Metabolic indicators for detecting *in situ* anaerobic alkylbenzene degradation

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Abstract

Monitoring programs for intrinsic bioremediation of fuel hydrocarbons require indicators that can convincingly demonstrate *in situ* metabolism. In this evaluation of potential indicators of *in situ* anaerobic alkylbenzene metabolism, laboratory and field data are reviewed for two classes of aromatic acids: (i) benzylsuccinate, *E*-phenylitaconate, and their methyl homologs, and (ii) benzoate, and methyl-, dimethyl-, and trimethylbenzoates. The review includes previously unpublished field data from a hydrocarbon-contaminated site in Fallon (Nevada), at which both classes of metabolites were detected in groundwater. The two classes of compounds were evaluated with respect to specificity (i.e., unique biochemical relationship to a specific alkylbenzene), stability, and generation as degradation intermediates versus dead-end products; recent developments in the biochemistry of anaerobic toluene and xylene degradation were incorporated in this evaluation. In general, benzylsuccinates/*E*-phenylitaconates are superior to benzoates in terms of their very high specificity to their parent hydrocarbons and their lack of commercial and industrial sources. They are also uniquely indicative of anaerobic conditions. All of the benzoates, benzylsuccinates, and *E*-phenylitaconates are relatively stable chemically and (with the exception of benzoate) biologically under anaerobic conditions, based on the limited data available. Although benzoate, benzylsuccinate, and *E*-phenylitaconate are intermediates of anaerobic toluene mineralization to carbon dioxide, their methyl homologs can be either mineralization intermediates or cometabolic dead-end products of alkylbenzenes, depending on the bacteria involved. Benzoates are far more commonly reported in field studies of hydrocarbon-contaminated aquifers than are benzylsuccinates and *E*-phenylitaconates, although it is not clear whether this is an accurate representation of the relative occurrence of these metabolites at contaminated sites, or whether it instead reflects the limited range of target analytes used in most field studies to date.

Introduction

Fuel hydrocarbon contamination of groundwater is a pervasive problem at federal and commercial facilities throughout the US. As of 1996, over 317,000 releases from leaking underground fuel tanks (LUFT) had been confirmed nationwide (US EPA 1998). The leaking fuel from storage tanks is estimated to have affected groundwater in approximately 40% of the reported cases (US House of Representatives 1988). Surface spill accidents and landfill leachate intrusion are additional sources of groundwater contamination by gasoline, aviation fuel, and other refined petroleum derivatives. The hydrocarbons of greatest regulatory concern in fuel-contaminated groundwater are known as BTEX (an acronym for benzene, toluene, ethylbenzene, and the three xylene isomers), which are among the most toxic and water-soluble constituents of gasoline and other fuels (Sittig 1985; Coleman et al. 1984).

Bioremediation is one of a limited number of options for restoring fuel-contaminated aquifers. Toluene and other alkylbenzenes are readily degradable in aerobic surface water and soil systems (Gibson & Subramanian 1984; Smith 1990); however, in the subsurface environment, contamination by organic com-

pounds often results in the complete consumption of available oxygen by indigenous microorganisms and the development of anaerobic conditions (e.g., Lee et al. 1988). Given the cost and technical difficulty associated with introducing oxygen into some aquifers, *in situ* bioremediation performed by indigenous, anaerobic bacteria (intrinsic bioremediation) merits serious consideration as a cost-effective means of groundwater restoration. Indeed, natural attenuation (which entails intrinsic bioremediation as a primary component) is currently the most common treatment option for fuel-contaminated groundwater and is being used at over 15,000 sites in the US (US EPA 1998).

A major impediment to the widespread acceptance of *in situ* bioremediation is the difficulty of demonstrating that decreases in the concentrations of BTEX in groundwater truly represent biological metabolism of these compounds rather than abiotic processes such as sorption, dilution, or dispersion. Although regulatory agencies often require monitoring that can convincingly demonstrate *in situ* metabolism, they typically do not identify specific indicators for this purpose (US EPA 1999). A 1993 National Research Council (NRC) report on *in situ* bioremediation (NRC 1993) made general recommendations for demonstrating biodegradation using field measurements; among the variables listed in the NRC report were "by-products of anaerobic activity" and "intermediary metabolite formation". Metabolites relevant specifically to BTEX degradation were not identified.

A variety of field indicators have been proposed to document *in situ* hydrocarbon metabolism, including (i) benzoate and alkylbenzoates (Reinhard et al. 1984; Cozzarelli et al. 1990), (ii) benzylsuccinate, *E*-phenylitaconate, and their methyl homologs (Beller et al. 1995), (iii) short-chain aliphatic acids (Cozzarelli et al. 1994), and (iv) stable carbon isotope ratios of dissolved inorganic carbon (Aggarwal & Hinchee 1991; Landmeyer et al. 1996). Proposed indicators of dominant terminal electron-accepting processes in aquifers have included hydrogen concentrations in groundwater (Lovley & Goodwin 1988) as well as anaerobic respiration products (e.g., sulfide, methane, Fe^{2+}, and nitrite) in groundwater (Wiedemeier et al. 1995).

A set of ideal characteristics for indicators of *in situ* alkylbenzene metabolism (Table 1) was formulated and used to guide this review. In addition to the characteristics listed in Table 1, a prerequisite for metabolites to serve as indicators is that they be released to the extracellular medium such that they could be expected to occur in contaminated groundwater. This article focuses exclusively on metabolites that have retained the aromatic portion of the BTEX carbon skeleton [i.e., (i) and (ii) in the preceding paragraph], because these are the most specific indicators of BTEX metabolism that have been proposed to date. However, it is highly unlikely that monitoring data for a single kind of indicator will convincingly document anaerobic *in situ* bioremediation for all chemicals of concern. Accordingly, regulatory agencies and researchers have stressed the importance of monitoring programs that incorporate multiple, independent lines of evidence to document *in situ* bioremediation (NRC 1993; Weidemeier et al 1995; US EPA 1999; Madsen 1991).

Benzylsuccinate, *E*-phenylitaconate, and their methyl homologs

The structures of benzylsuccinate, *E*-phenylitaconate, and their methyl homologs are presented in Figure 1. For simplicity, the toluene metabolite originally identified as benzylfumarate and later shown to be *E*-phenylitaconate (Chee-Sanford et al. 1996) will be referred to only as *E*-phenylitaconate. Also note that all reports of methyl homologs of *E*-phenylitaconate, and nearly all reports of methyl homologs of benzylsuccinate, have been based on tentative identifications in the absence of authentic standards. For the sake of brevity, the phrase "tentatively identified" will be omitted from the following discussion when describing these compounds.

Laboratory studies

Benzylsuccinate and E-phenylitaconate (from toluene)

Our understanding of the metabolic significance of benzylsuccinate and *E*-phenylitaconate in anaerobic toluene degradation has evolved since they were first reported as anaerobic toluene metabolites in 1992 (Evans et al. 1992). They were observed to accumulate during anaerobic toluene degradation by several denitrifying bacteria (*Thauera aromatica* strains T1 and K172, *Azoarcus* sp. strain T, and *Azoarcus tolulyticus* strain Tol-4; Evans et al. 1992, Chee-Sanford et al. 1996, Seyfried et al. 1994) and sulfate-reducing strain PRTOL1 (Beller et al. 1992; 1996). Overall yields of benzylsuccinate and *E*-phenylitaconate ranged from 0.5 to 17% of the toluene consumed, but were typically <3% (Evans et al. 1992; Beller et al. 1996;

Table 1. Ideal characteristics for indicators of *in situ* alkylbenzene metabolism

