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Field metabolomics and laboratory assessments of anaerobic intrinsic bioremediation of hydrocarbons at a petroleum-contaminated site

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Summary

Field metabolomics and laboratory assays were used to assess the *in situ* anaerobic attenuation of hydrocarbons in a contaminated aquifer underlying a former refinery. Benzene, ethylbenzene, 2-methylnaphthalene, 1,2,4- and 1,3,5-trimethylbenzene were targeted as contaminants of greatest regulatory concern (COC) whose intrinsic remediation has been previously reported. Metabolite profiles associated with anaerobic hydrocarbon decay revealed the microbial utilization of alkylbenzenes, including the trimethylbenzene COC, PAHs and several *n*-alkanes in the contaminated portions of the aquifer. Anaerobic biodegradation experiments designed to mimic *in situ* conditions showed no loss of exogenously amended COC; however, a substantive rate of endogenous electron acceptor reduction was measured (55 ± 8 μM SO_4 day^{-1}). An assessment of hydrocarbon loss in laboratory experiments relative to a conserved internal marker revealed that non-COC hydrocarbons were being metabolized. Purge and trap analysis of laboratory assays showed a substantial loss of toluene, *m*- and *o*-xylene, as well as several alkanes (C_6–C_{12}). Multiple lines of evidence suggest that benzene is persistent under the prevailing site anaerobic conditions. We could find no *in situ* benzene intermediates (phenol or benzoate), the parent molecule proved recalcitrant in laboratory assays and low copy numbers of *Desulfobacterium* were found, a genus previously implicated in anaerobic benzene biodegradation. This study also showed that there was a reasonable correlation between field and laboratory findings, although with notable exception. Thus, while the intrinsic anaerobic bioremediation was clearly evident at the site, non-COC hydrocarbons were preferentially metabolized, even though there was ample literature precedence for the biodegradation of the target molecules.

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Introduction

The release of petroleum components to the terrestrial subsurface is recognized as a pervasive environmental and human health problem requiring environmental remediation (USEPA, 1999). Monitored natural attenuation is a relatively low-cost remedial option that has become more widely accepted as its efficacy has been repeatedly demonstrated since the early 1990s (Borden *et al*., 1995; Reinhard *et al*., 1997; Chapelle, 1999; Gieg *et al*., 1999; Phelps and Young, 1999; USEPA, 1999; Beller, 2002; Field, 2002; Roling and van Verseveld, 2002; Essaid *et al*., 2003; Griebler *et al*., 2004; Maurer and Rittmann, 2004; Rittmann, 2004; McKelvie *et al*., 2005). Natural attenuation involves multiple mechanisms of contaminant removal (e.g. volatilization, sorption, advection, dispersion) but relies on biodegradation of the contaminants of greatest regulatory concern (COC) by the indigenous microflora (NRC, 1993; USEPA, 1999). However, the COC are typically part of complex chemical mixtures consisting of hundreds or even thousands of other co-contaminants. Even though the individual COC may be inherently susceptible to biodegradation, it is important to determine if this potential is realized under the prevailing environmental conditions.

Many studies have demonstrated the complete destruction of individual hydrocarbons catalysed by isolated organisms or enriched microbial consortia under a variety of electron-accepting conditions. Recent literature attests

to the importance of anaerobic biodegradation of mono- and polycyclic aromatic, alicyclic, alkene and alkane hydrocarbons in diverse ecosystems (Reinhard *et al.*, 1997; Gieg *et al.*, 1999; Phelps and Young, 1999; Spormann and Widdel, 2000; Elshahed *et al.*, 2001; Widdel and Rabus, 2001; Gieg and Suflita, 2002; Phelps *et al.*, 2002; Martus and Puttmann, 2003; Chakraborty and Coates, 2004; Meckenstock *et al.*, 2004a; Townsend *et al.*, 2004; Gieg and Suflita, 2005; Young and Phelps, 2005; Callaghan *et al.*, 2006; Safinowski *et al.*, 2006; Widdel *et al.*, 2006; Prince and Suflita, 2007). The direct detection of signature metabolites produced during the anaerobic biodegradation of hydrocarbons is indicative of the metabolism of the corresponding parent hydrocarbon. However, there is no compelling reason to presume that the same metabolic potential is present in all environments.

Field metabolomics is a fast and interpretationally direct method that can make use of existing infrastructure such as monitoring wells at contaminated areas to identify signature metabolites (Gieg and Suflita, 2005). Other methods have been used to describe anaerobic hydrocarbon degradation in petroleum-contaminated environments such as compound-specific isotope analysis and quantitative PCR of functional genes. A recent study compared the inherent advantages and limitations of each of these techniques and concluded that no single method proved satisfactory under all circumstances (Beller *et al.*, 2008). To be sure, metabolite profiling can be limited by detection requirements, but when evident, provides definitive and compound-specific verification of *in situ* metabolism. Coupling this approach with laboratory biodegradation assessments can elucidate microbial community function as well as the bioremediation potential.

