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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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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