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Assessing *in situ* rates of anaerobic hydrocarbon bioremediation

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Summary

Identifying metabolites associated with anaerobic hydrocarbon biodegradation is a reliable way to garner evidence for the intrinsic bioremediation of problem contaminants. While such metabolites have been detected at numerous sites, the *in situ* rates of anaerobic hydrocarbon decay remain largely unknown. Yet, realistic rate information is critical for predicting how long individual contaminants will persist and remain environmental threats. Here, single-well push–pull tests were conducted at two fuel-contaminated aquifers to determine the *in situ* biotransformation rates of a suite of hydrocarbons added as deuterated surrogates, including toluene-d_8, *o*-xylene-d_{10}, *m*-xylene-d_{10}, ethylbenzene-d_5 (or -d_{10}), 1, 2, 4-trimethylbenzene-d_{12}, 1, 3, 5-trimethylbenzene-d_{12}, methylcyclohexane-d_{14} and *n*-hexane-d_{14}. The formation of deuterated fumarate addition and downstream metabolites was quantified and found to be somewhat variable among wells in each aquifer, but generally within an order of magnitude. Deuterated metabolites formed in one aquifer at rates that ranged from 3 to 50 μg l^{-1} day^{-1}, while the comparable rates at another aquifer were slower and ranged from 0.03 to 15 μg l^{-1} day^{-1}. An important observation was that the deuterated hydrocarbon surrogates were metabolized *in situ* within hours or days at both sites, in contrast to many laboratory findings suggesting that long lag periods of weeks to months before the onset of anaerobic biodegradation are typical. It seems clear that highly reduced conditions are not detrimental to the intrinsic bioremediation of fuel-contaminated aquifers.

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Introduction

Intrinsic bioremediation of hydrocarbon-contaminated environments is now more accepted as a cost-effective site management strategy (NRC, 1993; Beller, 2000; Stenuit *et al*., 2008). This is due to the increased understanding of the relevant physical, chemical and biological processes that contribute to the attenuation of contaminants and to the development of effective tools to assess and monitor *in situ* mitigation efforts (Griebler *et al*., 2004; Beller *et al*., 2008). It is well known that aquifers contaminated with fuel rapidly become anaerobic, so the natural attenuation of the component hydrocarbons must rely on the metabolic capabilities of the indigenous anaerobic microorganisms (e.g. Foght, 2008). Research in the last 20 years indicates that a variety of petroleum hydrocarbons, including monoaromatic, polycyclic aromatic (PAH), alicyclic and aliphatic hydrocarbons can be biodegraded by anaerobic microorganisms via a growing list of novel enzymatic mechanisms (e.g. Widdel *et al*., 2006; Heider, 2007; Foght, 2008). Such investigations have yielded useful approaches for obtaining convincing evidence for the anaerobic *in situ* biodegradation of hydrocarbons, including the detection of unambiguous signature microbial metabolites (Beller *et al*., 1995; Beller, 2000; Gieg and Suflita, 2002).

An important mechanism used by anaerobes to activate hydrocarbons involves the addition of the parent substrate across the double bond of fumarate (Fig. 1). Fumarate is a central metabolic C_4 dicarboxylic acid that is added to a hydrocarbon via a glycl radical enzyme (e.g. Widdel *et al*., 2006). This process was initially demonstrated with toluene-degrading denitrifying bacteria and resulted in the transient formation of benzylsuccinic acid (Fig. 1A; Biegert *et al*., 1996; Beller and Spormann, 1997). Subsequent work demonstrated that other alkylbenzenes (e.g. Fig. 1B and C), aliphatic and alicyclic alkanes (e.g. Fig. 1E and F), and PAHs may be degraded via fumarate addition reactions to form specific succinate metabolites

