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Depth-Resolved Quantification of Anaerobic Toluene Degraders and Aquifer Microbial Community Patterns in Distinct Redox Zones of a Tar Oil Contaminant Plume[▽]

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Microbial degradation is the only sustainable component of natural attenuation in contaminated groundwater environments, yet its controls, especially in anaerobic aquifers, are still poorly understood. Hence, putative spatial correlations between specific populations of key microbial players and the occurrence of respective degradation processes remain to be unraveled. We therefore characterized microbial community distribution across a high-resolution depth profile of a tar oil-impacted aquifer where benzene, toluene, ethylbenzene, and xylene (BTEX) degradation depends mainly on sulfate reduction. We conducted depth-resolved terminal restriction fragment length polymorphism fingerprinting and quantitative PCR of bacterial 16S rRNA and benzylsuccinate synthase genes (*bssA*) to quantify the distribution of total microbiota and specific anaerobic toluene degraders. We show that a highly specialized degrader community of microbes related to known deltaproteobacterial iron and sulfate reducers (*Geobacter* and *Desulfocapsa* spp.), as well as clostridial fermenters (*Sedimentibacter* spp.), resides within the biogeochemical gradient zone underneath the highly contaminated plume core. This zone, where BTEX compounds and sulfate—an important electron acceptor—meet, also harbors a surprisingly high abundance of the yet-unidentified anaerobic toluene degraders carrying the previously detected F1-cluster *bssA* genes (C. Winderl, S. Schaefer, and T. Lueders, Environ. Microbiol. 9:1035–1046, 2007). Our data suggest that this biogeochemical gradient zone is a hot spot of anaerobic toluene degradation. These findings show that the distribution of specific aquifer microbiota and degradation processes in contaminated aquifers are tightly coupled, which may be of value for the assessment and prediction of natural attenuation based on intrinsic aquifer microbiota.

The fate of organic contaminants in groundwater environments is controlled by a multitude of abiotic and biotic processes, such as transport, dilution, dispersion, and chemical or microbial degradation. The last is considered the only sustainable component of natural attenuation (9, 46). Due to large carbon loads and the low rates of oxygen replenishment to groundwater systems, oxygen is rapidly depleted upon contaminant impact (2). Therefore, within hydrocarbon-contaminated aquifers, anoxic contaminant plumes with distinct redox compartments are formed, where microbial guilds capable of using locally available electron donors and acceptors are active (9). Biodegradation processes occur at different rates in these redox zones (46), but it is still poorly understood which plume compartments are most relevant for net contaminant removal. Especially, overlapping countergradients of electron donors and acceptors are assumed to be hot spots of biodegradation (3, 12, 53, 54), a hypothesis that has recently been summarized as the "plume fringe concept" (4). Such redox gradients may be extant on very fine spatial scales; thus, a detailed characterization of redox species and intrinsic microbiota at appropriate spatial resolutions is a prerequisite for a better understanding of biodegradation processes.

It is especially relevant to ask how the spatial distribution of plume compartments and degradation processes is correlated to local microbial communities in general and whether the distribution of specific contaminant degraders can inform us about the occurrence and localization of the respective processes. Local microbial community composition has been shown to yield important insights into the microbes characteristic of different contaminated zones, as well as into their putative involvement in specific transformation processes in aquifers (1, 13, 27, 39). However, the monitoring of microbial capacities and their distribution at contaminated sites as a basis for assessing natural attenuation and for promoting biota-based site management options is still in its infancy. This may be attributed partially to the fact that conventional multilevel groundwater sampling is performed usually with a depth resolution of meters (24, 49). This spatial resolution has been suggested to be inadequate for truly assessing ongoing natural attenuation processes (57).

In this study, to address these questions, we have characterized microbial community distribution across a high-resolution depth profile of a tar oil-impacted aquifer at a former gasworks location, where hydrocarbon degradation has been reported to depend mainly on sulfate reduction (14, 59). We have specifically traced as a model system intrinsic populations of anaerobic toluene degraders by using their benzylsuccinate synthase

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TABLE 1. qPCR primer and TaqMan probe sets used in this study for the quantification of defined *bssA* gene copy numbers in sediment DNA extracts

Specificity (reference)	Primer or probe	5′–3′ sequence	Location[a]
F1-cluster *bssA* (58)	bssApd2f	CCT ATG CGA CGA GTA AGG TT	8235–8254
	bssApd2r	TGA TAG CAA CCA TGG AAT TG	8416–8435
	bssApd2h	TCC TGC AAA TGC CTT TTG TCT CAA	8295–8318
"*Aromatoleum aromaticum*" strain EbN1 *bssA* (23)	BssN2f	GGC TAT CCG TCG ATC AAG AA	7904–7923
	BssN2r	GTT GCT GAG CGT GAT TTC AA	8109–8128
	BssN2h	CTA CTG GGT CAA TGT GCT ATG CAT G	8005–8029

[a] Primer and probe locations are given according to *Thauera aromatica* K172 *bss* operon nucleotide numbering (25).

(*bssA*) genes as specific catabolic markers (58). Benzylsuccinate synthase (Bss) is the key enzyme of anaerobic toluene oxidation and has repeatedly been proven to be a valuable functional marker gene for unknown anaerobic toluene degraders (6, 55, 58). Thus, we have shown for the Flingern site in Düsseldorf, Germany, that local anaerobic toluene degraders are dominated by an as-yet unaffiliated lineage of environmental *bssA* genes, tentatively termed "F1-cluster" *bssA*, which has ~90% amino acid similarity to known geobacterial *bssA* genes (58). However, the identities of the degraders carrying these *bssA* genes, as well as their quantitative distribution and relevance for net toluene degradation in the contaminant plume, remain to be addressed.

