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The role of microbial populations in the containment of aromatic hydrocarbons in the subsurface

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Abstract

A survey of soil gases associated with gasoline stations on the Swan Coastal Plain of Western Australia has shown that 20% leak detectable amounts of petroleum. The fates of volatile hydrocarbons in the vadose zone at one contaminated site, and dissolved hydrocarbons in groundwater at another site were followed in a number of studies which are herein reviewed. Geochemical evidence from a plume of hydrocarbon-contaminated groundwater has shown that sulfate reduction rapidly developed as the terminal electron accepting process. Toluene degradation but not benzene degradation was linked to sulfate reduction. The sulfate-reducing bacteria isolated from the plume represented a new species, *Desulfosporosinus meridiei*. Strains of the species do not mineralise ^{14}C-toluene in pure culture. The addition of large numbers of cells and sulfate to microcosms did stimulate toluene mineralisation but not benzene mineralisation. Attempts to follow populations of sulfate-reducing bacteria by phospholipid signatures, or *Desulfosporosinus meridiei* by FISH in the plume were unsuccessful, but fluorescently-labeled polyclonal antibodies were successfully used. In the vadose zone at a different site, volatile hydrocarbons were consumed in the top 0.5 m of the soil profile. The fastest measured rate of mineralisation of ^{14}C-benzene in soils collected from the most active zone (6.5 mg kg^{-1} day^{-1}) could account for the majority of the flux of hydrocarbon vapour towards the surface. The studies concluded that intrinsic remediation by subsurface microbial populations in groundwater on the Swan Coastal Plain can control transport of aromatic hydrocarbon contamination, except for the transport of benzene in groundwater. In the vadose zone, intrinsic remediation by the microbial populations in the soil profile can contain the transport of aromatic hydrocarbons, provided the physical transport of gases, in particular oxygen from the atmosphere, is not impeded by structures.

Introduction

Of all the components of gasoline, some of the most mobile in the environment (i.e., most soluble and volatile), and thus more likely to be transported "off-site" (Figure 1) are among the most hazardous. For example, benzene is relatively soluble in water (1780 mg l^{-1}) and volatile (Henry's Law constant = 550 Pa m^3 mol^{-1}) and is a known carcinogen (Cohen 1996). One guideline "trigger value" for benzene in Australia, the concentration at which some action is required, is 600 μg l^{-1}, although the trigger value may change depending on the nature of the environment or when benzene is in the presence of "other contaminants" (Chapman & Warne 2000).

The Swan Coastal Plain of Western Australia, on which the city of Perth is located, has a major refinery and many small gasoline distribution sites. Significant hydrocarbon contamination occurs at the refinery and in a survey of gasoline station sites, 20% showed signs of aromatic hydrocarbon leakage to groundwater (Barber et al. 1991). The fate of dissolved and volatile hydrocarbons in groundwaters and soils in the vicinity of Perth, on the Swan Coastal Plain, have been studied because of the potential environmental and health risks associated with gasoline contamination. Because of the extent of contamination, intrinsic

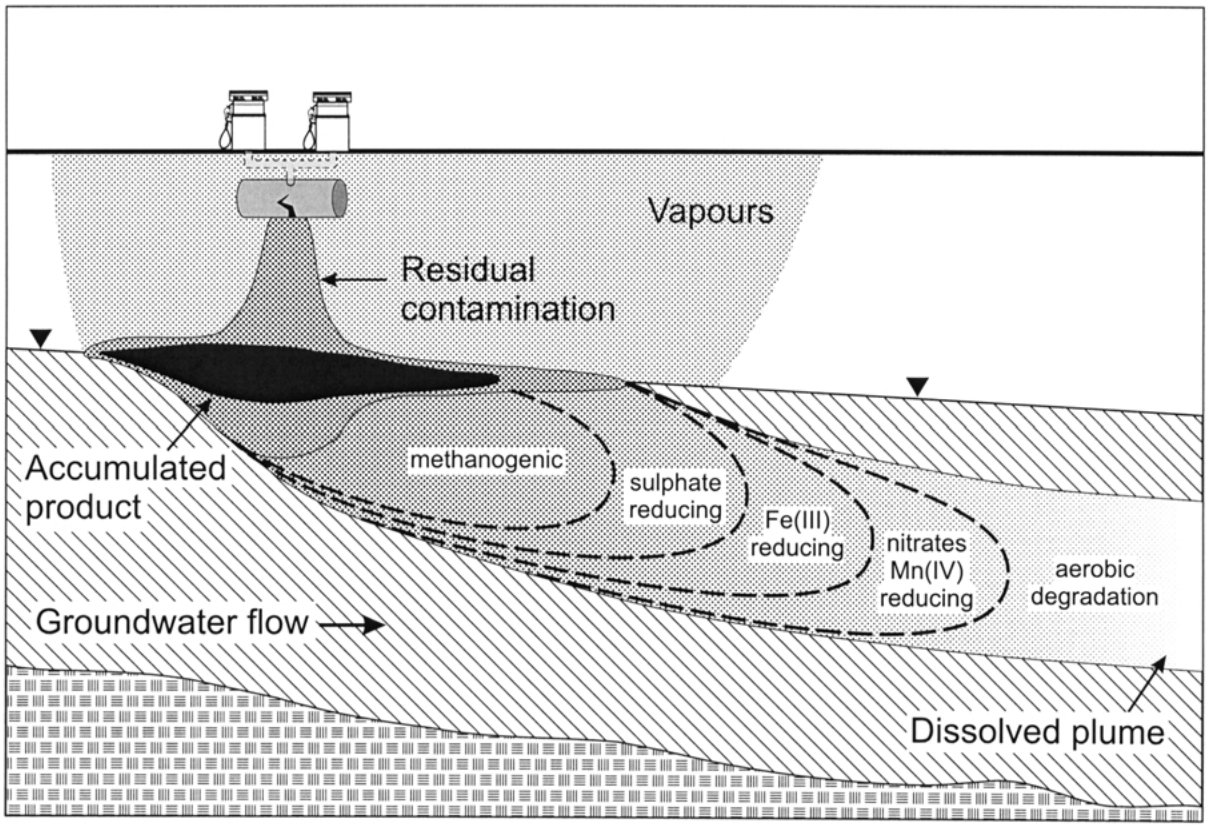

Figure 1. Typical distribution of aromatic hydrocarbons in the subsurface after spillage of gasoline.

remediation will, perhaps by default, play a significant role in contaminant removal and protection of the environment.