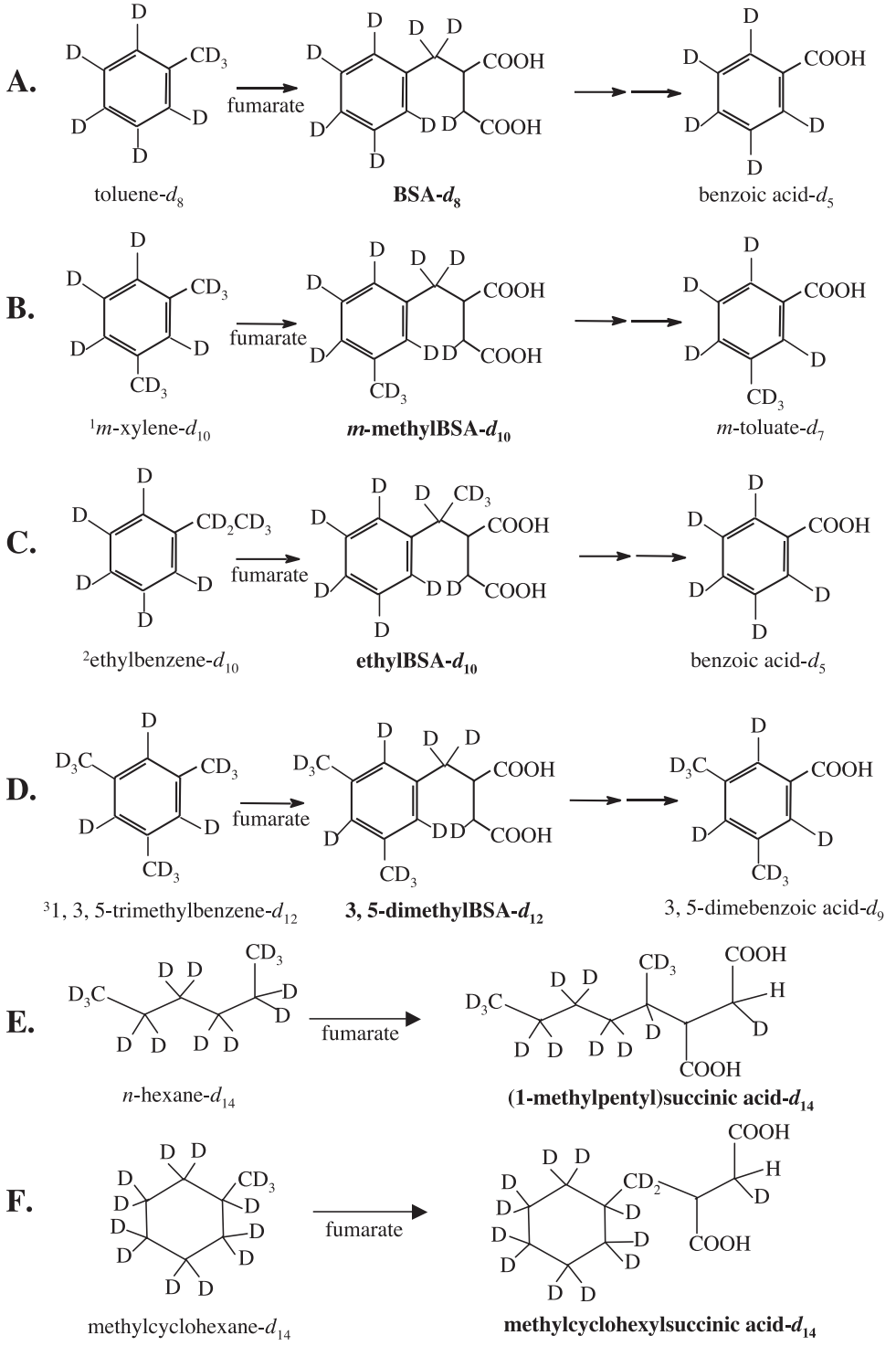

Fig. 1. Deuterated hydrocarbons used in this study and their corresponding fumarate addition and downstream metabolites assayed in push–pull tests by LC-MS-MS and/or GC-MS to elucidate rates of *in situ* hydrocarbon biotransformation. [1]Analogous pathway for the *o*-xylene-d_8 isomer. [2]Ethylbenzene-d_{10} was used in the Ft. Lupton tests, whereas ethylbenzene-d_5 was used in the Hickam tests. [3]Analogous pathway for the 1, 2, 4-TMB-d_{12} isomer.

(Krieger *et al*., 1999; Annweiler *et al*., 2000; Kropp *et al*., 2000; Elshahed *et al*., 2001; Rabus *et al*., 2001; Kniemeyer *et al*., 2003; Rios-Hernandez *et al*., 2003; Wilkes *et al*., 2003). Beller and colleagues (1995) initially proposed that the fumarate addition metabolites be used as signature indicators of *in situ* anaerobic hydrocarbon decay and they detected the respective products from toluene, *o*-xylene and *m*-xylene in a contaminated aquifer. Subsequent testing showed that the substrate-specific fumarate addition metabolites from these and other alkylbenzenes, alkanes and PAHs could be detected *in situ* at a variety of geologically and geographically diverse fuel-contaminated sites (Beller, 2000; Gieg and Suflita, 2002; Ohlenbusch *et al*., 2002; Martus and Püttman, 2003; Griebler *et al*., 2004; Ledin *et al*., 2005; McKelvie *et al*., 2005; Young and Phelps, 2005; Safinowski *et al*., 2006; Beller *et al*., 2008). Downstream metabolites from the fumarate addition intermediates (such as benzoate from toluene or toluates from xylenes) can also be detected in some aquifers (e.g. Cozzarelli *et al*., 1995; Gieg *et al*., 1999; Griebler *et al*., 2004). Despite their widespread detection, the *in situ* rates of anaerobic hydrocarbon metabolite formation are poorly understood. Lag periods of weeks to months prior to the onset of anaerobic hydrocarbon metabolism are commonly associated with laboratory experiments and suggest that field rates may be correspondingly slow (Edwards and Grbic'-Galic', 1994; Gieg *et al*., 1999). Rate information is crucial for accurate predictions of how long contaminants will remain environmental threats when relying on intrinsic bioremediation as a site management strategy.

One technique that can be used to quantitatively evaluate *in situ* microbial activities in aquifers is the single-well push–pull test (Istok *et al*., 1997). In this procedure, reactants of interest and a conservative tracer are mixed with water and injected, or 'pushed' into an aquifer using an existing well. Samples are withdrawn or 'pulled' from the same well and analyte concentrations are interpreted relative to the conservative tracer. Breakthrough curves for reactants, products and the tracer measured during the extraction phase are then used to calculate reaction rates for a given biotransformation process (Istok *et al*., 1997). Push–pull tests have been used most frequently to evaluate *in situ* respiration processes (Trudell *et al*., 1986; Istok *et al*., 1997; Haggerty *et al*., 1998; Kleikemper *et al*., 2002; Harris *et al*., 2006), reductive dechlorination (Hageman *et al*., 2001; 2004; Kim *et al*., 2006; 2008; Azizian *et al*., 2007) and microbial processes at heavy metal-contaminated sites (Senko *et al*., 2002; Istok *et al*., 2004; North *et al*., 2004). Only a handful of studies have evaluated the loss of injected hydrocarbons *in situ* using push–pull tests (Thierrin *et al*., 1993; Reinhard *et al*., 1997; 2005), but it is often unclear how much of the observed attenuation is actually due to biodegradation versus non-biological processes. To address this uncertainty, Reusser and colleagues (2002) used stable isotope labelled (deuterated) hydrocarbons in push–pull tests to evaluate their *in situ* biotransformation to the corresponding deuterated fumarate addition metabolites. The use of isotopically-heavy surrogates offers the advantage of detecting *in situ* hydrocarbon biotransformation reactions against a high background contaminant concentration and the labelled metabolites can unequivocally be attributed to biological processes. Indeed, Reusser and colleagues (2002) were able to measure the formation of the expected deuterated fumarate addition metabolites from toluene-d_8 and *o*-xylene-d_{10}, with formation rates ranging from 1 to 7 nM day^{-1} (approximately 0.2–1.6 μg l^{-1} day^{-1}). Since 1 mol of the parent hydrocarbon is converted to 1 mol of the corresponding fumarate addition metabolite, rates of formation conservatively reflect the rates of parent substrate decay.

We sought to assess whether these rates were generalizing to other fuel-contaminated aquifers by examining these same hydrocarbons (toluene-d_8 and *o*-xylene-d_{10}) along with an expanded suite of other hydrocarbons of varying water solubilities. In addition to measuring fumarate addition metabolite formation (Fig. 1), we assayed for additional downstream metabolites (e.g. toluates from xylenes, Fig. 1B) that might also be used to bolster evidence for the intrinsic rate of *in situ* hydrocarbon bioremediation. Push–pull tests were conducted at: (i) a well-studied gas condensate-contaminated aquifer located near Ft. Lupton, CO, USA (referred to as the Ft. Lupton site) where intrinsic bioremediation was previously documented (Gieg *et al*., 1999) and (ii) at a jet fuel-contaminated aquifer underlying Hickam Air Force Base, HI, USA (referred to as the Hickam site) where anaerobic hydrocarbon decay was suspected based on geochemical evidence (Parsons, 2005). Details about these sites are outlined in the *Experimental procedures*. We found that most of the deuterated test hydrocarbons were transiently converted to the expected isotopically-labelled fumarate addition and downstream metabolites within days at both sites and rate information could be reliably determined.

