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Applications and impacts of stable isotope probing for analysis of microbial interactions

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Abstract

Probing the interactions between microbes and their environment with stable isotopes became a powerful technique over the last years. While quadruple mass spectrometry or isotope ratio mass spectrometry (IRMS) require at least 300.000 bacterial cells, analysis at the single-cell level is possible with secondary ion mass spectrometry or Raman microspectrometry. The latter two techniques however need enrichments of more than 10 atom-% while IRMS can deal with 0.0001 atom-%. To find out who eats what one has to discern between the different species in a community. Several methods have been introduced to discern between the different taxa in microbial communities, e.g. by using fatty acids as biomarkers, density centrifugation of DNA/RNA or fluorescent *in situ* hybridization (FISH) with phylogenetic probes. While the biomarker approach can be coupled with the high sensitivity of the IRMS, the DNA approach gives in general a better phylogenetic resolution of the metabolic active microbes. A combination of both is the separation via coupling of FISH-probes to magnetic beads or fluorescent assisted cell sorting (FACS) of stained cells leading to fractions which can be analyzed by IRMS. Applying these techniques over a time course can reveal the metabolic kinetics and food webs. In this review the different methods are presented with examples and their advantages and disadvantages are discussed. An outlook on the combination of the various techniques and their applications in microbial ecology is given.

Keywords

Microbial interaction, stable isotope probing, isotope ratio mass spectrometry, flux analysis, microbial ecology

Introduction

Bacteria have evolved a remarkable spectrum of metabolic capabilities during more than 3.5 billion years of their existence on this planet. Furthermore, this is fostered by the fact that in nature bacteria species seldom occur as pure strains but used to live in complex microbial communities (Morris et al. 2013). These communities are changing and adapting, according to changes in environmental conditions but they are also resilient when the disturbance has passed. Microbial communities are able to use substrates not palatable for any of their members alone. They also have a considerable degree of work sharing between their members.

When the first eukaryotes developed they were faced with these complex and organized microbial communities and the only way of survival was to deal with their prokaryotic neighbors. The result was a highly sophisticated and fine tuned network of metabolic interactions. To understand these interactions with the aim to modulate them for our purposes is one of the prime goals in microbial ecology.

One elegant approach for the elucidation of metabolic activities and interactions of microorganisms present in a habitat is the incorporation of isotopically labeled substrates into their biomass by tracer pulse-chase experiments followed by the identification of the actively incorporating microorganisms.

A large number of reviews have been published on the identification of bacteria using substrates labeled with stable isotopes. These studies will be briefly discussed and important reviews cited but the main focus of this mini-review is on the metabolic interactions between microorganisms in communities and between microorganisms and their respective hosts using stable isotope probing (SIP). The discussion will be further focused on the stable isotopes of carbon.

The rise of stable isotope probing

Since almost one century isotopes have been used in biochemistry. At the beginning mainly radioactive isotopes were applied, e.g. in 1927 the oxidation of ^{14}C-propionate by *Escherichia coli* was reported (Wegener et al. 1927) or the incorporation of ^{14}C-glycine into proteins of a yeast was studied (Webb et al. 1950) only to give here two examples. Radioactive isotopes have also been used in ecological studies and one of the earliest reports was given by Tomes and Brian (1946) who reported on the tracing of the movements of *Agriotes* beetles in soil by applying radium sulfate to the beetles.

Because of the harmful effects of ionizing radiation and the security aspect and fostered the developing applications of ^{13}C-NMR spectrometry a shift from radioactive to stable isotope applications occurred. The first applications were probably in biosynthetic studies, e.g. in the biosynthesis of fungal metabolites using ^{13}C-NMR (Desaty 1968). However, the application in studies of metabolic fluxes has long been limited by the lack of highly sensitive instruments (e.g. ordinary GC-MS, Kouchii 1982) preventing the detection of natural fluctuations in stable isotope ratios.

For the precise measurement of isotope ratios standards are needed. This can be done by each laboratory by measuring a single carbon source, preferably carbon dioxide and reporting the differences of the samples against this standard. Such an approach is of very limited use because it does not allow comparisons of results from different laboratories. Because of this unacceptable limitation several attempts have been made to measure the exact isotope ratio of a carbonate and define this as a standard (Craig 1953). PeeDee belemnite, a South Carolinian carbonate rich in ^{13}C, served as standard. When it became exhausted it was replaced by Vienna PeeDee belemnite, defined by the International Atomic Energy Agency (IAEA) (Coplen 1995). Since this time ratios are usually not reported in atom-% but in the δ^{13}C scale which is defined as

$$\delta\ [‰] = ((R_{Sample}/R_{VPDB}) - 1) * 10^3 \qquad (1)$$

where R_{Sample} and R_{VPDB} are the $^{13}C/^{12}C$ isotope ratios corresponding to the sample and to the international standard Vienna PeeDee Belemnite, a South Carolinian carbonate rich in ^{13}C (R_{VPDB} = 0.0111802), respectively (Craig 1957).

There are a number of reports using natural differences in isotope ratios of substrates for monitoring of substrate use in microbes (Abraham et al. 1998) but for complex microbial communities larger differences in isotope ratios between the authochthonous pool and the added substrate are required which only enriched substrates can offer.

For the analysis of metabolic microbial interactions three important conditions have to be fulfilled:

- Sensitive instruments to detect the incorporation of the stable isotope
- Methods to discern between microbial species in a sample
- Fast analyses allowing sampling over time to determine the kinetics for incorporation

Instruments and techniques for the determination of isotopic ratios

Several instruments have been used to separate ^{12}C-labelled molecules from ^{13}C-labelled ones. Their main difference is in the precision, i.e. how accurate isotope enrichments can be measured, their sensitivity, i.e. how much material is needed for precise analyses, and, finally, their price.

