Anaerobic Biodegradation of Aromatic Hydrocarbons: Pathways and Prospects

Julia Foght

Biological Sciences, University of Alberta, Edmonton Alta., Canada

Key Words

Anaerobic hydrocarbon biodegradation • Benzylsuccinate synthase • Fumarate addition • In situ bioremediation • Benzene • Toluene • Polycyclic aromatic hydrocarbons • Naphthalene • Natural attenuation

Abstract

Aromatic hydrocarbons contaminate many environments worldwide, and their removal often relies on microbial bioremediation. Whereas aerobic biodegradation has been well studied for decades, anaerobic hydrocarbon biodegradation is a nascent field undergoing rapid shifts in concept and scope. This review presents known metabolic pathways used by microbes to degrade aromatic hydrocarbons using various terminal electron acceptors; an outline of the few catabolic genes and enzymes currently characterized; and speculation about current and potential applications for anaerobic degradation of aromatic hydrocarbons.

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Introduction

Aromatic hydrocarbons enter the global environment through human activities such as crude oil spillage, fossil fuel combustion and gasoline leakage as well as natural inputs like forest fire smoke and natural petroleum seepage. These hydrocarbons comprise simple aromatics like

benzene and toluene as well as polycyclic aromatic hydrocarbons (PAHs) from naphthalene to pyrenes, as well as myriad alkyl-substituted isomers. Annually, large inputs of such compounds impact both aerobic and anaerobic environments such as aquifers, surface freshwater bodies, soils, and terrestrial and marine sediments. Whereas aerobic biodegradation of both aromatic and saturated hydrocarbons has been well known and studied for many years, the documentation of anaerobic degradation is relatively recent (within the last two decades) and is constantly generating new insights. This activity is widespread, having been reported under nitrate-, iron-, manganese- and sulfate-reducing conditions as well as methanogenic conditions. Initial observations were limited to enrichment cultures and in situ sediments or ground waters, but a few pure cultures capable of anaerobic degradation of aromatic hydrocarbons have now been isolated and characterized. Such cultures have initiated the elucidation of degradative pathways, intermediates, and genes encoding key enzymes. At the same time, pathways and metabolites have been inferred from complementary studies of mixed populations using analytical chemical methods to demonstrate that laboratory results are relevant in situ. It is now clear that some key enzymatic steps in anaerobic hydrocarbon biodegradation involve novel biochemistry and versatile microbiota. For lists of organisms reported to degrade specific hydrocarbons, the reader is directed to reviews by Rabus [2005a] and Bonin et al. [2004].

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Julia Foght
Biological Sciences, University of Alberta
Edmonton, Alta. T6G 2E9 (Canada)
Tel. +1 780 492 3279, Fax +1 780 492 9234, E-Mail julia.foght@ualberta.ca

This review summarizes the literature on anaerobic biodegradation of monoaromatics and polycyclic aromatic hydrocarbons (PAHs) both in situ and in laboratory studies, documenting the known pathways, enzymes and genes, and reporting the prevalent terminal electron acceptor (TEA) conditions associated with each activity. This latter information is essential to understanding and manipulating biodegradation processes for potential application in situ.

Because the field is burgeoning, the literature on anaerobic hydrocarbon degradation has been reviewed quite recently; see, for example, Widdel and Rabus [2001] and Meckenstock et al. [2004b]. Even so, those reviews and sections of the current article likely will soon be outdated because of rapid progress in this field as new reports of substrates, pure cultures, enzyme activities and genome sequencing contribute to our understanding of anaerobic biodegradation. The scope of this review is limited to aromatic hydrocarbons such as those comprising crude oil and refined petroleum products; for saturated hydrocarbon biodegradation the reader is directed to the review by Widdel and Rabus [2001] and cited literature therein.

Substrates, Pathways and Terminal Electron Acceptors

Initial activation of hydrocarbons is crucial for anaerobic biodegradation, and four general enzymatic reactions are recognized: (1) addition of fumarate, catalyzed by a glycyl radical enzyme to yield aromatic-substituted succinates [reviewed by Widdel and Rabus, 2001]; (2) methylation of unsubstituted aromatics [Safinowski and Meckenstock, 2006]; (3) hydroxylation of an alkyl substituent via a dehydrogenase [Rabus and Widdel, 1995], and (4) direct carboxylation [Zhang and Young, 1997], which may actually represent a combination of reaction (2) followed by reaction (1). These activation reactions feed into pathways that result in ring saturation, β -oxidation and/or ring cleavage reactions, producing central metabolites such as benzoyl-coA that are eventually incorporated into biomass or completely oxidized [Harwood et al., 1999]. However, in the discussions below, it is important to note that documentation of substrate removal (without concomitant detection of CO₂ production or biomass increase) may not represent substantial oxidation of the substrate. In some cases the connection between substrate loss and product appearance was not closed experimentally, and partial oxidation to dead-end

products (co-metabolites) may have been misinterpreted as significant metabolism. When mineralization to CO₂ and/or production of CH₄ was measured, this result is reported; such conclusions about biodegradation are strengthened by the use of isotopically labeled (¹³C or ¹⁴C) substrates. Equally important is the reminder that many of the reports below have arisen from incubation of mixed cultures or undefined consortia in situ, and the observed degradation may result from the combined activities of consortium members.

Monoaromatics

BTEX components (benzene, toluene, ethylbenzene and xylene isomers) are the best-studied substrates of anaerobic biodegradation. Interest in these compounds results from the fact that they comprise a significant proportion of conventional fuels such as gasoline, are the most water-soluble of the aromatic hydrocarbons (and therefore most likely to be mobile in situ and biodegradable) and also are reported to have acute toxicity as well as long-term potential carcinogenic effects [Dean, 1985; Snyder, 2000]. Because BTEX compounds have different susceptibilities to anaerobic attack, they are discussed individually below. The major pathways for anaerobic BTEX degradation are presented in abbreviated form in figures 1 and 2. The reader is also directed to a recent mini-review [Chakraborty and Coates, 2004] on anaerobic biodegradation of monoaromatic hydrocarbons.

Benzene

Although benzene can be biodegraded aerobically, its thermodynamic stability dictates that enzymatic attack is very difficult to initiate under anaerobic conditions, commonly resulting in its persistence in anaerobic cultures [Langenhoff et al., 1996]. Its relatively high water solubility and known toxicity combined with apparent chemical and biological stability in situ [reviewed by Johnson et al., 2003] make it a priority pollutant despite its relatively low proportion in many petroleum contaminants. The occurrence and rate of biodegradation appears to be more site-specific for benzene than other BTEX components and is subject to inhibition by those co-contaminants [Johnson et al., 2003; Nales et al., 1998]; degradation is usually slow, incomplete and subject to long lag times [Edwards and Grbić-Galić, 1992].

Anaerobic benzene-degrading enrichment cultures are not very common [Johnson et al., 2003; Ulrich et al., 2005] and pure isolates even more rare [Coates et al., 2001a; Kasai et al., 2006]. It is ironic, therefore, that the first anaerobic cultures unequivocally demonstrating ar-

Fig. 1. Three of the anaerobic degradation pathways proposed for benzene. Square brackets indicate a postulated intermediate; broken arrows indicate multiple enzymatic steps; open arrows indicate further metabolism. **a** Hydroxylation to form phenol, cyclohexanone, or *p*-hydroxybenzoate and benzoyl-CoA [Caldwell and Suflita, 2000; Grbić-Galić and Vogel, 1987]. The hydroxyl donor under methanogenic conditions is postulated to be H₂O [Vogel and Grbić-Galić, 1986] or, under nitrate-reducing conditions with *Dechloromonas aromatica* RCB, a hydroxyl free radical [Chakraborty and Coates, 2005]. **b** Alkylation to form toluene, followed by fumarate addition to form benzylsuccinate and benzoyl-CoA [Coates et al., 2002; Ulrich et al., 2005]. The methyl donor may be methyltetrahydrofolate, S-adenosylmethionine or a cobalamin protein [Coates et al., 2002; Ulrich et al., 2005]. **c** Carboxylation to form benzoate (possibly through more than one enzymatic step) and benzoyl-CoA. The carboxyl donor is not likely bicarbonate [Phelps et al., 2001] but may be derived from benzene [Caldwell and Suflita, 2000].

omatic hydrocarbon degradation were benzene degraders; methanogenic cultures from Dunja Grbić-Galić's laboratory in the late 1980s [Grbić-Galić and Vogel, 1987; Vogel and Grbić-Galić, 1986] led the way in documenting anaerobic BTEX degradation and in proposing pathway intermediates.

In subsequent studies with enrichments, Edwards and Grbić-Galić [1992] measured 90% mineralization of ¹⁴C-benzene to ¹⁴CO₂, and although the actual TEA was not established in that study, sulfate was a likely candidate. Similar levels of mineralization were later documented under methanogenic [Kazumi et al., 1997], iron- [Anderson et al., 1998; Jahn et al., 2005; Kazumi et al., 1997; Lov-

ley et al., 1994], sulfate- [Kazumi et al., 1997; Lovley et al., 1995], and manganese-reducing conditions [Villatoro-Monzón et al., 2003]. Lovley et al. [1994] found that benzene degradation in sediments incubated in microcosms under iron-reducing conditions was enhanced in the presence of iron chelators such as nitrilotriacetic acid, humic acids, EDTA, etc. [Lovley et al., 1996] that increase bioavailability of that electron acceptor. However, no pure cultures of iron-reducing, benzene-degrading bacteria have yet been isolated [Botton and Parsons, 2006], and Phelps et al. [1998] noted that, despite long-term (3-year) enrichment of a sulfate-reducing culture with benzene as the sole carbon source, pure benzene-degrading

Fig. 2. Anaerobic pathways for degradation of alkylbenzenes. Broken arrows indicate multiple enzymatic steps and open arrows indicate further metabolism. **a** Toluene (xylene) activation by fumarate addition to form (methyl)benzylsuccinate, dehydration to (methyl)-*E*-phenyllitaconate and/or (methyl)-phenyllitaconyl-CoA, eventually forming (methyl)benzoyl-CoA [Heider et al., 1999; Leutwein and Heider, 1999]. The CoA donor may be succinyl-CoA [Heider et al., 1999]. **b** Ethylbenzene degradation under sulfate-reducing conditions via fumarate addition to form phenylethylsuccinate [Kniemeyer et al., 2003] leading to benzoyl-CoA. **c** Ethylbenzene degradation under nitrate-reducing conditions to form 1-phenylethanol, acetophenone, benzoylacetate and benzoyl-CoA [Ball et al., 1996; Kniemeyer and Heider, 2001; Rabus and Heider, 1998].

strains resisted isolation and the consortium remained quite complex, comprising at least 12 distinct phylotypes.

Early studies reported that benzene was not degraded under denitrifying conditions [Ball and Reinhard, 1996; Kazumi et al., 1997; Kuhn et al., 1988], and Anderson and Lovley [2000] found that addition of nitrate to a benzene-degrading consortium completely inhibited benzene degradation. However, Burland and Edwards [1999] conclusively linked benzene biodegradation to nitrate reduction in enrichment cultures, with 92–95% of ^{14}C -benzene recovered as $^{14}\text{CO}_2$ and the remainder presumably incorporated into biomass. Benzene oxidation in that study

also seemed to be more dependent on nitrate reduction to nitrite than complete reduction to dinitrogen gas (N_2), and benzene utilization corresponded to a lower cell yield than predicted by the free energy of the reaction. Similarly, Ulrich and Edwards [2003] found that benzene degradation under nitrate-reducing conditions was less efficient than predicted by the theoretical free energy available (table 1). The intermittent reports of benzene oxidation under denitrifying conditions appear to be site-specific and unpredictable; its degradation under these conditions may rely on the presence of specific consortium members capable of initiating attack on benzene rather than those metabolizing the oxidized intermedi-

Table 1. Stoichiometric equations and standard free energy changes for anaerobic degradation of selected aromatic hydrocarbons

Substrate	TEA	Equation	ΔG° (kJ/mol e-donor)	Source
Benzene	nitrate	$C_6H_6 + 6 NO_3 \rightarrow 6 HCO_3^- + 3 N_2$	-2,990	Ulrich and Edwards [2003]
		$C_6H_6 + 15 NO_3^- + 3 H_2O \rightarrow 6 HCO_3^- + 15 NO_2^- + 6 H^+$	-2,000	Ulrich and Edwards [2003]
Benzene	iron(III)	$C_6H_6 + 30 Fe(III) + 12 H_2O \rightarrow 30 Fe(II) + 6 CO_2 + 30 H^+$	n.r. ^a	Lovley et al. [1994]
		$C_6H_6 + 90 Fe(OH)_3 \rightarrow 6 HCO_3^- + 132 H_2O + 30 Fe_3O_4 + 6 H^+$	-2,660	Ulrich and Edwards [2003]
Benzene	chlorate	$C_6H_6 + 5 ClO_3^- \rightarrow 6 CO_2 + 5 Cl^- + 3 H_2O$	n.r.	Coates et al. [2002]
Benzene	sulfate	$C_6H_6 + 3 H_2O + 3.75 SO_4^{2-} \rightarrow 6 HCO_3^- + 1.875 HS^- + 1.875 H_2S + 0.375 H^+$	n.r.	Caldwell and Suflita [2000]
		$C_6H_6 + 3 H_2O + 3.75 SO_4^{2-} \rightarrow 6 HCO_3^- + 1.88 H_2S + 1.88 HS + 0.38 H^+$	-200	Ulrich and Edwards [2003]
Benzene	carbon dioxide	$C_6H_6 + 4.5 H_2O \rightarrow 2.25 CO_2 + 3.75 CH_4$	n.r.	Kazumi et al. [1997]
		$C_6H_6 + 6.75 H_2O \rightarrow 2.25 HCO_3^- + 3.75 CH_4 + 2.25 H^+$	-116	Ulrich and Edwards [2003]
Toluene	nitrate	$C_7H_8 + 7.2 NO_3^- + 7.2 H^+ \rightarrow 3.6 N_2 + 7.6 H_2O + 7 CO_2$	n.r.	Cunningham et al. [2001]
		$C_7H_8 + 7.2 NO_3^- + 0.2 H^+ \rightarrow 7 HCO_3^- + 3.6 N_2 + 0.6 H_2O$	-3,554	Heider et al. [1999]
Toluene	iron(III)	$C_7H_8 + 36 Fe(III) + 21 H_2O \rightarrow 36 Fe(II) + 7 HCO_3^- + 43 H^+$	n.r.	Cunningham et al. [2001]
		$C_7H_8 + 94 Fe(OH)_3 \rightarrow 7 FeCO_3 + 29 Fe_3O_4 + 145 H_2O$	-3,398	Heider et al. [1999]
Toluene	sulfate	$C_7H_8 + 4.5 SO_4^{2-} + 3 H_2O \rightarrow 7 HCO_3^- + 2.25 HS^- + 2.25 H_2S + 0.25 H^+$	-55	Edwards et al. [1992]
		$C_7H_8 + 4.5 SO_4^{2-} + 3 H_2O \rightarrow 7 HCO_3^- + 4.5 HS^- + 2.5 H^+$	-205	Heider et al. [1999]
Toluene	carbon dioxide	$C_7H_8 + 7.5 H_2O \rightarrow 4.5 CH_4 + 2.5 HCO_3^- + 2.5 H^+$	-131	Edwards and Grbić-Galić [1994]
Xylenes	iron(III)	$C_8H_{10} + 42 Fe(III) + 16 H_2O \rightarrow 42 Fe(II) + 8 CO_2 + 32 H^+$	n.r.	Botton and Parsons [2006]
Xylenes	sulfate	$C_8H_{10} + 5.25 SO_4^{2-} + 3 H_2O \rightarrow 8 HCO_3^- + 2.625 HS^- + 2.625 H_2S + 0.125 H^+$	-64	Edwards et al. [1992]
Xylenes	carbon dioxide	$C_8H_{10} + 8.25 H_2O \rightarrow 5.25 CH_4 + 2.75 HCO_3^- + 2.75 H^+$	-169	Edwards and Grbić-Galić [1994]
Ethylbenzene	nitrate	$C_8H_{10} + 8.4 NO_3^- + 0.4 H^+ \rightarrow 8 HCO_3^- + 1.2 H_2O + 4.2 N_2$	-4,148	Rabus and Widdel [1995]
Ethylbenzene	carbon dioxide	$C_8H_{10} + 5.25 CO_2 + 13.5 H_2O \rightarrow 8 HCO_3^- + 5.25 CH_4 + 8 H^+$	n.r.	Adapted from Zwolinski et al. [2000]
Naphthalene	nitrate ^b	$1/48 C_8H_{10} + (1 - f_s)/5 NO_3^- + [6(1 - f_s)/5 + f_s - 1] H^+ + f_s/20 NH_3 \rightarrow$	n.r.	Rockne et al. [2000]
		$[5/24 - f_s/4] CO_2 + (1 - f_s)/10 N_2 + f_s/20 C_5H_7O_2N + [11/60 - f_s/5] H_2O$		
		$1/48 C_8H_{10} + (1 - f_s)/2 NO_3^- + f_s/20 NH_3 \rightarrow$	n.r.	Rockne et al. [2000]
		$[5/24 - f_s/4] CO_2 + (1 - f_s)/2 NO_2^- + f_s/20 C_5H_7O_2N + [1/12 - f_s/10] H_2O$		
Naphthalene	carbon dioxide ^c	$C_8H_{10} + H_2O \rightarrow 5 CH_3COOH + 4 H_2$	-78	Christensen et al. [2004]
Phenanthrene	nitrate	$C_{14}H_{10} + 13.2 H^+ + 13.2 NO_3^- \rightarrow 14 CO_2 + 11.6 H_2O + 6.6 N_2$	-24	Tang et al. [2005]
Phenanthrene	sulfate	$C_{14}H_{10} + 8.25 SO_4^{2-} + 9 H_2O \rightarrow 14 HCO_3^- + 4.125 HS^- + 4.125 H_2S + 1.625 H^+$	-1.5	Tang et al. [2005]

^a Not reported.^b f_s is the fraction of electron donor coupled to cell synthesis.^c Partial reaction presented; subsequent acetoclastic methanogenesis is assumed.

ates, since pathway intermediates like benzoate are readily degraded with all TEAs. Caldwell et al. [1999] suggested, instead, that these sporadic observations are due to an indirect effect of nitrate-dependent oxidation of Fe(II) to Fe(III) in some anaerobic environments containing both nitrate and Fe(II), generating a more suitable TEA for benzene degradation. Recently, two pure cultures identified as *Dechloromonas* spp. strains RCB and JJ were isolated through their ability to mineralize benzene concomitant with nitrate reduction [Coates et al., 2001a]. Strain RCB, now identified as *Dechloromonas aromatica* RCB (GenBank accession number CP000089), can also oxidize benzene using chlorate or oxygen as TEAs. Although its genome has now been fully sequenced (http://genome.jgi-psf.org/draft_microbes/decar/decar.home.html), no genes or enzymes have yet been assigned

to initiating benzene-degrading activity in this or any other organism.

