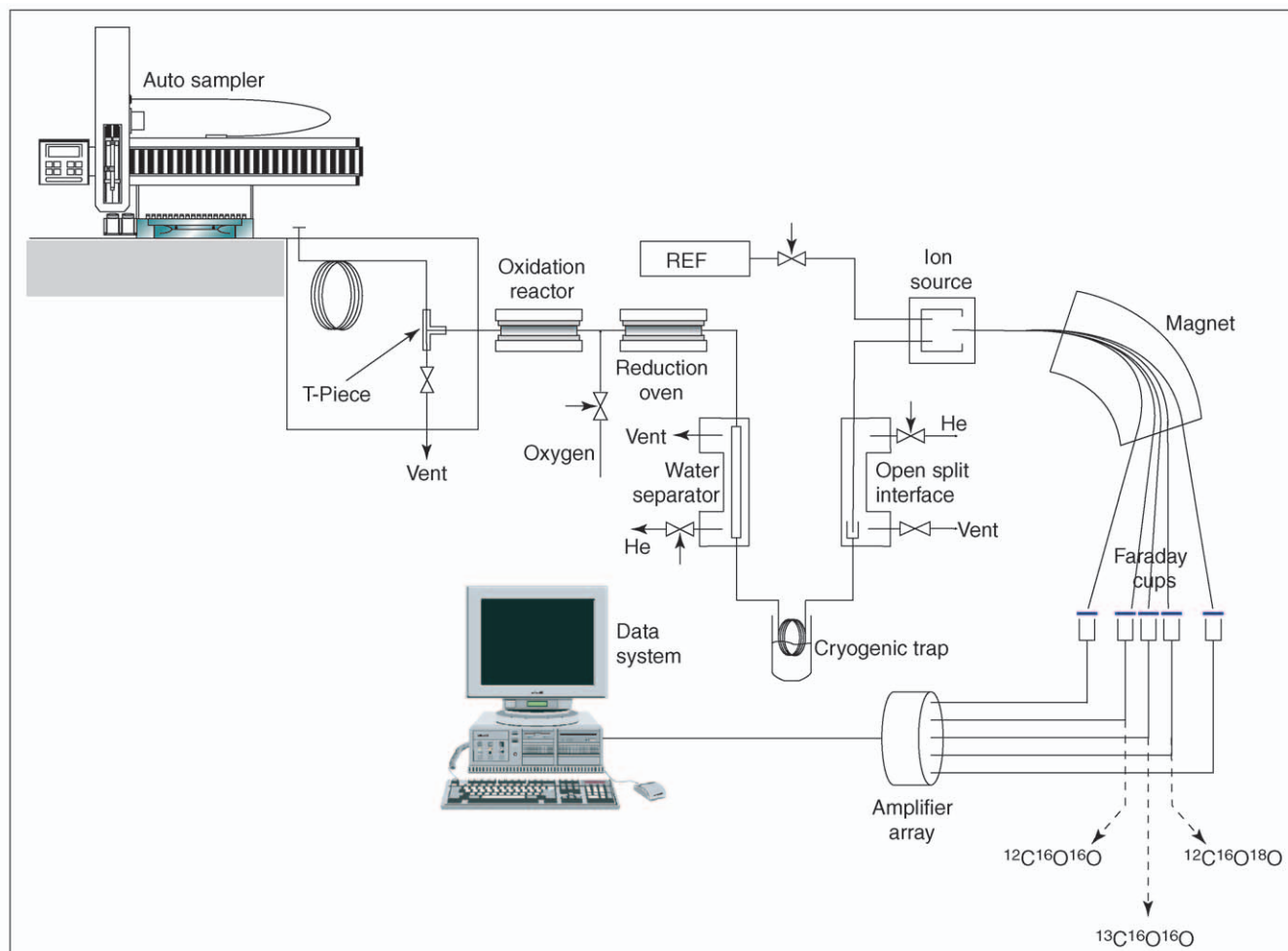
representing the contributions from all the major soil microbial groups. This extract was obtained during an investigation to assess the activities of methanotrophic bacteria. However, on the basis of this profile it is impossible to determine which PLFAs are contributed by methanotrophs and which derive from other classes of microorganism; this can only be revealed by incubating the soil with $^{13}\text{C}\text{H}_4$, then determining PLFA $\delta^{13}\text{C}$ values by GC/C/IRMS.

GC/C/IRMS instrumentation

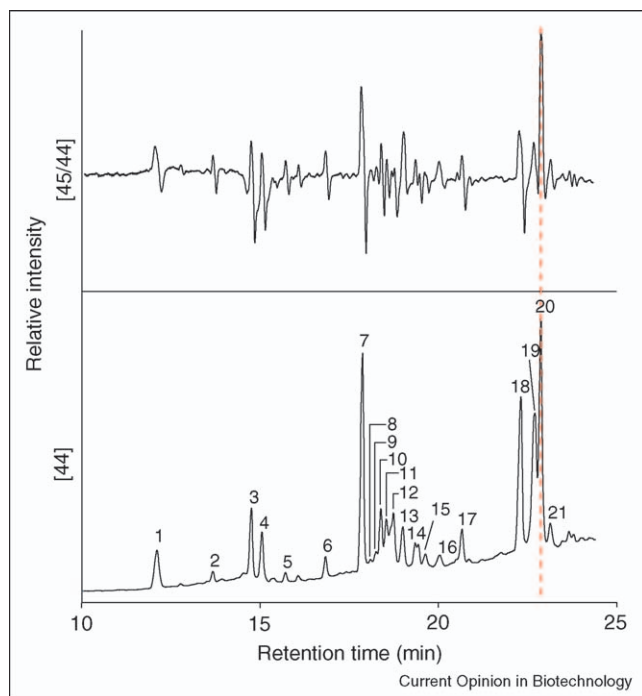
Isotope ratio monitoring GC/MS was first demonstrated by Matthews and Hayes [21] with Barrie and colleagues [22] coupling a GC via a combustion interface to a dual collector MS to produce the forerunner of today's GC/C/IRMS instruments (Figure 2). The prerequisite for

determination of accurate $\delta^{13}\text{C}$ values of individual compounds by GC/C/IRMS is good chromatographic separation. Peaks that are not fully resolved can be manually edited to obtain optimum values. In ^{13}C -labelling studies, closely eluting components may contain widely varying abundances of ^{13}C owing to utilization of ^{13}C -labelled substrate by specific members of the microbial community. This is apparent in Figure 3 in the mass/charge (m/z) 45/44 ratio trace, where the 18:1 ω 7c component exhibits a higher abundance of the m/z 45 versus m/z 44 (higher ratio of $^{13}\text{C}/^{12}\text{C}$) than the 18:1 ω 9c component. An important consideration when analysing highly ^{13}C -enriched compounds ($>10^3\%$) are possible carryover effects between GC analyses [19]. Separate columns and syringes are recommended for determinations of $\delta^{13}\text{C}$ values at natural abundance