At the site, we have collected sediment cores and installed a unique high-resolution multilevel monitoring well (3). Using fine-scale hydrogeochemical measurements as well as spatially resolved qualitative and quantitative molecular microbial community analyses, we show that a highly specialized degrader community, as well as a surprisingly high abundance of specific anaerobic toluene degraders, resides within the biogeochemical gradient zone underneath the plume core. These findings show that the distribution of specific aquifer microbiota and redox and degradation processes in contaminated aquifers are tightly coupled.

MATERIALS AND METHODS

Site description, sediment sampling, and biogeochemical measurements. Subsurface samples from a tar oil-contaminated former gasworks site in Düsseldorf-Flingern, Germany (14, 26, 59), were obtained from fresh sediment cores taken during the installation of high-resolution multilevel well (HR-MLW) 19222 in June 2005. A precise description of the well and its installation is detailed elsewhere (3). Triplicate sediment subcores were taken at different depths from the liners originating from between 5.7 and 12.7 m below ground surface (bgs) (the groundwater table was ~6.4 m bgs) by use of sterile 50-ml plastic tubes. Subsamples were immediately frozen on dry ice and stored at −20°C until DNA extraction. Groundwater samples for the analysis of contaminants and redox species were sampled from HR-MLW 19222 in September 2005. Dissolved toluene, sulfide, and ferrous iron in depth-resolved groundwater samples as well as sedimentary polycyclic aromatic hydrocarbons (PAHs) and ferrous iron were measured as described elsewhere (3).

Nucleic acid extraction. Independent DNA extracts from triplicate sediment subsamples were extracted from 19 depths between 5.7 and 12.7 m bgs. These depths covered all major redox compartments of the Flingern aquifer as well as one sample from the unsaturated zone (5.7 m) and one from the capillary fringe (6.3 m). DNA was extracted from freshly thawed ~1-g (~500-μl) aliquots of sediment material using a modification of a previously described protocol (35). Samples were suspended in 650 μl PTN buffer (120 mM Na_2HPO_4, 125 mM Tris, 0.25 mM NaCl [pH 8]) and incubated at 37°C for 30 min with 40 μl lysozyme (50 mg ml^{-1}) and 10 μl proteinase K (10 mg ml^{-1}). After the addition of 200 μl 20% sodium dodecyl sulfate, incubation was continued for 30 min at 65°C. Subsequently, the sediments were bead beaten (45 s at 6.5 ms^{-1}) with ~0.2 ml of zirconia-silica beads (a 1:1 mix of 0.1- and 0.7-mm diameter; Roth) in 2-ml screw-cap vials. Afterwards nucleic acids were sequentially purified by extraction with 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1) and 1 volume of chloroform-isoamyl alcohol (24:1) and precipitated with 2 volumes of 30% polyethylene glycol (17) by incubation at 4°C for 2 h and by centrifugation at 20,000 × *g* and 20°C for 30 min. All reagents were from Sigma, if not otherwise stated. For each single extract, two replicate extractions were pooled in 25 μl of elution buffer (Qiagen) and stored frozen (−20°C) until further analyses.

qPCR. Real-time quantitative PCR (qPCR) measurements were performed on a MX3000P qPCR cycler (Stratagene). For each sediment depth, the three independent DNA extracts from sediment subsamples were quantified in three different dilutions (1:5, 1:10, and 1:20) to account for the possibility of PCR inhibition in less diluted templates. Total bacterial 16S rRNA gene quantities were measured using a previously described Sybr green PCR (51) with minor modifications. We used standard *Taq* polymerase (Fermentas) assays in the presence of 0.1× Sybr green (FMC Bio Products) and 2 μl DNA template. Initial denaturation (94°C, 3 min) was followed by 50 cycles of denaturation (94°C, 15 s), annealing (52°C, 15 s), and elongation (70°C, 30 s). Subsequently, a melting curve was recorded between 55°C and 94°C to discriminate between specific and unspecific amplification products. An almost-full-length bacterial 16S rRNA gene amplicon of *Azoarcus* sp. strain T (22) genomic DNA was quantified using the PicoGreen double-stranded DNA quantification kit (Molecular Probes) and utilized as standard DNA for qPCR in a concentration range between 10^7 and 10^1 copies μl^{-1}.

For *bssA* qPCR, a TaqMan system for the previously identified F1 cluster of unidentified *bssA* genes (58) was developed (Table 1). We also developed an analogous assay for the *bssA* gene of "*Aromatoleum aromaticum*" strain EbN1 (23, 60). Primer and probe designs were conducted with Primer3 software v.0.3.0. (http://frodo.wi.mit.edu/). *bssA* qPCR was performed with the TaqMan universal master mix kit (Applied Biosystems), as specified by the manufacturer, using three-step thermal cycling (95°C, 10 min; and 50 cycles of 95°C, 15 s; 55°C, 20 s; and 72°C, 30 s) and 5′-6-carboxyfluorescein (FAM) and 3′-6-carboxytetramethylrhodamine dual-labeled probes (Biomers). A PicoGreen-quantified M13 amplicon of the F1-cluster partial *bssA* gene clone D12-03 (GenBank accession no. EF123678) previously retrieved from Flingern sediments and an almost-full-length *bssA* amplicon of "*A. aromaticum*" strain EbN1 were taken for standardization in concentrations between 10^7 and 10^1 copies μl^{-1}. PCR amplification efficiencies deduced from the slopes of standardization curves for different runs of 16S rRNA and *bssA* gene qPCR were 85% and 79% on average, respectively.