The pathway for anaerobic benzene degradation is the subject of considerable debate, with five mechanisms having been proposed for initiating anaerobic attack on benzene [reviewed by Coates et al., 2002]. Two of these pathways have little support in the literature: although the most common mode of initial activation of methyl-substituted aromatics is fumarate addition (see discussion below), it may be that the large activation energy required to remove hydrogen from the benzene ring precludes this mechanism for initiating benzene metabolism; and a proposed pathway involving initial attack by ring saturation has garnered little supporting evidence [Coates et al., 2002]. Three plausible pathways are presented in figure 1: pathway A – hydroxylation, producing phenol

[Grbić-Galić and Vogel, 1987] with subsequent carboxylation to the postulated intermediate *para*-hydroxybenzoate [Chakraborty and Coates, 2005] or ring reduction to yield cyclohexanone [Grbić-Galić and Vogel, 1987]; pathway B – methylation, producing toluene [Coates et al., 2002; Ulrich et al., 2005] followed by fumarate addition to produce benzylsuccinate; and pathway C – carboxylation, producing benzoate [Caldwell and Suflita, 2000], although this pathway may represent the sum of several enzymatic steps carried out by a consortium. All three pathways converge on benzoyl-CoA, a central intermediate in anaerobic oxidation of aromatic compounds, which is eventually oxidized to acetyl-CoA and carbon dioxide [Harwood et al., 1998]. Evidence has been reported for all three modes of attack, with phenol, benzoate and cyclohexanone consistently detected in cultures incubated under various anaerobic conditions [Caldwell and Suflita, 2000; Chakraborty and Coates, 2005; Grbić-Galić and Vogel, 1987; Phelps et al., 2001; Vogel and Grbić-Galić, 1986; Ulrich et al., 2005]. The source of the hydroxyl group of the phenol intermediate (fig. 1a) has been debated: H₂O was proposed as the donor in methanogenic cultures [Vogel and Grbić-Galić, 1986] but recent evidence points to a hydroxyl free radical as the donor used by *D. aromatica* RCB [Chakraborty and Coates, 2005]. Likewise, the methyl group donor in pathway B (fig. 1b) is unknown but may be S-adenosylmethionine, methyltetrahydrofolate or cobalamin [cited by Coates et al., 2002; Ulrich et al., 2005]. Activation of benzene by methylation may be analogous to the methylation of naphthalene proposed by Safinowski and Meckenstock [2006], discussed below.

Progress towards resolving the biochemistry of benzene degradation using pure cultures has been hampered in part by the fact that it is difficult to isolate hydrocarbon-degraders from methanogenic consortia because of the essential syntrophic interactions in such microbial communities. Whereas pure strains growing under nitrate- or sulfate-reducing conditions can in theory and in practice be enriched through utilization of benzene as the sole carbon source, in methanogenic consortia the organism initiating attack may not derive sufficient energy from benzene without a syntrophic partner to make the reaction thermodynamically favorable. That is, it may be energetically difficult to purify the benzene-oxidizing strain from a consortium using benzene itself. Instead of isolation, Ulrich and Edwards [2003] used physiological and nucleic acid techniques to characterize benzene-degrading methanogenic and denitrifying consortia. In the methanogenic culture they detected 16S rRNA gene se-

quences affiliated with uncultivated bacteria (predominantly the sulfate-reducing genus *Desulfosporosinus* and a delta-proteobacterial sequence) and uncultivated archaea (predominantly acetoclastic methanogens), whereas the nitrate-reducing consortium was dominated by bacterial sequences affiliated with denitrifying beta-proteobacteria similar to *Azoarcus* and *Dechloromonas*, with no archaea cloned. Certainly, more research is required to establish the prevalence of pathways and intermediates involved in anaerobic benzene biodegradation, and to identify the genes and enzymes responsible for this environmentally important activity. Another factor that has not been adequately addressed in the literature is the toxicity of benzene (or other BTEX components) to particular species, and how solvent sensitivity or tolerance could shift the species composition of mixed populations, thus influencing net biodegradative activity.

Toluene

In sharp contrast to the recalcitrance of benzene, toluene is the most readily degraded of the BTEX compounds [Langenhoff et al., 1996; Phelps and Young, 1999] under all TEA conditions. Its relative susceptibility to anaerobic degradation has yielded more pure isolates than any other aromatic substrate, and concomitantly the best resolution of catabolic pathways, enzymes and genes. Early studies demonstrated toluene degradation under denitrifying conditions using inocula from a variety of sources [Evans et al., 1991b]. Subsequently, toluene degradation was documented under manganese- [Cervantes et al., 2001; Langenhoff et al., 1996, 1997], iron- and sulfate-reducing [Beller et al., 1992a; Edwards et al., 1992] as well as methanogenic conditions [Langenhoff et al., 1996]. Toluene was readily degraded within 1–2 months under all redox conditions in these cultures; conversely, benzene was recalcitrant over the duration of the experiment (up to 525 days) [Langenhoff et al., 1996]. Similarly, toluene was preferentially degraded among BTEX components by oil sands enrichment cultures incubated under methanogenic conditions [Siddique et al., 2007]. There also have been intermittent reports of BTEX degradation under iron-reducing conditions in sediments incubated in the laboratory [Lovley et al., 1996; Villatoro-Monzón et al., 2003], as well as respiration with humic acids and the model quinone anthraquinone-2,6-disulfonate (AQDS) [Cervantes et al., 2001]. Addition of sub-stoichiometric amounts of humic acids enhanced toluene degradation linked to iron reduction in those studies, indicating that humus may be an adjunct electron shuttle and/or acceptor in situ [Gibson and Harwood, 2002; Lov-

ley et al., 1996] facilitating aromatic hydrocarbon biodegradation.

Elucidating the pathways used by toluene-degrading consortia is complicated by the syntrophic interactions assumed to be integral to anaerobic degradation, particularly methanogenesis. For example, a model toluene-degrading methanogenic consortium is postulated to comprise four physiological groups [Zwolinski et al., 2000]: a syntroph that initiates toluene oxidation, a homoacetogen that can reversibly oxidize acetate coupled to hydrogen generation, an acetoclastic methanogen and a hydrogenotrophic methanogen. Ficker et al. [1999] used molecular biological tools to characterize a methanogenic consortium maintained by transfer with toluene for 10 years. They detected and phylogenetically identified two dominant archaeal and two bacterial representatives: a bacterial sequence with no significant homology to any known genus (the presumptive toluene-degrader), a *Desulfotomaculum*-like sulfate-reducer (a presumptive homoacetogen), a *Methanosaeta* sequence type (acetoclastic archaeon), and a *Methanospirillum* sequence type (presumed hydrogenotrophic methanogen). Interestingly, there is general metabolic similarity between these results and those reported by Ulrich and Edwards [2003] from a benzene-degrading methanogenic consortium, discussed above. Presumably, initial hydrocarbon attack generates partially oxidized products such as fatty acids or alcohols that become available for fermentative and syntrophic species, eventually being made available to methanogens as acetate and/or $H_2 + CO_2$. As metagenomic analysis techniques become more accessible, biochemical modeling of consortia operating under different TEA conditions should enable discernment of catabolic pathways and interactions between metabolic types. In the meantime, the published catabolic pathways described below have been based on a relatively limited suite of pure isolates representing a few genera, and a handful of characterized enzymes.

Several pure denitrifying, toluene-degrading cultures have been isolated, including *Thauera aromatica* strains T1 [Evans et al., 1991a; Song et al., 1998] and K172 [Leutwein and Heider, 1999], *Thauera* sp. DNT-1 [Shinoda et al., 2004], *Azoarcus tolulyticus* strains Tol4 [Zhou et al., 1995] and ToN1 [Rabus and Widdel, 1995], *Azoarcus* spp. from diverse environmental sources [Beller and Sporrmann, 1997b; Fries et al., 1994], and *Dechloromonas aromatica* strains RCB and JJ [Coates et al., 2001a]. A few pure strains that couple iron reduction to toluene degradation have been isolated, such as *Geobacter metallireducens* GS-15 [Lovley and Lonergan, 1990; Lovley et al.,

1989, 1993] and *Geobacter grbiciae* strains TACP-2T and TACP-5 [Coates et al., 2001b]. Sulfate-reducing toluene-degrading isolates include *Desulfobacula toluolica* Tol2 [Rabus et al., 1993] and oXyS1 (related to the *Desulfobacteriaceae*) [Harms et al., 1999], as well as delta-proteobacterial strains TRM1 [Meckenstock, 1999] and PRTOL1 [Beller et al., 1996].

Toluene is the model for anaerobic biodegradation via the fumarate addition reaction (fig. 2a) mediated by benzylsuccinate synthase, a novel reaction elucidated for alkyl aromatics [Heider et al., 1999] and also saturated hydrocarbons [Widdel and Rabus, 2001]. Attack appears to be universally initiated by addition of the double bond of fumarate to the methyl group of toluene, yielding benzylsuccinate [Biegert et al., 1996; Leutwein and Heider, 1999]. This intermediate is then further oxidized to *E*-phenyllactate, and eventually benzoyl-coA [Biegert et al., 1996], a central aromatic metabolite that subsequently undergoes ring reduction, cleavage and oxidation to CO_2 [reviewed by Harwood et al., 1998].

Xylenes

Although all three xylene isomers appear to be biodegraded via the fumarate addition pathway (fig. 2a) analogous to toluene [Krieger et al., 1999], they have different susceptibilities to anaerobic biodegradation. Early conflicting reports of anaerobic xylene isomer degradation under nitrate-reducing conditions found that both *p*- and *m*- (but not *o*-) xylene were degraded [Kuhn et al., 1985], whereas Edwards et al. [1992] observed degradation of *p*- and *o*-xylene (*m*-xylene was not tested) under sulfate-reducing conditions after significant lag times. Early work by Zeyer et al. [1986] reported 80% mineralization of ring-labelled ^{14}C -*m*-xylene added to denitrifying river sediments. Several subsequent studies found that *m*-xylene is the most readily degraded isomer in mixed cultures [Beller et al., 1995] and in some cases its presence inhibits concomitant *o*- and *p*-xylene degradation [Meckenstock et al., 2004c; Morasch et al., 2004]. *o*-Xylene can be degraded by certain cultures [Edwards et al., 1992], and recently the first iron-reducing enrichment cultures were shown to oxidize *o*-xylene [Jahn et al., 2005] and *p*-xylene [Botton and Parsons, 2006]. Although *p*-xylene is often reported to be recalcitrant [Rabus and Widdel, 1995], it was degraded by mixed denitrifying enrichment cultures that were very selective, being unable to grow on benzene, ethylbenzene or *o*-xylene [Häner et al., 1995], and at least one sulfate-reducing enrichment culture recently was shown to degrade *p*-xylene via fumarate addition [Morasch and Meckenstock, 2005]. It is noteworthy

that the latter culture was enriched in the presence of Amberlite-XAD7 ion-exchange resin [Morasch et al., 2001], keeping the concentration of substrate (and possibly inhibitory metabolites) low during the initial stages of enrichment. This method of acclimatizing the inoculum to a recalcitrant substrate may prove useful for isolation of additional cultures.

Several pure cultures have been shown to utilize *m*-xylene for growth under nitrate- [Hess et al., 1997; Morasch et al., 2004; Rabus and Widdel, 1995] and sulfate-reducing [Harms et al., 1999] conditions, whereas only two pure cultures have been reported to mineralize *o*-xylene anaerobically, both under sulfate-reducing conditions: strain oXyS1 [Harms et al., 1999] and *Desulfotomaculum* sp. strain OX39 [Morasch et al., 2004]. Such isolates often show narrow substrate specificity, with mXyS1 degrading only *meta*- and oXyS1 preferring *ortho*-substituted aromatics [Harms et al., 1999]. No pure cultures utilizing *p*-xylene for growth have yet been reported. Such selectivity could account for the variable and site-specific patterns of xylene isomer degradation observed in situ and in enrichment cultures. It is also possible that *o*- and *p*-xylenes are being co-metabolized (via toluene) to dead-end products in some enrichment cultures rather than supporting growth [Beller, 2000].

Homologs corresponding to toluene fumarate addition metabolites have been detected in cultures incubated with xylenes (fig. 2a). For example, 4-methylbenzylsuccinate and 4-methylphenylitaconic acid were extracted from an enrichment culture incubated with *p*-xylene [Morasch and Meckenstock, 2005], and the expected 2-methylbenzylsuccinate homolog was detected in cultures co-metabolizing *o*-xylene [Beller and Spormann, 1997b].

Ethylbenzene

Ethylbenzene degradation has been reported in situ and in mixed cultures under sulfate-reducing [Elshahed et al., 2001] and nitrate-reducing conditions [Reinhard et al., 1997]. However, its recalcitrance varies with site and TEA: Villatoro-Monzón et al. [2003] observed that ethylbenzene degraded most rapidly of all BTEX compounds under iron-reducing conditions (threefold more rapidly than benzene) whereas Botton and Parsons [2006] failed to detect oxidation of ethylbenzene under iron-reducing conditions even though the same microcosms were able to degrade benzene, toluene and xylenes. Jahn et al. [2005] also reported ethylbenzene mineralization in two enrichment cultures incubated under iron-reducing conditions, but only after relatively long lag times, whereas Siddique et al. [2007] observed recalcitrance of ethylbenzene under

methanogenic conditions although toluene and xylenes were degraded.

Only five pure cultures utilizing ethylbenzene have been reported to date, of which four are nitrate reducers and one is a sulfate reducer, and all of which appear to have fairly restricted hydrocarbon substrate ranges. The first to be isolated were denitrifiers: strains EbN1 and PbN1 were originally affiliated with the genus *Thauera* [Rabus and Widdel, 1995] but strain EbN1 was recently fully sequenced [Rabus et al., 2005] and placed in the genus *Azoarcus* (although referred to as *Aromatoleum aromaticum* by Kloe et al. [2006]). *Thauera* sp. PbN1 also has been renamed *Azoarcus* sp. strain PbN1 [Kniemeyer and Heider, 2001]. *Azoarcus* sp. strain EB1 cannot grow on BTEX components other than ethylbenzene [Ball et al., 1996], whereas the denitrifying bacterium *D. aromatica* RCB is capable of degrading toluene as well as ethylbenzene [Chakraborty et al., 2005]. The ethylbenzene-mineralizing marine delta-proteobacterium strain EbS7 is the only sulfate-reducing pure culture reported to date, and its aromatic hydrocarbon substrate range also appears to be limited to ethylbenzene [Kniemeyer et al., 2003].

Two distinct pathways for anaerobic biodegradation of ethylbenzene have been documented. One is the classic fumarate addition reaction described for toluene and xylenes that produces the ethyl homolog of benzylsuccinate (fig. 2b), observed under sulfate-reducing conditions [Kniemeyer et al., 2003]. An alternate pathway observed in denitrifying organisms such as *Azoarcus* sp. strain EB1 [Johnson et al., 2001] is accomplished by hydroxylating the benzylic carbon to form 1-phenylethanol (fig. 2c), analogous to benzene attack (fig. 1a), with water donating the oxygen atom of the hydroxyl group [Ball et al., 1996]. Subsequent dehydrogenation produces acetophenone and eventually the central aromatic metabolite benzoyl-coA. The initial enzyme in this pathway, ethylbenzene dehydrogenase, has been characterized [Johnson et al., 2001; Kniemeyer and Heider, 2001] and is briefly discussed below. *Azoarcus* sp. EbN1, which degrades toluene by fumarate addition and ethylbenzene by hydroxylation, appears to use both pathways independently for initiating anaerobic oxidation of the two substrates [Heider et al., 1999; Rabus, 2005b].

Other Alkylmonoaromatic Substrates

Occasional reports occur in the literature documenting anaerobic degradation of alkyl-substituted monoaromatics other than BTEX compounds [reviewed by Beller, 2000], but frequently these are from in situ studies of

complex contaminants where anaerobic conditions are not unequivocally established and the mass balance of specific substrates and metabolites cannot be achieved. Tetramethylbenzenes found in JP-4 jet fuel are proposed to have been oxidized to the corresponding trimethylbenzoic acid isomers under sulfate-reducing conditions in a contaminated aquifer, apparently by methyl group oxidation [Cozzarelli et al., 1995; Namocatcat et al., 2003], and Martus and Püttman [2003] detected a variety of alkylated aromatic acids in groundwater contaminated with jet fuel and dominated by sulfate reduction. These intermediates, detected and characterized by gas chromatography with mass spectrometry (GC-MS), are postulated to be homologs of alkylbenzylsuccinates arising from metabolism of C₂- to C₇-benzenes. Harms et al. [1999] reported degradation of *m*- and *o*-ethyltoluene, and Prince and Suflita [2007] documented removal of ethyl-, propyl-, *iso*-propyl-, 1-ethyl-4-methyl- and two *iso*-butylbenzene isomers as well as *p*-xylene under methanogenic and especially sulfidogenic conditions by microbiota in contaminated sediments and groundwater.

