

# Improving protein extraction and separation methods for investigating the metaproteome of anaerobic benzene communities within sediments

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**Abstract** BTEX compounds such as benzene are frequent soil and groundwater contaminants that are easily biodegraded under oxic conditions by bacteria. In contrast, benzene is rather recalcitrant under anaerobic conditions. The analysis of anoxic degradation is often hampered by difficult sampling conditions,

limited amounts of biomass and interference of matrix compounds with proteomic approaches. In order to improve the procedure for protein extraction we established a scheme consisting of the following steps: dissociation of cells from lava granules, cell lysis by ultrasonication and purification of proteins by phenol extraction. The 2D-gels revealed a resolution of about 240 proteins spots and the spot patterns showed strong matrix dependence, but still differences were detectable between the metaproteomes obtained after growth on benzene and benzoate. Using direct data base search as well as de novo sequencing approaches we were able to identify several proteins. An enoyl-CoA hydratase with cross species homology to *Azoarcus evansii*, is known to be involved in the anoxic degradation of xenobiotics. Thereby the identification confirmed that this procedure has the capacity to analyse the metaproteome of an anoxic living microbial community.

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## Abbreviation

BTEX Benzene, toluene, ethylbenzene and xylenes

## Introduction

The extraction and purification of proteins for 2D-PAGE for the analysis of microbial communities

culturing soil (Benndorf et al. 2007) and anoxic aquifer material is still a challenge, thus hampering metaproteomic approaches. Sulphate and iron reduction leading to the formation of colloids containing reduced sulphur and iron species, elementary sulphur as well as organic humic like material complicate the extraction and purification of proteins for 2D-PAGE. Moreover, the biomass of anaerobic microbial communities is often low and therefore elevated strategies are required to extract sufficient amounts of proteins for further analysis.

Petroleum hydrocarbons such as benzene, toluene, ethylbenzene and xylenes (BTEX) are frequent soil and groundwater contaminants worldwide. All BTEX compounds are easily biodegraded under oxic conditions by bacteria (van Agteren et al. 1998). Contaminated plumes become, however, generally anoxic, due to the low water solubility and rapid microbial consumption of oxygen; under such conditions, benzene has been often described as recalcitrant (Johnson et al. 2003). Although benzene is the most mobile and most toxic BTEX compound (Dean 1985) microcosm studies suggest that anaerobic benzene degraders are not ubiquitous in subsurface sediments (Kazumi et al. 1997; Phelps and Young 1999; Weiner and Lovley 1998), and only a limited number of anaerobic benzene-degrading laboratory enrichment cultures have been established with different electron acceptors (Botton and Parsons 2006; Burland and Edwards 1999; Edwards and Grbicgalic 1992; Jahn et al. 2005; Kazumi et al. 1997; Lovley et al. 1995, 1996; Musat and Widdel 2008; Phelps et al. 1996; Phelps and Young 1999; Vogt et al. 2007; Weiner and Lovley 1998). The isolation of bacteria able to oxidize benzene in the absence of oxygen failed in most cases. Sulphate-reducing or methanogenic benzene-degrading enrichment cultures were still phylogenetically diverse after cultivation for many years (Phelps et al. 1998; Ulrich and Edwards 2003), suggesting that under strict anoxic conditions, benzene is degraded by microbial consortia rather than by single organisms. Also the biochemical pathway of anaerobic benzene degradation is currently elusive and might involve as first transformation step either a methylation (Ulrich et al. 2005), a hydroxylation (Caldwell and Suffita 2000; Ulrich et al. 2005), or a carboxylation (Musat and Widdel 2008).

A metaproteome approach would have the potential to analyse benzene degrading microcosms in

order to obtain information on the functionality of microbial communities in comparative studies as well as on de novo sequenced proteins with unknown functions. The metaproteomic approach has been successfully used to investigate the structure and function of oxic bacterial communities in different environmental systems (Kan et al. 2005; Park et al. 2008; Ram et al. 2005; Schulze et al. 2005; Wilmes and Bond 2004). A prerequisite is to isolate and purify protein fractions from slow growing cultures with low biomass and to overcome interferences from co-extracted sulphur, iron and humic like material.

In this work, we studied benzene degradation in an anoxic aquifer. The mineralization of benzene with sulphate as electron acceptor was quantified in sand- and lava granules-filled columns or in situ microcosms, using sulfidic groundwater of the site as inoculum (Vogt et al. 2007). The benzene-degrading bacterial community is attached to surfaces of the sand and lava particles, forming a biofilm. The phylogenetic composition of the biofilm is diverse, comprising of several taxonomically different phylotypes, as recently shown by means of 16S rRNA analysis (Kleinstaub et al. 2008).

For the present study, we modified the protocols for the analysis of the metaproteome of groundwater and soil samples (Benndorf et al. 2007). Several experimental challenges had to be handled: (1) attached cells had to be removed from the matrix of the lava granules; (2) Kleinstaub et al. (2006) expected low cell numbers and thus low protein contents due to low bacterial growth under the respective growth conditions; (3) the presence of contaminants from protein extracts which might interfere with 2D-PAGE, e.g. iron sulfides, elementary sulphur and charged colloids; (4) the identification of proteins from environmental samples where only sparse genetic information is available.

## Materials and methods

### Description of the field site

The examined contaminated aquifer is located on the site of a former coal hydrogenation and benzene production plant near Zeitz (Saxony-Anhalt, Germany). The contamination was caused by several leakages,

damages and accidents during the operation of the plant between 1960 and 1990. Natural attenuation (NA) processes at the site have been intensively investigated during the last years (Alfreider and Vogt 2007; Fischer et al. 2007; Godeke et al. 2006; Vieth et al. 2005; Vogt et al. 2007). An upper and a lower aquifer are separated by a lignite and clay layer. Both aquifers are heterogeneous and hydrogeologically connected due to discontinuities of the lignite-clay layer. The aquifers are composed of river gravel and sand sediments, which contain more than 95% quartz. The general groundwater flow direction is to the north. The groundwater of both aquifers is heavily contaminated, with benzene concentrations of up to 13 mM in the source zone, and characterized by anoxic conditions. Sulphate, reaching concentrations of 10 mM, is the main electron acceptor at the site (Vieth et al. 2005).