Quadrupole mass spectrometer

In a mass spectrometer the sample is first ionized, accelerated in an electric field, separated according to their individual mass, and finally detected. Different methods are used for each of these steps depending on the nature of molecules to be analyzed. Because the masses of the molecules or their fragments are detected these instruments can also give information of the amount of their stable isotopes. One ^{13}C atom in the molecule is detected one mass unit above the molecular ion (M+1 ion) and a molecule with n carbon atoms has consequently ions up to M+n. For molecules containing many carbons this becomes complicated and computer programs are used for calculating the isotope ratios. Seldom MS is used alone but in a combination with other separation techniques. The precision of the method may become rather good as van Eijk et al. reported about carefully optimized conditions of chromatography and detection to yield a precision of $\delta^{13}C$ +50 ‰ using HPLC-MS/MS (van Eijk et al. 2012). However, such a good precision still awaits demonstration in ecological studies. To open the field to proteins enzymatically digested proteins have been measured as oligopeptides and their isotope ratio has been assessed (Protein-SIP). Major challenges of protein-SIP are currently the difficulties to extract proteins from complex (environmental) matrices and the precision of above $\delta^{13}C$ +20.000 ‰ (Jehmlich et al. 2010). This seems to prevent currently novel insights into microbial interactions by this approach.

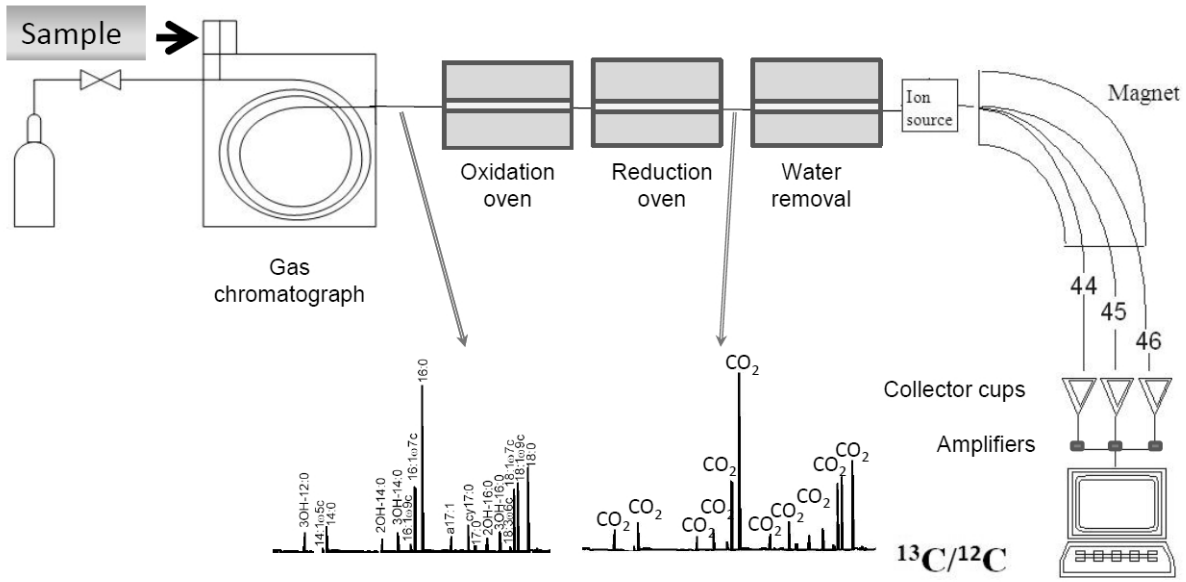

Figure 1: Scheme of carbon isotope ratio determination after gas chromatographic separation of a sample. The sample (here: fatty acid methyl esters) is injected into the gas chromatograph, the compounds separated followed by oxidation to CO_2, NO_x and water, the NO_x is then reduced to N_2 and finally the water separated using a Nafion membrane. During these steps the separation of the compounds is maintained allowing isotope determination for every separated compound. In the isotope ratio mass spectrometer ions of the masses *m/z* 44 ($^{12}C^{16}O^{16}O$), 45 ($^{13}C^{16}O^{16}O$ and $^{12}C^{17}O^{16}O$) and 46 ($^{13}C^{17}O^{16}O$) are collected. From these data $^{13}C/^{12}C$ is calculated.

Isotope Ratio Mass Spectrometer - IRMS

As an alternative to quadrupole MS specific Isotope Ratio Mass Spectrometer (IRMS) have been developed. Contrary to quadrupole MS, samples have to be introduced to the IRMS as pure gases, usually achieved by combustion in an elemental analyser coupled to packed column or gas chromatography with coupled catalytic oxidation, followed by reduction of NO_x and drying. The purity of CO_2 is critical for precise determinations of isotope ratios. In the IRMS ions of the masses *m/z* 44 ($^{12}C^{16}O^{16}O$), 45 ($^{13}C^{16}O^{16}O$ but also $^{12}C^{17}O^{16}O$), and 46 ($^{13}C^{17}O^{16}O$) are collected and their ratios are used for the determination of $\delta^{13}C$ (Figure 1). All measurements are compared to a standard gas which is measured before and after the sample. The precision achievable with such a device is close to ±0.000 001 $^{13}C/^{12}C$ and not achieved by any other technique discussed here (Table 1). For the ^{13}C-analysis of most compounds IRMS has to be coupled to a CO_2-generating analytical device. Most applications of continuous flow isotope ratio mass spectrometry use an elemental analyzer or a gas chromatography inlet system for compound-specific analysis. Coupling of HPLC with IRMS via an interface became possible since 2004 enabling compound-specific $\delta^{13}C$-analysis of non-volatile, polar compounds from complex mixtures. However, applications are still limited due to lack of compatibility with certain types of chromatography as well as limited flow rates and mobile phase compositions excluding, e.g. organic solvents and buffers (Godin, McCullagh 2011).

