

MiniReview

Stable isotopes and biomarkers in microbial ecology

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Received 28 September 2001; received in revised form 19 November 2001; accepted 15 January 2002

First published online 4 March 2002

Abstract

The use of biomarkers in combination with stable isotope analysis is a new approach in microbial ecology and a number of papers on a variety of subjects have appeared. We will first discuss the techniques for analysing stable isotopes in biomarkers, primarily gas chromatography-combustion–isotope ratio mass spectrometry, and then describe a number of applications in microbial ecology based on ¹³C. Natural abundance isotope ratios of biomarkers can be used to study organic matter sources utilised by microorganisms in complex ecosystems and for identifying specific groups of bacteria like methanotrophs. Addition of labelled substrates in combination with biomarker analysis enables direct identification of microbes involved in specific processes and also allows for the incorporation of bacteria into food web studies. We believe that the full potential of the technique in microbial ecology has just started to be exploited. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Biomarker; Phospholipid-derived fatty acid; Stable isotope analysis; Gas chromatography-combustion–isotope ratio mass spectrometry; ¹³C natural abundance; ¹³C labelling

1. Introduction

Microbial ecology addresses the identity and functioning of microorganisms in their natural environment. Although recent developments in molecular biology allow identification of microorganisms, the study of their functional aspects is generally either limited to laboratory isolates or involves measurements of fluxes. Direct links between microbial identity and biogeochemical processes are currently being determined using a number of culture independent techniques and stable isotope analysis of biomarkers provides one, but powerful approach.

Biomarkers are compounds that have a biological specificity in the sense that they are produced only by a limited group of organisms. A variety of compounds, such as fatty acids and ether lipids, are used in microbial ecology and related fields like organic geochemistry to detect different groups of organisms or their remains in natural or artificial ecosystems [1,2]. With the recent advance in analytical techniques, especially with the development of gas chromatography-combustion–isotope ratio mass spectrometry (GC-c–IRMS), it is now possible to analyse stable isotope

ratios of specific compounds including a number of biomarkers with excellent sensitivity with respect to both concentration and isotope content [3,4]. Since it became available around 1990, this technique has found wide application in organic geochemistry and medical sciences, and has become the standard method for this type of analysis. However, its use in microbial ecology, and ecology in general, is just beginning to be explored and in recent years there has been a steady increase in the application of the technique (e.g. [5–10]).

The GC-c–IRMS technique has traditionally been used to measure the natural variation in isotope ratios of single compounds due to isotope fractionation during primary production, respiration and assimilation [11]. This natural abundance approach can be used to study the source of the carbon assimilated by microorganisms [8,12] and may sometimes also be used to identify microbial populations involved in specific processes (e.g. methanotrophy [6]).

Stable isotopes have also been used extensively as tracers for rate measurements in microbial ecology (e.g. ¹⁵N uptake and regeneration, denitrification, nitrogen fixation, ¹³C fixation and respiration). The combination of deliberately added tracers and isotopic analysis of biomarkers provides the unique possibility to directly link microbial identity (biomarker), biomass (concentration of the biomarker) and activity (isotope assimilation). This approach

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is highly versatile since label can be added as either ^{13}C -bicarbonate or carbon dioxide [9,13], as ^{13}C -labelled organisms or their remains (such as plant litter or cultured algae [14,15]), or as any other organic substrate (e.g. ^{13}C -acetate or ^{13}C -toluene) to link processes to specific microbial populations [5,10,16–18].

This mini-review explores the potential of stable carbon-isotope (^{13}C) analysis of biomarkers to elucidate the functioning of microbial communities in extant ecosystems. We will introduce the biomarkers used in microbial ecology and the methods for analysing their isotopic content. We then review a number of instructive applications of the method and finally discuss directions of future developments.

2. Isotope analysis of biomarkers

2.1. Biomarkers

For microbial studies, biomarkers should ideally provide information on microbial identity and biomass, and therefore should have several characteristics [1,2]. For identification of microorganisms, specificity should be high in the sense that the biomarker is only produced by the organism of interest, otherwise interference from other microorganisms may occur. Specificity is however seldom absolute and then depends on the uniqueness of the biomarker, the relative abundance of the target organisms in the community and the concentration of the biomarker in the target. The specificity of the biomarker must be higher if the target organism forms only a small fraction of the community compared to major groups of organisms. Also, one would like to analyse a class of compounds in which markers for various groups of organisms can be found such as small subunit rRNA and membrane lipids like phospholipid-derived fatty acids (PLFA). Biomarker interpretation depends on known marker compositions of microbial isolates. Analysis of new isolates from previously uncultured groups, which will most likely appear in the near future, therefore remains important both for evaluation of currently used biomarkers and identification of potentially new specific markers.

For biomass determination, the biomarker should be present in relatively constant amounts in the organisms of interest. Biomarkers occurring in storage products should not be used if biomass has to be quantified because their content varies with the physiological condition of the organism. Also, biomarkers should rapidly turnover upon death of the organisms, as they should be associated with living biomass and not with the remains of organisms that have accumulated over time. This criterion is also the main difference between the use of the term biomarker in ecology and geology. Geologists refer to the term biomarker as molecules that not only are specific for certain source organisms, but also are relatively stable over geo-

logical time scales, so that they can be found in sedimentary archives. The rapid turnover criterion may however be less important in short-term labelling studies that rely on biomarker synthesis during the incubation period. The turnover of (ecological) biomarkers can actually be measured directly and very elegantly by application of ^{13}C -labelled biomarkers (e.g. [15]).

