Protein-based stable isotope probing (Protein-SIP) for simultaneous identification of bacterial species and determination of metabolic activity

Jana Seifert[1]*, Nico Jehmlich[1,2], Frank Schmidt[1,2], Martin Taubert[1], Martin von Bergen[1], Hans-Hermann Richnow[3] and Carsten Vogt[3]

[1]Helmholtz – Centre for Environmental Research - UFZ, Department of Proteomics, Leipzig, Germany, [2]Interfaculty Institute for Genetics and Functional Genomics, University of Greifswald, Germany, [3]Helmholtz Centre for Environmental Research - UFZ, Department of Isotope Biogeochemistry, Leipzig, Germany

e-mail: jana.seifert@ufz.de, martin.vonbergen@ufz.de

Introduction

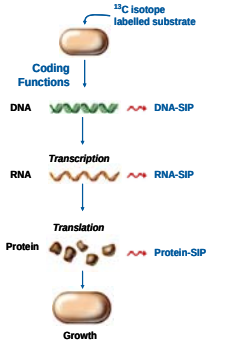


Fig. 1 Biochemistry of synthesis in the cell. (modified Pearson Prentice Hall, Inc. 2006)

In order to detect functional relationships within a microbial community, recently stable isotope probing (SIP) of proteins was developed. Metabolically active species assimilate the labeled substrate by synthesizing biopolymers like DNA, RNA and proteins and thereby incorporate stable isotopes (^{13}C or ^{15}N) into their biomass (Fig. 1)**. In contrast to nucleic acid targets, proteins give direct links between physiology and taxonomy, and low numbers of heavy isotope incorporation (~2%) can be determined.**

By applying matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) it is possible to analyze incorporation of stable isotopes into proteins by comparison of species-specific peak pattern with patterns obtained after growth on isotopically labeled carbon or nitrogen substrates. Two different 'shotgun proteomic' methods known as intact protein profiling (IPP) and shotgun mass mapping (SMM) were applied to analyze proteins of a pure culture to detect incorporation as a simple, fast, reliable and cost-efficient method. In a second experiment a defined mixed culture was used to show the specific metabolic activity of a single species in a microbial community comparing different incorporation levels (Fig. 2)**.**

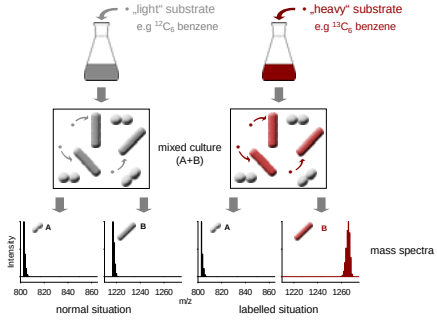


Fig. 2 Principle scheme of Protein-SIP to investigate to substrate consumption in a mixed culture

Pure cultures

Pseudomonas putida **was used to demonstrate ^{13}C or ^{15}N incorporation into proteins by employing Protein-SIP. For further simplification, species identification mass spectrometry approaches such as intact protein profiling (IPP) and shotgun mass mapping (SMM) were used [1]. Furthermore, ^{15}N-ammonium was used as a single source of nitrogen for Protein-SIP experiments.**

Intact protein profiling

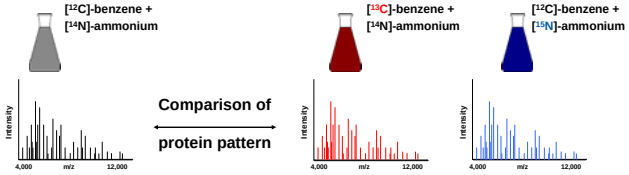


Fig. 3 Overview of intact protein profiling and the corresponding MS spectra of intact bacterial cells

MALDI-MS recorded spectra from intact proteins were compared with the reference spectra using the similarity tool from the MS-Screener software [2]. For this purpose, the mass shift of peaks resulting from metabolic incorporation of heavy stable isotopes (^{13}C or ^{15}N) was taken into account (Fig. 3)**. Thereby metabolic activity was detected by mass shifts and afterwards the mass shifts were quantified, resulting in an accuracy of ±9 atom % incorporation of heavy isotopes.**

