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Microbial community of a gasworks aquifer and identification of nitrate-reducing *Azoarcus* and *Georgfuchsia* as key players in BTEX degradation

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ABSTRACT

We analyzed a coal tar polluted aquifer of a former gasworks site in Thuringia (Germany) for the presence and function of aromatic compound-degrading bacteria (ACDB) by 16S rRNA Illumina sequencing, *bamA* clone library sequencing and cultivation attempts. The relative abundance of ACDB was highest close to the source of contamination. Up to 44% of total 16S rRNA sequences were affiliated to ACDB including genera such as *Azoarcus, Georgfuchsia, Rhodoferax, Sulfuritalea* (all Betaproteobacteria) and *Pelotomaculum* (Firmicutes). Sequencing of *bamA*, a functional gene marker for the anaerobic benzoyl-CoA pathway, allowed further insights into electron-accepting processes in the aquifer: *bamA* sequences of mainly nitrate-reducing Betaproteobacteria were abundant in all groundwater samples, whereas an additional sulfate-reducing and/or fermenting microbial community (Deltaproteobacteria, Firmicutes) was restricted to a highly contaminated, sulfate-depleted groundwater sampling well. By conducting growth experiments with groundwater as inoculum and nitrate as electron acceptor, organisms related to *Azoarcus* spp. were identified as key players in the degradation of toluene and ethylbenzene. An organism highly related to *Georgfuchsia toluolica* G5G6 was enriched with *p*-xylene, a particularly recalcitrant compound. The anaerobic degradation of *p*-xylene requires a metabolic trait that was not described for members of the genus *Georgfuchsia* before. In line with this, we were able to identify a putative 4-methylbenzoyl-CoA reductase gene cluster in the respective enrichment culture, which is possibly involved in the anaerobic degradation of *p*-xylene.

1. Introduction

In Germany, at least 1000 former gasworks sites are known (Mansfeldt, 2003), which are potential sources of pollutants that pose a risk to human health. The main contaminations at these sites derive from inappropriately stored coal tar, which is a complex mixture of mostly aromatic compounds (Zander, 1995). Especially BTEX (benzene, toluene, ethylbenzene, and xylenes) are of concern, since these low molecular weight toxic compounds are mobile with the groundwater flow. In general, the biodegradation of BTEX can occur under oxic conditions, or at reduced rates in the absence of oxygen with alternative electron acceptors such as nitrate, sulfate, Fe(III) or CO_2 (for reviews see Weelink et al., 2010; Fuchs et al., 2011). Contaminated aquifers are usually oxygen depleted and therefore facultative and/or obligate anaerobic bacteria are assumed to dominate the aromatic compound-degrading communities. All of the so far described isolates of aromatic compound-degrading anaerobes belong to the Proteobacteria or Firmicutes (for reviews see Widdel et al., 2010; Kleinsteuber et al., 2012; Boll et al., 2014; Lueders, 2017). Within the Proteobacteria, *Magnetospirillum* spp. (Alphaproteobacteria), *Azoarcus* spp. and *Thauera* spp. (both Betaproteobacteria) degrade aromatic compounds under nitrate-reducing conditions (Evans et al., 1991; Fries et al., 1994; Anders et al., 1995; Rabus and Widdel, 1995; Shinoda et al., 2005). The use of Fe(III) as electron acceptor was mainly reported for *Geobacter* spp. (Deltaproteobacteria) (Lovley and Lonergan, 1990; Coates et al., 2001), but also for *Georgfuchsia* spp. (Betaproteobacteria), which additionally uses nitrate or Mn(IV) as electron acceptor (Weelink et al., 2009). Sulfate is common for Desulfobulbaceae and Desulfobacteraceae (both Deltaproteobacteria) (Bak and Widdel, 1986;

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Meckenstock, 1999) as well as for *Desulfotomaculum* spp. and *Desulfosporosinus* spp. (Clostridia, Firmicutes) (Cord-Ruwisch and Garcia, 1985; Sun et al., 2014a). Members of the genus *Pelotomaculum* (Clostridia, Firmicutes) were often found in syntrophic consortia, in which aromatic compound degradation was observed under sulfate- or nitrate-reducing or fermenting conditions (for review see Kleinsteuber et al., 2012).

An important field of research focuses on the bioremediation of aromatic compound-contaminated sites by e.g. stimulating microbial attenuation processes (for reviews see Bamforth and Singleton, 2005; Farhadian et al., 2008; Perelo, 2010; Megharaj et al., 2011). For the design and monitoring of bioremediation approaches, it is important to identify the microorganisms present at the site and to predict their possible function in pollutant degradation (Majone et al., 2015). State of the art sequencing technologies allow an affordable in-depth analysis of 16S rRNA gene diversity (e.g. Langille et al., 2013; Henschel et al., 2015; Tan et al., 2015) and classified 16S rRNA sequences can be screened for the presence of potential aromatic compound-degrading bacteria; however, the corresponding microorganisms do not necessarily possess the genes for biodegradation (Junca and Pieper, 2004). To specifically detect aromatic compound-degrading anaerobes, a number of primers have been established that target functional genes of pathways involved in aromatic compound degradation (for review see von Netzer et al., 2016). These genes are (i) *bssA*, (ii) *bcr*/*bzd* or *bamB*, and (iii) *bamA* (Beller et al., 2002; Hosoda et al., 2005; Song and Ward, 2005; Winderl et al., 2007; Kuntze et al., 2008; 2011; Löffler et al., 2011; Staats et al., 2011). The *bssA* gene encodes a benzylsuccinate synthase that oxidizes alkyl side chains of substituted aromatic compounds by the addition of fumarate, a mechanism applied for the activation of most BTEX. The genes *bcr*/*bzd* or *bamB* code for ATP-dependent or -independent arylcoenzyme A reductases that catalyze the dearomatization of the aromatic ring. Together with *bamA*, which encodes a ring-cleaving hydrolase, *bcr*/*bzd* and *bamB* target the central catabolic pathway for anaerobic degradation of aromatic compounds such as petrochemicals, humic acids, lignin monomers, and aromatic amino acids (Carmona et al., 2009). The conserved *bamA* gene finds increasing application as functional gene marker, since it is suitable for the simultaneous detection of facultative and obligate anaerobic aromatic compound degraders and can be frequently PCR-amplified from different habitats. Sequences of *bamA* were either retrieved from field sites (Kuntze et al., 2011; Staats et al., 2011; Porter and Young, 2013; Verde et al., 2013; Ruan et al., 2016) or from microbial cultures growing with different electron acceptors (Kuntze et al., 2008; Higashioka et al., 2011; Li et al., 2012; Sun et al., 2014b).

In this study, 16S rRNA and *bamA* sequencing were applied for the functional description of a microbial community of a coal-tar polluted aquifer. This approach allowed a rapid and easy identification of potential aromatic compound-degrading bacteria as well as the prediction of prevailing electron-accepting processes in the aquifer. Further, a cultivation-based approach was used to identify microorganisms involved in the nitrate-dependent bioremediation of BTEX. This is the first combined application of deep 16S rRNA sequencing, *bamA* profiling, and cultivation attempts to characterize a microbial community of a BTEX-contaminated site.

