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

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 cm³) had...
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/heads pace 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 sacrificed 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 (BioGene). 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 bottom (100% denaturants correspond to 7 M urea and 40% w/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
absolutely 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 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 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

| Sample   | 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.
(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;
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. 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 methitica* 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

**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.
Pseudomonas 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-toluene 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 RNA 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.

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### 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   | 1a   | 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   | 12a  | Rhodoferax antarcticus Fryx1 | AY609198     | 98.4        |
|        | 13a  | Rhodoferax antarcticus Fryx1 | AY609198     | 98.9        |
|        | 14a  | Methylobacter sp. SV96 (Methane oxidizing bacterium) | AJ414655     | 99.8        |

*a Cloned bands*
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 phototrophic bacterium that grows anaerobically using either photosynthetic or fermentative metabolism, while Methylobacter 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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References

Aburto A (2007) Microbial diversity and factors affecting benzene degradation in a benzene-contaminated aquifer. PhD thesis, University of Essex, Colchester

Aburto A, Ball AS (2009) Bacterial population dynamics and separation of active degraders by stable isotope probing during benzene degradation in a BTEX-impacted aquifer. Rev Int Contam Ambie 25:147–156

Aburto A, Fahy A, Coulon F, Lethbridge G, Timmis KN, Ball AS, McGenity TJ (2009) Mixed aerobic and anaerobic microbial communities in benzene-contaminated groundwater. J Appl Microbiol 106:317–328

Agogue H, Casamayor EO, Bourrain M, Obernosterer I, Joux F, Herndl GJ, Lebaron P (2005) A survey on bacteria inhabiting the sea surface microlayer of coastal ecosystems. FEMS Microbiol Ecol 54:269–280

Alfreider A, Vogt C (2007) Bacterial diversity and aerobic biodegradation potential in a BTEX-contaminated aquifer. Water Air Soil Pollut 183:415–426

Alvarez PJJ, Vogel TM (1991) Substrate interactions of benzene, toluene, and p-xylene during microbial-degradation by pure cultures and mixed culture aquifer slurries. Appl Environ Microbiol 57:2981–2985

Arvin E, Jensen BK, Gundersen AT (1989) Substrate interactions during aerobic biodegradation of benzene. Appl Environ Microbiol 55:3221–3225

Atlas RM (1981) Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiol Rev 45:180–209

Cavalca L, Dell’Amico E, Andreoni V (2004) Intrinsic bioremediability of an aromatic hydrocarbon-polluted groundwater: diversity of bacterial population and toluene monooxygenase genes. Appl Microbiol Biotechnol 64:576–587

Chang SW, La HJ, Lee SJ (2001) Microbial degradation of benzene, toluene, ethylbenzene and xylene isomers (BTEX) contaminated groundwater in Korea. Water Sci Technol 44:165–171

Collins LD, Daugulis AJ (1999) Benzene/toluene/p-xylene degradation Part I Solvent selection and toluene degradation in a two-phase partitioning bioreactor. Appl Microbiol Biotechnol 52:354–359

CorseuilHX, Hunt CS, Ferreira dos Santos RC, Alvarez PJJ (1998) The influence of the gasoline oxygenate ethanol on aerobic and anaerobic BTX biodegradation. Water Res 32:2065–2072

Deeb RA, Alvarez-Cohen L (1999) Temperature effects and substrate interactions during the aerobic biotransformation of BTEX mixtures by toluene-enriched consortia and Rhodococcus rhodochrous. Biotechnol Bioeng 62:526–536

Eriksson M, Sodersten E, Yu ZT, Dalhammar G, Mohn WW (2003) Degradation of polycyclic aromatic hydrocarbons at low temperature under aerobic and nitrate-reducing conditions in enrichment cultures from northern soils. Appl Environ Microbiol 69:275–284

Fahy A (2003) Bacterial diversity and community dynamics in a benzene-contaminated sandstone aquifer. PhD thesis, University of Essex, Colchester

Fahy A, Lethbridge G, Earle R, Ball AS, Timmis KN, McGenity TJ (2005) Effects of long-term benzene pollution on bacterial diversity and community structure in groundwater. Environ Microbiol 7:1192–1199

Fahy A, McGenity TJ, Timmis KN, Ball AS (2006) Heterogeneous aerobic benzene-degrading communities in oxygen-depleted groundwaters. FEMS Microbiol Ecol 58:260–270

Fahy A, Ball AS, Lethbridge G, McGenity TJ, Timmis KN (2008a) High benzene concentrations can favour Gram-positive bacteria in groundwaters from a contaminated aquifer. FEMS Microbiol Ecol 65:526–533

Fahy A, Ball AS, Lethbridge G, Timmis KN, McGenity TJ (2008b) Isolation of alkali tolerant benzene-degrading bacteria from a contaminated sandstone aquifer. Lett Appl Microbiol 47:60–66