Shotgun mass mapping

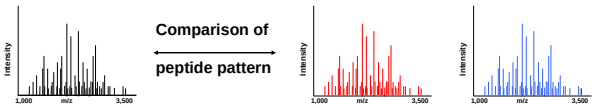


Fig. 4 Overview of intact protein profiling and the corresponding MS spectra of tryptic digested bacterial cells

Bacterial cells were proteolytically digested with trypsin and afterwards peptides were measured by MALDI-MS. The recorded spectra from tryptic peptides were compared (see IPP section) and mass-shifted peptides were detected resulting from a metabolic incorporation of stable isotopes into proteins (Fig. 4)**. Afterwards the incorporation of ^{13}C or ^{15}N were quantified, resulting in an accuracy of ±2 atom %. Additionally, species-specific peptide identifications yielded by Tandem MS fragmentations were obtained and could give evidences of the functional state of the culture (e.g. benzene 1,2- dioxygenase, catechol 1,2-dioxygenase and others).**

Defined mixed culture

The metabolic activity of ***Aromatoleum aromaticum*** **strain EbN1 was investigated using [$^{13}C_7$]-toluene as the labeled substrate [3]. Strain EbN1 is able to degrade toluene under denitrifying conditions forming benzoyl-coenzym A as the central intermediate [4].**

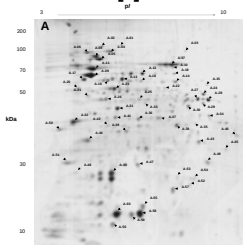


Fig. 5A 2DE separation of proteins from strain EbN1 grown on [$^{13}C_7$]-toluene

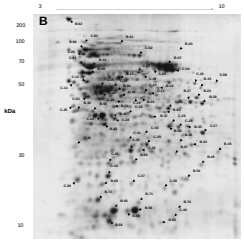


Fig. 5B 2DE separation of proteins from the mixed culture grown on [$^{13}C_7$]-toluene and non-labeled gluconate

The metabolic activity and the incorporation of ^{13}C from [$^{13}C_7$]-toluene in strain EbN1 was first investigated in an anaerobic batch with non-labeled toluene and [$^{13}C_7$]-toluene (Fig. 5A)**. In a second experiment strain EbN1 was cultivated with culture UFZ-1** (Fig. 5B) **which was enriched from a sludge using gluconate as the sole carbon source and showed no toluene degrading activity. The mixed culture was grown on non-labeled toluene,[$^{13}C_7$]-toluene and/or non-labeled gluconate.**

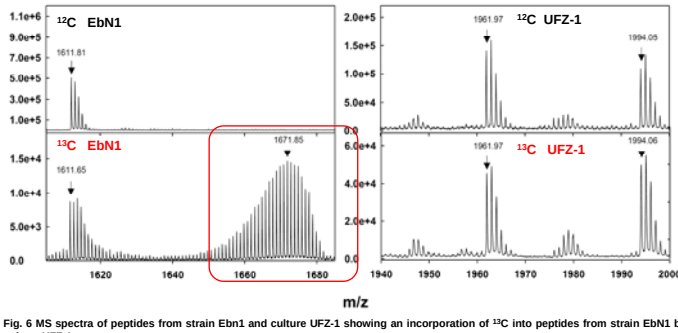


Fig. 6 MS spectra of peptides from strain Ebn1 and culture UFZ-1 showing an incorporation of ^{13}C into peptides from strain EbN1 but not in culture UFZ-1

The analyses of 38 proteins revealed a ^{13}C incorporation level of 92.3 ± 0.8% in the pure EbN1 culture. The analyses of the proteins originated from the mixed culture showed an exclusive incorporation of heavy carbon into peptides from strain EbN1 but not in peptides from the culture UFZ-1 (Fig. 6)**. The analyses of 19 proteins from strain EbN1 resulted in a 82.6 ± 2.3% ^{13}C incorporation level.**

Conclusions

Protein-SIP is a valuable method to identify active species within a bacterial consortium either with gel-based or shotgun proteomics. Future studies may use a combination of both methods to analyze microbial interactions and food chains regarding ^{13}C and ^{15}N incorporation into the whole protein interior or specific functional proteins. The analyses of subpopulations can be supported by fluorescence-assisted cell sorting.

