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ORIGINAL ARTICLE

Degradation of a benzene–toluene mixture by hydrocarbon-adapted bacterial communities

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Abstract We examined the rate of degradation of a benzene–toluene mixture in aerobic microcosms prepared with samples of an aquifer that lies below a petrochemical plant (SIReN, UK). Five samples exposed to different concentrations of benzene (from 0.6 to 317 mg l^{-1}) were used. Fast degradation (approx. 1–6 mg l^{-1} day^{-1}) of both contaminants was observed in all groundwater samples and complete degradation was recorded by the seventh day except for one sample. We also identified the microbial community in each of the samples by culture-independent techniques. Two of the less impacted samples harbour the aerobic benzene degrader *Pseudomonas fluorescens*, while *Acidovorax* and *Arthrobacter* spp. were found in the most polluted sample and are consistent with the population observed *in situ*. *Hydrogenophaga* was found in the deepest sample while *Rhodoferax* spp. were recovered in an alkaline sample (pH 8.4) and may also be implicated in benzene degradation. Time series analysis shows that each of the samples has a different community but they remain stable over the degradation period. This study provides new information on a well not previously studied (no. 309s) and confirms that adapted communities have the ability to degrade hydrocarbon mixtures and could be used in further bioaugmentation approaches in contaminated sites.

Keywords Benzene · Toluene · Degradation · *Hydrogenophaga* · Groundwater · Aquifer

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Introduction

Many contaminated subsurface environments are impacted by a mixture of the gasoline oxygenates, benzene, toluene, ethylbenzene, xylenes (BTEX), alkanes and polyaromatic hydrocarbons as well as a range of other contaminants. BTEX compounds are of particular interest because they are highly soluble in water and highly toxic. Several technologies are currently applied to remediate contaminated areas by the oil industry (Khan et al. 2004). However, BTEX contamination is still an issue of paramount importance, the better understanding of contaminated site communities and their particular behaviours towards different compound mixtures is critical in order to select the best bioremediation strategy (Hendrickx et al. 2005; Park and Sang 2007; Müller et al. 2009).

The growth of bacterial communities using BTEX as carbon sources has shown that bacteria are capable of degrading these compounds in contaminated and pristine sites (Fries et al. 1994). BTEX can be biodegraded in aerobic and anaerobic environments (Atlas 1981; Vogel and Grbic-Galic 1986) by a variety of bacteria very different from each other (Jindrová et al. 2002; Cavalca et al. 2004; Winderl et al. 2008). Communities that have been exposed to these pollutants are enriched in BTEX degrading bacteria.

Each contaminant has different resistance to microbial degradation. Some studies have revealed that the degradation rate of a BTEX compound can be influenced by the presence of another BTEX compound (Alvarez and Vogel 1991; Deeb and Alvarez-Cohen 1999; Littlejohns and Daugulis 2008). This interaction is due to many factors such as broad-spectrum enzymes with different affinities for each compound, repression and induction of genes involved in the degradation of one compound but not another, as well as the compound toxicity that can result in the death of degrading bacteria (Foght 2008).

We selected five samples (309d, 308i, DW3s, W18i, and 309s; Fig. 1) with different concentrations of benzene and toluene, depth and physicochemical characteristics (Table 1). Wells 309d, W18i, 308i and DW3s were previously studied for benzene degradation (Fahy et al. 2006; Aburto et al. 2009; Aburto and Ball 2009), while well 309s had not been studied before. Moreover, none of the bacterial communities of the SIReN aquifer had been studied during the degradation of a benzene–toluene mixture. This study compared five bacterial communities exposed to a benzene–toluene mixture.

Materials and methods

Site description

The SIReN site (UK) is an operational petrochemical plant, which overlies a major sandstone aquifer. The geology consists of four layers. Layer 1 is 2.8–8.5 m of sand with intermittent layers of gravel; layer 2 is 0.4–30 m of clay and sand lenses; layer 3 consists between 0 and 7.6 of weathered sands and gravels, and layer 4 is a deep aquifer, a continuous sandstone formation of at least 77 m thick. Styrene is the main contaminant in the soil but the major groundwater pollutants are BTEX. There are 76 monitoring wells over 26 locations across the site, mainly arranged in clusters of three to sample three depths at the same location. Monitoring studies have revealed that the most impacted areas have concentrations as high as 316 mg l^{-1} of benzene and 125 mg l^{-1} of toluene in different zones of the site (Jones et al. 2001).

