View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by University of Strathclyde Institutional Repository

Strathprints Institutional Repository

Ferguson, A.S. and Huang, W.E. and Lawson, K.A. and Doherty, R. and Gibert, O. and Dickson, K.W. and Whiteley, A.S. and Kulakov, L.A. and Thompson, I.P. and Kalin, R. and Larkin, M.J. (2007) *Microbial analysis of soil and groundwater from a gasworks site and comparison with a sequenced biological reactive barrier remediation process.* Journal of Applied Microbiology, 102 (5). pp. 1227-1238. ISSN 1364-5072

Strathprints is designed to allow users to access the research output of the University of Strathclyde. Copyright © and Moral Rights for the papers on this site are retained by the individual authors and/or other copyright owners. You may not engage in further distribution of the material for any profitmaking activities or any commercial gain. You may freely distribute both the url (http://strathprints.strath.ac.uk/) and the content of this paper for research or study, educational, or not-for-profit purposes without prior permission or charge.

Any correspondence concerning this service should be sent to Strathprints administrator: mailto:strathprints@strath.ac.uk

Journal of Applied Microbiology ISSN 1364-5072

REVIEW ARTICLE

Microbial analysis of soil and groundwater from a gasworks site and comparison with a sequenced biological reactive barrier remediation process

A.S. Ferguson[1,2], W.E. Huang[3,4], K.A. Lawson[1,5], R. Doherty[2], O. Gibert[2], K.W. Dickson[2], A.S. Whiteley[3], L.A. Kulakov[1,5], I.P. Thompson[4], R.M. Kalin[2] and M.J. Larkin[1,5]

1 QUESTOR Centre, David Keir Building The Queen's University of Belfast, Belfast, United Kingdom
2 EERC, School of Planning Architecture and Civil Engineering, David Keir Building, The Queen's University of Belfast, Belfast, United Kingdom
3 Molecular Microbial Ecology Section, Centre for Ecology and Hydrology – Oxford, Mansfield Road, Oxford, United Kingdom
4 Environmental Biotechnology Section, Centre for Ecology and Hydrology – Oxford, Mansfield Road, Oxford, United Kingdom
5 School of Biological Sciences, The Queen's University of Belfast, Belfast, United Kingdom

Keywords
degraders, gasworks, groundwater, microbial diversity, PAH, permeable reactive barrier, soil.

Correspondence
M.J. Larkin, School of Biological Sciences, The Queen's University of Belfast, David Keir Building, Belfast BT9 5AG, UK.
E-mail: m.larkin@qub.ac.uk

2006/1429: received 11 October 2006, revised 12 February 2007 and accepted 14 March 2007

doi:10.1111/j.1365-2672.2007.03398.x

Abstract

Aims: To investigate the distribution of a polymicrobial community of biodegradative bacteria in (i) soil and groundwater at a former manufactured gas plant (FMGP) site and (ii) in a novel SEquential REactive BARrier (SEREBAR) bioremediation process designed to bioremediate the contaminated groundwater.

Methods and Results: Culture-dependent and culture-independent analyses using denaturing gradient gel electrophoresis (DGGE) and polymerase chain reaction (PCR) for the detection of 16S ribosomal RNA gene and naphthalene dioxygenase (NDO) genes of free-living (planktonic groundwater) and attached (soil biofilm) samples from across the site and from the SEREBAR process was applied. Naphthalene arising from groundwater was effectively degraded early in the process and the microbiological analysis indicated a dominant role for *Pseudomonas* and *Comamonas* in its degradation. The microbial communities appeared highly complex and diverse across both the sites and in the SEREBAR process. An increased population of naphthalene degraders was associated with naphthalene removal.

Conclusion: The distribution of micro-organisms in general and naphthalene degraders across the site was highly heterogeneous. Comparisons made between areas contaminated with polycyclic aromatic hydrocarbons (PAH) and those not contaminated, revealed differences in the microbial community profile. The likelihood of noncultured bacteria being dominant in mediating naphthalene removal was evident.

Significance and Impact of the Study: This work further emphasizes the importance of both traditional and molecular-based tools in determining the microbial ecology of contaminated sites and highlights the role of noncultured bacteria in the process.

Introduction

From the nineteenth to mid-twentieth century, gas extraction from coal and oil has provided household heat, lighting and industrial fuel (Sanseverino *et al.* 1993). Gas production took place at manufactured gas plants (MGP) resulting in the generation of relatively large amounts of organic [polycyclic aromatic hydrocarbons (PAH), benzene, toluene, ethylbenzene, xylene and phenolic compounds] and inorganic (heavy metals, cyanides, sulfides

© 2007 The Authors

and ammonium compounds) waste products (Hatheway 2002). Prior to the introduction of environmental legislation, or indeed a comprehensive understanding of the detrimental effects to human and environmental health, waste products were naively reused and buried within the same sites, or released through accidental spills and leaks. Because of their current locations at valuable sites in cities and towns, site owners are looking to redevelop former manufactured gas plants (FMGP). However, the presence of such contaminants within soils, sediments and groundwater causes a concern and more economical methods to manage these risks are constantly sought.

A variety of treatment options are available that can employ physical, chemical or biological methodologies for the removal of contaminants (Nathanail *et al.* 2002). However, the clean-up of such sites using physical and chemical methods can prove to be problematical and expensive; particularly since the introduction of the EC landfill directive (Council Directive 1999/31/EC) that controls the amount of hazardous waste sent to landfill. On the other hand, bioremediation technologies that employ indigenous micro-organisms to degrade the contaminants *in situ* are more favoured. Natural communities of micro-organisms, in particular bacteria, have exhibited exceptional biochemical and physiological versatility in carrying out the biodegradation of many organic pollutants in either aerobic or anaerobic environments. Nevertheless, the bioremediation of complex organic and inorganic mixtures of contaminants that are present at FMGP sites is often a difficult task in practice. Pump-and-treat and *in situ* source oxidation techniques may not be effective in leading to complete site remediation. However, in combination, biological and engineering processes provide potentially novel solutions for risk management. Although still in its infancy, a strategy of risk management of FMGP sites through the installation of biological permeable reactive barriers (PRB) can be both an economically and environmentally feasible option in the remediation of such contaminated sites. In such systems, the natural groundwater flow from a site is allowed to pass, or is pumped through an engineered barrier (Carey *et al.* 2002) designed to encourage growth of biodegradative bacteria. In this study, the PRB reactor was a series of steel canisters containing sand (either aerated or not) or granulated activated carbon (GAC) that comprise the novel SEquential REactive BARrier (SEREBAR) bioremediation process. This process was designed to bioremediate the groundwater flowing through it. Although the overall process effectively removes organic contaminants such as naphthalene, it is not clear what the effective microbial community comprises of and how it might be distributed across the site and within the process itself. It is however clear that the complexity of the biodegradative bacterial community is likely to reflect the complexity of the composition of contaminants in the groundwater.

The primary waste product formed during coal pyrolysis is coal tar. Such tar waste can contain over 500–3000 distinct PAH compounds (Hatheway 2002), of which naphthalene (around 10% w/w) is often the dominant chemical species. Consequently, naphthalene is often used as a model compound for microbial PAH degradation studies (Fleming *et al.* 1993; Habe and Omori 2003) as it is the most mobile of the PAH in groundwater and is not readily degraded. As for all PAH, its rapid degradation depends on aerobic conditions that are, in turn, dependent on the initial incorporation or fixation of molecular oxygen into the molecule.