2. Material and methods

2.1. Sampling site

The sampling site was a former gasworks area located in Thuringia (Germany), which was not remediated so far. Coal tar derived pollutants are still leaking into the groundwater. The geology of the test field was analyzed by the JENA-GEOS®-Ingenieurbüro GmbH (Jena, Germany). The gasworks area is characterized by horizontal layers of an anthropogenic backfill at the top (2–3 m thickness) followed by alluvial loam (1 m thickness, Holocene), a layer of gravel (2–2.5 m thickness, Pleistocene) and sandstone sediment (start 5–6 m below ground, Rotliegend). The loam functions as aquiclude with low water permeability. In general, the loam hampers the infiltration of rainwater and pollutants from the anthropogenic backfill into deeper layers; however, it was partially removed below buildings located at the site. The layer of gravel represents the main water-bearing aquifer. The approximate groundwater flow rate is 10 cm per day. Groundwater was sampled from three wells (2 inch in diameter) originally designated as P2/98, RP 12/12 and P1/98 and renamed for convenience to A, B, and C, respectively. The wells are located downstream from the coal tar pit along a pollution gradient (Fig. 1). The wells have a depth of about six meter below ground with a 1 m screen section (5–6 m below ground; 1 mm pore size), which covers approximate half of the thickness of the aquifer. Groundwater samples were collected between 2012 and 2014 by JENA-GEOS®-Ingenieurbüro GmbH (Jena, Germany). Before sampling, groundwater was pumped for 10 min and discarded. Subsequently, the following parameters were determined on-site: oxygen, electrical conductivity, pH, redox potential and temperature. Further groundwater parameters and compound concentrations were determined by the Thüringer Umweltinstitut Henterich GmbH & Co. KG (Krauthausen, Germany): nitrate, DIN EN ISO 10 304-1 (ion chromatography); sulfate, DIN EN ISO 10 304-1 (ion chromatography); ammonium, DIN 38406-E5 (colorimetry); hydrogen carbonate, DIN 38 405-D8 (titration); BTEX, DIN 38 407-F9 (GC-MS); hydrocarbon oil index, DIN EN ISO 9377-2 (solvent extraction, GC); phenol index, DIN 38 409-H16-2 (distillation, colorimetry); PAH, DIN 38 4078-F39 (GC-MS); iron, DIN EN ISO 11885 (ICP-OES). The raw data are presented in Table S1.

2.2. Cultivation of microorganisms

For enrichment of microorganisms, groundwater was sampled from well A, B, and C, filled without leaving a headspace in sterile amber stained glass bottles and stored at 4 °C in the dark. For batch cultures, 100 ml groundwater was transferred into sterile 200 ml-serum flasks. The flasks were closed with butyl rubber stoppers and residual oxygen was removed by 30 alternating cycles of evacuation and gassing with sterile-filtered nitrogen (2 min each). Afterwards, the groundwater (100 ml) was amended with 0.5 ml 1 M $NaNO_3$, 0.1 ml of a single BTEX compound (prepared as 200 mM stock

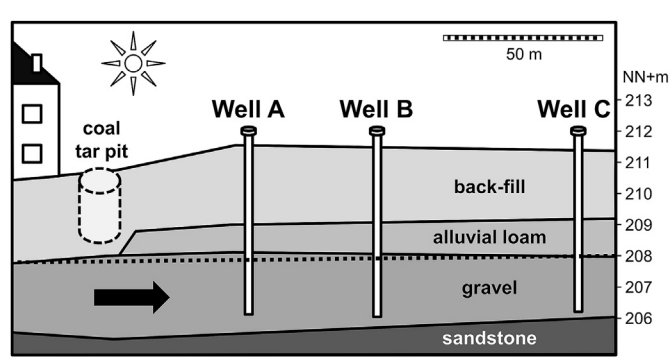


Fig. 1. Schematic cross-section of the Thuringia gasworks site showing the location of the coal tar pit and the three groundwater sampling wells A, B, and C. The dotted line indicates the water table. The arrow marks the groundwater flow direction. For further description of the geology, see section 2.1.

solutions of toluene, ethylbenzene, *p*-, *m*-, and *o*-xylene or 50 mM of benzene; each dissolved in 2,2,4,4,6,8,8-heptamethylnonane) and 1 ml of a potassium-phosphate buffered (100 mM, pH 7.5) supplement solution containing vitamins and trace metals (Mingo et al., 2016). The cultures were incubated in the dark at 24 °C with the bottleneck facing down and continuous shaking (100 rpm).

For further cultivation, an artificial mineral salt medium was used containing the following compounds (per liter ultra-pure water): 0.5 g NaCl, 0.5 g KH_2PO_4, 0.5 g $MgCl_2$ x 6 H_2O, 0.4 g NH_4Cl, 0.4 g KCl, 0.2 g $MgSO_4$ x 7 H_2O, and 0.1 g $CaCl_2$. The medium (95 ml) was transferred into 200 ml-serum flasks, which were closed with butyl rubber stoppers. Oxygen was removed by repeated degassing and flushing with nitrogen (25 cycles, 3 min each). After autoclavation, anaerobic sterile stock solutions (prepared in ultra-pure water) were added: 3 ml 1 M $NaHCO_3$ (CO_2-saturated), 0.5 ml 1 M $NaNO_3$, 0.5 ml vitamin solution, 0.1 ml SL-10 trace element solution, and 0.1 ml selenite-tungsten solution. The pH of the medium was adjusted to pH 7 by addition of 0.5 ml 1 M HCl. Single BTEX compounds (0.1 ml) were supplied from 1 M stock solutions prepared in 2,2,4,4,6,8,8-heptamethylnonane. The vitamin solution contained (per liter ultra-pure water): 8 mg *p*-aminobenzoic acid, 2 mg D(+)-biotin, 20 mg nicotinic acid, 10 mg Ca-D(+)-pantothenate, 30 mg pyridoxamine HCl, and 20 mg thiamine HCl (Pfennig, 1978; Widdel and Pfennig, 1981). The trace element solution was composed of 8.5 ml HCl (37%), 1.5 g $FeCl_2$ x 4 H_2O, 6 mg H_3BO_3, 190 mg $CoCl_2$ x 6 H_2O, 100 mg $MnCl_2$ x 4 H_2O, 70 mg $ZnCl_2$, 36 mg Na_2MoO_4 x 2 H_2O, 24 mg $NiCl_2$ x 6 H_2O, 2 mg $CuCl_2$ x 2 H_2O per liter ultra-pure water (Widdel and Pfennig, 1981; Widdel et al., 1983). The selenite-tungsten-solution contained (per liter ultra-pure water): 0.5 g NaOH, 3 mg Na_2SeO_3 x 5 H_2O, and 4 mg Na_2WO_4 x 2 H_2O. The medium was inoculated with 10 ml of a grown culture. Growth was followed by measuring the optical density at 578 nm and by determination of the protein concentration using the Roti®-Nanoquant reagent (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). Bovine serum albumin (>98% purity, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was used as standard.

2.3. Analytical methods

The concentration of BTEX was determined with a Clarus 500 gas chromatograph (GC) equipped with a flame ionization detector, a Turbo Matrix 40 headspace sampler (PerkinElmer, Rodgau, Germany) and a CP-PoraBOND Q fused silica column (25 m × 0.32 mm; Agilent Technologies, Waldbronn, Germany). Nitrogen (purity 5.0; Linde AG, Pullach, Germany) was used as carrier gas (pressure 100 kPa). For headspace sampling, the GC vial containing 1 ml sample from the aqueous phase of the medium and 20 μl nonane (10 mM) as internal standard was heated for 6 min at 95 °C in the headspace sampler before injection. The temperature of the injector and detector were 250 °C and 300 °C, respectively. The following temperature program was applied for the oven: 4 min at 150 °C followed by a gradient of 10 °C/min increasing to 280 °C and hold for 4 min. The retention times were as follows: benzene, 7.5 min; toluene, 10.6 min; ethylbenzene, 13.1 min; *p*-/*m*-xylene, 13.2 min; *o*-xylene, 13.6 min; nonane, 14.2 min. Aqueous solutions of BTEX (>99% purity; Merck KGaA, Darmstadt, Germany) prepared in ultra-pure water were used as standards (25–500 μM).

Nitrate and nitrite were quantified using a high performance liquid chromatography system (Merck-Hitachi) equipped with a L-6200 pump, a 2000A autosampler, a L-4500 diode array detector and a LiChrospher® 100 RP-18 (5 μm) LiChroCART® 125-4 column (Merck KGaA, Darmstadt, Germany). As eluent, 45% (v/v) methanol plus 0.3% (v/v) H_3PO_4 in ultra-pure water was used. A flow rate of 0.4 ml min^{-1} was applied. Signals were detected at 220 nm. Under these conditions, the retention times were: nitrate, 3.0 min; nitrite, 5.2 min. Nitrate was not retained by the column and eluted together with the injection peak. The area of the injection peak was determined in controls without nitrate and subtracted from nitrate areas. The concentrations of nitrate and nitrite determined via HPLC analysis were confirmed by colorimetric assays using a resorcinol based method for nitrate (Bosch Serrat, 1998) and the Griess Reagent System for nitrite (Promega GmbH, Mannheim, Germany). Mono-element standards in the highest available purity (CPAchem, Stara Zagora, Bulgaria) were used for calibration. Ammonium was determined using the Aquaquant® ammonium test kit (Merck KGaA, Darmstadt, Germany).