Spillage of gasoline in the environment usually results in transport of the majority of the contamination to the surface of the groundwater where it floats as a "light non-aqueous phase liquid" (LNAPL). The most toxic components of the gasoline, the aromatic hydrocarbons, follow one of two routes. They either slowly dissolve in the groundwater, and move in solution in the direction of groundwater flow, or they volatilise from the LNAPL and the groundwater into the gas phase in the vadose zone (Figure 1). As the contamination essentially moves in two planes, horizontally with the groundwater flow, and vertically through the soil profile, intrinsic remediation can be studied by following (1) the changes in the mass of contaminants along the flow paths, (2) the geochemical changes brought about by microbial metabolism along the flow paths and (3) the microbial activity and populations along the flow paths. A service station site with a discrete point source of contamination has been the focus of a number of studies on intrinsic remediation in groundwater. A site with extensive contamination over a wide area, a refinery site, has been the focus of studies on fate and transport of aromatic hydrocarbons in the vadose zone. These studies are reviewed here.

Intrinsic remediation of aromatic hydrocarbons in groundwater

Groundwater contamination and site characterisation

The Swan Coastal Plain is essentially a sand plain with a shallow unconfined aquifer with groundwater flow from the hills to the east and to the Indian Ocean to the west. Depths to groundwater vary by up to 1.8 m depending on the season. In the zone of groundwater table fluctuation, there is generally a cemented layer of iron oxyhydroxide-coated organic enriched sand. In the vicinity of the gasoline contaminated site, the groundwater velocity was between 100 and 170 m $year^{-1}$, effective porosity was between 0.26 to 0.3 m^3 m^{-3}, and the hydraulic conductivity ranged

from 8.6 to 29 m day^{-1} (Davis et al. 1999). Petroleum contamination of the aquifer from a leaking underground petroleum storage tank (UST) was discovered in 1990. Sample collection multiports (MP) (Davis et al. 1992) were installed that allowed water collection from 0.2 or 0.5 m depths at specific sites along the flow path of the contamination and the contamination plume was monitored over a five year period. There were minor variations in the position of the plume over the years, and typical maps of benzene concentration, toluene concentration and sulfate concentration in the groundwater are given in Figure 2, along with the positioning of the multiport sample sites.

In addition to toluene and benzene, the plume contained ethylbenzene, *m*- and *p*- xylene, *o*-xylene, 1,3,5-trimethyl benzene and naphthalene in significant concentrations. In April 1991, the benzene plume extended over 420 m from the site of initial contamination (past MP8), whereas the toluene plume was less than 250 m long (it had disappeared before MP7, Figure 2). *M*- and *p*-xylene and ethylbenzene were also detectable 420 m down the plume whereas *o*-xylene, 1,3,5-trimethylbenzene and naphthalene were not. Retardation coefficients due to sorption for benzene and toluene in aquifer material from this site are very similar, 1.02 and 1.05, respectively, (Thierrin et al. 1993). The limited retardation with respect to a conservative bromide tracer probably resulted from the very low organic carbon content in these sands (0.008–0.02%, Patterson et al. 1993). Truncation of the toluene plume with respect to the benzene plume (Figure 2) could not be explained in terms of the differences in the physical attributes of toluene versus benzene. The distribution of these two contaminants in the plume would be consistent with preferential biodegradation of toluene.

The concomitant loss of sulfate with the truncation of the toluene plume (Figure 2) provides geochemical evidence for toluene mineralisation linked to sulfate reduction as the terminal step in toluene mineralisation, especially as methane was not detected in the groundwater from within the plume (Davis et al. 1999). Groundwaters on the Swan Coastal Plain are generally anoxic. The concentrations of potential electron acceptors and their reduction products in the groundwater are given in Table 1. Analyses of cores from outside the plume showed that, at the depths at which the contamination occurred, extractible Fe(III) was at concentrations generally $<200\ \mu g\ g^{-1}$, and was not extractable in 0.5 M HCl, but was extractable in 5.0 M HCl (Prommer et al. 1999). This would suggest that most of the Fe(III) occurred in a highly crystalline form and was not available for microbial reduction (Prommer et al. 1999). The absence of appreciable bioavailable reducible iron is evidenced by the minimal amount of Fe^{2+} in the groundwater, either inside or outside the plume.

Table 1. Average concentrations of some potential electron donors and their reduction products both within and outside the hydrocarbon contamination plume (Davis et al. 1999).

Parameter	Within plume	Outside plume
NO_3^- (mg-N l^{-1})	0.05	0.18
NH_4^+ (mg-N l^{-1})	0.3	0.25
Fe^{2+} (mg l^{-1})	1.4	1.6
HCO_3^- (mg l^{-1})	74	14.3
SO_4^{2-} (mg S l^{-1})	10.8	27.5
H_2S (mg S l^{-1})	1.1	0.1

Minimal nitrate occurred in uncontaminated background groundwater near the site but appreciably more sulfate was available as a terminal electron acceptor for anaerobic respiration of the contaminants (Table 1). In the uncontaminated groundwater, nitrate concentrations averaged 0.18 mg N l^{-1} (ca. 0.01 mM) and sulfate concentrations averaged 27.5 mg S $^{-1}$ (ca. 0.86 mM). Stoichiometric equations for the oxidation of toluene using nitrate and sulfate are given in Equations (1) and (2) (see Borden et al. 1995; Edwards et al. 1992). Considerably more nitrate than sulfate is required to oxidise one mole of toluene. Given the average amount of each electron acceptor available in the groundwater, sulfate reduction will be the more important electron accepting process in this aquifer, despite the preferential use of nitrate as an electron acceptor by microbial populations conducting anaerobic respiration.

$$C_6H_5\text{—}CH_3 + 7.2\ H^+ + 7.2\ NO_3^- \rightarrow 7\ CO_2 + 7.6\ H_2O + 3.6N_2 \quad (1)$$

$$C_6H_5\text{—}CH_3 + 4.5\ SO_4^{2-} + 3H_2O \rightarrow 2.25\ H_2S + 2.25\ HS^- + 7\ HCO_3^- + 0.25\ H^+. \quad (2)$$

In the plume, the concomitant depletion of sulfate with toluene, and the lack of detectable methane suggests sulfate reduction is the most significant terminal electron accepting process for the dissolved components of gasoline in Perth's groundwaters. The increase

