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BACTERIAL POPULATION DYNAMICS AND SEPARATION OF ACTIVE DEGRADERS BY STABLE ISOTOPE PROBING DURING BENZENE DEGRADATION IN A BTEX-IMPACTED AQUIFER

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Key words: aerobic benzene degradation, SIP, groundwater, population dynamics

ABSTRACT

The activity and diversity of a groundwater bacterial community was studied during the degradation of benzene in samples from a BTEX-contaminated aquifer (SIReN, UK) through the use of denaturing gradient gel electrophoresis (DGGE), followed by excision and sequencing of dominant bands. Rapid aerobic benzene degradation occurred in all samples, with 60-70 % degradation of benzene. DGGE analysis revealed that unique, stable bacterial communities were formed in each sample. *Pseudomonas putida* and *Acidovorax delafieldii* were identified in groundwater samples 308s and W6s respectively, suggesting they are the important taxa involved in the degradation of benzene. Further work based on stable isotope probing (SIP) of RNA using ^{13}C benzene was carried out. Prominent bands were identified as *Acidovorax* and *Malikia* genera; the latter is very similar to the benzene-degrader *Hydrogenophaga*, which confirms the presence of active benzene degraders in the groundwater samples. The identification of the prominent communities provides knowledge of the bioremediation processes occurring *in situ* and the potential to enhance degradation. This study highlights the potential of combining community fingerprinting techniques, such as DGGE, together with SIP.

Palabras clave: degradación aeróbica de benceno, SIP, agua subterránea, dinámica de poblaciones

RESUMEN

El presente estudio investiga la dinámica de poblaciones durante la degradación de benceno en muestras de un acuífero contaminado con BTEX mediante la técnica de DGGE, obtención de bandas y su posterior secuenciación. Los resultados indican degradación aeróbica de benceno en todas las muestras y que las poblaciones son diferentes en cada muestra, pero se mantienen estables durante la degradación del contaminante. Las principales bandas obtenidas en el gel de poliacrilamida fueron secuenciadas e identificadas como *Pseudomonas putida* y *Acidovorax delafieldii* en las muestras 308s y W6s respectivamente, lo que sugiere que estos microorganismos son los principales degradadores

en ciertas muestras. Mediante el uso de isótopos estables se comprobó la existencia de microorganismos aerobios degradadores de benceno, los cuales fueron identificados como miembros de los géneros *Acidovorax* y presumiblemente *Hydrogenophaga* en otra de las muestras. La identificación de comunidades prominentes brinda información sobre los procesos que ocurren in situ y del potencial para aumentar la degradación. El presente estudio muestra el potencial de combinar técnicas como DGGE con isótopos estables.

INTRODUCTION

The contamination of groundwater represents a major hazard in terms of both environmental damage and public health; contaminants such as PAH (polyaromatic hydrocarbons), MTBE (methyl *tert*-butyl ether), and BTEX (benzene, toluene, ethylbenzene and xylenes) enter natural waters via wastewater effluents from petroleum refining industries, rainwater runoff from roads, accidental spills and leakages from underground storage tanks. The most soluble components of gasoline and diesel fuel are the BTEX compounds, making them common contaminants in a large number of aquifers. Among the BTEX compounds, benzene requires special attention because it is the most toxic, the most soluble (Sikkema *et al*. 1995) and carcinogenic (Dean 1985). Benzene is readily degraded aerobically, and recent studies have shown it is also degraded in the absence of oxygen (Phelps *et al*. 1998, Rooney-Varga *et al*. 1999, Caldwell and Suflita 2000, Villatoro-Monzón *et al*. 2003, Kasai *et al*. 2006). Further work is now required to elucidate the microorganisms responsible for benzene degradation in the various environments.

Stable isotope probing

Stable isotope probing (SIP) is used to identify microorganisms that assimilate a specific growth substrate. This is achieved by labelling the substrate with a stable isotope (the most common is ^{13}C) that will be incorporated into the microorganism's cellular biomarkers (e.g. lipids, DNA and rRNA) if they utilize the substrate. The labelled nucleic acids can then be resolved from the unlabelled ones by density gradient ultracentrifugation, specific genes amplified and separated in a denaturing gel, and sequenced. Thus, by sequencing the labelled nucleic acids, the microorganisms responsible for the substrate uptake can be identified and therefore link function to microbial identity. Although SIP can be performed with DNA or rRNA, the latter presents some advantages over DNA, such as a higher copy number and a higher turnover rate, which is a reflection of cellular activity independent of replication (Manefield *et al*. 2002a, Manefield *et al*. 2002b, Lueders *et al*. 2004, Dumont and Murrell 2005).