2.4. DNA extraction

Groundwater samples from well A, B, and C were taken with a scoop (3 liter each), centrifuged in sterile 500 ml-polypropylene bottles (18,600xg, 60 min, 10 °C) and concentrated to 10 ml aqueous sludge. DNA isolation was done with the RapidWater™ DNA isolation kit (Mo Bio Laboratories, Carlsbad, USA) according to the manufacturer's protocol with some modifications: The beads for three reactions were pooled into one 5 ml reaction tube. Aqueous sludge (0.75 ml) and 3 ml of solution RW1 from the kit were added to this tube. Cells were disrupted by heating at 65 °C for 10 min followed by mixing (30 hz^{-1}, 10 min, 4 °C) with a mixer mill MM 400 (Retsch GmbH, Haan, Germany). For each groundwater sample, two preparations were done in parallel. The supernatants of the two preparations were pooled and 6 ml solution RW2 was added. The mixture was applied to a single spin filter column. Further steps were performed according to the manufacturer's manual. A negative control was conducted by using the same method of DNA extraction, but by replacing the aqueous sludge with ultra-pure water.

DNA from microbial enrichments was extracted using the innuPREP Bacteria DNA kit (Analytik Jena AG, Jena, Germany). Samples (10 ml) were taken in the late exponential growth phase and were centrifuged for 45 min (4000xg, 10 °C). DNA extraction was performed according to the manufacturer's protocol.

The integrity of the genomic DNA from groundwater or enrichment cultures was confirmed by gel electrophoresis (0.8% (w/v) agarose gel in 1fold TAE buffer). Quantification was done using a Qubit® fluorometer and the Qubit® dsDNA BR assay kit (Thermo Fisher Scientific GmbH, Dreieich, Germany).

2.5. Illumina sequencing of 16S rRNA

The concentration of genomic DNA in environmental samples and enrichment cultures was normalized to 10 ng/μl with microbial DNA-free water (QIAGEN GmbH, Hilden, Germany). DNA samples were sent, together with a negative control (see section 2.4), to MR DNA® (Shallowater, TX, USA) for amplicon sequencing of the 16S rRNA gene (V3/V4 region). For initial PCR amplification, the primers S-D-Bact-0341-b-S-17 (5′-CCT ACG GGN GGC WGC AG-3′) and S-D-Bact-0785-a-A-21 (5‘-GAC TAC HVG GGT ATC TAA TCC-3‘) were used (Klindworth et al., 2012), which included a sample specific barcode attached to the forward primer using the bTEFAP® service based on barcoding processes originally described in Dowd et al. (2008). A DNA library was prepared according to the Illumina TruSeq DNA library preparation protocol. Paired-end sequencing was done on an Illumina MiSeq system using the Reagent Kit v3 following the manufacturer's guidelines. For analysis of Q25 merged sequence data, the MR DNA® pipeline (Shallowater, TX, USA) was used based on suggested and default QIIME methods (Caporaso et al., 2010). It included the following steps: joining of sequences, removing of barcodes, removing of sequences that are <150 bp, removing of sequences with ambiguous base calls,

denoising, de novo picking of operational taxonomic units (OTUs) defined by 97% nucleotide identity and removing of singleton sequences and chimeras. Taxonomical classification was done using a curated in-house database derived from RDP-II (Cole et al., 2005) and NCBI (O'Leary et al., 2016). A complete list of OTUs including E-values and percent nucleotide identity to the closest match is given as Supplementary Table S2. Sequences were submitted to the European Nucleotide Archive (http://www.ebi.ac.uk/ena) and are available under the study accession number PRJEB19661.

2.6. Preparation of bamA clone libraries

The *bamA* gene was amplified from genomic DNA isolated from environmental samples and enrichment cultures using the primers oah_f (5′-GCA GTA CAA YTC CTA CAC SAC YGA BAT GGT-3′) and oah_r (5′-CCR TGC TTS GGR CCV GCC TGV CCG AA-3′) published by Staats et al. (2011). The PCR was conducted in 25 μl reactions with 25 ng DNA, 0.2 μM of each primer and 5 U Taq DNA polymerase in reaction buffer A^+ containing 1.5 mM $MgCl_2$ (segenetic, Borken, Germany). For amplification, the following conditions were applied: 94 °C for 5 min, 39 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, final elongation at 72 °C for 8 min. The PCR product was analyzed on a 2% (w/v) agarose gel prepared in 1fold TAE. The expected size of the PCR product was approximately 350 bp. The band of interest was excised and purified using the Hi Yield® Gel/PCR DNA Fragment Extraction kit (Süd-Laborbedarf GmbH, Gauting, Germany). The *bamA* gene fragment was cloned into the pCR™4-TOPO® vector using the TOPO® TA cloning® kit (Thermo Fisher Scientific GmbH, Dreieich, Germany) and transformed into *E. coli* XL 1 Blue competent cells following the heat-shock procedure (Sambrook et al., 1989). Cells were streaked on LB-agar plates containing 100 μg ml^{-1} ampicillin. The presence of correct insert was confirmed by colony PCR. Therefore, single colonies were transferred to 20 μl sterile ultra-pure water. The suspensions were incubated for 5 min at 95 °C. After centrifugation, 2 μl of supernatant was used as template in the PCR reaction. M13R (5′-CAG GAA ACA GCT ATG AC-3′) and T7 (5′-TAA TAC GAC TCA CTA TAG GG-3′) were used as primers. The annealing temperature was 55 °C and 30 cycles were performed using the conditions described above. PCR products were analyzed on a 2% (w/v) agarose gel. Transformants, which contained an insert of the correct size, were sent to GATC Biotech AG (Konstanz, Germany) for plasmid preparation and Sanger sequencing using the T7 primer. Sequence data were analyzed with the software MEGA 7 (Kumar et al., 2016) using the following procedure: orientation of sequences in the same direction, trimming of plasmid and primer derived nucleotides and a MUSCLE alignment (Edgar, 2004) with *bamA* reference sequences of cultured bacteria (for accession numbers, see Table S5). Evolutionary relationships were evaluated by the maximum likelihood method based on the general time reversible model (Nei & Kumar, 2000) with gamma distribution and invariant sites (G+I). The phylogeny was tested using the bootstrap method and 1000 replicates. Nucleotide sequence identities with *bamA* reference sequences were calculated with the 'Sequence Identity Matrix' option in BioEdit (version 7.2.5; Hall, 1999). Sequences were submitted to the European Nucleotide Archive (http://www.ebi.ac.uk/ena) and are available under the accession numbers LT800504 - LT800686 (*bamA* of groundwater samples) and LT838365 - LT838395 (*bamA* of enrichment cultures, excluding redundant sequences).

2.7. Design of degenerated primers for the amplification of 4-methylbenzoyl-CoA reductase gene fragments by PCR

A BLASTP search (Altschul et al., 1990) against metagenomes derived from hydrocarbon-/coal-tar-polluted environments (JGI IMG system; Markowitz et al., 2012) was conducted using the amino acid sequence of the 4-methylbenzoyl-CoA reductase subunit C of *Magnetospirillum* sp. pMbN1 (MbrC; GenBank accession number: AIW63090) as template. Seven putative MbrC sequences were found with an identity on protein level of 43–68% to MbrC of *Magnetospirillum* sp. pMbN1. The corresponding gene sequences (for accession numbers, see Fig. 4A) were used in an alignment to design the *mbrC* specific primers mbrc317_FW (5′-TGT TYG TYA CCC AYC CKA TCT G-3′) and mbrc1031_RV (5′-AGA CCB GGY TCR CAC ATC TTG-3′). The PCR was conducted in 25 μl reactions with 25 ng DNA, 0.4 μM of each primer and 5 U Taq DNA polymerase in reaction buffer A^+ containing 1.5 mM $MgCl_2$ (segenetic, Borken, Germany). For amplification of the *mbrC* gene fragment, the following conditions were applied: 95° for 3 min, 30 cycles of 95 °C for 30 s, 53 °C for 30 s and 72 °C for 1 min, final elongation at 72 °C for 10 min. The homology of the amplicon amplified from the *p*-xylene-degrading enrichment culture (~700 bp) to *mbrC* was confirmed by Sanger sequencing (GATC Biotech AG, Konstanz, Germany).