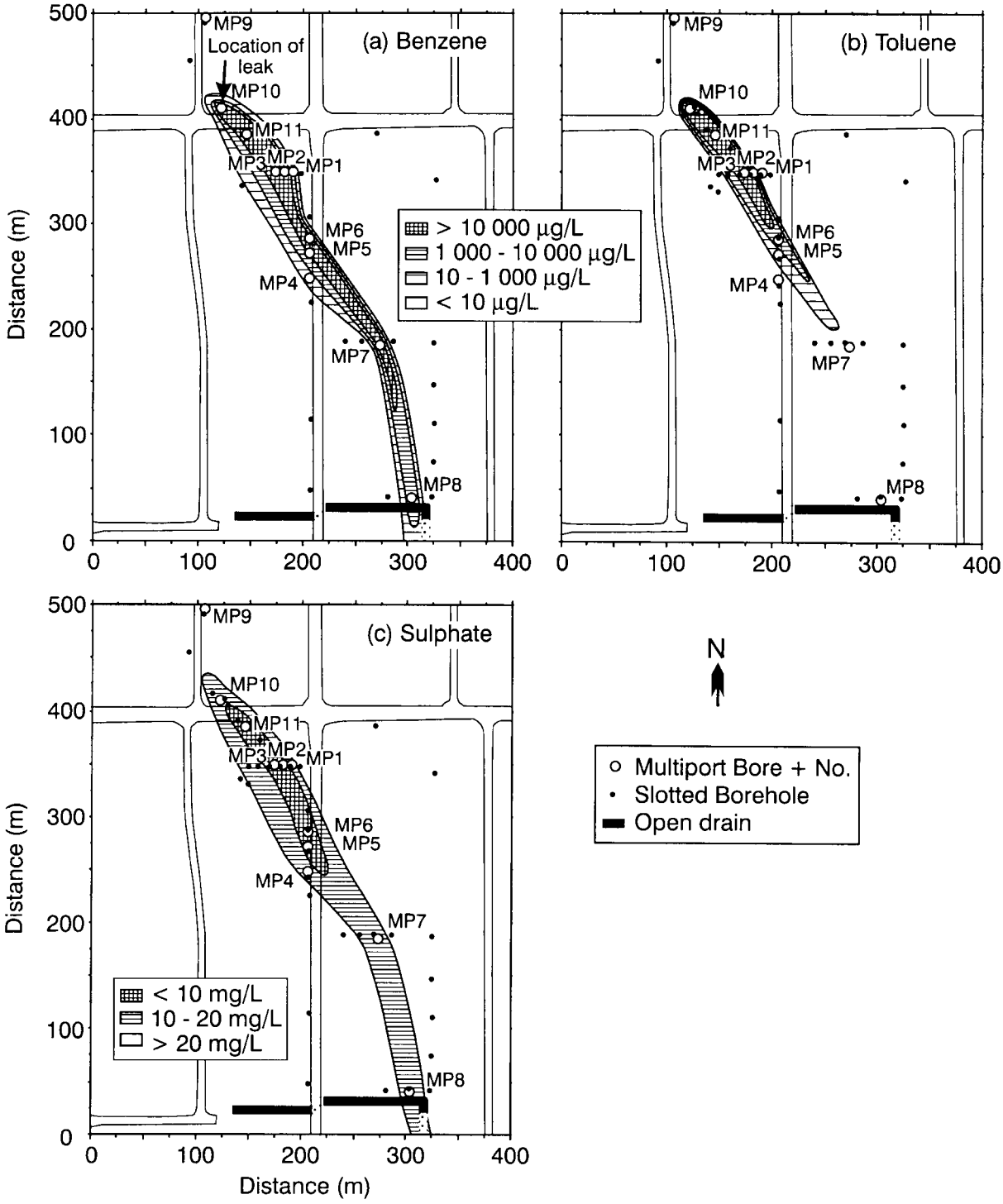

Figure 2. The position of multiport sample collection sites, and contours for concentrations of (a) benzene, (b) toluene, and (c) sulfate in groundwater after leakage of gasoline from an underground storage tank. (Reproduced from Davis et al. (1999) with the permission of the authors and Elsevier Science B.V.)

in the concentration of carbonate ions in the plume relative to the concentration in the background groundwater (Table 1) is also consistent with sulfate reduction (Equation (2)). What is disturbing, from a human and environmental health point of view, is that there was no evidence in this plume of benzene degradation linked to sulfate reduction (Figure 2).

Degradation in columns, and field tracer tests

Given the geochemical characteristics of the plume, and the inference that toluene but not benzene was undergoing anaerobic mineralisation, work was conducted in saturated columns of aquifer material in order to confirm or negate the field observations. In these studies, benzene was degraded under aerobic conditions, but not under nitrate-reducing or sulfate-reducing conditions over 133 days, even in the absence of other hydrocarbons (Patterson et al. 1993). Toluene rapidly degraded under nitrate-reducing conditions, within 31 days, and also showed concomitant degradation with sulfate depletion in groundwater that was not amended with nitrate (Patterson et al. 1993).

Thierrin et al. (1993) conducted in-plume tracer tests using the deuterated compounds, benzene-d6, toluene-d6, *p*-xylene-d10 and naphthalene-d8, and bromide as a conservative tracer. After dissolving the compounds in anoxic groundwater that had been collected from the site, they injected the water into the plume (near multiport 3, Figure 2) and monitored the breakthrough of the compounds 17 m down gradient from the injection bore. Degradation of benzene was not significant, as the breakthrough time and relative mass of benzene-d6 recovery was essentially the same as for the conservative tracer. The other compounds showed loss of mass relative to benzene and the tracer. Thierrin et al. (1993) used the analytical transport model SOLUTE to derive first order degradation rates for the plume from the hydrocarbon distribution data and the tracer test and the rates are compared in Table 2.

Table 2. Half-lives (days) for first order degradation rates of gasoline compounds derived from application of the analytical transport model SOLUTE to plume concentration data and data from an in field tracer test (Thierrin et al. 1993).

Compound	Tracer test	Plume data
Benzene	>800	>800
Toluene	100 ± 40	120 ± 25
Ethylbenzene	ND[1]	230 ± 30
p-xylene	225 ± 74	ND
m & *p*-xylene	ND	170 ± 10
o-xylene	ND	125 ± 10
1,3,5-trimethylbenzene	ND	180
Naphthalene	33 ± 6	160 ± 20

[1] ND = not determined by Thierrin et al. (1993).

