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Current Opinion in Biotechnology

Tracking activity and function of microorganisms by stable isotope probing of membrane lipids

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Microorganisms in soils and sediments are highly abundant and phylogenetically diverse, but their specific metabolic activity and function in the environment is often not well constrained. To address this critical aspect in environmental biogeochemistry, different methods involving stable isotope probing (SIP) and detection of the isotope label in a variety of molecular compounds have been developed. Here we review recent progress in lipid-SIP, a technique that combines the assimilation of specific ^{13}C-labeled metabolic substrates such as inorganic carbon, methane, glucose and amino acids into diagnostic membrane lipid compounds. Using the structural characteristics of certain lipid types in combination with genetic molecular techniques, the SIP approach reveals the activity and function of distinct microbial groups in the environment. More recently, deuterium labeling in the form of deuterated water (D_2O) extended the lipid-SIP portfolio. Since lipid biosynthetic pathways involve hydrogen (H^+) uptake from water, lipid production can be inferred from the detection of D-assimilation into these compounds. Furthermore, by combining D_2O and ^{13}C-inorganic carbon (IC) labeling in a dual-SIP approach, rates of auto- and heterotrophic carbon fixation can be estimated. We discuss the design, analytical prerequisites, data processing and interpretation of single and dual-SIP experiments and highlight a case study on anaerobic methanotrophic communities inhabiting hydrothermally heated marine sediments.

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Current Opinion in Biotechnology 2016, **41**:43–52

This review comes from a themed issue on **Analytical biotechnology**

Edited by **Hans-Hermann Richnow** and **Tillmann Lueders**

For a complete overview see the Issue and the Editorial

Available online 12th May 2016

http://dx.doi.org/10.1016/j.copbio.2016.04.022

Introduction

Microorganisms are key players in sediments and soils, mineralizing organic carbon and performing redox reactions. Based on taxonomic marker genes such as 16S rRNA an enormous phylogenetic complexity of these organisms has been revealed. To quantify the abundance of specific groups of microorganisms in the environment different approaches based on direct counting via fluorescence *in situ* hybridization (FISH [1]), real-time polymerase chain reactions (qPCR; [2]), or intact polar lipids (IPLs) [3] are used. Furthermore, metagenomic and metatranscriptomic approaches successfully identify metabolic potentials of single microorganism or microbial groups [4]. However, all these approaches cannot measure or estimate the activity and anabolic substrate turnover of specific microbial groups in a natural sample, and therefore stable isotope probing (SIP) has been designed. Here, specific bio-accessible compounds with artificially labeled isotopic compositions are added to samples, with metabolically active organisms incorporating these compounds. The fraction enriched in the heavy isotope (e.g., D = ^{2}H, ^{13}C or ^{15}N) is recovered in whole cells or in formed cell compounds (e.g., nucleic acids, proteins or lipids), which then allows the identification of active community members. In terms of lipid-SIP this approach is highly quantitative, as deviations from natural isotopic compositions are measured using precise isotope ratio mass spectrometry (IRMS). Lipids, however, do not possess the taxonomic specificity of nucleic acids, thus lipid-SIP often requires supplementary nucleic acid based information to fully complement the underlying community compositions.

The natural carbon isotope composition of lipids — expressed as δ^{13}C values relative to the internationally calibrated Vienna Peedee Belemnite (VPDB) standard — reveals first insights into the origin and pathways of biomass production. Heterotrophic organisms are usually limited by substrate availability, therefore their biomass largely reflects the isotopic composition of the substrate, with δ^{13}C values in the range of −15 to −27‰ vs VPDB [5,6]. On the contrary, autotrophic carbon fixation is accompanied by much stronger isotope effects, particularly for microorganisms from anoxic environments. For example, microbial lipids of archaea performing the anaerobic oxidation of methane (AOM) show δ^{13}C values of −100‰ and below [7,8,9]. Due to the high relative mass difference between the two stable isotopes of hydrogen (H and D), the microbial lipids are even stronger depleted in deuterium, reaching values down to −380‰ relative to the Vienna Standard Mean Ocean Water (VSMOW) standard [10].

SIP on polar/phospholipid derived fatty acids (PLFAs) has been first applied by Boschker and co-workers (1998)

to track carbon flow from methane and acetate into biomass of sedimentary marine microorganisms [11] and since then has been applied in several soil, peat and sedimentary environments, aquifers and water bodies. Over the past 15 years, lipid-SIP employing various carbon substrates (single-SIP) was extended to a variety of microbial lipid types, including the analysis of isoprenoid-based molecules, hopanoids and steroids characteristic for archaea [12,13,14•], bacteria [15,16,17], and eukaryotes [16,18], respectively. But only most recently a combined approach using both, inorganic carbon (IC) and deuterated water (D_2O) called dual-SIP was introduced, allowing the simultaneous analysis of hetero- and autotrophic carbon fixation without altering the substrate availability of the present microbial community [19••,20•].