The enzymes responsible for fixing oxygen into organic compounds are oxygenases that can be divided into two basic types; the mono-oxygenases (that incorporate a single oxygen atom as a hydroxyl group, sulfoxide or epoxide); and the dioxygenases (that incorporate two oxygen atoms as hydroxyl groups but also single oxygen atoms as hydroxyl or sulfoxide) (Gibson and Parales 2000; Ferraro *et al.* 2005). In the case of naphthalene, the initial attack on the molecule is through the action of a dioxygenase and this is considered to be the rate-limiting step in its aerobic degradation. The subsequent naphthalene degradation pathway can be divided into the upper pathway, the conversion of naphthalene through to salicylate which is generally the same in all naphthalene-degrading bacteria studied to date (Zhou *et al.* 2001; Huang *et al.* 2005); and lower pathway, the conversion of salicylate through to intermediates of the tricarboxylic acid cycle (TCA). The first metabolite produced by *Pseudomonas putida* G7 in the catabolism of naphthalene is *cis*-naphthalene dihydrodiol (Patel and Gibson 1974; Yen and Serdar 1998; Simon *et al.* 1993). This reaction is catalysed by the enzyme naphthalene dioxygenase (NDO) and the catalytic component NahA encoded by the *nahA* gene that resides in the upper pathway operon (Moser and Stahl 2001). Population profiling using 16S ribosomal RNA gene sequences allows overall shifts in the community structure to be deduced, however, the ability to determine whether genes encoding the degradation of key PAH such as the NDO genes, would allow a more detailed assessment of the degradation potential of the microbial communities present. Many studies, which utilize polymerase chain reaction (PCR) primer sequences for the detection of naphthalene-degrading bacteria, have focused their design on the NDO large alpha subunit gene (*nahAc*) of *P. putida* G7. As a result, such techniques can only detect G7-NDO-related sequences and may fail in the detection of more diverse sequences. These would include those sequences from *Burkholderia* RP007 (Laurie and Lloyd-Jones 1999), *Comamonas* spp. (Moser and

© 2007 The Authors

Stahl 2001), *Ralstonia* sp. U2 (Fuenmayor *et al.* 1998) and *Rhodococcus* spp. (Larkin *et al.* 1999).

We describe here a detailed characterization of the microbial communities associated with soil and groundwater from a FMGP site and the subsequent shifts in the microbial community when groundwater is treated in a biological PRB (SEREBAR). Using a combination of analysis of DNA from naphthalene-degrading isolates and direct analysis of DNA samples using primers for various NDO, we show that the dominant viable naphthalene degraders are *Pseudomonas* spp. but that NDO related to those from noncultivated *Comamonas* spp. are also present in the site.

Materials and Methods

Sample collection

Groundwater and soil samples were obtained from various locations across the site (Fig. 1). Triplicate soil samples, at various depths, were collected for microbial analysis in sterile 50-ml polypropylene containers. Prior to anaerobic groundwater collection in presterilized 1-l bottles, three borehole volumes were purged using a peristaltic pump (Watson Marlow 505S) to allow representative sample collection. Upon collection, samples were immediately stored at 4°C (for viable counts of culturable bacteria) and −20°C (for extraction of DNA). Enumeration of culturable bacteria occurred within 72 h of sampling. Additional sampling was carried out for full chemical characterization (MCERTS accredited laboratory).

Groundwater collection from locations in the SEREBAR reactor took place from sample ports positioned prior to the interceptor (inlet) and precanisters A, B, C, D, and E (Fig. 2). Time course samples were obtained after 2, 3, 5, 6, 9, 11 and 14 months of operation. Following a period of 7 months of operation, groundwater and biofilm samples were obtained from within canisters A through to F for microbial investigation. Some modifications of the interceptor took place during this time whereby the original design was replaced by a fully sealed unit.

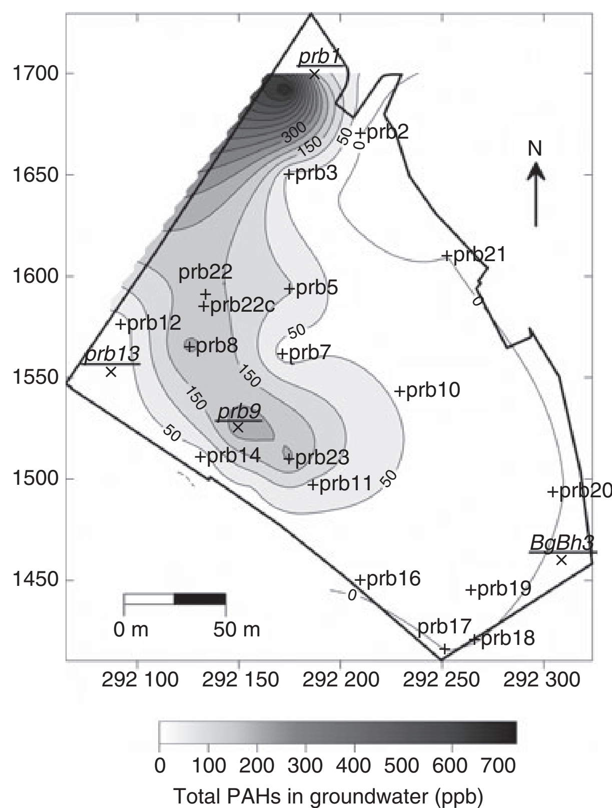

Figure 1 Distribution of polycyclic aromatic hydrocarbon (PAH) contaminants expressed as the concentration of a total of 16 PAH in groundwater at the site. Locations of boreholes associated with this study are indicated as permeable reactive barriers (PRB) numbered.

Enumeration and isolation of culturable bacteria

Total heterotrophic bacterial counts were carried out on R2A (Oxoid, Basingstoke, UK) agar. Microbial counts of bacteria that utilized substrates as sole carbon sources were carried out using M9 mineral salts medium. The substrates used were naphthalene, benzene, toluene, ethylbenzene, xylene, 1-methylnaphthalene, 2-methylnaphthalene, *m*-cresol, *p*-cresol, 1-naphthol and 2-naphthol in the vapour phase (added as a few crystals, in the case of solid substrates or 100 μl in a pipette tip, in the case of liquid substrates, to the lids of sealed petri dishes). Control plates with no added carbon sources were used to indicate little or no growth in the absence of a carbon source. Triplicate soil slurries from each soil core location and depth, or groundwater samples were serially diluted (10^0–10^{-6}), prior to plating and incubated at 25°C. Solid medium (M9) was prepared through the addition of Oxoid purified agar (18·0 g l^{-1}). Micro-organisms were isolated directly from the survey plates.

Total direct counts were performed using the LIVE/DEAD *BacLight* Baterial Viability Kit (Molecular Probes). Samples were filtered onto a black stained 0·1-μm cellulose nitrate filter (Whatman, Brentford, UK) and viewed using fluorescent microscopy.