Characteristic of metabolite	Significance
An unequivocal and unique biochemical relationship to the parent hydrocarbon	When found in groundwater, the compound can be definitively related to the metabolism of a specific alkylbenzene
No commercial or industrial uses	When found in groundwater, the compound can be definitively related to metabolism and not to anthropogenic releases of the compound itself
Biological and chemical stability	Stability increases the probability of detecting the compound in groundwater
Generation as an intermediate of mineralization rather than as a product of cometabolism	The goal of bioremediation is mineralization to innocuous products, not transformation to potentially toxic metabolites
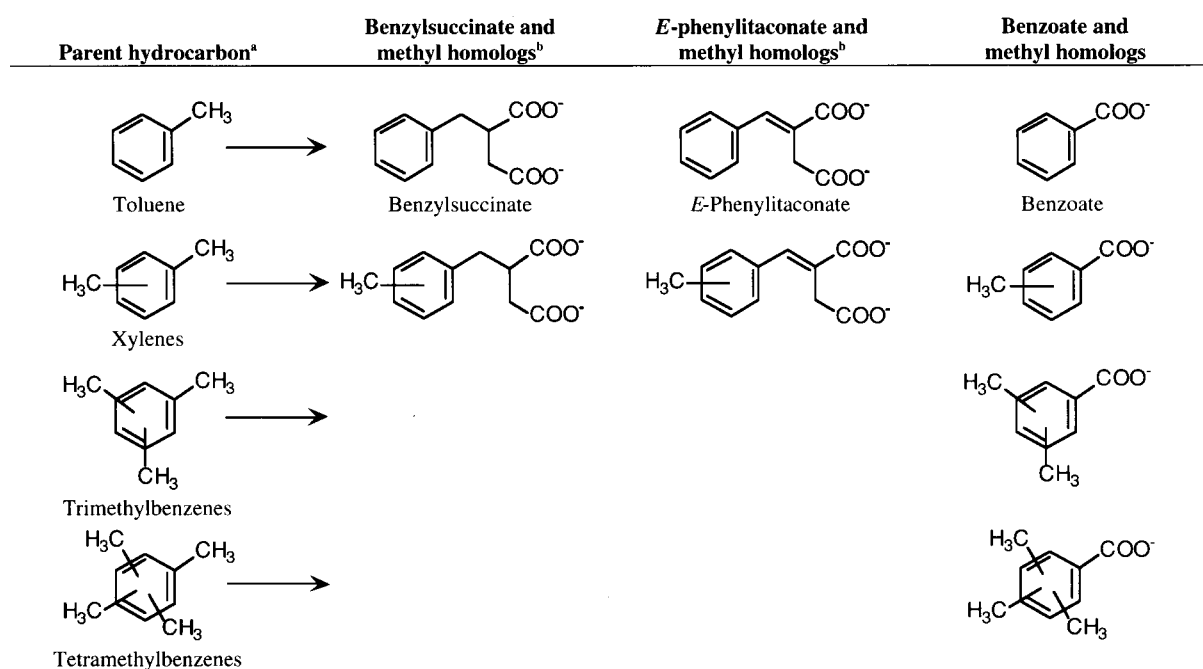
[a] With regard to the benzoates, the parent hydrocarbons shown are not necessarily the only possible metabolic precursors.

[b] Although benzylsuccinate and *E*-phenylitaconate have been identified as anaerobic toluene metabolites by GC/MS using authentic standards (Beller & Spormann 1997a), the methyl homologs have typically been tentatively identified based on consideration of GC retention times and mass spectral fragmentation patterns (see discussion in Beller et al. 1995 and Beller & Spormann 1997a).

Figure 1. Potential indicators of anaerobic alkylbenzene metabolism observed in laboratory and field studies.

Chee-Sanford et al. 1996; Seyfried et al. 1994). The fact that these compounds accumulated during toluene mineralization and could apparently not be metabolized when in the culture medium of toluene-grown cells led to the assumption that they were dead-end metabolites produced by a minor toluene transforma-tion pathway (Evans et al. 1992; Beller et al. 1992; Chee-Sanford et al. 1996).

However, *in vitro* studies of the novel benzylsuc-cinate synthase reaction (i.e., enzymatic benzylsuccin-ate formation from toluene and fumarate) have now provided strong evidence that benzylsuccinate is in fact the first intermediate of anaerobic toluene miner-

alization to CO_2 in denitrifying bacteria (Biegert et al. 1996; Beller & Spormann 1997a) and sulfate-reducing bacteria (Beller & Spormann 1997b; Rabus & Heider 1998). *In vitro* studies with toluene-grown, denitrifying *Azoarcus* sp. strain T have also demonstrated that *E*-phenylitaconate is an anaerobic toluene metabolite formed by dehydrogenation of benzylsuccinate (Beller & Spormann 1997a). Evidence that benzylsuccinate is the first intermediate of toluene mineralization to CO_2 includes the following two observations from *in vitro* experiments with toluene-grown cell preparations: (i) under appropriate conditions, benzylsuccinate can be converted to benzoate or benzoyl-CoA (Biegert et al. 1996; Beller & Spormann 1997a), which is a well-documented intermediate of anaerobic toluene degradation (see Benzoate section), and (ii) the *in vitro* rate of benzylsuccinate formation from toluene and fumarate is comparable to the *in vivo* rate of toluene consumption in the presence of nitrate (Beller & Spormann 1997a; 1999). Pathways of anaerobic toluene degradation that incorporate these recent findings have been proposed (Biegert et al. 1996; Beller & Spormann 1997a).

The finding that benzylsuccinate and *E*-phenylitaconate are intermediates of anaerobic toluene degradation rather than dead-end metabolites has strong implications for their use as metabolic indicators: they are indicators of mineralization (the desired result of bioremediation) rather than transformation. Furthermore, the accumulation of benzylsuccinate and *E*-phenylitaconate in the growth medium of various toluene-degrading laboratory cultures also has implications for their use as indicators: they are much more stable in extracellular medium than might be expected for degradation intermediates (e.g., in comparison to benzoate). This apparent stability is very likely a result of physiological limitations in transporting benzylsuccinate and *E*-phenylitaconate back into the cell once they have been released to the aqueous medium (by some undefined mechanism). As evidence of this transport limitation, note that benzylsuccinate cannot be metabolized by viable, toluene-grown cells in aqueous medium (Beller et al. 1992; 1996), but can be oxidized to *E*-phenylitaconate and benzoate during *in vitro* assays with cell-free extracts or permeabilized cells from toluene-grown cultures (Biegert et al. 1996; Beller & Spormann 1997a; 1998).

Methyl homologs of benzylsuccinate and *E*-phenylitaconate (from xylenes)

Use of methyl homologs of benzylsuccinate and *E*-phenylitaconate as metabolic indicators is more complicated than use of the unsubstituted compounds, primarily because a given methyl homolog may be either an intermediate or dead-end product of xylene metabolism, depending on the bacterial cultures involved. If a bacterium produces methyl homologs of benzylsuccinate and *E*-phenylitaconate from a xylene isomer that serves as a growth substrate, then these metabolites are probably intermediates. However, if the xylene isomer is not a growth substrate, then they are more likely to be dead-end metabolites. These observations are necessarily speculative because there are few detailed biochemical studies of anaerobic xylene metabolism and few available cultures capable of anaerobic growth on any xylene isomer. Only one anaerobic *o*-xylene-degrading bacterium has been isolated and no anaerobic *p*-xylene-degrading isolate has yet been reported (Harms et al. 1999; Heider et al. 1998).

A recent study of denitrifying *Azoarcus* sp. strain T, which can grow anaerobically on *m*-xylene or toluene, indicates that the initial reactions of anaerobic *m*-xylene degradation are analogous to those described for anaerobic toluene degradation by this and other bacterial species (Krieger et al. 1999). Thus, (3-methylbenzyl)succinate and *E*-(3-methylphenyl)itaconate are proposed initial intermediates in the anaerobic *m*-xylene mineralization pathway just as benzylsuccinate and *E*-phenylitaconate are in the anaerobic toluene mineralization pathway. Analogous to findings related to benzylsuccinate and toluene, although (3-methylbenzyl)succinate accumulated in the medium of *Azoarcus* sp. strain T grown on *m*-xylene (Beller et al. 1995), it could be oxidized to *E*-(3-methylphenyl)itaconate and *m*-toluate by permeabilized, *m*-xylene-grown cells under the appropriate *in vitro* conditions (Krieger et al. 1999).

To date, all other studies of anaerobic xylene metabolism that are relevant to methylbenzylsuccinates and related compounds have described reactions that appear to be cometabolic. For example, (2-methylbenzyl)succinate and *E*-(2-methylphenyl)itaconate (or a closely related isomer) were reported as products from the anaerobic *in vivo* transformation of *o*-xylene by denitrifying *Thauera aromatica* strain T1 and sulfate-reducing strain PRTOL1 (Evans et al. 1991; 1992; Beller et al. 1996); *o*-xylene did not serve as a growth substrate

for either organism. In experiments with toluene-grown strain PRTOL1 amended with *o*-xylene, >90 mol% of the *o*-xylene consumed was transformed to (2-methylbenzyl)succinate (Beller et al. 1996). This transformation could be considered cometabolic for two reasons: (i) it appears to involve an incomplete oxidation that provides no carbon and little or no energy to the cell and (ii) it may require the activity of an enzyme (benzylsuccinate synthase) involved in the metabolism of a growth substrate (toluene). The analogous transformation of *p*-xylene to (4-methylbenzyl)succinate by toluene-grown cells of strain PRTOL1 may also have been cometabolic, however, <5 mol% of the *p*-xylene consumed was transformed to (4-methylbenzyl)succinate (Beller et al. 1996).