Results

Laboratory incubations and the formation of deuterated metabolites

Sediments from the gas condensate-contaminated Ft. Lupton site were incubated with a suite of deuterated hydrocarbons under sulfate-reducing conditions in the laboratory in order to determine whether deuterated fumarate addition metabolites would form (Fig. 1). Our goal was to biologically generate deuterated hydrocarbon metabolites that could serve as authentic standards for

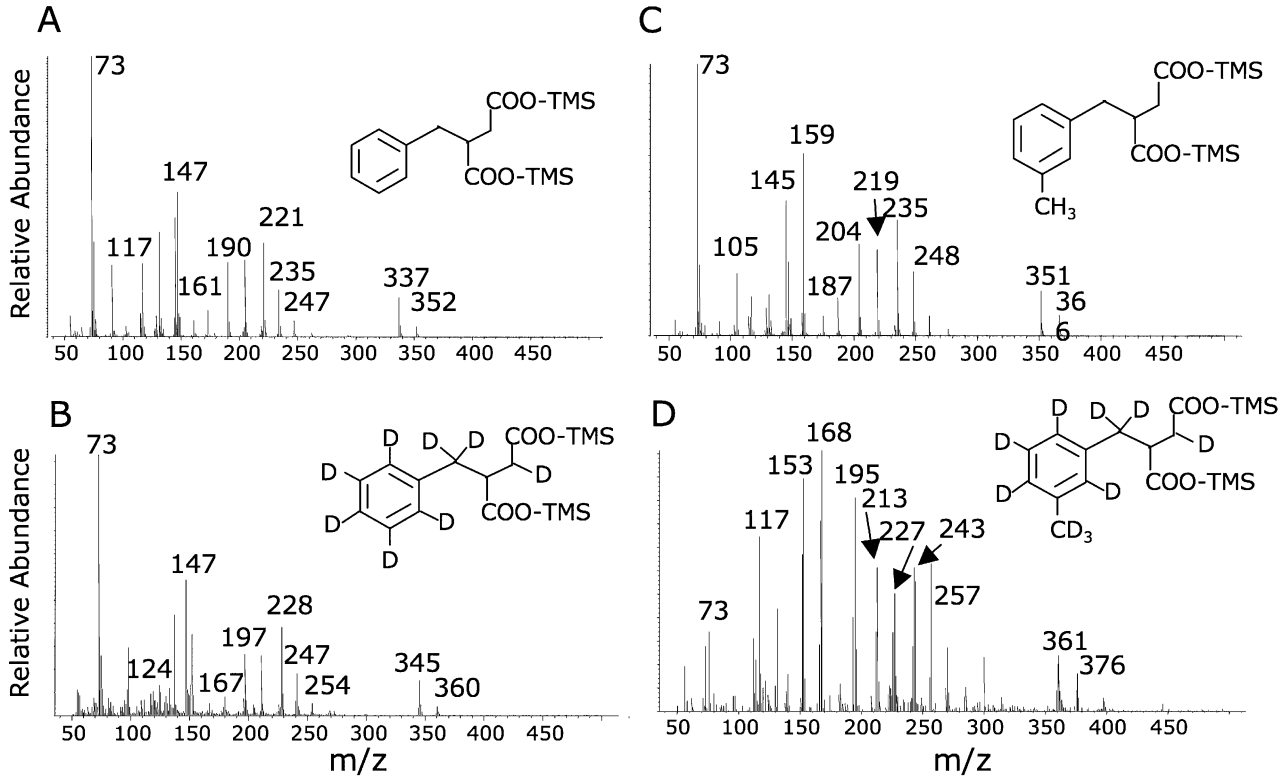

Fig. 2. Mass spectral profiles of (A) authentic benzylsuccinic acid, (B) deuterated (d_8) fumarate addition metabolite produced during the anaerobic decomposition of toluene-d_8 in laboratory incubations, (C) authentic *m*-methylbenzylsuccinic acid and (D) deuterated (d_{10}) fumarate addition metabolite produced during the anaerobic degradation of *m*-xylene-d_{10} in laboratory enrichments. Mass spectra and structures shown are of the trimethylsilylated derivatives analysed by GC-MS.

analytical comparisons with deuterated metabolites formed in field push–pull tests. We found that anaerobic microorganisms from the Ft. Lupton site could biodegrade the fully deuterated surrogates of toluene, *o*-xylene, *m*-xylene, 1, 2, 4-trimethylbenzene (1, 2, 4-TMB), 1, 3, 5-trimethylbenzene (1, 3, 5-TMB) and methylcyclohexane under sulfate-reducing conditions in laboratory incubations (data not shown). Evidence for biodegradation included concomitant parent substrate loss and sulfate consumption in stoichiometrically expected amounts, confirming previous observations (Elshahed *et al*., 2001). In the same incubations, we identified several deuterated metabolites indicative of the anaerobic biotransformation of the deuterated parent hydrocarbons (Fig. 1) using gas chromatography-mass spectrometry (GC-MS). We detected the formation of benzylsuccinate (BSA)-d_8 from toluene-d_8 and *m*-methylBSA-d_{10} from *m*-xylene-d_{10} based on comparisons with the GC-MS characteristics of unlabelled authentic standards wherein the mass ion and other fragment ions were shifted up by 8 or 10 mass units respectively (Fig. 2A–D). Benzoate-d_5 (from toluene-d_8) and *m*-toluate-d_7 (from *m*-xylene-d_{10}) were also detected (not shown). In the 1, 3, 5-TMB-d_{12}-amended incubations, we identified the predicted fumarate addition metabolite 3, 5-dimethylBSA-d_{12} as well as the downstream metabolite 3, 5-dimethylbenzoate-d_9 (Fig. 1D) by GC-MS. The MS profiles of unlabelled synthesized 3, 5-dimethylBSA and culture-generated 3, 5-dimethylBSA-d_{12} are compared in Fig. 3A and B, showing a shift upwards of 12 units in the resulting mass fragment ion for the d_{12}-labelled metabolite. Figure 3C further shows the MS of the downstream metabolite 3, 5-dimethylbenzoate-d_9 detected in the 1, 3, 5-TMB-d_{12}-degrading enrichment. From *o*-xylene-d_{10}- and 1, 2, 4-TMB-d_{12}-amended incubations, we could only detect the corresponding deuterated methyl- and dimethylbenzoates respectively (not shown). In the methylcyclohexane-d_{14}-amended incubations, the corresponding deuterated fumarate addition metabolite was tentatively identified (Fig. S1) by comparison with the mass spectral features of deuterated hexyl- and octylsuccinic acid (Gieg and Suflita, 2002). These culture-generated compounds were used as authentic standards to compare with deuterated metabolites formed during the field push–pull tests. Surprisingly, ethylbenzene-d_{10} was not metabolized in the laboratory incubations, although we previously observed its biodegradation and conver-