Table 1: Comparison of requirements, strengths and weaknesses of different SIP techniques

Technique	**Minimal cell numbers**	**Precision $^{13}C/^{12}C$**	**Phylogenetic resolution**	**Reference**
IRMS	300,000	0.000 001	Biomarker	Abraham unpub.
	Pros: High precision down to natural abundances; simultaneous analyses of several classes of biomolecules; easy purification		*Cons*: high cell numbers required; limited mainly to volatile biomolecules; phylogenetic resolution limited	
Nano-SIMS	1	0.001	FISH	Pett-Ridge, Weber 2012
	Pros: single-cell analysis; imaging; multi-isotope analyses; expensive machines		*Cons*: moderate enrichments required; low precision; sample preparation may damage 3D-structure	
MS, incl. protein-SIP	100,000	0.02	Biomarker; sequence	Jehmlich et al. 2010
	Pros: high flexibility concerning biomolecules; identification of compounds		*Cons*: high enrichments required; low precision; limited phylogenetic information; extensive sample preparation for proteins	
Raman	1	0.25	FISH	Wagner 2009
	Pros: single-cell analysis; imaging		*Cons*: very high enrichments required; very low precision	
DNA/RNA-SIP	100,000	>0.10	Sequence	Jehmlich et al. 2010
	Pros: high phylogenetic resolution; analyses of metabolic genes		*Cons*: high enrichments required; low precision; impurities may hamper separation	
FACS-SIP; magneto-FISH-SIP	300,000 (IRMS) 100,000 (RNA)	0.000 001 >0.10	Biomarker Sequence	Pawelczyk et al. 2011 Pernthaler et al. 2008
	Pros: independent from enrichment; availability of intact cells allow combination with many other techniques		*Cons*: as for IRMS or DNA/RNA-SIP; limited taxonomic resolution of probes or antibodies	

Secondary Ion Mass Spectrometer - SIMS

An alternative way of isotope ratio determination is the use of Secondary Ion Mass Spectrometer (SIMS). Here, the samples is sputtered by a narrow ion beam, usually Cs^+, which causes fragmentation of the target biomolecules leading to secondary ions, among them C_2^+. The isotope ratios of these small molecular ions are than determined (Orphan et al. 2001). A narrow ion beam allows resolutions at the level of single cells and their isotope mapping, a technique also called nano-SIMS (Stadermann et al. 1999). A very valuable overview over the technique with applications and its limitations has recently been published (Pett-Ridge, Weber 2012). SIMS has been further developed to Multi-isotope Imaging Mass Spectrometry (MIMS) allowing simultaneous visualization of several isotopes. MIMS is reported to be at least 1000 times more sensitive than ^{14}C autoradiography but is still orders less sensitive than GC-IRMS (Lechene et al. 2006) (Table 1).

Raman-Microspectroscopy

Raman-microspectroscopy allows the assessment of ^{13}C-enrichments at the single cell level. The Raman effect, discovered in 1928 by C. V. Raman, is the inelastic scattering of photons from a sample. Replacement of ^{12}C by ^{13}C causes in Raman spectra a shift of the resonance of the molecule. Over the last years several applications have been made exploiting this effect in SIP-experiments. Recording Raman spectra in a confocal microscope brought the resolution down to single-cell levels (Wagner 2009). If this technique is combined with taxa-specific FISH, isotope incorporation into single cells in microbial communities can be monitored. Quantification is very tricky but possible after performing careful calibrations; the analysis time per cell is usually 1 min (Huang et al. 2007). Quantification of $^{13}C/^{12}C$ ratios are usually obtained by comparing the Raman bands for phenylalanine at 967 cm^{-1} (^{12}C-phenylalanine) and 1003 cm^{-1} (^{13}C-phenylalanine). However, the application of Raman-SIP is not confined to these resonances and other resonances have been used where appropriate, e.g. the red shift of carotinoids (Li et al. 2012).

One disadvantage of Raman-SIP is the low precision of about $\delta^{13}C$ >+20.000 ‰. Although several suggestions have been made for improving the precision (Huang et al. 2010) no ecological applications have been reported up to now. The required high enrichments of more than 25 atom-% lead to long incubations which in turn can spoil the obtained results (Table 1). An example for this is the detection of phenylalanine incorporation into both reticulate and elementary bodies of intracellular *Protochlamydia amoebophila*. While reticulate bodies represent the intracellular life stage and are metabolically active, the elementary bodies form a spore-like stage considered to be metabolically inert. Because of the long incubation time required to get a significant signal (>100 h) it is not possible to conclude whether, contrary to expectations, elementary bodies are also metabolically active or reticulate bodies carrying the labeled phenylalanine switched into elementary bodies during the experiments (Haider et al. 2010).

Other detection methods

The use of ^{13}C-NMR in the analysis of isotopomer patterns for the elucidation of biosynthetic pathways should also be mentioned although few attempts have been made for applications in microbial ecology (Shimizu 2004). The application of multicollector inductively coupled mass spectrometer (ICPMS) has also been reported but this method seems to be currently at the level of pure chemical compounds and it is limited to the natural abundance range (Santamaria-Fernandez et al. 2008).

Discrimination between taxa to answer the question: Who eats what?