Figure 2



Generalised schematic of a GC/C/IRMS instrument configured for $\delta^{13}\text{C}$ analysis. Mixtures of compounds are separated by gas chromatography and combusted online to generate CO_2 and H_2O . H_2O is removed and the CO_2 introduced into a mass spectrometer equipped with a triple collector comprising three Faraday cups monitoring simultaneously m/z 44, 45 and 46, corresponding to $^{12}\text{C}^{16}\text{O}_2$, $^{13}\text{C}^{16}\text{O}_2$ and $^{12}\text{C}^{18}\text{O}^{16}\text{O}$, respectively. The output currents are amplified and integrated to allow calculation of $\delta^{13}\text{C}$ values. Reference CO_2 and fatty acids of known $\delta^{13}\text{C}$ values are utilized to monitor instrument performance and to standardise determinations.

Figure 3

The mass/charge ratio (m/z) 44 ion current (below) and instantaneous ratio of m/z 45/44 ions (above) recorded for PLFAs extracted from oak woodland soil following incubation with 10 ppm $^{13}\text{CH}_4$. It is possible to see (indicated by the dashed line) the higher m/z 45/44 ratio of 18:1 ω 7c (peak 20) compared with the 18:1 ω 9c (peak 19), indicative of the higher concentration of ^{13}C in this fatty acid. Peaks are numbered as in the caption for Figure 1.

and high enrichments; likewise septa and injector liners should be changed between the two types of analysis. A further important consideration when analysing highly ^{13}C -enriched PLFAs is the amplification range of the m/z 45 Faraday cup, as high ^{13}C abundances will saturate the detector when set to a range suitable for natural abundance determinations.

Conventional GC/MS instruments operating in the selected ion monitoring mode have been used to follow the fate of ^{13}C -labelled substrates into environmental microbial populations [8^{*},23]. However, complications arise in the selection of ions for monitoring in complex environments where target compounds are unlikely to be fully labelled, resulting in complex ion envelopes and, hence, greatly reduced detection limits compared with GC/C/IRMS.

^{13}C -Labelled PLFA distributions

The higher $\delta^{13}\text{C}$ values obtained for several PLFAs from a forest soil confirms incorporation of $^{13}\text{CH}_4$ (Figure 4a). Although this information is useful, a more valuable representation is obtained by calculating the concentra-

tion of ^{13}C in the various PLFAs (e.g. nanograms of ^{13}C per gram of soil), which can then be used to provide ^{13}C -PLFA 'fingerprint' distributions (Figure 4b) for chemotaxonomic assignments [2,9]. Rearranging the defining equation for $\delta^{13}\text{C}$ values yields Equation 1:

$$R_{\text{samp}} = \left(\frac{\delta^{13}\text{C}_{\text{samp}}}{1000} \times R_{\text{std}} \right) + R_{\text{std}} \quad (1)$$

From R_{samp} ($^{13}\text{C}/^{12}\text{C}$) the fractional abundance (F) of ^{13}C is calculated using Equation 2:

$$F = \frac{R}{R+1} = \frac{^{13}\text{C}}{^{13}\text{C} + ^{12}\text{C}} \quad (2)$$

By subtracting the fractional abundance of the unlabelled fatty acid from its fractional abundance following ^{13}C -labelling, the fraction of excess ^{13}C per carbon of the fatty acid is calculated. Multiplying this value by the concentration of carbon present in the target fatty acid (Equation 3) yields the total concentration of ^{13}C -labelled fatty acid.

$$\text{Amount of } ^{13}\text{C} = C(F_{\text{lab}} - F_{\text{unlab}}) \quad (3)$$

Where C is the concentration of carbon in the sample compound in ng (g matrix) $^{-1}$.

^{13}C -Labelling of lipids to investigate microbial communities in the environment