Microcosms and benzene-toluene monitoring

Groundwater was sampled on 15 April 2003; all samples (309d, W18i, 309s and 308i) were collected with a bladder type pump except DW3s that was collected with a peristaltic pump. Aerobic microcosms were prepared in triplicates by dispensing 20 ml of the groundwater from wells 309d, DW3s, W18i, 309s and 308i into 110-ml serum bottles. The groundwater samples had been previously stored in the laboratory at 12°C and no residual benzene or toluene were detected when measured by GC-FID. The microcosms were spiked with benzene and toluene to give each a concentration of 25 mg l^{-1} in order to have a medium concentration of the contaminants and observe which one is degraded preferentially when the starting concentration is the same. Microcosms were later crimp-sealed with PTFE-lined silicone septa. The amount of oxygen in this volume of headspace (90 cm^3) had

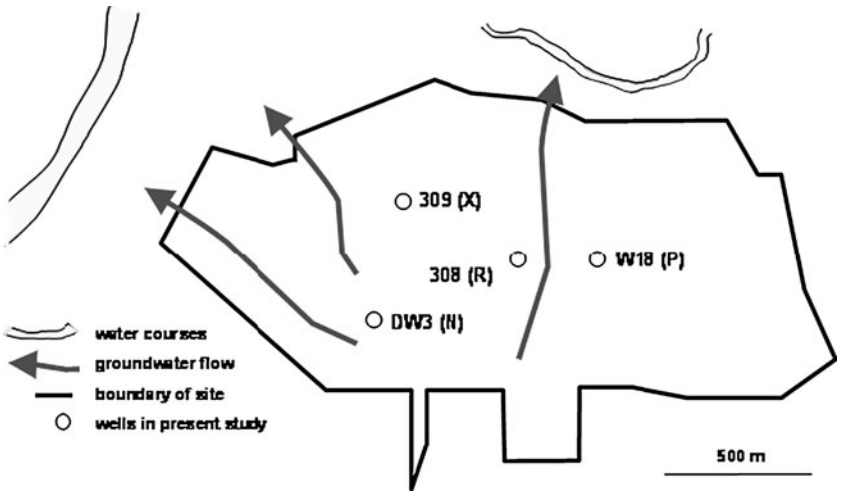

Fig. 1 Outline of the site SIReN, with general direction of the groundwater flow and location of wells sampled. All wells have an official name used in this study and in official reports (e.g. Jones et al. 2001), but in some papers (e.g. Fahy et al. 2005, 2006), a shorter code has been used (indicated in parentheses here). For well clusters at the same location, the relative depth is indicated in the text by s (shallow), i (intermediate) or d (deep) following the location name. For example, W18i analysed in this study was called Pi in (Fahy et al. 2005); it lies below W18s and above W18d

Table 1 Physical and chemical characteristics of wells sampled at the SIReN

Well	Layer	pH	DO (mg l^{-1})	Benzene (mg l^{-1})	Toluene (mg l^{-1})	Ethyl benzene	*m*- and *p*-xylene	*o*-xylene	Total PAHs	Total alkanes
309d	4	6.2	0.02	0.675	0.0002	12	23	11	0.14	13.3
308i	2	8.4	0.03	1.440	0.005	35	30	1	0.03	11.0
309s	1	6.1	0.00	3.607	0.003	85	33	34	3.16	12.6
DW3s	1	6.1	0.91	16.009	0.171	142	89	120	1.24	27.6
W18i	1	7.0	0.00	316.000	0.120	6,696	13	1	0.04	19.8

All values are in μg l^{-1} except where stated

Layer 1=sand and intermittent gravel; 2=clay; 3=sand and gravel; 4=sandstone

DO Dissolved oxygen, *PAH* polyaromatic hydrocarbons

Data from Shell Global Solutions measurements taken in April 2003 (G. Lethbridge, personal communication)

previously been shown to be sufficient to allow complete aerobic degradation of benzene at this concentration without depletion of oxygen (Fahy 2003).

Gas chromatography was used to measure the benzene and toluene concentrations in microcosms with a Unicam 610 Series GC fitted with a 4-mm internal diameter, glass packed column (10% apiezon on chromosorb W) and a flame ionization detector (FID). The temperatures were at the injector 250°C, column 155°C and detector 250°C, and detection was set at medium sensitivity. Hydrogen was used as carrier gas at a flow rate of 1.0 ml min^{-1}. Autoclaved microcosms were used as controls throughout the experiment. Standards and controls all had the same liquid/headspace ratio, and were held at the same temperature as the test samples. Microcosms were measured at time zero and every 2 days until hydrocarbons had been degraded. Degradation rates were calculated from linear regression of benzene or toluene concentrations curves by using Origin 7 (Originlab, Northampton, MA, USA).