The aim of this study was to obtain a profile of the bacterial community in several groundwater samples during benzene degradation and also to link the aerobic bacterial community in the groundwater sample DW3d to a specific function, i.e. benzene degradation. Sample DW3d was selected from the SIReN site for this experiment because it is heavily polluted (42.9 mgL^{-1}; **Table I**) with benzene (Earle *et al*. 2001) and the microorganisms thriving in it have been exposed to the contaminant for a long period,

TABLE I. PHYSICAL AND CHEMICAL CHARACTERISTICS OF WELLS SAMPLED AT THE SIReN

Well	Class[a]	pH	DO[b,c] (mgl^{1})	Benzene[c]	Toluene[c]	Ethyl Benzene[c]	*m*- & *p*-xylene[c]	*o*-xylene[c]	Total PAH	Total alkanes
W6s	Clean	5.3	0.00	<1	<1	<1	<1	<1	2.03	11.5
W18s	Clean	7.0	2.22	46	2	<1	<1	<1	0.56	12.2
308s	Low	10.2	0.16	15563	61	484	9	8	0.20	21.0
DW3s	Low	6.1	0.91	16009	171	142	89	120	1.24	27.6
DW3d	High	6.0	0.03	42969	1439	18390	1805	921	1.80	12.1

All values are in $\mu g\ l^{-1}$ except where stated
a. Class = Classification
b. DO = Dissolved Oxygen
c. Data from Shell Global Solutions measurements taken in April 2003 (G. Lethbridge, personal communication), except for benzene measurement from DW3d which was measured on the second sampling session as part of this study.

suggesting adaptation to the pollutant. Furthermore, previous clone libraries indicate the presence of aerobic hydrocarbon degraders such as *Polaromonas naphthalenivorans* (Aburto *et al*. 2009).

MATERIALS AND METHODS

Study site and sampling

The study site is a BTEX-contaminated aquifer located below an operational petrochemical plant known as SIReN (Site for Innovative Research in Natural Attenuation) in the UK. This study focuses on five wells showing different physicochemical characteristics and levels of contamination, nominally classified as high, low or clean (**Table I**). Details about the site can be found elsewhere (Earle *et al*. 2001, Fahy *et al*. 2005).

The groundwater used to prepare the microcosms was collected on the second sampling session (19/05/05) as described previously (Aburto *et al*. 2009). Samples DW3s, 308s and W18s were collected with a peristaltic pump, while a bladder type was used for DW3d and W6s. Groundwater was flushed from wells until pH, temperature and conductivity were stable. Samples were collected in 1 L glass bottles. The groundwater was refrigerated and protected from light during transportation to the laboratory.

Microcosms and benzene monitoring

The microcosms were prepared approximately eight hours after sampling by dispensing 60 mL of groundwater samples DW3d, DW3s, W18s, W6s and 308s in 110 mL serum bottles, and spiking with ^{13}C-benzene to a final concentration of 25 mgL^{-1}, which is about half of the last measurement *in situ*. These groundwater samples were selected in order to compare high contaminated, low contaminated, and clean samples (**Table I**). Microcosms were incubated in the dark at 12 °C (which is the *in situ* temperature) for seven days. 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.

DNA extraction and PCR

DNA was extracted at four different time points as described previously (Aburto *et al*. 2009) after the preparation of the microcosms. The time points were labelled: 0, 2, 5, and 7 days that correspond to a precise sampling time of 12, 48, 120 and 168 h respectively. Partial 16S rRNA gene fragments were obtained from PCR amplification with bacterial Muyzer primers 1. Forward with GC clamp (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG) and 2 Reverse (ATT ACC GCG GCT GCT GG) (*E. coli* position 341 to 534) (Muyzer 1993). The cycling conditions were as follows: an initial denaturation step of 94 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. Cycling was completed by a final elongation at 72 °C for 10 min.

DGGE

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 D Code Universal Mutation Detection System (Bio-Rad Hercules, CA, USA). Gels were stained for 15 min with 15 µL of SYBR Gold (Molecular Probes BV, Leiden, Netherlands) diluted in 150 mL of 1X TAE buffer. Stained gels were visualized in a GelDoc System (Bio Rad). 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. Bands 1, 4, 9, 12, 13 (**Fig. 1**) 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 a first instance to confirm presence of the insert and later with both forward and reverse Muyzer primers prior to sequencing and identification.