General considerations for lipid-based SIP

In general, microorganisms are strongly adapted to specific environmental conditions. To trace the activity of certain microorganisms in a natural sample, the physicochemical parameters of the experiment such as temperature, salinity, nutrients, pH and oxygenation should be controlled and closely mimic environmental conditions. In addition, the label uptake through potential food chains and trophic levels should be tracked by performing a time series, thus allowing insights into the interaction of multiple microbial groups across domains of life (e.g. [21,22••]). However, especially for energy-rich substrate combinations, the incubation time of the respective SIP experiment should be adjusted to prevent drastic shifts in community structures (i.e., limit cross-feeding). Thus, information about concentrations of organic substrates is mandatory in order to draw realistic conclusions about the importance and dominance of the microbial target group. Furthermore, we strongly recommend to pair lipid-SIP with detailed knowledge of the turnover of a given compound (i.e., measuring the $\delta^{13}C$ of produced IC for heterotrophic substrates) as well as the investigation of microbial community structures (i.e., 16S rRNA approaches). The knowledge on the predicted lipid biosynthesis pathways as well as metagenomic information will help to better understand the fate of the substrates in microorganisms and its turnover in the environment. Finally, lipid-SIP experiments should be scaled in a way that the membrane lipid concentrations are sufficient to measure its isotope ratios. For reasonable peak sizes (i.e., equivalent to 10 ng per lipid for $\delta^{13}C$ and ~100 ng per lipid for δD measurements) and relatively low label content ($\delta^{13}C < 500‰$; $\delta D < 5000‰$) technical precisions of 1‰ for carbon and of 10‰ for deuterium can be reached. However, abiotic label incorporation and natural variation between samples has to be taken into account. Hence, killed controls should be performed, measurements should be triplicated and only results with statistical significance should be considered.

From lipid extractions to isotope values

Lipid extraction often base on protocols by Bligh and Dyer [23] with modifications described by Sturt *et al.* [24], which are optimized for a high extraction efficiency of IPLs. To verify lipid yields, extractions involve internal standards that resemble structural analogues of components of interest. Isotope analysis via gas chromatography (GC)-IRMS requires cleavage of polar headgroups and carbon side chains, including derivatization reactions. Mild saponification followed by esterification or directly via acidic transesterification yields fatty acid methyl esters (FAMEs; [25,26]) from bacterial and eukaryotic membranes. Analysis of ether bound isoprenoid side chains from archaeal or ether bound alkyl side chains from bacterial lipids demands harsher cleavage by hydrogen iodide or boron tribromide treatments [27,28]. The latter reactions are followed by reduction of the intermediates by either lithium aluminium hydride, lithium triethylborohydride or H_2/PtO_2, yielding the corresponding isoprenoid branched or straight-chain hydrocarbons [27,29]. Cleavage reaction products are accessible for carbon and hydrogen isotope ratio measurements by GC-IRMS coupled to a combustion interface as described by Hayes and colleagues for carbon [30] and Burgoyne and Hayes for hydrogen [31]. Ratios are referenced against compounds of known isotopic composition and cross-checked with pulses of reference gas (i.e., CO_2 or H_2) that has been standardized before with internationally certified reference compounds [32]. In order to yield true isotopic compositions of the fatty acids ($\delta^{13}C_{FA}$ [Eq. (1)]; δD_{FA}; [Eq. (2)]) according to

$$\delta^{13}C_{FA} = [(C_n + 1) \times \delta^{13}C_{FAME} - \delta^{13}C_{MeOH}]/C_n \quad (1)$$

$$\delta D_{FA} = [(H_n + 3) \times \delta D_{FAME} - \delta^{13}D_{MeOH}]/H_n \quad (2)$$

isotopic composition of FAMEs ($\delta^{13}C_{FAME}$; δD_{FAME}) need to be corrected for the introduction of a methyl group ($\delta^{13}C_{MeOH}$; δD_{MeOH}) thereby taking the number of carbon (C_n) and hydrogen (H_n) atoms into account (cf. [6]).