References

[1] Jehmlich N, Schmidt F, Taubert M, Seifert, J, von Bergen M, Richnow HH, Vogt C. *Rapid Comm Mass Spectrom* (2009) 23,12,1871-1878
[2] Schmidt F, Schmid M, Mattow J, Facius A, Pleissner KP, Jungblut PR. *J Am Soc Mass Spectrom* (2003) 14, 9, 943-956
[3] Jehmlich N, Schmidt F, von Bergen M, Richnow HH, Vogt C. *ISME J*(2008) 2,1122-1133
[4] Widdel, F & Rabus, R *Curr Opin Biotechnol* (2001) 12, 259-276

HELMHOLTZ CENTRE FOR ENVIRONMENTAL RESEARCH – UFZ

Advances in Protein-based stable isotope probing

Decimal place calculation of ^{13}C incorporation to assess metabolic activity

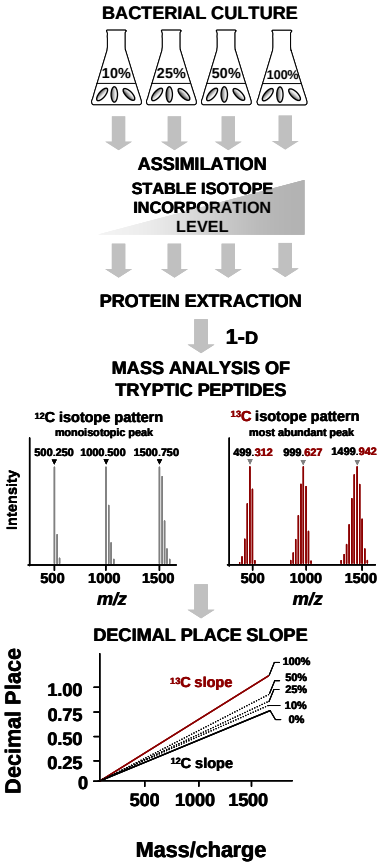
Nico Jehmlich[1] & Martin Taubert[1], Ingo Fetzer[2], Jana Seifert[1], Carsten Vogt[3]
Hans-Hermann Richnow[3], Frank Schmidt[1,4] and Martin von Bergen[1]

[1]Helmholtz Centre for Environmental Research - UFZ, Department of Proteomics, Leipzig, Germany, [2]Department of Environmental Microbiology,
[3]Department of Isotope Biogeochemistry, [4]Interfaculty Institute for Genetics and Functional Genomics, University of Greifswald, Germany

Overview

We describe a new way of determining the isotope incorporation of labeled carbon atoms (^{13}C) into proteins. Our method makes use of characteristic tryptic pattern of the decimal places (= the digits after the decimal point of the peptide masses) generated by liquid chromatography in combination with high resolution instruments such as linear ion trap LTQ-Orbitrap MS [3, 4]. The new method is independent from the peptide sequence which is important for the analysis of environmental samples where no sequence data is available.

Introduction & Methods



For biotechnological and environmental microbiology the identification of bacteria with certain functional abilities in microbial communities is of utmost importance. However, knowledge of structures, functions and activities of these communities is still rare. In order to detect functional relationships within a microbial community, recently metabolic stable isotope probing (SIP) of proteins was developed [1]. Employing isotopically (^{13}C or ^{15}N) labeled substrates, incorporation into the biomass can be detected. In order to measure the extent of stable isotope incorporation into proteins/peptides an alternative calculation method (decimal place slope calculation) was introduced.

The experimental workflow that was used for application of this method is shown in figure 1. In order to define the upper and lower limits for 0 and 100 atom % ^{13}C incorporation, a representative *in silico* data set of 89,773 theoretical tryptic peptides masses from *Mycobacterium tuberculosis* H37Rv were plotted against their decimal places. The practical incorporation was calculated using these $^{12}C/^{13}C$ reference slopes.