Microbial biomass and community structure – initial investigations

An initial brief examination of the microbial populations using fluorescent *in situ* hybridization probes, specific for *Bacteria* (EUB338) and species of the genus *Desulfovibrio* (SRB385) (Amann et al. 1990) was conducted on water drawn from a number of the multiports, both from within and outside the plume (S. Toze, pers. comm.). Few cells in the groundwater reacted with either stain, and considerable background autofluorescence prevented any definitive assessment of the microbial community structure.

The analysis of the fatty acids from membrane phospholipids has been extensively used in culture independent assessment of microbial communities (White et al. 1983), especially in soils and sediments, which are difficult environments to analyse definitively by molecular techniques. Phospholipid fatty acids (PLFA) were analysed in three cores from within and along the contamination plume (near MP11, MP3, and MP8; see Figure 2) and one core from outside the plume (near MP9; see Figure 2) (Franzmann et al. 1996). The PLFA were extracted from core material collected from depths of about 30 cm above the water table, at the water table, and from a depth within the contamination plume. Particulates were collected on large in-line filters (0.22 μm) from groundwater within the contamination plume from similar depth multiports adjacent to the sites of core collection. A summary of outcomes of the analyses is given in Table 3.

Of the groundwater samples taken, the sample that contained the lowest viable biomass was in the plume at MP11. This sample was from the site that contained the greatest amount of aromatic hydrocarbons (136 mg l^{-1}; Davis et al. 1999), whereas the greatest biomass in the groundwater occurred at MP3 where the water contains considerably less aromatic hydrocarbons (48 mg l^{-1}; Davis et al. 1999). Long et al. (1995) noted a drop-off in microbial biomass in subsurface samples that were exposed to high con-

Table 3. Phospholipid-derived fatty acid content extracted from different sediment cores or groundwater particulates inside and outside the contaminant plume. The positions of sample collections are shown in Figure 2.

Site	Depth (m)	PLFA content pmol (g dry wt.)$^{-1}$	Biomass[1] cell (g dry wt.)$^{-1}$	Water content (%)
Core material				
Near MP9[2]	3.00	209	5.4×10^6	11.9
	3.45[3]	66.3	1.7×10^6	11.1
	4.31	67.2	1.7×10^6	13.3
Near MP11	2.87	115	3.0×10^6	8.2
	3.35[3]	67.3	1.7×10^6	11.9
	4.17	47.3	1.2×10^6	15.4
Near MP3	3.80	77.4	2.0×10^6	15.5
	4.11[3]	157	4.1×10^6	16.5
	4.46	40.4	1.1×10^6	15.4
Near MP8	1.31	298	7.8×10^6	13.6
	1.50[3]	178	4.6×10^6	13.2
	5.39	32.8	0.9×10^6	15.3
Groundwater particulates		pmol ml^{-1}	Cells ml^{-1}	
MP9[2]	3.5	13.7	3.6×10^5	
MP11	4.0	3.0	0.7×10^5	
MP3	4.0	36.8	9.6×10^5	
MB8	5.0	6.1	1.2×10^5	

[1] Cell numbers are expressed in "stationary phase *E. coli* equivalent cells" (Franzmann et al. (1996).
[2] Data from a control site outside the plume and up-gradient from the contamination source.
[3] Sample taken at the depth of the groundwater.

centrations of petroleum hydrocarbons, which they defined as samples that contained "free product or non-aqueous phase liquid and contaminants dissolved in the groundwater in parts per million". According to Davis et al. (1999), the concentrations of benzene and toluene at MP11 were often comparable to concentrations in equilibrium with gasoline NAPL. The percentage of biomass attached to the aquifer substrate, as opposed to in the groundwater, can be calculated from the data in Table 4. In the vicinity of multiports MP9, MP11, MP3 and MP8, the percentage of biomass that was attached to the substrate was 98%, 99%, 96% and 98%, respectively. The percentage attached was always high, but greatest in cores taken from the region of highest contaminant concentration, MP11. It is generally considered that microorganisms in biofilms tolerate higher concentrations of toxic contaminants than "free living" microbial cells (Foley & Gilbert 1996).

Two phospholipid-derived fatty acids have been commonly used as signature fatty acids for the presence of sulfate-reducing bacteria. The fatty acids *i*17:1*c*9 and 10-methyl-16:0 are supposedly indicative of *Desulfovibrio* spp. and *Desulfobacter* spp., respectively. Neither signature was found in the PLFAs in core material collected from below the groundwater table in the zone of sulfate depletion (Franzmann et al. 1996). The fatty acid 10-methyl-16:0 was found in soil cores from outside the contaminant plume, either above or at the aquifer water table. A range of monomethylated fatty acids may be derived from actinomycetes that occur in aerobic soils (Zelles et al. 1995).

Enrichment and isolation of sulfate-reducing bacteria

Neither the initial FISH probing nor the analysis of phospholipid fatty acids of material from the contaminated site, provided evidence of populations of sulfate-reducing bacteria. Enrichments for sulfate-

Table 4. Average zeroth order mineralisation rates for ^{14}C-toluene and ^{14}C-benzene in groundwater collected from inside and outside the contamination plume (data from Robertson et al. 2000).

Treatment	Mineralisation rate in groundwater (mmol Ml^{-1} day^{-1}) from:			
	Inside plume		Outside plume	
	Toluene	Benzene	Toluene	Benzene
Sterile control	7.7×10^{-7}	1.9×10^{-9}	4.4×10^{-7}	2.2×10^{-9}
Non-amended groundwater	9.6×10^{-6}	2.7×10^{-8}	7.1×10^{-5}	5.2×10^{-8}
+ sulfate (5 mM)	1.7×10^{-5}	3.2×10^{-8}	1.2×10^{-4}	5.0×10^{-8}
+ sulfate (5 mM) + molybdate (2 mM)	2.3×10^{-6}	2.0×10^{-8}	1.6×10^{-6}	2.2×10^{-8}
+ sulfate (5 mM) + strain T2[1]	1.2×10^{-4}	3.1×10^{-8}	5.6×10^{-4}	4.7×10^{-8}

[1]T2 = a strain of *Desulfosporosinus meridiei* added as a 10% inoculum from a fresh culture.

reducing bacteria that contained toluene-in-paraffin oil or benzoate were prepared from anaerobic groundwater taken from the plume; from multiports MP11 and MP2 (Figure 2) (Robertson et al. 2000). Pure cultures of sulfate-reducing bacteria were isolated using roll tubes. Phylogenetic analysis showed that all of the cultures ($n = 8$) could be accommodated within the genus *Desulfosporosinus* in the Gram positive, low G + C, line of descent (see Figure 3). The strains were described as a new species, *Desulfosporosinus meridiei* (Robertson et al. 2001). The extraction of DNA for PCR amplification of the 16S rDNA from the strains generally required beating with zirconium beads and generally could not be obtained by chemical lyses (Robertson et al. 2000).