Toxicity assessment

Soil and groundwater toxicity was determined within 24 h of sample collection using the Microtox^{TM} Model

© 2007 The Authors

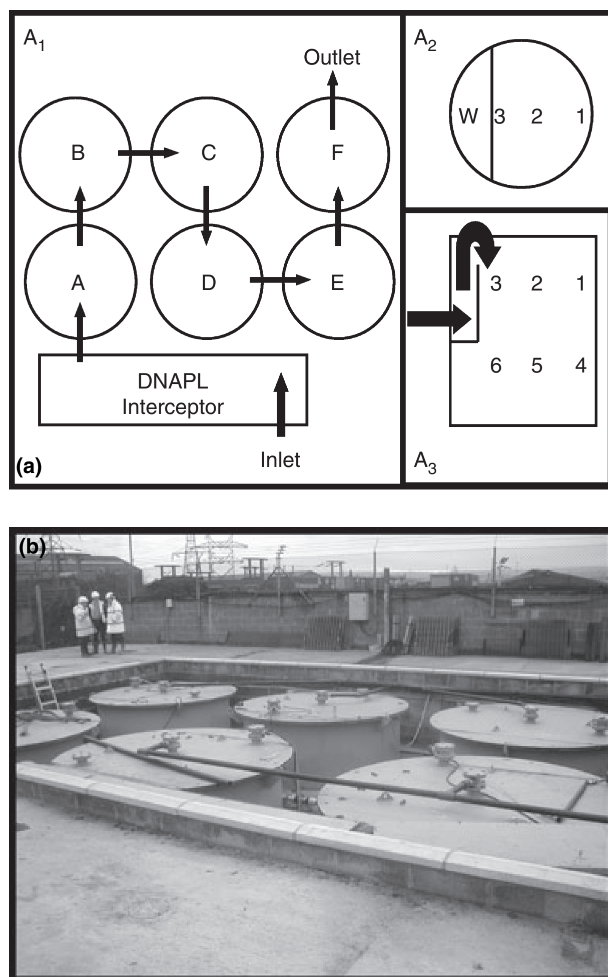

Figure 2 (a1) Plan view of SEquential REactive BARrier (SEREBAR) reactor. Arrows indicate direction of groundwater flow through the system. (a2) Plan view of single canister showing location of sampling. (a3) Cross-sectional view of a single canister showing sample locations. Sample positions: W, Weir groundwater collection, numbered locations are biofilm sample points, 1, 2, and 3 (surface); 4, 5, 6 (*c.* 1 m depth below surface). (b) *In situ* SEREBAR reactor. The inlet leads to a dense, nonaqueous phase liquid (DNAPL) interceptor tank.

500 Analyzer (Azur environmental Ltd., Wokingham, UK) (Ferguson *et al.* 2003).

DNA extraction, PCR amplification and denaturing gradient gel electrophoresis analysis (DGGE)

Groundwater samples (50 ml) were filtered through a 0·1-μm pore-size filter (Whatman), allowing concentration of microbial cells on the filter surface. Filters were incubated at 65°C for 30 min in 1-ml DNA extraction buffer [100 $mmol^{-1}$ Tris HCl (pH 8·0), 100 $mmol^{-1}$ sodium EDTA (pH 8·0), 100 $mmol^{-1}$ phosphate buffer (pH 8·0), (37·6 ml mol^{-1} K_2HPO_4, 2·4 ml mol^{-1} KH_2PO_4), 1·5 mol^{-1} NaCl, 1% cetyl-tri-methylammonium bromide]. Samples were transferred to Multimix 2 matrix tubes (BIO 101) and subjected to agitation in a FastPrep FP120 bead-beating system for 30 s at a speed of 5·5 m s^{-1}. Aqueous phase separation occurred following centrifugation (16 907 ***g*** for 30 s). Proteins were then precipitated (chloroform : isoamyl alcohol, 24 : 1 v/v) prior to repeat centrifugation at 16 907 ***g*** for 5 min. DNA was isolated by precipitation with isopropanol for 1–2 h, centrifuged at 16 907 ***g*** for 10 min, washed in 70% ethanol, dried, resuspended in RNase-free water and stored at −20°C.

Amplification of the V3 region of the 16S rRNA sequence from the regions 356 to 519 was achieved using PCR (PTC-220 DNA Engine Dyad Peltier Thermal Cycler; MJ Research Inc., Waltham, MA, USA) with the following primer pairs, 356 F (5′-CGCCCGCCGCGCCCCCGCCC CGGCCCGCCGCCCCCGCCCACTCCTACGGGAGGCAG C-3′) and 519R (5′-GTATTACCGCGGCTGCTG-3′). The GC-clamped products were separated on 10% (w/v) polyacrylamide gels with a 30–60% urea/formamide denaturing gradient (Whiteley and Bailey 2000). Denaturing gels were cast and run using the Ingeny PhorU2 system at 60°C and 200 V for 16 h. Gels were stained with SYBR gold nucleic acid stain (Molecular Probes) and visualized by ultraviolet (UV) trans-illumination. Images (of bands in excess of 50 μm) were acquired using a Versa-Doc Imaging System (Model 3000; BioRad Laboratories, Herts., UK). The DGGE gel was analysed by Phoretix 1D Advanced (version 5; Nonlinear Dynamic Ltd, Newcastle-upon-Tyne, UK) as per the manufacturer's instructions. A dendrogram was then generated to indicate the relative similarities of each of the bacterial communities.

Three different PCR primer sets (Moser and Stahl 2001) specific to *Pseudomonas*, *Comamonas* and *Rhodococcus* NDO genes (amplifying 994, 894 and 906 bp, respectively) were used to amplify the NDO large alpha subunit genes from DNA samples obtained from across the site and throughout the SEREBAR reactor. The primer sets employed were found to be specific for the NDO genes from each genera but it is not certain if they also could lead to amplification of NDO genes from other genera.

Site description, contaminants present and the SEREBAR process design

The SEREBAR site is at a FMGP currently used for gas storage and distribution. The site ceased operation as a gas production facility in the mid-twentieth century, following the increase in gas production from petroleum and North Sea gas. Various plant structures were decommissioned and demolished in 1980 allowing the west side

© 2007 The Authors

of the site to be redeveloped for commercial and residential usage. The site stratigraphy is composed of made ground (0·9–3·4-m thick), river terrace deposits, interbedded sand, silt and gravel, underlain by Permian breccio-conglomerates. Because of the highly impermeable nature of the weathered breccia, the contamination is mainly found within the made ground and river terrace deposits. Groundwater flow is from north to south across the site and Fig. 1 shows the site layout, including the sample points used for the various microbial analyses. In all, 100 different soil core samples were obtained, logged and stored.

The contaminants consisted of a mixture of PAH, benzene, toluene, ethylbenzene, xylene, phenols, cresols, complex cyanide and ammonium ions. Priority organic contaminants in the soil and groundwater included PAH (up to 1239 mg kg^{-1} and 0·04 mg l^{-1}, respectively) of which naphthalene was the dominant chemical species found in groundwater (Fig. 1); phenanthrene and flouranthene were the dominant species found in soils (data not shown). Two different contaminant profiles were observed across the site for both soil and groundwater matrices. In relation to the top soil (i.e. made ground), the distribution of the contaminants was widespread across the entire site reflecting the long and varied industrial history of the site. Contaminant hotspots within the soil do not serve as a major source for organic groundwater contamination. Instead, DNAPL (dense nonaqueous phase liquid) contamination from underground storage tanks and the bases of gas-holding tanks provided the main source for groundwater contamination.