Quantitative aspects of single and dual-SIP

During the lipid-SIP approach, isotope ratios are compared with the compound isotopic compositions of the original sample of the experiment (i.e., time point right before label addition — T_0). After the SIP experiment, positive deviations relative to the T_0 sample indicate label uptake into specific membrane lipids. However, rather than using the development of the $\delta^{13}C$ or δD values that derive from the mixture of originally present and experimentally labeled lipids, a translation into lipid production rates is recommended. Many single SIP studies converted the amount of ^{13}C-label incorporation into nanogram of label assimilation per PLFA per gram of sample, which results in specific chemotaxonomic fingerprints of microbial activity. Such calculations have been presented before (e.g. [33,34,35]). The calculation of lipid production based on the deuterium

assimilation ($prod_{LD}$) during dual-SIP experiments, however, is defined by Eq. (3):

$$prod_{LD} = \frac{F^{D}_{LD_{T_n}} - F^{D}_{LD_{T_0}}}{F^{D}_{Med} - F^{D}_{LD_{T_n}}} \times conc_{LD} \times \alpha_{H/D} \quad (3)$$

with the fractions of deuterium in the lipid (F^{D}_{LD}; derived from the abundances of D and H; $F^{D}_{LD} = D_{LD}/(H_{LD} + D_{LD})$) after an incubation interval (T_n) compared to the untreated sample (T_0), the deuterium fraction in the medium (F^{D}_{Med}) and the concentration of the lipid ($conc_{LD}$). All values are normalized to weight (i.e., per gram) and a time interval (i.e., per day, month or year). Moreover, a fractionation factor $\alpha_{H/D}$ has to be considered because deuterium is heavily discriminated during lipid biosynthesis. A $\alpha_{H/D}$ value is determined by comparing fractions of deuterium of lipid and medium (i.e., seawater or pore water: $\alpha_{H/D} = F^{D}_{Med}/F^{D}_{LD_{T_0}}$). For instance, a $\alpha_{H/D} = 1.32$ is retrieved for lipids with δD values of −250‰ produced in water medium with a δD value of 0‰ [19••,36]. For the assimilation of IC ($assim_{IC}$; in autotrophic microorganisms equaling the lipid production) the respective fractions of ^{13}C are used (Eq. (4)):

$$assim_{IC} = \frac{F^{13C}_{LD_{T_n}} - F^{13C}_{LD_{T_0}}}{F^{13C}_{DIC} - F^{13C}_{LD_{T_n}}} \times conc_{LD} \quad (4)$$

with the fractions of ^{13}C in the lipid (F^{13C}_{LD}) after an incubation interval (T_n) compared to the untreated sample (T_0) and the ^{13}C fraction in the DIC pool (F^{13C}_{DIC}). As isotope fractionation in carbon is comparably low (~20‰), a correction employing a fractionation factor is usually not necessary, but could be determined as described above.

To assess the relative importance of auto- and heterotrophy during the dual lipid-SIP approach, the ratio $R_{a/p}$ is defined as (Eq. (5)):

$$R_{a/p} = \frac{assim_{IC}}{prod_{LD}} \quad (5)$$

All anaerobic microorganisms assimilate substantial amounts of IC, which stays in the range of 5–30% of produced lipid carbon, resulting in $R_{a/p}$ values between 0.05 and 0.3 for their membrane lipids [19••]. Autotrophic microorganisms fix all their carbon from IC, hence $assim_{IC}$ and $prod_{LD}$ are similar, which leads to $R_{a/p}$ values of ≈1. Intermediate lipid $R_{a/p}$ values suggest production of either multiple microorganisms (mix of autotrophic and heterotrophic organisms) or by a single mixotrophic microbial group.

Recent single SIP studies

Since its early description in the late 1990s, lipid-SIP has been intensively applied to determine the turnover of methane, to track inorganic carbon fixation and to study degradation and assimilation of pollutants such as toluene in various environments [11,37,38]. Fatty acid labeling patterns derived from the calculation of ^{13}C-incorporation in these studies have been correlated with those from cultures, thus identifying the abundance of microbial players in the environment. For a detailed overview of many of these approaches published before 2006 we refer to the review by Evershed *et al.* [35].

