Identification of benzene-degrading Proteobacteria in a constructed wetland by employing in situ microcosms and RNA-stable isotope probing

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Abstract

Constructed wetlands (CWs) are effective ecological remediation technologies for various contaminated water bodies. Here, we queried for benzene-degrading microbes in a horizontal subsurface flow CW with reducing conditions in the pore water and fed with benzene-contaminated groundwater. For identification of relevant microbes, we employed in situ microcosms (BACTRAPs, which are made from granulated activated carbon) coupled with ¹³C-stable isotope probing and Illumina sequencing of 16S rRNA amplicons. A significant incorporation of ¹³C was detected in RNA isolated from BACTRAPs loaded with ¹³C-benzene and exposed in the CW for 28 days. A shorter incubation time did not result in detectable ¹³C incorporation. After 28 days, members from four genera, namely Dechloromonas, Hydrogenophaga, and Zoogloea from the Betaproteobacteria and Arcobacter from the Epsilonproteobacteria were significantly labeled with ¹³C and were abundant in the bacterial community on the BACTRAPs. Sequences affiliated to Geobacter were also numerous on the BACTRAPs but apparently those microbes did not metabolize benzene as no ¹³C label incorporation was detected. Instead, they may have metabolized plant-derived organic compounds while using the BACTRAPs as electron sink. In representative wetland samples, sequences affiliated with Dechloromonas, Zoogloea, and Hydrogenophaga were present at relative proportions of up to a few percent. Sequences affiliated with Arcobacter were present at < 0.01% in wetland samples. In conclusion, we identified microbes of likely significance for benzene degradation in a CW used for remediation of contaminated water.

Keywords Benzene degradation · Constructed wetlands · BACTRAPs · RNA-SIP · Betaproteobacteria

Introduction

Benzene is an important petroleum constituent and is thus a frequent contaminant in various environments where it may pose a serious ecotoxicological and human health risk (EEA 2014). To cope with these still ubiquitous contaminations, a suite of treatment approaches has been developed, including cost-effective bioremediation options where various microbes can degrade benzene aerobically or anaerobically (Farhadian et al. 2008). Many aerobic benzene-degrading microbes have been isolated, and their catabolism has been studied in great detail (Pérez-Pantoja et al. 2010). Anaerobic degradation proceeds much slower than its aerobic counterpart. Doubling times of laboratory cultures range from weeks to months, and biomass yields are low (Meekenstock et al. 2016). Identification of the particular benzene-degrading microbes and their degradation routes involved in situ will lead to a better assessment and restoration of contaminated sites.

Various stable isotope probing (SIP) approaches have been developed and applied to trace a chemical’s fate in a mixed microbial community and identify those microbes which thrive off the compound of interest (Neufeld et al.
Materials and methods

Chemicals

$^{13}$C$_{6}$-benzene (≥ 99 atomic percent) was obtained from Campro Scientific (Berlin, Germany). Granulated activated carbon (GAC) (type: AUF 540) as carrier material in the BACTRAPs was from Adako GmbH, Düsseldorf, Germany. All other chemicals were of analytical grade quality and were purchased from Sigma-Aldrich (Saint Louis, MO, USA), Roth (Karlsruhe, Germany), or Merck (Darmstadt, Germany).

Field-site description

The field site of this study was the pilot-scale treatment plant “compartment transfer” (CoTra) in Leuna (51° 18′30″ N, 12° 01′19″ E), Saxony-Anhalt, Germany. Previously, horizontal subsurface flow CW systems were built next to a former refinery and industrial site where the anoxic groundwater is strongly contaminated with benzene to investigate near-natural remediation strategies (Seeger et al. 2011a, b). Detailed descriptions of the CW systems are provided in those publications. In brief, the chosen CW (5 m length × 1.1 m width × 0.5 m depth) was filled with fine gravel (quartz, grain diameter of 2 to 3.2 mm) and planted with common reed, Phragmites australis (Fig. 1a). Inflow water contained benzene at a concentration of up to 7.5 mg L$^{-1}$ and was supplied untreated from the nearby located contaminated groundwater. Benzene concentrations of the inflow, along the flow path through the CW and the outflow were determined by head-space GC-MS ($n = 6$) as described before (Seeger et al. 2011b). Concentrations during this study were (mg L$^{-1}$) as follows: inflow, 5.70 ± 1.8; 1.25 m, 2.99 ± 2; 2.5 m, 2.24 ± 0.9; 3.75 m, 1.68 ± 1.4; and outflow, 1.53 ± 1.5. Other water characteristics along the flow path were determined ($n = 6$) as reported before and were of similar values as in the previous studies (Seeger et al. 2011a, b): circumneutral pH; TOC decreasing from around 32 to 21 mg L$^{-1}$; ammonium decreasing from around 50 to 25 mg L$^{-1}$; $E_{h}$ of around 0 mV in the inflow and around −100 mV in the CW and outflow; nitrate < 0.07 mg L$^{-1}$; sulfate decreasing from around 100 to 13 mg L$^{-1}$; and ferric and ferrous iron decreasing from around 2.7 to < 0.3 mg L$^{-1}$.