All of the major fatty acids that occurred in *Desulfosporosinus meridiei* (Robertson et al. 2000) also occurred in the phospholipid extracts of core material from the contaminated site (Franzmann et al. 1996). The cells lacked the fatty acid signatures often used as markers for sulfate reducing bacteria in environmental samples; *i*17:1*c*9 and 10-methyl-16:0. Members of the species also lacked the 16S rRNA hybridization sites for FISH probes that are commonly used for the detection of sulfate-reducing bacteria.

Microcosm tests

Microcosm tests with ring-labeled ^{14}C-benzene and ^{14}C-toluene showed that *Desulfosporosinus* strains were incapable of mineralizing either compound to $^{14}CO_2$ (Robertson et al. 2000). To test the role that *Desulfosporosinus meridiei* may have in benzene or toluene degradation, the rate of mineralisation of ^{14}C-benzene and ^{14}C-toluene was determined in a series of microcosms of collected groundwater, with different additions of sulfate, cells of *Desulfosporosinus meridiei* or molybdate as an inhibitor of sulfate reduction. The rate of toluene mineralisation was minimal in non-amended groundwater, but was enhanced by the addition of sulfate, and further enhanced by the addition of both sulfate and cells of *Desulfosporosinus meridiei* (Table 4). The rate of toluene mineralisation in the microcosm that received sulfate with molybdate was slower than the mineralisation rate in non-amended groundwater. The rate of benzene mineralisation in non-amended groundwater was minimal, and was not enhanced by the addition of sulfate or sulfate with cells of *Desulfosporosinus meridiei* (Table 4). Interestingly, groundwater collected from outside the contamination plume showed a greater capacity for toluene mineralisation than groundwater collected from within the contamination plume (Table 4). Although the sulfate addition, and further addition of an inoculum of sulfate-reducing bacteria greatly stimulated toluene mineralisation in the microcosms, the pure culture of sulfate-reducing bacterium that was added did not mineralise toluene. It is thus probable that *Desulfosporosinus meridiei* plays a similar role in this plume to the role methanogens play in toluene-degrading consortia. That is, they consume the hydrogen and organic acids produced by fermentative organisms from the toluene, thus preventing the accumulation of fermentation products that would otherwise make the further degradation of toluene thermodynamically impossible (Zwolinski et al. 2000). The mineralisation rates for toluene in the groundwater microcosms were considerably slower than degradation rates measured in the field (Davis et al. 1999). Microcosms experiments always contain artifacts, and should be used only to indicate the potential microbial processes. As the PLFA analysis showed that at least 98% of the microbial biomass was substrate bound, it is not surprising that the use of

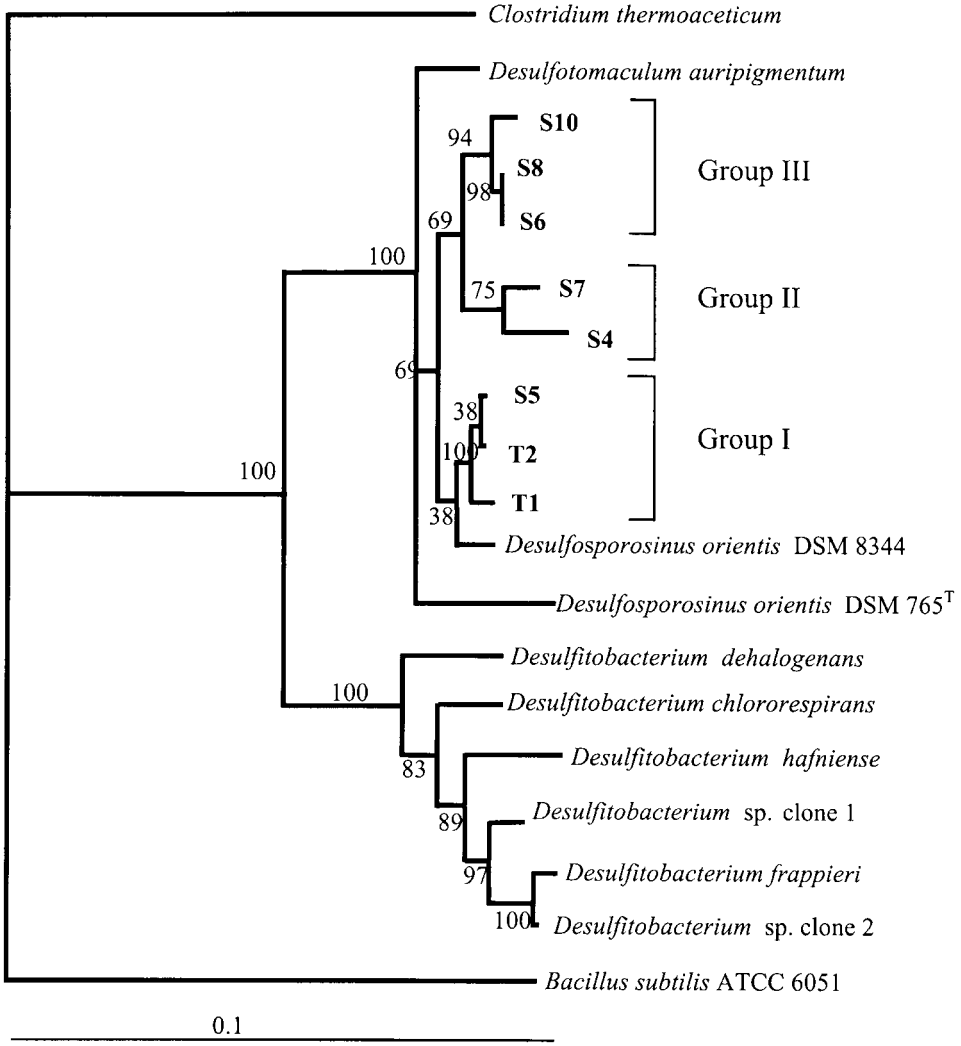

Figure 3. Phylogenetic tree of the 16S rRNA genes of sulfate-reducing strains from a BTEX contamination plume in Western Australia, and their near relatives. The tree was constructed using PHYLIP and the Jukes-Cantor measure of distance. Bootstrap values are indicated. Reproduced from Robertson et al. (2000), under copyright permission from Blackwell Science.

groundwater alone in microcosms would greatly underestimate the potential for microbial degradation of the contaminants.