For anaerobic, toluene-degrading bacteria (such as strain PRTOL1 and *Thauera aromatica* strain T1) that cannot grow on xylene isomers but can transform one or more of them to methylbenzylsuccinates, it seems plausible that the transformation is catalyzed by the toluene-activating enzyme, benzylsuccinate synthase. Furthermore, it has been suggested that *Azoarcus* sp. strain T, which can grow anaerobically on toluene or *m*-xylene, uses benzylsuccinate synthase to activate both aromatic hydrocarbons (Krieger et al. 1999). Indeed, *in vitro* studies with partially purified benzylsuccinate synthase from *Azoarcus* sp. strain T demonstrated that it can transform all 3 xylene isomers to their respective methylbenzylsuccinates (Beller & Spormann 1999). It follows that the inability of anaerobic, toluene-degrading bacteria to grow on certain xylene isomers may derive from their inability to efficiently oxidize methylbenzylsuccinates rather than an inability to carry out the biochemically challenging initial activation of xylenes to methylbenzylsuccinates (Beller & Spormann 1997a).

In summary, methyl homologs of benzylsuccinate and *E*-phenylitaconate may be intermediates of anaerobic xylene mineralization if produced by bacteria capable of growing on the corresponding xylene isomers; alternatively, they may be dead-end products of anaerobic xylene transformation if produced by bacteria capable of growing on toluene (and perhaps on different xylene isomers).

Field studies

A controlled-release experiment at Seal Beach (CA) was the first field study to document the occurrence of benzylsuccinate, *E*-phenylitaconate, and their methyl homologs in BTEX-contaminated groundwater and to propose them as indicators of *in situ* anaerobic alkylbenzene metabolism (Beller et al. 1995). The methods used in the Seal Beach experiment have been detailed elsewhere (Beller et al. 1995; Reinhard et al. 1997). Briefly, this study of *in situ* biodegradation involved a "slug test" with a single injection/extraction well and the controlled release of 5 aromatic hydrocarbons (BTEX, excluding *p*-xylene). The test slug consisted of approximately 840 L of Seal Beach groundwater that was (i) extracted from the aquifer, (ii) pumped through granular activated carbon (GAC) filters, (iii) deionized, (iv) purged with helium to remove oxygen and volatile aromatic compounds, (v) amended with bromide (for use as a conservative tracer), mineral salts, and aromatic compounds at 2 to 3 μM (225 to 300 μg/L) per component, and (vi) injected back into the same well to establish an aquifer test zone. Sulfate was the only electron acceptor supplied in this experiment.

To monitor concentrations of analytes, groundwater was pumped from the Seal Beach test zone at selected time intervals. Analysis of benzylsuccinate and related compounds involved diethyl ether extraction of 1 L of acidified groundwater, derivatization of the extracts with diazomethane, and analysis of the derivatized extracts by GC/MS (Beller et al. 1995). Of the metabolites addressed in this section, only benzylsuccinate was commercially available. Thus, tentative identification of *E*-phenylitaconate and methyl homologs of benzylsuccinate and *E*-phenylitaconate in Seal Beach groundwater was based principally on comparisons of the GC retention times and mass spectra of compounds found in groundwater extracts to those of metabolites produced by *Azoarcus* sp. strain T and sulfate-reducing strain PRTOL1 in laboratory experiments (Beller et al. 1995). Standards of *E*-phenylitaconate (Chee-Sanford et al. 1996) and (3-methylbenzyl)succinate (Krieger et al. 1999) have since become available and have made definitive identification possible for these compounds.

During the two-month Seal Beach experiment, three hydrocarbons (toluene, *o*-xylene, and *m*-xylene) were depleted in groundwater and the benzylsuccinate and *E*-phenylitaconate homologs corresponding to each of these hydrocarbons appeared in the groundwater (Beller et al. 1995; Figures 2 and 3). Trends in concentration data provide strong evidence that these distinctive metabolites were indeed associated with *in situ* alkylbenzene metabolism. None of the metabolites were detectable in the initial Seal Beach

groundwater sample (Figures 2 and 3). In general, as alkylbenzene concentrations decreased with time in Seal Beach groundwater, metabolite concentrations increased until the alkylbenzenes were depleted. In Figures 2 and 3, the concentrations of toluene, *o*-xylene, and *m*-xylene and of the associated metabolites are normalized to bromide and to the maximum concentration of each compound itself.

An interesting trend observed for the concentrations of all metabolites (Figures 2 and 3) is a decrease after the concentration maximum, suggesting a loss of these compounds by a mechanism other than simple dilution, which was corrected for by bromide normalization. If this decrease represents biodegradation of these products, this is in contrast to previously discussed laboratory observations of cultures in which these metabolites accumulated.

Of the metabolites observed in the Seal Beach study, the benzylsuccinate and *E*-phenylitaconate homologs derived from *o*-xylene were clearly predominant over those from toluene and *m*-xylene (Beller et al. 1995). Thus, it is possible that *o*-xylene was cometabolized in Seal Beach groundwater whereas toluene and *m*-xylene were mineralized (recall that, in laboratory studies, benzylsuccinate and *E*-phenylitaconate homologs released to the aqueous medium account for a small percent (e.g., <3 mol%) of their parent hydrocarbon when it is mineralized but approximately 100 mol% of their parent hydrocarbon when it is cometabolized). Although the *o*-xylene metabolites at their maximum concentrations constituted <5 mol% of the initial *o*-xylene concentration in groundwater (Beller et al. 1995), the actual yields of the metabolites would be higher than indicated by their maximum concentrations if, as suggested by Figures 2 and 3, the metabolites were being degraded or otherwise removed from the groundwater.

Although the Seal Beach study provided strong evidence that benzylsuccinate and related compounds could serve as indicators of *in situ* alkylbenzene metabolism, it was not entirely representative of conditions at other fuel-contaminated sites. For example, the injected groundwater contained a relatively simple contaminant mixture (5 of the BTEX compounds at relatively low concentrations), instead of having a complex, higher concentration mixture more characteristic of LUFT plumes. It is thus encouraging that fuel-contaminated groundwater from a site in Fallon (NV) was found to contain relatively high concentrations of benzylsuccinate and (2-methylbenzyl)succinate, as well as a number of alkylbenzoates (Figure 4). The

benzylsuccinates were identified by mass spectra (Figure 5) and GC retention times according to the approach described for the Seal Beach study (Beller et al. 1995). A Fallon groundwater sample from a different location contained (2-methylbenzyl)succinate and several alkylbenzoates, all of which were minor components of the total ion chromatogram (data not shown); benzylsuccinate was not detected in this second Fallon sample. Analytical procedures used for the Fallon samples were very similar to those used for the Seal Beach samples (Beller et al. 1995). Both Fallon samples were characterized by considerable BTEX contamination (>18,000 μg/L total BTEX in the sample represented in Figure 4 and ~200 μg/L in the second sample; Battelle 1998) and multiple indicators of anaerobic conditions (e.g., low dissolved oxygen concentration and redox potential; Battelle 1998).

Several comparisons of the Seal Beach and Fallon results are instructive. When considering benzylsuccinates and related compounds, metabolites from *o*-xylene were predominant at both field sites. The concentration of (2-methylbenzyl)succinate in the Fallon sample (Figure 4) was on the order of 1 μM (~220 μg/L), which is over an order of magnitude higher than the concentration observed in Seal Beach. The highly complex nature of contamination in the Fallon sample as compared to the Seal Beach samples (reflected in the noisy GC/MS baseline in Figure 4) resulted in difficulties in analyzing the Fallon sample. Most notably, the diazomethane derivatization that proved very effective in the Seal Beach study was ineffective in the Fallon sample; an alternative methylation involving Fischer esterification (i.e., heating the extract in the presence of methanol and sulfuric acid) was required for the Fallon samples. Even with the alternative derivatization method, the recovery of a surrogate compound (4-fluorobenzoate) was extremely poor (undetectable at 0.1 μM added concentration), whereas surrogate recovery averaged approximately 95% in the Seal Beach study (Beller et al. 1995). [This suggests that the (2-methylbenzyl)succinate concentration cited above for the Fallon sample may be a gross underestimate.]