Fig. 1 Flowchart of the experimental design to determine the metabolic incorporation of ^{13}C in protein/peptides by its decimal place slope in order to to distinguish active vs. non active species by artifical isotopomers measured by MS. In our study, *P. putida* ML2 grew in the presence of [$^{13}C_6$]-benzene (solely energy and carbon source) and as a control in the presence of [$^{12}C_6$]-benzene. Afterwards the proteins were extracted and subjected to 1-D gel electrophoresis, tryptic in-gel digested, and peptides were analyzed by nano-LC LTQ Orbitrap-MS. The difference of the isotopic pattern between $^{12}C/^{13}C$ peptides allowed the calculation of the ^{13}C incorporation within various labeling contents using the highest intensity ^{13}C peak by decimal place slope.

Calculation of Incorporation - Decimal Place

In order to proof the theoretical concept of decimal place slope calculation, a practical data set from peptides of *Pseudomonas putida* with assumed experimental incorporation of ^{13}C atoms are used. The exact measured peptide masses were plotted according to their decimal place, resulting in the given figure 2 (*panel left*).

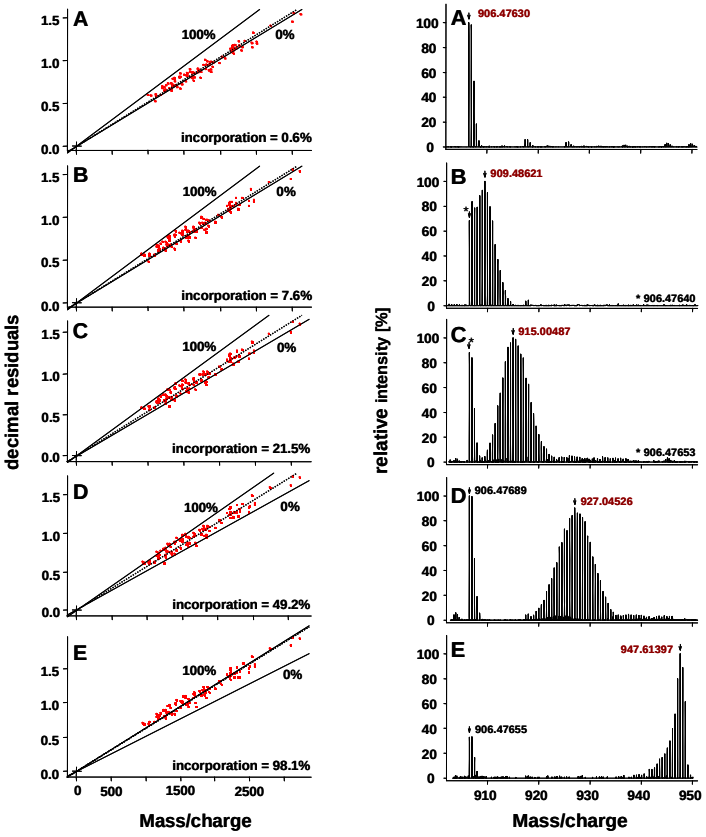


Fig. 2 ***Left panel*** Practical data set of *Pseudomonas putida* ML2 peptides measured by nano-LC LTQ Orbitrap-MS. In order to acquire an estimated incorporation error of <5 atom % about 100 peptide masses were needed. In each case, exactly 100 peptide masses (highest intensity peak) from 22 different proteins were plotted according their exactly mass and their decimal place. The slope pass through the data points was calculated by an R-script and resulted in the given figure (**A**-**E**) for the different substrate ratios (0 atom %, 10 atom %, 25 atom %, 50 atom % and 100 atom %). ***Right panel*** Averaged MS-spectra of one peptide from the benzene 1,2-dioxygenase ferredoxin-NAD(+) reductase subunit protein (*P. putida*) to demonstrate the differentially isotopic pattern of ^{12}C peptides and ^{13}C labeled peptides during the incorporation of different substrate ratios (**A**-**E**). The measured peptide with the sequence GIFAVGDVATWPLHSGGK has a mass charge of +2. In all substrate ratios the isotopomers changed due to their incorporation of heavy isotopes from the natural monoisotopic mass of 906.4763 (**A**) to the highest ^{13}C peak in the 10 atom % labeling experiment (**B**) with $[MH+H]^{+2}$ 909.48621. (**C**) The highest ^{13}C peak in the 25 atom % labeling experiment is $[MH+H]^{+2}$ 915.00487. (**D**) The highest ^{13}C peak in the 50 atom % labeling experiment is $[MH+H]^{+2}$ 927.04526. (**E**) The highest ^{13}C peak in the 100 atom % ^{13}C labeling experiment is $[MH+H]^{+2}$ 947.61397.