SIP using C_1 - compounds

In the following years, lipid-SIP has been used preliminary in freshwater habitats such as rice paddies, peats and soils, and largely methane has been utilized as ^{13}C-labeled carbon source (e.g., [17,22••,39] (Table 1)). With the increasing knowledge on lipid patterns of specific microorganisms, lipid ^{13}C-incorporation and production estimates could be better addressed relative to the active community members. For example, Shrestha and coworkers (2008) and Qiu and colleagues (2008) combined PLFA- and functional gene analysis or ribosomal nucleic acid (RNA)-SIP to identify and quantify the active methanotrophic community members within the rice rhizosphere [40•,41•]. Using a similar approach Chen and colleagues (2008) investigated methanotrophic bacteria in peatlands [42••], showing that different habitats (i.e., *Calluna* or *Sphagnum*/*Eriophorum*-covered moorland) are characterized by specific groups of *Methylocella* and *Methylocystis* methanotrophs. Employing an isotope switching (SIS) approach in a multi pulse-chase way, Maxfield and colleagues first added ^{13}C-methane to a landfill cover soil, and replaced it in the middle of the experiment by ^{12}C-methane [22••]. Their experiment resulted in a sequential assimilation of ^{13}C first by methanotrophic bacteria followed by secondary (Gram-negative and Gram-positive bacteria) and tertiary consumers (eukaryotes). SIS thus provides a valuable tool where total carbon dynamics (i.e., substrate incorporation, turnover and lipid decay) in complex environmental and biological matrices are accessible.

Multiple applications of IC ^{13}C-labeling were performed to track autotrophic community members. For example, in a IC ^{13}C pulse-chase *in situ* incubation, Miyatake and coworkers traced the carbon flow from microphytobenthos (i.e., diatoms) into bacteria such as Gammaproteobacteria, Bacteroidetes, and Deltaproteobacteria in intertidal marine sediments [43]. Employing the same approach, Boschker and colleagues [44••] related enhanced dark carbon fixation to sulfide-oxidizing Gammaproteobacteria that may act as important players in the coastal carbon cycle. In contrast, ^{13}C-IC assimilation via anapleurotic reactions of *Acidobacteriaceae*, next to chemolithoautotrophic utilization by methanogens, was suggested to be most important in a wetland mofette soil characterized by high emanations of CO_2 [45].

SIP using multiple and complex substrates

A variety of experiments focusing on bacteria and using a combination of ^{13}C-IC and other ^{13}C-labeled organic

Table 1

Representative studies employing SIP with various substrates and targeting membrane lipids

Labeled substrate	Studied environment	Targeted membrane lipid	Employed phylogenetic methods	Conclusions	Ref.
CH_4	Rice rhizosphere	PLFAs	RNA-SIP	Rice rhizosphere is dominated by Type I methanotrophs	[41•]
	Rice rhizosphere	PLFAs	pmoA-genes	Methanotrophic populations exhibit a pronounced spatial and temporal variations	[40•]
	Acidic peatlands	PLFAs	pmoA-based microarray	Diversity of methanotrophs in peatlands are greater than previously assumed	[42••]
	Peat moss	PLFAs, hopanoids		Type II methanotrophs are dominant in peat moss	[17]
	Submerged brown moss	PLFAs		Moss-associated methanotrophic bacteria reduce methane emissions in tundra	[39]
	Landfill cover soils	PLFAs		Sequential methane carbon assimilation by methanotrophs, Gram-negative and -positive bacteria and eukaryotes	[22••]
IC*	Desert soils	Bacterial and fungal PLFAs		Elevated CO_2 concentrations lead to shifts from bacterial to fungal carbon fixation	[46]
	Wetland mofette	PLFAs, isoprenoids	DNA-SIP	CO_2 promotes anaerobic and acidophilic microorganisms and alters carbon turnover in soils	[45]
	Potato plant and rhizosphere	PLFAs	RNA-SIP TRFLP, qPCR	Rapid organic carbon transfer from roots into saprotrophic and mycorrhizal fungi	[47]
	Intertidal sediments	PLFAs	16S rRNA Seq	Chemolithoautotrophic bacteria are important biomass producers in sediments	[44••]
	Intertidal sediments	PLFAs	Mag-SIP	Activity of diatoms and bacteria in intertidal sediments is tightly coupled	[48]
IC, CH_4	Seep sediments	PLFAs, isoprenoids		Metabolic coupling of ANME and partner bacteria, dominance of autotrophy	[49]
	Enrichment culture	PLFAs	Genome analysis	Dominance of autotrophic carbon dioxide fixation during growth of denitrifying methanotrophic bacteria	[50•]
IC, CH_4, methanol, acetate	AOM-dominated microbial mat	Isoprenoids		Co-occurrence of methanotrophy and methanogenesis in AOM systems	[51]
	Deep-sea sediments	Polar lipid subgroups		Archaeal lipid production rates imply accumulation of 2G-GDGT in marine sediments	[14•]
IC, CH4, acetate, glucose, leucine, Spirulina	Yellowstone streamer biofilm	PLFAs		Streamer biofilms thrive predominantly on exogenous organic material	[52]
IC, acetate, glucose	Iceland shelf sediments	GDGTs		Low metabolic activity in sedimentary Thaumarchaeota	[53]
IC, glucose, amino acids, pyruvate	Coastal hypoxic basin	PLFAs	16S rRNA gene Seq.	Chemoorganotrophic cable bacteria form consortia with autotrophic bacteria	[54••]
IC, propionate	Anammox bacteria, culture	PLFAs, ether lipids		Ladderane lipids are formed by a yet unknown pathway of biosynthesis	[55]
Acetate	Grassland soils			Soil microbial biomass response depend on the concentration of supplied energy sources	[56]
Glucose	Ectomycorrhizal fungi, culture	PLFAs		Position-specific ^{13}C-glucose labeling provides a framework for analyzing carbon flow and lipid biosynthesis in fungi	[57••]
	Soil	PLFAs		Incorporation of individual glucose ^{13}C-positions into PLFAs showed intensive recycling via gluconeogenesis	[58••]
	Deep-sea sediments	Isoprenoids glycerol units	Arch. 16S rRNA Seq. qPCR	Exclusive ^{13}C-labeling of glycerol backbone indicated recycling of membrane lipids by archaea	[59••]