Sequencing

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 (approx 15 ng) and 6 µL of water. Amplification conditions were: 25 cycles of (96 °C, 15 sec; 60 °C, 15 sec; 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 analyzer. Sequences were then processed with DNA Sequencing Analysis Software version 3.3.

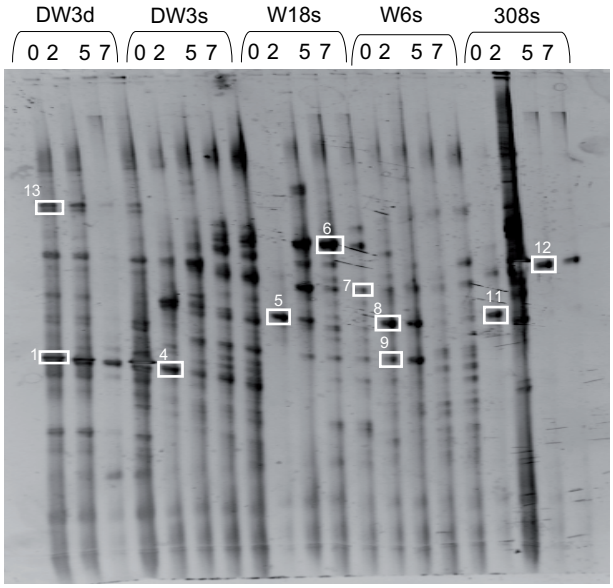

Fig. 1. DGGE gel showing the profile of the time series analysis carried out on microcosms spiked with ^{13}C benzene in the following samples: DW3d, DW3s, W18s, W6s and 308s. The numbers 0, 2, 5, and 7 indicate the day at which the community was analyzed

Stable isotope probing

The protocol used in this study is an adaptation of the method used by Manefield *et al.* (2002a). Microcosms containing 100 mL of groundwater (prepared eight hours after sampling) from well DW3d were spiked with the stable isotope ^{13}C-benzene (all carbon atoms labelled) to 25 mgL^{-1} as final concentration and incubated at 12 °C for 10 days. Nucleic acid extractions were performed at time 0 (4 hours after the ^{13}C benzene spike) and on the days 2, 5 and 7. RNA was isolated and quantified. 50 µL of template RNA (approx. 10 ng) was added to a mixture of 9.69 mL caesium trifluoroacetate (2.0 g mL^{-1}), 0.39 mL formamide and 1.37 mL H_2O in an 11.5 mL Sorvall ultracentrifugation tube (supplied by Kendro laboratories). The mixture was centrifuged at 35,000 rpm (119,500 x *g*), for 48 h in a Sorvall Discovery 90SE Ultraspeed Centrifuge, with a TST 41.14 rotor. It was later fractionated in 0.5 mL aliquots by piercing the bottom of the centrifuge tube with a needle to collect the fraction, and injecting sterile H_2O on top of the tube with the help of a peristaltic pump at a rate of 10 µL per second. A total of 20 fractions were collected per gradient, and refractive indices were measured. The RNA of each fraction was precipitated with isopropanol as follows: 1 mL isopropanol was added to each fraction and kept at –20 °C for 1 hour, centrifuged at 13,000 rpm for 30 min at 4 °C; the pellet was washed with 70 % ethanol, air dried and resuspended in 25 µL of DEPC treated H_2O. Each fraction was later subjected to reverse transcription and PCR amplification with Muyzer primers containing the GC-clamp. Appropriate fractions were selected and run in an acrylamide gel for community fingerprinting. Bands 1 to 7 were excised, cloned and reamplified with Muyzer primers (without the GC clamp) for sequencing. Sequences were compared with those in the EMBL database using a FastA search in order to identify the closest relatives.

For clarity, from now on, a sample spiked with ^{13}C-benzene will be called "heavy sample", while a sample with background concentrations of benzene will be referred as "light sample". In order to confirm the separation of labelled from the non-labelled RNA in sample DW3d, heavy and light gradients obtained from the isolate *Rhodococcus* Q71 were tested.