As an example, one peptide from the benzene 1,2-dioxygenase protein was taken in respect to the various substrate ratios to illustrate the differentially isotopic pattern between ^{12}C and ^{13}C peptides (Fig. 2 *panel right*). As expected, the heavy isotopomers shifted to a higher mass range due to their incorporation of heavy labeled carbon into the proteins [4].

Application in Community Proteomics

We conducted a model experiment for identifying the species responsible for anaerobic toluene degradation in an artificial mixed culture. The toluene degrading bacterium *Aromatoleum aromaticum* (strain EbN1) was grown together with the enrichment culture UFZ-1 under denitrifying conditions. Nitrate was added as electron acceptor, [$^{13}C_7$]-toluene and [$^{12}C_{12}$]-gluconate as carbon source. The latter was added because UFZ-1 is not able to grow on toluene.

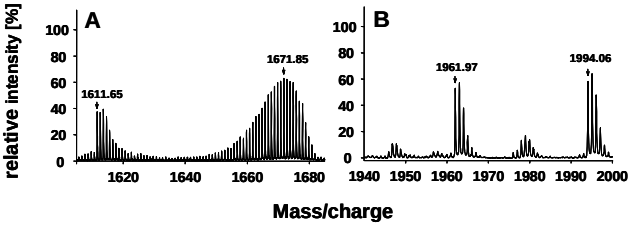


Fig. 3 Detail view of MALDI-TOF MS spectra. (**A**) Chaperone protein dnaK (*Aromatoleum aromaticum* strain EbN1) of the ^{13}C-toluene/gluconate culture provide incorporation of stable isotopes proved by MS. (**B**) Whereas no incorporation into peptides was detectable in the UFZ-1 culture. Example of the heat shock protein (*Pseudomonas stutzeri* A1501) of the ^{13}C-toluene/gluconate culture.

The ^{13}C incorporation was exclusively found in proteins from EbN1 at contents of about 85 atom % and not in proteins from the UFZ-1 culture (Fig. 3) [1,2]. Our results confirm the suitability and practicability of Protein-SIP experiments for carbon flux analysis in microbial communities. Labeled proteins were analyzed by 2-DE gel electrophoresis and MALDI-TOF MS for identification.

Conclusion

MS-analysis of microbial proteomes with high-resolution LC-MS to quantify the incorporation enabled us to identify the assimilation of an organic substrate on the protein level. Current studies have shown that this technique is suitable to elucidate the carbon flow in mixed bacterial communities and to identify pathways and key players of degradation [4]. This technique can be applied in several fields ranging from screening schemes for microorganisms with defined functions (e.g. proteolytic activities) to degradation of environmental harmful xenobiotics.

[1] Jehmlich N, Schmidt F, von Bergen M, Richnow HH, Vogt C. *ISME Journal* (2008) 2,1122-1133
[2] Jehmlich N, Schmidt F, Hartwich, M, von Bergen M, Richnow HH, Vogt C. *Rapid Commun. Mass Spectro* (2008) 22,2889-2897
[3] Fetzer I, Jehmlich N, Vogt C, Richnow HH, Seifert J, Harms H, von Bergen M, Schmidt F Bioinformatics (2009) submitted
[4] Jehmlich N, Fetzer I, Mattow J, Vogt C, Harms H, Thiede B, Richnow HH, von Bergen M, Schmidt F. Molecular & Cellular Proteomics (2009) revision.