Preparation of in situ microcosms

BACTRAPs were custom made essentially by following a previously described experimental set-up (Geyer et al. 2005). Briefly, GAC as carrier material was heated to 300 °C for 4 h to remove biogenic organic carbon residues and afterwards autoclaved at 121 °C for 20 min for rehydration (Schurig et al. 2015). To load the substrate, the
Air-dried granulate was transferred into a small desiccator, air was exchanged against nitrogen gas (N\textsubscript{2}), and an open vessel with 88 mg g\textsuperscript{-1} GAC of either [\textsuperscript{13}C\textsubscript{6}]-labeled or unlabeled benzene was placed inside. The desiccator was evacuated to 50 mbar and held at this pressure until the substrate was evaporated from the reservoir vessel. A third batch of GAC was treated the same way except that no benzene was added. Below, we refer to those GAC batches and the corresponding BACTRAPs as “\textsuperscript{13}C,” “\textsuperscript{12}C,” and “blank.”

For incubations of the in situ microcosms in the rhizosphere of the CW, two perforated and threefold segmented cages (stainless steel, 120 × 50 × 10 mm) were designed and framed in steel spades (Fig. 1b). The \textsuperscript{13}C, \textsuperscript{12}C, and blank GAC were separately transferred into the segments of two cages, which were exposed perpendicular to the flow direction, one for 6 and the other for 28 days at a depth of 150 mm, 1.5 m downstream of the systems’ inflow (Fig. 1c, d). At the beginning and at the end of the exposition time, representative wetland rhizosphere samples were taken from about 10 cm around the installed BACTRAP systems. A custom-made lance with closable sampling chamber was pushed to the sampling point (partly visible in Fig. 1c), and the aperture was opened. By moving the instrument mixed samples of gravel, sediments and roots were taken and after closing the aperture...
removed from the system. All BACTRAP and wetland samples were frozen immediately and stored at −80 °C until analysis.

**RNA extraction, isopycnic centrifugation, and cDNA synthesis**

We employed RNA-SIP (Lueders et al. 2016) for identification of active in situ benzene degraders. RNA from BACTRAPS was extracted from the GAC according to a modified protocol from Bombach et al. (2010). Three grams of GAC was distributed to twelve 2-mL bead-beating vials each filled with 0.2 mL of an equal-volume mixture of 0.1 and 1.0 mm zirconia beads (BioSpec Products, Bartlesville, OK, USA) and mixed with 300 μL sodium acetate buffer (50 mM Na-acetate at pH 5.3, 10 mM Na-EDTA), 200 μL of 200 mM tri-sodium phosphate solution, 50 μL 20% (w/v) sodium dodecyl sulfate solution and 450 μL phenol preheated to 65 °C. After incubation at 65 °C for 10 min, cells were disrupted by bead-beating twice at 6.5 m s⁻¹ for 60 s in a FastPrep-24 Instrument (MP Biomedicals, Santa Ana, CA, USA), frozen at −80 °C for 5 min, thawed again, and centrifuged at 16,100 relative centrifugal force (rcf) and 4 °C for 5 min. From each tube, 400 μL of the upper aqueous phase was transferred to Phase Lock Gel Heavy tubes (Eppendorf, Hamburg, Germany). Acetate buffer (200 μL) was added to each remaining phase, the mixtures were carefully vortexed and centrifuged again at 16,100 rcf and 4 °C for 5 min; 400 μL of each upper aqueous phase, combined with the 400 μL previously transferred phase was extracted with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1, v/v/v) and centrifuged at 16,100 rcf and 4 °C for 5 min. All extracted RNA was pooled and re-extracted with the clean-up protocol of the RNeasy Mini Kit including DNA on-column digestion (RNase-Free DNase Set, Qiagen, Hilden, Germany). The RNA was eluted in water, and its concentration and quality were determined by spectrophotometry (Nanodrop, Thermo Scientific, Wilmington, NC, USA) and Lab-on-a-Chip electrophoresis (Agilent Bioanalyzer, Agilent RNA 6000 Nano Kit, Agilent Technologies, Santa Clara, CA, USA).