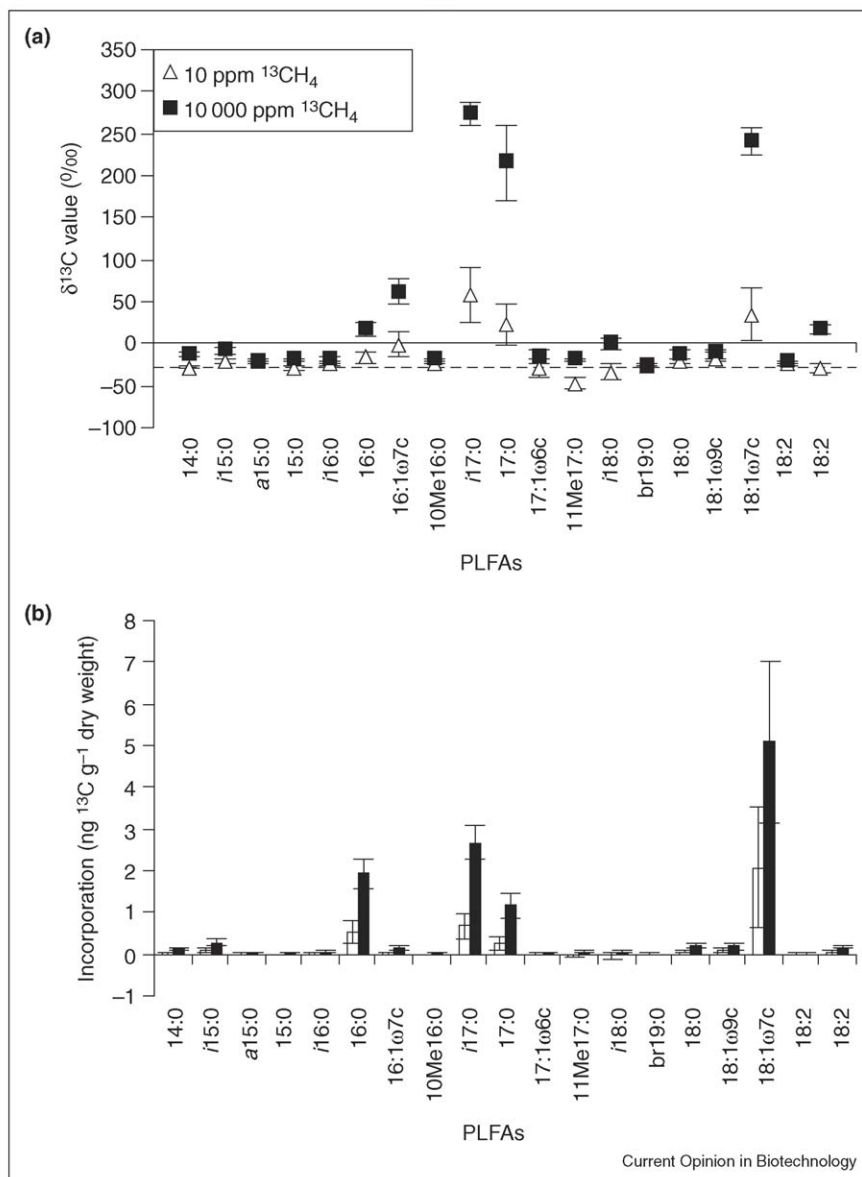
Table 2 provides a summary of the range of studies of environmental microbes involving lipids undertaken using ^{13}C -labelling and GC/C/IRMS. Clearly, PLFAs are the major lipids used; an advantage of using ^{13}C -labelling being the additional selectivity achieved compared with PLFA 'fingerprinting' alone.

Methanotrophic bacteria

A major area of utility of this methodology is the use of $^{13}\text{CH}_4$ to investigate methanotrophic bacteria in both aerobic and anaerobic environments, including sediments [9,10], soils [11,12,13^{*},14^{**},15^{*},16^{*}], microbial mat [17^{**}] and peat bogs [18^{**}]. A major advantage of using methane arises from its ease of addition to microcosms, providing the opportunity to target an important group of microorganisms. The major finding from studies performed to date are summarized in Table 2.

The ability to detect the effect of a changing environment on a given functional group of microorganisms would be of obvious advantage. Crossman *et al.* [13^{*}] used $^{13}\text{CH}_4$ incubations in laboratory microcosms to demonstrate variations in methanotrophic bacterial populations with depth through a landfill cover soil (Figure 5); type I methanotrophs were found to be more active in the surface layers, where concentrations of oxygen were highest and methane concentration low, whereas type II methanotrophs dominated in the deepest layers of the cap where methane concentrations were high and oxygen low. This approach was especially effective in investigat-

Figure 4



Raw and processed stable carbon isotopic information for PLFAs from a forest soil incubated with 10 and 10000 ppm $^{13}\text{CH}_4$. **(a)** $\delta^{13}\text{C}$ values of PLFAs. The dashed line represents the values for PLFAs extracted from control soils incubated with unlabelled CH_4 . **(b)** Absolute amount of ^{13}C incorporated into each of the PLFAs.

ing unculturable high-affinity methanotrophs in soils, revealing a novel type II methanotroph producing a br17:0 PLFA [11]. Additional bacterial markers (e.g hopanoids) can provide complementary chemotaxonomic information to PLFAs [12,14 \bullet]. Both classes of bacterial biomarker (Figures 6 and 7) indicated that the high-affinity methanotroph population in the forest soil highlighted above was related to type II methanotrophs, with PLFAs indicating a relationship to *Methylocapsa* and *Methylocella* genera of bacteria. In a further study of soil

methanotrophs, PLFA and gene probe techniques were adopted by Knief *et al.* [16 \bullet]. They reasoned that PCR detection of the *pmoA* gene, although an excellent functional gene marker, does not necessarily indicate the presence of a physiologically active methanotroph community. By contrast, the presence of ^{13}C -labelled PLFAs, following incubation of soils with $^{13}\text{CH}_4$, unambiguously confirms the presence of an active population. Significantly, different methanotrophic bacteria were shown to be present and active in different soils.

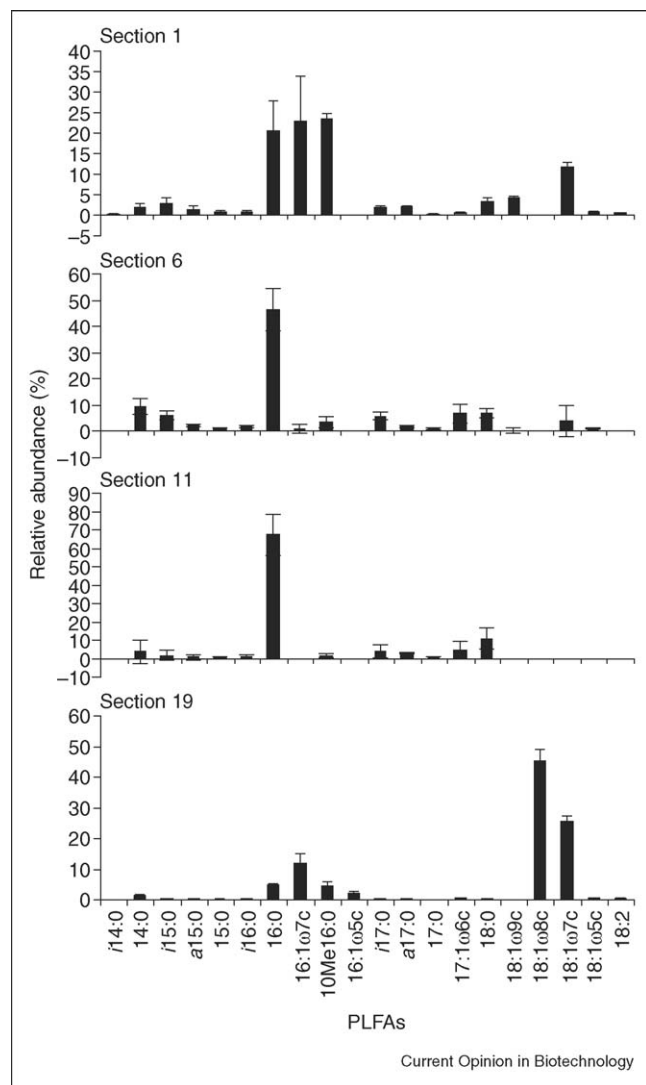
Table 2

¹³C-Labeling studies of environmental microbial communities.