The *Rhodococcus* isolate Q71 was grown on ^{12}C and ^{13}C-benzene separately. Gradients were prepared with RNA extracted from both conditions. RNA fractions were reverse transcribed, amplified with Muyzer primers and run in an agarose gel.

In the same way as with the *Rhodococcus* isolate, microcosms prepared with sample DW3d were amended separately with ^{12}C and ^{13}C-benzene. The gradients were centrifuged, fractionated and the refractive indices measured for each fraction (**Table III**). The fractions were treated as described for the *Rhodococcus* strain.

RESULTS

Changes in bacterial community during benzene degradation

Benzene degradation was detected from the second measurement at day 2 for most of the samples and until the final measurement on day 7 (**Fig. 2**) when the last microcosm was sacrificed for nucleic acid extraction. Generally, the rates of benzene degradation were similar for all samples (except the killed control), with a 60-70 % reduction in benzene concentration remaining after the 7 d incubation.

The DGGE pattern (**Fig. 1**) obtained from the different samples corresponds to each of the time points of benzene monitoring by gas chromatography (**Fig. 2**) and shows a stable community over time in most of the samples as well as different communities among the samples, which can also be seen in a dendrogram (**Fig 3**). All of the prominent bands in the gel were excised in order to identify them; however, several bands (n = 7) had poor sequence quality. Good quality sequences were retrieved from

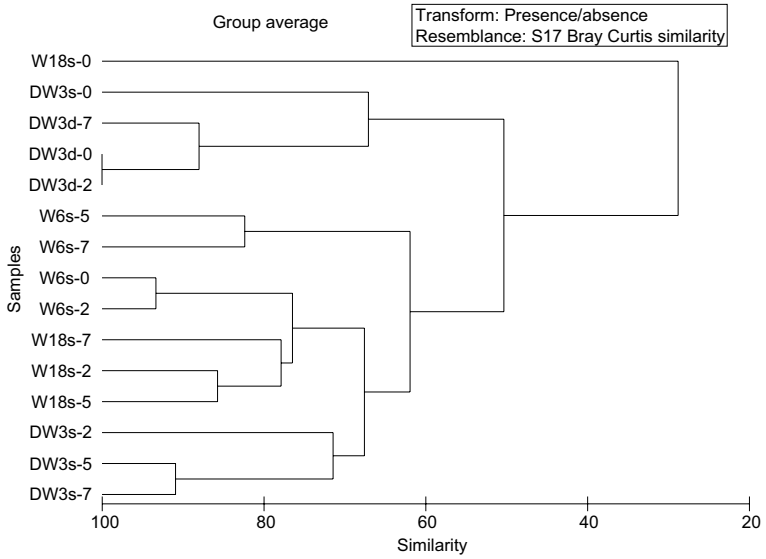

Fig. 3. Dendrogram showing the relationship between bacterial communities from five groundwater samples over seven days. The numbers 0, 2, 5, and 7 indicate the number of days at which the nucleic acids extraction was performed. Some time points had to be left out of the analysis since bands in the DGGE gel were very faint

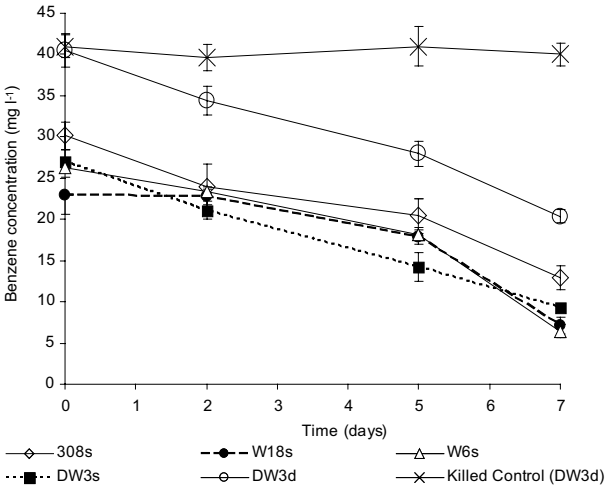

Fig. 2. Degradation of ^{13}C benzene in samples 308s, W18s, W6s, 308i, DW3s, DW3d. Error bars represent 1 SD of two samples. One representative control is shown (i.e. autoclaved groundwater from well DW3d)

the bands marked on the DGGE gel (**Fig. 1**) and their closest relatives are shown in **table II**.