We investigated whether a series of COC (benzene, ethylbenzene, 1,2,4-trimethylbenzene, 1,3,5-trimethylbenzene and 2-methylnaphthalene) were being metabolized in an aquifer underlying a former refinery site in Casper, WY (Fig. 1). During the almost eight decades of refinery operations, hydrocarbon contamination could be traced to a variety of releases (Brubaker *et al.*, 2003). Despite the complex nature of the contaminant mixture, most regulatory attention focused on benzene. Benzene was present throughout the site at concentrations exceeding 50 μM (4000 μg l^{-1}) (Brubaker *et al.*, 2003). Field metabolomics and laboratory biodegradation assays were used to garner evidence for the intrinsic remediation of all the COC. We found that while *in situ* microbial hydrocarbon metabolism was evident in the aquifer, not all COC were susceptible to anaerobic decay despite expectations from the literature. Generally, good agreement between the field and laboratory indications of anaerobic biodegradation was obtained. Several reasons for the relative recalcitrance of the COC are suggested.

Results

Metabolite profiling

A variety of signature metabolites associated with anaerobic hydrocarbon biodegradation were detected in groundwater from monitoring wells at the former refinery site, but not in samples from a background well (Table 1). Alkylbenzylsuccinic acid metabolites associated with the biodegradation of the xylene and C_3-alkylbenzene isomers (Table 1) were detected in seven of eight monitoring wells within the refinery area and one of the wells on the north side of the river where hydrocarbons were stored. Toluene degradation was also evident by the presence of benzylsuccinic acid on a single sampling occasion (Table 1). However, unlike the trimethylbenzene COC, no evidence for anaerobic ethylbenzene decay was obtained with the field metabolite profiling.

Metabolites associated with the anaerobic biodegradation of substituted naphthalenes were also detected at the site. The presence of the partial ring reduction metabolite 5,6,7,8-tetrahydro-2-naphthoic acid (rather than other isomeric components) was revealed by gas chromatography-mass spectrometry (GC-MS) in three of the 10 monitoring wells (Table 1). Another well indicated the presence of the unsubstituted naphthoic acid, while residues associated with methyl- and dimethyl naphthoic acids isomers could be found in MW-345. There was no evidence for the more reduced decahydronaphthoic acid in any of the wells, but most had a putative naphthalene metabolite with mass spectral features consistent with the presence of tetrahydronaphthoic acids. However, the mass spectral profiles exhibiting these features were not associated with retention times for the authentic standards for 1,2,3,4- or 5,6,7,8-tetrahydro-2-naphthoic acid. Notably, anaerobic naphthalene metabolism was not indicated in samples obtained from the monitoring well chosen for the biodegradation assays (MW-439).

Field evidence for the anaerobic biodegradation of pentane, hexane and a variety of C_5–C_9 unsaturated hydrocarbons (alkenes or alicyclic) was implicated based on the detection of the corresponding fumarate addition metabolites (Table 1). All wells, except MW-345, harboured one or more of these metabolites and a similar suite of compounds were observed in well MW-439 on both sampling occasions.

Sulfate reduction and hydrocarbon metabolism

Sulfate reduction could be measured in all aquifer material incubations regardless of COC amendment (Fig. 2). As replicate incubations varied, the rates were averaged with extremes (highest and lowest values) indicated. Given that hydrocarbon metabolism can sometimes require long incubation periods (Caldwell and Suflita,

