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Review article

Stable isotope probing approaches to study anaerobic hydrocarbon degradation and degraders

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Abstract

Stable isotope probing (SIP) techniques have become state-of-the-art in microbial ecology over the last ten years, allowing for the targeted detection and identification of organisms, metabolic pathways, and elemental fluxes active in specific processes within complex microbial communities. For studying anaerobic hydrocarbon degrading microbial communities, four stable isotope techniques have been used so far: DNA/RNA-SIP, PFLA-SIP, protein-SIP, and single cell-SIP by nanoSIMS or confocal Raman microscopy. DNA/RNA-SIP techniques are most frequently applied due to their most meaningful phylogenetic resolution. Especially using ^{13}C-labeled benzene and toluene as model substrates, many new hydrocarbon degraders have been identified by SIP, under various electron-acceptor conditions. This has extended the current perspective of the true diversity of anaerobic hydrocarbon degraders relevant in the environment. Syntrophic hydrocarbon degradation was found to be a common mechanism for various electron acceptors. Fundamental concepts and recent advances in SIP will be reflected here. A discussion how these techniques generate direct insights into intrinsic hydrocarbon degrader populations in environmental systems and how useful they are for more integrated approaches in the monitoring of contaminated sites and for bioremediation is given.

Introduction

Hydrocarbons are amongst the most frequently detected organic compounds in the environment due to their occurrence in crude oil and their global use in gasoline or chemical products. In most environments, aerobic catabolism of hydrocarbons is limited because of the low solubility and quick biological consumption of molecular oxygen. The discovery of anaerobic hydrocarbon degradation in various laboratory microcosm experiments (e.g. [Jones et al., 2008; Zengler et al., 1999] and in field-scale studies of biodegraded oil reservoirs [Aitken et al., 2004; Jones et al., 2008] and polluted aquifers [Wiedemeier et al., 1999] supports the hypothesis that anaerobic processes are likely to be responsible for *in situ* hydrocarbon biodegradation in the subsurface. Factors controlling *in situ* biodegradation and the specific microorganisms responsible remain however poorly understood.

Stable isotope probing (SIP) has become a central tool in microbial ecology. Summarized, the method allows detecting or even identifying active organisms in microbial communities by tracing the assimilation of carbon or essential nutrients like nitrogen labelled with stable isotopes. Although several SIP methods have been developed (see below), the general principle is similar: a microbial population or community is spiked with a substrate containing an artificially enriched ratio of heavy stable isotopes (the label), which is assimilated by distinct populations most active in substrate turnover. Eventually, the biomass label incorporation is qualitatively and quantitatively detected by isotope-sensitive analytical instruments. The great potential of SIP is reflected by the fact that the method allows tracking active organisms within extremely different habitats ranging from highly enriched laboratory microcosm to *in situ* applications, as well as from extremely slow- to fast-growing microbes (Figure 1). Besides detection and identification of key organisms, SIP enables to unravel fluxes of essential elements for biomass build-up (e.g., carbon, nitrogen, sulphur and oxygen), as well as substrate sharing or syntrophic relationships in microbial communities. Due to these methodological advantages, SIP turned out to be a key method for investigating hydrocarbon degrading microbial communities. The aim of this review is (i) to summarize studies in which SIP has been used to identify anaerobic hydrocarbon degraders, (ii) to introduce the used SIP

techniques (including recent advances in SIP technology) while discussing their potential to identify degraders of specific hydrocarbons, and finally (iii) discussing if knowledge gained from SIP studies may be used to improve concepts applied for the monitoring of contaminated sites and for bioremediation.

SIP methods

A number of SIP methods have been described which considerably differ in terms of sensitivity, precision and requirements [Abraham, 2014; Murrell and Whiteley, 2011] (Table 1). As assimilated stable isotopes are incorporated into the whole biomass, specific biomolecules ('biomarkers') are used for detecting and/or quantifying the flux of the label into biomass fractions, such as amino acids (AA) [Richnow et al., 2000], phospholipid derived fatty acids (PLFA) [Annweiler et al., 2000; Boschker et al., 1998], desoxyribonucleic acid (DNA) [Radajewski et al., 2000], ribonucleic acid (RNA) [Manefield et al., 2002], or proteins [Jehmlich et al., 2008]. Especially DNA, RNA and proteins are biomarkers of considerable taxonomic value, allowing establishing fundamental links between structure and function within microbial communities. Nano-secondary-ion-mass-spectrometry (nanoSIMS) in combination with *in situ* hybridization methods [Musat et al., 2012] or Raman microspectroscopy [Huang et al., 2007] represent further recent development to extend SIP approaches to the single-cell level.

SIP-techniques are extremely valuable for studying biodegradation in slow growing microbial communities, such as in anaerobic hydrocarbon degradation (Table 1 and 2).

The technical features and possible applications of the different SIP techniques have been extensively reviewed [Abraham, 2014; Evershed et al., 2006; Friedrich, 2006; Lueders, 2015; Musat et al., 2012; Neufeld et al., 2007a; Neufeld et al., 2007b; Radajewski et al., 2003; Seifert et al., 2012; von Bergen et al., 2013; Wagner, 2009; Whiteley et al., 2006] and are also summarized in a book [Murrell and Whiteley, 2011]. The different SIP-technologies can be differentiated by their performance and inherent technological features. A comparison of

technologies is advised for (i) scientists who seek the most suitable method for answering a specific research question, and (ii) for method developers to identify existing gaps and potential fields of development. For microbial ecology, the most relevant aspects for describing the functions and interactions within communities are phylogenetic coverage, sensitivity of isotope detection, and quantification of label incorporation (Figure 2). We consider these as first-order criteria.

PLFA-SIP is very sensitive to track isotope incorporation into cellular lipids, but has the lowest taxonomic resolution compared to the other SIP technologies (Table 1). Most lipids can only be roughly classified for certain groups of Bacteria, Archaea and eukaryotes [Neufeld et al., 2007b]. Protein-SIP has mostly been used for communities of intermediate complexity to date [von Bergen et al., 2013], but even more important than the mere number of species is their distribution of abundances. The detection of peptides by mass spectrometry is directly affected by the complexity of the sample; hence large differences in abundance may result in a failure to detect labeling of low-abundance taxa. The abundance can almost be neglected for DNA/RNA-SIP, where relevant sequences can be amplified after separation in gradient according to the density of DNA/RNA fragments posed by stable isotope labelling. Thus, DNA/RNA-SIP provides by far the best phylogenic resolution, where labeling can be screened for thousands of taxa per gradient, especially when next-generation sequencing of ribosomal genes or metagenomics is used [Aoyagi et al., 2015; Chen and Murrell, 2010]. At least for DNA-SIP, this technique allows not only identifying the main substrate assimilating phylotypes, but also of involved catabolic pathways [Grob et al., 2015; Kim et al., 2014a; Kim et al., 2014b; Pilloni et al., 2011; Winderl et al., 2010]. AA-SIP is sensitive for tracking microbial activity [Feisthauer et al., 2008; Richnow et al., 2000] but provides almost no taxonomic detail, as the composition of AA in biomass is not taxon-specific. Still, AA-SIP could be of great promise in the analysis of metabolic fluxes in defined species and biosynthetic pathways [Heinzle et al., 2008]. Secondary ion mass spectrometry and confocal Raman Microscopy allows SIP on a level of a single cell (see for a review Musat et al. 2012). Confocal Raman Microscopy allows SIP of ^{13}C and ^{15}N at a lateral resolution of about 1 µm however the minimum amount of label