The Fallon groundwater samples, although more difficult to analyze than the Seal Beach samples, suggest that benzylsuccinates may serve as useful indicators of *in situ* alkylbenzene metabolism under actual field conditions. Furthermore, Gieg et al. (1999) recently reported methylbenzylsuccinates (particularly the 3-methylbenzyl- isomer from *m*-xylene)
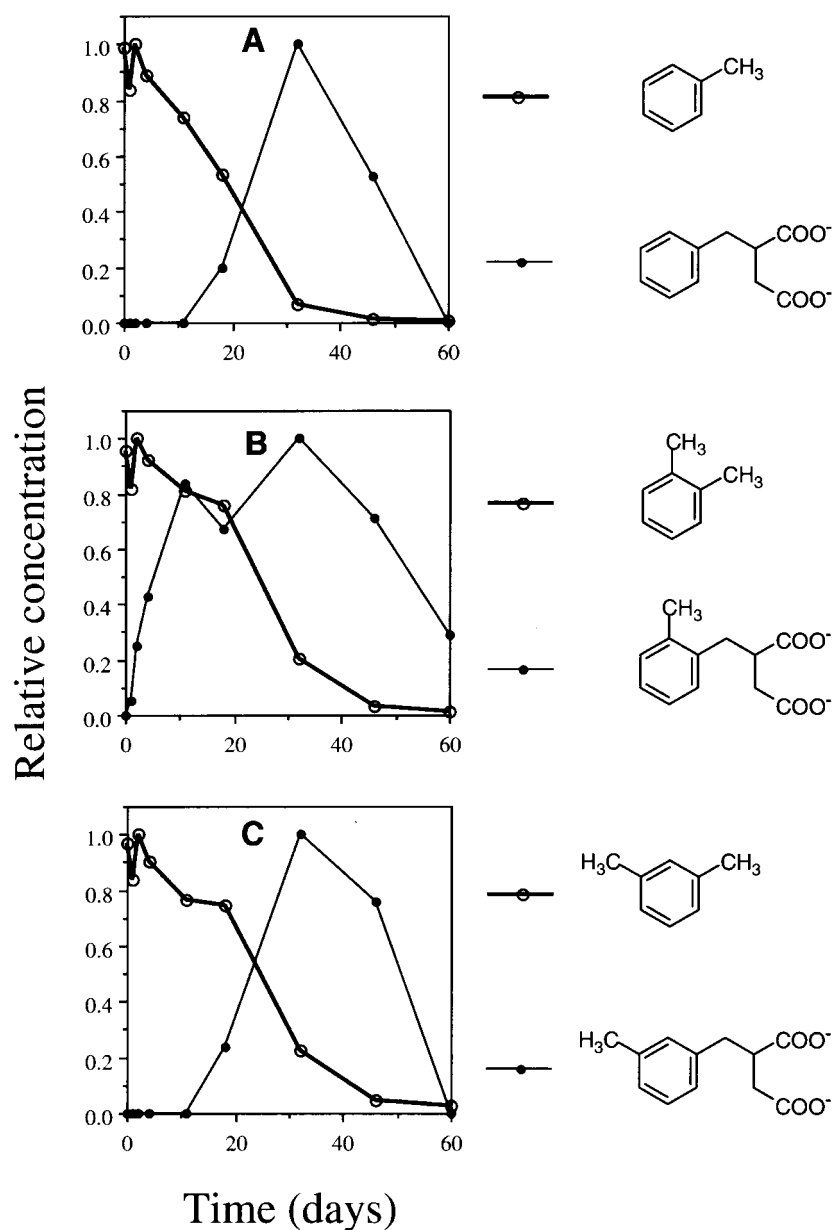
Figure 2. Normalized concentrations of alkylbenzenes and corresponding benzylsuccinate homologs in Seal Beach groundwater versus time: (A) toluene and benzylsuccinate, (B) *o*-xylene and (2-methylbenzyl)succinate, and (C) *m*-xylene and (3-methylbenzyl)succinate. All concentrations are normalized to bromide and to the maximum concentrations of the compounds themselves. Reprinted with permission from Beller et al. (1995). Copyright 1995 American Chemical Society.

in groundwater from a gas condensate-contaminated aquifer in Weld County, Colorado.

Finally, some additional compounds related to benzylsuccinates have been observed at very low concentrations in hydrocarbon-contaminated groundwater. At the Seal Beach site, 3-(*m*-toluyl)propionate was tentatively identified in groundwater and was

hypothesized to derive from anaerobic transformation of *m*-xylene (Beller 1995, Appendix). A related isomer, 3-(*p*-toluyl)propionate was tentatively identified in suspensions of toluene-grown, sulfate-reducing strain PRTOL1 cells that were amended with *p*-xylene (Beller et al. 1996). The formation of such compounds by the benzylsuccinate synthase-
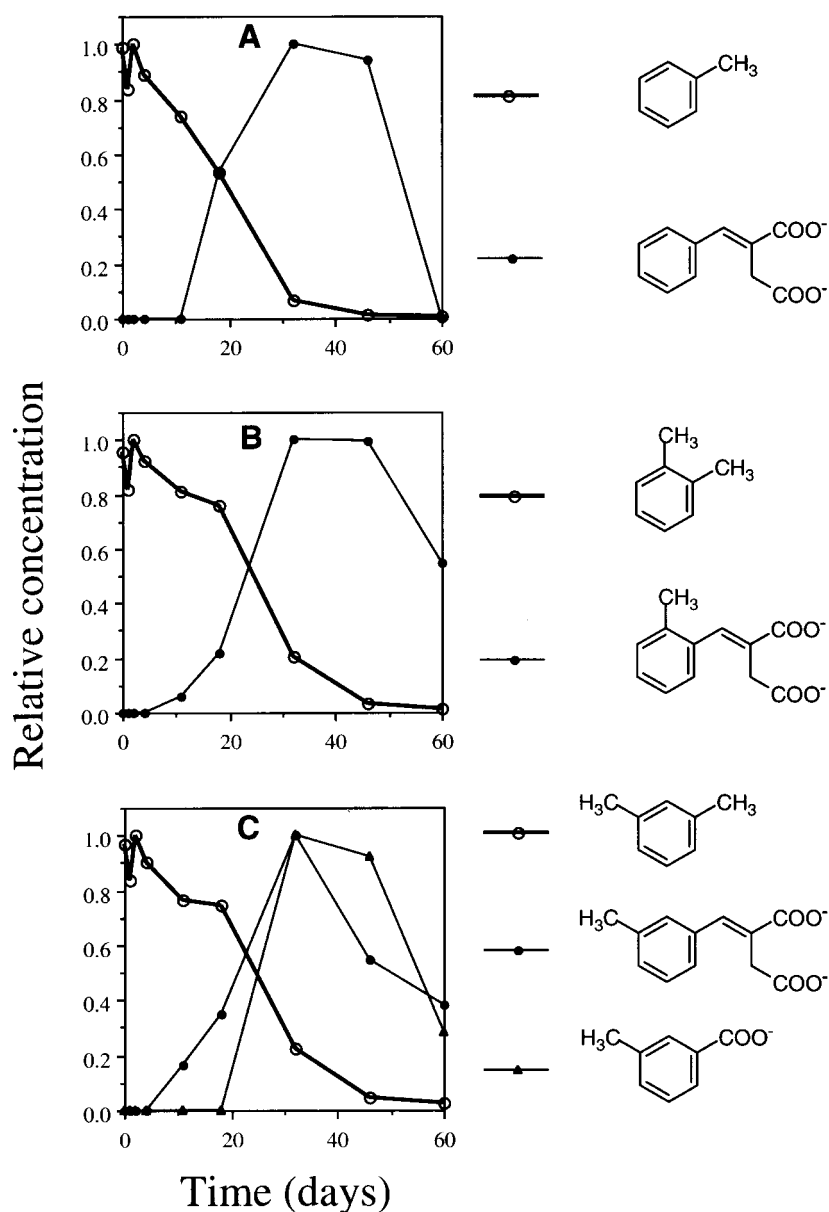
132

Figure 3. Normalized concentrations of alkylbenzenes and corresponding *E*-phenylitaconate homologs (or closely related compounds; see text) in Seal Beach groundwater versus time. *m*-Toluate is also included in C. All concentrations are normalized to bromide and to the maximum concentrations of the compounds themselves. Reprinted with permission from Beller et al. (1995). Copyright 1995 American Chemical Society.