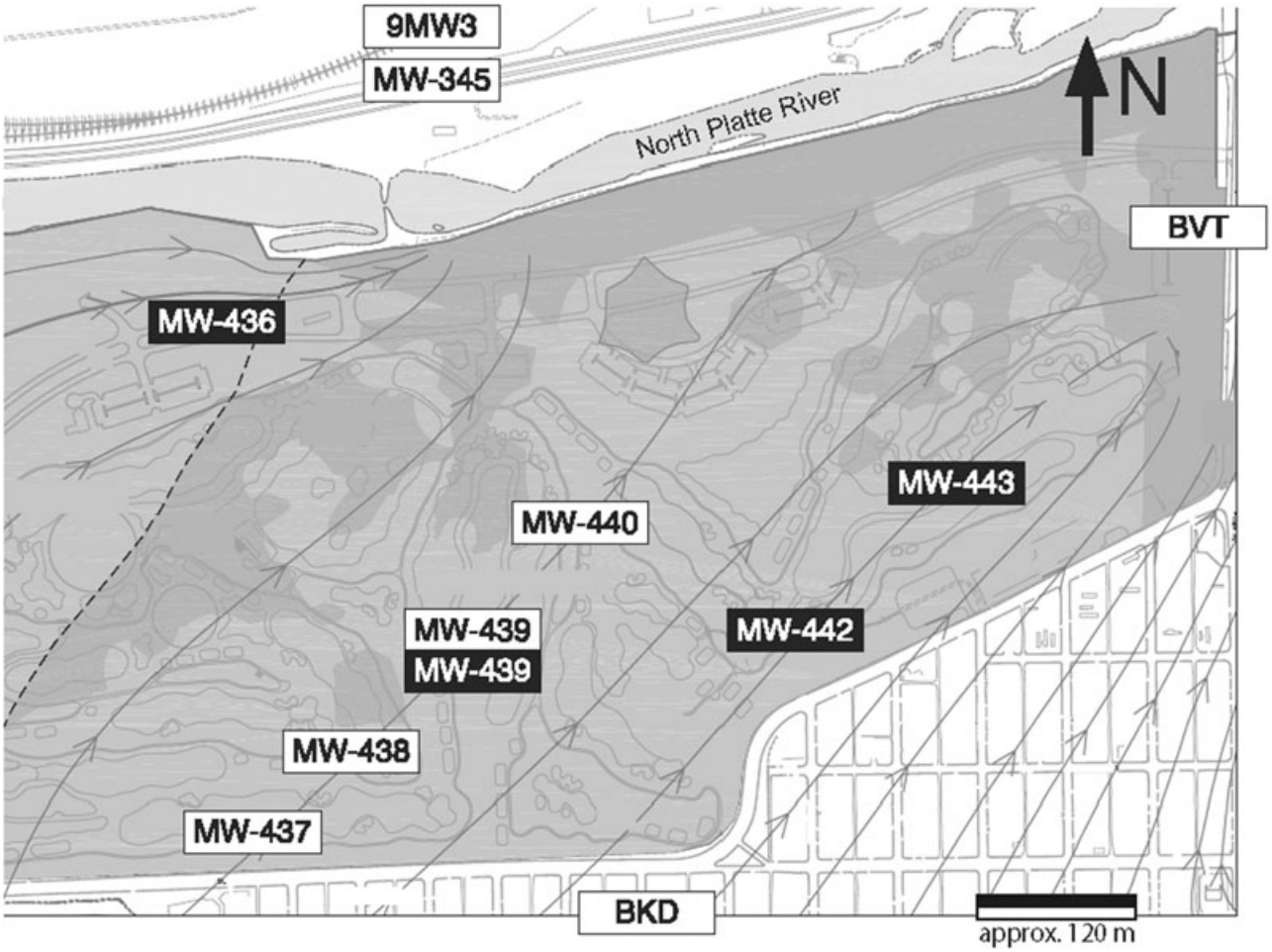

Fig. 1. Monitoring well locations (boxes) used for groundwater sampling at the former refinery site near the North Platte River in Casper, WY. Groundwater flow was in a north-eastern direction, towards the river. Samples were collected in February (white) and October (black) 2005 along transects throughout the site. Darker shaded regions represent active remediation zones and BVT represents a well used for bio-venting.

2000), sulfate was replenished (134 days) when the concentration of this anion fell to approximately 100 μM. The sulfate reduction rates in the COC-amended incubations were not significantly different before or after the sulfate replenishment or from the substrate-unamended control. Thus, the hydrocarbon addition did not inhibit background microbial activity. The average rate of sulfate loss for all incubations (excluding toluene) was 55.0 ± 8.0 μM day^{-1} (Fig. 2). Toluene, the positive control, stimulated sulfate reduction above the substrate-unamended control (Fig. 2).

The steady depletion of sulfate over the 250 days incubation suggested that other forms of organic matter were being consumed by the aquifer microflora. A purge and trap GC-MS analysis (Townsend *et al*., 2004; Prince and Suflita, 2007) of the residual hydrocarbons in non-sterile incubations was compared with those in sterile controls (Fig. 3) at the end of the experiment. Given the variation encountered in the sulfate depletion assays (above), quadruplicate incubations are depicted (Fig. 3). This determination revealed that *n*-alkanes, ranging from C_6 to C_{12}, were reduced or depleted by biodegradation in the aquifer material incubations. Similarly, significant losses were evident for toluene and *o*-xylene in all incubations, but there was no evidence for the removal of benzene or ethylbenzene. All but one of the four replicates showed a remarkably specific loss of 1,3-dimethyl and 1-ethyl-3-methylbenzenes (Fig. 3). Additional 1,3-disubstituted alkylbenzene biodegradation was noted with 1-methyl-3-propylbenzene but not 1-methyl-3-isopropylbenzene. Consistent with the sulfate reduction assay (above), there was no evidence for the anaerobic biodegradation of any of the COC in the incubations. Collectively, these findings argue that the anaerobic biodegradation of some of the endogenous hydrocarbons accounted for the background levels of sulfate consumption.

Table 1. Signature anaerobic metabolites of microbial hydrocarbon decay detected in groundwater monitoring wells as indicated by a closed circle (●).

Parent compound	Metabolite	Bkgd	436	437	438	439a	439b	440	442	443	BVT	9MW-3	345
	Alkylbenzylsuccinic acids												
Toluene	Benzylsuccinic acid					●							
Ethylbenzene	Ethylbenzylsucccinic acid												
m- or *o*-Xylene	Methylbenzylsuccinic acids				●	●	●		●		●		
p-Xylene				●	●	●	●	●	●		●		
C3 alkylbenzenes													
1,2,4-Trimethylbenzene	Dimethylbenzylsuccinic acids			●	●	●			●				
1,3,5-Trimethylbenzene				●			●	●	●				
1-Ethyl-3-methylbenzene				●	●	●	●						
Unassigned C3 alkylbenzenes*				●	●	●	●		●			●	
	Naphthoic acids												
Naphthalene or 2-methylnaphthalene	1- or 2-Napthoic acid									●			●
	Methylnaphthoic acid												●
	Dimethylnaphthoic acid												●
	1,2,3,4-Tetrahydro-2-naphthoic acid												
	5,6,7,8-Tetrahydro-2-naphthoic acid		●						●				●
	Unassigned tetrahydronaphthoic acids*		●	●	●			●	●	●	●	●	●
	Alkylsuccinic acids												
Alkanes (C_n)													
	C_5				●	●	●						
	C_6				●			●	●	●			
Unsaturated alkanes (C_{n-2})													
	C_5					●	●	●	●	●			
	C_6		●	●	●	●	●				●	●	
	C_7		●			●	●				●		
	C_8		●	●					●	●			
	C_9		●						●	●			

Monitoring well (MW) 439 was sampled in February (MW-439a) and October (MW-439b). * indicates matching mass spectral profiles only. BVT represents a well used for bio-venting.