Table 1 lists a number of biomarkers used in ecological studies that have been used in combination with stable isotope analysis. A further discussion on these and other biomarkers is found elsewhere (e.g. [1,2] and other references in this review). From Table 1, it is clear that individual biomarkers can be used to differentiate between major groups of microorganisms like bacteria, fungi and algae, with some further differentiation within these groups. For certain groups like sulphate reducers and methanotrophic bacteria, it is potentially possible to detect organisms at the genus level. Lipids found in biological membranes have received by far most attention as they show major differences between microbial groups and are relatively easily extracted from natural samples. The most commonly used membrane lipids are PLFA, which are found in bacteria and eukaryotes [1,2]. Environmental samples show a wide range of PLFA (between 30 and 50 different compounds, Fig. 1). Several of these can be used as specific biomarkers (Table 1). Other major advantages of using PLFA as biomarkers are that these compounds are turned over within days after cell death [1,15] and that a large set of pure-culture data are available for fatty acid patterns as they are widely used in taxonomy. Archaea can be detected and differentiated with ether lipids [19], though these ether-linked compounds are rather resistant towards degradation and may therefore not be associated with biomass. D-Amino acids have been used to detect bacteria in natural samples [12], but may also accumulate in the environment. Ergosterol has been used extensively to detect higher fungi [2], but so far not in combination with stable isotope analysis.

2.2. Stable isotope analysis and data handling

The current state-of-the-art method to study the isotopic composition of individual compounds is GC-c-IRMS. It comprises a GC equipped with a capillary column that is used to separate the compounds of interest at high resolution. The outlet of the column is attached to a miniature oxidation reactor where the organic molecules are combusted to CO_2 , N_2 and H_2O gas. A reduction reactor is included for ^{15}N analysis to convert oxidised nitrogen species to N_2 gas. Water is removed on-line and the purified CO_2 and N_2 are led into an isotope ratio mass spectrometer (IRMS). Because of its design, an IRMS measures the isotopic ratios between the heavy and light isotopes (e.g. $^{13}\text{C}/^{12}\text{C}$ for carbon and $^{15}\text{N}/^{14}\text{N}$ for nitrogen) and results are always calibrated against an international standard or derived reference material. De-

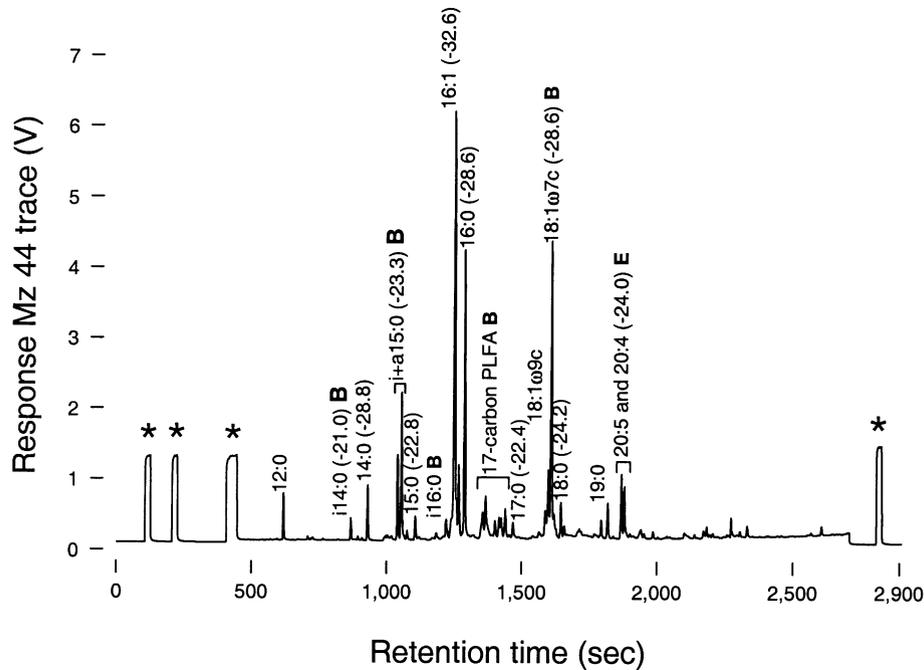


Fig. 1. An example of GC-c-IRMS analysis of PLFA extracted from salt-marsh sediment at Waarde, The Netherlands (after [8] with permission (Copyright 1999 by the American Society of Limnology and Oceanography, Inc.)). Indicated are detected PLFA with stable isotope ratios. Reference gas pulses used for calibrating stable isotope ratios are marked with stars and specific bacterial and eukaryote biomarkers with 'B' and 'E', respectively.

tailed descriptions of the GC-c-IRMS design and operation are found elsewhere [3,4]. Currently, GC-c-IRMS can be used to measure compound-specific stable isotope ratios of carbon, nitrogen and with modifications those of deuterium/hydrogen and oxygen. Especially for natural abundance work, individual biomarkers should be baseline-separated from other compounds, as it is necessary to use the whole peak for an accurate determination of the isotope ratio [3,4]. Many compounds have to be derivatised before GC analysis and isotope data should be corrected for the carbon atoms added during derivatisation [8,20]. The same IRMS that is used in combination with GC-combustion analysis can also be interfaced on-line with an elemental analyser (EA-IRMS) to determine

stable ratio isotopes of hydrogen, carbon, nitrogen, oxygen and sulphur in dissolved or particulate materials including macrophytes, animals, suspended matter, soils and sediments [4]. EA-IRMS with its high sample throughput and versatility is becoming increasingly popular in ecology as a method for stable isotope analysis.

The GC-c-IRMS can also be used to study the isotopic composition of non-biomarker compounds like sugars, amino acids, volatile fatty acids and xenobiotic compounds. The turnover and fate of these bacterial substrates may therefore be determined, as most of them are available in various stable isotope-labelled forms.