2.8. Genome walking

For genome walking, the method described for the Universal GenomeWalker™ kit (Clontech Laboratories, Mountain View, CA, USA) was followed. DNA libraries were prepared using the restriction enzymes DraI, EcoRV, and PvuII. The gene specific primers (upstream: 4MBR_GW_RV01: 5′-CCG CTC ATA CTC ATT GCG TAG-3′, 4MBR_GW_RV02: 5′-GGA TTC TGC GGA AGA TGC AG-3'; downstream: 4MBR_GW_FW01: 5′-GCT GTT GGA AGA TGT GAG CAC-3′, 4MBR_GW_FW02: 5′-GTG CAG CAC GAC AAC AAC AAG-3′) were derived from the 4-methylbenzoyl-CoA reductase gene fragment (see section 2.7). Specific PCR products of the EcoRV library were sequenced. For a second downstream walk, the primers 4MBR_GW_FW03 (5′-ATC GTC AAG CGC GAG ACC-3′) and 4MBR_GW_FW04 (5′-CTC GTA CTG CAT CAC GAC G-3′) were applied. The length of the entire fragment was 4167 bp. The open reading frames putatively encoding the subunits MbrA, MbrB, MbrC, and MbrD of a 4-methylbenzoyl-CoA reductase were aligned with reference sequences of benzoyl-CoA reductases and a 4-methylbenzoyl-CoA reductase using MUSCLE (Edgar, 2004). A phylogenetic tree was constructed using the maximum likelihood method based on the JTT matrix-based model (Jones et al., 1992) and 1000 bootstrap replicates (implemented in MEGA 7; Kumar et al., 2016). The sequence of the newly retrieved *mbrBCAD* gene cluster was deposited at the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under the accession number LT934314.

3. Results and discussion

3.1. The sampling site

The gasworks site studied here has an unsecured coal tar pit from which organic pollutants are leaking into the groundwater. A profile of the gasworks site and the location of the three groundwater sampling wells is depicted in Fig. 1. For a detailed description of the geology and the physico-chemical parameters see section 2.1 and Table S1. Median concentrations of selected groundwater parameters are given in the following: BTEX compounds were the major contaminants with highest concentrations close to the coal tar pit in well A (9.8 mg l^{-1}) and decreasing concentrations downstream in well B (1.7 mg l^{-1}) and C (0.16 mg l^{-1}). Oxygen concentrations were 0.6, 0.1, and 0.2 mg l^{-1} in well A, B, and C, respectively, reflecting the almost anoxic conditions of the aquifer. The concentration of ammonium, a waste product formed during gas production, was highest in the vicinity of the coal tar pit and

decreased further downstream (A: 23.7 mg l^{-1}, B: 14.9 mg l^{-1}, C: 5.1 mg l^{-1}). Probably due to ammonium oxidation, the concentration of nitrate was elevated in well A (13.1 mg l^{-1}) compared to B (1.4 mg ml^{-1}) and C (1.0 mg ml^{-1}). The concentration of sulfate was lowest in well A (71 mg l^{-1}) and increased further downstream (B: 317 mg l^{-1}, C: 561 mg l^{-1}), indicating sulfate-reducing conditions close to the coal tar pit (Schirmer et al., 2006). Dissolved iron, which is formed in the absence of oxygen by the reduction of insoluble Fe(III) to soluble Fe(II) was detected in well B and C (A: < 0.1 mg l^{-1}; B: 0.7 mg l^{-1}; C: 1.9 mg l^{-1}).

3.2. The microbial community of the aquifer with emphasis on aromatic compound-degrading bacteria

The microbial community of the aquifer, which was analyzed by 16S rRNA amplicon sequencing on an Illumina MiSeq platform (for a complete list of OTUs including the taxon affiliation, see Table S2; for a general summary of Illumina output data including the abundance of genera, see Table S3), was dominated by Proteobacteria with relative abundances of 78% (well A), 83% (well B) and 70% (well C), respectively (Fig. 2A). Within the Proteobacteria, the majority of sequences were classified as Betaproteobacteria (well A: 53%, well B: 35%, well C: 40%). Many Betaproteobacteria are facultative anaerobes frequently found in waste waters (Wagner et al., 2002; Nielsen et al., 2009; Wang et al., 2012). They can degrade a variety of organic compounds and seem to play a major role in the nitrate-dependent degradation of aromatic hydrocarbons (Parales, 2010). In well B, also Epsilonproteobacteria, mainly represented by *Arcobacter* spp., were highly abundant (36%). Its role in groundwater is not clear since free-living *Arcobacter* spp. have a versatile metabolism; however, they are often associated with sulfur and nitrogen cycling (Roalkvam et al., 2015). Their occurrence in contaminated environments was reported before (Gevertz et al., 2000; Collado et al., 2011; Levican et al., 2013). Other prominent phyla detected in all three groundwater wells included Actinobacteria (3.3–8.8%), Bacteroidetes (3.0–5.4%), and Firmicutes (2.8–6.7%).

The Illumina dataset was analyzed for the presence of aromatic compound-degrading bacteria (ACDB). Since the aquifer was oxygen-depleted, we focused on microorganisms, which have the potential to degrade aromatic compounds in the absence of oxygen via CoA thioesters using the benzoyl-CoA pathway (designated as oxygen-independent ACDB, short: no-ox ACDB). Furthermore, a literature search was conducted to identify taxa abundant in the aquifer that were previously associated with the degradation of aromatic compounds in oxygen-depleted environments, but for which the presence of the anaerobic benzoyl-CoA pathway was not yet described (for references and explanations, see Table S4). The majority of these taxa possess the oxygen-dependent box pathway for aromatic compound degradation as deduced from a BLAST analysis (data not shown) initially described by Rather et al. (2010). The box pathway is considered as an adaptation to low oxygen conditions (Fuchs, 2008). In this pathway, aromatic compounds are also degraded via CoA thioesters as intermediates, however, an oxygen-dependent dearomatization step is involved (for review see Fuchs et al., 2011). These taxa are designated as low-oxygen-