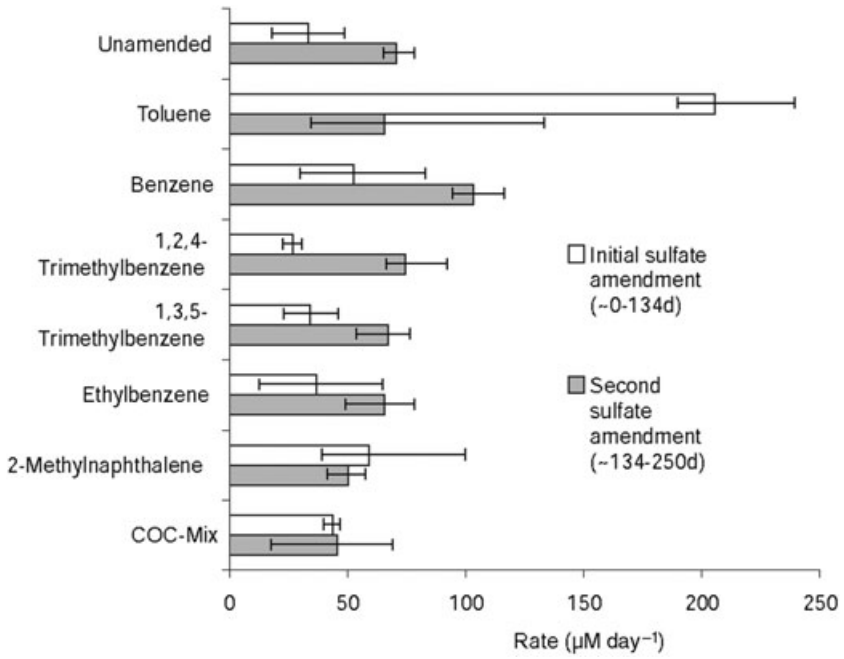

Fig. 2. Rates of sulfate reduction in aquifer material incubations. The average rate of sulfate reduction for the first sulfate amendment occurred at time zero until approximately 134 days (white bars). The second sulfate addition occurred at 134 days and average rates were calculated through 250 days (grey bars). Black marker bars demonstrate variability between samples, representing the highest and lowest rates of sulfate reduction observed among replicates.

General and specific biomass determination

We questioned if the chronic exposure of the resident microflora to hydrocarbons limited their population size and thus their ability to respond to the COC amendments. General biomass levels were determined by phospholipid fatty acids (PLFA) analysis on three samples that were COC-amended (benzene, 1,2,4-trimethylbenzene or 1,3,5-trimethylbenzene) and a fourth that was substrate-unamended. All samples harboured between 1.23×10^7 and 1.86×10^7 total microbial cells per gram (Table 2), and there was no significant difference in the levels of eukaryotes, sulfate-reducing bacteria, Proteobacteria, Firmicutes or general heterotrophs (not shown). The physiological status of the microflora in the samples was indicated by the ratio of *trans*- to *cis*- monoenoic fatty acids as well as the cyclopropyl fatty acids to their monoenoic precursors (White *et al*., 1996; Pinkart *et al*., 1997; White and Ringelberg 1997; Green and Scow, 2000). Gram-negative microorganisms typically increase their *trans*- fatty acids in the cell membrane when physiologically stressed with exposure to a toxic compound or starvation (Green and Scow, 2000). In the four samples analysed, the *trans*- to *cis*- ratios (Table 2) were in the range of 0.05–0.09 (16:1ω7t/16:1ω7c) and 0.13–0.16 (18:1ω7t/11:1ω7c). Ratios for *trans*- and *cis*- fatty acids of 0.1 indicate that the organisms are generally healthy and values in the range we observed are typical of contaminated sites (MacNaughton *et al*., 1999; Green and Scow, 2000). The ratios of cyclopropyl fatty acids/monoenoic precursors (Table 3) were < 1.0 (cy17:0/16:1ω7c) with the exception of the substrate-unamended sample (1.36), and 0.16–0.22 for cy19:/18:1ω7c. These values are typical of contaminated sites (Green and Scow, 2000), and do not suggest any dramatic toxic effects on the microbiota.

In addition, a molecular assay for the presence of *Desulfobacterium* cells was conducted as members of this

Table 2. Microbial biomass levels and community physiological status from *trans*- to *cis*- fatty acid ratios as well as cyclopropyl fatty acids to their monoenoic precursors in selected incubations as determined by PLFA analysis.

Sample	1	2	3	4
Amendment	Benzene	None	1,2,4-trimethylbenzene	1,3,5-trimethylbenzene
pmol PLFA ml^{-1}	929	659	720	613
Total biomass (cells g^{-1})	1.86×10^7	1.32×10^7	1.44×10^7	1.23×10^7
Physiological status				
cy17:0/16:1ω7c	0.65	1.36	0.72	0.69
cy19:0/18:1ω7c	0.22	0.22	0.19	0.16
16:1ω7t/16:1ω7c	0.05	0.09	0.07	0.06
18:1ω7t/18:1ω7c	0.15	0.13	0.16	0.15