Another method to study stable isotope composition of specific compounds is by ordinary quadrupole GC-mass spectrometry (MS), which is however a factor 100–1000 less accurate, and typically only changes of about 1% in ^{13}C content can be determined [13]. For ecological studies this generally means that artificially high concentrations of label have to be used before incorporation into biomarkers can be detected [21]. However, an advantage of GC-MS is that the intra-molecular distribution of label can be determined, which may provide important information on the degradation pathways of stable isotope-labelled tracers [22]. Some compounds are not, or not directly, amenable to GC analysis and their use in isotope biomarker studies requires other techniques. For instance biomarkers such as algal pigments, quinones and complex lipids, which are not readily analysed by GC methods due to their large molecular size and thermal instability, can be separated by high-performance liquid chromatography (HPLC). Preparative HPLC with off-line isotopic analysis has

Table 1
Examples of biomarkers used in microbial ecology [1,2]

Biomarker class	Organisms	Examples
PLFA	Bacteria and eukaryotes	Bacteria (i14:0, i15:0, a15:0, 18:1ω7c, cy19:0) ^a
		Algae (20:5ω3, 18:3ω3)
		Fungi (18:2ω6)
		Actinomycetes (10Me17:0, 10Me18:0)
		Sulphate reducers (i17:1, 10Me16:0)
		Methanotrophs (16:1ω8c, 18:1ω8c)
Sterols ^b	Eukaryotes	Higher fungi (ergosterol)
Hopanoic acids ^b	Bacteria	Cyanobacteria, methanotrophs
Ether lipids ^b	Archaea	Methanogens (hydroxy-archeols)
		Crenarchaea (cyclic tetra-ether lipids)
D-Amino acids ^b	Bacteria	D-Alanine

^aFatty shorthand notation as in [2].

^bDo not or only in some cases meet the fast turnover criterion.

been used for ^{13}C and ^{15}N analysis of pigments [23] and amino acids [24]. Solid-state ^{13}C -nuclear magnetic resonance is another technique that has been used in soils to detect label incorporation (e.g. [25]).

A major advantage of GC-c-IRMS is that very small changes in stable isotopic composition can be detected. Usually, stable isotope ratios are given in the δ -notation, which for carbon is defined as:

$$\delta^{13}\text{C} (\text{‰}) = \left(\frac{^{13}\text{C}/^{12}\text{C ratio}_{\text{sample}}}{^{13}\text{C}/^{12}\text{C ratio}_{\text{standard}}} - 1 \right) \times 1000 \quad (1)$$

The international standard for carbon is Vienna PeeDee-Belemnite ($^{13}\text{C}/^{12}\text{C}$ ratio $\equiv 0.0112372$). The typical precision that is obtained with a GC-c-IRMS system for as little as 1 nmol carbon is about 0.3‰, which means that changes in relative ^{13}C content of about 0.001% can be detected. This precision is necessary for natural abundance work where the maximum range of isotopic ratios is usually 20‰ or less, but it also means that very low label incorporations can be detected in tracer studies. Label additions in experiments utilising GC-c-IRMS methodology can therefore be minimised to concentrations close to or below those found in natural environments [5,17].

The δ -notation is based on isotope ratios, which is not very convenient for enriched samples [4]. Increases in isotope ratios ($\Delta\delta$ ratios) that are obtained in tracer work should be regarded as equivalent to increases in specific labelling, and do therefore not directly indicate the absolute amount of label that was incorporated into a certain biomarker [18]. Absolute amounts of label incorporated (^{13}C) are calculated from the product of biomarker concentration (C) and the increase in the fraction ^{13}C after labelling (F_t^{13}) relative to the control (F_c^{13}):

$$^{13}\text{C} = (F_t^{13} - F_c^{13}) \times C \quad (2)$$

The fraction ^{13}C can be calculated from the $^{13}\text{C}/^{12}\text{C}$ ratios (R) as:

$$F^{13} = R / (R + 1) \quad (3)$$

And R is calculated from the $\delta^{13}\text{C}$ ratios as measured with the IRMS equipment using the reverse of Eq. 1. Another effect of measuring isotope ratios rather than absolute amounts is that detectable amounts of label also depend on pool sizes or concentrations of the components to be analysed. For instance, labelling sediment with 10 μM ^{13}C -acetate gave a readily detected increase in $\delta^{13}\text{C}$ ratio of about 10‰ in some PLFA [5], but would not be detectable in the much larger sediment organic matter pool due to dilution.

A comparison of sensitivity between ^{13}C and ^{14}C labelling techniques should always include the pool size of the components of interest because ^{14}C measurements by counting are absolute, whereas ^{13}C analysis by IRMS pro-

vides ratio data. To give an example, Boschker et al. [5] found that labelling anoxic sediments with 10 μM uniformly labelled ^{13}C -acetate (99% ^{13}C) resulted in a 10‰ increase in δ value of the 16:0 PLFA, which corresponds to 0.1% of the added ^{13}C label. Approximately 2 ml of sediment was extracted for PLFA analysis and the 16:0 concentration was about 4 $\mu\text{g ml}^{-1}$. When ^{14}C -acetate with a specific activity of 2 GBq mmol^{-1} would have been used with a single GC injection of 0.1 μg 16:0 PLFA on column (which is about the maximum load for capillary GC columns), this would lead to approximately 0.3 Bq in the isolated 16:0 peak or to a scintillation count of 30 dpm above background. The absolute amount of 16:0 PLFA analysed could of course be increased by multiple GC injections or by injecting more of PLFA extract. The ^{13}C label enrichment and ^{14}C signal were both about 10 times the detection limit, showing that sensitivities of ^{13}C and ^{14}C labelling can be similar provided that small pools (microbial biomass in this example) are analysed.