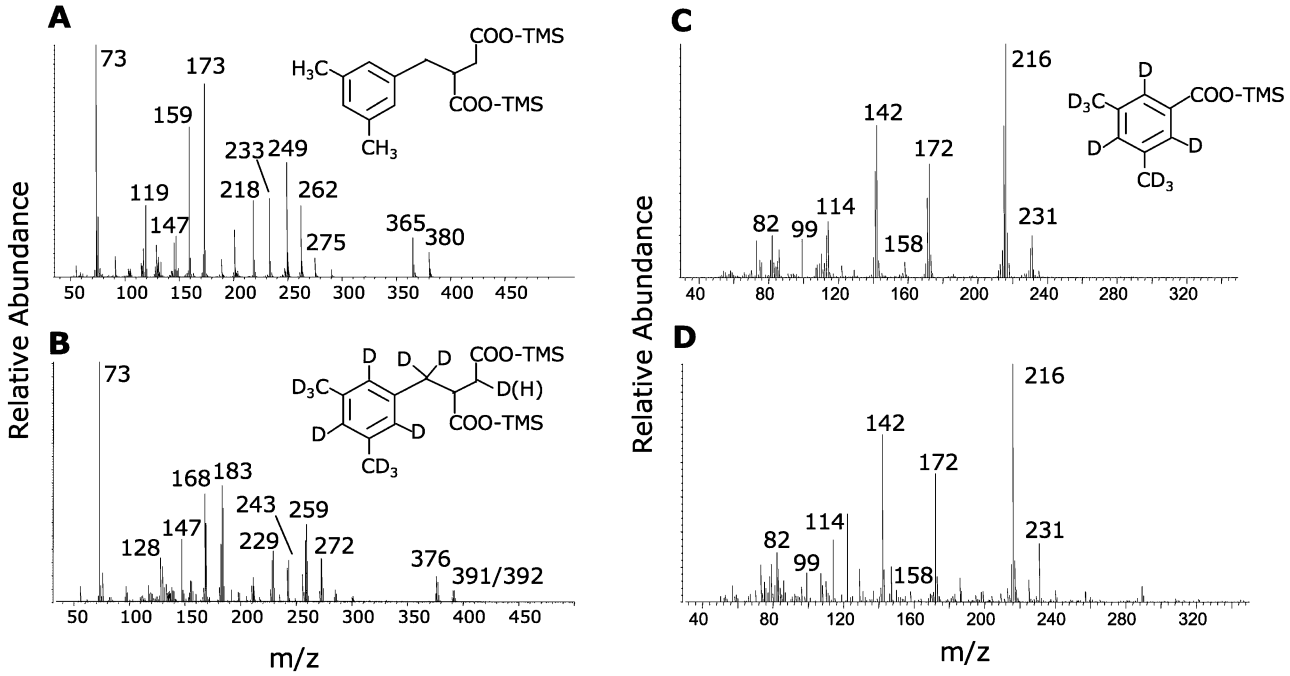

Fig. 3. Mass spectral profiles of (A) synthesized standard of 3, 5-dimethylBSA, (B) fumarate addition metabolite produced during the anaerobic degradation of 1, 3, 5-TMB-d_{12} in laboratory incubations, (C) 3, 5-dimethylbenzoate-d_9 produced during the anaerobic degradation of 1, 3, 5-TMB-d_{12} in laboratory enrichments and (D) metabolite detected during push–pull studies with deuterated hydrocarbons conducted at the Hickam site. Mass spectra and structures shown are of the trimethylsilylated derivatives analysed by GC-MS.

sion to the corresponding fumarate addition product using inoculum from the same aquifer (Gieg *et al*., 1999; Elshahed *et al*., 2001).

Unlabelled metabolites in the field

Prior to conducting push–pull tests, multiple groundwater samples were assayed for the presence of putative anaerobic hydrocarbon metabolites by GC-MS (Gieg and Suflita, 2002). At the Ft. Lupton site, the detection of fumarate addition metabolites associated with alkylbenzenes (*m*-xylene, *p*-xylene, ethylbenzene) and alkanes confirmed previous findings (Elshahed *et al*., 2001; Gieg and Suflita, 2002) and indicated that anaerobic hydrocarbon biodegradation was an ongoing process. Further, a fumarate addition metabolite was detected in several wells that matched the GC-MS characteristics of an authentic standard of 3, 5-dimethylBSA (Fig. 3A), indicating the *in situ* anaerobic biodegradation of 1, 3, 5-TMB at this site. The positive identification of 3, 5-dimethylbenzoate, a predicted downstream metabolite (Fig. 1D), also supported this observation (not shown). Numerous signature anaerobic metabolites were also identified at the Hickam site where anaerobic hydrocarbon biodegradation was inferred based largely on electron acceptor depletion data (Parsons, 2005). The putative metabolites included BSA, ethylBSA, *m*-methylBSA and 3, 5-dimethylBSA (Fig. 1; MS profiles as shown in Figs 2 and 3), at concentrations ranging from 4 to 20 μg l^{-1}, providing evidence that *in situ* anaerobic hydrocarbon biodegradation was also occurring at the Hickam site. Additional metabolites, such as benzoate, all toluate isomers and 3, 5-dimethylbenzoate (Fig. 1), were also found in a number of wells at concentrations of up to ~7 μg l^{-1}. In addition, a variety of C_5 to C_9 alkylsuccinates were identified in groundwater samples from this location, including (1-methylpentyl)succinate (from anaerobic *n*-hexane degradation, Rabus *et al*., 2001; Gieg and Suflita 2002) and a cyclic C_7-succinate, a putative metabolite of methylcyclohexane (Gieg and Suflita, 2002).

Field push–pull tests

Push–pull tests to assess the anaerobic biotransformation rates of toluene-d_8, ethylbenzene-d_{10}, *o*-xylene-d_{10}, *m*-xylene-d_{10}, 1, 2, 4-TMB-d_{12} and 1, 3, 5-TMB-d_{12} were conducted at seven locations within the contaminant plume at the Ft. Lupton site (numbered Tests 1–7). In four of these tests, the bromide tracer concentrations fell below detection within 3 days of the injection phase. As bromide serves to indicate the residence time of the injectate in the well and is used to account for dilution effects of all measured analytes, its detection is necessary in order to calculate *in situ* rates of hydrocarbon transformation. Bromide concentrations were measurable in the

Table 1. Production rates (μg l^{-1} day^{-1}) of deuterated metabolites in successful push–pull tests carried out at the Ft. Lupton and Hickam sites.