DNA extraction

Microbial community DNA was extracted from the microcosms as previously described (Manefield et al. 2002). Nucleic acids were extracted from the start of the degradation (2 days) and every 2 days until degradation was complete, one microcosm was sacrified for each DNA extraction. Cells were pelleted by centrifuging 20 ml of groundwater at 3,200 *g* for 10 min at 4°C, the pellet was resuspended in 0.5 ml potassium phosphate buffer 240 mM (pH 8.0) and transferred to a Lysis matrix B (Bio Gene). Next, 450 μl of Phenol:Chloroform:Isoamylalcohol (25:24:1, pH 8), mix was added and the matrix was then shaken in a bead beater at 2,000 rpm for 30 s. The mixture was centrifuged at 4°C for 60 s [RCF (×g)] and 450 μl from the aqueous top layer was collected. An equal volume of Chloroform:Isoamylalcohol (24:1) was added and mixed by inversion. A volume of 400 μl of the top aqueous layer was again collected and transferred to a new micro-centrifuge tube. Exactly 40 μl of 3 M sodium acetate pH 5.2 and 1 ml of ice-cold 100% ethanol were added to be incubated on ice for 30 min and later centrifuged at 15,000 *g* for 25 min at 4°C. The supernatant was discarded and the pellet washed with 70% ethanol in DEPC-treated water, and air dried for 30 min. The DNA was resuspended in 30 μl of DEPC water (Manefield et al. 2002).

PCR and DGGE

Environmental DNA was amplified under standard cycling conditions that were as follows: 94°C, 2 min; (94°C, 1 min; 55°C, 1 min; 72°C, 2 min)×30; 72°C, 10 min. Each 50 μl reaction contained: 1 μl DNA template (approximately 1 to 10 ng), 2.5 units of *Taq* DNA polymerase (Qiagen), 10×buffer (Qiagen), 10×alpha casein (1 mg ml^{-1}) (Sigma), 5 mmol dNTPs (Invitrogen, Carlsbad, CA, USA), 20 pmol each of forward and reverse primer, with Muyzer primers: forward with GC clamp (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG) and reverse (ATT ACC GCG GCT GCT GG) (*E. coli* position 341 to 534 of 16S rRNA gene) (Muyzer et al. 1993).

PCR products with a GC clamp were separated without purification in an 8% w/v polyacrylamide gel with a linear denaturing gradient, increasing from 40% at the top of the gel to 60% at the bottom (100% denaturants correspond to 7 M urea and 40% v/v formamide). The DGGE gel was run at 60°C for 5 h in a Dcode Universal Mutation Detection System (Bio-Rad Hercules, CA, USA). Gels were stained for 15 min with 15 μl of SYBR Gold (Molecular Probes, Leiden, The Netherlands) diluted in 150 ml of TAE buffer. Stained gels were visualised in a GelDoc System (BioRad).

Dominant bands were excised and incubated in 40 μl of elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA pH 8 and 0.1% w/v SDS) for 4 h at 37°C. DNA was precipitated with two volumes of

absolute ethanol, washed with 70% w/v ethanol and air-dried. The pellet was resuspended in 20 μl DEPC treated water. Some of the dominant bands in each set of microcosms were cloned in order to avoid sequencing co-migrating DGGE bands. The 16S rRNA gene fragment was reamplified with primer forward M13 and reverse M13 in the first instance to confirm presence of insert, and secondly with both forward and reverse Muyzer primers prior to sequencing and identification.

Cloning and sequencing

PCR products were cloned with a TOPO TA cloning kit (Invitrogen), as described in the manufacturer's instructions using One Shot TOP10 chemically competent *E. coli* cells. White colonies were selected from LB agar plates containing ampicillin (50 μg ml^{-1}) and X-Gal (20 μg ml^{-1}). The screening of inserts from transformants was performed by direct PCR amplification from colonies using primers M13 forward (GAC GTT GTA AAA CGA CGG CCA G) and M13 reverse (CAC AGG AAA CAG CTA TGA CCA TG). Heating at 94°C for 10 min preceded standard cycling conditions (see above).

Sequencing reactions contained 1 μl of 10 pmol μl^{-1} of the reverse primer 1389R (Invitrogen), 2 μl of Big Dye Terminator V2.0 Cycle Sequencing kit, 6 μl of 2.5× sequencing buffer, 5 μl of purified PCR product, and 6 μl of water. Amplification conditions were: 25 cycles of (96°C, 15 s; 60°C, 15 s; 60°C, 4 min). After sodium acetate and ethanol precipitation, the sequencing reaction products were run on a Perkin Elmer ABI PRISM 310 capillary electrophoresis automated genetic analyser. Sequences were then processed with DNA Sequencing Analysis Software version 3.3.