To correct for potentially distinct amplification/detection efficiencies of the different qPCR assays (i.e., Sybr green versus TaqMan PCR assays) and also for putative extraction/detection efficiencies of our general workflow, defined biomass amendments were evaluated for the Flingern sediments. For this, sediments were sterilized overnight at 180°C to eliminate intrinsic nucleic acids. Then, sediments were rewetted with sterile water and amended with defined cell numbers of a freshly grown, Sybr green-counted liquid culture of "*A. aromaticum*" EbN1 between 8×10^5 and 8×10^6 cells g^{-1} (wet weight) of sediment. Care was taken to adjust the sediments to the original water contents. Strain EbN1 carries four *rrn* operons and one *bss* operon per genome (23, 42). Nucleic acids were reextracted and quantified as described above. From the detected-versus-expected gene quantities, correction factors for the 16S rRNA and *bssA* gene counts directly obtained from Flingern sediments were inferred. Negative controls showed that heat sterilization destroyed over 99.99% of the initial 16S rRNA gene counts.

Microbial community fingerprinting. Terminal restriction fragment length polymorphism (T-RFLP) analysis of bacterial 16S rRNA gene amplicons was done as previously described (34) with primers Ba27f-FAM and 907r (40, 56) and MspI digestion. Primary electropherogram evaluation was performed using GeneMapper 5.1 software (Applied Biosystems). T-RF frequencies were inferred from peak heights (33). Signals with a peak height below 100 relative fluorescence units (41) or with a peak abundance contribution below 1% (36) were considered background noise and excluded from further analysis. The reproducibility of our T-RFLP assay was verified via replicate determination of T-RF abundances from two independent DNA extracts of three exemplary depths (6.3 m, 6.8 m, and 7.6 m bgs). T-RF abundances were highly reproducible, with an average standard deviation of 1.1% relative T-RF abundance (variations were between 0% and maximally 5.9% standard deviation for specific T-RFs). The Shannon-Wiener diversity index (H') was calculated as $H' = -\Sigma\, pi \ln pi$, where pi is the relative abundance of single T-RFs in a given fingerprint (19).

For statistics, T-RFLP data were evaluated as previously described (32, 43) using SYSTAT 10 software (SPSS, Inc.). Principal component analysis (PCA) was performed on T-RFLP relative peak abundance data. A covariance data matrix was extracted with pairwise deletion and no factor rotation. Data reduction provided a two-factorial PC ordination of the overall variance of the T-RFLP profiles. Additionally, a loading plot of inferred PC factors on specific T-RFs was generated to identify the T-RFs especially correlated to the discrimination of depth-resolved microbial community fingerprints in PCA ordination.

Cloning, sequencing, and phylogenetic analyses. Almost-full-length bacterial 16S rRNA gene amplicons were generated from the DNA extracts of four different depths (6.3 m, 6.8 m, 7.6 m, and 11.7 m) using the primer set Ba27f and 1492r (56). Amplicons were cloned and sequenced as previously described (58). Sequencing reads were manually assembled and checked for quality using SeqMan II software (DNAStar). All clones were subsequently screened for similarities to published sequences using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and integrated into an ARB database (31). All clone sequences were checked for chimeric nature with Chimera Check 2.7 of the Ribosomal Database Project II version 8.1 (http://rdp8.cme.msu.edu/html/) and by manual inspection of the alignment. From 151 clones, 6 were identified as chimeras and excluded from further analysis. For phylogenetic affiliation, trees including clones and closely related representative sequences (>1,400 bp) of cultivated and uncultivated species were constructed using neighbor-joining algorithms. Highly variable regions within the 16S rRNA were omitted from analysis by the application of a 50% base frequency filter. T-RFs of cloned sequences were predicted using ARB_EDIT4. For a representative set of clones, T-RFs predicted in silico were verified by direct T-RFLP analysis of cloned amplicons to precisely assign observed environmental T-RFs to cloned lineages.

Nucleotide sequence accession numbers. All clone sequences of this study were deposited with GenBank under accession numbers EU266776 to EU266920.

RESULTS

Depth distribution of contaminants and redox species in the plume. Sediment cores were taken at the tar oil-contaminated sandy aquifer in Düsseldorf-Flingern, Germany, in the course of the drilling and installation of an HR-MLW in June 2005 (3). Upon subsequent groundwater sampling in September 2005, the HR-MLW revealed steep gradients of contaminants and redox species over the depth transect. In Fig. 1, the depth-resolved distribution of the most prominent benzene, toluene, ethylbenzene, and xylene (BTEX) compound at the site, toluene, is displayed together with the reduced electron acceptors sulfide and ferrous iron in groundwater, as well as sedimentary PAHs and ferrous iron. In the upper 30 cm of the saturated zone, toluene concentrations averaged ~40 mg liter^{-1} and thus defined a highly contaminated plume core directly beneath the groundwater table. Toluene contributed approximately two-thirds of the total BTEX concentrations detected within these depths. Underneath this plume core, an ~65-cm-wide gradient zone, characterized by a strong decrease in contaminant concentrations and elevated levels of sulfide, was identified between 6.75 and 7.4 m bgs. These biogeochemical

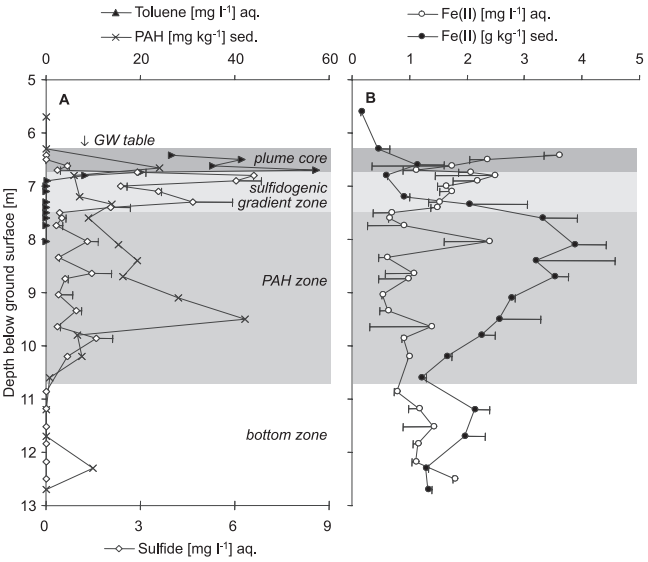

FIG. 1. Depth profiles of representative aromatic contaminants (A) and reduced electron acceptors (A and B) in sediment samples (sed.) and groundwater (aq.) from the Flingern aquifer. Samples were taken in June (sed.) and September (aq.) 2005. Error bars indicate the means of duplicate measurements, and the absence of error bars indicates single measurements. Plume and redox compartments were specified in accordance with the biogeochemical data. GW, groundwater.