The sequences retrieved from sample DW3d were closely related (97 % similar) to *Malikia granosa* and a bacterium isolated from soil (**Table II**). The organisms retrieved from the other groundwater samples (**Fig. 1**) included the following bacterial species: *Aquaspirillum autotrophicum* (band 7) in sample W18s, *Lactococcus lactis* (bands 5, 8, 11) in samples W18s, W6s and 308s, *Bacteriovorax stolpii* (previously known as *Bdellovibrio stolpii*; Baer *et al*. 2000) (band 6) in sample W18s, and *Pseudomonas putida* strain KT 2440 (band 12) in sample 308s. The sequences retrieved from bands 9 (sample W6s) and 13 (sample DW3d) matched with the organisms *Acidovorax delafieldii* and a bacterium found in a TEX mixture respectively; however, they were distant to this organism with 16S rRNA sequence similarity below 90 % for both sequences (**Table II**).

TABLE II. CLOSEST RELATIVES OF BAND SEQUENCES EXCISED FROM DGGE IN **FIG 1**, # CLONED BANDS

Sample	Band	Closest relative	Accession number	% Similarity
DW3d	#1	*Malikia granosa*	AJ627188	97
	#13	Bacterium isolated from oil	AB081542	80
DW3s	#4	*Malikia granosa*	AJ627188	91
W18s	5	*Lactococcus lactis*	AE006288	94
	6	*Bacteriovorax stolpii*	AJ288899	99
	7	*Aquaspirillum autotrophicum*	AB074524	99
W6s	8	*Lactococcus lactis*	AE006288	94
	#9	*Acidovorax delafieldii*	AF078764	86
308s	11	*Lactococcus lactis*	AE006288	94
	#12	*Pseudomonas putida* KT2440	AE016776	96

The use of stable isotope probing for detection of RNA from isolate Q71, *Rhodococcus erythropolis*

Stable isotope probing relies on the separation of labelled and non-labelled nucleic acids; therefore, the ultracentrifugation is critical and may be the most important step in the process. Gradients were centrifuged, fractionated and the refractive indices (**Table III**) indicated a good separation of the fractions.

The presence of amplicons in the first fractions (1 to 5) of the heavy sample for the isolate Q71 and their absence in the light sample suggests that the ultracentrifugation achieved the goal of separating the labelled from the non-labelled nucleic acids (**Fig. 4A, B**). This is supported by the appearance of bands only from the seventh fraction onwards in the light sample, except for a faint band on the second fraction. However, this band may be the product of experimental contamination as has been described previously (Manefield *et al*. 2002a), since no bands are visible in the adjacent fractions (1, 3, 4, 5, 6 in **Fig. 4B**).

The use of stable isotope probing for detection of RNA from environmental sample DW3d

On the basis of the successful results on the isolate Q71, it was decided to test the technique SIP on the environmental sample DW3d. The sample, designated as heavy, in fact contains a mixture of ^{13}C-labelled benzene (25 mgL^{-1}) and unlabelled benzene present in the groundwater, which is consistent with the presence of amplicons from all the fractions of the gradient. This is further supported by the lack of amplicons in the first fractions of the light sample and their presence from the sixth sample onwards (**Fig 5B**). Tentative evidence to support the separation of labelled and non-labelled DNA comes from the faint band in fraction 7, which is the zone where heavy and light nucleic acids are separated.

The presence of amplicons in all the fractions of the heavy sample was evident (**Fig. 5A**). This was expected since this sample contains heavy and light benzene; in contrast, amplicons are only visible from the sixth fraction onwards in the light sample (**Fig. 5B**). This suggests that fractions 1 to 5 in the heavy sample correspond to labelled DNA and is consistent with the result observed with the isolate Q71.

Fractions 1 to 5 and 6 to 10 of the heavy DW3d

TABLE III. REFRACTIVE INDICES OF THE ISOLATE Q71

Fraction	Isolate ^{13}C-*Rhodococcus* Q71 gmL^{-1} Density	Isolate ^{12}C-*Rhodococcus* Q71 gmL^{-1} Density
1	2.0100	2.0100
2	1.9969	1.9969
3	1.9969	1.9969
4	1.9969	1.9805
5	1.9696	1.9561
6	1.9561	1.9534
7	1.9291	1.9291
8	1.9291	1.9291
9	1.9157	1.9024
10	1.9157	1.9024
11	1.9051	1.9024
12	1.899	1.8990
13	1.8759	1.8891
14	1.8733	1.8785
15	1.8621	1.8759
16	1.8575	1.8627
17	1.8496	1.8627
18	1.5981	1.7217
19	1.1584	1.5981
20	1.1584	1.1584