Tracking Desulfosporosinus meridiei in the plume

The only sulfate-reducing species that was isolated from the plume was *Desulfosporosinus meridiei*, which would have evaded detection by the FISH technique used in the initial studies, and which did not contain the PLFA signatures usually indicative of sulfate-reducing bacteria. To examine the plume for the presence of *Desulfosporosinus meridiei*, both species-specific polyclonal fluorescent antibodies and FISH probes, as well as eubacterial FISH probes were prepared (Robertson 2000). The reaction of cultured cells to the polyclonal antibodies and the species-specific FISH probes are shown in Figure 4. The outcomes of the FISH and antibody probing are given in Table 5. Robertson's study was limited to the examination of groundwater due to the cost and other constraints on re-drilling for core material.

Although cultured cells of *Desulfosporosinus meridiei* produced a signal when probed with the oligonucleotide probe, the cells fluoresced faintly when compared with the signal produced by cells subjected to antibody probing (Figure 4). Cells of *Desulfosporosinus meridiei* were not detected in any

Table 5. Numbers of cells (cells ml^{-1}) in groundwater collected from four separate multiports from a hydrocarbon contaminated aquifer on the Swan Coastal Plain. Cell were detected after staining with DAPI, fluorescence *in situ* hybridisation probe EUB338, and fluorescent polyclonal antibodies raised against *Desulfosporosinus meridiei* strain T2. The percentage of the DAPI staining cells that stained with the probe EUB338 is also reported. Data from Robertson (2000).

Sample site	DAPI		EUB338		%EUB of total	Polyclonal	
	Mean	S.E.[1]	Mean	S.E.		Mean	S.E.
MP9[1]	2.6×10^7	9.2×10^6	2.2×10^6	1.1×10^6	8	4.0×10^3	2.2×10^3
MP11	2.4×10^7	4.6×10^6	5.4×10^6	4.3×10^6	23	4.6×10^3	2.3×10^3
MP16[3]	1.4×10^7	3.0×10^6	4.0×10^6	3.5×10^6	29	2.2×10^3	1.4×10^3
MP8	1.5×10^7	2.5×10^6	3.2×10^6	1.8×10^6	21	1.3×10^2	5.2×10^1

[1] S.E. = standard error.
[2] Multiport MP9 was external to the plume. Other sites were within the plume.
[3] MP16 was located 100 m down gradient from the source of contamination.

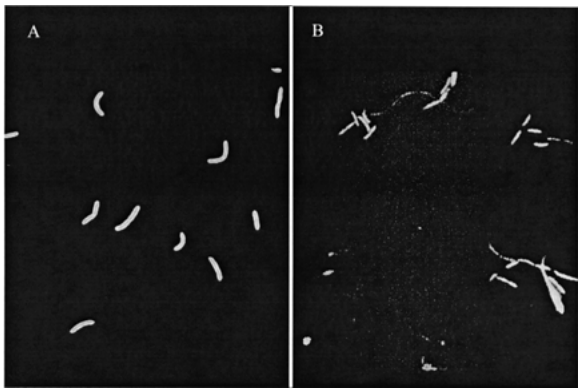

Figure 4. The reaction of fluorescent polyclonal antibodies (A) and fluorescence *in situ* hybridization probes (B) with cells from a culture of *Desulfosporosinus meridiei*.

groundwater sample by the FISH probe designed for *Desulfosporosinus meridiei. Desulfosporosinus meridiei* was detected by antibody probing, but numbers were less than 0.2% of the total population of cells (the numbers detected by DAPI staining). What is also of note, was that numbers of *Desulfosporosinus meridiei* were as great in water from outside the plume (MP9) as in groundwater drawn from inside the plume (MP11, MP16, MP8; Table 5). This is consistent with the observation that groundwater from outside the plume showed the same potential to mineralise toluene, under sulfate-reducing conditions, as water drawn from inside the plume.

At best, 29% of the DAPI-stained cells in the groundwater samples stained with the bacterial probe EUB338 (Table 5). The outcomes of the use of FISH probing of aquifer material have rarely been reported. Zarda et al. (1998) reported that between 3 to 42% of the cells that stained with DAPI in aquifer material contaminated with aromatic hydrocarbons, could be detected by FISH with probe EUB338. As pointed out by Zarda et al. (1998), detection rates after hybridization with probe EUB338 are used as an indication for the presence of metabolically active cells which contain sufficient amounts of rRNA coupled with sufficient cell permeability or permeabilisation to permit their detection. Watanabe et al. (2000) found that FISH was much poorer than PCR and denaturing gradient gel electrophoresis (DGGE) at detection of microorganisms in groundwater associated with crude oil storage facilities. In their study, labeled cells exhibited weak signals, which was attributed to low rRNA contents resulting from a groundwater residence time of 7 days in the cavities, and limited availability of electron acceptors.

The intensity of the signal obtained with FISH rapidly decreases with cell age, to less than 20% of the initial intensity by day 25 for some sulfate-reducing bacteria (Bade et al. 2000). In slow moving groundwater, in which the transport of nutrients to and products away from cells would be extremely slow, the average cell age must be much greater than in dynamic environments such as activated sludge, in which sludge age is usually less than 10 days, and in which the use of FISH probing is considerably more successful (Daims et al. 1999). However, Fredrickson et al. (1995) were able to successfully probe for delta proteobacteria in some deep subsurface samples from an aquifer with low hydraulic conductivity.

Desulfosporosinus meridiei could be detected in groundwater from the site by antibody probing and culture, but not by FISH probing. Given that Zarda et al. (1998) also showed a poor response by groundwater bacteria to FISH probing, its use for tracking

cells in contaminated aquifers would appear to be problematic.

Aromatic hydrocarbon attenuation in groundwater on the Swan coastal plain: Conclusions

Sulfate-reduction is the major terminal electron accepting process for the intrinsic remediation of plumes of aromatic hydrocarbons on the Swan Coastal Plain. Although toluene degradation can occur under methanogenic conditions, methanogenesis does not play a significant role on the Swan Coastal Plain even after considerable sulfate depletion in the groundwater. Benzene can be degraded under sulfate-reducing conditions (Lovley et al. 1995) but this does not occur significantly in the Swan Coastal Plain aquifer. The reason for this is unknown. However, benzene is rapidly degraded in Swan Coastal Plain aquifer material under aerobic or nitrate-reducing conditions. As benzene does not show natural attenuation by microbial populations in the Swan Coastal Plain aquifer, high concentrations persist in groundwater for large distances down-gradient from a source of contamination. These findings have serious implications for the management of gasoline-contaminated sites on the Swan Coastal Plain, especially as groundwater supplies about 70% of water used in the area.