In order to investigate the potential for benzene degradation of the lower sulphidogenic aquifer, ground water was percolated through two columns systems since 2002 under nearly in situ condition (Vogt et al. 2007). Each column system consists of four segments (each 25 cm in diameter and 6 m long) connected in series and filled either with coarse sand or with lava granules. The percolating groundwater contains 300  $\mu\text{M}$  benzene averages. Toluene, ethylbenzene and xylenes are present in trace amounts ( $\leq 1 \mu\text{M}$ ). Furthermore, 4 mM sulphate, 300  $\mu\text{M}$  sulfide, 120  $\mu\text{M}$  ammonium, 5  $\mu\text{M}$  orthophosphate, 150  $\mu\text{M}$  potassium, 2.3 mM magnesium, 2.2 mM sodium and 6.1 mM calcium are present. In both column systems, benzene is degraded under sulphate-reducing conditions since at least 3 years (Vogt et al. 2005).

#### Microbiological analyses

Lava granules or sand particles were removed from the top layer of each of the four lava granules-filled columns using a sterile spoon, after opening sample ports, as described elsewhere (Vogt et al. 2007). The material was immediately transferred into sterile 1 l bottles, which were subsequently filled with anoxic sulfidic groundwater derived from the column inflow, and closed with air-tight screw caps, to avoid oxidation processes caused by oxygen. The material was processed in the laboratory on the same day.

#### Cultivation of benzene community

Generally, the handling of the bottles filled with column material was conducted in an anoxic glove box (gas atmosphere 95% nitrogen, 5% hydrogen; Coy Laboratory Products Inc., Grass Lake, MI, US) to ensure anoxic conditions. For batch degradation experiments, the liquid phase of the bottles filled with lava granules (about 950 ml), comprising of anoxic groundwater, was replaced by a sulphate-containing (20 mM) mineral salt medium described elsewhere (Knoller et al. 2006). Usually, the medium was spiked with benzene (120  $\mu\text{M}$ ) or benzoate (700  $\mu\text{M}$ ) as carbon and energy sources, respectively. All mentioned solutions were sterilized by filtration or autoclaving and flushed with  $\text{N}_2$  before use. Bacterial cultures were incubated statically at ambient temperature in the dark.

#### Determination of benzene and sulfide concentrations

Benzene was analysed by automated headspace gas chromatography with a Varian 3800 gas chromatograph (Varian, Palo Alto, USA) equipped with a CP SIL 5 CB capillary column (film thickness, 0.12  $\mu\text{m}$ ; inside diameter, 0.25 mm; length, 25 m) and a flame ionization detector. The chromatographic conditions were as follows: injector temperature, 250°C (split 1:50); detector temperature, 260°C; and an oven temperature process program consisting of 70°C for 2 min, followed by an temperature increase at a rate of 10°C  $\text{min}^{-1}$  up to 90°C was followed by a further increase at a rate of 60°C  $\text{min}^{-1}$  up to 220°C was applied. Helium (1 ml  $\text{min}^{-1}$ ) was used as a carrier gas. Liquid test samples (diluted 1:10 or 1:20 in 1.6 mM  $\text{H}_2\text{SO}_4$ ; final volume, 10 ml) were prepared in 20 ml glass vials. For benzene equilibration for headspace analysis, the samples were agitated for 30 min at 70°C in a heated shaker (rotation regime, 250 rpm for 5 s and no rotation for 2 s) prior to analysis and 1 ml of each sample's headspace was injected. For calibration, diluted standards of benzene prepared from stock solutions were treated in the same way as the samples. The stock solutions were prepared in pure methanol.

Sulfide concentrations were analysed colorimetrically by the method of Cline (Cline 1969). The

fixation and preparation of samples was described elsewhere (Vogt et al. 2005).

### Protein extraction and purification

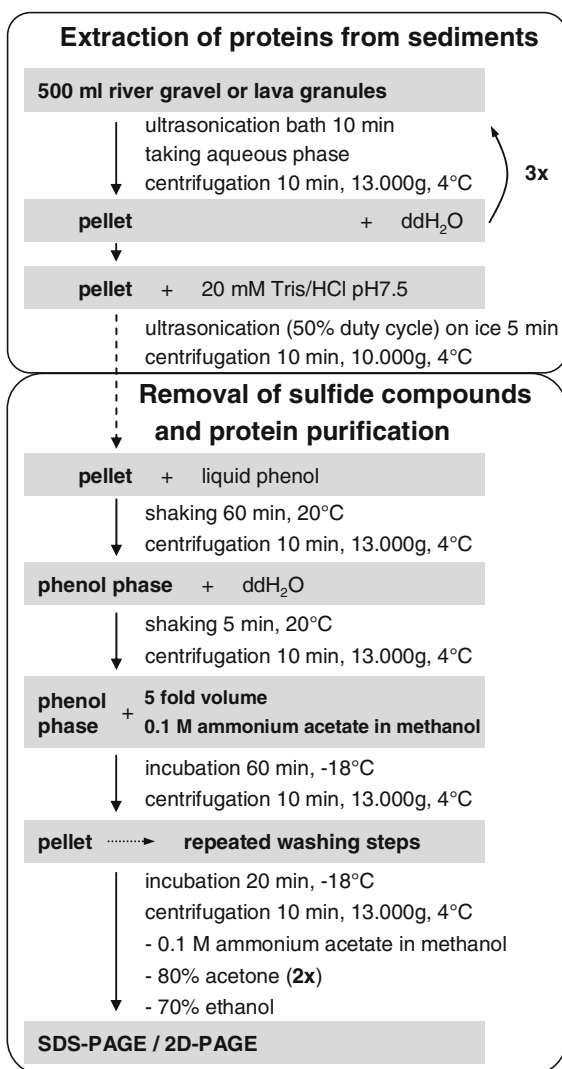
The cultivated samples were extracted and purified after following protocol (Fig. 1; Benndorf et al. 2007): 500 ml of the sediments (lava granules or coarse sand) were incubated for 10 min in an ultrasonication bath. The aqueous phase was taken and centrifuged (Biofuge fresco, Heraeus, Asheville, NC, USA) for 10 min at 13,000g and 4°C. This