While Eq. 2 gives unequivocal evidence for label assimilation by microorganisms, it does not provide a quantitative measure for label assimilation by the target organisms or community. Quantification in terms of biomass production requires a conversion factor from concentration of the biomarker to total biomass, i.e. gram of carbon biomarker per gram of carbon biomass. The total PLFA content of heterotrophic bacteria is relatively well constrained and varies between 0.073 and 0.038 g PLFA C/biomass C for aerobic and anaerobic communities, respectively [26]. However, labelling work is sometimes restricted to a few bacterial-specific compounds, and an additional conversion factor is required to relate the specific markers to the total bacterial PLFA. Fortunately, the relative amount of these specific biomarkers appears to be very constant in a wide range of bacterial dominated sediments ($28 \pm 4\%$ for the sum of i14:0, a15:0, i15:0, i16:0 and 18:1 ω 7c, [9]), although similar relationships are not yet available for terrestrial soils and pelagic systems. Middelburg et al. [9] and Moodley et al. [15] used this method to calculate total amounts of ^{13}C label incorporated into the bacterial biomass, and their results were in good agreement with other constraints on the bacterial community metabolism like carbon respiration rates.

3. Natural abundance studies

Natural abundance studies use the small differences in isotopic ratios as found in nature [11,27]. These isotope effects are caused by the preferential use of ^{12}C compared to ^{13}C in many biological and chemical processes, which is referred to as isotopic fractionation. Variation in $^{13}\text{C}/^{12}\text{C}$ ratios among primary producers occurs because of differences in inorganic substrate, fixation pathways, or environmental and physiological conditions (Fig. 2). For instance, most terrestrial macrophytes can be divided into

C3 plants with a $\delta^{13}\text{C}$ of around -27‰ and those with a C4 metabolism that show $\delta^{13}\text{C}$ values of around -14‰ . Marine phytoplankton (mainly C3 metabolism) has ratios around -21‰ as dissolved inorganic carbon (0‰) is more enriched than atmospheric carbon dioxide (-8‰) used by terrestrial vegetation. Heterotrophic organisms including many microbes in general show similar carbon-isotopic ratios as their food source(s) [28–30] (but see [31]). These differences in isotopic composition can be used to trace the origins of organic compounds in environments in which primary producers have different isotopic compositions such as coastal and estuarine ecosystems, rivers and lakes, and in terrestrial ecosystems undergoing a transition from C3 to C4 plants [27]. The main advantage of this approach is that a limited number of measurements can provide an independent and integrated view on organic matter cycling and food-web relationships.

3.1. Food source elucidation

The classical application for stable isotope analysis in ecology is to study the sources of the organic matter used by heterotrophic organisms based on the well established principle of ‘you are what you eat’ (Fig. 2). The use of biomarkers as representatives of biomass enables one to study carbon sources used by various types of microorganisms in complex ecosystems. Coffin et al. [29] developed a method to extract and analyse the stable isotopic composition of bacterial DNA extracted from water and sediment samples. Molecular 16S rRNA probes were used to check for contamination by eukaryote DNA. Pelz et al. [12] showed that the unique bacterial amino acid, D-alanine, has similar isotopic ratios as the bacterial substrate and it can therefore be used as bacterial biomarker in combination with GC-c-IRMS analyses to study bacterial carbon sources in soils and sediments.

A number of studies have been published on carbon sources used by bacteria in the sediments of salt marshes and seagrass beds [8,32–34]. Canuel et al. [35] showed by analysing ^{13}C ratios in a variety of biomarkers that salt marsh or seagrass-derived carbon was not important for bacteria in coastal sediment from Cape Lookout Bight, NC, USA. Creach et al. [32] applied the DNA extraction technique to salt-marsh sediments and their results indicate that rhizosphere bacteria predominantly used organic matter derived from local marsh plants. However, Boschker et al. [8] used ^{13}C ratios of bacterial PLFA to show that the contribution of local plant-derived material was highly variable among *Spartina* marshes and varied from being dominated by allochthonous material probably derived from phytoplankton to predominant use of locally produced *Spartina* material. In another study, isotope ratios of bacterial PLFA extracted from the sediment of several European *Zostera* beds showed no relationship with those of seagrass material but correlated well with ratios of an algal PLFA (20:5 ω 3) that is abundant in

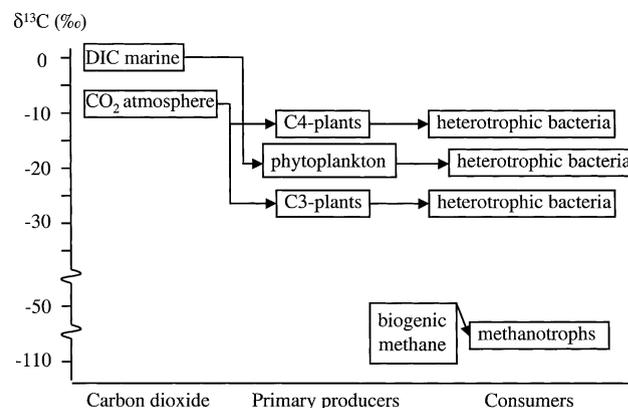


Fig. 2. Relationships between natural ^{13}C ratios in carbon dioxide pools, primary producers and consumers. Further description can be found elsewhere [27]. Heterotrophic organisms show little fractionation compared to their carbon sources (the ‘you are what you eat’ principle).

benthic diatoms [33]. Again this appeared to not hold for all seagrass beds as bacteria mainly used seagrass material in two tropical seagrass beds in Thailand [34]. These data do have major consequences for the carbon cycle in these coastal ecosystems, as they suggest that the high carbon mineralisation rates found in these ecosystems are often not directly driven by the input of local plant litter and that the importance of macrophyte production for sedimentary carbon cycling may have been overestimated previously. Moreover, nitrogen cycling and microbial community structure will also be affected as macrophytes and algal detritus differ widely in their composition and degradability.