Table 1 (*Continued*)

Labeled substrate	Studied environment	Targeted membrane lipid	Employed phylogenetic methods	Conclusions	Ref.
Amino acids	Soil	PLFAs		Position-specific ^{13}C-labeling allows insight into carbon utilization by various microbial groups	[60]
Toluene	Contaminated aquifer	PLFAs	16S rRNA TRFLP	Desulfocapsa related bacteria are main toluene-degraders in contaminated aquifers	[61]
Benzene	Benzene-degrading biofilm	PLFAs	Amino acid SIP	Transfer of pollutant carbon into multiple trophic levels including midge larvae	[21]
Beggiatoa	Mangrove sediments	PLFAs	18S rRNA Seq.	Chemolithoautotrophic bacteria play a minor role for mangrove grazers	[62]

* IC: Includes experiments using either bicarbonate or CO_2. Abbreviations: pmoA = gene that encodes for the alpha subunit of the particulate methane monooxygenase; TRFLP = Terminal restriction fragment length polymorphism; Mag-SIP = magnetic-bead-captured rRNA-SIP.

carbon compounds such as methane, acetate and glucose has been performed in the last years. Vasquez-Cardenas and colleagues [54••], for example, studied carbon fixation in sediments dominated by cable bacteria performing long-distance electron transport. The authors identified cable bacteria to be propionate assimilating heterotrophs, which support increased autotrophic carbon fixation by side communities. Schubotz and coworkers (2015) studied the carbon fixation in streamer biofilms and found a rather mixotrophic nature of most microorganisms in this community [52]. To minimize the effect of additional substrate availability, which is a crucial aspect during SIP studies using organic substrates, Dungait and colleagues [56] used trace amounts of ^{13}C-labeled glucose to disentangle active microbes in soils. Together with lipid patterns they were able to show that a large portion of glucose was metabolized by Actinobacteria and other Gram-positive bacteria.

Lipid-SIP on archaeal lipids was introduced by Wuchter and colleagues (2003) demonstrating that marine Crenarchaeota are autotrophs. In the following years, many lipid-SIP experiments have been designed to trace the activity of archaea, specifically of those performing AOM. For example, Blumenberg and coworkers [63] and Jagersma *et al.* [13] traced the assimilation of ^{13}C-methane into AOM communities, recovering it in anaerobic methanotrophic archaea (ANME) and their sulfate-reducing partner bacteria. Evidence that a large fraction of lipid carbon in ANME and their partner bacteria derives from IC was provided in follow-up experiments by Wegener and colleagues [49] using either ^{13}C-labeled methane or IC. Extension of this approach to additional ^{13}C-substrates such as methanol and acetate by Bertram *et al.* [51] gave rise to the question whether certain ANME may contain the metabolic capabilities to concomitantly function as methanogens.