within a cell required for detecting a spectral shift is about 10 atom% [Huang et al., 2007]. Confocal Raman Microscopy has been applied to analyze the assimilation of $^{13}C_{10}$-naphthalene by *Pseudomonas* spp. in groundwater samples (Raman–FISH, [Huang et al., 2004; Huang et al., 2007]). Time of flight secondary SIMS (TOF-SIMS) provide submicron lateral and a depth resolution below 1 nm and can be used for stable isotope probing [Cliff et al., 2002]. However, mass resolution is limited and allows a separation of isotopic species in the range of several atomic percent. The labelling of specific biomarkers is possible and the potential of TOF-SIMS seems to be underexplored.

In contrast, nanoSIMS offers an unique sensitivity to track isotope label and the option of multi-isotope measurements, it only holds a targeted phylogenetic resolution (for a review see Musat et al. (2012)), depending on à-priori probe-selection for the taxa suspected active. NanoSIMS analysis also allows for the quantification of label incorporation at natural abundance and can resolve the incorporation of less than 0.1 atom percent within individual cells and offers opportunities to track the isotope composition at natural abundance (see below) [Musat et al., 2012]. The single-cell capacity of nanoSIMS is a unique feature but the technique is challenging and the limited number of instruments implies a relatively low the accessibility.

The quantification of label incorporation is highly sensitive in PLFA-SIP and AA-SIP, namely in the range of about 0.1 atom percent [Boschker et al., 1998] and modern compound specific isotope mass spectrometry allows analyzing the isotope composition at natural abundance and can resolve an enrichment of 0.01 atom percent enrichment for carbon and nitrogen.

In contrast, protein-SIP requires more substantial label incorporation in the range of ~1 atom percent for carbon and nitrogen [Taubert et al., 2011; Taubert et al., 2013]. In contrast to other SIP approaches, the direct quantification of the ^{13}C incorporation of nucleic acids is challenging. Quantification is achieved by indirect methods based on the separation of “light” and “heavy” nucleic acids by density gradient centrifugation. This determination of labeling via buoyant densities has only a limited resolution, with detection limits mainly depending on

technical gradients fractionation, and usually estimated around ~20 atom percent [Lueders, 2015; Neufeld et al., 2007b].

The criteria of second-order are more technically orientated like sensitivity in terms of biomass (Table 1) or resource related aspects (costs in terms of instruments and maintenance). PLFA-SIP, DNA/RNA-SIP and protein-SIP have matured over the last decade which means that there are established protocols for sample preparation and analysis available [Jehmlich et al., 2010; Lueders, 2015; Neufeld et al., 2007a; Sachsenberg et al., 2015; Whiteley et al., 2007]. Hydrocarbons are a structurally very divers compound class and labelling of specific components for tracer experiments requires synthesis which can be costly and time consuming, especially more complex hydrocarbons such as isoprenoids, steroids, hopanoids, high molecular weight n-alkanes or polyaromatic hydrocarbons.

A further second-order criterion is related to the multitude of measurable isotopes and their simultaneous application. Usually, for the analysis of hydrocarbon degradation, only carbon (and potentially also hydrogen) isotopes are considered. However, due to the slow turnover especially of hydrocarbons of higher molecular weight, incorporation of labelled nutrients, e.g. as ^{15}N-ammonium can also be used as a general tracer of metabolic activity by growth [Krüger et al., 2008]. By nanoSIMS, it is possible to detect the incorporation of several isotopes simultaneously [Jaekel et al., 2013; Musat et al., 2012]. For DNA/RNA- and protein-SIP, ^{13}C, ^{15}N, ^{18}O and ^{2}H have been previously used [Cho et al., 2015; Justice et al., 2014; Schwartz, 2007; Taubert et al., 2013; Woods et al., 2011]. Most SIP experiments published to date have been based on one type of isotope labelling, although parallel labeling with ^{15}N and ^{2}H or ^{18}O and ^{2}H has recently been applied [Justice et al., 2014; Woods et al., 2011]. Woods and colleagues used ^{2}H and ^{18}O-labelled water to identify the growth of aerobic toluene assimilating organism in soil microcosms. In the approach of Justice and colleagues, ^{15}N was added via a specific nutrient, whereas the deuterium was used as a probe for general metabolic activity. A similar strategy was also combined with Raman spectroscopy in another recent study [Berry

et al., 2015]. The utilization of unspecific labelling for normalization of background metabolic activities will be of great importance in future studies.

Method developments in SIP techniques

The limited phylogenetic coverage of nanoSIMS might be overcome in the future by the steadily growing databases on species or phylum specific probes [Cole et al., 2014; Jaziri et al., 2014] or by coupling nanoSIMS with microarrays (Chip-SIP [Mayali et al., 2012]).

For protein-SIP, there is still upside potential with respect to the phylogenic coverage obtained by this method. The steadily increasing sensitivity of mass spectrometers in combination with purification steps of proteins or peptides that normalize the distribution of abundances could probably yield an increase of the detected species up to 500 per samples. This also requires an increased number of correctly annotated sequence databases. Currently, protein-SIP studies of microbial communities need to be accompanied by metagenome sequencing for satisfying identification of proteins; if a high number of (^{13}C-labelled) proteins can be identified, the method allows identifying primary and secondary degraders and their respective metabolic pathways (von Bergen et al., 2013).