catalyzed addition of fumarate to aromatic aldehydes has been discussed (Beller & Spormann 1999). Another potential fumarate addition product, 3-phenyl-1,2-butanedicarboxylic acid, was reported at trace concentration in groundwater from the Weld County (CO) site and in ethylbenzene-degrading, sulfate-reducing microcosms derived from that site (Gieg & Suflita 1999). Notably, this putative ethylbenzene metabolite was not detected in biochemical studies of anaerobic ethylbenzene degradation by denitrifying strain EbN1 or *Azoarcus* sp. strain EB1 (Ball et al. 1996; Johnson & Spormann 1999; Rabus & Heider 1998) or during *in vitro* tests of the substrate range of benzylsuccinate synthase from toluene-induced *Azoarcus* sp. strain T (Beller & Spormann, unpublished data).
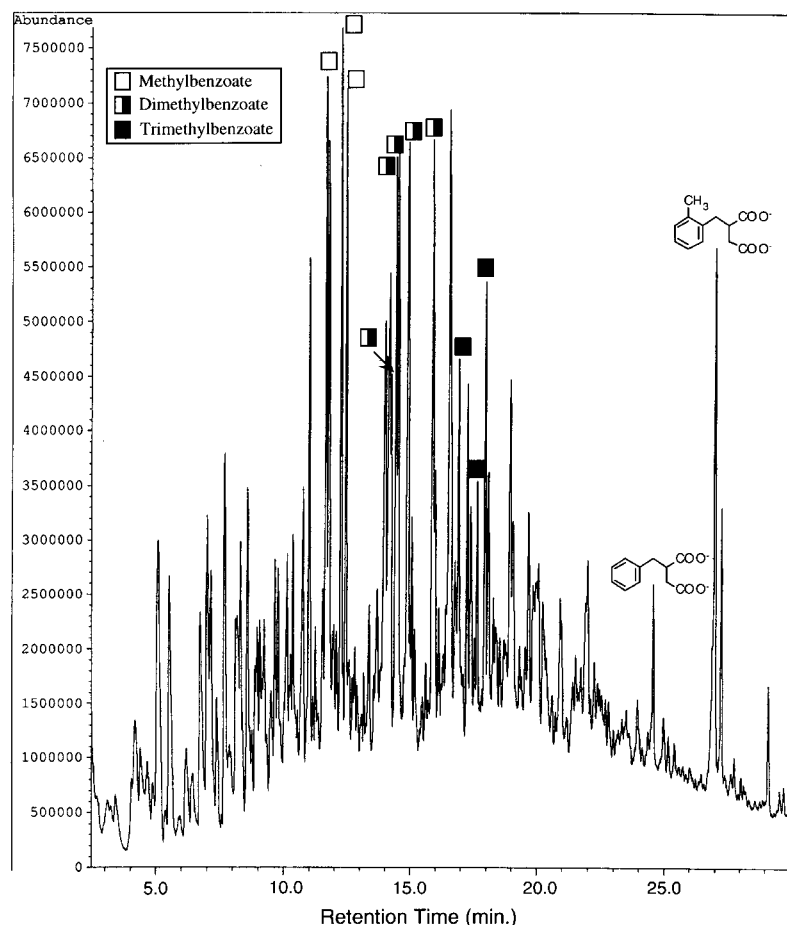
Figure 4. Total ion chromatogram representing a fuel-contaminated groundwater sample from Fallon (NV). The aromatic acids indicated in the chromatogram [benzylsuccinate, (2-methylbenzyl)succinate, and methyl-, dimethyl-, and trimethylbenzoates] were detected as methyl esters, as the sample extract was derivatized. Mass spectra corresponding to the peaks labeled as benzylsuccinate and (2-methylbenzyl)succinate are presented in Figure 5.

Advantages and limitations as indicators

Among the advantages of using benzylsuccinates and *E*-phenylitaconates as indicators of *in situ* anaerobic alkylbenzene metabolism, the most important is their extremely high specificity to their parent hydrocarbons and to anaerobic conditions (they have never been reported in studies of aerobic hydrocarbon degradation; e.g., Gibson & Subramanian 1984; Smith 1990; Chee-Sanford et al. 1996). In fact, these metabolites conform relatively well to all the ideal characteristics cited in Table 1, namely, (i) an unequivocal and unique biochemical relationship to their parent hydrocarbon [e.g., (2-methylbenzyl)succinate is related to *o*-xylene by a fumarate addition reaction and has no other known biochemical sources], (ii) no commercial or industrial uses, (iii) biological and chemical stability (although the Seal Beach study indicates that they may not be highly stable under field conditions), and (iv) generation as intermediates of mineralization rather than as products of cometabolism (although this may not apply to the methyl homologs in all cases). A primary limitation to the use of benzylsuccinates and *E*-phenylitaconates as metabolic indicators is that most of these compounds are not commercially available as standards. Thus, either custom synthesis of standards (e.g., Chee-Sanford et al. 1996) or use of metabolites from laboratory cultures as qualitative standards (e.g., Beller et al. 1995) is required.
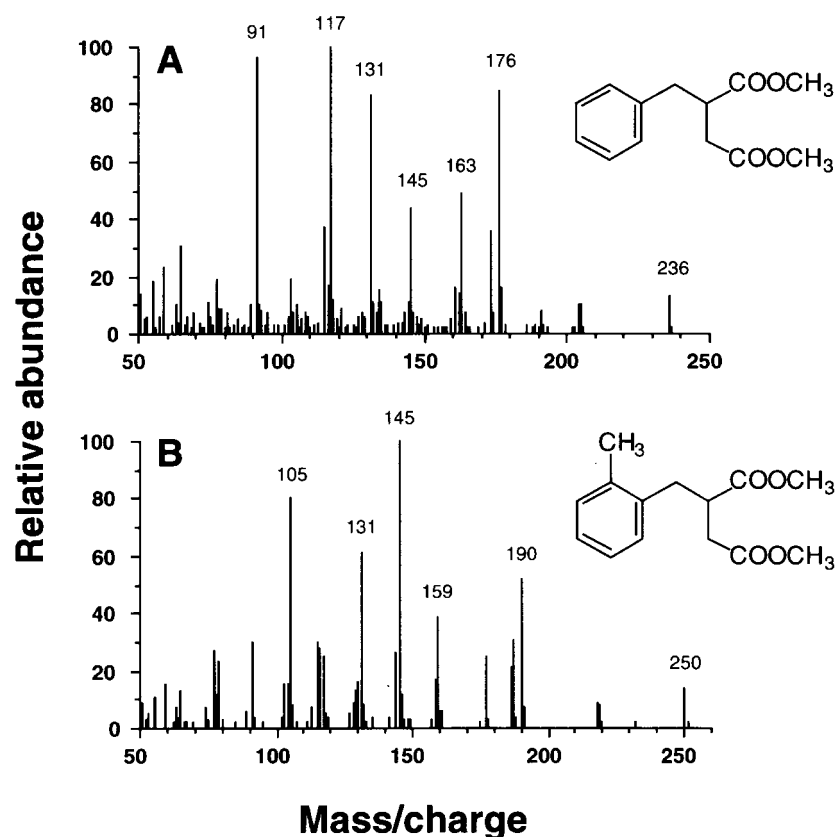
134

Figure 5. Mass spectra of dimethyl esters of benzylsuccinate (identified with an authentic standard) and (2-methylbenzyl)succinate (tentatively identified; see text) from the Fallon (NV) sample represented in Figure 4. For comparison, refer to Beller & Spormann (1997a) for the mass spectra of an authentic benzylsuccinate standard and (2-methylbenzyl)succinate produced as an anaerobic *o*-xylene metabolite by *Azoarcus* sp. strain T.

Benzoate and alkylbenzoates

The structures of benzoate, methylbenzoates (toluates), and di- and trimethylbenzoates are presented in Figure 1.

Laboratory studies

Benzoate

Laboratory studies of anaerobic toluene degradation by a variety of bacterial cultures have shown that benzoate (or its activated form, benzoyl-CoA) is an intermediate of anaerobic toluene mineralization to CO_2 (Beller et al. 1992; Biegert et al. 1996; Chee-Sanford et al. 1996; Frazer et al. 1993; Schocher et al. 1991; Seyfried et al. 1994). Detection of benzoate in the extracellular medium of toluene-degrading cultures has proven to be very difficult unless bacterial metabolism has been somehow retarded, for example by fluoro-

acetate addition or low-temperature incubation (Frazer et al. 1993; Schocher et al. 1991; Seyfried et al. 1994).

Benzoate (or benzoyl-CoA) is also a proven or proposed intermediate in the anaerobic degradation of a wide range of aromatic compounds other than toluene, including ethylbenzene, phenol, 4-hydroxybenzoate, *p*-cresol, benzyl alcohol, benzaldehyde, phenylacetate, 3-phenylpropionate, cinnamate, aniline, 3-chlorobenzoate, and phenylalanine (e.g., Heider & Fuchs 1997 and references therein; Dangel et al. 1991; Schneider et al. 1997; Elder et al. 1992; Ball et al. 1996). Thus, the presence of benzoate in contaminated groundwater does not definitively demonstrate anaerobic toluene degradation.