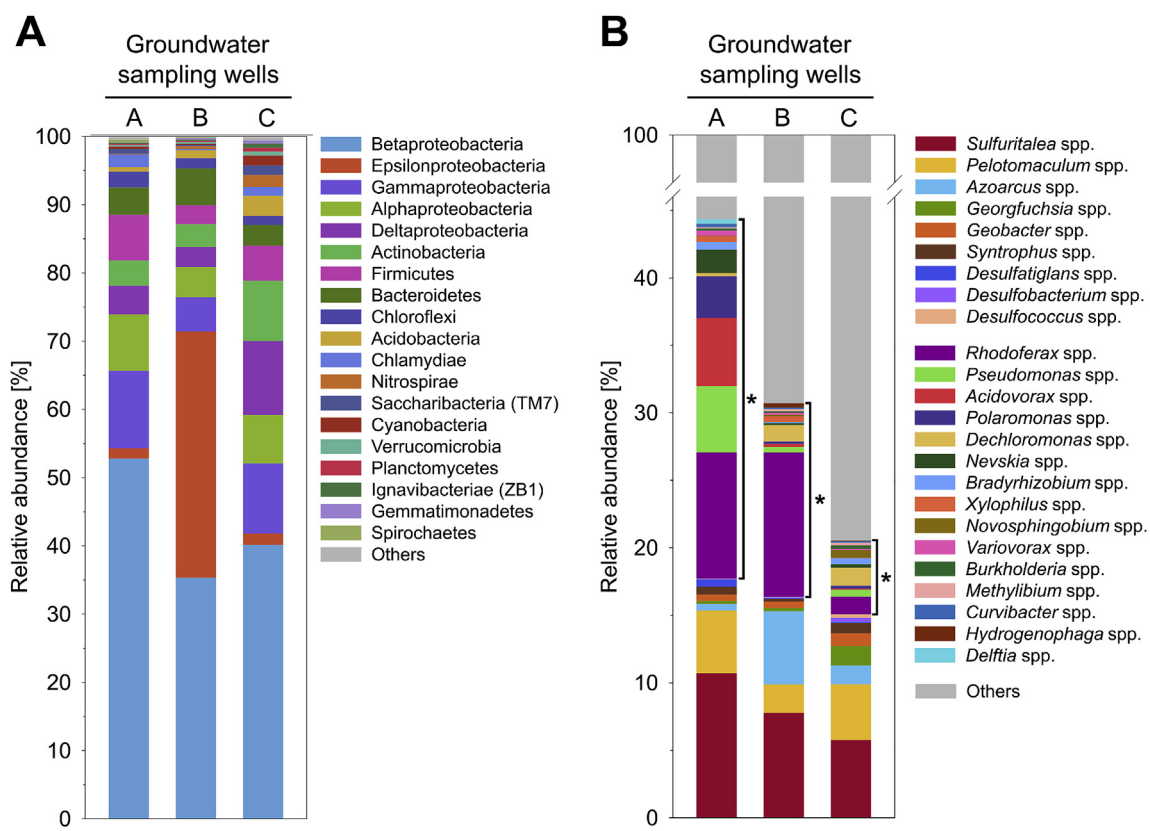


Fig. 2. (A) Relative abundance of bacterial phyla and proteobacterial classes expressed as percentage of total 16S rRNA sequences in groundwater samples. Phyla with a mean relative abundance below 0.15% were summarized as others. (B) Relative abundance of bacterial genera that were assigned as aromatic compound-degrading bacteria (ACDB) expressed as percentage of total 16S rRNA sequences in groundwater samples. ACDB with a mean relative abundance <0.1% as well as non-ACDB genera were summarized as others. The ACDB genera were sub-classified into low-oxygen-dependent (marked with an asterisk) and oxygen-independent ACDB. For explanations and references, see section 3.2 and Table S4, which also comprises the complete list of genera defined as ACDB. The data of this figure were obtained by 16S rRNA amplicon sequencing on an Illumina MiSeq platform and subsequent taxonomic classification with QIIME.

dependent ACDB (low-ox ACDB).

The highest relative abundance of total ACDB was detected in well A with 44% (Fig. 2B). The amount decreased in well B (31%) and C (21%). The number of ACDB was consistent with the concentration of BTEX in groundwater, which was highest in well A and lowest in well C. The most abundant no-ox ACDB in the aquifer were *Sulfuritalea* (well A: 10.7%, well B: 7.8%, and well C: 5.8%), *Pelotomaculum* (4.7%, 2.1%, and 4.1%), *Azoarcus* (0.5%, 5.4%, and 1.4%), *Georgfuchsia* (0.2%, 0.2%, and 1.4%), and *Geobacter* (0.5%, 0.5%, and 0.9%). The presence of *Sulfuritalea* spp. in all groundwater wells is noticeable. So far, the only pure culture of this genus is S*ulfuritalea hydrogenivorans* sk43H (Kojima and Fukui, 2011). It was isolated from a freshwater lake in Japan and is the closest relative of the strictly anaerobic ACDB *Georgfuchsia toluolica* G5G6. Aside from the ability of *S. hydrogenivorans* sk43H to degrade benzoate with nitrate (Kojima and Fukui, 2011), its role as ACDB was not investigated so far. This question will be addressed in future studies. Whereas the total amount of no-ox ACDB was roughly similar in all three sampling wells, a considerably increased abundance of low-ox ACDB including *Acidovorax, Polaromonas, Pseudomonas*, and *Rhodoferax* was observed in well A. This was in consistence with an increased oxygen concentration in this well (well A: 0.6 mg l^{-1}, well B: 0.1 mg l^{-1}, well C: 0.2 mg l^{-1}). This observation indicates that the increase of total ACDB in well A (Fig. 2B) is possibly caused by oxygen and not by BTEX concentrations. The availability of nitrate in well A (Table S1) might also positively affect the low-ox ACDB community as it was shown for *Pseudomonas sp.* (Svenningsen et al., 2016). The most abundant low-ox ACDB was *Rhodoferax* previously described to degrade benzoate with a variety of electron acceptors including Fe(III), nitrate, and oxygen (Finneran et al., 2003). Because of its versatility, the role of *Rhodoferax* in aromatic compound degradation is difficult to interpret and needs to be further elucidated. Other genera that were characteristic for the groundwater, but so far not described to degrade aromatic compounds, include *Arcobacter* (well A: 0.8%, well B: 32.4%, and well C: 0.5%), *Gallionella* (0.2%, 0.9%, and 11.3%), and *Giesbergeria* (18.3%, 0.1%, and <0.1%) (see also Table S3), all reported to commonly occur in ground- and wastewater (Rice et al., 1999; Grabovich et al., 2006; Hanert, 2006).

3.3. Application of the functional marker gene bamA

Since the taxa predicted to be involved in aromatic compound degradation based on 16S rRNA analysis do not necessarily possess the genes for biodegradation, *bamA* was applied as functional marker to target the anaerobic benzoyl-CoA pathway (Kuntze et al., 2008; 2011; Staats et al., 2011). With the *bamA* assay, no-ox ACDB are targeted, whereas low-ox ACDB cannot be detected. Fig. 3 shows a phylogenetic tree of the *bamA* nucleotide sequences recovered from clone libraries prepared with DNA from groundwater of well A, B, and C (see also Table 1). For comparison, *bamA* reference nucleotide sequences of isolated and enriched ACDB were included in the cladogram (Table S5). The obtained sequences were categorized into three clades: clade I comprises *Azoarcus-, Sulfuritalea-*, and *Georgfuchsia*-related *bamA* sequences. Clade II includes *Geobacter-*, *Magnetospirillum-*, and *Thauera*-related *bamA* sequences and clade III contains *bamA* sequences of various members of mainly sulfate-reducing/fermenting Deltaproteobacteria and versatile, often syntrophic Clostridia (Firmicutes). The majority of groundwater *bamA* sequences (116 of 183 clones, ≈ 63%) belonged to clade I. The *bamA* sequences within this clade were highly related to nitrate-reducing *Azoarcus* sp. EbN1 (≥93.6% identity) and *Sulfuritalea hydrogenivorans* sk43H (92.1% identity). This finding is in accordance with the 16S rRNA analysis, which revealed a high abundance of the no-ox ACDB-genera *Azoarcus* and *Sulfuritalea* in groundwater of the aquifer. Clade II includes 39 *bamA* sequences. A single sequence within this clade was related to *Magnetospirillum* sp. pMbN1 (94.2% identity). The remaining sequences either belonged to a cluster related to Fe(III)-reducing *Geobacteraceae* (86.5–88.5% identity), which were also identified by the 16S rRNA analysis, or showed similarity to phototrophic and nitrate-reducing *Alphaproteobacteria* (83.5–86.5% identity). The 28 sequences affiliated to clade III originated, with one exception, from sulfate-depleted groundwater of well A. These sequences were similar to sulfate-reducing/fermenting Deltaproteobacteria such as *Desulfatiglans anilini* (80.2% identity) and *Desulfomonile tiedjei* (77.1% identity) or were affiliated to syntrophic Firmicutes related to *Desulfotomaculum* sp. BICA1-6 (78.8% identity). Low similarities to reference sequences indicate the presence of so far uncharacterized members of Deltaproteobacteria and Firmicutes. The increased occurrence of these obligate anaerobic bacteria in well A was not apparent from analyzing the 16S rRNA sequences: the only dominant genus, which would group into clade III identified by 16S rRNA analysis was *Pelotomaculum*, however, 16S rRNA sequences affiliated to this genus were present in all sampling wells instead of being restricted to well A. This example highlights the advantage of combining 16S rRNA profiling with *bamA* analysis: while 16S rRNA sequencing allows a refined taxonomic classification, *bamA* is superior in identifying microbial degradation processes, in this case a sulfate-reducing/fermenting ACDB community in sulfate-depleted groundwater.