Thus, ample evidence exists for anaerobic biodegradation of (alkyl)monoaromatics in situ, in laboratory microcosms, and (for some substrates) in pure culture. Toluene has received the most attention because of its relative susceptibility to biodegradation and concomitant isolation of pure cultures, but considerable research is needed both in pure culture and in consortia for biodegradation of other monoaromatics, including multiply substituted benzene rings, as well as benzene itself.

Polycyclic Aromatic Hydrocarbons (PAHs)

To date, only a limited number of PAHs definitively have been shown to biodegrade anaerobically in situ or in microcosms containing soil, river sediment, aquifer material or marine sediment using nitrate, iron, manganese, sulfate or carbon dioxide as TEAs [reviewed by Meckenstock et al., 2004b]. These include the unsubstituted PAHs naphthalene and phenanthrene as well as some alkyl-PAHs. However, as noted by Safinowski et al. [2006], detailed information on anaerobic degradation of PAHs is scarce, and there is debate whether PAHs having three or more aromatic rings can support growth or whether they are only partially oxidized through co-metabolism with growth substrates [Ambrosoli et al., 2005; Meckenstock et al., 2004b; Safinowski et al., 2006; Sharak Genthner et al., 1997]. Cultivation of denitrifying or sulfate-reducing PAH-degrading pure cultures and enrichments has prov-

en difficult, and only within the last decade have a few pure cultures [Galushko et al., 1999] and stable mixed cultures been obtained to enable the study of anaerobic degradation pathways. For most PAHs only circumstantial evidence for anaerobic degradation currently exists and it is not clear whether substrate losses represent partial oxidation by individual organisms to generate transient or dead-end metabolites, or complete oxidation by sequential activity of consortium members, or a combination of these processes.

Unsubstituted PAHs: Naphthalene, Phenanthrene and Other PAHs

Mihelcic and Luthy [1988a, b] were the first to report loss of naphthalene from soil enrichments incubated with nitrate as the electron acceptor. In later studies, Bregnard et al. [1996] confirmed anaerobic naphthalene degradation by measuring mineralization of ¹⁴C-naphthalene under nitrate-reducing conditions in microcosms containing material from a chronically diesel-fuel contaminated aquifer. Coates et al. [1996a] detected ¹⁴C-naphthalene mineralization by sulfate-reducing marine harbour sediments and, interestingly, found that amendment of these sediments with insoluble iron oxides did not enhance contaminant degradation over the indigenous sulfate levels [Coates et al., 1996b], possibly due to the lower proportion of iron-reducers in the sediments compared with sulfate reducers. Subsequently, Bedessem et al. [1997] observed mineralization of ¹⁴C-naphthalene incubated with nine different enrichment cultures under sulfate-reducing conditions, although the lag times and degradation rates varied widely among enrichments. Nitrate-, manganese- and sulfate-reducing as well as methanogenic conditions also resulted in partial degradation of naphthalene in sediment columns [Langenhoff et al., 1996]. In these studies using continuous upflow columns infused with a mixture of benzene, toluene and naphthalene, the naphthalene was not measurably degraded under methanogenic or iron-reducing conditions and was only partially degraded with manganese (approx. 50%). Slightly more degradation was observed with nitrate or sulfate as TEA, with approximately 60% of ¹⁴C-naphthalene mineralized under sulfate-reducing conditions. Addition of benzoate as a co-substrate accelerated naphthalene removal under nitrate-reducing conditions, either by providing an additional electron donor for naphthalene ring reduction, by inducing appropriate genes, or by providing an additional carbon source for growth [Langenhoff et al., 1996]. Rockne et al. [2000] and Rockne and Strand [1998, 2001] found that naphthalene and phenan-

threne could be degraded by a denitrifying enrichment culture originally derived from creosote-contaminated soil. Radiolabel recoveries detected incorporation of carbon from ^{14}C -hydrocarbon into biomass and production of $^{14}\text{CO}_2$ confirmed metabolism [Rockne and Strand, 2001]. However, the degree of mineralization varied considerably between substrates, with only partial mineralization of naphthalene (17% oxidized to $^{14}\text{CO}_2$) versus 96% of phenanthrene; likewise, the proportion of PAH-carbon incorporated into biomass varied between substrates, with naphthalene contributing the most to biomass-carbon. Anderson and Lovley [1999] were the first to demonstrate naphthalene degradation in aquifers dominated by iron reduction, but they found that this activity also was site-specific.

Only a few pure naphthalene-degrading cultures have been isolated, including the sulfate-reducing marine delta-proteobacterium strain NaphS2 [Galushko et al., 1999] and three nitrate-reducing, naphthalene-degrading strains isolated from a marine sediment enrichment culture [Rockne et al., 2000], of which two were characterized: strain NAP-3-1 (phylogenetically related to *Pseudomonas stutzeri*) coupled partial naphthalene mineralization to complete denitrification whereas strain NAP-4-1 (related to *Vibrio pelagius*) reduced nitrate to nitrite. Both cultures produced a small amount of $^{14}\text{CO}_2$ from labeled naphthalene (initially 7–22%), with significant label (30–50%) incorporated into biomass.

Two pathways have been proposed for the initial anaerobic attack on naphthalene: carboxylation (fig. 3a) [Zhang and Young, 1997] versus methylation (fig. 3b) followed by fumarate addition (fig. 3c) [Safinowski and Meckenstock, 2006]. The two pathways converge at 2-naphthoic acid, and thereafter the aromatic rings are sequentially reduced, starting with the unsubstituted ring, to produce octahydronaphthoic acid [Annweiler et al., 2002; Phelps et al., 2002; Zhang et al., 2000] before ring cleavage or production of the dead-end product decahydronaphthoate. Under sulfidogenic conditions a third pathway (not shown), initiated via hydroxylation of naphthalene to naphthol, has been proposed but not verified [Bedessem et al., 1997].

Evidence for the carboxylation pathway (fig. 3a) comes from incubation of naphthalene-utilizing sediment cultures with ^{13}C -bicarbonate under sulfidogenic conditions. Zhang and Young [1997] observed that the label was incorporated into the carboxylic group of 2-naphthoic acid and inferred that the initial activation reaction was direct carboxylation of naphthalene, a conclusion echoed by Meckenstock et al. [2000]. However, because

the 2-naphthoic acid was detected in a mixed culture it possibly represents the product of sequential enzymatic steps rather than primary attack. Subsequently, Safinowski and Meckenstock [2006] examined the sulfate-reducing culture N47, enriched from a tar-oil-contaminated aquifer. The culture was observed by phase microscopy to comprise a single cell morphology but was not considered to be axenic, and a demonstrably pure isolate could not be obtained by dilution. The culture could utilize naphthalene or 2-methylnaphthalene as the sole carbon and energy source individually but not simultaneously. When parallel cultures were incubated with deuterated naphthalene or 2-methylnaphthalene, the same fumarate addition pathway metabolites predicted by the 2-methylnaphthalene pathway (fig. 3c) were detected in both cultures, suggesting that naphthalene is first methylated to form 2-methylnaphthalene (fig. 2b) then undergoes fumarate addition (fig. 3c). This is analogous to the proposed pathway for methylation of benzene to toluene before further metabolism (fig. 1b). Interestingly, these two reported modes of attack may actually represent a single pathway, since the methyl group may derive from bicarbonate via a reverse CO-dehydrogenase pathway [Safinowski and Meckenstock, 2006] eventually producing 2-naphthoic acid, consistent with Zhang and Young's observations but involving additional enzymatic steps. Despite the possible analogy to the benzene methylation pathway, Coates et al. [1997] found that sulfidogenic benzene-degrading sediments were unable to mineralize naphthalene, suggesting that the microbial populations were different and that initial enzymes for attack of these unsubstituted aromatics are substrate-specific.

Phenanthrene degradation has been observed under nitrate- and sulfate-reducing conditions in marine sediments [Tang et al., 2005; Zhang and Young, 1997]. By analogy to naphthalene (fig. 3a–c) the initial attack on phenanthrene may be carboxylation (as proposed by Zhang and Young [1997]), or methylation (as proposed by Safinowski et al. [2006]) with subsequent fumarate addition and oxidation to phenanthroic acid. Also, because some studies have documented concomitant naphthalene and phenanthrene degradation [Coates et al., 1997; Rockne and Strand, 2001; Zhang and Young, 1997], it might be assumed that the same enzymes effecting 2-ring naphthalene attack could be recruited for 3-ring phenanthrene oxidation in parallel pathways. However, when Chang et al. [2006] characterized the microbial communities in PAH-contaminated methanogenic marine sediments demonstrating naphthalene and phenanthrene degradation, they found that the two communities were

Fig. 3. Anaerobic pathways for degradation of naphthalene and methyl naphthalenes. Square brackets indicate a postulated intermediate; broken arrows indicate multiple enzymatic steps; open arrows indicate further metabolism. **a** Carboxylation of naphthalene to form 2-naphthoic acid and/or its CoA ester with bicarbonate as the likely carboxyl donor [Meckenstock et al., 2000; Zhang and Young, 1997]. Note that this activation may represent more than one enzymatic reaction. **b** Alkylation of naphthalene [Safinowski and Meckenstock, 2006] prior to oxidation via the 2-methylnaphthalene pathway. The methyl group may be generated from bicarbonate via a reverse CO-dehydrogenase reaction [Safinowski and Meckenstock, 2006]. **c** Activation of 2-methylnaphthalene via fumarate addition to form naphthyl-2-methylsuccinate, dehydration to naphthyl-2-methylenesuccinate and/or its CoA ester, followed by sequential saturation of the distal aromat-

ic ring of 2-naphthoate to form 1,4,5,6,7,8,9,10-octahydro-2-naphthoate, which is further metabolized either to the proposed dead-end metabolite decahydronaphthoate or the ring cleavage product *cis*-2-carboxycyclohexylacetate [Annweiler et al., 2002]. Note that Annweiler et al. [2002] and Greibler et al. [2004] depict the intermediates as the free acids because the CoA esters were not detected, likely due to hydrolysis during solvent extraction under alkaline conditions. However, the intracellular activated forms are likely the CoA esters, as Safinowski and Meckenstock [2004] have determined that CoA is introduced into the pathway after production of naphthyl-2-methylsuccinate. **d** Alkylation of 1-methylnaphthalene to form the postulated intermediate 1,2-dimethylnaphthalene, followed by fumarate addition and eventual co-metabolic formation of the presumptive dead-end product 1-methyl-2-naphthoic acid [Safinowski and Meckenstock, 2006].

distinct, suggesting that different species were responsible for naphthalene and phenanthrene degradation. Lack of pure cultures has hampered further elucidation of a phenanthrene pathway. McNally et al. [1998] used three *Pseudomonas* spp., isolated through their ability to degrade PAHs aerobically but also shown to reduce nitrate, to examine the degradation of phenanthrene, anthracene and pyrene under denitrifying conditions. They found that when the PAHs were provided at concentrations below their water solubility limit (so as to eliminate the effects of dissolution and mass transport) degradation occurred surprisingly quickly (within hours) and usually without a lag. However, because the substrate concentrations used in this study were very low (<0.05 to 1 ppm) and the cell density high (10^8 cells/ml), the possibility of substrate loss through partitioning into the cell membranes may be a factor, especially since degradation products were not determined to verify anaerobic oxidation. Thus, the pathway(s) for anaerobic phenanthrene degradation remain cryptic.

Other unsubstituted PAHs also have been shown to be removed by enrichments using nitrate or sulfate, including acenaphthene [Chang et al., 2002; Mihelcic and Luthy, 1988a, b; Yuan and Chang, 2007], fluorene and fluoranthene [Coates et al., 1997]. Coates et al. [1997] determined that ^{14}C -labelled naphthalene, phenanthrene, fluorene and fluoranthene (but not pyrene or benzo[a]pyrene) were mineralized to $^{14}\text{CO}_2$ by sulfate-reducing sediments from San Diego Bay. Rothermich et al. [2002] demonstrated that several indigenous as well as added PAHs were degraded in situ under sulfate-reducing conditions in harbor sediments. The substrates monitored comprised a suite of 14 PAHs having 2–5 rings, including naphthalene, phenanthrene, and the high molecular weight PAHs chrysene, pyrene and benzo[a]pyrene, among others, including alkyl-substituted naphthalenes discussed below. All substrates monitored eventually showed at least some depletion (9% for benz[a]anthracene to 89% for acenaphthene), with the smaller PAHs generally degrading faster than the larger PAHs. This study demonstrated for the first time that high molecular weight unsubstituted PAHs could be degraded under sulfate-reducing conditions [Rothermich et al., 2002]. However, because signature metabolites were not assessed, and because the larger PAHs were not radiolabeled for measurement of $^{14}\text{CO}_2$ evolution, it is not clear whether the PAH depletion was due to microbial growth resulting in complete mineralization, or to partial oxidation through co-metabolic processes, as has been suggested by Meckenstock et al. [2004b] and Safinowski et al. [2006].

More research involving mass balance of metabolites is required to resolve this question.

Although Meckenstock et al. [2004b] concluded that unsubstituted PAHs are not attacked under methanogenic conditions, Christensen et al. [2004] assessed the potential for PAH removal under methanogenic conditions, applying molecular modelling to calculate the free energy of reaction for naphthalene (and 1-methylnaphthalene) degradation using pathways proposed in the literature (table 1). Their calculations indicated that naphthalene oxidation should be thermodynamically feasible under methanogenic conditions. They then incubated naphthalene or 1-methylnaphthalene in microcosms inoculated with various anaerobic inocula. The mass of both substrates decreased in all microcosms at a rate proportional to temperature, up to 65°C for thermophilic enrichments. Similarly, Trably et al. [2003] measured removal of 13 unsubstituted PAHs from municipal sewage sludge in anaerobic stirred tank bioreactors under thermophilic methanogenic conditions and found that removal was enhanced at 55°C compared with 35°C and 45°C, particularly for the larger PAHs. However, in that study abiotic losses accounted for a significant portion of the total loss of the smaller PAHs at high temperatures, and no attempts were made to unequivocally demonstrate complete mineralization rather than partial co-metabolic oxidation, so the results must be interpreted cautiously.

Additional evidence was compiled by Chang et al. [2002] who followed the degradation of five 3-ring unsubstituted PAHs in soil under methanogenic, nitrate- or sulfate-reducing conditions. Over a period of 3 years, anaerobic cultures were established by periodic transfer with phenanthrene, and subsequently pure pyrene, anthracene, fluorene or acenaphthene were added individually or in combination for an additional year of enrichment with different TEAs. Significant removal of the PAHs (from 100% to approx. 60%) was observed for all compounds in the order phenanthrene > pyrene > anthracene > fluorene > acenaphthene, and PAH removal decreased in the order sulfate-reducing > methanogenic > nitrate-reducing conditions. Recently, Yuan and Chang [2007] documented removal of unsubstituted PAHs in anaerobic river sediment microcosms, again incubated under methanogenic, nitrate- or sulfate-reducing conditions. The order of degradation differed from the previous study, with degradation rates decreasing from acenaphthene > fluorene > phenanthrene > anthracene > pyrene, but similar order of TEA efficacy (sulfate-reducing > methanogenic > nitrate-reducing conditions). From

these microcosms, 12 strains were reported to degrade these PAHs anaerobically (although the TEA was not stated). The pure isolate with the best degradative ability, strain ER9, was identified morphologically and biochemically as a *Clostridium* sp. [Yuan and Chang, 2007]. Notably, in both these studies [Chang et al., 2002; Yuan and Chang, 2007] neither mass balance using radiolabelled substrates nor detection of characteristic metabolites were attempted, nor was cell growth or mineralization determined; therefore, even though the initial inoculum had been enriched by repeated transfer on phenanthrene which was likely utilized as a carbon source, the measured decrease in the other PAH concentrations arguably could be ascribed to co-metabolic oxidation, as proposed by Meckenstock et al. [2004b] and Safinowski et al. [2006]. Resolution of this controversy over utilization versus co-metabolism of unsubstituted PAHs requires more study using enrichment cultures, including metabolism under sulfidogenic and methanogenic conditions because much of the literature has been generated with denitrifiers. It also demands studies in which mass balance is achieved, to determine whether the PAHs are being completely mineralized and serving as growth substrates, or are merely being co-metabolized. And, finally, isolation of pure cultures that unequivocally oxidize these substrates anaerobically would allow pathways to be proposed and enzyme substrate ranges to be inferred.

Alkyl-PAHs: 2-Methylnaphthalene and 1-Methylnaphthalene

Whereas evidence for anaerobic attack on unsubstituted PAHs has been sparse until recently and the pathways are controversial, alkyl-PAHs appear to be degraded via the fumarate addition pathway. This distinction is analogous to the difference between benzene and alkylbenzene degradation discussed earlier in this review. Degradation of 2-methylnaphthalene, in particular, has been well documented [Annweiler et al., 2000, 2002] and presumptive pathways have been published that proceed through naphthyl-2-methylsuccinate and naphthylitaconate (naphthyl-2-methylsuccinate) followed by sequential saturation of the ring system and ring cleavage (fig. 3c). A pathway producing carboxylated 2-methylnaphthalene has been suggested [Sullivan et al., 2001] but not fully deduced (not shown). However, analogous to the differential susceptibility of xylene isomers, considerably more experimentation will be required to elucidate the biodegradability of 1-methylnaphthalene and its potential metabolites, as its degradation is not simply analogous to the more susceptible isomer 2-methylnaphtha-

lene. A co-metabolic pathway for 1-methylnaphthalene degradation has been proposed (fig. 3d) involving methylation at the 2-position, subsequent fumarate addition at that position, and eventual production of the dead-end product 1-methyl-2-naphthoic acid [Safinowski et al., 2006]. As could be predicted from this observation, preference for 2-methyl- over 1-methylnaphthalene as a carbon source has been reported [Meckenstock et al., 2000; Spormann and Widdel, 2000]. For example, the sulfate-reducing culture N47 could utilize 2-methylnaphthalene but could not grow on 1-methylnaphthalene [Annweiler et al., 2000; Meckenstock et al., 2000]. Galushko et al. [2003] noted the same isomer preference with a mixed culture and commented that the culture was also unable to grow on toluene, a methylnaphthalene homolog, further highlighting substrate specificity of anaerobic degradation enzymes and the potential for producing dead-end metabolites through co-metabolism. Notably, 1-naphthoic acid has been detected in field trials [Gieg and Suflita, 2002; Greibler et al., 2004], and might arise via a pathway analogous to that of 2-methylnaphthalene producing 2-naphthoic acid (fig. 3c), but this pathway has not been further explored.