Environment	¹³ C-labeled substrate	Biomarker	Detection method	Conclusions	Ref.
Laboratory microbial cultures	Natural abundance glycerol, glucose, mannose, lactose, complex medium	PLFA	GC/C/IRMS	Complex fractionation patterns varying with substrate and organism	[5]
Woodland and grassland soils	Universally labelled starch, xylose, vanillin and leaf litter	PLFA	GC/C/IRMS	Similar microbial groups responsible for degrading simple substrates in woodland and grassland soils, but different communities degraded complex substrates	[7*]
Estuarine sediments	[U- ¹³ C]acetate	PLFA	GC/C/IRMS	Acetate consumed by SRB similar to Gram-positive <i>Desulfotomaculum acetoxidans</i> and not by a population of Gram-negative <i>Desulfobactor</i> spp.	[9]
Estuarine sediments	H ¹³ CO ₃ ⁻	PLFA	GC/C/IRMS	Central role for microphytobenthos in moderating carbon flow in coastal sediments	[33]
Experimental soils	[1- ¹³ C]sodium acetate	PLFA, neutral lipids, glycolipids	GC/MS SIM	Incorporation greatest into PLFA; bacterial growth limited as low pH but occurred at pH 7 and 8	[23]
Rhizosphere rice paddy soil	¹³ CO ₂	PLFA	GC/C/IRMS	Microbial populations in rice soil differ in their response to plant photosynthate input	[31]
Rhizosphere grassland soil	¹³ CO ₂	PLFA	GC/C/IRMS	¹³ C-labelling showed fundamental differences in the way rhizodeposition was cycled through a microbial community during different stages of plant development	[30*]
Rhizosphere grassland soil	¹³ CO ₂	PLFA	GC/C/IRMS	Fungal and Gram-negative bacterial PLFAs showed most ¹³ C-enrichment. Liming did not affect assimilation or turnover rates of ¹³ C-label	[32**]
Sediments from petroleum-contaminated aquifer	[methyl- ¹³ C]toluene	PLFA	GC/C/IRMS	PLFAs resemble those of PHC-degrading <i>Azoarcus</i> spp.	[27]
Petroleum-contaminated groundwater	[2- ¹³ C] acetate	PLFA (FISH) ^a	GC/C/IRMS	Field-scale application of acetate to investigate carbon assimilation and mineralisation	[25]
Petroleum-contaminated aquifer water and sediment	[2- ¹³ C]acetate	PLFA (FISH) ^a	GC/C/IRMS	The main SRB degrading acetate in water was <i>Desulfotomaculum acetoxidans</i> and <i>Desulfobactor</i> in sediment	[26*]
Antarctic soil bacteria	¹³ C-labelled grass	Ergosterol, PLFA, NLFA	GC/MS SIM	Incorporation of ¹³ C increased over incubation period, but was not seen in PLFA and NLFA fractions	[8*]
Soil	Ring-labelled [¹³ C]toluene and [U- ¹³ C]glucose	PLFA	GC/C/IRMS	Specific labelling patterns for microbial PLFAs from ¹³ C-toluene incubation contrast with universal labelling of PLFAs from incubation with ¹³ C-glucose	[28]
Batch culture	[U- ¹³ C]toluene	PLFA	GC/C/IRMS	Quantified carbon flow along substrate-bacteria-protist food chain	[29*]
Soil	¹³ CH ₄	Hopanoids	GC/C/IRMS	Specific bacteriohopanoids labelled	[12]
Soil	¹³ CH ₄	PLFA	GC/C/IRMS	Identified new ambient methane-oxidising methanotroph similar to culturable type II	[11]
Upland soils	¹³ CH ₄	PLFA, DGGE	GC/C/IRMS	Different methanotrophs are present in different soils that oxidise atmospheric methane	[16*]
Landfill cover soils	¹³ CH ₄	PLFA	GC/C/IRMS	Changes in methanotrophic community from type I to type II with depth	[13*]
Soil	¹³ CH ₄	PLFA, hopanoids	GC/C/IRMS	Novel population of methane-oxidising bacteria related to type II methanotrophs, <i>Methylocapsa</i> and <i>Methylocella</i>	[15*]
Sediment/soil	¹³ CH ₄	PLFA	GC/C/IRMS	Shift in the composition of the methane-oxidising bacterial community in sediments/soils treated with ammonium	[10,15*]
Peat bog	¹³ CH ₄	Hopanoids, sterols (FISH)	GC/C/IRMS	Methanotrophic bacteria associated with <i>Sphagnum</i> mosses provide CO ₂ for photosynthesis	[18**]
Anaerobic oxidation of methane	¹³ CH ₄	PLFA, archaeal lipids	GC/C/IRMS	¹³ C uptake into specific lipids indicates that phylogenetically distinct microbes participate in the anaerobic oxidation of methane	[17**]

PHC, petroleum hydrocarbon contamination; SRB, sulfate-reducing bacteria.

Figure 5



Relative abundances of ^{13}C -labelled PLFAs extracted from four sections of the profile of a landfill cap following incubation with 10 000 ppm CH_4 containing 1% ^{13}C .

In contrast to soils, wetlands are an important natural source of methane. Indeed, a recent report involving the quantitative carbon isotopic analysis of ^{13}C -labelled hopanoids and sterols showed that endophytic methanotrophic bacteria provide a significant (10–15%) carbon source for the growth of *Sphagnum* mosses [18^{••}]. The use of fluorescence *in situ* hybridisation (FISH) analyses in conjunction with scanning electron microscopy (SEM) confirmed the presence of methanotrophic bacteria in stem leaves.