treatment was repeated for three times and the pellets were collected. Subsequently the combined pellets were dissolved with 20 mM Tris/HCl pH 7.5. The protein extraction was performed by ultrasonication on ice for a period of 5 min and 50% duty cycle (Ultrasonics sonifer 250, Branson, Danbury, CT, USA). The suspension was centrifuged for 10 min at 10,000g and 4°C. The pellets were mixed with liquid phenol (10 g phenol in 1 ml water) and shaken for 1 h at 20°C. Afterwards, the phases were separated by centrifugation (10 min at 14,000g). The proteins in the lower phenol phase were precipitated with the fivefold volume of 0.1 M ammonium acetate dissolved in methanol at -18°C for 1 h. In the following, the sample was centrifuged (10 min, 13,000g, 0°C) and the pellets were suspended by a sonication bath in 0.1 M ammonium acetate in methanol, incubated 15 min at -18°C and centrifuged again as described above. The pellets were successively washed in 0.1 M ammonium acetate in methanol, 80% acetone, 70% ethanol, each washing step including 15 min incubation and subsequent centrifugation.

The protein concentration was determined by a Bradford-assay whereby dilutions of bovine serum albumin (BSA) were used as a standard (Bradford 1976). Samples were stored at -20°C until further analysis.

### SDS-PAGE and 2D-PAGE of extracted proteins

Extracted proteins were separated by SDS-PAGE (Laemmli 1970) or 2D-polyacrylamide gel electrophoresis (2D-PAGE) (Görg et al. 2004; Hamdan and Righetti 2005; Wilkins et al. 1997). Before electrophoresis, the protein pellets were precipitated with fivefold ice cold acetone. The pellets were air-dried and mixed with sample buffer (Laemmli 1970), incubated for 5 min at 60°C and loaded on SDS-gels (4% stacking gel, 12% separating gel). Prior to 2D-PAGE, the resulting protein pellets were also air-dried and dissolved in DeStreak™ rehydration solution with 0.5% IPG (immobilised pH gradient) pH 4–7 or 3–10 non-linear (NL) buffer (v/v) (GE Healthcare, Uppsala, Sweden). In order to remove precipitates the solutions were centrifuged for 15 min at 62,000g (3K30, rotor 12154-H, Sigma laboratory centrifuges, Osterode, Germany) at ambient temperature and processed as described previously



**Fig. 1** Scheme of the extraction and purification of proteins from sediments

(Benndorf et al. 2006). After electrophoresis, gels were stained with colloidal Coomassie brilliant blue (Neuhoff et al. 1988) or with silver nitrate (Blum et al. 1987) and dried in a stream of unheated air. Due to the limited availability of sediment material, most gel experiments were only carried out once. The gels were scanned (Image Scanner II, GE Healthcare, Uppsala, Sweden) and analyzed with Delta2D software (version 3.3, Decodon, Greifswald, Germany). Spots with an threefold changed normalized volume were considered as amplified. Due to missing duplicates all changed spots were also evaluated manually and artefacts caused by local mismatching or by incorrect quantification due to false determination of the spot shape were removed.

#### Identification of proteins by nanoLC-ESI-MS

For identification of proteins from 2D-PAGEs of lava granules or coarse sand, the spots of interest were excised and digested overnight with trypsin (Santos et al. 2004). The extracted peptides were separated by reversed-phase nano-LC (LC1100 series, Agilent Technologies, Palo Alto, CA, USA; column: Zorbax 300SB-C18, 3.5 mm, 150  $\mu\text{m}$ , 0.075 mm; eluent: 0.1% formic acid, 0–60% acetonitrile) and analyzed by MS/MS (LC/MSD TRAP XCT mass spectrometer, Agilent Technologies). Database searches were carried out with MS/MS ion search (Mascot, Matrix Science, London, UK) against NCBIInr. Subsequent parameters were selected: all entries of NCBIInr (National Center for Biotechnology Information, Rockville Pike, USA), tryptic digestion, up to one missed cleavage site was allowed and for global modifications carbamidomethyl at cysteines and oxidized methionine were given as variable modifications. The search was restricted to peptides containing at least one charge and was conducted with following accuracies: peptide tolerance:  $\pm 1.2$  Da and MS/MS tolerance:  $\pm 0.8$  Da.

#### Identification of proteins by LTQ Orbitrap XL

For identification of proteins from 2D-PAGEs, the most abundant spots were excised and digested with trypsin as described above. The extracted peptides were separated by HPLC (column: C<sub>18</sub> column,

75  $\mu\text{m}$   $\times$  100 mm (LTQ Orbitrap XL), mobile phase A: 0.1% formic acid in water with 2% acetonitrile, mobile phase B: 0.1% formic acid in acetonitrile, flow rate: 300  $\text{nl min}^{-1}$ . Gradient: 5–35% B in 65 min) and analyzed by LTQ Orbitrap XL (Thermo Fisher Scientific, Waltham, USA) (Scigelova and Woffendin 2007). The instrument with nanospray ion source was operated as following: external calibration weekly, top 5  $\text{MS}^2$  (in the ion trap), 1 microscan, resolution (FT full scan): 30,000  $\text{m}/\Delta\text{m}_{50\%}$ ,  $\text{MS}^2$  threshold: 8,000 ( $\sim$  S/N 2:1), MS target:  $1\text{e}^6$ ,  $\text{MS}^2$  target:  $5\text{e}^3$ , exclusion mass tolerance: 10 ppm, charge state selection: 2+, 3+.