Townsend et al. [2003] extended observations of isomer preference to the ethyl- and dimethyl-substituted naphthalenes, incubating two crude oils in microcosms containing indigenous microbes from contaminated aquifer material. Interestingly, whereas the saturated hydrocarbons were degraded under both methanogenic and sulfate-reducing conditions, naphthalene and specific alkyl homologs were only degraded in the presence of sulfate, in the order naphthalene > 2-methylnaphthalene > 2-ethylnaphthalene > 2,6- and 2,7-dimethylnaphthalene (as co-eluting isomers). Other dimethylnaphthalenes as well as 1-methylnaphthalene and 1-ethylnaphthalene were not degraded during the 14-month incubation. That is, alkyl naphthalene degradation was both sulfate-dependent and homolog-specific. However, it is not clear from this experiment whether the homologs were utilized as the sole carbon source or co-metabolized because hydrocarbon loss was documented by GC without carbon balance. Morasch et al. [2004], however, noted that dimethylnaphthalenes (structural analogs of xylenes), were co-metabolized with *m*-xylene by *Desulfotomaculum* sp. strain OX39 to the corresponding methylnaphthoic acids but did not support growth of the culture, suggesting broad substrate specificity for the initial pathway enzymes but narrower specificity for lower pathway reactions.

Sulfur-, Oxygen- and Nitrogen-Containing Polycyclic Aromatic Heterocycles

Sulfate-reducing culture N47 [Safinowski and Meckenstock, 2006], the microscopically homogeneous (but not necessarily pure) culture described above, was used to deduce the co-metabolic transformation of the sulfur heterocycle benzothiophene. Initial experiments detected carboxybenzothiophene isomers during growth on naphthalene [Annweiler et al., 2001], and incubation with ^{13}C -bicarbonate suggested that the heterocycle was directly carboxylated, analogous to the pathway in figure 3a. However, later analysis [Safinowski et al., 2006] indicated that the initial activation involved methylation followed by fumarate addition to the methyl group, with eventual production of the carboxylic acid (analogous to 2-naphthoic acid production from naphthalene, discussed above; fig. 3b, c). In both studies, the same carboxybenzothiophene intermediate was detected, but the latter study implied additional enzymatic steps to produce that metabolite. In the presence of the growth substrates naphthalene or 2-methylnaphthalene, culture N47 also co-metabolized the oxygen-containing heterocycle benzofuran, producing the corresponding carboxylated metabolite without associated growth. Interestingly, some combinations of co-metabolic substrate (particularly acenaphthylene) and primary substrate inhibited growth of the mixed culture. Little has been published regarding anaerobic transformation of nitrogen heterocycles, with pyridine, quinoline, isoquinoline and indole reported to be susceptible and carbazole to be recalcitrant [as reviewed by Fetzner, 1998].

Thus, considerable research is required concerning anaerobic degradation of PAHs and heterocycles. This is particularly true for alkyl-PAHs because they represent a much larger proportion of contaminant hydrocarbons than do their unsubstituted homologs, and due to their molecular complexity and enzyme specificity are more recalcitrant to aerobic biodegradation (and possibly also to anaerobic degradation if multiply substituted with alkyl groups). The larger PAHs in particular have low aqueous solubility and therefore reduced bioavailability, thus contributing to environmental persistence. Elucidation of pathways, or even documentation of partially oxidized metabolites, would be useful for predicting the fate(s) and associated risks of PAHs in anaerobic environments.

Enzymes and Genes

Enzymes

The fumarate addition pathway appears to be predominant in anaerobic transformation of alkyl-monoaromatics [Biegert et al., 1996] and -PAHs [Annweiler et al., 2001], and may also be involved in catabolism of unsubstituted aromatics following methylation [Safinowski et al., 2006; Ulrich et al., 2005] (fig. 1, 3). The enzymatic hydrocarbon activation reactions forming benzylsuccinates from monoaromatics are discussed briefly below, but for comprehensive coverage of the fumarate addition reactions and corresponding enzymes, the reader is directed to reviews by Heider et al. [1999], Widdel and Rabus [2001], Boll et al. [2002] and Buckel and Golding [2006], and to reviews by Harwood et al. [1998] and Gibson and Harwood [2002] for reactions involving the central aromatic metabolite benzoyl-coA. No enzymes specific to attack on PAHs or alkyl-PAHs have been described, due at least in part to paucity of pure cultures to study.

The initial enzyme in the toluene pathway, benzylsuccinate synthase (Bss), belongs to a novel group of glycyl radical enzymes [Buckel and Golding, 2006]. Its activity, i.e. adding fumarate to the methyl group of toluene (fig. 2a), has been demonstrated in enrichments incubated under nitrate- [Beller and Spormann, 1997b; Biegert et al., 1996], iron- [Kane et al., 2002], and sulfate-reducing [Beller and Spormann, 1997a] as well as methanogenic conditions [Beller and Edwards, 2000; Washer and Edwards, 2007]. The enzyme isolated from *T. aromatica* K172 has been characterized [Leutwein and Heider, 2002] as has that from *Azoarcus* sp. strain T [Beller and Spormann, 1999]. The gene encoding the alpha subunit of the enzyme, *bssA*, has been detected in all anaerobic toluene-degrading isolates surveyed to date [Winderl et al., 2007], including *T. aromatica* K172 [Leuthner et al., 1998], *Azoarcus* sp. strain T [Achong et al., 2001] and *Geobacter metallireducens* [Kane et al., 2002], and recently was used to develop a functional gene marker to screen for anaerobic toluene degraders [Winderl et al., 2007].

Ethylbenzene dehydrogenase (EBD) catalyzes the initial attack in one pathway of ethylbenzene biodegradation (fig. 2c), producing 1-phenylethanol. This was the first enzyme shown to hydroxylate an aromatic hydrocarbon in the absence of molecular oxygen, deriving the hydroxyl group from water [Ball et al., 1996]. The membrane-associated enzyme of the denitrifying organism *Azoarcus* sp. strain EB1, which grows only on ethylbenzene and no other BTEX compounds, oxidizes a limited number of fluorinated and nonaromatic homologs of eth-

ylbenzene such as 4-fluoro-ethylbenzene and ethyldiene-cyclohexane, demonstrating a relatively broad substrate range, but does not transform either toluene or propylbenzene nor the saturated homolog ethylcyclohexane [Johnson et al., 2001]. The enzyme is produced only during growth of strain EB1 on ethylbenzene or the direct pathway metabolites 1-phenylethanol and acetophenone. In contrast, the enzyme isolated from *Azoarcus* sp. EbN1 is reported to be periplasmic [Rabus et al., 2002] and can hydroxylate propylbenzene with low efficiency [Kniemeyer and Heider, 2001]. For additional details the reader is directed to the paper on EBD crystal structure by Kloer et al. [2006] and to that of Rabus et al. [2002] for discussion of the corresponding genes (*ebdABC*). The ethylbenzene-degrading *Azoarcus* sp. strain PbN1 also grows on propylbenzene with the intermediates postulated to be analogous to those of ethylbenzene (i.e. phenylpropanol and propiophenone [Rabus and Widdel, 1995]); its initial enzyme also can hydroxylate propylbenzene [Kniemeyer and Heider, 2001].

Enzymes responsible for subsequent catabolism of central metabolites of the BTEX pathways (i.e. benzylsuccinates and benzoyl-coA) have been studied in denitrifying bacteria such as *T. aromatica* K172 [Leuthner and Heider, 2000; Leutwein and Heider, 1999, 2001, 2002], *Azoarcus* sp. EbN1 [Rabus et al., 2005] and the photosynthetic bacterium *Rhodospseudomonas palustris* [Egland et al., 1997]. Many of these central steps require activation of the free acid by addition of co-enzyme A. Recent studies on the activity of a new type of benzoyl-coA reductase [Wischgoll et al., 2005] suggest that *G. metallireducens* could be a model organism for coupling dearomatization to iron reduction [Carmona and Diaz, 2005].

The cellular location of pathway enzymes has been addressed by only a few researchers, even though this has implications for release of intermediates into the extracellular medium and therefore availability of metabolites or co-metabolites for further oxidation by other consortium members or for diffusion and mobility in the environment. Chakraborty and Coates [2005] proposed that hydroxylation of benzene occurs on the outer membrane or in the periplasm of *D. aromatica* RCB, allowing diffusion of phenol into the external medium during benzene degradation. Likewise, Rabus et al. [2002] have proposed a periplasmic location for ethylbenzene dehydrogenase in *Azoarcus* sp. EbN1. These locations would explain the appearance of pathway metabolites in culture supernatants and in the aqueous phase of contaminated environments, and limited uptake of the extracellular intermediates for further metabolism would explain accumulation of such

compounds in situ, as discussed later in this review in the section on 'Natural attenuation'. However, neither a mechanism nor a biological rationale for excreting potential growth substrates has been examined; this aspect of anaerobic hydrocarbon biodegradation requires considerable experimental attention.

Co-Metabolism

Co-metabolism (i.e. gratuitous partial oxidation of a substrate by an organism during growth on a different substrate) appears to be an important fate for several aromatic hydrocarbons. For this to occur, the initial enzymes in the pathways must have a broad substrate range, initiating attack on a variety of aromatics. Partially purified benzylsuccinate synthase (Bss) from *Azoarcus* sp. strain T was observed to catalyze fumarate addition to both toluene and *m*-xylene [Achong et al., 2001] as well as several non-hydrocarbon aromatics [Beller and Spormann, 1999]. In an analogous manner, Morasch et al. [2004] suggested that dimethylnaphthalenes are co-metabolized during growth of *Desulfotomaculum* sp. strain OX39 on *m*-xylene, producing the corresponding methylnaphthoic acids (although the fumarate addition products were not detected and the initial enzymes are unknown). Ethylbenzene dehydrogenase also transforms other aromatic and non-aromatic substrates [Johnson et al., 2001]. However, if the enzymes for the lower pathway reactions are more substrate-specific and unable to further transform those metabolites, as suggested by Beller and Spormann [1997b], it would account for the accumulation of metabolites characteristic of anaerobic biodegradation pathways [Beller, 2000]. For example, *D. aromatica* RCB completely mineralized toluene and ethylbenzene to CO₂, but only transformed *p*-xylene to an unidentified metabolite detected in the culture supernatant [Chakraborty et al., 2005]. Likewise, denitrifying *T. aromatica* strain T1 did not grow on *o*-xylene and only transformed it in the presence of toluene [Evans et al., 1991a]. Sulfate-reducing toluene-grown strain PRTOL1 co-metabolically transformed 90% of added *o*-xylene to the dead-end product 2-methylbenzylsuccinate [Beller et al., 1996], indicating the relative importance of co-metabolism in transformation of certain aromatics. Strain- and isomer-specific co-metabolism also seems to apply to PAHs: Saffinowski et al. [2006] found evidence for co-metabolism of 1-methylnaphthalene by culture N47 grown on either naphthalene or 2-methylnaphthalene, generating the presumptive dead-end product 1-methyl-2-naphthoic acid. Interestingly, they also found that neither three-ring compounds (i.e. fluorene, phenanthrene and anthracene)

nor biphenyl were co-metabolic substrates for culture N47, although, because phenanthrene co-metabolism has been reported by others [Safinowski et al., 2006; Zhang and Young, 1997] this observation may be specific to culture N47 [Safinowski et al., 2006]. The question of toxicity of presumptive dead-end co-metabolites to the microbiota has not yet been addressed. Obviously, as more pure cultures are isolated and their enzymes purified and characterized, the picture of aromatic co-metabolism and substrate specificity will become clearer. It is hoped that such future studies will also address the question of gene regulation (discussed below) regarding alkyl isomers and heterocyclic homologs in particular.

Gene Regulation

Evidence exists for both repression and induction of genes involved in anaerobic aromatic hydrocarbon degradation; however, often the evidence is based on the sequence of attack on isomers or a mixture of substrates by an enrichment culture rather than a pure isolate, complicating interpretation of the data.

Of the BTEX components, toluene is usually found to degrade most rapidly and with the shortest lag time [Meckenstock et al., 2004c; Siddique et al., 2007]. One explanation for this observation is repression by toluene of catabolic genes for other BTEX compounds. Meckenstock et al. [2004c] observed that the presence of toluene at concentrations $>20 \mu\text{M}$ inhibited *o*-xylene degradation in sulfidogenic sediment columns, and that *o*-xylene degradation did not begin until toluene was depleted (or was omitted from the culture). When tested with pure isolates, the phenomenon was found to be strain-specific: growth and *o*-xylene degradation by strain OX39 was sensitive to toluene, whereas toluene degradation by strain TRM1 (which does not degrade xylenes) was insensitive to the presence of *o*-xylene [Meckenstock, 1999; Meckenstock et al., 2004c]. The recalcitrance of benzene can be similarly explained: results from early studies indicated that benzene degradation by a methanogenic consortium was impeded by the presence of more readily utilized substrates [Edwards and Grbić-Galić, 1992; Phelps and Young, 1999] and benzene degradation in methanogenic aquifer columns was inhibited by the presence of toluene [Da Silva and Alvarez, 2004]. Likewise, a nitrate-reducing enrichment culture incubated with benzene plus toluene did not degrade benzene until toluene was depleted, whereas there was no inhibitory effect in a benzene-degrading methanogenic enrichment that was unable to degrade toluene [Ulrich et al., 2005]. In addition, nonhydrocarbon substrates can repress hydrocar-

bon degradation: for example, toluene and *o*-xylene degradation by a methanogenic consortium was inhibited by the presence of preferred electron sources (glucose, fatty acids, methanol, amino acids, etc.) [Edwards and Grbić-Galić, 1994], implying catabolite repression. Considering the potential permutations of genes and regulatory systems and the complex mixtures of substrates and metabolites typically found in contaminated sites, prediction of inhibitory regulatory effects (as opposed to general toxicity effects) is difficult and in any case would likely be site and strain specific.

Induction of monoaromatic catabolic genes also has been demonstrated. Prince and Suflita [2007] found that adding only 1 μl of gasoline (representing about 5% of the indigenous hydrocarbon) to microcosms containing a natural gas condensate enhanced biodegradation of aromatic compounds under methanogenic and, especially, sulfidogenic conditions. In particular, removal of *p*-xylene and ethyl-, propyl-, *iso*-propyl-, 1-ethyl-4-methyl- and two *iso*-butylbenzene isomers was enhanced when gasoline was added to cultures. In contrast, gasoline addition had little effect on biodegradation of alkanes or cycloalkanes, suggesting that the effect was a specific induction of aromatic catabolism genes. Similarly, overall PAH degradation was enhanced when a mixture of PAHs was incubated with anaerobic river sediment, versus individual substrates [Barbaro et al., 1992; Yuan and Chang, 2007].

Induction of toluene catabolic genes in the denitrifying organism *T. aromatica* [Heider et al., 1998] and *Thauera* sp. strain T1 involves multiple two-component regulatory systems [Coschigano and Young, 1997; Leuthner and Heider, 1998]. The metabolite benzylsuccinate, which is often found in the supernatant of *T. aromatica* cultures grown on toluene, may be an inducer of the toluene pathway (cited by Chakraborty and Coates [2004] as personal communication from P.W. Coschigano). Very recently, Washer and Edwards [2007] identified new putative benzylsuccinate synthase genes in a toluene-degrading methanogenic consortium. Their expression was upregulated in the presence of toluene but not the central metabolite benzoate, indicating specific induction by the primary substrate. The differential susceptibility of xylene isomers has been explained by Morasch et al. [2004] as a consequence of having distinct enzymes for initial attack on *m*-, *o*- and *p*-xylenes, encoded by genes that are differentially induced in *Desulfotomaculum* sp. OX39. Similarly, the specificity of strain oXyS1 for *ortho*-alkylbenzenes versus strain mXyS1 for *meta*-alkylbenzenes [Harms et al., 1999] could conceivably be due to specific

gene induction and/or to substrate specificity of the Bss enzyme.

The denitrifying organism *Azoarcus* sp. EbN1 is unique because it can grow on toluene and ethylbenzene using two different pathways: toluene is degraded via fumarate addition (fig. 2a) whereas ethylbenzene is hydroxylated (fig. 2c). Early studies [Champion et al., 1999] found that each pathway in EbN1 was separately and distinctly induced by its own substrate. The organization of toluene catabolic genes in EbN1 is similar to that of other denitrifying toluene degraders [Kube et al., 2004], and Kühner et al. [2005] found that two toluene pathway operons were specifically induced by exposure to toluene, whereas genes involved in ethylbenzene degradation were induced by both ethylbenzene and toluene, suggesting that toluene is a fortuitous inducer of the second pathway. The importance of such crossover induction is clear considering that environmental impact by a single contaminant is rare, and that most sites have multiple contaminant hydrocarbons. Full genome sequencing of EbN1 (GenBank accession number CR555306) ultimately revealed 10 presumptive pathways for anaerobic degradation (all but one converging at benzoyl-coA) and four pathways for aerobic biodegradation of aromatic compounds, plus paralogous genes as well as numerous transposable elements and multiple regulatory pathways [Rabus, 2005b], illustrating that regulation in this organism is probably more complex and versatile than can be discerned by simple incubation with individual substrates.