Blumenberg *et al.* [17^{••}] have extended ^{13}C -labelling studies of methanotrophs to an anaerobic methane-oxi-

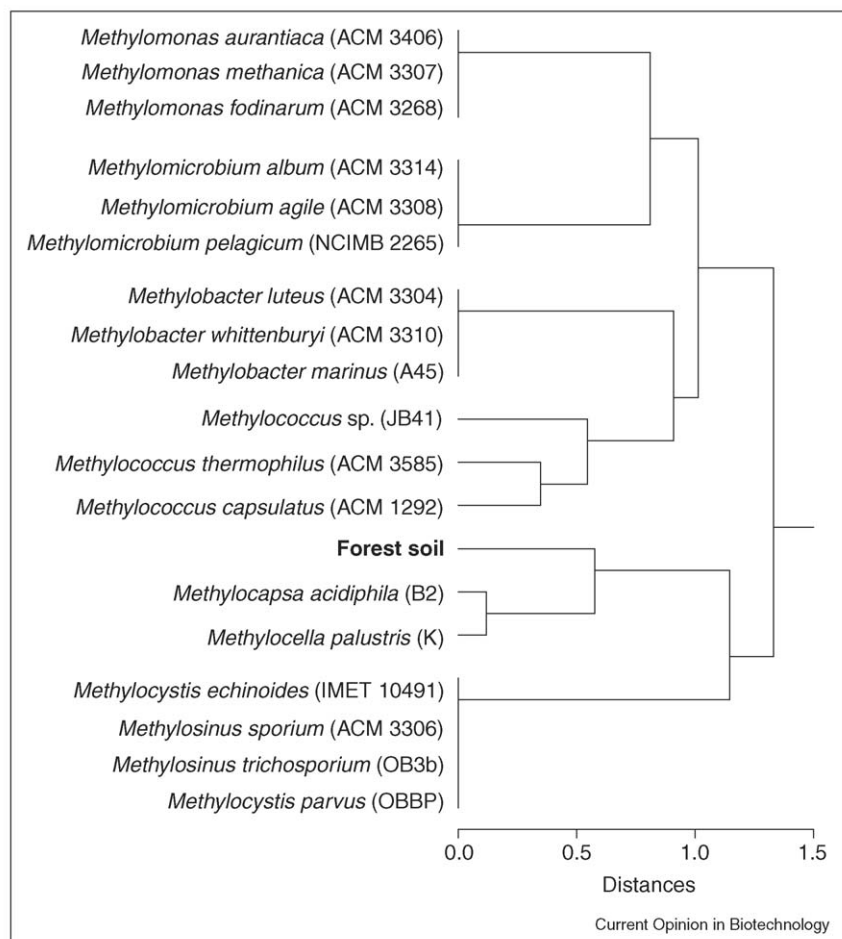
dising microbial mat. GC/C/IRMS analyses were performed on a range of compound classes following *in vitro* incubation with ^{13}C . Very significant differences were observed in the $\delta^{13}\text{C}$ values of the various bacterial and archaeal lipids. Largest differences were seen in the $\delta^{13}\text{C}$ values of bacterial fatty acids, archaeol, a mono-unsaturated archaeol and biphytanes. Incorporation of ^{13}C -label into a suite of polyunsaturated pentamethylcycosanolids indicated that methanotrophic archaea possess a biosynthetic pathway similar to that of methanogenic archaea. Moreover, greater uptake of ^{13}C into the lipids of sulfate-reducing bacteria (SRB) than those of archaea supports the hypothesis that autotrophic growth of SRB occurs on a methane-derived substrate supplied by the methanotrophs.

Utilizers of mineralisation products

Another important use of ^{13}C -labelling has been for investigations of bacterial communities utilizing the products of organic matter mineralisation. Boschker and co-workers [24] have shown through laboratory incubation of small anoxic/brackish sediment cores that ^{13}C -acetate and ^{13}C -propionate were utilized by different members of the microbial community. ^{13}C from acetate was recovered mainly from PLFAs with even numbers of carbon atoms (16:1ω7c, 16:0, 18:1ω7c), whereas fatty acids with odd numbers of carbon atoms (a15:0, 15:0, 17:1ω6, 17:0) were primarily labelled upon incubation with propionate. These findings clearly indicate that the two substrates were predominantly consumed by different specialized groups of SRB. The PLFA labelling pattern for the acetate consumers was similar to *Desulfotomaculum acetoxidans* and *Desulfotomaculum* spp., two acetate-consuming SRB, while those of the propionate consumers did not resemble any known strain.

Pombo and co-workers [25] have employed ^{13}C -labelling of PLFAs to trace the assimilation of ^{13}C -acetate in the mineralisation zone of a petroleum hydrocarbon-contaminated aquifer at the field scale. Some 500L of aquifer water were prepared containing 0.25 mM [2- ^{13}C]acetate (together with 0.5 mM NO_3^- and 0.5 mM Br^- , the latter being added as a conservative tracer) and injected into the aquifer. Samples were removed at 4 h, 23 h and 46 h and PLFA and FISH analyses suggested the presence of active denitrifiers. Label incorporation was seen in PLFAs and dissolved inorganic carbon after 4 h, with a high degree of labelling (>5000 ‰) occurring in certain PLFAs (especially mono-unsaturates) after 46 h. An analogous approach was adopted to investigate acetate-degrading SRB in the same aquifer [26[•]]. These studies emphasized the feasibility of using ^{13}C -labelled substrates in the field for biogeochemical investigations in a way that was unfeasible with radiocarbon tracers. However, it has been emphasized that laboratory experiments should be undertaken before advancing to field-scale experiments [27].

Figure 6



Comparison of the ¹³C-labelled PLFA distribution extracted from a forest soil following incubation with 10 ppm ¹³CH₄ with published PLFA compositions of pure cultures of methanotrophic bacteria [34,44]. Data used were the mole percentages of the PLFAs of pure cultures and the labelled PLFAs extracted from the soil. A hierarchical tree was produced by clustering analysis performed with the software package SYSTAT version 7 on Euclidean distances between the standardised data using averages.