Dendrograms

DGGE gel pictures were analysed by using the software Phoretix 1D as follows. Lanes were created manually, with a fixed width of 5% of the standard lane width. Each lane represented one groundwater sample or a specific time point. Background noise was subtracted by using the rolling ball algorithm with a radius of 50 pixels. First, automatic band detection was performed with a minimum slope of 75–100 and a noise reduction of 5, and peaks smaller than 2% of the maximum peak were discarded. The edge detection method was fixed to a width of 1. Then bands were assessed and corrected by eye, one by one. Bands were then matched to create a matrix containing presence or absence of band in each lane. The relatedness of the microbial communities was expressed as similarity coefficients. Sorensen's index of similarity was used to make pairwise comparison between different samples on a DGGE gel. Two bands were considered to be related if they migrated the same distance on the gel. Each sample was scored based on the presence or absence of band at the same position on the profile (lane) when compared to the profiles (lanes) of other samples on the gel. Dendrograms were also generated by subjecting the matrix to non-metric multidimensional scaling (NMDS) analysis using Primer software (version 6; Primer-E, Plymouth, United Kingdom).

Results

When groundwater samples were supplemented with the toluene and benzene mixture (25 mg l^{-1} each) fast degradation of both contaminants was observed with a constant rate; complete degradation of both contaminants was observed by the seventh day for all samples but 309d (Table 2, Fig 2). The DGGE profile of the bacterial communities during the degradation experiments shows that the community from each well was different, but for each well it remained stable during the period of incubation, suggesting that the degrading community is established early on (Fig. 3). Cluster analysis of the resulted DGGE gel confirmed this observation. Moreover, the dendrogram shows that there is 100% similarity between days 2 to 6 in samples 309d, 308i and W18i, while the community changes slightly on day 6 in samples 309s and DW3s (Fig. 4).

Excision of key bands from the DGGE gel (labelled on Fig. 3), followed by cloning (where stated) and sequencing was used to identify community members. The closest relatives to sequences obtained from sample 309d was *Hydrogenophaga flava* (Table 3). *Pseudomonas fluorescens* was identified as the closest relative to a specific band that appeared throughout the degradation process in samples DW3s and 309s (bands 3, 4 and 10). Moreover, a band showing 97.6% 16S rRNA gene sequence similarity to *Pseudomonas syringae* was also detected at all the time points in sample 309s, while *Pseudomonas mephitica*

Table 2 Rates of degradation in samples

Sample	Rate of degradation	
	Benzene mg l^{-1} day^{-1}	Toluene mg l^{-1} day^{-1}
309d	1.56±0.3	1.02±0.22
308i	6.20±1.41	6.41±0.21
309s	5.94±0.07	6.24±0.03
DW3s	3.67±0.42	3.62±0.25
W18i	5.44±0.13	3.57±0.53