The extracted RNA was fractionated by equilibrium density gradient centrifugation and gradient fractionation following a modified protocol of Lueders et al. (2004). To prepare the cesium trifluoroacetate (CsTFA) gradient, 750 ng of RNA per sample was mixed with up to 970 μL gradient buffer (0.1 M Tris-HCl at pH 8, 0.1 M KCl, 1 mM EDTA), 5.3 mL CsTFA (IllustraCsTFA, GE Healthcare, Chalfont St Giles, Great Britain), and 200 μL formamide. The mixture’s buoyant density (BD) was adjusted to 1.79 g mL⁻¹ (AR200 Automatic Digital Refractometer, Reichert Analytical Instruments, Depew, NY, USA) and transferred to Quick-Seal Polyclaromer tubes (Beckman Coulter, Brea, CA, USA). The tubes were centrifuged at 39,000 rpm (≈130,000 rcf) and 20 °C for 69 h in an ultracentrifuge (OPTIMA L-90K, Beckman Coulter) using a Near-Vertical Rotor (NVT 65.2, Beckman Coulter). Subsequently, they were fractionated in 12 fractions by displacement with water from below as described previously (Lueders et al. 2004). For each collected fraction, the density was determined using a refractometer. The RNA was recovered from the fractions by using the RNeasy Mini Kit (Qiagen, RNA clean-up protocol), eluted in water, and its concentration and quality were determined using the Nanodrop and Agilent Bioanalyzer. Fractionated RNA considered of sufficient quantity for sequencing was reverse-transcribed using the Omniscript Reverse Transcription Kit (Qiagen) with random hexamer primer (10 μM) or with the Sensiscript Transcription Kit (Qiagen) with the primer Uni909R (1 μM), 5′-CCG YGA ATT CTC AG-3′ (Kuntze et al. 2011) for bamA transcript quantification via quantitative reverse transcription PCR (RT-qPCR; see below), respectively.

RNA from wetland samples was extracted with the PowerSoil Total RNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer’s instructions. Briefly, cells in the sediment samples were lysed using silica carbide beads, lysis buffers, and phenol/chloroform/isoamyl alcohol. Total RNA was bound on a column matrix, washed and eluted in water. To remove potentially present residual DNA the extract was treated using the Ambion DNA-free Kit (Lifetechnologies, Carlsbad, CA, USA) followed by purification with the RNeasy Mini Kit (Qiagen). Extracted RNA was then transcribed into cDNA using the SuperScript III Reverse Transcriptase Kit (Invitrogen) with random hexamer primer (10 μM).

**Amplicon library preparation and Illumina sequencing**

Microbial community profiling was carried out by Illumina sequencing of 16S rRNA amplicons. For that, we used rhizosphere samples taken in the proximity of the BACTRAPS at days zero and 28, non-fractionated RNA from the BACTRAP incubated for 6 days, and those fractions of the BACTRAP incubated for 28 days with sufficient amounts of RNA for sequencing (closed symbols in Fig. 2). The latter included the fractions 12 to 7 of the 13C benzene-loaded BACTRAP (BD 1.742 to 1.769 g mL⁻¹), fractions 12 to 9 of the 12C-benzene-loaded BACTRAP (BD 1.742 to 1.769 g mL⁻¹) and the fractions 12 to 10 of the blank-BACTRAP (BD 1.747 to 1.764 g mL⁻¹). Amplicon library construction was performed following the general procedure of Camarinha-Silva et al. (2014). Briefly, the V1–2 region of the 16S rRNA-derived cDNA was amplified using the universal primers 27F, 5′-AGA GTT TGA TCM TGG CTC AG-3′ and 1492R, 5′-TAC GGY TCC AGG CTA TTG AT-3′.
Fig. 2 16S rRNA extracts, fractionated over buoyant density via isotopic centrifugation in CsTFA. Shown are the 16S rRNA density profiles of the BACTRAP compartments loaded with $^{13}$C (filled empty squares), $^{13}$C-benzene (filled and empty triangles), and unloaded blank (filled and empty circles), incubated for 28 days in the pilot-scale-constructed wetland. Fractions used for Illumina-based amplicon sequencing are indicated with closed symbols. Highest mean copy numbers enumerated with 16S rRNA-target RT-qPCR were $5.89 \times 10^8 \pm 3.34 \times 10^8$ copies mL$^{-1}$, $3.96 \times 10^8 \pm 3.04 \times 10^8$ copies mL$^{-1}$, and $9.17 \times 10^7 \pm 9.27 \times 10^7$ copies mL$^{-1}$ for the $^{12}$C-loaded, $^{13}$C-loaded, and blank BACTRAP, respectively. Lowest mean copy numbers (highest density fraction) were $2.18 \times 10^5 \pm 7.02 \times 10^3$, $3.94 \times 10^5 \pm 8.63 \times 10^3$, $3.96 \times 10^8 \pm 3.04 \times 10^7$, and $2.52 \times 10^5 \pm 3.02 \times 10^4$ copies mL$^{-1}$ for the $^{13}$C-loaded, $^{12}$C-loaded, and blank BACTRAP, respectively.

Sequencing data analysis

A custom pipeline described in detail in Camarinha-Silva et al. (2014) was used for further processing and analysis of sequence data. In brief, obtained sequences were first filtered according to specific quality requirements (discarding reads with low Phred scores, containing an N character, mismatches with primer or barcode sequences, or homopolymer stretches > 10). Subsequently the individual reads were trimmed to 120 nt by removing primer and barcode sequences, and the read pairs were combined to 240 nt-long sequences. In downstream analyses, they were clustered and filtered to consider exclusively phylotypes that (i) were present in at least one sample at a relative abundance > 0.1% of the total sequences of that sample or (ii) were present in at least five samples at a relative abundance > 0.01% for a given sample.