Toluene-degrading microorganisms

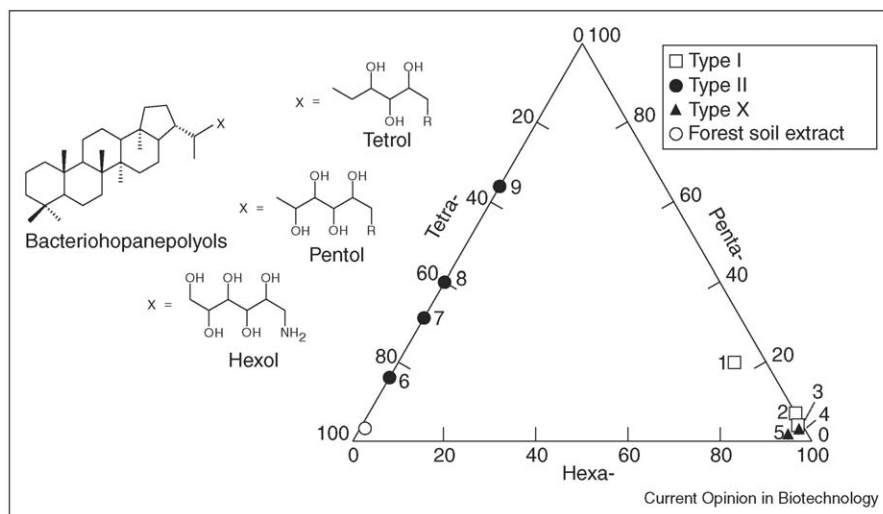
Several reports [27,28,29[•]] have focused on toluene as a microbial substrate, owing to its importance as a pollutant. Incubation of ¹³C-ring-labelled toluene with agricultural soil showed ¹³C to appear in only 16 of the 59 PLFAs extracted from the soil. A high degree of congruency (85%) was seen between the ¹³C-labelled PLFAs and those of the toluene-metabolising microorganisms isolated from the same soil. Interestingly, 91% of the total soil PLFAs were labelled when the same soil was incubated with ¹³C-glucose, confirming the power of coupling ¹³C tracers with PLFA analysis for investigating substrate metabolism in complex environments. The value of combining ¹³C-labeling of PLFAs with gene probe methods to study toluene-degrading microbial populations has also been demonstrated [27]. Additionally, using ¹³C-toluene as a substrate, Mauclair *et al.* [29[•]] provided

important insights into a substrate–bacteria–protist food chain. The stable carbon isotope values, together with biomass and biovolume determinations, were crucial in modelling carbon fluxes through the food chain.

Rhizosphere microbial biomass

A novel means of assessing the activities of rhizosphere microbial communities is to expose photosynthesising plants to ¹³CO₂ in the laboratory or field and then investigate the incorporation of ¹³C into microbial PLFAs obtained from rhizosphere soil. Three recent studies have exploited this approach [30[•],31,32^{••}]. Butler *et al.* [30[•]] pulse-labelled rye grass by exposure to ¹³CO₂ in laboratory microcosms, then employed stable carbon isotope analyses of PLFAs from the rhizosphere and bulk soil to investigate carbon dynamics at two stages during plant growth. The analyses showed most extensive labelling of

Figure 7



Percentages of each of the three main bacteriohopanepolyols extracted from various methanotrophic bacteria [35] and the labelled bacteriohopanepolyols extracted from a forest soil. The chemical structures of hexa-, penta- and tetra- bacteriohopanepolyols are shown on the left. Numbers refer to the methanotrophic bacterial species: 1, *Methylomonas* sp. (N1D1); 2, *Methylomicrobium album* (ACM 3314); 3, *Methylomonas methanica* (SI); 4, *Methylococcus capsulatus* (TRMC); 4, *Methylococcus capsulatus* (MC); 6, *Methylosinus sporium* (5); 7, *Methylosinus trichosporium* (PG); 8, *Methylocystis parvus* (OBBP); 9, *Methylosinus trichosporium* (OB3b).

the fungal PLFA 18:2 ω 6,9. Lu *et al.* [31] undertook similar microcosm experiments with rice plants. GC/C/IRMS of PLFAs showed ^{13}C to be incorporated rapidly into rhizosphere microbial PLFAs immediately following 6 h exposure to $^{13}\text{CO}_2$, confirming the tight coupling of rhizodeposition to microbial communities. Field-scale pulsing of $^{13}\text{CO}_2$ in plants in an upland grassland offered the potential to investigate carbon dynamics *in situ* [20,32**]. By quantifying the amount of ^{13}C in the rhizosphere, and in microbial PLFAs at two different time points, estimates were obtained of turnover rates for each of the PLFAs. The results indicate that liming (a common agricultural practice to increase soil pH and nutrient availability) had little effect on assimilation or turnover rates of the microbial biomass compared with non-limed controls. These investigations clearly show the potential to probe plant-microbe interactions using ^{13}C -labelling approaches.

Conclusions

^{13}C -labelling of microbial PLFAs provides an important new culture-independent approach for the study of environmental microorganisms. The methodology offers several advantages. Firstly, ^{13}C -labelled bacterial PLFAs confirm the presence of an active microbial community, which cannot be confirmed by genetic probes (e.g. PCR). Secondly, ^{13}C -labelling of a specific subset of PLFAs, as a result of incubation with specific substrates, highlights members of the microbial community consuming that substrate. Thirdly, quantified ^{13}C -labelled PLFA profiles can be searched against existing PLFA databases of cultured microorganisms to provide taxonomic informa-

tion. Lastly, variations in ^{13}C -labelled PLFA profiles between environments following treatment with a substrate indicate population-level differences.