A concern with the use of biomarkers as representatives of the whole biomass is the considerable range in isotopic carbon composition of individual biochemical fractions and compounds due to fractionation effects during synthesis reactions [11]. DNA and proteins show little fractionation relative to total biomass [28], but individual amino acids show a wide range of about 20‰ in single organisms [24]. Lipids are in general depleted in ^{13}C by $3\text{--}6\text{‰}$ compared to the total biomass [11]. When stable isotope data of biomarkers are used for source elucidation, corrections have to be made and isotopic fractionation factors should be taken into account with relevant control experiments [8,12,29]. Boschker et al. [8] showed that specific bacterial PLFA like i15:0 and a15:0 from a mixed culture were depleted by about $4\text{--}6\text{‰}$ relative to the substrate, and used this as a correction factor in determining bacterial carbon sources. This range is consistent with current theories on isotope fractionation in microbial fatty acids synthesis [11], and others have found similar factors (e.g. [28,36]). However, other PLFA were much more variable ($+4$ to -9‰ [8]) and Abraham et al. [31] also obtained variable fractionation factors for fatty acids from several bacterial strains growing on defined substrates. This large variation in isotope fractionation in some bacterial fatty acids is difficult to explain with current theories

of biosynthesis and isotope fractionation [11], and more work is needed.

3.2. Identification of populations

Some types of bacteria use carbon sources for growth with very specific ^{13}C signals or have a metabolism that results in characteristic isotopic ratios and these isotopic signatures can then be used for identification of these populations. Methanotrophic bacteria are a good example as the methane that is used for growth is usually highly depleted compared to other carbon substrates with ratios between -50 and -110‰ (Fig. 2). Moreover, methanotrophs fractionate against ^{13}C in their metabolism which adds another $0\text{--}20\text{‰}$ depletion [37,38]. One of the first applications of GC-c-IRMS was to show that certain hopanes in ancient sedimentary rocks had been produced by methanotrophs [3]. Also, methanotrophic symbionts in bivalves could be easily detected and described by their biomarker isotopic ratios (e.g. [39]). Other groups with specific isotopic signals are certain phototrophic bacteria that possess a reversed TCA cycle or 3-hydroxypropionate pathway for carbon dioxide fixation. The lipids of these bacteria have relatively high ^{13}C ratios (e.g. [11,40]).

An example of detecting methanotrophic bacteria in sediments taken from Lake Vechten, The Netherlands, is shown in Fig. 3. Based on their PLFA composition, mesophilic methane-oxidising bacteria can be divided into two groups: type I, which predominantly have series of mono-unsaturated, 16-carbon fatty acids, and type II, which contain mono-unsaturated, 18-carbon fatty acids [2]. Only 16-carbon fatty acids showed a clearly depleted carbon-isotopic signal in Lake Vechten sediments (Fig. 3), which indicated that type I methanotrophs were dominant. Cifuentes and Salata [36] used this type of data to estimate the relative abundance of methanotrophic bacteria.

Recently, advances have been made in the characterisation of the organisms involved in the anaerobic oxidation of methane, which is an important but poorly understood process in methane-rich marine sediments. It has now repeatedly been shown that these sediments contain highly ^{13}C -depleted archaeal ether lipids affiliated with methanogens [6,41–44]. This strongly suggests that the primary organisms involved in this process are methanogenic Archaea operating in reverse. A bacterial consortium consisting of an aggregate of methanogens surrounded by a layer of sulphate reducers belonging to the δ -Proteobacteria was found to dominate the bacterial community at a methane seep [45]. Recently, Orphan et al. [46] elegantly showed by analysing the microscale ^{13}C distribution within these microbial aggregates that the methanogens in these consortia are indeed the likely source of the highly depleted ether lipids. It is thought that the sulphate reducers consume an intermediate product of the methanogens in this symbiosis. A variety of highly ^{13}C -depleted bacterial compounds have also been found in methane-oxidising sediments

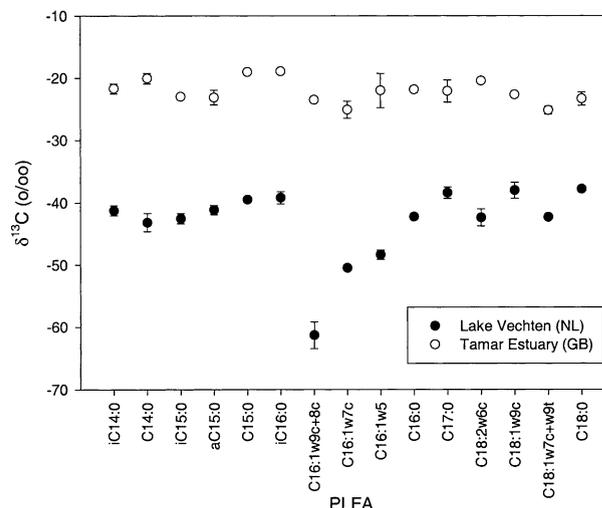


Fig. 3. Stable carbon-isotope ratios in PLFA extracted from the sediment of Lake Vechten, The Netherlands (0–1 cm depth, March 1995, end of non-stratified period). Data for the non-methanotrophic sediments of the Tamar estuary are added for comparison (0–1 cm depth, March 1995). Methane in Lake Vechten sediment had a $\delta^{13}\text{C}$ ratio of $-67.0 \pm 0.5\text{‰}$ as determined by GC-c-IRMS. Stable carbon-isotope ratio of sediment organic matter was different between sites (Vechten $-31.9 \pm 0.5\text{‰}$, Tamar $-21.9 \pm 0.1\text{‰}$), which may explain the generally more depleted ratios of PLFA in Lake Vechten. The more than average depletion in mono-unsaturated, 16-carbon PLFA (16:1w9c+8c, 16:1w7c and 16:1w5) in Lake Vechten suggests that these fatty acids were partially derived from methanotrophic bacteria.