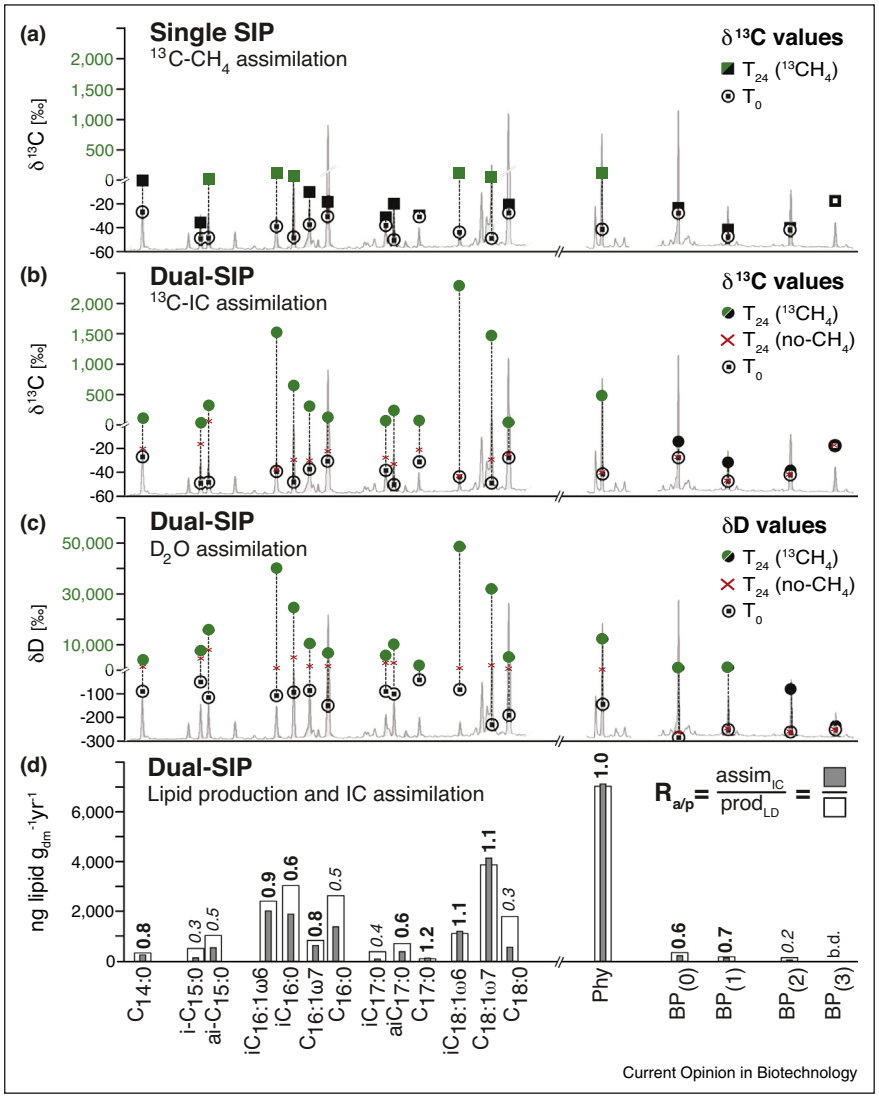
Focusing on the activity of archaea in sediments, several lipid-SIP studies were performed including different substrates and amendments. For example, Takano and coworkers [59••] carried out benthic *in situ*-labeling experiments in order to address the question whether sedimentary archaea are actively growing on organic substrates such as glucose. Whereas archaeal core lipid-derived hydrocarbons were not found to incorporate any of the ^{13}C-labeled glucose, the glycerol moieties of the ether lipids were strongly labeled. The authors concluded that the glycerol unit is synthesized *de novo*, while the isoprenoid unit is recycled from relic archaeal membranes. Subsequently, approaches by Lin *et al.* [14•] and Lengger *et al.* [53] used a wider suite of ^{13}C-labeled substrates (i.e., IC, acetate, glucose) and found a minor or lacking uptake into archaeal lipids, respectively. Based on low ^{13}C-incorporation and extremely slow turnover rates of archaeal lipids both studies concluded that benthic archaea are rather inactive and that sedimentary archaeal membrane components have either a long residence time or an allochthonous origin.

A recent development in the field of SIP employed ^{13}C-labeling at specific positions of sugars and amino acids [57••,58••,60], representing carbohydrates and proteins as two of the most abundant carbon pools within microorganisms and the environment. By applying the same molecule but carrying ^{13}C-label at a different C-position provides detailed information about carbon utilization and metabolic pathways such as gluconeogenesis as well as lipid biosynthesis in single microorganisms. Information about lipid biosynthetic pathways, however, could likewise be obtained by following the strength of label uptake into various fatty acids molecules and moieties such as performed by Rattray *et al.* (2009) targeting ladderane lipid biosynthesis of anaerobic ammonium oxidizing (anammox) bacteria [55]. These authors combined classical PLFA analysis with high-field ^{13}C nuclear magnetic resonance spectroscopy on isolated fatty acids and found that ring moieties of ladderane lipids are produced via a different route than straight-chain fatty acids.