An expansion of the applications of ^{13}C -labelling studies of microbes will be seen over the next few years. The currently available ^{13}C -labelled substrates provide considerable scope for new avenues of enquiry, with the possibility of expanding the range of specialist applications through the custom synthesis of ^{13}C -labelled compounds. Moreover, possibilities exist for enhancing the stable carbon isotopic analysis of PLFAs through the use of other lipid, and indeed non-lipid, biomarkers.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Amann R, Ludwig W, Schleifer KH: **Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation.** *Microbiol Rev* 1995, **59**:143-149.
2. Boschker HTS, Middelburg JJ: **Stable isotopes and biomarkers in microbial ecology.** *FEMS Microbiol Ecol* 2002, **40**:85-95.
3. Zelles L: **Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: a review.** *Biol Fertil Soil* 1999, **29**:111-129.
4. White DC, Davis WM, Nickels JS, King JD, Bobbie RJ: **Determination of the sedimentary microbial biomass by extractable lipid phosphate.** *Oecologia* 1979, **40**:51-62.

5. Abraham W-R, Hesse C, Pelz O: **Ratios of carbon isotopes in microbial lipids as an indicator of substrate usage.** *Appl Environ Microbiol* 1998, **64**:4202-4209.
6. Ekblad A, Hoögberg P: **Analysis of $\delta^{13}\text{C}$ of CO_2 distinguishes between microbial respiration of added C_4 -sucrose and other soil respiration in a C_3 -ecosystem.** *Plant Soil* 2000, **219**:197-209.
7. Waldrop MP, Firestone MK: **Microbial community utilization of recalcitrant and simple carbon compounds: impact of oak woodland communities.** *Oecologia* 2004, **138**:275-284.
This study revealed differences in the response of microbial communities in woodland and grassland habitats to complex and simple substrates.
8. Malosso E, English L, Hopkins DW, O'Donnell AG: **Use of ^{13}C -labelled plant materials and ergosterol PLFA and NLFA analyses to investigate organic matter decomposition in Antarctic soil.** *Soil Biol Biochem* 2004, **36**:165-175.
Reports the use of a ^{13}C tracer with GC/MS and selected ion monitoring to demonstrate differences in the activities of bacterial and fungal communities.
9. Boschker HTS, Nold SC, Wellsbury P, Bos D, de Graaf W, Pel R, Parkes RJ, Cappenberg TE: **Direct linking of microbial populations to specific biogeochemical processes by ^{13}C -labelling of biomarkers.** *Nature* 1998, **392**:801-805.
10. Nold SC, Boschker HTS, Pel R, Laanbroek HJ: **Ammonium addition inhibits ^{13}C -methane incorporation into methanotroph lipids in a freshwater sediment.** *FEMS Microbiol Ecol* 1999, **29**:81-89.
11. Bull ID, Parekh NR, Hall GH, Ineson P, Evershed RP: **Detection and classification of atmospheric methane oxidising bacteria in soil.** *Nature* 2000, **405**:175-178.
12. Crossman ZM, McNamara N, Ineson P, Evershed RP: **A new method for identifying the origins of simple and complex hopanoids in sedimentary materials using stable isotope labelling with $^{13}\text{CH}_4$ and compound specific stable isotope analyses.** *Org Geochem* 2001, **32**:359-364.
13. Crossman ZM, Abraham F, Evershed RP: **Stable isotope pulse-chasing and compound specific stable carbon isotope analysis of phospholipid fatty acids to assess methane oxidizing bacterial populations in landfill cover soils.** *Environ Sci Technol* 2004, **38**:1359-1367.
Reports the observed trends in type I to type II methanotrophs with soil depth, the results fit with known oxygen tolerances.
14. Crossman ZM, Ineson P, Evershed RP: **The use of ^{13}C labelling of bacterial lipids in the characterisation of ambient methane-oxidising bacteria in soils.** *Org Geochem* 2005, **36**:769-778.
Chemotaxonomic assignments of methanotrophs based on complementary analyses of hopanoids and PLFAs.
15. Crossman ZM, Ineson P, Evershed RP: **Investigation of the effect of ammonium sulfate on populations of ambient methane oxidising bacteria by ^{13}C -labelling and GC/C/IRMS analysis of phospholipid fatty acids.** *Soil Biol Biochem*: in press.
Contributes to the debate on the impact of inorganic fertilisers on methanotrophs.
16. Knief C, Lipski A, Dunfield PF: **Diversity and activity of methanotrophic bacteria in different upland soils.** *Appl Environ Microbiol* 2003, **69**:6703-6714.
A paper stressing the importance of ^{13}C -labelling of PLFAs for confirming active microbial populations.
17. Blumenberg M, Seifert R, Nauhaus K, Pape T, Michaelis W: **In vitro study of lipid biosynthesis in an anaerobically methane-oxidising microbial mat.** *Appl Environ Microbiol* 2005, **71**:4345-4351.
Reports a range of bacterial biomarkers investigated in an anaerobic methane-oxidising microbial mat.
18. Raghoebarsing A, Smolders AJP, Schmid MC, Rijpstra WIC, Wolters-Arts M, Derkson J, Jetten MSM, Schouten S, Sinninghe-Damsté JS, Lamers LPM et al.: **Methanotrophic symbionts provide carbon for photosynthesis in peat bogs.** *Nature* 2005, **436**:1153-1156.
 $^{13}\text{CH}_4$ -labelling of hopanoids and sterols indicate symbiotic methanotrophs in peat-forming vegetation.
19. Mottram H, Evershed RP: **Practical considerations in GC/C/IRMS of ^{13}C -enriched compounds.** *Rapid Commun Mass Spectrom* 2003, **17**:2669-2674.
Investigation of carry-over effects when highly ^{13}C -enriched compounds are present in a GC/C/IRMS analysis.
20. Ostle N, Ineson P, Benham D, Sleep D: **Carbon assimilation and turnover in grassland vegetation using an in situ $^{13}\text{CO}_2$ pulse labelling system.** *Rapid Commun Mass Spectrom* 2000, **14**:1345-1350.
21. Matthews DE, Hayes JM: **Isotope-ratio-monitoring gas chromatography-mass spectrometry.** *Anal Chem* 1978, **50**:1465-1473.
22. Barrie A, Bricout J, Koziet J: **Gas-chromatography – stable isotope ratio analysis at natural abundance levels.** *Biomed Mass Spectrom* 1984, **11**:439-447.
23. Arao T: **In situ detection of changes in soil bacterial and fungal activities by measuring ^{13}C incorporation into phospholipid fatty acids from ^{13}C acetate.** *Soil Biol Biochem* 1999, **31**:1015-1020.
24. Boschker HTS, de Graaf W, Koster M, Meyer-Reil LA, Cappenberg TE: **Bacterial populations and processes involved in acetate and propionate consumption in anoxic brackish sediment.** *FEMS Microbiol Ecol* 2001, **35**:97-103.
25. Pombo SA, Pelz O, Schroth MH, Zeyer J: **Field-scale ^{13}C -labeling of phospholipid fatty acids (PLFA) and dissolved inorganic carbon: tracing acetate assimilation and mineralisation in a petroleum hydrocarbon-contaminated aquifer.** *FEMS Microbiol Ecol* 2002, **41**:259-267.
26. Pombo SA, Kleikemper J, Schroth MH, Zeyer J: **Field-scale isotopic labelling of phospholipid fatty acids from acetate degrading sulfate-reducing bacteria.** *FEMS Microbiol Ecol* 2005, **51**:197-201.
Field-scale applications of acetate to investigate aquifer microbial biogeochemistry.
27. Pelz O, Chatzinotas A, Andersen N, Bernasconi SM, Hesse C, Abraham W-R, Zeyer J: **Use of isotopic and molecular techniques to link toluene degradation in denitrifying aquifer microcosms to specific microbial populations.** *Arch Microbiol* 2001, **175**:270-281.
28. Hanson JR, Macalady JL, Harris D, Scow KM: **Linking toluene degradation with specific microbial populations in soil.** *Appl Environ Microbiol* 1999, **65**:5403-5408.
29. Mauclair L, Pelz O, Thullner M, Abraham W-R, Zeyer J: **Assimilation of toluene along a bacteria-protist food chain determined by ^{13}C -enrichment of biomarker fatty acids.** *J Microbiol Methods* 2003, **55**:635-649.
Carbon flow modelled along a bacterial food chain.
30. Butler JL, Williams MA, Bottomley PJ, Myrold DD: **Microbial community dynamics associated with rhizosphere carbon flow.** *Appl Environ Microbiol* 2003, **69**:6793-6800.
Applications of laboratory mesocosm-based $^{13}\text{CO}_2$ pulse to investigate rhizosphere microbial carbon dynamics.
31. Lu Y, Murase J, Watanabe A, Sugimoto A, Kimura M: **Linking microbial community dynamics to rhizosphere carbon flow in a wetland rice soil.** *FEMS Microbiol. Ecol* 2004, **48**:179-186.
32. Treonis AM, Ostle NJ, Stott AW, Primrose R, Grayston SJ, Ineson P: **Identification of groups of metabolically-active rhizosphere microorganisms by stable isotope probing of PLFAs.** *Soil Biol Biochem* 2004, **36**:533-537.
Application of a novel field-based $^{13}\text{CO}_2$ pulse to investigate rhizosphere microbial carbon dynamics.
33. Middelburg JJ, Barranguet C, Boschker HTS, Herman PMJ, Moens T, Heip CHR: **The fate of intertidal microphytobenthos carbon: an in situ ^{13}C -labeling study.** *Limnol Oceanogr* 2000, **45**:1224-1234.
34. Dedysh SN, Khmelenina VN, Suzina NE, Trotsenko YA, Semrau JD, Liesack W, Tiedje JM: **Methylocapsa acidiphila gen. nov., sp. nov., a novel methane-oxidizing bacteria and dinitrogen-fixing acidophilic bacterium from Sphagnum bog.** *Int J Syst Evol Microbiol* 2002, **52**:251-261.