e-mail: nico.jehmlich@ufz.de, martin.taubert@ufz.de

Application of protein based stable isotope probing (Protein-SIP) to unravel anoxic benzene degradation

Martin Taubert[1], Jana Seifert[1], Martin von Bergen[1], Carsten Vogt[2] and Hans-Hermann Richnow[2]

[1]Helmholtz Centre for Environmental Research - UFZ, Department of Proteomics, Permoserstr. 15, D-04318 Leipzig, Germany
[2]Helmholtz Centre for Environmental Research - UFZ, Department of Isotope Biogeochemistry

Overview & Introduction

Microbial communities play a key role in the Earth's biogeochemical cycles. Additionally, many metabolic activities of microorganisms can only arise in communities and not in single microbial strains. Classical ways of analysing microorganisms, however, almost entirely rely on pure cultures, even though less than 1% of microbial species have been successfully grown in pure cultures so far. Efficient methods for analysing microbial communities are still scarce. To fill this gap, we expanded classical stable isotope probing (SIP) methods to metaproteomic analysis. Protein based stable isotope probing is a powerful tool capable of providing information on physiology and taxonomy of microbial communities, thus allowing to analyze functional relationships within them [1].

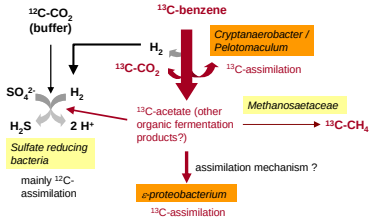


Fig. 1: Proposed composition of the investigated microbial community with hypothetical energy and carbon flow.

In our recent work, we applied Protein-SIP to a benzene degrading, sulfate reducing culture from a contaminated aquifer near Zeitz, Saxonia-Anhalt. Benzene degradation under sulfate reducing conditions is a process shown only in microbial communities so far. Its mechanisms and the exact course of degradation is still unknown. First clues on taxonomic composition of the community have been acquired by DNA stable isotope probing experiments ([2], see figure 1).

Fig. 2: Groundwater from Zeitz is pumped over these columns, allowing the attachment and growth of microorganisms at the gravels.

Methods

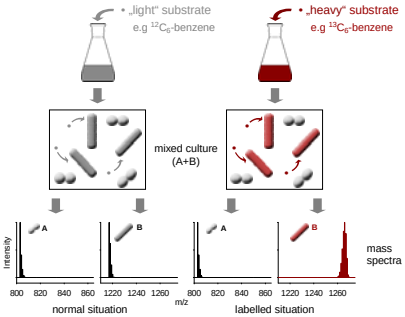


Fig. 3: Principle scheme of a protein SIP experiment with a mixed culture; incorporation of heavy isotopes into the biomass of species B is detected by a shift of peptide masses analyzed by mass spectrometry.

In protein SIP, nonradioactive heavy isotopes are used to label substrates, then the metabolisation and incorporation into proteins is analysed by high resolution mass spectrometry (LTQ Orbitrap, Thermo Fisher Scientific), which allows a determination of heavy isotope incorporation down to ~2% [3]. In this study, coarse sand material colonized with the benzene degrading microbial community was taken from columns shown in figure 2 and cultivated in 1 L glass bottles over up to 300 days under sulfate reducing conditions with ^{12}C- and ^{13}C-benzene as substrate. To monitor the cultivation, concentrations of sulfide and benzene as well as the isotope ratio of CO_2 in the headspace of the cultures were measured periodically. At successive time points, bottles were opened and prepared for further analysis. The workflow of sample preparation and analysis is shown in figure 4.

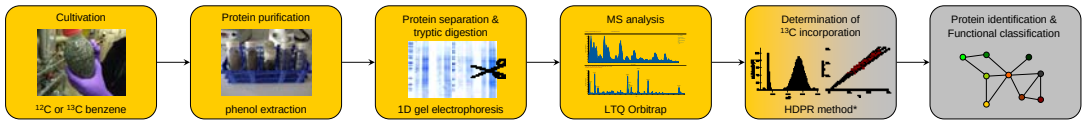


Fig. 4: Overview of the experimental workflow; boxes in grey color indicate unfinished or further intended work. *Calculation of incorporation was done applying a method based on the half decimal place rule for tryptic peptides. The HDPR method is independent of sequence information and uses the shift of a peptide mass decimal residuals on ^{13}C incorporation [4].