#### Data processing

The MS/MS spectra of peptides were extracted from raw LC MS/MS dataset using `extract_msn.exe` as part of software Xcalibur 2.04 (Thermo Fisher Scientific) and merged into Mascot Generic Format (\*.mgf) as a first step of analysis. Each dataset from every spot were searched with Mascot 2.103 (Matrix Science) using database MSDB from NCBI (Version from 08 September 2006; <ftp://ftp.ncbi.nih.gov/repository/MSDB/>). The search was performed covering the whole database, without taxonomy restrictions. In this approach only proteins with deposited genomic information could be identified.

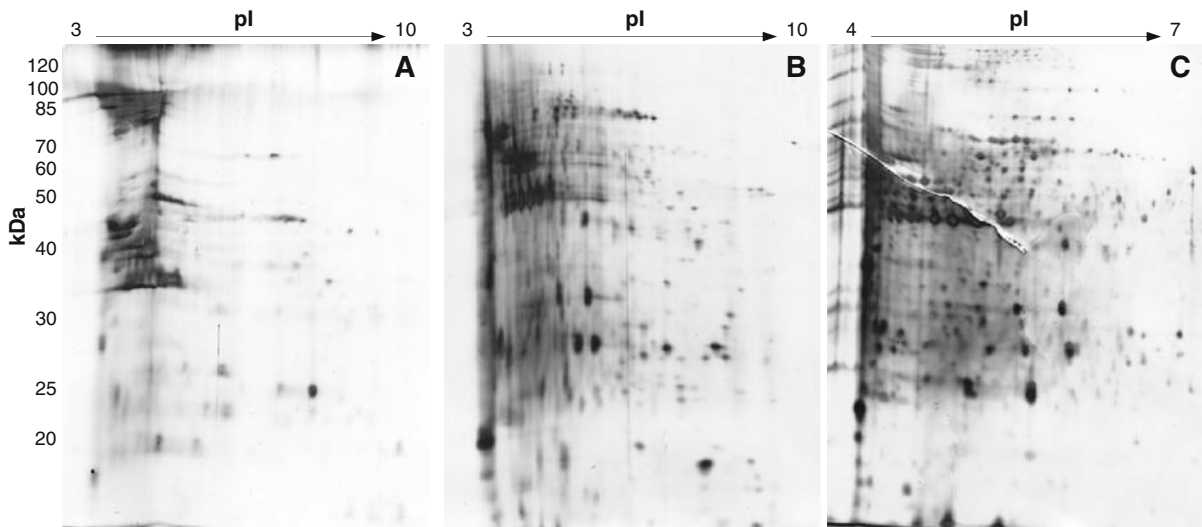
In the next step, all MS/MS spectra, which belong to those proteins clearly identified by Mascot were removed from the dataset using in-house software and were not submitted to the de novo sequencing run. This filtering step reduces the complexity of the dataset to avoid miss-interpretations in the homology searching procedure after de novo sequencing (Waridel et al. 2007). A new file (\*.mgf) containing all MS/MS spectra which were not clearly identified by Mascot, were processed by a special version of PepNovo (Frank and Pevzner 2005) called PepNovo2MSB (<http://peptide.ucsd.edu>) which provides MS-Blast compatible sequence prediction data. All sequence predictions of a dataset with a reliable PepNovo-score of at least 8.0 (Wielsch et al. 2006) were applied to the MS-Blast (Shevchenko et al. 2001) web form (<http://genetics.bwh.harvard.edu/msblast/>) and a homology search using default settings in database “nr95\_clean” and “Apply LC-MS/MS Presets” (Waridel et al. 2007) was performed.

## Results and discussion

### Improvement of protein extraction

Previously, we have developed a protocol for protein extraction from soil and groundwater samples. Our first task was the adaptation of the previously published protocol for protein extraction from lava granules. Most cells in our samples were located on the surface of the lava granules as grey coloured biofilm, suggesting that the cells were embedded in iron-sulfide or pyrite coatings, which were formed by sulfide produced by sulphate reduction and iron from the surrounding groundwater, as described elsewhere (Vogt et al. 2007). Since we expected low cell numbers and low protein concentrations due to the low cell yield coupled to benzene oxidation under sulphate-reducing conditions, we decided to dissolve the attached cells from the lava granules by repeated treatments with water in a sonication bath in advance of cell lysis. Afterwards, the detached and re-dissolved biofilm was concentrated by centrifugation and the containing cells were lysed with sonication. The following centrifugation step resulted in a dark, greyish precipitate which presumably contained most

of the iron sulfides, and a light yellow grey supernatant, indicating that a part of the sulfides had been oxidized by oxygen to sulphur or polysulfides during proceeding. Following standard protocols for sonication, the supernatant usually contained the protein of lysed cells, whereas the pellet contained intact cells or larger cell fragments. In order to track the fate of all proteins we decided to purify both fractions with phenol extraction followed by several washes with organic solvents. This method has been shown to remove huge amounts of interfering humic compounds from soil or groundwater. After dissolving the received pellets in SDS-sample buffer or in DeStreak™ rehydration buffer and loading on SDS-PAGE (not shown) and 2D-PAGE (Fig. 2a), respectively, we detected more than 90% of protein in the sediment fraction. The quality of separation, in particular in 2D-PAGES, was low due to smearing. Additionally, the proteins clustered in the acidic range. In order to exclude that some proteins of the supernatant were not extracted by phenol extraction, additional precipitation techniques, e.g. TCA or acetone precipitations were applied. However, the yields were lower as with phenol extraction and the separation in gels was decreased again. One reason for low