Following a thorough site investigation and treatability testing, the installation of the SEREBAR PRB took place in May 2004. Groundwater from the site flows into an interceptor tank, in which there is some passive aeration, and then in sequence to six steel treatment canisters (each 2·5-m diameter, 3·1-m height and 4·8-t weight; Fig. 2). The flow rates varied between 520 and 4000 l per day. The first four canisters were filled with sand to encourage microbial colonization. Artificial aeration of the groundwater occurred only within canisters C and D (air was sparged at 600 ml min^{-1}). Canisters E and F contained GAC which acted as a sorbent for any organic compounds that were not degraded by the PRB, and protected the outlet from accidental breakthrough of the contaminants. In order to ensure that the groundwater was directed into the system the SEREBAR design employed the funnel-and-gate principle for groundwater catchment and remediation. This was achieved through the installation of an impermeable sub-surface wall that channels the contaminant plume into the SEREBAR PRB (Carey *et al.* 2002). The sample positions and design of the SEREBAR PRB canister system are shown in Fig. 2.

Results

Enumeration of total bacteria and viable naphthalene-utilizing bacteria in groundwater and soil cores

A comprehensive survey of viable heterotrophic aerobic and facultative aerobic bacteria took place from the FMGP site (for both soil and groundwater samples) and throughout the SEREBAR PRB. Not surprisingly, both soils and groundwater harboured a significant number of heterotrophic bacteria and many appeared to be capable of growing on naphthalene. Enumeration of total heterotrophs and naphthalene degraders in the soil core samples is shown in Fig. 3a and indicate that the numbers varied across the site in relation to the distribution of the contaminants. Interestingly, no microbial growth was observed from samples PRB 1-3 and PRB 1-4 which were saturated with LNAPL (light nonaqueous phase liquid) and DNAPL, respectively. This borehole location was subsequently identified as a contaminant source zone because of its position close to an underground storage tank (Fig. 1). The results indicate that the majority of viable aerobic heterotrophic naphthalene utilizers in the soil

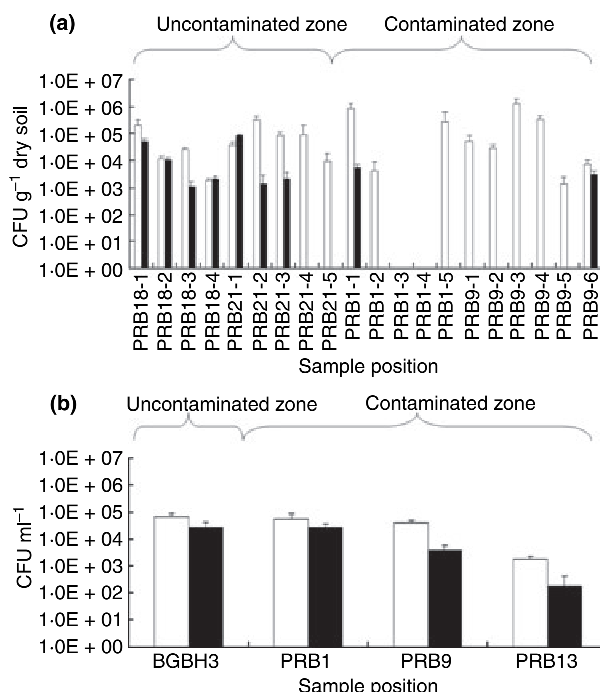

Figure 3 Distribution of total viable heterotrophic bacteria (□) and putative naphthalene utilizer colony counts (■) across the site from (a) soil samples and (b) groundwater samples. Soil samples prefixed with 1, made ground; 2, silt and 3–5, gravel. The counts are the means of triplicates with standard errors indicated.

© 2007 The Authors

Table 1 Enumeration of total micro-organisms, the percentage of living and dead cells by Baclight staining in groundwater samples (from locations indicated in Fig. 1) and the toxicity of the water samples by Microtox analysis

Sample position	Mean total counts per ml ($\times 10^6$) ± SD	% live	% dead	Live/dead SD	EC50 (% v/v)
BGBH 3	5·02 ± 0·20	82·8	17·2	3·6	100
PRB 9	1·53 ± 0·57	77·6	22·4	10·3	3·85
PRB 11	1·78 ± 0·26	93·0	7·0	2·9	4·43
PRB 13	2·35 ± 0·37	48·0	52·0	4·7	48·29

were present within the contaminant zone at locations PRB 18 and PRB 21.

In relation to groundwater samples, the total microbial counts are shown in Table 1. The highest viable count was at BGBH 3 ($5{\cdot}02 \times 10^6$ cells ml^{-1}), which is a relatively noncontaminated area. The lowest viable count was observed in water samples from PRB 9 ($1{\cdot}53 \times 10^6$ cells ml^{-1}) which corresponded to an area of high organic pollution within the main contaminant plume head (Fig. 1). As the biogeochemical conceptual model predicted the greatest percentage of live bacteria occurs at the plume fringe (PRB 11, Fig. 1). Correlation ($r = 0{\cdot}96$) between groundwater toxicity (as measured by Microtox assays) and total counts was apparent.

Enumeration of the viable heterotrophic and naphthalene-utilizing bacteria was carried out on groundwater samples from: (i) source zone (PRB 1), (ii) plume fringe (PRB 13), (iii) plume head (PRB 9) and (iv) a relatively noncontaminated zone (BGBH 3) (Fig. 3b). In contrast to the counts of viable bacteria from the soil cores, naphthalene utilizers appeared to be widely distributed across both the contaminated and noncontaminated areas. At this level it seems that where contaminant levels are high, and likely to be exerting toxic effects, naphthalene degradation may be dominated by one or two particular types of bacteria. In agreement with previous work carried out on an alternate FMGP (Ferguson *et al.* 2003), no correlation was observed between toxicity and microbial plate counts.

Enumeration of viable naphthalene-degrading bacteria in the SEREBAR canisters

Both biofilm (from the barrier media) and water samples were obtained from the SEREBAR reactor after 7 months of operation at a groundwater flow rate of 500 l per day. Enumeration of the total viable heterotrophic bacteria and naphthalene utilizers was carried out alongside a determination of the total organic carbon (TOC data not shown) and naphthalene concentration in water passing through the canisters. The results are shown in Fig. 4 and indicate that the naphthalene concentration declines early in the process (within the interceptor and canister A). This was mirrored by a concomitant decline in the TOC concentration from 9 to 6 mg l^{-1} (33%). As envisaged, the distribution of total viable heterotrophic and naphthalene-utilizing bacteria in samples obtained from the canisters was very heterogeneous, with the higher numbers broadly apparent within canister A. Canisters C and D were artificially aerated, whereas passive aeration of groundwater took place following flow of the groundwater over the interceptor weir. The extent of aeration explains the proliferation of naphthalene utilizers at these locations. However, it is notable that the interceptor was replaced soon after this point with a unit sealed from the atmosphere and in which the degree of aeration was unknown after this point. Consequently, the proliferation of viable naphthalene-utilizing micro-organisms was largely associated with the passively aerated interceptor water and with samples from canister A. This was clearly concomitant with the removal of naphthalene in the process. The bacteria cultivated from this study were further characterized as noted next.

Population diversity as assessed by 16s ribosomal RNA gene sequence DGGE analysis

The 16S ribosomal RNA gene DGGE profiles of amplified DNA from the top (Fig. 5a) and sub-soil (data not shown) core samples were generally different. This suggests that the depth and physical properties of the soil was a significant determinant of microbial community composition. The top soil was highly heterogeneous made ground whereas the sub-soil was predominantly naturally occurring silt.