The dual-SIP approach

In the past, autotrophic carbon fixation and heterotrophic activity has been independently investigated by

Figure 1



Shift of lipid carbon and hydrogen isotopic composition in a single **(a)**, in dual-SIP experiment **(b, c)** and lipid production/assimilation rates **(d)** between (T_0) and after 24 days of incubation with labeled substrates (T_{24}). **(a)** $\delta^{13}C$ values in a single SIP experiment with $^{13}CH_4$ (■) and **(b)** $\delta^{13}C$ and **(c)** δD values in a dual-SIP experiment with (●) and without (×) methane. Green symbols mark isotope values above 0‰ with different scales; grey background line: Sections of exemplary gas chromatograms. **(d)** Calculated rates of IC assimilation into lipids ($assim_{IC}$) and production of lipids ($prod_{LD}$) and their respective ratios ($R_{a/p}$) for only the 'plus methane'-experiment; bold = production by autotrophs, italic: production by hetero- or mixotrophs. Bacterial fatty acid nomenclature: $xC_{n:p\omega q;}$ where '*x*' defines a methyl branch at the terminal '*i* = *iso*' or subterminal '*ai* = anteiso' position, '*n*' refers to the carbon chain length, and '*p*'ω'*q*' to number of double bounds and position from the hydrophobic 'ω' end, respectively. Archaeal isoprenoid nomenclature: Phy = phytane derived from the glycerol diether lipid archaeol, and BP = biphytane derived from glycerol dibiphytanyl glycerol tetraethers (GDGTs), with index (*n*) indicating the number of cyclopentane rings (exception $BP_{(3)}$ with two cyclopentane and one cyclohexane ring).

^{13}C-uptake from IC and assimilation of various ^{13}C-labeled organic carbon sources, respectively. However, the addition of ^{13}C-labeled organic substrates supplies an extra energy source that likely stimulates a certain group of specialized heterotrophs. Hence, lipid-SIP experiments with single heterotrophic substrates are suitable to identify active microorganisms, but often fail to reproduce natural activity of the entire microbial population. SIP with IC, on the other hand, will also detect anapleurotic carbon fixation, i.e., to refuel critical intermediates in the metabolic machinery, by heterotrophic microorganisms. Depending on biochemical pathways and substrates, heterotrophic organisms have shown to assimilate IC between 1 and 30% [19••,64] and up to 50% in type II methanotrophic bacteria [65].

To overcome the limitations of single SIP, dual-SIP with D_2O combined with ^{13}C-IC was developed [19••]. D_2O labeling allows quantitative tracking of lipid production under *in situ* conditions without providing an additional energy source as suspected for carbon substrates. This approach facilitates the determination of total lipid production rates because the reduced proton carrier nicotinamide adenine dinucleotide phosphate (NADPH) introduces protons during all reduction steps of lipid synthesis. In both, bacteria and archaea, first the precursor acetyl-CoA is formed. In the bacterial fatty acid synthesis pathway the acetyl CoA is reduced with NADPH to butyryl-subunits, which by combination are elongated up to palmitate [66]. Archaeal isoprenoid lipid formation, on the contrary, uses the acetyl-CoA reduction with NADPH in the mevalonate pathway [67]. Abiotic exchange between lipids and water hydrogen is meanwhile inherently slow, as shown by the preservation of the lipid hydrogen isotope signal on geological time scales [68]. Moreover, it has to be considered that the addition of high amounts of D_2O (60–90%) slows down metabolic activity or is even toxic to microorganisms [69]. Microorganisms with low energy yields such as acetotrophic and hydrogenotrophic methanogens seem to be even more sensitive to D_2O. Here, only D_2O concentrations of $\leq$10% and $\leq$23%, respectively, did not affect methane production rates (Wegener, unpublished data).

The concurrent tracking of ^{13}C-IC assimilation and lipid production rates via D_2O can provide an estimate of the ratio between auto- and heterotrophic carbon fixation. The applicability of this approach has been demonstrated for the first time by Wegener and coworkers [19••] using cultures of *Desulfosarcina variabilis*. The dual-SIP approach was applied to study the metabolic activity of microorganisms in marine sediments and identified heterotrophic microorganisms being responsible for the high lipid production rates close to the sediment surface. The steeply declining lipid biomass production rates and the increase of autotrophic carbon fixation in deeper horizons suggested a microbial community that was less dependent on organic carbon sources. However, the strongest contribution of autotrophic carbon fixation so far was observed within the sulfate-methane transition zone, thus confirming the autotrophic nature of AOM communities. The specific lifestyle of AOM communities was later explored in much detail by Kellermann *et al.* applying the dual-SIP approach in hydrothermally-heated sediments from the Guaymas Basin [20•] (see case study below). Moreover, based on these experiments and archaeal lipid hydrogen isotope analysis of purified IPL fractions, Kellermann and coworkers most recently suggested that the tetraether biosynthetic pathway involves i) the production of intact phospho-archaeols, ii) condensation of two phospho-archaeol molecules to phospho-GDGTs and iii) progressive substitution of phospho- with glyco-headgroups in GDGTs [70•].