The *bamA* gene was also used by other researchers to target ACDB, but a deep 16S rRNA-based community profiling was not performed in these studies (e.g. Kuntze et al., 2011; Staats et al., 2011; Porter and Young, 2013; Verde et al., 2013; Sun et al., 2014b; Ruan et al., 2016). Sampling sites that were analyzed included contaminated soil or sludge, deep subsurface oil fields, and BTEX contaminated aquifers. Each site (or sample) was characterized by a specific '*bamA* community'. Some of the researchers suggested that the electron acceptor available in the habitat or sample had a major influence on the composition of the '*bamA* community'. According to this, the iron-reducing Geobacteraceae were the most abundant ACDB in the iron-containing aquifer analyzed by Staats et al. (2011). Strictly anaerobic Deltaproteobacteria and/or Firmicutes dominated the microbial communities in production waters of deep subsurface oil fields, where the presence of oxygen is unlikely (Verde et al., 2013; Ruan et al., 2016). The aquifer of the Thuringia gasworks site we have analyzed in this study was dominated by *bamA* sequences of presumably nitrate-reducing Betaproteobacteria indicating the importance of nitrate as electron acceptor for aromatic compound degradation at this site. This finding has considerable implications for the design of future bioremediation strategies and suggests the infiltration of nitrate as suitable approach to promote the indigenous microbial community in aromatic compound degradation. Previously, nitrate was successfully applied by other researchers as stimulating agent for *in situ* bioremediation (Cunningham et al., 2001; Eckert and Appelo, 2002; Rivett et al., 2008; Xu et al., 2014; 2015). Besides the occurrence of nitrate-reducing Betaproteobacteria in all sampling wells, additional *bamA* sequences affiliated to strictly anaerobic Deltaproteobacteria and Firmicutes were present in the aquifer, but mainly restricted to the highly contaminated, sulfate-depleted well A. These strictly anaerobic microorganisms are often involved in sulfate reduction or fermentation. The utilization of sulfate as electron acceptor would explain the observed decrease in the sulfate concentration in well A. In addition, well A was characterized by an increased abundance of low-ox ACDB, which require at least small amounts of oxygen for aromatic compound degradation (see Fig. 2B). At a first glance, the presence of nitrate-reducing, strictly anaerobic and low-ox ACDB in the same well is

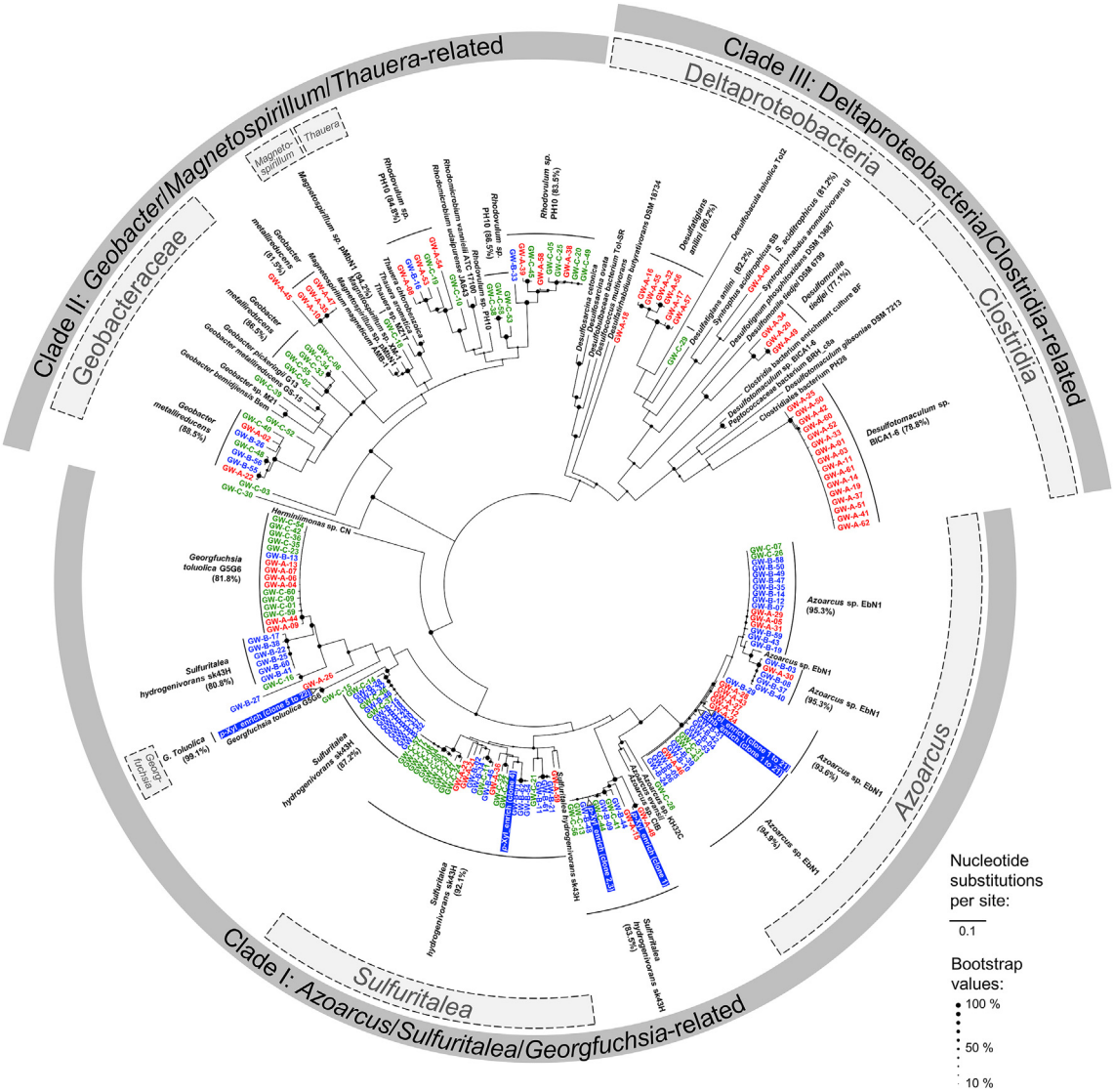


Fig. 3. Phylogenetic tree of *bamA* gene fragments constructed by using the maximum likelihood method based on the general time reversible model. The *bamA* nucleotide sequences were retrieved from groundwater samples of well A (red, GW-A-no.), well B (blue, GW-B-no.), and well C (green, GW-C-no.) as well as from nitrate-reducing enrichment cultures (highlighted in blue) utilizing toluene (Tol_enrich), ethylbenzene (Ethb_enrich) or *p*-xylene (*p*-Xyl_enrich) as electron donor. For classification, *bamA* nucleotide sequences of known aromatic compound-degrading bacteria (ACDB) were included in the cladogram. The *bamA* sequences within the three clades were further assigned to specific groups of ACDB based on nucleotide identities to reference sequences (given as median values in parentheses). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

contradictory to the assumption that contaminant plumes are separated into distinct redox zones (Lovley, 1997). However, recent findings indicate that different redox processes can occur in parallel, especially close to the source of contamination at the plume fringe, where no limitation of the electron donor is expected (Meckenstock et al., 2015). The excess of electron donor in well A would also explain why microorganisms using energetically less favorable electron acceptors such as sulfate or CO_2 can compete in this well against microorganisms that utilize e.g. oxygen, nitrate or iron. In conclusion, our results provide further evidence that *bamA* profiling is a valuable method to predict electron-accepting processes in environmental samples and a future application is suggested to assist in the design and/or monitoring of bioremediation approaches, preferentially by the use of next-generation sequencing techniques.