Results

Cultivation

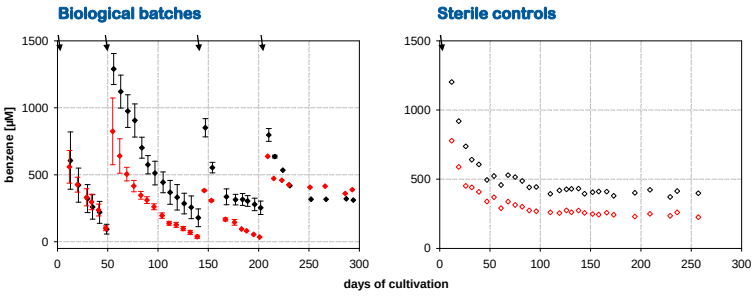


Fig. 5: Depletion of benzene during the cultivation; ◆ cultures with ^{12}C-benzene, ◆ cultures with ^{13}C-benzene, ◇/◇ sterilized control with ^{12}C/^{13}C-benzene. Arrows indicate timepoints of benzene addition (15 µl per timepoint and bottle). An average rate of benzene degradation of 7.76 µM/d ± 0.24 µM/d (^{12}C) resp. 5.28 µM/d ± 0.34 µM/d (^{13}C) was calculated for a representative time range between day 90 and day 145.

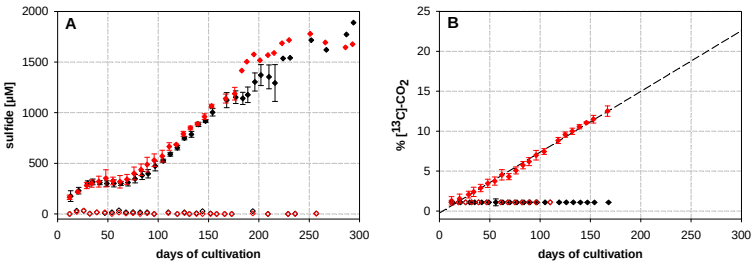


Fig. 6: Increase (A) of sulfide concentration and (B) of [^{13}C]-CO_2 ratio during the cultivation; ◆ cultures with ^{12}C-benzene, ◆ cultures with ^{13}C-benzene, ◇/◇ sterilized control with ^{12}C/^{13}C-benzene. (A) An increase in sulfide of 9.72 µM/d ± 0.14 µM/d (^{12}C) resp. 9.55 µM/d ± 0.67 µM/d (^{13}C) was calculated for the time range between day 90 and day 145. On average, 1.25 mol ± 0.05 mol resp. 1.81 mol ± 0.01 mol sulfide were formed per mol of benzene consumed. (B) An increase of ^{13}C-CO_2 was observed in cultures spiked with ^{13}C-benzene only. On average, 88.1 % ± 5.3 % of the carbon from degraded benzene was released as CO_2. These values also fit well with previous observations (see [2]).

Stable isotope probing

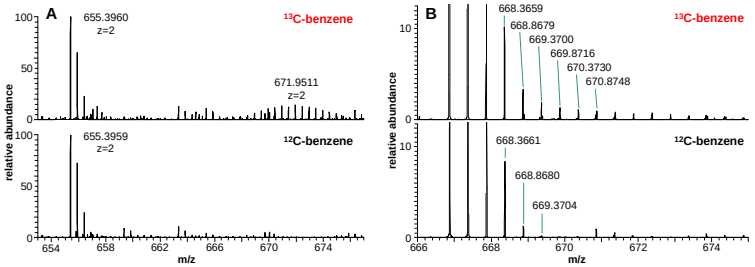


Fig. 7: Mass spectra showing high and low ^{13}C incorporation. (A) Example of a peak shift (m/z 655.40 to m/z 671.95) due to high heavy isotope incorporation. The shifted peak shows typical pattern of heavy isotope incorporation with left- and right-tailing. In the control spectrum, no shifted peak is visible. (B) Example of a peak (m/z 666.8614, z=2) with enhanced right-tailing due to low heavy isotope incorporation. Much more isotopic peaks (up to m/z 673.3831) are detectable than expected from the control spectrum.