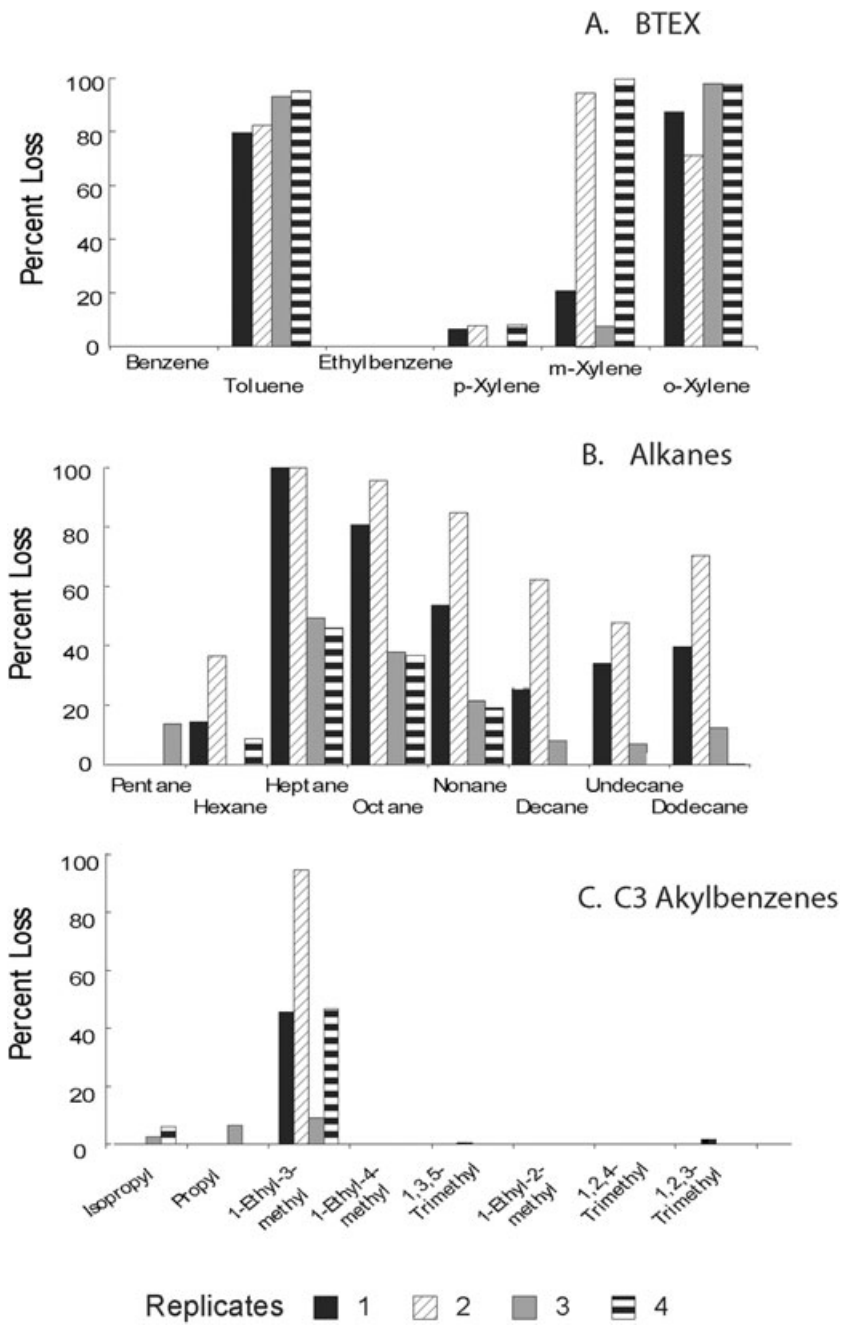

Fig. 3. Select hydrocarbon loss in substrate-unamended incubations by GC-MS analysis (after a 250 day incubation). Four replicates are represented for each analysis. Hydrocarbon loss other than COC is depicted as a percentage total loss.

genus (clone OR-M2, GenBank AY118142) have been implicated in anaerobic benzene metabolism (Da Silva and Alvarez, 2007). Real-time quantitative PCR analysis revealed relatively low copy numbers of the 16S rRNA gene sequence associated with this putative benzene-degrading sulfate-reducing bacterium (~1×10^3 cells per gram of soil). In fact, the concentration of benzene-degrading bacteria determined by this method was less than 1% of the total bacterial population in the sample and comparable to negative control samples in the reported assay (Da Silva and Alvarez, 2007).

Discussion

Field and laboratory evidence was used to assess the anaerobic intrinsic remediation of selected hydrocarbons in a petroleum-contaminated aquifer underlying a closed refinery. We have used the term metabolomics as we believe it to be conceptually consistent with existing definitions (Nicholson and Lindon, 2008). Typically, this approach seeks to characterize and quantify small metabolites in biological samples and is used mostly in comparative fashion – wild-type versus mutant, healthy

Table 3. Comparison of biodegradation indications based on field metabolomic profiling, endogenous hydrocarbon decay assays and exogenous COC-amended incubations.