The influence of PAH contamination on the composition of the exposed soil microbial community across the site was assessed by cluster analysis of 16S ribosomal RNA gene DGGE profiles (Fig. 5a). This shows that the degree of contamination within the top soil at each location of the site was a key factor in dictating the composition of the soil microbial communities. Bacterial communities, based on DGGE analysis largely clustered according to the contours of the total PAH concentrations (Fig. 5a), providing stronger evidence that PAH concentration was a more important factor in determining community composition than spatial location. This reflects the highly selective effects of the contaminants in the site on the microbial communities.

As high levels of contamination are likely to have a significant selective pressure on the microbial community, it is unsurprising that highly contaminated soil, e.g. PRB 21 and 5 in Fig. 5a has much less microbial diversity than

© 2007 The Authors

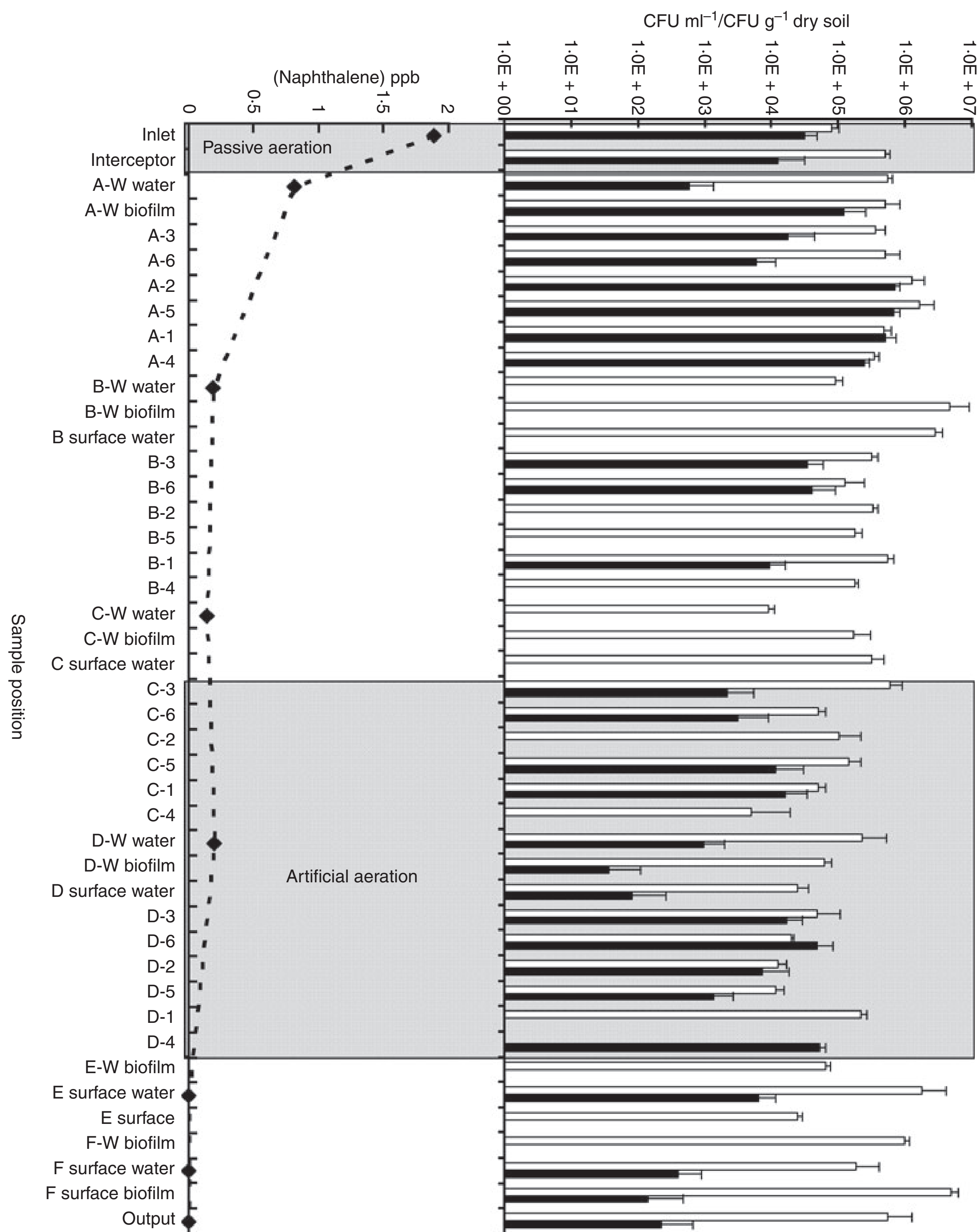

Figure 4 Concentration of naphthalene at various locations across the SEquential REactive BARrier (SEREBAR) reactor. Distribution of total viable heterotrophic (□) and naphthalene utilizers (■) for water and biofilm samples throughout the SEREBAR reactor. The counts are the means of triplicates with standard errors indicated. Samples were taken after 7 months of operation. Groundwater flow was 520 l per day.

© 2007 The Authors

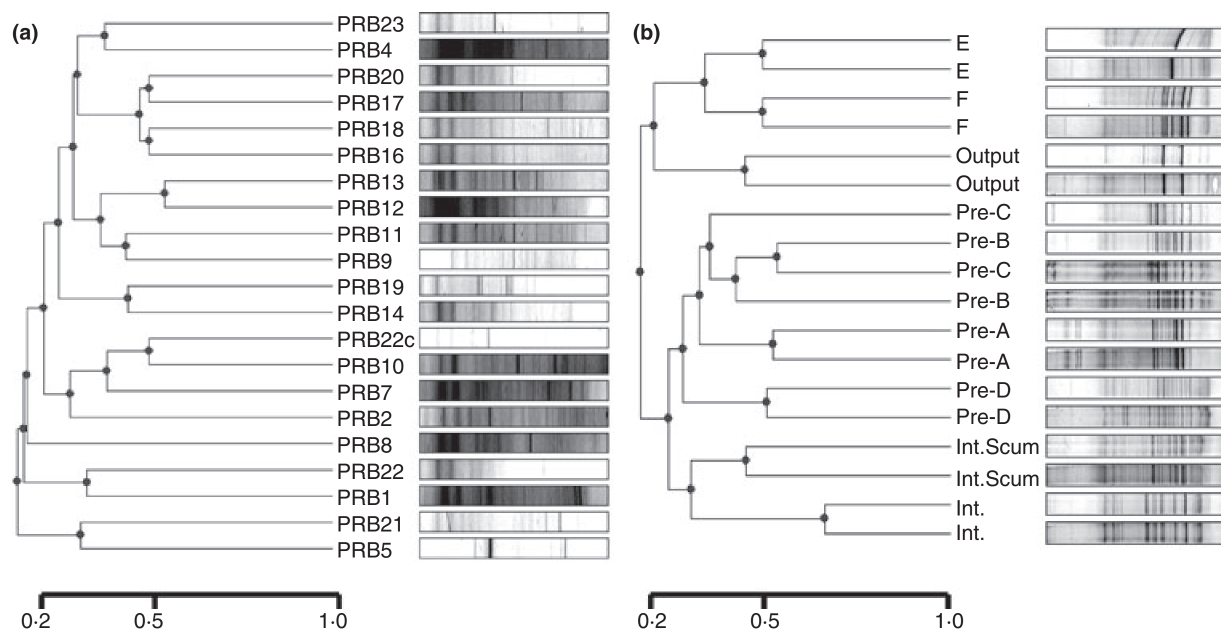

Figure 5 Diversity of 16S ribosomal RNA gene denaturing gradient gel electrophoresis (DGGE) profiles of DNA from (a) top soils (i.e. made ground) from permeable reactive barrier (PRB) locations indicated in Fig. 1 across the site, and (b) water samples taken after 7 months operation from the SEquential REactive BARrier (SEREBAR) reactors. Letters indicate the canisters from which samples were taken as in Fig. 2. Pre- indicates that the samples were taken from the inlet to the canisters. Int- indicates that samples were taken from the dense, nonaqueous phase liquid (DNAPL) interceptor, either as liquid or as a surface 'scum'.

lesser contaminated soils, e.g. PRB 4, 8, 12, 13, 14, 16, 17 and 18 in Fig. 5a. Interestingly, at the fringe of the concentration profile for soil contamination, which has an intermediate level of contamination, the microbial diversities were also relatively high, e.g. PRB 1, 2, 7, 10, 11 and 20 in Fig. 5a. This indicates that these intermediate levels of contamination may not be toxic enough to inhibit microbial metabolism. Taken together the results provide an indication of what concentrations of contaminant may effectively be tolerated and/or remediated by the bioremediation process.