PAH catabolic genes also appear to be induced. In studies with pure denitrifying isolates, McNally et al. [1999] found that the presence of naphthalene enhanced both phenanthrene and pyrene degradation, whereas phenanthrene apparently inhibited pyrene degradation, although these observations were not confirmed with metabolite analysis. Safinowski and Meckenstock [2006] noted that there was no lag phase when enrichment culture N47 was transferred from naphthalene as carbon source to 2-methylnaphthalene, indicating that induction of new genes was not required. However, the converse was not true, and after growth on 2-methylnaphthalene the culture exhibited a lag phase of almost 100 days before growth resumed on naphthalene. This observation is consistent with the degradative pathways shown in figure 3, where an additional methylation step (fig. 3b) is required to activate naphthalene for fumarate addition (fig. 3c); induction of the requisite gene might account for the lag upon transfer from 2-methylnaphthalene to naphthalene. It is less apparent how this observation would

support the existence of pathway 3a because induction of several upper pathway enzymes presumably would be required upon shift from naphthalene to 2-methylnaphthalene, yet the culture exhibited no lag phase. Notably, because culture N47 is not a confirmed pure culture [Safinowski and Meckenstock, 2006], these results are subject to alternative interpretations including interplay between two or more microbial partners in a consortium. Other combinations of substrates also resulted in partial or complete inhibition of degradation by culture N47, showing yet again that strain- and substrate-specific effects may contribute to explaining site-specific field observations.

Regulation of catabolic genes involved in central metabolism of aromatic intermediates such as benzoate has been investigated in *Azoarcus* spp. [Barragán et al., 2005; Durante-Rodríguez et al., 2006; López Barragán et al., 2004], *Thauera* spp. [Tropel and van der Meer, 2004] and the anaerobic phototroph *Rhodospseudomonas palustris* [Egland and Harwood, 1999]. For more detail, the reader is directed to a recent review on transcriptional regulation of aromatic degradation pathways [Tropel and van der Meer, 2004].

Thus, from the relatively limited number of pure cultures available for study, a complex and strain-specific picture of enzyme substrate specificity, pathway redundancy, and gene regulation emerges. Most of these observations arose from toluene-degrading denitrifying isolates, and without more evidence from additional diverse organisms, particularly PAH-degraders, it will remain difficult to formulate general rules for aromatic hydrocarbon catabolic enzyme specificity or gene expression.

Current and Potential Applications

Natural Attenuation and Bioremediation

Natural attenuation is a strategy for managing decontamination of soils, sediments and groundwaters, in which intrinsic physical and biological processes result in amelioration of the contamination. Biological processes typically comprise the major contributor to this treatment method. The term 'bioremediation' usually implies active intervention at the contaminated site to enhance biodegradation processes through, for example, addition of limiting nutrients (biostimulation) or of competent microbes (bioaugmentation). Because hydrocarbon-contaminated sites commonly include anaerobic sectors (due to oxygen consumption during organic carbon metabolism), the importance of anaerobic biodegradation pro-

cesses at such sites is obvious. Understanding the underlying principles discussed in previous sections of this review may enable prediction and manipulation of microbial processes to ensure or accelerate natural attenuation. Numerous reports have been published documenting natural attenuation of hydrocarbons in anaerobic aquifers, groundwater and sediments; the reader is directed to reviews by Widdel and Rabus [2001] and Meckenstock et al. [2004b].

Demonstration of natural attenuation requires at least three lines of evidence [Smets and Pritchard, 2003]: documented loss of the contaminants from the site; laboratory data (e.g. microcosms or microbial enumeration methods) indicating the presence of competent microbiota in situ; and evidence of appropriate microbial activity in situ (e.g. production of predicted metabolites, depletion of TEAs and/or accumulation of reduced TEA products). However, demonstrating anaerobic biodegradation in situ by measuring depletion of parent compounds can cause major technical problems because of difficulty distinguishing between losses due to biological transformation versus abiotic processes such as sorption, dilution or volatilization. To complement and augment depletion measurements, Beller et al. [1995] proposed that specific anaerobic metabolites ('signature metabolites') produced and excreted during degradation of the substrate be used as indicators of biodegradation. Ideally, anaerobic biomarkers are intermediates highly specific to the substrate's degradation pathway (i.e. are not products of metabolism of other compounds or abiotic processes); are produced only anaerobically; are not normally found in the environment unless the substrate is present; are water soluble so that they can be recovered from groundwater or saturated sediments; and are relatively stable chemically so that they can be detected, but are also relatively biologically labile so that their presence is an indicator of recent rather than relict metabolism [Phelps and Young, 2002]. Through studies with pure cultures and substrates it has been possible to identify candidate biomarkers such as those shown in figures 1–3, then correlate them with metabolites detected in situ [Beller et al., 1995]. The (alkyl)benzylsuccinates (i.e. the products of fumarate addition) fit these criteria and have been adopted as potentially universal signature metabolites for anaerobic biodegradation of BTEX compounds. In contrast, the (alkyl)benzoates (i.e. lower pathway intermediates) are not sufficiently specific because they can be formed aerobically or by metabolism of nonhydrocarbons like aromatic amino acids [Meckenstock et al., 2004b]. As new substrates and degradative pathways are identified, it is

clear that we are still adding to the catalogue of potential biomarkers for anaerobic metabolism [Chakraborty and Coates, 2005].

Technical difficulties with inconsistent detection and identification of signature metabolites still hamper characterization of anaerobic biodegradation in situ, due to their low concentrations (typically orders of magnitude lower than the parent hydrocarbons [Caldwell and Suflita, 2000; Griebler et al., 2004]), heterogeneity [Kazumi et al., 1997] and their transient nature due to biodegradation by other members of the consortium [Beller, 2000]. In addition, authentic standards of many hypothetical intermediates are not commercially available, preventing incontrovertible identification in culture extracts, groundwater, etc. Several current methods involve solvent extraction of relatively large volumes of groundwater with subsequent derivatization and GC-MS analysis [Elshahed et al., 2001; Gieg and Suflita, 2002; Gieg et al., 1999]. Although well-established and definitive, these analyses can be costly and time-consuming. New sophisticated methods are being developed and combined to streamline analysis of signature metabolites, including liquid chromatography (LC)/MS/MS [Beller and Kane, 2003] combined with stable carbon isotope (^{13}C) [Griebler et al., 2004; Meckenstock et al., 2004a; Reusser et al., 2002] or deuterated (^2H) [Ward et al., 2000] substrates, solid-phase extraction (SPE) [Alumbaugh et al., 2004; Leddin et al., 2005] and direct injection methods coupled with LC/MS/MS to increase sensitivity of detection.

The appearance of signature metabolites in the aqueous phase in situ or in culture medium is intriguing, because it is not clear why pathway intermediates that are potential growth substrates should be found outside the cell. Beller [2000] noted that benzylsuccinate and *E*-phenylitaconate, products of toluene metabolism (fig. 2a), accumulate in growth medium and are more stable than expected (in comparison with, for example, benzoate), and speculated that after they leave the cell they are not taken up efficiently from the extracellular medium. For example, benzylsuccinate was not metabolized by toluene-grown cells [Beller et al., 1992b, 1996] but could be oxidized to ϵ -phenylitaconate and benzoate by permeabilized cells [Beller and Spormann, 1998; Biegert et al., 1996]. Phelps et al. [2001] observed that exogenous benzoate was utilized less efficiently than benzene by a benzene-degrading enrichment culture, and suggested that it indicates inefficient uptake of benzoate by the cells. Similarly, Safinowski and Meckenstock [2006] observed that the fumarate addition pathway metabolites of 2-methylnaphthalene (a carbon source for culture N47) ac-

cumulated in the medium with incubation. They proposed that the metabolites are continuously excreted from the cells and, once released, cannot be taken up again by culture N47 for metabolism. This raises the question of how efficiently the culture can grow on 2-methylnaphthalene if a proportion of the substrate is excreted as metabolites that cannot be re-acquired as carbon source. It also begs the question of the mechanism (and biochemical rationale) of export of catabolic pathway metabolites, although this is also observed during aerobic growth on aromatic hydrocarbons and heterocycles. However, it is necessary to remember that the signature metabolites, at least, are typically found at low concentrations in the aqueous phase [Griebler et al., 2004] and probably represent only a small proportion of the total oxidized substrate. Obviously, further investigation of enzyme location(s), metabolite export and uptake is warranted as more pure cultures are isolated and enzymes characterized. The implications for prediction, implementation and detection of natural attenuation processes are important for at least two reasons: excretion of polar metabolites without re-uptake will mobilize the aromatic skeleton because of increased water solubility compared with the parent hydrocarbon and may either enhance bioavailability in situ or increase mobility of the metabolite; conversely, excretion with re-uptake and further metabolism (by the same or different organisms in a consortium) may enhance biodegradation.

Hydrocarbon-impacted environments usually experience complex mixtures of contaminants that can influence the overall activity of the microbial community [Zheng et al., 2001]. In a survey of various aquatic sediments incubated under four different redox conditions, Phelps and Young [1999] found that degradation of a mixture of BTEX compounds was site and TEA specific: whereas toluene was degraded in all enrichment cultures under all conditions, benzene degraded only in sulfidogenic cultures from one site, and pristine sediments generally did not degrade BTEX whereas chronically contaminated sediments usually yielded active cultures. In cultures amended with gasoline (which contains a large proportion of aliphatic hydrocarbons in addition to aromatics) BTEX degradation was slower and incomplete [Phelps and Young, 1999], indicating that degradation of complex mixtures is influenced by site microbiota, redox conditions and the substrate itself. This observation could be explained by either inhibition of BTEX degradation by the other hydrocarbons in the gasoline (through toxicity, gene expression effects, or shifts in the microbiota composition), or to their preferential degradation over BTEX

compounds [Phelps and Young, 1999]. The negative effect of gasoline on BTEX degradation in the aquatic sediments contrasts sharply with the stimulatory effect of gasoline on aromatic hydrocarbon degradation noted by Prince and Suflita [2007] in microcosms under methanogenic and especially sulfidogenic conditions, discussed earlier. These conflicting observations emphasize the unpredictability of site-specific responses to mixed substrates and the need for new, comprehensive approaches that do more than simply document substrate depletion.

Enhancing Natural Attenuation

The type and concentration of TEAs available in a contaminated environment will affect the outcome of natural attenuation or bioremediation. Common indigenous TEAs in impacted environments include nitrate, iron, sulfate and carbon dioxide, as discussed above, and in some cases also perchlorate and chlorate [Chakraborty et al., 2005], quinones [Cervantes et al., 2001] and humic acids [Cervantes et al., 2001; Lovley et al., 1996] (although, to date, the latter TEAs have only been linked to biodegradation of certain aromatics like toluene). Understanding the potential role of various TEAs in anaerobic biodegradation can inform remediation strategies such as amendment with TEAs to enhance natural attenuation.

Sulfate, for example, has several potential advantages as a TEA amendment [Anderson and Lovley, 2000]: unlike O₂, sulfate is not consumed by abiotic reactions with ferrous iron or sulfide; it does not form iron oxide precipitates in situ that can cause plugging; it is more soluble than O₂ and therefore can be added at higher concentrations, and furthermore accepts twice as many electrons as O₂; and it can be applied to groundwater at higher levels than nitrate, which is potentially toxic. The disadvantage is that some contaminated sites may lack the microbes that initiate attack on benzene under sulfate-reducing conditions [Weiner and Lovley, 1998]. Notwithstanding that potential limitation, Weiner et al. [1998] showed in laboratory microcosms, then Anderson and Lovley [2000] demonstrated in situ that adding sulfate as a TEA to a petroleum-contaminated aquifer stimulated benzene oxidation (whereas addition of nitrate completely inhibited benzene degradation in preliminary experiments [Anderson and Lovley, 2000]). These effects were noted despite the fact that the sediments, contaminated with hydrocarbons for more than 50 years, were methanogenic when the TEAs were injected. This indicates that the potential for anaerobic degradation coupled to sulfate reduction persisted regardless of prevailing TEAs, and

points to a possible role for sulfate-reducers activating aromatic hydrocarbons in methanogenic consortia.

Schreiber and Bahr [2002] added nitrate to a petroleum-contaminated aquifer and detected biodegradation of toluene, ethylbenzene and *m*- and *p*-xylenes but not benzene over a 60-day monitoring period. Interestingly, the stoichiometry of nitrate reduced to TEX oxidized was greater than predicted, and may have resulted from oxidation of other organics in the aquifer at the expense of the added nitrate. Ball and Reinhard [1996] also observed nonstoichiometric reduction of nitrate when amending microcosms containing BTEX. This common phenomenon can complicate calculation of TEA demand for natural attenuation. Phenanthrene biodegradation in marine sediments was enhanced two- to threefold by the addition of controlled-release TEAs, specifically nitrate as nitrocellulose and sulfate as gypsum [Tang et al., 2005]. This approach may lead to refined 'capping' strategies in marine harbour sediments, where a slow-release TEA would be incorporated directly into otherwise undisturbed contaminated sediments, thus avoiding the issues associated with multiple applications of highly soluble, potentially inhibitory TEAs, particularly nitrate [Tang et al., 2005].

Cunningham et al. [2001] enhanced in situ bioremediation of BTEX-contaminated groundwater by combining the advantages of nitrate and sulfate through amendment with both TEAs. The combination of TEAs accelerated the natural attenuation of the petroleum hydrocarbon contaminants; nitrate was used preferentially and so was rapidly consumed near the injection well, but sulfate had an effect outside the denitrifying zone. Degradation of xylene isomers appeared to be linked specifically to sulfate reduction, validating the choice of amending with two TEAs rather than just nitrate. Benzene was the most recalcitrant contaminant in situ but eventually showed evidence of biodegradation after approximately 15 months. This study illustrates how understanding the potential diversity of in situ anaerobic processes and adjusting the remediation method to suit the contaminants and the indigenous microbial community can be used to relieve the limitations encountered by injection of a single TEA.

The stimulatory effect of providing nutrients, such as fixed nitrogen and/or phosphate, has not been as thoroughly studied under anaerobic conditions as under aerobic conditions. However, at least two cases show the benefit of fertilizing nutrient-poor anaerobic environments contaminated with diesel fuel: Cross et al. [2006] observed enhanced anaerobic degradation when contami-

nated groundwater microcosms were amended with nutrients, specifically ammonium, nitrate and phosphate, and Powell et al. [2006] noted the stimulatory effect of nutrients (nitrate, ammonium, calcium, sulfate and phosphate) on denitrifying hydrocarbon degraders in nutrient-poor Antarctic soils.

Whereas biostimulation through TEA addition has been studied, bioaugmentation with bacteria capable of anaerobic degradation is virtually untested. Da Silva and Alvarez [2004] inoculated flowthrough aquifer columns with enrichment cultures and demonstrated increased benzene degradation under methanogenic conditions only in the bioaugmented columns, but this activity required a very long acclimation period and the observation was not verified in the field. It is possible that versatile degraders such as *D. aromatica* RCB, which can mineralize BTEX components under aerobic, nitrate-, perchlorate- and chlorate-reducing conditions [Chakraborty et al., 2005], may be valuable as bioaugmentation agents in certain applications. However, in general, added microbes are at a disadvantage in competition with the indigenous microbiota and, even under aerobic conditions, successful bioaugmentation trials are sparse.

Wastewater and Sewage Sludge Processing

Processing of hydrocarbon-containing industrial wastewaters and municipal sewage sludge is another area that may benefit from increased understanding of anaerobic biodegradation. Soil-wash fluids from a wood preserving site containing both pentachlorophenol (PCP) and PAHs necessitated an integrated waste management system of soil washing and anaerobic bioremediation to deal with both classes of compounds [Miller et al., 1998]. Removal of contaminants under anaerobic conditions was demonstrated with a simulated waste stream containing PCP (99.8% removal) and four model PAHs. Naphthalene and acenaphthene were removed efficiently (86% and 93% removal, respectively), although negligible removal of pyrene and benzo(b)fluoranthene was measured. In a recent study, Siddique et al. [2007] documented methanogenic removal of BTEX and other hydrocarbons (both aromatic and aliphatic) from naphtha in a slurry of oil sands tailings waste, without prior laboratory enrichment. Although high concentrations of an artificial mixture of BTEX or of naphtha inhibited methanogenesis in the microcosms, lower concentrations similar to those normally present in the tailings waste supported methane production and resulted in hydrocarbon depletion and methane production in the microcosms. Methanogenesis in the large volume tailings ba-

sins, sustained by anaerobic hydrocarbon biodegradation, apparently is responsible for daily emission of millions of liters of methane at this site. It is possible that anaerobic pretreatment of the tailings to remove hydrocarbons with capture of the produced CO₂ and methane could reduce greenhouse gas emissions from the current open system.