		Fumarate addition metabolites					Downstream metabolites		
Site	Test#	BSA-d_8	*o*-Me BSA-d_{10}	*m*-Me BSA-d_{10}	Ethyl-BSA-d_x[a]	3,5-diMe BSA-d_{12}	*o*-toluate-d_7	*m*-toluate-d_7	bzt-d_5
Ft. Lupton	Test 2	19.1	7.2	9.2	9.1	det	159.4[b]	28.3	22.8
	Test 5	50.2	*nd*	28.9	365.6[b]	*nd*	29.2	39.4	31.2
	Test 7	*nd*	*nd*	*nd*	2.9	*nd*	7.6	14.6	*nd*
Rate summary Ft. Lupton				3–50				8–39	
Hickam	Test 2	0.74	0.054	0.026	0.13	*nd*	0.68	*nd*	0.19
	Test 3	0.11	0.051	*nd*	*nd*	*nd*	0.27	*nd*	*nd*
	Test 4	0.94	9.5	0.58	1.30	0.1	0.99	2.52	0.064
	Test 5	3.68	15.9	0.46	12.76	*nd*	0.56	1.41	*nd*
	Test 7	0.38	0.68	0.11	0.64	*nd*	1.94	1.52	*nd*
	Test 8	0.11	0.14	*nd*	0.11	*nd*	0.30	0.21	*nd*
	Test 9	15.4	0.62	1.73	100.4[b]	*nd*	1.09	0.15	*nd*
Rate summary Hickam				0.03–15				0.1–2.5	

a. For the Ft. Lupton experiment, ethylbenzene-d_{10} was used, so the resulting fumarate addition metabolite detected was d_{10}-labelled; for the Hickam experiment, ethylbenzene-d_5 was used, so the fumarate addition metabolite detected was d_5-labelled.
b. Unusually high rates relative to those measured in other tests.
Rates were determined based on regression of initial time points.
bzt, benzoate; det, metabolite detected, but formation rate could not be calculated; *nd*, metabolite not detected.

remaining tests (Tests 2, 5 and 7), so metabolite formation rate data could be obtained (Table 1). Deuterated fumarate addition intermediates and expected downstream metabolites (Fig. 1, Table 1) were detected for all of the injected hydrocarbons, except for those from 1, 2, 4-TMB-d_{12}, by comparison of liquid chromatography-tandem mass spectrometry (LC-MS-MS) profiles of the pulled water samples with the biologically generated standards from the laboratory incubations (described above; spectral characteristics for LC-MS-MS analysis are shown in Table S1). Such detection provided strong evidence for their anaerobic biodegradation under prevailing conditions. Figure 4 shows representative extraction-phase breakthrough curves for the deuterated BSA (A) as well as for deuterated downstream metabolites (B) that were transiently formed during one test at the Ft. Lupton site (Test 5). The formation rates for each metabolite (Table 1) were determined based on the concentrations measured at initial time points (as shown in Fig. 4A). At the Ft. Lupton site, the rates of formation of the deuterated fumarate addition products and deuterated downstream metabolites were quite consistent, ranging from 3 to 50 μg l^{-1} day^{-1} and 8 to 39 μg l^{-1} day^{-1} respectively (Table 1). Notable exceptions to these ranges were observed in Test 2, where the rate of formation of *o*-toluate-d_7 was near 160 μg l^{-1} day^{-1} and in Test 5, where ethylBSA-d_{10} formed at 366 μg l^{-1} day^{-1} (Table 1). The metabolites ethylBSA-d_{10} and *o*-methylBSA-d_{10} were poorly resolved using the LC-MS-MS method, so this inordinately high rate of metabolite formation may reflect a portion of the combined concentrations of both compounds. The fumarate addition metabolite of 1, 3, 5-TMB-d_{12}, 3, 5-dimethylBSA-d_{12}, was also detected in some Test 2 samples by LC-MS-MS at concentrations reaching 65 μg l^{-1}, but was not measurable at enough time points to obtain accurate rate information. For the same reason, the rate of formation of the downstream metabolite 3, 5-dimethylbenzoic acid-d_9, although clearly detected by GC-MS (Fig. 3D), could not be determined.

The bromide concentration rapidly dropped to below detection limit within the first day of the extraction phase in two of the nine wells examined at the Hickam site. However, hydrocarbon transformation rate data could be obtained from the remaining seven wells. The rates of deuterated metabolite formation at the Hickam site are shown in Table 1. The fumarate addition metabolites from toluene-d_8, *o*-xylene-d_{10} and ethylbenzene-d_5 formed at rates ranging from 0.11 to 16 μg l^{-1} day^{-1}, with the exception of ethylBSA-d_5 that was formed at a rate of 100 μg l^{-1} day^{-1} in Test 9 (Table 1). The fumarate addition metabolite associated with *m*-xylene-d_{10} metabolism was formed at comparatively low rates (only up to 1.7 μg l^{-1} day^{-1}). The expected downstream metabolites benzoate-d_5, *o*-toluate-d_7 and *m*-toluate-d_7 were also formed at lower rates relative to the fumarate addition reactions and ranged from 0.06 (the lower detection limit) to 2.5 μg l^{-1} day^{-1}. Examples of the transient formation of deuterated metabolites in Test 9 at the Hickam site are shown in Fig. 4C and D. Overall, the rates of formation of metabolites at the Hickam site were lower than those measured at the Ft. Lupton site (Table 1). The *in situ* biotransformation of 1, 3, 5-TMB-d_{12} was observed in one test only (Test 4), wherein 3,5-dimethylBSA-d_{12} was formed at a rate of 0.1 μg l^{-1} day^{-1}. Throughout the duration of the test, the concentration of this metabolite continued to increase (not shown). Its downstream