Methyl-, dimethyl-, and trimethylbenzoates

Benzoate has received far more study as an anaerobic metabolite than the alkylbenzoates. In the context of anaerobic xylene metabolism, methylbenzoates (toluates) have been regarded as both intermediates of

135

mineralization and as dead-end products of cometabolism, primarily the latter. Generalizations about the role of toluates are limited by the fact that biochemical studies of xylene degradation have focused only on *m*-xylene-degrading cultures. *m*-Toluate was reported as an intermediate of *m*-xylene mineralization in studies involving cell suspensions of *m*-xylene-degrading, denitrifying *Azoarcus* sp. strain T (Seyfried et al. 1994). As was the case for benzoate detection in anaerobic toluene-degrading cultures, *m*-toluate detection in anaerobic, *m*-xylene-degrading cultures typically required retarding the degradation rate, for example with fluoroacetate or low-temperature incubation (Seyfried et al. 1994). It is reasonable to assume that *o*- and *p*-toluate will be found to be intermediates of anaerobic *o*- and *p*-xylene mineralization in cultures capable of anaerobic growth on these hydrocarbons, but such pure cultures are either unavailable (for *p*-xylene) or only very recently available (for *o*-xylene; Harms et al. 1999).

To date, *o*- and *p*-toluates have been reported as presumed dead-end products of *o*-or *p*-xylene cometabolism by anaerobic, toluene-degrading cultures. For example, *p*-toluate was found to accumulate as a presumed dead-end product of *p*-xylene transformation in cell suspensions of denitrifying and sulfate-reducing cultures (Beller et al. 1996; Rabus & Widdel 1995; Biegert & Fuchs 1995) and *o*-toluate was observed to be a cometabolic transformation product of *o*-xylene in denitrifying cultures (Arcangeli & Arvin 1995; Jorgensen et al. 1995). In studies of anaerobic xylene transformation by toluene-grown cultures in which all three xylene isomers were tested under the same conditions, yields of *p*-toluate were markedly higher than yields of *o*- or *m*-toluate (Beller et al. 1996; Biegert & Fuchs 1995).

Reports of dimethylbenzoates and trimethylbenzoates as products of anaerobic metabolism of trimethylbenzenes or tetramethylbenzenes, respectively, are exceedingly rare. Laboratory studies of this topic are limited by the absence of isolated bacterial cultures that can grow anaerobically on tri- or tetramethylbenzenes. In anaerobic microcosm experiments containing sediment and groundwater from a crude oil-contaminated aquifer, trimethylbenzoate isomers were detected during the depletion of tetramethylbenzene isomers (Cozzarelli et al. 1994). However, to a large extent, the interpretation of di- and trimethylbenzoates as indicators of *in situ* alkybenzene metabolism appears to be based on analogy to the relationship of toluates to xylenes.

Field studies

Benzoate and alkylbenzoates were first suggested as metabolic indicators of *in situ* alkylbenzene metabolism by Reinhard et al. (1984) in their study of leachate plumes from two landfills in Ontario. This concept was more fully elaborated by Cozzarelli et al. (1990) in a study of an oil-contaminated, anoxic aquifer located near Bemidji, MN. Since 1984, benzoate and/or alkylbenzoates have been reported in groundwater or aquifer solids at 14 hydrocarbon-contaminated sites, including the Fallon (NV) site discussed in this article (Reinhard et al. 1984; Barbaro et al. 1992; Beller et al. 1995; Cozzarelli et al. 1990; 1994; 1995; Fang et al. 1997; Gieg et al. 1999; Kampbell et al. 1996; Levine et al. 1997; Schmitt et al. 1996; Wilson et al. 1990). Benzoate and alkylbenzoate concentrations have not always been reported in these studies, but the observed concentrations of benzoates in groundwater at various sites have been as high as 53 μM ($\sim$6500 μg/L) for benzoate (Cozzarelli et al. 1994), $\sim$2.1 μM ($\sim$290 μg/L) for *o*-toluate (Cozzarelli et al. 1995), 5.1 μM ($\sim$770 μg/L) for total dimethylbenzoates (Cozzarelli et al. 1994), and $\sim$1.6 μM ($\sim$260 μg/L) for 2,4,6-trimethylbenzoate (Cozzarelli et al. 1995). Note that the very high benzoate concentration of 53 μM (Cozzarelli et al. 1994) was from a site contaminated by wood-preserving process wastewater that included diesel fuel, so anaerobic toluene degradation was probably not the only potential source of benzoate in the groundwater. It is difficult to make any generalizations about the relative composition of benzoates in groundwater at these 14 sites because the sites vary so widely in terms of contaminant sources and biogeochemical and hydrogeological conditions.

Notably, for the few studies in which benzylsuccinate and related compounds were target analytes along with benzoates, both groups of compounds were typically detected. For example, in the Fallon (NV) sample represented in Figure 4, methyl-, dimethyl-, and trimethylbenzoates (tentatively identified based on mass spectra) were prominent groundwater constituents along with benzylsuccinate and (2-methylbenzyl)succinate. In the controlled release study at Seal Beach (CA), *m*-toluate (identified with an authentic standard) was observed along with benzylsuccinate, *E*-phenylitaconate, and their methyl homologs (Beller et al. 1995 and Figure 3C). Recall that the only methylbenzenes present in the groundwater in the Seal Beach experiment were toluene,

Table 2. Summary of benzylsuccinates, *E*-phenylitaconates, and benzoates as indicators of *in situ* anaerobic alkylbenzene metabolism

Compound or compound class	Unique biochemical relationship to parent[a]	No commercial/ industrial use[a]	Biological/ chemical stability[a]	Intermediate vs. by-product[a]	Reported in field studies
Benzylsuccinate	+++	+++	++[c]	+++	Beller et al. (1995; Seal Beach, CA) Beller (this study; Fallon, NV)
Methylbenzylsuccinates	+++	+++	++[c]	++[d]	Beller et al. (1995; Seal Beach, CA) Gieg et al. (1999; Weld County, CO) Beller (this study; Fallon, NV)
E-phenylitaconate	+++	+++	++[c]	+++	Beller et al. (1995; Seal Beach, CA)
Methyl-*E*-phenylitaconates	+++	+++	++[c]	++[d]	Beller et al. (1995; Seal Beach, CA)
Benzoate	+	+	+	+++	Reinhard et al. (1984; Woolwich and North Bay, Ontario) Cozzarelli et al. (1990; Bemidji, MN) Wilson et al. (1990; Traverse City, MI) Cozzarelli et al. (1994; Bemidji, MN; Pensacola, FL) Cozzarelli et al. (1995; Galloway, NJ) Schmitt et al. (1996; Dusseldorf, Germany) Fang et al.(1997; Wurtsmith AFB, MI) Gieg et al. (1999; Weld County, CO)
Methylbenzoates (toluates)	++	++	++[c]	++[d]	Reinhard et al. (1984; Woolwich, Ontario) Cozzarelli et al. (1990; Bemidji, MN) Wilson et al. (1990; Traverse City, MI) Barbaro et al. (1992; Borden, Ontario) Cozzarelli et al. (1994; Bemidji, MN) Cozzarelli et al. (1995; Galloway, NJ) Beller et al. (1995; Seal Beach, CA) Levine et al. (1997; Tyndall AFB, FL) Fang et al.(1997; Wurtsmith AFB, MI) Gieg et al. (1999; Weld County, CO) Beller (this study; Fallon, NV)
Di- and trimethylbenzoates	++[b]	+++	++[b]	++[b]	Reinhard et al. (1984; Woolwich and North Bay, Ontario) Cozzarelli et al. (1990; Bemidji, MN) Wilson et al. (1990; Traverse City, MI) Cozzarelli et al. (1994; Bemidji, MN; Galloway, NJ) Cozzarelli et al. (1995; Galloway, NJ) Kampbell et al. (1996; Patrick AFB, FL) Levine et al. (1997; Tyndall AFB, FL) Fang et al.(1997; Wurtsmith AFB, MI) Gieg et al. (1999; Weld County, CO) Beller (this study; Fallon, NV)

[a]The headings are defined more fully in Table 1. The ratings range from + (least favorable) to +++ (most favorable).
[b]Very limited data available.
[c]These compounds are chemically stable and appear to be biologically stable in extracellular medium in laboratory studies (see text); however, they may be biologically depleted in the field (Beller et al. 1995).
[d]These compounds could be intermediates or by-products of anaerobic xylene metabolism, depending on the metabolic capabilities of the bacteria involved (see text).

m-xylene, and *o*-xylene, so the range of possible metabolites was limited. In a study of a gas condensate-contaminated aquifer in Weld County (CO), benzoate, and methyl- and dimethylbenzoates were observed along with methylbenzylsuccinates (Gieg et al. 1999). However, in a study at Tyndall Air Force Base (FL), methyl-, dimethyl-, and trimethylbenzoates were found in jet fuel-contaminated groundwater, whereas benzylsuccinate and *E*-phenylitaconate were looked for but not found (Levine et al. 1997). One cannot use the Tyndall AFB study (Levine et al. 1997) to definitively compare benzoates and benzylsuccinates as metabolic indicators because methylbenzylsuccinates were apparently not target analytes and no potential toluene metabolites of any kind were found (i.e., neither benzoate, benzylsuccinate, nor *E*-phenylitaconate was detected).