Foght J (2008) Anaerobic biodegradation of aromatic hydrocarbons: pathways and prospects. J Mol Microbiol Biotechnol 15:93–120

Fries MR, Zhou HJ, Chee-Sanford J, Tiedje JM (1994) Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Appl Environ Microbiol 60:2802–2810

Greene EA, Kay JG, Jaber K, Stehmeier LG, Voordouw G (2000) Composition of soil microbial communities enriched on a mixture of aromatic hydrocarbons. Appl Environ Microbiol 66:5282–5289

Hendrickx B, Dejonghe W, Boënne W, Brennerova M, Cernik M, Lederer T, Bucheli-Witschel M, Bastiaens L, Verstre et W, Top EM, Diels L, Springael D (2005) Dynamics of an oligotrophic bacterial aquifer community during contact with a groundwater plume contaminated with benzene toluene, ethylbenzene, and xylenes: an in situ mesocosm study. Appl Environ Microbiol 71:3815–3825

Hiraiishi A, Hoshino Y, Satoh T (1991) Rhodococcus fermentans gen. nov., sp. nov., a phototrophic purple nonsulfur bacterium previously referred to as the “Rhodocyclus gelatinosus-like” group. Arch Microbiol 155:330–336

Ho CH, Applegate B, Banks MK (2007) Impact of microbial/plant interactions on the transformation of polycyclic aromatic hydrocarbons in rhizosphere of Festuca arundinacea. Int J Phytoremediation 9(2):107–114

Jindrová E, Chocová M, Demnerová K, Brenner V (2002) Bacterial aerobic degradation of benzene, toluene, ethylbenzene and xylene. Folia Microbiol 47:83–93

Jones D, Lethbridge G, McCarthy P, Thomson S (2001) Project SIREN: Phase 2a conceptual site model & groundwater model R&D technical report P2-208/TR/2. Environment Agency, UK

Khán FI, Hussain T, Hejazi R (2004) An overview and analysis of site remediation technologies. J Environ Manage 71:95–122

Lee EY, Jun YS, Cho KS, Ryu HW (2002) Degradation characteristics of toluene, benzene, ethylbenzene, and xylene by Stenotrophomonas maltophilia T3-e. J Air Waste Manage 52:400–406

Littlejohns JV, Daugulis AJ (2008) Kinetics and interactions of BTEX compounds during degradation by a bacterial consortium. Process Biochem 43:1068–1076

Manefield M, Whiteley AS, Griffiths RI, Bailey MJ (2002) RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. Appl Environ Microbiol 68:5367–5373

Mikesell MD, Kuokkula JI, Olsen RH (1993) Metabolic diversity of aromatic hydrocarbon-degrading bacteria from a petroleum-contaminated aquifer. Biodegradation 4:249–59

Mongodin EF, Shapir N, Daugherty SC, DeBoy RT, Emerson JB, Shvartzbeyn A, Radune D, Vamathevan J, Riggs F, Grinberg V, Khouri H, Wackett LP, Nelson KE, Sadowsky MJ (2006) Secrets of soil survival revealed by the genome sequence of Arthrobacter aurescens T1C1. PLoS Genet 2:e214

Müller S, Vogt C, Laube M, Harms H, Kleinesteuber S (2009) Community dynamics within a bacterial consortium during growth on toluene under sulfate-reducing conditions. FEMS Microbiol Ecol 70:586–596

Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis
analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700
Park JY, Sang BI (2007) Change of sludge consortium in response to sequential adaptation to benzene, toluene, and o-xylene. J Microbiol Biotechnol 17:1772–1781
Shim H, Yang ST (1999) Biodegradation of benzene, toluene, ethylbenzene, and o-xylene by a coculture of Pseudomonas putida and Pseudomonas fluorescens immobilized in a fibrous-bed bioreactor. J Biotechnol 67:99–112
Trotsenko YA, Doronina NV, Li TD, Reshetnikov AS (2007) Moderately haloalkaliphilic aerobic methylobacteria. Microbiology 76:253–265
Vogel TM, Grbic-Galic D (1986) Incorporation of oxygen from water into toluene and benzene during anaerobic fermentative transformation. Appl Environ Microbiol 52:200–202
von Wintzingerode F, Selent B, Hegemann W, Göbel UB (1999) Phylogenetic analysis of an anaerobic, trichlorobenzene-transforming microbial consortium. Appl Environ Microbiol 65:283–286
Winderl C, Anneser B, Griebler C, Meckenstock RU, Lueders T (2008) Depth-resolved quantification of anaerobic toluene degraders and aquifer microbial community patterns in distinct redox zones of a tar oil contaminant plume. Appl Environ Microbiol 74:792–801