[43,44], and are probably in part derived from the symbiont sulphate reducers. However, the ^{13}C -depleted biomarkers from both bacterial and archaeal origins are often different between sediments and not all bacterial biomarkers detected are found in δ -Proteobacteria [44,47], which may indicate that other groups of microorganisms are also involved in the anaerobic oxidation of methane.

4. Labelling studies

The possibility of combining stable isotope labelling studies with biomarker analysis offers interesting, unprecedented possibilities to separately study the activities of different groups of microorganisms. The availability of stable isotope-labelled compounds is improving quickly and, although not yet as diverse as for radioisotope labels, a large variety of compounds can be purchased or produced by cultivating organisms on labelled substrates. Stable isotopes as labels do not suffer from legal restrictions and health problems associated with radioisotopes and can be used for instance directly in the field (e.g. [9]). This has the advantage that carbon and nitrogen transformations can be studied within the complexity of ecosystems and without the artefacts associated with incubations of subsamples. We have subdivided this part of the review ac-

ording to the form of the label used as different types of information are gained.

4.1. Linking population structure with specific microbial processes: labelling with specific ^{13}C compounds

Microbial ecologists are increasingly attempting to obtain direct links between microbial identity and biogeochemical processes for they are required to further our field (e.g. [5,48]). The use of stable isotope-labelled substrates in combination with biomarker analysis offers the unique opportunity to quantify and identify in an integrated way the degradation rates and pathways of the substrate, and the organisms involved [17,18]. The basic idea behind this approach is that a portion of the added stable isotope tracer is incorporated into the biomass of the metabolically active populations, which can be detected in a variety of biomarkers. By comparing the biomarkers that are labelled with known biomarker compositions of microorganisms, active populations can be identified. A major strength of the method is that the full biomarker fingerprint of an organism can be used in labelling studies and hence researchers are not restricted to using individual specific biomarkers, which are only found in a limited number of genera. This greatly extends the use of biomarker identification in natural environments [5]. The approach however depends on the availability of biomarker fingerprints from isolates, which are not available for many microbes in the environment. In addition to identification, estimates of growth rates and yields of functional sub-populations might be obtained, since polar lipid synthesis is closely linked to growth in microorganisms [1].

The mineralisation of organic matter in anoxic sediments is a stepwise process, in which several low molecular intermediates produced by fermentative bacteria play an important role. Organisms like sulphate reducing bacteria subsequently consumed these intermediates. The bacterial populations involved in the consumption of acetate, the main intermediate in most sediments, were studied by Boschker et al. [5,18] in a number of sediments where sulphate reduction was the dominant process. Typical biomarkers for sulphate reducing bacteria (Table 1) contained only minor amounts of label and ^{13}C -labelled acetate was mainly traced in even-numbered PLFA (16:1 ω 7c, 16:1 ω 5, 16:0 and 18:1 ω 7c). The acetate labelling pattern resembles PLFA compositions of *Desulfotomaculum acetoxidans* and recently isolated *Desulfofrigus* strains, which are both acetate consuming sulphate reducers. Acetate is probably directly degraded to CO_2 by sulphate reducers. However, several pathways are known for propionate degradation, the second most important intermediate. Propionate is degraded either directly or with acetate as an intermediate. Using ^{13}C -labelled propionate, Boschker et al. [18] showed that propionate was directly taken up from the pore water of anoxic sediment without the production of intermediate acetate. Propionate uptake was not affected by acetate

additions and the propionate labelling pattern of PLFA (mainly a15:0, 15:0, i16:0, 17:1 ω 6c and 17:0) was almost completely different from acetate, which strongly suggests that different bacterial populations are involved in the consumption of acetate and propionate. The complete labelling pattern with propionate did not resemble any of the known strains, which suggests that it may belong to an unknown type of sulphate reducer. This was one of the first studies where niche differentiation was directly shown in complex microbial communities.

Several studies on methanotrophic bacterial populations are available where ^{13}C labelling [5,17,49,50] or ^{14}C labelling [51,52] was used in combination with biomarker analysis. Methanotrophy in soils is a major sink for atmospheric methane. Typical ambient methane concentrations are however so low that known strains of methanotrophic bacteria would not be able to sustain measured methane oxidation rates. Bull et al. [17] applied ^{13}C -methane pulses to a forest soil and labelling patterns of PLFA suggested that the active populations at low methane concentrations were bacteria related but not similar to known type II methanotrophs. At higher methane concentrations, PLFA from both types I and II were labelled, which suggested that different populations of bacteria specialised at growth on different methane concentrations are present in soils. Similar results have been obtained with ^{14}C labelling and PLFA analysis for a range of soils by Roslev et al. [51]. Labelling of hopanoic acids specific for methanotrophs could also be shown in forest soils [50].

Labelling with ^{13}C -bicarbonate can be used to detect active primary producers like algae and chemoautotrophic bacteria [53,54]. An example of ^{13}C -bicarbonate labelling is shown in Fig. 4 for the upper, brackish part of the Scheldt estuary, a turbid and highly heterotrophic estuary on the Belgium–Dutch border. Due to the high ammonium loading of the Scheldt estuary, nitrification rates are among the highest reported. The results in Fig. 3 clearly show that it was possible to differentiate between photoautotrophic carbon fixation by algae and bacterial chemoautotrophy. Algal-derived, poly-unsaturated PLFA were a predominant feature of the labelling pattern in the light incubations (Fig. 4A) and the simple dark incorporation pattern without poly-unsaturated compounds was clearly bacterial (Fig. 4B). Based on this type of data it may be possible to calculate growth rates for these individual groups [54,55], with algae for instance further divided in green algae (18:3 ω 3) and diatoms (20:5 ω 3).