Obtained operational taxonomic units (OTUs) and their abundances were used in rarefaction analysis with R software using the VEGAN package (Oksanen et al. 2013). Chao1 richness estimators and Shannon diversity indices were calculated with the EstimateS software package (Colwell 2013). All OTUs were assigned a taxonomic affiliation based on naïve Bayesian classification (RDP classifier) (Wang et al. 2007) with an assignment cut-off of 70% confidence threshold. Dissimilarities in the various community structures were analyzed based on Bray-Curtis distance matrices using the R software package.

Calculation of OTU-specific enrichment factors in labeled 16S rRNA

To identify OTUs derived from cells which had incorporated $^{13}$C label from benzene, enrichment factors (EFs) were computed as previously done in RNA-SIP (Kramer et al. 2016) using the following equation:

$$EF = \frac{\text{heavy}^{13}\text{C}/\text{light}^{13}\text{C}}{-\text{heavy}^{12}\text{C}/\text{light}^{12}\text{C}}$$

where “heavy $^{13}$C” and “heavy $^{12}$C” were the relative abundance of a specific phylotype in fractions with a BD of 1.769 g mL$^{-1}$, and “light $^{13}$C” and “light $^{12}$C” the same for fractions with a BD of 1.742 g mL$^{-1}$. EF values > 0.5 were considered a result of isotopic labeling of the 16S rRNA of the particular OTU.

Quantitative reverse transcription PCR

Synthesized cDNA was analyzed via SYBR Green-based RT-qPCR, performed with a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Total bacterial 16S rRNA was quantified with the universal primers 519F, 5'-CAG CCG CCG TAA NWC-3' (Lane 1991) and Uni909R, 5'-CGA ATT CMT TTR AGT-3' (modified from Wang and Qian 2009) (= RT-qPCR I). Geobacteraceae 16S rRNA was determined with the primers Geo564F, 5'-AAG CGT TGT TCG GAW TTA T-3' (Etchebehere and Tiedje 2005) in a first amplification reaction. In a second and a third reaction, the primers were extended with specific sequences to insert sample-tracking barcodes and indices and mandatory process-specific Illumina sequences (see further details in Camarinha-Silva et al. 2014). Non-template controls were free of any amplification products after all three PCR assays. PCR amplicons were purified, quantified with the Quant-iT PicoGreen dsDNA reagent and kit (Invitrogen, Darmstadt, Germany), and prepared for Illumina sequencing exactly as described (Camarinha-Silva et al. 2014). Equimolar amounts of the amplicon libraries were 250 nt paired-end sequenced on a MiSeq System (Illumina, San Diego, CA, USA). Image analysis and base calling were accomplished using the Illumina Pipeline (version 1.7).
YTC CTG RC-3'), which were developed for detection of benzene-degrading anaerobes including Geobacter species (Kuntze et al. 2011) (= RT-qPCR III). In addition to template cDNA, PCR reaction mixtures contained 0.2 μM of each primer in RT-qPCR I (519F and Uni909R) and 0.3 μM in RT-qPCR II (Geo562F and Geo840R) and RT-qPCR III (SP9 and ASP1), and 1× of the KAPA SYBR FAST qPCR MasterMix (PEQLAB Biotechnology GmbH, Erlangen, Germany) (volume of 11.5 μL). PCR cycle parameters were as follows: initial denaturation at 95 °C for 2 min, followed by 40 cycles composed of denaturation at 95 °C for 3 s, annealing at 52 °C (qPCR I), respectively, 57 °C (qPCR II) and 59 °C (qPCR III) for 20 s and extension at 72 °C for 20 s. At the end of the qPCR analysis, a DNA melting curve was recorded by short denaturation at 95 °C for 3 s, heating at 60 °C for 1 min and then increasing the temperature to 95 °C by 0.3 °C min⁻¹. Standard curves were prepared using a cloned 16S rRNA gene fragment (27F-1378R) of Pseudomonas putida mt-2 for RT-qPCR I, a purified 16S rRNA gene fragment (27F-1492R) of Geobacter metallireducens GS-15 amplified during PCR for RT-qPCR II, and a bamA gene isolated from a toluene-degrading consortium of a planted fixed-bed reactor (Lünsmann et al. 2016a), cloned and re-amplified for RT-qPCR III. Standard curves were linear (R² > 0.999) over at least six orders of magnitude and amplification efficiency was > 85% for RT-qPCR I and II and 70–80% for RT-qPCR III. No signals were detected in controls without template.

**Nucleotide sequence accession number**

The sequence data were deposited at NCBI under BioProject ID/SRA accession number PRJNA483932.