Hydrocarbon	Field profiling	Endogenous activity	Hydrocarbon amendment
Benzene	○	○	○
Toluene (positive control)	●	●	●
Ethylbenzene	○	○	○
Xylene	●	●	NA
1,2,4-Trimethylbenzene	●	○	○
1,3,5-Trimethylbenzene	●	○	○
1-Ethyl-3-methylbenzene	●	●	NA
2-Methylnaphthalene	●	○	○
Various alkanes (saturated)	●	●	NA
Various alkanes (unsaturated)	●	–	NA

Closed circles (●) reflect positive indications of biodegradation, while open circles (○) indicate that no activity was evident. Toluene served as a positive control and degradation was observed with all procedures. No amendment (NA) indicates that non-COC hydrocarbons were not specifically evaluated by exogenous addition.

versus diseased organism, etc. We have extended this approach to the ecosystem level and compared a contaminant-free background aquifer to areas of the same aquifer impacted by spilt hydrocarbons. The detection of signature metabolites in groundwater samples is compelling evidence that hydrocarbon biodegradation was an ongoing process. Moreover, the identification of the metabolites implicates the parent hydrocarbons undergoing biotransformation (Elshahed *et al.*, 2001; Gieg and Suflita, 2002; Wilkes *et al.*, 2003; Gieg and Suflita, 2005). We detected a variety of putative metabolites at multiple locations in contaminated portions of the aquifer, but none was found in the background well (Table 1). Based on the identity of the metabolites, we infer that several alkylbenzenes, naphthalenes, polynuclear aromatic compounds, alkanes and potentially alicyclic hydrocarbons were undergoing anaerobic biodegradation in the shallow aquifer on both sides of the river. Most metabolite signatures were found on the south side of the river, the area where refinery operations were centred. The lack of alkylsuccinic acid metabolites in water samples taken from the north side of the river probably reflects the different hydrocarbons present in that area, the differential attenuation of alkane/alicyclic hydrocarbons or both. In one well, an almost identical suite of metabolites was detected at multiple samplings over different times of the year (Table 1, MW-439a, MW-439b), suggesting that seasonal fluctuations did not substantially influence ongoing microbial metabolism in the aquifer.

There was general agreement between the field metabolic profiling and the loss (or recalcitrance) of the parent hydrocarbons in laboratory incubations (Table 3). Nevertheless, there were also notable differences. For instance, the alkylsuccinate metabolites detected in the field suggested that pentane and hexane were biodegraded, while the alkane loss patterns in laboratory biodegradation assays showed that higher-molecular-weight hydrocarbons (i.e. C_7–C_{12}) were preferentially utilized by the aquifer microflora (Fig. 3). Several reasons can be advanced to account for these observations. First, the lack of consistent pentane or hexane loss indications in the laboratory assay most likely reflects the analytical limit of the purge and trap analysis. Second, if the lower-molecular-weight hydrocarbons were preferentially utilized by the microflora relative to the higher-molecular-weight *n*-alkanes, a steady re-supply of the former from the NAPL known to be in the aquifer may differentially impact metabolism of the latter compounds. However, as the C_5–C_6 hydrocarbons get depleted from the laboratory incubations, the higher-molecular-weight *n*-alkanes could then be more amenable to microbial attack.

Similarly, while field and laboratory assays suggest that the COC were largely resistant to anaerobic biodegradation there are also subtle differences. Metabolites associated with the anaerobic biodegradation of benzene, ethylbenzene and 2-methylnaphthalene have been reported in a number of studies (Rabus and Heider, 1998; Caldwell and Suflita, 2000; Kniemeyer *et al.*, 2003; Meckenstock *et al.*, 2004a; Ulrich *et al.*, 2005; Safinowski and Meckenstock, 2006; Safinowski *et al.*, 2006). The benzene metabolites benzoate and phenol were not found in this study, but benzylsuccinic acid was detected in at least one well (Table 1), implying that toluene was anaerobically metabolized. This conclusion was supported when toluene was used as a positive control in the laboratory biodegradation assays (Fig. 2). Recent evidence suggests that toluene may be a putative metabolite in anaerobic benzene degradation (Ulrich *et al.*, 2005). However, as toluene is a frequent component in petroleum, its detection cannot be reliably construed as evidence for benzene metabolism.

The detection of dimethylbenzylsuccinic acids (or isomeric counterparts) in most of the wells (Table 1) suggests that C_3-alkylbenzenes were anaerobically transformed in the aquifer. To see if these signals could be attributed to the metabolism of 1,3,5- or 1,2,4-trimethylbenzene COC, we synthesized the corresponding fumarate addition products. The same was done for 1-ethyl-3-methylbenzene as it was the only C_3-alkylbenzene implicated in the laboratory biodegradation assay. Gas chromatographic analysis revealed a characteristic suite of peak(s) with identical mass spectral profiles (Fig. S1). Comparison of the GC-MS characteristics of the synthesized standards to the field metabolites allowed us to confirm that the two COC as well as 1-ethyl-3-methylbenzene were metabolized in the field. However, only the latter could be confirmed in the laboratory assay (Fig. 3). Following the synthesis of the authentic C_3-alkylbenzylsuccinic acids, we were able to positively identify a putative fumarate addition metabolite resulting

from the degradation of 1,2,4-trimethylbenzene in MW-439 during the first, but not the second sampling, while the opposite temporal appearance of a fumarate-addition metabolite was observed for 1,3,5-trimethylbenzene. Those peaks showing the same mass spectral profile but different GC retention times that could not be matched to 1,2,4- and 1,3,5-trimethylbenzene or 1-ethyl-3-methylbenzene were identified as unassigned C_3-alkylbenzylsuccinates.

Of course, failure to detect a metabolite does not necessarily mean that the parent substrate is recalcitrant. However, the general lack of COC metabolism was also confirmed by the laboratory biodegradation assays. These compounds were added at 50 p.p.m. C (500–600 μM) so that their anaerobic biodegradation would easily be manifest by an increased level of electron acceptor consumption relative to the substrate-unamended controls. However, none of the COC amendments or the COC mixture stimulated sulfate reduction above the COC-free controls (Fig. 2). The rate of sulfate loss, while variable, was not substantially different in any of the incubations, suggesting that the addition of the COC amendments was not inhibitory to the resident microflora.