The approach is not limited to natural organic substrates, but can also be used to study microbes involved in the decomposition of xenobiotic and toxic substances [7,10,16,20]. Hansen et al. [16] showed that PLFA labelling patterns of a soil incubated with ^{13}C -toluene were highly specific and 85% similar to a toluene-metabolising actinomycete isolated from the same soil. As toluene degradation is widespread among microorganisms, it was surprising that the labelling pattern suggested that only a subset

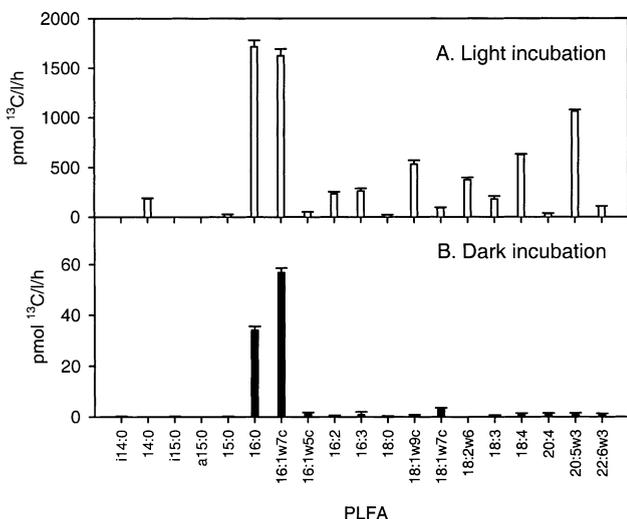


Fig. 4. Results of a ^{13}C -bicarbonate labelling study in the Scheldt estuary, Belgium (April 1998). Incorporation of label into PLFA was studied in light (A) and dark incubations (B). Dark incorporation was fully sensitive to nitrification inhibitors (N-serve with chlorate, data not shown).

of the microbial community was involved in toluene degradation. Similarly, toluene degradation in a denitrifying aquifer could be linked to *Azoarcus* spp. by a combination of ^{13}C labelling of PLFA and molecular techniques [10]. Pelz et al. [7] were able to elucidate the metabolic pathways and populations in a pollutant degrading bacterial community by studying the degradation of ^{13}C substrates and their incorporation into PLFA. Other applications in environmental studies include tracing the fate of ^{13}C -labelled bacteria in aquifers [56,57] and in situ quantification of contaminant decomposition based on isotopic changes of the remaining contaminant pool (e.g. [58]).

4.2. Coupling between primary producers and heterotrophic bacteria: labelling with ^{13}C carbon dioxide

Labelling experiments with ^{13}C -bicarbonate have been used to determine the autotrophic organisms involved in CO_2 uptake (see Section 4.1) and also to trace the transfer of carbon from autotrophic to heterotrophic organisms.

Middelburg et al. [9] presented an illustrative case how the fate of intertidal microphytobenthos carbon can be elucidated with in situ pulse-chase ^{13}C labelling experiments. At the beginning of low tide, exposed tidal sediments were sprayed with ^{13}C -bicarbonate and carbon fixation was measured as the incorporation of ^{13}C in the bulk organic pool as well as in PLFA. The labelling period ended after 4 h upon submergence of the sediments because ^{13}C -bicarbonate was flushed away by the tides and benthic microalgae became light limited. At the end of the labelling period PLFA found in diatoms (e.g. 16:2w4, 20:5w3 and 22:6w3) became strongly enriched in ^{13}C , but there was also already some ^{13}C enrichment in bacterial biomarkers (i14:0, i15:0, a15:0, i16:0 and 18:1w7c). A

mass balance calculation for fixed carbon at the end of the labelling period indicated that 59% was incorporated in diatoms, about 2% in bacteria and the remaining 39% of the fixed carbon was in other compartments (likely extracellular polymeric substances [59]).

The fate of this fixed carbon was subsequently followed over a 4-day period (the chase). Enrichment of bacterial biomarkers in ^{13}C peaked after 1–2 days and then decreased again. Middelburg et al. [9] attributed this rapid (within 4 h) and steady (over the first 24 h) labelling of bacteria to efficient growth of heterotrophic bacteria on extracellular carbon exudates of the benthic microalgae. This explanation requires experimental validation (e.g. by measuring the intermediate appearance of ^{13}C in extracellular carbohydrates or by following the fate of added ^{13}C -labelled extracellular carbohydrates), but it provides a feasible mechanism for intimate algal–bacterial coupling in surface sediments and is consistent with recent studies indicating rapid consumption of exudates [60].

Similarly, Boschker et al. [33] labelled *Zostera marina* plants, a temperate seagrass, to study transfer of root exudates to rhizosphere bacteria. They could however not detect any label transfer to bacterial PLFA in the rhizosphere after 24 h of labelling despite a clear ^{13}C signal in the roots. A limited transfer of root exudates is in agreement with natural isotope ratios of bacterial PLFA extracted from a range of *Zostera* beds, which show a minor contribution of seagrass material as a bacterial carbon source ([33], see Section 3.1).