For many applications it is sufficient to that a given substrate is degraded and its carbon atoms are incorporated into biomass. However, this is not sufficient for understanding the interactions of the different members of microbial communities. Here, one needs to know which species degrades a

given substrate. To answer this question one has to discern between the individual taxa in the microbial community under study. Several approaches to achieve this goal have been taken and applied to a large diversity of environmental samples.

Biomarkers: Taxa-specific biomolecules

Fatty acids are used for decades to discern between bacterial species, hence, they have been applied as biomarkers to discriminate between microbial taxa in SIP-experiments. Because of their fast degradation in dead cells polar lipid fatty acids (PLFA) are usually used in these experiments (Evershed et al. 2006). In several incorporation studies we compared PLFA with cellular fatty acids and we did not see significant differences in the kinetics of their labeling, however, this may depend on the communities under study. PLFA-SIP has been applied in many studies. To find out whether toluene is degraded in an anoxic sediment under denitrifying conditions and who are the main degraders microcosms were incubated with [methyl-^{13}C]toluene. The most enriched PLFAs were 16:1ω7c, 16:1ω7t, 16:0, cy17:0, and 18:1ω7c and also found in *Azoarcus* spp. and related species. The presence and bloom of *Azoarcus* in the contaminated sediments was further confirmed by FISH (Pelz et al. 2001). A similar approach has been taken by Tillmann et al. who used [U-^{13}C]-2,2'-dichlorobiphenyl to detect degraders of this barely degradable polychlorinated biphenyl congener. Again, not a single PLFA but a characteristic set of fatty acids were labeled which all fit to *Burkholderia* species. Although *Burkholderia* isolates from the contaminated soil were the only one assimilating biphenyl, none of the isolates were able to use 2,2'-dichlorobiphenyl indicating a complex metabolic network in the soil (Tillmann et al. 2005). Labelling of rice roots with $^{13}CO_2$ and analysis of PLFA in the soil revealed that in the rhizosphere mainly Gram-negative and eukaryotic microorganisms assimilated root-derived carbon, whereas Gram-positive microorganisms became relatively more important in the bulk soil (Lu et al. 2007). This is consistent with the results from pulsing annual ryegrass with $^{13}CO_2$ at different time periods and analyzing the incorporation into PLFA revealing the highest labeling of the fungal 18:2ω6,9 PLFA followed with those of Gram-negative bacteria associated with 16:1ω5 PLFA (Butler et al. 2003).

To elucidate the food web of chironomid larvae in sediments it was incubated with ^{13}C-methane and the PLFA of bacteria and larvae analysed. Sediment PLFA-SIP profiles showed the main incorporation of ^{13}C into species of the genus *Methylobacter* but incorporation into type II methane-oxidizing bacteria was negligible. Chironomid larvae mechanically separated from the sediment were also enriched and showed even some PLFA characteristic for *Methylobacter* revealing them as their food source (Deines et al. 2007). From another study an explanation for the low labeling of type II methane-oxidizing bacteria might be found. In this study microcosms supplemented with mineral salts medium in addition to ^{13}C-methane showed on the one hand rapid incorporation of the label into the biomass but a less diverse population and no label in type II methane-oxidizing bacteria (Cebrón et al. 2007). This experiment highlights again the dangers coming from unnaturally high substrate concentrations which, however, are often required for sufficient enrichment of nucleic acids for analyses but carefully selected experimental conditions can avoid this bias (Qiu et al. 2008). In an interesting combination of fatty acid and ether lipids isotope ratios and FISH-SIMS Orphan et al. used the natural ^{13}C-depletion of methane to elucidate the fate of methane in consortia of metabolically interdependent bacteria and archaea. Because the etherlipids showed δ^{13}C of -104‰ and lower, Archaea of the ANME-2 phylogenetic cluster have been identified as the prime consumer of methane, a conclusion also confirmed by FISH-SIMS (Orphan et al. 2001).

Pelz et al. studied a microbial consortium consisting of four bacteria species degrading 4-chloro-salicylate. While only one strain was able to grow on the substrate, the presence of the others allowed this strain tolerance of much higher substrate concentrations. Because SIP-experiments could not use PLFA as biomarkers because the two *Pseudomonas* species in the consortium could not be discerned in this way, the researchers developed a different approach. They separated the cells by

immunocapture using strain specific antibodies. From the separated cells the fatty acids we extracted and their isotope ratios analyzed. The experiments revealed that one species assimilated a toxic metabolite from the 4-chloro-salicylate degradation and protected in this way the community from poisoning (Pelz et al. 1999).

Fatty acids were not the only biomarkers applied in SIP-studies. Instead of fatty acids ether-lipids have also been used as biomarkers enlarging the field of taxa in SIP studies to Archeae (Niemann, Elvert 2008). A rather unusual approach was based the distinct natural $\delta^{13}C$ labeling pattern of amino acids from different taxa. Here it has been shown that the isotopic ratio of amino acids from these taxa could be discriminated using multivariate analyses (Larsen et al. 2013). However, no application in carbon flux studies has been made and it remains to be demonstrated that the determined differences still hold in such applications.

Proteins or peptides can also serve as biomarkers to discern groups of microbes. Exploiting the technical progress made in proteomics a SIP-coupling has been proposed (Jehmlich et al. 2010). Protein-SIP has recently been applied in microbial ecology but despite considerable efforts it could not reach the phylogenetic or metabolic resolutions achievable with DNA- or PLFA-SIP (Tauber et al. 2012).