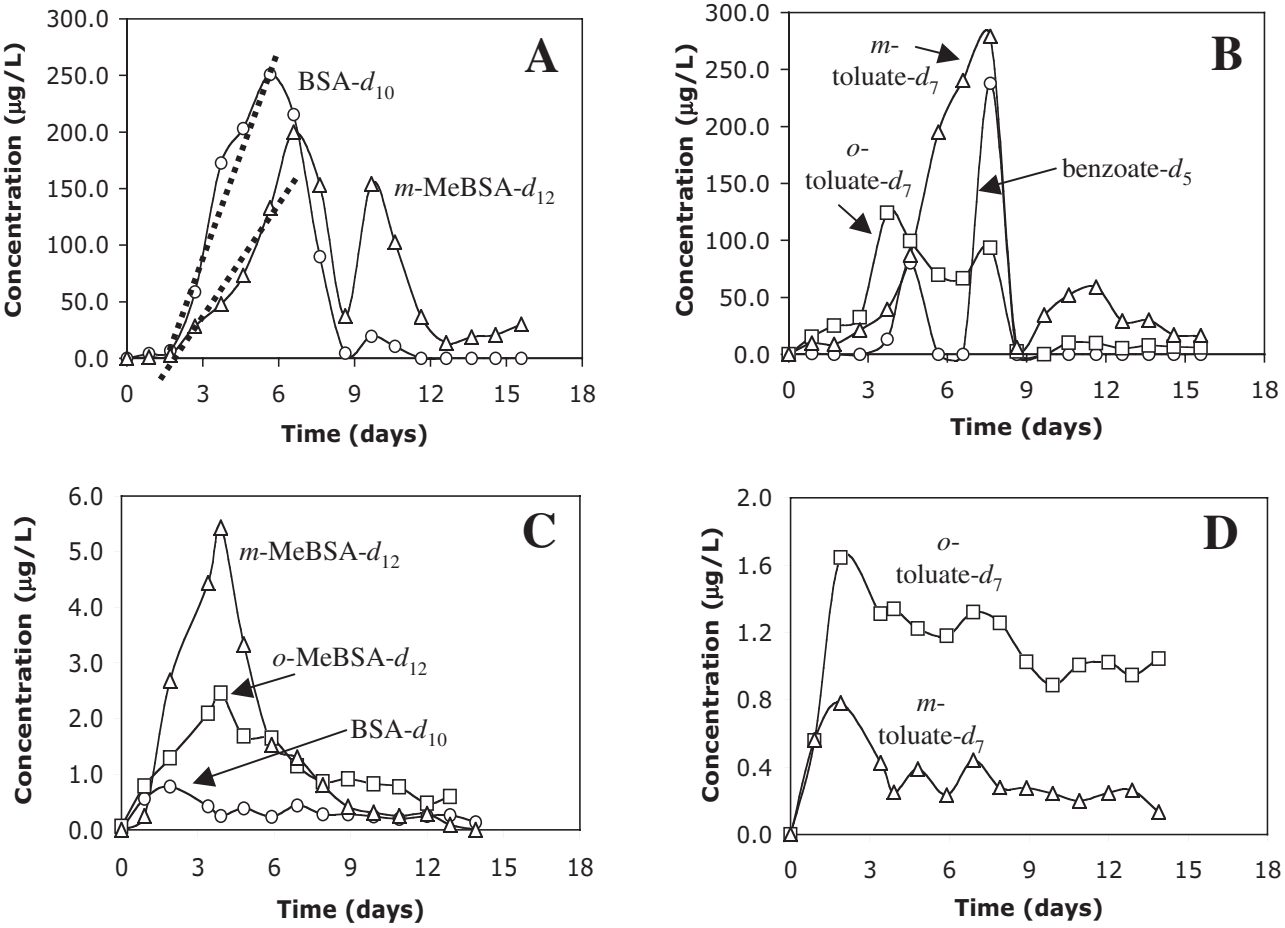

Fig. 4. Formation of deuterated alkylbenzylsuccinic acids (A, C) and additional downstream metabolites (B, D) over time in push–pull tests carried out at the Ft. Lupton (A, B) and Hickam (C, D) sites as measured by LC-MS-MS (normalized to bromide concentrations). Regression analysis to determine rates of formation were carried out using initial time points only (e.g. dotted lines in A).

metabolite, 3, 5-dimethylbenzoate-d_9, was also detected in some samples by GC-MS (Fig. 3D).

At the Hickam site, we tried to extend our ability to measure field biodegradation rates of low-water-solubility hydrocarbons by using *n*-hexane-d_{14} and methylcyclohexane-d_{14} as model aliphatic and alicyclic alkanes respectively. As noted above, the anaerobic biodegradation of these compounds was implicated by the identification of the corresponding unlabelled fumarate addition metabolites in several of the wells prior to our field procedures. Within the time frame of our experiments (up to 21 days), we were unable to detect the expected deuterated fumarate addition metabolites. However, when we analysed the water samples used in the field injection procedure to confirm the substrate concentration actually added to the wells, we were unable to detect *n*-hexane-d_{14} and we found only 3% (10 µg l^{-1} rather than the expected 300 µg l^{-1}) of intended amount of methylcyclohexane-d_{14}. Clearly, these substrates were not delivered as intended and therefore, it is not surprising that the corresponding deuterated metabolites were also not found.

Discussion

The rates of *in situ* anaerobic biotransformation of a variety of important alkylbenzenes associated with fuel mixtures spilled into aquifers were determined at two geographically and geologically distinct sites. In a previous proof-of-concept study, Reusser and colleagues (2002) showed that deuterated hydrocarbons could be used as surrogates in push–pull tests to observe the formation of corresponding deuterated fumarate addition metabolites. In their study, the biotransformation of two hydrocarbons was assessed in a total of four wells and the corresponding fumarate addition metabolites formed at rates of 1–7 nM day^{-1} (approximately 0.2–1.6 µg l^{-1} day^{-1}). Here, we expanded the number of hydrocarbons (to seven) and wells (to sixteen), and downstream metabolites were also assayed in an effort to obtain additional field anaerobic hydrocarbon biodegradation rates, examine the subsequent stages of decomposition and extend the field procedures to more hydrophobic substrates. Several of the fumarate addition metabolites we detected in the push–

pull tests had previously been detected in hydrocarbon-contaminated groundwater (such as BSA from toluene, methylBSA from xylene and ethylBSA from ethylbenzene; Beller *et al.,* 1995; Gieg and Suflita 2002). Here, we additionally found that 1, 3, 5-TMB can be anaerobically biotransformed to 3, 5-dimethylbenzylsuccinic acid and subsequently to 3, 5-dimethylbenzoic acid, as observed in both laboratory incubations and in groundwater samples. Further, we found that methylcyclohexane is converted to its fumarate addition product, methylcyclohexylsuccinic acid (tentatively identified in laboratory studies only). These observations extend the range of hydrocarbons known to be anaerobically biodegraded via a fumarate addition reaction.

The rates of metabolite formation in successful tests conducted at the Ft. Lupton site ranged from 3 to 50 $\mu g\ l^{-1}\ day^{-1}$, whereas the comparable rates garnered from the Hickam site were lower overall, ranging from 0.03 to 15 $\mu g\ l^{-1}\ day^{-1}$. The rates of metabolite formation of 0.2–1.5 $\mu g\ l^{-1}\ day^{-1}$ as determined by Reusser and colleagues (2002) in two other hydrocarbon-contaminated aquifers are within the ranges measured here. We found that the monoaromatic acid metabolites were generally formed at rates similar to alkylbenzylsuccinic acids within each site, thus additional downstream metabolites (Fig. 1) are also useful indicators of *in situ* biodegradation activity. As can be seen in Fig. 4, the majority of anaerobic metabolites assayed for were detectable within 2–3 days following injection. This observation demonstrated that the resident anaerobic microbial populations do not require long lag periods before the onset of metabolism and that anaerobic and highly reducing environmental conditions do not limit *in situ* metabolism.

The rates of *in situ* metabolite formation ranged from being reproducible to varying by two orders of magnitude among various wells within the same site (Table 1). For example, at the Ft. Lupton site, rates of BSA-d_8 formation from toluene-d_8 were within the same order of magnitude, ranging from 19 to 50 $\mu g\ l^{-1}\ day^{-1}$, and the rates of downstream metabolite formation were very similar overall. In contrast, rates of BSA-d_8 formation in the Hickam site as calculated from seven tests spanned across two orders of magnitude, from 0.1 to 15 $\mu g\ l^{-1}\ day^{-1}$ (Table 1). This difference in rates is presumably due to the heterogeneous nature of the subsurface environment where requisite microorganisms, electron acceptor availability, nutrient levels, hydrocarbon concentrations and surrounding hydrogeological phenomena can be highly variable. Such heterogeneity may also explain some usually high rates of deuterated metabolite formation that we measured in some of the tests from both sites (Table 1).