**Fig. 2** 2D-gel map of proteins extracted from sediment samples grown on benzene under different conditions. **a** After extraction and purification of the sample the received pellet was dissolved in DeStreak™ rehydration buffer and loaded on 2D-PAGE (1th dimension: pH 3–10; 2nd dimension: 12% acrylamide). **b** For further improvement the sample was

submitted to filtration with a molecular weight cut-off of 100 kDa and applied to 2D-PAGE (1th dimension: pH 3–10; 2nd dimension: 12% acrylamide). **c** In order to improve the resolution a narrower pH-gradient from 4 to 7 was chosen instead of 3–10 as in **a** and **b**. The gels were stained with silver nitrate

separation and vertical smearing in SDS-PAGE and 2D-PAGE was the presence of grey precipitates in the sample slots of the SDS-PAGE. Presumably, iron-sulfide or pyrite are able to adsorb proteins by ionic interactions because also the binding of positively charged phosphatidylcholine (Widler and Seward 2002) by negatively charged pyrite (Zhang et al. 2003) has been reported. The extraction with liquid phenol seems to decrease the interaction between pyrite and proteins since proteins have a higher solubility in liquid phenol than in water. Therefore, we decided to continue with the phenol extract of the sediment fraction that contained most protein, and to add a high speed centrifugation step in order to remove the precipitates. Nevertheless, the quality of 2D-PAGE was not significantly improved, although the amount of precipitates in sample wells of SDS-gels was decreased. For further improvement the samples were submitted to filtration with a molecular weight cut-off of 100 kDa. This treatment caused significant improvement of separation (Fig. 2b), in particular in the low and medium molecular range, but at the same time a loss of high molecular weight proteins was observed. Thus, this step was omitted and finally the use of a narrower pH-gradient from 4 to 7 was tried (Fig. 2c). The observed improvement of separation quality in the acidic range seemed to be worth to lose some basic proteins. This phenomenon is not only explainable by longer separation distance in the acidic range. We suppose that some of the contaminants persisted in the pH 3–10 strips at the anodic side and hampered separation were removed from pH 4 to 7 strips. Based on the improved protocol, several samples were analysed, although vertical smearing occurred in the acidic range with increased sample loads.

#### Protein patterns from different sediments represent the different communities

First, two solid samples collected directly from the benzene-degrading columns were analyzed; the solids consisted either of coarse sand or lava granules. Previous studies concerning the community structure attached to the different solids revealed significant differences (Kleinstaub et al. 2008). The predominant organisms colonizing the lava granules were related to the taxa *Nitrospira*, *Clostridia* and *Delta-proteobacteria*. The bacterial community attached to the sand particles was composed of *Delta-* and

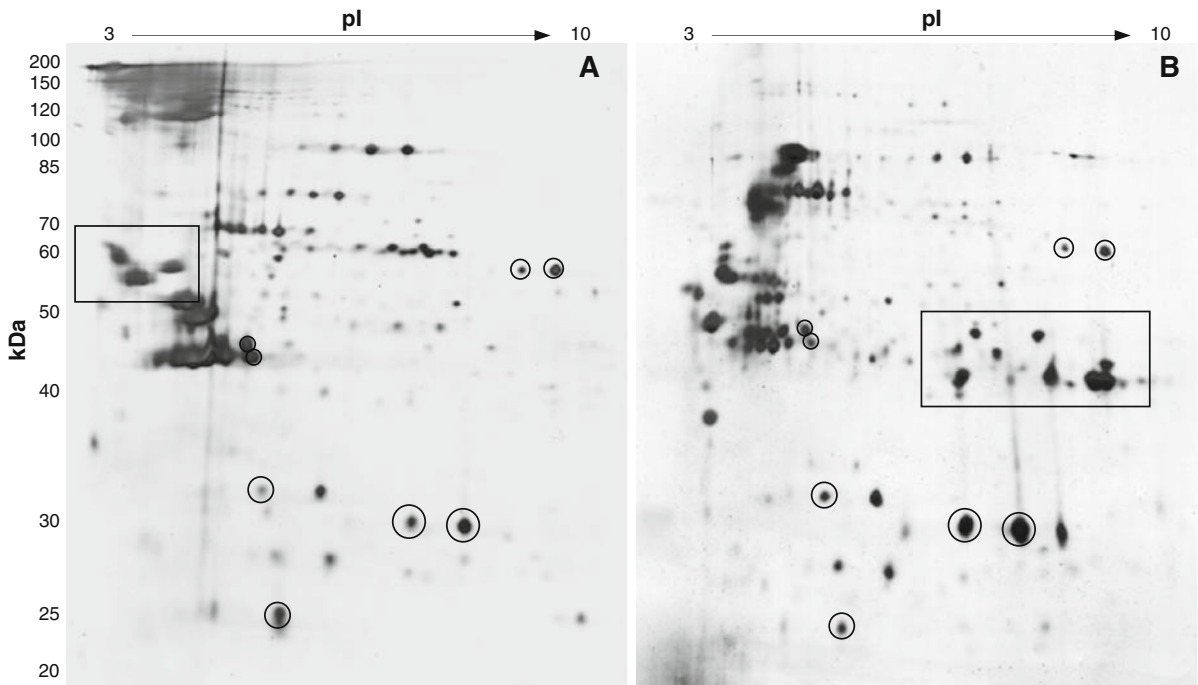
*Epsilonproteobacteria*, *Clostridia*, *Chloroflexi*, and *Actinobacteria*. Therefore, huge differences between the 2D-PAGEs were expected. Both minerals have different surface area for biofilm formation. In order to adjust the protein concentration to the same level, samples deriving from different volumes of sediment were loaded. Our hypothesis, that different communities are reflected by different protein compositions, was confirmed. Both gels showed nearly the same spot volumes and spot numbers (about 240) whereas a high percentage of spots with different locations were located (Fig. 3). In order to facilitate the virtual comparison, we marked some of the spots present in both gels. Because both communities capable of degrading benzene were inoculated by the same groundwater flow it could be suggested that some common proteins are related to benzene metabolism. Other spots may reflect the adaptation of the communities to the sediment matrix forming the surface of lava granules or sand. However, it cannot be excluded that the differences in protein pattern are artefacts to some extent due to selectivity of our sample preparation procedure.