Instead of fatty acid biomarkers or amino acids ribosomal RNA would be a much better biomarker for coupling metabolic activity to phylogeny. Capture of rRNA independent from their enrichment by biotin-labelled, taxa-specific oligonucleotide probes and streptavidin-coated paramagnetic beads (magneto-FISH) has been reported for 16S rRNA by MacGregor et al. (2002) and optimized by Miyatake et al. (2009). Instead of 16S rRNA Tillmann used ^{13}C-enriched 23S RNA because of its larger size requiring less bacterial cells for the analysis (Tillmann 2004). Magneto-FISH has been applied to purify syntrophic anaerobic methane oxidizing ANME-2c archaea and physically associated microorganisms from deep-sea marine sediment after incubation with $^{15}N_2$ for 6 months. Analyses of ^{15}N-incorporations by FISH-SIMS revealed nitrogen fixation within the aggregates confirming predictions from metagenomic analysis (Pernthaler et al. 2008). Unfortunately, the enrichment of rRNA is much slower than that of fatty acids causing a severe limitation of this method in pulse-labeling experiments but the method is a good alternative for density gradient enrichment.

One of the most important limitations of the use of biomarkers for SIP is their limited specificity. Usually it is not possible to identify a single molecule which is specific for the bacteria under study. Instead, often a set of biomarkers can be identified, e.g. several fatty acids, which are characteristic for the taxon. Some genera are notorious for their lack of unique fatty acids (e.g. *Pseudomonas*) while others are causing much less problems (Evershed et al. 2006). To identify biomarkers for bacteria taxa for which no species have yet been cultivated is challenging and results from cultures grown in the laboratory cannot be blindly transferred to bacteria from environmental samples. Another complication is the need to find biomarkers which can be volatilized intact in order to separate them in a gas chromatograph. Although interfaces coupling HPLC with IRMS are available this approach is burdened by severe solvent limitations.

Isolation of ^{13}C-DNA/RNA by density gradient centrifugation (DNA-, RNA-SIP)

An alternative to using taxa-specific molecules is the analysis of ^{13}C-enriched nucleic acids. The ^{13}C-enriched nucleic acid is separated from the non-enriched one by centrifugation in a density gradient. This is usually done in a caesium chloride/ethidium bromide gradient for visualization of the separated bands of nucleic acids (Radajewski et al. 2000). A detailed protocol also including critical steps and an improved procedure of DNA extraction after density centrifugation (Neufeld et al. 2007) and excellent and critical overviews over DNA-SIP have been published (Dumont, Murrell 2005; Friedrich 2006).

DNA-SIP has the advantage that it allows coupling between phylogenetic identification of substrate degraders with their function. The addition of $^{13}CH_4$ to sediments of a soda lake and analysis of the ^{13}C-enriched DNA identified type I methanotrophs as the main degraders forming 50% of all 16S rRNA and 100% of all *pmoA* clones (Lin et al. 2004). Using DNA-SIP and analyzing their 16S rRNA genes an interesting approach has been taken to elucidate the preference of microbes for two different substrates or the combination of both (Singleton et al. 2007). It turned out that some bacteria degraded the substrates simultaneously or sequentially while others assimilated only one of the substrates.

An unusual application of DNA-SIP has been taken to elucidate in a bacteria-host interaction the production of adenosine after stressing *E. coli* cells with human β-defensin. Adenosine is an immunomodulator and it was shown that some *E. coli* strains produce considerable amounts after being stressed with the antimicrobial peptide human β-defensin. To answer the question whether adenosine is synthesized *de novo* at defensin stress or release from already existing RNA, cells were fed with [U-^{13}C]-glucose before their exposure to defensin and compared to those fed with the same labeled substrate during defensin stress. Analysis of the formed adenosine showed ^{13}C-enriched adenosine only from cells exposed before the defensin stress confirming that already existing but not *de novo* synthesized adenosine was released (Estrela et al. 2013).

Manefield et al. (2002) focused on RNA instead of DNA and isolated ^{13}C-enriched rRNA by density gradient centrifugation from a bioreactor fed with ^{13}C-phenol. It was then analyzed by reverse transcription-PCR and denaturing gradient gel electrophoresis. RNA-SIP has been applied for the elucidation of the fate of $^{13}CO_2$ in rice fields leading to the identification of the still not cultivated Rice Cluster I Archaea as the main consumer and producer of methane (Lu, Conrad 2005). With high ^{13}C-enrichments and optimal ^{13}C-RNA-separation protocols even active community members comprising only a tiny fraction of all cells can be detected. It has been shown that *Desulfosporosinus* species, although constituting only 0.006% of the total community, were the main sulfate reducer in a peatland (Pester et al. 2010). A combination of rRNA- and mRNA-SIP (Pernthaler, Amann 2004) has been applied to unravel the role bacterial species in the degradation of naphthalene-degrading groundwater microbial community. While rRNA was used for the identification of the active degraders, ^{13}C-enriched mRNA confirmed the involvement of naphthalene dioxygenases. Two *Pseudomonas* sp., *P. fluorescens* and *P. putida*, and an *Acidovorax* sp. were identified as main degraders. This was confirmed at the single-cell level by FISH-Raman-SIP. While the two *Pseudomonas* species could be isolated the more active *Acidovorax* species eluded all isolation attempts (Huang et al. 2009).