The errors correspond to fitting errors

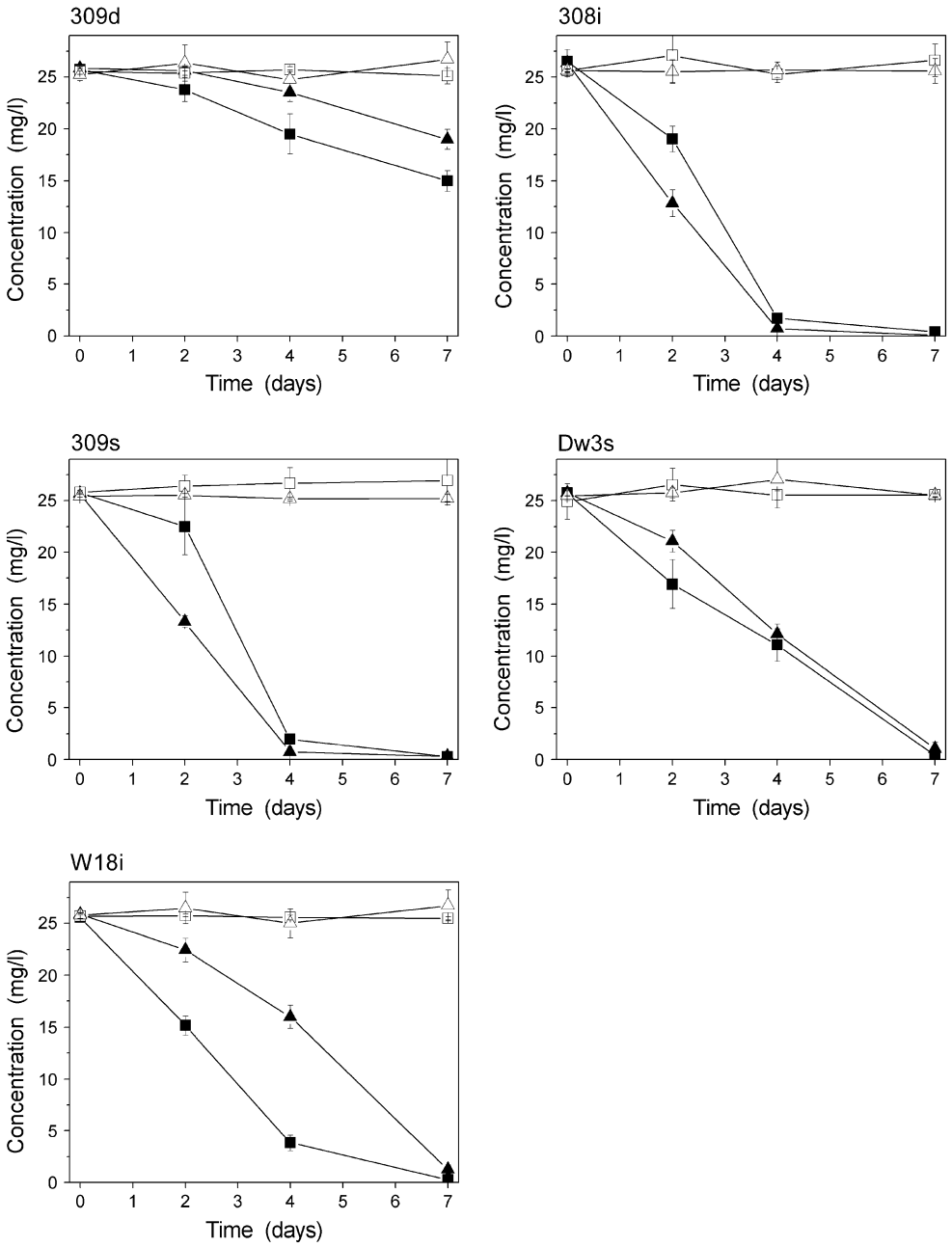

Fig. 2 Degradation of benzene–toluene mixtures by aerobic microcosms of groundwater samples. Benzene (■), toluene (▲), sterile controls of benzene (□) and toluene (Δ). The presented data are the means of triplicate experiments; *error bars* represent standard deviation of triplicates

(bands 5 and 6), *Acidovorax* sp. (bands 7 and 8) and *Arthrobacter* sp. were the closest relatives to the sequences retrieved from sample W18i. *Rhodoferax antarcticus* and *Methylobacter* sp. were the closest relatives to the bands sequenced from sample 308i.

Discussion

The microcosms for this study were prepared with groundwater samples from the wells 309d, 308i, 309s, DW3s and W18i at the SIReN site. All locations were exposed to hydrocarbons *in situ*. Upon addition of the hydrocarbons benzene and toluene to microcosms, the lack of a lag phase and the fast degradation of both hydrocarbons can be explained by the fact that the groundwater had developed an aerobic bacterial community readily adapted to the hydrocarbons. Several factors, such as the availability of organisms as well as enzymes and the presence of other substrates, have been shown to influence the relative rates of degradation of benzene and toluene, including the enhanced degradation of benzene in the presence of toluene (Alvarez and Vogel 1991; Arvin et al. 1989; Lee et al 2002).

Degradation rates observed in this study are consistent with other studies of degradation (Corseuil et al. 1998;