**Results**

**Fractionation of RNA showed incorporation of benzene-derived ¹³C into biomass**

In order to trace microbial benzene degradation on the BACTRAPs via incorporation of ¹³C, RNA was extracted and fractionated via isopycnic centrifugation. BACTRAP incubation for 6 days in the CW did not lead to observable ¹³C incorporation into RNA as the BD profiles of the RNA extracts of the ¹³C-BACTRAP and ¹²C-BACTRAP were essentially identical (data not shown). After 28 days of incubation, the BD profile of the RNA extract from the ¹³C-BACTRAP was shifted to a higher density compared with the ¹²C-control and the blank, which revealed that ¹³C from benzene had been used as carbon source by some microbes (Fig. 2). 16S rRNA copy numbers were much lower yet still appreciable in the fractions of the blank microcosm. This bacterial colonization of the unloaded carrier suggested that carbon sources from the surrounding pore water were used.

**Microbial community structures on BACTRAPs and in the nearby rhizosphere**

Microbial community profiling was carried out by Illumina sequencing of 16S rRNA amplicons. A total of 321,582 sequence reads of a length of 240 bp were obtained after quality filtering. The number of reads in all 18 samples ranged from 6724 to 28,058 per sample. These high-quality reads comprised a total of 1001 OTUs. The number of OTUs per sample and the calculated species richness (non-parametric Chao1 estimator) and species diversity (Shannon index) are reported in Table S1. Chao1 estimator and rarefaction curves (Fig. S1) showed that the read numbers obtained were high enough to reach satisfactory diversity coverage of the microbial community. The OTUs affiliated to 19 phyla (98.6% of all sequences), 22 classes (76%), 42 orders (71%), 77 families (59%), and 133 different genera (47%). A list of phylogenetic affiliations of the obtained sequences is provided in Table S2. A tree based on Bray-Curtis similarities revealed that the BACTRAP communities from days 6 to 28 formed two groups which were substantially different from the communities of the rhizosphere (Fig. S2).

As expected, the wetland community was composed of many phylotypes typically found in (semi-)aquatic habitats and soils, most of which belonged to the Proteobacteria (roughly 80% relative abundance) and Actinobacteria (up to 10% relative abundance) (Fig. 3). Acidobacteria, Bacteroidetes, Chloroflexi, and Nitrospirae made up between 1 and 5% each of the total wetland community. In all BACTRAP communities, members of Proteobacteria were still the most abundant phylum (around 71 and 79% of sequence reads at days 6 and 28, respectively) albeit with pronounced differences to the wetland communities at lower
taxonomic levels: Alphaproteobacteria were less abundant on the BACTRAPS (about 5 vs. about 20% relative abundance in the wetland samples) while Deltaproteobacteria and Epsilonproteobacteria were enriched in the BACTRAP samples (about 31 vs. about 15% in wetland samples for Deltaproteobacteria and about 2 vs. about 0.1% for Epsilonbacteria). The relative abundance of Betaproteobacteria was not substantially different in the various communities (around 25%); however, in the wetland samples most of the Betaproteobacteria belonged to the Burkholderiales while Rhodocyclales were dominant on the BACTRAPS.

Furthermore, Actinobacteria made up only about 1% of the BACTRAP communities while Firmicutes (about 0.3% in the wetlands vs. about 4% on the BACTRAPS) and Fusobacteria (<0.1% in the wetlands samples vs. about 17% in the BACTRAP samples after 6 days and about 4% after 28 days) were strongly enriched. Most Firmicutes sequences affiliated with the family Clostridiaceae (mostly OTU_0245, genus Clostridium sensu stricto, and OTU_0257 without further classification), and the family Carnobacteriaceae (mostly OTU_0237, genus Trichococcus). There were no Firmicutes sequences indicating the presence of Peptococcaceae, which have been identified previously as anaerobic benzene degraders (Kunapuli et al. 2007; Herrmann et al. 2010; van der Zaan et al. 2012; van der Waals et al. 2017). All of the Fusobacteria sequences affiliated with the family Fusobacteriaceae (mostly OTU_0287, 0288, 0289, no further classification). Relative abundances of Acidobacteria, Chloroflexi, and Nitrospirae were about the same or slightly lower in the BACTRAPS as compared with the wetland samples. The abundance of Bacteroidetes did not differ much between BACTRAP and wetland communities.