data indicated that this "sulfidogenic gradient zone" (Fig. 1) was a "hot spot" of BTEX degradation within the plume at the time of the sampling. Previous investigations indicated that sulfate reduction dominated contaminant degradation at the site (3, 14, 59). The gradient zone was also characterized by slightly elevated concentrations of ferrous iron in groundwater (Fig. 1B), but distinction to other zones was not as clear. Readily extractable ferric iron, however, was not detectable in significant amounts in sediments below the capillary fringe (data not shown). All BTEX compounds were below the detection limit under a depth of ~8 m.

Beneath the strongly sulfidogenic gradient zone, increased microbial activities were still inferable from free sulfide and especially sedimentary ferrous iron concentrations. Contaminants other than BTEX, i.e., PAHs, were found sorbed to the sediments down to ~10 m bgs, but also below that depth (Fig. 1A). This points toward a distinct, mainly PAH-contaminated zone (~7.5 m to ~10.7 m). Naphthalene and fluorene contributed the major mass of the PAHs identified by EPA standards. Free sulfide was below the detection limit in depths beneath 10.2 m. We thus defined the remaining sampling depth down to the underlying aquitard (~10.7 m to 12.7 m) as a still reduced, but less contaminated, bottom zone of the Flingern aquifer.

Quantitative distribution of bacteria and anaerobic toluene degraders. We previously detected microbes carrying an as-yet unaffiliated type of putative deltaproteobacterial *bssA* gene (termed the F1 cluster) that dominated the community of anaerobic toluene degraders in the Flingern aquifer (58). To correlate the quantitative distribution of the F1-cluster microbes to those of contaminant and redox species and total bacterial populations, depth-resolved qPCR quantifications were conducted. First, however, it was necessary to validate the

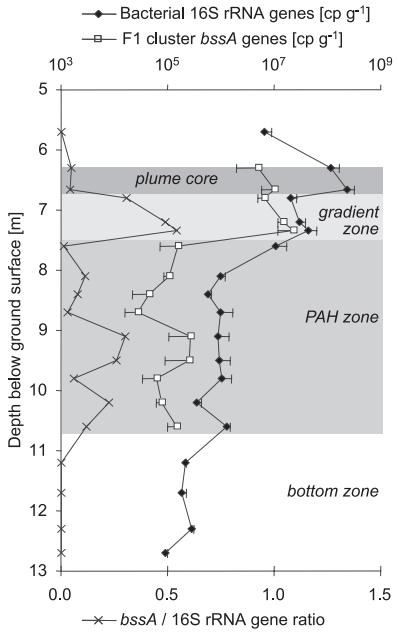

FIG. 2. Depth distribution of bacterial 16S rRNA genes and *bssA* genes of the F1 cluster of unknown anaerobic toluene degraders (58) as measured via qPCR. Shown are the means of gene cp g^{-1} sediment (wet weight) ± standard errors of three independent DNA extracts for each depth.

comparative extraction/detection efficiencies of the employed qPCR assays (16S rRNA gene Sybr green PCR versus *bssA* TaqMan PCR). For this, we added defined cell numbers of the denitrifying anaerobic toluene degrader "*Aromatoleum aromaticum*" EbN1 to heat-sterilized Flingern sediments. While expected *bssA* abundances were almost absolutely recovered at ~8×10^6 added cells g^{-1} sediment, 16S rRNA gene copy numbers were significantly underrepresented by a factor of ~6. The inferred correction factor was 5.5 ± 0.6 for the expected-versus-detected 16S rRNA/*bssA* gene frequencies over several orders of magnitude of cell biomass amendment. This factor was used to deduce corrected gene quantities from field qPCR measurements (Fig. 2).

Bacterial rRNA genes were detected in all depths and peaked at a maximum of $2.4 \pm 0.8 \times 10^8$ copies per g sediment (cp g^{-1}) directly within the plume core (6.65 m bgs). 16S rRNA gene quantities were generally above 10^7 cp g^{-1} in the upper zones of the aquifer (plume core and sulfidogenic zone) and dropped drastically in the PAH and bottom zones. Abundance distributions varied least within the PAH zone at a depth between 8.7 m and 9.8 m. In contrast to bacterial rRNA genes, the quantitative distribution of *bssA* genes peaked at $2.3 \pm 0.1 \times 10^7$ cp g^{-1} within the sulfidogenic gradient zone but not within the plume core. The sulfidogenic zone was generally characterized by high *bssA*/16S rRNA gene ratios, ranging between 0.3 and 0.5 (Fig. 2). Moreover, F1-cluster *bssA* genes were not detectable by TaqMan PCR (detection limit, ~5×10^3 cp g^{-1}) in sediments deeper than 11 m or above 6 m. Thus, their detection was highly correlated to saturated anaerobic contaminated sediments. Furthermore, by *bssA*-targeted T-RFLP fingerprinting with a previously published general primer set (58), we could show that the F1 cluster of *bssA* genes strongly dominated all *bssA* populations detected in the Flingern aquifer (data not shown).