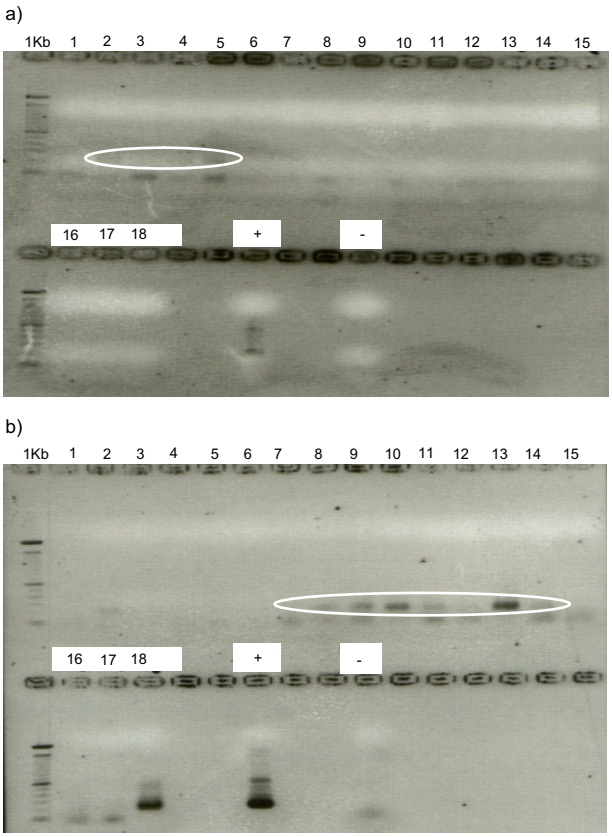

Fig. 4. PCR products from fractions collected from two gradients of isolate *Rhodococcus* Q71. **A**: the isolate was spiked with ^{13}C benzene. **B**: the isolate was spiked with ^{12}C benzene. Fraction 1 corresponds to the bottom of the tube (most dense fraction); 18 corresponds to the top of the tube (least dense fraction)

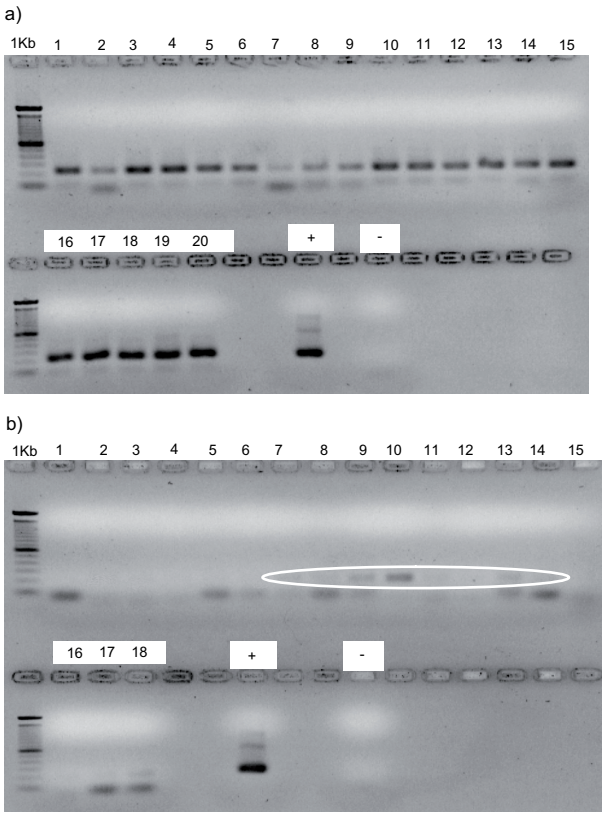

Fig. 5. PCR products from fractions collected from two gradients of groundwater sample DW3d. **A**: the groundwater sample was spiked with ^{13}C benzene. **B**: groundwater sample spiked with ^{12}C benzene. Fraction 1 corresponds to the bottom of the tube (most dense fraction); 18 corresponds to the top of the tube (least dense fraction)