Case Study: Tracing metabolic activity, function and carbon sources in anaerobic methanotrophic communities by single and dual-SIP approaches.

The case study we present in Figure 1 used incubations with (i) ^{13}C-labeled methane (16%), (ii) ^{13}C-IC (10%), D_2O (3%) and non-labeled methane, and (iii) labeling as applied in (ii) but without methane. The samples were incubated in a time series for up to 24 days at 37 °C (for details see [20•]). Microbial lipids were extracted and analyzed as described above. The single-SIP approach shows ^{13}C-methane carbon uptake for both, archaeal and bacterial lipids (Figure 1a). However, both lipid groups show even higher methane-dependent ^{13}C-IC uptake (Figure 1b), which strongly decreases in the absence of methane (displayed with red crosses in Figure 1b and c). These results provide strong evidence for the central role of methane as energy source but also for its minor importance as direct carbon source for the AOM communities. To fully assess the role of IC fixation the same lipids were analyzed for deuterium uptake (Figure 1c). To quantitatively compare *de novo* lipid production from ^{13}C-IC and D_2O uptake from water, isotope values are converted into IC assimilation rates ($assim_{IC}$) and lipid production rates ($prod_{LD}$) as described above. In the presence of methane, $R_{a/p}$ values of ~1 for specific bacterial fatty acids (i.e., $iC_{16:1\omega6}$, $iC_{18:1\omega6}$, $C_{18:1\omega7}$) and archaeal isoprenoid hydrocarbons (i.e., phytane), confirmed their production by microorganisms functioning as autotrophs (Figure 1d). Bacterial lipids with $R_{a/p}$ values lower than 0.6 are likely not only produced by AOM partner bacteria but also by co-occurring heterotrophic background communities.

Conclusions and future perspectives

In the past two decades, SIP of microbial lipids has been established as an essential, quantitative and culture-independent approach to study the activity as well as the carbon substrate turnover of microorganisms in various environments. The previously forecasted expansion of lipid-SIP applications [35] including the widening of the traceable lipid portfolio has been met. Approaches such as SIS, dual and position-specific SIP opens the field towards an extended understanding of microbial activity, metabolic function, lipid biosynthesis and carbon flow in any system holding active microbial communities. The limited phylogenetic resolution of lipid-SIP has been encountered by additional taxonomic nucleic acid based approaches to identify the active microbial community members. In addition, the sensitivity and specificity of lipid-SIP can be further increased by focusing on IPL classes derived from solely live and metabolically active microorganisms which are purified by preparative, multidimensional liquid chromatography [70•]. By excluding non-labeled, extracellular bulk lipids, the detection limit of isotope analysis is potentially lowered, which in turn allows SIP applications to microbial communities with lower activities that so far were inaccessible to current

lipid-SIP approaches. Lipid-SIP has not been recognized by applied sciences, but its application would allow quantitative insights into microbial activity during *in situ* microcosm experiments involving natural and stimulated biodegradation of organic pollutants (e.g., Fischer *et al.*, this issue [71]). We also hypothesize that future D_2O labeling approaches may combine ^{13}C-labeling of organic carbon sources other than methane or IC. In these experiments, the lipid deuterium signal could be used to identify the influence of the organic carbon compound on the overall lipid production relative to a control sample without organic substrate addition. Finally, to fully understand the fate of organic carbon substrates, ^{13}C-labeled metabolic intermediates should be monitored by coupled liquid chromatography - IRMS techniques, thus accessing the full complexity during lipid biosynthesis and of carbon transfer between various microbial groups.

Acknowledgements

The study was supported by the Deutsche Forschungsgemeinschaft (through the Research Center/Excellence Cluster MARUM - Center for Marine Environmental Sciences; M.E. and G.W.), the Gottfried Wilhelm Leibniz Price awarded to Antje Boetius (G.W.) and the National Science Foundation (Grant No. OCE-1046144; M.Y.K.).

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