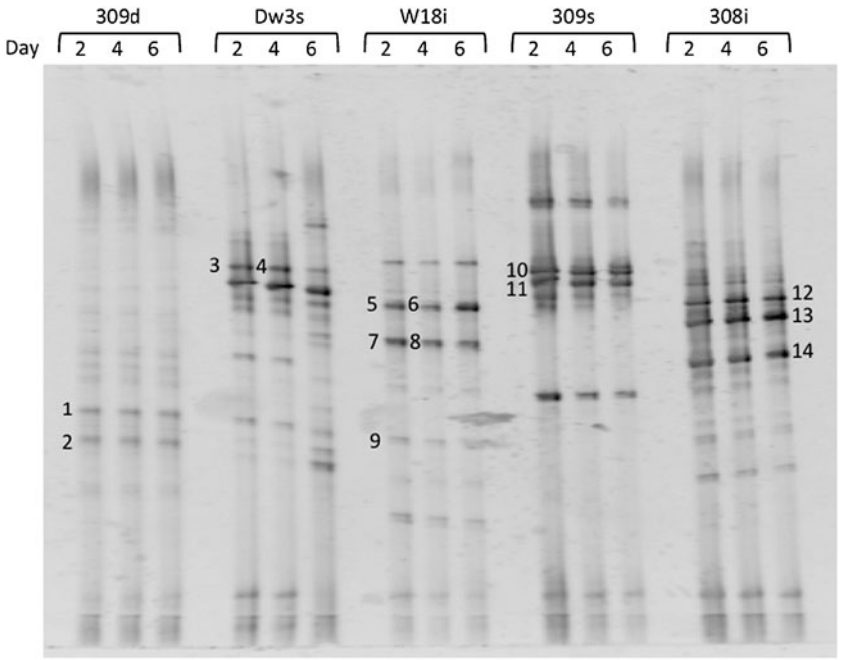

Fig. 3 DGGE gel showing the bacterial communities over time in microcosms of groundwater samples in wells 309d, DW3s, W18i, 309s and 308i inoculated with a mixture of benzene and toluene. Closest relatives of band 1: *Hydrogenophaga flava*, bands 3+4: *Pseudomonas fluorescens* Pf-5, bands 5+6: *Pseudomonas mephitica* ATCC, bands 7+8: *Acidovorax* sp. A-07-20, band 9: *Arthrobacter* sp. 14III, band 10: *Pseudomonas fluorescens* P69, band 11: *Pseudomonas syringae* B728a, bands 12+13: *Rhodoferax antarcticus*, band 14: *Methylobacter* sp. SV96

Collins and Daugulis 1999; Chang et al. 2001); however, in studies with hydrocarbon mixtures, a preferential degradation of toluene over benzene has been documented (Deeb and Alvarez-Cohen 1999, Chang et al. 2001); in this study, the degradation rates of benzene and toluene were very similar. This indicates (1) the presence of certain microbes that have the ability to degrade both hydrocarbons, or (2) the presence of different types of microbes, each of them with the ability to degrade benzene and toluene, respectively. The concentration of toluene *in situ* was much lower (below 1 mg l^{-1}) than those in the microcosms (25 mg l^{-1}), unlike the *in situ* concentrations of benzene (Table 1). Degradation of both hydrocarbons showed a constant rate. This is consistent with data obtained by Fahy and

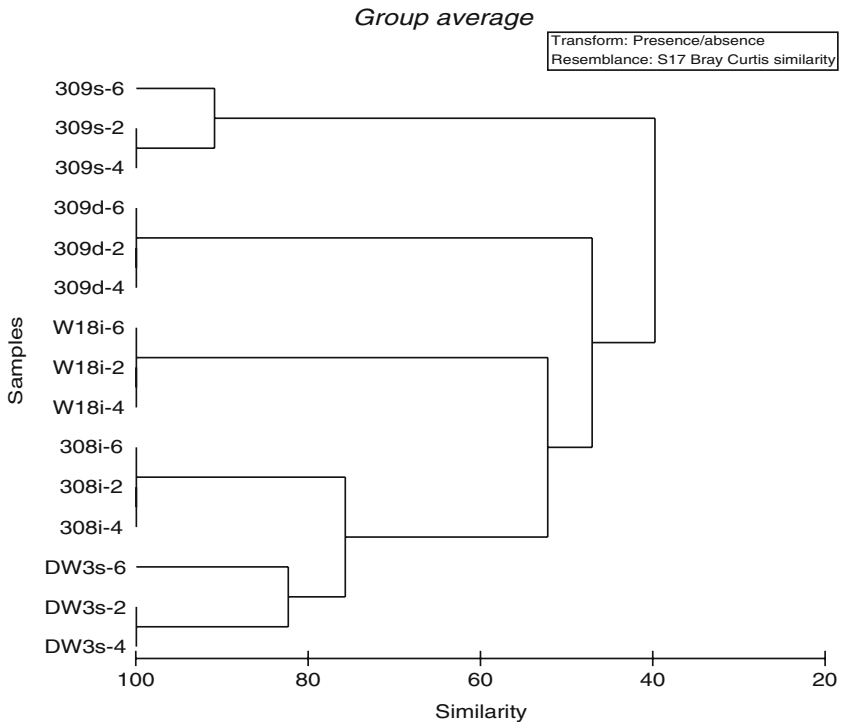

Fig. 4 Relationship between the composition of bacterial communities from five groundwater samples 309d, DW3s, W18i, 309s, 308i over 6 days. Relative depths of wells are indicated by: *d* deep, *i* intermediate, *s* shallow The dendrogram was created using Bray Curtis similarity which treats the data on a presence/absence basis and equates to the Sorensen coefficient

Table 3 Phylogenetic relationships based on 16S rRNA gene sequence of DGGE separated DNA bands from groundwater microcosms inoculated with a mixture of benzene and toluene