Similarly, domestic sewage sludge might be pretreated to remove hydrocarbons before diversion to other purposes such as application to agricultural soil [Trably et al., 2003]. Chang et al. [2003] incubated samples from municipal and petrochemical sludge with a suite of five PAHs and found that degradation was slower in the municipal sludge, possibly due to the presence of more susceptible, competing organic compounds in the municipal sludge, or conversely the presence of a more competent microbiota in the petrochemical sludge. They also found that sulfate-reducing conditions were superior to methanogenic and denitrifying conditions for PAH removal. However, nitrate and sulfate are not usually practical or desirable TEAs for sewage sludge bioprocessing, and methanogenic conditions are considered more practical, even though evidence for PAH removal under these conditions is currently scarce. To test the ability of sludge to degrade PAHs under methanogenic conditions, Christensen et al. [2004] enriched cultures from diverse sources: a wastewater treatment plant; digested manure and industrial food waste; leachate from a municipal landfill; or contaminated soils from gasoline stations. They found that each inoculum was able to degrade naphthalene and 1-methylnaphthalene, but the contaminated soil enrichments performed the best. Trably et al. [2003] also observed PAH losses from municipal sludge incubated as mesophilic and thermophilic enrichments and found that bioaugmentation with an adapted inoculum enhanced PAH degradation. This limited number of studies indicates the potential for waste stream processing although more research in this area is required, especially demonstrating mass balance to document complete oxidation of the hydrocarbons.

Electricigens

Geobacter metallireducens strain GS-15 is a toluene-degrading iron reducer. It is also an 'electricigen', capable of transferring electrons from central metabolic redox reactions to an external artificial anode, thereby generating electricity [Lovley, 2006] (although this has been demonstrated only with benzoate as the electron donor, not toluene [Bond et al., 2002]). *G. metallireducens* GS-15 has also been grown on toluene in syntrophic culture with

Wolinella succinogenes [Meckenstock, 1999], and hence theoretically could participate in syntrophic hydrocarbon degradation. Therefore, mixed microbial populations that include *G. metallireducens* using an artificial anode as an electron acceptor may prove to be valuable not only for bioremediation applications in contaminated sediments but also for concurrent low-level electricity generation [Lovley, 2006]. This potential would be increased by discovery of new electricigens with innate hydrocarbon-degrading abilities, or by genetic engineering of known electricigens to introduce the appropriate anaerobic catabolic genes, once available.

Petroleum Reservoirs

Head et al. [2003] have reviewed the literature on deep subsurface oil reservoirs and support the proposition that heavy oils have arisen through anaerobic biodegradation of conventional oils over geologic time, occurring in reservoirs with a water interface and an in situ temperature $\leq 80^{\circ}\text{C}$ [Aitken et al., 2004; Larter et al., 2006; Röling et al., 2003] or perhaps higher [Spark et al., 2000]. Deep subsurface biodegradation, involving aliphatic as well as aromatic hydrocarbons, is generally deleterious to the economic value of the oil, resulting in increased oil density and viscosity, sulfur content, acidity and metals, and decreased saturated and aromatic hydrocarbons corresponding to the extent of in situ biodegradation [Larter et al., 2006]. Thus, archaic anaerobic biodegradation has had huge economic impacts on fossil fuel quality and crude oil recovery worldwide.

Detection of anaerobic naphthalene signature metabolites, specifically 2-naphthoate and partially reduced 2-naphthoates (fig. 3c), during a screen of 77 degraded oil samples from around the world lends more specific support to the inference that anaerobic hydrocarbon biodegradation has occurred in a large proportion of oil reservoirs [Aitken et al., 2004; Magot et al., 2000]. Despite this circumstantial evidence, no pure culture has yet been isolated that exhibits the ability to degrade hydrocarbons anaerobically under in situ reservoir conditions [Aitken et al., 2004; Röling et al., 2003]. Nor have the succinate derivatives from fumarate addition pathways been confirmed yet in crude oils [Aitken et al., 2004], possibly because these polar metabolites partition into the aqueous phase [Suflita et al., 2004], but they have been detected in production water from oil fields [Semple and Foght, unpubl. obs.].

Sulfidogenesis appears to dominate in sulfate-containing reservoirs (e.g. offshore wells), especially those undergoing waterflooding for secondary recovery. It is

assumed that indigenous hydrocarbons support this detrimental sulfide generation ('souring') [Rueter et al., 1994], perhaps by members of the family *Desulfobacteriaceae* [Rabus et al., 1996]. Although pure aromatic hydrocarbon-degrading cultures demonstrating this activity have not yet been isolated, Chen and Taylor [1997] successfully enriched a thermophilic consortium from the produced water of an Alaskan oil field that could metabolize BTEX components to unknown water-soluble products concomitant with sulfide production, suggesting the potential for such activity in situ. Nitrate has been added to reservoirs as an alternate TEA for biological control of souring and has proved useful in some cases [Sunde and Torsvik, 2005; Telang et al., 1997]; again, it is assumed that a portion of the oil in situ serves as carbon and energy source for the nitrate-reducers but this awaits proof. Certainly, Rabus et al. [1999] found that a succession of BTEX- and aliphatic-degrading bacteria grew on crude oil under nitrate-reducing conditions, and that degradation by the community exceeded that observed with individual strains and compounds. In contrast, Kodama and Watanabe [2003] isolated sulfide-oxidizing, nitrate-reducing bacterial strains from underground oil storage facilities, but these strains apparently could not grow directly on crude oil as carbon source, so their importance in oil reservoirs is currently unknown. Nor has the possible role of biological iron reduction in reservoirs been well-addressed yet [Birkeland, 2004; Röling et al., 2003].

In reservoirs low in available sulfate, methanogenesis appears to be the primary TEA process and is thought to have contributed over geological time to methane gas associated with heavily biodegraded petroleum such as the Canadian oil sands deposits [reviewed by Head et al., 2003]. It may be possible to exploit anaerobic activity in situ (through biostimulation or bioaugmentation) to produce methane from reservoirs with otherwise economically unrecoverable oil, such as wells that have undergone extensive waterflooding and are marginal producers [Sufliita et al., 2004]. The contribution of aliphatic hydrocarbons to anaerobic degradation is likely to be more important in such environments because of the higher mass ratio of alkanes to aromatics in most crude oils. Thus, the potential exists to control reservoir souring (and associated metal corrosion in production facilities) or to enhance energy production via in situ methanogenesis through judicious manipulation of anaerobic hydrocarbon biodegradation in oil reservoirs. Alternatively, oil reservoirs may be sources of isolates capable of anaerobic biodesulfurization [Marcelis et al., 2003] to improve crude oil quality through removal of organic sulfur from sulfur heterocycles.

Future Directions

The field of anaerobic biodegradation of aromatic hydrocarbons is still very young, and much of the literature to date is based on field observations or the use of enrichment cultures rather than pure cultures incubated under defined conditions. For practical purposes, the former studies are perhaps more informative, as the field trials account for the effects of multiple substrates, diverse TEAs, and mixed indigenous microbiota subject to shifts in species composition. However, these results are recognized as being site- and contaminant-specific, complicating inference of the fundamental principles of anaerobic biodegradation. In contrast, the few published pure culture-pure substrate studies are useful for elucidating pathways, but do not necessarily reflect the potential for synergy or competition in situ.

Increasingly, molecular biology methods are being applied to fundamental anaerobic biodegradation questions to help clarify gross observations and predict degradation potentials, for example: 16S rRNA gene clone libraries of naphthalene-degrading consortia [Hayes and Lovley, 2002]; real-time PCR targeting of specific phyla known to degrade aromatics [Beller et al., 2002; Da Silva and Alvarez, 2007]; denaturing gradient gel electrophoresis (DGGE) to characterize hydrocarbon-degrading consortia [An et al., 2004; Hendrickx et al., 2005; Kasai et al., 2005]; fluorescent in situ hybridization (FISH) with phylogenetic probes [Christensen et al., 2004]; and DNA microarrays and proteomics to study alkylbenzene degradation [Kühner et al., 2005]. As more is learned about the genes encoding anaerobic hydrocarbon degradation, the use of functional gene probes may accelerate the assignment of genes to archetypal pathways and the screening of diverse environments and enrichment cultures for marker genes. Application of sophisticated chemical analyses such as LC/MS/MS [Beller, 2002] and stable isotope analysis [Griebler et al., 2004; Wilkes et al., 2000] to this field of study will result in elucidation of new pathways. Coupling stable isotope analysis of metabolites with stable isotope probing (^{13}C -incorporation into nucleic acids for subsequent isolation, amplification and cloning) should provide a synergy between chemical and biological approaches.

Examination of gene regulation and enzyme specificity will provide information for modeling of complex biodegradation processes in situ. However, such future endeavors are based on the assumption that we will be able to detect, isolate and cultivate novel degraders for study; more emphasis is needed on isolation and characteriza-

tion of archetypal strains to complement the few existing isolates available (mainly *Azoarcus* and *Thauera* spp.). The development of molecular probes for functional genes that mediate anaerobic biodegradation, or the use of stable isotope probing with DGGE and subsequent screening of colonies [Kasai et al., 2006] may expedite

this undertaking. Then it may be feasible to use diagnostic techniques to determine which TEA(s) would be most beneficial at a specific site given its particular contaminants and indigenous microbiota, thus taking laboratory observations to application in the field. This emerging field is ready to blossom.

References

- Achong GR, Rodriguez AM, Spormann AM: Benzylsuccinate synthase of *Azoarcus* sp. strain T: Cloning, sequencing, transcriptional organization, and its role in anaerobic toluene and m-xylene mineralization. *J Bacteriol* 2001;183:6763–6770.
- Aitken CM, Jones DM, Larter SR: Anaerobic hydrocarbon biodegradation in deep subsurface oil reservoirs. *Nature* 2004;431:291–294.
- Alumbaugh RE, Gieg LM, Field JA: Determination of alkylbenzene metabolites in groundwater by solid-phase extraction and liquid chromatography-tandem mass spectrometry. *J Chromatogr A* 2004;1042:89–97.
- Ambrosoli R, Petruzzelli L, Minati JL, Marsan FA: Anaerobic PAH degradation in soil by a mixed bacterial consortium under denitrifying conditions. *Chemosphere* 2005;60:1231–1236.
- An Y, Joo Y, Hong I, Ryu H, Cho K: Microbial characterization of toluene-degrading denitrifying consortia obtained from terrestrial and marine ecosystems. *Appl Microbiol Biotechnol* 2004;65:611–619.
- Anderson RT, Lovley DR: Naphthalene and benzene degradation under Fe(III)-reducing conditions in petroleum-contaminated aquifers. *Bioremed J* 1999;3:121–135.
- Anderson RT, Lovley DR: Anaerobic bioremediation of benzene under sulfate-reducing conditions in a petroleum-contaminated aquifer. *Environ Sci Technol* 2000;34:2261–2266.
- Anderson RT, Rooney-Varga JN, Gaw CV, Lovley DR: Anaerobic benzene oxidation in the Fe(III) reduction zone of petroleum-contaminated aquifers. *Environ Sci Technol* 1998;32:1222–1229.
- Annweiler E, Materna A, Safinowski M, Kappler A, Richnow HH, Michaelis W, Meckenstock RU: Anaerobic degradation of 2-methylnaphthalene by a sulfate-reducing enrichment culture. *Appl Environ Microbiol* 2000;66:5329–5333.
- Annweiler E, Michaelis W, Meckenstock RU: Anaerobic cometabolic conversion of benzo-thiophene by a sulfate-reducing enrichment culture and in a tar-oil-contaminated aquifer. *Appl Environ Microbiol* 2001;67:5077–5083.
- Annweiler E, Michaelis W, Meckenstock RU: Identical ring cleavage products during anaerobic degradation of naphthalene, 2-methylnaphthalene, and tetralin indicate a new metabolic pathway. *Appl Environ Microbiol* 2002;68:852–858.
- Ball HA, Reinhard M: Monoaromatic hydrocarbon transformation under anaerobic conditions at Seal Beach, California: Laboratory studies. *Environ Toxicol Chem* 1996;15:114–122.
- Ball HA, Johnson HA, Reinhard M, Spormann AM: Initial reactions in anaerobic ethylbenzene oxidation by a denitrifying bacterium, strain EB1. *J Bacteriol* 1996;178:5755–5761.
- Barbaro JR, Barker JF, Lemon LA, Mayfield CI: Biotransformation of BTEX under anaerobic, denitrifying conditions: field and laboratory observations. *J Contam Hydrol* 1992;11:245–272.
- Barragán MJL, Blázquez B, Zamarró MT, Manchego JM, García JL, Díaz E, Carmona M: BzdR, a repressor that controls the anaerobic catabolism of benzoate in *Azoarcus* sp. CIB, is the first member of a new subfamily of transcriptional regulators. *J Biol Chem* 2005;280:10683–10694.
- Bedessem ME, Swoboda-Colberg NG, Colberg PJS: Naphthalene mineralization coupled to sulfate reduction in aquifer-derived enrichments. *FEMS Microbiol Lett* 1997;152:213–218.
- Beller HR: Metabolic indicators for detecting in situ anaerobic alkylbenzene degradation. *Biodegradation* 2000;11:125–139.
- Beller HR: Analysis of benzylsuccinates in groundwater by liquid chromatography/tandem mass spectrometry and its use for monitoring in situ BTEX biodegradation. *Environ Sci Technol* 2002;36:2724–2728.
- Beller HR, Ding W, Reinhard M: Byproducts of anaerobic alkylbenzene metabolism useful as indicators of in situ bioremediation. *Environ Sci Technol* 1995;29:2864–2870.
- Beller HR, Edwards EA: Anaerobic toluene activation by benzylsuccinate synthase in a highly enriched methanogenic culture. *Appl Environ Microbiol* 2000;66:5503–5505.
- Beller HR, Kane SR: Advances in monitoring in situ biodegradation: use of state-of-the-art mass spectrometric and molecular techniques. *Soil Sed Contam* 2003;12:691.
- Beller HR, Kane SR, Legler TC, Alvarez PJJ: A real-time polymerase chain reaction method for monitoring anaerobic, hydrocarbon-degrading bacteria based on a catabolic gene. *Environ Sci Technol* 2002;36:3977–3984.
- Beller HR, Grbić-Galić D, Reinhard M: Microbial degradation of toluene under sulfate-reducing conditions and the influence of iron on the process. *Appl Environ Microbiol* 1992a;58:786–793.
- Beller HR, Reinhard M, Grbić-Galić D: Metabolic by-products of anaerobic toluene degradation by sulfate-reducing enrichment cultures. *Appl Environ Microbiol* 1992b;58:3192–3195.
- Beller HR, Spormann AM: Benzylsuccinate formation as a means of anaerobic toluene activation by sulfate-reducing strain PRTOL1. *Appl Environ Microbiol* 1997a;63:3729–3731.
- Beller HR, Spormann AM: Anaerobic activation of toluene and o-xylene by addition to fumarate in denitrifying strain T. *J Bacteriol* 1997b;179:670–676.
- Beller HR, Spormann AM: Analysis of the novel benzylsuccinate synthase reaction for anaerobic toluene activation based on structural studies of the product. *J Bacteriol* 1998;180:5454–5457.
- Beller HR, Spormann AM: Substrate range of benzylsuccinate synthase from *Azoarcus* sp. strain T. *FEMS Microbiol Lett* 1999;178:147–153.
- Beller HR, Spormann AM, Sharma PK, Cole JR, Reinhard M: Isolation and characterization of a novel toluene-degrading, sulfate-reducing bacterium. *Appl Environ Microbiol* 1996;62:1188–1196.
- Biegert T, Fuchs G, Heider J: 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* 1996;238:661–668.
- Birkeland N: The microbial diversity of deep subsurface oil reservoirs. *Stud Surface Sci Catal* 2004;151:385–403.
- Boll M, Fuchs G, Heider J: Anaerobic oxidation of aromatic compounds and hydrocarbons. *Curr Opin Chem Biol* 2002;6:604–611.