Recently, method developments were described for DNA/RNA-SIP: ultrahigh-performance liquid chromatography-tandem mass spectrometry was used to separate all five nucleobases, allowing determining the ^{13}C incorporation with at least 1.5 atom% ^{13}C above natural abundance [Wilhelm et al., 2014]. Furthermore, the method can be run with up to three orders of magnitudes less sample material compared to conventional methods for direct analyses of nucleic acids [Rangel-Castro et al., 2005] and can be used for both DNA- and RNA-based SIP studies. Another method development was described as CHIP-SIP where RNA-SIP, phylogenetic microarrays and nanoSIMS were combined to increase the sensitivity of ^{13}C and ^{15}N incorporation and to determine the phylogenetic identity of the ‘heavy’ cells. The method requires a sample-specific microarray and nanoSIMS equipment [Mayali et al., 2012]. However, so far, no other application was described for CHIP-SIP. Generally, a combination of techniques should be used in order to cover (i) the full number of active species (e.g., by

210 'conventional' DNA/RNA-SIP), (ii) secondly obtain functional metabolic information on key
211 species in the investigated process (e.g., by protein-SIP, nanoSIMS, DNA/RNA-SIP in
212 combination with metagenomics), and (iii) reveal metabolic interdependency by quantitative
213 analysis of the carbon flux (e.g., by protein-SIP, nanoSIMS).

214

215 **SIP studies for studying anaerobic BTEX degradation**

216 BTEX (benzene, toluene, ethylbenzene, xylenes) compounds have been frequently used as
217 model compounds for the study of anaerobic aromatics degradation activities and the
218 organisms mediating these activities are of considerable environmental relevance. Especially
219 anaerobic benzene and toluene degraders have been intensively investigated, which will be
220 summarized in the following.

221 ***Benzene***

222 Benzene is the most persistent BTEX compound under anoxic conditions [Vogt et al., 2011].
223 Only a few isolated pure cultures capable of anaerobic benzene degradation are established
224 for nitrate-reducing [Coates et al., 2001; Kasai et al., 2006] and recently for iron-reducing
225 conditions [Holmes et al., 2011; Zhang et al., 2012b]. Besides those, a fair number of laboratory
226 enrichment cultures has been established under diverse electron-accepting conditions
227 (summarized in [Meckenstock and Mouttaki, 2011; Vogt et al., 2011]), and in some of them,
228 the microbes assimilating ^{13}C during benzene degradation were identified by SIP. The results
229 of DNA or RNA-SIP studies suggest a broad phylogenetic diversity of organisms involved in
230 anaerobic benzene degradation: *Betaproteobacteria* assimilated carbon from benzene under
231 nitrate- [Kasai et al., 2006; van der Zaan et al., 2012] or sulfate-reducing conditions [Liou et
232 al., 2008], *Deltaproteobacteria* under sulfate-reducing [Oka et al., 2008] or methanogenic
233 conditions [Noguchi et al., 2014; Sakai et al., 2009], same as *Actinobacteria* [Noguchi et al.,
234 2014], *Alphaproteobacteria* and *Peptococcaceae* under nitrate-reducing, iron-reducing or
235 sulfate-reducing conditions [Herrmann et al., 2010; Kunapuli et al., 2007; Liou et al., 2008; van

236 der Zaan et al., 2012]. Syntrophic benzene degradation was postulated in cultures dominated
237 by *Peptococcaceae* due to their typical fermenting lifestyle and the labelling of more than one
238 phylotype in those enrichments [Herrmann et al., 2010; Kunapuli et al., 2007; van der Zaan et
239 al., 2012]. Syntrophic degradation of benzene by *Peptococcaceae* and *Betaproteobacteria*
240 under nitrate-reducing conditions was recently also reported in a metatranscriptomic study
241 [Luo et al., 2014].

242 Apart from these studies under controlled laboratory conditions, a DNA-SIP experiment where
243 ^{13}C-labelled benzene was dosed directly into undisturbed sediments at a coal-tar waste-
244 contaminated field site has been reported. Here, phylotypes belonging to a wide diversity of
245 taxa within the *Alpha-*, *Beta-*, *Delta-* and *Gammaproteobacteria*, as well as the *Bacteroidetes*,
246 were found ^{13}C-labelled (Liou et al., 2008*). In situ*-assimilation of ^{13}C-labelled benzene was
247 also detected by usage of BioSep® beads incubated in groundwater monitoring wells of
248 hydrocarbon-contaminated aquifers and subsequent PLFA-SIP [Geyer et al., 2005; Stelzer et
249 al., 2006]; due to the generally limited phylogenetic value of lipids, an identification of the
250 responsible microorganisms was however not possible.

251 A sulfate-reducing benzene-degrading freshwater enrichment culture was intensively
252 investigated, which was originally enriched in a sand- or lava-filled column system percolated
253 with benzene-containing sulfidic groundwater taken from a contaminated aquifer [Vogt et al.,
254 2007]. A specific feature of this culture is its essential attachment to sand particles [Vogt et al.,
255 2011], hindering on the one hand a specific enrichment of benzene assimilating organisms,
256 but reflecting on the other hand a native anaerobic benzene-degrading microbial community
257 as most microorganisms in aquifers are sessile [Griebler and Lueders, 2009]. In a first study
258 based on clone libraries and terminal restriction fragment length polymorphism (T-RFLP)
259 fingerprinting of PCR-amplified 16S rRNA genes was shown that a phylotype affiliated to the
260 genera *Cryptanaerobacter/Pelotomaculum* (belonging to the family *Peptococcaceae*)
261 increased in different benzene degrading communities after repeated benzene-spiking,
262 indicating a major role in benzene degradation [Kleinsteuber et al., 2008]. Assimilation of

benzene by the *Cryptanaerobacter/Pelotomaculum* phylotype was subsequently confirmed in a DNA-SIP experiment; the organism was also shown to be the most abundant in the consortium [Herrmann et al., 2010]. Notably, DNA of a phylotype belonging to the *Epsilonproteobacteria* was enriched in ^{13}C. Furthermore, phylotypes affiliated to sulfate-reducing *Deltaproteobacteria* were shown to be abundant, but not ^{13}C-labelled. Analysis of produced ^{13}C-CO_2 revealed that 95% of the added ^{13}C-labelled benzene was mineralized, while only a small amount was additionally converted to ^{13}C-labelled methane. Thus, most of the benzene-C was actually not assimilated. Considering the relatively low amount of energy available by mineralizing benzene with sulfate as electron acceptor ($\Delta G' = -185$kJ per mol benzene), it was concluded that the DNA-SIP approach was not sensitive enough to detect benzene assimilation for all members of this complex, putatively growth-limited syntrophic consortium.

For this reason, benzene assimilation by the consortium was further traced by a protein-SIP approach [Taubert et al., 2012]. Apart from optimized protein extraction from mineral sediments [Benndorf et al., 2009], the support of this protein-SIP study by shotgun metagenomics was vital to increase in the number of identified proteins [Taubert et al., 2012]. Proteins showing the highest and fastest ^{13}C-incorporation from ^{13}C-benzene were predominantly affiliated to members of the *Firmicutes* (to which the *Peptococcaceae* belong), confirming the conclusions drawn by the DNA-SIP study that a *Cryptanaerobacter/Pelotomaculum* phylotype is the primary benzene assimilating organism. However, a significant amount of ^{13}C was also detected in proteins belonging to sulfate-reducing *Deltaproteobacteria*, demonstrating for the first time that this group was assimilating C from benzene, too. Indeed, the protein incorporation pattern indicated a feeding on metabolites of primary benzene oxidation rather than directly thriving on benzene [Taubert et al., 2012]. Finally, proteins belonging to taxa within the *Bacteroidetes/Chlorobi* were also ^{13}C-labelled, but to a much lower extent. This suggests a secondary role of this group in the culture, e.g. as scavengers of dead biomass. Notably, labelled proteins belonging to the

290 *Epsilonproteobacteria* could not be detected, which could be due to missing corresponding
291 gene sequences in the respective metagenome.