There are some reports on the limitations of RNA/DNA-SIP. Detection limit is 10^5 to 10^6 cells (Rangel-Castro et al. 2005) and RNA enrichments of 0.1-0.2 atom% were reported being to too low for a meaningful analysis (Griffiths et al. 2004). This is consistent with the notion that density gradient centrifugation requires considerable enrichment of RNA (Friedrich 2006) preventing the detection of slow or low-level incorporation of the substrates (Table 1). Furthermore, no distinct band can be visible after density gradient centrifugation although active degraders might be present. This can be caused by low incorporation rates which are not sufficient for the formation of a distinct ^{13}C-DNA band. Another possibility is that the populations might have been highly active but small, and the low amount of labeled DNA formed might not be visible. The addition of a carrier-^{13}C-DNA (Gallagher et al. 2005) or a highly sensitive protocol involving the fluorometric quantification of the nucleic acid (Lueders et al. 2004) allowed the detection at much lower amounts. However, such an approach cannot solve the problem of low isotope incorporation into DNA causing insufficient separation in the density gradient. When using smaller amounts of ^{13}C one has to make sure that the DNA is indeed ^{13}C-labelled and not simply GC rich which also increases the density of DNA (Cupples et al.

2007). As the noise to signal ratio will be greater contaminations will also become more prominent, a problem intrinsic to all labeling experiments.

As already mentioned for Raman-SIP high incorporation rates in general often require long incorporation time which in turn increases the risk of cross-feeding. The search of actively methane oxidizing bacteria in the Movile cave using ^{13}C-methane identified also Proteobacteria which are not known for methane oxidation (Hutchens et al. 2004). One explanation might be that these bacteria where simply not known for this metabolic activity but more likely is that they have been labeled by cross-feeding on metabolites of ^{13}C-methane or predation of methane-degraders. A similar conclusion has been drawn by Dumon et al. who saw labelling of *Betaproteobacteria* by DNA-SIP but not by RNA-SIP and concluded that cross-feeding was primarily detected by DNA-SIP (Dumon et al. 2011). A simple time series could have helped here to decide between these possibilities because food chains can clearly be discerned by their kinetics of isotope incorporation (Mauclaire et al. 2003).

Comparison PLFA-SIP and DNA-SIP

Feeding anaerobic, sulfate-reducing marine sediments with ^{13}C-glucose at low concentrations (100 µM) resulted in a broad range of PLFA, rapidly labeled within 12 h. The identity of the glucose-using community remained unclear since neither characteristic fatty acids nor differences between ^{12}C- and ^{13}C-16S rRNA were found. Contrary to this widespread incorporation of glucose carbon into the microbial community the fatty acids labeled by ^{13}C-acetate were more characteristic comprising mainly branched uneven-numbered fatty acids. The 16S rRNA gene library contained sequences of *Desulfobacter* sp. but also a broad range of uncultured *Bacteria.* In contrast ^{13}C-DNA contained 16S rRNA genes of the candidate division JS1 and *Firmicutes*. This study demonstrated that PLFA- and DNA-SIP can be used together with low concentrations of ^{13}C-substrate and overlapping incubation times providing complementary information on the identity of active members of prokaryotic communities, however, not all results were identical (Webster et al. 2006).

The complementarity of PFLA- and RNA-SIP has been demonstrated in a study of CO_2 fixation at a pelagic redoxcline. Analysis of the isotope ratios of microbial PLFA along a depth profile revealed a significant enrichment slightly below the redoxcline pointing to high dark CO_2 fixation rates in this layer of up to 37%. RNA-SIP of water samples taken at different depths and amended with ^{13}C-bicarbonate led to the identification of *Sulfurimonas* and *Pseudomonas* species as the main bacteria responsible for dark CO_2 fixation. Analyses of the PLFA of ^{13}C-labelled samples originating from the CO_2 fixation maximum showed the enrichment of the same PLFA also found enriched *in situ*, linking the information derived from ^{13}C natural abundance analysis with the phylogentic identification obtained from RNA-SIP studies (Glaubitz et al. 2009).

Labelling with taxa-specific probes followed by isotope ratio analyses

Fluorescence *in situ* hybridization (FISH) with horseradish peroxidase labeled oligonucleotide probes and tyramide signal amplification, also known as catalyzed reporter deposition FISH (CARD-FISH), has been improved to facilitate enzyme penetration and reduce cell loss (Pernthaler et al. 2002), nevertheless SIMS using CARD-FISH remained problematic. Replacing tyramides by fluoro-tyramides led to halogen *in situ* hybridization coupled to secondary ion mass spectrometry (HISH-SIMS) which allows a simultaneous detection of ^{13}C, ^{15}N and ^{19}F-ions with nano-SIMS. The ^{19}F-signal can then be used for the identification of individual cells for which isotopic ration can then be computed. An interesting result coming from the application of this method was that large differences in isotopic ratios were observed between individual cells of the same species or even cells attached to each other. Such a diversity of isotope ratios obvious reflects differences in metabolic status or it may even reveal different types of metabolism (Musat et al. 2008).