It has proved difficult to define the microbial community structure responsible for the attenuation of plumes of aromatic hydrocarbons on the Swan Coastal Plain. FISH probing, which has yielded so much information on microbial communities in many environments, seems of little value in anaerobic groundwater environments.

"In closing, it seems prudent to recognize that understanding the identity and activity of microorganisms in the subsurface, and in all other habitats, is a major scientific challenge" (Madsen 2000).

Intrinsic remediation of aromatic hydrocarbons in the vadose zone

A second major exposure pathway for carcinogenic aromatic hydrocarbons from a contaminated subsurface is through vapour transport through the soil to the surface. It is not optimal to study this phenomenon in thin plumes that result from small point-source contamination from damaged or leaking underground storage tanks such as those that occur on the Swan Coastal Plain (Figure 2). Therefore the fate of hydrocarbon vapours has been studied on the Swan Coastal Plain at a refinery site where widespread LNAPL contamination over a large surface area of the shallow aquifer has occurred. The widespread contamination allows study of the transport and biodegradation of volatile hydrocarbons in the vertical plane without appreciable "edge effects" on the vapour transport from the groundwater table towards the surface.

Soil gases at the field site

The field site was a lightly grassed expanse that had experienced hydrocarbon groundwater contamination for probably more than 20 years. Residual LNAPL occurred at the groundwater-vadose interface, and free phase hydrocarbon accumulated in wells drilled in the study area. To enable the collection of gases from the soil profile, a number of multiport installations that consisted of bundles of 2-mm i.d. nylon tubes were installed at 0.25 m intervals to below the groundwater-soil interface in early 1999. Profiles of volatile hydrocarbons, mostly aromatic hydrocarbons [namely benzene, toluene, isomers of xylene, and 1,3,5-trimethylbenzene] and the soil gases oxygen and carbon dioxide, have been measured at the site over an annual cycle (Davis et al. 2000). The results from two of those profiles are shown in Figure 5.

The profiles changed greatly on a seasonal basis, and the depth penetration of oxygen into the soil was highly dependent upon the water content in the soil profile (Davis et al. 2000). The Swan Coastal Plain receives little rain over the Australian summer, 162 mm from October to April, however, considerable precipitation occurs during the Australian winter months (609 mm from May to September). The increase in the water content in the soil, reduces the air filled porosity, especially in a moisture retaining layer at about 0.1 to 0.3 m below the ground surface, which restricts vapour and oxygen transport. Thus in wet periods, aromatic hydrocarbons penetrate much higher in the soil profile, and oxygen penetration is shallower (Figure 5, Davis et al. 2000).

Biodegradation in the vadose zone

Despite seasonal variability in the profiles of aromatic hydrocarbons and oxygen in the soil profile, the presence of oxygen coincided with an absence of aromatic hydrocarbons (Figure 5; Davis et al. 2000). Field analyses of groundwater samples have indicated that the amount of BTX in groundwater is directly related to the availability of dissolved oxygen. Aromatic hydrocarbons are absent from groundwater in the vicinity of

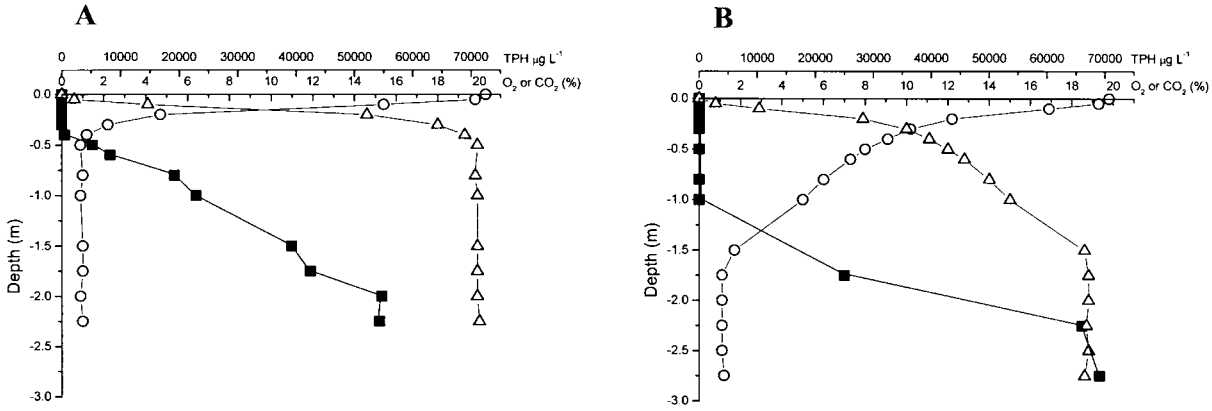

Figure 5. Profiles of soil gases over depth in the vadose zone above gasoline contaminated water, at the end of the wet season (A) and dry season (B) (Davis et al. 2000). Oxygen = ○, carbon dioxide = △. Total Petroleum Hydrocarbon (TPH) = ■.

contaminated sites if the dissolved oxygen content is greater than 0.9 mg l^{-1} (Chiang et al. 1989).

Zeroth order oxygen consumption rates can be determined from steady state oxygen versus depth profiles, provided the effective diffusion coefficient for oxygen in the soil is known. In some cases this may be problematic as, diffusion may be markedly affected by factors such as moisture content (Davis et al. 2000). The stoichiometric conversion of oxygen depletion data to hydrocarbon degradation may also be problematic as other oxidisable organic matter or oxidisable inorganic reduced species (sulfur gases, Fe(II), NH_3) may account for some of the oxygen consumption (Davis et al. 1998). From oxygen consumption profiles, Davis et al. (1998) determined a hydrocarbon degradation rate of between 110 to 170 mg-hexane l^{-1} day^{-1}. With an average air-filled porosity of 0.49 m^3 m^{-3} and a bulk density of 1340 kg m^{-3}, this equates to degradation rates of between 41 to 64 mg $(kg\ day)^{-1}$. These rates are high when compared with rates determined in other studies that have measured biodegradation of vapours from petroleum products during active bioventing as reported by Hoeppel et al. (1991) [up to 10 mg $(kg\ day)^{-1}$ for JP-4 at Hill AFB; 2–20 mg $(kg\ day)^{-1}$ for JP-4 at Tyndall AFB; 8 mg $(kg\ day)^{-1}$ for diesel in The Netherlands; 3 mg $(kg\ day)^{-1}$ for JP-5 at Patuxent River, Maryland; 5 mg $(kg\ day)^{-1}$ for JP-5 at Fallon, Nevada; 1 to 10 mg $(kg\ day)^{-1}$ for JP-5 at Eilson AFB, Alaska]. To determine an unambiguous mineralisation rate for volatile aromatics, Franzmann et al. (1999) used ^{14}C-ring-benzene in microcosms with core material from a range of depths in the soil profile to measure the potential for the soil microbial community for benzene mineralisation. The concentrations of aromatic hydrocarbons, oxygen and carbon dioxide in the soil profile at the time of sample collection are given in Table 6, as are the mineralisation rates, and estimates of the microbial biomass at different depths within the soil profile.