292 A comparative labelling experiment using ^{13}C-CO_2 and non-labelled benzene as carbon
293 sources further revealed, that the *Cryptanaerobacter/Pelotomaculum* phylotype and the
294 *Deltaproteobacteria* fixed large amounts of CO_2 (up to 50%) during benzene assimilation. In a
295 parallel study, spiked acetate and hydrogen strongly inhibited benzene degradation and
296 mineralization in the consortium, which is in accordance with the assumed syntrophic model
297 [Rakoczy et al., 2011]. In conclusion, the protein-SIP study confirmed the assumption that
298 benzene is syntrophically degraded by the consortium; benzene is primarily attacked and
299 fermented by the *Cryptanaerobacter/Pelotomaculum* phylotype, producing fermentation
300 products – e.g., acetate and hydrogen – which are used by sulfate-reducing
301 *Deltaproteobacteria* and other organisms. Protein-SIP turned out to be an advantageous
302 method for detecting and identifying the microbial interactions and carbon transfer
303 mechanisms in a slow-growing anaerobic hydrocarbon degrading community.

304 ***Toluene***

305 Toluene is – compared to other aromatic hydrocarbons – readily degradable under anoxic
306 conditions and therefore has been used as model compound for studying anaerobic
307 hydrocarbon degradation since many years. Several pure culture isolates capable of anaerobic
308 toluene degradation under various electron acceptor use are available [Weelink et al., 2010],
309 and their degradation pathway initiated by the addition of an activated methyl-group to
310 fumarate catalysed by the benzylsuccinate synthase (Bss) is well understood [Heider, 2007].
311 The gene encoding the alpha subunit of Bss (*bssA*) is used as a specific catabolic marker,
312 capable of detecting a wide diversity of anaerobic toluene degraders in anoxic terrestrial and
313 marine environments [von Netzer et al., 2013]. Recent developments are summarized in
314 another publication of this thematic issue [von Netzer et al., submitted]. As we will summarize
315 here, SIP has been used as an effective tool for identifying active anaerobic toluene degraders
316 in distinct microbial communities. Additionally, methodological developments in SIP have been

317 tested using toluene as (anaerobic) model substrate. For example, the toluene-degrading
318 nitrate reducer *Aromatoleum aromaticum* EbN1 has been applied as genome-sequenced
319 [Rabus et al., 2005] model organisms in a proof-of-principle study for introducing protein-SIP
320 as a tool to identify distinct hydrocarbon-degraders and their functions in complex microbial
321 communities [Jehmlich et al., 2008].

322 *Identification of anaerobic toluene degraders under sulfate-reducing conditions*

323 A considerable number of studies focus on the identification of toluene degraders under
324 sulfate-reducing conditions. The microbial community within a groundwater monitoring well of
325 a BTEX contaminated aquifer was first investigated by PLFA-SIP using toluene-amended *in*
326 *situ* microcosms (Bactraps) and then RNA-SIP in subsequent laboratory incubations. One
327 phylotype related to the *Desulfobulbaceae* (*Deltaproteobacteria*) was found to be the main
328 organism assimilating C from toluene [Bombach et al., 2010]. Similar phylotypes were also
329 detected as main toluene consumers by DNA-SIP in sulfate-amended laboratory incubations
330 of sediment samples directly taken from a tar-oil contaminated aquifer [Pilloni et al., 2011]. In
331 this study, the sensitivity of the DNA-SIP approach was improved by barcoded amplicon
332 pyrosequencing of bulk DNA extracts. *Desulfobulbaceae*-affiliated phylotypes were also
333 identified by DNA-SIP as dominant toluene degraders in sulfate-reducing enrichment cultures
334 obtained from oil sands tailing ponds [Abu Laban et al., 2015] and from sediments of a former
335 gas compressor site [Sun and Cupples, 2012]. This suggests a central role of this lineage for
336 toluene degradation under sulfate-reducing conditions in freshwater environments. Also within
337 the *Deltaproteobacteria*, a phylotype related to the *Syntrophobacteraceae* was recently shown
338 to dominate toluene degradation in sulfate-amended digester sludge microcosms as detected
339 by DNA-SIP [Sun and Cupples, 2012].

340 A second major taxon involved in sulfate-driven toluene metabolization identified by DNA-SIP
341 was again within the Gram-positive *Peptococcaceae*. Phylotypes affiliated to
342 *Desulfosporosinus* assimilated toluene under sulfate-reducing conditions in laboratory
343 microcosms containing tar-oil contaminated sediment [Pilloni et al., 2011; Winderl et al., 2010]

or agricultural soil [Sun and Cupples, 2012]. Notably, similar phylotypes assimilated ^{13}C from labelled toluene also under methanogenic conditions [Abu Laban et al., 2015; Fowler et al., 2014; Sun et al., 2014], indicating a functional versatility of these taxa being capable of toluene degradation under both sulfate reduction or fermentation. Such functional versatility has been also assumed for anaerobic benzene degrading *Peptococcaceae* as discussed above [Vogt et al., 2011].

Identification of anaerobic toluene degraders under nitrate- or iron-reducing conditions

A number of *Betaproteobacteria* were identified by DNA-SIP to metabolize toluene while respiring nitrate. This supports the assumption that *Betaproteobacteria* are important aromatics degrader under nitrate-reducing conditions, as already suggested by the isolation of such BTEX-degrading denitrifying *Betaproteobacteria* [Weelink et al., 2010]. In nitrate-amended microcosms prepared from agricultural soil, new phylotypes within the *Comamonadaceae* were shown capable of assimilating C from toluene [Sun and Cupples, 2012]. In contrast, well-known hydrocarbon degraders affiliated to the genus *Thauera* were identified as denitrifying toluene degraders in parallel microcosms amended with granular sludge [Sun and Cupples, 2012]. Coal tar waste-contaminated sediments have been subjected to a DNA-SIP / metagenomics approach under nitrate-reducing conditions using ring ^{13}C-labelled toluene [Kim et al., 2014b]. A *Herminiimonas* (*Burkholderiales*, *Betaproteobacteria*) phylotype was identified as key toluene degrader in heavy DNA, and its catabolic pathways for toluene and other aromatics degradation was successfully reconstructed from the metagenome. By the same methodological approach, a phylotype affiliated to *Desulfuromonas* was detected as toluene metabolizing organism in crude oil contaminated flat tidal sediments incubated in laboratory microcosms under iron-reducing conditions; degradation pathways for several aromatic hydrocarbons could also be reconstructed from the metagenome [Kim et al., 2014a]. In an earlier SIP study using comparative electron acceptor amendment, taxa within the *Rhodocyclaceae* (related to *Georgfuchsia* spp.) and the *Peptococcaceae* (*Thermincola* spp.) were identified as potential toluene-degraders under ferrihydrite amendment, excluding

371 a respective catabolic function for the abundant *Geobacter* spp. observed *in situ* [Pilloni et al.,
372 2011].