Various other aromatic acids have been reported in field studies of hydrocarbon-contaminated groundwater, including phenylacetate and/or tolylacetate (Cozzarelli et al. 1990; 1994; 1995; Gieg et al. 1999; Reinhard et al. 1984; Schmitt et al. 1996), phenol [a potential anaerobic benzene metabolite (Vogel & Grbic-Galic 1986); Schmitt et al. 1996; Wilson et al. 1990], and cresols (Schmitt et al. 1996; Wilson et al. 1990). None of these compounds has been definitively shown to result from anaerobic alkylbenzene metabolism (Heider et al. 1998). In particular, phenylacetate does not appear to be an anaerobic ethylbenzene metabolite based on biochemical studies conducted with denitrifying bacteria (Ball et al. 1996; Rabus & Heider 1998; Johnson & Spormann 1999).

Advantages and limitations as indicators

Perhaps the greatest advantage of benzoates as metabolic indicators of *in situ* anaerobic alkylbenzene metabolism is that they have been observed at a variety of hydrocarbon-contaminated field sites. Benzoates conform moderately well to the four ideal characteristics of metabolic indicators cited in Table 1. Three of the attributes (a unique biochemical relationship to a parent hydrocarbon, no commercial or industrial uses, and biological and chemical stability) apply poorly to benzoate but apply fairly well to the more highly substituted alkylbenzoates. Benzoate (or benzoyl-CoA) is clearly an intermediate of mineralization, but methyl-, dimethyl-, and trimethylbenzoates can be either intermediates or dead-end products of aromatic hydrocarbon metabolism. Unfortunately, the formation of alkylbenzoates from the anaerobic metabolism of di-,

tri-, and tetramethylbenzenes is not well understood. A disadvantage of benzoates as metabolic indicators of anaerobic metabolism is that some of them (benzoate and the toluates) are demonstrated intermediates of aerobic alkylbenzene metabolism (Gibson & Subramanian 1984; Smith 1990). Indeed, aerobic alkylbenzene metabolism must be considered as a possible explanation for finding benzoates but not benzylsuccinates/*E*-phenylitaconates in hydrocarbon-contaminated groundwater.

Conclusions

The evaluation of benzoates, benzylsuccinates, and *E*-phenylitaconates as indicators of *in situ* anaerobic alkylbenzene metabolism is summarized in Table 2. The table includes reports of these indicators in field studies of hydrocarbon-contaminated aquifers, as well as subjective ratings of the indicators with respect to the ideal characteristics cited in Table 1. The rationales supporting the subjective ratings of the indicators have been discussed in the text. In general, benzylsuccinates/*E*-phenylitaconates are superior to benzoates in terms of their very high specificity to their parent hydrocarbons and their lack of commercial and industrial sources. They are also uniquely indicative of anaerobic conditions. All of the benzoates, benzylsuccinates, and *E*-phenylitaconates are relatively stable chemically and (with the exception of benzoate) tend to have comparable biological stability under anaerobic conditions, based on our limited current knowledge. Although benzoate, benzylsuccinate, and *E*-phenylitaconate are intermediates of anaerobic toluene degradation, their methyl homologs can be either intermediates or cometabolic byproducts of alkylbenzenes, depending on the bacteria involved. The distinction between a given compound acting as an intermediate or cometabolite may not be discernible under field conditions, and may not always be of primary concern. Under field conditions in hydrocarbon-contaminated aquifers, compounds generated as dead-end cometabolites by a given bacterial species could serve as substrates for other bacterial species and ultimately could be mineralized by indigenous bacterial consortia. This may explain the eventual depletion of benzylsuccinates, *E*-phenylitaconates, and *m*-toluate observed in the Seal Beach study (Figures 2 and 3). Finally, it is clear that benzoates are far more commonly reported in field studies of hydrocarbon-contaminated aquifers than

are benzylsuccinates and *E*-phenylitaconates. Whether this is an accurate representation of the relative occurrence of these metabolites at contaminated sites, or whether it instead reflects the range of target analytes used in field studies to date, will require more field investigations that include benzoates, benzylsuccinates, and *E*-phenylitaconates as target analytes.

Acknowledgments

References

Aggarwal PK & Hinchee RE (1991) Monitoring *in situ* biodegradation of hydrocarbons by using stable carbon isotopes. Environ. Sci. Technol. 25: 1178–1180

Arcangeli J-P & Arvin E (1995) Cometabolic transformation of *o*-xylene in a biofilm system under nitrate reducing conditions. Biodegradation 6: 19–27

Ball HA, Johnson, HA, Reinhard M & Spormann AM (1996) Initial reactions in anaerobic ethylbenzene oxidation by a denitrifying bacterium, strain EB1. J. Bacteriol. 178: 5755–5761

Barbaro JR, Barker JF, Lemon LA & Mayfield CI (1992) Biotransformation of BTEX under anaerobic denitrifying conditions: Field and laboratory observations. J. Contam. Hydrol. 11: 245–272

Battelle (1998) Assessment of intrinsic remediation at six installation restoration program sites, Naval Air Station Fallon, Fallon, Nevada: Summary of the March and September 1997 Sampling Events. January 20, 1998, Interim Draft Progress Report. Battelle, Columbus, OH

Beller, HR (1995) Ph.D. Thesis, Stanford University, Stanford, CA

Beller HR, Ding W-H & Reinhard M (1995) Byproducts of anaerobic alkylbenzene metabolism useful as indicators of *in situ* bioremediation. Environ. Sci. Technol. 29: 2864–2870

Beller HR, Reinhard M & Grbic-Galic D (1992) Metabolic by-products of anaerobic toluene degradation by sulfate-reducing enrichment cultures. Appl. Environ. Microbiol. 58: 3192–3195

Beller HR & Spormann AM (1997a) Anaerobic activation of toluene and *o*-xylene by addition to fumarate in denitrifying strain T. J. Bacteriol. 179: 670-676

Beller HR & Spormann AM (1997b) Benzylsuccinate formation as a means of anaerobic toluene activation by sulfate-reducing strain PRTOL1. Appl. Environ. Microbiol. 63: 3729–3731

Beller HR & Spormann AM (1998) Analysis of the novel benzylsuccinate synthase reaction for anaerobic toluene activation based on structural studies of the product. J. Bacteriol. 180: 5454–5457

Beller HR & Spormann AM (1999) Substrate range of benzylsuccinate synthase from *Azoarcus* sp. strain T. FEMS Microbiol. Letters 178: 147–153

Beller HR, Spormann AM, Sharma PK, Cole JR & Reinhard M (1996) Isolation and characterization of a novel toluene-degrading, sulfate-reducing bacterium. Appl. Environ. Microbiol. 62: 1188–1196

Biegert T & Fuchs G (1995) Anaerobic oxidation of toluene (analogues) to benzoate (analogues) by whole cells and by cell extracts of a denitrifying *Thauera* sp. Arch. Microbiol. 163: 407–417

Biegert T, Fuchs G & Heider J (1996) Evidence that anaerobic oxidation of toluene in the denitrifying bacterium *Thauera aromatica* is initiated by formation of benzylsuccinate from toluene and fumarate. Eur. J. Biochem. 238: 661–668

Chee-Sanford JC, Frost JW, Fries MR, Zhou J & Tiedje JM (1996) Evidence for acetyl coenzyme A and cinnamoyl coenzyme A in the anaerobic toluene mineralization pathway in *Azoarcus tolulyticus* Tol-4. Appl. Environ. Microbiol. 62: 964–973

Coleman WE, Munch JW, Streicher RP, Ringhand HP & Kopfler FC (1984) The identification and measurement of components in gasoline, kerosene, and No. 2 fuel oil that partition into the aqueous phase after mixing. Arch. Environ. Contam. Toxicol. 13: 171–178

Cozzarelli IM, Baedecker MJ, Eganhouse RP & Goerlitz DF (1994) The geochemical evolution of low-molecular-weight organic acids derived from the degradation of petroleum contaminants in groundwater. Geochim. Cosmochim. Acta 58: 863–877