- Bond DR, Holmes DE, Tender LM, Lovley DR: Electrode-reducing microorganisms that harvest energy from marine sediments. *Science* 2002;295:483–485.
- Bonin P, Cravo-Laureau C, Michotey V, Hirschler-Réa A: The anaerobic hydrocarbon biodegrading bacteria: an overview. *Ophelia* 2004;58:243–254.
- Botton S, Parsons JR: Degradation of BTEX compounds under iron-reducing conditions in contaminated aquifer microcosms. *Environ Toxicol Chem* 2006;25:2630–2638.
- Bregnard TP, Höhener P, Häner A, Zeyer J: Degradation of weathered diesel fuel by microorganisms from a contaminated aquifer in aerobic and anaerobic microcosms. *Environ Toxicol Chem* 1996;15:299–307.
- Buckel W, Golding BT: Radical enzymes in anaerobes. *Ann Rev Microbiol* 2006;60:27–49.
- Burland SM, Edwards EA: Anaerobic benzene biodegradation linked to nitrate reduction. *Appl Environ Microbiol* 1999;65:529–533.
- Caldwell ME, Suflita JM: Detection of phenol and benzoate as intermediates of anaerobic benzene biodegradation under different terminal electron-accepting conditions. *Environ Sci Technol* 2000;34:1216–1220.
- Caldwell ME, Tanner RS, Suflita JM: Microbial metabolism of benzene and the oxidation of ferrous iron under anaerobic conditions: implications for bioremediation. *Anaerobe* 1999;5:595–603.
- Carmona M, Díaz E: Iron-reducing bacteria unravel novel strategies for the anaerobic catabolism of aromatic compounds. *Mol Microbiol* 2005;58:1210–1215.
- Cervantes FJ, Dijkstra W, Duong-Dac T, Ivanova A, Lettinga G, Field JA: Anaerobic mineralization of toluene by enriched sediments with quinones and humus as terminal electron acceptors. *Appl Environ Microbiol* 2001;67:4471–4478.
- Chakraborty R, Coates JD: Anaerobic degradation of monoaromatic hydrocarbons. *Appl Microbiol Biotechnol* 2004;64:437–446.
- Chakraborty R, Coates JD: Hydroxylation and carboxylation: two crucial steps of anaerobic benzene degradation by *Dechloromonas* strain RCB. *Appl Environ Microbiol* 2005a;71:5427–5432.
- Chakraborty R, O'Connor SM, Chan E, Coates JD: Anaerobic degradation of benzene, toluene, ethylbenzene, and xylene compounds by *Dechloromonas* strain RCB. *Appl Environ Microbiol* 2005b;71:8649–8655.
- Champion KM, Zengler K, Rabus R: Anaerobic degradation of ethylbenzene and toluene in denitrifying strain EbN1 proceeds via independent substrate-induced pathways. *J Mol Microbiol Biotechnol* 1999;1:157–164.
- Chang BV, Chang SW, Yuan SY: Anaerobic degradation of polycyclic aromatic hydrocarbons in sludge. *Adv Environ Res* 2003;7:623–628.
- Chang BV, Shiung LC, Yuan SY: Anaerobic biodegradation of polycyclic aromatic hydrocarbon in soil. *Chemosphere* 2002;48:717–724.
- Chang W, Um Y, Holoman TRP: Polycyclic aromatic hydrocarbon (PAH) degradation coupled to methanogenesis. *Biotechnol Lett* 2006;28:425–430.
- Chen C, Taylor RT: Thermophilic biodegradation of BTEX by two consortia of anaerobic bacteria. *Appl Microbiol Biotechnol* 1997;48:121–128.
- Christensen N, Batstone DJ, He Z, Angelidaki I, Schmidt JE: Removal of polycyclic aromatic hydrocarbons (PAHs) from sewage sludge by anaerobic degradation. *Water Sci Technol* 2004;50:237–244.
- Coates JD, Anderson RT, Lovley DR: Oxidation of polycyclic aromatic hydrocarbons under sulfate-reducing conditions. *Appl Environ Microbiol* 1996a;62:1099–1101.
- Coates JD, Anderson RT, Woodward JC, Phillips EJP, Lovley DR: Anaerobic hydrocarbon degradation in petroleum-contaminated harbor sediments under sulfate-reducing and artificially imposed iron-reducing conditions. *Environ Sci Technol* 1996b;30:2784–2789.
- Coates JD, Bhupathiraju VK, Achenbach LA, McInerney MJ, Lovley DR: *Geobacter hydrogenophilus*, *Geobacter chapellei* and *Geobacter grbiciae*, three new, strictly anaerobic, dissimilatory Fe(III)-reducers. *Int J Syst Evol Microbiol* 2001b;51:581–588.
- Coates JB, Chakraborty R, Lack JG, O'Connor SM, Cole KA, Bender KS, Achenbach LA: Anaerobic benzene oxidation coupled to nitrate reduction in pure culture by two strains of *Dechloromonas*. *Nature* 2001a;411:1039–1043.
- Coates JD, Chakraborty R, McInerney MJ: Anaerobic benzene biodegradation: a new era. *Res Microbiol* 2002;153:621–628.
- Coates JD, Woodward J, Allen J, Philp P, Lovley DR: Anaerobic degradation of polycyclic aromatic hydrocarbons and alkanes in petroleum-contaminated marine harbor sediments. *Appl Environ Microbiol* 1997;63:3589–3593.
- Coschigano P, Young L: Identification and sequence analysis of two regulatory genes involved in anaerobic toluene metabolism by strain T1. *Appl Environ Microbiol* 1997;63:652–660.
- Cozzarelli IM, Herman JS, Baedeker MJ: Fate of microbial metabolites of hydrocarbons in a coastal plain aquifer: the role of electron acceptors. *Environ Sci Technol* 1995;29:458–469.
- Cross KM, Biggar KW, Semple K, Foght J, Guigard SE, Armstrong JE: Intrinsic bioremediation of diesel-contaminated cold groundwater in bedrock. *J Environ Eng Sci* 2006;5:13–27.
- Cunningham JA, Rahme H, Hopkins GD, Lebron C, Reinhard M: Enhanced in situ bioremediation of BTEX-contaminated groundwater by combined injection of nitrate and sulfate. *Environ Sci Technol* 2001;35:1663–1670.
- Da Silva MLB, Alvarez PJJ: Enhanced anaerobic biodegradation of benzene-toluene-ethylbenzene-xylene-ethanol mixtures in bioaugmented aquifer columns. *Appl Environ Microbiol* 2004;70:4720–4726.
- Da Silva MLB, Alvarez PJJ: Assessment of anaerobic benzene degradation potential using 16S rRNA gene-targeted real-time PCR. *Environ Microbiol* 2007;9:72–80.
- Dean BJ: Recent findings on the genetic toxicology of benzene, toluene, xylenes and phenols. *Mutat Res* 1985;154:153–181.
- Durante-Rodríguez G, Zamorro MT, García JL, Díaz E, Carmona M: Oxygen-dependent regulation of the central pathway for the anaerobic catabolism of aromatic compounds in *Azoarcus* sp. strain CIB. *J Bacteriol* 2006;188:2346–2354.
- Edwards EA, Grbić-Galić D: Complete mineralization of benzene by aquifer microorganisms under strictly anaerobic conditions. *Appl Environ Microbiol* 1992;58:2663–2666.
- Edwards EA, Grbić-Galić D: Anaerobic degradation of toluene and o-xylene by a methanogenic consortium. *Appl Environ Microbiol* 1994;60:313–322.
- Edwards EA, Wills LE, Reinhard M, Grbić-Galić D: Anaerobic degradation of toluene and xylene by aquifer microorganisms under sulfate-reducing conditions. *Appl Environ Microbiol* 1992;58:794–800.
- Egland PG, Harwood CS: BadR, a new MarR family member, regulates anaerobic benzoate degradation by *Rhodospseudomonas palustris* in concert with AadR, an Fnr family member. *J Bacteriol* 1999;181:2102–2109.
- Egland PG, Pelletier DA, Dispensa M, Gibson J, Harwood CS: A cluster of bacterial genes for anaerobic benzene ring biodegradation. *Proc Natl Acad Sci USA* 1997;94:6484–6489.
- Elshahed MS, Gieg LM, McInerney MJ, Suflita JM: Signature metabolites attesting to the in situ attenuation of alkylbenzenes in anaerobic environments. *Environ Sci Technol* 2001;35:682–689.
- Evans PJ, Mang DT, Kwang Shin Kim, Young LY: Anaerobic degradation of toluene by a denitrifying bacterium. *Appl Environ Microbiol* 1991a;57:1139–1145.
- Evans PJ, Mang DT, Young LY: Degradation of toluene and m-xylene and transformation of o-xylene by denitrifying enrichment cultures. *Appl Environ Microbiol* 1991b;57:450–454.
- Fetzner S: Bacterial degradation of pyridine, indole, quinoline, and their derivatives under different redox conditions. *Appl Microbiol Biotechnol* 1998;49:237–250.

- Ficker M, Krastel K, Orlicky S, Edwards E: Molecular characterization of a toluene-degrading methanogenic consortium. *Appl Environ Microbiol* 1999;65:5576–5585.
- Fries MR, Zhou J, Chee-Sanford J, Tiedje JM: Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. *Appl Environ Microbiol* 1994;60:2802–2810.
- Galushko A, Minz D, Schink B, Widdel F: Anaerobic degradation of naphthalene by a pure culture of a novel type of marine sulphate-reducing bacterium. *Environ Microbiol* 1999;1:415–420.
- Galushko AS, Kiesele-Lang U, Kappler A: Degradation of 2-methylnaphthalene by a sulfate-reducing enrichment culture of mesophilic freshwater bacteria. *Polycyclic Aromat Compd* 2003;23:207–218.
- Gibson J, Harwood CS: Metabolic diversity in aromatic compound utilization by anaerobic microbes. *Ann Rev Microbiol* 2002;56:345–369.
- Gieg LM, Suflita JM: Detection of anaerobic metabolites of saturated and aromatic hydrocarbons in petroleum-contaminated aquifers. *Environ Sci Technol* 2002;36:3755–3762.
- Gieg LM, Kolhatkar RV, McInerney MJ, Tanner RS, Harris SH, Sublette KL, Suflita JM: Intrinsic bioremediation of petroleum hydrocarbons in a gas condensate-contaminated aquifer. *Environ Sci Technol* 1999;33:2550–2560.
- Grbić-Galić D, Vogel TM: Transformation of toluene and benzene by mixed methanogenic cultures. *Appl Environ Microbiol* 1987;53:254–260.
- Griebler C, Safinowski M, Vieth A, Richnow HH, Meckenstock RU: Combined application of stable carbon isotope analysis and specific metabolites determination for assessing in situ degradation of aromatic hydrocarbons in a tar oil-contaminated aquifer. *Environ Sci Technol* 2004;38:617–631.
- Häner A, Höhener P, Zeyer J: Degradation of *p*-xylene by a denitrifying enrichment culture. *Appl Environ Microbiol* 1995;61:3185–3188.
- Harms G, Zengler K, Rabus R, Aeckersberg F, Minz D, Rosselló-Mora R, Widdel F: Anaerobic oxidation of *o*-xylene, *m*-xylene, and homologous alkylbenzenes by new types of sulfate-reducing bacteria. *Appl Environ Microbiol* 1999;65:999–1004.
- Harwood CS, Burchhardt G, Herrmann H, Fuchs G: Anaerobic metabolism of aromatic compounds via the benzoyl-CoA pathway. *FEMS Microbiol Rev* 1999;22:439–458.
- Hayes LA, Lovley DR: Specific 16S rDNA sequences associated with naphthalene degradation under sulfate-reducing conditions in harbor sediments. *Microbial Ecol* 2002;43:134–145.
- Head IM, Jones DM, Larter SR: Biological activity in the deep subsurface and the origin of heavy oil. *Nature* 2003;426:344–352.
- Heider J, Boll M, Breese K, Breinig S, Ebenau-Jehle C, Feil U, Gad'on N, Laempe D, Leuthner B, Mohamed ME, Schneider S, Burchhardt G, Fuchs G: Differential induction of enzymes involved in anaerobic metabolism of aromatic compounds in the denitrifying bacterium *Thauera aromatica*. *Arch Microbiol* 1998;170:120–131.
- Heider J, Spormann AM, Beller HR, Widdel F: Anaerobic bacterial metabolism of hydrocarbons. *FEMS Microbiol Rev* 1999;22:459–473.
- Hendrickx B, Dejonghe W, Boenne W, Brennerova M, Cernik M, Lederer T, Bucheli-Witschel M, Bastiaens L, Verstraete W, Top EM, Diels L, Springael D: Dynamics of an oligotrophic bacterial aquifer community during contact with a groundwater plume contaminated with benzene toluene, ethylbenzene, and xylenes: an in situ mesocosm study. *Appl Environ Microbiol* 2005;71:3815–3825.
- Hess A, Zarda B, Hahn D, Häner A, Stax D, Höhener P, Zeyer J: In situ analysis of denitrifying toluene- and *m*-xylene-degrading bacteria in a diesel fuel-contaminated laboratory aquifer column. *Appl Environ Microbiol* 1997;63:2136–2141.
- Jahn MK, Haderlein SB, Meckenstock RU: Anaerobic degradation of benzene, toluene, ethylbenzene, and *o*-xylene in sediment-free iron-reducing enrichment cultures. *Appl Environ Microbiol* 2005;71:3355–3358.
- Johnson HA, Pelletier DA, Spormann AM: Isolation and characterization of anaerobic ethylbenzene dehydrogenase, a novel Mo-Fe-S enzyme. *J Bacteriol* 2001;183:4536–4542.
- Johnson SJ, Woolhouse KJ, Prommer H, Barry DA, Christofi N: Contribution of anaerobic microbial activity to natural attenuation of benzene in groundwater. *Engin Geol* 2003;70:343–349.
- Kane SR, Beller HR, Legler TC, Anderson RT: Biochemical and genetic evidence of benzylsuccinate synthase in toluene-degrading, ferric iron-reducing *Geobacter metallireducens*. *Biodegradation* 2002;13:149–154.
- Kasai Y, Takahata Y, Hoaki T, Watanabe K: Physiological and molecular characterization of a microbial community established in unsaturated, petroleum-contaminated soil. *Environ Microbiol* 2005;7:806–818.
- Kasai Y, Takahata Y, Manefield M, Watanabe K: RNA-based stable isotope probing and isolation of anaerobic benzene-degrading bacteria from gasoline-contaminated groundwater. *Appl Environ Microbiol* 2006;72:3586–3592.
- Kazumi J, Caldwell ME, Suflita JM, Lovley DR, Young LY: Anaerobic degradation of benzene in diverse anoxic environments. *Environ Sci Technol* 1997;31:813–818.
- Kloer DP, Hagel C, Heider J, Schulz GE: Crystal structure of ethylbenzene dehydrogenase from *Aromatoleum aromaticum*. *Structure* 2006;14:1377–1388.
- Kniemeyer O, Heider J: Ethylbenzene dehydrogenase, a novel hydrocarbon-oxidizing molybdenum/iron-sulfur/heme enzyme. *J Biol Chem* 2001;276:21381–21386.
- Kniemeyer O, Fischer T, Wilkes H, Glöckner FO, Widdel F: Anaerobic degradation of ethylbenzene by a new type of marine sulfate-reducing bacterium. *Appl Environ Microbiol* 2003;69:760–768.
- Kodama Y, Watanabe K: Isolation and characterization of a sulfur-oxidizing chemolithotroph growing on crude oil under anaerobic conditions. *Appl Environ Microbiol* 2003;69:107–112.
- Krieger CJ, Beller HR, Reinhard M, Spormann AM: Initial reactions in anaerobic oxidation of *m*-xylene by the denitrifying bacterium *Azoarcus* sp. strain T. *J Bacteriol* 1999;181:6403–6410.
- Kube M, Heider J, Amann J, Hufnagel P, Kühner S, Beck A, Reinhardt R, Rabus R: Genes involved in the anaerobic degradation of toluene in a denitrifying bacterium, strain EbN1. *Arch Microbiol* 2004;181:182–194.
- Kuhn EP, Colberg PJ, Schnoor JL, Wanner O, Zehnder AJB, Schwarzenbach RP: Microbial transformations of substituted benzenes during infiltration of river water to groundwater: laboratory column studies. *Environ Sci Technol* 1985;19:961–968.
- Kuhn EP, Zeyer J, Eicher P, Schwarzenbach RP: Anaerobic degradation of alkylated benzenes in denitrifying laboratory aquifer columns. *Appl Environ Microbiol* 1988;54:490–496.
- Kühner S, Wöhlbrand L, Fritz I, Wruck W, Hultschig C, Hufnagel P, Kube M, Reinhardt R, Rabus R: Substrate-dependent regulation of anaerobic degradation pathways for toluene and ethylbenzene in a denitrifying bacterium, strain EbN1. *J Bacteriol* 2005;187:1493–1503.
- Langenhoff AAM, Brouwers-Ceiler DL, Engelberting JHL, Quist JJ, Wolkenfelt JGPN, Zehnder AJB, Schraa G: Microbial reduction of manganese coupled to toluene oxidation. *FEMS Microbiol Ecol* 1997;22:119–127.
- Langenhoff AAM, Zehnder AJB, Schraa G: Behaviour of toluene, benzene and naphthalene under anaerobic conditions in sediment columns. *Biodegradation* 1996;7:267–274.
- Larter S, Huan B, Adams J, Bennett B, Jakanola O, Oldenburg T, Jones M, Head I, Riediger C, Fowler M: The controls on the composition of biodegraded oils in the deep subsurface. II. Geological controls on subsurface biodegradation fluxes and constraints on reservoir-fluid property prediction. *AAPG Bull* 2006;90:921–938.
- Ledin A, Reitzel LA, Bjerg PL: Quantitative determination of toluene, ethylbenzene, and xylene degradation products in contaminated groundwater by solid-phase extraction and in-vial derivatization. *Int J Environ Anal Chem* 2005;85:1075–1087.