4.3. The role of heterotrophic bacteria in benthic food web studies: labelling with ^{13}C -organic matter

Radiocarbon-labelled organic substrates (algae, macrophyte litter, dissolved organic matter) have been used extensively to study microbial decomposition of these substrates and assimilation and uptake of particulate organic matter by meio- and macrofauna groups. It is clear that ^{13}C can be substituted for ^{14}C , but with the additional advance of compound-specific isotope analysis and in situ experimentation. Blair et al. [61] have pioneered the use of ^{13}C -labelled algae in situ experiments to trace the fate of phytodetritus in ocean margin sediments. Using a submersible they added ^{13}C -labelled *Chorella* and followed the fate over a 1.5-day period. The benthic community responded rapidly as reflected in the rapid appearance of ^{13}C in ΣCO_2 (microbial+fauna respiration) and in meio- and macrofauna, and the mixing of the label to greater depth. The bacterial contribution was not evaluated except for respiration.

Sun et al. [14] also applied ^{13}C -labelled *Chorella* in their study of algal lipid degradation by bacteria under oxic and anoxic conditions and in the presence and absence of *Yoldia limatula*, a protobranch bivalve. Using a GC–MS technique [22], they followed the fate of three major fatty acids (16:1w7, 16:0 and 18:1w9) and phytol, the esterifying al-

cohol of chlorophyll *a*. They identified the formation of two major ^{13}C -labelled compounds: a uniformly ^{13}C -labelled C16 alcohol that was a likely transformation product from phytol and partially ^{13}C -labelled i15:0 fatty acid indicative of de novo synthesis of bacterial biomass [22]. Oxic conditions and the presence of the bivalve accelerated the degradation of algal lipids in sediments [14]. Label incorporation in higher organisms and respiration of ^{13}C were not reported.

Moodley et al. [15] used ^{13}C -labelled *Chorella* to study the utilisation of phytodetritus by bacteria and foraminifers in intertidal estuarine sediments. The response was rapid: about 5% of the added carbon was respired to CO_2 within 6 h and bacteria assimilated 2–4% of the added carbon within 12 h. Bacterial assimilation was assessed via the incorporation of ^{13}C in PLFA (i14:0, i15:0, a15:0, i16:0 and 18:1 ω 7c). The dominant foraminifer *Ammonia* responded very rapidly and ingested about 5% of the added carbon within 12 h. Although this was the first study to report both respiration and uptake by bacteria and foraminifers, it did not include other faunal groups.

5. Future perspectives

In this review we have mainly discussed the use of ^{13}C as an isotope tracer. Other isotopes that are amendable to compound-specific isotope analysis (^{15}N , D/H) would also give a wide range of possibilities to study the microbial transformations and fate of these elements. For instance, ^{15}N -labelled compounds have been used to study the competition between phytoplankton and bacteria for various forms of nitrogen. The differentiation between bacteria and phytoplankton in these studies is mainly based on size fractionation by filtration or on the use of inhibitors. Specific biomarkers would certainly aid in the interpretation of the results, if selective labelling of these compounds could be shown. Also, multiple isotope studies (^{13}C , ^{15}N , ^{34}S) have been widely used in natural abundance work to constrain organic matter sources used by larger organisms [27]. The use of both organic and inorganic forms of nitrogen and sulphur by microorganisms could be studied with specific biomarkers containing these elements if methods for analysing stable sulphur isotopes in biomarkers become available.

GC-c-IRMS has been used mostly to study lipids as biomarkers, which almost exclusively contain only carbon, hydrogen and oxygen. The use of other biomarker classes could be interesting for isotopes of elements other than those found in lipids. Certain D-amino acids are specifically produced by bacteria and their use has already been discussed above. DNA and proteins are potentially powerful targets if specific types can be isolated from environmental samples as they contain most of the stable isotopes that are used in ecological studies. Recently, ^{13}C labelling was used in combination with density gradient centrifuga-

tion to isolate heavy, ^{13}C -labelled DNA from active populations in a soil sample [62]. Molecular techniques were then used to characterise the isolated DNA and to identify the active bacteria. It should however be noted that labelling intensity must be very high before the density gradient centrifugation becomes effective. The incubation techniques used in that study should be described as an enrichment with its potential problems of selection for populations not representative of those active at natural substrate concentrations.

HPLC-IRMS would greatly broaden the types of biomarkers that can be analysed. Several attempts have been published to directly couple HPLC with high precision isotope analysis [4]. Commercial machines are however not available and sensitivity in terms of amounts of carbon needed is still rather low, so further development of these machines is needed.

For food web studies it is now possible to study bacteria and other microorganisms like microalgae and fungi through biomarkers and larger organisms like nematodes and foraminifers can mostly be hand-picked and analysed directly [9,15]. This provides unprecedented opportunities to cover the complete food web and will ultimately result in a better integration of microbial and general ecological approaches of carbon processing. However, intermediate-sized organisms like flagellates and ciliates are too small for hand-picking, which makes it difficult to integrally study the microbial loop and the classical food web by this approach. The use of biomarkers is therefore an interesting option. A candidate biomarker for these protists is tetrahymanol, which is produced in bacterivorous flagellates and ciliates as a steroid analogue [63]. Its use in combination with stable isotope analysis needs however to be demonstrated.

Several recent studies have shown that a combination of quantification and identification by isotope analysis of biomarkers and phylogenetic analysis by molecular techniques is highly effective in linking structural and functional aspects of microbial communities (e.g. [10,46,52]). As microbial communities are generally very complex, it is expected that this or similar combinations of techniques will provide powerful approaches to elucidate the functional relationships among the members of these communities.

Acknowledgements

The Fungal-Bacterial Interactions project of the Vernieuwingsfonds of the Royal Netherlands Academy of Arts and Sciences (KNAW) and the project EURO-TROPH (EVK3-CT-2000-00040) under the ELOISE programme of the European Union supported this publication. Publication number 2921 of the Netherlands institute of Ecology (NIOO/CEMO). We thank three anonymous reviewers for their constructive comments.

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