Identification of benzene degraders on the in situ microcosms

To determine which taxa played a key role in benzene turnover, we searched for sequence abundance profiles in the fractionated RNA of the BACTRAP communities of day 28 that matched the following two criteria: (i) a shift to higher density fractions in RNA from the $^{13}$C-loaded BACTRAP as compared with RNA from the $^{12}$C-loaded compartment as shown by an EF value > 0.5 (Kramer et al. 2016), and (ii) a relative abundance > 1% in the highest density fraction of the 13C-BACTRAP from day 28. The first criterion was matched by 269 OTUs, out of which 6 OTUs remained after applying the second criterion. The excluded 263 OTUs with an EF > 0.5 had a combined relative read abundance of 13.2%. The remaining 6 OTUs affiliated with the betaproteobacterial genera Dechloromonas, Zoogloea (both Rhodocyclales), and Hydrogenophaga (Burkholderiales), as well as the epsilonproteobacterial genus Arcobacter (Fig. 3). Sequence reads of these OTUs had a combined relative abundance of 52% in the highest density fraction (Table S2). Dechloromonas was represented by OTU_0536 (EF = 1.89; relative abundance of 24.5%), which had a sequence identical to that of the D. hortensis MA-1 and D. denitrificans ED1-type strains and accounted for about 91% of all reads affiliated with that genus (Fig. S3). While almost all other OTUs affiliated with Dechloromonas had EF values > 0.5, the relative abundance in the highest density fraction was 0.36% at the most. Among the sequence reads indicating the presence of Zoogloea spp., about 81% belonged to two sequence types, namely OTU_0585 (EF = 5.39; 13.2% relative abundance; identical in sequence to Z. ramigera ATCC 19544T) and OTU_0586 (EF = 2.22; 10.1% relative abundance; 99.6% sequence identity to Z. caeni EMB 43T) (Fig. S4). The remaining OTUs affiliated with Zoogloea and having an EF value > 0.5 had at most a relative abundance of 0.31%. The genus Hydrogenophaga was represented by OTU_0482 (EF = 2.35; 2% relative abundance; 98.8% sequence identity to H. flava CCUG 16581T) and OTU_0466 (EF value of 1.17; relative abundance of 1.2%, 99.2% sequence identity to H. flava CCUG 16581T) (Fig. S5). These two OTUs together comprised 78.3% of all Hydrogenophaga sequences in the highest density fraction. All other OTUs affiliated with Hydrogenophaga had a relative abundance of at most 0.6% in the highest density fraction (Fig. 4). The genus Arcobacter was represented by OTU_0771 (EF value = 11.0; 1.27% relative abundance in the highest density fraction; 99.2% sequence identity to A. aquimarinus CECT8442 T) (Fig. S6). Sequences grouped into OTU_0771 made up 82.8% of all sequences affiliated with Arcobacter.

On the BACTRAP compartments incubated for 6 days Dechloromonas, Zoogloea, Hydrogenophaga, and Arcobacter sequence types had relative total abundances of 7.5 to 13%, 0.6 to 2.5%, 2.5 to 3.6%, and 0.13 to 0.37%, respectively. In the wetland samples, the relative abundance of Zoogloea, Hydrogenophaga, and Dechloromonas ranged between 0.05 and 4.9%. The relative abundance of the Arcobacter phylotype was <0.01% in the wetland samples (Table S2).

Several other taxa described previously as capable of benzene degradation or detected in SIP studies with ex situ microcosms targeting benzene transformation (Aburto and Ball 2009; Aburto and Peimbert 2011; Jechalke et al. 2013; Kunapuli et al. 2007; Liou et al. 2008) were present on the BACTRAPS and in the wetland samples, namely Acinetobacter, Arthrobacter, Acidovorax, Azorarcus, Desulfovibacteraceae, Pseudomonas, and Rhodoferax. However, all of them occurred with an abundance of less than 1% in any sample and were not enriched for $^{13}$C.
Geobacter colonized the BACTRAPs but apparently did not metabolize benzene

Up to around 30% of all sequences in the BACTRAP communities affiliated to the genus Geobacter (Table S2). Geobacter can be common in sub-surface zones where iron reduction and benzene degradation occur concomitantly (Rooney-Varga et al. 1999), and some Geobacter strains have been described as anaerobic benzene degraders (Zhang et al. 2012). However, almost all OTUs affiliated with Geobacter had an EF value around zero or in the negative range (Fig. S7a). The few OTUs with an EF value > 0.5 had a combined relative abundance of 0.37%. In addition, Geobacteraceae 16S rRNA was assayed by RT-qPCR (Cummings et al. 2003) in all fractionated RNA samples from exposure day 28. This analysis supported the Illumina sequencing-based enumeration, although 16S rRNA copy numbers of Geobacteraceae were up to three times lower than those measured for Geobacter via Illumina sequencing (Fig. S7b). Thus, there was no evidence that Geobacter had incorporated the $^{13}$C label. Furthermore, we tested whether Geobacter might have used benzene as electron-donor while using a different or even no C-source (i.e., no benzene-based anabolism). Presumably, anaerobic degradation of benzene by Geobacter occurs via the benzoyl-CoA pathway (Zhang et al. 2013). Hence, we RT-qPCR-assayed the extracted RNA for the presence of bamA transcripts, encoding oxocyclohex-1-ene-1-carbonyl-coenzyme A hydrolase, which is an enzyme of this pathway (Fuchs et al. 2011). The bamA gene has previously been used as a molecular marker to query contaminated sites for the presence of anaerobic bacteria capable of degrading aromatic hydrocarbons and the used primers were shown to be appropriate to amplify Geobacter bamA genes (Kuntze et al. 2011). No evidence was obtained for the presence of bamA transcripts on the BACTRAPs. In the wetland samples the total relative abundances of Geobacter were < 0.2%. Likewise, there was no evidence for label incorporation by Carnobacteriaceae, Clostridiaceae, and Fusobacteriaceae.