Depth-resolved shifts in bacterial communities. Structural shifts in depth-resolved microbial communities were assessed via T-RFLP fingerprinting (Fig. 3). Fingerprinting data indicated strong changes in the diversity and composition of bac-

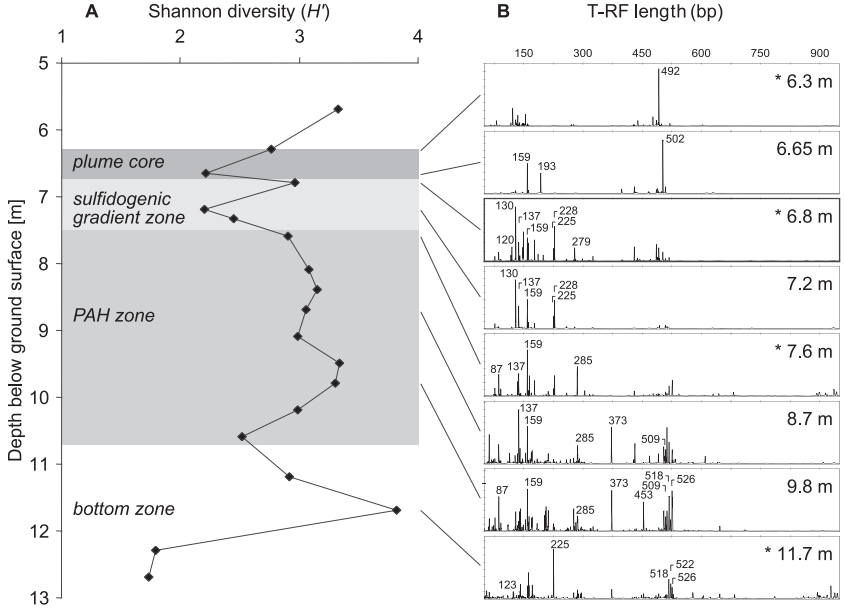

FIG. 3. Depth-resolved 16S rRNA gene T-RFLP fingerprinting of bacterial community structures in plume compartments of the Flingern aquifer. (A) Shannon-Wiener diversity index (*H'*) calculated for the entire T-RFLP data set. (B) Representative T-RFLP electropherograms of selected depths. Community patterns marked by an asterisk were subsequently selected for cloning. Selected characteristic T-RFs mentioned in the text are indicated by their base pair lengths.

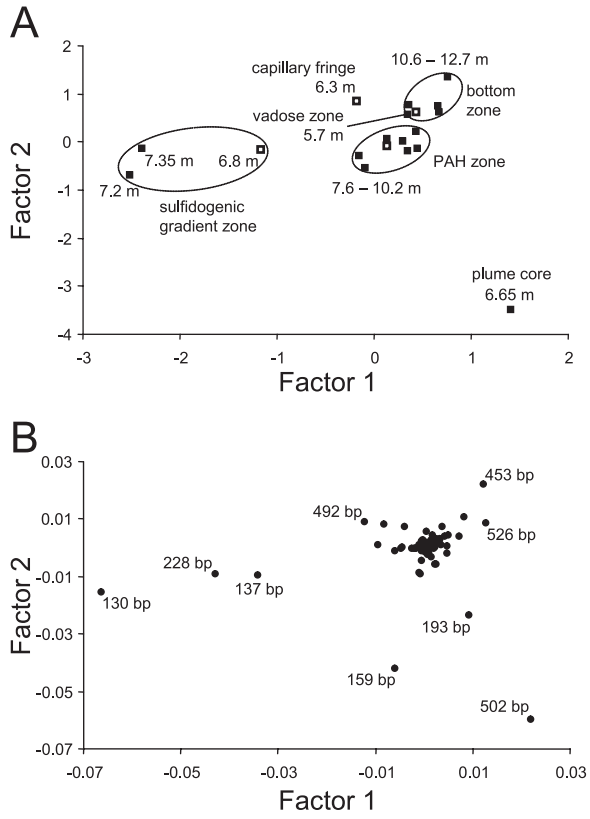

FIG. 4. (A) PC ordination of the overall variance in depth-resolved bacterial community composition as analyzed by T-RFLP fingerprinting. The depths at which specific fingerprints were retrieved are indicated next to the ordination points. Communities marked by an open square were subsequently selected for cloning. Inferred PC factors 1 and 2 accounted for 21.3% and 18% of total variance, respectively. (B) Loading plot of inferred PC factors on specific T-RFs. The identities (bp) of selected T-RFs with characteristic factor loading are indicated.

terial communities detectable by fingerprinting. The Shannon-Wiener diversity indexes (H') as inferred from relative T-RF abundances dropped to local minima within the highly contaminated plume core (6.6 m bgs), in the sulfidogenic gradient zone (7.2 m), and between the PAH and bottom zones (10.6 m) (Fig. 3A). As with absolute gene quantities, diversity indexes varied least throughout the PAH-contaminated zone. H' reached both the absolute maximum and minimum within the bottom zone.

Not only the diversity, but also the structural composition (i.e., the identity [bp] and relative abundance of detected T-RFs) of fingerprints, indicated that significant community shifts were occurring with depth. To better visualize these population shifts and to identify specific T-RFs characteristic for respective zones, PCA of T-RFLP data was conducted (Fig. 4A). The percentage of total community variability explained by the two primary PC factors inferred was only ~40%. This finding emphasizes the large variations in depth-resolved community structures evident already by visual inspection of the fingerprints (Fig. 3). The resulting limitations of reducing this variability to only a few virtual factors clearly caution the capability of our two-factorial PCA to explain overall microbial community variability in the Flingern aquifer. Nevertheless, data reduction clearly grouped fingerprints from different plume zones into distinct clusters and thus was able to recover some of the most relevant variations. Especially, populations from the sulfidogenic zone were separated in ordination from other samples, indicating distinct populations in these strata. Communities from the PAH and bottom zones were more related to each other and formed two adjacent clusters in PCA. Outliers were observed for the capillary fringe and especially the plume core, while the vadose zone community clustered together with the PAH and bottom zone samples.