Push–pull test methodology and resulting rate calculations are based on several assumptions, which include: (i) that injected solutions are well mixed with the surrounding groundwater, (ii) that the tracer used has the same transport properties as the analytes of interest and (iii) that there is some degree of spatial and temporal uniformity of *in situ* reactions (Haggerty *et al*., 1998; Burbery *et al*., 2004). In a study investigating rates of sulfate reduction in a fast-flowing aquifer, Burbery and colleagues (2004) reported on several limits of push–pull tests as measures of *in situ* microbial activity, including the lack of suitability for sites with high groundwater velocities. We also found this to be the case, as in four of the seven push–pull tests at the Ft. Lupton site, the bromide tracer was undetectable within 2 days following the injection presumably due to rapid groundwater migration along preferential flow paths. Without the tracer, we could not normalize the resulting information and obtain suitable rate measurements. The site heterogeneity offers a reasonable explanation for our findings, as we observed good tracer retention and rates could be determined from tests done in other wells located only about 5 m away from the unsuccessful tests.

Analysis of groundwater samples from the Hickam site prior to the push–pull tests showed that various alkanes and cyclic alkanes were undergoing anaerobic biodegradation to their respective alkylsuccinates. However, our current practice depends on the delivery of the substrates to aquifer in an aqueous phase during the injection phase of the push–pull tests. Our hydrocarbon measurements of samples taken during the injection phase showed that little or none of the *n*-hexane-d_{14} or methylcyclohexane-d_{14} was actually delivered in the intended manner. This is not a limitation of the push–pull test methodology *per se*, but shows that ways to ensure the delivery of less water-soluble but prevalent hydrocarbon contaminants warrant further investigation.

Despite some inherent limitations with push–pull tests, we were able determine *in situ* rates of anaerobic hydrocarbon biotransformation. In the field, the metabolites are certainly formed and consumed simultaneously. Thus, the net rate of metabolite formation likely reflects both processes and provides inherently conservative estimates of hydrocarbon metabolism. Based on this study and that of Reusser and colleagues (2002), we propose that the observed *in situ* rates of metabolite formation represent extremes (e.g. ranging from 0.1 to $> 10\ \mu g\ l^{-1}\ day^{-1}$) that could be useful for modeling purposes.

Experimental procedures

Site descriptions

Single-well push–pull tests were conducted at two hydrocarbon-contaminated sites. The first, located near Ft. Lupton, CO, USA (referred to as the Ft. Lupton site), consists of a shallow sandy aquifer overlying an active natural gas

production field in an agricultural basin where gas condensate is co-produced. An underground storage tank containing gas condensate leaked in the 1970s, causing BTEX and other hydrocarbons (mainly in the C_5 to C_{15} range) to spill into the underlying aquifer. The hydrocarbon contamination is confined around the water table level, approximately 1.4 m below surface to a 20 m × 15 m area. The mean hydraulic conductivity was calculated to be 2.4×10^{-5} m s^{-1}. A detailed description of this site and evidence that anaerobic intrinsic bioremediation of hydrocarbons is occurring here has been previously published (Gieg *et al*., 1999), including metabolic evidence for the *in situ* anaerobic biotransformation of alkylbenzenes and alkanes (Elshahed *et al*., 2001; Gieg and Suflita, 2002). Geochemical measurements taken over several years showed that sulfate reduction and/or methanogenesis are the predominant electron-accepting processes in the contaminated portion of this aquifer. Upstream, uncontaminated groundwater contains approximately 2 mM (~200 mg l^{-1}) sulfate as a potential electron acceptor (Gieg *et al*., 1999). Alkylbenzene concentrations (toluene, ethylbenzene, xylenes, trimethylbenzenes) ranged from undetectable to 124 μg l^{-1} in the contaminant plume prior to the push–pull tests.

The second site comprises an approximately 65 acre portion of an aquifer underlying a residential and commercially developed portion of Hickam Air Force Base, Oahu, Hawaii, USA (the Hickam site). The subsurface of this area consists of about 0.9–3 m of sediment (sand, clay and silt) underlain by volcanic tuff till about 9 m below surface through which the groundwater flows mainly through fractures (Parsons, 2005). The water table ranges from about 2 to 6 m below surface and the formation is characterized by a mean hydraulic conductivity of 1.8×10^{-3} cm s^{-1}. Groundwater flows at variable rates in a westerly direction and ultimately discharges into the Pacific Ocean. The area served as a fuel storage and transportation facility from 1940 to 1974 and as a result of these activities, the underlying aquifer became contaminated (at an unknown time) with leaded aviation gasoline and jet fuels. Most of the underground infrastructure relating to these activities was removed or closed in place, but remaining fuel components (including BTEX) continue to form a light non-aqueous phase layer that chronically contaminates surrounding groundwater to various extents. Numerous remedial actions have been implemented at the site since the mid-1980s with varying degrees of success, although multi-phase extraction units installed around the site had been effective at removing over 10 000 gallons of fuel as of 2005 (Parsons, 2005).

The feasibility of natural attenuation was investigated as a remedial option for parts of the site based on groundwater measurements suggesting that *in situ* hydrocarbon biodegradation might be occurring (Parsons, 2005). Such observations included a stable hydrocarbon plume, decreasing BTEX concentrations, and changes in electron acceptors and end-product formation (i.e. sulfide and methane) (Parsons, 2005). One subsection of the underlying aquifer was selected by the operators and contractors of the Hickam Air Force Base site for our studies, encompassing an area of about 90 m × 110 m. As of June 2005, groundwater concentrations of toluene, ethylbenzene and xylene in the area ranged from less than 1 μg l^{-1} up to 12, 290 and 32 μg l^{-1} respectively (Parsons, 2005).

Push–pull test procedures

The push–pull experimental methods used were similar for both sites and generally followed a previously published protocol (Reusser *et al*., 2002). Briefly, the injection solutions consisted of approximately 250 l of water amended with bromide (final concentration of 100 mg l^{-1}) as a non-reactive, non-sorbing tracer (Istok *et al*., 1997; Reusser *et al*., 2002) and isotopically heavy (deuterated) hydrocarbons (0.2–2.5 mg l^{-1}, depending on water solubility). Test solutions were made anaerobic by flushing with argon for 1 h and injected into groundwater wells at a target rate of 1 ml min^{-1} (~250 min for the injection process). Oxygen levels of the test solutions were monitored throughout the injection phase to ensure anaerobicity using a commercially available test kit. At least five samples were collected at intervals during the injection phase to determine the concentration of each analyte added to a particular well. For both the 'push' and 'pull' phases of each experiment, samples were taken for hydrocarbon, metabolite and anion analyses. Samples for deuterated hydrocarbon analysis were collected in acid-preserved 40 ml VOA vials with no headspace. Samples for anion determinations were collected in 7 ml glass vials, and samples for metabolite analysis were collected into acid-preserved 250 ml glass bottles with Teflon-lined lids. All samples were kept cold immediately following sampling, shipped to the laboratory on ice and kept refrigerated prior to analyses.