The results shown in Fig. 5b illustrate the diversity of 16S ribosomal RNA gene sequences in water samples taken from a point where they enter each of the SEREBAR PRB canisters (designated pre-). The DGGE profiles arising from each of two-replicate samples were tightly clustered and indicated the reproducibility of the 16S ribosomal RNA gene-PCR-DGGE methodology in this case. Although it is likely that all of the biodegradative microorganisms entering the SEREBAR PRB originated from the same source in the groundwater and inceptor water, the microbial community that developed downstream in the canisters varied considerably. This shows a highly flexible adaptation of the communities to the various niches in the process. Clearly the microbial communities readily changed with variations in aeration and composition of the support medium within the canisters (Fig. 5b). Generally, the DGGE profiles indicated that the microbial communities appeared to be clustered into three defined groups. Those in: (i) the interceptor biofilm scum and interceptor water, (ii) the water entering (designated pre-) canisters A, B, C and D and (iii) the output water from canisters E, F and the final process output (Fig. 5b). This is generally in accordance with the orientation of groundwater flow through the system. The profiles of samples from the interceptor biofilm scum and interceptor water grouped together simply because they were maintained under similar conditions (Fig. 5b). Samples arising from precanister A (unaerated) and precanister D (after aeration) also differed (Fig. 5b). Those from precanisters B and C grouped together as they both were from the unaerated canisters (Fig. 5b). Unlike the other canisters, GAC was the support medium in canisters E and F which clearly led to a very different DGGE profile and hence a different microbial community when compared with that associated with the sand canisters (A–D) (Fig. 5b).

Population changes within the SEREBAR reactor canisters

Throughout the first year of operation (July 2004–July 2005) both conventional microbiological techniques in conjunction with molecular genetic analyses were conducted on water samples obtained from the entry to each of the SEREBAR PRB canisters (designated pre-). During this period, the groundwater flow through the PRB was

© 2007 The Authors

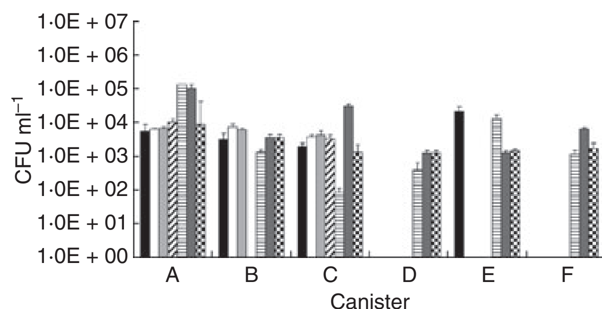

Figure 6 Total viable heterotrophic counts of bacteria using naphthalene as the sole carbon source across the SEquential REactive BARrier (SEREBAR) reactor after 2 ■, 3 □, 5 ▤, 6 ▧, 9 ▥, 11 ■, and 14 ▩ months of operation. Groundwater flow rate was increased from 520 to 4000 l per day after 7 months. The counts are the means of triplicates with standard errors indicated.

changed from the initial flow rate of 520 l per day (July 2004–December 2004) to 4000 l per day (February 2005–May 2005) before being reduced to 1700 l per day.

Under the low flow rate conditions (520 l per day), the enumeration of viable heterotrophic bacteria and also those as colonies appearing to use naphthalene as the sole carbon source (Fig. 6) revealed a general decrease in microbial numbers from inlet to outlet. An increase in groundwater flow through the SEREBAR PRB resulted in an overall apparent increase in viable microbial numbers present within the rear section of the reactor (outlets of canisters C and D). DGGE analysis also revealed changes in the bacterial community structure across the SEREBAR PRB over time and these were largely in response to changes in groundwater flow rates (data not shown). These results indicated that there may have been some movement of micro-organisms, initially present within the front section of the SEREBAR PRB, to the latter canisters after an increase in the flow rate. All flow rates tested to date were well within the possible range of the PRB operational limits.

Characterization of naphthalene-utilizing bacteria isolated from the SEREBAR PRB canisters

Isolates (29) of naphthalene-utilizing bacteria, arising from samples taken from the interceptor and SEREBAR PRB canisters after 7 months, were subjected to further analysis. The data shown here (Table 2) represent the range of colony types found only in the interceptor and in canister A where there was a higher population of bacteria that was concomitant with the significant biodegradation of naphthalene. The results indicate that the majority of the bacterial isolates were members of the genus *Pseudomonas* as evidenced by 16S ribosomal RNA gene sequencing. This conclusion was further reinforced by the detection of NDO sequence by PCR using primers demonstrated to be specific for the archetypal *Pseduomonas* NDO. However, it is notable that five of the isolates were members of the genus *Rhodococcus* and possessed an NDO sequence typical of a *Rhodococcus* NDO. More importantly, however, there were no isolates detected that were identified as being related to *Commomonas* or *Ralsotona* or indeed possessing an NDO of this group. PCR analysis using primers specific for NDO sequences typical of those from *Pseudomonas*, *Commomonas* and *Rhodococcus* genera were applied to DNA samples obtained from various locations within the SEREBAR PRB. It is notable that initial attempts to detect these sequences in samples obtained prior to the 14-month sampling period failed to detect such sequences. Nonetheless, Fig. 7 indicates that there were NDO sequences typical of those from *Comamonas* and *Pseudomonas* species present in the samples from the canisters at this stage. Surprisingly, it seemed that *Comomonas* type NDO sequence was thoroughly distributed in the system.