A key to answer that question would be to identify the proteins and to compare their taxonomical origin with the community data based on 16S rRNA. Some spots were cut from both silver stained gels, but their identification failed due to the low amount of sample and to sparse database information available for this kind of anaerobic communities. More details concerning the success of protein information will be discussed in “Protein identification by MS”. In further experiments two strategies were followed, the extraction of huge amounts of sediment (up to 5 l, not shown here) or the cultivation of the benzene community in batch-cultures in the laboratory.

The improved extraction protocol might support in the future the application of Protein-SIP applications, in which the metabolisation of a substrate containing stable isotopes of carbon and incorporation thereof into proteins helps to identify the metabolic active species (Jehmlich et al. 2008a, b).

#### Induction of proteins after growth on different substrates

After establishing a suitable protocol for protein extraction of lava granules and sand particles and showing a first proteomic comparison of the



**Fig. 3** 2D-gel electrophoresis from two solid samples that were collected directly from the benzene-degrading columns. In **a** lava granules and in **b** coarse sand was used as matrix. The gels were run with immobilized pH gradients from 3 to 10 pH and separated in the second dimension by PAGE (12%

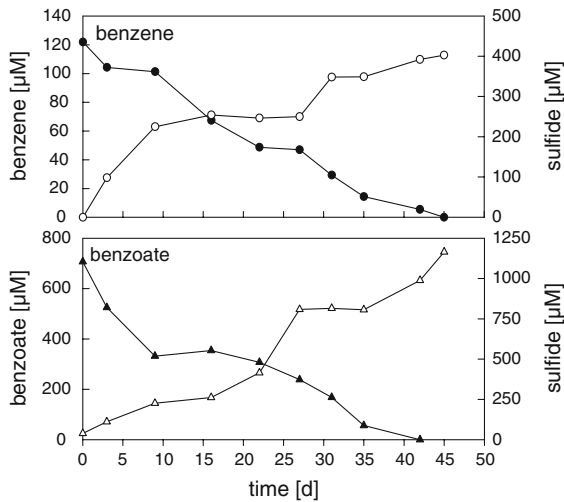
acrylamide) and stained with silver nitrate. The differences in the spot patterns of both communities were highlighted by *rectangles*. Both gels show nearly the same spot volumes and spot numbers (about 240, same spot locations were indicated by *circles* to demonstrate similarities

respective microbial communities, we focused in further experiments on the search for enzymes that are involved in the upper pathway of benzene metabolism under sulphate-reducing conditions. For that purpose, the volcanic sediment was chosen, since the protein yield per volume was higher. Two batch-cultures with benzene and benzoate and with volcanic sediment as inoculum were prepared. Growth was followed by the disappearance of the substrates and by the formation of sulfide. Both compounds were degraded within 45 days. The degradation of both compounds started immediately, and the formation of sulfide indicated that sulphate was used as electron acceptor (Fig. 4). Direct parameters for growth, e.g. cell numbers or protein concentrations, could not be determined due to the attachment of cells to the lava granules. After 45 days, the complete sediment of the batch-cultures (800 ml) was submitted to sample preparation and the extracted proteins were loaded on 2D-PAGEs.

The extraction of a threefold volume of lava granules in comparison to the volume used for

preparation of Fig. 2 as well as the additional cultivation in batch-cultures increased the total protein yield and enabled us to detect the proteins in the resulting gels with colloidal CBB stain (Fig. 5). However, loading higher protein amounts had a slightly negative effect on the separation quality, in particular in the acidic range some vertical streaks were observed again. The protein pattern after growth on benzene and benzoate containing ~450 spots appear to be very similar, only 23 were amplified in benzene grown community and 11 spots were amplified in benzoate grown community. Some gel regions with changed spots are highlighted in Fig. 5 with boxes. Although only one gel per community could be analyzed due to the limited amount of sample, the high percentage of unchanged spots in both communities gives an indication for the accuracy of the sample extraction procedure. However, reproducible quantification of the differences usually requires biologically independent replicates. However, an option for increasing quality of quantification in case of low amount of sample and missing