The loading plot of inferred PC factors (Fig. 4B) helped us to identify the specific T-RFs responsible for the distinct PCA ordination of fingerprints. Hence, the sulfidogenic zone samples were characterized by high relative abundances of especially the 130-bp T-RFs, but also the 137- and 228-bp T-RFs. Also, the 225- and 279-bp fragments were abundant in these communities (Fig. 3B), but not PCA discriminators from other depths. The ordination of the plume core fingerprint was correlated to high abundances of the 502- and 193-bp T-RFs, but also the 159-bp T-RFs. The last was ordinated between the plume core, sulfidogenic zone, and PAH zone samples and thus represents an important constituent of all these communities, as confirmed also by the visual inspection of electropherograms (Fig. 3B). Fragments characteristic for the PAH zone were not detectable in PCA ordination, since most remaining T-RFs clustered near zero in ordination space. Nevertheless, their high relative abundances allowed us to identify the 87-, 137-, 159-, 285-, 373-, 509-, 518-, and 526-bp fragments as important within this zone (Fig. 3B).

Bacterial 16S rRNA clone libraries from four depths. To phylogenetically characterize the distinct microbial assemblages intrinsic to the different plume compartments and to identify putative community members represented by the identified characteristic T-RFs, four clone libraries were constructed. Overall, we sequenced 145 full-length bacterial 16S rRNA gene clones from the depths of 6.3 m bgs (capillary fringe), 6.8 m bgs (sulfidogenic zone), 7.6 m bgs (PAH zone), and 11.7 m bgs (bottom zone). The relative phylum-level composition of the clone libraries revealed surprisingly significant distinctions in local community assembly, as summarized in Table 2. *Alphaproteobacteria* were found almost exclusively at the capillary fringe (6.3 m), where they dominated the library together with members of the *Betaproteobacteria*. *Deltaproteobacteria*, which were frequent in all other libraries, were not detected at 6.3 m. Members of the *Betaproteobacteria*, *Deltaproteobacteria*, and *Clostridia* appeared especially frequently in the library of the sulfidogenic gradient zone (6.8 m). Members of the *Deltaproteobacteria*, *Actinobacteria*, and *Chloroflexi* were abundant in the PAH zone library, while *Betaproteobacteria* were missing there. The bottom zone library (11.7 m) contained the most even distribution of major bacterial phyla (Table 2).

A considerable diversity of clones closely related to defined genera or lineages well-known to be capable of characteristic catabolic or respiratory activities was detected. Sequences related to known methylotrophs, sulfur- and ferrous iron-oxidizers were found especially at the capillary fringe. The sulfidogenic zone library contained high frequencies of clones between 95 and 97% related to *Geobacter chapellei*, a well-known

TABLE 2. Relative phylum-level compositions of depth-resolved bacterial 16S rRNA gene clone libraries and selected genus- or lineage-specific clone frequencies

Phylogenetic affiliation[b]	% of clones in indicated library at depth (bgs) of[c]:				T-RF length (bp)[a]	
	D10 6.3 m	D12 6.8 m	D15 7.6 m	D25 11.7 m	Predicted	Measured
Alphaproteobacteria	**31**			**3**		NA
Methylocystis related	3					NA
Betaproteobacteria	**26**	**19**		**14**	123/492	120/492
Thiobacillus related	6			6		NA
Gallionella related	6	6			123/125	120/122
Gammaproteobacteria	**9**					NA
Beggiatoa related	6				138	135
Deltaproteobacteria		**26**	**16**	**11**		NA
Desulfocapsa related		6			162	159
Geobacter related		19			132	130
Syntrophus related			7	6	127/509	123/509
Desulfobacterium related				6	166	164
Bacteroidetes		**6**		**14**	91	87
Nitrospirae	**3**		**7**	**8**		NA
Magnetobacterium related			5	8	290	285
Bacilli		**10**		**8**	137	133
Clostridia	**14**	**29**	**2**	**3**		NA
Sedimentibacter related		19			280	279
Uncultured *Peptococcaceae*	6				282	281
Desulfosporosinus related		3			227	225
Desulfotomaculum related				3	214	ND
Actinobacteria	**3**	**3**	**16**	**11**		NA
Rubrobacter related			7	6	131/162	128/160
Chloroflexi	**3**		**23**	**17**		NA
Uncultured I				14	518	518
Uncultured II	3		21	3	373/454/523	373/453/522
Dehalococcoides related			2			NA
TM6	**9**					NA
OP5			**7**		230	224
OP10			**7**	**3**		NA
TM7/OP11		**3**	**9**			NA
Others	**3**	**3**	**12**	**8**		NA

[a] Characteristic T-RF lengths (bp) predicted from the sequence data for all or a major portion of the clones of a given affiliation are indicated together with T-RF lengths actually measured in T-RFLP analysis. Values separated by a slash indicate more than one characteristic T-RF for a lineage. NA, not applicable; ND, not determined.

[b] The naming of phyla without cultivated representatives is in accordance to reference 44.