For the Ft. Lupton site, the push–pull tests were carried out in May 2004. Groundwater from an uncontaminated portion of the aquifer (containing ~200 mg l^{-1} or ~2 mM sulfate) was used to prepare the injection solution. Seven groundwater wells in the contaminated portion of the aquifer were used for push–pull tests (designated Tests 1–7). The suite of hydrocarbons in the field tests included toluene-d_8, ethylbenzene-d_{10}, *o*-xylene-d_{10}, *m*-xylene-d_{10} (amended at a final target concentration of 2.5 mg l^{-1}), 1, 2, 4-TMB-d_{12} and 1, 3, 5-TMB-d_{12} (1 mg l^{-1} final target concentration). Samples were removed daily for 18 days following the injection phase.

For the Hickam site, push–pull tests were carried out in May 2006. Tap water was used as the injection fluid. The suite of hydrocarbons (and their final target concentrations) included toluene-d_8, ethylbenzene-d_5 (ring-labelled), *o*-xylene-d_{10}, *m*-xylene-d_{10} (all added to approximate a 2 mg l^{-1} final concentration), 1, 3, 5-TMB-d_{12} (1 mg l^{-1}), *n*-hexane-d_{14} (0.2 mg l^{-1}) and methylcyclohexane-d_{14} (0.3 mg l^{-1}). The latter two compounds, representative of alkanes and cyclic alkanes, respectively, were included to determine whether such low-water-solubility hydrocarbons (14–18 mg l^{-1}) could also be used in push–pull biotransformation assays. Nine wells were used for push–pull tests to determine the *d*-hydrocarbon biotransformation rates at this site (designated Tests 1–9). Samples were removed daily for 15–21 days following the injection of the test solution.

Laboratory incubations

Contaminated sediments collected from the Ft. Lupton site were used to establish laboratory biodegradation assays in order to generate deuterium-labelled metabolites for use as authentic analytical standards. The microbial populations at this site were previously shown to biotransform toluene, ethylbenzene and all three xylene isomers to the respective

fumarate addition metabolites under sulfate-reducing conditions (Elshahed *et al*., 2001). Incubations were prepared in an anaerobic glove bag (containing 5% H_2 in N_2) by mixing 25 g sediments with 40 ml of cysteine sulfide-reduced groundwater sampled from an uncontaminated, upgradient well at the site (containing ~2 mM sulfate). The headspaces of incubations were exchanged with 20% CO_2 in N_2 prior to adding 2 μl of one of the following hydrocarbons: toluene-d_8, *o*-xylene-d_{10}, *m*-xylene-d_{10}, ethylbenzene-d_{10}, 1, 3, 5-TMB-d_{12}, 1, 2, 4-TMB-d_{12} or methylcyclohexane-d_{14} (15–18 μmol, or ~28 μg l^{-1}). Triplicate non-sterile incubations and duplicate autoclaved (sterile) controls were established for each hydrocarbon added. Incubations to which no exogenous hydrocarbons were added served as controls to assess background levels of sulfate reduction. Hydrocarbon and sulfate concentrations were monitored over time. When approximately 50–75% of the hydrocarbon substrate was consumed, a portion of the culture supernatant was removed and analysed (below) by GC-MS for the presence of deuterated metabolites. Remaining supernatants were then analysed by LC-MS-MS in order to serve as authentic standards. Deuterated (1-methylpentyl)succinate, the known fumarate addition metabolite of *n*-hexane (Rabus *et al*., 2001), was generated by incubating an alkane-utilizing sulfate-reducing isolate with *n*-hexane-d_{14} as previously described (Gieg and Suflita, 2002).

Chemical analysis

Ion chromatography was used to measure bromide and sulfate concentrations in the field samples (Reusser *et al*., 2002) and sulfate in laboratory incubations (Elshahed *et al*., 2001). Groundwater samples collected from each site prior to conducting push–pull tests were extracted with ethylacetate, derivatized by silylation and analysed by GC-MS as previously described (Gieg and Suflita, 2002) in order to determine the presence of hydrocarbon metabolites. Samples from laboratory incubations were prepared in the same way to look for deuterated metabolites from the appropriate deuterated substrates. The LC-MS-MS was used to detect and quantify the formation of deuterated anaerobic metabolites in the field tests in a manner similar to that previously described (Alumbaugh *et al*., 2004). However, the solid-phase extraction step was omitted and replaced with high-volume liquid injections (0.5 ml). Samples were injected onto a *Targa* column (Higgins Analytical, 150 mm × 2.1 mm) and analytes were separated using a 45–70% methanol/acetate buffer (10 mM, pH 3.7) gradient at a flow rate of 0.2 ml min^{-1}. The minimum detection limits for the putative metabolites using the high-volume injection technique ranged from 0.05 to 0.15 μg l^{-1}. The ion transitions (*m/z*, precursor ion $[M\text{-}H]^-$ and product ion $[M\text{-}H\text{-}COO]^-$) used for identification and quantification of putative metabolites by LC-MS-MS are shown in Table S1. Quantification of deuterated metabolites detected in field samples was based on calibrations performed with unlabelled authentic standards. The fumarate addition metabolites from *o*-xylene (*o*-methylBSA), *m*-xylene (*m*-methylBSA), ethylbenzene (ethylBSA) and 1, 3, 5-TMB (3, 5-dimethylBSA) were synthesized as previously described (Bickford *et al*., 1948) whereas BSA, *o*-toluate, *m*-toluate, 3, 5-dimethylbenzoate and benzoate were commercially available. Deuterated authentic standards were biologically generated from deueterated hydrocarbons using laboratory enrichments (as described above) and were used to determine LC-MS-MS retention times and mass spectral information. In some cases, GC-MS analysis was used to confirm or enhance LC-MS-MS metabolite results from field samples. Hydrocarbon concentrations in the laboratory incubations were measured by headspace analysis on a GC with flame ionization detection as previously described (Gieg *et al*., 1999).

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Supporting information

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

Fig. S1. Mass spectral profile of a putative TMS-derivatized deuterated fumarate addition metabolite of methylcyclohexane-d14 detected in laboratory incubations.
Table S1. LC-MS-MS properties used for the analysis of anaerobic hydrocarbon metabolites. Quantification was based on calibrations prepared with unlabelled authentic standards (greyed entries in table).

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