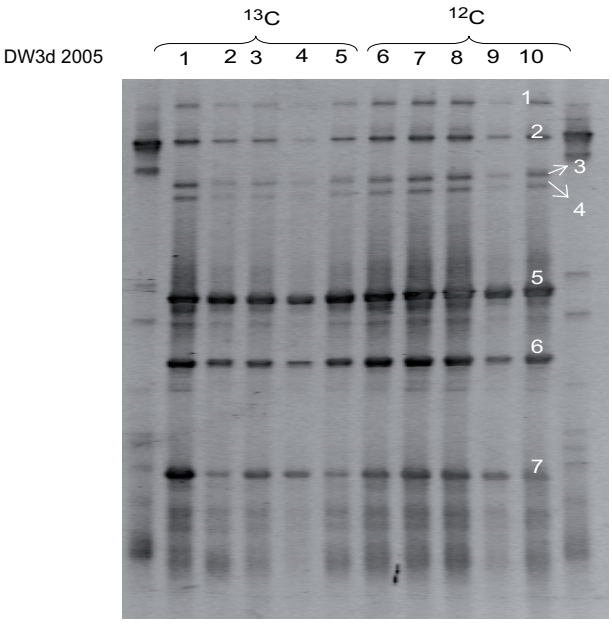

Fig. 6. DGGE gel showing the profile of fractions 1-10 from groundwater sample DW3d obtained after gradient ultra centrifugation in order to separate ^{13}C benzene from ^{12}C benzene, Time point 0: 4 hours after the ^{13}C benzene spike. DNA in fractions 1-5 is suspected to have incorporated ^{13}C benzene. Far left and right lanes contain a reference ladder

sample were assumed to represent the heavy and light DNA respectively, and were subjected to DGGE analysis. The profile obtained for both sets of fractions was very similar, and bands 1 to 7 were identical (**Fig. 6**). Based on 16S rRNA sequences retrieved from the bands excised from the gel, species like *Malikia granosa* and *Acidovorax* sp. KSP1 (**Table IV**), with sequence similarities of 92 and 91 %, respectively, were present.

DISCUSSION

Changes in bacterial community during benzene degradation

Rapid aerobic benzene degradation was observed for most of the groundwater samples (**Fig. 2**), which confirms the presence of adapted aerobic benzene degraders in each of the samples, in spite of the low amounts of oxygen *in situ* in wells DW3d, DW3s, high pH (10.2) in 308s and low benzene concentrations in samples such as W18s and W6s (**Table I**; Earle *et al*. 2001).

The DGGE profile (**Fig. 1**) suggests that the communities remained quite stable over 7 days, yet each groundwater community was different from each other. The relationship of these communities can also be observed in a dendrogram depicting the dissimilarity among these groundwater samples (**Fig. 3**). Most of the different time points cluster according to the groundwater sample and not the time point, confirming that each groundwater sample harbours different communities and that they do not change significantly over time. The only time point that was out of any cluster was W18s-0. However, this is understandable because the DGGE profile for this time point only shows one band along the lane (**Fig. 1**, band 5) while the successive time points for that sample include several bands in each lane. Furthermore, the rate of benzene degradation was the slowest for this sample. This suggests that the community changed dramatically in this sample from time 0 to time 2. However, it remained stable for successive time points. Samples DW3d and DW3s share five bands; one of those bands was identified as the microorganism *Malikia granosa* in both samples (**Fig. 1**, Bands 1 and 4).

Malikia granosa and *Malikia spinosa* are poly-

hydroxyalkanoate and polyphosphate accumulating bacteria (Spring *et al*. 2005) and are related to the *Hydrogenophaga* species; this is supported by the previous detection of similar clones in this groundwater sample (DW3d) (Aburto *et al*. 2009). Furthermore, isolates of a benzene-degrading *Hydrogenophaga* strain in another location (308s) of the same site have been obtained (Fahy *et al*. 2008). And after sequences analysis, 99 % percent similarity was shown between bands 1, 4 and the *Hydrogenophaga* isolate. One of the sequenced bands from groundwater sample W18s was related, with a 16S rRNA gene sequence similarity of 99 % to the organism *Aquaspirillum autotrophicum*. This is an aerobic hydrogen-oxidizing bacterium that contains one or more hydrogenase enzymes that bind hydrogen and use it either to produce ATP or for reducing power for autotrophic growth (Madigan 2000). This bacterium has been previously isolated from a eutrophic freshwater lake, and it can grow autotrophically or heterotrophically (Aragno 1999).