[c] The libraries at 6.3, 6.8, 7.6, and 11.7 m contained 35, 31, 43, and 36 clones, respectively. Division-level percentages (given in bold) include the genus- or lineage-specific percentages (nonbold).

deltaproteobacterial iron reducer (10). Furthermore, clones affiliated with the *Desulfocapsa*-related sulfate-reducing strain TRM1 (38) were found in this library. For these two lineages, signature T-RFs of 130 and 159 bp were inferable (Table 2). The library was also characterized by a high ratio of *Clostridia*. These *Sedimentibacter*- and *Desulfosporosinus*-related *Clostridia* were represented within the 279- and 225-bp T-RFs, respectively. *Desulfobacterium*-related clones (164-bp T-RF) and *Syntrophus*-related clones (123 and 509 bp) were detected only in the libraries from the one and two deepest samples, respectively. Clones related to the unusual iron reducers of the genus "*Candidatus* Magnetobacterium" (285 bp) within the *Nitrospirae* (50) were detected only in samples deeper than the sulfidogenic zone. Other observed signature T-RFs for the sampled metacommunity were 87 bp (different members of the *Bacteroidetes*), 120 and 492 bp (different *Betaproteobacteria*), 133 bp (different *Bacilli*), 224 bp (OP5 candidate division), and 373, 453, 518 and 522 bp (different uncultured *Chloroflexi*).

Rarefaction analyses of the bacterial communities retrieved within the clone libraries showed that coverage of the libraries was by far insufficient, at both 95% sequence similarity (at the genus level) and at the phylum-level diversity cutoff (data not shown). This was expected, but the aim of this study was not to fully cover the diversity of the distinct microbial communities retrieved from the different plume compartments but to highlight the most significant distinctions.

DISCUSSION

Geochemical and microbial zonation in the Flingern plume. In this study, we provide a fine-scale molecular characterization of the distinct microbial assemblages found within defined contaminant and redox compartments of the Flingern BTEX plume. We link microbial data based on nucleic acid extracts of aquifer sediments to groundwater and sediment geochemical data obtained from the same site, an approach which has previously been shown to provide valuable insights into aquifer microbiota/process correlations (5, 13, 18). The present study regards these couplings in fine spatial resolution, with sampling intervals ranging between 5 and 15 cm in zones of special interest and between 30 and 60 cm in deeper zones. Conventional multilevel wells usually have a spatial resolution in the range of meters (13, 24, 49, 57), but geochemical gradients formed by microbial activities can be expected to prevail at much smaller scales within contaminated aquifers (3, 12, 53). Thus, low-resolution groundwater samplings may fail to identify the defined strata where electron donors and acceptors meet and provide only insufficient information on the biogeochemical and microbiological characteristics of such hot spots of degradation.

With this high-resolution sampling, we were able to identify a zone of strong sulfidogenic activities underneath the actual BTEX plume core, a zone in which sulfate-dependent degradation activities are likely to be of special relevance (3). qPCR analysis (Fig. 2) showed that this zone was characterized by significantly increased absolute and relative abundances of the yet unidentified microbes represented by the F1-cluster *bssA* genes previously detected at the site (58). The quantitative distribution of this catabolic gene by depth did not correspond to that of rRNA genes. In the core of the plume, where absolute rRNA gene abundance was highest, the inferred *bssA*/16S rRNA gene ratio was only ~0.04. Within the next few decimeters, this ratio increased to values between ~0.3 and ~0.5, concomitant to the observed increase of sulfide. This increase points toward the establishment of a highly specialized anaerobic toluene degrader community underneath the actual plume core. These findings support the presumed "plume fringe concept," which postulates that biodegradation of groundwater contaminants occurs mostly within the biogeochemical gradients surrounding contaminant plumes (3, 4, 12, 53, 54). Here, we provide supportive field data for anaerobic toluene degradation proceeding specifically via Bss. The importance of aerobic toluene degradation at the capillary fringe, or of other degradation and fermentation processes that may have sustained the high total biomass counts in the plume core, however, cannot be explained with our present data and requires further attention.

Furthermore, we must caution that although the measured distribution profiles of both rRNA and *bssA* genes can be expected to be reliable, the absolute gene counts and hence also specific gene ratios may yet be biased. We have corrected for putative extraction and detection biases by analyzing defined biomass amendments of strain EbN1. However, while the inferred correction factor may hold true for strain EbN1, it may still be biased for F1-cluster organisms. In fact, the corrected relative *bssA*/rRNA gene ratios of up to 0.5 in the sulfidogenic zone still seem quite high, assuming that oligotrophic groundwater microbiota will typically have one or two *rrn* operons and that degraders will carry one *bss* operon. Nevertheless, the depth-resolved differences in the distribution of both markers will remain constant, clearly emphasizing the increased relative abundance of anaerobic toluene degraders within the sulfidogenic zone.

Identification of zone-specific microbiota. We applied complementary fingerprinting and sequencing strategies to unravel the correlations between local microbial community patterns and biogeochemical processes in different compartments of the Flingern plume. Within the upper parts of the contaminated aquifer (capillary fringe, plume core, and sulfidogenic zone), where bacteria were generally more abundant than in the deeper zones, repeated significant shifts and drops in community diversity were observed on very small scales. This corroborates the existence of distinct local communities in these strata which are specifically selected for by the local contaminant and redox regimen. It is currently not known on what time scales such specialized assemblages establish or how reactive they are to hydraulic dynamics or inputs of distinct electron acceptors (18, 37). Nevertheless, this spatial selection of distinct communities may be very relevant for contaminant degradation.

Direct spatial correlations between aquifer microbial community structure and contaminant scenarios have been previously recognized (1, 13, 16, 27). However, correlations between aquifer geochemistry and microbial communities are extremely complex, and statistical tools are needed to unravel these relations (1, 39, 47). Here, we were able to actually identify distinct aquifer microbiota characteristic for the resolved plume compartments. This identification relies on a satisfactory precision in linking T-RFs observed in environmental fingerprints to T-RFs of cloned sequences. Generally, incongruencies of $\pm$ 2 to 3 bp between predicted and measured T-RFs have to be taken into account (21, 28, 33), even if T-RFLP electrophoresis and T-RF sizing conditions are optimized with utmost caution (52). Therefore, we verified predicted T-RFs for representative clone sequences. Average differences between predicted and measured T-RFs in our analyses were -2.6 ± 1.7 bp (Table 2).