Discussion

It is typical of such contaminated sites that there is considerable chemical heterogeneity distributed around the contaminated plume, its source and in associated soils. Such heterogeneity provides, in turn, considerably heterogeneous niches for microbial communities to exist. This is amply reflected in the numbers and diversity of the microbial communities dispersed across soil and groundwater at the SEREBAR site and provides important information in further defining plume geometry and architecture. The variations noted here show substantial changes in community structure over relatively small geographical locations. The main conclusion drawn at an early stage in this project was that there was a complex community of bacteria distributed across the site, with many isolates capable of utilizing naphthalene. The results concerning numbers and diversity relative to the main plume, intermediate fringes of the plume and the relatively noncontaminated areas, indicate the role enforced by pollutant concentration and toxicity on the community that was present. Although it is uncertain in this case what role the bacteria might play in the degradation of contaminants *in situ*, the definition of a contaminant plume should take into account the microbial activity it supports (or does not). This data is a vital decision-making tool in order to appraise the remediation options available. The optimal engineering design and positioning of the SEREBAR PRB required knowledge of microbial plume architecture. The SEREBAR PRB was positioned at the head of the plume fringe where the viably active microbial population was uppermost, indicating the potential for rapid colonization of the SEREBAR PRB with micro-organisms capable of

© 2007 The Authors

Table 2 Characterization of naphthalene-utilizing isolates, phylogeny, substrate range and naphthalene dioxygenase (NDO) type

		Substrate utilization*											NDO Type**		
Location	Isolate	N	B	T	E	X	1-MN	2-MN	*m*-C	*p*-C	1-N	2-N	P	C	R
Gram-negative rods (*Pseudomonas* genus by partial 16S ribosomal RNA gene sequencing – data not shown)															
Preinterceptor	PI-1	+	−	−	−	−	−	+	−	−	+	+/−	+	−	−
	PI-2	+	−	−	−	−	−	+	−	−	+	+/−	+	−	−
	PI-3	+	−	−	−	−	−	+	−	−	+	−	+	−	−
	PI-4	+	−	−	−	−	−	+	−	−	+	+	+	−	−
	PI-5	+	−	−	−	−	−	+	−	−	+	+/−	+	−	−
	PI-7	+	−	−	−	−	−	+	−	−	+	+	+	−	−
	PI-8	+	−	−	−	−	−	+	−	+	+	−	+	−	−
Interceptor	Int-1	+	−	−	−	+/−	−	+	−	−	−	−	+	−	−
	Int-3	+						+	+	+	+	+	+	−	−
Canister A	ZVI-5	+	+	+	−	−	−	+	−	−	+	+/−	+	−	−
	ZVI-9	+	+	+	−	−	−	+	−	+	+	+	+	−	−
	ZVI-11	+	+	+	−	−	−	+	−	−	+	+	+	−	−
	ZVI-12	+	+/−	+/−	−	−	−	+	+	+	+	+	+	−	−
	ZVI-13	+	−	−	−	−	−	+	−	−	−	−	+	−	−
	ZVI-14	+	+	+	−	−	−	+	−	−	+	+	+	−	−
	ZVI-15	+	−	−	−	−	−	+	+	−	+	−	+	−	−
	ZVI-16	+	+	+	−	−	−	+	+	−	+	−	+	−	−
	ZVI-17	+	−	−	−	−	−	−	−	−	+	+	+	−	−
	ZVI-19	+	−	−	−	−	−	+	+	−	−	+	+	−	−
	ZVI-20	+	+/−	+/−	−	−	−	−	+	−	+	+	+	−	−
	ZVI-27	+	+	+	−	+/−	−	+	−	−	+	+	+	−	−
	ZVI-28	+	−	−	−	−	−	−	−	−	+	−	+	−	−
	ZVI-29	+	−	−	−	−	−	+	−	+	+	−	+	−	−
	ZVI-30	+	−	−	−	−	−	+	−	−	+	−	+	−	−
	ZVI-31	+	+	+	−	−	−	+	−	−	+	−	+	−	−
Gram-positive cocci (*Rhodococcus* genus by partial 16S ribosomal RNA gene sequencing – data not shown)															
Canister A	ZVI-1	+	+/−	+/−	+/−	−	−	−	+	−	+	+		−	+
	ZVI-4	+	+/−	+	+/−	−	+/−	−	−	−	+	+		−	+
	ZVI-7	+	−	+	+	−	−	−	+	−	+	+/−		−	+
	ZVI-8	+	+/−	+	+/−	−	−	−	+	−	+	+/−		−	+

*Substrates utilized for growth: N, naphthalene; B, benzene; T, toluene; E, ethylbenzene; X, xylene; 1-MN, 1-methylnaphthalene; 2-MN, 2-methylnaphthalene; *m*-C, *m*-cresol; *p*-C; *p*-cresol; 1-N, 1-naphthol; 2-N, 2-naphthol. Those scored + are those which showed clear growth in comparison with the control (inoculation of substrate-free R2A); +/− showed growth that was just greater than that of the control; – showed no growth on the substrate after incubation for 7 days.

**indicates + or – PCR product amplified using NDO primers specific for: P, *Pseudomonas* type; C, *Comomonas* type and R, *Rhodococcus* type.

using the contaminants as a carbon and energy source. The principle was that the installation of underground slurry walls in the site would funnel the contaminant plume and direct it through the treatment canisters before being released uncontaminated from the site. It is therefore not surprising that the operators exercised considerable caution in the design and deployed several canisters in series. Notably, this included two canisters distally located and containing GAC to provide a considerable margin of safety. The distribution of microbial numbers and the concomitant removal of naphthalene (also TOC) at the early stages of the system illustrates that the process was probably overdesigned for the flow rates planned at that stage. Clearly, the system functions with the evident development of a very complex microbial community from the outset. Although flow rates were increased at a later stage and then returned to a lower level, it appears that the perturbation was capable of being amply contained and suggests that the SEREBAR PRB functions well within the possible range of operational limits. The results obtained from viable counting methodology and from DGGE separation of 16S ribosomal RNA gene sequences only reveals some of the complexity associated with such a community. It is clear that very diverse populations inhabited a variety of niches in the interceptor channel and canister A where most of the organic components were degraded. It is also notable that the initial canisters were not artificially aerated and that any aeration was passive

© 2007 The Authors

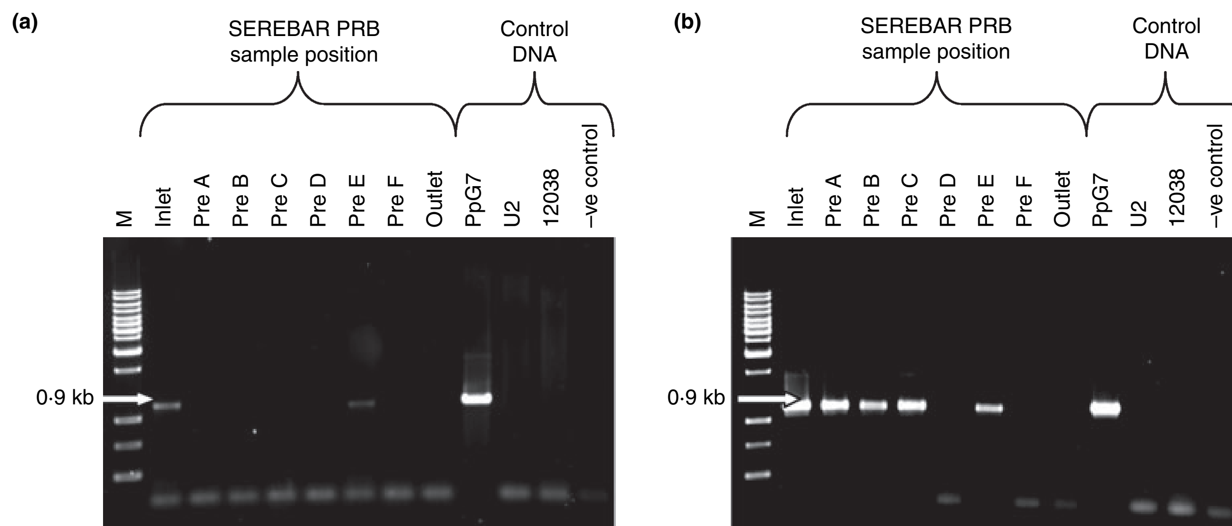

Figure 7 (a) *Pseudomonas* and (b) *Comamonas* naphthalene dioxygenase (NDO) detection using polymerase chain reaction (PCR) in DNA samples from the SEquential REactive BARrier (SEREBAR) canisters. Water samples were taken following 14 months of operation. Groundwater flow was 1000 l per day. Control DNA from strains – U2, *Ralstonia* U2; PpG7, *Pseudomonas Putida* pPG7; 12038, *Rhodococcus* 12038 are indicated (amplifying 994, 894 and 906 bp, respectively). M indicates size markers with 0.9 kb shown.