373 *Identification of toluene degraders under methanogenic conditions*

374 Only a few cultures mineralizing toluene to methane and carbon dioxide have been described
375 to date (summarized by Sun et al., 2014). Three of them were recently investigated by DNA-
376 or RNA-SIP. In methanogenic microcosms of an agricultural soil, DNA of a phylotype affiliated
377 to *Desulfosporosinus* (*Peptococcaceae*) was enriched in heavy DNA, indicating its central role
378 in methanogenic toluene degradation [Sun et al., 2014]. A similar phylotype became dominant
379 - together with archaeal *Methanosaeta* - in heavy DNA of methanogenic microcosms enriched
380 from oil sand tailings ponds [Abu Laban et al., 2015]. A highly enriched methanogenic toluene-
381 degrading culture derived from a gas-condensate contaminated aquifer was examined by
382 RNA-SIP [Fowler et al., 2014], where also a phylotype related to *Desulfosporosinus* was
383 identified as key toluene-assimilating organism. Besides the dominant *Desulfosporosinus*,
384 phylotypes affiliated to several other taxa (*Acidobacteria*, *Actinobacteria*, *Syntrophaceae*,
385 *Desulfovibrionales*, *Chloroflexi*) were also shown to assimilate ^{13}C from labelled toluene to a
386 lesser extent [Fowler et al., 2014; Sun et al., 2014]. This supports the concept of complex
387 syntrophic relationships ongoing in methanogenic BTEX degradation.

388

389 ***Xylene isomers***

390 A few pure cultures have been reported to grow anaerobically with *m*-xylene or *o*-xylene using
391 nitrate, iron(III) or sulfate as electron acceptor [Weelink et al., 2010]. In the cultures tested so
392 far, xylene is also activated via fumarate addition by Bss. The key players and enzymatic
393 disposition of an *m*-xylene-degrading sulfate-reducing culture enriched from BTEX-
394 contaminated groundwater have been elucidated by DNA-SIP and protein-SIP using partially
395 ^{13}C-labelled *m*-xylene [Bozinovski et al., 2012; Herrmann et al., 2009]. These studies showed
396 that a phylotype affiliated to the *Desulfobacteriaceae* was the main *m*-xylene assimilating

organism due to the dominance of its 16S rRNA gene in heavy DNA, and the high percentage of identified ^{13}C-labelled proteins affiliated related to this family. Nevertheless, only a limited number of proteins could be identified due to missing protein and/or genomic entries in public databases, hampering the detection of complete degradation pathways and possible functions of further observed phylotypes (e.g. *Epsilonproteobacteria*, *Deltaproteobacteria*, *Bacteroidetes* and *Treponema*) in this consortium. Thus, a metagenome of the culture was sequenced which significantly improved the identification of (^{13}C-labelled) proteins upon degradation of ^{13}C-*m*-xylene [Bozinovski et al., 2014]. The upper pathway for *m*-xylene transformation to 3-methylbenzoyl-Coenzyme A was fully elucidated for the *Desulfobacteriaceae* phylotype, including fumarate addition as initial enzymatic step. Also enzymes of the lower pathway starting from 3-methylbenzoyl-CoA leading to ring reduction and cleavage resulting in 4-methyl-glutaryl-CoA was almost completely identified. The expressed genes showed similarities to genes of methylnaphthalene degrading species like NaphS2 and N47. Thus, a benefit of this study was an increase of the number of reference sequences of genes for alkylated aromatics degradation by a metagenome. Unfortunately, these protein-SIP studies provided no deeper insight into the role of other bacterial groups within the community, like the *Epsilonproteobacteria*, which are probably involved in secondary metabolism. Besides *m*-xylene, also other xylene isomers (*o*-xylene, *p*-xylene) and ethylbenzene belong to the BTEX compounds often found as contaminants in the environment. To the best of our knowledge, no SIP study was performed with anaerobic cultures or oxygen-limited natural sites where these aromatics are present and actively degraded. For the time being, Herrmann et al. (2009b) showed different characteristics (*bssA* sequences and isotope fractionation factors) for cultures degrading different xylene isomers.

N-alkane degradation

Short and long-chain alkanes can be completely degraded to CO_2 under sulfate-reducing and methanogenic conditions [Kniemeyer et al., 2007; Zengler et al., 1999]. This mineralisation was proven for the first time using ^{13}C-labelled n-hexadecane [Zengler et al., 1999] and

transformation into lipids of the same enrichment culture were demonstrated by PLFA-SIP [Feisthauer et al., 2010]. So far, only limited information about key organisms and biochemical pathways are available and only a few pure cultures degrading *n*-alkanes have be described [Callaghan et al., 2012; Davidova et al., 2006; Widdel and Grundmann, 2010]. In recent years, SIP has been used to study the microbial communities involved in the degradation of hexadecane under methanogenic conditions [Cheng et al., 2013; Morris et al., 2012] as well as under sulphate-reducing conditions [Kleindienst et al., 2014]. Cheng et al. (2013) studied a consortium originating from an oilfield and incubated for 4 years at 35°C, by DNA-SIP with ^{13}C-hexadecane. Two dominant phylotypes belonging to the *Syntrophaceae* (closest relative *Smithella propionica*) and *Methanoculleus receptaculi* were identified in heavy DNA.

Morris et al. (2012) used protein-SIP with ^{13}C-labelled hexadecane and ^{13}C-labelled fatty acids (palmitate, stearate) to describe the active microbial fraction influenced by the presence and absence of residual oil. Unfortunately, hexadecane incubations were not evaluated by protein-SIP, as these samples yielded insufficient amounts of extracted proteins. In all other incubations, hydrogenotrophic and aceticlastic methanogens were labelled to an equal extend (58-77% ^{13}C). Here, direct *vs.* indirect ^{13}C-utilization was shown for different community members by the analysis of the peptide´s mass spectra. In addition, also in this study labelling of *Syntrophaceae* was observed, as ^{13}C-labelled proteins affiliated to *Synthrophus* sp. (74% ^{13}C) were identified in the incubations with palmitate and residual oils.