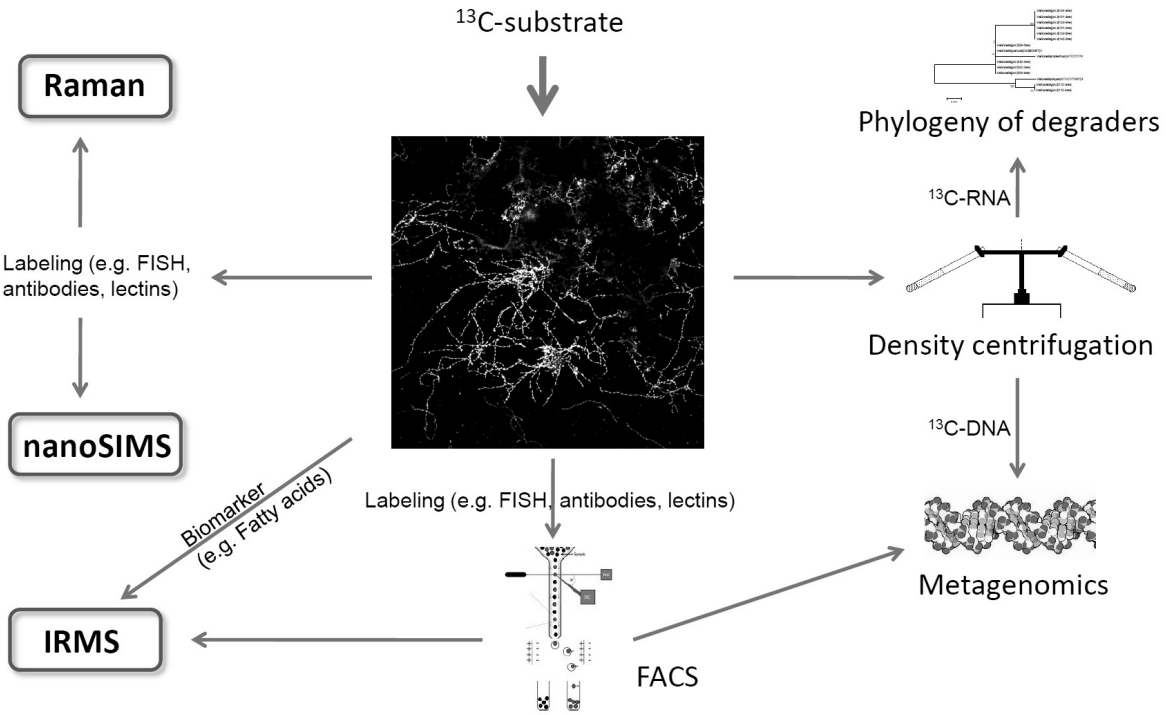

Figure 2: Scheme showing the most frequently applied methods of stable isotope probing. ^{13}C-substrate is added to a microbial community; samples are taken after certain time points and i) ^{13}C-nucleic acids are separated via gradient centrifugation for further analyses, ii) cells are sorted by fluorescent assisted cell sorting (FACS) using either their autofluorescence (e.g. green fluorescent protein (GFP)), FISH-labeling or stained antibodies and their biomolecules are analyzed by IRMS or the isotope labeling of cells is determined by Raman microspectrometry or nanoSIMS after FISH-labeling. Often combinations or these methods are applied in studies of microbial interactions.

Taxa-specific cell sorting followed by IRMS

Instead of the identification of the metabolic active microbes by i) the incorporation of the label into biomarkers, ii) the phylogenetic analysis of enriched nucleic acids or iii) the isotopic analysis of FISH-labeled cells by SIMS or Raman spectroscopy one can also separate the cells independent from their ^{13}C-incorporation and investigate the labeling of the separated cells. Such an approach allows the coupling to IRMS and the analysis of incorporations close to natural abundances, which is several orders lower than the enrichments required for DNA-SIP, Raman-microscopy or SIMS. To achieve this goal the microbial communities are first labeled by taxa-specific probes and the labeled cells are then separated. Specific staining of cells can be achieved by FISH-probes or with strain-specific antibodies and the labeled cells can then be sorted by fluorescence-activated cell sorting (FACS) (Pawelczyk et al. 2011). An alternative to FACS sorting is the application of magnetic beads as has been implemented in Magneto-FISH (Pernthaler et al. 2008). Immobilization of strain-specific antibodies and separation of cells by immunocapture is another alternative to achieve the separation of intact and labeled cells (Pelz et al. 1999).

Cells separated in this way can then be studied by all varieties of genomics, transcriptomics, proteomics, but also by IRMS, provided that sufficient cells can be separated to fulfill the requirements of these methods. For GC-IRMS analysis the limit is currently at 300.000 bacterial cells (Abraham, unpublished), now achievable with modern multicolor, high-speed FACS and highly sensitive IRMS machines. A more severe limitation is the selectivity of available FISH-probes and the specificity of antibodies (Figure 2).

Kinetics of ^{13}C-incorporation reveal microbial interactions

For the elucidation of microbial interactions the techniques described above have to be flexible and fast enough to allow sampling along gradients and at several time points. The data obtained from such analyses allow monitoring of the incorporation of the label over time leading to kinetics of incorporation, flux studies and the identification of scavenging organisms revealing food webs. A nice overview of the application of SIP in studies of plant-microbe interactions has been published (Prosser et al. 2006).

The time course of the enrichment of biomarkers has been applied for carbon flux studies. The abyssal sea floor is characterized by severe limitations of organic carbon which can arrive in pulses of phytodetritus. To assess the fate of such a food pulse ^{13}C-labeled phytodetritus was exposed for 23 days at 4800 m water depth and samples were taken at several time points. From the individual samples macrofauna, nematodes, and Foraminifera were separated from the sediment and their PLFA extracted. Contrary to expectations the fastest labeling occurred in the PLFA of the macrofauna, varying between the different taxa, probably due to the fact that other than bacteria these organisms are able to ingest the labeled biomass directly. Nematodes and Foraminifera showed labeling after 8 days, earlier than the bacteria in the samples. The incorporation of ^{13}C in bacterial PLFA was high after 23 days but confined to the surface layer pointing to low rates of vertical mixing. This experiment demonstrated the role of macrofauna in the material processing and the delayed onset of bacterial degradation (Witte et al. 2006).

Data of isotope enrichments can be applied in models of food webs and carbon flux in a food chain has been quantified using PLFA-biomarkers in a model system where toluene is degraded by a *Pseudomonas* species which is predated by the protozoa *Vahlkampfia* sp. Bacterial fatty acids 16:1ω7c and 18:1ω7c showed enrichment immediately after [U-^{13}C]-toluene addition while the protozoal 20:3ω6c and 20:4ω6c had a lag phase. The model derived from incorporation data and biovolumes showed that 28% of the toluene was incorporated into bacterial biomass and 12% of bacterial carbon appeared in protozoal biomass (Mauclaire et al. 2003).