35. Rohmer M, Bouvier-Nave P, Ourisson G: **Distribution of hopanoid triterpenes in prokaryotes.** *J Gen Microbiol* 1984, **130**:1137-1150.
36. Balkwill DL, Leach FR, Wilson JT, McNabb JF, White DC: **Equivalence of microbial biomass measures based on membrane lipid and cell wall components, adenosine triphosphate, and direct counts in subsurface aquifer sediments.** *Microbiol Ecol* 1988, **16**:73-84.
37. Lechevalier H, Lechevalier MP: **Chemotaxonomic use of lipids – an overview.** In *Microbial Lipids*, vol 1. Edited by Ratledge C, Wilkinson SG. Academic Press, London, pp. 869-902; 1988.
38. Brennan PJ: **Mycobacterium and other actinomycetes.** In *Microbial Lipids*, vol 1. Edited by Ratledge C, Wilkinson SG. Academic Press, London, pp. 204-298; 1988.
39. Haack SK, Garchow H, Odelson DA, Forney LJ, Klug MJ: **Accuracy, reproducibility, and interpretation of fatty acid methyl ester profiles of model bacterial communities.** *Appl Environ Microbiol* 1994, **60**:2483-2493.
40. Kroppenstedt RM: **The genus *Nocardiopsis*.** In *The Prokaryotes* 2. Edited by Balows A, Trüper HG, Dworkin M, Harder W, Schleifer KH. Springer, Berlin, pp. 1139-1156; 1992:1139-1156.
41. O'Leary WM, Wilkinson SG: **Gram-positive bacteria.** In *Microbial Lipids*, Vol 1. Edited by Ratledge C, Wilkinson SG. Academic Press, London, pp. 117-202; 1988.
42. Ratledge C, Wilkinson SG: *Microbial Lipids*. Academic Press, London; 1988.
43. Dobbs FC, Guckert JB: ***Callianassa trilobata* (crustacea: thalassinidea) influences abundance of meiofauna and biomass, composition, and physiologic state of microbial communities within its burrow.** *Mar Ecol Prog Ser* 1988, **45**:69-79.
44. Bowman JP, Sly LI, Nichols PD, Hayward AC: **Revised taxonomy of the methanotrophs: description of *Methylobacter* gen. nov., emendation of *Methylococcus*, validation of *Methylosinus* and *Methylocystis* species, and a proposal that the family *Methylococcaceae* includes only the group I methanotrophs.** *Int J Syst Bacteriol* 1993, **43**:735-753.
45. Lösel DM: **Fungal lipids.** In *Microbial Lipids*, vol 1. Edited by Ratledge C, Wilkinson SG. Academic Press, London, pp. 699-806; 1988.