A very comprehensive SIP study was recently performed to dissect alkane degradation in marine sediments [Kleindienst et al., 2014]. Two different marine sediment samples were incubated with ^{13}C-butane and ^{13}C-dodecane, were after DNA-, rRNA-, and protein-SIP as well as CARD-FISH were applied to identify active microbial community members. A dominance of different *Desulfobacteraceae* (*Desulfosarcina*/*Desulfococcus* clade) was found in heavy nucleic acids fractions. Protein-SIP identified proteins belonging to alkane degradation, beta-oxidation and the reverse Wood-Ljungdahl pathway upon including the draft genome sequence of *Desulfosarcina* sp. BuS5 in the search database. Similar phylotypes has also been identified

as key propane or butane degraders in marine enrichment cultures in a related study [Jaekel et al., 2013]. Here, primary degraders were identified and carbon assimilation rates were determined by combining halogen *in situ* hybridization and nanoSIMS analyses.

Polycyclic aromatic hydrocarbon degradation

Polycyclic aromatic hydrocarbons (PAHs) are very slowly mineralized under anaerobic conditions due to their non-polar nature resulting in limited bioavailability. Most studies regarding the microbiology of anaerobic PAH degradation are referring to naphthalene as model substrate, the simplest and best water-soluble PAH (reviewed by [Meckenstock and Mouttaki, 2011]). Owing to the low growth rates of anaerobic PAH degraders, identifying primary PAH degraders in complex consortia by SIP is difficult to perform as long incubation times generally increase the risk of labelling secondary degraders. Thus, only one study has been published so far where SIP was used for the characterization of anaerobic PAH degraders. Zhang and colleagues identified three phylotypes affiliated to different genera of the *Proteobacteria* for assimilating anthracene under methanogenic conditions in microcosms prepared with aquifer sediment taken from a landfill leachate-contaminated site [Zhang et al., 2012a]. Recently, naphthalene mineralization by several sulfate-reducing enrichment cultures gained from groundwater or sediment samples of different PAH-contaminated terrestrial sites was shown by monitoring the production of ^{13}C-labelled carbon dioxide from ^{13}C-labelled naphthalene added as substrate [Kümmel et al., 2015]. However, the putative primary naphthalene degraders in most of the cultures were affiliated to strain N47 within the *Desulfobacteriaceae*, a known sulfate-reducing naphthalene degrader [Meckenstock and Mouttaki, 2011], which were identified by combining 'classical' 16S rRNA gene sequencing and metaproteome analyses without SIP.

In addition, some work has been done to prove anaerobic naphthalene assimilation directly in groundwater monitoring wells or sediments of PAH contaminated sites by using *in situ* microcosms spiked with ^{13}C-labelled naphthalene. While this approach was successfully applied to identify key aerobic naphthalene assimilating phylotypes at an oxic PAH-

contaminated aquifer [Herbst et al., 2013], attempts to demonstrate assimilation of naphthalene-C under strictly anoxic conditions have not been successful yet, probably due to the very limited growth of respective microbes.

Characterization of anaerobic hydrocarbon degraders by SIP – chances, limits, and environmental implications

The long list of respective studies summarized in Table 2 demonstrates that SIP is a powerful tool to detect and characterize anaerobic hydrocarbon degraders in a wide range of settings. In most studies published so far anaerobic hydrocarbon degrading microbial communities were characterized by DNA- or rRNA-SIP, likely due to the comparably “low-tech” approach and much earlier establishment compared to other SIP techniques, as well as the excellent and undirected taxonomic precision. As these technologies have now clearly “come of age”, it seems timely to reflect on the more conceptual implications of respective result on the understanding of anaerobic hydrocarbon degradation in the environment.

Hydrocarbon degrading communities - from specific consortia to in situ conditions

Many SIP studies of anaerobic hydrocarbon degradation have been performed in laboratory microcosms under controlled redox conditions, using previously established enrichment cultures or specific consortia, or by incubating complex environmental samples (Table 2). Thus, microbes assimilating C from a wide range of aliphatic and aromatic hydrocarbons have been characterized. As a rule of thumb, the higher the molecular mass of the hydrocarbon, the less bioavailable it is and thus the slower the growth of respective degraders under anoxic conditions. This has led to considerable incubation times, facilitating the incorporation of label by mutualistic or commensalistic secondary organisms, which are not directly involved in the primary attack and degradation of the substrate. Here, compared to classical DNA- or rRNA-SIP, more recent and more sensitive SIP methods such as protein-SIP or nanoSIMS may actually be essential for further elucidating the functioning of such extremely slow growing anaerobic hydrocarbon degrader communities.

Only a small number of anaerobic hydrocarbon-related SIP studies to date have actually been performed *in situ* (Table 1). In complex environmental settings, it will always be more difficult to directly relate the assimilation of ^{13}C from labeled hydrocarbons to a specific respiratory process. Small amounts of oxygen potentially intruding the investigated compartments may strongly influence such *in situ* experiments, as most hydrocarbons are readily attacked by the ubiquitous mono- or dioxygenases of aerobic catabolic pathways [Head et al., 2006; Leahy and Colwell, 1990].

Syntrophic and commensalistic relationships

Microbial sharing of substrates is now recognized to play a key role in anaerobic hydrocarbon degradation [Kleinsteuber et al., 2012; Weelink et al., 2010], not only under methanogenic conditions as classically understood [Foght, 2008; Heider et al., 1999]. In SIP, an involvement of syntrophy was initially suggested for distinct iron-reducing [Kunapuli et al., 2007] as well as sulphate-reducing, benzene-degrading enrichments [Herrmann et al., 2010; Taubert et al., 2012]. As mentioned above, both cultures were dominated by degraders within the *Peptococcaceae*. The exchange of molecular hydrogen or electrons between the primary benzene-oxidizing *Peptococcaceae* and syntrophic *Deltaproteobacteria* has been suggested to occur in both systems. More recently, van der Zaan and colleagues (2012) reported syntrophic benzene degradation by primary degraders within the *Peptococcaceae* even for a denitrifying consortium (not based on SIP results). The cultures would readily switch to the use of ferric iron and sulphate as alternative electron acceptors, suggesting that the same primary degraders could interact with distinct respiratory guilds within the consortium.