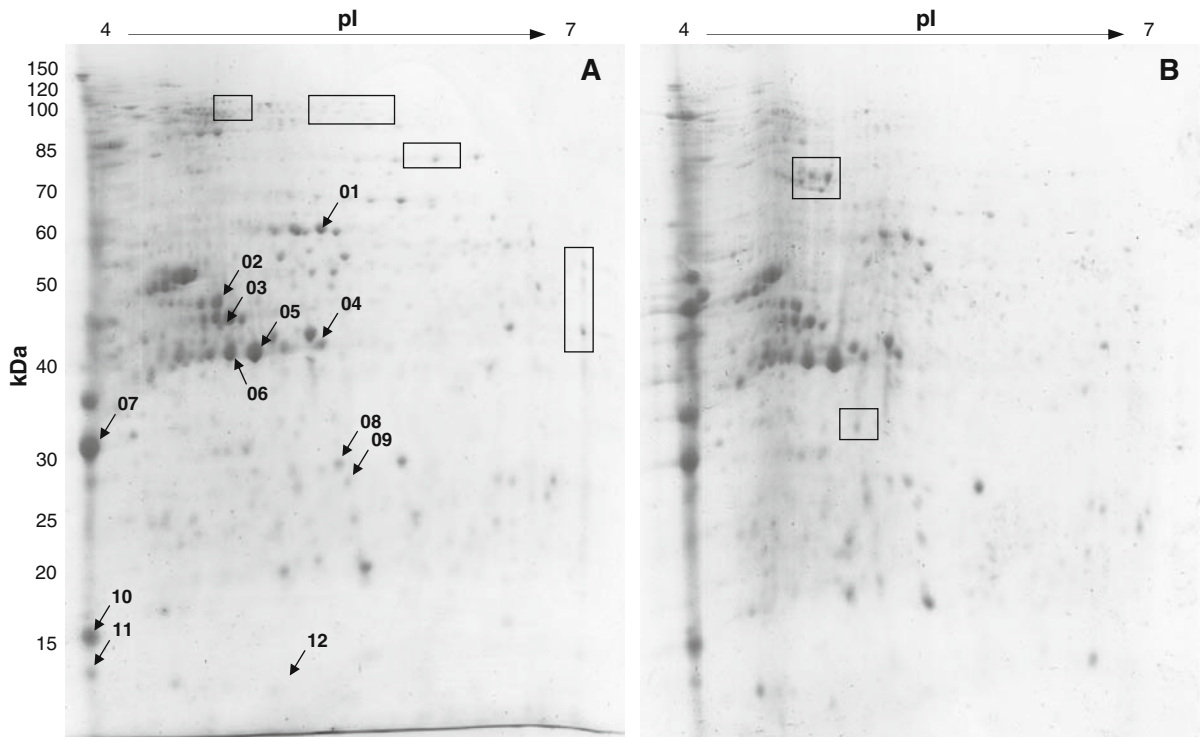
**Fig. 4** Benzene or benzoate consumption and sulphide production in microbial communities growing on benzene and benzoate. *Upper part:* Time course of benzene consumption (*solid circles*) and sulphide production (*open circles*) by the microbial community. *Lower part:* Control experiment with benzoate as carbon and energy source, the benzoate consumption is shown in *solid triangles* and the sulphide production by *open triangles*

replicated samples is to divide one sample into technical replicates and use more sensitive approaches like 2D fluorescent difference gel electrophoresis (2D-DIGE). Furthermore, the co-separation of two samples in one gel would facilitate the comparison of protein patterns and minimize the number of artefacts. However, the residual contaminations might interfere with the labelling of proteins with fluorescence dyes.

Assuming that the unknown enzymes of the benzene pathway are inducible by benzene, which is a typical characteristic of catabolic pathways, the amplified spots may be related to benzene degradation. They were submitted for tandem mass spectrometric analysis (MS/MS).

Protein identification by MS

Identification of proteins is essential for understanding the observed differences in the protein pattern from both sediments and for depicting the novel enzymes involved in benzene metabolism. Using the nanoLC ESI-IonTap MS and Mascot as search



**Fig. 5** 2D-gel electrophoresis of the sediment from the cultivated batch-cultures (800 ml) stained with colloidal CBB. The protein patterns after growth on **a** benzene and **b** benzoate appear to be highly similar, differences are indicated by *rectangles*

**Table 1** Identified proteins in the metaproteome profile of the microbial benzene community

No. Spot label	Protein name (seq. cov. %)	Gene ID of hits	Species homology	Functional group	Mowse score (MS/MS)	Total score BLASTp	Matched peptides	Sequence of peptides	Sherenga score
01	01-ESI ATP-synthase F1 alpha subunit (4)	89337108	<i>Desulfitobacterium hafnense</i>	Energy metabolism	94		3		
02	02-ESI ABC-transporter	22776991	<i>Oceanobacillus ithyensis</i> HTE831	ABC transporters		60	1	NLGFIIHQ	132
03	03-ESI Ompa-like protein precursor (5)	7978530	<i>Actinobacter radioresistens</i>	Metabolism of cofactors and vitamins	81		2		
04	04-ESI Acyl-CoA dehydrogenase, short-chain specific	51893615	<i>Symbiobacterium thermophilum</i> IAM 14863	Xenobiotics biodegradation and metabolism		62	1	SIV[E/V]GTSNIQK	152
05	05-ESI Regulatory protein (4)	71908523	<i>Dechloromonas aromatics</i>	Transcription factors	58		2		
06	06-ESI Phosphate ABC transporter, ATPase subunit	120602953	<i>Desulfovibrio vulgaris</i> subsp. <i>vulgaris</i> DP4	ABC transporters		66	1	IIGVGYGDY	180
07	07-ESI UDP-3-o-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase	49613257	<i>Erwinia carotovora</i> subsp. <i>atroseptica</i> SCRI1043	Glycan biosynthesis and metabolism		57	1	IGAFSAFK	112
08	08-ESI Enoyl-CoA hydratase (3)	33592544	Uncultured archaeon	Xenobiotics biodegradation and metabolism	66		1	WXQGITXXMEK	157
09	09-ESI Enoyl-CoA hydratase	10635042	<i>Azoarcus evansii</i>	Xenobiotics biodegradation and metabolism		64	1	YXEGXXAFMEK	169
10	10-ESI Conserved hypothetical protein	60494934	<i>Bacteroides fragilis</i> NCTC 9343	Membrane transport		57	1	LDDGDNAR	97
11	11-ESI Transcriptional regulatory protein	56380345	<i>Geobacillus kanstophilus</i> HTA426	Transcription factors		57	1	IXSTSGDPI	93

Using the nanoLC ESI-IonTap MS and Mascot as search engine (Mascot, <http://www.matrixscience.com>) against all bacterial species of NCBInr, four out of 70 analyzed proteins reached a MOWSE score that indicated significance. Further parameters given here are gene ID, species homology, mowse score or total score BLASTp as well as the number of peptides. These were in good accordance with the position of the spot in the gel. The identified proteins were classified by their function using KEGG database (Kyoto Encyclopedia of Genes and Genomes, <http://www.genome.jp/kegg/>) seq. cov. sequence coverage %

**Table 2** Identified proteins in the metaproteome profile of the microbial benzene community by using the LTQ Orbitrap XL MS and Mascot as search engine (Mascot, <http://www.matrixscience.com>), respectively, against all bacterial species of NCBIInr