Soil from a depth of 0.25 m showed the greatest potential for mineralisation of ^{14}C-benzene 83 $\pm$ 13 μmol $(kg\ day)^{-1}$ [which is equivalent to 6.5 $\pm$ 1 mg $(kg\ day)^{-1}$] (Table 6). Soil from this depth was exposed to a plentiful supply of hydrocarbon vapour and oxygen, 11.3 mg l^{-1} and 13%, respectively. At a depth of 0.5 m, the oxygen content dropped to 3% and approached the range that may be limiting for total petroleum hydrocarbon (TPH) mineralisation (2–5%; Hickey 1995). The microbial population at this depth showed a slightly less propensity for hydrocarbon mineralisation. At the surface, the microbial population was not exposed to measurable concentrations of TPH (i.e., $<$1 mg l^{-1}) but showed considerable propensity to mineralise aromatic hydrocarbons. In fact, the surface microbial population mineralised hydrocarbons at about the same rate as measured in surface soils from the Swan Coastal Plain that were not associated with hydrocarbon contamination (23 to 42 μmol $(kg\ day)^{-1}$; Franzmann et al. 1998). Further evidence for the lack of hydrocarbon exposure to the microbial population in the surface soil was the relative lack of *trans* isomer monounsaturated fatty acids in the phospholipid fraction extracted from this soil (Table 6). Monounsaturated *trans* isomer fatty acids are enriched in the phospholipids of aerobic microbial populations when exposed to aromatic hydrocarbons (Heipieper et al. 1994). There was little propensity for anaerobic mineralisation of benzene in

Table 6. Concentrations, in the gas phase, of volatile total petroleum hydrocarbons (TPH), oxygen and carbon dioxide; first and zeroth order ^{14}C-hydrocarbon mineralisation rates; biomass; and the percentage of PLFA monounsaturated fatty acids with *trans* isomerisation, in soil from different depths in the vadose zone above hydrocarbon contaminated groundwater on the Swan Coastal Plain. Data from Franzmann et al. (1999).

BGS[1] (m)	TPH (μg l^{-1})	CO_2 (%)	O_2 (%)	$t_{1/2}$ (days)	Min. rate (μmol (kg day)$^{-1}$)	Cell No. (cells g^{-1})	% *trans*
0	0	0	20	26 ± 7	52 ± 13	$2.0 \pm 0.7 \times 10^8$	0.6
0.05	662	0	20				
0.25	11384	3.2	13.8	11 ± 1	83 ± 13	$1.4 \pm 0.4 \times 10^8$	55
0.5	14387	11	3	72 ± 16	27 ± 6	$1.1 \pm 0.3 \times 10^8$	29
0.75	12357	1.2					
2.75	9933	12.6	1.2	$(6.3 \pm 1.1 \times 10^4)$[2]	0.04 ± 0.01	$2.7 \pm 1.3 \times 10^7$	0

[1] BGS = below ground surface.
[2] Unlike the other samples, this sample was incubated anaerobically.

soil deep within the profile ($t_{1/2}$ = 173 years, Table 6). Simple modeling showed that, provided the zeroth order mineralisation rates were used, the total flux of hydrocarbon between depth 0.5 m and the surface could be accounted for by the rate of aerobic hydrocarbon mineralisation in that zone (Franzmann et al. 1999).

At the end of the wet season, when the vadose soils contained considerably more water, TPH was much higher in the profile (Figure 5). This occurred because greater water saturation in the soil profile limited the penetration of oxygen from the surface (Davis et al. 2000). Intrinsic remediation of aromatic vapours in the vadose zone above gasoline-contaminated water seems to generally occur, provided there are no impediments to the transport of gases. With greater saturation of the soil, TPH was higher in the profile (Figure 5). Moseley and Meyer (1992) showed that above hydrocarbon-contaminated groundwater, greater accumulation of soil gas aromatic hydrocarbons occurred under paved areas and building structures than under non-built-up areas. This resulted in substantial leakage of benzene into the building structures and the crawl space beneath the floors. For intrinsic remediation of hydrocarbon vapours in the soil profile above contaminated groundwater, natural ventilation of the soil profile must not be impeded, since this will slow biodegradation and the containment of vapours within the vadose zone.

Conclusions

Except for the transport of benzene in groundwater, intrinsic remediation by microbial populations prevents the extensive transport of aromatic hydrocarbons in the aquifer or the vadose zone on the Swan Coastal Plain. This is consistent with the findings of many studies conducted in many different aquifers.

The transport of benzene in the groundwater on the Swan Coastal Plain is of concern, however addition of oxygen or nitrate in the benzene plume could be undertaken as a final remediation measure. It remains unclear why benzene mineralisation can occur under sulfate-reducing conditions in some aquifers but not others. Although the fate of the aromatic hydrocarbons, and the microbial processes involved in their mineralisation can be inferred through geochemical analysis and transport modeling, measurement of microbial activities using radio-labeled compounds increased our understanding of the processes and potential rates of mineralisation of the pollutants. The analysis of microbial populations through phospholipid-derived signatures and biomass estimates provided insight into the microbial populations in the soil profile above hydrocarbon-contaminated groundwater (Table 6), however signature analysis in the contaminated groundwater plume provided little useful information (Franzmann et al. 1996).

The use of fluorescently labelled polyclonal antibodies for tracing specific bacterial strains of *Desulfosporosinus meridiei* in the groundwater was greatly superior to the use of rRNA-directed FISH probes. The methodology for the application of FISH is rapidly improving through the use of new dyes, probes and image analysis techniques (Daims et al. 1999), however the successful use of FISH to describe microbial communities in slow moving groundwaters has not been demonstrated and will perhaps remain a practical challenge for some time.

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