Also some secondary phylotypes have been described to be notoriously present in specific hydrocarbon degrading anaerobic enrichment cultures, although probably not being involved in the primary syntrophic degradation. For example, *Spirochaetes* have been regularly observed in sulfate reducing naphthalene degrading enrichment cultures [Kümmel et al., 2015; Selesi et al., 2010], and phylotypes affiliated to the *Epsilonproteobacteria* were detected in several BTEX-degrading sulfate reducing enrichment cultures [Bozinovski et al., 2012;

Bozinovski et al., 2014; Herrmann et al., 2010; Pilloni et al., 2011]. It is currently unclear whether these relationships also exist under *in situ* conditions or whether they are supported by special conditions in the microcosms ('cultivation artefacts'). However, commensalistic or mutualistic relationships in microbial communities are generally poorly understood but expected to be of marked ecological relevance [Morris et al., 2013]. Here, SIP offers unmatched opportunities for studying such interactions.

Screening for new functions by SIP

Data obtained by sequencing the metagenome of heavy DNA fractions in SIP or analyzing associated metaproteomes can be vital to screen for new functions and physiological interactions [Grob et al., 2015]. Recent progress in bioinformatics allows the coupling of these data to reconstruct microbial community networks as demonstrated for an aerobic naphthalene degrading community [Tobalina et al., 2015]. Here, specific degradation pathways in two enrichment cultures were traced, for which differential pathway organizations had been hypothesized. Bioinformatics modelling based on experimental data from SIP will become more important in future studies and will help to better understand microbial community interactions like syntrophy in anaerobic hydrocarbon degradation. Another strategy to identify novel catabolic gene clusters was introduced by Wang et al. (2012). They used DNA-SIP to access active microbial fractions of an aerobic ^{13}C-labelled naphthalene-degrading culture, using this for metagenomics and a biosensor-based genetic transducer (BGT) technique (named SMB toolbox) [Wang et al., 2012]. The adaption of this method to anaerobic degradation pathways and degraders may be a valuable tool for advanced bio-resource mining.

Environmental implications – benefits of SIP for bioremediation

As summarized above, SIP has generated considerable advances into the diversity and ecology of anaerobic hydrocarbon-oxidizing microbes in the environment. It is worthwhile to consider how such advances can become evident on the levels of monitored natural attenuation or bioremediation [Madsen, 2006; Manefield et al., 2004; Uhlik et al., 2013]. The

identification of dominating degrader populations at a given site is a prerequisite for the development of targeted molecular quantification assays, based either on ribosomal or catabolic marker genes. Although not only for anaerobic degraders, SIP has indeed allowed for the identification and affiliation of novel catabolic genes relevant in biodegradation at contaminated sites [Jeon et al., 2003; Leigh et al., 2007; Pilloni et al., 2011; Wang et al., 2012] and even for the heterologous expression and functional characterization of an aerobic PAH degradation gene previously identified in SIP [Singleton et al., 2012]. Also for the design of site-specific bioremediation strategies, the prior identification, localisation, and quantification of intrinsic degraders can guide decision-making. SIP-based insights can support targeted amendments of electron acceptors or nutrients, or even bioaugmentation. For examples, the discovery of a novel benzene-degrading denitrifier in SIP [Kasai et al., 2006] has motivated the evaluation of its applicability in bioaugmentation [Kasai et al., 2007]. Finally, understanding the reactivity of intrinsic degraders to biostimulation has also been facilitated by SIP [Singleton et al., 2013]. In essence, SIP is capable of providing direct knowledge on intrinsic hydrocarbon degrader populations in diverse environmental systems. This is an important step forward towards more integrated concepts in contaminated site monitoring and bioremediation.

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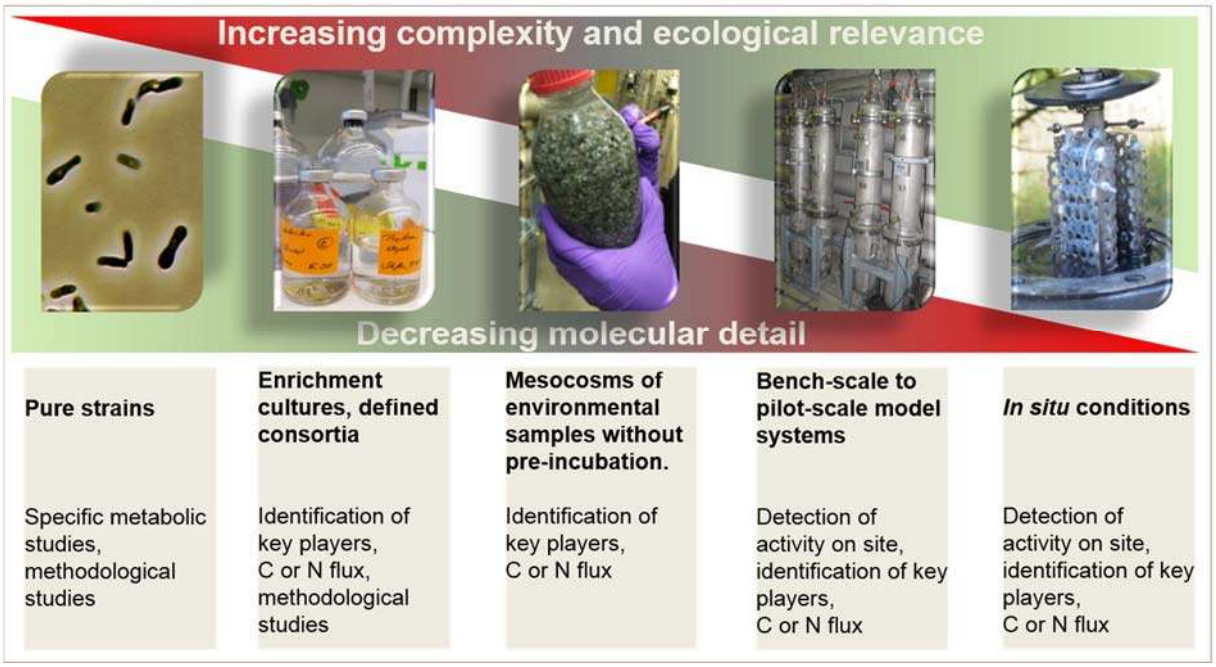

Figure 1 Differences in complexities of SIP studies and their characteristic features.

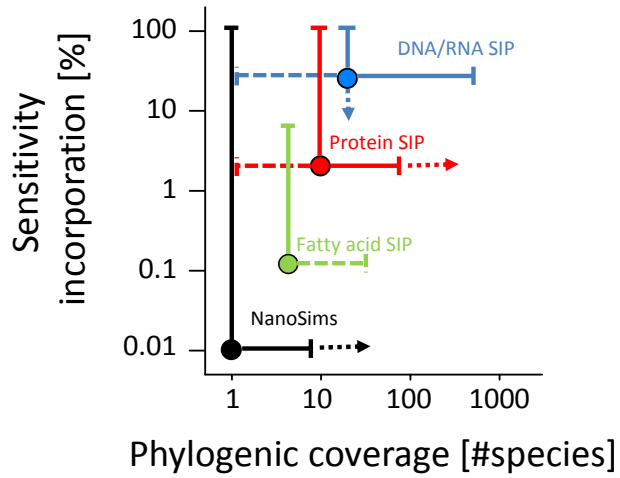

Figure 2 Inherent features of different stable isotope approaches. The parameters of first order (sensitivity of incorporation vs phylogenic coverage) are shown. Filled circles represent the the position for which the approaches has been used so far and the error bars with solid lines the ranges for which the method is also useful. The error bars with dashed lines show potential application and the arrows indicate actual developments in the respective approaches.