No.	Spot label	Protein name (seq. cov. %)	Gene ID of hits	Species homology	Functional group	Mowse score (MS/MS)	Total score BLASTp	Matched peptides
01	01-LTQ	ATP synthase F1, alpha subunit (6)	106895264	<i>Clostridium</i> sp. OhLAs	Energy metabolism	123		3
02	02-LTQ	Conserved hypothetical protein (8)	117579390	<i>Gramella forsetii</i> KT0803		224		4
03	03-LTQ	Outer membrane protein precursor (OmpA-like) (15)	50083922	<i>Acinetobacter</i> sp. ADP1	Metabolism of cofactors and vitamins	233		7
04	04-LTQ	Enolase (3)	27902665	<i>Methylobacterium extorquens</i>	Carbohydrate metabolism	83		1
04	04-LTQ	Acyl-CoA dehydrogenase	77995041	<i>Carboxydothermus hydrogenoformans</i> Z-2901	Xenobiotics biodegradation and metabolism		479	7
05	05-LTQ	Acyl-CoA dehydrogenase	85722956	<i>Syntrophus aciditrophicus</i> SB	Xenobiotics biodegradation and metabolism		199	3
05	05-LTQ	Acyl-CoA dehydrogenase	77995041	<i>Carboxydothermus hydrogenoformans</i> Z-2901	Xenobiotics biodegradation and metabolism		322	4
07	07-LTQ	Butyryl-CoA dehydrogenase	110819152	<i>Rhodococcus</i> sp. RHAI	Xenobiotics biodegradation and metabolism		188	3
06	06-LTQ	Acyl-CoA dehydrogenase	77995041	<i>Carboxydothermus hydrogenoformans</i> Z-2901	Xenobiotics biodegradation and metabolism		76	1
07	07-LTQ	Porin outer membrane protein	86281089	<i>Rhizobium etli</i> CFN 42	Metabolism of cofactors and vitamins		146	2
09	09-LTQ	Dienoyl-CoA hydratase	56314488	<i>Azoarcus</i> sp. EbN1	Xenobiotics biodegradation and metabolism		168	2
12	12-LTQ	Thioredoxin	118413975	<i>Campylobacter fetus</i> subsp. fetus 82-40	Nucleotide metabolism		178	3
		Thioredoxin	71848231	<i>Dechloromonas aromatica</i> RCB	Nucleotide metabolism		141	2

Further parameters given here are gene ID, species homology, mowse score or total score BLASTp as well as the number of peptides. These were in good accordance with the position of the spot in the gel. The identified proteins were classified by their function using KEGG database (Kyoto Encyclopedia of Genes and Genomes, <http://www.genome.jp/kegg/>)

All species used for cross-species homology indicated an anaerobic lifestyle or species able to degrade aromatic compounds  
seq. cov. sequence coverage %

engine, four out of 70 analyzed proteins reached a MOWSE score that indicated significance (Table 1). However, the sequence coverage and the numbers of peptides were low. With respect to enoyl-CoA hydratase (08-ESI), the ATP-synthase F1 alpha subunit (01-ESI), the ompA-like protein precursor (03-ESI) and the regulatory protein (05-ESI) were present with at least two peptides and Mascot scores of 66, 94, 81 and 58, respectively.

All four proteins could be linked with one of the following entries: species with anaerobic lifestyle, species able to degrade aromatics and enzymes potentially linked with anaerobic benzene metabolism. The identity of 08-ESI was confirmed by de novo sequencing from the same set of results with a peptide showing 72% identity to enoyl-CoA hydratase from an uncultured *archaeon*. From another spot (09-ESI) where Mascot search completely failed, a short sequence could be derived, that matches also to a putative enoyl-CoA hydratase of *Azoarcus evansii*, a species well-known for anaerobic degradation of toluene (Harwood et al. 1999). Enoyl-CoA hydratase is involved in benzoate metabolism (Harwood et al. 1999).

These results indicated that benzoate might be a metabolite of benzene degradation, as presumably observed by several authors (Boll et al. 2002; Chakraborty and Coates 2005). Whereas this fact was encouraging, the low percentage of identified spots requires further attention. One reason could be the relatively low intensity of spots in the CBB gels. Another reason is indeed the sparse availability of genome data that makes de novo sequencing necessary as shown for proteins 04-ESI and 05-ESI. Therefore, a subset of 12 samples was submitted to an OrbiTrap-MS and to Mascot searches (Table 2).

The following data processing of not clearly identified spectra by PepNovo (Frank and Pevzner 2005) and the search of received sequence tags by MS-Blast resulted in a significant improved identification, e.g. enoyl-CoA hydratase (09-LTQ), the ATP-synthase F1 alpha subunit (01-LTQ), the ompA-like protein precursor (03-LTQ), which were found with Mascot values that were in average two times higher than those found on the basis of ESI-MS/MS. In addition, further identifications were achieved, e.g. thioredoxin (12-LTQ) and acyl-CoA dehydrogenase from *Carboxydotherrmus hydrogenoformans* (04-LTQ). The enzymes of the family of acyl-CoA dehydrogenase

are also involved in benzoate metabolism (Harwood et al. 1999).

In conclusion, the selection of a more sensitive MS in combination with a more sophisticated search algorithm resulted in a considerably higher chance to identify low abundant proteins from environmental samples.

## Concluding remarks

Current results clearly demonstrate that metaproteome analysis has potential to analyse environmental samples containing species that cannot be cultivated as pure cultures in the laboratory. The improvement and adaptation of the extraction procedure to new environmental samples with respect to proteins extraction and separation is a requirement to analyse anoxic, slow growing microbial communities containing low cell numbers and low amounts of biomass. Nevertheless, metaproteome analysis provides access to biochemical processes in situ (Barreiros et al. 2003) and has the potential to contribute to the elucidation of metabolic pathways actually taking place within bacterial communities, e.g. the anaerobic benzene degradation pathway. Future technical developments should aim on increasing percentage of identified proteins.

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