The background sulfate respiration in all incubations suggested that some form of organic matter other than the COC was being metabolized over the course of the experiment. This was confirmed by purge and trap GC showing that a variety of alkanes and alkylated aromatic hydrocarbons were depleted from COC-unamended incubations (Fig. 3). This result suggests that there may be some preferential utilization of hydrocarbons at the former refinery site and that the COC, while inherently amenable to anaerobic intrinsic remediation, may simply not be attacked in deference to other substrates.

Mechanistically, the lack of COC biodegradation may be a result of metabolic interference by other compounds in the complex hydrocarbon mixture in the aquifer. Previous studies have demonstrated a significant lag or inhibition in the degradation of benzene, toluene, ethylbenzene or xylene when one or more of those hydrocarbons were added to laboratory incubations degrading a single BTEX component under a variety of electron-accepting conditions (Evans *et al*., 1991; Edwards and Grbic-Galic, 1994; Meckenstock *et al*., 2004b). It has also been shown that once degradation occurred, the microbial enrichments could be inhibited by the presence of other organic acids and alcohols (Edwards and Grbic-Galic, 1994), or co-metabolites as demonstrated with polycyclic aromatic hydrocarbons (Safinowski *et al*., 2006). Thus, the incomplete biodegradation of various hydrocarbons or the presence of their metabolic breakdown products could potentially interfere with the biodegradation of the COC.

This point notwithstanding, the biomass levels measured in COC-amended and -unamended samples (Table 2) were comparable to what has been found in hydrocarbon-contaminated aquifers (Green and Scow, 2000), and not significantly different at the end of the experiment. The physiological status of the organisms in this study suggests the Gram-negative microorganisms may be slightly stressed, presumably by the hydrocarbon contamination. However, laboratory incubations have also been shown to elicit similar stress responses (Green and Scow, 2000). The results of the PLFA analyses suggest there were active microbial populations in the samples and that the COC amendments were unlikely to be more inhibitory than the background hydrocarbons.

Yet, another possible reason for the lack of significant anaerobic biodegradation of the COC could be low numbers or the inherent lack of specific types of catalytic microorganisms. Consistent with the relative recalcitrance of benzene in the biodegradation assays, we observed low copy numbers of the 16S rRNA gene representing *Desulfobacterium* sp. clone OR-M2. While we recognize that our assay was narrow, we focused on this organism because it has specifically been implicated in the biodegradation of benzene under sulfidogenic and methanogenic conditions (Ulrich and Edwards, 2003; Da Silva and Alvarez, 2007) and molecular probes for its quantification were available. However, these probes are unlikely to cross-react with more distantly related *Desulfobacteriacea,* including two other clones, SB-21 and BznS295, obtained from marine benzene-degrading, sulfate-reducing bacterial enrichments (Phelps *et al*., 1998; Musat and Widdel, 2008). To our knowledge, there are no known pure cultures capable of benzene metabolism under comparable anaerobic conditions, but such organisms clearly exist as anaerobic metabolism of this contaminant has been documented under a variety of terminal electron-accepting conditions. Recently, a nitrate-reducing, benzene-degrading *Azoarcus* was isolated from a BTX-contaminated aquifer (Kasai *et al*., 2006). However, nitrate is not known to be an important potential electron acceptor in the aquifer and when nitrate was used as an experimental variable in our experiments, not only was benzene loss not evident, but background hydrocarbon loss also ceased (data not shown).

In summary, we observed excellent corroboration between field and laboratory findings (Table 3) in terms of both the types of hydrocarbons that were subject to intrinsic remediation and those that were not. The COC, while known to be susceptible to anaerobic biodegradation in other systems, proved largely recalcitrant in laboratory assays and positive field indications, while present, were transitory. The study suggests that the extrapolation of anaerobic biodegradation information from one site to another must be made with caution, and whenever possible, existing literature information must be supplemented with direct experimental evidence. Based on our current findings, we would not predict that anaerobic

biodegradation of COC will contribute significantly to natural attenuation at the site.

Experimental procedures

Field description

The former oil refinery is located south of the North Platte River in Casper, WY (Fig. 1). The site is underlain by shale bedrock approximately 9–12 m below the ground surface. The subsurface is composed of highly permeable Quaternary alluvium of medium to coarse sand, with some gravel and cobble, while the surface is dominated by silt and clay. The water table is located between 1.5 and 4.5 m below the surface and hydraulic conductivity is between 61–106 m day^{-1}. Petroleum releases into the subsurface included fuel gas, liquid propane gas, motor/aviation gasoline, fluid cracking unit coke, heavy fuel oil, kerosene and distillates, asphalt and other residual components that resulted in a relatively uniform distribution of non-aqueous phase liquid over approximately 90% of the site. Residual hydrocarbons spanned depths of 0.6 m above to 1.2 m below the water table surface.

The hydrocarbon plume migrated with the prevailing groundwater flow towards the river (Fig. 1). Over 460 000 m^3 of contaminated sediment and more than 50 million litres of non-aqueous phase liquid has been removed to date. A > 2600 m sheet pile wall was installed on the south bank of the river as a contaminant migration barrier. Monitoring wells were selected along the groundwater flow for sampling purposes (Fig. 1).