Sample	Band	Closest relative (notes)	Accession no.	% Similarity
309d	1[a]	*Hydrogenophaga flava*	AJ420328	100.0
DW3s	3, 4	*Pseudomonas fluorescens* Pf-5	CP000076	92.5
W18i	5, 6	*Pseudomonas mephitica* ATCC	AB021388	93.4
	7, 8	*Acidovorax* sp. A-07-20 (Deg. Polycyclic aromatic hydrocarbons)	AY136528	96.6
	9	*Arthrobacter* sp. 14III	AY576707	93.4
309s	10	*Pseudomonas fluorescens* P69 (Phenol and cresol contaminated area)	AY973265	94.5
	11	*Pseudomonas syringae* B728a	CP000075	97.6
308i	12[a]	*Rhodoferax antarcticus* Fryx1	AY609198	98.4
	13[a]	*Rhodoferax antarcticus* Fryx1	AY609198	98.9
	14[a]	*Methylobacter* sp. SV96 (Methane oxidizing bacterium)	AJ414655	99.8

[a] Cloned bands

collaborators (2008b) where degradation of benzene and toluene by *Pseudomonas* and *Hydrogenophaga* isolated from the SIReN aquifer was observed.

Sequencing of DGGE bands revealed the identity of some of these degraders. Bands 1 and 2 (Fig. 3) were predominant in sample 309d. However, only band 1 produced good quality sequences after cloning. The sequences were similar to the strain *Hydrogenophaga flava* which could be one of the hydrocarbon degraders in this sample. This is supported by the detection of this organism in parallel microcosms using the same groundwater as in the present study but degrading benzene, mixtures of benzene–toluene and benzene–naphthalene (Aburto 2007).

Two bands (3 and 4 in Fig. 3) at different time points but with the same profile on the acrylamide gel proved to be the same organism in sample DW3s. This organism was the strain *Pseudomonas fluorescens* Pf-5, and is a well-known benzene and toluene degrader (Greene et al. 2000). The strains *Pseudomonas mephitica* (band 5+6), *Acidovorax* sp. (band 7+8) and *Arthrobacter* sp. (band 9) were the closest match to the most dominant bands in sample W18i (Table 3). The *Acidovorax* strain was found as part of enrichment from northern soils (Ellesmere Island, Nunavut, Canada) capable of PAHs degradation (Eriksson et al. 2003), suggesting that this strain is also capable of monoaromatic hydrocarbon degradation. More important, a benzene-degrading consortium which was dominated by *Acidovorax* spp. was detected in groundwater sample W18i, and t-RFLP community profiles indicate that the community is very similar to that *in situ* suggesting that members of the *Acidovorax* can degrade benzene in the SIReN aquifer (Fahy et al. 2006). The *Arthrobacter* strain obtained had been found on the sea surface microlayer of coastal systems but has not been linked to benzene or toluene degradation (Agogue et al. 2005); however, several species of these genera such as *Arthrobacter oxydans* (Greene et al. 2000) and the strain HCB (Alvarez and Vogel 1991) can degrade these contaminants if oxygen is available.

The rapid degradation of both contaminants in sample 309s is consistent with *Pseudomonas fluorescens* P69 and *Pseudomonas syringae* B728a as the closest matches for the bands obtained on the denaturing gel. The former strain had been found in a phenol and *p*-cresol contaminated environment while the second is highly similar to the hydrocarbon degrader *Pseudomonas putida* KT2440. Thus, the degradation in this sample is enhanced by the presence of different organisms each with the ability to mineralise not only benzene and toluene mixtures but a wide variety of hydrocarbons, and is consistent with the low benzene concentration *in situ* (Table 1).

There were three predominant bands in sample 308i (Fig. 3) that were excised and cloned; two of them matched with *Rhodoferax antarcticus* (band 12+13) while the other was close to *Methylobacter* sp. (band 14). The former has been detected in the same area of the site but higher up in sample 308s (well 308 shallow sample), (Fahy et al. 2006), and the *Rhodoferax* clones from the most contaminated wells in SIReN are similar to many others from environmental clone libraries. Notably for example, DW3d4 (AM491923) (Aburto et al. 2009) has 99.1% 16S rRNA gene similarity to the most abundant clones from the most contaminated groundwater in a benzene-contaminated aquifer near Zeitz, Germany (Alfreider and Vogt 2007), suggesting a role for *Rhodoferax* in benzene degradation. Further support comes from the appearance of DGGE bands from *Rhodoferax* (and *Dechloromonas*) in a flow-through sand-packed column, only in the location where, and at the time when, benzene was being degraded (Aganbi et al., unpublished). Moreover, *Rhodoferax ferrireducens* has been detected previously in clone libraries from samples DW3d and W18i (Aburto et al.

2009) in the SIReN site and has been linked to hydrocarbon degradation previously (Eriksson et al. 2003). *Methylobacter* sp. had been found in the arctic but is not associated to hydrocarbon degradation. The match of *Rhodoferax antarcticus* to different bands along the same lane must correspond to several heterogeneous copies of 16S rRNA gene, since the two bands were cloned prior to sequencing, avoiding the possibility of sequencing mixtures of organisms.