Table 1: SIP techniques used for characterizing anaerobic hydrocarbon degrading microbial communities

SIP technique	Minimal cell numbers	Detection limit for stable isotope incorporation (^{13}C)	Phylogenetic coverage	Suitability for studying microbial communities of different complexity
DNA/RNA-SIP	~ 500 ng nucleic acids	20 at.%[1]	High (target molecule: 16S rDNA or 16S rRNA)	Pure cultures, specific consortia, undefined enrichment cultures, environmental samples, in situ
PFLA-SIP	> 10^6 [2]	< 0.1 at.%[2]	Low (target molecules: lipids)	Pure cultures, specific consortia, undefined enrichment cultures, environmental samples, in situ
Protein-SIP	> 10^5 [2]	1 at.%[3]	Intermediate (target molecules: proteins oft the whole proteome)	Pure cultures, specific consortia, undefined enrichment cultures, environmental samples, in situ
Nano-SIMS	1	0.1 at.%[4]	Intermediate (target molecule: 16S rRNA)	Pure cultures, specific consortia, undefined enriched cultures

[1] Neufeld et al., 2007b, Lueders, 2015; [2] Jehmlich et al. 2010; [3] Taubert et al. 2011, 2013; [4] Abraham, 2014

Table 2: Overview of hydrocarbon degradation studies based on stable isotope probing

	Dominant electron acceptor process	Sample type	Target bio-molecule	Target gene	Detection method	Reference
Specific consortia, enrichment cultures						
^{13}C-benzene	Iron reduction	Soil	DNA	16S rRNA gene	T-RFLP	[Kunapuli et al., 2007]
	Sulfate reduction	Marine sediment	DNA	16S rRNA gene	T-RFLP	[Oka et al., 2008]
	Methanogenesis	Soil	DNA	16S rRNA gene	DGGE	[Sakai et al., 2009]
	Methanogenesis	River sediment	DNA	16S rRNA gene	T-RFLP, pyroseq.	[Noguchi et al., 2014]
^{13}C-toluene	Nitrate reducing	Pure culture + enrichment from lake sediment	Protein	Whole genome	MALDI-MS/MS	[Jehmlich et al., 2008]
	Methanogenesis	Aquifer	RNA	16S rRNA gene, *bssA*	DGGE, sequencing, qRT-PCR	[Fowler et al., 2014]
	Methanogenesis	Soil, waste water treatment	DNA	16S rRNA gene, *bssA, bamA*	T-RFLP, sequencing, qPCR	[Sun et al., 2014]
	Methanogenesis, Sulfate reduction	Oil sand tailing ponds	DNA	16S rRNA gene, *bssA, dsrB*	T-RFLP, clone libraries, sequencing	[Abu Laban et al., 2015]
	Sulfate reduction	Groundwater	RNA	16S rRNA gene	T-RFLP	[Bombach et al., 2010]
^{13}C-*m*-xylene	Sulfate reduction	Groundwater	Protein	Whole metagenome	LC-MS/MS	[Bozinovski et al., 2012; Bozinovski et al., 2014]
^{13}C-palmitate, ^{13}C-stearate, ^{13}C-hexadecane	Methanogenesis	Oil field	Protein	Whole genome	LC-MS/MS	[Morris et al., 2012]
^{13}C-hexadecane	Methanogenesis	Oil field	DNA	16S rRNA gene	T-RFLP, cloning, sequencing	[Cheng et al., 2013]
^{13}C-propane, ^{13}C-butane	Sulfate reduction	Marine sediments	RNA	16S rRNA gene	nanoSIMS, FISH	[Jaekel et al., 2013]
Microcosms						
^{13}C-benzene	Nitrate reduction	Groundwater	RNA	16S rRNA gene	DGGE	[Kasai et al., 2006]
	Nitrate; sulfate; methanogenesis; aerobic respiration	Soil slurries	DNA	16S rRNA gene	T-RFLP	[Liou et al., 2008]
	Sulfate reduction	Sand from a percolation column exposed in a	DNA	16S rRNA gene	T-RFLP	[Herrmann et al., 2010]

		BTEX-contaminated aquifer				
	Nitrate reduction	Soil	DNA	16S rRNA gene	DGGE	[van der Zaan et al., 2012]
	Sulfate reduction	Sand from a percolation column exposed in a BTEX-contaminated aquifer	Protein	Whole metagenome	LC-MS/MS	[Taubert et al., 2012]
^{13}C-toluene	Sulfate reduction	Aquifer	DNA	16S rRNA gene, *bssA*	T-RFLP	[Winderl et al., 2010]
	Sulfate reduction; iron reduction	Aquifer	DNA	16S rRNA gene, *bssA*	T-RFLP, pyro-SIP	[Pilloni et al., 2011]
	Sulfate reduction; nitrate reduction	Soil, sediment, sludge	DNA	16S rRNA gene, *bssA*	T-RFLP	[Sun and Cupples, 2012]
	Nitrate reduction	Sediment	DNA	16S rRNA gene; Whole metagenome	T-RFLP, pyrosequencing	[Kasai et al., 2006]
	Iron-reduction	Tidal flats	DNA	16S rRNA gene; Whole metagenome	T-RFLP, pyrosequencing	[Kim et al., 2014a]
	Nitrate reduction	Sediment	DNA	16S rRNA gene; Whole metagenome	T-RFLP, pyrosequencing	[Kim et al., 2014b]
^{13}C-anthracene	Methanogenesis	Aquifer	DNA	16S rRNA gene, *bssA*	T-RFLP	[Zhang et al., 2012a]
^{13}C-butane / ^{13}C-dodecane	Sulfate reduction	Marine sediments	DNA, RNA, protein	16S rRNA gene, whole genome	T-RFLP, LC-MS/MS	[Kleindienst et al., 2014]
In situ						
^{13}C-benzene	Not determined	Aquifer, in situ-microcosms	PLFA	-	-	[Geyer et al., 2005]
	Not determined	Aquifer, in situ-microcosms	PLFA	-	-	[Stelzer et al., 2006]
	Not determined	Sediment	DNA	16S rRNA gene	T-RFLP, cloning, sequencing	[Liou et al., 2008]