The sulfidogenic zone sediments were characterized by high relative abundances of especially the 130-bp T-RFs, but also the 137-, 159-, 225-, and 228-bp T-RFs (Fig. 3 and 4). Via sequence data from the respective clone libraries, we could clearly affiliate the 130-bp T-RF to the *Geobacter* relatives detected only in the sulfidogenic zone library. It is striking that these well-known iron reducers were not retrieved in libraries from other zones of the Flingern aquifer. The 130-bp T-RF, however, was detected also in some fingerprints of the PAH zone, albeit at low frequencies. Furthermore, microbes affiliated with the *Desulfocapsa*-related sulfate-reducing contami-

nant degrader TRM1 (38) (159-bp T-RF), as well as clostridial sulfate reducers (45) and fermenters (7) (225 and 279 bp), were abundant in this zone. Unfortunately, a clear affiliation of the 137- and 228-bp fragments to clones retrieved within the sulfidogenic zone was not possible. Although these T-RFs were predicted for different *Bacilli* and *Clostridia* within our clone libraries (Table 2), they could not be verified by in vitro T-RFs. Thus, these apparent constituents of the Flingern community remain unidentified at present, potentially due to undersampling, and may possibly even represent fingerprinting artifacts (15). Along the same line, we must state that not all important T-RFs observed were recovered within our four libraries. Again, this may be an effect of our small clone libraries or of the different reverse primers used for both approaches. Certainly, this is also attributed to the fact that we did not clone and sequence amplicons from all relevant sediment depths, such as the plume core.

Nevertheless, the specific microbes characteristic for the sulfidogenic zone (deltaproteobacterial *Geobacter* and *Desulfocapsa* relatives and sulfate-reducing and -fermenting *Clostridia*) were in clear contrast to those characteristic for the capillary fringe (e.g., *Alphaproteobacteria* and *Betaproteobacteria*, the latter especially represented by the 492-bp T-RF) and for deeper zones of the Flingern aquifer (e.g., *Bacteroidetes*, 87-bp T-RF; *Magnetobacterium* relatives, 285-bp T-RF; and *Chloroflexi*, 373-, 454-, 518-, and 526-bp T-RFs). High frequencies of deltaproteobacterial 16S rRNA genes within contaminated aquifer sediments have previously been described, especially for the iron reducers of the *Geobacter* genus (11, 27, 29, 30). Here, however, we show that close relatives of these well-known contaminant degraders codominate a sulfidogenic gradient zone together with relatives of deltaproteobacterial and clostridial sulfate reducers and fermenters, which may point toward a colocalization or an overlap of the respective redox processes in the Flingern aquifer.

Putative affiliation of the F1-cluster *bssA* genes. The yet unaffiliated F1-cluster *bssA* genes (58) relatively and absolutely dominated in the sulfidogenic gradient zone. Thus, it seems fair to assume that the corresponding microbes will have also been detected in respective 16S rRNA gene analyses. We have previously identified the F1-cluster *bssA* genes as ~90% related to known geobacterial *bssA* and only ~75% related to the TRM1 *bssA* gene (58). At the same time, it was shown for a distinct site that our employed general *bssA* primer set is well capable of detecting typical geobacterial *bssA* genes when they are present (58). These facts allow the formulating of the following hypotheses on the putative affiliation of the F1-cluster *bssA* genes.

They may belong to the detected *Geobacter* relatives, which also have their respective clone and T-RF abundance distribution maxima in this zone. This would then imply that these local populations carry *bssA* genes more distinct from typical geobacterial *bssA* genes (20, 58). Concomitantly, the dominance of these specific degraders in the sulfidogenic gradient zone would imply that toluene degradation in these strata may not be as directly linked to sulfate reduction (or to typical sulfate reducers) as previously assumed (3, 14, 59). Up to now, no *Geobacter* isolate has been shown capable of reducing sulfate. However, some can reduce elemental sulfur (8) and thus could also be responsible for sulfide production. Nevertheless, at present we cannot exclude the possibility that the Flingern *Geobacteraceae*, if they carry the F1-cluster *bssA* genes, may well have the means of transferring electrons from toluene to sulfate. Although still a preliminary speculation, this possibility is supported by ongoing enrichment experiments in our laboratory, where Flingern toluene degraders carrying the F1-type *bssA* gene were detected in both sulfate-reducing as well as iron-reducing enrichment microcosms (U. Kunapuli, C. Windeerl, R. U. Meckenstock, and T. Lueders, work in progress).

Alternatively, the F1 *bssA* genes could also stem from other deltaproteobacterial sulfate reducers found within the gradient zone. Hence, these sulfate reducers would carry *bssA* genes distinct from that of strain TRM1 (58), which would be acceptable, since the possibility of the horizontal gene transfer of such catabolic genes can also not be excluded (48, 58). At the same time though, this would imply that none of the detected *Geobacter* relatives carry *bssA* genes, which otherwise would have been detected. Regarding the specific allocation of these microbes to the BTEX degradation hot spot and also the closer phylogenetic affiliation of the F1 *bssA* genes to known geobacterial sequences, the first scenario seems more likely at present. This, however, must clearly be verified in detail by our ongoing investigations.

In summary, we show that a highly specialized degrader community, including a surprisingly high abundance of anaerobic toluene degraders, resides within the sulfidogenic gradient zone underneath the contaminant plume core of the Flingern aquifer. These findings support the "plume fringe" hypothesis, according to which the fringes of contaminant plumes are the actual hot spots of contaminant degradation (3, 4, 53, 54). Also, these findings substantiate that the distribution of specific microbes and redox and degradation processes in contaminated aquifers are tightly coupled and that locally established biotic degradation capacities may be relevant controls of contaminant degradation in aquifers.

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