A constant band appearing at time zero for samples W18s, W6s and 308s (bands 5, 8 and 11; **Fig. 1**) was related, with a 94 % 16S rRNA similarity, to *Lactococcus lactis*. This organism, as indicated by its name, produces lactic acid as a sole fermentation product; it is an aerotolerant anaerobe able to grow even in the presence of oxygen (available in sample W18s; **Table I**). Another constant band appearing throughout the second to the seventh day in sample W18s was identified with a 16S rRNA gene sequence similarity of 99 % to *Bacteriovorax stolpii* (band 6; **Fig. 1**), previously known as *Bdellovibrio stolpii* (Baer *et al*. 2000). This organism is known for its ability to invade other bacteria and live parasitically on the host, until the host's death when a new generation of the *B. stolpii* is released (Kadouri and O'Toole 2005, Lambert *et al*. 2006, Rogosky *et al*. 2006). They can be found in diverse environments such as marine and freshwaters, sewage and soil.

A good quality sequence was obtained from sample 308s (**Fig. 1**; band 12); this band was closely related to *Pseudomonas putida* strain KT2440, which is a well known hydrocarbon degrader. This organism contains a large number of dioxygenases and at least four pathways for the degradation of hydrocarbons (Jiménez *et al*. 2002). Therefore, this bacterium is likely to carry out benzene degradation in spite of the high pH (10.2) and this is also supported by the isolation of benzene-degrading *Pseudomonas* species from the same well (Fahy *et al*. 2008).

It can be concluded that each of the groundwater samples harbours different communities that tend to remain stable throughout the degradation of the pollutant and that rapid benzene degradation suggests the presence of acclimated aerobic degraders in the groundwater samples studied here.

SIP

The community fingerprint of sample DW3d obtained by DGGE resulted in an identical profile for heavy and light nucleic acids (**Fig 6**). This is explained by the availability of both types of substrates (heavy and light) in the sample and it is clear that they were making use of both. Moreover, benzene was by far the main growth substrate present in the microcosm at concentrations far higher than any other, ruling out the possibility of another substrate for the microbes to feed on (**Table I**). The results in **figure 6** demonstrate that those organisms appearing in DGGE profiles shortly after the onset (time point 0: 4 hours after the ^{13}C benzene spike) of benzene degradation are likely to be directly involved in benzene degradation.

The most prominent bands were related to the strains *Malikia granosa* and *Acidovorax* sp. KSP1 (**Table IV**). Both species had been detected in different activated sludges elsewhere; *Malikia granosa* is a polyhydroxyalkanoate and polyphosphate accumulating bacteria (Spring *et al*. 2005) closely related to the *Hydrogenophaga* genera and, on this basis and the fact that several *Hydrogenophaga* strains have been detected and isolated from this and other groundwater samples of the same site, we strongly suggest that these bands are in fact benzene-degrading *Hydrogenophaga* strains identified as *Malikia* due to the shortness of the amplicon. *Acidovorax* strain KSP1 is a denitrifying bacterium capable of degrading the polyhydroxyalkanoate: poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (Khan *et al*. 2002).

TABLE IV. CLOSEST RELATIVES OF BAND SEQUENCES EXCISED FROM DGGE IN **FIG. 6**

Band	Closest relative	Accession number	% Similarity
1	*Malikia granosa*	AJ627188	92.1
2	*Malikia spinosa*	AB077038	91.7
3	*Acidovorax* sp. KSP1	AB076842	91.0
4	*Acidovorax* sp. KSP1	AB076842	91.0
5	*Malikia spinosa*	AB077038	92.0
6	Beta proteobact Wuba 72	AF336361	89.0
7	*Malikia spinosa*	AB077038	92.0

CONCLUSIONS

Fast aerobic degradation of benzene confirms the presence of acclimated microbes *in situ* that readily utilize the oxygen when it is available to degrade benzene; in order to support this, a few strains have been isolated from the same source (308s) (Fahy *et al*. 2008) as well as detected in previous clone libraries (Fahy *et al*. 2006). The population dynamics study shows different communities for each of the samples. As for the identification of benzene degraders by SIP in one of the samples, we succeeded in the separation of the labelled RNA and identification of the prominent bands from the heavy fractions; however, it is necessary to increase the certainty in the identification of those degraders by obtaining and sequencing a larger amplicon. Nevertheless, this study shows that stable isotope probing was useful in order to link microbial identity to function in a contaminated sample.

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