Although the microorganisms in samples 309d and 308i are not consistent with the *Pseudomonas* spp. observed *in situ* (Aburto et al. 2009) or in microcosms (Fahy et al. 2006), they are also benzene degraders and this suggests that *Hydrogenophaga* or *Rhodoferax* strains are favoured when both contaminants are present at the same concentration. In contrast, the microorganisms detected in well W18i are consistent with those observed *in situ* (Aburto et al. 2009) and in microcosms with high benzene concentrations (Fahy et al. 2008a). Moreover, the present study identified some of the prominent bacterial communities in samples 309s and DW3s, adding novel information to the microbiology of the site and complementing previous studies.

In summary, we identified aerobic bacteria that can survive in microaerobic or even anaerobic conditions by diverse metabolisms. It is clear that all five samples harbour very different bacterial populations (Fig. 4) and this was expected due to the high heterogeneity of the samples *in situ* (Table 1). Two of the less impacted samples (DW3s and 309s) harbour *P. fluorescens* which can be easily cultured, and it has shown its ability to degrade benzene in aerobic and microaerobic conditions (Mikesell et al. 1993; Shim and Yang 1999); both of these are shallow samples and contain polycyclic aromatic hydrocarbons (PAH), while it has been reported that *P. fluorescens* is able to degrade PAH in the rhizosphere (Ho et al. 2007). *Acidovorax* and *Arthrobacter* spp. were found in the most polluted sample (W18i). *Acidovorax* is a facultative litoautotrophic that can use molecular hydrogen and has been found in anaerobic sites contaminated with toluene (Winderl et al. 2008), while *Arthrobacter* is found in diverse environmental samples; it is characterized by its ability to survive under stressful conditions induced by starvation, ionizing radiation, oxygen radicals, and toxic chemicals (Mongodin 2006). *Hydrogenophaga* was found in the deepest sample (309d); it is a facultative autotrophic bacteria that can perform anaerobic nitrate respiration and has been found in anaerobic sediments contaminated with chlorobenzene (von Wintzingerode et al. 1999). *Rhodoferax* sp. and *Methylobacter* sp. were recovered in an alkaline sample (308i; pH 8.4). *Rhodoferax* is a facultative photoheterotrophic bacterium that grows anaerobically using either photosynthetic or fermentative metabolism, while *Mehtylobacter* is a facultative methylotrophic. Although *Rhodoferax and Methylobacter* are not unique to alkali environments, it is well known that they tolerate environments with high pH (Hiraishi et al. 1991; Trotsenko et al. 2007). In this study, we corroborate that very different communities of bacteria can degrade BTEX compounds.

In all cases, microcosm conditions had higher concentrations of oxygen, benzene and toluene than *in situ*; however, only the sample from DW3s changed its structure while the stability of the bands throughout time in the other four samples indicates that the community does not change from the onset to the end of the degradation period (Figs. 3 and 4). This suggests that the communities were already adapted to both pollutants and to the presence of oxygen, although *in situ* dissolved oxygen was negligible. Previous studies of benzene degradation using microcosms with samples of some of these wells indicate that populations are capable of rapid aerobic benzene degradation. This is due to the presence of facultative aerobes responding promptly to oxygen ingress (Fahy 2006; Aburto and Ball 2009; Aburto et al. 2009). Fahy et al. (2008a) studied samples of the same aquifer in aerobic microcosms with high concentrations of benzene (100 mg L^{-1}) and these experiments showed population changes in a few days (from Gram-negative to Gram-positive bacteria); moreover, very closely related *Arthrobacter* and *Rhodococcus* species were found in different samples after they were adapted to high concentrations of benzene.

Conclusion

The addition of a benzene–toluene mixture to adapted microbial communities results in a rapid and concomitant degradation of both contaminants (7 days) with rates of degradation of 1 to 6 mg l^{-1} day^{-1}; these rates are independent of the level of contamination to which the samples were exposed. The absence of a lag phase in all the samples and the stability of populations during the degradation period (except for DW3s) suggest that the aerobic degradation mechanism for benzene and toluene was already available. The identification of several possible hydrocarbon degraders such as strains of *Hydrogenophaga*, *Rhodoferax*, *Pseudomonas* and *Arthrobacter* in most of the samples confirms the adaptability of the microbial community and suggests the presence of these microorganisms *in situ* and also the capability to degrade a wide range of contaminants if proper conditions arise. It is also clear that communities are different from each other in each of the wells and that these adapted bacteria could be used for bioaugmentation on other contaminated sites.

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