and was provided by aeration at the inlet to the interceptor. Nevertheless, this stage of the process appears to degrade most of the contaminants. The population size of aerobic naphthalene-utilizing bacteria did rise again in the aerated canisters C and D and this provided a reserve of capacity in the process if for any reason the flow rates or concentration of contaminants increases. The issue for a microbiological study is determining which organisms are responsible for the degradation observed and what this might reveal about their physiology in the process. Throughout this study it was assumed that the flow rates and contaminant concentration requirements meant that very slow anaerobic degradation of compounds such as naphthalene may be too slow. Thus, some aeration and oxygen would be required to achieve adequate degradation rates. The aim was not to provide optimal oxygenation, but to test the hypothesis that passive flow rates and oxygenation would achieve the objective and operate in the interests of low energy consumption and long-term sustainability as the site is developed. It is likely that some anaerobic catabolism has taken place and this would be reflected in the overall community structure. A main conclusion from the point of view of the microbiology is that *Pseudomonas* spp. appeared to dominate the isolates from viable bacterial counts. Substrate ranges and 16S ribosomal RNA gene sequencing indicated that those cultivated were all different strains but that they all possessed sequences typical of a *Pseudomonas* NDO. They were accompanied by a small number of different *Rhodococcus* strains possessing NDO sequences typical of this genus. It comes as no surprise therefore the *Pseudomonas* NDO sequences were detected in DNA samples from the SEREBAR process itself. Of some surprise however, was that no NDO sequence typical of *Rhodococcus* could be detected despite considerable effort and repeated attempts. Instead, it was clear that the PCR amplifications revealed a sizeable copy number of NDO sequence related to the *Comamonas* type. This clearly indicates that cultivation methods applied to a considerable number of strains had failed to yield a strain of anything related to *Comamonas*. The conclusion is that the process was inhabited by, in this instance, noncultured bacteria of this type and that they may be the key organisms in the success of the SEREBAR process. This is currently the subject of further investigation.

Acknowledgements

SEREBAR Team, Parsons Brinkerhoff, National Grid Property Holdings Ltd., BBSRC-LINK grant references BRM19108 and BRM19109. EPSRC Industrial CASE Studentship (KA), First Faraday, DTI.

References

Carey, M.A., Fretwell, B.A., Mosley, N.G. and Smith, J.W.N. (2002) *Guidance on the use of permeable reactive barriers for remediating contaminated groundwater*. National Groundwater & Contaminated Land Centre report NC/01/51. Bristol, UK: Environment Agency ISBN 1 85705 665.

© 2007 The Authors

Ferguson, A.S., Doherty, R., Larkin, M.J., Kalin, R.M., Irvine, V. and Ofterdinger, U.S. (2003) Toxicity assessment of a former manufactured gas plant. *Bulletin of Environ Contamin Toxicol* **71**, 21–30.

Ferraro, D.J., Gakhar, L. and Ramaswamy, S. (2005) Rieske business: Structure-function of Rieske non-heme oxygenases. *Biochem Biophys Res Commun* **338**, 175–190.

Fleming, J.T., Sanseverino, J. and Sayler, G.S. (1993) Quantitative relationship between naphthalene catabolic gene frequency and expression in predicting PAH degradation in soils at town gas manufacturing sites. *Environ Sci Technol* **27**, 1069–1074.

Fuenmayor, S.L., Wild, M., Boyes, A.L. and Williams, P.A. (1998) A gene cluster encoding steps in conversion of naphthalene to gentisate in *Pseudomonas* sp. strain U2. *J Bacteriol* **180**, 2522–2530.

Gibson, D.T. and Parales, R.E. (2000) Aromatic hydrocarbon dioxygenases in environmental biotechnology. *Curr Opin Biotechnol* **11**, 236–243.

Habe, H. and Omori, T. (2003) Genetics of polycyclic aromatic hydrocarbon metabolism in diverse aerobic bacteria. *Biosci Biotechnol Biochem* **67**, 225–243.

Hatheway, A.W. (2002) Geoenvironmental protocol for site and waste characterization of former manufactured gas plants; worldwide remediation challenge in semi-volatile organic wastes. *Eng Geol* **64**, 317–338.

Huang, W.E., Wang, H., Huang, L.F., Zheng, H.J., Singer, A.C., Thompson, I.P. and Whiteley, A.S. (2005) Chromosomally located gene fusions constructed in *Acinetobacter* sp. ADP1 for the environmental detection of salicylate. *Environ Microbiol* **7**, 1339–1348.

Larkin, M.J., Allen, C.C.R., Kulakov, L.A. and Lipscomb, D.A. (1999) Purification and characterization of a novel naphthalene dioxygenase from *Rhodococcus* sp. strain NIC-MB12038. *J Bacteriol* **181**, 6200–6204.

Laurie, A.E. and Lloyd-Jones, G. (1999) The *phn* genes of *Burkholderia* sp. strain RP007 constitute a divergent gene cluster for polycyclic aromatic hydrocarbon catabolism. *J Bacteriol* **181**, 531–540.

Moser, R. and Stahl, U. (2001) Insights into the genetic diversity of initial dioxygenases from PAH-degrading bacteria. *Appl Microbiol Biotechnol* **55**, 609–618.

Nathanail, J.F., Bardos, P. and Nathanail, C.P. (2002) *Contaminated Land Management Ready Reference*. Nottingham: EPP & Land Quality Press.

Patel, T.R. and Gibson, D.T. (1974) Purification and properties of (+)-*cis*-naphthalene dihydrodiol dehyrdrogenase of *Pseudomonas putdida*. *J Bacteriol* **119**, 879–888.

Sanseverino, J., Werner, C., Fleming, J., Applegate, B., King, J.M.H. and Sayler, G.S. (1993) Molecular diagnostics of polycyclic aromatic hydrocarbon biodegradation in manufactured gas plant soils. *Biodegradation* **4**, 303–321.

Simon, M.J., Osslund, T.D., Saunders, R., Ensley, B.D., Suggs, S., Harcourt, A., Suen, W., Cruden, D.L. *et al.* (1993) Sequences of genes encoding naphthalene dioxygenase in *Pseudomonas putida* strains G7 and NCIB9816-4. *Gene* **127**, 31–37.

Whiteley, A. and Bailey, M.J. (2000) Bacterial community structure and physiological state within an industrial bioremediation system. *Appl Environ Microbiol* **66**, 2400–2407.

Yen, K. and Serdar, C.M. (1998) Genetics of naphthalene catabolism in *Pseudomonads*. *Crit Rev Microbiol* **15**, 247–268.

Zhou, N., Fuenmayor, S.L. and Williams, P.A. (2001) *Nag* genes of *Ralstonia* (formally *Pseudomonas*) sp. strain U2 encoding enzymes for gentisate catabolism. *J Bacteriol* **183**, 700–708.

© 2007 The Authors