Discussion

Detailed mass balancing had indicated that microbial transformation is paramount for the fate of benzene in model CWs (Seeger et al. 2011a, b). In this study, microbial degradation of benzene on BACTRAPs placed within a CW was demonstrated by the incorporation of $^{13}$C into RNA. RNA-SIP coupled to Illumina 16S rRNA amplicon sequencing revealed Dechloromonas, Zoogloea, Hydrogenophaga, and Arcobacter as benzene degraders on the BACTRAPs. The first three genera have been associated previously with benzene metabolism. We are not aware of any publication in
which *Arcobacter* was described as (potential) benzene degrader. *Dechloromonas aromatica* strain RCB is a facultative anaerobe for which benzene oxidation coupled to the reduction of oxygen, chloride, or nitrate was reported (Coates et al. 2001). Several studies indicated *Zoogloea* as an important benzene degrader in microbial communities (Kasai et al. 2006; Takahata et al. 2006; Weelink et al. 2007; Jechalke et al. 2013). Interestingly, RNA-SIP on microcosms derived from a microoxic and BTEX-contaminated aquifer showed that several members of the *Rhodocyclaceae*, including *Dechloromonas* and *Zoogloea*, incorporated $^{13}$C from labeled toluene (Bradford et al. 2018). Phylotypes closely related to *Hydrogenophaga* (*Comamonadaceae, Burkholderiales*) were abundant in aerobic microcosms generated from benzene-contaminated hypoxic groundwater samples (Fahy et al. 2006; Aburto et al. 2009) and were tentatively identified as benzene degraders by RNA-SIP coupled with denaturing gradient gel electrophoresis (DGGE) (Aburto and Ball 2009). The benzene-degrading *Hydrogenophaga* sp. strain Rs71 was isolated from the same aquifer (Fahy et al. 2006). A phylotype closely related to this strain had incorporated carbon from $^{13}$C-benzene in a SIP experiment with biofilm material (Jechalke et al. 2013). Similarly, employing DNA-SIP coupled with terminal restriction fragment length polymorphism (T-RFLP) with a microcosm derived from soil contaminated with benzene a phylotype was identified that affiliated with *Polaromonas*, another member of the family *Comamonadaceae* (Xie et al. 2011).

The absence of an RNA abundance maximum at a BD of 1.82 g mL$^{-1}$ (Lueders et al. 2016) in RNA extracts obtained from BACTRAPS where $^{13}$C benzene had been applied suggested that no or only little highly labeled RNA was present. This could have had two reasons. Firstly, the exposure time was relatively short. However, while a longer exposure time can result in incorporation of more label it can also obscure insight into the contaminant-degrading community due to cross-feeding (Neufeld et al. 2007), i.e., microbes not degrading benzene could become labeled with $^{13}$C due to incorporation of metabolites and degrading biomass from benzene degraders. Secondly, the availability of unlabeled organic carbon in form of plant-derived dissolved organic carbon (DOC) at the site limited the degree of RNA labeling of the various benzene-degrading populations due to mixed substrate use at low concentrations. In a previous BACTRAP experiment at a BTEX-contaminated groundwater site, mixed substrate use was deduced for benzene degraders based on much less relative label incorporation compared with that of toluene degraders (Geyer et al. 2005). Recently, evidence was presented that a member of the genus *Hydrogenophaga* was involved considerably in mineralization of plant-derived DOC in an alluvial aquifer (Jewell et al. 2017). Thus, especially *Hydrogenophaga* might have used additional substrate(s) from the rhizosphere while catabolizing labeled benzene, which might have resulted in a reduced incorporation of $^{13}$C compared with *Dechloromonas* and *Zoogloea*. Furthermore, mixed substrate use was demonstrated for toluene-degrading *Burkholderiales*, including *Hydrogenophaga*, in hypoxic CW model systems (Lünsmann et al. 2016a, b). In these systems, toluene was transformed predominantly via a single biochemical pathway involving toluene 4-monoxygenase (T4MO), which is adapted to low oxygen partial pressure (Martínez-Lavanchy et al. 2015; Lünsmann et al. 2016a). In addition to effectively capturing oxygen released by roots, toluene-transforming *Burkholderiales* took advantage of diurnal fluxes of labile root exudates by synthesizing polyhydroxyalkanoate in rhythm with the day-night regime (Lünsmann et al. 2016b). These microbes and their metabolic properties were identified in protein-SIP experiments in which $^{13}$C-toluene was added directly to the habitat, i.e., excluding the potential surface property bias of BACTRAPS. Importantly, T4MO can also use benzene as substrate (Tao et al. 2004). The encoding *moaABCDEF* genes are present in the genome of *Hydrogenophaga* sp. T4 (GenBank accession AZSO1000048), showing that aerobic benzene transformation capability via T4MO is indeed present in this genus.