Species discrimination by biomarker fatty acids is not always possible and alternative approaches are needed. One of them is the staining of cells by strain-specific antibodies followed by sorting of the cells using fluorescence assisted cell sorters (FACS). From the sorted cells PLFA have been extracted and their isotope ratios determined. For the experiments the 4-chlorosalicylate degrading microbial consortium (Pelz et al. 1999), already described above, was used; for labeling the toxic first intermediate of the degradation, [U-^{13}C]-4-chlorocatechol, was added to the consortium. To determine the kinetics of the degradation, samples were taken over several time points. While immunocapture did not allow such a sampling procedure with acceptable efforts FACS-sorting proved to be able to provide 20 million bacterial cells needed for IRMS-analysis. Improvement of both FACS-sorting and IRMS-sensitivity reduce this amount nowadays to about 300.000 – 500.000 cells (Abraham, unpublished). The enrichment curves of fatty acids from two most abundant members of the community showed good fits to pseudo-first order kinetics. While the primary degrader *Pseudomonas reinekei* incorporated the substrate faster than strain *Achromobacter spanius* the maximal incorporation of the latter was almost three times higher than in *P. reinekei*. Because *P. reinekei* has a low LD_{50} for 4-chloro-catechol *A. spanius* protects the entire consortium against this toxin by still degrading the metabolite when its concentrations are already too toxic for *P. reinekei* (Pawelczyk et al. 2011). The approach involving FACS-sorting is not limited to antibody staining but is also possible for FISH-labeled cells or fluorescent (e.g. GFP-labeled) bacteria.

An interesting finding from PLFA-SIP concerning a directed biosynthesis of fatty acids in *Mycobacterium bovis* BCG has been briefly reported. The analyses of fatty acids from lipid fractions after feeding with traces of ^{13}C-acetate revealed the maximum of incorporation after 1.5 days in

saturated fatty acids longer than 18:0 only in the glycolipid fraction. The same fatty acids from the other lipid fractions had their maximum only after 3 days, similar to all other fatty acids. This can be interpreted that the long-chain fatty acids are preferably attached to glycolipids highlighting again the special role glycolipids play for mycobacteria (Abraham and Gutierrez 2012).

Assessment of the kinetics of carbon incorporation is not limited to IRMS-analysis but has also been shown with DNA-SIP but here much higher incorporation levels are required. Rice roots were incubated anaerobically under an atmosphere of $H_2/^{13}CO_2$ or $N_2/^{13}CO_2$ with phosphate or carbonate as buffer medium and the nucleic acids were fractionated in a density gradient at different time points. Different active methanogenic populations were detected on the rice roots changing with H_2 concentrations and the type of buffer used in the system (Lu et al. 2005).

Outline of trends and prospects

What is the best method for the analysis of microbial interactions using stable isotope probing? Unfortunately, there is not a clear answer to this question because all methods have strengths and weaknesses. The answer, therefore, is that the best method is the one fitting best to the specific questions raised and the specific conditions of the system under study. There are, however, some general aspects which should be considered:

- Lowest possible addition of substrate to minimize the disturbance of the microbial community: To determine carbon fluxes one has to add substrate to the system. In order to keep this disturbance as low as possible highly sensitive methods are required for the detection of small incorporations. Furthermore, there have been reports that the isotope fractionation of many enzymes lead to distortions of flux rates of carbon at high ^{13}C-levels causing bias in incorporation kinetics (Fang et al. 2004).

- Fast analysis to allow time series: To follow the flow of carbon in microbial communities several samples at different time points have to be analysed. Preparation of the samples and separation of the labelled compounds and their analyses requires a considerable amount of time and efforts. Fast and highly sensitive instruments help to keep the work load tolerable.

- Sound phylogenetic resolution of the active members of the community: When analysing carbon fluxes in microbial communities discerning between the different taxa is usually essential. Protocols delivering high phylogenetic resolutions are required, e.g. RNA-SIP, FACS-separation of stained cells, etc.

- Affordable equipment: When planning SIP-experiments one should be aware that significant results require sophisticated instruments. Most of these instruments are expensive ranging from 100.000 to 5 Mio. Euro and their operation demands highly skilled and experienced personal.

Over the next years progress concerning the application of novel biomarkers in SIP-studies and a broader use of HPLC-IRMS can be expected. Even with the heavy constrains current HPLC-IRMS systems possess, many more compounds should be accessible for carbon flux studies. The use of the HPLC-IRMS interface has to compete with sophisticated derivatizations of the same compounds making them accessible for GC-IRMS analyses. Independent from the question whether these compounds were separated by HPLC or GC prior to isotope determinations these additional molecules will broaden our knowledge in carbon flux studies of microbial communities.

Further reductions in the amount of compounds required for isotope ratio determination together with faster sorting facilities will not only speed the whole process of the analysis but will also open

entirely new fields for such studies currently inaccessible because of inherent scarcity of sample material. One area which will definitely prosper from this progress will be host-microbes interactions with special focus on host-pathogen interactions.

Many new techniques have been reported but most of them still await their applications in bacteria interaction studies. Optimized combinations of these techniques and their adaptation to the communities under study will give significant new insights and for sure many surprises in microbial interactions bringing us closer to the understanding of the role of currently uncultured bacteria and host-microbe interactions.

Conflict of interest

The author declares that he has no conflict of interest.

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