3.4. Groundwater enrichment cultures

The involvement of nitrate-reducing ACDB in the degradation of aromatic compounds at the site was verified by cultivation attempts. Anoxic groundwater of well A, B, and C was amended with nitrate as electron acceptor and single BTEX compounds as electron donor. Under the conditions applied, ethylbenzene, toluene, *p*- and *m*-xylene were degraded (Table 2). The consumption rates and the lag phases differed among the BTEX tested and the groundwater wells used for sampling (Table 2). Toluene and ethylbenzene were degraded fastest in all groundwater samples with consumption rates ranging from 4 to 7 $\mu mol\ d^{-1}$. The degradation started either immediately (well B) or after 4–7 days of incubation (wells A and C). For *p*- and *m*-xylene, consumption started after prolonged lag phases of 2 and 5 weeks in groundwater obtained from well A and B, respectively. The average consumption rate was 1.4 $\mu mol\ d^{-1}$ for *p*-xylene and 0.6 $\mu mol\ d^{-1}$ for *m*-xylene. Higher consumption rates

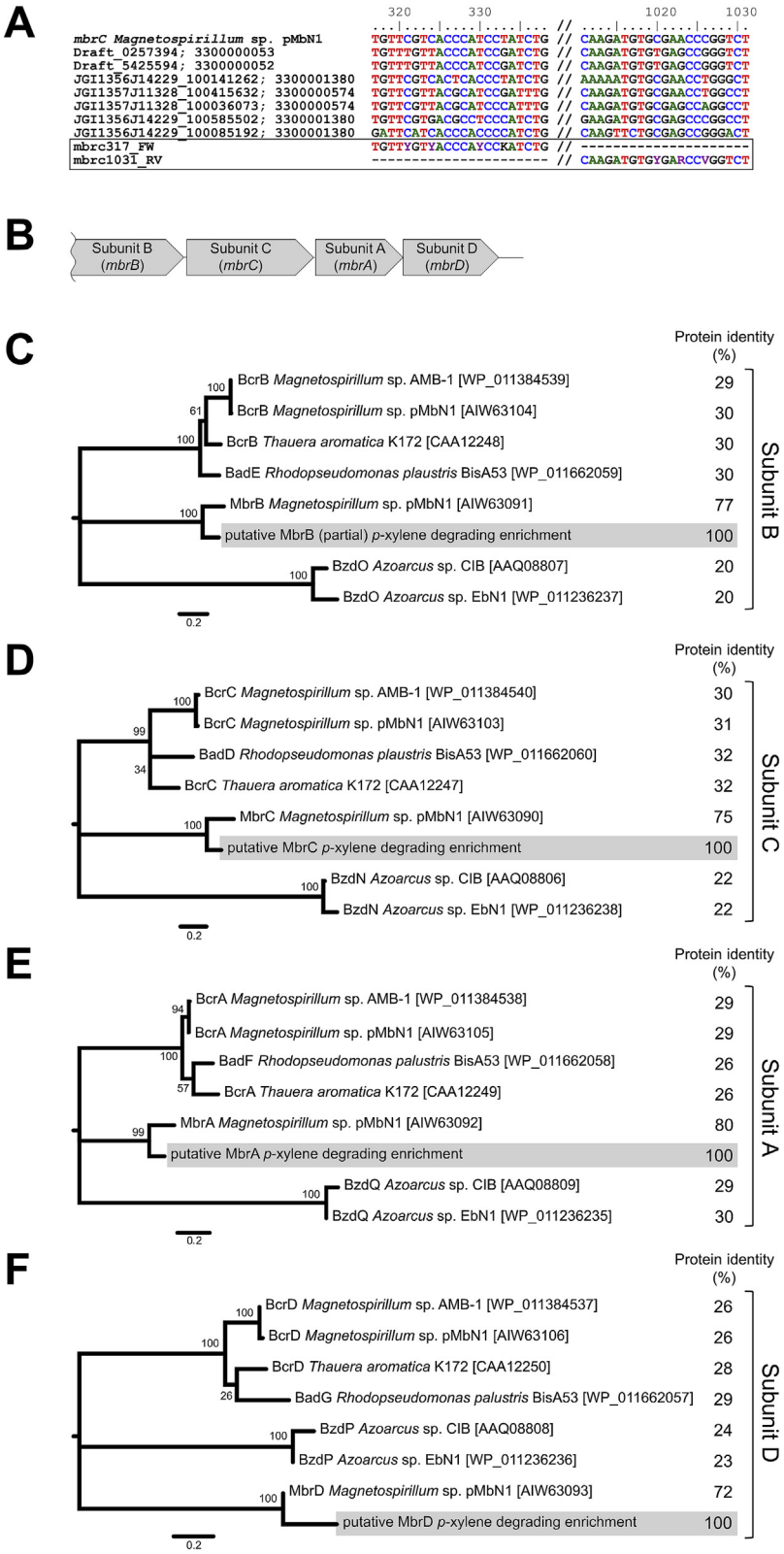
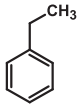
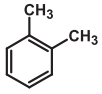
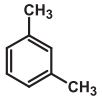
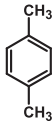


Fig. 4. Identification of a gene region encoding a putative 4-methylbenzoyl-CoA reductase (*mbrBCAD*) in the *p*-xylene-degrading enrichment culture. (A) Sequence alignment of *mbrC* of *Magnetospirillum* sp. pMbN1 with seven putative *mbrC* genes retrieved from metagenomic databases (IMG Gene ID and IMG Genome ID given in the figure) and the *mbrC*-specific degenerated primer sequences used for amplification of the respective gene fragment. The nucleotide numbers indicated are based on *mbrC* of *Magnetospirillum* sp. pMbN1. (B) The putative *mbrBCAD* gene-cluster of the *p*-xylene-degrading enrichment culture. The gene organization is identical to *Magnetospirillum* sp. pMbN1. For subunit B, only a partial gene region was obtained by genome walking. (C-F) Phylogenetic trees showing the relatedness of the four putative subunits MbrA-D from the *p*-xylene-degrading enrichment culture (EMBL accession number: LT934314) to MbrA-D of *Magnetospirillum* sp. pMbN1 as well as to corresponding subunits of several characterized benzoyl-CoA reductases (BcrA-D, BadD-G, BzdN-Q) from model organisms. Protein sequences were aligned with MUSCLE and the trees were constructed using the maximum likelihood method, both implemented in MEGA 7.

Table 1
Relative abundance in percent and absolute number (in parenthesis) of affiliated *bamA* nucleotide sequences obtained from clone libraries prepared with DNA isolated from groundwater of well A, B, and C as well as from enrichment cultures of well B amended with nitrate as electron acceptor and either toluene (Tol), ethylbenzene (Ethb) or *p*-xylene (*p*-Xyl) as electron donor. The taxonomic affiliation is based on clustering and nucleotide identity of obtained *bamA* sequences with *bamA* reference sequences (see also Fig. 3).

Affiliated clades	Groundwater well			Enrichment culture		
	Well A	Well B	Well C	+Tol	+Ethb	+*p*-Xyl
Clade I	**37.1** (23)	**91.8** (56)	**61.7** (37)	**100** (21)	**100** (21)	**100** (22)
Azoarcus	16.1 (10)	41.0 (25)	6.7 (4)	100 (21)	100 (21)	0 (0)
Sulfuritalea	6.5 (4)	14.8 (9)	23.3 (14)	0 (0)	0 (0)	4.5 (1)
Georgfuchsia	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	81.8 (18)
unclassified	14.5 (9)	36.1 (22)	31.7 (19)	0 (0)	0 (0)	13.6 (3)
Clade II	**19.4** (12)	**8.2** (5)	**36.7** (22)	**0.0** (0)	**0.0** (0)	**0.0** (0)
Geobacteraceae	3.2 (2)	4.9 (3)	15.0 (9)	0 (0)	0 (0)	0 (0)
Magnetospirillum	0 (0)	0 (0)	1.7 (1)	0 (0)	0 (0)	0 (0)
unclassified	16.1 (10)	3.3 (2)	20.0 (12)	0 (0)	0 (0)	0 (0)
Clade III	**43.5** (27)	**0.0** (0)	**1.7** (1)	**0.0** (0)	**0.0** (0)	**0.0** (0)
Deltaproteobacteria	17.7 (11)	0 (0)	1.7 (1)	0 (0)	0 (0)	0 (0)
Clostridia	25.8 (16)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Table 2
Consumption of BTEX in groundwater samples originating from well A, B, and C using nitrate as electron acceptor.