The general importance of *Burkholderiales* for aromatic hydrocarbon degradation in the environment (including the rhizosphere where various members of this order can be quite abundant) has been assumed previously based on several SIP experiments with ex situ microcosms as well as characterization of isolates (Denef 2007; Herbstr et al. 2013; Pérez-Pantoja et al. 2012).

In our CW samples, sequences indicating the presence of *Hydrogenophaga*, *Zoogloea*, and *Dechloromonas* comprised a significant amount of the total sequences (Table S2). Their relative abundances were actually rather substantial given that the particular plant in the CW, *P. australis*, can provide ample amounts of bioavailable organic carbon in form of root exudates (Zhai et al. 2013; Saad et al. 2016). Thus, benzene was just one of many carbon and electron donors available for heterotrophic bacteria in the rhizosphere. While it is virtually impossible to determine precisely the flux of plant-derived DOC into the rhizosphere under field conditions, and thus the contribution of benzene to total DOC, we would like to argue that benzene constituted only a small fraction of the biodegraded DOC in the present CW based on the following estimation. In earlier experiments with the same wetland although four times the benzene load in the inflow, microbiobally catalyzed load reduction was determined to be about 0.5 g benzene m$^{-2}$ day$^{-1}$ (equivalent to about 50% removal, as in this work) (Seeger et al. 2011b). Hence, for this study, we assume a load reduction of about 0.125 g benzene m$^{-2}$ day$^{-1}$ due to microbial transformation. For similar wetlands planted also with *P. australis*, Saad et al. (2016) estimated that about 4.5 g plant-derived DOC m$^{-2}$ d$^{-1}$ was used for dissimilatory sulfate reduction and denitrification. As
that calculation did not consider DOC used by aerobic heterotrophs supported by radial oxygen loss (ROL), the total amount of microbially converted DOC derived from plants must have been even higher.

The BACTRAP in situ enrichment system is advantageous over ex situ microcosms in the laboratory where complex environmental conditions and interactions are often lost. However, BACTRAP analyses are not without bias either as the surface properties of the activated carbon are different from the available surfaces in the habitat. Thus in principle it could have been that Dechloromonas, Zoogloea, Hydrogenophaga, and Arcobacter were simply the ones best suited among all benzene degraders present in the CW to reach and colonize the BACTRAPs. This may have been particularly the case for Arcobacter which was barely present in the CW, while members of this genus were found on or isolated from microbial fuel cells (Fedorovich et al. 2009; White et al. 2009). The presence of Geobacter on the BACTRAPs exemplified further that preferential colonization may have occurred, and apparently even independent of turnover of the target substrate. Geobacter is known to be able to grow well on graphite in microbial fuel cells (Bond and Lovley 2003). Geobacter (and other phylotypes temporally abundant on the BACTRAPs such as Fusobacteriaceae, Trichococcus, and Clostridiaceae) may have used the GAC in this experiment for anodic oxidation of unlabeled, plant-derived carbon sources from the rhizosphere. The corresponding cathodic reaction is uncertain. While acknowledging any potential colonization bias we nevertheless take the fact that Dechloromonas, Zoogloea, and Hydrogenophaga were labeled and abundant on the BACTRAPs as prima facie evidence that these microbes were significant benzene degraders in the CW, too.

It is worthwhile to consider the in situ molecular mechanism of benzene transformation by Dechloromonas, Zoogloea, and Hydrogenophaga as it has implications for design and operation of CWs. Members of these genera are aerobes or facultative anaerobes using certain inorganic oxygen concentrations of > 10 mg L\(^{-1}\) in the wetland (Seeger et al. 2011a). As the bulk pore water of the wetland was anoxic (\(E_h\) around \(-100\) mV) essentially all oxygen released must have been consumed by metabolic activities. We assume that some of that oxygen was consumed during aerobic benzene degradation. In addition to \(O_2\), nitrate generated via nitrification was potentially also available (Seeger et al. 2011a, b), and Dechloromonas, Zoogloea, and Hydrogenophaga might have used both terminal electron acceptors simultaneously. Yet, the biochemical activation of benzene occurred probably via oxygenases, which are documented to be encoded in the genomes of Dechloromonas, Zoogloea, and Hydrogenophaga isolates, while known genes for anaerobic degradation of aromatic compounds are absent (Salinero et al. 2009; Gan et al. 2017; Muller et al. 2017). Thus, benzene transformation was apparently carried out by aerobic microbes that necessitate only small concentrations of oxygen for metabolic transformations. Oxygen has been viewed to be one of the limiting factors for benzene removal in CWs (Seeger et al. 2011a, b).

Consequently, the most efficient technical approaches to improve benzene removal in CWs might be those that enhance oxygen transfer into the rhizosphere (Nivala et al. 2013).

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Compliance with ethical standards

Conflict of interest All Authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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