- Leuthner B, Heider J: A two-component system involved in regulation of anaerobic toluene metabolism in *Thauera aromatica*. FEMS Microbiol Lett 1998;166:35–41.
- Leuthner B, Heider J: Anaerobic toluene catabolism of *Thauera aromatica*: the *bbs* operon codes for enzymes of β oxidation of the intermediate benzylsuccinate. J Bacteriol 2000;182:272–277.
- Leuthner B, Leutwein C, Schulz H, North P, Haehnel W, Schiltz E, Schgger H, Heider J: Biochemical and genetic characterization of benzylsuccinate synthase from *Thauera aromatica*: a new glycol radical enzyme catalysing the first step in anaerobic toluene metabolism. Mol Microbiol 1998;28:615–628.
- Leutwein C, Heider J: Anaerobic toluene-catabolic pathway in denitrifying *Thauera aromatica*: Activation and β -oxidation of the first intermediate, (R)-(+)-benzylsuccinate. Microbiology 1999;145:3265–3271.
- Leutwein C, Heider J: Succinyl-CoA:(R)-benzylsuccinate CoA-transferase: An enzyme of the anaerobic toluene catabolic pathway in denitrifying bacteria. J Bacteriol 2001;183:4288–4295.
- Leutwein C, Heider J: (R)-benzylsuccinyl-CoA dehydrogenase of *Thauera aromatica*, an enzyme of the anaerobic toluene catabolic pathway. Arch Microbiol 2002;178:517–524.
- Lpez Barragn MJ, Carmona M, Zamarro MT, Thiele B, Boll M, Fuchs G, Garca JL, Daz E: The *bzd* gene cluster, coding for anaerobic benzoate catabolism in *Azoarcus* sp. strain CIB. J Bacteriol 2004;186:5762–5774.
- Lovley DR: Bug juice: harvesting electricity with microorganisms. Nat Rev Microbiol 2006;4:497–508.
- Lovley DR, Baedeker MJ, Lonergan DJ, Cozzarelli IM, Phillips EJP, Siegel DI: Oxidation of aromatic contaminants coupled to microbial iron reduction. Nature 1989;339:297–300.
- Lovley DR, Coates JD, Blunt-Harris EL, Phillips EJP, Woodward JC: Humic substances as electron acceptors for microbial respiration. Nature 1996;382:445–448.
- Lovley DR, Coates JD, Woodward JC, Phillips, Elizabeth JP: Benzene oxidation coupled to sulfate reduction. Appl Environ Microbiol 1995;61:953–958.
- Lovley DR, Giovannoni SJ, White DC, Champagne JE, Phillips EJP, Gorby YA, Goodwin S: *Geobacter metallireducens* gen. nov. sp. nov., a microorganism capable of coupling the complete oxidation of organic compounds to the reduction of iron and other metals. Arch Microbiol 1993;159:336–344.
- Lovley DR, Lonergan DJ: Anaerobic oxidation of toluene, phenol, and *p*-cresol by the dissimilatory iron-reducing organism, GS-15. App Environ Microbiol 1990;56:1858–1864.
- Lovley DR, Woodward JC, Chapelle FH: Stimulated anoxic biodegradation of aromatic hydrocarbons using Fe(III) ligands. Nature 1994;370:128–131.
- Magot M, Ollivier B, Patel BKC: Microbiology of petroleum reservoirs. Antonie Van Leeuwenhoek Int J Gen Mol Microbiol 2000;77:103–116.
- Marcelis CLM, Ivanova AE, Janssen AJH, Stams AJM: Anaerobic desulphurisation of thiophenes by mixed microbial communities from oilfields. Biodegradation 2003;14:173–182.
- Martus P, Puttmann W: Formation of alkylated aromatic acids in groundwater by anaerobic degradation of alkylbenzenes. Sci Total Environ 2003;307:19–33.
- McNally DL, Mihelcic JR, Lueking DR: Biodegradation of three- and four-ring polycyclic aromatic hydrocarbons under aerobic and denitrifying conditions. Environ Sci Technol 1998;32:2633–2639.
- McNally DL, Mihelcic JR, Lueking DR: Biodegradation of mixtures of polycyclic aromatic hydrocarbons under aerobic and nitrate-reducing conditions. Chemosphere 1999;38:1313–1321.
- Meckenstock RU: Fermentative toluene degradation in anaerobic defined syntrophic cocultures. FEMS Microbiol Lett 1999;177:67–73.
- Meckenstock RU, Annweiler E, Michaelis W, Richnow HH, Schink B: Anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. Appl Environ Microbiol 2000;66:2743–2747.
- Meckenstock RU, Morasch B, Griebler C, Richnow HH: Stable isotope fractionation analysis as a tool to monitor biodegradation in contaminated aquifers. J Contam Hydrol 2004a;75:215–255.
- Meckenstock RU, Safinowski M, Griebler C: Anaerobic degradation of polycyclic aromatic hydrocarbons. FEMS Microbiol Ecol 2004b;49:27–36.
- Meckenstock RU, Warthmann RI, Schafer W: Inhibition of anaerobic microbial *o*-xylene degradation by toluene in sulfidogenic sediment columns and pure cultures. FEMS Microbiol Ecol 2004c;47:381–386.
- Mihelcic JR, Luthy RG: Degradation of polycyclic aromatic hydrocarbon compounds under various redox conditions in soil-water systems. Appl Environ Microbiol 1988a;54:1182–1187.
- Mihelcic JR, Luthy RG: Microbial degradation of acenaphthene and naphthalene under denitrification conditions in soil-water systems. Appl Environ Microbiol 1988b;54:1188–1198.
- Miller KM, Suidan MT, Sorial GA, Khodadoust AP, Acheson CM, Brenner RC: Anaerobic treatment of soil wash fluids from a wood preserving site. Water Sci Technol 1998;38:63–72.
- Morasch B, Annweiler E, Warthmann RJ, Meckenstock RU: The use of a solid adsorber resin for enrichment of bacteria with toxic substrates and to identify metabolites: Degradation of naphthalene, *o*-, and *m*-xylene by sulfate-reducing bacteria. J Microbiol Methods 2001;44:183–191.
- Morasch B, Meckenstock RU: Anaerobic degradation of *p*-xylene by a sulfate-reducing enrichment culture. Curr Microbiol 2005;51:127–130.
- Morasch B, Schink B, Tebbe CC, Meckenstock RU: Degradation of *o*-xylene and *m*-xylene by a novel sulfate-reducer belonging to the genus *Desulfotomaculum*. Arch Microbiol 2004;181:407–417.
- Nales M, Butler BJ, Edwards EA: Anaerobic benzene biodegradation: a microcosm survey. Bioremed J 1998;2:125–144.
- Namocatcat JA, Fang J, Barcelona MJ, Quibuyen ATO, Abrajano Jr TA: Trimethylbenzoic acids as metabolite signatures in the biogeochemical evolution of an aquifer contaminated with jet fuel hydrocarbons. J Contam Hydrol 2003;67:177–194.
- Phelps CD, Battistelli J, Young LY: Metabolic biomarkers for monitoring anaerobic naphthalene biodegradation in situ. Environ Microbiol 2002;4:532–537.
- Phelps CD, Kerkhof LJ, Young LY: Molecular characterization of a sulfate-reducing consortium which mineralizes benzene. FEMS Microbiol Ecol 1998;27:269–279.
- Phelps CD, Young LY: Anaerobic biodegradation of BTEX and gasoline in various aquatic sediments. Biodegradation 1999;10:15–25.
- Phelps CD, Young LY: Metabolic biomarkers for detecting anaerobic PAH biodegradation in groundwater and sediments. Soil Sed Contam 2002;11:1023.
- Phelps CD, Zhang X, Young LY: Use of stable isotopes to identify benzoate as a metabolite of benzene degradation in a sulphidogenic consortium. Environ Microbiol 2001;3:600–603.
- Powell SM, Ferguson SH, Snape I, Siciliano SD: Fertilization stimulates anaerobic fuel degradation of Antarctic soils by denitrifying microorganisms. Environ Sci Technol 2006;40:2011–2017.
- Prince RC, Suflita JM: Anaerobic biodegradation of natural gas condensate can be stimulated by the addition of gasoline. Biodegradation DOI:10.1007/s10532-006-9084-4.
- Rabus R: Biodegradation of hydrocarbons under anoxic conditions; in Ollivier B, Magot M (eds): Petroleum Microbiology. Washington, ASM Press, 2005a, pp 277–299.
- Rabus R: Functional genomics of an anaerobic aromatic-degrading denitrifying bacterium, strain EbN1. Appl Microbiol Biotechnol 2005b;68:580–587.
- Rabus R, Fukui M, Wilkes H, Widdel F: Degradative capacities and 16S rRNA-targeted whole-cell hybridization of sulfate-reducing bacteria in an anaerobic enrichment culture utilizing alkylbenzenes from crude oil. Appl Environ Microbiol 1996;62:3605–3613.

- Rabus R, Heider J: Initial reactions of anaerobic metabolism of alkylbenzenes in denitrifying and sulfate-reducing bacteria. *Arch Microbiol* 1998;170:377–384.
- Rabus R, Kube M, Beck A, Widdel F, Reinhardt R: Genes involved in the anaerobic degradation of ethylbenzene in a denitrifying bacterium, strain EbN1. *Arch Microbiol* 2002;178:506–516.
- Rabus R, Kube M, Heider J, Beck A, Heitmann K, Widdel F, Reinhardt R: The genome sequence of an anaerobic aromatic-degrading denitrifying bacterium, strain EbN1. *Arch Microbiol* 2005;183:27–36.
- Rabus R, Nordhaus R, Ludwig W, Widdel F: Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. *Appl Environ Microbiol* 1993;59:1444–1451.
- Rabus R, Widdel F: Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. *Arch Microbiol* 1995;163:96–103.
- Rabus R, Wilkes H, Schramm A, Harms G, Behrends A, Amann R, Widdel F: Anaerobic utilization of alkylbenzenes and n-alkanes from crude oil in an enrichment culture of denitrifying bacteria affiliating with the β -subclass of proteobacteria. *Environ Microbiol* 1999;1:145–157.
- Reinhard M, Shang S, Kitanidis PK, Orwin E, Hopkins GD, Lebron CA: In situ BTEX biotransformation under enhanced nitrate- and sulfate-reducing conditions. *Environ Sci Technol* 1997;31:28–36.
- Reusser DE, Istok JD, Beller HR, Field JA: In situ transformation of deuterated toluene and xylene to benzylsuccinic acid analogues in BTEX-contaminated aquifers. *Environ Sci Technol* 2002;36:4127–4134.
- Rockne KJ, Chee-Sanford JC, Sanford RA, Hedlund BP, Staley JT, Strand SE: Anaerobic naphthalene degradation by microbial pure cultures under nitrate-reducing conditions. *Appl Environ Microbiol* 2000;66:1595–1601.
- Rockne KJ, Strand SE: Biodegradation of bicyclic and polycyclic aromatic hydrocarbons in anaerobic enrichments. *Environ Sci Technol* 1998;32:3962–3967.
- Rockne KJ, Strand SE: Anaerobic biodegradation of naphthalene, phenanthrene, and biphenyl by a denitrifying enrichment culture. *Water Res* 2001;35:291–299.
- Röling WFM, Head IM, Larter SR: The microbiology of hydrocarbon degradation in subsurface petroleum reservoirs: perspectives and prospects. *Res Microbiol* 2003;154:321–328.
- Rothermich MM, Hayes LA, Lovley DR: Anaerobic, sulfate-dependent degradation of polycyclic aromatic hydrocarbons in petroleum-contaminated harbor sediment. *Environ Sci Technol* 2002;36:4811–4817.
- Rueter P, Rabus R, Wilkes H, Aeckersberg F, Rainey FA, Jannasch HW, Widdel F: Anaerobic oxidation of hydrocarbons in crude oil by new types of sulphate reducing bacteria. *Nature* 1994;372:455–458.
- Safinowski M, Griebler C, Meckenstock RU: Anaerobic cometabolic transformation of polycyclic and heterocyclic aromatic hydrocarbons: evidence from laboratory and field studies. *Environ Sci Technol* 2006;40:4165–4173.
- Safinowski M, Meckenstock RU: Enzymatic reactions in anaerobic 2-methylnaphthalene degradation by the sulphate-reducing enrichment culture N 47. *FEMS Microbiol Lett* 2004;240:99–104.
- Safinowski M, Meckenstock RU: Methylation is the initial reaction in anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. *Environ Microbiol* 2006;8:347–352.
- Schreiber ME, Bahr JM: Nitrate-enhanced bioremediation of BTEX-contaminated groundwater: parameter estimation from natural-gradient tracer experiments. *J Contam Hydrol* 2002;55:29–56.
- Sharak Genthner BR, Townsend GT, Lantz SE, Mueller JG: Persistence of polycyclic aromatic hydrocarbon components of creosote under anaerobic enrichment conditions. *Arch Environ Contam Toxicol* 1997;32:99–105.
- Shinoda Y, Sakai Y, Uenishi H, Uchihashi Y, Hiraishi A, Yukawa H, Yurimoto H, Kato N: Aerobic and anaerobic toluene degradation by a newly isolated denitrifying bacterium, *Thauera* sp. strain DNT-1. *Appl Environ Microbiol* 2004;70:1385–1392.
- Siddique T, Fedorak PM, MacKinnon M, Foght JM: Metabolism of BTEX and naphtha compounds to methane in oil sands tailings. *Environ Sci Technol* 2007;41:2350–2356.
- Smets BF, Pritchard PH: Elucidating the microbial component of natural attenuation. *Curr Opin Biotechnol* 2003;14:283–288.
- Snyder R: Overview of the toxicology of benzene. *J Toxicol Environ Health [A]* 2000;61:339–346.
- Song B, Young LY, Palleroni NJ: Identification of denitrifier strain T1 as *Thauera aromatica* and proposal for emendation of the genus *Thauera* definition. *Int J Syst Bacteriol* 1998;48:889–894.
- Spark I, Patey I, Duncan B, Hamilton A, Devine C, McGovern-Traa C: The effects of indigenous and introduced microbes on deeply buried hydrocarbon reservoirs, north sea. *Clay Minerals* 2000;35:5–12.
- Spormann AM, Widdel F: Metabolism of alkylbenzenes, alkanes, and other hydrocarbons in anaerobic bacteria. *Biodegradation* 2000;11:85–105.
- Suflita JM, Davidova IA, Gieg LM, Nanny M, Prince RC: Anaerobic hydrocarbon biodegradation and the prospects for microbial enhanced energy production. *Stud Surf Sci Catal* 2004;151:283–305.
- Sullivan ER, Zhang X, Phelps C, Young LY: Anaerobic mineralization of stable-isotope-labeled 2-methylnaphthalene. *Appl Environ Microbiol* 2001;67:4353–4357.
- Sunde E, Torsvik T: Microbial control of hydrogen sulfide production in oil reservoirs; in Ollivier B, Magot M (eds): *Petroleum Microbiology*. Washington DC, ASM Press, 2005, pp 201–213.
- Tang YJ, Carpenter S, Deming J, Krieger-Brockett B: Controlled release of nitrate and sulfate to enhance anaerobic bioremediation of phenanthrene in marine sediments. *Environ Sci Technol* 2005;39:3368–3373.
- Telang AJ, Ebert S, Foght JM, Westlake DWS, Jenneman GE, Gevertz D, Voordouw G: Effect of nitrate injection on the microbial community in an oil field as monitored by reverse sample genome probing. *Appl Environ Microbiol* 1997;63:1785–1793.
- Townsend GT, Prince RC, Suflita JM: Anaerobic oxidation of crude oil hydrocarbons by the resident microorganisms of a contaminated anoxic aquifer. *Environ Sci Technol* 2003;37:5213–5218.
- Trably E, Patureau D, Delgenes JP: Enhancement of polycyclic aromatic hydrocarbons removal during anaerobic treatment of urban sludge. *Water Sci Technol* 2003;48:53–60.
- Tropel D, Van Der Meer JR: Bacterial transcriptional regulators for degradation pathways of aromatic compounds. *Microbiol Mol Biol Rev* 2004;68:474–500.
- Ulrich AC, Beller HR, Edwards EA: Metabolites detected during biodegradation of $^{13}\text{C}_6$ -benzene in nitrate-reducing and methanogenic enrichment cultures. *Environ Sci Technol* 2005;39:6681–6691.
- Ulrich AC, Edwards EA: Physiological and molecular characterization of anaerobic benzene-degrading mixed cultures. *Environ Microbiol* 2003;5:92–102.
- Villatoro-Monzón WR, Mesta-Howard AM, Razo-Flores E: Anaerobic biodegradation of BTEX using Mn(IV) and Fe(III) as alternative electron acceptors. *Water Sci Technol* 2003;48:125–131.
- Vogel TM, Grbić-Galić D: Incorporation of oxygen from water into toluene and benzene during anaerobic fermentative transformation. *Appl Environ Microbiol* 1986;52:200–202.
- Ward JAM, Ahad JME, Lacrampe-Couloume G, Slater GF, Edwards EA, Lollar BS: Hydrogen isotope fractionation during methanogenic degradation of toluene: potential for direct verification of bioremediation. *Environ Sci Technol* 2000;34:4577–4581.
- Washer CE, Edwards EA: Identification and expression of benzylsuccinate synthase genes in a toluene-degrading methanogenic consortium. *Appl Environ Microbiol* 2007;73:1367–1369.

- Weiner JM, Lovley DR: Anaerobic benzene degradation in petroleum-contaminated aquifer sediments after inoculation with a benzene-oxidizing enrichment. *Appl Environ Microbiol* 1998;64:775–778.
- Weiner JM, Lauck TS, Lovley DR: Enhanced anaerobic benzene degradation with the addition of sulfate. *Bioremed J* 1998;2:159–173.
- Widdel F, Rabus R: Anaerobic biodegradation of saturated and aromatic hydrocarbons. *Curr Opin Biotechnol* 2001;12:259–276.
- Wilkes H, Boreham C, Harms G, Zengler K, Rabus R: Anaerobic degradation and carbon isotopic fractionation of alkylbenzenes in crude oil by sulphate-reducing bacteria. *Org Geochem* 2000;31:101–115.
- Winderl C, Schaefer S, Lueders T: Detection of anaerobic toluene and hydrocarbon degraders in contaminated aquifers using benzylsuccinate synthase (*bssA*) genes as a functional marker. *Environ Microbiol* 2007;9:1035–1046.
- Wischgoll S, Heintz D, Peters F, Erxleben A, Sarnighausen E, Reski R, Van Dorselaer A, Boll M: Gene clusters involved in anaerobic benzoate degradation of *Geobacter metallireducens*. *Mol Microbiol* 2005;58:1238–1252.
- Yuan SY, Chang BV: Anaerobic degradation of five polycyclic aromatic hydrocarbons from river sediment in Taiwan. *J Environ Sci Health [B]* 2007;42:63–69.
- Zeyer J, Kuhn EP, Schwarzenbach RP: Rapid microbial mineralization of toluene and 1,3-dimethylbenzene in the absence of molecular oxygen. *Appl Environ Microbiol* 1986;52:944–947.
- Zhang X, Young LY: Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia. *Appl Environ Microbiol* 1997;63:4759–4764.
- Zhang X, Sullivan ER, Young LY: Evidence for aromatic ring reduction in the biodegradation pathway of carboxylated naphthalene by a sulfate reducing consortium. *Biodegradation* 2000;11:117–124.
- Zheng Z, Breedveld G, Aagaard P: Biodegradation of soluble aromatic compounds of jet fuel under anaerobic conditions: Laboratory batch experiments. *Appl Microbiol Biotechnol* 2001;57:572–578.
- Zhou J, Fries MR, Chee-Sanford JC, Tiedje JM: Phylogenetic analyses of a new group of denitrifiers capable of anaerobic growth on toluene and description of *Azoarcus tolulyticus* sp. nov. *Int J Syst Bacteriol* 1995;45:500–506.
- Zwolinski MD, Harris RF, Hickey WJ: Microbial consortia involved in the anaerobic degradation of hydrocarbons. *Biodegradation* 2000;11:141–158.