BTEX		Well A		Well B		Well C	
		lag phase[a] [d]	consumption rate[b] [μmol d^{-1}]	lag phase [d]	consumption rate [μmol d^{-1}]	lag phase [d]	consumption rate [μmol d^{-1}]
Benzene		>140	0	>140	0	>140	0
Toluene		7	5	<1	5	7	4
Ethylbenzene		7	5	<1	7	4	4
o-Xylene		>140	0	>140	0	>140	0
m-Xylene		25	0.5	28	0.6	>140	0
p-Xylene		12	1.5	28	1.3	>140	0

[a] Days before first observation of BTEX degradation and nitrate reduction.
[b] Average consumption rate of BTEX calculated between first observation of BTEX utilization and its complete degradation.

for *p*-xylene than for *m*-xylene are noteworthy, since earlier studies implied that *p*-xylene and other *p*-alkylated monoaromatics are particularly recalcitrant in comparison to their *meta*- or *ortho*-substituted homologs (Rabus et al., 1996; 1999; Wilkes et al., 2000). The nitrate/BTEX-treated groundwater of well B was used as inoculum to obtain stable enrichment cultures in a defined mineral salt medium amended with nitrate and either toluene, ethylbenzene, *p*- or *m*-xylene. Enrichment cultures were obtained with toluene, ethylbenzene and *p*-xylene (for representative growth curves and microphotographs, see Fig. S1). In the presence of *m*-xylene, no stable culture was achieved, possibly caused by the accumulation of nitrite or toxic *m*-xylene concentrations. The 16S rRNA and *bamA* profiling revealed that toluene and ethylbenzene favored the enrichment of *Azoarcus* species (analyzed after 14 transfers into fresh medium): the relative abundance of *Azoarcus*-affiliated 16S rRNA sequences increased approximately 16fold from 5.4% in groundwater to about 85% in the enrichment cultures (see Table S3). In accordance, all *bamA* gene fragments recovered from these enrichment cultures showed highest similarity to *Azoarcus* sp. EbN1 (Fig. 3; sequences designated as Tol/Ethb_enrich highlighted in blue). In groundwater of well B, which was used for the enrichment, *Azoarcus* spp. showed a considerably higher abundance than in the other two wells. This result, in combination with the observation that the genus *Azoarcus* seems to be adapted to the

degradation of toluene and ethylbenzene, led to the assumption that well B is a hotspot of *Azoarcus*-dependent toluene and ethylbenzene degradation. This finding also explains the enrichment of *Azoarcus*-dominated cultures without lag phases from this well (Table 2). In contrast, the *p*-xylene-degrading enrichment culture (analyzed after 5 transfers into fresh medium) was dominated by *Georgfuchsia* spp. with a relative abundance of 60.8% of total 16S rRNA sequences (see Table S3). This corresponds to a 250fold enrichment compared to its abundance in groundwater of well B (0.24%). Also *Azoarcus* (4.4%), *Sulfuritalea* (3.8%), *Geobacter* (2.8%), and *Rhodoferax* (1.1%) were detected, however, relative abundances were similar to those initially detected in groundwater samples. In accordance to 16S rRNA-based community analysis, the majority of *bamA* gene fragments retrieved from the *p*-xylene-degrading enrichment culture were closely related to *Georgfuchsia toluolica* G5G6 (99.1% identity; Fig. 3, sequences designated as *p*-Xyl_enrich (clones 5 to 22) highlighted in blue). *Georgfuchsia*-related 16S rRNA sequences were considerably less abundant in the aquifer than *Azoarcus*-related sequences (Fig. 2B). For *bamA*, *Georgfuchsia*-related sequences were even below the detection limit (Table 1). This suggests that the here enriched *Georgfuchsia* sp. is rather rare in the aquifer, which also explains the prolonged lag phases observed for *Georgfuchsia*-dominated enrichments with *p*-xylene (Table 2).

3.5. Identification of a putative 4-methylbenzoyl-CoA reductase in the p-xylene-degrading enrichment culture

Whereas the genus *Azoarcus* is well-known for the degradation of toluene and ethylbenzene under denitrifying conditions (e.g. Fries et al., 1994; Rabus and Widdel, 1995; Zhou et al., 1995; Ball et al., 1996), *Georgfuchsia* spp. were so far not reported to degrade *p*-xylene. The only isolated representative within this genus, *Georgfuchsia toluolica* G5G6, was described to grow with ethylbenzene and toluene, but no growth was observed with *p*-xylene when nitrate was applied as electron acceptor (Weelink et al., 2009). Under the conditions we applied, *p*-xylene degradation was coupled to the reduction of nitrate to nitrite (Fig. S1). As reported for the type strain, in our study nitrate could be replaced by Fe(III) as electron acceptor (data not shown). The ability of the enriched *Georgfuchsia* sp. to degrade *p*-xylene suggests the presence of a novel type of class I benzoyl-CoA reductase specific for aromatic ring reduction of the *p*-methylated benzoyl-CoA intermediate previously found in the Alphaproteobacterium *Magnetospirillum* sp. pMbN1 (Lahme et al., 2012). To check for the presence of genes putatively encoding this novel type of enzyme, degenerated primers targeting the 4-methylbenzoyl-CoA reductase subunit C (*mbrC*) were developed and applied on DNA of the *p*-xylene-degrading enrichment culture. A ~700 bp fragment was amplified. The translated protein sequence showed 75% identity to the MbrC subunit of *Magnetospirillum* sp. pMbN1. Genome walking was used to identify the gene region containing *mbrA*, *mbrB*, *mbrC*, and *mbrD*. The results are shown in Fig. 4. All subunits showed highest protein identity (72–80%) to the corresponding Mbr subunits of *Magnetospirillum* sp. pMbN1. The identity to conventional class I benzoyl-CoA reductase subunits was equal or below 32%. These data indicate the presence of a benzoyl-CoA reductase specific for *p*-alkylated substrates in the *p*-xylene-degrading enrichment culture dominated by *Georgfuchsia* sp. However, it cannot be excluded that other genera present in this culture (e.g. *Sulfuritalea*, *Geobacter*) are involved in the degradation of *p*-xylene. The occurrence of a specific 4-methylbenzoyl-CoA reductase is rare in so far characterized ACDB (Rabus et al., 2016). Nevertheless, the genetic potential to utilize *p*-alkylated aromatic compounds is established at the Thuringia gasworks site, which is supported by higher degradation rates observed for *p*-xylene than for *m*- and *o*-xylene (Table 2). So far, a few enrichment cultures have been described to degrade *p*-xylene under different electron-accepting conditions (Häner et al., 1995; Morasch and Meckenstock, 2005; Nakagawa et al., 2008; Rotaru et al., 2010). Among these, a highly enriched, nitrate-reducing culture was described, which was dominated by a *Denitratisoma*-related phylotype, a close relative of *Georgfuchsia toluolica* G5G6 (Rotaru et al., 2010). The only isolated *p*-xylene-degrading bacterium reported so far uses sulfate as electron acceptor and is related to the genus *Desulfosarcina* (Deltaproteobacteria) (Higashioka et al., 2012). Our study provides a new *p*-xylene-degrading enrichment culture dominated by *Georgfuchsia* sp. Ongoing studies aim for the isolation of the microorganism(s) responsible for the degradation of *p*-xylene. This might allow further insights into the pathways involved in the anaerobic degradation of *p*-xylene and other *p*-alkylated monoaromatics, a microbial feature which is still to date rarely reported.

4. Conclusion

Deep 16S rRNA and *bamA* clone library sequencing were combined to characterize the microbial community of a coal tar polluted aquifer with emphasis on aromatic compound-degrading bacteria. The importance of nitrate as electron acceptor for aromatic compound degradation at the site was confirmed by the enrichment of nitrate-reducing toluene-, ethylbenzene- and *p*-xylene-degrading microorganisms from groundwater of the gasworks site. A close relative of *Georgfuchsia toluolica* G5G6 was found to be involved in the degradation of *p*-xylene, a compound, which is rarely observed to be degraded under anoxic conditions. The methods applied here allowed the identification of hotspots for aromatic compound degradation and the prediction of the prevailing electron-accepting processes in the aquifer, which are valuable information to predict and/or monitor the outcome of bioremediation.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.watres.2017.12.040.

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