Historical groundwater geochemistry indicated that the steady-state dissolved hydrogen values in plume areas were 1–2 nM and that sulfate concentrations decreased from 25 mM at the southern edge of the site to 3–4 mM along transects (Fig. 1). These data suggested that sulfate reduction was a dominant terminal electron-accepting process at the site.

Metabolite profiling

Groundwater samples were collected from hydrocarbon-impacted and background wells in February and October 2005 and analysed for signature anaerobic metabolites as previously described (Elshahed *et al.*, 2001; Gieg and Suflita, 2002; 2005). The collected groundwater was immediately acidified in the field with 50% HCl to a pH < 2 and kept at 4°C until analysed. Groundwater samples (1 l) were extracted with ethyl acetate, dried over anhydrous Na_2SO_4, concentrated by rotary evaporation under a flow of N_2, derivatized with *N,O*-bis(trimethylsilyl)trifluoroacetamide (Pierce Chemical Co., Rockford, IL) and analysed by GC-MS (Elshahed *et al.*, 2001). Anaerobic metabolites were identified using derivatized authentic standards (Elshahed *et al.*, 2001; Gieg and Suflita, 2002; 2005).

To resolve the alkylbenzylsuccinate isomers,1,2,4-, 1,3,5-trimethylbenzene and 1-ethyl-3-methylbenzene were synthesized and similarly derivatized as before (Elshahed *et al.*, 2001). Increased chromatographic resolution was achieved by using a different column (DB-5ms 20 m × 0.18 mm i.d., 0.18 μm film, Agilent Technologies, Foster City, CA) and the GC oven temperature was held at 45°C (5 min) then increased to 140°C (20°C min^{-1}), then to 180°C (1°C min^{-1}), to a final temperature of 270°C (30°C min^{-1}).

Laboratory assessments

Sediments and groundwater were collected from a single location at the site (MW-439, Fig. 1) to construct aquifer material incubations. Approximately 50 ± 1.0 g of sediment collected from a depth of 6 m (within the hydrocarbon smear zone) and 75 ± 1.0 ml of groundwater were dispensed into sterile 160 ml serum bottles in an anaerobic chamber (5% H_2 in N_2). Groundwater was reduced prior to use with Na_2S (0.005%) and amended with resazurin (0.001%) as a redox indicator (Townsend *et al.*, 2003). The serum bottles were closed with butyl rubber stoppers, crimped with aluminum seals and given an N_2 headspace. The aquifer material incubations were stored in the dark at room temperature for approximately 30 days to equilibrate and to allow for the removal of endogenous levels of electron acceptors before being amended with COC, sulfate or other treatments as indicated.

Hydrocarbon and sulfate analyses

Substrate-amended incubations received 500–600 μM (50 p.p.m. carbon) of benzene, ethylbenzene, 1,2,4-trimethylbenzene, 1,3,5-trimethylbenzene, 2-methylnaphthalene or a mix of these compounds. Toluene (50 p.p.m. carbon) was added as a positive control. All substrates were added to the incubations as neat compounds with the exception of 2-methylnaphthalene that was dissolved in methanol. Heat-killed controls were autoclaved for 20 min on three consecutive days prior to substrate addition. For most laboratory incubations, hydrocarbon loss was monitored by headspace GC analysis [50 μl, 45°C (5 min), to 90°C (4°C min^{-1})]. Utilization of 2-methylnaphthalene was analysed by HPLC equipped with a reversed-phase C_{18} column (250 mm 4.6 mm, 5 μm particle size; Alltech, Deerfield, IL) and a UV detector (260 nm). The mobile phase (1 ml min^{-1}) consisted of acetonitrile : phosphoric acid (70:30). Losses of non-COC hydrocarbons from several incubations was assessed by purge and trap GC-MS as previously reported with 2,2,4-trimethylpentane as the conserved internal marker (Townsend *et al.*, 2004; Prince and Suflita, 2007).

Sodium sulfate (4 mM) was added to the aquifer slurries at the start of the experiment and re-amended when levels were at or below 100 μM. Initial concentrations were selected to mimic *in situ* sulfate levels. Sulfate reduction activity was quantified by ion chromatography (DX-500, AS4A anion exchange column, DIONEX, Sunnyvale, CA) as previously described (Townsend *et al.*, 2003).

Total and benzene-degrading biomass

Following 250 days, 60 ml samples were analysed for total biomass by PLFA determination (Microbial Insights, Rockford, TN). DNA extracted from microcosm sediment (1 ± 0.5 g) was used to detect and quantify total bacteria 16S rRNA and bacteria that are closely associated with benzene degradation under strongly anaerobic (methanogenic and sulfidogenic conditions) as described by Da Silva and Alvarez (2007).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. The fumarate addition product(s) of 1,2,4-trimethylbenzene, 1,3,5-trimethylbenzene or 1-ethyl-3-methylbenzene were synthesized and exhibited similar mass spectral profiles when derivatized (TMS) and analysed as indicated in the text (A). The resulting isomers could be distinguished based on the number of products and their respective gas chromatographic retention times (B). Peaks associated with the fumarate-addition product of 1,2,4-trimethylbenzene (black arrows), 1,3,5-trimethylbenzene (white arrow) and 1-ethyl-3-methylbenzene (grey arrows) are based on assignments made with individual C3-alkylbenzene parent compounds.

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