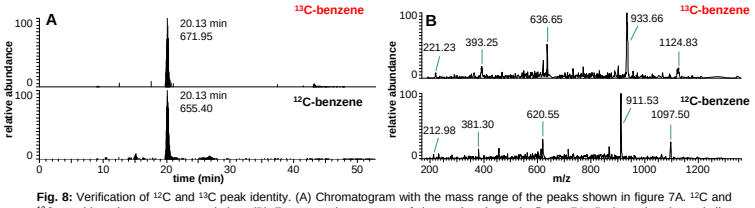


Fig. 8: Verification of ^{12}C and ^{13}C peak identity. (A) Chromatogram with the mass range of the peaks shown in figure 7A. ^{12}C and ^{13}C peptides show concurrent elution. (B) Fragmentation spectra of the peaks shown in figure 7A. Both peaks show similar fragmentation pattern except for the mass shift due to heavy isotope incorporation, hence indicating identity of the peptides sequence.

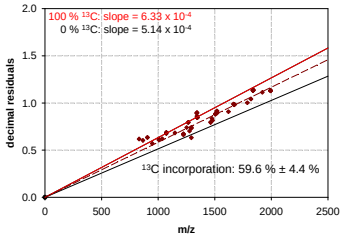


Fig. 9: Determination of heavy isotope incorporation by half decimal place slope calculation. The black slope and the red slope show the theoretical limits of 0 % and 100 % of ^{13}C incorporation, respectively. The masses of 59 peptide peaks showing high ^{13}C incorporation (see figure 7A) were plotted versus their decimal residuals. Linear fitting based on robust linear model resulted in a slope of 5.84 x 10^{-4}, yielding a heavy isotope incorporation of 59.6 % ± 4.4 %. (For additional information on HDPR calculation see [4])

Discussion & Outlook

As shown by the results of the cultivation, we successfully proofed metabolisation of ^{13}C-benzene to ^{13}C-CO_2 under sulfate reducing conditions. Furthermore, the incorporation of the heavy isotopes into biomass was shown by mass spectrometric analysis of tryptic peptides. A distinction between peptides showing high incorporation of stable isotopes and peptides showing low incorporation was possible. This indicates dissimilar origin of the according peptides from species on different trophic levels of the community, as suggested in figure 1. These findings show that protein based stable isotope probing is able to give insight into the functional relationships between organisms in natural ecosystems.

For further analysis, specifically high throughput identification of peptides, protein sequence information of the microbial community is needed. Therefore, the preparation of a metagenome from extracted DNA of the culture using next-generation 454 sequencing is about to be performed, allowing an in-depth metaproteomic analysis of the microbial community.

References:

[1] Jehmlich N, Schmidt F, von Bergen M, Richnow HH, Vogt C. *ISME Journal* (2008) 2,1122-1133
[2] Herrmann S, Kleinsteuber S, Chatzinotas A, Kuppardt S, Lueders T, Richnow HH, Vogt C. *Environ Microbiol.* (2009) [Epub ahead of print] PMID: 19840104
[3] Jehmlich N, Schmidt F, Hartwich M, von Bergen M, Richnow HH, Vogt C. *Rapid Commun Mass Spectrom.* (2008) 22(18), 2889-2897
[4] Jehmlich N, Fetzer I, Seifert J, Mattow J, Vogt C, Harms H, Thiede B, Richnow HH, von Bergen M, Schmidt F. *Mol Cell Proteomics.* (2010) [Epub ahead of print] PMID: 20064840

Deutsche Forschungsgemeinschaft DFG
dfg priority programme 1319
HIGRADE
HELMHOLTZ CENTRE FOR ENVIRONMENTAL RESEARCH – UFZ

e-mail: martin.taubert@ufz.de