Cozzarelli IM, Eganhouse RP & Baedecker MJ (1990) Transformation of monoaromatic hydrocarbons to organic acids in anoxic groundwater environment. Environ. Geol. Water Sci. 16: 135–141

Cozzarelli IM, Herman JS & Baedecker MJ (1995) Fate of microbial metabolites of hydrocarbons in a coastal plain aquifer: the role of electron acceptors. Environ. Sci. Technol. 29: 458–469

Dangel W, Brackmann R, Lack A, Mohamed M, Koch J, Oswald B, Seyfried B, Tschech A & Fuchs G (1991) Differential expression of enzyme activities initiating anoxic metabolism of various aromatic compounds via benzoyl-CoA. Arch. Microbiol. 155: 256–262

Elder DJE, Morgan P & Kelly DJ (1992) Anaerobic degradation of *trans*-cinnamate and ω-phenylalkane carboxylic acids by the photosynthetic bacterium *Rhodopseudomonas palustris*: evidence for β-oxidation mechanism Arch. Microbiol. 157:148–154

Evans PJ, Ling W, Goldschmidt B, Ritter ER & Young LY (1992) Metabolites formed during anaerobic transformation of toluene and *o*-xylene and their proposed relationship to the initial steps of toluene mineralization. Appl. Environ. Microbiol. 58: 496–501

Evans PJ, Mang DT, Kim KS & Young LY (1991) Anaerobic degradation of toluene by a denitrifying bacterium. Appl. Environ. Microbiol. 57: 1139–1145

Fang J, Barcelona MJ & West C (1997) The use of aromatic acids and phospholipid-ester-linked fatty acids for delineation of processes affecting an aquifer contaminated with JP-4 fuel. In: Eganhouse RP (Ed) Molecular Markers in Environmental Geochemistry (pp 65–76). American Chemical Society, Washington, DC

Frazer AC, Ling W & Young LY (1993) Substrate induction and metabolite accumulation during anaerobic toluene utilization by the denitrifying strain T1. Appl. Environ. Microbiol. 59: 3157–3160

Gibson DT & Subramanian V (1984) Microbial degradation of aromatic hydrocarbons. In: Gibson DT (Ed) Microbial Degradation of Organic Compounds (pp 181–252). Marcel Dekker, Inc., New York

Gieg LM, Kolhatkar RV, Tanner RS, Harris SH, Sublette KL & Suflita JM (1999) Intrinsic bioremediation of petroleum hydrocarbons in a gas condensate-contaminated aquifer. Environ. Sci. Technol. 33: 2550–2560

Gieg LM & Suflita JM (1999) Anaerobic biodegradation of alkylbenzenes in a hydrocarbon-contaminated aquifer. Poster presen-

tation at the 99th General Meeting of the American Society of Microbiology.

Harms G, Zengler K, Rabus R, Aeckersberg F, Minz D, Rosselló-Mora R & Widdel F (1999) Anaerobic oxidation of *o*-xylene, *m*-xylene, and homologous alkylbenzenes by new types of sulfate-reducing bacteria. Appl. Environ. Microbiol. 65: 999–1004

Heider J & Fuchs G (1997) Anaerobic metabolism of aromatic compounds. Eur. J. Biochem. 243: 577–596

Heider J., Spormann AM, Beller HR & Widdel F(1998) Anaerobic bacterial metabolism of hydrocarbons. FEMS Microbiol. Reviews 22: 459–473

Johnson HA & Spormann AM (1999) *In vitro* studies on the initial reactions of anaerobic ethylbenzene mineralization. J. Bacteriol. 181: 5662–5668

Jorgensen C, Jensen BK & Mortensen E (1995) Transformation of *o*-xylene to *o*-methylbenzoic acid by a denitrifying enrichment culture using toluene as the primary substrate. Biodegradation 6: 141–146

Kampbell DH, Wiedemeier TH & Hansen JE (1996) Intrinsic bioremediation of fuel contamination in ground water at a field site. Journal of Hazardous Materials 49: 197–204

Krieger CJ, Beller HR, Reinhard M. & Spormann AM (1999) Initial reactions in anaerobic oxidation of *m*-xylene by the denitrifying bacterium *Azoarcus* sp. strain T. J. Bacteriol. 181: 6403–6410

Landmeyer JE, Vroblesky DA & Chapelle F (1996) Stable carbon isotope evidence of biodegradation zonation in a shallow jet-fuel contaminated aquifer. Environ. Sci. Technol. 30: 1120–1128

Lee MD, Thomas JM, Borden RC, Bedient PB, Wilson JT & Ward CH (1988) Biorestoration of aquifers contaminated with organic compounds. CRC Critical Reviews in Environmental Control 18: 29–89

Levine AD, Libelo EL, Bugna G, Shelley T, Mayfield H & Stauffer TB (1997) Biogeochemical assessment of natural attenuation of JP-4-contaminated ground water in the presence of fluorinated surfactants. The Science of the Total Environment 208: 179–195

Lovley DR & Goodwin S (1988) Hydrogen concentrations as an indicator of the predominant terminal electron-accepting reactions in aquatic sediments. Geochim. Cosmochim. Acta 52: 2993–3003

Madsen EL (1991) Determining *in situ* biodegradation. Environ. Sci. Technol. 25: 1662–1673

National Research Council (1993) *In situ* Bioremediation: When Does it Work? National Academy Press, Washington, DC

Rabus R & Heider J (1998) Initial reactions of anaerobic metabolism of alkylbenzenes in denitrifying and sulfate-reducing bacteria. Arch. Microbiol. 170: 377–384

Rabus R & Widdel F (1995) Conversion studies with substrate analogues of toluene in a sulfate-reducing bacterium, strain Tol2. Arch. Microbiol. 164: 448–451

Reinhard M, Goodman NL & Barker JF (1984) Occurrence and distribution of organic chemicals in two landfill leachate plumes. Environ. Sci. Technol. 18: 953–961

Reinhard M, Shang S, Kitanidis PK, Orwin E, Hopkins GH & Lebron CA (1997) *In situ* BTEX biotransformation under enhanced nitrate- and sulfate-reducing conditions. Environ. Sci. Technol. 31: 28–36

Schmitt R, Langguth H-R, Püttmann W, Rohns HP, Eckert P & Schubert J (1996) Biodegradation of aromatic hydrocarbons under anoxic conditions in a shallow sand and gravel aquifer of the Lower Rhine Valley, Germany. Org. Geochem. 25: 41–50

Schneider S, Mohamed ME-S & Fuchs G (1997) Anaerobic metabolism of L-phenylalanine via benzoyl-CoA in the denitrifying bacterium *Thauera aromatica*. Arch. Microbiol. 168:310–320

Schocher RJ, Seyfried B, Vazquez F & Zeyer J (1991) Anaerobic degradation of toluene by pure cultures of denitrifying bacteria. Arch. Microbiol. 157: 7–12

Seyfried B, Glod G, Schocher R, Tschech A & Zeyer J (1994) Initial reactions in the anaerobic oxidation of toluene and *m*-xylene by denitrifying bacteria. Appl. Environ. Microbiol. 60: 4047–4052

Sittig M (1985) Handbook of Toxic and Hazardous Chemicals and Carcinogens, 2nd ed. Noyes Publications, Park Ridge, NJ

Smith MR (1990) The biodegradation of aromatic hydrocarbons by bacteria. Biodegradation 1: 191–206

US Environmental Protection Agency (1998) Office of Underground Storage Tanks web site. http://www.epa.gov/swerust1/fsclenup.htm (last updated 4/15/98)

US Environmental Protection Agency (1999) Use of Monitored Natural Attenuation at Superfund, RCRA Corrective Action, and Underground Storage Tank Sites. OSWER Directive 9200.4-17P, Final, April 21, 1999, US EPA, Washington, DC

U.S. House of Representatives (1988) "Underground storage tanks: Hearing before the Subcommittee on Energy and Agriculture of the Committee on Small Business", November 18, 1987; U.S. Government Printing Office: Washington, DC

Vogel TM & Grbic-Galic D (1986) Incorporation of oxygen from water into toluene and benzene during anaerobic fermentative transformation. Appl. Environ. Microbiol. 52: 200–202

Wiedemeier T, Wilson JT, Kampbell DH, Miller RN & Hansen JE (1995) Technical protocol for implementing intrinsic remediation with long-term monitoring for natural attenuation of fuel contamination dissolved in groundwater. Air Force Center for Environmental Excellence, San Antonio, TX

Wilson BH, Wilson JT, Kampbell DH, Bledsoe BE & Armstrong JM (1990) Biotransformation of monoaromatic and chlorinated hydrocarbons at an aviation gasoline spill site. Geomicrobiology Journal 8: 225–240